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Short Communication

Nypa Palm Frond Degradation Utilizing Cellulolytic Lactic Acid Bacteria Isolated from Nypa Palm Worm (*Namalycastis rhodochorde*)

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ABSTRACT

Cellulolytic lactic acid bacteria (LAB) isolated from the gut of nypa palm worms demonstrated cellulase enzyme activity. The primary objective of this study was to investigate the cellulose degradation capabilities of nypa palm frond of three LAB treatments and varying bacterial concentrations (2 %, 3.5 %, and 5 %) over a duration of 30 days. The results indicated that the NrLtG2 isolate exhibited superior degradation capabilities, resulting in a reduction of cellulose content ranging from 20.14 % to 26.39 %. In contrast, the NrLtC2 and NrLtC4 isolates displayed lower degradation abilities, with reductions ranging from 12.50 % to 15.63 %.

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Nypa palm worms (*Namalycastis rhodochorde*) represent a specific category of soil macrofauna that exclusively inhabit mangrove forest ecosystems characterized by the Nypa palm trees (*Nypa fruticans*). The worm is utilized by locals as fishing bait and as a feed source for fish, owing to their significant economic value attributed to their rich amino acid and fatty acid composition. The increasing demand for Nypa palm worms within these communities, coupled with the direct exploitation of this resource, poses a threat to their populations in natural habitats (Junardi 2008; Junardi & Riyandi 2020).

Anticipatory measures aimed at reducing the presence of *N. rhodochorde* in the environment are implemented through ongoing cultivation practices of *N. rhodochorde*. Junardi and Riyandi (2020) conducted experiments on feed that improved the efficacy of *N. rhodochorde* and facilitated the addition of segments using EM4 fermentation of nypa palm fronds. Previous research by Yanti et al. (2020) identified ten distinct types of cellulolytic LAB present in the gastrointestinal tract of *N. rhodochorde*. Among these, three isolates exhibited a high cellulolytic index, encoded NrLtC 2, NrLtC 4, and NrLtG 2. The capacity of these indigenous isolates to produce cellulase enzymes in degrading CMC renders them particularly suitable for application as biodegradation agent of nypa palm fronds for nypa palm worm feed in cultivation.

Microbes that degrade Nypa palm fronds can improve the nutritional content needed for *N. rhodochorde* in cellulolytic LAB cultivation media (Setyawati et al. 2021). These microbes are commonly found in isolation in the gastrointestinal tract of nypa palm worms, providing a potential solution. The lack of research on the cellulolytic LABs ability from the gastrointestinal of Nypa palm worms to degrade Nypa palm fronds underscores the necessity for this study. This research is necessary to assess the effectiveness of isolates in degrading nypa palm fronds. Feeding *N. rhodochorde* with the available nutrients is essential for achieving maximum yields. This research aims to determine the rate of the cellulose degradation activity of cellulolytic bacteria isolated from the digestive tract of Nypa palm worms when exposed to Nypa palm fronds. This research was undertaken to provide foundational support for the development of bacterial isolates as agents for the biodegradation of cellulose fibers and to explore their potential as components of probiotic feed, specifically targeting the biodegradation of Nypa palm frond fibers for the cultivation of nypa palm worms.

Nypa palm leaf fronds were collected from the secondary mangrove area of Sungai Kakap Village, Kubu Raya Regency, West Kalimantan. Three isolates of LAB with cellulolytic activity against CMC were isolated by Yanti et al. (2020) from the nypa palm worm, which was utilized in the degradation process. This study employed an experimental design comprised two treatments: I1 = NrLtC2, I2 = NrLtC4, and I3 = NrLtG2, alongside three concentrations of the isolate: K1 = 2 %, K2 = 3.5 %, and K3 = 5 % with three replications. The three isolates were cultivated in de Man, Rogosa, and Sharpe (MRS) broth media for 24 hours. The treatment starter suspension was prepared using a dilution method, which involved counting the number of colonies with the standard plate count method until the concentration reached 10^7 CFU ml⁻¹.

The dried Nypa palm fronds weighed 100 grams and were placed into a container with 1250 ml of distilled water. The three starter isolates were put into containers with bacterial concentrations of 2 %, 3.5 %, and 5 %, respectively, each with three repetitions. The sample was then incubated at room temperature in an airtight environment for 30 days (Egwuatu & Appreh 2018). During fermentation, bacterial density was observed every week. Following fermentation process, alterations in the cellulose content of palm leaf fronds, bacterial density, and pH levels of the substrates were assessed by comparing measurements taken before and after treatment (Yusnia et al.

2019). The degraded cellulose content was determined using the following formula:

$$\text{KST (\%)} = \frac{(\text{KSO (\%)} - \text{KSi (\%)})}{\text{KSO (\%)}}$$

Information: KST = Degraded cellulose content; KSo = Initial cellulose content; KSi = Final cellulose content

Determining cellulose content was conducted by modifying the Datta Method (2024). One gram of dry sample was added to 150 ml of distilled water and refluxed at 100 °C using a water bath for an hour. The results are filtered to obtain a residue and then washed with 300 ml of hot water. The residue was then dried in an oven until it reached a constant weight (weight b). Subsequently, the residue was combined with 150 ml of 1 N H₂SO₄ and refluxed in a water bath for 1 hour at a temperature of 100 °C. The results were filtered and washed with 300 ml of neutral solution, and the residue was dried until it reached a constant weight (weight c). The dry residue was added to 10 ml of 72 % H₂SO₄ and soaked at room temperature for 4 hours. The residue was mixed with 150 ml of 1 N H₂SO₄ and refluxed at 100 °C using a water bath for 1 hour. The residue was filtered and washed with 400 ml of distilled water until neutral. The residue is then heated in an oven at 105 °C until the weight is constant and weighed (weight d). Next, the residue is ashed and weighed (weight e) (Chesson 1981). Determination of cellulose content was carried out using the formula:

$$\text{Cellulose content (\%)} = (\text{c} - \text{d}) / \text{a} \times 100 \%$$

The analysis result showed that the initial cellulose content in the fiber of nypa palm fronds was 48 %. Following treatment with cellulolytic bacteria over a 30-day incubation period, the cellulose content exhibited a reduction ranging from 12.50 % to 26.39 %. The results of the ANOVA analysis indicated that the treatment effects of various bacterial types and bacterial suspension concentrations did not differ significantly from the control group, which underwent natural degradation without sterilization of the nypa palm fronds. However, an examination of the percentage values for cellulose reduction revealed that treatment I3K1 (isolate NrLtG4 at a concentration of 2 %) achieved the most substantial decrease in cellulose content, amounting to 26.39 %. In contrast, Treatments I1K1 (isolate NrLtC2 at a concentration of 2 %) and I1K2 (isolate NrLtC2 at a concentration of 3.5 %) demonstrated only a modest reduction in cellulose content, with decreases of 12.50 %. Notably, isolate NrLtG2 exhibited the highest level of cellulose reduction among the tested isolates (Table 1).

The bacterial isolates NrLtC2 and NrLtC4, which were isolated from coelomic fluid, and NrLtG2, isolated from the gastrointestinal tract, were selected as starters because previous studies showed that they had a high cellulolytic index (>2) (Yanti et al. 2020; Setyawati et al. 2021). The bacterial isolate NrLtG2 exhibited the highest cellulolytic index value when compared to the isolates NrLtC2 and NrLtC4. The elevated cellulolytic activity observed both in vitro in carboxymethyl cellulose (CMC) media and in the direct degradation of nipah fronds can be attributed to the NrLtG2 isolate, which was sourced from the intestines of worms that were in direct contact with nipah fibers due to their deposit feeding behavior. The bacterial isolate NrLtG2, residing in the digestive tract, plays a crucial role in the processes of food digestion and nutrient absorption, as these processes occur within the digestive system. In contrast, coelomic fluid typically contains nutrients that support the surrounding tissues; consequently, the microbial activity within the coelomic fluid is generally less pronounced in terms of depolymerization activities (Nakagawa et al. 2017). Bacteria can enter the digestive tract

primarily through feeding activities, particularly during the deposit feeding process. However, in species such as nypa palm worm and several other annelids, bacteria may also colonize the coelomic cavity. The presence of bacteria in the coelomic cavity and fluid is believed to result from the movement and transfer of bacteria from the intestines. Research conducted by Yakkou et al. (2021) and Zhong et al. (2022) demonstrated the colonization of bacteria isolated from the coelomic fluid of earthworms and probiotic *Bacillus* from coelomic fluid of peanut worm (*Sipunculus nudus*). However, there is a lack of detailed information regarding the mechanisms by which bacteria infiltrate the coelomic cavity, which serves a critical role in the immune system of the worm. Bacteria in the gastrointestinal tract can ferment food ingredients that are not digested by the body, such as fiber, and produce compounds such as short-chain fatty acids that can be utilized by the body (Holscher 2017; Nogal et al. 2021; Dar et al. 2021).

Table 1. Cellulose content after 30 days of incubation.

Treatments	Initial Cellulose Content (%)	Final Cellulose Content average (%)	Cellulose Reduction average (%)
Control		40.33±1.15	15.97±1.41
I ₁ K ₁		42.00±2.00	12.50±2.17*
I ₁ K ₂		42.00±1.00	12.50±2.08*
I ₁ K ₃		40.50±3.06	15.63±3.51
I ₂ K ₁		40.00±1.00	16.67±2.08
I ₂ K ₂		38.83±2.75	19.10±3.00
I ₂ K ₃	48,00 ± 0.00	36.83±4.37	23.26±5.15
I ₃ K ₁		35.33±2.00	26.39±2.36**
I ₃ K ₂		38.33±0.58	20.14±0.77
I ₃ K ₃		36.33±2.08	24.31±2.43

Footnotes: The treatment consists of a combination of bacterial strains (I₁K₁ = NrLtC2 with concentration 2 %; I₁K₂ = NrLtC2 with concentration 3.5 %; I₁K₃ = NrLtC2 with concentration 5 %; I₂K₁ = NrLtC4 with concentration 2 %; I₂K₂ = NrLtC4 with concentration 3.5 %; I₂K₃ = NrLtC4 with concentration 5 %; I₃K₁ = NrLtG2 with concentration 2 %; I₃K₂ = NrLtG2 with concentration 3.5 %; I₃K₃ = NrLtG2 with concentration 5 %). The asterisk (*) denotes the lowest reduction, while the double asterisk (**) the highest reduction.

The bacterial isolates added during incubation in all treatments experienced increased cell growth until the 4th week. Cell growth occurs exponentially, with the highest increase in the 3rd and 4th weeks. The highest cell growth occurred in the I₂K₂ (NrLtC4 isolate with concentration 3.5 %) treatment which reached a cell number of 8.7×10^9 CFU ml⁻¹ in the 4th week, while the lowest increase was observed in the I₃K₂ (NrLtG4 isolate with concentration 3.5 %) treatment, with a cell number of only 2.8×10^9 CFU ml⁻¹ in the 4th week (Figure 1).

The medium used to test nypa palm frond degradation did not show a significant change in pH value. The pH value remains neutral from before incubation until completion on day 30, ranging between pH 6.00 and 7.00 (Figure 2). Changes in the degree of acidity occur in tiny increments. The data regarding the number of bacterial colonies demonstrate an inverse relationship with the pH value of the substrate. Specifically, an increase in the number of cells corresponds with a decrease in the pH value of the substrate observed during the fourth week (Figures 1 and 2). However, the reduction in the substrate's pH has not been substantial enough to act as an inhibitor of LAB growth. This phenomenon is believed to be influenced by the suboptimal breakdown of cellulose within the degradation system, as well as a relatively low fermentation rate of organic acids (Othman et al. 2017).

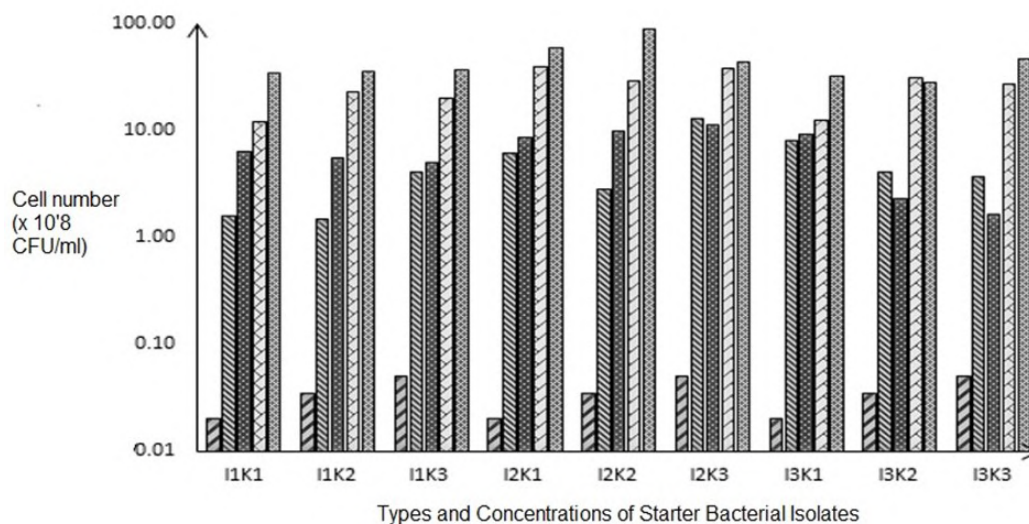


Figure 1. Growth of bacterial isolates during incubation, isolate NrLtC2 (I1), isolate NrLtC4 (I2), isolate NrLtG2 (I3), 2 % (K1), 3.5 %, and (K2), 5 % concentration (K3), in the week 0 , 1st week , 2nd week , 3rd week , 4th week . The treatment consists of a combination of bacterial strains (I1K1 = NrLtC2 with concentration 2 %; I1K2 = NrLtC2 with concentration 3.5 %; I1K3 = isolate NrLtC2 with isolate concentration 5 %; I2K1 = NrLtC4 with concentration 2 %; I2K2 = NrLtC4 with concentration 3.5 %; I2K3 = NrLtC4 with concentration 5 %; I3K1 = NrLtG2 with concentration 2 %; I3K2 = NrLtG2 with concentration 3.5 %; I3K3 = NrLtG2 with concentration 5 %).

Based on the conducted research, it can be concluded that the cellulolytic lactic acid bacteria isolates designated as NrLtC2, NrLtC4, and NrLtG2 possess the capability to degrade nypa palm fronds. It is suspected that these bacteria originate from the interaction between the digestive tract and cellulose substrates, which are consistently present due to the deposit-feeding activity of nypa palm worms (Setyawati et al. 2021). However, the degradation efficiency of these three isolates remains relatively low, with degradation rates ranging from 12.50 % to 26.39 % over a period of 30 days (Table 1). This phenomenon is believed to result from variations in the types of cellulose-degrading enzymes produced by the three bacterial isolates, which have not yet been thoroughly investigated. Additionally, the presence of other complex compounds may directly inhibit the cellulose degradation process. Tamunaidu and Saka (2011) characterized the chemical composition of nypa palm, which includes fronds, shells, husks, and leaves, analyzing components such as cellulose, hemicellulose, lignin, starch, protein, extractives, and inorganic constituents for each part. The total chemical composition revealed that the cellulose and hemicellulose contents ranged from 28.9 % to 45.6 % and 21.8 % to 26.4 %, respectively, with hemicellulose being predominantly composed of glucuronoxylan. The lignin content was found to be between 19.4 % and 33.8 %. In addition to these primary chemical components, starch, protein, and extractives were also present in significant quantities, ranging from 2 % to 8 %. Among the three isolates, NrLtG2 exhibited the highest degradation activity, achieving a cellulose degradation rate of up to 26.39 %, whereas the NrLtC2 isolate demonstrated the lowest degradation activity, with a cellulose degradation rate of less than 20 % (Table 1).

The degradation ability of the bacterial isolate illustrates the potential of indigenous cellulolytic LABs as a biodegradation agent in the production of feed for nypa palm worm cultivation. However, the results obtained in this study were suboptimal, as the degradation percentage remained below 39.57 %, a benchmark established by *Bacillus* DC-11 in the research conducted by Li et al. (2023). Datta (2024) observed that cellulose degradation is a gradual

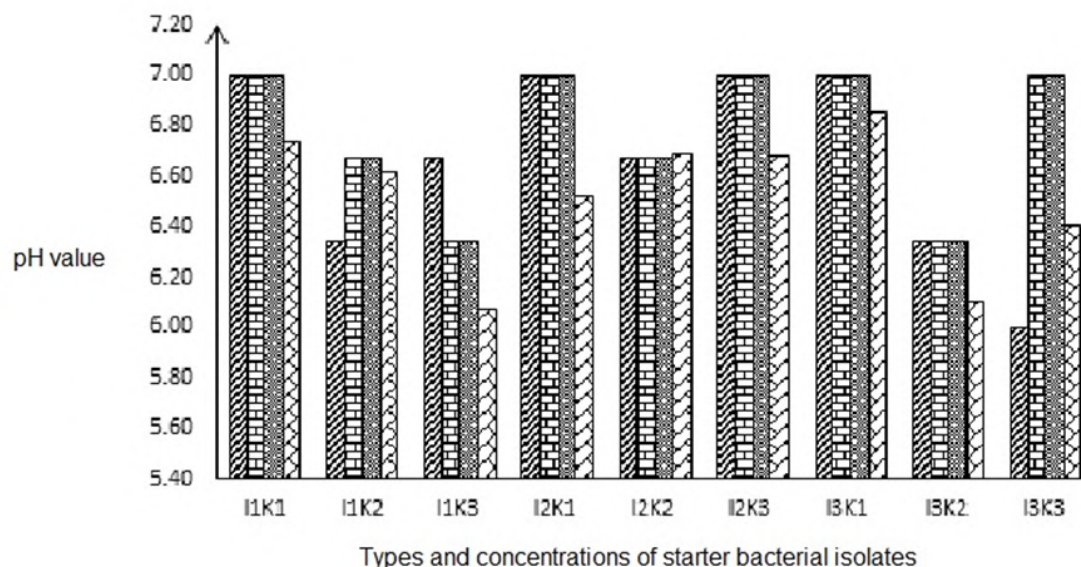


Figure 2. Changes in the pH value of the media during the incubation process in the 1st week , 2nd week , 3rd week , and 4th week . The treatment consists of a combination of bacterial strains (I1K1 = NrLtC2 with concentration 2 %; I1K2 = NrLtC2 with concentration 3.5 %; I1K3 = NrLtC2 with concentration 5 %; I2K1 = NrLtC4 with concentration 2 %; I2K2 = NrLtC4 with concentration 3.5 %; I2K3 = NrLtC4 with concentration 5%; I3K1 = NrLtG2 with concentration 2 %; I3K2 = NrLtG2 with concentration 3.5 %; I3K3 = NrLtG2 with concentration 5 %).

process influenced by a multitude of factors that can affect the degradation process both directly and indirectly. These factors encompass the availability of nitrogen, temperature, aeration, moisture content, pH, lignin content, as well as the concentration and activity of cellulases. Therefore, further research is necessary to determine the optimal concentration of LAB, the appropriate combination of lignin-degrading agents, and other compounds present in nypa palm fronds. Additionally, system is required to enhance the fermentation performance for Nypa palm fronds.

AUTHOR CONTRIBUTION

R.K; T.R.S; A.H.Y; and A.W. designed the research, collected and analyzed the data, supervised all the process, and wrote the manuscript.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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Short Communication

Protein Isolation and Purification of Sea Cucumber (*Holothuria* sp. and *Stichopus* sp.) from Sepanjang Beach, Yogyakarta

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ABSTRACT

Holothuria sp. and *Stichopus* sp. at Sepanjang Beach, Yogyakarta were studied for protein isolation. The crude protein concentrations in the body wall and intestine of *Holothuria* sp. were 2.42% and 2.13%, while *Stichopus* sp. had 4.03% and 4.01%, respectively. Proteins identified through SDS-PAGE analysis included collagen, softenin, and actin. Purified protein concentrations in *Holothuria* sp. were $3.72 \pm 0.04 \mu\text{g mL}^{-1}$ (body wall) and $4.09 \pm 0.05 \mu\text{g mL}^{-1}$ (intestine), while *Stichopus* sp. had $3.98 \pm 0.08 \mu\text{g mL}^{-1}$ and $9.34 \pm 0.48 \mu\text{g mL}^{-1}$, respectively. This study supports nutraceutical development, identifying potential health-enhancing supplements and drugs.

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Sea cucumbers are marine organisms with high economic value due to their nutritional content. Dried sea cucumbers contain 69-82 % protein, 1.7 % fat, 8-8.9 % water or moisture, 4.8 % fibre and 1 % lipid (Maskur et al. 2024; Ibrahim et al. 2015), while fresh sea cucumbers contain 87.78 % moisture, 65.53 % protein, and 1.76 % fat (Fawzya et al. 2015). Sea cucumbers offer a variety of health benefits and serve as a popular source of medicinal ingredients. Furthermore, sea cucumbers have various biological activities such as antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, and neuro-protectant. The protein content in sea cucumbers can be used as a therapy for various diseases (Ovchinnikova 2019; Al-Khayri et al. 2022). Fortunately, Indonesia has abundant sea cucumbers that spread widely across the beaches, especially in Yogyakarta. *Holothuria scabra* and *Stichopus hermannii* are two species commonly used as a health-promoting agent in traditional Asian medicine. Its extract has several health benefits, such as suppressing inflammation, promoting wound healing, and improving immunity (Pranweerapaiboon et al. 2021). Moreover, the abundance, underutilization, and potential of cultivating marine species such as sea cucumbers have led to extensive research into using them as a protein source (Guo et al. 2020). However, there hasn't been much research on protein isolation and purification of *Holothuria* sp. and *Stichopus* sp. from Sepanjang Beach, Yogyakarta.

Amalia et al. (2015) reported the abundance of several sea cucumber species in Sepanjang Beach, Yogyakarta, including *Holothuria* sp., *Stichopus* sp., and *Actinopyga* sp. They are also commonly found in several regions, including Tomini, Gorontalo (Daud et al. 2023; Olii et al. 2024), Raja Ampat, Papua (Handayani et al. 2017), Saparua Island, Central Maluku (Lewerissa 2014), Sulawesi (Hisam et al. 2022), and Nyamuk Island, Central Java (Mustagfirin et al. 2021). Additionally, it is cultivated in Karimun Jawa, Central Java (Widianingsih et al. 2024), Sangihe Islands, Sulawesi (Tarimakase et al. 2020), Southeast Maluku (Tomatala et al. 2020), and Kupang, East Nusa Tenggara (Menge et al. 2023). These species have several compounds that have therapeutic properties such as bioactive peptides, collagens, vitamins, minerals, fatty acids, collagen, gelatine, and amino acids. Protein hydrolysates from sea cucumber also have several benefits for health, such as antioxidant, neuroprotective, antiaging, anti-inflammatory, and anticancer (Pangestuti & Arifin 2018). This has proven that the proteins from sea cucumber have biological activity and has significant potential as a health-promoting agent (Man et al. 2023). Given the health benefits of sea cucumbers, it is essential to investigate their protein properties for potential health applications. This research aimed to isolate and to purify proteins from *Holothuria* sp. and *Stichopus* sp. found on Yogyakarta's beaches using anion exchange chromatography, targeting negatively charged proteins common in marine organisms due to their acidic amino acids (Senadheera et al. 2020). This study lays the groundwork for future research into sea cucumber proteins.

Holothuria sp. and *Stichopus* sp. were collected in January 2023 from Sepanjang Beach, Gunungkidul, Yogyakarta, Indonesia, extending from the coastline to the intertidal zone. The fresh weight of the sea cucumbers obtained varies from 100 to 150 grams. The body wall and intestine were pulverised using a grinder that had previously added liquid nitrogen. The sample was subsequently immersed in absolute ethanol (1:2 w v⁻¹) for the defatting. This research employed modified protein isolation approach based on Wang et al. (2020). Body wall and intestine samples of *Holothuria* sp. and *Stichopus* sp. were added to the lysis buffer with a ratio of 1:2. Lysis buffer consisted of 100 mM NH₄HCO₃, 6 M urea, and 0.2 % SDS, and protease inhibitors were added in a ratio of 1:100 between protease inhibitor and lysis buffer were used. Samples were extracted using ultrasonic-assisted extraction for 5 minutes at a frequency of 300 Hz. After the isolation process, the samples

were subjected to freeze-drying.

Protein content was determined using Bradford assay and Bovine Serum Albumin (BSA) as a standard, in accordance with procedure of Samah (2019) with optimisation. Samples were read with spectrophotometer at λ 595 nm. The molecular weight of the protein was confirmed using sodium dodecyl sulphate-polyacrylamide gel-electrophoresis (SDS-PAGE) with gradient gel concentration 4-12 % and Tris-Glycine SDS as a running buffer. This protocol referred to Smith (2011) with minor modifications. Protein purification was conducted using anion exchange chromatography by HiTrap Q HP and referred to factory protocol by GE Healthcare with series number 71-7149-00 AP. The start buffer was prepared using Tris – HCl 0,02 M pH 8, then an elution buffered with Tris – HCl 0,02 M pH 8 combined with NaCl 1 M (1:1 v v⁻¹). Gradient buffers were also prepared using NaCl 0.5; 1; 1.5; 2; and 2.5 M, and Tris – HCl from the start buffer was added about 100 μ L per 5 mL. The eluted protein was quantified with Bradford Assay.

Protein from sea cucumber was soaked in lysis buffer and protease inhibitor and isolated using the sonication method. Sonication, a widely used technique in protein extraction, employs ultrasonic waves to disrupt the cell membrane and facilitates the release of proteins (Kim et al. 2013). The primary function of ultrasound is to create bubble cavitation within the biological material, and the subsequent release of energy as these bubbles burst impacts the cell membranes, leading to their disruption (Kadam et al. 2015).

The sample identification was conducted based on observable morphological characteristics in accordance to identification guidebook by Wirawati et al. (2019). The results indicated that the collected sea cucumber samples are *Holothuria* sp. and *Stichopus* sp. (Supplementary Figure 1), further studies are needed for detailed species identification.

The measurement of protein content referred to bovine serum albumin (BSA) as standard with an R-squared value close to 1 (Supplementary Data 1). The protein contained in the freeze-dried powder from *Holothuria* sp. and *Stichopus* sp., as shown in Table 1. The samples were subjected to freeze-drying to remove moisture, thereby stabilising the protein and preventing denaturation and aggregation. Freeze-drying removes water by converting it into vapor through sublimation, reducing the risk of protein instability caused by hydration stress (Chen et al. 2021).

This study represented a preliminary investigation into the isolation and purification of proteins from *Holothuria* sp. and *Stichopus* sp. collected at Sepanjang Beach, Gunungkidul, Yogyakarta. The study focused solely on protein isolation and purification. Further comprehensive research is needed to identify specific protein types and their benefits. The research aimed to determine the protein yield obtained through isolation, to characterise the type of protein based on molecular weight using SDS-PAGE, and to quantify purified proteins through anion exchange chromatography. These processes facilitated the initial identification of proteins from *Holothuria* sp. and *Stichopus* sp. as well as the factors affecting these processes. This information might enhance future research on specific protein types and their potential therapeutic activities.

The results indicated that *Stichopus* sp. possesses a higher protein content, around 4 % ($20 \mu\text{g mL}^{-1}$) in both the body wall and intestine, compared to *Holothuria* sp. at 2.4 and 2.1 % in the body wall and intestine, respectively. Some studies have reported that *Stichopus* sp. protein content is 34.33 % (Ridhowati et al. 2018) and 41.3 % (Nguyen et al. 2022). Other study revealed that *Holothuria scabra* has a protein content of 4.96 %, while *Holothuria leucospilota* has a protein content of 10.06 % (Yunita et al. 2017). The results indicated that interspecies variations can influence the protein content in sea cucumbers.

Table 1. Crude protein content isolated from *Holothuria* sp. and *Stichopus* sp.

Sample	Crude sample mass (mg)	Freeze dried protein mass (Mean $\pm$ SE, mg)	Freeze dried yield (%)	Protein content (Mean $\pm$ SE, $\mu\text{g mL}^{-1}$)
BH	3000	46.2 $\pm$ 4.47	1.54	12.11 $\pm$ 0.00029
IH	3000	43.31 $\pm$ 4.39	1.40	10.64 $\pm$ 0.00032
BS	3000	31.2 $\pm$ 4.64	1.04	20.17 $\pm$ 0.00071
IS	3000	39.8 $\pm$ 3.52	1.32	20.06 $\pm$ 0.00024

Note BH: Body wall *Holothuria* sp., IH: Intestine *Holothuria* sp., BS: Body wall *Stichopus* sp., IS: Intestine *Stichopus* sp.

Additionally, the protein concentration found in this study differed significantly from that reported in some literatures. The differences in protein analysis methods may be the cause of this discrepancy. Apriliani et al. (2024) used proximate analysis to measure protein content and to find the total protein. This study, on the other hand, used an extraction method with a lysis buffer that included 100 mM NH_4HCO_3 , 6 M urea, 0.2 % SDS, and protease inhibitors to isolate only specific proteins. Therefore, optimising the type of solvent used is necessary to maximise the protein yield. Jafari et al. (2020) stated that protein content and yield during extraction can be influenced by factors such as the solvent and concentration used, sea cucumber species, weight-to-solvent ratio, extraction time, and temperature. Besides that, changes in the conformation of biomolecules in general can cause changes in shape that can adapt to various solvent conditions, namely causing adaptation to the solvent environment, such as changing a polar solvent to a non-polar one. This flexibility additionally affects the function of the compound in vivo. Water is a common solvent in many biological conditions, however molecular stability can also be achieved in non-polar solvents. Protein is a molecule composed of amino acids, half of the amino acids are hydrophobic, namely molecules that avoid contact with water, and also half of the amino acids are hydrophilic, molecules that have direct contact with water. The interaction between the protein and the solvent induces conformational changes in the protein, subsequently affecting its adaptation to the solvent (Meyer et al. 2013; Fomthum & Giacometti 2023).

In addition to these factors, seasonal variations and food availability can also influence the protein concentration in sea cucumbers. Seasonal changes impact temperature, which in turn affect food source availability and metabolic activity in sea cucumbers, potentially altering protein levels or amino acids content (Feng et al. 2021). Phytoplankton, a food source for sea cucumbers, was found to increase during the winter, so the availability of phytoplankton can influence protein content in sea cucumbers (Deng et al. 2019; Kingsolver et al. 2015). In this study, sampling was conducted in January 2023, during which Sepanjang Beach was still experiencing summer, resulting in limited food availability and affecting the protein content in the sea cucumbers. Torreno et al. (2023) reported that the body wall extracts of *Stichopus horrens* contained elevated levels of structural, diacylated phosphatidylcholines (PCs), suggesting their potential involvement in the synthesis of proteins crucial for the structural integrity and functioning of the sea cucumber's body. Their specific metabolic pathways generate essential bioactive compounds necessary for their survival and environmental adaptation.

The result of SDS-PAGE analysis showed that several proteins were detected in different molecular weights and some patterns are formed between the body walls and the intestine. The results of SDS-PAGE analysis shown in Figure 1. indicated that the *Holothuria* sp. body wall has various protein patterns with molecular weights of around 12, 17, 39, 49 kDa with the thickest band at 12 and 49 kDa. Besides that, intestine *Holothuria* sp. just

showed two bands with molecular weights of about 14 and 18 kDa with the thickest band at 14 kDa.

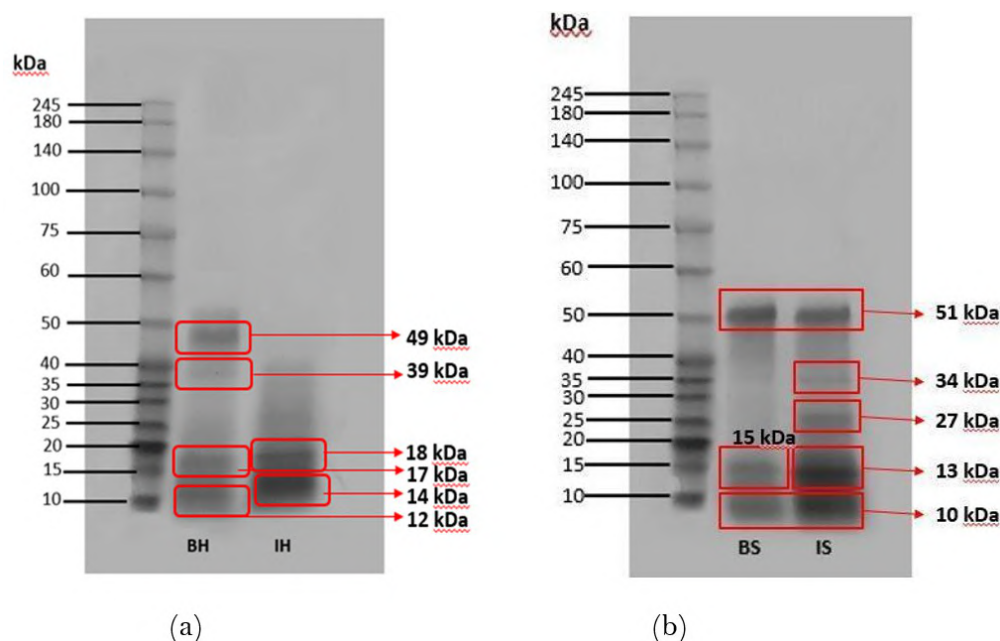


Figure 1. SDS-PAGE pattern of the protein isolated from *Holothuria* sp. (a), *Stichopus* sp. (b). BH: Body wall *Holothuria* sp.; IH: Intestine *Holothuria* sp.; BS: Body wall *Stichopus* sp.; IS: Intestine *Stichopus* sp.

The body wall of *Stichopus* sp. has proteins with molecular weights of around 10, 15, and 51 kDa, while the intestine of *Stichopus* sp. showed proteins of around 10, 13, 27, 34, and 51 kDa. The type of protein with a molecular weight of 12-18 kDa is probably collagen hydrolysate protein. This result aligns with Yusro et al. (2020), who stated that collagen hydrolysates obtained from gold sea cucumber have molecular weight with a range of approximately 14.4 to 25 kDa. Sea cucumbers have a high collagen content because their body walls are predominantly composed of collagen-forming amino acids such as glycine, proline, and hydroxyproline (Senadheera et al. 2020; Gustini et al. 2022). At 27 and 34 kDa, the probably protein detected is softenin. Takehana et al. (2014) indicated that the protein with a molecular weight of around 20-30 kDa is softenin. Softenin is a novel protein that makes the sea cucumber connective tissues softer by inhibiting fibril interactions. At 39, 49, and 51 kDa, the probable protein detected is actin. Truong and Le (2019) reported that the actin from sea cucumbers is detected at 40 - 50 kDa. Actin is the protein that forms most animal muscles and presents significant potential for various biological and biomedical uses due to its versatility and essential role in cellular functions (Haarer et al. 2023; Hatano et al. 2020).

Stichopus sp. exhibits a higher protein content than *Holothuria* sp. due to their differences in their metabolic processes. The metabolism of *Stichopus* sp. likely resulted in increased proteins for defense, digestion, and the formation of the outer layer of the sea cucumber (Torreno et al. 2023). In this research, it is showed that the body wall and intestine for both *Holothuria* sp. and *Stichopus* sp. have different patterns. The observed difference pattern may result from varying amino acid compositions that play role in proteins structure (Cuevas-Acuña et al. 2019). Chemical modification including glycosylation or ubiquitination could affect the gel mobility and cause retarded gel results. Other modifications such as phosphorylation, may also influence the mobility of the samples and the formation of bands on SDS-PAGE. Meanwhile, hyperphosphorylation may influence the background or smear observed in the gel (Stanley 2011; Guan et al. 2015; Liu et al. 2015).

After measuring the protein concentration using the Bradford assay, purification was performed using Anion Exchange Chromatography. Anion Exchange Chromatography facilitates protein purification by separating proteins according to their charge differences, achieving high resolution for proteins with equal charges but varying affinities (Kadakeri et al. 2020). Some of the proteins were eluted in low concentrations of salt and some in high salt concentrations. Protein of body wall's *Holothuria* sp. was abundant in 1.5 and 2.5 M NaCl elution gradients with concentrations of $3.72 \mu\text{g mL}^{-1}$ and $1.03 \mu\text{g mL}^{-1}$, respectively. In addition, the intestine of *Holothuria* sp. contained high protein concentrations of $4.09 \mu\text{g mL}^{-1}$ in 0.5 M NaCl and approximately $0.2 \mu\text{g mL}^{-1}$ in 1.5 M NaCl (Figure 2). Besides that *Stichopus* sp.'s body wall, proteins were eluted at 0.5 M NaCl with a concentration around $3.98 \mu\text{g mL}^{-1}$ and 2 M NaCl with a concentration around $3.07 \mu\text{g mL}^{-1}$. In contrast, for *Stichopus* sp.'s intestine, proteins were eluted at 0.5 M NaCl with concentration around $1.24 \mu\text{g mL}^{-1}$ and 1 M NaCl with a concentration around $9.34 \mu\text{g mL}^{-1}$ (Figure 3).

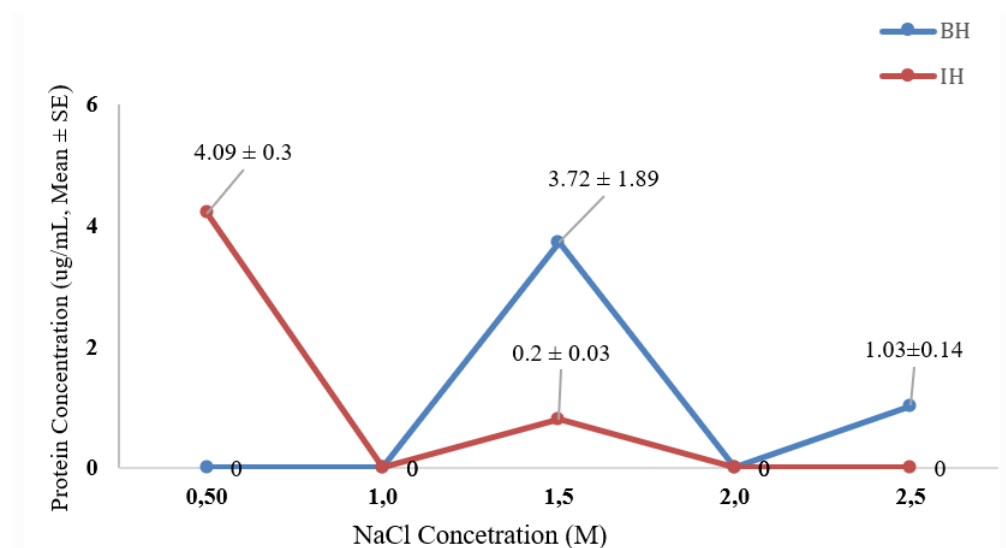


Figure 2. Result of Anion Exchange Chromatography from BH: Body wall *Holothuria* sp.; IH: Intestine *Holothuria* sp. The data is presented in the form of Mean $\pm$ SE.

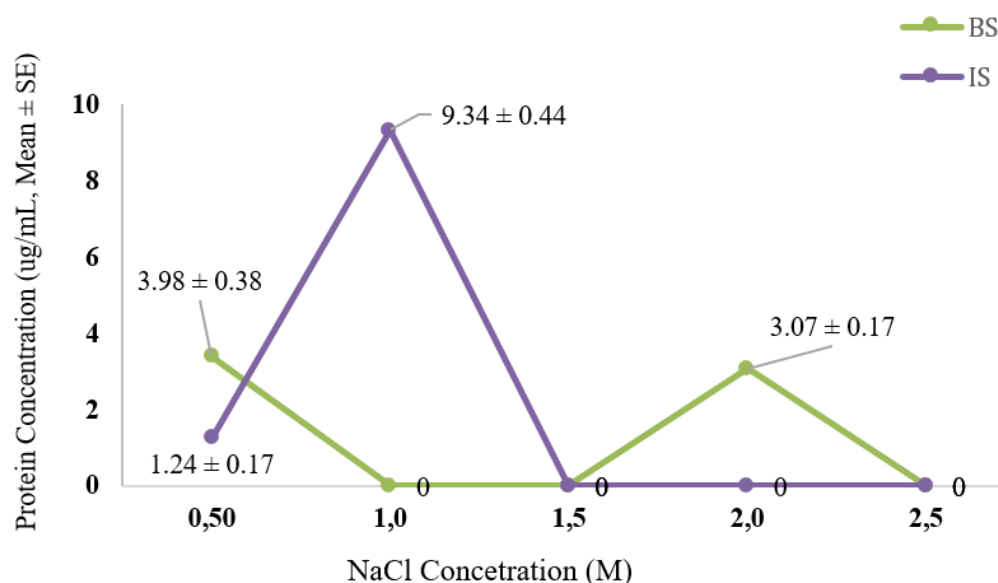


Figure 3. Result of the Anion Exchange Chromatography from BS: Body wall *Stichopus* sp.; IS: Intestine *Stichopus* sp. The data is presented in the form of Mean $\pm$ SE.

The purified protein of *Holothuria* sp.'s body wall and intestine showed the highest concentration with NaCl elution at 1.5 and 0.5 M. Meanwhile, the

body wall and intestine of *Stichopus* sp. showed the highest purified protein concentrations with NaCl elution at 0.5 and 1 M. These results indicated that NaCl 0.5; 1; and 1.5 M can be an effective choice as elution in protein purification of both *Holothuria* sp. and *Stichopus* sp. using Anion Exchange Chromatography. As an alternative, it is necessary to re-optimize with either lower or higher NaCl concentrations to get optimum results. The different concentrations of purified protein from each NaCl elution showed that proteins isolated from both organs have different ionic negativity and could be eluted with different NaCl concentrations. Protein will be eluted by gradient NaCl concentration from low to high, respectively. NaCl with low concentration elute protein with a low ionic charge and NaCl with high concentration elute protein with a higher negative charge (Shire 2015). Salt (NaCl) is used as an eluent in Anion Exchange Chromatography. This NaCl dissociates into Na^+ and Cl^- ions in solution, and Cl^- competes with proteins that have a negative charge to bind to the positive stationary phase. Negatively charged proteins that cannot bind due to disruption of Cl^- bonds will dissolve and elute (Acikara 2013).

Proteins from *Holothuria* sp. and *Stichopus* sp. had been extracted. The protein yield in the freeze-dried extract is approximately 1.54 % in the body wall and 1.40 % in the intestine of *Holothuria* sp. *Stichopus* sp. exhibits protein yields of 1.04 % in the body wall and 1.32 % in the intestine. The results of this research indicated that *Stichopus* sp. has a higher protein content, at 4 %, in both the body wall and the intestine, compared to *Holothuria* sp. The most abundant proteins detected by SDS-PAGE analysis were collagen, softenin, and actin. After purification using Anion Exchange Chromatography, the protein concentrations in *Holothuria* sp. are $3.72 \mu\text{g mL}^{-1}$ in the body wall and $4.09 \mu\text{g mL}^{-1}$ in the intestine. Concentration purified proteins in *Stichopus* sp. were about $3.98 \mu\text{g mL}^{-1}$ in the body wall and $9.34 \mu\text{g mL}^{-1}$ in the intestine. The result of this research needs further confirmation and development to understand the detailed characteristics of sea cucumber proteins, using advanced technologies such as Liquid Chromatography High-Resolution Mass Spectrometry (LC-HRMS), and to test their activities for various applications.

AUTHOR CONTRIBUTION

B.K.A., S.L.U., and T.R.N. designed the study. B.K.A. and S.L.U. conducted the laboratory work, analysed the data, and wrote the manuscript. L.H., Z.R., and Y.A.P. were the supervisors. T.R.N. was the supervisor and corresponding author. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest to disclose.

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Short Communication

Evaluation of Krusty/Homer and SPG1/SPG2 Primer Pairs in Identification of Six Begomoviruses Commonly Found in Indonesia

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ABSTRACT

Fourteen symptomatic crops and weeds from Magelang, Klaten, Sleman, and Bantul Regencies were tested against begomoviruses using Krusty/Homer and SPG1/SPG2 primer pairs. The acquired nucleotide sequences were involved in phylogenetic analysis which clustered isolates into six species. Furthermore, three recombinant isolates were also identified. Protocol for molecular characterization of prevalent begomoviruses using both primer pairs was initiated in this report. Molecular data of the obtained 14 isolates added important information on the genetic diversity of Indonesian isolates.

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Begomovirus, a genus within the family Geminiviridae, has single-stranded DNA genomes encapsidated in twinned particles approx. 22×38 nm in size. Its members are persistently transmitted by whitefly (*Bemisia tabaci*), and pose significant threats to agricultural productivity, especially in the tropical and subtropical regions (Fiallo-Olivé & Navas-Castillo 2023). Similar situation in Indonesia, begomoviruses are still real concerns for the cultivation of economically valuable *Solanaceae*, *Cucurbitaceae*, and *Fabaceae* crops (Santosa & Somowiyarjo 2023; Wahyono et al. 2023). Thus, accurate detection and thorough molecular characterization of these viruses are crucial for disease management in the country (Subiastuti et al. 2019).

Universal primer pairs such as Krusty/Homer (KH) (Revill et al. 2003) and SPG1/SPG2 (SPG) (Li et al. 2004) have been widely employed for detection of begomoviruses. These primers enable the amplification of a broad spectrum of begomovirus genomes, facilitating the identification up to species level in a single assay. Moreover, these primer pairs have been effectively proven in different crops and geographic regions. KH primer pair is developed to target ± 580 -base pair (bp) of partial *AV1* gene (coat protein/CP gene) of the begomovirus genome which is a highly conserved region (Figure 1), allowing for detection of diverse species (Revill et al. 2003). Meanwhile, SPG primer pair is designed to amplify ± 912 bp partial fragment of *TrAP* and *Rep* genes of begomoviruses (Figure 1). Therefore, not only having a high degree of specificity and sensitivity but also SPG covers up to one third of DNA-A segment, making it a valuable tool for studying variations in the viruses (Li et al. 2004).

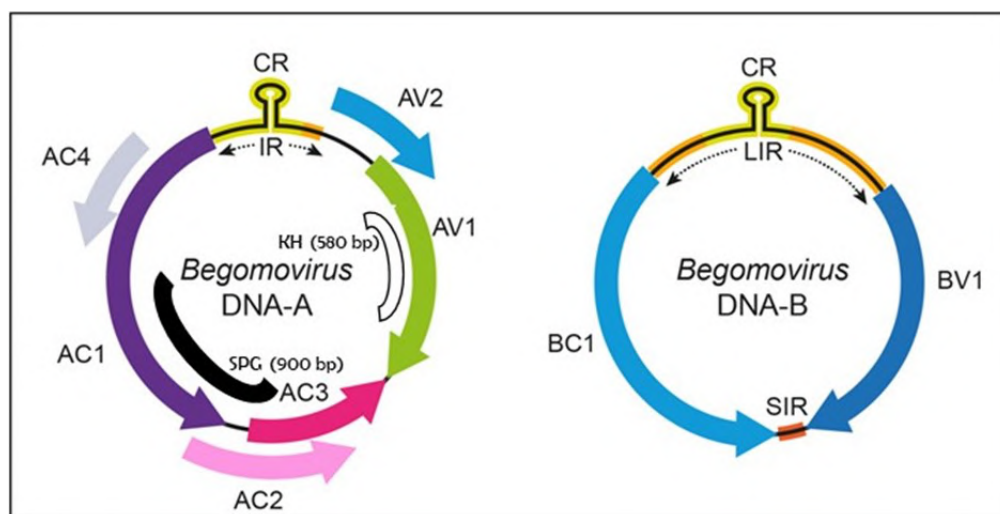


Figure 1. Schematic provided by ICTV (<https://ictv.global>) shows bipartite genome of begomovirus with bidirectional genome organization of AV1, AV2, AC1, AC2, AC3, and AC4 genes in DNA-A, and BV1 and BC1 genes in DNA-B, respectively. Approximate positions of regions within DNA-A segment of begomovirus genome amplified by Krusty/Homer primer pair (white area) and SPG1/SPG2 primer pair (black area).

In Indonesia, applications of KH and SPG primers has led to the identification of several major begomovirus species. For instance, tomato yellow leaf curl Kanchanaburi virus (TYLCKaV), squash leaf curl China virus (SLCCNV), pepper yellow leaf curl Indonesia virus (PepYLCIV), and papaya leaf curl virus (PaLCV) have been detected respectively in eggplant (*Solanum melongena*), squash (*Cucurbita pepo*), chili pepper (*Capsicum annuum*), and papaya (*Carica papaya*) cultivated in regions such as Java, Lampung, Bali, and Bengkulu using SPG primer (Kintasari et al. 2013; Wiratama et al. 2015; Sutrawati et al. 2021; Selangga & Listihani 2021; Akin et al. 2023). Additionally, mung bean yellow mosaic India virus (MYMIV), PepYLCIV, TYLCKaV, and

SLCCNV were reported in *Solanaceae*: chili pepper and eggplant, *Cucurbitaceae*: angled luffa (*Luffa acutangula*) and squash, and *Fabaceae*: mung bean (*Vigna radiata*) crops in Yogyakarta, Southeast Sulawesi, Lampung, and Central Java using KH primer (Wilisiani et al. 2014; Subiastuti et al. 2019; Giovanni et al. 2020; Mulyadi et al. 2021; Taufik et al. 2023; Akin et al. 2023; Santosa et al. 2024). Therefore, these viruses were also shown to be widespread and infecting different plant groups in Indonesia (Wiratama et al. 2015; Listihani et al. 2019; Sutrawati et al. 2021; Taufik et al. 2023).

By enabling rapid and accurate identifications of multiple begomovirus species, KH and SPG primers play crucial roles in the management of virus-induced diseases in crops. Despite covering different regions, these primers have only been used separately in molecular detection protocols and have never been involved in the molecular characterization of the same isolate in Indonesia. This study aims to evaluate both universal primer pairs in the molecular characterization of six begomovirus species widespread in Indonesia. It also seeks to expand our understanding of the genetic diversity of isolates infecting important horticultural commodities and weeds at observed locations.

Leaves of horticultural crops and weeds with conspicuous begomovirus symptoms were collected during field surveys in Magelang, Klaten, Sleman, and Bantul Regencies in January – June 2024. Samples were purposively taken based on viral symptom appearances then brought to Phytopathology Laboratory, Universitas Gadjah Mada to be kept at -4 °C until further analysis shortly.

Total DNA from each collected samples were extracted using Genomic DNA Mini Kit for Plant following standard steps suggested by the manufacturer (Geneaid Biotech Ltd., Taiwan). PCRs for each sample were subsequently done twice each using either of two universal primer pairs for begomoviruses detection: Krusty (F 5'-CCNMRDGGHTGTGARGGNCC-3')/Homer (R 5'-SVDGCRTGVGTRCANGCCAT-3') targeting $\pm$ 580 bp of partial *AV1* gene (Revill et al. 2003) and SPG1 (F 5'-CCCCCKGTGCGWRAATCCAT-3')/SPG2 (R 5'-ATCCVAAAYWTYCAGGGAGCT-3') amplifying $\pm$ 900 bp partial *AC1* and *AC2* genes (Li et al. 2004). Each PCR reaction was prepared in a volume of 40 μ l: 2 μ l (10 pmol μ l⁻¹) each of reverse primer and forward primer, 20 μ l of MyTaq HS Red Mix (Bioline, Germany), 4 μ l of DNA template, and 12 μ l of PCR-grade water. The PCR cycle is organized as follows pre-denaturation step at 96 °C for 3 minutes, followed by 35 cycles of denaturation at 96 °C for one minute, annealing 55 °C for KH and 59 °C for SPG, each lasting 1 minute, elongation at 72 °C for 1 minute, and lastly final elongation at 72 °C for 10 minutes.

PCR products were loaded into wells of agarose gel stained with Florosafe DNA Staining (1st BASE, Malaysia), and then electrophoresis for the gel was run for 50 minutes at 50 V. The appearance and size of PCR bands were examined under a UV transilluminator (Optima Inc., Japan). Successful amplified PCR products were submitted to LPPT UGM for Sanger sequencing bidirectionally. Nucleotide sequences of partial *AV1*, and partial *AC1* and *AC2* genes of each isolate were expected to be established by this strategy. Determination of species of isolates was performed using nucleotide BLAST online software (<https://blast.ncbi.nlm.nih.gov>) which paired the new nucleotide sequences with those available in NCBI GenBank database. Nucleotide sequences of the novel isolates were then registered in GenBank to obtain unique accession numbers.

Begomovirus isolates with complete genome sequences registered in GenBank were aligned with and then trimmed according to the lengths of sequences of the newly obtained isolates using ClustalW algorithm in

MEGA11 software (Tamura et al. 2021). Two phylogenetic trees, for partial *AV1* gene and partial *AC1* and *AC2* genes, were constructed with Maximum Likelihood (ML) statistical method and Tamura-Nei (Tamura & Nei 1993) parameter model implemented in MEGA11. Statistical significance of each branch was tested with 1000 bootstrap replicates.

Possible recombination event on the genome of isolates positioned in different species groups of the constructed phylogenetic trees was scanned using Recombination Detection Program (RDP v5.30) (Martin et al. 2021). Only event detected by at least five of algorithms (Bootscan, MaxChi, Chimaera, 3Seq, Siscan, GENECONV, and RDP) suited in the software was considered convincing with significance of Bonferroni-corrected *p* value of < 0.05 (Martin et al. 2021).

In total, 14 samples: 4 chili peppers (*Capsicum annuum*), 1 tomato (*Solanum lycopersicum*), 1 eggplant (*Solanum melongena*), 3 squash (*Cucurbita pepo*), 2 melons (*Cucumis melo*), 2 yardlong bean (*Vigna unguiculata* ssp. *sesquipedalis*), and 1 billy goat weed (*Ageratum conyzoides*) were collected during field surveys then tested for begomovirus infection.

Results of PCR and the subsequent electrophoresis indicated begomovirus infection in all 14 tested samples by appearance of bands with specific sizes for KH primers and for SPG primers, respectively. BLAST analysis on the obtained sequences identified six begomoviruses: ageratum yellow vein China virus (AYVCNV), tomato yellow leaf curl Kanchanaburi virus (TYLCKaV), pepper yellow leaf curl Indonesia virus (PepYLCIV), squash leaf curl China virus (SLCCNV), mung bean yellow mosaic India virus (MYMIV), tomato leaf curl New Delhi virus (ToLCNDV) among the 14 isolates. GenBank accession nos. PP874885-94 and PQ112501-4 for partial *AV1* gene and nos. PP874895-904 and PQ112505-8 for partial *AC1* and *AC2* genes were assigned to the obtained sequences (Table 1). Symptoms on the begomovirus infected samples including chlorosis, mosaic, blisters, and dwarfing (Figure 2).

The laboratory work presented here identified a diverse species of *Begomovirus* infecting 13 commercial crops and 1 weed in four regencies in Java Island of Indonesia. All sampled plants exhibited clear and severe symptoms. ToLCNDV was previously detected in cucumber (*Cucumis sativus*) grown in Klaten (Listihani et al. 2019). Nidianti et al. (2023) recently found SLCCNV infection on a melon cultivar screened for breeding program in Sleman. Another recent report recorded PepYLCIV and SLCCNV in Magelang Regency, infecting chili pepper and squash, respectively (Santosa et al. 2024). Therefore, begomoviruses were shown to be still major problem hampering cultivation of different horticultural commodities and inciting economical losses in the surveyed areas.

KH and SPG primers were demonstrated to be conveniently used in PCR for molecular identification of six species of *Begomovirus*. As results, nucleotide sequences of two different genome regions (*AV1*, and *AC1* and *AC2*) of 14 isolates of the six begomoviruses were obtained using Sanger method in this study, and then the sequences were registered in NCBI GenBank.

Phylogenetic analysis of the obtained sequences formed two separate trees based on the genomic regions. Both trees clustered isolates into different phylogroups according to their respective species. The newly obtained isolates generally have a closer genetic relationship with previously reported Indonesian isolates, as shown by their share of common basal nodes, than isolates from other country. Two melon isolates from Bantul regency (MIBTL and MIBTL2) were in SLCCNV group in *AV1* tree but positioned in ToLCNDV group in *AC1-AC2* tree, which indicated recombination in their genomes and thus the necessity to further analysis using RDP software (Figure 3).

Table 1. Begomovirus isolates identified in this report

No.	Isolate name	Species ^a	Host	Origin	Genbank accession no.	
					Partial AV1 gene	Partial AC1 and AC2 genes
1.	ACSMN	AYVCNV	<i>Ageratum conyzoides</i>	Sleman	PP874885	PP874895
2.	TrMGG	TYLCKaV	eggplant	Magelang	PP874886	PP874896
3.	ToKLN-Kr	TYLCKaV	tomato	Klaten	PP874887	PP874897
4.	CbKLN-Kr	PepYLCIV	chili pepper	Klaten	PP874888	PP874898
5.	CbKLN-K	PepYLCIV	chili pepper	Klaten	PP874889	PP874899
6.	CbMGG	PepYLCIV	chili pepper	Magelang	PP874890	PP874900
7.	CbSMN	PepYLCIV	chili pepper	Sleman	PP874891	PP874901
8.	LbSMN	SLCCNV	squash	Sleman	PP874892	PP874902
9.	KPMGG	MYMIV	yardlong bean	Magelang	PP874893	PP874903
10.	KPSMN	MYMIV	yardlong bean	Sleman	PP874894	PP874904
11.	LbSMN ₂	SLCCNV-ToLCNDV ^β	squash	Sleman	PQ112501	PQ112505
12.	MIBTL	SLCCNV-ToLCNDV ^β	melon	Bantul	PQ112502	PQ112506
13.	MIBTL ₂	SLCCNV-ToLCNDV ^β	melon	Bantul	PQ112503	PQ112507
14.	LbSMN ₃	ToLCNDV	squash	Sleman	PQ112504	PQ112508

^aageratum yellow vein China virus = AYVCNV; tomato yellow leaf curl Kanchanaburi virus = TYLCKaV; pepper yellow leaf curl Indonesia virus = PepYLCIV; squash leaf curl China virus = SLCCNV; mungbean yellow mosaic India virus = MYMIV; tomato leaf curl New Delhi virus = ToLCNDV. ^βRecombinant isolates.

Recombination in the *AC1* and *AC2* regions of genomes of MIBTL and MIBTL₂ was detected by RDP, Bootscan, MaxChi, Chimera, and 3Seq algorithms in RDP thus can be deduced as significant. WMK, a SLCCNV isolate from Indonesia, was revealed as the major parent of the two isolates while 17MY167A, a ToLCNDV isolate from Malaysia, was the minor parent. The RDP analysis also found a strong recombination signal in the genome of LbSMN₂ with support from RDP, GENECONV, MaxChi, Chimaera, SiScan, and 3Seq algorithms. A SLCCNV isolate from Timor-Leste (T4D) was suggested to be the major parent while its minor parent was LbSMN₃, a ToLCNDV isolate from Indonesia.

MBTL and MBTL₂ isolates were included in SLCCNV cluster in KH tree while positioned in ToLCNDV group in SPG tree, strongly indicating recombination. MBTL and MBTL₂ isolates which showed almost identical genetic sequences, at least in the observed regions, originated from two melon plants separated only around 25 m each other in the same greenhouse in Bantul Regency. These two were the only plants showing clear viral symptoms among >300 individuals in the greenhouse. RDP analysis confirmed MBTL and MBTL₂ were SLCCNV recombinant isolates which received donor from ToLCNDV. Scan using RDP further detected a recombination signal in the genome of LbSMN₂, which was also a SLCCNV isolate recipient of genetic donor from ToLCNDV. This recombination was unlikely observable if only one of primer pairs applied in the molecular diagnostic. Both SLCCNV and ToLCNDV are prone for recombination, and interspecies genetic exchange between them have been reported to occur naturally (Venkataravanappa et al. 2018). Another study demonstrated a case of intraviral recombination in the genome of an Indonesian ToLCNDV isolate

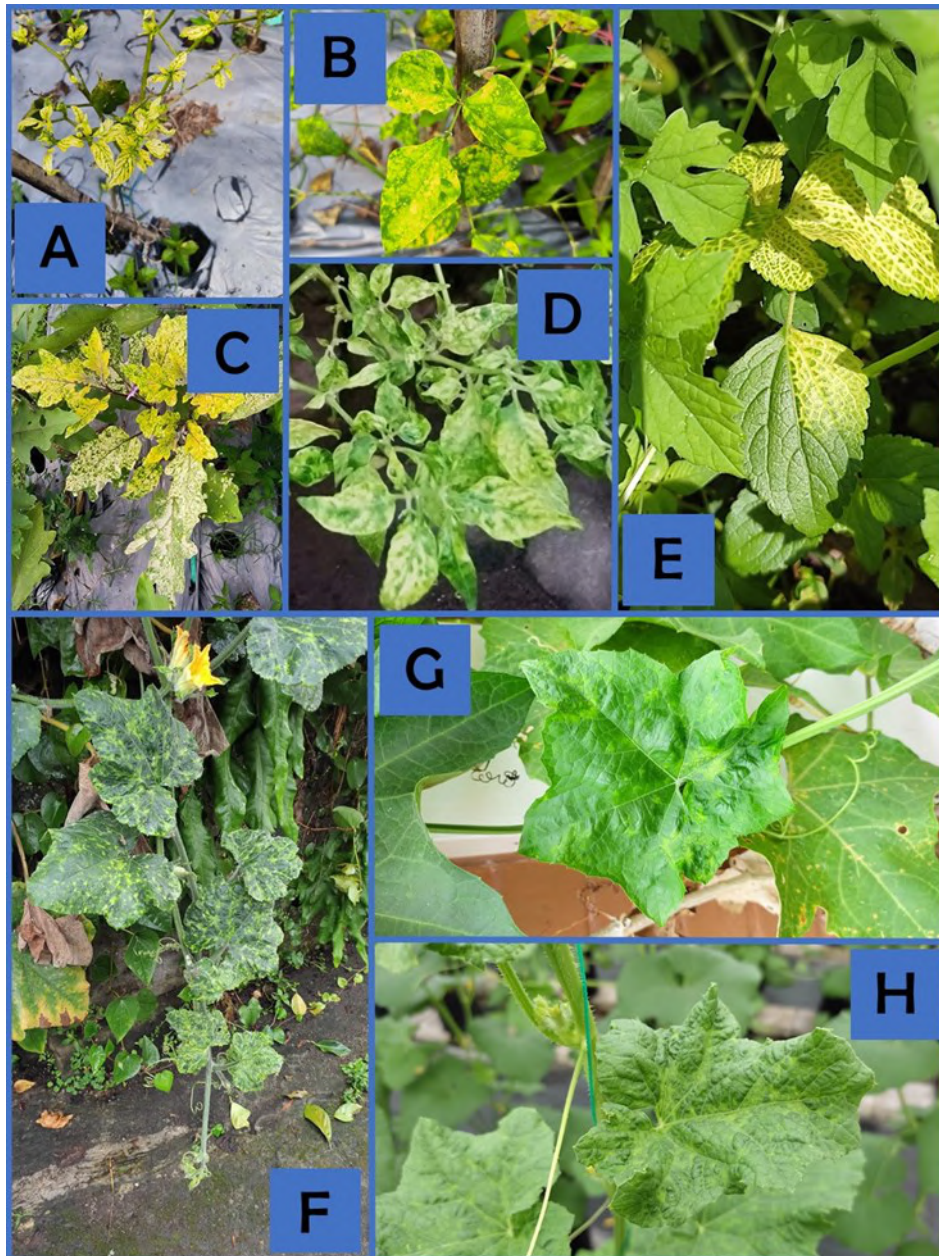


Figure 2. Variation of disease symptoms on horticultural crops caused by different begomoviruses reported in this study. A. Chlorosis and stunting on chili pepper infected by PepYLCIV isolate CbMGG in Magelang; B. Chlorosis on yardlong bean infected by MYMIV isolate KPMGG in Magelang; C. Chlorosis on eggplant infected by TY-LCKaV isolate TrMGG in Magelang; D. Chlorosis on chili pepper infected by PepYLCIV isolate CbSMN in Sleman; E. Vein chlorosis on *Ageratum conyzoides* infected by AYVCNV isolate ACSMN in Sleman; F. Severe mosaic and blisters on squash infected by recombinant SLCCNV-ToLCNDV isolate LbSMN2 in Sleman; G. Severe mosaic on squash infected by ToLCNDV isolate LbSMN3 in Sleman; H. Mosaic on melon infected by recombinant SLCCNV-ToLCNDV isolate MlBTL2 in Bantul.

(Wilisiani et al. 2019). In begomoviruses, recombination may act as a mechanism that ensures the evolution path is stay adaptive to new hosts or changing environmental constraints (Vo et al. 2022).

Protocol for detection and molecular characterisation of begomoviruses using KH and SPG has been established in this report but both primer pairs need to be tested against other species to complement the efficacy data of these primers. Nucleotide sequences covered by both primers provided important genomic data of isolates, including recombination events. Therefore, future studies on identification of begomoviruses are recommended to use both primer pairs to reduce false negative in case a distinct recombinant breakpoint could not be detected by one of the primers. Additional nucleotide sequences of 14 begomovirus isolates improved our understanding of the ge-

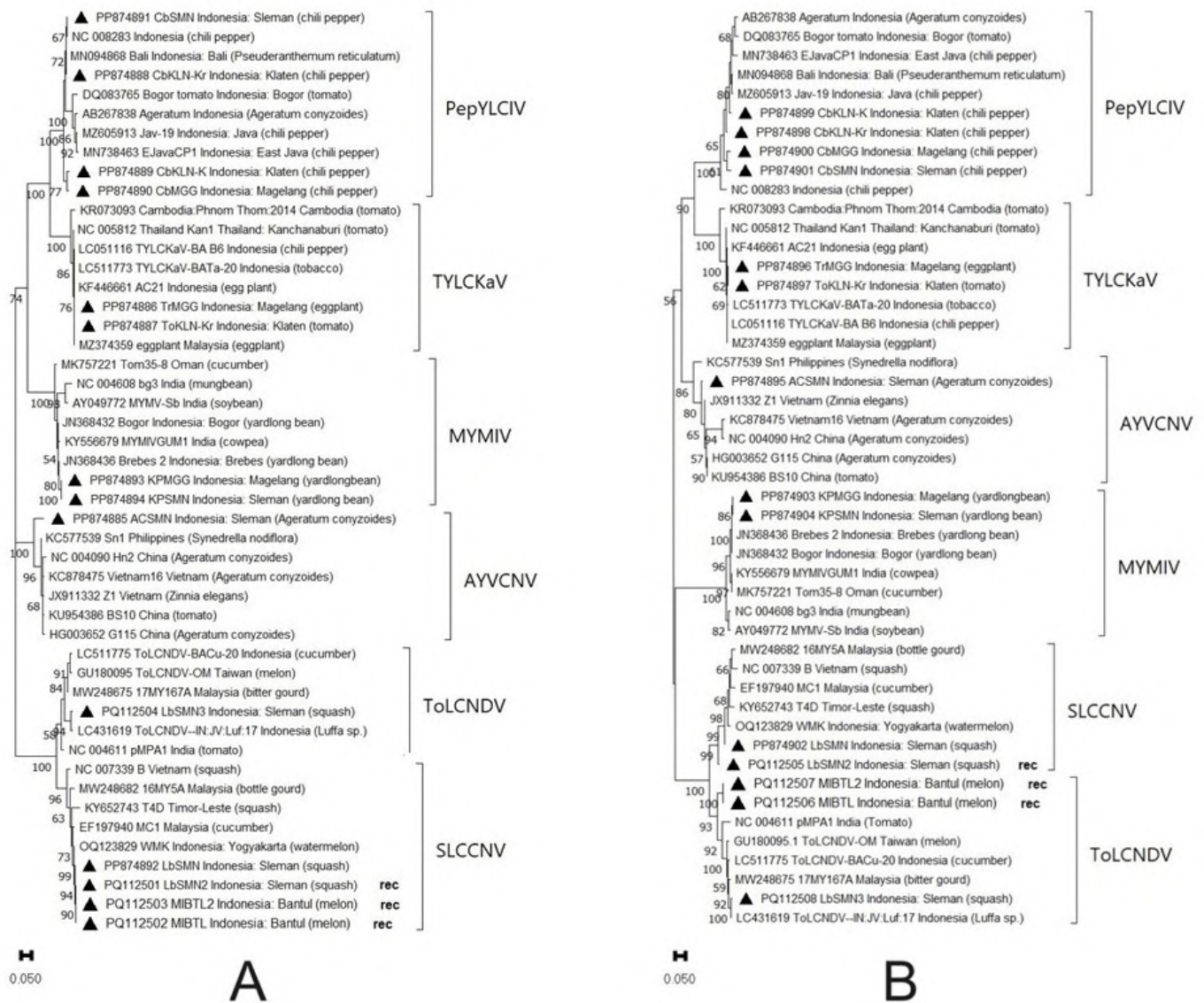


Figure 3. Phylogenetic trees based on nucleotide sequences of (A) ± 552 bp partial *AV1* gene targeted by Krusty/Homer primers and (B) ± 893 bp partial *AC1* and *AC2* genes covered by SPG1/SPG2 primers of isolates of six begomovirus species. The trees were generated in MEGA11 software by the Maximum-Likelihood method based on Tamura-Nei-parameter's model (1000 bootstrap replicates, only >50 % values were shown). Isolates reported here were marked with black triangles. SLCCNV and ToLCNDV recombinant isolates were marked with 'rec'. Ageratum yellow vein China virus = AYVCNV; tomato yellow leaf curl Kanchanaburi virus = TYLCKaV; pepper yellow leaf curl Indonesia virus = PepYLCIV; squash leaf curl China virus = SLCCNV; mungbean yellow mosaic India virus = MYMIV; tomato leaf curl New Delhi virus = ToLCNDV

onomic variance of Indonesian isolates. Further surveys are needed to study our local recombinant isolates that potentially cause outbreaks in the future, as have been observed in other countries (Moriones et al. 2017).

AUTHOR CONTRIBUTION

N.K.D. and H.A.Z. collected samples, conducted field observation, and performed laboratory research. A.S. performed laboratory research and wrote the draft manuscript. R.S. collected samples and conducted field observation. A.S.S. and M.A.H.A. analysed the data and revised the manuscript. A.I.S. designed and supervised the research, analysed the data, and wrote the draft manuscript.

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CONFLICT OF INTEREST

Authors have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Short Communication

Characteristics of Habitat and Diet of the Southern River Terrapin (*Batagur borneoensis*) in the Conservation Area on the Coast of Aceh, Indonesia

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ABSTRACT

The Southern River Terrapin (*Batagur borneoensis*) is an endangered aquatic species with a critically low population, found only in specific habitats. Surveys revealed that it forages, basks, and mates in freshwater to brackish rivers, while nesting occurs in sandy coastal areas. Its diet consists of crustaceans (41 %), vegetation (41 %), and gastropods (18 %), indicating a primarily carnivorous diet that includes fruits, flowers, leaves, shoots, and aquatic vegetation. Various crustaceans and gastropods were also observed as food sources. Conservation efforts are essential to preserving its habitat and food supply, ensuring the survival of this endemic species.

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The eastern coastal region of Aceh Province is home to one of the largest mangrove ecosystems in Southeast Asia, covering an area of 8,000 hectares (Safuridar et al. 2022). This mangrove area plays a crucial ecological role for various aquatic animals, including fish, shrimp, and the Southern River Terrapin (*Batagur borneoensis*). The mangrove vegetation and root structures, such as stilt roots, pneumatophores, and buttress roots, are highly beneficial as breeding grounds, nurseries, shelters, and foraging areas for various aquatic animals (Mawardi et al. 2023). The high nutrient composition in this mangrove ecosystem, derived from the decomposition of leaves and organic matter, makes this coastal area a potential habitat with a complex food chain (Mawardi et al. 2024).

The Southern River Terrapin (*Batagur borneoensis*) is an endemic aquatic species belonging to the class Reptilia and family Geoemydidae. According to The Red List of Threatened Species (IUCN), *B. borneoensis* is categorised as an endangered endemic aquatic species (Shepherd et al. 2021). Its population in its natural habitat is currently declining due to ecological pressures, illegal hunting by collectors, and threats from climate change and predators (Saputra et al. 2023; Suriadi et al. 2024). Currently, studies reported that the population of this species is only found in the aquatic regions of Asia, including Indonesia, Malaysia, and Bangladesh (Saputra et al. 2020; Bristy et al. 2021). The unique habitat characteristics and the abundance of available food in specific areas are the main reasons this aquatic species can only live and reproduce sustainably in suitable coastal regions (Guntoro et al. 2020).

The coastal region of Aceh Province plays a crucial role as a natural habitat for *B. borneoensis*. The suitable environmental characteristics of this coastal area are key factors in supporting the population of this endemic species. The extensive mangrove ecosystem provides abundant food, while the calm river flows and the wide sandy beaches create an ideal habitat for *B. borneoensis* (Saputra et al. 2023). To ensure the survival of this endemic coastal species in its habitat, conservation efforts are essential (Serdiati et al. 2023). The Yayasan Satucita Lestari Indonesia (YSLI) is one of the leading organisations in the conservation of *B. borneoensis* in the coastal region of Aceh Province. Routine patrols are conducted to prevent the hunting of *B. borneoensis* eggs and adults and to educate the community about preserving the coastal environment as a habitat for various species, including endangered endemic animals (Mawardi et al. 2022).

This research aims: 1) to identify the habitat characteristics of *B. borneoensis*, including its foraging areas, basking spots, nesting sites, and nursery zones; 2) to identify the types of food consumed by *B. borneoensis* in its natural habitat in the coastal waters of Aceh, and; 3) to maintain a stable food supply during conservation efforts. The abundance of food and suitable habitats are crucial components for the survival of this endemic species in its natural environment.

The research was conducted in the coastal area of Seruway, Aceh Province, from April to July 2024. Activities involved tracing the river currents and coastal edges for *B. borneoensis* to determine habitat characteristics and identify the types of food consumed by this endemic species. Habitat characteristics and food types were identified at four locations within the survey was conducted daily, once a week, using a boat from 7:00 AM to 6:00 PM. The identification of habitat characteristics was carried out by observing the activities of *B. borneoensis* at four designated locations, including basking, feeding, mating, nesting, and nursery areas. All observed data were then recorded accordingly. Similarly, the types of food were identified based on field observations of what *B. borneoensis* consumed in its habitat, and all kinds of food eaten were comprehensively recorded. The four research locations along the coastal area of Seruway, Aceh Province, can be specifically seen in Figure 1. Param-

ters measured included water pH using a pH meter (Extech PH100), water salinity using a Hand Refractometer (RHS-10ATC), and water temperature using a Mercury Thermometer (-10°C to 110°C). All data on food types and physicochemical parameters of the aquatic environment were analysed descriptively in percentage form using Microsoft Excel.

