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Short Communication

Karyomorphological Study on 21 Shallot Cultivars (*Allium cepa* L. var. *ascalonicum*) Released by BALITSA Indonesia in 1984–2018

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ABSTRACT

Shallot (*Allium cepa* L. var. *ascalonicum*) is a vegetable commodity with consistently increasing demand each year in Indonesia. BALITSA Research Centre released 21 superior cultivars between 1984 and 2018 to support production, but there were no research on their karyotype characteristics. The 21 cultivars were studied during prometaphase mitosis using the squash method to identify chromosomal variations. All cultivars shared the same chromosome number, $2n = 16$, but exhibited differences in chromosome size and shape, most of which were metacentric, submetacentric, and subtelocentric. The findings will inform breeding programs in Indonesia to improve shallot quality.

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The genus *Allium* (shallot) is one of the most species-rich genera of monocots worldwide (Kobřlová et al. 2024), with 1,200 species identified mostly in the northern hemisphere (Govaerts et al. 2021; G. Kim et al. 2023; Ahmad et al. 2024). There are more than 100 *Allium* species, of which 50 % are endemic to the Greek flora (Dimopoulos et al. 2016). One among the many diverse *Allium* plants, shallots. They have been cultivated since 3,200–2,700 BC, evidenced by the discovery of Egyptians and Ancient Greeks artefacts such as shallot paintings on statues, monuments, and stones. This evidence is further supported by Ancient Greek relics about shallot cultivation from 4,000 years ago.

Shallots (*Allium cepa* L. var. *ascalonicum*) are widely known and cultivated in many countries, including throughout Indonesia. In Indonesia, shallots are among the most popular and economically important horticultural crops (Mallor et al. 2011). Their high value stems are coming from the diverse applications in daily life, spanning culinary, medicinal, and agricultural uses. Culinarily, shallots are widely used as a key seasoning to enhance the flavour of various traditional and modern dishes (Ricciardi et al. 2020; Liao et al. 2022). Medicinally, they have long been utilised in traditional practices as a natural antibiotic to reduce fever, treat ulcers, manage diabetes, lower blood sugar and cholesterol levels, and prevent the hardening of blood vessels (Alfiyyah et al. 2020; Putra et al. 2021; Karavelioğlu & Hoca 2022; Mardani et al. 2023). These health benefits are largely attributed to bioactive compounds such as alliin and allicin, which exhibit bactericidal, antimicrobial, anticancer, and anti-inflammatory properties (Marefati et al. 2021; Alam et al. 2023; Habibah et al. 2023; Savitri et al. 2023). In agriculture, shallots are also valued for their by-products, which can be used as organic fertilizer (Banu 2020; Rinzani et al. 2020) and as natural pesticides to help manage pests in an environmentally friendly manner (Nilan et al. 2019; Alivianingsih et al. 2020; Mulyati 2020). This wide range of benefits highlights the strategic importance of shallots not only for food and health but also for sustainable agricultural practices.

Due to high demand, increasing shallot production is important to support Indonesia's food security. The great need and an increase in population results in an increasing demand for shallots every year (Wospakrik 2017). Efforts to overcome these problems require shallot breeding initiatives. Seeds have the potential to become a high-quality genetic resource material through the application of breeding programs (Singh et al. 2020). The initial step is to obtain primary genetic information of these plants through karyotype analysis. It is needed to support genetic studies as well as enhance the efficiency of breeding activities. It is also utilised to distinguish individuals from each species. From 2004 to 2018, the Vegetable Crops Research Institute (BALITSA Research Centre) has released 21 superior shallot cultivars to increase shallot production in response to the growing demand in Indonesia. However, the karyotype diversity of the 21 shallot cultivars remains understudied. Therefore, this study aims to identify the size of arm lengths and chromosome shapes of the 21 shallots cultivars based on cytological characteristics. It is expected to add insights into knowledge in supporting the shallot plant breeding program to improve the quality of shallots produced in Indonesia, as well as information on genetic resources and material for the preparation of shallot libraries.

The research objects used were 21 shallot cultivars from Indonesia, released by the Vegetable Crops Research Institute (BALITSA Research Centre) from 1984 to 2018. Of these, 20 cultivars are collections from Balitsa, including BM1 Bima Brebes, BM1 Maja Cipanas, BM3 Kuning, BM4 Kramat 1, BM5 Kramat 2, BM6 Sembrani, BM7 Katumi, BM8 Pikatan, BM9 Trisula, BM10 Pancasona, BM11 Mentas, BM12 TSS Agrihorti 1, BM13 TSS Agri-

horti 2, BM14 Violetta 1 Agrihorti, BM15 Violetta 2 Agrihorti, BM16 Violetta 3 Agrihorti, BM18 Ambassador 1 Agrihorti, BM19 Ambassador 2 Agrihorti, BM20 Ambassador 3 Agrihorti and BM21 Ambassador 4 Agrihorti and one cultivar BM17 Super Philip, was a collection from the Bima Agriculture Office of West Nusa Tenggara. These 21 cultivars sampled were selected because they have been certified by BALITSA Research Centre and are cultivated in various shallot centers in Indonesia. All of the shallot cultivars were then brought to Laboratory Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia for further chromosome investigation.

The chromosome preparation of the 21 shallot cultivars analysed in this study was carried out based on the protocol described by (Hillis & Moritz 1996). Shallot bulbs from each cultivar were germinated in petri dishes at the Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia. Root tips were collected and pre-treated in 0.03 % colchicine solution at 4 °C for 24 hours. Following this, the samples were fixed in 45 % acetic acid at 4 °C for 15 minutes, hydrolysed in 1 N hydrochloric acid at 55 °C for 3 to 5 minutes, and then squashed using 1 % aceto-orcein. Prepared slides were examined under an Olympus BX-41 microscope.

Chromosome measurements were carried out using Ideokar v.1.3 software (Mirzaghaderi & Marzangi 2015). Classification of mitotic prometaphase chromosomes was based on centromere position according to the criteria proposed by (Levan et al. 2009): metacentric chromosomes had a centromeric index ranging from 37.50 to 50.00, submetacentric from 25.00 to 37.49, subtelocentric from 12.50 to 24.99, and telocentric from 0 to 12.49. The resulting data on chromosome size and centromere position were used to assemble karyograms using Ideokar v.1.3, while the use of CorelDRAW Graphics Suite 2018 v.20.1.0.708 program (Arisuryanti & Wibowo 2016) to edit the karyotype.

Mitotic analysis in this study focused on specific morphological characteristics of prometaphase chromosomes, including chromosome size and centromeric index. Data were analyzed through chromosome observations made during the metaphase (Mercado et al. 2020; Pinky et al. 2022). The mitotic activity in the 21 shallot cultivars examined predominantly occurred between 09:00 and 10:00 a.m. (WIB – Western Indonesian Time). The somatic prometaphase chromosomes displayed a diploid number of $2n = 16$ (Figure 1), consistent with a previous finding conducted by (Arisuryanti et al. 2009). Although the 21 shallot cultivars exhibited the same diploid chromosome number, differences were observed in chromosome size and centromeric index, resulting in variation in the karyotype formula (Figure 1 and Table 1).

As shown in Figure 1 and Table 1, five distinct karyotype formulas were identified among the shallot cultivars: 16M, 14M+2SM, 12M+4SM, 10M+6SM, and 10M+4SM+2ST. The majority of cultivars (a total of eight) exhibited the 16M karyotype, including BM2 Bima Brebes, BM10 Pancasona, BM13 TSS Agrihorti 2, BM14 Violetta 1 Agrihorti, BM15 Violetta 2 Agrihorti, BM19 Ambassador 2 Agrihorti, BM20 Ambassador 3 Agrihorti, and BM21 Ambassador 4 Agrihorti. The presence of exclusively metacentric chromosomes in these eight cultivars indicated the absence of chromosomal mutations. In general, plant species that have not undergone extensive cultivation tend to retain a fully metacentric karyotype, reflecting chromosomal stability.

The 14M+2SM karyotype was identified in three cultivars: BM3 Kuning, BM11 Mentas, and BM18 Ambassador 1 Agrihorti. Another eight cultivars exhibited the 12M+4SM karyotype, namely BM1 Maja Cipanas, BM4 Kramat 1, BM5 Kramat 2, BM6 Sembrani, BM7 Katumi, BM9 Trisula, BM16 Violetta 3 Agrihorti, and BM17 Super Phillip. Additionally, the 10M+6SM and 10M+4SM+2ST karyotypes were each observed in a single

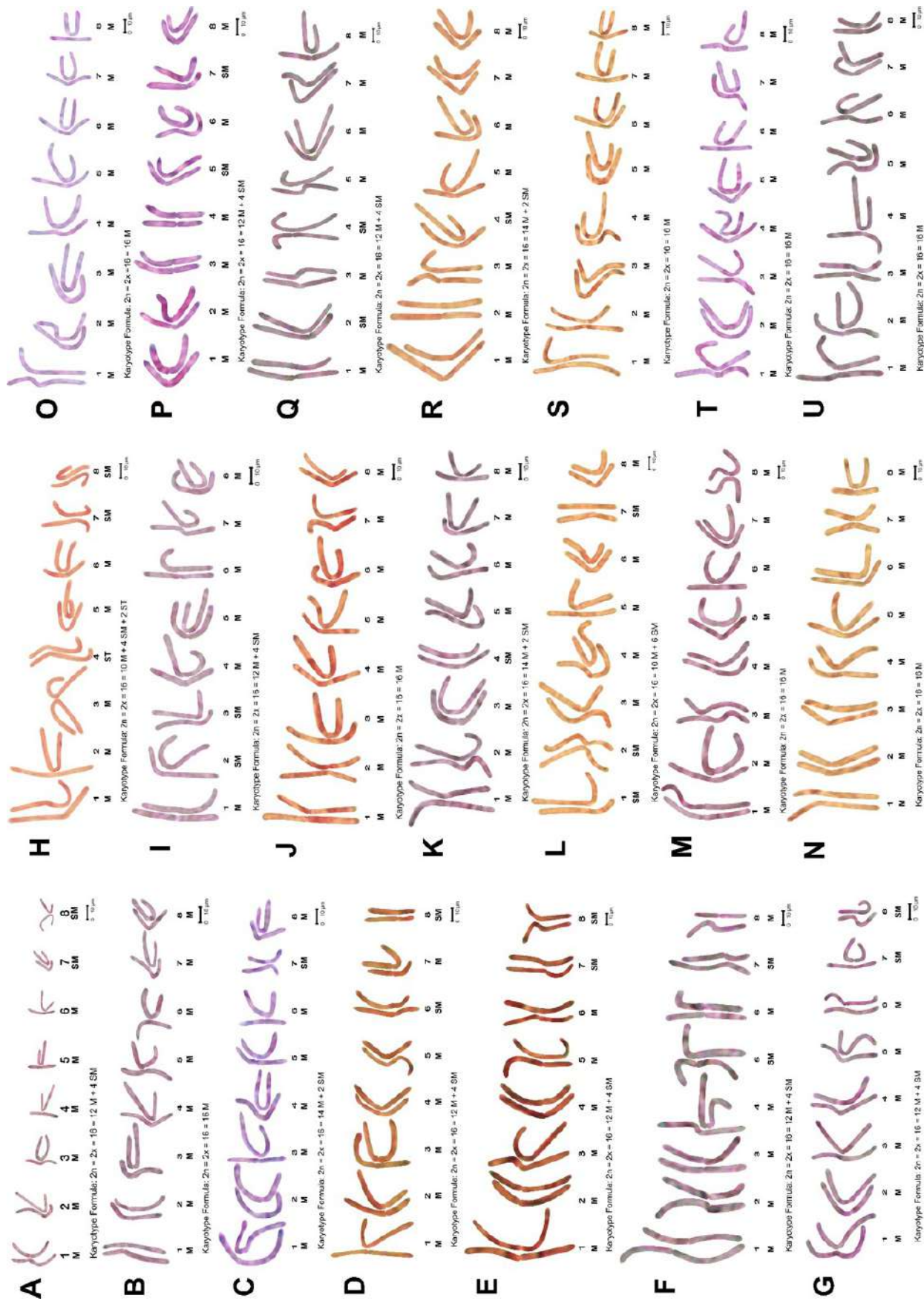


Figure 1. Photomicrophotographs of karyotypes of the 21 shallot cultivars from Indonesia: A. BM1 Maja Cipanas; B. BM2 Bima Brebes; C. BM3 Kuning; D. BM4 Kramat 1; E. BM5 Kramat 2; F. BM6 Sembrani; G. BM7 Katumi; H. BM8 Pikatan; I. BM9 Trisula; J. BM10 Pancasona; K. BM11 Mentas; L. BM12 TSS Agrihorti 1; M. BM13 TSS Agrihorti 2; N. BM14 Violetta 1 Agrihorti; O. BM15 Violetta 2 Agrihorti; P. BM16 Violetta 3 Agrihorti; Q. BM17 Super Philip; R. BM18 Ambassador 1 Agrihorti; S. BM19 Ambassador 2 Agrihorti; T. BM20 Ambassador 3 Agrihorti; and U. BM21 Ambassador 4 Agrihorti. The scale bar represents 10 μ m.

Table 1. Chromosome size and karyotype formula of the 21 Indonesian shallot cultivars.

Shallot Cultivars	Chromosome length (μm)			Centromere Index Score (NIS)	Chromosome Length Ratio (RLK)	Karyotype Formula
	Short Arm [p (μm)]	Long Arm [q (μm)]	Total Length [p+q (μm)]			
BM1 Maja Cipanas	2.34 ± 0.03 – 8.65 ± 0.06	4.04 ± 0.04 – 8.79 ± 0.02	6.74 ± 0.08 – 17.45 ± 0.07	30.73 ± 2.47 – 49.60 ± 0.12	1.02 ± 0.01 – 2.27 ± 0.26	2n = 2x = 16 = 12M+4SM
BM2 Bima Brebes	4.25 ± 0.12 – 7.89 ± 0.15	5.41 ± 0.03 – 10.35 ± 0.08	10.16 ± 0.11 – 17.23 ± 0.07	38.79 ± 0.43 – 48.50 ± 0.86	1.06 ± 0.04 – 1.58 ± 0.03	2n = 2x = 16 = 16M
BM3 Kuning	3.00 ± 0.02 – 8.77 ± 0.13	3.90 ± 0.07 – 10.75 ± 0.10	7.65 ± 0.25 – 19.52 ± 0.23	32.15 ± 0.21 – 49.21 ± 0.23	1.03 ± 0.01 – 2.11 ± 0.02	2n = 2x = 16 = 14M+2SM
BM4 Kramat 1	2.17 ± 0.09 – 6.23 ± 0.10	4.26 ± 0.20 – 7.29 ± 0.10	6.70 ± 0.19 – 13.52 ± 0.00	29.55 ± 1.16 – 48.41 ± 0.00	1.07 ± 0.00 – 2.39 ± 0.13	2n = 2x = 16 = 12M+4SM
BM5 Kramat 2	2.71 ± 0.05 – 8.38 ± 0.10	5.91 ± 0.13 – 10.61 ± 0.16	8.93 ± 0.14 – 18.99 ± 0.26	30.35 ± 0.08 – 49.75 ± 0.21	1.01 ± 0.01 – 2.30 ± 0.01	2n = 2x = 16 = 12M+4SM
BM6 Sembrani	3.13 ± 0.10 – 9.48 ± 0.10	5.59 ± 0.10 – 14.47 ± 0.07	9.68 ± 0.20 – 23.95 ± 0.17	30.48 ± 0.91 – 49.96 ± 0.00	1.00 ± 0.00 – 2.28 ± 0.10	2n = 2x = 16 = 12M+4SM
BM7 Katumi	2.43 ± 0.06 – 8.06 ± 0.10	4.60 ± 0.07 – 8.19 ± 0.06	8.69 ± 0.02 – 16.25 ± 0.16	27.55 ± 0.62 – 49.60 ± 0.13	1.02 ± 0.01 – 2.63 ± 0.08	2n = 2x = 16 = 12M+4SM
BM8 Pikatan	3.15 ± 0.07 – 9.75 ± 0.04	6.61 ± 0.07 – 14.49 ± 0.10	10.16 ± 0.02 – 24.24 ± 0.07	20.94 ± 0.23 – 48.53 ± 0.52	1.06 ± 0.02 – 3.78 ± 0.05	2n = 2x = 16 = 10M+4SM+2 ST
BM9 Trisula	4.44 ± 0.08 – 7.99 ± 0.10	5.98 ± 0.07 – 11.91 ± 0.07	11.11 ± 0.17 – 19.35 ± 0.00	32.36 ± 0.05 – 47.91 ± 0.03	1.09 ± 0.00 – 2.09 ± 0.00	2n = 2x = 16 = 12M+4SM
BM10 Pancasona	4.03 ± 0.10 – 8.76 ± 0.07	5.60 ± 0.09 – 10.29 ± 0.13	9.63 ± 0.19 – 18.52 ± 0.14	38.67 ± 0.58 – 49.56 ± 0.12	1.02 ± 0.00 – 1.59 ± 0.04	2n = 2x = 16 = 16M
BM11 Mentas	4.83 ± 0.05 – 8.53 ± 0.05	6.01 ± 0.07 – 10.48 ± 0.10	10.89 ± 0.01 – 19.01 ± 0.05	34.91 ± 0.65 – 49.82 ± 0.15	1.01 ± 0.00 – 1.86 ± 0.05	2n = 2x = 16 = 14M+2SM
BM12 TSS Agrihorti 1	4.07 ± 1.10 – 7.17 ± 0.06	6.61 ± 0.07 – 12.56 ± 0.06	11.28 ± 0.00 – 19.37 ± 0.01	33.44 ± 0.38 – 48.90 ± 0.62	1.04 ± 0.03 – 1.99 ± 0.03	2n = 2x = 16 = 10M+6SM
BM13 TSS Agrihorti 2	3.61 ± 0.06 – 8.53 ± 0.06	4.45 ± 0.03 – 8.58 ± 0.05	8.06 ± 0.09 – 17.11 ± 0.11	40.44 ± 0.79 – 49.85 ± 0.03	1.01 ± 0.00 – 1.47 ± 0.05	2n = 2x = 16 = 16M
BM14 Violetta 1 Agrihorti	4.37 ± 0.05 – 7.29 ± 0.14	4.90 ± 0.07 – 8.63 ± 0.11	9.27 ± 0.12 – 15.32 ± 0.09	41.14 ± 0.17 – 49.34 ± 0.61	1.03 ± 0.03 – 1.43 ± 0.01	2n = 2x = 16 = 16M
BM15 Violetta 2 Agrihorti	4.57 ± 0.06 – 11.01 ± 0.13	5.70 ± 0.09 – 11.19 ± 0.06	10.55 ± 0.05 – 22.20 ± 0.19	39.42 ± 0.05 – 49.58 ± 0.15	1.02 ± 0.01 – 1.54 ± 0.00	2n = 2x = 16 = 16M
BM16 Violetta 3 Agrihorti	1.99 ± 0.03 – 5.75 ± 0.06	3.29 ± 0.03 – 6.74 ± 0.08	5.29 ± 0.05 – 11.96 ± 0.13	33.72 ± 0.20 – 49.95 ± 0.05	1.00 ± 0.00 – 1.97 ± 0.02	2n = 2x = 16 = 12M+4SM
BM17 Super Phillip	2.95 ± 0.02 – 5.79 ± 0.10	4.58 ± 0.07 – 7.73 ± 0.05	7.53 ± 0.09 – 12.85 ± 0.00	34.26 ± 0.06 – 49.58 ± 0.07	1.02 ± 0.00 – 1.92 ± 0.00	2n = 2x = 16 = 12M+4SM
BM18 Ambassador 1 Agrihorti	4.06 ± 0.10 – 11.35 ± 0.04	5.33 ± 0.05 – 11.89 ± 0.10	9.39 ± 0.15 – 22.84 ± 0.14	35.90 ± 0.15 – 49.69 ± 0.13	1.01 ± 0.01 – 1.79 ± 0.01	2n = 2x = 16 = 14M+2SM

Description: M = metacentric; SM = submetacentric; ST = subtelocentric; T = telocentric.

Table 1. Contd.

Shallot Cultivars	Chromosome length (μm)			Centromere Index Score (NIS)	Chromosome Length Ratio (RLK)	Karyotype Formula
	Short Arm [p (μm)]	Long Arm [q (μm)]	Total Length [p+q (μm)]			
BM19 Ambassador 2 Agrihorti	2.48 ± 0.08 – 10.45 ± 0.03	2.93 ± 0.06 – 12.32 ± 0.06	5.41 ± 0.14 – 21.33 ± 0.08	38.61 ± 0.02 – 48.99 ± 0.04	1.04 ± 0.00 – 1.59 ± 0.00	2n = 2x = 16 = 16M
BM20 Ambassador 3 Agrihorti	4.47 ± 0.10 – 8.70 ± 0.08	5.63 ± 0.05 – 11.88 ± 0.10	10.10 ± 0.15 – 19.15 ± 0.07	37.96 ± 0.30 – 48.55 ± 0.03	1.06 ± 0.00 – 1.63 ± 0.02	2n = 2x = 16 = 16M
BM21 Ambassador 4 Agrihorti	2.89 ± 0.06 – 6.28 ± 0.05	4.43 ± 0.05 – 7.85 ± 0.04	7.32 ± 0.11 – 13.72 ± 0.12	39.48 ± 0.23 – 49.42 ± 0.01	1.02 ± 0.00 – 1.53 ± 0.01	2n = 2x = 16 = 16M

Description: M = metacentric; SM = submetacentric; ST = subtelocentric; T = telocentric.

cultivar: BM12 TSS Agrihorti 1 and BM8 Pikatan, respectively. The presence of submetacentric and subtelocentric chromosomes among these cultivars suggests the occurrence of chromosomal mutations, specifically pericentric inversions that involve the centromere. This type of mutation results in a reversed chromosome segment that spans both the short (p) and long (q) arms, as well as the centromere itself. A similar phenomenon was reported by (Arisuryanti et al. 2018) in two garlic (*Allium sativum* L.) cultivars, which exhibited submetacentric chromosomes due to frequent selection practices.

Cytological analysis also revealed diversity in chromosome size (Table 1). The results showed that the shortest lengths of chromosome short arms, long arms, and total chromosome length were observed in the BM16 Violetta 3 Agrihorti cultivar. In contrast, other cultivars exhibited varying lengths of short arms, long arms, and total chromosome size. The twenty-one shallot cultivars released by the Vegetable Crops Research Institute (BALITSA Research Centre) possess a diploid chromosome number of 2n = 16, which are classified into five distinct karyotype types: 16M, 14M+2SM, 12M+4SM, 10M+6SM, and 10M+4SM+2ST. The chromosome size and chromosome shape among these twenty-one cultivars exhibited considerable variation. This variation in chromosome size indicated the presence of genetic diversity among the 21 shallot cultivars examined. Genetic variation is a critical factor in breeding programs, as it enables the development of high-quality shallot varieties.

The use of cytological characteristics is considered critical for species identification (Y. Kim et al. 2023), in understanding plant evolution (Hosseini & Yaghoobi 2024), and hybrid populations (Sayadi et al. 2021). Therefore, the chromosome or karyotype structures presented in this study may be utilised for the classification of *Allium* species (Maragheh et al. 2019; Saensouk & Saensouk 2021). However, some members of the genus *Allium*, such as *Allium sativum*, *Allium fistulosum*, and *Allium cepa*, possess the same diploid chromosome number (2n = 16) and also share similar karyotype structures despite belonging to different species (Guetat et al. 2015; Arisuryanti et al. 2009; Arisuryanti et al. 2018; Abdali & Miri 2020). Therefore, molecular approaches are necessary to accurately determine cultivar identity, which in turn facilitates more effective breeding programs (Peruzzi et al. 2017; Hao et al. 2023). With the availability of new cytogenetic baseline data from the twenty-one shallot cultivars released by the Vegetable Crops Research Institute (BALITSA Research Centre), further research is needed. This includes analyses of the relationships between karyotype and phenotypic traits such as growth, disease resistance, and bulb quality; studies on the influence of karyotype on breeding outcomes and genetic selection; and the development of kar-

yotype-based genetic markers for shallot breeding and selection programs. Therefore, this study did not only provides novel cytogenetic baseline data but also opened avenues for integrated and applied follow-up research.

AUTHORS CONTRIBUTION

DM designed the research, collected plant samples, performed laboratory work, analysed the data, and wrote the manuscript. P, TA, BSD designed the research, supervised all the processes from the field work to laboratory analysis, and wrote the manuscript. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declared we have no conflicts of interest to disclose.

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Short Communication

The Use of Spool Tracking Technique to Monitor the Movement of Juvenile Asian Water Monitor Lizard

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ABSTRACT

Tracking animal movement is essential for conservation but often financially challenging. This study tested a low-cost spool tracking method using juvenile Asian water monitors (*Varanus salvator*) housed in a dome enclosure. Tail-mounted spools were more effective than back-mounted ones, allowing greater movement and fewer complications. Lizards were active during the day, preferred vegetated microhabitats, and often basked or soaked. Importantly, tail-mounted spools caused no injuries or entanglement, indicating this method is a viable, non-invasive option for monitoring juvenile lizard movement in resource-limited contexts, with potential application to other species.

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Understanding animal movement is fundamental for studying ecology and behaviour, both of which are vital for effective wildlife habitat and corridor management (Allen & Singh 2016). However, tracking animal movement remains a significant challenge (Kernohan et al. 2001). Researchers have developed a wide range of methods, from direct observational techniques (Altmann 1974) and mark-recapture studies (Lagrange et al. 2014) to sophisticated technologies such as radio telemetry, GPS tracking, RFID chips, geotags, radiolocation devices, and even georeferenced social media data (Rutz & Hays 2009; Benson 2010; Cagnacci et al. 2010; Kays et al. 2015; Miller et al. 2019; Gracanin & Mikac 2022). While these tools have greatly advanced our capacity to monitor wildlife, they are often prohibitively expensive and difficult to access, particularly in biodiversity-rich regions with limited financial and logistical resources. As more accessible alternative, spool tracking—which involves attaching a spool of thread to an animal—provides a simple yet effective method for monitoring the fine-scale movement of small animals over short distances (Sutherland et al. 2008).

Monitor lizards (*Varanus* spp.) among the largest members of the Varanidae family, are known for their relatively slow movement compared to other lizards (Bennett 1995). These reptiles are primarily diurnal becoming active in the morning to forage and bask and resting at night (Imansyah et al. 2007). Adults often seek shelter under trees, in burrows, or within dense vegetation, while juveniles more often use trees as resting sites—likely a strategy to avoid predation, including from larger conspecifics (Bennett 1995). The juvenile stage in monitor lizards is characterised by rapid developmental changes and heightened vulnerability, often associated with increased selective pressures (Sumner 2006). During this period, juveniles exhibit more arboreal behaviour than adults (Law et al. 2016), which has implications for movement patterns and habitat use that are not yet well understood.

Previous studies have utilised spool tracking techniques to investigate the movement and activity patterns of monitor lizards, such as *Varanus olivaceus* (Bennett 2014) and *V. bitatawa* (Law et al. 2016). However, to date, no such studies have been conducted in Indonesia despite its high diversity of *Varanus* species. To address this gap, we carried out a controlled experiment using spool tracking to monitor the movement of juvenile water monitor lizards (*V. salvator*) in captivity. The objectives of this study were: (1) to identify the most effective and least intrusive spool attachment position, and (2) to evaluate the behavioural responses of juvenile lizards to spool tracking within a controlled environment.

The study assessing the effectiveness of spool tracking device attachment in monitor lizards was conducted in a dome-shaped enclosure located at the Dramaga Campus of Institut Pertanian Bogor, which functioned as a controlled habitat. The experiment took place over a one-week period, from January 22 to 28, 2020. The dome, situated within the Rectorate Park, measured 11.5 meters in diameter and 7.2 meters in height, with a total surface area of 415.26 m². It was constructed from a series of semicircular iron frames covered with fine, perforated wire mesh and enclosed by a circular wall at the base (Figure 1a). The floor was cemented, with an opening located on the western side of the structure (Figure 1b).

For the purposes of the study, the enclosure was divided into two different habitat types: open and vegetated areas. The vegetated sections were dominated by understory plant species including *Asystasia gangetica*, *Derris elliptica*, *Clidemia hirta*, and *Rhoe discolor*. As there was no natural water source within the dome, water was supplied via an open container placed at the centre of the enclosure during the observation period.

We used yarn bobbin cocoons manufactured by Danfields (Leigh, UK), consisting of two thread types: a brown thread weighing 2.6 g with a length

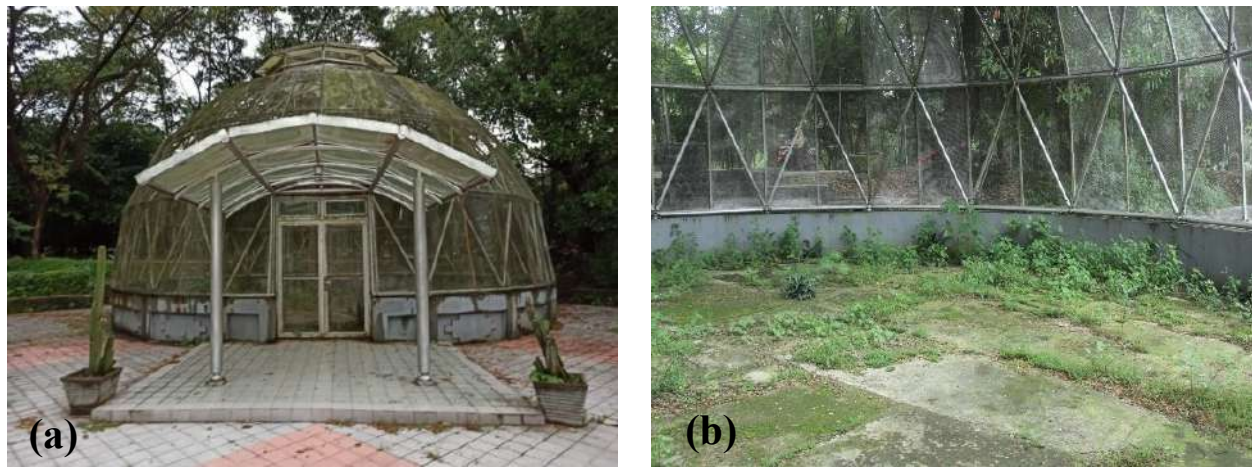


Figure 1. (a) The dome used for experiment and (b) vegetation inside dome.

of 70 m and a white thread weighing 2.2 g with a length of 120 m. The use of two types of threads was necessary to ensure an adequate length for the experiment. The 70-meter thread was initially insufficient to cover the entire required distance, so a 120-meter thread was added to ensure continuous unraveling during the tracking process. This combination ensured that the tracking device functioned effectively throughout the study. The threads were coiled without a central spool, then to unravel smoothly as the animal moved. Prior to data collection, we measured the snout-vent length (SVL) and body mass of each individual. Three juvenile water monitor lizards were used in this study, each with an approximate SVL of 40 cm and body mass around 100 g (Table 1). The animals were sourced from a local hunting group in Bogor (see [Yudha et al. 2022](#) for details on hunting practices).

Before the experiment began, the lizards were acclimatized in the laboratory for one week. During this period, they were housed individually in 30 × 60 cm plastic containers and fed crickets twice daily - one in the morning and again in the late afternoon, following the feeding protocol described by Byers (1999). Routine care was also maintained throughout the acclimation phase, including daily bathing between 07:00 and 12:00 and basking opportunities provided between 08:00 and 15:00.

Table 1. Spool attachment position in juvenile water monitor lizards (*Varanus salvator*) used in the experiment, snout-vent length (SVL), body mass, thread mass and thread length.

ID	Position of spool	SVL (cm)	Body mass (g)	Thread mass (g)	Thread length (m)
BA1	Back area	29.6	63	2.2	120
BA3	Tail	28.7	60	2.2	120
BA2	Control (no spool)	25.4	56	-	-

The spool tracking device was attached to two juvenile water monitor lizards at different body positions: one on the dorsal surface (back) and the other at the base of the tail (Figure 2). A third individual served as a control was not fitted with any device. The yarn used in this study was coiled internally within the bobbin, allowing it to unravel smoothly during movement. To minimise the risk of entanglement, the bobbin was securely fastened with tape (Figure 3a). The thread—made of soft cotton and typically used for embroidery—was selected for its fine texture and flexibility, reducing the risk of injury to the animals. In the event of an escape, the lightweight material

was expected to detach easily and safely. In accordance with Bennett (2000), the weight of the thread was kept below 5 % of the lizard's body mass, not exceeding 3 g in any case.



Figure 2. Juvenile water monitor lizard with tracking device positioned at the base of the tail.

The thread was attached either to the mid-dorsal region or base of the tail, with careful positioning to avoid interference with limb movement (Figure 3b and 3c). The free end of the thread was tied to a fixed point were selected randomly within the enclosure, such as a tree or branch, to facilitate unravelling during movement (Figure 3d and 3e). The animals were released into the dome enclosure, and data collection was conducted over two consecutive days for each treatment. In the first trial, the back-mounted lizard and the control individual were released together, followed by the tail-mounted lizard and the same control in the second trial. Observations were made every three hours between 06:00 to 18:00 with each session lasting 30 minutes. The observation intervals were set every three hours to avoid excessive disturbance to the animals and to ensure that enough thread remained for the tracking process. This timing was chosen based on the fact that the lizards were not highly active throughout the entire day, allowing for meaningful observations without frequent interruptions. During these periods, we recorded behavioural responses and spatial use. Movement distance was estimated by measuring the length of the unraveled thread.

During the experiment, we recorded several parameters: the animals' behavioural responses (measured by the number of movements, inferred from the length of thread unraveled), spatial location within the enclosure, total movement distance for each treatment, and the microhabitat characteristics within the dome. The spool attachment position that did not hinder natural movement was considered the most suitable for potential application in tracking juvenile Komodo dragons in the wild.

Upon completion of data collection, all experimental animals were released IPB University campus, which is part of their natural habitat. This study was conducted under an ethical permit issued by the IPB Animal Ethics Commission (Permit No. 175-2020 IPB).

Animal responses to the spool tracking device and microhabitat use were described qualitatively. To evaluate differences in average movement between treatments, movement data were analyzed using a one-way ANOVA. Movements of less than 0.05 m were considered negligible, indicating that the animal remained largely stationary.

Results showed a trend of greater daily movement in lizards with the

spool attached to the tail compared to those with the device attached on the back. A significant difference in total movement distance was found between treatments ($F_{10,3} = 19.44$; $P = 0.04$) (Table 2). Tail-mounted individuals displayed notable activity, including climbing vegetation up to 2.5 meters in height and attempting to escape via the wire enclosure. All individuals predominantly moved along the perimeter of the dome, with limited use of the central area.

Table 2. Daily movement distances of water monitor lizards over two days, based on spool attachment position.

Variable	Tail	Back side	Mean $\pm$ SE
Total movement (m)	167.70	90.30	129.00 $\pm$ 38.70
Mean movement per day (m day ⁻¹)	83.85	45.15	64.50 $\pm$ 11.30



Figure 3. Attachment and use of the spool tracking device on juvenile water monitor lizards (*Varanus salvator*): (a) bobbin cocoon thread used in the experiment; (b) spool attached to the mid-dorsal region; (c) spool attached to the base of the tail; (d) free end of the thread secured to a fixed point (tree) to enable unraveling; (e) movement path of the lizard indicated by the trailing thread.

Table 3 summarizes the average activity frequencies of juvenile water monitor lizards under different treatments, recorded at three-hour intervals from 06:00 to 18:00 over two days. Lizards with the spool device mounted on the tail exhibited a higher activity frequency (mean = 13.5, SE = 1.50) compared to those with the device attached to the back (mean = 10.5, SE = 1.32), suggesting that tail-mounted devices may interfere less with natural movement. Control groups without any device attachment (Control-1 and Control-2) recorded the highest activity frequencies, with means of 12.0 (SE = 1.41) and 14.5 (SE = 1.55), respectively. This pattern suggests a slight reduction in activity due to the presence of the spool device, regardless of position, although tail-mounted devices appear to have a lesser impact. Overall, the mean activity frequency across all treatments was 12.81 (SE =

0.74), reinforcing that while tracking devices may affect activity to some degree, proper placement—particularly at the tail—can help maintain behavior levels closer to those of unencumbered individuals.

Table 3. Average movement of juvenile water monitor lizards under different treatments, based on 3-hour interval observations from 06:00 to 18:00 over two days.

Spool location	Frequency of activity	Mean	Std. Error
Dorsal region	20	10.5	1.32
Control-1	23	12.0	1.41
Tail	26	13.5	1.50
Control-2	28	14.5	1.55
Total	97	12.81	0.74

Most of the experimental animals (76 %) were more active in vegetated areas than in open spaces (24 %). Water monitor lizards ventured into open areas primarily to bask in the sun and to soak in the water provided there. However, the majority of other activities—such as exploration and resting—occurred in vegetated zones, particularly under shaded areas or near small holes.

The results of this study demonstrated that the position of the spool tracking device significantly influences the movement behaviour and overall well-being of juvenile water monitor lizards (*Varanus salvator*). Lizards fitted with tail-mounted spools exhibited greater daily movement distances compared to those with back-mounted devices, with the difference being statistically significant. This suggests that the tail-mounted configurations better in supporting natural locomotion and exploratory behaviour. The enhanced mobility observed in these individuals may be due to reduced interference with limb function and balance—both of which are essential for a semi-arboreal species like *V. salvator*.

Importantly, individuals with the tail-mounted spool device retained the ability to climb vertical structures up to 2.5 meters and engaged in complex behaviours, such as escape attempts, indicating that their physical capabilities were not notably restricted. This contrasts with the back-mounted configuration, which in one case led to thread entanglement and temporary immobilisation. Although such entanglement was not fatal in this study, the incident underscores the potential risk associated with improper device positioning. Similar findings were reported by Susanto (2011), who noted fatal consequences in smaller amphibians due to thread entanglement, particularly when using coarser thread materials. Additionally, Gourevitch and Downie (2018) evaluated tree frog tracking methods and highlighted similar concerns regarding entanglement risks, particularly when using poorly secured or incorrectly positioned devices. These studies emphasised the importance of careful device placement to minimise harm to the animals. The use of finer, smoother cotton thread in our study likely mitigated some of these risks, but it does not eliminate the potential for adverse outcomes when the device is mounted dorsally.

Behavioural observations further supported the preference for tail mounting spool devices. Although all individuals remained active during the daylight hours, consistent with the species' diurnal activity patterns (Byers 1999), lizards in the tail-mounted group showed slightly higher activity frequency. These individuals also explored vertical structures and peripheral areas more extensively, suggesting that the tail-mounted configuration is less disruptive to natural behaviours. Furthermore, the preference for vegetated zones over open spaces observed in most individuals aligns with previous ecological findings (Pah 2003), indicating that the experimental conditions closely approximated the species' natural habitat preferences.

Initial signs of discomfort—such as tail-wagging and hind limb scratching were observed in both treatment groups but diminished rapidly, indicating a capacity for habituation. Nonetheless, device design and placement remain critical. Ensuring at least a 3 cm clearance from the hind limbs appears to be a simple yet effective modification to prevent interference with movement.

In conclusion, the tail-mounted spool configuration offers a more reliable and animal-welfare-conscious method for tracking juvenile water monitor lizards. It allows for natural movement, reduces the risk of entanglement, and provides robust movement data. Future studies aiming to monitor free-ranging individuals in natural habitats should consider adopting this attachment strategy to minimize stress and maximize data quality.

AUTHORS CONTRIBUTION

F.E.S collected and analysed the data and wrote the manuscript in Bahasa Indonesia. M.D.K designed the research, supervised all the process and wrote the translation of manuscript in English. Y.A.M supervised all the process.

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CONFLICT OF INTEREST

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Short Communication

Flight Activities and Pollen Load of *Geniotrigona thoracica* in Tanah Laut Regency, South Kalimantan, Indonesia

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ABSTRACT

Geniotrigona thoracica is a stingless bee with a high potential for honey production and pollination. This study analysed its flight activity and pollen diversity in Tanah Laut, South Kalimantan. Flight activity was recorded at four time intervals, while pollen was collected from foragers and nest cells. Environmental factors (temperature, light intensity, humidity) were measured and analysed using ANOVA and GLM. Results showed stable flight activity with a peak at 06:00–08:00. Temperature only affected bees returning without pollen. Herbaceous plants dominated pollen sources, supporting pollination and providing essential nutrients for colony sustainability.

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Geniotrigona thoracica is a species of stingless bee widely recognized for its high honey production, particularly in Southeast Asia. This species possesses a relatively large body size, ranging from 7.97 to 8.35 mm, and constructs honey pots with diameters of approximately 6–9 mm (Saufi & Thevan 2015; Purwanto et al. 2022). These morphological traits are thought to enhance its storage capacity and foraging efficiency, thereby increasing overall productivity. Beyond honey production, *G. thoracica* also yields other economically valuable products such as propolis and bee bread, which are rich in bio-active compounds and widely used in traditional medicine. In addition to its economic value, *G. thoracica* serves vital ecological roles as a pollinator for various flowering plant species in natural and agricultural landscapes (Agussalim et al. 2017, 2020; Chuttong et al. 2022). Its ability to adapt to managed meliponiculture systems while providing ecosystem services makes this species a promising candidate for sustainable apiculture development and biodiversity conservation.

The daily flight activities of *G. thoracica* are not limited to foraging alone but encompass a range of essential behaviours that support colony survival. Worker bees engage in foraging trips to collect pollen as a protein source, nectar as a carbohydrate source, and resins for nest construction and defense (Nunes-Silva et al. 2010; Leonhardt et al. 2011). These resources are critical for larval development, adult nutrition, and the structural integrity of the colony. In addition to resource collection, bees also leave the nest to remove waste material, including dead individuals, excreta, and remnants of brood cells (Nunes-Silva et al. 2013). Such waste management behaviour is essential for preventing disease and maintaining nest hygiene. The division of labour and time allocation for different tasks outside the nest reflect a high degree of social organization, typical of eusocial insects such as stingless bees (Leonhardt et al. 2011). Understanding these flight-related behaviours is crucial for interpreting the ecological interactions between *G. thoracica* and its surrounding environment.

Environmental conditions significantly influence the flight patterns and foraging efficiency of stingless bees. In particular, abiotic factors such as temperature, light intensity, humidity, and resource availability can directly affect the stingless bees decision to leave the nest and the duration of their foraging trips (Gadhiya & Pastagia 2019). In the case of *G. thoracica*, temperature influences the metabolic energy required for flight, while light intensity may affect the bees' ability to navigate and detect flowers. Humidity also plays a role in pollen viability and nectar concentration, which in turn can impact floral attractiveness. These environmental variables vary throughout the day and across different seasons, thus shaping the temporal patterns of bee activity. For stingless bees, optimal foraging often occurs within a narrow range of environmental conditions, and extreme weather can restrict flight or reduce resource collection (Kartikasari et al. 2023). Therefore, understanding how these factors interact with bee behaviour is essential for meliponiculture management, particularly when designing artificial nests or placing colonies in agricultural landscapes. This study aims to analyse the flight activity patterns of *G. thoracica* and the diversity of pollen from forage plants. The findings are expected to provide a valuable reference for beekeepers in designing feeding strategies and implementing appropriate management practices to optimize honey production. The importance of this research lies in its potential contribution to improving sustainable stingless beekeeping, enhancing honey yield, and promoting biodiversity through pollination services. Although previous studies have examined the foraging behaviour and ecological roles of stingless bees, comprehensive research that combines flight activity analysis with pollen diversity identification in *G. thoracica* remains limited. This state-of-the-art study integrates behavioural and botanical data to better understand the

ecological interactions of this species. The novelty of this research lies in its dual approach to evaluating both temporal flight patterns and floral resource usage, offering new insights that can inform habitat enrichment and targeted conservation strategies.

This study was conducted in June 2024 at a stingless bee farm in Tanah Laut Regency, South Kalimantan Province, Indonesia ($3^{\circ}19'00.01''$ S, $115^{\circ}40'59.99''$ E) (Figure 1). This study utilized a range of tools and materials to support both fieldwork and laboratory analysis. Insect nets with a diameter of 30 cm and fine nylon mesh were used to capture foraging worker bees. Collected pollen was stored in 1.5 mL microcentrifuge tubes (Eppendorf type). Pollen was removed from the bees' corbiculae using sterile stainless-steel tweezers (12 cm in length), while pot-pollen was collected using sterile plastic spatulas (10 cm in length). Environmental variables were measured using a digital thermo-hygrometer (HTC-1 model; temperature accuracy $\pm 1^{\circ}\text{C}$, humidity accuracy $\pm 5\%$) and a lux meter (LX1010B model; measurement range up to 50,000 lux; accuracy $\pm 5\%$) for light intensity. Bee activity was recorded using a smartphone video camera (1080p resolution) mounted on a tripod, and individual bees were counted manually using a hand counter clicker. Data were analyzed using R software version 4.2.0, applying Oneway ANOVA and General Linear Models (GLM). For pollen identification, the acetolysis method was employed following the standard procedure by Erdtman (1972), using acetic anhydride and concentrated sulfuric acid as reagents. Pollen identification was based on references from the Pollen Flora of Taiwan (Huang 1972) and the Australian Pollen and Spores Atlas (APSA 2017).

Pollen foraging activity was observed through three specific behaviours: bees flying out of the nest, returning to the nest with pollen, and returning to the nest without pollen. Bee activity was recorded during four times intervals: 06:00–08:00, 09:00–11:00, 13:00–15:00, and 16:00–18:00. Each colony was observed and recorded for 6 minutes per interval. The number of bees engaged in each activity were determined from video recordings and quantified using a hand counter. Data were analysed using Oneway ANOVA to evaluate significant differences in activity across the time intervals. The measured environmental conditions included light intensity, humidity, and temperature. The analysis aimed to determine the effect of environmental conditions on bee pollen-foraging activity. Additionally, Oneway ANOVA was performed to test for significant differences ($P < 0.05$) in each environmental condition across the time intervals. Environmental data were analysed using a General Linear Model (GLM) with a Gaussian distribution in R software (Paradis 2005) to evaluate their influence on bee foraging activity. A Oneway ANOVA was performed for each environmental factor at each time interval to determine significant differences ($P < 0.05$).

Pollen collection was conducted from the pollen basket on the legs and the pollen pots of *G. thoracica*. Bees were captured using insect nets, and the



Figure 1. Sampling map sites in Tanah Laut Regency, South Kalimantan Province, Indonesia.

pollen was extracted using sterile tweezers. The bee pollen was placed into 1.5 mL tubes, and the bees were subsequently released. Three individual bees per colony (10 colony) were captured for each time interval. Additionally, pollen was collected from the pollen pots using a sterile spatula and placed into 1.5 mL tubes. Pollen was taken from three pollen pots per colony. Pollen samples were stored in 1.5 mL tubes for further analysis. This approach was intended to obtain more comprehensive results regarding the pollen diversity of *G. thoracica*. Pollen diversity was analysed using the acetolysis method (Erdtman 1972), which is a standard procedure for preparing pollen samples for identification. Pollen identification referred to the Pollen Flora of Taiwan (Huang 1972) and the Australian Pollen and Spores Atlas (<https://apsa.anu.edu.au/>) (APSA 2017).

The number of *G. thoracica* individuals flying out of the nest did not differ significantly ($P = 0.09$) across the observation time intervals. However, between 06:00–08:00, it was observed that *G. thoracica* likely leaves the nest in large numbers to forage. This is evident from the peak activity of *G. thoracica* returning to the nest with pollen during this period (Figure 2). This finding is consistent with the observations of Atmowidi et al. (2018) reported that the foraging activity of three stingless bee species i.e., *T. laeviceps*, *H. itama*, and *L. terminata* in Sumber Klampok Village, West Bali, typically begins in the morning. Although the species observed in that study differ from *G. thoracica*, their foraging patterns exhibit a similar peak activity period in the early hours of the day. Stingless bees employ various communication methods during foraging, including the use of pheromones and vibrational signals (Hrncir et al. 2013). According to Willmer and Stone (2004), from sunrise until 08:00, many flowers release nectar, which leads to a high number of bees foraging during this time. *G. thoracica* leaves the nest between 09:00–11:00 and 13:00–15:00, likely in search of nectar. Nectar is an essential resource for the bee colony, as a larval feed preparation and for mixing organic compounds within the nest. Worker bees will drop nectar into the honey pot (Oskin & Ovsyannikov 2019). *G. thoracica* leaves the nest between 16:00–18:00, likely to forage for nectar, as evidenced by the higher number of bees returning to the nest without pollen compared to those returning with pollen (Figure 2). The results of this study are consistent with the daily foraging activity of *Apis* sp. reported by Erwan (1999) in Nyalindung, Sukabumi, Indonesia, where foraging activity was higher between 16:00–18:00 than between 09:00–15:00. Although Erwan (1999) focused on *Apis* sp. rather than stingless bees such as *G. thoracica*, the similarity in peak foraging times suggests that environmental factors such as nectar availability and temperature may influence foraging behavior across different bee taxa. This supports the notion that both *Apis* sp. and stingless bees optimize their foraging schedules in response to external environmental conditions.

Foraging activity is carried out by worker bees to search for and locate forage sources, which are then communicated to other worker bees (Kartikasari et al. 2023). According to Sriagtula and Sowmen (2018), plants with varying flowering seasons can influence the availability of sufficient forage resources for bees throughout the year. Bees can harvest pollen and nectar to store in the nest when plants are abundant. The availability of forage in the nest is used to meet daily feeding needs and stored for times when forage resources are scarce in the field. The results presented in Table 1 support this behaviour, showing that *G. thoracica* collected pollen from a wide range of plant species, including 17 species from 12 families. This indicates that worker bees actively explore and communicate diverse floral sources to ensure colony nutritional needs are met. The predominance of herbaceous plants in the pollen spectrum suggests that these plants are more accessible and preferred due to factors such as flower density, blooming period, and proximity to the

nest. The diversity of pollen collected reflects effective recruitment and communication among foragers, consistent with the typical foraging strategies of stingless bees, which rely on pheromone trails and resource marking to guide nestmates to profitable floral patches. The difference in the number of individuals leaving and returning to the nest may be related to variations in foraging duration, task allocation among worker bees, or the loss of individuals during foraging trips. Some bees may spend longer periods outside collecting resources from distant or abundant floral patches, while others return earlier after shorter foraging bouts, leading to the observed discrepancy between outgoing and incoming counts (Gadhiya & Pastagia 2019).

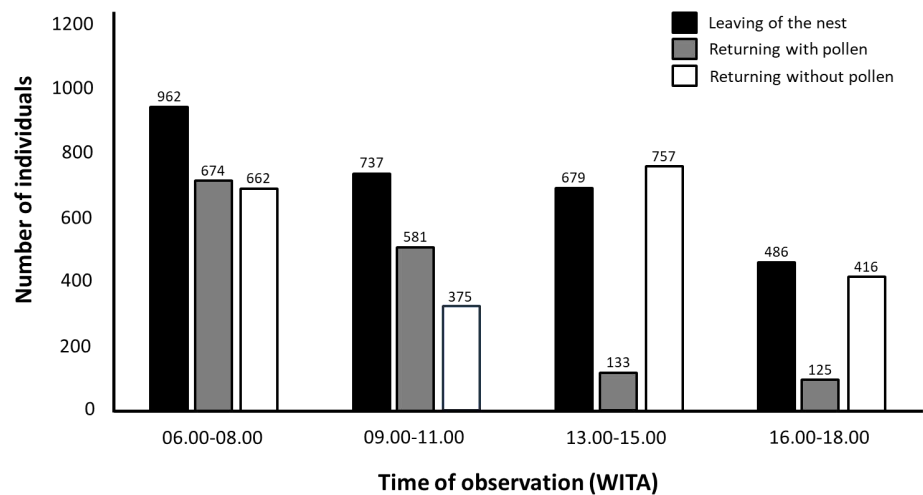


Figure 2 . Flight activities of *G. thoracica* workers.

The results of the General Linear Model (GLM) with a Gaussian distribution showed that light intensity, temperature, and humidity did not significantly affect the flight activity of *G. thoracica* leaving the nest ($P > 0.05$). These three environmental factors also had no significant effect on the activity of *G. thoracica* returning to the nest with pollen ($P > 0.05$). This outcome may be attributed to the behavioral flexibility and physiological adaptation of *G. thoracica* to the local environmental conditions in the study area. The stingless bee may have adjusted its foraging behavior to operate within a favorable microclimate range, thereby reducing the apparent impact of these environmental variables on certain activities. Additionally, stingless bees such as *G. thoracica* do not use the waggle dance to communicate foraging information like *Apis* sp. Instead, they rely on the use of pheromone trails and resource marking behaviours to recruit nestmates to profitable food sources (Hrncir et al. 2013). This pheromone-based recruitment system allows for efficient foraging even under variable environmental conditions, which may explain why light intensity and humidity showed no significant effect in this study. In contrast, temperature had a significant impact on the activity of *G.*

Table 1. Analysis of the influence of environmental factors on the activity of *G. thoracica*.

Environmental factors	Leaving of the nest		Returning with pollen		Returning without pollen	
	Coefisien estimate	P value	Coefisien estimate	P value	Coefisien estimate	P value
Light intensity	-4.81x10 ⁻⁶	0.99	3.12x10 ⁻⁴	0.53	-6.40x10 ⁻⁴	0.11
Temperature	-0.93	0.60	-2.39	0.11	2.41	*0.04
Humidity	0.09	0.92	0.51	0.63	0.02	0.95

* Significant at $P < 0.05$ (general linear Gaussian model test).

thoracica returning to the nest without pollen ($P = 0.04$), possibly due to the energetic cost of flight increasing under high temperature stress.

The stingless bees foraging activity decreased as a result of the daytime rise in ambient temperature. Erwan et al. (2020) stated that temperature is one of the key factors influencing the activity of *Trigona* species both inside and outside the nest. Bees instinctively regulate their flight based on energy efficiency, and as ambient temperature rises, the energy demand for flight increases (Ulanda et al. 2024). In contrast, light intensity and humidity showed no significant effect on the foraging activity of *G. thoracica* in this study. This may be due to the bees' behavioral adaptation to local microclimatic conditions near the nest entrance, which remain relatively stable throughout the day, especially in shaded or semi-forested environments where stingless bees typically nest.

Worker bees of *G. thoracica* will stay at the entrance of the nest to warm up their bodies before flying. If environmental conditions do not permit leaving the nest, such as during rain or strong winds, the worker bees will not engage in activities to meet the needs within the nest (Stabentheiner et al. 2007). Among the many worker bees, some are tasked with collecting nectar, some with collecting pollen, and others with collecting both pollen and nectar simultaneously (Budiarsa et al. 2023). Pollen collection activity is more than resin collection in the morning, but when it is noon resin collection activity is more than pollen collection. This is because in the morning generally available pollen is more than in the afternoon. Peak foraging activity in *Trigona* spp. in Serawak (Malaysia) occurs at 10:30 am. Worker bees carry more pollen in the morning (07:30) than nectar, but in the afternoon (14:30), workers carry more nectar than pollen (Nagamitsu & Inoue 2002). The highest activity of *T. laeviceps* in strawberry plantations occurs at 13:30-14:00 WIB (Harahap et al. 2013).

Foraging activities for pollen, nectar, and water depend on weather conditions and the colony needs. Air temperature, humidity, and light intensity are widely recognized as environmental factors that can influence bee foraging activity. However, in this study, the General Linear Model (GLM) results showed that light intensity and humidity did not significantly affect the foraging activity of *G. thoracica* ($P > 0.05$). This apparent contradiction may be explained by differences in species-specific responses and local environmental stability. While studies on other stingless bee species, such as *Tetragonula laeviceps* (Harahap et al. 2013), have shown variable correlations between weather factors and foraging behavior, *G. thoracica* in this study area may be well adapted to microclimatic conditions near the nest that buffer against fluctuations in light and humidity. Furthermore, stingless bees rely more on pheromone trails and spatial memory than on visual cues, which could reduce their dependence on external light conditions. Similarly, high humidity may not pose a limitation under stable tropical conditions where relative humidity remains within an acceptable range for foraging. Therefore, while humidity and light intensity are recognized as influential factors in broader ecological contexts, their effects may not always be significant depending on species behaviour, nest location, and environmental constancy.

In this study, the number of *G. thoracica* individuals leaving the nest was consistently higher than the number returning, especially during peak foraging hours. This discrepancy may be attributed to several factors (Figure 2). Firstly, foraging bees may spend varying amounts of time outside the nest depending on the availability and distance of floral resources. As a result, the number of returning individuals at any given time may not directly correspond to those that previously departed. Secondly, environmental stressors such as high temperature during midday (as observed in this study) may reduce the success rate of foraging trips, causing bees to return without pollen

or prolong their foraging duration. This is supported by Ulanda et al. (2024), who noted that elevated ambient temperatures increase the energetic cost of flight, potentially reducing foraging efficiency. Additionally, some bees may engage in tasks other than pollen collection, such as scouting or water foraging, which might not be accounted for in pollen return observations. Similar findings were reported by Harahap et al. (2013), where stingless bees showed asynchronous departure and return patterns due to task specialization and environmental conditions. Thus, the observed difference between outgoing and returning foragers highlights the complex nature of stingless bee foraging behavior and the influence of temporal and environmental factors on their activity.

Bees and flowering plants share a mutualistic relationship in which plants provide nectar and pollen as food sources, while bees assist in the pollination of flowers (Agussalim et al. 2017). The factors that attract bees to visit flowers include color, scent, and the size of the pollen (Putri et al. 2025). Pollen is the primary protein source for bees, and its composition depends on the plant species producing it. High-nutrient pollen is crucial for the growth and physiological development of worker bees. The protein in pollen is essential for the development of potential queen bees, workers, and males. Adequate protein intake is important for the development of body tissues, muscles, and glands in worker bees. Worker bees must collect pollen from a variety of plants because the nutritional content of each pollen type varies, thus meeting the protein needs of the entire colony (Thakur & Nanda 2020). According to observations by Asma et al. (2019), 25 % of honey bees leave the nest to forage are collecting pollen.

Based on morphological character observations of pollen, 17 species from 12 plant families were identified: Arecaceae, Cannaceae, Caricaceae, Compositae, Convolvulaceae, Leguminosae, Moraceae, Muntingiaceae, Myrtaceae, Poaceae, Solanaceae, and Rubiaceae (Table 2). This number is lower than the 26 plant species identified in a similar study on *G. thoracica* conducted at a beekeeping farm in Pesawaran Regency, Lampung Province (Sidik et al. 2022). The difference in pollen diversity may be attributed to regional variations in vegetation, land use, or flowering phenology. In both studies, herbaceous plants were the dominant source of pollen, indicating a consistent foraging preference among *G. thoracica* populations. Herbaceous plants typically have longer or staggered blooming periods, grow in large numbers in clustered habitats, and possess simpler floral structures that match the mouth morphology of stingless bees, making them easier and more efficient to forage (Sun & Frelich 2011; Dujardin et al. 2011). Moreover, these plants are often located closer to the ground and nearer to the nest, reducing energy costs during foraging.

Although the dominance of herbaceous plants was also reported in *G. thoracica* colonies in Banda Aceh (Putri et al. 2025), some studies, such as Agussalim et al. (2017), found greater use of tree pollen, particularly in agroforestry environments. This variation suggests that floral resource composition is highly influenced by local habitat types. Furthermore, Sidik et al. (2022) recorded pollen from families such as Euphorbiaceae and Piperaceae, which were absent in the present study, reinforcing the idea that landscape diversity and sampling season significantly affect the range of floral sources. The number and variety of plants visited by stingless bees influence the type of honey produced: bees that forage on diverse species tend to produce multifloral honey, whereas those foraging on limited sources may produce monofloral honey with a dominant pollen type (Chauhan et al. 2017). Overall, these findings confirm that while stingless bees generally prefer herbaceous plants, the exact pollen profile they collect is shaped by environmental context and local floral availability.

Table 2. Types of pollen collected by *G. thoracica*.

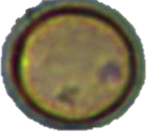
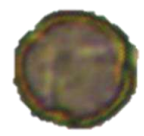
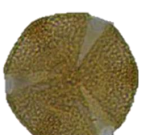
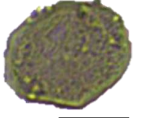
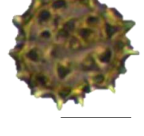
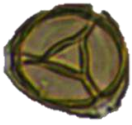
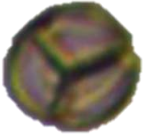
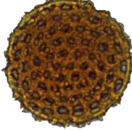
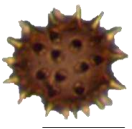
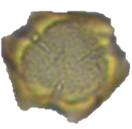
Plant species	Family	Habitus	Size (µm)	Aperture	Exine ornamentation	Predominant pollen type	Polar view
<i>Artocarpus</i> sp.	Moraceae	Tree	20.40-40.65	Tricolporate	Echinate	Important Minor Pollen Type	
<i>Cocos nucifera</i> L.	Arecaceae	Tree	20.52-25.15	Tricolpate	Echinate	Important Minor Pollen Type	
<i>Carica papaya</i> L.	Caricaceae	Tree	20.63-24.02	Tricolpate	Reticulate	Important Minor Pollen Type	
<i>Caesalpinia pulcherrima</i> (L.) Sw.	Leguminosae	Tree	23.00-30.33	Tricolporate	Rugulate	Important Minor Pollen Type	
<i>Melaleuca leucadendra</i> (L.) L.	Myrtaceae	Tree	28.41-30.76	Tricolporate	Reticulate	Secondary Pollen Type	
<i>Muntingia calabura</i> L.	Muntingiaceae	Tree	15.22-20.64	Tricolpate	Reticulate	Secondary Pollen Type	
<i>Senna siamea</i> (Lam.) H.S. Irwin & Barneby	Leguminosae	Herb	20.45-30.71	Tricolporate	Echinate	Important Minor Pollen Type	
<i>Mimosa invisa</i> Colla	Leguminosae	Herb	14.66-25.10	Tricolporate	Echinate	Important Minor Pollen Type	
<i>Canna hybrida</i> L.	Cannaceae	Herb	28.40-30.42	Tricolpate	Reticulate	Important Minor Pollen Type	
<i>Bidens pilosa</i> L.	Compositae	Herb	22.10-30.50	Tricolporate	Echinate	Secondary Pollen Type	
<i>Spermacoce ocimoides</i> Burm.f.	Rubiaceae	Herb	15.63-25.55	Tricolporate	Reticulate	Secondary Pollen Type	
<i>Echinochloa colona</i> (L.) Link	Poaceae	Herb	26.74-35.44	Tricolporate	Rugulate	Important Minor Pollen Type	

Table 2. Types of pollen collected by *G. thoracica*.

Plant species	Family	Habitus	Size (µm)	Aperture	Exine orna- mentation	Predomina nt pollen type	Polar view
<i>Solanum melongena</i> L.	Solanaceae	Herb	18.50-26.07	Tricolpate	Reticulate	Secondary Pollen Type	
<i>Mimosa pudica</i> L.	Leguminosae	Herb	15.10-25.72	Tricolporate	Echinate	Important Minor Pollen Type	
<i>Ipomoea obscu- ra</i> (L.) Ker Gawl.	Convolvu- laceae	Herb	25.62-30.91	Tricolpate	Reticulate	Important Minor Pollen Type	
<i>Cosmos sulphu- reus</i>	Compositae	Herb	20.00-32.61	Tricolpate	Reticulate	Secondary Pollen Type	
<i>Capsicum annu- um</i> L.	Solanaceae	Herb	19.25-25.35	Tricolporate	Reticulate	Secondary Pollen Type	

In conclusion, this study shows that although the activity of *G. thoracica* flying out of the nest did not differ significantly throughout the observation period, peak activity occurred in the morning between 06:00 and 08:00, when many individuals were foraging for pollen. Nectar foraging was also observed during other periods, with lower peaks in the afternoon. Environmental factors such as temperature, humidity, and light intensity did not significantly affect the flight activity of *G. thoracica*, except for temperature, which influenced the activity of returning without pollen. The diversity of plants visited by *G. thoracica* was dominated by herbaceous plants, which are more accessible and have longer blooming periods, contributing to the diversity of pollen collected and the type of honey produced. The mutualistic relationship between *G. thoracica* and flowering plants supports the pollination process and ensures a sustainable food resource for the stingless bees.

AUTHORS CONTRIBUTION

M.T. collected and analysed the data and wrote the manuscript. H.P. analysed the data and wrote the manuscript. H.P., S.S, and T.A. designed the research and supervised all the process.

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CONFLICT OF INTEREST

There is no conflict of interest regarding the research or the research funding.

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Research Article

Micropropagation of Superior Variety of Vanilla (*Vanilla planifolia* Andrews) Through *In Vitro* Indirect Organogenesis

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ABSTRACT

Two varieties of *Vanilla planifolia*, var. Sovania Agribun and Hivania Agribun, were nationally released in 2021. These varieties are categorised as superior due to their greater resistance to stem rot diseases compared to existing varieties. Micropropagation is a viable method for producing disease-free vanilla seedlings. This study aimed to identify the optimal combination of auxin and cytokinin for callus initiation and regeneration into plantlets of the superior vanilla variety, Hivania Agribun. The research used a completely randomised design with two factors: explant type and hormone combinations. A combination of 2,4-D, at a higher concentration than kinetin, was used to induce callusing, while a combination of 2,4-D with a higher concentration of kinetin was applied for callus proliferation; both steps were performed in the dark. The treatments with 2,4-D (0.5–1.5 mg L⁻¹) and kinetin (0.1 mg L⁻¹) were most effective in inducing callus formation on the first node and apical shoot explants. The combination of 2,4-D (0.1 mg L⁻¹) and kinetin (0.5–1 mg L⁻¹) was effective for callus proliferation and the formation of organogenic callus. Organogenic callus regeneration was achieved under illumination, with the medium containing BAP (0.5 mg L⁻¹) producing the most abundant regenerating shoot buds and shoots.

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INTRODUCTION

Propagation of vanilla plants is typically done vegetatively using tendril cuttings. However, propagating plants by that way requires a large amount of plant material, especially if extensive planting or rejuvenation is needed. Furthermore, according to Hu et al. (2019), long-term propagation by cuttings can lead to genetic erosion, which resulted in low genetic diversity and reduced the ability of vanilla plants to cope with environmental changes (Elestad et al. 2022). Another issue is disease susceptibility, particularly to pathogenic fungi. *Fusarium oxysporum* f.sp. *vanillae* has long been identified as the most dominant fungus responsible for causing stem rot diseases (SRD) (García et al. 2015).

In 2021, we under the Indonesian Agricultural Research and Development Agency released two varieties of *Vanilla planifolia*, named Sovania Agribun and Hivania Agribun. These varieties are classified as superior due to their higher resistance to SRD compared to existing varieties. The Official Letter of Decision of the Republic Indonesia Minister of Agriculture No. 115/Kpts / KB.020 / 12/ 2022 mentioned that *V. planifolia* var. Hivania Agribun was the result of hybridisation between the parents Vania 1 and Vania 2, both previously developed by the Research Institute for Spices and Medicinal Plants (Balittro) in Bogor. The vanillin content of Hivania Agribun is 2.87 %. Its unique characteristics include straight pods of equal size from base to tip, small flowers, and leaves positioned at a 45° angle to the stem/vine. This variety exhibits stable growth and is capable of adapting to a wide range of environmental conditions (Shukri 2023).

In vitro plant multiplication can serve as an alternative method to increase vanilla seed production. The balance between auxin and cytokinin is known to regulate callus formation and organogenesis (Zhao et al. 2021). The auxin 2,4-dichlorophenoxyacetic acid (2,4-D) is used to induce callus initiation by promoting the dedifferentiation of explant cells through cell division (Özkul et al. 2016; Anhuñi et al. 2023). In general, cytokinin 6-furfurylaminopurine (kinetin) stimulates cell division and controls cell morphogenesis, while BAP (6-benzylaminopurine) is commonly used to promote shoot development and to increase the percentage of shoot regeneration from callus (Hardjo et al. 2023). In *Vanilla planifolia* Vania 1, Ajijah et al. (2010) achieved callus induction rates of up to 60 % using 0.5 mg L⁻¹ kinetin combined with 2–3 mg L⁻¹ 2,4-D. Meanwhile, Erawati et al. (2021) reported that a combination of 0.5 mg L⁻¹ BAP and 2 mg L⁻¹ kinetin was effective in enhancing shoot proliferation from nodal cultures of vanilla.

This study aims to identify the optimal combination of auxin and cytokinin for callus formation and its regeneration in the propagation of the Hivania Agribun variety of vanilla.

MATERIALS AND METHODS

Plant Materials

The genetic material used was stem cuttings of *Vanilla planifolia* (Andrews) var. Hivania Agribun. It belongs to the Research Institute for Spices and Medicinal Plants (ISMCRI). Shortcuttings of one single leaf node were planted on a sandy planting medium in polybags and maintained in a greenhouse. They were sprayed with fungicide Propineb 70 % (2 g L⁻¹) every two days, three times starting from one week before new shoots were collected for tissue culture works. It was done as a standard procedure to minimise fungal contamination.

Methods

Explant Sterilisation

Explant material was taken by cutting the 3rd segment of the new shoot. The leaves on the 1st and 2nd nodes were removed, leaving the base of the leaf.

External sterilisation was done by soaking the explants in a liquid detergent solution for 30 minutes. The next procedure followed Ajijah et al. (2010) with some improvements. The explants were then rinsed in running tap water and soaked again in a solution of 70 % Propineb fungicide (2 g L⁻¹) and 20 % Streptomycin sulfate bactericide (2 g L⁻¹) for 60 minutes, respectively. The explants were then rinsed with sterile distilled water before taking them to the sterilisation stage in the laminar airflow hood. The explants were then soaked successively in 70 % ethanol for 1 minute, 20 % NaOCl, 10 % NaOCl, and 10 % Iodopovidon solution for 5 minutes each. This sterilisation ended with rinsing the explants three times using sterile distilled water in every successive sterilisation process.

Callus Induction

Sterilised explants were divided into three types: the apical shoot tip, the first node, and the second node. Each explant was slightly wounded in several areas and cultured horizontally on MS basal medium with nine different treatments—one control and eight combinations of plant growth regulators (PGRs). The treatments used in this study were adapted from the work of Ajijah et al. (2010) on the Vania 1 variety. The combinations consisted of 2,4-D (0.5; 1.0; 1.5; 2.0 mg L⁻¹) and kinetin (0.1; 0.3 mg L⁻¹). Each treatment consisted of 3 types of explants, each with seven replications. Each replication in a 75 mL glass vessel contained one explant. The cultures were placed in a dark room with a temperature of ± 25 °C. The parameters observed were the time of callus' first appearance, the percentage of explants producing callus, and the visual callus texture.

Callus Proliferation

The callus originated from the best induction media, giving rise to viable (greenish-white) callus, was multiplied by slicing it into several portions, 0.4 to 0.6 cm in diameter, and each portion was cultured on a growing medium with a combination of 2.4 D (0.1 mg L⁻¹) and increased kinetin concentrations (0.5; 1.0; 1.5 mg L⁻¹). Each treatment combination was made in five replications; each replication consisted of two calli. The cultures were maintained in the dark. The parameters observed were the visual shape of the callus and the increase in callus volume by measuring every week the length, width, and height (p x l x t) compared to the initial size of the callus portion.

Adventitious Shoot Regeneration

Callus from the best-performing treatments were transferred to the regeneration stage. The selection criteria for optimal callus were based on visual assessment, including viability, texture (compact to slightly friable), and a diameter of ≥ 0.7 cm. Each callus cluster was divided and subcultured in 3 regeneration media, which was MS supplemented with BAP (0.1; 0.3; 0.5 mg L⁻¹), instead of kinetin. Four replicates for each treatment, and each replicate consisted of two callus clumps. The cultures were kept in a dark room for the first week, then moved under the light at 800-1100 lux for 16/24 hours. The parameters observed were the initial shoot emergence time and the average number of shoots and shoot buds per callus explant.

Histology Incision

The incision was made by cutting the callus samples radially using a razor blade. The incision results were placed on a glass slide, given a drop of distilled water, then covered with a slip glass. Samples were observed using a compound light microscope (Olympus CX33) and photographed with an Indomikro HDMI camera at 40 - 100x magnification.

Data analysis

The results of visual observations of the callus were analysed descriptively, while the quantitative data were statistically analysed using a factorial completely randomised design (CRD) with a Two-Way Analysis of Variance (ANOVA). If significant differences were found, the analysis was followed by the Duncan's Multiple Range Test (DMRT) at a 5 % level of confidence, using Statistical Product and Service Solutions (SPSS) software, version 26.0.

RESULTS AND DISCUSSION

Callus Induction

Callus formation began with the swelling of the explant on the induction medium. This swelling occurred as a result of the explant's absorption of nutrients and water from the medium. Wounding the explant triggered callus formation as a natural response of the tissue to heal the wound. Callus formation was first observed between the second and fifth week after planting. The calli were primarily compact and yellow. Statistically, both the explant type and the combination of 2,4-D with kinetin significantly influenced callus formation, although no interaction between these two factors was observed (Table 1). The best explant types for inducing callus were the first node and the shoot tip, with average percentages of 46.03 % and 42.86 %, respectively.

Table 1. Percentage of explants *Vanilla planifolia* var. Hivania Agribun producing callus in various combinations of 2,4-D and kinetin according to explant type*

Induction treatment**	% explants producing callus			Average
	Shoot tip	1 st node	2 nd node	
DK0 (D0Kin0)	0	14.29	0	4.76 ^b
DK1 (D0.5Kin0.1)	71.43	57.14	0	42.86 ^a
DK2 (D0.5Kin0.3)	28.57	42.86	42.86	38.10 ^a
DK3 (D1Kin0.1)	57.14	71.43	0 0.00	42.86 ^a
DK4 (D1Kin0.3)	28.57	57.14	14.29	33.33 ^{ab}
DK5 (D1.5Kin0.1)	71.43	28.57	42.86	47.62 ^a
DK6 (D1.5Kin0.3)	57.14	71.43	14.29	47.62 ^a
DK7 (D2Kin0.1)	42.86	28.57	28.57	33.33 ^{ab}
DK8 (D2Kin0.3)	28.57	42.86	0	23.8 ^{ab}
Average	42.86 ^a	46.03 ^a	15.87 ^b	

*31 weeks after planting (WAP). **D=2,4-D at 0, 0.5, 1.0, 1.5, and 2.0 mg L⁻¹; Kin=kinetin at 0, 0.1, 0.3 mg L⁻¹. The numbers followed by the same letter in the same column and row indicate not significantly different according to the DMRT at $\alpha = 5$ %.

The percentage of callus formation in Hivania Agribun was observed in response to treatments with 2,4-D at concentrations of 0.5–1.5 mg L⁻¹, in combination with kinetin at 0.1 and 0.3 mg L⁻¹. The combination of 2 mg L⁻¹ 2,4-D with the same kinetin concentrations resulted in a lower percentage of callus formation. The addition of 2,4-D to the induction medium enhances cell wall permeability, inducing dedifferentiation, stimulating swelling, and altering callus color (Yaowachai et al. 2023). The presence of kinetin contributes to increased callus cell proliferation and differentiation through cell division (Sankepally & Singh 2016). Several callus turned brown after a few weeks in the induction medium. Tissue browning can be attributed to the oxidation of phenolic compounds by polyphenol oxidase (PPO) activity (Pompili 2023). The accumulation of oxidised phenolic compounds damages the cells, leading to cell death (I'anutushshoimah et al. 2020).

Callus Proliferation

The calli developed from the seven induction treatments were transferred to the callus proliferation stage. Rapid callus growth occurred between the third

and eighth weeks after subculture. The yellow, compact-textured callus continued to proliferate, and its yellow color became paler. Statistical analysis of variance showed that the origin of the callus (induction media) and the combinations of 2,4-D with kinetin at high concentrations (proliferation stage) had no significant effect on callus enlargement, nor did the interaction between treatments. However, the data presented in Figure 1 show that the most significant increase in volume occurred in the DK1 callus, which received treatment c (0.1 mg L^{-1} 2,4-D + 1.5 mg L^{-1} kinetin), followed by the DK7 callus, which received treatment b (0.1 mg L^{-1} 2,4-D + 1.0 mg L^{-1} kinetin).

Within ten weeks of observation, it was found that the combination of 2,4-D (0.1 mg L^{-1}) and elevated kinetin concentrations (0.5 , 1.0 , 1.5 mg L^{-1}) produced proliferative callus, as evidenced by an increase in callus size. A lower auxin level combined with high cytokinin levels can synergistically stimulate optimal callus cell division and shoot regeneration (Ramabulana et al. 2021). Ajijah et al. (2010) reported much higher concentration of 2,4-D ($2\text{--}3 \text{ mg L}^{-1}$) in combination with 0.5 mg L^{-1} kinetin that provided the most prolific calli in Vania 1 variety. Vania 1 is one of the parent plants from which Hivania Agribun was derived. At this stage, we observed that some calli became crumbly, but most maintained a white-yellow, compact texture with a smooth white surface. This white surface later transformed into a more watery callus.

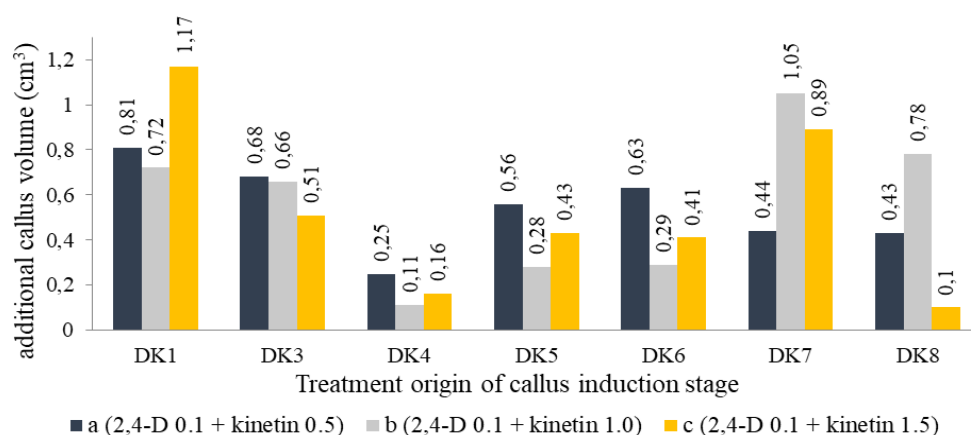


Figure 1. Increase in callus volume of *Vanilla planifolia* var. Hivania Agribun in the proliferation media (a, b, c) based on the origin of the induction media at 10 weeks after planting (WAP).

The callus produced at this stage emitted a fragrant aroma, which may indicate the presence of specific secondary metabolites of vanilla. According to Efferth (2019), the formation of secondary metabolites can be influenced by external factors, such as the pH of the culture medium, temperature, light, and culture aeration. Maintaining cultures in agar media containing certain nutrients and vitamins can also act as a trigger. High concentrations of ammonium ions or low phosphate levels stimulate the formation of secondary metabolites in *in vitro* cultures (Efferth 2019). The presence of oxidative stress, pathogen attacks, and physical and chemical stimuli serves as an elicitor to modulate gene expression and induce enzyme synthesis, which promotes the production of secondary metabolites, such as flavonoids, terpenoids, alkaloids, phenylpropanoids, and polypeptides (Al-Khayri et al. 2023), as demonstrated by Hasibuan et al. (2021), who reported that a *Diaporthe* fungal filtrate-derived elicitor enhanced quinidine production in Quina cell cultures.

Adventitious Shoot Regeneration

During the regeneration stage, the callus color changed to green, indicating the formation of chlorophyll in the presence of light, a process also promoted by cytokinin (BAP). Light is an abiotic factor that significantly affects plant

morphogenesis and growth. In addition to its role in photosynthesis, light can influence the expression of genes that control plant metabolism, which can impact growth and development, a process known as photomorphogenesis (Weremczuk et al. 2021). Figure 2 illustrates the callus regeneration into shoots.

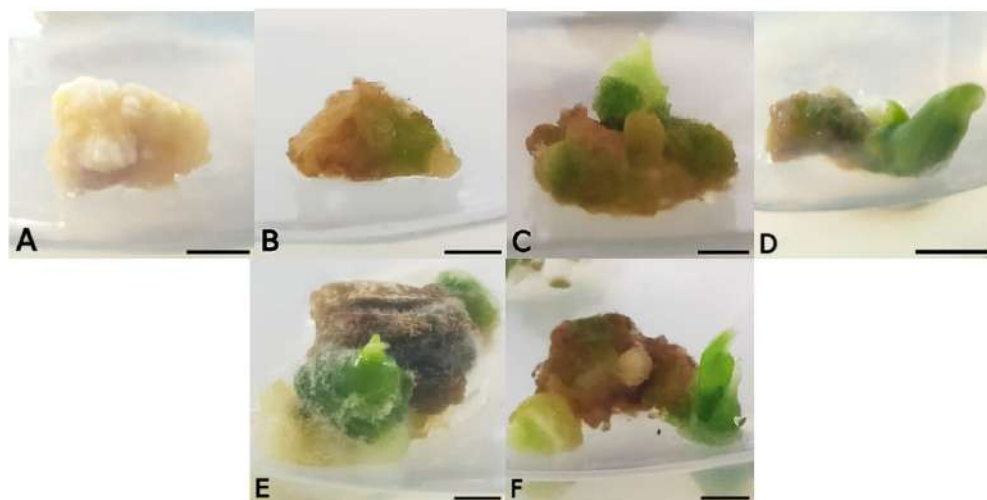


Figure 2. Callus regeneration of *Vanilla planifolia* var. Hivania Agribun to adventitious shoots on the media with BAP. (A) Callus on the day of subculture; (B) Callus turning green; (C - F) Adventitious shoots from callus, aged 4 WAP in regeneration stage. Scale = 5 mm.

According to Cortleven and Schmuelling (2015) and Cortleven et al. (2016), cytokinins directly regulate chlorophyll biosynthesis genes through the process of light harvesting during the transition from etioplasts to chloroplasts. Signals from cytokinin receptors activate the expression of the HEMA1, and the other genes. These genes encode the enzyme GluTR (Glutamyl-tRNA Reductase), which catalyzes the synthesis of 5-aminolevulinic acid (ALA). ALA is then converted into protochlorophyllide. The presence of light will activate NADPH-dependent protochlorophyllide oxidoreductase, facilitating the regeneration of chlorophylls. The formation of chlorophyll, beginning with ALA synthesis, marks the initiation of the phytochromobilin pathway, which originates from phytochrome apoproteins (Sanchez-Barcelo et al. 2016). Phytochromes are synthesised in the cytosol; the inactive form, Pr, acts as a photoreceptor that mediates photomorphogenic responses to red light. Upon exposure to light, Pr is converted to the active Pfr form, which then moves to the nucleus. The photoactivated phytochrome suppresses the activity of constitutive photomorphogenic 1 (COP1), thereby promoting photomorphogenesis (Sanchez-Barcelo et al. 2016).

BAP in combination with NAA or 2,4-D promotes callus induction and multiplication, stimulates shoot emergence, and increases the number of proliferated shoots and shoot length in vanilla (Chai et al. 2024) and in Phalaenopsis orchid (Sarmah et al. 2024). In another herbaceous plant, *Curculigo orchoides*, Umar et al. (2025) reported the highest callus formation and regeneration when using a combination of BAP and IBA. Other auxins, such as 2,4-D and picloram, resulted in lower culture development. According to the statistical analysis of variance, there was an interaction between the previous treatments (induction + proliferation media) and various concentrations of BAP (Table 2). The callus treatments DK3(a)B 0.5 and DK1(c)B 0.5 resulted in the highest number of shoots (36.25 and 26.25, respectively), with DK1(c)B 0.5 being the most effective in producing potential buds that developed into shoots (28.75). The development of adventitious shoots on the callus is shown in Figure 3.

Table 2. Regeneration of adventitious shoots of *Vanilla planifolia* var. Hivania Agribun as influenced by the previous treatments on callus and BAP, observed eight weeks after subculture.

Previous callus treatment	BAP concentration (mg L ⁻¹)	Average Number of shoots	Average Number of shoot buds	Shoot emergence time (weeks)
DK1(a)	B _{0.1}	1.25 ^{eph}	0.00 ^c	7
	B _{0.3}	2.50 ^{def}	0.00 ^c	5
	B _{0.5}	3.75 ^{def}	11.25 ^{bc}	5 – 8
DK1(b)	B _{0.1}	0.00 ^f	0.00 ^c	—
	B _{0.3}	5.00 ^{def}	0.00 ^c	4 – 6
	B _{0.5}	17.50 ^{bcd}	17.50 ^b	4 – 5
DK1(c)	B _{0.1}	0.00 ^f	0.00 ^c	—
	B _{0.3}	3.75 ^{def}	0.00 ^c	4 – 5
	B _{0.5}	26.25 ^{ab}	28.75 ^a	4 – 6
DK3(a)	B _{0.1}	16.25 ^{bcd}	7.50 ^{bc}	3 – 8
	B _{0.3}	3.75 ^{def}	1.25 ^c	4
	B _{0.5}	36.25 ^a	7.50 ^{bc}	2 – 7
DK3(b)	B _{0.1}	1.25 ^{ef}	0.00 ^c	7
	B _{0.3}	20.00 ^{bc}	7.50 ^{bc}	2 – 7
	B _{0.5}	0.00 ^f	0.00 ^c	—
DK3(c)	B _{0.1}	1.25 ^{eph}	0.00 ^c	7
	B _{0.3}	0.00 ^f	0.00 ^c	—
	B _{0.5}	0.00 ^f	0.00 ^c	—
DK5(a)	B _{0.1}	3.75 ^{def}	0.00 ^c	6 – 7
	B _{0.3}	20.00 ^{bc}	12.50 ^{bc}	4 – 5
	B _{0.5}	11.25 ^{cdef}	2.50 ^c	4 – 8
DK5(b)	B _{0.1}	1.25 ^{eph}	1.25 ^c	6
	B _{0.3}	0.00 ^f	0.00 ^c	—
	B _{0.5}	2.50 ^{def}	0.00 ^c	4
DK5(c)	B _{0.1}	0.00 ^f	1.25 ^c	8
	B _{0.3}	2.50 ^{def}	2.50 ^c	4
	B _{0.5}	11.25 ^{cdef}	1.25 ^c	4 – 8
DK7(a)	B _{0.1}	0.00 ^f	0.00 ^c	—
	B _{0.3}	0.00 ^f	0.00 ^c	—
	B _{0.5}	0.00 ^f	0.00 ^c	—
DK7(b)	B _{0.1}	0.00 ^f	0.00 ^c	—
	B _{0.3}	0.00 ^f	0.00 ^c	—
	B _{0.5}	5.00 ^{def}	0.00 ^c	6 – 8
DK7(c)	B _{0.1}	0.00 ^f	0.00 ^c	—
	B _{0.3}	0.00 ^f	0.00 ^c	—
	B _{0.5}	0.00 ^f	0.00 ^c	—

The numbers followed by the same letter in the same column indicates that it is not significantly different according to the DMRT at $\alpha = 5\%$.

The ANOVA revealed significant effects of BAP alone on the number of shoots. The highest number of shoots and shoot buds was obtained with BAP at 0.5 mg L⁻¹ (Table 3). Callus regeneration also depends on the condition of the callus at the time it was first cultured. Two regeneration pathways are recognized in *in vitro* culture: organogenesis and somatic embryogenesis. The latter is more common in woody plants, such as *Falcataria moluccana* (Sunandar et al. 2017) and *Coffea arabica* (Avila-Victor et al. 2023). In this study, regeneration followed the organogenetic pathway. The condition of the callus, depending on which part of the source it was cut from, influences its capacity to respond during the regeneration stage. Most DK7 (D2Kin0.1) calli (Table 2) turned brown and showed no proliferation or differentiation. The failure to regenerate shoots from DK7 callus may be due to the high levels of 2,4-D (2 mg L⁻¹) and cytokinin (kinetin and BAP), which resulted in the accumulation of phenolic compounds when the callus was exposed to light.

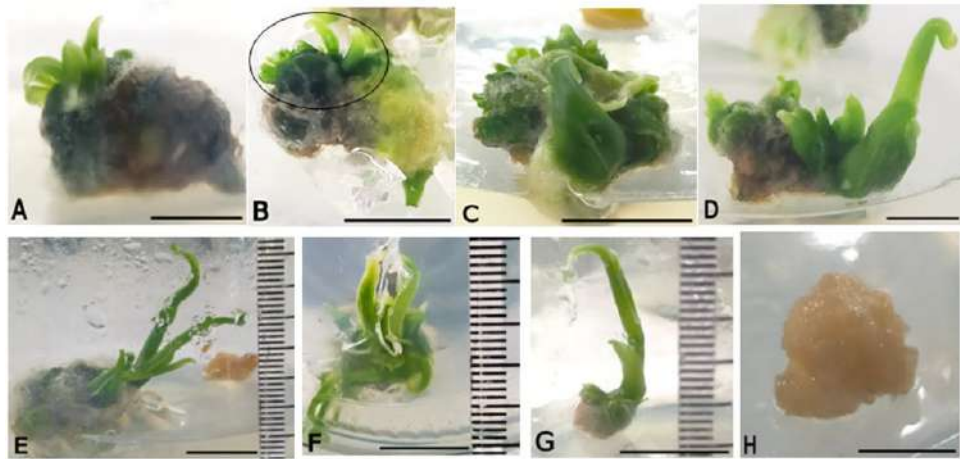


Figure 3. Growth and development of adventitious shoots of *Vanilla planifolia* var. Hivania Agribun on regeneration media, eight weeks after subculture. Developing shoots in the treatments DK1(c)B0.5 (A), DK1(a)B0.5 (B), DK1(b)B0.5 (C), DK3(a)B0.5 (D), elongating shoots (E-G), non-organogenic callus (H). Scale = 10 mm.

BAP is the most commonly used cytokinin in *in vitro* plant multiplication. Erawati et al. (2021) achieved the highest number of shoots through direct organogenesis from vanilla nodal cultures using a combination of 0.5 mg L⁻¹ BAP and 2 mg L⁻¹ kinetin. Similarly, Rahmah et al. (2023) reported the highest percentage of nodal explants developing shoots, as well as the greatest shoot number in the Vania 2 variety, when cultured with 1.5 mg L⁻¹ BAP and 0.1 mg L⁻¹ IBA.

Table 3. Average number of shoots and shoot buds regenerated from callus of *Vanilla planifolia* var. Hivania Agribun based on BAP concentration.

BAP Treat- ment	Number of shoots	Number of shoot buds	Shoot emergence time (weeks)
B _{0.1}	2.08 ^b	0.83 ^b	3 – 8
B _{0.3}	4.79 ^b	1.98 ^b	2 – 7
B _{0.5}	9.48 ^a	5.73 ^a	2 – 8

The numbers followed by the same letter in the same column indicates that it is insignificantly different according to the DMRT test at α = 5 %.

Histological Observation

Observations of some incised callus at 40x magnification showed that they are green, indicating the presence of chlorophyll in the regenerating callus cells (Figure 4A). The shoot meristem contained some leaf primordia, and vascular tissue had differentiated. In contrast, chlorophyll was absent in the non-organogenic area of the callus, which had a compact, slightly crumbly texture and was pale white (Figure 4B). However, this callus exhibited some areas with developing shoots in the meristematic zone.

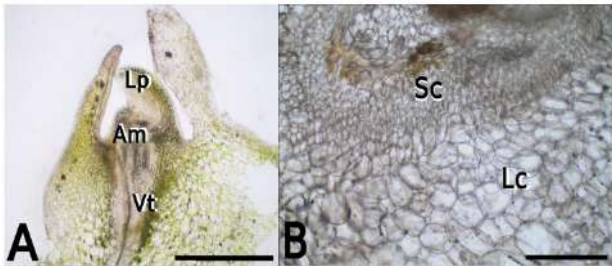


Figure 4. Histological sections of shoot and callus. Structure of adventitious buds from regenerating callus (A), non-regenerated callus (B). Lp = Leaf primordia, Am = Apical meristem, Vt = Vascular tissue, Sc = Small cells (meristematic), and Lc = Large cells (non-organogenic). Scale = 100 μm (A), 50 μm (B).

According to Hernandez et al. (2020), the organogenic structure of vanilla callus begins with callus proliferation, forming a heterogeneous structure with two main areas: broad, vacuolated cells (non-organogenic) and small, well-demarcated cells with very dense nuclei, characteristic of meristematic cells. These latter cells then develop into shoot apical meristems, forming a meristematic dome covered by young leaf primordia. During the regeneration stage, several peripheral areas of the organogenic callus will also form mitotic zones, differentiating into additional shoot apical meristems.

CONCLUSION

The shoot tip and first node are the most responsive explants. The combination of the auxin 2,4-D at higher concentrations (0.5–1.5 mg L⁻¹) with the cytokinin kinetin (0.1 mg L⁻¹) can induce callus formation in vanilla plants. The combination of a lower concentration of 2,4-D (0.1 mg L⁻¹) and elevated kinetin (0.5–1 mg L⁻¹) stimulates callus proliferation and accelerates organogenic callus formation. Single BAP at 0.5 mg L⁻¹ was the most effective for regenerating shoots on callus that had been previously treated with 0.5–1 mg L⁻¹ 2,4-D and 0.5–1.5 mg L⁻¹ kinetin during callus induction and proliferation. These results will support the distribution of this superior vanilla variety to farmers in order to increase the quality and quantity of vanilla bean production in Indonesia.

AUTHOR CONTRIBUTION

E.H. initiated, designed, and supervised the research, and prepared the manuscript; M. carried out all the lab works and wrote the final report; D.R. designed, supervised and revised the manuscript; A.S. analysed the data and R.Y. wrote and arranged the manuscript.

CONFLICT OF INTEREST

All the authors declare that there is no competing interest or financial relationship that could be construed as a potential conflict of interest.

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Research Article

Exploring Phylogenetic Relationships Among Some Bamboo Genera in Indonesia Using cpDNA *trnL-trnF* Intergenic Spacer Sequences: An In-silico Analysis

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ABSTRACT

Classifying bamboo genera remains challenging. DNA barcoding, using cpDNA *trnL-trnF* intergenic spacer (IGS) region, offers a promising solution. This study evaluated *trnL-trnF* IGS region's ability to construct phylogeny relationships among various bamboo genera using in-silico analysis. An in-silico analysis of various Indonesian bamboo genera (*Bambusa*, *Chloothamnus*, *Dendrocalamus*, *Dinochloa*, *Gigantochloa*, *Neololeba*, *Schizostachyum*, and *Thyrsostachys*) was performed using data from the National Center for Biotechnology Information. Phylogenetic analysis was conducted using Neighbor-Joining, Maximum Parsimony, and Maximum Likelihood methods (bootstrap = 1000), with *Widjajachloa* as an outgroup. Clustering patterns were visualized with a heatmap. Morphological traits were examined for comparison with the phylogenetic results. Neighbor-Joining analysis failed to differentiate bamboo genera. Maximum Parsimony analysis identified three distinct clades: *Dinochloa*, *Schizostachyum*, and "BDG+CNT" (comprising *Bambusa*, *Dendrocalamus*, *Gigantochloa*, *Chloothamnus*, *Neololeba*, and *Thyrsostachys*). *Dinochloa* and *Schizostachyum* clades exhibited strong phylogenetic support, while the "BDG+CNT" clade showed limited resolution. Maximum Likelihood analysis also identified three distinct clades: *Chloothamnus*, *Schizostachyum*, and "BDG+DNT" (comprising *Bambusa*, *Dendrocalamus*, *Gigantochloa*, *Dinochloa*, *Neololeba*, and *Thyrsostachys*). *Schizostachyum* clade exhibited strong phylogenetic support, while the *Chloothamnus* and "BDG+DNT" clades showed limited resolution. Morphologically, *Dinochloa* are climbing bamboos that differ from typical bamboos. *Schizostachyum* possess long internodes compared to the short of the "BDG+CNT" or "BDG+DNT" clades. Heatmap analysis revealed a distinct clustering pattern for *Schizostachyum* compared to other genera. The *trnL-trnF* IGS region cannot fully distinguish all bamboo genera in the phylogenetic tree, although it has the potential to differentiate genera such as *Dinochloa* and *Schizostachyum*, which aligns with their easily recognizable morphological characteristics.

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INTRODUCTION

Bamboo belongs to the family Poaceae (grasses) and the subfamily Bambusoideae (Ritonga et al. 2023). Bamboo exhibits interesting characteristics as it can either be clumping or running (dispersed) (Damayanto 2024). Bamboos with sympodial rhizomes tend to grow in clumps, while those with monopodial rhizomes grow in a dispersed (Widjaja 2001). The bamboo culms in Indonesia are generally cylindrical, segmented, and hollow, featuring hard, woody walls (Damayanto 2024). Each node of a bamboo culm can produce branches that bear leaves. Each leaf on the bamboo has a leaf sheath that typically includes auricles and a ligule, although sometimes the auricles are not clearly visible (Ritonga 2023). Inflorescences of bamboo are spikelets (Janzen 1976). The fruits of most bamboo are caryopsis (dry fruit), occasionally with a thickened pericarp resembling a berry (McClure 1966; Wong 2004).

Naturally, bamboo can be found on every continent in the world except Europe and Antarctica (Clark et al. 2015). In Indonesia, bamboo can be found in almost all regions (Widjaja 2001). Bamboo can grow from lowlands to mountainous areas at elevations of up to 4300 metres above sea level (asl) (Clark 2006). Based on the author's (IPGPD) exploration across Indonesia, bamboo demonstrates the capability to thrive at elevations ranging from 1 to 1900 metres asl. For example, bamboo can be found at elevations of 1–212 metres asl on Weh Island, Sumatra (Ritonga et al. 2023). Additionally, in the Maluku Islands, bamboo is found growing at elevations of 1–1100 metres asl (Robiah et al. 2022), while in the Lesser Sunda Islands, bamboo is found growing at elevations of 5–1700 metres asl (Damayanto 2024).

There are approximately 1670 bamboo species and 125 genera worldwide (Soreng et al. 2017). Of these, 175 species and 24 genera are found in Indonesia (Damayanto & Fefirenta 2021). This number will continue to rise as more bamboo species are newly published in Indonesia (see Widjaja 2023; Ritonga et al. 2025a; Widjaja et al. 2025). Most bamboo species, including those in Indonesia, rarely flower (Janzen 1976), which complicates identification. To overcome this challenge, DNA barcoding approaches are essential, as they can be used in phylogenetic studies to assist in species clustering, thereby facilitating the identification process. As a preliminary step, in-silico studies can help identify the most promising gene regions (molecular markers) for future research. While in-silico phylogenetic studies have been conducted on plants such as Fabaceae (Sikdar et al. 2018), *Oryza sativa* L. (Guha et al. 2019), Cucurbitaceae (Ho & Nguyen 2020), *Coelogyne* (Pratiwi et al. 2023), *Curcuma* (Sahu et al. 2024), Moraceae (Ngo et al. 2024), and *Bambusa* (Pandey & Bhargava 2024), none have focused specifically on bamboo species in Indonesia.

Several studies on the phylogeny of bamboo at the family, subfamily or genera levels have successfully used the chloroplast DNA (cpDNA) *trnL-trnF* intergenic spacer (IGS) region (Qiang et al. 2005; Sungkaew et al. 2009a; Hodkinson et al. 2010; Zhou et al. 2017). The consistent maternal inheritance and largely non-recombining nature of cpDNA make it a valuable marker for tracing maternal lineages and evolutionary relationships (Olmstead & Palmer 1994). Moreover, the conserved gene content and structure of cpDNA make it a reliable tool for studying and clarifying evolutionary relationships among plant groups (Xie et al. 2020). Although cpDNA is generally conservative, the non-coding regions of cpDNA, e.g. the *trnL-trnF* IGS regions, are often associated with mutational hotspots (Drábková et al. 2004) and exhibit a high mutation rate (Sakka et al. 2013), which can be used in phylogenetic analyses (Taberlet et al. 1991).

The rapid mutation rate in cpDNA's non-coding regions generates sufficient sequence variation to determine relationships both between and within species of plants (Taberlet et al. 1991). Furthermore, the universal primers for cpDNA non-coding regions, developed by Taberlet et al. (1991), have shown

consistent amplification across diverse plant lineages, making them applicable to bamboo as well. Therefore, the cpDNA *trnL-trnF* IGS region holds potential as a molecular marker for phylogenetic analysis of bamboo genera. This study evaluated the *trnL-trnF* IGS region's ability to construct phylogenetic relationships among various bamboo genera using in-silico analysis.

MATERIALS AND METHODS

In-silico and phylogenetic analyses

This study employed an in-silico method using cpDNA *trnL-trnF* IGS sequence data from various bamboo genera or species that can be found in Indonesia. The gene sequence data were sourced from the National Center for Biotechnology Information (NCBI) GenBank (<https://www.ncbi.nlm.nih.gov/>) in 2024. *Widjajachloa producta* (Pilg.) K.M.Wong & S.Dransf. (Poaceae) (Wong & Dransfield 2016) was used as an outgroup for the analysis. The sequence data, extracted in FASTA format, were summarized by GenBank accession number and species name.

We searched GenBank using a specific query format: “Genus” and “*trnL-trnF*”. This search was performed individually for each species using the NCBI Nucleotide database. The search results were then carefully reviewed to identify sequence entries corresponding to the *trnL-trnF* IGS. Only sequences with complete or nearly complete gene coverage, accurate species identification, and proper taxonomic classification were selected for inclusion in the dataset. Once suitable entries were identified, the GenBank accession numbers and associated species names were recorded. The selected sequences were then downloaded in FASTA format using the “send to” function available on each sequence’s GenBank entry page. In cases where multiple sequences were selected, accession numbers were compiled into a list and used in the NCBI Batch Entrez tool to download the sequences in bulk. All FASTA-formatted sequences were then organized into a single dataset for alignment and analysis. The dataset also included an outgroup taxon, *W. producta*, which was retrieved using the same procedure. In total, 41 sequences were collected and used for phylogenetic reconstruction, with accession numbers and species names summarized in Table 1.

The sequence data were aligned using the MUSCLE program (Edgar 2004) in the Molecular Evolutionary Genetics Analysis (MEGA) software v. 11 for Windows (https://www.megasoftware.net/download_form) (Tamura et al. 2021). The alignment results were then trimmed at both ends to ensure uniformity. The edited sequences were saved in FASTA format and used to construct phylogeny trees using both distance-based (Neighbor-Joining) (Saitou & Nei 1987) and character-based (Maximum Parsimony and Maximum Likelihood) analyses in MEGA v. 11, with a bootstrap value of 1000 (Felsenstein 1985). The phylogeny trees were then presented and described descriptively. The bootstrap value categories used refer to Sungkaew et al. (2009a, 2009b). Bootstrap values are high if $\geq 85\%$, moderate if 75–84%, weak if 50–74%, and very weak if $< 50\%$ (Sungkaew et al. 2009a, 2009b).

Heatmap analyses

A heatmap was generated to visualize clustering patterns. DNA sequence data from the phylogenetic analysis were employed to construct the heatmap. DNA sequence data were converted into numerical values and analysed using the online software Heatmapper (2024) (<http://www.heatmapper.ca/expression/>). The heatmap was then described descriptively.

Morphological observation

Morphological observations of bamboo genera were conducted by examining herbarium specimens at Herbarium Andalas University (ANDA) and Herbari-

Table 1. Metadata of cpDNA *trnL-trnF* IGS sequences of several bamboo species extracted from the National Center for Biotechnology Information.

Species	Accessions	Collections/Vouchers
<i>Bambusa multiplex</i> (Lour.) Raeusch. ex Schult. & Schult.f.	KT226866	zmy028
	KT226865	YHQ-013
	DQ137347	KUN:YHQ013
<i>Bambusa vulgaris</i> Schrad. ex J.C.Wendl.	JX428443	VNMNB000276
	JX428442	VNMNB000274
	JX428441	VNMNB000273
	JX428432	VNMNB000241
	JX428431	VNMNB000240
	JX428430	VNMNB000239
	JX428429	VNMNB000238
	JX428428	VNMNB000237
	JX428427	VNMNB000235
	JX428426	VNMNB000234
	JX428425	VNMNB000229
	KT226876	zmy029
<i>Chloothamnus elatus</i> (Holtum) Widjaja	KT226943	KMBG1215
<i>Dendrocalamus asper</i> Backer ex K.Heyne	KT226892	12234
	HM448968	SS&AT 110704-1/THNHM,KUFF
	EU434057	BAM6 1
<i>Dendrocalamus giganteus</i> Munro	KT226900	12201
	EU434058	BAM6 45
<i>Dinochloa malayana</i> S.Dransf.	KT226918	KMW200503
	EU434067	SD 1412/KEW
	DQ137355	KUN:KMW200503
<i>Dinochloa scandens</i> Kuntze	DQ137356	KUN:WEA200502
<i>Gigantochloa apus</i> (Schult.f.) Munro	KT226921	zmy020
<i>Gigantochloa atter</i> (Hassk.) Kurz ex Munro	KT226923	AUS200901
<i>Neololeba atra</i> (Lindl.) Widjaja	EU434078	Hodkinson NSW32/TCD
<i>Schizostachyum blumei</i> Nees	DQ137366	KUN:WEA200501
<i>Schizostachyum brachycladum</i> (Kurz ex Munro) Kurz	DQ137368	KUN:DZL200201
<i>Schizostachyum zollingeri</i> Steud.	EU434088	SS&AT 090704-1/THNHM & KUFF
	DQ137370	KUN:KMW200501
<i>Thyrsostachys siamensis</i> Gamble	DQ137336	KUN:YHQ024
	EU434069	S&AT 020704-3/THNHM & KUFF
	KT226963	12175
<i>Widjajachloa producta</i> (Pilg.) K.M.Wong & S.Dransf.	KM251339	UASWS1035
	KM251344	UASWS1040
	KM251343	UASWS1039
	KM251342	UASWS1038
	KM251341	UASWS1037
	KM251340	UASWS1036

um Bogoriense (BO). Herbarium abbreviations followed Thiers (2025). High-resolution images of bamboo specimens from global herbaria, available online through the Global Biodiversity Information Facility (<https://www.gbif.org>), Kew Data Portal (<https://data.kew.org>), and JSTOR Global Plants (<https://plants.jstor.org>), were also reviewed (Damayanto & Irsyam 2022). Additionally, photographs of fresh specimens were taken. The data were then used as a reference to compare the phylogeny tree with the morphological characteristics of bamboo genera.

RESULTS AND DISCUSSIONS

Among the trees generated, the Neighbor-Joining (NJ) analysis yielded the

best tree based on minimal total branch length, with a cumulative value of 0.98893930. This value reflects the sum of all branch lengths, representing the overall evolutionary distance among the taxa included in the analysis. Evolutionary distances were calculated using the Maximum Composite Likelihood method (Tamura et al. 2004) and are expressed as the number of base substitutions per site. Gaps and missing data were removed, resulting in a final dataset of 811 positions. Meanwhile, the Maximum Parsimony (MP) analysis produced a tree with a consistency index of 0.988839, a retention index of 0.997737, and a composite index of 0.986601 for all sites, with parsimony-informative sites approximately 51 %. The MP tree was generated using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei & Kumar 2000) with a search level of 1, starting from initial trees obtained by adding sequences randomly (10 replicates). Gaps and missing data were excluded (complete deletion option), resulting in a final dataset of 811 positions.

The Maximum Likelihood (ML) analysis, based on the Tamura-Nei model and performed on 41 nucleotide sequences, produced a tree with the highest log likelihood (-2187.12). The initial tree for the heuristic search was automatically generated by applying Neighbor-Joining and BioNJ algorithms to a pairwise distance matrix, estimated using the Maximum Composite Likelihood approach, and then selecting the topology with the superior log likelihood. All positions containing gaps and missing data were eliminated, resulting in a final dataset of 811 positions.

The analysis of NJ (Figure 1), MP (Figure 2), and ML (Figure 3) with 1000 bootstrap replicates each produced different phylogenetic trees. NJ analysis failed to effectively differentiate bamboo genera. However, *Bambusa multiplex* and some *Schizostachyum* species formed separate clusters, each with a bootstrap support of 61 %. Nevertheless, according to the criteria established by Sungkaew et al. (2009a, 2009b), a bootstrap value of 61 % is considered weak. Therefore, the clustering of these taxa cannot be considered reliable.

In contrast, the MP analysis identified three distinct clades: *Dinochloa*, *Schizostachyum*, and “BDG+CNT”, the latter including *Bambusa*, *Dendrocalamus*, *Gigantochloa*, *Chloothamnus*, *Neololeba*, and *Thyrsostachys*. The clades for *Dinochloa* and *Schizostachyum* were monophyletic and displayed strong phylogenetic support, with bootstrap values of 90 % and 87 %, respectively. However, the “BDG+CNT” clade exhibited limited resolution, with bootstrap values below 70 %, indicating less clear separation among the genera within this group. The “BDG+CNT” clade was not monophyletic at the genus level, as species from different genera were intermixed. Interestingly, all *B. multiplex* clustered together in the MP phylogenetic tree with a bootstrap support of 60 %, mirroring the result from the NJ analysis. However, this bootstrap value of 60 % is considered weak, as noted by Sungkaew et al. (2009a, 2009b).

The “BDG+CNT” clade in the MP tree does not differentiate between different genera. This is consistent with studies reporting that the genera *Bambusa*, *Dendrocalamus*, and *Gigantochloa* (known as “BDG” complex) are closely related and difficult to separate (Goh et al. 2010). The “BDG” complex is not limited to these three main genera but also includes *Maclurochloa*, *Melocalamus*, *Oreobambos*, *Oxytenanthera*, *Phuphanochloa*, *Pseudoxxytenanthera*, *Thyrsostachys*, and *Vietnamosasa* (Zhou et al. 2017; Liu et al. 2020). Consequently, the grouping of *Thyrsostachys* within the “BDG+CNT” clade in the MP tree in this study is unsurprising. Similarly, *Chloothamnus* and *Neololeba* are nested within the “BDG+CNT” clade, suggesting that they share close evolutionary relationships with other “BDG” members and may not represent distinct monophyletic lineages in this analysis.

Although Fu (2024) conducted a phylogenetic study on the subtribe Bambusoideae and proposed that all species within the genera *Dendrocalamus*, *Gigantochloa*, and *Thyrsostachys* are synonymous with *Bambusa* and that all

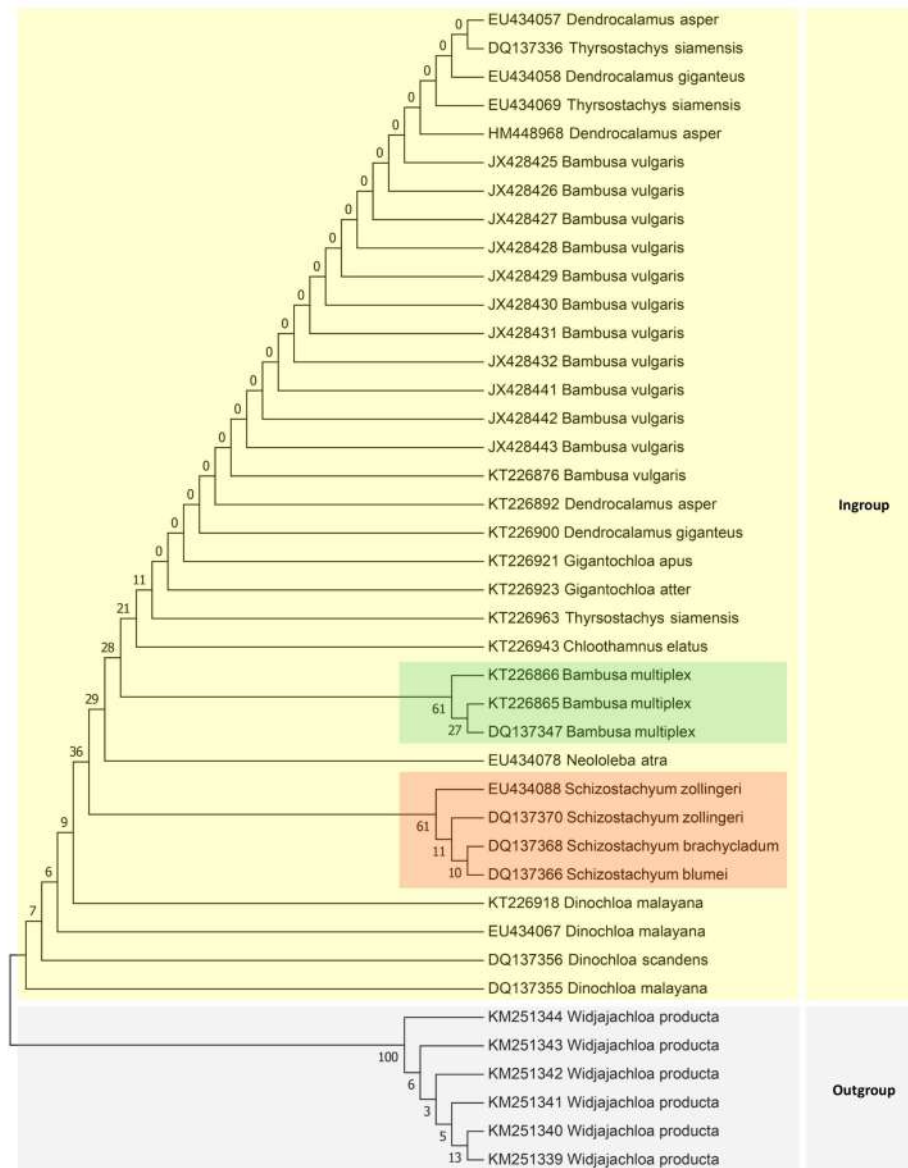


Figure 1. The phylogenetic tree using cpDNA *trnL-trnF* IGS region of several bamboo species was constructed using Neighbor-Joining analysis with a bootstrap value of 1000. The bootstrap results, showing taxa clustering percentages, are displayed next to branches.

species within *Neololeba* are synonymous with *Dinochloa*, the present study continues to treat these genera as distinct taxonomic entities. Fu's (2024) taxonomic treatment of bamboos has been met with considerable disagreement among researchers (Zhi-Qiang Zhang 2025, personal communication). Fu's (2024) proposed classification remains problematic and, in the present author's view, requires further validation through comprehensive and integrative analysis. In particular, the method of justification employed is not widely adopted, and the conclusions appear to have been generalised prematurely from a limited sample size, without incorporating additional characters or alternative analytical approaches. For instance, in the present study, *Chloothamnus* forms a distinct clade separate from *Dinochloa*, indicating that Fu's (2024) proposal to synonymise *Neololeba* with *Dinochloa* may be less applicable or not well supported in this context.

The ML tree revealed three major clades: *Chloothamnus*, *Schizostachyum*, and "BDG+DNT" (comprising *Bambusa*, *Dendrocalamus*, *Gigantochloa*, *Dinochloa*, *Neololeba*, and *Thyrsostachys*). The *Chloothamnus* clade formed a distinct group, separate from the other clades, but with very weak bootstrap support (36 %) at its branch, suggesting a poorly supported phylogenetic posi-

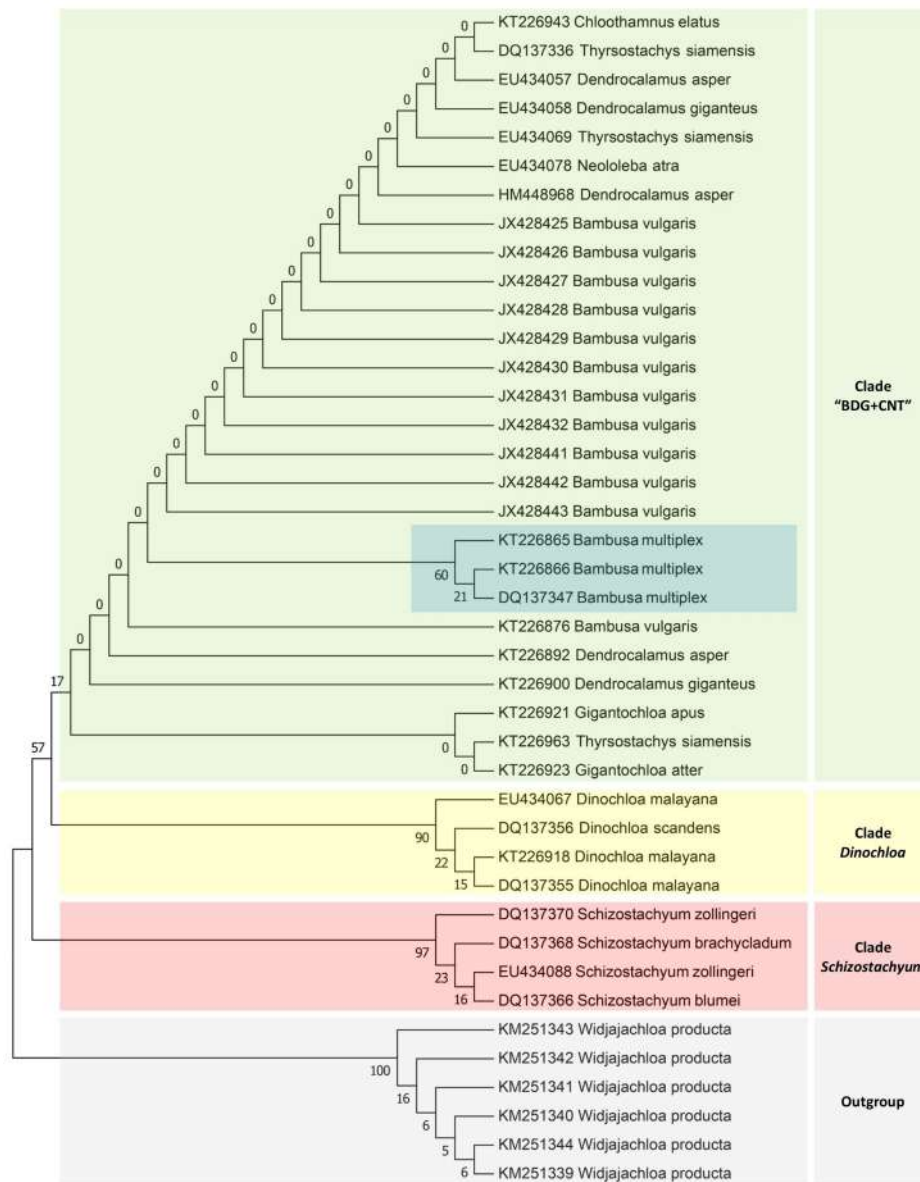


Figure 2. The phylogenetic tree using cpDNA *trnL-trnF* IGS region of several bamboo species was constructed using Maximum Parsimony analysis with a bootstrap value of 1000. The bootstrap results, showing taxa clustering percentages, are displayed next to branches.

tion. The *Chloothamnus* clade, as a singleton taxon, does not allow assessment of monophyly but forms a distinct lineage. In contrast, the *Schizostachyum* clade was monophyletic and strongly supported with a high bootstrap value (92 %), indicating a close relationship among its members and clear differentiation from other bamboo genera.

On the other hand, the “BDG+DNT” clade showed very weak overall bootstrap support (29 %), and the internal branches within this clade were also generally poorly supported (mostly 0 %), indicating limited resolution or poorly defined relationships among the included genera. The “BDG+DNT” clade was intermixed across the tree, indicating non-monophyly at the genus level. Interestingly, a subclade of *B. multiplex* was identified within the “BDG+DNT” clade, although it received weak bootstrap support (65 %). This *B. multiplex* subclade in the ML tree is consistent with the grouping observed in the NJ tree. Meanwhile, the *Dinochloa* subclade clearly separated the genus *Dinochloa* from the other genera within the “BDG+DNT” clade. This subclade was strongly supported with a bootstrap value of 94 %, indicating that *Dinochloa* is phylogenetically distinct and that the tested *Dinochloa* species share a close evolutionary relationship.

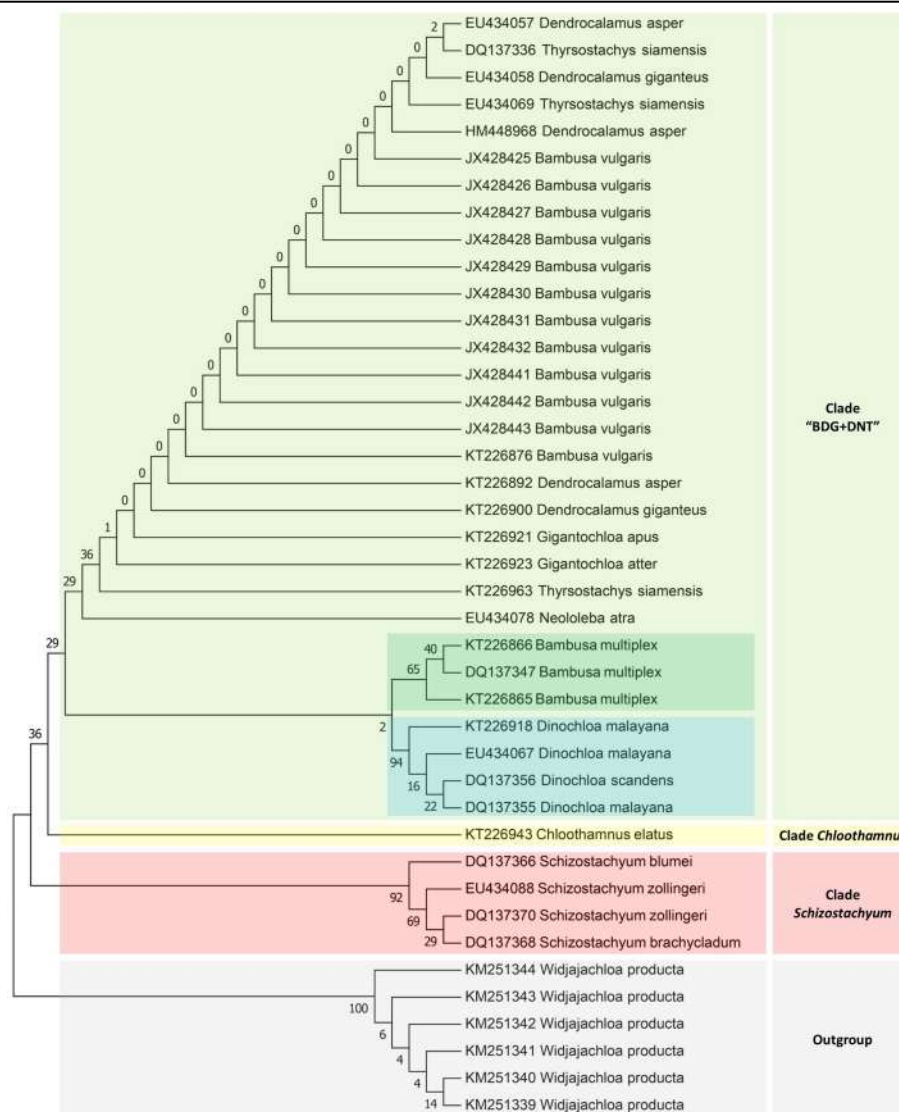


Figure 3. The phylogenetic tree using cpDNA *trnL-trnF* IGS region of several bamboo species was constructed using Maximum Likelihood analysis with a bootstrap value of 1000. The bootstrap results, showing taxa clustering percentages, are displayed next to branches.

The discrepancies between the MP, ML, and NJ analyses can be attributed to their different approaches to phylogenetic reconstruction. MP, a character-based method, meticulously examines individual character state changes to infer evolutionary relationships. The most parsimonious trees are the ones with the fewest character changes, provided no character changes revert to a previous state (Farris 1970). MP's detailed analysis of character data offers a more accurate depiction of evolutionary history, especially for complex relationships. On the other hand, ML is a model- and character-based phylogenetic method (Felsenstein 1973). It identifies the tree (topology and branch lengths) that most probably produced the observed sequence data, given a specific evolutionary model (e.g., Tamura-Nei). ML calculates the total 'likelihood' for each potential tree by multiplying the probabilities of observing each character site under that tree and model (Ludwig et al. 2011). Heuristic algorithms search for the tree with the highest log-likelihood (Kato et al. 2001).

In contrast, NJ, a distance-based method, relies on overall genetic similarity between taxa to construct a phylogenetic tree. This method works on the principle of identifying pairs of operational taxonomic units (OTUs, or neighbors) that reduce the overall branch length at each clustering stage, beginning with a star-like tree structure (Saitou & Nei 1987). By collapsing character information into pairwise distances, NJ may inadvertently obscure

subtle character state changes, potentially leading to less resolved or even misleading topologies.

The differences in these methods, therefore, can lead to different results, as seen in this study. Character-based methods, such as MP and ML, evaluate evolutionary changes at individual nucleotide or amino acid positions, offering detailed insights into complex divergence patterns (see [Farris 1970](#); [Felsenstein 1973, 2004](#); [Ludwig et al. 2011](#)). In contrast, distance-based methods like NJ summarise sequence similarity into pairwise distances, constructing trees by minimising overall branch lengths ([Saitou & Nei 1987](#); [Felsenstein 2004](#)). Although computationally efficient, distance-based approaches may oversimplify data and obscure subtle evolutionary signals. Consequently, the choice of method can substantially influence tree topology and interpretation.

