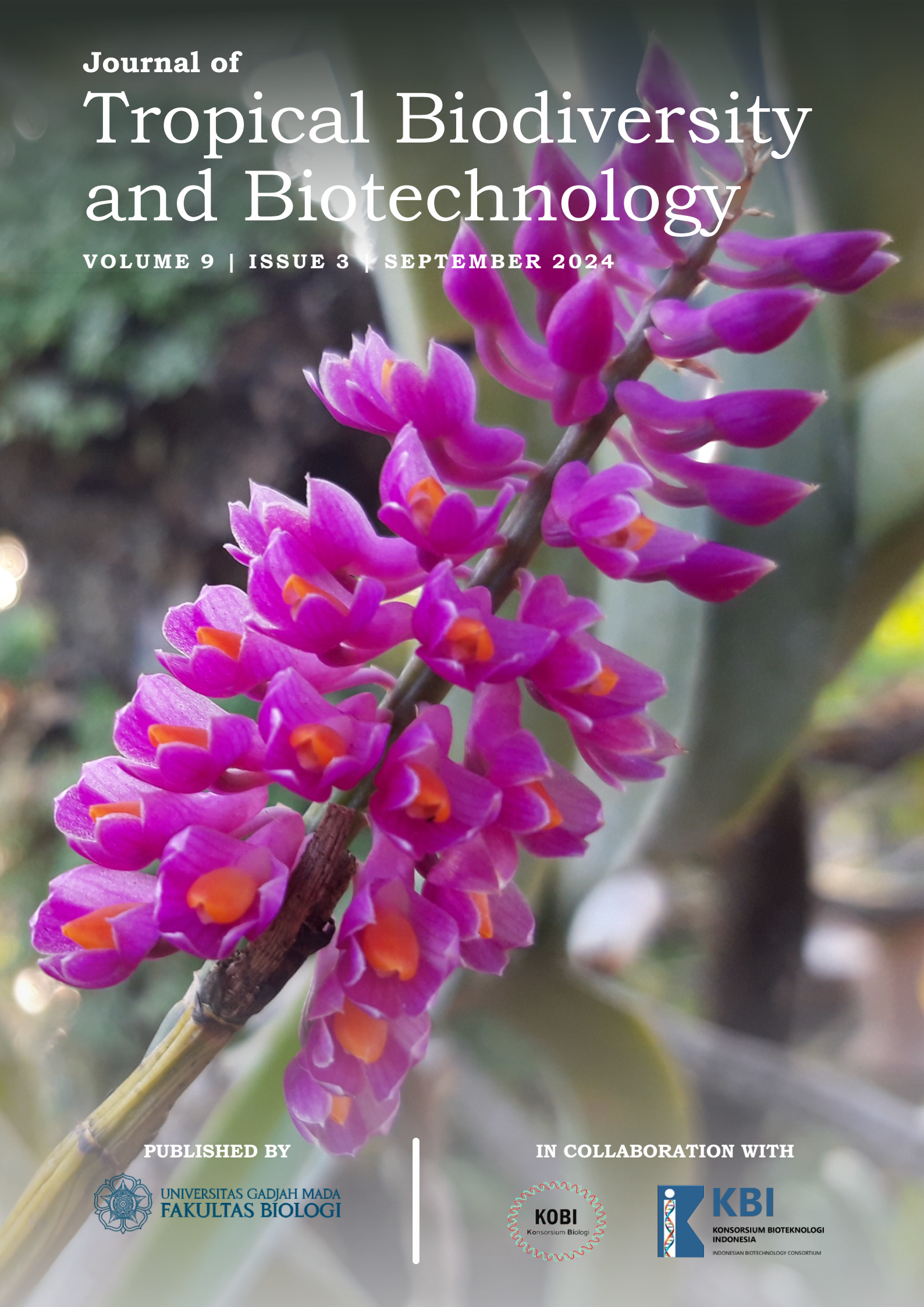

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Short Communications

Edaphic Characteristics of *Rafflesia* Habitats in Indonesia: Implications for Conservation and Propagation

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ABSTRACT
Rafflesia, a holoparasitic and endophytic plant, depends on its host, *Tetrastigma* spp., for survival, thus highlighting the critical interdependence between these species. Given the endangered status of *Rafflesia* due to anthropogenic pressures and narrow distribution, comprehensive conservation efforts are crucial. Ecological data on edaphic conditions, particularly the presence of the host, are important for effective conservation strategies. This study assessed soil properties across *Rafflesia* habitats on Sumatera, Borneo, and Java islands, revealing similarities in pH, carbon, nitrogen, cation exchange capacity (CEC), while the soil texture varied. These findings contribute valuable insights for informed conservation initiatives, both in-situ and ex-situ.

Rafflesia is a unique and endemic plant species of Indonesia. It has single biggest flower among flowering plants species in the world. Despite being large in size, *Rafflesia* is actually a holoparasitic and endophytic plant without vegetative organs such as stems, leaves roots or any photosyntentic systems (Molina et al. 2014; Mursidawati et al. 2020). Hence its life cycle, survival and establishment depend on its host, which is known as *Tetrastigma* spp. (Vitaceae). Thus, the distribution of the *Rafflesia* spp. in the forest are entirely dependent on the existence of its host species and its supporting plants. Because these plants are influence each other, all plant forms (lifeforms) and their habitat must be equally conserved (Adnan & Hadisusanto 2023).

In Indonesia, *Rafflesia* can be found on three of the largest islands: Sumatra, Borneo, and Java, as well as on some smaller surrounding islands like Nusa Kambangan (van Steenis et al. 1954) and Mursala Island (Mahyuni et al. 2016). All the *Rafflesia* species in Indonesia are considered endangered due to their unique biological and ecological characteristics, as well as the impact of human activities (Hidayati & Walck 2016; Lestari & Susatya 2022). Therefore, ex-situ conservation of *Rafflesia* is strongly recommended as a solution, especially because it can be challenging to access *Rafflesia* in its natural habitat for research purposes (Lestari & Susatya 2022). Wicaksono et al. (2016) also proposed the artificial or human intervention cultivation of *Rafflesia* spp.

To propagate or conserve *Rafflesia* in ex-situ area, ecological data which provide conditions required for its growth are needed, especially the edaphic conditions where the host exists (Hayati et al. 2021). In previous study, Wahab et al. (2021) mentioned that the presence of *Tetrastigma* sp. in the different *Rafflesia* habitats has its own relationship with the soil and is not influenced by one factor. Therefore, a more comprehensive study related to the edaphic habitat of various *Rafflesia* species must be carried out.

In Indonesia, studies related to the edaphic habitat of *Rafflesia* are still limited and mostly focused on aspects like soil pH. For example, Ramadhani et al. (2017) found that the area where *Rafflesia arnoldi* is found are usually have temperatures between 25 and 29 degrees Celsius, humidity levels of 95%, and an acidic pH of 5.5, with the soil consists of sandy clay soil with varying percentages of sand, silt, and clay. *R. patma* in Bojonglarang Jayanti Nature Reserve grows in more acidic soil with a pH of 5.5 and pH KCl at 4.8, with soil classified as sandy clay soil with different percentages of sand, silt, and clay, with the organic nutrient content is higher compared to the *Tetrastigma* plot, while the inorganic nutrient content or mineral soil elements in the *Rafflesia* plot are lower (Ali et al. 2015). *R. patma* in Leweung Sancang live in regosol soil, sandy loam, with loose and well-drained soil, acidic to neutral soil pH, very high organic C and Ca content, total N, Mg, high CEC, very low available P, and moderate K and Na (Priatna et al. 1989). *R. patma* in Ciletuh also grows on acidic soils with pH ranging from 5.8 to 7 (Triana et al. 2017). Laksana et al. (2018) found that the types of soil in the permanent plots of *R. zollingeriana* in Krecek are latosol soil, generally dark reddish in colour, and with a clay texture, with soil pH considered neutral, as it is close to 7. Rambey et al. (2023) observed that the habitat of *R. meijeeri* in Batang Gadis National Park Pagar Gunung indicating a neutral soil pH as it is close to 7 overalls (6.8 – 6.9). But there are also studies that do not address soil acidity. In addition, Renjana et al. (2022) mentioned that soil organic carbon was the most important variable affecting the occurrence of the host plants of *R. arnoldii.*

However, these studies only provide partial information, and more comprehensive research is needed to fully understand the specific edaphic conditions preferred by different *Rafflesia* species. Therefore, this study aims to assess the physical and chemical properties of soil at three distinct locations where three different *Rafflesia* species are found on different islands in Indonesia: Java, Sumatera, and Borneo. By gaining deeper insights into the physical and chemical properties of the soil in the natural habitat of these *Rafflesia* species, we can identify the factors that contribute to their growth and survival. The information of edaphic characteristics also plays a crucial role in determining the suitable locations for these species to thrive and reproduce, as well as their susceptibility to environmental changes and disturbances. This knowledge can be used to develop effective conservation strategies, such as habitat restoration or management, to support the survival of these unique species in their natural environments.

The study was conducted in three different islands of Indonesia where four populations of *Rafflesia* spp. were identified (Table 1). These study locations comprise Tempursari-Lumajang in East Java, Palak Siring Kemumu in Bengkulu, Sumatera island and Kayan Mentarang National Park in North Kalimantan, which is located in the island of Borneo (Figure 1).

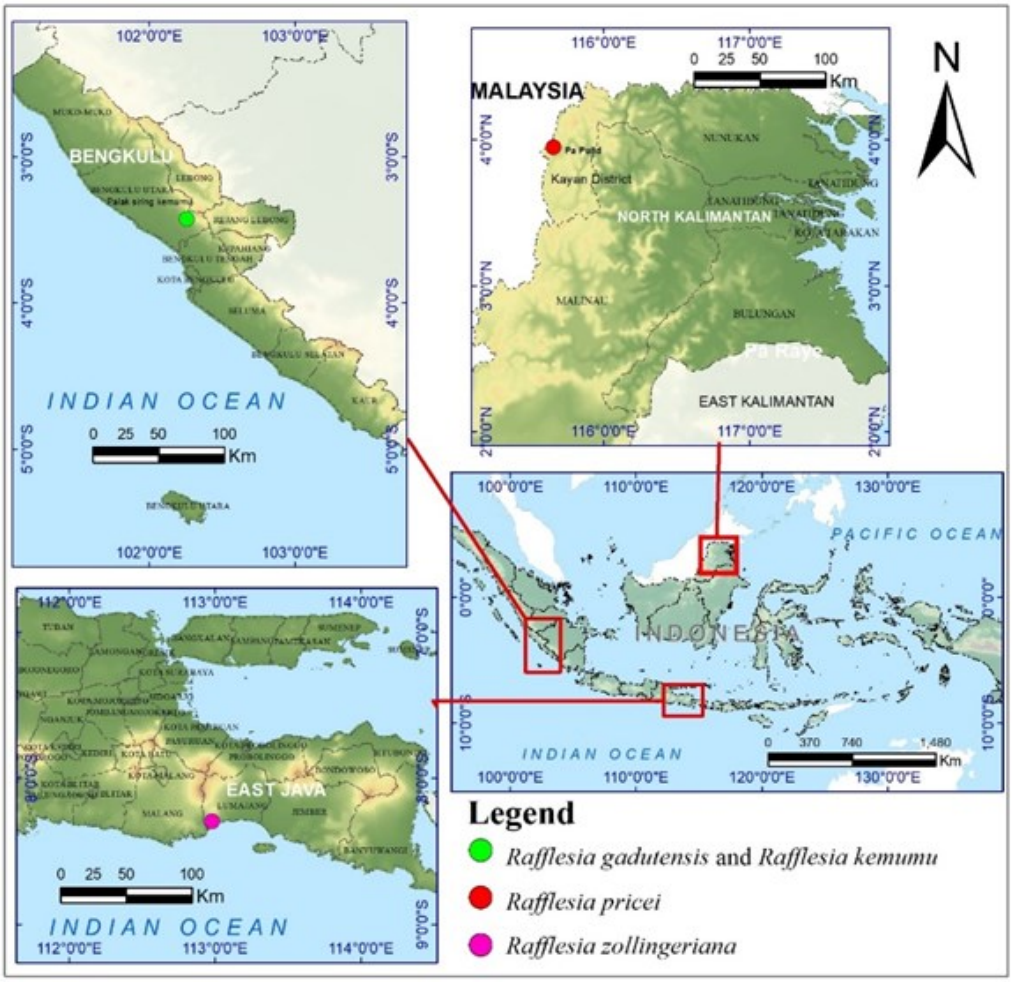

Figure 1. Map of Three *Rafflesia* Habitats in Three Island of Indonesia.

The first sample was collected from Argosari, a protected forest of Perhutani, that adjacent to human settlement in Tempursari, Lumajang, East Java, located at 450 m above sea level (asl) (Table 1). The new record for *R. zollingeriana* in East Java was detected from this area (Lestari & Susatya 2022).

Palak Siring Kemumu, Bengkulu Utara was habitat for two species *Rafflesia*: *R. gadutensis* and *R. kemumu* (Susatya et al. 2017). This area is lowland protected forest that also functioning as ecotourism object, located at 290 m a.s.l in Arga Makmur, Arma Jaya, Bengkulu Utara (Figure 2).

Kayan Mentarang National Park is lies between Malinau and Nunukan Regency, North Kalimantan, Indonesia. This national park is reported as habitat for *R pricei* (Jayasilan et al. 2004; Lestari et al. 2020). The soil sample was collected from *R. pricei* habitat in hillside of montane forest at 60^0 slopes, at 1277 m a.s.l. in Pa' Pulid forest near to Pa' Api, Nunukan, Kalimantan Utara.

Table 1. The study sites of four species of *Rafflesia*.

Location	Status	*Rafflesia* Species	Host Species
Tempursari, Lumajang	Conservation area	*R. zollingeriana*	*T. rafflesiae*
Palak Siring Kemumu	Conservation area for Ecotourism	*R. gadutensis*, *R. kemumu*	*T. leucostaphylum* and *T. pedunculare*
Kayan Mentarang National Park	National park	*R. pricei*	*Tetrastigma.* sp.

Figure 2. (A) *R. kemumu* blooms in Palak Siring, North Bengkulu (Picture by Bara); (B) Survey of *R. gadutensis* habitat; (C) First day blooming of *R. zollingeriana* in Lumajang (Picture by DL); (D) Survey of *R. zollingeriana* habitat. (E) Post blooming of *R. pricei* in Kayan Mentarang National Park (Picture by DL); (F) Survey of *R. pricei* habitat.

Soil sampling was carried out based purposive random sampling method in three different sites. Soil samples were collected from 0-20cm depth. After taken from the plots, the soil sample then air dried and analysed in Indonesian Coffee and Cocoa Research Institute (ICCRI) laboratory. The parameters soil were soil chemical and physical property. The chemical properties consist of soil pH, organic carbon (%), total nitrogen content (%), Phosphorus (ppm), K, Ca, Na, Mg (me $100g^{-1}$), Electron Exchange Capacity (CEC) (me $100g^{-1}$) and Base Saturation (%). Additionally, the observation of soil physical property focused on the soil texture.

Soil pH determine in water (1:2.5) using pH meter, organic carbon used wet oxidation method by Walkey and Black. Phosphorus extraction by Bray and Olsen method and K, Ca, Mg, Na, CEC and Base saturation through leaching with NH_4SO_4. Soil parameters ranking based on Indonesian Soil Research Institute (Balittanah 2009). The determination of soil texture was using the soil texture triangle (Figure 3). The percentage of each particles obtained then applied to the soil texture triangles and cross point between the percentage of particles to determine the texture class.

Based on our results, there were variations in soil texture and chemical properties among the three habitats of *Rafflesia*. The pH level in all three locations was found to be slightly acidic, ranging from 5.8 to 6. The Palak Siring Kemumu site had higher levels of total carbon and nitrogen content, with measurements of 7.67% and 0.69%, respectively, in comparison to the Tempursari-Lumajang and Kayan Mentarang Nation-

al Park areas. Nevertheless, according to Balai Penelitian Tanah (Indonesian Soil Research Institute) (Balittanah 2009), the soil carbon percentage in the three *Rafflesia* habitats was classified as high to very high, while the nitrogen content was classified as medium to high.

Figure 3. USDA Soil Texture Triangle (Groenendyk et al. 2015).

The ratio of carbon to nitrogen (C/N) in the soil can be influenced by various factors such as the type of vegetation, organic matter, and microbes. The optimum C/N ratio for plant growth is between 10-20. Our results showed that the C/N ratio in the three *Rafflesia* habitats was within this range, indicating that the soil has a higher level of decomposition and a faster release of nutrients (Kitayama et al. 1998; Eiland et al. 2001; Li et al. 2020)

In terms of phosphorus content, the Kayan Mentarang National Park site had the highest value at 96 ppm, which is considered very high, while the other two locations had lower levels with Palak Siring Kemumu at 13 ppm and Tempursari-Lumajang at 20 ppm (Table 2). In contrast, the Palak Siring Kemumu site had the highest base saturation at 99.3 %, followed by Tempursari-Lumajang at 86.99%, both of which were

Table 2. Soil Texture and Soil Chemical properties of three habitats of *Rafflesia* spp.

Location	Soil texture			Texture class	pH	C (%)	N (%)	C/N	P_2O_5	CEC	Sulphur	Base Saturation	Na
	Sand	Clay	Silt										
Tempursari, Lumajang	56	24	20	Sandy Clay loam	5.8	4.43	0.32	14	20	29.29	216	86.99	0.22
Palak Siring Kemumu	29	30	41	Clay Loam	6	7.67	0.69	11	13	28.54	58	99.3	0.19
Kayan Mentarang National Park	9	37	54	Silty clay loam	6	6.13	0.57	11	96	21.83	211	24.28	0.14

categorized as very high. The Kayan Mentarang National Park site had the lowest base saturation at 24.28%. Additionally, the cation exchange capacity (CEC) in all three habitats of *Rafflesia* spp. was high, with the highest value found in the Tempursari-Lumajang location at 29.29 me $100g^{-1}$, followed by Palak Siring Kemumu at 28.54 me $100g^{-1}$ and Kayan Mentarang National Park at 21.83 me $100g^{-1}$.

Moreover, the sulphur content varied among the three locations, with the highest content observed in the Tempursari-Lumajang location at 216, followed by Kayan Mentarang National Park at 211 and the lowest content in the Palak Siring Kemumu location at 58. The sodium content found at the low level. The highest sodium content was observed in the soil of the Palak Siring Kemumu location at 0.22 me $100g^{-1}$, followed by Tempursari-Lumajang at 0.19 me $100g^{-1}$ and Kayan Mentarang National Park at 0.14 me $100g^{-1}$. Sodium serves as an essential cation for plants, but excessive concentrations can be detrimental to plant growth, leading to root damage, compromised water and nutrient absorption, as well as disruptions in metabolic and cellular functions (Chang & Dregne 1955; Subbarao et al. 2003).

The soil texture classes of the habitat of four species *Rafflesia* also varied. The Tempursari-Lumajang location exhibited a sandy clay loam texture class, whereas the Palak Siring Kemumu location, situated in Sumatera, had a clay loam texture class. On the other hand, the Kayang Mentarang National Park location, located on the island of Borneo, had a silty clay loam texture class. These differences in soil texture can have implication for the existing of *Rafflesia* species and its host.

The soil texture affects the ability of soil to hold the available water and nutrients for plants thus directly affecting the plants growth. In Lumajang area, *R. zollingeriana* found in the sandy clay loam soil texture with higher percentage of sand in the soil. The previous studies reported that the *R. zollingeriana* found in Meru Betiri National Park, East Java found in soil with a high percentage of clay, sandy clay and sandy loam (Zuhud 1988; Laksana et al. 2018). Furthermore, previous study in the same national park, Maulidiyan et al. (2019) reported that *R. zollingeriana* occurred in an inclined rocky terrain, leading to the development of root buttresses in its host plant, *Tetrastigma* spp.

The two species of *Rafflesia*; *R. gadutensis* and *R. kemumu* found in clay loam, while the *R. pricei* found in silty clay loam soil texture. The silty clay loam soils tend to have moderate to high water-holding capacity and can retain water for relatively long periods of time compare to the sandy clay loam soil. The water uptake of *Rafflesia* is entirely dependent on its host plant, and therefore, the soil texture affects the water intake required for both the host plant and *Rafflesia*. The sandy clay loam, clay loam, and silty clay loam soil textures are considered have the ability to meet the water requirements of *Tetrastigma* and *Rafflesia*. Sandy clay loam soils are typically well-draining and can hold water for moderate periods, while clay loam soils, with their higher proportion of clay particles, have a higher water-holding capacity and are generally considered to be some of the most productive and versatile soils. Silty clay loam soils show a balance between water-holding capacity and drainage (Saxton et al. 1986; Andrenelli et al. 2016). Furthermore, *Rafflesia* required the soil texture that can support good drainage, which prevents waterlogging, root damage and *Rafflesia* decaying. The soil should able to hold onto sufficient moisture to meet the needs of the host and *Rafflesia*, but also allow excess water to drain away relatively quickly.

Calcium (Ca), magnesium (Mg), and potassium (K) are essential soil nutrients for the growth of plants including the host of *Rafflesia*:

Tetrastigma spp., although they are required in smaller quantities compared to other nutrients. The result showed that Potassium concentrations varied slightly among the study sites, ranging from 0.9 me $100g^{-1}$ to 1.79 me $100g^{-1}$ (figure 4). Calcium concentrations were highest at Palak Siring Kemumu, with a value of 22.92 me $100g^{-1}$, followed by Tempursari, Lumajang, with a value of 17.98 me $100g^{-1}$, and Kayan Mentarang National Park, with a value of only 1.73 me $100g^{-1}$. The magnesium content also varied across the study sites, with the highest concentration found at Tempursari, Lumajang (6.16 me $100g^{-1}$), and the lowest concentration found at Kayan Mentarang National Park.

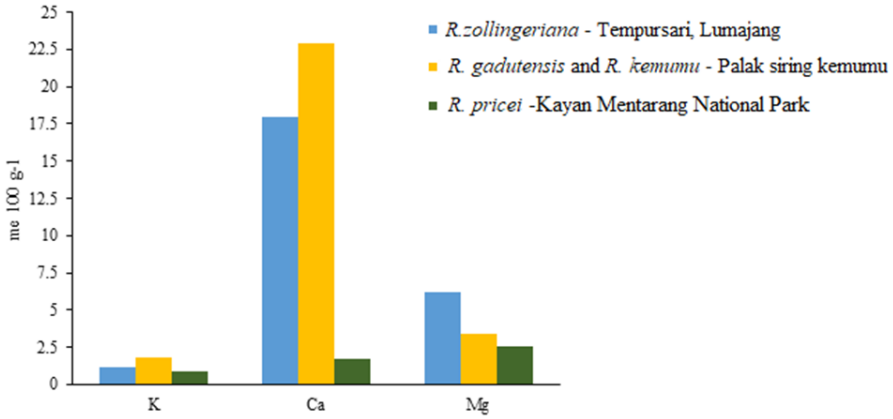

Figure 4. The Exchangeable Pottasium, Calsium and Magnesium content in the soil.

The Kayan Mentarang National Park, where *R. pricei* was discovered, exhibited the lowest base saturation. Low base saturation refers to a condition where the relative proportion of exchangeable base cations compared to the total soil cation exchange capacity (CEC), is relatively low (Juo et al. 1976; Chapman 2016). This indicates a reduced proportion of positively charged ions such as calcium, magnesium, potassium, and sodium in relation to the overall ion-holding capability of the soil. Moreover, the calcium content within the soil of Karang Mentarang was comparatively lower than that found in the other two locations. As a consequence of these distinctive soil attributes, the nutritional requirements of distinct *Tetrastigma* and *Rafflesia* species may exhibit variations contingent upon their respective geographic placements.

Variations in the edaphic characteristics were observed among populations of the same *Rafflesia* species. These differences can be due to multiple factors, including variations in elevation, geographical region, and the geological composition of soil formation. For instance, *R. zollingeriana* that found in Meru Betiri National Park, was encountered in soil of the Latosol type, characterized rich iron and aluminium oxide (Laksana et al. 2018). Previous study mentioned that this species tends to thrive in soils exhibiting a moderate to high Cation Exchange Capacity (CEC), high base saturation, and a high to very high concentration of potassium (K), sodium (Na), calcium (Ca), and magnesium (Mg). The carbon content of these soil ranges from moderate to high, while total nitrogen (N) content varies from low to moderate, accompanied by notably low phosphorus (P) levels (Zuhud 1988). The disparities in location and elevation contribute to variations in soil chemical character within the habitat of *R. zollingeriana* and its host plant. In Meru Betiri National Park, *R. zollingeriana* was found at elevations ranging from 0 to 300 meters above sea level (Zuhud 1988; Lestari et al. 2014), while in the Lumajang region

of East Java, it was encountered at elevations of 450 meters above sea level (Lestari & Susatya 2022).

Edaphic variation also found in *R. gadutensis* habitat. Despite its presence in Palak Siring Kemumu, *R. gadutensis* also distributed within West Sumatera (Meijer 1984; Meijer 1997; Nais 2001; Rahma et al. 2017). Based on the observation of Akhriadi et al. (2010), the population of *R. gadutensis* could be found elevations ranging from 350 to 750 meters above sea level, specifically within old secondary rainforest in West Sumatera. Additionally, the presence of *R. gadutensis* populations have been recorded on the diminutive island of Mursala, proximate to Sibolga along the western coastline of North Sumatra (Mahyuni et al. 2016). Meanwhile, *R. kemumu* is a new species of *Rafflesia* found in the lowland forests of Bengkulu (Susatya et al. 2017) and there has been no study regarding the presence of *R. kemumu* in areas other than Bengkulu.

This study has confirmed that each species of *Rafflesia* requires a distinct set of soil characteristics in its edaphic habitat. Despite variations between the habitats studied, we observed that the soil pH was slightly acidic, while carbon and nitrogen levels ranged from moderate to high. These findings emphasize the essential significance of nutrient-rich soil and a resilient cation exchange capacity (CEC) in facilitating the growth of both the host plant and *Rafflesia*. As such, these pivotal factors must be considered in the both in situ and ex-situ conservation initiatives aimed at safeguarding and nurturing *Rafflesia* populations. By acknowledging and integrating these soil-related insights, we can enhance the efficacy and success of conservation efforts for these remarkable species.

In terms of in-situ conservation, environmental changes, such as soil compaction and erosion, can adversely affect the growth and survival of *Tetrastigma*, directly impacting *Rafflesia* as well. Given the high sensitivity of *Rafflesia* to environmental changes, especially when its host plant is compromised, it is necessary to maintain the original edaphic conditions of both the host and *Rafflesia* undisturbed.

Due to the fact that the selection of an appropriate host for *Rafflesia* is determined by intricate physiological compatibility rather than a single factor alone (Renjana et al. 2022), in terms if ex situ conservation, it becomes crucial to replicate all abiotic and biotic factors present in natural habitat of *Rafflesia* as closely as possible to improve host compatibility with *Rafflesia*, to enhance the success of *Rafflesia* propagation.

The soil characteristics from the three locations reflect fertile soil with an adequate supply of macronutrients for the growth of the host plant. Our ongoing study has indicated that the combination of soil, compost, and fermented bamboo leaves present the potential medium for the cultivation of *Tetrastigma*, providing fertile soil and a favorable texture. However, further investigation is required to determine the optimal proportions for this media composition.

In addition to soil composition, the water and nutrient requirements of the soil are critical factors influencing the growth of both *Tetrastigma* and *Rafflesia*. This correlation arises because the development of the *Rafflesia* bud intricately tied to the host plant vine, acquiring water and nutrients (Nais 2001; Iman et al. 2021). Consequently, more comprehensive studies are imperative to identify the specific essential nutrients essential for the growth and development of the *Rafflesia*. This detailed observation will contribute to valuable insights towards creating an optimal environment that supports the thriving coexistence of *Tetrastigma* and *Rafflesia*.

AUTHOR CONTRIBUTION

Conceptualisation, D.L, RI and F.A.S.; validation and data curation, F.A.S and D.L., writing—original draft preparation, F.A.S.; writing, review and editing, F.A.S, D.L and R.I; F.A.S and D.L are the main authors of this publication. All author has reviewed and approved the final draft of the manuscript for publication.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Short Communications

Fantastic Macrofungi in Poncokusumo District, Bromo Tengger Semeru National Park (TNBTS) Area and Their Habitat Characteristics

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ABSTRACT
Bromo Tengger Semeru National Park (TNBTS) is a conservation area in East Java with an abundance of biodiversity, including macrofungi. This study aims to identify the macrofungal species existing in Poncokusumo district, TNBTS area, and their habitat characteristics for a further sustainable study of fungi. This study used a purposive sampling method by opportunistic exploration. Identification of macrofungi is conducted by morphological analysis and habitat characterization. The study identified 15 macrofungal species categorized as Ascomycota and Basidiomycota, that were distinct in their habitat characteristics. The 15 macrofungal species inhabited leaf litter, wood litter, soil, and bamboo.

Macrofungi belong to the kingdom Fungi, which is notable for its crucial functions in ecosystems. Numerous species serve as essential decomposers and animal and human food sources (Tang et al. 2015). Most macrofungi produce fleshy, colloidal fruiting bodies that represent sexual reproductive structures. Fungi have an easy presence especially in places with sufficient humidity and in environments conducive for their growth. Some fungi even grow and associate with other organisms, whether it be through mutualism, antagonism, or parasitism. Macrofungi constitute a type of fungi as they are visible to the naked eye without the aid of a microscope and possess distinctive colorations and fruit body shapes. Macrofungi can be round like umbrellas, balls, bird's nests, and trumpets, with striking colors, that microfungi do not possess. Therefore, macrofungi are called fantastic and interesting to study.

A major number of macrofungi are members of Basidiomycota or Ascomycota, while a few are members of Zygomycota. Their fruiting bodies are located either above or below the ground (Mueller et al. 2007; Tang et al. 2015). Approximately 6,000 out of 100,000 fungi species in the world can produce visible fruiting bodies (Ainsworth 2008). Macro-

fungi have parasitic, symbiotic, or saprophytic lives. Symbiotic macrofungi cannot reproduce independently. Therefore, they need host partners to disperse and reproduce (Tang et al. 2015).

Bromo Tengger Semeru National Park (TNBTS) is a conservation area spanning four different regencies in East Java Province, namely Probolinggo, Pasuruan, Malang, and Lumajang Regencies. This area has at least three ecosystem types, namely montane, sub-montane, and sub-alpine ecosystems (Anesta et al. 2020). These ecosystems provide perfect habitats for macrofungi to live, some being rare macrofungi (Anesta et al. 2020; Majumdar et al. 2022). Previous studies showed that changes in the number of species and conveyance extension of species separately, includes in specific changes in macrofungal communities due to climate change (Gong et al. 2012; Zotti & Pautasso 2013). Some other factors such as precipitation, CO_2 level, air temperature, soil pH, and habitat are known to affect the existence of macrofungi (Ferris et al. 2000; Lindblad 2001; Salerni et al. 2001; Chen et al. 2018; Rudolph et al. 2018; Taniguchi et al. 2018; Kotowski et al. 2021; Rakić et al. 2022; Song et al. 2022).

Fungi have a huge biodiversity, which is growing in databases with the increasing sensitivity of identification tools used by scientists. Hawksworth and Cowell (1991) reported that there are 1,500,000 types of fungi in the world and still counting. About 80% of fungi are of microscopic types, while the remaining 20% are macroscopic fungi. In Indonesia, macroscopic fungi make up only 0.15% of the total fungi worldwide (Retnowati et al. 2019).

The exploration of macrofungal species in the TNBTS area is still limited. Several studies have only focused on their use and relation to ethnobotany (Haryati & Azrianingsih 2012; Indriyani et al. 2012). Numerous macrofungi have been identified in several forests and national parks in Indonesia, including Bukit Danau Forest. Five orders, 13 families, and 17 genera of macrofungi have been documented in this location (Salmiah et al. 2020). In another national park, Danau Sentarum National Park, 23 species from 7 orders and 12 families were discovered, with the Polyporaceae family being the most abundant (Juarsih et al. 2023). Given this limitation, this study aims to conduct macrofungal species identification and habitat characterization. The data generated from this study are expected to serve as basic data for further studies of fungi in this area.

This study was conducted in five locations in Poncokusumo District, Malang Regency (part of the buffer zone of the TNBTS area), from October to December 2022 (Figure 1, Table 1). Poncokusumo District houses a typical lowland forest at an elevation of less than 1,000 meters above sea level. This area is typically hilly and mountainous. The average annual precipitation and temperature are about 2,300 mm and 21.7 ^{o}C, respectively. The highest precipitation is about 423 mm during the wet season in December, and the relative humidity of this area is about 82% (Santikayasa et al. 2017).

Sample collection was performed using the field survey method, involving surveying the forest area directly (Firdaushi & Basah 2018). The samples obtained were photographed and identified based on their morphological characteristics. Species identification was conducted based on Suryani and Cahyanto (2022), involving the use of expert validation, Khalid Hafazallah, from Generasi Biologi Indonesia. Morphological identification was also carried out by observing such characteristics as cap color, shape and size of the cap and stipe, cap edge, and stipe height (Putra & Dwi 2022). We also recorded and measured the habitat conditions and such factors as temperature and humidity, light intensity, an soil pH using a thermo-hygrometer, a Lux meter, and a soil tester, respectively.

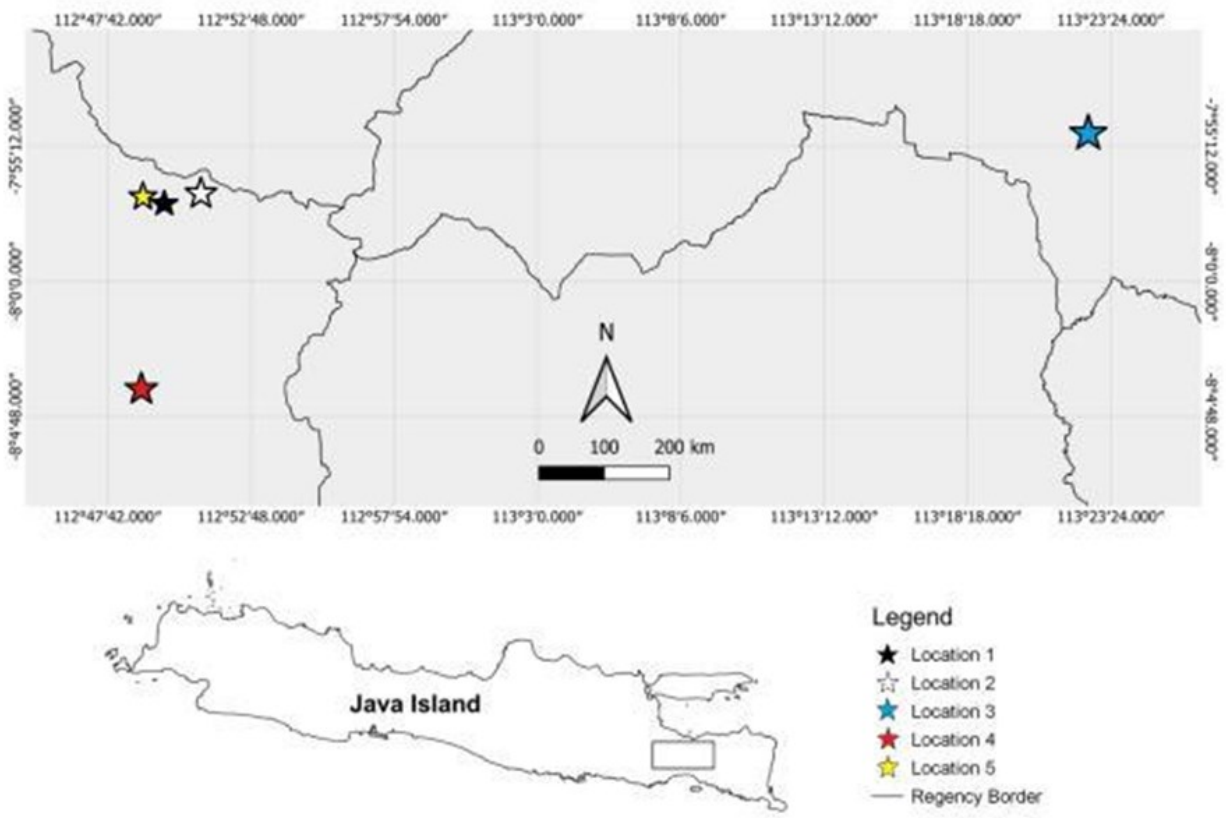

Figure 1. Location of the study area.
(the focus of study area is in the rectangle, Taman Nasional Bromo Tengger Semeru)

A total of 15 macrofungal species were discovered across five locations in Poncokusumo, TNBTS area, including 14 Basidiomycota species and a species of Ascomycota, as listed in Table 2. Notably, eight species were found inhabiting wood litter, while the rest were observed in leaf litter, soil, and bamboo (Table 3).

According to the data presented in Table 2, there were four species of macrofungi found in Sampling Location 1, namely *Lepista sordida* (Schumach.) Singer, *Lactocollybia* cf. *epia* (Berk. & Broome) Pegler, *Leucocoprinus fragilissimus* (Ravenel ex Berk. & M.A. Curtis) Pat., and *Dacryopinax* (Fr.) McNabb. Meanwhile, four species were found in Sampling Location 2, namely *Collybiopsis* aff. *ramealis* (Bull.) Millsp., *Trametes* cf. *villosa* (Sw.) Kreisel, *Pseudocolus fusiformis* (E. Fisch.) Lloyd, and *Morchella* cf. *galilaea* Masaphy & Clowez. Only two species were discovered in Sampling Location 3, namely *Ganoderma aplanatum* (Pers.) Pat. and *Auriculariales nigricans* (Sw.) Birkebak, Looney & Sánchez-García. Another two species were discovered in Sampling Location 4, namely *Trametes hirsute* (Wulfen) Lloyd and *Lentinus arcularius* (Batsch) Zmitr. Three species were found in Sampling Location 5, namely *Schizophyllum commune* Fr.:Fr, *Coprinopsis* cf. *kubickae* (Pilát & Svrček) Redhead, Vilgalys & Moncalvo, and *Coprinellus disseminatus* (Pers.) J.E. Lange.

The habitat of each macrofungal species is presented in Table 3. The macrofungi live in a habitat with temperatures ranging from 25 to 31 °C and neutral to acidic pH conditions (Table 4). The greatest diameter was found in *Ganoderma aplanatum* (Pers.) Pat., and the smallest was found in *Leucocoprinus fragilissimus* (Ravenel ex Berk. & M.A. Curtis) Pat., *Coprinopsis cf. kubickae* (Pilát & Svrček) Redhead, Vilgalys & Moncalvo, and *Dacryopinax aurantiaca* (Fr.) McNabb. The diameter of *Pseudocolus*

Table 1. Sampling location and habitat type of macrofungal in TNBTS area.

Location	Coordinate	Habitat Type	
Loc-1	-8°02'48.0"S 112° 49'33.8"E	Bamboo forest, far from settlements and close to cliffs	
Loc-2	-8°03'03.2"S 112° 49'08.5"E	Natural forest, dominated by shady big trees and far from settlements	
Loc-3	-8°05'15.10"S 112° 81'85.1"E	Natural forest, dominated by shady big trees and close to PDAM water channels	
Loc-4	-8°03'20.4"S 112° 48'43.9"E	Pine forest and close to the stream	
Loc-5	-8°03'03.2"S 112° 49'08.5"E	Plantation, dominated by *Hibiscus tiliaceus*, *Melia azedarach*, *Paraserianthes falcataria*, and *Samanea saman* trees, near settlements	

Table 2. List of macrofungi species found in the TNBTS area.

No.	Species	Genus	Family	Ordo	Division	Number found
1.	*Leucocoprinus fragilissimus* (Ravenel ex Berk. & M.A. Curtis) Pat.	*Leucocoprinus*	Agariceae	Agaricales	Basidiomycota	2
2.	*Schizophyllum commune* Fr.:Fr	*Schizophyllum*	Schizophyllaceae	Agaricales	Basidiomycota	52
3.	*Lactocollybia* cf. *epia* (Berk. & Broome) Pegler	*Lactocollybia*	Marasmiceae	Agaricales	Basidiomycota	3
4.	*Coprinopsis* cf. *kubickae* (Pilát & Svrček) Redhead, Vilgalys & Moncalvo	*Coprinopsis*	Psathyrellaceae	Agaricales	Basidiomycota	47
5.	*Coprinellus disseminatus* (Pers.) J.E. Lange	*Coprinellus*	Psathyrellaceae	Agaricales	Basidiomycota	19
6.	*Lepista sordida* (Schumach.) Singer	*Lepista*	Tricholomataceae	Agaricales	Basidiomycota	2
7.	*Collybiopsis* aff. *ramealis* (Bull.) Millsp.	*Collybiopsis*	Omphalotaceae	Agaricales	Basidiomycota	5
8.	*Pseudocolus fusiformis* (E. Fisch.) Lloyd	*Pseudocolus*	Phallaceae	Phallales	Basidiomycota	3
9.	*Dacryopinax aurantiaca* (Fr.) McNabb	*Dacryopinax*	Dacrimycetaceae	Dacrymycetales	Basidiomycota	1
10.	*Trametes hirsuta* (Wulfen) Lloyd	*Trametes*	Polyporaceae	Polyporales	Basidiomycota	4
11.	*Lentinus arcularius* (Batsch) Zmitr.	*Lentinus*	Polyporaceae	Polyporales	Basidiomycota	2
12.	*Trametes* cf. *villosa* (Sw.) Kreisel	*Trametes*	Polyporaceae	Polyporales	Basidiomycota	2
13.	*Ganoderma applanatum* (Pers.) Pat.	*Ganoderma*	Ganodermataceae	Polyporales	Basidiomycota	8
14.	*Auricularia nigricans* (Sw.) Birkebak, Looney & Sánchez-García	*Auricularia*	Auriculariaceae	Auriculariales	Basidiomycota	67
15.	*Morchella* cf. *galilaea* Masaphy & Clowez	*Morchella*	Morchellaceae	Pezizales	Ascomycota	1

Table 3. Species of macrofungal found in different characteristics of habitat in TNBTS area.

Name of species	Divison	Place of live characteristics	Locality				
			1	2	3	4	5
Collybiopsis aff. *ramealis* (Bull.) Millsp.	Basidiomycota	Leaf litter		+			
Pseudocolus fusiformis (E. Fisch.) Lloyd	Basidiomycota	Leaf litter		+			
Lepista sordida (Schumach.) Singer	Basidiomycota	Leaf litter	+				
Trametes hirsuta (Wulfen) Lloyd	Basidiomycota	Wood litter				+	
Ganoderma aplanatum (Pers.) Pat.	Basidiomycota	Wood litter			+		
Auricularia nigricans (Sw.) Birkebak, Looney & Sánchez-García	Basidiomycota	Wood litter			+		
Lentinus arcularius (Batsch) Zmitr.	Basidiomycota	Wood litter				+	
Trametes cf. *villosa* (Sw.) Kreisel	Basidiomycota	Wood litter		+			
Schizophyllum commune Fr.:Fr	Basidiomycota	Wood litter					+
Coprinopsis cf. *kubickae* (Pilát & Svrček) Redhead, Vilgalys & Moncalvo.	Basidiomycota	Wood litter					+
Lactocollybia cf. *epia* (Berk. & Broome) Pegler.	Basidiomycota	Wood litter	+				
Coprinellus disseminatus (Pers.) J.E. Lange	Basidiomycota	Soil					+
Morchella cf. *galilaea* Masaphy & Clowez.	Ascomycota	Soil		+			
Leucocoprinus fragilissimus (Ravenel ex Berk. & M.A. Curtis) Pat.	Basidiomycota	Soil	+				
Dacryopinax (Fr.) McNabb	Basidiomycota	Bamboo	+				

Note: Bamboo forest (1), natural forest and far from settlements (2), natural forest and close to PDAM water channels (3), pine forest (4), and plantation (5).

fusiformis (E. Fisch.) Lloyd was immeasurable because of the fungus' irregular shape. As for the color of the macrofungi, variations were observed between species (Table 4).

The macrofungal species found in Poncokusumo, TNBTS area, are described in detail as follows:

***Collybiopsis* aff. *ramealis* (Bull.) Millsp.** This species was observed in leaf litter under specific environmental conditions (Figure 2A). These conditions include a temperature of 26 °C, acidic pH, low light intensity, and a humidity level between 50 and 65%. This species has a white stipe that remains the same size from its base to tip, growing up to 3 cm in height. Its umbrella-shaped cap starts white with a brownish center and widens as it matures. The surface of the cap is smooth and has split edges, revealing its tight gills and rings. The diameter of the cap spans roughly 2.5–3 cm. *Collybiopsis* aff. *ramealis* (Bull.) Millsp. is a notable species that exhibits a unique characteristic of thriving independently on wooden branches with firmly anchored roots. This distinctive behavior sets it apart from other species (Amin et al. 2019).

***Pseudocolus fusiformis* (E. Fisch.) Lloyd**. This species was observed in leaf litter at a temperature of 26 °C, with a preference for neutral soil pH, low light intensity, and humidity levels ranging from 50 to 65%. This species can also be found in soil substrates, grasslands, and bushes (Pasaylyuk et al. 2018). The fruiting body of this species is both loose and stiff, featuring a soft tip that ultimately ruptures to become the thallus. The thallus resembles an octopus tentacle, featuring a rough surface and greyish-brown mucus that emits a pungent odor; therefore, this mushroom is often called devil's finger (Pasaylyuk et al. 2018). With three arms and an orange hue, the thallus can grow up to 8 cm in height, while the mycelium is white, smooth, and unassuming (Figure 2B).

Table 4. Species name, abiotic factors, and morphological characteristics of macrofungal.

No.	Species	Temperature (°C)	pH	Diameter (cm)	Colour
1.	*Leucocoprinus fragilissimus* (Ravenel ex Berk. & M.A. Curtis) Pat.	26	Acidic	2	White
2.	*Schizophyllum commune* Fr.:Fr	31	Neutral	2.5	Brownish-grey
3.	*Lactocollybia* cf. *epia* (Berk. & Broome) Pegler	30	Neutral	3	White
4.	*Coprinopsis* cf. *kubickae* (Pilát & Svrček) Redhead, Vilgalys & Moncalvo	27	Neutral	2	White-brown
5.	*Coprinellus disseminatus* (Pers.) J.E. Lange	27	Acidic	3	White
6.	*Lepista sordida* (Schumach.) Singer	25	Neutral	6	Purple
7.	*Collybiopsis* aff. *ramealis* (Bull.) Millsp.	26	Acidic	2.5	White with brownish center
8.	*Pseudocolus fusiformis* (E. Fisch.) Lloyd	26	Neutral	Immeasurable	Orange
9.	*Dacryopinax aurantiaca* (Fr.) McNabb	26	Neutral	2	Orange
10.	*Trametes hirsuta* (Wulfen) Lloyd	26	Acidic	3	Cream to brown
11.	*Lentinus arcularius* (Batsch) Zmitr.	27	Acidic	4	Dark brown and light brown
12.	*Trametes* cf. *villosa* (Sw.) Kreisel	30	Neutral	3	Brown center
13.	*Ganoderma aplanatum* (Pers.) Pat.	30	Neutral	34	Brown
14.	*Auricularia nigricans* (Sw.) Birkebak, Looney & Sánchez-García	26	Neutral	7	Reddish-brown
15.	*Morchella* cf. *galilaea* Masaphy & Clowez	25	Acidic	3	Brown

Figure 2. Macrofungi species (Basidiomycota) observed in leaf litter. **A.** *Marasmius collybiopsis* aff. *ramealis* (Bull.) Millsp., **B.** *Pseudocolus fusiformis* (E. Fisch.) Lloyd, **C, D.** *Lepista sordida* (Schumach.) Singer.

***Lepista sordida* (Schumach.) Singer**. This species is a type of macrofungi that boasts a striking purple hue. It is typically found in leaf litter. Its stipe is both stiff and loosely striped (fibrillose), its cap is wide and smooth, and its lamellae are branched to the edges (stipe margins) (Figure 2C). The cap edge is notched (umbonate) (Figure 2D). This species was observed in environments with a temperature of 25 °C, a humidity level of 85%, and a neutral pH value of 6. The stipe diameter is 6–10 cm. This species possesses a mycelium. This species is distributed in Indonesia (especially Java Island), Sri Lanka, Thailand, America, and Switzerland. It is often found in compost soil, grass, and gardens (Retnowati 2019).

***Trametes hirsuta* (Wulfen) Lloyd**. This species was observed in weathered wood with a habitat temperature of 26 °C, acidic soil pH of 5, moderate light intensity, and 70% humidity. This species forms semi-fan-shaped fruiting bodies with irregular brackets, which lack a cup (Figure 3A). As the fruiting body ages, its color changes from cream to brown, and it has a rough surface with long hair (villose). The fruiting body is directly attached to the substrate with a pseudo-root type, and its back is porous.

***Trametes* cf. *villosa* (Sw.) Kreisel**. This particular species is a *Trametes* species found in a habitat with a temperature of 30 °C, neutral pH of 6, moderate light intensity, and 75% humidity. The fruiting body of this macrofungus is semi-fan-shaped with irregular brackets, and it lacks a pileus. It has a brown center and cream-colored edges, with a firm texture and a rough surface (Figure 3B). The fruiting body has dimensions of 3 cm in height and 5 cm in diameter, with a slightly grooved edge that is directly attached to the substrate and porous in the back (Figure 3C). The *Trametes* species can be found living in groups, overlapping each other in soil habitats, in weathered wood colonies, or even growing solitarily (Norfajrina et al. 2021).

***Ganoderma applanatum* (Pers.) Pat**. This macrofungal species was observed in wood at a habitat temperature of approximately 30 °C. This species exhibits a fruiting body with a distinctive fan-shaped appearance, boasting a rough and grooved surface with a hard texture (Figure 3D). With a diameter of approximately 34 cm and a height of 19.4 cm, the body is brown in coloration, devoid of any visible cap or gills, and possessing a slightly curved edge. Notably, the lower portion of the body is white and features a fine porous texture (Figure 3E).

***Auricularia nigricans* (Sw.) Birkebak, Looney & Sánchez-García**. This species typically thrives in woodland environments characterized by moderate intensity of light and air humidity at approximately 80%. The fruiting body of this mushroom possesses a soft, rubbery, or chewy texture and features a lobe-like shape with a smooth surface and dense, fuzzy hair. Typically, this reddish-brown macrofungus lacks a stipe and is directly attached to the substrate (Figure 3F). It measures between 5 and 12 cm in diameter and between 3 and 7 cm in height. This species also thrives in colonies distinguished by a pseudo-rhizoid root type. In addition, this species is well known as the ear mushroom due to its fruiting body's resemblance to a human ear. It is included as an edible mushroom. It can also serve as a heartburn remedy and a means of reducing pain associated with internal injuries (Norfajrina et al. 2021).

***Lentinus arcularius* (Batsch) Zmitr**. This species was found in woodlands with a habitat temperature of 27 °C, acidic pH, moderate light intensity, and 82% humidity. It possesses a rigid, brown stipe that is hollow and rough in texture, standing 3.5 cm tall (Figure 3G). The stipe is centrally located and narrows at both the base and apex. The cap is dark

brown along the edges and light brown in the center, with a wide, flat-stipe shape, a densely hairy (pubescent) surface, and a diameter of 4 cm. The lamellae are crossed (anastomosed), attached to the stipe's mycelium, devoid of rings, and possess crossed hymenophores (Figure 3H). The Polyporales in this study, i.e., *Trametes hirsuta*, *Lentinus arcularius*, *Trametes* cf. *villosa*, and *Ganoderma aplanatum*, are known to have similar characteristics as saprophytes on weathered wood and as parasites on living wood (Rahma 2018).

***Schizophyllum commune* Fr.:Fr**. This species was found in wood habitats with a temperature of 31 °C, neutral pH of 6.2, and low light intensity. Its fruiting body is fan-shaped, elastic, covered in tightly packed velutinous hairs, and brownish-grey in surface appearance (Figure 3I). With a diameter of 2.5 cm and a height of 2 cm, this macrofungus lacks a stalk and has a flat, rimose edge. Additionally, it does not possess any gills or lamellae, but it has a flat regular bar. This species grows independently on wooden substrates, and its propagation occurs through the lumen of various vessels, xylem strings, fibers, and tracks (Fuziyanti et al. 2022). It is known by various local names in Indonesian regions, including Java, Sulawesi, Tidore, and Halmahera (Fitri et al. 2022). *S. commune* is an edible mushroom that can be eaten as a traditional food and a medicinal mushroom as an anti-inflammatory (Dewi et al. 2022). It can be cultivated on sawdust of jackfruit wood, *ketapang* (*Terminalia catappa*) wood, and *rambutan* (*Nephelium lappaceum*) wood (Mahardhika et al. 2022). According to a previous study, *S. commune* grows in 92 species of wood as its substrates (Yusran et al. 2023), including *H. tiliaceus*, *M. azedarach*, *P. falcataria*, and *S. saman* that were found in the fifth location of this study.

***Coprinopsis* cf. *kubickae* (Pilát & Svrček) Redhead, Vilgalys & Moncalvo**. This white-brown species is commonly found as colonies in wooded areas with a temperature of 27 °C, neutral soil pH of 6.3, high light intensity, and 77% humidity. This macrofungus has a stipe texture that is not hard, has a smooth surface, and can reach a height of up to 5 cm. The cap is minute, convex, and half-round, with undulating edges and a diameter of approximately 2 cm (Figure 3J, Figure 3K). Unlike some others, this species does not have any gills but instead has a mycelium. In addition, some *Coprinopsis* species are known to have the potential as food ingredients (Vantamuri & Kaliwal 2017).

***Lactocollybia* cf. *epia* (Berk. & Broome) Pegler**. This species was observed in a habitat characterized by a temperature of 30 °C, neutral soil pH (6.6), and moderate light intensity, such as weathered wood (Figure 3L). It displays a stiff, loosely attached stipe that maintains an equal shape from base to tip, situated in the center of the growth. The stipe surface is smooth, reaching 4 cm in height. It has a white convex-shaped cap 3 cm in diameter with a smooth surface texture but notched or umbonate at the edge (Figure 3M). Moreover, this species possesses a mycelium with no ring, and the gynophore is packed with regular lamellae. This species of mushroom boasts a unique aroma and is highly coveted for its nutritional benefits (Norfajrina et al. 2021). It is a popular choice for community cultivation due to its rich concentration of amino acids, vitamins, and minerals (Susan & Retnowati 2018).

***Coprinellus disseminatus* (Pers.) J.E. Lange**. This species can be found in soil with a habitat temperature of 27 °C, acidic pH of 5.6, moderate light intensity, and 62% humidity. It is characterized by a cylindrical stipe shape with a smooth white surface and a 6 cm height. Its cap shows an umbrella with a small parabolic shape (Figure 4A). This species boasts a soft texture and regular blade (translucent striate), with a stipe diame-

Figure 3. Macrofungi species (Basidiomycota) observed in wood litter. **A.** *Trametes hirsuta (Wulfen) Lloyd*, **B,C.** *Trametes* cf. *villosa* (Sw.) Kreisel, **D,E.** *Ganoderma aplanatum* (Pers.) Pat., **F.** *Auricuralia nigricans* (Sw.) Birkebak, Looney & Sánchez-García, **G,H.** *Lentinus arcularius* (Batsch) Zmitr., **I.** *Schizophyllum commune* Fr. **J,K.** *Coprinopsis* cf. *kubickae* (Pilát & Svrček) Redhead, Vilgalys & Moncalvo., **L,M.** *Lactocollybia* cf. *epia* (Berk. & Broome) Pegler.

ter of 3 cm. Moreover, its lamellae exhibit luminophore properties. This species can live in a variety of substrates such as soil, dead tree trunks, leaf litter, and dirt. However, it is only safe to consume this species during its early stages of growth as mature ones contain dangerous toxins (Mahardika et al. 2021).

***Morchella* cf. *galilaea* Masaphy & Clowez**. It was found in soil with a temperature of 23 °C, high light intensity, 75% humidity, and pH of 5. The stipe is soft, round, hollow, and smooth, with a height of 10 cm and brown color. The cap is brown, wavy, and devoid of a ring (Figure 4B). *Morchella*, both wildly grown and cultivated, is edible and utilized as a food ingredient (Putra 2021).

***Leucocoprinus fragilissimus* (Ravenel ex Berk. & M.A. Curtis) Pat**. This species was found in soil with acidic pH, low light intensity, and a temperature of 26 °C. It has a smooth, bone-white stipe that is the same size from base to tip, reaching a height of 5 cm. The wide umbrella-shaped cap is white with a yellow centre, 2 cm in diameter, and smooth in the surface with finely serrated edges. The blade sticks to the base (Figure 4C). In addition, it has a mycelium, rings, and a gynophore in the form of dense lamellae. This species is typically solitary or found in scattered groups. It is not edible and known for medical use (Priskila et al. 2018; Putra et al. 2022).

***Dacryopinax aurantiaca* (Fr.) McNabb**. This species is commonly found in weathered bamboo with habitat temperatures ranging from 20 to 26 °C, neutral pH, low light intensity, and high humidity. Its fruit body takes the form of a thallus with a rubbery, jelly-like texture and an irregular shape. The surface is smooth, with no rings, hymanophores, or lamellae, and the thallus's color is orange. The fungus attaches directly to the substrate and can reach a height of 3–4 cm. *Dacryopinax* belongs to the Dacrymycetales family and is commonly found on dead wood and bamboo that has been exposed to sunlight. Due to its jelly-like texture, it is often referred to as the jelly mushroom (Norfajrina et al. 2021).

Figure 4. Macrofungi species observed in soil and bamboo. **A.** *Coprinellus disseminatus* (Pers.) J.E. Lange. (Basidiomycota), **B.** *Morchella* cf. *galilaea* Masaphy & Clowez. (Ascomycota), **C.** *Leucocoprinus fragilissimus* (Ravenel ex Berk. & M.A. Curtis) Pat. (Basidiomycota), **D.** *Dacryopinax aurantiaca* (Fr.) McNabb. (Basidiomycota)

In conclusion, Poncokusumo District of TNBS houses four different types of habitat characteristics, namely bamboo forest, natural forest, pine forest, and plantation area. A total of 15 macrofungal species were discovered across five locations in there, including 14 Basidiomycota species and a species of the Ascomycota division. Eight species were found inhabiting wood litter, while the rest were observed in leaf litter, soil, and bamboo. Microscopic and biochemical tests on the macrofungal samples obtained are needed for further study.

AUTHOR CONTRIBUTION

All authors contributed equally to writing the manuscript. A.S.S.A con-

ducted the research, including data collection, while H.M., R.F.D., and H.S supervised the study. K.H and R.F.W.P validated the species data.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Short Communications

New Record of A Freshwater Prawn *Macrobrachium sundaicum* in Selat Panjang Island, Riau Province, Indonesia

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ABSTRACT
A freshwater prawn *M. sundaicum*, is an obligate species to acidic peat swamp. Up to the present, *M. sundaicum* has only been reported in the West Kalimantan, the Riau Archipelago, and Jambi Provinces in Indonesia. The aim of this research is to determine the distribution and habitat preferences of peat swamp prawn in Selat Panjang Island, Riau Province, Indonesia. The samples were collected in seven peat swamp rivers by hand net. The study yielded one hundred specimens. The acidic peat swamp is a perfect habitat for *M. sundaicum*. This study provided the basic information about peat swamp prawn in Selat Panjang Island, especially their distribution and habitat preferences.

The Indonesian freshwater shrimp and prawns are consisted of Atyidae and Palaemonidae families of the Decapoda order (Wowor et al. 2004; De Grave et al. 2015). The freshwater shrimps and prawns have unique life style, such as landlocked and amphidromous (Wowor & Choy 2001; Closs et al. 2003; Joy & death 2004). This style affects the distribution of the species (Wowor et al. 2009; Thuesen et al. 2011). Freshwater shrimps and prawns play an important role in their environment, and they are as decomposers maintain ecosystem balance (Wowor et al. 2004) and they act as bio-indicators of the quality of the aquatic environment (Wowor et al. 2009; Taufik 2011).

The freshwater prawn genus *Macrobrachium* comprises 261 valid species (WoRMS 2024). This genus is widely distributed and inhabited various types of habitat from swamps, reservoirs, lakes, lowland up to mountains rivers (Wowor et al. 2004) and can adapt to extreme environment such as water with a low pH (Ng 1992; Wowor 1999; Wowor & Choy 2001; Wowor et al. 2009). Low pH water in peat swamp causes limitation to the organisms that can survive. One of the species

that can survive in this specific environment is *Macrobrachium sundaicum*. The distribution of *M. sundaicum* species has been reported in West Kalimantan (Kapuas Hulu Regency; Pontianak on Sekadau; Rivers Kepayang at Anjungan; Rivers Mungan (Sarawak); Rivers Bejit (between Balai Ringin and Simunjan), Sumatra (Riau Archipelago (Natuna and Kundur island); Riau Province (Bengkalis); Jambi (Arang-Arang Lake), Peninsular Malaysia (Trengganu; Pahang; Johor State), Singapore (Neon Soon stream near Seletar reservoir), and southern Thailand (Wowor & Ng 2010). The presence of the peat swamp rivers in Riau Province including in Selat Panjang Island, raise the questions of the presence of *M. sundaicum*. So, the aims of this research were to determine the distribution and the habitat preferences of *M. sundaicum*.

The research was conducted in seven peat swamp rivers in Selat Panjang Island, Riau Province, Indonesia (Table 1) (Figure 1).

Table 1. Research locations.

River	Village	District	Coordinate
Sai Kundur	Kundur	Tebing Tinggi Barat	0° 57' 10.7994"S; 102° 33' 32.3994"E
Sai Karet	Kundur	Tebing Tinggi Barat	0° 57' 3.5994"S; 102° 33' 3.6"E
Pagar	Kundur	Tebing Tinggi Barat	0° 57' 0"S; 102° 33' 3.6"E
Kayu	Kundur	Tebing Tinggi Barat	0° 57' 3.5994"S; 102° 33' 7.2"E
Sawit	Kundur	Tebing Tinggi Barat	0° 56' 31.1994"S; 102° 33' 50.3994"E
Pinang	Kundur	Tebing Tinggi Barat	0° 57' 14.3994"S; 102° 33' 43.1994"
Pakis	Kundur	Tebing Tinggi Barat	0° 57' 50.3994"S; 102° 33' 57.6"E

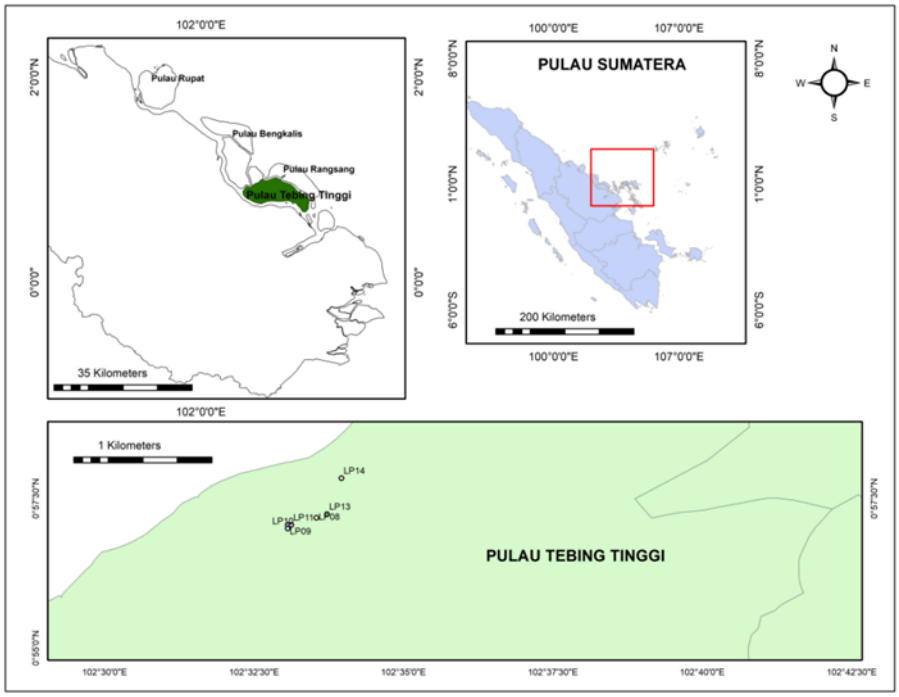

Figure 1. Sampling map sites in Selat Panjang Island, Riau Province.

The sampling was conducted by purposive sampling in October 2022. Sampling started at 05.00-11.00 pm using a hand net. The water temperature, water current, pH, canopy, substrate, the presence of

aquatic plant, and surrounding environment are the abiotic parameters measured and observed. The samples were preserved in 96% alcohol. The specimens are deposited in Museum Zoologicum Bogoriense (MZB), Research Centre for Biosystematics and Evolution, National Research and Innovation Agency (BRIN) Indonesia. The Global Position System (GPS) with GLONASS system of smartphone. Samples of the freshwater prawns were identified at the Research Centre for Biosystematics and Evolution, BRIN Cibinong in Bogor. Identification key by Wowor et al. (2004) based on morphological characteristics was used to determine the identity of the species.

Only one species was identified from the sampled locations, i.e, *M. sundaicum*. A total of 100 individuals consisting of 55 males, 34 females, and 11 ovigerous females were obtained (Table 2).

The life coloration of *M. sundaicum* the overall body is reddish-brown, with black stripes along the body. The color of the species is generally influenced by the color of the substrate. The color of the species resembles the color of the water where they coloration inhabit.

Prawn coloration morphologically, the obtained specimens are very similar to the original description of *M. sundaicum* described by Wowor & Ng (2010). The specimens from Selat Panjang Island have short rostrum, with tip not extending beyond distal end of scaphocerite but extending beyond distal end of third segment of antennular peduncle or tip slightly extending beyond distal end of scaphocerite in young specimens. The rostrum was armed dorsally with at least with 9-12 teeth (mode 11), 4 teeth completely postorbital (3 or 4 in other specimens, mode 4). Ventral carina with 4–6 teeth (mode 5). Second pereiopods dissimilar in shape, unequal in size, robust and fingers covered by soft dense pubescence especially in adult specimens. Second pereiopods with carpus shorter than chela and merus subcylindrical (Figure 2). This finding shows the presence of *M. sundaicum* in Selat Panjang Island, Riau Province is new record for this species.

Figure 2. *Macrobrachium sundaicum* in Selat Panjang Island, Riau Province.

Table 2. Samples gained from the sampling locations in Selat Panjang Island, Riau Province, Indonesia.

River	Species	Male	Female	Ovigerous female	Number of individuals
Sai Kundur	*M. sundaicum*	13	5	2	20
Sai Karet	*M. sundaicum*	11	13	1	25
Pagar	*M. sundaicum*	4	6	0	10
Kayu	*M. sundaicum*	5	6	1	12
Sawit	*M. sundaicum*	2	0	2	4
Pinang	*M. sundaicum*	13	1	4	18
Pakis	*M. sundaicum*	7	3	1	11

Habitat characteristic data was taken in the form of vegetation and canopy conditions. Conditions of abiotic factor include temperature, water current, and pH (Table 3). The highest pH at Sai Kundur River (4.6) and the lowest at Pinang River (3.9). The highest water temperature at Pakis and Pagar Rivers (27°C) and low water temperature in Sai Karet River (25°C). The water velocity was fast in Pagar and Kayu Rivers and slow in Sai Karet, Pinang, and Pakis Rivers. The highest canopy coverage is in Sai Kundur, Sawit, and Pakis River.

Based on this study, *M. sundaicum* is found in peat swamp rivers in Selat Panjang Island. The water comes from undisturbed forest and no anthropogenic activities. The characteristic of the habitat of the seven sampling sites varied which affecting the number of specimens obtained. The high density of *M. sundaicum* was found in Sai Kundur, Sai Karet, and Pinang rivers. The high density of prawn is related to the substrate on which they live. The substrate with leaf litter does not only provide as food source but also serves as a hiding place for the prawns. The high abundance of the shrimp is related to the large amount of accumulated vegetation debris on the bottom of the stream which in turn provides food sources for the shrimp (Bentes et al. 2011). The middle density was found in Pagar and Kayu Rivers, characterized by mud substrate and ferm roots. The hard ferm roots do not provide adequate hiding place and the root is also hard to be clanged by *Macrobrachium* spp. Annawaty et al. (2016) noted that the hard and sparse nature of water plant root is not suitable for *Caridina* spp. to cling around, making it less prefer habitat for the prawn. Besides that, low density was observed in Sawit River with mud substrate only. The mud substrate does not provide hiding place for the prawns so this kind of substrate is not prefer by the prawns. *Macrobrachium sundaicum* is found more often in habitats with aquatic plants. Based on Wowor et al. (2004) the presence of aquatic plants in water bodies can support the survival rate of the prawns. The dominant aquatic plants at the study site are grass. Besides that, *M. sundaicum* can be found in river with acidic water, riparian forests, hanging roots and leaf litter (Cai 2016).

Table 3. Samples gained from the sampling locations in Selat Panjang Island, Riau Province, Indonesia.

River	pH	Water temperature (°C)	Water current	Canopy	Environment around sampling site	Presence of aquatic plant	Substrate
Sai Kundur	4.6	26	Middle	70%	Rubber, Traditional village Pterydophyta, Grass	+	Mud, leaf litter, ferm roots, dead wood
Sai Karet	4.5	25	Slow	10%	Oil palm, Rubber, traditional village	+	Mud, leaf litter, dead wood
Kayu	4.4	26	Fast	30%	Pterydophyta, Traditional village	-	Mud, trailing roots
Sawit	4.0	26	Middle	70%	Oil palm, Pterydophyta	-	Mud
Pinang	3.9	26	Slow	50%	Oil palm, Rubber	+	Mud, leaf litter
Pakis	4.1	27	Slow	70%	Oil palm	+	Mud, leaf litter

*Information:
(+): present of aquatic plant
(-): not present of aquatic plant

The water color in all sampled rivers has dark tea-colored to almost black on reflected light with a pH between 3.9 to 4.7. The water temperature varied between 25 to 27°C. These unusual water conditions fit very well with the biological needs of *M. sundaicum*. According to Wowor & Ng (2010), the *M. sundaicum* species is most abundant in riparian rivers with cool and acidic water with a pH range of 4.5–6.0. However, it can also live at high water temperatures which varies between 24.5–35.5°C with low oxygen (Johnson 1967; Ng 1993).

AUTHOR CONTRIBUTION

All authors have contributed to the writing. LP was in charge of sampling, analysing the data and writing the manuscript. DY-F, AF. DW and A designed the study, supervised laboratory work, and wrote the manuscript.

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CONFLICT OF INTEREST

In this research there is no conflict of interest.

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Short Communications

Herpetofauna and Their Potential Threats in Karimata Island, Indonesia

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ABSTRACT
Karimata Island is an island about 100 km west of Borneo, causes geographical isolation and generally always shows an impact on the diversity of animal communities that are less, one of the communities affected is herpetofauna. Herpetofauna is very important in an ecosystem so it is necessary to conduct a survey. The survey was conducted from April 1 to April 7, 2023 in Betok Jaya Village, Karimata Island which was divided in 3 observation areas based on habitat type using the Visual Encounter Survey method. Herpetofauna found consisted in 22 species divided into 5 species of amphibians and 17 species of reptiles with a total of 43 individuals. Herpetofauna located adjacent to human areas is vulnerable to various disturbances such as maritime transportation activities, household waste pollution and land clearing, which can be a threat to the herpetofauna community of Karimata Island.

Karimata Island as an island that is separated about 100 km west of the mainland of Borneo. The separation of an island according to Barquero et al. (2010) and Losos & Ricklefs (2009) results in geographic isolation and generally always shows an impact on the diversity of animal communities that are less, one of the communities affected is herpetofauna. According to Christoffel & Lepczyk (2012) herpetofauna plays an important role in an ecosystem in the food chain that can control the population and as a bio-indicator of environmental change.

The existence of herpetofauna is important to know through surveys conducted both short and long term. Few surveys have been conducted in Karimata Island, the last of which found 26 species of herpetofauna in Padang Village (Arifin et al. 2011). The survey was limited to the eastern Karimata Island. Thus, it is necessary to survey herpetofauna on the western side of the island, which is in Betok Jaya Village, west of Karimata Island, to add information about the herpetofauna diversity and identify the threats to herpetofauna of Karimata Island.

The herpetofauna survey was conducted for 7 days from April 1 to April 7, 2023 on the western side of Karimata Island in Betok Jaya Village, North Kayong, West Kalimantan, Indonesia. Karimata Island has an area of up to 156.25 km^2 with a mountainous landscape of Karimata Island consisting of various habitat types with the highest peak being Mount Cabang (1,030 m asl) (KLHK 2016). Herpetofauna observations

were divided into 3 locations separated by habitat type (Figure 1). Location 1 is along the coast with coral reef habitat in Betok Jaya Hamlet (1° 35′8.06″S; 108°47′50. 08″E), location 2 is a mangrove forest area in Kelumpang Hamlet (1°38′40.91″S; 108°49′49.34″E), and location 3 is a secondary forest area in Kelumpang Hamlet (1°38′52.10″S; 108°50′5.75″E).

Herpetofauna found were documented using a camera and identified directly in the field with herpetofauna identification books (Das 2010; Inger et al. 2017; Das et al. 2022). Herpetofauna identified are then recorded and released back into their habitat and descriptive analysis is carried out based on the location of the observation.

The method used for herpetofauna surveys is the Visual Encounter Survey method for rapid assessment with limited time available (Ackley et al. 2009; Zakaria et al. 2022). Surveys were conducted in the morning and evening with a maximum observation time limit of 3 hours from 6–9 a.m and 6–9 p.m. Identification was carried out by matching of morphological characteristics and all herpetofauna encountered were subsequently released back into their natural habitat. During the observation, tools such as stationery, Garmin 64s GPS, grab stick, head lamp, and snake hook were used.

Herpetofauna found during observations at the three locations belonged to 22 species with a total of 43 individuals (Table 1), including 5 species of amphibians and 17 species of reptiles. A survey conducted by Arifin et al. (2011) found 26 species that were higher than the current survey results. Many factors can affect the species richness of an area, one of the most influential in this survey according to Kusrini (2019) is the observation time and methods used. The relatively faster observation

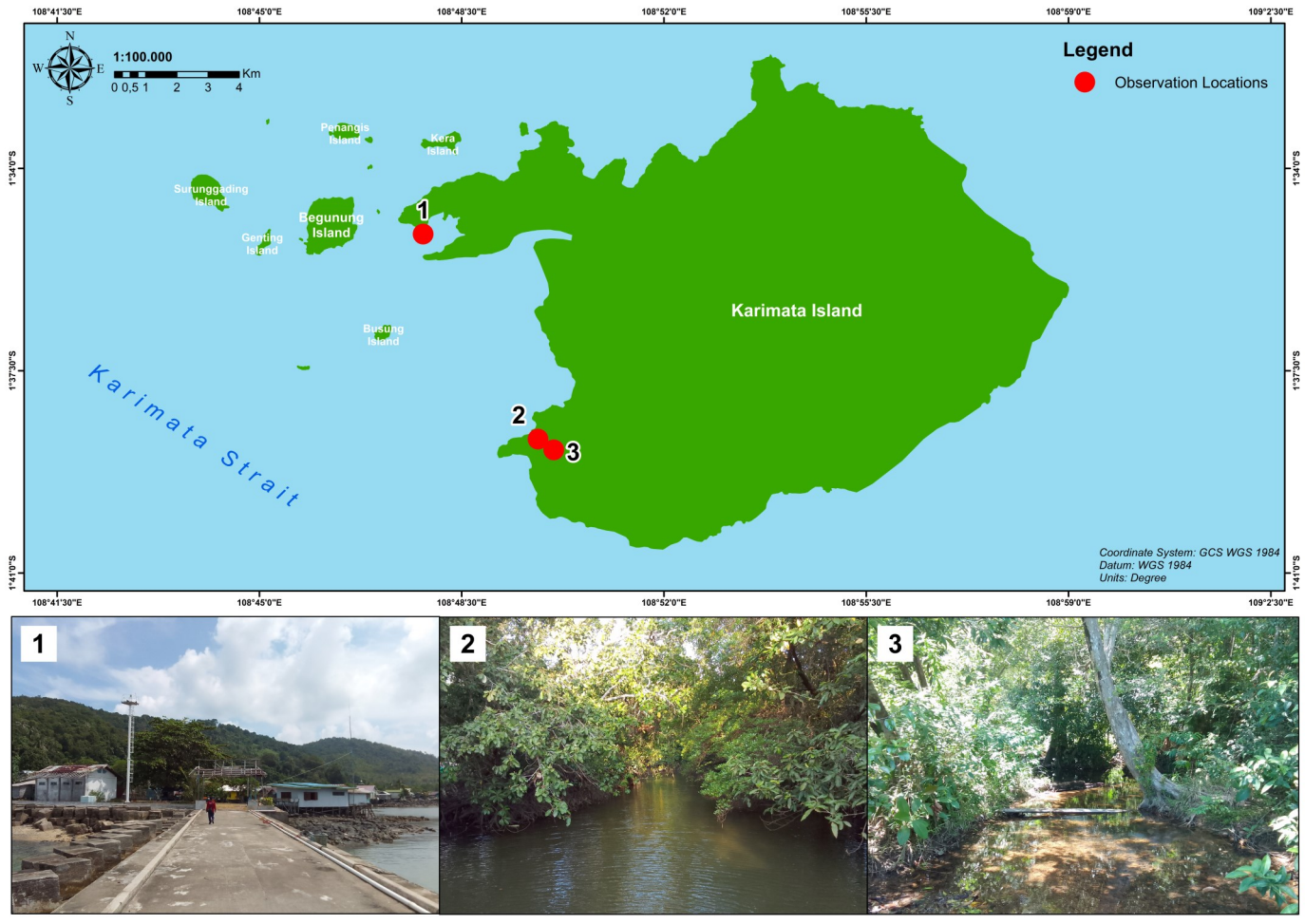

Figure 1. Herpetofauna survey map along with the environmental setting of the observation locations in Karimata Island.

Table 1. Herpetofauna found during observation in Karimata Island.

No	Family	Species	Location			n
			1	2	3	
Amphibians						
1	Dicroglossidae	*Fejervarya cancrivora*		√		5
2		*Limnonectes paramacrodon*			√	2
3		*Limnonectes* sp.			√	1
4	Ranidae	*Chalcorana* cf. *raniceps*			√	3
5		*Pulchrana baramica*			√	1
Reptiles						
1	Agamidae	*Draco* sp.		√		2
2	Colubridae	*Boiga drapiezzii*			√	1
3		*Dendrelaphis haasi*			√	1
4		*Xenochrophis trianguligerus*			√	1
5	Crocodylidae	*Crocodylus porosus*		√		2
6	Elapidae	*Laticauda colubrina*	√			1
7	Gekkonidae	*Cyrtodactylus* sp.			√	1
8		*Gehyra mutilata*		√		1
9		*Gekko gecko*		√		5
10		*Hemidactylus frenatus*		√		2
11		*H. platyurus*		√		2
12	Homalopsidae	*Cerberus schneiderii*		√		2
13		*Homalopsis buccata*		√		1
14	Scincidae	*Eutropis multifasciata*	√	√	√	6
15	Typhlopidae	*Indotyphlops braminus*		√		1
16	Varanidae	*Varanus salvator*		√		1
17	Viperidae	*Tropidolaemus subannulatus*		√		1
Total						43

Notes: n: number of individuals

time and only using one observation method had a big impact on the species richness found.

A total of 13 herpetofauna species that had never been found in previous surveys are new records for the herpetofauna diversity of Karimata Island (Table 2). Some herpetofauna were only found in one of the observation locations such as *L. colubrina* in location 1. *L. colubrina* was found in the morning actively moving around the coral reef. This is in line with the statement from Heatwole (1999) and Lane et al. (2010) which explained that these snakes are often found in coral reefs, coral islands, high seas, and coastal areas.

All observation locations adjacent to areas of human activity according to Urbina-Cardona et al. (2006) are very vulnerable to various disturbances that have an impact on reducing the quality of herpetofauna habitat (Figure 2). Location 1, which is adjacent to Betok Jaya Harbor, is often visited by ferries and various fishing boats. The activities of anchored ships can unwittingly damage coral reef ecosystems as found by Flynn (2015) and Nama et al. (2023) caused by anchor dropping and ship waste pollution. This can be seen from the condition of the coral reefs around location 1 which has been marked by coral bleaching. Thus, there is a need for proper management of ship activities and planned coral reef restoration actions should be implemented.

Location 2, which is the estuary of the Kelumpang River, is often used as a garbage dump by villagers. Trash can be a threat to herpetofauna, for example varanid lizard can be stuck in discarded drinks cans (Zdunek & Kolenda 2022) and microhabitat pollution by garbage

Table 2. Checklist of herpetofauna species that have been found on Karimata Island.

Family	Species	Observation result	
		Arifin et al. 2011	This survey
Amphibians			
Dicroglossidae	*Fejervarya cancrivora*	√	√
	Limnonectes ingeri	√	
	Limnonectes malesianus	√	
	Limnonectes paramacrodon	√	√
	Limnonectes sp. +		√
Megophryidae	*Leptolalax* cf. *gracilis*	√	
Ranidae	*Hylarana* (*Chalcorana*) cf. *raniceps*	√	√
	Pulchrana baramica +		√
	Staurois guttatus	√	
Rhacophoridae	*Philautus* sp.	√	
Reptiles			
Agamidae	*Draco* sp.	√	√
	Gonocephalus liogaster	√	
Colubridae	*Ahaetulla prasina*	√	
	Boiga drapiezzii +		√
	Dendrelaphis haasi +		√
	Gonyosoma oxycephalum	√	
	Oligodon purpurascens	√	
	Xenochrophis trianguligerus +		√
Crocodylidae	*Crocodylus porosus* +		√
Elapidae	*Laticauda colubrina* +		√
Gekkonidae	*Cnemaspis kendallii*	√	
	Cyrtodactylus sp.	√	√
	Gehyra mutilata +		√
	Gekko monarchus	√	
	Gekko gecko	√	√
	Hemidactylus frenatus	√	√
	H. platyurus +		√
Geoemydidae	*Batagur affinis*	√	
	Orlitia borneensis	√	
Homalopsidae	*Cerberus rynchops*	√	
	Cerberus schneiderii +		√
	Homalopsis buccata +		√
Scincidae	*Emoia atrocostata*	√	
	Eutropis multifasciata	√	√
	Lygosoma (*Subdoluseps*) *bowringii*	√	
	Tropidophorus beccarii	√	
Typhlopidae	*Indotyphlops braminus* +		√
Varanidae	*Varanus salvator* +		√
Viperidae	*Tropidolaemus subannulatus*	√	√

Notes: +: new record

dumping site (Lubis et al. 2008; Botejue & Wattavidanage 2012). If no waste management is implemented on Karimata Island, not only the local herpetofauna is affected, but also large-scale habitat destruction can occur. Therefore, there is need for active socialization about waste management and even providing integrated waste bins to minimize the damage caused.

The convertion into areca nut (*Areca catechu*) plantations around location 3 can have an impact on the herpetofauna community, especially due the loss of habitat for herpetofauna in terms of diversity and complexity of natural forest. The loss of natural forest can significantly

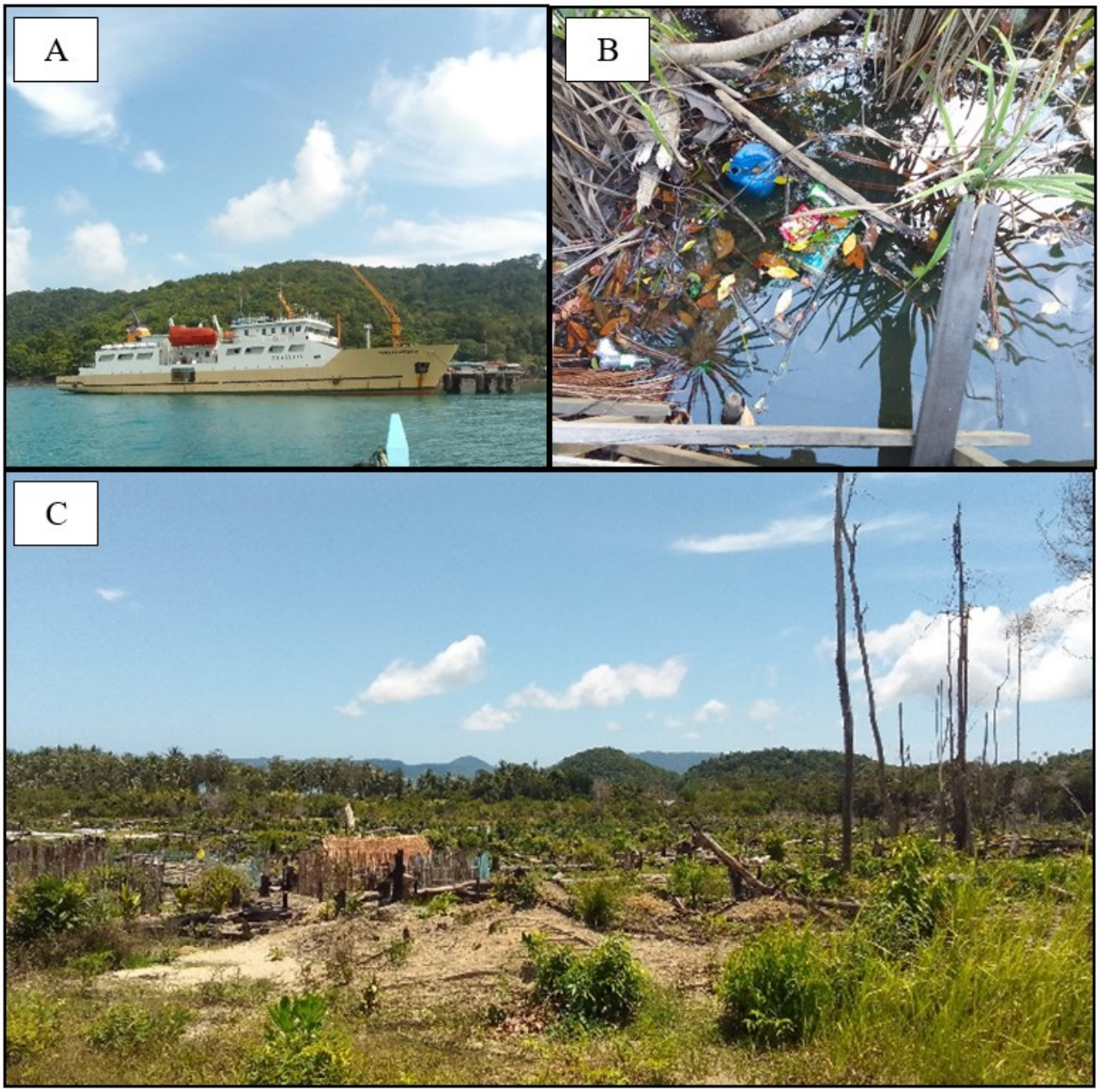

Figure 2. Potential threats encountered during the survey for herpetofauna on Karimata Island (A) Location 1; (B) Location 2; (C) Location 3.

affect the heterogeneity of habitat (Lehtinen & Ramanamanjato 2006; Ghosh & Basu 2020). Findings by Kwatrina et al. (2019) and Wanger et al. (2010) showed that low herpetofauna diversity due to forest conversion. Thus, regular evaluation and monitoring is needed so that it does not spread to other areas.

In conclusion, herpetofauna found during the survey were 22 species divided into 5 species of amphibians and 17 species of reptiles. The population of herpetofauna found from Karimata Island is potentially threatened due to maritime transportation activities, waste pollution, and land clearing for plantations.

AUTHOR CONTRIBUTION

FWP, OF and ML collected and analyzed the data, FWP validated, analysed the data, and wrote 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

Ecological Study of *Bidens pilosa* in Bandung, West Java, Indonesia

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ABSTRACT
Bidens pilosa has been widely distributed from tropical to temperate regions and is often reported as a weed in agriculture. It readily thrives in various environments, naturally spreading to open areas and artificial ecosystems, establishing new populations, emphasising the need for ecological studies to prevent its invasive potential. In this study, we focused on population study of *B. pilosa* and its distribution in Bandung as urban area. Survey was conducted using 1x1 m quadrate plots in eight locations (24 plots). Individual number of *B. pilosa*, the number of flowers in each individual, coordinates, and altitude of each plot were recorded to describe population structure and map this population in Bandung. Air temperature (°C), humidity (%), light intensity (Lux), and soil water content (%) were measured. Individual number and environmental condition are analysed using cluster analysis and PCA, then mapped using IDW (Inverse Distance Weighting). The highest population in AR (652 ind), followed by CG (626 ind), TR (253 ind), PA (135 ind), CW (78 ind), NR (39 ind), PU (28 ind), and PR (20 ind). On average, each *B. pilosa* individual produces 61 inflorescences, indicating a mature population with all developmental stages present across all locations. Ordination plots shows that *B. pilosa* has wide range of environmental condition from open to shade area with various environmental condition. Open areas, settlements, and agriculture host dense *B. pilosa* populations, and its biological traits suggest it may become invasive without proper control.

INTRODUCTION
Alien plants, also known as non-native or exotic species, originate from different areas and can thrive in new environments. When these foreign plants reach high populations and spread extensively, they become invasive, leading to ecological disturbances and contamination of ecosystems. Consequently, invasive species pose a significant threat to biodiversity and native species worldwide (Sunaryo & Tihurua 2012; Tjitrosedirdjo 2012).

One such species is *Bidens pilosa*, a cosmopolitan herbaceous plant native to Central and Tropical America. It has spread globally, especially in tropical and subtropical climates, often as a contaminant in agricultural seeds (US Forest Service 2018; Handayani et al. 2021). Concerns have arisen about *B. pilosa* becoming an invasive alien species that is difficult to control. It is listed among the 2000 exotic plant species in Indonesia

and identified as invasive, also significantly affecting more than 30 crops in over 40 countries (Setyawati et al. 2015; WIKTROP 2023). The spread of *B. pilosa* in Indonesia has been facilitated by its presence as a weed from Africa and its prevalence in agricultural and plantation areas (Siagian et al. 2017; Paiman 2020). Additionally, *B. pilosa* has invaded several conservation areas in Indonesia, including Bromo Tengger-Semeru National Park, Lake Kalimpa'a Lore Lindu National Park, and National Baluran Park, where it has become the dominant foreign plant species (Megawati et al. 2017; Padmanaba et al. 2017; Abidin et al. 2019).

Urban environments have also been invaded by *B. pilosa*, particularly in Bandung, the largest and the most populous metropolitan city in West Java. Research by Rahmawati (2022) has revealed that *B. pilosa* is the most common invasive foreign herbaceous plant in Bandung. The proliferation of foreign plants in urban areas demands attention due to potential direct impacts on ecology, economy, and public health (Gaertner et al. 2017; Potgieter et al. 2022). *B. pilosa*'s presence in urban ecosystems poses a threat to local biodiversity, as it produces allelochemical compounds that inhibit the growth of other plants (Xuan & Khanh 2016). Moreover, the distribution of *B. pilosa* in urban areas near conservation sites can disrupt the structure and function of these areas, leading to biotic homogenisation (Gaertner et al. 2017). The plant also serves as a host and vector for plant parasites and viruses, reducing the productivity of various food crops (Galon et al. 2015; GISD 2023). Furthermore, *B. pilosa* can be a source of allergens or phototoxic toxins, causing allergic reactions in humans (Gaertner et al. 2017; PFAF 2022).

The availability of resources resulting from human activities and minimal competition and disturbance contribute to the proliferation of *B. pilosa* (Paiman 2020). The presence of roads, railroad crossings, canals, and waterways in urban areas acts as corridors that facilitate the rapid spread of *B. pilosa* propagules (Säumel & Kowarik 2010; Meek et al. 2010; Gaertner et al. 2017). Addressing the invasion of *B. pilosa* is essential to prevent future challenges. However, a comprehensive understanding of the interactions between biotic and abiotic factors determining the level of invasiveness is still lacking. Therefore, gathering ecological data, such as environmental information, distribution patterns, and the specific invasive potential of *B. pilosa* in Bandung, is crucial. Such information can help formulate effective strategies to mitigate the impact of this invasive alien species on both urban and conservation areas. By taking prompt and informed action, it is possible to protect native biodiversity and preserve the delicate balance of ecosystems in the face of invasive threats like *B. pilosa*.

MATERIALS AND METHODS

Materials

GPS was employed to record the coordinates and elevation of observation sites. Microclimate parameters, including air temperature (°C), air humidity (%), and light intensity (Lux) were measured using a sling psychrometer and a lux meter. Each observation plot underwent five repetitions, yielding 120 data points. Soil moisture content (%) was determined through core sampling, involving the collection of wet soil samples and subsequent drying at 105°C for 24 hours. Soil moisture content was calculated by subtracting the weight of the dry soil sample from the weight of the wet soil sample (after drying), dividing by the weight of the wet soil sample, and then multiplying by 100%.

Methods

Study area and period

This study was conducted between July 2022 until October 2022 in several locations in the administrative area of Bandung City, West Java Province, Indonesia. The research site was determined according to Rahmawati (2022), which identified sampling coordinates where significant colonies of *B. pilosa* were present (Figure 2). The geographic coordinates of the study area are located in S6°49' - 7°18' and E107°14' - 107°56'. Each observation site shows the results of measurements of different microclimatic and environmental settings. The study areas encompass altitudes ranging from 300 to 970 meters above sea level (asl). Air temperature values are between 21.5 - 27°C, relative humidity is between 45 - 90%, light intensity is between 200 - 1100 Lux (Table 1). Each observation site possesses a distinct environmental setting, encompassing agricultural landscapes, residential areas, open spaces, rice fields, transportation routes (including roads and railways), and riverbanks (Figure 3).

Data collection

Random sampling encompassed eight observation sites, each with three 1 x 1 meter square plots, totalling 24 plots. These plots were strategically positioned in areas with *B. pilosa* populations, and their coordinates and elevation were recorded using handheld GPS. Population structure and inflorescent count data were collected during three phases: seedling, reproductive, and non-reproductive stages. The count included main plants, excluding rhizomes that develop into new individuals, with each clump considered as one individual. Each individual is characterized by clear morphological features as shown in Figure 1, with reproductive individuals identified by those that have flowered or flowers that have developed into achenes.

For flower count, three random samples of flowering adult individuals were selected from each plot. *B. pilosa* inflorescences contain two types of florets: white ray flowers and yellowish disc flowers, with the count focused on ray flowers, representing one flower. Microclimatic parameters are measured to analyse the relationship between environmental factors and the population of *B. pilosa*. These measurements also sup-

Table 1. Habitat characteristics of *Bidens pilosa* at eight study sites in Bandung, West Java, Indonesia.

Study sites	Environmental Setting	Temperature (°C)	Relative Humidity (%)	Light Intensity (Lux)	Altitude (m asl)	Coordinates	
						X	Y
Cigadung (CG)	Contour residental area	23.61 – 25.83	48 – 65	870 – 1085	780	107.62237500	-6.87258056
Panyileukan Rail Road (PR)	Dry open area without canopy	23.33 – 24.17	72 – 82	270 – 380	625	107.70618889	-6.94688056
Tol Road Edge (TR)	Windy open area	24.44 – 26.11	50 – 80	236 – 407	600	107.70455833	-6.96681667
GBLA-near River (NR)	Humid riverbanks	24.72 – 26.39	65 – 88	289 – 610	615	107.70455833	-6.96681667
Panyileukan (PA)	Rice fields and settlement	24.72 – 25.83	59 – 72	632 – 845	651	107.71350000	-6.93407778
Cipadung Wetan (CW)	Flat residential areas	25.28 – 26.94	60 – 80	535 – 760	679	107.71288056	-6.93217778
Arcamanik (AR)	Open spaces	22.22 – 23.61	75 – 86	202 – 228	700	107.6764222	-6.91053056
Padasaluyu Utara (PU)	Agricultural landscapes	21.67 – 22.50	73 – 82	149 – 463	966	107.5933528	-6.84853889

port the creation of prediction maps for the distribution of *B. pilosa*.

Figure 1. Morphology of *B. pilosa*: flowers (1), *achene* (2), leaf (3), dan stem (4).

Data analysis

Population structure

Population structure, which pertains to the distribution of individuals by age and sex, plays a pivotal role in determining population size changes over time. While sex differentiation can be challenging in certain organisms like plants, age structure becomes a critical determinant of population growth. Younger individuals are more likely to contribute to population growth as they reach reproductive maturity, while older individuals face higher mortality rates, impacting overall population dynamics. Odum's (1993) population structure analysis involves examining the population size across various life stages, represented in diagram form. These diagrams can take different shapes:

A. An expansive pyramid shape signifies a growing population, dominated by the young.
B. A bell-shaped polygon denotes a stable population where no age group dominates.
C. A jug or urn shape suggests a declining population, with a prevalence of older or unproductive individuals.

These models visually depict the distribution of individuals across age or life stages, providing valuable insights into *B. pilosa*'s population dynamics.

Relationship between population and ecological factors

Principal Component Analysis (PCA) is employed to elucidate complex relationships between observed variables, specifically between *B. pilosa* populations seize and microclimatic parameters. This analysis seeks to identify essential data patterns while reducing complexity by transforming the data into a new coordinate system. The PCA analysis was conducted using IBM SPSS Statistics 26.

Cluster analysis groups observed objects (*B. pilosa* populations at each observation site) into smaller clusters based on the similarity of observed variables, resulting in a dendrogram representation. These variables include number of populations, temperature, relative humidity, light intensity, and altitude. The average linkage method is used for clustering, which groups based on the average distance between observations. The criterion used compares the average distance within a cluster to that of other clusters. This method follows the research by Paramadina et al. (2019) and was conducted using IBM SPSS Statistics 26.