The results of the observations in the research area showed that *B. borneensis* was predominantly found in freshwater river streams and mangrove water areas, accounting for 84.62 %, while only 15.38 % were found in coastal areas. This endemic species predominantly lives in water, only coming ashore or onto trees at specific times to bask (Hernawan et al. 2019). The abundance of aquatic vegetation and mangrove plants provides an ideal habitat for the terrapin, serving not only as a food source but also as a basking sites and shelter from predators and other threats. Observations show that terrapin are seen predominantly basking on the branches of *Sonneratia caseolaris* and *Rhizophora mangle*. These plants are favoured by the terrapin, as they provide both basking spots and food resources. The specific habitat characteristics of *B. borneensis* are detailed in Table 1.

According to Table 1, the main habitat of *B. borneensis* includes river flows and mangrove ecosystem areas. The habitat characteristics of this species are dominated by *Sonneratia caseolaris* and various aquatic plants such as *Eichhornia crassipes*. The terrapin predominantly inhabits mangrove ecosystems in coastal regions, which are also occupied by species like *Rhizophora mangle*, *Casuarina equisetifolia*, and other aquatic species (Mallick et al. 2021; Mim et al. 2022). In addition to providing food, the plant vegetation in this habitat also serves as a basking site and shelter for the terrapin (Bristy et al. 2021). *B. borneensis* exhibits unique behaviour when nesting; it migrates to

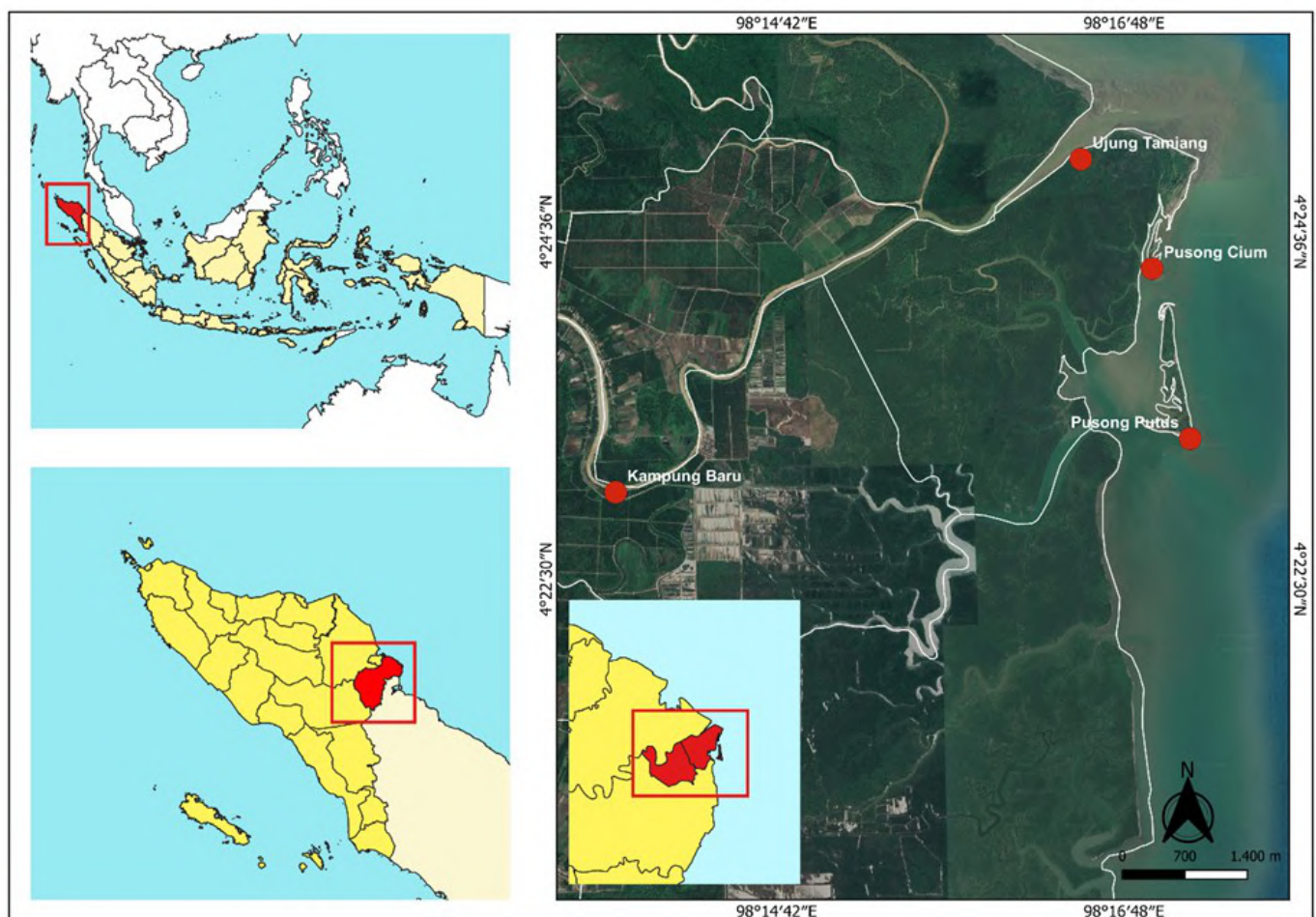


Figure 1. Research locations for habitat characteristics and diet of *B. borneensis* in the coastal area of Aceh Province.

Table 1. Habitat characteristics of *B. borneensis* in the coastal area of Aceh Province.

Location	Water Salinity (°C)	Water pH	Water Temperature (°C)	Plant Vegetation	Habitat Type
Kampung Baru	0.00-5.00	6.3	28	<i>Sonneratia caseolaris</i> , <i>Eichornia crassipes</i> , <i>Rhizophora mangle</i> .	Foraging, basking, mating, and nursery areas
Ujung Tamiang	15-21	6.5	28.5	<i>Avecennia germinans</i> , <i>Ipomoea imperati</i> , <i>Casuarina equisetifolia</i> .	Nesting & spawning
Pusung Cium	32-35	6.8	29	<i>Scaevola taccada</i> , <i>Casuarina equisetifolia</i> , <i>Avecennia germinans</i> .	Nesting & spawning
Pusung Putus	25-32	6.7	29	<i>Ageratina adenophora</i> , <i>Casuarina rosea</i> , <i>Casuarina equisetifolia</i> .	Nesting & spawning

sandy coastal areas to select suitable and safe locations for laying eggs (Figure 2). During nesting, the terrapin digs a hole in the sandy beach and, after laying eggs, covers it with sand to protect it from predation and human exploitation (Spitzweg et al. 2018; Guntoro et al. 2020).



Figure 2. (a) Habitat of *B. borneensis* in mangrove ecosystem, Ujung Tamiang; (b) Nesting site on the beach, Pusung Cium.

B. borneensis is a carnivorous species that consumes a variety of aquatic and mangrove vegetation. Fruits and flowers are the most dominant plant organs eaten by the terrapin, coming from various mangrove plant species and aquatic animals. Aquatic plants, such as leaves, shoots, and roots, are also part of its diet. The observations and surveys conducted in its natural habitat along the coastal areas of Aceh Province successfully recorded and collected data on various species of gastropods and crustaceans living in both aquatic and coastal areas, which form the regular diet of this endemic species. Observations collected various aquatic animal species as food for *B. borneensis* (Table 2).

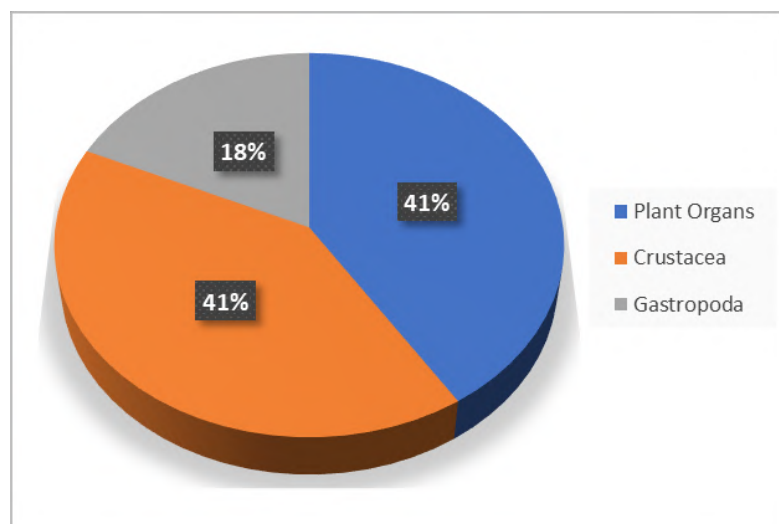
Table 2 shows that the most dominant food sources for *B. borneensis* in its habitat are plant vegetation and species of crustaceans. Based on observations, various species of gastropods also serve as a potential and nutrient-rich food source, with most of the gastropods consumed by the terrapin belonging to the Family Neritidae. Fruits and flowers of plants are the most dominant plant organs consumed by *B. borneensis*, followed by leaves and shoots. When the terrapin is in freshwater river areas, roots and tubers of aquatic plants are the most dominant food sources (Saputra et al. 2023). It is crucial to protect various plant species that serve as both habitat and food sources for the terra-

Table 2. Types of Food Consumed by *B. borneoensis* in Its Habitat at the Research Location.

Family	Species Names	Organs			Organs & meat
		Leaves/shoots	Fruits/flowers	Tuber/root	
Plants					
Lythraceae	<i>Sonneratia caseolaris</i>	-	+	-	-
Convolvulaceae	<i>Ipomoea aquatica</i>	+	+	+	-
	<i>Ipomoea imperati</i>	+	+	+	-
Ontederiaceae	<i>Pontederia crassipes</i>	-	-	+	-
Vitaceae	<i>Cayratia trifolia</i>	-	+	-	-
Fabaceae	<i>Cercis siliquastrum</i>	+	+	-	-
Rhizophoraceae	<i>Rhizophora mangle</i>	+	+	+	-
Acathaceae	<i>Avecennia germinans</i>	+	+	-	-
Pandanaceae	<i>Pandanus odorifer</i>	-	+	-	-
Crustacea					
Penaeidae	<i>Penaeus marguensis</i>	-	-	-	+
	<i>Penaeus monodon</i>	-	-	-	+
	<i>Penaeus setiferus</i>	-	-	-	+
		-	-	-	+
Sergestidae		-	-	-	+
Mysidae		-	-	-	+
Palaemonidae		-	-	-	+
Ocypodidae		-	-	-	+
		-	-	-	+
Gastropoda					
Neritidae		-	-	-	+
		-	-	-	+
		-	-	-	+
Naticidae		-	-	-	+

pin, as this vegetation plays a vital role in the sustainable survival of this endemic species (Tshewang et al. 2021).

In general, the regular diet of *B. borneoensis* in its natural habitat consists of various plant vegetation, different species of crustaceans, and gastropods found in freshwater, brackish, and coastal areas. Plant organs and crustacean species are the most dominant foods consumed by this terrapin, each constituting 41 % of the diet, while gastropod species contribute the least at 18 % (Figure 3). Adequate food sources and suitable environmental conditions are key factors for the survival of various animal species, including *B. borneoensis*. Environments with abundant food sources positively impact the high biodiversity of the area (Talukdar et al. 2021; Mawardi et al. 2024).

**Figure 3.** Diet composition of *B. borneoensis* in its habitat at the research location.

The research results aimed at identifying the types of food based on animals closely related to *B. borneoensis* (Karyadi et al. 2023) reported that the diet of the turtle (*Heosemys spinosa*) in the conservation area at Bengkulu University includes various types of plants, vegetables, gastropods, annelids, and various insect species. The dietary data for *Heosemys spinosa* can be seen in Table 3.

Table 3. The diet of the turtle (*Heosemys spinosa*) in the conservation area at Bengkulu University.

Food Groups	Type of Food (Organism)
Plants	Taro leaves
	Banana fruit
	Papaya fruit
	Vegetables
	Other herbal plants
Gastropods	Various species of snails
Insects	Various species of insects
Annelids	Various species of earthworms

Morphologically, *B. borneoensis* has two pairs of legs with webbed feet that function as propulsion and swimming tools in strong current areas, such as river flows and coastal regions. When it is time to lay eggs, *B. borneoensis* migrates from freshwater or brackishwater to the coastline (Figures 4a & 4b). The nesting behaviour of this endemic species is similar to that of sea turtles; it seeks a suitable location to create a nest, then digs a hole on the beach to lay its eggs. After laying the eggs, the terrapin covers them with sand to protect them from predation and human exploitation (Spitzweg et al. 2018; Saputra et al. 2020).



Figure 4. (a) *B. borneoensis* species (b) Nest and eggs of *B. borneoensis* on the beach.

Research conducted in various countries shows that the population of *B. borneoensis* in the wild is now endangered due to habitat degradation and, the exploitation of eggs and adults for commercial purposes, and as collectibles by collectors (Rahayu et al. 2021). The exploitation of eggs by local communities harms the regeneration of this endemic species, leading to a decline in the population of *Batagur borneoensis* in its natural habitat, as well as predation by natural predators in the surrounding area (Suriadi et al. 2024). The situation facing the population of *Batagur borneoensis* must be addressed collectively by the government, academics, and the public to ensure the conservation of this species (Hernawan et al. 2019; Guntoro et al. 2020).

Environmental degradation in the coastal areas of Aceh Province, which serve as the primary habitat for *B. borneoensis*, represents a serious threat to

the conservation of this species (Stanford et al. 2020). Increasing coastal community activities, such as fishermen using river flows as transportation for fishing, and the clearing of new lands for agriculture and plantations, have significantly disrupted the habitat of *B. borneoensis* (Saputra et al. 2021). Serious efforts from various parties and stakeholders are required for sustainable coastal management to protect the habitat of aquatic biota and endangered endemic species.

AUTHORS CONTRIBUTION

A.L.M. designed the research activities, coordinated the research, and wrote reports and scientific articles. T.H.W.A. conducted field observations on the habitat characteristics and diet of *B. borneoensis*. M.K. assisted with data collection and analysis of the habitat and diet of *B. borneoensis*.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest regarding this research.

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Short Communication

Metabarcoding of Freshwater Green Microalgae from Two Urban Streams in the Philippines

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ABSTRACT

Green microalgae (Chlorophyta) are found worldwide in various environments. Currently, there are few studies on Philippine freshwater green microalgae and traditional identification methods using morphology and life cycle characters are time consuming and may be unreliable. This study used eDNA and Illumina sequencing to sample algal communities in two urban streams situated in a local university campus and found 39 chlorophyte genera. Three markers were used and each marker detected taxa not found by the other markers, suggesting that a multimarker approach might be best for future metabarcoding studies and that other gene markers should be tested for utility.

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Green microalgae (Chlorophyta) are ubiquitous dwellers of terrestrial and aquatic ecosystems, found in both freshwater and marine ecosystems, and are commonly used for water quality assessments. They are a diverse lineage with an estimated 5000 undescribed species (Guiry 2012). The study and identification of algae have traditionally relied on morphology and culture characteristics, such as cell shape, life cycle, and other physical characteristics (Zou et al. 2016). However, these characters often exhibit plasticity and are not always reliable, especially with regard to cryptic taxa (Cianciola et al. 2010).

Studies of freshwater green microalgae in other countries have recently started using metabarcoding as a tool for surveying biodiversity (Jacobs-Palmer et al. 2021; Bukin et al. 2022). Similar to traditional DNA barcoding (Zou et al. 2016), metabarcoding utilises short gene sequences for molecular identification and can identify multiple species from a single sample (Kezlya et al. 2023). Metabarcoding, using multiple DNA markers for rapid biodiversity assessments, has proven effective and multiple metabarcoding projects for green algae have been conducted internationally (Groendahl et al. 2017; Jacobs-Palmer et al. 2021; Bukin et al. 2022).

There have been several limnological surveys in the Philippines that also identified freshwater green microalgae, such as Papa and Mamaril's (2011) survey of Taal Lake and a microalgal survey of the Rio Grande de Cagayan (Baleta et al. 2016). However, these studies have mostly relied on morphology for identification, and to date, no metabarcoding studies have been published for Philippine freshwater green microalgae.

This study presents the first application of metabarcoding to Philippine freshwater green microalgae sampled from urban streams. Results from traditional, culture-based identification are also briefly discussed.

Water sampling from urban streams in the University of the Philippines Institute of Biology Arboretum (AR; 14°39.00'N, 121°4.14'E) and along the University Avenue (UA; 14°39.24'N, 121°3.6'E) was conducted in February 2022 and March 2023, respectively. Samples were collected using one-litre Pyrex glass bottles that had been bleached, and washed with distilled water, and then autoclaved for sterility. Two replicates of three litres each were collected at each site. Surface and subsurface (< 5 cm depth) water were collected; however, both streams were shallow (< 0.5-metre depth at the sampling site) and gently flowing, so some sediment might have mixed in.

Water samples were vacuum-filtered using 20 µm nylon sieves (Sigma Aldrich, USA) to capture environmental DNA. DNA extraction from the sieves was performed by mashing the sieves using glass beads in 700 µL of lysis buffer (Zymo Research, USA) and a bead basher (Disruptor Genie, Scientific Industries, USA) at 328 × g (3,000 rpm) for 10 minutes. The resulting mixture was centrifuged at 20,000 × g for 10 minutes and the supernatant was collected. DNA purification was performed using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) following the manufacturer's protocols, with only the bead bashing step modified as previously indicated. Samples were then sent to MacroGen Inc. (Seoul, South Korea) for library preparation and Illumina MiSeq 250 base-pair (bp) paired-end sequencing. Primers with proven utility for algal metabarcoding, such as the V4 and V9 regions of the 18S nuclear gene and the 23S plastid gene (Bukin et al. 2022; Kezlya et al. 2023) were used for DNA amplification.

For both sampling locations, the V4 and V9 regions (Table S1) of the 18S gene were selected as standard primers due to their ease of amplification and relatively small (less than 600 bp for V4, and less than 200 bp for V9) product size. For the AR samples, the 23S markers were also tested for their utility in metabarcoding (Table S1). The 23S marker could not be tested at both sites due to funding constraints.

For downstream analysis, reads from both replicates at each sampling

site were pooled. Raw Illumina reads were filtered and trimmed using *Cutadapt* v4.4 (Martin 2011) to remove short reads, primers, and Illumina adapters. Filtered reads were analysed using the *dada2* v1.16 pipeline (Callahan et al. 2016) in R v4.3. Merged reads for the V4 and V9 markers were aligned to the SILVA database v132 (Callahan 2018). A custom database was made for 23S by downloading chlorophyte sequences from GenBank. Sequences were formatted according to the SILVA database for use in the *dada2* pipeline. Bootstrap support for database matching was set to the *dada2* package's default 50 % threshold.

For GenBank, the search terms for 23S were “((((Chlorophyta) OR Chlorophyta [Organism]) AND 23S[All Fields]) AND 100:10000[SLen] NOT “shotgun” [All Fields]”. Also, 23S sequences from two chloroplast genomes (MF197536 and NC_045059) were added to the final database.

The resulting classifications for V4, V9, and 23S were filtered to retain only Chlorophyta sequences using the *phyloseq* R package v1.41 (McMurdie & Holmes 2013). Graphs illustrating which green algal genera were identified by each marker and diversity index computations were generated in Microsoft Excel. Pooled Illumina reads were uploaded to the Sequence Read Archive of GenBank (Table S2).

Water samples from both locations were also plated on Bold's Basal Medium with 1 % kanamycin and 1 % nystatin. Cultured algae were viewed under light microscopy for morphological examination, and identity was further confirmed using the *tufA* marker (Table S1).

Reads from the V4 primers, the majority of which range from 477 to 518 bp, resulted in 1659 Amplicon Sequence Variants (ASVs). Of these, 216 were classified as Phylum Chlorophyta sequences when aligned to the SILVA database (v132), and 102 ASVs were further classified down to genus level, resulting in 26 genera (Figure 1A) among 19 families. The remaining 114 chlorophyte sequences were not matched to genus level and were discarded for further analysis.

The V9 primers yielded 2967 ASVs. A total of 82 ASVs were classified as Phylum Chlorophyta while 18 ASVs were classified down to genus level, resulting in 15 genera (Figure 1B) distributed among 14 families.

Processing of reads from the 23S primers resulted in 1185 ASVs. 45 of these ASVs were non-chlorophyte eukaryotes, while an additional ASV was classified as a Prasinodermophyte. Of the remaining 1139 chlorophyte ASVs, 102 were classified down to genus level by the *dada2* classifier, with nine genera distributed among eight families from the AR samples (Figure 1C). The 23S marker was not used for the University Avenue stream samples; therefore, there are no results from that site.

A total of 36 chlorophyte genera were detected by V4, V9, and 23S (Table 1). Some overlaps in taxon detection exist among each marker. *Oedogonium* and *Chlamydomonas* were detected by all three markers, while 10 other taxa were detected by at least two markers. The remaining 24 are unique to a specific marker, with 15 of those detected only by V4, five by 23S, and four by V9.

Samples from the Arboretum were somewhat more diverse as compared to the University Avenue based on Shannon H-values (Table 2) for both V4 and V9 primers. There is however a concern that the Shannon value for V4 is due to the overamplification of *Spermatozopsis* sequences, with 65 of the 102 chlorophyte ASVs matched to this genus. This is possibly a result of PCR bias, given that no other marker showed the same over abundance for this species in this study. The 65 ASVs for *Spermatozopsis* were matched to over 59,000 reads, with the next most abundant genus, *Micractinium*, comprised of only two ASVs with 449 matches (Figure 1A). Removing *Spermatozopsis* counts results in a nearly equal Shannon index value for AR and UA for the

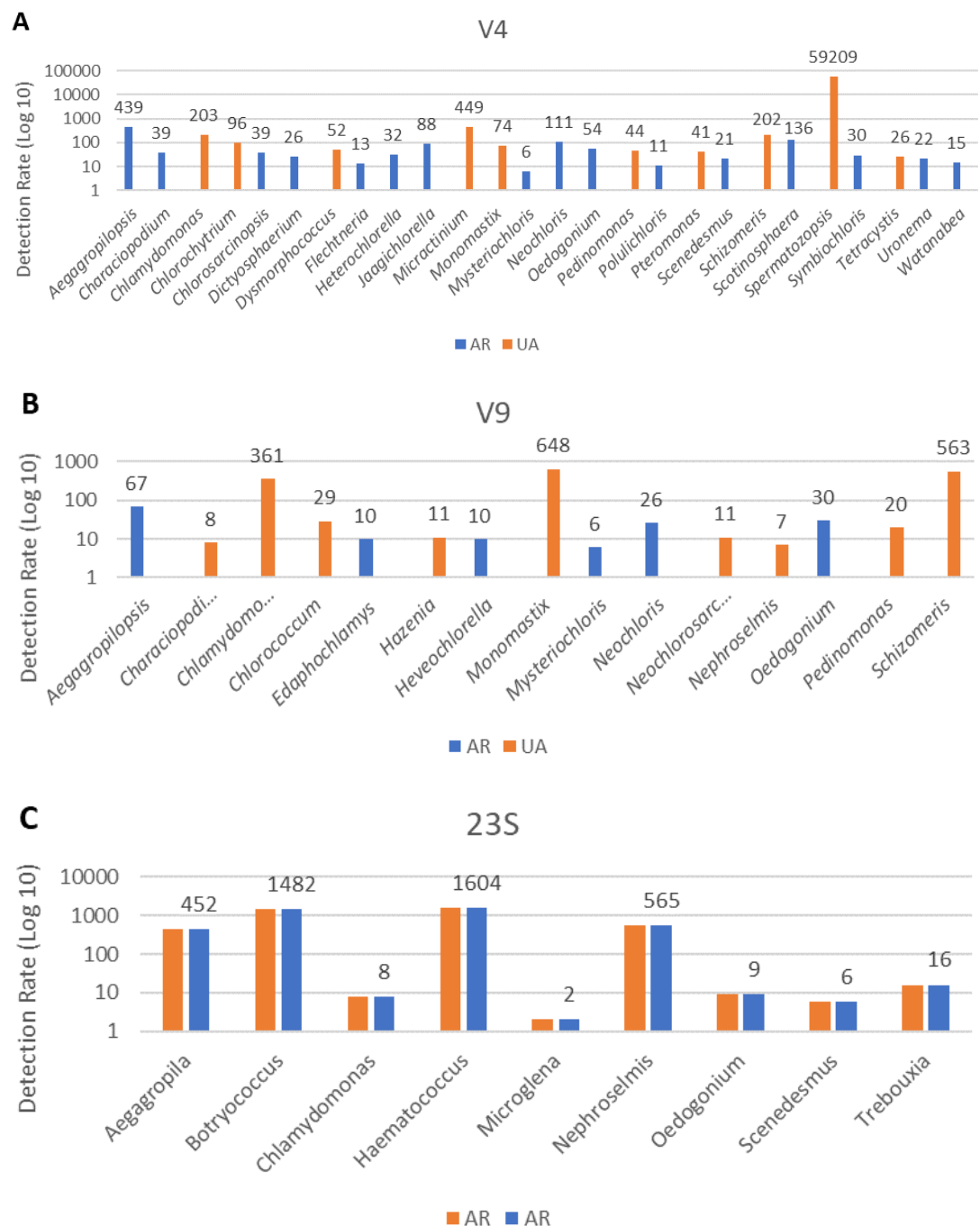


Figure 1. Chlorophyte genera detected by each marker. The Y-axes represent the detected ASVs, converted to logarithmic (log 10) scale to better visualise less represented taxa. The absolute number of matched ASVs for each genus is displayed atop their corresponding bar. Blue bars represent taxa found in the Arboretum, while orange bars represent taxa found near the University Avenue.

A. Chlorophyte genera detected and classified by V4 marker.

B. Chlorophyte genera detected and classified by V9 marker.

C. Chlorophyte genera detected and classified by 23S marker. This marker was only used in the Arboretum.

V4 marker (Table 2).

The V4 marker is the most productive for both eukaryotic surveys in general and for algal classification in particular, detecting nearly 1700 eukaryotic ASVs and classifying 26 chlorophyte genera. It also has the highest Shannon H-values among three markers, even with *Spermatozopsis* excluded. Meanwhile, the V9 primers classified fewer ASVs to the genus level but still resolved 15 chlorophyte genera, while detecting a significantly greater number of eukaryotic ASVs. The results presented here are consistent with previous studies (Tragin et al. 2018; Piredda et al. 2017) which found that the V4

primer provided better taxonomic resolution to genus level than V9. However, the latter classified more eukaryotic ASVs at higher taxonomic levels. These findings are in contrast to the results of Bradley et al. (2016), who found that the V9 primers better captured the community composition of mock samples than the V4 primers.

Table 1. Chlorophyte genera detected by each marker.

	V4		V9		23S
	AR	UA	AR	UA	AR
<i>Aegagropila</i>					✓
<i>Aegagropilopsis</i>	✓		✓		
<i>Botryococcus</i>					✓
<i>Characiopodium</i>	✓			✓	
<i>Chlamydomonas</i>		✓		✓	✓
<i>Chlorochytrium</i>		✓			
<i>Chlorococcum</i>				✓	
<i>Chlorosarcinopsis</i>	✓				
<i>Dictyosphaerium</i>	✓				
<i>Dysmorphococcus</i>		✓			
<i>Edaphochlamys</i>			✓		
<i>Flechtneria</i>	✓				
<i>Haematococcus</i>					✓
<i>Hazenia</i>				✓	
<i>Heterochlorella</i>	✓		✓		
<i>Jaagichlorella</i>	✓				
<i>Micractinium</i>		✓			
<i>Microglena</i>					✓
<i>Monomastix</i>		✓		✓	
<i>Mysteriochloris</i>	✓		✓		
<i>Neochloris</i>	✓		✓		
<i>Neochlorosarcina</i>				✓	
<i>Nephroselmis</i>				✓	✓
<i>Oedogonium</i>	✓		✓		✓
<i>Pedinomonas</i>		✓		✓	
<i>Polulichloris</i>	✓				
<i>Pteromonas</i>		✓			
<i>Scenedesmus</i>	✓				✓
<i>Schizomeris</i>		✓		✓	
<i>Scotinosphaera</i>	✓				
<i>Spermatozopsis</i>		✓			
<i>Symbiochloris</i>	✓				
<i>Tetracystis</i>		✓			
<i>Trebouxia</i>					✓
<i>Uronema</i>	✓				
<i>Watanabea</i>	✓				
Total taxa detected	16	10	6	9	9

The lower resolution in V9 may derive from a less reliable database (Piredda et al. 2017). This study used the SILVA database for both 18S markers, specifically version 132 (Callahan 2018). Unfortunately, this database is no longer updated, having been published in 2018. The latest version of the SILVA database, v138.1, is no longer appropriate for use with eukaryotes

(McLaren & Callahan 2021). A more up-to-date database might have resulted in different classification rates for both V4 and V9.

Table 2. Shannon diversity values (H-values) for each marker and site.

Marker	AR	UA
V4	2.090	0.132(1.807 ¹)
V9	1.479	1.305
23S	1.308	N/A ²

The 23S primer, being designed specifically for chlorophytes, detected over a thousand Chlorophyte ASVs though only 102 ASVs were classified to genus level. This marker found the least number of chlorophyte genera, but with the caveat that it was only used in one site. The primer possibly has greater utility for metabarcoding of Chlorophyta and other algal groups, given its universality among organisms with plastids and its relatively short length of around 400 base pairs that make it suitable for Illumina high-throughput sequencing. Notably, over half of the nine genera detected by this marker were not found by the 18S markers, suggesting that targeted markers should be used to complement more universal markers that might be too conserved to differentiate lower taxonomic levels (Bradley et al. 2016; Hajibabaei et al. 2019).

Unfortunately, the available database for the 23S marker is currently limited. A search on GenBank using the keywords indicated earlier returned only 768 results, with two additional sequences from complete chloroplast genomes manually added; moreover, no publicly available sequences in the BOLD. The taxonomic coverage of the GenBank sequences also leaves much to be desired, with 51 labelled as "uncultured *Trebouxia*" and another 133 representing only four species. Expanding the database of 23S sequences could increase the utility of the primer for environmental DNA surveys.

Some of the genera detected by metabarcodes have been detected in the Philippines based on previous biodiversity surveys, such as *Chlamydomonas*, *Cladophora*, *Haematococcus*, *Oedogonium*, *Pandorina*, and several species of *Scenedesmus* (Papa & Mamaril Sr. 2011; Baleta et al. 2016; Ederosas & Jumanawan 2016; Tingson & Tamayo-Zafaralla 2018; Senados et al. 2021), lending support to the findings of the current metabarcoding workflow.

Since metabarcoding may not capture all biodiversity present at the time of sampling, and gaps may exist in the reference databases (Groendahl et al. 2017), traditional culture-based and morphological identification was also performed. Streak plating of water samples from the AR on Bold's Basal Medium detected several green microalgae genera, such as *Chlorococcum* (Figure 2A), and *Asterarcys* (Figure 2B) (Guiry & Guiry 2024). The identity of these genera was confirmed through traditional DNA barcoding using the *tufA* marker. *Asterarcys* was not detected by metabarcoding, while streak plating did not capture many of the algal genera detected using eDNA. Thus, both genetic and traditional identification methods should be considered in future Philippine algal surveys.

The results of this study demonstrate the potential of metabarcoding for studying Philippine freshwater green microalgae and identifying taxa for biological surveys. The V4 and V9 regions of the 18S gene show promise as markers for rapid biodiversity surveys of eukaryotes using environmental DNA, including Philippine chlorophytes, withas both have detected and clas-sified over a dozen green algae genera detected and classified by each marker. The 23S marker was not tested on other eukaryotes, and its utility for chloro-phytes remains somewhat limited due to a relatively small database compared to 18S genes. However, expanding the available reference sequences in Gen-

Bank and other databases, particularly those from Philippine taxa, might make this a more attractive marker in the future, given its reliable amplification in this study. Due to the varied taxa detected by each marker, a multi-marker approach might work best for Philippine metabarcoding studies (Bradley et al. 2016; Hajibabaei et al. 2019).

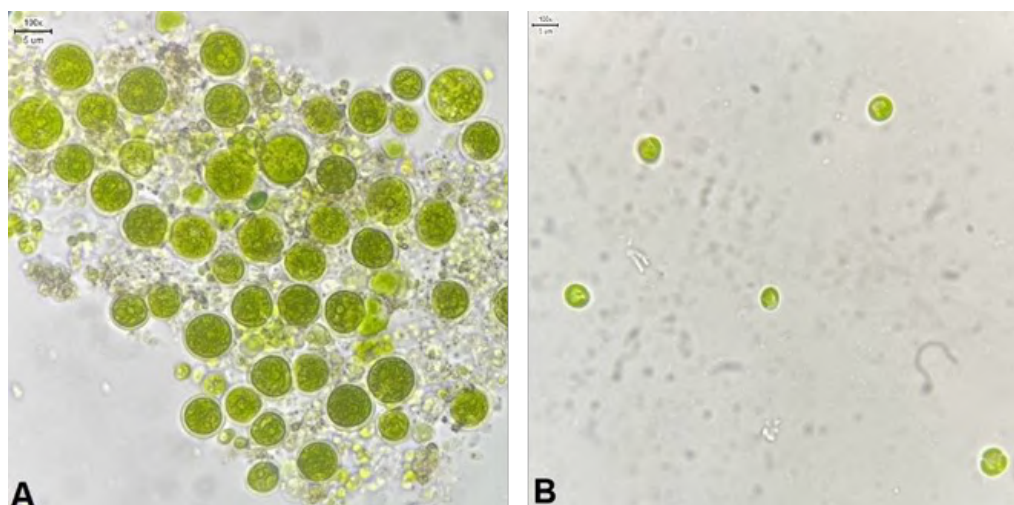


Figure 2. Algae cultured from water samples, viewed at 100X magnification. **A.** Clusters of unicellular *Chlorococcum* sp. isolated from the University Avenue sampling site. Scale bar = 5 µm. **B.** Isolates of unicellular *Asterarcys* sp. from the Arboretum sampling site. Scale bar = 5 µm.

AUTHOR CONTRIBUTION

G.C.L.Q., L.R.S.S., and W.D.M. conceptualised the study. L.R.S.S. and W.D.M. supervised the research. G.C.L.Q. and W.E.P. collected the data. G.C.L.Q. analysed the data and drafted the initial version of the manuscript. All authors revised and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest related to their participation in this study.

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Research Article

Whole-Genome Analysis and Tolerance Assessment on *Bifidobacteria*-like Bacteria Isolated from Breast Milk and Infant Fecal Samples in Indonesia

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ABSTRACT

Probiotics are living microorganisms that can provide health benefits to the host when they are present in the gastrointestinal tract in sufficient quantities. Probiotics should be able to survive the harsh conditions in the gastrointestinal tract. This study sequenced whole genomes of *Bifidobacteria*-like isolates (i.e., BS2-PS1, BS2-PS2, BS2-PB5, and BR2-12) and assessed their abilities to resist a low-pH environment and bile salt. Molecular identification through whole-genome sequencing indicated that the bacteria isolated from breast milk were identified as *Bifidobacterium breve* (BS2-PS1 and BS2-PS2) and *Lactocaseibacillus paracasei* subspecies *paracasei* (BS2-PB5), whereas the bacterial isolate isolated from infant feces was identified as *Lactiplantibacillus plantarum* (BR2-12). BS2-PS1 and BS2-PB5 exhibited resilience upon exposure to bile salts at concentrations of 0 %, 0.3 %, and 0.5 %, with survival observed even at the highest concentration (0.5 %) after 3 h of incubation. The ability to withstand bile salt was mediated by various genes (e.g., *bsh*, *cbh*, *dps*, *glf*, *cfb*, or *nagB*) found in genomes of tested isolates. Upon exposure to pH 5 for 3 h, BS2-PS1, BS2-PS2, and BR2-12 exhibited acid resistance as well. The ability to withstand low pH could be mediated by the presence of relevant genes (e.g., *argC*, *argH*, *dapA*, *pyk*, *pyrG*), as well as genes that encode the F₀F₁-ATPase enzyme, such as *atpC*, *atpB*, *atpE*, *atpF*, *atpH*, *atpA*, *atpG*, and *atpD*, in their genomes. These results highlighted the advantages of integrating *in vitro* and whole-genome data to evaluate the potential of bacterial isolates for further development as probiotic candidates.

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INTRODUCTION

Probiotics are well known for the many health benefits they offered. According to the Food and Agriculture Organisation of the United Nations (FAO) and World Health Organisation (WHO), probiotics are living microorganisms that can provide health benefits to their hosts if present in the gastrointestinal tract in sufficient quantities (Hill et al. 2014). Some of the benefits include reducing the risk of diarrhea and acting as an immunomodulator (Virk et al. 2024). The popularity of probiotic products has been on the rise globally due to the growing public awareness of their benefits. In 2018, the sales of probiotic products reached 8,618.9 million euros, and 9,401.6 million euros in 2021. Furthermore, the average annual growth of probiotic products is expected to reach 8.7 % in the period 2021–2030 (Europe International Probiotics Association 2023).

Lactobacillus and *Bifidobacterium* are commonly used probiotics that can be isolated from breast milk and infant fecal samples (Bazireh et al. 2020). Breast milk is crucial for infant growth and development. It contains key immunological elements (e.g., antibodies) and contributes to the development of the infant's immune system. Human Milk Oligosaccharides (HMOs) function as prebiotics, promoting the growth of beneficial bacteria in the digestive tract, such as *Bifidobacterium* species (spp.) (Ojo-Okunola et al. 2018; Yi & Kim 2021). A review on microbial communities in breast milk from 44 studies showed that a core microbiome of seven to nine bacterial genera was mainly found, including *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Pseudomonas*, *Corynebacterium*, *Enterococcus*, *Acinetobacter*, *Rothia*, *Cutibacterium*, *Veillonella*, *Bacteroides*, and *Bifidobacterium* spp. (Zimmermann & Curtis 2020). In line with these findings, *Bifidobacteria* are mainly found in breast-fed infants owing to the HMOs growth-stimulating effect (Lawson et al. 2020). Furthermore, analysing infant feces can provide valuable insights because it is made of metabolic digestive waste from the large intestine. Thus, microorganisms in the feces of infants reflect the bacteria present in the large intestine, of which they normally included *Bifidobacterium* spp., *Bacteroides* spp., *Clostridia* spp., and *Faecalibacterium* spp. (Martinez et al. 2013).

Dosan et al. (2024) had previously isolated and characterised 10 *Bifidobacteria*-like bacteria from breast milk (BS2-MB1, BS2-PB3, BS2-PB5, BS2-PS1, and BS2-PS2) and infant fecal samples (BR1-M1, BR1-B1, BR2-5, BR2-6, and BR2-12) collected in Indonesia. Based on their sugar fermentation patterns, while five isolates (BR1-B1, BS2-PB3, BR2-12, BS2-PS1, and BS2-PS2) had a pattern of *B. adolescentis*, two (BR2-5 and BR2-6) and three isolates (BR1-M1, BS2-MB1, and BS2-PB5) exhibited similar fermentation patterns to *B. longum* subspecies *longum* and *B. breve*, respectively (Dosan et al. 2024). However, sugar fermentation patterns have very limited sensitivity and specificity for microbial species identification. Nonetheless, this limitation can be solved through proper molecular identification, that is, via 16S rRNA or whole-genome sequencing. Four isolates (BR2-5, BR2-6, BS2-PB3, and BS2-PS1) were subsequently subjected to 16S rRNA sequencing. Unsurprisingly, the results of molecular identification were different from the carbohydrate fermentation patterns, in which the 16S rRNA sequencing indicated that while BR2-5 and BR2-6 were very similar to *B. animalis* subsp. *lactis*, BS2-PB3 and BS2-PS1 were very similar to *B. breve* (Dosan et al. 2024). Furthermore, the genome of BS2-PB3 had been sequenced and confirmed to be *B. breve*. This isolate was also observed to possess various genes supporting its potential probiotic use (Marbun et al. 2024).

Probiotics must withstand the challenging environment of the gastrointestinal system to affect the health of hosts who consumed them (Skoufou et al. 2024). We had recently investigated the abilities of various bacteria isolated from breast milk samples (*Bifidobacteria*-like bacteria and *Lactiplantibacil-*

lus plantarum) to survive under various stresses encountered in the gastrointestinal tract (e.g., exposure to low pH and bile acids; Sunardi et al. 2023; Dosan et al. 2024). Therefore, we decided to sequence whole genome of four selected *Bifidobacteria*-like isolates (i.e., BS2-PS1, BS2-PS2, BS2-PB5, and BR2-12) and to assess their abilities to resist low pH and bile salt as ones of probiotic characteristics in this study.

MATERIALS AND METHODS

Culture isolation and maintenance

This study was conducted in the Fundamental Biology and the Advanced Biology Laboratories of the Department of Biology, Universitas Pelita Harapan, Indonesia. Isolates BS2-PS1, BS2-PS2, and BS2-PB5, as well as isolate BR2-12, were previously isolated from breast milk and infant fecal samples, respectively (Dosan et al. 2024). The medium used to cultivate the isolates was trypticase phytone yeast (TPY), with the following formulation for 1 L: 17.6 g of tryptic soy broth (TSB) (HiMedia Laboratories, India), 1 mL of Tween 80 (Sigma-Aldrich, USA), 5 g of peptone (Liofilchem, Italy), 5 g of glucose (Sigma-Aldrich, USA), 2.5 g of yeast extract (HiMedia Laboratories, India), 0.5 g of L-cysteine (NOWFoods, Canada), and 2 g of KH_2PO_4 (Sigma-Aldrich, USA). When the solid medium was desired, the formulation was supplemented with 15 g of bacteriological agar (HiMedia Laboratories, India). The culture media that were inoculated with each isolate was incubated in anaerobic condition using Oxoid™ AnaeroGen™ 2.5 L sachet (Thermo Scientific, USA) at 37 °C for 48 h.

Morphological identification

The morphological identification on the shape, margin, colour, and elevation of bacterial colonies was conducted as described by Dosan et al. (2024). The cellular identification was executed using the Gram staining method.

Molecular identification via whole-genome sequencing

Genomic DNA of BS2-PS1, BS2-PS2, BS2-PB5, and BR2-12 were extracted and sequenced using Oxford Nanopore Technology. Filtering was done using Filtlong v0.2.1 (<https://github.com/rrwick/Filtlong/releases/tag/v0.2.1>), and the results were transformed using Samtools v1.19.2 (<https://github.com/samtools/samtools>). Furthermore, quality of reads was evaluated using Nanoplot v1.42.0 (<https://github.com/wdecoster/NanoPlot/releases/tag/v1.42.0>). Reads correction and assembly were performed using Canu v2.2 (<https://github.com/marbl/canu/releases/tag/v2.2>) and Flye v2.9.2-b1795 (<https://github.com/fenderglass/Flye>), respectively. The assembled sequences were polished four times using Racon v1.5.0 (<https://github.com/lbcb-sci/racon/releases/tag/1.5.0>) and three times using Medaka v1.11.1 (<https://github.com/nanoporetech/medaka/releases/tag/v1.11.1>). The mapping was performed using Minimap2 2.26-r1175 (<https://github.com/lh3/minimap2/releases>) and the quality was determined using Quast v5.0.2 (<https://github.com/ablab/quast>) and Qualimap v2.3 (<https://github.com/EagleGenomics-cookbooks/QualiMap>). The annotation was performed using PGAP v6.6 (<https://github.com/ncbi/pgap/releases>).

The complete genome sequence of all four isolates had been submitted to GenBank. The assembled whole genomes were subsequently labelled with dFAST v1.6.0 annotation tool (<https://dfast.ddbj.nig.ac.jp/dfc/>) for genomic prediction and functional characterisation from the isolates (Tanizawa et al. 2016). Furthermore, the visualisation of whole genomes of the isolates was performed with Proksee (<https://proksee.ca/>) to retrieve circular map of the assembled genome (Grant et al. 2023). Identification of the isolates was subsequently conducted using TYGS webserver (<https://tygs.dsmz.de/>) and vis-

ualized with iTOL ([https:// itol.embl.de/](https://itol.embl.de/)) (Meier-Kolthoff & Göker 2019; Letunic & Bork 2021). To measure similarity between genomes, whole-genome average nucleotide identity (ANI) was calculated with FastANI v1.34 (<https://github.com/ParBLiSS/FastANI/releases/tag/v1.34>) (Jain et al. 2018).

Identification of genes related to bile salt and acid tolerance

The identification of genes associated with bile salt and acid resistance was conducted as described by Valdez-Baez et al. (2022). Potential genes for bile salt and acid resistance mechanisms in *Bifidobacterium*, *Lacticaseibacillus*, and *Lactiplantibacillus* spp. were identified through a review of publications (Wang et al. 2021; Schöping et al. 2022; Shimizu et al. 2023; Contente et al. 2024) and shown in Table 1. Using the Basic Local Alignment Search Tool (<https://www.ncbi.nlm.nih.gov/blast/>), the sequences of these potential genes were compared to the genes of the bacterial isolates BS2-PS1, BS2-PS2, BS2-PB5, and BR2-12. For these alignments, a minimum identity percentage of 70 % was used.

Table 1. Acid- and bile-salt-resistance-associated genes in *Bifidobacterium*, *Lacticaseibacillus*, and *Lactiplantibacillus* spp.

Species	Gene	Mechanism	Reference
<i>Bifidobacterium</i> spp.	<i>bshA</i>	Taurine-conjugated bile salt hydrolase	O’Flaherty et al. 2018; Engevik et al. 2021; Flórez et al. 2021; Singhal et al. 2022; Kim et al. 2022; Min et al. 2022; Rossi et al. 2022; Schöping et al. 2022; Vestergaard et al. 2022; Li et al. 2024
<i>Lacticaseibacillus</i> spp.	<i>bshB</i>	Taurine-conjugated bile salt hydrolase	
<i>Lactiplantibacillus</i> spp.	<i>bshC</i>	Glycosyl-conjugated bile salt hydrolase	
	<i>atpB</i>	ATP synthase F0 sector subunit a	
	<i>atpE</i>	ATP synthase F0 sector subunit c	
	<i>atpF</i>	ATP synthase F0 sector subunit b	
	<i>atpH</i>	ATP synthase F1 sector subunit δ	
	<i>atpA</i>	ATP synthase F1 sector subunit α	
	<i>atpG</i>	ATP synthase F1 sector subunit γ	
	<i>atpD</i>	ATP synthase F1 sector subunit β	
	<i>atpC</i>	ATP synthase F1 sector subunit ε	
<i>Bifidobacterium</i> spp.	<i>cbh</i>	Breakdown bile salt	Wu et al. 2021; Reyes-Castillo et al. 2023
	<i>argC</i>	Tolerate acid environments	
	<i>argH</i>	Tolerate acid environments	
	<i>dapA</i>	Tolerate acid environments	
	<i>cfa</i>	Cyclopropane-fatty-acyl-phospholipid synthase	
	<i>nagB</i>	Glucosamine-6-phosphate deaminase	
<i>Lacticaseibacillus</i> spp.	<i>dltB</i>	D-alanyl-lipoteichoic acid biosynthesis protein DltB	Kim et al. 2022; Vestergaard et al. 2022
	<i>cfa</i>	Cyclopropane-fatty-acyl-phospholipid synthase	
	<i>ppaC</i>	Putative manganese-dependent inorganic pyrophosphatase	
	<i>brpA</i>	Biofilm regulatory protein A	
	<i>ltaS1</i>	Lipoteichoic acid synthase 1	
<i>Lactiplantibacillus</i> spp.	<i>pyk</i>	Pyruvate kinases involved in acid resistance	Singhal et al. 2021; Min et al. 2022; Contente et al. 2024
	<i>pyrG</i>	CTP synthase	
	<i>cbh</i>	Choloylglycine hydrolase	
	<i>dps</i>	DNA protection during starvation protein	
	<i>glf</i>	UDP-galactopyranose mutase	
	<i>cfa</i>	Cyclopropane-fatty-acyl-phospholipid synthase	

spp., species (plural).

Bile salt tolerance

The resistance assay to bile salt was conducted as described by González-Vázquez et al. (2022) and Dosan et al. (2024). A 10 % (v v⁻¹) bacteria liquid culture was inoculated into three vials containing TPY broth supplemented with bile salts at concentrations of 0 %, 0.3 %, and 0.5 % (w v⁻¹). Each treatment was incubated for 3 h at 37 °C under obligate anaerobic condition. Subsequently, the cell count for each treatment was determined by inoculating 50 µL of liquid culture with various dilution factors using the spread plate method on TPY agar. The plates were then incubated for 48 h at 37 °C under obligate anaerobic condition. Following incubation, the colony-forming unit (CFU) was counted. The cell count (log CFU mL⁻¹) was calculated as follows:

$$\text{Number of cells} \left(\frac{\log \text{CFU}}{\text{mL}} \right) = \log_{10} \left(\frac{\text{CFU} \times \text{dilution factor}}{\text{volume}} \right)$$

Acid tolerance

The resistance test to low pH was conducted as described by Kanwal et al. (2021) and Dosan et al. (2024). A 10 % (v v⁻¹) liquid culture was inoculated into three vials containing TPY broth with their pH were adjusted using hydrochloric acid (HCl), resulting in pH 2, 5, and 7.5. The pH 7.5 was used as control. Each treatment was incubated for 3 h at 37 °C under obligate anaerobic condition. Subsequently, the cell count for each treatment was calculated by inoculating 50 µL of liquid culture with various dilution factors utilising the spread plate method on TPY agar. The plates were incubated for 48 h at 37 °C under obligate anaerobic condition. After incubation, the CFU was counted using the above-mentioned formula for the bile salt tolerance.

Statistical analysis

Data were analysed and presented with GraphPad Prism version 10.2.3. Statistical significance for results of bile salt and pH testing were calculated using the Kruskal–Wallis test. Upon p-values <0.05, Dunn's multiple comparison test was performed. A p-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Morphological identification

To be grouped into taxonomic classifications according to their morphological characteristics, bacteria are identified morphologically, which allow quick distinction and simplify the identification. Additionally, genetic and biochemical assessment are frequently added to morphological identification as a basis for confirmation and in-depth analysis (Khawaldeh et al. 2017). The morphological identification for each isolate is presented in Figure 1. All isolates exhibited similar colony morphology on TPY agar: round shape, white, and convex elevation. Furthermore, all isolates were Gram-positive bacteria, which are evidenced by the purple colour. They are classified as Gram-positive due to the presence of a thick peptidoglycan matrix, which cause the absorption of crystal violet and displaying a purple colour (Harris 2022). The morphological characteristics of all isolates also showed small rod-shaped cells, with branching (bifid) shapes ranging from "V" to "Y." These findings are in compliance with those by Khushboo et al. (2023) which stated that *Bifidobacterium* spp. exhibited distinctive bifid shapes.

Molecular identification

The four genomes were annotated using the dFAST annotation tool (Table 2). Among the four isolates, BR2-12 had the longest genome size, i.e., 3,332,336 base pairs (bp), while BS2-PS1 and BS2-PS2 had the highest GC contents (58.9 %). The tool also predicted that BR2-12 had the highest num-

ber of coding sequences (3,141) and RNA (16 rRNA + 70 tRNA). Of note, *Lb. plantarum* strain BR2-12 was identified to have a clustered regularly interspaced short palindromic repeats. The Proksee webserver (<https://proksee.ca/>) was utilised to depict the circular genome graph of each isolate (Figure 2). Phylogenomic analysis was conducted to reconstruct the evolutionary history of organisms. The analysis, performed using TYGS (Figure 3) on *B. breve* BS2-PS1 and BS2-PS2, indicated that both strains were closely related to *B. breve* NCTC11815 and JCM 1192. The same analysis also revealed *Lc. paracasei* subsp. *paracasei* BS2-PB5 was closely related to *Lc. paracasei* subsp. *paracasei* JCM 8130, 363.501388 and IBB3423. Of note, *Lb. plantarum* BR2-12 was observed to be closely related to several strains of *Lb. plantarum* (UNQLp11, DSM 20174, and SRCM100442) and *Lacticaseibacillus* spp. This finding was not surprising because both *Lactiplantibacillus* and *Lactica-seibacillus* spp. belong to the same *Lactobacillaceae* family (Zheng et al. 2020).

The ANI values of BS2-PS1 and BS2-PS2 indicated that they belong to *B. breve* with 99 % identity. Of note, BS2-PS1 and BS2-PS2 shared a similarity of 99 %. The ANI values of BS2-PB5 indicated that it belongs to *Lc. paracasei*

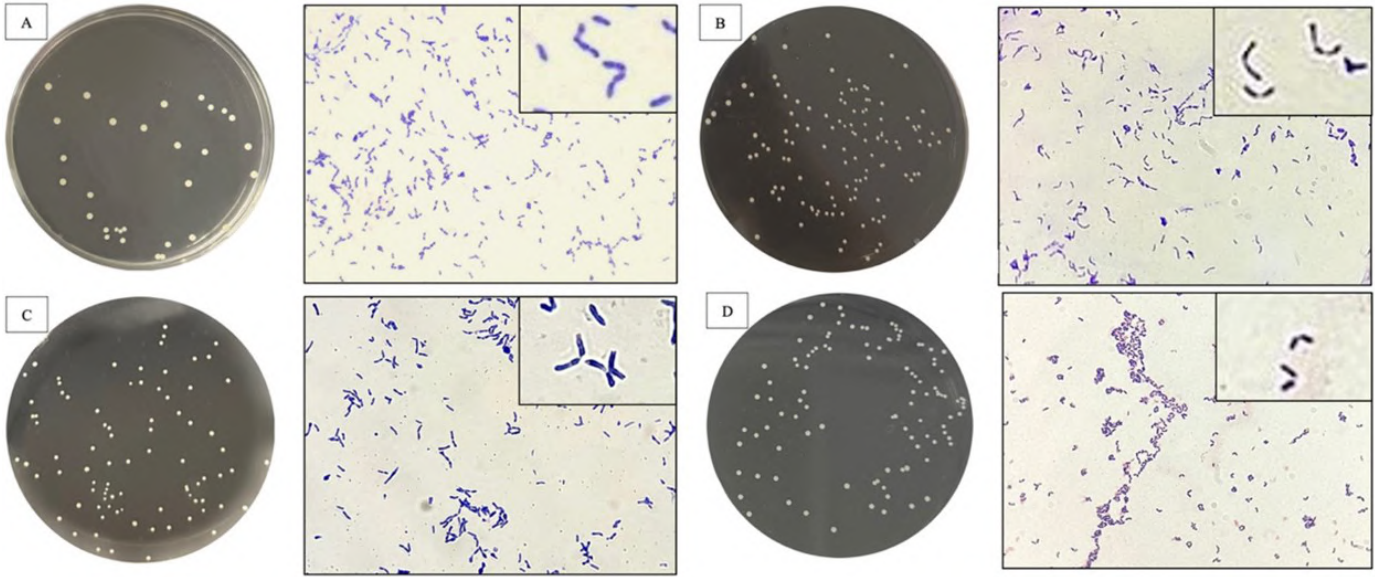
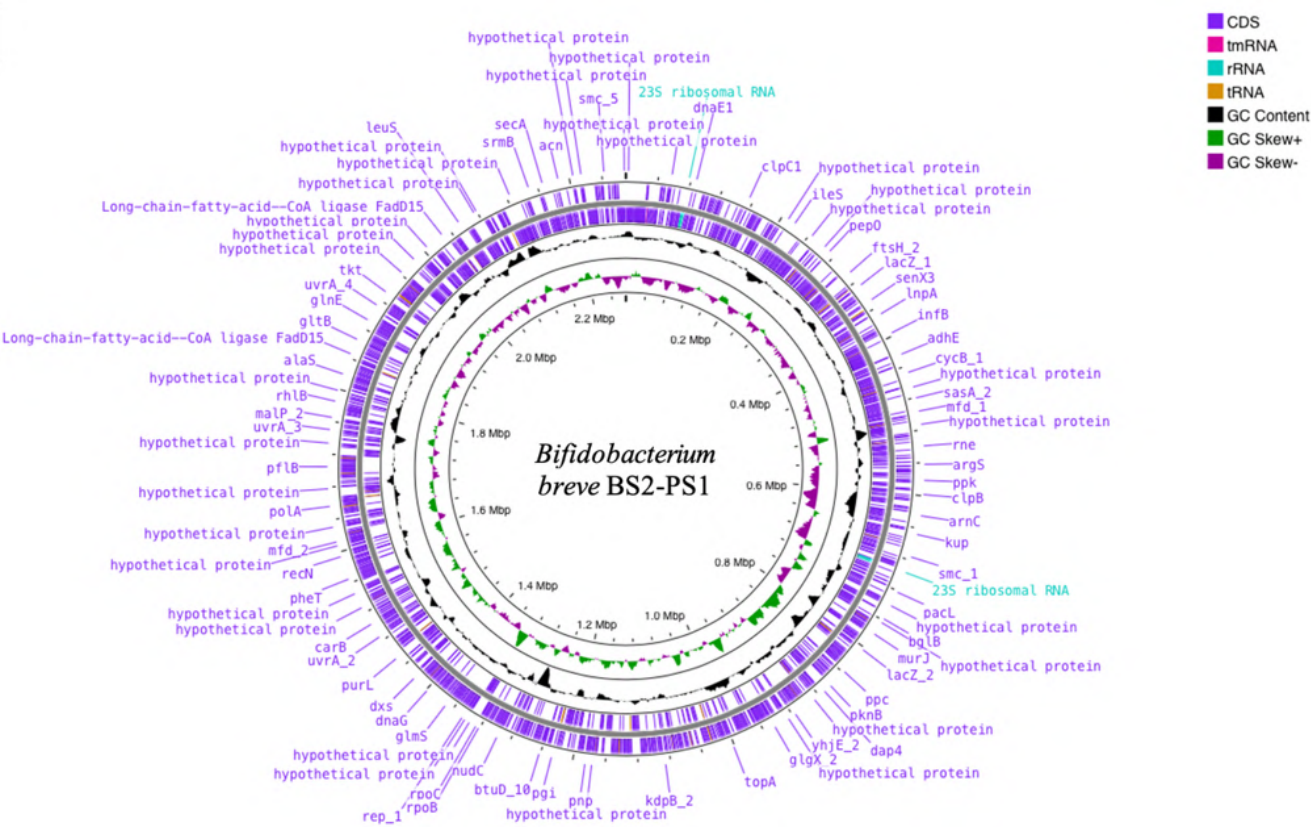


Figure 1. Visualisation of colony and cellular morphologies of the selected four isolates of *Bifidobacteria*-like bacteria. The left column shows the colony morphologies of (A) BS2-PS, (B) BS2-PS2, (C) BS2-PB5, and (D) BR2-12. The right column shows the cellular morphology of each corresponding isolate upon Gram staining using a light microscope with a magnifying power of 1000x.

Table 2. Genome annotation statistics of BS2-PS1, BS2-PS2, BS2-PB5, and BR2-12 isolates using the dFAST annotation tool.

Features	BS2-PS1	BS2-PS2	BS2-PB5	BR2-12
Species	<i>B. breve</i>	<i>B. breve</i>	<i>Lc. paracasei</i> subsp. <i>paracasei</i>	<i>Lb. plantarum</i>
Genome size (bp)	2,269,504	2,269,407	2,943,595	3,332,336
Contig	1	1	2	4
GC content (%)	58.9	58.9	46.5	44.5
N50 (bp)	2,269,504	2,269,407	2,905,540	3,218,310
L50	1	1	1	1
CDS	1,986	1,997	2,835	3,141
Total RNA	58 (4 rRNA + 54 tRNA)	58 (4 rRNA + 54 tRNA)	74 (15 rRNA + 59 tRNA)	86 (16 rRNA + 70 tRNA)
CRISPR	0	0	0	1

A



B

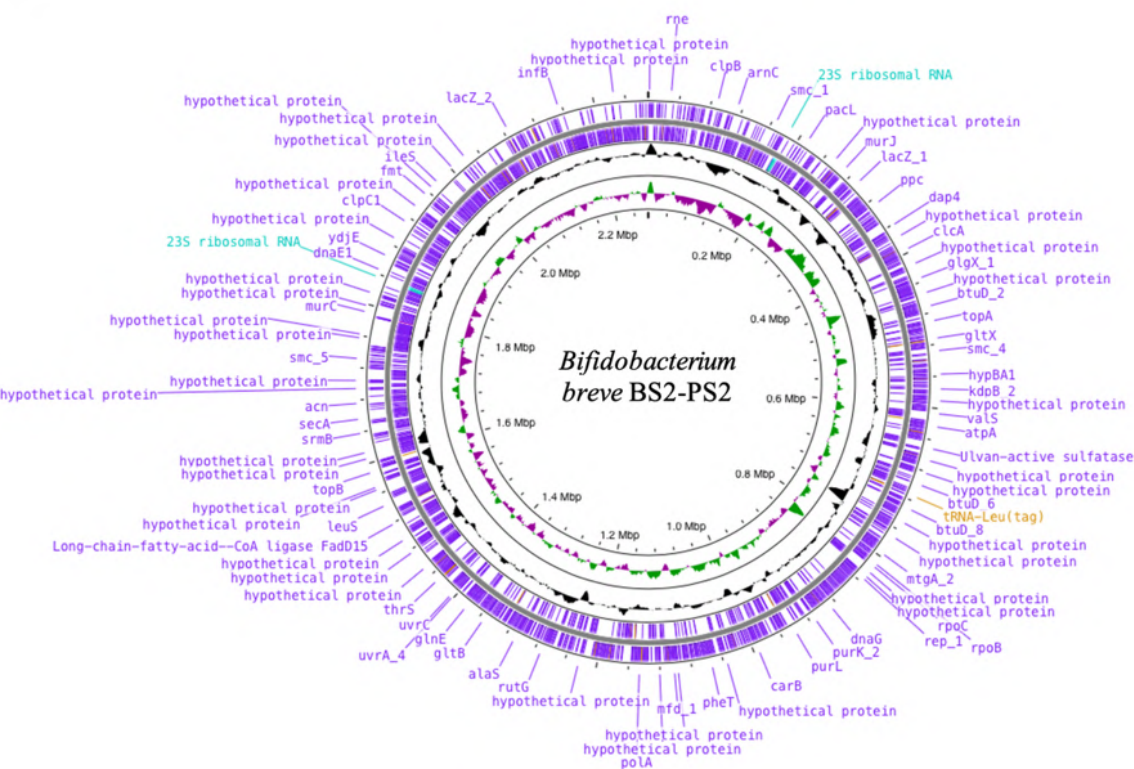
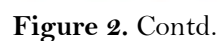


Figure 2. Visualisation of *B. breve* strain BS2-PS1 and BS2-PS2, *Lc. paracasei* subsp. *paracasei* strain BS2-PB5 and *Lb. plantarum* strain BR2-12 whole genomes. The visualization was performed using Proksee (<https://proksee.ca/>). Various genes, with hypothetical genes labeled by locus location, are depicted on the outermost violet circle. The first two outermost circles illustrated forward and reverse coding sequences (CDS), with hypothetical genes labelled as the locus location. The CDS was supplemented with tRNAs (brown), rRNAs (blue), and tmRNAs (pink). The black circle represents the GC content, whereas the green and dark purple circles represent the GC skew. The fifth innermost circle shows the genome size of (A) *B. breve* strain BS2-PS1, (B) *B. breve* strain BS2-PS2, (C) *Lc. paracasei* subsp. *paracasei* strain BS2-PB5, and (D) *Lb. plantarum* strain BR2-12.



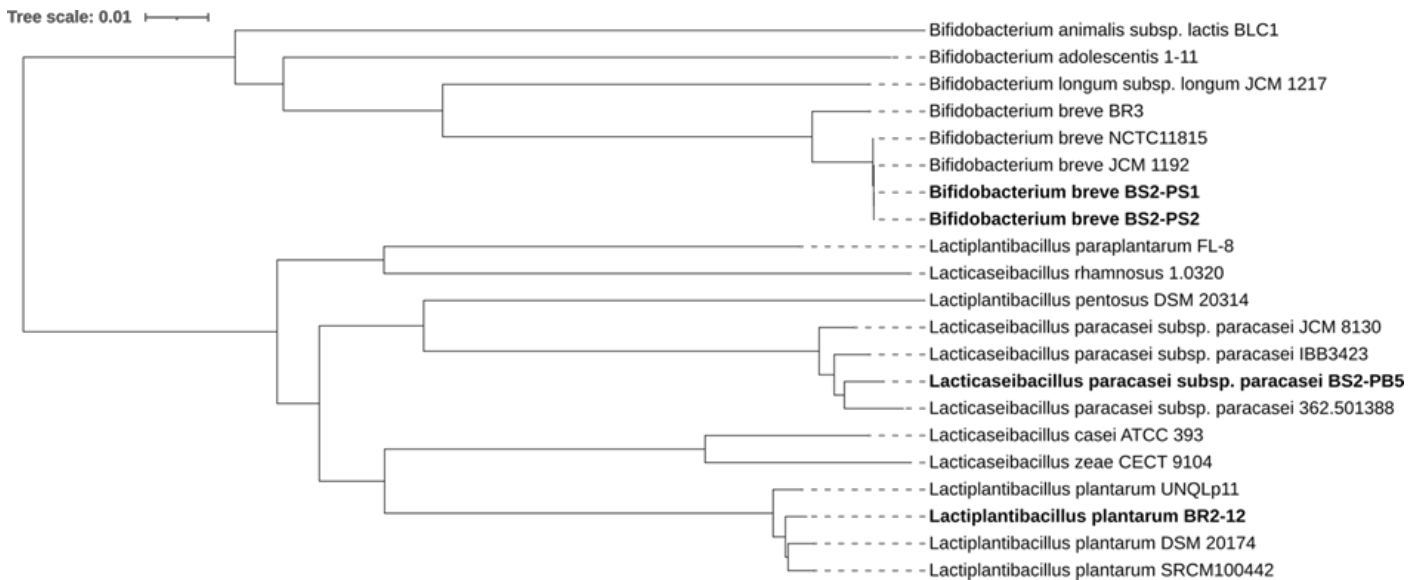


Figure 3. Phylogenetic tree of *B. breve* strain BS2-PS1 and BS2-PS2, *Lc. paracasei* subsp. *paracasei* strain BS2-PB5, and *Lb. plantarum* strain BR2-12. The comparisons of *B. breve* BS2-PS1, *B. breve* BS2-PS2, *Lc. paracasei* subsp. *paracasei* BS2-PB5 and *Lb. plantarum* BR2-12 with other strains of *Bifidobacterium* spp., *Lactocaseibacillus* spp., and *Lactiplantibacillus* spp. were carried out in the TYGS webserver (<https://tygs.dsmz.de/>) and were visualized by iTOL (<https://itol.embl.de/>). The tree was generated using TYGS, in which the MASH algorithm was used to compare all chosen genomes in the TYGS database. All pairwise comparisons were conducted using GBDP, and accurate intergenomic distances were deduced using the algorithm trimming and distance formula d5. The intergenomic distances were used to construct a balanced minimum evolution tree with branch support using FASTME 2.1.6.1, which included SPR postprocessing. Each branch support was inferred from 100 pseudo-bootstrap replicates. The tree was then visualised using iTOL.

with 98 % identity, whereas the ANI values of BR2-12 indicated that it belongs to *Lb. plantarum* with 99 % identity. This result is aligned with molecular identification using whole-genome sequencing, indicating that while isolates BS2-PS1 and BS2-PS2 were *B. breve*, BS2-PB5 and BR2-12 were *Lc. paracasei* subsp. *paracasei* and *Lb. plantarum*, respectively.

N50 was related to the median and mean lengths of a set of sequences, representing the length of the shortest read in the group of longest sequences, which account for at least 50 % of the nucleotides in the set of sequences. L50 was related to N50, indicating the number of sequences that when they arranged from longest to shortest are needed to reach or exceed 50 % of the total assembly size. bp, base pairs; GC, guanine–cytosine; CDS, coding sequence; RNA, ribonucleic acid; CRISPR, clustered regularly interspaced short palindromic repeats.

Tolerance to bile salt exposure

Bile salts contain bile acids and salts synthesised in the liver and stored in the gallbladder. Bile salts are essential for the digestion and absorption of dietary fats in the small intestine. Probiotics should be able to withstand bile salts as they disrupt the structure of cell membranes and damage DNA because they act as a natural detergent (Urdaneta & Casadesús 2017; Hagi et al. 2020). The average concentration of bile salts is approximately 0.3 % in the human intestinal tract, whereas concentrations as high as 0.5 % can be found in the duodenum (Grimm et al. 2014). Therefore, resistance to bile salts is important for the survival of probiotics in the digestive system. The growth response of all isolates to various concentrations of bile salts is shown in Figure 4.

Upon comparison between exposure to 0.3 % and no bile salts, the isolates BS2-PS1, and BS2-PB5 showed a reduction of 41 % and 8 %, respectively. Upon comparison between exposure to 0.5 % and no bile salts, the isolates BS2-PS1 and BS2-PB5 showed a reduction of 70 % and 47 %, respectively.

Indeed, the CFU reduction of BS2-PS1 upon exposure to 0.5 % of bile salt was statistically significant when compared to a condition without bile salt ($p = 0.0219$). Intriguingly, no substantial reduction on BS2-PS2 and BR2-12 was observed upon bile salt exposure. These were plausibly due to the poor growth of both isolates; hence their survivability rates could not be assessed. Taken together, these findings demonstrated that the isolates BS2-PS1 and BS2-PB5 were able to survive exposure to 0.3 % and 0.5 % of bile salts for 3 h, in which BS2-PB5 demonstrated a stronger resilience.

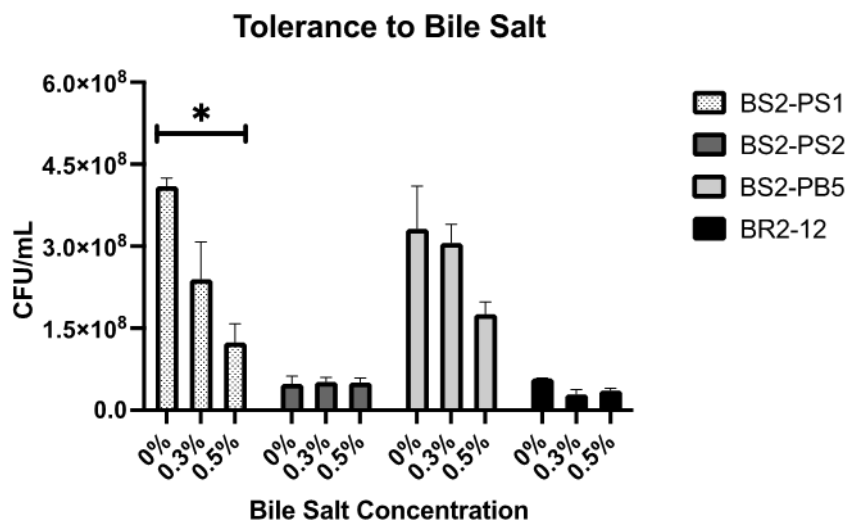


Figure 4. The bile salt tolerance of *B. breve* BS2-PS1 and BS2-PS2, *Lc. paracasei* subsp. *paracasei* BS2-PB5 and *Lb. plantarum* BR2-12 strains. Each isolate was cultured at 37 °C in TPY broth supplemented with either 0.3 % or 0.5 % (w v⁻¹) of bile salt. The control was the condition without any bile salt. Upon incubation for 3 h under obligate anaerobic condition, each culture was sampled and cultured on TPY agar for enumeration. The median of CFU mL⁻¹ is displayed as a bar graph. The upper error bar indicates the maximum value. Results of each bacterial strain were analysed individually using the Kruskal–Wallis test with p-values <0.05 considered statistically significant. Results of *B. breve* strain BS2-PS1 were subsequently analysed using Dunn’s multiple comparison test to determine which comparison between two groups was significantly different. An asterisk sign indicates p-values <0.05. The values were obtained from three experiments.

The ability to withstand bile salt stems from the presence of relevant gene(s), such as *bsh*, *cbh*, *dps*, *glf*, *cfa*, or *nagB* (Wall et al. 2014; Albarracin et al. 2022). Those genes confer bile salt resistance by breaking it down and increasing the tolerance to bile salt. One of the well-known enzymes that reduce bile salt toxicity is Bile Salt Hydrolase (BSH), encoded by the *bsh* gene. BSH enzyme catalyzes the hydrolysis of conjugated bile salts into deconjugated bile acids by breaking the bond between bile acid and amino acid. Therefore, BSH can decrease the overall concentration of bile salts, reducing their toxicity to bacterial cells (Bourgin et al. 2021). The breakdown of bile salts can reduce its toxicity through various mechanisms. It reduces membrane damage by limiting the penetration of bile salts into bacterial cells. It also minimises the DNA damage by lowering the concentration of bile salts, while the hydrolysis products, such as amino acids, provide nutrients that promote bacterial growth and survival (Jarocki et al. 2014; Song et al. 2019). As shown in Table 3, while BS2-PS1 and BS2-PS2 possess the same genes (i.e., *bsh*, *cbh*, *dps*, *glf* and *cfa*), BR2-12 has four of the five genes (i.e., *bsh*, *cbh*, *dps*, and *cfa*). It was of interest that among these three isolates, BS2-PS1 exhibited the highest resistant phenotype *in vitro*, suggesting that the expression of bile acid-resistant genes in BS2-PS1 was better than in BS2-PS2 and BR2-12. However, as BS2-PS2 and BR2-12 did not grow well in this assay, further studies are

Table 3. Bile resistance genes identified in the isolates.