The DNA sequence pattern observed in the heatmap (Figure 4) aligns relatively well with the clustering patterns found in the MP analysis. A clear and distinct separation is evident between the outgroup species (*W. producta*) and *Schizostachyum* spp. On the other hand, *Dinochloa* spp. also exhibits a

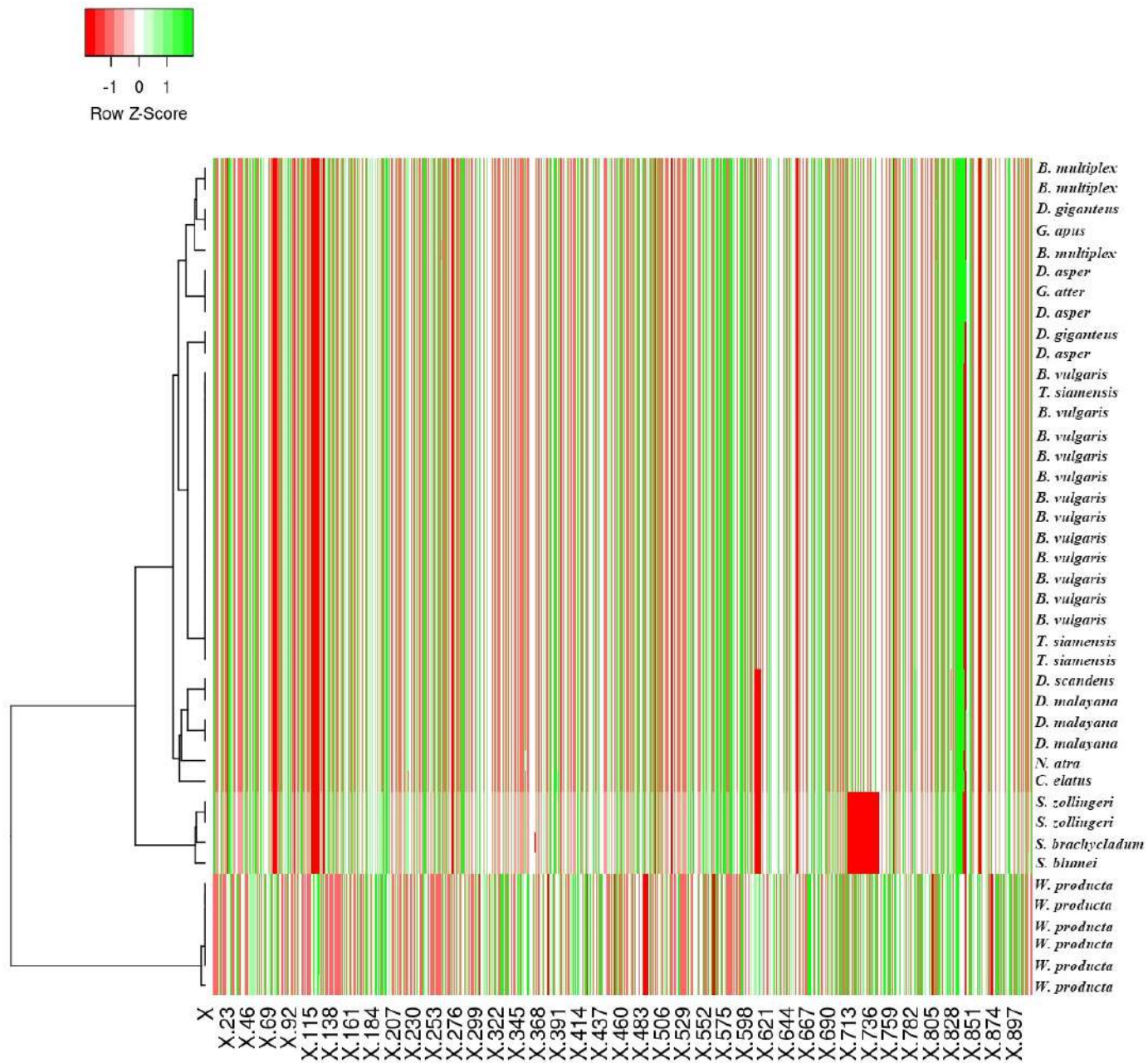


Figure 4. The heatmap of several bamboo species was constructed using cpDNA *trnL-trnF* IGS region.

unique pattern but remains clustered with *Neololeba* (*N. atra*) and *Chloothamnus* (*C. elatus*). The DNA sequence pattern for *Schizostachyum* is particularly distinct, as illustrated by the sequence pattern in the region between X.713–X.736 in Figure 4, which is noticeably different from other genera. This uniqueness suggests that the cpDNA *trnL-trnF* IGS region in *Schizostachyum* could serve as a genus-specific DNA barcode, similar to the findings for *Curcuma* reported by Sahu et al. (2024).

We tried to generate DNA barcodes of *Schizostachyum* species (*S. blumei*, *S. brachycladum*, and *S. zollingeri*) utilizing the cpDNA *trnL-trnF* IGS region as a target (Figure 5). This process was facilitated by an online application available at <http://biorad-ads.com/DNABarcodeWeb>. At a glance, these DNA barcodes appear to exhibit a variety of colours due to interpolation. Colour interpolation occurs when coloured objects are close together, causing our brains to naturally try to “smooth out” the transitions between adjacent colours. In reality, there are only four colours representing each DNA nucleotide. Each nucleotide, adenine (A), cytosine (C), guanine (G), and thymine (T), was assigned a specific colour: green for A, blue for C, black for G, and red for T (Figure 5).

Morphologically, members of the *Dinochloa* clade exhibit distinct characteristics, notably their climbing habit (Figure 6C), which differentiates them from typical bamboo species with erect (Figures 6A, 6D, and 6E) or scrambling culm (Figure 6B) (Damayanto 2017, 2018; Damayanto et al. 2021; Ritonga et al. 2024). Moreover, *Dinochloa* can also be easily identified by its “girdle” at the node, a rough part that is a remnant of the culm sheath (Figure 7D), which is a distinctive feature of this genus (Damayanto 2024; Ritonga et al. 2025b). Consequently, *Dinochloa* can be easily differentiated from other bamboo genera. In contrast, the *Schizostachyum* clade is characterised by relatively long internodes (Muzakki 2020), typically 1–1.5 metres long (Figure 6E) and exceptionally thin culm walls setting them apart morphologically from the members of “BDG+CNT” clade in MP analysis or “BDG+DNT” clade in ML analysis, which possess shorter internodes (Figures 6A and 6D) and relatively thicker culm walls.

These morphological distinctions align with the molecular clustering based on cpDNA *trnL-trnF* IGS, making both *Dinochloa* and *Schizostachyum*

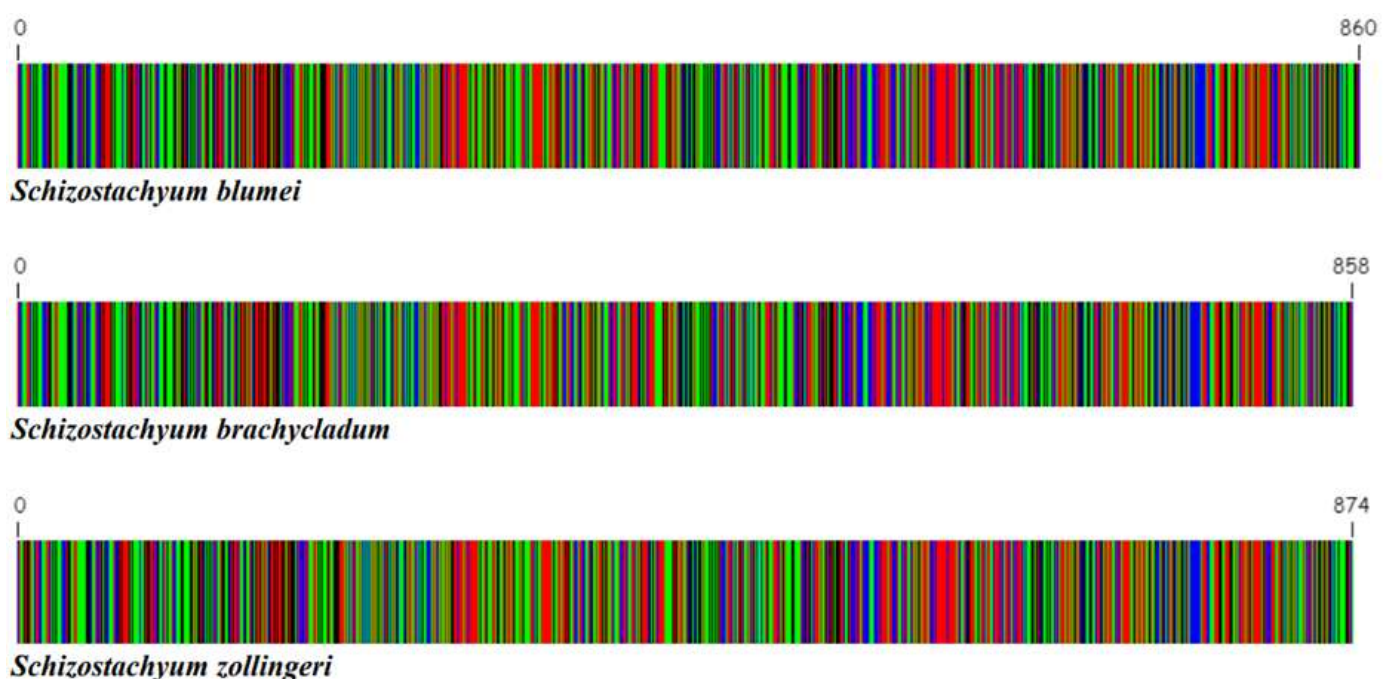


Figure 5. DNA Barcodes of three species of *Schizostachyum* (*S. blumei*, *S. brachycladum*, and *S. zollingeri*) using cpDNA *trnL-trnF* IGS region. Nucleotide bar colors: A = green, T = red, G = black, and C = blue.



Figure 6. Habit of several bamboo genera in Indonesia. (A) *Bambusa* with erect and slightly zigzag culms, (B) *Chloothamnus* with scrambling culms, (C) *Dinochloa* with climbing culms, (D) *Gigantochloa* with erect culms, (E) *Schizostachyum* with erect and long internode culm. Photos: I Putu Gede P. Damayanto (A, C, D, E), Lulut Dwi Sulistyaningsih (B).

readily identifiable through morphological and molecular means. A comparison of morphological characteristics for all genera used in this study is presented in Table 2. Representative species for each genus are illustrated in Figure 7.

Overall, the *trnL-trnF* IGS region cannot fully distinguish all bamboo genera in the phylogeny tree, although it has the potential to differentiate genera such as *Dinochloa* and *Schizostachyum* with relatively high bootstrap values, which aligns with their easily recognisable morphological characteristics.

CONCLUSIONS

The phylogenetic tree from the Neighbor-Joining analysis using the *trnL-trnF* IGS region failed to effectively differentiate bamboo genera. The phylogenetic tree from the Maximum Parsimony analysis using the *trnL-trnF* IGS region identified three distinct clades: *Dinochloa*, *Schizostachyum*, and “BDG+CNT” (comprising *Bambusa*, *Dendrocalamus*, *Gigantochloa*, *Chloothamnus*, *Neololeba*, and *Thyrsostachys*). Maximum Likelihood analysis identified

Table 2. Comparison of morphological characteristics of several bamboo genera in Indonesia.

Genera	Morphological characteristics						
	Habit	Culms	Nodes	Internodes	Branches	Culm sheaths	Inflorescence
<i>Bambusa</i>	Clumping; erect	Slightly zigzag	Ornaments absent	Relatively short (less than 1 m long)	A dominant branch with several smaller branches at each node	Usually covered with hairs; auricle conspicuous; blade usually broadly triangular	Raising in the branches
<i>Chloothamnus</i>	Clumping; scrambling	Straight at the base, then scrambling	Ornaments absent	Relatively short (less than 1 m long)	A dominant branch with several smaller branches at each node	Usually covered with hairs; auricle conspicuous; blade usually linear	Raising in the branches
<i>Dendrocalamus</i>	Clumping; erect	Relatively straight	Aerial roots in the lower to the upper part	Relatively short (less than 1 m long)	A dominant branch with several smaller branches at each node	Usually covered with hairs; auricle usually conspicuous; blade usually triangular	Raising in the branches
<i>Dinochloa</i>	Clumping; climber	Zigzag	Girdle present	Relatively short (less than 1 m long)	A dominant branch with several smaller branches at each node. The primary branch dormant and develops into a full-size culm when the apex is broken.	Glabrous or covered with hairs; auricle usually with bristles; blade usually broadly ovate to ovate-lanceolate	Raising in the branches
<i>Gigantochloa</i>	Clumping; erect	Relatively straight	Short aerial roots from the basal nodes	Relatively short (less than 1 m long)	A dominant branch with several smaller branches at each node	Usually covered with hairs; auricle usually firm and low; blade usually ovate-oblong	Raising in the branches
<i>Neololeba</i>	Clumping; erect to scrambling	Relatively straight	Ornaments absent	Relatively short (less than 1 m long)	A dominant branch with several smaller branches at each node	Usually covered with hairs; auricle conspicuous or lobe-like; blade triangular	Raising in the branches
<i>Schizostachyum</i>	Clumping; erect	Relatively straight	Ornaments absent	Relatively long (usually more than 1 m long)	Several subequal slender branches	Usually covered with hairs; auricle usually conspicuous; blade triangular, lanceolate to linear	Raising terminally on leafy branches
<i>Thyrsostachys</i>	Clumping; erect	Relatively straight	Ornaments absent	Relatively short (less than 1 m long)	A dominant branch with several smaller branches at each node	Usually covered with hairs; auricle small or inconspicuous; blade usually lanceolate	Raising terminally on leafy or leafless branches



Figure 7. Morphological characteristics of several bamboo genera in Indonesia. (A) *Bambusa vulgaris*, (B) *Chloothamnus schmutzii*, (C) *Dendrocalamus asper*, (D) *Dinochloa sepang*, (E) *Gigantochloa atter*, (F) *Neololeba atra*, (G) *Schizostachyum brachycladum*, (H) *Thyrsostachys siamensis*. Photos: Muhammad Azli Ritonga (A, C, E, G, H), Lulut Dwi Sulistyaningsih (B), I Putu Gede P. Damayanto (D, F).

three distinct clades: *Chloothamnus*, *Schizostachyum*, and “BDG+DNT” (comprising *Bambusa*, *Dendrocalamus*, *Gigantochloa*, *Dinochloa*, *Neololeba*, and *Thyrsostachys*). The *trnL-trnF* IGS region cannot fully distinguish all bamboo genera in the phylogenetic tree, although it has the potential to differentiate genera such as *Dinochloa* and *Schizostachyum* with relatively high bootstrap values, which aligns with their easily recognizable morphological characteristics.

AUTHOR CONTRIBUTION

M.A.R. and I.P.G.P.D. were jointly responsible for the conceptualization of the study, compiled and analysed the data, provided photographic documentation, created visual materials, prepared the initial draft of the manuscript, and carried out revisions. S., N., and T.M. were actively involved in reviewing and refining the manuscript. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest but are accountable for the article's content and composition.

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Research Article

Diversity and Ecological Significance of Edible and Inedible Macrofungi in Turgo National Park, Mount Merapi

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ABSTRACT

This study documented macrofungal diversity across three forest tracks—Turgo, Bingungan, and Tritis—within Turgo National Park, located on the slopes of Mount Merapi, Indonesia in 2017. A total of 133 macrofungal specimens were collected, with species richness varying by site: 48 species in Turgo Forest, 38 in Bingungan, and 52 in Tritis. Diversity, assessed via the Shannon-Weiner index, ranged from 2.01 in Turgo to 2.521 in Tritis, indicating moderate to high ecological diversity. Sørensen similarity indices revealed minimal overlap between forest communities (<0.1), suggesting high habitat specificity. Morphogroup analysis identified agarics as the dominant type, with ecological importance values peaking at 274 % in Turgo Forest. Other morphogroups, including jelly, polypores, club, and cup fungi, varied in presence and abundance among sites. Vegetation composition appeared to influence fungal distribution, with Bingungan Forest showing the highest plant diversity but lower fungal diversity than the coniferous forest in Tritis. A total of 26 macrofungi were identified as edible, inedible, or medicinal, including a prominent local edible species, *Auricularia delicata*, and a wild *Cordyceps* specimen associated with an insect host. These findings highlight the ecological distinctiveness of each site and the potential nutritional, medicinal, and conservation value of macrofungi in tropical volcanic forests.

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INTRODUCTION

Macrofungi have played an essential role in human civilization for thousands of years, contributing to various industries, including food, pharmaceuticals, and bioremediation. The term "macrofungi" refers to fungi that produce sporocarps, commonly known as fruiting bodies, which are visible to the naked eye and generally macroscopic in size (DaSilva 2005; Mueller et al. 2004). Macrofungal diversity is categorised into distinct morphogroups based on fruiting body structures, such as agarics, boletes, coral fungi, club fungi, tooth fungi, ball-shaped fungi, cup-disc fungi, and bracket fungi, most of which belong to the phyla *Ascomycota* and *Basidiomycota* (Lindsay et al. 2013; Mueller et al. 2004). The ecological significance of macrofungi lies in their role as decomposers, symbionts, and nutrient cyclers within forest ecosystems (Molina et al. 1993, 2001). Macrofungi exhibit various survival strategies, including saprophytism, parasitism, and epiphytism (Jacobs 1983), but their most critical ecological function is their symbiotic relationship with plants through mycorrhizal associations, acting as the main decomposers in forest communities (Niego et al. 2023). These interactions enhance nutrient cycling, promote forest resilience, and contribute to overall ecosystem stability (Stamets 2006). Given their ecological importance, understanding macrofungal diversity is crucial for assessing forest health, species-specific interactions with vegetation, and broader ecological dynamics. The edibility of macrofungi is another key factor in the study of macrofungal diversity. Edible macrofungi are those that are non-toxic and suitable for human consumption, whereas inedible macrofungi may be toxic, tough, or otherwise unsuitable for consumption. However, some inedible species, such as *Ganoderma lucidum* (lingzhi mushroom), possess medicinal properties and have been widely used in traditional medicine. According to the Food and Agriculture Organization (FAO), a total of 1009 edible macrofungal species have been documented worldwide, including 880 species used as food, 249 species with medicinal applications, and 88 species classified as both edible and medicinal (Boa 2008). Despite Indonesia's rich biodiversity, research on macrofungal diversity remains limited. Compared to other Southeast Asian countries, such as Malaysia and Vietnam, Indonesia lacks a well-documented macrofungal database, hindering the development of a comprehensive inventory of fungal resources, even though some wild mushrooms with local names and their uses have been documented (Putra et al. 2022). This research gap has constrained efforts to maximise the sustainable utilisation of local macrofungal species. In contrast, macrofungi play a significant socio-economic role in other regions. In China, for instance, wild edible macrofungi are harvested across 22 provinces for food, storage, sale, and cultivation, with an annual production volume reaching millions of tonnes (Li & Xu 2021). Well-known commercialised species include *Lentinula edodes* (shiitake), *Pleurotus* spp. (oyster mushrooms), *Auricularia polytricha* (wood ear), *Agaricus bisporus* (button mushrooms), *Flammulina velutipes* (enokitake), *Pholiota nameko* (nameko), and *Hypsizygus marmoreus* (shimeji), collectively contributing to an estimated production volume of 4,350,000 tons in China (Hall et al. 2003). Similarly, in India, wild edible macrofungi are directly harvested from forests and sold in local markets. A study by Khaund and Joshi (2013) identified 11 species collected by the Khasi community in Meghalaya, highlighting the importance of a well-documented macrofungal database for local economies and ecological conservation.

Recognising this research gap, our study aimed to establish a foundational macrofungal dataset from three forested areas within Turgo National Park, Indonesia. Turgo Village, located in Hargobinangun, Pakem, Sleman, Yogyakarta, lies on the slopes of Mount Merapi within the conservation area of Mount Merapi National Park (Taman Nasional Gunung Merapi, TNGM). This region, classified as a volcanic forest ecosystem, experiences a tropical

climate (Type A) with annual rainfall ranging from 875 mm to 2,527 mm, with the wettest months occurring between November and May (Prasetya & Aminatun 2019a). Following the 2010 eruption of Mount Merapi, forests in the area have undergone continuous ecological succession (Akmalia et al. 2020). Reports suggest that the region is showing signs of ecological recovery, with increasing flora and fauna diversity, including endemic Javanese orchids. However, the macrofungal diversity in these forests remains largely unexplored.

Our study provides baseline macrofungal data from three forested areas within Turgo National Park, focusing on edible and inedible macrofungal diversity, diversity and similarity indices of macrofungal ecosystems, species distribution mapping from 2017, and ecological significance analysis based on macrofungal morphogroups. These findings are essential for future research in ethnomycology, fungal taxonomy, and macrofungal ecology, ultimately contributing to a better understanding of fungal biodiversity and its ecological and socio-economic potential.

MATERIALS AND METHODS

Study area

The study area for macrofungal sampling was located in Turgo Village, Purwobinangun, Pakem Sub-district, Sleman District, Yogyakarta, Indonesia. Sampling was conducted across three distinct forest tracks: Turgo Forest, Bingungan Forest, and Tritis Forest.

Sampling of Macrofungi

Sampling was conducted along predetermined tracks based on variations in elevation. The sampling method followed an adaptive plot-based approach (Prayudi et al. 2019), using plots of 10 m × 10 m, with each sampling area comprising a total of 10 plots. The first sampling was conducted in the Turgo Forest at an elevation of 1,034–1,070 m, with coordinates ranging from 7° 35.114'S to 7° 34.995'S and 110° 25.179'E to 110° 25.247'E, on May 6, 2017. The second sampling took place in the Bingungan Forest at an elevation of 1,169–1,186 m, with coordinates from 4° 36.422'S to 4° 36.450'S and 916° 19.06'E to 916° 18.70'E, on May 20, 2017. The third sampling was carried out in the Tritis Forest at an elevation of 1,026–1,059 m on June 3, 2017. Each forest track was sampled once, during a single visit. Sampling was conducted simultaneously with microclimatic data collection, including air humidity, air temperature, atmospheric pressure, and light intensity. Each species was recorded for its presence in each plot, assigned a specimen code, and selected mature sporulating samples were collected for species identification.

Macrofungi preservation

The collected macrofungal specimens were wrapped in aluminium foil and placed in a cooler box filled with ice cubes (Leonard 2010) to keep them moist. Some specimens (not all) were preserved using an FAA solution in 100 mL vial bottles, as recommended by Deepak et al. (2014). The preservation solution consisted of a mixture of 96 % alcohol, 4 % formalin, and distilled water (aquadest) in a 25:5:70 mL ratio.

Photographing Macrofungi

A DSLR camera equipped with a macro lens was used to capture high-resolution images of the macrofungi. Several types of photographs were taken for each specimen to ensure thorough documentation: (1) the mushroom in its natural habitat and on its substrate, (2) the specimen with its sample label for identification, and (3) multiple views including the cap (top view), the gills or pores (bottom view), and the entire fruiting body. These images provide criti-

cal visual references for identification and serve as valuable documentation for future research and comparison.

Characterisation and identification

Characterisation was conducted by first grouping the collected specimens into morphogroups based on their macroscopic features. For the agaric group, detailed morphological characteristics, including cap (pileal margin, surface texture, and shape), lamellae (attachment, shape, and margin), stipe (surface, shape, context, and presence of an annulus), volva type, stipe base, and spore print colour were observed. These observations followed the standardized data sheet format recommended by Mueller et al. (2004).

Identification was carried out on the same day as specimen collection to ensure accuracy in recording fresh morphological features. Morphological characterization followed the guidelines outlined in Chapter 8 of Biodiversity of Fungi: Inventory and Monitoring Methods (Mueller et al. 2004). Additionally, several field guides and taxonomic references, including McKnight and McKnight (1987), Fischer and Bessette (1992), Fergus and Fergus (2003), and Boa (2004) were referred, to match our specimens with known species. For further verification, identifications were confirmed through consultation with expert mycologists where possible.

Data analyses

The collected data were analysed by calculating density, frequency, and presence number to determine importance values. Multiple indices were used to compare diversity across the different tracks, including the Shannon-Weiner diversity index. Additionally, Sørensen's similarity index was applied to assess the overlap and similarity of fungal communities among the three forests.

For vegetation structure analysis, canopy coverage was calculated along the sampling plots using the following equation: (Number of individuals of canopy species $X \div$ Total counted specimens of canopy in sampling site) $\times$ 100 %.

To assess the potential influence of environmental variables on macro-fungal diversity across the three forest tracks, a Pearson correlation analysis using PAST (Palaeontological Statistics Software, version 5.2), developed by Hammer et al. (2001) was conducted.

RESULTS AND DISCUSSION

Diversity and ecological analysis of macrofungi

A total of 133 macrofungal specimens were collected, with species abundance varying across the three forest tracks. Turgo Forest area recorded 48 species, Bingungan Forest recorded 38 species, and Tritis Forest recorded 52 species.

Table 1 presents the measured microclimate data across the three macrofungi sampling tracks. The results indicate that macrofungi were found within a temperature range of 19 °C to 25 °C, with air humidity levels ranging from 60 % to nearly 90 %. Atmospheric pressure varied between 650 atm and approximately 950 atm. Light intensity measurements showed substantial variation, with values ranging from as low as 38 lux to a maximum tolerance level of 7,570 lux.

Table 2 illustrates the relationship between species diversity and elevation. The highest number of species was recorded in the lowest-elevation forest, which had a diversity index of 2.521. In comparison, the highest-elevation forest had a diversity index of 2.51, while the mid-elevation Turgo Forest had a diversity index of 2.01. These diversity indices fall within the medium-to-high range, indicating that all three forest tracks maintain relatively healthy ecosystems.

Table 1. Microclimatic data recorded each track of macrofungal sampling.

Tracks	Elevation (m)	Humidity (%)	Air Temperature (°C)	Atmospheric Pressure (atm)	Light intensity (lux)
Turgo Forest	1034 – 1070	73 – 86	23 – 24.5	668 – 674	38 – 362
Bingungan Forest	1169 – 1186	69 – 76	19 – 21	704	353 – 7570
Tritis Forest	1026 – 1059	63 – 80	19 – 21	938	99 – 2664

Table 2. Diversity index and Similarity index of macrofungal communities each track and comparing among them.

Tracks	Shannon-Weiner Index	Sorensen Index Turgo	Sorensen Index Bingungan	Sorensen Index Tritis
Turgo Forest	2.01	1	0.046511628	0.08
Bingungan Forest	2.51	0.046511628	1	0.1
Tritis Forest	2.521	0.08	0.1	1

Regarding similarity indices, the results show that all three tracks exhibited low similarity values, suggesting that each forest has distinct ecosystem components. The greatest difference in macrofungal community composition was observed between Bingungan Forest and Turgo Forest, with a similarity index of 0.0465. Conversely, the closest similarity was found between Tritis Forest and Bingungan Forest, with a similarity index of 0.1. These findings highlight the uniqueness of macrofungal communities within each forest, despite their moderate species diversity.

In Figure 1, among the measured environmental variables, temperature was the only factor significantly correlated with macrofungal diversity ($r = -1.00$, $p < 0.05$). This perfect negative correlation suggests that within the observed microclimatic range, higher temperatures are consistently associated with lower macrofungal species richness. While humidity and light intensity also showed strong correlations ($r = -0.91$ and $r = +0.74$, respectively), these were not statistically significant, indicating that their influence on diversity could not be conclusively confirmed within the scope of this study.

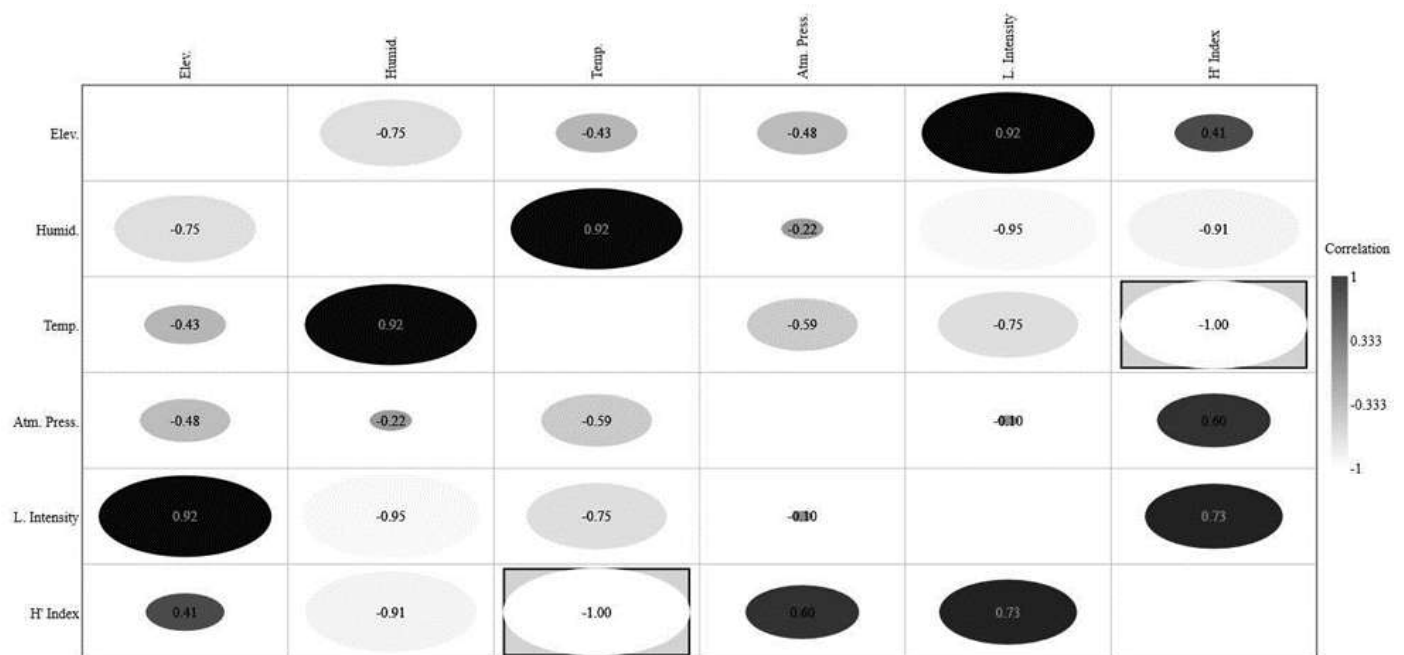


Figure 1. Correlation matrix showing the Pearson correlation coefficients between environmental variables and the macrofungal diversity index across three forest tracks in Turgo National Park, Mount Merapi. Ellipse shape and colour indicate the strength and direction of the correlation: darker tones represent positive correlations, lighter tones indicate negative correlations, and ellipse width reflects correlation strength; while ellipse with boxed plot shows statistically significant $p < 0.05$.

The histograms in Figure 2 illustrate the importance values of each macrofungal morphogroup across the three sampling tracks. These importance values were calculated as the sum of relative density, relative frequency, and relative diversity. The results indicate that the significance of each morphogroup varies across the different forest tracks. In Bingungan Forest, agarics exhibited the highest importance value at 138 %, followed by jelly fungi at approximately 89 %, and then clubs, cups, and tooth fungi. A similar pattern was observed in the other two forest tracks, where agarics also had the highest importance values—268 % in Tritis Forest and 274 % in Turgo Forest. Bingungan and Tritis Forests shared similar macrofungal morphogroups, including agarics, jelly fungi, polypores, clubs, tooth fungi, and cup fungi. However, Turgo Forest showed a distinct composition, lacking jelly and tooth fungi but containing coral fungi, a morphogroup that was not found in the other tracks. These findings highlight the ecological uniqueness of each forest and suggest variations in environmental factors influencing macrofungal community composition.

Table 3 presents the differences in vegetation structure across the three forest sampling tracks. In Tritis Forest, four plant species were identified, with bamboo species (*Bambusa* sp. 1 and *Bambusa* sp. 2) being the dominant vegetation. However, canopy cover data for this forest could not be measured due to missing records. In Turgo Forest, four plant species were also recorded, with *Schima wallichii* (Puspa) as the dominant species. In Bingungan Forest, a greater diversity of vegetation was observed, with nine plant species identified. The dominant species in this area were *Macaranga triloba* (Tutup Ijo) and *Ficus variegata* (Gondang). These findings highlight variations in plant composition across the three forests, which may influence macrofungal diversity and distribution.

In terms of morphological characteristics, the macrofungi found in Turgo belong to various morphogroups, including agarics (characterized by a hood-like cap), brackets (attached to the substrate, typically porous), jelly fun-

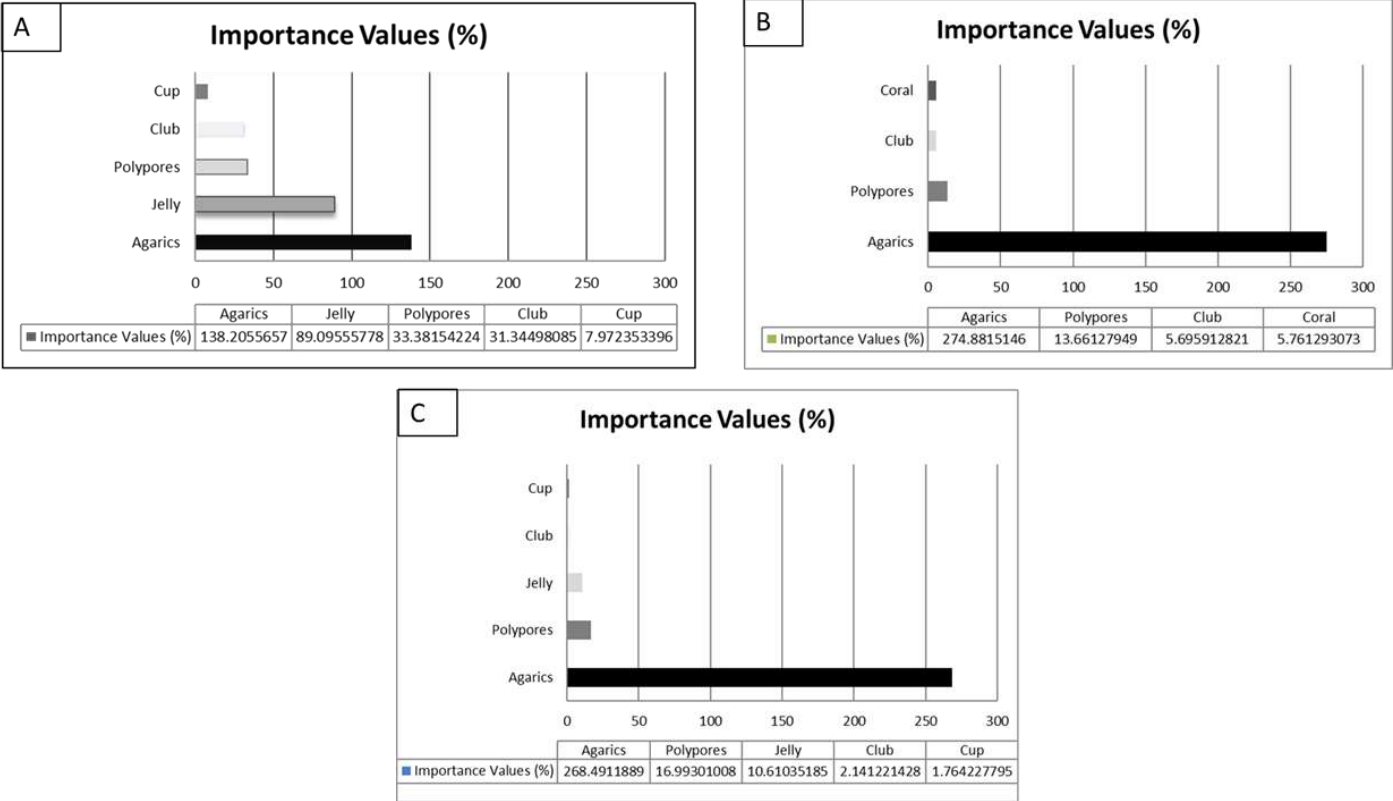


Figure 2. Importance values of macrofungal morphogroups in three forest tracks of Turgo National Park: (a) Bingungan Forest, (b) Turgo Forest, and (c) Tritis Forest.

Table 3. Vegetation Structure Percentages in each sampling forest.

Tritis Forest	Turgo Forest	Bingungan Forest
<i>Pinus merkusii</i> (uncounted)	<i>Schima wallichii</i> (57 %)	<i>Macaranga triloba</i> (30 %)
<i>Schima wallichii</i> (uncounted)	<i>Cinchona pubescens</i> (30 %)	<i>Ficus ariegata</i> (24 %)
<i>Bambusa</i> sp1. (uncounted)	<i>Pinus merkusii</i> (10 %)	<i>Altingia exelsa</i> (14 %)
<i>Bambusa</i> sp2. (uncounted)	<i>Altingia exelsa</i> (3 %)	<i>Syngium polyanthum</i> (5 %)
		<i>Albizia</i> sp. (5 %)
		<i>Cinchona pubescens</i> (5 %)
		<i>Erythrina</i> sp. (5 %)
		<i>Cinnamomum</i> sp. (5 %)
		<i>Castanea</i> sp. (5 %)

Table 4. Distribution of macrofungi in three forests of Turgo National Park based on morphogroups.

Morphogroups	Turgo Forest	Bingungan Forest	Tritis Forest	Total	Identified by species	Identified by genus	Unidentified
Agarics	38	15	38	91	10	37	44
Polypores	5	13	6	24	13	2	9
Jelly	0	2	3	5	5	0	0
Club	3	2	1	6	5	0	1
Cup	0	4	1	5	5	0	0
Coral	2	0	0	2	1	0	1
Total	48	36	49	133	39	39	55

gi (with a gelatinous texture), club fungi (cylindrical in shape), cup fungi (cup-shaped), and coral fungi (cylindrical with branching structures). Table 4 presents a total of 133 macrofungal morphogroups collected during the survey and their distribution across each forest track, of which 78 were successfully identified at the genus and species levels, while 55 specimens remained unidentified. Among the identified macrofungi, eight species were classified as edible: *Auricularia delicata*, *Auricularia auricula*, *Tremella mesenterica*, *Tremella fuciformis*, *Coprinellus disseminatus*, *Polyporus tricholoma*, *Coprinellus micaceus*, *Polyporus arcularius*, and *Auricularia cornea*. Twelve species were classified as inedible due to their texture, toxicity, or other properties: *Xylaria hypoxylon*, *Lachnellula occidentalis*, *Stereum ostrea*, *Stereum sanguinolentum*, *Dacryopinax elegans*, *Postia ptychogaster*, *Phillipsia subpurpurea*, *Marasmius capillaris*, *Marasmiellus candidus*, *Gloiocephala epiphylla*, *Lactarius vinaceorufescens*, and *Coprinus lagopus*. Six species were identified as medicinal due to their bioactive properties: *Ganoderma applanatum*, *Xylaria polymorpha*, *Daldinia concentrica*, *Microporus xanthopus*, *Trametes versicolor*, and *Cordyceps* sp. Additionally, several unidentified specimens were classified at the genus level, including *Mycena*, *Marasmius*, *Marasmiellus*, *Leucocoprinus*, *Lepiota*, *Gymnopus*, *Hygrocybe*, *Tubaria*, *Trogia*, *Hypholoma*, *Xeromphalina*, *Lichenomphalia*, *Clavaria*, *Pterula*, *Crepidotus*, *Psathyrella*, *Pluteus*, *Leucoagaricus*, *Laetiporus*, *Pulvinula*, *Neofavolus*, *Aleurodiscus*, *Lactarius*, *Favolaschia*, *Laccaria*, *Lactocollybia*, *Coprinus*, and *Campanella*.

Figure 3 illustrates the different types of macrofungal morphogroups documented in this study, while Figure 4 displays photographs of selected specimens that were identified to the genus or species level. Specimens that could not be identified to either genus or species were labelled as OTUs (Operational Taxonomic Units).

Table 5 represents the identified macrofungi recognized as either edible

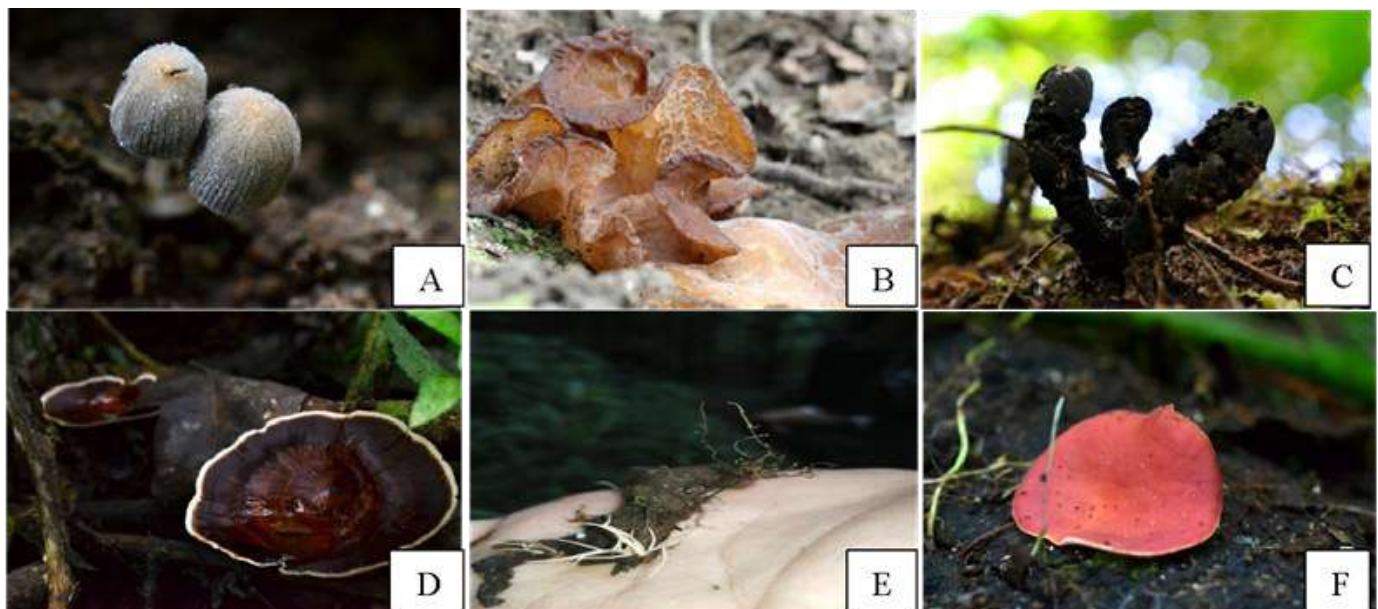


Figure 3. Representative morphogroups of Macrofungi that found in three forest of Turgo National Park, Mount Merapi: A) *Coprinellus micaceus* (agarics), B) *Auricularia delicata* (jelly), C) *Xylaria polymorpha* (club), D) *Microporus xanthopus* (Polypores), E) *Pterula* (coral), F) *Phillipsia subpurpurea* (cup).



Figure 4. Diversity of macrofungi that have been identified by genus or species name: 1) *Mycena* sp1. 2) *Crepidotus* sp1. 3) *Mycena* sp2. 4) *Gymnopus* sp1. 5) *Mycena* sp3. 6) *Auricularia delicata* 7) *Aleurodiscus* sp1. 8) *Xylaria polymorpha* 9) *Neofavolus* sp1. 10) *Neofavolus* sp2. 11) *Xylaria hypoxylon* 12) *Leucoagaricus* sp1. 13) *Microporus xanthopus* 14) *Pluteus* sp1. 15) *Marasmiellus* sp1. 16) *Leucocoprinus* sp1. 17) *Mycena* sp4. 18) *Tubaria* sp1. 19) *Campanella junghuhnii* 20) *Lepiota* sp1. 21) *Hemimycena* sp1. 22) *Cordyceps* sp1. 23) *Tubaria* sp2. 24) *Marasmius* sp1. 25) *Mycena* sp5. 26) *Gymnopus* sp2. 27) *Gloiocephala epiphylla* 28) *Mycena* sp6. 29) *Mycena* sp7. 30) *Coprinus lagopus* 31) *Hygrocybe* sp1. 32) *Trogia* sp1. 33) *Xeromphalina* sp1. 34) *Tubaria* sp3. 35) *Daldinia concentrica* 36) *Coprinellus* sp1. 37) *Coprinellus disseminatus* 38) *Lichenomphalia* 39) *Ganoderma applanatum* 40) *Hygrocybe* sp2. 41) *Gymnopus* sp3. 42) *Trametes versicolor* 43) *Psathyrella* sp1.

Some specimens that have not been identified either by species or genus: 44) OTU1 45) OTU2 46) OTU3 47) OTU4 48) OTU5 49) OTU6 50) OTU7 51) OTU8 52) OTU9 53) OTU10 53) OTU11 54) OTU12.



Figure 4. Contd.



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or medicinal. Edible macrofungi are those suitable for human consumption, while medicinal macrofungi contain bioactive compounds with potential health benefits and are used in alternative medicinal treatments. A unique local species, *Auricularia delicata*, is known as "jamur keprok" by the people of Turgo. This species was found exclusively and abundantly in forested bay areas at the highest sampling site. *Auricularia delicata* belongs to the ear mushroom family (*Auriculariaceae*), characterised by its gelatinous, ear-shaped, and jelly-like structure. From an ecological perspective, jelly fungi hold significant importance. In Bingungan Forest, jelly macrofungi had the second-highest ecological importance value after agarics, with values of 47.4 % and 82.6 %, respectively. Three species of jelly fungi were recorded in the Bingungan Forest between May and June: *Auricularia delicata*, *Dacryopinax elegans*, and *Tremella fuciformis*. Among them, *Auricularia delicata* exhibited the highest frequency of occurrence, with an ecological significance value of 44.2 %.

Another interesting finding was a specimen of wild *Cordyceps* that infects the body of an unidentified dead caterpillar. Although this specimen could not be classified to species level, as most *Cordyceps* species have been described, this *Cordyceps* is also categorised as a potential medicinal mushroom.

The spatial distribution of macrofungi across the three forest tracks in Turgo National Park, Mount Merapi (Figure 5, 6, 7) was mapped using GIS and drone-assisted visualisation technology. These maps precisely represent the GPS-based locations where each specimen was found and collected, providing a clear spatial context for understanding macrofungal diversity and habitat specificity.

Discussion

This study aims to explore and document the diversity and ecological roles of macrofungi in the tropical volcanic forest ecosystem of Turgo National Park, Mount Merapi, Indonesia. The specific objectives are: 1) To assess the species richness and diversity of macrofungi across three distinct forest tracks—Turgo, Bingungan, and Tritis—using quantitative diversity indices (e.g.,

Table 5. The identified mushrooms’s category of edibility.

Macrofungi species	Common Name	Category	References
<i>Auricularia auricula</i>	Wood ear	Edible	Zhao et al. 2019
<i>Auricularia delicata</i>	Jelly fungus	Edible	Wang et al. 2016; Wang et al. 2023
<i>Auricularia cornea</i>	Cloud ear	Edible	Drewinski et al. 2024
<i>Tremella mesenterica</i>	Witch’s butter	Edible	Hsu et al. 2014
<i>Tremella fuciformis</i>	Snow fungus	Edible	Mineroff & Jagdeo 2023
<i>Coprinellus disseminatus</i>	Fairy inky cap	Edible	Novakovic et al. 2016, 2018
<i>Coprinellus micaceus</i>	Glistening inky cap	Edible	Wang et al. 2021
<i>Polyporus tricholoma</i>	bristly tropical cup	Edible/medicinal	Vieira et al. 2008
<i>Polyporus arcularius</i>	Spring polypore	Edible/medicinal	Yen et al. 2022
<i>Ganoderma applanatum</i>	Artist’s fungus	Medicinal	Chen et al. 2021
<i>Xylaria polymorpha</i>	Dead man’s finger	Medicinal	Jang et al. 2007; Hacıoglu et al. 2011
<i>Daldinia concentrica</i>	Cramp ball	Medicinal	Benie et al. 2008
<i>Micoporus xanthopus</i>	Yellow-footed polypore	Medicinal	Ayu et al. 2019
<i>Trametes versicolor</i>	Turkey tails	Medicinal	Ajibola et al. 2024
<i>Cordyceps</i> sp.	-	Medicinal	Turk et al. 2022

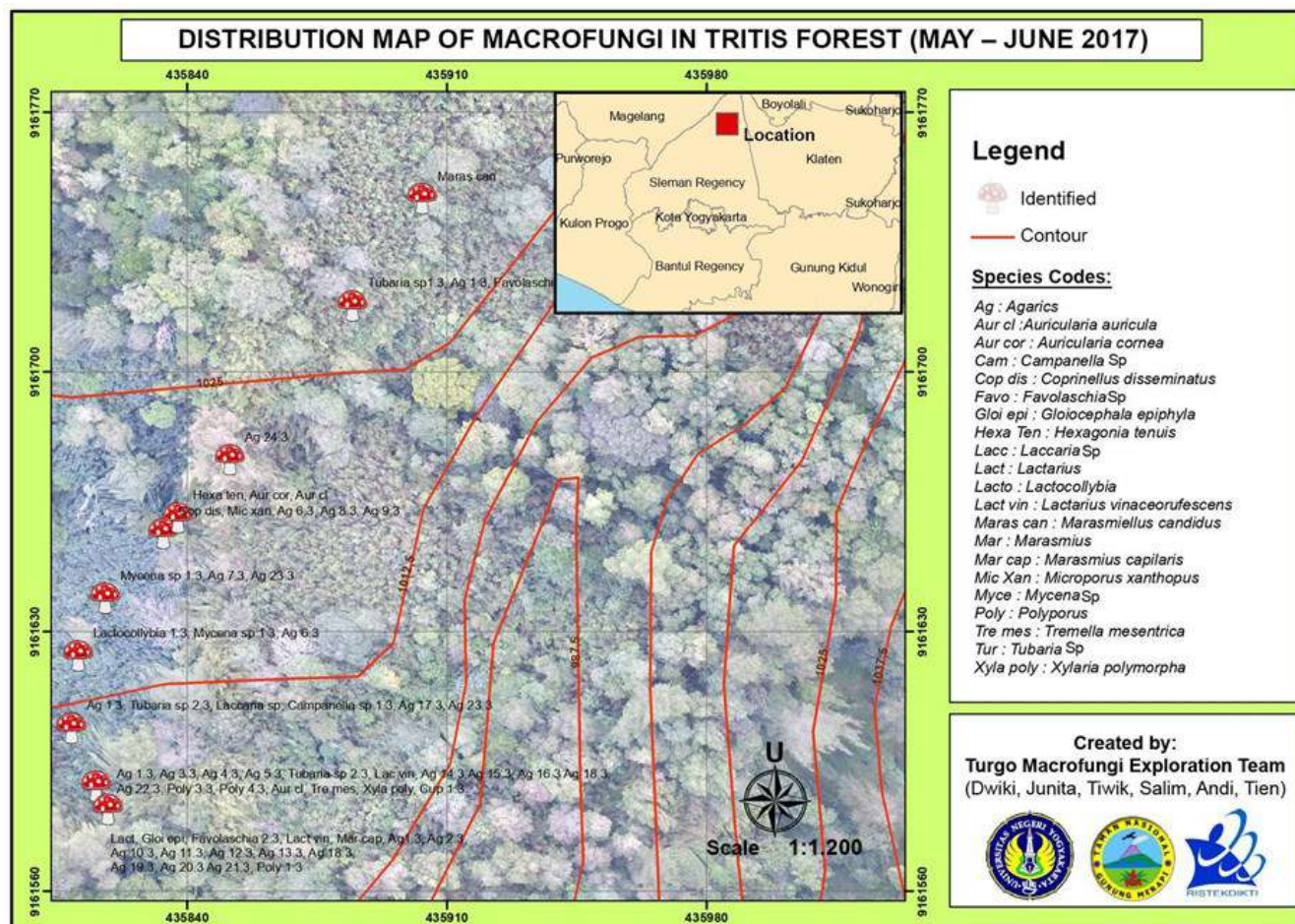


Figure 5. Representative distribution map of macrofungi using GPS coordinates sampling in Tritis forest.

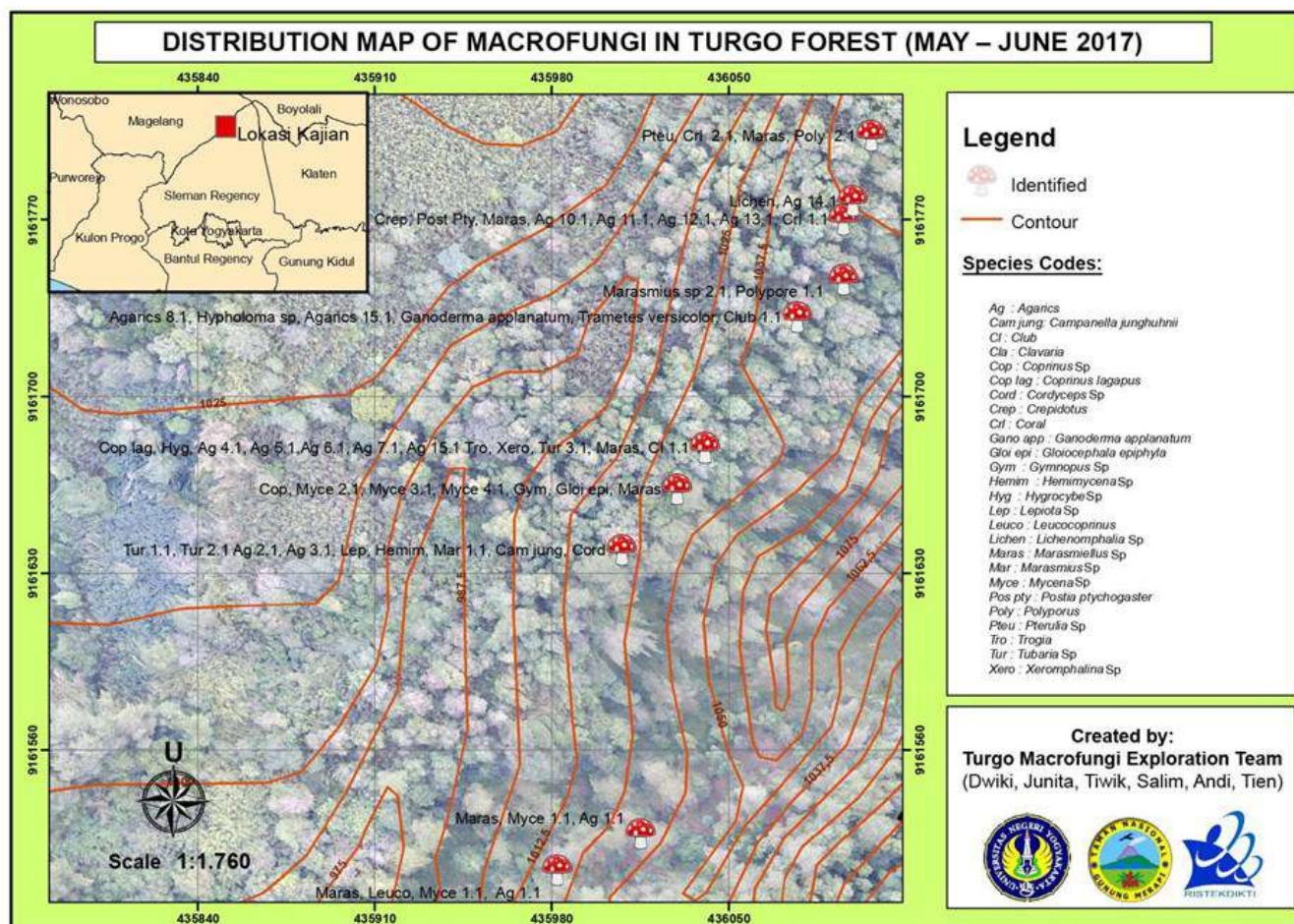


Figure 6. Representative distribution map of macrofungi using GPS coordinates sampling in Turgo forest.

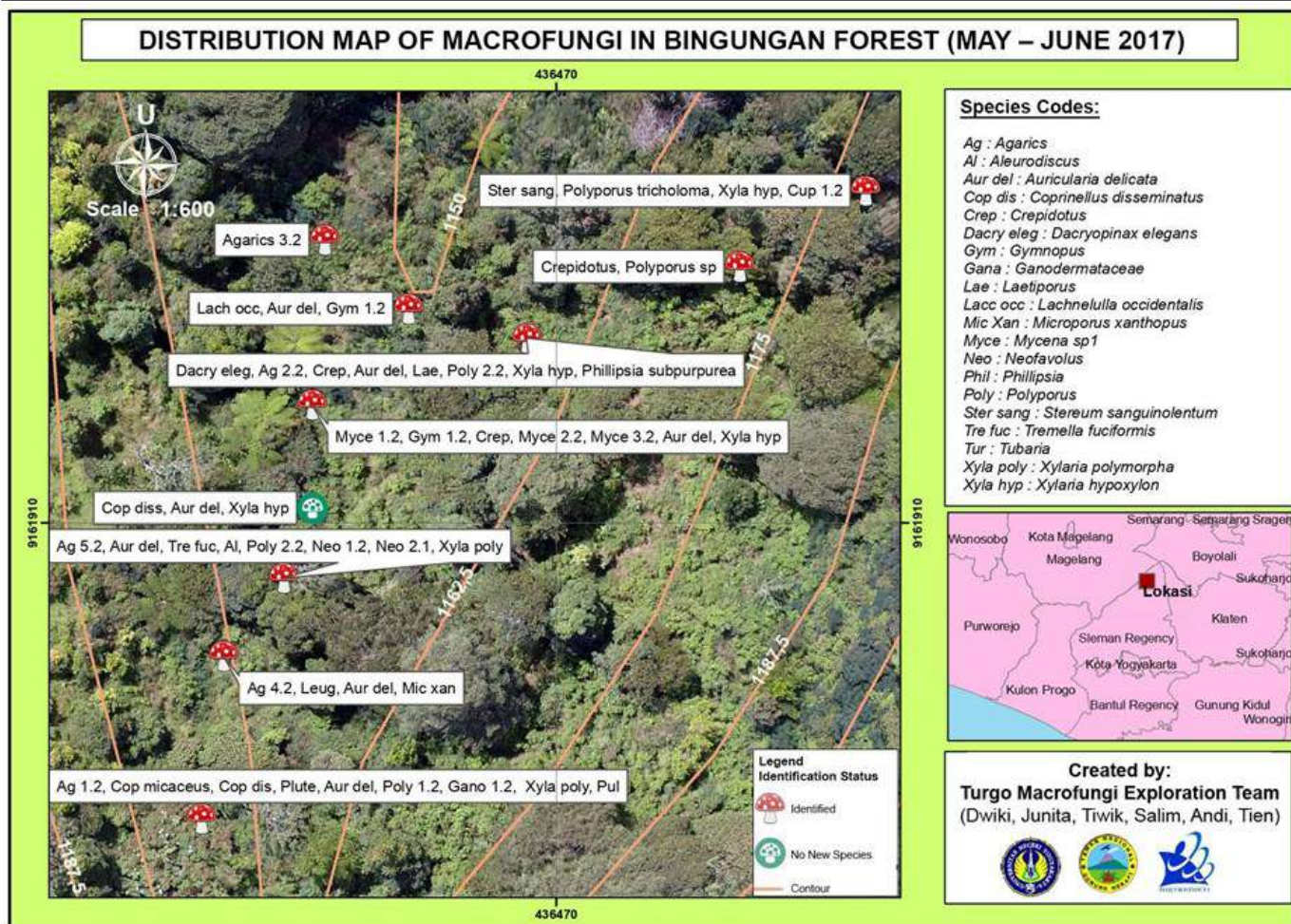


Figure 7. Representative distribution map of macrofungi using GPS coordinates sampling in Bingungan forest.

Shannon-Wiener Index). 2) To compare the similarity and composition of macrofungal communities between forest types by analysing Sørensen's similarity index and morphogroup distribution. 3) To examine the influence of environmental variables, such as elevation, vegetation structure, and microclimatic factors, on macrofungal diversity and distribution. 4) To identify and classify macrofungal species based on edibility, medicinal value, and ecological importance, including the documenting rare or culturally significant species such as *Auricularia delicata* and wild *Cordyceps*. Lastly, 5) to map the spatial distribution of macrofungi using GPS and GIS tools to better understand habitat specificity and inform future conservation efforts.

Exploration of macrofungi in Indonesia has been widely conducted; however, comprehensive ecological, physiological, and molecular documentation remain limited. Most research to date has focused primarily on diversity and morphological characteristics in specific regions, such as tropical tourist parks in North Sumatra (Nurhayat et al. 2021), karst forest areas (Armadhan et al. 2023), and several national parks (Putra et al. 2019; Yusran et al. 2022; Yafa et al. 2022). Additionally, macrofungal exploration has contributed to the identification of new species on small islands (Retnowati et al. 2022) and has also been conducted in palm plantation areas (Ekyastuti et al. 2023), among other locations.

Despite various efforts, the distribution of macrofungal diversity in Indonesia remains inadequately documented, and research on their ecological roles within ecosystems is still scarce. Consequently, macrofungal exploration remains underappreciated, except for certain edible species traditionally consumed by local communities. Unlike the well-established conservation initiatives for plants and animals, macrofungal conservation is still in its early stages. However, efforts have been gaining momentum, particularly in the

United Kingdom, other European countries, and tropical regions of the United States, such as Oregon, which serves as a global hub for mycological research.

Macrofungal conservation is intrinsically linked to the vegetation types that support their growth and fruiting, highlighting the strong ecological interactions between macrofungal abundance and habitat niches. This study adopts a conservation and management approach for forest macrofungi based on successful models implemented in the Pacific Northwest of the United States—Oregon, Washington, and Idaho. This conceptual framework, as outlined by Molina et al. (2001) and Molina et al. (2011), emphasises the critical role of macrofungal populations and communities in ecosystem functioning. Macrofungal diversity patterns are commonly analysed through diversity indices, assessments of similarity based on vegetation differences, and dominance patterns within specific sampling locations (Mueller et al. 2004; Prayudi et al. 2019). Furthermore, macrofungal biodiversity exploration using GPS to track survey inputs was incorporated (Figures 6, 7, and 8). As highlighted by Halme et al. (2012), this approach enhances long-term research efforts by facilitating the monitoring of diversity and population dynamics, particularly for wood-inhabiting macrofungi. Additionally, previous studies have aided in the targeted identification of macrofungal species. Notably, *Auricularia delicata* and *Xylaria polymorpha* exhibit a living strategy that maintains stable habitats throughout the year. Although these species are capable of colonizing other decayed or living wood nearby, they consistently remained within their initially observed range (Prasetya & Aminatun 2019a, 2019b).

To explore another ecological aspect, macrofungal sampling tracks based on elevation and vegetation structure were intentionally used to assess their influence on species diversity within macrofungal communities. In terms of elevation, comparing macrofungal communities across three sampling sites: the lowest-elevation area (Tritis Forest), the mid-elevation area (Turgo Forest), and the highest-elevation track (Bingungan Forest). Our findings indicate no significant differences in diversity among these sites. We propose that elevation may not directly influence macrofungal diversity within our study setting, as the lack of significant variation in elevation among our sampling sites—each categorised as mid-elevation forest—may account for the absence of significant differences in fungal community diversity indices (Chen et al. 2024). Classical ecological theories suggest that species diversity declines with increasing elevation due to harsher environmental conditions (Shen et al. 2020). In a linear trend, this principle may apply to fungal diversity and abundance (Wang et al. 2014). In terms of vegetation, our findings align with previous studies, which indicate that macrofungal diversity is higher in tropical coniferous forests than in tropical deciduous forests (O'Hanlon & Harrington 2012). However, these patterns may vary over time and space, as explained by Han et al. (2023), highlighting the importance of long-term monitoring to obtain a clearer picture of macrofungal biodiversity patterns. Furthermore, microclimatic factors such as humidity, precipitation, and temperature are key determinants of fungal activity in both temperate and tropical forest ecosystems (Durrall et al. 2006; Gómez-Hernández et al. 2012). Although inability to assess the significance of vegetation structure to the diversity of macrofungi in our study, records of some microclimatic ranges in which various macrofungi were located, climatic variables such as temperature and moisture are correlated with fungal abundance and richness in ecosystems (Talley et al. 2002).

Correlation analysis (Figure 1) provided further insights into these microclimatic influences. Temperature showed a perfect negative correlation with macrofungal diversity ($r = -1.00$, $p < 0.05$), suggesting that higher temperatures within the measured range suppress fungal richness. This result

supports ecological models proposing that fungi in tropical montane environments are adapted to cooler microclimates (Wang et al. 2014; Gómez-Hernández et al. 2012). Humidity ($r = -0.91$) and light intensity ($r = 0.74$) were also strongly correlated with diversity but were not statistically significant, possibly due to the limited number of plots or collinearity among variables. The negative trend with humidity may reflect moisture saturation, which can reduce oxygen availability or increase microbial competition. Meanwhile, the positive correlation with light intensity suggests a potential benefit of partial canopy openness in promoting species richness (O'Hanlon & Harrington 2012).

Overall, temperature emerged as the most reliable predictor of macrofungal diversity in this study. These findings emphasize the importance of microclimate monitoring and the need for larger datasets to validate ecological relationships.

To analyse fungal biodiversity patterns across the three forest sites, we employed Shannon-Wiener's diversity index and Sørensen's dissimilarity index. Based on Magurran's book published in 1988, both indices effectively differentiate fungal communities among sites and are sensitive to sample size, making them suitable for estimating fungal diversity patterns (Magurran 1988). These indices have also been applied in other macrofungal ecology and biodiversity studies (Ruiz-Almenara et al. 2019; Amenan et al. 2020; Joshi et al. 2022).

In addition to exploring macrofungal ecology, their edibility was also investigated. Prior to conducting our research, inquiries with local citizens about known or unknown wild mushrooms consumed in the area were performed. One identified species was wild *Auricularia delicata*, which produced an abundant fruiting body population limited to Bingungan Forest. This macrofungus is widely consumed in East Asia, Africa, Brazil, and India; in China, it has been successfully cultivated from wild specimens (Wang et al. 2016, 2023). Recent studies have also demonstrated its medicinal potential, particularly its antimicrobial activity (Wangkheirakpam et al. 2018). Among the three study sites, other edible *Auricularia* species and other jelly fungi (*Tremella mesenterica* and *Tremella fuciformis*) were spotted, although their fruiting body populations were small and not site-specific, unlike *Auricularia delicata*.

In Bingungan Forest, in addition to the *Auricularia delicata* population, another intriguing macrofungus, *Xylaria polymorpha*, which exhibited a large abundance of fruiting bodies, was observed. The widespread distribution of *Xylaria polymorpha* is attributed to its ability to colonise decayed wood over a broad range (Berger 1985; Rogers & Callan 1986). Despite its persistence across varying weather, seasons, and dry conditions (Prayudi et al. 2019; Chuzho & Dkhar 2019) this species has been studied for its antimicrobial and cytotoxic effects, indicating potential for antibiotic development (Jang et al. 2007; Hacıoglu et al. 2011). Another resilient Ascomycota species, *Daldinia concentrica*, has been investigated for its cytotoxic effects on cancer cells (Trung et al. 2019) and antimicrobial activity (Goswami et al. 2020). From the bracket fungi group, we identified several species with medicinal potential: *Ganoderma applanatum*, known for its benefits in managing diabetes, hyperlipidaemia, and hepatic degeneration (Hossain et al. 2021); *Trametes versicolor*, explored as a potential colorectal cancer therapeutic drug (He et al. 2022); and *Microporus xanthopus*, which possesses antimicrobial and antioxidant properties (Sholola et al. 2022). Additionally, among polypores, *Polyporus tricholoma*, recognised for its antimicrobial activity and edibility (Vieira et al. 2008) and *Polyporus arcularius*, known for its antioxidant and antimicrobial properties (Yen et al. 2022), were found. Among the most widely distributed agarics, the confirmation of edibility or medicinal properties of two species:

Coprinellus disseminatus, used for flavouring foods and known for its antioxidant and antiproliferative effects (Novaković et al. 2016, 2018), and *Coprinellus micaceus*, which has a high potassium content and pleasant flavour but is toxic when consumed with alcohol due to "Coprinus syndrome" (Michelot 1992). Unexpectedly, we discovered a wild *Cordyceps* species during our study. *Cordyceps* is known to contain cordycepin, a bioactive compound that has been used for centuries in traditional medicine for health and therapeutic purposes (Ashraf et al. 2020; Tapingkae et al. 2024). This compound, along with other bioactive constituents, has been extensively studied for its potential biomedical applications, including anticancer, antioxidant, anti-inflammatory, anti-ageing, immunomodulatory, antimicrobial, immunosuppressive, hypolipidemic, hypoglycaemic, neuroprotective, and fertility-enhancing properties (Shrestha et al. 2012; Shweta et al. 2023). Given the significance of these findings, further research is necessary to elucidate the molecular taxonomy, medicinal properties, ecological distribution, and specific habitat of this wild *Cordyceps* species.

Our study highlights the ecological importance of macrofungi, their potential conservation significance, and their medicinal and nutritional values. Continuing exploration using advanced molecular techniques and long-term monitoring is essential for a comprehensive understanding of macrofungal biodiversity, particularly in tropical forests where their roles remain underappreciated. Future research should integrate ecological, conservation, ethnomycological, and pharmacological perspectives to maximise the potential of macrofungi in sustaining both ecosystems and human well-being.

CONCLUSION

This study reveals that the Turgo tropical forest ecosystem on Mount Merapi supports a medium to high level of macrofungal diversity, with agarics emerging as the most ecologically dominant morphogroup. The variation in species richness across different forest types—coniferous, deciduous, and mixed—highlights the strong influence of vegetation structure on macrofungal distribution. In contrast, elevation appeared to have limited impact on fungal diversity due to the narrow altitudinal range of the study sites.

Importantly, the research identified several macrofungal species with nutritional, medicinal, and ecological significance, including *Auricularia delicata*, *Xylaria polymorpha*, and a wild *Cordyceps* specimen. These findings underscore the potential value of macrofungi for food, traditional medicine, and ecosystem functioning. However, the presence of many unidentified specimens points to a significant gap in taxonomic knowledge and highlights the urgent need for further exploration using molecular techniques.

Overall, this study contributes essential baseline data for future ecological, ethnomycological, and conservation-focused research on macrofungi in tropical volcanic ecosystems. It also highlights the necessity of integrating local biodiversity knowledge with systematic scientific investigation to support sustainable forest management and fungal conservation initiatives in Indonesia.

AUTHOR'S CONTRIBUTION

D.P. and I.S. contributed to all efforts for data collection, analysis, figure preparation, and manuscript writing. T. Aminatun provided overall supervision for the study, conceptualising the research framework and developing the idea exploring the relationship between ecological patterns and mushroom edibility within the ecosystem.

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CONFLICT OF INTEREST

We confirm that there are no conflicts of interest related to this study.

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Research Article

Molecular Identification and Phylogenetic Analysis of Andong in Bali Using matK Based DNA Barcoding

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ABSTRACT

Andong (*Cordyline fruticosa*) is a tropical plant with high ethnobotanical and cultural value. Despite its traditional usage, its taxonomic classification is still under debate, as various literature sources place it in different families, such as Liliaceae, Agavaceae, and Asparagaceae. This discrepancy indicates that morphology-based identification alone is insufficient for accurate classification. This study aims to determine the genetic identity and phylogenetic relationship of andong in Bali using matK gene-based DNA barcoding. DNA was isolated from andong leaves, amplified by PCR, sequenced, and then analysed using BLAST and phylogenetic tree reconstruction. The resulting 862 bp consensus sequence showed 99.8 % similarity to *Cordyline fruticosa* (OQ289248.1) from the USA. The phylogenetic tree constructed using the Neighbour-Joining method grouped andong in the same clade as that species, supported by a bootstrap value of 71 % and a genetic distance of 0.001. These findings supported the classification of andong in the family Asparagaceae, as it is genetically more closely related to species in this family than to those in other families. This study confirmed that DNA barcoding using the matK gene is effective for taxonomic clarification and the conservation of local plant genetic resources.

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INTRODUCTION

Cordyline fruticosa, locally known as andong, is a tropical plant native to Indonesia with significant ethnobotanical and cultural importance, especially in Bali. This species naturally grows in various regions across the Indonesian archipelago and has long been part of traditional practices, making it an important element of Indonesia's native biodiversity. In Bali, it is commonly cultivated as an ornamental plant and frequently used in traditional ceremonies such as *Tumpek Landep*. Its leaves are also used in various medicinal preparations to treat wounds, nosebleeds, digestive disorders, and hemorrhoids (Hildasari & Hayati 2021; Prayitno et al. 2024). In many regions of Indonesia, the plant also carries symbolic meanings in traditional ceremonies. In Bali, for instance, its leaves are used in the *tumpek landep* ceremony as part of the *ban-ten* offering (Sudarsana & Santha 2024), while in Kerinci, andong is incorporated in the *kenduri seko* ritual as a spiritual symbol (Santosa et al. 2020).

Although widely used, the taxonomic classification of andong remains inconsistent in scientific literature. Some literature classifies the species under the Liliaceae family (Pasorong et al. 2015; Santosa et al. 2020; Hildasari & Hayati 2021; Shabira et al. 2024), while others place it in Agavaceae (Hakim 2014; Naher et al. 2019; Elfita et al. 2021) or Asparagaceae (Nahor et al. 2020; Raslan et al. 2021). This inconsistency suggests that morphology-based classification may be insufficient, especially considering that plant morphological traits are often influenced by environmental factors, age, and growth conditions (Suparman 2012; Ruwaida et al. 2009).

With the advancement of molecular biology, DNA-based identification methods such as DNA barcoding have become increasingly utilised to support taxonomic classification, phylogenetic analysis, and the conservation and sustainable use of genetic resources. One of the most commonly used genes in plant DNA barcoding is the *matK* gene, which is derived from chloroplast DNA. This gene has a high substitution rate, spans 1500 base pairs (bp), and is frequently used to assess both intraspecific and interspecific genetic diversity (Selvaraj et al. 2008; Harnelly et al. 2018). The *matK* gene, with its high substitution rate, has been widely used for plant DNA barcoding both at interspecific and intraspecific levels in various studies, including those on orchids (*Vanda tricolor*), and melons (*Cucumis melo*) (Rosida 2022; Retnaningati 2017).

Therefore, this study aims to investigate the taxonomic classification and genetic relationships of *C. fruticosa* based on DNA barcoding using the *matK* gene. The results are expected to contribute scientific insights that support the identification and sustainable utilisation of biodiversity.

MATERIALS AND METHODS

Materials

Fresh leaves of andong were collected from a home garden in Jl. Basuki Rahmat, Sumerta Kelod, Denpasar, Bali (8°40'08.9"S, 115°14'07.3"E). Other reagents included 96 % ethanol, *matK* primers (forward and reverse), distilled water, etidium bromide (EtBr), Quick-DNA Magbead Plus Kit (Zymo Research, D4082), ddH₂O, My Taq HS Red Mix (2X), 1 g of agarose, parafilm paper, and 100 ml of TBE buffer.

The tools used in this study were a PCR machine, agarose gel, Eppendorf tubes, electrophoresis machine, vortex machine, measuring cylinder, mortar and pestle, analytical balance, centrifuge, micropipette, microwave, UV transilluminator, magnetic stand, capillary electrophoresis system, MEGA 11 (Molecular Evolutionary Genetic Analysis) software, BioEdit Sequence software, and access to the NCBI (National Centre for Biotechnology Information) website.

Methods

This study involved several stages, including total DNA isolation, followed by PCR amplification of the *matK* gene, electrophoresis and DNA visualisation, DNA sequencing, BLASTN analysis, and phylogenetic tree reconstruction.

DNA Isolation

Using the Quick-DNA Magbead Plus Kit, DNA was extracted from 50 mg of powdered leaf tissue. The leaf powder was then transferred into tube, and 750 µl of DNA/RNA Shield buffer was added. The mixture was vortexed for 1 minute at maximum speed and sealed tightly. The tube was then centrifuged at 10,000 x g for 1 minute, and 400 µl of the supernatant was transferred into a new tube. Next, DNA purification was carried out by adding 400 µl of Quick-DNA MagBinding Buffer and 33 µl of MagBinding Beads to the 400 µl of digested sample. The sample mixture was incubated for 10 minutes, then transferred to a magnetic stand until the beads separated from the solution, and the supernatant was discarded. The sample was washed with 500 µl of DNA Pre-Wash Buffer, then centrifuged, and this washing step was repeated. After that, the sample was washed again by adding 900 µl of g-DNA Wash Buffer and centrifuged twice. The sample was then incubated at 55 °C for 10 minutes, followed by the addition of 50 µl of DNA Elution Buffer, and shaken with a shaker at room temperature for 5 minutes. The sample was then transferred back to the magnetic stand until the beads separated from the solution, and the eluted DNA was transferred to a new tube. Furthermore, a quantitative test of DNA was carried out to determine the purity and concentration of DNA samples measured using a nanodrop.

Amplification of *matK* Gene by PCR

DNA amplification was carried out using the PCR technique with 2x MyTaq HS Red Mix. The primer pairs used were *matK* consisting of *matK*-F primer (5'-CGT ACA GTA CTT TTG TGT TTA CGA G-3') and *matK*-R primer (3'-ACC CAG TCC ATC TGG AAA TCT TGG TTC-5'). The PCR reaction component contains 25 µL consisting of 9.5 µL ddH₂O, 12.5 µL My Taq HS Red Mix (2X), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 1 µL template DNA. PCR stages in this study began with setting the initial pre-denaturation temperature of 95 °C for 1 minute and continued with 35 cycles, namely denaturation 95 °C for 10 seconds, annealing 52 °C for 15 seconds, extension 72 °C for 15 seconds, and storage temperature at the final stage 4 °C.

DNA Electrophoresis and Visualization

The amplified product was analysed using 1 % TBE agarose gel electrophoresis. Agarose was mixed with TBE buffer, heated until fully dissolved, cooled to 60 °C, and poured into a plate until solidified. Once the gel had solidified, it was transferred to an electrophoresis chamber filled with buffer. A total of 2 µL of the amplified product and a DNA ladder (1 µL loading dye with 2.5 µL DNA ladder) were loaded into the wells, then electrophoresed at 135V for 25 minutes. After electrophoresis, the gel was stained with 2 µL of ethidium bromide solution, and the resulting DNA bands were observed using a UV transilluminator.

DNA sequencing analysis

Sequencing was performed by PT Genetics Science Indonesia. BioEdit was used to align and construct a consensus sequence, which was then compared with GenBank entries via Basic Local Alignment Search Tool (BLAST) on the website (<http://blast.ncbi.nlm.nih.gov>).

Data analysis

DNA sequences obtained from GenBank were aligned to determine the level of similarity between the DNA sequences of the samples studied and the sequences that have been registered in the GenBank database. Genetic distance analysis was performed to measure the genetic variation between the sequences being compared. The phylogenetic tree was then reconstructed using MEGA 11 software with the Neighbour-Joining Tree method, Kimura 2-parameter evolutionary model, and bootstrap test with 1000 replicates to estimate the accuracy and stability of the phylogenetic reconstruction results

RESULTS AND DISCUSSION

Figure 1 presents the andong (*C. fruticosa*) plant used as the research sample. Morphological documentation was conducted as an initial step before molecular analysis to ensure the specimen's identification and to provide visual reference for further DNA barcoding and phylogenetic studies.



Figure 1. Andong plant used as the sample in this study collected from a home garden in Sumerta Kelod, Denpasar, Bali.

The concentration of andong DNA was $39.7 \text{ ng } \mu\text{l}^{-1}$, with an $A_{260}/_{280}$ purity ratio of 1.87. According to Dewanata and Mushlih (2021), DNA is considered pure if the absorbance ratio is between 1.8 and 2.0. DNA purity is determined by dividing the absorbance value at 260 nm by the absorbance value at 280 nm. A purity ratio below 1.8 indicated contamination with proteins, phenols, or other substances, while a ratio above 2.0 suggests RNA contamination (Mushlih 2019). Based on these results, the DNA sample meet the required purity standards and was therefore suitable for the next stage of analysis. DNA amplification of andong plants using matK primers, as shown in Figure 2, was successful, with a 1 % TBE agarose gel electrophoresis revealing a clear DNA band at 862 bp. The amplification results indicated a successful process, as evidenced by the absence of smear and a distinct banding pattern.

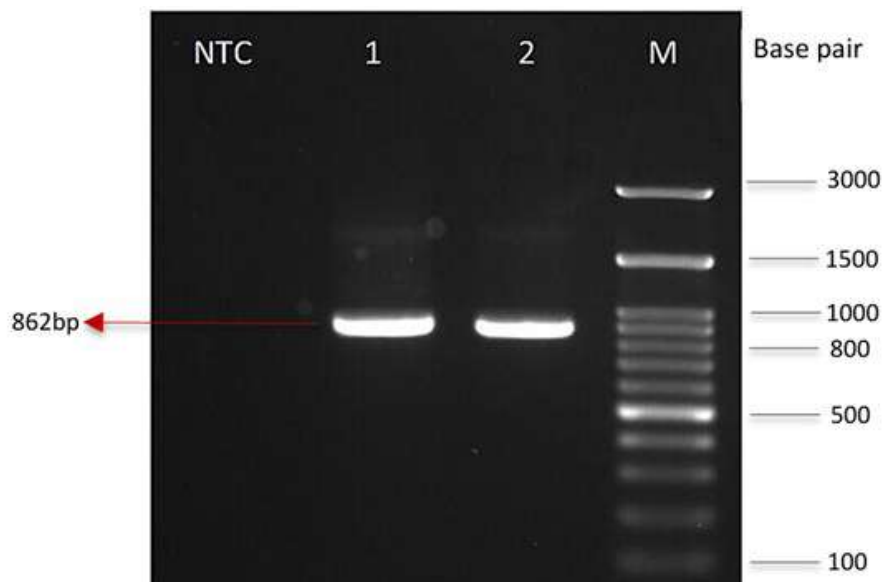


Figure 2. Visualisation of electrophoresis results of PCR products with matK primers. NTC = negative control; 1 = Andong DNA sample; 2 = positive control; M = DNA marker.

In this study, the DNA sequencing process of andong plants resulted in a forward sequence of 840 bp and a reverse sequence of 829 bp. Through the contig process using BioEdit software, a consensus sequence with a length of 862 bp was resulting representing the nucleotide base sequence of the andong plant shown in Table 1. The sequencing results were registered with NCBI and obtained accession number PV172161.

The consensus sequence was analysed using the BLAST (Basic Local Alignment Search Tool) program. Information from BLAST results in the form of data: Percentage identity, E-value, and Query Coverage. Percentage identity is a value that shows the percentage of matches between andong plant DNA sequences and sequences in the database, which illustrates the level of similarity between sequences. Meanwhile, E-value (Expected Value) is a number that indicates how often a match can occur by chance in a database. The smaller the E-value, the less likely it is that the match occurred just by chance, the more trustworthy the result is. Query Coverage is the percentage of nucleotide length that aligns between andong plant sequences and sequences in the database (Sogandi 2018; Rahmawati & Shabrina 2024).

The BLAST results, presented in Table 2, showed the top 10 GenBank entries, with percentage identity ranging from 96.52 % to 99.87 %. The andong plant from Bali exhibited the highest similarity of 99.87 % with *Cordyline fruticosa* (OQ289248.1) from the United States, indicating a very close genetic relationship. Furthermore, andong plants were 99.6 % similar to *C. cannifolia* (JX903594.1) and *C. stricta* (OQ290052.1), and 99.4 % similar to *C. pumilio* (JQ435553.1) and *C. australis* (KX229865.1). The most similar GenBank sequences showed comparable Max Scores, Total Scores, Query Coverage close to 100 %, E-values near 0, and Percentage Identity close to 100 %.

Based on the 10 sequences obtained from the BLAST alignment, further analysis was conducted to assess the level of similarity and genetic distance. This analysis involved the andong plant sequences, and 10 sequences from GenBank.

The analysis results showed that the andong plant had a very high similarity level with *C. fruticosa* (99.8 %), indicating the possibility that they belong to the same species (Table 3). Other species within the *Cordyline* genus, such as *C. cannifolia* and *C. stricta*, also exhibited high similarity levels, ranging from 99.4 % to 99.6 %. Meanwhile, species from other genus, such as *Asparagus*, had lower similarity values, ranging from 96.3 % to 96.4 %. Accord-

Table 1. DNA sequencing results of andong plants.

Forward sequences (840 bp)	ATCTTGGTTCAAATTCTTCAATGCTGGATTCAAGATGTTTCCTTTCTT- GCATTTATTGCGATTCTTTCTTCACGAATATCATAATTGGAATAGTCTTCTCA TTACTCAGAAGAAATCTATTTACGTTTTTTTCAAAAAGAAAATAAAAAGATTATTT CGGTTCCCTATACAATTCTTATGTATTTGAATGTGAATTTTTATTAGTTTTTA TTCGTAAACAATCTTCTTATTTACGATTAACATCTTCTGGAACCTTTCTTGAG CGAACACATTTCTATGGAAAAATAGAACATCTTCAAATAGAACATTTTATAGT AGTATGTCGTAACATTTTTTCATAGAACCCTATGGATCTTCAAAGATCCTTTCA TGCATTATGTTCAATATCAAAGAAAAGCAATTCTTGCTTCAAAGGGGACTTAT CTTCTGATGAAGAAATGGAAATATCATTTTTGTCAATTTCTGGCAATATTATTT TCACTTTTGGTCTCAACCGTATAGGATCCATATAAAACCAATTATCAAACATTC TTTCTATTTTCTGGGTATCTTTCAAGTCTACTAATACATTTTTTCGGCGGTAA GGAATCAAATGTTAGAGAATTCTTTTCTAATAGATACCGTTACTAAGAAATTT GATACCATAGTCCCGGTATTCTTCTTATTGGATCCTTGGCTAAAGCTAAATT TTGTACCGTATCGGGCCATCCTATTAGTAAGCCGATCTGGGCCGATTATCGG ATTCTGATATTCTTGATCGATTCTGGTCCGATATGTAGAAATTTTTCTCATTAT CACAGTGGATCCTCAAAAAAACAGGGTTTGTATCGAATAAAGTATATACTCGA
Reverse sequences (829 bp)	GATGTTTCCTTTTTTGCATTTATTGCGATTCTTTCTTCACGAATATCATAATT- GGAATAGTCTTCTCATTACTCAGAAGAAATCTATTTACGTTTTTTTCAAAAAGAA AATAAAAGATTATTTTCGGTTCCTATACAATTCTTATGTATTTGAATGTGAATT TTTATTAGTTTTTATTCGTAAACAATCTTCTTATTTACGATTAACATCTTCTG GAACCTTTCTTGAGCGAACACATTTCTATGGAAAAATAGAACATCTTCAAATA GAACATTTTATAGTAGTATGTCGTAACATTTTTTCATAGAACCCTATGGATCTT CAAAGATCCTTTTCATGCATTATGTTCAATATCAAAGAAAAGCAATTCTTGCTT CAAAGGGGACTTATCTTCTGATGAAGAAATGGAAATATCATTTTTGTCAATTTT TGGCAATATTATTTTCACTTTTGGTCTCAACCGTATAGGATCCATATAAAACCA ATTATCAAACATTTCTTTCTATTTTCTGGGTATCTTTCAAGTCTACTAATAC ATTTTTTCGGCGGTAAAGGAATCAAATGTTAGAGAATTCTTTTCTAATAGATACC GTTACTAAGAAATTTGATACCATAGTCCCGGTATTCTTCTTATTGGATCCTT GGCTAAAGCTAAATTTTGTACCGTATCGGGCCATCCTATTAGTAAGCCGATCT GGGCCGATTATCGGATTCTGATATTCTTGATCGATTCTGGTCCGATATGTAG AAATTTTTCTCATTATCACAGTGGATCCTCAAAAAAACAGGGTTTGTATCGAA TAAAGTATATACTTCGACTTTTCGTGTGCTAGAACTT
Consensus se- quences (862 bp)	ATCTTGGTTCAAATTCTTCAATGCTGGATTCAAGATGTTTCCTTTCTT- GCATTTATTGCGATTCTTTCTTCACGAATATCATAATTGGAATAGTCTTCTCA TTACTCAGAAGAAATCTATTTACGTTTTTTTCAAAAAGAAAATAAAAAGATTATTT CGGTTCCCTATACAATTCTTATGTATTTGAATGTGAATTTTTATTAGTTTTTA TTCGTAAACAATCTTCTTATTTACGATTAACATCTTCTGGAACCTTTCTTGAG CGAACACATTTCTATGGAAAAATAGAACATCTTCAAATAGAACATTTTATAGT AGTATGTCGTAACATTTTTTCATAGAACCCTATGGATCTTCAAAGATCCTTTCA TGCATTATGTTCAATATCAAAGAAAAGCAATTCTTGCTTCAAAGGGGACTTAT CTTCTGATGAAGAAATGGAAATATCATTTTTGTCAATTTCTGGCAATATTATTT TCACTTTTGGTCTCAACCGTATAGGATCCATATAAAACCAATTATCAAACATTC TTTCTATTTTCTGGGTATCTTTCAAGTCTACTAATACATTTTTTCGGCGGTAA GGAATCAAATGTTAGAGAATTCTTTTCTAATAGATACCGTTACTAAGAAATTT GATACCATAGTCCCGGTATTCTTCTTATTGGATCCTTGGCTAAAGCTAAATT TTGTACCGTATCGGGCCATCCTATTAGTAAGCCGATCTGGGCCGATTATCGG ATTCTGATATTCTTGATCGATTCTGGTCCGATATGTAGAAATTTTTCTCATTAT CACAGTGGATCCTCAAAAAAACAGGGTTTGTATCGAATAAAGTATATACTTCG ACTTTCGTGTGCTAGAACTT

ing to the criteria established by Drancourt et al. in Afrianti et al. (2023), a similarity value of ≥ 99 % indicated that two sequences originate from the same species, whereas values between 97 % and 99 % suggest similarity at the genus level. If the similarity value falls within the 89-93 % range, the sequences likely belong to different families.

The genetic distance analysis also revealed an evolutionary relationship pattern consistent with the similarity results (Table 4). The lowest genetic distance value (0.001) was found between the andong plant and *C. fruticosa*,

Table 2. List of samples obtained from NCBI GeneBank.

Species	Max Score	Total Score	Percentage identity	E-value	Query cover	Accession number	Source
<i>Cordyline fruticosa</i>	1439	1439	99.87 %	0.0	90 %	OQ289248.1	USA
<i>Cordyline cannifolia</i>	1576	1576	99.65 %	0.0	100 %	JX903594.1	Korea
<i>Cordyline stricta</i>	1447	1447	99.62 %	0.0	92 %	OQ290052.1	USA
<i>Cordyline pumilio</i>	1565	1565	99.42 %	0.0	100 %	JQ435553.1	UK
<i>Cordyline australis</i>	1548	1548	99.41 %	0.0	99 %	KX229865.1	UK
<i>Asparagus officinalis specimen_voucher</i>	1426	1426	96.52 %	0.0	100 %	AB646504.1	Thailand
<i>Asparagus cochinchinensis</i>	1426	1426	96.52 %	0.0	100 %	KY457631.1	Taiwan
<i>Asparagus officinalis</i>	1426	1426	96.52 %	0.0	100 %	HQ180855.1	USA
<i>Asparagus oligoclonos</i>	1426	1426	96.52 %	0.0	100 %	JF972921.1	Korea
<i>Eustrephus latifolius</i>	1426	1426	96.52 %	0.0	100 %	JQ435554.1	UK

Table 3. Similarity percentage of andong plants with species in GenBank.

No	Species	Similarity percentage (%)										
		1	2	3	4	5	6	7	8	9	10	11
1	Andong	ID										
2	<i>C. fruticosa</i>	99.8	ID									
3	<i>C. cannifolia</i>	99.6	99.7	ID								
4	<i>C. stricta</i>	99.6	99.7	100	ID							
5	<i>C. pumilio</i>	99.4	99.6	99.6	99.6	ID						
6	<i>C. australis</i>	99.4	99.6	99.6	99.6	100	ID					
7	<i>A. officinalis specimen_voucher</i>	96.4	96.6	96.6	96.6	96.8	96.8	ID				
8	<i>A. cochinchinensis</i>	96.4	96.6	96.6	96.6	96.8	96.8	100	ID			
9	<i>A. officinalis</i>	96.4	96.6	96.6	96.6	96.8	96.8	100	100	ID		
10	<i>A. oligoclonos</i>	96.4	96.6	96.6	96.6	96.8	96.8	100	100	100	ID	
11	<i>E. latifolius</i>	96.3	96.4	96.4	96.4	96.6	96.6	94.6	94.6	94.6	94.6	ID

Table 4. Genetic distance of andong plants with species in GenBank.

No	Species	Genetic Distance										
		1	2	3	4	5	6	7	8	9	10	11
1	Andong	ID										
2	<i>C. fruticosa</i>	0.001	ID									
3	<i>C. cannifolia</i>	0.003	0.002	ID								
4	<i>C. stricta</i>	0.003	0.002	0.000	ID							
5	<i>C. pumilio</i>	0.005	0.003	0.003	0.003	ID						
6	<i>C. australis</i>	0.005	0.003	0.003	0.003	0.000	ID					
7	<i>A. officinalis specimen_voucher</i>	0.036	0.034	0.034	0.034	0.031	0.031	ID				
8	<i>A. cochinchinensis</i>	0.036	0.034	0.034	0.034	0.031	0.031	0.000	ID			
9	<i>A. officinalis</i>	0.036	0.034	0.034	0.034	0.031	0.031	0.000	0.000	ID		
10	<i>A. oligoclonos</i>	0.036	0.034	0.034	0.034	0.031	0.031	0.000	0.000	0.000	ID	
11	<i>E. latifolius</i>	0.037	0.035	0.035	0.035	0.034	0.034	0.055	0.055	0.055	0.055	ID

indicating a very close genetic relationship. Other species within the *Cordyline* genus, such as *C. cannifolia* and *C. stricta*, had slightly larger genetic distances (0.002-0.003) but still demonstrated close relationships. In contrast, species from the *Asparagus* genus exhibited higher genetic distances (≥ 0.031), reflecting a more distant taxonomic relationship. The smaller the genetic distance between two species, the higher their similarity, signifying a closer evolutionary relationship. Conversely, a greater genetic distance suggested a more distant relationship, indicating differences at a higher taxonomic level, such as genus or family.

Based on the similarity and genetic distance analysis, it can be concluded that the andong plant has a very close genetic relationship with *C. fruticosa*, strongly suggesting that it is a variety of this species. Minor differences in nucleotide sequences may be due to environmental factors, ecological differences, or natural genetic variation within populations. These findings suggested that the andong plant in Bali may represent a locally adapted variant of *C. fruticosa*.

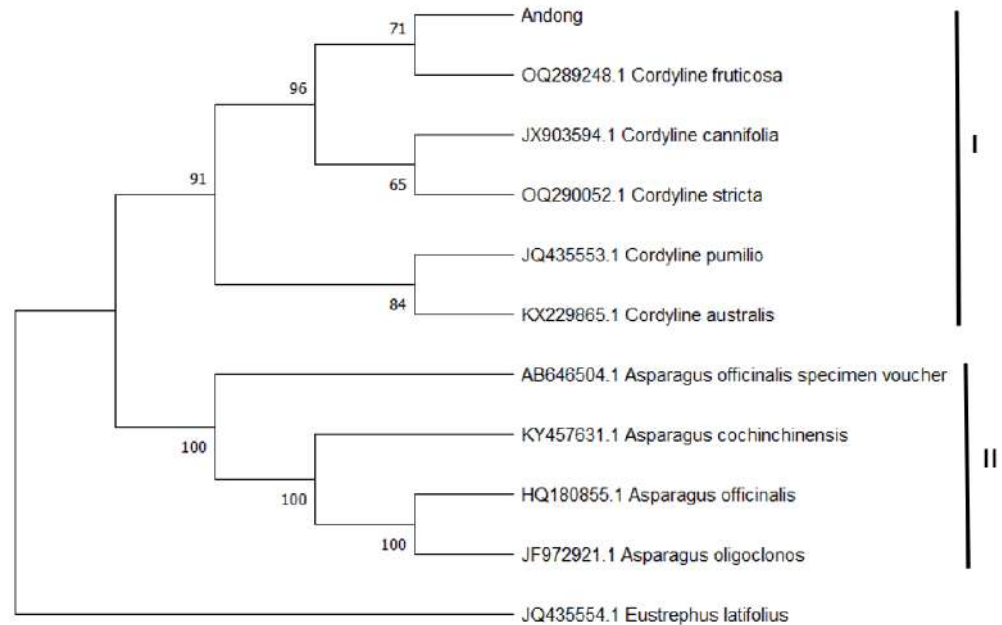


Figure 3. Phylogenetic tree of andong based on matK gene using the Neighbor-Joining method with 1000 bootstrap replications.

Phylogenetics is a method used to analyse evolutionary or phylogenetic relationships between organisms (Ayu et al. 2021). Phylogenetic analysis using the Neighbour-Joining method with a 2-parameter Kimura model and a bootstrap of 1000 replicates successfully reconstructed the phylogenetic relationship of andong plants in the Asparagus family (*Asparagaceae*). The numbers on the branches of the phylogenetic tree represent bootstrap values, which indicate the accuracy of the formed branching.

The phylogenetic reconstruction using the Neighbour-Joining (NJ) method based on matK gene sequences resulted in the separation of taxa into two major groups in the form of an unrooted tree (Figure 3). The first group consisted of the genus *Cordyline*, including the andong sample, while the second group consisted of the genus *Asparagus*. *Eustrephus latifolius* occupied the outermost branch with the greatest genetic distance compared to all other taxa, indicating the highest level of genetic divergence in this analysis according to the distance patterns generated by the NJ method (DeSalle & Tessler 2023).

The reconstruction showed that the andong sample from Bali clustered with *C. fruticosa* (OQ289248.1) with a bootstrap value of 71. Although this represents a moderate level of confidence, the relationship is supported by a very low genetic distance of 0.001 and a BLAST similarity of 99.8 percent which molecularly confirmed their close relationship. According to Rosidiani et al. in Anafarida and Badruzsaufari (2020), a bootstrap value between 70 and 100 percent indicated a stable clade with minimal likelihood of structural changes. Therefore, the grouping of the andong sample with *C. fruticosa* can be considered reliable.

The andong sample and *C. fruticosa* subsequently clustered with *C. canifolia* (JX903594.1) and *C. stricta* (OQ290052.1) with a bootstrap value of 96

percent, forming a sub cluster with strong genetic affinity within the genus *Cordyline*. *C. pumilio* (JQ435553.1) and *C. australis* (KX229865.1) joined in the next branch with a bootstrap value of 84 percent, indicating that all *Cordyline* species included in the analysis share close genetic relationships.

The genus *Asparagus* formed a separate group consisting of *A. officinalis*, *A. cochinchinensis*, and *A. oligoclonos* with bootstrap values of 100 percent for all internal branches, indicating very strong genetic similarity within the genus. The branch lengths in this phylogenetic tree represent genetic distances among species, where longer branches indicated greater genetic divergence while shorter branches indicated closer relationships (Oktafia & Badruzsaufari 2021). The clear separation between the *Cordyline* and *Asparagus* clusters reflects significant genetic differences at the family level, providing insight into their genetic relationships.

The position of *Cordyline* clustering within the same major group as the genus *Asparagus* supports the placement of *Cordyline* within the family *Asparagaceae* as recognized in modern taxonomic literature. These findings provided molecular evidence that the andong sample from Bali belongs to *C. fruticosa* and shared close genetic relationships with members of the family *Asparagaceae*, reinforcing the current taxonomic classification compared to older literature that placed *Cordyline* in *Liliaceae* or *Agavaceae*.

CONCLUSIONS

MatK-based DNA barcoding successfully clarified the taxonomic status of andong in Bali. The high sequence similarity with *Cordyline fruticosa* and its consistent clustering in the phylogenetic tree support its classification within the *Asparagaceae* family (*Asparagaceae*). This method proves effective for the genetic characterisation and conservation of local plant resources. However, further research using different primers is recommended to validate and strengthen these findings.

AUTHOR CONTRIBUTION

L.S.S performed laboratory works (collecting samples, DNA extraction, PCR amplification, agarose gel electrophoresis, data analysis, and writing manuscript). I.K.S. and I.W.W. designed the research and supervised all the processes. I.G.P.W., I.G.B.P., T.A.P. agreed to the final manuscript.

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CONFLICT OF INTEREST

The author declares no conflict of interest with any party.

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Research Article

Monitoring a Local Snake Community in an Agricultural Area in Yogyakarta, Indonesia: Its Diversity, Feeding Habits, and Potential Threats

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ABSTRACT

Snakes play a vital role in ecosystems as mesopredators, serving as both predators and prey. They contribute to ecological balance by regulating prey populations and supporting higher trophic levels. Despite their ecological significance, snake populations are increasingly threatened by anthropogenic disturbances, including habitat loss and the construction of artificial structures such as cemented water canals. In this study, we conducted a field survey of snake biodiversity in Kampung Satwa, Sumberagung Village, Yogyakarta, Indonesia, from November 2024 to January 2025, using the Visual Encounter Survey method. A total of 67 individuals from 14 snake species were recorded. The most abundant species were *Ptyas korros*, *Dendrelaphis pictus*, and *Ahaetulla prasina*. Dietary analysis revealed that *Fejervarya lim-nocharis*, a frog species frequently found in the area, would be a major prey item for the abundant snake species. Considering that toxic chemicals can easily accumulate in these prey species, potential biomagnification could pose a potential risk to the snake community. Additionally, 14 individuals were found near or within cemented water canals, indicating a risk of entrapment, particularly for species with limited climbing ability. One case of roadkill was also documented. These findings highlight the vulnerability of snake populations to human-made structures and underscore the need for continued biodiversity monitoring at population levels and the implementation of community-based conservation measures to mitigate future threats.

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INTRODUCTION

Snakes are part of the class Reptilia characterised by rigid scales covering the body, vestigial limbs, a brille as an eyelid substitute, and a lack of a tympanum. This group inhabits various habitats, from primary forests to human settlements. Each species of snake exhibits distinct ecological traits and dietary preferences, even when coexisting within the same habitat (Das 2010). Most snake species are considered mesopredators in various ecosystems, which are not only important predators, but also serve as prey for a variety of large animals. Thus, snakes provide a wide-ranging support to ecosystems, such as regulating prey populations and providing nutrition as prey to higher trophic levels (Voris & Murphy 2002; Geary et al. 2018).

Despite their ecological importance, snakes face significant threats from human activities. These include habitat loss and fragmentation caused by land-use changes and the construction of artificial structures, which can negatively impact snake populations and biodiversity (Graitson et al. 2020). One such structure is the cement-lined water canal, which, although essential for human use, can lead to animal entrapment, injuries, and habitat degradation (Reading et al. 2010; Geary et al. 2018; Kourides et al. 2024). Regular monitoring provides valuable insight into local snake populations and their prey within specific areas, serving as a foundation for conservation efforts. Such monitoring is also essential for assessing the impacts of human activities on snake populations and for informing strategies to maintain a sustainable environment (Lind et al. 2005).

Studying snake natural history is important to understand the ecosystem restoration and conservation (Dayton 2003). Indonesia hosts a rich reptile diversity, including many endemic snakes, mainly due to its favourable climate conditions and complex geological history. Approximately 190 snake species are endemic to the region, highlighting the urgent need for conservation efforts to prevent further habitat degradation and exploitation (How & Kitchener 1997; Das 2010). However, the natural history of most of these species remains poorly understood. This lack of information underscores the need to document their natural history to enhance our understanding and improve species conservation strategies (Greene 2005).

Kampung Satwa, located in Yogyakarta Province of Java, is a place where many inhabitants keep exotic animals for recreational and educational purposes. The surrounding area consists predominantly of agricultural fields and human settlements (Ministry of Tourism of the Republic of the Indonesia 2024; Kampung Satwa 2025). This area and its surroundings comprise three hamlets: Sumberagung, Sumberrahayu, and Summersari. The landscape is dominated by rice fields, intersected by cement-lined water canals and interwoven with human settlements, resulting in significant habitat fragmentation. This fragmentation is further exacerbated by the use of pesticides in local farming practices, which may have detrimental effects on the local snake population. This structure potentially has a negative effect on the local snake population. A previous short-term monitoring study conducted in Kampung Satwa's surroundings recorded ten snake species (Andhika 2022). These populations are likely influenced by ongoing human activities, offering an opportunity to better understand the challenges faced by snake populations in agricultural landscapes in Indonesia. In this study, we conducted a follow-up survey to expand the existing records of the local snake fauna in Kampung Satwa, document the natural history of each species, and assess the potential impacts of human-made structures, such as cemented water canals, on snake populations.

MATERIALS AND METHODS

Survey Period

Sampling was conducted in Kampung Satwa, Kedung Banteng Village, Moyudan Regency, Special Region of Yogyakarta, from 1 November 2024 to 24 January 2025. The study area spans from Kedung Banteng-Kaliduren Street in the north to the Setran-Klangon Street intersection in the south, covering three main districts: Summersari, Sumberagung, and Sumberrahayu, as shown in Figure 1. The total area surveyed covers approximately 8.4 km².

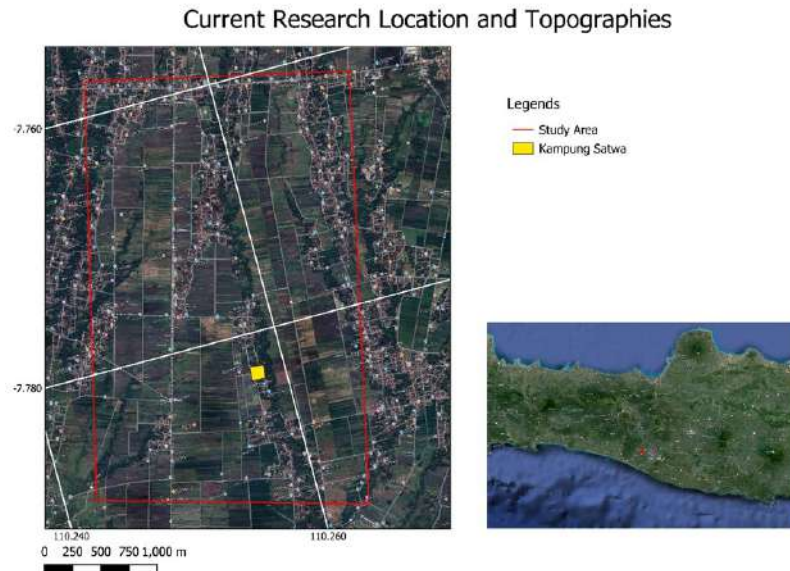


Figure 1. Sampling area used in this research.

Sampling Methods

Sampling was conducted using the opportunistic Visual Encounter Survey (VES) method within the corresponding area (Figure 1), both during the day and at night. Surveys were carried out once a week from November 2024 to early January 2025, and increased to seven times per week in mid-January, resulting in a total of 19 observations. Each sampling session lasted approximately five hours. Individuals were captured using a hook or snake tongs and identified based on Das (2010). Capture locations were geotagged using Google Maps. For each individual, the distance to the nearest water canal was visually estimated, with a maximum approximation of 5 metres when a canal was present. Photographic documentation was conducted using a DSLR camera. All captured specimens were then measured using a measuring tape and calliper to record snout-to-vent length (SVL) and total length (TL). Following the morphological measurements, stomach contents were obtained through forced regurgitation and identified to the lowest possible taxonomy level. Faecal samples were also collected by gently palpating the posterior part of the snake's body until defecation occurred. Any unidentified stomach contents and faecal samples were preserved in 70 % ethanol for later identification. Some of the captured snakes were also released to their corresponding capture point, while the remainder were given to Kampung Satwa's manager to keep for educational purposes.

RESULTS AND DISCUSSION

Snake Fauna

A total of 67 individuals, representing 14 snake species, were documented during this study (Table 1). The most abundant species was *Ptyas korros* (Javan rat snake; 14 individuals), followed by *Dendrelaphis pictus* (Common bronzeback; 11 individuals), *Ahaetulla prasina* (Gunther's whip snake; 10 indi-

viduals), and *Lycodon capucinus* (Island wolf snake; 6 individuals). The rarest species observed were *Coelognathus flavolineatus* (Yellow-striped trinked snake) and *Cylindrophis ruffus* (Common pipe snake), with only one individual each. All species in this sampling were encountered using the haphazard sampling method, which involves opportunistically collecting all individuals encountered and is well-suited to provide descriptive data. This method is particularly useful in poorly studied areas or when the target species has an unknown distribution. However, a limitation of this method is its lack of statistical validity, as it introduces selection bias that ignores the random sampling principle (Hall et al. 2013; Smith et al. 2017).

Compared to other studies that conducted in different landscapes on Java Island, the present site is characterised by a high frequency of *Ptyas korros* and provides multiple habitats for various snake species, known to occupy a range of ecological niches (Table 1). Due to its proximity to an urban area, some species that adapted to the urban environment were also consistently encountered, such as *Dendrelaphis pictus*, *Lycodon capucinus*, *Ptyas korros*, and *Naja sputatrix*. This type of landscape exhibits a low frequency of aquatic snake species, likely due to the lack of natural water bodies such as rivers in the surrounding area. In contrast, studies conducted in more water-rich environments have reported a high frequency of aquatic species such as *Rhabdophis subminiatus* and *Enhydryis enhydryis* (Yudha et al. 2016; Khoerunisa et al. 2022).

The number of snake species in the present study is higher than that reported by Andhika (2022), which documented only eight species (Table 1). The increase in species number may be attributed to the longer sampling duration (three months vs. one month), given that both surveys were conducted during the same rainy season. One species recorded in Andhika (2022), *Fowlea melanzostus*, was not detected in the present study. This species is typically associated with freshwater habitats (Das 2010). Its absence may be due to the high frequency of rainfall during the study period, which likely resulted in elevated water levels and fast current, potentially limiting the movement and occurrence of this aquatic snake.

Snake Dietary Information

In total, eight stomach content and ten faecal samples were obtained (Table 2). The present study revealed that *Ahaetulla prasina*, *Ptyas korros*, and *Dendrelaphis pictus* fed on *Fejervarya limnocharis*, one of the prey species frequently found at the present site (Figure 2). These three snake species are the most commonly captured in Kampung Satwa, suggesting that the abundance of the frog species at the site may primarily support these snake populations, and thus a large part of the snake community. Another abundant colubrid species recorded in this study was *Lycodon capucinus*, which stomach content included Scincidae scales and body parts of Blattodea. The species is known to prey primarily on frogs and lizards (Das 2010). The presence of Blattodea remains may be the result of secondary ingestion, suggesting that the snake consumed a skink that had recently fed on these insects. To the best of our knowledge, the present observation of *Ahaetulla prasina* feeding on frog prey is the first record of the species' feeding habits, which are considered to feed on mammals and birds (Tran et al. 2021).

The present study documented that *Bungarus candidus* preys on a rice eel, *Monopterus* sp. (Figure 3). This finding is consistent with previous reports that documented eels in the diet of this species, in addition to other prey such as snakes, *Ptyas korros*, and small mammals like rats (Luu & Ha 2018). We also found *Boiga dendrophila* that preying on *Lonchura* species, an abundant finch in the area. This dietary observation is consistent with its arboreal lifestyle, which provides greater access to avian prey. The diet of snakes often

Table 1. Snake fauna in Kampung Satwa.

Family	Species	Habitat Type (Das 2010)	TL (mm)	SVL (mm)	Weight (g)	Ref.
Colubridae	<i>Ahaetulla prasina</i>	Arboreal	621 ± 26 (10)	409 ± 159 (10)	9.4 ± 8.8 (10)	1, 2
	<i>Boiga dendrophila</i>	Arboreal and Terrestrial	1740 ± 2 (2)	1433 ± 7 (2)	770 ± 7 (2)	1, 2
	<i>Coelognathus flavolineatus</i>	Arboreal and Terrestrial	1609 (1)	1295 (1)	308.5 (1)	1, 2
	<i>Dendrelaphis pictus</i>	Arboreal	491 ± 19 (11)	326 ± 124 (11)	6.7 ± 7.5 (11)	1, 2
	<i>Lycodon capucinus</i>	Terrestrial and arboreal	453 ± 14 (6)	388.8 ± 122 (6)	19.01 ± 13.1 (6)	1, 2
	<i>Ptyas korros</i>	Terrestrial and arboreal	1002 ± 49 (14)	720 ± 421 (14)	200.6 ± 193.9 (14)	1, 2
	<i>Xenochrophis vittatus</i>	Aquatic	466 ± 17 (2)	348 ± 129 (2)	19.4 ± 18.2 (2)	1, 2
	<i>Fowlea melanzostus</i>	Aquatic	-	-	-	2
Cylindrophidae	<i>Cylindrophis ruffus</i>	Subfossorial	398 (1)	390 (1)	28.0 (1)	1, 2
Elapidae	<i>Bungarus candidus</i>	Terrestrial	1564 ± 7 (2)	1416 ± 58 (2)	954.5 ± 258 (2)	1, 2
	<i>Bungarus fasciatus</i>	Terrestrial	1564 ± 04 (3)	1416 ± 42 (3)	954.5 ± 36.1 (3)	1, 2
	<i>Naja sputatrix</i>	Terrestrial and arboreal	1291 ± 89 (3)	828 ± 427 (3)	520.4 ± 436.7 (3)	1, 2
Homalopsidae	<i>Homalopsis buccata</i>	Aquatic	240 ± 14 (6)	200 ± 115 (6)	12.8 ± 2.4 (6)	1, 2
Typhlopidae	<i>Rampotyphlops braminus</i>	Subfossorial and arboreal	53 ± 17 (2)	48 ± 129 (2)	1.3 ± 1 (2)	1, 2
Xenopeltidae	<i>Xenopeltis unicolor</i>	Subfossorial	1028 (1)	945 (1)	483 (1)	1, 2

Notes: Morphological characteristics of snakes caught in the present study are shown in Mean ± SD and sample sizes are shown in the parentheses. The categorisation of habitat type of each species based on Das (2010) and the categorisation of species encounter reference will be written in (1) as current study and (2) as Andhika (2022).

exhibits geographical variations, depending on the prey community (Luiselli et al. 2005). The present observation emphasizes the importance of collecting dietary information at the population level to better understand food web dynamics and to support environmental health monitoring.

Dietary information on the local snake community can provide valuable insights for developing effective conservation strategies. *Fejervarya limncharis*, a common prey species, is known to carry pesticide residues such as organochlorine and organophosphate on its body, which may pose health risks to its predators (Kusrini 2005). Given that a large part of the snake community in this study relies on these prey, preserving the current habitat is crucial to maintaining viable prey populations for these snakes. Although several species, such as *Lycodon capucinus*, *Ptyas korros*, and *Naja sputatrix*, have high adaptability in urban habitats, other species that depend heavily on prey associated with rice fields may be vulnerable to habitat changes. Thus, maintaining rice field ecosystems as snake habitats is also a potential solution for sustainable snake conservation (Halstead et al. 2019; Khoerunisa et al. 2022; Borzée et al. 2025).

Table 2. Gut contents obtained from collected snakes. Snake individual counts and number of prey items from each individual snakes are shown in the parentheses.

Species	Sex	SVL (mm)	Stomach Contents	Fecal Sample
<i>Ahaetulla prasina</i>	Male	647	<i>Fejervarya limnocharis</i> (1)	-
<i>Boiga dendrophila</i>	Male	1378	<i>Lonchura</i> sp. (1)	Estrildidae bird
<i>Lycodon capucinus</i>	Male	331	-	Blattodea insect, Scincidae scales
<i>Dendrelaphis pictus</i>	Female	278	<i>Fejervarya limnocharis</i> (1)	-
<i>Dendrelaphis pictus</i>	Female	280	<i>Fejervarya limnocharis</i> (1)	-
<i>Ptyas korros</i>	Male	1214	<i>Fejervarya limnocharis</i> (1)	-
<i>Ptyas korros</i>	Male	547	<i>Fejervarya limnocharis</i> (1)	-
<i>Ptyas korros</i>	Female	426	<i>Fejervarya limnocharis</i> (1)	-
<i>Bungarus candidus</i>	Female	580	<i>Monopterus</i> sp. (1)	-

**Figure 2.** *Ptyas korros* preying on two individuals of *Fejervarya limnocharis*.**Figure 3.** *Bungarus candidus* preying on *Monopterus* sp.

Artificial Disturbances

In 67 snake observations in the present study, 14 snakes were found near water canals (within a proximity of 5 meters, Table 3). Some of them were found on the water canal bank, while others were swimming in the structure.

Table 3. Snake species, individuals, and distance to the water canals.

Species	Individuals	Distance from Water Canal (m)
<i>Boiga dendrophila</i>	2	0; 2
<i>Coelognathus flavolineatus</i>	1	1.5
<i>Lycodon capucinus</i>	1	0.1
<i>Ptyas korros</i>	3	1; 0.5; 0.1
<i>Bungarus candidus</i>	2	0.1; 2
<i>Bungarus fasciatus</i>	1	0.5
<i>Naja sputatrix</i>	1	2
<i>Homalopsis buccata</i>	2	0
<i>Rhampotyphlops braminus</i>	1	0
Total Encounters	14	

Bungarus candidus, *Bungarus fasciatus*, *Ptyas korros*, and *Boiga dendrophila* individuals were found near the water canal, and one individual of *Bungarus fasciatus* was found swimming in the water canal. *Homalopsis buccata*, considered an aquatic species, was also found inside the canal. Although considered as such, this species is also prone to this disturbance through indirect water pollution resulting from increased anthropogenic activity. The design and function of a water canals often do not support the ecological requirements of aquatic fauna, as they differ significantly from natural water bodies in terms of environmental parameters. Consequently, such artificial habitats may fail to sustain populations of aquatic species, potentially leading to local declines or disappearance (Lin et al. 2020; Khan et al. 2023). Considering that these water canals have cemented vertical walls that can reach over 1.5 metres in height and 1 metre in width, they pose a significant barrier for snakes that fall into them. Escape may be particularly difficult due to interspecific variation in climbing ability, while some species may be able to climb out, others may become trapped. In addition to posing a direct threat to individual snakes, these structures also contribute to habitat fragmentation, thereby limiting animal movement and distribution (Sheehy et al. 2016; Gallego-García & Sarasola 2024). It should be noted that the species near water canals does not appear to correlate with each species' capture rate (Table 1; Table 3), which suggests that the entrapment rate would be highly species-specific.

Previous studies have shown that the existence of water canals poses challenges to several animal taxa. Based on García (2009), the water canals system caused the mortality of 17 species, including fish, amphibians, herpetofauna, and birds, with a total of 134 individuals during one-year study. Thus, the present observations suggest that the presence of the water canals would threaten the population, especially for specific species which have a wide home range area to fulfil their biological needs such as mammals and reptiles. Moreover, in the study area, numerous additional water canals are currently under construction. The presence of water canals not only affects snakes but also impacts various biological aspects of terrestrial mammals, including their distribution area and behavioural patterns (García 2009; Vörösmarty et al. 2010; Hamilton et al. 2025). Therefore, immediate actions are urgently needed to mitigate further population decline, particularly for species inhabiting areas near water bodies. One promising approach is the implementation of community-based monitoring programs aimed at rescuing entrapped individuals, reporting problematic structures, and installing escape routes to facilitate the movement of snakes. In parallel, continuous monitoring of snake biodiversity is essential to assess current and emerging threats, and to serve as indicators of broader environmental health (Beaupre & Douglas 2009; Kourides et al. 2024).

The continued encroachment of human settlements into natural habitats poses several threats such to snake populations, including roadkill inci-

dents and human-snake conflict. Although such a structure may not immediately cause large-scale population declines, it can restrict snake movement and fragment habitat. This, in turn, may lead to increased inbreeding and lower overall population fitness. Road systems, in particular, directly impact snake populations by increasing mortality rates, especially during warmer and rainy seasons when snake activity is heightened. This threat is increasing annually, placing snake populations at greater risk of road-related fatalities. Additionally, climate change, particularly global warming, may alter snake activity patterns, potentially exacerbating roadkill rates (Schwartz et al. 2020; Liu 2025). Such risks were observed in this study, with one *Naja sputatrix* individual found as a roadkill victim (Figure 4).



Figure 4. Roadkilled *Naja sputatrix* in the sampling area.

CONCLUSIONS

A total of 14 snake species comprising 67 individuals were recorded in the current study, representing the snake fauna of Kampung Satwa. Based on the dietary analysis, toxin accumulation through contaminated prey may pose a potential threat to local snake populations. However, the most immediate threat appears to be the construction of cemented water canals, which can result in individual entrapment and mortality. Continued monitoring of the snake community is essential to evaluate future threats and to inform effective conservation strategies.

AUTHOR CONTRIBUTION

R.E. shared the idea and design, managed and guided the writing, and supervised the manuscript. A.P.N. designed the research, conducted the study, and analysed the snake data. S.A. contributed to the writing and revising the manuscript. T.K. conducted and supervised the field survey and revised the manuscript. All authors approved the manuscript for publication.

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CONFLICT OF INTEREST

There is no conflict of interest related to this research at any stage of manuscript preparation.

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Research Article

Analysis of Length/Width-Weight and Condition Factor Revealed Smaller Crab Sizes of *Thalamita crenata* (Rüpell, 1830) in Butuan Bay, Philippines

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ABSTRACT

The marine ecosystem of Butuan Bay, Philippines, supports a diverse array of crab species, including the *Thalamita crenata* (crenate swimming crab), which plays a vital role in coastal ecology and fisheries. The study examined *T. crenata* samples from sites with remaining occurrences in Butuan Bay, Philippines and aimed to understand the ecological and biological dynamics of *Thalamita crenata* in Butuan Bay by analysing their morphometric relationships, condition factors (CF), and spatial-sexual dimorphism. Morphometric data from Butuan Bay were analysed to determine growth relationships, condition factors, and spatial-sexual differences in size and marketable sizes between sexes and locations (Cabadbaran and Buenavista). Results showed significant spatial and sexual dimorphism, with larger crabs and higher median CF values ($p < 0.01$) found in Cabadbaran site than in Buenavista site. Male crabs were larger than females, consistent with crustacean patterns. The power model best described the weight-carapace length (WT-CL) and width (WT-CW) relationships, revealing negative allometry (WT-CL: $b = 0.3286$, $R^2 = 0.6820$, $p < 0.01$; WT-CW: $b = 0.3014$, $R^2 = 0.7478$, $p < 0.01$). The study highlights the influence of localised environmental factors and emphasizes the need for targeted conservation efforts, including enforcing size limits, protecting habitats, and regulating fishing practices. Restoration efforts and continued monitoring are essential to ensure the sustainable exploitation of *T. crenata* and maintain its ecological and economic importance.

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INTRODUCTION

Thalamita crenata, also known as crenate swimming crab is a common swimming crab that thrives in shallow seas throughout the tropical Indo-Pacific Region, Western Pacific, Indian Ocean, and Red Sea, as well as along the African Coast (Susanto & Irnawati 2014). *T. crenata* prefers brackish water, notably nearby river mouths and the extreme seaward fringe of mangrove swamps; it rarely lives in clear sea water, such as coral reefs (Cannicci et al. 1996). The species can be recognised by distinct six rounded lobes which are nearly equal sizes, located between the eyes along the anterior margin of the carapace (Motoh 1980). Additionally, it is identified by its olive to dark natural colour with its five fine sharp spines on the antero-lateral margin of the carapace—characterised by six rounded lobes between its eyes and five sharp spines along its sides (Pastor et al. 2024).

This species is frequently captured in intertidal zones (high tides) by traps and as bycatch in gillnets and is generally smaller than most other commercially targeted crab species (Muhd-Farouk et al. 2017). Nevertheless, it is collected by the local coastal communities and highly favoured for its flesh quality. Due to its abundance and the relative ease of capture compared to mud crabs, it constitutes a significant component of the daily diet among coastal towns in the Philippines. It was used to be commonly sold as fresh catch in traditional market and as local delicacy in food stalls (Pastor et al. 2024).

Ecological studies have shown that *T. crenata* is one of the predators in the food web of mangrove habitat contributing to the balance of the ecosystem it belongs (Eeckhaut et al. 2020). *T. crenata* is known to scavenge on carrion, with larger individuals often displacing smaller ones at feeding sites (McKillup & Mckillup 1996). Generally, crabs play an important role as prey, predators, or detritus feeders in the complex food web of coastal ecosystems (Sharifian et al. 2021). Overexploitation of this species would disrupt the ecosystem balance and has cascading effect to the food web in the aquatic habitat (Muhd-Farouk et al. 2017). In the Philippines, mangrove ecosystems have significantly decreased largely due to conversion into fishponds and overharvesting of mangrove woods (Natividad et al. 2014). The *T. crenata* thriving in mangrove ecosystems consequently declined along with its overexploitation as source of food. Yet there are limited studies of this species given its ecological importance and source of protein to local communities (Hamid et al. 2019). Studies in *T. crenata* in the Philippines were limited only as part of field guide for edible crustacea (Motoh 1980), indicator species of mangrove rehabilitation (Walton et al. 2007), checklist of commonly harvested crabs (Subang et al. 2020), and morphometric variations (Pastor et al. 2024).