Distribution map

A visual representation or distribution map was generated to give an overview of how the *Bidens pilosa* population is spread throughout Bandung city. This was achieved by employing the Inverse Distance

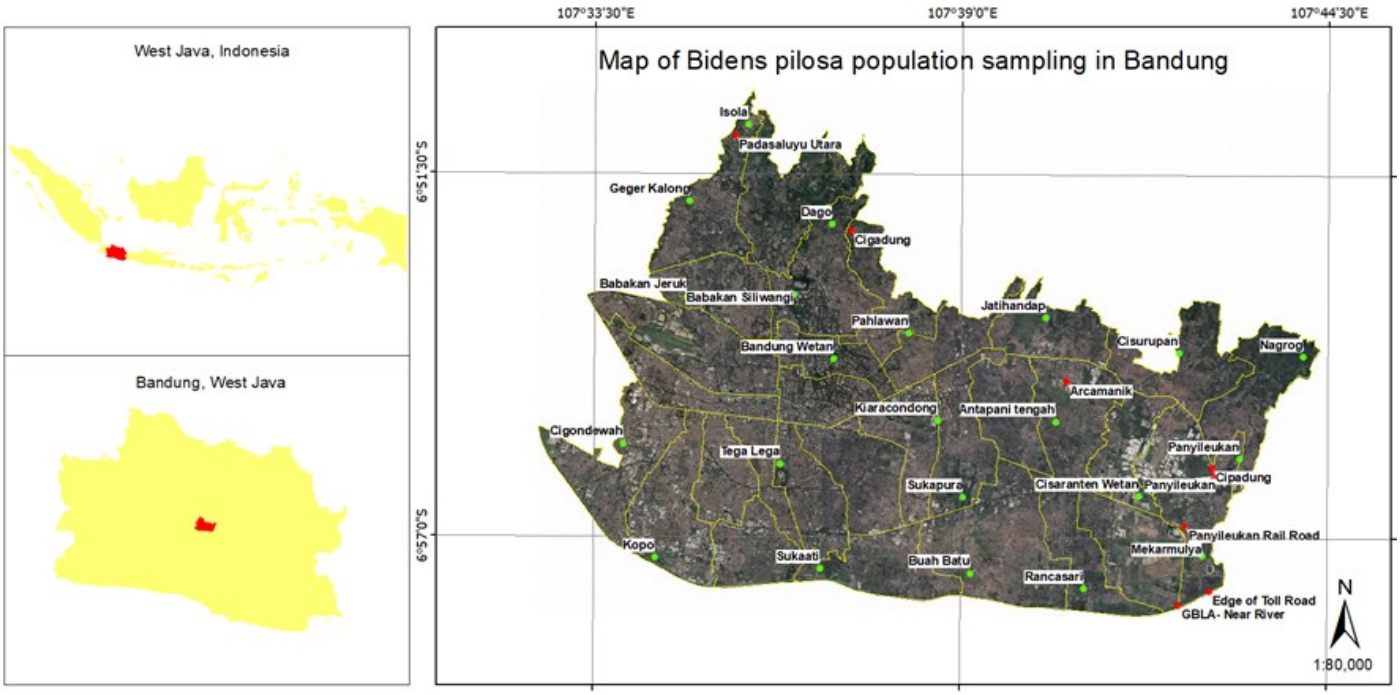

Figure 2. Map of study site in Bandung urban area, West Java, Indonesia; dot features represent site for sampling point collection. The sampling points conducted by Rahmawati (2022) are depicted with green dots, while the observation points in this study are represented by red dots.

Weighting (IDW) technique to create the distribution map for *Bidens pilosa*. The Inverse Distance Weighting (IDW) method assumes that each input point has a locally diminishing influence with distance. It is influenced by the inverse distance obtained from a mathematical equation, allowing us to adjust the relative influence of sample points. IDW interpolation is suitable for map creation when sample selection requires data on quantity, distribution, or density (Yudanegara et al. 2021).

The choice of the IDW method stems from the assumption that *B. pilosa* can be found across Bandung city due to its adaptability. Locations with significant *B. pilosa* concentrations are taken as indicative of broader areas where the plant could be present. Absence of *B. pilosa* at certain observation points is treated as a value of 0. The distribution map for *B. pilosa* in Bandung city was formulated by combining the total populations from each of the 8 observation sites, along with the population data for *B. pilosa* gathered by Rahmawati (2022) at these points. The IDW method involves estimating values at unsampled locations based on neighbouring data points, a process known as spatial interpolation. This distribution mapping was carried out using the ESRI ArcMap 10.8. software using IDW as an interpolation method. In the context of Geospatial Information Systems (GIS), interpolation is used to predict values in areas lacking direct measurements, aiding in map creation and value distribution projection across the observed region (Sejati 2019).

RESULTS AND DISCUSSION

B. pilosa has invaded Bandung city, with an average density of 76.2 individuals/m2 across all life stages (seedling, non-reproductive, and reproductive). *B. pilosa* was found in all observation sites with different environmental baselines and microclimatic conditions at each observation site (Table 1). Additionally, individual plants were capable of producing 20 to 120 inflorescences, resulting in an estimated annual seed yield of 2287 to 4575 per plant.

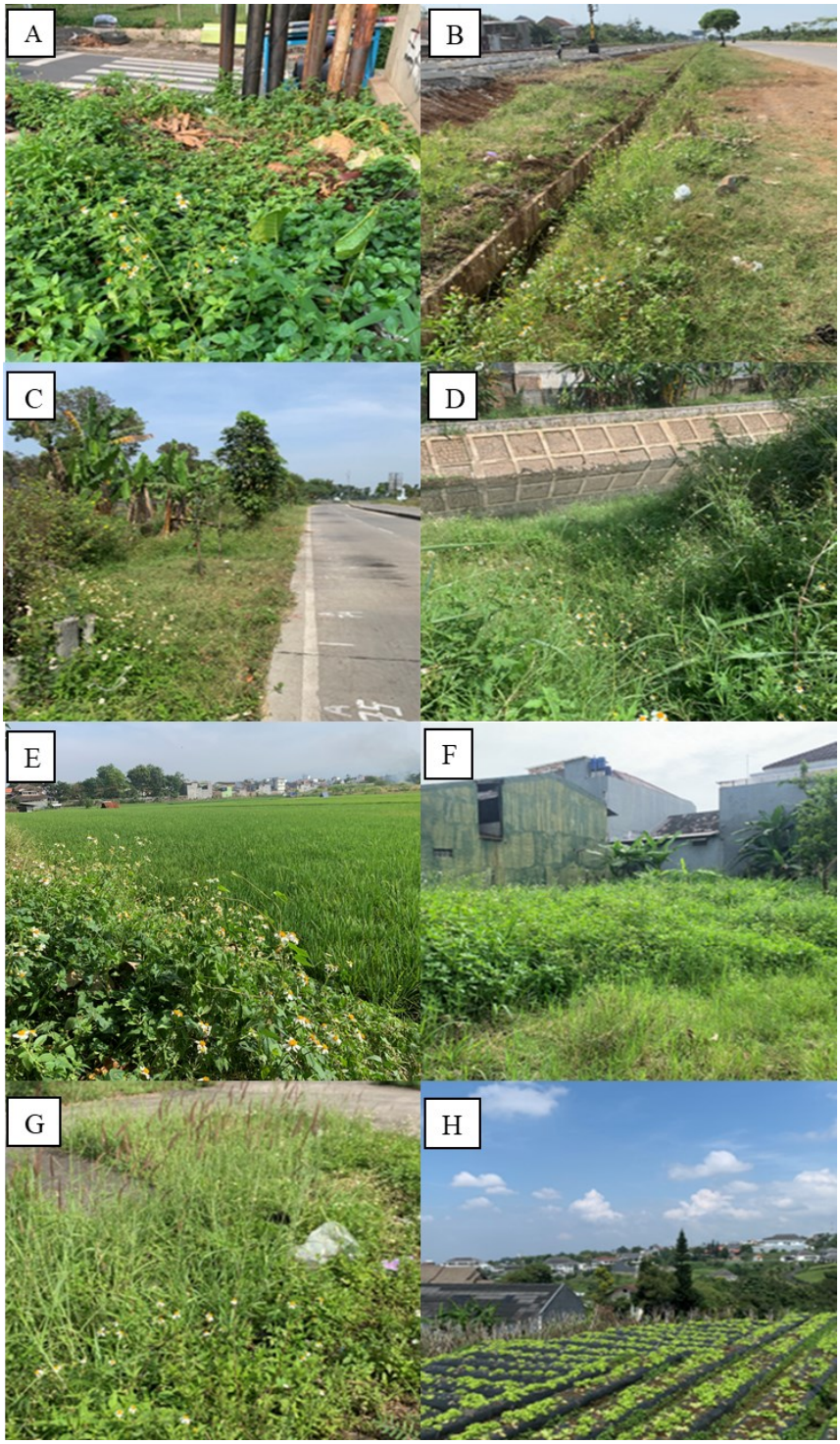

Figure 3. Various environmental settings can be observed at the eight research locations, listed from left to right: Cigadung/CG (A), Panyileukan Rail Road/PR (B), Edge of Toll Road/TR (C), GBLA-Near River/NR (D), Panyileukan/PA (E), Cipadung Wetan/CW (F), Arcamanik/AR (G), and Padasaluyu Utara/PU (H).

Population structures of *B. pilosa*

Based on our survey, the AR area exhibited the highest estimated number of *B. pilosa* individuals, with a total of 218 individuals per m^2, followed closely by the CG area with 209 individuals per m2. The areas with the highest number of individuals primarily consisted of individuals in the seedling stage. On the other hand, the PR area had the least estimated number of *B. pilosa* individuals, with only 7 individuals per m^2, and the PU area had 9 individuals per m^2 (Figure 3). In the PR area, the absence of seedlings was attributed to significant disturbance in the area,

potentially due to control measures implemented near the railroad tracks to avoid disruption. The population in the PU area was also limited, likely due to competition with cultivated plants in dry land agricultural areas, which were actively managed by farmers.

The number of individuals recorded within a 24 m² observation area was 1.831, resulting in an average of 76.2 individuals per m2. Among the eight observation points, the dominant class is seedlings, with a total of 1.211 individuals and an average of 151 seedlings per m². On the other hand, the non-reproductive phase has the lowest proportion, averaging 27 individuals per m² (Figure 4). Based on the description of Figure 5, the visualization of the population distribution is expected to resemble an expansive pyramid shape. This suggests that there is a higher abundance of individuals in the seedling stage compared to other stages, gradually decreasing as they progress into reproductive phases.

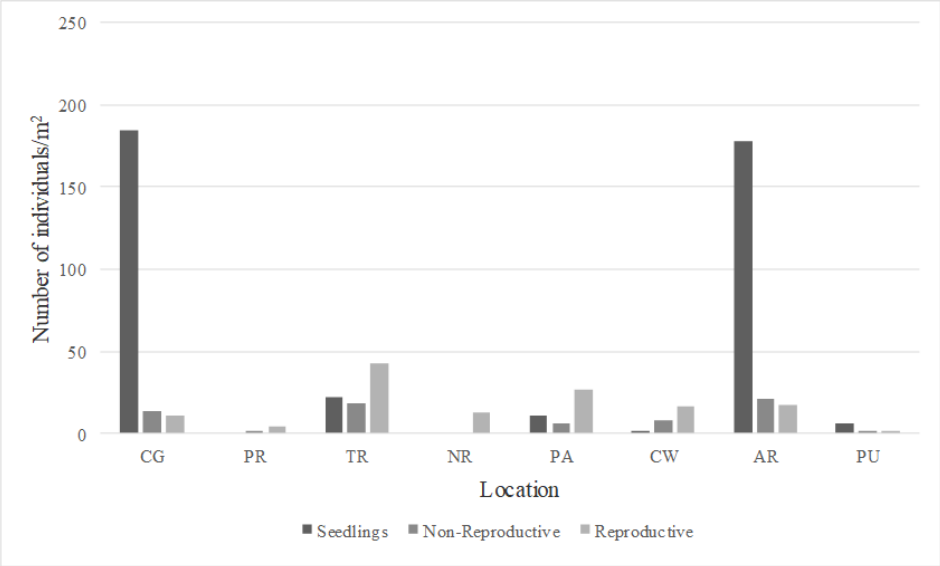

Figure 4. Population density of *B. pilosa* in research area.

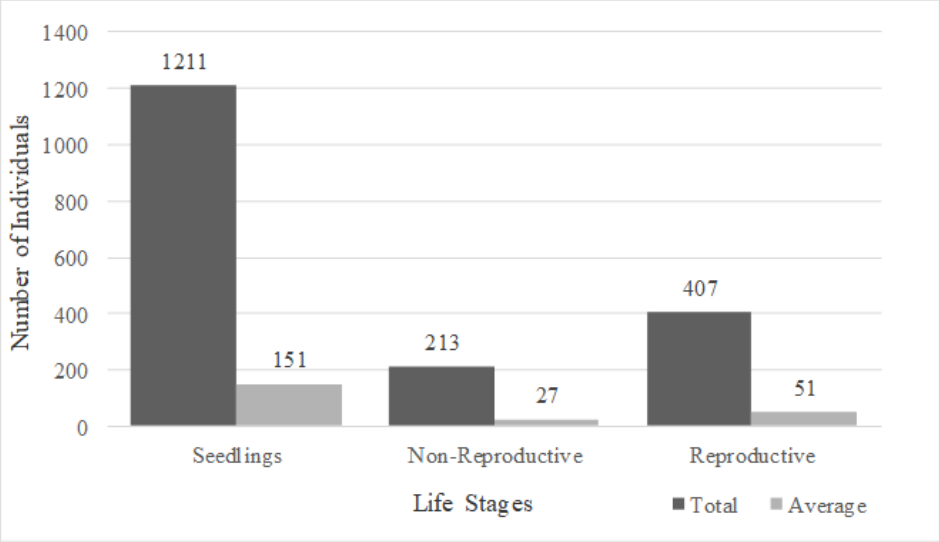

Figure 5. The proportion of the number of individuals between population classes.

Plant strategy

Referring to Table 2, the mean inflorescent yield per observed individual was 61. According to estimations derived from literature, a total of 4575 seeds per year can be produced from 61 flowers (assuming 4 generations

annually). Success germination of *B. pilosa* can reach up to 74% (US Forest Service 2018; GISD 2023). Indicating that it is possible for each mature individual to generate 424 to 847 new individuals per generation. Massive seed production and high survival rate of this species show a benefit of population establishment.

Table 2. Reproductive characteristic of *B. pilosa*.

	Inflorescent/ flowers (per ind)	Seed production (per year)	Recruitments
Field Survey	61	2287 – 4575	424 – 846,56
(Waterhouse 1994; US Forest Service 2018; GISD 2023)	80	3000 – 6000	555 – 1110

Proximity between populations and their correlation with climatic factors

Based on Figure 6, the results indicated the presence of two major clusters within the *B. pilosa* population in Bandung city. The first cluster encompasses the P_5/PA Population, P_6/CW Population, P_3/TR Population, P_4/NR Population, and P_1/CG Population. The second cluster consists of the P_2/PR Population, P_7/AR Population, and P_8/PU Population (Figure 6). Among these clusters, the populations at P_5/PA and P_6/CW sites exhibited the highest degree of proximity, primarily due to their close geographical distance. The first cluster is believed to have formed due to variations in air humidity, while the second cluster appears to be influenced by the proximity of air temperature and light intensity factors, as indicated by the PCA ordination displayed in Figure 7.

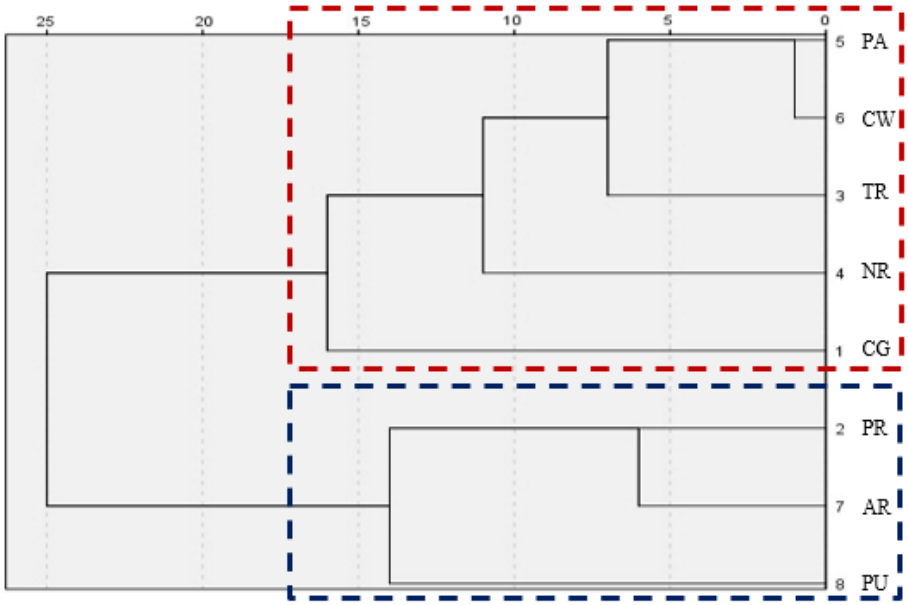

Figure 6. Clustering results for *B. pilosa* populations at eight observation sites; the red dashed rectangle is the first cluster, and the blue dashed rectangle is the second cluster.

Despite being included in the first cluster, the population at the P_1/CG site is located farthest away in the PCA ordination, suggesting a more distinct clustering pattern. This distinction is likely attributable to the population's growth in an environment characterized by the lowest humidity among all observed locations. On the other hand, the P_8/PU

site represents the most remote point in the ordination, indicating that the population at this site thrives in the most distinct environment compared to all other observation points. Notably, site P_8 has the highest altitude and lowest temperature (Table 1).

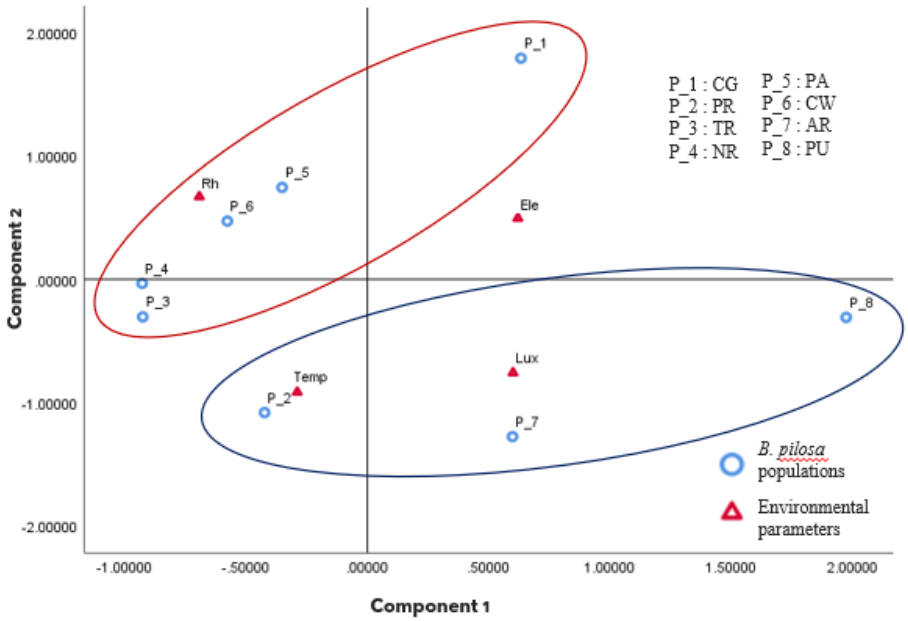

Figure 7. Result of PCA between *B. pilosa* populations and climatic factors; red circle is the first cluster, allegedly formed due to humidity factor; blue circle, The second cluster was allegedly formed due to the closeness of the air temperature and light intensity factors.

Distribution of *B. pilosa* by population density in the Bandung Area

B. pilosa thrives in diverse environments, forming large clusters, particularly in open areas with substantial human activity. The highest population estimates were observed in areas like rice fields, open residential spaces, and near dry land agriculture, highlighting its widespread presence in such environments (Figure 8).

Discussion

The locations with the highest number of *B. pilosa* individuals were predominantly in the seedling stage, indicating the presence of developing colonies. Such colonies, dominated by seedlings or tillers, tend to exhibit continued growth (Ansari et al. 2015). The height of individual seedlings reflects successful seed germination. The areas of Cigadung/CG and Arcamanik/AR had the highest seedling populations, suggesting widespread dispersal of *B. pilosa* seeds in these areas and favourable environmental conditions for germination, particularly due to relatively shaded light conditions compared to other locations. This finding is supported by Chauhan et al. (2019), who emphasized the influence of environmental factors, including light, on germination.

The Railroad/PR and GBLA-Near River/NR areas exhibited low populations of seedlings, likely due to relatively high temperatures and humidity compared to other locations, which hampered the germination process. Temperature and humidity are significant factors that impact seed germination. Certain herbaceous weed seeds have a broad temperature range and can easily germinate under various conditions, on the other hand, some seeds necessitate a specific temperature range to initiate germination. Additionally, weed seeds exhibit varying humidity requirements depending on their specific type (Chauhan et al. 2019). In the Padasaluyu/PU area, the small population of *B. pilosa* is suspected to result

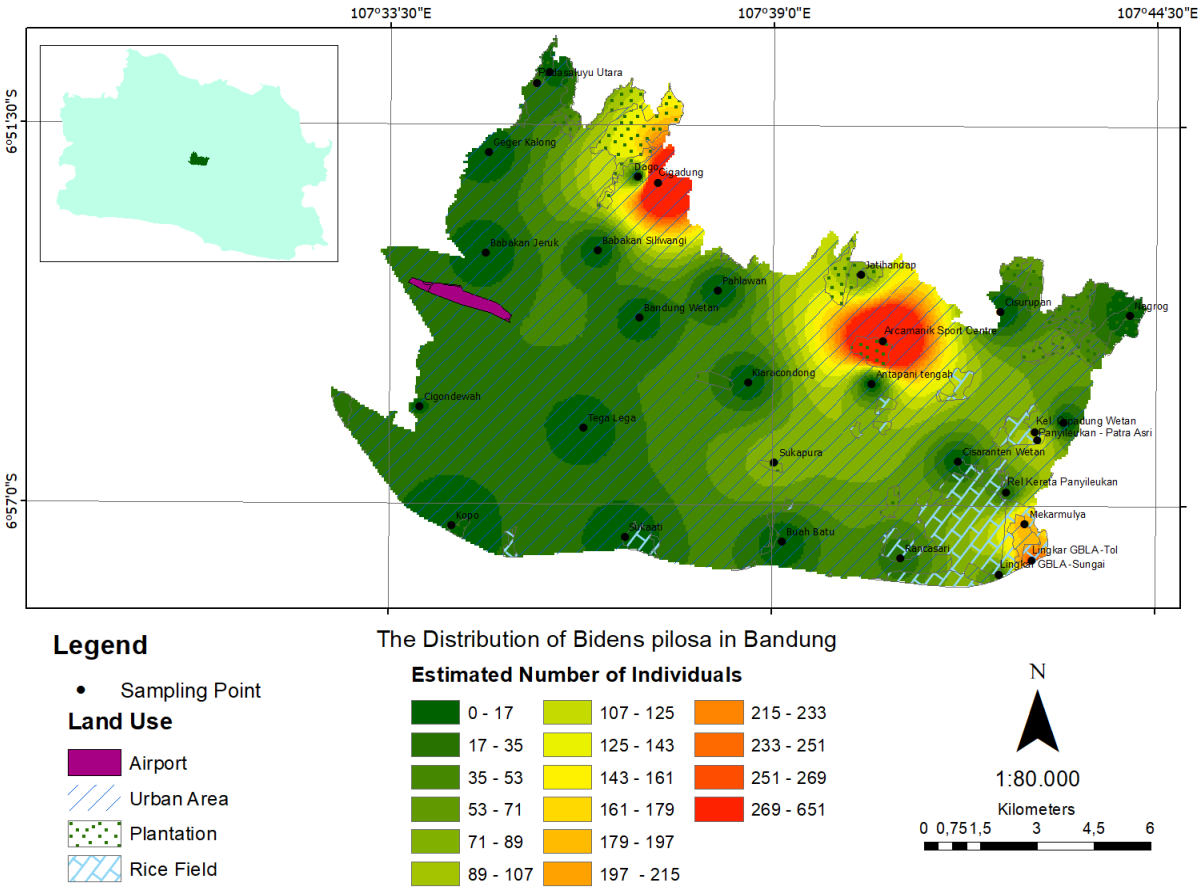

Figure 8. The overlay of the distribution of *Bidens pilosa* on the land use map, provides insights into the plant's preferences for specific habitats within the city of Bandung.

from seedlings being unable to survive due to competition with cultivated plants. Sebastian (2022) explained that biotic interactions, both between different species and within the same species, play a significant role in population growth, particularly at the seedling stage when plants have not yet fully adapted to their environment, making them relatively unstable.

The growth phase of a population is critical, with the seedling phase dominating in the case of *B. pilosa* due to its adaptability (LiYi et al. 2012). Population structure is influenced by factors such as birth rate, mortality, habitat, and human activity (Subahar 1998; Susanto & Halang 2019). *B. pilosa* exhibits a remarkable capacity to generate a substantial number of new individuals, estimated at approximately 555 to 1110 per generation per plant (Waterhouse 1994; US Forest Service 2018; GISD 2023). Environmental factors significantly impact its growth, particularly during the seedling stage (Sebastian 2022).

Flowers play a vital role in the survival of plants as they are essential for the reproductive process. The success of reproduction, including flowering, fruit maturity, and seed production, depends greatly on the developmental stages during the generative phase. Any failure at these stages can lead to reduced seed productivity and hinder the successful production of new offspring (Owens et al. 1991; Hidayat 2010). The flowering process is influenced by various factors, both internal and external. According to Hidayat (2010), internal factors such as genetics and hormones, as well as external factors like light, water, and temperature, significantly impact the flowering process and flower production.

B. pilosa exhibits adaptability to a diverse range of environmental factors, enabling its growth in various conditions. In Bandung, the colonies of *B. pilosa* thrive in multiple locations, each characterized by distinct

environmental settings (Figure 7). Notably, these colonies show a preference for open areas that offer specific environmental conditions conducive to their growth and establishment (Figure 8). According to Pebriani (2017), *B. pilosa* is capable of growing in diverse habitat types, including disturbed areas, naturally disturbed environments, and those influenced by human intervention. It exhibits adaptability to a wide range of habitats, such as gardens, dry land agriculture, grasslands, vacant land, disturbed areas, open spaces in urban regions, and even roadside areas (Mahmoud et al. 2015; Silalahi et al. 2021). *B. pilosa* demonstrates tolerance to low humidity conditions, enabling its growth in relatively dry areas. Moreover, it can withstand moderately severe droughts and generally prefers locations with annual rainfall ranging from 500-3500 mm (Galinato et al. 1999; Rojas-Sandoval 2018). The species also exhibits a broad altitude range, spanning from lowlands to highlands up to 3,600 meters above sea level. It demonstrates a wide temperature tolerance, thriving in temperatures above 15°C and below 45°C. However, it is susceptible to frost, although its roots can survive and regenerate even after exposure to temperatures as low as -15°C (GISD 2023). Additionally, Ash-shiddiqqiyah et al. (2021) support this adaptability, highlighting *B. pilosa*'s ability to grow across various altitudes, ranging from lowlands (0 -100 m asl) to 200 m asl in Semarang City. These findings collectively demonstrate *B. pilosa*'s remarkable adaptability to diverse environmental settings within the city of Bandung.

B. pilosa, thriving in areas with high human activity, spreads through epizoochory, aided by hooked structures on its fruit (Paiman 2020). It readily colonizes disturbed habitats, whether natural or human-induced (Pebriani 2017). New introductions can cover distances exceeding 100 meters in less than 50 years (Richardson et al. 2000). The current distribution map of *B. pilosa* in Bandung requires further refinement using Ground Control Points (GCPs) for precise mapping (Susetyo & Gunarso 2018).

The population of *B. pilosa* in Bandung exhibits invasive characteristics, invading various land use systems due to factors like germination capability, competitive tolerance, canopy cover, and management practices (Tjitrosoedirdjo et al. 2016a). Its high reproductive potential, with over 1000 seeds/m2 and rapid growth from mature individuals, makes it a prolific invader (Tjitrosoedirdjo et al. 2016b). *B. pilosa* disperses widely across altitudes and environments, except for densely built urban areas, suggesting space constraints (McClure & Bartuska 2007). Prioritising control in colonized locations is essential to prevent further spread, given the complexities of urban ecosystems and the challenges posed by invasive species interactions (Gaertner et al. 2017). Managing these challenges in urban environments is crucial for future urban ecology and sustainability.

CONCLUSIONS

In conclusion, the estimated number of individual *Bidens pilosa* found in Bandung is around 1,831 individuals with an average of 76.2 individuals/m2. The most dominant is the young individual stage or seedlings (151 individuals/m2), then the reproductive stage (51 individuals/m2), and the non-reproductive stage (27 individuals/m2). Each individual can produce as many as 61 florets per individual. *B. pilosa* exhibits a remarkable tolerance to a wide range of environmental and climatic conditions, enabling its extensive distribution throughout Bandung, particularly in open areas with substantial human activity. It is anticipated that the population of *B. pilosa* in Bandung will experience exponential growth due to

the high number of new recruits observed in all surveyed plots. Consequently, it is imperative to implement effective control measures to prevent the invasive establishment of this alien species within the region of Bandung.

AUTHOR CONTRIBUTION

D.P.O. designed the research, collected and analysed the data also wrote the manuscript. D.R. also designed the research, funded, and supervised all the process.

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CONFLICT OF INTEREST

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

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Research Article

Bioprospecting and Molecular Identification of Amylase and Cellulase Producing Thermophilic Bacteria from Sediment of Nglimut Hot Springs, Kendal Regency

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ABSTRACT

The utilisation of enzymes in the industry has brought numerous benefits and advantages to production processes. Enzymes serve as biocatalysts, efficiently catalyzing reactions and hydrolysis in biochemical processes. However, there are challenges in applying enzymes in the industry, particularly concerning enzyme stability. The obstacle encountered in the production processes involving industrial enzyme applications is the low stability of enzymes when used at high temperatures. Heat-sensitive enzymes undergo damage or denaturation. Thermophilic microorganisms are chosen because they hold the potential to produce thermophilic enzymes. The thermophilic enzymes exhibit better heat stability compared to other enzymes, making them an effective alternative for future industrial production processes. This study aims to isolate thermotolerant bacteria from Nglimut Hot Spring sediment, screen for cellulase- and amylase-producing isolates, and molecularly identify the best isolate using *16S rRNA* barcode. The results show that 22 bacterial isolates were found in the sediment of a hot spring; TS-14 was the best isolate in producing amylase, with the highest average amylolytic index of 2.38, whereas TS-15 had the highest cellulolytic index of 2.11. Based on *16S rRNA* identification, TS-14 showed an homological identity of 79% with *Bacillus amyloliquefaciens*, while TS-15 had a 100% homological identity with *Bacillus licheniformis*. These results were important as the first step of screening bacterial potential to produce thermophilic enzymes that could be applied in the downstream processing in future industrial and biotechnology companies.

INTRODUCTION

Thermophilic enzymes have significant potential across various industrial processes due to their exceptional stability, functional capability under high temperatures, and ability to withstand structural alterations. These enzyme varieties find widespread application in sectors such as the chemical industry, food production, pharmaceuticals, paper manufacturing, and textiles. Currently, enzymes derived from microorganisms are more fre-

quently utilised within industrial contexts. An enzyme earns the "thermostable" label when it demonstrates a high transition or denaturation temperature while maintaining extended functionality at this elevated temperature. In this context, the temperature considered high exceeds the boundary of thermophilic growth (>55°C). Typically, extracellular enzymes exhibit heightened stability as they remain unaffected by intracellular factors like compatible solutes. Furthermore, enzymes can be influenced by additional factors, including pH, water content, and temperature. As a result, thermophilic enzymes present an alternative for industrial applications that require robust enzymes capable of enduring extreme environmental conditions, owing to their reliability under such circumstances (Turner 2007).

Nglimut Hot Spring in Gonoharjo, Kendal Regency, Central Java, Indonesia, was chosen because it harbors a diverse range of microorganisms that thrive in high-temperature environments. The presence of plantations and other biodiversity in the area surrounding Mount Ungaran further contributes to the potential of the microorganisms living in the hot spring to produce thermophilic enzymes. The coordinates of the Nglimut Hot Spring are within 110°19'47.3"E to 110°20'12.3"E longitude and 7°08'56.9"S to 7°09'42.1"S latitude, at an elevation of 700 meters above sea level. Research conducted by Emianto (2011) indicated that the reservoir temperature in Gonoharjo is approximately 207.53°C, but the actual reservoir temperature might be higher or lower than the calculated value. This difference is attributed to the fact that many dissolved elements in the geothermal fluid near the surface precipitate, especially Na-K-Ca elements, causing differences in chemical content between the fluid sample and the fluid in the reservoir (Emianto 2011).

Bacteria originating from hot springs are microorganisms capable of thriving in elevated temperatures, from 45°C to temperatures exceeding 100°C. Groups of bacteria derived from these hot spring environments can generate enzymes characterized by inherent stability at high temperatures and resilience against changes in physical and chemical conditions. An illustrative instance of such enzymes is cellulase, as pointed out by Khalil (2011). The attention directed towards these bacteria from hot springs is primarily due to their potential as a source of robust enzymes that retain their functionality in high-temperature environments. Worth mentioning is the cellulase enzyme, which holds substantial commercial importance. Cellulase enzymes exhibit considerable promise in the conversion of agricultural cellulosic materials into glucose feedstocks and their role in bioethanol production (Mohammad et al. 2017).

Cellulase enzymes are primarily synthesized by fungi, bacteria, and protozoa, which facilitate the hydrolysis of cellulose in a process called cellulolysis. The significance of cellulase enzymes lies in their diverse range of applications. Industries such as textiles, food production, detergents, leather, and paper manufacturing require stable and functional enzymes under extreme pH and temperature conditions. Additionally, cellulase enzymes play a crucial role in biomass fermentation for biofuels, altering fibers, and even in pharmaceutical applications. Bacterial organisms tend to outpace fungi in cellulase production due to their faster growth rate. Noteworthy bacterial genera that exhibit cellulolytic properties encompass *Cellulomonas* sp., *Pseudomonas* sp., *Bacillus* sp., and *Micrococcus* sp. (Shanmugapriya et al. 2012).

Amylase is a commercial enzyme that makes up 25% of the world's enzyme market needs (Reddy et al. 2003). Amylase can hydrolyze amylum and produce glucose. Amylase originating from thermophilic bacteria can have high thermostability, it may be stable in the presence of sub-

stances that can denature enzymes and stable in an acidic environment, so it has high commercial value for its use in industrial processes and biotechnology. Thermostable amylase is increasingly used in industrial processes and biotechnology (Sianturi 2008). Various industrial processes that use amylase include the food industry, fermentation, textiles, paper, detergents, and pharmaceuticals (de Souza & de Oliveira Magalhães 2010).

The production of amylase is affected by temperature, pH, enzyme concentration, substrate concentration, and inhibitor effects (Poedjiadi et al. 2006; Soeka et al. 2015). This study aims (i) to screen for potentially thermophilic enzymes, especially amylase and cellulase, from sediment of Nglimut hot springs, Kendal Regency, (ii) to determine the optimum condition for enzyme production by temperature variation via qualitative assay, and (iii) to identify the best cellulase and amylase-producing isolate through 16S rRNA barcode. The data provided significant information as fundamental research of the potential thermophilic enzyme from sediment of hot springs, which was previously never disclosed. This study improves our understanding of the basic qualitative assay and is important as the first step of screening the potential thermophilic enzymes, which could later be optimized using the quantitative assay and applied in downstream processing in future industrial and biotechnology companies.

MATERIAL AND METHOD

Sampling site

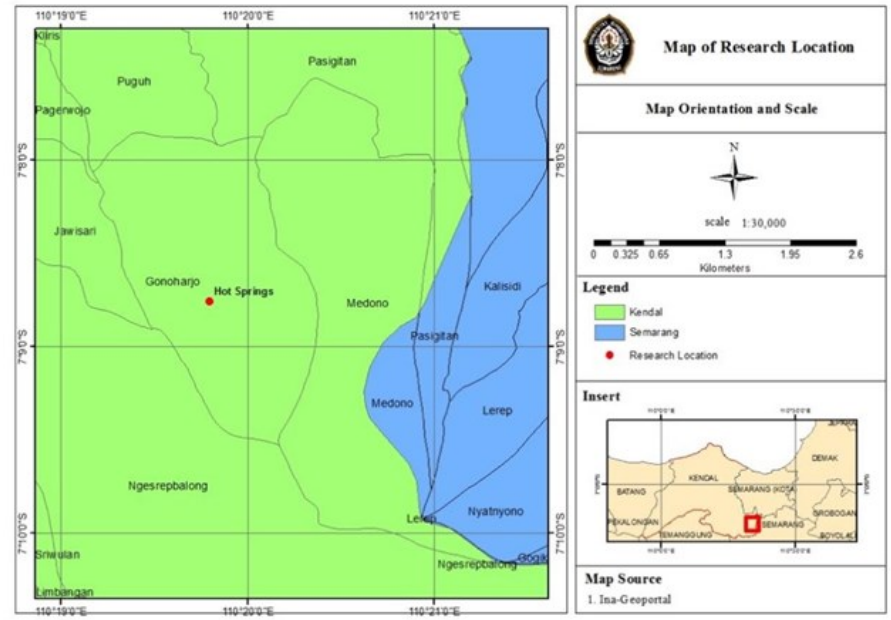
The Nglimut, Gonoharjo, Kendal Regency had the longitude coordinates 110°19'47.3"E to 110°20'12.3"E and latitude coordinates 7°08'56.9"S to 7°09'42.1"S with the temperature approximately of 45-50°C. Temperature differences occur during summer and rainy seasons. The research was carried out by collecting sediment samples from the Nglimut hot springs in Gonoharjo, Kendal Regency (Figure 1). Samples were taken and placed in a hot water flask so that the temperature is maintained until reaching the laboratory for further testing. The bacterial isolation, cellulase and amylase enzyme screening, temperature and pH variation assay, as well as molecular identification using *16S rRNA* gene markers were done in this study.

Bacterial Isolation

Isolation of thermophilic bacteria was done on Nutrient Agar (NA) (Hi Media, India) and Thermus Agar (TA) (Hi Media, India) media. The screening process of isolating thermophilic bacteria was taking a 1-gram sample of the hot spring sediment, placing it into 9 milliliters of sterile distilled water, followed by employing a serial dilution method to create dilutions ranging from 10^{-1} to 10^{-7}. Each dilution (1 mL) was introduced onto NA and TA media using the spread plate technique. Subsequently, incubating was done at a temperature of 45°C for 48 hours. The colonies grown in the media were then observed for colony morphology. The gram staining was performed for microscopic observation (Khalila et al. 2020).

Cellulase Screening

Cellulase activity screening was done on CMC Agar media, made by mixing 1.36 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 2 g NaCl, 1 g $(NH_4)_2SO_4$, 0.01 g $FeSO_4.7H_2O$, 3 g CMC, 1 g yeast extract, and 15 g agar powder into 1 L of distilled water in an Erlenmeyer flask (Naresh et al. 2019). All thermophilic bacterial isolates were tested for cellulase activity at 45°C, 50°C,



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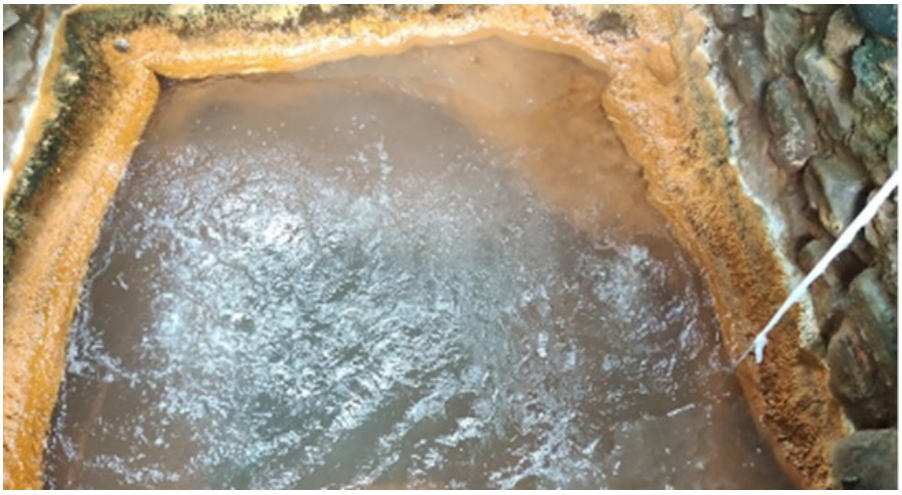


B

Figure 1. Sampling Map of research location in Nglimut Hot Spring (**A**) , Nglimut Hot Spring, Gonoharjo (**B**).

and 55ºC in duplicates. The cellulase enzyme test followed the procedure from Naresh et al. (2019). A sterilized paper disc was placed on top of the enzyme test media. Each liquid culture of bacterial isolates was inoculated onto the paper disc and then incubated for 48 hours. The test results were then calculated using the cellulolytic index equation (Naresh et al. 2019):

$$\text{Cellulolytic index} = \frac{(\text{Clear zone diameter} - \text{bacterial colony diameter})}{\text{bacterial colony diameter}}$$

The isolates of thermophilic bacteria that produce the highest cellulase activity were then identified molecularly to determine their identity.

Amylase Screening

Amylase activity screening was done by making an amylase selective medium with 2 g of yeast extract, peptone 5 g, $MgSO_4.7H_2O$ 0.5 g, NaCl 0.5 g, $CaCl_2.2H_2O$ 0.15 g, starch 10 g, and agar 20 g. The ingredients were put into a glass beaker, and sterile distilled water was added to a volume of 1 L. Afterwards, it was incubated for 48 hours at 45ºC, 50ºC, and 55ºC in duplicates. Sterilized paper discs were placed on the enzyme test media. Each liquid culture of bacterial isolates was inoculated onto the paper discs and incubated for 48 hours. After 48 hours of incubation, the media around the colonies were covered with an iodine solution. Positive results

are indicated by the presence of a clear zone around the colony (Zuraidah 2020). The amylolytic index is measured using the following formula:

$$\text{Amylase index} = \frac{(\text{Clear zone diameter} - \text{bacterial colony diameter})}{\text{bacterial colony diameter}}$$

Enzyme Production Characterisation

Characterization of amylase and cellulase production was carried out by incubation at various temperatures of 40°C, 45°C, 50°C, and 55°C for 48 hours. This characterisation aims to determine the optimal temperature of bacteria in producing thermophilic enzymes. Enzyme characterization was performed by preparing cellulase enzyme screening media, CMC Agar media, as described above. The bacterial isolates used in the experiment were selected based on the largest clear zone index values from each previous enzyme screening. Bacterial isolates TS-14 and TS-15 were inoculated from liquid media, with 5 µL each, onto paper discs placed on the enzyme characterization test media. The results were indicated by the presence of clear zones around the bacterial colonies.

DNA Extraction and *16S rRNA* Molecular Identification

The DNA extraction was performed following the Insta-Gene Protocol using Bio-Rad InstaGene Matrix kit (USA), for bacterial DNA extraction (Gray et al. 2014). The DNA concentration was checked using a nanodrop spectrophotometer (Thermo Fisher Scientific Nanodrop 2000 Spectrophotometer, USA). DNA was amplified using a PCR thermocycler (Labnet MultiGene OptiMax Thermal Cycler, USA). The amplification of genomic DNA was done using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and primers 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Gislin et al. 2018). The PCR mastermix was 50 µL consisting of 25 µL MyTaq PCR Kit, 2 µL Forward primer, 2 µL Reverse primer, 19 µL ddH_2O, and 2 µL sample. The result of PCR was then electrophoresed (Mupid-EXu Electrophoresis, Japan) using a 1% Agarose Gel stained with FloroSafe Stain (1st BASE, Singapore) in 1X TAE Buffer for 30 min with a strength of 100 volts. The band of the DNA target was compared with the 1 KB DNA Marker (Promega, United States). The visualisation of the DNA bands uses Gel Documentation tool (UVITEC UVIDOC HD2, United Kingdom). The amplicon was then purified and continued with the sequencing process (Genetika Science, Jakarta). The sequencing outcomes were compared with the information available on GenBank using the Basic Local Alignment Search Tool (BLAST) program on the NCBI website (www.ncbi.nlm.nih.gov). This was done to acquire similar results for the sequences. After aligning the sequences, the phylogenetic tree was analyzed using the software Molecular Evolutionary Genetics Analysis 11 (MEGA 11). The phylogenetic tree was constructed using the Neighbor-Joining Tree and the Kimura-2 model parameters. To establish the connection between thermophilic bacteria and other bacterial species, this process was repeated 1000 times with bootstrap replication.

RESULTS AND DISCUSSION

Twenty-two thermophilic bacterial isolates of sediment samples from Nglimut Hot Spring were obtained on TA medium. Different macroscopic and microscopic characteristics are shown in Figure 2.

The positive results of the screening test conducted on the thermophilic bacterial isolates TS 1 - TS 22 were evidenced by the appearance of a clear zone around the colonies after iodine exposure. The clear zone indicated the presence of amylase activity (Table 1).

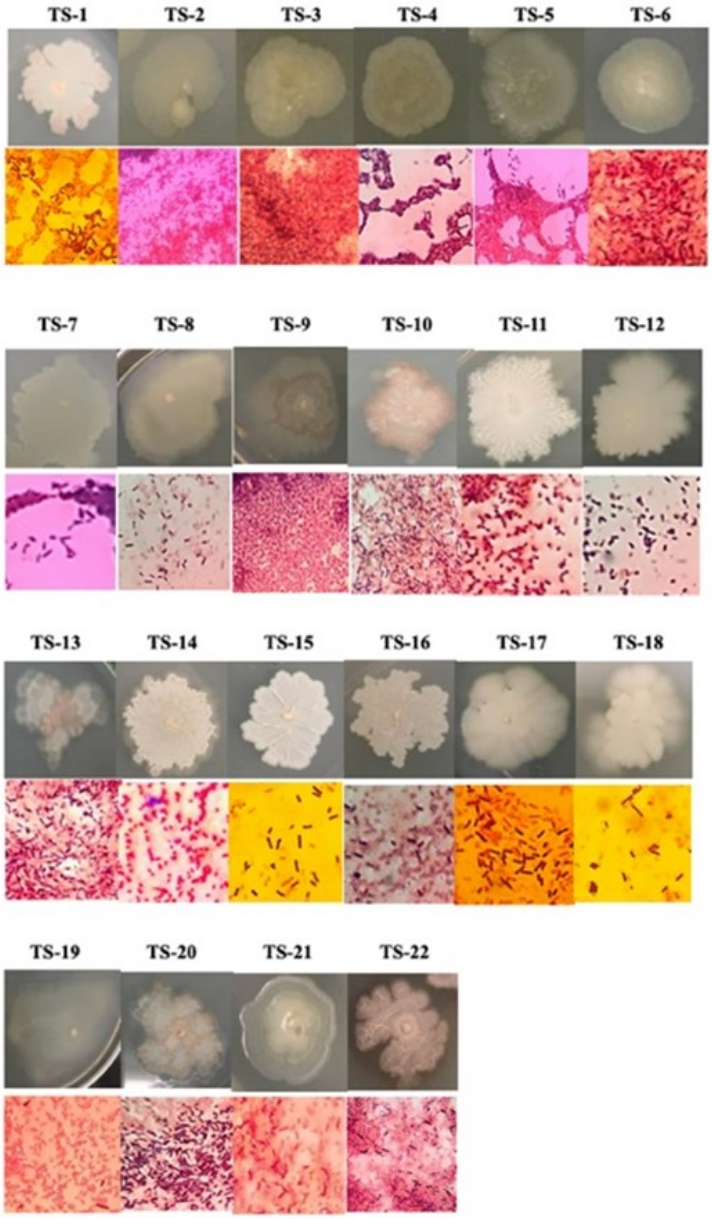


Figure 2. Macroscopic and Microscopic characterization of thermophilic bacterial isolates TS1-TS22 with 100X magnification.

Table 1 shows a compilation of 22 bacterial isolates sourced from hot spring deposits. Furthermore, 15 isolates were positive in producing amylase, while 7 isolates were positive for cellulase production. The clear zone formed indicates amylase activity in dismantling starch molecules in the growth medium. The appearance of a clear zone around the bacterial colony can be attributed to the disintegration of starch mediated by amylase, thus preventing the formation of complexes between starch and iodine (Octarya et al. 2011).

The amylolytic index can be calculated based on the diameter of the clear zone. This is in line with Zuraidah et al. (2020) and Mawati et al. (2021), stating that after incubation, the clear zone formed on each paper disc was observed and measured with a vernier caliper. TS 14 isolate had the highest average amylolytic index of 2.38. This indicates that the TS 14 isolate could produce higher amylase than the other isolates. The TS 14 isolate was further tested for its amylase activity by treating it with variations in temperature to determine the optimal cultivation temperature for the production of amylase. In addition, molecular identification was carried out on the TS 14 isolate to determine the species of the isolate.

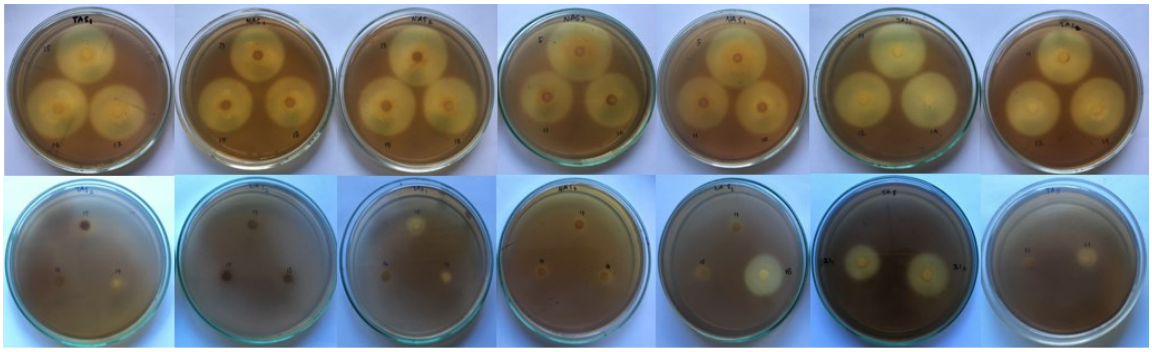
Table 1. Screening of Potential Amylase and Cellulase Production from TS1-TS22.

Code	Macroscopic and Microscopic Characterization							Enzyme Production	
	Colony Forming	Margin	Elevation	Color	Texture	Cell Forming	Gram	Amylase	Cellulase
TS-1	Irregular	Lobate	Flat	Pinkish white	Dry	Rod	+	+	-
TS-2	Round	Smooth	Flat	Yellowish white	Moist	Rod	-	-	-
TS-3	Irregular	Lobate	Flat	Yellowish white	Mucoid	Rod	+	-	-
TS-4	Irregular	Irregular	Flat	Yellowish white	Mucoid	Rod	+	-	-
TS-5	Irregular	Irregular	Flat	Clear white	Moist	Rod	+	-	-
TS-6	Round	Smooth	Umbonate	White	Mucoid	Rod	+	-	-
TS-7	Irregular	Irregular	Flat	White	Mucoid	Rod	+	-	-
TS-8	Irregular	Irregular	Flat	White	Dry	Rod	+	+	-
TS-9	Irregular	Irregular	Flat	Pinkish white	Mucoid	Rod	+	+	-
TS-10	Irregular	Irregular	Raised spreading edge	Pink	Mucoid	Rod	+	+	-
TS-11	Irregular	Irregular	Flat	Milky white	Dry	Coccus	+	+	+
TS-12	Irregular	Irregular	Flat	Milky white	Dry	Rod	+	+	+
TS-13	Irregular	Irregular	Raised spreading edge	Pinkish white	Mucoid	Rod	+	+	
TS-14	Irregular	Irregular	Raised spreading edge	Milky white	Dry	Coccus	-	+	+
TS-15	Irregular	Lobate	Raised spreading edge	Milky white	Dry	Rod	+	+	+
TS-16	Irregular	Irregular	Raised spreading edge	Milky white	Dry	Rod	+	-	+
TS-17	Irregular	Irregular	Flat	Milky white	Dry	Rod	+	+	+
TS-18	Irregular	Irregular	Flat	Milky white	Dry	Rod	+	+	-
TS-19	Irregular	Lobate	Flat	Milky white	Dry	Rod	+	+	-
TS-20	Irregular	Irregular	Flat	Milky white	Dry	Rod	+	+	-
TS-21	Irregular	Irregular	Flat	White	Dry	Rod	+	+	+
TS-22	Irregular	Irregular	Flat	Pinkish white	Mucoid	Rod	+	+	-

Figure 3 shows that a temperature of 40ºC was the best condition for treatment interaction. This is in line with Konsula (2004), and Fitriani (2013), stating that thermophilic *Bacillus* spp. produce extracellular thermostable *alpha*–amylase with an optimum growth temperature of 40ºC.

In the screening of thermophilic amylase, TS-14 obtained the highest index value of 2.37 at a temperature of 40°C, while temperatures of 45°C, 50°C, and 55°C were tested. Figure 3 illustrates the results of the screening of thermophilic amylase, with an index value of 1.62 at 45°C, 0.73 at 50°C, and no activity observed at 55°C. This is due to the nature of thermophilic amylases produced by microorganisms with a maximum temperature tolerance of 50°C (Mohammad et al. 2017).

All thermophilic bacteria isolates were tested for cellulase activity at 40ºC, 45ºC, 50ºC, and 55ºC. The results show that 7 isolates were capable of producing cellulase (Figure 3). Qualitative assessment of cellulase activity in thermophilic bacterial isolates is seen from the clear zone



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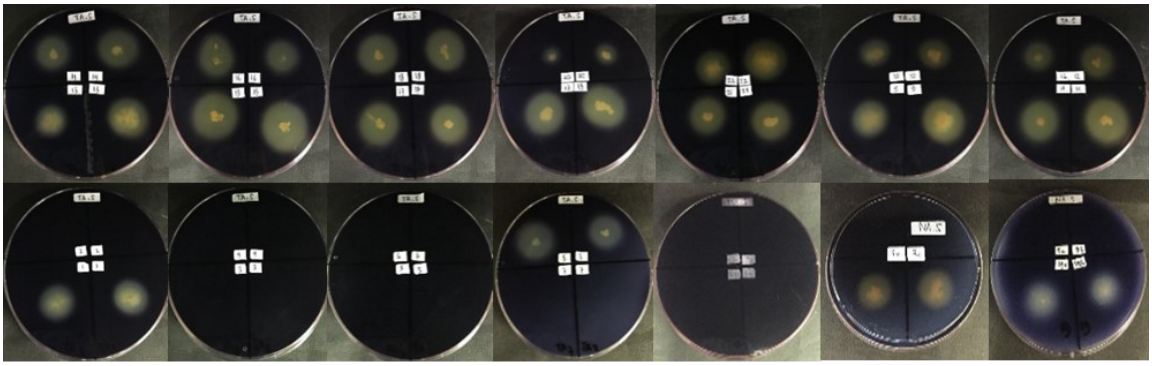

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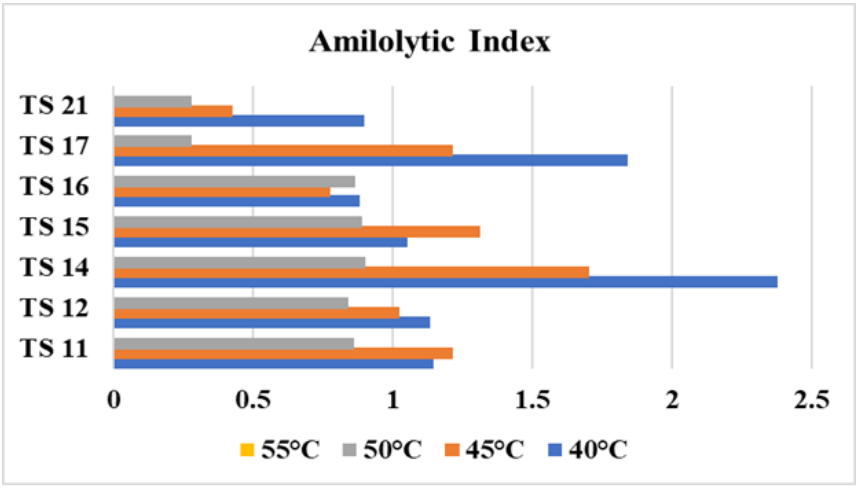

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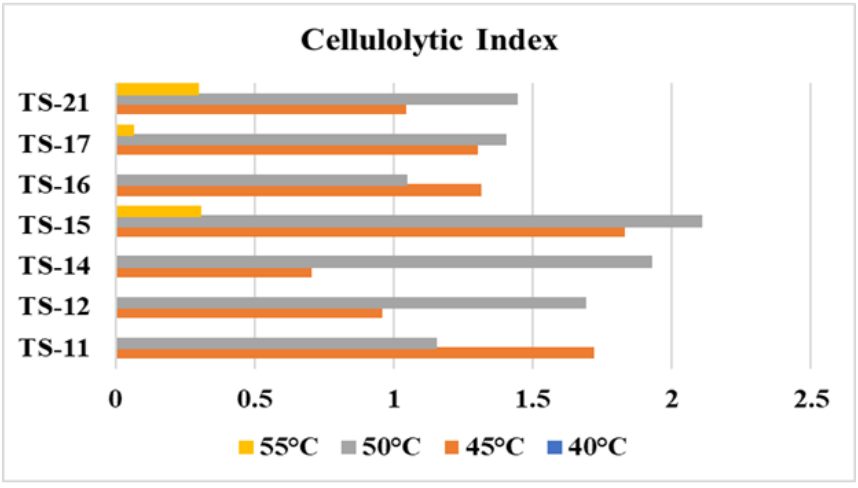

D

Figure 3. Characterisation of cellulase production (**A**). Characterisation of amylase production (**B**). Amylolytic index based on difference temperature (**C**). Cellulolytic index based on difference temperature (**D**)

produced around bacterial colonies. The appearance of a clear zone around the colony on CMC media is the result of cellulose breakdown by bacteria that have cellulolytic abilities (Khalila et al. 2020).

The clear zone was then calculated with the cellulolytic index, and the results were averaged. The clear zone around the colony was measured to select the highest cellulase producer (Figure 3D) (Shaikh et al. 2013). The highest cellulolytic index of each temperature was isolate TS 15 with an index value of 1.83 at 45°C, 2.11 at 50°C, and 0.31 at 55°C. At 40°C, no enzyme activity is observed because this temperature is not within the optimal range for thermophilic microorganisms to generate cellulase. According to Gilter's classification, thermophilic microorganisms exhibit enzyme production at a minimum temperature of 45°C and a maximum temperature of 70°C (Akour 2019). This indicates that the TS 15 isolate was the best producer of cellulase enzymes and would be further identified molecularly.

Identification of the 16S rRNA gene was carried out using an amplification of the genomic DNA with primers 27F and 1492R primers. The 16S rRNA gene is part of the prokaryote genome, which has conserved parts and a hypervariable region that makes it valuable for the identification of bacterial species. The electrophoretic PCR product of the TS 14 and TS 15 isolate (Figure 2) shows an amplicon with a size of 1500 bp.

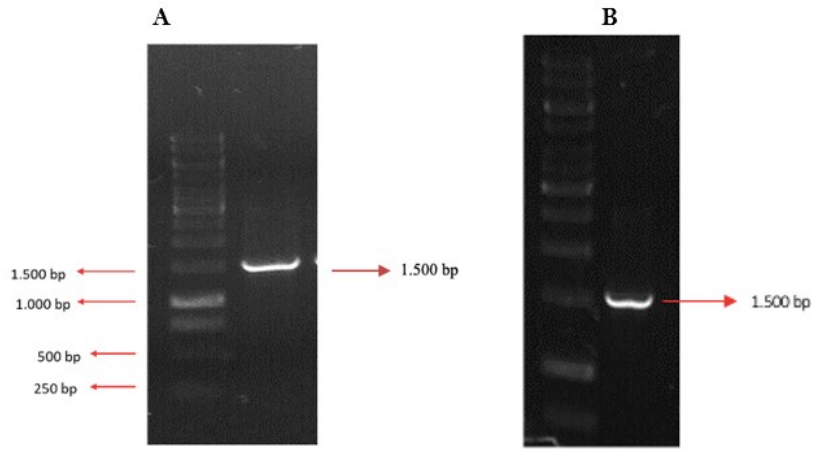

Figure 4. The gel electrophoresis result of the PCR-amplified product of the *16S rRNA* gene obtained from TS 14. (**A**), The gel electrophoresis findings for the PCR-generated product of the *16S rRNA* gene obtained from TS 15. (**B**)

The visualization of PCR amplification using Gel Electrophoresis showed white bands with a length of approximately 1500 bp depicted in Figure 4. The visible band on the doc gel indicates successful amplification. This is in line with Noer (2021), stating that the 16S rRNA gene sequence length is about 1500 bp and consists of conserved regions, relatively large genes, with interspecific polymorphisms to exhibit statistically valid measurement differences. The PCR products of both TS Sequencing results in the form of forward and reverse sequences were then edited using Bioedit software to become consensus sequences.

The phylogenetic tree of TS-14 and TS-15 isolates was made using MEGA X software, constructed using the Neighbor-joining tree method, and tested using the Bootstrap method with a value of 1,000 replications. According to Telle et al. (2011), bootstrap analysis is a method to test how well the model data set is. The bootstrap value is indicated by numbers next to the branches of the phylogenetic tree. A neighbor-joining tree is an approach used to construct a tree illustrating kinship relationships, relying on the nearness of evolutionary distances.

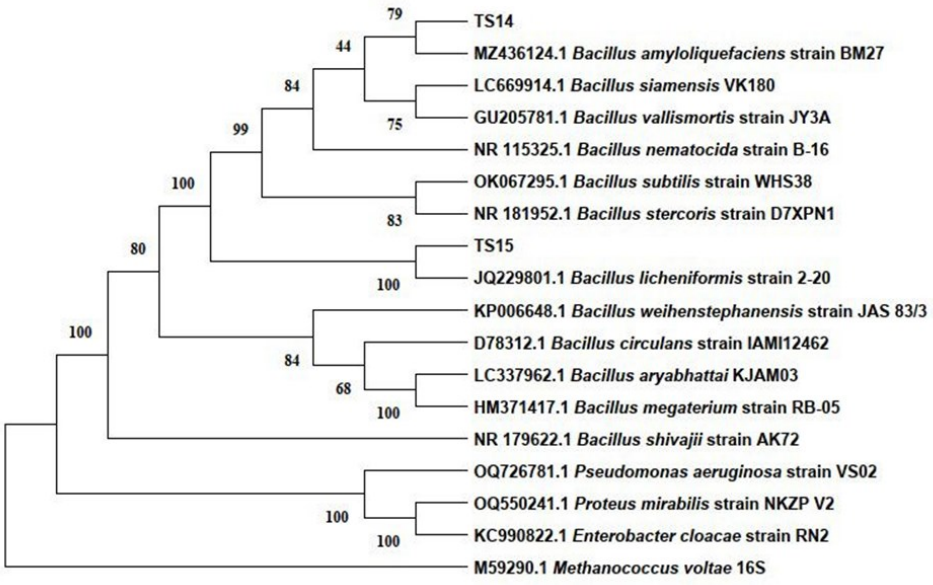

Figure 5. TS-14 and TS-15 isolate sequence phylogenetic tree.

The phylogenetic tree construction is shown in Figure 5. It shows that TS-14 is related to *Bacillus amyloliquefaciens* with a bootstrap value of 79%. The formation of amylase activity of TS-14 was influenced by the temperature, with an optimal value of 40°C. This was in accordance with the results of Ningsih et al. (2012), stating that the amylase enzyme produced by *B. amyloliquefaciens* has an optimum temperature of 30-60°C.

The TS-15 consensus sequences were matched against data in the Genbank on the BLAST program within the NCBI site. The results show that TS-15 isolates had a 100% similarity of its bootstrap value with *B. licheniformis*. A similarity percentage of 99% indicates that the query sequence with the database sequence is the same sequence and has similarities at the species level (Shofa et al. 2019). The bootstrap value shows close kinship if it has a high value, which is more than 70% (Widyadnyana et al. 2015). It has been commonly reported that cellulases can be produced by *B. licheniformis* at temperatures ranging from 30 to 60°C (Karim 2015). The genetic relationship is described by the value of the genetic distance, where the lower the genetic distance, the closer the genetic relationship (Butet el al. 2019).

CONCLUSION

The screening of thermophilic enzyme-producing bacteria from sediment of hot springs in Nglimut, Gonoharjo, Central Java resulted in two promising isolates. TS14, which has the highest potential of amylase formation, was molecularly identified as *B. amyloliquifaciens* and has an amylase index of 2.38 at 40°C. TS 15 exhibits the highest potential for manufacturing thermophilic cellulase enzyme with a cellulase index of 2.11 at 50°C and is molecularly identified as *B. licheniformis* species. This finding is the first attempt to screen and optimize the amylase and cellulase enzymes via qualitative assay. In the future, it will be exciting to test the potential isolates with other enzyme activity and also the quantitative assay to determine the specific activity of the enzyme.

AUTHORS CONTRIBUTION

A.B., N., and W.W. designed the study; J.S., R.S.M. and A.R.M. carried out the laboratory work; D.W., L.H., and L. analysed data and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests that could have appeared to influence the work reported in this paper.

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Research Article

Mining GATA Transcription Factor Encoding Genes in The Cocoa Tree (*Theobroma cacao* L.) Suggests Their Potential Roles in Embryo Development and Biotic Stress Response

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ABSTRACT

GATA transcription factors (TFs) are widely recognized as significant regulators, characterized by a DNA-binding domain that consists of a type IV zinc finger motif. This TF family has been widely investigated in numerous higher plant species. The purpose of the present work was to comprehensively analyze the GATA TF in cocoa plant (*Theobroma cacao* L.) by using various bioinformatics tools. As a result, a total of 24 members of the GATA TFs have been identified and annotated in the assembly of the cocoa plant. According to phylogenetic analysis, these TcGATA proteins were classified into four distinct groups, including groups I (10 members), II (seven members), III (five members), and IV (two members). Next, our investigation indicated that the TcGATA proteins in different groups exhibited a high variation in their physicchemical features due to their different protein lengths, gene structures, and conserved motif distributions, whereas the TcGATA proteins in the same clade might share the common conserved motifs. Additionally, the gene duplication of the *TcGATA* genes in the cocoa plant was also investigated. Of our interest, the relative expression levels of the *TcGATA* genes were investigated according to available transcriptome databases. The results exhibited differential expression patterns of all *TcGATA* genes in various developmental stages of zygotic and somatic embryogenesis, indicating that these *TcGATA* genes divergently function during various developmental stages of the zygotic and somatic embryos. Moreover, *TcGATA* genes were differently expressed under *Phytophthora megakarya* treatment across different points of treatment and cocoa varieties. To sum up, our findings could provide a basis for a further deep understanding of the GATAs in the cocoa plant.

INTRODUCTION

Theobroma cacao L. (2n = 2x = 20), origin in Amazonian lowland rainforests, was domesticated over 1,500 years ago (Motamayor et al. 2002) and

has been grown in more than 50 countries in the world (Diaz-Valderrama et al. 2020; Jaimez et al. 2022). The chocolate tree is considered an economically important species due to its use in the cosmetics, and confectionery industries (Tan et al. 2021), chocolate production (Figueira & Scotton 2020), and medicinal benefits (Pucciarelli 2013). Chocolate tree plantations are often grown in agroforestry ecosystems alongside other fruit and commercial crops, thus providing lasting economic and environmental benefits (Guiltinan et al. 2008). Furthermore, the cocoa tree supplies essential livelihoods for 40 - 50 million people worldwide (Wickramasuriya & Dunwell 2018). It would be significant to investigate the growth and developmental processes of this important chocolate-producing tree at the molecular level. Although the cocoa tree has been viewed as an experimental organism with some limitations in the research field (Figueira & Scotton 2020), its genome was an excellent resource permitting accelerated progress in plantation, breeding, and allowing the understanding of the biochemistry of this tree crop (Motamayor et al. 2013).

Plants are exposed to a variety of environmental stresses that reduce and limit crop productivity. To adapt and survive under these adverse conditions, plants are equipped with specific genes that confer tolerance to such stresses and also regulate their developmental processes. The link between these stress-response mechanisms and gene regulation is predominantly facilitated by transcription factors (TFs). Briefly, TFs are proteins that bind to specific DNA sequences, thereby controlling the transfer of genetic information from DNA to mRNA, playing a crucial role in turning genes on or off in response to environmental stimuli. Among them, GATA has been regarded as one of the ubiquitous TF families in plants that plays an essential role in many processes of plant development, metabolism and signal transduction (Reyes et al. 2004; Behringer & Schwechheimer 2015; Schwechheimer et al. 2022), and abiotic stress signalling (Gupta et al. 2017; Zhang et al. 2021; Zhao et al. 2021). Specifically, the GATA proteins shared a highly conserved type IV zinc finger motif (Schwechheimer et al. 2022) followed by a basic region facilitating DNA binding (Merika & Orkin 1993; Teakle et al. 2002; Reyes et al. 2004; Behringer & Schwechheimer 2015; Schwechheimer et al. 2022). Due to the large number of plant genomes available, the GATAs have been investigated in numerous dicotyledonous and monocotyledonous species, such as *Arabidopsis thaliana* (Teakle et al. 2002; Kim et al. 2021a), soybean (*Glycine max*) (Zhang et al. 2015), rice (*Oryza sativa*) (Gupta et al. 2017), apple (*Malus* × *domestica*) (Chen et al. 2017), grape (*Vitis vinifera*) (Zhang et al. 2018), cotton (*Gossypium* spp.) (Zhang et al. 2019), chickpea (*Cicer arietinum*) (Niu et al. 2020), pepper (*Capsicum annuum*) (C Yu et al. 2021), sweet cherry (*Prunus avium*) (Manzoor et al. 2021), cucumber (*Cucumis sativus*) (Zhang et al. 2021), potato (*Solanum tuberosum*) (R Yu et al. 2021), four Rosaceae species (Manzoor et al. 2021), purple false brome (*Brachypodium distachyon*) (Peng et al. 2021), *Populus* (Kim et al. 2021b), wheat (*Triticum aestivum*) (Du et al. 2022; Feng et al. 2022), foxtail millet (*Setaria italica*) (Lai et al. 2022), and peanut (*Arachis hypogaea*) (Li et al. 2023). Among them, the function of GATA TFs have been well-investigated in many plants (Gupta et al. 2017; Zhu et al. 2020; Zhang et al. 2021; C Yu et al. 2021; Feng et al. 2022; Li et al. 2023; Le et al. 2023). Unfortunately, even though the genome of the cocoa tree has been published recently (Motamayor et al. 2013), the characterization of the GATAs of this important economical species has not been described.

In this current study, we aimed to conduct a systematic investigation of the GATA TFs in the cocoa genome by using bioinformatics ap-

proaches. Here, we performed genome-wide identification and characterization of the cocoa GATAs. To establish the evolutionary relationship of GATA TFs in cocoa with other plants, we also performed a comparative genomic analysis. Finally, the expression levels of the *GATA* genes were investigated by using previous RNA-Seq datasets. The obtained results will provide a cornerstone to understand various plant TF characteristics including evolutionary insights.

MATERIALS AND METHODS

Identification and annotation of GATA family in cocoa tree

To identify all putative members of the TcGATA family in the cocoa genome, a TBLASTN search (Gertz et al. 2006) in the NCBI, Phytozome (Goodstein et al. 2012) and PlantTFDB databases (Jin et al. 2017) has been conducted against the recent genome of this species (BioProject accession: PRJEB14326) (Motamayor et al. 2013) using well-characterized AtGATA proteins from *A. thaliana* (Teakle et al. 2002) as queries (cut-off value < 10e-4). The Pfam database (Mistry et al. 2021) was then used to confirm all potential candidates that included the conserved GATA zinc finger domain (Pfam accession: PF00320). Afterward, the full-length protein, coding DNA (CDS) and genomic DNA (gDNA) sequences and the corresponding identifier of each TcGATA member were collected for further analyses.

Characterization of GATAs in cocoa tree

The exon/intron organization of *TcGATA* genes was constructed from the CDS and gDNA of each TcGATA member by using Gene Structure Display Server v2 (GSDS) (Hu et al. 2015) and the physicochemical features of the TcGATA proteins were calculated by the Expasy Protparam online tool (Gasteiger et al. 2003; Gasteiger et al. 2005) as previously described (Niu et al. 2020; Wang et al. 2021). Sub-cellular localization prediction of TcGATA proteins was performed by using the SherLoc2 program (Briesemeister et al. 2009). The gene ontology of *TcGATA* genes, including biological functions, cellular content, and molecular functions, was estimated by NETGO 2.0 (Yao et al. 2021) with scores higher than 0.8. The conserved motifs in the GATA TFs were screened by using the MEME web-based tool (Bailey et al. 2006).

Phylogeny and gene duplication analysis of the GATAs in cocoa tree

To generate the phylogenetic tree, the MAFFT program (Katoh & Standley 2013) was used to align the full-length protein sequences of TcGATA members from cocoa and well-characterized GATA proteins from other higher plant species, including *A. thaliana* (Teakle et al. 2002; Kim et al. 2021a), apple (Chen et al. 2017), *Populus* (Kim et al. 2021b), grape (Zhang et al. 2018) and rice (Gupta et al. 2017). The Maximum likelihood (ML) phylogenetic tree was generated using MEGA version 11 software (Tamura et al. 2021) with the bootstrap test replicated 1000 times.

Gene duplications were determined as previously described (Guo et al. 2015). The ratio between Ka (the number of nonsynonymous substitutions per non-synonymous site) and Ks (the number of synonymous substitutions per synonymous site) values were calculated by using MEGA version 11 (Tamura et al. 2021) and DNASp version 6 tools (Rozas et al. 2017).

Analysis of the expression profiles of the *GATA*s in cocoa

The expression features of *TcGATA* genes were detected at different de-

velopmental stages of zygotic and somatic embryos by investigating data in a public database (GEO accession: GSE55476) (Maximova et al. 2014) available from the NCBI GEO (Barrett et al. 2013). Additionally, the expression profiles of the *TcGATA* genes under pathogen infection were investigated by analyzing the previous microarray atlas (GEO accession: GSE116041) (Pokou et al. 2019). Relative expression values of *TcGATA* genes were estimated using *Actin 11*, the most stable expressed gene in various cocoa tissues (Pinheiro et al. 2011), as a reference gene, following the previous description (Cao 2022). Up- and down-regulated genes were defined by a fold-change cut-off (|fold-change| ≥ 1.5-fold) between 6, 24, and 72 hours after inoculation (hai) and 0 hai.

RESULTS AND DISCUSSION

Identification and annotation of the TcGATA proteins in cocoa tree

A total of 24 putative *TcGATA* genes were identified in the cocoa genome (Table 1), along with their annotations, like Phytozome locus and their corresponding sequences. Finally, we assigned these 24 GATA full-length protein sequences to TcGATA01 to TcGATA24 based on their physical location on the genome (Table 1).

Recently, there has been a significant effort to identify and characterize GATA TFs in various higher plant species, including both dicotyledonous and monocotyledonous plants (Table S1). Compared to other plants, the TcGATA family found in the cocoa genome is larger than in sweet cherry (18 genes) (Manzoor et al. 2021), *Ophiorrhiza pumila* (18

Table 1. Summary of the TcGATAs in cocoa.

Gene	Phytozome locus	Gene size	Protein size	MW	pI	AI	GRAVY	SCL
TcGATA01	Thecc.01G024200	1414	389	42.57	5.84	54.94	-0.70	Nucl
TcGATA02	Thecc.01G034600	875	249	27.78	8.47	41.16	-0.86	Nucl
TcGATA03	Thecc.01G136600	747	248	28.43	5.54	61.29	-0.55	Golgi, Extra, Vacu, Nucl
TcGATA04	Thecc.01G308700	489	119	13.25	9.98	64.79	-0.76	Nucl
TcGATA05	Thecc.01G385800	939	273	30.63	7.71	46.92	-0.89	Nucl
TcGATA06	Thecc.02G040700	2410	238	26.42	7.66	80.71	-0.52	Nucl
TcGATA07	Thecc.02G076600	1071	322	35.51	5.52	55.12	-0.62	Nucl
TcGATA08	Thecc.02G128000	5007	353	38.95	5.00	62.41	-0.73	Nucl
TcGATA09	Thecc.02G128100	3563	313	33.18	5.01	62.27	-0.58	Nucl
TcGATA10	Thecc.03G242400	4664	538	59.88	6.52	70.67	-0.66	Nucl, Cyto
TcGATA11	Thecc.04G215200	1572	243	26.93	8.43	57.82	-0.75	Nucl
TcGATA12	Thecc.04G294500	642	147	16.08	9.76	63.06	-0.94	Nucl, Cyto
TcGATA13	Thecc.05G293000	1121	302	33.38	8.79	66.19	-0.67	Nucl, Cyto
TcGATA14	Thecc.05G319600	1047	171	18.64	10.14	61.58	-0.61	Nucl, Cyto
TcGATA15	Thecc.06G060500	9887	308	33.85	6.59	60.71	-0.85	Nucl
TcGATA16	Thecc.06G097700	1193	320	34.94	6.14	55.22	-0.67	Nucl
TcGATA17	Thecc.08G072900	4282	299	32.11	5.69	63.95	-0.57	Nucl
TcGATA18	Thecc.08G073000	3928	355	38.47	4.69	67.52	-0.68	Nucl
TcGATA19	Thecc.09G046000	1493	363	39.92	5.88	57.47	-0.71	Nucl
TcGATA20	Thecc.09G053800	1562	311	34.00	9.21	57.81	-0.67	Nucl, Cyto
TcGATA21	Thecc.09G089800	1189	302	33.64	9.15	59.83	-0.84	Nucl, Cyto
TcGATA22	Thecc.09G218100	2714	255	28.15	9.04	45.57	-1.11	Nucl, Cyto
TcGATA23	Thecc.09G342100	2326	342	37.04	8.75	60.38	-0.63	Nucl
TcGATA24	Thecc.10G075500	1460	341	37.09	6.67	65.51	-0.57	Nucl

Note: -: No information, protein size (amino acid residues), MW: Molecular weight (kDa), AI: Aliphatic index, pI: Iso-electric point, GRAVY: Grand average of hydropathicity, SCL: Sub-cellular localization, Nucl: Nuclear, Cyto: Cytoplasm, Golgi: Golgi apparatus, Vacu: Vacuolar, Extra: Extracellular

genes) (Shi et al. 2022), castor bean (*Ricinus communis*) (19 genes), grape (19 genes) (Zhang et al. 2018), Japanese apricot (*Prunus mume*) (20 genes), and peach (*Prunus persica*) (22 genes) (Manzoor et al. 2021), sugar beet (*Beta vulgaris*) (16 genes) (Le et al. 2023). However, it is significantly smaller than in other plants, such as *A. thaliana* (30 genes) (Reyes et al. 2004), *P. bretschneideri* (Manzoor et al. 2021), apple (35 genes) (Chen et al. 2017), seven *Populus* spp. (33 to 40 members), *G. hirsutum* (87 genes) (Zhang et al. 2019), and wheat (79 members) (Feng et al. 2022). These comparisons reveal that the number of GATA members varies greatly across different plant species.

Analysis of the physical and chemical features of the TCGATA proteins in cocoa

The predicted full-length amino acid sequences of 24 TcGATA members in cocoa were analyzed using the ExPaSy Protparam tool (Gasteiger et al. 2003, 2005). The investigation provided information on the physical and chemical properties of the TcGATAs in cocoa, which are summarized in Table 1. The full-length of the predicted protein sequences encoded by the 24 TcGATAs varied from 119 (TcGATA04) to 538 amino acid residues (TcGATA10). The weights of the TcGATAs ranged from 13.25 kDa (TcGATA04) to 59.88 kDa (TcGATA07). The theoretical isoelectric point (pI) values of the TcGATAs were distributed between 4.69 (TcGATA18) and 10.14 (TcGATA14), with 12 TcGATAs being acidic (pI values ranging from 4.69 to 6.67) and the remaining sequences being basic (pI values varying from 7.71 to 10.14). The aliphatic index (AI) values of the TcGATAs ranged from 41.16 (TcGATA02) to 77.86 (TcGATA06). Additionally, the grand average of hydropathicity (GRAVY) values for all members of the TcGATAs in cocoa were less than 0, indicating that the TcGATAs were hydrophilic proteins (Table 1).

The obtained results were in agreement with the previously comprehensive analysis conducted on the general characteristics of GATA TFs in various higher plant species. For instance, GATAs in Rosaceae woody species, like *Pyrus bretschneideri*, *Prunus avium*, *P. mume*, and *P. persica*, were reported to range from 119 to 548 amino acid residues in full-length sequences and from 12.99 to 60.23 kDa in molecular weight, respectively (Manzoor et al. 2021). In grapes, the protein full-lengths of GATA TFs ranged from 109 to 386 amino acid residues (Zhang et al. 2018), while apples had GATAs with amino acid residues varying from 90 to 1161 (9.9 to 129.74 kDa) (Chen et al. 2017). Additionally, seven *Populus* species were found to possess a total of 389 predicted GATA proteins, with sequence lengths ranging from 82 to 791 amino acid residues, except for PtsGATA29, which had only 46 amino acid residues (Kim et al. 2021b). Additionally, the pI values of GATA TFs in higher plant species were found to a wide range from acidic to base, with pI scores in four Rosaceae woody species ranging from 4.71 to 10.07 (Manzoor et al. 2021), and peanut (*Arachis hypogaea)* ranging from 4.75 to 10.21 (Li et al. 2023), respectively. These varying pI scores are due to their different protein lengths. Interestingly, the GRAVY values of all members of GATAs in apples (Chen et al. 2017), four Rosaceae species (Manzoor et al. 2021), and peanuts (Li et al. 2023) were evidently negative, indicating that these GATAs may be hydrophilic (Schwechheimer et al. 2022). Overall, the physical and chemical properties of TcGATAs in cocoa, and possibly other plant species, were highly variable based on the study results. The dissimilarity of the physical and chemical properties proposed that GATAs might play different functions in plants.

Phylogenetic analysis, Gene structure and Conserved Motif of the GATAs in cocoa

A phylogenetic tree comprising all of the 24 TcGATAs and well-characterized GATAs from *A. thaliana* (Teakle et al. 2002; Kim et al. 2021a), grape (Zhang et al. 2018), and *P. trichocarpa* (Kim et al. 2021b) has been constructed in order to clarify the phylogenetic relationships of the TcGATAs in cocoa (Figure 1).

According to the ML estimation, 24 TcGATA proteins were divided into four different groups, namely groups I, II, III, and IV, respectively, as shown in Figure 1. Specifically, group I had the largest number of cocoa TcGATA proteins (10 TcGATA members), followed by group II (seven TcGATA members), and group III (five TcGATA members). Group IV had the least number of cocoa TcGATA proteins, with only two members, including TcGATA06 and TcGATA10, respectively (Figure 1).

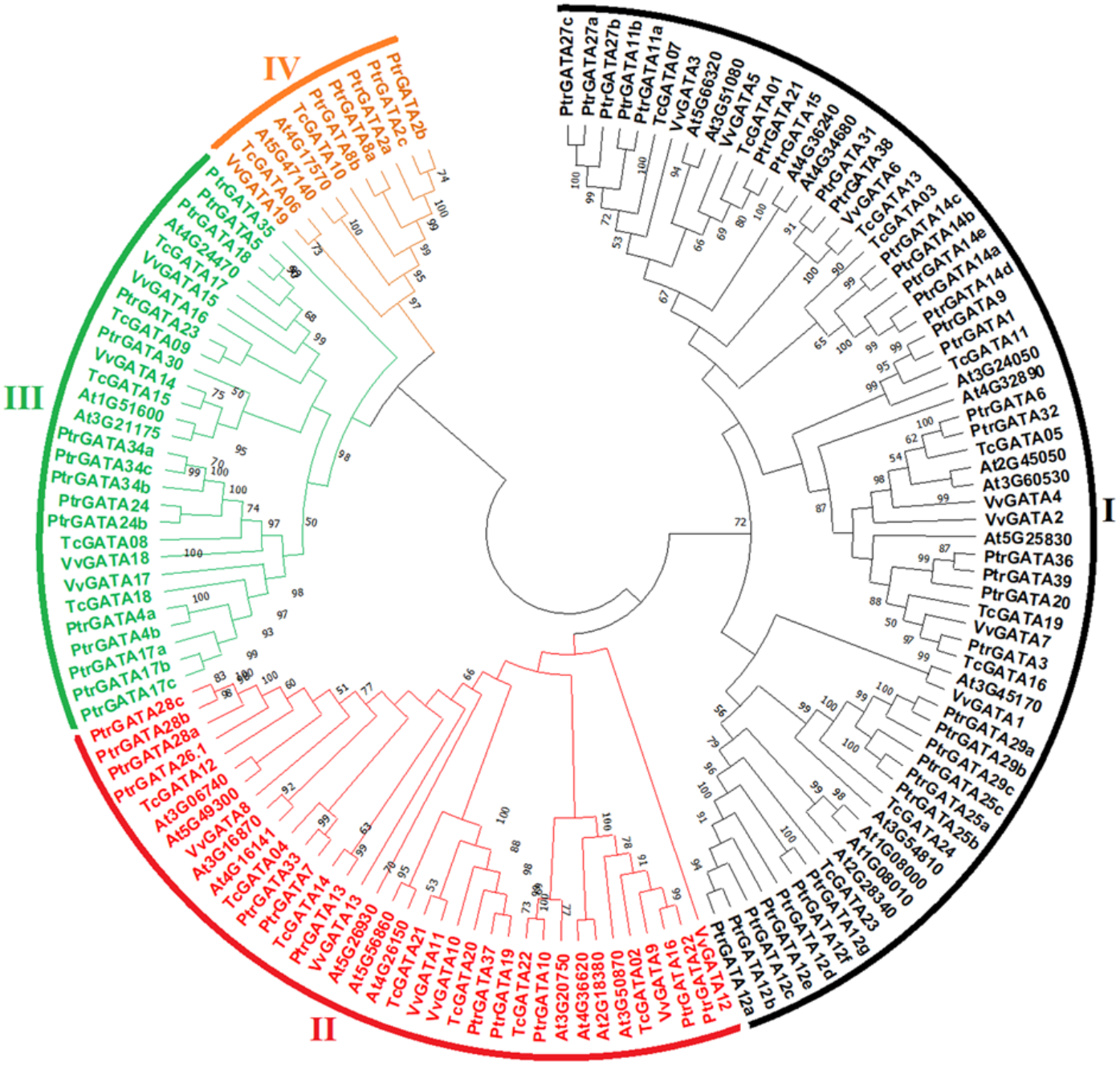

Figure 1. Phylogenetic tree of GATA family from *Arabidopsis thaliana* (At), *Populus trichocarpa* (Ptr), *Vitis vinifera* (Vv), and *Theobroma cacao* (Tc).

Previously, the classification of GATA TFs had been established for different higher plant species (Table S1). GATA TFs from many a large number of dicotyledonous plants were also classified into four main clades, like *A. thaliana* (Teakle et al. 2002), four Rosaceae species (Manzoor et al. 2021), peanuts (Li et al. 2023), and grape (Zhang et al. 2018). Out of four clades, clades I and IV contained the largest and smallest number of GATAs, respectively. Particularly, 49 GATA TFs found in potato (*Solanum tuberosum*) were divided into five groups, group II had the largest number of GATA proteins (15 GATA members), followed by groups IV (13 GATA members), V (10 GATA members), III (eight GATA members), and I (only three GATA members) (R Yu et al. 2021). But the phylogenetic tree was constructed from only the 49 GATAs of potato. Therefore, the classification of potato GATAs might need further comparison with other species. The clade V, VI, and VII GATAs were reported only in rice with two, four, and two members, respectively (Gupta et al. 2017).

The exon/intron arrangement of the cocoa *TcGATA* genes was then examined. The results showed that newly discovered cocoa *TcGATA* family genes have exon counts ranging from 1 to 11 (Figure 2). Interestingly, the *TcGATA* genes in the same clade may share the seminar gene organization (Figure 1, 2). For example, eight (out of ten) members in group I included two exons, except for *TcGATA03* had only one exon, and *TcGATA01* contained three exons (Figure 2). Seven (out of nine) *TcGATA* members of group II contained three exons, while two others had two exons (*TvGATA02* and *TcGATA04*). In group III, a majority member had seven exons, whereas two remaining members had 10 (*TcGATA18*) and 11 exons (*TcGATA08*) (Figure 2). Two group IV-belonging *TcGATA* genes included four (*TcGATA06*) and eight (*TcGATA10*) exons, respectively (Figure 2). Our findings confirmed the wide range of variability in exon numbers of *GATA* genes identified in four Rosaceae species (from 1 to 10 exons), peanut (Chen et al. 2017), grape (from 1 to 11 exons) (Zhang et al. 2018), and *Populus* species (from 1 to 12 exons) (Kim et al. 2021b). The unique gene architecture of TcGATA15 and TcGATA17, characterized by their extremely short exons and

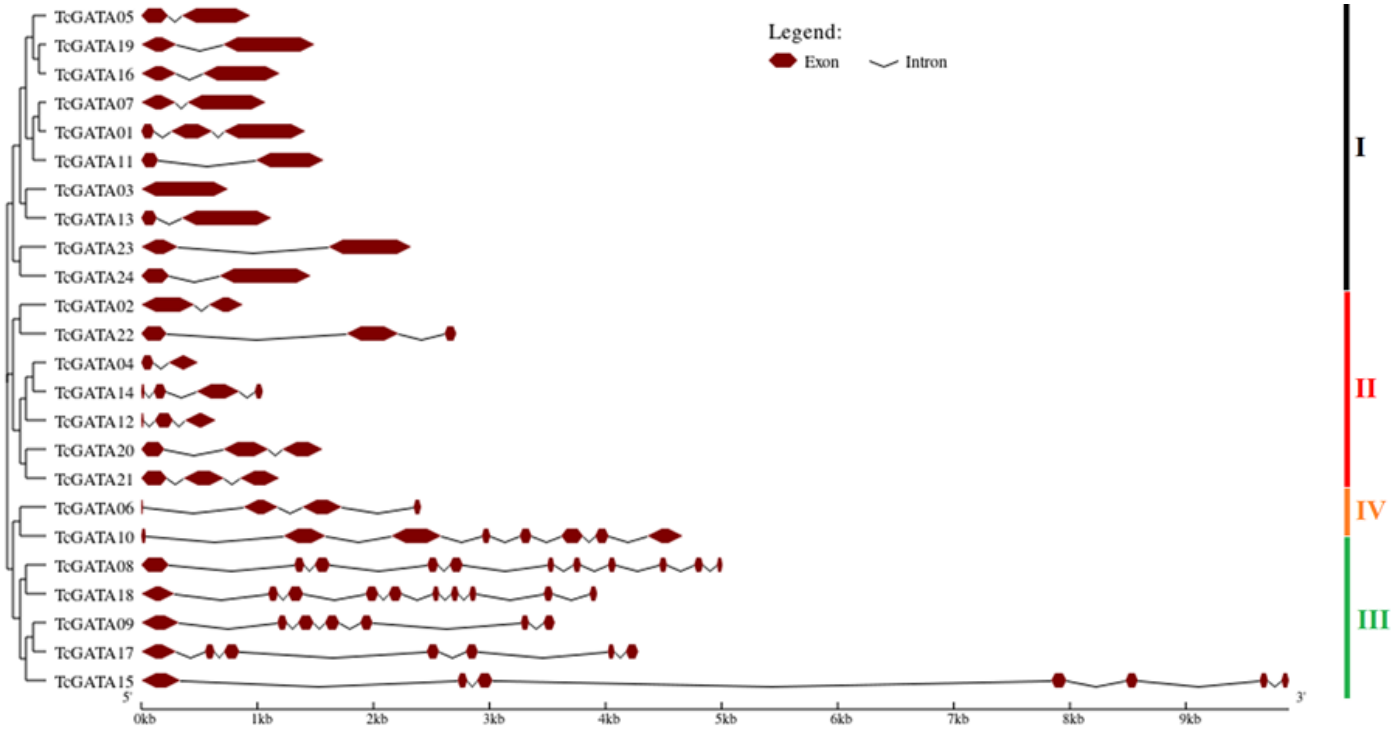

Figure 2. Gene exon/intron organizations of the *GATA* family in cocoa.

long introns, offers fascinating insights into evolutionary processes. This atypical structure, contrasting with more common gene architectures, aligns with theories suggesting that the evolution of introns is linked to alternative splicing and the resulting functional diversity in proteins. The presence of long introns in these two genes could potentially delay transcriptional output, providing a mechanism for the suppression of gene expression under adverse conditions. This hypothesis aligns with the broader concept that gene architecture can be an adaptive trait in evolution, where specific structural features, like long introns, may confer selective advantages in response to environmental challenges. The divergence in the gene structure of the *GATA* family of cocoa suggests that the *TcGATA* genes underwent an evolutionary change, which might have generated the functional separation of the *GATA* family and might enable genes to have new functions that can help plants better adapt to environmental changes (Fan et al. 2014).

The evolutionary relationship and classification of the TcGATA family were validated by analyzing their conserved motifs predicted and confirmed by the MEME program (Bailey et al. 2006) (Figure 3). The conserved motif 1 was present in all TcGATA proteins, and the majority of members of the same TcGATA group exhibited similar patterns. Group IV had the lowest number of conserved motifs (1), while group I had the highest (6). However, some proteins had distinct conserved mo-

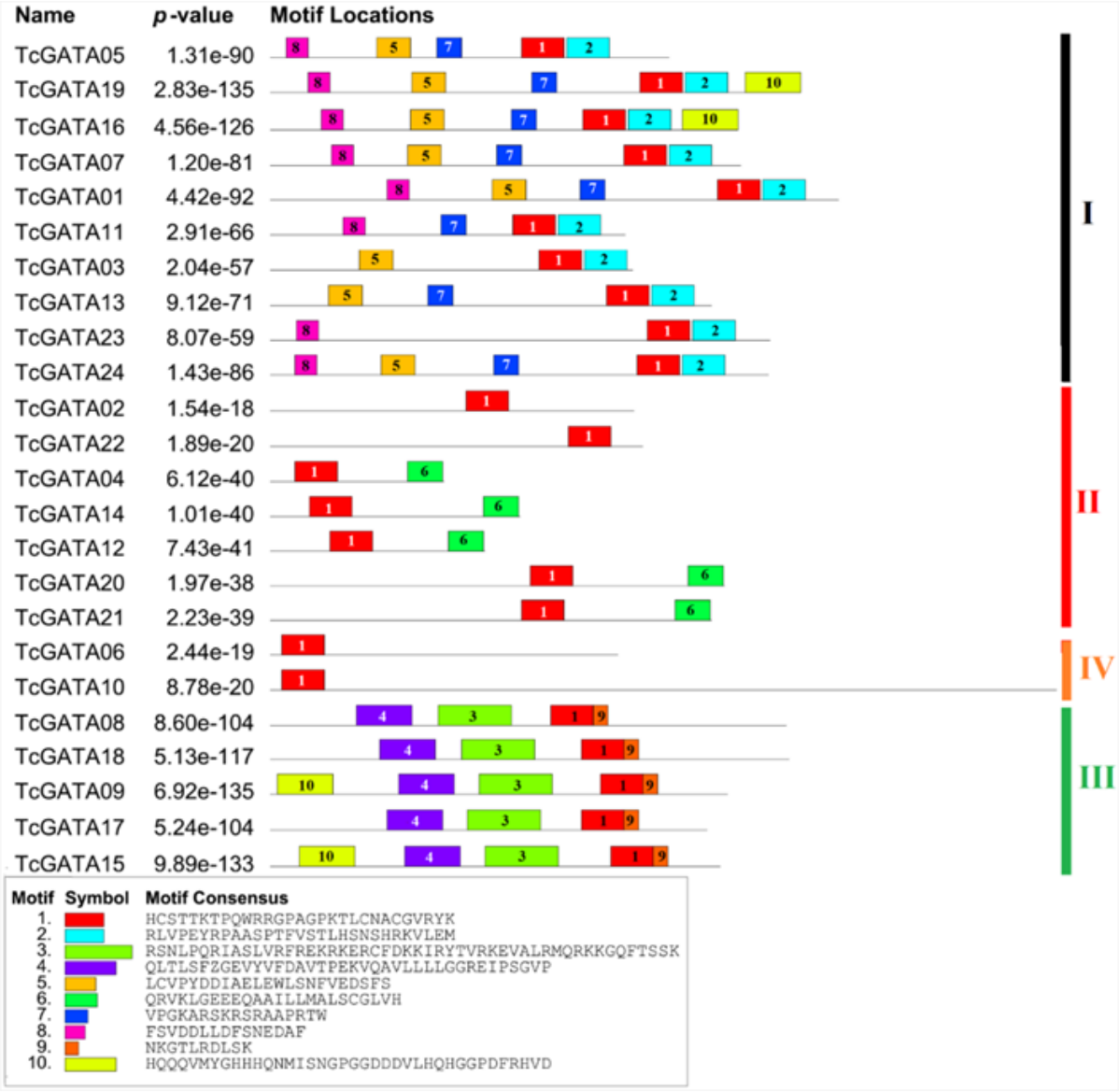

Figure 3. Conserved motifs of the GATA family members in cocoa generated by MEME.

tifs across different groups. For instance, motif 1 was unique to all groups. All members of group I contained motifs 2, 5 except for two genes (TcGATA11 and TcGATA23), motif 7 except for two genes (TcGATA03 and TcGATA23), and motif 8 except for two genes (TcGATA03 and TcGATA13). In addition, motif 10 was found in two members, TcGATA16 and TcGATA19, respectively. For group II, motif 6 was detected in five (out of seven) members (TcGATA04, TcGATA12, TcGATA14, TcGATA20 and TcGATA21). All members of group III contained motifs 3, 4, and 9. Moreover, motif 10 was recorded in two genes, TcGATA09 and TcGATA15, respectively. The common motif detected in all TcGATA was the zinc finger loop (C-X2-C-X18–20-CNAC) domain. GATA members in groups I, II, and IV had the C-X2-C-X18-CNAC conserved domain, while the group III members harboured the C-X2-C-X20-CNAC domain (Figure 6). Additionally, the conserved amino acid motif TPQWRXGPXGXKTL was identified between the second and third cysteine residues in the C-X2-C-X18-CNAC zinc finger loop of group I while the conserved amino acid motif TX2T-PLWRXGPXGPKXL was detected between the second and third cysteine residues in the C-X2-CX18-CNAC zinc finger loop of group II. Moreover, the conserved amino acid motif GXSX3TPXMRRGPXGPRXL was detected between the second and third cysteine residues in the C-X2-CX20-CNAC zinc finger loop of group III and the conserved amino acid motif GX2STPLWRNGPPEK-PVL was identified between the second and third cysteine residues in the C-X2-CX18-CNAC zinc finger loop (Figure 4).