Isolate	Gene	Mechanism	Identity (%)	Gene ID
BS2-PS1	<i>bsh</i>	Bile salt hydrolase	99.6	89503319
	<i>cbh</i>	Breakdown bile salt	84	13069843
	<i>dps</i>	DNA protection during starvation protein	99	69566565
	<i>glf</i>	UDP-galactopyranose mutase	98.6	8950422
	<i>cfa</i>	Cyclopropane-fatty-acyl-phospholipid synthase	72.3	6653298
BS2-PS2	<i>bsh</i>	Bile salt hydrolase	99.6	89503319
	<i>cbh</i>	Breakdown bile salt	84	13069843
	<i>dps</i>	DNA protection during starvation protein	99	69566565
	<i>glf</i>	UDP-galactopyranose mutase	98.6	8950422
	<i>cfa</i>	Cyclopropane-fatty-acyl-phospholipid synthase	72.3	6653298
BS2-PB5	<i>nagB</i>	Glucosamine-6-phosphate deaminase	86.4	45549971
BR2-12	<i>bsh</i>	Bile salt hydrolase	100	77216564
	<i>cbh</i>	Breakdown bile salt	99.5	14532344
	<i>dps</i>	DNA protection during starvation protein	99.5	77216492
	<i>cfa</i>	Cyclopropane-fatty-acyl-phospholipid synthase	99.6	77218098

required to investigate this speculation. Interestingly, BS2-PB5 only had one relevant gene (*nagB*), but it displayed a similar level of resistance phenotype to BS2-PS1. Therefore, the actual mechanism of *nagB* to confer resistance to bile salt in future studies should also be investigated.

Tolerance to acidity

Another criterion to consider is the resistance of probiotics to acidity in the gastrointestinal tract. The pH ranges from pH 2 (in stomach) and pH 4–5 (in small intestine) to pH 7–7.4 (in large intestine) (Henze et al. 2021). Hu and Li (2018) suggested that low pH reduces the cell growth by potentially activating the G1 cell cycle arrest. Mendonça et al. (2022) also indicated that low pH can disturb the proton motive force and other cellular processes, which leads to cell damage and death. Therefore, tolerance to acidity is a fundamental capability that probiotics possess to provide benefits to their host. The growth response of all isolates to various pH is illustrated in Figure 5.

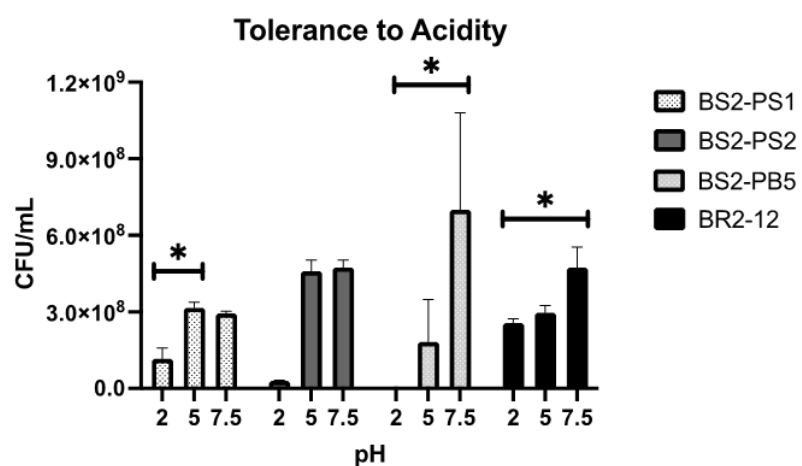


Figure 5. The acid tolerance of *B. breve* BS2-PS1 and BS2-PS2, *Lc. paracasei* subsp. *paracasei* BS2-PB5, and *Lb. plantarum* BR2-12 strains. Each isolate was cultured at 37 °C in TPY broth with their pH adjusted to 2 and 5. The control was a condition with pH 7.5. Upon incubation for 3 h under obligate anaerobic condition, each culture was sampled and cultured on TPY agar for enumeration. The median of CFU mL⁻¹ is displayed as a bar graph. The upper error bar indicates the maximum value. Results of each bacterial strain were analysed individually using the Kruskal–Wallis test, with a p-value <0.05 considered statistically significant. Results of *B. breve* BS2-PS1,

Lc. paracasei subsp. *paracasei* BS2-PB5, and *Lb. plantarum* BR2-12 strains were subsequently analysed using Dunn's multiple comparison test to determine which comparison between two groups differed significantly. An asterisk sign indicated a p-value <0.05. The values were obtained from three experiments.

Compared to results for pH 7.5, *B. breve* BS2-PS1 and BS2-PS2 strains were interestingly not affected by pH 5. This finding is in contrast with the results of *Lc. paracasei* subsp. *paracasei* BS2-PB5 and *Lb. plantarum* BR2-12 strains in pH 5, in which both isolates underwent substantial reduction in their cellular counts (74 % and 38 % reduction, respectively). However, upon exposure to pH 2 for 3 h, all isolates contracted substantial reduction in their cellular counts. Specifically, the isolate BS2-PB5 and BR2-12 contracted significant reduction when compared to their cellular counts in pH 7.5 ($p = 0.0190$ and $p = 0.0219$, respectively). These findings indicated that all tested isolates were substantially reduced by a very low pH during 3 h of exposure. In compliance with the results of Mendonça et al. (2022), bacterial growth significantly decreased at a pH <4, and no growth or metabolic activity was observed at a pH <3. Nevertheless, it had been reported that the duration of normal gastric emptying was significantly faster than 3 hours because the meal retention within the stomach could vary between 30–90 % at the first hour of intake, and the meal retention was subsequently ≤ 10 % at the 4th hour of intake (Abell et al. 2008), suggesting that the transit time of ingested probiotics should not last longer than 3 h among normal individuals. Hence, the effects of stomach acid on cellular viability should arguably be less substantial than our findings due to a shorter duration.

Table 4 shows that BS2-PS1, BS2-PS2, and BR2-12 isolates were able to withstand low pH (2 and 5) due to the presence of several genes (e.g., *argC*, *argH*, *dapA*, *pyk*, or *pyrG*) (Yin et al. 2012; Tang & Matsuda 2024). In addition, the presence of eight subunit genes that encode the F₀F₁-ATPase enzyme, such as *atpC*, *atpB*, *atpE*, *atpF*, *atpH*, *atpA*, *atpG*, and *atpD* (Wu et al. 2022; Kingkaew et al. 2023), would confer resistance to acid as well. Although the presence of all eight subunit genes is not necessary for encoding the F₀F₁-ATPase enzyme, several subunits (e.g., α , β , and c subunits) are crucial for building this enzyme (Xu et al. 2015). The BS2-PS1, BS2-PS2, and BR2-12 isolates were found to possess all eight subunit genes of this enzyme. The F₀F₁-ATPase enzyme mediates an important role in maintaining the pH balance within the cell when exposed to acidic conditions. F₀F₁-ATPase functions by transferring proton (H⁺) from the cytoplasm to the extracellular environment with the aid of energy generated by ATP synthesis reactions. By pumping protons out of the cell, F₀F₁-ATPase helps maintain intracellular pH balance under acidic conditions (Aoi & Marunaka 2014; Tran et al. 2021). Interestingly, although BS2-PB5 exhibited moderate levels of resistance to pH 5 (but not to pH 2), its whole-genome sequencing revealed the absence of any gene mediating acid resistance. Nonetheless, it had been reported that *Lc. paracasei* could survive in pH as low as 5 (Wu et al. 2021). Thus, there might be an unidentified mechanism of acid resistance in *Lc. paracasei*.

CONCLUSIONS

The whole-genome analyses of tested four isolates demonstrated that they were from three different species, that is, *B. breve* (BS2-PS1 and BS2-PS2), *Lc. paracasei* subsp. *paracasei* (BS2-PB5), and *Lb. plantarum* (BR2-12). Different degrees of sensitivity to bile salt (for isolates BS2-PS1 and BS2-PB5) and low pH exposures (for all isolates) were observed, of which the differences could be contributed by the presences of certain genes, such as *bsh*, *nagB*, *atpD*, and *atpC*. Thus, our findings demonstrate the benefits of combining *in vitro* and whole-genome data in assessing potentials of bacterial isolates to be further developed as probiotic candidates.

Table 4. Acid resistance genes identified in the isolates.

Isolate	Gene	Mechanism	Identity (%)	Gene ID
BS2-PS1	<i>atpC</i>	ATP synthase F1 sector subunit ϵ	98.6	14789701
	<i>atpB</i>	ATP synthase F0 sector subunit a	100	89502650
	<i>atpE</i>	ATP synthase F0 sector subunit c	96.4	89502651
	<i>atpF</i>	ATP synthase F0 sector subunit b	100	12168982
	<i>atpH</i>	ATP synthase F1 sector subunit δ	100	12168983
	<i>atpA</i>	ATP synthase F1 sector subunit α	99.7	89502654
	<i>atpG</i>	ATP synthase F1 sector subunit γ	100	12168985
	<i>atpD</i>	ATP synthase F1 sector subunit β	98.1	89502656
	<i>argC</i>	Tolerate acid environments	99.7	89503025
	<i>argH</i>	Tolerate acid environments	100	89503035
	<i>dapA</i>	Tolerate acid environments	99.8	89502831
BS2-PS2	<i>atpC</i>	ATP synthase F1 sector subunit ϵ	98.6	14789701
	<i>atpB</i>	ATP synthase F0 sector subunit a	100	89502650
	<i>atpE</i>	ATP synthase F0 sector subunit c	96.4	89502651
	<i>atpF</i>	ATP synthase F0 sector subunit b	100	12168982
	<i>atpH</i>	ATP synthase F1 sector subunit δ	100	12168983
	<i>atpA</i>	ATP synthase F1 sector subunit α	99.8	89502654
	<i>atpG</i>	ATP synthase F1 sector subunit γ	100	12168985
	<i>atpD</i>	ATP synthase F1 sector subunit β	98.2	89502656
	<i>argC</i>	Tolerate acid environments	99.7	89503025
	<i>argH</i>	Tolerate acid environments	100	89503035
	<i>dapA</i>	Tolerate acid environments	99.8	89502831
BR2-12	<i>atpC</i>	ATP synthase F1 sector subunit ϵ	100	77215740
	<i>atpB</i>	ATP synthase F0 sector subunit a	100	77215747
	<i>atpE</i>	ATP synthase F0 sector subunit c	100	77215746
	<i>atpF</i>	ATP synthase F0 sector subunit b	99.8	77215745
	<i>atpH</i>	ATP synthase F1 sector subunit δ	100	77215744
	<i>atpA</i>	ATP synthase F1 sector subunit α	100	77215743
	<i>atpG</i>	ATP synthase F1 sector subunit γ	100	14531356
	<i>atpD</i>	ATP synthase F1 sector subunit β	99.9	77215741
	<i>pyk</i>	Pyruvate kinases involved in acid resistance	99.9	77218256
	<i>pyrG</i>	CTP synthase	99.6	77217110

AUTHOR CONTRIBUTION

T.F.H., M.S., T.T.J., and J.J. designed the study. T.F.H., A.B.R., I.S.K., and R.T.L. conducted the experiments and collected the data. M.S., T.T.J., and J.J. supervised the study. T.F.H., J.S.P., and J.J. analysed the data and drafted the manuscript. All authors approved the final version.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Assessing Indigenous Ureolytic Bacteria Isolated from Gua Damai Limestone for Microbially Induced Calcite Precipitation (MICP)

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ABSTRACT

Low porosity and non-aggregated soil are significant global concerns, presenting substantial environmental hazards. This study determined the capacity of native ureolytic bacteria found in limestone to stabilise soil through the process of microbially induced calcite precipitation (MICP). Six pure bacterial isolates obtained from limestone in Gua Damai, Batu Caves, Selangor were qualitatively assessed for urease production. The isolate S4C4, identified as *Bacillus tropicus* strain NTF4, demonstrated the highest urease activity at 821.654 U mL⁻¹. This isolate precipitated 37.15 ± 9 mg mL⁻¹ of CaCO₃ after 96 hours of incubation and XRD analysis confirmed the biocementation of organic soils treated by *B. tropicus* strain NTF4, primarily forming calcite and vaterites. Significant calcite polymorph presence in soil samples is attributed to a longer treatment duration which promotes crystal development and stability. Harnessing indigenous limestone ureolytic bacteria with high urease activity presents a promising avenue for green soil bio-stabilisation. This approach potentially unlocks sustainable and scalable applications of microbial-induced calcite precipitation (MICP) in large-scale geo-engineering projects.

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INTRODUCTION

Surface erosion, fluvial erosion, erosion along riverbanks, and mass movement erosion, which is essentially the force of gravity moving a loose combination of soil and rock particles downslope, are examples of the geological processes that cause excessive soil removal and soil deterioration (Yusof et al. 2022). Soil erosion occurs under typical conditions that have existed for millions of years, leading to the creation of new soils (Ahmad et al. 2020). Heavy rains, erosive conditions, poor land management techniques, and construction methods are frequently the causes of landslides (Paramanathan et al. 2021), with excessive grazing, deforestation, and unsuitable farming methods the main causes of increased soil erosion (Retallack 2021). The application of geotechnical techniques, such as chemical grouting like polyurethane, lignosulfonates, and acrylamides, to control or reduce the risk of soil structure collapse is not only expensive but also poses a hazard to human health and the environment (Van Paassen et al. 2010) and thus, many countries have banned their use. Consequently, attention has focused on the search for more passive and environmentally friendly geotechnical techniques for soil structure based on common soil microbes found in soil (DeJong et al. 2015).

The biomineralisation process, also known as microbially induced calcium carbonate precipitation (MICP), is an environmentally friendly technique that has emerged in the fields of geotechnical and civil engineering. It serves as a viable and sustainable alternative for ground improvement, surpassing existing remediation technologies in enhancing the soil's mechanical properties (Naveed et al. 2020). MICP occurs naturally as a byproduct of microbial metabolic activity via a variety of mechanisms including urea hydrolysis, photosynthesis, sulphate and nitrate reduction, and other biochemical processes facilitated by microbes that raise the carbonate saturation state (Leeprasert et al. 2022). The most extensively used method is calcium carbonate (CaCO_3) precipitation by urease-producing bacteria through urea hydrolysis (Castro-Alonso et al. 2019; Naveed et al. 2020). CaCO_3 formation between the soil particles can help cement the gaps leading to improved geochemical characteristics, particularly the friction angle, cohesion, bending strength, and stiffness (Jiang et al. 2019).

Numerous microorganisms demonstrate urease activity such as *Sporosarcina pasteurii*, a non-pathogenic, endospore-producing bacterium, which thrives in harsh conditions with a basic pH of 9.0 (Chen et al. 2022). However, it is crucial to isolate and study other potential urease-producing bacteria, such as the genera *Bacillus*, *Geobacillus*, *Lysinibacillus*, *Pararhodobactor*, and *Psychrobacillus*, to produce bioresources suitable for a variety of climates and conditions (Yoshida et al. 2010; Ghosh et al. 2019). Suitable bacteria are typically present in challenging environments, especially those with high alkalinity, nutritional scarcity, and intense shearing forces. MICP is only possible if the bacteria survive (Leeprasert et al. 2022) and depends on pH, the amount of dissolved inorganic carbon and calcium, as well as the presence of nucleation sites (Erdmann & Strieth 2022) essential for consistent and continuous calcium carbonate production. Significant calcium precipitation must support the expression of high ureolytic activity by these bacteria for MICP applications (Erdmann & Strieth 2022).

The current study found that cave-dwelling bacteria are prospective sources of urease with the ability to induce calcite crystals (Komala & Khun 2013; Soon et al. 2014). Only native ureolytic bacteria found in limestone caves can produce energy by fixing carbon dioxide into organic compounds (Tomczyk-Żak & Zielenkiewicz 2015), contributing to their growth and survival. Limestone comprises about 50 % carbonate minerals (Fairbridge et al. 1967), predominantly consisting of calcite and aragonite. Thus, a significant diversity of ureolytic bacteria capable of performing MICP is expected in the

limestone, as well as the potential to isolate novel ureolytic bacteria. However, there are few published studies on the exploitation of microbial diversity in these regions, therefore this study evaluated the MICP activity of indigenous ureolytic bacteria isolated from limestone.

MATERIALS AND METHODS

Isolation of ureolytic bacteria from limestone

Limestone samples were collected from Gua Damai, Batu Caves, Selangor (3° 14'52"N 101°41'14"E), at a depth of 5–10 cm using a mechanical drill, chisels, and mallet. All samples were crushed using a mortar and pestle before being mixed with sterile distilled water. The samples were preserved before completing the appropriate bacterial isolation (Omoregie et al. 2018) using calcium carbonate precipitation (CCP) agar. Cultures were incubated at 28 °C ± 0.5 °C for 7 days before being transferred to CCP Minimal Medium Agar and incubated at 28 °C ± 0.5 °C for 24 hours (Aliyu et al. 2023a). The colonies were screened for urease and preserved as a stock culture. The long-term storage of pure ureolytic bacterial colonies was performed according to the modified methods adapted from Bibi et al. (2018).

Urease activity

The pure isolates were streaked and incubated on Christensen's urea agar (pH 6.8) at 28 ± 0.5 °C for 120 hours and observed every 6 hours (Burbank et al. 2012). The change in colour from orange to pink determined a pH increase indicating urease production. To target potential high-urease producers, only isolates that changed colour within 48 hours were selected for further analysis (Wei et al. 2015). The selected isolates were incubated in urea broth (pH 6.8) and shaken at 180 rpm for 120 hours at 28 ± 0.5 °C (Aliyu et al. 2023a). Urease activity was determined by measuring the amount of ammonia released from urea using the phenol hypochlorite assay method described by Kim and Youn (2016).

Calcium carbonate production

Calcium carbonate production was determined by the acid test whereby 10 % HCl was dropped onto the precipitated CaCO₃ and the bubble of carbon dioxide gas released signals the presence of carbonate minerals. modified method adapted from Wei et al. (2015) and described by Aliyu et al. (2023a) was used to assess the calcium carbonate production by the selected isolates.

Isolate identification

Molecular identification of the selected isolates was based on 16S ribosomal RNA sequencing.

Molecular identification of the selected isolates was based on 16S ribosomal RNA sequencing. The entire 16S ribosomal RNA gene was amplified from bacterial DNA using universal primers universal primers 27F (5' - AGAG-TTTGATCCTGGCTCAG- 3') and 1492R (5' - GGTTACCTTGTTAC-GACTT- 3') (Wu et al. 2014). The PCR cycling conditions were set with an initial denaturation at 94 °C for 2 minutes, followed by 25 cycles of denaturation at 98 °C for 10 seconds, annealing at 53 °C for 30 seconds, and extension at 68 °C for 1 minute. The forward and reverse sequencing outcomes are merged and refined to create a comprehensive full-length sequence. The nucleotide Basic Local Alignment Search Tool (BLAST) sequence from the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/nuccore/NR_115714.1; https://www.ncbi.nlm.nih.gov/nuccore/NR_157734.1) was utilised to compare the genes for identification. Neighbour-joining trees were created using the Kimura 2-parameter model using MEGA version 7.0 with bootstraps of 1000

replicates after repeated alignment of genes with their closed taxa using the CLUSTAL W method (Kumar et al. 2016).

MICP in soil

The bioremediation ability of the indigenous bacteria from limestone was assessed using the modified method of Bibi et al. (2018). Approximately 50 mL of urea medium containing 0.4 g of sterilised farm soil, 20 g L⁻¹ of urea, and 3.7 g L⁻¹ of CaCl₂·2H₂O were used to inoculate ureolytic bacteria isolates with an initial bacteria concentration of 0.1 at an optical density of 600 nm. The control treatment contained only 0.4 g of sterilised farm soil (black organic soil with a silky texture; pH 4.1 to 4.5) and 3.7 g L⁻¹ of CaCl₂·2H₂O in 50 mL of urea media. Both mixtures were inoculated at 28 ± 0.5 °C and consistently shaken at 120 rpm for 30 days. The soil samples were then collected, washed three times with distilled water, and the pellets were oven-dried at 40 ± 0.50 °C for 48 hours. All samples were crushed into evenly distributed particles for X-ray diffraction (XRD) analysis.

X-ray diffraction analysis

The dried treated soil samples were subjected to XRD analysis to confirm calcium carbonate deposition (Dhami et al. 2016) using a Shimadzu 6000 diffractometer to elucidate the crystalline phase composition and differentiate between amorphous and crystalline forms of CaCO₃ (calcite, vaterite, or aragonite). The Cu K α radiation and a Panalytical Empyrean reflectometer were used at an ambient temperature of 28 °C. The exploration range (2 θ) was modified at a speed of 2.00 (deg min⁻¹) from 20° to 80° with the Cu anode set at 0.0530°, 30 kV, and 30 mA as the step sizes, voltages, and currents, respectively.

RESULTS AND DISCUSSION

Six ureolytic bacterial isolates were isolated from limestone samples (pH 8.69 to 8.92) (Table 1). Most MICP studies reported that ureolytic bacteria were found in slightly basic environments (Navneet et al. 2011) because they need them to break down urea and make ammonium for the MICP process (Prah et al. 2011). Urea (20 g L⁻¹) was added to the CCP media to prepare a selective medium that only supports the growth of ureolytic bacteria that can tolerate higher urea concentrations (Wei et al. 2015). This modification also produced a distinct and unique odour resembling ammonia gas from the culture plates, therefore, the bacterial activity is responsible for the urea decomposition.

Three isolates changed the agar colour to pink, with isolate S1C5 changing the agar colour in 18 hours, followed by isolates S4C4 and S5C3 (Table 1), indicating the presence of ureolytic bacteria. When urea is hydrolysed by urease, it releases ammonia (NH₃) and carbonic acid (H₂CO₃) which generate hydroxide ions, leading to a pH increase (Mekonnen et al. 2021). Soon et al. (2014) found that ureolytic bacteria thrive within a pH range of 7.5 to 9.5, which aligns with the optimal conditions for MICP. However, ensuring optimal pH conditions is challenging because the solubility of calcium ions at higher pH levels decreases significantly, limiting the bacteria's ability to acquire and utilise these ions (Intarasoontorn et al. 2021). The ureolytic bacteria use glucose and peptone in Christensen's urea agar to grow quickly (Dortey et al. 2020). However, only isolates S1C5 and S4C4 were subjected to further evaluation due to their ability to produce a colour change within less than 48 hours, indicating their high adaptability and potential to establish independent populations (Aliyu et al. 2023a).

Long-term observation revealed that S1C5 and S4C4 achieved the greatest urease activity after 96 hours (Figure 1) and the urease activity of S4C4 exceeds 500 U mL⁻¹, which is comparable to the activity observed in

isolates obtained from cement (Achal et al. 2010) and limestone (Kshetri & Ningthoujam 2016). The exponential rise in urease activity after 24 hours correlated to bacterial population expansion and increased urease enzyme synthesis (Omoriegie et al. 2019). Despite a decrease in urease activity in both isolates after 120 hours, possibly due to factors such as insufficient nutrition, metabolic inhibition, cell death, and enzyme breakdown (Jiang et al. 2016), isolate S4C4 maintained urease activity of at least 2.8 % after 96 hours, thus, was selected for further analysis.

Table 1. Duration for urea agar’s colour change for urease activity indication.

Isolates	pH of limestone sample	Hours
S5C1	8.69	Colour unchanged
S5C2	8.69	Colour unchanged
S5C3	8.69	72
S4C4	8.92	24
S1C5	8.78	18
S1C6	8.78	Colour unchanged

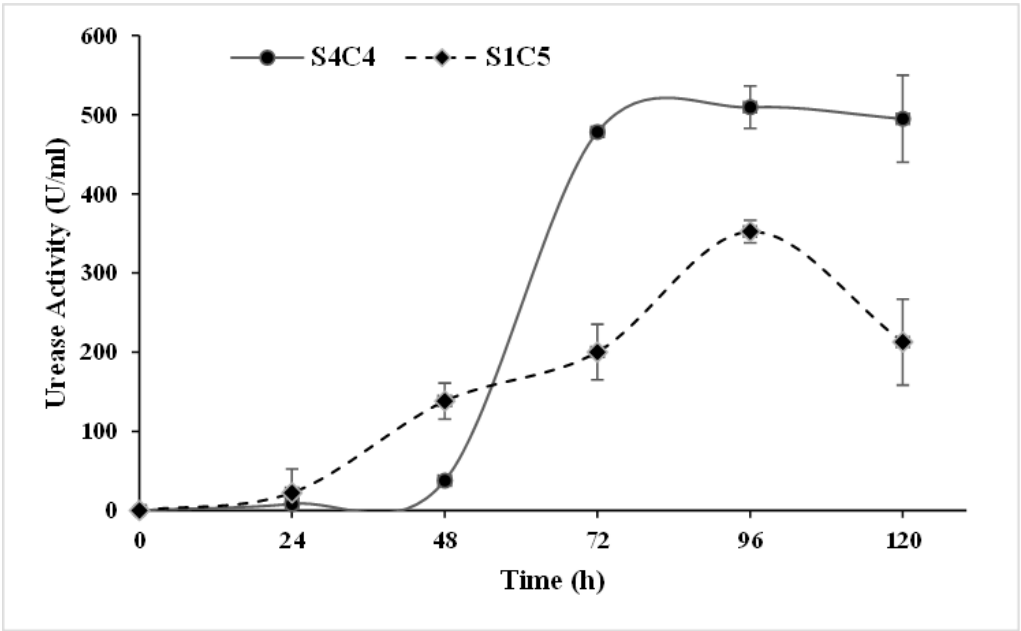


Figure 1. Urease activity of S1C5 and S4C4 in Christensen’s urea broth media based on the absorbance of phenol hypochlorite assay at OD626nm.

Phylogenetic research utilising neighbour joining (unrooted trees) indicated that S4C4 was affiliated with the genus *Bacillus*, exhibiting 99.9 % similarity to *B. tropicus* strain MCCC 1A01406. Thus, isolate S4C4 was known onwards as *Bacillus tropicus* strain NTF4 (Figure 2), a Gram-positive bacterium that produces urease and can generate endospores under conditions of low nutrition or temperature (Thakur et al. 2021; Shen et al. 2022). Previous studies have identified *B. tropicus* as having urease activity in limestone, which is commonly observed in cave materials (Elmanama & Alhour 2013; Omoriegie et al. 2019). *Bacillus* sp. possesses cell walls with many layers that are structurally adapted to endure abiotic stress and their urease production can be induced, constitutive, or repressed (Ibarra-Villarreal et al. 2021). Several *Bacillus* species such as *B. licheniformis*, *B. sphaericus*, *B. megatarium*, *B. thuringiensis*, *B. cereus*, *B. paramycoides*, and *B. pseudomycoides* produce urease and cause calcite precipitation (Helmi et al. 2016; Moravej et al. 2018; Mukherjee et al. 2019; Algaifi et al. 2020; Aliyu et al. 2023b).

Bacillus tropicus strain NTF4 produced up to 37.15 mg mL⁻¹ of precipi-

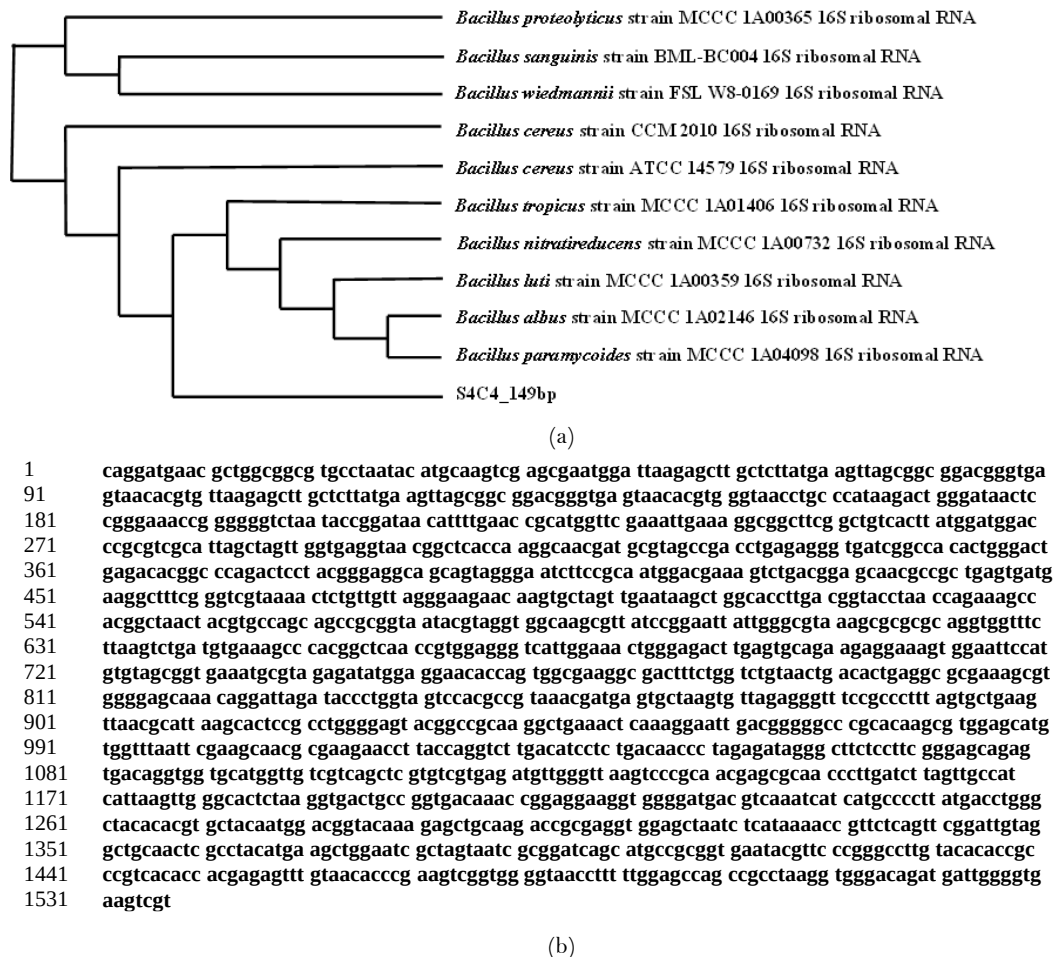


Figure 2. (a) Phylogenetic Tree – Neighbour Joining (Unrooted Tree) by NCBI Blast Tree Method, as compared to known species for isolate S4C4; (b) S4C4 16S ribosomal RNA, partial sequence.

tates containing CaCO_3 (Richardson et al. 2014), confirming its capability for MICP. There have been extensive reports that bacterial isolates possess the potential to produce calcite precipitates (Al Qabany et al. 2011) and significantly influence the CaCO_3 crystal size, morphology, form, and biochemistry making the selection of bacteria critical for successful solidification (Seifan & Berenjian 2019). Despite *B. tropicus* strain NTF4 CaCO_3 precipitation being significantly lower than that of *B. lentus* and *B. diminuta* (Wei et al. 2015), extrinsic factors like media, physicochemicals, and reactant ions can mitigate this effect (Aliyu et al. 2023b). There are differences between species in the amount of CaCO_3 precipitation suggesting that there may be differences in how active urease is utilised. Urease catalyses urea hydrolysis liberating carbonate (CO_3^{2-}) ions, so higher urease activity may more CO_3^{2-} to accumulate in the culture medium, which in turn encourages more CaCO_3 to precipitate. A steady amount of urease activity in the microenvironment also helps make more dissolved CO_2 in the form of HCO_3^- or CO_3^{2-} and ammonium ions, which raises the pH (Zaghloul et al. 2021).

The untreated soil sample's XRD 2 Theta diffraction angles reveal vaterite peaks at 21.29° , 27.13° , 40.74° , and 42.67° , along with distinct calcite peaks at 46.19° , 60.35° , and 68.28° (Figure 3), suggesting the formation of CaCO_3 crystals in line with Aliyu et al. (2023b) who reported that vaterite and calcite had likely been formed in the soil sample before the test. This is because farm soil is usually urea-rich and has a variety of in situ ureolytic bacteria. Also, sudden changes in temperature, salinity, or pressure can promote vaterite formation (Zhu & Dittrich 2016; Oral & Ercan 2018). According to Reeksting et al. (2020), decreased urease levels can affect the shape of the

crystals and the changes that happen during biomineralisation, and the lack of urea and urease activity could cause vaterite crystals to form with unique properties.

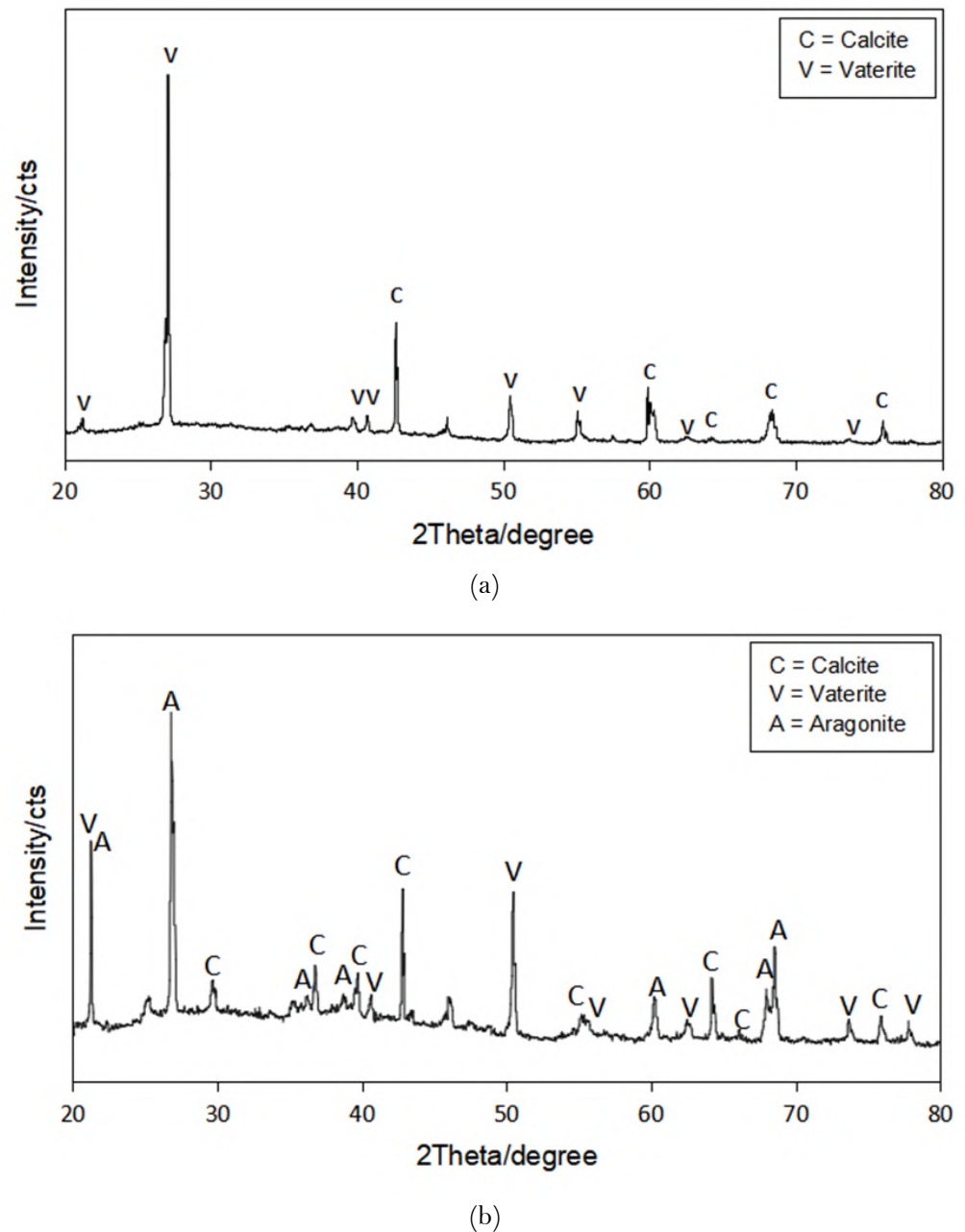


Figure 3. XRD spectra of (a) uninoculated soil sample and (b) *B. tropicus* inoculated soil sample after 30 days of incubation period.

All three CaCO_3 crystalline polymorphs were precipitated with calcite becoming the main precipitate by *B. tropicus* strain NTF4. The XRD spectra confirmed that *B. tropicus* strain NTF4 precipitated more calcites than the control, with distinct peaks at 29.72° , 42.81° , 55.14° , 66.13° , 68.01° , and 75.93° and several vaterite and aragonite peaks were also detected (Figure 3b). *Bacillus tropicus* strain NTF4 also precipitated aragonite, and over time, aragonite will gradually convert into calcite through recrystallisation (Kontoyannis & Vagenas 2000). Aragonite being less stable with a faster dissolution rate is more likely to transform into calcite than vaterite, lowering its concentration in the soil (Chen et al. 2022).

Reeksting et al. (2020) found a correlation between urease activity and the rapid precipitation of vaterite crystals which later transformed into calcite crystals. *Bacillus tropicus* with significant urease activity enhanced the for-

mation of kinetically preferred vaterite crystals (Kakelar et al. 2016). CaCO_3 precipitation began with the formation of metastable spherical vaterite crystals before transformational disintegration and dissolution formed calcite crystals (Al-Thawadi & Cord-Ruwisch 2012). Vaterite is rarely found in the environment despite its crucial role as a building block for calcite production (Mwandira et al. 2017). However, the degree of supersaturation in the environment significantly influences the rate of transformation from vaterite to calcite. According to Spanos and Koutsoukos (1998), the rate at which vaterite dissolves and calcite crystallises is similar when the supersaturation ratio is between 1.2 and 1.5. However, the pH greatly influences any changes in the supersaturation conditions (Oral & Ercan 2018).

Studies by Bang et al. (2010) and Achal et al. (2013) reported that calcite was the main form of biomineralisation, whereas Akyol et al. (2017) found that vaterite was the most common form of CaCO_3 . The dominant calcium carbonate polymorphs in the ureolysis-driven MICP in this study were in line with most previous studies which also identified calcite and vaterite as the most frequently precipitated forms (Al-Thawadi & Cord-Ruwisch 2012; Algaifi et al. 2020). Calcite is generally considered better than vaterite in MICP because it is more stable than at low solution supersaturations (Rajasekar et al. 2021) and the presence of urease enzymes from *B. tropicus* strain NTF4 favours calcite formation over vaterite. Calcite also reigns supreme in MICP applications due to its superior thermodynamic stability (Chang et al. 2017) and ability to offer better cementation with improved mechanical properties (Xu et al. 2024). The interfacial energy during the transformation of amorphous calcium carbonate (ACC) to vaterite is lower than ACC to calcite, which promotes more vaterite formation compared to calcite. However, this alone is not sufficient to offset the advantages of calcite in terms of stability and crystallinity (Yi et al. 2021). Future comprehensive investigations of these factors should be conducted to optimise MICP. Nevertheless, the present study demonstrated dense calcium carbonate formation by *B. tropicus* strain NTF4 on and within soil grains, highlighting their potential as ureolytic bacterial agents for soil stabilisation via MICP.

CONCLUSION

Bacillus tropicus strain NTF4, an indigenous bacterium that produces urease, causes biocementation by causing calcium carbonate to develop and solidify in the soil. Most precipitation occurred within 48 hours and the precipitated CaCO_3 consisted predominantly of calcite and vaterite. This indigenous strain exhibited notable resistance to elevated urea levels and demonstrated alkaline tolerance showcasing its intriguing capabilities as an agent for biocementation.

AUTHORS CONTRIBUTION

All authors collaborated to carry out this work. AWAS and NTF performed the study, analysed the data and drafted the manuscript. RG and NAA carried out data validation and draft review. MM designed, oversaw the study, also reviewed the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

A Streamlined Plant DNA Extraction Method with Liquid Nitrogen-Free Approach

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ABSTRACT

Molecular technique such as Polymerase Chain Reaction (PCR) is essential in various research fields. The amplification process of plant DNA can be challenging due to the presents of metabolites that can inhibit the polymerase enzyme, as well as expensive procedures or time-consuming laboratory work required. Most of the protocol involved liquid nitrogen, which is not always accessible, especially in laboratories with limited resources. Consequently, this study proposed an alternative protocol free from liquid-nitrogen usage, that was designed to be efficient in the DNA extraction from dry and fresh leaf samples across 40 plant species belonging to 27 different families. The DNA obtained from all the samples showed concentrations greater than 50 ng μL^{-1} , with the quality indexes in the acceptable range ($A_{260}/_{280}$: 1.50-2.21, $A_{260}/_{230}$: 0.60-2.20). The efficacy of this method was demonstrated by successful PCR amplification using *rbcL* primer, validating the DNA suitability. This protocol can be considered a good option to be used both with fresh and dried plant leaves. Moreover, the absence of liquid nitrogen usage in the protocol could decrease the laboratory cost considerably and turning it into a more easily replicable method to be used in laboratories with limited resources.

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INTRODUCTION

Molecular techniques play crucial role in various research fields, including genetic diversity, genetic mapping, evolutionary, breeding, and population studies (Demeke & Jenkins 2010; Tamari & Hinkley 2016). The initial step involves obtaining genetic material such as DNA or RNA in optimal quantity and quality for amplification process by Polymerase Chain Reaction (PCR) is very essential. However, obtaining high-quality genetic material from plants can be challenging due to the presence of natural metabolites that can inhibit the PCR techniques (Abdel-Latif & Osman 2017). Consequently, the effectiveness of the DNA extraction process has often been a limiting factor (Abdel-Latif & Osman 2017). Normally, DNA extraction follows three main steps i.e.: (1) membrane disruption; (2) separation and purification of DNA from other components such as proteins and lipids; and (3) concentration and purification of DNA (Shetty 2020). Yet, the specific reagents and techniques employed in each step may vary depending on the type of sample being analysed. In the case of samples derived from plants, there are different factors that must be considered in DNA extraction protocol standardisation such as the presence of secondary metabolites, including high amounts of polyphenols and polysaccharides (Csaikl et al. 1998; Demeke & Jenkins 2010; Shepherd & McLay 2011; Chabi Sika et al. 2015; Inglis et al. 2018; Aboul-Maaty & Oraby 2019). They can interfere the DNA activity and the subsequent PCR analyses (Shepherd & McLay 2011). Because of these potential inhibitors, several protocols are developed using CTAB buffer along high salt concentrations, preventing the solubility of polysaccharides (Rezadoost et al. 2016). Meanwhile, polyvinylpyrrolidone (PVP) is used to be added to remove polyphenols by breaking the bond between DNA, RNA and phenolics, preventing loss of DNA and increasing DNA yield (Labra et al. 2001; Rezadoost et al. 2016; Abdel-Latif & Osman 2017). As well, the addition of β -mercaptoethanol has been used helping to reduce tannins and other polyphenols (Hwang & Kim 2000; Labra et al. 2001; Marín et al. 2021). In addition, many protocols indicate the use of phenol to separate cellular molecules and debris from the DNA, however, it requires special containment tools to avoid risks for the user and the environment (Sahu et al. 2012). Therefore, DNA extraction protocols are very often adjusted to particular plant species or plant tissues to obtain high-quality genetic material for downstream analyses (Csaikl et al. 1998; Shepherd & McLay 2011; Chabi Sika et al. 2015; Inglis et al. 2018; Aboul-Maaty & Oraby 2019; Marín et al. 2021). In this work, because of the more common availability, only β -mercaptoethanol was chosen as a good enough tool to avoid contaminants in the DNA isolation procedure.

There are specific commercial kits for plant DNA extraction however, they usually represent high costs, and even the traditional procedures include reagents that are not very common in laboratories with limited resources, adding the fact that they occasionally show limitations in terms of the amount of DNA obtained. Moreover, for plant tissue samples, most of the previous methods involve the use of liquid nitrogen at the first step of the DNA extraction process, not only to break cell but also as a good strategy to deactivate harmful enzymes preventing DNA damage (Porebski et al. 1997; Sharma et al. 2002; Kotchoni et al. 2011; Healey et al. 2014; Mayjonade et al. 2016; Afshar-Mohammadian et al. 2018; Lear et al. 2018; Serna-Domínguez et al. 2018; Quiñones et al. 2024). This reagent is well known because of its efficient processing of specimens, such as animals, fungi, and microorganisms samples (Rogers & Bendich 1988; Höss & Pääbo 1993; Lee et al. 2003; Jan Kieleczawa 2006; Varma et al. 2007; Asghar et al. 2015; Peñafiel et al. 2019). However, it is hazardous and expensive (Scharf et al. 2020) and its storage requires specific conditions that unavailable in most laboratories. Despite give the advantages, the use of liquid nitrogen may not be universally applicable or

replicable, especially in laboratories with limited resources.

Nowadays, there are numerous DNA protocols developed in the interest of obtaining suitable DNA by reducing costs and time. Determining “the best protocol” is strenuous and unfair, as each method has its own advantages and limitations depending on the research conditions (Mace et al. 2003; Hollingsworth et al. 2009; Demeke & Jenkins 2010; Zhang et al. 2010; Yanisko et al. 2011; Ahari et al. 2012; Fazekas et al. 2012; Hasan et al. 2012; Sahu et al. 2012; Jinlu et al. 2013; Parveen et al. 2016; Tamari & Hinkley 2016; Abdel-Latif & Osman 2017; Esfandani-Bozchaloyi et al. 2019; Paul et al. 2019). Even though there is a wide variety of standardised protocols available, most of them are limited to specific experimental units tailored for their projects. These protocols constraints a challenge when it used for different species, especially for the next-generation sequencing analysis that required high quality and quantity DNA from many different taxa simultaneously (Särkinen et al. 2012; Shetty 2020). In addition, the PCR analysis should be performed for assessing the DNA suitability. The amplification process using barcoding primers is one of the methods that could be used to validate the purity of DNA, especially since the basis for this study is species molecular identification, particularly when it is focused on unstudied areas, by supplementing the morphological techniques (Kool et al. 2012; Bezeng et al. 2017; Pathak et al. 2018; Amin et al. 2020; Mavrodiev et al. 2021). In plant study, approximately 2000 species have been described by molecular (barcoding) and morphological techniques every year (Prance 2001; Ng’ang’a 2019) and many of them were not clearly described in terms of distribution, ecology, threats, and benefits (Bebber et al. 2010; Ruas et al. 2022). For barcoding purposes, the *rbcL* plastid region has already reported as an important region to be used, which after the evaluation of several markers was adopted by the Plant Working Group (PWG) of the Consortium for the Barcoding of Life (CBOL) as one of the standard plant DNA barcodes, not excluding the use of supplementary markers that could be required depending on the aim of the research (Quandt et al. 2004; Anderson et al. 2006; Kress & Erickson 2007; Pirie et al. 2007; Taberlet et al. 2007; Haider 2011; Hollingsworth et al. 2011; Vere et al. 2015; Harnelly et al. 2018; Volenzo & Odiyo 2020; Ho et al. 2021).

Consequently, it is key to look for a feasible and inexpensive plant DNA extraction method that can be used as widely as possible. Considering common protocols, their advantages, and disadvantages, this research achieved its aim of showing a successful standardised protocol without liquid nitrogen, RNase and PVP, for plant DNA extraction, suitable for PCR amplification in molecular identification activities, reducing the use of expensive or not commonly reagents available in limited resources laboratories.

MATERIALS AND METHODS

Genetic materials

The plant samples of 40 different species were collected in Napo Province, Eastern Ecuador. For lab analyses, as many as 2 to 4 healthy-young leaves per species were used in this study, being sure that at least 1 cm² per two leaves were available. Half of the total collected leaves for each species were stored in labelled Ziploc bags and frozen at -20 °C using ice packs. the remaining leaves were stored in paper envelopes with silica gel for at least seven days, to be used as dry material. The samples were transported to the Molecular Biology and Biochemistry Laboratory of Universidad Regional Amazónica Ikiam for further analysis. Samples were collected under MAATE-ARSFC-2022-2415 and MAATE-CMARG-2023-0833 permits.

DNA Extraction

To isolate the total DNA content from leaves (fresh and dry leaves) the protocol consisted of several steps, avoiding the use of liquid nitrogen, polyvinylpyrrolidone (PVP) and RNase, i.e.

- (1) Grind ~1 cm² of leaves sample with 800-1000 µL CTAB 2 % (w v-1) (Solvent: 0.1M Tris HCl, 1.4M NaCl, and 0.02M EDTA pH 8) using a sterile mortar and pestle. Transfer the mix to a labelled 1.5 mL micro-tube.
- (2) Add 8-10 µL of β-mercaptoethanol and barely mix by vortex for 5 seconds. Then, a spin to get down any drop from tube walls.
- (3) Incubate the samples for 60 minutes at 60 °C. Stir gently every 15 minutes.
- (4) Lay down the sample at room temperature for 2 minutes.
- (5) Add 500 µL of chloroform: isoamyl alcohol (24:1) solution. Mix gently for 10 seconds by a vortex.
- (6) Centrifuge during 15-20 min at 15 000 rpm.
- (7) Transfer the supernatant (~600µl) to a newly labelled 1.5 mL microtube and add chilled isopropyl alcohol (~600 µl) considering a comparison of 1:1 (supernatant: isopropyl alcohol). Mix the samples by inversion.
- (8) Incubate the samples for 30 minutes at -20 °C.
- (9) Centrifuge for 10 minutes at 10 000 rpm and carefully discard the supernatant (liquid phase) by inversion.
- (10) Add 600 µL of 70 % ethanol and stir by inversion until seeing the pellet is floating.
- (11) Centrifuge for 10 minutes at 5000 rpm and carefully completely discard the ethanol.
- (12) Repeat steps 10 and 11 until you get clear ethanol in the tube.
- (13) Let samples dry overnight at room temperature to eliminate every alcohol residue. NOTE: Dry process can be improved by letting the inverted tube on a sterile paper towel overnight.
- (14) Finally, elute the DNA using 50-100 µL of ultrapure water and stir gently.

The quantity and quality of the DNA extracted were evaluated using spectrophotometer Nanodrop ND-ONEC-W (Thermo Scientific™) using 2 µL of each sample. The total DNA concentration was measured at 260 nm, and its quality was registered considering 50 µL of dilution factor and the comparison between A₂₆₀/230 and A₂₆₀/280. Also, an agarose gel electrophoresis of the total DNA was performed to estimate the size of molecules and the integrity of the genetic material obtained.

DNA suitability assay

In order to verify the suitability of the DNA extracted in activities such as molecular identification, the amplification of the *rbcLa* region was performed by PCR technique. A reaction of 10 µL as final volume was prepared using 1 µL of 10X Buffer, 0.8 µL of 25 mM MgCl₂, 0.2 µL of Taq, 2 µL of 10 mM dNTPs, 0.8 µL of each 10 µM primers (*rbcLaF*: 5'-ATGTCACCACAAACAGAGACTAAAGC 3'; *rbcLaR*: 5'-GTAAAATCAAGTCCACCRGC 3'), and 1 µL of 50ng µL⁻¹ DNA template. PCR was performed with an initial temperature of 94 °C for 2 minutes, followed by 35 cycles, consisting of an initial denaturation of 93 °C for 1 minute, an annealing temperature of 55 °C for 45 seconds, and an extension of 72 °C for 1 minute. At the end of cycles, a final extension temperature of 72 °C for 10 minutes was applied. The PCR products were then separated using 2 % agarose electrophoresis.

Data analysis

Analyses were conducted using R software (Version 4.3.3). First, density plots and Levene's tests were employed to assess the distribution and homogeneity of the datasets (DNA concentration and quality indexes), respectively. Mann-Whitney U test (for non-normally distributed datasets) was performed to compare the DNA concentrations and quality indexes between the two types of sample material (fresh and dry leaves).

Subsequently, multivariable Generalized Linear Models (GLMs) were used to examine the effects of sample-material (fresh and dry leaves) and plant family on the DNA concentration and quality indexes. Data transformation techniques such as logarithmic, square root, and inverse, were employed to achieve the best fit before the analysis. GLM with Gamma distribution using a logarithm link function was performed and, Q-Q plots of model residuals were used to test the conformity between the empirical vs. the given theoretical distribution.

RESULTS AND DISCUSSION

The implementation of an efficient DNA extraction process is imperative to achieve optimal results in terms of quantity and quality. The DNA extraction process is essential to achieve good amplification that lets the researchers study its genetic content. The DNA concentration needed for a successful PCR assay depends on the minimum requirements of the polymerase used (Bartlett & Stirling 2003). It is commonly in a range of 5-50 ng μL^{-1} for genomic DNA, and 0.1-1 ng μL^{-1} for plasmids, considering a final volume of PCR reaction of 50 μL (Steitz 1998; Bartlett & Stirling 2003; Van Pelt-Verkuil et al. 2008). The protocol performed in this study showed good DNA concentrations obtained from fresh and dry leaves of 40 plant-samples of 27 different families, collected in Napo Ecuador. The DNA concentration was greater than 50 ng μL^{-1} for all samples (Table 1).

Moreover, the data analysis showed no statistically significant differences in DNA concentration ($p=0.93$) between the two sample types (fresh and dry) (Table 2). In addition, GLMs analysis determined a non-significant impact of the type of material ($Pr(>Chisq)=0.99$), but a significant impact of the plant family factor ($Pr(>Chisq)=0.004$) in the DNA concentration (Table 3).

Furthermore, the quality indices (A260/A280 and A260/A230) of fresh and dry leaves showed statistically significant differences between them (Table 2), which was supported by GLMs analysis that demonstrated the significant influence of type material on A260/A280 and A260/A230 values (Table 3).

On the other hand, the plant family demonstrated a significant impact on the A260/A230 index ($Pr(>Chisq)=0.03$), however, it did not exhibit a significant effect on the A260/A280 quality index ($Pr(>Chisq)=0.66$) (Table 3). Additionally, the Q-Q plots employed depicted that the residuals of our models are aligned well with the expected theoretical distribution, as seen in Figure S1.

It is important to mention that the fact that DNA quantity was above 50 ng μL^{-1} for all samples (Table 1), instead of a problem just means that a previous DNA dilution step is necessary to reach a good PCR, and more DNA concentration is available if other assays are planned. Also, the total yield of DNA was higher than the reported in another study that consider DNA extraction processes based on CTAB (Mavrodiev et al. 2021).

Regarding the DNA quality, the ratio of A260/280 is commonly used considering an ideal range value of 1.8-2.0 (Hollingsworth et al. 2009; Desjardins & Conklin 2010; Pereira et al. 2011; Abdel-Latif & Osman 2017). This parameter also showed no statistically significant differences between

Table 1. Quantity and quality of DNA measured by spectrophotometry (Nanodrop ONE) in dry and fresh leaves of 40 plant-samples of 27 different families, collected in Napo Ecuador.

No.	Plant family	Scientific name	Samples			Fresh leaves				Dry leaves							
			Order	Spanish common name	English common name	Concentration (ng μ L ⁻¹) _*	$\pm$ SD	A ₂₆₀ / ₂₈₀ [*]	$\pm$ S D	Concentration (ng μ L ⁻¹) [*]	$\pm$ SD	A ₂₆₀ / ₂₈₀ [*]	$\pm$ S D / ₂₃₀ [*]				
1	Acanthaceae	<i>Fittonia albivenis</i> (Veitch) Brummitt.	Lamiales	Tortuga roja	Red Ner-ve Plant	323,04	215,36	2,04	0,12	1,35	0,13	752,89	286,32	1,91	0,04	1,51	1,03
2	Amaranthaceae	<i>Iresine herbstii</i> Hook. ^a	Caryophyllales	Molleja	Bloodleaf	489,89	157,99	2,05	0,07	1,55	0,48	1250,64	1377,84	1,69	0,49	2,03	0,08
3	Anacardiaceae	<i>Mangifera indica</i> L. _{ab}	Sapindales	Mango	Mango	576,60	412,29	2,11	0,08	1,96	0,30	285,04	92,53	2,10	0,01	1,83	0,04
4	Aquifoliaceae	<i>Ilex guayusa</i> Loes. ^a	Aquifoliales	Guayusa	Guayusa	520,78	217,91	2,07	0,01	1,97	0,11	573,84	231,63	1,79	0,49	1,62	1,00
5	Araceae	<i>Monstera deliciosa</i> Liebm. _{ab}	Alismatales	Costilla de Adán	Swiss-cheese Plant	1095,60	1110,54	2,00	0,13	1,89	0,77	268,42	138,10	1,82	0,03	1,15	0,07
6	Asparagaceae	<i>Cordylone fructicosa</i> (G.Forst.) Endl. ^a	Asparagales	Banderilla	Ti plant	400,10	34,56	1,86	0,25	1,85	0,52	802,13	683,56	1,81	0,34	0,60	0,07
7	Bignoniaceae	<i>Crescentia cujete</i> L. _{ab}	Lamiales	Pilche	Calabash tree	927,42	896,88	1,76	0,48	1,63	0,58	462,13	215,04	1,88	0,30	1,32	0,48
8	Bixaceae	<i>Bixa orellana</i> L. _{ab}	Malvales	Achiote	Lipstick-tree	171,92	142,84	2,02	0,04	2,00	0,27	522,72	321,60	1,82	0,22	1,70	0,26
9	Caricaceae	<i>Carica papaya</i> L. ^a	Brassicales	Papaya	Papaya	711,62	13,89	2,16	0,05	2,10	0,23	1127,96	697,42	2,10	0,06	1,92	0,09
10	Cyatheaceae	<i>Cyathea arborea</i> (L.) Sm. ^a	Cyatheales	Helecho gigante	West just treefern	1222,23	1795,67	2,15	0,08	1,90	0,32	216,02	90,03	1,75	0,23	1,48	0,85
11	Cyclanthaceae	<i>Carludovica palmata</i> Ruiz & Pav. ^a	Pandanales	Paja toquilla	Panama hat plant	542,48	183,10	2,05	0,04	1,96	0,34	336,17	138,75	1,87	0,07	1,05	0,19
12	Euphorbiaceae	<i>Codiaeum variegatum</i> (L.) A.Juss. ^a	Malphighiales	Croton	Croton	816,44	792,47	2,10	0,04	2,00	0,30	961,36	65,92	2,00	0,22	1,99	0,01
13	Euphorbiaceae	<i>Manihot esculenta</i> Crantz. _{ab}	Malphighiales	Yuca	Cassava	403,91	394,18	2,08	0,10	2,00	0,22	754,60	55,61	2,10	0,04	1,77	0,01
14	Euphorbiaceae	<i>Croton lechleri</i> Muell. Arg. ^a	Malphighiales	Sangre de drago	Dragon's Blood	1787,72	1358,89	1,94	0,14	2,15	0,01	4438,61	379,84	1,97	0,01	1,61	0,01
18	Fabaceae	<i>Cedrelinga cate-niformis</i> (Ducke) Ducke	Fabales	Chuncho	Tornillo	136,79	56,53	1,82	0,22	1,22	0,02	170,06	0,81	1,89	0,01	1,50	0,01
15	Fabaceae	<i>Mimosa pudica</i> L. ^a	Fabales	Dormilona	Sensitive plant	631,80	660,42	2,15	0,00	1,99	0,17	156,76	67,49	2,00	0,13	1,17	0,08

Table 1. Contd.

No.	Plant family	Scientific name	Samples			Fresh leaves					Dry leaves						
			Order	Spanish common name	English common name	Concentration (ng μL ⁻¹)*	±SD	A260/280*	±S D	A260/230*	±SD	Concentration (ng μL ⁻¹)*	±SD	A260/280*	±S D	A260/230*	±SD
16	Fabaceae	<i>Arachis pintoi</i> Krapov. & W.C.Greg. ^b	Fabales	Maní forrajero	Yellow peanut plant	255,34	120,97	2,03	0,04	1,76	0,61	202,64	47,57	1,50	0,38	1,58	0,29
17	Fabaceae	<i>Inga edulis</i> Mart. ^a	Fabales	Guaba	Ice-cream bean	170,57	180,69	1,99	0,20	2,10	0,04	286,05	156,32	2,10	0,01	1,78	0,54
19	Fabaceae	<i>Brownia grandiceps</i> Jacq. ^a	Fabales	Cruscaspi	Rose of Venezuela	263,61	173,57	2,16	0,03	2,00	0,29	486,59	178,00	2,10	0,10	1,79	0,02
20	Heliconiaceae	<i>Heliconia rostrata</i> Ruiz & Pav. ^{a,b}	Zingiberales	Heliconia lorito	Hanging Lobster's Claw	372,28	97,90	1,99	0,16	1,60	0,51	328,35	244,13	1,76	0,26	0,98	0,38
21	Lamiaceae	<i>Mentha × piperita</i> L. ^{a,b}	Lamiales	Menta panga (kichwa)	Peppermint	449,70	241,64	2,04	0,12	1,87	0,03	2105,34	1579,88	2,10	0,15	1,77	0,05
22	Lamiaceae	<i>Ocimum basilicum</i> L. ^{a,b}	Lamiales	Albahaca	Basil	123,53	98,20	2,02	0,28	1,95	0,01	327,65	281,19	1,84	0,05	1,03	0,37
23	Lauraceae	<i>Persea americana</i> Mill. ^{a,b}	Lamiales	Aguacate	Avocado	1132,32	1421,50	2,11	0,07	1,97	0,35	108,62	2,38	2,10	0,09	0,75	0,01
24	Malpighiaceae	<i>Banisteriopsis caapi</i> (Spruce ex Griseb.) C.V.Morton. ^a	Malpighiales	Ayahuasca	Soul vine (yage)	183,97	72,80	1,92	0,01	1,53	0,00	86,46	1,33	1,88	0,00	1,26	0,01
25	Malvaceae	<i>Theobroma cacao</i> L. ^a	Malvales	Cacao	Cacao	416,06	117,68	2,10	0,01	1,60	0,15	225,73	98,64	2,04	0,04	1,33	0,28
26	Malvaceae	<i>Theobroma bicolor</i> Humb. & Bonpl. ^a	Malvales	Cacao blanco	Macambó tree	141,48	5,55	2,02	0,07	1,77	0,36	183,81	42,21	2,03	0,01	1,86	0,44
27	Musaceae	<i>Musa x paradisiaca</i> L. ^b	Zingiberales	Plátano	Banana	449,53	135,23	2,20	0,01	1,53	0,67	253,48	204,98	1,71	0,50	1,07	0,28
28	Myrtaceae	<i>Eugenia stipitata</i> ^a	Myrtales	Arazá	Arazá	304,56	95,59	2,11	0,01	2,02	0,26	200,40	66,86	2,10	0,02	1,69	0,47
29	Myrtaceae	<i>Syzygium malaccense</i> (L.) Merr. & L.M.Perry. ^{a,b}	Myrtales	Pumarosa	Malay apple	212,10	240,60	2,04	0,09	1,51	0,07	572,92	482,78	1,63	0,39	1,54	0,02
30	Myrtaceae	<i>Psidium guajava</i> L. ^{a,b}	Myrtales	Guayaba	Common guava	1413,80	1383,67	2,10	0,02	1,58	0,51	466,98	76,20	2,04	0,02	1,57	0,07

Table 1. Contd.

No.	Samples				Fresh leaves				Dry leaves								
	Plant family	Scientific name	Order	Spanish common name	English common name	Concentration (ng μL^{-1})*	$\pm\text{SD}$	A260/280*	$\pm\text{S D}$	A260/230*	$\pm\text{SD}$	Concentration (ng μL^{-1})*	$\pm\text{SD}$	A260/280*	$\pm\text{S D}$	A260/230*	$\pm\text{SD}$
31	Oxalidaceae	<i>Averrhoa carambola</i> L. ^{a,b}	Oxalidales	Carambola	Copoazu	611,98	314,47	2,14	0,03	2,20	0,29	1663,19	1213,98	2,13	0,04	2,08	0,04
32	Piperaceae	<i>Piper aduncum</i> L. ^a	Piperales	Matico	Spiked pepper	453,99	161,76	2,11	0,05	2,01	0,20	1311,74	758,55	2,12	0,02	1,80	0,69
33	Piperaceae	<i>Piper peltatum</i> L.	Piperales	Maria panga	Largeleaf fingertree	785,62	913,39	1,98	0,17	1,47	0,69	347,55	25,54	2,17	0,04	1,93	0,42
34	Rubiaceae	<i>Borojoa patinoides</i> Cuatrec. ^a	Gentianales	Borojo	Borojo	559,87	276,31	1,94	0,17	1,53	0,25	179,86	50,13	2,01	0,07	0,91	0,08
35	Rubiaceae	<i>Coffea arabica</i> . ^{a,b}	Gentianales	Café	Coffee	133,90	51,96	1,68	0,23	1,13	0,06	163,90	51,96	1,82	0,23	1,40	0,03
36	Sapindaceae	<i>Nephelium lappaceum</i> L. ^{a,b}	Sapindales	Rambután/Achotillo	Hairy Lychee	146,15	37,04	2,03	0,09	1,43	1,03	271,33	1,44	1,89	0,03	1,53	0,04
37	Sapotaceae	<i>Pouteria caimito</i> Radlk. ^{a,b}	Ericales	Avio	Star Apple	439,18	185,92	2,04	0,08	1,82	0,16	235,96	91,52	2,10	0,09	1,68	0,27
38	Solanaceae	<i>Solanum quitoense</i> Lam. ^a	Solanales	Naranjilla (cocona)	Lulo (Quechua)	226,42	141,14	2,02	0,15	1,93	0,65	829,40	289,08	2,05	0,04	1,69	0,14
39	Solanaceae	<i>Cestrum nocturnum</i> L. ^a	Solanales	Caballero de la noche	Night jessamine	540,84	302,46	1,98	0,21	1,84	0,61	970,96	242,55	1,95	0,13	1,87	0,70
40	Zingiberaceae	<i>Hedychium coronarium</i> J.Koenig.	Zingiberales	Lirio de arroyo	White ginger lily	239,16	64,74	1,97	0,10	1,13	0,16	263,09	112,26	1,99	0,07	1,79	0,34

*Mean of three replicates. ^a High content of polyphenols reported in literature. ^bHigh content of polysaccharides reported in literature.

Black text: Gymnosperms. Blue text: Angiosperms. Green highlighted: Eudicots. Yellow highlighted: Magnoliids. Salmon highlighted: Monocots. Blue highlighted: Pteridophyta

Table 2. Statistical differences in DNA extraction between fresh and dry leaves from 40 plant-samples of 27 different families, collected in Napo Ecuador

Variable	Mean Dry leaves ng μL^{-1} ($\pm$ SD)	Mean Fresh leaves ng μL^{-1} ($\pm$ SD)	<i>p</i> -value
Concentration	628 ($\pm$ 765.73)	519.61 ($\pm$ 380.57)	0.93
A260/A280	1.94 ($\pm$ 0.16)	2.02 ($\pm$ 0.11)	0.02*
A260/A30	1.52 ($\pm$ 0.37)	1.77 ($\pm$ 0.28)	0.002**

Significance: $p < 0.1$: ‘*’, $p < 0.01$: ‘**’, $p < 0.001$: ‘***’.

Table 3. Statistical significance of the impact of the type of material (fresh and dry leaves) and plant family on DNA quantity and quality of 40 plant-samples of 27 different families, collected in Napo Ecuador.

Variable	Data transformation	Factor	DF	Chisq	<i>Pr</i> ($>$ Chisq)
Concentration	Log10	Material	1	1.22	0.99
		Plant Family	26	2.89	0.004 **
A260/A280	Inverse	Material	1	6.61	0.008 **
		Plant Family	26	0.86	0.66
A260/A30	Sqrt	Material	1	3.13	0.0002 ***
		Plant Family	26	1.71	0.03 *

DF: Degrees of Freedom, Chisq: Chi-square statistical value, $Pr(>Chisq)$: P-value associated with Chi-squared statistical value. Significance: $p < 0.1$: ‘*’, $p < 0.01$: ‘**’, $p < 0.001$: ‘***’. All variables were treated by multivariable GLM with a Gamma distribution (Log link function).

the type of material (Table 2), and the values acquired for this index were from 1.50 to 2.20, which was expected because of the different possible metabolites present in samples depending on the plant families. For fresh material, twenty five samples were placing just in the ideal range, and fifteen showed values lightly above this range (until 2.20), which suggest the presence of RNA (Scientific 1975; O’Neill et al. 2011; Matlock 2015). It should happen because the protocol presented in this work avoided the use of RNase, which despite of its importance and efficiency, can be uncommon in laboratories with limited resources.

On the other hand, for dry material, twelve samples showed ideal values, five samples can still be labelled as “clean DNA” (>1.7) (O’Neill et al. 2011). Moreover, only two samples were below 1.7 with the lowest value at 1.50 for *Arachis pinto*, which can be related to its naturally high-protein nature molecule content (Hertentains & Ruiloba 2010; de Almeida Araújo et al. 2022) but also to the oxidation process of leaves when drying, which can result in the concentration of components identified as contaminants in the DNA quality evaluation by spectrophotometry (Sahu et al. 2012; Snoussi et al. 2021). It is also supported by GLMs analysis employed where the type of material demonstrated a relevant impact on the A260/280 ratio ($Pr(>Chisq) = 0.008$) (Table 3). In addition, one sample belonging to the gymnosperms group (*Cyathea arborea* (L.) Sm.) displayed a little lower A260/280 quality index in dry samples (1.48) than the values obtained by Jamaludin et al (2020) (1.90-1.99), who developed a specific CTAB-based protocol including polyvinylpyrrolidone (PVP) to reduce the problems of phenolics present in Cyatheales plant order (Sahu et al. 2012; Jamaludin et al. 2020). Yet, that is a clear example of a good protocol performed specifically for limited group of plant families, the opposite objective of this work which seeks to be global. Another important fact to take into account is that the accuracy of the spectrophotometer can slightly shift the values measured, especially for the A260/280 ratio where many times the 1 nm accuracy specification of equipment will result in ± 0.2 (Desjardins & Conklin 2010; Matlock 2015; Thermo Scientific 2020). It can also explain why some researchers do not take the ideal range as an entire strength and unbreakable parameter, instead sometimes work with values with few decimals of difference (Matlock 2015;

Thermo Scientific 2020). Although in spite of the absorbance at 280nm is a commonly used indicator of protein content, mainly due to tryptophan and tyrosin side chains coming from the sample (O'Neill et al. 2011), certain samples may be difficult to be successfully evaluated at that wavelength due to interference with ionic reagents used in the DNA extraction (O'Neill et al. 2011; Clark et al. 2015; Lucena-Aguilar et al. 2016). In those cases, it is recommended to evaluate the samples at a different wavelength such as 228nm as an alternative to identify the presence of peptide bonds (O'Neill et al. 2011).

Because of the reason exposed in the last paragraph, the other quality index (260/230) is often considered the more accurate index to estimate the DNA quality (O'Neill et al. 2011). Its value could indicate that organic compounds or chaotropic salts such as thiocyanate salt are in the DNA (Gupta et al. 2013; Zhong et al. 2013) and the ideal range is 2.0-2.2 (Thakuria et al. 2008; Antony-Babu et al. 2013; Santos et al. 2018). For this ratio, values obtained were lower than the 260/280 index, but all indices greater than 1.5 (Table 1) can be appraised as a guideline for good DNA quality (Desjardins & Conklin 2010). As well as in this protocol, variability and values below the ideal range for this index (2.0-2.2) are common in procedures with organic separation phases, since reagents such as phenol, guanidine HCl, carbohydrates and EDTA, commonly used in the DNA extraction process have absorbances close to ~230 nm (De Campos et al. 2017). Considering it, for this work, EDTA and carbohydrates can be related to a decrease of A_{260/230} values, the first one because it is part of the lysis buffer, however it was used for all samples and not all of them showed low values, so the second one reagent mentioned could be more related to it because it is often becoming problem in plant samples as a natural content (Scientific 1975; Matlock 2015). In contrast, values a little bit higher than the ideal range can be related to a slight variation in the pH, however, it is not always a problem because it does not necessarily act as a PCR inhibitor, and it is still considered an acceptable error range for this index (Wilfinger et al. 1997).

In general, the quality indexes obtained in the DNA extraction process are closely related to some factors such as the secondary metabolites present (Friar 2005; Sahu et al. 2012; Bailey et al. 2022). Its content is directly related to the plant family which by the data analysis employed in this study, revealed its great effect not only in the quality but also in the DNA concentration obtained (Table 3). Thus, our results can also be explained by the concentrations of β -mercaptoethanol (1 %), which is a reducing agent that breaks the disulfide bonds in the thiol groups of cysteine residues, altering the protein structure, eliminating ribonucleases released during cell lysis (Rezadoost et al. 2016; Spadoni et al. 2019; Schenk et al. 2023; Liu 2024). On the other hand, CTAB (2 %) is an amphipathic surfactant detergent that facilitates the lysis of both the cell wall and the plasma membrane (Križman et al. 2006; Krishnan et al. 2024). DNA extraction dissolves membrane molecules in an aqueous medium, forming micelles that encapsulate molecules such as phospholipids and proteins (Križman et al. 2006; Krishnan et al. 2024). Meanwhile, a high ionic strength caused using NaCl (1.4 M) promotes the formation of complexes between proteins and CTAB, allowing their removal during the separation of the organic phase (Heikrujam et al. 2020). Thereby, it facilitates the purification of DNA from polyphenols and proteins, so, a high concentration of those reagents can help to improve DNA extraction (Lodhi et al. 1994; Khanuja et al. 1999; Križman et al. 2006; Kool et al. 2012; Sahu et al. 2012; Arruda et al. 2017; Heikrujam et al. 2020; Krishnan et al. 2024). Additionally, it is interesting to mention that the DNA extraction of Laurales and Piperales (Magnolids), monocots, and eudicots have shown similar results in terms of purity (260/230: 1.8-2.0) with another procedure based on the CTAB method

by Doyle and Doyle (1987) utilizing a buffer with relatively large amounts of CTAB (3X) and sodium chloride (4M) (Mavrodiev et al. 2021). However, despite their methodology's decreased extraction time, it describes a protocol for use with EconoSpin All-In-One Silica Maxi Spin Columns (catalog no. 2040-050; Epoch Life Sciences, Missouri City, Texas, USA), which can be easily scaled for use with the Mini (Epoch 1910-050/250) or Midi (Epoch 050) Spin Columns by adjusting the amount of starting material and extraction buffer (Mavrodiev et al. 2021). In comparison, the protocol presented in this work showed similar results with fewer laboratory resources.