Analysis using LWR is an important aspect in fishery biological investigations, growth model of the species, ecology, and habitat suitability of crustacean populations (Susanto & Irnawati 2014; Jumawan et al. 2022). The link between length, width, and weight is essential to comprehending the biological and ecological dynamics of species, particularly crabs like *T. crenata*. The study on relationship evaluation makes it possible to estimate a crab's weight based on its parameters, which is a vital information for managing the population and conducting population surveys (Suryandari et al. 2018; Marchessaux et al. 2023). In morphometric studies, the weight relationship between length and width are two crucial factors to consider. *T. crenata* study was explored which emphasises that this type of crab has a fundamental length-weight relationship that is typical of many marine crabs, indicating that the crabs' weight increases with their growth. Presently, the information about the length width/weight relationship (LWR) of *T. crenata* in the Philippines is not fully understood. Conservation and environmental management initiatives benefit from the monitoring of the LWR values (Noori et al. 2015).

The health and status of a species can be evaluated through relationships. Using these factors, which can represent environmental conditions and food availability in their habitats and nutritional state of individuals can be evaluated (Jumawan et al. 2022). The condition factor (CF) provides details about a habitat's environmental quality to its organisms. Excessive deviations from expected condition parameters could indicate overfishing, pollution, or habitat degradation-related changes in habitat quality (Rohmayani et al. 2018).

There is limited information on monitoring of fisherman catch and sizes of *T. crenata* crabs in the Philippines. This is partly due to its smaller sizes and lower market value compared to mud crabs (*Scylla* spp.) and blue swimming crabs (*Portonius pelagicus*). Given its relatively small size, the *T. crenata* is still included as economically important crab species despite its lower price in the market (Susanto & Irnawati 2014; Muhd-Farouk et al. 2017). There are observations and anecdotal reports pertaining to decreasing marketable sizes of *T. crenata* sold in local markets in the Philippines. However, this claim needs to be verified and supported by data analysis. The intention of the study is also to conduct LWR and condition factor as part of investigating the marketable sizes of *T. crenata* sampled in the Philippines.

MATERIALS AND METHODS

Study Area

Crab samples were collected using traditional fisherman traps designed to catching *T. crenata* as marketable sizes. Collected *T. crenata* crabs, representing various size classes, were promptly transported to the laboratory for storage under cool conditions from two specific sites within Butuan Bay: Cabadbaran City and Buenavista, Agusan del Norte. These locations, with coordinates 9.1222° N, 125.5376° E and 8.7859° N, 125.3686° E, respectively (Figure 1), were selected for this study. Butuan Bay is a complex ecosystem influenced by both freshwater from the Agusan River and saltwater from the ocean. It is situated in northeastern Mindanao and opens into the Bohol Sea, also referred to as the Mindanao Sea (Navarro et al. 2022). The bay also faces environmental challenges such as heavy metal pollution (Elvira et al. 2016; Cabuga et al. 2020). Species identification was carried out using the field guide summary from SeaLifeBase (Ng 1998).

Species Identification, sex determination, and Measurement of Morphometric Indices

Species was identified based on existing taxonomic records. Sex was identified based on abdomen shape: males possess a V-shaped abdomen (Figure 2B), while females have a rounder abdomen (Figure 2C) (Ng 1992). Crab morphometrics were determined using a vernier caliper for measurements (mm) and a digital weighing scale for total weight (g). Specific measurements included maximum carapace width (CW), length (CL), frontal width (FW), natatory leg dactylus length (NDL) and width (NDW), cheliped dactylus length (CDL), palm cutting edge (CE), length (PL), and width (PW) (Figure 2) (Shahdadi et al. 2018; Asaduzzaman et al. 2021).

Regression Modeling of Morphometric Traits in *T. crenata*

Morphometric data, including carapace width (CW), length (CL), and weight (WT), were analysed to determine length-width and weight relationships. Data were categorised by sex (male, female) and pooled for comparative analysis. Exponential, linear, and power regression models were fitted to the data, with the model exhibiting the highest R² value selected to represent the crab's allometric growth (Jumawan et al. 2022). The preferred regression equation was graphically illustrated.

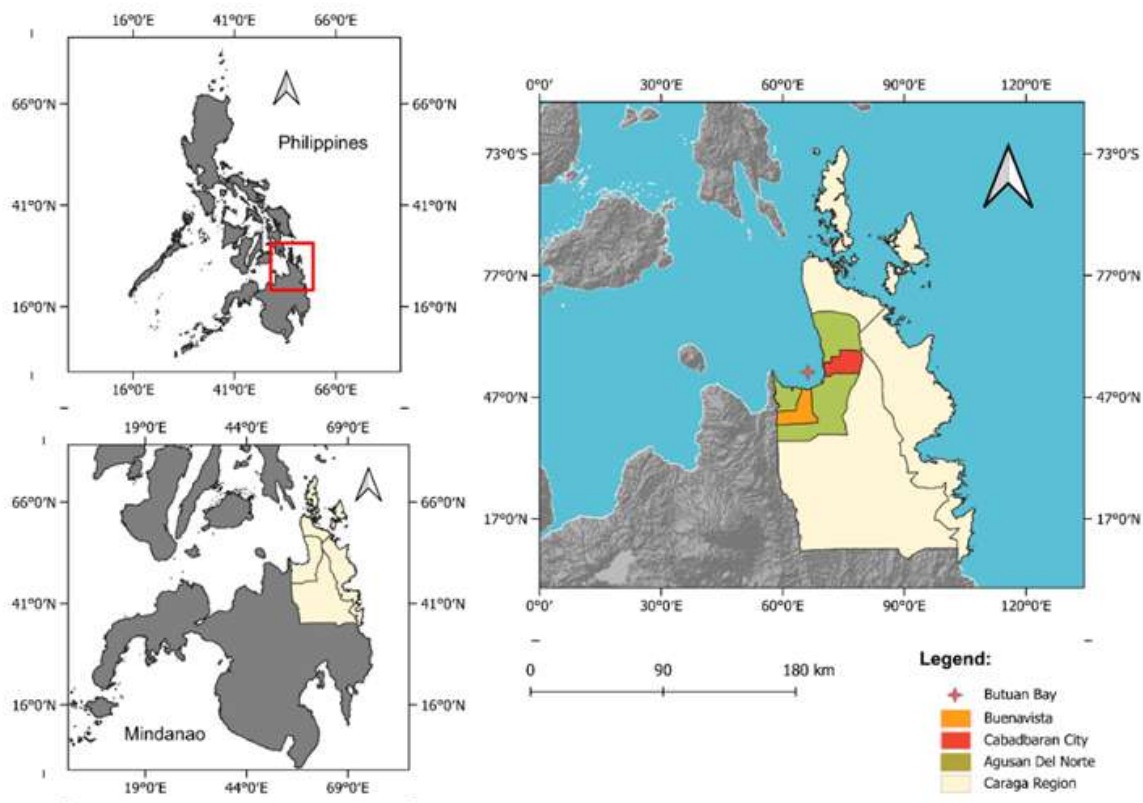


Figure 1. Map showing the sampling area in Butuan Bay.

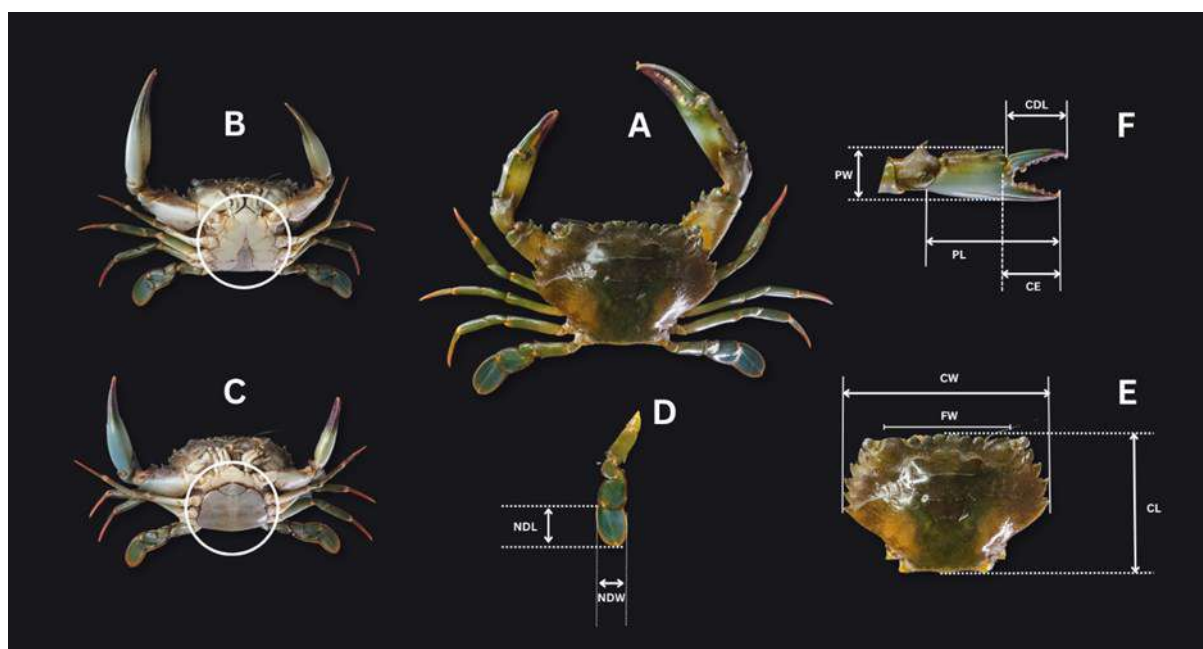


Figure 2. Actual collected sample of *T. crenata* with the studied morphometric indices. A) Dorsal view of the species; B) Ventral part showing the distinct V-shaped abdomen of male species; C) Female with distinct round shaped abdomen; D) natatory leg dactylus length (NDL) and natatory leg dactylus width (NDW); E) Frontal width (FW) carapace width (CW), and carapace length (CL); F) maximum palm width (PW), maximum palm length (PL), palm cutting edge (CE), and cheliped dactylus length (CDL).

Evaluation of length/width-weight relationships and Fulton's condition factor

The allometric growth equation, $Y = aX^b$, where Y represents weight (WT), X represents carapace width (CW) or length (CL), a is the y-intercept, and b is the allometric growth coefficient, was applied to describe growth patterns. Growth pattern was classified as isometric when $b = 3$, positively allometric

when $b > 3$, and negatively allometric when $b < 3$. The strength of the relationship between variables was assessed using the coefficient of determination (R^2). To compare length-weight relationships among female, male, and pooled data, the power equation was log-transformed into linear form $\log Y = a + b \log X$, and linear regression analysis was conducted. The significance of differences in relationships (CW, CL to weight) was tested using MANCOVA ($p < 0.05$). Condition factor (CF), calculated using the modified Fulton's formula ($CF = (100WT)/CW^b$) (Jumawan et al. 2022), evaluate the overall health of crabs. Chi square analysis was performed to compare between area and sex datasets. Differences in CF among sex and pooled groups were assessed using the Kruskal-Wallis test ($p < 0.05$). Statistical analyses and graph generation were performed using R software, JASP, and Microsoft Excel.

RESULTS AND DISCUSSION

Morphometrics, size and sex-specific distribution

The descriptive statistics presented in Table 1 provide a comprehensive overview of the morphometric traits of *T. crenata* crabs across different areas and sexes. The data revealed that crabs caught from Area 1 observed to be larger compared to those samples from Area 2, as indicated by the higher mean values for weight (WT), carapace width (CW), carapace length (CL), and other morphometric variables. Comparably, *T. crenata* male crabs exhibit a larger mean value for most traits compared to *T. crenata* female crabs. The values of standard deviation provide insights into each group's variability. A higher SD suggests greater variations in the traits within that group. As projected (Table 1), the higher standard deviation for weight in Area 2 indicates that there is more variability in weight among *T. crenata* crabs from Area 2 (Buenavista) than Area 1 (Cabasaran). Female body size is the principal determinant of reproductive output in the case of brachyuran crabs, considering the demographic pressure on fecundity being the primary selective mechanism influencing its annual reproductive effort (Somers 2020). Variability in the weight of crab samples can be attributed to sex, maturity, molt stage, and carapace form which significantly affect the weight of blue crabs, with males being heavier than females of equal width and mature males weighing more than immature females (Sundet 2014). The minimum and maximum value highlights the range of variations within each trait and group. For instance, the minimum CW is 30 mm, while 70 mm is recorded as the maximum CW value, indicating a wide range of sizes among the crabs. The carapace shape variation in crabs is explained by the interaction between sex and habitats, in a study of rocky shore crabs it was observed having more slender and lengthent carapaces than salt marsh individuals (Lezcano et al. 2015).

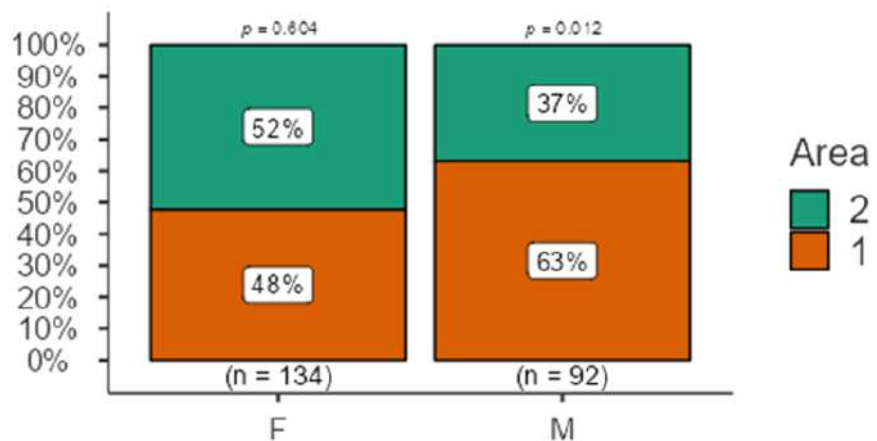
A bar chart was displayed to compare the categorical variables between Areas (dependent variable) and crab sex (grouping variable) (Figure 3). Chi-square analysis revealed higher values of *T. crenata* samples in Area 1 than Area 2 samples ($\chi^2 = 5.13$; $p = 0.024$). The female crab samples (52 %) were similar ($p = 0.604$) between Area 1 (52 %) and Area 2 (48 %). While the male crab samples were significantly higher ($p = 0.024$) in Area 1 (63 %) compared to Area 2 (37 %). These findings imply potential sex-specific preferences for various habitats or behaviours which resulted in differing distribution patterns between areas.

The observed sex-specific distribution patterns may have significant implications for *T. crenata* population dynamics. Female-dominated areas may experience increased reproductive output, while male-dominated areas may exhibit heightened competition for mates. These differences could influence growth rates, survival, and fecundity, ultimately impacting fisheries management and conservation strategies (Kuris 2020; Somers 2020; Nair et al. 2024). Additionally, the variation in distribution patterns between Area 1 and Area 2

Table 1. Descriptive statistics of collected *T. crenata* samples categorised into Areas (1-Cabadbaran, 2-Buenavista), sex, and pooled datasets.

Indices (mm)	Mean $\pm$ Standard Deviation					Range Values (min-max)				
	Area 1 (n=122)	Area 2 (n=104)	Female (n=134)	Male (n=92)	Pooled (N=226)	Area 1 (n=122)	Area 2 (n=104)	Female (n=134)	Male (n=92)	Pooled (N=226)
WT	33.53 $\pm$ 9.22	24.89 $\pm$ 12	26.6 $\pm$ 9.33	33.8 $\pm$ 12.9	29.55 $\pm$ 11.44	11-57	7-76	7-55	10-76	7-76
CW	54.77 $\pm$ 6.04	49.2 $\pm$ 7.64	50.5 $\pm$ 6.52	54.6 $\pm$ 7.86	52.21 $\pm$ 7.36	36-67	30-70	33-66	30-70	30-70
CL	38.54 $\pm$ 3.97	31.95 $\pm$ 5.3	34.4 $\pm$ 5.11	37.2 $\pm$ 6.05	35.51 $\pm$ 5.67	29-47	23-47	23-44	25-47	23-47
FW	33.46 $\pm$ 3.65	31.24 $\pm$ 5.86	31.8 $\pm$ 4.52	33.4 $\pm$ 5.32	32.44 $\pm$ 4.91	15-42	14-45	20-45	14-45	14-45
NDL	12.84 $\pm$ 2.06	12.32 $\pm$ 2.56	12.1 $\pm$ 2.23	13.3 $\pm$ 2.28	12.6 $\pm$ 2.31	8-17	1-20	1-19	8-20	1-20
NDW	8.71 $\pm$ 2.21	7.56 $\pm$ 1.95	7.63 $\pm$ 1.74	8.99 $\pm$ 2.47	8.18 $\pm$ 2.17	5-20	5-20	5-19	6-20	5-20
CDL	20.04 $\pm$ 2.87	15.99 $\pm$ 3.32	16.9 $\pm$ 3.08	20.1 $\pm$ 3.69	18.18 $\pm$ 3.68	13-28	9-25	9-23	12-28	9-28
CE	16.73 $\pm$ 3.66	14.46 $\pm$ 4.21	14.4 $\pm$ 2.85	17.5 $\pm$ 4.87	15.69 $\pm$ 4.08	12-45	8-30	9-30	8-45	8-45
PL	41.07 $\pm$ 6.98	35.75 $\pm$ 7.9	35.7 $\pm$ 5.85	42.8 $\pm$ 8.53	38.62 $\pm$ 7.86	14-58	10-60	10-49	14-60	10-60
PW	11.89 $\pm$ 2.31	12.7 $\pm$ 3.82	11.6 $\pm$ 2.94	13.3 $\pm$ 3.09	12.27 $\pm$ 3.11	8-19	7-34	7-34	8-24	7-34

may also be influenced by environmental factors, such as water quality, substrate composition, or food availability (Mokhtari et al. 2015; Pati et al. 2023). Moreover, understanding these patterns can inform spatial management approaches, ensuring sustainable exploitation of *T. crenata* populations. Future studies should investigate these potential drivers, incorporating multivariate analysis to disentangle the complex relationships between ecological factors and *T. crenata* distribution.

**Figure 3.** Comparison of crab samples between areas and sex as factors indicate significant differences using Chi-square analysis. (Area: $\chi^2 = 5.13$, $p = 0.024$).

This pattern was also observed in separate study on *Armases* crabs which also exhibited a sex-specific habitat choice and preference in food, which then potentially acted as key biotic vector or spatial subsidies across habitat borders (Hübner et al. 2015). Even male hermit crabs also play as better intraspecific competitors compared to females in the acquisition of vacant shells, which could allow them to grow better and larger than females (Plasman et al. 2023). Similar to observations in blue swimming crabs (*Portunus pelagicus*) from coastal Thailand (Hisam et al. 2020), our *T. crenata* samples exhibited ontogenetic habitat shifts, with juveniles predominantly

found in shallower waters and larger individuals occurring in deeper areas, suggesting a comparable pattern of habitat use during growth. Further research is required to explore its underlying ecological and behavioral factors influencing such variations.

The class sizes of *T. crenata* samples were categorised in three classes representing small, medium, and large, based on weight and carapace sizes. The comparative histograms visually depict the distribution of crab weight (Figure 4A), carapace width (Figure 4B), and carapace length (Figure 4C) across different size classes. Based on weight data it showed that small size ranged from 7-28 g, the medium size ranged from 29-51 g, and the large size ranged from 52-76 g. The carapace width values showed small size ranged from 30-43 mm, medium size ranged from 44-57 mm, and large size ranged from 58-70 mm. The carapace length values indicate small size ranged from 23-30 mm, medium size ranged from 31-38 mm, and large size ranged from 39-47 mm.

The results showed varying distributions across the three morphometric traits. For crab weight, the medium size class (29-51 g) has the highest number of individuals, with 104 crabs falling within this range. However, the small size class (7-28 g) has a slightly higher number of individuals, with 114 crabs, while the large size class (52-76 g) has the fewest number of crab samples, with only 8 individuals. In contrast, the distribution of carapace width is skewed towards the medium size class (44-57 mm), with 142 individuals falling within this range. The large size class (58-70 mm) has a notable number of individuals, with 58 crabs, while the small size class (30-43 mm) has the fewest number of crab samples, with only 26 individuals. The distribution of carapace length is more evenly spread across the three categories, with the medium size class (31-38 mm) having the highest number of crab samples, with 101 individuals, followed by the large size class (39-47 mm) with 67 crabs, and the small size class (23-30 mm) with 58 crabs. The prevalence of medium-sized crabs may reflect optimal environmental conditions, food availability, or reproductive strategies favouring this size class (Jaramillo et al. 2017). Factors influencing size-class distribution, such as habitat quality (Griffen & Norelli 2015), predation pressure (Noè et al. 2018), genetic factors (Mashar et al. 2017), and overfishing practices (Hamid & Wardiatno 2015), which warrant investigation.

While the abundance of medium-sized crabs may suggest optimal conditions, it's also essential to consider the limitations of the dataset. The comparison of size classes was only within the range of the actual collected samples, which may not represent the entire population or historical trends. In other words, the dominance of medium-sized crabs may not necessarily indi-

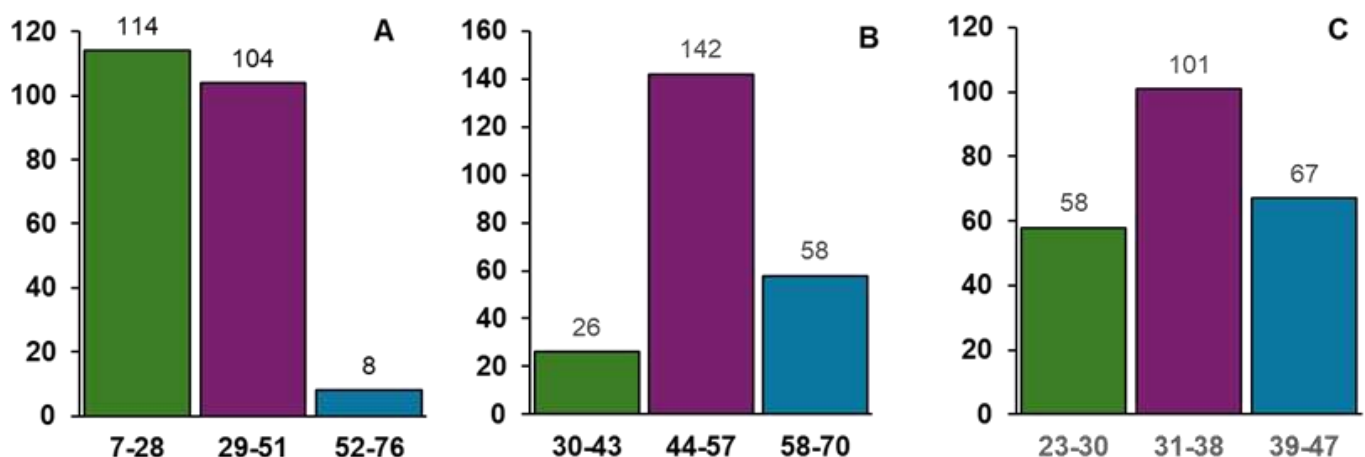


Figure 4. The histogram of the crab weight (A), carapace width (B), and carapace length (C) categorized into small, medium, and large classes.

cate optimal conditions, but rather a snapshot of the current population structure. It's possible that environmental or ecological factors may be limiting the growth or survival of larger crabs, leading to their underrepresentation in the sample. Furthermore, if there are existing records of more abundant larger sizes in the past, it could suggest that the current population is not thriving as well as it once did. This highlights the importance of considering historical data and long-term trends when interpreting population dynamics (Alberts-Hubatsch et al. 2016; Young & Elliott 2019).

Power regression model revealing allometric growth patterns

The results of fitting various data models of the correlation between body weight (WT) and carapace length (CL) alongside to weight and carapace width (CW) in the collected crabs was presented in Table 2. Data reveals that the relationship between the length/width and weight of *T. crenata* is best described by a non-linear power regression model. The WT-CL relationship, the power model ($y = 11.825x^{0.3286}$) provided the best fit with an $R^2=0.6820$. This suggests that approximately 68.2 % of the variation in weight can be illustrated by variations in the length of *T. crenata*'s carapace. The power model also suggested that the association between weight and carapace length is nonlinear, where weight increasing at a decreasing rate as carapace length increases. For the WT-CW relationship, the power model ($y = 19.076x^{0.3014}$) also suggested the best fit, with an $R^2=0.7478$, indicated that approximately 74.78 % of the variation in weight can be explained by variations in the width of carapace. This model, which outperforms both exponential and linear models, indicates a strong and highly significant correlation between the crab's weight and its carapace dimensions (width and length) for pooled, female, and male data sets.

Building on the non-linear power regression model, the study also utilized a linearized form of the power equation to furtherly analyse the length/width-weight relationship of *T. crenata* (Table 3). The linearised form confirmed the strong correlation between the weight and carapace dimensions ($p<0.01$), with high coefficients of determination (R^2) values for all data sets. For instance, the R^2 value for the weight-carapace width relationship 0.7478,

Table 2. Fitting data models to LWR data. Body weight to carapace length (WT-CL), and body weight to carapace width (WT-CW).

Relationship	Data Models	Equations	R^2 values
WT-CL	Linear	$y = 0.4018x + 23.636$	$R^2 = 0.6571$
	Exponential	$y = 24.868e^{0.0116x}$	$R^2 = 0.6206$
	Logarithmic	$y = 11.16\ln(x) - 1.3834$	$R^2 = 0.6749$
	Power	$y = 11.825x^{0.3286}$	$R^2 = 0.6820$
WT-CW	Linear	$y = 0.5489x + 35.985$	$R^2 = 0.7288$
	Exponential	$y = 37.686e^{0.0107x}$	$R^2 = 0.7003$
	Logarithmic	$y = 15.151\ln(x) + 2.1237$	$R^2 = 0.7391$
	Power	$y = 19.076x^{0.3014}$	$R^2 = 0.7478$

Table 3. Allometric growth patterns of *T. crenata* with the corresponding R^2 value and p-value.

Relationship	Linearised Power Function	a	b	R^2 value	p-value	Type of growth
WT-CL	$\ln(y) = 0.3286 \ln(x) + 2.469$	0.32	2.47	$R^2 = 0.6820$	$p<0.01$	negative allometry
WT-CW	$\ln(y) = 0.3014 \ln(x) + 2.949$	0.3	2.95	$R^2 = 0.7478$	$p<0.01$	negative allometry

indicated a strong fit. Similarly, the weight-carapace length relationship also showed high R^2 value (0.6820), further validating the robustness of the model.

The use of the linearised form not only reinforces the findings of the non-linear analysis but also provides a clearer understanding of the growth patterns (Jumawan et al. 2022). It highlights that the carapace dimensions increase at a slower rate compared to the weight, consistent with the negative allometric growth pattern observed. Hence, the linearized form of the power regression model provides a comprehensive and accessible way to analyse and to interpret the length/width-weight relationships in *T. crenata*. It confirms the non-linear nature of the growth patterns and offers valuable insights into the health and growth dynamics of this crab species in its natural habitat (Voje 2016).

The power regression models presented (Figure 5) illustrates the relationship between carapace length (CL), carapace width (CW), and weight in *T. crenata* crab samples. The model for the CW-Weight relationship ($y=11.825x^{0.3286}$, $R^2=0.682$) where a strong positive correlation between was observed from these two variables which implies that as the carapace width of a crab increases, its weight also directly increases. Similar patterns of the model for the CL-Weight relationship ($y=19.076x^{0.3014}$, $R^2=0.7478$) also illustrates a strong positive correlation which implies that a larger carapace length is directly associated with a greater weight. It was observed in the study from 2016, where carapace width has a positive growth pattern in weight for males, and a negative allometric pattern for females in the blue swimming crab, *Portunus segnis* (Hajjej et al. 2016). The R^2 values for both models demonstrate a substantial point of variation in weight made evidently through observed variations in carapace width and length. The strong relationships observed between carapace dimensions and weight highlight their potential utility in fisheries management, as reliable weight predictions can inform more efficient and sustainable harvesting practices while minimising bycatch (Mullowney et al. 2020). Although carapace width and length proved to be effective predictors of weight in this study, incorporating species-specific traits, environmental variables (e.g., temperature, salinity), and additional morphometric data could further refine predictive models and improve understanding of the biological and ecological factors influencing weight variation in *T. crenata*.

Crabs frequently display negative allometric growth, where carapace size increases more slowly than body weight during maturation. This pattern reflects energy allocation priorities, as the carapace's primary role in protection reduces the need for proportional scaling with body mass, which is instead driven by tissue and organ growth (Leite et al. 2014; Din et al. 2017). Sexual dimorphism is evident in many species: female mud crabs (*Scylla tranquebarica*) exhibit negative allometry, while males show positive allometry, gaining weight disproportionately relative to carapace size (Din et al. 2017). Similarly, in *Ucides cordatus*, females display negative allometric growth, whereas males grow isometrically, resulting in higher weight for equivalent carapace dimensions (Leite et al. 2014). These differences underscore sex-specific strategies in energy investment, often linked to reproductive roles.

Such patterns are influenced by life cycle stages; during maturation, energy may shift from growth to reproduction or survival, altering the condition factor (a health and energy reserve metric) and reinforcing negative allometry (Leite et al. 2014). These dynamics carry ecological and evolutionary consequences, such as stabilising selection on male genitalia for reproductive success (Voje 2016), though positive allometry can emerge in specific traits under environmental or biological pressures (Simpson et al. 2016).

Stress concentration and clamping behaviors play a critical role in de-

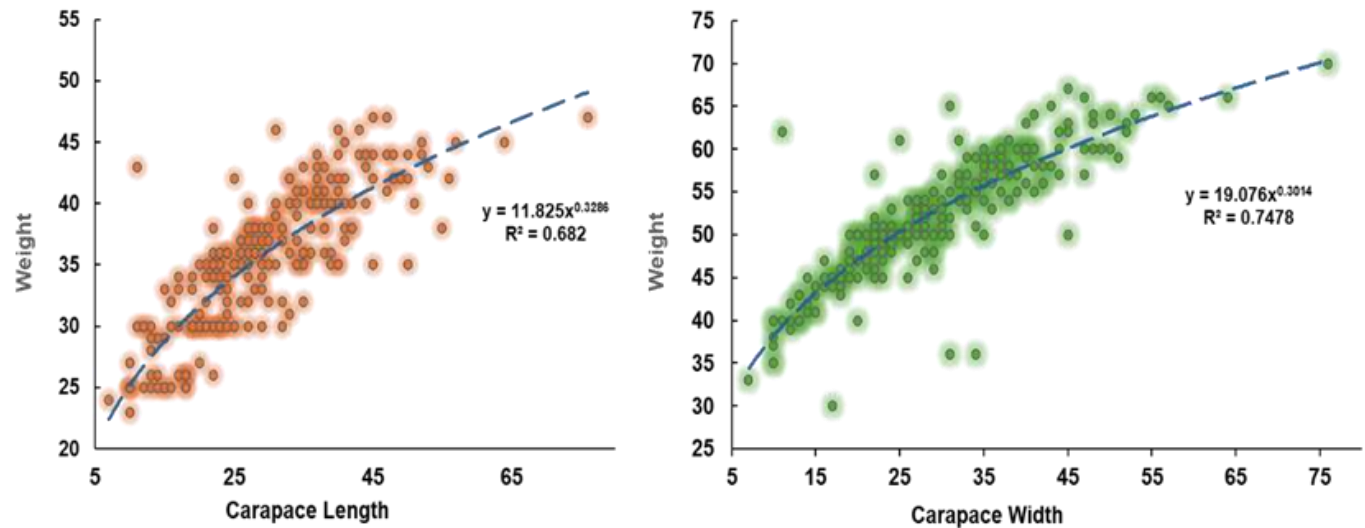


Figure 5. The power regression model of the length/width-weight of crab samples. The CW-CT and CW-CL relationship of pooled samples.

termining the effective bite force, with crabs adjusting their muscle force to avoid overload and potential damage to the claws—a trade-off against other positively or isometrically growing structures (Zhang et al. 2021). Energy allocation further prioritises muscles and reproductive organs over carapace expansion. Male spider crabs, for instance, may invest energy differentially: smaller males reproduce repeatedly, while larger males focus on a single reproductive event (Rosen et al. 2020). Similarly, in *Carcinus maenas*, males develop larger claws for competition, potentially inducing negative allometry in other body parts (Cothran & Thiel 2020).

Recent studies corroborate these trends. *Scylla* spp., *Callinectes sapidus*, and *Barytelphusa guerinii* all demonstrate slowed carapace growth relative to biomass gain, with sex-based variations in scaling exponents (Devi & Smija 2015; Waiho et al. 2016; Widigdo et al. 2017). *Goniopsis cruentata* further highlights ontogenetic shifts, with negative allometry across morphometric traits (Silva et al. 2024). This conserved growth strategy optimizes energy use; smaller carapaces suffice for protection, freeing resources for locomotion and reproduction. In *Uca tangeri*, claw size—critical for mating—exemplifies how negative allometry balances reproductive success and foraging efficiency (Moruf & Ojetayo 2017). Even digestive anatomy reflects this pattern, as stomach allometry in brachyuran crabs aligns with dietary adaptations to habitat resources (Griffen et al. 2018). Thus, negative allometry in crabs represents a multifaceted adaptation, balancing biomechanical constraints, reproductive strategies, and environmental pressures through differential growth trajectories.

Spatial, sexual dimorphism, and condition factors

The substantial F-value of 56.743 and p-value less than .001 for the "Area" parameter indicate that *T. crenata* from different regions display distinct morphological characteristics (Table 4). In the same manner, the significant F-value of 23.018 and p-value below .001 for the "Sex" parameter implied that male and female *T. crenata* exhibit differences in their morphological characteristics. The non-significant interaction effect between area and sex (F-value = 0.558, p-value = 0.83) suggested that the effects of these factors on morphometric traits are independent from each other, which simply means that the effect of area on morphology is consistent across both sexes and vice versa. Weight was validated as a significant covariate (F-value = 77.518, p-value < .001), which suggest that heavier crabs tend to have different morphologi-

Table 4. MANCOVA analysis of morphometric traits and weight as covariate compared between factors area and sex of crabs, with T Test to comparing the LWR of crab samples.

Parameters	Wilk's Lambda Value	F-value	df1	df2	p-value
Area	0.294	56.743	9	213	< .001
Sex	0.507	23.018	9	213	< .001
Area * Sex	0.977	0.558	9	213	0.83
Weight	0.234	77.518	9	213	< .001
Parameters	Morphometry	$\chi^2 = \text{Area 1}$	$\chi^2 = \text{Area 2}$	T Test Value	p-value
Area	Weight	33.5	24.9	-4.87	< .001
	CW	54.8	49.2	-4.26	< .001
	CL	38.5	32	-3.71	< .001
Sex	Morphometry	$\chi^2 = \text{Female}$	$\chi^2 = \text{Male}$	T Test Value	p-value
	Weight	26.6	33.8	-4.87	< .001
	CW	50.5	54.6	-4.26	< .001
	CL	34.4	37.2	-3.71	< .001

cal traits compared to less heavier crabs. Morphological traits in intertidal crabs are influenced by sexual, allometric, and habitat factors, with claw size being particularly prominent and carapace shape often associated with habitat preferences (Vermeiren et al. 2021). Vermeiren et al. (2021) also emphasised that morphological variation arises from an intricate interplay of environmental, genetic, and physiological factors, highlighting the combined effects of sex, growth patterns, and habitat conditions. Further research could investigate the specific mechanisms driving these effects and examine the potential ecological implications of morphological variation in crabs.

The observed spatial and sexual dimorphism in *T. crenata* morphology has significant implications for ecological and evolutionary studies. Distinct morphological traits may influence feeding behaviors, mating success, and predator avoidance strategies (Daly et al. 2021). Additionally, understanding these variations can inform conservation efforts, focusing on habitat preservation and sustainable fishing practices.

Analysis of the condition factor (CF) for different categories of *T. crenata* (Figure 6). CF is a metric used to assess the overall health and well-being of a crab, with higher values indicating better condition. The box plots show the distribution of CF values for each category, with the box representing the middle 50 % of the data, the whiskers extending to the minimum and maximum values, and dots representing outliers. The Kruskal-Wallis test results ($p < 0.01$) indicate significant differences in CF between some categories. Differences between areas could be attributed to environmental factors like food availability or water quality. While not highly significant, there might be subtle sex-specific differences in CF. Considering food availability is a critical environmental determinant influencing the distribution and behavior of crabs' species. Variations in crabs' habitat quality, namely: availability of prey, can significantly impact the reproductive success and morphological adaptations of crabs (Griffen & Norelli 2015), suggesting that food availability directly impacts the reproductive potential and overall fitness of crabs. Water quality, which includes salinity and sediment characteristics, also plays a key significant role in the distribution and habitat preference of crabs indicating water quality parameters are crucial in determining habitat suitability for various crab species (Mokhtari et al. 2015). Overall, higher CF values suggest well-nourished and healthy crabs, while lower values could indicate potential stress or nutritional deficiencies.

The data in Table 5 provides a comparative analysis of *T. crenata* size variations across Butuan Bay (Philippines), Panjang Island (Indonesia), and Setiu Wetlands (Malaysia), using carapace width (CW) and length (CL) as key metrics. Notably, crabs in Butuan Bay exhibit smaller average dimensions

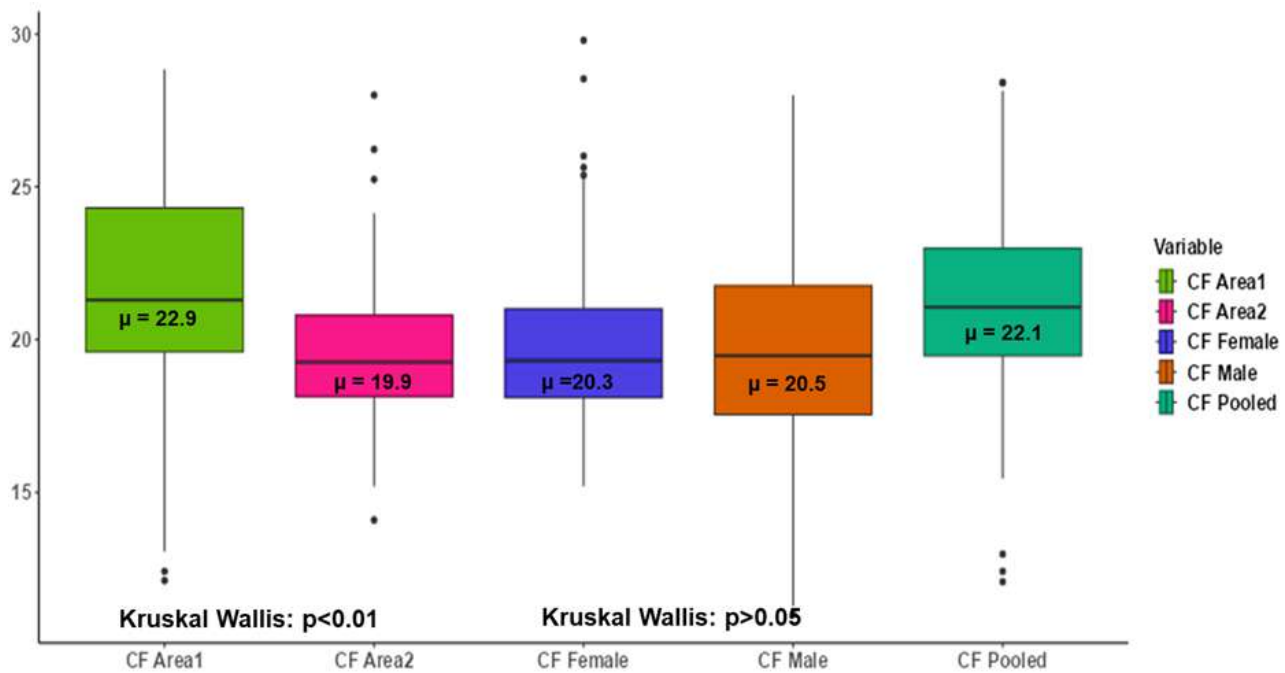


Figure 6. Condition factor of *Thalamita crenata*.

than those in other regions. Males in Butuan Bay measure 54.6 mm CW (max 70 mm) and 37.2 mm CL (max 47 mm), while females are smaller, averaging 50.5 mm CW (max 66 mm) and 34.4 mm CL (max 44 mm). In contrast, males from Panjang Island are significantly larger, with a mean CW of 59.53 mm (max 78 mm) and CL of 41.62 mm (max 55 mm), and females similarly surpass Butuan Bay specimens at 53.76 mm CW (max 70 mm) and 38.23 mm CL (max 57 mm). This trend is further emphasised by data from Setiu Wetlands, where males average 66.9 mm CW (max 81.9 mm)—markedly larger than Butuan Bay males—though CL data for this region is unavailable.

Pooled data for Butuan Bay, combining both sexes, reinforces the observed size disparity, with a mean CW of 52.21 mm (max 67 mm) and CL of 35.51 mm (max 47 mm), consistently lower than other locations. The inclusion of CL measurements strengthens evidence of potential size reduction, as differences persist across both metrics. These findings suggest environmental or anthropogenic pressures may be influencing growth patterns in Butuan Bay, warranting further investigation into factors such as habitat conditions, fishing practices, or genetic diversity.

The observed size reduction in Butuan Bay may stem from multiple stressors. Environmental degradation, such as pollution or habitat loss, could limit resource availability and impede growth. Overfishing is another critical concern, as selective harvesting of larger individuals may truncate population structure, leaving smaller, younger crabs to dominate the breeding pool (Pastor et al. 2024; Elvira et al. 2016). Over time, this pressure could drive genetic changes favouring smaller body sizes, as larger crabs are removed before reproducing. Smaller females may produce fewer eggs, reducing reproductive output and threatening population resilience. Such shifts in size structure could destabilise local ecosystems, altering predator-prey dynamics and diminishing the species' ecological role as a key intertidal engineer.

These comparison highlights the need for targeted conservation strategies. Mitigating environmental stressors through habitat restoration, enforcing size-based fishing regulations to protect mature individuals, and monitoring genetic diversity are essential steps to curb further decline. Future research should prioritise comprehensive data collection across all regions, including female specimens from Malaysia, to enable robust comparisons and clarify the mechanisms driving size differences.

Table 5. Comparison of mean sizes of CW and CL from previous studies in Indonesia and Malaysia.

Study Area	Dataset	Carapace Width (mm)			Carapace Length (mm)			Reference
		Mean	Min	Max	Mean	Min	Max	
Butuan Bay	Pooled	52.21	30	67	35.51	23	47	This Study
	Male	54.6	30	70	37.2	25	47	
	Female	50.5	33	66	34.4	23	44	
Panjang Island, Indonesia	Male	59.53	40	78	41.62	27	55	Susanto & Irnawati 2014
	Female	53.76	42	70	38.23	30	57	
Setiu Wetlands, Terengganu, Malaysia	Male	66.9	38.9	81.9	NA	NA	NA	Muhd-Farouk et al. 2017

CONCLUSION

This study on *Thalamita crenata* in Butuan Bay, Philippines, revealed significant sexual and spatial differences in morphometric relationships and condition factors based on 226 samples. Crabs from Cabadbaran (Area 1) were larger and in better condition than those from Buenavista (Area 2), with males generally larger than females, consistent with typical crustacean dimorphism. The weight–carapace length and width relationships showed negative allometry, and overall crab sizes were smaller compared to populations in Indonesia and Malaysia, likely due to overfishing, habitat degradation, or environmental stress. These findings highlight concerns for population sustainability and underscore the need for targeted management, such as size regulations, habitat protection, and improved fishing practices. Future studies should incorporate environmental variables (e.g., temperature, salinity) and genetic analyses to better understand the drivers of morphological variation and to support effective and localised conservation strategies.

AUTHOR CONTRIBUTION

JHJ conceptualised the study design, spearheaded the data analysis, and finalised the manuscript for publication. PJBP conducted the sampling and data collection and contributed to the discussion and data interpretation. RBC performed the literature review and participated in the discussion and data interpretation.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests, whether financial, personal, or professional, that could impact the validity or interpretation of the research findings presented in this article.

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Research Article

Leaf Anatomical Structure of Five True Mangrove Species in Pemogan Village, Denpasar – Bali

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ABSTRACT

Anatomy is an approach to study physiological processes that occurs in plants. Even though the mangrove community is known as one of the communities with the largest carbon stock, data regarding the anatomical structure of its leaves is still insufficient. Therefore, this research aims to determine the anatomical structure of leaves in several mangrove species and to compare those structures. Samples of mangrove species were obtained from Simbar Segara Batu Lumbang mangrove area in Pemogan Village, Denpasar City, Bali. The species are used i.e. (1) *Lumnitzera racemosa* (Combretaceae) in landward zone; (2) *Xylocarpus granatum* (Meliaceae) in landward zone; (3) *Aegiceras corniculatum* (Primulaceae) in middle zone; (4) *Rhizophora apiculata* (Rhizophoraceae) in middle zone; and (5) *Sonneratia alba* (Lythraceae) in seaward zone. Anatomical data was obtained by the free hand section method through the midrib and a paradermal method. The data was analysed descriptively, then continued with principal component analysis (PCA). Based on the results, *L. racemosa* and *S. alba* are species with amphistomatic (the other species were hypostomatic) and isobilateral leaves (the others were dorsiventral). These two characters are the dominant in PCA. Epidermal tissue, palisade mesophyll, sponge mesophyll, vascular bundles, and calcium oxalate crystals are evenly found in all species. *Rhizophora apiculata*, *A. corniculatum*, and *X. granatum* have hypodermal tissue as a dominant character in PCA. Secretory tissue and salt glands are only observed in *A. corniculatum*. The five species have three different stomata types, namely tetracytic (*L. racemosa*), anomocytic (*X. granatum*, *A. corniculatum*, *R. apiculata*), and staurocytic (*S. alba*).

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INTRODUCTION

Mangroves are plant communities that play an important role in mitigating climate change with their ability to absorb and to store large amounts of carbon. The average amount of carbon stock in the world's mangrove ecosystem is estimated around $115 \pm 7 \text{ Mg C ha}^{-1}$ as above ground carbon stock and $741 \pm 29 \text{ Mg C ha}^{-1}$ as below ground carbon stock (Kauffman et al. 2020). The amount of carbon stock in mangroves involves two main mechanisms, namely sequestrations through photosynthesis and sediment trapping by their root system. Specifically, in photosynthesis, the anatomical structure of mangrove leaves plays an important role in this process. The two main tissues in mangrove leaves that play a role in photosynthesis are palisade and spongy mesophyll which are differentiations of parenchymal tissues to carry out the assimilation process. Despite having these tissues, the assimilation process in mangroves requires additional structures to maintain their physiological conditions within the optimal range (Tatongjai et al. 2021). These additional structures are an essential mechanical adaptation to environmental stresses in the mangrove habitat (Tihurua et al. 2023).

Mangroves have a specific habitat which is in intertidal areas affected by sea tides. This habitat is difficult for the other plant species to grow because it has various environmental stresses, such as rapid wind movement, high water tides, periodic inundation, high light intensity, and fluctuations in water salinity. Related to the photosynthesis process in leaf, adaptation to these stresses can be observed at the anatomical level (Filartiga et al. 2022; Strock et al. 2022). Several anatomical characteristics of leaves related to the adaptation to environmental stresses in mangroves are the presence of thick hypodermal tissue, salt glands, secretory tissue, sclereids, and the arrangement of tissue in the leaves including their shape and thickness (Sánchez et al. 2021; Tatongjai et al. 2021; Tobing et al. 2022; Tihurua et al. 2023).

Not all mangrove species have similar tissue structure, because they do not always have the same niche. Mangrove niches can be simply divided based on zones, such as the landward zone, the middle zone, and the seaward zone. Each zone has different environmental conditions that tend to be gradual from landward to seaward (particularly the sediment from muddy to sandy and from low to high salinity), so that the species composition will change and form a unique ecotone (Wang et al. 2019; Irawan et al. 2021). In Bali, Kitamura et al. (1997) categorised mangrove species based on major distribution in landward, middle, and seaward zone. The landward zone commonly features *Bruguiera gymnorhiza*, *Ceriops tagal*, *Excoecaria agallocha*, *Lumnitzera racemosa*, *Sonneratia caseolaris*, and *Xylocarpus granatum*. In the middle zone, *Aegiceras corniculatum*, *Rhizophora mucronata*, *R. apiculata*, and *R. stylosa* are present. Conversely, *Sonneratia alba*, *Avicennia marina*, and *A. alba* are typically found in the seaward zone. Under certain conditions such as the sea sedimentation that affect the salinity fluctuation, several species can overlap and be found in other zones, such as *R. apiculata*, *S. alba*, *A. marina*, and *A. alba* which can be spread across various zones.

The mangrove area of Simbar Segara Batu Lumbang is located in Pemogan Village, Denpasar City and has all three mangrove zones. The mangrove area is relatively sustained because of the community whose actively conserves the mangroves with various activities and cleans up the trash that floated from the sea. Because this area reflects natural mangrove conditions, it is such an appropriate location for foundational research on mangrove ecosystems, particularly for investigating the anatomy of mangrove leaves. Study on the leaf anatomy of mangrove species in Bali is still insufficient, even though this anatomical structure is closely related to the physiological processes (Filartiga et al. 2022; Strock et al. 2022), especially in carbon absorption and adaptation to the environment that become a distinct ecosystem service in

mangroves. Therefore, as an initial step in compiling the database, this study aims to determine the anatomical structure of mangrove leaves in different zones in the Simbar Segara Batu Lumbang mangrove area of Pemogan Village, Denpasar, Bali.

MATERIALS AND METHODS

Study Site

The study was conducted in January-May 2023 in the Batu Lumbang area located in Pemogan Village, Denpasar. The Batu Lumbang area is utilised as one of the tourist attractions managed by the Simbar Segara Batu Lumbang fisherman group, where around 81 % of the area is mangrove forest (Mursyid et al. 2022). This study used an exploration method (Rugayah et al. 2004) by exploring the mangrove area in the Batu Lumbang area. The research location is shown in Figure 1 with a red line indicating the exploration route for sampling (south towards the sea) with more than 5 km. The exploration started at the coordinate S: -8.719185° , E: 115.201130° and ended at S: -8.733596° , E: 115.197620° . The landward zone of mangrove explored by foot, but for the middle zone to seaward zone we used canoe to reach the representative locations.

Sample Collections

The exploration in the mangrove area of Batu Lumbang obtained ten true mangrove species: *Aegiceras corniculatum*, *Avicennia marina*, *Bruguiera gymnorhiza*, *Ceriops tagal*, *Excoecaria agallocha*, *Lumnitzera racemosa*, *Rhizophora apiculata*, *R. mucronata*, *Sonneratia alba*, and *Xylocarpus granatum*. The identification was using Flora of Java volume 1 (Backer & van den Brink 1963) and mangrove database from Handbook of Mangrove in Indonesia: Bali and Lombok (Kitamura et al. 1997). Among those species, five species that resembled different families were chosen by prioritising their natural distribution zone (Table 1). Landward zone composed by *X. granatum* and *L. racemosa*, middle zone composed by *A. corniculatum* and *R. apiculata*, while the seaward zone represented by *S. alba*. Three leaves from three different individual were collected with the following criteria: (1) the leaves have fully bloomed; (2) the



Figure 1. Mangrove exploration routes in Batu Lumbang, Denpasar.

Table 1. Taxonomical data of sampled mangrove species. The accepted name obtained from POWO (<https://powo.science.kew.org/>), while the vernacular name refers Kitamura et al. (1997).

No	Family	Species	Vernacular Name	Coordinate sample
1.	Combretaceae	<i>Lumnitzera racemosa</i> Willd. Synonym: -	Saman-sigi, kedukduk, truntun, susup, lasi.	-8.722494°, 115.200861°
2.	Meliaceae	<i>Xylocarpus granatum</i> J.Koenig Synonym: <i>Carapa granatum</i> (J.Koenig) Alston; <i>Carapa indica</i> A.Juss.	Banang-banag, siri, nyirih, niri.	-8.720863°, 115.200865°
3.	Primulaceae	<i>Aegiceras corniculatum</i> (L.) Blanco Synonym: <i>Umbraculum corniculatum</i> (L.) Kuntze; <i>Rhizophora corniculata</i> L.	Kacangan, kacang-kacang, gedangan, klungkum.	-8.724433°, 115.200498°
4.	Rhizophoraceae	<i>Rhizophora apiculata</i> Blume Synonym: -	Bakau, bakau putih, man-gi-mangi.	-8.727390°, 115.200151°
5.	Lythraceae	<i>Sonneratia alba</i> Sm. Synonym: <i>Blatti alba</i> (Sm.) Kuntze	Prapat, bogem, pupat, prepat.	-8.729688°, 115.200921°

colour were dark green or darker than the young leaves; (3) in the fourth position from the shoot; and (4) there are no pest or disease infections. The collected leaves were cleaned in running water to remove dirt that stuck to the surface of the leaves. The leaves were then dried with tissue, put it into a sample bottle, and poured with 70 % alcohol until all parts of the leaves were completely submerged.

Anatomical Data Collections

Two methods were used for anatomical observation. First is the transversal section method through the midrib to observed the layers of leaf structure, and the second is paradermal section method and paradermal replica to observe the structure of the leaf epidermis. The transversal section used the free-hand section method according to Lux et al. (2015) with some modifications in clearing solution (using 5 % sodium NaClO) and staining solution (using 1 % safranin and 1 % fast green). The leaves were removed from the sample bottle, then dried with tissue. To facilitate cutting, the sample was clamped between carrots, then cut as thinly as possible using a cutter. The cut results were re-immersed in 70 % alcohol for 5 minutes, then the sample transferred to 5 % NaClO solution and soaked for 2-5 minutes. After the sample was transparent, it was then washed in 70 % alcohol for 2 minutes. The sample stained with 1 % safranin for 1 minute, then rinsed with 70 % alcohol for 2 minutes and continued with 1 % fastgreen staining for 1 minute. After staining, the sample were rinsed with 70 % alcohol for 1 minute, then observed using through microscope that equipped with Optilab. Scaling was done using Image Raster software.

Paradermal sections were made using the free hand section method also from samples that had previously been stored in 70 % alcohol, using modified method from Lux et al. (2015). The samples were removed from the bottle, then dried using tissue and then incisions were made on the upper and lower surfaces of the leaves. The incisions (samples) were soaked in 5 % NaClO solution for 5-10 minutes. The NaClO solution was then replaced with 70 % alcohol and soaked for 5 minutes. The samples were then stained with 1 % safranin for 3 minutes and then rinsed again with 70 % alcohol. The samples were then placed on a glass object and added with one drop of aquadest and then covered with a cover glass. Another method, namely a paradermal replica, was made by applying clear nail polish to the surface of the leaves that had previously been cleaned with tissue (without cutting). The nail polish that had been applied was waited for 10 minutes to dry. After that, clear tape was attached to the nail polished part, then the tape was pulled and attached to the glass object. The preparations produced from both methods were then ob-

served on a microscope that equipped with an Optilab device, while the scaling and cell counting were carried out using Image Raster software. The paradermal replica was easier than paradermal sections, but not all species were suitable for this method. Three repetitions were used in every samples. The observed characters consisted of stomatal type and stomatal index calculated using the formula retrieved from Poole and Kurschner (1999):

$$\text{Stomatal Index (SI)} = \frac{\Sigma S}{\Sigma S + \Sigma E} \times 100 \%$$

Annotations:

ΣS = Number of stomata in one field of view

ΣE = Number of epidermal cells in one field of view

Data Analysis

The data obtained were analysed descriptively to determine the anatomical characteristics of the mangrove leaves. The data were presented as leaf anatomy images and comparison tables. The comparison table summarises all observable characters, both synthetic characters and diagnostic characters of the mangrove species. In addition, further analysis was carried out in the form of Principal Component Analysis (PCA) to determine the characters that are unique to the anatomy of mangrove leaves. Character scoring uses a numerical taxonomic approach by considering the Adansonian principle according to Sokal and Sneath (1963). All the numerical analysis conducted in Multi-Variate Statistical Package (MVSP v.3.13.) software by Kovach Computing Services.

RESULTS

Leaf anatomical structure of mangrove in Batu Lumbang

Lumnitzera racemosa

Lumnitzera racemosa leaves are succulent leaves with a not very prominent leaf vein. Based on a transverse section through the midrib (Figure 2a), the observed tissues consist of upper epidermal, upper palisade mesophyll, spongy mesophyll, vascular bundles, lower palisade mesophyll, and lower epidermal, while the non-protoplasmic components observed are drusen-type of calcium oxalate crystals. The presence of upper and lower palisade mesophylls indicates that this leaf is an isobilateral type. Although both palisade mesophyll tissues consist of two layers of cells, the cells in the upper palisade mesophyll are longer than the lower. The vascular bundle was in arc shape with collateral position.

The characteristics of the stomata are shown in Figure 2b1 and 2b2. The stomata presence on the upper (adaxial) and lower (abaxial) sides of the leaf indicate that *L. racemosa* has amphistomatic leaves. The type of stomata is determined by the shape and position of the subsidiary cells that flank the guard cells. The type of stomata in *L. racemosa* is tetracytic, because the guard cells are surrounded by four subsidiary cells (two in lateral and two in poles of guard cells). The SI value in *L. racemosa* is $9.57 \pm 1.38 \%$ on the adaxial surface and $6.42 \pm 0.91 \%$ on the abaxial surface.

Xylocarpus granatum

Transversal section of *X. granatum* (Figure 3a) shows the leaf structure consisting of upper epidermal, 2-3 layers of hypodermal, 2-3 layers of palisade mesophyll, spongy mesophyll, vascular bundles, and lower epidermal, while the non-protoplasmic components observed are drusen-type of calcium oxalate crystals. The vascular bundle has an arc shape, have a small addition vascular bundle above it, and surrounded by lignified cells, the same type as reported by Tihurua et al. (2023). Based on the characteristics of the stomata in Figure 3b1 and 3b2, the type of stomata in *X. granatum* is anomocytic which

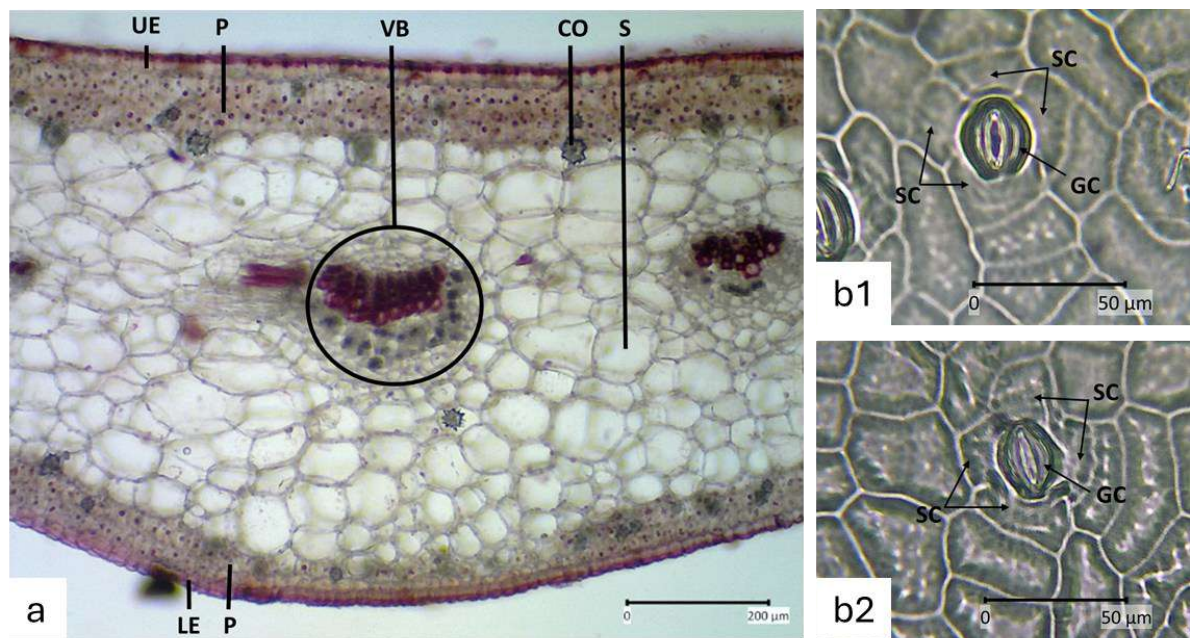


Figure 2. Leaf anatomical structure of *L. racemosa* (a) transversal section through the midrib, (b1) replica paradermal of the adaxial surface, (b2) replica paradermal of the abaxial surface. Annotations: UE: upper epidermal, LE: lower epidermal, P: palisade mesophyll, S: spongy mesophyll, VB: vascular bundle, CO: calcium oxalate crystal (drusen-type), GC: guard cell, SC: subsidiary cell.

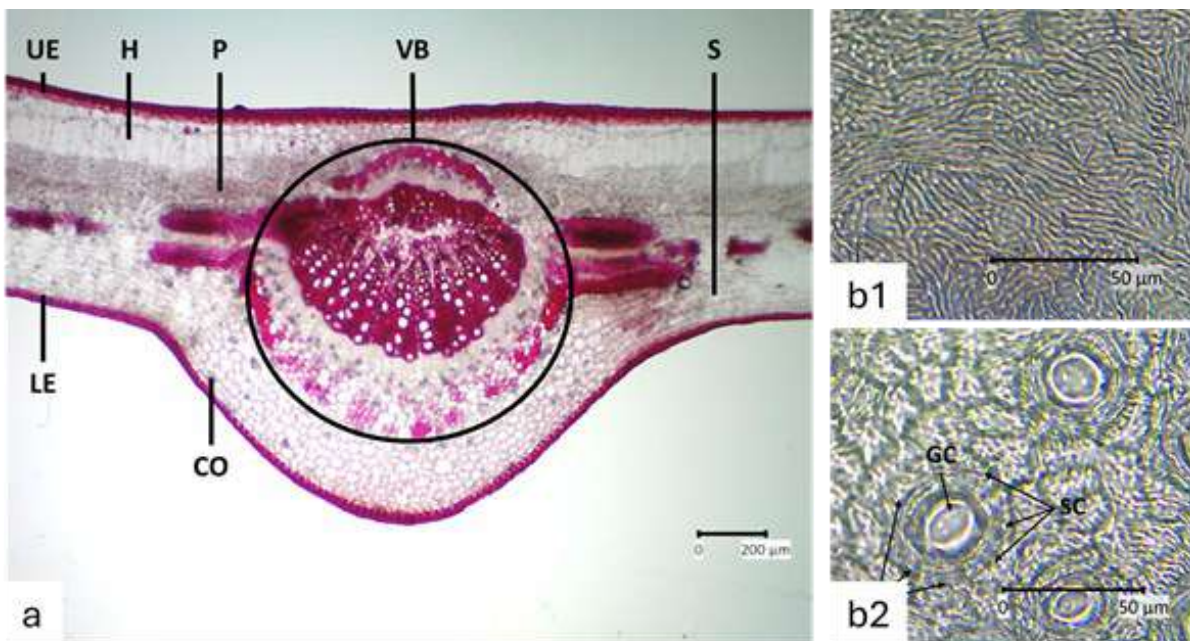


Figure 3. Leaf anatomical structure of *X. granatum* (a) transversal section through the midrib, (b1) replica paradermal of the adaxial surface, (b2) replica paradermal of the abaxial surface. Annotations: UE: upper epidermal, LE: lower epidermal, H: hypodermal, P: palisade mesophyll, S: spongy mesophyll, VB: vascular bundle, CO: calcium oxalate crystal (drusen-type), GC: guard cell, SC: subsidiary cell.

is the guard cells surrounded by subsidiary cells which have the same shape and size with the epidermal cells. Stomata are only found on the lower surface (abaxial) which indicate the leaf of this species is a hypostomatic. The SI value in *X. granatum* is 13.95 ± 2.25 %.

Aegiceras corniculatum

The anatomical structure of *A. corniculatum* (Figure 4a) shows a structure consisting of upper epidermal tissue, 3-4 layers of hypodermal, 2-3 layers of palisade mesophyll, spongy mesophyll, vascular bundles, secretory tissue, salt

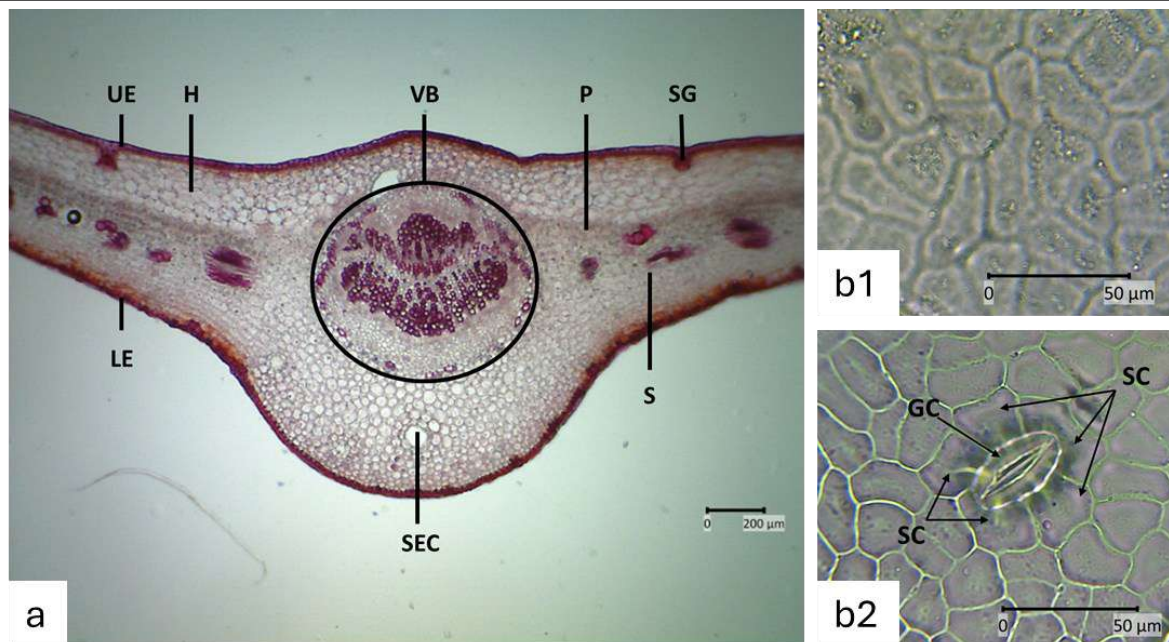


Figure 4. Leaf anatomical structure of *A. corniculatum* (a) transversal section through the midrib, (b1) replica paradermal of the adaxial surface, (b2) replica paradermal of the abaxial surface. Annotations: UE: upper epidermal, LE: lower epidermal, H: hypodermal, P: palisade mesophyll, S: spongy mesophyll, VB: vascular bundle, SEC: secretory tissue, SG: salt gland, GC: guard cell, SC: subsidiary cell.

glands, and lower epidermal. The vascular bundle resembles that of *X. granatum*, forming an arc vascular bundle with an additional vascular bundle on the upper part of the arc, as observed by Tihurua et al. (2023). Based on the characteristics of the stomata in Figure 4b1 and 4b2, the type of stomata in *A. corniculatum* is anomocytic which is the guard cells surrounded by subsidiary cells which similar to the epidermal cells. The SI value in *A. corniculatum* is 8.27 ± 0.67 %.

Rhizophora apiculata

Leaf transversal section through the midrib of *R. apiculata* (Figure 5a) is arranged by upper epidermal tissue, 4–7 layers of hypoderms, 2–3 layers of palisade mesophyll, spongy mesophyll, vascular bundles, sclereids, and lower epidermal. In addition, there is drusen-type of calcium oxalate crystals. The vascular bundle composed by two arc which bend outside, and a small vascular bundle addition in the centre. Sometimes it also resembles separated vascular bundles that form closed ring with additional vascular bundle in the centre (Tihurua et al. 2023). In addition, there are also cork warts in the spongy mesophyll to the lower epidermis. Based on the characteristics of the stomata in Figure 5b1 and 5b2, the type of stomata in *R. apiculata* is anomocytic which is the guard cells surrounded by subsidiary cells which have the same shape and size with the epidermal cells. Stomata are only found on the lower surface (abaxial), so it is called a hypostomatic leaf. The SI value in *R. apiculata* is 4.71 ± 0.45 %.

Sonneratia alba

The leaves of *S. alba* are succulent with fairly clear midrib. This species has leaf with a layer of upper and lower epidermal tissue, 3–4 layers of upper and lower palisade, sponge, vascular bundles, sclereids, and drusen-type of calcium oxalate crystals (Figure 6a). The vascular bundle composed by two flat arcs of xylem and phloem, similar as other *Sonneratia* species reported by Tihurua et al. (2023). The upper arc tends to smaller than the lower arc. The presence of two upper and lower palisade mesophylls indicates that this leaf is

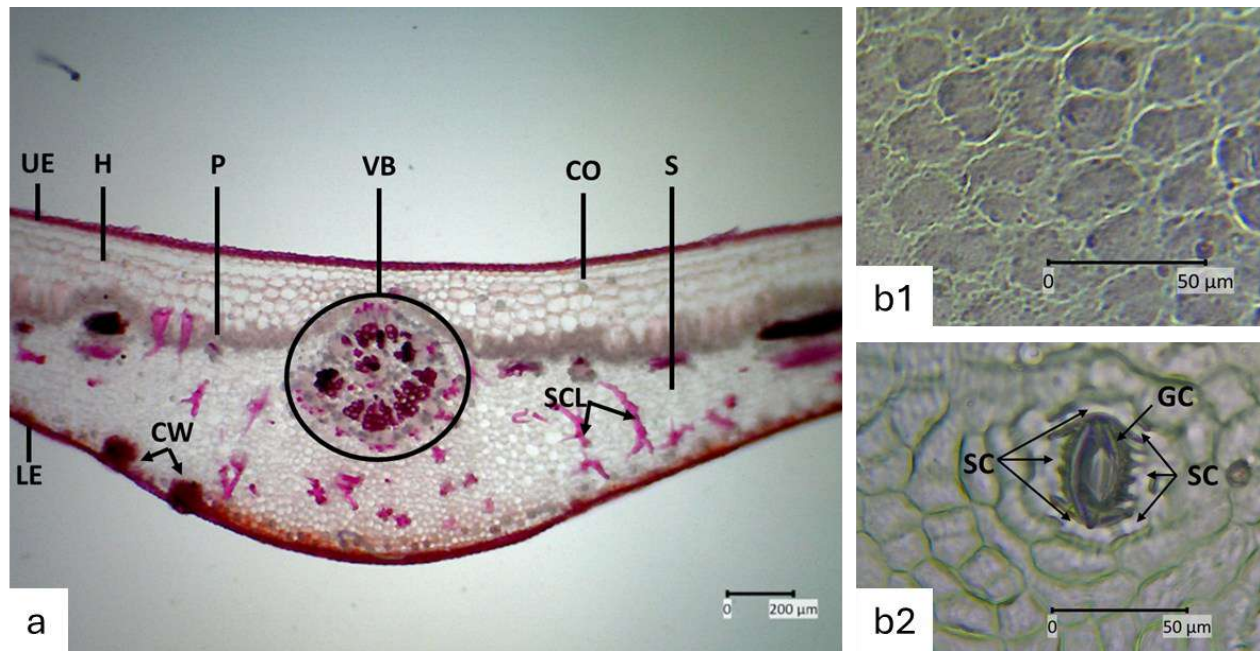


Figure 5. Leaf anatomical structure of *R. apiculata* (a) transversal section through the midrib, (b1) replica paradermal of the adaxial surface, (b2) replica paradermal of the abaxial surface. Annotations: UE: upper epidermal, LE: lower epidermal, H: hypodermal, P: palisade mesophyll, S: spongy mesophyll, VB: vascular bundle, CO: calcium oxalate crystal (drusen-type), SCL: sclereids, CW: cork warts, GC: guard cell, SC: subsidiary cell.

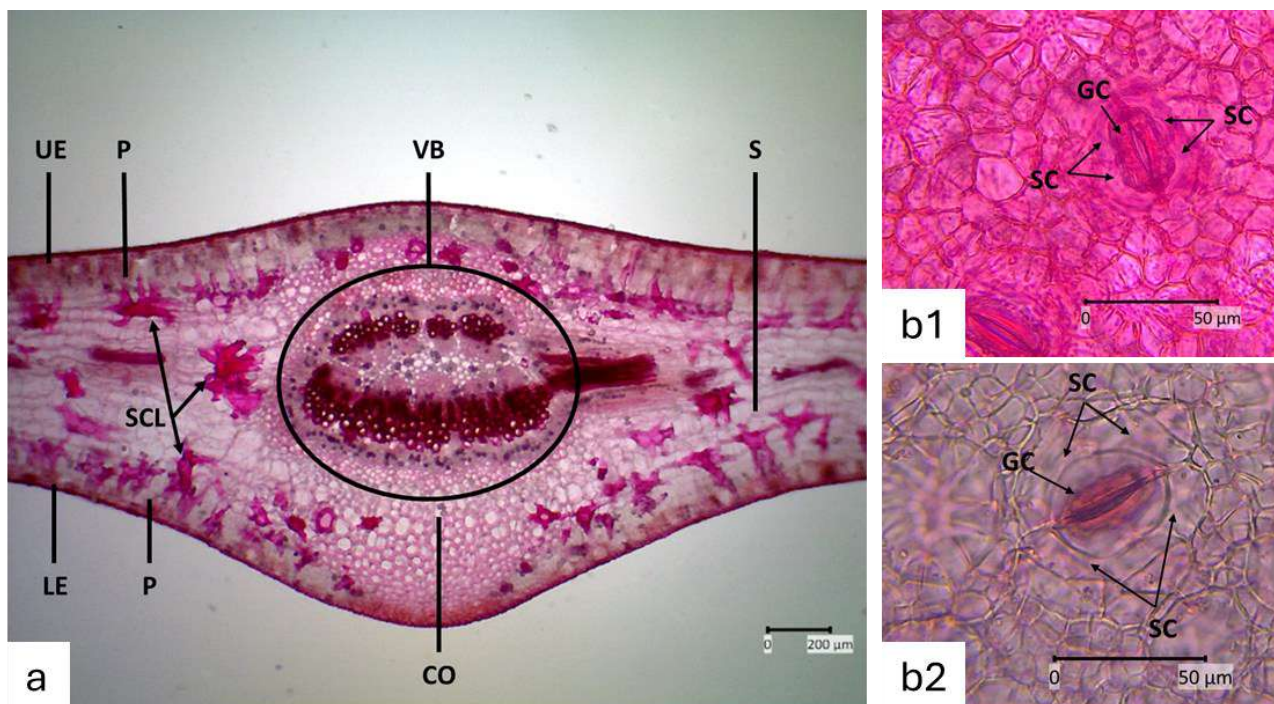


Figure 6. Leaf anatomical structure of *S. alba* (a) transversal section through the midrib, (b1) paradermal of the adaxial surface, (b2) paradermal of the abaxial surface. Annotations: UE: upper epidermal, LE: lower epidermal, P: palisade mesophyll, S: spongy mesophyll, VB: vascular bundle, CO: calcium oxalate crystal (drusen-type), SCL: sclereids, GC: guard cell, SC: subsidiary cell.

an isobilateral. Based on the characteristics of the stomata (Figures 6b1 and 6b2), the type of stomata in *S. alba* is staurocytic because of the guard cells surrounded by 4 subsidiary cells in perpendicular positions, but parallel to the guard cells which giving an X-position of subsidiary cells. This species is grouped in to amphistomatic leaf as stomata are found on the upper (adaxial) and lower (abaxial) surfaces. The SI value in *S. alba* was 2.56 ± 0.52 % on the adaxial surface and 1.90 ± 0.25 % on the abaxial surface.

Structural comparison of leaf anatomical traits in mangrove

Comparison of anatomical structures of mangrove leaves is shown in Table 2. Among the 15 characters, *R. apiculata* and *S. alba* have the most characters (11 characters) while *X. granatum* has the fewest characters (9 characters). In terms of character, salt glands and secretory tissue are the rarest which is only observed in *A. corniculatum*. A principal component analysis (PCA) was carried out to determine the presence of unique anatomical characters in a species. Of the 15 characters, selection was carried out by eliminating characters with the same value, so that 11 characters were remained. After that, scoring was carried out by giving a score or value to the character and was presented in the form of a scoring table as shown in Table 3. The results of this scoring were used to carry out PCA. Based on the PCA results, 4 axes are needed to obtain a cumulative score of 100 %, while the 3 main axes have obtained a cumulative percentage value of the variance of 91.48 % as shown in Table 4. Pankhurst (1991) stated that the accuracy of the PCA results is classified as good or appropriate if the cumulative value on the three main axes is more than 80 %.

Visualisation of PCA results is in the form of a scatter plot with vectors which represent the eigenvalues (Figure 7). The scatter plot is made with axis 1 and axis 2 with a cumulative percentage of 81.75 %, which means that the PCA results accommodate 81.75 % of the variation in the data. The value of axis 1 (57.05 %) is higher than axis 2 (24.70 %), so the priority of cluster separation is more determined by axis 1 or the x-axis than by axis 2 or the y-axis. In general, there are two main clusters formed, namely cluster-I (blue) consisting of *S. alba* and *L. racemosa*, and cluster-II (red) consisting of *R. apiculata*, *A. corniculatum*, and *X. granatum*. The characters that are responsible in grouping the cluster-I are K3 (lower palisade mesophyll), K11 (stomatal type) which overlapping with K3, and K9 (adaxial stomatal index). These two characters are found in *S. alba* and *L. racemosa*, while the other three species do not have this character. The character K9 (adaxial stomatal index) is only found in amphistomatic leaves, while *R. apiculata*, *A. corniculatum*, and *X. granatum* have hypostomatic leaves. The character K3 (lower palisade mesophyll) only found in isobilateral leaves, while the three species in cluster-II have dorsiventral leaves. Another character is K11 (stomatal type) which is tetracytic in *L. racemosa* and staurocytic in *S. alba*, while the three species in cluster-II have anomocytic type. Mangrove species with dorsiventral leaves have thick hypodermal, so K1 (upper hypodermal) is the main vector that groups cluster-II.

DISCUSSION

The anatomical structure of mangrove leaves resembles the anatomical structure of dicotyledonous plants, but with slight additions and modifications to the constituent tissues. In the epidermal tissue, there is a thick cuticle layer on the upper and lower surfaces of the leaves. The cuticle is an exudate in the form of wax produced by the epidermal tissue to prevent excessive evapotranspiration (Sánchez et al. 2021). This is a common adaptation mechanism found in plants with habitats that have high light intensity (Chorchuhirun et al. 2020; Sánchez et al. 2021).

In some species, such as *X. granatum*, *A. corniculatum*, and *R. apiculata*, there is a hypodermal layer just below the upper epidermal tissue. The hypodermal is a derivative of the epidermal tissue which is generally composed of large and elongated cells. The hypodermal plays a role in preventing excessive water loss due to environmental stress such as high light intensity and salinity by preventing osmotic shock, so that it can maintain tissue osmotic pressure (Chorchuhirun et al. 2020; Sánchez et al. 2021).

The tissue below is palisade mesophyll. In dorsiventral leaves, this tis-

Table 2. Leaf anatomical comparison of the observed mangrove species.

No	Character	Species				
		LR	XG	AC	RA	SA
1	Upper epidermal	+	+	+	+	+
2	Upper hypodermal	-	2—3 layers	3—4 layers	4—7 layers	-
3	Upper palisade mesophyll	2 layers	2—3 layers	2—3 layers	2—3 layers	3—4 layers
4	Vascular bundle	+	+	+	+	+
5	Sponge mesophyll	+	+	+	+	+
6	Lower palisade mesophyll	2 layers	-	-	-	3—4 layers
7	Lower epidermal	+	+	+	+	+
8	Salt gland	-	-	+	-	-
9	Secretory tissue	-	-	+	-	-
10	Calcium oxalate crystal	+	+	-	+	+
11	Sclereid	-	-	-	+	+
12	Cork warts	-	-	-	+	-
13	Adaxial stomatal index	9.57 ± 1.38 %	-	-	-	2.56 ± 0.52 %
14	Abaxial stomatal index	6.42 ± 0.91 %	13.95 ± 2.25 %	8.27 ± 0.67 %	4.71 ± 0.45 %	1.90 ± 0.25 %
15	Stomatal type	Tetracytic	Anomocytic	Anomocytic	Anomocytic	Staurocytic

Annotations: (+) indicating present, (-) indicating absent, LR=*Lumnitzera racemosa*, XG=*Xylocarpus granatum*, AC=*Aegiceras corniculatum*, RA=*Rhizophora apiculata*, SA=*Sonneratia alba*.

Table 3. Characters selections and scores of observed leaf anatomical characteristics.

Code	Character	Species				
		LR	XG	AC	RA	SA
K1	Upper hypodermal [0 = absent, 1 = 2—3 layers, 2 = 3—4 layers, 3 = >4 layers]	0	1	2	3	0
K2	Upper palisade mesophyll [0 = 2 layers, 1 = 2—3 layers, 2 = >3 layers]	0	1	1	1	2
K3	Lower palisade mesophyll [0 = absent, 1 = 2 layers, 2 = >2 layers]	1	0	0	0	2
K4	Salt gland [0 = absent, 1 = present]	0	0	1	0	0
K5	Secretory tissue [0 = absent, 1 = present]	0	0	1	0	0
K6	Calcium oxalate crystal [0 = absent, 1 = present]	1	1	0	1	1
K7	Sclereid [0 = absent, 1 = present]	0	0	0	1	1
K8	Cork warts [0 = absent, 1 = present]	0	0	0	1	0
K9	Adaxial stomatal index [0 = absent, 1 = <5 %, 2 = >5.1 %]	2	0	0	0	1
K10	Abaxial stomatal index [0 = 0—5 %, 1 = 5.1—10 %, 2 = >10.1 %]	1	2	1	0	0
K11	Stomatal type [0 = anomocytic, 1 = tetracytic, 2 = staurocytic]	1	0	0	0	2

Table 4. Eigenvalues for each axis in PCA.

Value	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	3.65	1.58	0.69	0.48
Percentage	57.05	24.70	10.72	7.52
Cumulative percentage	57.05	81.75	92.48	100.00

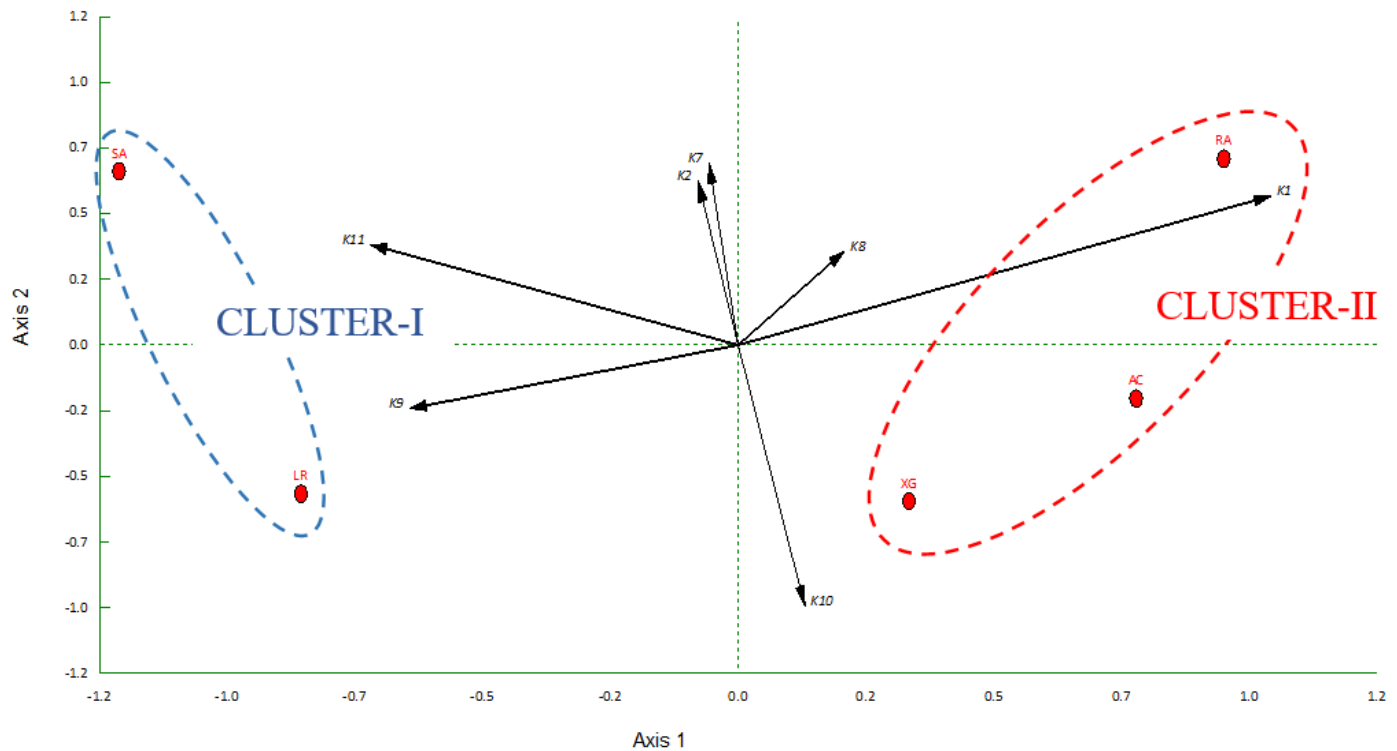


Figure 7. Scatter plot of PCA based on observed leaf anatomical structures in selected mangrove species.

sue is only found in the upper part, while the lower part occupied by spongy mesophyll tissue. Species with dorsiventral leaves are *X. granatum*, *A. corniculatum*, and *R. apiculata*, while others (*L. racemosa* and *S. alba*) are isobilateral. Indirectly, this shows the relationship between mangrove species with and without hypodermal structures. Species *X. granatum*, *A. corniculatum*, and *R. apiculata* have dorsiventral leaves with hypodermal layers, while *L. racemosa* and *S. alba* have isobilateral leaves without hypodermal. This hypodermal character is one of the cluster separation factors in PCA analysis as shown in Figure 7. Since the hypodermal related to the water stress by salinity and light intensity, *X. granatum* as a landward species have lesser hypodermal layers than *A. corniculatum* and *R. apiculata* which adapted in middle zone with higher salinity. Differences in the hypodermal layer were strongly influenced by genetic factors of each species. However, when related to environmental stress, a thicker hypodermal layer frequently found in a species which exposed to high levels of sunlight. Karabourniotis et al. (2021) stated that the presence of the hypodermal layer reduces the energy from potentially destructive sunlight, allowing the mesophyll tissue below to obtain optimal energy for photosynthesis. So, among *A. corniculatum* and *R. apiculata*, the hypodermal in *R. apiculata* were denser as an adaptability in full light intensity than in *A. corniculatum*.

Based on their function, palisade and spongy mesophyll tissues are responsible for the photosynthesis process with slightly different priorities. Palisade mesophyll tends to play a role in processes which involving sun light, so the density of chloroplasts is very high in the column-shaped palisade cells (Sánchez et al. 2021; Tobing et al. 2022). In contrast to spongy mesophyll with polygonal or irregular cells with air space between cells that are responsible in air circulation. Despite their different functional priorities, these two tissues show adaptive responses to environments stressed by high light intensity and salinity. The adaptation is by forming a dense cell arrangement (in palisade mesophyll, the cells are very slender and dense) with few air chambers (air chambers in spongy mesophyll are fewer and smaller than in majority of dicotyledonous species) to increase photosynthetic efficiency and prevent excessive transpiration (Parida et al. 2004; Sánchez et al. 2021; Tatongjai et

al. 2021).

To prevent the leaf structure from being damaged by strong winds, there are additional structures in the form of sclereids that are spread across the hypodermal and mesophyll tissues to provide mechanical support to the leaf structure. Not only as a supporting structure, sclereids are also able to prevent a decrease in turgor pressure while preventing herbivores (Vinoth et al. 2019; Tatongjai et al. 2021). These sclereids were observed in *R. apiculata* and *S. alba* in form of astrosclereid and rhizosclereid. Astrosclereids are star-shaped sclereids with elongated and relatively uniform branches, while rhizosclereids have several branches that are longer than others (Rao & Bhupal 1973; Tatongjai et al. 2021). Additionally, in the hypodermal and mesophyll tissues, drusen-type calcium oxalate crystal within idioblasts can be observed. Calcium oxalate crystals originate from the accumulation of metabolic waste that is generally found around the phloem tissue, then spreads to other tissues (Tatongjai et al. 2021). Physiologically, calcium oxalate crystals play a role in detoxifying heavy metals (Franceschi & Nakata 2005) and also as a source of CO₂ for photosynthesis (Tooulakou et al. 2016). In several taxonomic studies, the characteristics and distribution of calcium oxalate crystals are one of the main diagnostic characters for distinguishing between species (Raeski et al. 2023).

In addition, there are also structures in the form of secretory tissue and salt glands. Of the five species, these two structures are only found in *A. corniculatum*. Secretory tissue is characterised by the presence of epithelial cells around the cavity, while the salt glands are epidermal derivatives that distributed found on the adaxial and abaxial surfaces of the leaves (Naskar et al. 2021). The salt glands in *A. corniculatum* are one of the unique structures, which causes the leaves of this species have fine salt grains on the surface of the leaves. With a habitat that has relatively high salinity compared to fresh water, mangrove species have several salt excretion mechanisms which are classified into salt secretion, salt exclusion, and salt accumulation (Parida & Jha 2010). The mechanism in *A. corniculatum* is called salt secretions because salt excretion is carried out through secretory tissue, including salt glands (Parida & Jha 2010; Naskar et al. 2021). In *Bruguiera*, *Rhizophora*, *Sonneratia*, and *Xylocarpus*, salt excretion is carried out by the salt accumulation method by accumulating salt in cells in leaf tissue which are then shed at certain periods or when mature (Parida & Jha 2010; Tihurua et al. 2023). Another mechanism is salt exclusion by carrying out ultrafiltration on the roots which is commonly found in most mangrove species (Parida & Jha 2010).

In *R. apiculata*, there is a structure in the form of cork warts. This structure develops from the abaxial surface of the leaf and plays an important role in air circulation from the leaves to the roots in anoxic environments (Evans & Bromberg 2010; Naskar et al. 2021). Naturally, this structure can be found in various plant species, including mangroves. The PCA results in Figure 7 show the K8 vector (cork warts) which leads to *R. apiculata*. This indicated that this study agreed to Tihurua et al. (2023) which stated that cork warts is a distinguished anatomical characteristic for *Rhizophora*.

The common epidermal derivative found in leaves is stomata. These five mangrove species have different types of stomata. Our findings for *S. alba* (stauromycytic and anomocytic type), *R. apiculata* (anomocytic type), *X. granatum*, and *A. corniculatum* (anomocytic type) align with previous research (Tihurua et al. 2020, 2023; Sabandar et al. 2021). While for the *L. racemosa*, we found different types of stomata according to the previous study. Tihurua et al. (2020) found anomocytic and anisocytic type for *L. racemosa*, while in this research have observed tetracytic type.

Based on their position, leaves with stomata found on the upper (adaxial) and lower (abaxial) surfaces of the leaves, such as in *L. racemosa* and

S. alba. While, species with stomata only on the lower (abaxial) surface are found in *X. granatum*, *A. corniculatum*, and *R. apiculata*. The separation of species clusters based on the presence of stomata was also confirmed through PCA analysis (Figure 7). Based on the stomatal index (SI) value, there is a gradual pattern based on its life zone. This is due to the sensitivity of stomata to environmental changes, so that the SI value and other stomatal characteristics indirectly indicate environmental conditions (Zhu et al. 2018; Khan et al. 2022). In hypostomatic leaves, *X. granatum* living in the landward zone has the highest SI (13.95 ± 2.25 %), followed by *A. corniculatum* (8.27 ± 0.67 %) in the middle zone, and the lowest in *R. apiculata* (4.71 ± 0.45 %) in the middle zone facing the sea. The high SI value (K10 = abaxial stomatal index) in *X. granatum* causes the position of this species in the PCA diagram separated from *R. apiculata* and *A. corniculatum*. Similar things are also found in amphistomatic leaves which is the SI value of *L. racemosa* in the landward zone is always higher than *S. alba* in the seaward zone on the adaxial and abaxial surfaces. Another thing found in this study is the SI value on the adaxial surface which is always higher than the abaxial surface. In *L. racemosa*, the SI value on the adaxial surface was 9.57 ± 1.38 %, while on the abaxial surface it was 6.42 ± 0.91 %. In *S. alba*, the SI value on both surfaces was very small with 2.56 ± 0.52 % on the adaxial surface and 1.90 ± 0.25 % on the abaxial surface.

The SI analysis was popular due to the sensitivity in environmental changes, even in species level. In this study, the SI value related to the water stress due to the salinity and light intensity, similar to the effect in hypodermal layers. The landward species (*X. granatum*) facing lower salinity fluctuation, resulting in higher SI value. On the contrary with *R. apiculata* that adapted in high salinity fluctuation, which developed a lower SI value. The lower SI value generally reflects a strategy to conserve water by reducing transpiration, although this often comes at the cost of limiting CO₂ uptake and photosynthesis, unless compensated by other anatomical or physiological adjustments. The other factors that also related to the water regulation and gas exchange were the stomatal density and stomatal conductance (Royer 2001; Hetherington & Woodward 2003). The SI value also related to the leaf types. The landward species such as *X. granatum* and *L. racemosa* have different preferences. Hypostomatic leaf in *X. granatum* have SI 13.95 ± 2.25 %, while in amphistomatic leaf of *L. racemosa* have lower SI but occur in both side of the leaf surfaces to compensate that (adaxial 9.57 ± 1.38 %, abaxial 6.42 ± 0.91 %). It was the same between *R. apiculata* and *S. alba*.

CONCLUSIONS

The anatomical structure of leaves in five mangrove species belonging to five different families has different compositions between species. Based on the presence of stomata, *L. racemosa* and *S. alba* are species with amphistomatic leaves, while the others are hypostomatic leaves. Based on tissue symmetry (marked by the presence of lower palisade mesophyll), *L. racemosa* and *S. alba* are isobilateral leaves, while the others are dorsiventral leaves. Epidermal tissue, palisade mesophyll, spongy mesophyll, vascular bundles, and calcium oxalate crystals are evenly found in all species. In *R. apiculata*, *A. corniculatum*, and *X. granatum*, there is hypodermal tissue below the upper epidermal tissue. Secretory tissues and salt glands are only observed in *A. corniculatum*. Sclereids as a supporting tissue are found in *S. alba* and *R. apiculata*. The five species have three different stomata types, namely tetracytic (*L. racemosa*), anomocytic (*X. granatum*, *A. corniculatum*, *R. apiculata*), and staurocytic (*S. alba*). Based on the PCA results, the grouping of *L. racemosa* with *S. alba* is caused by leaves that are amphistomatic (there are stomata on the adaxial surface of the leaf/K-9) and isobilateral (have lower palisade mesophyll/K-3), while the grouping in *R. apiculata*, *X. granatum*, and *A. corniculatum* is predom-

inantly caused by the presence of hypodermis (K-1).

AUTHOR CONTRIBUTION

I.M.S.W. designed the research, collected and analysed the data, wrote the manuscript, and supervised all the process; N.K.R.P. collected samples and anatomical data, analysed the data, and wrote the manuscript; N.M.G. designed the research, wrote, and proofread the manuscript.

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CONFLICT OF INTEREST

Please state any conflict of interest regarding the research or the research funding.

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Research Article

Assessment of Fruit Production of Main Tree Species of Kon Chu Rang Nature Reserve, Gia Lai Province, Vietnam

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ABSTRACT

To carry out propagation work on the main tree species in the indigenous forests of Vietnam (Kon Chu Rang nature reserve), the primary tasks were to observe their development and fruiting, determine morphometric indicators, and apply processing methods to identify hidden defects and damage. Taxonomy and morphometric indicators of the main tree species were studied. The effects of sulfuric acid at various concentrations on the fruits of hard-shelled species with prolonged germination periods were studied. The X-ray method was employed to determine whether fruits and seeds were damaged by pests and diseases. The elements contained in the soil samples were determined, and methods for storing and sowing seeds of the trees under study were recommended. Due to the loss of a large amount of forest resources, the monsoon tropical forests of Vietnam require replenishment; the main task of forestry is to study the seed productivity of tree species, which plays a huge role in accelerating the development of nursery farming. Using the scarification method with sulfuric acid, it was established that the species *Ormossia balansae*, *Dialium cochinchinense*, and *Dipterocarpus kerrii* responded positively to a 2 % concentration, and growth differed from other experimental options by 2-3 cm along the length of the seedling.

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INTRODUCTION

Reproduction and seed production play a key role in the restoration of Vietnam's forests after fires and deforestation. These characteristics determine the ability of forest ecosystems to regenerate. Restoration of forests depends on the availability of seeds of tree species and their ability to germinate under changed conditions. High reproduction and seed productivity help to minimise losses in biodiversity. This, in turn, contributes to preserving the ecological functions of forests, such as climate regulation, soil conservation, maintenance of the water regime, and provision of a natural habitat for many animal species.

In the context of the establishment of new economic ties and market relations, the problems of rational use and protection of forests are becoming particularly relevant not only in Vietnam but throughout the world (Šálek & Vřlupek 2012; Luong 2014; Dang 2020; Ngo et al. 2021). Long-term mismanagement of forests has led to significant deterioration of the national forest fund (Khuc et al. 2018; Trong et al. 2020; Huong & Cuong 2022).

In 2010, Gia Lai province (the location of the Kon Chu Rang Nature Reserve) had 673,541 hectares of natural forest, constituting more than 43 % of its total area (D.C. Nguyen et al. 2022). By 2022, the region had lost 4,08 thousand hectares of natural forest, equivalent to 2,80 million tons of CO₂ emissions (Tuan et al. 2022; Hop et al. 2023). Between 2001 and 2022, Gia Lai Province lost 931 ha of forest due to fires. The year with the largest loss of forestland due to fires during this period was 2016, when fires destroyed 530 hectares of forest, representing 2.9 % of all forest losses that year.

From 2001 to 2022, Gia Lai Province lost 38,200 hectares of monsoon rainforest, accounting for 23 % of its total forest loss during the same period. The total area of tropical monsoon forest in the study region declined by 11 % over the study period.

Understanding seed productivity is essential for forest restoration in Vietnam for the following reasons: determining the optimal sowing time, calculating the required number of seeds, selecting suitable tree species, developing seed storage and treatment methods, planning reforestation activities, and monitoring and evaluating effectiveness. In tropical conditions, many species' seed productivity aspects remain insufficiently studied, underscoring the relevance of this research topic. The study of seed productivity in woody plants is a main task of modern forestry, playing a crucial role not only in planting establishment but also in identifying adaptive capabilities. Tropical plant species generally do not exhibit seed dormancy period; their ripeness and maturity stages coincide. In reproductive biology, the fruiting rhythm of wild plants is rarely studied.

The quantitative and qualitative characteristics of fruits and seeds can vary significantly under the influence of meteorological, edaphic, biocenotic, and age factors, as well as depending on other individual characteristics of the plants being studied.

Observing the fruiting of main tree species in Vietnam's indigenous forests will help this issue by identifying new related species that are poorly distinguished by morphological characteristics. Short-term observations, combined with soil sampling, can help identify phenological phase shifts in the species that make up the observed community.

Seasonal observations make it possible to divide the plant community into synusia (homogeneous ecological groups belonging to the same tier and having similar development rhythms). An important and urgent task is to identify patterns and factors rhythms in plant communities depend. This can be solved by long-term observations of the complete cycles of plant growth and development, from the emergence of seedlings to an adult fruit-bearing.

The purpose of this research is to observe the development and fruiting

of main tree species in the indigenous forests of the Republic of Vietnam (Kon Chu Rang Nature Reserve), determine their morphometric parameters and processing methods, and identify hidden defects and damage for forest nursery creation.

MATERIALS AND METHODS

The field studies

The field studies were conducted in the Kon Chu Rang Nature Reserve (Gia Lai Province, Vietnam) on the Central Highlands Vietnam plateau (geographic coordinates: latitude 48.648232, longitude 44.435462). Geobotanical studies were conducted along the lines and at temporary standard sites. The main objects of observation included species (floristic) composition (Trong et al. 2020; Nagahama et al. 2023) and quantitative and qualitative relationships among plants.

Plant species names were based on literary sources (Phan et al. 2021). The names of families and genera of flowering plants were verified using taxonomy (Takhtajan 1967) and data from the International Plant Names Index (IPNI.org) database. Routes were created and navigated using waypoint map with a Garmin GPS device (GPSMAP 65). Multiband technology and advanced GNSS support (GPS, GLONASS, Galileo, QZSS, and IRNSS) provided optimal accuracy in mountainous terrain and dense forests.

The collected fruits and seeds were weighed using a laboratory scale SPS602F Ohaus (600 g x 0.01 g), and taxonomic characteristics along the survey routes were established using an Insize 1214-1004 (0-1000 mm) measuring fork and a Nikon Forestry Pro device. Soil samples were taken using a drill to a depth of 150 cm in increments of 10 cm (Phan et al. 2021); the study of soil samples was carried out in the soil analysis laboratory of the Science and Agroecology Center of the Russian Academy of Sciences.

During studies of fruiting and determination of fruit and seed maturity, the following phases were recorded: sprouts (from seeds and fruits), unripe fruits, mature fruits, fruit shedding, shell colour, and pulp.

Signs of seed heterogeneity were identified according to the criteria presented in Figure 1.

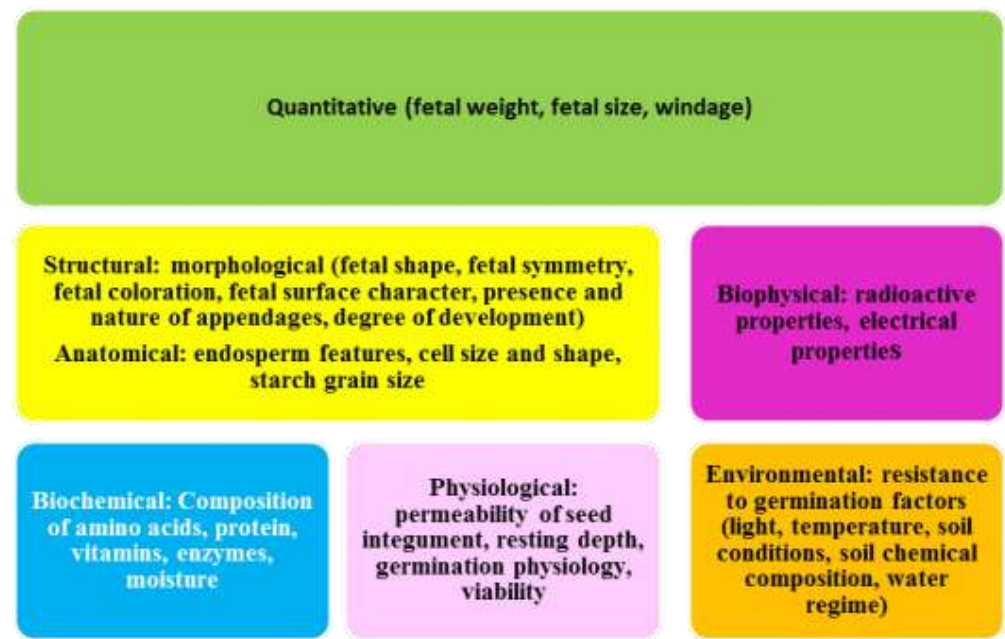


Figure 1. Signs of seed heterogeneity according to the category.

Under the conditions of the Kon Chu Rang Nature Reserve, the total area of the experimental nursery was 36 m². A pipeline was mounted at the

top of the pressure network with sprayers, enabling the production of a fog-like stream. This watering method did not displace seeds during surface sowing (Figure 2).

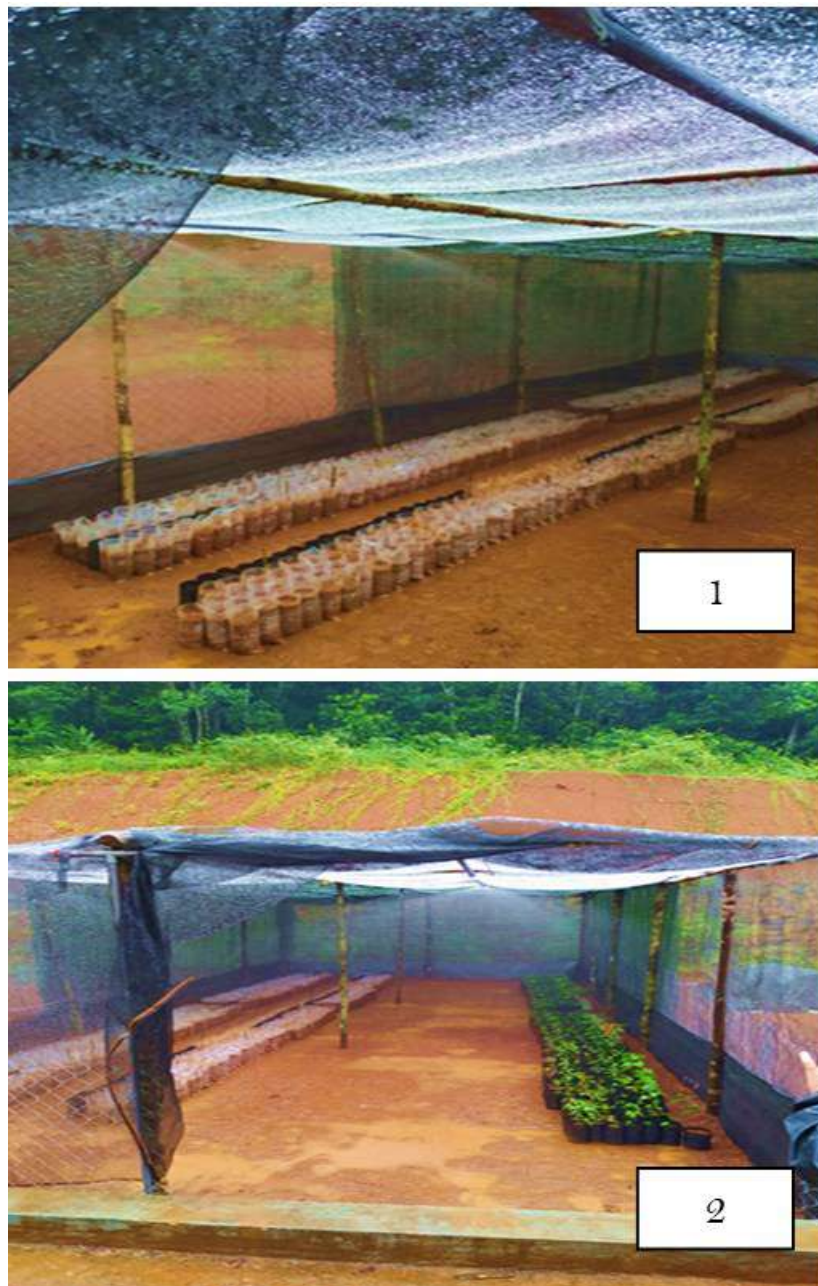


Figure 2. A pipeline with sprayers in an experimental nursery: 1 – general view of seed containers, 2 – general view of containers with seedlings.

A Saike Digital SK2009H2 digital biological trinocular microscope was used for observation. We studied weather conditions at a local weather station (Vietnam) and the Climate Monitor website (Pogodaiklimat.ru).

The study of the main bioecological characteristics of species valuable for reforestation enabled identification of the most promising species and selection of seeds for creating a collection area in the nursery. To isolate seeds from the external environment, they were bagged and pre-dried. This prevented free water from entering cells, which could cause a significant increase in respiratory rate. To avoid the effects of high temperatures, seeds were stored in a refrigerator.

Seeds sown in containers at the Kon Chu Rang Nature Reserve nursery in November 2022 were placed evenly in sunny and shaded areas. During the seed germination observations, it was established that a key classification cri-

terion for plants is their relationship to sunlight, both during the initial and subsequent growth.

The laboratory and radiographic studies

Laboratory and radiographic studies were conducted at the Laboratory Selection, Seed Production, and Nursery Production of the Science and Agroecology Center of the Russian Academy of Sciences (Volgograd, Russian Federation; geographic coordinates latitude 48.648232, longitude 44.435462) during 2022–2023.

Hidden defects in fruits and seeds were examined using a multifunctional mobile X-ray unit (PRDU) with the following technical specifications: ambient dose rate at a distance of 0.1 m from the chamber surface $1.0 \mu\text{Sv hour}^{-1}$, anode voltage of 50 KV; intermittent operating mode, X-ray exit angle of 45° , exposure time of 6 s. After treatment, seeds were placed in an IMA-1000THV climatic chamber and a KS-200 SPU-2 climatostat for further germination observation.

For chemical scarification of seeds, an experiment was carried out with sulfuric acid H_2SO_4 with a density of 1.84 % and concentration of 95 % in the following exposures: control (H_2O), option 1 - H_2SO_4 2 % (1.12–1.15 ml/100 ml of water), option 2 - H_2SO_4 3 %. (1.98–2 ml/100 ml water).

The concentration and duration of seed treatment with sulfuric acid depend on the thickness of the seed coat of the studied plant species.

Descriptive statistics included regression analysis to model relationships between multiple independent variables using Equation 1:

$$y = b_0 + b_1 x_1 + b_2 x_2 + \dots + b_n x_n \quad (1)$$

Where:

y – dependent variable,

$x_1, x_2, \dots$ – independent variable,

$b_0, b_1, b_2, \dots$ – coefficients of independent variables.

To compare the two groups, the Student's criterion was used as a variant of the analysis of variance, which is calculated using the equation 2:

$$t = \frac{XD - sD}{sD / \sqrt{n}} \quad (2)$$

Where:

n – sample size,

XD – the average value for the measurement difference,

sD and XD – standard deviation and standard error of the mean difference.

The standard error was calculated by dividing the standard deviation of the sample by the square root of the number of sample elements using the equation 4:

$$mM = s / \sqrt{n} \quad (3)$$

Where:

s – standard error,

n – the number of elements.

Processing of the experimental results was performed using the following programs: Statistica, Excel, and MathLab (Takhtajan 1967; Vu 2017; Bui et al. 2022; Zimmer et al. 2022; Nagahama et al. 2023).

RESULTS AND DISCUSSION

The Kon Chu Rang Nature Reserve occupies 15,900 hectares (159,000,000 m^2). The reserve's topography is mountainous, with elevation decreasing from north to south (<https://konchurang.vn/>). The altitude ranges from ap-

proximately 900 to 1200 m. The vegetation consists predominantly of evergreen monsoon tropical forests, occupying 98 % of the reserve area (Chong et al. 2009; Q.V. Nguyen et al. 2022a). The floristic composition includes 546 species, of which 121 are medicinal (Nagahama et al. 2023; Zimmer et al. 2022; Q.V. Nguyen et al. 2022b). The climate of the study region is tropical monsoon (Figure 3, 4, and 5) (Earthdata n.d.; D.C. Nguyen et al. 2022).

During the research period (November to March), the following climatic characteristics were noted: humidity on the plateau averaged 79 %, precipitation was 99 mm (4 % of the total annual norm). During the research period, air temperatures ranged from 23 to 27 °C during the day, and 13 to 16 °C at night, with daylight lasting 12 hours and 2 minutes. Soil sample analysis from the reserve indicated that humus content decreased with increasing depth, with the highest humus content (5.05 %) found in the 20-30 cm layer. The content of chloride and sulfate increased at depths of 110-120 cm, indicating low salinity and pollution levels. Increasing pH with soil depth indicates a shift from neutral to alkaline conditions, necessitating amendments such as gypsum, sulfur, or ferrous sulfate to reduce alkalinity (Table 1).

During a route survey of the territory of the Kon Chu Rang nature reserve, the authors identified and collected fruits and seeds of valuable tree species promising for subsequent reforestation (Table 2, Figure 6).

The status of some species requires an increase in their numbers to counter the threat of extinction (IUCN n.d.; Vu 2017).

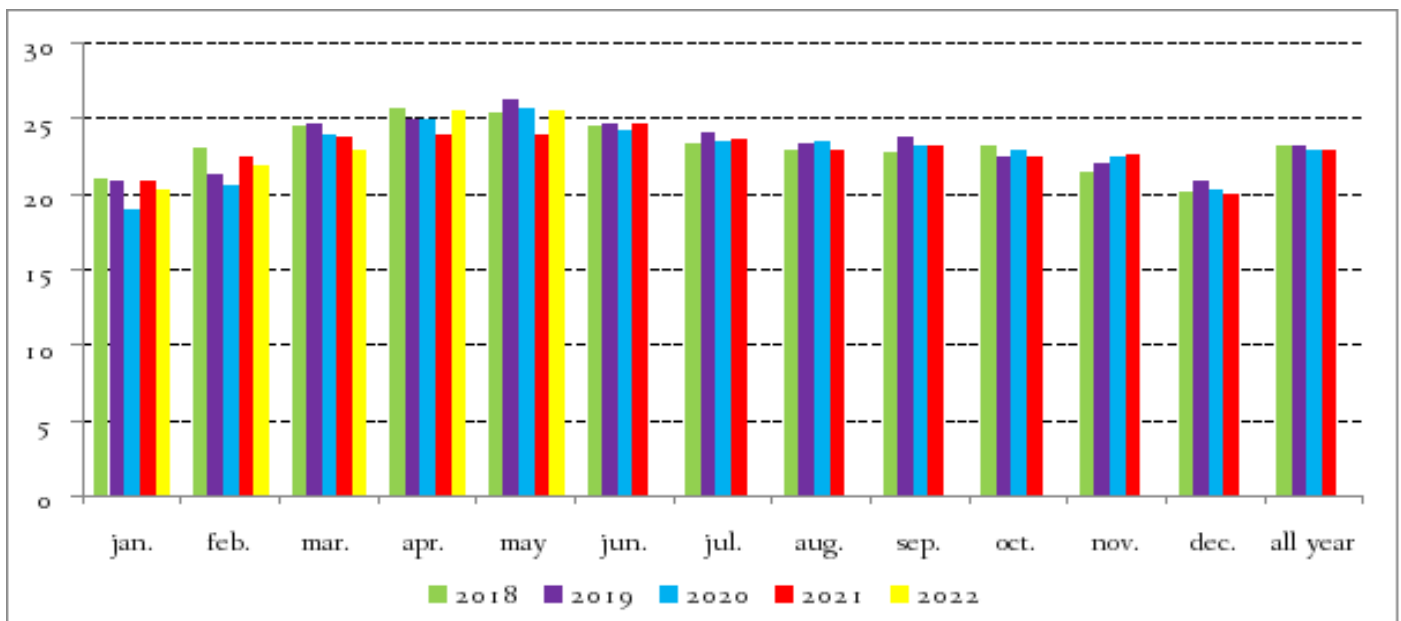


Figure 3. Average annual air temperature (°C) in Gia Lai Province, Vietnam.

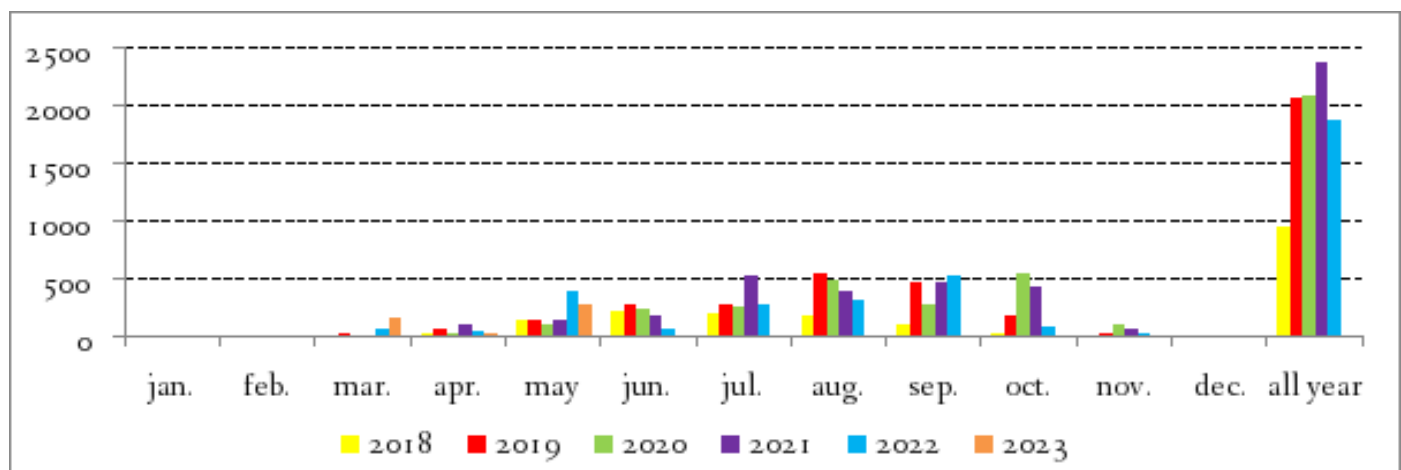


Figure 4. Monthly and annual precipitation amounts (mm) in Gia Lai province, Vietnam.

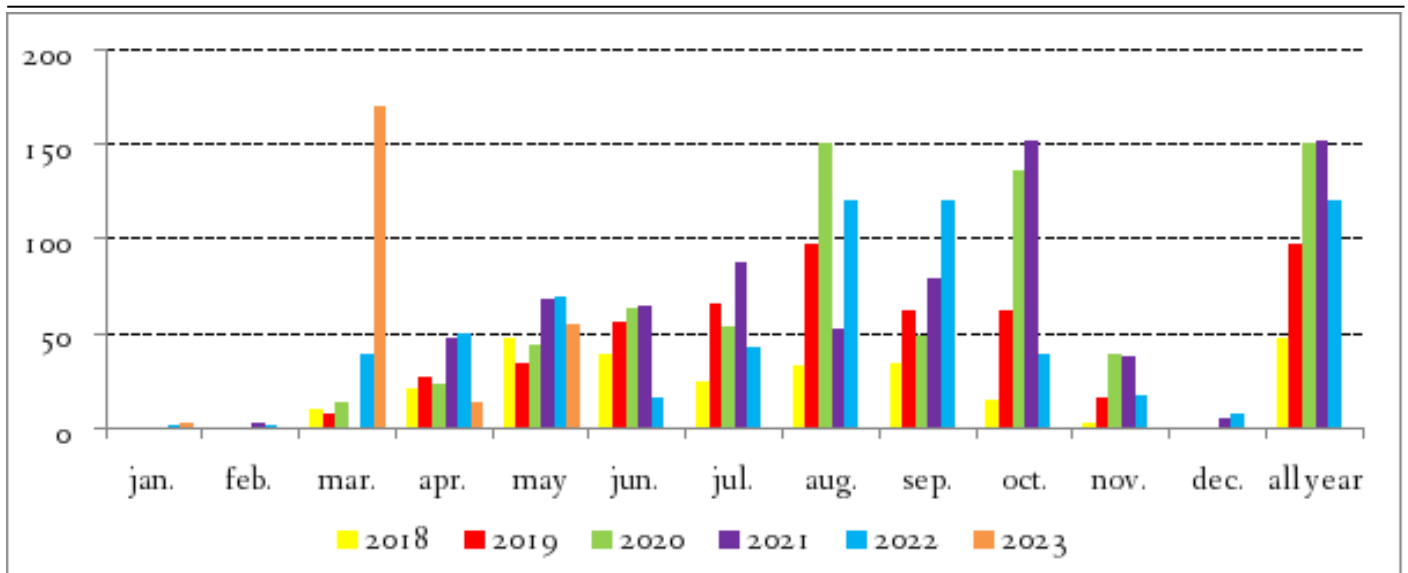


Figure 5. Maximum daily precipitation amount (mm) in Gia Lai province, Vietnam.



Figure 6. Seeds collected during species identity analyses.

Thus, *Parashorea stellata* is a vulnerable species due to habitat loss, causing range shifts. In Malaysia, approximately 805,000 mature trees within permanent protected forests. The lifespan of this species is around 100 years. Over the past 300 years, the populations have declined by 30-50 % due to habitat loss from agricultural expansion and clear-cutting. *Dipterocarpus kerrii* is also endangered., with approximately 224,000 mature trees in Malaysia's permanent conservation forests (Ashton 1982). The subpopulation is scattered, and the species' lifespan is approximately 100 years. Over the past 300 years, populations have declined by 30-50 % due to timber exploitation (Kuznetsov et al. 2003; Kuznetsov & Kuznetsova 2021; Kuznetsov et al.

Table 1. Results of soil sampling on the territory of the Kon Chu Rang nature reserve.

Indicators	Units	Analysis results											
		Ground depth, cm											
		110-120	100-110	90-100	80-90	70-80	60-70	50-60	40-50	30-40	20-30	10-20	0-10
Chloride	mg kg ⁻¹	32.0	20.6	20.5	26.1	14.5	18.8	41.7	12.9	26.0	29.0	14.7	25.1
Fluoride	mg kg ⁻¹	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Total phosphorus	%	0.116	0.149	0.158	0.208	0.196	0.208	0.152	0.090	0.076	0.089	0.124	0.121
Phosphate	mg kg ⁻¹	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3
Formate	mg kg ⁻¹	2.65	2.10	1.95	2.70	< 1	1.55	3.85	2.00	3.95	4.20	< 1	1.25
Sulfate	mg kg ⁻¹	31.3	25.2	21.5	20.5	13.8	14.4	19.2	8.8	11.4	94	4.10	7.0
Mobile phosphorus	mln ⁻¹	0	0	0	0	0	0	0	0	0	0	0	0
Mobile potassium	mln ⁻¹	98	77	63	8.1	30	26	29	32	60	76	102	111
Humus	%	0.78	0.67	1.12	0.99	1.26	1.59	1.87	2.22	2.95	5.05	4.69	5.80
Oxalate	mg kg ⁻¹	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3
Nitrate	mg kg ⁻¹	10.0	8.8	8.5	7.9	7.8	9.1	17.7	22.1	43.6	60.9	116	132
Sodium	mg kg ⁻¹	401	7.6	21.9	15.2	12.7	9.4	16.7	5.60	6.3	8.3	8.7	14.2
Magnesium	mg kg ⁻¹	13.8	1.98	3.50	2.85	2.35	1.90	3.27	1.55	2.30	3.65	5.85	7.8
Calcium	mg kg ⁻¹	32.1	7.9	20.6	11.0	9.2	7.3	12.3	6.2	12.5	11.3	15.4	22.2
Potassium	mg kg ⁻¹	14.1	3.50	12.9	7.1	6.05	4.55	6.05	5.50	9.1	13.6	26.5	30.3
pH	pH	7.3	6.1	6.2	6.1	6.3	6.2	6.1	6.2	6.1	5.9	5.8	5.7
Acetate	mg kg ⁻¹	3.65	3.10	< 3	5.5	< 3	3.05	5.95	4.05	8.1	12.3	8.2	13.4
Ammonium	mg kg ⁻¹	< 2	< 2	< 2	< 2	2.10	< 2	< 2	< 2	< 2	2.43	2.90	3.99
Nitrite nitrogen	mg kg ⁻¹	0.061	0.059	0.058	0.046	0.043	0.040	< 0.037	0.039	0.039	< 0.037	0.040	< 0.037
Nitrogen of nitrates	mln ⁻¹	7.6	5.4	3.9	4.1	4.0	4.4	4.6	5.5	12.9	16.0	> 23	> 23
Ammonium exchange	mln ⁻¹	< 5/0	8.1	< 5	< 5	< 5	< 5	< 5	< 5	< 5	0.12	0.13	> 0.3

Table 2. Information of dominant trees for collecting seeds.