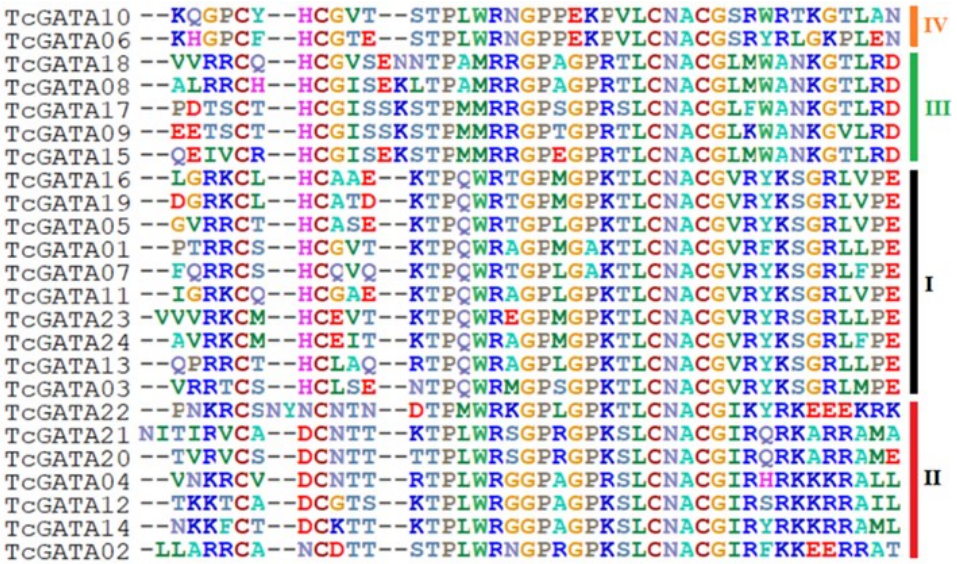

Figure 4. Alignments of GATA domains of all identified TcGATA family members in cocoa tree.

Our findings showed that the motif distributions of TcGATA proteins were comparable within each subfamily. The presence of the same motif in all groups or in each group suggested that they might have fundamental functions. The conserved GATA domains and motifs found between the second and third cysteine residues in the C-X2-CX18-20-CNAC zinc finger loop found in different groups of TcGATAs were consistent with conserved structures previously identified uin peanut (Li et al. 2023), chickpea (Niu et al. 2020), and *Populus* species (Kim et al. 2021b). The examination of gene architectures and conserved motifs reveals that GATA members within a group exhibit relatively high conservation properties in various species and that members among groups exhibit reasonably high conservation properties.

Physical distribution and gene duplication of the GATAs in cocoa

The distribution of the 24 *TcGATA* genes across the cocoa genome was investigated in this study. Results showed that the *TcGATA* gene family was distributed randomly across the genome (Figure 5). The quantity of *TcGATA* genes differs across various chromosomes, with chromosomes 9 and 16 containing the largest number of *TcGATA* gene distributions with five members, followed by chromosome 2 with four members, and chromosomes 4, 5, 6, and 8 with two members each (Figure 5). It is noteworthy that chromosomes 3 and 10 each only had one *GATA* gene, while chromosome 7 had no *TcGATA* gene (Figure 5).

As an intriguing aspect of this research, the duplication events that occurred in the *TcGATA* gene family in cocoa were predicted as previously described (Niu et al. 2020), with details provided in Figure 5 and Table 2. Three duplicate genes were found in the *TcGATA* family, with nucleotide similarities ranging from 53.3 (*TcGATA08* and *TcGATA18*) to 57.3% (between *TcGATA09* and *TcGATA17*). These findings indicate that whole genome duplication (WGD) and segmental duplication (SD) events played a significant role in the expansion of the *TcGATA* gene family. Additionally, the Ka/Ks ratios for the three duplicated genes were all less than 1, ranging from 0.26 (*TcGATA08* and *TcGATA18*) to 0.30 (*TcGATA20* and *TcGATA21*), indicating that the *TcGATA* genes were under strong purifying selection.

These findings showed a similar trend in chromosome distribution and evolution of *GATA* genes in many plant species. The random distribution of *GATA* genes across the genome was reported in seven *Populus* species (Kim et al. 2021b), four Rosaceae species (Manzoor et al. 2021) and chickpea (Niu et al. 2020). Interestingly, there was no tandem duplication observed in the *TcGATA* family, while two WGD and one SD events accounted for the duplication events in the *TcGATA* family (Table 2). This result confirmed that the WGD and SD events played a significantly important role in the evolution of the *GATA* genes compared to tandem duplication events, as also observed in chickpeas (Niu et al. 2020), *Populus* species (Kim et al. 2021b), grape (Zhang et al. 2018), and perhaps many other plants (Zhang et al. 2019; Li et al. 2023).

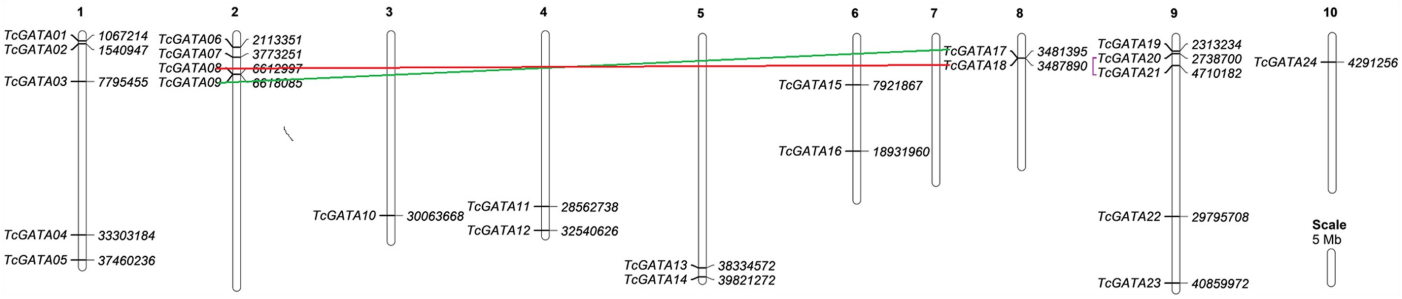

Figure 5. The chromosomal distribution of *TcGATA* genes in the cocoa genome. The red lines indicated the duplication events. The chromosome number is indicated above for each chromosome.

Table 2. Prediction of the duplication events in the Tc*GATA* gene family in cocoa.

Duplicated gene	Duplicated gene	Duplication event	Similar level (%)	Ka	Ks	Ka/Ks
TcGATA08	*TcGATA18*	WGD	53.3	0.342	1.31	0.26
TcGATA09	*TcGATA17*	WGD	57.3	0.363	1.35	0.27
TcGATA20	*TcGATA21*	SD	55.2	0.441	1.47	0.30

Note: WGD: Whole genome duplication, SD: Segmental duplication, Ka: the number of nonsynonymous substitutions per non-synonymous site, Ks: the number of synonymous substitutions per synonymous site.

Gene ontology analysis of the GATAs in cocoa

In this study, gene ontology (GO) analysis was used to annotate the probable roles of the TcGATA TFs. Appropriately, 24 TcGATAs were then categorized into 55 functional groups and divided into three main ontologies, including cellular component, biological process, and molecular function (Figure 6). As a result, in the cellular component category, all 24 TcGATAs anticipated their function in the nuclear, intracellular organelle, while only one member awaited the role in the intracellular, non-membrane-bounded organelle (Figure 6). The GO analysis also indicated that all TcGATAs were distributed in the nucleus (Figure 6), which was also confirmed by the sub-cellular localization prediction by the SherLoc2 tool (Table 1). All TcGATAs were localized in the nuclear compartment followed by the cytoplasm (seven out of 24) (Figure 6). While only one member of the TcGATA was predicted to localize on the Golgi apparatus, vacuolar, plasma membrane, or extracellular (Table 1).

Figure 6. GO analysis involving in molecular function, biological processes, and cellular components of TcGATAs investigated by NETGO 2.0.

It has been thought that the determination of the sub-cellular localization of proteins can provide insight into their potential roles (Goodin 2018). In the molecular function category, all TcGATA proteins were predicted to act as TFs (DNA binding, nucleic acid binding, and sequence-specific DNA binding) (Figure 6). Under the biological process annotation, all TcGATAs were associated with biological processes, and 18 out of 24 TcGATAs anticipated their function in the regulation of biological processes. In addition, 11 out of 24 TcGATAs were predicted to function in response to stimuli. These obtained results were also in agreement with the previous reports that the 32 PbGATAs of Chinese white pear anticipated their functionality in DNA binding, and nucleic acid binding TF (Manzoor et al. 2021).

Expression patterns of the cocoa TcGATAs

In this study, of our interest, the expression pattern of the *TcGATA* genes in different stages of cocoa embryo development was investigated (Figure 7). In general, most *TcGATA* genes were expressed in all stages of embryo development, except for *TcGATA14* (Figure 7). The expressed *TcGATA* genes exhibited different expression levels during different developmental stages of the embryo (Figure 7). Sixteen *TcGATA* genes were differentially expressed during zygotic embryo maturation, with 11 *TcGATA* genes displaying higher expression levels in the mature zygotic embryo tissues than in other developmental stages, including *TcGATA03*, *TcGATA05*, *TcGATA07*, *TcGATA11*, *TcGATA22* (group I), *TcGATA04*, *TcGATA20*, *TcGATA21* (group II), *TcGATA08*, *TcGATA18* (group III), and *TcGATA10* (group IV), respectively (Figure 7). However, four *TcGATA* genes belonging to group I showed lower expression levels in mature zygotic embryo samples than in early developmental stages, including *TcGATA02*, *TcGATA03*, *TcGATA23*, and *TcGATA24* (Figure 7). Similarly, five *TcGATA* genes exhibited higher expression at mature (M-SE) than late torpedo (LT-SE) developmental stages of somatic embryogenesis, including *TcGATA05*, *TcGATA07*, *TcGATA11*, *TcGATA04*, and *TcGATA18*, respectively. However, *TcGATA16* showed a lower expression level at M-SE than LT-SE developmental stages of somatic embryogenesis (Figure 7). At the same developmental stages, differential gene expression between zygotic and somatic embryogenesis was recorded. At the torpedo stage, four *TcGATA* genes (*TcGATA02*, *TcGATA03*, *TcGATA16*, and *TcGATA23*, respectively) were more expressed in zygotic embryos compared to somatic embryo while three other genes (*TcGATA04*, *TcGATA07*, and *TcGATA10*, respectively) were less expressed. At the mature stage, 11 *TcGATA* genes (*TcGATA01*, *TcGATA03*, *TcGATA04*, *TcGATA08*, *TcGATA09*, *TcGATA17*, *TcGATA18*, *TcGATA20*, *TcGATA21*, *TcGATA22*, and *TcGATA23*, respectively) had higher expression levels in zygotic embryos compared to somatic embryo while three other genes (*TcGATA07*, *TcGATA16*, and *TcGATA19*, respectively) had lower expression levels (Figure 7). Overall, the expression of *TcGATA* genes in embryogenesis suggested that this transcription family played an important role in the seed development of cocoa. The differential expression patterns of different genes in various developmental stages of zygotic and somatic embryogenesis indicated that different *TcGATA* genes divergently function during various developmental stages of the zygotic and somatic embryos. Despite the large number of reports of the genome-wide analysis of GATAs in plants, the function of this family in embryogenesis has been poorly communicated. Earlier, the GATA factor HANABA TARANU was reported to be required to position the proembryo boundary in the early embryo of *A. tha-*

liana (Nawy et al. 2010). On the other hand, the expression of two *GATAs* (*GATA NITRATE-INDUCIBLE CARBON-METABOLISM-INVOLVED* and *CYTOKININ-RESPONSIVE GATA1*) in *Arabidopsis* seedlings has been described (Chiang et al. 2012). In addition, the BME3 (Blue Micropylar End 3) GATA TF has been previously described as a positive regulator of *Arabidopsis* seed germination (Liu et al. 2005). So, our findings provided evidence that indicated the function of *GATAs* in embryo development in plants. Moreover, further deep investigation might be required to explore the role of GATAs in the seed development of the seed crop species.

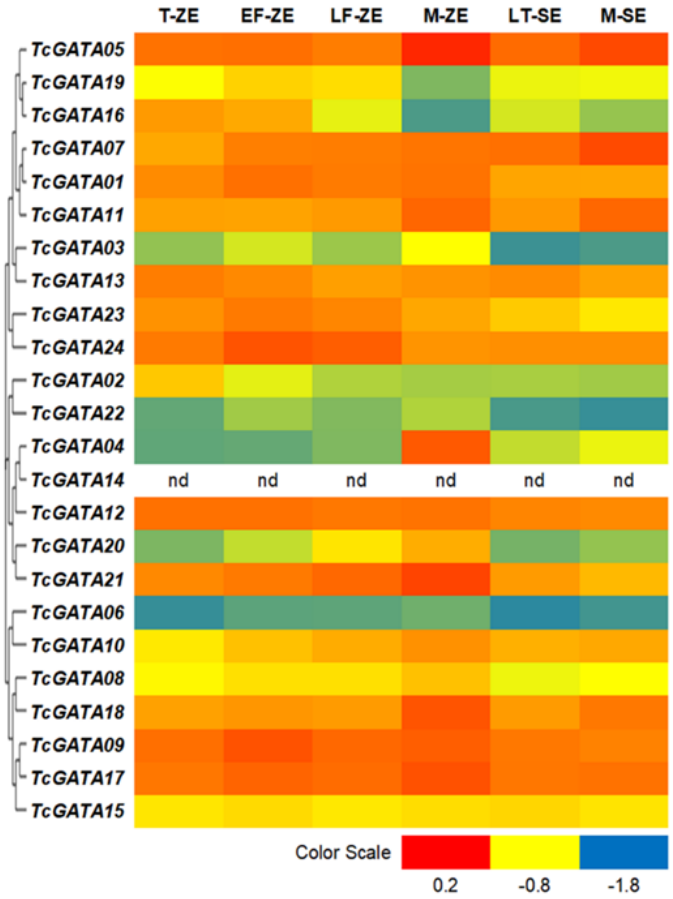

Figure 7. Expression patterns of the *T. cacao GATA* genes during Zygotic (ZE) and Somatic Embryo (SE) maturation. Values represented log2 of the relative expression level of *TcGATA* genes per expression level of *Actin 11* gene which was the most stable expressed gene in various tissues (Pinheiro et al. 2011). T-ZE: Torpedo zygotic embryo, EF-ZE: Early-full zygotic embryo, LF-ZE: Late-full zygotic embryo, M-ZE: Mature zygotic embryo, LT-SE: Late Torpedo somatic embryo, M-SE: Mature somatic embryo, nd: non-determined.

A significant number of *TcGATA* genes showed differential expression under *Phytophthora megakarya* treatment across different points of treatment and cocoa varieties (Figure 8). Particularly, the expression of *TcGATA22* was not detected in any treatments of both Nanay and Scavina genotypes. At 6 hours after inoculation (hai), only two genes, *TcGATA06* and *TcGATA23*, showed an increase in relative expression level in the Nanay genotype. However, in the Scavina genotype, four genes, including *TcGATA05*, *TcGATA08*, *TcGATA12* and *TcGATA18*, were up-regulated by *P. megakarya* treatment, whereas *TcGATA03* and *TcGATA04* were down-regulated. At 24 and 72 hai, in Nanay genotype, five genes, including *TcGATA04*, *TcGATA05*, *TcGATA13*, *TcGATA17*, and *TcGATA19*, were down-regulated by *P. megakarya* treatment, and only

two genes, *TcGATA06* and *TcGATA07*, were up-regulated. Differently, in the Scavina genotype, eight genes, including *TcGATA01*, *TcGATA04*, *TcGATA05*, *TcGATA06*, *TcGATA12*, *TcGATA16*, and *TcGATA20*, were up-regulated. Moreover, at 72 hai, most of the expressed *TcGATA* genes were up-regulated by *P. megakarya* treatment, except for *TcGATA03*, which was down-regulated and four genes, *TcGATA07*, *TcGATA10*, *TcGATA15*, and *TcGATA19*, which were not regulated by *P. megakarya* treatment (Figure 8). In summary, *TcGATA* genes showed different expression patterns in the susceptible (Nanay) and resistant (Scavina) cocoa genotypes under *P. megakarya* treatment at different time points (6, 24, and 72 hours) after inoculation. These discovered results indicated that *TcGATA* genes function differently under *P. megakarya* treatment in various genotypes of cocoa tree. The increase in relative expression level from 6 hai to 72 hai in the tolerance genotype contributed to explaining the function of *TcGATA*s in the biotic stress response in cocoa. In the literature, expression pattern analysis exhibited that *GATA* genes responded to diverse abiotic stresses, such as high temperature, salinity, cold, and drought treatments, in many plants, such as rice (Gupta et al. 2017), wheat (Feng et al. 2022), *oilseed rape* (Zhu et al. 2020), cucumber (Zhang et al. 2021), and pepper (R Yu et al. 2021). However, knowledge about the function of *GATA*s in the biotic response was limited until recently. For example, overexpression of *TaGATA1* showed high resistance to *Rhizoctonia cerealis* in wheat (Liu et al. 2020). A further detailed investigation into the role of *TcGATA* in *Phytophthora* might be necessary, as cocoa undergoes significant annual losses to the water mold *Phytophthora* spp. (Oomycetes) (ranging between 20 and 25% of global losses) (Adeniyi 2019).

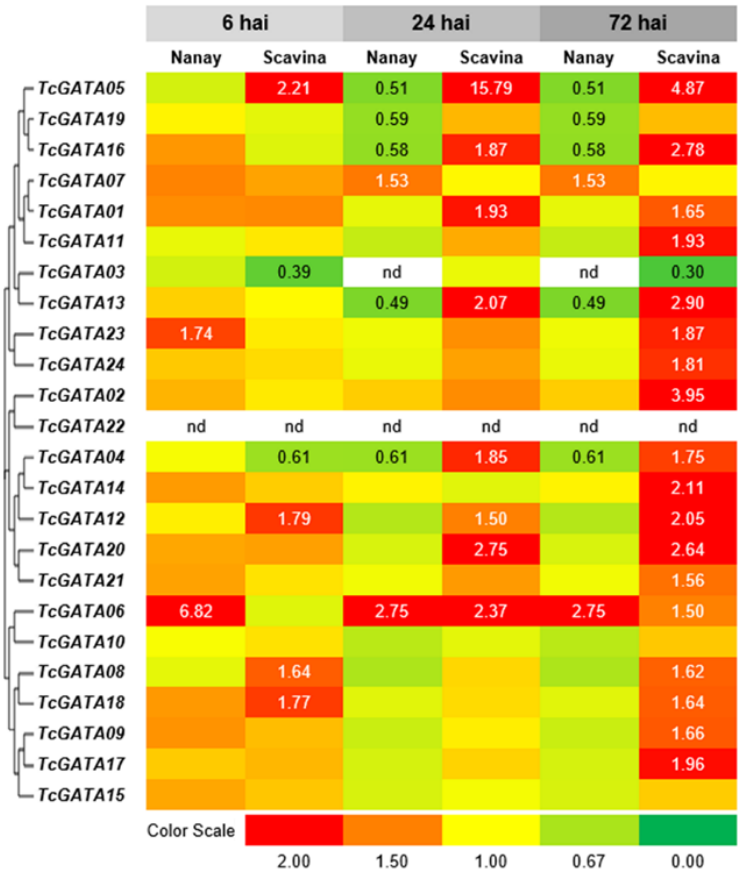

Figure 8. Expression patterns of the *TcGATA* gene family under inoculation of *Phytophthora megakarya*. hai: hour after inoculation, Nanay: Nanay variety (susceptible genotype), Scavina: Scavina (tolerance genotype), nd: non-determined.

CONCLUSIONS

This present study focused on the identification and characterization of the GATA TF family in cocoa tree. A total of 24 *TcGATA* genes were identified in the assembly of cocoa. By using various tools, the physicobiochemical features, gene structure, and conserved motifs of the TcGATA proteins were analyzed. The gene expression patterns of the *TcGATA*s were investigated during the development of zygotic and somatic embryos. Moreover, their expression patterns under inoculation with *P. megakarya* were also analyzed. The results provide valuable information for further understanding the different functions of *TcGATA*s during seed development and in response to *P. megakarya* in cocoa plants. Additionally, these findings offer insightful information for comparative genomics studies in plants based on the characterization, evolution and expression of GATA gene family.

AUTHOR CONTRIBUTION

N.T.B.C. contributed to the research design, data collection and analysis, and preparation of the first draft of the manuscript, T.M.L. contributed to data collection, H.D.C. contributed to data collection and analysis, and preparation of the first draft of the manuscript. T.T.T.H. contributed to data collection and analysis. L.T.M.T. contributed to data collection and analysis, H.V.L. contributed to the research design, data collection and analysis, Q.T.X.V. contributed to data collection and analysis, H.H.P. contributed to data collection and analysis, V.T.T. contributed to data collection, P.B.C. contributed to the research design, data collection and analysis, and preparation and editing of the manuscript and to supervise all the process.

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CONFLICT OF INTEREST

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

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APPENDICES

Table S1. Number of GATA genes in each group of some plant species used in genome-wide identification of the GATA gene family.

Plant genome names	Number of each group of GATA genes							Total	Ref.
	I	II	III	IV	V	VI	VII		
Arabidopsis thaliana	14	11*	3	2	0	0	0	30	(Reyes et al. 2004)
Arachis hypogaea	26	13	6	0	0	0	0	45	(Li et al. 2023)
Brassica napus	36	43	10	7	0	0	0	96	(Zhu et al. 2020)
Glycine max	30	17	9	8	0	0	0	64	(Zhang et al. 2015)
Gossypium arboreum	20	13	8	5	0	0	0	46	(Zhang et al. 2019)
Gossypium hirsutum	36	25	16	10	0	0	0	87	(Zhang et al. 2019)
Gossypium raimondii	19	14	8	5	0	0	0	46	(Zhang et al. 2019)
Malus domestica	20	8	4	3	0	0	0	35	(Chen et al. 2017)
Populus deltoides	18	9	9	2	0	0	0	38	(Kim et al. 2021b)
Populus euphratica	18	12	8	2	0	0	0	40	(Kim et al. 2021b)
Populus pruinosa	17	11	7	2	0	0	0	37	(Kim et al. 2021b)
Populus tremula	17	7	8	1	0	0	0	33	(Kim et al. 2021b)
Populus tremula x alba	18	10	8	2	0	0	0	38	(Kim et al. 2021b)
Populus tremuloides	17	7	9	2	0	0	0	35	(Kim et al. 2021b)
Populus trichocarpa	18	10	9	2	0	0	0	39	(Kim et al. 2021b)
Populus trichocarpa	18	10	9	2	0	0	0	39	(Apuli et al. 2020)
Prunus avium	6	7	4	1	0	0	0	18	(Manzoor et al. 2021)
Prunus mume	7	7	5	1	0	0	0	20	(Manzoor et al. 2021)
Prunus persica	8	6	6	2	0	0	0	22	(Manzoor et al. 2021)
Pyrus bretschneideri	13	11	6	2	0	0	0	32	(Manzoor et al. 2021)
Ricinus communis	7	7	4	1	0	0	0	19	(Ao et al. 2015)
Solanum lycopersicum	14	9	4	3	0	0	0	30	(Yuan et al. 2018)
Solanum tuberosum	3	15	8	13	10	0	0	49	(R Yu et al. 2021)
Ophiorrhiza pumila	7	5	5	1	0	0	0	18	(Shi et al. 2022)
Vitis vinifera	7	6	5	1	0	0	0	19	(Zhang et al. 2018)
Oryza sativa	8	9	3	1	2	4	2	29	(Gupta et al. 2017)
Phyllostachys edulis	12	13	6	0	0	0	0	31	(Wang et al. 2020)
Setaria italica	14	8	4	2	0	0	0	28	(Lai et al. 2022)
Triticum aestivum	21	23	31	4	0	0	0	79	(Du et al. 2022)
Triticum aestivum	35	21	12	4	0	0	0	79	(Feng et al. 2022)
Total	**483**	**342**	**217**	**85**	**2**	**4**	**2**	**1131**	

*This number is from the recent analysis (Kim et al. 2021b).

***In the case of four species, different classification, group A, B, C, and/or D, was used so that it is also omitted (group A: 15 GATA genes, group B: 5 GATA genes, group C: 7 GATA genes, and group D: 1 GATA genes in *Brachypodium distachyon* (Peng et al. 2021), group A: 12 genes, group B: 9 genes, group C: 4 genes, and group D: 3 genes in *Capsicum annuum* (C Yu et al. 2021), group A: 17 GATA genes, group B: 5 GATA genes, and group C: 3 GATA genes in *Cicer arietinum* (Niu et al. 2020), group A: 11 genes, group B: 9 genes, group C: 4 genes, and group D: 2 genes in *Cucumis sativus* (Zhang et al. 2021)).

In case of *Zea mays*, different classification, goup I, II, III, IV, V, and VI, was used so that it is also omitted (group I: 5 genes, group II: 0 gene, goup III: 7 genes, goup IV: 3 genes, group V: 5 genes, group VI: 3 genes (Jiang et al. 2020)

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Research Article

Cryptic Diversity of Barred Mudskippers, *Periophthalmus argentilineatus* (Valenciennes, 1837), from the Southern Coast of Java and East Lombok, Indonesia inferred by *COI* Mitochondrial Gene

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ABSTRACT

The Barred Mudskipper (*P. argentilineatus*) is an amphibious fish species that displays fully terrestrial behaviour during low tides. Previous studies have indicated the existence of cryptic species of the barred mudskipper, leading to difficulties in taxonomic identification due to similarities in morphological characteristics. Therefore, this study aimed to generate DNA barcodes for Indonesian barred mudskipper populations. We collected ten specimens from Clungup Beach and Kondang Bandung Beach, representing our samples. Additionally, we incorporated 25 previously collected *COI* sequences from Indonesia into our analysis. The mitochondrial *COI* gene was amplified using PCR and analysed using various bioinformatic programs. This study provides evidence for the presence of three genetically distinct clades (A, B, and C) within the *P. argentilineatus* population in Indonesia, with a deep genetic divergence of 2.41% to 6.12%. Clade A showed a high genetic divergence of 5.51-6.12%, suggesting the presence of a cryptic species consistent with previous studies. The high level of haplotype diversity and low nucleotide diversity observed in each clade suggest a population bottleneck followed by a rapid expansion. The lack of geographical separation in the haplotype network analysis indicates that gene flow between populations may have been facilitated by glaciation events in the past. These findings contribute to a better understanding of the biodiversity of the barred mudskipper species in Indonesia and will aid in the accurate identification of cryptic species. This study highlights the importance of using molecular techniques to complement morphological identification in understanding the evolution and diversity of mudskipper fish species.

INTRODUCTION

Mudskipper is an amphibious fish that exhibits fully terrestrial behaviour during low tides (Polgar et al. 2017; Xinxin et al. 2018). This definition applies to genera: *Periophthalmus* Bloch & Schneider, 1801, *Boleophthalmus* Valenciennes, 1837, *Scartelaos* Swainson, 1839, *Periophthalmodon* Bleeker, 1874, and *Zappa* Murdy, 1989 (Murdy 1989; Polgar et al. 2010). These genera were classified into the subfamily Oxudercinae based on morpho-

logical, osteological, and eco-ethological characteristics (Murdy 1989). Several molecular studies, however, have indicated that Oxudercinae is paraphyletic relative to the gobiid of subfamily Amblyopinae (worm-eel gobies) and that both subfamilies are members of the '*Periophthalmus* lineage' of gobionelline-like gobies (Agorreta & Rüber 2012; Agorreta et al. 2013). As a result, both subfamilies previously included in the family Gobiidae underwent a major revision and both subfamilies are placed in a separate family, Oxudercidae (Nelson et al. 2016; Kuang et al. 2018; McCraney et al. 2020).

The genus *Periophthalmus* Bloch & Schneider, 1801 is widely recognized as the most diverse genus among mudskipper genera. In recent years, several new species within the genus *Periophthalmus* have been discovered, resulting in a total of 20 valid species (Murdy 1989; Murdy & Takita 1999; Darumas & Tantichodok 2002; Larson & Takita 2004; Jafaar & Larson 2008; Jaafar et al. 2016; Fricke et al. 2023). One such species is *Periophthalmus argentilineatus* (Valenciennes, 1837), commonly known as the barred mudskipper, which is distributed throughout a wide range of geographical regions, including the Red Sea and the east coast of Africa, and extending eastward to Southern Japan, Australasia, and Oceania, up to the Samoa Islands (Murdy 1989). In Indonesia, this fish species can be found on seven main islands, such as Sumatera, Java, Kalimantan, Sulawesi, Lesser Sunda, Moluccas, and Papua (Pormansyah et al. 2019).

Barred mudskippers generally inhabit mangrove swamps, tidal mudflats, and estuaries. This fish is a carnivorous and opportunistic feeder on various types of prey such as insects, crustaceans, fish eggs, and polychaetes worms (Kruitwagen et al. 2007). Barred mudskipper possesses two distinct dorsal fins and pelvic fins that are not interconnected or fused for less than 1/3 the length of the inner rays. Additionally, the presence of a pelvic frenum is either absent or only visible under magnification, as reported by Murdy (1989) and Polgar (2014). These fish are also widely recognized for their remarkable abilities to move, climb, and skip around in the water, a characteristic attributed to their powerful pectoral fins (Murdy 1989; Khaironizam & Norma-Rashid 2002; Kottelat 2013).

The molecular study conducted by Polgar et al. (2014) using both nuclear (*rag1*) and mitochondrial markers (*D-loop* and *16S* rRNA) suggested the existence of at least three distinct cryptic or pseudo-cryptic species in *P. argentilineatus*. Furthermore, according to the personal observation by Polgar (2014), these three cryptic species include one that is morphologically consistent with *P. sobrinus* Eggert (found in the Red Sea and South Africa), another that is consistent with *P. vulgaris* Eggert (observed from Sri Lanka to West Sumatra, the Sunda Islands, Sulawesi, the Philippines, the Moluccas, West Papua, and Northern Australia), and a third that consistent with *P. argentilineatus* Valenciennes (found from West Sumatra to the Sunda Islands, Southeast Borneo, and the Moluccas). Another study conducted by Aji and Arisuryanti (2021) using *COI* mitochondrial gene as a DNA barcoding marker also showed a suspected cryptic species of *P. argentilineatus* with a genetic divergence of 5.46-5.96% from Baros Beach, Special Region of Yogyakarta, Indonesia.

Due to the limitations of morphological identification methods of cryptic species, molecular genetic approaches for species identification have been used and developed in recent years. The primary purpose of DNA barcoding is to provide for the rapid identification of potentially unknown species, including cryptic species (Hebert et al. 2003a). DNA barcoding method can facilitate the discovery of cryptic species that are

still difficult to analyse using traditional approaches. The mitochondrial cytochrome c oxidase subunit I (*COI*) gene has been widely accepted as a reliable, universal animal species-level barcode for the vast majority of the animal kingdom (Hebert et al. 2003b). The *COI* gene has been used to detect and differentiate multiple cryptic species of fish such as the Swamp eel (Arisuryanti et al. 2016), Neotropical fish (Melo et al. 2016), Pearl cichlid (Souza et al. 2017), Cardinalfish, Pilot fish, Pacific rudderfish (Huo et al. 2017), and Pomfret (Li et al. 2019).

This study aimed to generate DNA barcodes for the barred mudskipper populations from Clungup and Kondang Bandung Beach in East Java, Indonesia, using the cytochrome c oxidase subunit I (*COI*) gene. We also aimed to investigate the genetic diversity and relationships among the barred mudskipper from these two populations and other regions in Indonesia, providing valuable insights into the evolutionary history and conservation status of this species.

MATERIALS AND METHODS

Sample Collection

Ten barred mudskipper fish specimens were collected from two different locations, Clungup Beach and Kondang Bandung Beach (Figure 1). The sampling of the barred mudskipper fish was obtained using a hand net. The collected samples were then thoroughly cleaned and documented. The documentation of the collected barred mudskipper fish specimens involved the use of a digital camera to photograph each sample. The digital photographs served to record essential details such as size and any notable external features. Each mudskipper fish sample was placed in a zip-lock bag, stored in a cool-box, and then transported to the Laboratory of Genetics and Breeding, Faculty of Biology, UGM. The samples were preserved in 99% ethanol and stored at -20°C for further analysis. To gain a more comprehensive analysis, we also included 25 previously registered *COI* sequences of the barred mudskipper from various locations in Indonesia. These locations included Bogowonto Lagoon (MT439598-MT439600) (Arisuryanti et al. 2018), Tekolok Estuary (MW514015-MW514024) (Rha'ifa et al. 2021), Baros Beach (MZ606679-MZ606687) (Aji & Arisuryanti 2021), and Pasir Mendit Beach (OQ804359-OQ804361) (Febrianti et al. 2023) (Table 1). The inclusion of these samples aimed to enhance the analysis of the genetic diversity and relationships among the barred mudskipper populations from different regions in Indonesia.

DNA Extraction

DNA extraction from fish tissue muscle (fillet) was performed using the Qiagen DNEasy Blood and Tissue kit (QIAGEN, Valencia, CA, USA). Each sample was mixed with 180 µL of ATL buffer in a 1.5 ml tube. The fillet was mechanically disrupted with ethanol-sterilized scissors and then added with 20 µL Proteinase K (600 mAU/mL). The sample was then thoroughly mixed and underwent an overnight incubation at 50°C, with periodic tube inversion during the initial three hours. After incubation, the samples were vortexed, and a solution consisting of 200 µL of AL buffer and 200 µL of cold absolute ethanol was added. After further mixing and centrifugation, the resulting mixture was processed through a spin column and successively washed with 500 µL of AW-1 buffer and 500 µL of AW-2 buffer. Following centrifugation, the spin column was transferred to a new microtube, subjected to a 2-minute incubation at 50° C, and eluted with 250 µL of AE buffer. The eluted DNA solution was then stored at -20°C for the following analysis.

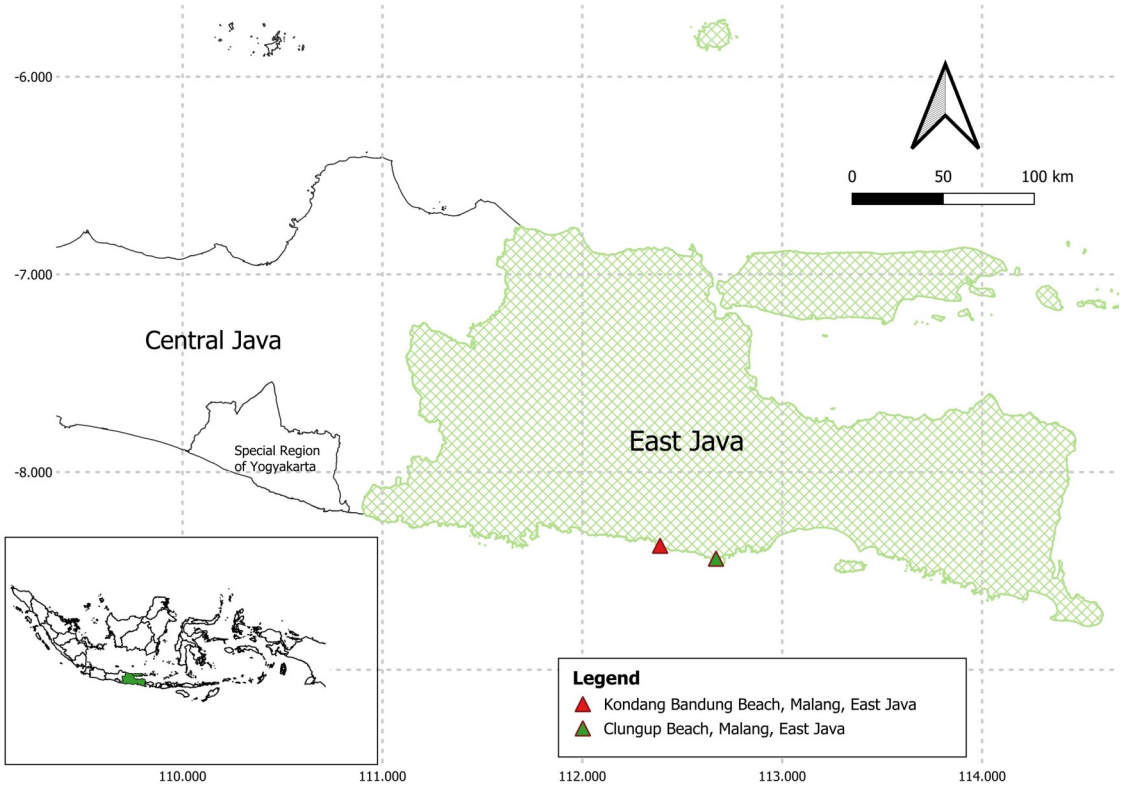

Figure 1. Sampling sites of barred mudskipper populations.

Table 1. Sample location, sample code, geographic reference, and sample size of Indonesian barred mudskippers used in this study.

Location	Sample Code	Latitude (S)	Longitude (E)	Sample Size (N)	References
Kondang Bandung Beach, East Java	MSK	8°22'20.0"	112°23'19.5"	4	This Study
Clungup Beach, East Java	MSC	8°26'15.1"	112°40'07.0"	6	This Study
Pasir Mendit Beach, Yogyakarta	MSP	7°53'39.6"	110°01'10.1"	3	Febrianti et al. 2023
Bogowonto Lagoon, Yogyakarta	MSB	7°53'58.1"	110°01'54.2"	3	Arisuryanti et al. 2018
Baros Beach, Yogyakarta	MBR	8°00'27.4"	110°17'02.2"	9	Aji & Arisuryanti 2021
Tekolok Estuary, West Nusa Tenggara	MSL	8°20'30.0"	116°42'31.0"	10	Rha'ifa et al. 2021

Barcode Marker Amplification

The *COI* mitochondrial gene was amplified in the T100 Thermal-Cycler (Biorad) using two universal primers for fish, FishF2 (5'-TCGACTAATCATAAAGATATCGGCAC-3') and FishR2 (5'-ACTTCAGGGTGACCGAAGAATCAGAA-3') (Ward et al. 2005). The PCR reaction was carried out in a 25 µL reaction volume containing 5-50 ng of genomic DNA, 12.5 µL MyTaq HS Red Mix (Bioline), 1 mM $MgCl_2$, 0.6 µM of forward primer and 0.6 µM of reverse primer, and 5.5µL double distilled water (ddH_2O), and 3 µL DNA template. Negative control was established by omitting the template DNA from the reaction mixture to assess the effectiveness of the DNA amplification. The PCR thermal profile followed Arisuryanti et al. (2020) and consisted of: 2 min of pre-denaturation at 95°C, followed by 35 cycles of denaturation at 95° C for 15 sec, annealing at 50°C for 30 sec, and extension at 72°C for 30 sec, with a final extension of 5 min at 72°C.

Electrophoresis and Sanger Sequencing

The electrophoresis of PCR products was performed on a 1% agarose gel stained with Florosafe (Bioline) and buffered with Tris-acetate EDTA (TAE) at 100 volts for 25 minutes. The gel was visualized under ultraviolet light. All amplification products were then transported to LPPT UGM for purification and sequencing. Sanger sequencing reactions were performed on each specimen using both forward and reverse primers.

Sequence Editing & Verification

The DNA sequencing results were edited in GeneStudio and verified using the DNASTAR program with SeqMan and EditSeq (DNASTAR Inc. Madison, USA). The consensus sequencing results were examined using the Nucleotide BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Identification Engine in BOLD (https://www.boldsystems.org/index.php/IDS_OpenIdEngine). The species verification of barred mudskipper was determined by achieving a high BLAST identity percentage paired with the E-value 0.0 and integrating similarity results from the BOLD database to ensure a comprehensive similarity assessment.

Sequence Alignment, Genetic Diversity & Genetic Distance

The *COI* sequence alignment of the barred mudskipper was completed using Opal on Mesquite v.3.51 program (Maddison & Maddison 2018) and ClustalW on the MEGAX program (Kumar et al. 2018). The DnaSP 6.12.01 program was used to calculate genetic diversity (Rozas et al. 2017). The divergences among the major clades were calculated using MEGAX program (Kumar et al. 2018) under the Kimura-2 Parameter (K2P) model.

Phylogenetic Relationship

The phylogenetic tree was constructed using the Neighbor-Joining and Maximum Likelihood methods with Kimura-2 Parameter (K2P) substitution model and 1000 bootstraps replications in MEGAX program (Kumar et al. 2018) and Bayesian Inference using the BEAST program (Suchard et al. 2018). The Akkaike Information Criterion (AIC) implemented in jModelTest 2.1.10 (Darriba et al. 2012) was used to determine the best fit evolutionary model. The most suitable sequence substitution model for this research is HKY with gamma (HKY + G) on the Akaike Information Criterion (AIC). To estimate the posterior probability distribution, the Markov Chain Monte Carlo (MCMC) method was used for 10 million generations with a sampling frequency of every 1,000 generations. The consensus trees were then visualized in FigTree 1.4.4 (Rambaut 2019).

Haplotype Network & Principal Coordinate Analysis

The haplotype network was generated using PopART v1.7. (Leigh & Bryant 2015) and the Principal Coordinate Analysis (PCoA) was carried out in GenAlEx 6.5 (Peakall & Smouse 2012).

RESULTS

Sequence alignment

A fragment length of 579 bp was successfully generated from the *COI* sequence of this research. The length of the *COI* sequences remained at 579 bp even after adding two additional sequences i.e. *P. novemradiatus* (KU692765) and *B. boddarti* (KU692378) as the outgroups of the phylogenetic tree. The *COI* sequences of *P. argentilineatus* from Clungup Beach

have been deposited in GenBank under accession number PP593608-PP593613 whereas the *COI* sequences of *P. argentilineatus* from Kondang Bandung Beach have been deposited in GenBank under accession number PP593621-PP593624.

Nucleotide Composition

The nucleotide composition of six populations of the barred mudskipper was found to be different, except for the populations found at Pasir Mendit Beach and Bogowonto Lagoon, which had the same composition of C and A (as shown in Table 2). There were small variations in the percentages of T, C, A, and G nucleotides among the populations, with differences ranging from 0.60% to 0.26%. Additionally, the total composition of A and T was found to be greater than the composition of G and C. The GC content, or the proportion of guanine and cytosine, varied slightly among all populations, ranging from 42.31% to 42.70%.

Table 2. Mean of nucleotide composition (%) of *COI* mitochondrial gene among six populations of Indonesian barred mudskippers in this study.

Population	T	C	A	G	A+T	G+C
Pasir Mendit (MSP)	32.47	25.79	25.22	16.52	57.69	42.31
Bogowonto (MSB)	32.41	25.79	25.22	16.58	57.63	42.37
Baros (MBR)	31.95	26.25	25.41	16.39	57.36	42.64
Kondang Bandung (MSK)	31.87	26.38	25.43	16.32	57.30	42.70
Clungup (MSC)	32.30	26.05	25.22	16.44	57.51	42.49
Tekolok (MSL)	32.38	25.98	25.09	16.55	57.48	42.52

Phylogenetic Tree and Genetic Distance

A total of 37 sequences were used in the phylogenetic analysis, including 2 samples from GenBank as outgroups: *P. novemradiatus* (KU692765) *and B. boddarti* (KU692378). The phylogenetic tree topology of the NJ, ML, and BI trees was found to be identical, therefore only the BI tree that was presented in this study (Figure 2). The phylogenetic tree showed three distinct clades, Clade A (n=18), Clade B (n=9), and Clade C (n=8). The lowest genetic distance was 2.41% between clade B and C, while the highest was 6.12% between clade A and C (Table 3).

Table 3. Mean percentage nucleotide sequence divergence of a 579 bp fragment of the *COI* mitochondrial gene among Indonesian barred mudskippers in this study.

	Clade A	Clade B	Clade C
Clade A			
Clade B	5.51 (5.24-6.00)		
Clade C	6.12 (5,62-6,97)	2.41 (1.76-2.84)	

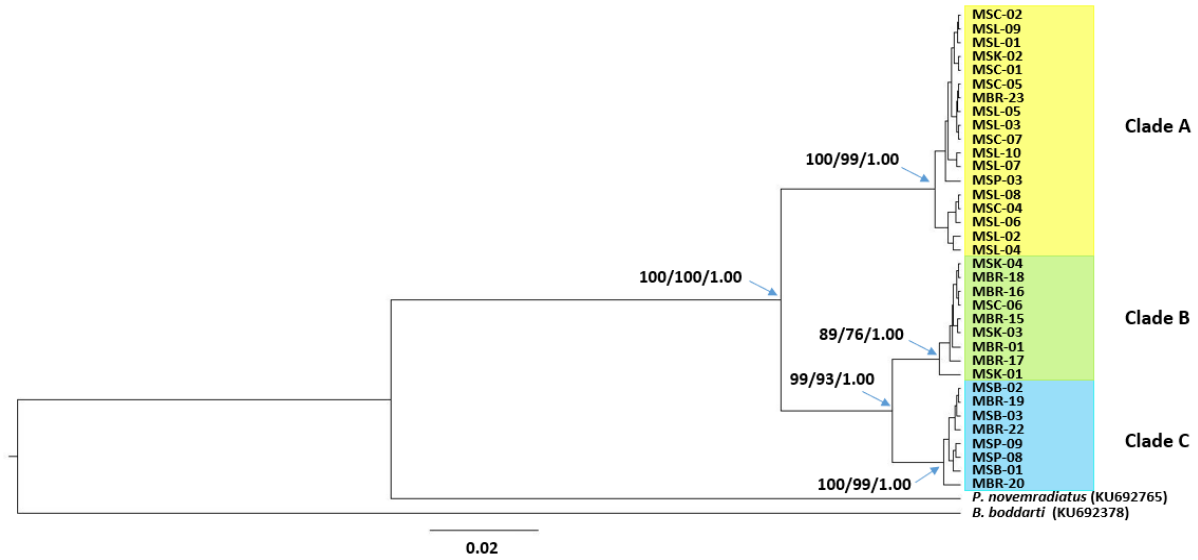

Figure 2. Phylogenetic tree of Indonesian barred mudskippers (*P. argentilineatus*) and outgroup inferred from *COI* mitochondrial gene sequences (579 bp) based on Neighbour-Joining (NJ), Maximum-Likelihood (ML), Bayesian Inference (BI) topology. The number of each node represents bootstraps for NJ and ML and posterior probabilities for Bayesian Inference.

Genetic diversity of Indonesian barred mudskippers based on the *COI* gene

The thirty-five barred mudskipper *COI* sequences revealed 49 variable sites, 40 parsimony informative sites, and 18 haplotypes. No insertions, deletions, or stop codons in the sequences were detected. The transitional pairs (si= 43) were more frequent than trans-versional pairs (sv= 8). The overall mean of haplotype diversity (Hd) was 0.884±0.043 and nucleotide diversity (π) was 0.03223±0.00158. The highest haplotype diversity was found in Clade C (0.884±0.043), while the lowest was found in Clade A (0.634±0.127). For the nucleotide diversity, the highest was detected in Clade A (0.00293±0.00073), whereas the lowest was in Clade B (0.00192±0.00063) (Table 4). The polymorphic sites were visualized in three distinct colours, representing three different clades: Clade A (green), Clade B (yellow), and Clade C (blue) (Table 5-6). A unique polymorphic site, number 297, was detected with different nucleotides in each clade: Clade A (nucleotide C), Clade B (nucleotide A), and Clade C (nucleotide G).

Haplotype Network and Principal Coordinate Analysis (PCoA)

Both the haplotype network and PCoA (Figure 3 & 4) revealed three distinct clusters and haplogroups representing three different clades, but there was a lack of clear separation by geographical region, indicating overlap among the three genetically divergent lineages. Haplogroup A consisted of 7 haplotypes (HA1-HA7), Haplogroup B consisted of 5 haplotypes (HB1-HB5), and Haplogroup C consisted of 6 haplotypes (HC1-HC6). Haplogroup A and B were separated by 27 mutation points, while Haplogroup B and C were separated by 10 mutation points. The most widespread haplotype, HA1 (n = 11), was shared among 4 populations (Tekolok Estuary, Baros Beach, Bogowonto Lagoon, and Clungup Beach).

DISCUSSION

In this study, we present evidence of the existence of three genetically distinct monophyletic clades (A, B, and C) within the *P. argentilineatus* species from six different populations in Indonesia. Using maximum sup-

Table 4. The genetic diversity of barred mudskippers based on the *COI* mitochondrial gene.

Sample	Number of Samples	Haplotype Number	Haplotype Diversity	Nucleotide Diversity
Clade A	18	7	0.634±0.127	0.00293±0.00073
Clade B	9	5	0.722±0.159	0.00192±0.00063
Clade C	8	6	0.893±0.111	0.00271±0.00052
All Populations	35	18	0.884±0.043	0.03223±0.00158

Table 5. Polymorphic sites of Indonesian barred mudskippers inferred from *COI* gene (site 06 to 297).

	Nucleotide sites number	06	19	26	34	54	63	66	87	93	111	114	147	180	219	222	228	234	237	246	252	261	279	288	297
CLADE A	MSC-01	G	C	G	T	T	C	T	A	C	C	A	T	A	C	G	T	T	T	T	A	C	G	A	C
	MSC-02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSC-04	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	MSC-05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSC-07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSK-02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-01	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-02	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-04	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-06	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	MSL-07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	MSL-09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-10	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MBR-23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSP-03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CLADE B	MSK-01	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	A
	MSK-03	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MSK-04	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MBR-01	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MBR-15	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MBR-16	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MBR-17	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MBR-18	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
	MSC-06	A	.	.	C	C	T	C	.	T	T	.	C	.	T	A	C	C	A	C	.	T	A	C	.	A
CLADE C	MSB-01	A	.	T	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G
	MSB-02	A	.	.	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G
	MSB-03	A	G	.	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G
	MSP-08	A	.	.	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G
	MSP-09	A	.	.	.	C	T	C	.	T	T	.	C	G	T	A	C	C	A	.	G	T	A	C	.	G
	MBR-19	A	.	.	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G
	MBR-20	A	.	.	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G
	MBR-22	A	.	.	.	C	T	C	.	T	T	.	C	.	T	A	C	C	A	.	G	T	A	C	.	G

port from bootstraps (100% in NJ/ML) and posterior probability (1.00 in BI), we were able to construct an accurate phylogenetic tree. Our analysis revealed a deep genetic divergence of 2.41-6.12% among these clades, with Clades B and C being considered conspecific based on the intraspecies genetic distance threshold of 3.5% proposed by Zemlak et al. (2009). Clade A, however, was found to have a genetic divergence of 5.51-6.12%, suggesting the presence of a cryptic species. This finding is consistent with previous genetic studies of *P. argentilineatus* from East African and Indo-Malayan populations using nuclear markers (*rag1*) and mitochondrial markers (*D-loop* and *16S* rRNA) which revealed the existence of at

Table 6. Polymorphic sites of Indonesian barred mudskippers inferred from the *COI* gene (site 333 to 573).

	Nucleotide sites number	333	335	336	337	339	399	432	438	441	450	453	456	457	478	489	498	513	516	517	528	531	543	561	573
C	MSC-01	A	T	T	C	A	T	T	C	T	C	A	C	C	C	T	G	C	A	T	T	T	T	T	T
L	MSC-02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A	MSC-04	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.
D	MSC-05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E	MSC-07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSK-02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A	MSL-01	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-02	.	.	C	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.
	MSL-03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-04	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.
	MSL-05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-06	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	.	A	.	.	.	.	.	.	.
	MSL-07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-08	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.
	MSL-09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSL-10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MBR-23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	MSP-03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.
C	MSK-01	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	.	C
L	MSK-03	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	.	C
A	MSK-04	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	.	C
D	MBR-01	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	.	.	.	C
E	MBR-15	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	G	C	.	.	C
	MBR-16	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	.	C
B	MBR-17	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	C	C
	MBR-18	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	.	C
	MSC-06	G	.	.	T	.	C	C	.	.	T	G	.	T	.	.	.	A	C	C	.	C	.	.	C
C	MSB-01	G	C	.	T	G	.	C	T	.	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
L	MSB-02	G	C	.	T	G	.	C	T	C	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
A	MSB-03	G	C	.	T	G	.	C	T	C	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
D	MSP-08	G	C	.	T	G	.	C	T	.	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
E	MSP-09	G	C	.	T	G	.	C	T	.	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
	MBR-19	G	C	.	T	G	.	C	T	C	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
C	MBR-20	G	.	.	T	G	.	C	T	.	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C
	MBR-22	G	C	.	T	G	.	C	T	C	T	.	T	T	T	.	.	A	C	C	.	.	C	.	C

least three molecularly distinct cryptic or pseudo-cryptic species (Polgar et al. 2014). Furthermore, Polgar (2014) proposed that one of these cryptic species is morphologically consistent with *P. sobrinus* Eggert, one is morphologically consistent with *P. vulgaris* Eggert, and one is morphologically consistent with *P. argentilineatus* Valenciennes. Our discovery highlights the occurrence of cryptic species with preserved morphologies in the genus *Periophthalmus*, a genus with a long evolutionary history of more than 30 million years under strong stabilizing selection in *Periophthalmus* habitats (Polgar et al. 2014). Additionally, the use of a different molecular marker in this study (*COI*) compared to previous studies by Polgar et al. (2014) using *rag1*, *D-loop*, and *16S* markers and personal observation of Polgar (2014) yielded the same conclusion, further demonstrating the robustness of our findings. This highlights the importance of using multiple markers and approaches in species delimitation for a more comprehensive understanding of the diversity within a genus.

We discovered an overall high level of haplotype diversity (Hd ≥ 0.5) and nucleotide diversity ($\pi \geq 0.01$) in six different populations of Indonesian barred mudskipper. In contrast, each clade of A, B, and C sepa-

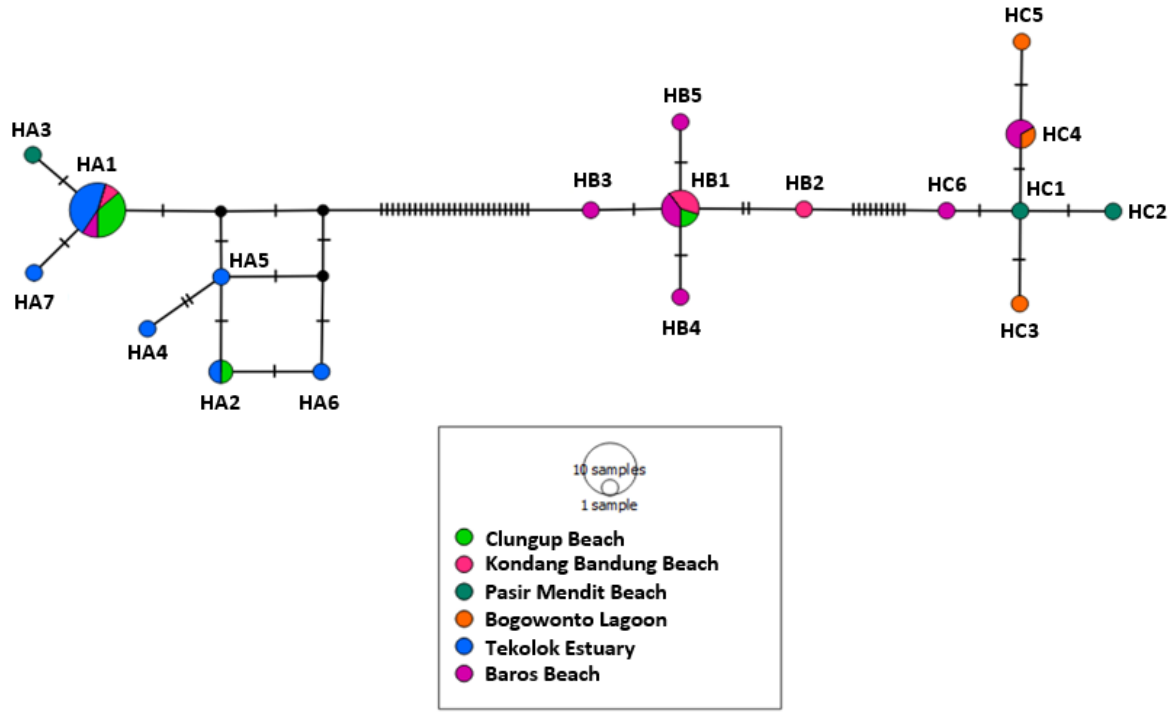

Figure 3. Median-joining haplotype network of Indonesian barred mudskippers based on *COI* mitochondrial gene sequences (579 bp). The size of the circles reflects the number of samples. Lines connecting haplotypes show evolutionary routes between haplotypes, whereas short stripes represent mutation points between haplotypes. Each colour represents six different populations of barred mudskipper in this study.

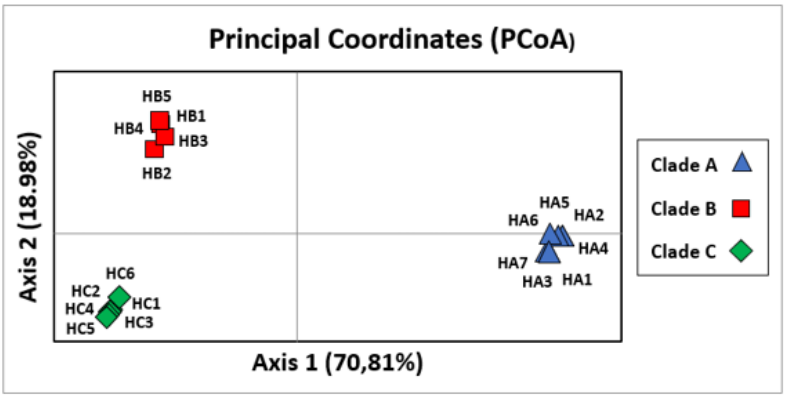

Figure 4. Principal Coordinate of Analysis (PCoA) of Indonesian barred mudskippers based on *COI* mitochondrial gene sequences (579 bp).

rately showed a high level of haplotype diversity (Hd ≥ 0.5) and low nucleotide diversity ($\pi \leq 0.01$), which is consistent with the pattern of a population bottleneck followed by a rapid expansion (Grant & Bowen 1998) and may reflect the evolutionary history of the barred mudskipper. The ratio of transitions to transversions in this study is similar to that observed in other mtDNA studies of teleost fish (Bingpeng et al. 2018; Wu et al. 2018), which suggests that the molecular evolution of the *COI* gene in barred mudskippers is consistent with that of other fish. The T>C>A>G nucleotide composition pattern is also consistent with other fish families that have been studied using the *COI* gene as a DNA barcoding marker (Bingpeng et al. 2018; Wu et al. 2018; Linh et al. 2019). An interesting finding of our study was the identification of a unique polymorphic site (number 297) in the *COI* gene, which could serve as a genetic marker for each barred mudskipper clade.

The haplotype network analysis revealed a lack of clear separation by geographical region, indicating an overlap of the three genetically divergent lineages. Haplotype HA1, included in haplogroup A, was the most widespread haplotype (n = 11) and was shared among four populations (Tekolok Estuary, Baros Beach, Bogowonto Lagoon, and Clungup Beach). These four populations are relatively far apart, with the greatest distance between Bogowonto Lagoon (Java) and Tekolok Estuary (Lombok, Lesser Sunda) being over 700 km, which implies that there should be little or no gene flow between these populations. Based on this result, we hypothesize that in the past, there was a spatial linkage that connected all of these populations. There are many possibilities that could be influenced, for example by historical events, such as sea level or ocean currents, which facilitate the dispersal and gene flow between distant populations (Miller et al. 2005; García-De León et al. 2018). Additionally, the Sunda Shelf, which covers a large area of western Indonesia, may have played a crucial role in this process. This shallow marine shelf has been known to have experienced significant changes in sea level in the past and may provide a suitable habitat for the mudskippers. The amphibious nature of the mudskipper populations would have allowed them to migrate and establish themselves on both islands during periods of low sea level. Once sea levels rose again, the connection was lost, but the mudskipper population had already spread and established itself on both islands, leading to the shared genetic haplotype observed in our study. This hypothesis has been proposed by multiple studies to explain the gene flow between the mainland and the Sunda Islands of various fish populations (Dodson et al. 1995; Nelson et al. 2000; McConnell 2002). Further studies, such as population genetic modelling and historical demographic analysis, are needed to reveal this hypothesis and to gain a deeper understanding of the evolutionary history of the Indonesian barred mudskipper.

CONCLUSIONS

This study provides evidence for the presence of three genetically distinct clades (A, B, and C) of barred mudskipper populations in Indonesia, with a deep genetic divergence of 2.41% to 6.12% among the clades. This genetic divergence further suggests the presence of cryptic species within the genus *Periophthalmus*, which is consistent with previous studies on the genus. The high level of haplotype diversity and low nucleotide diversity observed in each clade is indicative of a population bottleneck followed by a rapid expansion. Furthermore, the lack of clear separation by geographical region in the haplotype network analysis suggests that historical events, such as sea level changes or oceanic currents, may have facilitated gene flow between distant populations in the past. These findings highlight the importance of using molecular approaches in species delimitation and the need for further studies to gain a deeper understanding of the evolutionary history of the Indonesian barred mudskipper.

AUTHOR CONTRIBUTION

The laboratory work for this study was conducted by a team of researchers including KWA, HH, IPS, FAR, and DF. They were responsible for collecting samples, extracting DNA, amplifying it using PCR, analysing the results using agarose gel electrophoresis, and writing the manuscript. The overall design and planning of the study, and writing the manucript were led by TA and DSP, who also supervised the entire process.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest. The authors are responsible for the article's content and writing.

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Research Article

SiDREB2-based SNAP Marker-Assisted and Multi-Trait Selection in The Early Generation of Foxtail Millet (*Setaria italica* L. Beauv.)

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ABSTRACT

Setaria italica L. or foxtail millet is known for its nutritious grains and adaptability to unfavorable environmental conditions. High productivity, early heading, medium stature, and tolerance to drought- or salinity stress are among the breeding objectives for foxtail millet. The objective of this study was to select F_3 families of foxtail millet from the cross of Botok-10xICERI-6 by weighted selection index and assisted by *SiDREB2*-based SNAP marker. Genotyping of 178 F_3 families using the *SiDREB2*-based SNAP marker resulted in 29 A/A genotypes, 121 A/G genotypes, and 28 G/G genotypes. Further evaluation was conducted on 48 F_3 families consisting of 27 A/A genotypes and 21 A/G genotypes in an augmented randomized complete block design together with their parental genotypes (Botok-10xICERI-6) and three check genotypes (Mauliru-2, NTB-1, and Toraja). Plant height and heading time had high broad-sense heritability, whereas grain weight per plant had a moderate broad-sense heritability. Ten potential F_3 families were selected based on a weighted selection index with 20% intensity, comprised of seven A/G genotypes and three A/A genotypes with a weighted selection index ranging from 0.84 to 3.76. The F_3 family with pedigree numbers B10I6-15-136, B10I6-15-161, and B10I6-15-70 with A/A genotypes are considered putative transgressive segregants and could be continued to the next generation for further breeding process.

INTRODUCTION

Millet is a group of underutilized small-seeded cereals from the Panicoidae subfamily commonly cultivated in areas with water scarcity (Panchal et al. 2023). India, African countries, and China are the top three global millet producers (FAO 2021). One of the major millet produced globally is foxtail millet (*Setaria italica* L. Beauv) which ranks second after pearl millet (*Pennisetum glaucum*) (Panchal et al. 2023). The other members of millet include barnyard millet, finger millet, kodo millet, little millet, and proso millet (Saini et al. 2021). Foxtail millet is

considered a functional food due to its nutritional benefits, including its low glycemic index and high contents of protein, dietary fiber, and antioxidant in its grain (Arora et al. 2023). Additionally, several health benefits have been reported for foxtail millet, including cancer (Zhang & Liu 2015) and cardiovascular disease (Jali et al. 2012) prevention. Broad adaptation of foxtail millet to unfavorable environmental conditions, including drought (Xiao et al. 2021) and salinity (Ardie et al. 2015; Han et al. 2022) has increased the importance of this species in marginal areas. Despite the remarkable benefits of this species, foxtail millet is not a popular food crop in Indonesia.

Recombination breeding through hybridization is a conventional yet useful strategy for generating a superior variety (Al-Khayri et al. 2019). However, the self-pollinated nature, floral morphology, tiny size of the flower, and anthesis behavior are the main challenges in the hybridization of foxtail millet (Moharil et al. 2019; Nagaraja et al. 2023) leading to no Indonesian superior variety of foxtail millet has been released to date. Nugroho (2020) induced male sterility in foxtail millet by warm water treatment to facilitate artificial hybridization of Indonesian local foxtail millet genotypes, namely Botok-10 and ICERI-6. Botok-10 is a local foxtail millet genotype from East Nusa Tenggara with relatively high potential productivity but with tall plants, and late heading time. Meanwhile, ICERI-6 is one of the foxtail millet collections in the Indonesian Cereals Research Institute (ICERI) with moderate plant height, and early heading time but low potential productivity (Ratnawati et al. 2024). Furthermore, molecular assessment using the *SiDREB2*-based SNAP marker categorized ICERI-6 as a tolerant genotype, while Botok-10 as a sensitive genotype to salinity or drought stress (Widyawan et al. 2018). A suitable selection strategy is necessary to identify progenies with high productivity, moderate plant height, early heading, and tolerance to drought or salinity stress from the recombination of Botok-10xICERI-6.

One of the selection methods commonly applied for multiple traits is weighted index selection, in which relative weights are used for traits of interest (Moeinizade et al. 2020). A weighted index selection based on productivity, heading time, and plant height was used to select superior F_2 individuals from the crosses of ICERI-5 x Botok-10 (Sintia et al. 2023). However, this method was not able to identify F_2 individuals potentially tolerant to drought or salinity stresses. Plant abiotic tolerance evaluation requires proper experimental design and replications for an accountable result (Negrão & Julkowska 2020). Therefore, phenotypic selection for abiotic stress tolerance is impractical to be performed in the early generation of a segregating population. Marker-assisted selection (MAS) with proper molecular markers is expected to overcome such challenges in early-generation selection (Hasan et al. 2021).

The dehydration-responsive element binding (DREB) is a plant transcription factor involved in the complex regulatory tolerance mechanisms to drought and salinity stresses in many plants (Singh & Chandra 2021). A *DREB2* homolog in foxtail millet, *SiDREB2*, was reported to possess single nucleotide polymorphism (SNP) at the 558^{th} nucleotide (an A/G substitution), and this SNP was further associated with drought tolerance in foxtail millet (Lata et al. 2011). A *SiDREB2*-based single nucleotide amplified polymorphism (SNAP) marker was further developed by Widyawan et al. (2018) to estimate the tolerance level of foxtail millet to drought or salinity. As part of foxtail millet breeding through hybridization, the objective of this study was to select

F_3 families from the cross of Botok-10xICERI-6 by a weighted selection index and assisted by *SiDREB2*-based SNAP marker.

MATERIALS AND METHODS

SiDREB2-based SNAP marker-assisted selection of F_3 family derived from Botok-10xICERI-6 cross

Genetic materials

The parental genotypes Botok-10 (a local foxtail millet genotype from East Nusa Tenggara, Indonesia) and ICERI-6 (a collection of Indonesian Cereals Research Institute, ICERI), and 178 F_3 families from the cross of Botok-10 and ICERI-6 were used as genetic materials in this experiment. Seeds from each parental genotype and F_3 family were sown in two tray holes with ten seeds per hole in seedling trays containing compost and manure (1:1, v/v). The shoot parts of 14-day-old seedlings were harvested as a bulk sample (10-20 seedlings per F_3 family number) and were preserved in a 2 mL microtube containing 700 µL CTAB (Cetyl-Trimethyl Ammonium Bromide) buffer at -20°C for further DNA isolation.

Total Genomic DNA isolation and DNA amplification.

The CTAB method (Doyle & Doyle 1990) was used to extract total genomic DNA from the shoot parts of 14-day-old seedlings with slight modification namely, we exclude the use of 0.2% (v/v) 2-mercaptoethanol in the lysis buffer. The *SiDREB2*-based SNAP markers consisted of two forward primers and one reverse primer as listed in Table 1. The PCR reaction with a total volume of 10 µL consisted of genomic DNA (2.5 µL, 12 ng.µL^{-1}), forward (SD2-558-SNP-A or SD2-558-SNP-G) and reverse primer (SD2-558-SNP-Rev) (2.5 µL, 10 pmol), and 5.0 µL of 2× PCR mix (KAPA2G Fast HotStart ReadyMix, Sigma-Aldrich, Germany). The PCR was performed using Esco's Swift Maxi Thermal Cycler (Esco Technologies, Singapore) following the PCR profile reported by Ratnawati et al. (2024).

Analysis of molecular data

Successful amplification using forward primers (SD2-558-SNP-A or SD2-558-SNP-G) and reverse primer (SD2-558-SNP-Rev) resulted in a 300 bp amplicon. Amplicons were analyzed by electrophoresis at 90 volts for 40 minutes in 1x TAE buffer on 1.5% (w/v) agarose gel. The agarose gels were immersed in ethidium bromide solution (0.5 µg.mL^{-1}) prior to gel visualization using a UV transilluminator (AlphaImager® Mini). The *SiDREB2*-based SNAP marker-assisted selection was conducted by evaluating the presence of a 300 bp band for the A allele, G allele, or both A and G alleles in particular F_3 family (Figure 1). The band specific for the G allele appeared in the female parent genotype (Botok-10), while the band specific for the A allele appeared in the male parent (ICERI-6).

Table 1. The *SiDREB2*-based SNAP marker used in this experiment.

Primer name	Nucleotide sequence (5'-3')	Tm (°C)	Primer type
SD2-558-SNP-G	GCAAGTCCGTGGAGGTACTACAG	58.8	Forward
SD2-558-SNP-A	AAGTCCGTGGAGGTACTGCAA	58.3	Forward
SD2-558-SNP-Rev	AGGAACTCAACACACAGGACAACT	57.9	Reverse

Source: Widyawan et al. (2018)

Weighted index selection of Botok-10xICERI-6 derived F_3 family

Plant materials

Fifty F_3 family numbers were further selected from the above 178 F_3 families for further field evaluation. These 50 F_3 families consisted of 29 A/A genotypes and 21 A/G genotypes based on *SiDREB2*-SNAP marker. However, two F_3 family numbers with A/A genotypes failed to grow in the field and further analyses were conducted on the remaining 48 F_3 family. Five check genotypes used include the parental genotypes (Botok-10 and ICERI-6) and three local genotypes (Toraja, NTB-1, and Mauliru-2). The Toraja genotype originated from Sulawesi, the NTB-1 genotype originated from West Nusa Tenggara (NTB-1), and the Mauliru-2 genotype originated from East Nusa Tenggara.

Procedure

The experiment was conducted from January to May 2023 in the Cikabayan Bawah Experimental Station of IPB University, Bogor, Indonesia (6°33'24.23"S, 106°43'33.4"E). The agro-climates conditions during this experiment were recorded to be 21.43°C average temperature, 87.82% average humidity, and 1,100 mm per month average rainfall (BMKG 2023). This experiment was arranged in an augmented randomized complete block design with five replicates. Each replicate was a 40 m x 0.8 m size block consisting of ten F_3 family numbers and five check genotypes. Each F_3 family number and check genotype were planted in three rows, resulting in 45 planting rows per block. Each row consisted of eight plants, with plant spacing of 75 cm x 10 cm.

Seeds were subjected to hot water treatment to reduce the risk of seed-borne fungi as described by Parlindo et al. (2022). Treated seeds were then directly sown in planting holes containing 3% Carbofuran. Fertilizers of SP-36 (150 kg.ha^{-1}) and KCl (75 kg.ha^{-1}) were applied two weeks after planting (WAP), while urea was applied two times at 2 and 6 WAP with the rate of 150 kg.ha^{-1} at each application. A plant net was installed at 2 WAP to prevent crop loss due to birds.

The observation was conducted for 11 characters according to the UPOV descriptor (UPOV 2013) on the following characters: plant height (cm), the length and width of flag leaf (cm), stem diameter (mm), heading time (DAP), harvest time (DAP), 100-grain weight (g), the length (cm) and weight (g) of main panicle, main panicle grain weight (g), and grain weight per plant (g).

Data analysis

The obtained phenotypic data were analyzed for variance components estimates, phenotypic coefficient of variation (PCV), genotypic coefficient of variation (GCV), broad-sense heritability (h^2_{bs}), and weighted selection index using SAS, Minitab 19, and Microsoft Excel software. The variance component estimates include phenotypic variance (σ^2_p), environmental variance (σ^2_e), and genetic variance (σ^2_g) as described by Mahmud & Kramer (1951). The PCV and GCV were divided into three categories according to Knight (1979), namely low (0-10%), moderate (10 -20%), and high (>20 %). The classification of broad-sense heritability followed the classification of Stansfield (1991): high (50% ≤ h^2_{bs} < 100%), moderate (20% ≤ h^2_{bs} < 50%), and low (0 ≤ h^2_{bs} < 20%). An equation reported by Sintia et al. (2023) was used to calculate the weighted selection index (SI) as follows: SI = – plant height – heading time + (3 x grain weight per plant).

Scatter plots were built using Microsoft Excel based on the selection index (Y-axis) and means of standard deviation of the three

targeted traits (X-axis). The mean of the standard deviation of five check genotypes is indicated by the vertical dashed line, while the mean of SI calculated from 48 F_3 families is shown by the horizontal dashed line. Only 25 F_3 families with a minimum of 12 observable plants per family were mapped in the plot.

RESULTS AND DISCUSSION

SiDREB2-based SNAP marker-assisted selection of F_3 family derived from Botok-10xICERI-6 cross

Molecular markers have been widely used to improve abiotic stress tolerance in crops (Younis et al. 2020). Our study suggests that marker-assisted selection using the *SiDREB2*-based SNAP marker is a simple method to select potentially drought- or salinity-tolerant lines in an early segregating population. Figure 1 shows the representative visualization of amplicons using a particular primer pair. The A/G genotype was indicated by 300 bp amplicons produced by both SD2-558-SNP-A/Rev and SD2-558-SNP-G/Rev primer pairs. The A/A genotype only showed the 300 bp amplicon produced by SD2-558-SNP-A/Rev primer pair, while the G/G genotype only showed the 300 bp amplicon produced by SD2-558-SNP-G/Rev primer pair. The 300 bp amplicons produced by the SD2-558-SNP-A primer indicate tolerant genotypes, while amplicons produced by the SD2-558-SNP-G primer indicate sensitive genotypes (Widyawan et al. 2018; Ratnawati et al. 2024). Drought- or salinity-tolerance estimation using *SiDREB2*-based SNAP markers on 178 F_3 families derived from Botok-10xICERI-6 cross resulted in 29 A/A genotypes, 121 A/G genotypes, and 28 G/G genotypes.

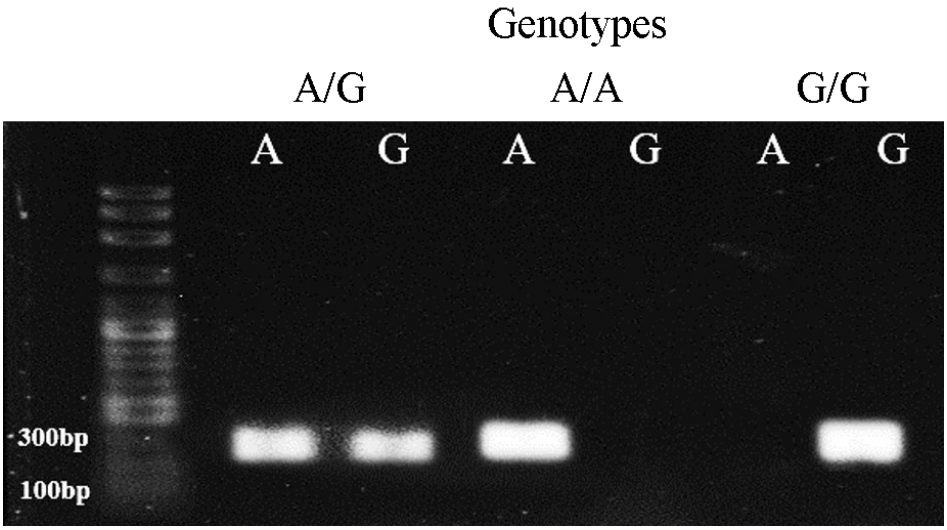

Figure 1. Representative gel electrophoresis result of A/G, A/A, and G/G genotypes using the *SiDREB2*-based SNAP marker.

There were no significant differences in plant height, heading time, and grain weight per plant between the A/A, A/G, and G/G genotypes (Table 2), indicating that there were no associations between the 558th base variation of the *SiDREB2* gene and the observed phenotypic traits at the F_2 generation under non-stress conditions. The selection index calculated based on the three previously mentioned traits showed that the A/A genotype's selection index ranged between -3.69 to 12.67, while the A/G and G/G genotypes' selection index ranged from 5.79 to 15.39 and -5.69 to 11.13, respectively. Lata et al. (2011) reported that *SiDREB2* gene expression increased under drought or salinity conditions, indicating that the effect of the *SiDREB2* allele would be more

pronounced under stress conditions. Therefore, the effect of the 558th base variation of the *SiDREB2* gene needs to be further evaluated under stress- and no-stress conditions in the later generation. Further field evaluations were then conducted on 50 F_3 family numbers having the A allele (A/A or A/G genotypes) with the highest selection index from the 178 F_3 family numbers evaluated above.

Weighted index selection of Botok-10xICERI-6 derived F_3 family

Some of the major traits targeted in the foxtail millet breeding program are high yield, early heading time, medium stature, and tolerance to drought/ salinity stress. Shorter plants and earlier heading times in comparison to the female parent (Botok-10) were observed in the F_3 population (Table 3). Furthermore, the F_3 population showed higher grain weight per plant than the male parent (ICERI-6), indicating that there are potential segregants with higher yields in the F_3 population. The F_3 population showed higher average values of the length and width of flag leaf, and 100-grain weight than both parents, while the remaining traits showed average values between the two parents.

The F_3 population in this study showed a lower standard deviation for plant height than the two parents, while the standard deviation for heading time, and grain weight per plant were in between the two parents. This indicates that although the phenotypic variation for these target traits was lower than at least one of the parental genotypes, further selection is still necessary for more uniform performances in the next generation.

In order to better understand the extent of genetic variability in the F_3 population, the variance components, phenotypic and genotypic coefficient of variation, and broad-sense heritability were calculated and presented in Table 4. Traits with high GCV values indicate high levels of

Table 2. The effect of A/A, A/G, and G/G genotypes on plant height, heading time, and grain weight per plant of F_2 generation derived from the Botok-10xICERI-6 cross.

Traits	Genotype			Kruskal-Wallis test
	A/A	A/G	G/G	
Plant height (cm)	69.30	69.28	69.21	ns
Heading time (DAP)	122.43	122.83	127.75	ns
Grain weight per plant (g)	13.20	13.47	14.50	ns

Note: DAP: days after planting, number of F_3 families: A/A=29, A/G=121, G/G = 28; ns = not significant

Table 3. Mean value and standard deviation of Botok-10, ICERI-6, and F_3 population from the cross of Botok-10 and ICERI-6.

Traits	Mean and standard deviation		
	Botok-10	ICERI-6	F_3 (Botok-10xICERI-6)
Plant height (cm)	211.04 ± 26.68	116.36 ± 21.67	125.30 ± 20.24
Flag leaf length (cm)	31.74 ± 5.21	34.03 ± 4.80	35.53 ± 6.40
Flag leaf width (cm)	2.34 ± 0.37	2.36 ± 0.41	2.46 ± 0.52
Stem diameter (mm)	6.29 ± 0.83	5.24 ± 1.08	5.80 ± 1.15
Heading time (DAP)	95.69 ± 6.58	64.90 ± 2.61	76.08 ± 4.89
Harvest time (DAP)	127.00 ± 0.00	112.03 ± 2.95	109.78 ± 6.17
100-grain weight (g)	0.22 ± 0.07	0.26 ± 0.09	0.30 ± 0.10
Main panicle length (cm)	22.26 ± 3.24	21.28 ± 3.05	21.46 ± 5.19
Main panicle weight (g)	11.36 ± 3.63	2.47 ± 0.65	4.83 ± 0.54
Main panicle grain weight (g)	7.49 ± 3.09	1.36 ± 0.38	3.56 ± 1.86
Grain weight per plant (g)	7.49 ± 3.09	1.47 ± 0.44	3.74 ± 2.00

Note: DAP: days after planting

genetic variability, whereas traits with low GCV values demonstrate low levels of genetic variability. Meanwhile, the extent of the differences between the PCV and GCV implies the relative significance of genetic and environmental influences on a given trait, with large differences indicating a significant environmental influence and small differences indicating a significant genetic influence (Xu 2021). Therefore, successful selection for targeted traits can be expected from traits with high GCV and with minimum differences between PCV and GCV. Moderate GCV and a small difference between PCV and GCV (6.64) were recorded for plant height, while low GCV and a small difference between PCV and GCV (2.54) were recorded for heading time. Grain weight per plant showed high GCV and a relatively greater difference between PCV and GCV (20.47). These results indicate that environmental influence was more pronounced for grain weight per plant, while genetic influence was more dominant for plant height and heading time. Moreover, moderate to high GCV estimates indicate that selection can be performed based on plant height and grain weight per plant, while the heading time was relatively less varied between F_3 families. Sintia et al. (2023) also reported moderate to high GCV estimates for the plant height and grain weight per plant of an F_2 population derived from the ICERI-5xBotok-10 cross of foxtail millet.

Heritability also needs to be considered in determining effective selection traits. A high value of broad-sense heritability on a particular trait indicates that the total variability of the trait is under genetic control, and selection based on this trait would be advantageous for trait improvement (Schmidt et al. 2019). As shown in Table 4, plant height and heading time have high broad-sense heritability, while grain weight per plant has moderate broad-sense heritability. A previous study of an F_2 population derived from the ICERI-5xBotok-10 cross of foxtail millet by Sintia et al. (2023) showed high broad-sense heritability for grain weight per plant and moderate broad-sense heritability for plant height and heading time. Meanwhile, Anuradha and Patro (2020) reported that flowering time, plant height, and grain yield had the highest heritability values based on their study on eight foxtail millet genotypes in India. These different heritability estimates might be due to different parental genotypes as well as different breeding generations. Altogether, the PCV, GCV, and broad-sense heritability values in this study indicate that a weighted selection index based on the three main target traits would be effective and can be performed accordingly.

Table 4. Variance component, phenotypic coefficient of variation, genotypic coefficient of variation, and broad-sense heritability of F_3 population from the cross of Botok-10 and ICERI-6.

Traits	σ^2_p	σ^2_e	σ^2_g	PCV (%)	GCV (%)	h^2_{bs} (%)	Category of h^2_{bs}
Plant height (cm)	986.423	628.802	532.288	25.024	18.382	53.961	High
Flag leaf length (cm)	37.554	23.222	20.783	17.326	12.889	55.341	High
Flag leaf width (cm)	0.071	0.053	0.033	10.978	7.461	46.192	Moderate
Stem diameter (mm)	0.768	0.720	0.248	15.083	8.568	32.271	Moderate
Heading time (DAP)	65.597	38.097	38.082	10.672	8.132	58.055	High
Harvest time (DAP)	78.324	67.285	29.729	8.037	4.951	37.957	Moderate
100-grain weight (g)	0.004	0.003	0.002	21.511	15.823	54.105	High
Main panicle length (cm)	22.304	27.641	2.341	22.028	7.137	10.497	Low
Main panicle weight (g)	8.456	6.275	3.924	60.456	41.184	46.407	Moderate
Main panicle grain weight (g)	4.541	3.629	1.920	60.025	39.030	42.280	Moderate
Grain weight per plant (g)	4.441	3.641	1.811	56.648	36.181	40.792	Moderate

Note: σ^2_p: phenotypic variance, σ^2_e: environmental variance, σ^2_g: genetic variance, PCV: phenotypic coefficient of variation, GCV: genotypic coefficient of variation, h^2_{bs}: broad-sense heritability, DAP: days after planting

A weighted index selection with an intensity of 20% of 48 F_3 families from the cross of Botok-10 x ICERI-6 resulted in ten F_3 families (Table 5). The top ten F_3 families comprised three A/A genotypes and seven A/G genotypes, with the selection index ranging from 0.84 to 3.76. The selection indexes of the top ten F_3 families were higher than the parental genotypes and all check genotypes, except the Mauliru-2 genotype. The Mauliru-2 genotype showed a considerably high selection index (3.03). Ratnawati et al. (2024) also identified Mauliru-2 as a potential high-yielding genotype compared to the other seven Indonesian foxtail millet genotypes. Given that Mauliru-2 has the G allele for the *SiDREB2* gene, this genotype is potentially developed further as a superior foxtail millet variety through pure line selection for non-stressed areas.

The variability between F_3 individuals within a particular F_3 family can be seen from the mean standard deviation of the three target traits. The top ten F_3 families still showed a greatly varied mean standard deviation from 4.64 to 13.35. The F_3 family with a high selection index and a low mean standard deviation is desirable to be selected and can be classified as putative transgressive segregants. Considering the check genotypes were planted in five blocks, while each F_3 family number was planted in only one block, the mean standard deviation of the check genotypes could be used as a suitable comparison to identify putative transgressive segregants in the F_3 population derived from Botok-10xICERI-6 cross. The scatter plot in Figure 2 shows the distribution of 25 F_3 families based on their selection index and the mean standard deviation of the three target traits used to develop the selection index. Genotypes in quadrants (I) and (IV) are those with selection index values lower than the mean selection index of all F_3 families observed, thus they were considered not potential to be selected further. Meanwhile, genotypes in quadrants (II) and (III) are those with selection index values higher than the mean selection index of all F_3 families observed. The F_3 family with pedigree numbers B10I6-15-136, B10I6-15-161, and B10I6-15-70 with A/A genotypes are considered putative transgressive segregants since they are located in quadrant (I). These three F_3 families have a higher selection index than the mean selection index of all F_3 families and a lower mean standard deviation than the mean standard

Table 5. Weighted index selection results in the F_3 population of Botok-10xICERI-6 cross.

Genotype	F_3 family	Mean and standard deviation			Selection index	Mean SD
		Heading time (DAP)	Plant height (cm)	Grain weight per plant (g)		
A/G	B10I6-15-177	75.34 ± 4.32	83.60 ± 19.49	5.21 ± 3.32	3.76	9.36
A/G	B10I6-15-104	62.11 ± 12.47	86.99 ± 13.70	3.79 ± 1.93	3.45	9.75
A/G	B10I6-15-48	72.47 ± 5.19	117.84 ± 22.89	5.09 ± 2.41	2.79	10.38
A/A	B10I6-15-136	79.17 ± 3.06	139.56 ± 9.50	6.17 ± 2.05	2.65	5.00
A/G	B10I6-15-180	70.19 ± 6.50	127.91 ± 30.96	4.90 ± 1.57	2.48	13.35
A/G	B10I6-15-74	71.71 ± 4.28	136.56 ± 18.45	5.00 ± 1.49	2.11	8.40
A/A	B10I6-15-161	79.95 ± 1.80	129.26 ± 9.30	5.24 ± 2.28	1.51	4.64
A/A	B10I6-15-70	83.94 ± 4.29	188.62 ± 9.72	6.84 ± 4.05	1.23	6.50
A/G	B10I6-15-237	75.69 ± 4.17	115.14 ± 27.86	4.19 ± 1.77	1.07	11.52
A/G	B10I6-15-57	66.48 ± 7.73	125.04 ± 26.21	3.37 ± 1.94	0.84	12.23
G/G	Botok-10	95.69 ± 6.27	211.04 ± 25.62	7.49 ± 2.96	-0.31	11.62
A/A	ICERI6	64.90 ± 2.51	116.36 ± 20.81	1.47 ± 0.42	-1.45	7.91
G/G	Mauliru-2	83.25 ± 3.98	135.96 ± 11.10	6.74 ± 2.37	3.03	5.82
G/G	NTB-1	78.27 ± 4.87	126.06 ± 18.91	3.57 ± 1.93	-0.62	8.57
G/G	Toraja	69.72 ± 1.57	97.02 ± 15.62	2.74 ± 1.43	0.41	6.20

Note: DAP: days after planting; Mean SD = mean of the standard deviation of the three target traits

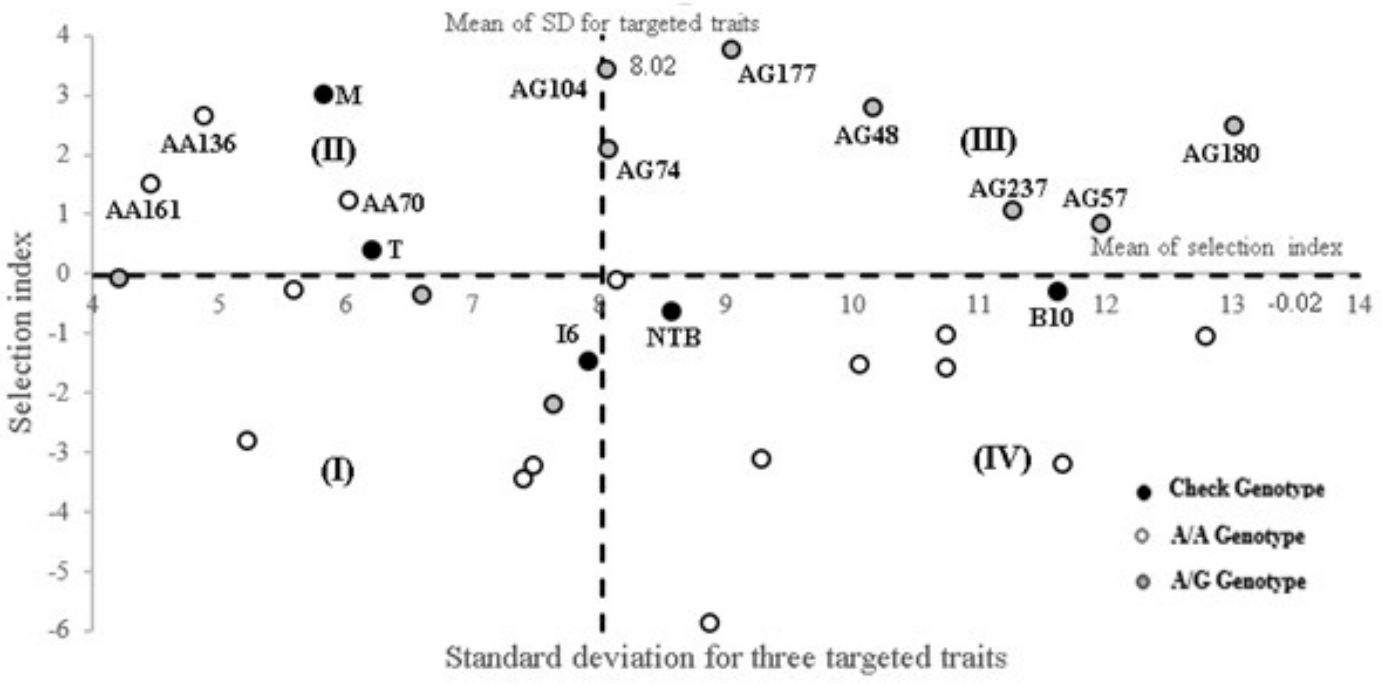

Figure 2. Scatter plot of 25 F_3 foxtail millet family numbers derived from Botok-10xICERI-6 cross. B10 = Botok-10; I6 = ICERI-6; M = Mauliru-2, T= Toraja.

deviation of the check genotypes. Moreover, these three F_3 families have A/A genotypes that indicate their potential tolerance to drought/salinity stress. The Mauliru-2 genotype is also located in quadrant (II), confirming its potential as a superior foxtail millet variety. Although it is located in quadrant (III), the pedigree number B10I6-15-177 with A/G genotype showed the highest selection index. Therefore, F_4 families with A/A genotype generated from F_3 individuals in this pedigree also potential to be evaluated further.

CONCLUSIONS

Multiple-traits selection using *SiDREB2*-SNAP marker combined with weighted selection index on F_3 families of foxtail millet from the cross of Botok-10xICERI-6 identified 10 potential F_3 families with the highest selection index. Three F_3 families with A/A genotypes (pedigree numbers B10I6-15-136, B10I6-15-161, and B10I6-15-70) are considered putative transgressive segregants and are recommended to be continued to the next generation for further breeding process.

AUTHOR CONTRIBUTION

L.K.S.B. performed the genotyping and phenotyping experiments at the F_3 generation, analyzed the data, and wrote the manuscript; D.D.S. conducted experiments on the F_2 generation; W.B.S. and S.W.A. designed research, supervised the research and data analysis, script writing and editing.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Reproductive Behavior and Parental Role of Giant Gourami (*Osphronemus goramy* Lacepède, 1801)

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ABSTRACT
The giant gourami (*Osphronemus goramy* Lacepede, 1801), a popular aquaculture species in Southeast Asia, exhibits unique cooperative biparental care behaviour. To support captive breeding efforts, this study aimed to visually document the reproductive activity of giant gourami, elucidate each stage in detail, and provide insights into the distinct parenting roles of males and females. Underwater cameras were used to observe a breeding pair of gourami in a pond for five days, conducted three times with different pairs during different spawning periods. The male and female contributions to nest building were quantitatively analysed using the T-test, while their parental care involvement was qualitatively assessed and statistically analysed using the Mann-Whitney U test. The results revealed three main phases of giant gourami reproduction: pre-spawning (including adaptation, nest building, and courtship), spawning and fertilisation, and post-spawning with parental care. Our observation confirmed the biparental tendency, with males being more involved in pre-spawning activities and females taking on a prominent role in post-spawning care. In conclusion, males focused on mating preparations and courtship, while females invested more in parental care.

INTRODUCTION
Anabantoids or labyrinth fish (order: Anabantiformes, suborder: Anabantoidei) are a group of ray-finned fish classified by the presence of an air-breathing organ. Members of this order consist of facultative and obligate air-breathing fish, whose vascularized additional breathing organs help them obtain oxygen directly from the air (Mendez-Sanchez & Burggren 2019). Due to their physiology and air-breathing nature, anabantoid fish can thrive in seemingly unfavourable aquatic environments, particularly in shallow and oxygen-deprived waters. Their typical habitat choices have led to the evolution of unique behaviours related to the utilization of their labyrinth organ (Huang & Lin 2016). These behaviours are particularly evident in their reproductive processes, where mating behaviours vary across different genera (Rüber et al. 2006).

Most anabantoids exhibit intricate mating processes, incorporating specific courtship behaviours, nest building, and parental care in most

species. There are two main known modes of parental care in anabantoid fish: mouthbrooders and nest builders, with a few members showing free-spawning tendencies, such as the genus *Anabas* (Zworykin 2012). The predominant behaviour in the group is nest building, which can be categorised into three main types: bubble-nest builders, as observed in most small gourami fish; substrate nest spawning, seen in the genus Sandelia; and nest construction using plant materials, as observed in the genus Osphronemus (Rüber et al. 2004; Rüber et al. 2006; Tate et al. 2017).

Giant gourami is a native species found in the Southeast Asia region. The fish is highly valued as a commercial species in Indonesia, Malaysia, Thailand, and Philippines (Ling 1977; Yap 1999; Rimmer et al. 2013). Its habitat is similar to that of other anabantoid relatives, as it inhabits slow-moving waters such as rivers, lakes, swamps, and other isolated bodies of water (Ismail et al. 2018). Giant gourami is also equipped with a labyrinth organ, allowing it to tolerate low-oxygen environments and even stay out of water for short periods (Cole et al. 1999). Their ability to adapt to harsh environments is reflected in their reproductive behaviour, as extra care is necessary for their offspring to survive in low-oxygen and confined waters filled with potential predators.

By nature, the fish is a nest builder and exhibits a parental care system that involves contributions from both parents. This trait persists even when the fish is bred in captivity (Slembrouck et al. 2020). Farmers have recognised the nest-building and parental care nature of giant gourami and traditionally provide nest supports, such as adding baskets or holes in the breeding pond, for the fish to construct their dwellings (Kristanto et al. 2019). Previous studies have addressed practical methods and analyses regarding breeding techniques (Arifin et al. 2013; Nafiqoh & Nugroho 2013; Amornsakun et al. 2014; Slembrouck et al. 2019). However, the intricacies of such breeding behaviour have been seldomly studied, including the extent of parental role assignment for each parent.

Our study aims to provide a more comprehensive understanding of giant gourami's reproductive behaviour, focusing on the pre-spawning phase, through the mating process, and up to the parental egg care in the post-spawning phase. Several studies have previously used underwater video to capture the behaviours of fish in the wild, including *Lepomis auritus* (Martin & Irwin 2011), *Moapa coriacea* (Ruggirello et al. 2020), and *Thymallus arcticus* (Kupferschmidt et al. 2019). Additionally, previous studies have employed video recording as a tool to investigate the mating behaviors of zebrafish *Danio rerio* (Zempo et al. 2021) and American poeciliid *Brachyhaphis olomina* (Garita-Alvarado et al. 2018) in greater detail. Our methods similarly involve video recordings using underwater cameras to visually capture and provide evidence of the mating behavior of giant gourami. The resulting output would offer visual evidence of the intricate phases of mating behavior that have been discussed by local farmers and other practitioners for years.

We also identify the extent of reproductive roles performed by each brood parent by collecting quantitative data based on their behaviour. Quantitative description of fish behaviour has been employed for anabantoid fish studies previously by Miller & Hall (1968). The analyses were also recently employed for aquaculture studies (Settle et al. 2018; An et al. 2021). By observing the mating process, we seek to elucidate the behaviour of both sexes in each phase, from courting to caregiving periods. Furthermore, we emphasize the analysis of the distinctive roles played by each sex throughout the entire reproductive period. Behavioural studies such as steps of mating behaviour could be a stepping stone to further investigate their neurobiological functions regarding to their trigger and

response to environmental and social cues (Mes et al. 2018; Tripp et al. 2020). Understanding fish behaviour is essential for advancing intelligent aquaculture system and enhancing breeding efficiency (Du et al. 2022). Basic knowledge about the behavioural intricacies of mating process in fish is ultimately a prerequisite to develop an intensive and commercial level aquaculture system (Araujo et al. 2022).