The elimination of any type of columns uses and equipment for automatic DNA extraction means that the difference in values obtained for DNA concentration and quality between samples could be closely associated with the skills of the human operator. So, it is important to be very careful in some steps such as the separation of the organic from the aqueous phase where we have to take supernatant (aqueous phase) that contains the DNA, leaving the interphase, lipids, and debris at the bottom of the tube (organic phase) (McKiernan & Danielson 2017; Heikrujam et al. 2020). If a little interphase or organic phase is recovered and mixed with the aqueous phase, the index value of A₂₆₀/A₂₈₀ could decrease because of the organic compounds present, resulting in low-quality DNA. Also, to improve the value of the A₂₆₀/A₂₃₀ ratio, despite the importance of ethanol in the washing steps of the protocol, it is crucial to be sure that all of it is removed from the DNA pellet because it also could be a strong PCR inhibitor (Schrader et al. 2012). In addition, we realise that using dry material yielded slightly higher mean values than fresh material for DNA concentration and quality indexes (Table 2).

On the other hand, the agarose gel electrophoresis of total DNA extracted showed thin and thick bands that act as an indicator of the molecular weight (Lee et al. 2012) (Figure 1 and Figure 2). For fresh material, we can identify a band above 10kb, which corresponds to genomic DNA, and marked pattern of two lower bands between 1k and 2k bp, which can correspond to RNA molecules such as 28S and 16S ribosomal, respectively (Figure 1). It is mentioned considering as a reference another study where the same pattern was identified after the use of the CTAB-LiCl extraction method (Vennapusa et al. 2020). The visualisation of these bands is possible because the method extracts the total nucleic acids from the tissue, as RNase is not used. Furthermore, a clear difference in DNA integrity between fresh and dry material (Figure 1 and Figure 2). Even fresh material displayed expected bands, many of them show smears, suggesting DNA degradation (Zhang et al. 2004; Schwessinger 2023). For dry material samples integrity observed was lower with a big smear in almost every sample (Figure 2). This fuzzy effect can be related to some factors including the presence of impurities in the sample such as high protein and salt concentrations, which can help to explain the results (Scientific 2022). However, it can also be closely related to DNA degradation because avoiding liquid nitrogen affects the good condition of the genetic material (Quiñones et al. 2024).

Nevertheless, all samples examined in this study demonstrated acceptable DNA concentration and quality indexes for both fresh and dry material (Table 2). It was obtained even with 34 of the 40 considered species having at least one report about their significant to high content of polyphenols, and, 15 of 40 which have reported their remarkable content of polysaccharides (Table 1) (Wollgast & Anklam 2000; Jiménez-Escrig et al. 2001; Torres et al. 2002; Sotelo et al. 2010; Thitilertdecha et al. 2010; Gómez et al. 2012; Sahu et al. 2012; Saffoon et al. 2014; Khanam et al. 2015; Gan et al. 2018; Hendawy et al. 2018; Olvera et al. 2018; Ahmed et al. 2019; Galviz-Quezada et al. 2019; Herrera-Calderon et al. 2019; Ijaz et al. 2019; Ribeiro de Sousa et al. 2019; Suleiman 2019; Gonçalves et al. 2020; Jamaludin et al. 2020; Lima et al. 2020; Savi et al. 2020; Spórna-Kucab et al. 2020; Asy'ari Hasbullah & Rini Umiyati 2021;

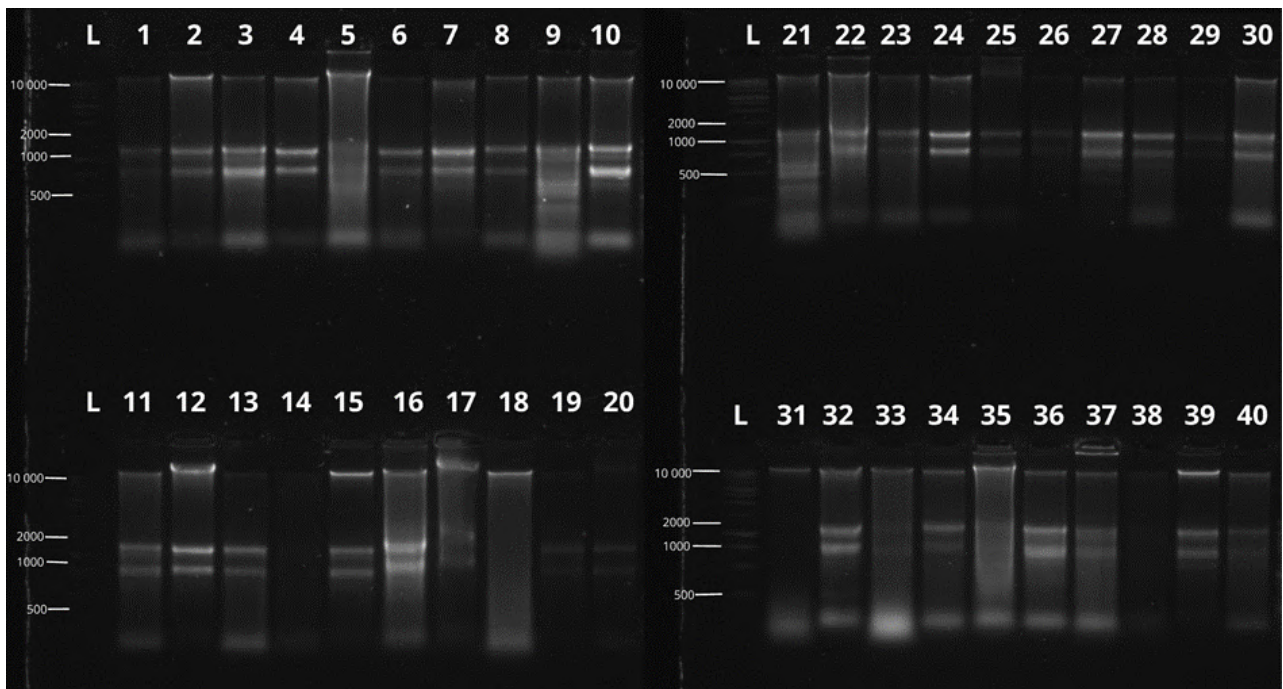


Figure 1. Agarose gel (1 %) electrophoresis of the genomic DNA extracted from fresh leaves. L: 1Kb ladder. Wells are labelled with numbers (1-40) following the species listed in Table 1.

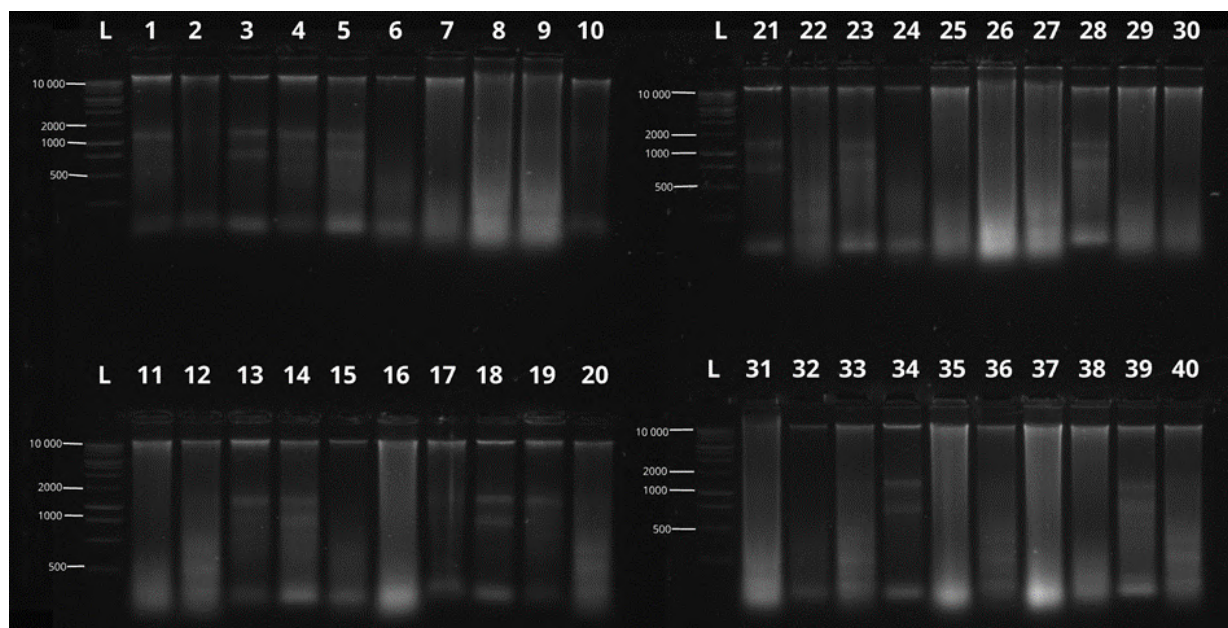


Figure 2. Agarose gel (1 %) electrophoresis of the genomic DNA extracted from dry leaves. L: 1Kb ladder. Wells are labelled with numbers (1-40) following the species listed in Table 1.

Fernandes de Araújo et al. 2021; Nguyen Thi et al. 2021; Simpson et al. 2021; Abd Elkader et al. 2022; Haggag 2022; Korany et al. 2022; Dk et al. 2023; Kim & Iida 2023; Obregon et al. 2023; Song et al. 2023; Zhou et al. 2023). Furthermore, it is important to highlight the applicability of data analysis techniques like GLM, which in addition to its contribution to understanding how really variables affect, does not always necessitate the normality of predictors. It stands out in its utility in real-world biological data applications (Bolker et al. 2009; Keselman et al. 2016).

The success of the extraction method in obtaining good-quality genomic DNA can suggest its interesting applicability. It should be noted that PCR could be one of the keys to determining the suitability of the genetic material (Bolker et al. 2009; Schrader et al. 2012; Keselman et al. 2016; Handy et al. 2020). According to it, the PCR assay showed a successful amplification of

the *rbcLa* region for the total of samples without differences between dry and fresh material used, with a band of ~550pb as expected (Figure 3 and Figure 4).

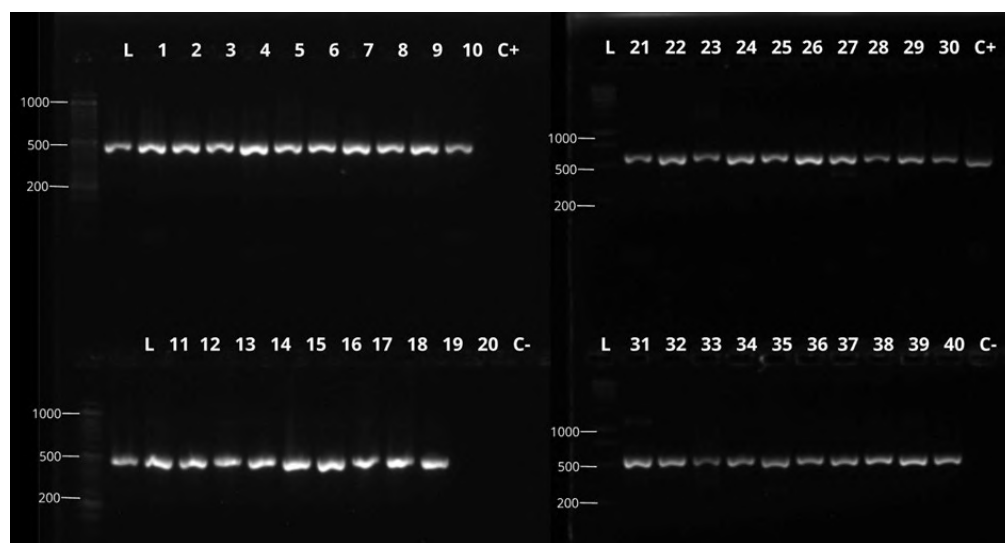


Figure 3. Agarose gel (2 %) electrophoresis of the amplification of *rbcLa* region using DNA from fresh leaves. L: 100bp ladder. Wells are labelled with numbers (1-40) following the species listed in Table 1.

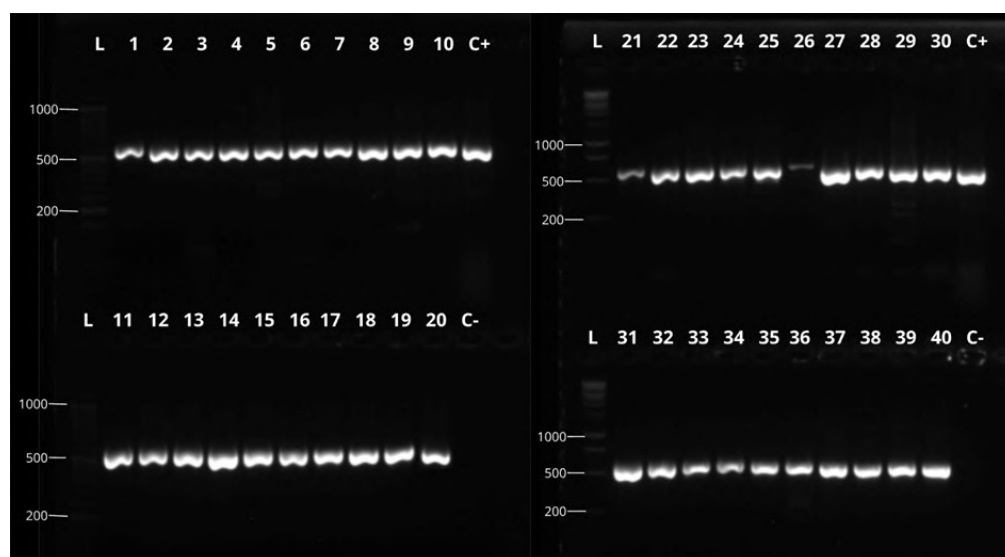


Figure 4. Agarose gel (2 %) electrophoresis of the amplification of *rbcLa* region using DNA from dry leaves. L: 100bp ladder. Wells are labelled with numbers (1-40) following the species listed in Table 1.

So, these results probe the suitability of DNA extracted for PCR activities in molecular plant species identification, suggesting that this protocol can be used for fresh or dry plant samples. Moreover, without using liquid nitrogen in the protocol, the cost of the DNA extraction process decreases considerably and turning it into a more easily replicable method to be used in laboratories with limited resources.

Finally, it is weighty to emphasise the importance of evaluate the efficiency of this procedure when using herbarium material or long storage samples, as well as evaluate its applicability to obtain DNA that can be uses for more complex techniques that involves enzymes restriction, Next Generation Sequencing (NGS) analysis, and the use of other type of molecular markers such as RFLPs or SSRs, in order to those methods can ask for more rigorous DNA requirements.

CONCLUSION

The protocol presented in this work demonstrated its applicability for molecular identification aims, and it can be useful in extracting DNA from at least 27 plant families, even groups of high phenolic content. We promote the application of this protocol for different purposes to evaluate its broad applicability. Meanwhile, the protocol is a good option, especially in academic fields and laboratories with limited resources.

AUTHOR CONTRIBUTION

NEDLMS and AOM designed the research. AOM and AAZ collected the plant material. NEDLMS, AOM, AAZ and JCC processed the samples until get results. NEDLMS and AOM analysed the data. NEDLMS, AOM, AAZ, JCC, EF, SL and MCP wrote the original draft and agreed to the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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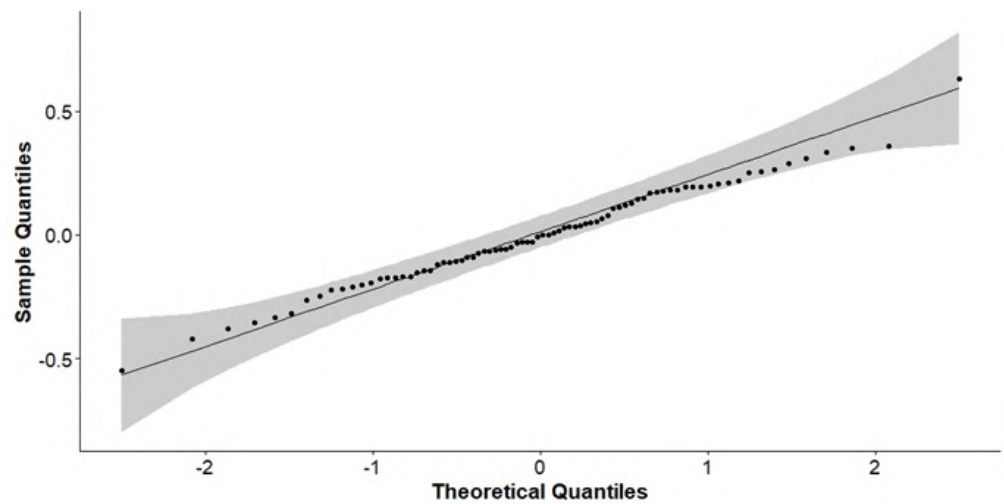
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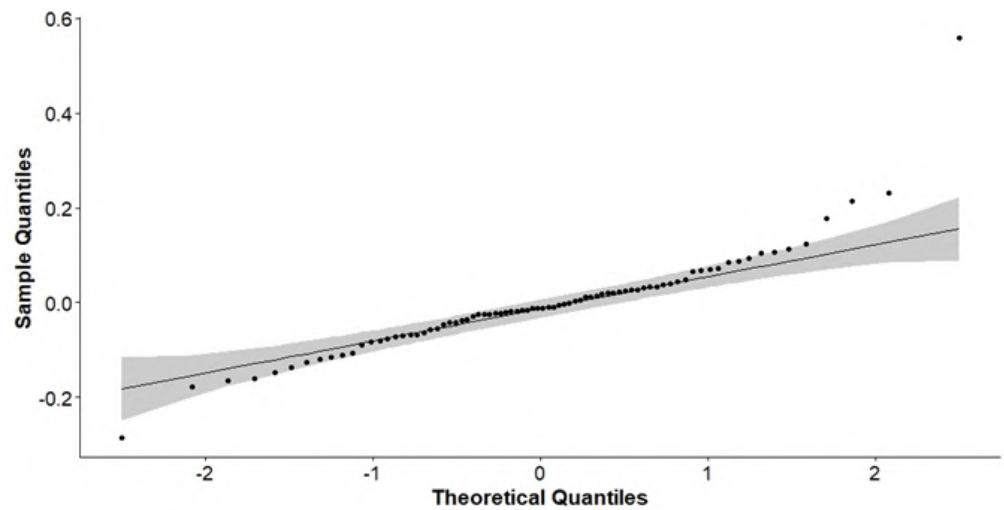
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APPENDICES

A.



B.



C.

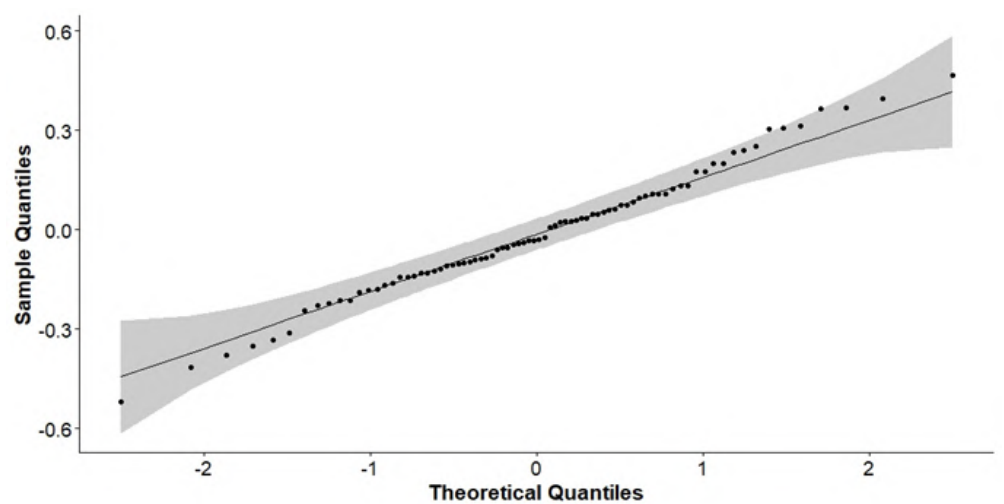


Figure S1. Q-Q Plots for residual models of DNA quantity and quality of 40 plant-samples of 27 different families, collected in Napo Ecuador. A: DNA Concentration Model. B: A260/280 Model. C: A260/230 Model.

Research Article

Identification and Characterisation of Endophytic Bacteria in Rice Bean (*Vigna umbellata*)

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ABSTRACT

Rice bean (*Vigna umbellata*) is an underutilised legume with unexplored endophytic microbiome. Identification and characterisation of these endophytes are critical in understanding their roles in plant's growth, health, and productivity. In this study, twelve morphologically and biochemically distinct bacterial endophytes were isolated from rice bean roots. Sequence analysis of the 16S rDNA of the isolates revealed that members of Proteobacteria, including *Stenotrophomonas* sp., *Shinella* sp., *Roseomonas* sp., *Pantoea dispersa*, and *Serratia marcescens* dominated the root tissues of rice bean. The remaining isolates were found to be members of Actinobacteria, Bacteroidetes, and Firmicutes. The *in vitro* assays showed the potential abilities of the endophytic *Stenotrophomonas* sp., *Shinella* sp., *Microbacterium gilvum*, *Serratia marcescens*, and *Bacillus qingshengii* in indole acetic acid production, exopolysaccharide production, and phosphate solubilisation. Overall findings suggest diverse and potentially multifunctional endophytes in rice bean that can be incorporated into agricultural practices and crop improvement programs.

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INTRODUCTION

Rice bean (*Vigna umbellata*) is an annual, diploid legume member of family Fabaceae (Verma et al. 2022) that produces yellow flowers and small edible beans encased in pods. This fast-growing plant is a multipurpose legume that is more commonly cultivated in the South, Southeast, and East Asia, which are considered as its place of origin (Pattanayak et al. 2019). It is mainly grown for its protein- and micronutrient-packed seeds (Pattanayak et al. 2018) and also utilised for fodder and green manure (Dhillon & Tanwar 2018). However, when it comes to global production rice bean is nowhere close to its relative species, mung bean and cowpea, which are the most important domesticated *Vigna* species (Verma et al. 2022). In some countries in Southeast Asia, including The Philippines, this legume is traditionally cultivated in remote areas for the farmers' sustenance and livelihood. As a legume, rice bean is suitable in mixed-cropping systems as a rich nitrogen source, which could aid in improving soil fertility.

Unlike other notable *Vigna* species the potential of rice bean is only recognised in recent years; thus, scientific knowledge about this crop is still evolving as recent and ongoing studies uncover and offer new insights into its significance. It is characteristically known for its high biomass and grain yield potential and possess tolerance to abiotic and resistance to pest and disease, traits that are essential to thrive in harsh conditions (Guan et al. 2022). The intricate relationship between host plants' notable agronomic and resilience traits and their association with complex micro-ecosystem such as endophytes are increasingly recognised.

Plants possess a complex microbiome with diverse bacteria that colonise various organs and tissues and promote plant growth and fitness. Endophytes are either transmitted through seeds demonstrating plant growth promotion activities once the seeds have germinated or are recruited from soil (Verma & White 2018). These beneficial microorganisms have been demonstrated to play an important role in (i) facilitating nutrient-transfer symbioses for the uptake and utilization of nutrients, (ii) promoting plants growth, development, and production, (iii) alleviating oxidative stress, (iv) providing protection in plants from diseases and pests, and (iv) inhibiting growth of weeds (White et al. 2019).

The advancement in molecular techniques has made it possible to uncover complex microbial communities residing in the tissues of legumes and other host plants. Some of the current techniques employed that propel the field of plant microbiomes include DNA genome sequencing to determine the genes encoded by these plant growth-promoting bacteria (PGPB); transcriptomics, proteomics, and metabolomics that are used to understand the plant gene expression in association with PGPB; PGPB genome editing; and development of encapsulated microbial inoculants (Gamalero et al. 2022). Accumulating evidence revealed the diverse microbial endophytes contributing to the productivity and resilience of plants, which include members of several phyla such as Actinobacteria, Acidobacteria, Bacteroidetes, Deinococcus-thermus, Firmicutes, Proteobacteria, and Verrucomicrobia (Rana et al. 2020). Proteobacteria are the most dominant, well investigated group followed by Firmicutes and Actinobacteria, with *Bacillus*, *Pseudomonas*, *Burkholderia*, *Rhizobium*, and *Klebsiella*, as the dominant genera in most leguminous and non-leguminous plants (Rana et al. 2020).

However, the microbial community associated with rice bean is unexplored, which limits our understanding of the intricate relationships between these microorganisms and their host. Therefore, identifying its culturable endophytic bacterial community is essential for gaining valuable insights into the endophytic bacterial taxa with functional characteristics vital to the growth and development of plants. This information is vital in developing

strategies such as integration of endophytic inoculants into agricultural practices and crop improvement programs for enhancing crop resilience and productivity.

MATERIALS AND METHODS

Plant Materials and Sampling

Healthy seeds of rice bean accessions obtained from the germplasm collection of the Center for Studies in Biotechnology, Cebu Technological University were surface-sterilised and allowed to germinate in sterile petri plates lined with moistened filter paper. About ten sprouts per treatment in three replications were sown in the experimental pot at the experimental nursery area of Cebu Technological University Barili Campus under natural day-night conditions. The soil medium used was collected from the areas where accessions were sampled. All soil samples were mixed equally to create a composite soil sample.

Endophytic Bacteria Isolation

Root samples of rice bean at vegetative stage that were collected in triplicates were surface-sterilised using a 50 ml solution containing 2 % sodium hypochlorite (NaClO) and Phosphate Buffer “Saline” (PBS – 1.44 g of Na₂HPO₄, 0.24 g of KH₂PO₄, 0.20 g of KCl, 8.00 g of NaCl, pH 7.4) for 25 min. Subsequently, the samples were washed with 50 ml 2 % sodium thiosulfate (Miché & Balandreau 2001) three times for 10 min. The roots were rinsed eight times with sterile distilled water before 1-hour rehydration in 100 mL sterile deionised water at room temperature. The efficiency of the sterilisation process was confirmed by plating 100 µL of the final rinse on nutrient agar (NA) and was incubated for six days at 28 °C. The root samples were weighed and macerated in 1 mL PBS buffer. The suspensions were used for counting and isolation from the dilution (1: 10, v v⁻¹) in PBS to 10⁻³ dilution as described by Lopez et al. (2016); subsequent dilutions, in three replica vials for each dilution, were inoculated on trypticase soy agar (TSA).

Morpho-cultural and Biochemical Characterisation

Serial dilutions of extracted suspensions were performed up to threefold and 100 µL of the last dilutions were spread on Nutrient Agar (NA) plates using a sterile spreader. The cultures were incubated for 24 hours at room temperature. Colonies were characterised by margin, colour, elevation, texture, and shape. The isolates were then differentiated based on the structural property of their cell wall using the Gram Stains kit (Himedia, Maharashtra, India).

The starch hydrolysis test was performed using the iodine test described in Dunican and Seeley (1962), while the catalase activity of the strains was evaluated using the H₂O₂ solution drop method for bubble production (Zhu et al. 2022). Moreover, carbohydrate fermentation and CO₂ production assay using different sugars including glucose, maltose, and sucrose, was done following the protocol described in Reiner (2012). Yellow cultures indicate positive fermentation results while gas production (visible air bubbles in the inverted Durham tube) indicates positive CO₂ production (Reiner 2012). In addition, the antibiotic sensitivity assay using disc diffusion assay (Sarker et al. 2014) was carried out for amoxicillin, chloramphenicol, cephalixin, and doxycycline.

***In vitro* assay for plant growth promotion property**

Screening for IAA Production

All strains were screened for Indole-3-acetic acid (IAA) production as described by Loper and Schroth (1986). Absorbance was measured at 530 nm after 30 min at room temperature. The concentration of IAA from each cul-

ture medium was calculated against the pure IAA standard curve (where: $y = 0.0098x + 0.0245$; $R^2 = 0.9972$).

Screening for Phosphate-solubilising Activity

The phosphate-solubilizing activity was determined qualitatively following the protocol of Nautiyal (1999) with modification. A clear halo formed around the colonies indicates phosphate-solubilising activity.

Screening for Exopolysaccharides (EPS) Production

Exopolysaccharides (EPS) production was tested as described previously in Zlosnik et al. (2008). Production of EPS was monitored visually according to the morphology of the colony and the viscous aspect.

PCR and Sequencing

A pure culture of isolates was grown on NB for 24 hrs. After incubation, the samples were centrifuged for 15 min at 1100 rpm to harvest the pellet. The supernatant liquid was discarded while the pellets were added with 100 μ L sterilized deionized water. The total genomic DNA was extracted using an InstaGene™ Matrix DNA extraction kit (Bio-Rad Laboratories). Amplicons of the 16S rRNA region of each of the isolates were generated using polymerase chain reaction (PCR) with the use of the universal primer pairs 27F (5'-A G A G T T T G A T C C T G G C T C A G - 3') and 1492R (5'-G G T T A C C T T G T T A C G A C T T - 3'). A Single-pass sequencing was carried out on ABI 3730XL (Applied Biosystems, Carlsbad, CA; Macrogen, Inc., South Korea) using the primers 785F (5'-G G A T T A G A T A C C C T G G T A - 3') and 907R (5'-C C G T C A A T T C M T T T R A G T T T - 3').

Analysis and deposition of 16S rDNA sequences

The sequence consensus for each isolate was inferred with Genious Prime 2024 (version 11.0.20.1) software and queried against the non-redundant nucleotide database of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/blast/>) using Basic Local Alignment Search Tool (BLAST). Nucleotide sequence hits with at least 99 % similarity were identified for each isolate and downloaded for further analysis to verify identity. Generated 16S rDNA sequences from this study were deposited in GenBank, NCBI database.

RESULTS AND DISCUSSION

A total of 12 cultivable, distinct bacteria based on morpho-cultural characteristics, (Supplementary Table 1) were isolated from root samples of rice bean. Characteristics of bacterial endophytes based on Gram classification, starch hydrolysis, catalase activity, carbohydrate fermentation (glucose, maltose, and sucrose), CO₂ formation, and antibiotic sensitivity reaction are summarised in Table 1. Gram staining test revealed that 58.3 % of the isolates are Gram-negative and 41.7 % are Gram-positive. All isolates except VU-26 hydrolyzed starch, indicating that most produce exoenzyme amylase that breaks down starch into simpler sugar forms such as monosaccharides. Catalase production was also confirmed in 58.33 % of the isolates. Catalase facilitates cellular detoxification by catalysing the decomposition of hydrogen peroxide into water and hydrogen and has been implicated in regulating cellular signaling pathways for growth and apoptosis (Rasheed 2024). The antibiotic sensitivity assay also showed that all isolates were susceptible to cephalexin and doxycycline (Table 1), which are widely used broad-spectrum antibiotics (Wang et al. 2020). In addition, six isolates (50 %) are susceptible to all four antibiotics.

A bonitur scale was generated to identify bacterial isolates with plant-growth promotion potential (El-Sayed et al. 2014), as shown in Table 2. Re-

sults show that all strains are capable of producing IAA, with the highest level exhibited by VU-02 followed by VU-03, VU-15, VU-18, and VU-30. The remaining strains produced IAA with levels below $<50 \mu\text{g mL}^{-1}$. Auxin IAA is one of the most essential phytohormones that regulate growth and developmental processes in plants (Egamberdieva et al. 2017). Microbes colonising the rhizosphere of plants can produce this substance, which plants can use for cell division, differentiation, and enhancement of the host's uptake of minerals and nutrients from the soil (Shokri & Emtiazi 2010).

Likewise, the ability of beneficial bacteria to break down fixed and insoluble soil phosphorus into usable form that can be absorbed by plants is essential for growth and metabolism, resulting in an improved yield (Pan & Cai 2023). In the current study, VU-18 and VU-30 produced substantial phosphorous solubilisation abilities, while minimal activities were detected for VU-20, VU-24, VU-26, and VU-30. In plants, phosphorus uptake will increase in root and stem development, improve seed formation, and increase crop maturity and nitrogen fixation (Billah et al. 2019). In addition, phosphorus is critical in various stages of the photosynthetic process, including ATP synthesis (Khan et al. 2021).

Moreover, two isolates, including VU-02 and VU-03 produced substantial levels of Exopolysaccharides (EPS). EPS is a microbial polysaccharide synthesised by bacteria. It is involved in cell communication and nodule development in legumes and is thus crucial in nitrogen fixation (Liu et al. 2017). In addition, EPS produced by endophytic bacteria can alleviate salinity stress as it can bind with Na thus decreasing the quantity of Na ions available for plant uptake (Kumar et al. 2020).

A homology search of the 16S rDNA sequences using the Basic Alignment Search Tool (BLAST) in NCBI revealed the identity of the isolates, which were then deposited in GenBank, NCBI database and were given accession numbers OR342355 to OR342366. The phylogenetic tree showed that half (50 %) of the isolates belong to Phylum Proteobacteria with six members, followed by Firmicutes with three members (25 %), Actinobacteria with two members (16.67 %), and Bacteroidetes (8.33 %) (Figure 1). Despite the low number of endophytes analyzed, the affinity of isolates demonstrates significant diversity.

The homology search revealed that the phylum Proteobacteria, which dominated the roots of rice bean is composed of *Stenotrophomonas* sp. (OR342355) (100 % homology), *Shinella* sp. (OR342356) (99.50 %), *Roseomonas* sp. (OR342357) (100 %), two strains of *Pantoea dispersa* (both 99.86 %) (OR342362 and OR342364), and *Serratia marcescens* (OR342365) (99.93 %). The three members of Firmicutes include *Bacillus altitudinis* (OR342358) (99.93 %), *Bacillus qingshengii* (OR342366) (99.87 %), and *Bacillus* sp. K2DN333 (OR342363) (99.93 %). The Actinobacteria include *Microbacterium gilvum* (OR342359) (99.79 %) and *Microbacterium nanhaiense* (OR342360) (99.65 %), and the only member of Bacteroidetes isolated in this study is *Sphingobacterium mucilaginosum* (OR342361) (99.18 %) (Table 3).

Overall, *Stenotrophomonas* sp. (OR342355) has the highest assessment value (5) marked with the highest IAA level produced among isolates and a substantial level of EPS production, followed by *Shinella* sp. (OR342356), *Microbacterium nanhaiense* (OR342360), and *Serratia marcescens* (OR342365). This finding is consistent with previous report on the PGP and stress tolerance activities of *Stenotrophomonas* sp. isolated from field-grown poplar, which was found to produce IAA and detected to have active genes for auxin biosynthesis pathways and spermidine pathway (Ulrich et al. 2021). Likewise, inoculation with *Shinella* sp. has been reported to improve the growth of sugarcane, marked by an increase in dry weight (Taulé et al. 2016). Moreover, *Serratia marcescens* was found to participate in nitrogen cycling via denitrification-

Table 1. Biochemical characteristics of endophytic bacteria in rice bean

Bacterial Code	Isolate	Gram reaction	Starch hydrolysis	Catalase activity	CO ₂ Formation	Carbohydrate Fermentation				Antibiotic sensitivity			
						Glu-cose	Malt-ose	Su-crose	Amoxicillin	Chloramphenicol	Cephalexin	Doxycycline	(30µg disc ⁻¹)
VU-02	-	-	+	-	-	-	-	-	R	R	S	S	
VU-03	-	-	+	-	-	+	+	+	S	S	S	S	
VU-06	-	-	+	-	-	-	-	-	R	R	S	S	
VU-12	+	+	+	+	-	-	-	-	S	S	S	S	
VU-15	+	+	+	-	+	+	+	+	S	R	S	S	
VU-18	+	+	+	-	+	-	+	-	R	S	S	S	
VU-20	-	-	+	+	+	-	-	+	S	S	S	S	
VU-24	-	-	+	+	-	-	+	-	S	S	S	S	
VU-26	+	+	-	+	-	-	-	-	I	I	S	S	
VU-29	-	-	+	+	-	+	+	+	I	S	S	S	
VU-30	-	-	+	+	+	+	+	+	S	S	S	S	
VU-31	+	+	+	+	-	-	-	-	S	S	S	S	

(+) = positive reaction; (-) = negative reaction
(R) = Resistant, (I) = Intermediate Resistant, (S) = Susceptible

Exopolysaccharides production (0 = no EPS production; 1 = low EPS production; 2 = average EPS production; 3 = high EPS production)

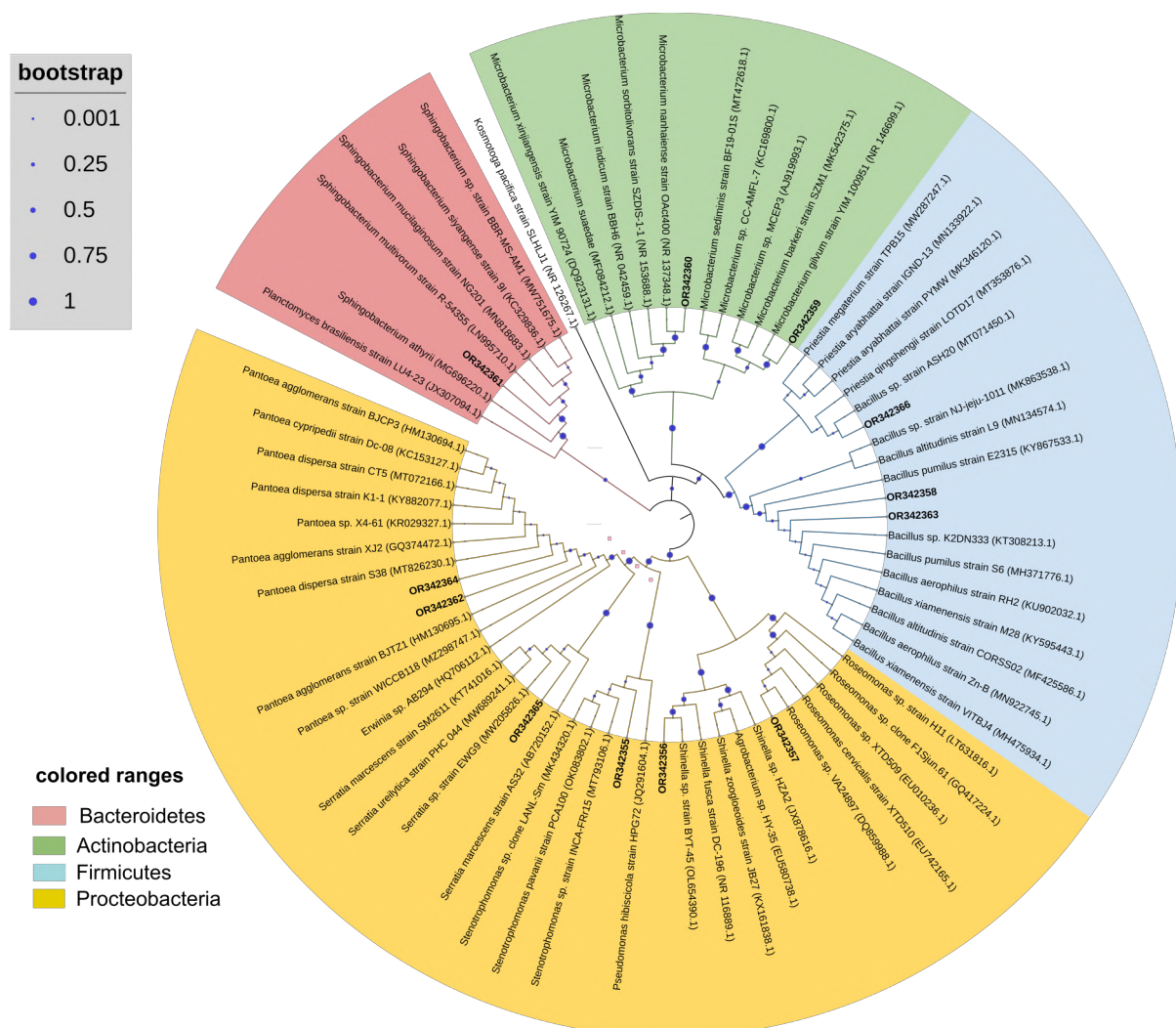


Figure 1. Phylogenetic tree constructed from 16S rRNA sequences using the maximum likelihood method based on the Tamura-Nei model (Tamura & Nei 1993) in MEGA 11 (Tamura et al. 2021) with 1000 bootstrap replicates. The analysis involved 72 nucleotide sequences, including the outgroup, *Kosmotoga pacifica* strain SLHLJ1(NR 126267.1). The phylogenetic tree was visualized in Interactive Tree of Life version 6.8.1 (Letunic & Bork 2021). GenBank accession numbers of nucleotide sequences are shown along with the name of the bacterial species.

Table 3. The nearest phylogenetic relative of bacterial isolates based on the 16S RNA sequencing.

Isolate's Accession Code	Nearest Phylogenetic Neighbor	Homology (%)	E - value	Family	Phylum
VU-02 (OR342355)	<i>Stenotrophomonas</i> sp. strain INCA-FRr15 (MT793106.1)	100	0	<i>Xanthomonadaceae</i>	Proteobacteria
VU-03 (OR342356)	<i>Shinella</i> sp. strain BYT-45 (OL654390.1)	99.50	0	<i>Brucellaceae</i>	Proteobacteria
VU-06 (OR342357)	<i>Roseomonas</i> sp. VA24897/2006 (DQ859988.1)	100	0	<i>Acetobacteraceae</i>	Proteobacteria
VU-12 (OR342358)	<i>Bacillus altitudinis</i> strain CORSS02 (MF425586.1)	99.93	0	<i>Bacillaceae</i>	Firmicutes
VU-15 (OR342359)	<i>Microbacterium gilvum</i> strain YIM 100951 (NR_146699.1)	99.79	0	<i>Microbacteriaceae</i>	Actinobacteria
VU-18 (OR342360)	<i>Microbacterium nanhaiense</i> strain OAct400 (NR_137348.1)	99.65	0	<i>Microbacteriaceae</i>	Actinobacteria
VU-20 (OR342361)	<i>Sphingobacterium mucilaginosa</i> strain NG201 (MN818683.1)	99.18	0	<i>Sphingobacteriaceae</i>	Bacteroidetes
VU-24 (OR342362)	<i>Pantoea dispersa</i> strain S38 ITI (MT826230.1)	99.86	0	<i>Enterobacteriaceae</i>	Proteobacteria
VU-26 (OR342363)	<i>Bacillus</i> sp. K2DN333 (KT308213.1)	99.93	0	<i>Bacillaceae</i>	Firmicutes
VU-29 (OR342364)	<i>Pantoea dispersa</i> strain K1 -1 (KY882077.1)	99.86	0	<i>Enterobacteriaceae</i>	Proteobacteria
VU-30 (OR342365)	<i>Serratia marcescens</i> strain AS32 (AB720152.1)	99.93	0	<i>Enterobacteriaceae</i>	Proteobacteria
VU-31 (OR342366)	<i>Bacillus qingshengii</i> strain LOTD17 (MT353876.1)	99.87	0	<i>Bacillaceae</i>	Firmicutes

DNRA-nitrification pathway, which is essential to sustain plant growth and development (Hamada & Soliman 2023). However, no studies have yet explored the potential of *Microbacterium nanhaiense* as a plant growth-promoting endophyte in plants.

CONCLUSIONS

Overall findings of this study revealed the presence of cultivable bacterial endophytes in rice bean with potential plant growth-promotion activities. However, the evaluation for potential PGP properties in this study is limited to a few assays. To confirm these initial findings, additional relevant PGP screenings, including assessments of abiotic stress tolerance, biotic resistance, and influence on morphological and yield traits of crops, must be explored. Furthermore, in-depth studies uncovering the mechanisms of such roles in host plants and other crops are necessary to fully understand the extent of their beneficial effects and their potential utilisation for crop improvement.

AUTHOR CONTRIBUTION

M.C.N. designed the research, analysed some datasets, wrote some parts of the manuscript, and supervised all the processes; J.H.R. performed the experiments, analysed some datasets, and wrote some parts of the manuscript; and M.L.D.P. aided in designing the research, supervised all the processes, and proofread the manuscript.

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CONFLICT OF INTEREST

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

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APPENDICES

Supplementary Table 1. Morphological characterization of isolates based on colony.

Accession	Morpho-cultural characteristics				
	Margin	Color	Elevation	Texture	Form
OR342355	undulate	white	flat	dry	round
OR342356	entire	white	convex	dry	punctiform
OR342357	entire	pink	convex	slimy	round
OR342358	filamentous	white	flat	slimy	rhizoid
OR342359	lobate	yellow	flat	slimy	rhizoid
		green			
OR342360	lobate	yellow	umbonate	slimy	rhizoid
OR342361	entire	milky	flat	slimy	round
OR342362	erose	white	flat	dry	irregular
OR342363	entire	white	umbonate	dry	round
OR342364	erose	white	flat	slimy	rhizoid
OR342365	entire	red	convex	slimy	round
OR342366	entire	white	umbonate	dry	round

Research Article

DNA Barcoding, Prey Spectrum Analysis, and Vegetative Propagation of *Nepenthes mirabilis* × *rafflesiana*, A Rarely Sighted Pitcher Plant Hybrid from Peninsular Malaysia

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ABSTRACT

A solitary female *Nepenthes* hybrid (*Nepenthes mirabilis* × *rafflesiana*), bearing 20-cm tall reddish pitchers, was discovered in the last remaining peat swamp forest in Johor, Peninsular Malaysia. The nothospecies (natural hybrid) is considered a very rare occurrence and has been designated as a taxon of high ecological importance, underscoring the urgent need for in-depth study and targeted conservation initiatives. To confirm its genetic identity, DNA sequencing was conducted, and the phylogenetic analysis of the internal transcribed spacer region (ITS1-5.8S-ITS2) indicated a strong genetic relationship between the hybrid (accession no. OR741796) and *N. rafflesiana*. Consistent with this, the ITS1 secondary structure of the hybrid exhibited a conserved folding structure similar to that of *N. rafflesiana*. It was recorded that the nothospecies primarily preys on hymenopterans from the family Formicidae, including at least seven genera. Furthermore, mosquito larvae from the genus *Toxorhynchites* were observed in both upper and lower pitchers, highlighting the pitcher plant's importance as a crucial breeding site for the predatory elephant mosquito. Given that only one living specimen of the nothospecies was discovered, we optimised a shoot-based vegetative propagation method that allowed several clones to be produced, which will be vital for the success of future reintroduction efforts. This is the first report to simultaneously address the molecular, ecological, and horticultural aspects of a *Nepenthes* natural hybrid in Peninsular Malaysia.

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INTRODUCTION

Plant taxonomists have primarily directed their attention towards true species, while *Nepenthes* nothospecies (natural hybrids) and intergrades are underexplored and occasionally regarded as biological outliers. Furthermore, these hybrids are challenging to resolve—through both traditional taxonomy and modern phylogenetics (Scharmann et al. 2021). *Nepenthes* nothospecies are generally fertile, capable of recurrent hybridisation, a form of adaptive radiation, and may possess unique attributes (in both ornamental and scientific aspects) (Rosli et al. 2021; Scharmann et al. 2021). According to Blanckaert and Bank (2018), hybridisation may also result in the displacement or even the creation of entirely new species, a concern that is particularly relevant in the animal kingdom. However, we believe that the occurrence of the *Nepenthes* hybrids in a particular ecosystem reflects an evolutionary advantage driven by a combination of natural and anthropogenic factors. It is imperative to thoroughly document this occurrence for a comprehensive understanding of its dynamics.

The ecological study of *Nepenthes* natural hybrids from Peninsular Malaysia remains a relatively unexplored topic. In 2021, a peculiar reddish *N. mirabilis* × *rafflesiana*, which is the focal subject of our study, was discovered in the Ayer Hitam Utara Forest Reserve (AHUFR) administered by the Johor State Forestry Department (Figure 1). Its taxonomic designation as a nothospecies was predetermined based on its clear intermediate features between those of its parental species, *N. mirabilis* and *N. rafflesiana* (Ghazalli et al. 2021). This taxon is considered a rarity despite the widespread occurrence of its two progenitors in the lowland and hilly habitats of Peninsular Malaysia (Tamizi et al. 2023b). Even within the AHUFR, only one living specimen *N. mirabilis* × *rafflesiana* was discovered. Clarke (2001) briefly mentioned *N. mirabilis* × *rafflesiana* in his monograph '*Nepenthes* of Sumatra and Peninsular Malaysia'; however, details regarding its characteristics and specific locality were not mentioned. We estimate that this population is likely located in the northern part of Selangor State. Unfortunately, this particular population is currently threatened by land conversion and development, as it is not situated within any forest reserve. Mansur (2007) performed diversity research on *Nepenthes* in Kalimantan, Indonesia, and included *N. mirabilis* × *rafflesiana* in the listing, albeit no taxonomy information was provided. Here, we did not focus on the formal description or taxonomical treatment of Johor's *N. mirabilis* × *rafflesiana*. Instead, we analysed its DNA barcode and prey preferences, along with the rooting potential of shoot cuttings. This integrated approach is crucial for gaining a comprehensive understanding of the biological and ecological aspects of the hybrid, thereby supporting conservation efforts such as the reintroduction programme. Throughout this article, we refer to this specific hybrid by the taxonomic label '*N. mirabilis* × *rafflesiana* AHUFR'. With the treatment of the Johor's type as a taxon of conservation priority, our findings are projected to deepen our understanding of this nothospecies, forming a critical basis for the formulation of targeted future conservation strategies.

MATERIALS AND METHODS

Sample collection from the study site

A single shoot about 25 cm long with three petiolated laminae (leaves) was excised from the sole *N. mirabilis* × *rafflesiana* AHUFR plant and kept in an air-tight bag. The bag was filled with some water to ensure freshness of the material and prevent wilting during transportation. This shoot sample would be subsequently used for the DNA study and vegetative propagation. A soil sample was taken from the plant's base, and its pH was measured using a portable pH probe (HI 8424) from Hanna Instruments (Woonsocket, RI, USA). The precise locations (coordinates) of the studied *Nepenthes* in the for-

est reserve were recorded but are not disclosed in this article for conservation purposes. The map of the study site was sourced from the NASA/NGA Shuttle Radar Topography Mission (SRTM) datasets provided by the U.S. Geological Survey's EROS Data Center (<https://www.usgs.gov/centers/eros>) and underwent additional editing using PhotoScape v3.7 (<http://www.photoscape.org/>).

DNA purification and ITS isolation from the studied specimen

Genomic DNA was extracted from *N. mirabilis* × *rafflesiana* AHUFR leaves using the DNeasy Plant Mini Kit (Qiagen, Germany), following the manufacturer's protocol. The barcode region, ITS1-5.8S-ITS2 (~600bp), was then PCR-amplified from the DNA using universal ITS1-2 primers (ITS_F: 5'-AGGAGAAGTCGTAACAAGGTT; ITS_R: 5'-GATGCAACCTTGGCCTT) and Q5 High-Fidelity DNA polymerase (New England Biolabs, UK). Thermocycler settings included an initial denaturation at 98 °C for 30 seconds, followed by denaturation at 98 °C for 10 seconds, annealing at 61 °C for 15 seconds, extension at 72 °C for 20 seconds, and a final extension at 72 °C for 2 minutes. Subsequent to gel purification using the QIAquick Gel Extraction Kit (Qiagen, Germany), each PCR product was subjected to Sanger sequencing in two reactions using the aforementioned primers. The resulting reads were manually edited and aligned using BioEdit Sequence Alignment Editor (Raleigh, NC, USA). The sequenced DNA barcode was further analysed using the Nucleotide Basic Local Alignment Search Tool (BLASTn) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to ascertain its identity. Ultimately, the sequences were submitted to the GenBank® database (<https://www.ncbi.nlm.nih.gov/genbank/>).

ITS acquisition, sequence analysis, and ITS1 secondary structure prediction

The ITS of *Nepenthes* native to Peninsular Malaysia were downloaded from the GenBank database, and information on the specimen provenance and voucher data are provided in Table 1. These ITS sequences (including an out-group taxon) were aligned using ClustalW multiple alignment tool and manually trimmed using Molecular Evolutionary Genetics Analysis (MEGA11) software (Pennsylvania State University, PA, USA). A phylogenetic analysis was then computed with 1000 bootstrap replications in the same software using the Maximum Likelihood (ML) method based on the Tamura-Nei model (Tamura & Nei 1993). Following this, the ITS sequences of *N. mirabilis* × *rafflesiana* AHUFR and its parental species (*N. mirabilis* and *N. rafflesiana*) were multiple-aligned and exported as a FASTA file for custom visualisation using BioEdit software.

The boundaries of ITS1, 5.8S, and ITS2 from *N. mirabilis* × *rafflesiana* AHUFR and its parental species were determined by comparing them with the ITS sequences of *Nepenthes* characterised by Alejandro et al. (2007, 2008). Subsequently, the first internal transcribed spacer (ITS1) foldings were queried for minimum free energy (MFE) secondary structures using the RNAfold server (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>) (Gruber et al. 2008; Tieng et al. 2023). The resulting models were downloaded in JavaScript Object Notation (JSON) format and custom-visualised using the forna (force-directed RNA) tool (<http://rna.tbi.univie.ac.at/forna/>) for this publication (Kerpedjiev et al. 2015).

Analysis of pitcher fluids and captured prey

The sampling of the pitchers' digestive fluids, conducted in February 2023, followed the methodology outlined by Ristiawan and Hikmat (2022) and Mo-

Table 1. Specimen provenance of the taxa used for the phylogenetic analysis with *Ancistrocladus robertsoniorum* serves as an outgroup (Meimberg et al. 2010). The *Nepenthes* samples are originating from Peninsular Malaysia (PM) or greenhouse-grown specimens cultivated at the Botanical Garden of the University of California-Berkeley (UCBG), Bogor Botanical Garden (BBG), and Komunitas Tanaman Karnivora Indonesia (KTKI).

No.	Taxon	Specimen Voucher / Material	Origin	GenBank Accession No.	Source
1.	<i>Nepenthes albomarginata</i>	TR10	UCBG	HM204892	Renner & Specht 2011
2.	<i>Nepenthes ampullaria</i>	KRB27100901	BBG	AB675914	Alamsyah & Ito 2013
3.	<i>Nepenthes benstonei</i>	INBIOSIS-N BEN-2011	Bkt. Bakar, Kelantan, PM	JX042560	Bunawan et al. 2017
4.	<i>Nepenthes domei</i>	MD1 12423	Terengganu, PM	OR722473	This study; Ghazalli et al. 2020
5.	<i>Nepenthes gracilis</i>	KRB27100902	BBG	AB675882	Alamsyah & Ito 2013
6.	<i>Nepenthes malayensis</i>	MDIAATNMAL001 / MDI 12242	Terengganu, PM	MN347033	Tamizi et al. 2020a
7.	<i>Nepenthes mirabilis</i>	INBIOSIS-N MIR-2011	Rawang, Selangor, PM	JX042556	Bunawan et al. 2017
8.	<i>Nepenthes rafflesiana</i>	INBIOSIS-N RAF-N-2011	Mersing, Johor, PM	JX042557	Bunawan et al. 2017
9.	<i>Nepenthes rafflesiana</i>	TR25	UCBG	HM204904	Renner & Specht 2011
10.	<i>Nepenthes sanguinea</i>	BL4110906	KTKI	AB675898	Alamsyah & Ito 2013
11.	<i>Nepenthes sanguinea</i>	MDIBN1 Np05A	Fraser's Hill, Pahang, PM	OR722474	This study
12.	<i>Nepenthes sanguinea</i>	MDIBN1 Np05B	Cameron Highlands, Pahang, PM	OR722475	This study
13.	<i>Nepenthes</i> × <i>intermedia</i>	TR20	UCBG	HM204899	Renner & Specht 2011
14.	<i>Nepenthes mirabilis</i> × <i>rafflesiana</i> AHUFR	MDIBN1 Np0902A	AHUFR, Johor, PM	OR741796	This study
15.	<i>Ancistrocladus robertsoniorum</i>	GB-8	East Africa	GQ443551	Meimberg et al. 2010

ran (1999), with some modifications. Digestive fluids from several lower and upper pitchers of the studied *Nepenthes* plants at AHUFR were decanted into sterile 50 mL conical centrifuge tubes. The pitchers were then refilled with sterile water without exceeding the hip level of the pitchers to compensate for the fluid lost. The pH of the digestive fluid from opened pitchers was measured using a pH probe from Eutech Instruments (model pH700). The fluid was then filtered through a filter paper and the contents were retained and preserved in 70 % ethanol for prey analysis. At the laboratory, the samples were analysed on a grid-marked Petri dish and classified with the aid of a dissecting microscope. The identification of prey taxa (ants and other terrestrial invertebrates) was carried out by the insect taxonomist and according to Bolton (1994) and Maryati (1999). We identified all captured prey up to the Order level. Insects from the Order Hymenoptera and larvae from the Order Diptera could be identified down to the genus level because most of the specimens were intact. Nonetheless, for some taxa, classification below the family level was not possible due to the digestion or dismemberment of the prey invertebrates by the fauna residing and feeding inside the pitchers.

Vegetative propagation of shoot cuttings

The primary shoot cutting previously acquired from the site was first washed with an ample volume of tap water followed by sterile water. The leaves (except for the young apical leaves) were partly removed and the stem was further cut using a sharp sterile blade leaving only three petioles (Figure 5A). A sterile, moistened sphagnum mix (three parts dry sphagnum to one part perlite) was prepared and placed in an orchid pot (ø 7 cm). The shoot cutting was firmly inserted into the sphagnum mix and encased within a clean, transparent bag. This setup was then placed in an area with 50 % shade in the greenhouse, and the bag was kept sealed for one month to maintain 100 % moisture, which promotes root growth. Ensuring the cleanliness and sterility of the materials and equipment used was essential to prevent excessive fungal contamination and, consequently, enhance the survival rate of the new plant.

Once the cutting had successfully rooted and produced a new leaf blade (usually about after one month), the bag was gradually punctured with ten holes (ø 1 cm). This step was taken to acclimatise the young plant to the ambient humidity. After the plant had acclimatised (within two weeks), the rooted shoot was then transplanted into a larger pot, with more sphagnum-perlite mix added. To provide stability to the newly rooted shoot, a support pole was installed, and the plant continued to be grown under 50 % sunlight shade for approximately a year. Watering was carried out once every two days to prevent the sphagnum from drying out. When the plant reached one year of age, a 25 cm shoot was excised, and the entire rooting protocol was repeated to obtain new clones.

RESULTS

Ecological notes

The Ayer Hitam Utara Forest Reserve (AHUFR) (elevation *ca.* 40 m above sea level) is the largest and last remaining peat swamp forest in Johor (Muhammad Jais et al. 2022). A very small portion of the forest was mined in the past and now a total of 3795.84 hectares has been gazetted as a permanent forest reserve (Figure 1) (Muazam et al. 2022). It is home to many Peninsular Malaysian lowland *Nepenthes*, with at least four species and five nothospecies have been recorded within the study site (Ghazalli et al. 2021). The site experienced a slight human disturbance; hence, the phenology of these *Nepenthes* was unpredictable and sporadic. The sole specimen of *N. mirabilis* × *rafflesiana* AHUFR was determined to be a female plant based on the presence of an unpollinated infructescence, and its fecundity has not been determined. *Nepenthes mirabilis* × *rafflesiana* AHUFR (Figure 2) was discovered growing as a terrestrial climbing plant in clay-like laterite soil (pH = 5.2) close to a body of blackwater swamp, and occasional flooding has been observed at this site. A few taxa of *Nepenthes*, including *N. ampullaria*, *N. gracilis*, *N. mirabilis*, *N. rafflesiana*, *N. ×hookeriana*, and *N. ×trichocarpa*, were observed growing in close proximity to the nothospecies. These *Nepenthes* plants thrived well in open areas within the forest reserve as well as at the forest margin.

Phylogenetics inference of *N. mirabilis* × *rafflesiana* AHUFR and ITS1 secondary structure characterisation

The ITS region (ITS1-5.8S-ITS2) was sequenced and deposited to the GenBank® database (accession no. OR741796). Nucleotide search of the ITS using Nucleotide Basic Local Alignment Search Tool (BLASTn, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) validated the isolated DNA sequence belonged to the genus *Nepenthes* and the top five taxon hits sharing the highest ITS identity similarity were *N. ×intermedia*, *N. rafflesiana*, *N. gracilis*, and *N. mirabilis* (Table 2). The ITS was used to compute the phylogenetic position of *N. mirabilis* × *rafflesiana* AHUFR among 13 Peninsular Malaysian *Nepenthes*



Figure 1. Location of the Ayer Hitam Utara Forest Reserve (AHUFR) in the state of Johor, Peninsular Malaysia. The forest reserve is an isolated and protected peat swamp forest, gazetted as a state park by the government of Johor. An old mining site within the forest reserve is visible from the satellite, an area where various *Nepenthes* taxa could be spotted.



Figure 2. The sole living specimen of *Nepenthes mirabilis* × *rafflesiana* at Ayer Hitam Utara Forest Reserve (AHUFR) discovered in 2021. **A.** Lower pitchers exhibiting the gross shape of *N. mirabilis* but with the lid and colouration of *N. rafflesiana*; **B.** The mature plant can produce lower pitchers exceeding 20 cm tall; **C.** An upper pitcher (20 cm tall); **D.** The inner margin of the peristome is serrated with fine teeth, a trait inherited from *N. rafflesiana*; **E.** Habit of *Nepenthes mirabilis* × *rafflesiana* (AHUFR) showing basal and upper stems with petiolated leaves; **F.** Unpollinated female inflorescence of *N. mirabilis* × *rafflesiana* AHUFR. Image credits: Amin Asyraf Tamizi, Salasiah Mohamad, and Siti Noratikah Mustafa.

accessions. This analysis revealed the formation of two well-supported *Nepenthes* clades (bootstrap value > 90 %). *Nepenthes sanguinea*, *N. malayensis*, *N. domiei*, *N. benstonei*, and *N. albomarginata* formed the highland (HL) and intermediate-highland (I-HL) clade, while *N. ampullaria*, *N. gracilis*, *N. mirabilis*, and *N. rafflesiana* formed the lowland (LL) clade. *Nepenthes mirabilis* × *rafflesiana* AHUFR was clustered together with *N. ×intermedia* (a hybrid of *N. rafflesiana* of an ambiguous lineage) under the same branch within the LL clade, indicating these two are closely related (Figure 3A). Despite being an interspecific hybrid, *N. mirabilis* × *rafflesiana* AHUFR (along with *N. ×intermedia*) emerged as a sister branch to *N. rafflesiana*, rather than *N. mirabilis*. Furthermore, the ITS sequence of *N. mirabilis* × *rafflesiana* AHUFR showed a much higher sequence similarity with that of *N. rafflesiana* with only two nucleotide mismatches found for the entire 516 bp length (Figure 3B). Whereas, there were four nucleotide mismatches and an indel on the ITS between the nothospecies and *N. mirabilis*.

The ITS1 secondary structures of *N. mirabilis* × *rafflesiana* AHUFR and its parental species were further studied. This was accomplished by transcribing the respective ITS1 nucleotide sequence into an RNA molecule, and we utilised several bioinformatics tools to predict and compute its folding structure. The ITS1 secondary structures of *N. mirabilis* × *rafflesiana* AHUFR and its parental species, generated with optimal minimum free energy (MFE), are illustrated in Figure 4. All of the ITS1 secondary structures consisted of two long and two short helices radiating from a large central loop. Conserved helix structures were observed between the ITS1 of *N. mirabilis* × *rafflesiana* AHUFR and that of *N. rafflesiana*. In contrast, the structure of ITS1 in *N. mirabilis* exhibited a different conformation on both Helix 1 and Helix III. Overall, the generated ITS1 structures displayed a common core.

Characteristics of pitcher fluids and prey composition

In this segment of the study, we conducted an analysis of the prey composition of *N. mirabilis* × *rafflesiana* AHUFR and related taxa. A total of 15 samples were examined, comprising upper and lower pitchers of the nothospecies, *N. mirabilis*, and *N. rafflesiana* (Table 3). It is noteworthy that, in all the studied *Nepenthes*, the pitcher fluids were strong to slightly acidic with levels ranging from pH 1.74 to 6.17 (Table 3). It is intriguing to note that several mature pitchers, including *N. mirabilis* × *rafflesiana* AHUFR upper pitcher 2, exhibited a diminished acidic pH, reaching 6.17.

In terms of prey composition, our result is in common with findings from previous studies reported by Moran (1999), Chin et al. (2014), and Marina et al. (2018). The pitchers trapped a wide range of arthropod prey. Overall, a total of 278 individuals of trapped fauna from six orders of arthropods were

Table 2. Top Peninsular Malaysian species search hits for ITS of *N. mirabilis* × *rafflesiana* AHUFR (query length = 512 bp) in Nucleotide BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sampling locality of *Nepenthes* from Peninsular Malaysia (PM) is indicated next to the taxon name; otherwise, isolate numbers are provided.

	Scientific Name (Isolate / Origin)	Total / Max Score	Query Cover (%)	Identity Simi- larity (%)	GenBank Ac- cession No.
1.	<i>Nepenthes</i> × <i>intermedia</i> (TR20)	933	100	99.61	HM204899
2.	<i>Nepenthes rafflesiana</i> (Mersing, PM)	928	100	99.41	JX042557
3.	<i>Nepenthes rafflesiana</i> (TR25)	922	100	99.22	HM204904
4.	<i>Nepenthes gracilis</i> (KRB27100902; Sabah; UKM Bangi, PM)	911	100	98.83	KP978789; AB675882; JX042555
5.	<i>Nepenthes mirabilis</i> (Rawang, PM)	902	100	97.46	JX042556

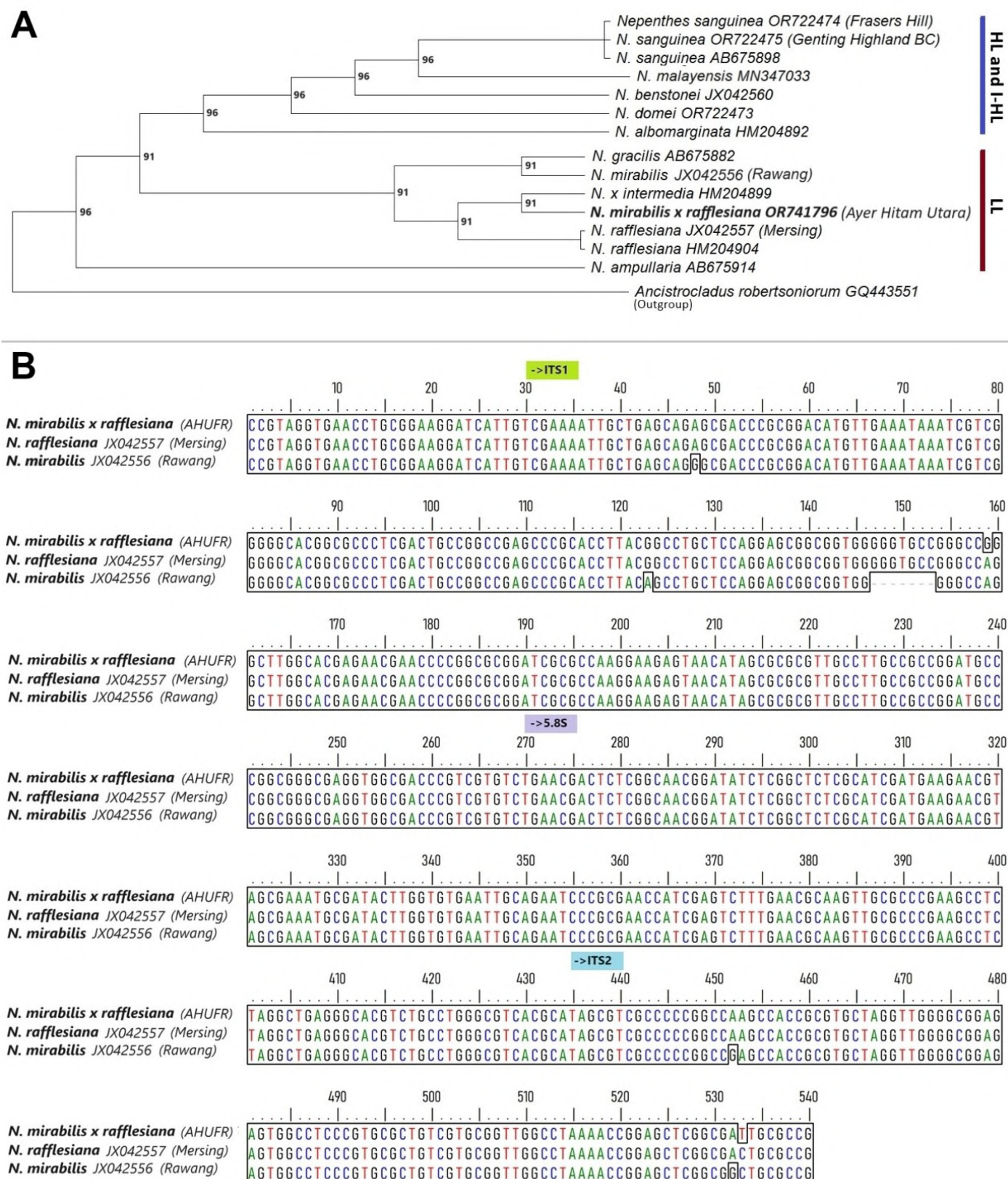


Figure 3. Phylogenetic analysis and alignment of ITS sequences. **A.** The tree displays the phylogenetic relationships of 14 *Nepenthes* taxa and accessions, including the studied taxon, and was inferred using Maximum Likelihood (ML) in MEGA11. Bootstrap values (%), based on 1000 replicates, are shown close to the nodes; **B.** Comparative ITS sequence alignment of *N. mirabilis* × *rafflesiana* AHUFR (OR741796) with its parental species, *N. mirabilis* (JX042556) and *N. rafflesiana* (JX042557). Non-consensus nucleotides are indicated by the outline, and the ITS1, 5.8S, and ITS2 boundaries are highlighted.

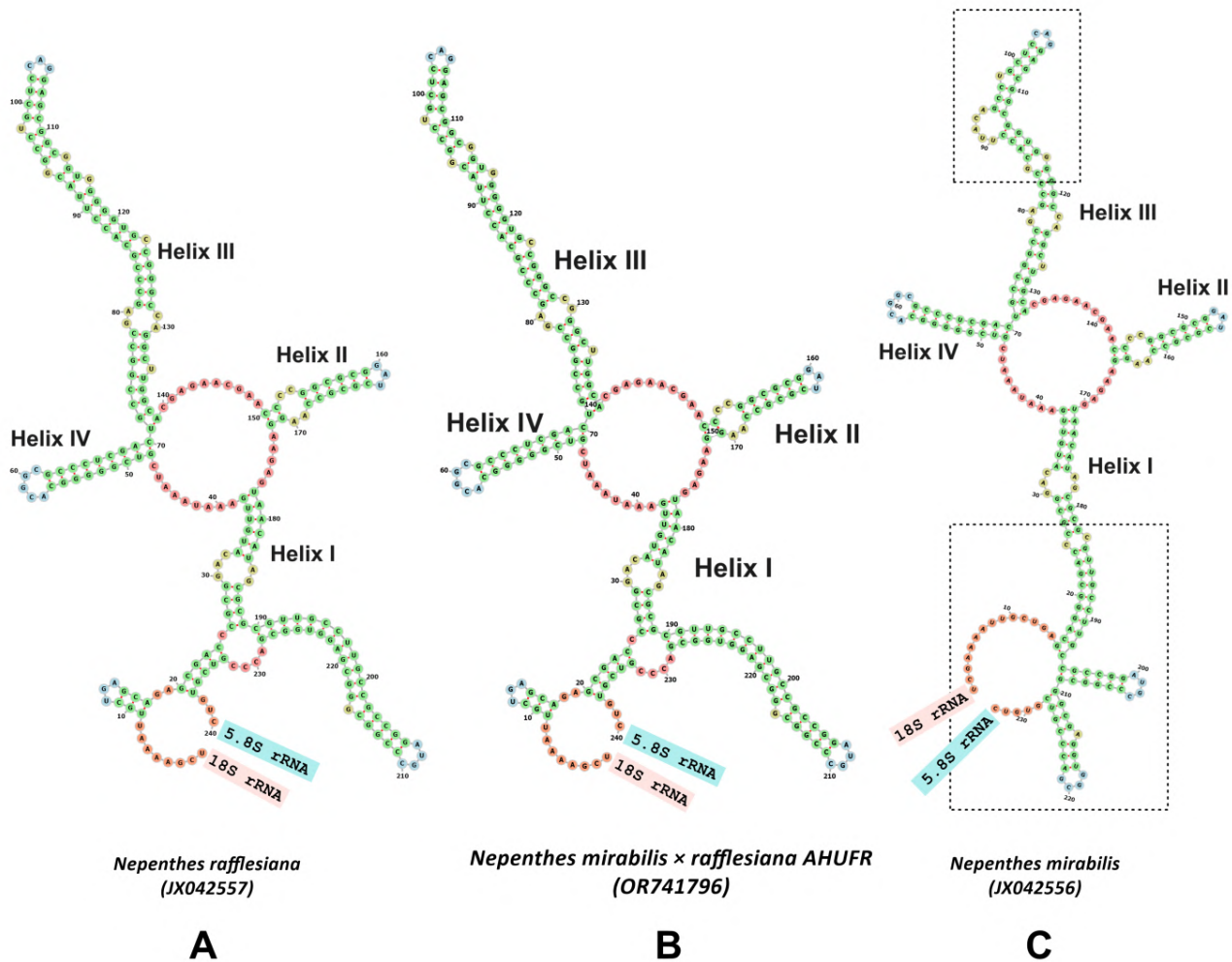


Figure 4. Inferred secondary structure models of the first internal transcribed spacer (ITS1) in 18S-5.8S pre-rRNA molecule for **A.** *Nepenthes rafflesiana* (JX042557), **B.** *N. mirabilis* × *rafflesiana* AHUFR (OR741796), and **C.** *N. mirabilis* (JX042556). The models were computed by an energy-minimisation approach using the RNAfold web server and final adjustments of folded structures were performed with forna tool. The dotted-line boxes indicate non-conserved fold structures of the ITS1 from *N. mirabilis* when compared with those of ITS1 from *N. mirabilis* × *rafflesiana* (AHUFR) and *N. rafflesiana*.

found in the traps of three taxa. Most of the arthropods are from the class Insecta. Table 3 shows the pitcher fluid pH values and Table 4 enlists the trapped prey of the three *Nepenthes* taxa. Insects from the order Hymenoptera recorded the highest number of individuals in all *Nepenthes* taxa. Several genera of ants identified in *N. mirabilis* × *rafflesiana* AHUFR were *Cerapachys* (raider ant), *Paraparatrechina*, *Polyrachis* (spiny ant), and *Crematogaster* (Saint Valentine ant). Living dipteran mosquito larvae from the genera *Tripteroides*, *Aedes* (tiger mosquito), *Malaya*, and *Toxorhynchites* (elephant mosquito) were also found in the pitchers of the studied nothospecies. Interestingly, the elephant mosquito larvae can be found in both *N. mirabilis* × *rafflesiana* AHUFR and *N. rafflesiana*. Meanwhile, mosquito larvae from the genus *Tripteroides* can be found in both *N. mirabilis* × *rafflesiana* AHUFR and *N. mirabilis*. Drowned spiders (Araneae) were only found in *N. mirabilis* × *rafflesiana* AHUFR. Most of the invertebrates found in pitcher plants are considered as prey except for the infauna from the order Diptera. The dipteran larvae were observed to be thriving and using the pitchers to obtain nutrients and as their habitat to complete the life cycle. Table 4 enlists the prey/infauna composition of the nothospecies, *N. mirabilis* × *rafflesiana* AHUFR, and its parental species, *N. mirabilis* and *N. rafflesiana*, from the study site.