TT	Species	Family	GPS		Fruit/Seed collection time
			E	N	
1.	<i>Endospermum chinense</i> Beth.	Euphorbiaceae	14° 29' 15.37"	108° 32' 28.36"	Nov.- Dec.
2.	<i>Lithocarpus pachylepsis</i> A. Camus.	Fagaceae	14° 29' 15.37"	108° 32' 28.36"	Jul.- Oct.
3.	<i>Dialium cochinchinense</i> Pierre.	Fabaceae	14° 29' 15.37"	108° 32' 28.36"	Jul. - Nov.
4.	<i>Endospermum chinense</i> Benth.	Euphorbiaceae	14° 29' 16.32"	108° 32' 23.76"	Nov.- Dec.
5.	<i>Dialium cochinchinense</i> Pierre.	Fabaceae	14° 29' 16.32"	108° 32' 23.76"	Jul.- Nov.
6.	<i>Castanopsis acussilata</i> (Blume.) A. DC.	Fagaceae	14° 29' 17.87"	108° 32' 22.5"	Jul.- Oct.
7.	<i>Bischofia javanica</i> Blume.	Euphorbiaceae	14° 29' 17.87"	108° 32' 22.5"	Jun.- Aug.
8.	<i>Reevesia pubescens</i> Mast.	Malvaceae	14° 29' 19.03"	108° 32' 21.74"	May - Jun.
9.	<i>Dialium cochinchinense</i> Pierre.	Fabaceae	14° 29' 19.48"	108° 32' 21.54"	Jul. - Nov.
10.	<i>Machilus parviflora</i> Meisn.	Lauraceae	14° 29' 20.87"	108° 32' 20.59"	Sept. - Febr.
11.	<i>Nephelium hypoleucum</i> Kurz.	Sapindaceae	14° 29' 22.82"	108° 32' 18.77"	Apr. - May
12.	<i>Dialium cochinchinense</i> Pierre.	Fabaceae	14° 29' 22.82"	108° 32' 18.77"	Jul.- Nov.
13.	<i>Ormosia balansae</i> Drake.	Fabaceae	14° 29' 35.65"	108° 32' 40.01"	Sept. - Oct.
14.	<i>Engelhardtia chrysolepis</i> Hance.	Juglandaceae	14° 29' 59"	108° 32' 44.2"	Dec. - May
15.	<i>Michelia mediocris</i> Dandy.	Magnoliaceae	14° 29' 57.85"	108° 34' 21.05"	Aug.- Oct.
16.	<i>Engelhardtia chrysolepis</i> Hance.	Juglandaceae	14° 29' 58.84"	108° 34' 17.93"	Dec. - May
17.	<i>Michelia mediocris</i> Dandy.	Magnoliaceae	14° 30' 0.13"	108° 34' 16.45"	Aug. - Oct.
18.	<i>Schima wallichii</i> (DC.) Korth.	Theaceae	14° 30' 3.46"	108° 34' 14"	Aug.- Oct.
19.	<i>Calophyllum calaba</i> L. var. bracteatum (Wight) P. Stevens	Clusiaceae	14° 30' 6.67"	108° 34' 11.81"	Apr. - May, Jul. - Sept.
20.	<i>Michelia mediocris</i> Dandy.	Magnoliaceae	14° 30' 7.65"	108° 34' 11.29"	Aug.- Oct.
21.	<i>Garuga pierrei</i> Guillaumin.	Burseraceae	14° 30' 11.15"	108° 34' 9.82"	Apr.- May., Sept. - Oct.
22.	<i>Endospermum chinense</i> Benth.	Euphorbiaceae	14° 30' 22.75"	108° 34' 11.4"	Nov.- Dec.
23.	<i>Mastixia arborea</i> (Wight.) C. B. Clarke.	Cornaceae	14° 30' 34.58"	108° 34' 16.79"	Feb.- May
24.	<i>Endospermum chinense</i> Benth.	Euphorbiaceae	14° 30' 37.03"	108° 34' 8.81"	Nov. - Dec.
25.	<i>Calophyllum calaba</i> L. var. bracteatum (Wight) P. Stevens	Clusiaceae	14° 30' 37.08"	108° 34' 18.96"	Apr., May, Jul., Sept.
26.	<i>Podocarpus neriifolius</i> D. Don.	Podocarpaceae	14° 30' 41.59"	108° 34' 21.79"	Aug.
27.	<i>Parashorea stellata</i> Kurz.	Dipterocarpaceae	14° 30' 45.9"	108° 34' 22.3"	Oct. - Dec.
28.	<i>Dipterocarpus Kerrii</i> King.	Dipterocarpaceae	14° 30' 7.45"	108° 34' 31.3"	Feb.
29.	<i>Dacrydium elatum</i> Roxb. Wall. ex Hook.	Podocarpaceae	14° 28' 16.4"	108° 32' 9.53"	Jun.
30.	<i>Dacrycarpus imbricatus</i> (Blume) de Laub.	Podocarpaceae	14° 28' 9.07"	108° 32' 36.1"	Aug. - Sep.

2025). The abundance of *Dacrycarpus imbricatus* and *Dacrydium elatum* are estimated to be declining. In Vietnam, these species are assessed as nationally vulnerable due to overexploitation for timber and habitat destruction for other purposes, such as rubber production (Thomas et al. 2007; Kim et al. 2015; Vu et al. 2021). In Malaysia and Indonesia, *D. elatum* primarily grows in peat bog drained for oil palm (*Elaeis guineensis* Jacq.) plantations, a valuable source of palm oil.

The studied tree species were categorised by height into three groups: 1 – tall (*Parashorea stellata*, *Dipterocarpus kerrii*, *Dacrydium elatum*, *Dacrycarpus imbricatus*, *Castanopsis acussilata*, *Bischofia javanica*, *Calophyllum calaba*, *Shima wallichii*), 2 – medium-sized (*Endospermum chinense*, *Dialium cochinchinense*, *Engelhardtia chrysolepis*, *Machilus parviflora*, *Nephelium hyroleucum*, *Ormosia balansae*, *Michelia mediocris*, *Mastixia arborea*, *Lithocarpus pachylepsis*), and 3 – shorter-growing (*Garuga pierrei*, *Reevesia pubescens*) Table 3.

Significant differences in germination and subsequent development were observed depending on whether the seed containers were placed on the sunny or shady side (Table 4).

Table 3. Indicators of growth and fruit size of tree species on the territory of the Kon Chu Rang nature reserve.

TT	Species	Taxacion			
		Tall, m		Fruit, mm	
		L	D	L	D
1	<i>Endospermum chinense</i> Beth.	6 ± 0.01	35 ± 0.16	8 ± 0.03	10 ± 0.01
2	<i>Lithocarpus pachylepis</i> A. Camus.	10 ± 0.10	20 ± 0.11	25 ± 0.25	65 ± 0.40
3	<i>Dialium cochinchinense</i> Pierre.	20 ± 0.18	35 ± 0.21	20 ± 0.14	15 ± 0.15
4	<i>Castanopsis acussilata</i> (Blume.) A. DC.	30 ± 0.31	40 ± 0.19	1 ± 0.01	8 ± 0.02
5	<i>Bischofia javanica</i> Blume.	15 ± 0.11	40 ± 0.10	8 ± 0.07	10 ± 0.03
6	<i>Reevesia pubescens</i> Mast.	10 ± 0.08	18 ± 0.11	35 ± 0.20	25.4 ± 0.24
7	<i>Machilus parviflora</i> Meisn.	23 ± 0.17	30 ± 0.24	15 ± 0.10	13 ± 0.07
8	<i>Nephelium hyroleucum</i> Kurz.	25 ± 0.18	30 ± 0.18	30 ± 0.18	50 ± 0.31
9	<i>Ormosia balansae</i> Drake.	22 ± 0.15	30 ± 0.19	13 ± 0.11	17 ± 0.11
10	<i>Engelhardtia chrysolepis</i> Hance.	20 ± 0.11	35 ± 0.27	48 ± 0.20	55 ± 0.22
11	<i>Michelia mediocris</i> Dandy.	18 ± 0.14	25 ± 0.19	5.8 ± 0.03	5 ± 0.02
12	<i>Schima wallichii</i> (DC.) Korth.	37 ± 0.24	47 ± 0.24	20 ± 0.16	30 ± 0.35
13	<i>Calophyllum calaba</i> L. var. <i>bracteatum</i> (Wight) P. Stevens	30 ± 0.28	40 ± 0.29	6 ± 0.04	16 ± 0.14
14	<i>Garuga pierrei</i> Guillaumin.	8 ± 0.02	15 ± 0.18	10 ± 0.08	16 ± 0.10
15	<i>Mastixia arborea</i> (Wight.) C. B. Clarke.	20 ± 0.19	25 ± 0.15	25 ± 0.17	32 ± 0.31
16	<i>Parashorea stellata</i> Kurz.	40 ± 0.24	50 ± 0.28	8 ± 0.03	10 ± 0.01
17	<i>Dipterocarpus kerrii</i> King.	45 ± 0.35	50 ± 0.21	20 ± 0.16	160 ± 0.61
18	<i>Dacrydium elatum</i> Roxb. Wall. ex Hook.	8 ± 0.08	40 ± 0.34	40 ± 0.32	30 ± 0.35
19	<i>Dacrycarpus imbricatus</i> (Blume) de Laub.	43 ± 0.29	50 ± 0.21	50 ± 0.39	60 ± 0.41

Table 4. Seed germination and seedling growth depending on placement in the nursery, March 2023, Kon Chu Rang nature reserve, Vietnam.

Family	Sunny side		Shadow side	
	Germination (%)	Growth (cm)	Germination (%)	Growth (cm)
Fagaceae	96.5 ± 4.9	28.0 ± 1.2	108.7 ± 3.5	35.0 ± 2.4
Fabaceae	42.8 ± 3.1	14.0 ± 1.8	71.4 ± 1.9	23.0 ± 2.0
Myrtaceae	87.6 ± 3.8	15.0 ± 1.1	70.0 ± 2.4	11.0 ± 0.9
Sapindaceae	92.0 ± 1.2	19.0 ± 2.1	81.2 ± 3.0	20.0 ± 1.2
Nyssaceae	0.0	0.0	0.0	0.0
Meliaceae	30.0 ± 1.9	8.0 ± 1.0	60.0 ± 1.6	10.0 ± 0.4
Dipterocarpaceae	0.0	0.0	174.0 ± 6.3	15.0 ± 0.6

The appearance of healthy shoots was observed in species belonging to the *Fagaceae*, *Myrtaceae*, and *Sapindaceae* families. These species also exhibited significant growth ranging from 7 to 20 cm. Seedlings growth up to 8 cm on the shady side of the nursery were obtained from valuable species such as *Parashorea stellata* and *Dipterocarpus Kerrii* of the *Dipterocarpaceae* family.

At the same time, seeds of some species, particularly *Mastixia arborea* and *Ormosia balansae*, failed to germinate. Inspection of containers with ungerminated seeds suggests a prolonged dormancy, indicating the need for further observation of stratification and germination timing.

Seedling growth in containers also varied depending on whether they were placed on the sunny or shady side. At the same time, the growth differences of seedlings were generally insignificant, except for *Dacridium elatum*, which died completely on the sunny side (Table 5).

Seed germination and seedlings survival are significantly influenced by sowing methods. Studies show that treatment with 2 % sulfuric acid promotes faster seedling emergence in the initial period. However, no significant differences in height were observed, possibly due to species-specific growth rhythms (Table 6).

This method of treating hard-coated seeds may accelerate germination and, combined with full-rate organo-mineral fertilisation, enables high yields

Table 5. Growth of seedlings depending on placement in the nursery, March 2023, Kon Chu Rang nature reserve, Vietnam.

Family	Seedling growth, cm	
	Sunny side	Shadow side
Fagaceae	11.0 ± 0.04	2.0 ± 0.01
Lauraceae	3.0 ± 0.03	3.0 ± 0.01
Euphorbiaceae	3.0 ± 0.01	6.0 ± 0.01
Magnoliaceae	4.0 ± 0.02	5.0 ± 0.04
Podocarpaceae	8.0 ± 0.04	12.0 ± 0.07
Myrtaceae	4.0 ± 0.01	4.0 ± 0.02

Table 6. Dynamics of growth in seedlings depending on the concentration of sulfuric acid for seed treatment.

Experi- ment	Observation dates, growth, cm								
	30.11	15.12	1.01	15.02	30.03	1.04	15.05	01.06	3.07
	2022	2022	2023	2023	2023	2023	2023	2023	2023
<i>Ormosia balansae</i>									
H ₂ SO ₄ 2 %	1.0 ± 0.01	1.5 ± 0.02	1.6 ± 0.02	1.6 ± 0.02	1.7 ± 0.03	1.7 ± 0.02	8.7 ± 0.4	9.4 ± 0.5	10.7 ± 0.5
H ₂ SO ₄ 3 %	1.3 ± 0.02	1.5 ± 0.01	1.7 ± 0.02	1.7 ± 0.03	1.8 ± 0.03	1.8 ± 0.04	9.5 ± 0.5	10.1 ± 0.6	11.0 ± 0.4
Control	1.1 ± 0.01	1.3 ± 0.01	1.3 ± 0.02	1.4 ± 0.01	1.4 ± 0.02	1.5 ± 0.02	8.1 ± 0.4	9.1 ± 0.6	9.9 ± 0.5
<i>Dialium cochinchinense</i>									
H ₂ SO ₄ 2 %	1.0 ± 0.01	1.6 ± 0.02	1.8 ± 0.02	1.8 ± 0.02	1.9 ± 0.02	1.9 ± 0.03	16.4 ± 0.7	18.9 ± 0.7	21.0 ± 0.6
H ₂ SO ₄ 3 %	1.0 ± 0.01	1.5 ± 0.02	1.7 ± 0.02	1.8 ± 0.02	1.8 ± 0.02	1.9 ± 0.03	15.0 ± 0.6	16.7 ± 0.6	18.8 ± 0.5
Control	1.1 ± 0.01	1.4 ± 0.01	1.5 ± 0.02	1.5 ± 0.02	1.6 ± 0.01	1.7 ± 0.03	13.9 ± 0.4	15.1 ± 0.5	17.6 ± 0.4
<i>Dipterocarpus kerrii</i>									
H ₂ SO ₄ 2 %	1.1 ± 0.01	1.4 ± 0.01	1.7 ± 0.02	2.1 ± 0.04	2.7 ± 0.02	4.3 ± 0.03	5.8 ± 0.2	6.9 ± 0.3	10.0 ± 0.3
H ₂ SO ₄ 3 %	1.0 ± 0.01	1.2 ± 0.01	1.4 ± 0.02	1.7 ± 0.03	2.5 ± 0.01	4.1 ± 0.02	5.1 ± 0.1	7.2 ± 0.4	8.3 ± 0.2
Control	1.1 ± 0.01	1.2 ± 0.01	1.6 ± 0.01	2.4 ± 0.02	2.6 ± 0.02	4.1 ± 0.02	5.0 ± 0.2	6.8 ± 0.4	9.1 ± 0.3

of standard seedlings at one year. The use of soil and climatic resources is determined by agricultural technology. Modern agricultural technology in forest nurseries enables regulation of water and nutrient regimes at near-optimal levels for plant growth and development. Other natural resource factors essential for plant growth (e.g., heat and light) are difficult to regulate outdoors and currently constitute limiting factors for forest nursery productivity (Figure 7).

The studies established that the biological temperature requirements of individual species are constant, independent of growing conditions and geographical location. Thus, if species require a biological temperature sum of 24–90 °C in Vietnam, then to grow them in Volgograd (Russia), where the temperature sum is below this requirement, limits the growth potential of annual seedlings. In the arid zone, low early-autumn temperatures (<10 °C artificially) end the growing season before growth potential is fully realised.

Dipterocarpus kerrii. fruits are considered ripe when they turn light brown. After falling, seeds rapidly lose viability; thus, they should not be recommended to be stored for more than 10–15 days, and they must also be kept in damp sand.

For hard-shelled seeds, a 3 % sulfuric acid solution is recommended. For softer shells, the concentration is reduced to 2 % to prevent shell rupture,

which allows acid penetration that destroys the embryo. At lower concentrations, acid acts as a growth stimulant; concentrations above 3 % are toxic (Al-Hadedy et al. 2024). To accelerate germination, the seeds can be treated with 2 % H_2SO_4 solution. Before sowing, fruits are soaked in clean water for 6 hours after lionfish have been removed. When sowing on a light substrate, plants should be watered sufficiently until seeds crack or germinate, but no longer than five days post-sowing, after which they can be transplanted into containers.

For transplanting, fruits must be placed horizontally within the container. The soil mixture for nursery sowing comprises 90 % zonal soil, 9 % manure, and 1 % superphosphate, filling up to two-thirds of the container volume. Containers should measure 9x13 cm or 10x15 cm, and have 4–6 drainage holes. Containers are arranged in rows one metre wide and as long as nursery conditions allow, with 40–50 cm row spacing. Crops require regular care: weeding the grass and loosening the soil every 10–15 days.

If planting occurs during the rainy season, soil loosening should be done 3–4 days after the rain. Shading is maintained 70–75 % during the first month, 40 % in the second, 20 % in the third, and completely removed in the fourth month. To control pests, seedlings must be sprayed with pesticides twice monthly; recommended preparations are Benlat 0.05 % and Bookdo at of 0.5–1.0 % concentration.



Figure 7. Stages of scarification and seed germination in the Science and Agroecology Center, Russian Academy of Sciences: 1, 2 – containers with seeds in sulfuric acid; 3 – seeds of *Ormosia balansae* sprouted after the experiment; 4 – containers with seeds after being placed in sulfuric acid; 5, 6, 7, 8 – successful germination of seeds of experimental plants.

Mastixia arborea: The fruits are blue-violet drupes. No germination was observed after the seed dispersal. Collected seeds can be dried and stored in a refrigerator or in a dark, cool storage room.

Seeds are collected from mature trees exhibiting good growth and development, a dense crown, and no signs of pests or disease damage. Seeds are sown in the nursery containers after one day of soaking and in the greenhouse after five days of soaking.

These seeds do not respond to sulfuric acid treatment intended to accelerate germination. Sowing substrate consists of zonal soil, and the container should be filled to 2/3 of the total volume. Containers should have dimensions of 9x13 cm or 10x15 cm, with 4–6 holes. The plants are lined up in rows 1 m wide, with a length depending on the specific conditions of the nursery. The row spacing is 40–50 cm.

Pest control was performed with Katera 50 EC at 0.75 mL L⁻¹ water, and disease control with Cymanil 720 WP at 3 g L⁻¹ water. Upon seedling emergence, liquid complex NPK fertilizers are applied. Pest and disease treatments are applied every 15–20 days; combined treatments (two chemicals in one solution) are possible. Fertilisers are applied every 20 days. Five months after sowing, treatment was carried out with the fungicide Sat Khuan (pH 6, Cu content – 15 mg L⁻¹), along with root feeding of plants with Nem at a dosage of 20 g/10 l of water. Watering with these preparations should be repeated after seven days.

Ormosia balansae: Seeds can be stored in cool, dark places. Before direct planting in the nursery (light substrate), it is recommended to pour boiling water over the seeds and sow them in containers after they swell, typically after one day.

Unswollen seeds are recommended to be re-soaked in boiling water for five days before sowing in sandy soil in a greenhouse. To accelerate germination, treat seeds with 3 % H₂SO₄. Seedlings reaching 8–10 cm should be transplanted into nursery containers. The substrate for sowing seeds and seedlings consists of zonal soil, with containers filled to 2/3 of the total volume. Fresh seed germination rate was 50 %.

Dialium cochinchinense. Seeds are collected from mature healthy trees with dense crowns and no pest or disease damage. Collected seeds may be dried and stored in a refrigerator or cool, dark storage.

Seeds are planted in containers at the nursery after a day of soaking in warm water. Sowing substrate consists of zonal soil, and the container should be filled to 2/3 of the total volume. To accelerate germination, H₂SO₄ can be treated at a concentration of 2 %.

Pest and disease treatments, as well as fertiliser applications, are consistent with those described for other species. Using a multifunctional mobile X-ray installation (PRDU) at Science and Agroecology Center of the Russian Academy of Sciences, the authors examined hidden fruit and seed defects — including microdamage by insect pests, cracks, mechanical defects, and internal germination — providing comprehensive, reliable information on internal structure and damage presence.

Thus, studies identified areas of seeds that have symptoms of physiological necrosis – for example, areas of black tissue, cotyledons with isolated areas of necrosis or damage caused by fungi, bacteria, insects, or mechanical damage, as well as cotyledons with natural pigmentation, without visible defects.

Poor fruit condition in *Lithocarpus pachylepsis* and *Mastixia arborea* has varying effects depending on damage type, including reduced seedling vigour, chlorosis, chromosomal damage, and ultimately decreased germination lead-

ing to seedling mortality.

In cases of widespread visible seed mould, germination rates should be calculated every two days. Dead and diseased seeds, which can be sources of infection for healthy seeds, should be removed and their number should be noted at each count (Figure 8 and 9).

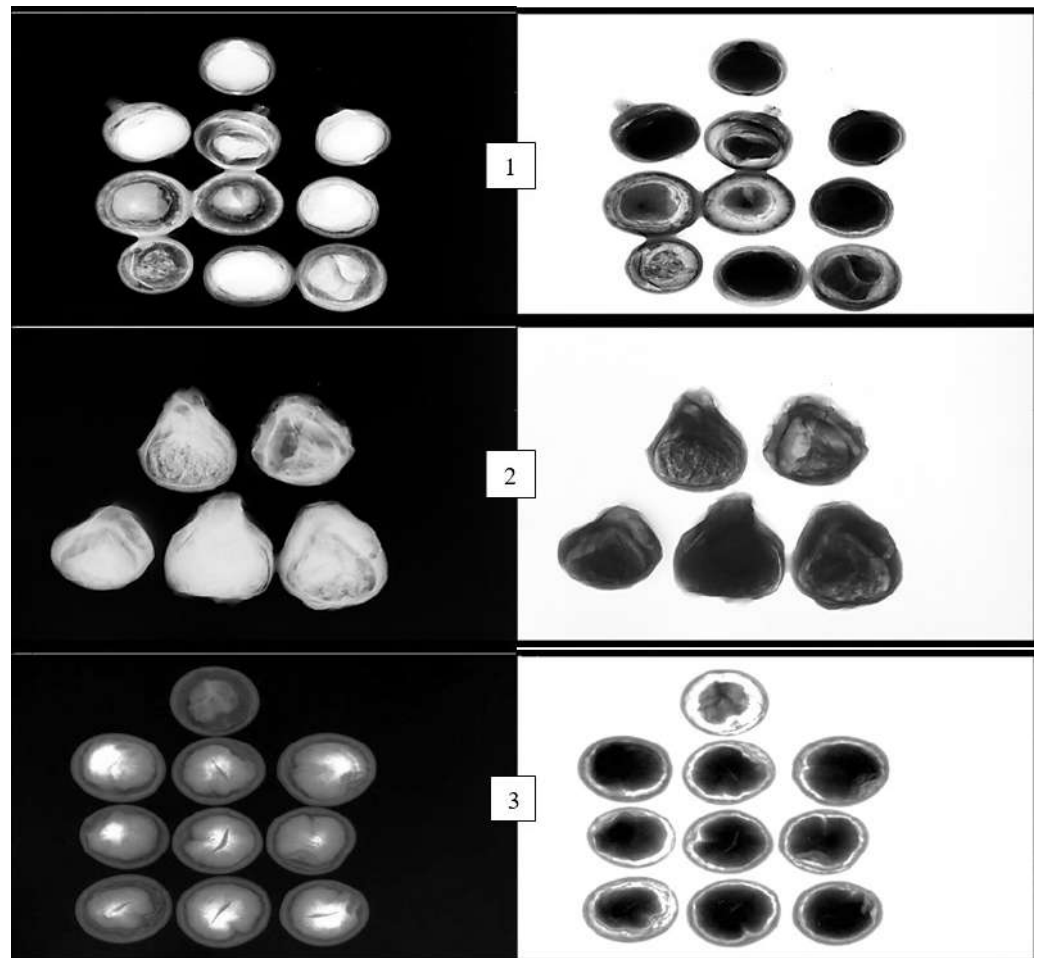


Figure 8. Seeds of some tree species under negative and positive conditions, X-ray duration 6 sec. (exposure time) 1) *Lithocarpus pachylepis*, 2) *Dipterocarpus kerrii*, 3) *Michelia mediocris*.

Research on growing seedlings of the main species of indigenous trees of Vietnam is particularly important for studying and selecting species capable of resisting the aggressive effects of tropical climate factors (Nagahama et al. 2023). Seedling cultivation, nursery management, biodiversity conservation (gene pool), study of nature and climate transformation, ecosystem restoration and management, and biosystem protection are relevant to Vietnam (Trong et al. 2020; Dang 2020).

Conservation and restoration of tropical forest ecosystems currently face numerous difficulties, as tropical forests have complex structures; most are secondary forests influenced by anthropogenic factors, making natural restoration difficult (Kuznetsov et al. 2025; Ngo et al. 2021). Considering the data obtained as a result of studying the growth, development, and reproduction methods of tree and shrub species, it is possible to develop the nursery base of the industry, the main task of which will be the cultivation of high-quality planting material with optimal conditions for its cultivation (Šálek & Výlupek 2012; Khuc et al. 2018). This will enable standardised yields of planting material per unit area, ensure high survival rates when transplanting seedlings to forest-cultural areas, promote subsequent growth, and address urgent challenges related to forest reproduction measures, species composi-

tion improvement, productivity enhancement, and the preservation of biodiversity as well as historical, cultural, and natural heritage within forest fund lands (Luong 2014).

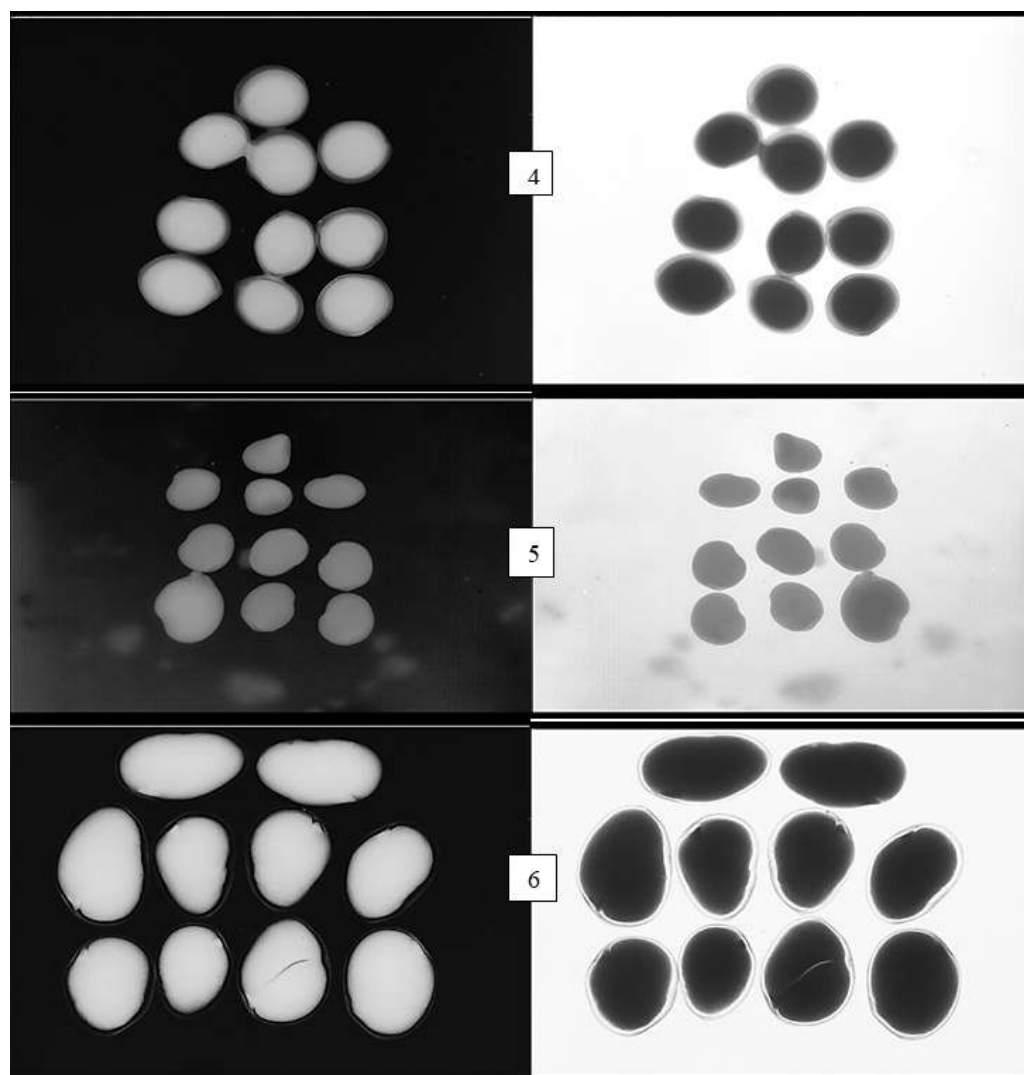


Figure 9. Seeds of some tree species under negative and positive conditions, X-ray duration 6 sec. (exposure time): 4) *Ormosia balansae*, 5) *Dialium cochinchinense*, 6) *Mastixia arborea*, negative - magnification factor 2.1 ERMF, shutter speed 50 seconds, positive - magnification factor 3.5 ERMF, shutter speed 50 seconds.

CONCLUSIONS

The soil of the Kon Chu Rang Nature Reserve contains a small amount of humus, most of which is found in the upper layers. Increased alkalinity can affect plant growth and development without the use of agrotechnical measures such as gypsum or the addition of ferrous sulfate. It has been established that some tree species (*Parashorea stellate*, *Dipterocarpus kerrii*, *Dacrycarpus imbricatus*, *Dacrydium elatum*) endemic to the reserve are endangered and require population increases through reproduction, including seed propagation.

Large, medium, and small-fruited species are distinguished by variations in their morphological characteristics, as evidenced by the high correlation coefficient. Low humidity and air temperature are necessary for seed storage. When sowing seeds, the relationship between the species and sunlight should be considered.

The fastest-growing plants were members of the *Fagaceae* family, whose seedlings had higher rates of germination, vitality, and growth compared to others. Further study of other species is required due to the large sample size. Studies on methods for treating seeds with a hard shell have shown that it is

optimal to treat seeds with 2 % sulfuric acid to accelerate germination, which has made it possible to provide recommendations for the cultivation of individual species.

AUTHOR CONTRIBUTION

A.S.S. and S.A.E. were responsible for literature searching, selecting and coding references, drafting the manuscript, synthesising content, and revising the final version. D.H.C. and T.T.T.H. conceptualised the draft, analysed reference coding, and reviewed the entire manuscript. A.V.S. prepared the work plan, defined bibliographic search parameters, conceptualised the draft, and reviewed the manuscript. A.I.B., A.N.K., and A.M.P. selected relevant references, analysed reference coding, and reviewed the manuscript analysis.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest regarding the publication of this study. Furthermore, ethical issues including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been fully observed by the authors.

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Research Article

Metagenomics Profile of Bacterial Community and Its Biocatalytic Activities of The Cultured Bacteria in Liquid of Pitcher Plant (*Nepenthes Adrianii*)

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ABSTRACT

Nepenthes, commonly known as pitcher plants, are carnivorous plants that catch insects. One species, *Nepenthes adrianii*, is endemic to Mount Slamet in Central Java. The bacterial community present in the fluid of *Nepenthes* pitchers is supposed to play a crucial role in digesting trapped insects, converting them into nutrients for the plant. This study investigated the metagenomics profile of the bacterial community found in the fluid of *N. adrianii*, collected from two different altitudes, and evaluated the biocatalytic activity of cultured bacteria. Next-generation sequencing (NGS) was performed on the V3-V4 hypervariable region of the 16S rRNA gene. Additionally, biocatalytic screening—including cellulolytic, amylolytic, proteolytic, and chitinolytic tests—was conducted by growing bacteria on basal media, each supplemented with 1 % of carboxymethyl cellulose (CMC), starch, skim milk, and colloidal chitin. The formation of a clear zone in the media indicated bacteria with biocatalytic activity, which were subsequently identified molecularly using the 16S rRNA gene. The results indicated that the four most abundant bacterial phyla were Proteobacteria, Firmicutes, Bacteroidota, and Actinobacteria, with Proteobacteria having the highest abundance in all altitudes. Bacterial diversity in the fluid of *N. adrianii* showed high with a Shannon diversity index exceeding three, along with several dominant bacterial species. Bacterial isolates confirmed to have proteolytic activity included *Leifsonia aquatica*, *Bacillus tequilensis*, and *Bosea lupini*. Cellulolytic activity was attributed to *Pseudomonas* sp. Amylolytic activity was linked to *Pseudomonas* sp. and a Bacillaceae bacterium, while *Pseudomonas aeruginosa* exhibited chitinolytic activity.

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INTRODUCTION

Nepenthes is a group of genera from the Nepenthaceae family commonly known as pitcher plants. Nepenthes are famous for their unique pitcher-shaped structures at the tip of their leaves, used to catch insects and other small animals and as a nutrient source. Nepenthes had a high species diversity; in 2021, 168 species were successfully identified. Nepenthes can be found in Indonesia, some of which are *Nepenthes adrianii*, *Nepenthes gymnamphora*, *Nepenthes mirabilis*, *Nepenthes ampullaria*, *Nepenthes raflesiana*, and *Nepenthes spathulata*. *N. adrianii* is particularly notable as it is considered rare, only growing in specific locations in the highlands, and is endemic to Mount Slamet (Batoro & Wartono 2017).

Nepenthes species can thrive as an epiphyte, which often leads to nutrient deficiencies, prompting the plant to adapt by modifying its leaves into a specialized structure known as a digestive pitcher. This pitcher, often referred to as a pitcher, not only serves as a trap but is also equipped with complex digestive fluids. The structure of the Nepenthes pitcher has five distinct leaf zones, including the base of the leaf, tendrils, digestive zone, wax zone, and leaf cap or lip. When insects or small animals enter the pitcher, they first encounter the wax zone, which has a slippery surface that makes it difficult to escape, ultimately leading them into the fluid at the bottom of the pitcher. The plant produces digestive enzymes within the digestive zone, which assist in breaking down the trapped prey (Dkhar et al. 2020). Meanwhile, other studies have also shown a symbiotic interaction between the microbial community in the digestion of trapped prey. The fluid within Nepenthes plants acts as a micro-ecosystem that hosts various bacterial and arthropod communities. Research conducted by Chan et al. (2016) on Nepenthes fluid isolated from Malaysia identified 616 species of bacteria belonging to 18 different phyla through metagenomic analysis. The dominant bacterial phyla in this sample were Proteobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia, and Planctomycetes. These five phyla are closely related to the processes involved in the decomposition of organic matter. Similarly, a study by Kanokratana et al. (2016), which examined Nepenthes fluid from Thailand, identified 20 phyla, with Bacteroidetes and Actinobacteria being the most prevalent. Additionally, Takeuchi et al. (2015) studied four Nepenthes species and found 17 phyla with 195 genera, again predominantly featuring Proteobacteria and Bacteroidetes.

Several studies indicate that Nepenthes produce antibacterial compounds, allowing only certain bacteria to grow well within their fluid. Research conducted by Chan et al. (2016) showed the presence of enzymatic activity from successfully cultured microorganisms. Enzymatic activities in bacteria isolated from Nepenthes fluid are proteolytic, amylolytic, cellulolytic, xylanolytic, and chitinolytic enzymes. In addition, some bacteria that live in Nepenthes fluid have chitinase and lipase enzyme activities, supporting the hypothesis of a symbiotic relationship between these bacteria and the carnivorous plants (Morohoshi et al. 2011).

The bacterial community and biocatalytic activity are influenced by geographic location and altitude. Variations in these factors affect climate and temperature conditions, which in turn influence the types of insects or prey captured and alter the composition of pitcher plant fluid (Gilbert et al. 2020a, 2020b). Despite their importance, the role of bacteria in the digestive processes of Nepenthes pitcher fluid has not been extensively studied. This research aims to investigate the metagenomic profile of bacteria found in the fluid of *N. adrianii* and to assess the biocatalytic activity of bacteria that can grow in culture media. The findings of this study enhanced our understanding of how these bacteria contribute to prey degradation.

MATERIALS AND METHODS

Study area and sample collection

Pitcher plant fluid samples (*N. adriani*) were collected from pitcher plants around the Mount Slamet hiking trail, Central Java, Bambang route at two different altitudes. The first altitude is around 900 meters above sea level (m asl) to post one at an altitude of 1947 m asl. While the second altitude, sampling at higher altitudes was taken from the post two hiking trail with an altitude of between 2000 and 2500 m asl. The pitcher fluid was put into a 15 mL sterile centrifuge tube. Pitcher samples were collected in triplicate at each altitude. Then the tube is wrapped to avoid contamination during the trip to the laboratory. Samples that have been collected into centrifuge tubes are stored in an ice box during the trip. In the lab, the pitcher fluid is preserved by storing at low temperatures (4 °C in refrigerator).

DNA extraction and metagenome sequencing

Total genomic DNA was extracted as much as 250 µl obtained from each *N. adriani* pitcher fluid sample that were homogenized, followed by centrifugation, and collecting the remaining microbial DNA or cell into one tube with the DNA extraction protocol using ZymoBIOMICS DNA Microprep (Zymo Research Corp). Genomic DNA samples were stored in sterile microcentrifuge tubes and sealed tightly sent to Novogene for sequencing with the Next Generation Sequencing (NGS) platform Illumina Inc. (USA). Amplification and sequencing were performed on the V3-V4 hypervariable region with specific primers (515F-806R) 515F GTGCCAGCMGCCGCGGTAA; 806R GGACTACHVGGGTWTCTAAT with 250 bp paired-end readings. The library preparation of bacterial community samples was carried out following the NEBNext Ultra™ DNA Library Prep Kit protocol for Illumina. All DNA samples were then quantitatively checked by Qubit instrument and qPCR before being loaded into the Illumina sequencer platform.

Screening for amylolytic, proteolytic, cellulolytic, chitinolytic bacteria

Screening of amylolytic, proteolytic, cellulolytic, chitinolytic activity was carried out by growing pitcher plant fluid bacteria on basal media by adding 1 % starch (Merck) for amylolytic, skim milk (nzmp) for proteolytic, carboxymethyl cellulose (CMC) (Himedia) for cellulolytic, and colloidal chitin for chitinolytic adjusted to pH 7.0 (Aryati et al. 2016). The composition of basal media is 0.2 g NaNO₃, 0.05 g MgSO₄, 0.005 g K₂HPO₄, 1 mg FeSO₄, 2 mg CaCl₂, 2 mg MnSO₄ and 2 % agar adjusted to pH 7 (Sari et al. 2016). A sample of *N. adriani* pitcher plant fluid of 0.1 mL was grown on screening media using the spread plate method. Furthermore, the culture was incubated at 28 °C for 7 days.

The formation of a clear zone in the basal media supplied with a specific substrate for bacterial growth indicates a positive result when the bacteria exhibit biocatalytic activity. The formed clear zone occurs because the bacteria growing in the media utilize the available substrate by hydrolysing the its bonds. The ability of bacteria to degrade polysaccharides with different levels of complexity was tested using starch and CMC substrates. The ability of bacteria to degrade chitin was tested using a colloidal chitin substrate, and the ability to hydrolyse peptide bonds in proteins was tested using a skim milk as a substrate.

In amylolytic screening media that has been treated with iodine, the iodine binds to starch and produces a dark blue color in the substrate area. However, if the bacteria hydrolyze the starch into glucose, the glucose will not bind to iodine, resulting in a clear area surrounding the inoculum (Gopinath et al. 2017; Tan et al. 2023; Nimatuzahroh et al. 2024). Meanwhile, cellulose degradation by cellulolytic bacteria is characterized by congo red

discoloration, which forms a light yellow zone or clear zone around the colony on cellulose agar (Chan et al. 2016; Dewiyanti et al. 2022; Ni'matuzahroh et al. 2024).

Preparation of colloidal chitin, 10 g of shrimp chitin powder (shrimp shell) is mixed with 100 mL of concentrated HCl to form a thick slurry, which is then cooled in ice water to control heat. The slurry is stirred every 5 minutes for 30 minutes, becoming less viscous over time. After hydrolysis, it is diluted with 2 L of chilled distilled water while stirring, forming a white suspension. The mixture is filtered using an LCCM-based apparatus. The CC paste trapped in the filter is washed multiple times (3–5 repetitions) with distilled water until a near-neutral pH is achieved (Koteshwara 2021).

Calculation of biocatalytic enzyme activity index using the formula:

$$\text{Enzyme activity index} = \frac{\text{clear zone diameter (mm)} - \text{colony diameter (mm)}}{\text{colony diameter (mm)}}$$

The clear zone indicates the ability of each bacterium to hydrolyze the substrate, which is measured by the enzymatic activity index. Extracellular enzyme activity demonstrates a strong response when the ratio of extracellular enzyme to colony is greater than or equal to 2. If the extracellular enzyme ratio falls between 1 and 2 (i.e., $1 < x < 2$), the activity is considered moderate. Conversely, if the extracellular enzyme ratio is less than 1 (i.e., $x < 1$), the response is regarded as weak (Choi et al. 2005).

Molecular identification of biocatalytic bacteria

Bacterial isolates were grown on Nutrient Broth (NB) media and incubated on a shaker for 24 hours. The culture was centrifuged at 12,000x g for 10 minutes to harvest the cells and the supernatant was discarded. According to the manufacturer's instructions, DNA extraction was performed using Quick-DNA™ Fungal/Bacterial Miniprep (Zymo Research Corp). After obtaining the DNA genome, PCR was performed with 50 µL of reaction volume consisting of 25 µL of master mix (GoTaq® Green master mix), 2 µL of 10 pmol forward primer, 2 µL of 10 pmol reverse primer, 2 µL of (100 ng) DNA template, and 19 µL of Nuclease-Free Water. The PCR program is based on research by Zhang et al. (2020) with slight modifications, including initial denaturation at 94 °C for 5 minutes, denaturation at 94 °C for 1 minute with 35 cycles, annealing at 57°C for 1 minute, elongation at 72 °C for 2 minutes and extension at 72 °C for 10 minutes, after which the temperature was lowered and ended at 4 °C. Universal primers 16S rRNA 27F (5' AGATTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'). The primers amplify the DNA template with an amplicon length of approximately 1460 bp. An aliquot of 3 µL of PCR product was verified by 1 % agarose gel electrophoresis (dos Santos et al. 2019).

Data analysis

For metagenomics: Adapter and PCR primer sequences from the paired-end reads were removed using Cutadapt (Martin 2011). DADA2 was used to correct sequencing error, remove low quality sequences and chimera errors (Callahan et al. 2016). The Amplicon Sequence Variants (ASVs) data was used for taxonomic classification against SILVA (silva_nr99_v138.1) database. Downstream analysis and visualisation were performed using packages in RStudio (R version 4.3.2) (<https://www.R-project.org/>). Data visualisation, including histogram charts, venn diagram, alfa diversity (Simpson diversity, Shannon diversity, and Inv-Simpson) was performed to illustrate the taxonomic composition and distribution across samples. Diversity indices, including Shannon, Simpson, and inv-Simpson, were calculated using statistical packages in R Studio (version 4.2.3) (<https://www.R-project.org/>) to assess the alpha diversity of microbial communities within the pitcher fluid samples.

Biocatalytic activity was analyzed through an experimental approach using bacterial isolates cultured from pitcher fluid samples. enzymatic activity tests were conducted qualitatively on specific media: skim milk agar for proteolytic activity, starch agar for amylolytic activity, chitin agar for chitinolytic activity, and cmc agar for cellulolytic activity. each test was performed in triplicate to ensure the validity and consistency of the results. the formation of clear zones around bacterial colonies was used as a positive indicator of enzymatic activity, and the test results were analysed descriptively. For biocatalytic bacterial identification: Forward and reverse sequences from Sanger sequencing were assembled using BioEdit. Subsequently, the 16S rRNA gene sequences of bacterial isolates were analyzed with the BLAST program, which searched for similarities in the GenBank database on the NCBI website (<https://blast.ncbi.nlm.nih.gov/>).

RESULTS AND DISCUSSION

Metagenomics profiles of bacterial communities

The *N. adrianae* pitcher fluid represents an extreme microhabitat that offers a unique environment for specific microbial communities, including bacteria. Altitude is a key environmental factor that can influence the distribution and characteristics of microorganisms due to variations in temperature, air pressure, humidity, and resource availability. Therefore, comparing the bacterial communities at low and high altitudes provides valuable insights into how environmental conditions affect the structure of these microbial communities.

Next-generation sequencing (NGS) analysis of *N. adrianae* pitcher fluid at two different altitudes showed variations in the relative abundance of the bacterial community. The relative abundance of the bacterial community of the *N. adrianae* pitcher fluid is displayed through a histogram (Figure 1). In both datasets, bacteria from the Proteobacteria phylum dominate with the most significant proportion (more than 50 %), followed by phyla such as Firmicutes, Bacteroidota, Actinobacteriota, and Verrucomicrobiota in smaller amounts. The dominant Proteobacteria phylum in both locations reflects its role as the main bacterial phylum in the pitcher fluid ecosystem, which decomposes organic compounds. These results are consistent with previous studies conducted by Chan et al. (2016), Kanokratana et al. (2016), Sickel et al. (2016), and Gilbert et al. (2020b), which showed that the phyla Proteobacteria, Firmicutes, Bacteroidota, and Actinobacteriota were the dominant phyla found in pitcher plant fluid. At the genus level, bacteria from the genus *Pseudomonas* were the most dominant at lower altitudes, with a proportion of about 33 %, followed by genera such as *Rhodopsedomonas* and *Acidosoma* and other genera in lower numbers. At higher altitudes, bacteria from the genus *Rhodospirillum* were the most dominant, with a proportion of about 11 %, followed by genera such as *Clostridium sensu stricto* 3 with a proportion of about 8 %.

The dominance of Proteobacteria can be explained by the ability of members of this phylum to adapt to environments with varying nutrient availability (Zhou et al. 2020). Proteobacteria, especially *Pseudomonas*, which dominated at lower altitudes, are known as highly metabolically flexible bacteria, both as decomposers of organic matter and producers of secondary metabolites, also able to survive in extreme environmental conditions such as high organic compound content or low pH (Chauhan et al. 2023). Meanwhile, the dominant genus *Rhodospirillum* at high altitudes reflects its ability to adapt to environmental stress conditions such as low temperatures and limited light. The presence of the genus *Clostridium sensu stricto* from the phylum Firmicutes at both altitudes indicates its essential role in the anaerobic degradation process, which is relevant for the function of the *Nepenthes* pitcher in digesting prey.

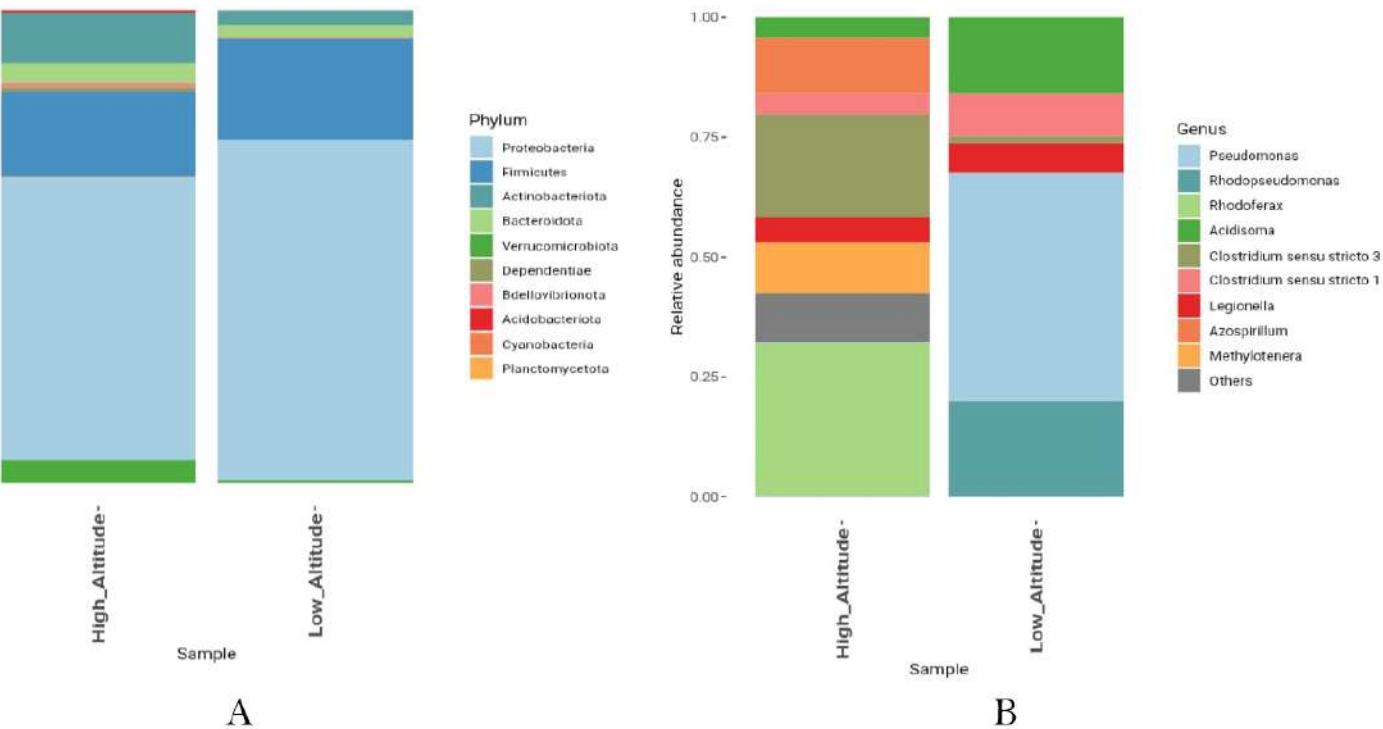


Figure 1. Visual histogram percentage relative abundance bacteria in the pitcher plant fluid of *N. adrianii* (A) phylum taxa, (B) genus taxa.

Differences in community composition at two altitudes indicated the influence of environmental factors on the structure of microbial communities (Zakavi et al. 2022). Higher altitudes have lower temperatures, lower oxygen pressure, and limited carbon sources. The results of this study revealed that *Nepenthes* pitcher fluid microbes play an essential role in supporting plant ecological functions, especially in organic degradation and nitrogen provision. These differences were shown at each altitude, reflecting the adaptation of microbes to specific environmental conditions.

The number of bacterial species observed in the pitcher fluid at both altitudes which is stated as had a number of unique Amplicon Sequence Variants (ASVs) and shared ASVs detected shown with a Venn diagram (Figure 2).

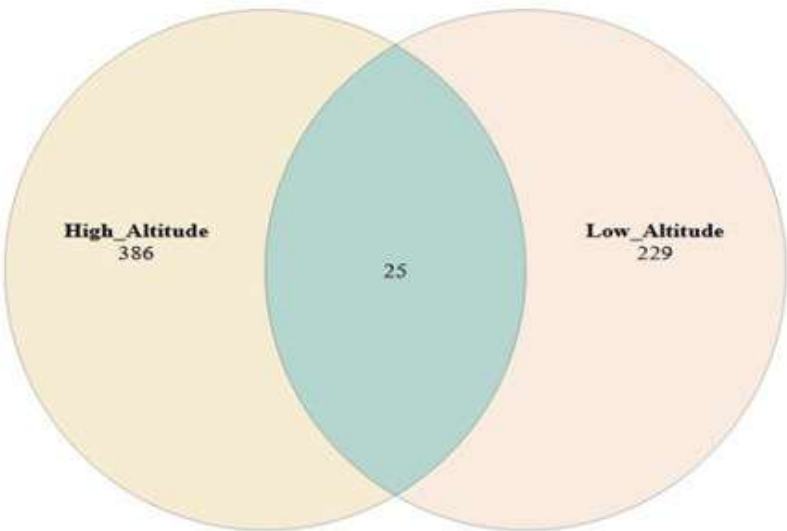


Figure 2. Venn diagram illustrating the unique number of bacterial taxa from each altitude and both in the pitcher fluid.

NGS 16S rRNA amplicon metagenomic sequencing analysis revealed the presence of 25 shared (ASVs) in both pitcher fluid samples, indicating a core microbial community that is adaptable to varying altitude conditions. However, each elevation exhibited unique microbial diversity, with samples from higher elevations showing 386 unique species, while samples from lower elevations had 229 unique species. This variation in diversity is likely influenced by the differing environmental conditions at each altitude. These findings aligned with the research conducted by Zakavi et al. (2022), which examined bacterial communities across different altitudes. Their study suggested that altitude is a significant factor influencing microbial community composition. Although microbiome diversity tends to increase with altitude, highland bacteria are also more resistant to heat and changes in pH. The presence of unique ASVs in each sample indicate microbial niche specialization, where each species has specific adaptations to capitalise on the resources available in its environment.

Bacterial diversity in the liquid of pitcher plant *N. adrianii*

The liquid of the pitcher plant is known to have a diversity of bacteria that have their roles in the unique habitat. Species diversity in the bacterial community of pitcher plant fluid is expressed as alpha diversity. Alpha diversity was analysed based on the Shannon and Simpson indices. The Shannon index measures species diversity, with higher values indicating greater diversity. In contrast, the Simpson index assesses species evenness and indicated the dominance of certain species within a community (Table 1). The diversity of bacteria in the *N. adrianii* liquid observed at two different altitudes is classified as high, as indicated by the Shannon index value exceeding 3 (Waskita et al. 2024).

Based on the alpha diversity analysis of the data, several metrics are used to measure the diversity of the microbial community, which is stated as the number of observed ASVs, including the Shannon index, the Simpson index, and the inverse Simpson. Each metric provides a different perspective on the structure and complexity of the microbial community. The highland samples had a total of 411 ASVs, while the lowland samples had a total of 254 ASVs. This higher species richness may reflect more heterogeneous environmental conditions, such as microhabitat variations, lower temperatures, or nutrient availability, allowing more species to flourish. Conversely, fewer ASVs observed in the lower plains may be due to environmental homogeneity, such as more stable temperatures or high humidity, which may favor the dominance of certain species.

The Shannon index value is 4.33 at higher altitudes and 3.08 at lower altitudes. The Shannon index measures biodiversity by assessing the number of species and their relative distribution. The higher value at elevated altitudes suggested that the microbial community is more evenly distributed, indicating more balanced representation of species than at low altitudes.

Simpson index value is 0.9705, which is close to 1. This means that even though the community at a high altitude is rich in species and has a considerable Shannon value, showing even more diversity, the Simpson index indicated the presence of fairly dominant species. So, even though this community is rich, the dominance of certain species still occurs. Simpson value at low altitude is 0.8606, lower than the high altitude. This value indicates that species dominance also existed, but the level of dominance was relatively lower compared to the high altitude. The dominance of certain species can occur since of more significant environmental pressures, e.g., lower temperatures or more limited nutrient levels, so only highly adaptive species can become dominant. Lower dominance at lower altitudes may be due to more stable or uniform environmental conditions, allowing more species to share resources relatively more evenly.

Table 1. Microbiome alpha diversity shows the diversity of a single microbial community sample, taking into account the number and abundance of different species.

Liquid of pitcher plant	Number of Species	Shannon Index	Simpson Index	Indesks Inv. Simpson
High Altitude (2.000-2.500 m asl)	411	4.33	0.97	33.95
Low Altitude (900 -1.947 m asl)	254	3.08	0.86	7.18

This metric indicated the number of species that dominate the community. Higher values at high altitudes indicated a community with more diverse structure. High values indicated that the microbial community has many species with equal distribution. This reflects an environment that supports complex ecological interactions. Low inverse Simpson values suggested that a few species dominated the community; one or a few species have a much more extensive coverage than the other species. During the sampling exploration, we noted variations in environmental conditions (Table 2).

There were different temperature and humidity conditions, whereas, at higher altitudes, the temperature and humidity were lower compared to lower altitudes. Meanwhile, the light intensity at lower altitudes had lower intensity. Differences in environmental condition, such as altitude, air temperature, air humidity, and light intensity, can significantly influenced the diversity of bacteria in pitcher plant fluid (Chou et al. 2014; Kanokratana et al. 2016; Gilbert et al. 2020a, 2020b).

Biocatalytic activity of bacteria cultured from pitcher plant fluid

Nepenthes plants can produce their digestive enzymes, as found in the study of An et al. (2002); the study reported that high expression of hydrolytic enzymes was found in the digestive zone of the pitcher. Meanwhile, other studies also showed a symbiotic interaction between the microbial community in the pitcher fluid and plants in the digestion of trapped prey (Bittleston 2018). Biocatalytic test showed the presence of clear zones on the screening media, indicating that the isolated bacteria from the pitcher plant fluid produced enzymes capable of breaking down specific substrates. This proves the presence of biocatalytic activity in the cultured bacteria (Figure 3).

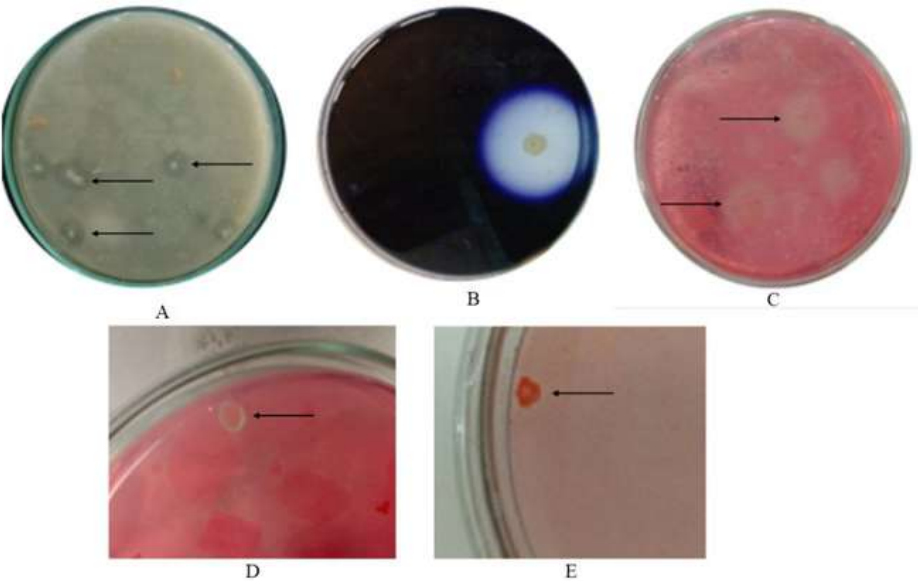


Figure 3. Extracellular enzyme activities showing clear zones shown in the arrows area. (A) proteolytic, (B) amylytic, (C) and (D) cellulolytic, (E) chitinolytic.

The formation of a clear zone on the screening media consisting of amylytic, cellulolytic, proteolytic, and chitinolytic activities indicated that the

Table 2. Environmental condition.

Altitude	Air temperature (°C)	Relative air humidity (%)	Light intensity (lux)
High Altitude (2.000-2.500 m asl)	20.15 ± 0.06	89.75 ± 0.52	2088 ± 218.2
Low Altitude (900 -1.947 m asl)	25.25 ± 1.4	97.25 ± 1.1	1251 ± 34.5

bacteria in the pitcher plant have all four types of biocatalytic activities. Bacteria exhibiting biocatalytic activity found in the pitcher fluid of *Nepenthes* are believed to be closely linked to the ecological function of the pitcher as a trapping and digestive system for prey. The pitcher fluid naturally contains various digestive enzymes (An et al. 2002), such as proteases, lipases, and chitinases, which aid in the breakdown of trapped insect body components. Metagenomic analysis revealed a significant diversity of bacterial communities, including genera known to produce hydrolytic enzymes. The presence of these bacteria supports the hypothesis that they function as symbionts contributing to prey decomposition and nutrient provisioning for the plant. Detected biocatalytic activities include proteolytic activity, which is essential for breaking down protein-rich insect prey. These bacteria are likely involved in the hydrolysis of insect proteins, facilitating nitrogen release for plant uptake. Amylolytic activity, which assists in the degradation of starch and other polysaccharides, along with chitinolytic activity, which is responsible for breaking down the chitinous exoskeletons of insects, further highlights the role of bacteria in degrading structural components of the prey. Additionally, cellulolytic activity suggested a broader enzymatic profile, enabling the breakdown of cellulose originating from plants or insect sources. The presence of these enzyme-producing bacteria indicated a synergistic relationship between *Nepenthes* and its microbiota, where microbial metabolism complements the plant's endogenous enzymes, enhancing the breakdown of complex organic matter and optimizing nutrient acquisition in nutrient-poor environments.

Bacterial Isolates of *N. adrianii* fluid with biocatalytic activity have different abilities in hydrolyzing substrates, as indicated by differences in the biocatalytic index of each isolate (Table 3). Analysis of the biocatalytic index of bacterial isolates from *N. adrianii* sac fluid showed variations in enzymatic activity in the isolates.

Variations in biocatalytic indices indicated different enzymatic abilities of bacteria in the *Nepenthes* pitcher fluid. Proteolytic isolates (P1-P5) tend to have a high biocatalytic index, reflecting the critical role of these bacteria in digesting proteins from insects entering the pitcher. The highest index in isolate P3 (9 ± 0.82) indicated a dominant proteolytic activity. Isolates with cellulolytic activity (C1 and C2) have significant differences in indices, with C2 (3.9 ± 0.62) showing a better ability to degrade cellulose than C1 (0.3 ± 0.03). This suggests that cellulose in the pitcher plant environment can affect the colonization of certain bacteria. Low chitinolytic activity (K1 and K2) may indicate a supporting role in the decomposition of insect chitin, with indices still below 1. Differences in biocatalytic index values, which indicate the ability of bacteria to degrade substrates, can arise from various factors. These factors included pH levels, incubation temperature, the quantity or quality of organic material or substrates (Rutu et al. 2017; Piotrowska-Długosz et al. 2022), the duration of incubation time, and the presence or absence of enzyme cofactors such as Mn^{2+} in the substrate (Rutu et al. 2017). In contrast, amylolytic activity in isolates A2 and A3, although not reaching proteolytic, indicated that these bacteria are also involved in the degradation of complex carbohydrates such as starch. These biocatalytic indices suggested that bacteria in the *Nepenthes* pitcher fluid have enzymatic characteristics that support the function of the pitcher as an insect trap. The dominant proteolytic activity corresponds to the central role of the pitcher in digesting proteins from the

Table 3. Biocatalytic index of cultivable bacterial isolates from the Pitcher fluid of *N. adriani*.

Isolate code	Activity	Biocatalytic index (mean $\pm$ SD)
P1	Proteolytic	3.25 $\pm$ 0.5
P2	Proteolytic	3 $\pm$ 0.0
P3	Proteolytic	9 $\pm$ 0.82
P4	Proteolytic	5.88 $\pm$ 0.63
P5	Proteolytic	1.84 $\pm$ 0.19
C1	Cellulolytic	0.3 $\pm$ 0.03
C2	Cellulolytic	3.9 $\pm$ 0.62
K1	Chitinolytic	0.3 $\pm$ 0.14
K2	Chitinolytic	0.63 $\pm$ 0.25
A1	Amylolytic	0.7 $\pm$ 0.18
A2	Amylolytic	1.83 $\pm$ 0.35
A3	Amylolytic	3.95 $\pm$ 0.33

insect body, while cellulolytic, chitinolytic, and amylolytic activities reflect additional abilities to digest other components such as insect exoskeleton (chitin) and plant compounds that may enter the pitcher fluid. Differences in indices between isolates may reflect microbial adaptation to specific environmental conditions in the pitcher fluid.

Molecular Identification of bacteria with biocatalytic activity

The electrophoregram indicates that the PCR technique using the primers 27F and 1492R successfully amplified the 16S rRNA gene from bacterial isolates found in pitcher plant fluid. A distinct DNA band observed in the range of 1500 bp confirms that the bacterial genomic DNA was effectively extracted and purified for use as a template. The successful amplification of the 16S rRNA gene demonstrates that the primers used are specific to the target gene and that the PCR conditions were appropriately optimized.

BLAST analysis of bacterial isolates from pitcher plant fluid revealed a high level of similarity, with an identity percentage of 99 % to 100 % and a query cover ranging from 99 % to 100 %. The bacterial isolates identified through this analysis were *Leifsonia aquatica*, *Bacillus tequilensis*, and *Bosea lupini*. Additionally, *Pseudomonas* sp. demonstrated proteolytic activity, while *Pseudomonas aeruginosa* exhibited chitinolytic activity. Cellulolytic activity was confirmed in *Pseudomonas* species, specifically *Pseudomonas* sp. strain GL24, while a *Bacillaceae* bacterium was identified as having amylolytic activity (Table 4).

Leifsonia aquatica, *Bosea lupini*, and *Bacillus tequilensis* were bacteria with proteolytic activity found in *N. adriani* pitcher fluid. These proteolytic bacteria aid in the digestion of proteins from captured insects, breaking them down into simpler elements that the plant can absorb. Additionally, protease play a crucial role in the ecological balance, particularly in maintaining the nitrogen cycle (Razzaq et al. 2019). Isolate P1, in addition to having similarities with *L. aquatica*, also has similarities with *Leifsonia* sp. NaF-BtI-2 found in the human oral cavity and *L. xyli* and *Lysinimonas* sp. LM-2018 found in soil and plants.

The BLAST search results on NCBI isolate P3 showed that the 16S rRNA sequence analyzed had high similarity to several bacterial species from the genus *Bosea*. The highest identity was found in *Bosea lupini*, with an identity percentage of 99.71 % and a maximum score of 2512. In addition, other species, such as *Bosea vestrisii* and *Bosea* sp. Tri-39, both of which were also found in root nodules, also showed a high level of similarity with identity percentages between 99.71-99.78 %. These results indicate that the isolate analysed is most likely a member of the genus *Bosea*, which belong to the α -proteobacteria group. The genus *Bosea* is known to have diverse habitats and is often found in soil and air and as part of plant microbiota. A high identity

Table 4. Identifies *N. adrianii* fluid bacteria isolates based on the 16S gene sequence—the identification based on BLAST similarity searching with the Genbank database.

Isolate Code	Biocatalytic Activity	Description	Max Score	Total Score	Query Cover	E Value	Identity Percent-age	Acc Len	Accession
P1	Proteolytic	<i>Leifsonia aquatica</i> strain A1 16S ribosomal RNA gene, partial sequence	2560	2560	99 %	0.0	99.71 %	1493	JX010948.1
P2		<i>Bacillus tequilensis</i> strain A80 16S ribosomal RNA gene, partial sequence	2636	2636	99 %	0.0	99.93 %	1455	OP435775.1
P3		<i>Bosea lupini</i> strain R-45681 16S ribosomal RNA, partial sequence	2512	2512	99 %	0.0	99.71 %	1444	NR_108514.1
K1	Chitinolytic	<i>Pseudomonas aeruginosa</i> strain QK-4 16S ribosomal RNA gene, partial sequence	2615	2615	100 %	0.0	99.93 %	1432	MH746107.1
C1	Cellulolytic	<i>Pseudomonas</i> sp. GC04 16S ribosomal RNA gene, partial sequence	2614	2614	100 %	0.0	99.93 %	1426	KP671493.1
C2		<i>Pseudomonas</i> sp. strain GL24 16S ribosomal RNA gene, partial sequence	2601	2601	99 %	0.0	99.79 %	1432	OR647672.1
A1	Amylolytic	<i>Bacillaceae bacterium</i> strain AFS099849 16S ribosomal RNA gene, partial sequence	2627	2627	99 %	0.0	99.79 %	1508	OP986711.1
A2		<i>Pseudomonas</i> sp. strain GL24 16S ribosomal RNA gene, partial sequence	2615	2615	100 %	0.0	100.00 %	1432	OR647672.1

percentage (>99 %) indicates a close phylogenetic relationship with the species found in the database (Syah et al. 2024). This finding suggested that the microorganisms in the sample may play a role in specific environmental processes, such as the nitrogen cycle or interactions with plants. Isolates C1 and C2 were identified in the genus *Pseudomonas*, a group of gram-negative bacteria with high metabolic diversity. *Pseudomonas* is often found in the environment, including soil, air, and plants. Some BLAST results, such as *Pseudomonas koreensis*, also show closeness, indicating a more specific species. This species is known as an environmental bacteria with the ability to degrade certain organic compounds. *Pseudomonas* sp. have cellulolytic activity. Both belong to the proteobacteria phylum widely found in animal digestion system, where they help degrade organic matter into easily absorbable nutrients. *Pseudomonas* sp. was also confirmed to have cellulolytic activity in *N. mirabilis* (Chan et al. 2016).

Chitinolytic activity in identified bacteria has low enzymatic activity. Chitinolytic bacteria in *N. adrianii* fluid identified in this study were *Pseudomonas aeruginosa*. *P. aeruginosa* can be found in soil where it plays a role in biodegradation. Research conducted by Folders et al. (2001) showed that *P. aeruginosa* has chitinase activity with its ability to inhibit the growth of *Fusarium oxysporum* and *Rizoctonia solani* fungi. Chitinolytic bacteria in *N. adrianii* plants degrade chitin in prey, such as insects, converting it into simpler molecules to be utilized for their nutrition by *N. adrianii* plants. The high abundance of bacteria and biocatalytic activity indicate that bacteria living in *Nepenthes* fluid have a high possibility of degrading prey into simpler molecules that plants can utilize to meet their nutritional needs.

CONCLUSION

This study found that the habitat of *Nepenthes adrianii* at different altitudes affected the diversity of bacteria in its pitcher fluid, with higher altitudes showing greater bacterial diversity. Metagenomic analysis revealed that the fluid contains a rich community dominated by Proteobacteria, Firmicutes, Bacteroidota, and Actinobacteriota. Several cultured bacterial isolates demonstrated enzymatic activities including proteolytic, cellulolytic, chitinolytic, and amylolytic as evidenced by clear zones on selective media. Notably, *Leifsonia aquatica*, *Bacillus tequilensis*, and *Bosea lupini* showed strong proteolytic activity, while *Pseudomonas* sp. exhibited both cellulolytic and amylolytic activity, and *Pseudomonas aeruginosa* showed chitinolytic activity. These findings highlighted the metabolic potential of pitcher-associated bacteria and enhanced our understanding of how these microbes contributed to prey degradation and nutrient acquisition in *N. adrianii*.

AUTHOR CONTRIBUTION

A.A.N., A.S. and S. designed the research. A.A.N. and A.S. carried out the laboratory work. A.A.N., N.E. and A.S. analysed the data. A.A.N., A.S., and A.P. wrote the manuscript.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest during the research

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Research Article

Type and Morphological Character of Local Cloves (*Syzygium aromaticum* (L.) Merr. & L. M. Perry) from North Maluku Province, Indonesia

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ABSTRACT

The North Maluku Province is renowned for being the original region where clove distribution started. This region comprises several island groups with high clove diversity. The research aimed to investigate and to recognise the morphological diversity of local cloves in North Maluku Province. The study employed a survey method, which included exploring six islands, namely Ternate, Tidore, Halmahera, Moti, Bacan, and Kasiruta, and led to the discovery of 13 local clove accessions. These accessions are Afo, Zanzibar Merah, Sikotok, Siputih, Rica, Maluku, "Gagang Panjang" clove, Sibela I, Sibela II, Forest Clove, White Zanzibar, Bogor clove, and "Biasa" clove. Based on Hierarchical Cluster Analysis (HCA), all clove accessions were morphologically characterised and classified into two groups with a dissimilarity coefficient of 49 %. The first group is the cultivated type of cloves that consists of local Afo cloves, Sibela I, Sibela II, Siputih, Sikotok, Rica, Red Zanzibar, "Gagang Panjang" clove, White Zanzibar, Bogor cloves, "Biasa" cloves, and Maluku cloves. On the other hand, the second group is the wild-type clove group, namely Forest Cloves. The characteristics of local clove accessions based on Principle Component Analysis (PCA) showed a total diversity of 52.5 %.

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INTRODUCTION

The Maluku Islands, consisting of Maluku Province and North Maluku Province in Indonesia, are considered the centre of origin of cloves in the world (Sundari et al. 2019; Tamnge & Yusnaini 2021; Mahulette et al. 2022; Naziella et al. 2022). The Maluku Archipelago region is believed to contain significant clove diversity due to its origin as a clove distribution centre (Alfian et al. 2019; Hariyadi et al. 2020; Kembauw et al. 2021a, 2021b; Wattimena et al. 2023). Previous explorations into clove diversity have been conducted in Maluku Province, leading to the identification of several local clove varieties, including Tuni, Boiselang, Red Zanzibar, White Zanzibar, Bogor, Jinten, Tae, Damar, Forest Clove, and Raja (Mahulette et al. 2022). This information supports the claim that North Maluku Province possesses a high clove diversity, as it was also part of the original clove distribution centre (Sundari et al. 2019; Tamnge & Yusnaini 2021). The existence of Afo cloves on Ternate Island in North Maluku Province, which are over 350 years old, serves as historical evidence that this area is indeed part of the centre of origin for global clove distribution (Sundari et al. 2019; Tamnge & Yusnaini 2021; Aboe 2022).

North Maluku Province is among the top 10 provinces in Indonesia in terms of clove production (Ditjenbun 2020). The province produced an average of 4,260 tons of clove, contributing to about 3.21 % of the national production. The clove area in North Maluku Province is expected to reach 26,502 hectares (BPS Maluku Utara 2022). Although the clove commodity area in this province is less vast than that in other central provinces of Indonesia, the region has high clove productivity due to many superior local clove varieties and agro-climatic suitability. To discover more about the diversity of local clove varieties in North Maluku Province, it is essential to carry out exploration activities on each island group to identify the existence of local clove accessions. This exploration should be followed by identifying and characterising local clove accessions based on their morphological features.

According to Anisa et al. (2022) and Hartati et al. (2022), carrying out morphological characterisation in plant breeding programs is crucial as it is the initial stage in identifying high-yielding genotypes. This type of characterisation helps gather information about identity, diversity, and relationships between plant species (Hernosa et al. 2022; Ustari et al. 2023). The diversity obtained at the morphological level is significant in selecting plant genotypes with high yield potential and creating a database of plant species diversity to develop varieties (Diaguna et al. 2022; Dini et al. 2023; Wattimena et al. 2023).

The North Maluku Province comprises multiple clusters of islands with distinct agro-climatic features. These islands are believed to hold a rich diversity of cloves as they were the first areas of clove distribution. However, more information is needed on the diversity of clove in each island group within the province. It is crucial to conduct an exploration and morphological characterisation of local cloves on each island group in North Maluku to gather comprehensive information about the diversity of clove in the entire Maluku Islands region.

MATERIALS AND METHODS

In this study, 13 different clove accession groups clove types were analysed for their characteristics. These accessions were collected from 6 islands in North Maluku Province, Indonesia, where clove plants are commonly found. The islands include Ternate, Tidore, Halmahera, Moti, Bacan, and Kasiruta. The plants were sampled and measured between July and September 2023, and only those that were over 15 years old, had a uniform crown shape, and were free from pests and diseases were selected for characterisation. From the 13 types of cloves characterised five sample plants were selected from each type of clove, so 65 plants were measured. Each plant sample was then

taken, with 10 samples of leaves, flowers, fruits, and seeds for measurement. The measured morphological characteristics included crown morphology, branching, leaves, and flower buds. To measure these characteristics, equipment such as a Haga meter for tree height, measuring tape for crown width and stem circumference, ruler for leaf length and width and flower length, ImageJ software for leaf area, digital caliper for flower diameter, RHS 2015 colour chart for color scale of stems, leaves, and flowers, and GPS for plant sample coordinates were used.

A survey method was utilised in this study, with purposive sampling for measurement. A total of 54 morphological characters were measured based on the Tropical Fruit Descriptors (IPGRI) (1980) and modified by Mahulette et al. (2022). The resulting measurements were then used to create a dendrogram for grouping and determining each clove accession's distinguishing characteristics. Hierarchical Cluster Analysis (HCA) was used for dendrogram creation, while Principle Component Analysis (PCA) was used to determine character traits. Differences in morphology between different clove types were analysed using the LSD test. R Stat 3.1.0 software was used for HCA and PCA, and STAR 2.0.1 software was used for the LSD test.

RESULTS AND DISCUSSION

Exploration of local cloves in North Maluku

Local clove exploration was conducted on six islands in North Maluku Province, Indonesia, resulting in the discovery of several unique groups of local clove accessions. Figure 1 displays the distinct accession groups found on each island. Ternate Island had five local clove accessions: Afo, Red Zanzibar, Sikotok, Siputih, and Rica. Tidore Island also had five accession: Maluku Cloves, Gagang Panjang Cloves, Red Zanzibar, Afo, and Sikotok. Bacan Island identified five local clove accessions: Sibela I, Sibela II, Forest clove, White Zanzibar, and Red Zanzibar. Kasiruta Island had three local clove accessions: Red Zanzibar, Bogor, and Biasa. Halmahera Island had four local clove accessions: Afo, Red Zanzibar, Sikotok, and Maluku cloves. Moti Island had three local clove accessions: Maluku, Zanzibar Merah, and Sikotok. Overall, the exploration discovered 13 local clove accessions across the six islands, as outlined in Table 1.

Upon research, it was discovered that Afo cloves are primarily distributed in the Mount Gamalama area of Ternate Island, their original distribution location. Ternate Island also boasts a significant distribution of Red Zanzibar, Sikotok, Siputih, and Rica cloves. Red Zanzibar cloves can also be found on other explored islands, such as Tidore Island, Bacan Island, Kasiruta Island, Halmahera Island, and Moti Island. Sibela I and Sibela II cloves are mainly distributed in the Sibela Mountain area on Bacan Island, their original distribution area. Biasa cloves are primarily distributed on Kasiruta Island, while Maluku cloves are mainly distributed on Halmahera Island. Moreover, other cloves are present, such as Gagang Panjang cloves on Tidore Island, Forest and White Zanzibar cloves on Bacan Island, and Bogor cloves on Kasiruta Island. However, their distribution is less significant.

It has been observed that most local clove accessions are of the cultivated type and possess an aromatic profile. However, the non-aromatic forest cloves found on Bacan Island are an exception to this trend. Among the aromatic group of cloves are Afo cloves, Red Zanzibar, Sikotok, Siputih, Rica, Maluku cloves, Gagang Panjang, Sibela I, Sibela II, White Zanzibar, Bogor cloves, and Biasa cloves. According to Biernasiuk et al. (2023), Gengatharan et al. (2023), and Salsabila et al. (2023), cultivated cloves are known for their strong aroma, which is attributed to high levels of eugenol. Conversely, non-aromatic cloves, like Forest cloves, have a milder aroma due to low levels of eugenol, as reported by Mahulette et al. (2020, 2021) and Aziz et al. (2023).

Recent research has discovered a variety of local cloves that stand out

Table 1. Accession of local cloves on six islands in North Maluku Province, Indonesia.

Accession	Distribution	Type
Afo*)	Ternate, Tidore, Halmahera Island	Cultivated type)/aromatic
Red Zanzibar *)	Ternate, Tidore, Bacan, Kasiruta, Halmahera and Moti Island	Cultivated type)/aromatic
Sikotok*)	Ternate, Tidore, Halmahera, and Moti Island	Cultivated type)/aromatic
Siputih*)	Ternate Island	Cultivated type)/aromatic
Rica	Ternate Island	Cultivated type)/aromatic
Maluku	Tidore, Halmahera, and Moti Island	Cultivated type)/aromatic
Gagang Panjang	Tidore Island	Cultivated type)/aromatic
Sibela I	Bacan Island	Cultivated type)/aromatic
Sibela II	Bacan Island	Cultivated type)/aromatic
Forest Clove	Bacan Island	Wild type)/non-aromatic
White Zanzibar	Bacan Island	Cultivated type)/aromatic
Bogor	Kasiruta Island	Cultivated type)/aromatic
Biasa	Kasiruta Island	Cultivated type)/aromatic

Note:*) indications have been released as superior varieties.

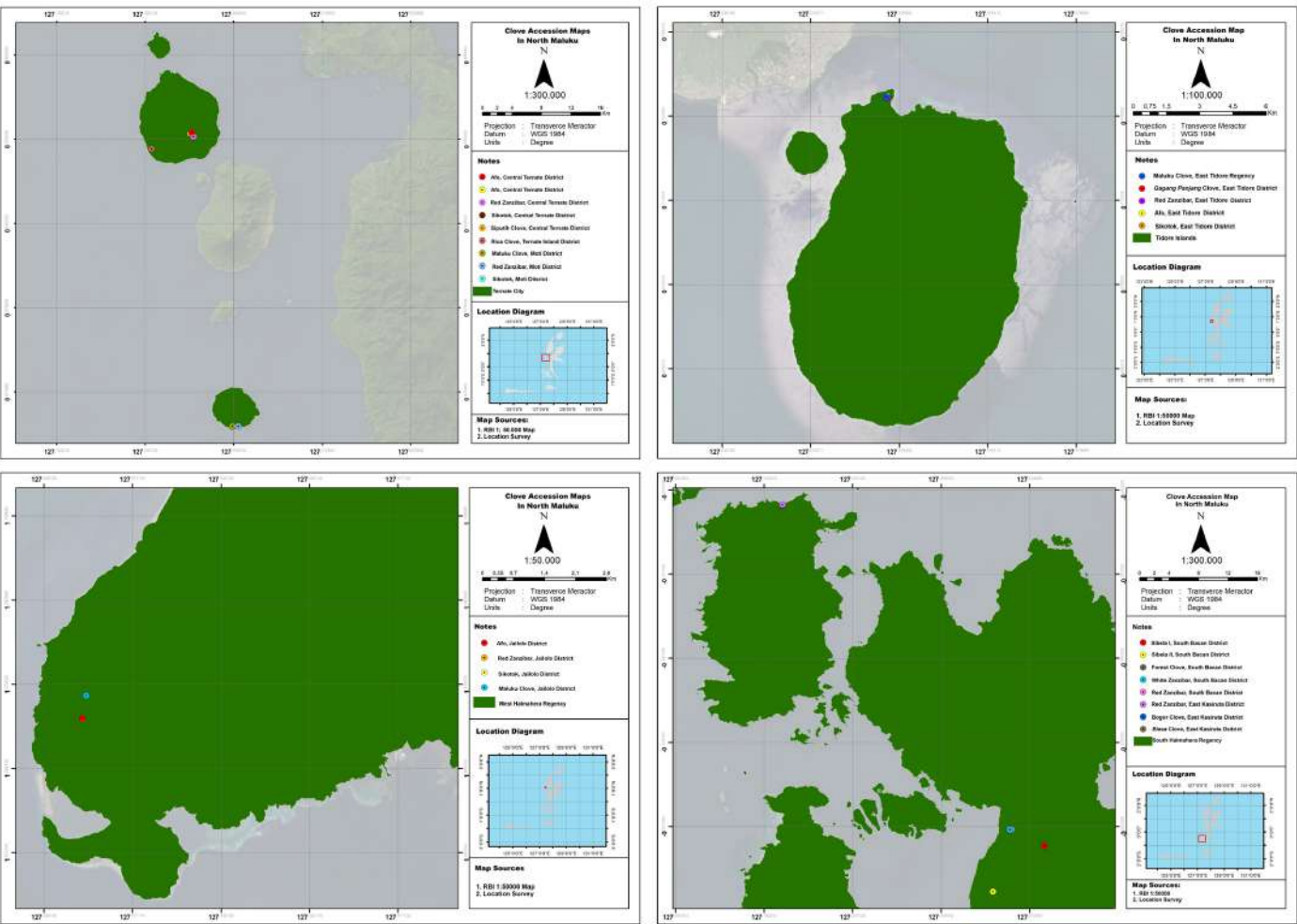


Figure 1. Research location at the six island in North Maluku.

as superior, including Afo, Red Zanzibar, Sikotok, and Siputih. Furthermore, studies have revealed clove variants, such as Red Zanzibar and White Zanzibar, which differed only in the colour of their shoots. Moreover, Sibela I, and Sibela II cloves have unique variants that vary in flower size, leaf size, and crown shape. According to Mahulette et al. (2022) and Wattimena et al. (2023), cloves can exhibit variations in their populations despite belonging to the same species. It is due to cross-pollination over time, which can lead to the emergence of new variants within the population (Mahulette et al. 2019a, 2019b, 2020; Hariyadi et al. 2019).

The study showed that the morphological diversity of cloves discovered in North Maluku Province differed significantly from previous explorations conducted in Maluku Province (Sundari et al. 2019; Mahulette et al. 2022). This exploration shows that only Red Zanzibar, White Zanzibar, Bogor, and Forest cloves share similar genotypes in Maluku Province. However, Afo Clove, Sikotok, Siputih, Rica, Maluku Clove, Gagang Panjang Clove, Sibela I, Sibela II, and Biasa Clove were exclusively identified in North Maluku Province. These findings confirmed that Maluku and North Maluku Province (Maluku Archipelago) possess remarkable morphological diversity in clove distribution. The high morphological diversity in these two provinces further supports the thought that they are the centre of origin cloves worldwide.

Morphological diversity of North Maluku local clove accessions

North Maluku clove accessions from exploration were differentiated by measuring several morphological characters. The results of the different tests showed significant differences in several morphological characteristics such as leaf length, leaf width, flower length, flower diameter, flower weight, and number of flowers per set (Table 2).

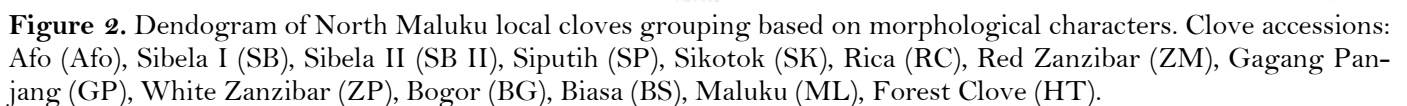
Through morphological character measurements, it is evident that the Forest Clove accessions boast the largest leaf and flower sizes among all local North Maluku clove accessions. However, despite its larger size, the number of flowers per set of the Forest clove accession is relatively less than the Biasa and Gagang Panjang clove accessions. The shortest leaf length was observed in the Bogor clove and was not significantly different from the leaf length of the Sikotok and Rica accessions. Similarly, the Rica clove exhibited the shortest leaf width and was not significantly different from the leaf width of the Sikotok. The Sibela I and Sibela II had the shortest flower bud length. However, it was similar to the flower length of the Gagang Panjang clove, Bogor cloves, and Biasa cloves. The Biasa and Gagang Panjang cloves had the highest number of flowers per arrangement. Despite having more flowers per set, the Gagang Panjang clove had the lowest flower diameter and weight. These morphological variations among the different accession groups could be attributed to genetic differences. North Maluku Province is a part of the original distribution area of cloves, and various accessions of cloves with varying morphological characters can be found there. According to Kayoumu et al. (2022) and Qiao et al. (2022), genetic differences in a plant population cause numerous morphological differences. Environmental factors and cultivation techniques also contribute to the diversity of a plant population (Ezin et al. 2022; Qiao et al. 2022).

Grouping of North Maluku local clove accessions based on Hierarchical Cluster Analysis (HCA)

A Hierarchical Cluster Analysis (HCA) was conducted on 13 different clove types in North Maluku to categorise them based on 44 morphological traits. Each type was represented by five plants, resulting in 65 plants analysed (Figure 2). HCA is commonly used to group plants with similar traits (Hartati et al. 2022; Mahulette et al. 2022). This grouping can assist in identifying plant groups with high agronomic potential, particularly in terms of production characteristics. The widespread use of HCA in plant breeding to gain insights into plant diversity (Ezin et al. 2022; Ustari et al. 2023). This information is valuable in plant breeding, particularly when selecting parents for crossbreeding.

Hierarchical Cluster Analysis (HCA) revealed that all the local clove accessions can be divided into two large groups with a dissimilarity coefficient of 49 %. The first group includes almost all local clove accessions, such as Afo cloves, Sibela I, Sibela II, Siputih, Sikotok, Rica, Red Zanzibar, Gagang Panjang, White Zanzibar, Bogor cloves, Biasa cloves, and Maluku cloves. On

Note: Numbers followed by the same letter in the same column are not significantly different in the 5 % α LSD test.



-6-

plants with distant kinship, which exhibit more diverse characteristics.

Group I

Group I based on Hierarchical Cluster Analysis (HCA), consisted of 12 local clove accession groups with 72 % similarity (28 % dissimilarity). All local clove accession group members are cultivated type cloves from the aromatic group. This group consists of Afo cloves, Sibela I, Sibela II, Siputih, Sikotok, Rica, Red Zanzibar, Gagang Panjang, White Zanzibar, Bogor cloves, Biasa Cloves, and Maluku cloves (Figure 3-9).

The Afo clove (Figure 3A) is a highly similar group within the population, with a 98 % clustering rate. Distinguished by its unique stem that divides at the base, the Afo clove boasts an average height of 35.04 m, a conical crown shape, and a 45° branching angle. The lowest branch height of this group is 1.34 m, and its leaves are elliptical, with a length of 12.75 cm, a width of 4.61 cm, and an index of 2.79, resulting in a leaf area of 35.32 cm². The old leaves of the Afo clove exhibit a deep yellowish green/green group/N134B colour, while the shoots have a moderate yellowish pink/greyed-orange group/N170D colour. The flower buds of this group are flat funnels, with an average of 19.04 flowers per series, a length of 1.84 cm, and a diameter of 3.99 mm. The buds weigh 0.30 g and have a light greenish-yellow/yellow group/4C color.

The Sibela I clove (Figure 3B) accession group shares a 96 % similarity level. The most notable feature of this group is the smaller size of their flower buds compared to other clove accessions, though they are slightly larger than those of the Sibela II group. Additionally, like the Afo clove, the main stem of this group divides at the base. These accessions have an average tree height of 30.20 m with a slightly rounded crown shape, a divided main stem, a lower branch height of 1.58 m, and a branching angle of 45°. Compared to the Sibela II group, this group's leaves and flower buds are slightly larger. The elliptical leaves measure 12.84 cm in length and 5.07 cm in width, with a leaf size index of 2.54 and a leaf area of 66.95 cm². The leaf colour is deep yellowish green/green group/N134B, while the shoot color is brilliant yellow-green/yellow-green group/ 149A. The flat funnel-shaped flowers have 9.40 per series, and the ripe flower buds are 1.65 cm long, 4.24 mm in diameter, and weigh 0.24 g. They are a brilliant yellow-green/yellow-green group/150C in color.

The Sibela II clove (Figure 4C) accession groups are clustered with a 95 % similarity rate. This group has a few notable characteristics, such as a conical crown, slightly smaller leaves, and flower buds, compared to the Sibela I clove accession group. On average, the trees in this group have a height of 34.23 m, with a dividing main stem, conical crown, lowest branch height of 1.34 m, and a branching angle of 45°. The leaves are elliptical, with a length of 9.52 cm, a width of 3.75 cm, and an index of 2.55. They have a leaf area of 34.08 cm² and a dark leaf color of deep yellowish green/green group/N134A. The shoot colour is moderate yellowish pink/greyed-orange group/N173D. The flower buds of this group are flat funnel-shaped, with a flower length of 1.60 cm, a diameter of 4.24 mm, and a weight of 0.25 g. Members of this group tend to have 9.40 flowers per series and have a brilliant yellow-green/yellow-green group/150C flower color.

The Siputih clove (Figure 4D) accession groups clustered with a similarity level of 94 %. One identifying feature of this group is the bright yellow hue of its leaves and flower buds, which set it apart from other groups in the population. The trees in this accession group have an average height of 29.84 m and a cone-shaped crown with a main stem that does not divide. The lowest branch height is 1.32 m, and the branching angle is 45°. The leaves are elliptical, measuring 9.78 cm in length and 3.86 cm in width. The leaf size index is 2.52, with a leaf area of 26.70 cm². The older leaves are more vibrant

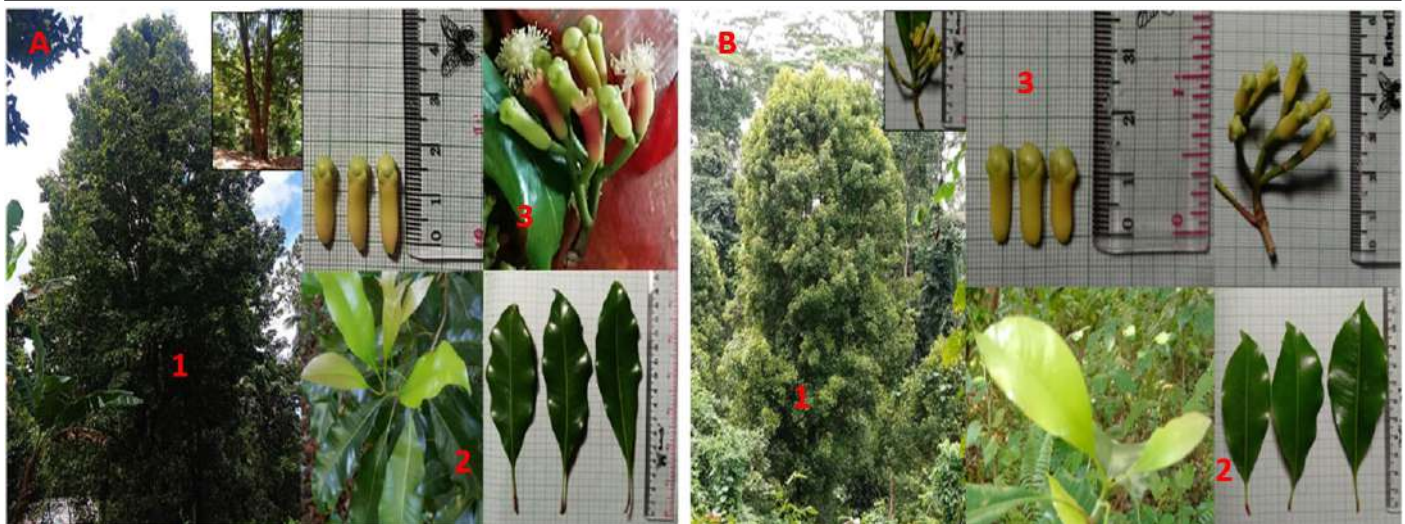


Figure 3. Afo clove (A), Sibela-I clove (B). Tree (1), Leaves (2), Flower Bud (3).

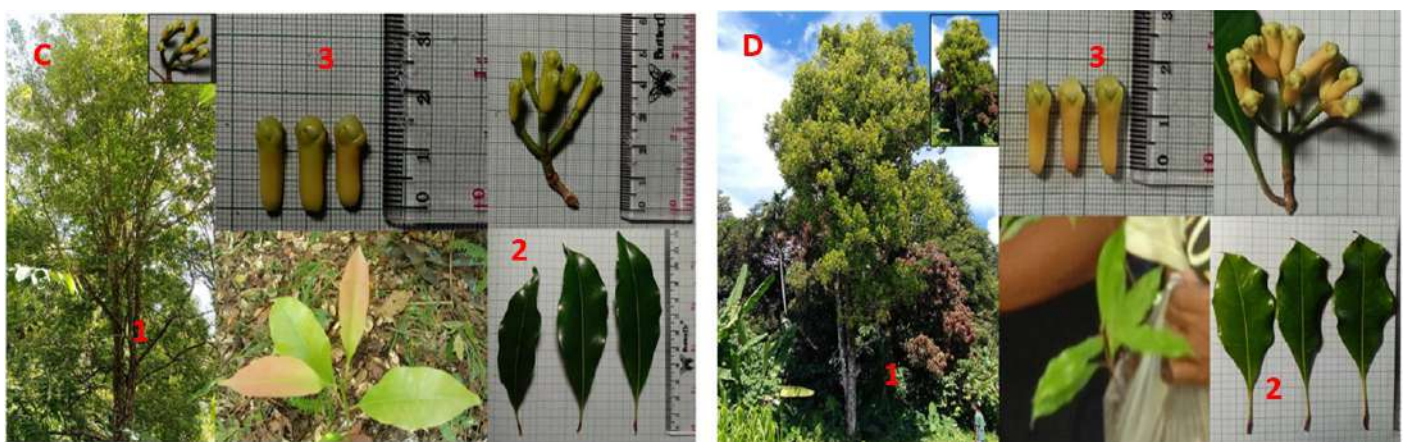


Figure 4. Sibela II Clove (C), Siputih clove (D). Tree (1), Leaves (2), Flower Bud (3).

than other clove accessions, such as the strong yellow-green/yellow-green group/N144C, while the shoots have a grayish reddish orange/greyed-orange group/174D. The mature flower buds of this group are flat and funnel-shaped, with a length of 1.79 cm, a diameter of 4.44 mm, and a weight of 0.32 g. Each set contains an average of 16.20 flowers, and the color of the ripe flower buds is light yellow/yellow group/12C.

The Sikotok clove (Figure 5E) accession group shares a 98 % similarity in clustering. This group is characterized by its small leaves, short handle, and easily falling flower buds. On average, members of this group have a tree height of 20.14 m, with a conical crown and an undivided main stem. The lower branch is typically at a height of 1.10 m, with a branching angle of 45°. The elliptical-shaped leaves have a length of 9.11 cm, a width of 3.54 cm, a leaf size index of 2.58, and a leaf area of 25.29 cm². The old leaves are a strong yellow-green/green group/143C, while the shoots are a brilliant yellow-green/yellow-green group/154C. When picked ripe, the flower buds of this group are flat funnel-shaped, with an average of 7.20 flowers per arrangement. The flower bud is 1.77 cm long and 3.95 mm in diameter, weighing 0.26 g, and appears in a brilliant yellow-green/yellow-green group/154C color.

The Rica clove (Figure 5F) accession group is clustered with 95 % similarity. These cloves display a morphology similar to Sikotok cloves, though they differ in their branching direction, which curves downwards. The mature plucked flowers of this group are known for their reddish color and do not fall off easily. Trees belonging to this group have an average height of 20.80 m with a slightly rounded crown shape and a main trunk that does not divide.

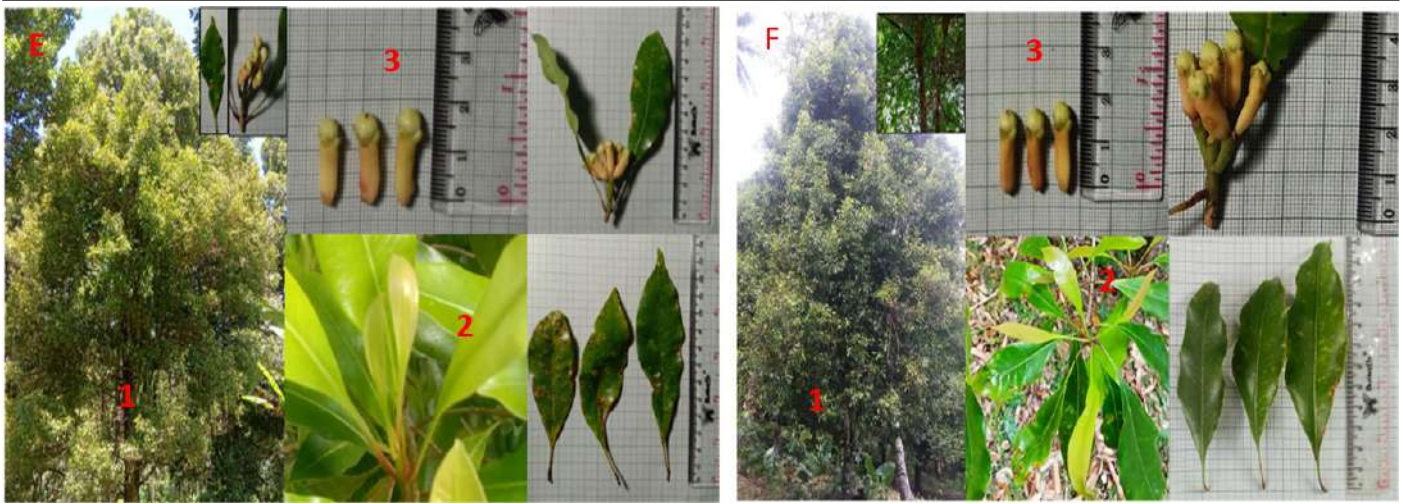


Figure 5. Sikotok Clove (E), Rica Clove (F). Tree (1), Leaves (2), Flower Bud (3).

The lower branches have a height of 1.63 m, and their branching angle is 90°. The leaves of this group are elliptical, with a length of 9.40 cm, a width of 3.24 cm, a leaf size (index) of 2.90, and a leaf area of 25.28 cm². The color of the old leaves is a strong yellow-green/green group/143C, and the shoot color is a moderate reddish orange/grey-red group/179C. This group's picked ripe flowers (flower buds) are flat funnel-shaped with a length of 1.81 cm, a diameter of 4.11 mm, and a weight of 0.34 g. The number of flowers per series is 17.00, and the color is light yellow-green/yellow-green group/154D.

The Red Zanzibar clove (Figure 6G) accession group has a 94 % similarity cluster. This group's most distinctive feature is the reddish hue of its shoots and flower buds and the higher number of flowers per series. On average, this accession has a tree height of 35.84 m, a conical crown, a lower branch height of 1.26 m, an undivided main stem, and an angle of branching of 45°. The leaves are elliptical, with a length of 12.73 cm, a width of 5.27 cm, a leaf size (index) of 2.42, a leaf area of 51.01 cm², and a deep yellowish green/green group/N134B leaf colour, with a strong red/red group/53B shoot colour. Members of this group have an average of 23.80 flowers per arrangement, shaped like a flat funnel. The flower buds have a length of 2.07 cm, a diameter of 4.24 mm, a flower weight of 0.30 g, and a moderate reddish orange/greyed-red group/178C colour.

The Gagang Panjang clove (Figure 6H) accession group is characterised by a 93 % similarity in clustering. This group is easily distinguished by its longer flower stalks and buds that appear greener than other accession groups. Trees in this group have an average height of 20.60 m, with a cone-shaped crown and a non-dividing main trunk. The lower branches are at a height of 1.34 m, and the branching angle is 45°. Leaves in this group are elliptical, 12.17 cm in length and 4.91 cm in width. The leaves have a leaf size (index) of 2.48, with a leaf area of 41.65 cm². The dark leaf colour is a deep yellowish green/green group/N134B, while the shoot colour is a moderate yellowish pink/greyed-red group /179D. Picked ripe flowers (flower buds) are flat funnel-shaped, with approximately 26.00 flowers per set. The flowers are 1.75 cm long, 3.28 mm in diameter, weigh 0.21 g, and are a brilliant yellow-green/yellow-green group/149B.

The White Zanzibar clove (Figure 7I) accession group is similar, at 96 %. These accessions have a similar morphology to the Red Zanzibar clove but with fewer red shoots and flowers. On average, this accession has a tree height of 27.80 m with a cone-shaped crown. The main trunk does not divide; the lowest branch height is 1.16 m, and the branching angle is 45°. The leaves are elliptical, measuring 12.62 cm in length and 4.84 cm in width. Their leaf size (index) is 2.61, with a leaf area of 46.78 cm². The dark leaf colour is a viv-

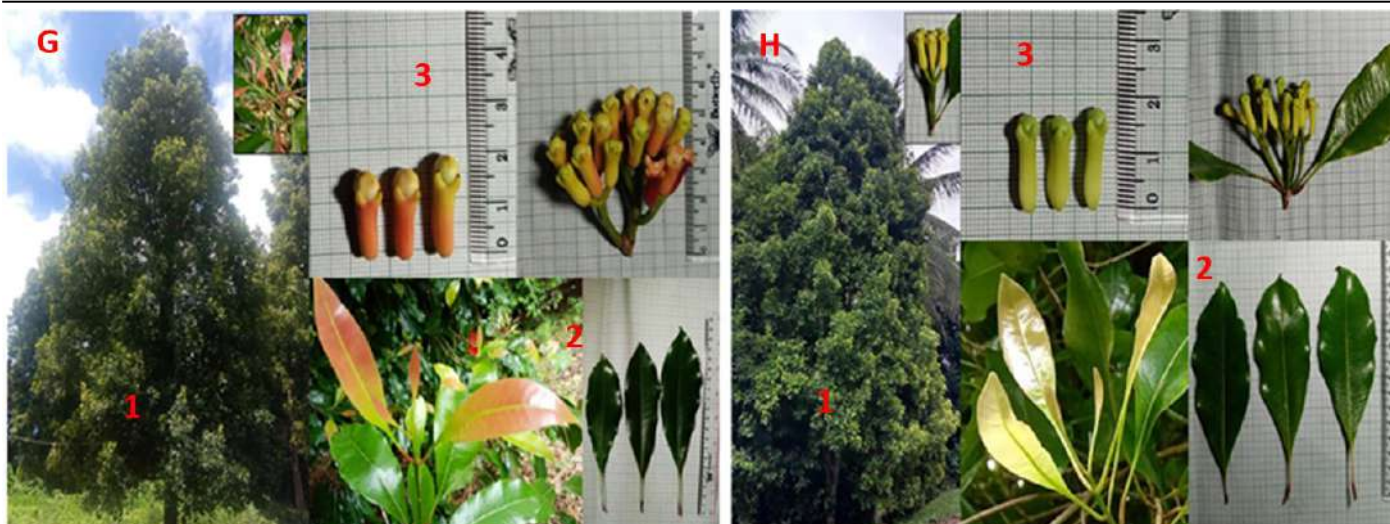


Figure 6. Red Zanzibar clove (G), Gagang Panjang clove (H). Tree (1), Leaves (2), Flower Bud (3).

id yellowish-green/green group/140A, and the shoot colour is a moderate yellowish pink/greyed-orange group /N170D. The ripe flower buds are flat funnel-shaped, with a length of 2.03 cm, a diameter of 4.08 mm, and a weight of 0.30 g. Each arrangement contains an average of 21.40 flowers, coloured brilliant yellow-green/yellow-green group/154C.

The Bogor clove (Figure 7J) accession group shares a 94 % similarity in clustering. These clove plants have a similar morphology to Red Zanzibar cloves but with slightly smaller leaves and flowers. Their trees average 18.00 m in height, with a conical crown and a main stem that does not divide. The lower branches start at 1.60 m in height, with a branching angle 45°. The leaves in this group are elliptical, measuring 8.70 cm in length, 4.77 cm in width, and with an index size of 1.83. The leaf area is 42.19 cm², with a deep yellowish green/green group/141B color. The shoot color is moderate reddish orange/greyed-red group/179C. The flower buds are flat funnel-shaped, 1.69 cm in length, and 5.25 mm in diameter. Each series has an average of 12.80 flowers, with a brilliant greenish yellow/yellow-green group/151D color.

The Biasa clove (Figure 8K) accession group shares a 95 % similarity in its characteristics. One of the most notable traits of this group is the similarity in size and color of their leaves to cloves. The Tuni variety is typically cultivated in the Maluku Province and has a cone-shaped crown with more flowers per set than other group members. On average, this clove grow to a height of 17.80 m, have a cone-shaped crown, a single trunk, the lowest branch height of 1.39 m, and a branching angle of 45°. The elliptical leaves of this group measure 9.76 cm in length and 4.83 cm in width, have a leaf size index of 2.27, a leaf area of 29.84 cm², and exhibit a strong yellow-green/yellow-green group/N144C colour. The shoots of these trees are light yellow-green/yellow-green group/154D. Each arrangement produces around 26.40 flowers, with ripe flowers harvested in a flat funnel and exhibiting a brilliant yellow-green/yellow-green group/154C colour. The flowers have a length of 1.76 cm, a diameter of 4.83 mm, and weigh 0.33 g.

The Maluku clove (Figure 8L) accession group is 96 % similar in the cluster. Its members share distinct characteristics such as a slightly rounded crown shape and thicker, rounded leaves resembling wild-type cloves, yet with a spicier aroma than cultivated cloves. The average height of these trees is 21.60 m, with a non-dividing main stem, a slightly rounded crown shape, and a 90° branching angle. The ovate-shaped leaves have a length of 10.23 cm, a width of 5.32 cm, and a leaf size (index) of 1.92. They boast a leaf area of 48.78 cm², a deep yellowish green/green group/141 leaf colour, and a moderate yellowish pink/greyed-shoot colour, orange-group/N173D. Ripe flowers

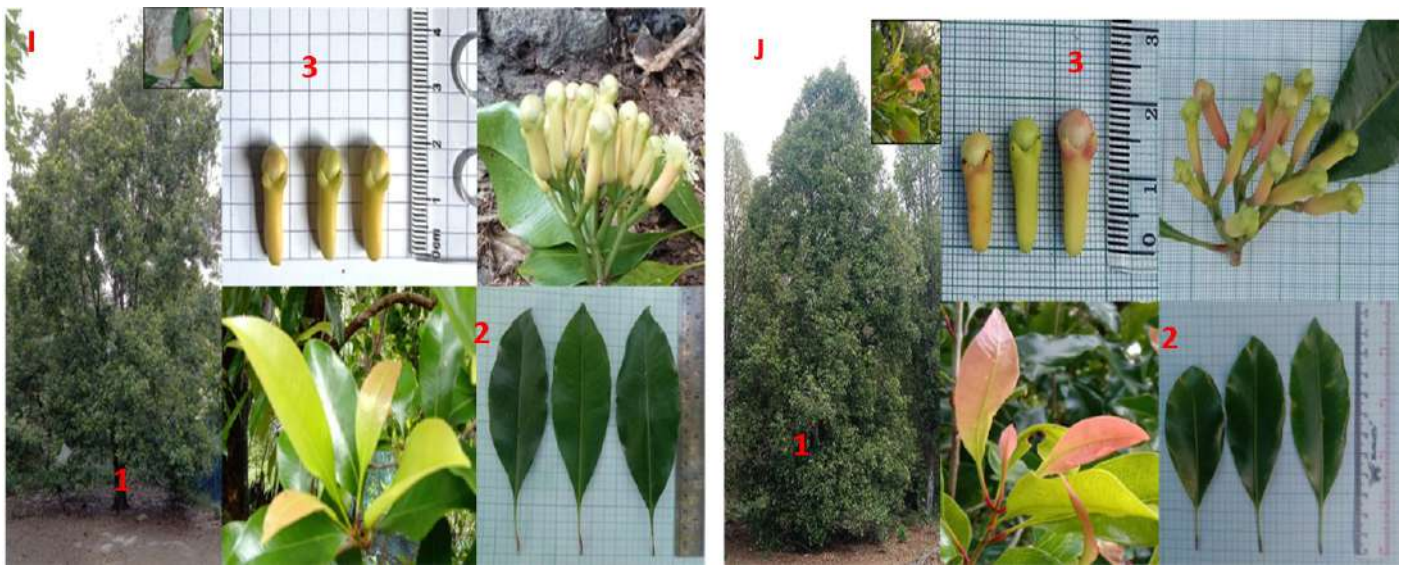


Figure 7. White Zanzibar clove (I), Bogor clove (J). Tree (1), Leaves (2), Flower Bud (3).



Figure 8. Biasa Clove (K), Maluku Clove (L). Tree (1), Leaves (2), Flower Bud (3).

(flower buds) have a flat funnel shape, with 13.20 flowers per series. The flower buds measure 1.85 cm in length, 4.93 mm in diameter, weighing 0.32 g, and have a brilliant yellow-green/yellow-green group/154C colour.

Group II

Based on Hierarchical Cluster Analysis (HCA), Group II comprises Forest clove (Figure 9) accessions that cluster with 94 % similarity (Figure 15). These accessions are included in the wild-type cloves from the non-aromatic group. The distinguishing characteristics of this clove group are its rounded crown, large leaves and flowers, oval and thicker leaves, and a less spicy aroma in both flowers and leaves. The trees of this accession group have an average height of 35.14 m, a rounded crown shape with an undivided main stem, and a branching angle of 90°. The members of this group have the largest oval-shaped leaves with a leaf length of 18.45 cm, a width of 8.89 cm, a leaf size index of 2.08, a leaf area of 144.13 cm², and a dark yellow-green/yellow-green group/144A leaf colour. The shoot colour is a moderate yellowish pink/grayed-orange group/N173D. This group's picked ripe flowers (flower buds) are the largest, with a length of 2.36 cm, a diameter of 6.66 mm, a weight of 0.80 g, and a light yellow-green/yellow-green group/154D colour. The number of picked ripe flowers (flower buds) of members of this group is 13.58 flowers and is round and funnel-shaped.



Figure 9. Forest clove (M). Tree (1), Leaves (2), Flower Bud (3).

Characteristics of North Maluku local clove based on Principal Component Analysis (PCA)

The characteristics of local North Maluku cloves were examined through Principle Component Analysis (PCA) on 65 local clove accessions, using data from 39 diverse morphological characters. According to Greenacre et al. (2022) and Jolliffe (2022), PCA effectively identifies the most influential factors from many variables with high variability. According to Mahulette et al. (2022) and Wattimena et al. (2023), PCA is commonly used to identify key characteristics of a plant group. In this case, one character stood out as having the greatest impact among all those analysed. Furthermore, as noted by Mahulette et al. (2022) and Ustari et al. (2023), defining characters are independent factors that can be distinguished from the larger set of variables analysed. The PCA results revealed a total diversity of 52.5 %. Vectors with a red scale indicated the identifying characters (Figure 10a). The vectors close to each accession represented its characteristic traits (Figure 10b).

Group I PCA results showed that Afo clove's distinguishing feature was the main stem division, while Sibela I and Sibela II cloves had spiciness and leaf aroma. Siputih had a ripe flower colour, Sikotok had larger leaves, and Rica cloves had a branching direction. Red Zanzibar cloves had the number of flowers per series, canopy width, and stem circumference. Long Handle cloves had longer flower stalks, White Zanzibar cloves had longer leaf stalks, Bogor cloves had shoot colour, Biasa cloves had bark color, and Maluku cloves had crown shape and branching angles. Group II / Forest Clove had characteristics such as lower branch height, leaf length, leaf width and area, leaf tip shape, leaf thickness, flower length, diameter, and weight.

CONCLUSIONS

North Maluku Province has identified 13 local clove accessions: Afo, Zanzibar Red, Sikotok, Siputih, Rica, Maluku, Gagang Panjang Clove, Sibela I, Sibela II, Forest cloves, White Zanzibar, Bogor cloves, and Biasa cloves. All clove accessions were categorised into two groups with a dissimilarity coefficient of 49 %. Based on Principle Component Analysis (PCA), each clove accession's characteristics were identified. The PCA results showed the characteristics that contribute the most to the grouping, where the characteristic attributed

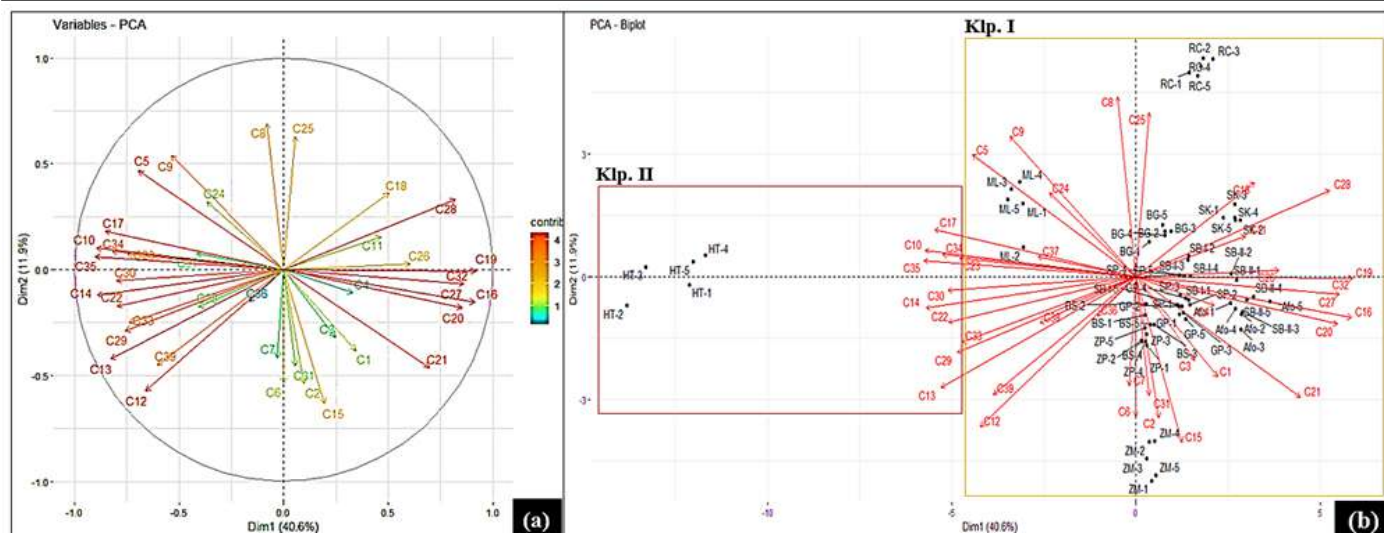


Figure 10. Characteristics of local clove accession groups in North Maluku based on Principle Component Analysis (PCA). Morphological characters, C1-C39; Clove accessions, Afo (Afo), Sibela I (SB), Sibela II (SB II), Siputih (SP), Sikotok (SK), Rica (RC), Red Zanzibar (ZM), Gagang Panjang (GP), White Zanzibar (ZP), Bogor (BG), Biasa (BS), Maluku (ML), Forest Clove (HT).

of Afo cloves obtained are the main stem that divides, Sibela I and Sibela II cloves are the spiciness and aroma of the leaves. Siputih cloves are the colour of ripe flowers, Sikotok is the size of the leaves, and Rica cloves are the branching direction. Red Zanzibar cloves are the number of flowers per arrangement, canopy width, and stem circumference; long-handled cloves are the length of the flower stalk; White Zanzibar cloves are the length of the leaf stalk; Bogor cloves are the colour of the shoots; Ordinary cloves are the colour of the bark, and Maluku cloves are the shape of the crown and the angle of the branching. Forest cloves, as wild-type cloves, are indicated to have characteristic characters in the height of the lowest branch, leaf length, leaf width, leaf area, leaf tip shape, leaf thickness, flower length, flower diameter, and flower weight. These 13 clove accessions prove North Maluku is the centre of origin and has high clove diversity. This accession can be used for future variety development.

AUTHOR CONTRIBUTION

All authors contributed to the published article's research, data collection, data analysis, and writing.

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CONFLICT OF INTEREST

There is no conflict of interest in this research.

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Research Article

Characterisation of CONSTANS Gene Family from the Amaranthaceae Family and Their Response to Adverse Environmental Conditions Based on Genome-Wide Identification and Transcriptome Analysis

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ABSTRACT

The CONSTANS (CO) transcription factor plays a key role in regulating photoperiodic flowering by integrating environmental signals and activating downstream flowering genes. However, no comprehensive information on the CO transcription factor family has been reported in *Amaranthus* species. In this study, we performed a genome-wide identification, characterisation, and expression analysis of the CO gene family in six *Amaranthus* species, including *A. hypochondriacus*, *A. palmeri*, *A. cruentus*, *A. hybridus*, *A. tuberculatus*, and *A. tricolor*. A total of 12 to 13 CO genes were identified in each species, and their chromosomal positions, gene structures, and physicochemical properties were analysed. Phylogenetic analysis categorised these CO genes into three distinct groups, revealing evolutionary relationships with CO genes from *Arabidopsis thaliana* and sugar beet. Gene structure analysis showed considerable diversity in exon-intron organisation, indicating functional differentiation within the CO family. Additionally, transcriptome analysis using RNA-Seq data demonstrated tissue-specific and stress-induced expression patterns, particularly under drought and herbicide treatments. This study provides the first comprehensive insight into the CO transcription factor family in *Amaranthus* species, offering a foundation for future research on photoperiodic flowering regulation and stress-response mechanisms in these species.

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INTRODUCTION

The Amaranthaceae family, commonly known as the Amaranth family, comprises a diverse group of globally distributed plants with significant agricultural and ecological importance. This family includes *Amaranthus hypochondriacus*, *A. palmeri*, *A. cruentus*, *A. hybridus*, *A. tuberculatus*, and *A. tricolor*. Originating primarily from the Americas, many of these species have been cultivated for their nutritional value since ancient times (Caselato-Sousa & Amaya-Farfán 2012). For instance, *A. hypochondriacus* and *A. cruentus* are renowned for their high-protein grains and are staple crops in various cultures, while *A. tricolor* is widely grown in Asia as a leafy vegetable rich in vitamins and minerals (Soriano-García & Aguirre-Díaz 2019; Baraniak & Kania-Dobrowolska 2022). On the other hand, *A. palmeri* and *A. tuberculatus* are notable for their adaptability and have become pervasive agricultural weeds that challenge crop production due to their resistance to common herbicides. Understanding how these plants sense photoperiod information to predict impending environmental changes and precisely regulate flowering time under favourable conditions at the molecular level is essential (Pulvento & Sellami 2021). Investigating these mechanisms will not only advance our understanding of plant developmental biology but also provide practical benefits for improving crop yields, controlling weed species, and adapting agricultural practices to the challenges of climate change (Roeber et al. 2021b; Jan et al. 2023). Moreover, insights gained from such studies could contribute to broader agricultural advancements by informing breeding programmes aimed at improving stress resilience and productivity in other crop species (Roeber et al. 2021a; Baguma et al. 2023).

CONSTANS (CO) is a pivotal transcription factor in plants that plays a central role in regulating photoperiodic flowering by integrating environmental light cues with the plant's internal circadian clock (Kim et al. 2008; Zhang et al. 2023). Structurally, CO is characterised by two conserved B-box zinc finger domains at its N-terminus, which are involved in protein-protein interactions and may contribute to DNA-binding specificity (Dahal et al. 2022). These domains are crucial for forming functional protein complexes that regulate downstream genes (Dahal et al. 2022; Zhang et al. 2023). At its C-terminus, CO contains a CONSTANS, CO-like, and TOC1 domain, which is essential for nuclear localisation and mediates interactions with other circadian clock components (Wenkel et al. 2006). Functionally, CO acts as a transcriptional activator of the *FLOWERING LOCUS T* gene, promoting the transition from vegetative growth to flowering in response to favourable photoperiod conditions, particularly under long-day environments (Zhang et al. 2023). As a key gene in the photoperiod pathway (Kim et al. 2008; Zhang et al. 2023), understanding the CONSTANS transcription factor in *A. hypochondriacus* is necessary to elucidate how this species perceives and responds to photoperiodic signals at the molecular level. To date, the CO transcription factor family has been investigated in various plant species, including barley (*Hordeum vulgare*) (Griffiths et al. 2003), rice (*Oryza sativa*) (Griffiths et al. 2003), Arabidopsis (*Arabidopsis thaliana*) (Griffiths et al. 2003), mango (*Mangifera indica*) (Liu et al. 2022), Chinese white pear (*Pyrus bretschneideri*) (Wang et al. 2017), grapevine (*Vitis vinifera*) (Wang et al. 2019), sugar beet (*Beta vulgaris*) (Chia et al. 2008), *Chrysanthemum lavandulifolium* (Fu et al. 2015), ginkgo (*Ginkgo biloba*) (Yan et al. 2017), and quinoa (*Chenopodium quinoa*) (Tran et al. 2025). However, there is no report on the CO transcription factor family in *A. hypochondriacus*.

Studying CO in this context will shed light on the unique regulatory mechanisms employed by *A. hypochondriacus* and contribute to optimising flowering time and enhancing agronomic traits in this nutritionally important and stress-resilient crop. Such insights are crucial for enhancing yield, adapt-

ing cultivation practices to shifting climatic conditions, and ultimately contributing to global food security.

This study aims to conduct a comprehensive genome-wide identification, characterisation, and expression analysis of the CO transcription factor family in six *Amaranthus* species. By investigating their gene structure, evolutionary relationships, physicochemical properties, and expression patterns across various tissues and environmental conditions, this study seeks to provide insights into the functional roles of CO genes in photoperiodic flowering and stress responses. The findings aim to enhance our understanding of the CO transcription factor family in the Amaranthaceae family and contribute to future crop improvement strategies.

MATERIALS AND METHODS

Database search of CO genes

To identify CO genes within the Amaranthaceae family (Singh et al. 2023), we conducted a protein-based search strategy. Firstly, well-known CO protein sequences from *A. thaliana* (Griffiths et al. 2003) were obtained from TAIR (<http://www.arabidopsis.org/>) and used as queries in a local BLASTP search against the predicted proteomes of six *Amaranthus* species, including *A. hypochondriacus* (Sunil et al. 2014), *A. palmeri* (Montgomery et al. 2020), *A. cruentus* (Ma et al. 2021), *A. hybridus* (Montgomery et al. 2020), *A. tuberculatus* (Montgomery et al. 2020), and *A. tricolor* (Wang et al. 2023). All predicted proteins were then analysed using HMMER (<https://www.ebi.ac.uk/Tools/hmmer/>) (Potter et al. 2018) with a conserved CO domain downloaded from the Pfam database (<http://pfam.xfam.org/>) (Mistry et al. 2021), applying an E-value threshold between 0.0001 and 0.001. The filtered protein sequences were designated as CO transcription factors of Amaranthaceae species for further evaluation.

Characterisation of CO transcription factors

To analyse the physicochemical characteristics of CO transcription factors, we employed the ProtParam tool available on the ExPASy server (Gasteiger et al. 2003, 2005) as previously described (La et al. 2022; H.D. Chu et al. 2024). The amino acid sequences of the CO proteins were retrieved from the proteome assemblies of the respective *Amaranthus* species and input into ProtParam (Gasteiger et al. 2003, 2005) for analysis. This tool calculated various physicochemical parameters, including molecular weight, theoretical isoelectric point, aliphatic index, and grand average of hydropathicity.

Categorisation of CO transcription factors

To investigate the evolutionary relationships of CO transcription factors among six *Amaranthus* species (*A. hypochondriacus*, *A. palmeri*, *A. cruentus*, *A. hybridus*, *A. tuberculatus*, and *A. tricolor*), we constructed a phylogenetic tree using the Maximum Likelihood method as previously described (La et al. 2022; H.D. Chu et al. 2024). Multiple sequence alignment was performed using ClustalW (Thompson et al. 2002; Larkin et al. 2007) to obtain the alignment of conserved domains. The aligned sequences were then used to build the phylogenetic tree employing the Maximum Likelihood approach implemented in MEGA X software (Kumar et al. 2018). To assess the statistical support of the phylogenetic groupings, a bootstrap analysis with 1000 replicates was conducted as previously described (Cao 2022; La et al. 2022; N.T.B. Chu et al. 2024).