We hope that our results will establish a solid foundation of knowledge on giant gourami breeding. The development and improvement of new fish breeding techniques require a thorough understanding of the complete reproductive process of the fish. Therefore, it is crucial to build a knowledge base from the ground up, particularly regarding the natural behaviours of endemic fish such as the giant gourami.

MATERIALS AND METHODS

Broodstock selection and preparation

The experiment was conducted in fish farm owned by a local gourami breeder and farmer in Duku village, Minggir district, Sleman Regency, in The Special Region of Yogyakarta. The giant gourami broodstock was also obtained from said farm. Three pairs of giant gourami were selected and prepared for mating. The parent fish's qualities were evaluated using Indonesian standards for giant gourami broodstock (SNI 2000) which stipulates that the fish must be in good health, have a complete organ structure, is lack physical abnormalities in the external morphology, including operculum and genitalia and vibrant external coloration. Male and female broodstock weighed 2 - 3 kg and 1.5 - 2 kg respectively and were both regarded as sexually mature. The male body length ranged between 40 - 50 cm, while females had 35 - 40 cm length. The body colour of mature male giant gourami was vivid red and black, with a pointed stomach section, a regular scale pattern, and active mobility. Mature female broodstock was characterised by having a rounded stomach, the edges of the scales were slightly opened, and slower movement compared to the males.

Breeding pond preparation

The giant gourami broodstocks were reared in a rectangular concrete pond with dimensions of 2 m length, 1.5 m width, and 1.5 m height filled with 1 m water level for observation, amounting to about 3 m^3 water volume in the pond (Figure 1). The experimental pond was prepared with nest-building materials and a pair of underwater cameras (Brica B-PRO5 α Edition mark II with 16 MP CMOS sensor, fixed focus angle lens, and aperture of F/2.8, f=2.9 mm). Nest materials were prepared with a braided bamboo basket and dry palm fibre to facilitate the fish in constructing their nest. The materials were put on top of a woven bamboo table inside the pond. The pond was filled with water after the basket, bamboo table, and observation cameras were installed, before adding the fish.

The broodstocks were initially reared in separate rearing pond before being transferred to the experimental pond. We measured the temperature in the initial rearing pond, with temperature at 27 – 28 ºC and pH at 7.2. The experimental pond had 28 - 30 ºC water temperature and pH at 7.4. The pond was filled with water from a nearby irrigation canal, leading to reduced visibility in the video recording due to turbidity.

The underwater camera was prepared as depicted in Figure 2. The camera was submersible and held inside a structure of PVC pipe and supported with two bamboo stakes, mounted on a concrete block. Two sets of cameras were prepared in the pond, one for capturing activities in the nest and one for capturing activities around the bamboo table. Both cam-

eras were set to capture a video with a 1920 x 720 pixels ratio in 30 frames per second. The lens covered around 30 - 40 cm field of vision.

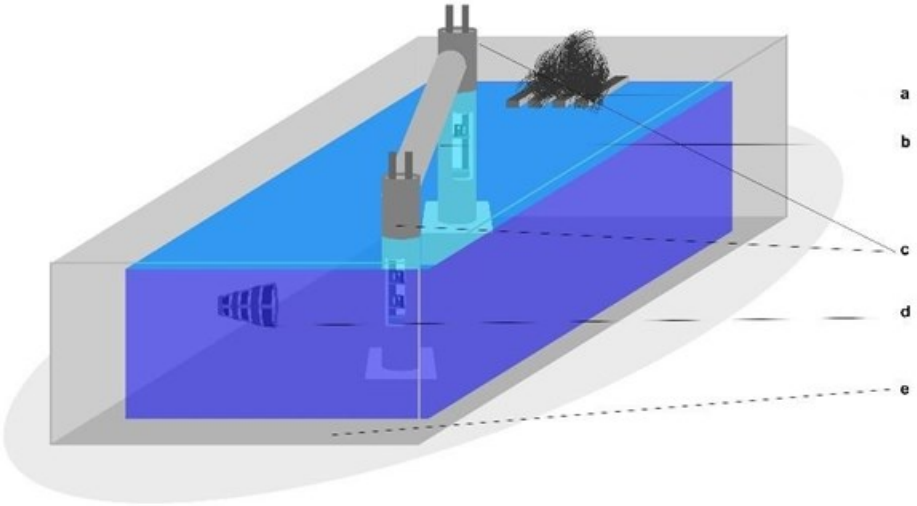

Figure 1. Observation pond design: a. Woven bamboo table, b. PVC pipe to mount the camera, c. Underwater camera, d. Bamboo basket for nesting, e. Breeding pond area.

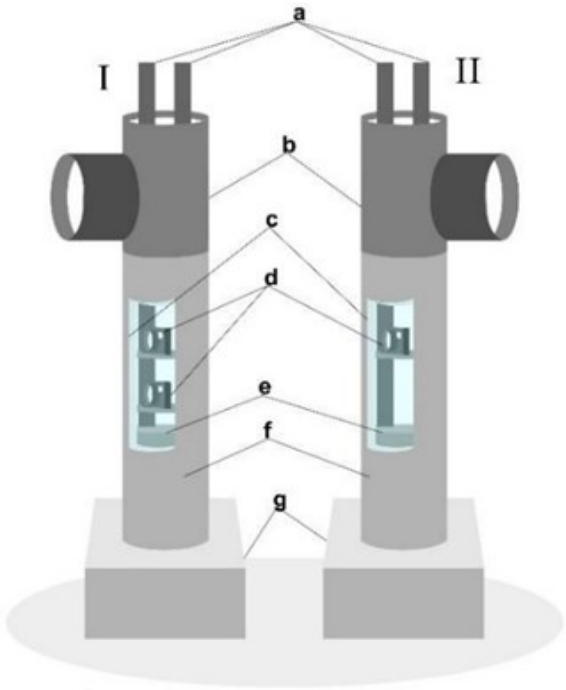

Figure 2. Underwater camera design: a. Bamboo stakes for supporting the structure, b. T-shaped PVC pipe fitting, c. 3 mm Acrylic, d. Underwater camera, e. Styrofoam, f. Four-inch PVC pipe, g. Concrete block for support. Camera I is facing toward the nest, and camera II is facing toward the bamboo table.

Observation of reproductive behaviour

All observations were conducted in three replications, using a separate pond for each breeding process. Each pair of broodstock was reared in separate ponds, with one breeding cycle in one pond considered as one replication. In total, three broodstock pair was observed in three separate ponds. Data were collected in the form of video recordings from two cameras in each pond, facing both the nest and bamboo table (Figure 2). All pairs reproduced naturally without hormonal induction. Our observation is limited to the adaptation, courtship phase, and until the early stage of parental behavior (egg guarding).

The observation period started when the fish were reared inside the pond and lasted until the post-spawning period when the parents took care of the eggs. To study the significance of gender roles in nest construction, we observed the fish twice: in the morning between 08.00 - 11.00 AM and in the afternoon between 02.00 - 05.00 PM. Data were collected every 15 minutes, four times in the span of one hour, both in the morning and afternoon period.

For parental care analysis, we observed the behaviour of both male and female fish while tending to their egg in the nest. Observation was conducted four times in the span of one hour only at the last day. In captive breeding, the nest containing the eggs is typically removed from the breeding pond to be hatched in separate container. Hence, the parental care observation was only conducted at day + 1 post fertilisation. The data were collected based on qualitative scoring: 1 – Seldom (The parent never swam directly in front of the nest or being next to the egg at least once in the observation period); 2 – Occasional (The parent visited the nest more than once or spent less than half of the observation period next to the nest); 3 – Frequent (The parent frequently visited the nest or spent more than half of the observation period next to the nest); 4 – Always (The parent never left the nest).

Data analysis

We conducted both qualitative and quantitative analyses in this study. Qualitative data were collected based on reproductive behaviour such as courtship, mating, and parental care observed within the pre-spawning, spawning, and post-spawning period. Data were collected from three ponds as three replications. The descriptive analysis was performed for every behaviour during the pre-spawning and spawning periods. Quantitative analysis was conducted on the frequency of exhibited nest building behaviour between parents. The results of the quantitative data were statistically analysed using T-test. Parental care behaviour of each parent was scored and statistically analysed using non-parametric test.

RESULTS

The overview of the mating process

In this study we observed three pairs of giant gourami each in different observation pond. Generally, we classified the pattern of mating behaviour into five phases as depicted in Figure 3, which are adaptation, courtship, nest-building, spawning, and parental care.

The initial adaptation phase started as soon as the pair was transferred into the new pond from the previous broodstock pond. At this point the fish scours the area of the pond, finding the potential nest location, and the building materials (ijuk on top of the bamboo table). The phase continued with courtship interactions between the pair, which lasted around three hours. Following the successful courtship, the pair constructed the nest with each defined role. The fish behaviour throughout the mating process are recorded in the ethogram (Table 1). The behaviour pattern of the pair of broodstock is further elaborated in the following sections.

Adaptation and Courtship

The initial phase of the pre-spawning process is to adapt the fish to the new pond habitat. The most noticeable difference from the previous rearing habitat was the reduced light penetration to the water column due to turbidity. This, however, does not seem to hamper the fish's locomotion and interaction, as they recognized the nest (bamboo basket) and the area

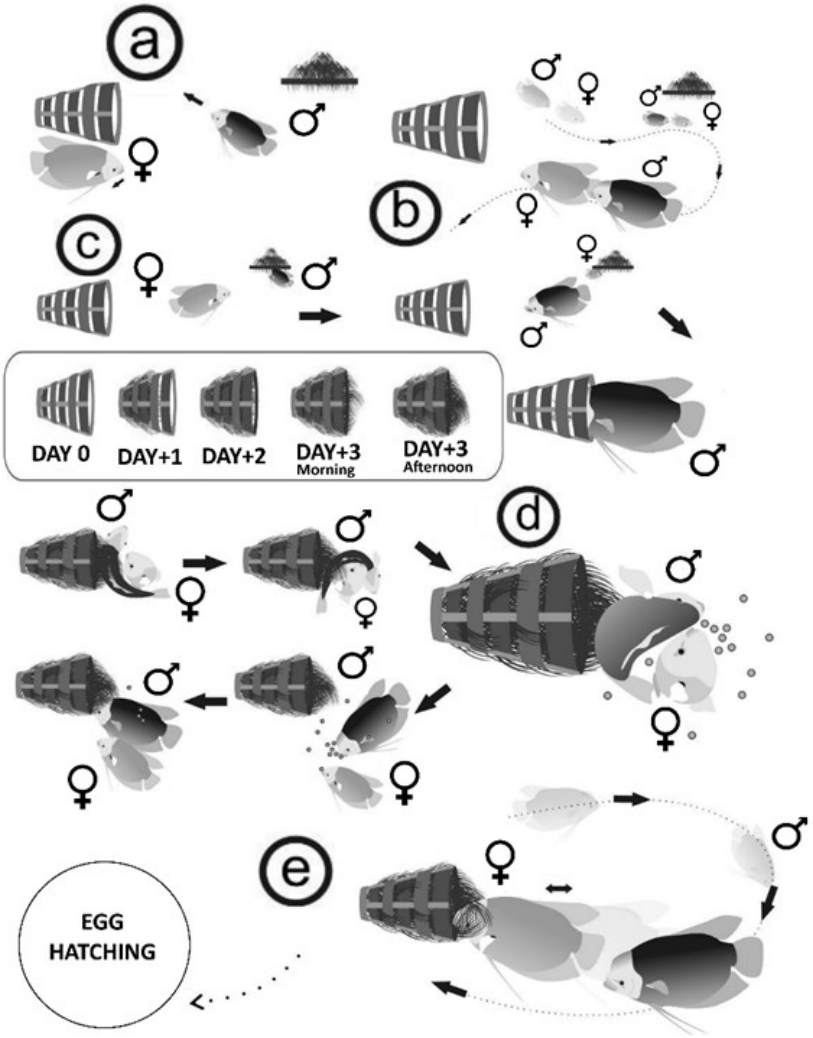

Figure 3. The mating and parental care process in *Osphronemus gorami* pair. (a) Adaptation phase; (b) Courtship phase; (c) Nest-building phase; (d) Fertilization/Spawning phase; (e) Parental care phase.

Table 1. Ethogram of giant gourami mating behaviour. The information is split between the male and female broodstock.

Phase	Male	Female
Adaptation	• Swimming around the basket • Scouting the place where the ijuk fibre is located	• Swimming around the basket • Swimming in and out of the basket
Courtship	• Showing aggressive swimming pattern near the female • Aggressively chase the female while occasionally flaring operculum • Actively syncing swimming movement with the female	• Slowly swims away from the male, occasionally stops • Actively swimming away from the male, circling the pond • Female shows appeasement by slowing down
Post-Courtship	• Swimming in pair with the female, following their movement • Following the female from behind • Swimming outside of the basket, circling the perimeter	• Swimming in pair with the male around the pond • Female actively swimming in search for the basket, swimming around it • Female enters and prepares the basket by cleaning the inside with pectoral and tail fin
Nest Building	• Actively swim in the direction of the bamboo table • Transported the ijuk fibres with its mouth to the inside of the basket while arranging it	• Female got out from the basket, swimming behind the male • Occasionally picked the ijuk fibres, just swims behind the male, or stayed inside the basket to arrange the fibres

Table 1. Contd.

Phase	Male	Female
Spawning / Fertilisation	• Male swims circling the female just outside the basket after it was fully filled with fibres • Performed anabantoid embrace by skewing his body into U-shape, wrapped around the female • While bending, the male expelled the sperm to fertilize the eggs • Gathered the eggs and put them inside the nest with the female	• Female stayed just outside the basket, leaning its body towards the direction of the male • Performed a receptive pose by leaning the body towards the male, while it embraces • Eggs were expelled while being embraced by the male • Helped gathering the eggs with the male
Parental Care	• Mainly circled outside the perimeter of the nest. • Seldomly swim near the nest	• Swim at the entrance of the nest • Constantly fanning the nest with its pectoral fin.

around it. Male fish were seen swimming around the entire pond during this phase, whereas female fish were seen swimming in circles around the bamboo basket that had been prepared as a potential nesting area and entering and exiting the basket (Figure 4). It was observed that in the adaptation phase, the fish surveyed the area of their habitat to identify and establish their territorial area before the mating phase.

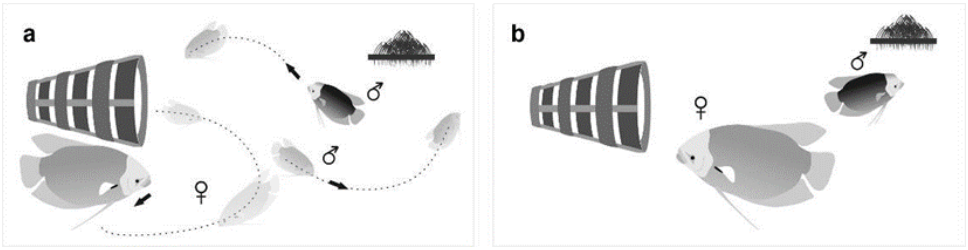

Figure 4. Adaptation phase: a. Male broodstock scours the habitat while female broodstock swims in and out of the nest, and checks the perimeter of the nest. b. Both broodstock identify the present objects in the vicinity of the pond.

The second phase of the pre-spawning behaviour was courtship, illustrated in Figure 4. Three steps of courtship behaviour were observed in this study: Identification, courting by the male fish and courting response from the female fish. In the first step, the male fish exhibited aggressive swimming behaviour while the female fish were swimming around and re-entering the bamboo basket in sequential order. Male courting starts when the male fish swim aggressively towards the female fish, effectively chasing the female around the habitat area. The aggressive swimming behaviour was also accompanied by the fish frequently opening its mouth and operculum. Subsequently, the female fish gave a response to the male chase and swims more slowly. In this step, the movement of the male slowed and gradually synchronized with the female's swimming pace.

The two fish then swim in pair around the habitat, around the nest territory, and in and out of the bamboo basket, with the male fish supposedly guarding the female (Figure 5). This behaviour continued until the male fish started searching for materials to build their nest.

Nest building

After the female was courted and the fish formed a pair, the fish started to swim around the habitat to search for nest-building materials. Palm fibres or "Ijuk" were provided on the woven table for the fish to build their nest (Figure 6). The fibres were picked by the fish by swallowing and saving them in their mouth, subsequently transported into their bamboo basket nest.

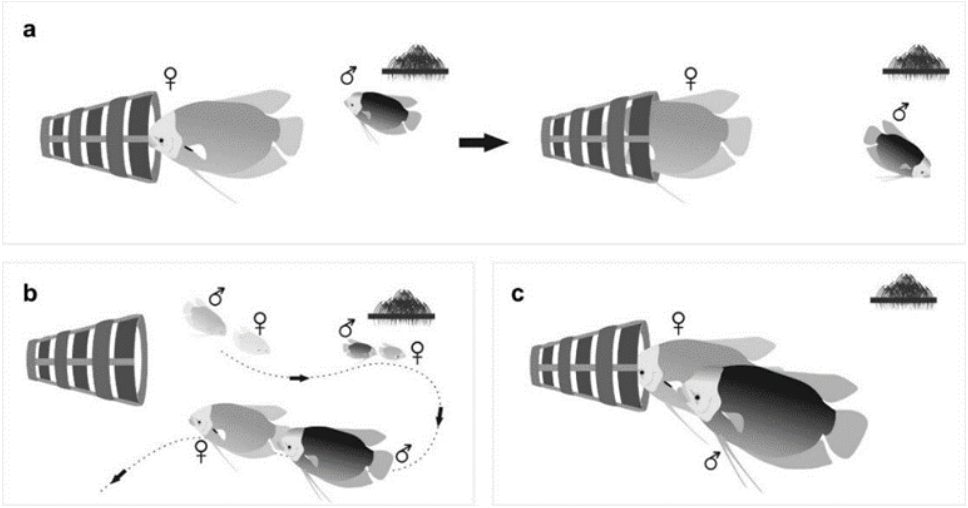

Figure 5. Courtship phase: a. Both broodstock identifying their potential mate. b. Female fish swims around while male fish court the female by chasing it throughout the pond area. c. Male fish synchronize their movement with the female, following it swimming around the nest.

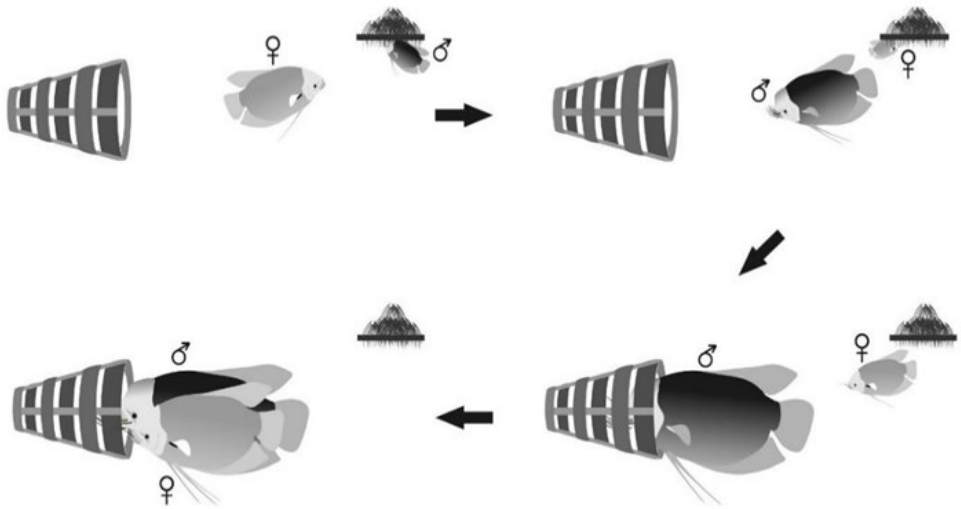

Figure 6. Nest construction. The basket acted as a hole for the nest. Male fish first initiate the nest building by picking up palm fiber from the woven table. Female fish followed the male and occasionally assisted in gathering the materials.

The fibres were expelled from the fish's mouth, noticeable by the presence of bubbles from their mouth, and arranged inside the basket. The fibres were arranged in a circular pattern akin to a bird nest and started from the innermost of the basket to the outer parts.

The nest-building behaviours were exhibited by both male and female fish (Figure 7). The male fish however clearly showed a more dominant role in the process. The role of picking nest materials and building was predominantly observed in the male fish, while the female fish mainly followed the male while only occasionally picking up the nest materials and help arranging the nest.

The process of nest building was continuous for three observation days (Figure 8). Observation on the first day of the nest building showed that the bamboo basket started to be scarcely filled with palm fibres in the afternoon. Afternoon observations on the second day revealed that the fibres had already filled the interior of the basket but had not yet covered the exterior or the entrance part of the nest. The basket was filled to the entrance in the morning of the third day, with materials transporting activities significantly reduced compared to the days before. In the after-

noon, female fish was occasionally seen neatly arranging fibres by pulling loose fibres and pushing them into the inner part of the basket.

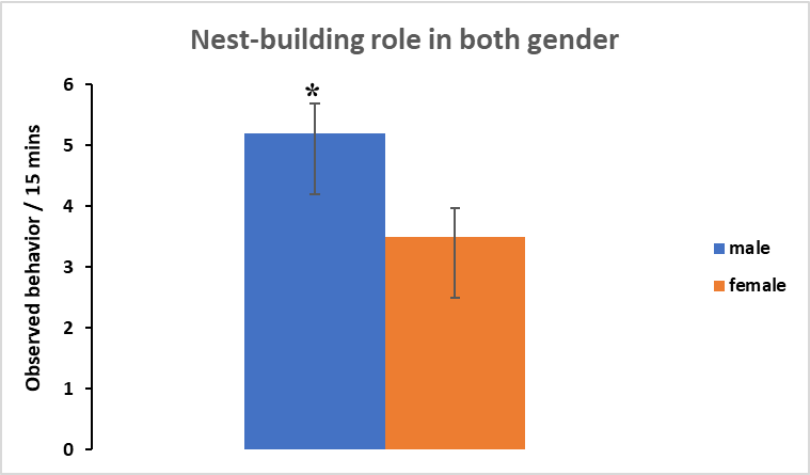

Figure 7. Comparison of the average frequency of nest building between male and female parents (3 replications) observed every fifteen minutes. The difference between both data is statistically significant *) T-test p value = 0.008, $p < 0.05$).

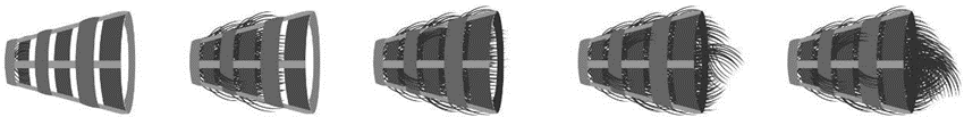

Figure 8. Nest construction process: a. The bamboo basket for nesting at the start of the mating period (D0), b. The bamboo basket started to be filled with palm fibers (D+1 afternoon), c. Partially filled nest (D+2 afternoon), d. Filled nest (D+3 morning), e. Completed nest filled with fibers (D+3 afternoon).

Spawning

The spawning process of giant gourami was observed after the nest was established. The whole spawning ritual is illustrated in Figure 9. The ritual started with the male fish swimming encircling the female fish, which was followed by the female who skewed its body angle until it leaned on the body of the male fish. The male in return arched its body, forming an upside-down "U" shape, and wrapped it around the female's body. At this point, the female expelled the eggs and externally fertilised by the male fish.

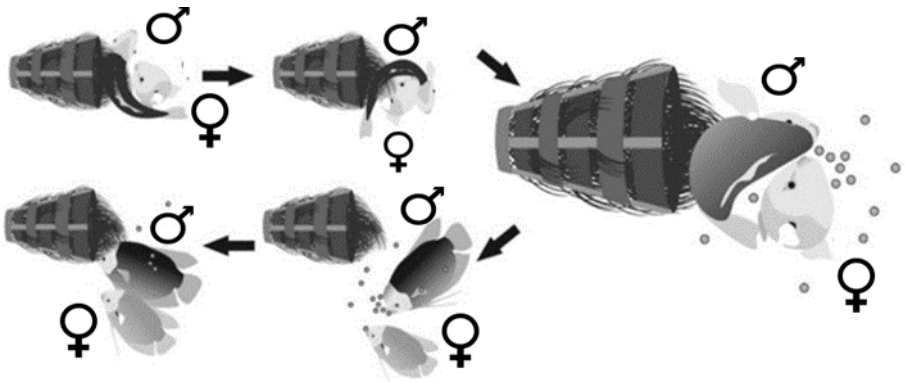

Figure 9. Giant gourami spawning process: Right after the completion of the nest building, both fish entwined their body with the female fish released the eggs, and subsequently fertilized by the male. The fertilized eggs were then moved to the nest by both parents.

The fertilization process occurred twice in one spawning cycle. The floating eggs were then subsequently gathered by both parents and car-

ried to the nest. In our observation, the spawning and fertilization process occurred between 4.00 - 5.00 PM.

Post-spawning

The reproductive behaviour observed after the spawning period is parental care. The giant gourami exhibited parental care behaviour in the form of nest guarding and egg care (Figure 10). The male fish predominantly guard the territory by swimming around their nest area. The female fish mostly swim near the entrance of the nest while fanning the nest with its pectoral fin.

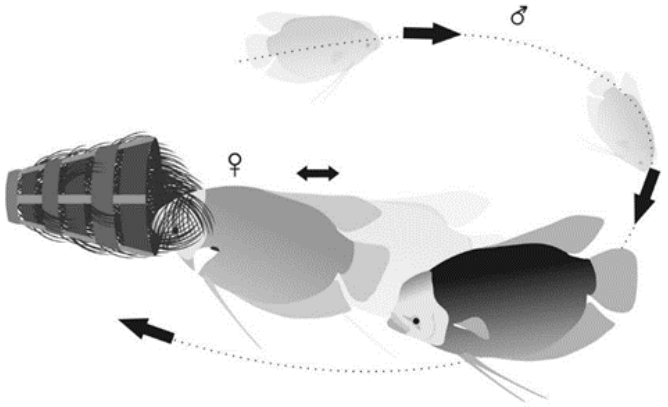

Figure 10. Egg care and parental behaviour: Female fish predominated the role of egg care by fanning the egg from the entrance of the nest using its pectoral fin, while male fish mostly swims around in the vicinity of the nest in a protective manner.

Based on the observation, both parents had different roles in parental care. Figure 11 showed the overall presence of each parent near the nest. The difference between male (median = 2) and female (median = 4) is statistically significant (Mann-Whitney U test p value < 0.05, U = 0). The female fish primarily hovers close to the nest opening and stays in the vicinity. The male fish only occasionally swims up to the nest to examine the eggs before swimming away. It is assumed that the male fish also supposedly guards the nest but covers a wider area around their territory. The female fish largely served as the caregiver because she was closest to the eggs and occasionally fanned the nest.

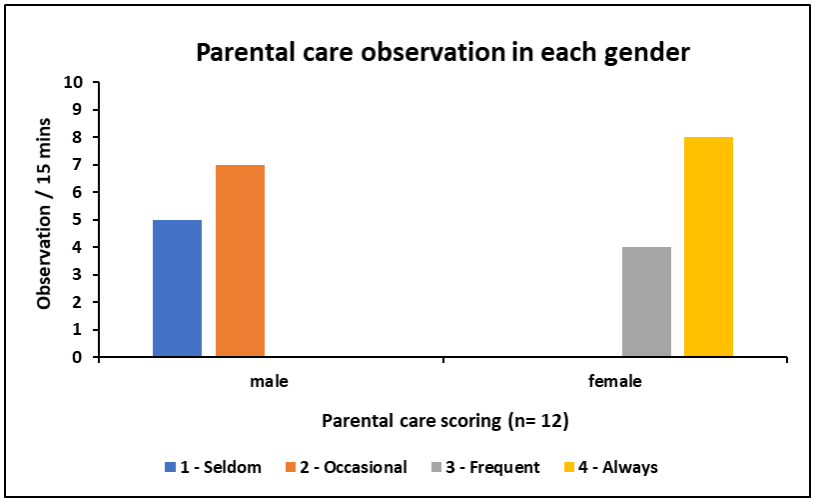

Figure 11. Comparison of the average frequency of parental care behaviour between male and female parent (3 replications) observed every fifteen minutes. The behaviour which was interpreted as parental care are swimming and caretaking activities in the vicinity of the nest. The scoring criterias are: (1) Seldom,

which the parent never swam directly in front of the nest or being next to the egg at least once in the observation period; (2) Occasional, which the parent visited the nest more than once or spent less than half of the observation period next to the nest; (3) Frequent, which the parent frequently visited the nest or spent more than half of the observation period next to the nest; (4) Always, which the parent never left the nest. The statistical difference between both gender is significant ($n = 12$, Mann-Whitney U test p value = 0.00; $p < 0.05$)

DISCUSSION

Timeline of reproductive behaviour

The findings of this study shed light on the reproductive behaviour of *Osphronemus goramy* in a captive environment. The observed behaviours can be categorized into different phases, including pre-spawning phases of adaptation, courtship, and nest building, spawning, and post-spawning parental care. By examining each phase, we can gain insights into the reproductive strategies and roles of male and female fish in this species.

The overall mating process takes about five days (Figure 12). The pre-spawning phase started on day 1 after the fish were acclimatized. The courtship process was observed in the first afternoon and nest building was observed starting the next day when the fish started gathering fibre material. The nest-building process comprised most of the pre-spawning phase, in which the fish took three days to finish. The spawning process, which includes embracing rituals and fertilization, happened right after the nest was finished in the afternoon of the fourth day. On the fifth day, the eggs were already placed and settled inside the nest and both parents already assumed their respective parental roles, guarding and fanning the nest.

Our result is consistent with previous reference regarding to steps of giant gourami mating, where the whole process could take about a week and nest building process took about 3 days to finish (Tanjung & Jhonly 2015). Each stage of the reproductive process saw distinct behaviours from both parents. In the pre-spawning stage, we saw male-centric behaviour, but in the post-spawning stage, caring behaviour was predominately the responsibility of the female.

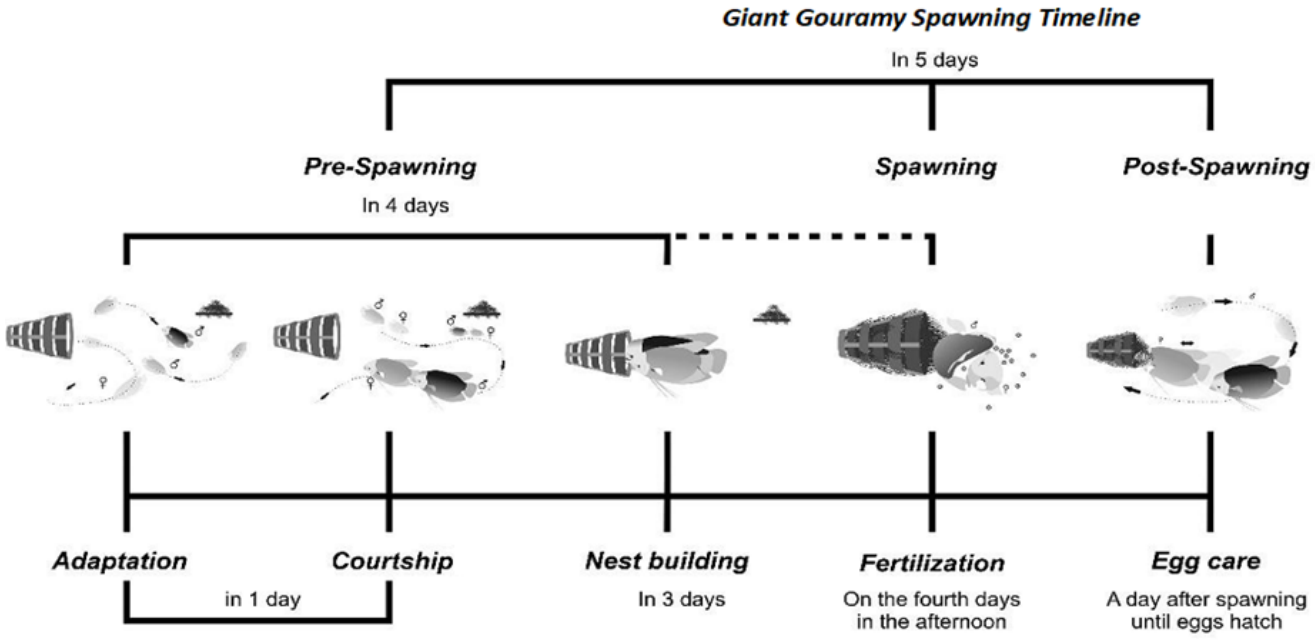

Figure 12. Timeline of the entire reproductive process of giant gourami pair in five days period. The adaptation and courtship rituals were observed on the first day, followed by nest building which took three days to complete. On the fourth day, spawning occurred and was subsequently followed by parental care (egg) on the fifth day.

Rüber et al. (2006) documented four main modes of reproductive behaviour in anabantoid fish: Free-spawning without parental care (genus Anabas, Helostoma, Ctenopoma), mouthbrooding (genus Ctenops, Spheerichtys, and several Betta species including *Betta macrostoma*), substrate spawning with male parental care (*Sandelia capensis*), and bubble nesting with parental care. Among the members of the anabantoids, bubble nesters are the most common, exhibited in at least five genus (Betta, Trichopsis, Trichogaster, Colisa, Macropodus). In fish, there are generally three types of nest construction: excavating burrows and other substrates for egg-placement, piling up walls with materials such as sands, pebbles, animal materials, and plant matter, and constructing cemented-nest with materials glued together by kidney secretions (Navarrete-Fernández et al. 2014). In this case, giant gourami exhibited piling up nest construction, filling the walls of a hole with plant materials for depositing eggs. This behaviour is also unique in the Osphronemid family (Rüber et al. 2006; Arifin et al. 2020; Slembrouck et al. 2020).

Generally, the giant gourami displayed similar stages and behaviour as other anabantoid fish. Males tend to show aggressive display to entice the female, accompanied by chasing the female (Bronstein 1982; Miller & Jearld 1983). The initial response of the female is avoidance and fleeing for several hours until it showed response of appeasement / submission by slowing down and swims with the male (Bronstein 1982). However, compared to other nest-building anabantoids, particularly in the family of Osphronemidae, the giant gourami performed courtship before building nest. Other nest builder members from the gourami family such as Trichogaster, Trichopsis, and several species of Betta including Betta splendens had the males build a nest first before performing courtship (Liengpornpan et al. 2006; Saha et al. 2017; Lichak et al. 2022). The activity and behaviour of both parents and how it varies between the sexes will be further discussed in the section that follows.

Pre-spawning phase

The early pre-spawning rituals we observed in this study were adaptation and courtship behaviour. Preeminent behaviours were observed in both male and female fish. The male directly shows aggressive behaviour towards the female, including opercular flares accompanied by frequent mouth-openings, circling and chasing the female throughout the area, synchronised swimming, and leading-to-nest movement.

The first prominent display of courtship by the male is the opercular flare, in which the fish puffed up the gills and opened the operculum wide. This behavioural pattern is apparent in the early courtship where the male showed aggressive swimming in front of the female and later while chasing the female around. The opercular flare signifies the intensity of the male to attract the female (Goddard & Mathis 1997). This type of aggressive behaviour is also displayed by other anabantoid fish to compete between males for female attraction and deter intruders from their territory (Forsatkar et al. 2016). The act of flaring is taxing to their physiology, as it prevents water from passing through gills making the fish experience a hypoxic state (Abrahams et al. 2005; Castro et al. 2006). To the female fish, the display of intensity could signal the health and virility of the prospective male, and a show of fierceness and aggressivity would give an impression of a strong potential mate although eventually, the preferences differ between species (Dzieweczynski et al. 2014). Male fitness and vitality would be defined by the display of aggressiveness and opercular flare, indicating a preferable mate with ideal genetic resources.

The female fish occasionally displayed fleeing behaviour in court-

ship, in which the males chase it throughout the pond area while flaring its operculum and opening its mouth. The males increased their intensity of aggressive behaviour when the females are shown to be unreceptive (Rainwater & Miller 1968), hence the male may chase the female relentlessly until it showed a positive signal or gesture of appeasement by swimming slower. By that time, the movement of the male also slowed and eventually synchronized with the female's swimming movement. The paired swimming would lead to the male enticing the female to swim in the direction of the nest. In giant gourami, the receptive female swim beside the male as if it was escorted by the male towards the nest and then circles and swims in and out of the nest. Similar escorting behaviour was also observed in other anabantoid fish in the family Belontiidae, where the female returns to the nest with the male by swimming in a zig-zag pattern (Hall & Miller 1968; Miller & Jearld 1983).

After successfully courting the female and leading it into the nest area, the giant gourami male started the nest-building phase, or in this case adding plant materials into the prepared braided conical bamboo basket, which acts as its nest. The palm fibres or "ijuk" were used as a structure for the eggs to latch inside the nest. The bamboo basket, is typically prepared by giant gourami farmers, particularly in Indonesia, as a nesting frame, while palm fibres are provided inside the pond area as nest-building material (FAO 2023). The courted female fish were led into the nest area where the female familiarize herself with it by circling and swimming in and out from the bamboo basket. Both fish were then searched for plant materials to fill the basket. This behaviour was predominantly observed in males, actively seeking palm fibres and bringing them to the nest. The fish accomplishes this by swallowing the plant matter, exhaling it with bubbles, and depositing it inside the nest. Females also participate in nest-building albeit seldom.

In this study, bamboo basket was provided as a safe nest location for the fish and also serves as the supporting structure of the nest. Palm fibres are supplied as a substitute for fibrous vegetation materials that the fish actively seek in their natural habitat. The provision of palm fibres or ijuk also speeds up the nest-building process, since the fish does not have to spend the extra effort of biting stems and leaves of vegetation to obtain the fibrous materials. The well-covered nest cushioned with plant fibres is the haven for storing the eggs. Both sexes performed spawning after the process of building the nest was complete.

Spawning and egg-care

Spawning started when the male performed a circling movement and curved its body, clasping around the female's body. The clasping behaviour itself is not exclusive to anabantoids, as it is also observed in other families of fish such as cyprinids (Family: Cyprinidae) which the male clasped to the female body with inclination to one side, keeping the vent area close, and subsequently eggs and milt were released (Maurakis et al. 2001; Kottelat et. al. 2006; Jacob 2013; Chacko & Sekharan 2022). In anabantoids, it had a different pattern of embracing and clasping, as the male bends into a reverse U-shape and wraps around the female. At that moment, eggs were expelled by the female straight into the vicinity of the male's urogenital opening, where it emits sperm and subsequently fertilized the eggs. The bent portion of the male body exerts pressure on the lateral region of its testes like a lever to release sperm (Hayakawa & Kobayashi 2009). The behaviour, known as anabantoid embrace, is regarded as a distinctive spawning method of anabantoid fish (Rainwater & Miller 1968; Chandran et al. 2013; Tate et al. 2017).

The female parent's egg-guarding and egg-fanning action is the most prevalent parental care behaviour that we have seen in this study. This behaviour is the most common parental care in nest-building teleosts (Ishimatsu et al. 2018), and is generally exhibited by anabantoid fish (Jaroensutasinee & Jaroensutasinee 2001; Mitra et al. 2006). The female fish fans its pectoral fin in the direction of the nest to create water movement, removing impurities from the nest and ventilating the eggs (van Lieshout et al. 2013). The level of dissolved oxygen in the water is crucial to the survivability and growth of the embryo inside the egg. Oxygen gas is diffused into the egg through the egg's outer layer (Kranenbarg et al. 2000). The rate of oxygenation is dependent on the level of ambient DO and the velocity of the water current (O'Brien et al. 1978). The fish egg had a capsule-like layer called chorion that protects the embryo and the egg from external perturbation, however, it also hinders oxygen diffusion into the egg (White & Seymour 2011). The fanning process by the parent fish ensures the water circulation inside the nest, removing stagnated water and oxygenizing the water around the eggs, and the resulting current helps with oxygen diffusion into the eggs (Green & McCormick 2004).

Being a fish with additional air-breathing apparatus in the form of a labyrinth organ, anabantoids are physiologically well-equipped to thrive in a low-oxygen environment. The hypoxic environment, in the form of stagnant and confined waters, is the kind of habitat that anabantoid fish calls home, including giant gourami. Thus, the general breeding and reproductive strategies are also adapted to the hypoxic condition. This trait is exhibited by the members of anabantoids with parental care behaviour by creating bubble-nest, mouth-brooding, and constructing nests on substrates such as vegetations; although several species free-spawns (Tate et al. 2017). Parental care is a plesiomorphic condition in anabantoids, and the evolutionary transition from one form of parental care to others is a common occurrence among the genera (Rüber et al. 2004). The form of bubble-nest building behaviour was indicated to have independently evolved into distinct types, most prominently occurring in the genus *Osphronemus* and *Microtenopoma* (Rüber et al. 2006). In the case of giant gourami (*Osphronemus goramy*), it is interesting to note that the fish is a nest builder by nature; but it deviates from the common method of creating bubble-nest, as observed within their relatives, in favour of constructing a submerged nest with vegetation materials. Divergence in evolutionary behaviour may also alter the role of parental care between male and female fish.

Parental role

Giant gourami generally had a biparental role, and parental care is mainly observed to be the female's role. In our observation, the propensity of a biparental role was already apparent in the pre-spawning period, where female fish also contributed to nest building aside from the male albeit not as significant. The role is, however, reversed in the post-spawning parental care period. The female fish took a more prominent role in tending the eggs while the male assumed the role of a nest guard, swimming in the outer vicinity of its territory. The type of parental role that we encounter is different compared to other anabantoids, especially bubble-nest builders. In paradise fish (*Macropodus opercularis*), the egg care behavior is mostly paternal, with the male fanning and creating bubbles to keep the eggs stay afloat (Cole et al. 1999; Huang & Chang 2011; Rácz et al. 2021). A similar male-oriented role was also observed in *Trichogaster tricopterus*, where it also chased out the female from the vicinity of the nest

before retrieving and tending the eggs (Kramer & Liley 1971; Kramer 1973; Pollak et al. 1981). The female is usually chased out by the male because of their tendency of eating the eggs (Kramer 1973).

Cooperation between two parents is the defining characteristic of biparental care behaviour. A different parent is inherently assigned to a specific role in providing care. In the case of giant gourami, females tended to eggs and fry while the males typically assumed the guarding role and had less direct interaction with their offspring. The sex-specific role between female and male giant gourami is also typically present in other biparental caregivers, for instance in biparental convict cichlids, male fish had more tendency to leave their eggs to chase out intruders (Itzkowitz et al. 2001). The pattern is similar as the female took a more active role in the egg/fry care compared to the male, which is also reflected in our results where post-spawning egg care is typically displayed by females.

The assignment of parental roles in both parents could be rooted in the difference in investment in their reproductive process. There is an asymmetry in gamete size and reproductive energy requirement in both sexes, as males typically produced smaller, less energy-demanding gametes (sperm) and females generate bigger and high-energy gametes (egg) but more metabolically costly (Sutton & Wilson 2019). The result is that individuals with larger reproductive stakes will invest more in parental role, while sex with lower parental investment will likely spend more effort in competing for reproductive chances (Trivers 1972). The notion that gamete size would directly affect reproductive decisions has been criticised in recent studies (Jennions & Kokko 2010; Liker et al. 2015) for reproductive decision also depends on other factors such as the current environmental situation; however, the theory applies regarding parental investment in giant gourami, relating to how much investment each parent had spent for their reproductive needs. For instance, females spent much metabolic energy on producing and laying eggs, while males spend their energy on courtship and competition.

The assumption that female fish spend more energy to create eggs suggests that they had bigger stakes in spawning than male fish. This might prompt the female fish to expend more effort to protect their significant investment through more frequent, hands-on care for the eggs. This condition leads to the assumption that females choose mates who are perceived as vigorous and fit in terms of reproduction, which is reflected in the courtship ritual, in which the males must compete for mating chances with females (Trivers 1972). Females are choosier because the courtship and spawning ritual could be costly for them, as a male's display of intensity could mean aggravated assault on the female, which could reduce their reproductive capability (Krasnec et al. 2012).

Our findings show that males made significant pre-spawning investments, particularly in courtship rituals and nest building. In theory, a part of their investment is already paid off in the form of successful reproductive chance, thus their stake in post-spawning care is arguably lower. Furthermore, because gamete production requires less energy, men recover faster than females, who need time to produce eggs (Kokko & Jennions 2008). As males consume less energy for reproductive needs, their post-spawning activity may be directed toward actions that might facilitate future mating. Previous findings have found that males with territorial dominance are more reproductively active (Limberger 1983; Maruska & Fernald 2010; Kustan et al. 2012) and they are more likely to obtain more reproductive success (Paull et al. 2010; Yokoi et al. 2016). Protecting territorial dominance may be the favoured mode of behaviour because it offers a better trade-off than investing energy in providing direct care for the eggs, such as constant fanning.

The behavioural pattern of the male also supports polygamous mating. This breeding strategy involves a male mate with multiple females, and it may favour increased breeding success in males. Our present work does not represent the polygamous nature of male giant gourami because reproductive activity was observed using a 1:1 sex ratio. Commercial breeders, on the other hand, have been known to use many females in a breeding pond, with a 1:3 male-to-female ratio (Slembrouck et al. 2019; Arifin et al. 2020; Slembrouck et al. 2020). The increased male ratio has been known to result in competition between males for courting females, and could result in aggravated aggression between fish that could potentially reduce the breeding efficiency (Arifin et al. 2020). Due to the nature of our methodology, we are unable to provide information on fish behaviour in situations when there are several females and several male competitors. Future studies should explore the various parental roles and behaviour that may arise in those conditions.

CONCLUSION

Overall, our findings revealed that giant gourami exhibits complex mating behaviours, including specific courtship rituals and nest building. Their biparental care trait is reflected in both male and female parents actively participating in the reproductive process. The parental role was assigned differently, as male-dominated behaviours occurred in the pre-spawning phase while the female-centred role was exhibited in direct egg care after spawning. The difference in the parental role might be influenced by each gender's reproductive stake, as the male is more involved in the courting process, fertilization, and territorial behaviour; while the female invested more energy in producing and laying eggs, thus had a higher stake in the parental care phase. The egg care behaviour of constantly fanning the eggs by the female may be an evolutionary response to low-oxygen environments. In the end, our research highlights the importance of understanding the natural behaviours of giant gourami's effective breeding. By unravelling the intricacies of their reproductive processes, we provide a foundation for the development of improved breeding techniques.

Further studies could focus on investigating the genetic factors influencing reproductive behaviour in giant gourami, as well as the impact of environmental factors on their breeding success. The physical and chemical factors such as water quality parameters could also affect the mating process of the fish. In addition, specific social behaviour regarding competition and reproductive fitness in multiple-pair situations should be investigated. This would provide insight into the degree to which parental role in the face of rivals, the propensity for polygamy, and the precise length of time each parent needs to recover from reproduction before being ready for the next spawning cycle. Basic behavioural knowledge should be built from the ground up as it would serve as a reference to develop and improve breeding strategies for commercial giant gourami production in the future.

AUTHORS CONTRIBUTION

T.I.J. designed the process, performed data collection and analysis, and wrote initial manuscript draft. I.H. conceptualized and supervised the study. H.B. performed data analysis, wrote and edited the manuscript. D.W.K.S. designed and supervised the study, wrote and edited the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Effect of Different Concentrations and Combinations of Benzyl Aminopurine and Indole-3-Butyric Acid on Micropropagation of *Vanilla Planifolia*

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ABSTRACT
Micropropagation of explants *in vitro* has potency to address propagule demands for promoting large-scale vanilla production. Plant growth regulators (i.e., cytokinin, auxin) are necessary for the plant micropropagation success. Objective of this study is to determine the shoot multiplication and root development of *Vanilla planifolia* under the influence of different concentrations and combinations of BA and IBA for micropropagation. Sixty stem nodal segments of *Vanilla planifolia* were cultured on MS medium supplemented with IBA (0, 0.5, or 2.0 mg/L) and combined with BA (0, 0.5, 1.0, or 2.0 mg/L). Shoot multiplication and root induction were measured after 60 days of culture. The results show that the MS medium with 1 mg/L IBA hindered shoot growth, while the medium containing 1 mg/L BA yielded the highest number or weight of shoots per explant. For the root development, supplementing the medium with 0.5 mg/L or 1 mg/L IBA improved root length or number of roots per explant, respectively. This research establishes a valuable approach for vanilla micropropagation by utilising low concentrations of plant growth regulators and a rapid protocol. This paves the way for significant advancements in large-scale commercial production.

INTRODUCTION
Vanilla planifolia, commonly named 'flat-leaved vanilla', is a species of vanilla orchid, native to Mexico, Central America, Colombia, and Brazil (Sinha et al. 2008). According to Royal Botanical Gardens, Kew (RBG Kew 2023) vanilla is one of the most popular flavors in the world, and the second most expensive spice after saffron because of the intensive labor required to grow and produce its pods (fruits). Vanilla plants have been introduced worldwide to tropical areas including Cambodia (Ramachandra Rao & Ravishankar 2002; McGregor 2004; Cameron 2011). The pod or tiny seeds of vanilla are used as flavoring agents (vanillin) in food, including ice cream, chocolate, liquor, soft drinks and candies, and for other purposes, e.g., cosmetics and pharmaceuticals as well.

Vanilla is perennial climbing orchid with a thick, cylindrical, succulent green stem and an evergreen vine that can reach up to 15 m in length (Janarthanam & Seshadri 2008). Stem cutting is the common

propagation method for this plant (Anuradha et al. 2013; Baqueiro-Peña & Guerrero-Beltrán 2017; Arya et al. 2021), with labour intensive and time consuming (Kalimuthu et al. 2006). Rapid micropropagation is needed to meet demands of the vanilla world market that remaining consists about 70% of synthetic vanilla extract, which is produced from chemical components (DMNP 2023). Micropropagation has become the cornerstone of a massive commercial plant propagation industry, with hundreds of laboratories around the globe utilizing this technique.

Several studies on multiplication of *V. planifolia* have been done, using different parts of vanilla plant, e.g., callus culture, protocorms, shoot tips, stem nods, root tips, shoot tips (e.g. Philip & Nainar 1986; Giridhar & Ravishankar 2004; Kalimuthu et al. 2006; Janarthanam & Seshadri 2008), and different supporting materials, light intensity or media types (e.g., Kunwanlop et al. 2018; Erawati et al. 2021). Plant growth regulators (PGRs), known as plant hormones, are essential for plant micropropagation because they play critical role in directing the development and multiplication of plant cells and tissues in a controlled environment. PGRs influence the way plant tissues develop into specific structures like shoots or roots; for instance, cytokinin (e.g., Benzyl Aminopurine – BA, Kinetin) promote shoot multiplication, while auxin (e.g., Naphthaleneacetic Acid – NAA, Indole-3-Butyric Acid – IBA) can stimulate root initiation (Bhatla & Lal 2023). Previous studies on the effects of cytokinin and auxin on *V. planitolia* micropropagation investigated the use of various combinations, such as BA or kinetin with NAA supplemented MS medium (George & Ravishankar 1997; Ayele et al. 2017; Izzati et al. 2013), BA and kinetin with MS medium (Erawati et al. 2021), and BA alone with Gamborg's B5 medium (Kunwanlop et al. 2018). de Oliveira et al. (2013) report the influence of BA on shoot multiplication of *V. planifollai*, and IBA on rooting in double-phase culture system.

However, there are no reported studies specifically investigating the combined effects of BA and IBA concentrations on the micropropagation of *V. planifolia*. The objective of the present study was to determine the effects of different concentrations and combinations of BA and IBA on shoot multiplication and root induction of vanilla plants (*Vanilla planifolia)*, using stem nodal segments for *in-vitro* culture.

MATERIALS AND METHODS

Experimental design

The experiment was conducted at the AgroBio4Cam (ABC) Laboratory (latitude: 13.0897004 N, longitude: 103.233008 E) in Battambang, Cambodia, from February 20th to April 22nd, 2023. ABC, a private company established in Battambang, Cambodia in 2021 with Registration No: KH/88412/22 under the Ministry of Commerce, provided the laboratory facilities. *Vanilla planifolia* explants were obtained from a commercial farm in Siem Reap and brought to the ABC laboratory in 2022.

A complete randomised design was used for this experiment, with 12 treatments and 5 replicates, one cutting in each culture vessel or replicate. Combinations of different concentrations of IBA and BA were prepared for each treatment to test their effects on shoot and root initiation of *in vitro* propagation of vanilla plants (*V. planifolia*). Combinations of different concentrations of IBA and BA were prepared for each treatment to test their effects on shoot and root initiation of *in vitro* propagation of vanilla plants (Table 1).

Table 1. Detailed treatments of the effect of BA (Benzyl Aminopurine) and IBA (Indole-3-Butyric Acid) on shoot and root multiplication of vanilla plants (*Vanilla planifolia*) micropropagation.

Treatments	Plant growth regulators (mg/L)	
	BA	IBA
T0	0	0
T1	0.5	0
T2	1	0
T3	2	0
T4	0	0.5
T5	0.5	0.5
T6	1	0.5
T7	2	0.5
T8	0	1
T9	0.5	1
T10	1	1
T11	2	1

Culture conditions

This study employed stem nodal segments (2 – 3 cm in length with a single axillary bud) from healthy young shoots of *V. planifolia* for micropropagation. Following sterilisation protocols established by Abebe et al. (2009), explants were first washed with a 3 g/L detergent solution (three times, 10 minutes each) followed by a 30-minute fungicide soak (3 g/L copper hydroxide). After rinsing with sterile distilled water (three times), explants were transferred to a laminar flow cabinet and sequentially treated with 70% alcohol (5 minutes) and 0.1% mercuric chloride (5 minutes), with thorough rinsing with sterile water after each step.

Following sterilization, individual explants were cultured aseptically on basal MS medium (Murashige & Skoog 1962) supplemented with 3% sucrose, 0.4% agar (gelling agent), and various combinations of IBA (0, 0.5, or 2.0 mg/L) and BA (0, 0.5, 1.0, or 2.0 mg/L). Prior to agar addition, the medium pH was adjusted to 5.7 ± 0.1 using 1 N KOH or HCl. The medium was sterilized by autoclaving at 121°C for 20 minutes. Each explant was inoculated into a 250 mL vessel containing 50 mL of the designated medium. Cultures were incubated in a plant growth room at 25 ± 1°C under a 16-hour photoperiod provided by cool white, fluorescent light (1,000 – 2,000 lux) for 60 days (Figure 1). Daily observations were made, and growth and development measurements were recorded after 60 days of culture.

Statistical analysis

To evaluate plant growth and development across all treatments and the control, the following parameters were measured after 60 days in culture, including shoot formation time (days to first appearance), number of shoots per explant, shoot fresh weight, shoot length, number of leaves per shoot, number of roots per explant, root length, and root fresh weight.

The root-to-shoot length ratio was calculated by dividing root length by shoot length. To assess mean differences across multiple groups, one-way analysis of variance (ANOVA) was used for normally distributed data. For data with non-normal distributions, the Kruskal-Wallis test (Kruskal & Wallis 1952) was employed. Post-hoc analysis for

both tests involved Tukey's Honestly Significant Difference (HSD) test (Tukey 1949) to compare all pairwise means at $\alpha = 0.05$. All statistical analyses were performed using R statistical software version 3.6.3 (R Core Team 2020), and data visualization was accomplished with the 'ggplot2' R package (Wickham 2011).

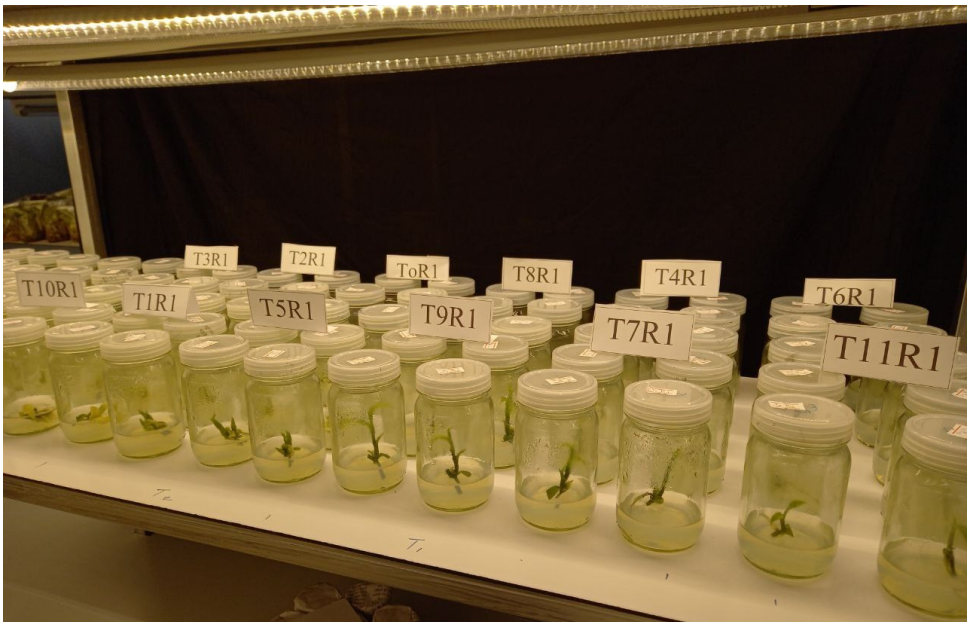

Figure 1. The vanilla plants (*Vanilla planifolia*) *in vitro* culture in the culture room of the AgroBio4Cam laboratory were observed at 40 days after inoculation.

RESULTS AND DISCUSSION

All *in vitro* cultures of *Vanilla planifolia* were free of contamination (Figure 1 & 2). Application of different BA and IBA concentrations and combinations significantly affected shoot and root growth and development of *V. planifolia* micro-propagation (Figure 3 & 4; $P < 0.001$).

Shoot Initiation and Multiplication

Supplementation with BA alone led to the earliest shoot development observed within 7 – 8 days (Figure 3a). Conversely, media lacking plant growth regulators or containing only IBA delayed shoot development. Interestingly, the greatest shoot length (5.37 cm) was achieved on medium containing 1 mg/L IBA (Figure 3b), while the medium with 1 mg/L BA resulted in the highest number (4.93 shoots per explant) and weight (828 mg) of shoots per explant (Figure 3c, d). The control group (medium without plant growth regulators) exhibited poorly developed shoots.

This study suggests that cytokinin alone (BA) can promote single shoot regeneration from *V. planifolia* nodal explants cultured on MS media containing 1 mg/L IBA with 0 or 0.5 mg/L BA. In contrast, shoot multiplication required supplementation with both BA (1 mg/L) and IBA (0.5 mg/L) in the MS medium. These findings align with Zuraida et al. (2013), who reported the greatest shoot number and length for *V. planifolia* cultured on MS medium with 1 mg/L BA for stem nodal segments, or 2 – 3 mg/L BA for shoot apex explants (cultured for 45 days). Erawati et al. (2020) found that adding 3 mg/L BA to the MS medium yielded the best results for *V. planifolia* multiplication, with 3 – 4 shoots emerging per explant, each measuring 2 – 2.5 cm in length, at 28 days after inoculation.

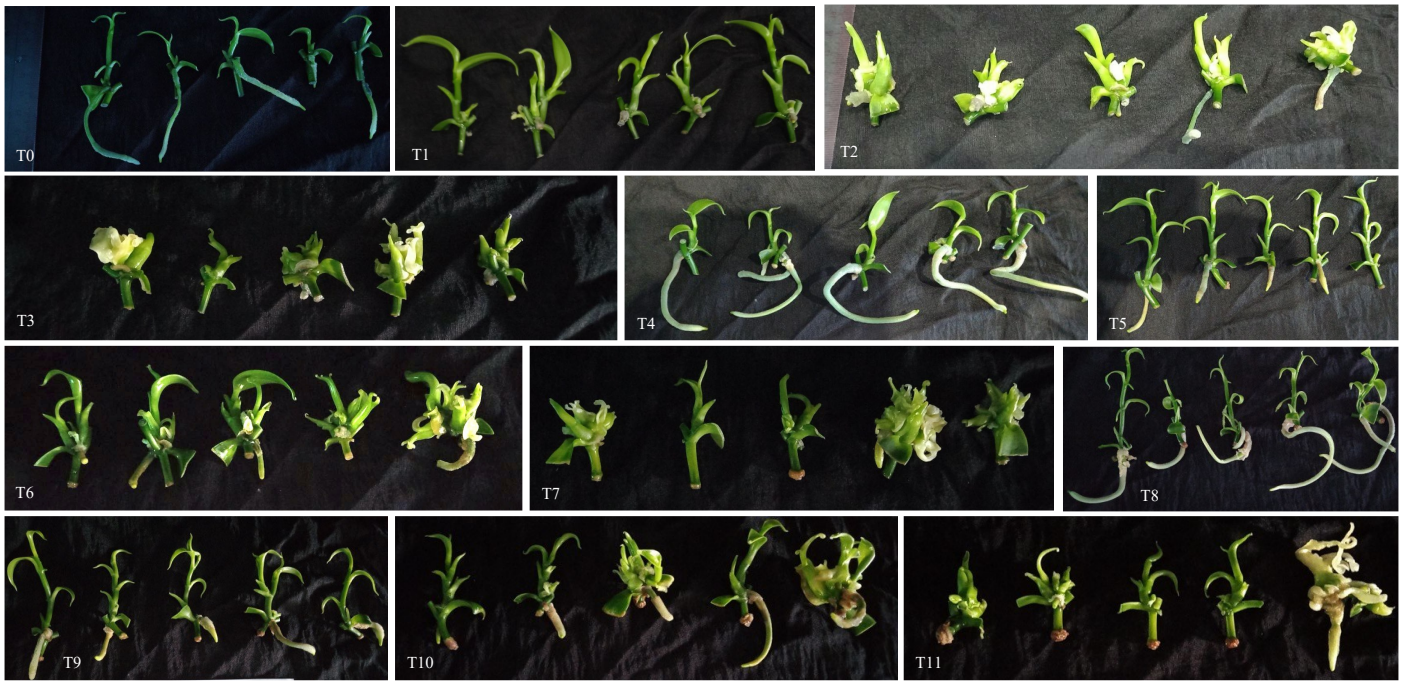

Figure 2. Effect of different BA and IBA concentrations and combinations on the propagation of Vanilla (*Vanilla planifolia*) after 60 days of *in vitro* culture.
Note: T0: 0 mg/L BA + 0 mg/L IBA; T1: 0.5 mg/L BA + 0 mg/L IBA; T2: 1 mg/L BA + 0 mg/L IBA; T3: 2 mg/L BA + 0 mg/L IBA; T4: 0 mg/L BA + 0.5 mg/L IBA; T5: 0.5 mg/L BA +0.5 mg/L IBA; T6: 1 mg/L BA + 0.5 mg/L IBA; T7: 2 mg/L BA + 0.5 mg/L IBA; T8: 0 mg/L BA + 1 mg/L IBA; T9: 0.5 mg/L BA + 1 mg/L IBA; T10: 1 mg/L BA + 1 mg/L IBA; and T11: 2 mg/L BA + 1 mg/L IBA.

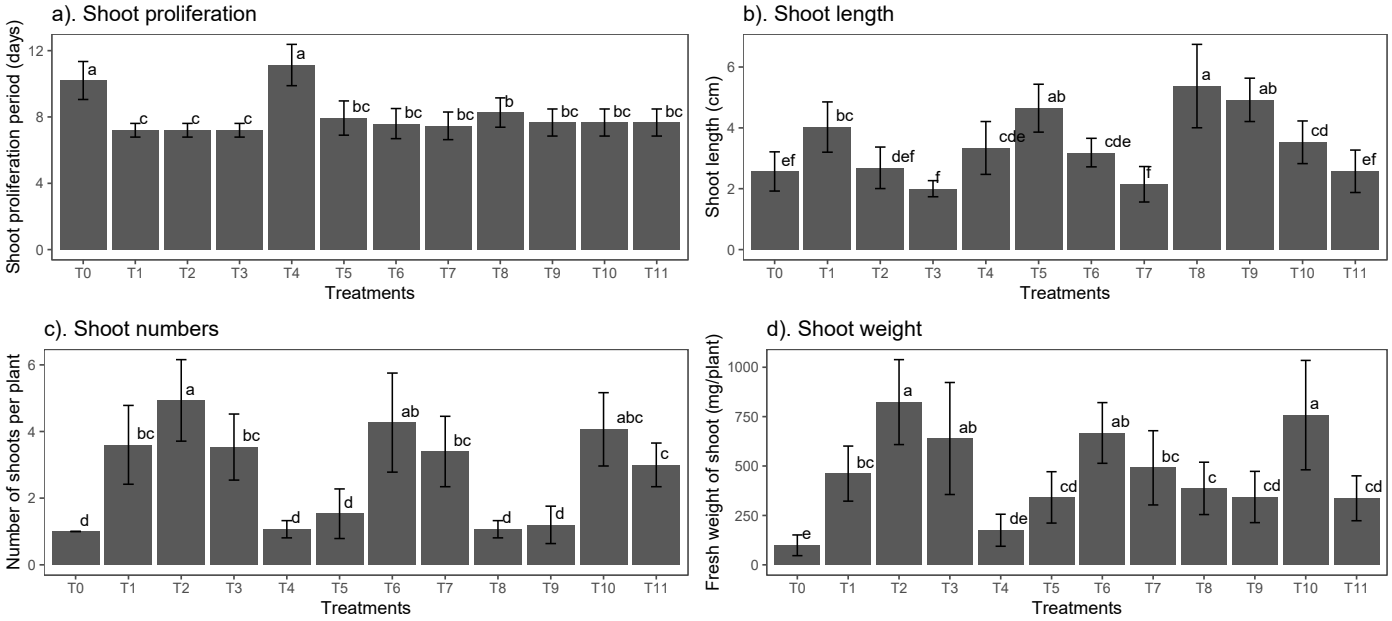

Figure 3. Effects of BA and IBA on shoot initiation and multiplication in vanilla plants (*Vanilla planifolia*) micropropagation: a). period of shoot proliferation (days); b). shoot length (cm); c). shoot number per explant; and d). shoot weight per explant (mg). Bars represent standard deviation. Different letters (a, b, c, d, e, f) within each panel indicate significant differences ($P < 0.05$) according to Tukey's HSD test.

Conversely, Erawati et al. (2021) reported the highest number of shoots (6 shoots per explant) for *V. planifolia* micropropagation using MS medium supplemented with 0.5 mg/L BA and 2 mg/L Kinetin at 56 days after inoculation. Prabaninggar et al. (2021) found that BA at concentrations of 1, 2, or 3 mg/L had no effect on shoot proliferation in *in vitro* microcuttings of *V. planifolia*. Conversely, Ayele et al. (2017) reported that the combination of 2 mg/L BA and 0.5 mg/L NAA yielded the best re-

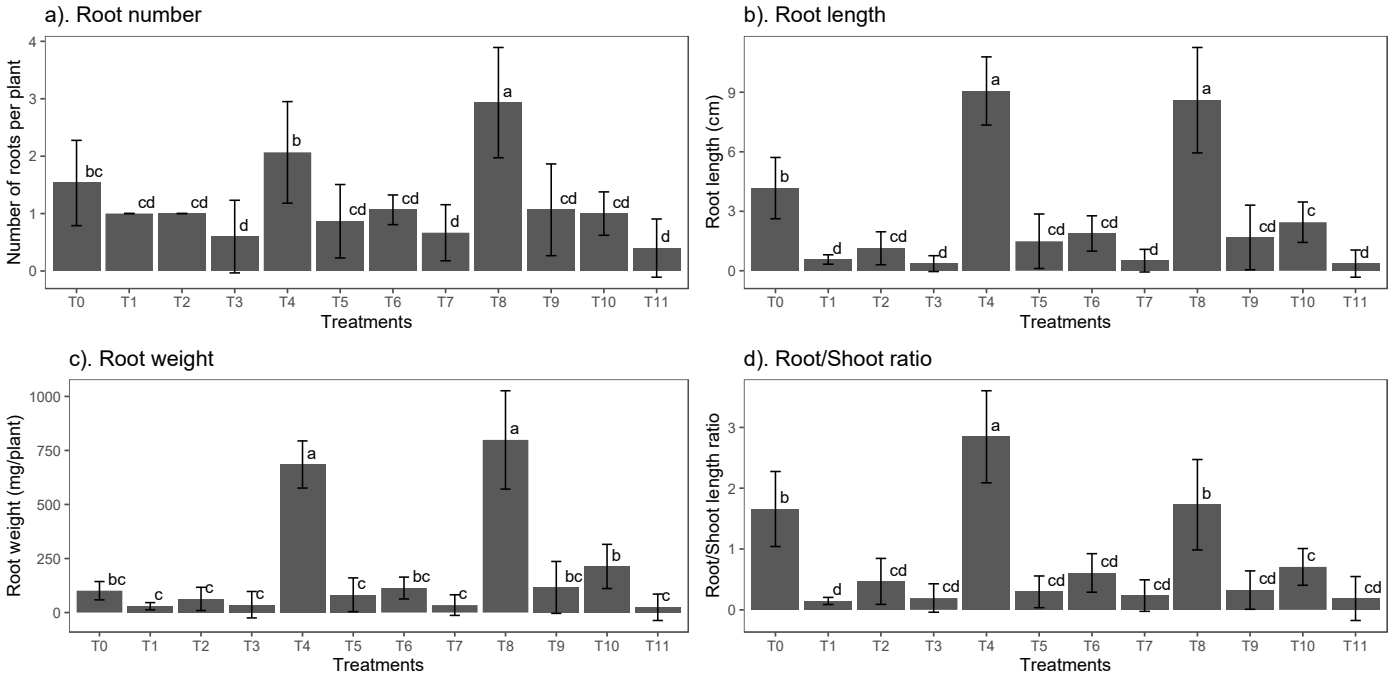

Figure 4. Effects of BA and IBA on the root growth and development: a). root number per explant; b). root length (cm); c). root weight per explant (mg); and d). root-to-shoot length ration. Bars represent standard deviation. Different letters (a, b, c, d) within each panel indicate significant differences ($P < 0.05$) according to Tukey's HSD test.

sults for *V. planifolia* shoot multiplication. Notably, this treatment produced the highest number of shoots (5.33) and the longest shoots (4.9 cm) after five weeks of culture. Other studies by Sharma & Bora (2017) reported that MS medium supplemented with 3 mg/L BA and 1 mg/L NAA was most effective for maximum shoot bud differentiation of *V. planifolia* cultured from nodal segments. This medium resulted in the highest number of multiple shoots (11.6 per explant). Abebe et al. (2009) reported that for *V. planifolia* cultured on MS medium for 45 days, the combination of 1 mg/L BA and 1.5 mg/L Kinetin produced the maximum shoot multiplication (4.17 shoots per explant). Higher BA concentrations in the medium resulted in a decrease in shoot numbers for *V. planifolia*. However, supplementing liquid MS medium containing 1 mg/L BA with 15% coconut water improved shoot multiplication. Furthermore, research by de Oliveira et al. (2013) suggests that lower BA concentrations can enhance proliferation rates of vanilla plants in a double-phase *in vitro* culture system. Tan et al. (2011) observed the highest shoot multiplication with 9.6 shoots per explant and a shoot length of 4.7 cm, achieved using this approach.

Root Growth and Development

The medium supplemented with 1 mg/L IBA yielded the greatest number of roots per explant (2.93) (Figure 4a). For root length and weight, the medium containing either 0.5 mg/L or 1 mg/L IBA resulted in the highest values (9.07 cm and 799 mg per explant, respectively) (Figure 4b, c). Notably, the control group exhibited the least root growth. These findings suggest that auxin plant growth regulators can improve root growth and biomass. Explants were also able to develop roots on MS medium even without the addition of any plant growth regulators. The medium containing 0.5 mg/L IBA yielded the greatest root-to-shoot length ratio (Figure 4d).

This study found that auxin was necessary for improved root growth and biomass, although roots could grow on MS medium without auxin supplementation. Kunwanlop et al. (2018) reported similar findings

in their studies on two vanilla species. They observed the highest root induction in Gamborg's B5 medium (Gamborg et al. 1968) supplemented with 1 mg/L BA, or 2 mg/L BA for *V. planifolia* micropropagation. Previous studies (Abebe et al. 2009; Zuraida et al. 2013) reported that root induction for *V. planifolia* micropropagation on MS medium did not necessarily require plant growth regulators. However, Ayele et al. (2017) found that half-strength MS medium supplemented with 0.5 mg/L IAA produced the highest number (4.0 roots per plantlet) and longest roots (6.1 cm) among the treatments they tested. In their study on *V. planifolia* micropropagation, Tan et al. (2011) found that 1 mg/L NAA in MS medium yielded the maximum root number, with 2.9 roots per explant. Besides plant growth regulators, other factors can influence shoot and root induction in vanilla plant micropropagation. These factors include medium type (solid or liquid), light intensity, plant hormone type, and more. Several studies have explored these influences (e.g. Srean et al. 2011; Tan et al. 2011; Bello-Bello et al. 2016; Sidek et al. 2018).

CONCLUSION

Our research established a streamlined method for large-scale production of vanilla plants. The investigation of different cytokinin (BA) and auxin (IBA) concentrations and combinations has revealed several options for plant hormone application in the *in vitro* culture of stem nodal segments for micropropagation of vanilla plants (*V. planifolia*). For shoot multiplication, using only 1 mg/L BA in the solid MS medium yielded the best results. Conversely, the highest root induction was achieved with 0.5 mg/L IBA in the same medium. This study demonstrates that single plant growth regulators can be effective for micro-propagating vanilla stem nodal segments. Supplementation with either 0.5 mg/L or 1 mg/L IBA promotes greater root length or more roots per explant, respectively. This research establishes a straightforward protocol for large-scale vanilla plant production. The key lies in using low concentrations of plant growth regulators, along with the rapid protocol developed. This approach can be effectively applied to various stages of vanilla micropropagation, paving the way for significant advancements in commercial production.

AUTHORS CONTRIBUTION

S.K. & C.H. designed the research and executed the experiment, S.K. analysed the data and wrote original draft preparation, S.R., S.R. & P.S. reviewed and edited the manuscript.

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CONFLICT OF INTEREST

The authors state that there was no conflict of interest in this research.

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Research Article

Spongia officinalis -Associated *Pseudomonas fluorescens* as a Reservoir of Bioactive Compounds: A Novel Source of Natural Anticancer Compounds

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ABSTRACT

Marine sponges are important sources of chemical variety and repository of biodiversity. In this study, the microbial communities found in the *Spongia officinalis* that was taken from the Kanyakumari coast in India were explored. We identified, characterised, and evaluated the bioactive potential of the sponge-associated bacteria. A total of 12 bacterial isolates were obtained, primarily consisting of gram-positive rods (7 isolates) and some gram-negative rods (2 isolates), and cocci (1 isolate). Among these KKS6 showed tremendous radical scavenging activity (85.16 ±1%) with a minimum inhibitory concentration as 167.26±0.1 µg/mL at the highest concentration when compared to other extracts. With an IC50 value of 55.32 g/mL, this isolate also displayed impressive anticancer activity against HeLa cells. The screened isolate was identified as *Pseudomonas fluorescens* strain using 16S rRNA sequencing. This discovery emphasises the importance of bacteria associated with *Spongia officinalis* as a source of bioactive compounds with medicinal potential. This study highlights the novel findings of diverse microbial communities found in *Spongia officinalis* and their potential for use in biotechnology and medication development. *Pseudomonas fluorescens* was found to be a prolific generator of bioactive byproducts, including strong antioxidants and anticancer agents, which could be a potent drug molecule in future anticancer research.

INTRODUCTION

The marine environment is the largest source of unexplored chemical richness, which has piqued the interest of health scientific communities (Karthikeyan et al. 2022). The tremendous marine biodiversity, which consists of some 230,000 known species and the bioactives constitutes a vast reservoir of anticancer drugs, with a market value estimated to be within USD 5.69 trillion (Wang et al. 2020). Notably, bacteria located within these sponges have been discovered to have a critical role in the manufacture of bioactive chemicals with various pharmacological characteristics (Srinivasan et al. 2021). Marine sponges have a great diversity of bacterial populations which are assumed to be deeply engaged in their

host sponges' metabolic activities (Taylor et al. 2007; Brinkmann et al. 2017).

The search for novel chemotherapeutic drugs has prompted scientists to investigate the isolation, identification, and characterisation of these metabolites, with a particular focus on their anticancer action (Mohan et al. 2022). The marine environment offers a unique and mostly untapped resource for such compounds, the complicated symbiotic connections between marine sponges and their accompanying bacteria hold the possibility of revealing a treasure trove of pharmacologically relevant chemicals. This has sparked a surge of interest in studying the isolation and identification of these bacterial strains, as well as the processes through which they synthesise physiologically active chemicals (Fenical 1993).

The Demospongiae *Spongia officinalis*, is a rich source of secondary metabolites, particularly sterols, terpenoids and phenol (Migliuolo et al. 1990; Manzo et al. 2011). It has also been demonstrated that *S. officinalis* supports a substantial and varied bacterial community. Moreover metabolites retrieved from *Spongia officinalis* have proven to show a wide variety of medical potential (Stabili et al. 2008; Prastiyanto et al. 2022).

The current work conducts a thorough investigation into the isolation, identification, and characterisation of bacterial symbiont within marine sponge *Spongia officinalis*, with a focus on the bioactive metabolites of symbiont bacteria and their potential as an anticancer lead candidate.

MATERIALS AND METHODS

Collection of sponges from coastal area

In the southernmost point of peninsular India, Kanyakumari (N 8° 5' 5.694", E 77° 32' 30.4656), marine sponge was collected by SCUBA diving at a depth of ten feet by random sampling. With latex gloves on, the samples were cut using a dive knife. The pieces were then placed into different plastic sample collection bags, and then transported within two hours to a laboratory for further processing. The salinity, temperature, pH, and TDS (Total Dissolved Solids) were determined to be 31.24%, 29.8°C, 7.89 and 312 mg/L respectively (Jin et al. 2014).

Processing of sponges

Sponge tissues were cleaned with sterile and were cut with a sterile scalpel blade from the inner mesohyl. The sterile 1-cm^3 sponge sample was pulverised vigorously for two to three minutes in a sterile mortar with 9 mL of sterile sea water (Cheng 2017).

Isolation of bacteria from sponge sample

The homogenized sponge sample was serially diluted to a concentration of 10^{-6} in sterile seawater, and 100 µl aliquots were plated onto Zobell marine agar medium. The plates were incubated at 37°C for 48 hrs. Colonies were selected from isolation plates and re-streaked at least twice to achieve pure cultures from single colonies from plates that were visually considered to be from single cultures. Cultures were incubated at 37°C in zobell marine broth made with artificial seawater (Webster & Hill 2001).

Morphological identification of sponge associated bacteria

A method suggested by (Photolo et al. 2020) was applied to determine the shape and gram stain reaction. To determine morphological traits including form and Gram stain reaction, pure colonies were subjected to Gram staining. Using a compound light microscope with a 100x magnification, Gram stain slides were examined.

Extraction of crude secondary metabolite from bacteria

The pure colonies of bacterial isolates were inoculated into 10 mL of zobell marine broth and incubated at 37 °C with shaking for three days to create seed cultures. In a 250 mL conical flask, 50 mL of zobell marine broth was inoculated with about 1 mL of these seed cultures. These were incubated for three days at 37°C in a shaking incubator (Kennedy et al. 2009). Bacterial cells were removed during the preparation of extracts by centrifugation at 3000 x g for 15 min. By employing liquid-liquid chromatography with ethyl acetate as a solvent, the cleared culture broth was used to isolate metabolites. The extract was evaporated in a rotary evaporator set at 40 °C and 90 rpm in order to extract the crude metabolites. The leftover material was recondensed. After that, the concentrated crude extract was kept at 4 °C for further research (Arasu et al. 2014).

Preliminary screening of chemical compounds

Standard qualitative chemical analysis was conducted on metabolite extract of isolates to detect the presence of chemical compounds such as phenol, alkaloid, flavonoid, terpenoid, steroid, saponins, tannins and glycoside (Harborne 1973).

Bioactivity of sponge associated microbial metabolite

Antioxidant activity of extracted metabolite

A sterile 96-well plate was used to test the metabolite capacity to scavenge DPPH free radicals. The extract was blended (1:1 v/v) with DPPH (0.02 mg/mL) at various doses and incubated at 25 °C in the dark for 30 minutes. The absorbance was measured at 517 nm using an ELISA plate reader (The Infinite F50 Plus-with Magellan data analysis software). Ascorbic acid (AA) was utilised as thestandard. The percent inhibition of DPPH radical was measured by using the formula: [A0 − A1 / A0] × 100 where, A1 and A0 equal the absorbance of the control and the test, respectively and median inhibitory concentration (IC50) was calculated (Tsilo et al. 2020).

Anticancer activity- MTT assay

HeLa cells were seeded in Roswell Park Memorial Institute (RPMI) 1640 medium, and the initial number of cells was counted using a microscope. After being trypsinised, the cells were centrifuged. A hemocytometre was used to count the cells after the supernatant was removed and the pellet was resuspended in 1 mL of complete media. A 96-well plate containing 2×10^4 cells was then seeded with 100 µl of the medium and cultured overnight to promote cell adhesion. Following that, 100 µL of test material extracts were added to each well at varied concentrations (512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 µg/mL). The cells were then kept at 37°C for another 24 hours. The cells were examined under a microscope after 24 hours. Each well was treated with 5-mg/mL 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT). To dissolve the formazan crystals, a stop solution of dimethyl sulfoxide (DMSO) was then given to each well. An ELISA plate reader (The Infinite F50 Plus-with Magellan data analysis software) was used to measure the cell viability at 570 nm (Elmanama et al. 2020).

Biochemical and molecular identification of bacteria

The bacterial isolates were identified using methyl red, indole, vogues proskauer, urease,citrate utilisation, TSI and oxidase test (Chelossi et al. 2004). The DNA isolation was carried out by phenol-chloroform methodIn brief, a homogenization buffer containing proteinase K and

SDS was used for cell lysis. The DNA-rich aqueous phase was then obtained through phase separation using phenol, chloroform, and isoamyl alcohol, and was further precipitated with isopropanol. The extracted DNA was redissolved in a solution containing Tris and EDTA and kept at -20°C until use (Wright et al. 2017). 16S rRNA sequencing was used to identify the isolated DNA from the bacterial isolate. The template for PCR amplification was genomic DNA. For 16S rRNA gene amplification primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') were used. The following conditions were used for the PCR process: initial denaturation at 94°C for 5 min, then 40 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 3 min, with a final extension at 72°C for 10 min. dNTPs (0.2 mM each), 1 reaction buffer (20 mM Tris pH 8.4, 50 mM KCl), MgCl2 (1.7 mM), primers (0.1 M each), and Taq DNA polymerase (1.25 units) were included in the PCR reaction mixture (50 µl). Amplification was done using a Thermocycler system (HiMedia Laboratories Private Limited). Agarose gel electrophoresis was used to evaluate the PCR products (Kennedy et al. 2009).The PCR fragments were sequenced by Genetic analyser (Sanger DNA Sequencer). The 16S rRNA gene sequence was evaluated using BLAST NCBI GenBank database to identify similar bacterial sequences. The phylogenetic tree was created using MEGA 7 software, according to Neighbor Joining method.

RESULTS AND DISCUSSION

Sponge identification isolation of bacteria

Marine sponge, *Spongia officinalis* (class Demospongiae) (Figure 1) was identified at the Department of Zoology, University of Madras. Demosponges have reportedly been found to contain a significant amount of pharmaceutically relevant bioactive chemicals (Krishnan & Keerthi 2016). The total colony-forming units of sponge's sample were analysed four days after initial inoculation. Totally 12 different bacterial colonies were isolated from processed marine sponge sample on the basis of unique colonial characteristics on Zobell marine agar plate. The 12 isolates were labelled as KKS01, KKS02, KKS03, KKS04, KKS05, KKS06, KKS07, KKS08, KKS09, KKS10, KKS11, KKS12 respectively.

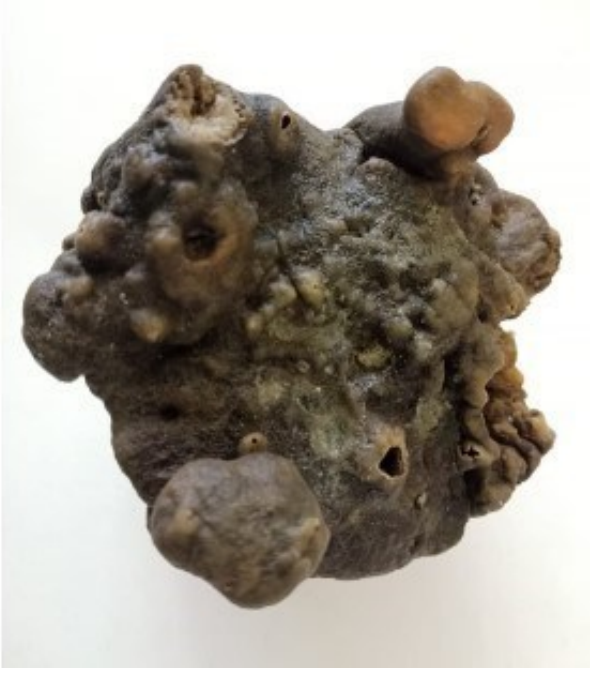

Figure 1. *Spongia Officinalis* (class Demospongiae).

Morphological identification of metabolites

Morphological helps in partial identification of microorganism. Gram staining and colony morphology (colour, form, margin, elevation) results of all the 12 isolates were represented in Table 1 and Figure 2. Colony morphological characteristics of the isolates showed that all the isolate have shown varied morphologies. Gram staining results revealed that gram positive strain dominated the isolates extracted from *Spongia officinalis*.

Table 1. Colony Morphology and Gram Staining of Selected Isolates.

S.NO	Isolate	Colony Morphology	Gram Reaction
1	KKS01	Orange, irregular, convex and entire	Gram positive rod
2	KKS02	White, punctiform, convex, smooth	Gram positive rod
3	KKS03	Yellow, circular, flat, wavy	Gram positive rod
4	KKS04	Creamy white, round, convex, entire	Gram negative rod
5	KKS05	Yellowish white, irregular, raised, curled	Gram positive rod
6	KKS06	Yellowish green, round, raised and smooth	Gram negative rod
7	KKS07	Pale white, flat, convex, entire	Gram positive rod
8	KKS08	White, circular, raise, entire	Gram positive rod
9	KKS09	Off white, irregular, convex, wavy	Gram negative rod
10	KKS10	Orange, circular, flat, entire	Gram negative rod
11	KKS11	Yellow, punctiform, convex, entire	Gram negative cocci
12	KKS12	Glossy white, circular, raised, wavy	Gram positive rod

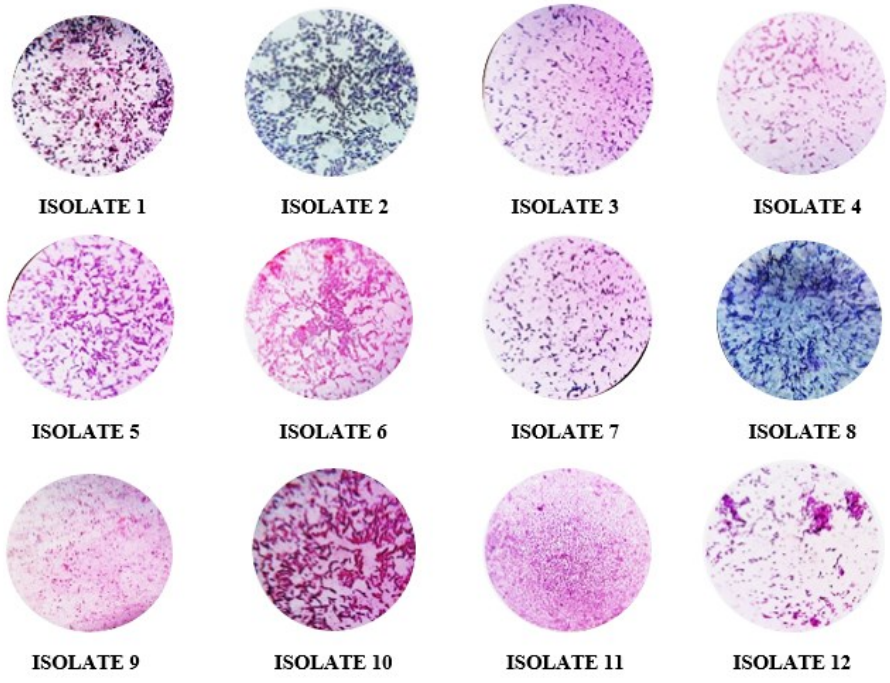

Figure 2. Gram Staining of Bacterial Isolates.

Extraction of secondary metabolites from bacteria using liquid-liquid extraction method

Potential bacterial strains were produced using the flask method using Zobell marine broth for 3 days. To determine a good yield on alternate days, culture was analysed. Broth contains a higher yield of secondary metabolites based on the quantity of yield at the fifth day of culture. The crude ethylacetate of 12 strains extract was subjected to preliminary bioactive compound screening. The production capacity of secondary metabolites often depended on a stationary phase of bacterial development, during which there was a steady overall rate of bacterial growth and death. Active secondary metabolite with antioxidant capacity was screened further by DPPH assay.

Preliminary screening of bioactive compounds

Qualitative chemical analysis

The qualitative chemical analysis of ethylacetate extract of 12 isolates are represented in table 2. Table 2 explains the 8 different bioactive metabolites present in ethylacetate extracts of isolates by qualitative analysis. Different bioactive compounds were present in different isolates. Commonly, phenol and flavonoid were unanimously present in almost all isolates except isolates KKS03 and KKS12. Triterpenoids from several sea sponges have been reported to have cytotoxic action (Li et al. 2013). Marine sponges contain alkaloids, steroids, terpenoids, phenols, and saponins that have been linked to a variety of bioactivities, including antibacterial and antioxidant properties (Riguera 1997; Bhakuni & Rawat 2006; Tangman et al. 2015). From the result, it was evident that all these chemical compounds either individually or in combination contribute to various health benefits.

Screening of anticancer potential metabolites

DPPH assay

Reactive Oxygen Species (ROS) overproduction is a condition that leads to oxidative stress. Oxidative stress, which has been connected to several

Table 2. Phytochemical analysis of ethylacetate extract of selected isolates.

Isolate	Phenol	Flavanoid	Alkaloid	Tannins	Saponnin	Glycoside	Steroid	Terpenoid
KKS01	++	++	-	-	+	-	-	-
KKS02	+++	++	-	-	++	-	-	
KKS03	-	-	++	-	-	-	-	+
KKS04	++	++	+	-	-	-	-	-
KKS05	+++	++	-	-	-	-	-	-
KKS06	+++	+++	-	+	-	-	++	+++
KKS07	-	-	-	++	++	-	++	-
KKS08	+++	+++	-	-	-	-	-	-
KKS09	+	+	-	-	-	++	++	-
KKS10	+	+++	-	+	-	-	-	-
KKS11	+	+	-	-	-	-	-	-
KKS12	-	-	+++	-	++	-	++	-

Note: '-' Indicates absence, '+' Indicates slight presence, '++' Indicates moderate presence, '+++' Indicates High Presence.

health problems, including cancer, is defined as a disparity among the body's enzymatic antioxidants and the rate at which free radicals are produced. According to a number of recent studies, bacterial metabolites function as antioxidants by producing certain compounds that can scavenge oxygen radicals. The ethyl acetate extracts from 12 bacterial isolates have been tested for antioxidant activity by DPPH assay. Isolate KKS02, KKS04, KKS05, KKS07, KKS08, KKS09, KKS10, KKS11 and KKS12 did not show good antioxidant activity. Figure 3 and 4 represents the percentage inhibition of ascorbic acid and ethyl acetate extract of 12 isolates against DPPH to analyse the antioxidant activity.

Figure 3 shows the percentage inhibition of standard ascorbic acid at 1000µg/mL was found to be 90.32±0.2, while the IC_{50} value was observed to be 99.05±0.3 According to Figure 4, the percentage inhibition was observed to be 80.73±0.1, 52.86±1, 73.01±0.8, 52.10±0.9, 53.85±0.9, 85.16±1, 53.73±0.9, 42.70±0.8, 41.35±0.3, 35.98±0.3, 45.44±0.5, 39.13±0.2 at 1000µg/mL for KKS01, KKS02, KKS03, KKS04, KKS05, KKS06, KKS07, KKS08, KKS09, KKS10, KKS11 and KKS12 respectively. In addition, IC_{50} values of KKS01, KKS02, KKS03, KKS04, KKS05, KKS06, KKS07, KKS08, KKS09, KKS10, KKS11 and KKS12 were found to be 237.24±0.6, 887.08±0.7, 322.92±0.8, 897.83±0.8, 832.26±0.8, 167.26±1, 740.90±0.6, 1144.67±0.7, 1202.92±0.5, 1372.31±0.4, 1056.52±0.6, 1341.30±0.5 respectively. Among these KKS6 showed tremendous radical scavenging activity (85.16 ±1%) with minimum inhibitory concentration of 167.26±0.1 µg/mL at highest concentration when compared to other extracts. In this study, the metabolite extracted from KKS6 isolates showed strong antioxidant activity. According to earlier research, *Bacillus licheniformis* established antioxidant molecules from shrimp waste that were highly effective antioxidants (Kumar et al. 2013). Similarly, Extracts of *Actinobacteria* isolated from soil or marine sediments have been proven in studies to exhibit potent antibacterial and antioxidant properties (Rao & Rao 2013; Shivale et al. 2018). The antioxidant capacity of the crude metabolites would have been enhanced by the presence of bioactive chemicals. As the bioactive metabolites demonstrated a potential response in scavenging the free radicals, it may be used as a useful medication to treat pathological illnesses caused by free radicals, such as cancer (Arunachalam & Appadorai 2013).

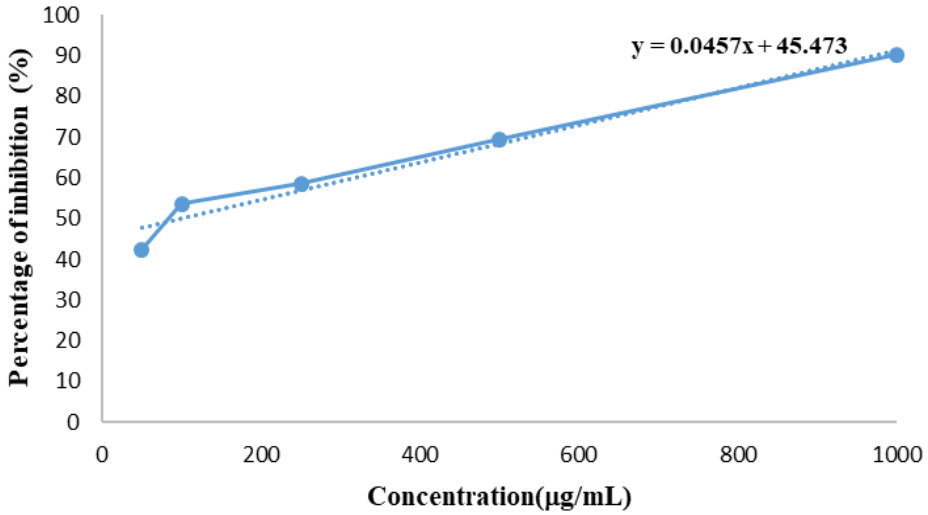

Figure 3. Percentage of inhibition (DPPH ASSAY).

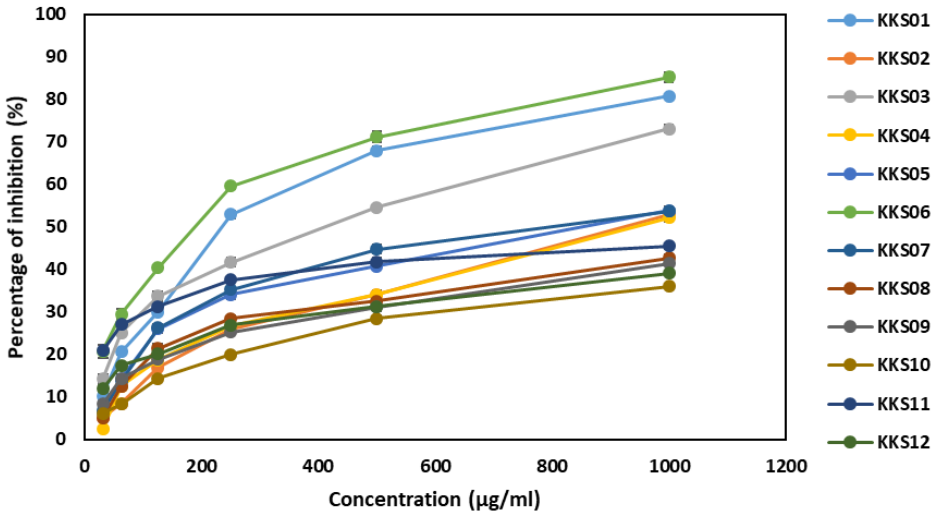

Figure 4. Percentage of inhibition (DPPH ASSAY).

Anticancer activity against HeLa cell line

To combat the problem of drug resistance and the negative side effects linked to several proven synthetic anticancer treatments, the search for new anticancer compounds from natural sources is essential. The remarkable capacity of marine sponge derivatives to impede the spread of malignancies has led to intensified research activities aimed at discovering innovative anticancer medicines. (Zhang et al. 2017; Ćetković et al. 2018). In terms of the variety of their bioactive metabolites, marine sponges are a "gold mine," and they could produce future medicines for a number of serious, globally prevalent diseases (Koopmans et al. 2009).