Table 3. The pH values of pitcher fluid of the three *Nepenthes* taxa in AHUFR.

Taxa	Types of pitchers	Condition of the pitchers	pH values	pH average
<i>N. mirabilis</i> × <i>rafflesiana</i>	Upper pitcher 1	Opened	2.41	4.84 (acidic)
	Upper pitcher 2	Opened	6.17	
	Lower pitcher 1	Opened	5.73	
	Lower pitcher 2	Opened	5.92	
	Lower pitcher 3	Opened	3.95	
<i>N. mirabilis</i>	Upper pitcher 1	Opened	5.32	3.86 (acidic)
	Upper pitcher 2	Opened	4.25	
	Upper pitcher 3	Opened	1.74	
<i>N. rafflesiana</i>	Upper pitcher 1	Opened	2.32	3.98 (acidic)
	Upper pitcher 2	Opened	4.16	
	Upper pitcher 3	Opened	5.44	
	Upper pitcher 4	Unopened	3.40	
	Lower pitcher 1	Opened	4.19	
	Lower pitcher 2	Opened	4.79	
	Lower pitcher 3	Opened	3.55	

Table 4. Prey/infauna composition of the studied *Nepenthes* in AHUFR. *N. m* × *r* = *Nepenthes mirabilis* × *rafflesiana* AHUFR; *N. m* = *Nepenthes mirabilis*; *N. r* = *N. rafflesiana*.

Orders	Families	Genera	<i>N. m</i> × <i>r</i> upper pitchers	<i>N. m</i> × <i>r</i> lower pitchers	<i>N. m</i> upper pitchers	<i>N. r</i> upper pitchers
Hymenoptera	Formicidae	<i>Componotus</i>	-	-	-	✓
		<i>Meranoplus</i>	-	-	-	✓
		<i>Cerapachys</i>	✓	✓	-	-
		<i>Crematogaster</i>	✓	✓	✓	-
		<i>Dolichoderus</i>	✓	✓	-	-
		<i>Myrmecaria</i>	✓	✓	-	-
		<i>Paratrechina</i>	✓	✓	✓	-
		<i>Paraparatrechina</i>	✓	✓	-	-
		<i>Polyrachis</i>	✓	✓	-	-
Diptera	Culicidae	<i>Tripteroides</i>	✓	✓	✓	-
		<i>Aedes</i>	✓	✓	-	-
		<i>Malaya</i>	✓	✓	-	-
		<i>Toxorhynchites</i>	✓	✓	-	✓
	Corethrellidae	unidentified	-	-	✓	-
	Ceratopogonidae	<i>Dasyhelea</i>	-	-	✓	-
	unidentified	unidentified	-	✓	-	-
Araneae	unidentified	unidentified	-	✓	-	-
Orthoptera	unidentified	unidentified	-	✓	-	✓
Coleoptera	unidentified	unidentified	-	✓	✓	-
Hemiptera	Membracidae	unidentified	-	✓	-	-

Regenerative and rooting potential of cut shoots

In this part of the study, we successfully rooted and grew the primary shoot cutting excised from the original *in situ* plant of *N. mirabilis* × *rafflesiana* AHUFR into a mature plant bearing both lower and upper pitchers (Figure 5). Rooting was achieved within one month, with the first pitcher appearing approximately a month later. After a year, a second shoot cutting was excised from the primary clone, resulting in the successful production of a second clone. Subsequently, more clones were created. As of the writing of this article, 10 clones have been successfully produced using the vegetative propagation method, all with a 100 % recovery rate. Since these clones originated from a single upper stem, specifically the apical shoot, the initially produced traps were aerial (or upper) pitchers and had a consistent colouration similar

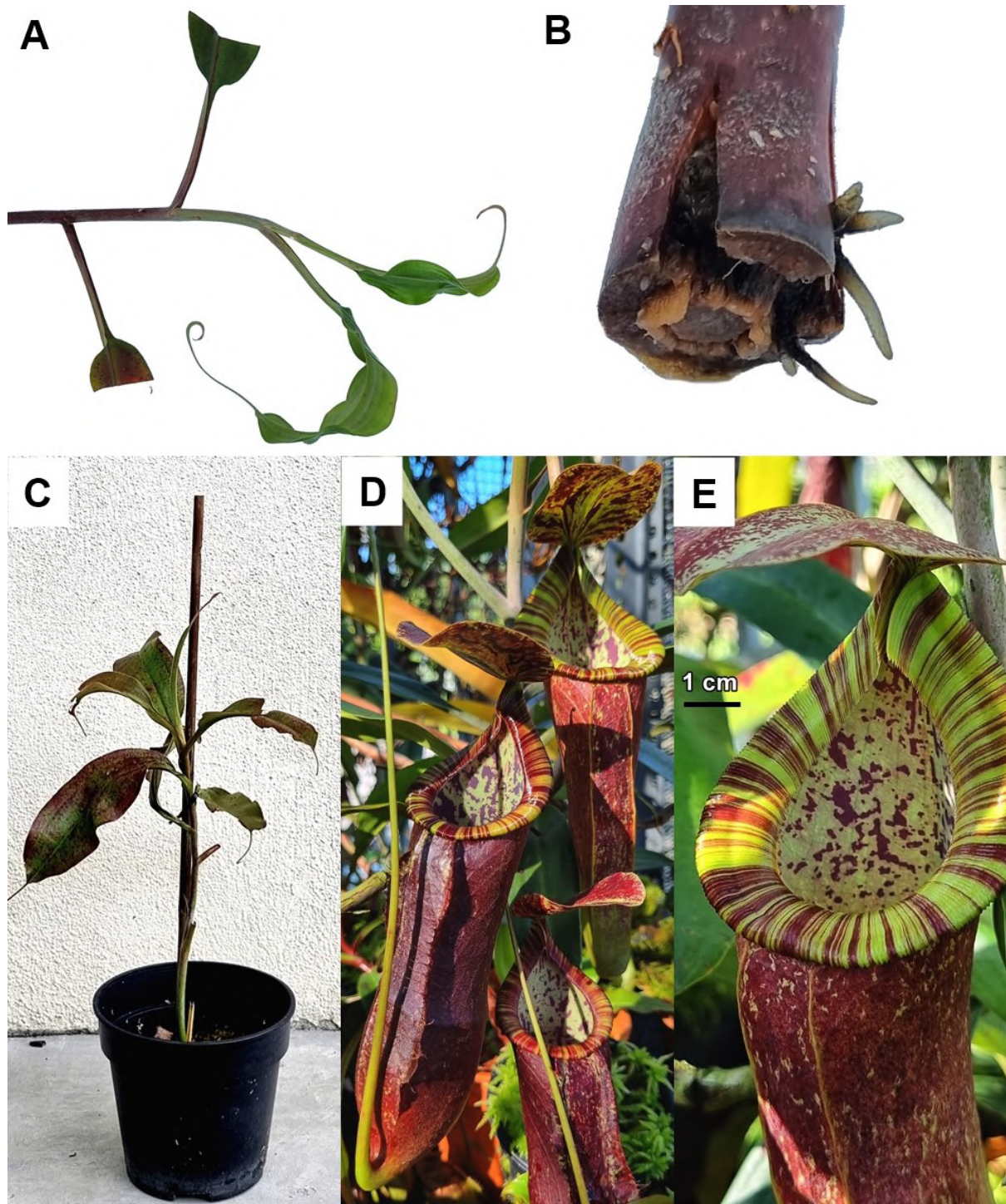


Figure 5. Shoot-based propagation of *N. mirabilis* × *rafflesiana* AHUFR. **A.** A shoot containing three internodes is harvested from the mother plant and prepared for rooting; **B.** Roots start to protrude from the bottom of the cut after a month; **C.** The new plant is transplanted to a bigger pot to encourage root and leaf formations; **D.** Two lower pitchers and one upper pitcher from the primary two-year-old clone; **E.** An upper pitcher exhibiting the flattened peristome typical of *N. mirabilis*, combined with the toothed and striped peristome features of *N. rafflesiana*. Image credits: Amin Asyraf Tamizi.

to that of the original mother plant in AHUFR. Currently, the primary clone, now two years old, has just started to produce a basal stem bearing lower pitchers (Figure 5D).

DISCUSSION

Nepenthes pitcher plants are gaining much interest in the scientific field, recognised for their potential as a source of novel compounds and as an inspiration for biomimetic applications (Miguel et al. 2018); hence, conservation and

documentation of the naturally occurring taxa become priorities for researchers and conservators. Drawing insights from a decade-long *in situ* observation, our study revealed that natural hybridisation and introgression of *Nepenthes* from Peninsular Malaysia can occur in both natural and anthropogenic habitats (Tamizi et al. 2020a, 2020b, 2023a). Currently, there are 19 published species of *Nepenthes*, with seven new species named between 2020 and 2023 (namely *N. berbulu*, *N. domei*, *N. latiffiana*, *N. limiana*, *N. malayensis*, *N. sericea*, and *N. ulukaliana*), recorded from Peninsular Malaysia (Tan et al. 2022; Golos et al. 2023; Lim et al. 2023; Tamizi et al. 2023b). Another taxon, provisionally labelled as *N. Pesonawangsa223* (Tamizi et al. 2023b), is regarded as a potential new species from the region. Meanwhile, the actual number of *Nepenthes* nothospecies occurring in the peninsula could be underestimated and only a handful of these have been described and assigned with binomial nomenclatures, indicating the lack of study (Masters 1881; Danser 1928; Clarke 2001; Adam & Hamid 2007; McPherson & Robinson 2012; Tamizi et al. 2020b). Clarke (2001) recorded a sum of natural hybrids (and putative hybrids) originating from Peninsular Malaysia; however, scientific names were not designated to these nothospecies. Recently, we encountered three undescribed nothospecies of *N. benstonei*—*N. ampullaria* × *benstonei*, *N. benstonei* × *gracilis*, and *N. benstonei* × *rafflesiana*—bringing the total to 17 nothospecies in Peninsular Malaysia (Tamizi et al. 2023a). Although hybrids have been deliberately produced by the horticultural community and collectors, these are, nonetheless, beyond the taxonomic interest.

Molecular taxonomy of *Nepenthes* remains a fascinating subject, especially when it comes to the phylogenetic inference of closely related taxa and nothospecies, which can be sometimes challenging to resolve solely through morphology (Murphy et al. 2020). Sequence variations in the ITS of fungi and eukaryotes have been widely reported, and researchers have leveraged these variations for taxon delimitation including over 20,000 plant species (Masaoka & Kobayashi 2005; Kaplan & Fehrer 2007; Hodač et al. 2014; Belyakov et al. 2022; Letsiou et al. 2024). Besides, the availability of over 250 ITS sequence accessions in the GenBank nucleotide database (<https://www.ncbi.nlm.nih.gov>) enhances the dataset's representation, enabling more accurate comparisons between query sequences and reference sequences. In a previous study, we differentiated *N. malayensis* from *N. sanguinea* and *N. domei* from *N. benstonei* using the ITS1-5.8S-ITS2 region, treating each of these species as distinct taxa (Tamizi et al. 2020a; Ghazalli et al. 2020). It is worth noting that the ITS sequence of *N. sanguinea* deposited by Alamsyah and Ito (2013) (GenBank accession no. AB675898) shows complete conservation (100 %) with the ITS sequences of *N. sanguinea* accessions (OR722474 and OR722475) from two distinct populations we sequenced in this study. A similar finding is observed for three distinct *N. gracilis* accessions (KP978789, AB675882, and JX042555) (Table 2), despite their different sources. Bunawan et al. (2017) utilised *trnL* and ITS sequences to deduce the phylogeny of several Peninsular Malaysian *Nepenthes* taxa, and the resulting tree generally supports a cladistic clustering of highland and lowland *Nepenthes*, similar to our finding (Figure 3A). We concluded that the ITS serves as a highly reliable DNA region for *Nepenthes* molecular classification, offering at least a confident species resolution. However, this work demonstrates its utility in deducing the phylogenetic affinity of the nothospecies. The ITS sequence search for *N. mirabilis* × *rafflesiana* AHUFR against the database enlisted *N. ×intermedia* and *N. rafflesiana* as the top taxa with the closest hit, followed by *N. gracilis* and *N. mirabilis*. *Nepenthes* × *intermedia* (syn. *N. ×dominii*) is a horticultural hybrid of an ambiguous parentage (Masters 1882; Bednar 1985; Riedel et al. 2007). According to the notes and illustrations provided by Masters (1882) and Bednar (1985), it is evident that *N. ×intermedia* is a derivative

of *N. rafflesiana*. Therefore, its high sequence similarity and close positioning with *N. mirabilis* × *rafflesiana* AHUFR on the phylogenetic tree are strongly justified. In brief, the parentage of *N. rafflesiana* in *N. mirabilis* × *rafflesiana* AHUFR is supported through molecular and phylogenetic analysis. Conversely, *N. mirabilis*, the other putative parent, consistently appeared lower than *N. gracilis* in the alignment list (Table 2). This case, however, raises the possibility of introgression from *N. gracilis* due to historical hybridisation events. It is important to note that such inference may not always hold true as it is imperative to complement the molecular data analysis with a thorough morphological examination for a more comprehensive assessment. Additionally, the ITS region being compared was only 512 bp long, and extending the coverage would provide much better resolution. In accordance with the brief morphological analysis of *N. mirabilis* × *rafflesiana* AHUFR, none of the observed characteristics indicated the introgression of any major traits from *N. gracilis* (decurrent leaf attachment, angular stem, reduced peristome, and rounded lid), which would otherwise be noticeable. Such traits, though slightly diluted, were previously observed in many *N. gracilis* nothospecies including *N. nothosp. Timur* (B_aliasiiL421) (*N. gracilis* × *benstonei*), *N. ×neglecta*, *N. ×trichocarpa*, and *N. ×setiuensis*. Henceforth, we strongly accept that *N. mirabilis* × *rafflesiana* AHUFR is strictly an interspecific hybrid of *N. mirabilis* and *N. rafflesiana*. The nothospecies displayed a strong resemblance to *N. mirabilis* especially on the gross shape of the pitchers, position of the hip close to the pitcher midsection, formation of fimbriate leaf margin, and the structure of the stem and leaves (petiolated). Meanwhile, the toothed peristome, relatively large pitcher size, glaucous stem and leathery leaves are the traits attributed to *N. rafflesiana*.

Based on the sequence alignment, the 5.8S and ITS2 regions were found to be highly conserved in *Nepenthes*; therefore, the ITS1 region was selected for secondary structure analysis to better deduce its taxonomic affiliation, due to its higher sequence variability. Nafisi et al. (2023) and Saidon et al. (2023) also reported that ITS1 exhibited a higher degree of accuracy and conveyed more phylogenetic information. Furthermore, the article by Saidon et al. (2023) is the only report on the use of ITS secondary structure to discriminate *Nepenthes* prior to this study. In line with phylogenetic inference, the ITS1 secondary structure of *N. mirabilis* × *rafflesiana* AHUFR highly resembled that of *N. rafflesiana*, with some variations observed in *N. mirabilis*. This observation further supports the close genetic affinity of the nothospecies with *N. rafflesiana*. Beyond plant systematics, the ITS1 sequence and its secondary structure have previously been employed to elucidate low-level phylogenetics in diverse organisms, including ciliates, dinoflagellates, fungi, insects, and animals (Wang et al. 2007; Hoshina 2010; Coleman 2013; Koetschan et al. 2014; Kumar et al. 2018; Thornhill & Lord 2010).

Our study on the pitchers of *N. mirabilis* × *rafflesiana* AHUFR and its parental species has revealed some interesting findings. Generally, *Nepenthes* pitcher acidity typically falls within the pH range of 2 to 6 (Takeuchi et al. 2011; Biseau et al. 2013; Gilbert et al. 2022). Our data from the three studied taxa align with this trend, with one exception: a pitcher from *N. mirabilis* reached an exceptionally acidic level, measuring as low as pH 1.74. The interior glands possess the ability to secrete acid-dependent digestive enzymes (chitinases and proteases) to digest prey and this is further aided by the presence of acidity-specialised bacteria, as well as mutualistic infauna, which break down large prey fragments, particularly insects, into smaller particles (Higashi et al. 1993; Biseau et al. 2013; Bazile et al. 2015). As mentioned earlier, the acidic pitcher fluid is conducive to digestive enzyme reactions, but the shift towards neutrality may be attributed to the depletion of digestive fluid and enzymes during prey digestion. Nevertheless, it is important to

acknowledge that the pH value of the pitcher fluid may also vary depending on the specific taxon, the accumulation of captured prey over time, the mixing with rainwater, and pH modification by pitcher infauna (Takeuchi et al. 2011). We then report the prey and infauna found in the pitchers of *N. mirabilis* × *rafflesiana* AHUFR. In terms of the prey spectrum of the studied nothospecies, the most abundant prey captured were mostly from the class Insecta. This finding is not surprising as insects are known to be the most attracted to pitcher plants due to their nectar secretion and pitcher colouration (Moran 1999). *Nepenthes mirabilis* × *rafflesiana* AHUFR was recorded to have an insatiable appetite for insects belonging to the order Hymenoptera, consistent to studies reported by Marina et al. (2018) and Setiawan et al. (2022). However, the frequency of prey organisms attracted to the pitchers depends on factors such as their abundance in the area, mobility and the presence of pull or push factors influencing their movement towards the pitchers. Research has suggested that the extrafloral nectar secreted by *N. rafflesiana* from the glands around the pitcher's mouth, along with the sweet fragrance produced, might act as attractants for its prey (Moran 1999)—similar may be true for its hybrid in question, *N. mirabilis* × *rafflesiana* AHUFR. In comparison with the other parental species, *N. mirabilis*, we observed the presence of ant prey from the genera *Crematogaster* and *Paratrechina* in the pitchers of *N. mirabilis* × *rafflesiana* AHUFR as well. In contrast, no overlapping insect orders were found between *N. rafflesiana* and the studied nothospecies. This lack of overlap may be attributed to various factors, including differences in pitcher colouration, nectar quality and quantity, as well as spatial considerations. There was no lepidopteran insect found in pitchers observed during this study, while records have shown insects of this order can be found in pitcher plants, although in lower numbers (Marina et al. 2018). This may be caused by their flight ability and habits, as they are more attracted to the flowers as compared to the extrafloral nectars produced by the pitchers. This study provides the first insight into the prey spectrum of *N. mirabilis* × *rafflesiana* growing in AHUFR.

In general, the invertebrates discovered in this study function as prey for *Nepenthes*, with some also serving as symbionts, notably the dipteran larvae (mosquitoes). The genus diversity of mosquito larvae in *N. mirabilis* × *rafflesiana* AHUFR exceeded that in its parental species, possibly due to its average pH being a bit closer to neutral. This heightened diversity is particularly noteworthy, especially considering that the larvae of most mosquito species are typically found in water within the pH range of 3.3 to 10.5, as mentioned by Nikookar et al. (2017). It is worth highlighting that the presence of larvae of an unidentified *Toxorhynchites* mosquito raises intriguing prospects for further investigation. This particular mosquito group is acknowledged as an important natural predator of other mosquitoes and has been observed residing within *Nepenthes* pitchers. (Tsukamoto 1989; Clarke 1998; Lim et al. 2019; Maquart et al. 2023). Although *N. mirabilis* × *rafflesiana* AHUFR exhibits genetic proximity to *N. rafflesiana*, the pitcher architecture and insect-trapping traits of this nothospecies align more closely with those of *N. mirabilis*. The results from this study may still be preliminary, more data can be obtained once more *N. mirabilis* × *rafflesiana* AHUFR individuals are discovered within the forest.

As noted earlier, the sole plant of *N. mirabilis* × *rafflesiana* AHUFR is female and this individual is considered a taxon of high conservation priority. Previously, Siti-Suhaila and Norwati (2021) micropropagated a horticultural hybrid, *Nepenthes* 'Viking' × 'Miranda', using a temporary immersion bioreactor system. The method took more than 16 months, from seed explant preparation to acclimatisation, to regenerate and establish new plantlets with approximately 10 cm long leaves. The reported success rate for this microprop-

agation was 70 %, which is considered high. Alternatively, the vegetative propagation (macropropagation) method that relies on cuttings from mature plants to create new individuals is deemed a much easier way to produce new clones; however, this approach remains underexplored for local *Nepenthes*. Sani et al. (2000) found it inefficient, reporting an overall rooting success rate of less than 50 % even with the use of rooting hormones. Additionally, Bahadur et al. (2007) briefly mentioned that the method is difficult. In a previous study, we conducted a preliminary test in using stem cuttings to vegetatively propagate *N. albomarginata*, *N. ampullaria*, *N. gracilis*, *N. limiana*, *N. mirabilis*, *N. rafflesiana*, *N. sanguinea*, *N. × neglecta* (*gracilis* × *mirabilis*), *N. × trichocarpa* (*ampullaria* × *gracilis*), and *N. benstonei* × *gracilis* (Tamizi et al. 2023a). It was determined that macropropagation using apical shoot cuttings are effective for generating new plants. Building upon this, we utilised shoot cuttings as the starting material to successfully generate multiple clones of *N. mirabilis* × *rafflesiana* AHUFR. Both of our previous and current studies have proven that producing new *Nepenthes* plants from stem cuttings, particularly shoots, is simpler than previously thought.

CONCLUSION

This study aimed to examine the genetic background and further affirming the taxonomic position of the sole living *N. mirabilis* × *rafflesiana* from Johor's last peat swamp forest. The ITS DNA barcode deposited in the GenBank® database will serve as a crucial reference for future population authentication and surveys. We also emphasise the reliability of the ITS region as a robust DNA barcode for determining species limitations in future studies. While there could be more individuals of *N. mirabilis* × *rafflesiana* AHUFR yet to be discovered, the studied specimen has been listed as one of the Johor's iconic plant taxa entitled for conservation priority. The insight into prey preferences gleaned from our data provides a fundamental understanding of the ecological requirements of local *Nepenthes*, while the development of a feasible vegetative propagation method instils optimism for potential future reintroduction programmes aimed at preserving this remarkable nothospecies. Further investigations are needed to discover various aspects of this natural hybrid, encompassing genomic studies, assessments of fecundity, analyses of phytochemical composition, exploration of ecological interactions, and extensive population surveys. These future studies will provide invaluable information and contribute to the conservation and understanding of *N. mirabilis* × *rafflesiana* AHUFR.

AUTHORS' CONTRIBUTIONS

A.A.T., S.M., and A.A.A.R. contributed to the conception and design of the study. All authors (A.A.T., S.M., A.A.A.R., S.N.M., M.S.H., and M.Z.Z.) were involved in material preparations and data collecting process. A.A.T., S.M., and A.A.A.R. conducted the data analyses and prepared the initial draft of the manuscript. Subsequently, all authors provided comments on later versions of the manuscript. The final version was read and approved by all authors.

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CONFLICT OF INTEREST

The authors have no competing interests to declare.

DATA AVAILABILITY

All data generated, accessed, or analysed during this study are included in this published article. The ITS nucleotide sequences have been deposited in GenBank® (www.ncbi.nlm.nih.gov/genbank/).

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES

During the preparation of this work, ChatGPT (a language model developed by OpenAI) was strictly employed to enhance language and readability. Subsequently, the authors thoroughly reviewed and edited the content as necessary, taking full responsibility for the publication's content.

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Research Article

Bioremediation and Detoxification of the Textile Dye Methyl Blue by *Trichoderma asperellum* LBKURCC1 with Laccase Activity

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ABSTRACT

Textile production generates effluent contaminated by 10-15 % dyes used. Fungal-mediated remediation presents an ecologically acceptable and inexpensive method for treating dye effluents. *Trichoderma asperellum* LBKURCC1 isolated from Riau cacao rhizosphere produces laccase. Laccase can catalyse the degradation of several textile dyes. This study aims to investigate *T. asperellum* LBKURCC1 ability to bioremediate and to detoxify the textile triphenylmethane dye, methyl blue (MB). *T. asperellum* LBKURCC1 cultures in minimal media with pHs of 4.5, 5.5 and 6.5 were incubated (24 hours, room temperature) with 50-ppm MB solutions. MB decolorisation analysis was conducted by examining the UV-Vis absorption spectra of MB after 24 hours treatment. It was also determined if the degradation was contributed by enzymatic biodegradation or by biosorption processes. The contribution of laccase activity to the bioremediation process was assessed by monitoring laccase activity in fungal treated MB solution. Acute toxicity of MB to *Artemia salina* larvae was determined pre- and post-fungal treatment. The results showed that decolorisation of MB by *T. asperellum* LBKURCC1 occurred at all tested pH levels, but at different rates for different pH. The highest decolorisation rate was at pH 4.5 (85 % per 24 hours). Enzymatic biodegradation was the higher contributor to the decolorisation, compared to mycelia biosorption. Laccase activity was induced by MB and the highest activity was found at pH 4.5. MB toxicity to *A. salina* larvae was eliminated by the fungal biodegradation. This study confirms the potential of *T. asperellum* LBKURCC1 life cultures in the degradation of MB, eliminating its toxicity.

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INTRODUCTION

During textile dyeing, an average of 10–15 % of the dye goes to waste effluents (Juárez-Hernández et al. 2021; Boer et al. 2004). Synthetic dyes present in textile effluent pose a hazardous environmental threat. Water quality is degraded by dyes in the aquatic ecosystems. Dyes reduce the penetration of sunlight, thus hampering photosynthetic activity, and generation of oxygen. Synthetic dyes also increase the Chemical Oxygen Demand (COD) for degradation of these dyes, causing further reduction of oxygen availability for aquatic lives (Plácido et al. 2016). Members from the triphenylmethane family of synthetic dyes are also known to be mutagenic and difficult to degrade naturally (Sinha & Hanamghar 2019). A triphenylmethane dye widely used in the textile industry is methyl blue (MB) (Figure 1), also known as cotton blue, acid blue 93 or Helvetia blue, having the chemical formula $C_{37}H_{27}N_3Na_2O_9S_3$ (Singh et al. 2020). Due to the negative impacts on the environment, waste effluents containing methyl blue, or other triphenylmethane dyes must be removed or degraded before being released into natural waters (Dahlena et al. 2022).

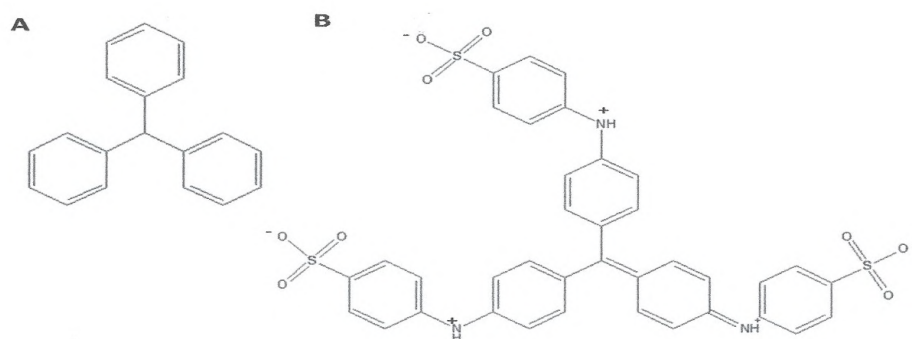


Figure 1. A) General structure of triphenylmethane dyes. B) Structure of methyl blue

Various chemical and physical methods have been developed to remove or to degrade synthetic textile dyes from effluents, among others adsorption methods (Eteba et al. 2023; Nadew et al. 2023; Procópio et al. 2023), chemical methods (Sandani et al. 2021), photocatalysis (Siraj et al. 2021), electrocoagulation (Mutambyi et al. 2023) and Fenton oxidation (Drăgoi et al. 2021). However, several methods are costly and can create secondary pollutants, such as sludge, which would require additional treatment (Sar et al. 2024). An ecologically acceptable and relatively inexpensive alternative for textile dye removal, decolourisation and/or degradation is using biological methods, employing life cultures of microorganisms, or enzymatic treatments (Shanmugam et al. 2017; Al-Tohamy et al. 2022; Kumar et al. 2024). Various fungal strains can decolorise textile dyes through enzymatic biodegradation and biosorption by fungal mycelia. *Aspergillus flavus* mycelia could decolorize 77 % of a 200 mg L⁻¹ solution of the textile dye acid black 52 in 50 hours (Ghosh et al. 2017), and 100 % of a triphenylmethane dye malachite green (MG) solution (150 mg L⁻¹) within 6–8 days (Barapatre et al. 2017). Several laccase producing species from the genus *Trichoderma* have been reported to degrade synthetic textile dyes. A marine isolated *Trichoderma harzianum* strain TSK8 could degrade 89 % of MG in ten days (Saravanakumar & Kathiresan 2014). Live culture mycelia of *Trichoderma viride* and *Trichoderma virens* could decolorize 75 % to 93 % of 50 ppm MG and an azo dye congo red in 24 hours (Argumedo-Delira et al. 2021). A rapid growing species of *Trichoderma*, *T. tomentosum* could decolorise 99 % textile effluents containing 85.5 ppm of the azo-dye acid red 3R in three days (He et al. 2018). Other examples

are the bioremediation of the dyes methylene blue, malachite green and safranine by *Aspergillus terreus* YESM 3 (Mohammed & Mabrouk 2020), and direct black 22 by *Penicillium chrisogenum* ERK1 (Lanfrancioni et al. 2022). The bioremediation of these dyes are closely linked to production and activity of ligninolytic enzymes such as manganese peroxidase (EC 1.11.1.13) (Ali et al. 2022), lignin peroxidase (EC 1.11.1.14) and laccase (EC 1.10.3.2) (Chatha et al. 2017; Arora et al. 2022). These enzymes catalyse oxidation reactions of toxic dyes, degrading them into less harmful compounds (Asses et al. 2018; Adenan et al. 2022; Anita et al. 2022).

Trichoderma asperellum strain BPLMBT1 has been reported to produce laccase, which when purified, effectively degrade the recalcitrant triphenylmethane dye crystal violet (Shanmugam et al. 2017). *Trichoderma asperellum* LBKURCC1 is a biocontrol strain, isolated from healthy cacao tree rhizosphere soil in a plantation located at Pekanbaru city area, Riau, Island of Sumatra, Indonesia (Nugroho et al. 2018). We then asked the question whether our Indonesian endogenous strain could also produce laccase and be developed for bioremediation purposes. In a previous study, we observed that the mycelia of this filamentous ascomycetes fungi effectively decolorise the azo dye direct violet 51 (Nugroho et al. 2018). Additionally, we found that *T. asperellum* LBKURCC1 produces laccase when cultured in a rice stalk solid-state fermentation medium (Rahayu et al. 2019; Sellyna et al. 2020). The crude laccase extracted from this solid-state fermentation medium demonstrated the ability to degrade methylblue (Dahlana et al. 2022). Based on these findings, *T. asperellum* LBKURCC1 life cultures emerge as promising candidates for direct application in bioremediation of textile effluents containing methyl blue dye. Using life cultures directly offers advantages over isolated enzymes, eliminating the need for fermentation production and enzyme extraction, which can be costly. The use of fungal life cultures in bioremediation can be further optimised by employing immobilised cells (Couto 2009). However, before employing these life cultures, it is essential to assess whether the dye can induce extracellular laccase production, and the effectiveness of using life cultures in the bioremediation process with various types of dyes. Therefore, the objective of this research was to determine the efficacy of *T. asperellum* LBKURCC1 life cultures in decolorising a methyl blue solution, and to evaluate the dye's ability to induce extracellular laccase production, thereby accelerating the specific dye degradation and dye removal from the environment. Since some dye degradation products are still toxic or can cause acute toxicity in aquatic animals, in this study we also evaluated the toxicity of methyl blue before and after treatment with *T. asperellum* LBKURCC1 life cultures.

MATERIALS AND METHODS

Materials

Microorganism

The fungal strain *Trichoderma asperellum* LBKURCC1 was obtained from the Universitas Riau Biochemistry Laboratory Culture Collection facility in Pekanbaru, Indonesia, where it is maintained on PDA slants, supplemented with 0.005 % citric acid (Nugroho et al. 2023). Five days old, rejuvenated cultures on agar slants were used for bioremediation experiments, as described in the appropriate method subsection. This fungal strain was originally isolated from the rhizosphere of healthy cacao plants in a cacao plantation in Kecamatan Rumbai, Pekanbaru, Riau Province, Sumatera, Indonesia (Nugroho et al. 2018). The species identity of the strain was confirmed by phylogenetic analysis using ITS (Internal Transcribe Spacer region of the ribosomal RNA) and *tef1* sequences. GenBank Accession numbers for these sequences are KY203853 (ITS) and KY213959 (*tef1*) (Nugroho et al. 2018).

Chemicals and consumables

Methyl blue or MB (also known as acid blue 93; cotton blue; water blue) was from Merck Sigma-Aldrich Co. (CAS No. 28983-56-4; MW 799.8). Glass fiber circles GF/C for filtering and separation of mycelia from culture media was from Whatman™ with the pore size of 1.2 µm (Cat. No. 6780-2504). For culture media chemicals were biochemical grade: Ammonium sulfate ((NH₄)₂SO₄), magnesium sulfate (MgSO₄), Potassium dihydrogen phosphate (KH₂PO₄), glucose, yeast extract, potato, dextrose, Tween-20, Copper sulfate heptahydrate (CuSO₄·7H₂O). Chlortetracycline was pharmaceutical grade. Other compounds employed in this study were analytical grade.

Methods

Bioremediation of the textile dye methyl blue by fungal life culture experiments

Trichoderma asperellum LBKURCC1 spores were obtained from five days old, rejuvenated agar slants, by suspending in sterile water, and filtered using sterile glass wool. Spore concentration was determined using a table previously made to correlate O.D. 660 nm to life spores (Sawitri 2010). Approximately 9 x 10¹² spores were spread on PDA petri dishes and allowed to grow into lawns by incubation at room temperature (± 30 °C), for three days. Two plugs (1 cm in diameter) were aseptically plugged from the mycelium lawn and inoculated into 30 mL mineral media in each 100 mL flask. Mineral media (MM) for the experiments had the following composition for each 1L media: 1 g (NH₄)₂SO₄, 2 g MgSO₄, 0.6 g KH₂PO₄, 22.3 g yeast extract, 1 g Tween-20, 0.046 g CuSO₄·7H₂O (Nugroho et al. 2023). The MM media were prepared in appropriate buffers for experiment at pH 4.5, 5.5 and 6.5. These flasks were assigned as fungal methyl blue bioremediation test flasks. The flasks were incubated for two days at room temperature (± 30 °C) with shaking at 120 rpm. At this point the logarithmic phase of fungal growth had been reached, and 750 µL of 0.002 g mL⁻¹ methyl blue were added to each test flask, bringing the concentration of methyl blue to 50 ppm, following dye decolourisation experiments described for textile dyes of the azo, triphenylmethane and anthraquinone group of dyes (Adnan et al. 2017; Argumedo-Delira et al. 2021; Ben Ali et al. 2020; Nugroho et al. 2023). At such a concentration, methyl blue has an average absorbance at its visible light peak (594 nm), of 1 to 1.5 (depending on pH of the solution). Methyl blue was added together with 150 µL (5 g L⁻¹) chlortetracycline solution to prevent bacterial growth. The test flasks were then further incubated at room temperature (± 30 °C) with shaking at 120 rpm. For spectrophotometric and enzyme activity analysis, from each test flask aliquots were taken at different time points within a period of 24 hours. These aliquots were then centrifuged 10 minutes at 13,000 rpm, to separate any debris and fungal spores that can interfere with the supernatant spectrophotometric assays (Nugroho et al. 2023). Spectrophotometric analyses were done using a UV-Vis Agilent Cary 60 spectrophotometer, that measured the absorbance of the solution at 320 nm to 800 nm. Light photography of supernatant aliquots was taken for colour documentation and visual comparison between samples. Abiotic control methyl blue flasks were methyl blue dye in MM media without adding fungal plugs. For brevity these abiotic control flasks will further be referred to as controls. Controls had the same treatment as the fungal bioremediation test flasks. MM media alone served as blanks for the spectrophotometric assays. All were conducted in triplicate experiments.

Biosorption of methyl blue by fungal mycelia assay

To determine the quantity of the methyl blue dye that was absorbed by the

fungal mycelia after 24 hours of incubation in the test flasks, mycelia were filtered under vacuum with GF/C glass fibre filters, following methods described previously for the study of reactive black 5 bioremediation by life cultures of *Trichoderma asperellum* LBKURCC1 (Nugroho et al. 2023). To extract any of the dye absorbed by the fungal biomass, 30 mL of 1 M NaOH pH 8 was added to the collected mycelia in a flask and shaken for 7 hours at room temperature ($\pm 30^\circ\text{C}$). The NaOH extract was separated from the mycelia by centrifugation, and absorbance of the separated filtrate was measured at a wavelength of 320–800 nm. Mineral media alone served as blanks for the spectrophotometric assays.

Quantitative analysis of bioremediation, enzymatic biodegradation, and biosorption

Percentages of bioremediation (R), biosorption (S), and biodegradation by enzymes produced by life fungal cultures (D) were calculated using the equations (1), (2) and (3), as described previously by Adnan et al. (2017) and Nugroho et al. (2023), with slight modification, that is the area under peaks were calculated from 530 nm to 670 nm, as characteristics of methyl blue visible light absorption peaks.

$$R (\%) = \frac{A_c - A_t}{A_c} \times 100 \quad (1)$$

R: bioremediation percentages (%). A_c : area under the absorption spectrum curve of control flasks starting at the wavelength where the differences between controls and fungal bioremediation tests were observed. A_t : area under the absorption spectrum curve of fungal bioremediation test flasks at the same wavelength used to measure A_c (Nugroho et al. 2023).

$$S (\%) = \frac{A_s}{A_c} \times 100 \quad (2)$$

S: biosorption percentages (%). A_s : area under the absorption spectrum curve of NaOH extract supernatant at the same wavelength used to measure A_c .

$$D (\%) = R - S \quad (3)$$

D: enzymatic biodegradation percentages (%). R: total dye bioremediation is achieved through both absorption (S) and enzymatic degradation (D).

Determination of laccase activity

Enzyme activity assay was investigated to determine laccase activity during degradation. It was performed using the supernatant obtained from the culture media and measured at the indicated time points. Laccase activity was determined using a microplate reader (Berthold Technology TriStar LB 941, Germany), with ABTS as a substrate for laccase, in which the change of oxidized ABTS absorbance at 405 nm as a function of time was measured. The enzymatic reaction mixture contained 180 μL sodium acetate buffer (0.05M, pH 5.5), 10 μL of 5 mM ABTS and 10 μL of enzyme, and the reaction mixture was incubated at 30°C for 5 minutes. The enzyme activity was calculated based on the extinction coefficient for oxidized ABTS at 405 nm (ϵ_{405}), that is $36.8 \text{ mM}^{-1}\text{cm}^{-1}$ (Wang & Subramani 2017). The quantity of enzyme required for the oxidation of one μmol of ABTS per minute (U L^{-1}) was defined as one unit of laccase activity.

Artemia salina toxicity tests

Acute toxicity tests were performed by adding the treated dye to *Artemia salina* (brine shrimp) nauplii. This Brine Shrimp Lethality Test (BSLT) is often used to test toxicity of dyes that are present in dye effluents, as a rapid and general screening of toxic compounds (Sani et al. 2018; Saravanakumar & Kathiresan 2014). Solutions of methyl blue (MB) dye in mineral media (MM)

treated with *T. asperellum* LBKURCC1 life cultures for 24 hours from the methyl blue bioremediation experiments (section 2.2.1), were added to vials containing freshly hatched nauplii (Instar I) in reconstituted sea water initially at varying concentrations. Blanks consisting only of MM media were also tested. Due to high salts in the MM media, 100 % of nauplii died in 24 hours when the concentration of blanks was higher than 0.4 % (v v⁻¹). Therefore, to test the toxicity of the MB before (undegraded) and after bioremediation (degraded), concentrations of 0.2 and 0.4 % (v v⁻¹) from the bioremediation test were used for BSLT. The treated nauplii were then incubated for 24 hours. After 24 hours, the number of still living and the number of dead larvae were each counted (Latha et al. 2007). The survival rate in percentages were calculated as percentages of life larvae to total number of larvae in each experiment. MB in MM treated with *T. asperellum* LBKURCC1 is denoted as “Degraded MB”. The same experiments were done using MB in MM media denoted as “Undegraded MB” and likewise done with only MM media denoted as “Blanks”. All toxicity tests were repeated 5 (five) times.

RESULTS AND DISCUSSION

Methyl blue or with the trade name cotton blue, Helvetia blue or acid blue 93, is a member of the triphenylmethane group of dyes (Dahlen et al. 2022). The complex aromatic structure of triphenylmethane dyes make them difficult to degrade, so that they can easily accumulate in the natural environment, causing toxic effects on living organisms (Taharchaouche et al. 2023). The employment of fungi as bioremediation agents for biosorption and biodegradation of dyes is the appropriate choice as this method is ecologically acceptable, relatively inexpensive, and easy to implement (Munck et al. 2018). In this study we have investigated the ability of *T. asperellum* LBKURCC1 life cultures to de-colorize methyl blue.

Decolourisation of methyl blue by *T. asperellum* LBKURCC1 life cultures

Figure 2 illustrates the results of a study on methyl blue dye decolourisation using live *T. asperellum* LBKURCC1 mycelia after 24 hours at pH 4.5, 5.5, and 6.5. Decolourisation was compared among mineral media (MM) blanks, abiotic controls (MM with added dye only), and test reactions (MM with added dye and live mycelia). For brevity the abiotic controls will be referred to as controls. At pH 4.5, decolourisation occurred at a higher rate, evident from the disappearance of the blue color in the test reactions after 6 hours of incubation. The greenish and dark yellow color observed after 6 and 24 hours of incubation in the test reactions at pH 4.5 is due to the color of the MM media, which is yellow. Compared to controls, there could be seen mark differences in color at pH 4.5 after 6 hours incubation. At pH 5.5, the blue color disappeared after 24 hours of incubation in the test reactions, and mark difference between the test and control color could be observed. However, no visual difference could be observed by the naked eye at pH 6.5 in test reactions after 24 hours incubation, compared to time zero. There was also no visible difference at pH 6.5 that could be observed between the test reaction and the controls color from time zero, until 24 hours incubation. This is because at times zero of pH 6.5, methyl blue has already been partially oxidised and therefore changed colour from aqua blue to a greenish hue. It is therefore difficult to the naked eye to distinguish between controls and test reaction at pH 6.5.

To confirm the visual results, a more quantitative analysis of methyl blue decolorization by *T. asperellum* LBKURCC1 mycelia was conducted by examining the UV-Vis absorption spectra of methyl blue after 24 hours of treatment with the fungus at room temperature and compared to the controls. The absorption spectra (Figure 3) demonstrate that decolourisation of methyl

blue by *T. asperellum* LBKURCC1 life cultures occurred at all tested pH levels, but at different rates for different pH. This is evident from the differences in spectrum profiles of methyl blue at pH 4.5, 5.5, and 6.5 between controls (blue lines) and *T. asperellum* LBKURCC1 treated test solutions of the dye (orange lines). The absorption maximum wavelength of methyl blue at 594 nm, observed in controls, disappeared in the test solutions, indicating that the fungal life cultures successfully decolourised (bioremediated) methyl blue. The rate at which this 594 nm peak disappeared was higher at pH 4.5 compared to the other pHs.

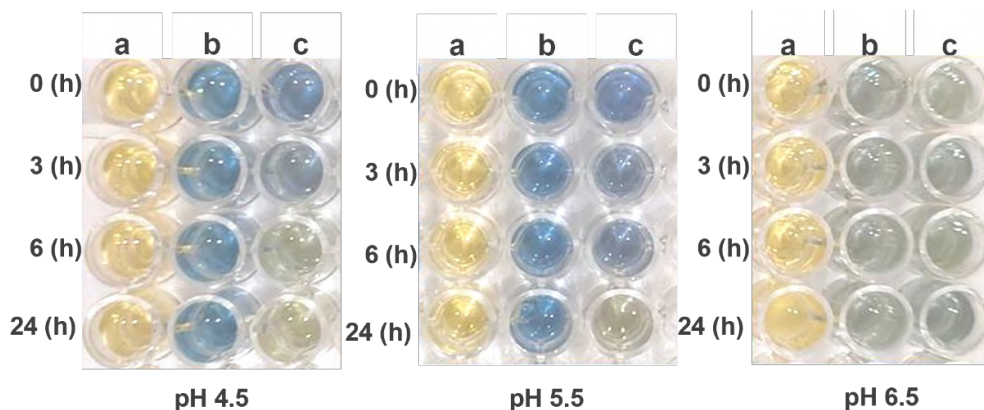


Figure 2. Visual colour results of methyl blue decolourisation at room temperature by *T. asperellum* LBKURCC1 at the indicated time points and pH. a = MM blanks; b = controls (methyl blue in MM); c = test reactions (methyl blue in MM with added *T. asperellum* LBKURCC1 live cells).

Bioremediation, biosorption and enzymatic biodegradation of methyl blue by *T. asperellum* LBKURCC1 live cultures

Dye bioremediation by fungal life cultures may result from biosorption by fungal mycelia or enzymatic biodegradation through secreted enzymes. To assess the contribution of biosorption and enzymatic biodegradation to methyl blue bioremediation by *T. asperellum* LBKURCC1 life cultures, we determined the bioremediation, biosorption and enzymatic biodegradation percentage after 24 hours of incubating the dye with fungal cells, as described in the Materials and Methods section.

Table 1 presents the percentages of methyl blue bioremediation, biosorption, and enzymatic biodegradation after 24 hours of incubation with *T. asperellum* LBKURCC1 life cultures. The highest bioremediation achieved after 24 hours was at pH 4.5, reaching an average of 85 % decolourisation. At pH 5.5, bioremediation did not reached half of that observed at pH 4.5 and was not significantly higher than that at pH 6.5. The results also indicated a minimal contribution of biosorption to the decolourisation process. It can be deduced from these results that more than 99 % of methyl blue bioremediation by *T. asperellum* LBKURCC1 cells was attributed to enzymatic biodegradation. Enzymatic biodegradation was preferably at pH 4.5 compared to the other pHs, which may reflect on the enzyme kinetic properties, and the fungal growth robustness.

Bioremediation in the decolourisation of a dye gives the total dye removal due to biosorption by cells or mycelia, and biodegradation due to enzymatic catalysed reactions, such as oxidation of the dye by laccase. A study conducted by (Arslantaş et al. 2022) investigated the decolourisation of basic red 18 using various fungal species, primarily through biosorption. When a high amount of dye removal is due to biosorption, this causes another problem because the fungal mycelia would have to be disposed and cannot be re-used. In our study, the biosorption of methyl blue by *T. asperellum*

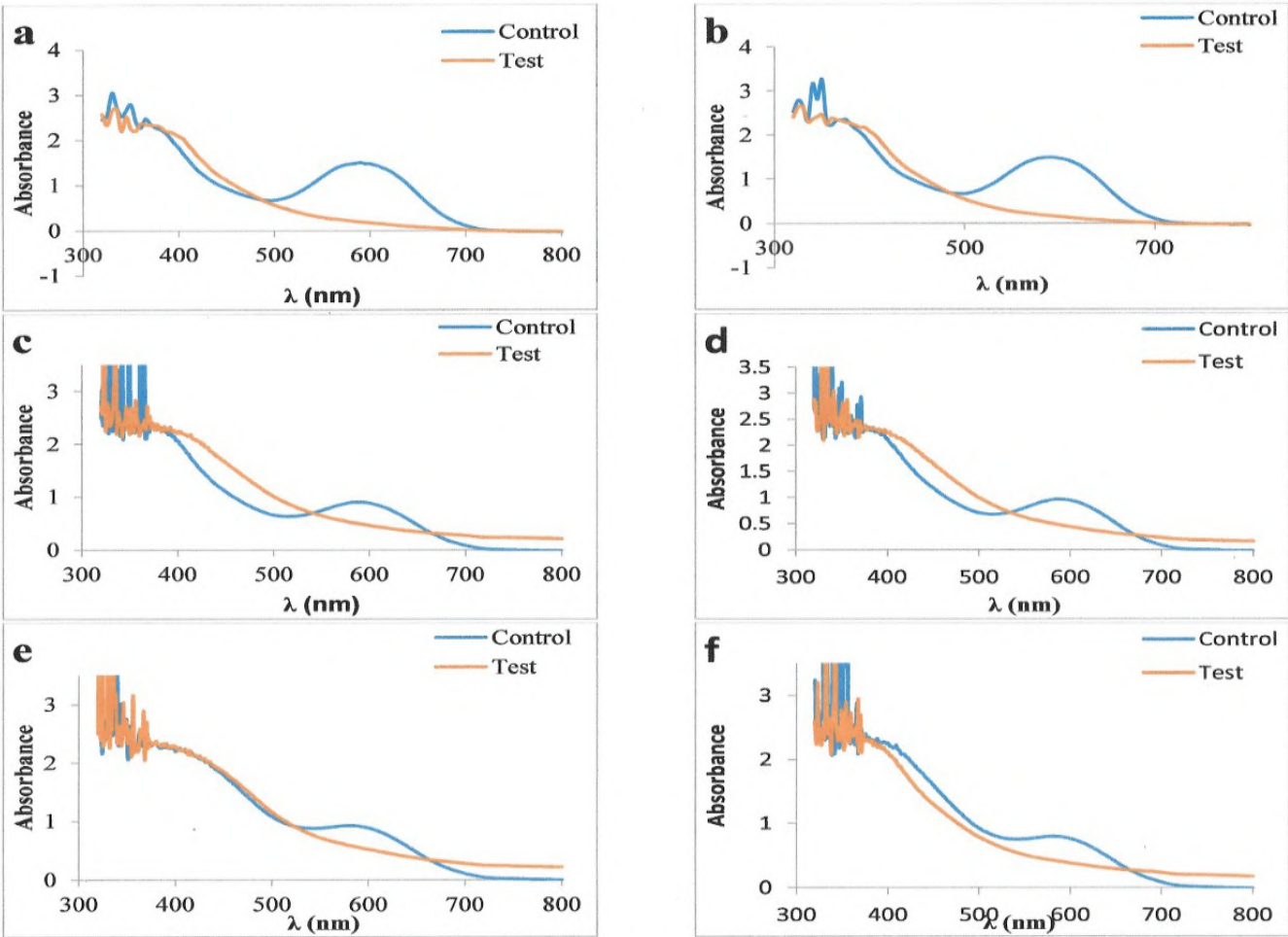


Figure 3. Duplicate results of UV-Vis scanning spectra of methyl blue after 24 hours treatment with *Trichoderma asperellum* LBKURCC1 cell, incubated at room temperature, and pH 4.5 (panels a & b); pH 5.5 (panels c & d); or pH 6.5 (panels e & f). Blue solid lines = Control solution of methyl blue in MM media. Orange solid lines = Test solution of methyl blue in MM media treated with *T. asperellum* LBKURCC1 life cultures.

Table 1. Average percentage values for bioremediation (R), biosorption (S), and biodegradation (D) of methyl blue by *T. asperellum* LBKURCC1 live cultures at various media pH, after 24 hours incubation†

pH	Bioremediation (R%)	Biosorption (S%)	Biodegradation (D%)
4.5	85 ± 1 (a)	0.2 ± 0.01 (b)	85 ± 1 (a)
5.5	36 ± 2 (b)	0.4 ± 0.01 (a)	35 ± 2 (b)
6.5	33 ± 10 (b)	0.0 ± 0.00 (c)	33 ± 10 (b)

†Average of triplicate experiments followed by standard deviation (SD) values. The mean value followed by the same letter in parentheses in a column is not statistically different according to the Duncan Multiple Range Test at $p > 0.05$ significance level.

LBKURCC1 was found to be minimal, as indicated in Table 1. Instead, biodegradation mediated by enzymatic activity played a predominant role in the decolourisation and bioremediation of methyl blue by *T. asperellum* LBKURCC1 cells. Since only a small amount of methyl blue is adsorbed by *T. asperellum* LBKURCC1 mycelia, the fungal mycelia can be effectively reused for the continuous biodegradation of methyl blue dye, particularly in an immobilized cell format.

Induction of *T. asperellum* LBKURCC1 laccase activity by methyl blue
A potential enzyme involved in the oxidation of methyl blue during the decol-

ourising process is the oxido-reductase laccase. To assess if *T. asperellum* LBKURCC1 excretes laccase into the media upon incubation with methyl blue, we measured laccase activity every hour for the first six hours and at 24 hours from the initiation of adding methyl blue to the fungal cultures in the MM media. Laccase activity at time zero was negligible at pH 4.5 but was detected at low activity in cultures grown at pH 5.5 and pH 6.5. The results (Figure 4) indicate that methyl blue induces production of laccase by the fungi at all pH tested, with a rise in activity at 1 hour after methyl blue addition to the fungal cultures in MM media. During the initial two hours after the addition of methyl blue, laccase production, as indicated by its activity, was highest at pH 4.5, followed by pH 5.5. Laccase activity at pH 4.5 experienced a slight decrease at 3 hours but then steadily increased, reaching 87 U mL⁻¹ at 6 hours, and continuing to rise to 118 U mL⁻¹ at 24 hours. Laccase activity at pH 5.5 peaked at 4 hours, reaching a maximum of 77 U mL⁻¹ and remained present at 69 U mL⁻¹ after 24 hours, suggesting relatively stable activity but at a lower level compared to pH 4.5. Laccase activity at pH 6.5 was relatively unstable, displaying negligible activity at various time points. At pH 6.5, the maximum laccase activity was only 37 U mL⁻¹ after 6 hours of incubation with the dye, with no detectable laccase activity after 24 hours. These results show that methyl blue induces production of laccase by *T. asperellum* LBKURCC1 at all pH tested, however activity and stability of the enzyme differs at the different pHs. Activity and stability of *T. asperellum* LBKURCC1 laccase were highest at pH 4.5, and lower at pH 5.5 and pH 6.5, which is in line with the methyl blue bioremediation and enzymatic biodegradation percentages results shown in Table 1. Therefore, it can be deduced that the activity of *T. asperellum* LBKURCC1 laccase has a significant role in the enzymatic biodegradation and bioremediation of methyl blue by live cultures of *T. asperellum* LBKURCC1.

Our findings that production of *T. asperellum* LBKURCC1 is induced by a synthetic dye such as methyl blue, is in line with findings by other researchers of *Trichoderma* laccase induction by other kinds of textile dyes. Decolorization experiments of the triphenylmethane dye, malachite green, by the marine *Trichoderma harzianum* TSK8 revealed a strong correlation between dye degradation and laccase production, which was induced by the dye (Saravanakumar & Kathiresan 2014). Likewise, another study showed that the anthraquinone dyes alizarin red S and quinizarine green SS also induced production of laccase by *Trichoderma lixii* F21. Here too there was also a strong correlation between laccase production and degradation of these anthraquinone dyes. When the laccase inhibitor sodium azide was added to the decolourisation medium, there was a sharp decrease in both of the anthraquinone dye degradation by *T. lixii* F21 (Adnan et al. 2017).

Induction of *T. asperellum* LBKURCC1 laccase by methyl blue for 24 hours resulted in a much higher laccase activity (118 U mL⁻¹), when compared to the 24 hours induction of *T. harzianum* TSK8 laccase by malachite green (3.99 U mL⁻¹). This may explain why it took ten days to bioremediate 89 % of malachite green by *T. harzianum* TSK8 live cultures (Saravanakumar & Kathiresan 2014). In comparison, it took only 1 day for *T. asperellum* LBKURCC1 to achieve 85 % of methyl blue bioremediation. Likewise, induction of *T. lixii* F21 laccase by the anthraquinone dyes after 24 hours resulted in a laccase activity of only 15 U L⁻¹ (0.015 U mL⁻¹), which again may explain the relatively slow degradation of the anthraquinone dyes by live cultures of *T. lixii* F21. It took 7 days to reach 78 % bioremediation of alizarin red S and 98 % of quinizarine green S by live cultures of *T. lixii* F21 (Adnan et al. 2017).

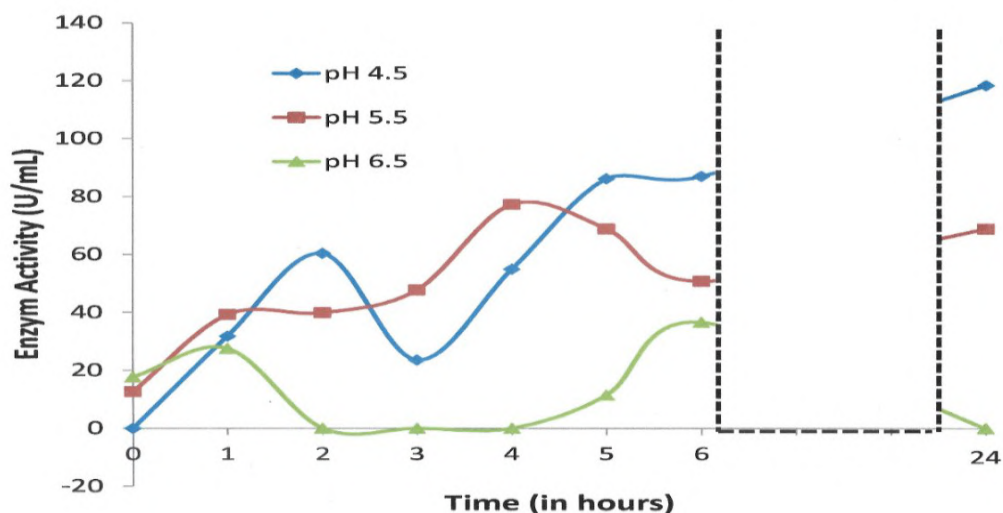


Figure 4. Laccase activity detected at the indicated time points in the culture media during bioremediation of methyl blue by *T. asperellum* LBKURCC1 at 30 °C and pH 4.5 (filled diamonds, ◆), 5.5 (filled squares, ■), or 6.5 (filled triangles, ▲).

Removal of methyl blue toxicity by enzymatic biodegradation

The Brine Shrimp Lethality Test (BSLT) using *Artemia salina* nauplii is a well-established test for acute toxicity of various compounds. It is often used to test for compounds released into bodies of water that may harm aquatic life (Carballo et al. 2002). Compounds that are toxic against *A. salina* often indicate toxicity to other aquatic organisms, and so is often used to evaluate textile dye toxicity (Morshed et al. 2018; Ardiati et al. 2024). Toxicity tests of degraded and undegraded methyl blue (MB) dye were measured against brine shrimp (*A. salina*) nauplii and compared to blanks in which only minimal media was added to the reconstituted sea water. The results of the toxicity tests are shown in Figure 5. The survival rate of shrimp larvae in the blanks alone was in the average range of 70 % to 82 %, possibly due to some factor in the minimal media not compatible with the larvae metabolism. Survival rate of the larvae decreased significantly ($p < 0.05$) when undegraded MB dye was exposed to it. In contrast, survival rate of the larvae exposed to degraded MB was the same ($p \geq 0.05$) as that of the blanks, at all pH tested. It thus can be concluded MB dye is toxic to *A. salina* larvae, and that treatment of MB dye solution with live cultures of *T. asperellum* LBKURCC1 can detoxify MB. Apparently 30 to 40 % bioremediation of MB at pH 5.5 and 6.5 by *T. asperellum* LBKURCC1 cells is sufficient for the detoxification of MB concentration used in the study (50 ppm).

The findings of this study align with prior research demonstrating the correlation between bioremediation of the triphenylmethane dye malachite green and the production of laccase by a marine *Trichoderma harzianum* strain TSK8 in media containing malachite green. *T. harzianum* TSK8 achieved an 89 % degradation of a 750-ppm malachite green solution within ten days (Saravanakumar & Kathiresan 2014). Likewise, removal of phenolic substances from pharmaceutical waste waters by the fungi *Trametes versicolor* was also linked to the presence of laccase activity (Bernats & Juhna 2018). These results confirm the presence of laccase is important for the bioremediation of various dyes and chemicals by fungi.

Fungi are recognized producers of other ligninolytic enzymes, other than laccase, that have been implicated in textile dye bioremediation. These other enzymes are lignin peroxidase (LiP; EC 1.11.1.14) (Singh et al. 2021) and manganese peroxidase (MnP; EC 1.11.1.13) (Pedroza et al. 2007; Kumar & Arora 2022). However, the utilization of these enzymes necessitates the presence of peroxide. In a study on the bioremediation of anthraquinone dyes,

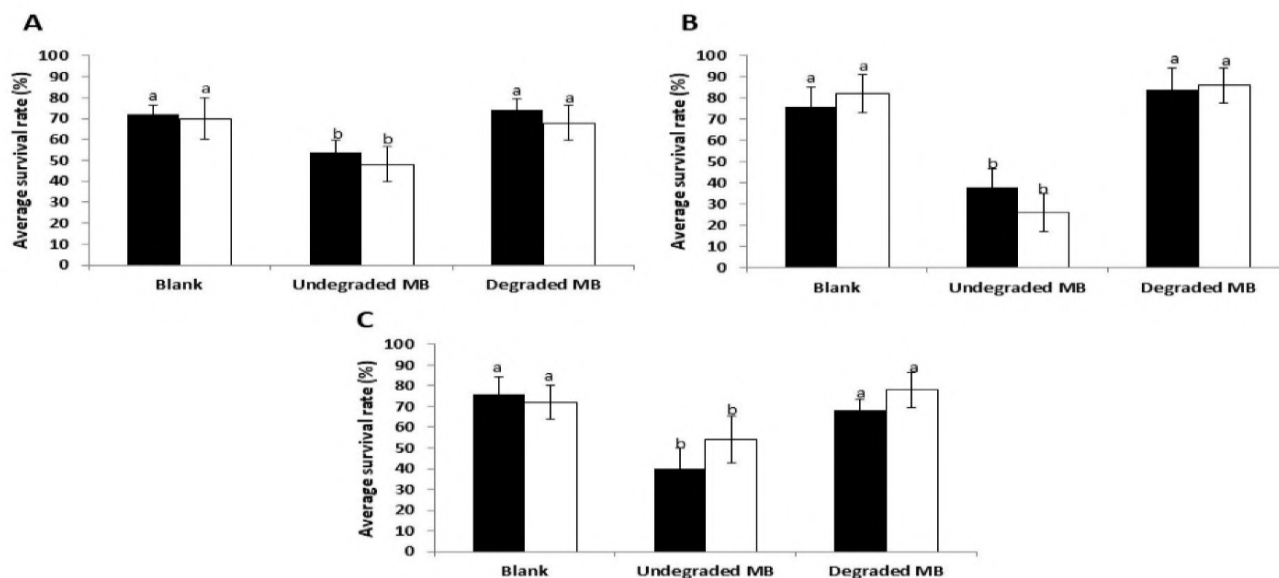


Figure 5. Average survival rate of *A. salina* in salt water added with MM media (Blank), MM media with MB dye (Undegraded MB) or MM media with MB dye treated by *T. asperellum* LBKURCC1 cells (Degraded MB) at different pHs: A) pH 4.5; B) pH 5.5; C) pH 6.5. Concentration of sample: 2 mL L⁻¹ (black bars); 4 mL L⁻¹ (white bars). Difference in alphabet depicted on top of each bar of the same color shows a statistically significant difference in averages ($p < 0.05$) as determined by the Duncan Multiple Range Test. Standard deviation of each average is shown by error bars.

Adnan et al. (2017) found that *Trichoderma lixii* F21 life cultures exhibited no LiP or MnP activity, with laccase being the dominant enzyme factor. Another study on *Marasmius cladophyllus* UMAS MS8 demonstrated that laccase activity induced by the azo dye remazol brilliant blue R contributed to its degradation, while no MnP activity was observed, and only trace amounts of LiP activity were detected (Sing et al. 2017). Given these findings, LiP and MnP activity were not assessed in the methyl blue bioremediation experiments in this study.

Our prior research established that *T. asperellum* LBKURCC1 produces laccase in a solid-state fermentation system using rice stalks as the inducer (Rahayu et al. 2019; Sellyna et al. 2020). Crude laccase extracts (0.014 U mL⁻¹) from this system degraded 60 % of a 50-ppm methyl blue solution after a 4-day reaction (Dahlana et al. 2022). However, enzyme production and extraction, even utilizing agricultural waste, can be time-consuming and relatively expensive. This study demonstrates the feasibility of directly employing *T. asperellum* LBKURCC1 cultures for methyl blue bioremediation, thus eliminating the need for separate enzyme production and extraction. The cell culture system also exhibits a higher methyl blue biodegradation rate than our previous study using crude laccase enzyme extracts.

This study contributes valuable data to the database of fungi and their capabilities to decolourise or biodegrade specific dyes, focusing on laccase induction. There is a diverse efficiency of laccase activity from different sources towards a wide range of dyes (Jeon & Lim 2017), as well as a diverse efficiency of various fungi in bioremediation of various dyes (Tang et al. 2022). As an example, in this study *T. asperellum* LBKURCC1 at pH 4.5 in 24 hours could bioremediate 85 % of a 50-ppm methyl blue solution. This is significantly higher than our previous study in which the same fungal strain at pH 4.5 after 24 hours could only bioremediate 5 % of a 50-ppm solution of the azo-dye reactive black 5 (Nugroho et al. 2023). Considering this, the selection of an appropriate strain for a specific dye biodegradation necessitates a comprehensive database for various applications. In this study we have shown that *T. asperellum* LBKURCC1 can produce laccase by induction of methyl blue dye.

Methyl blue dye is then degraded by the laccase produced in the media, causing decolourisation. The degradation products of methyl blue by *T. asperellum* LBKURCC1 laccase apparently is non-toxic, as demonstrated by the BSLT assay.

The cost-effectiveness of utilizing live microorganism cultures for textile dye effluent biodegradation can be further optimized by employing immobilised cells instead of free cells. Cell immobilisation enables cell reuse, reducing cell recycling and recovery costs (Alam et al. 2021). Additionally, immobilised cells allow the utilisation of microbial consortiums, or mixtures of fungi and bacteria for bioremediation of various substances simultaneously (Pereira et al. 2021). Future developments involving immobilised *T. asperellum* LBKURCC1 cells in conjunction with other dye-degrading fungi hold promise for cost-effective and efficient systems to biodegrade mixtures of various textile dyes.

CONCLUSIONS

T. asperellum LBKURCC1 Life cultures effectively bioremediate the triphenylmethane textile dye methyl blue. Following a 24-hour incubation of the life cultures with 50 ppm of methyl blue at pH 4.5, an average of 85.2 % of the dye was successfully remediated. Notably, over 99 % of methyl blue bioremediation by *T. asperellum* LBKURCC1 mycelia can be attributed to enzymatic biodegradation, specifically through laccase activity. The induction of *T. asperellum* LBKURCC1 laccase production in response to methyl blue occurs across a pH range of 4.5 to 6.5. However, the highest and most stable laccase activity was observed at pH 4.5. Toxicity testing on *Artemia salina* showed that degradation of methyl blue by *T. asperellum* significantly ($p < 0.05$) eliminated its toxicity. These findings underscore the potential of *T. asperellum* LBKURCC1 as a promising candidate for direct fungal-mediated bioremediation of wastewater containing the triphenylmethane dye methyl blue.

AUTHOR CONTRIBUTION

A.D.: Methodology, validation, visualisation, data collection and analysis, and original draft writing. Y.N.: Supervision and formal analysis. I.: Methodology and validation. N.W. and D.K.: Data collection, analysis, and graphical production. Y.: Supervision and validation. T.T.N.: Conceptualisation, research designation, drafts and final paper revision, review, and supervision, Principal Investigator of project and corresponding author. The final manuscript was read and approved by all authors.

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CONFLICT OF INTEREST

The authors state that there is no conflict of interest regarding the research or the research funding.

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Research Article

Bioactivity of Volatile Compounds Present in *Crinum zeylanicum* Leaf Extracts Identified by GC-MS for the Control of *Cercospora malayensis* Isolates of Okra (*Abelmoschus esculentus* L.) *In Vitro*

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ABSTRACT

Cercospora malayensis induced leaf spot is a dangerous okra disease that reduces crop yields. The aim of this study is to use leaf extract from *Crinum zeylanicum* to regulate the growth of *Cercospora malayensis* *in vitro*. The pathogen was isolated from okra leaves from the Meyomessala and Akonolinga localities that had typical disease symptoms. Phytochemical screening and GC-MS analysis of *C. zeylanicum* leaf extracts were carried out. The mycelial growth and germination of *C. malayensis* isolates 1 and 2 were evaluated with concentrations of 15, 30, 60, and 120 $\mu\text{L mL}^{-1}$, fungicide (3.33 g L^{-1}), and control. Minimum inhibition concentrations (MIC₅₀ and MIC₉₀) were evaluated. The results showed the presence of alkaloids, phenols, terpenoids and sterols in the leaf extracts. In acetone (AcE), methanol (ME) and aqueous (AqE) extracts, 32; 39 and 10 chemical compounds, respectively were found by GC-MS analysis. The most prevalent biochemically active compounds were n-Hexadecanoic acid (35.04 %), Cis-Vaccenic acid (31.76 %), Quinoline-7-carboxylic acid, 2-phenyl-, methyl ester (26.63 %), 9,12-Octadecadienoic acid (Z,Z)-methyl ester (16.89 %) and 9,12-Octadecadienoic acid (Z,Z) (10.83 %). AqE, AcE and ME extracts at 120 $\mu\text{L mL}^{-1}$ inhibited 100 % of mycelial growth and conidial germination of isolates 1 and 2 compared to the control. The lowest MICs (MIC₅₀ and MIC₉₀) were 6.79 and 10.98 $\mu\text{L mL}^{-1}$ for isolate 1 and 7.48 and 11.22 $\mu\text{L mL}^{-1}$ for isolate 2, respectively. *C. zeylanicum* it is possible to use extracts for their volatile biochemical substances in a *C. malayensis* control program.

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INTRODUCTION

Okra (*Abelmoschus esculentus* L. Moench.) is a plant species grown in tropical, subtropical and warm temperate regions (Saifullah & Rabbani 2009). It is a vegetable plant belonging to the Malvaceae family with controversial origins (West Africa, Ethiopia, South Asia) and cultivated for its succulent fruit as well as its rich nutrient content (Kahlon et al. 2007). Global okra production is projected to begin at around 9 million tons in 2021; Africa produces around 3.5 million tons (FAOSTAT 2024). Over the last ten years, Cameroon has recorded the highest okra fruit yield in 2016 with an estimated average of 78 763 tons, compared with 2022, with an estimated average of 77631.82 tons (FAOSTAT 2024). This decline in fruit yield production is due to non-compliance with good agricultural practices, the application of soil fertility, low-yielding varieties and biotic and abiotic stress (Dubey & Bhagat 1998; Ali & Hossain 2000).

Several diseases, such as fungi, hamper okra production (Raid & Palmateer 2006). Among fungal diseases, *Cercospora* leaf spot is one of the major diseases (Jukte et al. 2016). The genus *Cercospora* belongs to the Mycosphaerellaceae family, with two different *Cercospora* species including *C. malayensis* and *C. abelmoschi* that can cause the disease on economically important plants (Braun et al. 2014). Cercosporiosis with *C. malayensis* has been documented in tropical and subtropical Asian countries and also in West Africa where okra is grown, particularly during the rainy season (Beilharz et al. 2002; Braun & Freire 2004; Hernández-Gutiérrez & Dianese 2009). Disease symptoms are generally visible on the plant's leaves. All stages of plant development are affected by the disease. Infection begins when conidia reach the underside of okra leaves. The conidia germinate and emit mycelia that penetrate the leaf parenchyma through the stomata (Souza et al. 2015). The pathogen's mycelium spreads into the leaf parenchyma, damaging cells and causing their necrosis through the production of photosensitive toxins that degrade cell walls (Steinkamp et al. 1979; Daub 1982). This process resulted in the appearance of small, irregular, pale-brown spots on okra foliage. Leaf spots can enlarge and coalesce as the disease progresses on the plant, forming larger, irregular lesions leading to yellowing, wilting, defoliation and loss of vigour, resulting in yield losses ranging from 45 to 80 % (Chai et al. 2021).

Synthetic fungicides are abused by growers to control the disease in the field. In the long term, they give rise to resistance phenomena, cause environmental pollution and are toxic to humans (Watanabe-Akanuma et al. 2005; Perera et al. 2005). In the pursuit of better, non-polluting and health-promoting substitutes, biological control using natural substances of plant origin appears to be an alternative for protecting field crops (Ambang et al. 2011). The use of extracts from plants that are high in secondary metabolites (terpenoid compounds, phenolics, nitrogen compounds and saponins) as biopesticides has proved effective in managing crop diseases (Toka et al. 2023; Dida et al. 2024). Several studies have demonstrated the biopesticidal properties of certain plants (Mboussi et al. 2018; Atindo et al. 2020; Manga et al. 2021; Essomé et al. 2022). *Crinum zeylanicum* (Linn.) is a monocotyledonous perennial herb in the Amaryllidaceae family (Refaat et al. 2012). Like most plants, phytochemical studies of *Crinum* genus organs have identified biological activities (Tram et al. 2002). The purpose of this study is to use leaf extract from *Crinum zeylanicum* to regulate the growth of *Cercospora malayensis* *in vitro*.