Structural analysis of CO genes

To analyse the gene structure of the CO gene family in *A. hypochondriacus*, we employed the GSDS tool (Hu et al. 2015) as previously described (Chu et al.

2018; Tran et al. 2025). The exon-intron arrangements were visualised by comparing full-length genomic sequences with their corresponding coding sequences. First, the phylogenetic tree of the CO gene family in *A. hypochondriacus* was constructed to guide the structural arrangement, ensuring that genes from closely related clades were analysed in a comparative context. The input genomic and coding sequences were aligned, and the GSDS tool (Hu et al. 2015) was used to map the distribution and number of exons and introns in each gene.

Transcriptome analysis of CO genes

To analyse the transcriptome of CO genes in *Amaranthus* species, recent RNA-Seq datasets reported in previous studies (Clouse et al. 2016) were obtained from the Phytozome (Goodstein et al. 2012) and AGRDB databases (Singh et al. 2023). Specifically, expression data and treatment time points for *A. hypochondriacus* were retrieved from a previous transcriptome study, which profiled gene expression across various tissues and under drought conditions (Clouse et al. 2016). For *A. palmeri* and *A. tuberculatus*, time-course expression profiles in response to glufosinate and mesotrione herbicide treatments, respectively, were obtained, providing processed gene expression data linked to specific herbicide exposure durations (Salas-Perez et al. 2018; Kohlhase et al. 2019). Expression levels of CO genes were calculated using FPKM or RPKM metrics for each dataset to ensure accurate normalisation of transcript abundance across different conditions. The heatmaps were generated using R scripts. All figures were created using R software and Adobe Illustrator.

RESULTS AND DISCUSSION

Genome-wide identification of the CO transcription factor family in the Amaranthaceae family

To identify the CO gene family in the genomes of *Amaranthus* species, we conducted comprehensive database searches using CO protein sequences from *A. thaliana* (Griffiths et al. 2003). After filtering with HMMER (Potter et al. 2018), all putative members of the CO gene family in the reference genomes of *A. hypochondriacus*, *A. palmeri*, *A. cruentus*, *A. hybridus*, *A. tuberculatus*, and *A. tricolor* were collected. As expected, a total of thirteen, thirteen, thirteen, twelve, twelve, and twelve members of the CO transcription factor family were found in *A. hypochondriacus*, *A. palmeri*, *A. cruentus*, *A. hybridus*, *A. tuberculatus*, and *A. tricolor*, respectively. Interestingly, we observed that the number of CO genes varied slightly among the six *Amaranthus* species, with some having thirteen members and others twelve. This variation may be attributed to species-specific gene duplication or loss events, differences in genome assembly completeness, or annotation quality across species. Such discrepancies are commonly reported in comparative genomic studies and indicate natural evolutionary divergence within the same plant family (Lespinet et al. 2002). These annotations of the CO transcription factor family in six Amaranthaceae species are presented in Tables 1 and 2.

All members of the CO gene family in *A. hypochondriacus*, *A. palmeri*, *A. cruentus*, *A. hybridus*, *A. tuberculatus*, and *A. tricolor* were located and designated based on their physical position on chromosome as *AhypCOL01* to *AhypCOL13*, *ApalCOL01* to *ApalCOL13*, *AcrucOL01* to *AcrucOL13*, *AhybCOL01* to *AhybCOL12*, *AtubCOL01* to *AtubCOL12*, and *AtrCOL01* to *AtrCOL12*, respectively (Tables 1 and 2). Of particular interest, thirteen members of the CO gene family in *A. hypochondriacus* were identified and mapped across sixteen chromosomes (Figure 1). Specifically, *AhypCOL01* and *AhypCOL02* were located on chromosome 1, *AhypCOL03* and *AhypCOL04* were found on chromosome 2, and *AhypCOL07* and *AhypCOL08* resided on chromosome 9. Five CO genes, including *AhypCOL05*, *AhypCOL06*, *AhypCOL09*, *AhypCOL10*, and

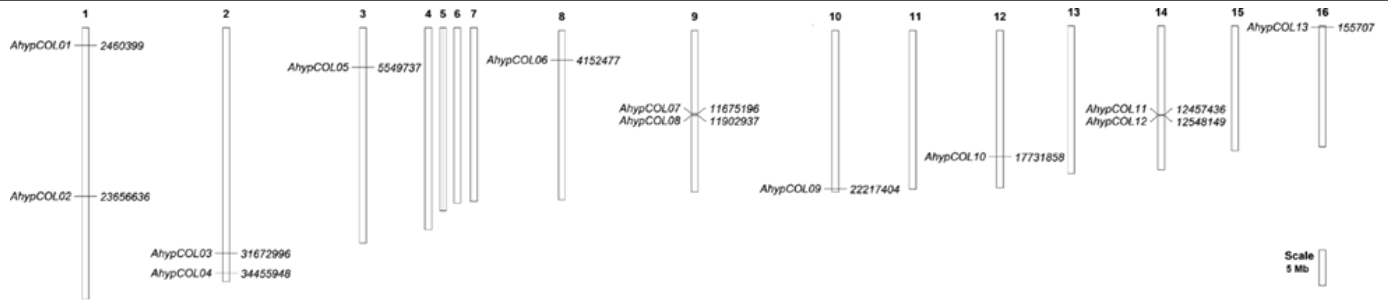


Figure 1. Physical localization of thirteen members of the CO gene family in *Amaranthus hypochondriacus*.

Table 1. Summary of the CO transcription factor family in *Amaranthus hypochondriacus*.

Gene name	Locus name	Genomic full -length (bp)	Protein full length (aa)	Molecular weight (kDa)	pI	GRAVY	Aliphatic index
AhypCOL01	AH000260	5334	410	44.84	5.26	-0.49	65.66
AhypCOL02	AH001280	6951	406	44.66	5.16	-0.57	62.73
AhypCOL03	AH004287	4715	318	35.73	4.35	-0.61	70.47
AhypCOL04	AH004578	4746	495	55.03	6.22	-0.53	75.86
AhypCOL05	AH005226	2857	426	41.10	5.80	-0.65	66.81
AhypCOL06	AH012867	2783	383	43.17	5.45	-0.65	69.48
AhypCOL07	AH014367	2774	392	44.96	5.28	-0.81	66.12
AhypCOL08	AH014379	1214	375	41.08	5.39	-0.34	72.32
AhypCOL09	AH016613	2005	370	41.07	5.46	-0.58	57.76
AhypCOL10	AH019184	1239	363	39.69	5.73	-0.52	57.93
AhypCOL11	AH020994	1228	377	41.89	5.52	-0.41	70.40
AhypCOL12	AH020998	2958	378	43.36	5.15	-0.95	59.02
AhypCOL13	AH022740	858	284	32.18	5.41	-0.67	62.89

AhypCOL13, were situated on chromosomes 3, 8, 10, and 16, respectively. Chromosome 14 also harboured two *CO* genes, namely *AhypCOL11* and *AhypCOL12*.

Previously, the number of members of *CO* gene families has been reported in various crop species. In *A. thaliana*, sixteen *CO* family genes have been identified and annotated (Griffiths et al. 2003). Similarly, the isolation of sixteen *CO* genes from rice has been reported (Griffiths et al. 2003). In the case of Chinese white pear, fifteen *CO* genes have been identified in the recent assembly (Wang et al. 2017). At least eleven *CO* genes have been reported in the *C. lavandulifolium* genome (Fu et al. 2015). Furthermore, nineteen and thirty-one candidate homologous *CO* genes have been identified in ginkgo (Yan et al. 2017) and mango (Liu et al. 2022), respectively. This variation in the number of *CO* genes among different species highlights the complexity and evolutionary dynamics of the *CO* gene family, suggesting that the expansion or contraction of these genes may play a crucial role in regulating flowering time and adaptation to diverse environmental conditions across plant taxa.

Determination of physicochemical properties of the CO transcription factor family in the Amaranthaceae family

To provide insights into the structural stability, solubility, and overall behaviour of the proteins under physiological conditions, we analysed the protein sequences of the *CO* transcription factor family in six *Amaranthus* species. As a result, the physicochemical features of the *CO* transcription factor families in the Amaranthaceae were described in Tables 1 and 2.

Firstly, we focused on the general parameters of the *CO* transcription factor in *A. hypochondriacus*. As shown in Table 1, the physicochemical analysis of the *AhypCOL* proteins in *A. hypochondriacus* revealed notable variations in their structural properties. The protein lengths ranged from 284 (*AhypCOL13*) to 495 amino acids (*AhypCOL04*), highlighting a diversity in

Table 2. Summary of the CO transcription factor family in other *Amaranthus* species.

Gene name	Locus name	Protein full length (aa)	Molecular weight (kDa)	pI	GRA-VY	Aliphatic index
<i>ApalCOL01</i>	-	354	38.29	4.55	-0.43	64.80
<i>ApalCOL02</i>	Apalm008716.1	299	33.38	4.13	-0.52	73.01
<i>ApalCOL03</i>	Apalm008959.1	488	54.33	6.16	-0.59	73.93
<i>ApalCOL04</i>	Apalm009074.1	481	53.61	6.49	-0.60	71.56
<i>ApalCOL05</i>	Apalm011240.1	429	48.18	5.63	-0.56	68.62
<i>ApalCOL06</i>	Apalm027054.1	376	42.19	5.29	-0.66	68.96
<i>ApalCOL07</i>	Apalm031030.1	394	45.05	5.29	-0.81	69.26
<i>ApalCOL08</i>	Apalm031052.1	376	41.06	5.38	-0.38	71.62
<i>ApalCOL09</i>	Apalm034219.1	371	41.24	5.44	-0.59	57.87
<i>ApalCOL10</i>	Apalm039160.1	362	39.64	5.73	-0.55	57.27
<i>ApalCOL11</i>	Apalm043787.1	377	41.86	5.11	-0.40	71.67
<i>ApalCOL12</i>	Apalm043796.1	384	44.18	5.16	-0.97	59.11
<i>ApalCOL13</i>	Apalm048372.1	295	33.65	5.36	-0.76	54.24
<i>AcrucOL01</i>	Acrue000328.1	410	44.84	5.26	-0.50	65.66
<i>AcrucOL02</i>	-	354	38.34	4.53	-0.44	66.16
<i>AcrucOL03</i>	Acrue005567.1	484	53.65	5.85	-0.55	74.36
<i>AcrucOL04</i>	Acrue005876.1	299	33.43	4.08	-0.52	73.65
<i>AcrucOL05</i>	Acrue010732.1	426	48.06	5.74	-0.66	66.81
<i>AcrucOL06</i>	Acrue024490.1	383	43.13	5.52	-0.65	69.74
<i>AcrucOL07</i>	Acrue025858.1	379	41.38	5.39	-0.34	72.35
<i>AcrucOL08</i>	Acrue025882.1	392	44.96	5.33	-0.82	66.12
<i>AcrucOL09</i>	Acrue029840.1	371	41.27	5.44	-0.59	57.60
<i>AcrucOL10</i>	Acrue034394.1	362	39.60	5.73	-0.53	58.09
<i>AcrucOL11</i>	Acrue038814.1	377	41.81	5.63	-0.41	70.40
<i>AcrucOL12</i>	Acrue038825.1	378	43.31	5.06	-0.94	60.05
<i>AcrucOL13</i>	Acrue043364.1	285	32.34	5.41	-0.67	62.67
<i>AhybCOL01</i>	Ahybri000228.1	410	44.84	5.26	-0.50	65.66
<i>AhybCOL02</i>	-	354	38.34	4.53	-0.44	66.16
<i>AhybCOL03</i>	Ahybri004189.1	318	35.73	4.35	-0.61	70.47
<i>AhybCOL04</i>	Ahybri004868.1	426	48.12	5.70	-0.65	67.72
<i>AhybCOL05</i>	Ahybri012553.1	383	43.17	5.45	-0.66	69.48
<i>AhybCOL06</i>	Ahybri014249.1	391	44.81	5.39	-0.81	66.55
<i>AhybCOL07</i>	Ahybrind2_09	377	41.48	5.61	-0.33	74.01
<i>AhybCOL08</i>	Ahybri016450.1	371	41.27	5.44	-0.59	57.60
<i>AhybCOL09</i>	Ahybri018927.1	362	39.60	5.73	-0.53	58.09
<i>AhybCOL10</i>	Ahybri021005.1	377	41.89	5.52	-0.41	70.40
<i>AhybCOL11</i>	Ahybri021008.1	378	43.43	5.15	-0.95	60.05
<i>AhybCOL12</i>	Ahybri022681.1	285	32.34	5.41	-0.68	62.67
<i>AtubCOL01</i>	Atube000282.1	410	44.90	5.42	-0.50	66.85
<i>AtubCOL02</i>	Atube001848.1	401	44.20	5.12	-0.59	63.04
<i>AtubCOL03</i>	Atube005520.1	318	35.81	4.38	-0.64	66.51
<i>AtubCOL04</i>	Atube005822.1	439	49.31	5.90	-0.60	74.19
<i>AtubCOL05</i>	Atube006401.1	426	47.91	5.84	-0.59	67.49
<i>AtubCOL06</i>	Atube018650.1	391	44.63	5.50	-0.76	70.03
<i>AtubCOL07</i>	Atube018664.1	376	41.08	5.66	-0.37	72.39
<i>AtubCOL08</i>	Atube021699.1	370	41.07	5.38	-0.58	57.76
<i>AtubCOL09</i>	Atube024664.1	362	39.69	5.72	-0.55	57.54
<i>AtubCOL10</i>	Atube027408.1	377	41.80	5.40	-0.42	69.05
<i>AtubCOL11</i>	Atube027414.1	387	44.26	5.18	-0.88	60.18
<i>AtubCOL12</i>	-	297	33.59	5.95	-0.69	61.75
<i>AtrCOL01</i>	LOC130806235	410	45.03	5.47	-0.45	70.41
<i>AtrCOL02</i>	LOC130805640	406	44.74	5.13	-0.57	62.76
<i>AtrCOL03</i>	LOC130820990	325	36.65	4.58	-0.69	67.45
<i>AtrCOL04</i>	LOC130820595	484	54.02	6.03	-0.59	73.55
<i>AtrCOL05</i>	LOC130808794	429	48.23	5.55	-0.56	72.26
<i>AtrCOL06</i>	LOC130812668	384	43.32	5.53	-0.74	65.49
<i>AtrCOL07</i>	LOC130814712	393	45.15	5.29	-0.81	64.94

Table 2. Contd.

Gene name	Locus name	Protein full length (aa)	Molecular weight (kDa)	pI	GRA-VY	Aliphatic index
<i>AtrCOL08</i>	LOC130814700	376	40.89	5.37	-0.34	71.62
<i>AtrCOL09</i>	LOC130804412	370	41.08	5.38	-0.58	57.76
<i>AtrCOL10</i>	LOC130803863	360	39.42	5.66	-0.56	55.69
<i>AtrCOL11</i>	LOC130824516	377	41.91	5.33	-0.43	66.50
<i>AtrCOL12</i>	LOC130824521	377	43.31	4.14	-0.90	58.17

Note: -: No information. Blank entries under the "Locus name" column indicate cases where locus information was not available in the corresponding genome annotations or databases at the time of analysis. The authors thank the reviewer for pointing this out. The blank rows in Table 2 indicate cases where locus information was not available in the reference genome or annotation databases at the time of analysis. The authors have rechecked the datasets to confirm this and have added a note in the table caption for clarification.

size that may correlate with functional specialisations among different CO family members. Correspondingly, the molecular weights of these AhypCOL proteins varied between 32.18 (AhypCOL13) and 55.03 kDa (AhypCOL04), which is consistent with the differences observed in their amino acid sequence lengths. The theoretical isoelectric points of the CO transcription factors were calculated to range from 4.35 (AhypCOL03) to 6.22 (AhypCOL04), indicating a spectrum of net charge distributions at physiological pH. Analysis of the aliphatic index showed values ranging from 57.76 (AhypCOL09) to 75.86 (AhypCOL04). The aliphatic index is a positive factor for the thermostability of globular proteins; thus, higher values imply greater stability across a range of temperatures. The grand average of hydropathicity values was found to be between -0.34 (AhypCOL08) and -0.95 (AhypCOL12). A negative grand average of hydropathicity values of the AhypCOL transcription factors in *A. hypochondriacus* indicated that these proteins were generally hydrophilic as previously described (Kyte & Doolittle 1982), which aligns with their expected localisation in the aqueous cellular environment and their involvement in protein-DNA and protein-protein interactions essential for transcriptional regulation.

Next, the physicochemical analysis of the CO transcription factor families in the five remaining *Amaranthus* species, including *A. palmeri*, *A. cruentus*, *A. hybridus*, *A. tuberculatus*, and *A. tricolor*, also exhibited remarkable property variations. The protein lengths of the CO transcription factors ranged from 295 (ApalCOL13) to 488 (ApalCOL03), 285 (AcrucOL13) to 484 (AcrucOL03), 285 (AhybCOL12) to 426 (AhybCOL04), 297 (AtubCOL12) to 439 (AtubCOL04), and 325 (AtrCOL03) to 484 (AtrCOL04) amino acids across the *Amaranthus* species, including *A. palmeri*, *A. cruentus*, *A. hybridus*, *A. tuberculatus*, and *A. tricolor*, respectively. Correspondingly, the molecular weights of these CO proteins varied between 33.38 (ApalCOL02) and 54.33 (ApalCOL03) kDa in *A. palmeri*, 32.34 (AcrucOL13) and 53.65 (AcrucOL03) kDa in *A. cruentus*, 32.34 (AhybCOL12) and 48.12 (AhybCOL04) kDa in *A. hybridus*, 33.59 (AtubCOL12) and 49.31 (AtubCOL04) kDa in *A. tuberculatus*, and 36.65 (AtrCOL03) and 54.02 (AtrCOL04) kDa in *A. tricolor*. Interestingly, the theoretical isoelectric points of all CO transcription factors in the five remaining *Amaranthus* species were less than 7.0 (acidic), ranging from 4.13 (ApalCOL02) to 6.49 (ApalCOL04) in *A. palmeri*, 4.08 (AcrucOL04) to 5.85 (AcrucOL03) in *A. cruentus*, 4.35 (AhybCOL03) to 5.73 (AhybCOL09) in *A. hybridus*, 4.38 (AtubCOL03) to 5.95 (AtubCOL12) in *A. tuberculatus*, and 4.14 (AtrCOL12) to 6.03 (AtrCOL04) in *A. tricolor*. The grand average of hydropathicity values of all CO transcription factors in *A. palmeri*, *A. cruentus*, *A. hybridus*, *A. tuberculatus*, and *A. tricolor* was found to be negative. This finding indicates that these proteins are generally hydrophilic. Additionally, analysis of the aliphatic index revealed values ranging from 54.24 to 73.93 in the ApalCOL transcription factor family in *A. palmeri*, 57.60 to 74.36 in the

Acrucol transcription factor family in *A. cruentus*, 57.60 to 74.01 in the AhybCOL transcription factor family in *A. hybridus*, 57.54 to 74.19 in the AtubCOL transcription factor family in *A. tuberculatus*, and 55.69 to 73.55 in the AtriCOL transcription factor family in *A. tricolor*. The aliphatic index reflects the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine) and is considered a positive indicator of protein thermostability. Proteins with higher aliphatic index values are generally more stable at elevated temperatures and under extreme environmental conditions (Ikai 1980). This suggests that CO transcription factors with higher aliphatic index values may maintain functional integrity under stress. Although there is no strict universal threshold, aliphatic index values above 70 are often associated with enhanced thermostability in plant proteins (Ikai 1980).

The characteristics of the CO transcription factor families in higher plant species have also been investigated previously. For example, the CO transcription factor family in mango exhibited sequence lengths ranging from 492 to 1,536 base pairs, resulting in proteins composed of 163 to 511 amino acids (Liu et al. 2022). These proteins have molecular weights between 18.11 and 56.63 kDa and predicted isoelectric points from 3.93 to 8.85 (Liu et al. 2022). The predicted open reading frames of the CO genes in the grapevine ranged from approximately 1,044 bp for VviCOL4 to 1,425 bp for VviCOL14b (Wang et al. 2019). The corresponding polypeptides varied in length from 347 to 474 amino acids, resulting in calculated molecular masses between approximately 38.00 and 51.43 kDa (Wang et al. 2019). In the case of *C. lavandulifolium*, the CO transcription factor family sizes have been reported to range from 337 to 434 amino acid residues (Fu et al. 2015). Additionally, the protein size of the CO transcription factor family in Chinese white pear ranged from 340 and 488 amino acid residues (Wang et al. 2017), and from 253 to 575 amino acid residues in quinoa (Tran et al. 2025). In summary, the physicochemical properties of the CO transcription factors, including protein length, molecular weight, isoelectric point, aliphatic index, and grand average of hydropathicity values, provide valuable insights into their structural stability and functional potential. These parameters lay the groundwork for further structural and functional analyses, contributing to a deeper understanding of how CO proteins regulate flowering time in response to photoperiodic signals.

Classification of the CO transcription factor family in the Amaranthaceae family

To assess the relationships of CO transcription factors in the Amaranthaceae family, an unrooted phylogenetic tree of the CO proteins from six *Amaranthus* species and well-characterised CO proteins from *A. thaliana* (Griffiths et al. 2003) and sugar beet (Chia et al. 2008) was successfully constructed. As a result, Figure 2 presents the Maximum Likelihood-based phylogenetic tree of all members of the CO transcription factor families in the Amaranthaceae family and relatives.

All members of the CO transcription factor families isolated from six *Amaranthus* species were categorised into three groups through phylogenetic analysis comparing CO proteins from *A. thaliana* (Griffiths et al. 2003) and sugar beet (Chia et al. 2008). Particularly, a total of twenty-nine members of the CO transcription factor families in the Amaranthaceae family were placed in Group I, with three members in each CO transcription factor family in six *Amaranthus* species, including AhybCOL07, AhybCOL09, AhybCOL10 from *A. hybridus*, AhypCOL08, AhypCOL10, AhypCOL11 from *A. hypochondriacus*, Acrucol07, Acrucol10, Acrucol11 from *A. cruentus*, ApalCOL08, ApalCOL10, ApalCOL11 from *A. palmeri*, AtubCOL07, AtubCOL09, AtubCOL10 from *A. tuberculatus*, and AtriCOL08, AtriCOL10, AtriCOL11 from *A.*

tricolor, assigned to subgroup Ia. Meanwhile, eleven members of the CO transcription factor families in six *Amaranthus* species, particularly AhubCOL08, AhybCOL12 from *A. hybridus*, AhypCOL09, AhypCOL13 from *A. hypochondriacus*, AcruCOL09, AcruCOL13 from *A. cruentus*, ApalCOL09, ApalCOL13 from *A. palmeri*, AtubCOL08, AtubCOL12 from *A. tuberculatus*, and AtriCOL09 from *A. tricolor*, were positioned in subgroup Ib. Next, two members in each CO transcription factor family in six *Amaranthus* species, including AhybCOL06 and AhybCOL11, AhypCOL07 and AhypCOL12, AcruCOL08 and AcruCOL12, ApalCOL07 and ApalCOL12, AtubCOL06 and AtubCOL11, and AtriCOL07 and AtriCOL12 from *A. hybridus*, *A. hypochondriacus*, *A. cruentus*, *A. palmeri*, *A. tuberculatus*, and *A. tricolor*, respectively, were grouped in group II. Finally, the remaining thirty-four members of the CO transcription factor families in *A. hybridus*, *A. hypochondriacus*, *A. cruentus*, *A. palmeri*, *A. tuberculatus*, and *A. tricolor* were classified under group III.

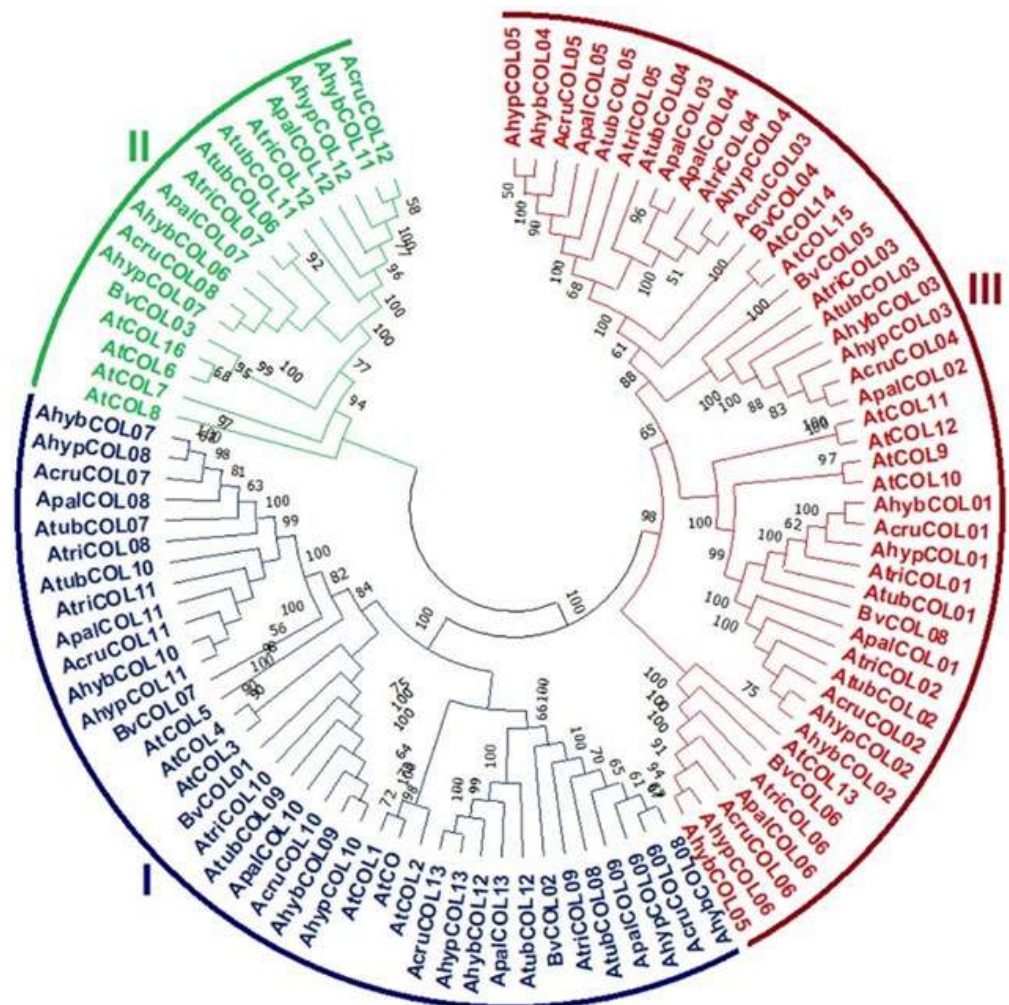


Figure 2. Categorization of the CO transcription factor families in the Amaranthaceae family.

Previously, the classification of the CO transcription factor families in higher plant species was reported. The twelve CO genes isolated from grapevine were categorized into three groups through phylogenetic analysis (Wang et al. 2019). For example, three members, including VviCOL2, VviCOL4, and VviCOL5, were placed in group I, while VviCOL16a and VviCOL16b were classified under group II, and VviCOL9a, VviCOL9b, VviCOL11a, VviCOL11b, VviCOL13, VviCOL14a, and VviCOL14b were grouped in group III (Wang et al. 2019). Furthermore, to investigate the evolutionary relationships among CO proteins, a phylogenetic tree was constructed using sixty members of the CO transcription factor families from *A.*

thaliana, grapevine, and mango (Liu et al. 2022). As expected, these CO proteins were clearly divided into three distinct clades, which largely reflected their structural differences (Liu et al. 2022). This classification has been reported to be similar to that of the CO transcription factor families in other crop species, such as barley (Griffiths et al. 2003), rice (Griffiths et al. 2003), Chinese white pear (Wang et al. 2017), *C. lavandulifolium* (Fu et al. 2015), ginkgo (Yan et al. 2017), and quinoa (Tran et al. 2025).

Gene structure analysis of the CO transcription factor family in *Amaranthus hypochondriacus*

The gene structure of the CO transcription factor family in *A. hypochondriacus* was analysed to investigate the exon-intron organization and potential structural variations among the gene family members. Using the GSDS tool (Hu et al. 2015), full-length genomic sequences were aligned with their corresponding coding sequences to identify the arrangement of exons and introns. The results revealed significant variation in the number of exons among different CO genes in *A. hypochondriacus* (Figure 3)

The gene structure analysis showed that some *AhypCOL* genes possessed multiple exons. In contrast, only one member of the CO gene family, namely *AhypCOL13*, had no introns, reflecting the evolutionary divergence within the CO gene family in *A. hypochondriacus*. This could be a genuine biological feature, as intronless genes are commonly found in plant genomes and are often associated with gene duplication events, such as retrotransposition or intron loss through evolution (Chen et al. 2023). For instance, CO genes in closely related clades of the phylogenetic tree exhibited similar exon-intron patterns, indicating possible conservation of gene structure in evolutionarily related CO family members in *A. hypochondriacus*. Conversely, variations in exon-intron distribution in more distantly related genes could indicate functional diversification across different CO family members in *A. hypochondriacus*. In particular, the number of exons in the CO genes in *A. hypochondriacus* ranged from one (*AhypCOL13*) to five (*AhypCOL04*). Among them, a large number of the CO genes in *A. hypochondriacus*, particularly six out of thirteen members, contained two exons, while five (out of thirteen) members of the CO genes in *A. hypochondriacus* had four exons. These findings highlight the structural diversity within the CO transcription factor family in *A. hypochondriacus*, which may contribute to the regulation of photoperiodic flowering in response to environmental cues.

Recently, the genomic organisation of the thirty CO genes in mango has shown significant variation (Liu et al. 2022). Specifically, three *MiCOL* genes had four introns, eight contained three introns, nine had two introns, ten possessed only one intron, and *MiCOL11* was entirely devoid of introns (Liu et al. 2022). Regarding the CO genes in grapevine, *VviCOL9a*, *VviCOL11a*, *Vvi-*

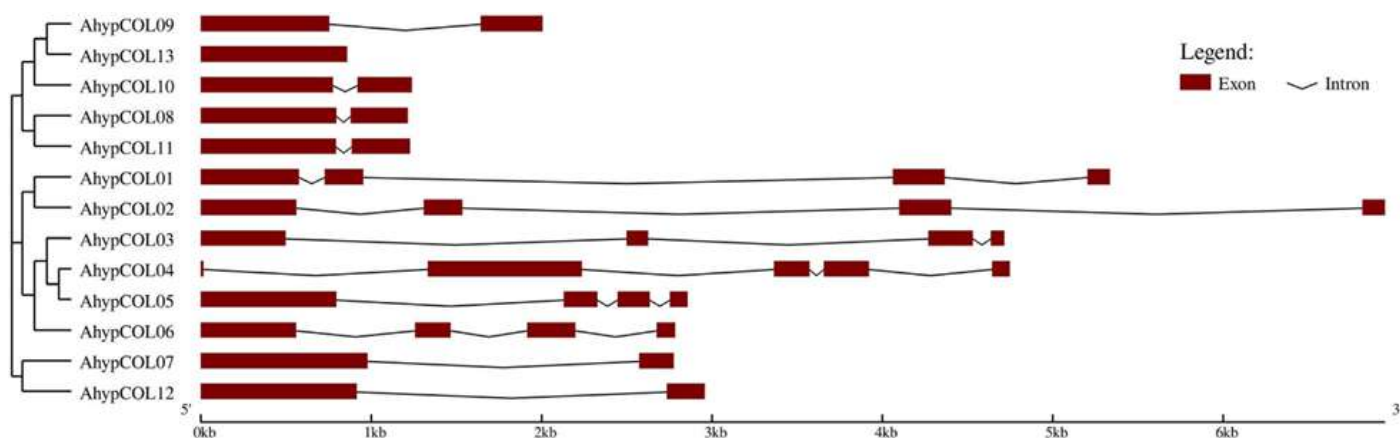


Figure 3. Determination of gene structure of the CO transcription factor family in *Amaranthus hypochondriacus*.

COL11b, *VviCOL13*, *VviCOL14a*, and *VviCOL14b* each possessed three introns. In contrast, *VviCOL2*, *VviCOL4*, *VviCOL5*, *VviCOL16a*, and *VviCOL16b* were found to contain only one intron (Wang et al. 2019). Taken together, these structural differences highlight the diversity within the CO gene family in *A. hypochondriacus* and possibly in other crop species.

Expression patterns analysis of the CO transcription factor family in the Amaranthaceae family

As a main part of this study, we investigated the expression patterns of the CO gene family in several species belonging to the Amaranthaceae family. As a result, four RNA-Seq datasets from *A. hypochondriacus*, *A. palmeri*, and *A. tuberculatus* were analysed to assess the expression patterns of the CO gene family in major tissues under various conditions. Figures 4 and 5 described the expression patterns of the CO gene family in *A. hypochondriacus*, *A. palmeri*, and *A. tuberculatus*.

In *A. hypochondriacus*, the expression patterns of the *AhypCOL* gene family varied across major organs during the growth and development of plants. We found that three CO genes, including *AhypCOL09*, *AhypCOL10*, and *AhypCOL11*, were highly expressed in leaf tissues, while only *AhypCOL10* was exclusively expressed in stem and floral samples (Figure 4A). Two CO genes, *AhypCOL09* and *AhypCOL10*, were up-regulated in drought-treated tissues. Furthermore, it has been reported that *AhypCOL09* was mainly expressed in stem, root, flower, and mature seed samples, while *AhypCOL10* was exclusively expressed in the main organs, such as leaf, stem, root, flower, and mature seed (Figure 4B).

In *A. palmeri*, seven (out of thirteen) members of the CO gene family were detected in their expression levels in glufosinate-treated plants. In particular, three CO genes, including *ApalCOL03*, *ApalCOL07*, and *ApalCOL11*, were not altered in both glufosinate-sensitive and glufosinate-resistant plants under treatments (Figure 5A). Two CO genes, including *ApalCOL09* and *ApalCOL10*, were induced in glufosinate-sensitive and/or glufosinate-resistant plants under treatments. *ApalCOL13* expression decreased in glufosinate-resistant plants (~ -2.12 -fold), whereas *ApalCOL04* showed contrasting expression levels in glufosinate-sensitive and glufosinate-resistant plants by ~ -1.98 -fold and 2.41-fold, respectively. In *A. tuberculatus*, most CO genes (eleven out of twelve) exhibited their interesting expression patterns in leaves at 3, 6, 12, and 24 hours after mesotrione treatment (Figure 5B). For example, four CO genes, including *AtubCOL01*, *AtubCOL02*, *AtubCOL03*, and

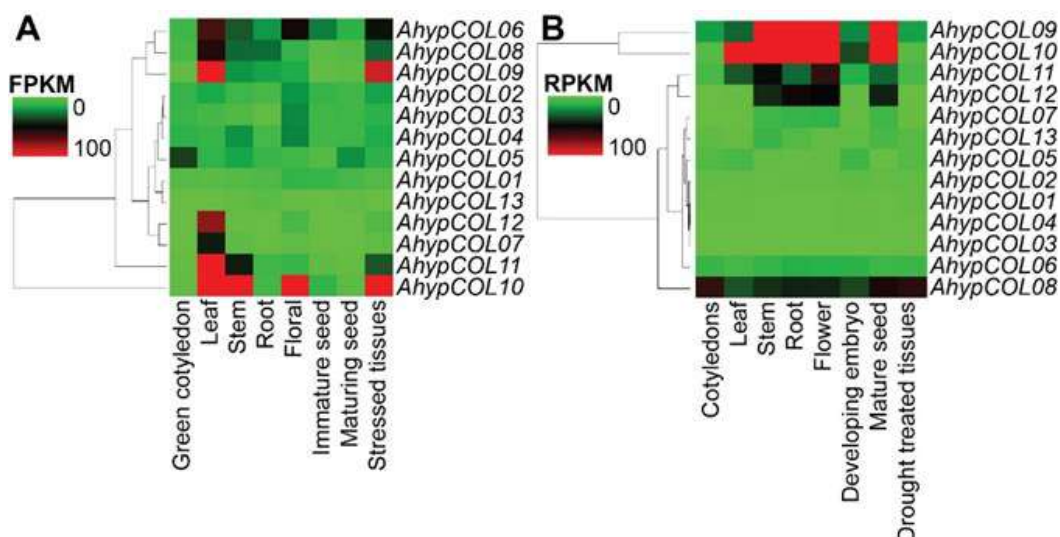


Figure 4. Expression patterns of the CO gene family in *Amaranthus hypochondriacus* based on (A) Phytozome and (B) AGRDB databases.

AtubCOL04, were down-regulated in leaves after three hours of treatment but were up-regulated in these examined tissues after 6 and/or 12 and/or 24 hours of treatment. Interestingly, *AtubCOL05* and two other *CO* genes, *AtubCOL08* and *AtubCOL09*, were induced and reduced in leaves under the treatments, respectively. Meanwhile, three *CO* genes, including *AtubCOL06*, *AtubCOL10*, and *AtubCOL11*, were up-regulated in leaves after 3 and/or 6 hours of treatment but down-regulated in these tissues after 12 and/or 24 hours. Taken together, our reanalysis of RNA-Seq datasets revealed that the *CO* gene family in three *Amaranthus* species was differentially expressed in various organs during the growth and development.

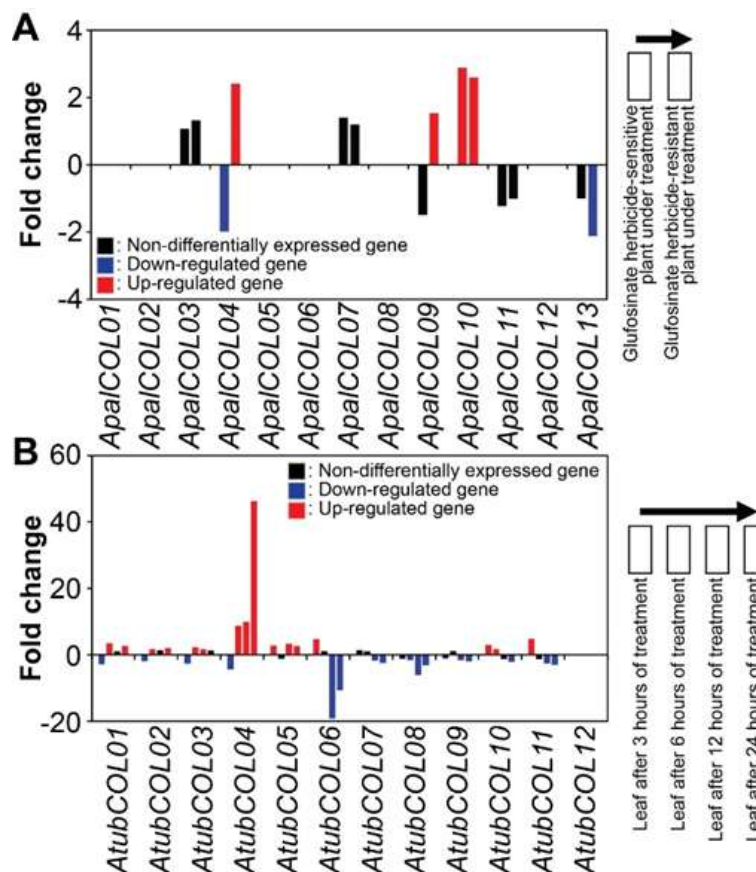


Figure 5. Expression patterns of the *CO* gene families in (A) *Amaranthus palmeri* and (B) *A. tuberculatus*.

CONCLUSION

In conclusion, the comprehensive genome-wide identification, structural analysis, and expression profiling of the CONSTANS (*CO*) transcription factor family in the *Amaranthaceae* family have provided significant insights into the genetic and functional diversity of these genes. The identification of twelve to thirteen *CO* genes across six *Amaranthus* species revealed their chromosomal localisation and structural diversity, with varying exon-intron arrangements suggesting functional diversification. Physicochemical analyses further highlighted structural differences, including variations in protein length, molecular weight, and isoelectric points. Phylogenetic analysis classified the *CO* transcription factor family into three major groups, aligning with known *CO* proteins from *A. thaliana* and sugar beet, thereby offering a framework for understanding evolutionary relationships within the *CO* gene family. Gene expression analysis across multiple RNA-Seq datasets demonstrated tissue-specific and stress-responsive regulation of *CO* genes, with certain members highly expressed under environmental stresses such as drought and herbicide treatments. This study could enhance our understanding of the role of *CO* transcription factors in regulating photoperiodic flowering and stress

responses in *Amaranthus* species, thereby contributing to future agricultural applications and crop improvement strategies.

AUTHOR CONTRIBUTION

X.D.V. designed the research, collected and analysed data, and prepared the first manuscript draft. P.B.C. co-designed the research, collected and analysed data, edited the manuscript, and supervised the study. Q.P.N., T.T.T.H., D.D.N., and Q.T.N.L. contributed to data collection, with T.T.T.H., D.D.N., and Q.T.N.L. also involved in data analysis. H.V.L. contributed to research design, data collection, and analysis. H.D.C. co-designed the research, collected and analysed data, edited the manuscript, and supervised the study

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CONFLICT OF INTEREST

The authors declare no conflicts of interest regarding the research or funding.

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Research Article

Relationship between Percentage of Live Coral Cover and Chaetodontidae Reef Fish in the Tejakula Waters of Buleleng Regency, Bali

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ABSTRACT

Tejakula District is one of the tourism development areas in Buleleng Regency, Bali, based on the condition of the waters, which are classified as good. The increasing population in this location has caused increased exploitation of coastal and beach areas, especially in coral reef areas. The coral covers in the Tejakula utilisation area has been damaged due to natural and human factors. This study aims to obtain information on the percentage of living coral reefs, the abundance of Chaetodontidae fish, and their relationships. The Underwater Photo Transect (UPT) method was used for coral reef data collection, and Chaetodontidae fish data were collected using the Underwater Visual Census (UVC). The CPCE 4.0 application was used for coral reef data processing. The Chaetodontidae fish data were calculated based on the number of individuals, and then identified the type of genus. The relationship between the percentage of coral cover and the abundance of Chaetodontidae fish was analysed using the Pearson correlation. Almost all stations in Tejakula Waters had weak category coral cover. The results on the abundance of Chaetodontidae fish found five genera: *Chaetodon*, *Parachaetodon*, *Heniochus*, *Forcipiger* and *Hemitaurys*. The relationship between the percentage of cover and fish in Tejakula Waters was in the weak category with $r = 0.091$. Supervision, monitoring, and attention to activities around Tejakula Waters are needed to ensure the coral reef ecosystem remains well maintained.

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INTRODUCTION

Indonesia is a country with several regions capable of developing in the tourism sector, one of which is the island of Bali, which is known as Indonesia's main tourist destination. Currently, tourism development in Bali is mostly focused on the southern regions such as Denpasar, Nusa Penida and Kuta (Jubaedah & Anas 2019). This is rarely carried out in the northern part, such as Tejakula District in Buleleng Regency. Astronomically, Tejakula sub-district is located at 8°56'LS - 115°8'BT with a sub-district area of 97.68 km². The entire area of this sub-district is divided into ten villages within Tejakula (Wisnawa 2014). In general, Tejakula waters are relatively healthy and support abundant marine biota in the coral reef area. The coral cover in the Tejakula utilisation area consists of fringing coral reefs and coral clusters or plates that grow around 50 to 100 metres above the tidal line. Coral grows on the bottom of fine black sand and coral fragments at a depth of 4 to 30 metres (Wisnawa 2014).

Coral reefs are ocean floor structures consisting of calcium carbonate (CaCO₃) deposits, produced by coral animals in symbiosis with lime-producing algae. Coral reefs have high ecological and economic functions (Falah et al. 2020). Coral reef ecosystems exhibit high biological productivity and provide sources of food, medicine, and construction materials. Ecologically, coral reefs serve as habitats, spawning grounds, nurseries, shelters for marine biota, and natural wave breakers. Recognising the enormous role and function of reefs for marine ecosystems, data and information as well as the condition of coral reefs in an area are very important to know and have for the benefit of sustainable management of coral reefs (Fahmi et al. 2018).

Reef fish are among the most numerous organisms associated with coral reefs and are found in all reef habitats. The lives of reef fishes are very dependent on the condition of coral reefs; if coral reefs are damaged, they lose their habitat (Mujiyanto et al. 2020). Reef fish are one of the leading fisheries commodities (Falah et al. 2020), so it is necessary to monitor them so that fish stocks in nature can be maintained sustainably (Mandolang et al. 2021). Reef fish monitoring aims to track fish populations and their relationship with reef health (Bintoro et al. 2023). Their presence makes coral reef ecosystems the most inhabited by aquatic biota. This high diversity is due to the variation in the main reef types, all of which are inhabited by coral fish species (Rembet et al. 2011; Sudarmaji & Efendy 2021)

Coral mining activities, fishing with toxic materials and explosives, the use of non-selective fishing gear and pollution that occurs in the sea anywhere on land are the main problems causing coral reef degradation (Titaheluw et al. 2015). The influence of changes in environmental quality due to resource exploitation activities in coral reef ecosystems can be identified by looking at physical, chemical and biological indicators (Jubaedah & Anas 2019). Biological indicators of changes in coral reef ecosystems included the presence of Chaetodontidae fish (Bintoro et al. 2023). Damage to coral reefs directly impacts reef fish, particularly those that depend on coral polyps as food (Rembet et al. 2018). One form of association between fish and coral that can be seen is coral-eating fish (corallivores) such as those from the families Chaetodontidae, Balistidae, and Tetraodontidae (Reese 1981).

Chaetodontidae, or butterflyfishes, characterised by colourful, lined body patterns and are a common coral reef family consisting of 120 species, typically found in pairs or small groups. Butterflyfishes are active during the day and hide in shelters close to the coral surface at night. Butterflyfishes are divided into 10 genera; 78 % feed on coral polyps, while others feed on small invertebrates, algae, or plankton (Mujiyanto et al. 2020). Butterflyfishes are indicator species, which reflect the health of coral reef ecosystems (Riansyah et al. 2018). Coral reefs greatly influence butterflyfish life by providing both shelter from predators and a food source. Reese (1981) stated that butterfly-

fishes can be used as indicators of the health of coral reef ecosystems because they like certain types of substrate which can describe the condition of coral reefs.

According to Citra and Purwanto (2022), although activities in the north of Bali are not as dense as in the south, Tejakula is a marine fisheries sector with a livelihood as fishermen and a tourism sector because its people depend on the potential of the coastal area supported by data from fishermen in Buleleng Regency, the most common were found in Tejakula District, as many as 1590 people in 2019. The population increase in coastal areas, particularly in Tejakula Waters, has led to greater exploitation and utilisation of resources, especially aquatic products such as coral reefs (Citra & Purwanto 2022). Resource exploitation is often conducted in environmentally unfriendly ways, placing pressure on coral reef resources. Some activities that are destructive to these resources come from destructive tourism, cultivation and fishing activities (destructive fishing) using cyanide poison. Water pollution from domestic and industrial waste also contributes (Moir et al. 2020). Some of the coral reefs in Tejakula Waters have degraded, leading to coral reefs destruction and decreased live coral cover. This directly impacts fish resources by reducing available space, shelter, and food, thereby affecting fish species closely associated with coral reefs. Therefore, this research aims to determine the relationship between the percentage of live coral cover and the abundance of fish in the Chaetodontidae in Tejakula Waters, Buleleng, Bali, using the Underwater Visual Census (UVC) method.

MATERIALS AND METHODS

Research Station

This research was conducted in December 2022 in Tejakula Waters, Buleleng, Bali, at six locations using the purposive sampling. Sampling is based on considerations or criteria required when collecting data. The determined locations were six stations: Bondalem 1, Bondalem 2, Tejakula, Tembok, Penutukan and Pacung (Figure 1).

Data Collection

Benthic Survey

Benthic cover data collection in this study were collected using the Underwater Photo Transect (UPT). Data collection used a 100-metre roll metre laid at a depth of 6–8 metres and conducted at each of the six predetermined station points. A rectangular frame measuring 44 × 58 cm was placed at each metre along the coordinate point. Photographs were taken using an underwater camera along the transect line, the photos were taken perpendicularly with a distance of 60 cm from the top of the frame starting from the 1st to the 100th metre with an interval of 1-metre between photos. Photographs with odd numbers were taken on the left side of the transect line and even numbers were on the right side of the transect (Figure 2) (Daud et al. 2021). The following illustration shows how to collect data in Figure 2.

Butterflyfishes Survey

The Underwater Visual Census (UVC) method was used to collect butterflyfishes data (English et al. 1997). Fish data were collected by drawing a 100 m transect line parallel to the edge of the coastline, then reef fish data were recorded by diving following the transect line that had been installed around the coral parallel to the coastline. Starting at one end of the transect, then dive and observe on the side of the transect 2.5 m to the right and 2.5 m to the left and 5 m above the transect until the next 5 m. Butterflyfishes were identified to genus level and recorded around coral reefs. Observations were repeated once to minimise data collection error (Figure 3) (Utomo et al. 2013). Data

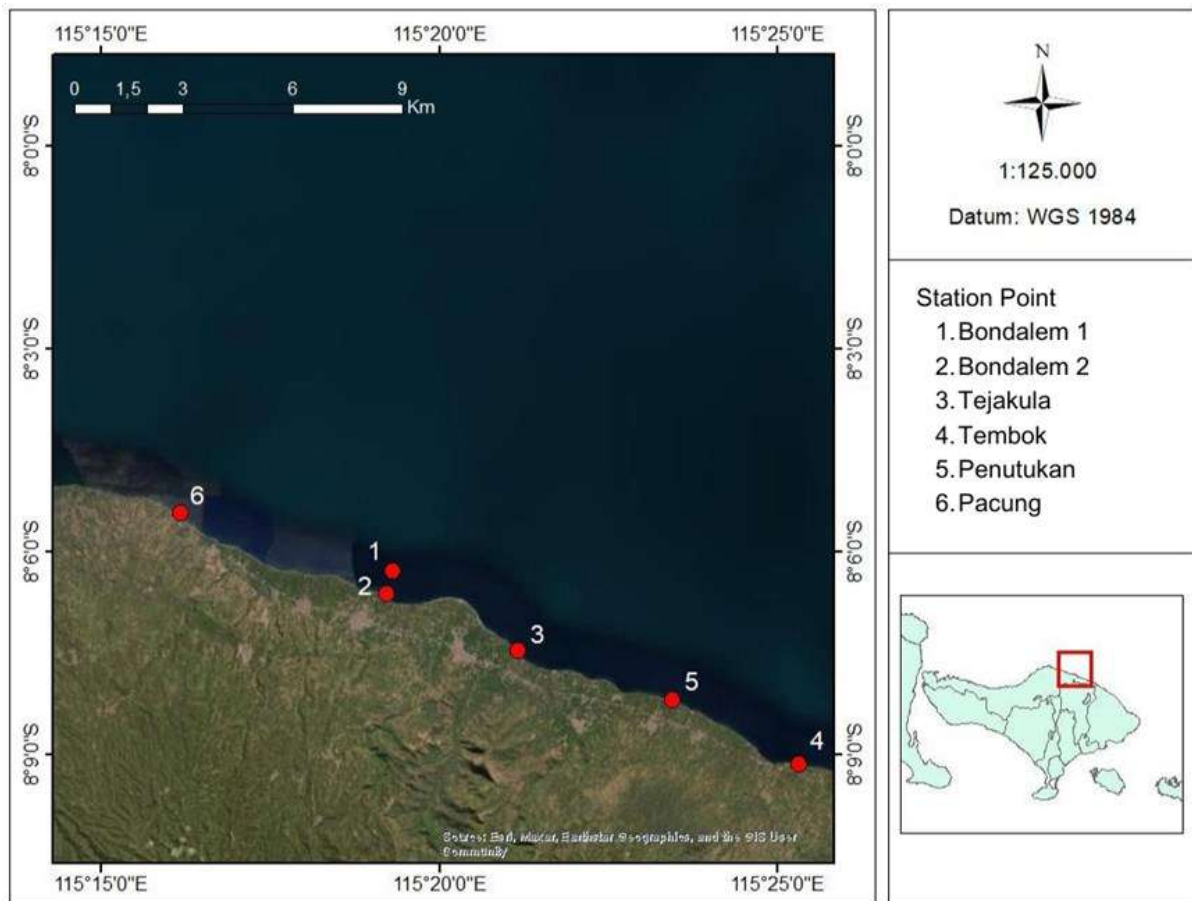


Figure 1. Research Location Map.



Figure 2. Placement frame which is marked with a red box with dimensions of 44 cm x 58 cm on roll meter 100 m long is used for Underwater Photo Transect (UPT) in coral reef ecosystems and Photos of the transects from the right and left sides.

collection was repeated four times, with repetitions every 25 metres along each transect. This repetition was conducted to minimise errors in data collection and to assess the number of fish associated with coral reefs.

Data Analysis

Percentage Analysis of Coral Cover (%)

The percentage of coral reef benthic cover was analysed using the basic category mode in CPCe software with 30 random points. The coral cover data obtained from the above calculation were then categorised according to the Coral Reef Health Index in Indonesia (Table 1).

Table 1. Coral reef condition categories (Giyanto et al. 2023).

Live Coral Cover	Criteria
>35 %	High
19-35 %	Moderate
<19 %	Low

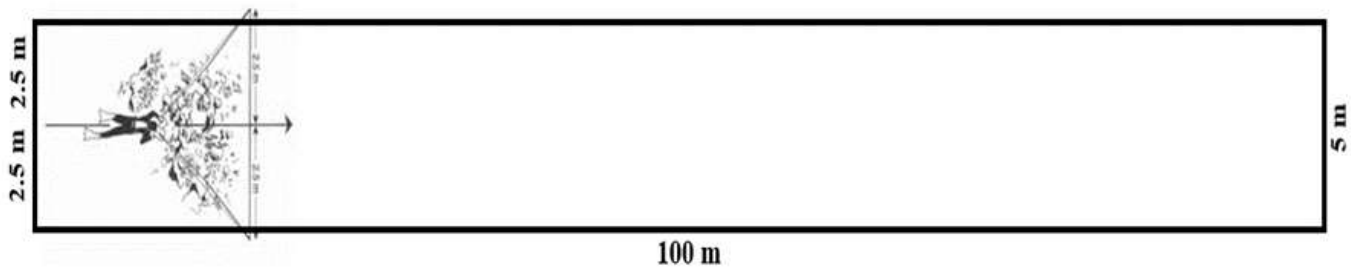


Figure 3. Reef fish data were collected using the Underwater Visual Census (UVC) method by drawing a 100 m transect line then recording the data and observing 2.5 m of the transect side to the right and left at a height of 5 m above (English et al. 1997).

Abundance of Butterflyfishes

The abundance of each type of fish at the research station was described by looking at the composition and abundance of the species. Species abundance was defined as the number of individuals of one species per meter quadrant in each research station. Abundance of butterflyfishes through visual census data collection 100 m long, 5 m wide ($100 \times 5 = 500 \text{ m}^2$).

Analysis of the Relationship between the Abundance of Butterflyfishes and the Percentage of Live Coral Cover

The relationship between butterflyfish abundance and coral cover percentage was analysed using Pearson correlation. The strength of the relationship between x and y values can be seen from the correlation coefficient (r) value which ranges from (-1) to (+1). The closer to 1 or -1, the stronger the relationship between the two variables (Table 2).

Table 2. Pearson correlation relationship categories.

r	Interpretation
0	No Correlation
0.01-0.20	Very weak correlation
0.21-0.40	Weak
0.41-0.60	Somewhat Weak
0.61-0.80	Sufficient
0.81-0.99	High
1	Very high

RESULTS AND DISCUSSION

Condition of Coral Cover in Tejakula Waters

This research was conducted at six research locations in Tejakula Waters, which had the following benthic cover (Figure 4). It can be seen that the most common benthic cover found in Tejakula Waters is rock, sand, rubble and dead coral.

The characteristics of the water substrate in Tejakula District are in the form of coral reefs, coral fractures, sand dominated by black sand, and muddy (Prasetia et al. 2023). Coral reef health may be directly or indirectly impacted by changes in water quality. At Bondalem 1 and Bondalem 2 stations, more rocks were found than hard corals, which fall into the low category, due to coral damage that resulted in rocks dominance (Figure 4). Rock includes large dead coral heads and solidified coral pieces (Duckworth & Wolff 2011). The damage to the coral is thought to be due to tourism activities in the coastal area and the decline in water quality in Bondalem (Citra & Purwanto 2022). According to Wibawa and Luthfi (2017), pollution from land will subsequently alter the water quality, potentially harming coral reefs.

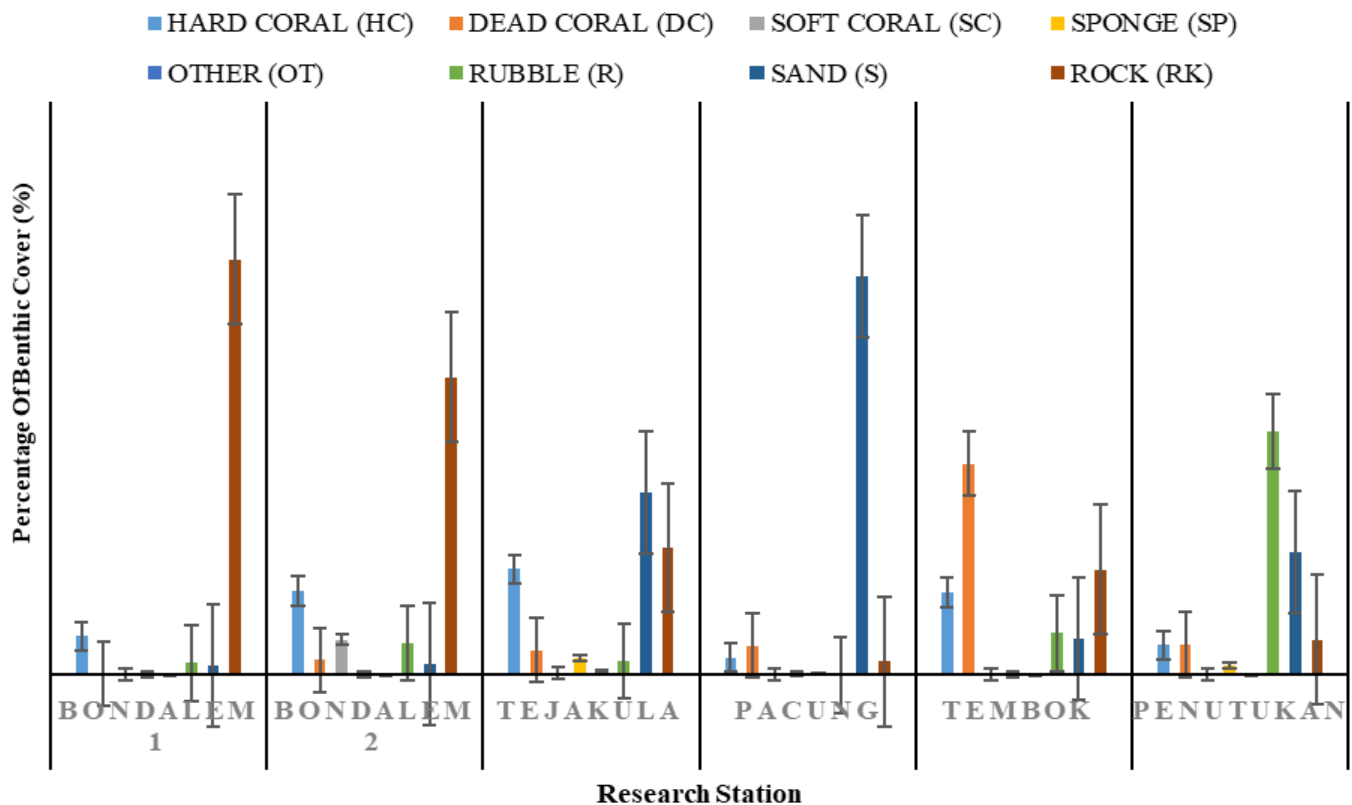


Figure 4. Amount of benthic cover in Tejakula Waters.

Sand at Pacung station was another typical benthic cover. This is due to high sedimentation caused by heavy ship activity, as most local people work as fishermen (Citra & Purwanto 2022). This is in accordance with Maharani et al. (2024), who stated that sedimentation on coral reefs can increase sand accumulation and reduce coral cover. According to Supriyadi et al. (2017), currents have the ability to influence sedimentation by carrying sediment at the water's bottom or on the surface.

There was a lot of rubble at Penutukan station, which is considered to be the result of coral reefs being directly harmed by human activity; the area is frequently passed by fishing boats and has intense community activities. Citra and Purwanto (2022) stated that Penutukan has fisheries commodities such as fish and squid, while tourism services include enjoying dolphin attractions, fishing, diving and snorkelling which can break the surrounding coral. Human activities such as fishing, gathering ornamental fish, using explosives, non-environmentally friendly gear, and unsustainable harvesting can all directly or indirectly harm coral reefs (Maharani et al. 2024).

Other than that, Tembok station had the most dead coral. Destructive human activities include diving, and the use of potassium for fishing. Coral reef utilisation activities by the Tembok community, such as ornamental fish and coral reef farming by coastal fishermen, are quite high (Citra & Purwanto 2022). If resource the extraction is not environmentally friendly, it can result in the death coral reefs in the waters near the Tembok station. In line with Anwar et al. (2014), there is a significant amount of anthropogenic (caused by human activity) harm and deterioration to coral reef ecosystems. Environmentally harmful resource extraction, such as the use of toxic compounds in fishing, is the cause of this damage.

The results of observations on the condition of live coral cover in Tejakula Waters, Buleleng, Bali are included in the low category with an average of 12.52 % (Figure 5).

Based on the six stations, Tejakula station has moderate coral cover conditions (22.14 %), while the others have low conditions (3.7-17.65 %)

(Figure 5). Although Tejakula is the most optimal location for utilizing coastal resources compared to other stations, the people of this village care about protecting their marine areas because it can affect the community's economy and regional development (Citra & Purwanto 2022). Tejakula station has unique potentials and appeal for domestic and international tourists. The planting of coral reefs in the Tejakula area has an important role in maintaining the marine ecosystem. This awareness can maintain the condition of coral reefs compared to other stations. Jubaedah and Anas (2019) attest to the fact that Bali's coral reef ecosystem is currently being conserved and managed as best it can by the local community, which helps the ecosystem grow.

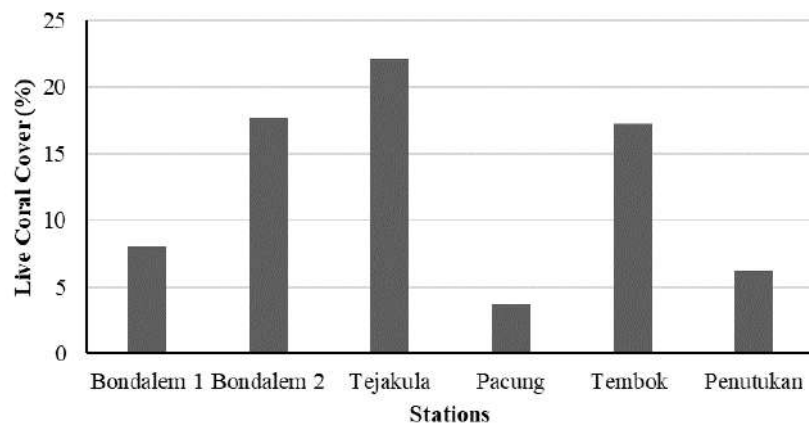


Figure 5. Percentage of live coral cover in Tejakula Waters, Buleleng Bali.

Abundance of Butterflyfishes

Based on the results of observations of butterflyfishes at six stations in Tejakula Waters, the abundance values at all stations were relatively low (Table 3). There were five genera, including *Chaetodon*, *Forcipiger*, *Heniochus*, *Parachaetodon* and *Hemitaurichthys*. The average distribution of Butterflyfishes species in Tejakula waters is 0.092 (ind m⁻²), found at each station can be seen in Figure 6.

Despite being present at every research station, the *Chaetodon* genus was most abundant at Tembok Station (0.136 ind m⁻²) and Penutukan (0.134 ind m⁻²). Even though the coral conditions at these locations were relatively poor, *Chaetodon* could still be found. The genus *Chaetodon*, known as the largest genus in Chaetodontidae, is a type of butterflyfish commonly found on coral and rocky reefs in tropical and subtropical ocean regions (Rondonuwu et al. 2017). In addition, some special species of *Chaetodon* can be found in damaged coral reef ecosystems. The presence of this genus can indicate dependence on the health of coral reefs (Devanya et al. 2022).

Additionally, this results in varying abundance at each station, which may correspond to the amount of live coral cover (Riansyah et al. 2018). The abundance of reef fish is not only influenced by the percentage of coral cover. Different reef contours, such as caves or walls, can support greater reef fish

Table 3. Abundance of Butterflyfishes.

Research Stations	Abundance (ind m ⁻²)				
	<i>Chaetodon</i>	<i>Forcipiger</i>	<i>Heniochus</i>	<i>Parachaetodon</i>	<i>Hemitaurtys</i>
Bondalem 1	0.030			0.006	
Bondalem 2	0.052		0.004	0.002	
Tejakula	0.072		0.004		
Pacung	0.038	0.004	0.002		
Tembok	0.136	0.014	0.028		0.004
Penutukan	0.134		0.020		

diversity. The abundance of reef fish is also influenced by biotic and abiotic factors. Biotic factors that can influence this include competition, predation, and the distribution of reef fish. Meanwhile, abiotic factors that influence such as current, depth and brightness. The complexity of coral reef ecosystems also influences fish abundance, including substrate variables, biodiversity, and oceanographic factors (Erdana et al. 2022).

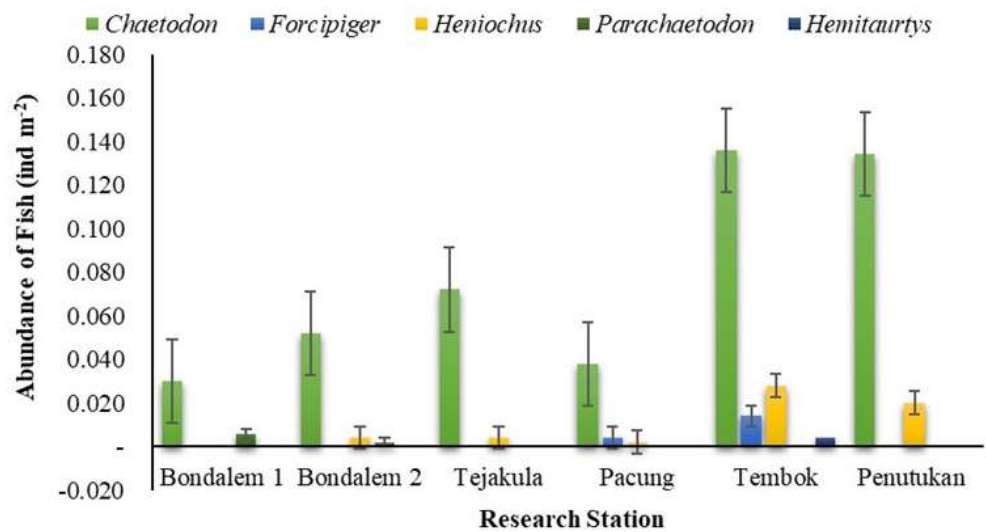


Figure 6. Abundance of butterflyfishes in Tejakula Waters, Buleleng Bali.

Relationship between Percentage of Live Coral Cover and Abundance of Butterflyfishes in Tejakula Waters, Buleleng Regency, Bali

Figure 5 and Figure 6 shows the findings from observations at six different locations in Tejakula Waters regarding butterflyfish abundance and the percentage of live coral cover. Stations with higher coral cover generally reported higher numbers of butterflyfish. Nonetheless, some results were inconsistent, such as Penutukan station, which had high fish abundance despite low coral cover. Invertebrate biota (worms, molluscs, soft corals) and algae, in addition to coral, are considered to be more abundant in this area and can be consumed by butterflyfishes.

Berumen and Pratchett (2008) stated that butterflyfishes generally feed on hydrozoans, sponges, polychaetes, crustaceans, algae, and corals, including *Scleractinia* and *Alcyonacea*. Suharti et al. (2018) stated that the butterflyfishes are more prevalent on coral reefs dominated by *Acropora* corals, a type of long branching colony. *Acropora* corals are home to a variety of fish species, such as *Chaetodon auriga*, *Chaetodon citrinellus*, and *Chaetodon octofasciatus*. The number and variety of butterflyfishes are similar to the types of coral found in the waters. Butterflyfishes typically consume *Acropora* and *Pocillopora* corals (Pratchett 2005). Since *Acropora* is the predominant coral genus in Tejakula Waters, as shown in Figure 7, the quantity of butterflyfishes in Tejakula Waters was comparatively directly correlated with the high level of coral cover.

According to the findings of the Pearson correlation study, there was a very weak association ($r = 0.091$) between the percentage of coral cover and the number of butterflyfishes (Figure 8). There was a positive association between the amount of coral cover and the number of butterflyfishes. This weak association is believed to be caused by changes in the water characteristics, which are often dynamic and can affect the coral reef ecosystem, which is the habitat for coral and other marine biota in Tejakula Waters, as well as the mobilization of butterflyfishes.

This is consistent with Adrian et al. (2020), who suggest that other factors, such as the hydro-oceanographic characteristics of the waters, may be responsible for the waters' instability due to the minimal presence of indicator fish groups. Suharti et al. (2018) state that butterflyfishes have the strongest

association with coral and are highly sensitive to changes in water conditions and damage to coral reefs. It also has a positive relationship with coral reefs, and a decline in the population of this species is considered a sign of changes in coral reef conditions. Similar findings were made by Adrian et al. (2020) in Pengunjung waters, Bintan Regency, where a minor correlation was also observed ($r = 0.276$). In the Pulau Koon marine conservation area, research by Erdana et al. (2022) also demonstrated a correlation (r) has a value of 0.0001. There is an extremely weak correlation between the amount of coral cover and fish abundance, as indicated by the Pearson correlation value of 0.001.

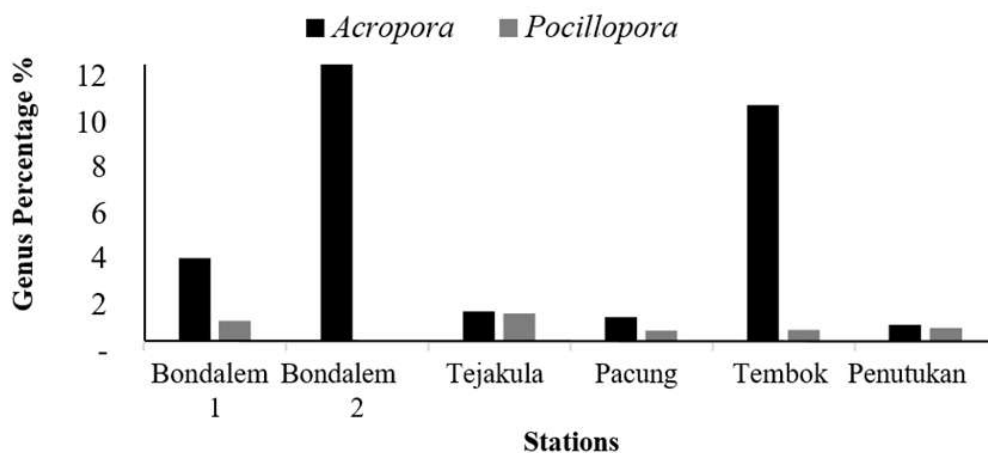


Figure 7. Percentage of coral genus in Tejakula Waters, Bali.

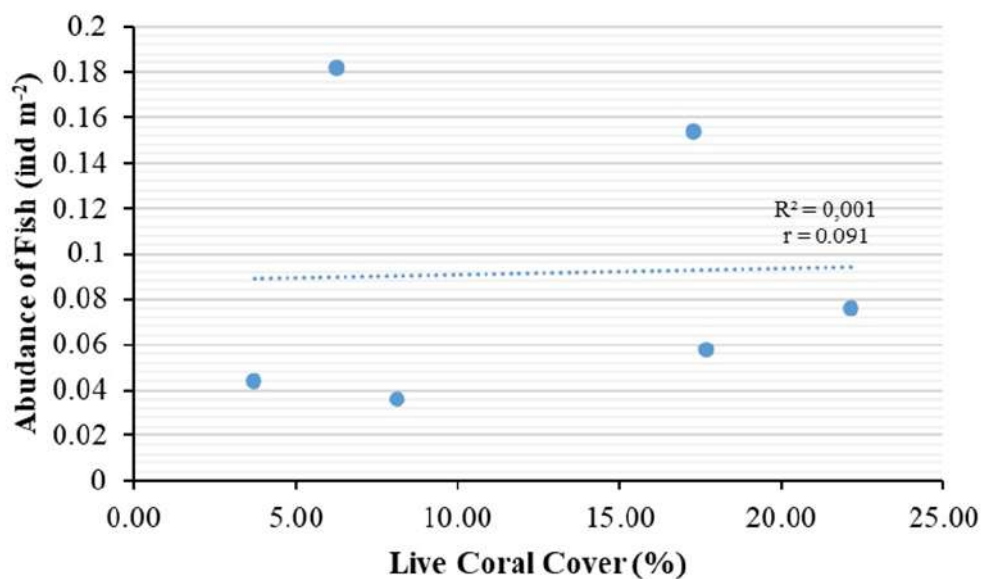


Figure 8. Relationship of butterflyfishes with live coral cover in Tejakula Waters.

CONCLUSIONS

The percentage of live coral cover in the waters of Tejakula, Buleleng, Bali falls into the low category. The average abundance of butterflyfishes was measured at 0.092 (ind m⁻²). There is relatively weak relationship between Tejakula Waters' coral cover and the number of butterflyfishes. Further research on the relationship between butterflyfish abundance and the percentage of live coral cover in Tejakula Waters, Buleleng, Bali, is recommended.

AUTHOR CONTRIBUTION

N.F.A. contributed to data collection, analysis, and writing the original draft. F.K contributed to wrote manuscript editing, and text proofreading. D.P contributed to main concepts, ideas, research equipment, and script revision. R.

contributed to the main concepts, idea, and script revision.

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CONFLICT OF INTEREST

The authors declare no competing interests regarding the research or the research funding.

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Research Article

Metabolic Adaptation of *Chlorella vulgaris* InaCCM49 to Cadmium-Salinity Stress: UPLC-MS/MS-Based Identification of Antioxidant Metabolites

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ABSTRACT

Chlorella vulgaris is a microalga species studied for its characteristics and potential applications since 1960. This study investigates the effect of combined salinity and cadmium stress on compound diversity, antioxidant activity, and pigment concentrations in *C. vulgaris* InaCCM49. Microalgae were cultivated in control and Cd-saline treatments (0.4 M NaCl and 95 μ M CdCl₂), followed by biomass harvesting, pigment determination, IC₅₀ measurement, and metabolomic analysis. Cd-salinity stress enhanced antioxidant activity, showing 51.8% lower IC₅₀ values (79.47 ppm) compared to controls (164.99 ppm), and increased carotenoid content to 1.919 mg g⁻¹. Meanwhile, chlorophyll a and b concentrations were notably higher in control treatment during the half-logarithmic phase. Partial Least Squares Discriminant Analysis revealed clear metabolomic differences between treatments. Stigmatellin Y, maltose, glycolipid 1-hexadecanoyl-3-(6'-sulfo- α -D-quinovosyl)-sn-glycerol, and phenolic derivative (2-phenoxy-3-pyridinyl)[3-(2-thienyl)-1H-pyrazol-1-yl] methanone exhibited VIP scores >15. Stigmatellin Y and glycolipids were highly synthesized in controls, whereas maltose and phenolic derivatives were elevated in Cd-saline treatment. Pheophorbide a and 3-oxo-nonadecanoic acid showed the strongest negative correlations with IC₅₀ (regression coefficients of -14.6047 and -13.9555), indicating key roles in antioxidant activity. This study represents the first comprehensive metabolomic analysis of *C. vulgaris* InaCCM49 under combined cadmium-salinity stress, revealing metabolic adaptation through enhanced synthesis of phenolic derivatives and pheophorbide a-mediated antioxidant responses.

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INTRODUCTION

Microalgae have been explored for commercial applications since the 1960s, with increasing interest due to their potential as sources of primary and secondary metabolites. Among these, *Chlorella vulgaris* is widely studied for its applications in biofuel production, bioremediation, cosmeceuticals, and as a nutritional supplement, owing to its rich composition of bioactive compounds including phenolic, flavonoids, and carotenoids, which contribute to its significant antioxidant capacity as measured by ABTS radical scavenging activity (Pantami et al. 2020; Coronado-Reyes et al. 2022). The ability of *C. vulgaris* to produce a variety of metabolites under different conditions makes it an excellent model organism for studying metabolic adaptations and bioactive compound synthesis. However, the biotechnological potential of microalgae is significantly influenced by environmental challenges that can either enhance or compromise their metabolic performance.

Environmental stress plays a crucial role in modulating the biosynthesis of protective metabolites, including antioxidants, in *C. vulgaris*. Several studies have demonstrated that abiotic stressors influence secondary metabolism by altering cellular defence mechanisms and triggering adaptive responses that affect overall metabolite production and antioxidant capacity (El-fayoumy et al. 2021; Hiremath & Mathad 2022; Wang et al. 2022; Zhang et al. 2023). Zhang et al. (2023) showed that UV-B and salt stress elevated phenolic and flavonoid levels in *C. vulgaris*, enhancing antioxidant enzyme activities, while Wang et al. (2022) found that nitrogen deprivation and salinity stress synergistically increased carotenoid and lipid antioxidant content, supporting the notion that combined abiotic stresses can substantially boost secondary metabolite production.

Among various abiotic stressors, the combination of heavy metal contamination and salinity presents a particularly complex challenge in coastal and industrial environments. Heavy metal exposure, particularly cadmium (Cd), has been reported to induce oxidative stress, which triggers metabolic adjustments affecting photosynthetic pigments, lipid metabolism, and antioxidant responses (Xu et al. 2024). Chlorophyll biosynthesis and degradation are significantly altered under Cd stress, leading to modifications in photosynthetic efficiency (Atabayeva et al. 2022). Similarly, carotenoid biosynthesis is upregulated as a defence mechanism against oxidative damage, while lipid metabolism undergoes substantial modifications with changes in key membrane lipids such as acetone mobile polar lipids (AMPL) and phospholipids (PL), accompanied by accumulation of stress-responsive fatty acids and lipid derivatives (Chia et al. 2013; Shi et al. 2020). The synergistic effects of combined Cd-salinity stress on metabolomic profiles remain largely unexplored despite their environmental relevance.

While individual effects of Cd or salinity stress on *C. vulgaris* metabolism have been studied, comprehensive metabolomic analysis of combined stress responses remain limited. Furthermore, strain-specific responses have received insufficient attention in metabolomics research. Unlike the Indonesian strain InaCCM49, strain UTEX 395 has been extensively characterised using a genome-scale metabolic model (iCZ843), demonstrating strong adaptability across trophic modes through upregulated amino acid, pigment, and lipid biosynthesis under nutrient stress. In contrast, CCAP 211/11B (equivalent to SAG 211-11B) exhibits high halotolerance, sustaining growth and lipid accumulation in seawater, indicating greater salinity resilience compared to UTEX 395. Given the complexity of metabolic changes induced by stress, untargeted metabolomics approaches, particularly chemometric techniques such as Partial Least Squares (PLS) regression, are effective for establishing correlations between metabolite profiles and biological activities, enabling identification of key metabolites contributing to stress tolerance and

antioxidant capacity (Hawrył et al. 2020; Umar et al. 2021).

This study focuses on investigating the effect of combined salinity and cadmium stress on compound diversity, antioxidant activity, chlorophyll, and carotenoid pigment concentrations in *C. vulgaris* InaCCM49, while evaluating compounds contributing to IC₅₀ values through UPLC-MS/MS and PLS analysis. By integrating untargeted metabolomics with multivariate statistical analysis, this research aims to elucidate key metabolic pathways underlying the dual stress response and identify bioactive compounds contributing to antioxidant potential. Understanding the metabolic adaptations of *C. vulgaris* InaCCM49 will provide insights into strain-specific biotechnological applications and the potential for producing bioactive compounds with significant antioxidant properties under environmentally relevant stress conditions.

MATERIALS AND METHODS

Materials

Chlorella vulgaris InaCCM49 was obtained from the Indonesian Culture Collection of Microorganisms (InaCCM), National Research and Innovation Agency (BRIN), Indonesia. The strain was maintained under standard laboratory conditions at pH 7.0 ± 0.2, 25 °C ± 2 °C, with continuous illumination of 50 μmol photons m⁻² s⁻¹ under a 12:12 h light:dark cycle, in BG-11 medium. Baseline measurements for cell density, chlorophyll content, carotenoid levels, and antioxidant activity were established from control cultures prior to stress treatment application.

Equipment and reagents used included: Low Speed Cryogenic Universal Centrifuge (Centrifuge 5430, Eppendorf), Vortex mixer (QL-901, Kylin-Bell Lab Instruments Co., Ltd China), Ultrapure water meter (Milli-Q Integral, Millipore Corporation, USA), Refrigerated Vacuum Concentrator (Maxi Vac-beta, Gene Company), Tissue Grinder (JXFSTPRP, Shanghai Xin Ning, China), Methanol (A454-4), Acetonitrile (A998-4) were all LCMS level (Thermo Fisher Scientific, USA), Ammonia formate (17843-250G, Honeywell Fluka, USA), Formic acid (50144-50 ml, Dimka, USA). Ultrapure water (18.2 MΩ·cm) was generated using a Milli-Q system (Millipore). Internal standards used were: d3-Leucine, 13C9-Phenylalanine, d5-Tryptophan, and 13C3-Progesterone.

Methods

Microalgae cultivation

A total of 20 % (v/v) of 6-day-old *C. vulgaris* InaCCM49 microalgae starter culture was cultivated in vitro in control treatment using artificial seawater media Blue Treasure™ added with sterile Walne nutrients as much as 1 mL L⁻¹. Artificial seawater was prepared using commercial salt (Blue Treasure SPS Sea Salt™, China) dissolved in ultrapure water. The seawater salt mixture at salinity of 35‰ contains: Na⁺ = 9300 mg L⁻¹, Cl⁻ = 17300 mg L⁻¹, Ca²⁺ = 430 mg L⁻¹, Mg²⁺ = 1400 mg L⁻¹, K⁺ = 360 mg L⁻¹, SO₄²⁻ = 2260 mg L⁻¹, Sr²⁺ = 9.0 mg L⁻¹, Rb²⁺ = 0.11 mg L⁻¹, Fe = 0.06 mg L⁻¹, Br⁻ = 20 mg L⁻¹, B = 4.0 mg L⁻¹, F⁻ = 0.8 mg L⁻¹, and trace elements. Cultivation conditions were of 25 °C, medium pH 7.5-8, continuous 24-hour irradiation, aeration with oxygen bubbling, 2 % of CO₂ levels, light intensity of 4000 lux, and salinity of 35 ‰. The nutrient solution was prepared according to Walne (1970) containing per litre: NaNO₃ 100.0 g, NaH₂PO₄·2H₂O 20.0 g, EDTA (disodium salt) 45.0 g, FeCl₃·6H₂O 1.3 g, MnCl₂·4H₂O 0.36 g, H₃BO₃ 33.6 g, and trace metal solution (TMS) 1.0 mL containing ZnCl₂ 2.1 g, CoCl₂·6H₂O 2.0 g, (NH₄)₆Mo₇O₂₄·4H₂O 0.9 g, and CuSO₄·5H₂O 2.0 g per 100 mL. The solution was acidified to clarify concentrated HCl and sterilised by autoclaving at 15 psi for 15 minutes. Unlike Xu et al. (2024), who investigated single cadmium stress on *Scenedesmus obliquus* with concentrations ranging from 0.005–10 mg

L⁻¹ (EC₅₀ = 0.41 ± 0.03 mg L⁻¹ after 96 hours), our study employed a combined stress approach by adding 0.4 M NaCl and 95 µM CdCl₂ (approximately 10.7 mg L⁻¹) to the cultivation medium from day 0 to simulate realistic coastal pollution conditions where cadmium contamination co-occurs with salinity stress, representing a supra-lethal dose designed to elicit strong metabolic responses. While Xu et al. (2024) monitored *Scenedesmus obliquus* responses every 24 hours over 96 hours during logarithmic growth phase, our extended sampling protocol captured metabolic dynamics across different growth phases based on preliminary IC₅₀ experiments at three critical timepoints: mid-exponential phase (5 days), late-exponential phase (8 days), and death phase (12 days). Metabolomic samples were collected in triplicate during the highest IC₅₀ phase (day 5) to capture peak metabolic stress responses when cellular adaptation mechanisms are most active. Microalgal growth under control and stress treatments was measured by calculating cell density using a Neubauer's chamber Haemocytometer, weighing dry biomass, and measuring optical density (OD) with a UV-Vis spectrophotometer at λ 680 nm every 24 hours until reaching the death phase (Gonzalez-Esquer et al. 2019).

Microalgae harvesting

The harvesting of microalgae in stress and control treatments was performed at three different growth phases, namely at half-logarithmic phase, end of logarithmic phase, and death phase. Phase selection was based on established protocols for capturing stress-adaptive metabolites across different physiological states, as metabolite profiles vary significantly during algal growth cycles under abiotic stress conditions (Gonzalez-Esquer et al. 2019). The half-logarithmic phase represents active cell division with high metabolic activity; the end logarithmic phase indicates nutrient limitation onset, and the death phase reflects maximum stress response, collectively providing comprehensive metabolomic insights into *C. vulgaris* adaptation mechanisms (Chen et al. 2020; Liu et al. 2021). Microalgae were separated from the medium by centrifugation technique at a speed of 3000 rpm for 10 minutes. A total of 1-10 mg of filtered biomass was then diluted with 25 mL of 0.5 M ammonium formate to remove salt residues from the cell culture. Furthermore, filtration was performed twice as a further purification step (Barten et al. 2022). The collected microalgal biomass was then freeze-dried until a dry powder was obtained.

Determination of chlorophyll a, chlorophyll b, and carotenoid

Four millilitres of microalgal culture were taken and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded, and the cell pellet was washed twice with 4 ml of distilled water to remove salt residues before being centrifuged again. The cell pellet was suspended in 4 ml of absolute methanol and centrifuged for 10 minutes at 3000 rpm. The supernatant was taken to measure its absorbance using a spectrophotometer. Absorbance was measured at λ 652 nm, λ 665 nm, and λ 470 nm using methanol as the blank. Concentrations of chlorophyll a, chlorophyll b, and carotenoid pigments were calculated using formulas from Table 1 (Lichtenthaler & Buschmann 2001).

Table 1. Calculation formula for chlorophyll a, chlorophyll b, and carotenoid contents.

Component	Calculation formula
C _a (µg mL ⁻¹)	16.72 A ₆₆₅ -9.16 A ₆₅₂
C _b (µg mL ⁻¹)	34.09 A ₆₅₂ - 15.28 A ₆₆₅
C _{x+c} (µg mL ⁻¹)	(1000 A ₄₇₀ - 1.63 C _a - 104.96 C _b) /221
C _a = chlorophyll a content, C _b = chlorophyll b content, C _{x+c} = total carotenoid content	

Metabolite extraction for metabolomic analysis

Fifty micrograms (50 µg) of samples, specifically A1 from the Cd-saline treatment and E3 from the control treatment, were weighed into 1.5 mL Eppendorf tubes, respectively. Each sample was soaked with 800 µL of a precooled extraction solution (methanol:water = 7:3, v/v) and 20 µL of Internal Standard 1 (IS1). The methanol-water extraction protocol was selected based on established methods for comprehensive polar metabolite recovery in UPLC-MS metabolomics studies (Salem et al. 2020; Sarkar et al. 2025). Homogenisation was performed using a weaving grinder at 50 Hz for 10 minutes, followed by water bath ultrasonication at 4 °C for 30 minutes. Subsequently, the samples were allowed to stand at -20 °C for 1 hour to enhance metabolite precipitation and extraction efficiency (Gorrochategui et al. 2021). The extracts were centrifuged at 14,000 rpm at 4 °C for 15 minutes. A volume of 600 µL of supernatant was filtered through a 0.22 µm membrane, and 20 µL of the filtered solution from each sample was combined to create a mixed quality control (QC) sample, following standard metabolomics protocols for microalgal stress studies (Liu et al. 2023).

This QC sample was used to assess the repeatability and stability of the LC/MS analysis. QC samples were injected at the beginning, middle, and end of each analytical batch, with additional QC injections every 10-15 study samples throughout the analytical sequence to monitor instrumental drift and system performance (Wang et al. 2022; Smith et al. 2023). Quality control acceptance criteria, following current metabolomics guidelines: metabolite features were required to show relative standard deviation (RSD) < 30 % for peak intensity across QC injections, RSD < 2 % for retention time, and mass accuracy < 10 ppm for reliable identification (González-Domínguez et al. 2024). Features not meeting these criteria were excluded from subsequent statistical analyses to ensure data quality and reliability. Both the filtered samples and the mixed QC samples were transferred to 1.5 mL sample vials for subsequent instrumental analysis.

UPLC-MS/MS analysis

Metabolite separation and detection were performed using a Waters UPLC I-Class Plus system coupled with a Q Exactive high-resolution tandem mass spectrometer (Thermo Fisher Scientific, USA). Each treatment group included three biological replicates (n=3) for statistical analysis. Chromatographic separation was conducted on Hypersil GOLD aQ Dim column (1.9 µm 2.1×100 mm, Thermo Fisher Scientific, USA), with mobile phase A consisting of 0.1 % formic acid in water and mobile phase B consisting of 0.1 % formic acid in acetonitrile. The column temperature was maintained at 40 °C. The gradient conditions were optimised based on established UPLC-MS metabolomics protocols (Want et al. 2012; Salem et al. 2020) and were as follows: 5 % B over 0.0-2.0 min, 5-95 % B over 2.0-22.0 min, held constant at 95 % B over 22.0-27.0 min, and washed with 95 % B over 27.1-30 min. The flow rate was 0.3 mL min⁻¹, and the injection volume was 5 µL.

Mass spectrometry conditions: Using Q Exactive (Thermo Fisher Scientific, USA), with primary and secondary mass spectrometry data acquisition following established untargeted metabolomics protocols (Gorrochategui et al. 2021; Sarkar et al. 2025). The scan range was 125–1500 m/z for positive ions and 100–1500 m/z for negative ions with a resolution of 70,000. The automatic gain control (AGC) target for MS acquisitions was set to 1×10⁶ with a maximum ion injection time of 100 ms. The top three precursors were selected for subsequent MS/MS fragmentation with a maximum ion injection time of 50 ms and resolution of 30,000, and the AGC was 2×10⁵. The stepped normalised collision energy was set to 20, 40, and 60 eV. ESI parameters were set as follows: Sheath gas flow rate was 40, Aux gas flow rate was 10,

positive-ion mode Spray voltage ($|KV|$) was 3.80, negative-ion mode Spray voltage ($|KV|$) was 3.20, Capillary temperature was 320 °C, Aux gas heater temperature was 350 °C.

Metabolite ion peak extraction and metabolite identification

Offline mass spectrometry data were imported into Compound Discoverer 3.3 (Thermo Fisher Scientific, USA) and analysed in combination with BMDB (BGI Metabolome Database), mzCloud, and ChemSpider online databases, generating a data matrix containing metabolite peak areas and identification results.

Metabolite identification and annotation followed the Metabolomics Standards Initiative (MSI) confidence levels to ensure reliable compound identification (Schymanski et al. 2014; Creek et al. 2024). Confidence levels were assigned as follows: Level 1 (identified metabolites) - confirmed with authentic reference standards matching exact mass, retention time, and MS/MS fragmentation patterns; Level 2 (putatively annotated compounds) - probable structures based on library spectral similarity and/or diagnostic evidence without reference standards; Level 3 (putatively characterised compound classes) - characterised to compound class level only; Level 4 (unknown compounds) - unidentified compounds with spectral or chromatographic information (Sumner et al. 2007; Blazenovic et al. 2018).

Only metabolites with MSI confidence levels 1 and 2 were included in final statistical analyses to ensure annotation reliability and reduce false positives. False discovery rate (FDR) control was applied at 5 % for multiple testing corrections during annotation scoring to maintain statistical rigor in metabolite identification (Tsugawa et al. 2015). Database matching scores were filtered using minimum spectral similarity thresholds (>70 % for mzCloud, >80 % for BMDB) and mass accuracy requirements (<5 ppm) to enhance identification confidence. The resulting data table was further analysed and processed.

Software Information: Compound Discoverer Version: v.3.3. Parameters: Parent ion mass deviation: <5 ppm; Mass deviation of fragment ions: <10 ppm; Retention time deviation: <0.2 min. Official Website: <https://mycompounddiscoverer.com/>

Antioxidant activity using the ABTS test

A total of 200 mg of dry microalgae samples were soaked in 15 mL of DCM:MeOH (v/v= 1:1) for 2x24 hours. Every 24 hours, the maceration solution was centrifuged at 4000 rpm for 10 minutes. The supernatant was put into a porcelain cup for evaporation to obtain the extract yield. Furthermore, the yield was weighed and dissolved in absolute methanol at a concentration of 10,000 ppm. Antioxidant was measured using ABTS reagent (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid) as follows. Samples from each treatment were prepared in concentrations of 1250, 625, 312.5, and 156.25 ppm, and subjected to antioxidant testing using the ABTS method. A total of 7.5 μ L of sample was added to a 96-well microplate, followed by 142.5 μ L of ABTS reagent. The mixture was homogenised and incubated for 30 min at 25 °C in darkness. Absorbance was measured at 745 nm, with each measurement performed in triplicate (Coulombier et al. 2020). Antioxidant activity was expressed as IC₅₀ values (concentration required to inhibit 50 % of ABTS radical), calculated from dose-response curves using regression analysis. A negative control (methanol blank) was included in each assay to establish baseline absorbance. Assay validity was confirmed through positive inhibition responses across the tested concentration range, consistent with established ABTS protocols for plant extracts (reference).

Data Analysis

The experimental results of IC₅₀, chlorophyll a (Chl_a), chlorophyll b (Chl_b), and carotenoids concentrations are expressed as means $\pm$ standard deviation (SD) of three replicates per treatment. One-way ANOVA was performed using R version 4.4.2, with significance levels tested at $p < 0.05$ (Tukey's HSD test).

The processed metabolite data and IC₅₀ values were analysed using partial least squares (PLS) regression to assess the correlation between metabolite profiles and antioxidant activity. Data preprocessing involved normalisation of metabolite data by TIC (Total Ion Count) and imputation of missing values. PLS analysis was performed with leave-one-out (LOO) cross-validation using the `pls` function in R. Regression coefficients were extracted and normalised to identify metabolites with the most significant influence on IC₅₀ values. The statistical analysis was conducted using R version 4.4.2, and results were visualised with bar plots representing the contributions of each metabolite.

In addition, the PLS-DA model and Variable Importance in Projection (VIP) scores were constructed to evaluate the relationships between metabolites and IC₅₀ values. These analyses were performed using MetaboAnalyst 6.0 (<https://www.metaboanalyst.ca>) from a dataset of 1843 metabolites obtained through UPLC-MS. For PLS-DA analysis, data preprocessing included normalisation and transformation to ensure suitability for multivariate analysis. VIP scores were calculated to identify key metabolites contributing significantly to the model's prediction. Analysis and visualisations were conducted using the MetaboAnalyst platform.

RESULTS AND DISCUSSION

Results

The experiment was designed with different treatments and growth phases, as shown in Table 2. The highest chlorophyll a and chlorophyll b contents were observed in the control treatment during the half-log phase (E1), at 18.886 mg g⁻¹ and 16.923 mg g⁻¹, respectively. Conversely, the lowest chlorophyll a and b concentrations were found in the control treatment during the death phase (E3), namely 0.909 mg g⁻¹ and 1.886 mg g⁻¹, respectively.

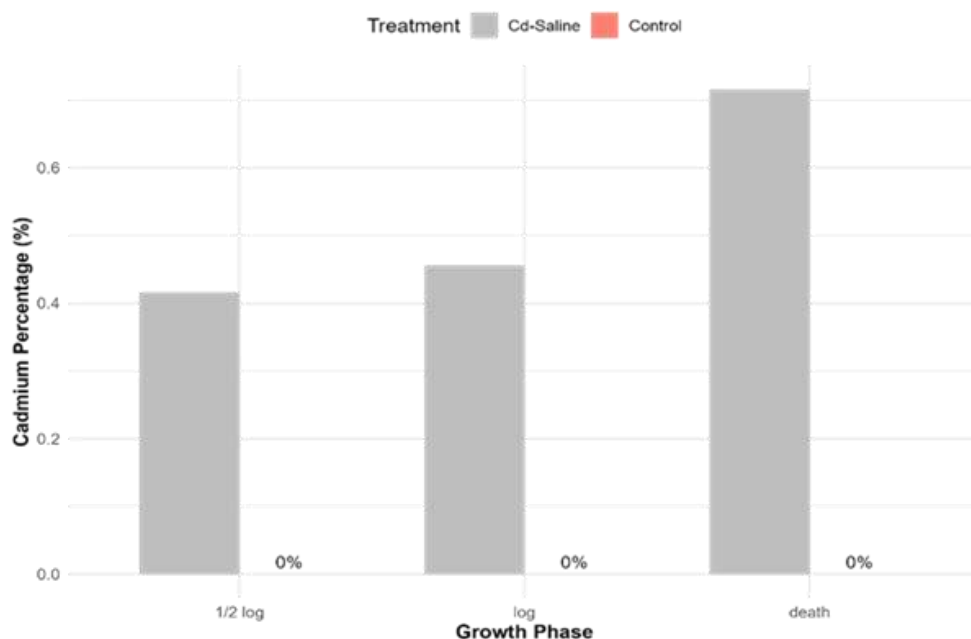
Total carotenoid concentrations showed variation across treatments and growth phases, with the highest level recorded in the Cd-saline treatment during the half-log phase (A1) at 1.919 mg g⁻¹. The carotenoid measurements represented the total carotenoid content, which may include various isomers such as β -carotene, lutein, zeaxanthin, and other xanthophyll derivatives that are known to have distinct antioxidant roles and stress response mechanisms in microalgae (Minhas et al. 2016; Sathasivam et al. 2019). Individual carotenoid isomer analysis would provide more detailed insights into specific stress adaptation pathways, as β -carotene primarily functions in photoprotection while lutein and zeaxanthin are involved in the xanthophyll cycle for dissipating excess light energy (Metting 1996; Lemoine & Schoefs 2010). The lowest carotenoid content was found in the control treatment during the log phase (E2) at 0.130 mg g⁻¹.

The Cd-saline treated samples demonstrated relatively lower IC₅₀ values compared to control treatments, indicating higher antioxidant activity. The strongest antioxidant activity was observed in the Cd-saline treatment during the half-log phase (A1) with an IC₅₀ value of 79.472 ± 3.3341 ppm (lower IC₅₀ values indicate stronger antioxidant content). The weakest antioxidant activity was recorded in the control treatment during the log phase (E2) with an IC₅₀ value of 387.262 ± 1.2601 ppm. Overall, the Cd-saline treatments exhibited enhanced antioxidant activity and total carotenoid content compared to control treatments, while chlorophyll levels (both a and b) were notably higher in the control treatment during the half-log phase (E1).

Table 2. Chlorophyll a, chlorophyll b, carotenoid content, and ABTS radical scavenging activities (IC₅₀) in extracts of *C. vulgaris* under Cd-saline treatments at different growth phases.

Treatment	Sample code	IC ₅₀ (ppm)	Chl_a (μg mL ⁻¹)	Chl_b (μg mL ⁻¹)	Carotenoids (μg mL ⁻¹)
Cd-saline, half-log phase	A1	79.472 ^d ± 3.3341	18.494 ^b ± 0.036	14.681 ^b ± 0.018	1.919 ^a ± 0.015
Cd-saline, log phase	A2	81.820 ^d ± 3.1634	4.300 ^d ± 0.003	3.752 ^d ± 0.001	0.625 ^b ± 0.001
Cd-saline, death phase	A3	195.352 ^b ± 4.2488	1.117 ^e ± 0.011	2.959 ^e ± 0.007	0.166 ^d ± 0.002
Control, half-log phase	E1	164.985 ^c ± 2.1452	18.886 ^a ± 0.078	16.923 ^a ± 0.009	0.649 ^b ± 0.006
Control, log phase	E2	387.262 ^a ± 1.2601	6.739 ^c ± 0.005	9.483 ^c ± 0.005	0.130 ^e ± 0.003
Control, death phase	E3	79.965 ^d ± 7.8958	0.909 ^f ± 0.001	1.886 ^f ± 0.010	0.313 ^c ± 0.024

Note: Values are presented as mean ± standard deviation (n = 3). Different superscript letters within the same column indicate significant differences according to one-way ANOVA followed by Tukey's HSD (Honestly Significant Difference) test at p < 0.05. P-values were adjusted for multiple comparisons using the Tukey HSD procedure, which controls the family-wise error rate across all pairwise comparisons within each measured parameter (Abdi & Williams 2010).

**Figure 1.** Cadmium accumulation percentages across different growth phases in *Chlorella vulgaris* under Control and Cd-saline treatments.

The cadmium accumulation percentages are visualised in Figure 1. The study demonstrated a clear trend in cadmium accumulation, highlighting distinct differences between the control and Cd-saline treatments across all growth phases. The control treatment consistently maintained 0 % cadmium accumulation. In contrast, the Cd-saline treatment showed a progressive increase in cadmium concentration, starting at 0.4165 % during the half-log phase, rising to 0.4560 % in the log phase, and reaching a peak of 0.7154 % in the death phase. Figure 2 presents a heatmap illustrating the correlations between cadmium percentage, pigment content, and IC₅₀ values. Correlation

analyses revealed several significant relationships. Notably, a strong negative correlation was observed between IC₅₀ values and chlorophyll content, indicating that samples with higher chlorophyll levels exhibited enhanced antioxidant activity, particularly during the half-log phase (E1).

Furthermore, a strong positive correlation was identified between cadmium accumulation and pigment content (chlorophyll and carotenoids), suggesting an adaptive response by *C. vulgaris* to cadmium stress, characterised by increased pigment production. Additionally, a very strong positive correlation was found between chlorophyll a and chlorophyll b levels, reflecting synchronised changes in these pigments across all treatments. This coordinated response underscores the close metabolic relationship between these chlorophyll types.

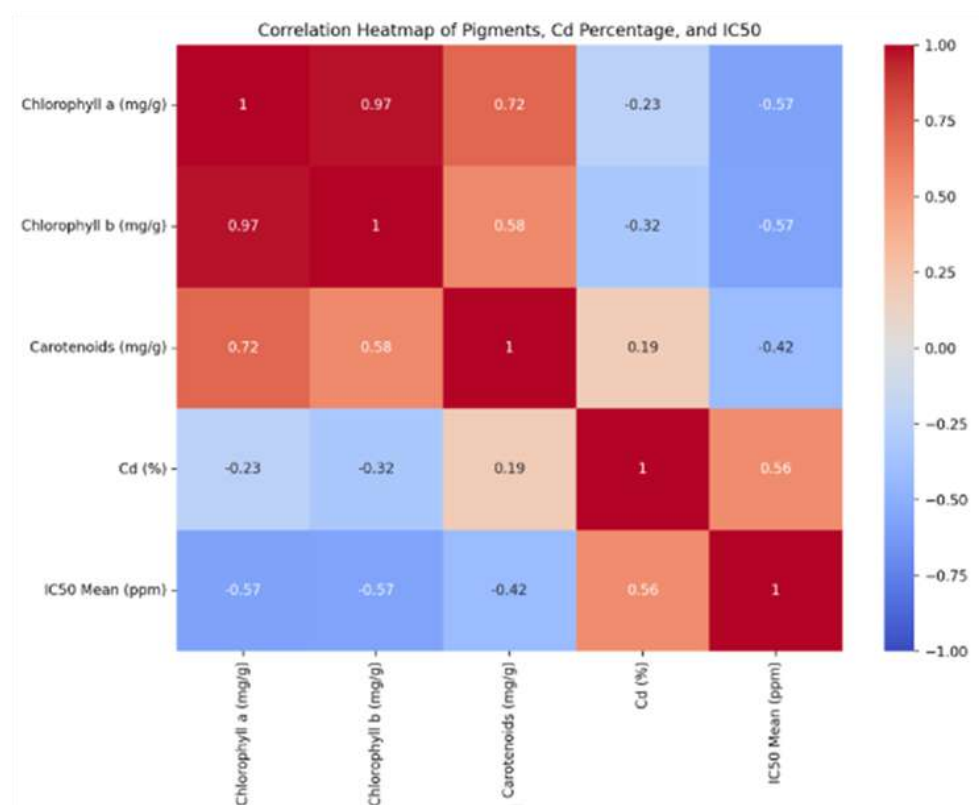


Figure 2. Heatmap showing the correlations between cadmium accumulation percentage, pigment content (chlorophyll a, chlorophyll b, and carotenoids), and IC₅₀ values across different growth phases. The red color represents a strong positive correlation, indicating a direct relationship between variables, while the blue color represents a strong negative correlation, indicating an inverse relationship.

Peak lists generated from positive mode LC-MS data were analysed using MetaboAnalyst, with blank solvents and media excluded to avoid skewing the results. A total of 1843 compounds were identified across the six samples, with each compound representing a unique combination of *m/z* values and chromatographic peak areas. The metabolomics analysis revealed apparent differences between the Cd-saline and control treatments, as shown in the PLS-DA plot (Figure 3). Samples from the Cd-saline treatment formed distinct clusters, separating clearly from the control samples. The distinct clustering pattern indicates significant biochemical differences between the two treatments, likely driven by stress-induced metabolic changes. Components 1 and 2 explained 75.1 % and 7.1 % of the variance, respectively, demonstrating the ability of PLS-DA to effectively distinguish between treatment groups. The chemical diversity observed in the Cd-saline treatment suggests an adaptive response to cadmium stress, potentially involving metabolites associated with antioxidant activity and other stress-related pathways. These findings

highlight the significant metabolic alterations in *C. vulgaris* under cadmium and salinity stress.

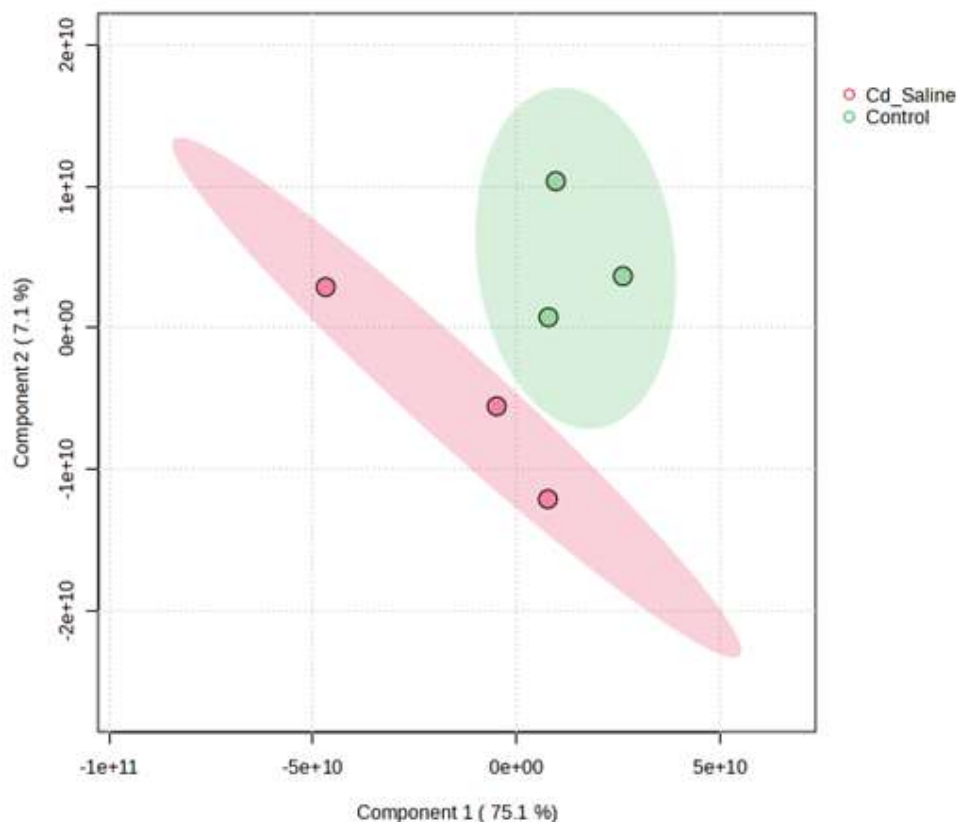


Figure 3. PLS-DA score plot illustrating the separation of metabolic profiles between Cd-saline and Control treatment groups. The distinct clustering of samples indicates significant biochemical differences driven by stress-induced metabolic changes. Components 1 and 2 accounted for 75.1 % and 7.1 % of the variance, respectively, highlighting the effectiveness of PLS-DA in distinguishing between the treatment groups.

To identify the key metabolites driving these differences, Variable Importance in Projection (VIP) scores were calculated and ranked (Figure 4). The pronounced separation observed in the PLS-DA plot emphasises the distinct metabolic profiles of *C. vulgaris* under Cd-saline and control conditions. The clustering pattern, supported by the high VIP scores of key metabolites, highlights the significant biochemical perturbations induced by cadmium and salinity stress. Notably, compounds such as stigmatellin Y, maltose, a glycolipid (1-hexadecanoyl-3-(6'-sulfo- α -D-quinovosyl)-sn-glycerol), and an aromatic organic compound ((2-phenoxy-3-pyridinyl)[3-(2-thienyl)-1H-pyrazol-1-yl]methanone) exhibited VIP (Variable Importance in Projection) scores exceeding 15. Although a threshold of VIP > 1.0 is commonly used to indicate biological relevance in PLS-DA models, exceptionally high values, such as those observed in this study, can occur due to strong discriminatory power between groups, substantial differences in metabolite abundance, or model scaling effects (Westerhuis et al. 2008; Chong et al. 2019). These elevated VIP scores emphasise the key role of these metabolites in distinguishing the metabolic response of *Chlorella vulgaris* under Cd-saline stress compared to control conditions. Stigmatellin Y and glycolipids were more abundant in the control group, while maltose and the aromatic organic compound ((2-phenoxy-3-pyridinyl)[3-(2-thienyl)-1H-pyrazol-1-yl]methanone) showed higher levels under Cd-saline treatment (VIP > 15).

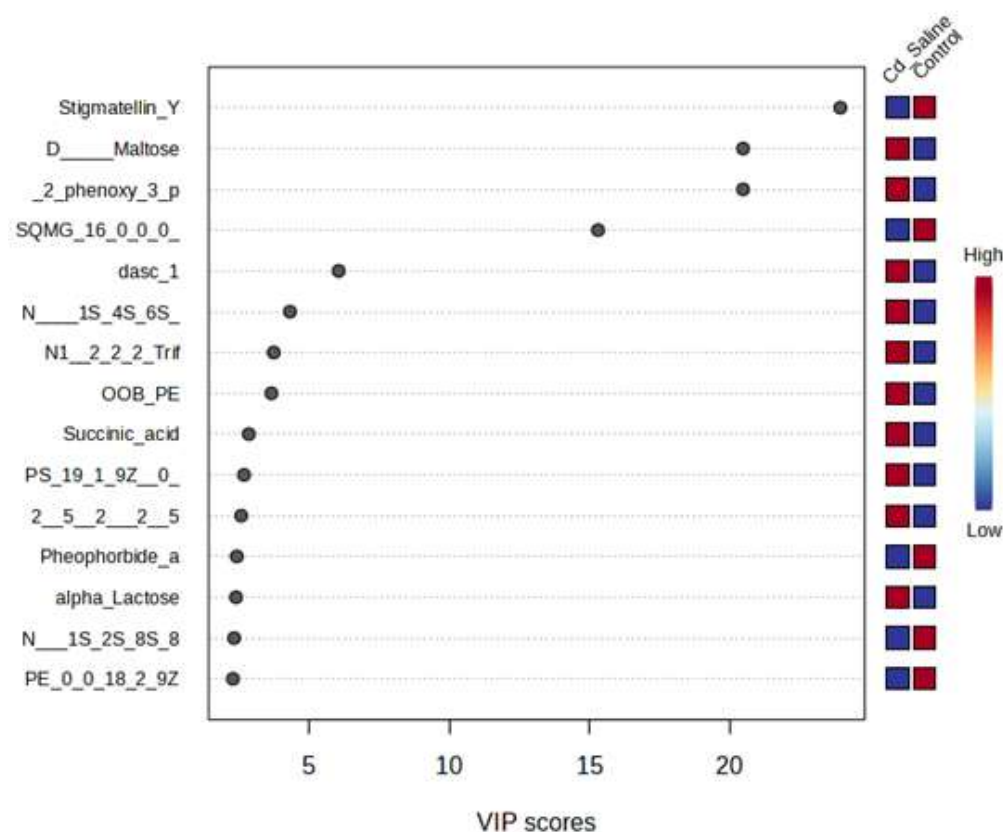


Figure 4. VIP scores of top metabolites identified through PLS-DA analysis ranked from highest to lowest. The heatmap on the right displays the relative concentrations of each metabolite in the Cd-saline and Control groups, with red indicating higher concentrations and blue indicating lower concentrations.

A Partial Least Squares (PLS) model was developed to analyse the relationship between UPLC-MS peak area data for each treatment and the biological activity assay results, represented by IC_{50} values (Pirouz 2006). The PLS model, constructed from spectral (UPLC-MS) data and antioxidant activity tests, explained a cumulative 57.93 % of variance in antioxidant activity, with LV-1 and LV-2 contributing 22.83 % and 35.10 %, respectively. The negative U PLS scores (factor-1) on the x-y relationship plot indicate a substantial contribution of individual samples to antioxidant activity. The weighted regression coefficients of metabolites in the PLS model are presented in Figure 5, illustrating their relative contributions to antioxidant activity. The loading plot provides insights into the metabolites that contribute most significantly to antioxidant activity. Table 3 lists the metabolites with significant contributions based on normalised regression coefficient values.

The most substantial negative peaks in the model were associated with pheophorbide a and 3-oxo-nonadecanoic acid, which had regression coefficient values of -14.6047 and -13.9555, respectively. These metabolites are predicted to play crucial roles in antioxidant activity, as evidenced by their direction and extreme positions on the loading plot. Significant negative regression coefficient values suggest metabolites with a major impact on biological activity. Among them, *pheophorbide a*, with the most negative regression coefficient (-14.6047), showed the highest contribution to antioxidant activity. Although pheophorbide a is a well-known chlorophyll degradation product, it has been reported to retain strong antioxidant potential due to its ability to quench singlet oxygen and scavenge free radicals (M. Saide et al. 2020). Similarly, metabolites such as 3-oxo-nonadecanoic acid and PG(16:1(9Z)/0:0) significantly contributed to biological activity, potentially through their involvement in lipid metabolism and antioxidant defence pathways. These findings support

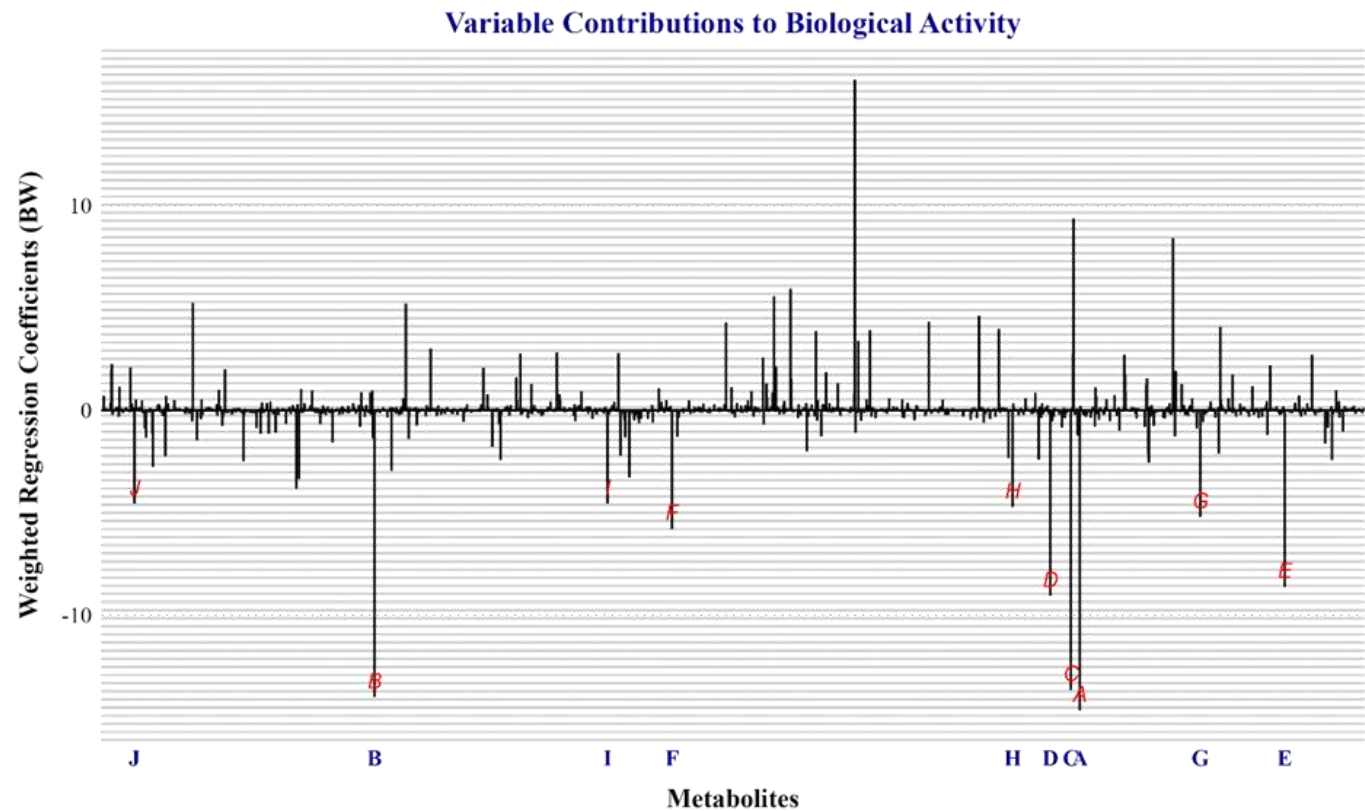


Figure 5. Weighted regression coefficients of metabolites in the PLS model correlating UPLC-MS data with antioxidant activity (IC₅₀ Values).

Table 3. The compounds identified in the methanol extracts of *C. vulgaris* using UPLC-MS/MS contributed to the antioxidant activity.

Metabolites	Label	Database	Classification	RT (Min)	CalcMz	Formula	BW
Pheophorbide a	A	KEGG	Chlorophyll derivative	20.146	593.2755	C ₃₅ H ₃₆ N ₄ O ₅	-14.6047
3-oxo- nonadecanoic acid	B	Lipidmaps	Fatty acid	13.884	313.27377	C ₁₉ H ₃₆ O ₃	-13.9555
PG(16:1(9Z)/0:0)	C	Lipidmaps	Phospholipid	13.783	481.25784	C ₂₂ H ₄₃ O ₉ P	-13.5928
PE(19:1(9Z)/0:0)	D	Lipidmaps	Phospholipid	15.863	494.32456	C ₂₄ H ₄₈ NO ₇ P	-9.00797
Erucamide	E	mzCloud	Lipid amide	23.826	338.34156	C ₂₂ H ₄₃ NO	-8.57737
Elatine	F	KEGG	Terpenoid alka- loid	12.655	693.33942	C ₃₈ H ₅₀ N ₂ O ₁₀	-5.74801
Stigmatellin Y	G	KEGG	Aromatic polyke- tide	14.118	483.27328	C ₂₉ H ₄₀ O ₆	-5.15258
OOB-PE	H	lipid maps	Phospha- tidylethanolamine derivative	15.271	562.31698	C ₂₇ H ₅₀ NO ₉ P	-4.66956
D-(+)-Maltose	I	KEGG	Disaccharide	0.873	365.10519	C ₁₂ H ₂₂ O ₁₁	-4.51065
(2-phenoxy-3- pyridinyl)[3-(2- thienyl)-1H- pyrazol-1-yl] methanone	J	mzCloud	-	0.861	365,1053	C ₁₉ H ₁₃ N ₃ O ₂ S	-4.51065

the notion that more negative regression coefficient values indicate greater influence on bioactivity, consistent with the principle that lower IC₅₀ values correspond to higher antioxidant capacity. Overall, the results highlight the dynamic metabolic adjustments of *Chlorella vulgaris* under stress conditions and underscore the role of specific metabolites in enhancing antioxidant potential.

Discussion

Temporal Dynamics of Antioxidant Response and Metabolic Flexibility

The antioxidant response of *C. vulgaris* to cadmium stress demonstrates a temporally coordinated cellular adaptation mechanism, with the strongest antioxidant activity (IC₅₀ = 79.472 ± 3.3341 ppm, where lower IC₅₀ values indicate higher antioxidant activity) observed during the half-log phase. This enhanced response during the exponential growth phase reflects the organism's metabolic flexibility, as cells in this phase possess maximum biosynthetic capacity and energy availability to rapidly deploy defensive mechanisms (Zhang et al. 2023). The heightened metabolic activity during exponential growth provides the necessary ATP and reducing equivalents to synthesise antioxidant compounds and activate enzymatic defence systems, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (Jun et al. 2003; Hameed et al. 2011).

Notably, our findings contrast with Cheng et al. (2016), who reported delayed antioxidant responses under similar cadmium exposure conditions. This discrepancy likely reflects strain-specific variations in stress response kinetics and metabolic capacity, as demonstrated by Bacova et al. (2019), who observed distinct epigenetic and metabolic responses between different microalgal species under cadmium stress. While Wang et al. (2022) observed peak antioxidant activity 48–72 hours post-exposure, our results demonstrate immediate activation within the first growth phase, suggesting that our *C. vulgaris* strain possesses more rapid stress-sensing and response mechanisms. This rapid response capability may confer ecological advantages in fluctuating environmental conditions where prompt adaptation is crucial for survival.

Cadmium Accumulation in Environmental Context

The observed cadmium accumulation of 0.7154 % (Figure 1) warrants evaluation against realistic environmental levels. Xu et al. (2024) reported cadmium concentrations ranging from 0.15–2.3 % in microalgae from contaminated freshwater systems, positioning our experimental levels within environmentally relevant ranges. However, the synergistic effects of cadmium-salinity stress create conditions more severe than single-stress scenarios, potentially explaining the pronounced metabolic responses observed. The combined stress likely disrupts cellular homeostasis through multiple pathways: cadmium interferes with essential metal enzymes while salinity imposes osmotic stress, collectively overwhelming cellular defence mechanisms and necessitating comprehensive metabolic reorganisation.

Photosynthetic Pigment Dynamics and Photoprotection

The progressive decline in chlorophyll content across growth phases reflects cadmium's direct interference with photosynthetic machinery. The identification of pheophorbide a (PPBa), a chlorophyll degradation product, suggests active chlorophyll catabolism under stress conditions. Beyond indicating photosynthetic damage, PPBa accumulation may serve photoprotective functions by dissipating excess light energy and preventing photo-oxidative damage (A. Saide et al. 2020). This dual role of PPBa—as both a stress marker and protective compound—exemplifies the complex nature of stress-induced metabolic changes, where degradation products can acquire new functional roles in

cellular defence.

The strong positive correlation between pheophorbide a and antioxidant activity (compared with [M. Saide et al. 2020](#)) likely stems from its ability to scavenge reactive oxygen species (ROS) and its role in modulating light-harvesting efficiency under stress. This relationship suggests that chlorophyll degradation, rather than being merely destructive, represents a regulated process that contributes to overall cellular antioxidant capacity.

Carotenoid-Mediated Stress Response

Carotenoid analysis revealed maximum concentrations of 1.919 mg g⁻¹ during the half-log phase under Cd-saline treatment, substantially higher than the 1.2 mg g⁻¹ reported by Shi et al. (2020) for *C. vulgaris* under cadmium-only stress. This elevated carotenoid accumulation under combined stress conditions indicates that salinity enhances carotenoid biosynthesis, possibly through increased expression of carotenoid biosynthetic genes or enhanced precursor availability. The temporal coincidence of peak carotenoid levels with maximum antioxidant activity underscores the critical role of these pigments in early stress response, functioning as primary (ROS) scavengers and membrane stabilisers.