HeLa cells were incubated with ethylacetate extract of KKS6which showed strong antioxidant activity among other metabolites for 48 hours to test whether growth could be inhibited at concentration between 512 to 1 µg/mL. Figure 5 represents the percentage of inhibition and minimum inhibitory of ethylacetate extract of KKS6 against HeLa cell line. The percentage inhibition was observed to be 35.28±0.1, 36.44±0.4, 37.84±0.3, 43.09±0.4, 55.14±0.8, 57.93±1.2, 70.03±2, 74.98±1.4, 85.51±3.9, 90.37±1.4 at concentration between 1 to 512 µg/mL respectively. Isolate KKS6 significantly inhibited growth of HeLa cell line, with IC_{50} values of 55.32±1.2 µg/mL. *Microbacterins* A and B, significantly inhibited the growth of human tumour cell lines such as Bel-7402, HCT-8, A549, A2780 and BGC 823 (Liu et al. 2015). Previous research has reported that the *B. velezensis* isolated from marine sediments hadanticancer properties against MCF-7 cell line (Mostafa et al. 2019). Lodopyridone, an alkaloid from a marine *Saccharomonospora* species, was discovered to be cytotoxic (IC50 = 3.6 M) to HCT-116 human colon cancer cells (Maloney et al. 2009). Hence from comparing the result with the current study, it was evident that crude metabolite extracted from *Spongia officinalis* bacteria can be a potent anticancer drug.

Biochemical characterization

The isolate KKS66 was grown in King's B medium and incubated at 37°C for 24 hours. Pure colony of isolate 6 (blue- green colonies) under UV light was depicted in Figure 6. Biochemical characterization of KKS6was carried out. Results revealed that KKS6 was all test except citrate and oxidase are negative as given in table 3. From the results it is evident the organism belongs to genus *Pseudomonas.*

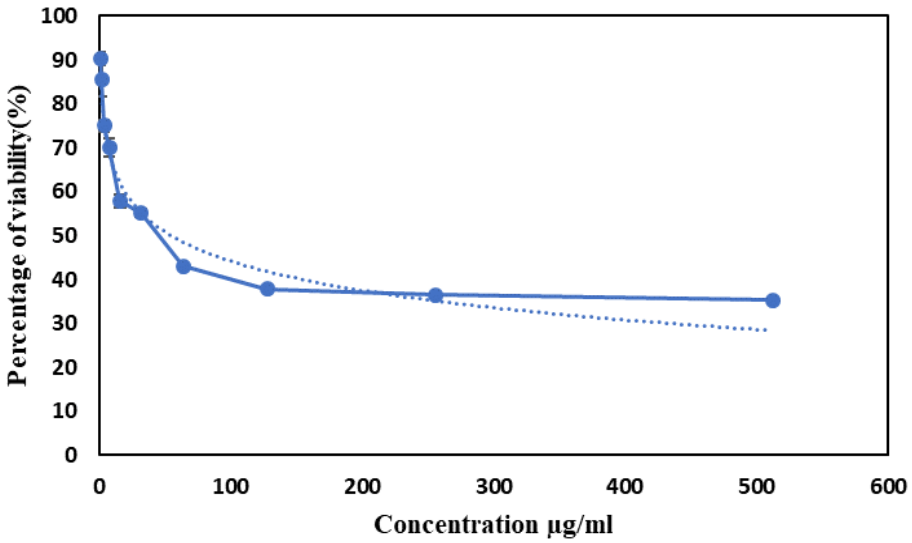

Figure 5. Percentage of viability (HeLa cell line).

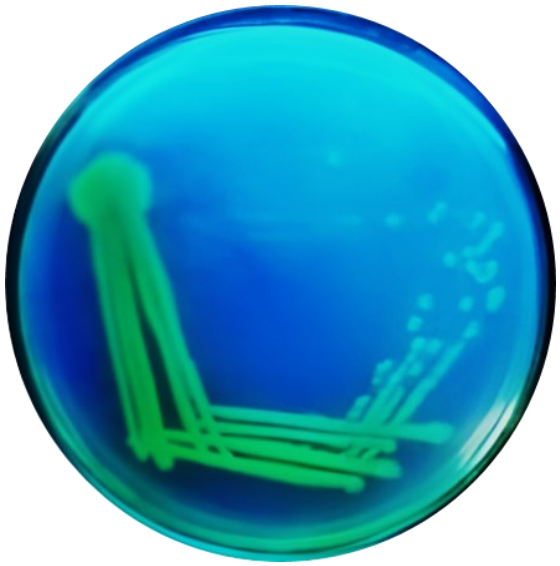

Figure 6. Pure colony isolation of KKS6.

Table 3. Biochemical characterization of KKS6.

Biochemical Test	Observation	Result
Indole Test	No pink or red color formation- The organism could not decompose tryptophane to indole	Negative
Methyl Red Test	No color change and hence no glucose fermentation	Negative
Voges Proskauer Test	No colour change and hence no production of acetylmethyl carbinol from glucose fermentation	Negative
Citrate Test	Change to blue color indicates the ability of organism to use citrate as sole carbon source	Positive
Tsi	Alkaline slant and bottom indicates no carbohydrate fermentation	Negative
Oxidase	The purple color change indicates the ability of organism to produce cytochrome c oxidase	Positive
Urease	No hydrolysis of urea indicate negative result	Negative

Molecular identification by 16s rRNA sequencing

DNA isolation was performed and PCR product was validated by agarose gel electrophoresis shown in Figure 7. The 16SrRNA partial sequence alignment database search of screened isolate shows 99% identity with *Pseudomonas fluorescens* strain by BLAST analysis. The phylogenetic tree was constructed with the aid of BLAST analysis (Figure 8). Hence the present study reveals that the query organism (KKS6) is identical to *Pseudomonas fluorescens* strain ATCC 13525.

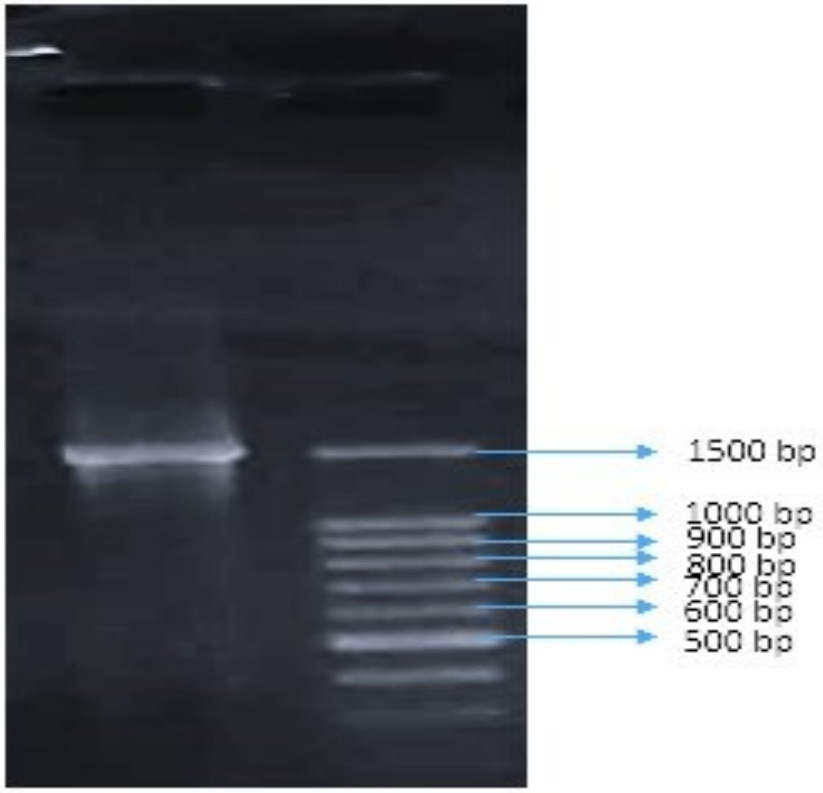

Figure 7. Agarose gel electrophoresis of PCR product (left- sample DNA: right -ladder.

CONCLUSION

In conclusion, the study identified 12 symbiont bacterial isolates from the marine sponge *Spongia officinalis*, with morphological characteristics re-

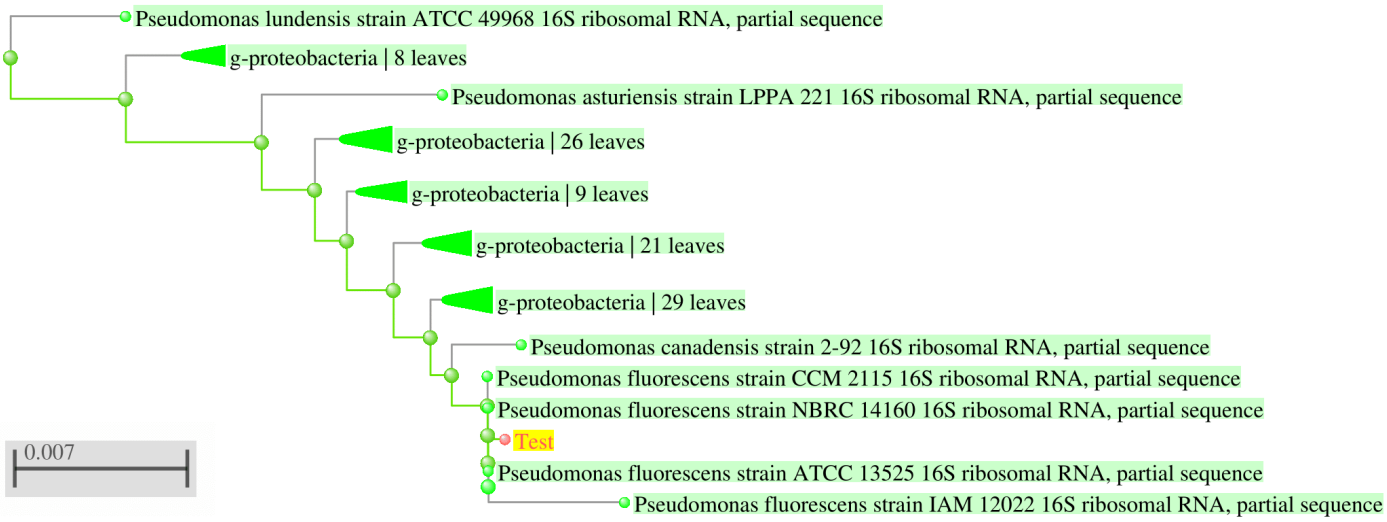

Figure 8. Phylogenetic tree (Neighbour – joining method- Kimura two-parameter (K2P) Model).

vealing predominant Gram-positive rods. Ethylacetate extracts exhibited diverse bioactive compounds, notably phenols and flavonoids. Isolate KKS6 showed remarkable antioxidant activity with an IC50 value of 167.26±0.1 µg/mL and significant inhibition of HeLa cell line growth with an IC50 value of 55.32±1.2 µg/mL compared to extracts. The 16s rRNA sequencing confirmed the organism to be *Pseudomonas fluorescens*. These findings suggest the potential of *Spongia officinalis*-associated symbiont bacteria, particularly *Pseudomonas fluorescens*, as a source of bioactive compounds with antioxidant and anticancer properties. The future study will focus on in-vivo studies to confirm the potential of these metabolites as potential drug candidate.

AUTHORS CONTRIBUTION

Conceptualization, M.J. Y.D. and U.S.; Data curation, M.J. and U.S.; Investigation, M.J. Y.D and U.S.; Supervision, M.J.; Validation, M.J. and Y.D.; Roles/Writing - original draft, M.J.; Writing - review & editing, M.J. and U.S.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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Research Article

Diversity of *Zingiber* Mill. (Zingiberaceae) in Peninsular Malaysia Including Short Remarks of an Undescribed Taxon

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ABSTRACT
Zingiber, a notable genus within the Zingiberaceae family, is widely distributed throughout Southeast Asia. It encompasses a total of at least 141 species on a global scale, with 25 native species and 30 known taxa identified specifically in Peninsular Malaysia. Of these known taxa, at least 7 are categorised as threatened, 5 are endemic, and the rest remain unassessed regarding their conservation status. This article provides a comprehensive checklist and taxonomic insights for all native *Zingiber* in Peninsular Malaysia. Remarkably, from the current fieldwork, the discovery of a peculiar *Zingiber* plant from the northern part of Peninsular Malaysia holds the potential to contribute additional records within this genus. Initially, this species resembles *Z. belumense* and *Z. purpureum* in their inflorescence colouration, displaying shades ranging from brownish maroon to dark purple with green bracts, but this newly proposed taxon stands out due to its combination of distinctive traits. An intriguing observation notes the presence of red sap when the leafy shoots were cut and needs further corroboration. This article establishes a provisional taxonomic designation for the newly discovered species, *Zingiber* sp. (Bahangense130). A comprehensive description supported by robust molecular phylogenetic evidence is currently underway, while brief notes and illustrative images of the proposed taxon are provided within this paper.

INTRODUCTION
Ginger has been exploited since ancient times in Chinese medication, Ayurvedic remedies, and even daily spices. It remains popular and recognised for its significance in potential health benefits, besides being a staple ingredient in many cuisines worldwide (Bhatt et al. 2013; Semwal et al. 2015; Dissanayake et al. 2020; Kumari et al. 2020) *Zingiber* Mill. was derived from the Sanskrit word *singabera* (Larsen et al. 1999). Locally known as *halia* in Malaysia and *jahe* in Indonesia, the genus *Zingiber* is prevalent among genera of the family Zingiberaceae, encompassing 141 species primarily in tropical and subtropical Asia. This principal and complex genus is categorised under the order Zingiberales with 53 genera and has more than 1200 species globally (Holttum 1950; Larsen et al. 1999; Kress et al. 2002; POWO 2023).

Peninsular Malaysia is rich and diverse with wild gingers (Holttum 1950). Earlier studies by pioneer researchers have provided decisive information on *Zingiber* in Peninsular Malaysia (Ridley 1924; Holttum 1950; Theilade 1996). In 1950, the genus *Zingiber* was revised by Holttum, who instigated the ambiguity of three complex species: *Z. gracile* Jack, *Z. griffithii* Baker, and *Z. puberulum* Ridl. (Holttum 1950). Forty-eight years later, Theilade raised varieties of *Z. gracile* to species rank: *Z. gracile* var. *aurantiacum* Holttum, *Z. gracile* var. *elatior* Ridl., and *Z. gracile* var. *petiolatum* Holttum, besides acknowledging a new taxon, namely *Z. fraseri* Theilade because of its arcuate leafy shoots (Holttum 1950). The latest study done in 2014 presented 6 new taxa while subsuming *Z. fraseri* Theilade under *Z. griffithii* var. *major* Holtt. and elevating *Z. besar* Lim & Meekiong to species status based on the morphological description (Lim & Meekiong 2014a). Nevertheless, after thoroughly investigating the correlated species, the authors retracted the previous classifications within the same year. *Zingiber fraseri* was then redetermined as *Z. besar* var. *fraseri* (Theilade) C.K.Lim & Meekiong (Lim & Meekiong 2014b). Consequently, *Z. besar* had been accepted as a synonym for *Z. fraseri* as the name was older than *Z. besar* (Govaerts 2016). Three established infraspecific names in the International Plant Names Index (IPNI) are *Z. fraseri* Theilade, *Z. fraseri* var. *major* (Ridl.) Govaerts, and *Z. fraseri* var. *nervifolium* (Meekiong and C.K.Lim) Govaerts.

Currently, 25 species with 30 taxa of *Zingiber* in Peninsular Malaysia have been identified and extensively distributed from southern to northern Peninsular Malaysia (POWO 2023; IPNI 2023). They dispersed at different elevations ranging from lowland and mid-mountain forest to upper montane forest (Holttum 1950; Theilade 1996; Lim & Meekiong 2014a; Lim & Meekiong 2014b). Meanwhile, some of the *Zingiber* species like *Z. longibracteatum* and *Z. chrysostachys* flourish well in the limestone hills in Perlis and Perak. Additionally, 11 species of *Zingiber* in Peninsular Malaysia are said to be overlap with those in Thailand (*Z. raja*, *Z. chrysostachys*, *Z. fraseri* var. *fraseri*, *Z. fraseri* var. *major*, *Z. longibracteatum*, *Z. multibracteatum*, *Z. petiolatum*, *Z. puberulum* var. *puberulum*, *Z. spectabile*, *Z. wrayi* var. *wrayi*, and *Z. wrayi* var. *halabala*) and one species with Myanmar (*Z. gracile*) (POWO 2023). The distribution patterns of the *Zingiber* species are influenced by a combination of ecological conditions, including soil nutrition, climate, and elevation since they share a biogeographical region (Ordonez et al. 2009).

This work offers insight into *Zingiber* in Peninsular Malaysia based on various published sources besides field observations and morphological examinations. Also, a potentially new taxon that resembles *Z. purpureum* Roscoe and *Z. belumense* C.K.Lim & Meekiong is described. Upon thorough examination, the inflorescence shape and the labellum vary. Furthermore, notable characteristics such as red sap were observed when the leafy shoots were cut, eventually making the species distinct among the *Zingiber* of Peninsular Malaysia.

MATERIALS AND METHODS

From September 2022 to September 2023, a series of fieldwork was primarily conducted at type localities of *Zingiber* in Peninsular Malaysia, including Penang, Pahang, Johor, and Perak. Fertile samples, representing plants bearing flowers and/or fruits, were meticulously collected for further analysis and preservation as herbarium specimens. Concurrently, sterile samples were also documented and collected as voucher specimens. Each type locality within forest reserves was constantly revisited following the flowering period of each taxon and precisely marked with

Global Positioning System (GPS) coordinates. However, for the newly proposed taxon, only the general area was stated in the paper (e.g., the mountain range's name), as we were concerned with its conservation status. Additionally, favourable sites for wild gingers, like humid, shady environments near streambanks and swampy areas in lowland forests and hilly slopes, were also observed. Nine specimens were collected from the wild, namely *Z. aurantiacum*, *Z. flaviflorum*, *Z. malaysianum*, *Z. griffithii*, *Z. gracile*, *Z. spectabile*, *Z. multibracteatum*, *Z. raja*, and *Zingiber* sp. (Bahangense130). The plant parts, including vegetative and floral parts, were examined and measured using a measuring tape. The floral parts, like the stigma and the surface of the ovary, were examined using a USB digital microscope (1000×) to observe the details. All collected specimens were identified and described morphologically.

Furthermore, all *Zingiber* species in Peninsular Malaysia were compared and identified from resources like the published protologues and studied herbarium specimens at the Forest Research Institute Malaysia (KEP). The conservation status of each *Zingiber* species was based on the IUCN Red List of Threatened Species (IUCN 2023). For herbarium specimens, collected samples were soaked in 70% ethanol, pressed, and dried in the oven at 50–60 °C for a week. They were then deposited in the herbarium of Universiti Tun Hussein Onn Malaysia.

RESULTS AND DISCUSSION

Zingiber of Peninsular Malaysia

Zingiber of Peninsular Malaysia prefers humid, shady environments; for instance, near creeks (*Z. griffithii*), riverbanks (*Z. raja*), and fresh swampy areas (*Z. puberulum*). Some of the *Zingiber* species, like *Z. malaysianum*, *Z. flaviflorum*, and *Z. multibracteatum* prosper in open ground with semi-shady environments. At the same time, *Z. gracile*, *Z. multibracteatum* and *Z. aurantiacum* are often found on hill slopes to steep hill slopes. Common *Zingiber* species such as *Z. spectabile* thrive at the roadsides of Fraser's Hill and along the pavements of the forest reserve in Taka Melor Eco Forest. Based on our observations in the wild, most *Zingiber* species in Peninsular Malaysia grow well in sandy loam soil, some with thick litter, but *Z. puberulum* thrives on rocks and peaty soil.

List of *Zingiber* in Peninsular Malaysia

A total of 25 species and 30 taxa of wild *Zingiber* in Peninsular Malaysia were described from 1950 to 2014 (Holttum 1950; Theilade 1996; Lim 2001; Lim 2003; Leong-Škorničková 2014; Lim & Meekiong 2014a; Lim & Meekiong 2014b). The data on the distribution, elevations, and conservation status of the genus *Zingiber* are tabulated in Table 1. Informative notes on each taxon are provided. A brief description of the probable new species, *Zingiber* sp. (Bahangense130) is also included.

Zingiber aurantiacum (Holttum) Theilade, Gard. Bull. Singapore 48: 232 (1996 publ. 1998)

Lectotype: Burkill & Holttum SFN. 8806. (SING). Peninsular Malaysia, Pahang, Fraser's Hill (Figure 1)

Distribution: Johor, Malacca, Negeri Sembilan, Pahang, Selangor

Description: See Holttum (1950), Theilade (1996), Lim (2003)

Notes: The Latin word *aurantiacum* means orange colour. The colour of the inflorescence bract is a discernible morphological characteristic of *Z. aurantiacum* species. The large plant, lengthy scape, and elongated ovoid inflorescence in orange with a green tinge at the apex of each bract, which matures to a reddish-pink hue, are distinguishing characteristics of

Table 1. Information of *Zingiber* in Peninsular Malaysia.

Species	Distribution	Elevations (m a.s.l)	IUCN Conservation status	Taxonomic Authority
Z. aurantiacum	PM	1,300 m	VU	(Holttum) Theilade
Z. angustifolium	PM	1,800 m	-	C.K.Lim & Meekiong
Z. belumense	PM	280 m	-	C.K.Lim & Meekiong
Z. chrysostachys	PT–PM	200–1,400 m	EN	Ridl.
Z. curtisii	PM–Perak		DD	Holttum
Z. elatius	PM–Penang	Up to 1,150 m	DD	(Ridl.) Theilade
Z. flaviflorum	PM	1,300 m	-	C.K.Lim & Meekiong
Z. fraseri var. *fraseri*	PT–PM	1,300 m	EN	Theilade
Z. *fraseri* var. *major*	PT–PM		-	(Ridl.) Govaerts
Z. fraseri var. *nervifolium*	PM		-	(Meekiong & C.K.Lim) Govaerts
Z. gracile	Myanmar–PM	30–450 m	DD	Jack
Z. griffithii	PM–Borneo	30–50 m	NT	Baker
Z. kelantanense	Unknown	200 m	-	C.K.Lim
Z. kunstleri	PM	150–950 m	LC	King ex Ridl.
Z. limianum	PM		-	Meekiong
Z. longibracteatum	PT–PM		-	Theilade
Z. malaysianum	PM		LC	C.K.Lim
Z. multibracteatum var. *multibracteatum*	PT–PM	1,300 m	NT	Holttum
Z. multibracteatum var. *viride*	PM–Pahang	1,800–2,000 m	-	Holttum
Z. nazrinii	PM		-	C.K.Lim and Meekiong
Z. petiolatum	PT–PM		VU	(Holttum) Theilade
Z. puberulum var. *chryseum*	PM–Johor		-	(Ridl.) Holttum
Z. puberulum var. *puberulum*	PT–PM	350 m	NT	Ridl.
Z. raja	PT–PM	250 m	EN	C.K.Lim & Kharukanant
Z. sabun	PM		-	C.K.Lim
Z. sulphureum	PM–Pahang		EN	Burkill ex Theilade
Z. spectabile	PT–PM	Up to 1,000 m	DD	Griffith
Z. wrayi var. *halabala*	T–PM		-	C.K.Lim
Z. wrayi var. *wrayi*	T–PM		EN	(Prain ex Ridl.) Ridl
Z. zerumbet	Tropical, subtropical Asia		DD	(L.) Roscoe ex. Sm.
Zingiber sp. (Bahangense130)	PM–Penang	114 m	-	Aimi Syazana & Salasiah

PM: Peninsular Malaysia, PT: Peninsular Thailand, T: Thailand, VU: Vulnerable, EN: Endangered, DD: Data Deficient, NT: Near Threatened, LC: Least Concern

this species. The identification of this species is facilitated by the prominent pulvinus, pale maroon-suffused ligule, and sheath. Initially classified alongside *Z. aurantiacum* as a variety of *Z. gracile*, Holttum failed to specify the type locality (Holttum 1950). Theilade subsequently selected the lectotype of *Z. aurantiacum* from Fraser's Hill (Theilade 1996). Based on observation, *Z. aurantiacum* is commonly found in Fraser's Hill. Furthermore, the pungent aromatic odour emitted upon crushing the leaves is a notable characteristic that sets it apart from related species such as *Z. petiolatum*, *Z. gracile*, and *Z. kelantanense*. *Zingiber aurantiacum* is classified as a montane plant due to its exclusive occurrence in higher montane regions. Based on the IUCN Red List of Threatened Species, this species is categorised as Vulnerable (VU) (criterion: B2ab(iii)) and the population trend is decreasing (Table 1).

Figure 1. *Zingiber aurantiacum*. (a) The inflorescence of *Z. aurantiacum* during the flowering period. (b) The inflorescence of *Z. aurantiacum* turns pink when it is fruiting. (c) The leaf sheath of *Z. aurantiacum* can be differentiated by the purple spots at the ligule and the sheath. (d–e) The habitat of *Z. aurantiacum* in Fraser's Hill.

Zingiber angustifolium C.K.Lim & Meekiong, Folia Malaysiana 15: 32 (2014)

Holotype: C.K. Lim L12417. (UKMB). Peninsular Malaysia, Negeri Sembilan, Gunung Berembun, 7th June 2014

Distribution: Johor, Malacca, Negeri Sembilan, Pahang, Selangor

Description: See Lim and Meekiong (2014a)
Notes: The terminology pertains to the unique, slender, and linear leaflets. The species' characteristic is comparable to that of other *Zingiber* species, including *Z. raja* and *Z. petiolatum*, in terms of their tall size distinguished by their unscented foliage and unicostate leaves. Moreover, the inflorescence resembles that of *Z. raja*; however, the flower distinguishes itself through its white coloration—in contrast to *Z. raja*, which possesses purple speckles along the labellum. Thus far, *Z. angustifolium* has been observed inhabiting verdant forests close to mountain peaks, where it grows sympatrically with wild ginger species such as *Alpinia scabra* and *Meistera ochrea* (Lim & Meekiong 2014a).

Zingiber belumense C.K.Lim & Meekiong, Folia Malaysiana 15: 26 (2014)
Holotype: C.K. Lim L12590. (UKMB). Peninsular Malaysia, Perak, Belum ("Titiwangsa Highpoint")
Distribution: Perak, Belum ("Titiwangsa Highpoint")
Description: See Lim and Meekiong (2014a)
Notes: The name signifies the type locality, Belum Forest Reserve, where this species is exclusively found. Therefore, *Z. belumense* is regarded as both unique and susceptible. This tiny ginger is distinguished by its slender fusiform inflorescence, comprising light cream flowers and brilliant green bracts that mature to dark brown or red. Observations revealed that this species exhibited more robust, 3 m-tall plants with arcuate leafy shoots. Notably, this species inhabits the slopes of bamboo forests and is sympatric with *Alpinia* species and *Z. multibracteatum* (type: Fraser's Hill, hilly slope), which are uncommonly observed in gingers. In addition, it is worth noting that the Titiwangsa Highpoint serves as the type locality for both *Iguanura belumensis* C.K.Lim and *Geostachys belumensis* C.K.Lim (Lim & Meekiong 2014a).

Zingiber chrysostachys Ridl., J. Straits Branch Roy. Asiat. Soc. 32: 129 (1899)
Lectotype: Ridley 19960. (SING). Peninsular Malaysia, Perak, Gerik
Distribution: Kedah, Perak
Description: See Holttum (1950), Theilade (1996)
Notes: Vegetatively, *Z. chrysostachys* is closely related to *Z. curtisii*. On the contrary, the inflorescence resembles the cultivated species *Z. ottensii* and *Z. spectabile* due to its inflexed bracts and red speckles on its labellum. However, in contrast to closely related species, *Z. chrysostachys* is a remarkably tiny plant and is the only small species with inflexed bract characteristics. The yellow inflorescence of *Z. chrysostachys* features in complete contrast to its purple peduncle. In terms of identification, the diagnostic characters are beneficial. This species thrives at mid-elevations in secondary and dry bamboo forests on limestone slopes in Perak and Kedah. *Z. chrysostachys* leaves, known as *lampoyang* or *lempui*, are historically used by Malay traditional healers to treat fever (Holttum 1950; Theilade 1996). Despite that, this Endangered (EN) species (criterion: B2ab (iii)) population is decreasing. The species is threatened by logging and harvesting as the young inflorescence is consumed as food (Table 1) (Ragsasilp et al. 2022; IUCN 2023).

Zingiber curtisii Holttum, Gard. Bull. Singapore 13: 54 (1950)
Lectotype: Ridley 19960. (SING). Peninsular Malaysia, Perak, Gerik
Distribution: Kedah, Perak
Description: See Holttum (1950), Theilade (1996)

Notes: The sterile plant of this species resembles that of *Z. chrysostachys* in appearance. However, the inflorescence grows longer and slenderer. The inflorescence bract is notably conspicuous due to its pale yellow-green colouration and a minor inflexion at its apex. In addition, the labellum and sidelobes of *Z. curtisii* are intricately patterned in a deep purple hue. In contrast, the anthers exhibit a profound purple hue (Holttum 1950; Theilade 1996). *Zingiber curtisii* population is stable and categorised as Data Deficient (DD) (Table 1).

Zingiber elatius (Ridl.) Theilade, Gard. Bull. Singapore 48: 227 (1998)
Lectotype: Ridley 9340. (K) [barcode K000255246], Peninsular Malaysia, Penang
Distribution: Johor, Penang, Perak, Selangor
Description: See Holttum (1950), Theilade (1996)
Notes: Initially, the species name *Z. elatius* was published as *Z. gracile* var. *elatior* or *Z. elatior*, both of which contained an erroneous grammatical Latin termination. Following the International Code of Nomenclature for Plants, Fungi, and Algae (ICN), the name was subsequently changed to *Z. elatius* (Leong-Škorničková 2014). Theilade (1996) stated that the specimen collection was deposited at SING; however, a comprehensive manual search of the Zingiberaceae collection and the Zingiberaceae type collection at SING yielded no trace of this specimen ever being included in the collection. A specimen that matched Ridley's initial description was discovered at the Kew Herbarium; it had been collected in Penang and possessed comparable morphological characteristics. The new lectotype is, therefore, designated by the barcode [K000255246] (Lim & Meekiong 2014a). *Zingiber elatius* is distinguished by its linear leaves and orange to red bracts slender fusiform inflorescence. According to the IUCN, *Z. elatius* is Data Deficient (DD) and the population trend is unknown (Table 1).

Zingiber flaviflorum C.K.Lim & Meekiong, Folia Malaysiana 15: 37 (2014)
Holotype: C.K. Lim L12539 (UKMB). Peninsular Malaysia, Pahang, Fraser's Hill (Figure 2)
Distribution: Common along the trail in Fraser's Hill
Description: See Lim and Meekiong (2014a)
Notes: *Flaviflorum* means yellow in Latin. The epithet denotes the flowers' pale yellow to light cream hue. The leaves emit a potent aroma and fragrance upon being crushed. In contrast to *Z. gracile*, this species is distinguished by its broader inflorescence, which features a yellow to light yellow flower and glossy green foliage. The inflorescence emerges from the base and the bract changes colour from green to pale pinkish as it ages to vibrant pink. Currently, documentation of *Z. flaviflorum* is limited to Fraser's Hill, suggesting the potential necessity for comprehensive monitoring and protection.

Zingiber fraseri var. *fraseri* Theilade, Gard. Bull. Singapore 48: 214 (1996 publ. 1998)
Holotype: Theilade 12 (AAU). Peninsular Malaysia, Pahang, Fraser's Hill
Distribution: Common along the trail in Fraser's Hill
Description: See Theilade (1996)
Notes: Lim and Meekiong (2014a) subsume *Z. fraseri* under *Z. griffithii* var. *major* and upgraded to a new name as *Z. besar* C.K. Lim & Meekiong. Following a comprehensive analysis of this species, Lim and Meekiong (2014b) reinstated the nomenclature *Z. fraseri* to designate a specific vari-

ety of *Z. besar* known as *Z. besar* var. *fraseri*. Later, Govaerts (2016) rectified this superfluous designation. The utilised variety name predates *Z. besar*; therefore, the correct names should be recombined under *Z. fraseri*. The International Plant Names Index (IPNI) lists three accepted infraspecific names: *Z. fraseri*, *Z. fraseri* var. *major*, and *Z. fraseri* var. *nervifolium*. The bract of the inflorescence is bright red (Theilade 1996), but no flowers were present at the time of collection. Although this species is related to *Z. griffithii*, its ovoid inflorescence and tapering pointed apex distinguish it. *Zingiber fraseri* has common characteristics of the genus *Zingiber* in Peninsular Malaysia, which possesses arcuate leafy shoots. Based on our research findings (the survey was carried out during the same month that Theilade conducted collection activities), the pointed apex inflorescence of *Z.* aff. *fraseri* suggests a possible close relationship with *Z. flaviflorum*. Additionally, the bract pigmentation resembles that of aged *Z. flaviflorum*. Nonetheless, a comprehensive investigation, including molecular analysis, is required. This species is native to Peninsular Malaysia. *Zingiber fraseri* has been assessed as an Endangered (EN) species (criteria: B1ab (iii) + 2ab (iii)), and the population trend for this species is decreasing (Table 1).

Figure 2. *Zingiber flaviflorum*. (a–d) Stages of blooming inflorescence starting at the end of May until the end of July. (e) Procumbent and erect habit of *Z. flaviflorum*. (f) Close-up of *Z. flaviflorum* flower. (g) Tall leafy stems of *Z. flaviflorum* in Fraser's Hill on open ground.

Zingiber fraseri var. *major* (Ridl.) Govaerts, Taiwania 61: 270 (2016)
Holotype: Ridley. (SING). Peninsular Malaysia, Pahang, River Tahan
Distribution: Peninsular Thailand to Peninsular Malaysia
Description: See Holttum (1950)
Notes: This variety has been collected in Pahang with a less hairy character, a short scape, and a short ovoid inflorescence with a rounded apex.

Zingiber fraseri var. *nervifolium* (Meekiong & C.K.Lim) Govaerts, Taiwania 61: 270 (2016)
Holotype: C.K. Lim L12903. (UKMB). Peninsular Malaysia, Pahang, Janda Baik
Distribution: Bukit Tinggi
Description: See Lim and Meekiong (2014b)
Notes: The epithet denotes the characteristics of the veined leaves. This species is predominantly observed in open areas and have been spotted in Terengganu, indicating a broader distribution. Furthermore, a distinguishing characteristic of *Z. fraseri* var. *nervifolium* over *Z. besar* is the lack of aroma (Lim & Meekiong 2014b). So far, these species have yet to be collected since the first encounter.

Zingiber gracile Jack, Malayan Misc. 1(1): 1 (1820)
Holotype: Curtis 075425. (SING). Peninsular Malaysia, Penang (Figure 3)
Distribution: Peninsular Malaysia and Myanmar
Description: See Holttum (1950) and Theilade (1996)
Notes: Based on the expansive investigation, *Z. gracile* has a distinct thin, scarious ligule, lanceolate leaves, a short spike, and a scape. Known as a small plant, *Z. gracile* can grow up to 3 m like other taller *Zingiber* species in Peninsular Malaysia, such as *Z. spectabile*. There are ambiguous specimens in the herbaria since many samples clustered under *Z. gracile* do not have those ligule characteristics. Additionally, there is another specimen awaiting examination that we believe is grouped within the *Z. gracile* complex, as it has a long and thin ligule. However, more specimens need to be collected to refine the classifications. The conservation status of *Z. gracile* is Data Deficient (DD) and its population trend is unknown (Table 1).

Zingiber griffithii Baker, J.D.Hooker, Fl. Brit. India 6: 246 (1892)
Holotype: Griffith 5731. (K) Peninsular Malaysia, Melaka (Figure 3)
Distribution: Peninsular Malaysia, Borneo
Description: See Holttum (1950) and Theilade (1996)
Notes: *Zingiber griffithii* thrives well in shady environments near small streams in humid conditions. Comparatively, the leaves of this species are broader and feature finely raised veins, in contrast to related species such as *Z. gracile* and *Z. puberulum*. Furthermore, compared to *Z. puberulum* and *Z. gracile*, the inflorescence bract of *Z. griffithii* is pink and considerably less robust (not tightly imbricated bract, pulpy). This characteristic distinguishes *Z. griffithii* among the *Zingiber* species found in Peninsular Malaysia. As stated in the herbarium specimen, the roots of *Z. griffithii* are used to quicken delayed childbirth. *Zingiber griffithii* is Near Threatened (NT) (criterion: B2b (iii)) and the population trend is decreasing (Table 1). Thus, it is crucial to protect and conserve the species.

Zingiber kelantanense C.K.Lim
Holotype: C.K. Lim L6206. (KEP). Peninsular Malaysia, Kelantan
Distribution: Peninsular Thailand, Peninsular Malaysia (Kelantan)

Description: See Lim (2003)
Notes: In 2003, C.K. Lim published a description of *Zingiber kelantanense* in which he enumerated all four closely related *Zingiber* taxa from Malaysia and Thailand. Despite a thorough manual examination of every *Zingiber* specimen in Peninsular Malaysia, the holotype L6206 for *Z. kelantanense* in KEP remains unlocated. Fresh living specimens need to be collected and further corroborated. This species resembles *Z. aurantiacum* and *Z. petiolatum*; however, in addition to its longer petiole and ligule, it possesses a lamina with conspicuous veins. At present, *Z. kelantanense* is exclusively observed in its type locality, Kelantan. Additionally, this species lacks a pungent odour (Lim 2003).

Zingiber kunstleri King ex Ridl., J. Straits Branch Roy. Asiat. Soc. 32: 127 (1899)
Holotype: Ridley 11449. (SING). Peninsular Malaysia, Perak, Taiping
Distribution: Peninsular Malaysia (Pahang, Perak, Terengganu)
Description: See Holttum (1950) and Theilade (1996)
Notes: It has been suggested that *Z. kunstleri* and *Z. wrayi* are closely related due to their deflexed inflorescence bracts. On the other hand, *Z. kunstleri* has lanceolate to linear leaves and a significantly larger inflorescence than *Z. wrayi*. Slashed rhizomes exhibit a distinctive purplish-lilac hue. This attribute could serve as a diagnostic feature for species identification. Holttum (1950) stated that *Z. kunstleri* labellum is a distinct shade of reddish brown, setting it apart from other *Zingiber* species found in Peninsular Malaysia. Ridley's description of *Z. kunstleri* was predicated upon Kunstler's field notes and drawings. The labellum, however, almost certainly requires correction. Most herbarium specimens have identical labellum as *Z. wrayi*: a yellow patch with a purple spot. Nevertheless, only two specimens exhibited the purple rhizome characteristic of *Z. kunstleri*; no flower description was provided. Therefore, a substantial amount of fieldwork focusing on its type locality is crucial and currently being conducted with thorough examinations on morphology and phylogenetic analysis to resolve the uncertainties of this species. *Zingiber kunstleri* is considered Least Concern (LC) and the population trend is stable (Table 1).

Zingiber limianum Meekiong, Folia Malaysiana 15: 20 (2014)
Holotype: C.K.Lim L12460. (UKMB). Peninsular Malaysia, Pahang, Bukit Tinggi
Distribution: So far, the species can only be found at the type locality. This species may be considered endemic to Bukit Tinggi.
Description: See Lim and Meekiong (2014b)
Notes: The epithet honours Datuk Seri Lim Chong Keat, whose pioneering efforts in biodiversity conservation established the population. *Zingiber limianum* is classified as a rare and endangered species due to its restricted distribution in the type locality, necessitating extensive monitoring and preservation. This species is distinguished by its ovate, dark-green, leathery adaxial leaves, which are maroon in colouration. The inflorescence bracts of this species are pink, yellow, and dark brown, and its flowers are bright yellow. Although they are comparable in appearance, *Z. limianum* and *Z. malaysianum* are easily differentiated due to their distinct leaf and inflorescence characteristics.

Zingiber longibracteatum Theilade, Nordic J. Bot. 19: 408 (1999)
Holotype: Maxwell 75-878. (AAU). Thailand, Peninsular region, Trang province

Distribution: Thailand and Peninsular Malaysia
Description: See Theilade (1999) and Lim (2001)
Notes: Its purple labellum and upright leafy shoots resemble those of the Thai *Zingiber* species *Z. newmanii*. However, it is distinguished by its longer bracts and bracteoles, longer crimson ligules, larger leaves ornamented with silky hairs beneath, and shorter inflorescence featuring the same colouration. This species is unique by not having the overlapping inflorescence bract for the genus *Zingiber* but being stipitate. The conservation status of *Z. longibracteatum* is Vulnerable (VU) (criterion: B2ab (iii)) while the population trend is decreasing (Table 1).

Figure 3. *Zingiber gracile* and *Z. griffithii*. (a–b) Leafy stems of *Z. gracile* besides the distinctive thin, scarious ligule. (c) Clump of *Z. griffithii* with adaxial raised veins. (d–f) Pink pulpy inflorescence of *Z. griffithii* near small streams.

Zingiber malaysianum C.K.Lim, Folia Malaysiana 3: 27 (2002)
Holotype: C.K.Lim L2843. (KEP). Peninsular Malaysia, Johor, Labis (Figure 4)
Distribution: Peninsular Malaysia (Johor)
Description: See Lim (2002)
Notes: Peninsular Malaysia is home to the rare *Z. malaysianum*, which has distinctively pale green rachis and reddish brown or liver-coloured foli-

age. However, due to its characteristics, it can be easily overlooked in shady forests. The inflorescence resembles that of *Z. citrinum* Ridl. (a *Z. griffithii* complex often seen in the Johor region) and is frequently yellow before turning pink. The inflorescence bract varies consistently between yellow, pink, and red. As per our observations, a limited population was identified within the restricted region of Labis Forest Reserve. Due to the recent deluge, certain *Z. malaysianum* plants were rendered nonviable in Bekok, Johor. Additionally, *S. klossii* var. *glomerata* and other *Zingiber* sp. are sympatric with *Z. malaysianum*; they are found in sandy loam with leaf litter. The IUCN classifies this species as Least Concern (LC) due to its stable population trend (Table 1).

Zingiber multibracteatum var. *multibracteatum* Holttum, Gard. Bull. Singapore 13: 57 (1950)

Holotype: Corner. SFN 33174. (SING). Peninsular Malaysia, Pahang, Fraser's Hill (Figure 4)

Distribution: Peninsular Malaysia (Kelantan, Pahang, Perak)

Description: See Holttum (1950) and Theilade (1996)

Notes: According to our observations, *Z. multibracteatum* is frequently observed on steep slopes in open ground. The species' height could be up to 3 m tall, and it is considered a large montane plant for the genus *Zingiber*. Vegetatively, *Z. multibracteatum* looks similar to *Z. puberulum* by its brownish velutinus leaf sheath but differs by its inflorescence besides labellum, which is closely similar to *Z. spectabile*. *Zingiber multibracteatum* is differentiated by its broad ovoid and dark maroon inflorescence, distinct velutinus petiole and ligule, and large flowers featuring labella speckled with purple cream. The fruit of *Z. multibracteatum* is in capsule and ovoid shape with three locules. Interestingly, the seeds are pink and can be seen from the outer part of the fruit. The conservation status of *Z. multibracteatum* is Near Threatened (NT), but the population trend is unknown (Table 1).

Figure 4. *Zingiber malaysianum* and *Z. multibracteatum* var. *multibracteatum*. (a–b) Unique raised veins leaves and reddish brown foliage *of Z. malaysianum*. (c–d) Inflorescence of *Z. multibracteatum* during fruiting. (e) Habitat of *Z. multibracteatum* in an open ground. (f) Notable brown velutinus leaf sheath of *Z. multibracteatum*.

Zingiber multibracteatum var. *viride* Holttum, Gard. Bull. Singapore 13: 58 (1950)
Holotype: Holttum 19967. (SING). Peninsular Malaysia, Cameron Highlands, Tanah Rata
Distribution: Peninsular Malaysia (Pahang)
Description: See Holttum (1950) and Theilade (1996)
Notes: *Z. multibracteatum* var. *viride* shares the majority of morphological attributes with *Z. multibracteatum* var. *multibracteatum*, which is found in Fraser's Hill, except for a few features, including broader leaves and cylindrical inflorescence. Concurrently, the inflorescence bract is light green and subtly broad, starkly contrasting with that of *Z. multibracteatum* var. *multibracteatum*. Both of these species flourish in the elevated montane forest environment.

Zingiber nazrinii C.K.Lim & Meekiong, Folia Malaysiana 15: 31 (2014)
Holotype: C.K.Lim L12483. (UKMB). Peninsular Malaysia, Perak, Bubu Forest Reserve
Distribution: Peninsular Malaysia, Perak
Description: See Lim and Meekiong (2014a)
Notes: *Zingiber nazrinii*'s incurved and pouchy bracts are comparable to the more substantial and elongated montane plant *Z. multibracteatum*. However, this particular species is distinguished by its white or creamy flower, unlike *Z. spectabile* and *Z. multibracteatum*, which have yellow-purple patches on their labellum. In addition, this diminutive ginger has broad, ovate leaves that are conspicuously veined, and shoots and leaf sheaths that are frequently hairy. Its inflorescence ranges from green to pink, and its cuspidate bracts are convex. This species from Perak thrives in moist environments near small streams and frequently shares its habitat with *Globba leucantha* and *Iguanura wallichiana* var. *major*. *Zingiber nazrinii* is not classified as a rare species due to its distribution in various regions of Perak, including Sg. Kejar and Royal Belum (Lim & Meekiong 2014a).

Zingiber petiolatum (Holttum) Theilade, Gard. Bull. Singapore, 13:63 (1950)
Holotype: Corner. SFN 31570. (SING). Peninsular Malaysia, Kedah
Distribution: Peninsular Thailand to Peninsular Malaysia
Description: See Holttum (1950) and Theilade (1996)
Notes: Certain distinguishing features set *Z. petiolatum* apart from *Z. aurantiacum*, including its classification as a higher montane plant for the latter. Our observations indicate that the leaf sheath of *Z. aurantiacum* is frequently purplish. In contrast, the foliage is glossier but has reduced rigidity compared to *Z. petiolatum*. In addition, *Z. aurantiacum* possesses a potent fragrance, whereas *Z. petiolatum* lacks any discernible aroma (Lim 2003). The name *petiolatum* may be misconstrued since numerous herbarium specimens are subsessile, even though the epithet refers to the species' longer petioles. Numerous samples with long petioles and ligules are classified under other taxa. *Zingiber petiolatum* is Vulnerable (VU) (criteria: B1ab(iii) +2ab(iii)), and the population trend is decreasing (Table 1).

Zingiber puberulum var. *chryseum* (Ridl.) Holttum, Gard. Bull. Singapore, 13:63, (1950)
Holotype: Ridley. 1330. (SING). Singapore, Stagmount
Distribution: Peninsular Malaysia (Pahang) and Singapore
Description: See Holttum (1950) and Theilade (1996)

Notes: Since its initial assemblage in 1908, *Z. puberulum* var. *chryseum* has remained undiscovered. It is said that its type locality in Singapore has been destroyed and burned for development one year after collection. This particular variety can be distinguished by the yellow colouration of the inflorescence bract, as opposed to the pink colour observed in *Z. puberulum* var. *puberulum*. The inflorescence, shape, and leaf dimensions are all comparable to those of *Z. puberulum*, except for the pale yellow inflorescence bract and the glabrous plant. Nevertheless, despite these two characteristics showing assurance, it is worth noting that the genus *Zingiber* in Peninsular Malaysia exhibits a colour variation (from yellow to pink as it ages). Moreover, ambiguity may result from establishing the variety without providing a comprehensive description, which should include essential features such as the labellum, dorsal corolla lobe, lateral corolla lobe, and even the vegetative parts. Consequently, further extensive investigation is required for this particular species.

Zingiber puberulum var. *puberulum* Holttum, J. Straits Branch Roy. Asiat. Soc. 32: 130 (1899)
Holotype: Ridley. SN. 1894. (K). Singapore, Bukit Timah
Distribution: Peninsular Thailand, Peninsular Malaysia (Penang, Perak, Terengganu, Pahang, Selangor, Johor)
Description: See Holttum (1950) and Theilade (1996)
Notes: Ridley recognises *Z. puberulum* within the Bukit Timah Forest Reserve. Although this species is closely related to *Z. griffithii* and *Z. petiolatum*, its larger leaves, smaller inflorescence, velutinus leaf sheath, and ligules distinguish it. In contrast, *Z. puberulum* var. *puberulum* has a diverse indumentum, and the ligule and leaf sheath are always hairy. Besides *Z. puberulum*, *Z. multibracteatum* also has the velutinus characteristic on the leafy sheath. However, *Z. puberulum* is often mistakenly identified since it has a prevalent characteristic of *Zingiber* in Peninsular Malaysia: a pink inflorescence. Therefore, all crucial parts need to be considered when examining the specimens. The conservation status of *Z. puberulum* is Near Threatened (NT) (criterion: B2b (iii)) with decreasing population trend (Table 1).

Zingiber raja C.K.Lim & B.Kharukanant, Folia Malaysiana 4: 69 (2003)
Holotype: C.K.Lim L6371. (KEP). Peninsular Malaysia, Perak, Belum Forest Reserve (Figure 5)
Distribution: Peninsular Thailand to Peninsular Malaysia
Description: See Lim (2003)
Notes: The epithet *raja* (king) is derived from the inflorescence, which is conspicuously erect and typically more prominent than the more common *Zingiber*. It is, therefore, referred to as the King of *Zingibers*. Furthermore, the designation of Upper Belum as an integral element of Royal Belum by Sultan Perak renders the name doubly appropriate (Lim & Meekiong 2014a). *Zingiber raja* is distinguished by its inflorescence being both more extensive and taller with orange-pinkish inflorescence bract; additionally, the flower is exquisitely designed with purple-yellow patches. Currently, *Z. raja* is confined to restricted regions in Malaysia, including Belum and Temenggor. This species flourishes in moist environments, amid streams, and on rocks. It is Endangered (EN) (criterion: B1ab (iii) + 2ab(iii)) and the population trend is decreasing (Table 1).

Zingiber sabun C.K.Lim, Folia Malaysiana 15: 25 (2014)
Holotype: C.K.Lim L12575. Peninsular Malaysia, Kedah, Bukit Palong
Distribution: Peninsular Malaysia (Perak)

Description: See Lim and Meekiong (2014a)
Notes: The epithet is the Malay word for soap since it emits a distinctive and recognisable scent. *Zingiber sabun* can be distinguished by the undulated or wrinkled leaves besides the colouration of the labellum, tinged with purple dots. It is further separated from other species by its distinctive property: the soap smell when the leaves are crushed. Additionally, the inflorescence's spike-like fusiform shape differs significantly from the other *Zingiber* found in Peninsular Malaysia.

Zingiber sulphureum Burkill ex Theilade, Bot. Mag. 12: 75 (1995)
Holotype: Haniff & Nur. SNF 8016. (K). Peninsular Malaysia, Pahang, Gunung Tahan
Distribution: Peninsular Malaysia (Gunung Senyum, Fraser's Hill)
Description: See Holttum (1950) and Theilade (1996)
Notes: Burkill used the epithet *sulphureum* to refer to the herbarium specimen, SNF 8016. This small ginger plant has ovate leaves similar to *Z. griffithii*, a tiny flower with sulphur-yellow bracts and a short calyx. It can be found at elevations between 50 and 1200 m a.s.l. in lowland forests and limestone hills. *Zingiber sulphureum* has been classified as Endangered (EN) with decreasing population trend (criterion: B1ab(iii) + 2 ab(iii)) (Table 1).

Zingiber spectabile Griffith., Not. Pl. Asiat. 3: 413 (1851)
Holotype: Griffith. 5762. (K). Peninsular Malaysia, Melaka (Figure 5)
Distribution: Peninsular Thailand and Peninsular Malaysia
Description: See Holttum (1950) and Theilade (1996)
Notes: The most widespread species of the genus *Zingiber* is *Zingiber spectabile*. It can be discovered in disturbed areas along pavements, roadsides, and trails up to 1,000 m a.s.l. Although this species may have a similar appearance to *Z. ottensii*, its distinctive features include an orange inflorescence with incurved bracts that form open pouches and a dark purple labellum speckled with yellow. At present, this is the largest Malayan *Zingiber* species. *Meistera ochrea* is found within the same area as *Z. spectabile* along the pavements to the Taka Melor waterfall. Based on the observation, the flowers of *Z. spectabile* open at 11 a.m. Locally known as *tepus tanah* or *bihip* in Indonesia, the pounded leaves of this handsome species can be used topically to treat burns. Besides, the water from the inflorescence can be dropped into infected eyes (Sharifi-Rad et al. 2017). *Zingiber spectabile* is classified as Data Deficient (DD) and the population trend is unknown (Table 1).

Zingiber wrayi var. *halabala* C.K.Lim, Folia Malaysiana 2: 50 (2001)
Holotype: Md. Nur 18569. (SING). Peninsular Malaysia, Pahang, Pulau Tioman
Distribution: Peninsular Thailand to Peninsular Malaysia (Perak, Kelantan, Terengganu, Pahang)
Description: See Lim (2003)
Notes: The holotype was chosen to commemorate one of Mohd Nur's initial specimens from Pulau Tioman's southernmost known position. The inside of the rhizome is creamy white and tastes sweet. The species' leaves and leafy stems have a strong anise scent. Compared to *Z. kunstleri*, both types of *Z. wrayi* exhibit procumbent and diminutive inflorescences. Uniquely, the inflorescence bract's colour varies, frequently being a dark coral red with decurved apices. Some plants also have inflexed bracts, but the ones in Halabala Forest Reserve have deflexed bracts (Lim 2003).

Figure 5. *Zingiber raja* and *Zingiber spectabile*. (a) The striking inflorescence and flower of *Z. raja*. (b) The habitat of *Z. raja* in Royal Belum. (c) Flower of *Z. spectabile* (sideview). (d) The inflorescence of *Z. spectabile* found at Taka Melor Eco Forest. € Close-up *Z. spectabile* flower, purple labellum with yellow speckles, arching stigma with cilia. (f) Arcuate leaf sheath of *Z. spectabile*.

Zingiber wrayi var. *wrayi* Prain ex Ridley, J. Straits Branch Roy. Asiat. Soc. 41: 31 (1904)
Holotype: Wray. 3735. (SING). Peninsular Malaysia, Perak, Upper Perak
Distribution: Peninsular Thailand to Peninsular Malaysia (Perak–Piah Forest Reserve)
Description: See Holttum (1950) and Theilade (1996)
Notes: The species can easily be identified because of the deflexed bracts and large leaves. Compared to *Z. kunstleri*, *Z. wrayi* is more petite, and its leaves are broader. This variety is reported to lack an anise aroma. Compared to *Z. wrayi* var. *halabala*, which has a sweet taste, and distinctly anise-scented leaves and stems, the rhizome of the species is scentless. In Thailand, the bract is more frequently curled outwards; in Malaysia, the bracts are inflexed with a pointy or sharp tip. The conservation status of *Z. wrayi* is Endangered (EN) with a decreasing population trend (Table 1).

Zingiber zerumbet (L.) Roscoe ex Sm., Exot. Bot. 2: 105 (1806)
Holotype: BRI-AQ0118904. (BRI)
Distribution: Cultivated in India, China, and throughout Southeast Asia
Description: See Holttum (1950) and Theilade (1996)

Notes: *Zingiber zerumbet* is widely cultivated in Peninsular Malaysia. It is closely related to *Z. ottensii*, but the bracts are green and the labellum is lemon yellow without any speckles. Traditionally, ripe noni (*Morinda citrifolia* L.) fruit and powdered *Z. zerumbet* rhizomes have been used to treat severe sprains. Water infused with powdered and filtered rhizome material is drunk to treat stomach ache, and the cooked and softened rhizome has been used to treat toothaches or cavities by pushing it into the hollow and leaving it there for however long as necessary (Sharifi-Rad et al. 2017). *Zingiber zerumbet* is categorised as Data Deficient (DD) and the population is still unknown (Table 1).

The undescribed taxon from Teluk Bahang, Penang

During the fieldwork at the type locality of *Z. gracile*, the first author encountered a new taxon blooming well along the trails on the slope in Teluk Bahang. At first glance, this unique taxon looked similar to *Z. purpureum* and *Z. belumense* However, a new taxon was proposed after a thorough examination based on the vegetative and reproductive parts of living specimens. Additionally, after consulting the collector (C.K. Lim, pers. comm.), it was clear that the new taxon differs from the related species. The examination was also based on high-quality digital images of herbarium specimens from AAU, K, and RBGE database.

Interestingly, many individuals of this taxon were observed in Penang Hill as well. The description and comparison with related species are presented in Table 2. A detailed description and complete information about this species will be accessible in the upcoming publication.

Zingiber sp. (Bahangense130) Aimi Syazana & Salasiah

Holotype: Aimi Syazana, AS0130 (UTHM). Peninsular Malaysia, Penang, Teluk Bahang, 2nd August 2023 (Figure 6)

Distribution: Penang Hill

Description: Perennial rhizomatous herb, 2.8–3.0 m tall. Leaf sheaths long trailing arching downward, pseudostem green, pubescent; ligule bifid and short, acute apices, sparsely pubescent at the edge of the apices, green; petiole prominent pulvinus, 0.5 cm to short; laminae elliptic, 19.5–43 cm × 3.5–9.5 cm, attenuate at base, aristate to caudate at apex, adaxially shiny green, abaxially shiny pale green, and compactly pubescent at midrib. Inflorescence decumbent, scape subterranean in the ground, creamish white with tinged brownish pink at the edge of the margin, glabrous; spike ovoid with pointed apex, 15.0–16.0 cm long, bract, 6.0 cm, ovate, brownish maroon-dark purple with green, apex acute, pink margin with prominent line, densely pubescent; floral tube 6.0 cm long, bracteole 2.9 cm long, oblong, apex obtuse; calyx 2.8 cm long, ovate, bifid, apex acute, translucent white; dorsal corolla lobe 2.5 cm long, oblong to ovate, apex acuminate, yellowish cream semi-translucent; lateral corolla lobe 2.5 cm long, oblong to obovate with deeply bifid, apex acuminate, yellowish cream semi-translucent; labellum yellowish cream semi-translucent, apices bluntly undulate deflexed. Fruit unknown.

Notes: This species, found in Penang, bears a strong resemblance to *Z. belumense* and Z. *purpureum* in terms of its brownish maroon to dark purple coloration with green bracts. However, it is distinguished through several characteristics (Figure 7). Notably, it can be readily discriminated by the strong aromatic odour, elliptic leaves, subterranean ovoid shaped inflorescence with pointed apex in brownish maroon-dark purple with green that often has pink margin, yellowish cream labellum with lateral staminodes. Moreover, the unique characteristic of red sap observed in the pseudostems of this newly proposed taxon warrants meticulous in-

vestigation to determine its consistency across various environmental conditions, despite its uniform occurrence within the plant clump from the same locality.

Additionally, more than 10 individuals of this taxon were observed in Penang Hill, and it thrives particularly well in sandy soil, although its potential uses remain unknown at this time. To reflect its discovery location, we provisionally named this taxon as *Zingiber bahangense*. Nevertheless, a comprehensive taxonomic description is currently in progress, along with the acquisition of molecular phylogenetic evidence.

Figure 6. *Zingiber* sp. (Bahangense130) Aimi Syazana & Salasiah. (a) The inflorescence of proposed new taxon. (b) Long trailing leafy shoots. (c) Decumbent inflorescence. (d) Red sap from the cut pseudostem.

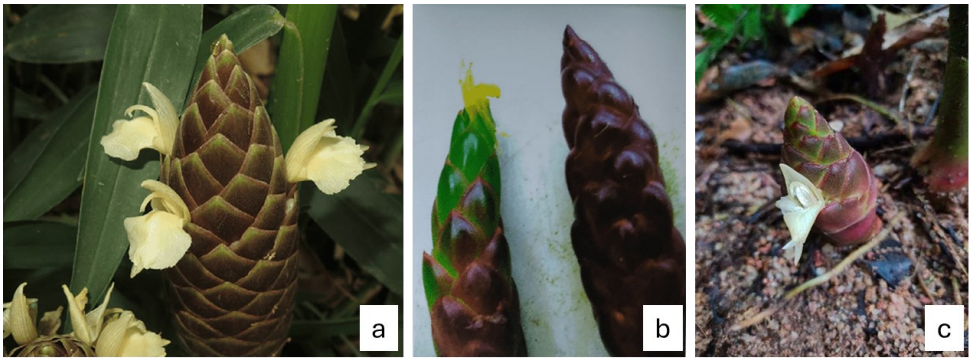

Figure 7. The closely related species to the undescribed taxon. (a) Inflorescence of *Z. purpureum* (Adopted from Bai et al. 2019). (b) The presence of coloration for *Z. belumense* inflorescence (Adopted from Lim & Meekiong 2014a). (c) A new undescribed taxon, *Zingiber* sp. (Bahangense130).

Table 2. Comparison of morphological characteristics on related *Zingiber* species: *Zingiber* sp. (Bahangense130), *Z. purpureum*, and *Z. belumense*.

	***Zingiber* sp.** (Bahangense130)	***Z. purpureum***	***Z. belumense***
Type	Teluk Bahang, Penang	India and Myanmar	Belum, Perak
Leaves	Long trailing arching downward, elliptic	Erect, linear, or narrowly lanceolate	Arcuate leaf arching downward, ovate
Inflorescence	Erect, decumbent. Scape 7–8 cm long, subterranean in the ground. Spike ovoid with pointed apex in brownish maroon-dark purple with green, pink margin, 15.0–16.0 cm long	Erect. Scape 20–30 cm long. Spike fusiform or cylindric ovate in dark red to maroon, purplish or almost brown bracts with greenish margins, 10–16 cm long	Decumbent and erect. Scape 12–30 cm long. Spike fusiform in bright green turning dark purple to red, 10–18 cm ´ 3–4.5 cm long
Labellum	Yellowish cream semi-translucent, apices bluntly undulate, deflexed	6 cm long. Pale yellow, midlobe broadly rounded, apex bilobed	2.5–2.7 cm long. Margin slightly wrinkled, apex deeply bilobed, tips acute
Scent	Strongly aromatic	Strongly aromatic	-
Other notable characteristics	Red sap is observed from the cut leaf sheath	Rhizome in lemon yellow colour	-

CONCLUSION

This paper presents the current taxonomic work for *Zingiber* in Peninsular Malaysia and some insights related to the *Zingiber* species. In addition to the current total of 25 *Zingiber* species and 30 taxa in the region, the first documentation of *Zingiber* sp. (Bahangense130) portrays the significant highlight of the potential 26th species of the genus. In this work, we present an account of its vegetative and floral characteristics, introduce a temporary taxon designation, and suggest a binomial name to secure priority for the authors who initially discovered this distinctive ginger. It is important to note that this article marks the beginning of our exploration, and extensive research on genus *Zingiber* of Peninsular Malaysia, including the new *Zingiber* sp. (Bahangense130), is actively underway.

AUTHOR CONTRIBUTION

All authors contributed to the research, including the collection, data analysis, and manuscript preparation.

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CONFLICT OF INTEREST

No conflict of interest.

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Research Article

Phytochemical Properties, Antioxidant, and Cytotoxicity Activity of Knobweed (*Hyptis capitata*) from South Sulawesi, Indonesia

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ABSTRACT

Hyptis capitata Jacq. known as Sumambu plants in Sulawesi, has phytopharmaceutical importance. *H. capitata* extracts were evaluated for their phytochemical properties, antioxidant activity, and cytotoxicity. Using the maceration yielded five types of extracts: root chloroform (RC), root methanol (RM), leaf chloroform (LC), leaf methanol (LM), and leaf ethanol (LE). Phytochemical properties were identified by qualifying procedure and digital image analysis for quantifying Red-Green-Blue (RGB) percentage and hex colour code. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was used to determine the half-maximal inhibitory concentration (IC_{50}). Cytotoxicity screening of each extract was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay against HeLa and 4T1 cells. Gas Chromatography-Mass Spectrometry (GC-MS) assay was used to identify the phytochemical compounds of the extracts with the most promising potential. Alkaloids were the major constituents of the phytochemicals of RC, RM, LE, LC, and LM. RM and LM have potency and weak free radical scavenging activities, with IC_{50} value 31.08 and 58.03 µg/mL, respectively. The IC_{50} of RC and RM against HeLa cells were 84.21 ± 0.63 and 172.10 ± 02.90 µg/mL, respectively. Meanwhile, the cytotoxicity of RC and RM against 4T1 cells were 86.42 ± 0.80 and 182.82 ± 7.00 µg/mL, respectively. It means RC and RM exhibit a moderate level of cytotoxicity in both HeLa and 4T1 cells. LM shows moderate cytotoxicity, but it is limited to 4T1 cells with an IC_{50} value of 181.86 ± 12.68 µg/mL. The cytotoxicity level of extracts was lower than doxorubicin. Campesterol, ferruginol, stigmasterol, cis-13-octadecenoic acid methyl esters, and methyl palmitate were predicted to play a role in the antioxidant activity and cytotoxicity of RC, RM, and LM. RC, RM, and LM possess the potential for development as anticancer agents. Moreover, RM shows promise as an antioxidant due to its notable radical scavenging activity. Further research is required to explore the cytotoxic effects of RC, RM, and LM on normal cells and to assess their toxicity in experimental animals.

INTRODUCTION

Knobweed (*Hyptis capitata* Jacq.) of the Lamiaceae family was not only native to its habitat in Central and South America but also has become

naturalised in subtropical and tropical regions of Asia and Australia (Lohr et al. 2016; Sumitha & Mini 2019; John et al. 2022). This plant has an ethnobotanical history that has been used in at least 16 countries in America, Europe, and Asia. When knobweed is applied as traditional medicine, the preparation method that is widely used is decoction (To'bungan et al. 2022a). In Indonesia, especially Sulawesi, *H. capitata* known as Sumambu or Sualang plants demonstrated anti-inflammatory properties (Audina et al. 2018; To'bungan et al. 2022a). In ethnopharmacology studies, *H. capitata* plants were found to be useful in treating diabetes mellitus, especially in Banggai, Central Sulawesi, where whole plants were used as one of ingredients in a traditional medicine preparation called jamu (Hartanti & Budipramana 2020). In a report by John et al. (2022), *H. capitata* was described as a traditional medicine used by local tribes for the treatment of asthma and fever. The leaves of this plant are also used by the Suku Anak Dalam tribe to treat wounds, both open wounds and internal wounds (Rupa et al. 2017). The pharmacological importances of this genus are for the treatment of respiratory ailments, nasal congestion, fever, liver disorders, gastrointestinal disorders, skin infections, and even human immunodeficiency virus is known (John et al. 2022). Based on this properties, *H. capitata* has been recognised as an ethnomedicinally important plant species, mainly due to its antiviral, cytotoxic, antioxidant, hepatoprotective, antimicrobial, anticancer, and anti-inflammatory properties (John et al. 2022; Sumitha et al. 2022).

Phytochemical analysis revealed that many bioactive compounds were found in this genus (John et al. 2022). *H. capitata* leaves have secretory structures forming glandular trichomes and idioblast cells (Rupa et al. 2017). The glandular trichomes contain terpenoids and alkaloids, whereas, the idioblast cells contain lipophilic compounds (Rupa et al. 2017). Terpenoid compounds such as limonene, eugenol, farnesol isomer A, d-nerolidol, coumarin, and neophytadiene could act as antimicrobial agents (Rupa et al. 2017). The composition of phytochemical compounds, especially terpenes, terpenoids, lignans, and flavonoids, varies widely among the species of *Hyptis*, depending on the geographical location, genetic, and climatic factors (John et al. 2022).

Previous study by To'bungan et al. (2022b) reported that the root chloroform extract of *H. capitata* has moderate cytotoxicity (IC_{50} 21-200 µg/mL) against T47D (34.90 ± 4.7 µg/mL) and WiDr (44.65 ± 12.07 µg/mL) cancer cells was. Based on its high cytotoxicity (13.8 ± 0.65 µg/mL), selectivity to T47D cells (3.71), and ability to induce apoptosis and antimetastatic, To'bungan et al. (2022b) concluded that the F2 fraction of *H. capitata* root chloroform extract has the highest potential as anticancer drug. The anticancer activity of the root chloroform extract was related to ferruginol, campesterol, and stigmasterol, which were able to induce apoptosis and inhibit cell migration (To'bungan et al. 2022b). *H. capitata* root methanolic extract has also been reported to have antiproliferative properties and the ability to induce apoptosis on WiDr cells associated with the phytochemical compounds chatecol, 9-hexadecanoic acid, hexadecanoic acid ethyl ester, methyl stearate, ferruginol, retinoic acid, campesterol, stigmasterol, and γ-sitosterol (To'bungan 2023). Acute toxicity and cytotoxicity of ethanolic leaf extract containing neophytadiene; hexadecanoic acid, methyl ester; hexadecanoic acid, ethyl ester; heptadecanoic acid, 16- methyl, methyl ester; phytol; ɤ-sitosterol; docosanoic acid, ethyl ester; and squalene were reported by To'bungan et al. (2022c).

No prior studies have been conducted to assess the cytotoxic effect of *H. capitata*, on cervical cancer cells (HeLa) and breast cancer cells (4T1). HeLa cells are cancer cells that are commonly used as a research

model for cervical cancer. Meanwhile, 4T1 cells was chosen as one of the cancer models, because it is more aggressive and can spontaneously metastasize from primary tumour to multiple distant (Kaur et al. 2012). Extract treatment that can inhibit cell viability will be a solution of metastatic breast cancers. Furthermore, the antioxidant activity of *H. capitata*, which naturally grows around Tana Lili, North Luwu, South Sulawesi, has not yet been investigated. Phytochemical compounds contained in *H. capitata* and thought to be involved in biological activity were traced using qualitative phytochemical tests and GC-MS (gas chromatography-mass spectrometry). Hence, the purpose of this study was to examine the anticancer potential of *H. capitata* in HeLa and 4T1 cells, along with its antioxidant activity, in order to lay the groundwork for its development as a cancer-fighting agent or novel source of natural antioxidants in the future.

MATERIALS AND METHODS

H. capitata taxonomic identification, harvesting and extraction

Taxonomic identification

H. capitata was collected by selective sampling from the Center for the Implementation of Agricultural Standards, Tana Lili, Luwu Utara, South Sulawesi, Indonesia (Figure 1). Identification was performed under the certification number 014535/S.Tb/III/2019 (To'bungan et al. 2022b) at the Laboratory of Plant Systematics, Faculty of Biology, Universitas Gadjah Mada, DIY. *H. capitata* was identified as the genus *Hyptis* with the NCBI reference code: txid204124 (Schoch et al. 2020).

Harvesting

H. capitata root and leaf parts were harvesting in the morning and prepared by washing and grouping. The roots and leaves were covered with black cloth and dried under the sun until a constant dry weight was obtained. Powder was prepared by grinding the dried roots and leaves. The resulting powder was sieved through a 44-mesh sieve. The sieved root and leaf powder were stored at room temperature (± 25°C) in containers filled with food grade silica gel.

Figure 1. Root (**a**), leaf (**b**), and shoot (**c**) parts of *H. capitata*. Personal records of To'bungan (2022). *H. capitata* was collected from its natural habitat in Tana Lili, Luwu Utara, South Sulawesi, Indonesia (2°36'46.6"S 120°35'28.5" E). Map of Indonesia and Southeast Asia generated by Mahardhika (2023).

Extraction

Extraction was performed by the maceration method, where the leaf and root mesh powder were treated with physical and chemical processing (solvent). Ratio of leaf and root powder with solvent is 1:5. The solvent-powder solution was prepared by weighing 100 g leaf and root powder. Absolute chloroform, absolute methanol, and absolute ethanol were the solvents used to prepare leaf extracts. Whereas, the solvents used to prepare root extracts were absolute chloroform and absolute methanol. Merck KGaA EMSURE®, Germany produced the solvents used in extraction. Different solvents were used to determine their phytoextraction efficacy. The maceration process was carried out for 48 h. It was stirred occasionally throughout the process. Each solution was filtered through a Whatman® Paper Filter 42 and dried in a porcelain cup at room temperature (± 25°C) until the evaporation was complete. The whole process resulted in root chloroform (RC), root methanol (RM), leaf chloroform (LC, leaf methanol (LM), and leaf ethanol (LE) extracts.

Phytochemical screening tests

Alkaloids

A stock solution was prepared by weighing 30 mg of each extract and dissolving it in 10 mL absolute chloroform. 300 µL ammonia was added to the stock solution and the mixture was homogenized. The homogeneous stock solution was divided into three groups of 2 mL each in accordance with the reagents. To each group, 1 mL sulfuric acid 2 M was added. The mixture was homogenized and left for 5 min. Two layers were formed and the bottom half of the layer was completely discarded. Groups I, II, and III were then treated with 1 mL Dragendorff's (Merck KGaA, Germany), Mayer's (Merck KGaA, Germany), and Wagner's (Merck KGaA, Germany) reagents, respectively. The formation of red-brown-orange (Dragendorff's), white (Mayer's), and brown (Wagner's) precipitates has been observed and documented as indicative of the presence of alkaloids.

Saponins

Extracts were weighed to 30 mg and dissolved in 5 mL aquadest. The solution was then homogenized. Foam formation was observed. The presence of saponins was indicated by a foam measuring 1-3 cm and lasting 15 min.

Flavonoids

Extracts were weighed to 0.1 g and dissolved in 5 mL 30% methanol. A stirring hot plate (Thermo Scientific™ Cimarec+™) was used to heat the solution at 250°C for 5 min. To each of the solutions, 250 µL concentrated sulfuric acid 2 N was added. The red coloration indicates that flavonoids are present.

Tannins

The solution from the saponins test was heated to 100°C using the Memmert® Waterbath WNB14. 250 µL $FeCl_3$ 10% was added to the solution. The color change to blue or turquoise black was observed to indicate the presence of tannins in the extracts.

Triterpenes and Steroids

Extracts were weighed to 0.1 g and transferred to a drop plate. To the extracts, 150 µL anhydrous sodium acetate (EMSURE®, Canada) and 50 µL concentrated sulfuric acid 2 N were added. The color change to red and green, respectively, was observed as an indicator of the presence of

triterpenes and steroids.

Antioxidant activity

DPPH radical scavenging assay (Abdulrahman et al. 2019)

Determination of maximum wavelength (λ) and absorbance (A)

8 mg DPPH (Sigma Adrich, Singapore) was dissolved in 200 mL EMSURE® absolute ethanol to prepare 40 µg/mL DPPH. The homogeneous DPPH reagent solution was incubated for 30 min at room temperature (± 25°C) in dark bottles covered with aluminum foil. EMSURE® absolute ethanol (AE) was used as a blank solution for the assay. A UV-Vis spectrophotometer (Thermo Fisher Scientific™ Genesys 10S UV-Vis, SN: 2L9L1019203) was used to determine the maximum wavelength (λ_{max}) and absorbance (A_{max}) of the DPPH solution. The entire procedure was performed in darkness. Light contamination was carefully avoided. The statistical design was three replications, repeated three times (each extract concentration tested was made in triplicate and also repeated three times).

Standard solutions

As a standard and positive control, ascorbic acid (AA) was used. 4 mg EMSURE® ascorbic acid was dissolved in 20 mL EMSURE® absolute ethanol to prepare a 200-ppm stock solution. The dilution series included five concentrations: 2, 4, 6, 8, and 10 µg/mL. 1 mL AA from each concentration was stored in the dark vials. To the AA solution, a 4 mL DPPH solution was added. Incubation was performed without light for 30 min at room temperature (±25°C). The absorbance of AA was measured using a UV-Vis spectrophotometer (Thermo Fisher™ Genesys 10S UV-Vis, SN: 2L9L1019203) at the maximum wavelength (λ_{max}) of the DPPH solution. The statistical design consisted of three replications, repeated three times for each concentration.

Extract solutions

The 200 µg/mL stock solution was prepared by dissolving 4 mg in 20 mL of EMSURE® absolute ethanol of the extracts consisting of RC, RM, LE, LC, and LM. The dilution series included five concentrations: 12.5, 25, 50, 100, and 200 µg/mL. To each stock solution of the respective extracts, 1 mL was stored in dark bottles and 4 mL DPPH solution was added. The incubation process was carried out in the absence of light for 30 min at room temperature (± 25°C). Absorbance of each extract was measured using a UV-Vis spectrophotometer (Thermo Fisher Scientific™ Genesys 10S UV-Vis, SN: 2L9L1019203) at maximum wavelength (λ_{max}) of DPPH solution (517 nm). For each concentration of the respective extracts, the statistical design consisted of three replicates repeated three times (each extract concentration tested was made in triplicate and also repeated three times).

In vitro cytotoxicity screening in HeLa and 4T1 cancer cells

HeLa and 4T1 cancer cells were obtained from the Laboratory of Parasitology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta. HeLa and 4T1 cancer cells were cultured in complete Roswell Park Memorial Institute 1640 (RPMI) medium (Gibco™, Canada), with 96 well plate at a density of 11 x 10^3 cells/well and 8 x 10^3 cells/well, respectively. The assay procedure was in accordance with the previously published protocol by To'bungan et al. (2022b). Ethics Committee of the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Ma-

da,Yogyakarta-Dr. Sardjito General Hospital certified the use of cancer cells with ref. KE/FK/0011/EC/2023 (validation number: 63b4ee136addb).

Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS (Thermo Scientific Trace 1310 Gas Chromatograph-ISQ LT Single Quadropole Mass Spectrometry) analysis was performed at the Laboratory of Integrated Research and Testing, Universitas Gadjah Mada, Yogyakarta, according to the procedure previously published by To'bungan et al. (2022b). The chromatogram, retention time (min), chemical equation, relative area (%), molecular weight, and phytochemical compounds of each extract were obtained (Supp. File 3). The retention time (min) is represented by the x-axis of the chromatograms, while the y-axis is explained as the relative area (%). The retention time is the time taken for the analytes to pass through the columns and reach the mass spectrometer detector. Relative area (%) is based on the number of counts taken by the mass spectrometer detector at the point of retention. Relative area (%) uses the target component peak area as a proportion of the total area of all detected peaks. Relative area (%) is used to approximate the concentration of phytochemical compounds identified during retention time. The extracts further analyzed by GC-MS were selected based on the lowest IC_{50} value of DPPH radical scavenging and cytotoxicity assay. The phytochemical compounds from each extract were identified using the National Institute of Standards and Technology (NIST) library similarity index and the NIST Chemistry WebBook, SRD 69 (https://doi.org/10.18434/T4D303). Phytochemical compounds were cross-referenced with National Library of Medicine (NLM)/NCBI PubChem and various credible sources for relevant therapeutic potential.

Statistical and digital image analysis

Absorbance of blank, DPPH, standard, and extracts were recorded. The following formula was used to calculate the absorbance of extracts~standard [1.1~1.2] and control [2]:

[1.1] Absorbance of extracts=Absorbance of extracts - Absorbance of blank

[1.2]Absorbance of standard=Absorbance of standard - Absorbance of blank

[2]Absorbance of control=Absorbance of DPPH - Absorbance of blank

The inhibition of extracts~standard [3.1~3.2] was calculated using the following formula:

[3.1] $$\%\ \text{Inhibition} = \left[\frac{(\text{Absorbance of control} - \text{Absorbance of extracts})}{\text{Absorbance of control}}\right] \times 100\%$$

[3.2] $$\%\ \text{Inhibition} = \left[\frac{(\text{Absorbance of control} - \text{Absorbance of blank})}{\text{Absorbance of control}}\right] \times 100\%$$

John et al. (2022)

The linear regression between $\log_e x$ concentration (X-axis) of extracts and standard with their respective inhibition percentage (Y-axis) was used to calculate the half-maximal inhibitory concentration (IC_{50}) of extracts~standard. To determine the reliability of the results, the r^2 value greater than 0.7 was used. The equation obtained from the linear regression [4] was used to calculate the IC_{50} as follows:

[4] $y = 50$

$x = IC50$

$\ln IC50 = \frac{(y-b)}{a}$

$IC50\ (\mu g/ml) = e^x$

Regression and data presentation were performed using the algorithm (Supp. File 2) in the RStudio/2023.03.1+446 ©2009-2023 Posit Software, PBC "Cherry Blossom" release (6e31ffc3, 2023-05-09) for Windows. IBM® SPSS® Statistics v. 21 was used to analyze the data for one-way analysis of variance (ANOVA) and Tukey's HSD *post hoc* analysis.

IC_{50} of cytotoxicity test obtained from linear regression analysis of log concentration. The concentration that inhibited cell growth by 50% (IC_{50}) was determined from the data obtained. In addition, the data were analysed using IBM® SPSS® Statistics v. 21 for one-way analysis of variance (ANOVA) followed by Tukey's HSD *post hoc* analysis.

Digital image analysis was performed in the environment of RStudio/2023.03.1+446 ©2009-2023 Posit Software, PBC "Cherry Blossom" release (6e31ffc3, 2023-05-09) for Windows using the color quantification algorithm (Supp. File 1). For further cross-referencing against a web-based database, digital image analysis was performed to translate the qualitative characteristics of each image into quantifiable measurements in the form of RGB percentage and hex color code. The highest value of the RGB color space and its corresponding hex color code were cross-referenced using a web-based colour database.

RESULTS AND DISCUSSION

Results

Phytochemical properties

Phytochemical compounds play a role in many biological activities, including antioxidant and cytotoxic (anticancer) activities. A qualitative search for phytochemical compounds provides a general description of the group of compounds predicted to be contained in each extract tested (Table 1). Subsequent colour quantification and cross-referencing confirmed the presence of alkaloids in the RC extract based on Dragendorff's and Mayer's reagents. Only Dragendorff's indicated alkaloids in the RM, LE, LC, and LM extracts. Due to its selectivity and specificity (Raal et al. 2020), Dragendorff's was the primary reagent for the identification of alkaloids. Dragendorff's (potassium bismuth iodide) consists of basic bismuth nitrate ($Bi(NO_3)_3$), tartaric acid, and potassium iodide (KI) (Raal et al. 2020). Alkaloid testing with Dragendorff's reagent used nitrogen to form coordinate covalent bonds with metal ion K^+, resulting in potassium alkaloid precipitates (Parbuntari et al. 2018; Sabdoningrum et al. 2021). When identifying with Dragendorff's reagent, the risk of false-positive results usually requires the use of other reagents. Raal et al. (2020) stated that the presence of other plant constituents, such as purines, proteins, betaines, and ammonium salts, might also yield positive results, so a positive result might not always indicate the presence of alkaloids. In addition, Dragendorff's reagent was not precipitate caffeine, strychnine, and brucine (Raal et al. 2020). To confirm the positive results in this study, Mayer's and Wagner's reagents were used in addition to Dragendorff's reagent. As a result, only RC extract was positive for both Dragendorff's and Mayer's reagents. Alkaloids could be confirmed as a constituent in the extracts of the *H. capitata*. However, possible false positives could be suspected from the negative results with Wagner's and Mayer's reagents.

Saponins were present in RM and LE extracts. Saponins are structurally complex steroidal and triterpenoidal amphiphatic glycosides that are widely produced by plants and have surfactant properties, forming a stable soap-like foam when shaken in aqueous solution (Shi et al. 2004; Faizal & Geelen 2013; Mugford & Osbourn 2013; Parbuntari et al. 2018; Rai et al. 2021; Sabdoningrum et al. 2021). The term saponin defines a

Table 1. Phytochemical properties of extracts.

Extracts	Alkaloid			Saponin	Flavonoid	Tannin	Triterpene/Steroid
	Dragendorff	Mayer	Wagner				
RC	(+)	(+)	(-)	(-)	(+)	(-)	(+ triterpenes)
RM	(+)	(-)	(-)	(+)	(+)	(+)	(+ triterpenes)
LE	(+)	(-)	(-)	(+)	(-)	(+)	(+ steroids)
LC	(+)	(-)	(-)	(-)	(-)	(-)	(+ steroids)
LM	(+)	(-)	(-)	(-)	(-)	(-)	(+ steroids)

Note: See Supp. File 1 for detailed color quantification procedures and results. Positive (+) and negative (-) annotations were validated by cross-referencing the highest RGB percentage and hex color code with a web-based color database. *H. capitata* extracts: root chloroform (RC), root methanol (RM), leaf ethanol (LE), leaf chloroform (LC), and leaf methanol (LM). Phytochemical screening was performed to determine alkaloids, saponins, flavonoids, tannins, and triterpenes/steroids in RC, RM, LE, LC, and LM extracts of *H. capitata* (Table 1).

group of high molecular weight glycosides that are composed of a glycan moiety linked to an aglycon, which is also referred to as genin or sapogenin (Faizal & Geelen, 2013; Rai et al. 2021). Triterpenes and sterols both originate from the mevalonate pathway with 2,3-oxidosqualene as a common precursor (Mugford & Osbourn 2013).

Tannins were present in RM and LE extracts. Plant tannins are a unique group of phenolic metabolites with a relatively high molecular weight ranging from 500 to 3000 Da that have the ability to form strong complexes with carbohydrates and proteins (Serrano et al. 2009; Singh & Kumar 2019). Tannins are composed of an aggregation of complex phytochemical compounds and secondary metabolites, such as hydroxyl groups.

Flavonoids were present in RC and RM extracts. Flavonoids are an important class of natural products. In particular, they belong to a class of plant secondary metabolites with a polyphenolic structure of benzo-□-pyrone (Kumar & Pandey 2013; Panche et al. 2016). Flavonoids are synthesised through the phenylpropanoid pathway, with the conversion of phenylalanine to 4-coumaroyl-CoA, which is ultimately entered into the flavonoid biosynthetic pathway (Ferreyra et al. 2012; Kumar & Pandey 2013).

Triterpenes were present in RC and RM extracts. Among these secondary metabolites, terpenes constitute a major class further subdivided into monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, and tetraterpenoids (Darshani et al. 2022). Triterpenes are 30-carbon secondary metabolites formed by the combination of six isoprene units in accordance with Ruzicka's isoprene rule (Darshani et al. 2022).

LE, LC, and LM extracts were found to contain steroids. Steroids which are derived from the terpenoid building block isopentenyl pyrophosphate are a subclass of terpenoids that contain a characteristic arrangement of four cycloalkane rings that are connected to each other (Tong 2013; Patel & Savjani 2015). Terpenoids are a large class of oxygenate terpene analogues.

Antioxidant activity

The maximum wavelength (λ_{max}) and the incubation time (T) corresponding to the maximum absorbance (A_{max}) of the 40 µg/mL DPPH solution were found to be 517 nm and 30 min, respectively. The assay was continued by following the obtained λ_{max} and incubation time (T) of the DPPH solution to determine the absorbance of the standard (AA) and extracts (RC, RM, LE, LC, and LM) at their respective concentrations. Radical scavenging activity is determined based on the high and low absorption values of the extracts tested.

Log$_e$x concentration and % inhibition

Absorbance is inversely proportional to % inhibition. The % inhibition of 48.002 ± 1.82(4%) at the concentration of 10 ppm confirms the highest value of AA. As the concentration increases, the % inhibition increases steadily. A significant (p<0.05) difference in % inhibition is observed among the concentrations based on *post hoc* analysis. The extracts also showed a similar trend of % inhibition. For all extracts, the highest % inhibition was observed at 200 µg/mL (Table 2). The % inhibition of RC, RM, LE, LC, and LM were 28.92 ± 4.97(17%), 93.791±1.25(1%), 41.06±0.37(1%), 37.99±0.59(2%), and 70.16±1.44(2%), respectively. Confirming the absorbance-based assumption, the highest % inhibition was observed in RM extract, followed by LM, LE, LC, and RC. *Post hoc* analysis showed significant (p<0.05) difference between % inhibition of RC, RM, LC, and LM. However, there was an insignificant difference in the % inhibition of LC and LE at the concentration of 200 µg/mL. Further confirming the absorbance-based assumption, a significant difference (p < 0.05) between concentrations was observed in RM extract.

Half-maximal inhibitory concentration (IC_{50})

Half-maximal inhibitory concentration (IC_{50}) is related to extract radical scavenging activity. The radical scavenging activity can be used to interpret the antioxidant activity. DPPH has been widely used as an *in vitro* assay for the antioxidant activity of plant extracts (Kiziltas et al. 2022; Klomsakul et al. 2022; Tariq et al. 2022; Vidhya et al. 2022). IC_{50} was calculated using the formula obtained from the linear regression between log_ex concentration and % inhibition. A series of optimisation was carried out based on the reliability of r^2 > 0.7. Based on the IC_{50} value, RM extract followed by LM had the minimum concentration for inhibiting at least half (50%) of the initial DPPH concentration (Table 3). The concentration and composition of phytochemical compounds may be responsible for the antioxidant activity of RM extract. As a standard, the IC_{50} value of AA showed the highest free radical scavenging activity. IC_{50} value of RM extract is classified as potent, while LM and AA are classified as weak and potent, respectively. *Post hoc* analysis showed the significant (p < 0.05) difference between each extract. Insignificant differences were observed for RC, LE, and LC. Meanwhile, RM and LM are significantly different from each other and from RC, LE, and LC.

Cytotoxicity assay in HeLa and 4T1 cells

HeLa and 4T1 cancer cells were used to evaluate the cytotoxicity of the extracts. DMSO and DOX were used as negative and positive controls, respectively. Cytotoxicity is represented by the IC_{50} value, which describes the minimum extract concentration that inhibits at least half (50%) of the viability of cancer cells (Nordin et al. 2018). The most potent extract in terms of cytotoxicity IC_{50} was RC, followed by RM extract (Table 4). Treatment with either RC or RM extract showed insignificant differences (p < 0.05) in both HeLa and 4T1 cells. In the 4T1 cells, the treatments led to more diverse result. LM extract presented countable cytotoxicity in 4T1 cancer cells. There was also a significant difference (p < 0.05) between the LC and the three extracts of RC, LM, and RM in the 4T1 cancer cell. The cytotoxicity of RC and RM extracts in HeLa cancer cells was in the category of moderate cytotoxicity. Meanwhile, RC, LM, and RM expressed moderate cytotoxicity in 4T1 cancer cells. In the 4T1 cells, the treatment of LC extract was categorised as non-toxic. To'bungan et al. (2022b) reported a moderate cytotoxicity of *H. capitata* root chloroform extract against T47D (34.90±4.7 µg/mL) and WiDR

Table 2. Radical scavenging inhibition of RC, RM, LE, LC, and LM.

Concentration (µg/mL)	% Inhibition ± SD (% CV)				
	RC	RM	LE	LC	LM
200	28.92±4.97(17%)	93.791±1.25(1%)	41.06±0.37(1%)	37.99±0.59(2%)	70.16±1.44(2%)
100	25.98±6.11(24%)	86.76±1.38(2%)	37.43±0.61(2%)	35.18±0.19(1%)	53.64±0.68(1%)
50	24.48±5.38(22%)	56.54±0.47(1%)	31.64±0.14(0%)	31.42±1.86(6%)	44.14±1.32(3%)
25	22.83±6.38(28%)	41.49±1.16(3%)	28.19±0.59(2%)	29.34±2.23(8%)	37.49±1.71(5%)
12.50	21.33±6.68(31%)	30.41±2.89(10%)	27.21±1.102(4%)	29.74±0.65(2%)	35.43±1.89(5%)

Table 3. Antioxidant IC_{50} from samples.

Samples	RC	RM	LE	LC	LM	AA
$\bar{x}$ IC_{50} (µg/mL)	$>100^{d}$	31.08^{b}	$>100^{d}$	$>100^{d}$	58.03^{c}	13.36^{a}
±SD	>100	1.035	91.97	>100	4.16	1.03
CV%	32	3	8	24	7	8

Note: One-way ANOVA followed by *post hoc* Tukey HSD produced mean ($\bar{x}$) from three replications. Superscripts with different letter described the significance ($p<0.05$) between IC_{50} (µg/mL) samples. SD = Standard Deviation; CV% = Coefficient of Variation. Value above a thousand written as >100. Ascorbic acid (AA) as standard and positive control. *H. capitata* extracts: root chloroform (RC), root methanol (RM), leaf ethanol (LE), leaf chloroform (LC), and leaf methanol (LM). IC_{50} value classification: <10 µg/mL (very potent), 10-50 µg/mL (potent), 50-100 µg/mL (weak), 100-250 µg/mL (very weak), and >250 µg/mL (inactive) (Reviana et al. 2021).

Table 4. Cytotoxicity IC_{50} against cervical cancer cells (HeLa) and breast cancer cells (4T1)

Treatments	$\bar{x}$ IC_{50} ± SD (µg/mL)	
	HeLa	4T1
RC	84.21 ± 0.63^{b}	86.42 ± 0.80^{b}
LC	>1000	548.33 ± 98.44^{c}
LM	>1000	$181,86 \pm 12,68^{b}$
RM	172.10 ± 2.90^{b}	182.82 ± 7.00^{b}
LE	>1000	>1000
DOX	1.22 ± 0.33^{a}	0.11 ± 0.05^{a}
DMSO	>1000	>1000

Note: One-way ANOVA followed by *post hoc* Tukey HSD produced mean ($\bar{x}$) from three replications. Superscripts with different letter described the significance ($p < 0.05$) between IC_{50} (µg/ml) of treatments. SD = Standard Deviation. Value above a thousand written as >1000. Dimethyl sulfoxide (DMSO) as negative control. Doxorubicin (DOX) as positive control. *H. capitata* extracts: root chloroform (RC), root methanol (RM), leaf ethanol (LE), leaf chloroform (LC), and leaf methanol (LM). Cytotoxicity categories based on the US National Cancer Institute (Hameed 2012; Srisawat et al. 2013)): very toxic $IC_{50} \leq 20$ µg/mL, moderate/toxic IC_{50} 21–200 µg/mL, weak IC_{50} 201–500 µg/mL, and non-toxic $IC_{50} \geq 500$ µg/mL.

(44.65±12.07 µg/mL) cancer cells. The methanolic extract of the *H. capitata* root has also been reported to have anti-proliferative properties and the ability to induce apoptosis on WiDR cells by To'bungan (2023). To identify and confirm the association of phytochemical compounds in RC, RM, and LM with antioxidant activity and cytotoxicity, the investigation was continued by GC-MS assay.

GC-MS assay

The RC, RM and LM chromatograms can be seen in Figure 2-4. GC-MS detected 116, 67 and 44 peaks, respectively. The predicted phytochemical compounds in each extract are presented in Table 5-6. The detected phytochemical compounds which are predicted to have antioxidant and cytotoxic activity, include campesterol, ferruginol, stigmasterol, cis-13-octadecenoic acid methyl esters, and methyl palmitate.

Root chloroform extract

As shown in Figure 2, 116 peaks were identified. RC extract consisted of six phytochemical compounds with RA higher than 1, representing most constituents. The identified phytochemical compounds were pregn-5-en-20-one, 3-hydroxy, (3ß,17a)-, methyl retinoate, ferruginol, quassin, stigmasterol, and campesterol (Table 5).

Figure 3 shows 67 peaks identified. The RM extract was composed of six phytochemical compounds with RA higher than 2, which represented the majority of the constituents. The phytochemical compounds identified were gibberellin A44 (GA44), ferruginol, cis-13-octadecenoic acid methyl ester, methyl palmitate, stigmasterol, and quassin (Table 5).

Figure 4 shows 44 peaks identified. The LM extract was composed of six phytochemical compounds with RA higher than 3 which represented the majority of the constituents. The phytochemical compounds identified were trans-13-octadecenoic acid methyl ester, ethyl linolenate, ethyl palmitate, methyl palmitate, neophytadiene, and methyl isostearate (Table 7).

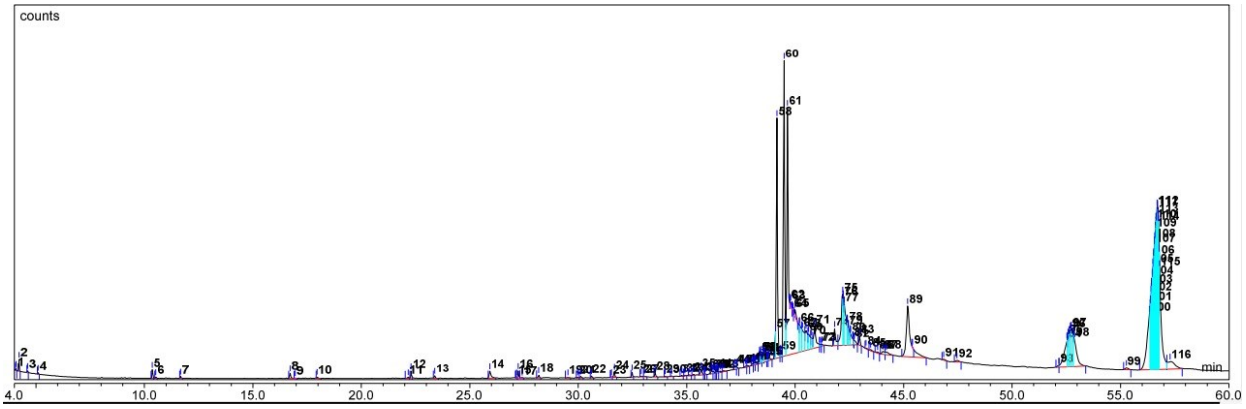

Figure 2. Chromatograms of *H. capitata* root chloroform extract (RC). GC-MS detects 116 peaks.

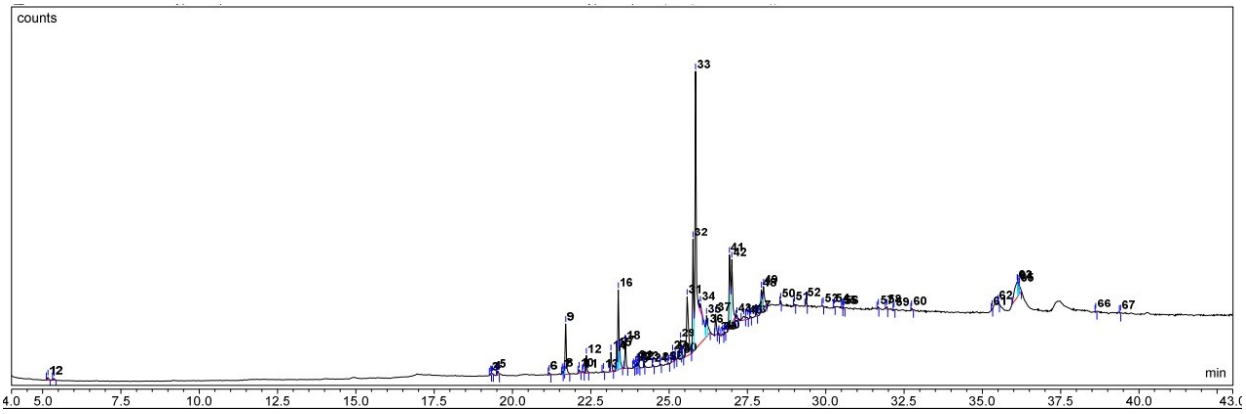

Figure 3. Chromatograms of *H. capitata* root methanol extract (RM). GC-MS detects 67 peaks.