MATERIALS AND METHODS

Isolation and identification of *Cercospora malayensis*

Pure isolates of *C. malayensis* were obtained on Potato Dextrose Agar (PDA) culture medium supplemented with Ampicillin 250 mg L⁻¹ and chlorampheni-

col (200 mg L⁻¹) from leaves collected in the locality of Akonolinga (4°12' N, 11°24' E) and Meyomessala (2°43' N, 11°42' E) showing typical symptoms of the disease. Diseased leaves were washed several times with distilled water and disinfected with 90 % alcohol and sodium hypochlorite (10 %) for 2 min. Leaf fragments measuring approximately 2 mm² were placed in the middle of the Petri dish poured with PDA media and incubated in the dark for eight days 25 ± 2 °C (Hasanin & Hashem 2020). Emerging white mycelia of each fragments were observed under a light microscope and moved to new Petri dishes containing PDA culture medium for purification until pure cultures were obtained. Identification of the pathogen was based on macroscopic and microscopic observations of the morphological characteristics of the mycelium, conidia and an identification key (Hashem & Farrag 2005).

Preparation of *Crinum zeylanicum* leaf extracts

Crinum zeylanicum leaves collected in the locality of Akonolinga (4°12' N, 11°24' E) were identified by an expert botanist from the National Herbarium. After three weeks of shade drying at room temperature, the leaves were processed into a powder with an electric grinder. The organic extracts were prepared by macerating of 500 g of powder in 5 L of solvent, represented here by Methanol and Acetone for 48 hours. The resulting mixes were filtered using filter paper and the filtrates concentrated in a rotary evaporator (Büchi R-200 Rotary Evaporator at 60 °C) until almost complete elimination of the extraction solvents. The extracts obtained were kept in a refrigerator at 4 °C until its needed (Vongsak et al. 2013). The aqueous extract of *C. zeylanicum* leaves was acquired by maceration of 50 g of powder in 100 mL of distilled water and filtered through a fine muslin cloth (Zibbu & Batra 2010).

Phytochemical screening of *Crinum zeylanicum* leaf extracts

The various extracts from *C. zeylanicum* leaves were subjected to phytochemical analysis to determine their chemical composition. Chemical compounds were identified by observing certain characteristics after testing each extract with specific dyes. The standard protocol described by Edeoga et al. (2005), Kumar et al. (2010), Tiwari et al. (2011) and Banu and Cathrine (2015) was used.

GC-MS analysis of *Crinum zeylanicum* leaf extracts

C. zeylanicum leaf extracts were analysed using a GC-MS apparatus (Agilent GC 7890A) coupled to a mass spectrometer (Agilent 5975C TAD VL MSD) equipped with an Elite-1 fused silica capillary column (30 m x 0.25 mm diameter, film thickness 0.25 µm) and helium as the carrier gas. The energy of the electronic ionisation system is 70 eV. After being kept at 150 °C for one minute at a rate of 20 °C min⁻¹, the oven reached a final temperature of 280 °C for 9 min. Total GC running time was 13 min. Separated constituents were identified by comparing their spectra with those of the National Institute Standards and Technology (NIST) database of over 62,000 models. The injected solution was prepared by mixing 1.5 g of anhydrous sodium acetate and 6 g of magnesium sulfate, autoclaved and cooled in a desiccator, with 10 mL of aqueous extract and 10 mL of acetonitrile. For the organic extract, 1 mL was mixed with 2 mL solvent (acetone or methanol) and 0.15 g magnesium sulfate. After that, the mixtures were vortexed and centrifuged at 4000 rpm (QuECHERS 2004). One microliter (1 µL) of each preparation was injected into the column.

In vitro test

From pure isolates 1 and 2 obtained from samples collected in the locality of Akonolinga and Meyomessala respectively, explants approximately 7 mm in

diameter were removed with a cork borer and placed in the middle of each Petri dish containing PDA medium poisoned with aqueous, methanol and acetone extracts at 15; 30; 60 and 120 $\mu\text{L mL}^{-1}$ concentrations to the synthetic fungicide with active ingredient Metalaxyl 80 g kg^{-1} and Mancozeb 640 g kg^{-1} at 3.33 g L^{-1} and to the control with no added extract or fungicide. From the stock solutions (500 $\mu\text{L mL}^{-1}$) of every extracted 50 mL of each extract and 100 mL of solvent, volumes of 1.8; 3.6; 7.2 and 14.4 mL were taken and added respectively to 28.2; 26.4; 22.8 and 15.6 mL of PDA for a final volume of 30 mL each, it was transferred at a rate of 10 mL per 90 mm Petri dish. For controls, a 10 mL solution of PDA medium was poured directly into each Petri dish. The medium enriched with the synthetic fungicide at the concentration of 3.33 g L^{-1} was prepared using the normal rate (50 g per 15 L of water). Tests were performed in triplicate for each pathogen. Incubation was carried out at $25 \pm 2^\circ\text{C}$, and mycelial growth of isolates was assessed from the second day after incubation (DAI) until the mycelia of each isolate completely filled the control Petri dishes, by taking measurements of the two perpendicular diameters traced on the Petri dishes back. The growth diameters were calculated and Inhibition Percentage (IP) was determined according to the formula used by Singh et al. (1993).

$$\text{IP (\%)} = (\text{Mgc} - \text{Mgt}) / \text{Mgc} \times 100$$

Where: IP = inhibition percentage; Mgc = mycelial growth in control; Mgt = mycelial growth in the treatment.

Evaluation of fungicidal or fungistatic activity

The fungicidal or fungistatic activity of *Crinum zeylanicum* leaf extracts was assessed by selecting Petri dishes in which the growth of pathogen isolates had been completely inhibited at the end of mycelial growth, and depositing them in a new culture medium containing no extract and/or fungicide. After 6 days, the Petri dishes were observed and if growth had resumed, the starting extract was qualified as fungistatic; if not, it was considered fungicidal (Toka et al. 2023).

Minimal inhibitory concentrations of the different extracts.

The Minimal Inhibitory Concentrations (MIC₅₀ and MIC₉₀) of the different extracts were ascertained by contrasting the Inhibitory Percentage values with the corresponding concentrations' (Ci) natural logarithm values (España et al. 2017).

$$\text{IP} = f(\ln \text{Ci}).$$

The inhibition percentage (IP) is determined for each treatment relative to the control after 8 days of mycelial growth catch, following the formula of Singh et al. (1993):

$$\text{IP (\%)} = (\text{Dc} - \text{Dt}) / \text{Dc} \times 100$$

Where: Dc = mean culture diameter measured in control; Dt = mean culture diameter measured in treatment.

The linear regression line $Y = ax + b$ derived from the $\text{PI} = f(\ln \text{Ci})$ function is used to determine MIC₅₀ and MIC₉₀, where Y = percentage inhibition, a = slope of line, MIC = ex and b = constant (Griffin et al. 2000).

Cercospora malayensis conidial germination test

The germination test was carried out with *C. malayensis* conidia obtained from a pure culture of isolates 1 and 2 aged 10 days contained in Petri dishes, by taking the mycelium of both isolates and mixing each in a quantity of sterile distilled water, then calibrated using the mallassez cell to obtain 2×10^4 conidia mL^{-1} (Fulano et al. 2016). The impact of extracts on conidial germination of *C. malayensis* isolates 1 and 2 compared with a synthetic fungicide was assessed by casting PDA culture medium supplemented with extracts and

fungicide onto slides. Conidial suspensions of 20 µL of *C. malayensis* were spread out on the slides using a micropipette in contrast to the control slides. Each preparation was repeated three times and kept in the dark at 25 °C for a minimum of 12 h. After 24 h the count of germinated conidia was made on a total of 100 conidia on three different zones of each slide using the photonic microscope (Widmer & Laurent 2006). Inhibition percentages (IP) of extracts against the two *C. malayensis* isolates were calculated applying the subsequent formula:

$$\text{IP (\%)} = (A-B)/A \times 100 \text{ (You et al. 2016)}$$

Where: A = Estimated number of conidial on control medium; B = Estimated number of conidial in the presence of the extract

Statistical analysis

Data collected for the parameters studied were subjected to a one-way ANOVA using R software version 4.0.1. Multiple comparison of means was determined by the Tukey HSD test when notable distinctions ($P < 0.05$) for one of the factors were found, and when normality of the data (Shapiro-Wilk test; $P > 0.05$) and homogeneity of variance (Bartlett test; $P > 0.05$) were verified.

RESULTS

Identification of *Cercospora malayensis*

Two pure isolates of *C. malayensis* were obtained from fragments of okra leaves collected in the localities of Akonolinga and Meyomessala (Figure 1). The two isolates were identified morphologically by macroscopic and microscopic characters. The macroscopic characteristics of the two isolates observed in Petri dishes showed whitish mycelia on the upper and lower surfaces with a cottony appearance (Figure 1 A,C). Under the light microscope, conidia were continuous and elongated, subaigul to obtuse at the apex and truncate to obconical at the base with 3 to 8 septa and slightly curved (Figure 1 B,D). These morphological traits aligned with those of *C. malayensis* F. Stevens & Solheim, the causal agent of Cercosporiosis of okra.

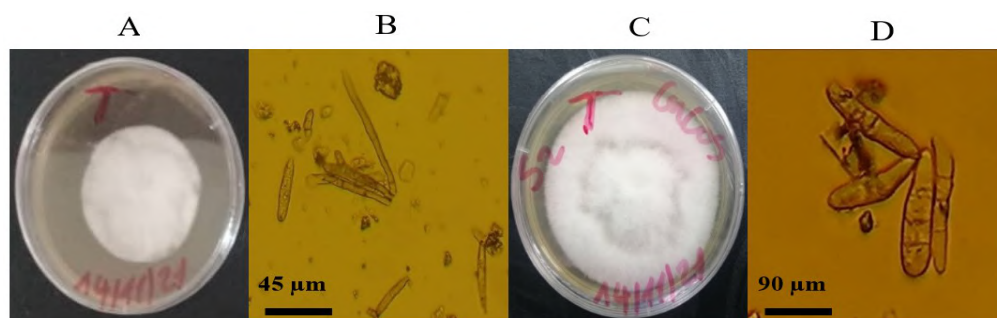


Figure 1. Pure isolates of *Cercospora malayensis*; A: Mycelium of *C. malayensis* isolate 1; B: Conidia of isolate 1 observed under a light microscope; C: Mycelium of *C. malayensis* isolate 2; D: Conidia of isolate 2 observed under a light microscope.

Phytochemical constituents of *Crinum zeylanicum* leaf extract

Phytochemical screening revealed the existence of several families of compounds, including terpenes, sterols, phenols, saponins, flavonoids, oils, sugars, saponins and alkaloids. Alkaloids, flavonoids, sterols and terpenes are most abundant in the aqueous and methanol extracts. Sugars and oils are present in trace in the methanol extract. The methanol and aqueous extracts are the richest in compound families. The acetone extract is the poorest in chemical compound family, as only terpenes, sterols and oils are present. Terpenes, sterols and oils are present in all four extracts. Quinone and saponins are absent in all extracts (Table 1).

Table 1. Various natural products in the different *Crinum zeylanicum* extracts.

Products	Acetone extract	Aqueous extract	Methanol extract
Terpenes	++	+	+
Sterols	++	+	+
Phenols	-	+	+
Quinons	-	-	-
Saponins	-	-	-
Flavonoids	-	++	++
Alkaloids	-	+++	+++
Oils	+	+	T
Sugar	-	+	T

- Absent, + present, ++ moderate present, +++ highly present, T trace presence (Jafri et al. 2022).

GC-MS analysis of the various extracts

The chromatographic profile of the different *C. zeylanicum* leaf extracts showed variable results depending on the extraction solvents used. Peaks represent majority, minority and ultra-minority compounds (Figure 2). Majority chemical compounds constitute the highest peaks. Chemical compounds identified by mass spectrometry are grouped according to their bioactivity, molecular weight, molecular formula, and retention time (RT). In the acetone extract, 32 chemical compounds were detected, the most representative being: Quinoline-7-carboxylic acid, 2-phenyl-, methyl ester (26.63 %), 9,12-Octadecadienoic acid (Z,Z) (10.83 %) and 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (10.37 %) (Table 2). In the methanol extract, 39 chemical compounds were detected (Table 3), the most abundant being: 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (16.89 %), Phytol (11.78 %), 3-Methyl-12-phenyl-8,9,10,12-tetrahydro-7H-benzo[b][4,7]phenanthroline-11-one (8.91 %), 9-Octadecenoic acid, (E)- (8.28 %). Finally, in the aqueous extract, n-Hexadecanoic acid (35.04 %) and Cis-Vaccenic acid (31.76 %) were the most abundant, with 10 chemical compounds detected (Table 4).

In vitro effect of *Crinum zeylanicum* seed extracts against *Cercospora malayensis*

The suppression of mycelial development varied significantly of *C. malayensis* isolates 1 and 2 between different concentrations of *C. zeylanicum* leaf extracts, fungicide and control (Figure 3). For *C. malayensis* isolate 1, concentrations of 60 and 120 $\mu\text{L mL}^{-1}$ inhibited mycelial growth by 100 % in both the methanol and acetone extracts. In contrast, the aqueous extract at 120 $\mu\text{L mL}^{-1}$ showed little inhibition (53.04 %). The synthetic fungicide inhibited 100 % of the mycelial growth of *C. malayensis* isolate 1, compared with the control, where the inhibition percentage was null (Figure 3A). For *C. malayensis* isolate 2, 100 % inhibition was observed with methanol extract of *C. zeylanicum* leaves at concentrations of 60 and 120 $\mu\text{L mL}^{-1}$. With the aqueous and acetone extracts, inhibition of 94.52 % and 100 % respectively was recorded at a concentration of 120 $\mu\text{L mL}^{-1}$ (Figure 3B). The synthetic fungicide inhibited 100 % of mycelial growth compared with the control, where growth was complete in Petri dishes after 8 days of incubation (Figure 4).

Minimum inhibition concentrations of different extracts

The correlation test between the parameters studied demonstrated the existing relationships and enabled us to determine the Minimum Inhibitory Concentrations (MICs). Regression lines obtained with isolate 1 and aqueous extract ($y = 12.076x - 2.2877$), acetone extract ($y = 33.371x - 49.508$) and methanol extract ($y = 25.95x - 14.907$) all showed positive slopes. Similarly, positive regression lines were obtained with isolate 2 and the methanol ($y = 32.016x - 42.135$), acetone ($y = 27.701x - 26.135$) and aqueous ($y = 24.605x -$

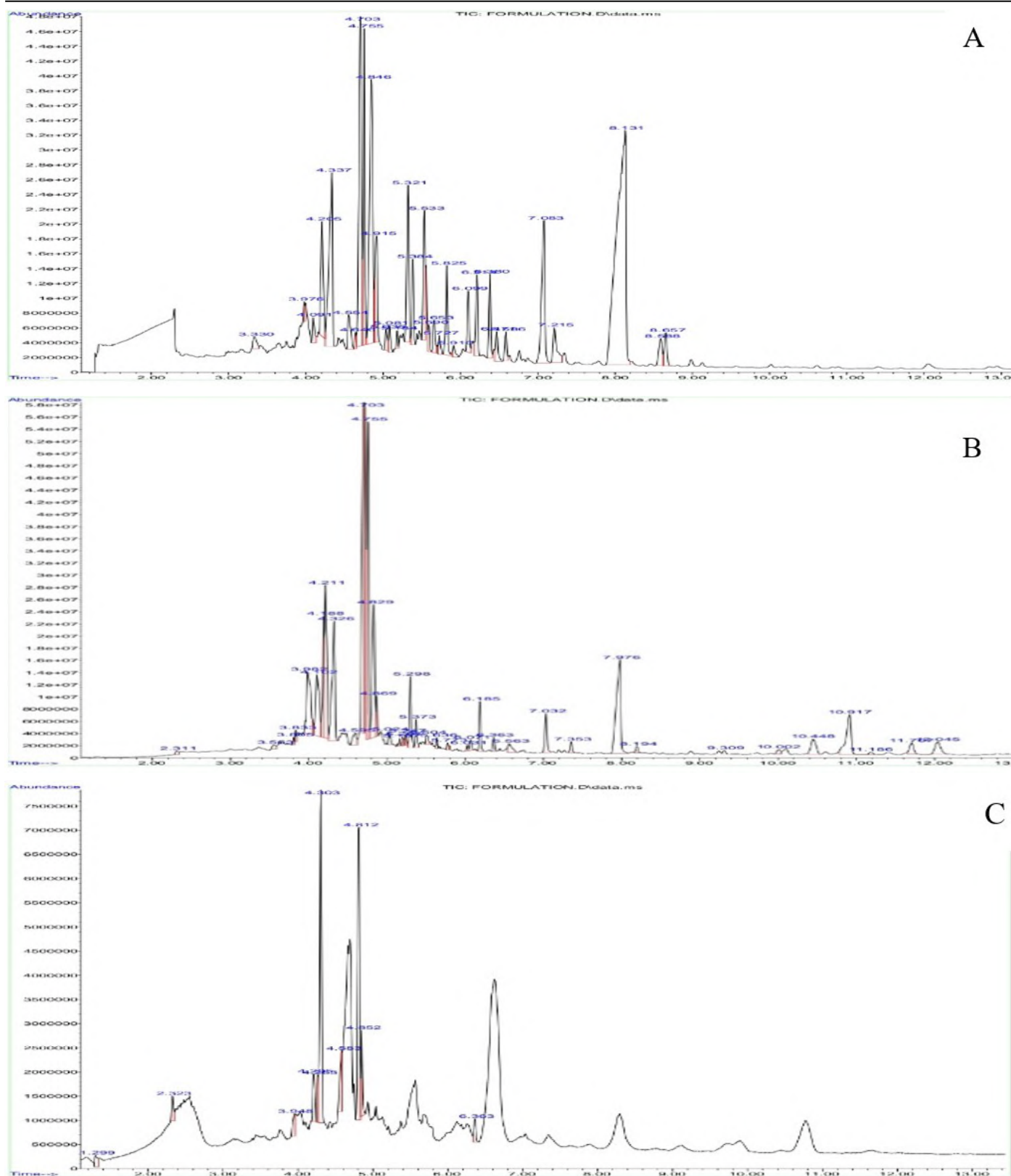


Figure 2. Chromatographic profile of *Crinum zeylanicum* leaf extract; A: Acetone extract, B: Methanol extract, C: Aqueous extract.

26.517) extracts of *C. zeylanicum* leaves. The lowest minimum inhibitory concentrations (MIC₅₀) were acquired with the methanol extract (6.79 $\mu\text{L mL}^{-1}$) for *C. malayensis* isolate 1 and with the acetone extract (7.48 $\mu\text{L mL}^{-1}$) for isolate 2. For MIC₉₀, low minimum inhibitory concentrations of 10.88 and 11.22 $\mu\text{L mL}^{-1}$ were obtained for the methanol extract of *C. zeylanicum* leaves for *C. malayensis* isolate 1 and 2 respectively. The highest minimum inhibitory concentrations (MIC₅₀ and MIC₉₀) were recorded with the aqueous extract

Table 2. Chemical compounds present in the acetone extract of *Crinum zeylanicum* leaves identified by GC-MS.

Peak	RT (min)	Area (%)	Name of compound	MF	MW
1	3.33	0.55	2-Methyl-Z,Z-3,13-octadecadienol	C ₁₉ H ₃₆ O	280.00
2	3.98	0.36	(-)-trans-Pinane	C ₁₀ H ₁₈	138.25
3	4.09	0.70	Bicyclo [3.1.1] heptane, 2,6,6-trimethyl-, [1S-(1.alpha., 2.beta., 5.alpha.)]-	C ₁₀ H ₁₈	138.25
4	4.21	3.66	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	286.46
5	4.34	7.44	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256.42
6	4.55	1.19	1H-Phenalen-1-one	C ₁₃ H ₈ O	180.20
7	4.65	0.35	2-Methyl-4'-methoxy-benzophenone	C ₁₅ H ₁₄ O ₂	226.27
8	4.70	10.37	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	C ₁₉ H ₃₄ O ₂	294.47
9	4.76	5.27	Phytol	C ₂₀ H ₄₀ O	296.00
10	4.85	10.83	9,12-Octadecadienoic acid (Z,Z)-	C ₁₈ H ₃₂ O ₂	280.45
11	4.92	2.71	2,3,8-Trimethyl-1H,9H-pyrrolo[3,2-H]quinolin-6-one	C ₁₄ H ₁₄ N ₂ O	226.27
12	5.04	0.56	Acridin-9-amine, 1,2,3,4-tetrahydro-5,8-dimethyl-	C ₁₅ H ₁₈ N ₂	226.32
13	5.08	0.58	1,1'-Biphenyl, 5-hydroxy-3,4'-dimethoxy-	C ₁₄ H ₁₄ O ₃	230.26
14	5.18	0.29	2,4,6-Trimethyldecane-1,3,10-triol	C ₁₃ H ₂₈ O ₃	232.36
15	5.32	3.74	Benzeneacetic acid, .alpha.-(acetyloxy)-2-methoxy-, methyl ester	C ₁₂ H ₁₄ O ₅	238.24
16	5.38	1.70	Propanedioic acid, 3-methoxyphenylhydrazono-	C ₁₀ H ₁₀ N ₂ O ₅	238.20
17	5.53	2.79	2-Propen-1-one, 1-(4-aminophenyl)-3-phenyl-	C ₁₅ H ₁₃ NO	223.27
18	5.59	0.46	4,5-Dihydro-N-(p-methoxyphenyl)-3-furamide	C ₁₂ H ₁₃ NO ₃	219.24
19	5.65	0.87	4-Isopropenylcyclohexanone	C ₉ H ₁₄ O	138.21
20	5.73	0.41	Mesembrine	C ₁₇ H ₂₃ NO ₃	289.40
21	5.83	2.01	Benzeneethanamine, N-(1-methylethylidene)-	C ₁₁ H ₁₅ N	161.24
22	5.91	0.33	2-[p-Cyanophenyl]-5-chlorobenzimidazole	C ₁₄ H ₈ ClN ₃	253.68
23	6.09	1.48	4H-1-Benzopyran-4-one, 2,3-dihydro-7-hydroxy-2-(3-hydroxyphenyl)-	C ₁₅ H ₁₂ O ₄	256.25
24	6.21	1.95	3-Hydroxy-4-nitrobenzaldehyde	C ₇ H ₅ NO ₄	167.12
25	6.38	1.81	5-(p-Aminophenyl)-4-(p-tolyl)-2-thiazolamine	C ₁₆ H ₁₅ N ₃ S	281.40
26	6.47	0.82	Acetyl-caranine	C ₁₈ H ₁₉ NO ₄	313.30
27	6.59	0.68	9,10-Anthracenedione, 2-hydroxy-	C ₁₄ H ₈ O ₃	224.21
28	7.08	5.66	Daniquidone	C ₁₅ H ₁₁ N ₃ O	249.27
29	7.22	1.65	1H-Indene, 1-(1,5-dimethyl-2-hexenyl)octahydro-7a-methyl-, [1R-[1.alpha.(1R*,2Z),3a.beta.,7a.alpha.]]-	C ₁₈ H ₃₂	248.25
30	8.13	26.63	Quinoline-7-carboxylic acid, 2-phenyl-, methyl ester	C ₁₇ H ₈ NO ₂	263.29
31	8.54	1.11	8-Hydroxy-6-methyl-8-phenyl-hexahydro-indolizin-5-one	C ₁₁ H ₁₂ N ₂ S	204.21
32	8.66	1.06	5-Ethoxy-6-methoxy-8-nitroquinoline	C ₁₂ H ₁₂ N ₂ O ₂	248.23

RT: retention time; MF: molecular formula; MW: molecular weight

(11.77 and 20.77 $\mu\text{L mL}^{-1}$) and (8.45 and 12.88 $\mu\text{L mL}^{-1}$) respectively, for isolate 1 and 2 of *C. malayensis* (Table 5).

Fungicidal and fungistatic activity of extracts

The fungicidal and fungistatic activity of *C. zeylanicum* leaf extracts was studied with Petri dishes at the highest concentration (C4 = 120 $\mu\text{L mL}^{-1}$), which showed total inhibition (Table 6). After 10 days of incubation, no renewed growth of *C. malayensis* isolates 1 and 2 was observed with explants taken from Petri dishes poisoned with methanol (MeE) and acetone (AcE) extracts of fungicidally active *C. zeylanicum* leaves. The aqueous extract (AqE) of *C. zeylanicum* leaves was fungistatic for *C. malayensis* isolates 1 and 2.

Effect of *Crinum zeylanicum* leaf extracts on germination of *Cercospora malayensis* conidia

The germination test for *C. malayensis* conidia demonstrates that there is a substantial difference ($P < 0.05$) between *C. zeylanicum* leaf extracts at different concentrations, the synthetic fungicide and the control (Figure 5). With *C. malayensis* isolate 1, low conidial germination of 46.56 %, 45.57 % and 42.22

Table 3. Chemical compounds present in the methanol extract of *Crinum zeylanicum* leaves identified by GC-MS.

Peak	RT (min)	Area (%)	Name of compound	MF	MW
1	2.31	0.23	Benzeneacetic acid, .alpha.-ethyl-, ethyl ester	C ₁₂ H ₁₆ O ₂	192.25
2	3.55	0.31	1,19-Eicosadiene	C ₂₀ H ₃₈	278.52
3	3.81	0.20	Methyl 13-methyltetradecanoate	C ₁₆ H ₃₂ O ₂	256.42
4	3.83	0.32	Tetradecanoic acid, 12-methyl-, methyl ester	C ₁₆ H ₃₂ O ₂	256.42
5	3.98	5.55	Bicyclo [3.1.1] heptane, 2,6,6-trimethyl-, (1.alpha.,2.beta.,5.alpha.)-	C ₁₀ H ₁₈	138.25
6	4.10	4.97	11-Hexadecen-1-ol, acetate, (Z)-	C ₁₈ H ₃₄ O ₂	282.46
7	4.12	3.69	Pentadecanoic acid, 14-methyl-, methyl ester	C ₁₇ H ₃₄ O ₂	270
8	4.21	7.71	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	286.45
9	4.33	7.94	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256.42
10	4.59	1.00	2-Butyloxycarbonyloxy-1,1,10-trimethyl-6,9-epidioxydecalin	C ₁₈ H ₃₀ O ₅	326.4
11	4.70	16.89	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	C ₁₉ H ₃₄ O ₂	294.47
12	4.76	11.78	Phytol	C ₂₀ H ₄₀ O	296
13	4.83	8.28	9-Octadecenoic acid, (E)-	C ₁₈ H ₃₄ O ₂	282.5
14	4.87	0.84	Octadecanoic acid	C ₁₈ H ₃₆ O ₂	282.5
15	5.02	0.57	Acridin-9-amine, 1,2,3,4-tetrahydro-5,7-dimethyl-	C ₁₅ H ₁₈ N ₂	226.32
16	5.17	0.18	3-Hydroxydecanoic acid	C ₁₀ H ₂₀ O ₃	188.26
17	5.20	0.27	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	C ₁₉ H ₃₂ O ₂	292.00
18	5.25	0.30	cis-5,8,11-Eicosatrienoic acid, methyl ester	C ₂₁ H ₃₆ O ₂	320.5
19	5.29	2.63	6-Amino-2,4-dimethylphenol	C ₈ H ₁₁ NO	137.18
20	5.37	0.98	Methyl 18-methylnonadecanoate	C ₂₁ H ₄₂ O	326.6
21	5.50	0.48	Tricyclo [3.3.3.0(1,5)] undec-6-ene-2,3,6-tricarbonitrile	C ₁₄ H ₁₃ N	223.27
23	5.77	0.25	1,19-Eicosadiene	C ₂₀ H ₃₈	278.52
24	6.03	0.20	Triacetyl acetate	C ₃₂ H ₆₄ O ₂	480.8
25	6.07	0.43	4H-1-Benzopyran-4-one,2,3-dihydro-7-hydroxy-2-(3-hydroxyphenyl)-	C ₁₅ H ₁₂ O ₄	256.25
26	6.19	1.67	Methyl 20-methyl-heneicosanoate	C ₂₃ H ₄₆ O ₂	354.6
27	6.36	0.51	Bis (2-ethylhexyl) phthalate	C ₂₄ H ₃₈ O ₄	390.6
28	6.56	0.71	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	C ₃₀ H ₅₀ O	426.7
29	7.03	1.89	3-Methyl-1-phenethyl-3,4-dihydro-isoquinoline	C ₁₈ H ₁₉ N	249.3
30	7.35	0.51	Tetracosanoic acid, methyl ester	C ₂₆ H ₅₀ O ₂	382.7
31	7.98	8.91	3-Methyl-12-phenyl-8,9,10,12-tetrahydro-7H-benzo [b] [4,7] phenanthrolin-11-one	C ₂₃ H ₂₀ NO ₂	340.4
32	8.19	0.33	Squalene	C ₃₀ H ₅₀	410.7
33	9.31	0.22	Stigmastan-6,22-dien, 3,5-dedihydro-	C ₂₉ H ₄₆	394.6
34	10.02	0.36	Thiazole, 4-[1,1'-biphenyl]-4-yl-2-methyl-	C ₁₆ H ₁₃ NS	251.3
35	10.45	1.65	Stigmasteryl tosylate	C ₃₆ H ₅₄ O ₃ S	566.9
36	10.92	4.3	Stigmasta-5,22-dien-3-ol, acetate, (3.beta.)-	C ₃₁ H ₅₀ O ₂	454.7
37	11.19	0.18	Ergosta-4,6,22-trien-3.beta.-ol	C ₂₈ H ₄₄ O	396.6
38	11.71	0.98	Stigmastan-3,5-diene	C ₂₉ H ₄₈	396.7
39	12.05	1.48	17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta [a] phenanthren-3-ol	C ₂₇ H ₄₆ O	386.7

RT: retention time; MF: molecular formula; MW: molecular weight.

% was recorded in the methanol extract at concentrations of 15, 30 and 60 $\mu\text{L mL}^{-1}$ respectively. With the aqueous extract, conidial germination was observed at 15 $\mu\text{L mL}^{-1}$ (88.88 %), 30 $\mu\text{L mL}^{-1}$ (87 %) and C3 = 60 $\mu\text{L mL}^{-1}$ (39.78 %), and with the acetone extract at 15 $\mu\text{L mL}^{-1}$ (29 %) and 30 $\mu\text{L mL}^{-1}$ (17.33 %). With *C. malayensis* isolate 2, germination rates of 37.11 %, 20.44 % and 16.88 % were recorded with the methanol extract at concentrations 15, 30 and 60 $\mu\text{L mL}^{-1}$ respectively. The aqueous extract recorded a germination of 95.55 %, 76.67 %, 32.22 % and 13.33 % at concentrations 15, 30 and 60 $\mu\text{L mL}^{-1}$ respectively. In general, the synthetic fungicide and 120 $\mu\text{L mL}^{-1}$ recorded no conidial germination compared with the control, where germination was 100 %. Conidial germination in the control, methanol extract and aqueous extract at 120 $\mu\text{L mL}^{-1}$ is shown in figure 6.

Table 4. Chemical compounds present in the aqueous extract of *Crinum zeylanicum* leaves identified by GC-MS.

Peak	R T (min)	A r e a (%)	Name of compound	MF	MW
1	1.299	1.91	Chlorfenapyr	C ₁₅ H ₁₁ BrClF ₃ NO ₂	407.61
2	2.323	2.81	Pentanoic acid	C ₅ H ₁₀ O ₂	102.13
3	3.948	2.88	Pentadecanoic acid	C ₁₅ H ₃₀ O ₂	242.40
4	4.205	5.37	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₃	286.456
5	4.263	4.03	cis-9-Hexadecenoic acid	C ₁₆ H ₃₀ O ₂	254.41
6	4.303	35.04	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256.42
7	4.583	7.77	Gamma.- Sitosterol	C ₂₉ H ₅₀ O	414.7
8	4.812	31.76	Cis-Vaccenic acid	C ₁₈ H ₃₄ O ₂	282.5
9	4.852	6.31	Octadecanoic acid	C ₁₈ H ₃₆ O ₂	282.5
10	6.363	2.11	Bis(2-ethylhexyl) phthalate	C ₂₄ H ₃₈ O ₄	390.6

RT: retention time; MF: molecular formula; MW: molecular weight

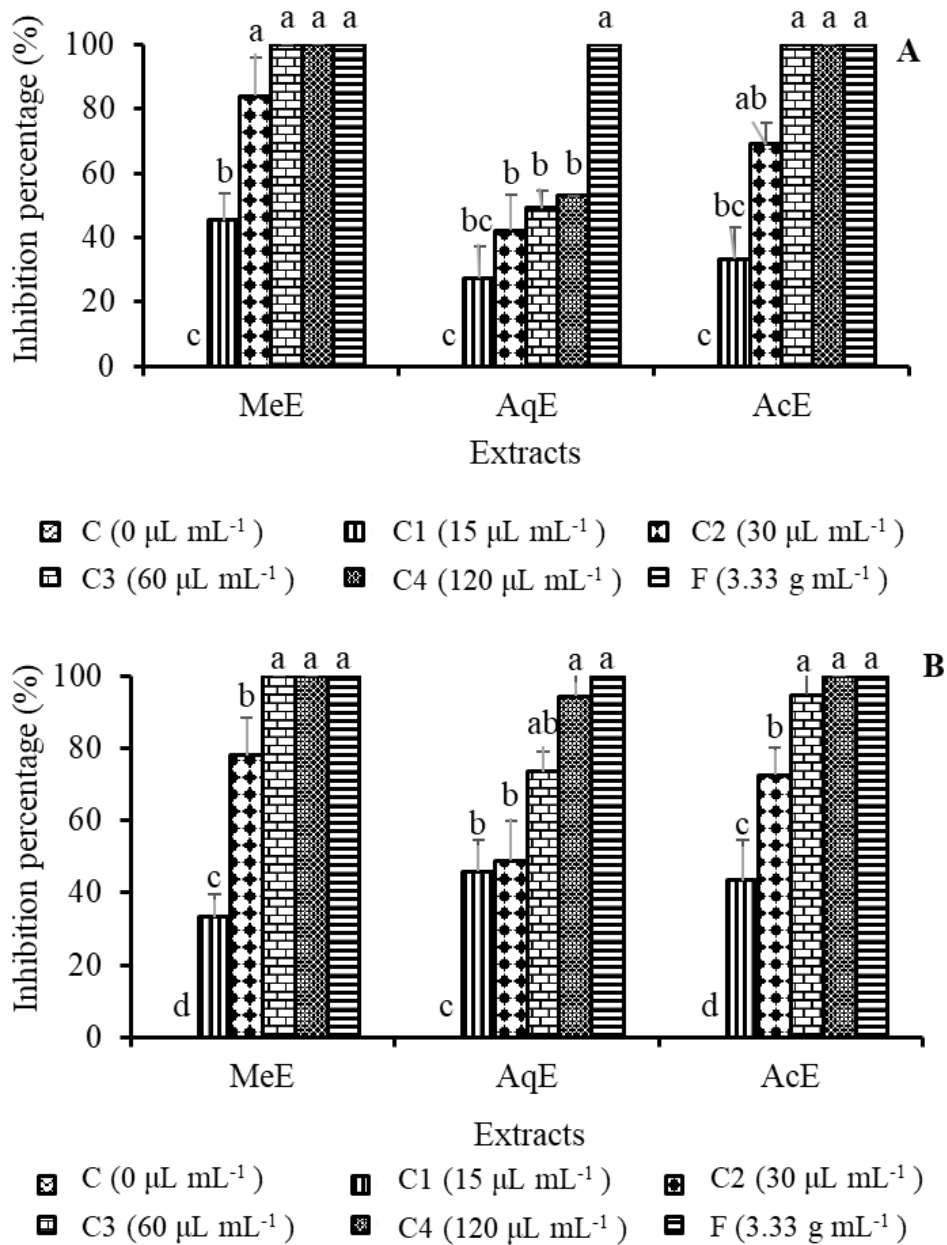


Figure 3. Inhibition of mycelial growth of *Crinum zeylanicum*; A: Isolate 1 obtained from leaves from Akonolinga; B: Isolate 2 obtained from leaves from Meyomessala; AqE: aqueous extract; AcE: acetone extract, MeE: methanol extract; F: fungicide; C: control.

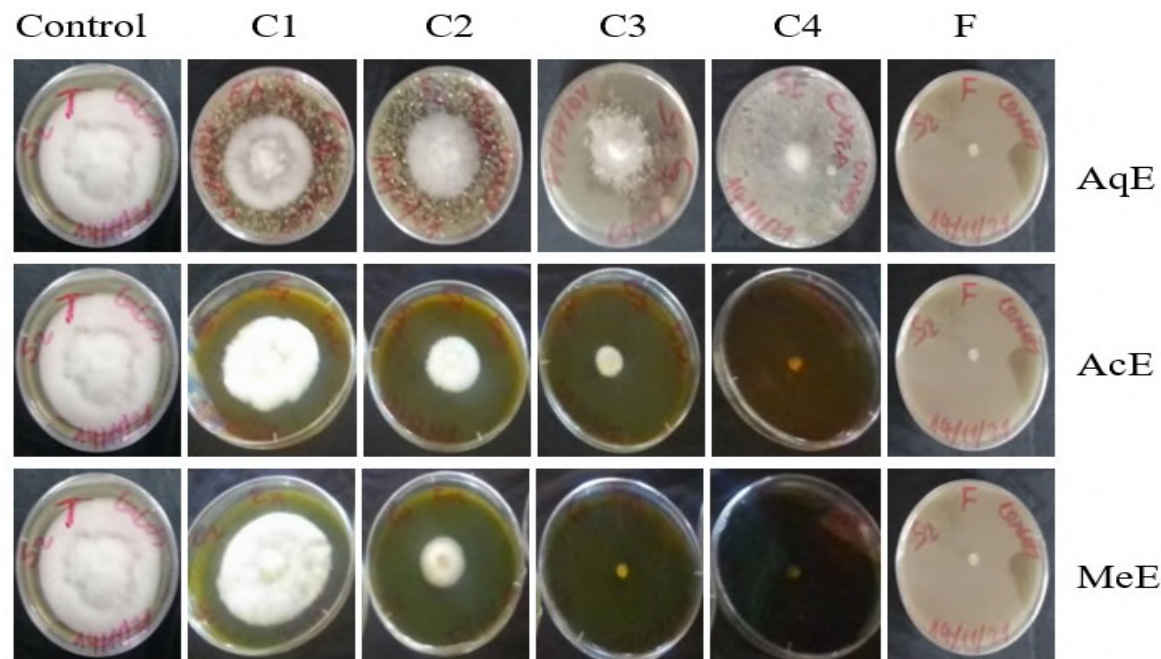


Figure 4. Inhibitory effect of organic and aqueous extracts of *Crinum zeylanicum* leaves on mycelial growth of *Cercospora malayensis* isolate 2 after 8 days incubation. C1: 15 $\mu\text{L mL}^{-1}$; C2: 30 $\mu\text{L mL}^{-1}$; C3: 60 $\mu\text{L mL}^{-1}$; C4: 120 $\mu\text{L mL}^{-1}$; F: 3.33 g L⁻¹ fungicide C: Control; AqE: aqueous extract; AcE: acetone extract, MeE: methanol extract; F: fungicide; C: control.

Table 5. Minimum inhibition concentration MIC 50 and MIC 90.

Pathogen agent	Extracts	MIC 50	MIC 90
<i>Cercospora malayensis</i> (Isolate 1)	Methanol extract	6.79	10.98
	Aqueous extract	11.77	20.77
	Acetone extract	8.10	11.36
<i>Cercospora malayensis</i> (Isolate 2)	Methanol extract	7.83	11.22
	Aqueous extract	8.45	12.88
	Acetone extract	7.48	11.39

Table 6. Fungicidal and fungistatic effect of *Crinum zeylanicum* leaf extracts

Agent pathogen	Extracts (120 $\mu\text{L mL}^{-1}$)	Activities
<i>Cercospora malayensis</i> (Isolate 1)	Methanol extract	Fungicide
	Aqueous extract	Fungistatic
	Acetone extract	Fungicide
<i>Cercospora malayensis</i> (Isolate 2)	Methanol extract	Fungicide
	Aqueous extract	Fungistatic
	Acetone extract	Fungicide

DISCUSSION

Pure isolates of *C. malayensis* obtained from samples collected in Akonolinga (Isolate 1) and Meyomessala (Isolate 2) showed morphological characteristics similar to those of *C. malayensis* F. Stevens & Solheim, the causal agent of Cercosporiosis of okra. In Petri dishes, the macroscopic characteristics of *C. malayensis* filaments show white, cottony mycelia on both the top and bottom surfaces. Indeed, Farrag (2011), Ju et al. (2020), Park et al. (2017), and Chai et al. (2021) describe *C. malayensis* mycelial filaments as off-white, flat and aerial colonies. Microscopic study shows continuous, elongated conidia, truncate to obconical at the base, with three to eight septa, and subacute to obtuse at the apex. Ju et al. (2020) working on the first report of *C. malayensis* causing leaf

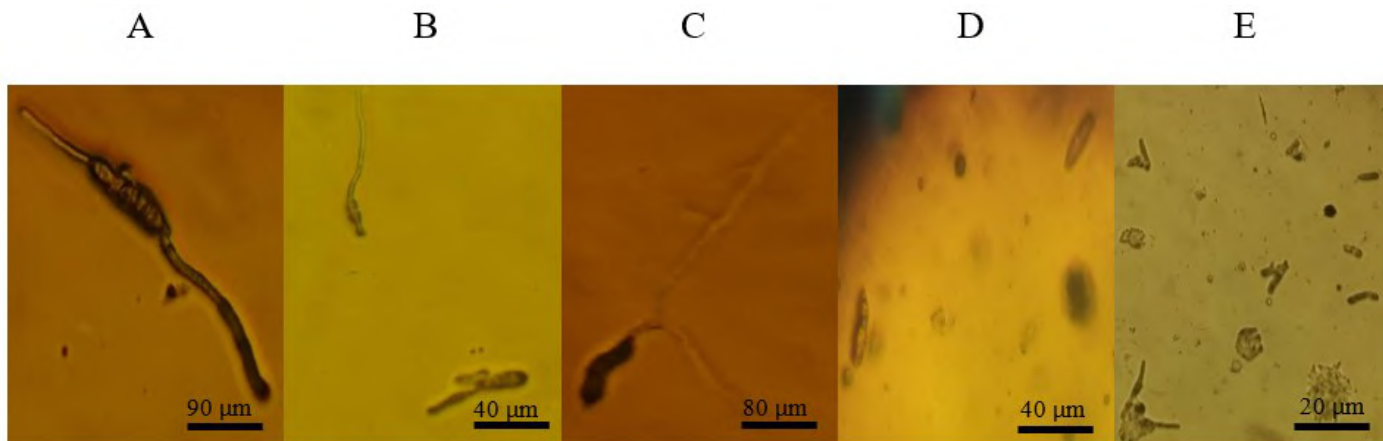


Figure 6. Effect of extracts on germination of *Cercospora malayensis* conidia; A, B and C: Germination of conidia in control; D: Conidia not germinated at concentration C4 = 120 $\mu\text{L mL}^{-1}$ in methanol extract and E: in aqueous extract of *Crinum zeylanicum* leaves.

spots on okra in Korea described the conidia as acicular, hyaline, subacute to obtuse at the apex, truncate to obconic at the base, 2 to 20 septate, with a swollen hilum at the tip, and measuring 34 to 280×3.4 to $6.5 \mu\text{m}$. Also, Crous and Braun (2003) and Świdarska-Burek (2015) present conidia as singly or in acropetal chains, amero to scolecosporeous and pigmented or hyaline.

Phytochemical screening of *C. zeylanicum* leaf extracts revealed the existence of multiple chemical families, including: terpenes, sterols, phenols, flavonoids, oils, sugars, saponins, alkaloids. These families of secondary compounds have been listed in numerous works as possessing antifungal activity (Rosen & Stein-Gold 2016; Ramírez-Gómez et al. 2019; Yusoff et al. 2020; Zulbayu et al. 2021; Sukdee 2023). Phenols, terpenes and flavonoids disrupt pathogen membranes, inactivate enzymes, bind to adhesins, and create complexes with the cell wall. Tannins bind to proteins, inhibit enzymes and alkaloids embed themselves in the cell wall (Gurjar et al. 2012). GC-MS results of *C. zeylanicum* leaf extracts show the presence of numerous chemical compounds that exhibit biocidal activity. The difference in the detection of chemical compounds in *C. zeylanicum* leaf extracts would stem from the polarity of the solvents used. Ngo et al. (2017) show that differences in solvent polarity can help explain differences in the solubility of plant active ingredients. The most dominant compounds being Quinoline-7-carboxylic acid, 2-phenyl-, methyl ester (26.63 %), 9,12-Octadecadienoic acid (Z,Z) (10.83 %) and 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (10.37 %) in acetone extract, 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (16.89 %), Phytol (11.78 %) in methanol extract and n-Hexadecanoic acid (35.04 %), Cis-Vaccenic acid (31.76 %) in the aqueous extract. Studies have shown that the compounds present in *C. zeylanicum* leaf extracts have antifungal, insecticidal and antibacterial activity (VasudhaUdupa et al. 2021; Toka et al. 2023; Dida et al. 2024).

Crinum zeylanicum leaf extracts tested at different concentrations against *C. malayensis* significantly inhibited its mycelial growth. Concentrations C3=60 $\mu\text{L mL}^{-1}$ and C4=120 $\mu\text{L mL}^{-1}$ of the methanol and acetone extract and C4=120 $\mu\text{L mL}^{-1}$ of the aqueous extract inhibited 100 % of the mycelial growth of isolates 1 and 2, as did the synthetic fungicide Metalaxyl 80 g kg^{-1} and Mancozeb 640 g kg^{-1} . Tram et al. (2002) reported on the biological activities of bulbs, leaves, roots and flowers of the *Crinum* genus. The authors showed that *C. zeylanicum* bulbs are rich in alkaloids, which regulate plant growth and are active against phanerogamic parasites. Also, the antifungal properties of leaf extracts from the leaves of this plant could be due to chemical compounds possessing biological properties (Berkov et al. 2011). Studies have shown that certain chemical compounds (n-Hexadecanoic acid, Octade-

canoic acid, Hexadecanoic acid, methyl ester, Bicyclo [3.1.1] heptane, 2,6,6-trimethyl-, 9,12-Octadecadienoic acid (Z,Z)- etc.) possess antifungal properties (Alawode et al. 2021; Vuerich et al. 2023). The precise way that chemical substance work present in plant extracts is unclear, but it is likely that these chemical compounds form complexes with polysaccharides and proteins associated with an outer layer of fungal cells, which can lead to destabilization of cell membrane function, resulting in pathogen death (Rongai et al. 2017). In contrast, Yoshimi et al. (2022) and Da et al. (2019) demonstrated that plant extracts rich in chemical compounds disrupt the synthesis of β -1,3-glucan, an integral part of many fungi's cell walls of many fungi.

The low minimal inhibitory concentrations (MIC50 and MIC90) confirmed the efficacy of *C. zeylanicum* leaf extracts. Jasso de Rodríguez et al. (2006) pointed out that the inhibitory capacity of plant extracts results from differences in the composition and concentration of bioactive compounds in the plant. The fungicidal and fungistatic activity of extracts at a concentration of 120 $\mu\text{L mL}^{-1}$ showed that only the aqueous extract was fungistatic, while the methanol and acetone extracts were fungicidal. Indeed, Boli et al. (2021), Toka et al. (2023), and Dida et al. (2024) report fungicidal activity with organic extracts of *Anona muricata*, *Azadirachta indica*, *Balanites aegyptiaca*, *Thevetia peruviana* against *Cercospora malayensis*, *Fusarium oxysporum* and *Phytophthora infestans*. *C. zeylanicum* leaf extracts inhibited conidial germination of *C. malayensis* isolates 1 and 2 with no germination at the concentration of 120 $\mu\text{L mL}^{-1}$ of organic, aqueous and synthetic fungicide extracts. Reports by Abayhne and Chauhan (2016) showed that the methanol extract and ethanol extract of *Cymbopogon citratus* and *Datura stramonium* directly inhibit germination of *P. infestans* zoospores. Rashid et al. (2004) also showed that aqueous extracts of *Azadirachta indica* inhibited germination of *Phytophthora infestans* sporangia. According to Baltussen et al. (2020), conidial germination is a fundamental stage in fungal development. Due to their high content of secondary compounds, the extracts render conidial germination conditions unfavorable, keeping them dormant.

CONCLUSION

The goal of this study is to use leaf extract from *Crinum zeylanicum* to regulate the growth of *Cercospora malayensis* *in vitro*. Extracts of *C. zeylanicum* leaves revealed the presence of terpenes, sterols, phenols, saponins, flavonoids, oils, sugars, and alkaloids. GC-MS analysis of the extracts unveiled a wealth of chemical compounds with biocidal activity. The extracts inhibited mycelial growth and conidial germination of *C. malayensis* similar to the synthetic fungicide at a concentration of 120 $\mu\text{L mL}^{-1}$. The lowest MICs were recorded with the methanol and acetone extracts. *C. zeylanicum* leaf extracts can be used for integrated crop disease control strategy.

AUTHOR CONTRIBUTION

Ndongo B., Conceptualisation; Akong M.E. Original draft preparation; Ngatsi P.Z., Formal Analysis data, Writing –Review- Editing; Kuate W.N.T., Methodology.

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CONFLICT OF INTEREST

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

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Research Article

Genetic and Morphometric Variation of the Genus *Nyctixalus* in Indonesia Based on mtDNA 16S rRNA Gene

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ABSTRACT

The genus *Nyctixalus* was first described by Boulenger in 1882. Previous systematic studies of this group primarily focused on its relationships with other rhacophorid frogs and did not explore inter- and intraspecific variation in detail. This study aimed to determine the position of Indonesian species within their genus. We used DNA barcoding of the mitochondrial 16S rRNA gene and conducted a morphological analysis. Mitochondrial genealogies were constructed based on NJ, ML, and BI analyses, while uncorrected p-distance was also calculated. The Mann-Whitney U-test and PCA were used to analyse variations among species for each character component. Dissimilarity may contribute to measurable group structure. DNA barcoding revealed that *Nyctixalus* forms a monophyletic group. The PCA analysis revealed that *N. margaritifer* population from Java formed a group, while *N. pictus* could not be separated on the scatter plot. DNA barcoding revealed that *Nyctixalus* forms a monophyletic group. The genetic distance within the population of *N. margaritifer* (0–1.52 %) and *N. spinosus* (0–0.2 %) showed a variation of intraspecific genetic distance. However, in the two allopatric populations of *N. pictus* Kalimantan and Sumatra (p-distance = 4.3–4.6 %), there are high variations (6.9–9 %) between populations from Sumatra and Malaysia. These findings suggest that *N. anodon* van Kampen, 1907, from Kayu Tanam, Sumatra Barat, Indonesia, represents a distinct species from *N. pictus* Peters, 1871, originally described from Sarawak, Malaysia.

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INTRODUCTION

Genus *Nyctixalus* was first described based on adult morphological specimens of *Nyctixalus margaritifer* obtained from the *East Indies* (Boulenger 1882). However, the systematic status of the genus was not renewed and subsequent studies focused on the division of the family Ranidae (Boulenger 1888; Boulenger & Robinson 1912). There was also no discussion or improvement of the systematics of the genus, although some investigations used the genus as part of the identification keys of the family Ranidae (Cope 1889; Palacký 1898; Gadow 1901; Roux 1905).

Initially, genus *Nyctixalus* consisted of species *N. margaritifer* (Boulenger 1882) and *N. robinsoni* (Annandale 1917) (Annandale 1917; van Kampen 1923). *N. robinsoni* has a morphology of pupil shape with the same horizontal line axis as the specimen *Philautus aurifasciatus* (Schlegel), but it is not re-registered as part of genus *Nyctixalus* (Smith 1931). The discovery of *Ixalus pictus* (Peters 1871), *I. flavosignatus* (Boettger 1893), *Rhacophorus anodon* (van Kampen 1907), and *Hazelia spinosus* (Taylor 1920), also led to changes in both the nomenclature and the number of species within the genus.

The *Nyctixalus*, which was initially transferred into the genus *Philautus*, also lacked vomer teeth (Smith 1931; Inger 1966). Similarities among *N. pictus*, *N. spinosus* and *N. margaritifer* include a warty dorsum, ossified scalp, and bony prominences along the frontoparietal region (Smith 1931; Inger 1966; Liem 1970). However, *N. margaritifer* can be easily distinguished from *N. pictus* and *N. spinosus* by toes half-webbed skin (Boettger 1893; Taylor 1920). In contrast, *N. margaritifer* has toes with a slight web at the base that extends as a narrow fringe along the sides (Boulenger 1882; van Kampen 1923).

Their life cycles are similar, eggs hanging on a wooden wall filled with water (Taylor 1962). Subsequently, the genus was re-classified as *Hazelia* (Taylor 1962; Liem 1970) and *Edwardtayloria* (Marx 1975). The BMNH specimen 1885.12.31.35, a male adult obtained from Mount Willis (Java) by V. Huegel, was designated as a neotype of *N. margaritifer* (Boulenger 1882). These species were further re-classified as *Nyctixalus* (Dubois 1981; Segura-Delorme et al. 2005).

Recent molecular studies that utilized various genes, including 12S rRNA, tRNA val, and 16S rRNA mtDNA (Poyarkov et al. 2015; Sivongxay et al. 2016; Poyarkov et al. 2018; Chen et al. 2019), as well as cytochrome oxidase subunit I (COI), nuDNA brain-derived neurotrophic factor (BDNF), rhodopsin (RHO), seventh in absentia (SIA), and tyrosinase (TYR) (Chunskul et al. 2021) suggested that there may be significant genetic divergence within the *N. pictus* species.

The mitochondrial 16S rRNA gene has been widely utilized for DNA barcoding in amphibian research around the world. This approach is favoured due to the extensive reference database available for amphibians, which is significantly rich in taxa (Hebert et al. 2003; Vences et al. 2012) and has proven effective in identifying anuran species (Dubeux et al. 2022). The primary aim of this study was to conduct a comprehensive analysis of *Nyctixalus* samples collected from various locations throughout its distribution range. This involved employing DNA barcoding techniques using the 16S rRNA fragment, alongside morphometric analysis to better understand the systematic relationships of species within the genus, particularly in the context of Indonesia.

MATERIALS AND METHODS

Materials

This study examined 15 adult specimens of *N. margaritifer* (nine from West Java, six from Central Java) and 20 specimens of *N. pictus* (three from Aceh, four from North Sumatra, two from West Sumatra, one from Jambi, four from Bengkulu, one from West Kalimantan, three from East Kalimantan, and two

from North Kalimantan) housed in Museum Zoologicum Bogoriense (MZB), National Research and Innovation Agency (BRIN), and a specimen from the Mt. Wilis East Java stored in Universitas Negeri Malang (Priambodo et al. 2021). A total of 31 partial 16S mtDNA sequences were analysed, including sequences from *Nyctixalus*, *Rhacophorus reinwardtii*, *Chiromantis doriae*, *Theloderma lacin*, *Theloderma horridum*, and *Theloderma pseudohorridum* (outgroup), as well as several populations of *N. margaritifer* (Table 1).

Methods

Morphological Analysis

Morphometric analysis included precise measurement of 24 characters using a dial calliper, calibrated to an accuracy of 0.01 mm (Matsui 1984; Matsui et al. 2013). The specific morphometric characters measured include: Snout-vent length (SVL): the distance from the tip of the snout to the vent. Tibia length (TL): the distance from the external margin of the knee joint to the external margin of the heel articulation. Tarsal length (TR): the distance from the external margin of the heel articulation to the proximal edge of the inner metatarsal tubercle. Femur foot length (F): The distance from the midpoint of the cloacal gap to the external margin of the knee joint, measured with the thighs and shins oriented perpendicular to the body axis First toe length (T1): the distance from the distal edge of the inner metatarsal tubercle to the tip of the first toe. Fourth toe length (T4): measured from the distal edge of the inner metatarsal tubercle to the tip of the fourth toe. Third toe length (T3): determined from the distal edge of the inner metatarsal tubercle to the tip of the third toe. Fourth toe disc diameter (TD4): the horizontal width of the fourth toe disk. Upper arm length (H) and forearm length (A): these measurements contribute to an understanding of limb proportions. Wrist width (Lt. m): this metric assesses the structural integrity of the limbs. Third finger length (F3): measured from the distal edge of the palmar tubercle to the tip of the third finger. Third finger disc diameter (F3D): the diameter representing the horizontal width of the third finger disk. Second finger length (F2): measured from the distal edge of the palmar tubercle to the tip of the second finger. Head length (HL): the distance from the tip of the head to the posterior margin of the tympanum. Head width (HW): the widest distance across the head, measured at the commissure of the jaws. Additionally, distances were measured for posterior eyes distance (EPD), inter-orbital distance (IOD), anterior eyes distance (EAD), inter-nostril distance (IND), eye-nostril distance (EN), eye diameter (EY), and horizontal tympanum diameter (TD) (Figure 1).

To compare the variations among the 24 characters, ratio values of morphometric datasets were calculated by dividing each measurement by the standard length (SVL) to minimize the body size influence (Munir et al. 2018). The Mann-Whitney U test assessed variance between species for each character, while principal components analysis (PCA) examined potential group structures. Characters were analysed using the Bartlett test for homogeneity of variance, with significance set at $p < 0.05$. Predictor variables from PCA were retained based on the Kaiser-Meyer-Olkin measure (>0.5) and eigenvalues (>1) (Kaiser 1960; Gabriel 1971). Mean $\pm$ standard deviation (SD) was calculated for 24 characters of each species, with statistical significance set at $P < 0.05$. All analyses were conducted using R software, ensuring precision and reliability in our results (R Core Team 2019).

Molecular work

Mitochondrial 16S rRNA gene sequences were obtained from five adult *N. margaritifer* specimens collected from Mt. Salak (West Java), Mt. Slamet (Central Java), and Mt. Wilis (East Java), as detailed in Table 1. DNA extraction, amplification, and sequencing were conducted following the procedures

Table 1. Samples mitochondrial 16S rRNA gene from *Nyctizalus* and outgroup species analysed along with voucher information, collection location, and GenBank accession number.

No.	Species	Specimen vouchers	Country	Accession No.	Source
In group					
1.	<i>N. pictus</i>	FMNH231094	Malaysia: Borneo, Sabah (Lahad Datu)	GQ204726	Meegaskumbura et al. 2011
2.	<i>N. pictus</i>			AF458135	Wilkinson et al. 2002
3.	<i>N. pictus</i>	FMNH 231095	Malaysia: Borneo, Sabah (Lahad Datu)	DQ283133	Frost et al. 2006
4.	<i>N. pictus</i>			AF268255	Wilkinson et al. 2002
5.	<i>N. pictus</i>	KUHE53517	Malaysia: Borneo, Sarawak, Bario	LC012863	Nguyen et al. 2015
6.	<i>N. pictus</i>	NMBE1056413	Malaysia: Borneo, Sarawak, Batang Ai_National Park	JN377342	Das & Haas 2010
7.	<i>N. pictus</i>	MVZ239460	Indonesia: Sumatra, Bengkulu	GQ204732	Meegaskumbura et al. 2011
8.	<i>N. pictus</i>	AH07001	Malaysia: Borneo, Sarawak, Mt. Mulu National Park	KU561880	Dever 2017
9.	<i>N. pictus</i>			GU154888	Das & Haas 2010
10.	<i>N. pictus</i>	ZFMK 61922	Indonesia: Borneo, West Kalimantan, Kapuas Hulu, Rantau Prapat	AF215349	Vences 1999
11.	<i>N. pictus</i>	ZCAK SEA0005	Indonesia	LC640580	Kambayashi et al. 2022
12.	<i>N. pictus</i>	MNHN 1999.7718	Thailand: Phang-Nga Province, Takva Thung District Raman Forest Park	AY880502	Segura-Delorme & Dubois 2004
13.	<i>N. pictus</i>	CAS247868	Myanmar: Tanintharyi	KU244380	Dever 2017
14.	<i>N. spinosus</i>	ACD 1043	Philippines: Mindanao	DQ283114	Frost et al. 2006
15.	<i>N. spinosus</i>	Nsp1	Philippines: Mindanao	KT461916	Poyarkov et al. 2015
16.	<i>N. spinosus</i>	ACA 940	Philippines: Mindanao	AF458136	Wilkinson et al. 2002
17.	<i>N. margaritier</i>	MZB.Amph.33355	Indonesia: West Java, Mt. Salak, Sukamantri Village, Taman Sari, Kabupaten Bogor	PP987095	This study
18.	<i>N. margaritier</i>	MZB.Amph.33356	Indonesia: West Java, Mt. Salak, Sukamantri, Taman Sari, Kabupaten Bogor	PP987094	This study
19.	<i>N. margaritier</i>	MZB.Amph. 33353	Indonesia: Central Java, Mt. Slamet, Kabupaten Banjarnegara District	PP986996	This study
20.	<i>N. margaritier</i>	MZB.Amph. 33354	Indonesia: Central Java, Mt. Slamet, Kabupaten Banjarnegara	PP987054	This study
21.	<i>N. margaritier</i>	KUHE:26135	Indonesia: Central Java, Mt. Ungaran	LC012864	Nguyen et al. 2015
22.	<i>N. margaritier</i>	BP UM11	Indonesia: East Java, Mt. Wilis, Sigogor Pupus Village, Ngebel Subdistrict, Ponorogo District	PP987108	Priambodo et al. 2021
23.	<i>N. margaritier</i>	NK2114	Indonesia: East Java, BTSNP, Senduro, Mt. Semeru	OQ555086.1	Hanifa et al. 2023
24.	<i>N. margaritier</i>	NK2122	Indonesia: East Java, BTSNP, Senduro, Mt. Semeru	OQ555087.1	Hanifa et al. 2023
25.	<i>Nyctizalus sp.</i>	CAS247492	Myanmar	KU561882	Dever 2017

Table 1. Contd.

No.	Species	Specimen vouchers	Country	Accession No.	Source
26.	<i>Nyctizalus</i> sp.	CAS247476	Myanmar	KU561881	Dever 2017
Out group					
27.	<i>C. doriae</i>	FMNH 255213	Laos: Huaphahn Province, Vieng Tong District, Phou Louey National Biodiversity Conservation Area, nr. Nam Puong River	DQ28315	Frost et al. 2006
28.	<i>R. reinwardtii</i>	US 16179	Indonesia: Patuha, Bandung, West Java	KX398930.1	O'Connell et al. 2016
29.	<i>T. lacin</i>	KUHE 19426	Thailand: Nakon Sri Tamarat	LC012859	Nguyen et al. 2015
30.	<i>T. horridum</i>	KUHE 52582	Malaysia: Negeri Sembilan, Kenaboi	LC012861	Nguyen et al. 2015
31	<i>T. pseudohorridum</i>	MZB:Amph 23883	Indonesia: Central Java, Nusa Kambangan Island	ON571716.1	Kurniawan et al. 2023

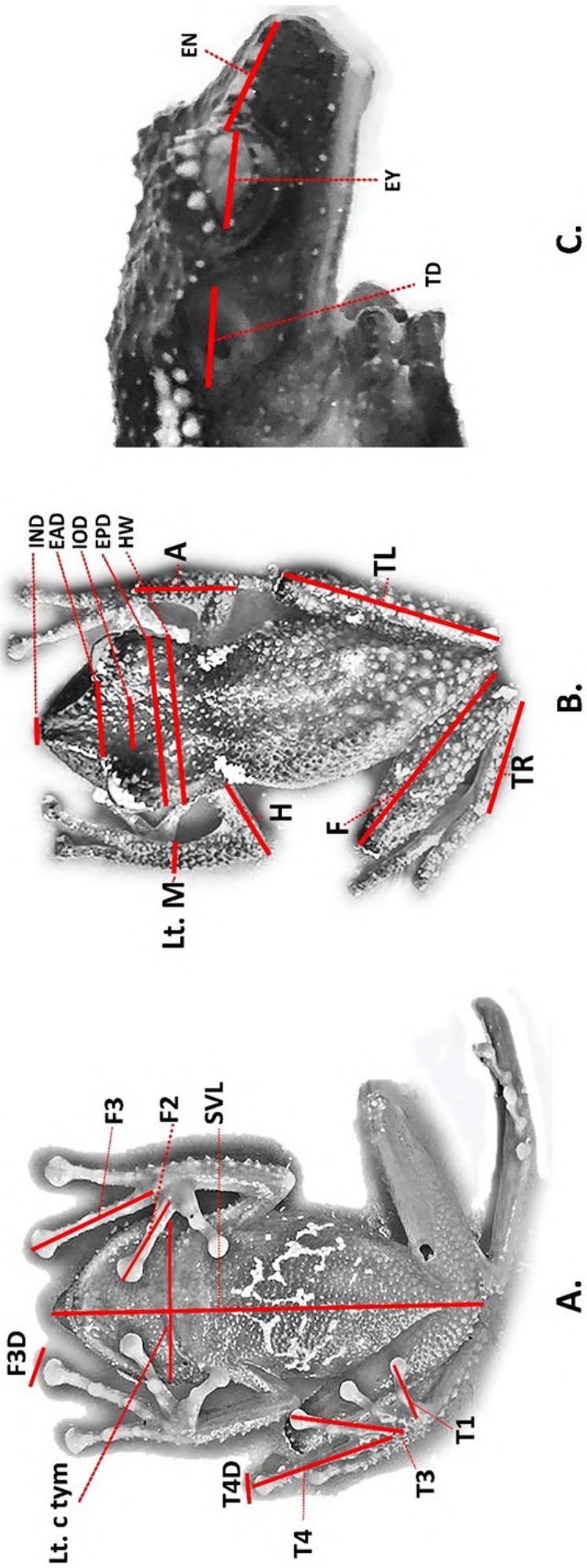


Figure 1. An illustration showing physical location for the 24 morphometric measurements (A) ventral, (B) dorsal, (C) lateral view. See the main text for the definitions of these characters.

outlined by Sambrook et al. (1989), Matsui et al. (2010), Hamidy et al. (2012), and Munir et al. (2018).

Total DNA was extracted from small amounts of tissue diluted in a buffer solution using the standard phenol-chloroform method. DNA amplifications of the 16S rRNA gene were carried out in polymerase chain reaction (PCR) using the primer set 16L-1 (CTGACCGTGCAAAGGTAGCGTAATCACT) and primer 16H-1 (CTCCGGTCTGAACTCAGATCACGTAGG) (Hedges 1994) to obtain a round 540 bp-length fragment of the gene. The PCR reactions were performed in 30 µL total volumes comprised of 1 µL DNA template, 3 µL PCR buffer (NH₂SO₄), 0.6 µL dNTP, 4 µL MgCL₂, 1 µL of each primer, 0.5 µL Bovine serum albumin (BSA), 0.2 µL *Taq* DNA polymerase with appropriate buffer and ddH₂O to volume.

The PCR process began with an initial denaturation phase at 94 °C for 5 minutes. This was followed by 33 amplification cycles, each consisting of three key phases: denaturation at 94 °C for 30 seconds, annealing the primer for 30 seconds at temperatures between 48 °C and 50 °C, and extension at 72 °C for 1 minute and 30 seconds. For DNA sequencing, BigDye® Terminator reagents from 1st Base Asia were used to sequence the PCR products in both directions with the same primers to ensure the accuracy. Sequence chromatograms were checked, edited, and assembled using ChromasPro software (Technelysium Pty Ltd., Tewantin, Queensland, Australia).

MAFFT was used for sequence alignment with E-INS-I parameters (Katoh et al. 2002). MEGA X performed the Neighbor Joining (NJ) analysis and calculated the uncorrected p-distance (Kumar et al. 2018). The NJ tree was generated with 1,000 replications using the two-parameter Kimura model, applying pairwise deletion for gaps (Kimura 1980). A genetic distance of ≥ 3 % was set for species differentiation (Fouquet et al. 2007). The Maximum Likelihood (ML) analysis was performed using the IQ-Tree Web Server, incorporating ultrafast bootstrap replicates (Trifinopoulos et al. 2016). The general time-reversible (GTR) model, featuring empirical base frequencies (+F) and four discrete gamma shape rate categories (+G4), was selected as the model of evolution that is based on the Bayesian Information Criterion (BIC).

A Bayesian Inference (BI) analysis was conducted using MrBayes version 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist et al. 2012). This analysis employed a Monte Carlo Markov Chain (MCMC) approach over 20 million generations, with sampling occurring every 1,000 generations. The GTR+G model was chosen based on the Akaike Information Criterion (AIC). Bootstrap values of 70 % or higher were considered significant for confirming the nodes (Huelsenbeck & Hillis 1993; Leaché & Reeder 2002; Minh et al. 2013). The optimal models for both maximum likelihood (ML) and Bayesian inference (BI) analyses were determined using jModelTest, ensuring reliable and accurate results (Posada 2008; Darriba et al. 2014). The resulting trees were visualized using FigTree. v1.4.3 (Rambaut 2016).

RESULTS AND DISCUSSION

The morphometric analysis results revealed no significant difference in morphological character between *N. pictus* specimens from Sumatra and Kalimantan ($P > 0.05$). A comparison of morphology character ratios between *N. margaritifer* and *N. pictus* from Sumatra, Indonesia, showed no significant differences ($P > 0.05$) in the characters T1, T4, T3, H, A, Lt. c. tym, EN, EY, TD, and HW. Similarly, the comparison morphology character ratios with *N. pictus* from Kalimantan presented no significant variances among characters T4, T3, H, F3D, A, EPD, IOD, IND, EN, EY, TD, and HW (Table 2).

Morphometric analysis indicated no significant differences in morphological characters between *N. pictus* specimens from Sumatra and Kalimantan ($P > 0.05$). A comparative analysis of morphological ratios between *N. margaritifer* and *N. pictus* from Sumatra revealed no significant differences ($P > 0.05$) in the characters T1, T4, T3, H, A, Lt. c. tym, EN, EY, TD, and HW. Furthermore, no significant differences were observed in the following morphological measurements among *N. pictus* specimens from Kalimantan: T4, T3, H, F3D, A, EPD, IOD, IND, EN, EY, TD, and HW as seen in Table 2.

The PCA analysis used characters based on the Kaiser-Meyer-Olkin (KMO) test results. The overall KMO value was 0.73, derived from the morphological characters SVL, TL, T1, TD4, left wrist (lt. M-wrist), F3, and F2. The Bartlett test results of the ratio of morphological characters showed that variances were equal across groups (homogeneous) as indicated by $P = 0.000$ ($P < 0.05$). PCA analysis used PC1 and PC2 components with eigenvalues > 1 , as shown in Table 3. The results showed a PC I of 53.41 %, which had positive contribution values for SVL, F3, and F2. Meanwhile, PC II of 15.32 % had positive contribution values for TL, T1, TD4, and Lt. M. These two principal components in Table 3 explained 68.73 % of the morphometric variation.

Furthermore, the PCA analysis showed that the *N. margaritifer* populations were separated from *N. pictus* from Kalimantan, while there was a slight overlap between the populations of both species from Sumatra. However, the *N. pictus* population from the two regions did not show a clear separation (Figure 2).

The alignment in this study consisted of 31 sequences (Table 1) with targeted gene fragment of ~542 bp. The phylogeny tree reconstruction using NJ, ML, and BI methods revealed no significant differences in tree topology (Figure 3, S1, S2), with consistent genetic distances and bootstrap values observed among the tree branches between populations.

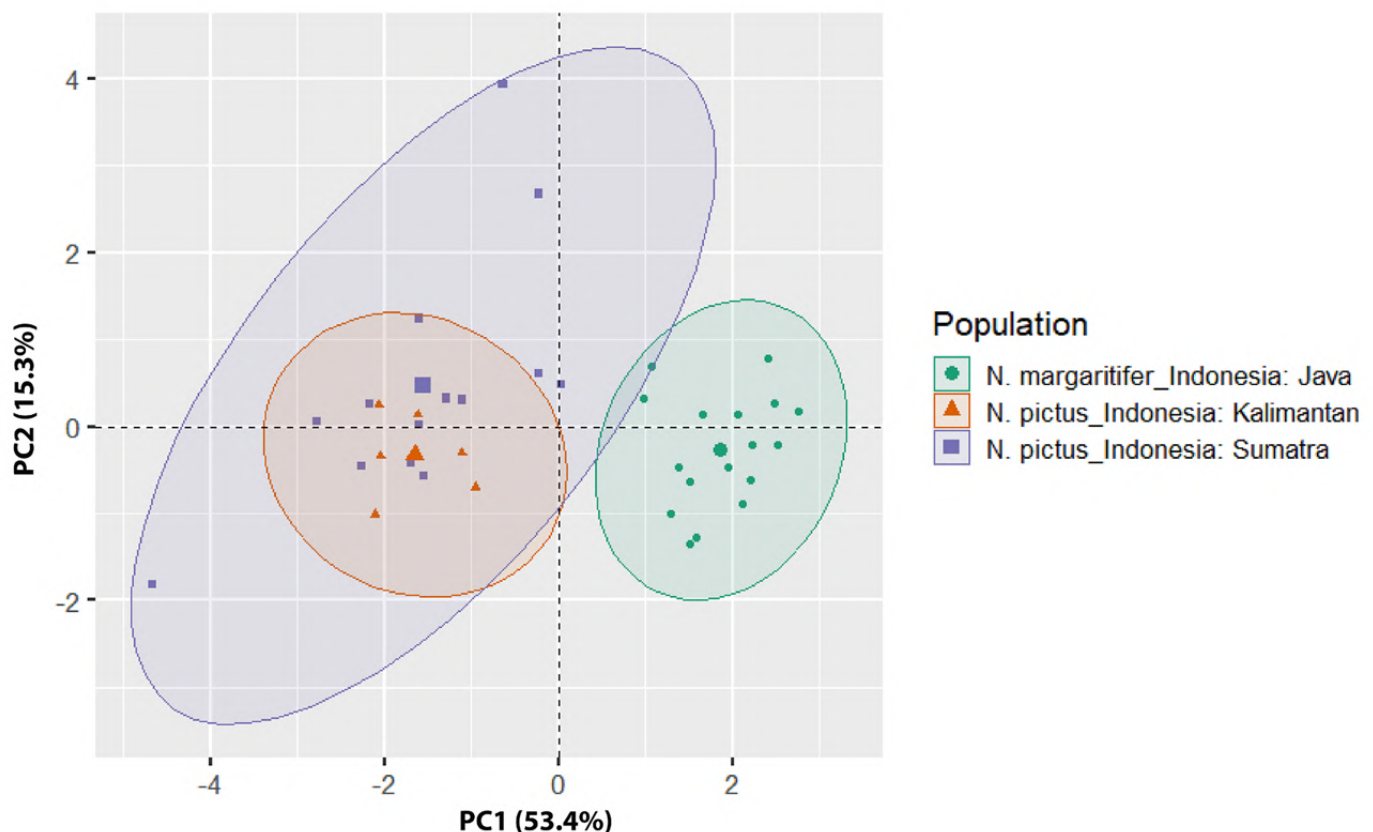


Figure 2. Principal Component Analysis plot on seven size-corrected morphological characters *Nyctixalus* from Indonesia.