Metabolic Pathway Integration and Osmotic Adjustment

Maltose Accumulation and Osmotic Regulation

The accumulation of maltose under Cd-saline stress reflects a sophisticated osmotic adjustment mechanism. As a compatible solute, maltose participates in cellular osmoregulation while maintaining enzyme functionality under osmotic stress. The elevated maltose levels likely result from activation of the trehalose-6-phosphate synthase pathway, which regulates sugar signalling and carbon partitioning during stress conditions. This osmolyte accumulation strategy aligns with recent findings by Reignier et al. (2024), who demonstrated similar osmolyte-based adaptation mechanisms in cyanobacterial communities under salt stress. This pathway not only produces osmolytes but also modulates carbon flux towards stress-protective compounds, demonstrating the interconnected nature of stress response mechanisms.

Secondary Metabolite Modulation

The depletion of stigmatellin Y in stressed samples, contrasting with its abundance in controls, suggests stress-induced reprioritisation of metabolic resources. Stigmatellin Y, associated with mitochondrial electron transport and ubiquinol-cytochrome c reductase activity ([von Jagow & Ohnishi 1985](#)), requires substantial metabolic investment for its biosynthesis. Under stress conditions, metabolic resources are redirected towards immediate survival mechanisms rather than secondary metabolite production. This finding contrasts with Mendes et al. (2024), who reported maintained stigmatellin Y levels in stress-tolerant microalgal strains, suggesting that stigmatellin Y reduction may serve as a biomarker for stress susceptibility in different strains.

Aromatic Compound Induction

The increased abundance of the aromatic organic compound ((2-phenoxy-3-pyridinyl)[3-(2-thienyl)-1H-pyrazol-1-yl]methanone) under stress conditions reflects activation of detoxification pathways. The pyridine and pyrazole rings in this compound can interact with key metabolic enzymes, potentially serving antioxidant functions or facilitating xenobiotic metabolism ([Nowicka 2022](#)). This compound's accumulation represents the organism's attempt to neutralise toxic effects through enhanced detoxification capacity.

Membrane Remodelling and Lipid Metabolism

The observed reduction in glycolipid levels, particularly (2S)-1-palmitoyl-3-O

-(6-sulfo- α -D-quinovopyranosyl)-glycerol (SQMG(16:0/0:0)), indicates extensive membrane remodelling under combined stress. Glycolipids play crucial roles in membrane stability, cell recognition, and osmotic regulation. Their depletion suggests that membrane restructuring prioritises basic membrane integrity over specialised functions, with increased presence of phosphatidylglycerol and phosphatidylethanolamine derivatives compensating for lost glycolipid functions.

Proposed Integrated Stress Response Model

Based on our findings, we propose a comprehensive model of metabolic pathway integration under combined Cd-salinity stress. The response begins with initial stress detection through rapid activation of ROS-scavenging systems and carotenoid biosynthesis, followed by osmotic adjustment via maltose accumulation through trehalose-6-phosphate synthase pathway activation. Simultaneously, membrane adaptation occurs through glycolipid reduction and phospholipid restructuring to maintain membrane integrity. Resource reallocation becomes evident as secondary metabolite synthesis (stigmatellin Y) decreases in favour of primary defence mechanisms, while detoxification enhancement proceeds through aromatic compound accumulation to neutralize toxic effects. Finally, photosynthetic adjustment involves controlled chlorophyll degradation with pheophorbide a accumulation for photoprotection, creating an integrated cellular response that maintains viability under combined stress conditions.

Methodological Considerations and Limitations

Several methodological limitations warrant acknowledgment. The untargeted metabolomics approach, while comprehensive, may miss low-abundance stress-responsive metabolites such as proline, which is commonly induced under abiotic stress but was not detected in our analysis. This absence likely reflects either concentrations below detection limits or matrix effects during LC-MS/MS analysis. Additionally, ionisation efficiency variations and matrix effects in LC-MS/MS analysis may lead to underestimation of certain metabolite classes.

The batch culture system employed may introduce temporal variations in nutrient availability and waste accumulation, potentially confounding metabolic responses attributed solely to cadmium-salinity stress. Future investigations would benefit from continuous culture systems and integration of multiple analytical platforms (NMR, GC-MS, targeted metabolomics) to achieve more complete metabolic profiling.

Environmental and Biotechnological Implications

The demonstrated cadmium accumulation capacity (0.7154 %) within environmentally relevant ranges, coupled with maintained antioxidant activity, positions *C. vulgaris* as a promising candidate for bioremediation applications. The strain's ability to rapidly activate defensive mechanisms while accumulating significant metal concentrations suggests potential for dual-purpose systems combining wastewater treatment with valuable biomass production.

The temporal specificity of antioxidant responses highlights the importance of harvest timing for maximising bioactive compound yields. The half-log phase emergence as the optimal harvest point for antioxidant activity provides practical guidance for industrial cultivation protocols.

CONCLUSIONS

This study successfully investigated the effects of combined cadmium-salinity stress on *C. vulgaris* InaCCM49, revealing significant enhancements in antioxidant capacity ($IC_{50} = 79.472$ ppm, 51.8% improvement over controls) and

carotenoid content (1.919 mg g^{-1}) during the half-log phase. UPLC-MS/MS coupled with PLS analysis identified key metabolites contributing to antioxidant activity, with pheophorbide a and 3-oxo-nonadecanoic acid showing the strongest correlations (regression coefficients of -14.6047 and -13.9555, respectively). Metabolomic profiling revealed distinct stress-responsive metabolites: maltose and phenolic derivatives accumulated under stress, while stigmatellin Y and glycolipids were depleted, indicating metabolic resource reallocation toward primary defence mechanisms. These findings demonstrate strain-specific metabolic adaptations of *C. vulgaris* InaCCM49 under combined stress, providing insights for optimizing algal cultivation in polluted environments and strategic harvest timing to maximize bioactive compound yields.

AUTHOR CONTRIBUTION

D.E. designed the research, collected the data, and wrote the manuscript. R.S.R. analysed the data and wrote the manuscript. A.E.R. collected and analysed the data. H.P. and N. supervised the entire process.

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CONFLICT OF INTEREST

The authors declare no conflict of interest regarding funding, the research process, or the writing of this article.

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Research Article

Genetic Diversity and Albumin Content of Striped Snakehead *Channa striata* in Indonesia: A Study on Farmed and Wild Fish from Five Main Islands

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ABSTRACT

Genetic characterisation and key traits of species are crucial in determining the direction of aquaculture development. The genetic diversity of the striped snakehead (*Channa striata*) across five main islands in Indonesia, both from aquaculture and wild populations, was explored through its partial mtDNA COI. Subsequently, this study examined the albumin content extracted from the meat and comprehensively aligned it with the variation in its genotype. Phylogenetic studies showed the Sumatran snakehead fish sample forms its own cluster and has a large genetic distance from other samples. Interestingly, the albumin content in the Sumatra samples was significantly higher (1.907 %) than in others (1.253–1.509 %). Further analysis of albumin content from the Borneo samples from the same taxa in the phylogenetic tree showed no significant difference between farmed and wild *C. striata*. The findings of this study are fundamental for initiating a selective breeding programme and developing conservation management.

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INTRODUCTION

More than forty species of the genus *Channa* are native to Malaysia, Indonesia, and Peninsular Asia (Courtenay & Williams 2004). One of them is the striped snakehead, *Channa striata* (Bloch 1793), which is well known in biomedical fields in many local Asian communities and is an important species in both fisheries and aquaculture. Froese and Pauly (2010) stated that *C. striata* is one of the most widely distributed snakehead species, with a native range covering southern China, Pakistan, most of India, southern Nepal, Bangladesh, Sri Lanka, and most Southeast Asian countries. A biodiversity assessment study in Bangladesh (Bashar et al. 2020) revealed that snakeheads decreased in the catch composition.

Albumin is an important component in biomedical applications and is abundantly found in *C. striata*. It is well known for its postoperative medicinal application to enhance wound healing and reduce postoperative pain and discomfort (Ab Wahab et al. 2015). In addition, local market demand for snakehead fish in Indonesia is greatly expanding due to its commercial value, its pleasant flavour in local food (especially in Borneo Island), and the biomedical benefits already known by local communities (Song et al. 2013). Due to high demand, striped snakehead has been cultured commercially in Indonesia since the 2000s.

The availability of *C. striata* in Indonesia mostly comes from the wild or capture due to the exploitation of open waters. The Ministry of Marine Affairs and Fisheries of the Republic of Indonesia (2018) stated that aquaculture contributes only 14 % of the total supply, although *C. striata* farming in Indonesia has already been carried out for a decade. There is limited genetic knowledge regarding the populations of *C. striata*, whereas this field is crucial to understanding and developing sustainable aquaculture, biotechnology, and conservation programmes. Studies conducted in Peninsular Malaysia (Ambak et al. 2006; Tan et al. 2012) and Mekong drainage (Adamson et al. 2010) highlighted that *C. striata* is a relatively non-migratory species, sharing common haplotypes among adjacent populations.

Hence, research on genetic variation and albumin content research in *C. striata* is needed to fundamentally support aquaculture activities, including domestication, breeding, and sustainable fisheries management. Furthermore, it can also contribute to pharmaceutical applications. This study focuses on *C. striata* samples of five main islands, spanning the northern to the western parts of Indonesia, examining the genetic characteristics and diversity inferred from the mtDNA cytochrome c oxidase subunit 1 (COI) gene. In addition, the albumin characteristics of *C. striata* from aquaculture and the wild are also covered in this study.

MATERIALS AND METHODS

Animal rights

The care and use of experimental animals complied with the UGM Ethics Committee animal welfare laws, guidelines, and policies as approved by the certificate No.00013/04/LPPT/I/2020.

Samples collection

Random samples of individuals (Table 1) were collected from a total of 13 locations distributed throughout Sumatra, Java, Borneo, Sulawesi, and Papua (Figure 1). Sampling locations were divided into five regions following the main islands of Indonesia.

Genome Isolation

Fin clips for DNA isolation were excised from the dorsal fin rays. These clips were approximately 2 cm² in size and preserved in 1.5 mL tubes containing



Figure 1. (a) Sampling sites of selected locations of farmed and wild *C. striata* from the five main islands in Indonesia and (b) Sampling sites of selected locations of Borneo Island. All sampling sites are indicated with .

Table 1. Sampling locality, coordinate, and collection date of *C. striata* in this study.

Island/Region	Location	Latitude (North)	Longitude (East)	Date Collected
Sumatra	Swamp - Palembang South Sumatra (1)	3° 04' 04"	104° 66' 48"	7.10.2019
Java	Lake - Ngawi, East Java (1)	7° 23' 55"	111° 34' 27"	20.8.2019
	Aquaculture - Yogyakarta Special Region (2)	7° 75' 42"	110° 43' 40"	19.10.2019
Borneo	Lake - Banjar, South Kalimantan (1)	3° 31' 11"	115° 00' 34"	6.8.2019
	Swamp - Hulu Sungai Selatan, South Kalimantan (2)	2° 66' 88"	115° 20' 17"	7.8.2019
	Estuary - Banjar, South Kalimantan (3)	3° 32' 44"	114° 77' 91"	8.8.2019
	Aquaculture Center - Banjar, South Kalimantan (4)	3° 29' 52"	114° 55' 58"	9.8.2019
	Aquaculture - Banjar, South Kalimantan (4.2)	3° 43' 12"	114° 88' 08"	10.8.2019
	Swamp - Barito Kuala South Kalimantan (5)	3° 00' 47"	114° 74' 67"	11.8.2019
	Littoral - Banjar South Kalimantan (6)	3° 45' 98"	114° 52' 84"	12.8.2019
	Swamp - Pulang Pisau, Central Kalimantan (7)	2° 64' 51"	114° 20' 63"	15.8.2019
Sulawesi	Swamp-Makassar South Sulawesi (1)	5° 08' 00"	119° 25' 04"	29.10.2019
Papua	Swamp -Merauke, Papua (1)	8° 28' 42"	140° 22' 51"	22.9.2019

95 % ethanol. Samples were stored at a temperature of -20 °C until use. The genome was isolated from fin clip samples using a TNES method (Wasko et al. 2003). A total of 24 samples were used for genome isolation, with 2 samples from each population (Borneo (7), Java (2), Sumatra (1), Sulawesi (1), and Papua (1)).

DNA amplification and sequencing

The COI gene from snakehead fish samples was amplified using combinations of the following forward and reverse primers: (COIF): 5'-

TCACACGTTGATTTTTCTCGACT-3' and (COIR): 5'-AATAAGCGCGTGTGTCAACG-3' (Hardaningsih et al. 2025). Amplification was conducted by Polymerase Chain Reaction (PCR) performed in a 50 mL reaction volume by using *My Taq* Extract-PCR Kit (Bioline®). Approximately 100 ng of genomic DNA, 1 mM of each primer, 2x *My Taq* Extract-PCR Kit, and Nuclease Free Water with a total reaction volume of 50 ml. The amplicons of approximately 906 bp were subsequently amplified under the following thermal conditions; initial denaturation at 94 °C (5 min), 35 cycles of 94 °C (30 sec), 50 °C (30 sec), 72 °C (1 min), and a final extension at 72 °C (5 min) before termination of the reaction at 12 °C (Hardaningsih et al. 2025). The PCR products were visualised on a 1 % agarose gel stained with Florosafe DNA stain (1st Base®). The PCR products were sent for DNA sequencing to 1st BASE Laboratories Sdn Bhd, Selangor, Malaysia. They were analysed using forward and reverse primers for the first batch, and only the forward primer for the next batch.

Data analysis

The sequences were aligned and compiled for editing using ClustalW implemented in MEGA v. 5.0 (Tamura et al. 2011). To ensure accuracy, the DNA strands were sequenced in both directions (COIF and COIR) for each individual in the first batch. Both DNA strands were checked for ambiguous bases by comparing them with chromatograms and editing them manually. BLAST (Basic Local Alignment Search Tools) analysis on the NCBI (National Center for Biotechnology Information) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=GeoBlast&PAGE_TYPE=blastSearch) is used to confirm species. The phylogenetic tree was constructed using Maximum Likelihood (ML) methods with the Kimura 2-parameter evolution model and 1000× bootstrap replications (Tamura et al. 2011).

Albumin extraction

The extraction method used in this study was carried out on raw fish meat using cold extraction (Asfar et al. 2019). A total of 39 samples were used for albumin extraction, with 3 samples from each population: Borneo (8), Java (2), Sumatra (1), Sulawesi (1), and Papua (1). Specimens weighing 200–500 g each were weighed, cleaned, and filleted. A total of 40 grams of meat from each fish was used for albumin extraction and stored at -25 °C until use. The fish fillets were then cut into small pieces, weighed, and blended using 0.9 % NaCl solution at a ratio of 1:1. Next, the homogenate was centrifuged at 2500 g at 4 °C for 40 minutes. The supernatant obtained was adjusted to a pH of 4.6 by adding HCl or NaOH, which is known to be the isoelectric point of fish albumin (Ramdhani et al. 2019). The homogenate was centrifuged at 2500 g at 4 °C for 45 minutes to form a precipitate, then incubated at 5-10 °C. The precipitate obtained was then dried by heating at 45 °C until it reached a constant weight. The albumin obtained was crushed to form a powder. The extraction was replicated three times for each sample.

Albumin content analysis

All values of albumin content were presented as means for each group. Albumin datasets were analysed statistically using Analysis of Variance (ANOVA), followed by the Tukey test at $\alpha = 0.05$. SPSS v. 20 was used for statistical analysis. An independent T-test was used to determine a statistically significant differences between the wild (Borneo 1, 2, 3, 6, 7) and farmed (Borneo 4.1 and 4.2) samples from the same haplotype.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was conducted to analyse the albumin protein molecular masses of the extracted *C. striata* according to Laemmli (1970) with modifications.

larities can be explained by common ancestry. Although the Sumatra samples were in a different clade from the other samples from main islands in Indonesia, they still share a common ancestor. Two sequences from NCBI were used as an outgroup. High bootstrap values supported the phylogenetic tree in each group branch. These data show that the phylogenetic tree construction has a high accuracy level, which confirming that *C. striata* from Indonesia shares a common ancestor.

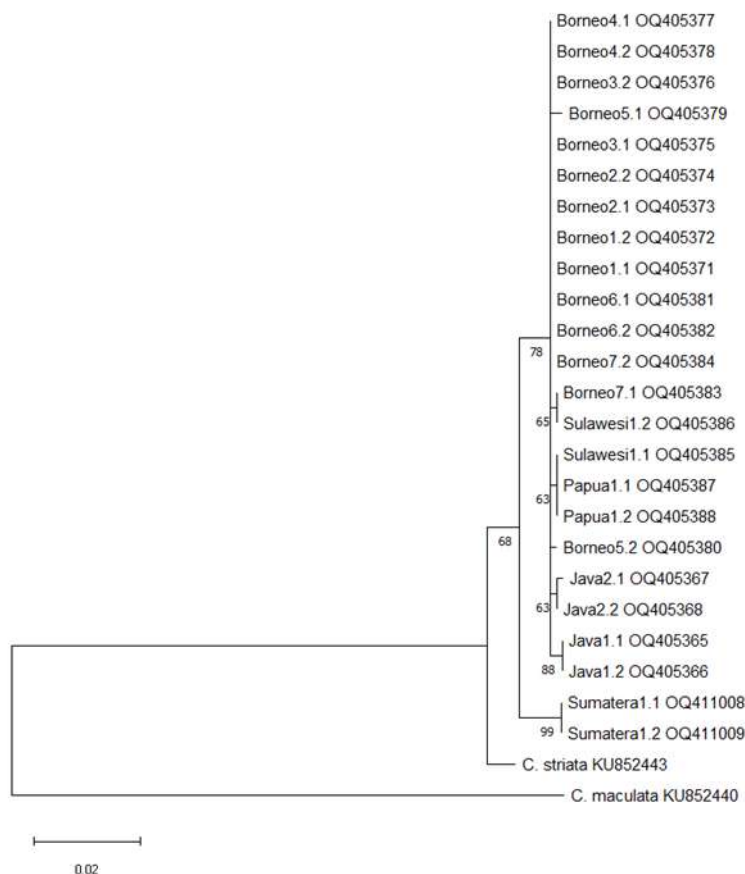


Figure 3. Phylogenetic tree of *C. striata* from the five main islands in Indonesia using Maximum Likelihood inferred from DNA sequences of the mitochondrial gene COI.

Albumin Content

Characterisation of albumin by SDS-PAGE showed that the albumin isolate consisted of seven protein bands with two major bands and five minor bands (Figure 4a). These protein bands ranged from 14 to 133 kDa. Two major bands were observed at 27 and 133 kDa, with one minor band at 14 kDa, and the three minor bands in the 25–39 kDa range. One minor band corresponds to the size of BSA at 66 kDa. This result aligns with Nugroho (2013), who conducted albumin isolation from *C. striata* by Bromocresol Green (BCG) and generated the same electrophoretic result band profiles, with two minor bands and five major bands in the range of 14–133 kDa. Meanwhile, other studies on albumin isolation in *Salmo salar* (Maillou & Nimmo 1993) resulted in a broad-spectrum fraction ranging from 46–145 kDa. Proteomic analysis of *C. striata* muscle tissue by Gam et al. (2006) found that the protein profiles of fish with different lengths and catch months did not differ significantly and were evenly distributed across a molecular masses from 10 to 205 kDa, detected in 16 protein bands. In addition, the identification of crude protein from *C. pleurophthalmus* by Aryani et al. (2020) showed 27 protein bands with molecular weights ranging from 10.202 to 134.432 kDa. Andreeva (2010)

stated that albumin identification in fish is rather difficult due to the atypical location of mobile plasma fractions in electropherograms, which do not correspond with the mobility of mammalian albumin. Low-molecular-weight fractions are often represented in electrophoresis by multiple bands or spots with unclear borders, overlapping the fractions. This statement leads to confusion in terminology, so some authors use the term albumin or albumin-like protein. All bands or fractions found in this study were considered total albumin, based on the broad spectrum of albumin in fish.

Figure 4b shows that the albumin content obtained in this study ranged from 1.253 to 1.907 % with an average value of 1.45 ± 0.15 %. The highest value was found in the Sumatra samples (1.907 ± 0.11 %), and the lowest in the Java 2 sample (1.253 ± 0.15 %). The Sumatra sample had the highest albumin content and was significantly different ($P < 0.05$) from the Borneo 4.1, Borneo 4.2, Borneo 6, Borneo 7, Java 2, Papua, and Sulawesi samples.

Genetic variation and albumin content analyses continued at the same taxonomic level, involving both wild and farmed *C. striata*. Based on the genetic variation and reconstruction of phylogenetic trees using mtDNA marker, all *C. striata* from the Borneo samples belonged to the same taxon, except for the sample labelled Borneo 5. Figure 4c shows that the albumin content of *C. striata* among the samples was not significantly different. The average albumin content of farmed *C. striata*, as represented by Borneo 4.1 and Borneo 4.2, was lower (1.20 ± 0.01 %) than that of wild *C. striata* (1.28 ± 0.05 %) but this difference was not statistically significant. This study indicates that *C.*

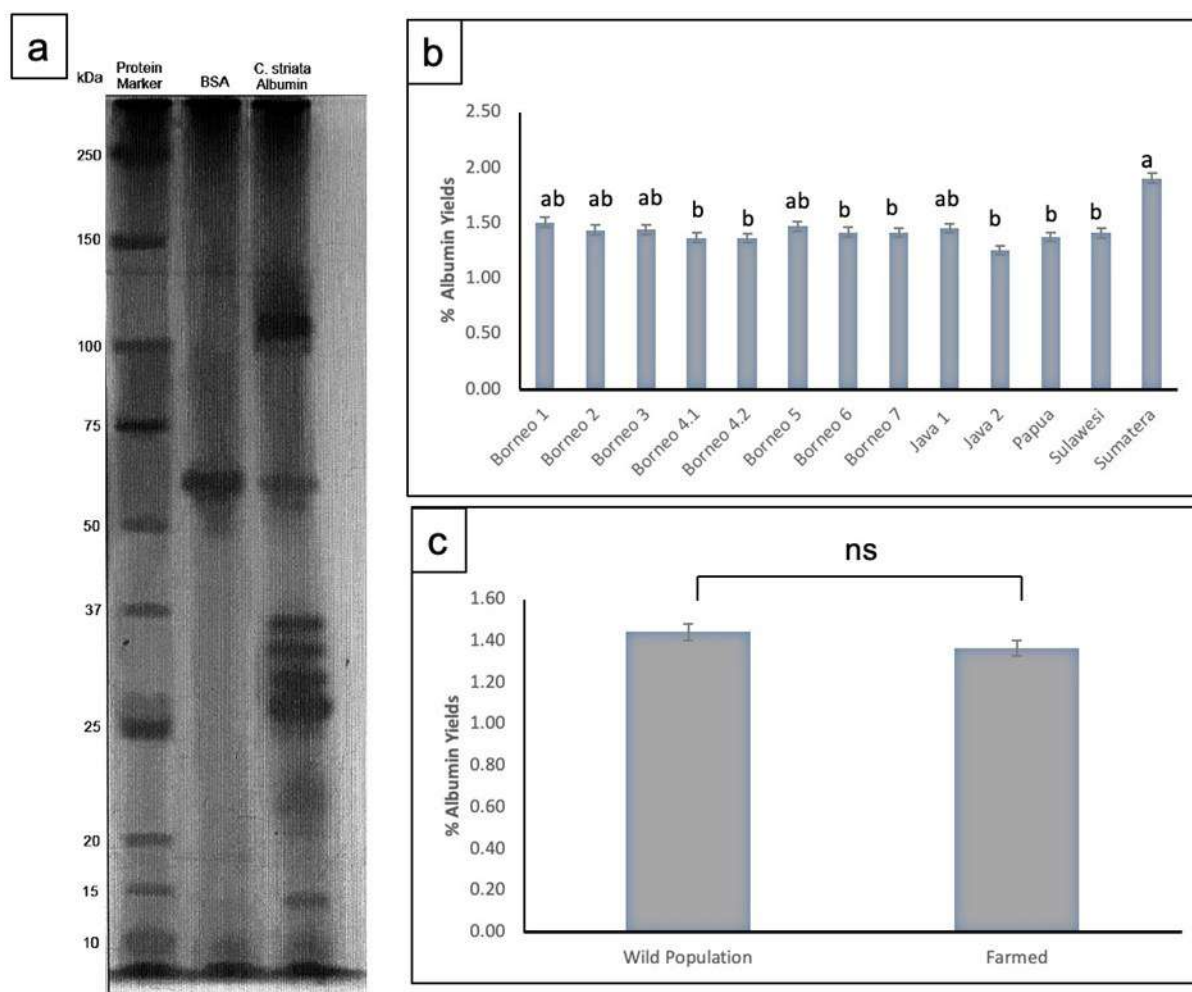


Figure 4. Albumin content of *C. striata* from the five main islands in Indonesia. (a) Analysis by Polyacrylamide Gel Electrophoresis of Albumin extracted from *C. striata* muscle compared with Bovine Serum Albumin and protein marker; (b) Albumin yield of *C. striata* from all locations. Significant differences ($P < 0.05$) shown by different superscripts; (c) Albumin concentration of *C. striata* from wild (Borneo 1, 2, 3, 6 and 7) and farmed (Borneo 4.1 and 4.2) under the same taxa samples ($P < 0.05$).

striata of the same genetic taxon, whether from aquaculture or the wild, have albumin contents that do not differ significantly. In addition, based on the proximate analysis (Table S1), the average total protein of *C. striata* meat was 17.07 ± 2.25 %, with the highest protein (21.3 %) found in the swamp sample from Borneo Island (Borneo 2). Furthermore, albumin in *C. striata* contributed 8.62 ± 1.45 % of the total protein.

In this study (Table S2), albumin extraction was also carried out on tilapia *Oreochromis niloticus*. The albumin yield of *O. niloticus* was lower than that of *C. striata* (0.57 ± 0.04 %). A previous study on the albumin concentration of various species by Fatma et al. (2020) found that *C. striata* had a higher albumin yield than *Decapterus russelli* and *Lates calcarifer*. This result suggests that albumin is abundantly found in *C. striata* and is higher than in certain fish species commonly found in brackish water and marine environments. Based on the evidence, this study proves that, using the same method and under the same circumstances, albumin extracted from *C. striata* muscle tissue is higher than that of *O. niloticus*, a common freshwater aquaculture commodity.

CONCLUSIONS

Based on the profile of genetic variations and albumin contents of *C. striata* in this study, it was found that *C. striata* from Sumatra are genetically formed in a different clade on the phylogenetic tree. The albumin content of *C. striata* in Indonesia varies; significant differences were found between the Sumatra samples and most Indonesian samples from Borneo, Java, Sulawesi, and Papua. There were no significant differences in the albumin content of *C. striata* from farmed and wild individuals of the same taxon. This research could serve as a foundational study and be considered in the development of aquaculture, e.g., broodstock selection, and the improvement of Indonesia's fisheries resource management. Furthermore, further studies on functional genomics can be developed from these findings. It is necessary to build up an understanding of the gene networks underlying important traits such as albumin content.

AUTHOR CONTRIBUTIONS

R.H., P., and D.W.K.S. performed experiments and/or data analyses. D.W.K.S. conceived the project and designed the experiments. R.H. and D.W.K.S. wrote the manuscript. All authors reviewed the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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APPENDICES

Table S1. Nutritional value of *C. striata* based on proximate analysis with the following methods: Kjeldahl for total protein; Soxhlet for total lipid, Gravimetry for ash and moisture; and by difference for carbohydrate

Samples	Ash (%)	Total Fat (%)	Carbohydrate (%)	Total Protein (%)	Moisture (%)
Borneo 1	1.37	0.8	2.8	18.06	76.97
Borneo 2	1.08	0.97	2.68	21.3	73.97
Borneo 3	0.91	1.65	2.23	16.38	78.83
Borneo 4.1	0.9	0.58	0.81	18.88	78.83
Borneo 4.2	0.7	0.47	1.21	17.31	80.31
Borneo 5	0.97	1.53	0.6	16.18	80.72
Borneo 6	1.03	0.73	1.54	18.59	78.11
Borneo 7	1.18	0.48	1.64	17.18	79.52
Java 1	1	0.54	1.55	18.64	78.27
Java 2	0.44	0.38	1.4	14.47	83.31
Papua	1.11	0.83	6.46	12.08	79.52
Sulawesi	1.23	0.18	2.65	16.65	79.29
Sumatra	0.72	0.53	0.67	16.24	81.84

Table S2. Yield of albumin extracted from *C. striata* muscle tissue. W0: initial weight; X Wt: Yield of albumin.

Samples	W0 (g)	X Wt (g)	% Yields
Borneo 1	40	0.604	1.509
Borneo 2	40	0.576	1.439
Borneo 3	40	0.576	1.441
Borneo 4.1	40	0.548	1.370
Borneo 4.2	40	0.548	1.369
Borneo 5	40	0.588	1.471
Borneo 6	40	0.567	1.418
Borneo 7	40	0.566	1.416
Java 1	40	0.583	1.457
Java 2	40	0.501	1.253
Papua	40	0.550	1.376
Sulawesi	40	0.565	1.412
Sumatra	40	0.763	1.907
Tilapia	40	0.228	0.570

Research Article

Upregulation of Glucomannan Synthesis Genes in Porang (*Amorphophallus muelleri* Blume) after Polyploidy Induction using Colchicine

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ABSTRACT

Inducing polyploidy in porang using colchicine has been explored and successfully enhanced the genetic variability of porang. However, the specific impact of polyploidy on the expression of the genes responsible for glucomannan synthesis in porang has not been extensively studied. Therefore, this study aims to evaluate the effect of polyploidy induction on the expression of genes responsible for glucomannan synthesis in porang. Porang shoot explants ($2n = 26$) were used for polyploidy induction. Polyploidy induction was carried out using colchicine added to the culture medium. The concentrations of colchicine used in this study were 0 %, 0.01 %, 0.02 %, 0.03 %, and 0.04 %. After 53 days in media without colchicine, explants were collected for flow cytometry analysis and RNA isolation. The application of colchicine at various concentrations induced tetraploid and mixoploid porang. Mutation induction using colchicine also increases the expression (upregulation) of the CSLA, GMPP, CSLD, and GMPP genes encoding glucomannan biosynthesis in porang. Tetraploid (0.01 % colchicine treatment) and mixoploid porang (0.02 % colchicine treatment) showed the highest expression of genes involved in glucomannan biosynthesis via both sucrose and invertase pathways.

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INTRODUCTION

Porang (*Amorphophallus muelleri* Blume) is a tuberous plant that belongs to the Araceae family. This tuberous plant is valued for its high content of glucomannan, a polysaccharide with various industrial (Zhang et al. 2022) and pharmaceutical applications (Ahmadi et al. 2022). Due to its versatile applications in the food, pharmaceutical, and industrial sectors, porang exports have steadily increased in recent years (Smith & Rahardjo 2021). This is also the result of the development of porang cultivation, both through intensification and extensification by the Indonesian government (Irianto et al. 2023). However, the continued use of porang seedlings only from the same source poses a risk to future porang cultivation because of reduced genetic diversity (Wahyudi et al. 2013), leading to vulnerability to pathogen attacks. Therefore, genetic modifications aimed at increasing genetic diversity, especially increasing glucomannan content, are needed.

Increasing glucomannan synthesis can be achieved via polyploidy induction. Polyploidy is a common phenomenon in plants (Anatskaya et al. 2022) and can lead to significant changes in their genetic (Manzoor et al. 2019) and physiological characteristics (Sabzehzari et al. 2019) and is also important in plant genetics and breeding (Touchell et al. 2020). Polyploidy induction has been reported to enhance the physiological activity of *Salvia officinalis*, leading to increased stress tolerance and higher content of secondary metabolites (Hassanzadeh et al. 2020).

The induction of polyploidy in porang using colchicine has been explored and shown to successfully enhance its genetic variability (Suyono et al. 2023). However, the specific impact of polyploidy on the expression of the genes responsible for glucomannan synthesis in porang has not been extensively studied. Previous research suggests that polyploidization can increase the overall metabolic activity in plants (Corneillie et al. 2019; Mohammadi et al. 2023), potentially influencing the biosynthesis of important compounds such as glucomannan.

The biosynthesis of konjac glucomannan (KGM) involves multiple steps, where various enzymes transform substrates such as sucrose into glucomannan. The process begins with photosynthates from the leaves transported to the tubers in the form of sucrose (Gille et al. 2011). Sucrose is then converted into glucomannan through the invertase (INV) pathway and the sucrose synthase (SUS) pathway. The sucrose pathway involves the cellulose synthase-like D (CSLD) and UDP-glucose pyrophosphorylase (UGP) genes, while the invertase pathway involves the cellulose synthase like-A (CSLA) and GDP-mannose pyrophosphorylase (GMPP) genes in its biosynthesis (Diao et al. 2014).

Detecting gene expression for glucomannan synthesis via sucrose and invertase pathways has been applied in various glucomannan-producing plants (Diao et al. 2014; Wu et al. 2021; Gao et al. 2022). High expression levels of CSLA, CSLD, UGP, and GMPP were observed during developmental stages 2 and 3 in *Amorphophallus konjac*, which are believed to play a major role in KGM biosynthesis (Gao et al. 2022). The enzyme UDP-glucose pyrophosphorylase (UGP) catalyses the formation of UDP (uridine diphosphate)-glucose, which functions as a substrate and glycosyl donor in the synthesis of β -glucan for the glucomannan chain (Yang et al. 2020). GDP-mannose pyrophosphorylase (GMPP) synthesises GDP-mannose, which is then transported to the Golgi apparatus and used as a substrate for the formation of CSLA and/or CSLD (Qi et al. 2022). Subsequently, the CSLD and CSLA genes encode the glycosyltransferase enzyme that synthesises β -1,4 glucan from UDP-glucose to form the main chain of cellulose and hemicellulose (glucomannan) (Yang et al. 2020). Therefore, CSLA, CSLD, UGP, and GMPP are commonly used to identify KGM biosynthesis in *Amorphophallus*.

Understanding the expression of genes related to glucomannan synthesis is important for evaluating the success of mutation induction using colchicine. This information is also critical for understanding the impact of induced mutations on glucomannan production, assessing the potential for improved yield or quality, and guiding further optimisation of genetic modification strategies. Therefore, this study aims to evaluate the effect of polyploidy induction on the expression of the glucomannan synthesis genes in porang (*A. muelleri* Blume).

MATERIALS AND METHODS

Research design and sample preparation

The experiment was conducted using a Completely Randomised Design (CRD) with five treatments and three replications, resulting in a total of 15 experimental units ($n = 15$). The factor used in this study was colchicine concentration with the following levels: 0.01 %, 0.02 %, 0.03 %, and 0.04 %. A control treatment without colchicine (0 %) was included as a comparison to evaluate the effect of colchicine application.

Porang shoot explants ($2n = 26$) were subcultured on Murashige and Skoog (MS) media at pH 6 with the addition of 2 mg L⁻¹ benzyladenine (BA). The explants were maintained at 21 °C under 24-hour fluorescent light of 1500 lux. Explants that had been cultured for 30 days were subsequently used for polyploidy induction.

Polyploidy induction

Young shoot explants of porang ($2n = 26$) were selected from in vitro cultures for polyploidy induction. Polyploidy induction was carried out using colchicine added to the culture medium. The stock solution was prepared at a concentration of 1 % by dissolving 280 mg of colchicine powder in 28 mL of distilled water. This stock solution was then diluted to obtain treatment solutions with concentrations of 0 %, 0.01 %, 0.02 %, 0.03 %, and 0.04 %. Colchicine added to a bottle containing 12.5 mL of MS media using a 0.22 µm Millipore filter. After 7 days under colchicine treatment, explants were transferred to MS medium without colchicine. After 53 days in media without colchicine, explants were ready for observation.

Ploidy level estimation using flow cytometry

A total of 25 mg of porang leaf explant was ground in a mortar containing 100 µL of LBO1 lysis buffer (15 mM Tris, 2 mM Na₂EDTA, 0.5 mM spermine.4 HCl, 80 mM KCl, 20 mM NaCl, 0.1 % (v/v⁻¹) Triton X-100 (pH 8)). The mixture was then incubated for 5 minutes at room temperature and filtered using a 30 µm nylon mesh to isolate the nuclei (Parsons et al. 2019). The filtrate was stained with 5 µL of propidium iodide fluorescent dye solution (50 µg mL⁻¹) (Ermayanti et al. 2018) and 2.5 µL RNase (20 mg mL⁻¹). The solution was then incubated on ice for 15 minutes and homogenised by vortexing. A of 15 sample was then analysed using a flow cytometer (Guava easyCyte), and the data were analysed using InCyte 3.1 software.

RNA Isolation and RT qPCR analysis

53-day-old explants were used for RNA extraction. Total RNA was extracted using RNeasy Pure Plant Plus Kit TIANGEN following the manufacturer's instructions. cDNA synthesis was performed using NEXscript RT 2X Master Mix. The mixture for cDNA synthesis consisted of 4 µL ddH₂O, 1 µL forward primer (50 µM), 1 µL reverse primer (50 µM), 4 µL RNA template, and 10 µL NEXscript RT 2X Master Mix. The mixture was then incubated at 53 °C for 45 minutes and continued at 95 °C for 5 minutes. The incubation was performed on a thermal cycler.

The RT-qPCR procedure was carried out following the HRM Analysis Kit (Evagreen) protocol from TIANGEN. The reaction system was prepared according to the protocol guide for the HRM Analysis Kit. The primers used for the RT-qPCR are listed in Table 1. The RT-qPCR procedure was carried out using a Bio-Rad CFX 96 with an initial denaturation at 95 °C for 2 minutes, denaturation of 95 °C for 10 seconds, and annealing 55-60 °C (Table 1) for 30 seconds, repeated for 40 cycles. The gene *elf-4a* served as an internal control.

Table 1. Primer sequence used for detection of glucomannan activity genes.

Primer	Sequence	Annealing (°C)
CSLD (Gao et al. 2022)	F: (5'-CGACTTCAAGATCTGCACCG-3') R: (5'-CTTCACCACCTCCTCCATGT-3')	58 °C
UGP (Coleman et al. 2007)	F: (5'-GCAACTTCAGATCTGCTTCTTG-3') R: (5'-TCCAATTTTCACACCAGATTTTG-3')	56 °C
CSLA 2 (Gao et al. 2022)	F: 5'-TGCTGAGGAGTGCTTGATGA-3' R: 5'-TGAAGCCGAAGAATGCATGG-3'	60 °C
GMPP 1 (Gao et al. 2022)	F: 5'-CTCCAGCAGTATCATCGGGT-3' R: 5'-AAATCTCATCGCCACATGC-3'	60 °C
<i>elf-4a</i> (Wang et al. 2017)	F: 5'-ACAAGATGAGGAGCAGGG-3' R: 5'-GGTGATAAGGACACGAGA-3'	55 °C

Statistical analysis

Flow cytometry data were analysed descriptively with InCyte 3.1 software, to observe the distribution of polyploid cells. Ploidy level was determined by comparing the peak curves between the control and the mutated porang. Gene expression analysis was carried out using Bio-Rad CFX Maestro 96 software with the Livak method (delta-delta Ct method). The relative expression of target genes (CSLD, UGP, CSLA2, and GMPP) was normalised to the housekeeping gene *elf-4a*. Analysis of variance (ANOVA), followed by Duncan test 95 % confidence, was performed on the relative expression data of target genes.

RESULTS AND DISCUSSION

Effect of colchicine on ploidy level

The application of colchicine with various concentrations (0 %, 0.01 %, 0.02 %, 0.03 %, and 0.04 %) increased the ploidy level of porang explants (*A. muelleri*) (Figure 1). Colchicine treatment induced the formation of both tetraploid ($4n = 4x = 52$) and mixoploid porang, in contrast to the diploid control ($2n = 2x = 26$) (Figure 1). However, most colchicine treatments resulted in mixoploid porang. Tetraploid porang was observed after 0.01 % colchicine treatment, whereas mixoploid porang resulted after 0.02 %, 0.03 %, and 0.04 % of colchicine treatment (Figure 1).

Colchicine treatments of 0.01-0.05 % are commonly used concentration and have been shown to produce tetraploid plants (Nilanthi et al. 2008; Wang et al. 2024). However, the response to colchicine differs depending upon the plant. For example, 0.01 % colchicine treatment successfully produced tetraploids in *Lilium regale* (Jeloudar et al. 2019), but 0.5 % colchicine is needed to induce tetraploid in *Trichosanthes dioica* (Hassan et al. 2020). Conversely, higher concentrations of colchicine have been associated with phytotoxic effects and reduced explant viability (Heo et al. 2016), underscoring the importance of optimising colchicine dosage for each species.

Several studies have confirmed the effectiveness of colchicine in producing polyploid plants with desirable traits. In fruit crops like blackberry (Sabooni et al. 2022) and bananas (Amah et al. 2019), colchicine treatment

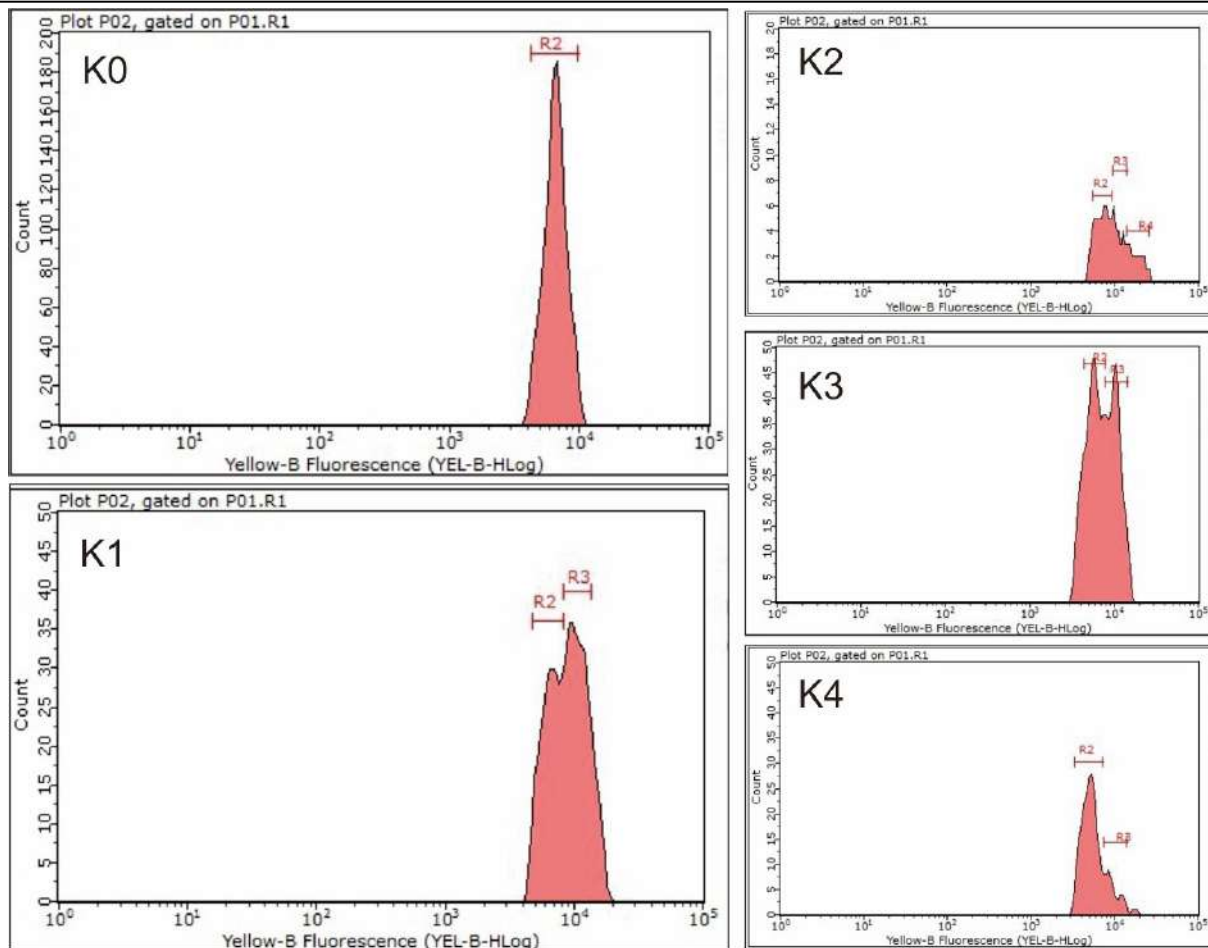


Figure 1. Ploidy category of porang (*A. muelleri*) after colchicine induction. K0: untreated porang (diploid peak (R2) of control porang close to channel 10000), K1: tetraploid porang after 0.01 % colchicine induction, K2: mixoploid porang after 0.02 % colchicine induction, K3: mixoploid porang after 0.03 % colchicine induction, K4: mixoploid porang after 0.04 % colchicine induction.

affects morphological characteristics and improves flavour profiles due to increased cell size and altered metabolic pathways. In ornamental plants, colchicine-induced polyploidy has enhanced flower size, colour intensity, and resistance to environmental stressors (Manzoor et al. 2019). In addition, colchicine-induced polyploidy also successfully changes the morphological characteristics (Widoretno et al. 2023) and genetic variability (Suyono et al. 2023) of porang.

Overall, colchicine is an effective mutagenic agent for chromosome doubling, providing agronomic benefits such as increased plant organ size and higher yield potential. However, despite its effectiveness, one significant challenge in its application is the occurrence of mixoploidy (Figure 1) (concentration 0.2-0.4 %). Mixoploidy refers to the presence of cells with different ploidy levels within the same organism, which can lead to uneven growth, developmental anomalies, and inconsistent trait expression (Marisa et al. 2012). This condition arises because colchicine treatments do not always result in uniform chromosomal duplication across all cells, particularly when the treatment duration, concentration, or method of application is not carefully optimised. This phenomenon occurs not only in porang but also in *Neolamarckia cadamba* (Eng et al. 2021), *Brassica* and *Capsicum annum* (Tammu et al. 2021), where mixoploid plants were produced after colchicine induction.

Mixoploidy is a common phenomenon in polyploidy induction using chemical agents such as colchicine (Tammu et al. 2021). In this study, colchicine concentrations of 0.02–0.04 % produced mixoploid plants, indicating partial success in chromosome duplication. However, the ploidy stability aspect of mixoploid plants has not been further studied. Mixoploidy stability is im-

portant to ensure that the induced genetic changes remain consistent during growth, regeneration, and both generative and vegetative reproduction. Mixoploid plants are also at risk of somatic instability, which is a change in ploidy proportions between tissues or over time due to uneven cell division (Marisa et al. 2012; Mehravi et al. 2022). In addition, selection of superior plants becomes difficult because gene expression and metabolite production can vary between plant parts (Marisa et al. 2012; Eng et al. 2021). Therefore, further evaluation of ploidy distribution between tissues and trait inheritance during the generative phase is crucial.

Although mixoploidy presents several limitations, it also holds potential applications, especially if it can be utilised vegetatively in cultivation systems. Several studies have shown that mixoploid plants can exhibit certain advantages, including tolerance to abiotic stresses such as drought and salinity (Mehravi et al. 2022). The presence of both diploid and polyploid cells may allow these plants to better cope with environmental challenges, thus increasing their survival rates under adverse conditions (Mondin et al. 2018). Mixoploid *Neolamarckia cadamba* has been shown to produce larger stomata and increased leaf area compared to its diploid counterparts, which can enhance photosynthetic efficiency and overall plant productivity (Eng et al. 2021). In addition, secondary metabolite production is higher in tissues with higher ploidy (Mohammadi et al. 2023).

In the context of porang, the vegetative cultivation system using tubers and bulbils (A'yun et al. 2019) allows for the practical use of mixoploids, as long as ploidy stability is maintained throughout the growth cycle. For this reason, it is necessary to carry out periodic ploidy monitoring using flow cytometry on various tissues, phenotypic evaluation under different field conditions to determine the consistency of growth, yield, and glucomannan content, and development of vegetative propagation protocols from tissues with dominant ploidy to produce stable and uniform clones (Barron et al. 2020).

Colchicine-induced gene expression involved in glucomannan synthesis

The expression of genes involved in glucomannan biosynthesis in this study was analysed in relation to the sucrose (SuS) and invertase (INV) metabolic pathways. Cellulose Synthase-Like D (CSLD) and UDP-glucose pyrophosphorylase (UGP) were involved in the sucrose pathway, whereas Cellulose Synthase Like-A (CSLA) and GDP-mannose pyrophosphorylase (GMPP) were associated with the invertase pathway. Colchicine induction with various concentrations (0.01 %, 0.02 %, 0.03 %, and 0.04 %) affected the activity of glucomannan synthesis via both sucrose (CSLD, UGP) and invertase (CSLA and GMPP) pathways in porang (*A. muelleri* Blume) (Figure 2). The increased expression of glucomannan biosynthesis genes observed after colchicine induction may be associated with ploidy changes in porang, specifically tetraploidy at 0.01 % and mixoploidy at 0.02 %.

Polyploid induction using colchicine increases the expression (upregulation) of the CSLA, UGP, CSLD, and GMPP genes involved in glucomannan biosynthesis in porang (*A. muelleri* Blume). However, polyploid induction using 0.01 and 0.02 % colchicine results in higher expression of genes related to glucomannan synthesis than other treatments (Figure 2). These results suggest that polyploid induction using colchicine at concentrations of 0.01 % (K1) and 0.02 % (K2) significantly enhances the expression of genes involved in glucomannan biosynthesis, namely CSLA, GMPP, and CSLD. The increased gene expression indicates a potential link between polyploidy and enhanced metabolic activity, leading to higher glucomannan accumulation in *A. muelleri*.

Furthermore, the elevated expression levels observed in K1 and K2 treatments suggest that chromosomal doubling may positively influence the

gene expression responsible for glucomannan synthesis. This effect could be attributed to increased gene dosage and regulatory changes in the expression of key biosynthetic enzymes (Madani et al. 2021). Comparing the different colchicine treatments, it is evident that the polyploidised plants exhibit higher expression of genes for glucomannan biosynthesis, which may have significant implications for breeding and commercial cultivation.

Colchicine treatment has also been shown to enhance glucomannan synthesis in various organisms, particularly in fungi and plants. In *Cunninghamella*, colchicine at concentrations up to 20 ppm accelerated sugar absorption and polysaccharide accumulation, indicating a potential increase in glucomannan synthesis (Salama & Naguib 1963). In *Arabidopsis*, the cellulose synthase-like A (CSLA) family, particularly CSLA2, CSLA3, and CSLA9, are crucial for glucomannan synthesis, with overexpression leading to increased glucomannan content (Goubet et al. 2009). Furthermore, the genome of *Amorphophallus konjac* reveals key genes involved in glucomannan biosynthesis, suggesting that genetic manipulation could further enhance production (Gao et al. 2022). These findings collectively suggest that colchicine may stimulate glucomannan synthesis through both metabolic pathways and modulating gene.

Although all genes showed increased expression after polyploidy induction, differences in expression levels between genes may reflect their regulatory positions or potential rate-limiting steps in the glucomannan biosynthesis pathway. Based on RT-qPCR results (Figure 2), the expression of CSLA and GMPP tended to increase significantly more than UGP and CSLD in the K1 and K2 treatments. This suggests that the invertase pathway likely plays a more dominant role in contributing to glucomannan biosynthesis in porang. However, biochemically, GMPP plays a crucial role as a producer of GDP-mannose, the primary sugar donor in glucomannan chain synthesis by glycosyltransferase enzymes (CSLA/CSLD) in the Golgi apparatus (Qi et al. 2022). Several previous studies have also shown that GMPP activity is a critical link in controlling carbon flow towards hemicellulose and storage polysaccharide biosynthesis (Wu et al. 2021; Gao et al. 2022). Low GDP-mannose availability due to limited GMPP expression can inhibit glucomannan chain elongation, even when CSLA expression is high. Therefore, GMPP can be considered the gene with the greatest potential to be the rate-limiting step in this biosynthetic pathway. Conversely, CSLA, as the primary catalyst in β -1,4-glucomannan chain formation, also plays an essential role. However, because its expression tends to be responsive to polyploidy treatment and is higher than that of GMPP, it is unlikely to be the primary limiting point under these treatment conditions.

Polyploidy induction has also been detected to affect increasing gene expression in several plants. Increasing the ploidy level from diploid to tetraploid has been shown to increase the expression of the podophyllotoxin (PTOX) gene in the leaves and stems of *Linum album* (Javadian et al. 2017). The increase in PTOX is also correlated with unregulated expression of several enzymes involved in PTOX biosynthesis, such as phenylalanine ammonia-lyase (PAL) and pinoresinol-lariciresinol reductase (PLR). In addition, several genes such as monoterpene synthase (MTS) and limonene synthase (LS) in *Citrus limon* have also been reported to be upregulated under tetraploid conditions. This increase aligns with the increased antioxidant activity observed in *Citrus limon* (Bhuvaneswari et al. 2020).

Although increased expression of glucomannan biosynthesis genes (CSLA, CSLD, UGP, and GMPP) after colchicine treatment in porang (*A. muelleri*) was detected in this research, the possible involvement of epigenetic regulation is also worth considering. One of the major epigenetic changes resulting from polyploidy is DNA methylation. In the autotetraploid plant

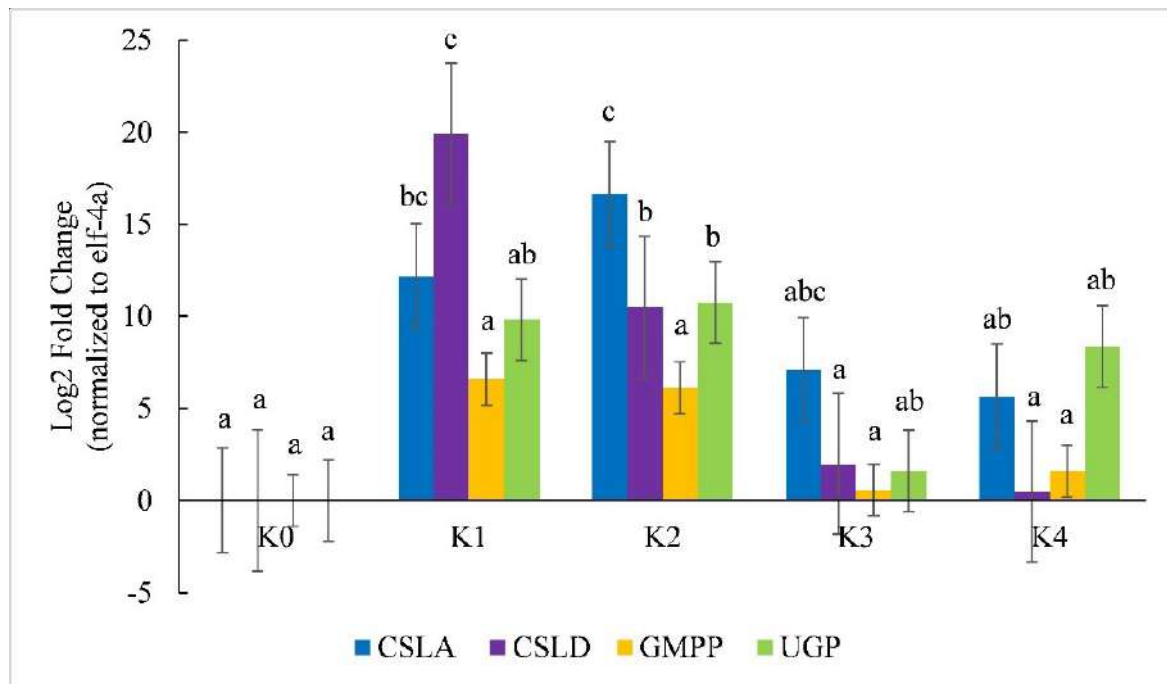


Figure 2. An increase in gene-related glucomannan synthesis after polyploid induction using colchicine. K0 = 0.00 %; K1 = 0.01 %; K2 = 0.02 %; K3 = 0.03 %; and K4 = 0.04 %. Different letters (a, b, c, d) indicate significant differences among groups ($p < 0.05$) based on ANOVA followed by Duncan test.

Brassica rapa, increased methylation was detected primarily at transposable elements (TEs) and gene regulatory regions (promoters and downstream regions), which can stabilise gene expression and prevent abnormal expression following “genomic shock” (Ma et al. 2025). Therefore, to test this hypothesis, further studies involving DNA methylation analysis are highly recommended. In addition, quantitative analysis of glucomannan content is also important to ensure that upregulation of this gene has a real impact on glucomannan biosynthesis.

The expression of CSLA, GMPP, CSLD and GMPP genes indicates that colchicine induction affects glucomannan biosynthesis through the invertase and sucrose pathways (Figure 3). Photosynthetically derived sucrose is synthesised in the leaves and transported to the tubers, where it can enter either the invertase pathway or the sucrose synthase pathway. In the invertase pathway, fructose-6-phosphate (Fru-6-P) is isomerised by phosphomannose isomerase (PMI) to generate mannose-6-phosphate (Man-6-P). Man-6-P is then converted to mannose-1-phosphate (Man-1-P) by phosphomannomutase (PMM) and further metabolised by GDP mannose pyrophosphorylase (GMPP), which catalyses the production of GDP mannose (GDP-Man) by GDP-mannose pyrophosphorylase (GMPP). The nucleotide sugars GDP-mannose, GDP-glucose, and ADP-glucose are transported into the Golgi apparatus, where they serve as substrates for glucomannan synthesis catalysed by CSLA and CSLD enzymes (Figure 3) (Qi et al. 2022).

Another advanced analysis such as transcriptomics may be needed to observe the effect of colchicine on gene expression profiles at a more comprehensive level. This approach allows for the identification of differentially expressed genes (DEGs) that are influenced by colchicine treatment, providing insights into the molecular pathways and biological processes affected (Zhou et al. 2017). Transcriptome analysis is also capable of detecting the differential expression of thousands of genes, particularly those involved in microtubule dynamics, cell division, and stress responses (Zhou et al. 2017). Furthermore, integrating transcriptomic data with proteomic and metabolomic analyses could offer a multidimensional understanding of colchicine's impact on cellular functions.

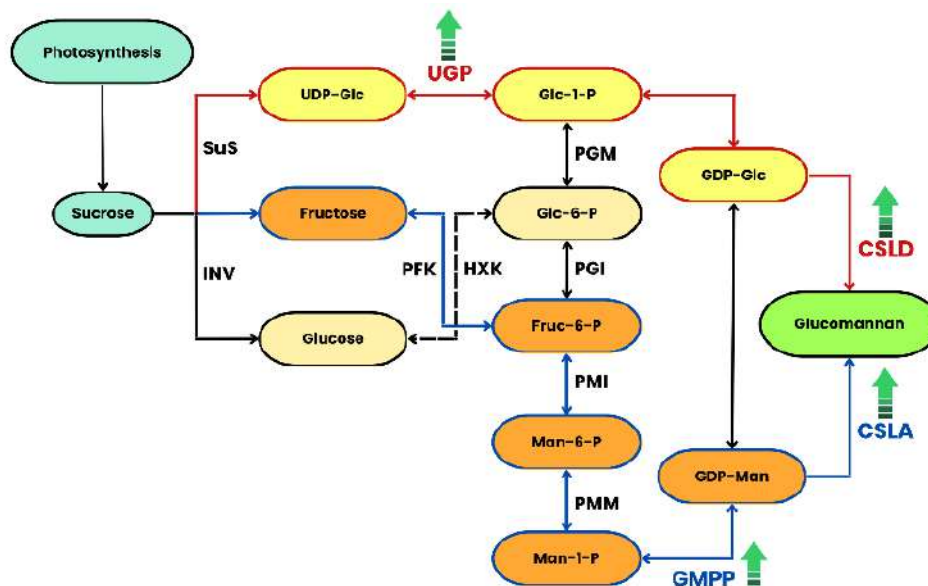


Figure 3. Pathway of glucomannan biosynthesis in porang.

CONCLUSIONS

Colchicine application in the range of 0.01 % to 0.04 % affects the ploidy level of porang explants, with 0.01 % inducing tetraploidy and higher concentrations (0.02–0.04 %) predominantly resulting in mixoploidy. Among all treatments, porang explants treated with 0.01 % and 0.02 % colchicine, resulting in tetraploid and mixoploid plants, respectively, showed the highest expression levels of genes involved in glucomannan biosynthesis through both the sucrose (CSLD, UGP) and invertase (CSLA, GMPP) pathways.

AUTHOR CONTRIBUTION

D.W. was responsible for the conceptualisation, methodology, data analysis, and writing of the original draft. S.Y.N. provided supervision, interpreted the data, and reviewed the manuscript. R.S.R. contributed to the experimental design, data collection, and manuscript review and editing. N.A.S., S.L.W. and D.R.K. conducted laboratory work and analysed data.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the research or its funding.

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Research Article

Records of Philippine Birds in the Critical Watersheds of Surigao del Norte, Northeastern Mindanao, Philippines

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ABSTRACT

The Philippines is recognised as a global biodiversity hotspot with exceptionally high bird endemism. However, many ecologically important regions, including those in Surigao del Norte, remain poorly studied. This study recorded birds in Baoy, Can-agtiw, and Agas-as watersheds with notes on diversity indices and notable and threatened species from August to September 2023. We utilised direct observation along established transect lines and mist-netting methods to record birds in the three watersheds. A total of 63 bird species belonging to 35 families and 14 orders were documented, including 33 Philippine endemics (52.38%). Overall diversity (H') was 3.101, and Baoy watershed recorded the highest number of individuals and richness (647 individuals with 44 spp.). Several locally threatened species were also documented, including two critically endangered species, i.e., Philippine hanging Parrot (*Loriculus philippensis*) and Blue-naped Parrot (*Tanygnathus lucionensis*); the endangered Mindanao Hornbill (*Penelopides affinis*); and five vulnerable species, i.e., Philippine Hawk-eagle (*Nisaetus pinskeri*), Writhed Hornbill (*Rhabdotorrhinus leucocephalus*), Philippine Dwarf Kingfisher (*Ceyx melanurus*), Southern Silvery Kingfisher (*Ceyx argentatus*), and the Mindanao Wattled Broadbill (*Sarcophanops steerii*). Although preliminary, the high number of endemic and threatened birds highlights the conservation value of these watersheds as important critical habitats. These findings emphasise the need for targeted conservation efforts, including immediate protection of these habitats and improving watershed monitoring strategies essential to safeguarding bird populations and ensuring long-term ecological sustainability.

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INTRODUCTION

The Philippines is among the world's most biodiverse nations and a recognised global hotspot, owing to its unique location and high species endemism in Southeast Asia (Mittermeier et al. 1999; Kittelberger et al. 2021). The country holds a record of over 743 species of birds, including 260 endemics. Of these, 182 are listed in the national conservation priority list (Wild Bird Club of the Philippines 2024) and 89 are globally threatened (IUCN 2024). Birds are integral to ecosystems, functioning as both predators and prey as well as contributing to ecological balance. They provide essential services such as pest control, seed dispersal, and ecosystem restoration (Mariyappan et al. 2023), while also serving as indicators of environmental health (Smits & Fernie 2013), particularly in vulnerable watersheds (Pacheco & Sanches Fernandes 2020).

Among the significant islands of the Philippines, Mindanao is regarded as a critical Endemic Bird Area (EBA). However, over the past decades, a significant portion of its tropical forest has experienced extensive deforestation, habitat degradation, and wildlife exploitation (Poffenberger & McGean 1993; Kittelberger et al. 2021). Extensive logging of Mindanao's dipterocarp-rich watersheds from the 1950s to 1970s caused severe upland erosion, lowland siltation, and increased flooding, effects that persist today (Poffenberger & McGean 1993). Adding to the pressure, the island is also widely recognised as a mining hub, where concessions extensively overlap with the critical remaining tropical rainforests (Paz & Mallari 2018). These threats degrade the habitats of many endemic, forest-dependent bird species, particularly those with narrow elevational ranges and larger body sizes, which are more prone to extinction (Kittelberger et al. 2021).

Surigao del Norte in Mindanao hosts three vital watersheds that sustain diverse wildlife and provide essential water resources and ecosystem services for local communities. The three critical watersheds (Baoy, Can-agtiw, and Agas-as) are situated in the Municipalities of Claver, Gigaquit, Malinomo, and Taganaan. These watersheds serve as the primary source of potable water and irrigation in these municipalities. Unlike other well-studied watersheds such as Agusan Marsh (Sucaldito-Salibad & Nuñez 2014), Bega Watershed (Calimpong & Nuñez 2015), and Lake Mainit Watershed (Lador & Seronay 2020), these three critical watersheds in Surigao del Norte remain unexplored in terms of bird studies. This knowledge gap is further underscored by the lack of formal protection and the limited management interventions. Thus, this study was carried out to record birds in these watersheds, with notes on biodiversity indices and highlighting notable and threatened species. Knowing the bird species and understanding bird ecology within watersheds contribute to effective conservation and management strategies. This study provides baseline information on vital bird species and the overall condition of forest ecosystems, which will be necessary in formulating local ordinances and establishing local conservation areas in the watersheds.

MATERIALS AND METHODS

The Study area and the Sampling stations

Surigao del Norte (1,952.62 km²) is located in the northeastern region of Mindanao, positioned between 125° 15' to 126° 15' east longitude and 9° 18' to 10° 30' north latitude. It is bordered to the north and east by the Pacific Ocean, while the south is bordered by the provinces of Agusan del Norte and Surigao del Sur. The study was conducted in three watersheds within Surigao del Norte, namely Baoy, Can-agtiw, and Agas-as (Figure 1 and Table 1). The wildlife Gratuitous Permit (GP) was secured from the Department of Environment and Natural Resources (DENR) (GP number R13-2023-58) prior to the survey.

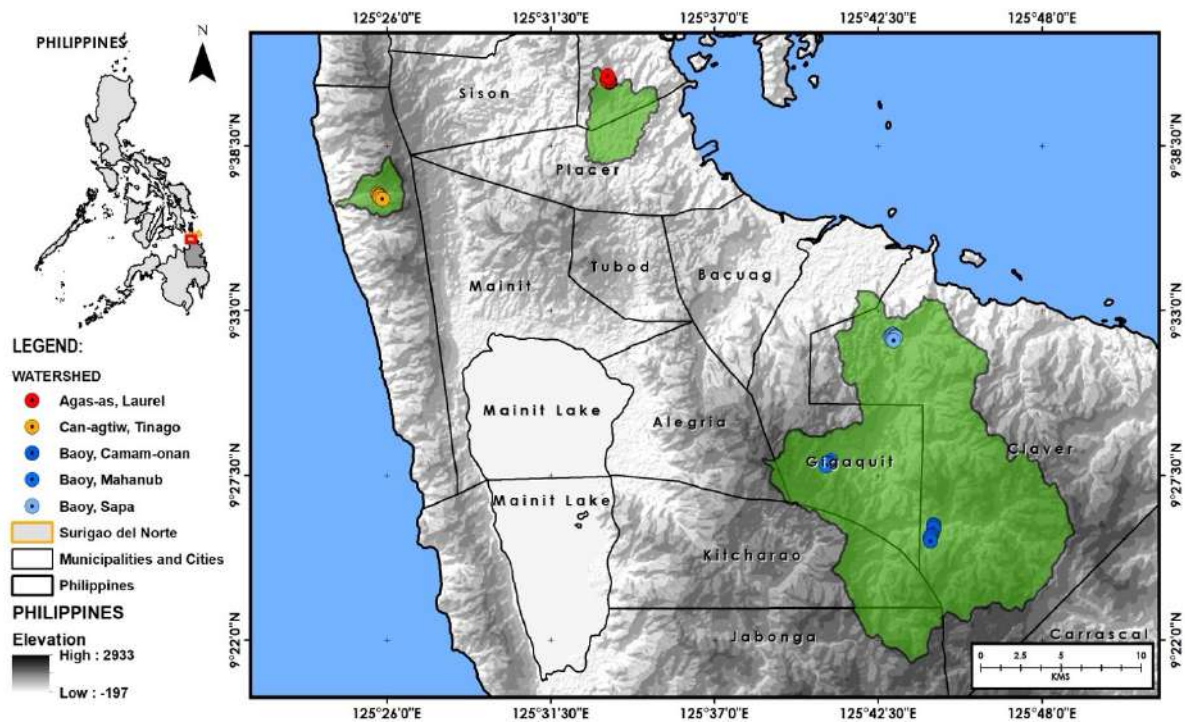


Figure 1. Map of the Surigao del Norte locating the Municipalities of the three watersheds: Claver and Gigaquit (Baoy), Tagana-an (Agas-as), and Malimono (Can-agtiw).

Table 1. Description of the sampling stations in Surigao del Norte, Caraga region, Philippines.

Watershed	Barangay	Municipality	Coordinates	Habitat Type
Baoy Watershed	Site 1: Camam-onan	Gigaquit	N 09°25'50" E 125°44'22"	Old-growth forest
	Site 2: Mahanub	Claver	N 9.465078 E 125.680559	Old-growth with patches of karst forest
	Site 3: Sapa	Claver	N 09°32.123 E 125°42.908	Agro-forest with patches of Karst forests
Can-agtiw Watershed	Tinago	Malimono	N 09°36.864 E 125°25.704	Secondary growth forest with patches of agro-forest
Agas-as Watershed	Laurel	Taganaan	N 09°40.609 E 125°33.478	Agro-forest

Data collection

Fieldwork was carried out from August to September 2023. Direct observations were conducted along established 2-km transects with a width of 100 m on either side (Gibbons & Gregory 2006; Sutherland 2006; Pitogo et al. 2024). At each sampling station, surveyed between 0500 to 0800 h and 1500 to 1740 h, coinciding with peak bird activity. Each site was surveyed twice on consecutive days, starting at the furthest end at dawn to minimize time-of-day bias with two to three observers. All birds seen or heard within a 50 m radius were documented. Binoculars, digital cameras, and telescopes were utilised to locate the birds at far distances within the area. Mist nets with three different mesh sizes were also used to capture birds: 6 x 12 m², 6 x 10 m², and 6 x 6 m² mist nets. The mist nets were strategically established along possible flyways and feeding grounds (fruit-bearing trees). The nets were opened from 0530 to 1730 h (Malaki et al. 2022) and were constantly checked every 30 minutes to 1 hour for possible catch, especially early in the morning and late in the afternoon, to avoid casualties. Species identification was aided by online photographic guides, bird call recordings, and books, including Birds of the Philippines (Allen 2020) and Guide to the Birds of the Philippines (Kennedy et al. 2000).

Data analysis

The Paleontological Statistical Software Package (PAST ver.4.03) was used to analysed species richness (n), number of individuals (N), Shannon-Wiener diversity (H'), evenness (E), and dominance (D). Community structure analysis was derived from mist-net captures. Community similarity was assessed using the Jaccard similarity index in Python 3.13.0, with values ranging from 0 (no similarity) to 1 (complete similarity).

RESULTS AND DISCUSSION

Bird species composition

The survey documented a total of 63 bird species from 14 orders belonging to 35 families (Appendix). The record constitutes 8.48 % of all recognised birds in the Philippines. Of the 63 recorded species, 31 species were captured using mist nets, and 50 species were recorded during the transect survey. The most represented orders are Passeriformes (20 families), and Coraciiformes and Piciformes (2 families). The order Passeriformes is by far the largest order of birds and is found on every continent, with the highest diversity in the tropics (Almeida et al. 2018). The family Columbidae (doves and pigeons) recorded the highest species richness (n = 7 spp.), followed by Alcedinidae (kingfishers, n = 5 spp.) and Nectariniidae (sunbirds and spiderhunters, n = 5 spp.). Columbids are remarkably adaptable, thriving across a wide range of environments. However, they are also among the most threatened bird families globally, with the Philippines recognised as a critical hotspot for Columbid diversity and conservation efforts (Walker 2007).

Among the records, 33 species (52.38 %) were endemic, while 30 species (47.62 %) were resident in the country. More importantly, six species were found to be restricted only to Mindanao (Figure 2). This includes the Writhed Hornbill (*Rhabdotorrhinus leucocephalus*), Philippine dwarf Kingfisher (*Ceyx melanurus*), Southern Silvery Kingfisher (*C. argentatus*), Mindanao Wattle-billed Broadbill (*Sarcophanops steerii*), Mindanao Blue Fantail (*Rhipidura supercilialis*), and White-browed Tailorbird (*Orthotomus nigriceps*), which is only restricted to the Caraga region and some parts of Davao del Sur.

Notable records in the Baoy watershed included a male Philippine Trogon (*Harpactes ardens*) observed in Brgy. Sapa (Figure 2A) and a female captured in Brgy. Camam-onan. This species, endemic to the Philippines and restricted to Basilan, Dinagat, and Mindanao, is currently undergoing a population decline (IUCN 2024). The Writhed Hornbill (*R. leucocephalus*) (Figure 2C), classified as Near Threatened by the IUCN and Vulnerable under the Philippine Red List (PRL), was observed in Sabang, where it was found in captivity at a local residence. Endemic bird species are key determinants of conservation strategies, as their restricted distribution heightens their vulnerability to environmental change (Alvarez-Alvarez et al. 2020), and the extinction of these species may initiate cascading effects that compromise overall biodiversity and ecosystem functioning (Gebremichael et al. 2022).

The results are comparable to the recorded bird species in Mts. Apo, Kitanglad, Hamiguitan, and Malindang (n = 65 spp.) (Nuneza et al. 2015) and in the Karst forest area in Bulacan (n = 63 spp.) (Duco et al. 2021). The species richness recorded across the three critical watersheds is likewise higher than those reported in previous studies, including the avifaunal assessment of eight waterfalls in Agusan del Sur (n = 59 spp.; Nuneza et al. 2018), Mt. Hamiguitan (n = 53 spp.; Ates et al. 2011), Mt. Pantaron in Bukidnon (n = 31 spp.; Salolog et al. 2021), and the mining areas of Agusan del Sur and Claver, Surigao del Norte (n = 47 spp.; Paz & Mallari 2018). These differences in recorded species richness may, in part, be influenced by variations in sampling effort, survey duration, and methodologies employed across studies.

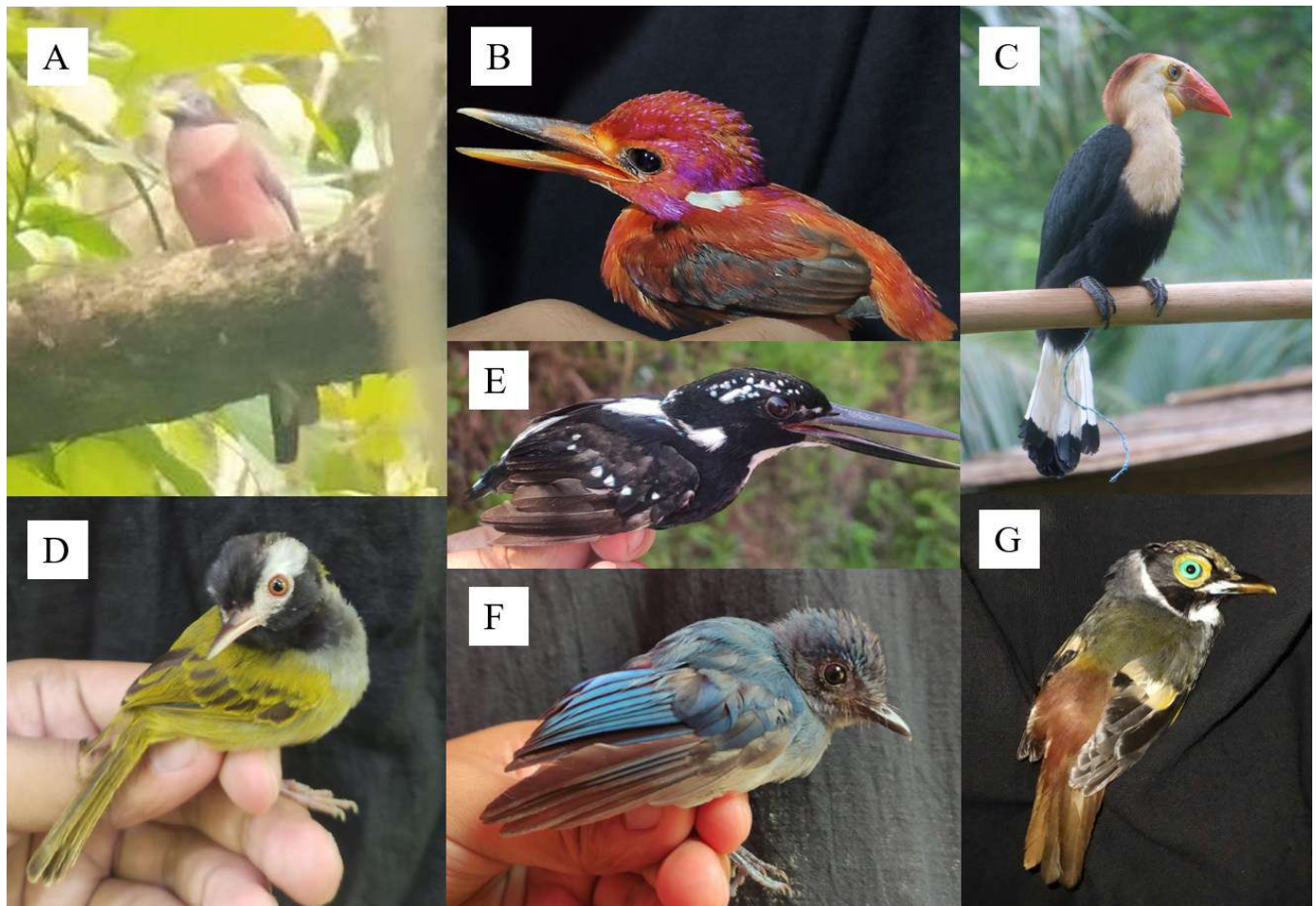


Figure 2. Notable birds restricted to Mindanao recorded in the three watersheds: (A) Male Philippine trogon, (B) Philippine Dwarf Kingfisher, (C) Wreathed Hornbill, (D) White-Browed Tailorbird, (E) Southern Silvery Kingfisher, (F) Mindanao Blue Fantail, and (G) Mindanao Wattled Broadbill.

Conservation status and Local distribution map of notable species

Among the documented species, four species are globally threatened under the IUCN Red List (6.35 %), and eight species are threatened under PRL (12.70 %). Two parrot species, the Philippine Hanging Parrot (*Loriculus philippensis*) and Blue-naped Parrot (*Tanygnathus lucionensis*), are classified as Critically Endangered, whereas the Mindanao Hornbill (*Penelopides affinis*) is listed as Endangered in the PRL (DENR Administrative Order (DAO) 2019-09 2019).

The spatial distribution of multiple threatened bird species across selected watersheds in Surigao del Norte, Philippines, is presented in Figure 3. Notably, the critically endangered, Philippine Hanging Parrot (*L. philippensis*) and the vulnerable Southern Silvery Kingfisher (*C. argentatus*) were recorded in all watersheds. The Baoy watershed recorded the highest number of threatened species ($n = 7$ spp.). Seven species were present in Camam-onan, while three species were present in Sapa, and none were recorded in Mahanub. The high number of threatened species in the Baoy watershed suggests its role as a suitable habitat with abundant resources. This is reinforced by its old-growth forest, interspersed with karst patches and agroforest areas, which provide diverse vegetation and structural complexity that support avian diversity. The Can-agtiw watershed supported five threatened species, whereas the Agas-as watershed recorded the fewest ($n = 3$ spp.). The lower number in Agas-as may be attributed to forest conversion to agriculture and increased anthropogenic disturbance.

The Baoy watershed is the largest surveyed site, which may contribute to the observed bias in the high number of species. However, records showed

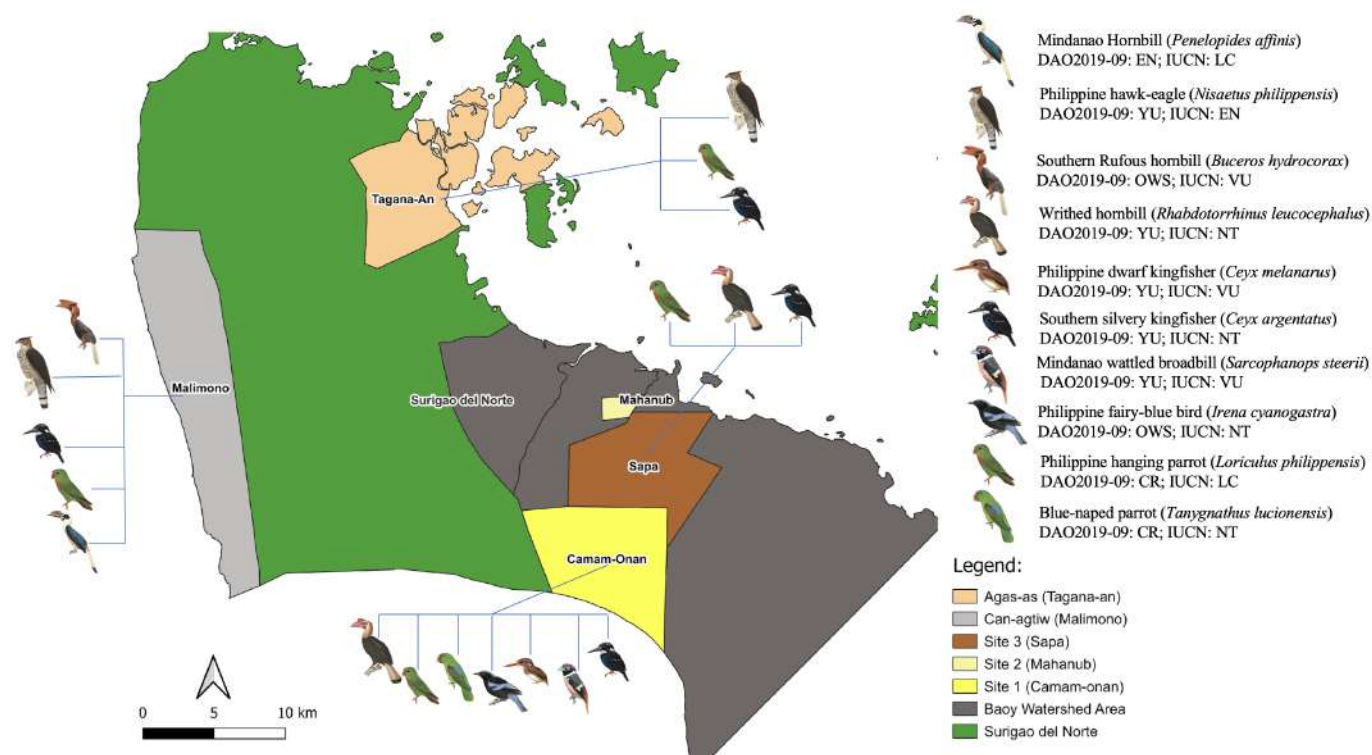


Figure 3. Local distribution map of notable and threatened species across the three watersheds. (PRL DAO2019-09: EN=Endangered, YU=Vulnerable, OWS=Other Wildlife Species, CR=Critically Endangered; IUCN: LC=Least Concern, EN=Endangered, VU=Vulnerable, NT=Near Threatened). Silhouettes from Birds of the World (2025).

several uncommon and threatened bird species, which may support the idea that uncommon birds thrive in undisturbed forests and are intolerant to forest disturbances (Arcilla & Strazds 2023). Although the Agas-as watershed also recorded a significant number of species, majority of the birds were common and abundant in disturbed areas, while some were not endemic to the area. Urban birds often exhibit smaller body size, broader dietary niches, and reduced territoriality, enabling them to exploit fragmented habitats and diverse food sources (Patankar et al. 2021). Furthermore, the higher elevation, particularly in old-growth forest areas, provides secure roosting sites for hornbills. Such environments reduce human disturbance, supporting their survival and shaping distinct evolutionary dynamics (Bastianelli et al. 2017; Laguë 2017).

Most of the species recorded were classified as Least Concern (87.30 %), indicating possible resilience to habitat disturbances and human activities. However, this current record of significantly high richness within these habitats also signifies the adaptive capacity of wildlife to coexist amid anthropogenic influences. Additionally, the cohabitation of globally and locally threatened species within these watersheds presents challenges for conservation efforts, as competition for resources, habitat degradation, and climate change may impact species differently, necessitating targeted management strategies (Tabora et al. 2023). This also underscores the importance of local conservation efforts in protecting watersheds and preserving threatened species, which play a vital role in maintaining biodiversity, ecosystem balance, and the overall health of the watershed.

Community structure and diversity metric

During the survey period, 31 bird species comprising 83 individuals were captured using mist nets. The most abundant in terms of number of individuals was the Yellow-vented Bulbul (*Pycnonotus goiaver*, N = 10), followed by the

Silvery Kingfisher (*C. argentatus*, $N = 8$), and the Philippine Bulbul (*Hypsipetes philippinus*, $N = 7$). It was also noted that *C. argentatus* and *P. goiaver* were present in all watersheds. *P. goiaver* is also a common and frequently encountered bird in lowland areas, especially in urban places such as Sapa and Laurel.

The community structure analysis in Table 2 showed that species richness (n) was highest in Site 1 of Baoy watershed ($n = 13$ spp.), while Agas-as watershed had the highest number of individuals ($N = 32$). The high species richness could be attributed to higher horizontal heterogeneity of vegetation (Carrasco et al. 2019). However, despite the high richness, most recorded birds were common lowland inhabitants such as *P. goiaver* and *Todiramphus chloris*, which are highly adaptable to anthropogenically altered environments. These species often thrive in urban landscapes, utilising them for foraging, nesting, and reproduction (Tanalgo et al. 2015). Similarly, other urban-adapted birds, including starlings, pigeons, and sparrows, dominate due to their ecological flexibility (Borgmann 2024). While urbanisation typically reduces overall biodiversity, these adaptable species proliferate in remaining niches, with non-native species occasionally establishing dominance, though they form only a minor portion of the avifauna (Thompson et al. 2022).

Species diversity ($H' = 2.48$) was highest at Site 1 in the Baoy watershed, likely due to its protection by local indigenous people and limited anthropogenic disturbance. The presence of old-growth and fruit-bearing trees (e.g., Dao, Fig) further supports high bird diversity, consistent with Batáry et al. (2014) and Joshi et al. (2012), who noted that tree diversity and vegetation complexity promote avian diversity. Similarly, Carrasco et al. (2019) reported a positive correlation between bird and plant species diversity, with denser canopies and varied vegetation supporting more bird species due to greater habitat complexity. Forests exhibiting diverse vegetation density levels, especially areas with native trees, tend to host a greater variety of bird species. Tall native tree species such as Dao (*Dracontomelon dao*), various Lauan (*Shorea* spp.), Magkuno (*Xanthostemon verdugonianus*) trees, and several Fig trees (*Ficus* sp.) are present across sampling sites. In contrast, the lowest diversity was recorded at Site 2 ($H' = 0.69$), partly due to heavy rainfall that reduced captures and bird observations during the survey. The overall dominance index was low ($D = 0.06$) and evenness was relatively high ($E = 0.72$), indicating few dominant species consistent with patterns influenced by elevation, habitat type, and disturbance (Alvarez-Alvarez et al. 2020).

The Jaccard similarity index revealed distinct patterns of bird species composition across the five sites (Table 3). The highest similarity (0.475) was observed between the old-growth forest (Baoy S1) and the agro-forest with patches of karst forest (Baoy S3), suggesting that modified landscapes adjacent to or interspersed with karst habitats may still retain a considerable proportion of forest-associated bird species. A moderate level of similarity was also found between Baoy S3 (agro-forest with karst patches) and Can-agtiw watershed (secondary growth forest with agroforest) (0.378), indicating that these structurally heterogeneous habitats support overlapping bird communities. In contrast, Agas-as (an agroforest habitat) exhibited relatively low similarity with old-growth habitats (0.185–0.222), suggesting a substantial turnover in species composition associated with land-use conversion.

Overall, these results indicate that while agroforestry systems and secondary forests retain a subset of forest species, old-growth and karst forest habitats harbour unique bird communities that are not well represented in more disturbed areas. This underscores the importance of conserving both intact and karst-associated forests to maintain avian biodiversity in the landscape.

Table 2. Diversity indices of captured birds in the three watersheds during the survey period.

Indices	Baoy			Can-agtiw	Agas-as	Total
	S1	S2	S3			
Species Richness (n)	13	2	8	10	11	31
Total number of individuals (N)	17	2	13	19	32	83
Species Dominance (D)	0.09	0.50	0.16	0.16	0.15	0.06
Species Diversity (H')	2.48	0.69	1.95	2.09	2.08	3.10
Species Evenness (E)	0.92	1	0.88	0.81	0.73	0.72

Table 3. Jaccard similarity index values showing pairwise similarity of bird communities across different watersheds in Surigao del Norte, Northeastern Mindanao, Philippines

	Baoy S1 (Camam-onan)	Baoy S2 (Camboayon)	Baoy S3 (Sapa)	Can-agtiw	Agas-as
Baoy S1	1.000	0.194	0.475	0.312	0.222
Baoy S2		1.000	0.241	0.176	0.185
Baoy S3			1.000	0.378	0.286
Can-agtiw				1.000	0.318
Agas-as					1.000

CONCLUSIONS

The inventory of watersheds in Surigao del Norte documented 63 species, including 33 Philippine endemics. Notably, eight species were threatened in the Philippine Red List, with *L. philippensis* and *C. argentatus* recorded in all watersheds. Despite their conservation importance, these watersheds are increasingly threatened by poaching, forest clearing for agriculture, expanding human settlement, logging, and the impacts of mining. Although the results are preliminary, the high number of endemic and threatened birds highlights the conservation value of these watersheds. The necessity of including conservation efforts in the watershed management plans is further emphasised by the cohabitation of native and endangered species. Consequently, the designation of this area as a Local Conservation Area is highly recommended to ensure the conservation of its invaluable biodiversity. Considering these findings, the study offers a detailed understanding of bird populations within the three watersheds. This information is vital for shaping targeted conservation and management strategies to preserve the Philippines' significant bird diversity.

AUTHOR CONTRIBUTION

S.C.R. designed the research, collected and analysed the data, and wrote the manuscript; E.G. designed the research, collected and analysed the data, and wrote the manuscript; J.J.A.G. collected and analysed the data; J.R. designed the research, analysed the data, and wrote the manuscript; R.S. designed the research, acquired the funding and supervised the entire process; C.N. designed the research, analysed the data, and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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APPENDICES

Table S1. Species composition, conservation status, endemism, and occurrence of birds across the surveyed watersheds of Surigao del Norte, Northeastern Mindanao, Philippines.

Taxa	Common Name	Conservation Status			Watersheds					
		DAO 2019-09	IUCN, Popu- lation Trend	Endemism	Baoy			Can- agtiw	Agas- as	
					S 1	S 2	S3			
Accipitriformes										
Accipitridae										
1.	<i>Haliastur indus</i> (Boddaert, 1783)	Brahminy Kite °	LC, ↓	Resident	+	-	+	+	+	+
2.	<i>Nisaetus pinsheri</i> (Preleuthner and Gamauf, 1998)	Philippine Hawk-eagle °	EN, ↓	Endemic	-	-	-	+	+	+
Bucerotiformes										
Bucerotidae										
3.	<i>Penelopides affinis</i> Tweeddale, 1877	Mindanao Hornbill °	LC, ↓	Endemic	-	-	-	+	+	-
4.	<i>Buceros mindanensis</i> Tweeddale, 1877	Southern Rufous Hornbill °	VU, ↓	Endemic	-	-	-	+	+	-
5.	<i>Rhabdotorrhinus leucocephalus</i> (Vieillot, 1816)	Wriathed Hornbill °	NT, ↓	Endemic	+	-	+	-	-	-
Caprimulgiformes										
Apodidae										
6.	<i>Collocalia troglodytes</i> Gray, 1845	Pygmy swiftlet °	LC, ↓	Endemic	+	+	+	+	+	+
Columbiformes										
Columbidae										
7.	<i>Chalcophaps indica</i> (Linnaeus, 1758)	Asian Emerald Dove °	LC, ↓	Resident	+	-	-	-	-	+
8.	<i>Macropygia tenuirostris</i> Bonaparte, 1854	Philippine Cuckoo dove °	LC, ↓	Resident	+	-	-	+	+	+
9.	<i>Streptopelia tranquebarica</i> (Hermann, 1804)	Red-collared Dove °	LC, ↓	Resident	-	-	-	-	-	+
10.	<i>Spilopelia chinensis</i> (Scopoli, 1786)	Spotted dove °	LC, ↑	Resident	-	-	-	-	-	+
11.	<i>Phapitreron leucotis</i> (Temminck, 1823)	White-eared Brown dove °	LC, -	Endemic	+	+	+	+	+	+
12.	<i>Ramphiculus occipitalis</i> (Gray, 1844)	Yellow-breasted Fruit Dove °	LC, ↓	Endemic	-	-	-	-	-	+
13.	<i>Geopelia striata</i> (Linnaeus, 1766)	Zebra Dove °	LC, -	Resident	-	-	+	-	-	-

Table S1. Contd.

Taxa	Common Name	Conservation Status			Watersheds				
		DAO 2019-09	IUCN, Popu- lation Trend	Endemism	Baoy				
					S 1	S 2	S 3	Can- agtiw as	Agas- as
Coraciiformes									
Alcedinidae									
14.	<i>Halcyon gularis</i> (Kuhl, 1820)	OWS	LC, ?	Endemic	+	+	+	+	+
15.	<i>Todiramphus chloris</i> (Boddaert, 1783)	OWS	LC, ↓	Resident	+	-	+	-	+
16.	<i>Ceyx melanurus</i> (Kaup, 1848)	YU	VU, ↓	Endemic	+	-	-	-	-
17.	<i>Ceyx argentatus</i> Tweeddale, 1877	YU	NT, ↓	Endemic	+	-	-	+	+
18.	<i>Pelargopsis capensis</i> (Linnaeus, 1766)	OWS	LC, ↓	Resident	+	-	+	-	-
Meropidae									
19.	<i>Merops philippinus</i> Linnaeus, 1766	OWS	LC, -	Resident	-	-	+	-	+
20.	<i>Merops americanus</i> P.L.S. Müller, 1776	OWS	LC, -	Endemic	+	+	+	-	-
Cuculiformes									
Cuculidae									
21.	<i>Cacomantis merulinus</i> (Scopoli, 1786)	OWS	LC, -	Resident	-	-	+	-	-
22.	<i>Eudynamys scolopaceus</i> (Linnaeus, 1758)	OWS	LC, -	Resident	+	-	+	-	-
23.	<i>Centropus melanops</i> Lesson, 1830	OWS	LC, ↓	Endemic	+	-	+	-	-
24.	<i>Centropus viridis</i> (Scopoli, 1786)	OWS	LC, -	Endemic	-	-	+	-	+
Galliformes									
Phasianidae									
25.	<i>Gallus gallus</i> (Linnaeus, 1758)	OWS	LC, ↓	Resident	-	-	-	+	+
Gruiformes									
Rallidae									
26.	<i>Hypotaenidia torquata</i> (Linnaeus, 1766)	OWS	LC, -	Resident	-	-	-	-	+
Passeriformes									
Artamidae									
27.	<i>Artamus leucorynchus</i> (Linnaeus, 1771)	OWS	LC, -	Resident	-	-	+	+	-
Campephagidae									
28.	<i>Lalage nigra</i> (Forster, 1781)	OWS	LC, ↓	Resident	-	-	+	+	+

Table S1. Contd.

Taxa	Common Name	Conservation Status			Watersheds				
		DAO 2019-09	IUCN, Population Trend	Endemism	Baoy		S3	Can- agtiw	Agas- as
Cisticolidae									
29.	<i>Orthotomus nigriceps</i> Tweeddale, 1878	OWS	LC, -	Endemic	-	-	-	+	+
Corvidae									
30.	<i>Corvus macrorhynchos</i> Wagler, 1827	OWS	LC, -	Resident	+	+	+	+	+
Dicaeidae									
31.	<i>Dicaeum trigonostigma</i> (Scopoli, 1786)	OWS	LC, -	Resident	-	-	-	-	+
32.	<i>Dicaeum austral</i> (Hermann, 1783)	OWS	LC, -	Endemic	-	-	+	+	+
Estrildidae									
33.	<i>Lonchura leucogastra</i> (Blyth, 1846)	OWS	LC, -	Resident	-	-	+	-	+
Eurylaimidae									
34.	<i>Sarcophanops steerii</i> (Sharpe, 1876)	YU	VU, ↓	Endemic	+	-	-	-	-
Hirundinidae									
35.	<i>Hirundo tahitica</i> Gmelin, 1789	OWS	LC, ?	Resident	+	+	+	+	-
Irenidae									
36.	<i>Irena cyanogastra</i> Vigors, 1831	OWS	NT, ↓	Endemic	+	-	-	-	-
Locustellidae									
37.	<i>Megalurus palustris</i> Horsfield, 1821	OWS	LC, ?	Resident	-	-	+	-	-
38.	<i>Cincloramphus timoriensis</i> (Wallace, 1864)	OWS	LC, -	Resident	-	-	+	-	+
Monarchidae									
39.	<i>Hypothymis azurea</i> (Boddaert, 1783)	OWS	LC, -	Resident	-	-	-	-	+
Nectariniidae									
40.	<i>Aethopyga bella</i> Tweeddale, 1877	OWS	LC, -	Endemic	+	-	-	-	-
41.	<i>Aethopyga pulcherrima</i> Sharpe, 1876	OWS	LC, -	Endemic	+	-	-	-	-
42.	<i>Cinnyris jugularis</i> (Linnaeus, 1766)	OWS	LC, -	Resident	+	-	-	+	+
43.	<i>Arachnothera flammifera</i> Tweeddale, 1878	OWS	LC, -	Endemic	-	-	-	-	+
44.	<i>Leptocoma sperata</i> (Linnaeus, 1766)	OWS	LC, -	Endemic	+	-	+	-	-
Oriolidae									
45.	<i>Oriolus chinensis</i> Linnaeus, 1766	OWS	LC, ↓	Resident	-	-	+	-	+

Table S1. Contd.

Taxa	Common Name	Conservation Status			Watersheds					
		DAO 2019-09	IUCN, Popu- lation Trend	Endemism	Baoy			Can- agtiw	Agas- as	
					S 1	S 2	S3			
Pachycephalidae										
46.	<i>Pachycephala philippinensis</i> (Walkden, 1872)	OWS	LC, ↓	Endemic	-	-	-	-	+	+
Passeridae										
47.	<i>Passer montanus</i> (Linnaeus, 1758)	OWS	LC, ↓	Resident	-	-	-	-	-	+
Pitidae										
48.	<i>Erythropitta erythrogaster</i> (Temminck, 1823)	OWS	LC, ↓	Endemic	+	-	-	-	-	-
Pycnonotidae										
49.	<i>Hypsipetes Philippinus</i> (Forster, 1795)	OWS	LC, -	Endemic	-	-	-	-	+	+
50.	<i>Hypsipetes everetti</i> (Tweeddale, 1877)	OWS	LC, -	Endemic	-	-	-	+	-	-
51.	<i>Pycnonotus goiavier</i> (Scopoli, 1786)	OWS	LC, ↑	Resident	-	+	+	+	+	+
52.	<i>Poliolophus urostictus</i> (Salvadori, 1870)	OWS	LC, -	Endemic	+	-	-	-	+	-
Rhipiduridae										
53.	<i>Rhipidura superciliaris</i> (Sharpe, 1877)	OWS	LC, -	Endemic	-	-	-	-	+	-
Sturnidae										
54.	<i>Sarcops calvus</i> (Linnaeus, 1766)	OWS	LC, ?	Endemic	-	-	-	-	+	-
Timaliidae										
55.	<i>Macronus striaticeps</i> Sharpe, 1877	OWS	LC, ↓	Endemic	-	-	-	-	+	-
Pelecaniformes										
Ardeidae										
56.	<i>Bubulcus ibis</i> (Linnaeus, 1758)	OWS	LC, ↑	Resident	-	-	-	+	-	-
Piciformes										
Megalaimidae										
57.	<i>Psilopogon haemacephalus</i> (Müller, 1776)	OWS	LC, ↑	Resident	-	-	-	+	+	+
Picidae										
58.	<i>Dryocopus javensis</i> Tristan, 1879	OWS	LC, ↓	Resident	-	-	-	-	+	-
Psittaciformes										
Psittacidae										
59.	<i>Loriculus philippensis</i> (Müller, 1776)	CR	LC, ↓	Endemic	+	-	-	+	+	+
60.	<i>Tanygnathus lucionensis</i> (Linnaeus, 1766)	CR	NT, ↓	Resident	+	-	-	+	-	-
61.	<i>Bolbopsittacus lunulatus</i> (Scopoli, 1786)	OWS	LC, -	Endemic	+	-	-	+	+	+

Table S1. Contd.

Taxa	Common Name	Conservation Status		Watersheds				
		DAO 2019-09	IUCN, Popu- lation Trend	Endemism	Baoy			Can- agtiw as
					S 1	S 2	S 3	
Strigiformes								
Strigidae								
62.	<i>Otus everetti</i> (Tweeddale, 1897)	OWS	LC, ↓	Endemic	-	-	+	-
Trogoniformes								
Trogonidae								
63.	<i>Harpactes ardens</i> (Temminck, 1826)	OWS	LC, ↓	Endemic	+	-	+	-

Legend: Conservation status: IUCN Red List of Threatened Species: LC-Least Concern, NT-Near Threatened, VU-Vulnerable, CR-Critically Endangered, EN-Endangered; DAO: OWS-Other Wildlife Species, YU-Vulnerable; Population Status: ↓-Decreasing, ↑-Increasing, “-”- Stable, ?-Unknown, “-”- captured, “-”-observed/ sighted; Occurrence: “+”- Observed, “-”-Not Observed.

Research Article

Deciphering Bacterial and Organic Acids Shift during Fermentation of *Porang* (*Amorphophalus oncophylus*) Tubers

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ABSTRACT

Porang serves as a valuable export commodity as it is a rich source of glucomannan. Fermentation can be an alternative way to increase glucomannan content. Bacterial shift during fermentation could determine the safety of fermented product. This study aims to decipher bacterial and organic acids shift during fermentation of *porang* (*Amorphophalus oncophylus*) tubers. The bacterial diversity during fermentation was determined using a metagenomic approach with amplification of the V1–V9 regions of the 16S rRNA gene, followed by sequencing on the Oxford Nanopore platform. Ultra Performance Liquid Chromatography (UPLC) was employed to detect acetic and lactic acid. It was observed that Pseudomonatoda (syn Proteobacteria) was the dominant phylum during fermentation while Gammaproteobacteria and Enterobacteriales were the dominant class and order, respectively. Enterobacteriaceae was the dominant family until 48 hours of fermentation and shifted with Pectobacteriaceae at 72 hours of fermentation. *Kalamiella piersonii*, *Pantoea dispersa*, *Acinetobacter calcoaceticus* had high relative abundance at 0 hours fermentation while *Enterobacter cloacae* complex, *Novosphingobium guangzhouense*, *Kluyvera intermedia* had high relative abundance at 24 hours of fermentation. The highest relative abundance within 48 hours of fermentation was observed for *Leuconostoc falkenbergense*, a lactic acid bacterium, followed by *Dickeya fangzhongdai*, *Enterobacter cloacae* complex. *Dickeya fangzhongdai*, a pectinolytic bacterium, was dominant at 72 hours of fermentation followed by *Massilibacillus massiliensis* and *Leuconostoc falkenbergense*. Lactic acid was detected at 72 hours of fermentation while acetic acid increased during fermentation. Since the lactic acid bacteria (*L. falkenbergense*) are abundant, *porang* tubers fermentation should be terminated at 48 hours fermentation for safety.

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INTRODUCTION

Porang flour contains glucomannan, which has been widely used in various applications for its safety, film-forming capacity, gel-forming characteristic, favourable biocompatibility, and environmental sustainability (N. Zhou et al. 2022). Common methods for processing *porang* tubers are physical (dry method) or chemical (wet method) approaches (Wardhani et al. 2020). However, processing *porang* tubers with chemical hydrolysis, enzymes, physical degradation and irradiation has disadvantages. For example, chemical degradation requires a long processing time and can cause environmental pollution (Chua et al. 2012; Jin et al. 2014), enzyme-based hydrolysis is associated with high expenses (Jin et al. 2014; Kumoro et al. 2020), and physical or irradiation methods necessitate complex equipment.

Processing *porang* tubers through fermentation may serve as an effective approach to produce fermented flour with improved nutritional and functional qualities (Darmadi et al. 2020; Cuamatzin-garcía et al. 2022). Fermentation treatment of *porang* tubers has been carried out with the addition of 5 to 25 % of 10^8 CFU mL⁻¹ *Lactobacillus plantarum*, but the variables observed have been limited to protein content, yield and total LAB in *porang* flour (Juliana et al. 2020). Supplementation with bacteria *Bacillus subtilis* and yeast *Saccharomyces cerevisiae* in processing *porang*'s tubers showed that fermented *porang* flour had higher glucomannan content, carbohydrate, and fibre content than *porang* flour without fermentation. Moreover, the fermented *porang* flour exhibited antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* and promoted the growth of *Lactobacillus acidophilus* (Helmi & Karsiningsih 2024).

The diversity of bacteria in fermented foods can influence their safety. The existing of Proteobacteria in fermented foods may indicate that the food does not meet safety standards for consumption (Keisam et al. 2019; Cason et al. 2020; Li et al. 2021). The presence of spoilage or pathogenic bacteria such as *Halanaerobium* in fermented seafood (Lee et al. 2014; Dai et al. 2018), *Salmonella*, *Listeria monocytogenes* in cheese (Escobar-Zepeda et al. 2016), *Escherichia*, *Shigella*, and *Klebsiella* in beer (Cason et al. 2020) made a consideration the correct time to stop fermentation. Previous studies revealed that dynamics population of bacteria occurred during fermentation of beer (Cason et al. 2020), shrimp paste or shrimp sauce (Lee et al. 2014; Phewpan et al. 2020; Li et al. 2021; Helmi et al. 2022), cheese (Fuka et al. 2013; De Pasquale et al. 2014).

The incorrect fermentation termination time and inappropriate fermentation processes, such as insufficient salt content in shrimp sauce, may influence the bacterial community dynamics. For instance, shrimp sauce fermented with suboptimal (low) salt concentrations can promote the proliferation of *Halanaerobium*, which leads to increased levels of trimethylamine (Lee et al. 2014). At first stage of fermentation of some fermented food such as shrimp paste and beer, Proteobacteria frequently had high abundance but decreased its abundance during fermentation (Dai et al. 2018; Cason et al. 2020). A decline in Proteobacteria accompanied by an increase in Firmicutes during fermentation are expected during fermentation to result desired and safety products. Dominance of lactic acid bacteria from the phylum Firmicutes, including *Lactobacillus* and *Leuconostoc*, plays a crucial role in determining food safety (Cason et al. 2020). Lactic Acid Bacteria (LAB) recognise as GRAS (Generally Recognised as Safe) (Plavec & Berlec 2020; Liu et al. 2021). LAB produces antibacterial agents namely bacteriocins, hydrogen peroxide, and organic acids that suppress the growth of pathogenic and spoilage bacteria (Köberl et al. 2019; Plavec & Berlec 2020; Ibrahim et al. 2021; Yap et al. 2022).

Bacterial diversity and metabolites in fermented food can define the good and poor of fermentation products (Hong et al. 2016). The balance be-

tween early heterofermentative LAB species (*Leuconostoc mesenteroides*) and later acid-tolerant *Lactobacillus plantarum* ensures optimal kimchi flavour and aroma, whereas increased oxygen or failure to suppress contaminant growth (e.g., coliforms, *Pichia*) can lead to gas accumulation, mucilaginous texture, and off-flavours (Lee et al. 2020; Kwon et al. 2025). In fermented shrimp or fish sauces, dominance of *Halanaerobium* during mid-stage fermentation is linked to elevated trimethylamine (TMA) formation whereas a more balanced microbial community—featuring genera like *Tetragenococcus*, *Lentibacillus*—produces favourable volatile compounds (e.g. pyrazines) and free amino acids, resulting in desirable sensory attribute (Lee et al. 2014; Du et al. 2019; Helmi et al. 2022). By investigating the shift of bacterial population during fermentation, it can be determined the correct time to stop the fermentation to avoid the availability of pathogenic or spoilage bacteria, so the safety of fermented food products can be enhanced.

A targeted metabolomic approach using chromatography has been conducted to characterize food. This approach has been applied to fermented foods to investigate the metabolites produced by microorganisms. In fermented foods, microorganisms generate metabolites as resulted products of enzymatic process to degrade the raw material (Gao et al. 2021). Some metabolites can determine the safety of food such as lactic acids, and acetic acids that play a role as antimicrobial compounds (Ibrahim et al. 2021; Zapašnik et al. 2022). In fermented foods, lactic and acetic acids contribute to an acidic atmosphere, lowering pH and consequently restricting the proliferation of spoilage microbes (Leeuwendaal et al. 2022; Sionek et al. 2023).

Evaluating the diverse of bacteria and metabolites during food fermentation can reveal the functional roles of bacteria in these products (Huang et al. 2018; Yang et al. 2020; Helmi et al. 2022; Wu et al. 2023). Various approaches have been used to assess the microbiome in fermented food namely culturable and unculturable dependent methods. Nowadays, sequencing of total DNA, including 16S rRNA genes, through metagenomic approaches is extensively utilised in environmental microbiology, especially following the emergence of high-throughput sequencing technologies (Nam et al. 2023). Oxford Nanopore Technology (ONT) offers long-read sequencing capable of covering the complete 16S rRNA gene (V1–V9 regions), providing a rapid, cost-effective, and high throughput process (Bahram et al. 2019; Santos et al. 2020). As all informative regions are represented, full-length 16S rRNA sequencing allows for more robust bacterial identification with higher taxonomic and phylogenetic resolution (Bahram et al. 2019).

The composition and diversity of bacterial communities during spontaneous fermentation of *porang* tubers had not been reported. Moreover, the organic acids produced as metabolites of microbial activity had also not been documented. This study incorporates a natural fermentation stage to explore its impact on microbial community shifts and biochemical transformation prior to flour processing. The objective of this study was to investigate the changes in bacterial populations and organic acids concentration during the fermentation of *porang* tubers. This study represents fundamental research aims at elucidating the role of bacteria during fermentation of *porang* tubers.

MATERIALS AND METHODS

Materials

Porang tubers, sterile distilled water, Plate Count Agar, DNA MiniPrep, primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), MyTaq HS Red Mix, agarose, molecular-grade water.

Methods

Fermentation of *porang* tubers

To investigate the microbial and biochemical dynamics during fermentation, a spontaneous fermentation process was applied to *porang* tubers. Fermentation was performed according to the method described by (Oyedeji et al. 2013; Ojokoh et al. 2015; Helmi et al. 2024). Fresh *porang* tubers (*Amorphophallus oncophyllus*) were manually peeled, sliced to a thickness of 1.5 mm and washed thoroughly with tap water. Following washing and draining, 1 kg of thinly sliced *porang* tubers was immersed in 2 L of sterile distilled water in a 3-L sterile plastic container. The container was covered with sterile aluminium foil and container cover. No inoculum was added, thus enabling the native microbiota present on the tuber surface and in the environment to initiate fermentation.

Fermentation was performed under ambient room temperature conditions (approximately 28–30 °C) and left undisturbed throughout the process. At the beginning of the fermentation (0 hour), immediately after soaking, the container was manually agitated for approximately 60 seconds in order to dislodge and to release surface-associated microorganisms from the *porang* tuber slices into the soaking medium. This step was crucial to ensure that the initial microbial community present in the fermentation liquid represented the indigenous microbiota of the *porang* tubers, rather than from the distilled water, which was sterilised prior to use. The soaking water was then collected aseptically for microbial and chemical analyses, serving as the baseline (0 h) sample for comparison with subsequent fermentation time points. Samples of 50 mL of soaking liquid were aseptically taken at 0 hours (prior to fermentation), and subsequently after 24, 48, and 72 hours of fermentation. The fermentation process was conducted in triplicate for each time point (0 h, 24 h, 48 h, and 72 h). For metagenomic analysis, equal volumes of soaking/fermentation water were obtained from the three replicates at each respective time point and composited to form a representative sample. Thus, four composite samples (one per time point) were analysed for bacterial community composition. Meanwhile, for total bacterial counts and organic acid analysis, samples from each replicate were analysed individually, resulting in triplicate data per time point. These samples were immediately stored at –20 °C until further analysis, which included measurements of organic acid profiles, total bacterial counts, and metagenomic analysis.

Total bacteria

Total bacteria were counted using total plate count method. Serial tenfold dilutions were performed until 10^{-7} , followed by spreading 100 µL of each diluted sample (10^{-3} – 10^{-7}) onto Plate Count Agar (Himedia-India). The plates were incubated at 25 °C for 24 hours, and visible colonies were enumerated to determine the bacterial population.

Organic acid analysis

The concentrations of organic acids (acetic and lactic acid) were determined using UPLC with a photodiode array (PDA) detector. The method was adapted from Wang et al. (2014) with modifications involving the use of UPLC that was operated under UPLC-optimized flow rate and pressure instead of conventional HPLC to enhance separation efficiency and reduce analysis time. The profiling of organic acids was conducted using a Waters Acquity UPLC H-Class system (Waters, Milford, MA, USA) equipped with a quaternary pump (UPQSM), autosampler injector, and PDA detector. Data were acquired and processed with Empower 3 software (Waters, 2010, Milford, MA, USA). Chromatographic separation of acetic and lactic acids was performed on a Waters Acquity UPLC Spherisorb Atlantis T3 C18 column

(4.6 mm × 250 mm × 5 µm) at room temperature, using a linear flow rate of 0.8 mL min⁻¹. The mobile phase consisted of 0.01 mol L⁻¹ sulfuric acid in water under isocratic conditions, and detection was carried out at 220 nm with the PDA detector.

Metagenomic analysis

Genomic DNA was isolated using a DNA MiniPrep kit (Zymogen USA Catalog 4300) following the manufacturer's instructions (Helmi et al. 2022, 2024). DNA concentration was determined using Qubit fluorometer (Helmi et al. 2024). PCR amplification utilized MyTaq HS Red Mix, 2X (Bioline, BIO-25048) with universal primers 27F (5'-AGAGTTTGTATC[A/C]TGGCTCAG-3') and 1492R (5'-G[C/T]TACCTTGTTACGACTT-3') (Suriani et al. 2023). A standard 25 µL PCR mixture consisted of 12.5 µL of 2× MyTaq HS Red Mix, 1 µL each of the 27F and 1492R primers (10 µM), 2 µL of template DNA (approximately 1–10 ng), and molecular-grade water to a final volume of 25 µL. The thermocycling program included an initial denaturation at 95 °C for 2 min, followed by 30–35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s, with a final extension step at 72 °C for 7 min. Amplification products (2 µL) were examined by electrophoresis on 1 % TBE agarose using a 1 kb DNA ladder (2.5 µL loaded) as a size marker.

The gDNA passed QC followed by Library preparation. Library preparation involved full-length 16S rRNA gene amplification, DNA end-repair, barcode ligation, and adapter attachment. Libraries were prepared using the PCR Barcoding Kit (Oxford Nanopore Technologies) following the protocol described by Ohta et al. (2023). DNA sequencing was subsequently performed using Oxford Nanopore Technologies sequencing kits. Sequencing was performed under MinKNOW (version 23.04.5). After sequencing, basecalling was conducted. Basecalling of the generated data was performed with Guppy v6.5.7 using the high-accuracy model (Wick et al. 2019). NanoPlot (v1.40.1) and NanoFilt (v2.8.0) were used for sequencing quality assessment and read filtering, respectively, while taxonomic classification was carried out with Centrifuge (v1.0.4) (De Coster et al. 2018; Nygaard et al. 2020). Sequencing quality control was performed, and reads were taxonomically classified using Centrifuge with a Bacteria and Archaea index constructed from the NCBI 16S RefSeq database (<https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/>) (Kim et al. 2016). Downstream analyses and visualizations were carried out using Pavian (<https://github.com/fbreitwieser/pavian>), Krona Tools (<https://github.com/marbl/Krona>), and RStudio version 4.2.3 (<https://www.R-project.org/>).

RESULTS AND DISCUSSION

Total bacteria during *porang* fermentation

During fermentation, the total bacteria count increased up to 48 hours and then decreased by 72 hours (Table 1). This decline may result from unsuitable conditions facilitating bacterial growth in the terminal phase such as high acidity, nutrient depletion, and the accumulation of metabolite compounds, like ethanol, bacteriocins, organic acids. The rise in acetic acid and lactic acid during fermentation led to higher of acidity (Zubaidah et al. 2020). Organic acids and acidity are known to inhibit microbial growth various fermentation including cassava, inasua, and Ambonese arrack (Mahulette et al. 2018; Mahulette & Astuti 2020). Alcohol accumulation also inhibit the microorganism (Mahulette et al. 2018; Mahulette & Astuti 2020; Helmi et al. 2024). Certain bacteria, including lactic acid bacteria and *Bacillus* species, are capable of producing bacteriocins that can suppress the growth of competing microorganisms. During the early stages of fermentation, bacteria use simple sugars to produce acids (Mahulette et al. 2018). LAB also utilise acidic compounds from

the degradation of carbohydrates as substrates for growth (Mahulette & As-tuti 2020).

Table 1. Total culturable bacteria of fermentation water during fermentation of *porang* tubers.

Organic acids	0 hour	24 hours	48 hours	72 hours
Total bacteria (log CFU mL ⁻¹)	4.85 ± 0.33	7.31 ± 0.19	8.05 ± 0.09	7.33 ± 0.11

Organic acids production and total bacteria during *porang* fermentation

Table 2 shows that acetic and lactic acid increased during fermentation. Acetic and lactic acid production by microorganisms involved in the fermentation process contributed to the acidification of the fermentation medium (Oyedepi et al. 2013; Oyinlola et al. 2016). No organic acids were detected at the start of fermentation (0 hours). At 24 hours, acetic acid was detected, which can be produced by either acetic acid bacteria (AAB) or lactic acid bacteria. Acetic Acid Bacteria (AAB) facilitate the oxidation of ethanol to acetic acid (Gomes et al. 2018). The increase in acetic acid showed that the glucose level in the fermentation water rose over time. Acetic acid production during fermentation can result from either glucose or ethanol conversion (Hata et al. 2023). The increasing acetic acid during fermentation was due to the increasing of simple sugar or ethanol during fermentation. Helmi et al. (2024), observed an increase in ethanol levels in *porang* fermentation up to 48 hours. The increase in simple sugars, such as glucose, during the late phase of fermentation promotes lactic acid formation. The metabolic activity of Lactic Acid Bacteria (LAB) results in the formation of lactic acid and other organic acids from glucose depending on whether they are homofermentative or heterofermentative categories. Homofermentative Lactic Acid Bacteria (LAB) generate only lactic acid, while heterofermentative LAB synthesise lactic acid, acetic acid, and, in some cases, ethanol (Mahulette et al. 2018; Sharma et al. 2020).

Table 2. The organic acids during fermentation of *porang* tubers revealed by UPLC.

Organic acids	0 hour	24 hours	48 hours	72 hours
Acetic acid (ppm)	nd	100.35 ± 0.80	119.91 ± 2.82	230.21 ± 6.57
Lactic acid (ppm)	nd	nd	nd	146.84 ± 1.82

nd=not detected

Metagenomic study

Table 3 showed that the extracted DNA metagenome from *porang* water fermentation had a sufficient concentration for PCR amplification. Agarose gel electrophoresis was employed to evaluate the quality of the amplified gDNA (PCR product), as shown in Figure 1. The results indicated that the PCR products were of good quality, displaying band approximately 1500 bp in size.

Table 3. The quantity of genomic DNA concentration during fermentation of *porang* tubers

Fermentation Samples (hrs)	Volume (µL)	Qubit Concentration (ng µL ⁻¹)
0	50	3.0
24	50	3.2
48	50	4.1
72	50	10.3

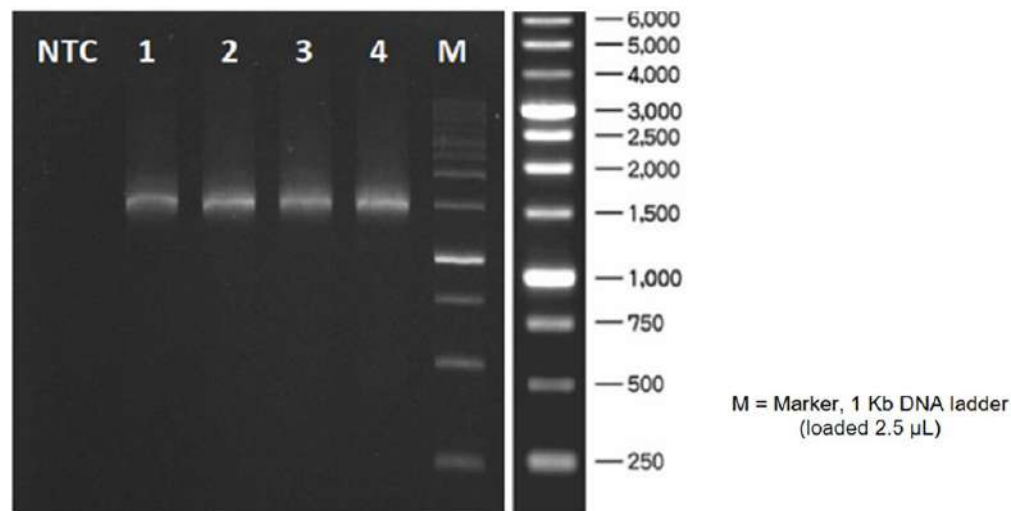


Figure 1. Agarose visualisation of PCR products from amplification of DNA meta-genome extracted from *porang* water fermentation. Symbols: NTC=No Template Control, 1=0 h, 2=24 h, 3=48h, 4=72h, M=marker.

Diversity index shows that sample of water fermentation at 72 hours fermentation had the lowest diversity index while 48 hours had the highest bacterial diversity index (Figure 2). According to Kim et al. (2017), bacterial diversity among fermented food samples has been compared using diversity indices through a culture-independent approach employing high-throughput sequencing of 16S rRNA genes. The diversity indices of Shannon, Simpson and Inv Simpson were highest at 48 hours of fermentation followed by 0 hour and 24 hours fermentation (Figure 2). This condition can be caused by at 48 hours fermentation, complex carbohydrate had been hydrolysed into simple sugars that facilitated bacterial growth. It can be assumed that some various carbon sources were available at 48 hours fermentation, so the diversity of bacteria also high at this time. In line with lactic acid concentration, at 0-, 24- and 48-hours fermentation cannot detect. At 0-, 24-, and 48- hours, lactic acid was not detected using the UPLC system under the current analytical conditions. The peak corresponding to lactic acid only appeared at 72 hours, indicating that its concentration in the earlier time points was likely below the detection limit of the instrument. Lactic acid was generated via glucose metabolism mediated by lactic acid bacteria during fermentation, reflecting active carbohydrate catabolism and the onset of anaerobic microbial activity.