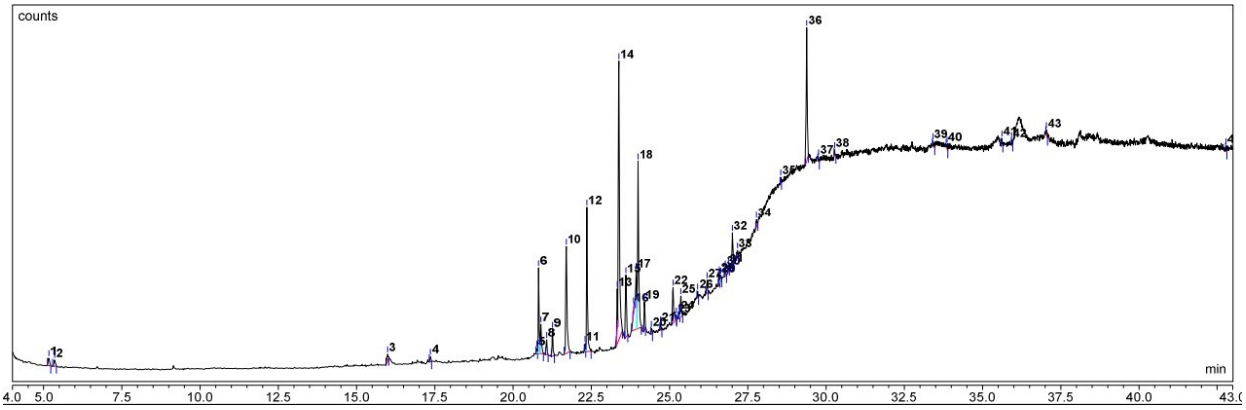

Figure 4. Chromatograms of *H. capitata* leaf methanol extract (LM). GC-MS detects 44 peaks.

Table 5. Identified phytochemical compounds of RC extract of *H. capitata*

No.	RT (min)	Chemical formula	Phytochemical compounds	RA (%)	Bioactivities	Classifications
1	10.36	$C_{11}H_{24}$	undecane	0.14	*X*	alkanes [MeSH]
2	11.67	$C_{10}H_{22}$	decane	0.03	*X*	alkanes [MeSH]
3	16.71	$C_{15}H_{32}$	2,7,10-trimethyldodecane	0.14	*X*	alkanes [ChEBI]
4	16.95	$C_{19}H_{40}$	6-methyloctadecane	0.01	*x*	alkanes [ChEBI]
5	17.96	$C_{17}H_{36}$	2,6,10-trimethyltetradecane	0.05	*v*	fatty acids [LOTUS]
6	22.29	$C_{16}H_{34}$	hexadecane	0.13	*ox*; *bx*	alkanes [MeSH]
7	25.92	$C_{13}H_{14}O_4$	1'-acetoxychavicol acetate	0.31	*v*	alcohols; benzyl alcohols [MeSH] carboxylic ester [ChEBI]
8	27.16	$C_{26}H_{54}$	3-ethyl-5-(2-ethylbutyl) octadecane	0.01	*ox*; *bx*	alkanes [ChEBI] fatty acids [LOTUS]
9	27.23	$C_{21}H_{44}$	2,6,10,15-tetramethylheptadecane	0.16	*x*	prenol lipids; sesquiterpenoids [HMDB0062787]
10	27.42	$C_{17}H_{32}O_2$	7-methyl-z-tetradecen-1-ol acetate	0.02	*cx*	carboxylic esters [ChEBI] fatty acids [LOTUS]
11	29.53	$C_{17}H_{32}O$	13-heptadecyn-1-ol	0.04	*v*	alcohols; fatty alcohols [ChEBI]
12	30.11	$C_{17}H_{36}O$	2-methyl-1-hexadecanol	0.05	*v*	fatty acids; fatty alcohols [LOTUS]
13	31.54	$C_{25}H_{44}N_2O_5S$	2-myristynoyl pantetheine	0.01	*x*	fatty acids; n-acyl amines [LOTUS]
14	32.91	$C_{18}H_{24}O_2$	methyl 5,8,11-heptadecatriynoate	0.03	*x*	fatty acids; methyl esters [CAS 56554-57-5]
15	33.05	$C_{18}H_{36}O_2$	ethyl palmitate	0.04	*ox*	fatty acids; palmitic acids [MeSH]
16	33.55	$C_{20}H_{40}O_2$	ethanol, 2-(9-octadecenyloxy)-, (z)-	0.03	*cx*	dialkyl ethers [CAS 5353-25-3]
17	36.42	$C_{26}H_{54}$	3-ethyl-5-(2-ethylbutyl) octadecane	0.06	*ox*; *bx*	alkanes [ChEBI] fatty acids [LOTUS]
18	37.23	$C_{37}H_{76}O$	1-heptatriacontanol	0.11	*v*	fatty acids; fatty alcohols [LOTUS]
19	38.36	$C_{16}H_{30}O_2$	9-hexadecenoic acid	0.20	*v*	fatty acids [MeSH]
20	39.09	$C_{20}H_{30}O_2$	podocarpa-8,11,13-triene-7ß,13-diol, 14-isopropyl-	0.47	*cx*; *bx*	terpenoids; podocarpane diterpenoids [LOTUS]
21	39.16	$C_{20}H_{30}O$	ferruginol	6.72	*v*	terpenoids; diterpenoids (C20) [KEGG: phytochemical compounds]
22	39.49	$C_{21}H_{30}O_2$	methyl retinoate	8.78	*v*	vitamins; vitamin A [MeSH] prenol lipids; retinoids; retinoid esters [HMDB0254612]
23	39.65	$C_{21}H_{32}O_2$	pregn-5-en-20-one, 3-hydroxy-, (3ß,17a)-	16.21	*v*	alcohols; secondary alcohols; 3beta-hydroxy steroids; pregnenolone [ChEBI]

Table 5. Contd.

No.	RT (min)	Chemical formula	Phytochemical compounds	RA (%)	Bioactivities	Classifications
24	39.79	$C_{20}H_{28}O_3$	16-hydroxymethyleneandrost-5-en-3-ol-17-one	0.06	*cx*	alcohols; secondary alcohols; 3beta-hydroxy steroids; dehydroepiandrosterone [ChEBI] terpenoids; androstane steroids [LOTUS]
25	41.82	$C_{20}H_{38}O_2$	paullinic acid	0.71	*x*	fatty acids [ChEBI]
26	42.97	$C_{69}H_{134}O_6$	tribehenin	0.25	*x*	fatty acids [MeSH]
27	45.41	$C_{22}H_{28}O_6$	quassin	5.16	*x*	terpenes [MeSH]
28	52.16	$C_{26}H_{44}O_5$	ethyl iso-allocholate	0.07	*v*	terpenoids; cholane steroids [LOTUS]
29	52.69	$C_{28}H_{48}O$	campesterol	1.31	*ox*; *cx*	alcohols; secondary alcohols; 3beta-hydroxy steroids [ChEBI] terpenoids; steroids [KEGG: phytochemical compounds]
30	56.75	$C_{29}H_{48}O$	stigmasterol	2.75	*v*	alcohols; secondary alcohols; 3beta-hydroxy steroids [ChEBI] terpenoids; steroids [KEGG: phytochemical compounds]

Note: RT = retention time; RA = relative area; *ox* = antioxidant/radical scavenging activity; *bx* = antibacterial/ antimicrobial; *cx* = cytotoxicity/anticancer/antiproliferative/anticarcinogenic; *x* = no activity reported with respect to *ox*, *bx*, and *cx*; *v* = *ox*, *bx*, and *cx* activities reported. See Supp. File 3.

Table 6. Identified phytochemical compounds of RM extract of *H. capitata*.

No.	RT (min)	Chemical formula	Phytochemical compounds	RA (%)	Bioactivities	Classifications
1	5.17	$C_{27}H_{44}O_3$	1,25-dihydroxyvitamin D3	0.29	*cx*; *bx*	vitamins; cholecalciferols [MeSH]
2	21.70	$C_{17}H_{34}O_2$	methyl palmitate	3.35	*ox*; *bx*	fatty acids [MeSH]
3	22.36	$C_{18}H_{36}O_2$	ethyl palmitate	0.93	*ox*	fatty acids [MeSH]
4	23.15	$C_{20}H_{30}$	7-isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene	1.22	*x*	terpenoids [LOTUS]
5	23.33	$C_{19}H_{34}O_2$	7,10-octadecadienoic acid, methyl ester	1.14	*x*	lineolic acids and derivatives [CAS 56554-24-6]
6	23.38	$C_{19}H_{36}O_2$	cis-13-octadecenoic acid, methyl ester	4.65	*cx*	fatty acids [MeSH]
7	23.43	$C_{19}H_{36}O_2$	10-octadecenoic acid, methyl ester	1.55	*ox*; *bx*	fatty acids [LOTUS]
8	23.60	$C_{19}H_{38}O_2$	methyl isostearate	1.5	*x*	fatty acids [LOTUS]
9	23.93	$C_{26}H_{44}O_5$	ethyl iso-allocholate	0.18	*v*	terpenoids; cholane steroids [LOTUS]
10	25.11	$C_{35}H_{68}O_5$	1,2-dipalmitoyl-rac-glycerol	0.54	*x*	glycerolipids [ChEBI]
11	25.36	$C_{24}H_{36}O_6$	8,14-seco-3,19-epoxyandrostane-8,14-dione, 17-acetoxy-3ß-methoxy-4,4-dimethyl-	1.17	*cx*	ketals [PubChem CID 550132]

Table 6. Contd

No.	RT (min)	Chemical formula	Phytochemical compounds	RA (%)	Bioactivities	Classifications
12	25.57	$C_{20}H_{30}O$	ferruginol	7.21	*v*	terpenoids; diterpenoids (C20) [KEGG: phytochemical compounds]
13	25.76	$C_{20}H_{26}O_5$	gibberellin A44 (GA44)	7.90	*x*	isoprenoids; terpenoids; diterpenoids; gibberellins [ChEBI]
14	26.00	$C_{20}H_{28}O_3$	16-hydroxymethyleneandrost-5-en-3-ol-17-one	0.73	*cx*	alcohols; secondary alcohols; 3beta-hydroxy steroids; dehydroepiandrosterone [ChEBI] terpenoids; androstane steroids [LOTUS]
15	26.20	$C_{32}H_{48}O_6$	dodecanoic acid, 1a,2,5,5a,6,9,10,10a-octahydro-5a-hydroxy-4-(hydroxymethyl)-1,1,7,9-tetramethyl-6,11-dioxo-1H-2,8a-methanocyclopenta[a]cyclopropa[e]cyclodecen-5-yl ester, [1aR-(1aa,2a,5ß,5aß,8aa,9a,10aa)]-	1.71	*bx*; *ox*	fatty acids; lauric acids [MeSH] fatty acids; dodecanoid acid [ChEBI] fatty acids [KEGG: phytochemical compounds]
16	26.24	$C_{24}H_{34}O_6$	butanoic acid, 1a,2,5,5a,6,9,10,10a-octahydro-5,5a-dihydroxy-4-(hydroxymethyl)-1,1,7,9-tetramethyl-11-oxo-1H-2,8a-methanocyclopenta[a]cyclopropa[e]cyclodecen-6-yl ester, [1aR-(1aa,2a,5ß,5aß,6ß,8aa,9a,10aa)]-	0.71	*cx*; *bx*	carboxylic acids; acyclic acids; butyric acid [MeSH] fatty acids [ChEBI] fatty acids [KEGG: phytochemical compounds]
17	26.59	$C_{32}H_{39}NO_{10}$	3-pyridinecarboxylic acid, 2,7,10-tris(acetyloxy)-1,1a,2,3,4,6,7,10,11,11a-decahydro-1,1,3,6,9-pentamethyl-4-oxo-4a,7a-epoxy-5H-cyclopenta[a]cyclopropa[f]cycloundecen-11-yl ester, [1aR-(1aR*,2R*,3S*,4aR*,6S*,7S*,7aS*,8E,10R*,11R*,11aS*)]-	0.07	*ox*	diterpenoids [CAS 51906-00-4]
18	28.01	$C_{22}H_{28}O_6$	quassin	2.48	*x*	terpenes [MeSH]
19	28.56	$C_{36}H_{69}NO_6Si_3$	methyl glycocholate, 3TMS derivative	0.13	*ox*	glycinated bile acids and derivatives [PubChem CID 22214169]

Table 6. Contd.

No.	RT (min)	Chemical formula	Phytochemical compounds	RA (%)	Bioactivities	Classifications
20	36.11	$C_{29}H_{48}O$	stigmasterol	3.26	*v*	alcohols; secondary alcohols; 3beta-hydroxy steroids [ChEBI] terpenoids; steroids [KEGG: phytochemical compounds]
21	38.62	$C_{16}H_{50}O_7Si_8$	1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyloctasiloxane	0.09	*x*	siloxanes [CAS 19095-24-0]

Note: RT = retention time; RA = relative area; *ox* = antioxidant/radical scavenging activity; *bx* = antibacterial/antimicrobial; *cx* = cytotoxicity/anticancer/antiproliferative/anticarcinogenic; *x* = no activity reported with respect to *ox*, *bx*, and *cx*; *v* = *ox*, *bx*, and *cx* activities reported. See Supp. File 3.

Table 7. Identified phytochemical compounds of LM extract of *H. capitata*

No.	RT (min)	Chemical formula	Phytochemical compounds	RA (%)	Bioactivities	Classifications
1	17.36	$C_{30}H_{48}O_2$	ergosta-5,22-dien-3-ol, acetate, (3ß,22E)- (brassicasterol)	0.28	*cx*; *ox*	terpenoids; cholestane steroids [LOTUS]
2	20.82	$C_{20}H_{38}$	neophytadiene	4.48	*ox*; *bx*	hydrocarbons; olefins; acyclic olefins [ChEBI] terpenoids; phytane diterpenoids [LOTUS]
3	21.70	$C_{17}H_{34}O_2$	methyl palmitate	8.28	*ox*; *bx*	fatty acids [MeSH]
4	22.36	$C_{18}H_{36}O_2$	ethyl palmitate	8.55	*ox*	fatty acids; palmitic acids [MeSH]
5	23.33	$C_{19}H_{34}O_2$	7,10-octadecadienoic acid, methyl ester	1.71	*x*	lineolic acids and derivatives [CAS 56554-24-6]
6	23.38	$C_{19}H_{36}O_2$	trans-13-octadecenoic acid, methyl ester	22.13	*cx*	fatty acid methyl esters [CAS 56554-47-3]
7	23.60	$C_{19}H_{38}O_2$	methyl isostearate	3.80	*x*	fatty esters; fatty acid methyl esters [CAS 5129-61-3] fatty acids [LOTUS]
8	23.85	$C_{26}H_{44}O_5$	ethyl iso-allocholate	0.60	*v*	terpenoids; cholane steroids [LOTUS]
9	24.00	$C_{20}H_{34}O_2$	ethyl linolenate	12.04	*x*	fatty acids [MeSH]
10	25.11	$C_{35}H_{68}O_5$	1,2-dipalmitoyl-sn-glycerol	2.68	*x*	fatty acids; diacylglycerols [LOTUS]
11	25.36	$C_{24}H_{46}O_2$	methyl 12-(2-octylcyclopropyl) dodecanoate	1.29	*ox*; *bx*	fatty acid esters; fatty acid methyl esters [HMDB0031018]
12	27.01	$C_{24}H_{36}O_6$	8,14-seco-3,19-epoxyandrostane-8,14-dione, 17-acetoxy-3ß-methoxy-4,4-dimethyl-	1.77	*cx*	ketals [PubChem CID 550132]
13	27.17	$C_{27}H_{40}O_4$	spirost-8-en-11-one, 3-hydroxy-, (3ß,5a,14ß,20ß,22ß,25R)-	0.43	*V*	11-oxosteroids [CAS 54965-96-7]
14	42.78	$C_{16}H_{50}O_7Si_8$	1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyloctasiloxane	0.26	*X*	siloxanes [CAS 19095-24-0]

Note: RT = retention time; RA = relative area; *ox* = antioxidant/radical scavenging activity; *bx* = antibacterial/antimicrobial; *cx* = cytotoxicity/anticancer/antiproliferative/anticarcinogenic; *x* = no activity reported with respect to *ox*, *bx*, and *cx*; *v* = *ox*, *bx*, and *cx* activities reported. See Supp. File 3.

Discussion

Secondary metabolites of plants expressed a certain therapeutic potential and represent phytochemical compounds with bioactive properties. *H. capitata* contains phytochemical compounds that can be used to treat certain health problems. The phytochemical compounds identified in *H. capitata* extracts were alkaloids, saponins, flavonoids, tannins, and triterpenes/steroids (Table 1.). However, the presence of all the phytochemical compounds was confirmed only in the RM extract. Alkaloids have antiproliferative, antibacterial, antioxidant potential which can be used to develop drugs (Dey et al. 2020). From a therapeutic point of view, alkaloids are particularly well-known as an anesthetic, cardioprotective, and anti-inflammatory agents (Heinrich et al. 2021). Saponins also possess a number of important pharmaceutical properties, such as anti-inflammatory, antifungal, antibacterial, antiparasitic, anticancer, and antiviral activities (Mugford & Osbourn 2013; Rai et al. 2021). Similar bioactivities have also been confirmed by *in vitro* assays that show tannins to have antiviral, antibacterial, enzyme inhibitory, antioxidant, radical scavenging, and antimutagenic properties (Serrano et al. 2009; Singh & Kumar 2019; Hilmi et al. 2021; Sabdoningrum et al. 2021).

Apart from alkaloids, saponins and tannins, flavonoids, triterpenes and plant sterols also have therapeutic properties. Flavonoids possess several beneficial qualities, such as anticarcinogenic, antioxidative, anti-inflammatory, and antiviral (Kumar & Pandey, 2013; Panche et al. 2016; Sabdoningrum et al. 2021). Triterpenes also have promising antioxidant and antidiabetic activities and inhibit the formation of advanced glycation end products, which are involved in the pathogenesis of diabetic nephropathy, embryopathy, neuropathy, or impaired wound healing (Nazaruk & Borzym-Kluczyk, 2015). In preclinical studies, triterpenes showed broad pharmacological effects including anticancer, antioxidant, anti-inflammatory, antiatherosclerotic, antiviral, hepatoprotective, and immunomodulatory activities (Renda et al. 2022). Plant steroids have many interesting medicinal, pharmaceutical, and agrochemical activities such as antitumor, anticancer, immunosuppressive, hepatoprotective, antibacterial, plant growth hormone regulator, sex hormone, antihelminthic, cytotoxic, and cardiotonic activities (Salvador et al. 2013; Patel & Savjani 2015).

The antioxidant activity in Table 3 shows that RM has antioxidant activity categorised as potent. In the same way, antioxidant activity of the ethanol extract of *H. capitata*, which grows in Samarinda, is also classified as potent (Kusuma et al. 2020). The present study shows that the free radical scavenging activity is supported by the content of antioxidant -compounds, namely alkaloids, tannins, flavonoids and triterpenes (Panche et al. 2016; Chai et al. 2019; Maisetta et al. 2019; Li et al. 2020). Antioxidants act by preventing oxidation of a molecule. Alkaloids act as primary antioxidants by donating hydrogen atoms to free radicals (Kumaradewi et al. 2021). Similar to alkaloids, tannins also act as primary antioxidants. However, they also function as secondary antioxidants (Amarowicz, 2006). Flavonoids as antioxidants can act as stimulants for internal antioxidant enzymes, counteract free radicals, inhibit free radical forming enzymes, and chelate metals (Prochazkova et al. 2011). Likewise, terpenoids act as radical scavengers (Luo et al. 2021). In addition to antioxidant activity, the anticancer potential of *H. capitata* extracts was also tested through *in vitro* cytotoxicity assay. Based on half inhibitory concentration (IC_{50}) of extracts in Table 4, it was known that RC, RM, and LM had moderate cytotoxic values. The phytochemical compounds of

each extract contributed to inhibiting the viability of cancer model cells, both Hela and 4T1. Saponins, flavonoids, triterpenoids and steroid compounds contained in the extracts were predicted to contribute to its cytotoxic activity. Saponins as well as triterpenoids, and flavonoids play role in triggering apoptosis, autophagy and are involved in cell cycle arrest (Bishayee et al. 2011; Kopustinskiene et al. 2020; Elekofehnti et al. 2021). Meanwhile, apart from increasing cellular antioxidant capacity, plant sterols are involved in inhibiting cell proliferation (Perens & Shahman 2005).

Analysis of phytochemical compounds by GC-MS on RC (Table 5), showed pregn-5-en-20-one, 3-hydroxy, (3ß,17a); methyl retinoate; ferruginol; quassin; stigmasterol, and campesterol had more RA from 1 %. Among phytochemical compounds, there was no reference on quassin bioactivity regarding antioxidant, and cytotoxicity. Meanwhile, the bioactivity of campesterol, such as antioxidant activity and cytotoxicity, has been confirmed by a number of records (Table 5). The remaining phytochemical compounds were confirmed for their bioactivities related to antioxidant, and cytotoxicity. The phytochemical compound of pregn-5-en-20-one, 3-hydroxy, (3ß,17a)- has already been identified by To'bungan et al. (2022b) in the chloroform extract of the root and its fraction of *H. capitata*, albeit in smaller amounts. The presence of ferruginol, stigmasterol, and campesterol is consistent with previous findings by To'bungan et al. (2022b).

Campesterol and stigmasterol, together with β-sitosterol, are the most abundant phytosterols, while ergosterols are less commonly found in plants. MTT assay of *Rhizophora apiculata* extract phytosterols against HeLa, MCF-7, and A549 demonstrate cytotoxicity and importance of aliphatic sterol moiety (Kurniawan et al. 2023). One of the three types of phytosterol isolated from *Rhizophora apiculata* have a moderate cytotoxic effect on HeLa, MCF-7, and A549 cells with IC_{50} values 71.20, 67.95, and 54.9µg/mL respectively (Kurniawan et al. 2023). The cytotoxicity of non-esterified stigmasterol has been reported to significantly affect human intestinal cell viability and proliferation through cell cycle arrest at the G_2/M phase and inhibition of DNA synthesis (Kasprzak et al. 2023). Stigmasterol also induces the apoptosis pathway by enhancement of intracellular reactive oxygen species (ROS) generation and caspase 3/7 activity of human intestinal cells (Kasprzak et al. 2023). Lee et al. (2021) obtained 14.58% ferruginol from GC-MS analysis of *Metasequoia glyptostroboides* with anticancer activities mediated by the presence of hydroxyl groups, including other biologically active components reported to be anticancer and/or antitumor against Hela cells. Terpene derivatives and terpenoids are the main classes of phytochemical compounds in RC extract. The phytochemical compounds found in RC extract were consistent with the findings in phytochemical screening. It also confirmed the cytotoxicity in HeLa and 4T1 cancer cells. However, it contradicted the finding in radical scavenging assay.

Phytochemical compound analysis by GCMS shows there were several compounds that were predicted to be contained in RM, namely gibberellin A44 (GA44), ferruginol; cis-13-octadecenoic acid methyl ester, methyl palmitate, stigmasterol, and quassin (Table 6). Among phytochemical compounds, there was no reference on quassin and gibberellin A44 (GA44) bioactivity in terms of antioxidant and cytotoxicity. Meanwhile, the bioactivities of methyl palmitate, as antioxidant activity by suppressing oxidative stress , have been confirmed by Hamed et al. (2020), and for cis-13-octadecenoic acid methyl ester, contained in *Curcuma longa*, is thought to be involved in its

anticancer properties (Anekwe et al. 2023). The remaining phytochemical compounds were confirmed for their bioactivities related to antioxidant, and cytotoxicity. The phytochemical compound ferruginol has already been identified by To'bungan et al. (2022b) in the chloroform extract of the root and its fraction of *H. capitata*, with a higher RA (%) in its fraction. To'bungan (2023) specifically reported the presence of ferruginol in the root methanolic extract of *H. capitata* and was thought to be involved in its cytotoxic activity against WiDr colon cancer cells. Terpene derivatives and terpenoids are the major classes of phytochemical compounds in RM extract. The phytochemical compounds found in RM extract are consistent with the findings in phytochemical screening and also radical scavenging assay. The radical scavenging activity of the RM extract was qualified as potent and could be highly correlated with the concentration of ferruginol, methyl palmitate, and stigmasterol. It also confirmed the cytotoxicity in *HeLa* and *4T1* cancer cells.

Trans-13-octadecenoic acid methyl ester, ethyl linolenate, ethyl palmitate, methyl palmitate, neophytadiene, and methyl isostearate are predicted to be contained in LM (Table 7). Among the phytochemical compounds, there was no reference on the bioactivity of ethyl linolenate and methyl isostearate in terms of antioxidant and cytotoxicity. Meanwhile, the bioactivities of methyl palmitate and neophytadiene, such as antioxidant activity have been confirmed by several records, and for ethyl palmitate, antioxidant activity has been reported. The bioactivities related to cytotoxicity were confirmed for the remaining phytochemical compounds of trans-13-octadecenoic acid methyl ester. Krishnamoorthy & Subramaniam (2014) classified trans-13-octadecenoic acid methyl ester as linoleic acid esters. Linoleic acid ester treatment reportedly enhances ER-mitochondrial contact formation (MERC) which in turn promoted calcium (Ca^{2+}) signaling, mitochondrial energetics, and $CD8^{+}$ T-cell (CTL) effector functions, thus improving antitumor activity (Lauson et al. 2023). The enhanced apoptotic effect of cisplatin in A549 cells when integrated with conjugated linoleic acid was also confirmed by Yuce et al. (2023).

The phytochemical compound trans-13-octadecenoic acid methyl ester has already been identified by To'bungan et al. (2022b) in the root chloroform extract of *H. capitata* with a lower RA (%). Fatty acid derivatives and fatty acid methyl esters were the major classes of phytochemical compounds in LM extract. The phytochemical compounds found in LM extract are consistent with the findings in phytochemical screening and also radical scavenging assay. The phytochemical compounds identified in the LM extract were also responsible for the cytotoxicity in the 4T1 cancer cells.

CONCLUSION

Both RC and RM showed moderate cytotoxicity to Both HeLa and 4T1 cells, whereas only LM showed moderate cytotoxicity to 4T1 cells. RM exhibited high antioxidant activity. Campesterol, ferruginol, stigmasterol, cis-13-octadecenoic acid methyl esters, and methyl palmitate are predicted to play a role in the cytotoxicity and antioxidant activity. RC, RM, and LM have the potential to be developed for anticancer purposes. In addition to being an anticancer, RM also has the potential to be developed as an antioxidant, based on its relatively radical scavenging activity. Further studies regarding the cytotoxic effects of RC, RM, LM on normal cells and their toxicity in experimental animals need to be investigated.

AUTHOR CONTRIBUTION

NT and SSW conceived, formulated, supervised, and designed the research. FNLH and IWSM performed the research and drafted the manuscript.

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CONFLICT OF INTEREST

There is no conflict of interest.

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Research Article

Sequence-Structure Comparative and Network-Based Prediction of Drought Gene Candidate Regulator in *Elaeis guineensis*

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ABSTRACT

Drought poses a significant threat to global food security, particularly impacting crops like oil palm. Selecting genes for genome editing to enhance drought tolerance presents formidable challenges. To ensure that the target gene is chosen correctly and results in the desired character, a pilot study is necessary to determine the target gene for knockout. Two genes drought-related, AtBRL3 and AtOST2, were scrutinized in this context. Aligned with the *Elaeis guineensis* genome, their neighbouring proteins and gene ontology were analysed to identify potential targets for genome editing. AtBRL3, identified as BRL1 (XP_010913986.1) in *E. guineensis*, exhibited 58.48% identity and 100% coverage. It interacts with 12 nodes, including BIR1, BRI1, and AT2G20050, crucial for signalling pathways and cellular responses. Molecular function analysis revealed kinase activity. AtOST2 showed high similarity to plasma membrane ATPase/HA1 (XP_010913679.1) in *E. guineensis*, with 87.46% identity and 100% query cover. It correlated with 14 genes associated with ABA stimulus, stomatal movement, and hormone response. EgBRL1 and EgHA1, resembling AtBRL3 and AtOST2, respectively, emerge as promising targets for developing drought-tolerant oil palm cultivars through gene editing. Nonetheless, further validation through in vitro gRNA target selection and in vivo conversion of OST2/BRL3-containing plasmids in oil palm calluses is indispensable to demonstrate their efficacy in conferring novel drought resistance traits.

INTRODUCTION

Abiotic stresses in oil palm plantations, such as drought, negatively affect growth and productivity. El Nino is one of the weather phenomena connected to drought. The tropical Pacific Ocean experiences El Nino, a significant oceanic warming episode, about every 6 years (Wang et al. 2017; Trenberth 2020). Most oil palm is grown in the tropical regions of Southeast Asia, Africa, and South America (Corley & Tinker 2008; Zhang et al. 2018), where water is crucial to the crop's growth. Oil palm is susceptible when exposes to drought for 90 days and requires about 2000 mm of rain per year to produce oil ideally (Corley & Tinker 2008).

To suppress the productivity losses during water stress situations,

oil palm needs to be more drought-tolerant. According to Adam et al. (2011), the generation of male inflorescences and female fruit bunch or flower abortion brought on by stressors both had an impact on the bunch's production. Through a multilocation test of progeny trials in both dry and wet environments, the conventional method of selecting palms for drought resistance has been done. Conventional breeding is a process of selective breeding where crops are chosen based on their superior performances. The most well-known traditional breeding techniques include hybridization, recurrent selection, mass selection, backcross breeding, and pure-line selection. It requires more time and is excessively reliant on a plant's genotype. But different extrinsic factors have an impact on a plant's phenotypes. However, selection based on phenotypic expression is mostly inaccurate. Breeders began incorporating numerous biological specialties into plant breeding as a result, and they created modern breeding techniques. High throughput phenotyping, genomic selection, markers-assisted breeding, and CRISPR-Cas9 are some of the most popular modern breeding techniques (Lamichhane & Sapana 2022). Our group have been harnessing the CRISPR/Cas9 technology to improve oil palm genetics for favourable traits, such as Ganoderma tolerant using transcriptomic approach (Putranto et al. 2019). To ensure that the target gene is chosen correctly and results in the desired character, a pilot study is necessary to determine the target gene for knockout. Thus, before we continue to designing sgRNA for further CRISPR experiments, a bioinformatics approach to select precise gene target was done.

Based from literature studies, two genes namely OST2 and BLR3 was chosen as candidate. In Arabidopsis, OPEN STOMATA 2 (OST2) (AHA1) is a key plasma membrane H+-ATPase involved in the stomata response (Merlot et al. 2007). Plant cells create proton gradients by the action of plasma membrane proton (H+)-ATPases, which activate a variety of secondary transporters that facilitate the uptake of ions and metabolites (Palmgreen 2001; Osakabe et al. 2014). The eleven Arabidopsis plasma membrane H+-ATPases, or AHA1–AHA11 (Baxter et al. 2003), are made up of transmembrane domains with ten helices that include phosphorylation and nucleotide-binding sites, as well as N- and C-terminal domains in the cytoplasm (Pedersen et al. 2007). The primary regulatory domain involved in H+-ATPase inhibition is the C-terminus; phosphorylation in this area and subsequent interaction with 14-3-3 proteins regulate activation (Svennelid et al. 1999). A study reported two dominant mutations in the ost2 locus result in constitutive activation of the proton pump by eliminating stomata responses to abscisic acid (ABA) (Pedersen et al. 2007). Additionally, a study documented that ost2_cripspr mutants exhibited a markedly higher degree of stomatal closure in conjunction with a lower amount of transcriptional water loss when evaluating the stomatal response under ABA-induced circumstances. The results showed that a mutation at the OST2 locus caused by CRISPR/Cas9 improved stomatal responsiveness, which in turn promoted drought tolerance (Joshi et al. 2020). While BRL3 gene is classified as leucine-rich repeat (LRR)-RLK family members of the BR-INSENSITIVE 1 (BRI1), bind directly to brassinosteroid (BR) hormones (Li et al. 1997; Wang et al. 2001; Kinoshita et al. 2005; Hothorn et al. 2011; She et al. 2011). Early BR signalling events (Gou et al. 2012) depend on BRI1's interaction with the co-receptor BRI1 ASSOCIATED RECEPTOR KINASE 1 (BAK1), which is triggered by ligand perception. The BRI1-EMS-SUPPRESSOR1 (BES1) and BRASSINAZOLE RESISTANT1 (BZR1) transcription factors (Yin et al. 2002; Wang et al. 2002; He et al. 2002) are primarily responsible for controlling the expression of certain BR-regulated genes. This BRI1–BAK1 heterodimerization

starts a signalling cascade of phosphorylation events. While BRs influence several developmental and environmental stress reactions in plants, there is ongoing debate regarding the precise function of BRs in stressful situations. While overexpressing the BR biosynthesis enzyme DWF4 and applying BRs exogenously both improve a plant's ability to withstand drought stress, suppressing the BRI1 receptor also produces drought-resistant phenotypes (Feng et al. 2015; Ye et al. 2017). Interestingly, interaction between the two pathways upstream of the BRASSINOSTEROID-INSENSITIVE 2 (BIN2) kinase has been reported (Zhang et al. 2009; Gui et al. 2016). ABA signalling suppresses the BR signalling pathway following BR perception. BRL3ox shoots showed higher concentrations of proline, GABA, and tyrosine while under drought stress. On the other hand, the most prevalent metabolites in the BRL3ox roots during the stress time course were trehalose, sucrose, myo-inositol, raffinose, and proline. Significantly, there has been prior research connecting all of these metabolites to drought tolerance (Fàbregas 2018).

Since both genes historically explored in model plant Arabidopsis, here we performed alignment to the reference genes with the genome database of oil palm (*E. guinensis*), protein modelling, and validation using Ramachandran plot. A networking analysis was also performed to support the data of the chosen target genes.

MATERIALS AND METHODS

Protein sequence collection and modelling

The *At*BRL3 and *At*OST2 sequences were collected from Uniprot database, with ID Q9LJF3 and ID A0A1P8AYX4, respectively. In order to select the best model protein of BRL3/OST2 from *Arabidopsis thaliana* and *Elaies guineensis*, we utilised Robetta prediction, with RoseTTA fold option chosen. Robetta's accuracy is primarily reliant on the presence of homologs, or homologous sequences, in the PDB, UniProt, and Uniclust sequence databases. In the supplementary information of the RosettaCM publication (Baek et al. 2021), a predicted confidence value that accounts for this is given for comparative modelling domains and was found to correspond with the actual GDT to native. The model prediction was then validated and compared by its secondary structure using Ramachandran plot by PROCHECK (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/).

Alignment and phylogenetic tree construction

The protein sequence of *At*BRL3 and *At*OST2 were globally aligned to *E. guineensis* protein database using the BLASTP program in NCBI (https://blast.ncbi.nlm.nih.gov/). The data retrieved were collected to build a phylogenetic tree. Neighbour-joining (NJ) approach and bootstrap 1000 were considered because of the high similarity score between proteins.

Networking analysis

To understand the neighbour protein interaction with *At*BRL3 and *At*OST2, a network analysis and gene ontology (GO) is performed with String-db (https://string-db.org/) (Szklarczyk et al. 2023) and the network was visualized with Cytoscape (Shannon et al. 2003).

RESULTS AND DISCUSSION

Sequence alignment and phylogenetic tree construction

Global alignment of *At*BRL3 revealed six similar proteins in *E. guineensis*: ID XP_010928900.1, XP_010913986.1, XP_010925081.1,

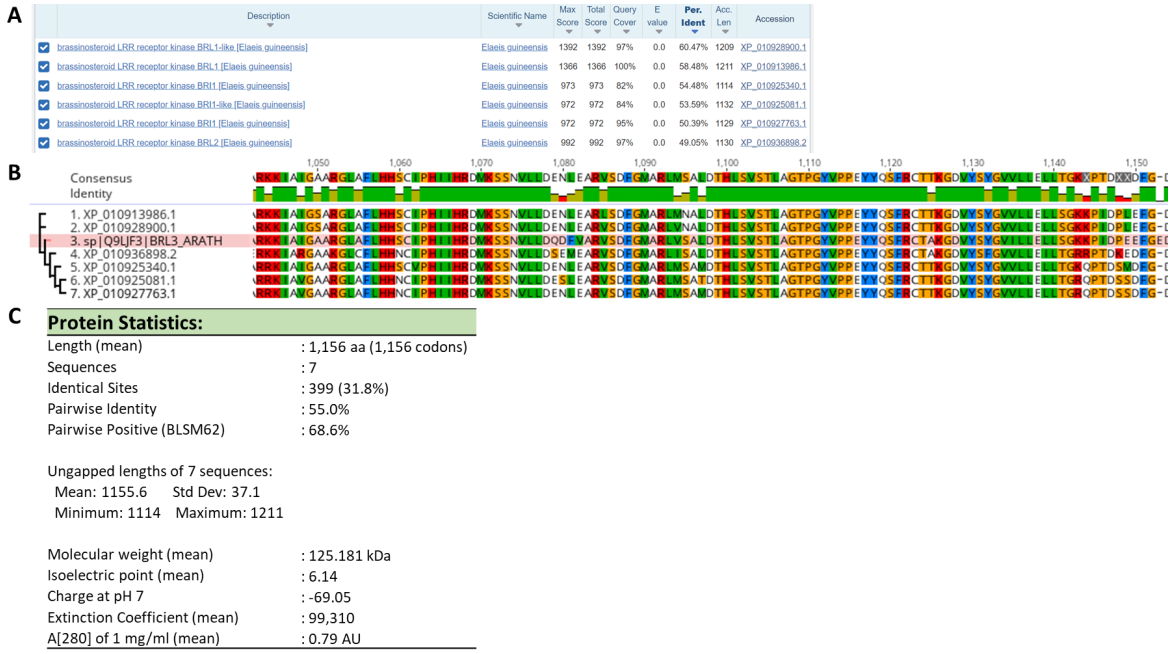


Figure 1. Global alignment of *AtBRL3* to *E. guineensis* protein database with query cover and similarity information (A), visualization of multiple alignment of six similar proteins *Eg*BRL1 and *At*BRL3 (B), detail of alignment data (C).

XP_010925340.1, XP_010927763.1, and XP_010936898.2. The coverage varied between 82-96% with the highest identity of 65.43% from ID XP_010928900.1 (Brassinosteroid LRR receptor kinase BRL1-like [*E. guineensis*]) (Figure 1A). Multiple alignment was performed by aligning six BRL1 protein retrieved from BLASTP with *At*BRL3 and shows 55.0% similarity (Figure 1C). Thus, the data initiate the finding of possible sequence similarity of *BRL3* in *E. guineensis*.

To support the data, six identical proteins of *Eg*BRL1 were then analysed to estimate its phylogenetic correlation with *At*BRL3 (Figure 2). Roughly, *At*BRL3 showed closeness with XP_010928900.1 and XP_010913986.1. This is in line with high similarity percentage results from BLASTP. Accordingly, ID XP_010913986.1 became the best candidate, with query cover 100%, of the closest protein to *At*BRL3 based on the sequence comparison.

Similar method was also performed to identify the similar proteins of *At*OST2. BLASTP data showed 12 similar proteins to *At*OST2: XP_010913679.1, XP_019707983.1, XP_010928676.1, XP_010929278.1, XP_010942146.1, XP_010914190.1, XP_010929115.1, XP_029118717.1, XP_010932230.1, XP_010933786.1, XP_010923303.1, and XP_010914191.1. All of the proteins showed high identical score of coverage, 100%. However, the identity percentage varied from the lowest 80.56% to 87.46% (Figure 3A). Next, multiple alignment was performed, visualised, and analysed. The alignment shows 85% similarity between *At*OST2 and 12 proteins plasma membrane ATPase in *Elaeis guineensis*. The sequence comparison data were then used for further analysis.

A general rule of thumb: two protein sequences are said to be homologous if they share more than 30% of their total lengths (far greater identity score is seen by accident in short alignments), although the 30% criterion ignores a lot of readily observable homologs. Taken together, the finding of *At*BRL3/*At*OST2 homologs passing the cut off score and considered have high homology. Also, protein sequence chosen as the template is correlated to the sensitivity of protein (and translated-DNA) than DNA: DNA similarity searches. Compared to protein-protein or

translated alignments, the evolutionary look-back time for DNA: DNA alignments is between 5 and 10 times shorter from Protein: DNA alignments. After more than 200–400 million years of divergence, DNA–DNA alignments hardly ever find homology; nevertheless, protein–protein alignments frequently find similarity in sequences that last shared an ancestor more than 2.5 billion years ago (Pearson 2013).

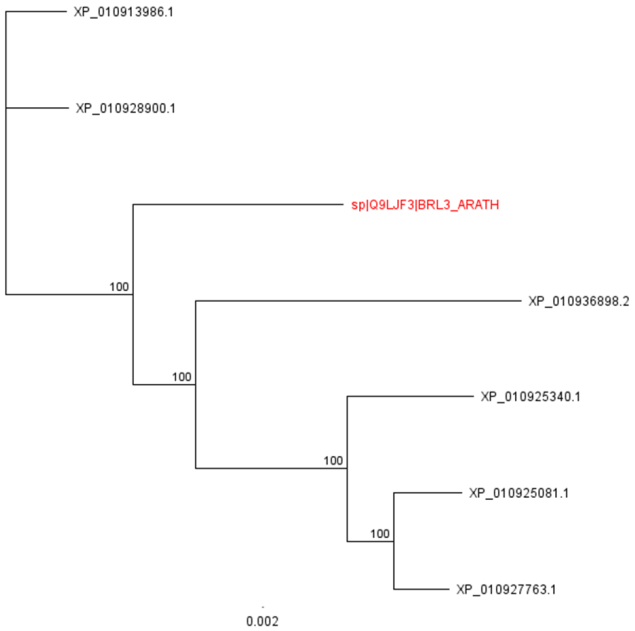
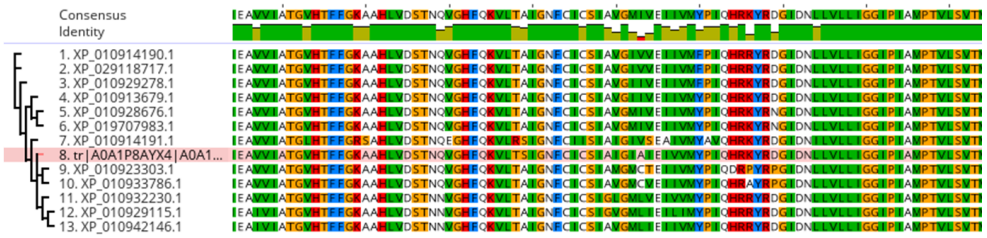


Figure 2. Phylogenetic construction of *At*BRL3 and six similar proteins in *E. guineensis* using Neighbour joining approach with 1000 bootstrap.

A

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
plasma membrane ATPase isoform X1 [Elaeis guineensis]	Elaeis guineensis	1559	1559	100%	0.0	86.33%	954	XP_010928676.1
plasma membrane ATPase isoform X2 [Elaeis guineensis]	Elaeis guineensis	1559	1559	100%	0.0	86.33%	885	XP_019707983.1
plasma membrane ATPase [Elaeis guineensis]	Elaeis guineensis	1556	1556	100%	0.0	87.46%	954	XP_010913679.1
plasma membrane ATPase [Elaeis guineensis]	Elaeis guineensis	1528	1528	100%	0.0	86.10%	953	XP_010929278.1
plasma membrane ATPase 4 [Elaeis guineensis]	Elaeis guineensis	1525	1525	100%	0.0	83.73%	950	XP_010942146.1
plasma membrane ATPase isoform X2 [Elaeis guineensis]	Elaeis guineensis	1510	1510	100%	0.0	85.36%	957	XP_010914190.1
plasma membrane ATPase 4 [Elaeis guineensis]	Elaeis guineensis	1501	1501	100%	0.0	82.82%	950	XP_010929115.1
plasma membrane ATPase isoform X1 [Elaeis guineensis]	Elaeis guineensis	1497	1497	100%	0.0	82.86%	985	XP_029118717.1
plasma membrane ATPase 4 [Elaeis guineensis]	Elaeis guineensis	1486	1486	100%	0.0	83.09%	953	XP_010932230.1
plasma membrane ATPase 3 [Elaeis guineensis]	Elaeis guineensis	1443	1443	100%	0.0	81.44%	956	XP_010933786.1
plasma membrane ATPase 3 [Elaeis guineensis]	Elaeis guineensis	1436	1436	100%	0.0	81.33%	956	XP_010923303.1
plasma membrane ATPase [Elaeis guineensis]	Elaeis guineensis	1432	1432	100%	0.0	80.56%	956	XP_010914191.1

B



C

Protein Statistics:	
Length (mean)	: 946 aa (946 codons)
Sequences	: 13
Identical Sites	: 621 (62.9%)
Pairwise Identity	: 85.0%
Pairwise Positive (BLSM62)	: 92.2%
Ungapped lengths of 13 sequences:	
Mean: 945.7 Std Dev: 27.2	
Minimum: 885 Maximum: 985	
Molecular weight (mean)	: 104.119 kDa
Isoelectric point (mean)	: 6.41
Charge at pH 7	: -60.63
Extinction Coefficient (mean)	: 114,029
A[280] of 1 mg/ml (mean)	: 1.10 AU

Figure 3. Global alignment of *At*OST2 to *E. guineensis* protein database with query cover and similarity information (A), visualization of multiple alignment from twelve similar plasma membrane ATPase proteins from *E. guineensis* and *At*OST2 (B), detail of alignment data (C).

The phylogenetics tree illustrates two different clusters, with bootstrap score 99.8 and 64.4 (Figure 4). *At*OST2 is located in the bigger cluster with others EgOST. However, phylogenetic tree shows XP_010913679.1 is the closest based on the genetic distance, supporting with highest score from BLASTP (100% query cover and 87.46% similarity).

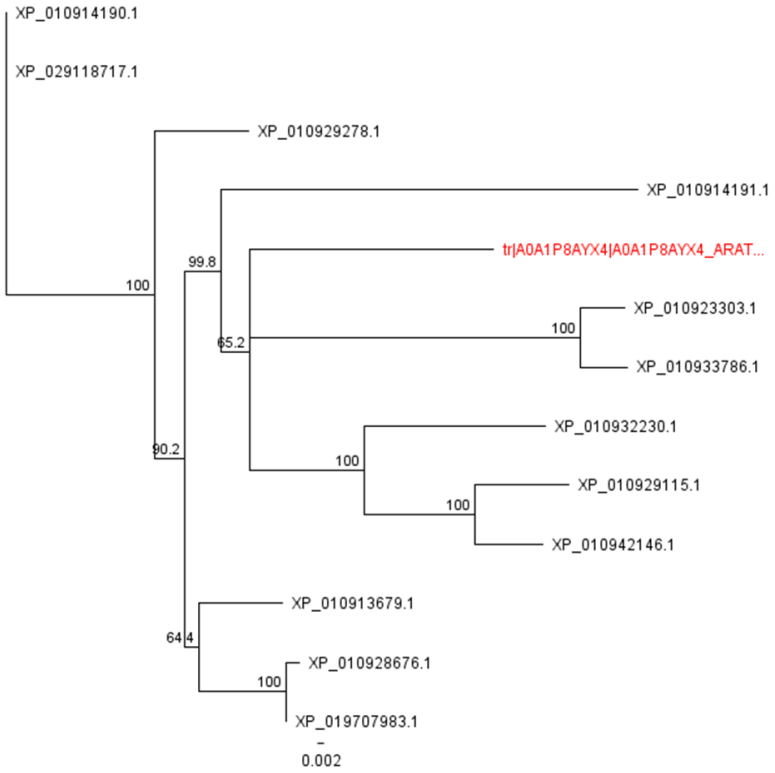


Figure 4. Phylogenetic construction of *At*OST2 and twelve similar proteins in *E. guineensis* using Neighbour joining approach with 1000 bootstrap.

Protein structure comparison of BRL3/OST2 in *A. thaliana* and *E. guineensis*

To compare the protein structure of BRL3/OST2, a protein modelling based on *ab initio* approach was performed. The sequence of *At*BRL3 (ID Q9LJF3) was modelled with RoseTTAFold, with confidence 0.76, from residue 1 to 1164 (Figure 5a). Coherently, the sequence of XP_ XP_010913986.1 (EgBRL1) was also modelled into 3D structure, with confidence score 0.75 (Figure 6a). The model protein was then validated by Ramachandran plot (Figure 5b, 6b, Table 1).

The *At*OST2 protein with ID A0A1P8AYX4 and XP_010913679.1 (EgHA1) were also modelled by RoseTTAFold by Robetta tools. AtOST2 was successfully modelled with confidence score 0.76 (Figure 7a), while 0.81 is the confidence score of EgHA1 the (Figure 8a).

Next, Ramachandran analysis was also performed to validate the protein modelling results (Table 1). The Ramachandran plot, which displays the mapping of pairs of torsion angles of the polypeptide backbone on the background of the "allowed" or predicted values, is one of the most helpful techniques for validating protein structures. Glycines and other amino acids, as well as various amino acids to a lesser extent, have substantially varied allowable areas of the Ramachandran plot (Wlodawer 2017). The data showed AtBRL3 and XP_010913986.1 have 82.7% and 82.1% most favoured regions, respectively. Even better, higher percentage was exhibit by modelled of AtOST2 and XP_010913679.1 94.2 and 94.9%, respectively.

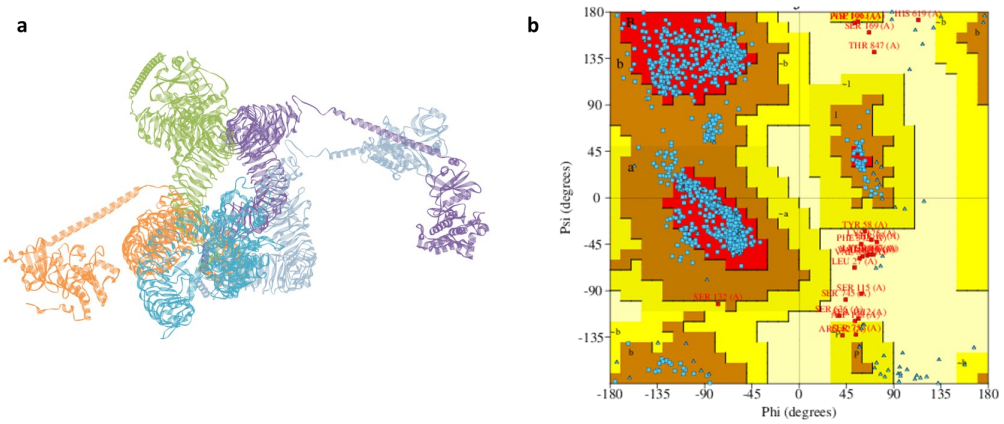


Figure 5. Protein modelling of AtBRL3 (a) and Ramachandran plot validation (b).

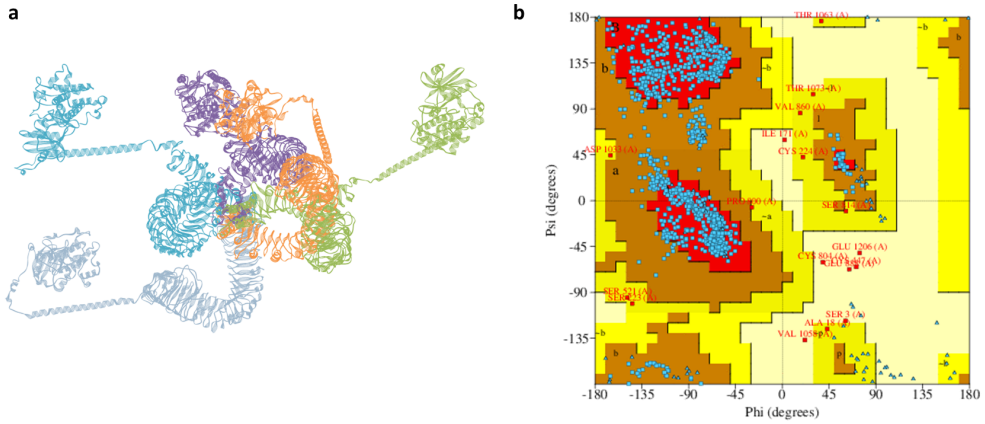


Figure 6. Protein modelling of XP_010913986.1 (a) and Ramachandran plot validation (b).

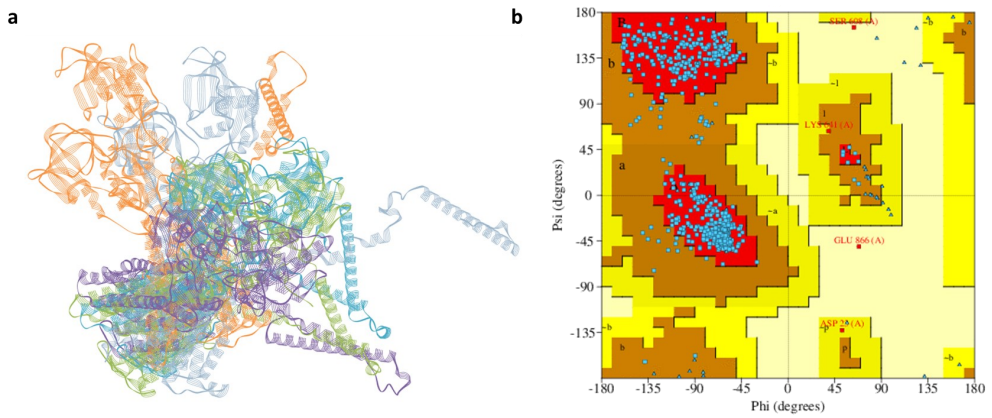


Figure 7. Protein modelling of AtOST2 (a) and Ramachandran plot validation (b).

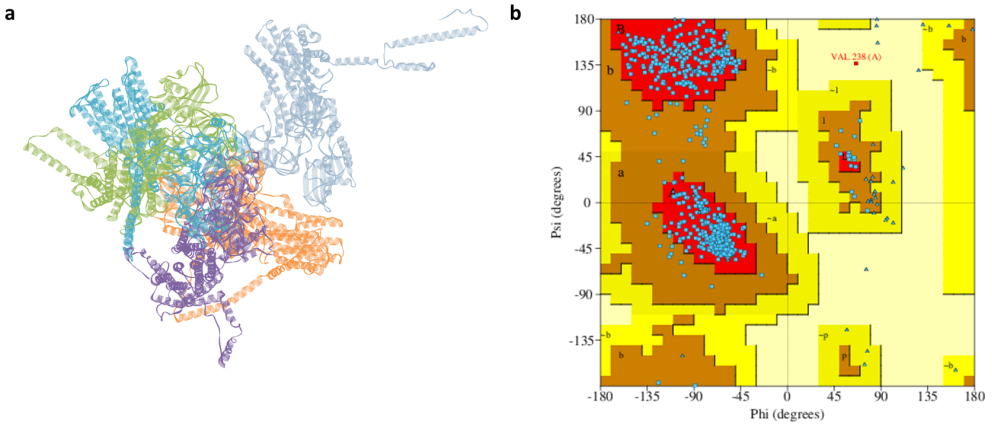


Figure 8. Protein modelling of XP_010913679.1 (a) and Ramachandran plot validation (b).

Table 1. Ramachandran plot score analysis.

Parameters	AtBRL3		XP_010913986.1		AtOST2		XP_010913679.1	
	No of residues	**%**	**No of residues**	**%**	**No of residues**	**%**	**No of residues**	**%**
Most favoured regions [A,B,L]	834	82.7	850	82.1	735	94.2	801	94.9
Additional allowed regions [a,b,l,p]	151	15	169	16.3	41	5.3	42	5
Generously allowed regions [~a,~b,~l,~p]	4	0.4	9	0.9	2	0.3	0	0
Disallowed regions [XX]	19	1.9	7	0.7	2	0.3	1	0.1
Non-glycine and non-proline residues	1008	**100**	1035	**100**	780	**100**	844	**100**
End-residues (excl. Gly and Pro)	1		1		2		2	
Glycine residues	105		110		68		71	
Proline residues	50		63		35		37	
Total number of residues	**1164**		**1209**		**885**		**954**	

In terms of secondary structure, *At*BRL3 structure consist of helix structure mostly in the N terminal, then followed by coil structure mixed with strand and helix in some part up to the C terminal. The secondary structure is slightly similar to the XP_010928900.1 protein model. In more detail, the helix structure of XP_010913986.1 initiate in the beginning of the model (AA 2-20 and 26-38) making the coil structure longer than the *At*BRL3 (Figure 9a and b). Moreover, the template available for modelling in both *At*BRL3 and XP_010928900.1 are different.

Whilst AtOST2 and XP_010913679.1 show obvious distinct secondary structures. AtOST2 consists of helix in the region AA 1-61, in between region appear coil structure in AA 21-27 (Figure 10a). It is contrast to the modelling of XP_010913679.1, which showing coil-helix-coil -helix repeatedly in the whole amino acids structure (Figure 10b).

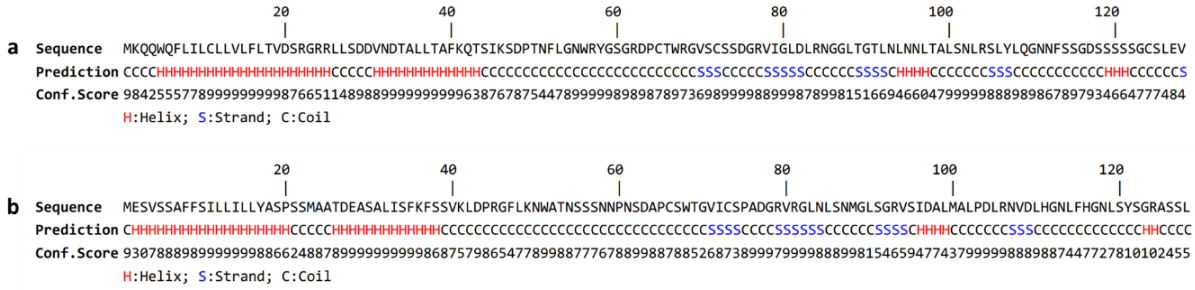


Figure 9. Secondary structure prediction of (a) *At*BRL3 and (b) XP_010913986.1

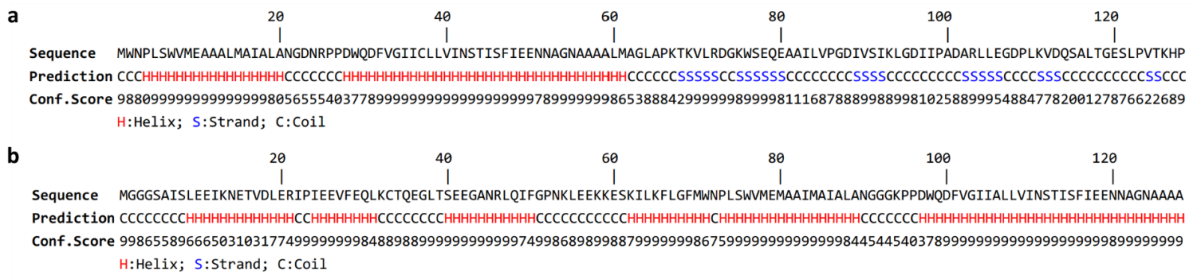


Figure 10. Secondary structure prediction of (a) *At*OST2 and (b) XP_010913679.1

Networking analysis

To explore the neighbour-gene of *At*BRL3 and *At*OST2 in the cellular level and analyse the gene ontology occur in the system, we performed networking analysis. When *At*BRL3 and *At*OST2 put in the network, there is no line associated with other genes related to proliferation and growth, such as AUX1, ARF15, TIR1, SKP1, ILR1 and oil biosynthesis-related genes such as FAD3, KASI, WRI1, FATB (Figure 11a & 12a).

However, different ways of data mining shows that *At*BRL3 alone is connected to the several proteins related to the defence mechanism against pathogen and growth signalling such as BAK1, BIR1, SERK4, and BRI1 (Figure 11b). A null allele of BAK1 exhibits a semi-dwarf phenotype and has decreased sensitivity to brassinosteroids (BRs), whereas overexpression of BAK1 causes elongated organ phenotypes. BRI1 interacts with serine/threonine protein kinase BAK1 both *in vitro* and *in vivo*. A severe dwarf phenotype similar to the phenotype of null bri1 alleles is produced by the expression of a dominant-negative mutant allele of BAK1. These findings show that BAK1 is a part of the BR signalling system (Li et al. 2002). Numerous studies have shown that BAK1 regulates dPCD (development-related programmed cell death) in an essential way. For instance, silencing *Gh*BAK1 in cotton (*Gossypium hirsutum*) results in high levels of cell death and increased ROS production, indicating that BAK1 controls cell death in a way that is conserved across a wide range

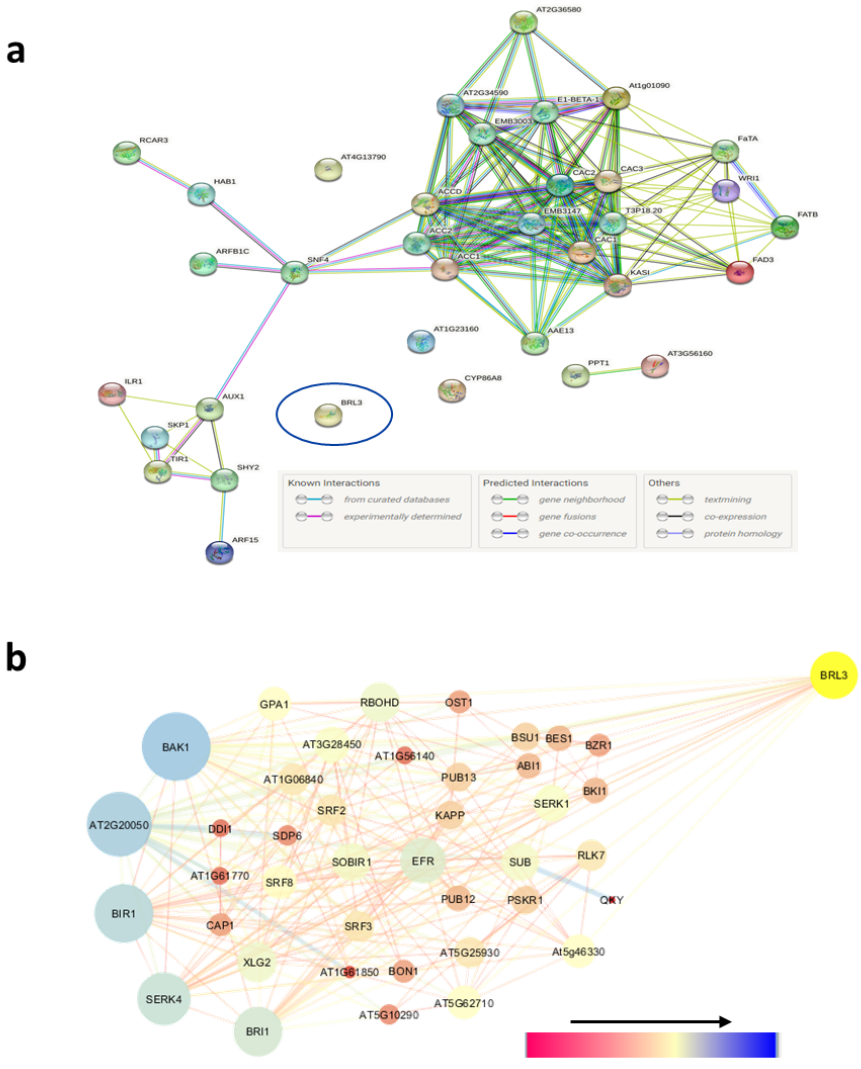


Figure 11. String-db analysis of *At*BRL3 and proteins related to proliferation, growth, and oil biosynthesis (a), Network analysis of BRL3 by Cytoscape (b).

of plant species (Gao et al. 2013, 2019). The networking data leads to the hypothesis that BRL3 gene is important for plant survival. Taken together, BRL3 as a target gene for editing for drought-tolerant oil palm is crucial to be kept or upregulated in the system. In *Arabidopsis*, overexpression of BRL3, a member of the brassinosteroid receptor family with increased vascularity, can increase resistance to drought stress. Overexpression of the drought-tolerant BRL3 receptor offers drought tolerance without hindering overall development, in contrast to loss-of-function mutations that result in drought resistance at the price of growth in the widely expressed BRI1 receptor (Fàbregas et al. 2018). Thus, the BRL3 activation is one of the alternatives to generate drought-tolerant oil palm.

In line with networking results, *At*OST2 is also correlated to proteins related to plant adaptation to abiotic stress such as HAB1, ABI5, PP2CA, PYL13 (Figure 12b). HAB1, one of the main PP2Cs from Clade A protein phosphatases, is a negative regulator of ABA signalling in *Arabidopsis*. The research suggests that PYL5 is a nuclear and cytosolic ABA receptor that directly inhibits clade A PP2Cs to activate ABA signalling. Furthermore, PYL5-mediated suppression of clade A PP2Cs can be used to achieve increased resistance to drought (Santiago et al. 2009). In the presence of ABA and abiotic stressors, the basic leucine zipper transcrip-

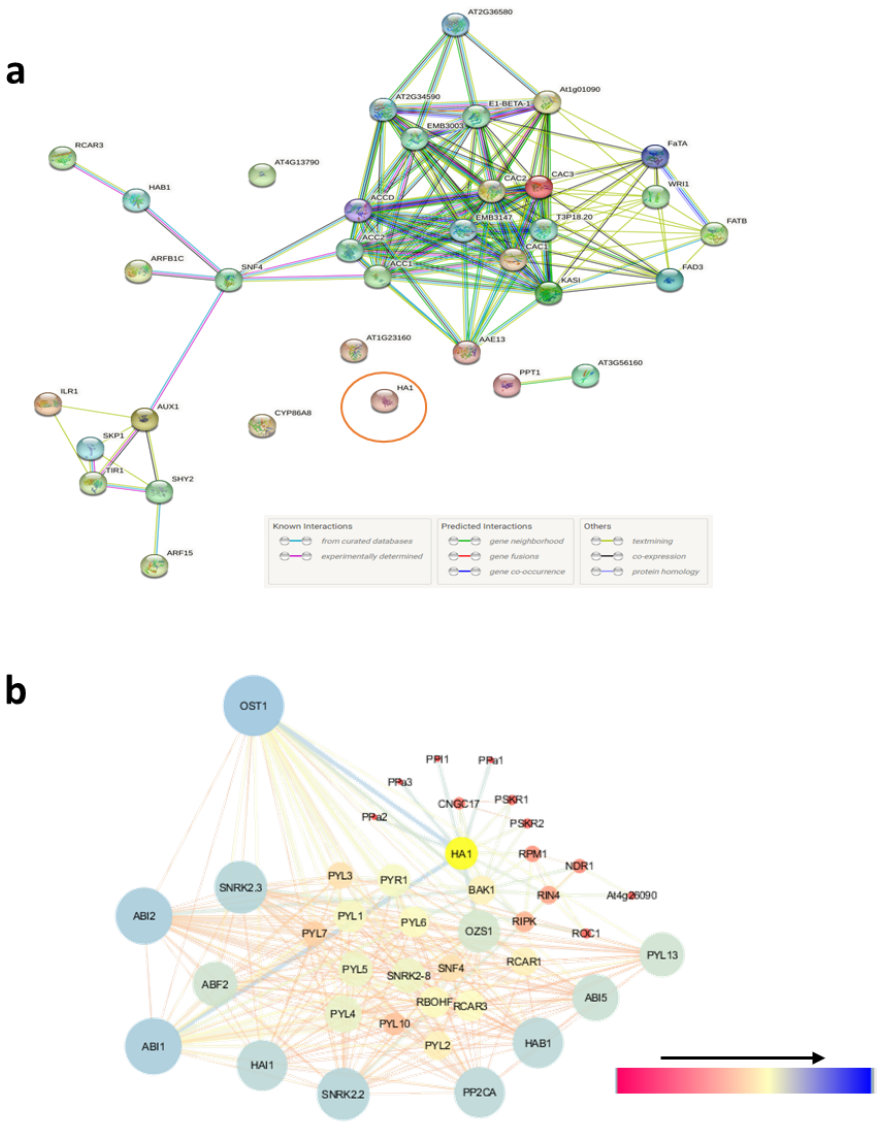


Figure 12. String-db analysis of *At*OST2/HA1 and protein related to proliferation, growth, and oil biosynthesis (a), Network analysis of *At*OST2 by Cytoscape (b)

tion factor known as ABA Insensitive 5 (ABI5) is essential for controlling seed germination and early seedling growth. ABI5 controls the expression of genes that have the ABSCISIC ACID RESPONSE ELEMENT (ABRE) pattern in their promoter region, contributing to the core ABA signalling that is made up of PYR/PYL/RCAR receptors, PP2C phosphatases, and SnRK2 kinases. The stress adaption genes, such as LEA proteins, are among the regulated targets (Skubacz et al. 2016). However, the correlation between OST2 and ABI5 is still unclear, especially on whether it plays as an inhibitor or activator. OST2 is one of the candidate target genes for genome editing to develop drought-tolerant oil palm by knocking it out, based on the previous research (Osakabe et al. 2016).

CONCLUSION

In terms of sequence comparison, BRL3 and OST2 are promising target for gene editing to generate drought-tolerant oil palm. This is supported by the high similarity of alignment from both genes in *A. thaliana* to *E. guinensis* and structure modelling protein shows favourable comparison. In addition, functional protein prediction *via* network analysis shows BRL3 and OST2 playing important roles in drought tolerance, *via* activation and or gene inhibition. However, to prove the effectivity of both genes to generate new variety of drought tolerance in oil palm, an in vitro (gRNA target selection) and in vivo (by transforming the plasmid containing OST2/BRL3 gene to the oil palm callous) approaches are inevitable.

AUTHORS CONTRIBUTION

G.W.P and R.A.P designed the research and supervised all the process, G.W.P, A.A.A, L.D.M and Y.S. collected and analysed the data. I.R, H.M, E.Y wrote the manuscript.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Research Article

Fern Species-Area Relationship in Urban Anthropogenic Islands in Slawi, Tegal, Central Java

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ABSTRACT
In anthropogenic islands as urban parks, the fern species richness and composition may be determined ecologically by the quality of habitat, including area greenness, or biogeographically by area size. As the development of the theory of island biogeography also includes man-made parks, it is feasible to test whether area-species relationship applies in these urban parks, and is more pronouncedly evident compared to another ecological factor, such as NDVI. Total species number and composition of 8 urban parks in a *kecamatan* (subdistrict) in Tegal Regency were collected and arranged in clustering methods to understand the similarity between parks. The similarity analysis result is important for the management of the parks in Slawi. The species richness data is subsequently tested using Pearson correlation and regression against NDVI and area sizes. The relation between NDVI and species richness is non-significant (p=0.058), while area size and species richness is significant (p=0.003). This signifies that the urban fern species richness is determined by area as biogeographical factor, compared to NDVI as ecological factor. This result is important for the purpose of designing and managing urban parks as evidently size is important in the effort of attracting native biodiversity into urban parks and in turn enhancing the well-being of urban population.

INTRODUCTION
The theory of island biogeography (MacArthur & Wilson 1963; MacArthur & Wilson 1967) was originally developed to understand the effect of island environment, including isolation, to species richness and species speciation. It was developed to understand the biodiversity occurring in newly formed oceanic islands in relation to distance to larger land masses, especially continents. The key factors involved in the original and recently developed theory (Whittaker & Fernández-Palacios 2007) are the size of island, the relative distance to neighboring island and mainland, the time of isolation, climate, and past relation with land masses. Along its development, this theory is not applied only in real island environment, but into several island-like environments, including human-derived environments (Gleditsch et al. 2023). This theory is also applied in differ-

ent sense rather than only real island, such as succulent vegetation in arid environment in Africa (Desmet & Cowling 2004) and completely submerge marine habitat (Neigel 2003). Human activities alter natural vegetation into anthropogenic habitats according to specific human needs, creating islands surrounded by human habitats. Human managers call these islands urban green spaces, which in- turn may take several forms including urban parks.

In these anthropogenic islands, wild species thrive from several sources, mostly from deliberate human introduction, followed by natural vegetation remnants, and natural vegetation new immigrants (Savard et al. 2000). While deliberate human introduction is sometimes thought of as unnatural, and vegetation remnants are mostly unlikely in small urban parks, entirely altered during construction, new immigrants are thought as natural and reflecting the vegetation capability in dispersal from nearby sources including nearby parks or nearby natural habitats. This component of urban park biodiversity is an interesting object of biodiversity study. In parks, this natural vegetation component interacts with human vegetation introductions, sharing a certain park area size. It is interesting to understand the species' richness and composition in these island urban habitats, as they vary in size and management.

Fern is a suitable plant group to test the interchange ability among populations, as they are mostly airborne, capable of long-distance dispersal, with the exception of several aquatic fern, Marsileales and Salviniales (Dassler & Farrar 2001; De Groot et al. 2012). A wild fern individual in an urban park is almost certainly a newly established individual, or can be called as a spontaneous fern, growing from airborne spores into minute gametophytes and subsequently into adult sporophytes (Sato 1982; Taylor et al. 2005); a process that makes them feasible subject of biogeography study. As ferns are mostly airborne, angiosperms are dispersed in various manners, from ballistic-anemochory to intricate zoochory. Variable dispersal methods in the angiosperms compared to the ferns makes the latter is ideal and rather homogenous group to study, in terms of dispersal and spontaneous colonization of an area.

Urban parks vary not only in size, but also in management. Several parks with variability in trees and shrubs; many with mostly grass; others with extensive tiling or concretes. The greener a park, the more likely to attract biodiversity, flora, fauna, and microorganisms, compared to those less green. A popular measure in quantifying the greenness, thus healthiness of a park, is NDVI (Normalized Difference Vegetation Index), a remote sensing tool. In a place with high NDVI, it is expected to encounter higher biodiversity, both in flora (Pau et al. 2012), and fauna (Seto et al. 2004). In small parks where source of biodiversity, as nearby forest or nearby mountain is neglectable, it is interesting to consider whether island biogeography most pronounced factor, the island size, or the greenness of an area, determined the species richness in an area.

MATERIAL AND METHODS

This research was conducted for 6 months from July to December 2022. The location of this research is in the 8 city parks in *Kecamatan* (Subdistrict) Slawi, Tegal Regency (Figure 1, Table 2). Eight parks are appointed based on the advice of the Tegal Regency Environmental Service (Dinas Lingkungan Hidup Kabupaten Tegal), as these parks are the most managed amongst 18 *kecamatan* (subdistrict) in Tegal Regency and have the feature of most urbanized surroundings. In appointing parks only in a single *kecamatan* (subdistrict), we deliberately reduce the effect of isolation from species immigration sources (like Mt. Slamet, 50 km

south), meaning all parks have relatively similar isolation to Mt. Slamet. Therefore, the biogeographic driver in determining species number of a park is solely park area size.

Fern censuses were done in the manner of exhaustive census, i.e., all fern individual possible for identification, within the perimeter of a park were included in the data collection. According to sizes, each park takes about 3—7 days observation to conclude all identifiable fern individuals in the data collection. As some ornamental ferns are commonly found as garden adornments, we limit our censuses to include only spontaneous ferns/vegetations (Robinson & Lundholm 2012). Consequently, individuals obviously planted in pots (as maidenhairs, *Adiantum*) or tied on tree trunks (as staghorns, *Platycerium*) were excluded. However, careful considerations were taken when dealing with adults, juveniles and (especially) sporelings of the escaping naturalized ornamental. These individuals, when clearly free living, even though from ornamental/planted parents, are included in the data collection. All individuals living within the park perimeter, including high up on the roof of tree branches or down below sewer system walls were recorded their species names and their habitats, as terrestrial (on ground), epiphytes (on trees), lithophytes (on rocks, concretes, walls, rooftiles or other stone-like materials), or combinations; and photographed. The photos were crosschecked with the description in *Flora of Malaya vol II. Ferns of Malaya* (Holttum 1966) and *Panduan lapangan paku-pakuan (Pteridophyta) di Taman Margasatwa Ragunan* (Agatha et al. 2019). Nomenclature follows POWO (2023). When it is thought to be difficult to identify through photos, herbarium specimens were taken to be identified in the lab. Herbarium sheets were deposited at the *Herbarium Biologi Universitas Negeri Jakarta* (JUNJ).

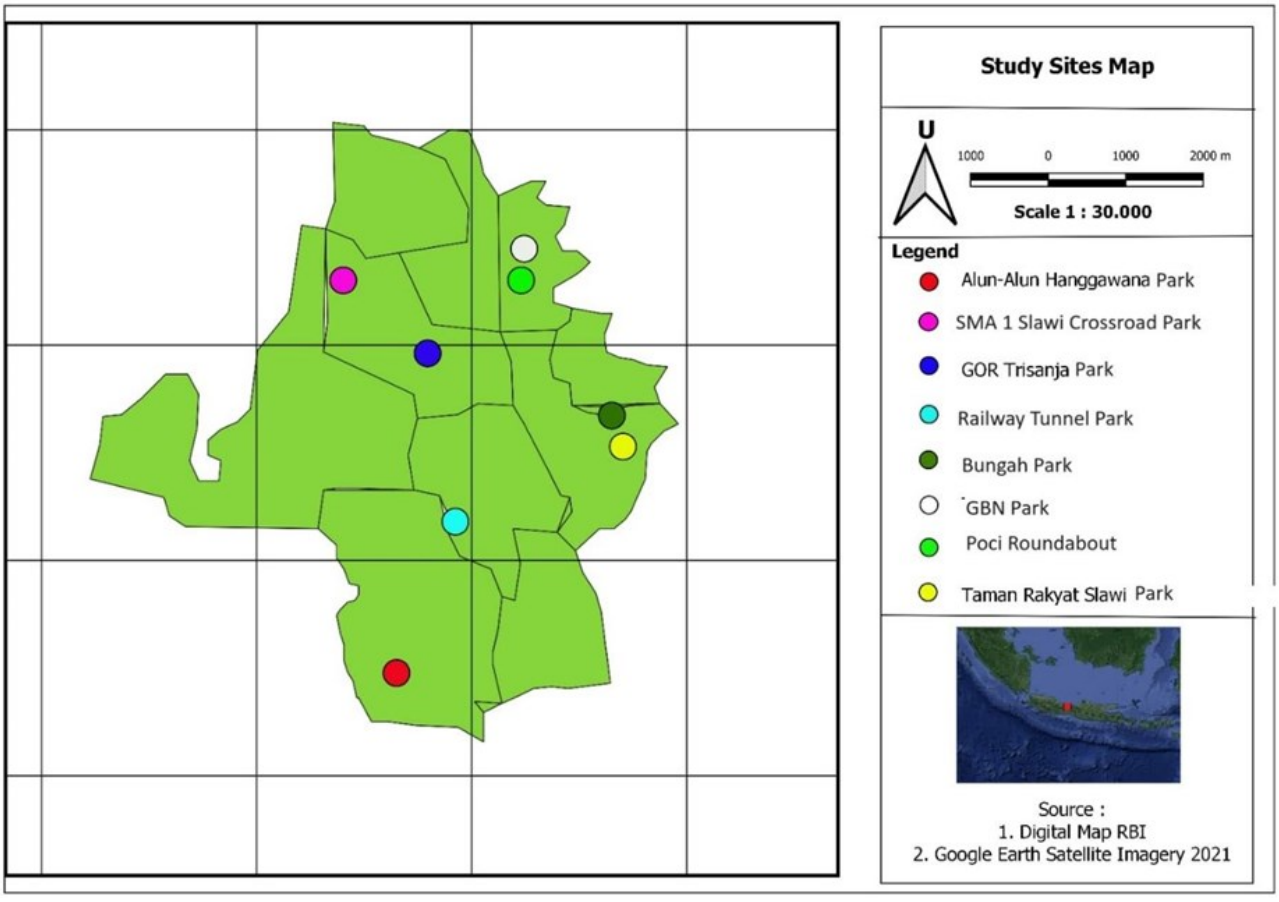

Figure 1. The study locations are at 8 urban parks in *Kecamatan* (Subdistrict) Slawi, *Kabupaten* (Regency) Tegal, Central Jawa.

Species encountered were arranged into a present-absent matrix against park names (Table 1). The matrix was then processed using clustering method in R 4.3.1 (R Core Team 2023). We used *Euclidean* for distance calculation, and *hclust* command in R for UPGMA dendrogram construction.

We obtained the exact area sizes of the parks from the official records deposited in the Tegal Regency Environmental Service. The Normalized Difference Vegetation Index (NDVI; Gessesse & Melesse 2019; Johansen & Tømmervik 2014) values for each park were calculated using QGIS 3.10 (QGIS.org 2023). Images of each park acquired from Landsat 8 of the year 2022 (USGS 2022) were made into raster layers. Data entried into QGIS was NIR (Near Infrared Reflectance) band spectral 5 and RED (visible red reflectance) band spectral 4. NDVI was obtained by calculating both bands in raster calculator using formula:

$$NDVI = \frac{(NIR - RED)}{(NIR + RED)}$$

To verify the NDVI result, we did an actual angiosperm census, covering the ornamental plants planted by park managements. All ornamental angiosperms living in each park were recorded carefully not to pass a single species. Large canopy arboreal species in the park such as *Magnolia champaca*, *Ficus benjamina* or *Dialium indum* or shrubby *Annona muricata* and *Wrightia religiosa* planted within a park (Appendix 1) may be important in determining park microhabitat for spontaneous fern species. However, this census did not cover the smaller spontaneous weedy angiosperms.

The NDVI values used in this study are the mean NDVIs, which were tested, along with ornamental angiosperm species number and park area sizes on Pearson's correlation and simple regression test against fern species numbers. Statistics tests were done in R 4.3.1.

RESULT AND DISCUSSION

Species number and habitat of urban Slawi ferns

Eight parks in Slawi, Tegal, Central Java, harbor 24 species belonging to 7 families of Leptosporangiate ferns (Table 1). No Lycophytes and Eusporangiate ferns were encountered. The 7 families in all parks are typical of urban environment, as no forest families such as Hymenophyllaceae (Proctor 2012) and Cyatheaceae (Lehnert et al. 2013) were found. All species and families encountered in Slawi is of the same species and families with other urban habitat in Java, as in Jakarta (Andayaningsih et al. 2013; Agatha et al. 2019). This indicates that urban Slawi park fern communities are unable to maintain continuity with its adjacent forest communities, as in neighboring Mount Slamet, about 50 km to the south, where fern species composition is much diverse (Budiana & Sukarsa 2012; Sungkono et al. 2012; Praptosuwiryo 2013; Sedayu et al. 2022). Ferns in urban Slawi perhaps stand as a distinct type of urban fern community different from forest community. In biogeography point of view, this means that urban Slawi, environment is highly managed according to anthropogenic needs that no forest species can immigrate and establish in eight parks, despite the ability of most ferns to disperse long distance using their airborne spores. In this sense, eight parks in Slawi, Tegal are justified to be defined as anthropogenic islands, with hypothetically, forests in the neighboring Mt. Slamet as the "mainland", where the genetic variability, i.e. spore rain of various fern species may be sourced.

Only 2 species found in all parks are terrestrial. As in parks, terrestrial habitat is reserved for managed ornamentals, trees, shrubs, and ground covers, it is logical that intensively managed parks harbor lowest

Table 1. Species, habitat, nativity and present-absent matrix of all fern species in 8 urban parks in Tegal.

NO	FAMILY	SPECIES	HABITAT	NATIVITY	GOR Trisanja Park	Poci Roundabout Park	Slawi GBN Park	Alun-Alun Hanggawana Park	Railway tunnel Park	Bungah Park	SMA 1 Slawi Crossroad Park	Taman Rakyat Slawi Park
1	Dennstaedtiaceae	*Microlepia speluncae (L.) T.Moore*	C	Native	1	0	0	0	0	0	0	0
2	Lygodiaceae	*Lygodium flexuosum (L.) Sw.*	A	Native	1	0	0	0	0	0	0	0
3	Nephrolepidaceae	*Nephrolepis biserrata (Sw.) Schott*	C	Native	1	0	0	0	0	0	0	0
4	Polypodiaceae	*Davallia solida (G.Forst.) Sw.*	B	Native	0	0	0	0	0	1	0	0
5	Polypodiaceae	*Drynaria quercifolia (L.) J.Sm.*	E	Native	1	1	1	1	1	1	1	1
6	Polypodiaceae	*Phymatosorus scolopendria (Burm.f.) Pic.Serm.*	D	Native	0	1	0	0	0	1	0	0
7	Polypodiaceae	*Pyrrosia longifolia (Burm.f.) C.V.Morton*	B	Native	1	0	0	1	0	0	0	1
8	Polypodiaceae	*Pyrrosia piloselloides (L.) M.G.Price*	B	Native	1	0	1	0	0	0	1	1
9	Polypodiaceae	*Pyrrosia stigmosa (Sw.) Ching*	B	Native	0	0	0	1	0	0	0	0
10	Pteridaceae	*Adiantum capillus-veneris L.*	C	Native	1	0	0	1	1	1	0	0
11	Pteridaceae	*Adiantum philippense L.*	C	Native	1	0	1	0	1	1	1	0
12	Pteridaceae	*Adiantum tenerum Sw.*	C	Alien (South America)	0	1	0	0	0	0	0	0
13	Pteridaceae	*Hemionitis tenuifolia (Burm.f.) Christenh.*	C	Native	1	1	0	0	1	1	0	0
14	Pteridaceae	*Pityrogramma calomelanos (L.) Link*	D	Alien (Central America)	0	0	1	1	1	0	0	1
15	Pteridaceae	*Pteris biaurita L.*	C	Alien (Central America)	1	0	1	0	0	0	0	1
16	Pteridaceae	*Pteris ensiformis Burm.f.*	D	Native	1	0	0	0	1	1	0	1
17	Pteridaceae	*Pteris tripartita Sw.*	C	Native	1	0	0	1	0	0	0	0
18	Pteridaceae	*Pteris vittata L.*	D	Native	1	1	1	1	1	1	1	1
19	Tectariaceae	*Tectaria angulata (Willd.) Copel.*	C	Native	1	0	0	0	0	0	0	0
20	Tectariaceae	*Tectaria siifolia (Willd.) Copel.*	C	Native	1	0	0	0	0	0	0	0
21	Thelypteridaceae	*Thelypteris dentata (Forssk.) E.P.St.John*	D	Native	0	0	1	1	0	0	0	0
22	Thelypteridaceae	*Thelypteris parasitica (L.) Tardieu*	C	Native	0	0	0	0	1	0	0	0
23	Thelypteridaceae	*Thelypteris subpubescens (Blume) K.Iwats.*	D	Native	1	0	0	1	1	1	0	1
24	Thelypteridaceae	*Thelypteris opulenta (Kaulf.) Fosberg*	A	Alien (Thailand)	1	0	0	0	0	0	0	0
Total	**7**	**24**			**17**	**5**	**7**	**9**	**9**	**9**	**4**	**8**

0= absent; 1: present; Habitat: A: Terrestrial; B: Epiphyte; C: Lithophyte; D: Terrestrial-Lithophyte; E: Epiphyte-Lithophyte

species of spontaneous vegetation, especially ferns. Furthermore, in competition with other spontaneous spermatophyte vegetation, ferns lower, because ferns intrinsically with inferior ecophysiological features, i.e., inefficiency in the two-stage life cycle (Watkins et al. 2007) and inferior vascular system (Watkins et al. 2010), compared to spermatophytic spontaneous vegetation. It is why, when spontaneous vegetation establishes in a suitable terrestrial habitat, ferns are less competitive compared to spermatophytes, hence lower species number in terrestrial habitat of a highly managed park.

Living on trees, the epiphytic lifestyle has provided less competition compared to terrestrial lifestyle. It is why more epiphytic species found in 8 parks (4 species). In Indonesia, fern species is second behind orchids as the most diverse vascular epiphytes (Böhnert et al. 2016), and sometimes number one exceeding orchids in succession environment as in Krakatau (Partomihardjo et al. 2004). Living on trees exposed epiphytes to low light and water scarcity. That is why only specialized species like orchids and ferns can live as epiphytes. And as lower competition occurs on trees, the diversity of epiphytic ferns is logically higher compared to the terrestrials.

Amongst all habitats, the lithophyte habitat has the highest fern diversity (11 species). As ferns are known as successive species, with the ability to thrive in difficult situations, it is not surprising that they are found in a higher number of species. This lifestyle also posed ferns on less competition with spermatophytes, as less spermatophytes are specialized in lithophyte lifestyle. Furthermore, we observe many ferns are found not only as (horizontal) lithophyte, but also as wall (vertical) lithophyte, meaning they anchored on a vertical stony substrate as sewer wall. This is double hindrance for the spermatophyte, as wall is not only stony -like substrate, but also permanent shade for the lithophytes. It is why many of our park ferns are found on rather dark and super humid sewer walls where no spermatophytes thrive. This phenomenon of ferns occupying man made solid substrate is not only in Indonesia, but also in India (Morajkar et al. 2015), and probably in other part of the world. Two other habitats are combination of terrestrial-lithophyte (6 sp.) and epiphyte-lithophyte (1 sp.). This confirms the importance of lithophytic lifestyle for ferns in competition with other spermatophyte spontaneous vegetation in urban setting, that the combination habitats always include lithophyte, and no combination as terrestrial-epiphyte encountered. Our finding may be similar to what was found in India where lithophytic ferns composed the most important life-forms, compared to other life forms, such as terrestrial, epiphyte and aquatic (Anjum et al. 2014).

We found 4 alien ferns (16.6%; Table 1) on our list of spontaneous ferns in eight parks in Slawi. Some introduced species as *Adiantum latifolium* (Muhaimin 2017), might easily established in urban green spaces including parks, however in low species number, as revealed by our observation. The reproduction pattern and ecophysiology of fern might be important in explaining why unlike in spermatophyte, where alien species is major contributor in urban species number (Gleditsch et al. 2023), alien ferns are not highly represented in this study.

Similarity between urban parks

The composition of fern species in each park is depicted in Figure 2., as a visualization of the data in Table 1. GOR Trisanja Park, the park with the most (17) species is placed on the basal most branch of the dendrogram. The second basal is Alun-Alun Hangawana Park, which harbors a similar number of species to Railway Tunnel Park and Bungah Park.

However, Alun-Alun Hangawana Park is placed second most basal in the dendrogram. This is due to the uniqueness of the park in harboring the only individual of *Pyrrosia longifolia* and *P. stigmosa*, which are absent in other parks. Both *P. longifolia* and *P. stigmosa* are epiphytic Polypods uncommon to urban habitats and encountered more in rural or forested area (Jannah et al. 2015; Salamah et al. 2020). This uniqueness is the reason why Alun-Alun Hanggawana Park is different from other parks, aside from GOR Trisanja park which species numbers exceed other parks. *Drynaria quercifolia*, a quite handsome epiphyte, is the most common species found in all parks and serves as the unifying species for all clusters.

Two major park clusters in Figure 2 are the cluster of [Taman Rakyat Slawi Park [Slawi GBN Park, SMA1 Slawi Crossroad Park]] and the cluster of [Poci Roundabout Park [Railway Tunnel Park, Bungah Park]]. Again, in these major clustering, species number is not the unifying factor, as the parks with lowest fern species (SMA 1 Slawi Crossroad Park and Poci Roundabout Park) are placed on a different cluster. Species composition is important in defining similarities between parks. This similarity analysis is important in terms of urban biodiversity management. Some wild species of ferns are quite handsome with ornamental potential and might be left alone in managed areas as additional ornamental plants. Similarity between parks might be a tool in defining which park is more impoverished or, alternatively, with unique species compared to others. This, along with species richness will serve an important recommendation to park managements on how to attract native vegetation to urban environment. In general, the occurrence of native plant species in an urban habitat will attract other native animal species; and native species in an urban habitat will suppress introduced alien species (Ossola & Niemelä 2017).

Relationship between NDVI, ornamental angiosperm species, area and fern species number

NDVI is a standardized measure in quantifying vegetation healthiness and has been used widely in conjunction with classical vegetation surveys. Several surveys have exhibited the correlation of NDVI with species richness, as in butterflies and birds (Seto et al. 2004) and in plants (Pau et al. 2012) including forest ferns (Oldekop et al. 2012). This can be interpreted as the higher the NDVI, which means the healthier the vegetation, the more species of plant live in the area, including ferns. This is not the case in ferns in Slawi's parks, as we found that there is no significant correlation between NDVI and fern species number (Table 2; Pearson r=0.691, p=0.058). This means that urban fern species number in Slawi parks is not determined by NDVI. Pau et al. (2012) reveals that while their research showed NDVI has positive correlation with the species number in Hawaiian dry forest species, they believe that the actual driver in determining species number are other abiotic factors as precipitation. This may also be the reason behind the non-significant relationship between NDVI and fern species number. However, this research does not propose any factor (as precipitation) possible as alternative factors.

The NDVI analysis is further confirmed by the i nsignificant Pearson's correlation (p=0.7) between the species number of angiosperms (Table 2, Appendix 1) planted in each park and the species number of ferns. It turns out that planted angiosperm species as the major associated species of fern community does not rule the species richness of the ferns in a park. NDVI is an indirect measurement of an area "greenness"; and when measured directly as the total richness of planted ornamental

angiosperms (Table 2), both measures are not significant correlates to the fern species richness. Indeed, our angiosperm survey did not cover total angiosperm species richness, as smaller spontaneous angiosperms, generally weedy species, were not covered. However weedy angiosperms are usually much smaller in posture compared to tree and shrubby angiosperm ornamentals which posture may determine the park microhabitat crucial, undoubtedly important for spontaneous fern community.

In our case, apparently the fern species number in Slawi parks is related to area, a major factor in island biogeography in determining species number. The Pearson correlation test for area versus species number is significant (Table 2; Pearson r=0.891, p=0.003), with simple regression values: R^2 =0.806, F(1)=24.907, p=0.002. This means that the area of a park is a determining 80.6% variance in a park species number, as plotted on Figure 3.

In island biogeography, isolation and area size are considered the most important drivers of the species number (MacArthur & Wilson 1967; Whittaker & Fernández-Palacios 2007). As we deliberately appoint our study areas in only a single *kecamatan* (subdistrict) (Figure 1), reducing the effect of isolation, we believe that our study is ideal in demonstrating the effect of urban anthropogenic island size to the diversity of spontaneous fern species, in urban environment. Anthropogenic islands are essentially biodiversity islands resulting from fragmentation of surrounding area into urban structure and infrastructure. Some urban specialists and rural or forest immigrants, including ferns, can utilize these islands and subsequently integrated into the park biodiversity. It is a general rule that the more species living in an urban habitat, the healthier the urban habitat is. In turn, healthier urban habitat will affect better

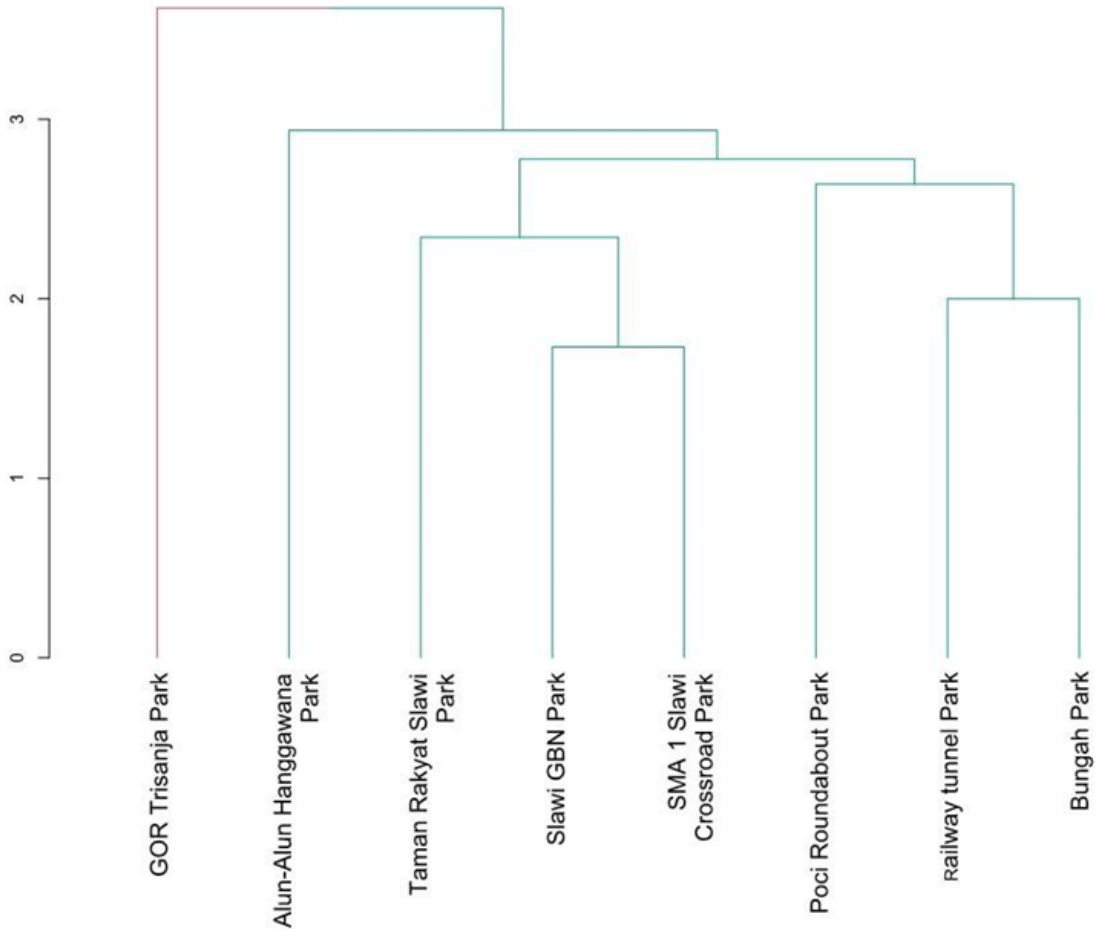

Figure 2. Park clustering based on fern species presence-absence.

human health and well-being (Brown & Grant 2005).