Table 2. Measurements morphological character male *N. margaritifer* and *N. pictus*. SVL (Mean ± SD, in mm). **P* < 0.05 See text for character abbreviations.

Body part	Measurement			U test		
	<i>N. margaritifer</i> Indonesia: Java (Nm_J) n = 17	<i>N. pictus</i> Indonesia: Sumatra (Np_S) n = 14	<i>N. pictus</i> Indonesia: Kalimantan (Np_K) n = 6	Nm_J vs Np_S	Nm_J vs Np_K	Np_S vs Np_K
SVL	42.03 ± 2.03	31 ± 2.02	31.87 ± 2.09	0.000*	0.000*	0.621
TL	22.02 ± 0.92	17.82 ± 1.01	18.53 ± 1.52	0.000*	0.001*	0.409
TR	12.54 ± 1.16	9.92 ± 0.84	10.22 ± 0.56	0.010*	0.021*	0.680
F	19.05 ± 1.51	15.55 ± 1.25	16.33 ± 1.3	0.000*	0.002*	0.284
T1	4.08 ± 0.26	2.9 ± 0.67	2.79 ± 0.14	0.234	0.006*	0.409
T4	12.66 ± 1.01	9.1 ± 0.94	9.59 ± 0.59	0.405	0.944	0.621
T3	9.3 ± 1.02	6.81 ± 0.57	7.05 ± 0.52	0.937	0.889	0.564
TD4	1.56 ± 0.19	0.96 ± 0.16	0.97 ± 0.17	0.004*	0.006*	0.741
H	8.24 ± 0.51	5.64 ± 0.82	5.96 ± 1.04	0.062	0.107	0.741
A	10.28 ± 0.89	7.42 ± 0.73	7.78 ± 0.99	0.190	0.674	0.621
lt. M	2.19 ± 0.27	1.32 ± 0.29	1.17 ± 0.17	0.002*	0.001*	0.099
F3	11.15 ± 0.73	6.89 ± 0.78	7.39 ± 0.78	0.000*	0.002*	0.284
F3D	2.2 ± 0.44	1.31 ± 0.25	1.7 ± 1.15	0.004*	0.123	0.934
F2	6.88 ± 0.64	4.37 ± 0.69	4.66 ± 0.81	0.000*	0.010*	0.869
HL	14.17 ± 1.43	8.1 ± 0.52	8.52 ± 0.92	0.000*	0.002*	0.680
Lt.c. tym	12.3 ± 0.89	9.41 ± 0.91	10.12 ± 0.66	0.062	0.003*	0.117
EPD	13.04 ± 0.89	9.21 ± 0.53	9.67 ± 0.8	0.015*	0.363	0.138
IOD	6.26 ± 0.49	4.4 ± 0.43	4.4 ± 0.26	0.017*	0.093	0.458
EAD	7.21 ± 0.47	5.62 ± 0.51	6.02 ± 0.5	0.012*	0.008*	0.409
IND	2.34 ± 0.39	1.52 ± 0.23	1.58 ± 0.22	0.017*	0.161	0.805
EN	5.06 ± 0.55	3.82 ± 0.4	4.14 ± 0.35	0.302	0.107	0.187
EY	4.81 ± 0.41	3.5 ± 0.34	3.32 ± 0.37	0.874	0.141	0.138
TD	3.04 ± 0.68	2.51 ± 0.27	2.48 ± 0.24	0.112	0.441	0.409
HW	14.74 ± 0.6	11.38 ± 1.25	11.64 ± 1.1	0.074	0.123	0.458

Table 3. Variabel PC1 and PC2 morphological character male *N. margaritifer* and *N. pictus*. Bold figures indicate the highest loadings.

Variable	PC1	PC2
Snout-vent length (SVL)	0.4647223	-0.32533093
Tibia length (TL)	-0.4100977	0.30999856
First toe length (T1)	0.2565834	0.69352190
Fourth toe disc diameter (TD4)	0.3160115	0.32218328
Wrist width (Lt. M)	0.3574805	0.36893181
Third finger length (F3)	0.4258582	-0.27430144
Second finger length (F2)	0.3755093	-0.04397765
Eingen value	1.9335	1.0357
% of varians	53.41	15.32
Cumulative varians (%)	53.41	68.73

The phylogeny tree revealed a significant discovery *Nyctixalus* (clade *Nyctixalus* genus) is in a monophyletic position against the outgroup. This finding, a cornerstone of our research, underscores the unique evolutionary path of the *Nyctixalus* genus. In the clade, genus *Nyctixalus* formed a monophyletic group divided into two subclades (NJBS = 88 %, UFB = 94 %, and BPP = 1.00). The subclade I consisted of *N. margaritifer* and *N. pictus* groups (NJBP = 90 %, UFB = 83 %, BPP = 0.96), while subclade II consisted of *N. spinosus* from the Philippines (NJBP = 100 %, UFB = 98 %, BPP = 1.00).

The population of *N. pictus* group exhibited six distinct lineages (NJBS = 90 %, UFB = 83 %, BPP = 0.96). The six lineages in *N. pictus* included *N. pictus* from Malaysia, specifically Sabah, Lahad Datu, Sarawak, Bario (Lineage 1, NJBS = 100 %, UFB = 97 %, BPP = 1.00), Mt. Mulu (Lineage 2, NJBS = 84 %, UFB = 87 %, BPP = 0.99), and Sarawak, Batang Ai, also recognized as true *pictus* (Lineage 6), Indonesia from Kapuas Hulu, West Kalimantan (Lineage 3, NJBS = 99 %, UFB = 94 %, BPP = 1.00) and Bengkulu, Sumatra (Lineage 4, NJBS = 100 %, UFB = 99 %, BPP = 1.00), Myanmar and Thailand (Lineage 5, NJBS = 99 %, UFB = 99 %, BPP = 1.00), and lineage 6 from Sarawak, Batang Ai, also known as true *pictus*.

In our results, *N. margaritifer* from Java demonstrated low genetic distances of only 0–1.5 % (Table 4) consistent with morphology characteristics. Our study results confirmed the aforementioned by Priambodo et al. (2021) and Hanifa et al. (2023). Furthermore, the rediscovery of *N. margaritifer* in Mt. Willis, East Java (Priambodo et al. 2021) also supported the identification of a topotype of *N. margaritifer* collected in Mount Willis (Java) by V. Huegel (Dubois 1981).

The genetic distances among *N. pictus* populations were substantial (*N. pictus* lineage 1–6, p-distance = 4.3–9.9 %; Table 4), strongly supporting their distinct species status. This finding is consistent with the established understanding that uncorrected p-distances exceeding 3 % between taxa often indicate distinct species (Fouquet et al. 2007). The diverse genetic distances observed among various lineages in the analyses can help clarify their distinct species relationships (Hamidy et al. 2011).

However, in the two allopatric populations *N. pictus* lineage 3 (Kalimantan) and 4 (Sumatra) (p-distance = 4.3–4.6 %), there are high variations (8.26–9.01 %) between population *N. pictus* from Sumatra and Malaysia (Table 4). It can be assumed that *N. anodon* van Kampen, 1907 from Kayu Tanam, Sumatra Barat, Indonesia, is a different species to *N. pictus* Peters, 1871 (type locality) from Sarawak, Malaysia, which has been recognized as a synonym of *N. pictus* (Smith 1931; Inger 1966).

The ancestral genus *Nyctixalus* is believed to have a significant connec-

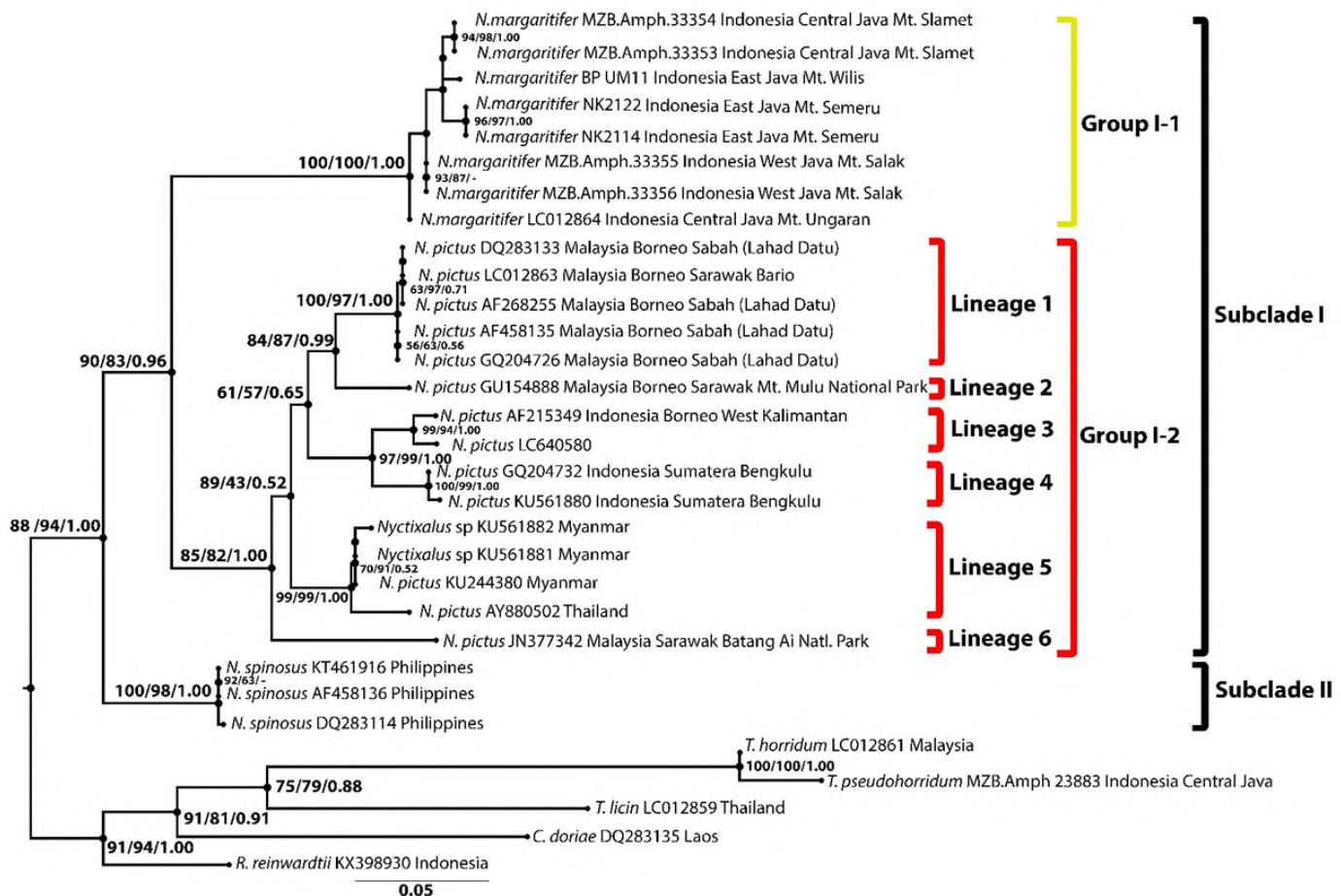


Figure 3. Maximum Likelihood phylogram showing the relationship within genus *Nyctixalus*. Values at the branch points to Neighbour Joining probability (NJB), Ultrafast Bootstrap Support (UFB), and Bayesian Posterior Probabilities (BPP).

tion to the formation of the original Borneo landmass, which was separated from the larger Sundaland area. This geological history is mirrored in another Bornean frog genus, *Leptobrachium*. In this case, the divergence between the Bornean and Philippine Clades and the other related clades from Sundaland and Asia is likely occurred during a critical period spanning the late Eocene, approximately 42 to 35 million years ago (MYBP), extending into the early Oligocene, roughly 35 to 29 million years ago (MYBP). Subsequently, the pattern is similar when the ancestor of *Leptobrachium lumadorum* from Mindanao split from entire Bornean species in early Miocene, 23– 14 MYBP (Matsui et al. 2010), and this event is likely responsible for splitting event of *N. spinosus* from the entire ancestor of *N. pictus* and *N. margaritifer*. The evolutionary history of *Nyctixalus* species provides crucial insights into the region's biodiversity and biogeography.

The ancestor *N. margaritifer* split from the common ancestor of *N. pictus* is earlier than diversification within lineages of *N. pictus*. The ancestor of *N. pictus* lineage 4 (Sumatra) split from *N. pictus* lineage 3 (Borneo) probably occurred during the Pleistocene glacial periods when all Sunda land islands were connected as one landmass (Inger & Voris 2001; Hall 2013). The high genetic variation in the *N. pictus* in Southeast Asia, especially Borneo Island, involves multiple events. The long-term continental connection between Borneo and the Malay Peninsula occurred episodically twice: once in the mid-Miocene and again in the early Pliocene (Voris 2000; Inger & Voris 2001; Inger 2005; Hall 2013). The event might have isolated the ancestor of *N. pictus* in Borneo from a common ancestor of *N. pictus* in Sumatra and Peninsular (Myanmar and Thailand).

The regions of Sumatra (Riyanto & Kurniati 2014; Hamidy & Kurniati

Table 4. Average uncorrected p-distances (%) based on 542 bp fragment of the mitochondrial 16S rRNA gene within genus *Nyctiaius*.

No	Species	1	2	3	4	5	6	7	8	9	10	11	12
1.	<i>N. pictus</i> lineage 1 (Malaysia: Borneo, Sabah (Lahad Datu) and Sarawak, Bario)	0 -0.19											
2.	<i>N. pictus</i> lineage 2 (Malaysia: Borneo, Sarawak, Mt. Mulu)	4.55-4.88											
3.	<i>N. pictus</i> lineage 3 (Indonesia: Borneo, West Kalimantan)	6.46 -7.33	6.25-7.13	1.55									
4.	<i>N. pictus</i> lineage 4 (Indonesia: Sumatra, Bengkulu)	6.94-7.64	7.56-7.84	4.27-4.63	0.41								
5.	<i>N. pictus</i> lineage 5 (Myanmar and Thailand)	4.83-7.64	5.93-7.65	7.21-10.59	6.59-9.41	0.57-2.95							
6.	<i>N. pictus</i> lineage 6 (Malaysia: Borneo, Sarawak, Batang Ai)	8.83-9.29	9.48	9.42-9.62	8.26-9.01	7.4-9.88							
7.	<i>N. margaritifer</i> (Indonesia: Java)	11.29-12.99	13.8-14.91	13.24-14.72	12.94-14.32	10.62-14.24	11.65-12.45	0 - 1.52					
8.	<i>N. spinosus</i> (Philippines)	11.3 - 12.13	13.91-14.17	12.09-12.97	12.03-13.22	10.77-13.53	10.78-11.08	11.39-13.83	0-0.2				
9.	<i>R. reindwardtii</i>	13.95-14.56	15.7	16.62-16.69	15.19-16.28	12.84-16.01	14.55	15.58-17.9	11.8-12.41				
10.	<i>C. doriae</i>	20.05-21.7	19.99	20.13-20.29	19.39-20.4	17.4-20.72	18.03	19.02-20.52	15.91-16.22	14.02			
11.	<i>T. licin</i>	15.57-16.58	17.85	17.5-17.45	18.09-18.5	15.39-17.56	20.12	18.61-20.37	17.11-17.69	15.48	19.61		
12.	<i>T. horridum</i>	20.43-21.68	24.34	24.05-24.77	23.56-24.46	19.03-22.86	23.56	20.5-22.2	18.37-19.16	18.77	20.5	17.06	
13	<i>T. pseudohorridum</i>	21.79-22.57	25.17	25.72-25.75	23.51-24.41	20.57-23.13	23.26	21.07-23.08	18.36-23.26	19.05	21.31	18.56	2.84

2015; Munir et al. 2018) and Borneo (Matsui 2015) remained unexplored and faced threats from anthropogenic activities (Eprilurahman et al. 2021). Therefore, further investigations must be conducted to evaluate the taxonomic status of selected *N. pictus* populations. These investigations should include studying the call and morphological characteristics to identify any new lineage of the *N. pictus* group. Conservation efforts are also recommended to preserve the species from extinction.

CONCLUSIONS

This study showed that *N. margaritifer* population in Java, Indonesia, had no morphological differences and genetic distance. The morphometric analysis showed minimal differences between *N. margaritifer* and *N. pictus* from Sumatra and Kalimantan in body ratios and measurements, including toe lengths (T3 and T4), upper arm length (H), forearm length (A), eye-nostril distance (EN), eye diameter (EY), tympanum diameter (TD), and head width (HW). Given the high uncorrected p-distances above 3 % between the *N. pictus* population in Sumatra and those in Malaysia, it can be assumed that *N. anodon* from Kayu Tanam, Sumatra Barat, Indonesia is a different species to *N. pictus* (type locality) from Sarawak, Malaysia. Therefore, additional research is necessary to determine the taxonomic status *N. anodon* from Kayu Tanam Sumatra and new lineages of *N. pictus* in Indonesia.

AUTHOR CONTRIBUTION

A.H., and R.I., designed the research. R.I. collected, analysed data, and wrote the manuscript. Speciment Mt. Wilis, East Java, Indonesia collected by A.M.K., B.P. Manuscript supervised by A.H., A.F., D.P-F.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in the preparation of this research article.

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APPENDICES

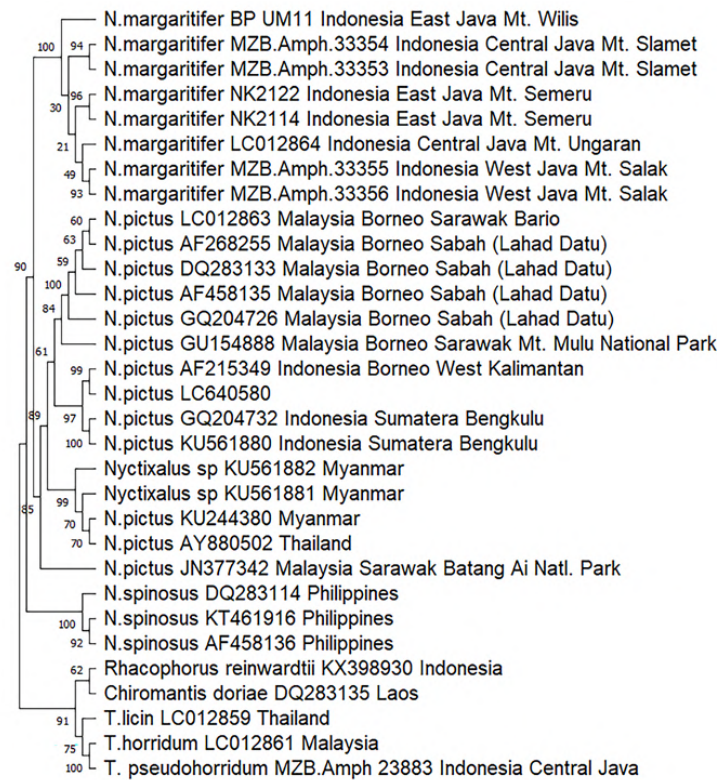


Figure S1. The neighbour-joining phylogram based on the studied 542 bp of the mitochondrial 16S rRNA gene. The value at the branch represents bootstrap support.

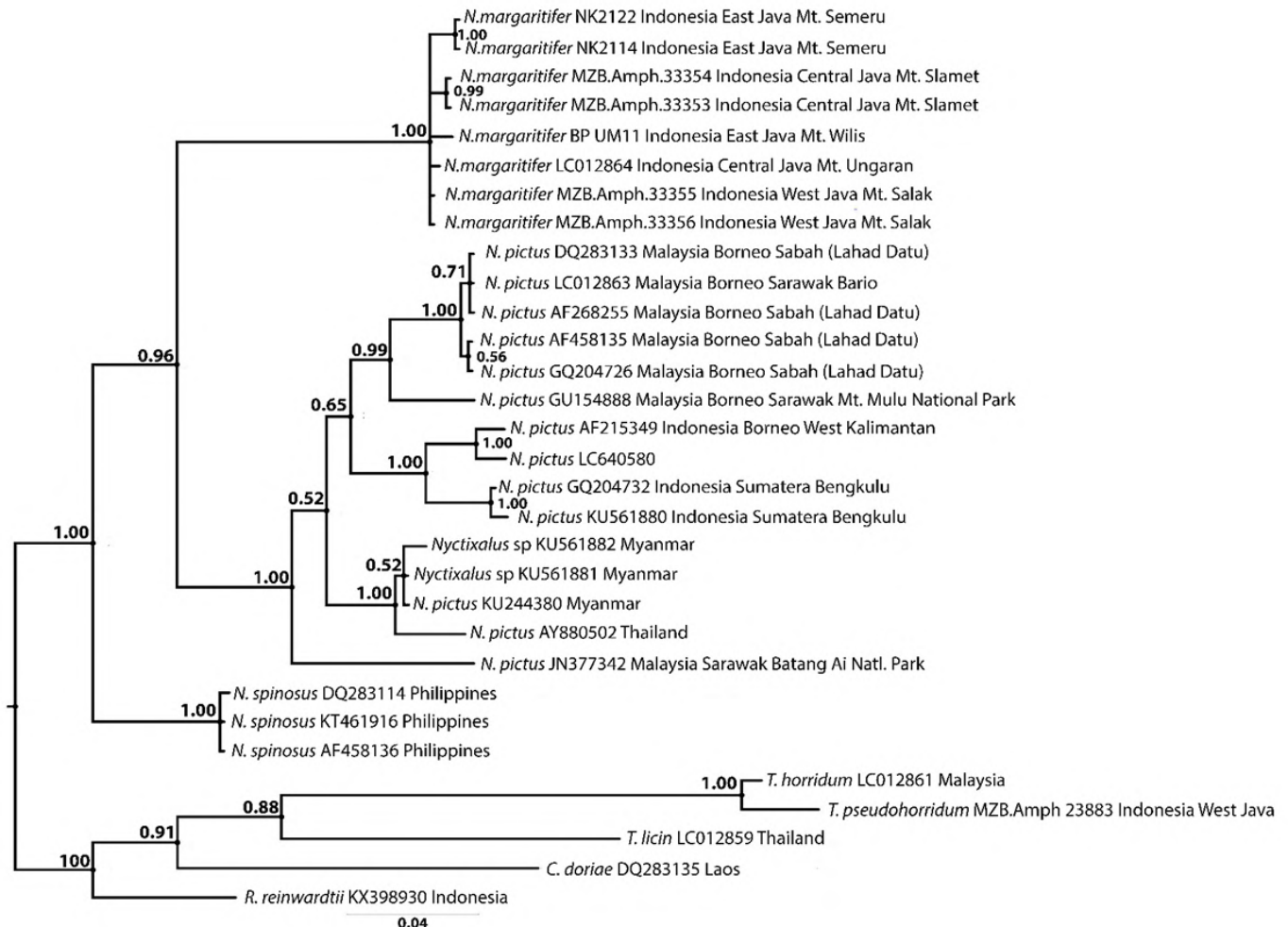


Figure S2. Bayesian consensus phylogram based on the studied 542 bp fragment of the mitochondrial 16S rRNA gene. Values at the branches indicate Bayesian Posterior Probabilities (BPP).

Research Article

Mandibular Characteristics to Determine Quaternary Proboscideans of Indonesia

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ABSTRACT

Proboscideans (order Proboscidea) are a group of mammals known for their distinctive proboscis or trunk. During the Pleistocene Epoch, proboscideans thrived globally, including the Indonesian Archipelago. Most taxonomic determination for proboscidean fossils primarily relies on molar analysis. Mandible analysis for determining Quaternary proboscidean taxa in Indonesia is still rarely utilized, despite its potential for taxonomic identification. Therefore, this research aims to identify the diagnostic characters of Quaternary proboscideans found in Indonesia based on their mandibular characteristics. Quantitative morphometrical analysis and qualitative morphological observations were conducted in determining the diagnostic characters of each taxon. Mandible specimens of modern Asian elephant (*Elephas maximus*) were also used as a comparison to fossil specimens. The values obtained by morphometric measurements were then analyzed using the Principal Component Analysis (PCA) method in PAST 4.12b. In addition, qualitative morphological characters obtained were described to further support the PCA results. The results showed that the mandibles of each taxon observed have their own distinguishable characteristics, thus can be used to determine the taxa of Quaternary proboscideans found in Indonesia.

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INTRODUCTION

Proboscideans (order Proboscidea) are commonly known by their distinctive proboscis or trunk. Today, this order is represented solely by the family Elephantidae, which includes three extant species. They are: Asian elephant (*Elephas maximus*), African savanna elephant (*Loxodonta africana*), and African forest elephant (*L. cyclotis*). Currently, the African savanna elephant and Asian elephant are considered endangered by the IUCN, while the African forest elephant is considered as critically endangered. Their populations are facing significant threats from habitat loss and fragmentation, poaching, wildlife trade, and human-elephant conflict (Riddle et al. 2010; Hammond et al. 2022; Sukmasuang et al. 2024).

During the Pleistocene Epoch, approximately 2.6 million to 11,700 years ago, large bodied mammals (megafauna) were widespread across the globe (Carlton 2019). Within the Indonesian Archipelago, fossil evidence dating back to the Pleistocene has revealed a diverse assemblage of megafauna, including giant tortoises, buffalos, hippopotamuses, tigers, hyenas, and giant pangolins (van den Bergh et al. 2001; Setiyabudi 2016). During this period, proboscideans also shaped the biodiversity of megafauna in the region. According to Wibowo et al. (2015), there are six genera of proboscideans from Pleistocene Indonesia: *Sinomastodon*, *Palaeoloxodon*, *Stegolophodon*, *Stegoloxodon*, *Stegodon*, and *Elephas*, with at least 14 described species. However, in the present day, only two proboscideans can be found in Indonesia: the Sumatran elephant (*E. m. sumatranus*) and a very small population of Bornean elephant (*E. m. borneensis*) in North Kalimantan (Riddle et al. 2010; Sharma et al. 2018; Feldhamer 2020).

The taxonomic determination of most extinct mammalian taxa primarily relies on cranial analysis, especially the teeth (Prothero & Williams 2017). Proboscideans have a unique type of molar termed lophodont (Feldhamer 2020). However, there are some differences and variations among the molars of proboscideans. The molars of three proboscidean families found in the Indonesian Archipelago each have their own characteristics. Gomphotheriidae members are characterized by low-crowned molars (brachyodont) with blunt and conical cusps arranged in transverse rows (lophs for upper molars; lophids for lower molars), termed as bunolophodont. Stegodontidae and Elephantidae members are characterized by lophodont molars in which the cusps and loph(id)s multiplied along with the thinning of the enamel to form more incised ridges. The difference between the two latter families is in their crown height. Elephantidae members evolved to have a heightened tooth crown, termed hypsodont, whereas Stegodontidae members retained the brachyodonty of gomphotheres (Zhang et al. 2017). Besides their crown height (hypsodonty), the increasing number of lophs is also a general indicator of more modern or progressive taxa (Sukumar 2003).

Proboscidean mandibles have been used to analyze their diets and feeding adaptations, mastication mechanisms, and evolutionary history (Todd 2010; Ferretti & Debruyne 2011; von Koenigswald 2016; Li et al. 2023). From a taxonomic perspective, the mandible could be a potential component in determining proboscidean taxa. Todd (2010) analyzed the mandibles of the three extant species from both sexes and found some characteristics that could be used as diagnostic characters. For Indonesian specimens, several mandibular characteristics of extinct proboscideans from Java, the Lesser Sunda Islands, and Sulawesi have been described by van den Bergh (1999). However, there is still no identification key on mandible specimens for Quaternary proboscideans of Indonesia. Identification through analyzing mandibular characteristics is also rarely conducted, despite the substantial number of specimens found at various paleontological sites in Indonesia. Therefore, this research aims to identify the diagnostic characters of Quaternary proboscide-

ans in Indonesia based on their mandibular characteristics through quantitative morphometrical analysis and qualitative morphological observation. These diagnostic characters were then made into an identification key that could be used as a practical guide, alternative to molar analysis.

MATERIALS AND METHODS

Materials

In this study, we examined 21 proboscidean mandibles from five institutions: Museum of Biology, Universitas Gadjah Mada (MB-UGM); Laboratory of Bioanthropology and Palaeoanthropology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (LBP-UGM); Laboratory of Mammals Biosystematics, Museum Zoologicum Bogoriense, National Research and Innovation Agency (MZB-BRIN); Bandung Geological Museum (MGB); and Sangiran Early Man Museum (MMPS). We analyzed the mandibles of the modern Asian elephant (*Elephas maximus*, n=8), *Elephas hysudrindicus* (n=1), *Sinomastodon bumiajuensis* (n=1), *Stegodon elephantoides* (n=1), *Stegodon trigonocephalus* (n=1), *Stegodon florensis* (n=2), *Stegodon sondaari* (n=1), *Elephas* sp. from Sangiran (n=1), and *Stegodon* sp. from Sangiran (n=5). The specimens used in this study were in a complete or near-complete condition from approximately the same age. The age estimation analysis was based on the research by Lee et al. (2011) on African savanna elephant (*Loxodonta africana*) age determination from their mandibular teeth.

Two specimens were treated as an exception in the qualitative morphological observation, namely *Elephas hysudrindicus* (EH) and *Stegodon florensis* (SF2), as both specimens had been reconstructed because of their damaged condition. The EH mandible underwent extensive reconstruction, leaving the teeth as the main visible part of the original fossil. The reconstruction itself was based on a Sumatran elephant (*Elephas maximus sumatranus*) specimen (U. P. Wibowo, personal communication, January 8, 2024). Additionally, the SF2 mandible underwent reconstruction as well, particularly on the posterior part of the mandibular ramus. Therefore, the morphological characteristics of both specimens could not be observed, since their original morphology had been altered by the reconstruction process.

Methods

The methods used in this study were quantitative morphometrical analysis based on van den Bergh (1999) with the addition of one new parameter in the form of a ratio (see parameter M26 in Table 1) and qualitative morphological observation. The morphometrical data were then analyzed using Principal Component Analysis (PCA) in PAST 4.12b. PCA was used to figure out specimens grouping and determine characters that could potentially be used as diagnostic characters. The qualitative data obtained from morphological observation was not included inside the PCA and treated separately as descriptive characters. Morphological observation was conducted on the angle between the mandibular ramus and the mandibular corpus, the orientation of mandibular condyles from lateral view, the shape of the mandibular condyles, and the curvature of the mandibular notch. The dominant mandibular characteristics for each taxon, obtained from both PCA and morphological observation, were then compiled to create a dichotomous key for a more practical identification guide.

RESULTS AND DISCUSSION

PCA Results on the Mandibles

Morphometrical parameters could be easily measured on complete and undamaged specimens. However, several specimens in this study were damaged or incomplete, preventing several parameters from being measured. Based on

Table 1. Morphometrical parameters used to determine the diagnostic characters of the mandibles.

No.	Code	Characters
Parameters based on van den Bergh (1999)		
1.	M1	Maximum length of the mandible in a plane defined by the basal points of support and parallel to the median plane
2.	M2	Total height of the mandible perpendicular to its basal plane
3.	M3	Total length of the mandibular ramus
4.	M4	Height between the basal plane and the coronoid apophyses
5.	M5	Height of the mandibular corpus measured at the level of the anterior border of the dental alveoli
6.	M6	Height of the mandibular corpus measured at the level of the anterior onset of the mandibular ramus
7.	M7	Length of the interalveolar crest
8.	M8	Height between the inferior border of the mandibular foramen and the base of the mandibular condyle
9.	M9	Horizontal distance of the mandibular ramus posteriorly of the posterior border of the mandibular condyle
10.	M11	Maximum width of the mandible measured between the external borders of the condyles
11.	M12	Width between the internal borders of the condyles
12.	M13	Largest (transverse) diameter of the condyle
13.	M14	Smallest (antero-posterior) diameter of the condyle
14.	M15	Width of the mandible at the level of the onset of the mandibular ramus
15.	M16	Width of the mandible between the external borders of the coronoid apophyses
16.	M17	Distance separating the internal borders of the mandibular corpus at the level of the onset of the mandibular ramus
17.	M18	Maximum transverse diameter of the mandibular corpus
18.	M19	Distance separating the internal borders of the mandibular corpus at the level of the anterior border of the dental alveoli
19.	M20	Minimum transverse diameter of the mandibular corpus
20.	M21	Antero-posterior length of the mandibular symphysis
21.	M22	Distance between the superior terminations of the interalveolar crests
22.	M23	Minimum distance between the internal borders of the mandibular corpus
23.	M24	Length of the occlusal wear surface
24.	M25	Maximum width of the occlusal wear surface
Parameter added in this study		
25.	M26	Ratio between the maximum width of the mandible (M11) against the maximum length of the mandible (M1)

the observation, the most frequently damaged parts were the tip of the mandibular symphysis, the mandibular condyles, the coronoid process, the mandibular ramus, and the anterior part of the molars. Because of that, parameters associated with the parts mentioned could not be measured thoroughly on several specimens. Therefore, the PCA method used in this study should minimize the effects of incomplete measurements data when analyzing the potential diagnostic characters and grouping the specimens to their respective taxa.

The PCA method produced a total variance value of 82.316 % from the sum of Principal Component 1 (PC1=71.258 %) and Principal Component 2 (PC2=11.058 %). The value of each Principal Component (PC) can be seen in Table 2. Furthermore, all morphometrical parameters, except for M9, were positively correlated with PC1, indicating a size-based grouping along the x-axis (see Figure 1). The grouping of specimens along the y-axis was potentially based on four significant parameters within PC2: M6, M12, M20, and M26 (see Figure 2). This is because the loading values of those parameters were either above +0.5 (M12 and M26) or below -0.5 (M6 and M20).

Based on the x-axis (PC1) in Figure 3, *Stegodon sondaari*, the smallest specimen observed, was placed on the far-left side of the x-axis. Meanwhile,

Stegodon elephantoides, the largest specimen observed, was placed on the far-right side of the x-axis. In the middle, there are specimens of medium sizes: *Elephas maximus*, *Stegodon trigonocephalus*, *Elephas* sp. from Sangiran, and *Stegodon* sp. from Sangiran. Therefore, the x-axis reflects the size differences among the observed specimens (see Figure 4 for size comparison of each taxon). On the y-axis (PC2), the *Stegodon-Sinomastodon* group was placed on the more positive axis, while the *Elephas* group was placed on the more negative axis. As mentioned before, the grouping of specimens along the y-axis was potentially influenced by parameters M6, M12, M20, and M26.

Table 2. Eigenvalue and % Variance for all Principal Components.

PC	Eigenvalue	% Variance
1	17.8144	71.258
2	2.76451	11.058
3	1.50689	6.0276
4	1.09713	4.3885
5	0.863605	3.4544
6	0.647223	2.5889
7	0.218398	0.87359
8	0.0878436	0.35137



Figure 1. Loading plots of PC1. The red line marks the threshold at +0.5.

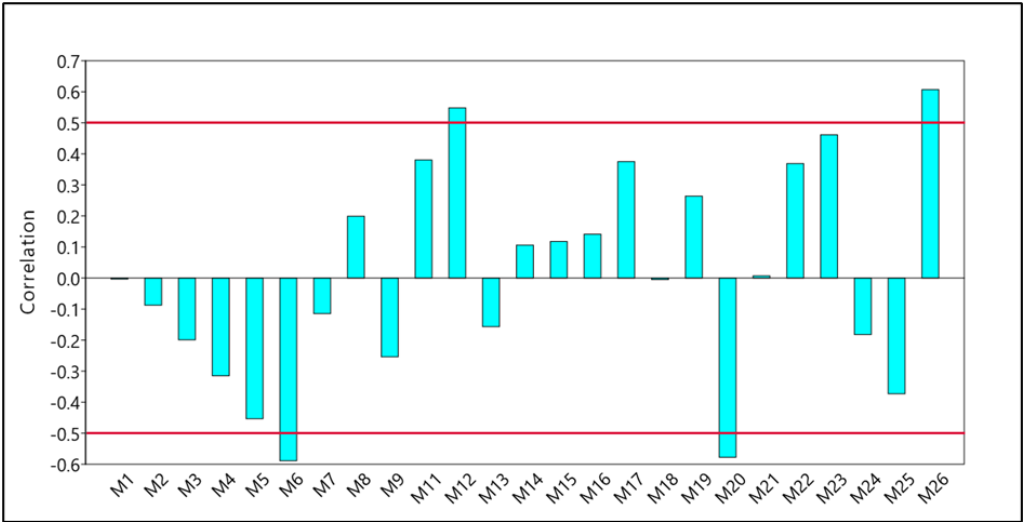


Figure 2. Loading plots of PC2. The red lines mark the threshold at +0.5 (upper) and -0.5 (lower).

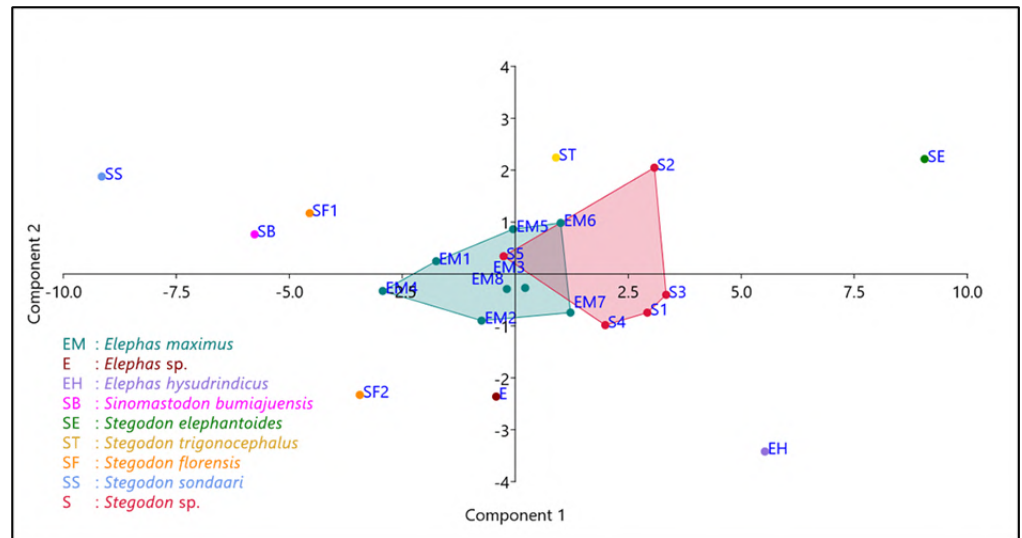


Figure 3. PCA results on the measured mandibles.

Mandibular Characteristics of Each Taxon

The *Stegodon-Sinomastodon* group in the PCA was characterized by a more positive correlation to M12 (width between the internal borders of the condyles) and M26 (ratio between the maximum width of the mandible (M11) against the maximum length of the mandible (M1)). From the dorsal view, the genus *Stegodon* has a wider mandible appearance compared to the genus *Elephas* or *Sinomastodon*. Therefore, increasing the measurement values of M12 and M26. Mandibles from the genus *Elephas* have an intermediate distance between the internal borders of the condyles when compared to the other two genera. Meanwhile, the only specimen of *Sinomastodon* mandible was missing its left condyle. Through shape and size estimation based on the right condyle, the *Sinomastodon* mandible likely has a shorter distance between the internal borders of the condyles than *Stegodon* or *Elephas*. Therefore, based on parameter M12, the distance between the internal borders of the condyles from smallest to largest, is as follows: *Sinomastodon*, *Elephas*, and *Stegodon* (see Figure 5).

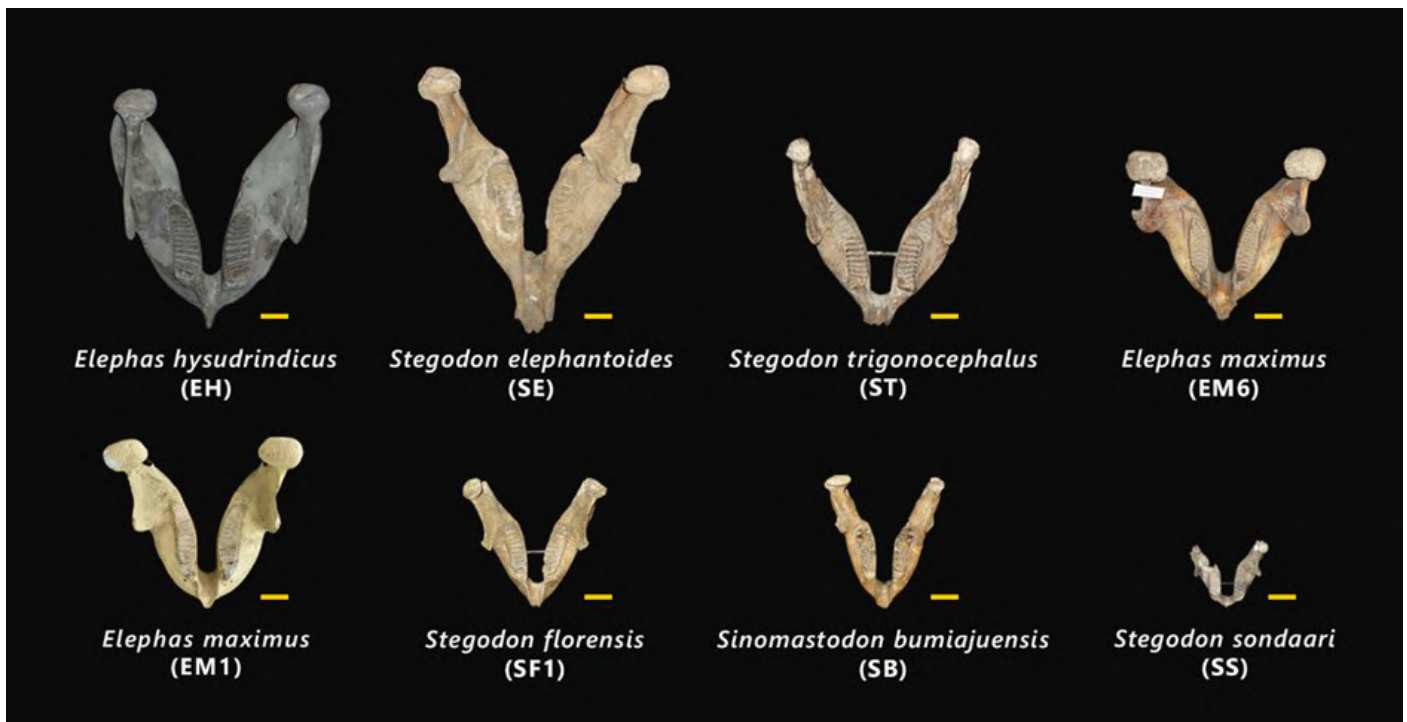


Figure 4. Dorsal view of the Quaternary proboscidean taxa observed in this study, represented by eight specimens. Scale bars: 10 cm.

The *Elephas* group in the PCA was characterized by a more positive correlation to M6 (height of the mandibular corpus measured at the level of the anterior onset of the mandibular ramus) and M20 (minimum transverse diameter of the mandibular corpus). Both parameters were potentially influenced by their molar type. As previously stated, members of the family Elephantidae, such as *Elephas*, possess high-crowned molars or hypsodont. To accommodate this feature, the Elephantidae members likely evolved a higher mandibular corpus, indicated by a positive correlation with parameter M6. In contrast to *Elephas*, *Stegodon* and *Sinomastodon* still possess low-crowned molars (brachyodont), which resulted in smaller M6 values. In addition, the buccal side of the mandibular corpus in the genus *Elephas* have a more “swollen” appearance than *Stegodon* and *Sinomastodon* specimens. This characteristic likely caused the M20 values of *Elephas* mandibles to be higher than the other two genera.

In this study, qualitative morphological observations were also conducted to determine other characters that could distinguish the mandibles of each taxon apart from the morphometrical parameters. The results showed that there are several characters that could be used to distinguish the mandible of Quaternary proboscideans of Indonesia at the genus level, namely the angle between the mandibular ramus and the mandibular corpus, the orientation of the mandibular condyles from lateral view, the shape of the mandibular condyles, and the curvature of the mandibular notch.

Measurement of the angle between the mandibular ramus and the mandibular corpus was done by drawing the horizontal line parallel to the molar surface and the vertical line along the direction of the masseteric fossa. From these measurements, the angle of *Elephas* mandibles tends to be at $\pm 90^\circ$ (right angle). Meanwhile, the genus *Stegodon* has a mandibular angle of $\pm 100^\circ$. The genus *Sinomastodon* showed the highest value of mandibular angle, which is 113.6° (Figure 6). These characters were also mentioned by van den Bergh (1999), who stated that the genus *Stegodon* has a wider mandibular angle than the genus *Elephas*. However, the publication did not mention the an-

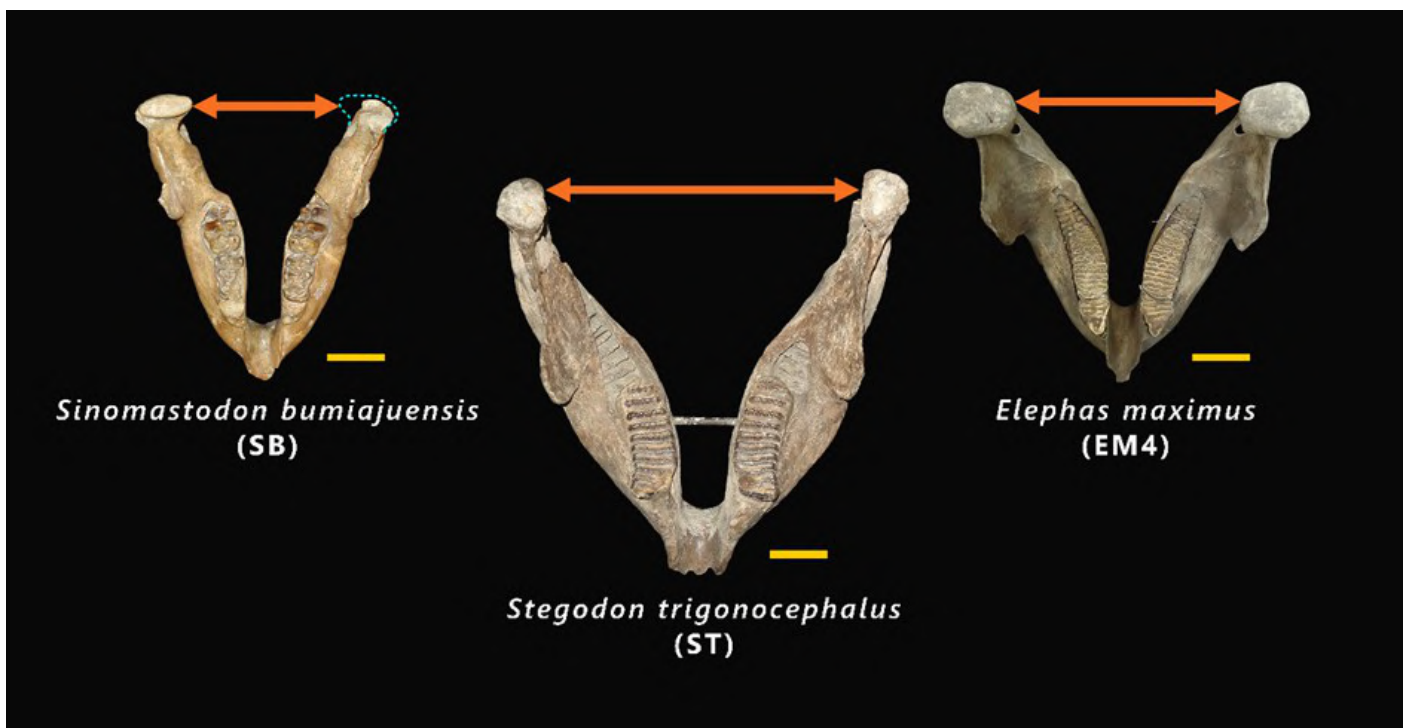


Figure 5. Dorsal view between the three genera of proboscideans found in Indonesia, represented by *Sinomastodon bumiajuensis*, *Stegodon trigonocephalus*, and *Elephas maximus*. The cyan dashed line on specimen SB is an estimation for the missing left condyle, while the orange lines in the three specimens illustrate the measurement of parameter M12. Scale bars: 10 cm.

gle value of those mandibles, nor the mandibular angle of the genus *Sinomastodon*. Therefore, this study provides new insights for the mandibular angle of Quaternary proboscideans found in Indonesia.

The orientation of the mandibular condyles also differs between the *Stegodon-Sinomastodon* group and the *Elephas* group. In Figure 6, the mandibular condyles of *Stegodon* and *Sinomastodon* face straight upwards. Meanwhile, the mandibular condyles of the genus *Elephas* tilt slightly to the anterior side. The shape of the mandibular condyles from each genus also showed differences. In the genus *Sinomastodon*, the condyle is rather oval and flattened antero-posteriorly. The genus *Stegodon* possesses condyles that are generally circular or oval. In dwarf stegodonts, namely *Stegodon florensis* and *S. sondaari*, the condyles tend to be slightly more circular than stegodonts from Java. The condyles of *Stegodon trigonocephalus* and *Stegodon* sp. from Sangiran showed a variety of shapes, such as rectangular (latero-medial) with blunt corners or oval. However, due to the majority of the condyles being damaged, the shape of the condyles for *Stegodon trigonocephalus* and *Stegodon* sp. from Sangiran could not be certainly determined. In *Stegodon elephantoides*, the condyles are relatively oval and antero-posteriorly wider than the condyle of *Sinomastodon bumiajuensis*. The condyles of the genus *Elephas* share a similar shape with those of a *Stegodon* sp. from Sangiran (S3), being rectangular (latero-medial) with blunt corners. However, the condyles of the genus *Stegodon* tend to be antero-posteriorly wider on the lingual side. In the genus *Elephas*, the condyles tend to be antero-posteriorly wider on the buccal side. The shape differences of the condyles between the three genera can be seen in Figure 7.

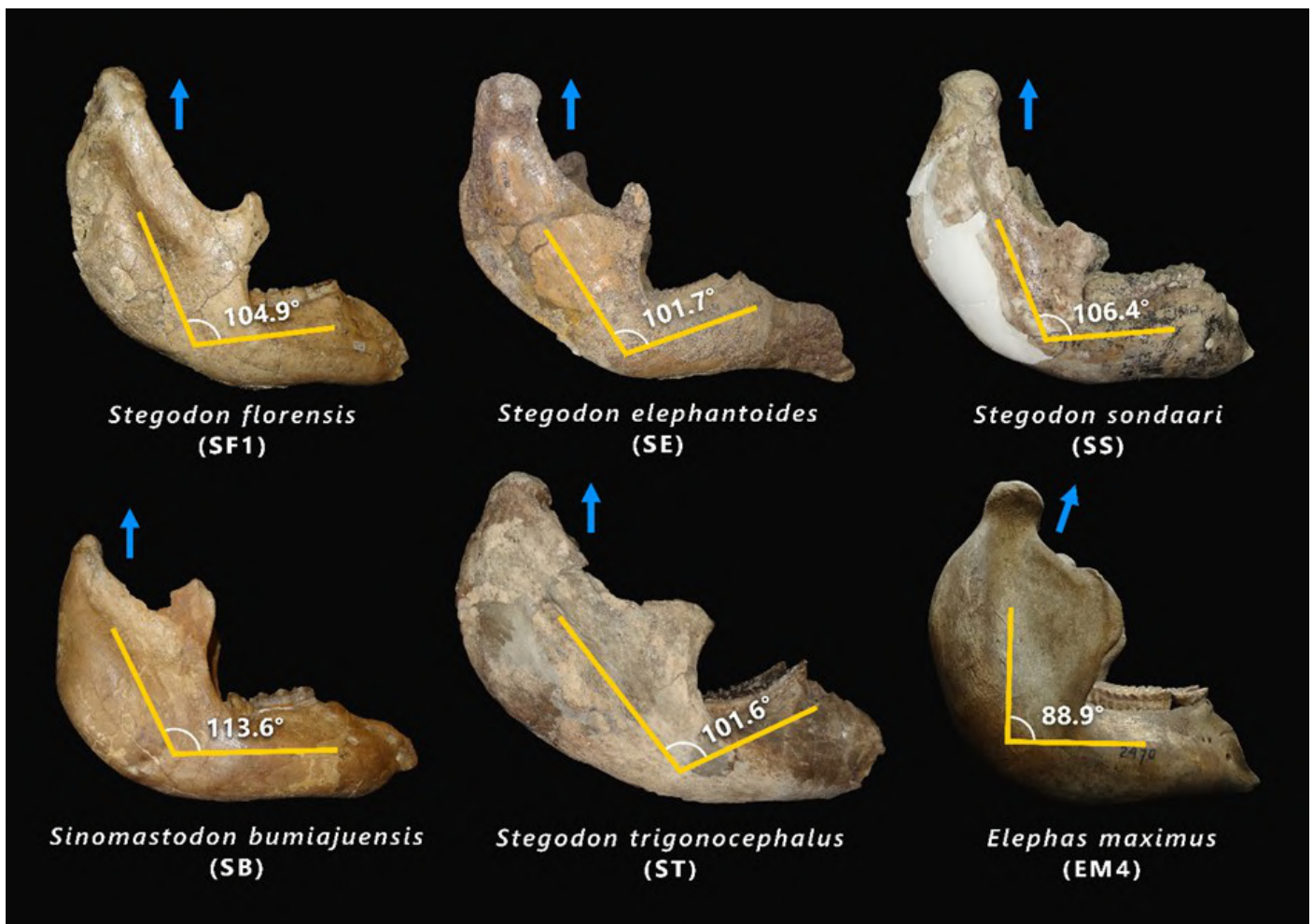


Figure 6. Mandibular angle (yellow lines) and mandibular condyles orientation (blue arrows) of six Quaternary proboscideans observed in this study. In specimens SE and SS, the photos used are of the flipped left buccal side due to the incomplete right buccal side. Specimens are not to scale.

The next observed characters of the genera were the curvature of the mandibular notch, which is the groove between the condylar process and the coronoid process (Figure 8). The genus *Sinomastodon* has a short curve and slightly curved upwards at the tip of the coronoid process. In the genus *Stegodon*, the curve tends to be longer. The direction of the curve at the tip of the coronoid process in the genus *Stegodon* also showed variations. In *Stegodon trigonocephalus*, the curve points directly to the anterior side, while in *S. elephantoides*, *S. sondaari*, and *S. florensis*, the curve points upwards and tends to form a “hook”. Several specimens of *Stegodon* sp. from Sangiran (S1, S2, S3, and S4) also showed variations between the two forms. Meanwhile, the curve in the genus *Elephas* is short and tends to curve directly to the anterior side. Although most members of the genus *Stegodon* possess a “hook-shaped” coronoid process, one modern *Elephas maximus* specimen (EM6) also has this character (Figure 9). Therefore, the “hook” shape of the coronoid process most likely could not be used as a distinguishing characteristic between the genera.

The characters mentioned above can already be used to distinguish *Sinomastodon bumiajuensis* and *Elephas maximus*. However, further descriptions are needed to confirm the diagnostic characters of the genus *Stegodon* at the species level due to its diversity. Based on our morphological observations on four species of the genus *Stegodon*, characters that can be used as diagnostic characters are the mandibular symphysis and specimen size.

According to van den Bergh (1999), the mandible of *Stegodon elephantoides* is very distinctive and can be distinguished from other members of the genus *Stegodon* by its elongated and downward-curved mandibular symphysis. *Stegodon elephantoides* is also suspected to be the largest *Stegodon* species found in Indonesia (van den Bergh 1999). In contrast, *S. sondaari* is the smallest member of the genus *Stegodon* discovered in Indonesia to date (van den Bergh 1999; van den Bergh et al. 2008; Meijer et al. 2010). Additionally, van den

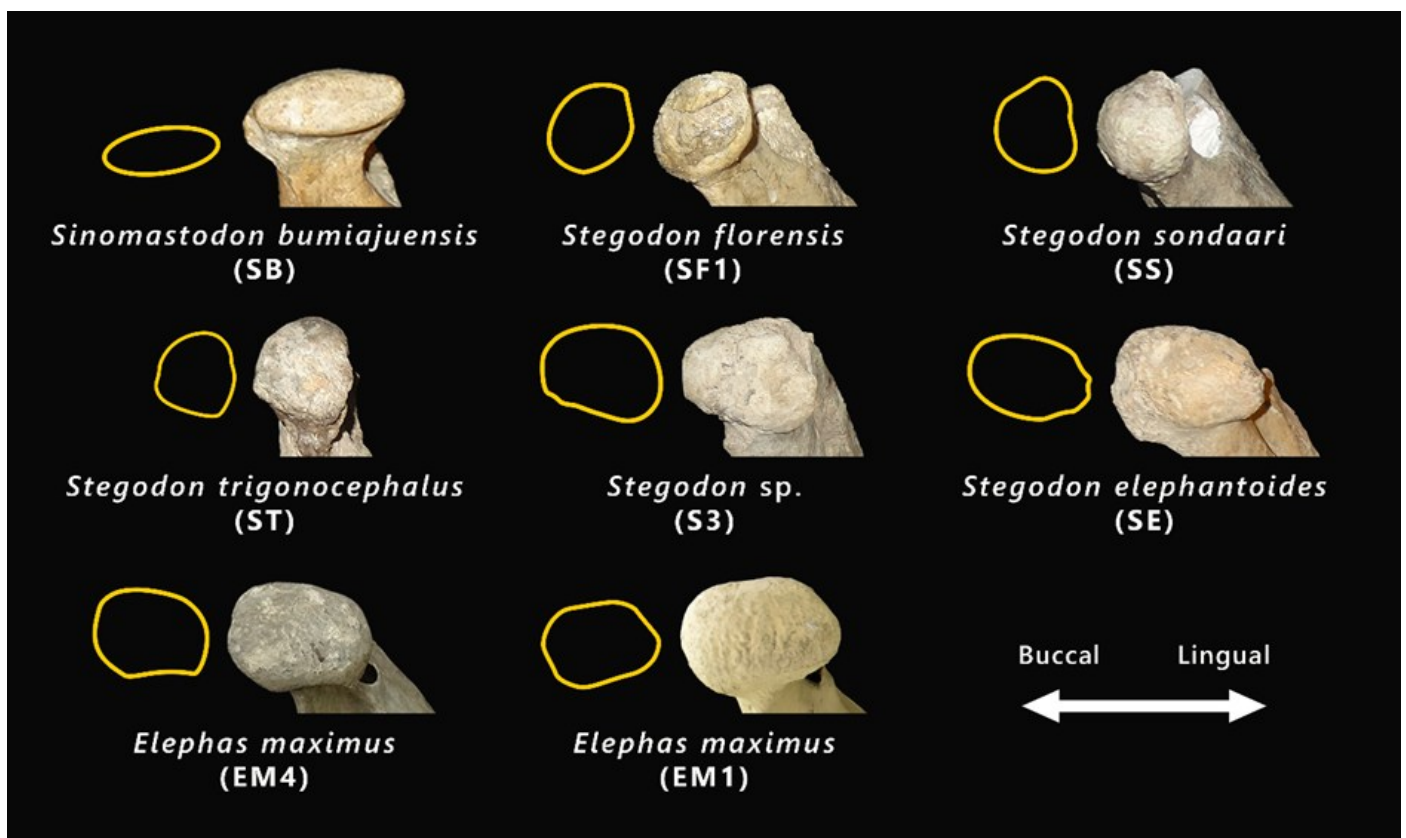


Figure 7. The shape of the mandibular condyles of six Quaternary proboscideans observed in this study. In specimens SS, SE, and EM1, the photos used are of the flipped left condyle due to the damaged right condyle. Specimens are not to scale.

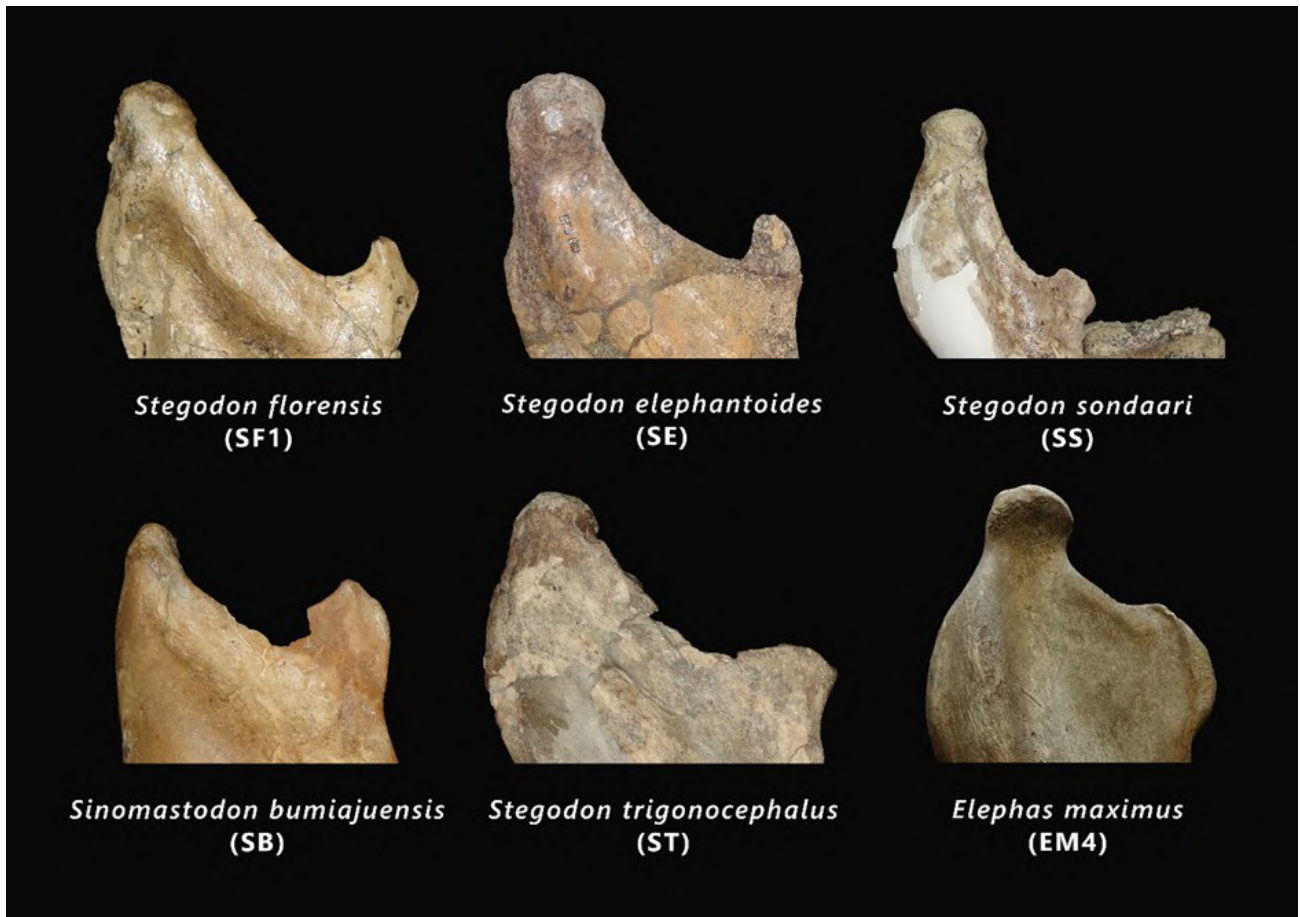


Figure 8. The curvature of the mandibular notch of six Quaternary proboscideans observed in this study. Specimens are not to scale.

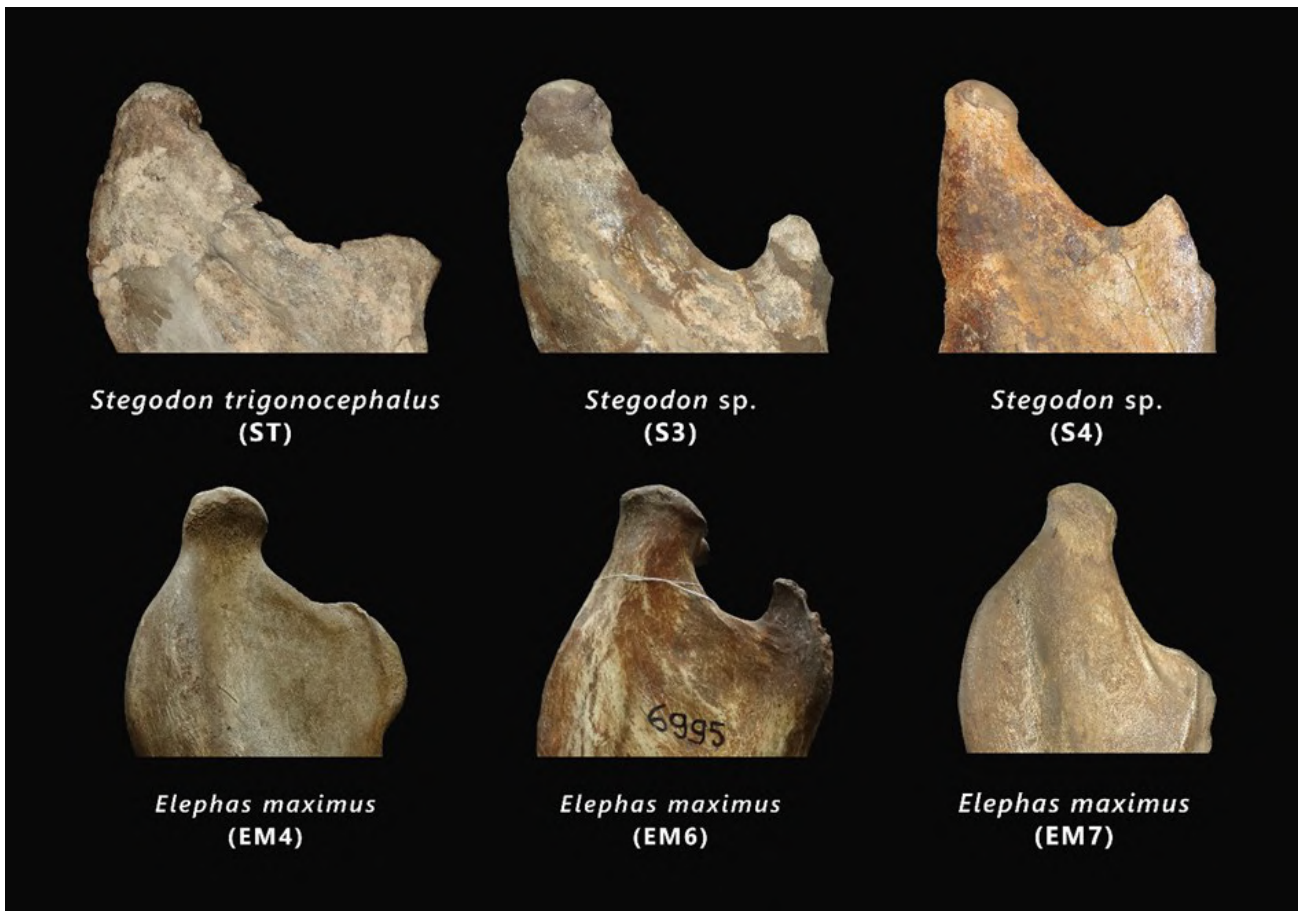


Figure 9. Variations on the curvature of the mandibular notch in *Stegodon trigonocephalus*, *Stegodon* sp. from Sangiran, and *Elephas maximus* observed in this study. Specimens are not to scale.

Bergh (1999) stated that the mandibular symphysis of *S. sondaari* is very short, since its anterior border is located right in between the anterior border of the alveoli, lacking the protrusion observed in other *Stegodon* species.

The mandibles of the last two members of the genus *Stegodon* in this study, namely *S. trigonocephalus* (including *Stegodon* sp. from Sangiran) and *S. florensis*, were difficult to distinguish, except for their size. Based on the observation, both species have a relatively short mandibular symphysis with a small protrusion to the anterior side, as stated in van den Bergh (1999). However, *S. florensis* is smaller than *S. trigonocephalus*, particularly *S. t. trigonocephalus*, at the same age range (van den Bergh 1999; van den Bergh et al. 2008). The smaller body size of *S. florensis* was the result of insular dwarfism, although not to the extreme degree as *S. sondaari*. When comparing the two species, it is important to ensure that the specimens belong to the same age range. Therefore, it is necessary to examine the molar characters first to determine their relative ages before comparing the size of the mandibles.

Diagnostic Characters of the Mandibles for Each Taxon

Based on the quantitative morphometrical measurements and qualitative morphological observations that have been conducted, it can be concluded that there are several characters which can be used as diagnostic characters of the mandibles for Quaternary proboscideans found in Indonesia. The characters can be seen in Table 3.

Based on the diagnostic characters in Table 3, we constructed a dichotomous key for a more efficient way in identifying Quaternary proboscideans of Indonesia. Since all the materials in this study were from complete or near-complete specimens, the dichotomous key is best be applied to specimens with similar conditions.

Table 3. List of mandibular diagnostic characters for each taxon observed in this study.

No.	Character	Gomphotheriidae	Stegodontidae				Elephantidae
		<i>Sinomastodon bumiajuensis</i>	<i>Stegodon elephantoides</i>	<i>Stegodon trigonocephalus</i> and <i>Stegodon</i> sp. (Sangiran)	<i>Stegodon florensis</i>	<i>Stegodon sondaari</i>	<i>Elephas maximus</i>
1.	The size of the mandible in general	Small	Very large	Medium to Large	Small	Very small	Medium
2.	The width of the mandible	Narrow	Wide	Wide	Wide	Wide	Intermediate
3.	The height of the mandibular corpus	Low	Low	Low	Low	Low	High
4.	The angle between the mandibular ramus and the mandibular corpus	Blunt angle (±110°)	Blunt angle (±100°)	Blunt angle (±100°)	Blunt angle (±100°)	Blunt angle (±100°)	Right angle (±90°)

Table 3. Contd.

No.	Character	Gomphotheriidae	Stegodontidae				Elephantidae
		<i>Sinomastodon bumiajuensis</i>	<i>Stegodon elephantoides</i>	<i>Stegodon trigonocephalus</i> and <i>Stegodon</i> sp. (Sangiran)	<i>Stegodon florensis</i>	<i>Stegodon sondaari</i>	<i>Elephas maximus</i>
5.	The orientation of the mandibular condyles from lateral view	Straight upwards	Straight upwards	Straight upwards	Straight upwards	Straight upwards	Tilt slightly to the anterior side
6.	The shape of the mandibular condyles	Oval and flattened antero-posteriorly	Oval and relatively wide antero-posteriorly	Rectangular (latero-medially) with blunt corners (antero-posteriorly wider at the lingual side), relatively circular or oval	Relatively circular	Relatively circular	Rectangular (latero-medially) with blunt corners (antero-posteriorly wider at the buccal side)
7.	The curvature of the mandibular notch	Short	Long	Long	Long	Long	Short
8.	The mandibular symphysis	Short; small anteriorly protruding rostrum	Elongated; long anteriorly protruding and curved downward-curved rostrum	Short; small anteriorly protruding rostrum	Short; small anteriorly protruding rostrum	Very short; no protruding rostrum	Short; small anteriorly protruding rostrum

Dichotomous Key to the Mandibles of Quaternary Proboscideans Found in Indonesia

- 1a. Narrow mandibular width; mandibular condyles oval and flattened antero-posteriorly.....*Sinomastodon bumiajuensis* (Gomphotheriidae)
- 1b. Intermediate to wide mandibular width; mandibular condyles oval, circular, or rectangular (latero-medially) with blunt corners.....**2**
- 2a. Blunt angle ($\pm 100^\circ$) between the mandibular ramus and the mandibular corpus; low mandibular corpus; long curve on the mandibular notch; mandibular condyles face straight upwards.....**3** (Stegodontidae)
- 2b. Right angle ($\pm 90^\circ$) between the mandibular ramus and the mandibular corpus; high mandibular corpus; short curve on the mandibular notch; mandibular condyles tilt slightly to the anterior side.....*Elephas maximus* (Elephantidae)

3a.	Very small to small mandible size.....	4
3b.	Medium to very large mandible size	5
4a.	Very short mandibular symphysis; no protruding rostrum.....	<i>Stegodon sondaari</i>
4b.	Short mandibular symphysis; small anteriorly protruding rostrum.....	<i>Stegodon florensis</i>
5a.	Elongated mandibular symphysis; long anteriorly protruding and downward-curved rostrum.....	<i>Stegodon elephantoides</i>
5b.	Short mandibular symphysis; small anteriorly protruding rostrum.....	<i>Stegodon trigonocephalus</i>

CONCLUSIONS

This research brings a novel approach to fossil identifications in Indonesia through mandible observations, especially for Quaternary proboscideans. Based on the results of this study, the diagnostic characters for identifying the mandibles of Quaternary proboscideans found in Indonesia are the size of the mandible in general, the width of the mandible, the height of the mandibular corpus, the angle between the mandibular ramus and the mandibular corpus, the orientation of the mandibular condyles from lateral view, the shape of the mandibular condyles, the curvature of the mandibular notch, and the mandibular symphysis. Further studies with more specimens from other proboscidean taxa not mentioned in this study are needed to make a complete identification key to all known Quaternary proboscideans from Indonesia and other parts of the world.

AUTHOR CONTRIBUTION

A.D.A. designed the research, collected and analyzed the data, and wrote the manuscript. D.S.Y. supervised the research process, proofread the manuscript, and gave advice during the research process.

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CONFLICT OF INTEREST

All authors declare that there is no conflict of interests.

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APPENDICES

Appendix 1. List of materials observed in this study. YO: years old; AEY: African Elephant Years.