Shannon–Weaver and Simpson diversity indices are widely applied to assess bacterial diversity based on operational taxonomic units (OTUs). Richness estimators such as ACE (Abundance-based Coverage Estimator) and Chao1 are commonly used to predict the expected number of OTUs in a sample, considering all identified bacterial species. Chao1 is a non-parametric estimator that infers species richness within a community, whereas ACE estimates species richness based on sample coverage, defined as the sum of the probabilities of the observed species (Kim et al. 2017). The Figure 2 shows that 0 hour had the highest richness of species in community followed by 24 hours fermentation. Some studies on kimchi (Jung et al. 2011), fermented rice wine (Liang et al. 2020) and fermented shrimp/fish sauces (Jeong et al. 2013; Jung et al. 2013) indicate that bacterial communities begin developing from day 0 of fermentation, with microorganisms originating from the raw materials (vegetables, rice, or seafood). The highest richness of species at 0 hour can be caused by the suitable condition for natural bacteria (autochthonous bacteria) from *porang* tubers. Fermentation of *porang* tubers reduced the richness of species. A decline in microbial richness during fermentation is likely driven by the accumulation of inhibitory metabolites, namely ethanol and organic acids. These metabolites can create stressful environmental conditions that

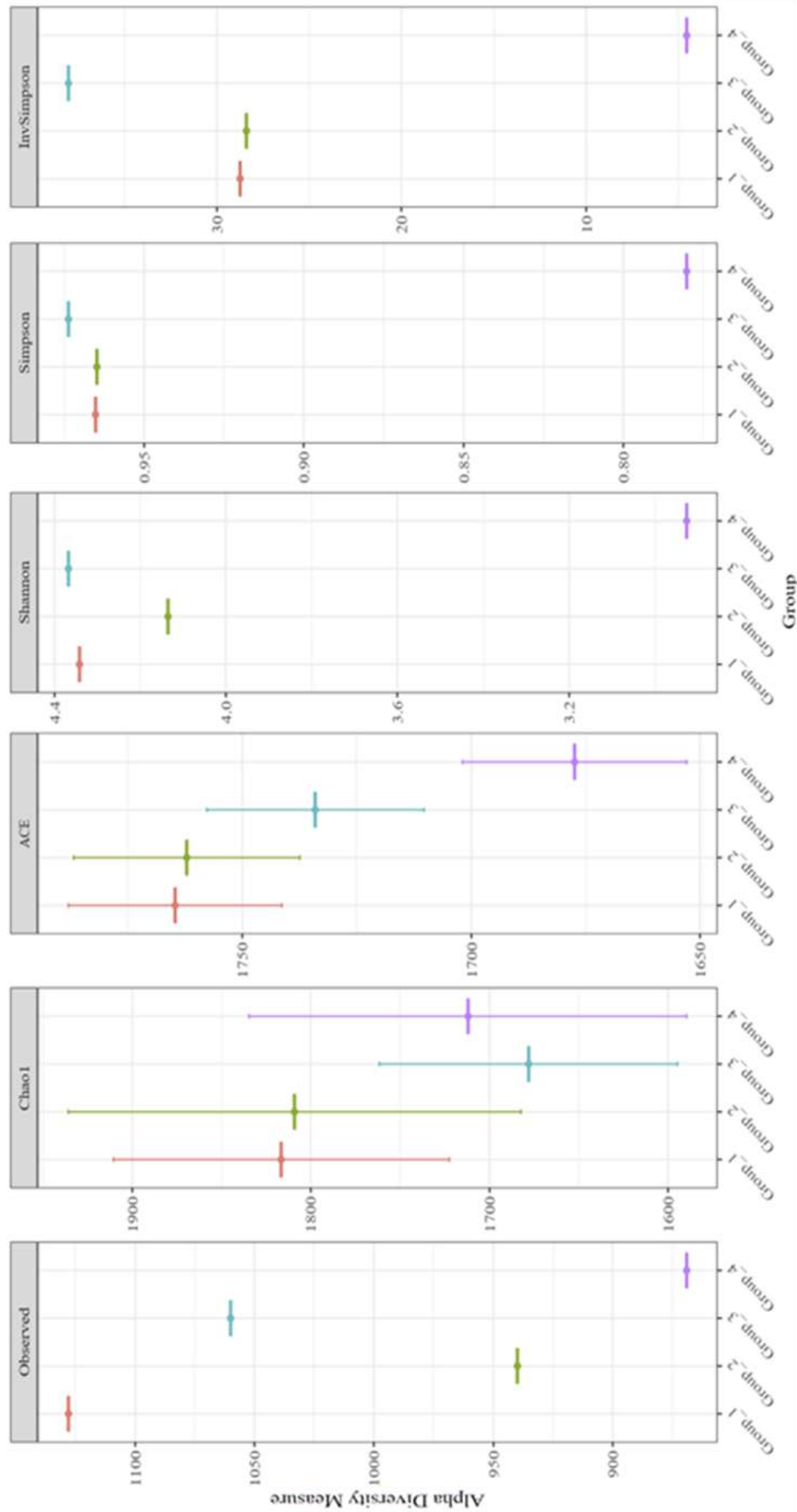


Figure 2. Alpha diversity of bacteria during fermentation of *porang* water fermentation. Group 1= 0 h fermentation, Group 2=24 h fermentation, Group 3=48 h fermentation, Group 4=72 h fermentation.

suppress the growth of certain microbial taxa. For instance, organic acids lower the pH, while ethanol disrupts membrane integrity, both of which can inhibit microbial diversity (Pérez-Gallardo et al. 2013; Köberl et al. 2019; Plavec & Berlec 2020; Ibrahim et al. 2021; Yap et al. 2022; Varize et al. 2022). In line with organic acids investigation (Table 2) increased during fermentation. Ethanol concentration increased during fermentation until 48 hours of fermentation *porang* tubers. At 72 hours fermentation, the ethanol decreased (Helmi et al. 2024). Ethanol can be changed to be acetic acid (Chapp et al. 2024).

Figure 3 shows relative abundance of bacteria during fermentation. During fermentation, the phylum Pseudomonadota (synonym: Proteobacteria) emerged as the dominant group of bacteria. This major phylum of Gram-negative bacteria exhibits remarkable diversity, including human-associated, soil-borne, pathogenic, and spoilage microorganisms (Cason et al. 2020; Li et al. 2021). The Pseudomonadota phylum was also reported to dominate the fermentation processes of various plants such as tomatoes and medicinal plants (Köberl et al. 2019; Cason et al. 2020; Lavefve et al. 2021; X. Zhou et al. 2022). After 48 hours of fermentation, a significant decline was observed in Proteobacteria, while Bacillota reached its peak abundance (Figure 3a). The phylum Bacillota (synonym Firmicutes) represents the major group of Gram-positive bacteria and encompasses a wide variety of Lactic Acid Bacteria (LAB) (Cason et al. 2020). The increased abundance of Bacillota during fermentation underscores the safety of fermented foods, as several species within this phylum are known to produce bacteriocins and organic acids. Conversely, a decline in Proteobacteria during fermentation is favourable, as it contributes to product safety by limiting spoilage and pathogenic microorganisms (Cason et al. 2020; Li et al. 2021). The phylum Bacteroidota (synonym Bacteroidetes), comprising Gram-negative, nonspore-forming, anaerobic or aerobic, and rod-shaped bacteria showed the highest abundance at the start of fermentation (0 hours). These bacteria are commonly found in soil, which explains their initial abundance in *porang* fermentation water, as the tubers were sourced directly from soil.

Class Gammaproteobacteria was the most abundant class observed during fermentation. As the largest and most metabolically diverse class within the phylum Proteobacteria, Gammaproteobacteria includes Gram-negative bacteria which are partly pathogenic to humans, animals and plants. This class has also been reported as dominant in the fermentation of brick Fu (Wu et al. 2023) and medicinal plants (Köberl et al. 2019).

At the family level, the bacterial composition varied over the course of fermentation. It was dominated by Enterobacteriaceae and Erwiniaceae at 0 hour of fermentation. By 24 hours and 48 hours of fermentation, Enterobacteriaceae remained the dominant family, while at 72 hours of fermentation, Pectobacteriaceae became predominant. Enterobacteriaceae, which is the dominated family from 0 to 48 hours, includes a diverse range of bacteria such as *Vibrio*, *Lactobacillus*, *Lactococcus*, *Yersinia* that commonly occurring in fermented foods. (Kergourlay et al. 2015). The abundance of Erwiniaceae at 0 hours, suggests the presence of plant-pathogenic bacteria originating from the *porang* tubers, as members of this family are primarily plant pathogens (Franco et al. 2022a). Erwinaceae was detected in fermented silage, barley (Franco et al. 2022a, 2022b). At 72 hours of fermentation, the dominance of the Pectobacteriaceae family indicates the presence of bacteria that are also pathogenic to plants (Oulghazi et al. 2020).

At the genus level, fermentation dynamics revealed shifts in bacterial dominance over time. At 0 hour, the genus *Pantoea* was predominant. At 24 hours it was dominated by *Enterobacter*. The *Pantoea* genus which dominates at 0 hours is a genus of plant pathogens (Jena et al. 2023). *Pantoea* part of *Er-*

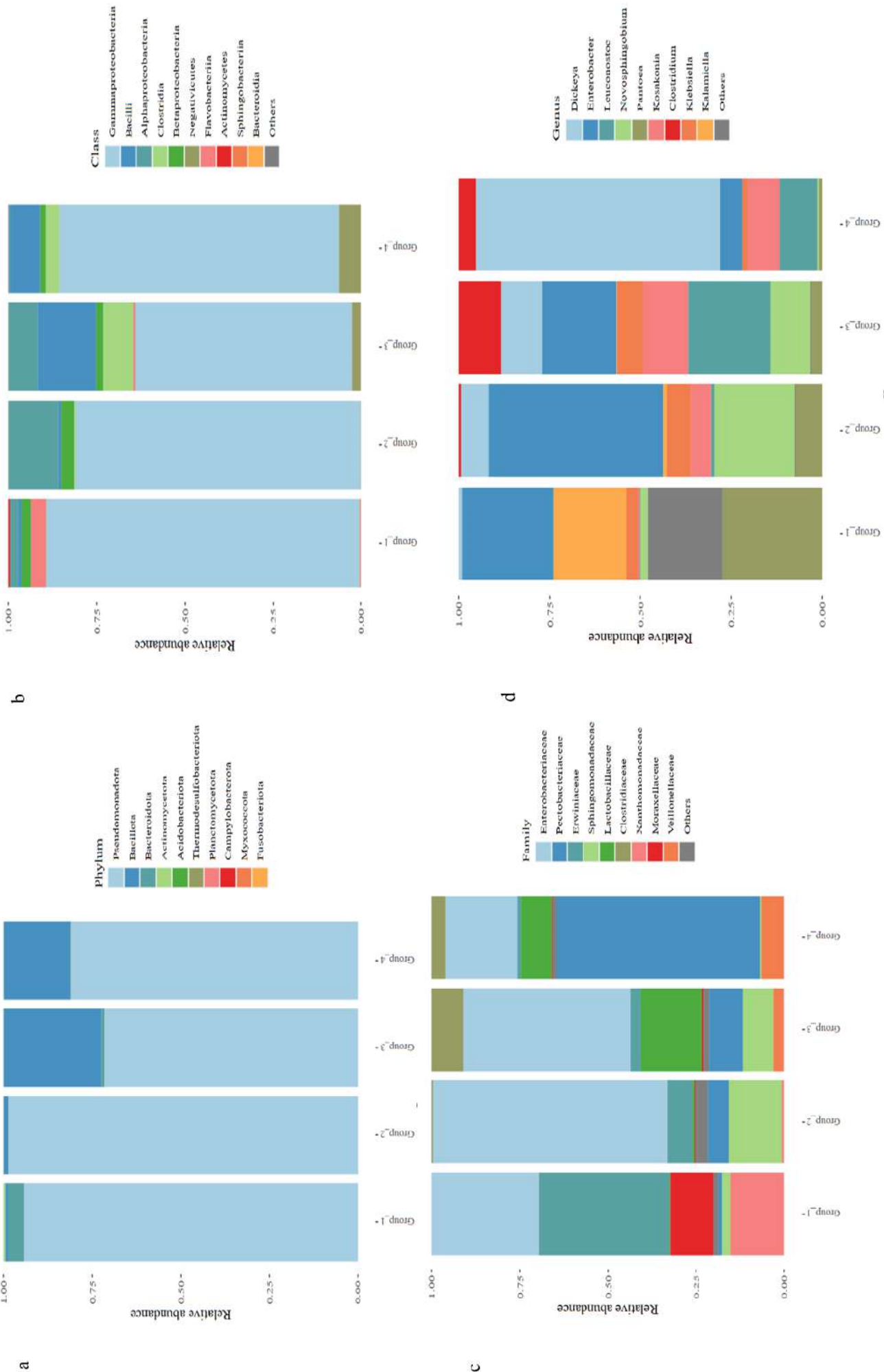


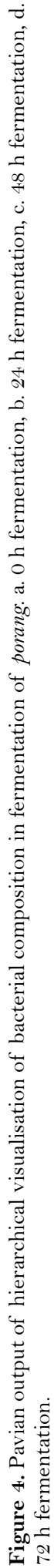
Figure 3. Relative abundance of bacteria during fermentation of *porang*: a: phylum level, b: class level, c: family level, d: genus level. Group 1=0 h, Group 2=24 h, Group 3=48 h, Group 4=72 h.

winiaceae family, includes plant pathogen such as *Pantoea piersonii* (synonym *Kalamiella piersonii*) and *Pantoea dispersa*. *Pantoea dispersa* has been reported to cause leaf, panicle, and grain blight in India (Jena et al. 2023) and has also been found in agarwood (Chhipa & Kaushik 2017) and during soy sauce fermentation (*inyu*) (Wei et al. 2013). At 24 hours, genus *Enterobacter* was dominated. Genus *Enterobacter* also found in some fermented foods such as Italian table oil (Benítez-Cabello et al. 2020), sufu (soy bean fermented food) (Huang et al. 2018), yoghurt and cheese. Some of genus *Enterobacter* are recognised as contaminant in dairy fermented foods and some of them are recognized as pathogen. The dominated of *Enterobacter* was correlated with sugars including glucose and fructose (Huang et al. 2018). It can be assumed that at 24 hours of *porang* tubers fermentation rich with glucose. At 48 hours of fermentation, it was dominated by *Leuconostoc* and at 72 hours of fermentation it was dominated by *Dickeya*. *Leuconostoc* is recognised as probiotic bacteria of some fermented foods (Fijan 2014). *Leuconostoc* was also dominated bacteria of jeotgal (Koo et al. 2016). Genus *Dickeya*, dominated bacteria at 72 hours, was also plant pathogenic (Oulghazi et al. 2020).

At Figure 4 shows that at species level at 0 hours was dominated by *Kalamiella piersonii*, *Pantoea dispersa*, *Acinetobacter calcoaceticus*. At 0 hours fermentation, *Kalamiella piersonii* which are in synonym with *Pantoea piersonii* was dominated at 0 hours fermentation. Another abundant bacterium at 0 hours was *Acinetobacter calcoaceticus*, a nonmotile Gram-negative coccobacillus found in soil, plants, and the human intestine. It grows under aerobic conditions, is catalase-positive, and oxidase-negative (Wu et al. 2018; Glover et al. 2022). The bacterial population at 0 hours predominantly consisted of plant pathogens and soil-associated bacteria. At 24 hours, the bacterial profile shifted with *Enterobacter cloacae* complex, *Novosphingobium guangzhouense* and *Kluyvera intermedia* becoming dominant. The *E. cloacae* had been detected in some foods including soy sauce (*inyu*) and tempeh (Wei et al. 2013; Billqys et al. 2023). As *E. cloacae* complex is a known contaminant in dairy products (Nasr-Eldin et al. 2023), it means that the pathogenic or spoilage bacteria present during the initial stages of fermentation (0–24 hours). Genus *Novosphingobium* is characterised by facultative anaerobic growth, motility, catalase and oxidase positivity, and starch hydrolysis capability (Belmok et al. 2023). According to Helmi et al. (2024), 24 hours' fermented *porang* tubers flour contain the highest starch content approximately 53.56 % while *Novosphingobium guangzhouense* has the capability to hydrolyse the starch. The highest starch content made the suitable substrate for this bacterium. *Kluyvera intermedia* is a bacterium that lives in soil, water and sewage. This bacterium is a gram negative, motile, facultative anaerobe bacterium. *Kluyvera intermedia* was found in ice stored sardine and mackerel and produced biogenic amine (Fadhlaoui-Zid et al. 2012).

Leuconostoc falkenbergense, *Dickeya fangzhongdai*, *Enterobacter cloacae* complex were dominated bacteria at 48 hours. *Leuconostoc falkenbergense* is a lactic acid bacterium which found in fermented string beans and traditional yogurt (Wu & Gu 2021). It is facultative anaerobe, capable of growing at pH 5 and utilise pyruvate to produce lactic acid from glucose and mannose (Wu & Gu 2021). *L. falkenbergense* caused lactic acid detected at 72 hours fermentation. It meant that at 72 hours fermentation, lactic acid had been accumulated. The dominance of *Leuconostoc falkenbergense* at 48 hours aligns with the detection of lactic acid, marking this stage as the ideal point of processing *porang* to be fermented flour.

At 72 hours of fermentation time point, the bacterial community was predominantly occupied by *Dickeya fangzhongdai*, *Massilibacillus massiliensis*, *Leuconostoc falkenbergense*. In contrast, *Dickeya fangzhongdai* is a plant pathogenic bacterium with pectinolytic activity isolated from pear tree (Tian et al.



2016). Glucomannan from konjac contains alkaloids, pectin, amino acids (Devaraj et al. 2019). The rich of pectin in glucomannan made the suitable substrate for *Dickeya fangzhongdai* to grow well. The abundance reduction of *L.falkenbergense* in this period showed the glucose reduction. It also supported that the amount of ethanol reduced at 72 hours fermentation (Helmi et al. 2024) while the acids accumulate (Table 1). The abundance increasing of *Dickeya fangzhongdai* showed that in this period, glucomannan had been degraded. The acids accumulation in this period can degrade glucomannan (Kumoro et al. 2018). *Massilibacillus massiliensis* is an anaerobic bacterium lacking catalase and oxidase activity (Tidjani Alou et al. 2017) was also detected, but its presence underscores the continued of microbial diversity and emphasises the potential risks associated with prolonged processing. The increase of lacking catalase and oxidase bacteria in fermentation showed that there was accumulation of toxic metabolite products (such as acids or ethanol), and depletion of oxygen in the end of fermentation.

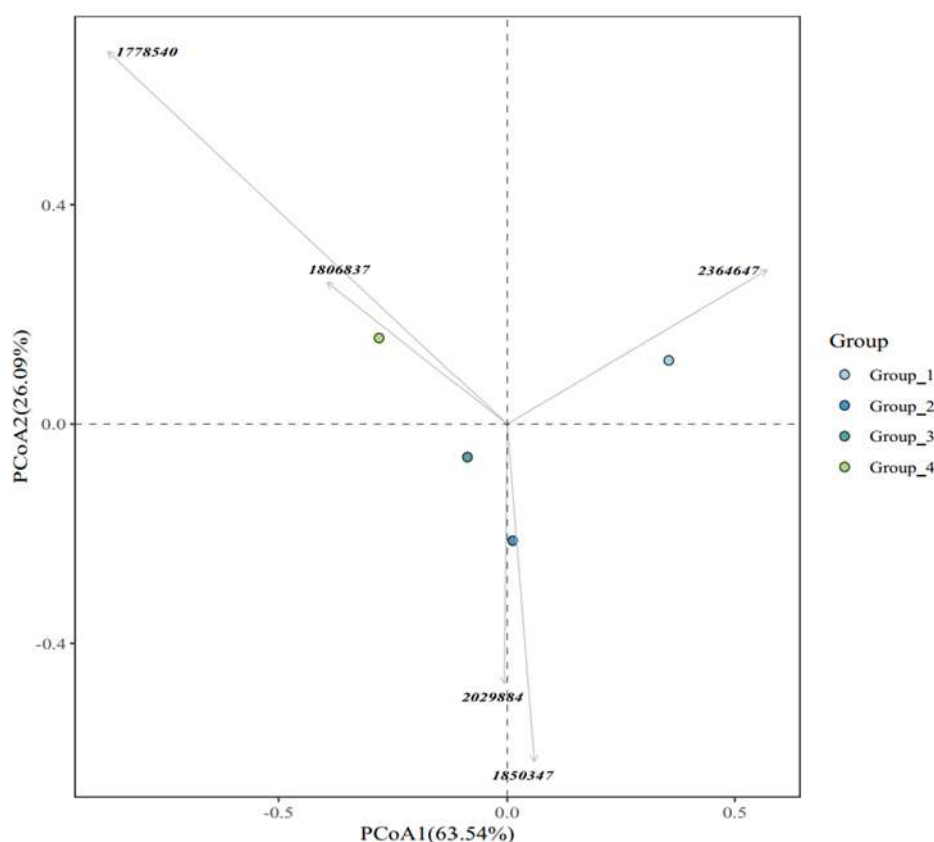


Figure 5. Clustering of samples based on Principle Coordinate Analysis (PCoA). Group 1=0 h, Group 2=24 h, Group 3=48 h, Group 4=72 h.

Figure 5 illustrates clustering of samples based on principal coordinate analysis (PCoA), demonstrating that fermentation time significantly impacts bacterial diversity. The 48- and 72-hour samples form one distinct cluster, while the 0- and 24-hour samples are grouped in another a separate cluster. This indicates that longer fermentation times lead to shifts in bacterial composition. The analysis of relative abundance of bacterial types, reveals that changes in fermentation time altered the dominant bacterial diversity. Since the abundance of lactic acid bacteria (*L. falkenbergense*) become prevalent by 48 hours, thus, to ensure the safety during *porang* tubers fermentation, the process needs to be halted at the 48-hour mark. This conclusion is further supported by previous studies on fungal dynamics during fermentation, which reported dominance by *Cyberlendra subsufaciens* and *Candida parapsilosis* at the same time point (Helmi et al. 2024). *Candida parapsilosis* is an ethanol produc-

er yeast while *Cyberlendra subsufaciens* is a higher ester production yeast which can give off a fruity aroma (Bellut et al. 2019). The abundance of *Cyberlendra subsufaciens* can improve the aroma of *porang* fermented flour.

CONCLUSIONS

Pseudomonatoda (syn Proteobacteria) was the dominated phylum during fermentation while Gammaproteobacteria and Enterobacteriales were the dominated class and order, respectively. Enterobacteriaceae was the dominant family until 48 hours fermentation and shifted with Pectobacteriaceae at 72 hours fermentation. *Leuconostoc falkenbergense*, a lactic acid bacterium, had the highest relative abundance 48 hours fermentation. Lactic acid was detected at 72 hours fermentation while acetic acid increased during fermentation. Considering the microbial dynamics, 48 hours of fermentation appears to be the optimal stopping point, given the dominance of beneficial lactic acid bacteria such as *Leuconostoc falkenbergense*.

AUTHOR CONTRIBUTION

H.H. conceived and designed the study, supervised all experimental procedures, and drafted the manuscript. R.K. collected the data, supervised the fermentation process, and contributed to manuscript writing. R.G.M. collected the data, supervised the organic acid analysis, and contributed to manuscript writing. E.K. performed data analysis and contributed to manuscript preparation. K.S. contributed to manuscript writing and performed proofreading.

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CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest associated with this research.

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Research Article

Mollusc Diversity from the Prehistory Context of Kompleks Ceruk Pajangan Archaeological Site (Java, Indonesia)

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ABSTRACT

Mollusc shells from prehistoric sites in a karst region offer insights into past biodiversity, human survival strategies, and the environments where they lived. This article covers mollusc study results from Kompleks Ceruk Pajangan that have not been studied before. In this study, we determine which species were exploited by the human inhabitants, and assess their palaeoenvironmental conditions. The study identified 3378 mollusc specimens across 16 families and 25 species, including 8 species from 5 families of bivalves, and 17 species from 11 families of gastropods. Kompleks Ceruk Pajangan exhibited low mollusc diversity and evenness, indicating human preference on how these molluscs were collected and discarded in the past. Rather than randomly, they were collected as food sources and these low indices reflect the site inhabitants' preferences towards freshwater molluscs. Additionally, a significant presence of molluscs from marine/estuarine habitats suggests that this site, 6000–8000 years ago, was much closer to the coastal area than at present.

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INTRODUCTION

Mollusc shells are particularly significant data in the archaeology of South-east Asia, where coastal and riverine environments have been central to human settlement and subsistence. Shell middens (an archaeological term for extensive deposits of discarded mollusc shells in cave sites) are abundant in the region and provide valuable evidence of dietary practices and resource exploitation by prehistoric communities (Szabó & Amesbury 2011). Furthermore, mollusc shells have been used in archaeological contexts to create tools, ornaments, and trade items, highlighting technological innovations and social interactions within and beyond the region (Campbell 2017; Ridout-Sharpe 2017; Bailey & Hardy 2021). Radiocarbon dating of mollusc shells has been crucial for archaeologists in establishing chronological frameworks for understanding the timing and development of human activities, including the transition from foraging to agriculture (Bellwood 2017).

Recent efforts have focused on analysing mollusc ecofacts from archaeological sites in the Rembang Zone, a karst area on the north coast of Java and Madura which stretches from Pati/Grobogan (Java) in the westernmost part to the Kangean Islands (Madura) in the easternmost part (van Bemmelen 1949; Wibowo & Alifah 2022; Wibowo et al. 2022a). In the Rembang Zone of Java, numerous mollusc shells were recovered from archaeological excavations at Gua (Cave) Kidang, Gua Bedug, and Kompleks Ceruk Pajangan (Pajangan Rockshelter Complex) (Figure 1). Of these three excavated sites, the mollusc shells from Kompleks Ceruk Pajangan are the only ones that have not been studied previously.

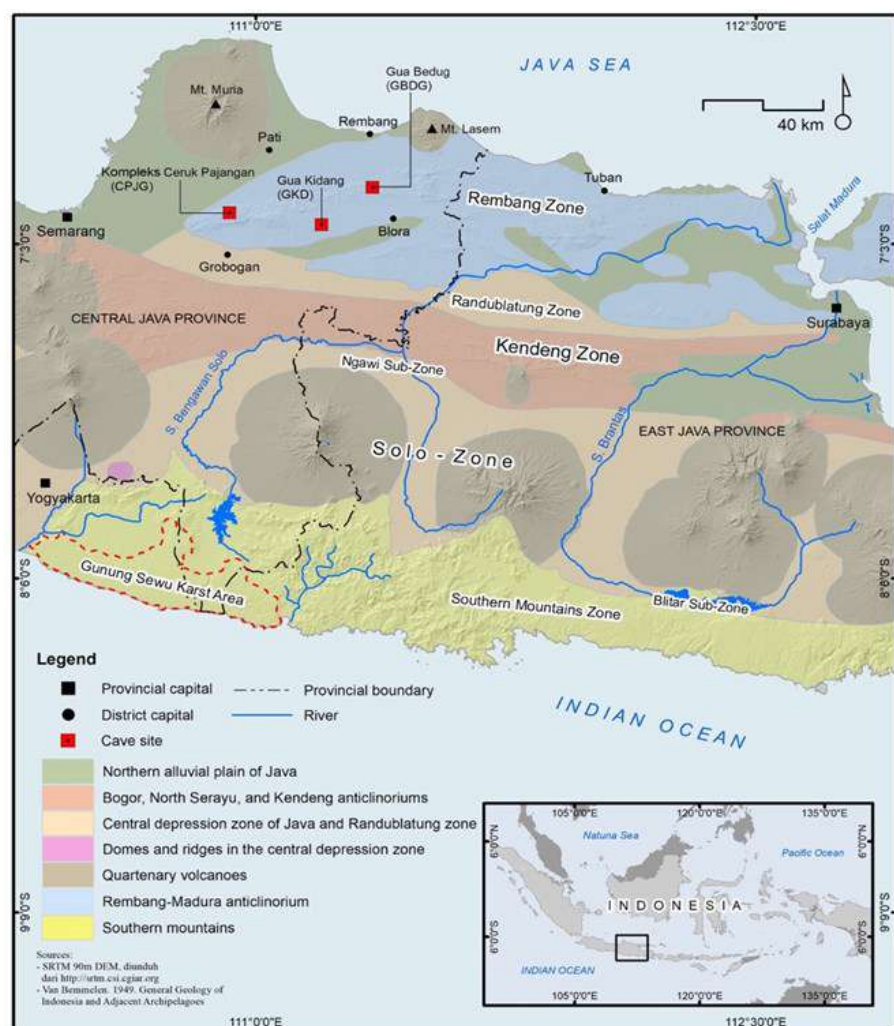


Figure 1. The location of Kompleks Ceruk Pajangan among other sites within the same karst area (Credits: Ahmad Surya Ramadhan and Hari Wibowo 2024).

This article provides a taxonomic identification of the mollusc shells from Kompleks Ceruk Pajangan along with their palaeoenvironmental contexts. This prehistoric occupation site was discovered in 2021 during a survey in Pati, Central Java, Indonesia (Wibowo et al. 2021, 2022a). An archaeological excavation at this site in 2022 yielded a total of 4787 mollusc ecofacts and artefacts (Tanudirjo et al. 2022). AMS Radiocarbon dating from this site was carried out using Unionidae freshwater molluscs, indicating human occupation from at least 6743-6302 cal BP to 8286 - 7869 cal BP (Wibowo & Fajari 2023).

The study of mollusc shells from Kompleks Ceruk Pajangan will complement studies of two other sites in the same karst area that have been previously analysed, Gua Kidang (Blora Regency) and Gua Bedug (Rembang Regency). These two sites were inhabited during a period relatively similar to that of Kompleks Ceruk Pajangan. The upper human occupation layer of Gua Kidang was dated to 5719-5578 BP cal BP (Wibowo et al. 2022b), while the lower layer date of 9440 ± 220 BP (11.275-10.188 cal BP) is considered as less reliable because using conventional dating methods (Nurani & Hascaryo 2012; Nurani et al. 2019). AMS radiocarbon dating for Gua Bedug are indicating that this site was inhabited from 6012 - 5895 cal BP to 8777 - 8542 cal. BP (Wibowo et al. 2022b, 2024, 2025). Current results of mollusc shell studies from these two sites show not only similarities but also differences, even though they are in the same karst area and are only 14-20 km apart.

A total of 1751 mollusc shells from Gua Kidang were analysed, most of which were identified only to the family level, consisting of 8 families (Kirana 2013). For Gua Bedug, the identification of 5845 mollusc ecofacts successfully classified them into 13 families, comprising 19 species (Sulistiyo & Wibowo 2023). Combined with previous studies from Gua Kidang and Gua Bedug, this article, which covers mollusc shells from Kompleks Ceruk Pajangan, aims to provide a clearer picture of the prehistory of the north coast of Java, 6000-8000 years ago. In particular, the mollusc diversity at these sites can offer insights into past environments and the subsistence strategies of the humans who inhabited them.

MATERIALS AND METHODS

Materials

The mollusc shell specimens studied in this research were collected during the 2022 archaeological excavation of Kompleks Ceruk Pajangan. All specimens were physically transported to the laboratory for analysis. The collection procedure adhered to standard archaeological excavation methods, using basic tools such as trowels and bamboo spatulas. Archaeological excavation is an irreversible process, as the same area of the ground cannot be excavated twice (Barker 1993). Due to this irreversible nature, detailed field recordings were taken to document the findings within each excavation unit.

Basic field recording methods were employed during the collection process. Mollusc shells recovered from excavation units were documented through field notes, drawings, and photographs. Within each excavation unit, shells were sorted and categorised by class (bivalves or gastropods), depth interval, and soil stratigraphy layer. Each category was stored and labelled separately in zip plastic bags, which were then placed in plastic containers for transportation to the laboratory. While packaging precautions were taken to ensure the physical integrity of the specimens, no additional measures were implemented to control storage temperature or humidity during transportation.

Ten excavation units were excavated across three sectors: Sector Pajangan (U14 T18, U18 T15, U18 T20, U18 T21, U16 T20), Sector Pajangan A (TP 1), and Sector Pajangan B (U6 T2, U6 T3, U5 T2, U5 T3) (see Figure 2). A total of 4787 mollusc shell specimens, both intact and fragmentary, were

collected from depths ranging from 0 cm to 270 cm relative to the secondary datum point (SDP = 198 m asl).

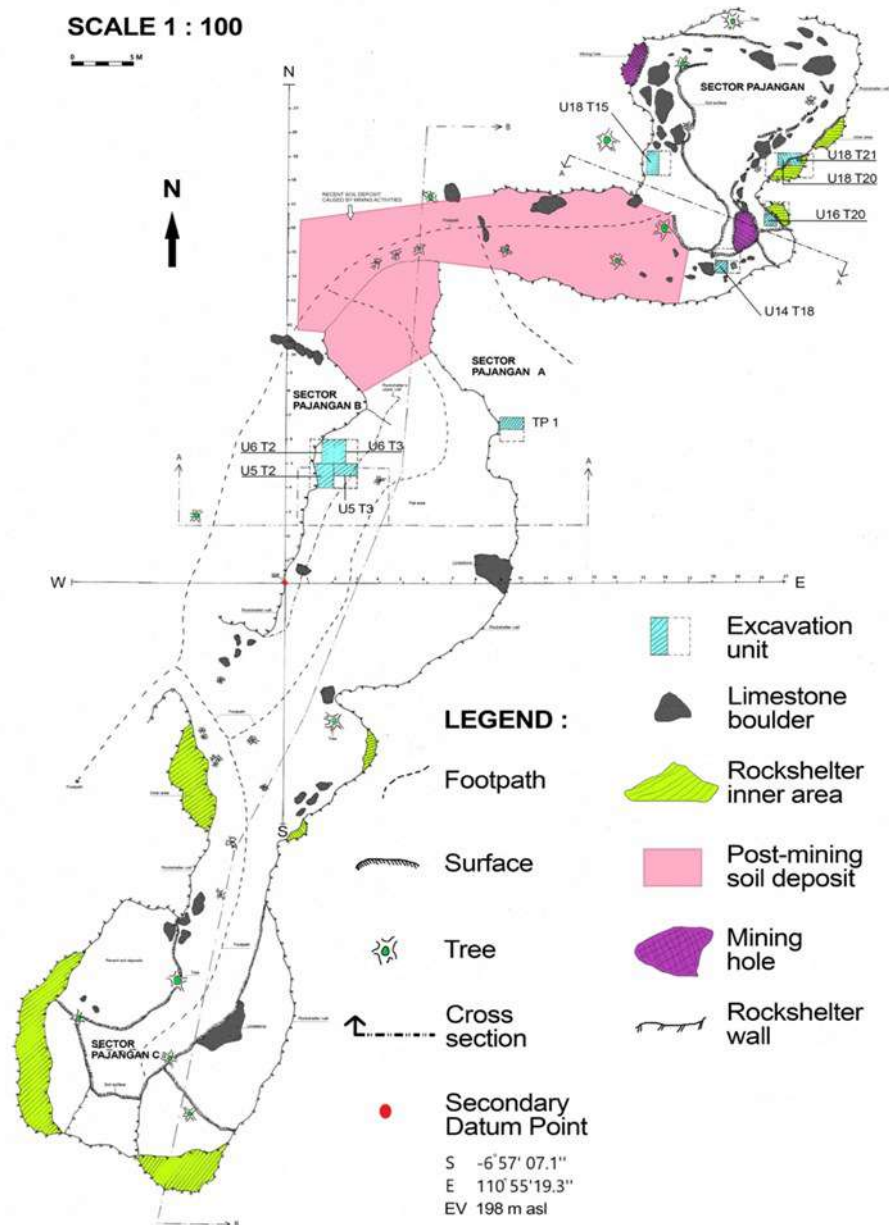


Figure 2. Site plan of Kompleks Ceruk Pajangan, excavation units are marked with light blue and Secondary Datum Point marked with red (Credits: Fadly Ginanjar and Hari Wibowo 2023).

To focus on the mollusc shells from the prehistoric context, not all collected specimens are presented in this study. Specimens from excavation units TP 1, U5 T3, and U6 T3 were excluded due to indications of stratigraphic disturbance caused by modern or contemporary activities. From the remaining seven units, specimens were further filtered by soil stratigraphy layers. Specimens collected from the surface or disturbed layers were also excluded. Consequently, of the original 4787 mollusc shells, only 3378 are included in this article.

Methods

The management and analysis of mollusc specimens followed the methods outlined by Sulistiyo and Wibowo (2023). The results of these analyses were then compared with literature on gastropods and bivalves from Indonesia, particularly Java, including works by Dharma (1992), Marwoto et al. (2020),

and van Benthem Jutting (1950, 1952, 1953, 1956). Specimen identifications were validated using mollusc databases such as the World Register of Marine Species (WoRMS Editorial Board 2024) and MolluscaBase (MolluscaBase eds. 2024) to ensure compliance with current nomenclature.

Morphometric measurement of the mollusc shells followed guidelines from Carpenter and Niem (1998) and Raup and Stanley (1971). Specimens were quantified using common zooarchaeological methods, including NISP (Number of Identified Specimens) and MNI (Minimum Number of Individuals), as described by Lyman (2018) and Harris et al. (2015). Taxonomic richness, diversity, and evenness were then assessed using the number of taxa (NTAXA), Simpson's diversity index (1-D), Shannon-Wiener diversity index (H'), and Shannon's evenness index (E) (Shannon & Weaver 1964). Diversity in this study adopts the category based on Irsyad et al. (2015): $H' < 1.5$ indicates low diversity, $1.5 \leq H' \leq 3$ indicates moderate diversity, and $H' > 3$ indicates high diversity.

Mollusc shell ecofacts were identified to the species level when possible to determine their habitats and reconstruct past environmental conditions. Four main habitat categories were defined in this study: freshwater, marine/estuary, forest litter, and arboreal. The freshwater habitat includes rivers, ponds, and lakes, while the marine/estuary habitat encompasses seas, coastal areas, and mangroves. Forest litter habitats refer to terrestrial environments where molluscs live on the forest floor, often among leaf litter, deadwood, or decomposing organic material. Arboreal habitats are for molluscs primarily living in trees or forested areas.

Mollusc shell presence at the site was classified as either anthropic or native. The anthropic category includes molluscs brought to the site through human activities in prehistoric times. Indicators for this category include habitat context and taphonomic observations. Molluscs transported from their natural habitats and accumulated at the site—either by humans or animals—were considered anthropic if taphonomic evidence, such as cut marks or purposeful breakage, suggested human involvement (Allen 2017). For instance, gastropod shells may display purposeful breakage near the apex or lip to extract the soft body (Hunt & Hill 2017), while bivalves may show prying marks along the hinge (Pickard et al. 2017). Gastropod opercula found detached from the aperture may also indicate efforts to extract the soft body for consumption (Allen 2017). The native category includes molluscs naturally inhabiting the site vicinity, with no evidence of human interference. These molluscs exhibit no taphonomic traces suggesting human activity.

RESULTS AND DISCUSSION

Initial investigations of land molluscs in the Sukolilo karst revealed that 17 % of the total known land snail species in Java are present in the area (Nurinsiyah 2014). From the Kompleks Ceruk Pajangan archaeological site, 3378 mollusc specimens with prehistoric contexts were analysed. These mollusc shells were identified as belonging to 16 families and 25 species. The bivalve class comprises 5 families and 8 species, while the gastropod class comprises 11 families and 17 species (see Table 1, Figures 3 and 4).

Mollusc Diversity at Kompleks Ceruk Pajangan

Of the 3378 mollusc shell specimens, 97.63 % ($n = 3298$) were successfully identified (NISP). Among these, the Minimum Number of Individuals (MNI) was determined to be 47.20 % ($n = 1557$). Observations show that the bivalve class is more often recovered in fragmented conditions due to the fragile nature of their shells. Bivalve mollusc shells are prone to breaking, either intentionally or naturally, after being discarded and buried in the soil (Glover & Kidwell 1993). Similarly, large terrestrial gastropod shells are also fragile and prone to breakage (Sulistiyo & Fakhri 2023). Shells that tend to remain intact

Table 1. Identification results of Kompleks Ceruk Pajangan mollusc specimens.

	Family/ Species	Habitat	Presence
BIVALVE	Cyrenidae		
	<i>Geloina expansa</i> (Mousson, 1849)	MR/ ES	A
	<i>Batissa violacea</i> (Lamarck, 1818)	MR/ ES	A
	Arcidae		
	<i>Tegillarca granosa</i> (Linnaeus, 1758)	MR/ ES	A
	Pectinidae		
	<i>Chlamys</i> sp. (Röding, 1798)	MR/ ES	A
	Placunidae		
	<i>Placuna</i> sp. (Lightfoot, 1758)	MR/ ES	A
	Unionidae		
GASTROPOD	<i>Pilsbryoconcha exilis</i> (I. Lea, 1838)	FW	A
	<i>Rectidens sumatrensis</i> (Dunker, 1852)	FW	A
	<i>Lens contradens</i> (I. Lea, 1838)	FW	A
	Potamididae		
	<i>Telescopium telescopium</i> (Linnaeus, 1758)	MR/ ES	A
	<i>Terebralia palustris</i> (Linnaeus, 1758)	MR/ ES	A
	Viviparidae		
	<i>Filopaludina javanica</i> (von dem Busch, 1844)	FW	A
	Ampullariidae		
	<i>Pila ampullacea</i> (Linnaeus, 1758)	FW	A
	Pachychilidae		
	<i>Sulcospira testudinaria</i> (von dem Busch, 1842)	FW	A
	Thiaridae		
	<i>Melanoides tuberculata</i> (O. F. Müller, 1774)	FW	A
	Camaenidae		
	<i>Amphidromus</i> sp. (Albers, 1850)	AR	N
	<i>Tricheulota zodiacus</i> (A. Férussac, 1821)	AR	N
	<i>Landouria</i> sp. (Godwin-Austen, 1918)	AR	N
	Ariophantidae		
	<i>Hemiplecta</i> sp. (Albers, 1850)	AR	N
	Achatinidae		
	<i>Paropeas achatinaceum</i> (L. Pfeiffer, 1846)	FL	N
	Cyclophoridae		
	<i>Opisthoporus corniculus</i> (Mousson, 1849)	FL	N
	<i>Cyclophorus perdix</i> (Broderip & G. B. Sowerby I, 1830)	FL	N
	<i>Cyclophorus</i> sp. (Montfort, 1810)	FL	N
	Dyakiidae		
	<i>Elaphroconcha javacensis</i> (Férussac, 1821)	FL	N
	<i>Elaphroconcha</i> sp. (Gude, 1911)	FL	N
	Trochomorphidae		
	<i>Trochomorpha</i> sp. (Albers, 1850)	FL	N

Note:

Habitat : (MR/ ES) Marine/ Estuary, (FW) Freshwater, (FL) Forest Litter, (AR) Arboreal

Presence : (A) Anthropic, (N) Native

exhibit a high NISP-to-MNI ratio, such as small terrestrial and aquatic gastropods (Table 2).

Based on species MNI, diversity and evenness were measured using the Shannon-Wiener diversity and evenness indices (Table 3). Overall, the site falls into the category of low diversity, with a diversity index of less than 1.5. Additionally, the bivalve class is observed to be more diverse than the gastropod class. Examining diversity by sectors, Sector Pajangan is more diverse than Sector Pajangan B. However, for the gastropod class, Sector Pajangan B is slightly more diverse than Sector Pajangan. The most diverse mollusc shells at this site were recovered from Sector Pajangan, specifically from unit U16 T20 for the bivalve class and unit U18 T15 for the gastropod class. All

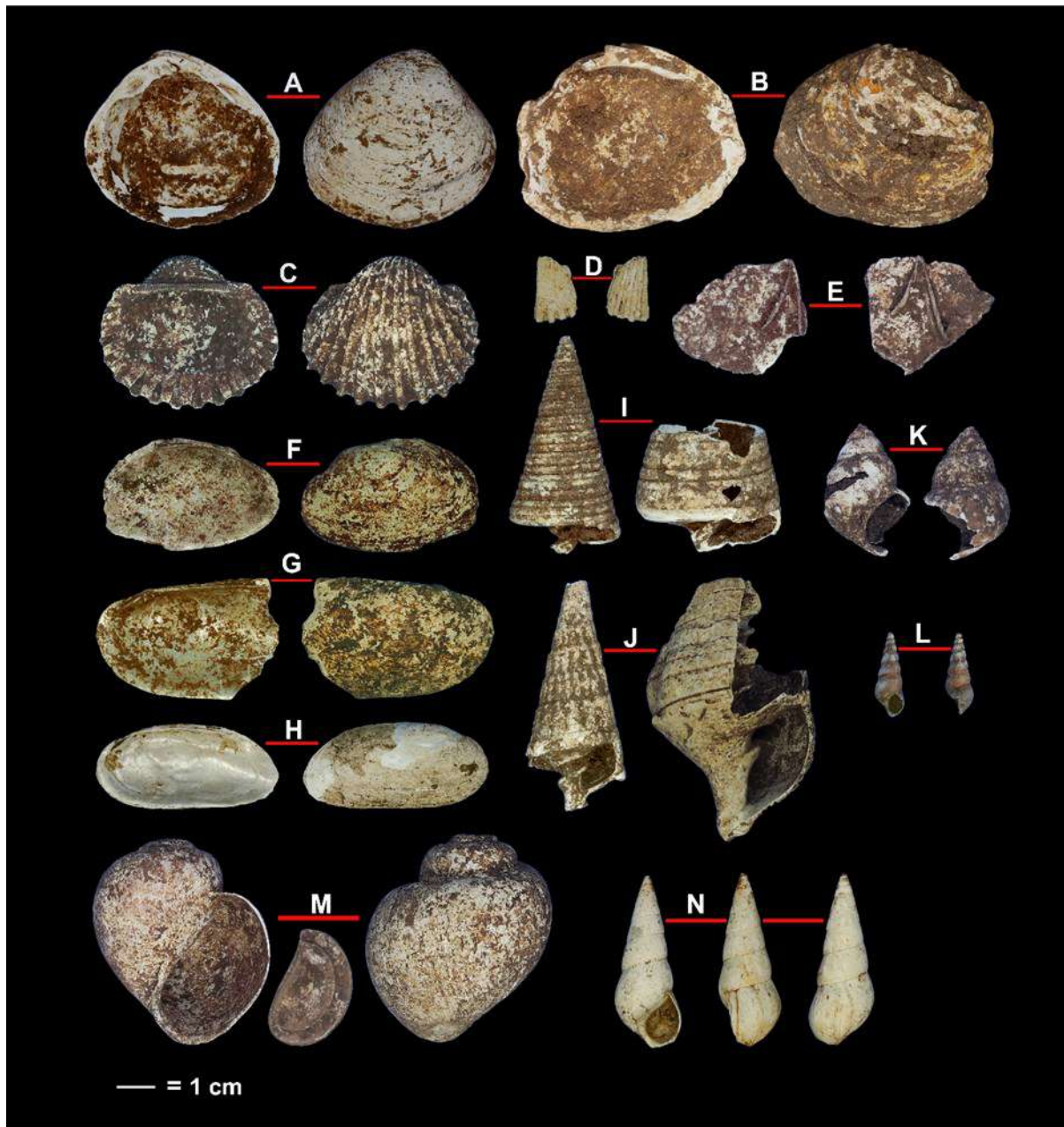


Figure 3. Aquatic bivalves and gastropods: A. *Geloina expansa*; B. *Batissa violacea*; C. *Tegillarca granosa*; D. *Chlamys* sp.; E. *Placuna* sp.; F. *Lens contradens*; G. *Pilsbryconcha exilis*; H. *Rectidens sumatrensis*; I. *Telescopium telescopium*; J. *Terebralia palustris*; K. *Filopaludina javanica*; L. *Melanoides tuberculata*; M. *Pila ampullacea*; N. *Sulcospira testudinaria* (Photo: Restu Budi Sulistiyo 2023).

excavation units in both sectors fall into the low diversity category for both mollusc classes, except for the gastropod diversity index of unit U18 T15, which marginally exceeds the minimum value for the moderate diversity category.

Regarding the evenness index, the bivalve class is overall more evenly distributed than the gastropod class. When divided by sectors, the evenness indices of gastropod and bivalve classes are relatively similar in Sector Pajangan B. In contrast, in Sector Pajangan, the difference between the two classes is more pronounced. The more balanced evenness results in Sector Pajangan B are influenced by the lower number of gastropods recovered in that sector, whereas in Sector Pajangan, the bivalve class was recovered in much smaller numbers compared to gastropods. This is further supported by the results of the Simpson diversity index (1-D). A value closer to 1 indicates higher diversity and a more even distribution of species. The values for both mollusc classes are similar in Sector Pajangan B, whereas a larger gap is observed in Sec-

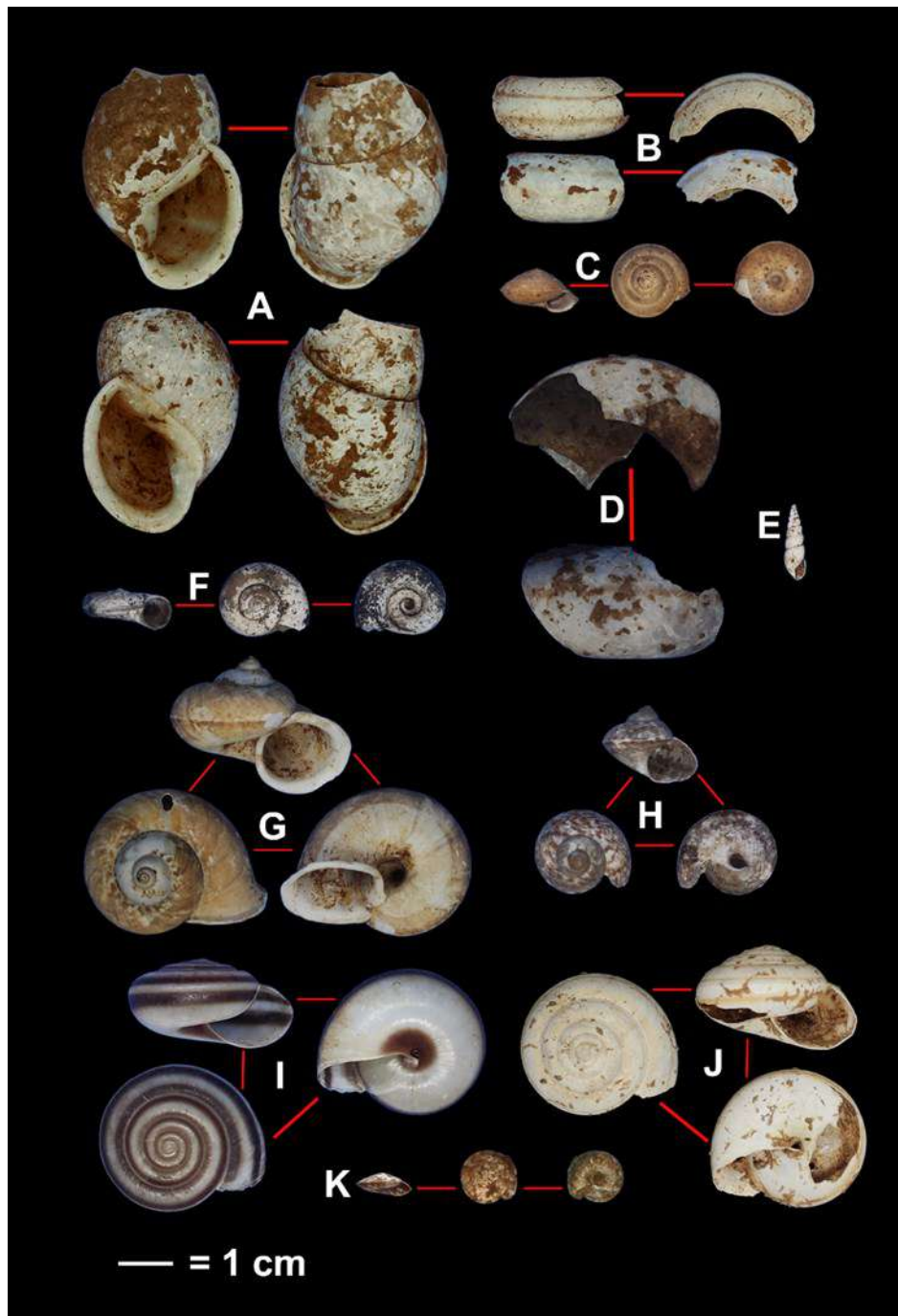


Figure 4. Terrestrial gastropods: A. *Amphidromus* sp.; B. *Tricheulota zodiacus*; C. *Landouria* sp.; D. *Hemiplecta* sp.; E. *Paropeas achatinaceum*; F. *Opisthoporus corniculatus*; G. *Cyclophorus perdis*; H. *Cyclophorus* sp.; I. *Elaphroconcha javacensis*; J. *Elaphroconcha* sp.; K. *Trochomorpha* sp. (Photo: Restu Budi Sulistiyo 2023).

tor Pajangan.

The low diversity and evenness indices may reflect the fact that these mollusc shells were products of human activity in prehistoric times. Rather than being randomly collected, they were likely gathered by the site's past inhabitants for specific purposes. These indices may also indicate a preference for certain types of molluscs by the site's inhabitants, leading to both low diversity and low evenness, particularly in the gastropod class.

The presence of molluscs at Kompleks Ceruk Pajangan and subsistence efforts

Of the 25 identified species, 14-or more than half- are associated with anthropic activities, meaning they were brought by humans to the vicinity of the rockshelter site (see Table 1 and 4). Previous studies have revealed that

Table 2. Number of Identified Specimens and Minimum Number of Individuals quantification result.

Family/ Species	NISP	%	MNI	%	Ratio
Cyrenidae	22	0.7	1	0.1	
<i>Geloina expansa</i> (Mousson, 1849)	422	12.5	123	7.9	29.1
<i>Batissa violacea</i> (Lamarck, 1818)	80	2.4	23	1.5	28.8
Arcidae	1	0.0	1	0.1	
<i>Tegillarca granosa</i> (Linnaeus, 1758)	1	0.0	1	0.1	100.0
Pectinidae					
<i>Chlamys</i> sp. (Röding, 1798)	1	0.0	1	0.1	100.0
Placunidae					
<i>Placuna</i> sp. (Lightfoot, 1758)	84	2.5	24	1.5	28.6
Unionidae	158	4.7	16	1.0	10.1
<i>Pilsbryoconcha exilis</i> (I. Lea, 1838)	202	6.0	103	6.6	51.0
<i>Rectidens sumatrensis</i> (Dunker, 1852)	8	0.2	8	0.5	100.0
<i>Lens contradens</i> (I. Lea, 1838)	10	0.3	10	0.6	100.0
Potamididae					
<i>Telescopium telescopium</i> (Linnaeus, 1758)	56	1.7	30	1.9	53.6
<i>Terebralia palustris</i> (Linnaeus, 1758)	50	1.5	33	2.1	66.0
Viviparidae					
<i>Filopaludina javanica</i> (von dem Busch, 1844)	5	0.1	4	0.3	80.0
Ampulariidae		0.0		0.0	
<i>Pila ampullacea</i> (Linnaeus, 1758)	1716	50.8	786	50.5	45.8
Pachychilidae					
<i>Sulcospira testudinaria</i> (von dem Busch, 1842)	292	8.6	292	18.8	100.0
Thiaridae					
<i>Melanoides tuberculata</i> (O. F. Müller, 1774)	1	0.0	1	0.1	100.0
Camaenidae	23	0.7	15	1.0	65.2
<i>Amphidromus</i> sp. (Albers, 1850)	17	0.5	12	0.8	70.6
<i>Tricheulota zodiacus</i> (A. Férussac, 1821)	55	1.6	20	1.3	36.4
<i>Landouria</i> sp. (Godwin-Austen, 1918)	1	0.0	1	0.1	100.0
Ariophantidae					
<i>Hemiplecta</i> sp. (Albers, 1850)	48	1.4	22	1.4	45.8
Achatinidae					
<i>Paropeas achatinaceum</i> (L. Pfeiffer, 1846)	5	0.1	5	0.3	100.0
Cyclophoridae	1	0.0	1	0.1	
<i>Opisthoporus corniculus</i> (Mousson, 1849)	2	0.1	2	0.1	100.0
<i>Cyclophorus perdix</i> (Broderip & G. B. Sowerby I, 1830)	19	0.6	9	0.6	47.4
<i>Cyclophorus</i> sp. (Montfort, 1810)	9	0.3	4	0.3	44.4
Dyakiidae					
<i>Elaphroconcha javacensis</i> (Férussac, 1821)	1	0.0	1	0.1	100.0
<i>Elaphroconcha</i> sp. (Gude, 1911)	1	0.0	1	0.1	100.0
Trochomorphidae					
<i>Trochomorpha</i> sp. (Albers, 1850)	7	0.2	7	0.4	100.0
Unidentified bivalve fragments	30	0.9			
Unidentified gastropod fragments	50	1.5			
NISP - MNI	3298	97.6	1557	47.2	
Total Number of Fragments	3378	100.0			

mollusc consumption was common among prehistoric populations (Stephens et al. 2008; García-Escárzaga & Gutiérrez-Zugasti 2021; Sulistiyo & Fakhri 2023; Sulistiyo & Wibowo 2023). This indicates that the inhabitants of this site collected molluscs from their original habitats, transported them to the site, consumed them, and discarded the shells, which were subsequently deposited in the soil.

Almost all freshwater aquatic molluscs in the anthropic category represent subsistence efforts by the prehistoric inhabitants, as they were transported to the site specifically for consumption. Several freshwater mollusc specimens also exhibit breakage patterns indicative of efforts to extract the soft

Table 3. Molluscs diversity and evenness measurements.

Unit	Bivalve				Gastropod			
	N-TAXA	1-D	H'	E	N-TAXA	1-D	H'	E
U18 T15	4	0.39	0.754	0.544	10	0.69	1.53	0.664
U18 T20	1	0	0	0	3	0.04	0.118	0.107
U18 T21	2	1	0.693	1	5	0.28	0.604	0.375
U16 T20	6	0.63	1.31	0.733	12	0.5	0.898	0.362
U14 T18	4	0.61	1.13	0.814	9	0.68	1.39	0.632
Sector Pajangan	7	0.7	1.4	0.722	16	0.53	1.14	0.412
U5 T2	5	0.67	1.23	0.763	4	0.59	1	0.721
U6 T2	3	0.37	0.661	0.601	3	0.41	0.687	0.625
Sector Pajangan B	5	0.55	1.03	0.641	6	0.58	1.1	0.613
Site Overall	8	0.69	1.39	0.668	17	0.53	1.16	0.409

Note: (N-TAXA) Number of Taxa, (1-D) Simpson's index, (H') Shannon-Wiener's index, (E) Shannon's evenness

Table 4. Species distribution in each excavation unit.

Species	U18 T15	U18 T20	U18 T21	U16 T20	U14 T18	U6 T2	U5 T2
<i>Geloina expansa</i> *	++	-	++	++	++	++	++
<i>Batissa violacea</i> *	-	-	++	++	++	-	++
<i>Tegillarca granosa</i> *	-	-	-	-	-	-	+
<i>Chlamys</i> sp.*	+	-	-	-	-	-	-
<i>Placuna</i> sp.*	-	-	-	++	++	++	++
<i>Lens contradens</i> *	-	-	-	++	-	-	-
<i>Pilsbryconcha exilis</i> *	++	++	++	++	++	++	++
<i>Rectidens sumatrensis</i> *	++	-	-	++	-	-	-
<i>Telescopium telescopium</i> *	++	-	++	++	++	++	-
<i>Terebralia palustris</i> *	-	-	++	++	++	-	-
<i>Turritella terebra</i> *	-	-	-	-	-	-	-
<i>Filopaludina javanica</i> *	-	-	-	+	+	-	-
<i>Pila ampullacea</i> *	++	++	++	++	++	++	++
<i>Sulcospira testudinaria</i> *	++	++	-	++	++	-	++
<i>Hemiplecta</i> sp.	+	+	-	+	-	-	-
<i>Melanoides tuberculata</i> *	-	-	-	+	-	-	-
<i>Amphidromus</i> sp.	-	-	-	+	+	-	+
<i>Tricheulota zodiacus</i>	+	-	-	+	+	-	-
<i>Landouria</i> sp.	+	-	-	-	-	-	-
<i>Lissachatina fulica</i>	-	-	-	-	-	-	-
<i>Paropeas achatinaceum</i>	-	-	-	-	-	-	+
<i>Opisthoporus corniculatus</i>	+	-	-	+	-	-	-
<i>Cyclophorus perdix</i>	+	-	+	+	-	-	+
<i>Cyclophorus</i> sp.	-	-	+	-	+	+	-
<i>Lagocheilus obliquistriatus</i>	-	-	-	-	-	-	-
<i>Elaphroconcha javacensis</i>	+	-	-	-	-	-	-
<i>Elaphroconcha</i> sp.	+	-	-	+	+	-	-
<i>Trochomorpha</i> sp.	+	-	-	+	-	-	+
<i>Apoecus</i> sp.	-	-	-	-	-	-	-

Note: (+) Present, (-) Absence, (*) Anthropic, (++) Abundant (as food).

body parts, likely for food (Hunt & Hill 2017). Furthermore, the mollusc shells at this site are relatively intact. If the accumulation and consumption of molluscs had been carried out by animals such as rodents or otters, the shells would typically be more heavily damaged or fragmented (Hunt & Hill 2017; Sulistiyo & Fakhri 2023).

The primary dietary molluscs include freshwater species from families such as Ampullariidae, Pachychilidae, and Unionidae. The gastropod *Pila ampullacea* is likely the main food source for the prehistoric inhabitants of Kom-

pleks Ceruk Pajangan, as its shells and opercula were recovered in abundance across all excavation units. Similarly, the gastropod species *Sulcospira testudinaria* was also found in large quantities and appears in nearly all units, except U18T21 and U6T2. From the bivalve class, the Unionidae family includes species such as *Pilsbryoconcha exilis*, *Rectidens sumatrensis*, and *Lens contradens*, with *P. exilis* being commonly found across all excavation units. Previous studies suggest that Unionidae bivalves were commonly consumed as food by prehistoric people (Corr 2000; Thomas 2002; Stephens et al. 2008; Faulkner 2011; Pickard et al. 2017; Sulistiyo & Wibowo 2023).

Within the same karst area, mollusc records are similar at the family level across different sites, but dietary preferences vary. At Gua Bedug and Gua Kidang, located within the same karst region, Unionidae bivalves and *Sulcospira testudinaria* gastropods were also identified as food sources (Kirana 2013; Sulistiyo & Wibowo 2023). However, dietary emphasis differs between sites. At Gua Kidang, Unionidae bivalves were the primary food source, followed by Camaenidae gastropods (Kirana 2013). At Gua Bedug, *Sulcospira testudinaria*, a species of Pachychilidae family was the most exploited food source, followed by Unionidae (Sulistiyo & Wibowo 2023). These differences may reflect local variations in mollusc availability between sites. However, as part of the broader karst region, the inhabitants of Kompleks Ceruk Pajangan, Gua Bedug, and Gua Kidang shared a similar subsistence practice of prioritising the exploitation of freshwater molluscs over other types.

The morphometric measurement of *Pila ampullacea* opercula provides valuable insights into the dietary habits of the Kompleks Ceruk Pajangan inhabitants. The operculum of *Pila ampullacea* is calcareous, making it harder and more likely to be preserved compared to the softer, corneous operculum of *Sulcospira testudinaria*, which decomposes easily. Corneous opercula are primarily composed of organic materials like keratin or similar proteinaceous substances. These materials are biodegradable and susceptible to enzymatic breakdown by microorganisms, fungi, and other decomposers in the environment. In contrast, calcareous opercula consist largely of calcium carbonate (CaCO_3), an inorganic mineral. This mineral structure is highly resistant to biological decay and can persist in the environment for extended periods under favourable conditions (Glover & Kidwell 1993; Kidwell & LaBarbera 1993).

Due to its composition and hardness, *Pila ampullacea* operculum is not edible, whereas the softer operculum of *Sulcospira testudinaria* may still be consumable. No *Sulcospira testudinaria* opercula were recovered from any excavation units at this site. However, this absence does not necessarily confirm their consumption, as they may have decomposed naturally. In contrast, *Pila ampullacea* opercula were found in abundance, suggesting that the prehistoric inhabitants consumed only the soft body parts of this species and discarded the opercula.

Interestingly, *Pila ampullacea* opercula were recovered from all seven excavation units, while their shells were found in only five. A particularly noteworthy observation, as reflected in Figure 5, is the abundance of *Pila ampullacea* opercula in units U18 T20 and U18 T21, where their shells were absent. This may indicate a food preparation process: the soft bodies of *Pila ampullacea* were likely extracted in a different area of the site and subsequently brought to units U18 T20 and U18 T21 for consumption, leaving only the opercula deposited in these units.

Both the shells and opercula of *Pila ampullacea* were most frequently found in unit U16 T20 in terms of both quantity and preservation. In this unit, the highest quantities and best-preserved *Sulcospira testudinaria* remains were also discovered. The vertical distribution of these two species suggests that *Pila ampullacea* was consumed alongside *Sulcospira testudinaria* (Figure 6).

Originating from the same freshwater habitat, *Sulcospira testudinaria* likely served as a complementary food source to *Pila ampullacea*.

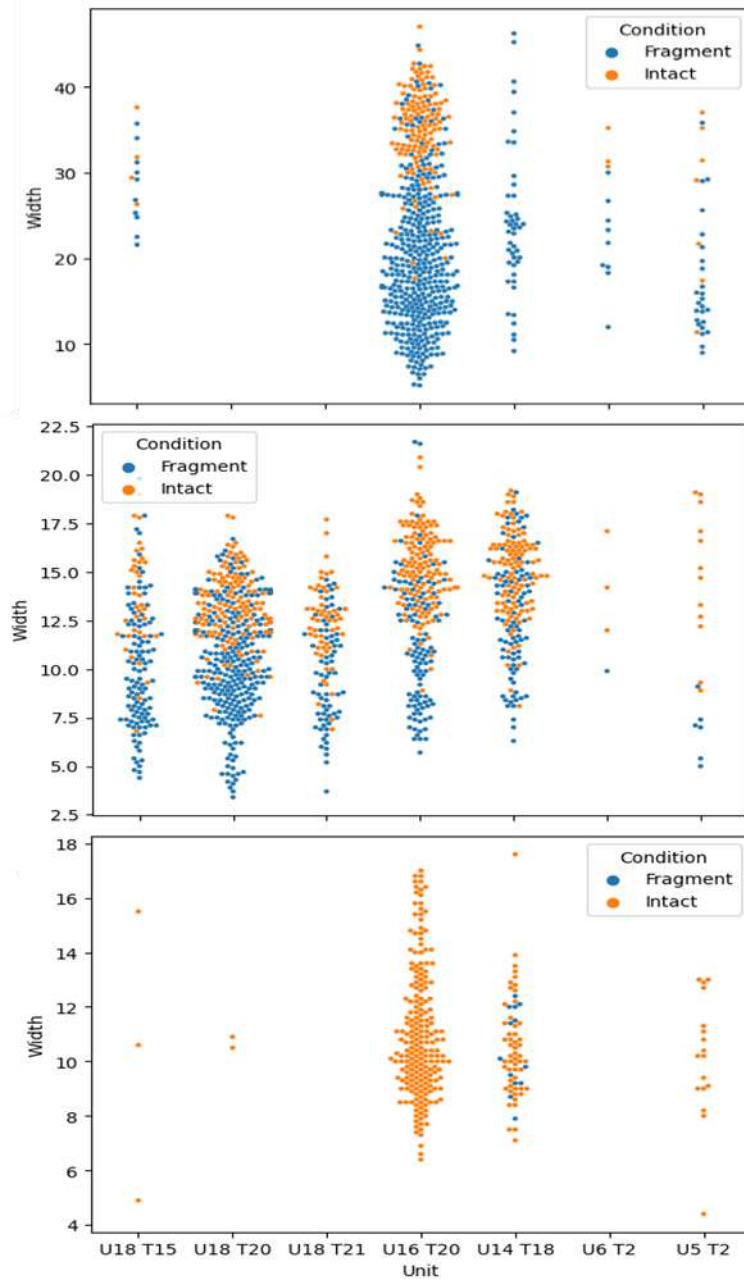


Figure 5. Swarmplot showing distribution of *Pila ampullacea* shells (above, $n=611$) and opercula (middle, $n=1105$), and *S. testudinaria* shells (below, $n=292$) across all units.

Besides freshwater molluscs, the inhabitants of Kompleks Ceruk Pajangan also consumed molluscs from marine and estuarine habitats. Common families in this habitat category include Cyrenidae, Placunidae, and Potamididae. Bivalves from the Cyrenidae family include the species *Geloina expansa* and *Batissa violacea*. *Geloina expansa* shells are prevalent in nearly all excavation units except U18T20, whereas *Batissa violacea* is less common. *Geloina expansa* was also found at Gua Bedug in fewer quantities (Sulistiyo & Wibowo 2023), and absent as a ecofacts at Gua Kidang (Kirana 2013). Another bivalve, *Placuna* sp., was identified, though its thin shells were highly fragmented. Gastropods from the Potamididae family, including *Telescopium telescopium* and *Terebralia palustris*, were recovered in edible size. *Telescopium telescopium* shells are abundant, while *Terebralia palustris* appears to be limited to only a few excavation units. All the molluscs mentioned above remain commonly

consumed today (Carpenter & Niem 1998). A unique pattern was observed in the Pajangan Sector, particularly in unit U14 T18, where marine and estuarine molluscs appeared to have been consumed more frequently than freshwater bivalves during a specific period (Figure 6).

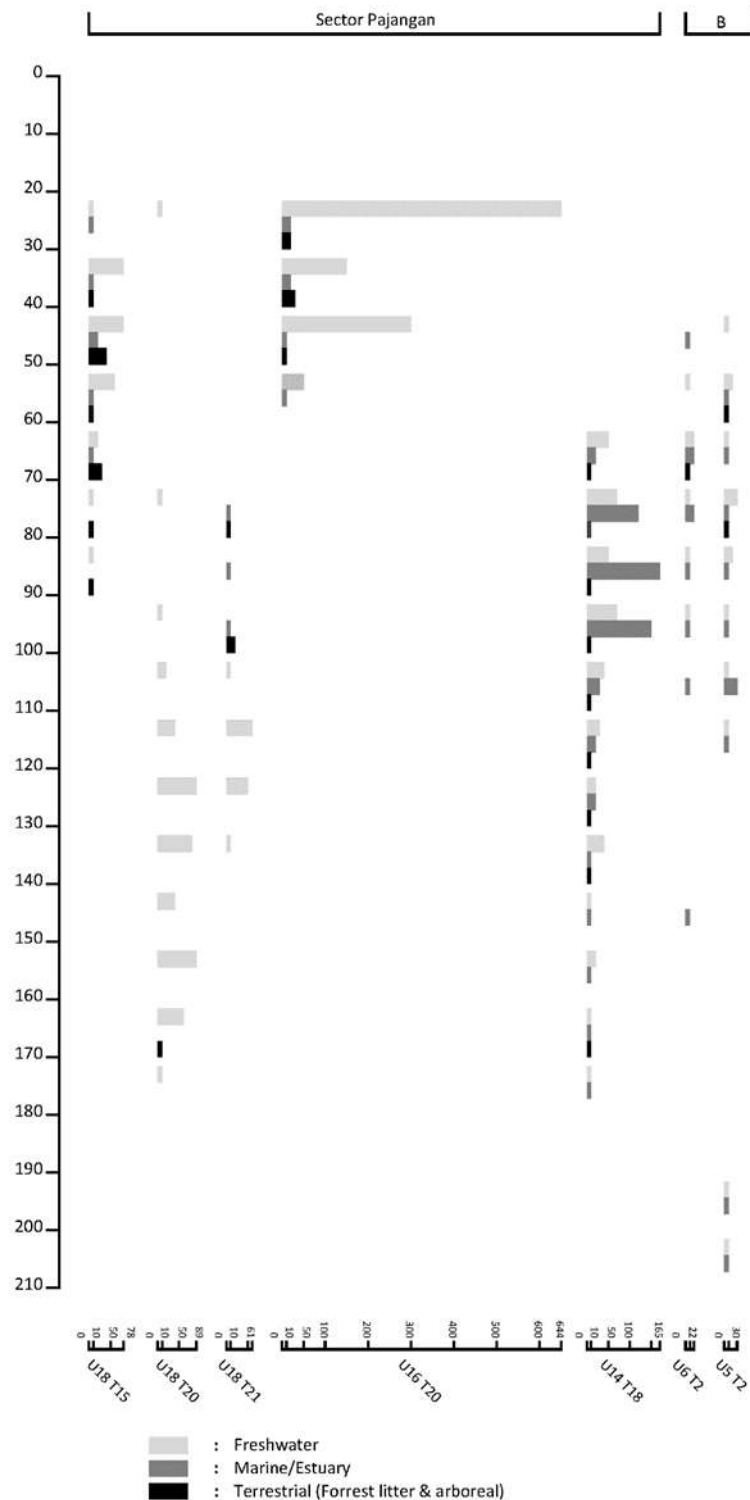


Figure 6. Barplot showing vertical distribution of mollusc shells based on habitat, excluding shells that are unidentified across units (in cm, n=3298).

In addition to aquatic molluscs, terrestrial gastropods such as *Amphidromus* sp. and *Hemiplecta* sp. are known from other studies to have been exploited by prehistoric peoples (van Benthem Jutting 1952; Kirana 2013; Sulistiyo & Fakhri 2023; Sulistiyo & Wibowo 2023). These land snails, although low in fat, are high in protein, making them better suited as supplementary rather than primary food sources. Land snails are often utilised when pre-

ferred food sources are scarce or unavailable (Hunt & Hill 2017). At Kompleks Ceruk Pajangan, the shells of *Amphidromus* sp. can still be observed as food from the cut apices, as seen in Gua Kidang (Kirana 2013) and Gua Bedug (Sulistiyo & Wibowo 2023). *Hemiplecta* sp. were found in highly fragmented conditions. This fragmentation prevents the identification of taphonomic markers that could confirm their consumption. While such fragmentation could result from human efforts to extract soft body parts as observed in other studies (Sulistiyo & Fakhri 2023), it may also have occurred naturally.

Certain other mollusc species appear to have been transported from aquatic environments by human activity but do not show evidence of consumption. Marine and estuarine species such as *Chlamys* sp. and *Tegillarca granosa* might have been accidentally collected by the inhabitants of Kompleks Ceruk Pajangan while gathering other molluscs. A similar explanation may apply to the freshwater species *Filopaludina javanica* and *Melanoides tuberculata*. Their presence at the site is minimal, and in their natural habitats, they are often found alongside other frequently gathered freshwater molluscs (Sulistiyo & Fakhri 2023; Sulistiyo & Wibowo 2023).

The Paleoenvironment surrounding Kompleks Ceruk Pajangan

Four main habitats are identified from the mollusc record, providing a clearer understanding of the surrounding environment at Kompleks Ceruk Pajangan 6000–8000 years ago. During this time, the mollusc evidence suggests that the area was characterised by forested landscapes, abundant freshwater resources, and close proximity to marine/estuarine environments. This is supported by the presence of 33 specimens from the forest litter habitat, 96 from arboreal habitats, 714 from marine/estuary habitats, and 2391 specimens from freshwater habitats (Figure 7).

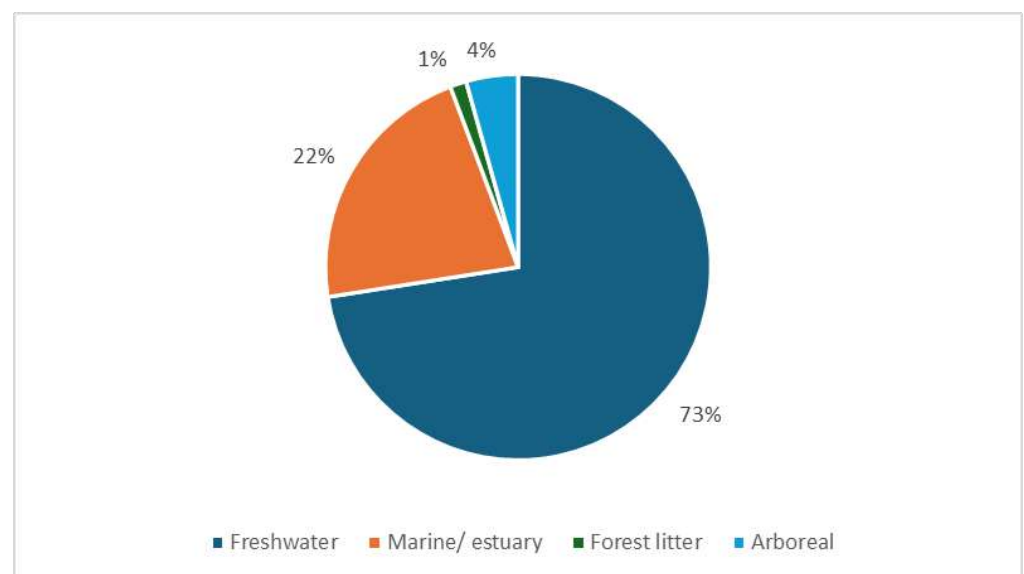


Figure 7. Habitat distribution of mollusc ecofacts in Kompleks Ceruk Pajangan (n=3298).

Given that this site is currently located 43 km from the modern coastline of northern Java, the abundance of marine/estuarine specimens is remarkable. This habitat is represented archaeologically by five species. The bivalve of *Geloina expansa* and gastropods of *Telescopium telescopium* and *Terebralia palustris* are commonly found in muddy substrates within mangroves or coastal mudflats. *Placuna* sp. and *Tegillarca granosa* also inhabit coastal environments such as lagoons, sheltered bays, and estuaries, while *Batissa violacea* resides in riverbanks and riverbeds, favouring fresh and brackish, often flowing water. Rather than indicating a broad foraging range for the prehistoric

inhabitants, the abundance of marine/estuarine specimens likely reflects significant changes in the coastline over at least 5000 years. Furthermore, the high degree of coastline changes on the north coast of Java was also been well documented in the historical records.

Historical records document dramatic shifts along the northern coast of Java. For example, areas such as Pati, Demak, and Kudus were once part of a strait that separated Mount Muria from Java. Large ships navigated this strait in 800 CE, and small boats could still traverse it until the 18th century, despite sedimentation-induced shallowing (De Graaf & Pigeaud 1986). It has been proposed that the ancient coastline followed the 25-metre contour line on modern topographic maps (Soekmono 1967), suggesting that low-lying areas between Semarang and Rembang were once submerged (Figure 8).

Considering the ancient coastline, during the occupation of Kompleks Ceruk Pajangan (6000-8000 years ago), this site was approximately 2,5 km from the shoreline. This close proximity explains the abundance of marine/estuarine molluscs compared to other sites in the same karst area. For instance, Gua Kidang yielded only three ecofact specimens of marine/estuarine molluscs (two from Marginellidae and one from Donacidae) (Kirana 2013). The other marine/estuarine molluscs of Cyrenidae (8) and Arcidae (1) at Gua Kidang were used as artefacts. Gua Bedug in total yielded 63 specimens, more than Gua Kidang and less than Kompleks Ceruk Pajangan. Gua Bedug is estimated to have been 14 km from the ancient coastline, and Gua Kidang 19 km. This trend suggests that the quantity of marine/estuarine molluscs diminishes with increasing distance from the coast.

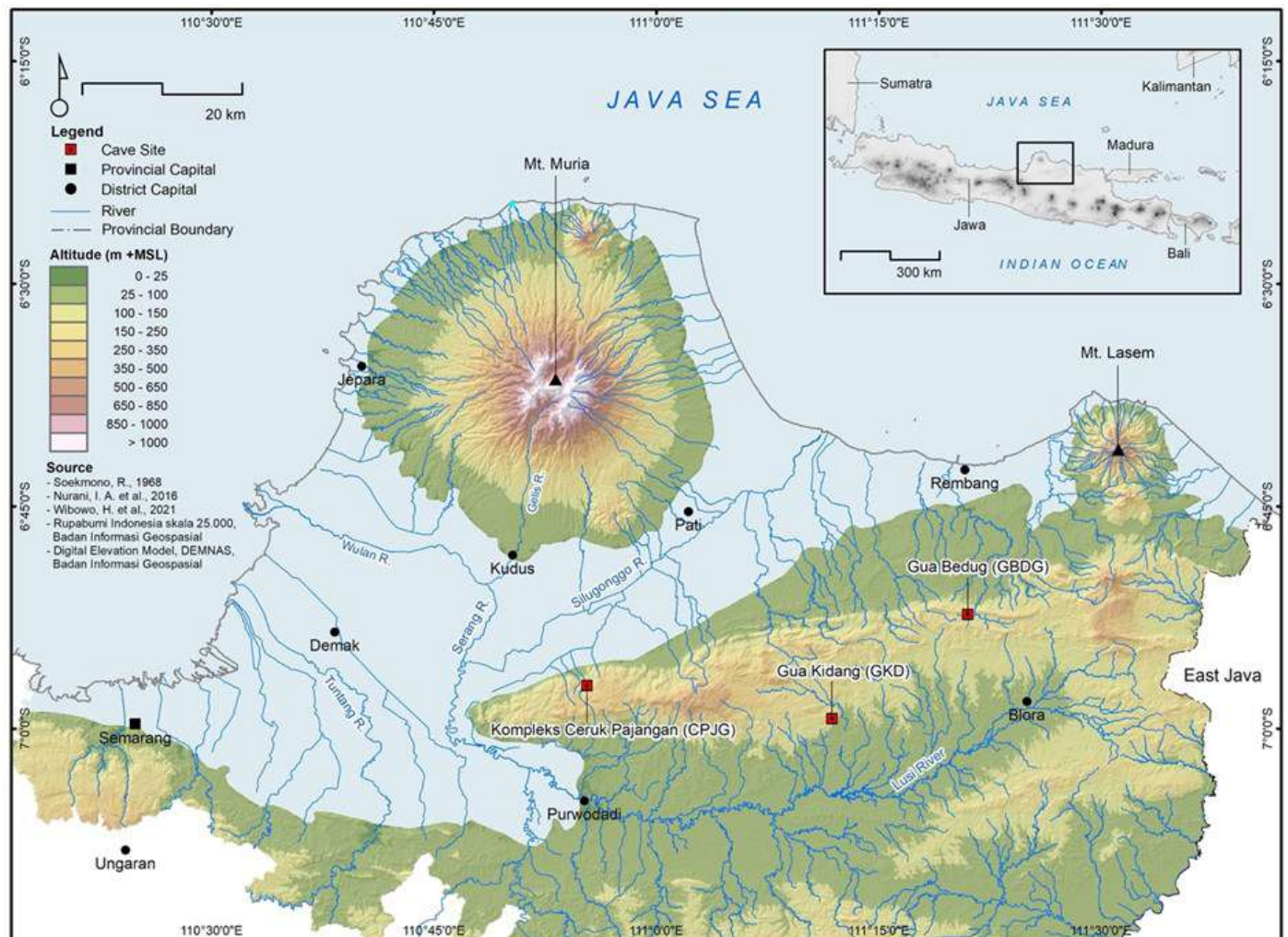


Figure 8. Ancient coastline along the Muria Strait, reconstructed based on Soekmono (1967) and overlaid with present-day river networks (Credits: Ahmad Surya Ramadhan and Hari Wibowo 2024).

Further mollusc data indicate that the ancient coastal area near Kompleks Ceruk Pajangan was surrounded by forested environments with plentiful freshwater resources. Forest environments are represented by molluscs from arboreal and forest litter habitats (Figure 9). Tree snail species such as *Amphidromus* sp., *Tricheulota zodiacus*, and *Hemiplecta* sp. depend on vegetation cover and forest canopy, while *Landouria* sp. relies on herbaceous plants but not heavily on forest litter (Heryanto 2017).

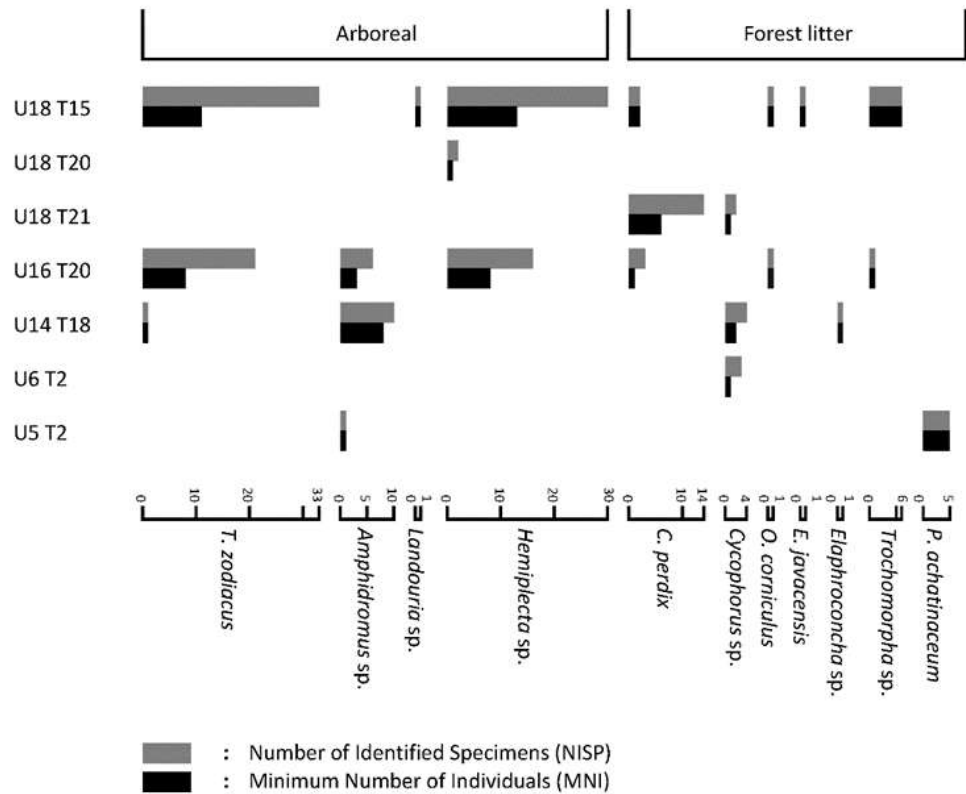


Figure 9. Barplot showing distribution of mollusc ecofacts based on arboreal and forest litter habitats across all units (n=165).

Species from Achatinidae, Cyclophoridae, Dyakiidae, Trochomorphidae, and Enidae families further indicate forest environments. These species prefer leaf litter and organic material on the forest floor, hiding in moist locations such as rocks or deadwood to maintain humidity (Hunt & Hill 2017; Sulistiyo & Fakhri 2023). The presence of these species at Kompleks Ceruk Pajangan likely reflects their attraction to organic waste left by human inhabitants. Notably, species like *Paropeas achatinaceum* and *Trochomorpha* sp. were found inside aquatic mollusc shells, indicating their entry into the site after these shells were discarded (Figure 10).

The palaeoenvironment around Kompleks Ceruk Pajangan included several streams that flowed into the ancient strait, which are now tributaries of the Silugonggo River (Figure 8). The abundance of freshwater resources is evidenced by the dominance of freshwater molluscs, which were a primary food source for the site's inhabitants (Figure 11). Species such as *Pila ampullacea* and *Sulcospira testudinaria* are epifaunal molluscs living on substrates in calm waters like ponds, swamps, and lakes (Isnainingsih & Listiawan 2010; Marwoto et al. 2020). Infaunal bivalves from the Unionidae family thrive in mixed mud and sand substrates in low-current waters, where they filter feed. Resilient species like *Melanoides tuberculata* and *Filopaludina javanica* are species that are currently commonly found. These species are more resistant to polluted water conditions (Marwoto et al. 2020).

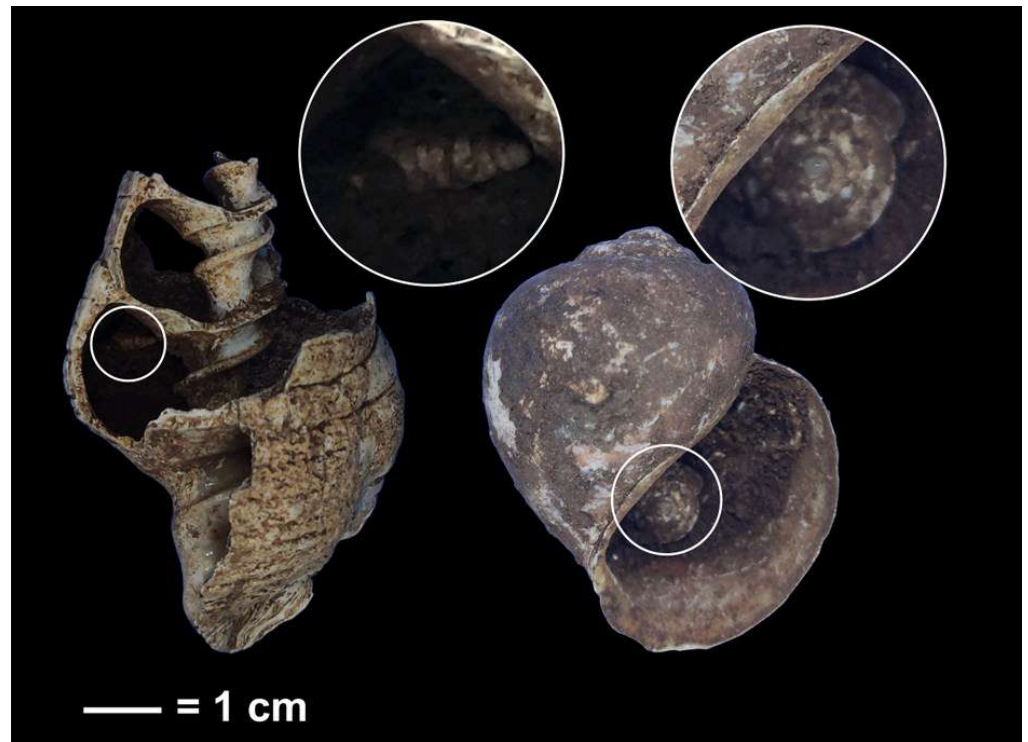


Figure 10. *Paropeas achatinaceum* inside *Terebralia palustris* shell (left) and *Trocomorpha* sp. inside *Pila ampullacea* shell (right) (Photo: Restu Budi Sulistiyo).

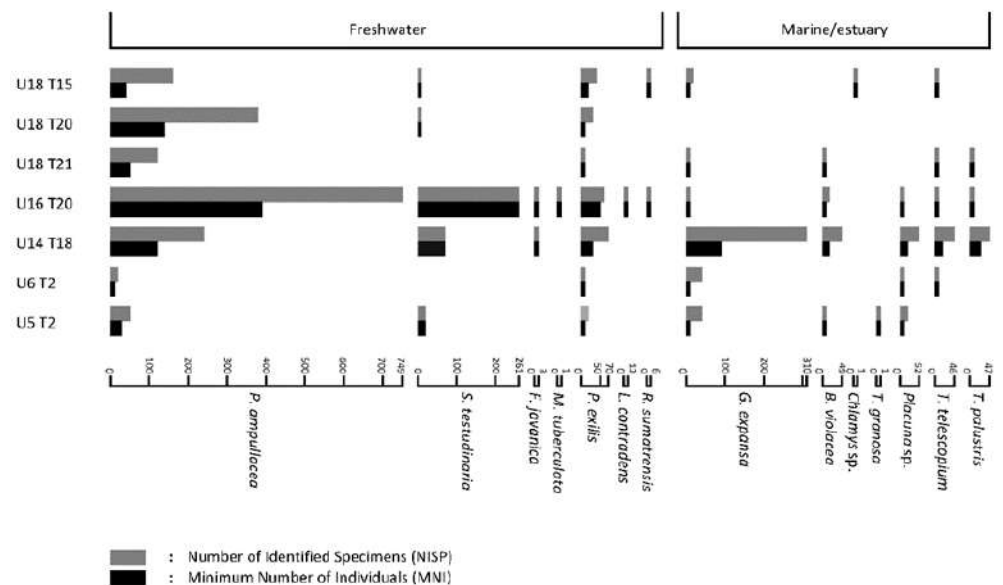


Figure 11. Barplot showing distribution of mollusc ecofacts based on their aquatic habitat across all units (n=2928).

CONCLUSIONS

Human activity significantly influenced the diversity of molluscs found in the archaeological record of Kompleks Ceruk Pajangan. Prehistoric preferences for gathering specific molluscs as food resulted in a low diversity and evenness index, as freshwater molluscs were favoured over other types. The abundance of marine/estuarine specimens supports previous hypotheses and historical data that suggest the presence of an ancient strait. Compared to other sites in the same karst area, these marine/estuarine specimens also highlight the role of proximity to natural resources in shaping past subsistence strategies. While freshwater molluscs were the primary food source, the proportion of consumed marine/estuarine molluscs increased in sites closer to the ancient coastline.

AUTHOR CONTRIBUTION

D.A.T., H.W., and S.N. designed the research and collected the data. H.W. supervised the entire process, contributed to the map, and wrote the initial manuscript. R.B.S. contributed to species identification, documentation, validation, analysed the data, and wrote the initial manuscript. D.A.T., C.R., and S.N. supervised, reviewed, and edited the initial manuscript. All authors have reviewed, revised, and proofread the final manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in any part of this research.

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Research Article

New Records and an Updated List of Amphibians from Ba Den Mountain, Tay Ninh Province, Vietnam

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ABSTRACT

Ba Den Mountain is one of the highest granitic mountains in southern Vietnam and hosts a great diversity of natural habitats and biodiversity potential. In the decades leading up to 2023, only 12 species of amphibians were recorded living on Ba Den Mountain, indicating an incomplete understanding of the amphibian diversity in the area. In 2023 and 2024, we conducted field trips to survey amphibians on Ba Den Mountain, Tay Ninh Province, Vietnam. Based on morphological examination, this study presents an updated checklist of 19 amphibian species from this area. Nine of them were recorded for the first time in Ba Den Mountain: *Glyphoglossus guttulatus*, *Microhyla butleri*, *Microhyla mukhlesuri*, *Micryletta erythropoda*, *Hylarana montosa*, *Hylarana erythraea*, *Chirixalus nongkhorensis*, *Kurixalus* sp., and *Theloderma vietnamense*. Three frogs were new provincial records: *Hylarana montosa*, *Chirixalus nongkhorensis*, and *Kurixalus* sp. Regarding its conservation status, *Glyphoglossus molossus* is listed as a Near Threatened species on both the IUCN Red List (2025) and Vietnam Red List of Threatened Species (IEBR 2025). The mountain's biodiversity is being threatened by tourism development without appropriate conservation measures in place. More surveys and further research are suggested to determine the impact of tourism not only amphibian survival but also on other animal and plant species.

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INTRODUCTION

Ba Den Mountain (known in English as “Black Lady Mountain”) is an isolated granitic outcrop situated amidst agricultural land in Tay Ninh Province, southern Vietnam, covering an area of 1,730 hectares (Pham et al. 2000; Nguyen 2019). It is one of the highest granite mountains in southern Vietnam, with its highest peak up to 986 metres above sea level. It is totally isolated and surrounded by flat agricultural land (Pham et al. 2000). Due to the heterogeneity of its landforms, this mountain has a great diversity of both natural habitats and biodiversity potential. In the herpetofauna list of Vietnam, Bourret (1942) recorded eight species of amphibians in Tay Ninh Province. Nguyen and Ho (1996) and Pham et al. (2000) listed 12 records of amphibians in Ba Den Mountain, Tay Ninh Province. Pham et al. (2024) recorded *Ocidozyga martensi* and analysed the feeding ecology of the species from the mountain. H.T.T. Nguyen et al. (2024) documented a new provincial record of *Microhyla ninhthuanensis* from this area as well as from Tay Ninh Province. Recently, Tran et al. (2025) observed a case of a leucistic tadpole of *Hoplobatrachus chinensis* in this area. Despite sporadic surveys over the past several decades, including recent efforts, no comprehensive assessment of the current amphibian diversity on Ba Den Mountain has been conducted.

In this study, we report nine new records and present an updated checklist for the amphibian fauna of Ba Den Mountain, Tay Ninh Province, Vietnam. Updating species lists is essential for improving our understanding of regional biodiversity, identifying the conservation status of species, taxonomic change, and detecting changes in species distribution over time, especially under the influence of habitat loss and climate change (Do et al. 2017; Krzikowski et al. 2022).

MATERIALS AND METHODS

Study Area and Field Survey

Ba Den Mountain (11°21'06"–11°24'37"N, 106°08'41"–106°11'28"E) is an isolated granitic outcrop situated amidst agricultural land in Tay Ninh Province, southern Vietnam (Figure 1). The highest point of the mountain is 986 metres above sea level, making it the highest mountain in southern Vietnam, south of the Central Highlands. The top of the mountain is dominated by secondary tropical monsoon forest dotted with large boulders, caves, and criss-crossed by streams.

Fieldwork on Ba Den Mountain was carried out during 28–30 September 2023, 26–29 February 2024, 12–14 April 2024, and 16–20 July 2024. Each survey night involved 2–3 researchers with one local people and approximately six person-hours of active search effort between 18:00–23:00 hours. Frogs were collected by hand. After photographing live frogs with a Canon T7 camera equipped with a 60 mm macro lens, each specimen was fixed in 70 % ethanol for 24 hours, and then transferred to 70 % ethanol for storage. Liver tissue samples were preserved for future molecular analysis but were not used in the present study. Together, preserved specimens and liver tissue samples were deposited at the Institute of Life Sciences Collection of Zoology (ILS), Ho Chi Minh City, Vietnam.

Morphological examination and identification

Morphological examination: Measurements commonly used in amphibian studies followed Luong et al. (2022, 2025) and were taken on preserved specimens using dial callipers to the nearest 0.1 mm, including: snout-vent length (SVL); head length (HL), distance from posterior corner of mandible to tip of snout; maximum head width (HW); rostral length (RL), distance from anterior corner of orbit to tip of snout; eye diameter (ED); distance from nostril to the tip of snout (NS); distance from anterior corner of orbit to the nostril

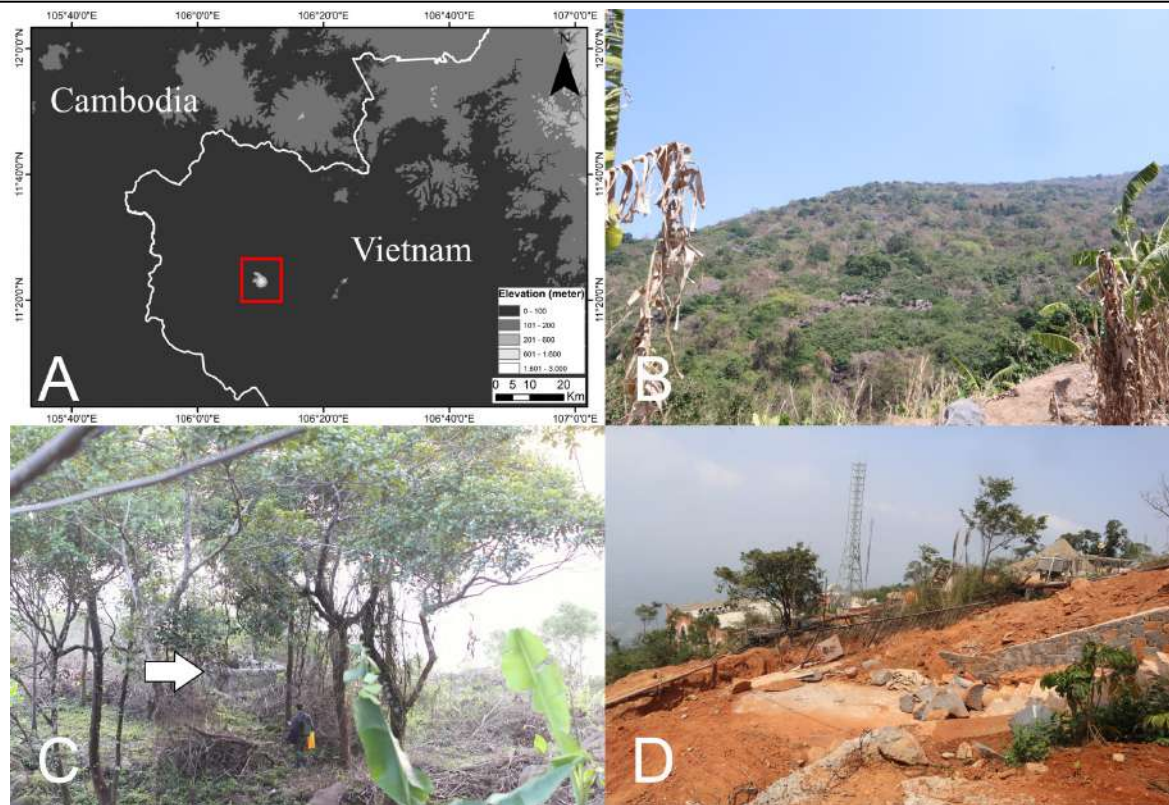


Figure 1. Ba Den Mountain: A. Location of Ba Den Mountain, Tay Ninh Province, Vietnam (red square), B. Habitat of Ba Den Mountain, C. A small artificial pool where *Theloderma vietnamense* was found (white arrow) on the top of mountain in April 2023, D. Deforesting for extensive construction on the top of mountain in February 2024.

(EN); interorbital distance (IOD); internasal distance (IND); maximum width of upper eyelid (UEW); distance between anterior corners of orbits (DAE); posterior margin of mandible to nostril (MN); posterior margin of mandible to anterior corner of orbit (MFE); posterior margin of mandible to posterior corner of orbit (MBE); distance between posterior corners of orbits (DPE); tympanum diameter (TD); distance from anterior margin of tympanum to posterior corner of orbit (TYE); upper arm length (UAL), distance from axilla to elbow; forearm length (FAL), distance from elbow to the tip of third finger; outer palmar tubercle length (OPT); medial palmar tubercle length (MPT); inner palmar tubercle length (IPT); femur length (FeL), from vent to knee; tibia length (TbL), distance from knee to tarsus; maximum tibia width (TbW); foot length (FoL), distance from tarsus to the tip of fourth toe; inner metatarsal tubercle length (IMT); outer metatarsal tubercle length (OMT). The webbing formula followed Savage and Heyer (1997). Sex was identified based on the presence of nuptial pads, internal vocal sac openings or examination of the gonads.

Species identification was based on the literature, including Bourret (1942), Tarkhnishvili (1994), Taylor (1962), Stuart et al. (2006), Hecht et al. (2013), Hasan et al. (2014), Poyarkov et al. (2015), Vassilieva et al. (2016), Sheridan and Stuart (2018), Hasan et al. (2019), Le et al. (2021), Poyarkov et al. (2021), Le et al. (2022), Luong et al. (2022), and V.D.H. Nguyen et al. (2024). Conservation status followed Vietnam Red List of Threatened Species (VNRL) (IEBR 2025), and the IUCN Red List of Threatened Species (IUCN 2025).

RESULTS AND DISCUSSION

Species composition

A total of 19 amphibian species representing six families and two orders were documented from Ba Den Mountain, Tay Ninh Province, Vietnam (Table 1). Among them, the family Microhylidae shows the highest diversity with seven

Table 1. List of amphibian species recorded in Ba Den Mountain, Tay Ninh Province, Vietnam.

Number	Species name	Previous records	IUCN (2025)	VNRL (2025)
Order Gymnophiona				
Familia Ichthyophiidae Taylor, 1968				
1	<i>Ichthyophis</i> cf. <i>kohtaoensis</i> Taylor, 1960 ⁺	1	DD	
Order Anura				
Familia Bufonidae Gray, 1825				
2	<i>Duttaphrynus melanostictus</i> (Schneider, 1799)	1, 5	LC	
Familia Dicroglossidae Anderson, 1871				
3	<i>Occidozyga lima</i> (Gravenhorst, 1829)	1, 5	LC	
4	<i>Occidozyga martensii</i> (Peters, 1867)	2, 5	LC	
5	<i>Fejervarya limnocharis</i> (Gravenhorst, 1829)	1, 5	LC	
6	<i>Hoplobatrachus chinensis</i> (Osbeck, 1765)	1, 4, 5		
Familia Microhylidae Günther, 1858 (1843)				
7	<i>Glyphoglossus guttulatus</i> (Blyth, 1856)*	5	LC	
8	<i>Glyphoglossus molossus</i> Günther, 1869	1, 5	NT	NT
9	<i>Kaloula pulchra</i> Gray, 1831	1, 5	LC	
10	<i>Microhyla butleri</i> Boulenger, 1900*	5	LC	
11	<i>Microhyla mukhlesuri</i> Hasan, Islam, Kuramoto, Kurabayashi, and Sumida, 2014*	5	LC	
12	<i>Microhyla ninhthuanensis</i> Hoang, Nguyen, Ninh, Luong, Pham, Nguyen, Orlov, Chen, Wang, Ziegler, and Jiang, 2021	3, 5		
13	<i>Micryletta erythropoda</i> (Tarkhnishvili, 1994)*	5	LC	
Familia Ranidae Batsch, 1796				
14	<i>Hylarana erythraea</i> (Schlegel, 1837)*	5	LC	
15	<i>Hylarana montosa</i> (Sheridan and Stuart, 2018)**	5	LC	
Familia Rhacophoridae Hoffman, 1932 (1858)				
16	<i>Chirixalus nongkhorensis</i> (Cochran, 1927)**	5	LC	
17	<i>Kurixalus</i> sp. **	5	LC	
18	<i>Polypedates megacephalus</i> Hallowell, 1861	1, 5	LC	
19	<i>Theloderma vietnamense</i> Poyarkov, Orlov, Moiseeva, Pawangkhanant, Ruangsuwan, Vassilieva, Galoyan, Nguyen, and Gogoleva, 2015*	5	LC	

(* = new records for Ba Den Mountain; ** = new records for Ba Den Mountain and Tay Ninh Province; + = species recorded in previous studies but not found during our surveys; 1 = Pham et al. (2000), 2 = Pham et al. (2024), 3 = H.T.T. Nguyen et al. (2024), 4 = Tran et al. (2025), 5 = This study; IUCN (2025) = IUCN Red List of Threatened Species; VNRL (2025) = Vietnam Red List of Threatened Species; DD = Data Deficient, LC = Least Concern, NT = Near Threatened).

species, followed by Dicroglossidae (four species), Rhacophoridae (four species), Ranidae (two species), Bufonidae and Ichthyophiidae (one species each).

Updating the list of amphibians by Pham et al. (2000), nine species are recorded for the first time from Ba Den Mountain, including *Glyphoglossus guttulatus*, *Microhyla butleri*, *Microhyla mukhlesuri*, *Micryletta erythropoda*, *Hylarana montosa*, *Hylarana erythraea*, *Chirixalus nongkhorensis*, *Kurixalus* sp., and *Theloderma vietnamense*.

New records in Ba Den Mountain, Tay Ninh Province, Vietnam

Microhylidae

Glyphoglossus guttulatus (Blyth, 1856) (Figure 2A)

Specimens examined (n=2): one adult male (ILS H.11178), 19 July 2024, Ba Den Mountain, Ninh Son ward, Tay Ninh province (11°23'12"N; 106°08'43"E); one adult female (ILS H.11177), 19 July 2024, Ba Den Mountain, Ninh Son ward, Tay Ninh Province (11°23'14"N; 106°08'34"E).

Diagnosis: morphological characteristics of the specimens match the descriptions of Taylor (1962) and Vassilieva et al. (2016). Head short, mouth narrow, eye small; canthus rostralis indistinct; vomerine teeth present. Tympanum indistinct, supratympanic fold present. Limbs short; interdigital webbing absent on hands and very shallow on feet. Inner metatarsal tubercle prominent, spade-shaped; outer metatarsal tubercle present. Skin slightly granulated. Body coloration varies from brown to orange with dark blotches with different sizes and shapes on back; ventral surface white.

Description: body moderate (SVL 33.08 mm in male; 36.95 mm in female), roughly triangular in shape; head wider than long (HL/HW 0.72–0.76); snout blunted dorsally; nostrils round, closer to the tip of snout than to eye (NS/EN 0.96–0.97); canthus rostralis indistinct, lore oblique, not concave; vomerine teeth present; pupil diamond-shaped; interorbital distance 1.30–1.46 times broader than internarial, and 1.37–1.53 times broader than upper eyelid; tympanum indistinct. Forelimbs slender (FLL 4.38–6.02 mm), hand length (HAL 14.81–17.52 mm), relative finger lengths $I < II < IV < III$, tips of fingers blunt-pointed; webbing absent; nuptial pad absent. Hindlimbs with thigh slender (FeL 15.81–16.56 mm), relative toe lengths $I < II < V < III < IV$, tips of toes pointed; webbing formula $I0 - 2II1\frac{1}{2} - 3III2 - 3\frac{3}{4}IV3\frac{3}{4} - 2V$; tibiotarsal articulation reaching behind the eye at mandible when leg adpressed along body; outer metatarsal tubercle present. Skin: dorsal skin slightly granulated; supratympanic fold present; venter smooth.

Colour in life: dorsal surfaces brown to orange with dark blotches on the back; a dark brown stripe runs along the upper part of the supratympanic fold; limbs with dark brown crossbars dorsally; venter white with throat darker in males.

Natural history notes: specimens were found between 08:30 pm to 09:00 pm on the ground near a lake at the base of the mountain. The surrounding habitat was mixed secondary forest of small hardwoods and shrubs.

Distribution: in Vietnam, this species has been reported from Kon Tum and Gia Lai southwards to Dong Nai and Ba Ria–Vung Tau provinces. Elsewhere, this species occurs in Cambodia, Laos, Myanmar, Malaysia, and Thailand (Nguyen et al. 2009; Vassilieva et al. 2016).

Microhyla butleri Boulenger, 1900 (Figure 2B)

Specimens examined (n=3): two adult females (ILS H.11133, 11138), 15 July 2024, Ba Den Mountain, Ninh Son ward, Tay Ninh Province (11°23'14"N; 106°08'37"E); one male ILS H.11179, 18 July 2024, Ba Den Mountain, Ninh Son ward, Tay Ninh Province (11°23'12"N; 106°08'43"E).

Diagnosis: morphological characteristics of the specimens match the descriptions of Bourret (1942), Hecht et al. (2013) and Luong et al. (2022). Body small, triangular; eye small; canthus rostralis indistinct; vomerine teeth absent. Tympanum indistinct, supratympanic fold present. Interdigital webbing absent on hands and shallow on feet; tips of fingers with notable grooves except the first. Inner and outer metatarsal tubercles present. Skin smooth with some smooth flattened pustules. Body brown; a double X-shaped marking present with white outline on back, ventral surface cream.

Description: body small, triangular (SVL 21.40 mm in male; 21.50–22.35 mm in females); head triangular, longer than wide (HL/HW 1.01–1.05); nostrils round, closer to the tip of snout than to eye (NS/EN 0.82–0.92); canthus ros-

tralis indistinct, lore oblique, not concave; vomerine teeth absent; pupil horizontal; interorbital distance 1.04–1.31 times broader than internarial, and 1.22–1.73 times broader than upper eyelid; tympanum indistinct. Forelimbs slender (FLL 3.67–3.81 mm), hand length (HAL 8.70–9.00 mm), relative finger lengths $I < II < IV < III$, tips of fingers pointed with notable grooves present on tips of three fingers II–IV; webbing absent; nuptial pad absent. Hindlimbs with thigh slender (FeL 10.47–10.87 mm), relative toe lengths $I < II < V < III < IV$; webbing formula $I2 - 2\frac{1}{2}II1\frac{3}{4} - 3III2\frac{1}{3} - 3\frac{1}{2}IV3\frac{1}{2} - 2\frac{1}{4}V$; tibiotarsal articulation reaching to the middle of eye when leg adpressed along body. Skin: dorsal skin smooth with some rather large smooth flattened pustules; supratympanic fold distinct; venter smooth with cloacal region granular.

Colour in life: dorsum and flank brownish with reddish tubercles; a double X-shaped marking on back, darker than ground colour, and outlined with a white band; a white stripe from eye to beginning of arm; limbs with dark transverse bars dorsally; venter cream with throat darker in males than in females.

Natural history notes: specimens were found between 07:30 pm and 08:30 pm on the ground under dry leaves. The surrounding habitat was mixed secondary forest of small hardwoods and shrubs.

Distribution: in Vietnam, this species has been reported from Bac Kan, Lao Cai, and Ha Giang southwards to Ho Chi Minh City, Dong Nai, and Ba Ria – Vung Tau provinces. Elsewhere, this species occurs in Cambodia, China, India, Indonesia, Laos, Malaysia, Myanmar, Singapore, Taiwan, and Thailand (Vassilieva et al. 2016; Frost 2025).

***Microhyla mukhlesuri* Hasan, Islam, Kuramoto, Kurabayashi, and Sumida, 2014 (Figure 2C)**

Specimens examined (n=2): one adult female (ILS H.11136) and one adult male (ILS H.11137) 18 July 2024, Ba Den Mountain, Ninh Son ward, Tay Ninh Province (11°23'12"N; 106°08'43"E).

Diagnosis: morphological characteristics of the specimens match the descriptions of Hasan et al. (2014) and Le et al. (2021). Body small, triangular; eye small; canthus rostralis indistinct; vomerine teeth absent. Tympanum indistinct, supratympanic fold present. Interdigital webbing absent on hands and shallow on feet; tips of fingers lacking grooves. Inner and outer metatarsal tubercles present. Skin smooth with some smooth flattened pustules. Body brown; a double X-shaped marking present on back, darker than ground colour without a white band outlined; ventral surface white.

Description: body small, robust, triangular (SVL 23.48 mm in female; 19.89 mm in male); head triangular, slightly longer than wide (HL/HW 1.03–1.08); snout rounded; nostrils round, closer to the tip of snout than to eye (NS/EN 0.73–0.79); canthus rostralis round, indistinct, lore sloping, and weakly concave; vomerine teeth absent; pupil horizontal; interorbital distance 1.11–1.28 times broader than internarial, and 1.36–1.45 times broader than upper eyelid; tympanum indistinct. Forelimbs slender (FLL 3.13–3.79 mm), hand length (HAL 8.05–8.23 mm), relative finger lengths $I < II < IV < III$, tips of fingers pointed, lacking grooves; webbing absent; nuptial pad absent. Hindlimbs with thigh slender (FeL 10.54–10.58 mm), relative toe lengths $I < II < V < III < IV$; webbing formula $I2 - 2\frac{1}{3}II2 - 3\frac{1}{3}III3 - 4IV4 - 3V$; tibiotarsal articulation reaching the posterior edge or middle of the eye when leg adpressed along body. Skin: dorsal skin smooth with some smooth flattened pustules; supratympanic fold distinct; ventral skin smooth.

Colour in life: dorsum light brown; a double X-shaped marking on the back, darker than ground colour without a white band outlined; flank with a black band from snout to groin, but interrupted at the post corner of eyes; limbs with dark transverse bars dorsally; venter whitish with a few very small

speckles on the throat.

Natural history notes: specimens were found between 07:30 pm and 09:00 pm on the ground under dry leaves. The surrounding habitat was mixed secondary forest of small hardwoods and shrubs.

Comment: Pham et al. (2000) reported *M. ornata* in Ba Den Mountain, Tay Ninh Province, Vietnam. However, this species is restricted to South Asia, and applied *M. fissipes* Boulenger, 1884 is applied for populations in China and South Asia (Nguyen et al. 2009). Recently, Yuan et al. (2016) discussed how Pleistocene climatic fluctuations and the Red River barrier shaped the genetic structure of the *Microhyla fissipes* complex (*M. mukhlesuri* and *M. fissipes*); and documented that *M. fissipes* is not found west or south of the Red River in Yunnan, China and Vietnam.

Distribution: in Vietnam, this species has been reported west and south of the Red River (Yuan et al. 2016; Frost 2025). Elsewhere, this species occurs in Bangladesh, Cambodia, China, India, Indonesia, Laos, Malaysia, Myanmar, and Thailand (Frost 2025).

***Micryletta erythropoda* (Tarkhnishvili, 1994) (Figure 2D)**

Specimens examined (n=2): two adult males (ILS H.11140, 11141), 15 July 2024, Ba Den Mountain, Ninh Son ward, Tay Ninh Province (11°23'14"N; 106°08'37"E).

Diagnosis: morphological characteristics of the specimens match the descriptions of Tarkhnishvili (1994), Poyarkov et al. (2021) and V.D.H. Nguyen et al. (2024). Body small, oval; eye small; canthus rostralis rounded; vomerine teeth absent. Tympanum distinct, round, supratympanic fold very indistinct. Interdigital webbing absent on hands and very shallow on feet; tips of fingers lacking grooves. Inner and outer metatarsal tubercles present. Skin smooth with tiny and flat tubercles posteriorly. Body brick red or pinkish with many black blotches; ventral cream.

Description: body small, oval (SVL 24.04–25.81 mm in males); head triangular, wider than long (HL/HW 0.91–0.95); snout obtuse, rounded dorsally; nostrils oval, closer to the tip of snout than to eye (NS/EN 0.59–0.64); canthus rostralis rounded, and distinct, lore slightly concave; vomerine teeth absent; pupil round; interorbital distance 1.16–1.27 times broader than internarial, and 1.17–1.35 times broader than upper eyelid; tympanum distinct, round. Forelimbs slender (FLL 4.24–5.19 mm), hand length (HAL 9.21–11.09 mm), relative finger lengths I<II<IV<III, tips of all fingers rounded; webbing absent; nuptial pad absent. Hindlimbs with thigh slender (FeL 8.69–8.96 mm), relative toe lengths I<II<V<III<IV; rudimentary web between toes II–III and III–IV; tibiotarsal articulation reaching to posterior edge of tympanum or middle of eye when leg adpressed along body; outer metatarsal tubercle present. Skin: dorsal skin smooth above, posteriorly scattered with tiny and flat tubercles on dorsum of body, flanks, and hindlimbs; supratympanic fold indistinct; ventral skin of body and limbs smooth.

Colour in life: dorsum brick red to pinkish with black blotches; a black patch present from snout to flank with white blotches below the eye and tympanum areas; venter cream with throat notably darker.

Natural history notes: specimens were found at 07:30 pm on grass next to a small puddle. The surrounding habitat was mixed secondary forest of small hardwoods and shrubs.

Comment: in the past, the *Micryletta* population on Ba Den Mountain, Tay Ninh Province was identified as *M. inornata* (Pham et al. 2000; Nguyen et al. 2009). Recently, *Micryletta erythropoda* was shown to be a species distinct from *M. inornata* based on molecular evidence and morphology from the type locality in Dong Nai Province (Poyarkov et al. 2018). Poyarkov et al. (2021) indicated the species' presence in various provinces of Vietnam, including Kon

Tum, Gia Lai, Dak Lak, Binh Phuoc, Ba Ria-Vung Tau, Tay Ninh, An Giang, and Kien Giang, without molecular or morphological evidence. V.D.H. Nguyen et al. (2024) recorded *M. erythropoda* in Dak Nong Province, Vietnam based on both molecular and morphological evidence. This study confirms the distribution of *M. erythropoda* in Ba Den Mountain, Tay Ninh Province, and suggests the previous record is misidentified. We noted the presence of an outer metatarsal tubercle (vs. absent in *M. inornata*), foot webbing rudimentary (vs. absent), supratympanic fold indistinct (vs. rather indistinct), lateral sides of head dark brown with white spots (vs. brown with silver white line along upper lip).

Distribution: in Vietnam, this species has been reported from the lowlands of southern Vietnam. Elsewhere, this species is possibly found in Cambodia and Laos (Frost 2025).

***Hylarana erythraea* (Schlegel, 1837) (Figure 2E)**

Specimen examined (n=1): one adult female (ILS H.11142), 15 July 2024, Ba Den Mountain, Ninh Son Ward, Tay Ninh Province (11°23'09"N; 106°08'34"E).

Diagnosis: morphological characteristics of the specimen match the descriptions by Hasan et al. (2019). The body is moderated in size. Canthus rostralis distinct, lore concave; vomerine teeth present. Tympanum distinct, round, supratympanic fold present. Interdigital webbing absent on hands and present on feet; tips of fingers and toes extend to disks with circummarginal grooves. Inner and outer metatarsal tubercles present. Skin smooth, dorsolateral folds present. Body green with a pair of distinct brown and white stripes along dorsolateral folds; ventral white.

Description: body moderated (SVL 52.2 mm); head triangular, longer than wide (HL/HW 1.18); snout pointed dorsally; nostrils round, closer to the tip of snout than to eye (NS/EN 0.64); canthus rostralis distinct, lore concave; vomerine teeth present; pupil horizontal; interorbital distance 0.90 times broader than internarial, and 0.97 times narrower than upper eyelid; tympanum distinct, rounded. Forelimbs slender (FLL 10.02 mm), hand length (HAL 26.06 mm), relative finger lengths I<II<IV<III, tips of fingers extend to small disks with circummarginal grooves; webbing absent; nuptial pad absent. Hindlimbs with thigh slender (FeL 26.08 mm), relative toe lengths I<II<V<III<IV, tips of toes extend to ogival-shaped disks with circummarginal grooves; webbing formula IO – 1II0 – 2III0 – 2IV2 – 0V; tibiotarsal articulation reaching between eye and nostril when leg adpressed along body; outer metatarsal tubercle present. Skin: dorsal skin smooth; supratympanic fold present; dorsolateral folds distinct and thick, from behind the eye to groin; venter smooth.

Colour in life: dorsal surfaces green with a pair of distinct brown stripes along the white inner side of the dorsolateral folds; limbs brown dorsally; venter white.

Natural history notes: specimen was found at 08:00 pm on the ground next to a big lake at the base of the mountain. The surrounding habitat was mixed by secondary forest of small hardwoods and shrubs, with some local refreshment stalls around the area to cater to tourists.

Distribution: in Vietnam this species has been reported from Lao Cai, and Da Nang southwards to Ba Ria – Vung Tau, and Kien Giang Provinces (Nguyen et al. 2009). Elsewhere, this species occurs in Brunei, Bangladesh, Cambodia, Indonesia, India, Laos, Malaysia, Myanmar, Singapore, Thailand, and the Philippines (Frost 2025).

***Hylarana montosa* (Sheridan and Stuart, 2018) (Figure 2F)**

Specimen examined (n=1): one adult male (ILS H.11085) 26 February 2024,

Ba Den Mountain, Ninh Son ward, Tay Ninh province (11°22'19"N; 106°10'01"E).

Diagnosis: morphological characteristics of the specimen match those described by Sheridan and Stuart (2018) and Le et al. (2022). The body is moderate in size. Canthus rostralis distinct, lore concave; vomerine teeth present. Tympanum distinct, round, supratympanic fold present. Interdigital webbing absent on hands and present on feet; tips of fingers and toes extend to disks with circummarginal grooves. Inner and outer metatarsal tubercles present. Skin smooth, dorsolateral folds present. Body brown with dark brown spots, dark stripes continue along dorsolateral folds; ventral surface has brown mottling.

Description: body moderated in size (SVL 50.02 mm); head triangular, longer than wide (HL/HW 1.09); snout pointed dorsally; nostrils round, closer to the tip of snout than to eye (NS/EN 0.90); canthus rostralis distinct, lore concave; vomerine teeth present; pupil horizontal; interorbital distance 0.59 times broader than internarial and 0.79 times narrower than upper eyelid; tympanum distinct, rounded. Forelimbs slender (FLL 8.91 mm), hand length (HAL 24.40 mm), relative finger lengths I<II<IV<III, tips of fingers extend to small disks with circummarginal grooves; webbing absent; nuptial pad present. Hindlimbs with slender thigh (FeL 25.39 mm), relative toe lengths I<II<V<III<IV, tips of toes extend to small disks with circummarginal grooves; webbing formula $I0 - 1\frac{1}{2}II0 - 2III0 - 2IV1 - 0V$; tibiotarsal articulation reaching posterior edge of nostril when leg adpressed along body; outer metatarsal tubercle present. Skin: dorsal skin finely granular; supratympanic fold present; flanks slightly glandular; dorsolateral fold distinct, from behind the eye to groin; venter smooth.

Colour in life: dorsal surfaces brown with dark brown spots; dark stripes run from snout to the groin, broken posterior to axilla, continuing along dorsolateral fold; ventral surface with brown mottling; limbs have dark brown cross-bars dorsally.

Natural history notes: the specimen was found calling at 08:30 pm in water of a puddle near a small stream. The surrounding habitat was mixed secondary forest of small hardwoods and shrubs.

Comment: Pham et al. (2000) reported *H. leptoglossa* in Ba Den Mountain, Tay Ninh Province, Vietnam. However, according to Frost (2025), *H. leptoglossa* only occurs in Myanmar, northeast India, Bangladesh, and adjacent western Thailand. In the past, the occurrence of this species in Vietnam was rejected. It was considered likely that the record was a misidentified *Rana nigrovittata* (now *Hylarana nigrovittata*) (Orlov et al. 2002; Frost 2025). Recently, Sheridan and Stuart (2018) redelimited the *Hylarana nigrovittata* complex, excluding *H. nigrovittata* from southern Vietnam and instead assigning southern records to *H. montosa*.

Distribution: in Vietnam, this species has been reported from Gia Lai, Lam Dong, and Ba Ria – Vung Tau Provinces (Sheridan & Stuart 2018; Le et al. 2022). This is the first record from Tay Ninh Province and confirms the distribution of *H. montosa* from Ba Den Mountain as well. Elsewhere, this species occurs in Cambodia, Laos, and Thailand (Sheridan & Stuart 2018; Frost 2025).

***Chirixalus nongkhorensis* (Cochran, 1927) (Figure 2G)**

Specimens examined (n=2): one adult male (ILS H.11164), 17 July 2024, Ba Den Mountain, Ninh Son ward, Tay Ninh province (11°23'13"N; 106°08'35"E); one adult male (ILS H.11182), 18 July 2024, Ba Den Mountain, Ninh Son ward, Tay Ninh Province (11°23'12"N; 106°08'43"E).

Diagnosis: morphological characteristics of the specimen match the descriptions of Taylor (1962), Stuart et al. (2006) and Vassilieva et al. (2016). The

body elongated with long slender limbs. Canthus rostralis distinct; vomerine teeth absent. Pupil horizontal. Tympanum distinct, round, supratympanic fold present. Interdigital webbing very shallow on hands and present on feet; tips of fingers and toes extend to disks with circummarginal grooves. Inner metatarsal tubercles present; outer metatarsal tubercles absent. Skin finely granular. Body yellow, brown pigments present less or more prominent; ventral surface uniform white.

Description: body elongated (SVL 24.04–25.81 mm); head triangular, depressed, slightly longer than wide (HL/HW 1.04–1.06); snout rounded; nostrils round, closer to the tip of snout than to eye (NS/EN 0.73–0.75); canthus rostralis distinct, lore concave; vomerine teeth absent; pupil horizontal; interorbital distance 1.19–1.33 times broader than internarial, and 1.45–1.59 times broader than upper eyelid; tympanum distinct, rounded. Forelimbs slender (FLL 3.90–4.42 mm), hand length (HAL 10.50–10.68 mm), relative finger lengths $I < II < IV < III$, tips of fingers extend to round discs, with circummarginal grooves; webbing formula $I2 - 2II2 - 2III2 - 2IV$ with webbing between fingers I–II and II–III less prominent than between finger III–IV; nuptial pad absent. Hindlimbs with slender thigh (FeL 10.41–13.28 mm), relative toe lengths $I < II < V < III < IV$; webbing formula $I2 - 2II1\frac{1}{4} - 2\frac{2}{3}III1\frac{1}{4} - 2IV1\frac{1}{2} - 1V$; tibiotarsal articulation reaching between eye and nostril when leg adpressed along body; outer metatarsal tubercle absent. Skin: dorsal and ventral skin finely granular; supratympanic fold present.