The relationship between area size and species number (Figure 3) explicitly illustrates the importance of park size in harboring biodiversity. We can see that on the left side where park sizes are smaller, the smaller number of ferns are observed. Conversely, at the furthest right point, GOR Trisanja Park which area is largest, harbors largest number of fern species.

It is interesting why NDVI, and further, the species number of ornamental angiosperms are not correlated to fern species richness as the area size. NDVI and ornamental angiosperm species are definitely measures of associated species cohabitate the spontaneous ferns in a park. Ferns in general need humid environment provided by other large trees, that a park with more plant species logically provides more niche for ferns. This may not be the case in urban parks, as there are areas managed and less managed (Sedayu et al. 2022). In the ornamental trees, bushes and shrubs are routinely managed. Pruning and cleaning may clear epiphytic gametophytes and young sporophytes from tree surfaces. It is why our census revealed more terrestrial and lithophytic ferns, independent of host tree habitat, compared to epiphytes. However, these terrestrial and lithophytic ferns may thrive in parks in areas less managed with suitable condition, especially humidity, such as man-made park's sewer wall or roofing. Indeed, study in forest environment revealed that tree species richness is not correlated to fern species richness, however tree species richness is correlated to epiphytic species richness (Williams-Linera et al. 2005).

Since our finding illustrates the importance of area size (compared to NDVI, the vegetation greenness), it is reasonable to propose that in managing urban green spaces, the size of a park or other green spaces be

Table 2. NDVI, park area (m2) and fern species number in 8 observed parks in Slawi.

NO	PARK NAMES	NDVI	Area (m²)	ANGIOSPERM SP. NOs	FERN SP. NOs
1	GOR Trisanja Park	0.331	74,174.81	6	17
2	Poci Roundabout Park	0.2139	4,400.00	12	5
3	GBN Park	0.2139	4,093.06	18	7
4	Alun-Alun Hanggawana Park	0.1687	15,426.18	18	9
5	Railway tunnel Park	0.2679	800	5	9
6	Bungah Park	0.3098	4,550.00	19	9
7	SMA 1 Slawi Crossroad Park	0.1337	228.02	6	4
8	Taman Rakyat Slawi Park	0.1309	4,000.00	7	8

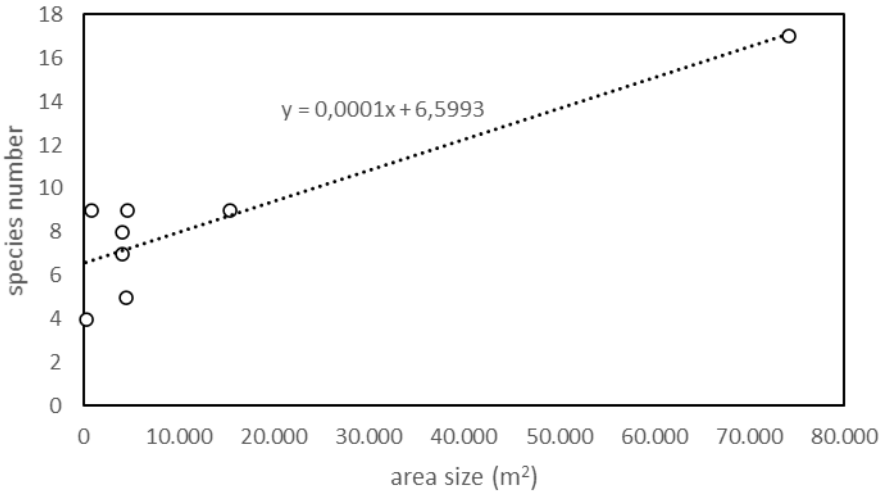

Figure 3. The area-species relationship of the fern community in 8 urban parks in Slawi. Trendline in dash line.

in a reasonable size to shelter many more urban spontaneous species. In Slawi, only 2 parks with size exceeded 1 ha (GOR Trisanja Park, 74,174.81 m^2 and Alun-Alun Hanggawana Park, 15,426.18 m^2), while a study advised that an affective park size in attracting biodiversity is about 1.08 ha (Yao et al. 2022). This is because, as a rule, the larger the anthropogenic island, as any other functional islands, the more species it can shelter.

CONCLUSION

The species of spontaneous ferns in 8 urban parks in Slawi are composed of 24 species. The species composition arranged in similarity clustering showed that species number and composition is important in defining parks uniqueness. The relationship between NDVI and species number is non-significant, while area-species is significant. This indicates that the fern species richness in urban parks is highly determined by the area size, as a biogeographical measure. To further invite more native biodiversity, it is advisable for park managements and designers to take account of species uniqueness and area size of a park into important design and management decision-making.

AUTHOR CONTRIBUTION

AS designed the research, wrote the manuscript, and supervised all the process. NP, A and MW collected field data, satellite imagery and literature collection. MIN is charged with data analysis, while LM is responsible for photo and specimen identification.

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CONFLICT OF INTEREST

We wish to declare that there is no conflict whatsoever among authors regarding this research and research funding.

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Appendix 1. The list of ornamental angiosperms in all 8 parks in Slawi, Tegal, Central Java.

No	Species	GOR Trisanja Park	Poci Roundabout Park	GBN Park	Alun-Alun Hanggawana Park	Railway tunnel Park	Bungah Park	SMA 1 Slawi Crossroad Park	Taman Rakyat Slawi Park
1	*Acalypha siamensis* Oliv. ex Gage	0	1	0	0	0	0	1	1
2	*Albizia chinensis* (Osbeck) Merr.	0	0	0	1	0	0	0	0
3	*Anacardium occidentale* L.	0	0	0	1	0	0	0	0
4	*Annona muricata* L.	0	0	1	0	0	0	0	0
5	*Averrhoa carambola* L.	0	1	1	1	0	0	0	0
6	*Bambusa bambos* (L.) Voss	0	0	0	0	0	1	0	0
7	*Bougainvillea spectabilis* Willd.	0	1	0	0	0	0	0	0
8	*Casuarina junghuhniana* Miq.	0	0	0	0	0	0	1	1
9	*Chrysalidocarpus lutescens* H.Wendl	1	0	0	1	0	1	1	0
10	*Dialium indum* L.	0	0	0	0	1	0	0	1
11	*Dracaena sanderiana* Mast.	0	0	1	0	0	0	0	0
12	*Erythrina crista-galli* L.	0	0	0	1	0	0	0	0
13	*Eugenia uniflora* L.	0	0	0	0	0	1	0	0
14	*Euphorbia tirucalli* L.	0	1	1	0	0	0	0	0
15	*Ficus benjamina* L.	0	0	1	1	0	1	0	0
16	*Handroanthus chrysotrichus* (Mart. Ex DC.) Mattos	0	0	0	0	0	1	0	0
17	*Heptapleurum arboricola* Hayata	0	0	0	0	0	1	0	0
18	*Hevea brasiliensis* (Willd.ex A.Juss.)	0	0	0	0	0	1	0	0
19	*Ixora* L.	0	0	0	0	0	1	0	0
20	*Lantana camara* L.	0	0	0	0	0	1	0	0
21	*Lilium superbu*m L.	0	0	1	1	0	1	0	0
22	*Magnolia champaca* (L.) Baill.ex Pierre	0	0	0	0	0	1	0	0
23	*Mangifera indica* L.	0	0	0	1	0	0	0	1
24	*Manilkara zapota* (L.) P.Royen	0	0	0	1	0	0	0	0
25	*Melaleuca leucadendra* (L.) L	0	0	1	1	0	0	0	0
26	*Mimusops elengi* L.	0	0	0	0	0	1	0	0
27	*Monoon longifolium* (Sonn.) B.Xue & R.M.K Saunders	0	1	1	1	1	0	0	1
28	*Mussaenda pubescens* Dryand.	0	0	0	0	0	0	1	0
29	*Nerium oleander* L.	0	0	0	0	0	0	1	0
30	*Phyllanthus acidus* (L.) Skeels	0	1	0	0	0	0	0	0

Appendix 1. Contd.

No	Species	GOR Trisanja Park	Poci Roundabout Park	GBN Park	Alun-Alun Hanggawana Park	Railway tunnel Park	Bungah Park	SMA 1 Slawi Crossroad Park	Taman Rakyat Slawi Park
31	*Plumeria rubra* L.	0	1	0	1	1	0	0	0
32	*Pometia pinnata* J.R.Forst.&G.Forst	0	0	1	0	0	0	0	0
33	*Psidium guajava* L.	0	0	1	0	0	0	0	0
34	*Pterocarpus indicus* Willd.	0	0	0	1	0	1	0	0
35	*Roystonea regia* (Kunth) O.F.Cook	0	0	0	0	0	0	0	0
36	*Ruellia tuberosa* L.	1	1	1	0	1	1	0	0
37	*Samanea saman* (Jacq.) Merr.	1	0	1	0	0	0	0	0
38	*Spondias dulcis* Parkinson	0	0	1	0	0	0	0	0
39	*Streblus asper* Lour.	1	0	1	1	0	1	0	0
40	*Swietenia mahagoni* (L.) Jacq	0	0	0	1	0	0	0	1
41	*Syzygium borbonicum* J.Gueho & A.J.Scott	0	1	0	1	0	0	0	0
42	*Syzygium cumini* (L.)	0	1	0	0	0	0	0	0
43	*Tabebuia heterophylla* (DC.) Britton	0	0	0	0	0	1	0	0
44	*Tabebuia rosea* (Bertol.) DC.	1	1	0	1	0	1	0	0
45	*Terminalia catappa* L	0	0	1	0	0	0	0	0
46	*Terminalia neotaliala* Capuron	1	1	1	1	1	1	1	1
47	*Wrightia religiosa* (Teijsm.&Binn.) Benth. Ex. Kurz	0	0	1	0	0	1	0	0
48	*Yucca gloriosa* L.	0	0	1	0	0	0	0	0
	TOTAL SPECIES	6	12	18	18	5	19	6	7
	Note: 0 = absent; 1 = present								

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Research Article

Diversity of Orchid species in the Tilu Mountains Region of Indonesia and the Potential for Phytochemistry

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ABSTRACT

Orchids are one of the largest and globally distributed plant families. Indonesia has the most types of orchids, estimated around 20% from the total species across the world. Mainly orchids used as an ornamental plant. This research aimed to elucidate another potential of orchids as possibly for herbal medicine plant. The potential of orchids as herbal medicine has been known for a long time, but there is lack of well-documented research. The research method used in this research is exploration on predetermined research sites that were conducted in Mount Tilu, West Java on 2022. Observation data of orchids were collected on the sites and the analysis was carried out in a qualitative descriptive approach by describing the data from the research results and comparisons were made through a literature review. Based on the research results, we found about 31 species from 28 genera consisting of 24 epiphytic orchids and 7 terrestrial orchids in the Mount Tilu Kuningan area, West Java. There are 4 endemic species categorised in Java; *Chilochista javanica*, *Crepidium koordesii*, *Crepidium junghuhnii*, and *Taeniophyllum biocellatum*.

INTRODUCTION

Orchids are one of the largest and globally distributed plant families (Chase et al. 2015; Christenhusz & Byng 2016; Zhang et al. 2022). Orchids are plants with a variety of shapes, colours and sizes so that these plants are very popular and are in great demand (Zhang et al. 2018; Tiwari et al. 2024). Until now the number of orchids in the world is 29,199 species that have been accepted (Govaerts et al. 2017), with several hundred new species that have been published every year, for example as many as 370 in 2013 and if estimated, the total number of orchids is about 31,000 species (Joppa et al. 2010). In Indonesia, orchid plants have the most types, namely around one fifth from the total species when compared to other types of flowering plants (Kusmana & Hikmat 2015), spread across nations including lowland and upland orchids (Yudaputra et al. 2024).

Generally, the use of orchids is usually used as an ornamental plant. In addition, orchids can also be used as decoration in the form of dried flowers and herbal medicine (De et al. 2014). The potential of orchids as herbal medicine has been known for a long time, but it is not very popular compared to ornamental plants (Sulistriarini 2008). The content of

alkaloids, flavonoids, glycosides, and other phytochemicals in orchids makes these plants have an important role as herbal medicinal ingredients (Jalal et al. 2008). Chinese society is known for the first-time using orchids as medicine. Then there is India, several countries in the Americas (Aztecs), Africa (Zulu), Europe (Greece), and Australia (indigenous people and Australian aborigines) also use orchids as medicine (Hossain 2011). The Batak Ethnic Community of North Sumatra (Karo, Simalungun, Toba) is known to also use orchids for traditional medicine (Aswandi & Kholibrina 2021).

On the island of Java, it is not known that there are publications that use orchids other than for the cultivation of ornamental plants, even though Java has many types of orchids. In the notes of Chomber (1990), states that the island has about 731 species with 231 endemics with a total spread in the West Java region of 642 species. Mount Tilu, Kuningan is an area in West Java which is known to have the potential to find orchids. The types of orchids on Mount Tilu had previously been collected by the Kuningan District Environmental Management Agency and the Indonesian Tropical Nature Institute in 2006, but only 5 species of epiphytic orchids were found (*Eria multiflora*, *Eria junghuhnii*, *Dendrochilum* sp., *Pholidota ventriculosa*, and *Appendicula pendula*).

MATERIALS AND METHODS

Research Geographical Sites

The research was conducted in the Mount Tilu Region to be precise in Cimara Village, Kuningan Regency, West Java in January 2022. Administratively, the Mount Tilu area is included in Karangkacana District and Cibingbin District. Located in the geographical position S 07° 06' 12.2"

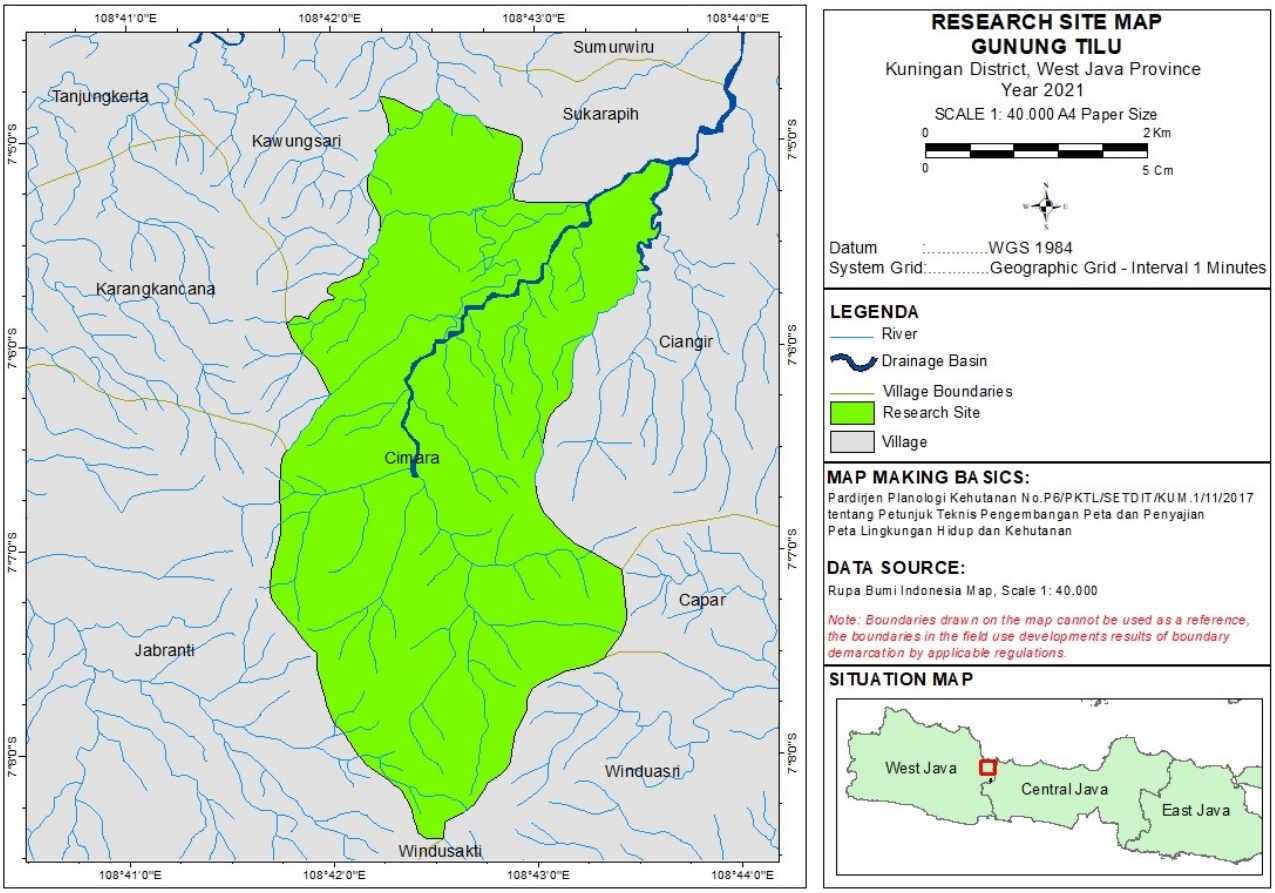

Figure 1. The research location of Mount Tilu, Cimara Village (VMJX+CM Cimara, Kuningan Regency, West Java).

and E 108⁰ 41' 47.7" to S 07⁰ 07' 48.1" and E 1080 41' 39.0" (Figure 1).

Mount Tilu is a group of mountains that has at least three highest peaks, namely Sukmana peak 1154 m, Mount Tilu peak 1076 m, and other peaks that are not known name 1112 m (Figure 2). The area consists of production forest (pine and teak) and secondary natural forest which is still a protected forest. Orchid data collection was carried out in both forest areas, which are production forest (Gagajahan Route) and secondary natural forest (Datarmuncang Route, Mungkal Bangkong Route, around Curug Manteng).

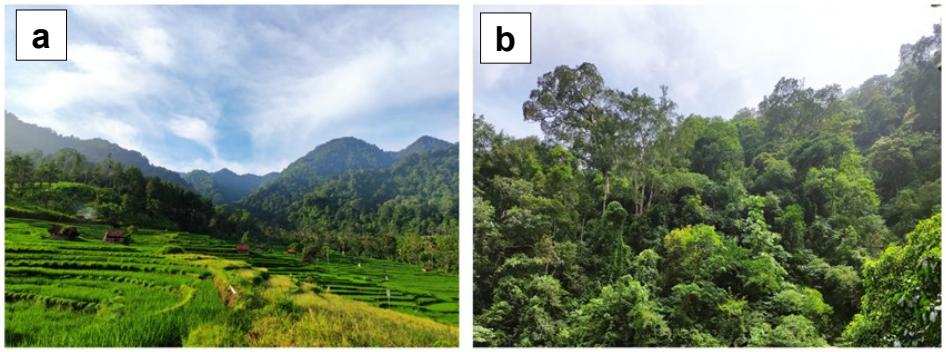

Figure 2. a). Mount Tilu have mountaintops; b). Secondary natural forest.

Materials

The tools and materials used in this research were documentation tools (Sony HX350 pro-summer camera), morphological measuring tools (ruler), GPS Locus Map, environmental parameter measuring tools (soil tester, thermometer, hygrometer, lux meter). The research exploration of orchids species in Mount Tilu were also use any equipment for human safety while tracing the mountain, such as special shoes, hat, special cloths, bag, and stick (Figure 3).

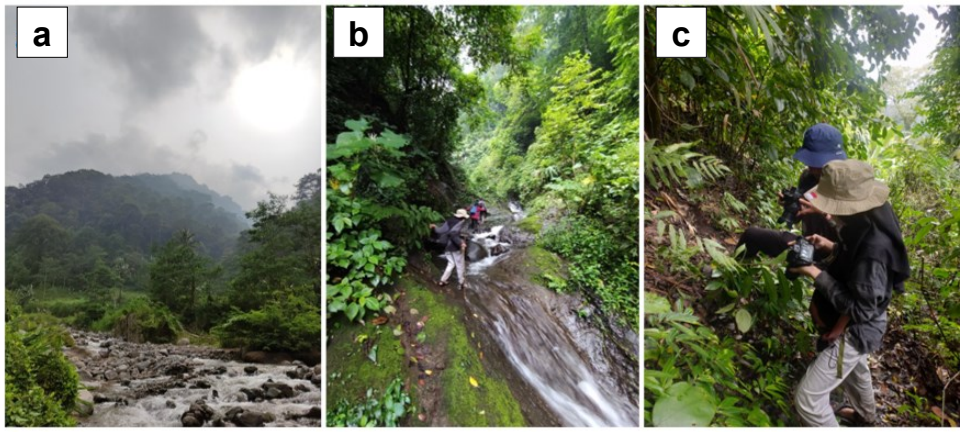

Figure 3. a) Research site around forest; b) Special wears and hat; c) Documentation tools used Sony HX350 pro-summer camera.

Methods and Data Analysis

The research method used is exploration on predetermined research paths. Data analysis was carried out in a qualitative descriptive manner by describing the data from the research results and comparisons were made through a literature review.

RESULTS AND DISCUSSION

The orchids on the Mount Tilu area

Based on the research results, there were 31 species from 28 genera con-

sisting of 24 epiphytic orchids and 7 terrestrial orchids in the Mount Tilu (Figure 4). Of all the species found, there are 4 species of orchids that fall into the endemic category of Java, namely *Chilochista javanica, Crepidium koordesii, Crepidium junghuhnii, dan Taeniophyllum bicelatum* (Table 1).

The results of a comparison of orchid finding data between a 2006 study by the Kuningan Regional Environmental Management Agency (BPLH) and the Indonesian Tropical Nature Institute (LATIN), the species found today are new species that have never been found before. However, 5 species of orchids (*Appendicula pendula, Eria junghuhnii, Eria multi-*

Table 1. Comparison of the findings of the types of orchids found in the Mount Tilu area.

No	Orchid Type	Habitat	2006	2022	Endemic
1	*Acriopsis liliifolia*	Epiphyte	-	1	-
2	*Aerides odorata*	Epiphyte	-	1	-
3	*Appendicula pendula*	Epiphyte	1	-	-
4	*Ascocentrum miniatum*	Epiphyte	-	1	-
5	*Bulbophyllum* sp.	Epiphyte	-	1	-
6	*Calanthe triplicata*	Terrestrial	-	1	-
7	*Chiloschista javanica*	Epiphyte	-	1	end. Java
8	*Coelogyne speciosa*	Epiphyte	-	1	-
9	*Coelogyne trinervis*	Epiphyte	-	1	-
10	*Cleisostoma discolor*	Epiphyte	-	1	-
11	*Dendrobium secundum*	Epiphyte	-	1	-
12	*Dendrobium setuarti*	Epiphyte	-	1	-
13	*Dendrochilum* sp	Epiphyte	1	-	-
14	*Eria junghuhnii*	Epiphyte	1	-	-
15	*Eria multiflora*	Epiphyte	1	1	-
16	*Flickingeria angulata*	Epiphyte	-	1	-
17	*Flickingeria* sp	Epiphyte	-	1	-
18	*Gastrochilus* sp	Epiphyte	-	1	-
19	*Geodorum densiflorum*	Terrestrial	-	1	-
20	*Habenaria reflexa*	Terrestrial	-	1	-
21	*Liparis barbata*	Terrestrial	-	1	-
22	*Liparis* sp	Epiphyte	-	1	-
23	*Luisia antennifera*	Epiphyte	-	1	-
24	*Crepidium junghuhni*	Terrestrial	-	1	end. Java
25	*Crepidium koordersii*	Terrestrial	-	1	end. Java
26	*Pholidota* sp	Epiphyte	-	1	-
27	*Pholidota imbricata*	Epiphyte	-	1	-
28	*Pholidota ventriculosa*	Epiphyte	1	-	-
29	*Polystachya concreta*	Epiphyte	-	1	-
30	*Rhyncostylis retusa*	Epiphyte	-	1	-
31	*Schoenorcis juncifolia*	Epiphyte	-	1	-
32	*Spathoglotis plicata*	Epiphyte	-	1	-
33	*Taenophyllum bicelatum*	Epiphyte	-	1	end. Java
34	*Vanda tricolor*	Epiphyte	-	1	-
35	*Zeuxine gracilis*	Terrestrial	-	1	-
36	*Zeuxine* sp	Epiphyte	-	1	-
	Amount		***5***	***32***	**4**

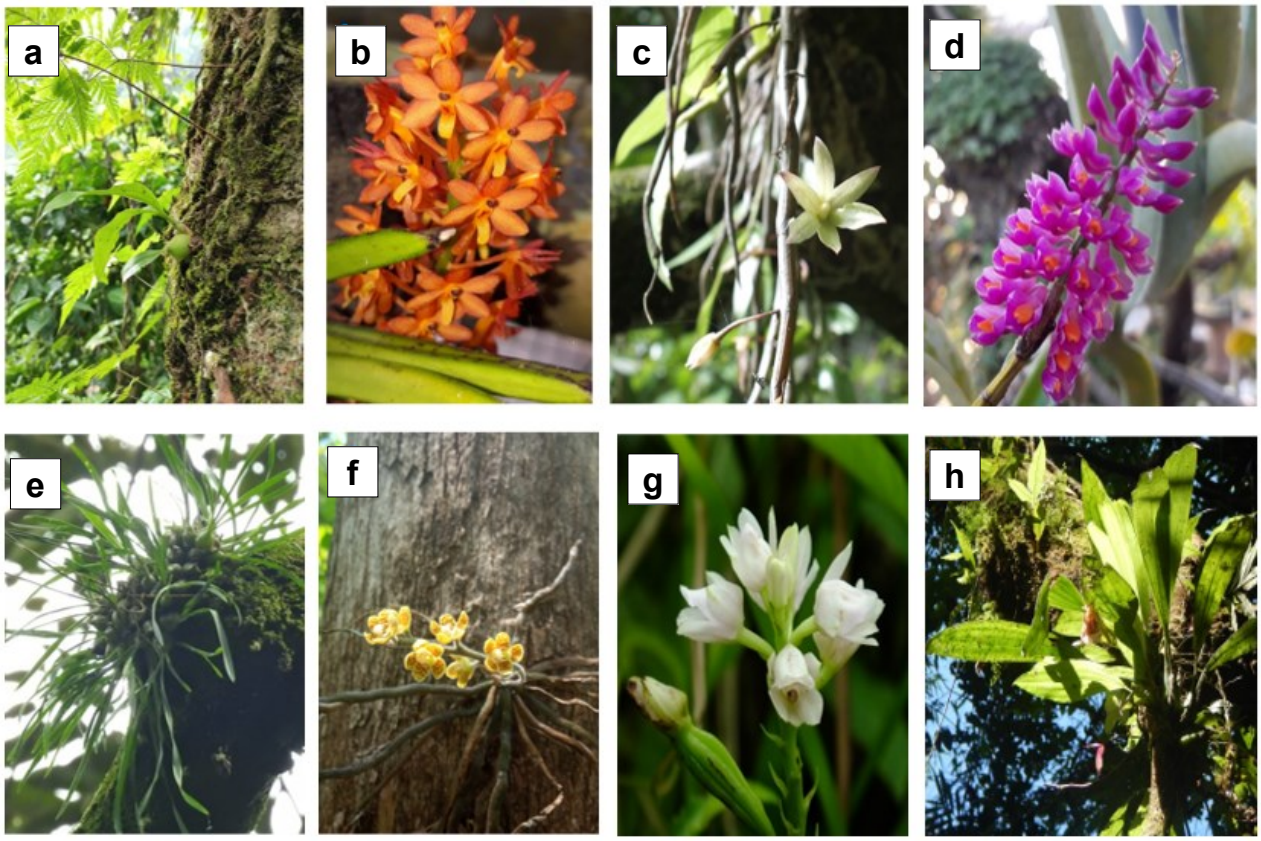

Figure 4. a). Orchids types in the Mount Tilu area; b). *Acrosentrum miniatum* c). *Dendrobium stuartii*; d). *Dendrobium secundum;* e). *Acriopsis liliifolia;* f). *Chilochista javanica*; g). *Geodorum densiflorum*; h). *Coelogyne speciosa*. (Source: Research exploration team 2022)

flora, and *Pholidota ventriculosa*) previously recorded were not found at all in the study site. This may have occurred due to differences in sampling locations (the research locations conducted by BPLH and LATIN were not specified), the observation area was not wide enough, the length of the study, or the species no longer existed at the study site.

Based on figure 5, of the 32 species of orchids found, the species that had a total count of >50 individuals, *Calanthe triplicata* (56 individuals), *Crepidium junghuhnii* (84 individuals), *Dendrobium stuartii* (67 individuals), *Flickingeria angulata* (343 individuals), *Flickingeria* sp. (64 individuals), *Pholidota* sp. (60 individuals). In addition, there are also several species that are found quite a lot like *Habenaria reflexa* (45 individuals), *Aerides odorata* (43 individuals), *Acriopsis liliifolia* (32 individuals), and *Zeuxine* sp (30 individuals). While the species with the least number of individuals were found among them *Ascocentrum miniatum*, *Chilochista javanica*, *Cleisostoma discolor*, *Coelogyne speciosa*, *Coelogyne trinervis*, *Crepidium koordesii*, *Geodorum densiflorum*, *Liparis barbata*, *Liparis* sp, *Schoenorcis juncifolia*, and *Spathoglotis plicata.*

Figure 6 shows the plots of orchids found at the study site. The distribution of orchids in the Mount Tilu area mostly tends to be well clustered epiphytic and terrestrial orchids, although some species are randomly distributed. There are three types of ecological distribution patterns of plants, namely clustered, random, and uniform (Borregaard et al. 2009). The distribution of clusters of orchids is caused by the environment that forms a microclimate for orchids to grow and reproduce. Usually at certain locations in an area, there are orchids found in large quantities and some are not. This is due to different environmental conditions within an area. The method of reproduction of orchids is also the cause of the clustered distribution pattern. Vegetative propagation of orchids uses

rhizomes or pseudobulbs which produce large numbers of new individuals and usually grow close to their parents. Apart from that generatively, orchids also produce thousands to millions of seeds. The existence of a host tree can catch the distribution of orchid seeds, so they don't fall too far and live close together (Kurniawan & Mustika 2021).

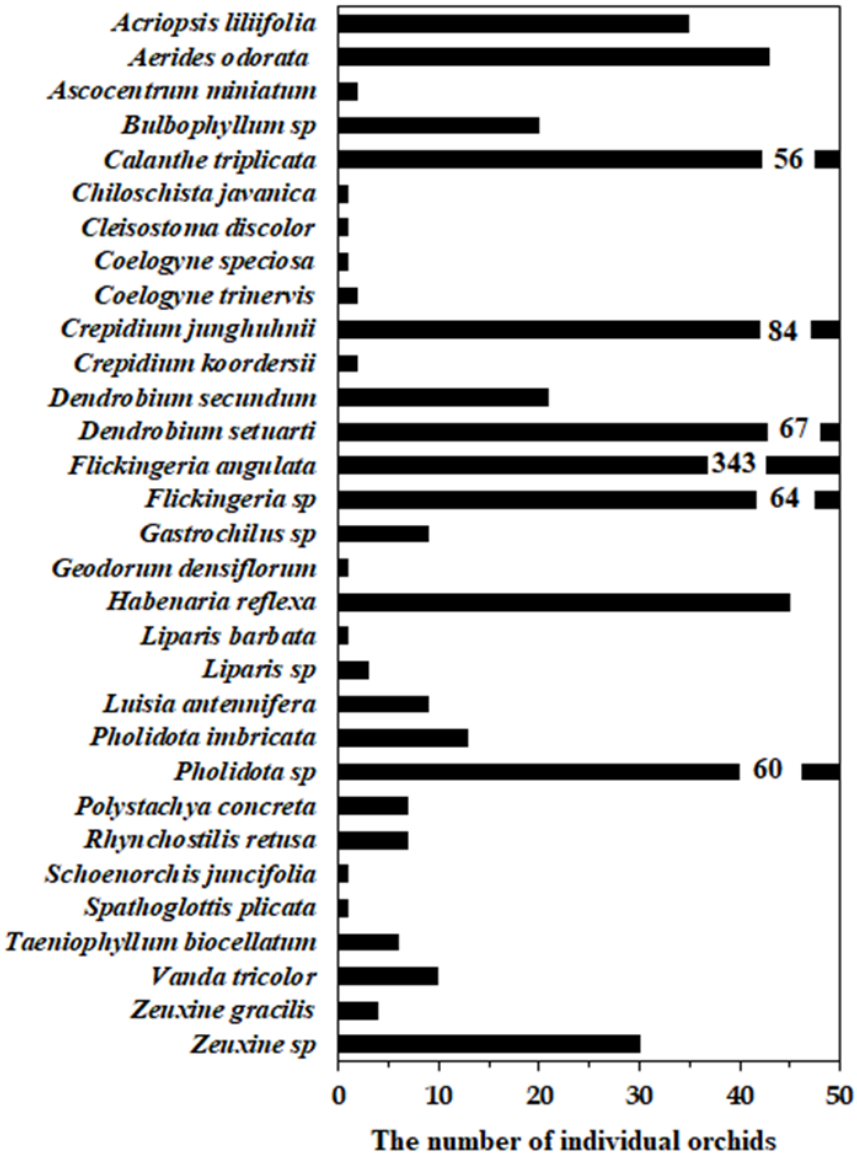

Figure 5. Number of individual orchid species in the Mount Tilu area.

When viewed based on the type of habitat, the proportion of found epiphytic orchids is greater (77%) than terrestrial orchids (23%) (Figure 7). This is because the condition of the host trees in the area has a large diameter with a dense canopy cover. Most of the epiphytic orchids are found in tree crowns, especially in large tree branches and in groups, although some are found singly. In addition, there are also those found on the main stem such *Coelogyne speciosa* dan *Dendrobium stuartii*. For other parts such as the base of the tree or the outermost branches, no epiphytic orchids were found. This is because at the base of the tree the condition is upright 90° making it difficult for the orchid to stick and get a little sunlight because it is blocked by the surrounding vegetation. Whereas the outermost branches will be exposed to high intensity direct sunlight, small branches, and the risk of being exposed to wind is also large, so they are not very suitable for orchid growth (Marsusi et al. 2001).

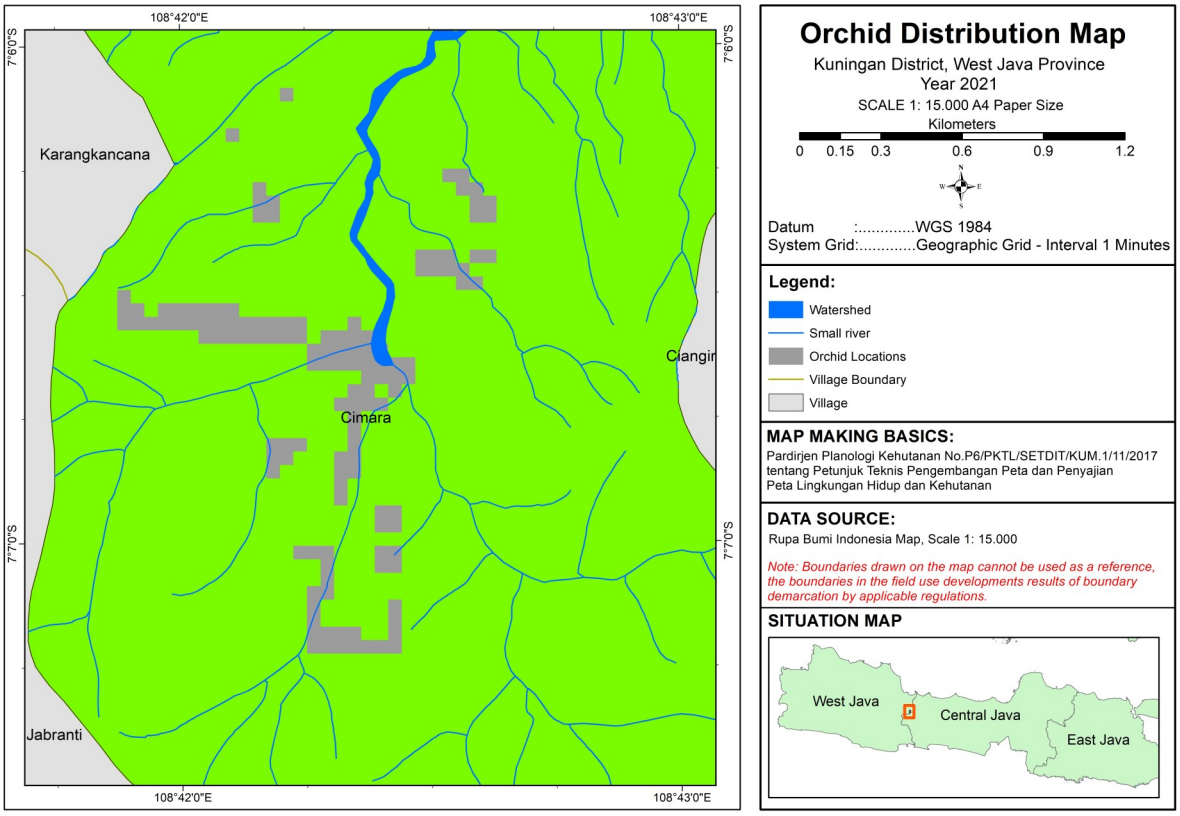

Figure 6. Map of orchid distribution findings in the Mount Tilu area, Kuningan, West Java shown with the grey colour.

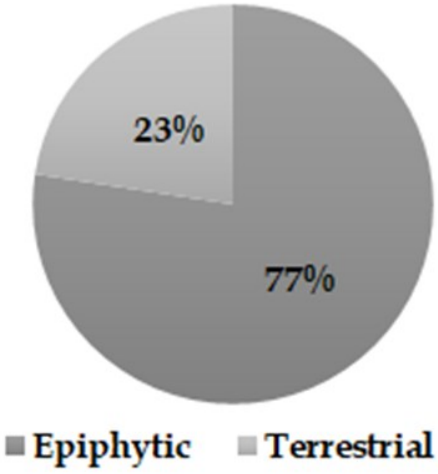

Figure 7. Comparison of the types of orchids found based on their habitat type.

The following are the types of host trees used by epiphytic orchids to grow and develop. The host trees found in the study area consisted of 10 species from 9 families (Table 2).

Each of these host trees, most of them have the characteristics of rough skin, cracks, easy to peel, and not too hard. Such tree conditions favour epiphytic orchids to grow. Rough tree bark occurs because the tree is able to hold water and humus in the cracks of the tree bark so that it becomes moist, making it suitable for the growth of orchids because these plants like high humidity. However, the roughness of tree bark is not a reference for the growth of epiphytic orchids, this is because there are several types of orchids that are able to live on smooth tree bark

(Bergstrom & Carter 2008; Yulia & Budiharta 2010). Like *Vanda tricolor* that grows on kedondong, *Acriopsis liliifolia* on durian trees, and *Pholidota imbricata* on a banyan tree. The three types of hosts at the study site appeared to have fairly smooth tree bark compared to other hosts. Then, the host trees that were found also did not have very dense canopies, so that was in accordance with the statement of Seitske et al. (2001), that orchids like trees that do not have too dense canopies so that the need for light will be fulfilled. In addition, several types of epiphytic orchids were also found on different types of hosts, indicating that these species have a high tolerance for different environments so that they are easy to adapt and grow on several types of hosts.

Table 2. Host tree species of epiphytic orchid habitat.

No	Family	Host Tree Type	Orchid Type
1	Anacardiaceae	*Spondias dulcis*	*Vanda tricolor*
2	Dilleniaceae	*Dillenia sp*	*Aerides odorata*
			Polystachya concreta
3	Flacourtiaceae	*Pangium edule*	*Aerides odorata*
4	Malvaceae	*Durio zibenthinus*	*Acriopsis liliifolia*
5	Meliaceae	*Swietenia mahagoni*	*Luisia antennifera*
			Taeniophyllum bicelatum
6	Moraceae	*Artocarpus heterophyllus*	*Ceologyne speciosa*
			Polystachya concreta
			Rhyncostilis retusa
7	Moraceae	*Ficus sp*	*Bulbophyllum sp*
			Flickingeria angulate
			Liparis sp
			Pholidota sp
8	Phyllantaceae	*Bischofia javanica*	*Flickingeria angulate*
			Luisia antennifera
9	Pinaceae	*Pinus merkusii*	*Airides odorata*
			Chilocista javanica
			Dendrobium stuartii
			Rhyncostilis retusa
10	Verbenaceae	*Tectona grandis*	*Pholidota imbricata*

Based on data on the composition of terrestrial orchids, the number found was very small, namely 8 species (Table 3). The condition of terrestrial orchids that were flowering at the study site facilitated the identification process at the species level. Although there are many orchids *Calanthe triplicata* found that are not flowering or in a state of fruiting. In addition to orchids *Habenaria reflexa* most of them are still in a state of budding, so you need to monitor them for a few days until the flowers bloom. The abundance of individual terrestrial orchids is very high and tends to cluster in one area in particular *Calanthe triplicata*, *Crepidium junghuhnii*, *Habenaria reflexa*, and *Zeuxine* sp (Figure 1). The clustering of terrestrial orchids in close proximity is due to the presence of stolons in the soil, so that new individuals grow not far from their parents

(Puspitaningtyas et al. 2003). Even so, the spread of ground orchids in a wide scope can also be through seeds.

Table 3. Terrestrial orchid habitat in Mount Tilu area.

No	Types of Terrestrial Orchids	Habitat
1	*Calanthe triplicata*	Soil litter
2	*Crepidium junghuhnii*	Soil litter and rocks
3	*Crepidium koordesii*	Soil litter
4	*Liparis barbata*	Soil litter
5	*Habenaria reflexa*	Soil litter
5	*Spathoglotis plicata*	Soil litter
6	*Zeuxine gracilis*	Soil litter
7	*Zeuxine sp*	Soil litter

The types of terrestrial orchids that are found are mostly in the shade of trees. But there are also orchids *Spathoglotis plicata* which can live with full light or no shade (Sadili & Sundari 2017), and these orchids are found on river banks with open environmental conditions. If you look at the specific conditions of their habitat, almost all of the terrestrial orchids that were found grew on soil litter except for *Crepidium junghuhnii* growing on large rocks (Table 3). The reason for terrestrial orchids being found and growing in soil with weathered litter content is that the source of nutrition for terrestrial orchids completely comes from the soil or litter. Meanwhile, the stone used to grow terrestrial orchids has moss, which causes high humidity, because the water is stored in the moss. Humid conditions will provide good water availability for terrestrial orchids or microorganisms associated with orchids (Tirta et al. 2010).

The richness of orchid species in the Tilu Mountain area cannot be separated from environmental influences such as humidity, temperature, pH, light intensity, and altitude. Measurement of environmental parameters was carried out in the time range 07.00-15.00 WIB. Based on the results of the average measurement of environmental parameters, this location is suitable for orchid growth (Table 4).

Table 4. Average measurement results of environmental parameters.

No	Environmental Parameters	Measurement Result (Average)
1	Soil moisture (%)	53
2	Humidity (%)	72
3	Temperature (^{o}C)	26
4	pH	5
5	Light Intensity (Lux)	2477
6	Place Altitude (m)	330-777

The average results of measuring soil moisture in the study area were 53% and 73% for air humidity. Orchids will grow optimally with 50 -80% humidity (Purwanto 2016). The results of temperature measurements were 26^{o}C. The ideal environmental temperature for orchid growth is 25-27^{o}C, with a minimum temperature of 21-23^{o}C. Daytime temperature 27-32^{o}C and night temperature 21-24^{o}C (Farokhah et al. 2018). Furthermore, for pH measurements of 5, where the ideal pH for orchids is 5-6 (Naik et al. 2014). For the measurement of light intensity

of 2,477 lux. The light requirements for orchids vary depending on the type. Some require direct light around 5000 lux, light (3000-5000 lux), medium (2000-3000 lux), and under shade (1000 lux) (Jacquemyn et al. 2007).

Based on the altitude, all types of orchids found on Mount Tilu are found at an altitude of 330-777 meters above sea level. Orchid diversity in Java is found at an altitude of 500-2000 m as much as 90%, in the lowlands 9%, and upland 1% (Yudaputra et al. 2024). Although at the research location orchid species were found at an altitude of 300 meters above sea level, these conditions indicate that orchids are not only found at an altitude of 500 meters above sea level. This is in accordance with the statement of Steenis (1975), that orchids can be found at altitudes below 500 meters above sea level or more than 2000 masl, and the diversity of orchid species will decrease. If viewed based on the influence of environmental parameters, it is clear here that these factors greatly influence the diversity of orchids.

Potential Utilisation of Orchids in the Mount Tilu Area

In Indonesia, the use of orchids is mostly for ornamental plants. Even internationally, orchids play an important role in the cut flower industry because of their attractiveness, long shelf life, high productivity, proper blooming season, easy packing and transportation. Orchids account for a large part of the global floricultural trade as cut flowers and as pot plants and are estimated to account for around 10% of the international trade in fresh cut flowers (De et al. 2014; Zhang et al. 2022).

If we look at the use of orchids as ornamental plants, these plants play a very important role in supporting the community's economy. Unfortunately, there are still many people who tend not to know the various types of natural orchids so that their use is very limited. As is the case with the local people on Mount Tilu, no one has used orchids either for economic needs, just for decoration, or for health. Communities tend to take advantage of more promising forest products such as wood, rubber latex, medicinal plants, or honey from bees. Public knowledge of natural orchids is also very minimal, this is evidenced by the fact that they do not know if there are terrestrial orchids. They know that orchids are plants that attach to host trees. But there was lack of public knowledge about orchids causes the utilization of orchids in the area is also still lacking.

Although all types of orchids in the Mount Tilu area have the potential to be ornamental plants, most of them have small flowers, the colours are not as attractive as *Acriopsis liliifolia*, *Cleisostoma discolor*, and *Polystachya concreta*. In fact, orchids must meet several criteria in order to sell well in the market to meet consumer tastes such as flower shape, size, color, resistance, number of flowers per stalk, and fragrance. Based on the results of the study, there are several types of orchids that have beautiful flowers, one of them *Vanda tricolor* (Figure 8). These orchids are very popular among lovers of ornamental plants, but now their number in nature is decreasing. While there are other types that have quite interesting flowers *Ascocentrum miniatum* popular enough to be commercialized, *Coelogyne speciosa*, *Coelogyne trinervis*, *Aerides odorata*, *Rhyncostilis retusa*, and *Calanthe triplicata*. As for *Chilochista javanica* (ghost orchid), this orchid is very unique because it has no leaves and the flowers are quite beautiful even though they are small. With the beauty of the flowers from these types of orchids, it has the potential to be developed as a hybrid (hybrid) and cultivated to fulfil the demand for ornamental plants.

In fact, the use of orchids is not only limited as ornamental plants, some are used as herbal medicine. In Indonesia itself, the Batak Ethnic

Figure 8. *Vanda tricolor*. (Source: Research team 2022)

Table 5. Potency of orchids on Mount Tilu as medicine.

No	Orchid type	Used Part	Treatment	Reference
1	*Aerides odorata*	Fruit, leaves	The fruit is mashed to heal wounds, the leaves are made into juice to cure ear and nose ulcers.	Tsering et al. 2017
2	*Acriopsis liliifolia*	Pseudobulb	Pseudo tubers are used as a febrifuge in treating malaria and raising blood pressure, the juice of the bulbs is used as ear drops	Sulistiarini 2008; Hossain 2011
3	*Calanthe triplicata*	Roots, flowers, pseudobulbs	The roots and flowers are used as an analgesic for diarrheic and cavities. Pseudobulbs are used in indigestion.	Jalal et al. 2010; Yonzone et al. 2011
4	*Geodorum densiflorum*	Roots and tubers	The root paste is taken before meals to promote menstruation in women, and applied to wounds or insect bites. The tuber extract is administered orally for intermittent fever in cattle. The root powder is given orally to goats for diarrheic symptoms.	Hossain et al. 2009
5	*Pholidota imbricata*	Pseudobulb	Pseudobulb juice is applied to relieve pain in the nose, stomach and rheumatism. Pseudobulb paste is used to reduce fever, pain and swelling during arthritis. And pseudobulb powder can be used as tonic	Vaidya et al. 2000; Baral & Kurmi 2006; Yonzone et al. 2011; Panda & Mandal 2013 ; Subedi et al. 2013
6	*Rhyncostilis retusa*	Root	The root is effective against rheumatism, asthma, tuberculosis, cramps, epilepsy, vertigo, kidney stones, menstrual disorders	Tsering et al. 2017
7	*Spathoglotis plicata*	Whole plant	A decoction of the plant is used for rheumatism and relieving internal heat.	De et al. 2014

community of North Sumatra also uses orchids as medicine. This is evidenced by the discovery of types of orchids that are traded in the main markets of Karo Regency, namely the Kabanjahe and Berastagi markets such as *Annoectochilus reindwardtii* (for fever, stamina enhancer, cancer, aphrodisiac), *Macodes petola* (aphrodisiac fever), *The nerves are folded* (aphrodisiac stomach ache), *Nervilia aragoana* (boils, fever, stamina enhancer), and *Dendrobium salacense* (stomach ache). The demand for these four orchids is high (except *Dendrobium salacense*), but its supply is low except *Nervilia Aragoana* moderate supply. In Kaban Tua Village (in Karo District) and Simbou Baro Village (Simalungan District) there are 5 species of orchids that are used as medicine, namely *Annoectochilus reindwardtii* (for fever medicine, stamina enhancer, aphrodisiac), *Godyera rubicunda* (diabetes mellitus, stamina enhancer), *Nervilia Aragoana* (fever, stamina enhancer), *Nervilia plicata* (fever, aphrodisiac), and *Phaius callosus* (diabetes mellitus). Whereas in Simbou Baro Village (Simalungan Regency) there are 2 species of orchids that are used as medicine namely *Dendrobium salacense* (abdominal pain) and *Macodes petola* (cancer, fever). The same thing happened to the people of Toba, North Sumatra, who used orchids as traditional medicine (Silalahi & Nisyawati 2015; Aswandi & Kholibrina 2021). Meanwhile in Sanggau Regency, West Kalimantan, it is known that *Plocoglottis lowii* it is also used as a medicine to detoxify various poisons in the body including psychotropic poisons (Normagiat et al. 2018).

Based on the explanation above, the potential for exploiting the wealth of orchids in the Mount Tilu area is actually unlimited. Utilisation for ornamental plants also has great potential to improve the community's economy. Although most types of orchids do not meet the criteria to meet consumer tastes, advances in hybridization technology, domestication and genetic engineering can increase the number and quality of orchids (Sadili & Sundari 2017). In addition, the potential for orchids on Mount Tilu to be used as medicine needs to be taken into account even though only a few types of orchids have been proven as medicines.

Seeing the potential utilisation of orchids in Mount Tilu, conservation activities should be carried out. Although all orchid species found are not included in the IUCN, CITES, and Indonesian Law No. P. 106 on protected plants and animals. With more than 28,000 species in the world, orchids are the second largest family after the most threatened Asteraceae (Chase et al. 2003; Chase et al. 2015). To date, there are 1098 orchid species listed on the IUCN Red List, and 48.7% of these are categorised as threatened (endangered 456 species, critically endangered 259 species, extinct 6 species, and vulnerable 240 species) by IUCN. So, the threat of orchid orchids does not only occur in Indonesia but also globally.

The threat is greatest due to habitat damage and climate change, but many orchids are also threatened due to wild harvesting for horticultural, food and medicinal purposes. Therefore, we need to take a possible approach to address threats on a broad scale. According to Fay (2018), orchid conservation efforts can be done by understanding orchid biology such as: 1. Systematics and genetics studies to identify and determine conservation priorities; 2. Conservation of habitat, e.g., host trees and physical conditions of the environment; 3. Understand pollinators and orchid pollination; 4. Understand the association with mycorrhiza, for the growth of orchid seeds. In addition, it can also control illegal harvesting for trade and carry out tissue culture propagation or conventional cultivation. Documenting and collecting orchids also help in contributing databases to conservation plans. Some of these efforts can be realized well if

elements of society and government support each other to make conservation efforts so that the orchid will not become extinct in the future.

CONCLUSION

Based on this research, we found 31 species from 28 genera consisting of 24 epiphytic orchids and 7 terrestrial orchids in the Mount Tilu Kuningan area, West Java. Furthermore, regarding the potential use of orchids on Mount Tilu, conservation activities should be carried out, although all types of orchids found are not included in the red list of IUCN, CITES, and Indonesian Law No. P. 106 concerning protected plants and animals. The use of orchids should be done wisely by carrying out conservation efforts so that these orchids will not become extinct in the future.

AUTHORS CONTRIBUTION

All authors have contributed equally to complementing this research. The contributions of each author are: BP designed, writing, collected, and analysed the data. TR and MLK assisted with collecting and analysing the data. AA carried out data processing using mapping analysis. DA assisted in the collection of data. LN assisted in all the collection data, writing, revised of the manuscript.

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CONFLICT OF INTEREST

There is no conflict of interest in this research.

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Research Article

Characterization of Flower's Color based on *CHS* Gene Structure in *Phalaenopsis* 'OX Queen' and *Dendrobium* 'Cheddi Jagan' Orchids

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ABSTRACT

Orchids (Orchidaceae) are ornamental plants known for their high aesthetic value attributed to the shapes, colours, and fragrances of their flowers. Two types of hybrid orchids with attractive flowers, namely the *Phalaenopsis* 'OX Queen' orchid and the *Dendrobium* 'Cheddi Jagan' boast attractive flowers were used in this research, because of the beauty of its flower colour. The objective of this research is to characterise the morphology of flower colour and *CHS* (*Chalcone Synthase*) gene content that induces flower colour. The method used in this research analyzing the flower's colour by using the RHS (*Royal Horticultural Society*) colour chart and molecular analysis by DNA genomic isolation and PCR amplification of gDNA for *CHS* gene specific primers. The results showed that purple colour is observed through the RHS, with *P.* 'OX Queen' coded as Deep Purple Pink (N73A) and *D.* 'Cheddi Jagan' coded as Strong Reddish Purple (N72C). The *CHS* gene can be amplified in *P.* 'OX Queen' 1,287 bp and *D.* 'Cheddi jagan' 3,731 bp. In both orchids, the results of amplification showed CHS motifs with conserved domains PLN03172 and PLN03170. The research results show that there is a significant difference in the morphology of the flowers of orchids. Purple colour is observed through the RHS, with *P.* 'OX Queen' coded as N73A and *D.* 'Cheddi Jagan' coded as N73C. The results showed that gDNA can be isolated by using CTAB method according to Murray and Thomson, and the *CHS* gene can be amplified by using CHS primers, resulting 1200 bp of *P.* 'OX Queen' and 2500 bp for *D.* 'Cheddi Jagan'. Through this study, preliminary data is expected to be obtained for future research, which is the formation of variegated flowers through editing the CRISPR/Cas9 genome in the *CHS* gene. This research is intended to support further studies on the formation of variegated flower patterns in *P.* 'OX Queen' and *D.* 'Cheddi Jagan', focusing on the *CHS* gene using CRISPR/Cas9 technique.

INTRODUCTION

Orchids are ornamental plants that have high diversity. In Indonesia, orchid diversity it's about 23% of the total of 30,000 orchid species in the

world and it is estimated around 5,000 species (Karoy et al. 2022). *Phalaenopsis* and *Dendrobium* orchids are two types of orchids that captivate attention with the beauty of their flower morphology (Semiarti et al. 2020) and widely used as parents of hybrid orchids (Li et al. 2021). The uniqueness of the orchid is the shape, colour and aroma of their flowers. *P.* 'OX Queen' is an orchid hybrid, resulting from a cross between *P.* 'Brother Success' as the female parent and *P.* 'Plum Rose' as the male parent (Royal Horticultural Society 2024a). Based on the Orchid Roots recognized by the Royal Horticultural Society, *D.* 'Cheddi Jagan' is also an orchid hybrid, resulting from a cross between *D.* 'Cheong Chee Yon' as the female parent and *D.* 'Genting Rose' as the male parent (Royal Horticultural Society 2024b). Hybrid orchids are popular because they have beautiful colours and are commonly used as decorative flowers both indoors and outdoors. *P.* 'OX Queen' and *D.* 'Cheddi Jagan' orchids were used in this study because both of them have plain purple flowers which will then be carried out *CHS* gene analysis on both orchid flowers.

Flower's colour is one of the things that causes plants to be attractive and in demand, the colour formed in this is produced through the biosynthesis of pigments which include anthocyanins, carotenoids and betalains. The *Chalcone Synthase* (*CHS*) gene encoded an enzyme responsible for initiating the flavonoid biosynthesis pathway in plants. CHS enzyme is a key in the flavonoid biosynthetic pathway that determines colour which contributes to flower petal colours, against UV radiations, attract pollinators, as well as plant growth and development (Kong et al. 2020; Jia et al. 2023). Flavonoids are responsible for pigmentation in flowers and fruits from orange to pink, red, violet and blue. The biosynthesis of flavonoids starts with the condensation of one molecule of p-coumaroyl-CoA and three malonyl-CoA molecules, which is catalyzed by CHS to produce naringenin chalcone (Sun et al. 2015). In sepals, petals, and flower labellums, anthocyanin pigments are the most commonly found pigments, anthocyanin pigments are divided into three colour groups such as pelargonidin which produces orange or red, cyanidin which produces pink, and delphinidin which produces purple or blue (Grotewold 2006; Pratama et al. 2023). Several studies related to the *CHS* gene such as on *Malus* Crabapple (Tai et al. 2014), *Paeonia suffruticosa* (Zhou et al. 2011), *Dahlia variabilis* (Ohno et al. 2018), *Phalaenopsis* orchids (Han et al. 2006) (Kuo et al. 2019), and many more. Research on the *CHS* gene in orchids was previously carried out by Linggabuwana et al (2024) was the inspiration for this research. The beautiful flower's colour of this hybrid orchid has sparked interest among researchers in characterization of the *CHS* gene which codes for flower coloration and can construct sgRNA that will be used in genome editing. In future research it is hoped that researchers can change the colour of orchid flowers from plain flower colours such as pink or purple to become variegated flowers on the marginal part, then the uniqueness of this flower can be developed on an industrial scale, so that it can increase its economic and aesthetic value.

Regarding identification, digital image processing techniques (Putra 2021) can be employed to accurately identify various orchid types based on the texture of their flowers. Molecular analysis serves as a complementary tool to morphological analysis, aiding in the development of new orchid varieties within plant breeding programs (Arif & Ratnawati 2018). Genetic research on orchids holds the potential to unveil valuable information concerning genetic diversity, floral development, and the species adaptations to their environment. The isolation of DNA from orchid flowers has become essential in understanding the genetic and bio-

logical aspects of the abundant orchid species in Indonesia. Furthermore, this molecular analysis using plant DNA supports increasingly important genetic conservation efforts, particularly in light of several orchid species being threatened by environmental changes and human activities. Currently, various DNA isolation techniques have been developed, tailored to the unique characteristics of orchids, such as their robust cell walls and specific chemical components within floral tissues (Arif & Ratnawati 2018). Research on the isolation of orchid flower DNA is crucial for comprehending the most efficient and reliable methods for obtaining high-quality DNA samples from various orchid species. The two main stages of flowering are called flower initiation and flower development. The onset of flowers is thought to be regulated by a number of biochemical and physiological processes. The growth of flowers involves the establishment of the different floral elements, which are represented in changed and carefully controlled expression of genes resulting in the development of many flower-specific mixtures (Herdenberger et al. 1990).

This research will study the experimental procedures carried out about characterisation of flower phenotypes and genotypes from *P.* 'OX Queen' and *D.* 'Cheddi Jagan'. Through this study, preliminary data is expected to be obtained for future research, which is the formation of variegated flowers through editing the CRISPR/Cas9 genome in the *CHS* gene.

MATERIALS AND METHODS

Materials

Plant Materials

Orchid plant that were used in this research were *P.* 'OX Queen' orchid flower and *D.* 'Cheddi Jagan' orchid Nambangan Orchid nursery owned by Hasan Sulaiman, S.P., located at 43 Telaga Warna Street, Rejowinangun, Nambangan, North Rejowinangun, Central Magelang Subdistrict, Magelang City, Central Java.

Morphological Analysis

Writing ruler (smallest scale is 1 mm), Canon SX430 IS camera (Japan), and identifying flower color based on the RHS Color Chart.

Genomic DNA Extraction

Chemical materials for isolation of plant genome DNA were 3% CTAB (containing 1 M Tris HCl, 0.5 M EDTA, 3% CTAB powder, isopropanol, isoamyl alcohol, NaCl, and pure water), PVP 1%, chloroform, absolute ethanol, 70% ethanol, TE buffer pH 8. The ingredients for the PCR reaction are 2x MyTaqTM HS Red Mix (Bioline), KOD FX Neo kit (Toyobo), pure water, specific primers for *Actin* and *CHS* genes for forward and reverse, presented in Table 1. below, and genome DNA of *Phalaenopsis* 'OX Queen' and *Dendrobium* 'Cheddi Jagan'.

Gene Amplification

The amplification of the orchid *P.* 'OX Queen' genome DNA was conducted using the *PaCHS2* primers. Meanwhile, the genome DNA of *D.* 'Cheddi Jagan' was amplified using *DcCHSII1* primers. The chemical materials for electrophoresis are agarose gel powder, TAE 1X, 6X loading dye (Geneaid), 100 bp DNA ladder (Geneaid), 1 kb DNA ladder (Geneaid), ddH2O, RedSafe™ Nucleic Acid Staining Solution (Intron Biotechnology), and visualised with tools UV transilluminator (Extragene UV3CL, Taiwan).

Table 1. List of primer used in this research

Primer	Sequence
PaCHS2 F	5'-AGATCTTTGCAATAATTTTAAAAAAAAATTC-3'
PaCHS2 R	5'-GTTTTATTCGAGGCTGAGTTTG-3'
DcCHSII1 F	5'-AAATAGCTGCCCACGCTCTTG-3'
DcCHSII1 R	5'-GCAAAAAAGATTCATAAGACTTCTTTATTA-3'
Actin F	5'-GTATTCCCTAGGATTGTTGGT-3' (accession number AY134752) (Semiarti et al. 2007)
Actin R	5'-CAGAGTGAGAATACCTCGTTTG-3' (accession number AY134752) (Semiarti et al. 2007)

Methods

Flower Morphological Observation

Three samples of *P.* 'OX Queen' and three samples of *D.* 'Cheddi Jagan' which has purple colours were used in this study. To observe orchid morphology, a writing ruler (smallest scale was 1 mm) is used to measure orchid habitus, size of roots, leaves, and flowers. An RHS Colour chart is used to determine flower colour codes, and a Canon SX430 IS camera (Japan) is used for documentation.

Genome DNA Extraction

The isolation of *P.* 'OX Queen' and *D.* 'Cheddi Jagan' flower genomic DNA was performed using a modified version of the Murray and Thompson method (Murray & Thompson 1980) using isopropanol. The DNA isolation results of *P.* 'OX Queen' and *D.* 'Cheddi Jagan' were visualized using 1% gel electrophoresis using the Mupid-exU Submarine electrophoresis system (Japan).

Amplification of *CHS* Gene

The results of DNA isolation were used as a template for amplifying the *CHS* and *Actin* gene sequences using the Polymerase Chain Reaction (PCR) method. The *CHS* gene was used to detect anthocyanin pigments (Linggabuwana et al. 2024), and the *Actin* gene was used as an internal positive control (Zhao et al. 2012). The composition of the reagents in the PCR process is presented in Table 2.

Table 2. Reagent Components for PCR Reaction of *CHS* and *Actin* gene.

Reagent Component	Volume (µL)
Genome DNA (ng/µL)	2
2x PCR Buffer (mM)	12.5
Forward primer (10 pmol/µL)	0. 75
Reverse primer (10 pmol/µL)	0. 75
ddH₂O (µL)	3.5
dNTPs (mM)	5
KOD Fx Neo (units/µL)	0.5
Total	25

The PCR process for the *PaCHS2* and *DcCHSII1* gene was carried out with a protocol of 30 cycles, pre-denaturation at 94°C for 2 minutes, denaturation at 98°C for 30 seconds, annealing temperature based on optimization results *P.* 'OX Queen' at 59°C, *D.* 'Cheddi Jagan' at 57°C and *Actin* at 51°C for 30 seconds, extension at 68°C time based on optimiza-

tion results *P.* 'OX Queen' for 40 seconds, *D.* 'Cheddi Jagan' for 2 minutes and *Actin* for 5 seconds and hold at 4°C.

Data Analysis

Phenotypic analysis was carried out by observing plant morphology including the flower's colour of the orchids. Genotype analysis was carried out on the *CHS* gene sequencing results of the orchids using bioinformatic tools that are the NCBI BLAST (BLAST: Basic Local Alignment Search Tool (nih.gov)) is used to align genome DNA obtained with the database, NCBI Conserved Domain Search (NCBI Conserved Domain Search (nih.gov)) is used to view conserved domains in both orchid sequences. The *CHS* gene motif can determine using the website Prosite Expasy (Expasy - PROSITE), Prosite Translate (Expasy - Translate tool) and Biomodel Sequence Massager (Sequence Massager (uah.es)) as well as the MultAlin (http://multalin.toulouse.inra.fr/multalin/) was used to align orchids sequences, ApE and UGENE applications are used to align orchids sequences with reference sequences and mRNA to determine the location of exons and introns in both orchids.

RESULTS AND DISCUSSION

Morphological Character of *Phalaenopsis* 'OX Queen'

Morphological observations of *P.* 'OX Queen' as displayed in Figure 1, there are 10 orchid flowers in a single cluster, predominantly displaying purple hues and accentuated by white edges along their margin. This is in accordance with van Tongerlo et al. (2021), that the *Phalaenopsis* orchid is famous for its enchanting beauty and abundant flowers. Each stalk has the potential to produce between 5 and 10 flowers simultaneously, with each flower lasting up to 3 months. This figure indicate that this orchid plant exhibits monopodial stem growth with a plant size of approximately 15-17 cm. The leaves of *P.* 'OX Queen' are dark green and lance-shaped, with a length of approximately 7-25 cm and a width of about 4-10 cm. The flowers are round with slightly overlapping petals and sepals. Fully bloomed flowers measure around 10-15 cm in length and 7-8 cm in width, displaying a plain purple color.

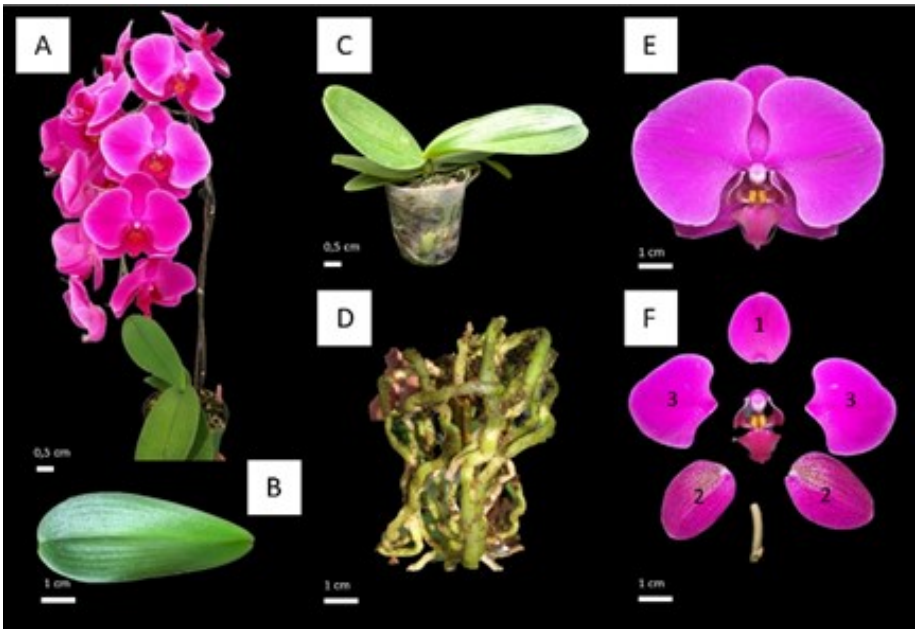

Figure 1. Habitus of *P.* 'OX Queen' hybrid. A= Habitus of Reproductive stage of *P.* 'OX Queen'; B= Leave; C= Vegetative Stage; D) Roots; E= Flowers from the front view; F= Flower parts consists of dorsal sepals (1), lateral sepals (2) and petals (3).

Morphological Character of *Dendrobium* 'Cheddi Jagan'

Morphological observations of *D.* 'Cheddi Jagan,' as presented in Figure 2, reveals that this orchid plant exhibits sympodial stem growth with a plant size of approximately 20-25 cm. The leaves of *D.* 'Cheddi Jagan' are dark green and lance-shaped, with a length of approximately 7-15 cm and a width of about 4-5 cm. Typically, each *D.* 'Cheddi Jagan' plant has four pseudobulbs, each measuring around 10-13 cm in height. The flowers are oval-shaped with slightly overlapping petals and sepals, and their texture resembles velvet or velvety material. Fully bloomed flowers measure approximately 7-8 cm in length and 5-6 cm in width, and they are of a plain purple color. The pollen is yellow with a white operculum. De et al. (2015) states that the *Dendrobium* genera have sympodial characteristics and have pseudobulbs, these pseudobulbs can be long, short or swollen. The inflorescences are terminal or subterminal with various sizes and colours, this is what makes the *Dendrobium* orchid a popular orchid used as a decorative flower indoors, as a plant in a pot, or as a cut flower.

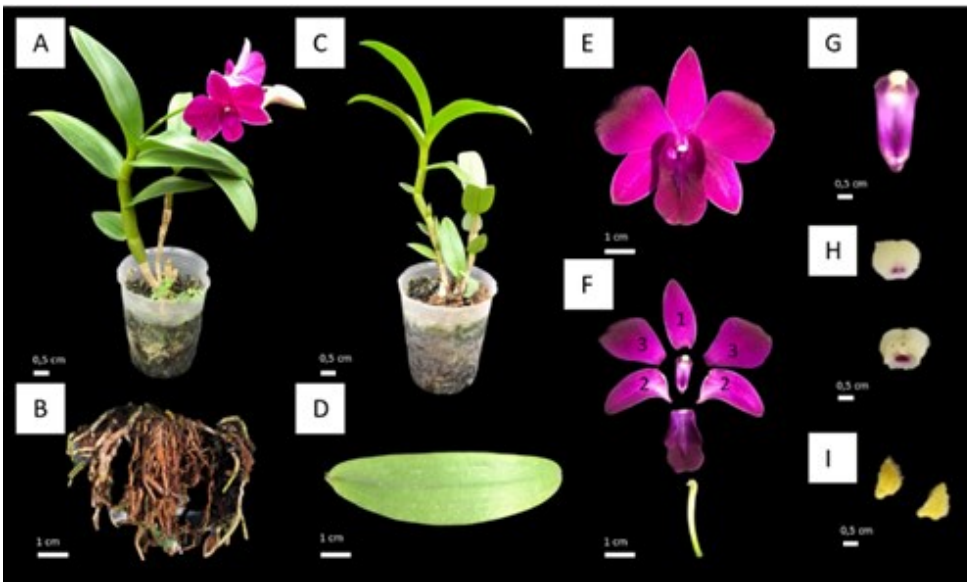

Figure 2. Habitus of Reproductive stage of *D. 'Cheddi Jagan'*. A= Habitus *D.* 'Cheddi Jagan'; B= Roots; C= Vegetative Phase; D= Leave; E= Flowers from the front view; F= Flower parts consists of dorsal sepals (1), lateral sepals (2) and petals (3); G= Columna; H= Operculum; I= Pollinia.

Flower Morphology of *P.* 'OX Queen'

Table 3. Colour identification of *P.* 'OX Queen' hybrid flower based on RHS Colour Chart.

Flower Sample	Colour Code	Colour Identification Based on RHS Colour Chart
	Red purple Group N73 A	

Morphology Flower of *Dendrobium* 'Cheddi Jagan'

Table 4. Colour identification of *Dendrobium* 'Cheddi Jagan' hybrid flower based on RHS Colour Chart.

Flower Sample	Colour Code	Colour Identification Based on RHS Colour Chart
	Red purple Group N72 C	

Table 3 displays the results of flower colour identification based on the RHS colour chart for *P.* 'OX Queen,' which is characterized by a purple colour with code N73A colour card group or Deep Purplish Pink colour (sRGB: R207, G116, and B171),. while the, *D.* 'Cheddi Jagan' that displayed in Table 4 is identified with a purple colour denoted by code N72C colour card group which means Strong Reddish purple (sRGB: R193, G104, and B160). Codes A to D or the first, second, third, and fourth colour chips are respectively lighter in colour but have the same colour hue (Voss 2001). *P.* 'OX Queen' and *D.* 'Cheddi Jagan' both exhibit purple colours, but differ in the nuances of the flower colour. *D.* 'Cheddi Jagan' appears to have a deeper shade of purple compared to *P.* 'OX Queen'.

Genotypic Analysis

Analyze of DNA and PCR

Based on the qualitative DNA (Figure 3) tested using gel electrophoresis, it was determined that the DNA sample sizes of *P.* 'OX Queen' and *D.* 'Cheddi Jagan' were both greater than 10,000 bp.

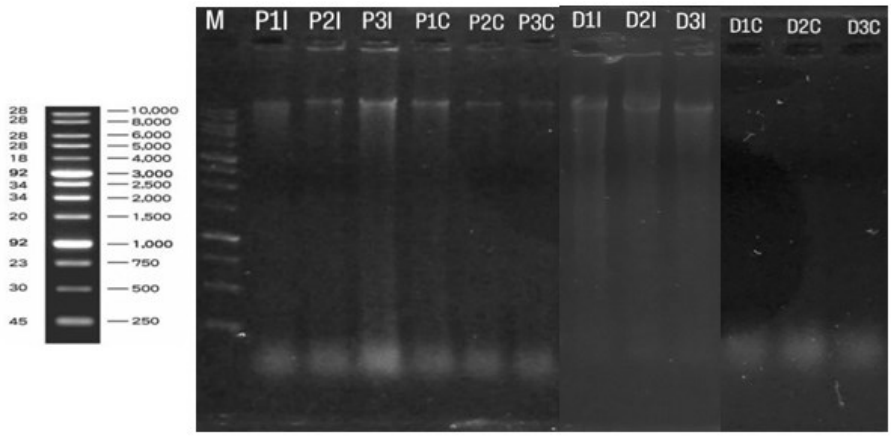

Figure 3. DNA genome visualization from *P.* 'OX Queen' (P1-P3) and *D.* 'Cheddi Jagan' (D1-D3).

Based on Table 5, the purity of the DNA produced in the *P.* 'OX Queen' orchid is included in the good category, whereas in the *D.* 'Cheddi Jagan' orchid the DNA purity produced is still not good due to the presence of contaminants in the DNA sample. DNA isolation methods in different plants can produce different purities and concentrations due to various factors, including the chemical content in the plant, the efficiency

of DNA separation or purification, and the DNA precipitation process (Rizko et al. 2020). According to Green and Sambrook (2019), the purity ratio value of A280/A260 should be in the range of 1.8–2.0. A purity ratio of A260/A280 below this range indicates that there is protein contamination.

Table 5. Quantity analysis of *P.* 'OX Queen' (P1-P3) and *D.* 'Cheddi Jagan' (D1 -D3) using NanoDrop Spectrophotometers.

Sample ID	Conc.	Units	A260/A280	A260/A230
P1	113.016	ng/µL	1.92	1.085
P2	132.076	ng/ µL	1.848	1.079
P3	120.8	ng/ µL	1.996	0.998
D1	73.407	ng/ µL	1.517	0.564
D2	138.732	ng/ µL	1.309	0.438
D3	134.57	ng/ µL	1.398	0.400

Meanwhile, based on the A260/230 purity ratio value, a sample has good DNA purity if it has an A260/230 purity ratio value ranging from 1.8-2.0 for DNA (Dwiyani et al. 2016). Based on orchid DNA electrophoresis, electropherogram results were obtained with a size of more than 10 kb. According to Waluyo et al. (2013), a good DNA band in an electropherogram is if the DNA band is clearly visible and has uniform length of base pairs without any smearing. The quantity of orchid DNA was determined using the UV spectrophotometric method at wavelengths of 260 and 280 nm (Semiarti et al. 2020).

Amplification *of CHS* gene Fragmen in *P.* 'OX Queen' and *D.* 'Cheddi Jagan'

The results of DNA isolation were used as a template for amplifying the *CHS* and *Actin* gene sequences using the PCR method. The *CHS* gene was used to detect anthocyanin pigments (Linggabuwana et al. 2024) and this was used to determine the specifications of the two orchid genera and the *Actin* gene was used as an internal positive control (Zhao et al. 2012).

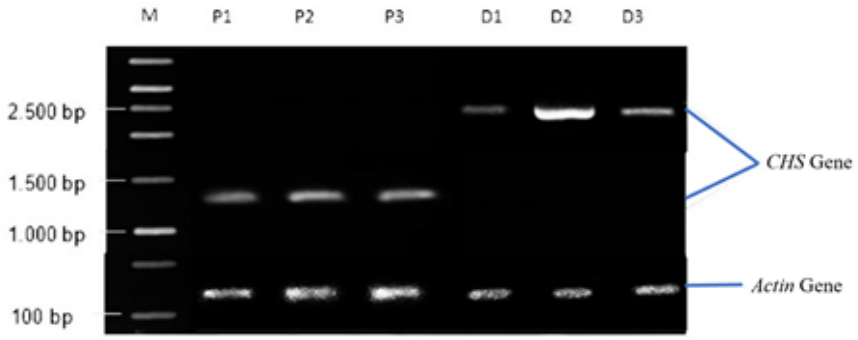

Figure 4. Detection of *CHS* and *Actin* gene in *P.* 'OX Queen' and *D.* 'Cheddi Jagan' purple zone using the PCR method.

Figure 4 shows the PCR results of the *CHS* gene in *P.* 'OX Queen' of 1,287 bp and in *D.* 'Cheddi Jagan' of 3,731 bp. These results were validated by primary *Actin* as a housekeeping gene and positive control. The *actin* primer has a size of 114 bp, showing the successful amplification of the expected DNA fragment. The *actin* primer shows strong and clear band intensity, conforming the presence of target DNA in the sample. These results strengthen confidence in validity and reliability of the data resulting from PCR analysis, and ensure that the amplification of target

and control DNA has proceeded as expected.

Based on the qualitative DNA test using gel electrophoresis, it was determined that the DNA sample sizes of *P.* 'OX Queen' and *D.* 'Cheddi Jagan' were both bigger than 10,000 base pairs. Modification of the Murray and Thompson method with the addition of isopropanol aims to precipitate DNA. Heikrujam et al. (2020) stated that apart from precipitating DNA, isopropanol can also dissolve non-polar solvents such as chloroform so that it can remove impurities in DNA and produce good quality DNA. This is in accordance with research by Sari and Restanto (2022) namely that PVP is used to bind phenolic components and avoid oxidation. The genome DNA isolated by Murray and Thompson method (1980) were used for amplification of *CHS* and *Actin* genes.

The sequence results obtained showed that the number of base pairs was different from the target size of the primers used, possibly because the primers were prepared based on reference sequences from different orchid species contained in the database. As a next step, mapping and characterization of the *CHS* sequence can be carried out in both the *P.* 'OX Queen' orchid and the *D.* 'Cheddi Jagan' orchid to see differences in the size of the DNA sequence. Several factors that need to be considered to get PCR results that match expectations are the quantity and quality of DNA isolation results. Good quality DNA has a purity between 1.8 – 2.0 and a concentration above 100 ng/µL based on measurements with a spectrophotometer. *Actin* serves as a housekeeping gene used as an internal positive control (Yonindi et al. 2022). Based on the visualized DNA results via gel electrophoresis, a 114 bp DNA band from the Actin gene amplification can be observed.

CHS gene Motifs Analysis

The DNA results obtained from Oxford Nanopore Technology sequencing were 1.287 bp for *P.* 'OX Queen' and 3.731 bp for *D.* 'Chedi Jaggan'. It can be seen in Figure 5 which shows a schematic of the *CHS* gene in the DNA sequence of orchid flowers. The sequence of the *CHS* gene in *P.* 'OX Queen' is at bases 1.171 to 1.300, while in *D.* 'Chedi Jaggan' is at bases 2.731 to 2.860. In this base there is a *CHS* motif encoded in both orchid flowers.

Figure 5 shows the results of aligning the sample sequence with the database sequence. The *P.* 'OX Queen' consensus was aligned with the '*Phalaenopsis* hybrid cultivar *Chalcone Synthase* gene' with accession number AY825502.1, while the *D.* 'Cheddi Jagan' consensus was aligned with the '*Dendrobium catenatum* unplaced genomic scaffold' with accession number NW_021318618.1. In *P.* 'OX Queen' and *D.* 'Cheddi Jagan', the presence and conservation of the CHS domain suggest their capability to produce flavonoids. The CHS conserved domain in different orchid species can contribute to the identification of genetic variations that may influence the flavonoid biosynthesis and consequently, the phenotypic traits such as flower's colour.

In Table 6, it can be seen that for *P.* 'OX Queen', two sequences were found that were related to the *CHS* gene. The first sequence is registered in super families PLN03172 and accession number cl30448, while the second sequence is registered in super families PLN03170 and accession number cl30450. These two sequences had very low significance values (1.20e-19 and 4.69e-163), indicating that the *CHS* gene is very conservative and important in flavonoid synthesis in *P.* 'OX Queen'. Meanwhile, for *D.* 'Cheddi Jagan', there are three sequences related to the *CHS* gene. The first two sequences are registered in super families PLN03172 and accession number cl30448, while the third sequence is registered in

Table 6. The CHS Conserved Domain of *P.*'OX Queen' and *D.* 'Cheddi Jagan'.

Sample	Name	Accession	Description	Interval	E-value
P. 'OX Queen'	PLN03172 super family	cl30448	chalcone synthase family protein; Provisional	5-205	1.20e-19
	PLN03170 super family	cl30450	chalcone synthase; Provisional	294-1283	4.69e-163
D. 'Cheddi Jagan'	PLN03172 super family	cl30448	chalcone synthase family protein; Provisional	103-222	1.35e-07
	PLN03170 super family	cl30450	chalcone synthase; Provisional	1186-1272	6.12e-04
	PLN03172 super family	cl30448	chalcone synthase family protein; Provisional	281-1207	0,00E+00
	PLN03170 super family	cl30450	chalcone synthase; Provisional	198-278	4.92e-10

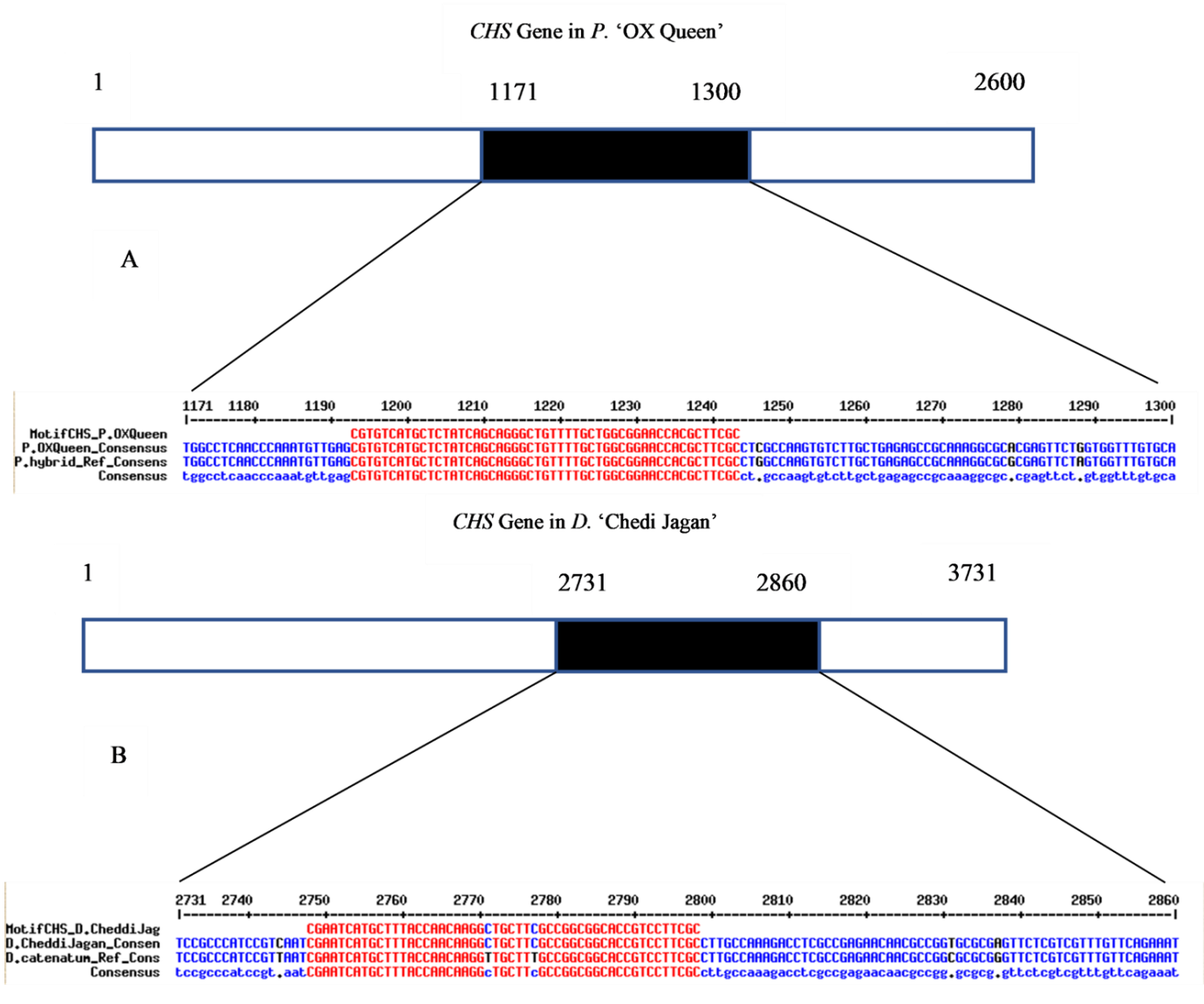

Figure 5. MultAlin alignment of the *CHS* gene in (A) *P.* 'OX Queen' and (B) *D.* 'Cheddi Jagan'.

super families PLN03170 and accession number cl30450. The first sequence has a fairly low significance value (1.35e-07), while the second and third sequences have a higher significance value (6.12e-04 and 0.00E+00). This suggests a variation in *CHS* gene sequences between *P.* 'OX Queen' and *D.* 'Cheddi Jagan'. So, these results indicate that the *CHS* gene plays a role in flavonoid biosynthesis in both orchid species, but the level of conservation and significance may vary between different species. This is in accordance with the statement from Hartono et al. (2021) that the smaller the E value (large negative exponent value), the higher the level of significance. Thus, the results indicate that the sequence match to the protein family or domain determined in the analysis is highly significant.

Based on the analysis of amino acid motifs shown in Figure 6., it was found that in *P.* 'OX Queen' there is a CHS protein motif in amino acid sequences 538 to 554 while in *D.* 'Cheddi Jagan' has a CHS protein motif in amino acid sequences 658 to 674. Apart from the CHS protein, in *P.* 'OX Queen' there is the EGF-1 (EGF-like domain signature 1) protein which is located at sequence 149 to 160, it is also shown in Figure 5A. with a red box. The *CHS* gene encodes the enzyme CHS which is an enzyme that is important in flavonoid biosynthesis. This protein accumulates anthocyanins in plant organs such as roots, stems, leaves and flowers (Linggabuwana et al. 2024). These pigments accumulate in vacuoles and their stability depends on intravacuolar conditions which include pH and pigment concentration (Luo et al. 2017). Anthocyanin accumulation is formed through phenylpropanoid pathways and catalysed by a number of enzymes including CHS, chalcone-flavanone isomerase (CHI), flavanone-3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose: flavonoid 3-O glycosyltransferase (UFGT) (Liu et al. 2022).

The morphological and molecular analysis of *P.* 'OX Queen' and *D* 'Cheddi Jagan' orchids aim to lay the groundwork for CRISPR/Cas9-mediated genome editing, particularly in sgRNA determination. This research utilizes CRISPR/Cas9 in floricultural crops, focusing on enhancing flowering traits like colour modification, prolonging shelf life, initiation and development of flowers, and altering ornamental foliage colour through genome editing. Researchers aim to comprehensively under-

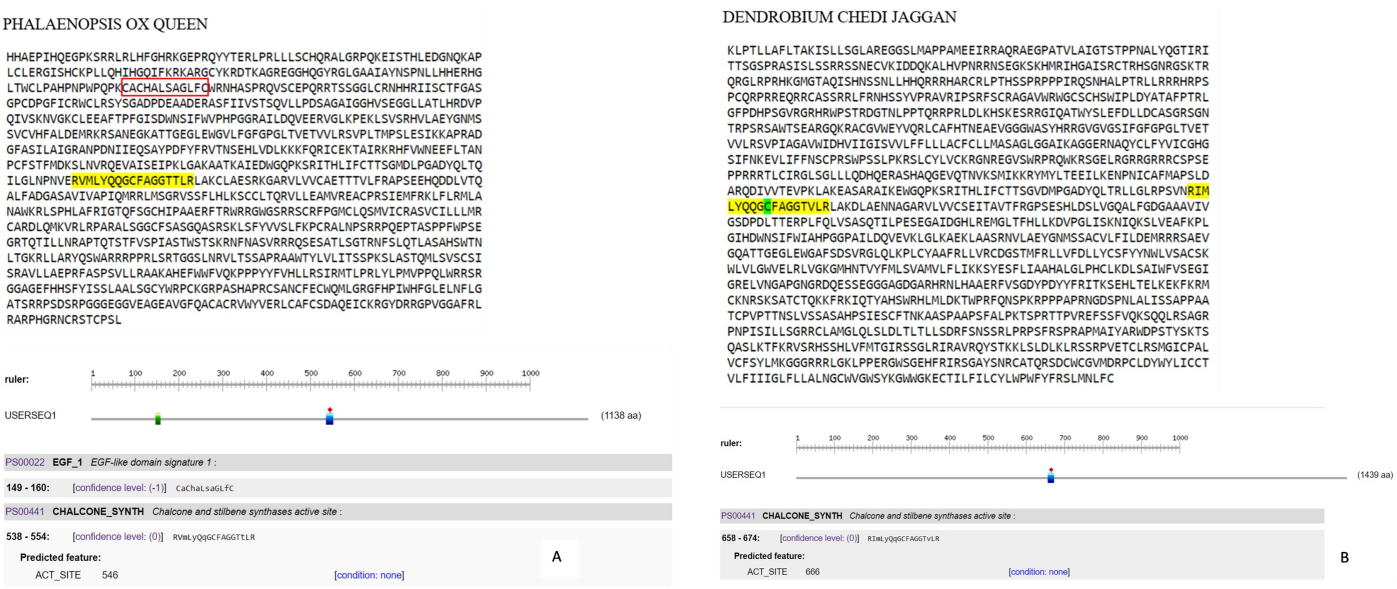

Figure 6. Amino acid motifs in CHS protein of *P.* 'OX Queen' (A) and *D.* 'Cheddi Jagan' (B) from PROSITE Analysis.

stand the morphological and molecular aspects of both orchid species. Morphological analysis identifies targetable unique traits, while molecular analysis delves into genetic details and gene expression (Arif & Ratnawat 2018).

This experiment provides crucial insights for price genome editing, emphasizing effective sgRNA identification and design, results are expected to deepen understanding of the genetic basis of *P.* 'OX Queen' and *D.* 'Cheddi Jagan', facilitating more targeted genome editing for variegated flower creation, primarily targeting the CHS protein function essential for flower coloration.

CONCLUSION

This research found that the *CHS* gene fragment was successfully isolated from the genomes of two hybrid orchids *Phalaenopsis* 'OX Queen' and *Dendrobium* 'Cheddi Jagan' which have plain pink and purple flowers, apparently in both orchids there are CHS protein with the CHS family domain motif that function for flavonoid synthesis, but they have slight differences in the DNA structure of the *CHS* gene. This indicates the possibility that the results of the phenotypic analysis of flower colour and *CHS* gene structure can be used to determine target sequences for sgRNA to create *Phalaenopsis* and *Dendrobium* orchids with variegata patterned of flower's color using the CRISPR/Cas9 Genome editing system.

AUTHOR CONTRIBUTION

E.S. designed the research and supervised all the process, Y.R.H. and E.G. responsible for laboratory activities, phenotypic and molecular data analysis and writing the manuscript, P.D.K assisted in the wet lab and dry lab activities and manuscript proofreading and A.P. conducted data and manuscript proofreading.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this research.

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Research Article

Detection of Entomological Origin of Honey Sold in Indonesia Based on *16S rRNA* Gene Analysis

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ABSTRACT
Honey is known for its various benefits for health, cosmetic ingredients, and other industrial materials. Especially, during the Covid-19 pandemic, many people consume honey to maintain body endurance. In Indonesia, the honey produced is dominated by *Apis mellifera* honey. With a cheaper price and a larger quantity, *A. mellifera* honey is often offered as forest honey or stingless bee honey to get more profit. Therefore, this study aims to determine the entomological origin of honey claimed as forest honey and stingless bee honey sold in the Indonesian market using the detection of *16S rRNA* gene amplicon. This study tested 30 samples of forest honey and 30 samples of stingless bee honey. DNA that has been isolated from honey samples was amplified by PCR using *16S rRNA* primers. The results from the sequence analysis showed that nine of honey samples were identified as honey fraud. Two samples were confirmed as falsification of the origin of honey-producing bees and four honey samples were confirmed as honey mislabelling. From this study it can be concluded that, it is possible to determine the entomological origin of honey molecularly by sequencing the *16S rRNA* gene. Therefore, this method can be used to identify honey fraud that may occur on the market.

INTRODUCTION
Honeybees (Apidae: Apini; *Apis*) and stingless bees (Apidae: Meliponini) belong to group of insects with a high diversity and wide distribution. As many as eight of the nine species belonging to the genus *Apis* in the world and about 46 species of stingless bees are widespread throughout Indonesia (Engel 2012; Kahono et al. 2018). The variety of honey bee species and stingless bees in Indonesia is also supported by Indonesia's geographical landscape, diverse landscape topography and environment, and the history of Indonesia's complex geological formation (Hall 2009). Honey bees and stingless bees are groups of bees that produce honey (Kahono et al. 2018; Gratzer et al. 2019; Buchori et al. 2022).

According to Codex Alimentarius (FAO 2019) honey is defined as a natural substance in the form of a sweet liquid produced by honey bees not only from plant nectar but also derived from plant secretions or plant -sucking insect excretions. Since a long time ago, Indonesian people have believed that honey, one of the traditional medicines, can cure various

diseases. Honey is beneficial both for health, cosmetics, and other industrial ingredients. In addition, honey can also act as an antioxidant, anti-inflammatory, anti-diabetic, and immune guard (Samarghandian et al. 2017). Therefore, during the Covid-19 pandemic, honey is sought after by the public to increase their body immunity to avoid the virus.

The highest honey production in Indonesia is produced in Java, 81.06% of the national production (Badan Pusat Statistik 2020). Mostly, it is produced by *Apis dorsata* and *Apis mellifera*. It has also been widely developed to increase honey production (Buchori et al. 2022). Honey production is also increasingly produced by grazing in Sumatra on industrial forest, *Accacia* and *Eucalyptus* (Pribadi 2016). Based on information from market players, some of *Apis mellifera* honey is forfeited as forest honey (honey from *Apis dorsata* or *Apis* spp) or stingless bee honey. Because the forest honey and stingless bees honey price is higher than *Apis mellifera* honey.

Honey fraud is a problem that often occurs. Based on APIMONDIA, honey fraud acts include dilution of honey with artificial syrup, premature harvesting of honey, use of ion-exchange resins, artificial feeding of honey-producing bees, and falsification or mislabeling of the origin of honey (geographical or botanical). This action can be considered fraud because, to gain more profit, honey is sold with a quality that does not meet global standards (APIMONDIA 2019). Therefore, it is necessary to have an effective method of determining the entomological origin of honey to avoid such fraud. Molecular identification of honey has been carried out using *16S rRNA*.

Research conducted by Kek et al. (2017) by detecting the *16S rRNA* and *CO1* genes, it was possible to determine the origin of honey from 14 samples obtained directly from the forest and one sample of commercial honey. In addition, research by Zhang et al. (2019) and Raffiudin et al. (2023) can also determine the origin of honey based on the presence of the *Major Royal Jelly Protein 2* (*mrjp2*) gene. However, previous research was still limited to honey samples from *A. mellifera* and *A. cerana*. Considering that the honey sold in the Indonesian market comes from various species of honey-producing bees, not only *A. mellifera* and *A. cerana* (Engel 2012; Kahono et al. 2018), therefore, this research is expanded on honey traded on the Indonesian market, marketed as forest honey and stingless bee honey.

MATERIALS AND METHODS

Materials

The tools used in the preparation of honey samples were a 50 mL falcon tube, 1.5 mL Eppendorf tube, semi-analytical balance (Ohaus), vortex, measuring cup, -4°C freezer, centrifuge (GyroZen), water bath, micropipette, pipette tips, and NanoDrop. For PCR analysis, the tools used were a PCR tool (Applied Biosystems™ 2720 thermal cycler), Spindown Mini Centrifuge, micropipette, 0.2 mL PCR tube, pipette tips, measuring cup, Erlenmeyer flask, spatula, semi-analytical balance, microwave, electrophoresis tool, UV transilluminator, and a smartphone camera.

The materials used in this study included 30 honey samples each claimed by the sellers or beekeepers to be forest honey (H1-H30) and stingless bees honey (K1-K30), absolute ethanol, sterile distilled water, DNA extraction kit FavorPrepTM Tissue Genomic DNA Extraction Mini Kit 100 Prep (Proteinase K) (animal tissue, Blood, Cell, fungus, bacteria), Gotaq® Green Master Mix Promega, primer (Table 1), agarose 1.5%, aquabidest, Nuclease Free Water (NFW), Fluorosafe, Geneaid 100 bp DNA Ladder and Tiangen 100bp DNA Ladder, and TBE buffer.

Table 1. Primers used in this study.