Colour in life: dorsal and lateral surfaces yellow with less or more prominent brown pigments; upper lip lighter than the ground colour; ventral surface immaculate white, margin of chin with dark spots; limbs with blurry crossbars.

Natural history notes: specimens were found at 09:00 pm on a small branch of a tree near a large lake at the base of the mountain. The surrounding habitat was mixed secondary forest of small hardwoods and shrubs.

Distribution: in Vietnam, this species is known from Lao Cai, Gia Lai, Dong Nai, Binh Thuan, Ba Ria – Vung Tau, and Kien Giang Provinces (Vassilieva et al. 2016; Le et al. 2022). This is the first record of *C. nongkhorensis* from Ba Den Mountain as well as from Tay Ninh Province. Elsewhere, this species occurs in Cambodia, China, India, Laos, Malaysia, Myanmar, and Thailand (Frost 2025).

***Kurixalus* sp. (Figure 2H)**

Specimen examined (n=1): one juvenile (ILS H.11017) 30 September 2024, Ba Den Mountain, Ninh Son ward, Tay Ninh Province ($11^{\circ}23'41''N$; $106^{\circ}10'29''E$).

Diagnosis: morphological characteristics of the specimen match the descriptions of genus *Kurixalus* as Nguyen et al. (2020), and Messenger et al. (2022) with small body size (SVL <50 mm), snout tip pointed, poorly developed webbing on fingers; moderately developed webbing on toes; tips of digits enlarged to discs, all bearing circummarginal grooves, serrated dermal fringes present on forearm and tarsus.

Description: body small (SVL 18.57 mm); head triangular, wider than long (HL/HW 0.96); snout pointed; nostrils round, closer to the tip of snout than to eye (NS/EN 0.71); canthus rostralis distinct, lore concave; vomerine teeth absent; pupil horizontal; interorbital distance 1.28 times broader than internarial, and 1.69 times broader than upper eyelid; tympanum distinct, rounded. Forelimbs slender (FLL 4.41 mm), hand length (HAL 8.75 mm), relative finger lengths $I < II < IV < III$, tips of fingers extend to disks round discs, with circummarginal grooves; webbing formula $I2 - 2\frac{1}{2}II2 - 3\frac{1}{2}III2 - 2IV$ with webbing between finger I–II and II–III less prominent than between finger III–IV; nuptial pad absent. Hindlimbs with thigh slender (FeL 8.9 mm), relative toe lengths $I < II < III < V < IV$; webbing formula $I1\frac{1}{2} - 2II1 - 2\frac{1}{4}III1 - 2IV2 - 1V$;

tibiotarsal articulation reaching between eye and nostril when leg adpressed along body; outer metatarsal tubercle absent. Skin: dorsal and ventral skin with very small tubercles; supratympanic fold present.

Colour in life: dorsal and upper lateral surfaces brown with some dark spots; lower lateral surface orange; ventral surface orange except throat and chest white with dense dark small spots.

Natural history notes: the specimen was found around 11:30 pm on tree leaves approximately two metres above the ground at an elevation of 252 m above sea level on the mountain. The surrounding habitat was mixed secondary forest of small hardwoods and shrubs.

Comment: our specimen is similar to *K. banaensis* and *K. montokarwai* but it was juvenile. The lateral fringes on its limbs and its infra-cloacal tubercles were less developed, which are key characteristics used to separate *K. banaensis* from *K. montokarwai* as Nguyen et al. (2014). Further surveys are needed to collect adult specimens to more precisely identify the species.

Distribution: in Vietnam, the *Kurixalus* genus is known from the north to southernmost Binh Phuoc Provinces (Nguyen et al. 2014; Frost 2025). This is the first record of *Kurixalus* species for both Ba Den Mountain and Tay Ninh Province. Elsewhere, this genus occurs in China, Myanmar, Thailand, Cambodia, Philippines, Japan, Malaysia, Indonesia, and Taiwan (Nguyen et al. 2020; Frost 2025).

***Theloderma vietnamense* Poyarkov, Orlov, Moiseeva, Pawangkhanant, Ruangsuwan, Vassilieva, Galoyan, Nguyen, and Gogoleva, 2015 (Figure 2I)**

Specimens examined (n=2): one adult male (ILS H.11000) and one adult female (ILS H.11003), 22 April 2023, Ba Den Mountain, Thanh Tan commune, Tay Ninh Province (11°22'54.7"N; 106°10'08.6"E).

Diagnosis: morphological characteristics of the specimens match the descriptions of Poyarkov et al. (2015). Body elongated. Canthus rostralis distinct, rounded; vomerine teeth absent. Pupil horizontal. Tympanum distinct, round, supratympanic fold present. Interdigital webbing very shallow on hands and present on feet; tips of fingers and toes extend to disks with circummarginal grooves. Inner metatarsal tubercles present; outer metatarsal tubercles absent. Skin rough with many calcified, white-tipped small pearly asperities. Body brown with numerous bluish-white asperities; a distinct dark-brown chevron of an inverted U-shaped presents on the back; ventral dark blackish-brown with bluish-white thin reticulations forming a network-like pattern.

Description: body elongated (SVL 29.36 mm in male; 34.52 mm in female); head triangular, longer than wide (HL/HW 1.08–1.11); snout truncated on dorsal view; nostrils oval, closer to the tip of snout than to eye (NS/EN 0.40–0.55); canthus rostralis relatively distinct, rounded; vomerine teeth absent; pupil horizontal, and diamond-shaped; interorbital distance 1.31–1.32 times broader than internarial, and 0.91–1.09 times broader than upper eyelid; tympanum distinct, rounded. Forelimbs slender (FLL 4.48–6.60 mm), hand length (HAL 14.99–13.02 mm), relative finger lengths I<II<IV<III, tips of fingers extend to wide disks round discs, with circummarginal grooves; webbing formula $I2 - 2II1\frac{1}{2} - 3III2 - 2IV$; nuptial pad present in male and absent in female. Hindlimbs with slender thigh (FeL 15.93–17.74 mm), relative toe lengths I<II<III<V<IV; webbing formula $I1 - 2II1 - 2III1 - 2\frac{1}{4}IV2 - 1V$; tibiotarsal articulation reaching between eye and nostril when leg adpressed along body; outer metatarsal tubercle absent. Skin: dorsal skin very rough with many calcified, white-tipped small pearly asperities, and dense on forehead and on the area posterior to tympanum, skin not co-ossified to skull; supratympanic fold present; ventral skin of thighs and posterior of belly is granular but smooth on chest and throat.

Colour in life: dorsum is brown with irregular brownish-black spots and covered with numerous bluish-white asperities; a distinct dark-brown chevron of an inverted U-shaped presents on the centre of the back with a rusty-brown anterior edge patch; a dark brown pelvic band presents on flank each side; rear of the dorsum, body flanks and dorsal side at the base of hindlimbs and around the tibiotarsal articulation have creamy-white background; forelimbs and hindlimbs have blackish-brown bands and dark brick-reddish discs dorsally; ventral side is dark blackish-brown with bluish-white thin reticulations forming a network-like pattern, with bluish speckles on the throat.

Natural history notes: specimens were found attached to the inner wall of a small artificial pool at an elevation of 951 m above sea level on the mountain-top. The surrounding habitat was a heavily disturbed mixed secondary forest of small hardwoods and shrubs.

Distribution: in Vietnam, this species is known from southern and central Vietnam (Poyarkov et al. 2015; Frost 2025). Elsewhere, this species occurs in Cambodia, Laos, and Thailand (Ginal et al. 2023; Frost 2025).

Taxonomic changes

Ichthyophis glutinosus: Pham et al. (2000) reported *I. glutinosus* from Ba Den Mountain, Tay Ninh Province, Vietnam. However, this species is distributed only in central and southern Sri Lanka (Frost 2025). Orlov et al. (2002) suggested that *I. glutinosus* population from Vietnam is actually *I. bannanicus*. Recently, Nishikawa et al. (2021) synonymised *I. bannanicus* with *I. kohtaoensis* based on molecular evidence. Therefore, we suggest that the previous record



Figure 2. New records of amphibian on Ba Den Mountain: **A.** *Glyphoglossus guttulatus* (ILS H.11178), **B.** *Microhyla butleri* (ILS H.11138), **C.** *Microhyla mukhlesuri* (ILS H.11137), **D.** *Micryletta erythropoda* (ILS H.11140), **E.** *Hylarana erythraea* (ILS H.11142), **F.** *Hylarana montosa* (ILS H.11085), **G.** *Chirixalus nongkhorensis* (ILS H.11182), **H.** *Kurixalus* sp. (ILS H.11017), and **I.** *Theloderma vietnamense* (ILS H.11003).

of *I. glutinosus* by Pham et al. (2000) should be re-identified as *Ichthyophis* cf. *kohtaoensis* but requires further study to determine the exact species.

Microhyla palmipes: Pham et al. (2000) reported *M. palmipes* in Ba Den Mountain, Tay Ninh Province, Vietnam. Bain and Nguyen (2004) considered the occurrence of this species in Vietnam unjustified and excluded it from the herpetofauna of Vietnam. According to Frost (2025), *M. palmipes* is only known from Sumatra, Nias, Java, and Bali, Indonesia. In our surveys, we did not encounter any *Microhyla* species that look similar to *M. palmipes*. Therefore, we suggest re-identify the previous record of *M. palmipes* by Pham et al. (2000) as *Microhyla* cf. *palmipes* on the list of amphibian species in Ba Den Mountain, Tay Ninh Province, Vietnam.

Polypedates leucomystax: Pham et al. (2000) reported *P. leucomystax* in Ba Den Mountain, Tay Ninh Province, Vietnam. Kuraishi et al. (2012) indicated that this forms part of the *P. leucomystax* complex and documented the distribution of *P. mutus* and *P. megacephalus* in Vietnam. Liu et al. (2018) reported on call characteristics of *P. mutus* and *P. megacephalus* and compared differences of morphology between them. The specimens from Ba Den Mountain should be identified as *P. megacephalus* by characteristics including small reticulate structures on the back; absence of black spots on the sides of the body, with spots arranged in reticulate patterns along the posterior; and tibiotarsal articulation reaching between the eyes and nostrils when legs are adpressed along the body.

CONCLUSION

We recorded nine additional species of amphibians from Ba Den Mountain, Tay Ninh Province: *Glyphoglossus guttulatus*, *Microhyla butleri*, *Microhyla mukhlesuri*, *Micryletta erythropoda*, *Hylarana montosa*, *Hylarana erythraea*, *Chirixalus nongkhorensis*, *Kurixalus* sp., and *Theloderma vietnamense*. Among these, *Hylarana montosa*, *Chirixalus nongkhorensis*, and *Kurixalus* sp. are reported from Tay Ninh Province for the first time. Our new records bring the total amphibian diversity of Ba Den Mountain to 19 species.

In terms of conservation status, *Glyphoglossus molossus* is listed as Near Threatened species in both the IUCN Red List (2025) and Vietnam Red List of Threatened Species (IEBR 2025). During surveys, some threats to the wildlife inhabiting the mountain were observed including extensive deforestation and construction projects. In fact, the specimens of *Theloderma vietnamense* that we observed were gathered in a small artificial pool in a deforested area with extensive construction underway (Figure 1C). Unfortunately, the pool and surrounding area were destroyed one year later (Figure 1D). Ba Den Mountain is considered an unprotected area by local authorities, allowing for development projects like the one observed. With unchecked development, we believe the frogs' habitat atop the mountain is imperilled.

AUTHORS CONTRIBUTION

T.G.T designed the research, collected and analysed the data, and wrote the manuscript. N.V.H.D designed the research and supervised the entire process. N.T.T, N.T.N, and N.T.P.T reviewed the manuscript.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest in any part of this research.

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Research Article

Fucoxanthin-Rich *Padina australis*: Anti-Ageing Properties Through Enzyme Inhibition

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ABSTRACT

The brown seaweed, *Padina australis* Hauck (PAH), known for its high carotenoid content, has been shown to mitigate skin ageing by inhibiting collagenase activity. This study aims to evaluate the inhibitory effects of PAH on collagenase and tyrosinase enzymes. The study begins by extracting the seaweed using ethanol. Afterwards, it is fractionated with ethyl acetate to obtain ethyl acetate-soluble and ethyl acetate-insoluble compounds (EAS and EAI, respectively). The crude extract and both fractions were then subjected to assays to determine their collagenase and tyrosinase inhibitory activities. The results demonstrated that the isolation exhibited the highest inhibition of both enzymes, followed by the EAS, with the EAI showing the least activity. Thin-layer chromatography (TLC) analysis of both fractions revealed retardation factors (R_f identical) to the standard fucoxanthin. TLC densitometry analysis quantified fucoxanthin concentrations at $42.36 \pm 3.94 \mu\text{g mg}^{-1}$ in the extract and $99.104 \pm 7.76 \mu\text{g mg}^{-1}$ in the EAS. Furthermore, the study concluded that the extract, EAS, and isolate had IC_{50} values below $100 \mu\text{g mL}^{-1}$, categorising them as effective inhibitors. The high fucoxanthin content in the extract and fractions suggests significant potential for anti-ageing applications.

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INTRODUCTION

The global ageing population is rapidly increasing, leading to a higher prevalence of age-related health issues and a growing demand for effective anti-ageing solutions (Tamaz & Nino 2021). This demographic shift is accompanied by an urgent need to address the physiological and cosmetic aspects of ageing, which include skin deterioration, loss of elasticity, and the appearance of wrinkles (Rizzi et al. 2021). The skin, as the body's largest and most visible organ, plays a crucial role in protecting against environmental hazards and regulating body temperature. However, it is also highly vulnerable to the ageing process (Robert et al. 2012). Over time, the structural proteins collagen and elastin, which provide skin with its elasticity and firmness, gradually break down. This degradation leads to the common signs of ageing, such as wrinkles, sagging, and a loss of youthful plumpness (Tzaphlidou 2004). These visible changes are often accompanied by a decrease in the skin's ability to heal and retain moisture, making it more prone to dryness and irritation. Consequently, the quest for effective anti-ageing substances that can mitigate or reverse these effects is a major focus within dermatological research (Park et al. 2014).

Given the urgency to mitigate the effects of ageing, scientific investigations have increasingly focused on natural sources of bioactive compounds that can inhibit enzymes responsible for skin ageing (Mukherjee et al. 2011). Collagenase and tyrosinase are two key enzymes implicated in this process. Collagenase degrades collagen, a protein crucial for maintaining skin structure and firmness, while tyrosinase is involved in melanin production, leading to pigmentation issues (Marques et al. 2021). Additionally, these enzymes, when overactive, can lead to the degradation of essential skin proteins such as collagen and elastin, resulting in premature ageing and other skin issues (Srisuksomwong et al. 2023).

Marine organisms, especially seaweeds, are at the forefront of research for natural inhibitors due to their abundance of bioactive compounds. These compounds have shown potential in protecting against enzyme-related damage, offering a natural solution for skin care (Cao et al. 2020). Seaweeds' unique composition, including antioxidants, peptides, and minerals, contribute to their efficacy in skin preservation (Pangestuti et al. 2021). Since seaweed originates from the ocean, has evolved to withstand extreme conditions, surviving in demanding habitats where it endures powerful waves, high salt concentrations, intense sunlight, and shifting temperatures. To survive, they have evolved protective mechanisms by producing bioactive compounds such as antioxidants, peptides, and polysaccharides. These compounds help seaweeds cope with oxidative stress and environmental damage, and when applied to human skin, they offer similar protective benefits. Antioxidants can neutralise free radicals caused by UV exposure, while peptides and other bioactive molecules support skin regeneration and moisture retention. This resilience, developed in their natural marine habitat, translates into potent protective properties for human skin (Naser 2021), making seaweed-derived ingredients highly effective in skincare formulations. As such, they are increasingly being recognised for their therapeutic benefits and are being incorporated into a variety of skincare products aimed at enhancing skin integrity and appearance (Pereira 2018).

Researchers have taken particular interest in *Padina australis* Hauck (PAH), a brown seaweed, because of its remarkable ability to combat ageing. Among these marine organisms, the brown seaweed, *Padina australis* Hauck (PAH), has garnered attention for its potent anti-ageing properties. PAH is considered superior to other marine organisms due to its rich content of bioactive compounds such as fucoxanthin, phlorotannins, and polysaccharides, which provide potent antioxidants, anti-inflammatory, and anti-ageing prop-

erties. Research shows PAH exhibits strong collagenase and tyrosinase inhibitory activities, essential for skin protection and anti-ageing effects. Moreover, PAH is known to contain high levels of fucoxanthin, a carotenoid with powerful antioxidant capabilities (Thiyagarasaiyar et al. 2021). Fucoxanthin's antioxidant properties are closely associated with its ability to combat oxidative stress, a key factor in the ageing process (Guvatova et al. 2020). By protecting cells from oxidative damage and inhibiting the activity of collagenase and tyrosinase, fucoxanthin helps to maintain skin health and reduce signs of ageing (Chen et al. 2021). This study aims to explore the anti-ageing properties of fucoxanthin-rich PAH through its enzyme inhibition capabilities. By extracting and fractionating the bioactive components of this seaweed, their effectiveness in inhibiting collagenase and tyrosinase was assessed. This study aims to explore PAH's potential as a natural anti-ageing agent, focusing on its ability to inhibit collagenase and tyrosinase, key enzymes linked to skin ageing, to support the development of effective skincare solutions.

MATERIALS AND METHODS

Sample Collection

Padina australis brown seaweed was collected from the coast of Gunung Kidul, Yogyakarta. The collection was conducted during low tide, typically between 13:00-14:00 and 01:00-03:00. The seaweed was carefully harvested to ensure minimal damage to the samples and the surrounding ecosystem by placing in a covered bucket and promptly transferred it to a sealed cooler to maintain freshness. Upon arrival, it was washed thoroughly under running water to remove impurities, and the roots were discarded to eliminate any sand attached. The seaweed was then drained and immediately dried at a controlled temperature of 50 °C to preserve its quality.

Methods

Extraction and Fractionation

The collected PAH seaweed was thoroughly washed with seawater to remove any attached debris and sand. The cleaned seaweed was then dried at room temperature and ground into a fine powder to create PAH simplicial powder. A total of 500 g of this powdered seaweed was macerated with 3 L of ethanol for three days, with occasional stirring to enhance the extraction process. After the initial 3-day maceration, the seaweed underwent re-maceration with fresh ethanol for an additional three days to ensure maximum extraction of bioactive compounds (Mugiyanto et al. 2019).

The following procedure was adopted from methods previously developed by other researchers, with modifications. The ethanol extract from both maceration phases was then combined, filtered, and concentrated under reduced pressure using a rotary evaporator to obtain the crude extract. Subsequently, fractionation using ethyl acetate (EA) was carried out. The concentrated crude extract was dissolved in ethyl acetate, shaken vigorously, and then separated using a separating funnel, which had been allowed to settle beforehand. The ethyl acetate soluble fraction (EAS) and ethyl acetate insoluble fraction (EAI) were separated accordingly. The EAS and EAI, enriched with targeted bioactive compounds, were further concentrated under reduced pressure to obtain a more refined extract suitable for subsequent assays and analyses (Fajriyah et al. 2024).

Fourier Transform Infrared (FTIR) Analysis

For FTIR analysis, the extracted and fractionated samples of PAH were prepared by first drying a small amount of each sample to remove residual solvent. The dried samples were then directly analysed using an FTIR spectrometer. Spectra were recorded over a range of wavelengths typically from

4000 to 400 cm^{-1} . The FTIR spectra obtained were analysed to identify characteristic absorption bands corresponding to functional groups present in the samples (Waznah et al. 2022).

Isolation of Active Compounds

Isolation of active compounds from fractions exhibiting collagenase and tyrosinase inhibitory activity was conducted using preparative thin layer (Preparative TLC). Prior to compound separation, the mobile phase selection was crucial and based on solvent polarity, following methods outlined by Nurrochmad et al. (2018). The solvent system employed was methanol: chloroform with varying ratios (80:20 v/v), (60:40 v/v), (50:50 v/v), (40:60 v/v), and (20:80 v/v). Once an optimal mobile phase was determined, preparative TLC was employed to separate compounds. This method was chosen for its simplicity and cost-effectiveness compared to more complex techniques like HPLC or column chromatography. While HPLC might offer better resolution, preparative TLC provided sufficient resolution for the purposes of this study, allowing effective separation of compounds while maintaining practicality and reducing operational costs. The technique was particularly suited to our study's small-scale extraction and isolation objectives, with subsequent analysis confirming the efficacy of the separated compounds (Bhushan 2024; Mondal et al. 2024). Further, separation resulted in distinct bands on the TLC plate, each band was carefully scraped off and subsequently eluted with ethyl acetate. The eluates from each band were concentrated by evaporation to yield isolated compounds. The fucoxanthin isolated in this study was designated as ISO.

Concentration Analysis

Concentration analysis of the ethyl acetate extract and soluble fraction was performed to quantify the presence of fucoxanthin. Each sample was dissolved in 10 % DMSO to ensure solubility and consistency. A series of concentration standards was prepared for both the samples and the fucoxanthin standard (Sigma Aldrich). For thin-layer chromatography (TLC), 0.2 μL of each prepared sample was applied to a silica gel TLC plate using a Linomat 5 (Camag) applicator. The stationary phase was silica gel GF 60 254 with solvent front position was 90.0mm. The samples were eluted with a solvent mixture of chloroform: ethyl acetate: methanol (5:1:0.5) to separate the components effectively. After elution, the TLC plates were analysed using a Camag densitometer to measure the intensity of fucoxanthin bands (Duraisamy et al. 2023).

Bioassay for Collagenase Inhibiting Activity

The assessment of collagenase inhibitory activity was conducted using the Collagenase Activity Colorimetric Assay Kit (Sigma Aldrich, MAK 293). To initiate the assay, 10 μL of the sample and 10 μL of collagenase enzyme were combined in each well of a 96-well plate. The volume was then adjusted to 100 μL with assay buffer. Subsequently, 100 μL of substrate solution was added to each well, ensuring thorough homogenization of the contents. The plate was then incubated for a specified duration at 37 °C to allow enzymatic reactions to proceed. Following incubation, the absorbance of the reaction mixture was measured at 405 nm using a microplate reader. This procedure enabled quantification of collagenase activity inhibition by the samples, providing valuable insights into their potential as anti-aging agents (Zhang et al. 2013).

Bioassay for Tyrosinase Inhibiting Activity

The evaluation of tyrosinase inhibitory activity was performed using the Tyrosinase Inhibitor Screening Kit (Colorimetric, Sigma Aldrich MAK 257). In each well of a microplate, 20 μL of the sample was combined with 50 μL of tyrosinase enzyme solution and 30 μL of tyrosinase substrate. The contents

were thoroughly mixed and incubated for 10 minutes at 25 °C to allow for enzymatic reaction initiation. After the incubation period, the absorbance of the reaction mixture was measured at 510 nm using a microplate reader. Furthermore, for kinetic mode analysis, absorbance measurements were conducted over a period of 60 minutes to monitor the enzymatic reaction progress (Ishihara et al. 2018).

Data Analysis

The inhibitory activity of the samples against collagenase and tyrosinase enzymes was quantified by determining their IC₅₀ values, which represent the concentration required to inhibit 50 % of enzyme activity. Each experiment was conducted in triplicate, and the results were expressed as mean $\pm$ standard deviation (SD) to ensure reliability and consistency across multiple trials. Furthermore, statistical analysis was performed using SPSS version 2.0 with ANOVA to evaluate the significance of differences between groups. A p-value of less than 0.05 was considered statistically significant, indicating meaningful variations in inhibitory effects among the samples tested (White et al. 2022).

RESULTS AND DISCUSSION

Results

Characteristics and Harvesting Practices of Wild PAH

PAH specimens were collected from Gunung Kidul Beach, located between coordinates S8°9'2"E110°36'114" and S8°7'5"E110°32'52", during the months of October and November 2023. The collection took place biannually, specifically timed during low tide periods between 13:00-15:00 and 00:00-03:00, to optimise access to the specimens. The ambient seawater salinity was measured at 32.45 - 33.15 ppt. This natural habitat is free from any cultivation by local communities or governmental interventions. Morphologically, *Padina australis* is characterised by its wide, transparent brown thallus, forming sheet-like or filamentous structures, which are typical of this species. Figure 1 illustrates the appearance of the specimens collected in this study.



Figure 1. Morphological characteristics of PAH collected from Gunung Kidul Beach, showing its wide, transparent brown thallus structure.

Characterization Using FTIR

FTIR analysis was employed to identify functional groups present in the ethyl acetate soluble fraction and isolate of PAH compounds. Spectra were recorded in the range of 4000 to 500 cm⁻¹. As depicted in Figure 2, notable differences were observed in the spectral peaks between the fraction and isolate. Specifically, the presence of hydrogen bonds (O-H) was detected at wave-

numbers 3370 and 3384 cm^{-1} , with a broader peak observed in the isolate between wavenumbers 3200 and 3600 cm^{-1} compared to the fraction. Hydrocarbon bonds ($-\text{C}-\text{H}$) at wavenumber 2922 cm^{-1} exhibited a sharper peak in the isolate than in the fraction. Additionally, a carbonyl group ($\text{C}=\text{O}$) was identified at wavenumber 1718 cm^{-1} , along with characteristic fingerprint regions between 700–800 cm^{-1} . Moreover, the functional groups identified in the ethyl acetate-soluble fraction and isolate samples align with previous research findings by Yip et al. (2014), particularly noting typical fucoxanthin compound absorptions in the fingerprint region and an allenic group at wavenumber 1940 cm^{-1} . Variations in peak intensities between the fraction and isolate suggest the presence of interfering compounds in the fraction, influencing vibrational characteristics of each bond. These bands correspond to common functional groups associated with fucoxanthin and similar carotenoids, reflecting its complex molecular structure.

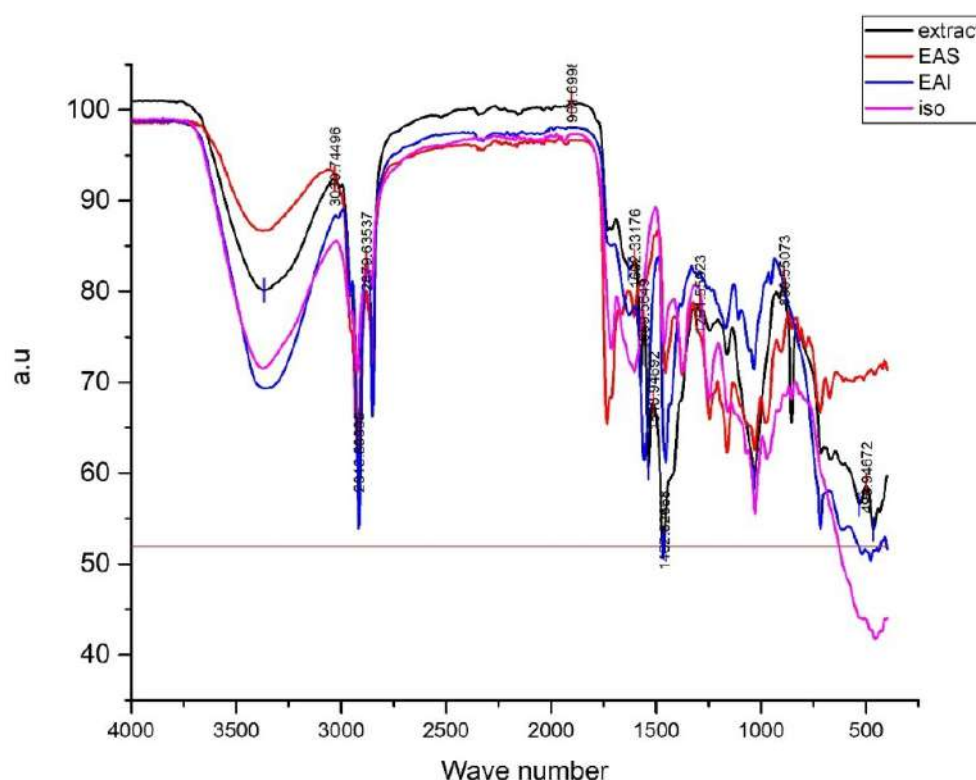


Figure 2. FTIR spectra showing characteristic absorption peaks of functional groups in the ethyl acetate: extract, soluble fraction (EAS), insoluble fraction (EAI) and isolate (iso) of PAH compounds.

Formulation Potential

Before performing densitometric thin-layer chromatography (TLC), the optimisation of the mobile phase was conducted using TLC. The results indicated that the mobile phase (chloroform: ethyl acetate: methanol, 5:1:0.5) provided excellent separation, as evidenced by distinct and well-separated bands. The separated compounds were then subjected to TLC alongside a fucoxanthin standard, with the resulting spot distances corresponding to those of the standard. The outcomes are presented in Figure 3A.

The extract, EAS, and isolate obtained from PAH in this study present promising prospects for further research. To facilitate this process, quantification of active compounds was conducted using TLC-densitometry, focusing on the extract and EAS. Fucoxanthin was employed as the standard chemical for comparative analysis, providing insights into the concentration and distribution of bioactive components within these samples. Figure 3 (B-D) shows the results of TLC-densitometry analysis of the extract and ethyl acetate-

soluble fraction compared with standards. Similar compounds were found between the extract, ethyl acetate-soluble fraction, and fucoxanthin standard at RF 0.8-0.9.

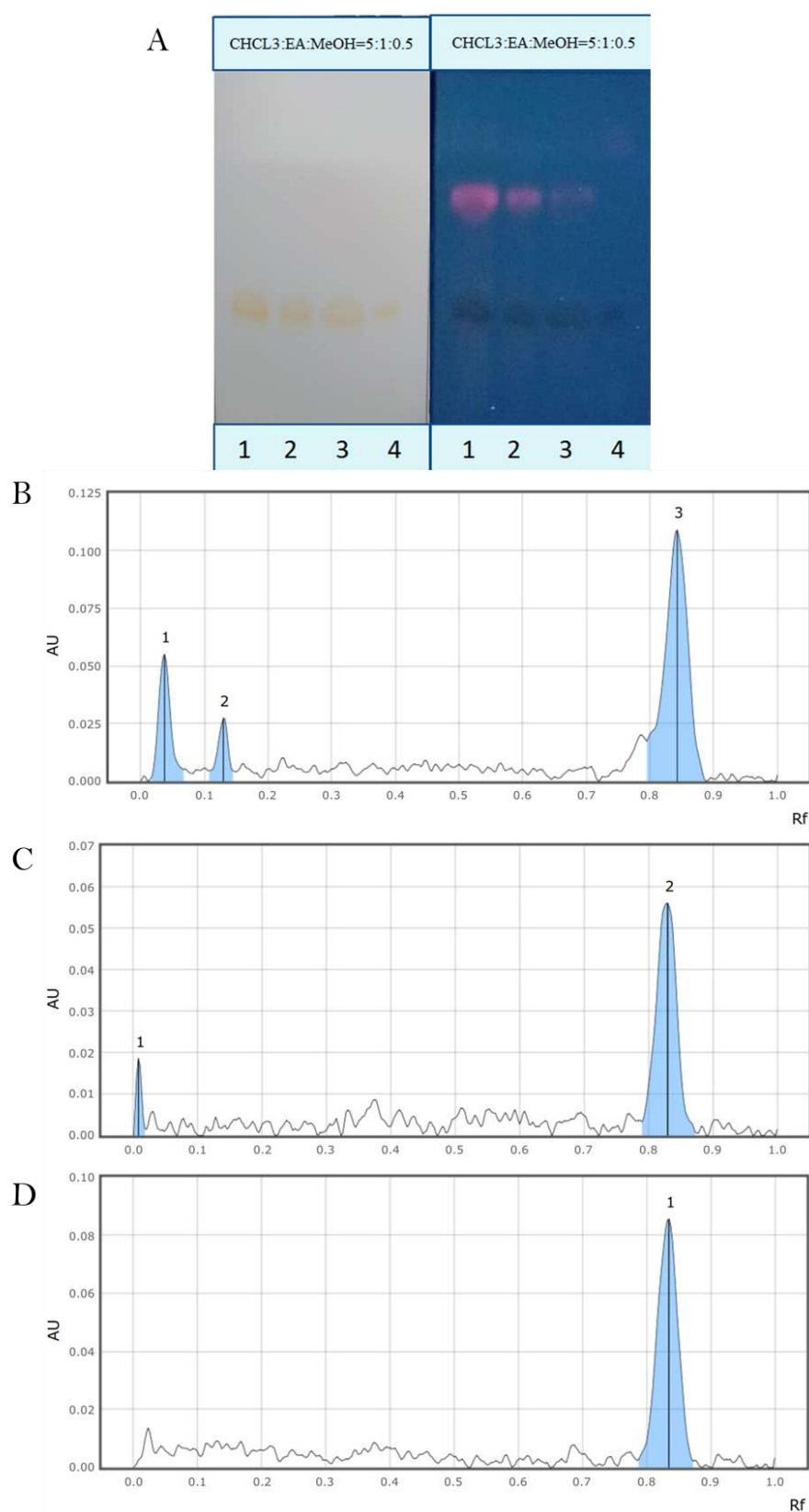


Figure 3. The figures represent the results of TLC and TLC-densitometry. (A) shows the TLC outcome using the solvent system chloroform: ethyl acetate: methanol (5:1:0.5), which achieved complete separation. The left panel displays the visualisation under visible light, while the right panel shows the same under UV light. The TLC-densitometry analysis of PAH extract (B), EAS (C), and a standard fucoxanthin from Sigma Aldrich (D) was conducted, with all samples read at a wavelength of 447 nm.

Quantification of Fucoxanthin Levels

The densitometry analysis conducted in this study provided area under the curve (AUC) data for both the samples and the fucoxanthin standards. A calibration curve was constructed using the AUC data of the fucoxanthin standards, yielding the equation $Y = 2.516 \times 10^{-8}X + 7.147 \times 10^{-4}$ with an R-squared value of 98.63 % (Figure S1). This calibration curve enabled the quantification of fucoxanthin content in the samples based on their respective AUC values. By applying this curve, the fucoxanthin levels in each replication of the samples could be accurately calculated. The quantification results for the extract and EAS are presented in Figure 4. The fucoxanthin levels were determined to be $42.36 \pm 3.94 \mu\text{g mg}^{-1}$ and $99.104 \pm 7.76 \mu\text{g mg}^{-1}$, respectively, in the extract and EAS.

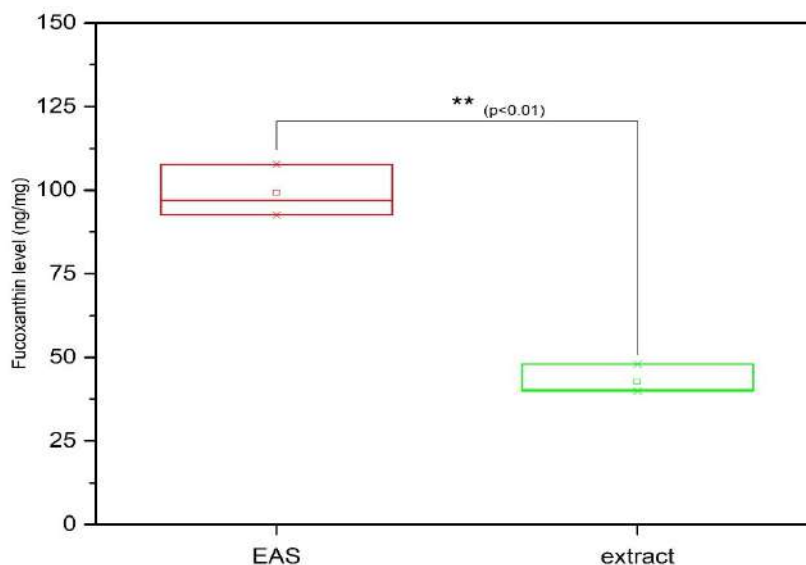


Figure 4. Fucoxanthin levels in PAH extract and EAS.

Collagenase Activity Inhibition

The collagenase activity inhibition assay revealed that the extract, EAS, isolates, and EAI of PAH exhibited varying degrees of inhibitory activity. The assay results, shown in Figure 5A, indicated IC_{50} values of $41.96 \pm 2.86 \mu\text{g mL}^{-1}$ for the extract, $34.57 \pm 0.99 \mu\text{g mL}^{-1}$ for the EA soluble fraction, $21.78 \pm 1.86 \mu\text{g mL}^{-1}$ for the isolate, and $-6.30 \mu\text{g mL}^{-1}$ for the EA insoluble fraction. The negative IC_{50} value of the EA insoluble fraction suggests that the activity of its chemical compounds differs significantly from the other samples. The isolate demonstrated the greatest inhibitory power, which can be attributed to the higher purity of its chemical compounds. This observation is consistent with the IC_{50} values of the extract and EA soluble fraction, where the fraction with higher inhibitory activity corresponds to a lower IC_{50} value.

Tyrosinase Inhibition Activity

The tyrosinase inhibition assay results for the various samples of PAH, including the extract, EAS, EAI, and the isolate, are indicative of their potential anti-tyrosinase activities. The IC_{50} values, which represent the concentration required to inhibit 50 % of tyrosinase activity, were determined as follows: $48.42 \mu\text{g mL}^{-1}$ for the extract, $29.47 \mu\text{g mL}^{-1}$ for EAS, $11.58 \mu\text{g mL}^{-1}$ for EAI, and $5.26 \mu\text{g mL}^{-1}$ for isolate (Figure 6B). These findings reveal that the isolate exhibited the highest tyrosinase inhibitory activity with the lowest IC_{50} value of $5.26 \mu\text{g mL}^{-1}$, suggesting that it is the most potent among the tested samples. Furthermore, the progressive increase in potency from the crude extract to the isolate indicates that the active compounds responsible for tyrosinase inhibition are more concentrated in the isolated form. This aligns with the

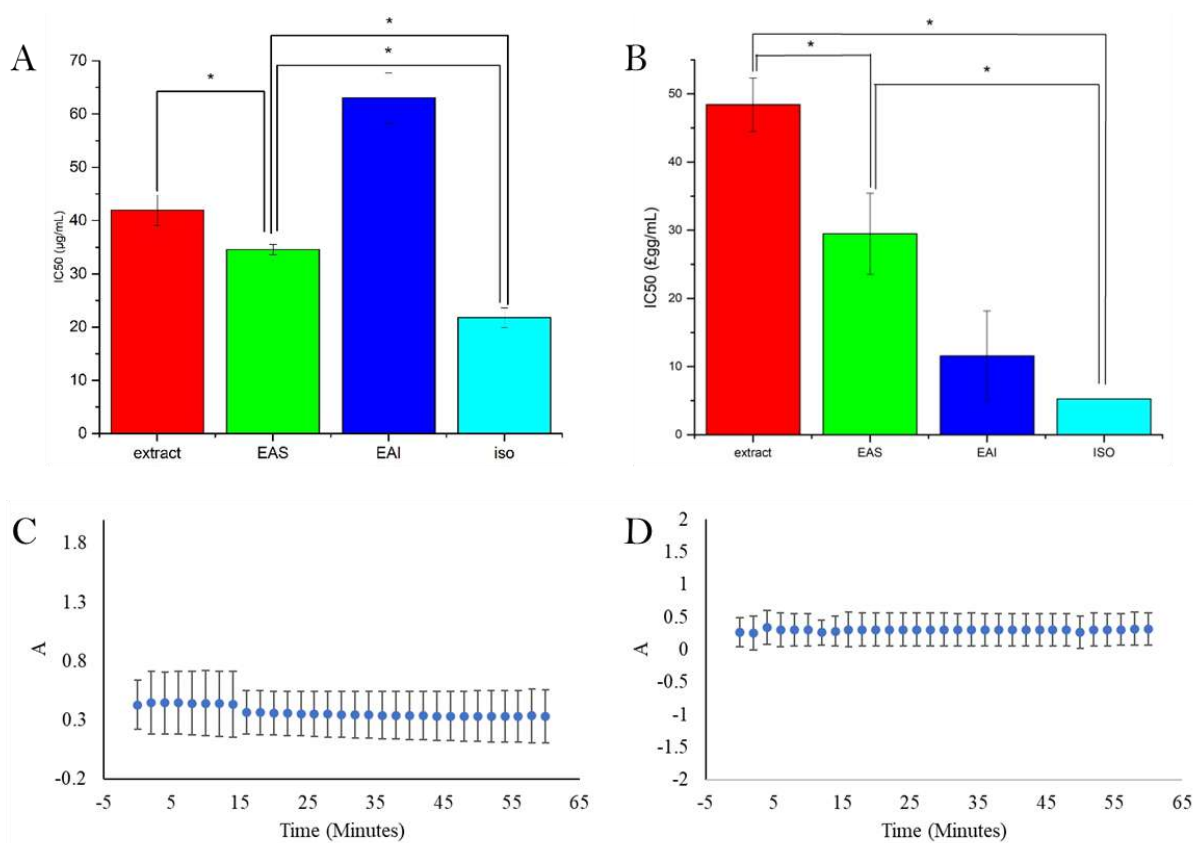


Figure 5. The results of the collagenase (A) and tyrosinase (B) inhibition assays were assessed in the extract, EAS, EAI, and isolated compounds (ISO). The effect of DMSO was illustrated in panels (C) and (D) for collagenase and tyrosinase inhibition, respectively, with a significance level of $p < 0.05$ indicated.

understanding that fractionation and isolation processes effectively enrich the active constituents, such as fucoxanthin, thereby enhancing their bioactivity (Bucar et al. 2013; Wen et al. 2019). These results highlight the potential of PAH as a source of natural tyrosinase inhibitors, which could be developed for anti-hyperpigmentation treatments and other cosmetic applications.

Discussions

Brown seaweed is composed of a diverse range of bioactive compounds, making it a valuable resource for various applications, particularly in the realm of health and cosmetics (Wang et al. 2020). The primary components include pigments, carbohydrates, phlorotannins, lipids, and low molecular weight organic compounds. The carbohydrate content is notably rich and includes mannitol, fucoidan, alginate, and laminaran. These carbohydrates contribute to the seaweed's structural integrity and offer various health benefits, such as antioxidant and anti-inflammatory properties (Huang et al. 2019). Furthermore, among the pigments, brown seaweed contains a significant amount of carotenoids, a group of pigments responsible for the yellow, orange, and brown colours in many plants. The most prominent carotenoid found in brown seaweed is fucoxanthin as confirmed in our study (Figure 3) (Miyashita et al. 2011).

Antioxidant and anti-inflammatory activities are crucial to anti-ageing, as oxidative stress and chronic inflammation accelerate skin aging. Fucoxanthin's strong antioxidant properties, as shown by Junopia et al. (2020) and Maeda et al. (2018), help prevent oxidative damage, while its anti-inflammatory effects, demonstrated by Zhao et al. (2017), reduce inflammation-related ageing (Zhao et al. 2017; Maeda et al. 2018; Junopia et al. 2020). Together, these properties enhance skin protection, reduce cellular damage, and support fucoxanthin's potential as an effective anti-ageing agent in skin-

care products. In this study, we successfully isolated fucoxanthin from PAH. The confirmation of the isolated fucoxanthin and EAS is evident from the functional group analysis depicted in the Figure 6. The presence of the characteristic allenic bond ($C=C=C$) in fucoxanthin was confirmed by absorption peaks at 1930 cm^{-1} and 929 cm^{-1} in the spectrum, corresponding to the fucoxanthin standard (Yip et al. 2014). Further analysis revealed the absorption peaks at 3380 cm^{-1} and 3013 cm^{-1} , indicating the presence of $-OH$ bonds, consistent with Purnomo's observations (Zailanie & Purnomo 2011). Additionally, we identified peaks at 2957 cm^{-1} and 2855 cm^{-1} , suggesting the presence of alkanes with $C-H$ bonds, further corroborating the structural characteristics of fucoxanthin. Moreover, our results included an absorption peak at 1732.54 cm^{-1} , which also pointed to the presence of ketones with $-C=O$ bonds, a finding that aligns with Purnomo's earlier work (Zailanie & Purnomo 2011). The peaks identified at 1455 cm^{-1} , 1434 cm^{-1} , 1378 cm^{-1} , and 1364 cm^{-1} indicate the presence of scissoring and bending alkanes with $-C-H$ bonds, reinforcing the structural integrity of the isolated fucoxanthin and aligning with the previous studies (Yip et al. 2014). A distinctive feature of our study was the confirmation of the characteristic allenic bond ($C=C=C$) in fucoxanthin, evidenced by absorption peaks at 1930 cm^{-1} and 929 cm^{-1} . This specific characteristic was previously unreported in the context of fucoxanthin isolated from PAH, highlighting a potential area for further research into its unique properties.

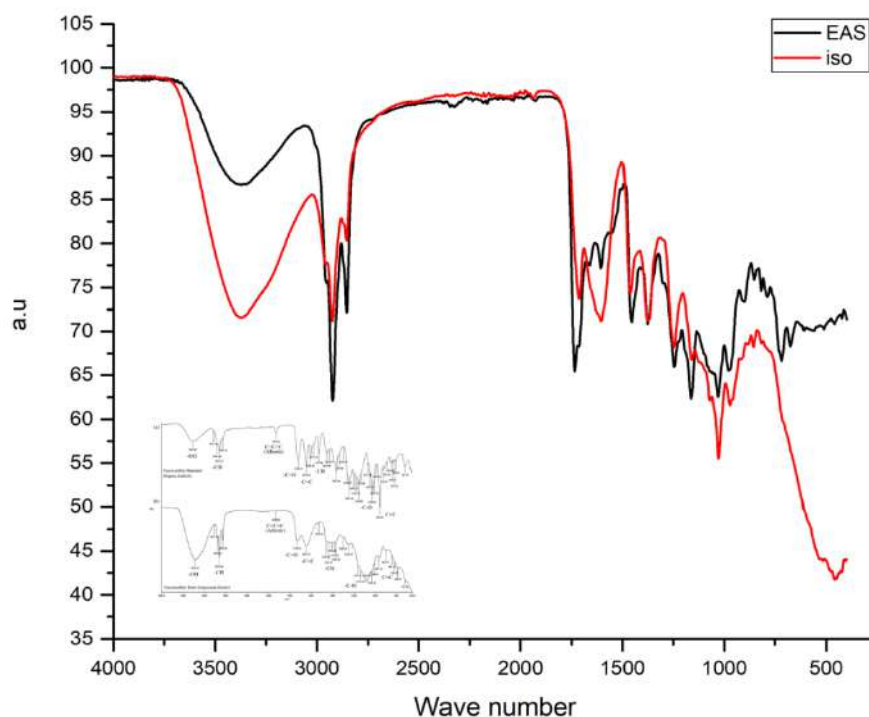


Figure 6. FTIR spectra of the EAS and isolate of PAH showing similar characteristic functional groups. Insert is the standard FTIR spectra of fucoxanthin by other study (Yip et al. 2014).

Moreover, the presence of esters with $-C-O$ bonds was confirmed by the absorption peak at 1033 cm^{-1} . These functional groups are characteristic of fucoxanthin. The TLC-densitometry analysis revealed that the fucoxanthin content in the EAS was approximately twice that of the extract (Figure 4). Specifically, the EAS contained $99.104\text{ }\mu\text{g mg}^{-1}$ of fucoxanthin, compared to $42.36\text{ }\mu\text{g mg}^{-1}$ in the extract. These results highlight the effectiveness of the ethyl acetate fractionation process in concentrating the bioactive compound, fucoxanthin. This significant increase in fucoxanthin concentration in the EAS underscores its potential for further development. Given the higher pu-

rity and concentration of fucoxanthin, the ethyl acetate soluble fraction exhibits promising potential for use in phytopharmaceutical applications. The enhanced fucoxanthin content in the EAS could lead to more effective formulations for anti-ageing and other health-related products, capitalising on fucoxanthin's well-documented antioxidant and anti-inflammatory properties (Neumann et al. 2019; Bigagli et al. 2021). Moreover, the successful isolation and concentration of fucoxanthin from PAH through ethyl acetate fractionation could streamline the production process in an industrial setting, making it a viable option for large-scale manufacturing of phytopharmaceutical products. This approach not only maximises the yield of the active compound but also enhances the efficacy of the final product, potentially leading to more robust therapeutic outcomes (Atanasov et al. 2015).

Human skin collagenase was initially isolated in active form from skin explant culture medium and subsequently as a proenzyme from monolayer fibroblast cultures. Interstitial collagenase (MMP-1) levels increase with age due to the fragmentation of collagen fibres and disorganisation of the collagen fibre arrangement in the dermis (Nurrochmad et al. 2018). Similarly, tyrosinase is a crucial enzyme in the process of melanogenesis. It contains two copper (II) ions as the main cofactors in the active site, coordinated by six protected histidine residues (Soradech 2016). Our fractionation results demonstrated a significant difference in IC_{50} values between the EAS and the crude extract, although the EAS still lagged behind the isolated fucoxanthin in terms of inhibitory activity. Specifically, the collagenase inhibition assay revealed that the IC_{50} values for the EAS were substantially lower than those of the crude extract, indicating a higher concentration of the active compound (fucoxanthin) in the EAS compared to EAI. The higher efficacy of isolated fucoxanthin compared to the EAS and crude extract can be attributed to its greater purity and concentration. Isolated fucoxanthin is present in a purer form, enhancing its bioavailability and interaction with target enzymes like collagenase and tyrosinase (Bucar et al. 2013). In contrast, EAS and crude extracts are complex mixtures that include non-active components which can dilute fucoxanthin's efficacy. The presence of these other compounds may create competitive interactions that reduce fucoxanthin's effectiveness by interfering with its binding to enzyme active sites, resulting in higher IC_{50} values for the extracts (Atanasov et al. 2015). The isolation process eliminates secondary metabolites that may hinder specific inhibitory activities, allowing the isolated fucoxanthin to exert a more potent effect.

Moreover, the ethyl acetate fractionation process was effective in concentrating fucoxanthin, as evidenced by the lower IC_{50} values for collagenase inhibition and the higher fucoxanthin content in the EAS. These findings suggest that the EAS is a more potent inhibitor of collagenase and tyrosinase, making it a promising candidate for further development into phytopharmaceutical products aimed at combating skin ageing and hyperpigmentation. This enrichment of fucoxanthin in the EAS highlights its potential utility in anti-ageing formulations, supporting its application in both therapeutic and cosmetic industries. Previous research has shown fucoxanthin inhibits collagenase and elastase, enzymes responsible for the degradation of collagen and elastin, which are essential for maintaining skin elasticity and structure (Freitas et al. 2020; Landa-Cansigno et al. 2023). Additionally, studies have reported its tyrosinase inhibitory activity, which helps in reducing hyperpigmentation by controlling melanin synthesis in the skin (Chen et al. 2015; Nurrochmad et al. 2018). These enzyme-inhibitory properties are crucial in delaying skin ageing processes such as wrinkling and age spots. The bioactivity of fucoxanthin, specifically in collagenase and tyrosinase inhibition, aligns with its role in EAS, emphasising its relevance in anti-aging treatments. These findings support its potential application as a key ingredient in cosmeceutical products, driving the development of natural anti-aging solutions based on

seaweed-derived compounds.

CONCLUSION

This study demonstrates that the EAS fraction and isolated compounds from PAH exhibit significant collagenase (IC_{50} : $41.96 \pm 2.86 \mu\text{g mL}^{-1}$ extract, $21.78 \pm 1.86 \mu\text{g mL}^{-1}$ ISO) and tyrosinase (IC_{50} : $5.26 \mu\text{g mL}^{-1}$ ISO) inhibition, highlighting their potential as a natural anti-aging agent. Future research should investigate how environmental factors (e.g., salinity, temperature) affect PAH's bioactive content and whether compound optimisation (e.g., fucoxanthin synergies) could enhance cosmeceutical efficacy, paving the way for pharmaceutical and cosmetic applications.

AUTHORS CONTRIBUTION

W. designed the study, collected and analysed the data, and wrote the manuscript. R.M. provided consultation on data analysis. N.F. provided consultation on the research process. E.P.S. supervised the entire research process.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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APPENDICES

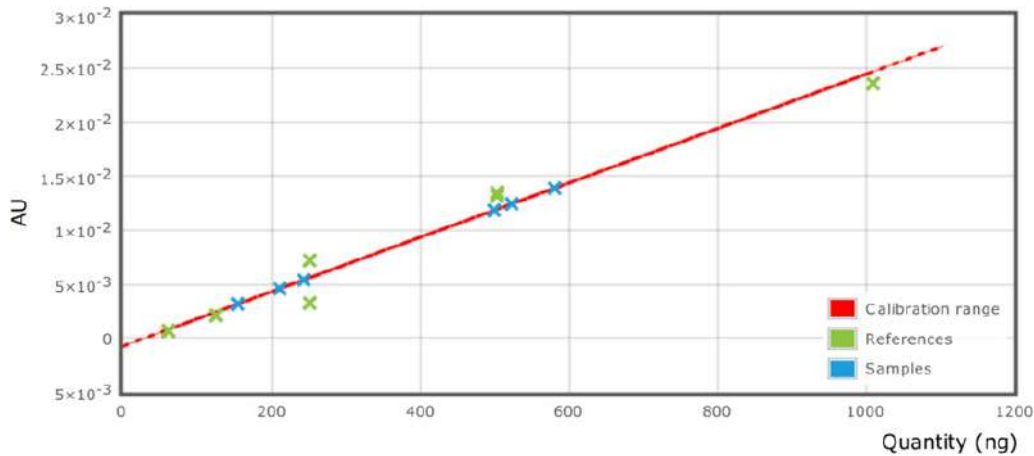


Figure S1. Calibration curve of fucoxanthin standard by using CAMAG at 447 nm.

Research Article

Metabolic Response of Extremophile *Halochlorella Rubescens* on Carbohydrate Biosynthesis Cultivated Under High Carbon-Rich Condition

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ABSTRACT

Microalgae cultivation under elevated carbon dioxide (CO₂) concentrations may negatively affect biomass production and intracellular carbohydrate biosynthesis, posing a major challenge for large-scale industrial applications. Despite the growing interest in carbohydrate-based bioproducts derived from microalgae, the underlying mechanisms regulating carbohydrate accumulation under high CO₂ conditions remain insufficiently understood. This study investigated the effects of CO₂ concentrations ranging from 0.04% to 25% on the growth performance, morphological characteristics, and carbohydrate productivity of *Halochlorella rubescens*. The highest biomass concentration (0.65 g L⁻¹) and carbohydrate productivity (16.34 mg L⁻¹ d⁻¹) were achieved at 5% CO₂, indicating that moderate CO₂ enrichment enhances carbon assimilation and carbohydrate accumulation. However, exposure to higher CO₂ levels altered cellular morphology and influenced the expression of key proteins involved in carbohydrate biosynthesis. Transcriptomic and proteomic analyses revealed significant enrichment of genes associated with carbon fixation, photosynthesis, starch synthesis, and carbohydrate metabolic pathways under elevated CO₂ conditions. Notably, the upregulation of photosynthesis-related proteins (ATP synthase, Photosystems I and II, and chlorophyll a-b binding proteins), Calvin cycle enzymes (ribulose phosphate, glyceraldehyde-3-phosphate, and ribose-5-phosphate), and starch biosynthesis enzymes (starch synthase and ADP-glucose pyrophosphorylase) was observed at 5% CO₂. These findings indicate coordinated regulation of multiple carbon metabolic pathways during cultivation under enriched CO₂. Overall, this study identifies key metabolites and regulatory targets involved in carbohydrate biosynthesis in *H. rubescens*, providing valuable insights for strain improvement and genetic engineering strategies in microalgal biotechnology.

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INTRODUCTION

Microalgae is a photosynthetic microorganism that can be abundantly found in the environment. This microorganism can be used in many applications, including pharmaceuticals, phycoremediation, renewable energy, and agriculture. It can be classified into different major groups, and extremophile microalgae has been reported to have advantages in synthesizing novel metabolites (Varshney et al. 2015; Malavasi et al. 2020). It is known that this microalga can perform photosynthesis for biomass production and synthesis of other valuable biological macromolecules, such as lipids, carbohydrates, and proteins when cultivated under optimum conditions (Soru et al. 2019; Tan et al. 2020). In addition, extremophile microalgae is resistant to a wide range of abiotic factors that could affect its growth. The role of extremophilic microalgae in synthesizing valuable macromolecules is made possible by their ability to maintain their metabolism under acidic or alkaline pH conditions, elevated temperatures, high light intensity, CO₂ and metal concentrations levels (Varshney et al. 2015). Their resistance capability allows them to be cultivated in extreme conditions without compromising the temperature and light intensity fluctuation. Moreover, cultivation under extreme conditions could also reduce contamination by undesired microorganisms during the process.

Cultivation of microalgae under extreme or elevated conditions could also affect its biochemical composition. Several studies have demonstrated that the shift from atmospheric CO₂ with high CO₂ concentration during microalgae cultivation significantly affects biochemical composition, especially in lipid, carbohydrate, and protein content in microalgal biomass. Kandasamy et al. (2021) indicated that varying dissolved CO₂ in microalgae cultivation significantly affected their protein and lipid content. However, no change in carbohydrate content was observed when the cultivation was provided with CO₂. Another study also indicated that the cultivation of *Chlamydomonas reinhardtii* using 0.8 % CO₂ via a stepwise-feeding approach showed a drastic change in biochemical compositions in its cell (Nakanishi et al. 2021). It is important to consider the changes of microalgal biochemical compounds during cultivation as those compounds will be further used for other applications, such as biodiesel and any fine chemical production, including bioethanol, bio-butanol and biohydrogen, via a fermentation process.

Formation of microalgae metabolite during cultivation involves a complex reaction. Generally, the cultivation using CO₂ involves substrate intake machineries which biophysical or biochemical carbon concentrating mechanisms (CCMs) are employed in microalgae which involves synergistic reaction of Carbonic anhydrase (Cas) enzyme and bicarbonate transporter (BCTs) converting carbon dioxide into inorganic carbon forms and transporting them across the microalgae cell membranes (Mackinder 2018; Chunzhuk et al. 2023). A few studies have shown that the distribution of CCM in microalgae strains is strain-dependent. A study by Wei et al. (2019) on the transcriptomic analysis of *Nannochloropsis oceanica* indicated that distinct upregulations of genes related on CCM which different with *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum* for cultivation under low CO₂ concentration. In addition, previous proteomic analysis studies have reported that a significant alteration was observed in microalgae and cyanobacteria cultivated under CO₂-replete conditions. A proteomic analysis of *P. tricornutum* cultivated under different CO₂ concentration reveal that exposure of this microalgae under high CO₂ concentration has enhanced the expression of proteins related to central carbon metabolism such as Calvin cycle, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle and glycolysis (Wu et al. 2021). The study also suggested that cultivation under elevated CO₂ conditions has improved microalgal growth rate and metabolite such as fatty acid, lipids, pigment, insoluble sugar production yield as well as other energy storage mate-

rials.

While the microalgae metabolite synthesis, especially lipid fraction under cultivation using high CO₂, is widely understood, information on the underlying molecular modulations involved in the sequestration of CO₂ and carbohydrate biosynthesis of extremophile microalgae particularly *Halochlorella rubescens* is scarce. Since proteins play a crucial role in regulating cellular functions, understanding their abundance can offer valuable insights into the intracellular changes occurring during environmental stress, such as high CO₂ conditions. However, the existing study has primarily focused either on the effects of high CO₂ on CO₂ concentrating mechanisms (CCM) or on extracellular proteins, which highlights the lack of comprehensive proteomics studies under high CO₂ conditions. Moreover, carbohydrate biosynthesis shares common carbon precursors with lipid synthesis, which its biosynthesis mechanisms are poorly defined especially for *H. rubescens* cultivated under elevated CO₂ condition.

Thus, this study aims to evaluate the influence of CO₂ concentration on the accumulation of carbohydrate polymer in *Halochlorella rubescens*. The effect of cultivation under elevated carbon conditions on the microalgae response will be evaluated based on their gene expression and proteomic analysis. This aspect of analysis on the microalgae strain has never been attempted in previous investigations and would offer more information regarding metabolic changes in the microalgae cells cultivated under CO₂-elevated conditions. The information obtained could be useful to gain a deeper insight into the relationship between high CO₂ concentration and carbohydrate accumulation, which will lead to improved strategies for biofuel production from microalgae.

MATERIALS AND METHODS

Microalgae and cultivation condition

A freshwater microalgae *Halochlorella rubescens* was used throughout this study. The selected microalgae was maintained in 1L - Modified algae growth (MLA) medium that consists of (g L⁻¹): magnesium sulphate (0.49 g L⁻¹), sodium nitrate (1.7 g L⁻¹), di-potassium phosphate (0.4 g L⁻¹), calcium chloride (0.03 %). The medium was sterilized using 0.22 µm Millipore filter. For this experiment, the batch culture was performed by cultivating the culture in an illuminated incubator chamber with 0.3 L min⁻¹ supplemented with different CO₂ concentrations such as 0.04, 5, 15 and 25 % (vvm) intermittently under light with a photon intensity of 450 µmol m⁻² s⁻¹. The microalgae was cultivated at 30.0 ± 2.0 °C and the microalgae biomass was harvested when it reached stationary stage. For the harvesting process, the microalgae was centrifuged at 4500 rpm for 15 min and the pellet obtained was then rinsed several times using distilled water. The washed pellet was subjected to drying process at 60 °C for 24 h. The dried microalgae biomass obtained was kept and used for further analysis.

Growth monitoring

In order to evaluate the performance of microalgae under different CO₂ concentration, the experiment was conducted by cultivating the microalgae in medium supplemented intermittently with 0.04 %, 5 %, 15 % and 25 % CO₂ for 1 hr every 12 hr per day. The microalgae growth was monitored using UV Spectrophotometry (Shimadzu UV1700, Japan) every 24 hr for 14 days. The microalgae cell was expressed as cell dried weight by converting the OD value according to the following equation:

$$CDW_{H.chlorella} = 0.2845 (OD_{680}) + 0.0016 \quad (1)$$

The maximum biomass production and biomass productivity were calculated according to the following equation:

$$P = \frac{X_f - X_0}{t_f - t_0} \quad (2)$$

Where, X_f is cell concentration (g L^{-1}) at a time t_f (days), P is microalgal biomass productivity ($\text{g L}^{-1} \text{d}^{-1}$), and X_0 is initial cell concentration (g L^{-1}) at time t_0 (days). In this study, the carbohydrate productivity was also calculated according to the following equation:

$$C = M \times P \quad (3)$$

where C is the carbohydrate content (mg L^{-1}), P is microalgal biomass productivity ($\text{g L}^{-1} \text{d}^{-1}$).

Biochemical composition

The biochemical compounds including lipid, carbohydrate and protein of microalgal biomass cultivated using different concentration was analysed using Blight and Dyer, Phenol sulphuric acid and Lowry method respectively (Kassim & Tan 2017; Li et al. 2021).

RT-PCR analysis

Total RNA of the microalgae biomass cultivated in different CO_2 concentration was performed as per described by Torres et al. (2021) with slide modification. The total RNA were extracted by GF-1 Total RNA Extraction Kit (Brand Vivantis; Malaysia). For this study, a total of 100 mg of harvested microalgae was mixed with RNase-free and 400 μL of buffer TR followed centrifugation at 10,000 rpm for 3 min. The lysate obtained was transferred into a homogenisation column and centrifuged again under similar conditions. The flow-through was mixed with 350 μL of 80 % ethanol and mixed thoroughly prior to subjected for RNA isolation steps. Next, a total of 650 μL of sample was transferred into a RNA binding column and centrifuged at 10,000 rpm for 1 min and the flow-through obtained was discharged. The sample column was added with 500 μL buffer and centrifuged again at 10,000 rpm for 1 min. After centrifugation, the flow-through was added with 70 μL DNase I digestion mix and incubated at room temperature for 15 mins followed by 500 μL of inhibitor removal buffer. The sample was centrifuged and 500 μL of wash buffer was added into the RNA isolated and centrifuged as above for 1 min. The flow through was discarded and this process was repeated twice. Lastly, 40-60 μL of RNase-free water was added directly onto the membrane and centrifuged as above. The RNA eluent was stored at -20°C or -80°C for further analysis. The quantity of RNA obtained was determined by Thermo Nano Drop2000. The concentration of RNA was measured and standardized to 10 μg before starting the cDNA synthesis process.

The cDNA was synthesized using cDNA synthesis kit. For this process, the gene expression of the selected gene was evaluated using ViPrimePLUS One Step Taq RT-qPCR and SYBR Green Mastermix kit. Briefly, the standardized RNA was added with 1 μL of random hexamer, 1 μL of 10mM dNTPs mix, and top-up to 10 μL with nuclease-free water. The mixture was incubated at 65°C for 5 min and chilled on ice for 2 min. Then, this RNA mixture was added with the 10 μL of the cDNA synthesis mix and incubated at 42°C for 60 min. Finally, the reaction was terminated at 85°C for 5 min and the synthesized cDNA was stored at -20°C for further used. The target genes were amplified using ViPrime PLUS One Step Taq RT-qPCR MasterMix for rubisco (Ribulose biphosphate carboxylase), ISA (Isoamylase), AGB (1,4-alpha glucan), PGM (Phosphoglucomutase), SP (Starch phosphorylase), Accc (Acetyl Coenzyme A Carboxylase), and housekeeping gene 18S rRNA. The primers shown in Table 1 were synthesized by Apical Scientific

Sdn. Bhd. The threshold cycle (Ct) values were obtained from the reaction and logarithmically transformed. Delta Ct (Δ Ct) values were calculated using 18S rRNA, and delta delta Ct ($\Delta\Delta$ Ct) values relative to the determined expression level.

Table 1. Primer sequence used for RT-PCR.

Primer	Forward	Reverse
18S rRNA	5'-ATC AAC CTG ACA AGG CAA CC - 3'	5' - CCT GCG TAA TTT GAC TC - 3'
Rubisco	5'-CTG CGT ACA CCA GCA ACG- 3'	5' - CCC AGT ACC GGT TGT CGT A - 3'
ISA	5'- CCA CCG CCT CTG TCA ACT - 3	5' - CGT TGG CCT CAT TGT GCT - 3'
AGB	5'-TGA TGT GCT TCC TCA CCC T - 3'	5'-TCC AAA GTG TCC TGG TCC T - 3'
PGM	5'-GAC TCC AAC ATT GCG AAG AT - 3'	5'-GGA AGA GGT CGG TCA GGT - 3'
SP	5'-AGC CTG TAT CTG CAG GTT GC - 3'	5' - TTG CAG GTT GGT TGT TTG G - 3'
Accc	5'-CAA TCA TGA TCA AGG CTA CCG - 3'	5' - AGG AAC TCG CTC TCG TCC T - 3'

Note: Rubisco= Ribulose biphosphate carboxylase. ISA=soamylase. AGB=1,4-alpha glucan, PGM= Phosphoglucomutase, SP= Starch phosphorylase, ACCC= Acetyl Coenzyme A Carboxylase.

Protein extraction and proteomic analysis

Protein of microalgae was extracted using borax/PVPP/phenol (BPP) protocol. In this process, a total of 1 g dried microalgae biomass was re-suspended in 3 mL of ice-cold 100 mM Tris (pH 8) extraction buffer. The buffer contained of the following: 100 mM EDTA, 50 mM borax, 50 mM vitamin C, 1 % PVPP 199 w/v, 1 % Triton X-100 v/v, 2 % b-mer-captoethanol v/v and 30 % sucrose w/v. The sample was vortexed 10 min and the sample was added with two-volume of Tris-saturated Phe (pH 8). The mixture was the centrifuged at 15000g for 15 min. The upper layer obtained was removed and subjected for precipitation using ammonium sulfate saturated-methanol and was incubated at 22 °C for at least 6 h. After incubation period, the purification process was repeated for two times and the washed sample was air-dried and recovered by using lysis buffer prior to be stored at -80 °C. The peptide mixture obtained was further resuspended in Buffer A (20 mM ammonium formate in ultrapure water, pH = 10.0) followed by separation at high pH with a linear gradient of Buffer B (20 mM ammonium formate in 80 % acetonitrile, pH = 10.0) within 40 min. The peptide solution was then dried in a centrifugal evaporator (CentriVap Concentrator, Labconco, MO, USA). Peptide clean-up–The dried peptide pellet was resuspended in 200 µL of 0.1 % formic acid (FA) and incubated at ambient temperature with slight agitation for 4 h. The bound peptides on the C18 membrane disks were sequentially eluted with 50 % ACN in 0.1 % FA for 2.5 h.

The peptide mixture was analysed by online nanospray liquid chromatography (EASY-nLC) 1200 System (Thermo Scientific, MA, USA) and Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer system (Thermo Scientific, MA, USA) with a spray voltage of 1800 V in positive mode. The primary data was acquired using Xcalibur (Ver. 4.1.31.9) (Thermo Scientific, MA, USA) and were clustered using Proteome Discoverer (Ver. 2.2) (Thermo Scientific, MA, USA). Functional and pathway analysis of the identified proteins was carried out using Blas2GO software, as well as the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes

(KEGG) databases.

Statistical analysis

All the experiment was performed in triplicate and were calculated calculate the mean $\pm$ standard error. Statistical analysis was performed according to one-way ANOVA analysis using Minitab 16 software. The level of statistical significant of each parameters was defined with a value of $P < 0.05$.

RESULTS AND DISCUSSION

The growth profile of *H. rubescens* cultivated using different CO₂ concentrations was evaluated. Figure 1 shows that the maximum biomass concentration of 0.65 g L⁻¹ was obtained when *H. rubescens* was cultivated using 5 % CO₂. The biomass productivity and specific growth rate of the microalgae cultivated under this condition are 0.593 g L⁻¹ d⁻¹ and 0.25 d⁻¹. An insignificant difference in the growth of *H. rubescens* in 5 % and 15 % CO₂ is also observed. The result signifies that *H. rubescens* has a wide range of tolerance to CO₂ concentration. Changes in morphological characteristics of *H. rubescens* cultivated using different CO₂ concentrations were also assessed. Figure 2 depicts significantly changes in *H. rubescens* morphology when the cultivation was supplied with high CO₂ concentration. Figure 2 shows that obvious differences are observed for *H. rubescens* when exposed to CO₂. The round shape of *H. rubescens* became elongated when the cells were exposed to high CO₂ concentrations. This observation indicates that the presence of CO₂ not only affect the growth of microalgae but also the shape and cell structure of *H. rubescens*.

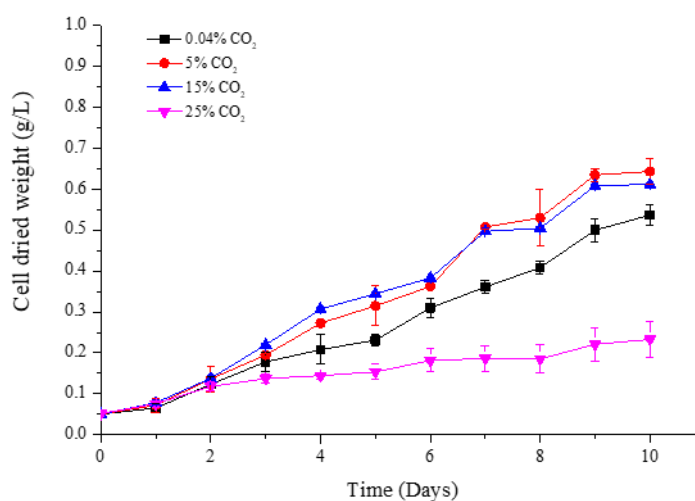


Figure 1. Growth profile of *H. rubescens* cultivated with different CO₂ concentrations.

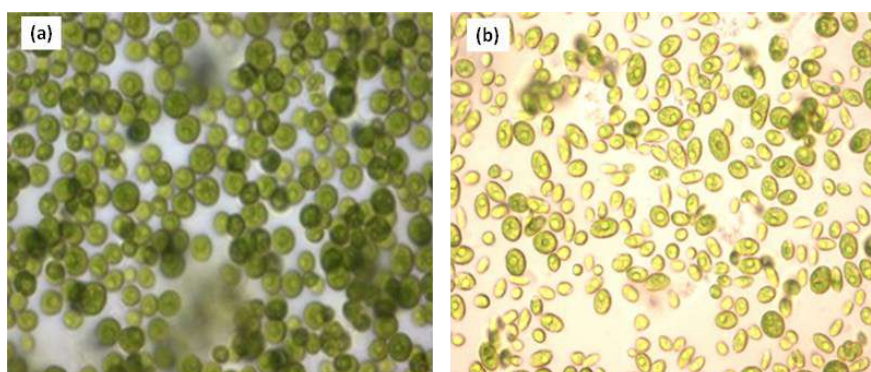


Figure 2. Morphology characteristic of *H. rubescens* under light microscope cultivated using different CO₂ concentration. (a) Cultivation under normal condition 0.04 % CO₂ (b) Cultivation under 5 % CO₂

Biochemical composition cultivated under different CO₂ concentrations

The study indicated that supplementation of high CO₂ concentration during the cultivation of *H. rubescens* could significantly affect biochemical composition in the cells. Table 2 shows the biochemical composition of *H. rubescens* cultivated using air and 15 % CO₂. Carbohydrate content is significantly affected by the presence of CO₂ during the cultivation process. Cultivation using higher concentrations of CO₂, i.e., at 5 % and 15 %, yielded 39.45 ± 0.33 % and 40.56 ± 0.42 % of carbohydrate content. The highest carbohydrate content was obtained when the cultivation was performed using 25 % CO₂, with 46.49 ± 0.63 %. However, cultivation of *H. rubescens* under this condition could inhibit microalgal growth, leading to low carbohydrate productivity. This study indicated that the highest carbohydrate productivity was achieved for *H. rubescens* cultivated using 5 % CO₂ followed by 15 % CO₂ with 16.34 mg L⁻¹ d⁻¹ and 15.22 mg L⁻¹ d⁻¹ respectively. Low biomass observed in microalgae cultivated under high CO₂ concentration (> 25 % CO₂) could be attributed to several factors, such as pH imbalance, toxicity, and osmotic stress. Exposing microalgae to high CO₂ could lead to overaccumulation of intracellular CO₂, which can interfere with metabolic pathways and cellular functions. Moreover, stress conditions will affect the uptake of nutrients and shift the microalgae to accumulate more metabolite in the cells, ultimately impairing growth (Singh & Singh 2014).

Table 2. Biochemical composition of *H. rubescens* cultivated using different CO₂ concentrations.

Compound (% of dried bio- mass)	0.04 % CO ₂	5 % CO ₂	15 % CO ₂	25 % CO ₂
Carbohydrate	33.86 ± 0.22	39.45 ± 0.33	40.56 ± 0.42	46.49 ± 0.63
Protein	42.88 ± 0.13	56.78 ± 0.22	54.20 ± 0.44	28.36 ± 0.21
Lipid	13.27 ± 0.13	3.77 ± 0.22	5.23 ± 0.15	25.16 ± 0.11

This study also found that further cultivation under higher CO₂ could also increase protein content. Nevertheless, cultivation using more than 15 % CO₂ leads to low protein production, which corresponds to low biomass production during the cultivation under this condition. High CO₂ conditions can lead to increased energy demand to maintain cellular homeostasis and pH regulation. The increased energy demand can divert resources away from protein synthesis, reducing the protein content. A similar outcome was observed in the lipid content of *H. rubescens*, which exhibited a similar trend when cultivated at high CO₂ concentrations.

Differentially expressed genes related to carbon fixation pathway and carbohydrate biosynthesis

For this study, six main targeted genes that are possibly involved in carbohydrate accumulation for cultivation in normal and elevated conditions were evaluated. The genes are ribulose biphosphate carboxylase (*rbcS*), isomaltase (*ISA*), 1,4-α-glucan (*AGB*), phosphoglucomutase (*PGM*), starch phosphorylase (*SP*), and acetyl-CoA-carboxylase (*ACC*). The evaluation of gene expression involved in carbohydrate biosynthesis was performed using RT-PCR, and the amplification curves are presented in Figure 3. The results show that cultivation of *H. rubescens* using high CO₂ significantly affected the expression of important genes in carbon fixation and carbohydrate metabolic pathways. Higher expressions of ribulose bisphosphae carboxylase-oxygenase (Rubisco) and *SP* are observed in *H. rubescens* when the cultivation was performed under

elevated CO₂ concentration. The *rbcS* is a gene that encodes the ribulose 1,5 biphosphate carboxylase or oxygenase related to photosynthesis and carbon fixation in microalgae cells (Wang et al. 2015; Peng et al. 2016), while the *SP* gene is related to the starch biosynthesis pathway. The SP enzyme is a key enzyme in starch biosynthesis in photosynthetic microorganisms and plants (Busi et al. 2014). This present study also indicates a significant expression of the *AGB* gene. High expression of both genes indicates high glycogen synthesis and formation of alpha (1,6) branch linkages found in polysaccharides when *H. rubescens* was cultivated under elevated CO₂ concentration.

Another important gene in the metabolite biosynthesis in microalgae is *ACC*. The upregulation of this gene corresponds to the accumulation of lipid bodies in microalgae (Li et al. 2015; Su et al. 2021). Cultivation of *H. rubescens* using high CO₂ concentration, mainly at 25 % CO₂, significantly affects the expression of ACCase genes, implying the enhancement of acetyl-CoA conversion to malonyl-CoA, which is the main precursor for lipid biosynthesis in microalgae. This finding correlates with the lipid content analysis, which demonstrates a higher lipid content when the microalgae was cultivated using 25 % CO₂ compared to other CO₂ concentrations (Table 1). High lipid content in the microalgae cultivated under elevated CO₂ concentration could be attributed to the stress environment that led to the formation of lipids as energy and protection mechanisms for cell survival under that condition (Huang et al. 2022). However, although the presence of high CO₂ could improve the production and biosynthesis of lipids, this condition could also lead to a disruptive effect on cell growth (Aratboni et al. 2019). Therefore, according to this study, the presence of elevated CO₂ in the cultivation system could affect the expression of main genes related to carbon fixation and also other biochemical component biosynthesis in the microalgae biomass.

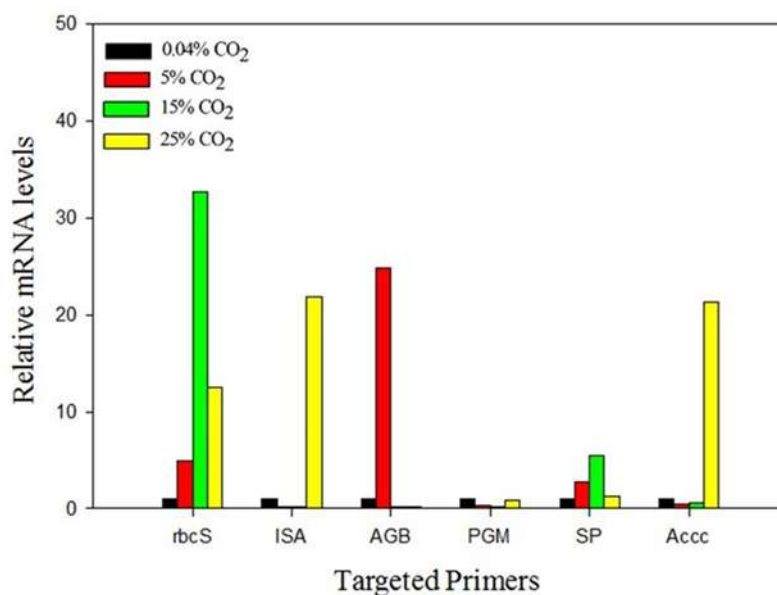


Figure 3. Expression of major genes related on carbohydrate biosynthesis for *H. rubescens* cultivated using different CO₂ concentration.

Proteomic data analysis

Proteomic analysis of *H. rubescens* under elevated CO₂ and ambient conditions was performed using LC-MS. The analysis revealed 1940 and 3060 proteins under normal and high CO₂ concentration conditions (Figure 4). The Venn diagram analysis of filtered protein datasets shows that 1593 proteins are unique to the control, whereas 2713 proteins are unique for *H. rubescens* cultivated using high CO₂ concentration. It is noted that 347 proteins were commonly detected for microalgae cultivated using air and high CO₂ concentra-

tion. These common proteins are likely to be associated with essential biological activity pathways, such as the central (energetic), Kelvin cycle, and glycolysis pathways.

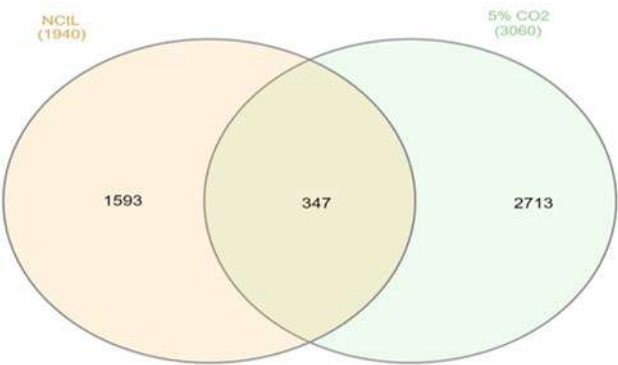


Figure 4 Venn diagram of identified proteins from cultivation of *H. rubescens* using air (NCtL) and 5 % CO₂.

Gene ontology (GO) enrichment analysis

Molecular function Gene Ontology (GO) enrichment analysis was performed to assess the functional distribution of transcripts in the entire annotated *H. rubescens* cultivated using ambient air and high CO₂ concentration (Figure 5). This study found that 896, 1412, and 1047 proteins acquired from *H. rubescens* cultivated in the control conditions are assigned to the biological processes, molecular function, and cellular components. Meanwhile, 698, 1008, and 737 proteins acquired from the cultivation using high CO₂ are assigned to these groups, respectively.

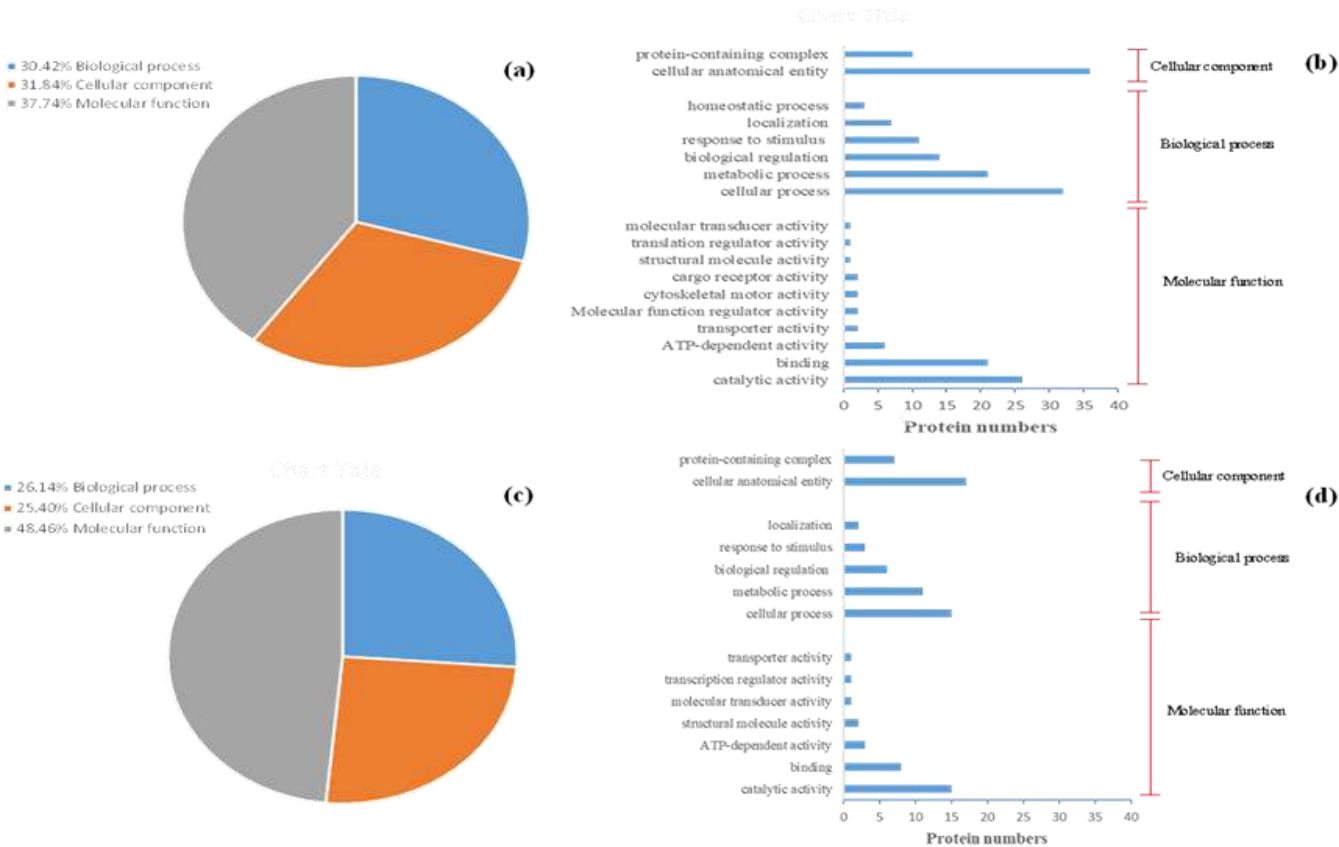


Figure 5. Gene Ontology (GO) classification and functional annotations on protein of *H. rubescens* cultivated under different conditions. (a) and (b) Cultivation under normal condition, (c) and (d) Cultivation under 5 % CO₂ concentration.

Effect of CO₂ concentration on main carbohydrate biosynthesis protein

Cultivation of *H. rubescens* using different CO₂ concentrations could significantly affect the growth, gene expression, and carbohydrate content. Further proteome analysis was performed to identify related proteins and enzymes in microalgae cultivated under these conditions. This proteomic study indicates a significant change in several proteins and enzymes concerning the inorganic carbon pathway, photosynthesis and carbon fixation, and carbohydrate metabolisms pathway (Table 3).

As photosynthetic microorganisms, microalgae harvest light energy, CO₂, and water into energy and biomass through photosynthesis. The process involves a complex light-harvesting unit that will provide energy-rich molecules for the metabolic activity of microalgae. Interestingly, proteomic analysis in this study indicates that the expression of several proteins related to photosynthesis, including light-harvesting complex (Lhc), cytochromes, reaction center of photosystem I (PS-I) and photosystem-II (PS-II), were increased when exposed to high CO₂ concentration. Similar observations have been reported of *Chlorella pyrenoidosa*, *Trebouxia* sp., and *Phaeodactylum tricornutum*. The studies stated that the increase in chlorophyll content enhanced photosynthetic efficiency when environmental CO₂ concentration was elevated (Xia & Kun-Shan 2005; Sehnal et al. 2014; Wu et al. 2021). More chlorophyll a/b binding proteins were identified in *H. rubescens* cultivated in high CO₂ concentration compared to those cultivated using normal air. Intermittent exposure of *H. rubescens* to CO₂ also showed an increased expression of ATP synthase in the thylakoid membrane since this protein would use proton gradient to synthesize ATP for further reaction in microalgae cells. This study also indicates that strong regulation of these proteins under high CO₂ concentration stimulates photosynthesis and improves biomass production.

During cultivation, microalgae consume CO₂ for photosynthesis, and it can be performed in different ways. Generally, eukaryotic microalgae will convert CO₂ into dissolved inorganic carbon (Ci) in the surrounding aqueous medium to support photosynthesis (Mondal et al. 2016). The conversion process involves specific enzymes, such as carbonic anhydrase, to form Ci prior to being channeled into the microalgae cells. The modes of Ci uptake are diverse in these organisms, ranging from diffusive CO₂ uptake to the active transport of HCO₃⁻ and CO₂, and many have an external carbonic anhydrase to facilitate HCO₃⁻ use.

In this study, higher level of Rubisco expression was observed in *H. rubescens* when the cultivation was performed under high CO₂ concentration. The increase of protein expressed under this condition indicated that more Ci was fixed and ribulose-1,5-biphosphate (RuBP) and was catalyzed to form two molecules of 3-phosphoglycerate (3PG). An increase of 3PG in microalgae cultivated in high CO₂ increased carbon fix towards the glycolysis reaction. Higher expression of the sedoheptulose-1,7-bisphosphatase (SBPase) enzyme was also noted, which is an important enzyme involved in the Calvin cycle. The abundance of this protein enables higher photosynthetic rates and stimulates biomass and yield. A similar observation was noted for the transketolase enzyme. There are higher expression in protein related to transketolase, which is related to the pentose phosphate pathway and Calvin cycle of photosynthesis. This finding agrees with the study that explained high biomass production and CO₂ fixation under a similar condition (Heureux et al. 2017).

An increased level of glyceraldehyde-3-phosphate (G3P) was also observed in *H. rubescens* cultivated in high CO₂ concentration. G3P is a converse enzyme that plays an important role in the glycolytic pathway. The high presence of G3P indicates that more glyceraldehyde-3-phosphate was produced and channeled to the glycolysis reaction, which leads to the production of more ATPs to support microalgae growth.

Table 3. List of detected protein and function classification in *H. rubescens* cultivated under normal and high elevated CO₂ concentration.

Protein accession number	Hypothetical function	Log ₂ FC	Up/ Down Regulation
A2SY36	Chlorophyll a-b binding protein, chloroplastic (Fragment) OS=Tetrademus obliquus OX=3088 PE=2 SV=1	0.137	Up
A2SY33	Chlorophyll a-b binding protein, chloroplastic OS=Tetrademus obliquus OX=3088 PE=2 SV=1	0.169	Up
A0A383WCU3	Chlorophyll a-b binding protein, chloroplastic OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS15739 PE=3 SV=1	0.584	Up
A0A383VL30	Chlorophyll a-b binding protein, chloroplastic OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS5542 PE=3 SV=1	0.736	Up
A0A383WJC7	Chlorophyll a-b binding protein, chloroplastic OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS17720 PE=3 SV=1	2.322	Up
Photosystem			
A0A249RWS1	Photosystem II CP47 reaction center protein OS=Tetrademus obliquus OX=3088 GN=psbC PE=3 SV=1	0.099	Up
A0A140HAJ2	Photosystem II CP43 reaction center protein OS=Bracteacoccus minor OX=50037 GN=pcbC PE=3 SV=1	0.321	Up
A0A0S2LQU8	Photosystem II D2 protein OS=Bracteacoccus giganteus OX=50039 GN=psbD PE=3 SV=1	0.263	Up
A0A2Z5DY77	Photosystem II CP47 reaction center protein OS=Chlamydomonas sp. UWO 241 OX=1653778 GN=psbB PE=3 SV=1	0.362	Up
A0A2R4PAT8	Photosystem II protein D1 OS=Haritina reticulata OX=183314 GN=psbA PE=3 SV=1	0.415	Up
A0A140H9J3	Photosystem I iron-sulfur center OS=Neochloris aquatica OX=3099 GN=psaC PE=3 SV=1	0.485	Up
A0A2Z4MA45	Photosystem I iron-sulfur center OS=Halochlorococcum sp. NIES-1838 OX=2249730 GN=psaC PE=3 SV=1	0.585	Up
A0A383V9R0	Photosystem II reaction center Psb28 protein OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS2905 PE=3 SV=1	-1.584	Down
Calvin cycle			
A0A2R4PAN7	Ribulose biphosphate carboxylase large chain OS=Haritina reticulata OX=183314 GN=rbcL PE=3 SV=1	0.234	Up
A0A6B9VTQ3	Ribulose biphosphate carboxylase large chain OS=Pithophora roettleri OX=926344 GN=rbcL PE=3 SV=1	0.263	Up
A0A6G6CV56	Ribulose biphosphate carboxylase small chain OS=Ettlia sp. YC001 OX=1191503 GN=rbcS2 PE=2 SV=1	1.415	Up
A0A383W0L6	Glyceraldehyde-3-phosphate dehydrogenase OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS11361 PE=3 SV=1	1	Up
A0A411H938	Glyceraldehyde-3-phosphate dehydrogenase OS=Ettlia sp. YC001 OX=1191503 GN=GAPDH PE=2 SV=1	0.906	Up
A0A2Z4THB7	Triosephosphate isomerase 2 OS=Tetrademus obliquus OX=3088 PE=3 SV=1	0	NC
A0A383WAG4	Fructose-biphosphate aldolase OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS14242 PE=3 SV=1	0.321	Up
A0A383WJL3	Transketolase OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS17672 PE=3 SV=1	0.415	Up
A0A383VTM0	Ribose-5-phosphate isomerase OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS9166 PE=3 SV=1	-7.643	Down
A0A087SRH0	Ribulokinase OS=Auxenochlorella protothecoides OX=3075 GN=F751_3834 PE=3 SV=1	-6.644	Down

Table 3. Contd.

Protein accession number	Hypothetical function	Log ₂ FC	Up/ Down Regulation
Starch metabolism			
A0A2V0PAZ7	ADP-glucose pyrophosphorylase OS=Raphidocelis subcapitata OX=307507 GN=Rsub_06969 PE=3 SV=1	0.585	Up
A0A383W111	Starch synthase, chloroplastic/amyloplastic OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS11531 PE=3 SV=1	8.966	Up
A8HT27	1,3-beta-glucan synthase OS=Chlamydomonas reinhardtii OX=3055 GN=FAP228 PE=3 SV=1	6.644	Up
A0A6T8WKD8	Starch synthase, chloroplastic/amyloplastic OS=Chlamydomonas leiostraca OX=1034604 GN=CLEI1391_LOCUS21077 PE=3 SV=1	6.644	Up
D8TY18	Alpha-amylase OS=Volvox carteri f. nagariensis OX=3068 GN=VOLCADRAFT_74960 PE=3 SV=1	6.644	Up
Glycolysis			
A0A6U3F903	Phosphoglycerate mutase (2,3-diphosphoglycerate-independent) OS=Mantoniella antarctica OX=81844 GN=MANT1106_5236 PE=3 SV=1	-6.644	Down
A0A383W0L6	Glyceraldehyde-3-phosphate dehydrogenase OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS11361 PE=3 SV=1	1	Up
A0A411H938	Glyceraldehyde-3-phosphate dehydrogenase OS=Ettlia sp. YC001 OX=1191503 GN=GAPDH PE=2 SV=1	0.906	Up
A0A6U3KH96	Pyruvate kinase OS=Mantoniella antarctica OX=81844 GN=MANT1106_22042 PE=3 SV=1	0	NC
A0A6S8PZE8	Aconitase_C domain-containing protein OS=Dunaliella tertiolecta OX=3047 GN=DTERR00134_22399 PE=4 SV=1	6.644	Up
A0A383VDH1	Pyruvate dehydrogenase E1 component subunit alpha OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS4161 PE=4 SV=1	7.644	Up
D8TGZ9	Pyruvate dehydrogenase E1 component subunit beta OS=Volvox carteri f. nagariensis OX=3068 GN=VOLCADRAFT_108817 PE=4 SV=1	0	NC
A0A383WP41	Isocitrate dehydrogenase [NADP] OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS19466 PE=3 SV=1	0	NC
Q00Z61	Oxoglutarate/iron-dependent dioxygenase OS=Ostreococcus tauri OX=70448 GN=OT_ostta11g00880 PE=4 SV=1	6.643	Up
A0A383W3J2	Malate dehydrogenase OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS12416 PE=3 SV=1	1.322	Up
A0A6T8DN68	Citrate synthase (Fragment) OS=Nephroselmis pyriformis OX=156128 GN=NPYR00342_LOCUS12143 PE=3 SV=1	6.644	Up
A0A6V2BER8	Citrate synthase OS=Tetraselmis striata OX=3165 GN=TSSTR00447_LOCUS24856 PE=3 SV=1	6.644	Up
A0A6U2C1Y1	Citrate synthase OS=Chlamydomonas euryale OX=1486919 GN=CEUR00632_957 PE=3 SV=1	6.644	Up
A0A383WH81	Isocitrate dehydrogenase [NAD] subunit, mitochondrial OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS17018 PE=3 SV=1	6.644	Up
Q00Z61	Oxoglutarate/iron-dependent dioxygenase OS=Ostreococcus tauri OX=70448 GN=OT_ostta11g00880 PE=4 SV=1	6.644	Up
A0A383VJ66	Uncharacterized protein OS=Tetrademus obliquus OX=3088 GN=BQ4739_LOCUS5696 PE=3 SV=1	6.644	Up
A0A061SCG4	Acetyl-CoA carboxylase (Fragment) OS=Tetraselmis sp. GSL018 OX=582737 GN=ACAC PE=4 SV=1	-6.644	Down

Several marked changes in proteins expression that related to carbohydrate metabolisms and starch synthesis were also observed when *H. rubescens* was cultivated in high CO₂ concentration. Many proteins, including ADP-glucose pyrophosphorylase (AGPase), starch synthase, pyruvate synthase (PK), fructose-1-6-biphosphate aldolase (FAB), glucose-1-phosphate adenylyltransferase (SAT), are involved in the starch synthesis pathway (Xu et al. 2022; Ran et al. 2019). The production of a high amount of protein related to starch synthesis, including AGPase and starch synthase in *H. rubescens*, suggests that a high accumulation of starch content occurred in the microalgae cultivated in this condition. However, no changes in the level of pyruvate dehydrogenase were observed in microalgae cultivated in normal and high CO₂ concentrations.

In order to gain a further understanding of specific metabolisms, a possible metabolic pathway involved during the cultivation under high CO₂ has been constructed according to protein expression obtained from the analysis (Figure 6). There are two possible metabolic pathways associated with carbohydrate biosynthesis in *H. rubescens* during cultivation using high CO₂ concentration. The two main metabolites that possibly play a crucial role during the process are glyceraldehyde-3-phosphate and pyruvate. The increased level of AGPase could enhance the synthesis of ADP glucose, leading to an increased amount of starch in the microalgae. On the contrary, reduced levels of pyruvate kinase and pyruvate dehydrogenase could lower the production of acetyl-CoA, leading to a decline in lipid production. The finding from this study has supported the biochemical composition profiling which indicated that reduction lipid content in *H. rubescens* when the microalgae was cultivated under higher CO₂ concentration. There is no correlation between transcriptomic and proteomic on Accc expression could contributed by post-transcription and translation regulation.

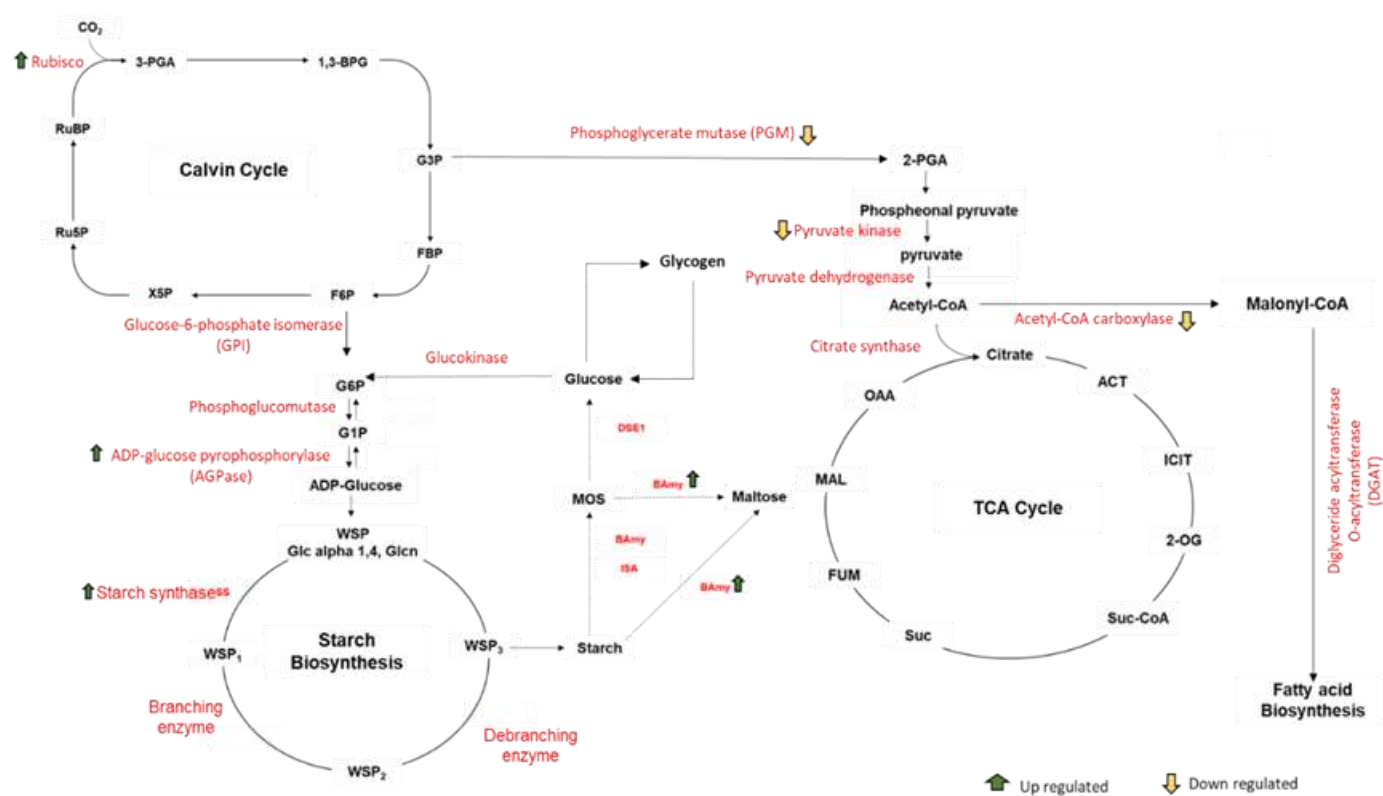


Figure 6. Putative metabolic pathway of *H. rubescens* cultivated using elevated CO₂ concentration.

CONCLUSION

This transcriptomic and proteomic study revealed new insight on carbon metabolisms, carbon fixation and carbohydrate biosynthesis for *H. rubescens* cultivated using different CO₂ concentrations. This study indicated that providing high CO₂ concentration could affect physiochemical characteristic of microalgae. In addition, expression of different genes and protein related to photosynthetic carbon fixation, cell division, biosynthesis carbohydrate, fatty acids, secondary metabolites and chlorophyll for *H. rubescens* cultivated under elevated CO₂ condition. Several important metabolites that act as main precursor for accumulation of carbohydrate and lipid in microalgae was also identified. The finding provides a crucial information for future strain improvement via mutagenic selection and metabolic dissection for better understanding on metabolic changes especially in carbon biofixation and accumulation of targeted compound in *H. rubescens*.

AUTHORS CONTRIBUTION

M.A.K and T.K.M designed the research, collected, analysed data and wrote the manuscript. B.Y.C.L and M.N.I process, collected and analysed data. M.A.K, M.H.A.B, M.N.I and J.L supervised the research process.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest during the research.

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Research Article

Vegetation Analysis and Species Diversity in the Protection Block and Natural Forest of KGPA Mangkunagoro I Grand Forest Park, Central Java, Indonesia

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ABSTRACT

Grand Forest Park (GFP) is a conservation area designated for biodiversity preservation and native and non-native fauna collections. Comprehensive data on vegetation diversity is crucial for scientific knowledge and effective conservation management. This study analysed tree species diversity and community structure in two locations: the GFP Mangkunagoro I Conservation Block and the BKPH Lawu Utara Natural Forest in Karanganyar, Central Java. Vegetation analysis was performed across four growth stages: seedlings, saplings, poles, and trees. The results revealed that the GFP Conservation Block hosts 42 tree species from 34 families. In this area, *Helicia robusta* (locally known as Cebongan) dominated the seedling (IVI 174.60) and sapling (IVI 73.79) stages, while *Quercus* sp. (Pasang) dominated the pole stage (IVI 36.23), and *Oenanthe javanica* was most prominent in the tree stage (IVI 60.29). Conversely, the BKPH Lawu Utara Natural Forest recorded 24 tree species from 23 families. Dominance in this area was characterised by *Helicia robusta* in the seedling stage (IVI 41.26), but transitioned to *Quercus* sp., which dominated the sapling (IVI 52.93), pole (IVI 42.91), and tree stages (IVI 115.80). These findings illustrated distinct patterns of species dominance and composition between the conservation block and natural forest, providing essential baseline data for forest management in the Lawu region.

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INTRODUCTION

The Grand Forest Park (GFP) is a specialised conservation area designated for biodiversity preservation, research, and education, housing both native and non-native species (Republic of Indonesia 1990). Beyond its recreational appeal, similar to the Batur Geopark in Bali (Mihardja et al. 2023), the primary value of a GFP lies in its vegetation diversity, which serves as the foundation for biological resource development and floristic conservation (Malik et al. 2021; García-Navas et al. 2022). These forests act as critical wildlife habitats and provide essential ecosystem services, including watershed protection, soil fertility maintenance, and microclimate regulation (Fikadu & Argaw 2021; Basuki et al. 2022; Wardah et al. 2022; Gelelcha et al. 2023).

GFP KGPA Mangkunagoro I, located in Karanganyar, Central Java, possesses significant biophysical potential and diverse germplasm (Cahyono et al. 2021). However, this area faces mounting anthropogenic pressure due to the high dependence of surrounding communities on forest resources (Rahman et al. 2021; He et al. 2022). Despite its importance, the comprehensive identification of its vegetation diversity remains incomplete. Furthermore, the ecological stability of this region is not only determined by the GFP itself but also by the adjacent natural forests, such as the BKPH Lawu Utara managed by Perum Perhutani. Both areas represent different management regimes that collectively sustain the Lawu mountain ecosystem.

Understanding the diversity, structure, and composition of tree species in both the GFP Mangkunagoro I and the BKPH Lawu Utara natural forest is vital for local managers. Previous studies have often focused solely on protected blocks, overlooking the comparative state of adjacent managed natural forests. Therefore, this study aims to provide a detailed analysis of vegetation status in the GFP Conservation Block and the BKPH Lawu Utara Natural Forest. These findings are expected to serve as a scientific baseline for forest managers to maintain species composition and to guide reforestation efforts with ecologically appropriate species in both conservation and production forest zones.

MATERIALS AND METHODS

Study area

The research was conducted in the Grand Forest Park (GFP) Mangkunagoro I, Karanganyar, Central Java. Data collection and vegetation analysis were carried out in two distinct management regimes: the GFP Conservation Block and the adjacent Natural Forest of BKPH Lawu Utara (managed by Perum Perhutani) (Figure 1).

Data Collection

The study utilised primary vegetation data collected through field surveys. A systematic sampling with a nested plot design was employed across both locations. We recorded tree diameter at breast height (DBH), total height, and the number of individuals for each species. The growth stages and their respective plot sizes were classified as follows:

- a. Seedlings: Individuals from sprouts to tillers with a height of <1.5 m, measured in 2×2 m subplots.
- b. Saplings: Individuals with a height of ≥ 1.5 m and a DBH of <10 cm, measured in 5×5 m subplots.
- c. Poles: Individuals with a DBH of 10 to <20 cm, measured in 10×10 m subplots.
- d. Trees: Mature individuals with a DBH of ≥ 20 cm, measured in 20×20 m plots.

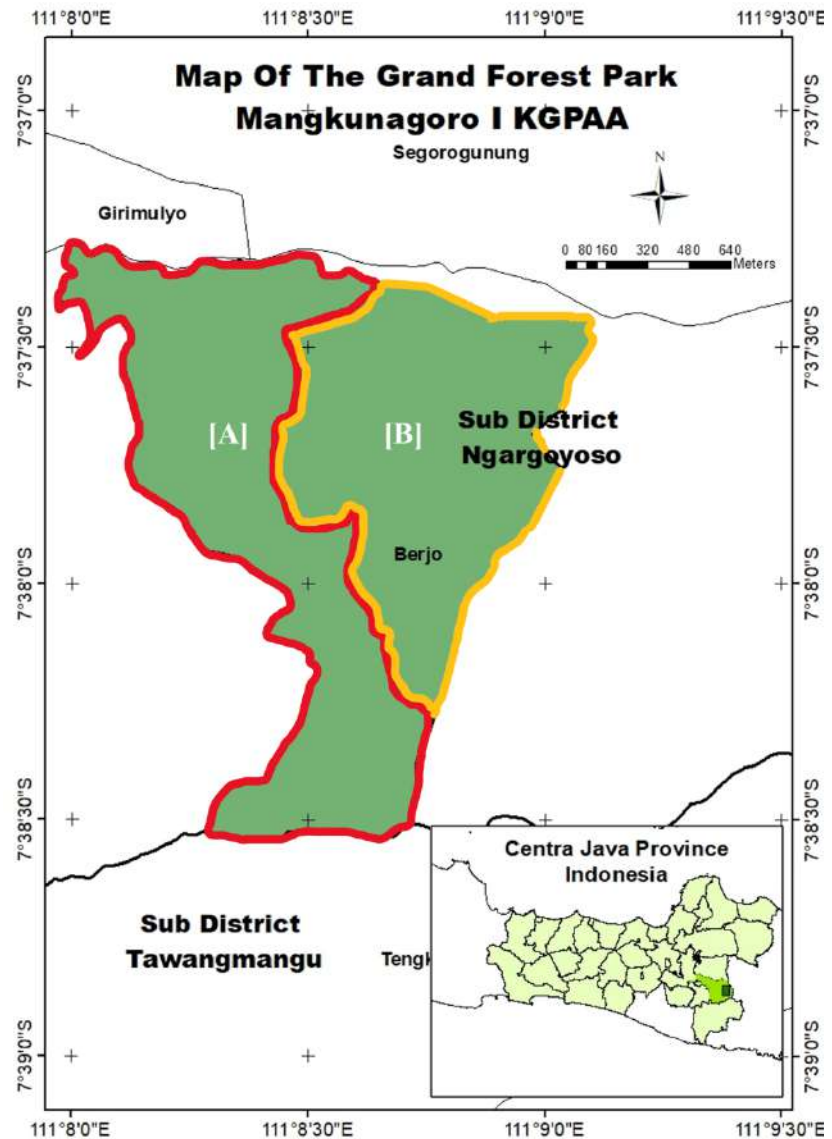


Figure 1. The study area is located in the GFP Conservation Block [B] and the BKPH Lawu Utara Natural Forest of Perum Perhutani [A], Karanganyar, Central Java.

Data Analysis

The data obtained from the field survey were analysed to determine the vegetation structure and diversity in both study locations. The analysis included the following indices:

1. Species Richness Species richness was calculated using the Margalef's Richness Index (R_1) (Margalef 1958) to determine the variety of species in each location:

$$R_1 = \frac{S - 1}{\ln N}$$

Where S is the total number of species and N is the total number of individuals.

2. Species Diversity and Evenness To assess the stability of the ecosystem, the Shannon-Wiener Diversity Index (H') was applied (Shannon & Weaver 1949; Magurran 2004):

$$H' = - \sum_{i=1}^S (P_i \ln P_i)$$

Where P_i is the proportion of the i th species calculated as n_i/N , with n_i repre-

senting the number of individuals of a species and N being the total number of individuals in the community. Furthermore, the Evenness Index (E) was used to measure the distribution of individuals among species (Pielou 1966):

$$E = \frac{H'}{\ln S}$$

The value of E ranges from 0 to 1, where a value closer to 1 indicates a more uniform distribution of species.

3. Importance Value Index (IVI) The ecological role and dominance of species in the community were determined using the Importance Value Index (IVI) (Curtis & McIntosh 1950). For the tree stage, IVI is the sum of Relative Density (RD), Relative Frequency (RF), and Relative Dominance (RDo). For seedlings, saplings, and poles, IVI is the sum of RD and RF (Soerianegara & Indrawan 1998):

$$IVI = RD + RF + RDo$$

Where:

Relative Density (RD) = (Number of individuals of a species) $\times$ 100 %

Relative Frequency (RF) = (Frequency of a species) $\times$ 100 %

Relative Dominance (RDo) = (Basal area of a species) $\times$ 100 %

4. Community Similarity To compare the species composition between the GFP Conservation Block and the BKPH Lawu Utara Natural Forest, the Sorensen's Similarity Index (IS) was used (Sorensen 1948; Magurran 2004):

$$IS = \left(\frac{2w}{a + b} \right) \times 100\%$$

Where w is the number of species common to both locations, a is the number of species in area A, and b is the number of species in area B.

RESULTS AND DISCUSSION

Vegetation Composition and Diversity Indices

The vegetation inventory across two management blocks revealed a significant difference in taxonomic richness. In the GFP Conservation Block, we identified 42 tree species belonging to 34 families (Table 1). Meanwhile, the BKPH Lawu Utara Natural Forest showed a lower richness with 24 tree species from 23 families (Table 2).

Community Structure and Species Dominance

The importance of each species within the community was assessed using the Importance Value Index (IVI). In the GFP Conservation Block (Table 3), the community structure is heavily dominated by *Helicia robusta* (Cebongan) in the early regeneration stages, reaching an IVI of 174.60 in the seedling stage. This dominance gradually decreases as the trees mature, with *Oenanthe javanica* (Pampung) becoming more prominent in the tree stage with an IVI of 60.29.

In contrast, the BKPH Lawu Utara Natural Forest (Table 4) exhibited more stable dominance pattern by the Fagaceae family. *Helicia robusta* (Cebongan) remained present in the seedling stage (IVI 41.26), but the subsequent growth stages (sapling, pole, and tree) are dominated by *Quercus* sp. (locally known as Pasang). The IVI of *Quercus* sp. increased significantly at the tree stage, reaching 115.80, indicating its role as the primary canopy-forming species in this natural forest block.

Table 1. Tree species and their families and tree significance index based on growth rate in the GFP Protection Block (Raw data source: GFP KGPA Mangkunagoro 1).

Local Name	Scientific name	Family	IVI Block GFP Protection (%) Phase			
			Seedlings	Saplings	Poles	Trees
Akasia	<i>Acacia decurrens</i> (J.C.Wendl.) Willd.	Fabaceae			1.55	2.07
Bagan	<i>unidentified</i>	<i>unidentified</i>		15.49	7.75	4.18
Bertupuk	<i>unidentified</i>	<i>unidentified</i>		5.16	4.65	4.53
Bintami	<i>Cupressus</i> sp.	Cupressaceae				15.04
Cale	<i>Ficus fistulosa</i> Reinw. ex Blume	Moraceae		56.08	35.62	24.69
Cebongan	<i>Helicia robusta</i> (Roxb.) R.Br. ex Blume	Proteaceae	174.60	73.79	13.72	3.64
Cuwut	<i>unidentified</i>	<i>unidentified</i>			5.31	8.39
Dempul	<i>Glochidion rubrum</i> Blume	Apocynaceae			9.30	23.72
Ganitri	<i>Elaeocarpus angustifolius</i> Roxb. ex- G. Don	Elaeocarpaceae				1.60
Gulma	<i>unidentified</i>	<i>unidentified</i>				7.18
Jebukan	<i>Galearia filiformis</i> (Blume) Boerl.	Pandanaceae			13.06	16.65
Kebak	<i>Ficus alba</i> Reinw. ex Blume	Moraceae			1.55	2.01
Kendit	<i>unidentified</i>	<i>unidentified</i>				1.05
Kengkeng	<i>Polinia ciliata</i> trin.	Poaceae				3.43
Kesambi	<i>Schleichera oleosa</i> (Lour.) Oken	Sapindaceae				1.03
Kina	<i>Cinchona pubescens</i> Vahl	Rubiaceae			19.22	4.26
Lotrok	<i>Wenlandia dasythyrsa</i> Miq.	Rubiaceae				2.09
Ndokndokan	<i>Xanthophyllum vitellinum</i> (Blume) D.Dietr.	Polygalaceae				6.84
Nogosaren	<i>Mesua ferrea</i> L.	Calophyllaceae				2.89
Nyampuh	<i>Actinodaphne procera</i> Ness	Lauraceae				4.77
Pampung	<i>Oenanthe javanica</i> (Blum) DC.	Apiaceae			1.55	60.29
Pacalkidang	<i>Aglaia heptandra</i> Coord. & Valetton	Meliaceae				1.16
Pasang	<i>Quercus</i> sp.	Fagaceae			36.23	40.49
Pinus	<i>Pinus merkusii</i> Jungh. & de Vriese	Pinaceae			2.87	10.02
Puspa	<i>Schima wallichii</i> (DC.) Korth.	Theaceae			1.55	7.69
Renik	<i>Eurya acuminata</i> DC.	Pentaphylacaceae			14.61	6.54
Sarangan	<i>Castanea argentea</i> (Bl.) Bl.	Fagaceae			4.65	23.85
Sembung	<i>Blumea balsamifera</i> (L.) DC.	Asteraceae				1.08
Tanen	<i>Celtis</i> sp.	Cannabaceae			1.55	3.20
Walikputih	<i>Acer laurinum</i> Hassk.	Sapindaceae			1.55	3.43
Wangwung	<i>unidentified</i>	<i>unidentified</i>				1.69
Wilodo/Cale	<i>Ficus fistulosa</i> Reinw. ex Blume	Moraceae			3.10	1.00
Apit	<i>unidentified</i>	<i>unidentified</i>	12.70	42.09	1.55	
Cemara	<i>Casuarina junghuhniana</i> Miq.	Casuarinaceae		7.39	5.97	
Ampel	<i>Bambusa</i> sp.	Poaceae	12.70			
Ekaliptus	<i>Eucalyptus</i> sp.	Myrtaceae			1.55	
Imer	<i>Flueggea virosa</i> (Roxb. ex Willd.) Royle	Phyllanthaceae			1.55	

Table 1. Contd.

Local Name	Scientific name	Family	IVI Block GFP Protection (%) Phase			
			Seedlings	Saplings	Poles	Trees
Jowor	<i>Actinorhysis calapparia</i> (Blume) H. Wendl. & Drude ex Scheff.	Arecaceae			1.55	
Kayu manis	<i>Glycyrrhiza glabra</i> L.	Fabaceae			2.22	
Rasamala	<i>Altingia excelsa</i> Noronha	Altingiaceae			1.55	
Suren	<i>Toona sinensis</i> (A. Juss.) M. Roem.	Meliaceae			1.55	
Talok	<i>Mallotus moritzianus</i> Müll. Arg.	Euphorbiaceae			3.10	

Table 2. Tree species and their families and tree importance value index based on growth rate and measuring plots in the North Lawu BKPH Natural Forest Block (Raw data source: GFP KGPA Mangkunagoro 1).

No.	Local name	Scientific name	Family	BKPH North Lawu Natural Forest Block, IVI (%) Phase			
				Seedlings	Saplings	Poles	Trees
1	Anggrung	<i>Trema orientale</i> (L.) Blume	Cannabaceae				3.24
2	Pasang	<i>Quercus</i> sp.	Fagaceae	22.16	52.93	42.91	115.80
3	Cebongan	<i>Helicia robusta</i> (Roxb.) R.Br. ex Blume	Proteaceae	41.26	10.80	5.30	
4	Preh	<i>Ficus retusa</i> L.	Moraceae	12.32	3.95		
5	Dempul	<i>Glochidion rubrum</i> Blume	Apocynaceae				6.31
6	Ganitri	<i>Elaeocarpus ganitrus</i> Roxb. ex- G. Don	Elaeocarpaceae		3.95		2.79
7	Puspa	<i>Schima wallichii</i> (DC.) Korth.	Theaceae	24.19	16.59	23.31	25.33
8	Jebungan	<i>Galearia filiformis</i> (Blume) Boerl.	Pandanaceae		7.9		13.07
9	Kengkeng	<i>Polinia ciliata</i> trin.	Poaceae		3.95	27.01	20.96
10	Pampung	<i>Oenanthe javanica</i> (Blume) DC.	Apiaceae			7.15	14.10
11	Putek	<i>unidentified</i>	<i>unidentified</i>		3.95		4.82
12	Jenggiti	<i>Eurya acuminata</i> DC.	Pentaphyllaceae	5.54	12.25	5.30	8.18
13	Walikputih	<i>Acer laurinum</i> Hassk.	Sapindaceae				8.38
14	Cuwut	<i>unidentified</i>	<i>unidentified</i>	5.54		10.60	
15	Puspa	<i>Schima wallichii</i> (DC.) Korth.	Theaceae	24.19	16.59	23.31	25.33
16	Piji	<i>Areca pumila</i> Blume	Arecaceae		7.90		
17	Renik	<i>unidentified</i>	<i>unidentified</i>	5.54	12.25		
18	Nagasari	<i>Mesua ferrea</i> L.	Calophyllaceae	10.63	11.20	10.86	3.92
19	Pinus	<i>Pinus merkusii</i> Jungh. & de Vriese	Pinaceae		3.95		40.94
20	Kidangan	<i>Phyllanthus indicus</i> (Dalzell) Müll. Arg.	Phyllanthaceae				
21	Klembir	<i>unidentified</i>	<i>unidentified</i>		11.85	5.30	6.63
22	Nyampuh	<i>Actinodaphne procera</i> Nees	Lauraceae	11.08	3.95		
23	Lotrok	<i>Wenlandia dasythyrsa</i> Miq.	Rubiaceae		7.50	17.75	
24	Tlutup	<i>unidentified</i>	<i>unidentified</i>	5.54			2.27

Regeneration and Successional Patterns

A high IVI in a community indicated species' superior ability to utilise environmental resources compared to its associates (Wrońska-Pilarek et al. 2023). Based on the data in Table 3 and Table 4, both locations showed healthy regeneration status, characterised by the presence of dominant species across various growth stages.

The extreme dominance of *Quercus* sp. in BKPH Lawu Utara suggests a climax community where this species has successfully adapted to the extreme monsoon conditions and environmental stressors (Yin et al. 2023). For the GFP, the shift from *Helicia robusta* to other species in the tree stage suggested more dynamic successional process. These findings provide critical baseline data for forest managers; ensuring the protection of *Quercus* mother trees in BKPH and maintaining the enrichment planting in GFP which is essential for the long-term sustainability of the Karanganyar forest ecosystem.

CONCLUSION

This study demonstrated significant differences in vegetation composition and community structure between the GFP Mangkunagoro I Conservation

Table 3. Species diversity index and species uniformity index as well as richness index from Marglef, based on the growth rate of protected forest blocks and BKPH North Lawu forest blocks.

Size	GFP Protection Block				
Plot (m ²)	S	N	R1	H'	E
2x2	2	17	0.35	0.224	0.323
5x5	6	45	1.31	1.468	0.819
10x10	28	152	5.37	2.260	0.678
20x20	32	289	5.47	2.762	0.797
Size	North Lawu BKPH Natural Forest Block				
2x2	7	34	1.70	1.501	0.771
5x5	19	108	3.84	2.221	0.754
10x10	22	137	4.27	2.314	0.749
20x20	18	244	3.09	1.994	0.690

Note: S (Sorensen's Similarity Index), N (Importance Value Index), R1 (Margalef's Richness Index), H' (Species diversity), E (Evenness Index).

Table 4. Tree species similarity index in the GFP protected area and the North Lawu BKPH natural forest block based on growth rate.

Size	Amount Species	Quantity	IS
20x20	In Protection block (A)	32	0.22
	In the North Lawu BKPH Natural Forest block (B)	18	
	In both blocks (C)	13	
	In Protection block (A)	28	
10x10	In the North Lawu BKPH Natural Forest block (B)	22	0.16
	In both blocks (C)	9	
	In Protection block (A)	6	
5x5	In the North Lawu BKPH Natural Forest block (B)	19	0.36
	In both blocks (C)	2	
	In Protection block (A)	2	
2x2	In the North Lawu BKPH Natural Forest block (B)	7	0.52
	In both blocks (C)	1	
	In Protection block (A)	2	

Block and the BKPH Lawu Utara Natural Forest. The GFP area exhibited higher species richness (42 species) compared to the natural forest of BKPH Lawu Utara (24 species), reflecting its role as a biodiversity conservation and collection zone. In terms of dominance, *Helicia robusta* characterised the early successional stages in both locations, while the climax stage in the natural forest was heavily dominated by *Quercus* sp. (IVI 115.80). These findings suggested that while both areas showed healthy natural regeneration, different management strategies are required. Conservation efforts should prioritise the protection of climax species like *Quercus* sp. in natural forests, while GFP management should focus on maintaining high taxonomic diversity through enrichment plantation and habitat preservation.

AUTHORS CONTRIBUTION

H.D.R. Conceptualisation, Methodology, Writing – original draft. B.H. Supervision, Validation. P. Project administration, Resources. C.Y.L. Data curation, Investigation. A.W. Formal analysis. S.A.C. Software, Visualisation. N.P.N. Methodology, Validation. A.W.N. Investigation. A.I. Supervision. I.A. Writing & review. F.M.H.N. Data curation. D.P. Investigation. M.N. Formal analysis. M.S. Investigation, Methodology, Writing – review & editing. W. Resources, Data curation. I.L.P.P. Review

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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