Primer	Sequence	Gene Marker	Product size (bp)	Reference
LR13107-F	TGG CTG CAG TAT AAC TGA CTG TAC AAA GG	16S rRNA *Tetragonula cf. pagdeni*	496	Thummajitsakul et al. 2013
LR12467-R	GAA ACC AAT CTG ACT TAC GTC GAT TTG A			

Methods

Sample collection

Samples were obtained from honey sold in online and offline shops directly from beekeepers in Klaten and Magelang that the seller claimed either as forest honey or stingless bee honey.

Sample preparation

The honey sample preparation method was carried out according to Thummajitsakul et al. (2013) with some modifications. A sample of 12.5 g of honey was put into a 50 ml falcon tube. Aquadest were added to the sample until it reached 50 ml. The honey solution is homogenized with a vortex. Samples were incubated at 40°C for 30 minutes in a water bath. The samples were centrifuged at 5000 rpm at 20°C for 20 minutes to precipitate the pellets. The supernatant was discarded, and the pellet was redissolved with distilled water. The honey sample was centrifuged at 5000 rpm at 20°C for 20 minutes to obtain pellets. 500 ul of aquabidest was added to the pellet and transferred to a 1.5 mL PCR tube. The sample can be extracted using Favorgen Tissue Genomic DNA Extraction Mini Kit 100 Prep (Proteinase K) (animal tissue, Blood, Cell, fungus, bacteria).

Polymerase Chain Reaction (PCR)

DNA amplification was carried out by PCR using primers LR13107-F and LR12647-R to detect the presence of bee DNA based on the *16S rRNA* gene, according to Thummajitsakul et al. (2013). The results of DNA isolation were amplified using an Applied Biosystems™ 2720 Thermal Cycler PCR machine with a reaction volume of 25 µL. Each reaction volume contains 12.5 µL GoTaq® Green Master Mix, 5.5 µL NFW, 1.75 µL forward primer, 1.75 µL reverse primer, and 3.5 µL template DNA. PCR conditions for DNA amplification are adjusted to predenaturation at 95°C for 2 minutes, (denaturation at 95°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 30 s with 35 cycles), and final extension at 72°C for 5 minutes.

Data visualization and analysis

PCR products with an amplicon of the *16S rRNA* gene of honey bees and stingless bees were electrophoresed on 1.2% agarose gel. PCR products showing positive electrophoresis results were sequenced at Integrated Laboratory for Research and Testing (LPPT) of Universitas Gadjah Mada. The sequencing results obtained from LPPT UGM were edited with MEGA X software. The sequences were then saved in FASTA format for analysis using NCBI Nucleotide BLAST (BLAST-n) page (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLST-n results show the sequence results that are most similar to the consensus sequences.

RESULTS AND DISCUSSION

The initial stage for molecular testing is the extraction of honey samples to obtain bee DNA contained in honey. The bee DNA in honey is an environmental DNA (eDNA). eDNA is a DNA fragment released by an organism into the environment. eDNA can be derived from nuclear or mi-

tochondrial DNA, which can be found in the cellular or extracellular matrix. Faecal secretions, mucus, gametes, loose hair and skin, and carrion organisms are some of the sources of DNA found in the environment (Barnes & Turner 2016). Many uses of bee eDNA in honey have been carried out, such as to detect the presence of parasites and pathogens and to determine the origin of nectar and pollen obtained by honey bees (Matsuzawa et al. 2020; Ribani et al. 2020). In addition, it has also been carried out to determine the entomological origin of honey (Kek et al. 2017; Zhang et al. 2019).

The success of DNA extraction is indicated by the good quality in terms of DNA concentration and the purity of the DNA obtained. Based on this study, the DNA concentration obtained ranged from 1.31 to 2181.71 ng/nL with A260/A280 ratio average is 2.06 and A260/A230 ratio average is 1.91. This indicates that most of the DNA has good purity with low level of protein and organic component contamination in DNA. DNA with good purity has an A260/A280 ratio ranging from 2.0-2.2 and an A260/A230 ratio ranging from 1.8-2.0. DNA. The purity and concentration of DNA affect PCR efficiency (Lucena-Aguilar et al. 2016).

DNA is amplified using the Polymerase Chain Reaction (PCR) method. PCR is a method used to replicate DNA in vitro, making it possible to obtain multiple copies of DNA fragments from samples. In general, PCR reagents consist of template DNA, Taq polymerase, primers, dNTPs, and buffer solutions (Kadri 2019). In this study PCR used primer to detect *16S rRNA* gene sequences.

The probability of the presence *16S rRNA* gene in a genome preparation is greater, it is because the ribosomal RNA have more DNA copies (Hori & Engel 2023). The characteristic of the *16S rRNA* gene is that it is easy to isolate, over time it may change its sequence, so that detection among distant species is possible with this gene. In addition, this gene is also composed of various parts that are conserved (Byrne et al. 2018). PCR using primers from mitochondrial DNA shows a relatively faster evolution than nuclear DNA. In addition, the number of DNA copies inside the cell is high, so it can be used to differentiate between animal species (Kim et al. 2017).

The *16S rRNA* gene was detected in honey samples using the primer LR13107-F/LR12647-R (Thummajitsakul et al. 2013). The PCR was divided into two batches and repeated twice. The results of the first batch of PCR (Figure 1) showed that there were 13 forest honey samples (H1, H2, H3, H5, H6, H7, H8, H9, H10, H12, H13, H14, H15, H16) and five samples stingless bees honey (K17, K18, K19, K21, K25) is positive. Meanwhile, in the second repetition (Figure 2), it showed that 12 forest honey samples (H1, H2, H3, H5, H6, H7, H10, H12, H13, H14, H15, H16) were positive, and five samples of stingless bee honey (K17, K18, K19, K21, K25) were positive. The bands appearance indicates that the sample contains the *16S rRNA* gene from honey bees or stingless bees. Based on these results, the appeared band was around 500bp.

Batch 2 PCR results (Figure 3) showed that nine samples of forest honey were positive (H17, H19, H20, H22, H25, H27, H28, H29, H30) and eight samples of positive stingless bee honey (K1, K2, K5), K6, K7, K9, K13, K14). Meanwhile, the second repetition in Figure 4 shows that ten samples of forest honey were positive (H17, H18, H19, H21, H22, H23, H24, H26, H28, H30) and eight samples of positive stingless bee honey (K1, K2, K3, K5, K9, K15). The band's appearance indicates that the sample contains the *16S rRNA* gene from honey bee or stingless bee. It showed the band that appears is around 500 bp in size.

Based on the result from PCR batch 1 and PCR batch 2, the num-

ber of positive samples are different in the first repetition and second repetition. In PCR batch 1, the first repetition of samples H8 and H9 showed positive results with the appearance of a DNA band, but in the second repetition the DNA band did not appear. The same thing also happened to the results of PCR batch 2. The absence of DNA bands in the PCR results may be due to the small number of DNA copies (copy number) in the sample. This is because the source of bee DNA in honey is eDNA, which does not only contain bee DNA in honey. Then, if it is related to the concentration of the DNA extraction results, even though it has quite good purity, there is a DNA concentration that is too low or too high. When the DNA concentration gets higher, the inhibitor concentration also increases. It can inhibit replication process in PCR. Meanwhile, when the DNA concentration is too low, too few DNA copy numbers are formed to be visualized.

Possible causes of DNA bands that initially appeared then did not reappear were DNA samples that had been stored for too long after extraction which affects DNA stability but depends on buffer composition used (Röder et al. 2010), repeated freeze-thawing, DNA samples kept at room temperature for too long, and inefficient purification of DNA samples, resulting in residues nuclease is still present in the sample. The more freeze-thawing cycles, the greater the degradation rate of genomic DNA. The concentration of DNA stored at 100 mg/mL was slightly more stable during freeze-thawing than DNA stored at 10 mg/mL. Thus, it can be said that the stability of DNA not to be degraded during the freeze-thawing phase is better when the DNA has a concentration of more than 100 mg/mL (Shao et al. 2012).

The research data showed that it was possible to determine the entomological origin of honey molecularly by detecting the *16S rRNA* gene. However, it is necessary to sequence the *16S rRNA* gene to know which species are identified because the size of the DNA amplicon is the same in all honey bee and stingless bee samples around 500bp.

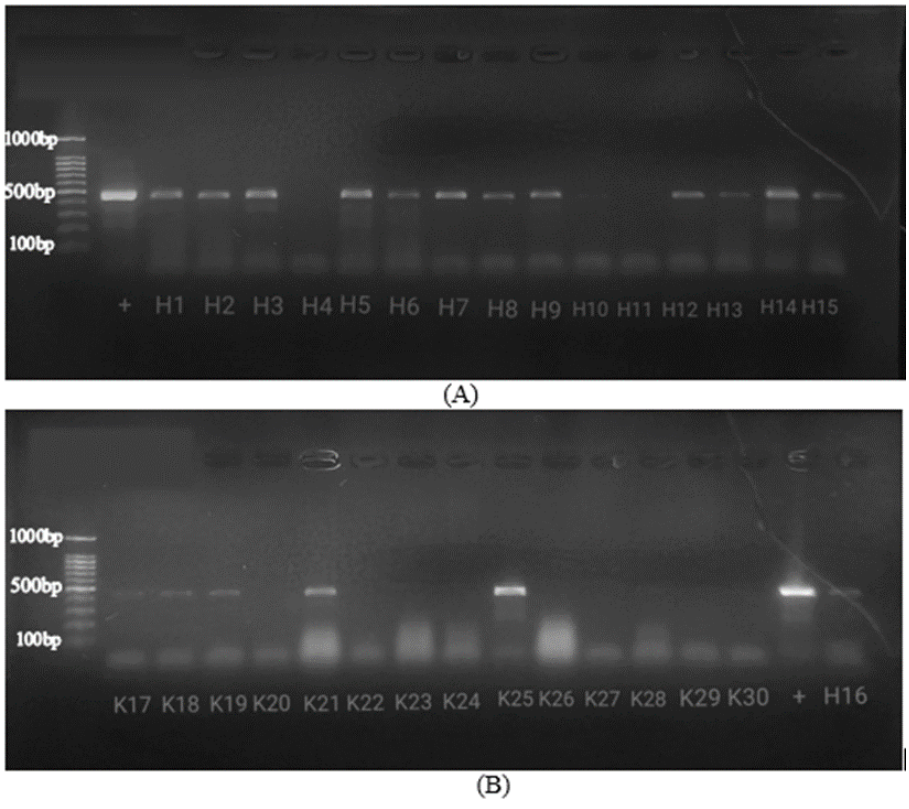

Figure 1. Results of Batch 1 DNA amplification of forest honey (A) and stingless bees honey (B) with primer LR13107-F/LR12647-R for the first repetition.

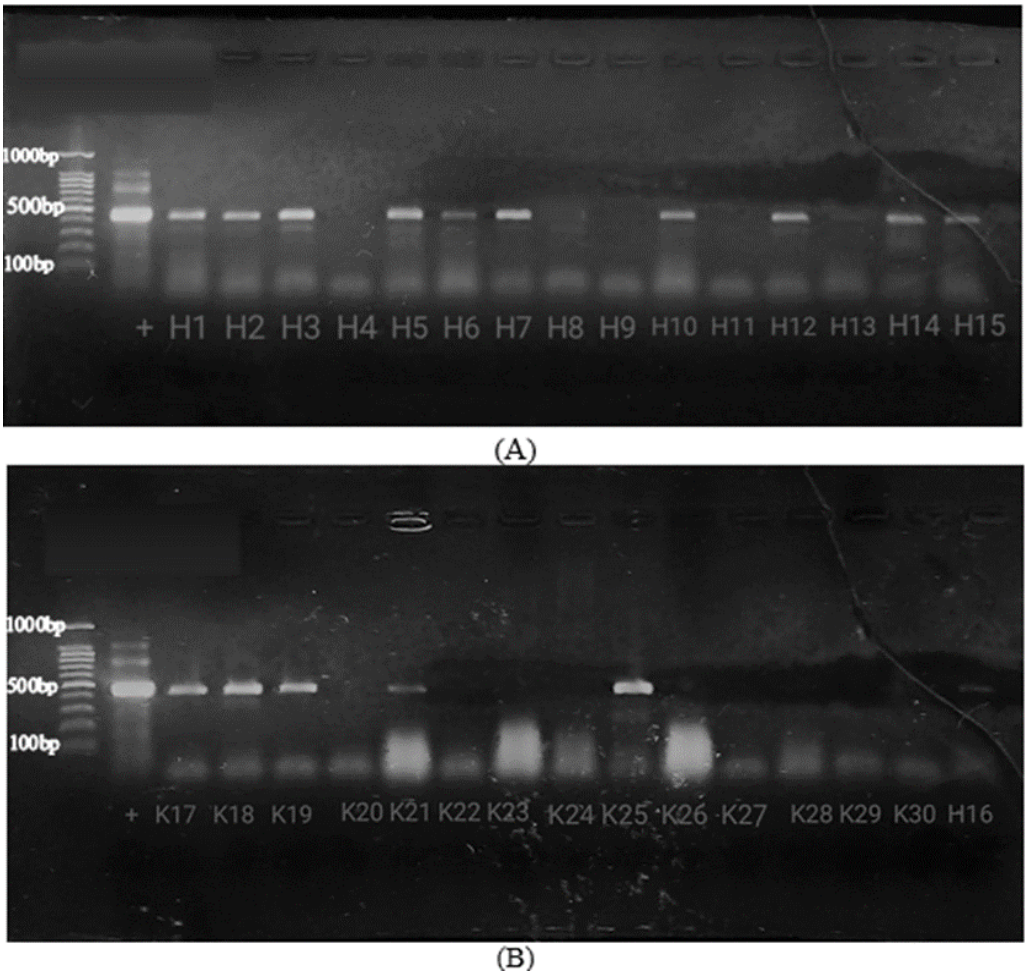

Figure 2. Results of Batch 1 DNA amplification of forest honey (A) and stingless bees honey (B) with primer LR13107-F/LR12647-R for the second repetition.

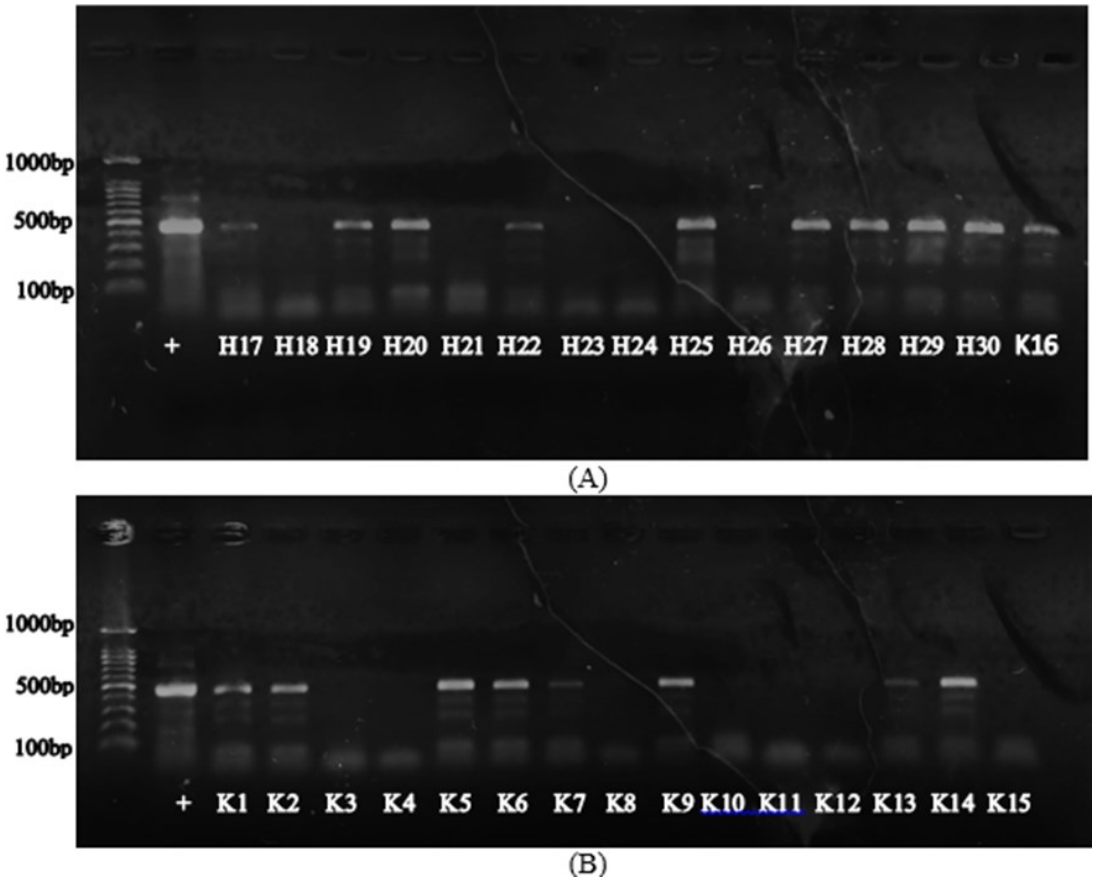

Figure 3. Results of Batch 2 DNA amplification of forest honey (A) and stingless bees honey (B) with primer LR13107-F/LR12647-R for the first repetition.

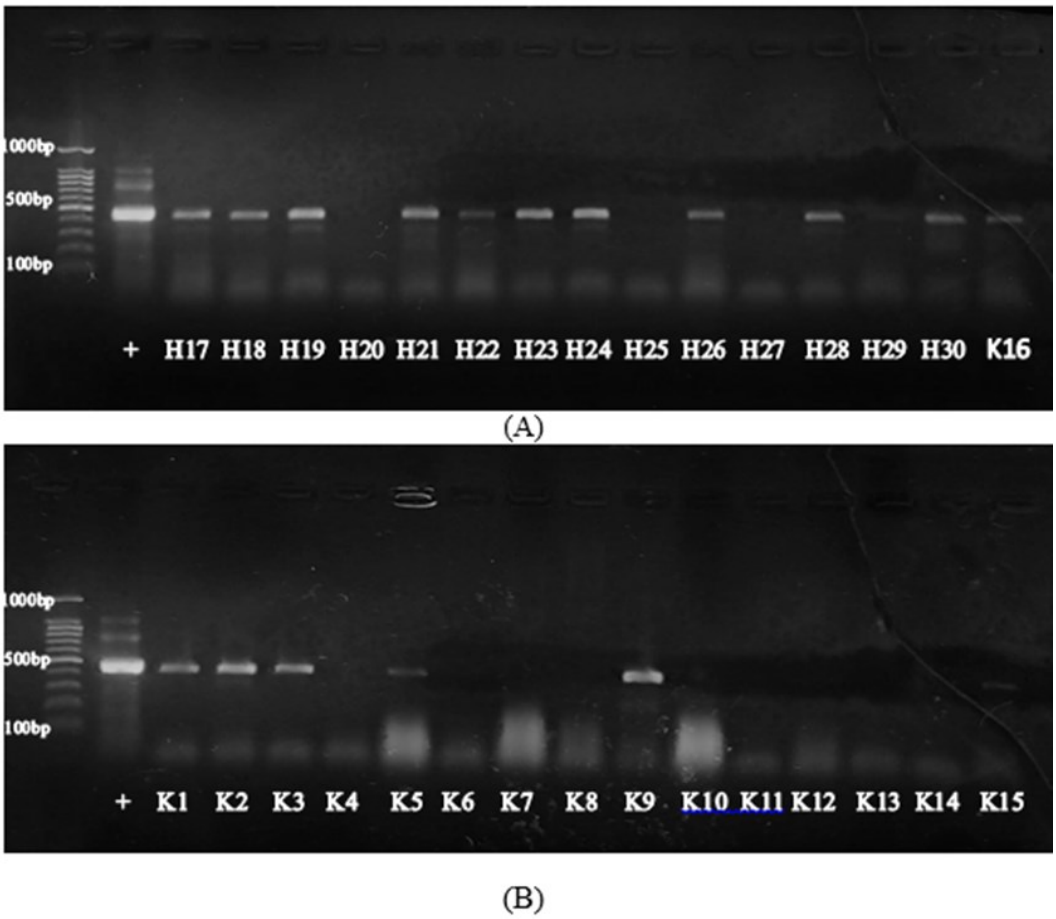

Figure 4. Results of Batch 2 DNA amplification of forest honey (A) and stingless bees honey (B) with primer LR13107-F/LR12647-R for the second repetition.

Using the 16S rRNA gene method, a genus is declared similar if it has 97% similarity and is said to be a species if the similarity is 99% (Petti 2007). Table 2. shows that the highest percent identity is for samples H19 and H25 at 100%, so it can be said that 100% probability that the honey samples come from *A. mellifera*. The H28 sample shows 99% percent identity, so it can be said that 99% probability that the honey sample comes from *A. cerana*. Meanwhile, the lowest percent identity is shown by the K25 sample of 96.33%. From these results, the sample has a similarity of 96.33% with *T. laeviceps*.

From nine samples that were sequenced, it was shown that six samples of honey did not match with the seller's claims. Based on Table 2. samples of forest honey (H16, H19, H20, H25, H27, and H28) resulted in contradiction between the seller's claims and the identification results. Samples H16, H20, and H27 labelled as forest honey samples claimed as honey from *A. dorsata*, while the identification results showed that it came from stingless bees. Meanwhile, samples H19 and H25, which claimed to be honey from *A. dorsata*, were identified as honey from *A. mellifera*. Table 2 shows the percent identity of the two samples is 100%. Thus, it can be said with certainty that both samples were honey derived from *A. mellifera*. This finding implied that there was an act of falsification of the origin of honey. This is one of honey fraud because honey from *A. mellifera* bees on the market has a much lower selling value than forest honey (Zhang et al. 2019). The seller gains more profit by selling honey produced by *A. mellifera* bees as forest honey.

Based on the same Table 2. mislabelling honey was also detected. Therefore, the honey-producing bees from the honey sample may have a close relationship with the identified species.

Table 2. BLAST-n results of positive sample sequences.

Sample Code	Identification	Claim	*Accession Number*	*Query Cover*	*E Value*	*Percent Identity*
H16	*Tetragonula cf. pagdeni*	*Apis dorsata* honey	DQ790437.1	99%	0.0	96.78%
H19	*Apis mellifera*	*Apis dorsata* honey	AP018434.1	95%	0.0	100%
H20	*Heterotrigona itama*	*Apis dorsata* honey	KU571761.1	100%	0.0	96.88%
H25	*Apis mellifera*	*Apis dorsata* honey	MN714160.1	100%	0.0	100%
H27	*Heterotrigona itama*	*Apis dorsata* honey	KU571761.1	97%	0.0	97.91%
H28	*Apis cerana*	*Apis dorsata* honey	AP017984.2	99%	0.0	99%
K5	*Tetragonula cf. pagdeni*	Stingless bees honey	DQ790437.1	100%	0.0	97.22%
K9	*Tetragonula cf. pagdeni*	Stingless bees honey	DQ790437.1	99%	0.0	96.60%
K25	*Tetragonula laeviceps*	*Tetragonula laeviceps* honey	KU571748.1	100%	0.0	96.33%

CONCLUSIONS

Based on this research, it can be concluded that, it is possible to determine the entomological origin of honey molecularly by sequencing the *16S rRNA* gene. Two honey samples, namely H19 and H25, committed fraud by claiming honey from *A. mellifera* honey bees as forest honey specifically *A. dorsata* honey. Four samples claimed to be honey from *A. dorsata*, were mislabelled, namely forest honey H16, H20, and H27, that were identified as stingless bees honey, and the sample number H28, identified as honey from *A. cerana*.

AUTHOR CONTRIBUTION

A.N. collected and analysed the data and wrote the manuscript, H.P. designed the research, corrected the manuscript and supervised all the processes.

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CONFLICT OF INTEREST

The authors declared there are no conflicts of interest regarding the research.

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Research Article

The Diversity of Ants (Hymenoptera: Formicidae) on Industrial Forest in Sungai Merah Village, Sarolangun, Jambi with Its Identification Key

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ABSTRACT
Ants have a very important role in an ecosystem. The insects act as decomposers, pollinators, soil aerators, pest controllers, and predators. Their role is very varied so that they can be easily found in various ecosystems, one of which is the rubber and oil palm ecosystem. The ant's diversity in this forest eventually will affect the productivity of the land. The aim of this study is to determine the diversity and role of ants in the rubber and oil palm plantations in Sungai Merah Village. Both of the plantations dominate the industrial forest in Jambi. The method used in this study was purposive random sampling. Observation plots were installed in the ecosystem of rubber and oil palm plantations; each ecosystem had 4 plots consisting of 9 units of pitfall traps, and 9 units of bait traps. The results of this study show that 15 species of ants are found in the ecosystem of rubber and oil palm plantations. Furthermore, the collected ants consisted of 39.972 individual ants belonging to 12 genera and 5 subfamilies. Ants establish in the ecosystem of rubber and oil palm plantations in Sungai Merah Village have an important role either as predators (*Crematogaster* spp., *Odontomachus rixosus*, *Odontoponera tranversa*, *Pheidole huberi*, *Tetraponera rufonigra*, *Tapinoma melanocephalum*, *Camponotus* spp. and *Colobopsis moeschi*), or foragers such as ants from the genera *Anoplolepis*, *Camponotus*, *Monomorium* and *Polyrhachis*. As predators, *Oecophylla smaragdina*, *Camponotus* spp. and *Crematogaster* spp. also play a role as biological control agents in the ecosystem.

INTRODUCTION
Jambi is one of the rubber and palm oil-producing provinces in Indonesia. In 2017 the statistics agency of Jambi (BPS Jambi) reported that there were at least 669.000 ha of rubber plantations and ± 497.000 ha of oil palm plantations spread across 11 regencies. One of the regencies which have a sizable area of rubber and oil palm plantations is Sarolangun regency.

Geographically Sungai Merah Village is located in Pelawan Subdistrict, Sarolangun Regency, Jambi Province, and based on zoogeography this village is included into the Indomalayan realm, also called as oriental realm by biogeographers (Worboys et al. 2010). Majority population in this village works as rubber farmers and oil palm farmers. The area of rubber plantations in Pelawan-Singkut sub-district ± 31.631 ha

and oil palm plantations area ± 2.769 ha (Hidayat & Yohanes 2007). Furthermore, the area of this plantation area will continue to increase over time with the opening of new land and converting plantations from rubber plantations to oil palm plantations or from other plantations. Therefore, this conversion will cause a change in habitat and affect biodiversity, including ant diversity.

The ecosystems of rubber and oil palm plantations have different characteristics. The litter covers most of the land surface, preventing the growth of weeds or other wild vegetation, but sometimes wild vegetation is also found in this ecosystem (Harwanto et al. 2020). There are various species of wild vegetation in the oil palm plantations (Bilkis et al. 2022) because the litter in this ecosystem does not cover the land surface like the litter in rubber plantations (Roza 2018). The characteristics of the ecosystem will affect organisms, including ants.

Ants are social insects from the order Hymenoptera and the Formicidae family. They have very important roles as pollinators, predators of pests, parasitoids of pests, weed killers, scavengers, decomposers, soil builders, and food providers. Insects are also useful in medicines and have aesthetic and scientific values (Rawal 2020). Because of their very varied role, ants can easily be found in various ecosystems, one of which is the rubber and oil palm plantation.

The diversity of ant species in rubber and oil palm plantations in Sungai Merah Village has never been reported. Therefore, it is necessary to conduct a study on the diversity of ant species found in rubber and oil palm plantations in Sungai Merah Village, specifically its species identification, diversity, and abundance of ant species and to find out the role of these ants based on literature study.

MATERIALS AND METHODS

Materials

The tools used in this study were plastic cups, plastic plates, plastic mica, sample bottles, tweezers, brushes, stationery, small shovels, gloves, bamboo poles, soil meters, plastic bags, thermometer, soil tester, soil thermometer, hygrometer, digital microscope, and camera. Meanwhile, the materials used were research subjects (ants), 70 and 96% alcohol, fish, sugar solution, and cotton.

Methods

Time and Place of Research

The study was conducted from June 2021 – December 2022, which took place in rubber and oil palm plantations located in Sungai Merah Village, Pelawan sub-district, Sarolangun regency Jambi Province, Laboratory of Entomology, Faculty of Biology, Gadjah Mada University and Insect Laboratory of the National Research and Innovation Agency (BRIN).

Collection Method

This study used a purposive random sampling. Sampling was conducted in 2 different ecosystems, in each ecosystem there were 4 plots with a size of 20 x 20 m^2, each plot was installed with 9 units of pitfall traps, 9 units of sugar solution bait traps, and 9 units of fish bait traps.

Pitfall trap

Pitfall traps are used to catch ants that are on the ground. A total of 9 units of pitfall traps were installed in each plot with 10 m between the pitfall traps. Pitfall traps are made of plastic cups with a diameter of 10 cm and a height of 15 cm. The trap was immersed in the soil and the sur-

face of the glass was parallel to the land surface. Each trap was filled with 1/4 of 70% alcohol. Furthermore, to prevent water from entering the trap, a cover made of mica plastic was used which was attached to a bamboo pole. Traps were set for 1 x 24 hours, and sampling was conducted once every 1 week for 3 consecutive weeks. In addition, the ants that were caught were then collected and then put into a sample bottle and labelled (Swift & David 2001).

Bait trap

Bait trap is an ant sampling technique by using bait. The baits used in this study were fish and sugar solution. The bait was put into a flat plate which had a diameter of 20 cm, then the plate was placed around the pitfall trap which was ± 25 cm. Sampling with this technique was conducted within 3 - 4 hours. This sampling was conducted once a week for 3 consecutive weeks. The collected samples were then put into sample bottles and labelled modified by (Wielgoss et al. 2010).

Measurement of Environmental Parameters

Measurement of environmental parameters was conducted at each sampling plot, which included measurements of air temperature, soil temperature, air humidity, soil moisture and soil pH.

Sample Identification

The collected samples were then identified at the Entomology Laboratory of the Biology Study Program, Faculty of Biology, Universitas Gadjah Mada and the Insect Laboratory of the National Research and Innovation Agency (BRIN), referred to the book Identification Guide to The Ant Genera of The World (Bolton 1994) and Identification Manual for Bornean Ants (Hashimoto & Rahman 2003).

Data Analysis

The Shannon-Wiener diversity index (H') was calculated using the following formula.

$$H' = -\sum_{i=1}^{n} Pi\ ln\ ln\ Pi$$

To calculate the evenness index (E) of ant species, the following formula was used.

$$E = \frac{H'}{ln\ ln\ (s)}$$

RESULTS AND DISCUSSION

Based on the study which had been conducted, 15 species of ants had been identified in the rubber and oil palm plantations. The collected ants consisted of 39.972 individual ants belonging to 12 genera and 5 subfamilies. The numbers of ant species in the rubber plantations were 14 species with a total of 17.216 individuals while in the oil palm plantations there were 11 species of ants with a total of 22.756 individuals (Table 1).

Based on the study which had been conducted, we found 15 species of ants belonging to five subfamilies and 12 genera.

Subfamily Ponerinae

The subfamily has 14 genera (Nazarreta 2017) but in this group the abdomen slightly curved, mandible varies from linear to triangular, only consists of two genera that are *Odontoponera* and *Odontomachus* (Figure

Table 1. The species, number of individuals and the role of ants in the rubber and oil palm plantations

No	Species	Sampling Location								Role	References
		Rubber Plantations				Oil Palm Plantations					
		1	2	3	4	1	2	3	4		
1	*Anoplolepis gracilipes*	0	2632	1132	8	2376	425	236	1619	Foragers	Haneda & Yuniar 2020
2	*Camponotus arrogans*	1	2	0	0	9	0	0	0	Foragers, Predator	Haneda & Yuniar 2020; Borbély & Nagy 2022
3	*Camponotus dolichoderoides*	650	33	15	1	36	138	15	37	Foragers, Predator	Haneda & Yuniar 2020; Borbély & Nagy 2022
4	*Colobopsis moeschi*	3	24	1	0	0	0	0	0	Foragers, Predator	Haneda & Yuniar 2020; Borbély & Nagy 2022
5	*Camponotus* sp. 1	0	0	0	0	7449	0	3136	4928	Foragers, Predator	Haneda & Yuniar 2020; Borbély & Nagy 2022
6	*Crematogaster* sp. 1	190	0	54	19	0	457	0	7	Predator, Predator	Sholih et al. 2019
7	*Crematogaster* sp. 2	152	286	55	11	0	72	5	0	Predator, Predator	Sholih et al. 2019
8	*Monomorium* sp. 1	9	100	89	8	25	26	18	26	Foragers, harvester	Haneda & Yuniar 2020
9	*Odontomachus rixosus*	3	17	0	2	3	49	10	7	Predator	Haneda & Yuniar 2020
10	*Odontoponera transversa*	23	72	16	25	44	156	31	92	Predators	Cao et al. 2022
11	*Oecophylla smaragdina*	1916	0	1367	0	0	0	0	0	Predator	Pierre & Idris 2013
12	*Pheidole huberi*	0	10	2	5	208	0	8	0	Seed harvester, omnivore, predator, scavenger	Haneda & Yuniar 2020
13	*Polyrhachis proxima*	11	4	8	0	0	0	0	0	Foragers	Haneda & Yuniar 2020
14	*Tapinoma melanocephalum*	234	24	47	7931	0	28	0	1080	Invasive ants, predator	Rubiana et al. 2015
15	*Tetraponera rufonigra*	24	0	0	0	0	0	0	0	Predator	Pawar 2014

1). Characteristics of subfamily Ponerinae are tangible frontal lobes, sting at the end of the abdomen, mesosoma attached to the abdomen through a single petiole, second segment of slightly curved, mandible varies from linear to triangular.

Characteristics *Odontoponera* mandibles are triangular, frontal lobes are narrowly separated posteromedian portion of clypeus, and anterior clypeal margin is armed with 7-9 of distinct teeth (Nazarreta et al. 2021). There is one species of ant from the genus *Odontoponera* that was found in this study; *Odontoponera transversa* has characteristics short mandible and triangular, body has fine hair and is quite thick, rounded head and has strokes like fingerprint (Figure 1a).

Characteristics *Odontomachus* mandibles long and straight, the top of head has V-shaped lines, and upper-front of the head is sometimes with shallow grooves (Nazarreta et al. 2021). There is one species of ant from genus *Odontomachus* that was found; *Odontomachus rixosus* has characteristics elongated mandible and straight, body has spare fine hairs, head oval, gaster oval and tapered (Figure 1b).

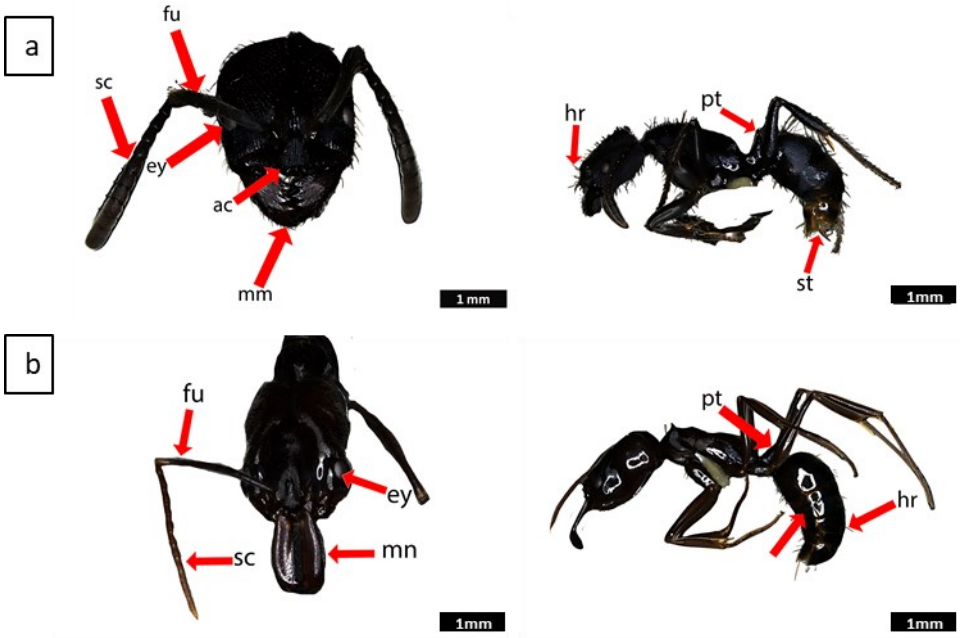


Figure 1. The ants form subfamily Ponerinae (a) *Odontoponera trasnversa*, (b) *Odontomachus rixosus*, (fu) funiculus of antenna, (sc) scape of antenna, (mn) mandible, (ey) eyes, (ac) anterior clypeal, (hr) hair, (st) sting, (pt) petiole, (g) gaster.

Subfamily Myrmicinae

The subfamily has 44 genera (Nazarreta 2017), but we found only three genera; *Crematogaster*, *Monomorium* and *Pheidole* (Figure 2). Characteristic subfamily Myrmicinae has two petioles, a pair of compound eyes which are small and round, the pronotum (the first segment of the mesosoma) is fused with the mesonotum (the second segment of the mesosoma).

Characteristic genus *Crematogaster* post-petiole attached to gaster, gaster viewed from above is roughly heart-shaped (Nazarreta et al. 2021). There are two species of ants that belong to the genus *Crematogaster* was found in this study; *Crematogaster* sp. 1 has characteristic gaster triangularis, rounded head and lot of fine hair, dark-black body (Figure 2a). Meanwhile, *Crematogaster* sp. 2 is characterised by an elongated oval gaster, elongated oval head and spare fine hairs, brownish body (Figure 2b).

Characteristics of the genus *Monomorium* antennae has 3 segment club, post-petiole at most is only slightly wider than long, and front mar-

gin of the clypeus has a single central elongated seta (Nazarreta et al. 2021). There is one species of ant that belongs to the genus *Monomorium*; *Monomorium* sp. 1 has the characteristics triangular mandible and elongated, elongated antennae, eyes clearly visible, antennae 10 segments (Figure 2c).

Pheidole has characteristic antennal scrobes absent, head behind the eye without elongated groove, pronotum forming a high dome like arch (Nazarreta et al. 2021). There is one species of ant that belongs to the genus *Pheidole* was found in this study; *Pheidole huberi* has triangular mandible and short, antenna 12 segments and short, head has strokes like fingerprints (Figure 2d).

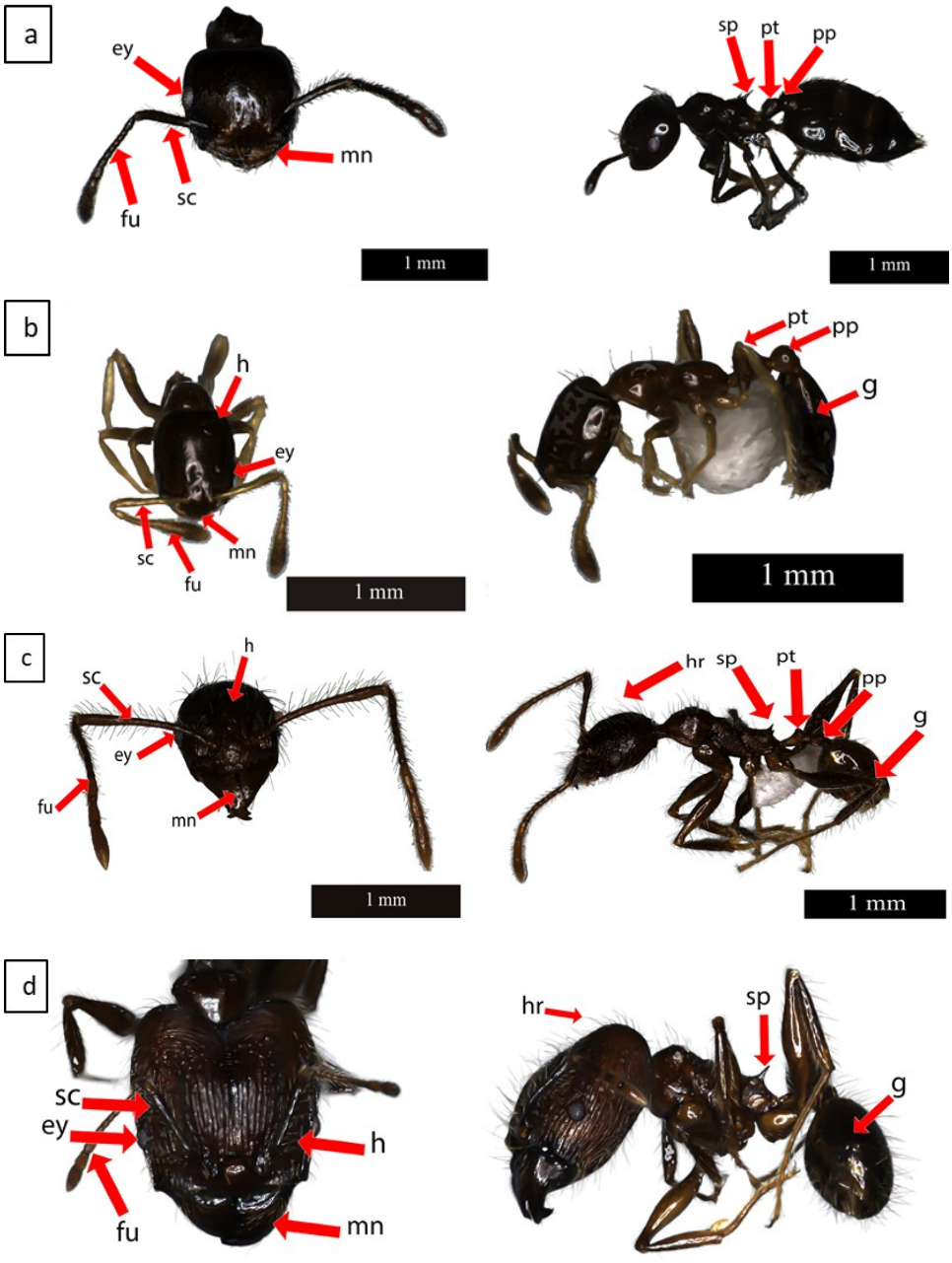


Figure 2. The ants from subfamily Myrmicinae (a) Crematogaster sp. 1, (b) Crematogaster sp. 2, (c) Monomorium sp., (d) Pheidole huberi, (ey) eye, (sc) scape of antenna, (fu) funiculus of antenna, (mn) mandible, (pt) petiole, (pp) postpetiole, (sp) spine, (h) head, (g) gaster, (hr) hair.

Subfamily Pseudomyrmecinae

Only has one genus *Tetraponera*. Characteristics subfamily Pseudomyrmecinae a pair of compound eyes large and elongated, two petioles, pronotum connected to mesonotum by flexible joints (Nazarreta 2017). There is one species was found in this study; *Tetraponera rufonigra* has characteristics head oval and black, thorax red and curved, gaster black (Figure 3).

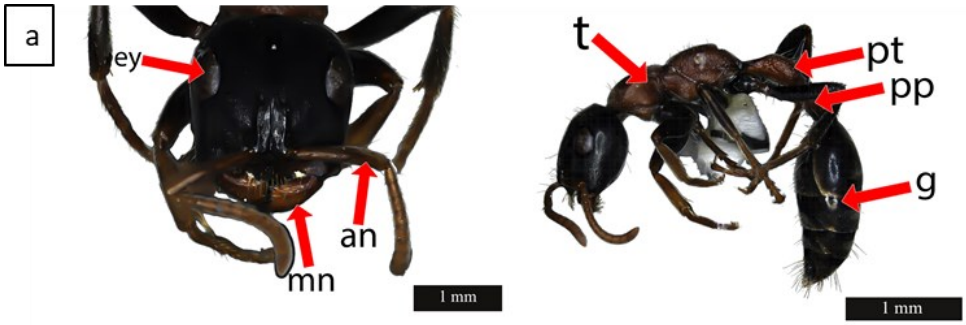


Figure 3. The ant from subfamily Pseudomyrmicinae (a) Tetraponera rufonigra, (mn) mandible, (an) antenna, (ey) eye, (pt) petiole, (pp) post-petiole, (g) gaster, (t) thorax.

Subfamily Dolichorinae

The subfamily has eight genera (Nazarreta 2017), but only genus *Tapinoma* was found in this study. Characteristic subfamily Dolichoderinae has one petiole, acidopore shaped like a slit without any hair around it. Characteristics genus *Tapinoma* petiole is overhung by the first gastral segment, gaster with 4 visible tergites, and the fifth tergite segment is reflected below the fourth (Nazarreta et al. 2021). Only one species was found in this study; *Tapinoma melanocephalum* has characteristic antenna 12 segments, mandible triangular, abdomen consists of 4 segments (Figure 4).

Figure 4. The ant from subfamily Dolichorinae (a) Tapinoma melanocephalum, (ey) eye, (mn) mandible, (sc) scape of antenna, (fu) funiculus of antenna, (pt) petiole, (g) gaster.

Subfamily Formicinae

The subfamily consists of 18 genera (Nazarreta 2017), but only five genera were found in this study; *Polyrhachis*, *Oecophylla*, *Anoplolepis*, *Camponotus*, and *Colobopsis*. Characteristic subfamily Formicinae, one petiole, has acidopore (reduction of the sting) which has short hairs on the edges.

Characteristics genus *Polyrhachis*; antenna 12 segments, mandible subtriangularis, first segment of the gaster smaller than half of the total length of the gaster (Nazarreta et al. 2021). There is only one species of ant that belongs to the genus *Polyrhachis* that is *Polyrhachis proxima*. Characteristics *Polyrhachis proxima* spine on mesosoma (pronotum),

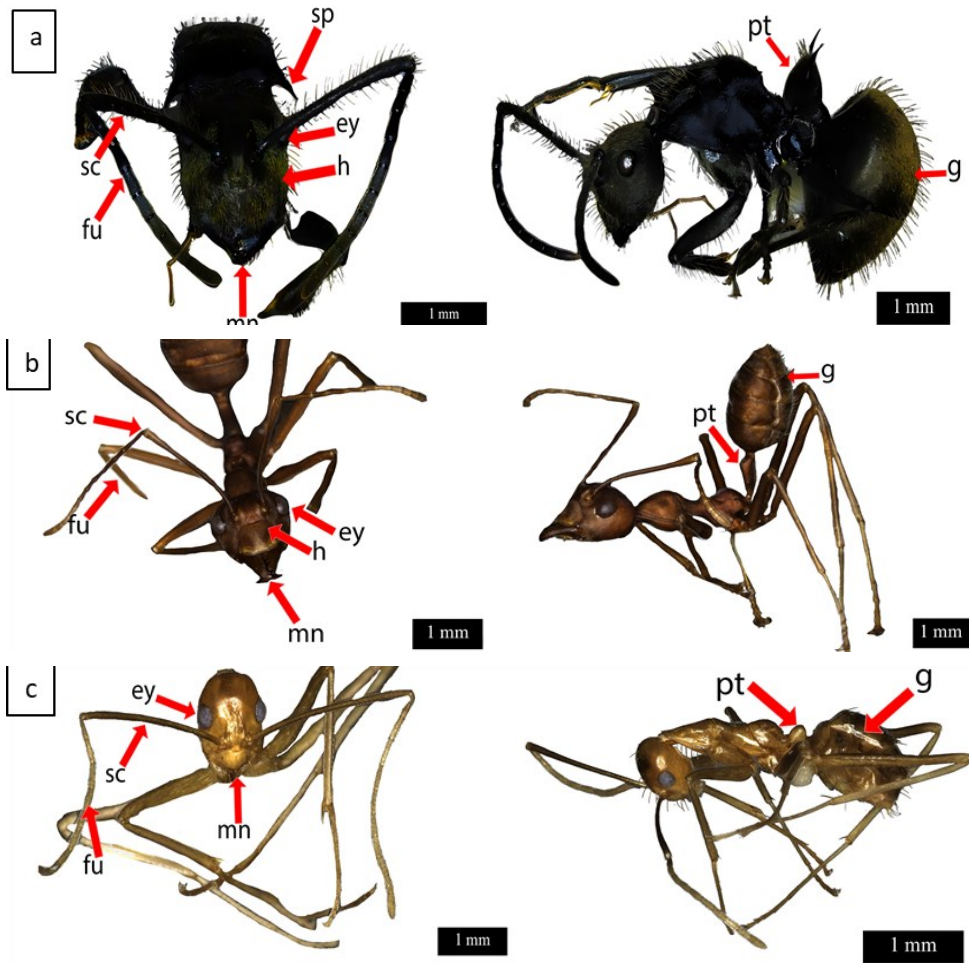
whole body lot of fine hair, body pitch black, gaster round and tapered (Figure 5a).

Characteristics of the genus *Oecophylla* antenna 12 segments, mandible subtriangularis, reduced petiole. Species of ant that belongs to the genus *Oecophylla* that is *Oecophylla smaragdina* has characteristics head without hair, elongated triangular mandible, small eyes that do not extend beyond the sides of the head (Figure 5b).

Characteristics *Anoplolepis* antenna 12 segments, mandible subtriangular, and pronotum elongated. There is one species found in this study, that belongs to the genus *Anoplolepis*; *Anoplolepis gracilipes* has characteristics head oval without hairs, pronotum elongated, long antennal scape (Figure 5c).

Characteristics of *Camponotus* antenna12 segments, mandible subtriangular, antennal socket separate from the clypeus. From this study was found 3 species of ants belonging to the genus *Camponotus* that are *Camponotus* sp. clypeus not clearly visible, whole body black (Figure 5d). *Camponotus arrogans* clypeus clearly visible, head and gaster solid black, mesosoma brown (Figure 5e). *Camponotus dolichoderoides* head heart-shaped, a lot of fine hairs, brown body (Figure 5f).

Characteristic *Colobopsis* having a strongly impressed metanotal groove, raised dorsal face of the propodeum, and compound eyes placed in a relatively posterior position on the head. There is one species was found in this study; *Colobopsis moeschi* has characteristic head oval, there are fine and sparse hairs, and has pale brown body, antennal insertions well separated, occur at about the midlength of the frontal carinae, and clypeus relatively narrow (Ward & Boudinot 2021) (Figure 5g).



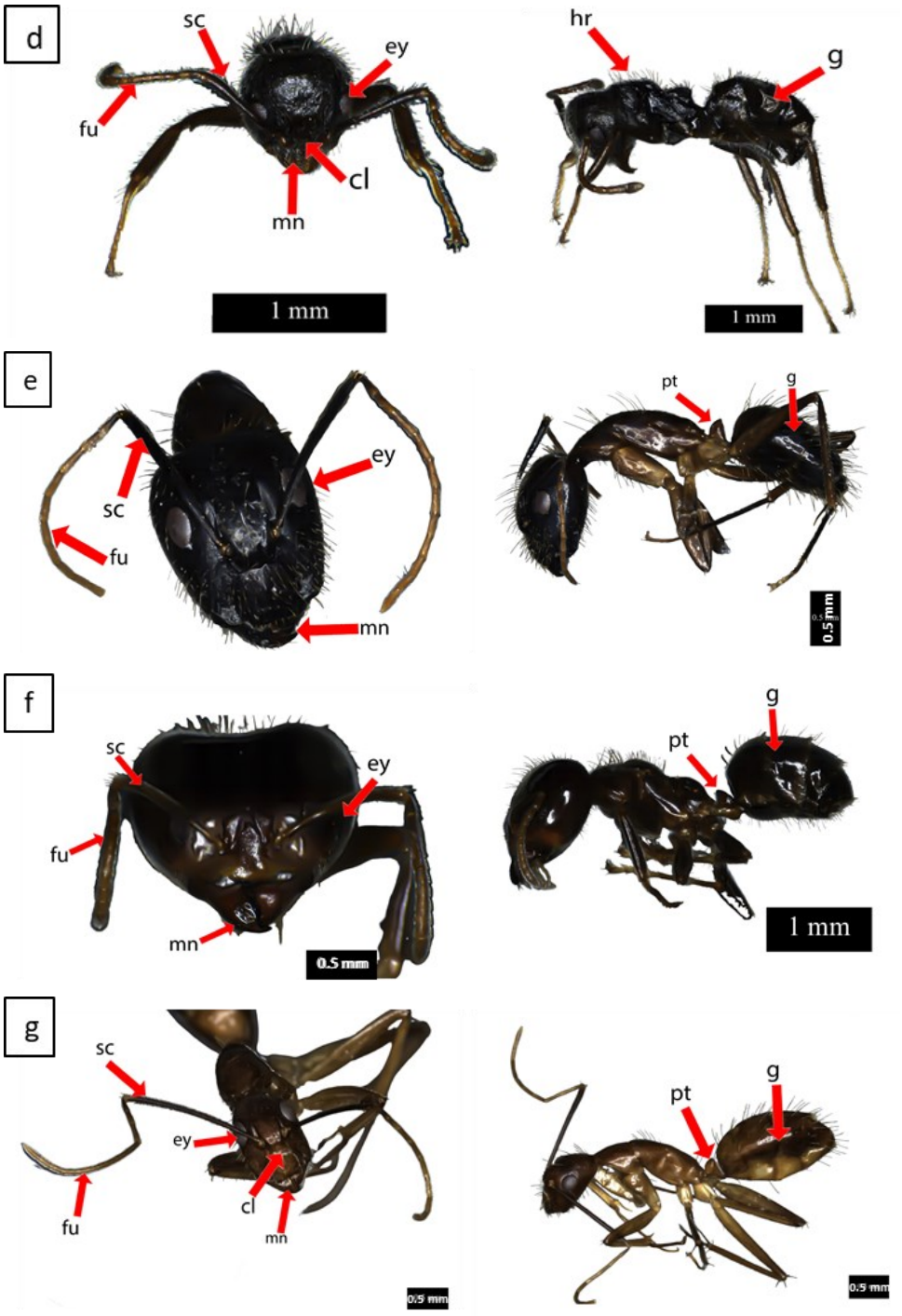


Figure 5. The ants from subfamily Formicinae (a) Polyrhachis proxima, (b) Oecophylla smaragdina, (c) Anoplolepis gracilipes, (d) Camponotus sp., (e) Camponotus arrogans, (f) Camponotus dolichoderoides, (g) Colobopsis moeschi, (mn) mandible, (sc) scape of antenna, (fu) funiculus of antenna, (ey) eye, (sp) spine, (g) gaster, (pt) petiole, (cl) clypeus, (hr) hair (h) head.

Based on the described morphological features, an identification key modified from Nazarreta et al. (2021) to the collected ant species is constructed as follows.

IDENTIFICATION KEY TO ANT SPECIES FROM SUNGAI MERAH VILLAGE SAROLANGUN JAMBI

1. a. Sting present **2**
   b. Sting absent **4**

2. a. Mesosoma attaches to abdomen through single segment (petiole), second segment of abdomen slightly curved, mandible varies from straight (linear) to triangular (Figure 1). .....**Ponerinae (5)**
   b. Mesosoma attaches to abdomen through two segments, has petiole and post petiole ***3***
3. a. Eyes present compound eyes small and round, two petioles, pronotum (first segment of mesosoma) fused with mesonotum (second segment of mesosoma) (Figure 2)..... ..... **Myrmicinae (6)**
   b. Eyes present very large and elongated, two petioles, pronotum connected to mesonotum by flexible joints (Figure 3). .....**Pseudomyrmicinae**; ***Tetraponera rufonigra***
4. a. Has petiole, acidopore (a reduction of the sting) which is shaped like a slit without any hair around it (Figure 4). ... .**Dolichorinae (9)**
   b. Has petiole, acidopore (reduction of the sting) which on the edges there are short hairs (Figure 5). ..... **Formicinae (10)**
5. a. Elongated mandible and straight, body has spare fine hairs, head oval, gaster oval and tapered (Figure 1b). .... ***Odontomachus rixosus***
   b. Short mandible and triangular, body has fine hair and is quite thick, rounded head and have strokes like fingerprints (Figure 1a). ..... ***Odontoponera transversa***
6. a. Antenna 10-11 segments, post-petiole attached to upper gaster (heart-shaped when viewed from above). **7**
   b. Antenna 10 - 12 segments, post-petiole attaches to front gaster (when viewed from above the gaster does not have shape like a heart). **8**
7. a. Gaster triangularis, head rounded and lot of fine hair, dark-black body (Figure 2a) ..... ***Crematogaster* sp. 1**
   b. Elongated oval gaster, elongated oval head and spare fine hairs, brownish body (Figure 2b). ..... ***Crematogaster* sp. 2**
8. a. Triangular mandible and elongated, elongated antennae, eyes clearly visible, antennae 10 segments (Figure 2c). .... ***Monomorium* sp.**
   b. Triangular mandible and short, antenna 12 segments and short, head has strokes like fingerprints (Figure 2d). .... ***Pheidole huberi***
9. a. Antenna 10-11 segments.
   b. Antenna 12 segments, mandible triangular, abdomen consists of 4 segments (Figure 4a). .... ***Tapinoma melanocephalum***
10. a. Antenna 9-11 segments, acidopore clearly visible **11**
    b. Antenna 12 segments, acidopore clearly visible, mandible consists of less than 10..... **12**
11. a. Head oval no hairs, pronotum elongated, long antennal scape (Figure 5c). .....***Anoplolepis gracilipes***
    b. Head no hair, elongated triangular mandible, small eyes do not extend beyond the sides of the head (Figure 5b). .... ***Oecophylla smaragdina***

12. a. Has spines on mesosoma (pronotum), whole body a lot of fine hair, body pitch black, gaster round and tapered (Figure 5a). ..... ***Polyrhachis proxima***
    b. No spines on mesosoma, varies of color **13**
13. a. Head rounded **14**
    b. Head not rounded **15**
14. a. Clypeus clearly visible, head and gaster solid black, mesosoma brown (Figure 5e). ..... ***Camponotus arrogans***
    b. Clypeus not clearly visible, whole body black (Figure 5d). ..... ***Camponotus* sp.**
15. a. Head heart-shaped, lot of fine hairs, brown body (Figure 5f). ..... ***Camponotus dolichoderoides***
    b. Head oval, there are fine and sparse hairs, body pale brown, antennal insertions well separated, occur at about the mid-length of the frontal carinae, and clypeus relatively narrow (Figure 5g). ..... ***Colobopsis moeschi***

The study showed that several species of ants can be found in rubber and oil palm plantations. There are several species of ants which can only be found in rubber plantations, and there are several species of ants which can only be found in oil palm plantations. According to Majeed et al. (2021) ants are cosmopolitan insects, and they can be found in various ecosystems, such as forests, wetlands, water sources, and dry land. Differences in the composition of ant species in a habitat are caused by differences in the response of ants to the habitat. Based on the research, it shows that *Camponotus* sp. is a species which exist only in the oil palm plantations, *Colobopsis moeschi*, *Oecophylla smaragdina*, *Polyrhachis proxima*, and *Tetraponera rufonigra* are the ant species which exist only in the rubber plantations. According to Nazarreta (2017) ants which can only be found in a particular ecosystem, depending on their ability to adapt to that environment, these types of ants are usually referred to as unique species. Therefore, *Camponotus* sp. is a unique species in the oil palm plantations while the unique species in the rubber plantations are *Colobopsis moeschi*, *Oecophylla smaragdina*, *Polyrhachis proxima*, and *Tetraponera rufonigra*.

Similar research on ant diversity had been conducted by Haneda and Yuniar (2015) which was conducted in Bungku Village, Jambi Province. Based on his research, as many as 50 species of ants were identified, belonging to 33 genera and 6 sub families. Siriyah (2016) had conducted research in the Seasonal Forest of Baluran National Park, East Java in which 40 species of ants were identified belonging to 19 genera and 4 subfamilies. Nazarreta et al. (2020) had been conducted a similar a study in Bukit Dua Belas National Park and Bukit Harapan National Park, based on their research it was reported that there are 76,641 ants belonging to 177 species from 7 subfamilies and 54 genera. Compared to these data, the diversity of ants in Sungai Merah Village is lowest.

The ants from genus *Camponotus* were the most numerous in this study, both in terms of the number of species and the number of individuals in both ecosystems. *Camponotus* has a wide distribution so it is very common to find them in large numbers. Ants of genus *Camponotus* can build their nests underground, in dead tree branches, in tree trunks, and plant roots. Ants of this genus are considered opportunistic and generalist in their nesting habits and food sources. Several species of this genus exhibit dietary habits that are eating plants as well as insect exudate, fallen fruit, and insects (Ronque et al. 2018). The ants from genus *Campono-*

tus are known to use crumbs as a source of food; besides, they are also known to be able to eat other insects which have died (Haneda & Yuniar 2020).

Ants from genus *Camponotus* can help control pests, Borbély & Nagy (2022) stated that *Camponotus* ants have a mutualistic symbiotic relationship with apple plants. The disturbing symbiosis of ants and fleas can cause a tremendous decrease in the abundance of fleas due to increased pressure on natural enemies on flea colonies. Moreover, ants from genus *Camponotus* are also known to have a symbiotic mutualism with various plants, since the ants come to the plants to look for nectar, at the same time the ants control pests in these plants (Agrawal & Rastogi 2010). Based on this point, ants from genus *Camponotus* can be used as an effective method which supports environmentally friendly pest control.

The study found several species of ants that have a role as predators such as, *Crematogaster* spp, *Odontomachus rixosus*, *Odontoponera transversa*, *Pheidole huberi*, *Tetraponera rufonigra*, *Tapinoma melanocephalum* and *Camponotus* spp. Haneda & Yuniar (2020) reported that there are several ants that have the potential to be predators in an ecosystem, they are ants from the genus *Odontomachus*, and *Pheidole*. Based on the study of Sholih et al. (2019) *Crematogaster* is the ant that can control *Pseudococcus* sp., bugs on coconut plantations and it has good potential to be used as a predator. According to Rubiana et al. (2015) *Tapinoma melanocephalum* is an invasive ant that can be found in rubber and oil palm plantations, this ant replaces the role of the original ants, due to land conversion and becoming a predatory ant. Moreover, according to Cao et al. (2022) *Odontoponera transversa* is known as a predator. Apart from playing a role as predators, some ants also play a role as foragers such as, the ants from the genera of *Anoplolepis*, *Camponotus*, *Monomorium* and *Polyrhachis* (Haneda & Yuniar 2020). The predatory behaviour of *Oecophylla smaragdina* towards *Pteroma pendula* was confirmed by (Pierre & Idris 2013). *Oecophylla smaragdina* also known as predator of bagworms (*Metisa plana*) in oil palm plantations (Exélis et al. 2023). In rubber plantations, *Oecophylla smaragdina* is known as predator of beetle *Luprops tristis* (Aswathi et al. 2011).

Based on the number of individual ants collected, it shows that the number of individual ants in the rubber plantations is less than the number of individual ants in the oil palm plantations, due to habitat disturbance by humans. The frequency of human activities is higher in the rubber plantations than in the oil palm plantations. Haneda & Yuniar (2015) stated that the activities conducted by humans in rubber plantations have a higher intensity, almost every day rubber farmers conduct tapping activities of rubber trees whereas in the ecosystem of oil palm plantations farmers harvest palm fruit every 2 weeks and care routinely conducted within 4-6 months, relatively long intervals can provide an opportunity for disturbed ant communities to recover.

According to Yaherwandi et al. (2019) habitat modification and forest conversion into rubber and oil palm plantations changes the ant community structure. Habitat changes and disturbances can change the composition of ants by affecting changes in the interaction of tropical ecosystems and food webs. Conversing the land from forests into industrial forest affects the biodiversity of predatory insects, especially ants. The richness and diversity of ant species increase with temperature, and they decrease with decreasing rainfall and altitude. Ants are also sensitive to environmental disturbances. Therefore, ants are often used as bioindicators of health and ecosystem function (Nooten et al. 2019).

Table 2. Analysis of Diversity Index and Evenness Index.

Index Analysis	Sampling Location	
	Rubber Plantations	Oil Palm Plantations
Diversity (H')	1.44	0.99
Evenness (E)	0.54	0.41

The diversity index calculated using the Shannon – Wienner diversity index shows the result that the ant species diversity index in the rubber plantations is moderate with a value of 1.44 while the ant diversity index in the oil palm plantation is relatively low with a value of 0.99. The purpose of calculating this index is to determine the degree of diversity of an organism in an ecosystem. Furthermore, the parameters which are used to determine the diversity index value in an ecosystem are determined by the number of species and the relative abundance of species in an ecosystem. The results of the analysis which had been conducted show that the rubber plantations are more stable when compared to the oil palm plantations. It is not in line with the research which had been conducted by Yaherwandi et al. (2019) that the biodiversity of ants in oil palm plantations is higher than in rubber plantations, secondary forest, and primary forest. This discrepancy can be caused by several factors such as, temperature and humidity so that the diversity of ants in the rubber plantations is more stable.

In our study, low ant diversity in the oil palm ecosystem was affected by domination of one species of ant *Camponotus* sp. The species is only found in the palm oil plantations with the number of individuals reaching 15,513 which is equivalent to 90.1% of the total number of ants found in the rubber plantations. It corroborates the statement of Putra et al. (2021) that diversity is high if the number of individuals of each species found does not occur in inequality one of the species. Otherwise, if it is composed of only one species or only a few species, then the diversity is low. Diversity can also be said to be low if there are several species but with an unequal number of individuals. The existence of a high number of individuals in one species found can also cause a low value of the diversity index at that location.

Calculation of the evenness index shows that the evenness of the ant population in the rubber plantations is moderate with a value of 0.54 while the evenness of the ant population in the oil palm plantations is relatively low with a value of 0.41. It shows that the distribution of individuals in the rubber plantations is more even when compared to the oil palm plantations. Krebs (2014) stated that evenness values range from 0-1 and it is divided into several categories, such as, low community category with a value of less than 0.5; unstable category with a value of 0.5-0.75; and stable category with a value of 0.75-1.

The results of the t-test show that the diversity (H') and evenness (E) of ants in each plot in the rubber and oil palm plantations do not show a significant difference. It shows that there is no significant difference in the factors which support the existence of ants in the two ecosystems. In addition, factors which support ants to live and develop in an ecosystem include sufficient food sources, places to nest and microclimate (Andersen 1995).

Environmental parameter measurements showed that ants can still be found in an ecosystem that have soil temperatures in the range of 25.6 – 27°C. air temperatures in the range of 27.7 – 31.7°C. soil moisture in the range of 36.7 – 73.3% and humidity in the range of 71 – 89%. Based on Riyanto (2007) the optimal and tolerance temperature range for ants in the tropics ranges from 25 – 32°C. Some ants leave the nest to find

Table 3. Measurement of environmental parameters in the rubber and oil palm plantations.

Plot	Environmental Parameters				
	Soil Temperature (°C)	Air temperature (°C)	Soil moisture (%)	Humidity (%)	pH
RP 1	26.3±2.1	28.7±3.6	40.0±17.3	81.0±9.2	6.3±0.3
RP 2	26.3±2.6	31.7±8.7	56.7±5.8	71.0±21.6	5.9±0.1
RP 3	25.6±2.6	28.3±4.1	46.7±5.8	88.7±3.1	6.3±0.1
RP 4	26.3±1.6	27.7±3.1	50.0±0.0	89.0±2.7	6.6±0.0
OPP 1	27.0±2.7	29.3±3.8	36.7±5.8	83.7±8.0	6.8±0.0
OPP 2	26.3±1.6	29.0±3.7	73.3±5.8	78.0±14.7	6.4±0.2
OPP 3	27.0±1.8	29.0±3.5	43.3±5.8	77.7±15.6	6.5±0.2
OPP 4	26.3±2.1	29.3±3.8	70.0±0.0	79.3±12.0	6.46±0.1

food at a surface temperature of 10 - 45°C. According to Latumahina et al. (2015) the average temperature and humidity which can affect the distribution and development of ants is at 27°C and 85% humidity. At a too high temperature, several physiological processes such as reproduction, metabolism, and respiration of ants will be disrupted. The air temperature suitable for the growth of ant ranges from 15 - 27°C, where higher temperatures are tolerable if there is a sufficient shade with optimal humidity. Ants are known to prefer a cool, humid, and not too hot air for their daily activities and reproduction (Shattuck 2000). In addition, the ideal pH value for ants to live and grow is 4.5 – 6.8 (Suin 1997).

The results of the ANOVA one way show that the environmental parameters which have a significant difference between the ecosystems were soil moisture and pH. It implied that soil moisture and pH have a role in the presence of ants in the ecosystem of rubber plantations and oil palm plantations in Sungai Merah Village.

CONCLUSIONS

Based on the study, 15 species of ants have been identified in the ecosystem of rubber plantations and oil palm plantations in Sungai Merah Village. Based on the diversity analysis (H') the diversity of ants in the rubber plantations ecosystem is more stable compared to the diversity of ants in the oil palm ecosystem. Based on the evenness analysis (E), the evenness of ants in the rubber plantations is medium and in oil palm plantations the evenness of ants is low.

The ants found in both of plantations have an important role for the ecosystem, some species play a role as predator (*Crematogaster* spp, *Odontomachus rixosus*, *Odontoponera transversa*, *Pheidole huberi*, *Tetraponera rufonigra*, and *Tapinoma melanocephalum*), meanwhile the ants from the genera *Anoplolepis*, *Camponatus* spp., *Monomorium*, and *Polyrhachis* play a role as foragers, *Oecophylla smaragdina*, *Camponotus* spp., *and Crematogaster* also known as biological control agents in the ecosystem.

AUTHOR CONTRIBUTION

Research concept, design, collected, data analysis, manuscript drafting: N.L.F., supervised all the processes, reviewed, and proofread the final manuscript: H.P., former supervisor, review research design: R.C.H.

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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Review Article

Utilisation of Snails for Wound Healing: A Review

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ABSTRACT
Snails exhibit remarkable adaptability, allowing them to flourish in diverse environmental conditions and resulting in thriving populations in specific regions. This abundance has led communities to harness snails for various purposes, including their use as animal feed, daily dietary source, and in traditional wound-healing practices with historical roots. The primary objective of this systematic review is to identify the snail species commonly employed in wound healing and evaluate the bioactivity of compounds derived from different snail species. This review was conducted using literature review method, drawing from international databases such as Scopus, and encompassed publications from 2013 to 2023. A total of 22 articles met the inclusion and exclusion criteria. Snail body parts that have been explored for wound-healing purposes include both the body and the shell, along with snail secretions, particularly their mucus. Various methods have been employed to extract mucus, involving manual stimulation of the snail's body, spraying with a saline solution (NaCl), application of electric shock, and the use of ozone gas through nebulisation. Prominent snail species found to be beneficial for wound healing include *Achatina fulica*, *Helix aspersa*, *Eobania desertorum*, *Helix lucurus*, *Cornu bistrialis*, *Theba pisana*, and *Megalobulimus lopesi*. These snail species demonstrate potential applications in the treatment of burns, excision wounds, incision wounds, and diabetic ulcers. Key compounds within snail secretions encompass mucopolysaccharides, polyphenols, peptides, and glycosaminoglycans. These compounds exert significant effects on haemostasis, inflammation control, cellular proliferation, and re-epithelialisation, significantly contributing to the wound healing process.

INTRODUCTION
Molluscs are an abundant group of animals that serve a vital part in the animal kingdom's trophic hierarchy. All of snail's part are useful especially its mucus, shell, and body without shell. Snail's mucus is a mucous fluid that shield the whole surface of the animal and secreted by particular salivary epidermal glands on the snails feet (Greistorfer et al. 2017). It was used by snails for various purposes, including movement, self-defense, identifying prey, and mating (Newar & Ghatak 2015).

Molluscs contain several thousands of bioactive chemicals. It includes peptides, terpenes, sterols, polypropionates, nitrogen compounds, fatty acid derivatives, macrolides, and alkaloids (Ulagesan et al. 2018). Snail mucus mostly consists of large polymers that are rich in carbohydrates and some small proteins that can relieve stomach pain because the

mucus can neutralise stomach acidity and gastroesophageal reflux (Benkendorff et al. 2015). Furthermore, snail flesh contains high levels of protein, vitamins, and omega-3-fatty acids. It has a healthy balance of essential amino acids, including lysine, isoleucine, leucine, and phenylalanine (Kehinde et al. 2020).

Snails have been used for generations to treat a wide range of medical situations (Benkendorff et al. 2015). The mucus obtained from snails is applied to the skin to manage dermatitis, inflammation, acne, calluses, and to speed up wound alleviating (Ulagesan et al. 2018). Additionally, it can be used in respiratory problems and stomach pain (El-Zawawy & Mona 2021). Several bioactive chemicals have been studied, especially for their cytotoxic, antimicrobial, antitumor, antileukemic, antineoplastic, and antiviral properties in this gastropods group (Ulagesan et al. 2018; El-Zawawy & Mona 2021).

Wound healing is a complicated process that restores impaired cells and tissue to normal. It is a biological response to injury that involves the activation of fibroblasts, macrophages, and endothelial cells. Apart from that, a proper integration of the biological and molecular mechanisms of cell migration as well as proliferation is also required (Ulagesan et al. 2018). The recovery process is divided into four phases: haemostatis, inflammation, proliferation, and remodeling. These phases serve as a framework for considering the fundamental concepts of wound healing. Through these considerations, medical professions can improve their ability to care for injured bodies and assist in healing complex tissues. Wounds that never heal encourage health workers to look for the main cause that has not been resolved (Pawar & Shamkuwar 2023). Healing chronic wounds requires patient-centered, comprehensive, evidence-based therapy, interdisciplinary, and cost-effective (Pawar & Shamkuwar 2023).

Because the large population of snails and their compound content have health potential, as well as the high number of injuries that occur in the world, a literature review about the use of snails for wound healing is required. Therefore, this literature review can be used as a source of information to find out the potential of snails for the health sector, especially healing various wounds.

METHODS

This article was written using the literature review method (Figure 1). This method required international indexed article sources obtained from the Scopus website. The keywords used in searching for article sources were wound and slime. There were inclusion and exclusion criteria for selecting articles based on the year the article was published, type of article, language, open access, and type of source. After that, selection was carried out based on related topics through screening of article titles and abstracts.

The inclusion criteria of this article were articles on the topic of wound healing, using primary data and publication year <10 years, 2013-2023. The articles used were original articles and full text indexed by Scopus Q1-Q4, as well as were open access. The selected articles must be in English.

The exclusion criteria for this article were topics on tumor cell line, tumor invasion, cancer prognosis, carcinogenesis, cancer growth, tumor growth, cancer staging, tumor xenograft, liver cell carcinoma, breast cancer, lung tumor, cancer cell, cancer survival, tumor marker, stomach cancer, cancer tissue, and colorectal cancer. Articles in the form of review journals, short reposts, and case reports are also not used in this article.

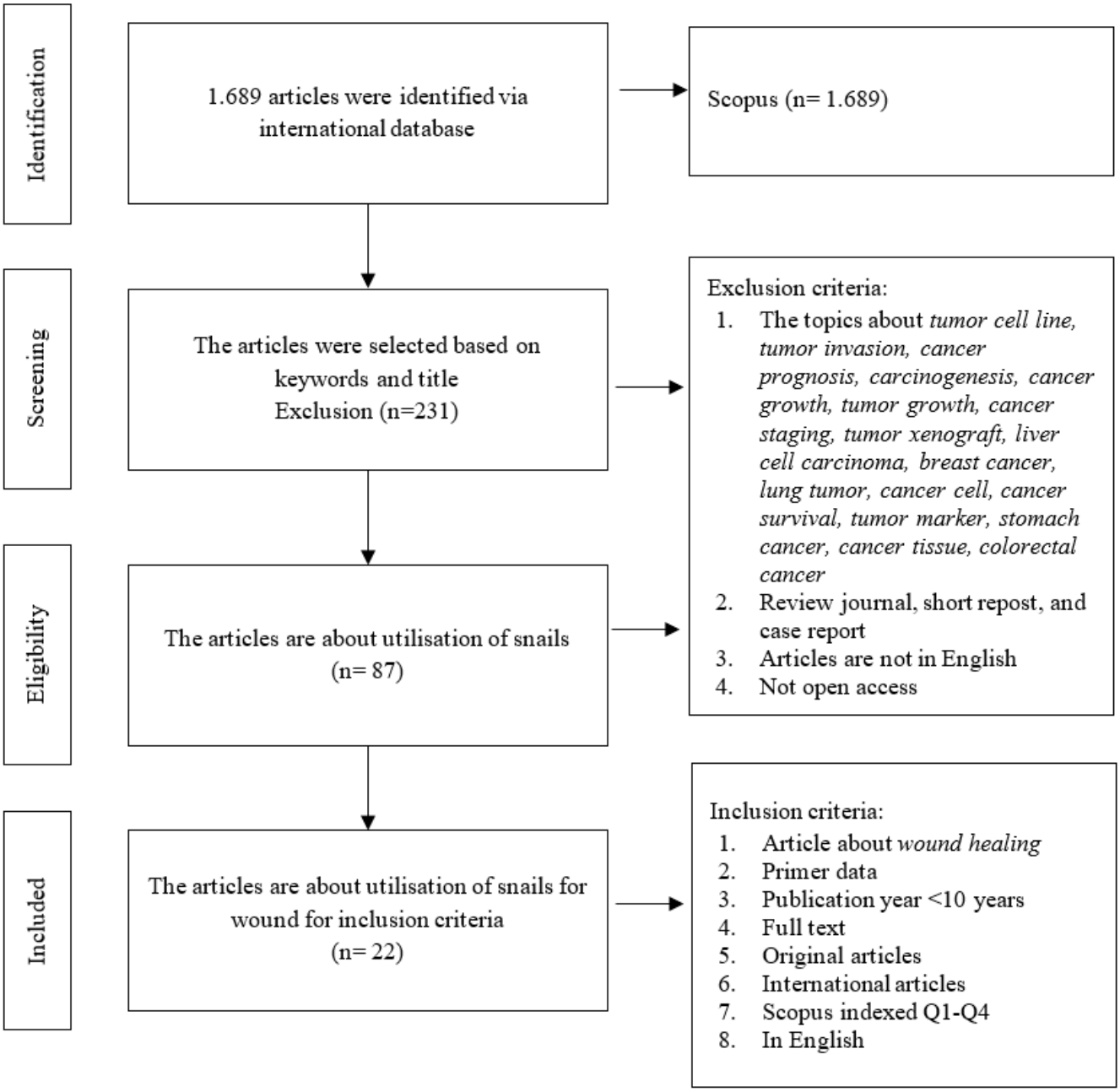

Figure 1. PRISMA diagram of article selection in systematic reviews.

In addition, this article did not use articles that are not in English and are not open access.

RESULTS AND DISCUSSION

In the identification stage, there were 1,689 articles found through international databases. The international database used in the article search is Scopus. In the screening stage, articles found in the identification stage were then selected based on keywords "wound" and "snail", as well as article titles, so that 231 articles were excluded from related topics. After that, at the eligibility stage, articles related to snail utilisation were reviewed. Then, at the included stage, articles related to the use of snails in wound healing with inclusion criteria were selected.

Snail mucus collection

Snail mucus can be obtained by various methods. The amount of mucus obtained differs depending on the species and method used. Once the mucus is collected, it can be stored in different ways as shown in Table 1.

Table 1. Snail's mucus collection methods.

No.	Species	Amount	How to collect mucus	How to store	Reference
1.	*Helix aspersa*	15 snails produced 100 mL of mucus	Manually stimulating the pedal glands in the legs	Sterilized with a 0.45µm membrane and stored at -80°C, lyophilized to store in a dry state	El-Zawawy & Mona 2021
		500 snails (about 10 kg) produced 600 mL of mucus	The snails were sprayed with 3% NaCl and waited for 45 minutes	After sterilized with a peristaltic pump and 0.2 µm filter, the samples were kept at 4°C or -80°C.	Gentili et al. 2020; Mencucci et al. 2021
2.	*Helix aspersa muller*	-	Stimulated manually with the tip of a sterile cotton swab	Sterilized with filters measuring 10 µm, 1 µm, and 0.22 µm, then stored at 4°C	Gugliandolo et al. 2021a
		-	Using ozone gas in a few hours	Directly used in the next process as soon as possible	Gubitosa et al. 2020
3.	*Eremina desertorum*	15 snails produced 100 mL of mucus	Manually stimulating the pedal glands in the legs	Sterilized with a 0.45µm membrane and stored at -80°C	El-Zawawy & Mona 2021
4.	*Achatina achatina*	-	The snail is euthanized with an electrical shock of 5 – 10 volts for 30 – 60 s	Lyophilised then stored at temperature 4°C	Nworah et al. 2022
5.	*Eobania vermiculuta*	-	Stimulated manually by a sterile needle	Lyophylised and stored at -20°C	El-Attar et al. 2022
6.	*Achatina fullica*	-	Touching and pressing the snail's body by a sterile glass stick	Centrifuged at 10×g for 5 minutes, stored at 4°C	Agustina et al. 2020
		-	Stimulated by an electric shock 5 - 10 volt for 30 – 60 s	Macerated 24 h at 40° C, processing through precipitation	Harti et al. 2018
		50 snails produced 550 – 600 mL of mucus	Stimulated by an electric shock 1.5 volt for 30 – 60 s	Stored in the freezer	Putri et al. 2020
		A snail produced 3 – 5 mL mucus	Swiped by the tip of a syringe	Directly used in the next process as soon as possible	Igaap et al. 2023

Snail mucus can be obtained by stimulating the snail pedal using the tip of a tool such as a sterile cotton swab and sterile needle (Gugliandolo et al. 2021a; El-Attar et al. 2022). The mucus that has been obtained is sterilised to maintain the pH of the mucus using various filter sizes in micrometers. This is also done to remove impurities and endotoxins that can hinder the injection of mucus for chemical characterisation (Gugliandolo et al. 2021a).

Apart from physical stimulation, to get mucus, snails can be sprayed with 3% NaCl. Giving low concentrations of NaCl can cause stress in the snails so that mucus can be produced, and the mucus is collected in sterile tubes. Mucus collection was carried out for 45 minutes. After that, the snails are given water and returned to their habitat. The process that takes place in this method does not cause death to the snails (Gentili et al. 2020). Electric shock with varying electrical intensities and durations, 1.5 – 10 volts for 30 – 60 seconds, can also be used to collect snail mucus (Putri et al. 2020; Nworah et al. 2022). Although this procedure was simple and effective, the snails that were employed will die.

For an advanced method, snail mucus can be obtained by natural

gases such as ozone. The mechanical extraction entailed the application of technology, which allowed us to obtain the mucus in a matter of hours. Specifically, the snails were put in a device that nebulized ozone for roughly an hour; the O_3 creates a kind of intensity that drives mucus production while minimising stress for the snails (Gubitosa et al. 2020).

The storage of snail's mucus can be achieved by either keeping fresh mucus at low temperatures or by lyophilizing it before processing and long-term storage. Longer lasting storage of mucus can be done by drying it via the lyophilisation method overnight. From this process a solid powder will be obtained which can be used for biological characterisation (El-Zawwy & Mona 2021). Fresh mucus is typically preserved in a freezer at temperatures ranging from -80°C to -20°C or in a refrigerator at 4°C.

Identification of snails for wound healing

This review has identified snail species with promising medicinal potential for wound healing. The utilised parts for wound healing include the body, shell, and mucus. Within the snail's body parts, a range of compounds with roles in wound healing have been discovered, and their biological effects are documented, as presented in Table 2.

Healing of excision wounds in mice can occur more quickly by Snail Secretion Filtrate (SSF) from *H. aspersa muller's* SSF can significantly increase the speed and percentage of wound closure. Based on histology, the re-epithelialisation process assisted by SSF was also better than that which was not given SSF. This is related to an increase in the amount of collagen in the wound area treated with SSF. Collagen is a part of the extracellular matrix which has a major involvement in wound healing at each phase (Fleck & Simman 2010). Apart from that, SSF also helps in the creation, deposition, and maturation of new collagen. This event is modulated by metalloproteinases (MMPs) which are key to wound matrix modification (Gugliandolo et al. 2021a).

Diabetes mellitus patients have a sluggish recovery of the wound process. Diabetes mellitus is a metabolic illness identified by chronic hyperglycemia that leads to a variety of consequences, including foot ulcers and poor wound healing. Despite receiving sufficient and prompt care, diabetic patients' wounds could remain for weeks. Fibroblast proliferation in the later stages of wound healing is associated with the recovery of structure and function at the wound site (Ulagesan et al. 2018). Diabetes impairs macrophage responses and the phenotypic transition from M1 to M2 (Deng et al. 2023).

The use of Cb-peptide ointment on diabetes-induced excisional wounds can accelerate wound contraction and re-epithelialisation. Diabetes-induced cuts also showed an increase in tensile strength when treated with Cb-peptide ointment compared to the control group due to a rise in collagen concentration and fiber stabilisation. This shows that collagen has an important function in wound healing (Ulagesan et al. 2018). Additionally, mucus *A. fulica* demonstrated a healing effect on diabetic wounds, which assists in encouraging the transformation of wound recovery from the inflammatory to the proliferative stage (Deng et al. 2023).

The active material composition of snail mucus contributed to its wound healing potential (Figure 2). Chemicals found in snail's mucus included achatin isolates and heparan sulfate. The achatin isolates were antibacterial and analgesic, and calcium aids in haemostasis. Snail mucus' antibacterial and antiinflammatory characteristics accelerated the inflammatory and proliferative stages of wound healing (Harti et al. 2018).

Table 2. Types of snails whose mucus is used in wound healing

No.	Species	Part of body	Utilisation	Compound	Effect	Reference
1.	*Achatina fulica*	Mucus	Burns, cuts	Protein, glycosaminoglycans, acharan sulfate, allantoin, metallic element	Increasing the number of basal epithelial cells	Putri et al. 2020; Song et al. 2021; Nworah et al. 2022; Deng et al. 2023
2.	*Helix aspersa*	Mucus	Excision wound	Thiophene, 3-(decyloxy) tetrahydro-1,1-dioxide, 4-(nonafluoro-tert-butyl) nitrobenzene, glycosaminoglycans, glycolic acid, allantoin, polyphenols, sugar, collagen, mucopolysaccharides	Antimicrobial, increases cell migration and speed of tissue repair, anti-inflammatory	Gentili et al. 2020; Gugliandolo et al. 2021a; El-Zawawy & Mona 2021
3.	*Helix aspersa muller*	Mucus	Wound repair, potential for skin damage caused by pollution, gastric ulcers	Glycolic acid, allantoin, polyphenols, mucopolysaccharide, hyaluronic acid, collagen, elastin, vitamin A, vitamin B, vitamin E, copper, nickel, chromium	Cell proliferation and migration, antimicrobials, skin protection, protects against 0_3 exposure by preventing oxidative damage and pro-inflammatory responses, regulation of inflammation	Trapella et al. 2018; Gentili et al. 2020; Gugliandolo et al. 2021b
4.	*Eremina desertorum*	Mucus	Wound	7-bromoheptyl ethyl ester, methyl 1,2-benzisothiazole-3-acetate, 3H-1,2,4-triazole-3-thione, 4,5-dihydro-4,5-diphenyl,	Antimicrobial, anti-inflammatory, cell proliferation and migration are regulated by TGF-β1 and VEGF gene expression	El-Zawawy & Mona 2021
5.	*Helix lucorum*	Mucus	Wound	Protein, glycosaminoglycans, allantoin, metallic element	Decreasing the blood loss in haemostasis	Deng et al. 2023
6.	*Cryptozona bistrialis*	Mucus peptides	Excision and incision wounds	Peptides	Antimicrobial, fibroblast proliferation, collagen synthesis	Ulagesan et al. 2018
7.	*Tibia curta*	The body of a snail without a shell	Excision wound	Fatty acids, sterols, alkanes, amino acids	Reduction of skin thickness	Ragi et al. 2016; Pawar & Shamkuwar 2023
		Shell	Excision wound	-	Reduction of skin thickness	Pawar & Shamkuwar 2023
8.	*Megalobulimus lopesi*	Shell	Diabetic ulcer	Calcium carbonate	Accelerates wound closure, stimulates angiogenesis, increases calcium concentration in the wound area, controls the expression of cytokines and growth factors	Andrade et al. 2018

Heparan sulfate, a component of snail mucus that affects fibroblast proliferation, was beneficial in accelerating wound healing by aiding in blood coagulation and fibroblast cell proliferation. Heparan sulfate also enhances angiogenesis by reducing vascular endothelial growth factor (VEGF) and decreasing the mitogenic activity of fibroblast growth factor (FGF) (Vieira et al. 2004; Harti et al. 2018).

Chitosan in *A. fulica* had the best results in vitro for lymphocyte proliferation activity, outperforming 100% snail's mucus and 5% snail mucus cream. Leukocytes and their differentiation provide body defenses for mice. White blood cells, or leukocytes, are some of the most active blood cells in the body's defense mechanism (Joe et al. 2004; González-Lamothe et al. 2009). White blood cells identify and eliminate infections during immunological reactions, as well as aiding in inflammation and healing (Rajakaruna et al. 2002; Fadillah & Santoso 2019). Snail's mucus and 5% chitosan can be used to create galenic anti-inflammatory lotions. In vitro, snail mucus creams and chitosan galenic formulations were helpful for lymphocyte proliferation and wound healing. (Harti et al. 2018).

Chronic wounds are treated with antibiotics, anti-inflammatory treatments, or a combination of the two, but some of these medications have a number of side effects. As a result, safer alternatives are required. Several investigations have been conducted to develop optimal clinical wound healing biomaterials (Ulagesan et al. 2018). In addition to the concentration of active substances such as snail's mucus, using ointments or gels as part of the preparation might prevent adverse effects by lowering the total dose required to achieve the aim (Goyal et al. 2016; Whittam et al. 2016; Refiani et al. 2021).

Mucus composition changes depending on species and mechanical factors such as temperature, light intensity, humidity, food supply, and soil conditions. The physical features of snails, like as colour and mucus viscosity, are also influenced by environmental factors. *H. aspera* snail mucus is colourless and thinner than *E. desertorum* (desert snail) mucus, which is somewhat hazy white and viscous. Desert snails' high viscosity mucus works as a barrier, reducing moisture loss and protecting them from bacterial diseases (El-Zawawy & Mona 2021).

H. aspersa muller's mucus extract promotes mammalian fibroblast survival, proliferation, and migration. Fibroblasts are the predominant cell type in granulation wound tissue. Fibroblasts play a vital role in wound healing by secreting growth factors that promote proliferation, angiogenesis, and matrix deposition (Ulagesan et al. 2018). The biological effects of snail's mucus on cell proliferation and migration may have consequences for wound healing and therapeutic drug development (Trapella et al. 2018). *H. aspersa muller*'s mucus contains mucopolysaccharide, polyphenols, hyaluronic acid, and other bioactive compounds, as well as minerals (Gugliandolo et al. 2021a).

The key compounds found in snails contributing to wound healing include the following:

a. Mucopolysaccharide

   H. aspersa muller's mucus contains mucopolysaccharide, which enhances mucus adhesion to the skin, and polyphenols, which have the potential to prevent oxidative damage. Moreover, this species' mucus can stimulate endogenous hyaluronate synthesis, boosting the skin's water binding capacity and viscoelasticity (Trapella et al. 2018; Gentili et al. 2020). Increasing mucus adhesion to the skin, which serves as a barrier, can protect epithelial cells from pollution (Gentili et al. 2020).

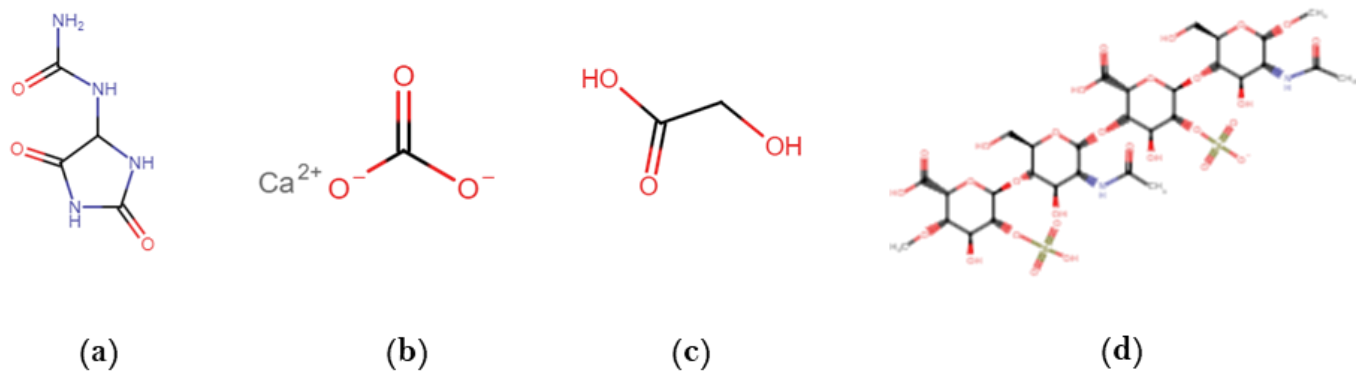

Figure 2. Chemical structure of compounds in snails that have functions in wound healing; allantoin (a), calcium carbonate (b), glycolic acid (c), acharan sulfate (d).

b. Polyphenols

Polyphenols in *H. aspersa*'s mucus can activate defense mechanisms such as the NRF2 pathway, triggering an antioxidant response capable of correcting tissue redox imbalances caused by O_3. Through the activation of NRF2, a mixture of natural compounds such as vitamin C, vitamin E, and ferulic acid can reduce ozone-induced oxidative stress in keratinocytes, RHE, and human skin, indicating that the harmful effects of ozone can be modulated by tissue antioxidant responses. Polyphenols can also help to prevent and treat pollution-induced cutaneous oxidative damage (Gentili et al. 2020).

c. Peptides

One of the finest examples is the use of tiny compounds that are both inexpensive and functional in increasing the production of endogenous wound healing agents such as peptides. Peptides are biomaterials that have many bioactivities related to wound healing (Ulagesan et al. 2018).

Peptide of *Cryptozona bistrialis* (Cb-peptide) significantly increased the response to migration in the scratch wound test. In addition, Cb -peptide also shown considerable cellular activity in diabetes-induced excisional wounds (using Alloxan), including maximum collagen deposition, blood vessel regeneration, and significant epithelialisation (Ulagesan et al. 2018).

d. Glycosaminoglycan

In general snail's mucous contains glycosaminoglycan with acharan sulfate sulfate. It has the repeated disaccharide units of →4)-2-acetamido-2-deoxy-α-D glucopyranose (1→4) -2-sulfo-α-L-idopyranosyluronic acid (1→(GicNAc-IdoA2SO3-) (Joo et al. 2005; Putri et al. 2020). Glycosaminoglycan molecules are composed of carbohydrates, uric acid, dissolved globular proteins, and oligoelements (calcium, copper, iron, and zinc). Glycosaminoglycans of snails (*A. fulica*) are related to the heparin sulfate family and serve to accelerate wound recovery by increasing blood coagulation and fibroblast cell proliferation (Vieira et al. 2004; Agustina et al. 2020).

Microbial infection is the main factor influencing the wound healing process (Ulagesan et al. 2018). To prevent infection from microbials, much research has been done on antimicrobial peptides because they have a wide biochemical diversity and specialisation regarding antiviral, antibacterial, antifungal, antiprotozoal, and antitumor or wound healing effects (Ulagesan et al. 2018). The antibacterial activity is also possessed by several types of snails as shown in Table 3.

Table 3. The antimicrobial activity of various snail's mucus extracts

No.	Species	Types of microbes	Reference
1.	*H. aspersa*	*Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Aspergillus niger, Rhizopus stolonifer, Trichoderma harzianum, Candida albicans*	El-Zawawy & Mona 2021
2.	*E. desertorum*	*P. aeruginosa, E. coli, S. aureus, A. niger, R. stolonifer, T. harzianum, C. albicans*	El-Zawawy & Mona 2021
3.	*C. bistrialis*	*S. aureus, P. aeruginosa, C. albicans, M. racemosus*	Ulagesan et al. 2018
4.	*Pomacea canaliculata*	*S. aureus, Methicilin-Resistant Staphylococcus aureus (MRSA), S. epidermidis, Corynebacterium sp.*	Nantarat et al. 2019
5.	*Lissachatina fulica*	*S. aureus, MRSA, S. epidermidis, Corynebacterium sp*	Nantarat et al. 2019
6.	*A. fulica*	*C. albicans, Penicillium chrysogenum, Aspergillus fumigatus, Hafnia alvei, Serratia mercescens, S. aureus*	Ulagesan & Kim 2018
7.	*C. bistrialis*	*C, albicans, P. chrysogenum, A. fumigatus, Mucor racemosus, P. aeruginosa, P. vulgaris, H. alvei, S. mercescens, S. aureus, Micrococcus luteus*	Ulagesan & Kim 2018
8.	*Pila globosa*	*A. fumigatus, M. racemosus, S. aureus*	Ulagesan & Kim 2018
9.	*Pila virens*	*C. albicans, A. fumigatus, P. chrysogenum, M. racemosus*	Ulagesan & Kim 2018
10.	*Bellamya dissimilis*	*M. racemosus*	Ulagesan & Kim 2018
11.	*Bithynia pulchella*	*P. chrysogenum*	Ulagesan & Kim 2018

Snail mucus' antibacterial activity is determined by the snail species, the extraction procedure, and the organism's resistance. *E. desertorum*'s mucus has strong inhibitory efficacy against certain resistant bacteria, including *E. coli, P. aeruginosa, S. aureus, A. niger, R. stolonifer, C. albicans*, and *T. harzianum. E. desertorum* mucus is more effective than *H. aspersa* mucus against resistant bacteria associated with burn wound infections (El-Zawawy & Mona 2021). Those microorganisms were found in various wounds, even acute and chronic wounds. Pathogens such as bacteria, fungi, and viruses can impair wound healing by a variety of processes, the most common of which are infection and inflammation at the wound site. In addition, they are related to delayed healing and complications (Bowler et al. 2001).

CONCLUSION

Based on the result of reviewing 22 articles, it was defined about snail's mucus collection methods, as well as types of snails, its components, and its biological activities. Snail mucus can be collected by stimulating the snail's body with friction, NaCl spray, electric shock, and nebulized ozone gas. Types of snails used in wound healing, namely *A. fulica, H. aspersa, E. desertorum, H. lucurus, C. bistrialis, T. curta*, and *M. lopesi*. The body parts of snails that can be utilized in wound healing are the whole body, mucus, and shell. The dominant component in snails are mucopolysaccharides, polyphenols, peptides, and glycosaminoglycans. The snails can be used for some wound type such as burns, excision wounds, incision wounds, and diabetic ulcers.

AUTHOR CONTRIBUTION

The study's conceptualisation was done by DF, PS, and RM, while DF carried out data analysis and manuscript preparation. The manuscript's content was reviewed and approved for publication by all the authors.

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CONFLICT OF INTEREST

There is no conflict of interest in this article.

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