No.	Taxa	Code	Institution	Collection Number	Bone/Fossil	Specimen's Age
Family Elephantidae						
1	<i>Elephas maximus</i>	EM1	MB-UGM	-	Bone	29 YO
2	<i>Elephas maximus</i>	EM2	MGB	-	Bone	15 YO
3	<i>Elephas maximus</i>	EM3	MGB	VR. 2080801	Bone	47±2 AEY
4	<i>Elephas maximus</i>	EM4	MZB-BRIN	2470	Bone	26±2 AEY
5	<i>Elephas maximus</i>	EM5	MZB-BRIN	6989	Bone	26±2 AEY
6	<i>Elephas maximus</i>	EM6	MZB-BRIN	6995	Bone	47±2 AEY
7	<i>Elephas maximus</i>	EM7	MZB-BRIN	6990	Bone	28±2 AEY
8	<i>Elephas maximus</i>	EM8	MZB-BRIN	-	Bone	15± AEY
9	<i>Elephas</i> sp.	E	MMPS	ELP/1489/BPSMPS/2013	Fossil	47±2 AEY
10	<i>Elephas blythindicus</i>	EH	MGB	-	Fossil	49±2 AEY
Family Stegodontidae						
11	<i>Stegodon elephantoides</i>	SE	MMPS	1487/ELP/BPSMPS/2013	Fossil	49±2 AEY
12	<i>Stegodon trigonocephalus</i>	ST	MGB	VR. 1070501/1070601	Fossil	28±2 AEY
13	<i>Stegodon florensis</i>	SF1	MGB	BL2012/T13/F562	Fossil	47±2 AEY
14	<i>Stegodon florensis</i>	SF2	MGB	MM2011/T24A/F174	Fossil	49±2 AEY
15	<i>Stegodon sondaari</i>	SS	MGB	TT2014/T1/F122-123	Fossil	47±2 AEY
16	<i>Stegodon</i> sp.	S1	MMPS	1195/ELP/BPSMPS	Fossil	45± AEY
17	<i>Stegodon</i> sp.	S2	MMPS	-	Fossil	45± AEY
18	<i>Stegodon</i> sp.	S3	MMPS	ELP/0293/BPSMPS/10	Fossil	47±2 AEY
19	<i>Stegodon</i> sp.	S4	MMPS	3234/ELP/BPSMPS/18	Fossil	70+ AEY
20	<i>Stegodon</i> sp.	S5	LBP-UGM	21409 (0101300)	Fossil	18±2 AEY
Family Gomphotheriidae						
21	<i>Sinomastodon bumiajuensis</i>	SB	MGB	K.135	Fossil	18±2 AEY

Research Article

Tree Architecture Models of the *Syzygium* Genus Collection at the Purwodadi Botanical Gardens

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ABSTRACT

There are many species of plants in the genus *Syzygium*. This genus is distinguished by its strong root system, evergreen, sprawling and complex tree crowns and a powerful branching system on the trunk. The vertical structure of *Syzygium* trees is distinguished by branching a large crown. Based on its characteristics, the genus *Syzygium* has a great potential in supporting the sustainability of ecosystem balance. The diversity of tree architecture possessed by the genus *Syzygium* allows *Syzygium* species to play an active role in soil and water conservation efforts. The aim of this study is to identify various architectural models of trees *Syzygium* species in the collection of Purwodadi Botanical Garden in order to assess their role in the ecosystem. The method used in this study was to measure the physical characteristics of trees, such as tree height, trunk diameter, and crown area, and to observe the shape of trees, such as stem growth patterns, branching patterns, and direction of branch development. As the result, of the 17 *Syzygium* tree species selected and in accordance with the research criteria, 7 of them have the Stone model, 3 species have the Scar-rone model, 2 species have the Attims model, 2 species have the Petit model, and 1 species each has the Koriba, Massart, and Troll models. Each variation of tree architecture model has different functions to the ecosystem.

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INTRODUCTION

Forest has an important role in the water cycle regulation, the carbon cycle role, and the habitat for world terrestrial biodiversity (Indrajaya et al. 2022). One of the botanical gardens in Indonesia that illustrates artificial dry lowland forest habitat is Purwodadi Botanical Garden (PBG) located in East Java. The genus *Syzygium*, a member of the Myrtaceae family, is one of the most abundant collections in the Purwodadi Botanical Garden (PBG), it grows naturally and can be found in both ex situ and in situ habitats surrounding the garden (Beech et al. 2017). PBG has an ecosystem that is still preserved. The abundance of tree collections and the similarity of tree conditions as in their natural habitat make Purwodadi Botanical Garden an optimal place for research.

Syzygium plays various roles in life (Aung et al. 2020, 2023; Ilham et al. 2022; Kristanti et al. 2022). *Syzygium* trees are characterised by varying heights, extensive branching systems, complex and shady crowns, the nature of roots that can withstand the river flow, as well as hold up or slows down the rate of river flow. Root systems join strong pedestal stems (Mudiana 2016; Yasin et al. 2022). In stratum C of the tropical forest ecosystem, *Syzygium* trees have a vertical structure that has quite a lot of branching and crowns (Utami & Putra 2020). Thus, the *Syzygium* species will create various models of tree architecture, thus potentially conserving soil and water for the benefit of the ecosystem, *Syzygium* have an important role in the stabilisation of the region along the banks of the river (Mudiana 2016). Preliminary research shows that there is no significant difference between the physical characteristics of *Syzygium* trees in their natural habitat, dry lowland forest in Gunung Baung Nature Tourism Park (TWA), and *Syzygium* trees collected in Purwodadi Botanical Garden (*ex situ*), so *Syzygium* research should be conducted at PBG.

Tree crowns in the *Syzygium* tree architecture model can clarify the rainfall-mitigating and time-lagging effect during rainfall, and thereby helps to control peak flow, flood, and erosion (Li et al. 2016). Tree felling makes tree architecture does not function optimally and puts water at risk of causing landslides and floods. Therefore, the ecological function of each species of *Syzygium* tree can be known by analysing architecture model. Tree architectural can describe those trees' ability to conserve the soil surface. Their model is a tree building model as a result of plant growth controlled by meris (Agung & Abban 2017).

The branching pattern of a tree derived from the morphological description of the crown at a certain stage of the tree is a model of tree architecture. According to Joye (2019), tree architecture describes the plant structure that occurs in all members of the same species. Environmental factors and growing sites also have an impact on the architectural state of each tree species. This results in different branching patterns, trunk shapes, and branching angles (Echereme et al. 2015). There are 23 widely recognised tree architecture models (Joye 2019).

Mudiana (2016) in his research on the *Syzygium* genus diversity in Pasuruan Regency, showed the results that this genus naturally grows in the forest of Mount Baung Pasuruan and along the river (Welang watershed). However, the researchers only collected data on *Syzygium* species. While Arlianty (2020) conducted research on tree architecture with various tree species on public senior high school, the results showed that there were 10 tree architecture models, Scarrone, Corner, Attims, Troll, Roux, Leeuwenberg, Koriba, Rauh, Aubreville and Stone. The tree architecture model that dominated the schools was the Troll model (35.7 %) with 15 species.

The innovative aspect of this research is the identification of *Syzygium* tree architecture models in the Purwodadi Botanical Garden collection as one

of the plant genera that has strong, extensive, and complex crown branching in several tree architecture models. It is very important to analyse the tree architecture model as a first step to find out how each species of *Syzygium* tree contributes to the ecosystem. The purpose of this study is to determine the various architectural models of *Syzygium* trees in the Purwodadi Botanical Garden collection and their services or functions to the ecosystem.

MATERIALS AND METHODS

Study Area

In the vicinity of the Purwodadi Botanical Garden (Figure 1), this study was carried out in December 2022. The purposeful sampling strategy was used to pick the trees. Through exploration and observation, measurements of physical characteristics of trees and examinations of the shapes of *Syzygium* genus trees in the Purwodadi Botanical Garden collection were used to gather data for tree architecture models.

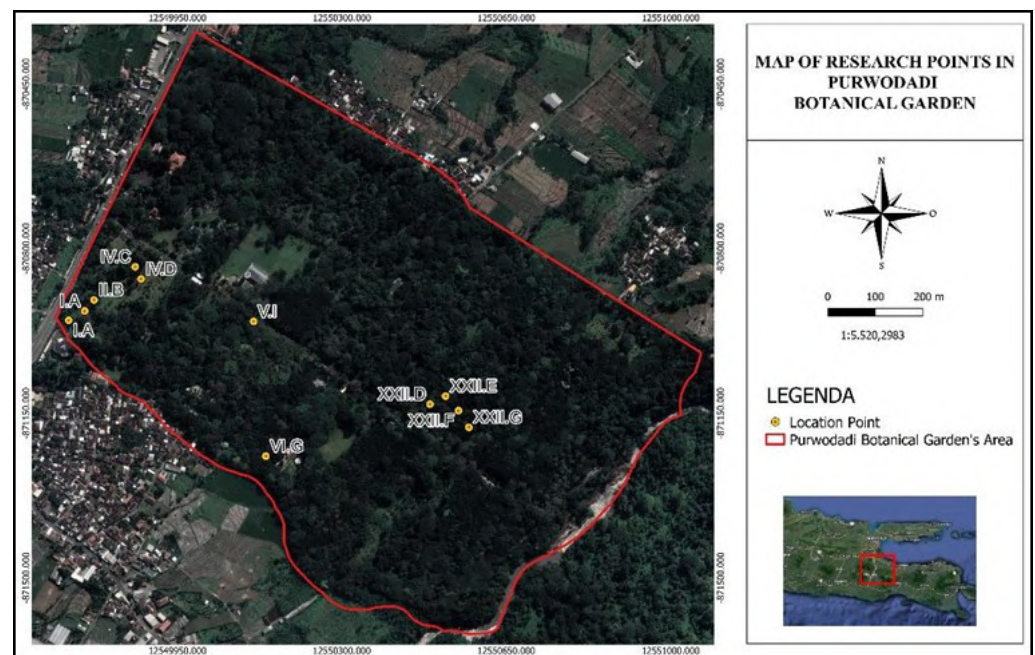


Figure 1. Map of research location points in Purwodadi Botanical Garden area.

Procedure

Measurement the physical factor of *Syzygium* tree

Measurements of tree physical factors include tree height, trunk diameter, and crown area (Figure 2). Tree height measurements were made using the *smartmeasure* application by reading the scale on the tool. Trunk diameter is measured using a measuring tape wrapped around the trunk with a height of 1.3 meters to get the circumference of the tree, then the circumference data is used for stem diameter analysis using the formula ($d = K/\pi$). Measurement of crown area was carried out by projecting the ends of the crown, from west to east and north to south on the ground [$(D1 = \text{West} + \text{East})$] + [$(D2 = \text{North} + \text{South})$]. The average length of these lines divided by 4 is considered the crown diameter ($D = D1 + D2/4$). Furthermore, the diameter of the existing crown can be used to determine the crown area (Crown area = $D = D1 + D2/4$) (Pretzsch et al. 2015).

Observation the Branching of Tree *Syzygium* Canopy

The architecture is the result of its adaptation to environment, branching architecture density is a highly variable, changing with age, position in canopy, access to light, plant vigour, disease, and water stress (Charles-Dominique et al. 2017). The architecture of trees can be approached from many viewpoints,

according to the vertical and horizontal characteristic (Seidel et al. 2019). Tree growth patterns, branching patterns, and the direction of branch growth points can all be observed (Figure 3). According to McGarry et al. (2016), there are three different types of stem growth patterns: dichotomy (main stem is divided into two equal sections), sympodial (main stem cannot be distinguished from branches), and monopodial (the axis of growth continues from a single point) (Figure 3a). Orthotropic stems grow vertically, while plagiotropic ones grow horizontally, over or below the soil surface (Gambino et al. 2016, Figure 3b). The angle produced between the main stem and the branch can be used to classify the direction of branch growth point: fastigiated, patens, horizontal, and declinatus (Nurdiana 2020, Figure 3c). Each species of *Syzygium* spp. is characterized by the data collected, which are then utilised to examine the tree architectural model.



Figure 2. Measurement of physical factors of *Syzygium* trees. (a) Illustration of tree height measurement using *smartmeasure*. (b) Measurement of tree diameter. (c) Illustration of crown area measurement (Hematang et al. 2021).

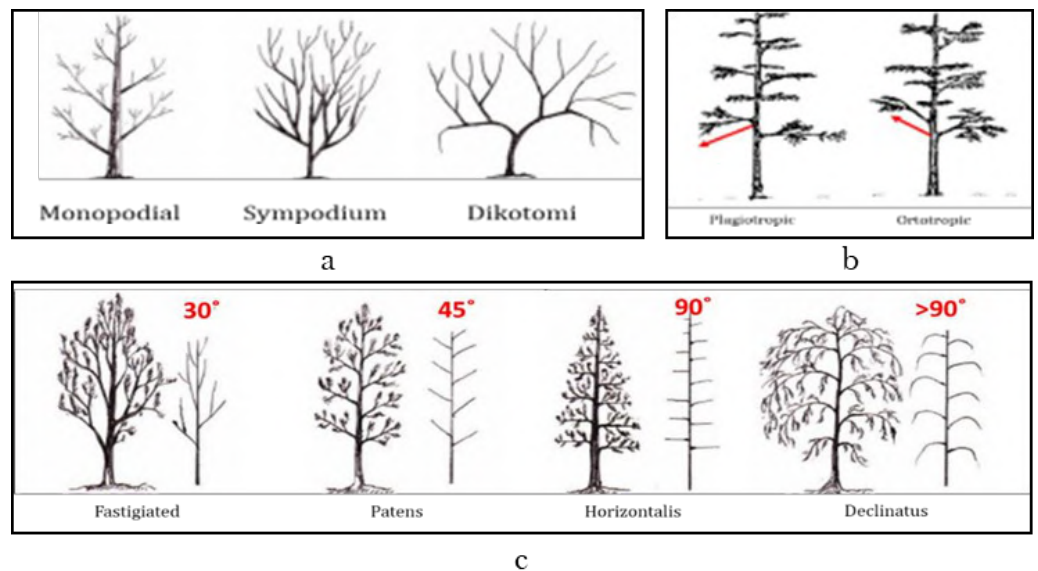


Figure 3. Variety of stem growth and branching models. (a) Trunk growth pattern. (b) Branching pattern. (c) Branch growth point direction (angle).

Tree Architecture Model Type Analysis

The analysis stage is carried out by documenting the image of each tree, then drawing illustrations through manual sketches, and also using artificial intelligence (AI) through the Dreamina Ai website to visualise the tree architecture model that has been observed.

RESULTS AND DISCUSSION

Syzygium Tree Collection of Purwodadi Botanical Garden

Trees are tall, bulky plants with a primary trunk that is at least 20 cm in diameter with the height of activity hole was 5.1 m (Pakkala et al. 2018). Trees also play key ecological and ecosystem roles, having major influences on hydrological cycles, nutrient cycles, disturbance regimes, and the distribution and abundance of populations of their own and other species (Lindenmayer & Laurance 2016). Table 1 lists 25 different *Syzygium* trees species, 17 of which visually resemble tree-level plants. Thus, one tree from each of the 17 species was selected, assessed for its physical characteristics, and its shape recorded to determine the tree architecture model.

Table 1. List of *Syzygium* spp. trees collection of Purwodadi Botanical Garden.

No.	Scientific Name	Total	Location/Vac.
1	<i>S. javanicum</i> Miq.	4	I A, IV D, V I, XXII D
2	<i>S. cumini</i> (L.) Skeels	2	II B, V I
3	<i>S. acuminatisimum</i> (Blume) DC.	2	IV C, XXII D
4	<i>S. pycnanthum</i> Merr. & L.M.Perry	2	XXII E, XXII F
5	<i>S. discophorum</i> (Koord & Valetton) Amshoff	2	XXII E, XXII F
6	<i>S. polycephalum</i> (Miq.) Merr. & L.M.Perry	1	V I
7	<i>S. jambos</i> (L.) Alston	1	VI G
8	<i>S. lineatum</i> (DC.) Merr. & L.M.Perry	1	XXII D
9	<i>S. racemosum</i> (Blume) DC.	1	XXII D
10	<i>S. polyanthum</i> (Wight) Walp.	1	XXII D
11	<i>S. formosum</i> (Wall.) Masam.	1	XXII D
12	<i>S. nervosum</i> A.Cunn. ex DC.	1	XXII D
13	<i>S. sexangulatum</i> (Miq.) Amshoff	1	XXII D
14	<i>S. malaccense</i> (L.) Merr. & L.M.Perry	1	XXII E
15	<i>S. creaghii</i> (Ridl.) Merr. & L.M.Perry	1	XXII E
16	<i>S. aqueum</i> (Burm.f.) Alston	1	XXII F
17	<i>S. garcinifolium</i> (King) Merr. & L.M.Perry	1	XXII G
Total		25	

Physical Factors of *Syzygium* spp. trees in Purwodadi Botanical Garden Collection

Measuring tree characteristics, such as height, trunk diameter, and crown area, is very important for determining the habitat or physical factors that influence each individual tree (Sirami & Manuhua 2022). The physical properties of each species of *Syzygium* are listed in Table 2, and they will be used to analyse the type under the "tree or pole" category. Tree height typically ranges from 6 to 21 meters. The diameter of the trunk then varies from 16 to 73.3 cm. While the diameter of the tree's crown varies from 4.6 to 57 meters.

Lowland forests are characterised by many dense tree-climbing plants, large buttressed trees, and many trees with tall. In the lowland forest, many tree species belong to the family Dipterocarpaceae, Lauraceae, Myrtaceae, Miristicaceae, and Ebenaceae (Nugraha & Kusmana 2022). According to Baker and Harper (2022) trees can be classified ecologically based on their stage of growth. The trunk diameter of young trees (poles) ranges from 10 to 20 cm, whereas the diameter of the tree category is greater than 20 cm. According to this idea, Table 2 results from measuring the *Syzygium* spp. tree's diameter indicate that the Purwodadi Botanical Garden's *Syzygium* tree collection falls under the umbrella of poles (young trees) and trees. According to their height, trees can be categorized into four groups: young small size, old small size, young big size, and old big size (Ali & Wang 2021). All of the *Syzygium* tree species now in existence are included in the three categories, according to the findings of this theory. Crown base height, diameter at breast height (usually measured at 1.3 m above ground), tree height, and

crown radius are some examples of tree-based measurement (Seidel et al. 2019).

Table 2. Data on physical factors of 17 *Syzygium* spp. tree species in Purwodadi Botanical Garden collection.

No.	Scientific Name	Measurement of Tree Physical Factors		
		Tree Height (m)	Stem Diameter (cm)	Tree crown area (m)
1	<i>S. acuminatisimum</i>	9	20.7	2.8
2	<i>S. aqueum</i>	7	17.5	7
3	<i>S. creaghii</i>	10	17.2	6.9
4	<i>S. cumini</i>	10	57.4	57
5	<i>S. discophorum</i>	8	20.7	2.5
6	<i>S. formosum</i>	8	16	4.6
7	<i>S. garcinifolium</i>	13	41	7.72
8	<i>S. jambos</i>	6	27	9
9	<i>S. javanicum</i>	11	29	14.2
10	<i>S. lineatum</i>	11	37.9	10.4
11	<i>S. malaccense</i>	8	26.8	6.4
12	<i>S. nervosum</i>	13	30.3	6.7
13	<i>S. polyanthum</i>	21	42	11.5
14	<i>S. polycephalum</i>	20	73.3	10.53
15	<i>S. pycnanthum</i>	9	25.5	6
16	<i>S. racemosum</i>	14	34.4	20
17	<i>S. sexangulatum</i>	12	29	17

Shape of *Syzygium* spp. tree in Purwodadi Botanical Garden Collection

There are several considerations in determining the architectural model of a tree, a handful of morphological characters, such as the position of sexual structures or the orientation of axis growth (Chomicki et al. 2017). *Syzygium* tree observations include branching patterns, branch growth points, and stem growth patterns. In general, the stem growth pattern of the *Syzygium* species is sympodial, with up to 8 species, and monopodial, with up to 8 species. There is one form of tree with a dichotomous stem growth pattern. These findings are shown in Table 3. Then, there are up to 14 different forms of orthotropic branching and up to 3 different plagiotropic branching patterns that make up the branching pattern of *Syzygium* trees species. In terms of branch development points, the *Syzygium* species typically has branch growth points of patterns, which can have up to 9 types, fastigiata, which can have up to 3, horizontal, which can have up to 4, and declinata, which can have up to 1 type (Table 3).

Plant branching patterns will create a tree architectural model, according to Shipunov (2023), there are three types of stem growth patterns: monopodial, sympodial, and dichotomous. According to Klimešová et al. (2019), monopodial branching occurs when the main stem is consistently visible, sympodial branching occurs when the main stem and branches cannot be distinguished, and according to Spencer et al. (2023), that dichotomy branching occurs when the branching divides into two equal portions (Shipunov 2018). There are two categories of branching patterns: orthotropic and plagiotropic. Plagiotropic is a branching pattern with a horizontal branch direction, whereas orthotropic is an upright branching pattern toward the vertical direction (Gambino et al. 2016). There are four types of branch growth points: patens, fastigiata, horizontal, and declinata. With respect to the main stem, fastigiata is created with an angle of less than 30 degrees, patens with a 45-degree angle, a 90-degree horizontally, and declinata with an angle greater than 90

degrees or drooping (Nurdiana 2020). To study the tree architecture model in each type of *Syzygium* tree, all patterns were applied.

Table 3. Observation data of 17 *Syzygium* spp. tree species in Purwodadi Botanical Garden collection.

No.	Scientific Name	Shape Observation			Tree Architecture Model
		Stem Growth Pattern	Branching	Branch Growth Point	
1	<i>S. acuminatisimum</i>	Monopodial	Orthotropic	Patens	Scarrone
2	<i>S. aqueum</i>	Sympodial	Orthotropic	Fastigiated	Stone
3	<i>S. creaghii</i>	Monopodial	Orthotropic	Fastigiated	Attims
4	<i>S. cumini</i>	Dichotomy	Orthotropic	Horizontalis	Koriba
5	<i>S. discophorum</i>	Monopodial	Plagiotropic	Horizontalis	Petit
6	<i>S. formosum</i>	Monopodial	Orthotropic	Patens	Scarrone
7	<i>S. garcinifolium</i>	Sympodial	Orthotropic	Patens	Stone
8	<i>S. jambos</i>	Sympodial	Orthotropic	Patens	Stone
9	<i>S. javanicum</i>	Sympodial	Orthotropic	Patens	Stone
10	<i>S. lineatum</i>	Sympodial	Orthotropic	Patens	Stone
11	<i>S. malaccense</i>	Monopodial	Plagiotropic	Declinatus	Massart
12	<i>S. nervosum</i>	Monopodial	Plagiotropic	Horizontalis	Petit
13	<i>S. polyanthum</i>	Monopodial	Orthotropic	Patens	Scarrone
14	<i>S. polycephalum</i>	Monopodial	Orthotropic	Patens	Attims
15	<i>S. pycnanthum</i>	Sympodial	Orthotropic	Patens	Stone
16	<i>S. racemosum</i>	Sympodial	Orthotropic	Fastigiated	Stone
17	<i>S. sexangulatum</i>	Sympodial	Orthotropic	Horizontalis	Troll

Architectural model of *Syzygium* spp. tree in Purwodadi Botanical Garden (PBG) collection

Purwodadi Botanical Garden has an ecosystem resembling a lowland forest with a dry climate that forms a landscape resembling a tropical forest. Tropical forests are full of trees with diverse trunk and canopy structures (tree architecture model) very similar to those in temperate climates (Heydari et al. 2020). Table 4 displays tree architecture models that were created using information from physical factor data analysis and branching pattern observations. Purwodadi Botanical Garden's *Syzygium* tree architecture collection has seven models, according to the data. Scarrone, Stone, Attims, Petit, Koriba, Massart, and Troll are a few of these models. Based on the available information, it is known that *Syzygium* trees own the Stone model the most, with a total of 7 different types of trees. The Attims model has two varieties of trees, the Petit model has two types of trees, the Scarrone model has three species of trees, the Koriba, Massart, and Troll models each have one type of tree (Table 4).

Based on the publication Tropical Trees and Forests by Helle et al. (1978) in Shipunov (2023), there are 23 models of tree architecture that are widely used. Two categories can be made up of the numerous tree architecture models. 1) Trees that are monocausal or have no branches, using the Holttum and Corner models. 2) The Tomlinson, Schoute, Chamberlain, McClure, Leeuwenberg, Koriba, Prevost, Fagerlind, Petit, Nozcran, Aubreville, Massart, Roux, Cook, Scarrone, Stone, Rauh, Attims, Mangelot, Champagnat, and Troll models are polyaxial trees, or trees having more than one branch. Each tree architecture model has a unique characteristic that is represented by the branching structure and trunk expansion. Although there was significant intraspecific variance in response to canopy variety in architectural traits is related to the functional (Loubota Panzou et al. 2018).

Table 4. Architectural model of 17 *Syzygium* spp. tree collection of Purwodadi Botanical Garden collection.

Tree Architecture Model	Scientific Name
Scarrone	<i>Syzygium acuminatisimum</i>
	<i>Syzygium formosum</i>
	<i>Syzygium polyanthum</i>
Stone	<i>Syzygium aqueum</i>
	<i>Syzygium garcinifolium</i>
	<i>Syzygium jambos</i>
	<i>Syzygium javanicum</i>
	<i>Syzygium lineatum</i>
	<i>Syzygium pycnanthum</i>
	<i>Syzygium racemosum</i>
Attims	<i>Syzygium creaghii</i>
	<i>Syzygium polycephalum</i>
Petit	<i>Syzygium discophorum</i>
	<i>Syzygium nervosum</i>
Koriba	<i>Syzygium cumini</i>
Massart	<i>Syzygium malaccense</i>
Troll	<i>Syzygium sexangulatum</i>

Scarrone Model

Syzygium. acuminatisimum, *Syzygium. polyanthum*, and *Syzygium. formosum*, trees which is presented in (Figure 4) feature a Scarrone tree architectural model with a monopodial trunk, orthotropic branching, and the growth point of the patens branch producing an angle of roughly 45°, according to data in Table 3. Scarrone is a tree architectural model with a sympodial or monopodial trunk, according to Shipunov (2023). The branching of the Scarrone model tree is all orthotropic. When the branch and main stem establish a about 45° angle, patens begin to grow (Nurdiana 2020).

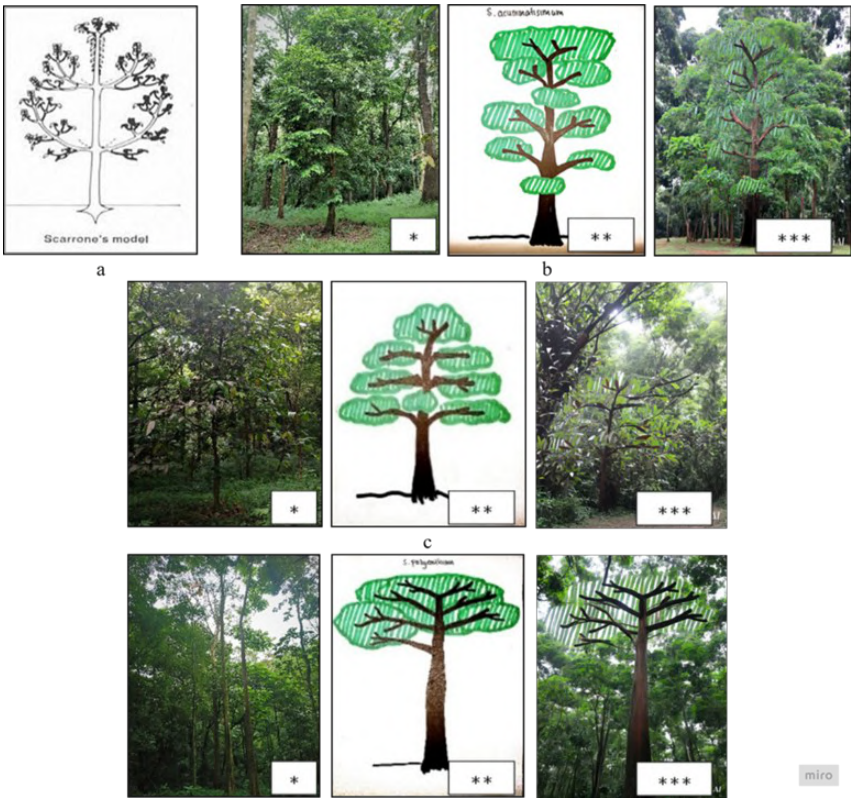


Figure 4. (a) Scarrone model (Tomlinson 1983), (b) *Syzygium acuminatisimum*, (c) *Syzygium formosum*, (d) *Syzygium polyanthum*. Description: * original picture, **manual illustration, ***AI illustration.

Stone Model

The observational data in Table 3 demonstrates that the Stone architectural model is the most prevalent of all models in the Purwodadi Botanical Garden's collection of *Syzygium* trees species. Sympodial stems with orthotropic branching patterns and stem development points ranging from fastigiated to patens are hallmarks of the Stone model in the data (Figure 5). According to the Stone model Shipunov (2023). The orthotropic branching and stems continue to develop sympodially. All trees have growing points that form an angle between 30 and 45 degrees, hence these points are fastigiated and rounded (Nurdiana 2020).

Attims Model

Observational data in Table 3 demonstrates that *Syzygium* trees species using the Attims model exhibit a distinctive monopodial stem growth pattern with fastigiated and patens branch growth points. Both species have an orthotropic pattern of branching (Figure 6). According to Shipunov (2023). The Attims model exhibits continuous growth, a monopodial trunk, and always orthotropic branching. Because the developing point in the Attims model forms an angle between 30° and 45°, it is patens and fastigiated (Nurdiana 2020).

Petit model

Observation data in Table 3, shows that *Syzygium discophorum* and *Syzygium nervosum* trees have *Petit's* architectural model. The stem growth pattern of both species is monopodial with plagiotropic branching and *horizontal* branch growth points (Figure 7). The *Petit* model has continuous growth with monopodial stems and orthotropic or plagiotropic branching (Shipunov 2023). The *horizontal* growth point indicates that the distance between the branch and the stem forms a 90° angle (Nurdiana 2020).

Koriba Model

The observation data in Table 3 shows that *Syzygium cumini* trees species have a *Koriba* architectural model characterised by dichotomous stem growth patterns, orthotropic branching patterns and horizontal branch growth points (Figure 8). The *Koriba* model has a stem that continues to develop sympodially with orthotropic branching (Shipunov 2023). The tree's growing point forms a 90° angle, which accounts for the horizontal growth point (Nurdiana 2020).

Massart Model

The characteristics of the *Massart* model owned by *Syzygium* trees species presented in Table 3 showed that the stem growth pattern is monopodial with plagiotropic branching patterns and *declinatus* branch growth points (Figure 9). The *Massart* model has a monopodial stem with branching that is always plagiotropic or orthotropic (Shipunov 2023). While the *declinatus* growing point is because the tree has a growing point that forms an angle >90° (Nurdiana 2020).

Troll model

The characteristics of the Troll model in *Syzygium sexangulatum* trees species presented in Table 3, show that the stem growth pattern is sympodial with orthotropic branching and *horizontal* branch growth points (Figure 10). The Troll model has a sympodial stem with plagiotropic or orthotropic branches (Shipunov 2023). The stem axis is *horizontal* (Nurdiana 2020).

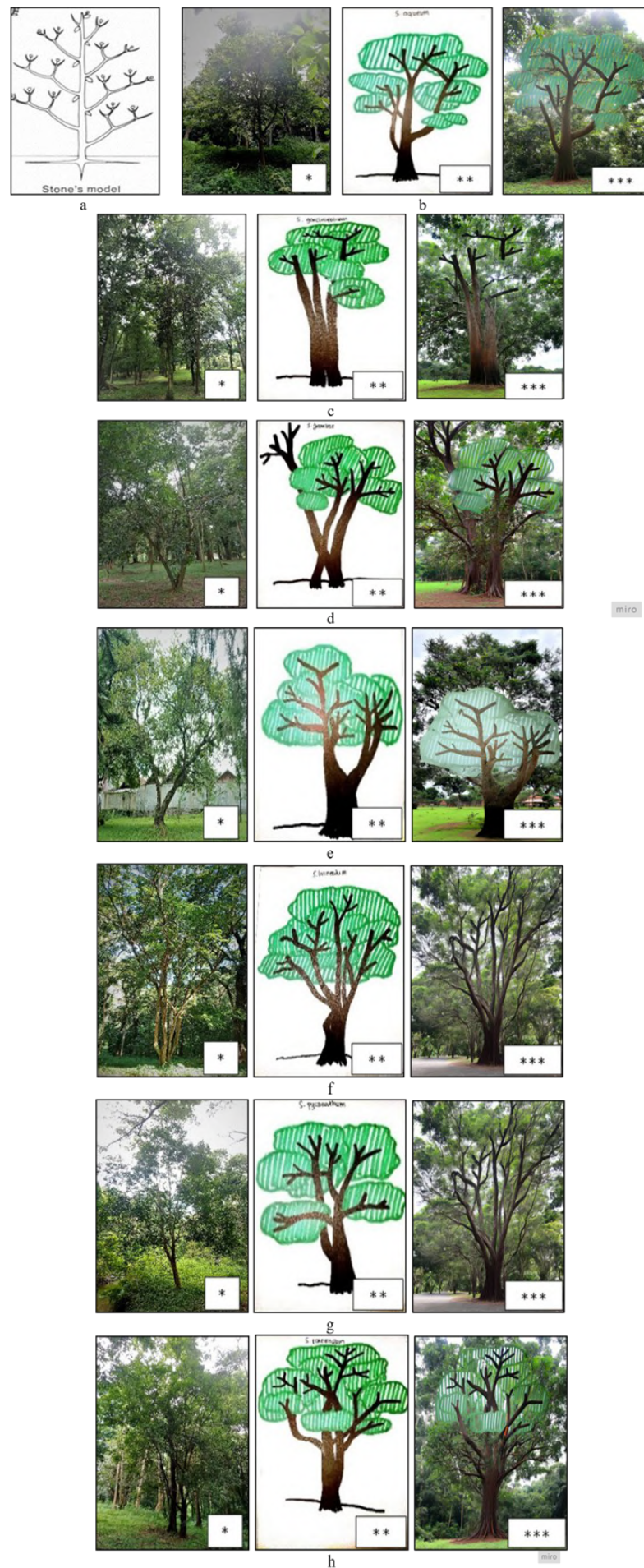


Figure 5. (a) Stone model (Tomlinson 1983), (b) *Syzygium aqueum*, (c) *Syzygium garcinifolium*, (d) *Syzygium jambos*, (e) *Syzygium javanicum*, (f) *Syzygium lineatum*, (g) *Syzygium pycnanthum*, (h) *Syzygium ramosum*.

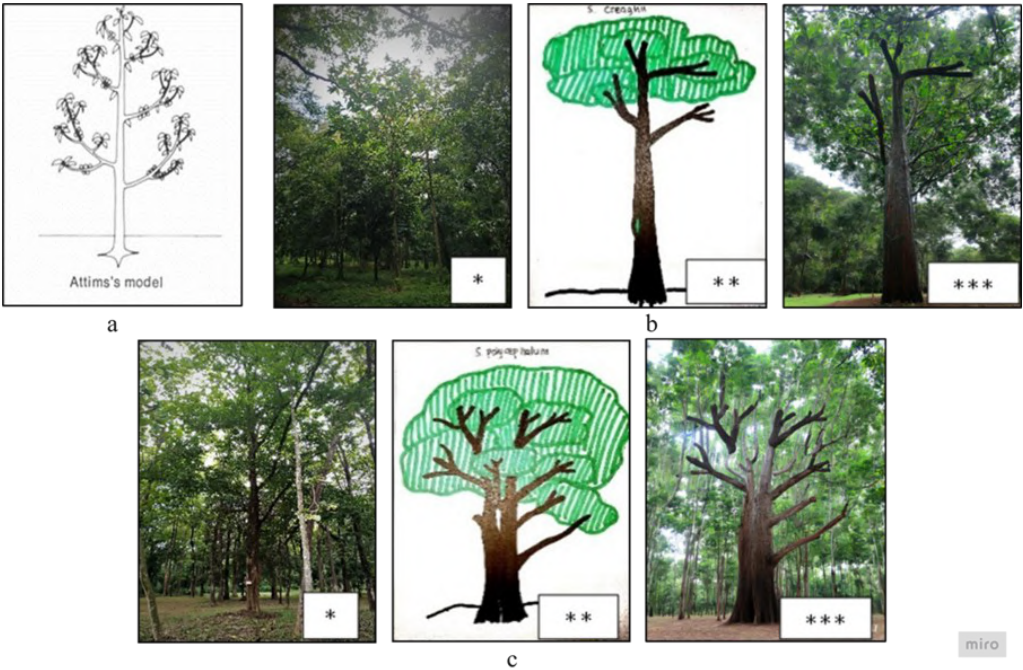


Figure 6. (a) Attimis model (Tomlinson 1983), (b) *Syzygium creaghii*, (c) *Syzygium polycephalum*. Description: * original picture, **manual illustration, ***Ai illustration.

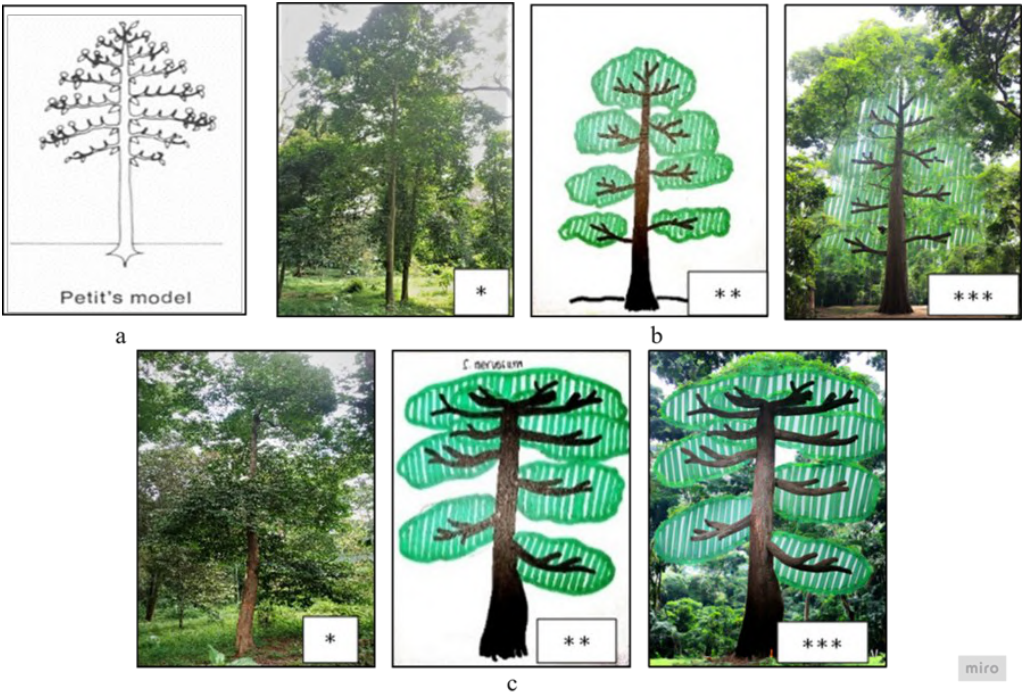


Figure 7. (a) Petit's model (Tomlinson 1983), (b) *Syzygium discophorum*, (c) *Syzygium nervosum*. Description: * original picture, **manual illustration, ***Ai illustration.

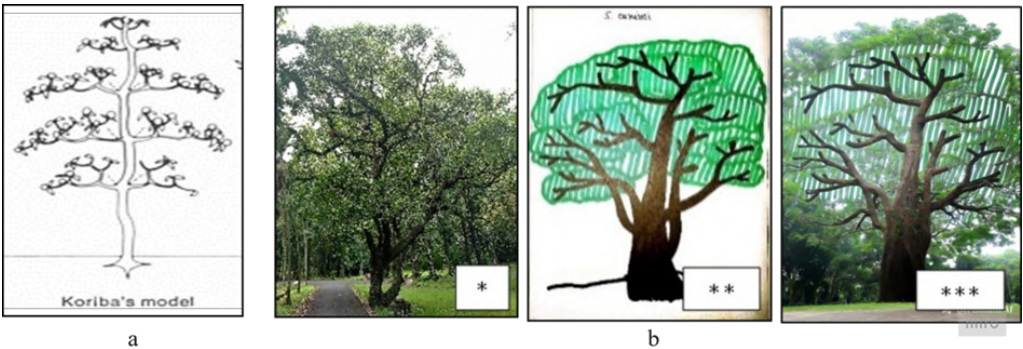


Figure 8. (a) Koriba model (Tomlinson 1983), (b) *Syzygium cumini*.

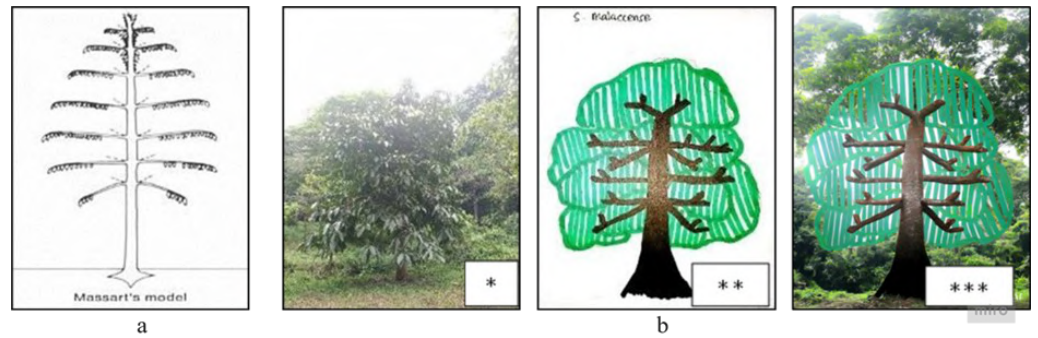


Figure 9. (a) Massart model (Tomlinson 1983), (b) *Syzygium malaccense*. Description: * original picture, **manual illustration, ***Ai illustration.



Figure 10. (a) Troll model Shipunov (2023), (b) *Syzygium sexangulatum*. Description: * original picture, **manual illustration, ***Ai illustration.

Potential Ecosystem Functions of *Syzygium* spp. Tree Architecture Models

Tree plays a fundamental role on forest ecosystems to the integrity of biotic and abiotic environmental influences. The adaptation effects apparently show in trees characteristic, structures and growth habits (Lin et al. 2018). The characteristics of the *Syzygium* species, which have deep and strong roots that can grasp the soil and avoid landslides, have demonstrated the ability of *Syzygium* trees species to promote ecological equilibrium. Additionally, *Syzygium* has a broad and intricate crown branching structure, which supports transpiration while reducing erosion because rainwater is absorbed in the tree crown before reaching the ground. Tree architecture model can help identify one of the various types of tree that support ecosystem balance (Lin et al. 2018; Fachruddin et al. 2020).

With orthotropic or upward-facing branching characteristics away from the ground surface and fastigiated and patens branching angles that form an angle of less than 90, *Syzygium* trees species in the Purwodadi Botanical Garden collection with Stone, Sccarone, and Attims architectural models have upward-facing branching tendencies. The traits of architectural models with these traits allow them to transpire more effectively than other models because they can hold more rainwater. Rainwater that lands on the tree canopy evaporates as soon as it hits the surface because of this. According to Song et al. (2020), the benefit of vegetation canopy has demonstrated that interception loss could account for 10–50 % of total rainfall in various natural and plantation forests. Erosion risk is decreased as a result.

Plagiotropic or orthotropic branching can be seen in the Koriba, Petit, Massart, and Troll models of *Syzygium* trees species that were gathered for this study, but all of them contain Horizontalis and Declinatus branching angles more than 90 degrees. By dangling and sloping downward, this architectural design creates a crown that leans toward the ground. In forests, the canopy changed rainfall kinetic energy and thus, modifying raindrop size and velocity, usually used indicator to express the potential of rainfall erosivity and predict soil erosion rates (Li et al. 2019)

The results of research conducted by Rahmania et al. (2024) showed that *Syzygium polyccephalum* species with attims tree architecture model and *Syzygium acuminatisimum* with sccarone tree architecture model had the highest interception value. Both have monopodial stems (distinguishable by branching), orthotropic branching (upward-facing branching), and patens stem growth direction (the distance between the main stem and branching forms a 45° angle). Trees with these characteristics have crowns that face upwards and away from the ground. This causes rainwater to evaporate immediately by the interception before falling to the ground. Interception is the rainwater on tree vegetation will be divided into two parts, some of which will evaporate and some will reach the ground through canopy escape and stem flow. So, it can support evaporation in the hydrological cycle in the ecosystem.

Although most species' architectural parameters overlapped and there was significant architectural variation within species, there was a clear correlation between a species' ranking in the probability distributions of architectural parameters and life history traits like adult stature and the amount of light needed for regeneration. According to these findings, architectural characteristics determines the level of productivity of a forest (Chantrain et al. 2018). The community structure of *Syzygium* in PBG is characterized by convergence and differentiation in tree design capable of supporting the hydrological cycle at the interception stage in the ecosystem.

CONCLUSION

There are 7 models of tree architecture in the *Syzygium* species collection of Purwodadi Botanical Garden. All *Syzygium* trees species have their own characteristics so that they can be grouped into tree architecture models. Of the 17 *Syzygium* tree species that met the selection criteria, 7 species have a Stone-model, 3 species have a Scarrone model, 2 species have Attims and Petit model, and 1 species each has Koriba, Massart, and Troll model. Each variation of tree architecture model has different functions to the ecosystem.

AUTHORS CONTRIBUTION

Rahmania, F. N., designed, supervised, conducted research sampling, analysed the research data, and drafted the research report, Prahardika, B. A., as a supervisor in the preparation of the design and discussion of research results, while Irawanto, R., as a field supervisor in sampling research data..

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CONFLICT OF INTEREST

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

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Research Article

Exploring Bivalve Community on the Southern Beaches of Bangkalan Madura Regency, Indonesia

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ABSTRACT

The southern beaches of Bangkalan Madura Regency are located in the Madura Strait, near the harbour of Surabaya. The areas have various substrates and mangrove vegetation, rich in invertebrates' diversity such as bivalve. This study aimed to identify the species, to analyse the diversity, and the importance value index of bivalve, as well as to describe the ecological characteristics on the southern beaches of Bangkalan Madura Regency, Indonesia. The sampling was conducted in the intertidal zone of the southern beaches of Bangkalan, namely Rongkang Beach, Kwanyar Beach, and Modung Beach. The quadrant transect method was applied for the sampling process using 270 plots of 1 x 1 m², located at upper intertidal, middle intertidal, and lower intertidal of beaches. Furthermore, samples were identified based on morphological characters and diversity was analysed using the Shannon-Wiener diversity index. The importance value index of each species was calculated and the ecological characteristics of the habitat were analysed by using Principal Component Analysis (PCA). The results showed that the South Beach of Bangkalan Madura Regency had 19 bivalve species from 10 families and 7 orders. Bivalve in this area were diverse, with a diversity index of 2.16. The highest importance value index was *Solen* sp. at 58.87 % followed by *Tegillarca granosa* at 30.34 %. Additionally, the PCA results showed that substrate pH, water pH, salinity, temperature, sand of substrate, and clay of substrate affected bivalve community. This showed that the southern beaches of Bangkalan were favourable for bivalve habitat.

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INTRODUCTION

Bivalves are benthic animals in shallow (littoral) or deep water (deep zone) living by sinking their bodies into the mud or sand substrate, sheltering behind rocks, wood, and marine plants. Some are attached to rocks, corals, and wooden pillars, or crawl freely on the surface of the substrate (Setyono 2006; Vaughn & Hoellein 2018; Abdulla-AI-Asif et al. 2021). The mussel group can be found in estuaries, rivers, mud flats, and rocky shores to the deep sea (Lim et al. 2018). Furthermore, bivalves are biological resources with high economic value, such as raw materials for food (Lim et al. 2018; Huang et al. 2023), crafts, as well as bioactive resources (Mona et al. 2021), and building materials (Sudhakaran & Poulose 2021). The species of bivalves are mostly consumable because of the ability to be directly processed into traditional food, such as *lorjuk rengginang* in Kamal District, Bangkalan (Kalsum et al. 2013; Susena & Yanuwadi 2015). The typical coastal community of Modung Beach, Bangkalan earns a living as bivalve fishermen and collectors for both consumption and trade, including *lorjuk* (bamboo clams), blood cockles, and oysters (Ambarwati et al. 2016). A previous study showed that *lorjuk* could be processed into various kinds of food (Wahyurini 2017). Apart from being an ingredient, bivalves play an important role in the environment, such as recycling organic matter, facilitating the mineralization process, and as bio-indicators of water quality because of sensitivity to changes in the aquatic environment (Zarkasyi et al. 2016; Vaughn & Hoellein 2018; Abdulla-AI-Asif et al. 2021). Some species have ecological roles as filter feeders and food for predators (Lim et al. 2018). The presence of bivalves is affected by several factors, including pollution, type of bottom substrate, composition of organic matter, predators, and competitors, as well as parameters of the aquatic environment (Dame 2012; Glaspie & Seitz 2017; Craeymeersch & Jansen 2019; Rahardjanto et al. 2020).

Studies on bivalve diversity have been conducted on the southern beaches of Madura Island. Ambarwati et al. (2016) listed 38 species of bivalves found on Modung Beach, while 8 species were reported in Barung Toraja Beach (Bening & Purnomo 2019). Another study found 15 species associated with lamp shells (Phylum Brachiopoda) in the Madura Strait (Rakmawati & Ambarwati 2020). Additionally, 21 species of bivalves were reported from the southern beaches of Pamekasan, Madura (Kurniawan et al. 2024). These studies showed that beaches on Madura Island had a high bivalve diversity.

The southern beaches of Bangkalan, Madura are covered by mangrove vegetation and has various substrates, namely muddy sand and rocky (Ramadhani et al. 2022; Ambarwati et al. 2024). This environmental condition provides potential habitats for bivalve community, located near a large harbour where local and international ships dock. Therefore, beach can have additional species from other areas, because aquatic animals such as bivalves spread easily due to larvae carried through ship ballast water. Some invasive species can spread to other places through ship ballast water (Arif et al. 2016). According to Irawan and Soegianto (2006), there were two additional orders and two crab species in the Madura Strait that had not been recorded in previous surveys in Indonesia. The addition of crab species is because the Madura Strait is a shipping traffic lane, allowing accidental migration through ship ballast water (Irawan & Soegianto 2006). This was supported by a study that showed *Mytella strigata*, a brackish water mussel from America was found to spread for the first time in Singapore through ship ballast water (Lim et al. 2018; Yip et al. 2021). Based on the described background, there is a need to explore bivalve community and their important contributions to the economy. Therefore, this study aims to identify the species, to analyse the diversity, and the importance value index of bivalve, as well as to describe the ecological

characteristics on the southern beaches of Bangkalan Madura Regency, Indonesia.

MATERIALS AND METHODS

Materials

This explorative study used 70 % alcohol to preserve the sample of bivalves.

Methods

Field Work

This study was conducted on the southern beaches of Bangkalan Madura Regency consisting of three stations, namely Rongkang Beach (7°09'52 "N and 112°50'30 "E), Kwanyar (7°09'52 "N and 112°50'58 "E), and Modung Beach (7°11'53 "N and 112°57'57 "E) (Figure 1). Rongkang Beach has rocky upper intertidal zone, without mangrove vegetation. Kwanyar Beach has thick mangrove vegetation and the substrate is muddy sand. Modung Beach has thin mangrove vegetation with muddy sand substrate. The sampling was conducted during the period when beach receded to the utmost low tide. Furthermore, the quadrant transect method was used by placing one transect line vertically on the coastline, which was divided into three intertidal, including upper, middle, and lower intertidal. A total of three quadratic plot sizes of 1 x 1 m² were placed in the middle of each intertidal area, as shown in Figure 2. Each station consisted of 10 transect lines, with a distance between transects of approximately 10 m. The substrate in the plot was dug using a trowel to collect the living bivalves. Initially, all bivalves found in the quadrant plots were cleaned and preserved using 70 % alcohol in collection bottles and labeled. The samples were deposited at the Laboratory of Animal Systematics, Universitas Negeri Surabaya, for further study.

The sample of substrates was taken from each intertidal area and kept in a plastic bag for further analysis in the laboratory. The composition of substrate was analysed using the sieving method (Test Sieve) and the salinity was measured using a hand refractometer (Atago, master-S28M, Japan). The pH and temperature of water were measured by using a pH56 meter (Milwaukee, Romania). The pH of substrate was measured by using a soil tester.

Identification

The observation was conducted on the morphological characteristics of bivalve such as shell shape, colour, and sculpture, including ligament colour, adductor muscle scars, umbo position, umbo colour, pallial lines, pallial sinuses, and dentition. Bivalve samples were identified using the Lamprell and Whitehead (1992), Lamprell and Healy (1998), Dharma (2005), Huber (2010, 2015), Tan et al. (2022), and the World Register of Marine Species (WoRMS) database website.

Data Analysis

Diversity data were analyzed based on the Shannon-Wiener diversity index (H') (Odum 1993):

$$H' = -\sum \frac{(ni)}{N} \ln \frac{(ni)}{N}$$

Where: H' : Diversity index; ni : Number of individuals of the i -th species; N : Total individual population. The results of the diversity index calculation are divided into three categories, namely $H' < 1$, $1 < H' < 3$, and $H' > 3$, representing low, medium, and high levels of species diversity, respectively.

The importance value index (IVI) was calculated by using the following formulas:

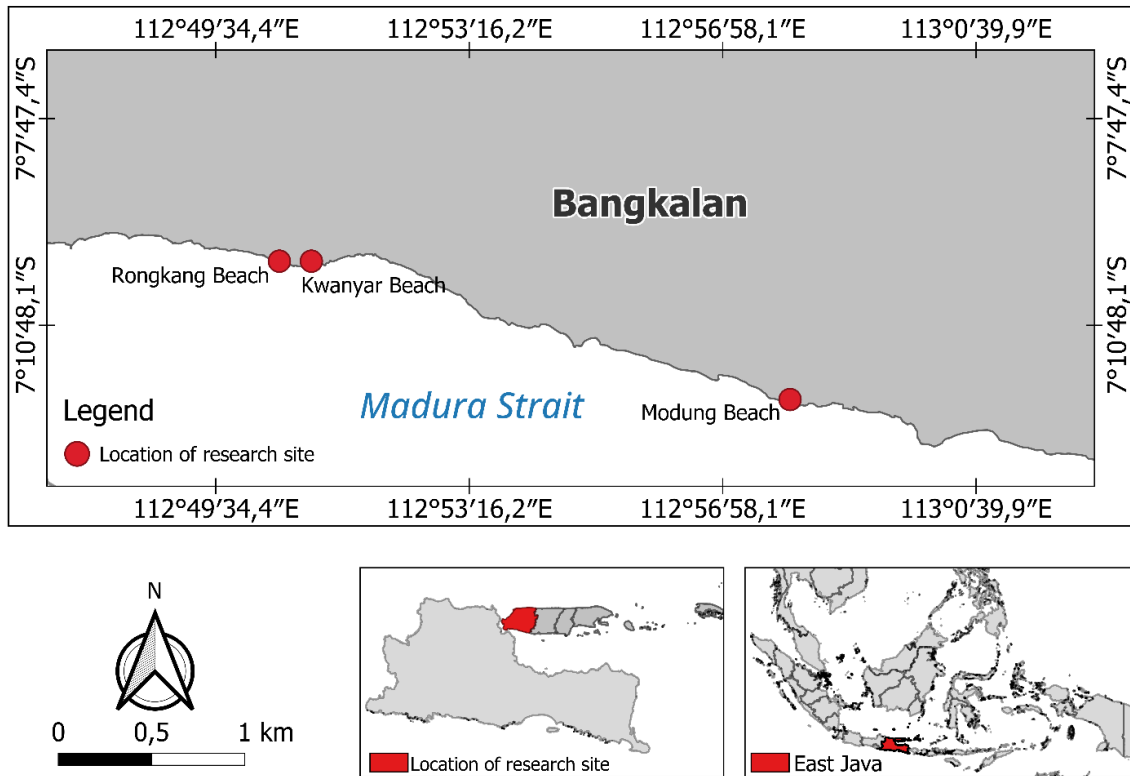


Figure 1. Bivalve sampling stations in the southern beaches of Bangkalan, Madura.

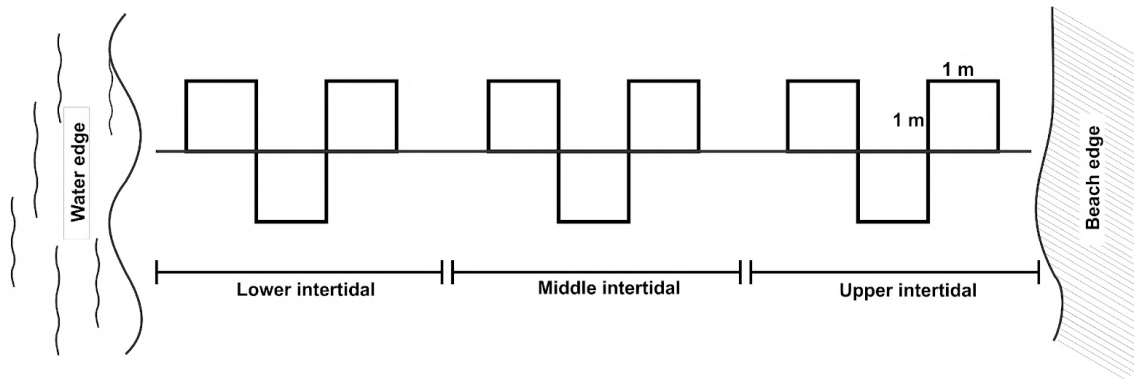


Figure 2. Bivalve sampling design of one transect line.

$$Di = \frac{Ni}{A}$$

$$DRi = \left(\frac{Di}{DN} \right) \times 100 \%$$

$$FRi = \left(\frac{Fi}{FN} \right) \times 100 \%$$

$$IVIi = DRi + FRi$$

Where: Di : density of individuals of the i species (ind m^{-2}), Ni : number of individuals of the i species, A : total area of sampling (m^2), DRi : relative density of i species (%), DN : total density of all species, FRi : relative frequency of i species (%), Fi : frequency of i species, FN : total density of all species, $IVIi$: importance value index of i species.

Ecological characteristics data were analysed using Principal Component Analysis (PCA). Several stages were carried out in PCA, including the calculation of Bartlett's test of sphericity, Kaiser-Meyer-Olkin (KMO), Measure Sampling Adequacy (MSA), communalities, and determination of the main factors formed.

RESULTS AND DISCUSSION

Bivalve Community

The southern beaches of Bangkalan had different substrate types, namely Rongkang Beach with dominant rocky substrate, while Kwanyar Beach and Modung Beach had sandy mud substrate. The identification results in Figure 3 showed that 19 bivalve species belonging to 10 families and seven orders were obtained in the southern beaches of Bangkalan. Among all samples collected, the order Veneroida had the most members, consisting of three families and seven species. The family with the largest number of species is Veneridae with four species, as shown in Table 1.

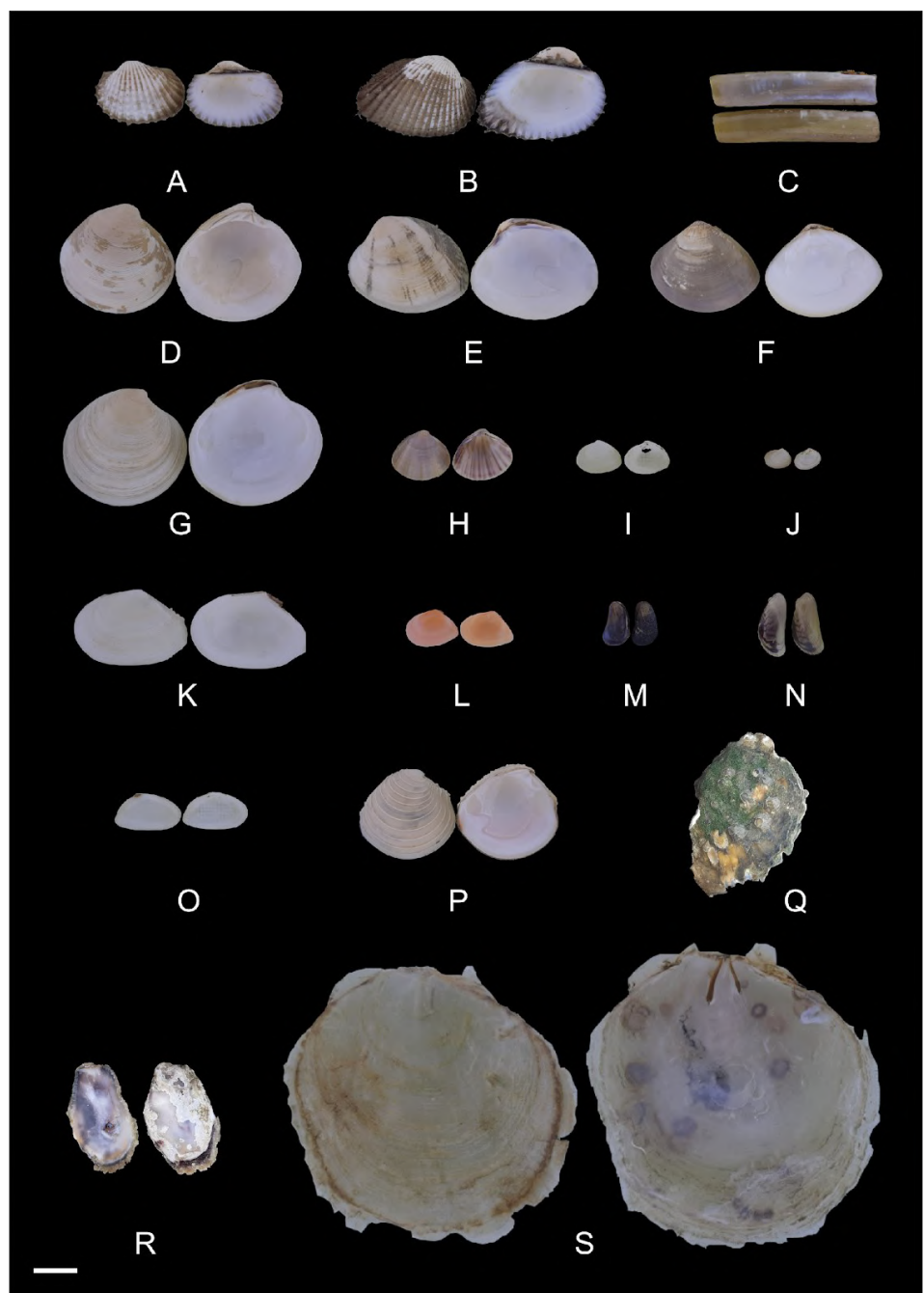


Figure 3. Bivalves on the southern beaches of Bangkalan Madura, A: *Tegillarca granosa* (Linnaeus, 1758), B: *Anadara rhomboidalis* (Schumacher, 1817), C: *Solen* sp., D: *Dosinia dilecta*

A. Adams, 1856, E: *Marcia hiantina* (Lamarck, 1818), F: *Meretrix* sp., G: *Semele cordiformis* (Holten, 1802) H: *Mactra incarnata* Reeve, 1854, I: *Heterocardia gibbosula* Deshayes, 1855, J: *Amarilladesma* sp., K: *Psammacoma gubernaculum* (Hanley, 1844), L: *Jitlada philippinarum* (Hanley, 1844), M: *Vignadula mangle* (Ockelmann, 1983), N: *Arcuatula senhousia* (W. H. Benson, 1842), O: *Tellinides timorensis* Lamarck, 1818, P: *Placamen isabellina* (R. A. Philippi, 1849), Q: *Crassostrea* sp., R: *Saccostrea* sp., S: *Placuna placenta* (Linnaeus, 1758). Scale bar: 10 mm.

The number of bivalves found on the southern beaches of Bangkalan is almost similar with the one in Pamekasan Madura (Kurniawan et al. 2024). However, the species composition on the two beaches was different. This study found new records that had not been reported in previous surveys conducted in Bangkalan Madura (Ambarwati et al. 2016; Rakmawati & Ambarwati 2020; Wahyuni et al. 2016) including *Placamen isabellina*, *Mactra incarnata*, *Amarilladesma* sp., *Semele cordiformis*, *Placuna placenta*, *Vignadula mangle*, and *Arcuatula senhousia*. One of the newly discovered records in the intertidal area of Bangkalan Beach was *Arcuatula senhousia*, which was previously known as *Musculita senhousia* and commonly found in the Madura Strait (Santi et al. 2021). The Asian mussel had spread to New Zealand, Australia, the Mediterranean, and the Pacific Beach of the United States (GISD 2024). The species could grow rapidly and lived in groups on soft substrates, with the ability to dominate benthic communities and exclude. Additionally, the species invaded the Oristano Lagoon-Gulf System (W Sardinia, Italy) at moderate densities (<1000 m²), without impacting sediment structure and local macrozoobenthic assemblages (Como et al. 2015).

Arcuatula senhousia has spread to West Africa, where there is accidental transportation with oysters or other bivalves traded for shellfish farming or carried by ship ballast water (Lourenco et al. 2018). This is supported by other studies where larvae of aquatic animals such as bivalve can spread through passive transport such as hull (biofouling) with ballast water (Phillips et al. 2012; Lim et al. 2018).

On the southern beaches of Bangkalan, certain groups of bivalves comprising the Veneridae, Arcidae, Solenidae, Mactridae, and Tellinidae families, coexist in the same habitat with lamp shells (Brachiopoda) (Rakmawati & Ambarwati 2020). The Veneridae species dominate the sampling area on the southern beaches of Bangkalan. This result was similar to the mollusk diversity in Modung Beach Bangkalan Madura, where most of bivalve species found were members of the Veneridae family (Ambarwati et al. 2016). Other studies reported that the Veneridae family was found in the Madura Strait with the highest number of species compared to others (Rakmawati & Ambarwati 2020). Moreover, substrate dominated by sandy mud is considered suitable for Veneridae (Rakmawati & Ambarwati 2020; Takar et al. 2023).

The majority of bivalves discovered on the southern beaches of Bangkalan were in the middle and lower intertidal areas. The species in the upper intertidal area were mostly the Ostreidae (*Saccostrea* sp. and *Crassostrea* sp.) and the Mytilidae (*Vignadula mangle* and *Arcuatula senhousia*) attached to rock and coral substrates. *Saccostrea* and *Crassostrea* attach their shells to the rock, while *Vignadula mangle* and *Arcuatula senhousia* attached using byssus.

In the middle and lower intertidal, bivalves were found to live by migrating (infauna). The species possessed long siphons that could support their lives as infauna (Zwarts & Wanink 1989; Poutiers 1998). Approximately all bivalves on the South Beach of Bangkalan were obtained at depths between 0-15 cm, but *Solen* sp. was found to be very deep at a depth of 20 cm. Each taxon has a distinctive morphology as a form of adaptation to its habitat (Ambarwati et al. 2016). For example, *Solen* sp. has a very deep pallial sinus that shows the size of the siphon and the ability to dig into the substrate. This species has a slender and small body with modified parts to dig the substrate faster (Trisyani et al. 2016).

Based on bivalve found on the southern beaches of Bangkalan, approximately all species can be consumed with high economic value. This causes a significant increase in the exploitation of bivalve by coastal community, particularly *Solen* sp. The species is often called *lorjuk* or bamboo clams, which is usually used as an ingredient for making *rengginang*, *lorjuk* rice cracker, and fried *lorjuk* (Kalsum et al. 2013; Susena & Yanuwadi 2015; Wahyurini 2017). In addition to *lorjuk*, clams that are often caught by coastal community are blood cockles (*Tegillarca granosa*), due to delicious taste and high economic value. Previous studies have shown that *Tegillarca granosa*, *T. nodifera*, and *Anadara rhomboidalis* are consumed by coastal community (Dharma 2023).

Bivalve Diversity Index

Bivalve diversity analysis was carried out to determine the distribution level on three stations and was calculated using the Shannon-Wiener diversity index formula. The diversity index value on the southern beach of Bangkalan was 2.16, which is classified as medium. Each station had a different value, where Rongkang Beach had the highest index value of 1.84, followed by Kwanyar and Modung at 1.41 and 1.21, respectively.

Based on the diversity index calculation ($H'=2.16$), bivalve on the southern beaches of Bangkalan was on the medium level. Moreover, the presence of species with high abundance, equal, or approximately equal, in community showed high diversity (Odum 1993). The calculation of the Shannon-Wiener diversity index ranged from 1.5 to 3.5, suggesting a moderate level (Magurran 2004). Generally, two factors that affect the diversity index value include the number of species and relative abundance (McNaughton & Wolf 1992).

Rongkang Beach had the highest diversity index value compared to Kwanyar and Modung. This was because the substrate of Rongkang Beach was more varied, with rocky predominating in the upper intertidal alongside sandy mud in the middle and lower intertidal. Additionally, this beach had several bivalve species with high relative abundance, including *Solen* sp., *Vignadula mangle*, *Arcuatula senhousia*, and *Saccostrea* sp.

The index value of bivalve diversity in Kwanyar Beach was 1.40, while the lowest was Modung at 1.21. This lower value was because there were suburban areas around beach with several activities including fishing. According to D'Souza and Shenoy (2023), human activities such as settlement and fishing could affect the diversity of macrozoobenthos including bivalve.

Solen sp. from the Solenidae family is a species that dominated the three stations, as shown in Figure 4. The high relative abundance value showed that the southern beaches of Bangkalan dominated by sandy mud substrate were a suitable habitat for *Solen* sp. Although sand can facilitate the movement or shift of biota, mud offers a stable substrate and serves as an attachment for organisms (Wahyuni et al. 2016; Craeymeersch & Jansen 2019). Therefore, a sandy mud substrate supports *Solen* sp. in growth and development (Wahyuni et al. 2017). These results were in line with previous studies, where *Solen* sp. dominated the waters of Bangkalan (Wahyurini 2017).

In three stations, the least common species were *Anadara rhomboidalis*, *Amarilladesma* sp., *Semele cordiformis*, and *Placuna placenta*, as shown in Figure 4. This study was conducted in the intertidal area, which contributed to the low distribution of *Placuna placenta*. Malacofauna studies at Selayung Beach, Tarakan City, North Kalimantan showed that *Placuna placenta* was commonly found in the subtidal area (Zainuddin et al. 2018). Since these clams are more valuable economically compared to others, over-exploitation can occur causing a decrease in population. Due to numerous human activities on Kwanyar and Modung Beach including fishing, these two places have potentially been overused.

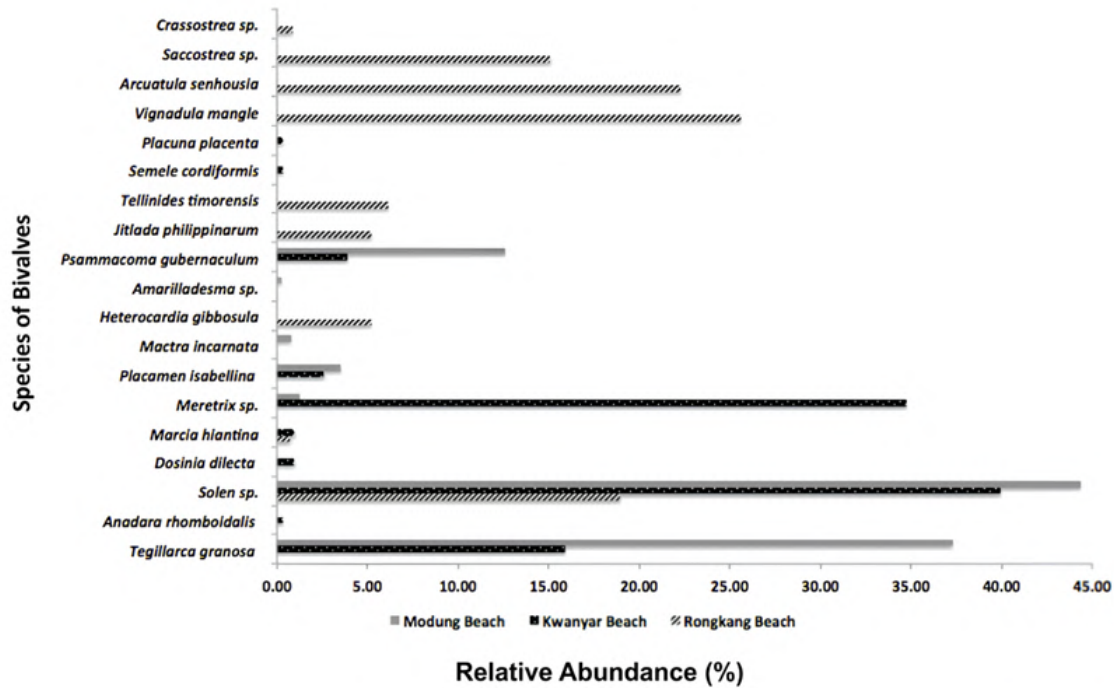


Figure 4. Bivalve species relative abundance (%) of each station.

Importance Value Index of Bivalve

Each bivalve species had different density and frequency values. The species with the highest density was *Solen* sp. at 1.78 ind m⁻² with a relative frequency of 30 %, as shown in Table 1. Meanwhile, the lowest was *Anadara rhomboidalis*, *Amarilladesma* sp., *Semele cordiformis*, and *Placuna placenta* comprising only one individual during the sampling. Based on the results, each species had a different distribution. *Solen* sp. was found on all beaches, while

Table 1. Bivalve species on the southern beaches of Bangkalan Madura Regency.

No.	Order	Family	Species	Ni	Ni/A (ind m ⁻²)	DRi (%)	F	FRi (%)	IVI (%)
1.	Arcida	Arcidae	<i>Tegillarca granosa</i> (Linnaeus, 1758)	197	0.73	11.82	0.37	18.52	30.34
2.			<i>Anadara rhomboidalis</i> (Schumacher, 1817)	1	0.00	0.06	0.00	0.19	0.25
3.	Adapedonta	Solenidae	<i>Solen</i> sp.	481	1.78	28.87	0.60	30.00	58.87
4.	Venerida	Veneridae	<i>Dosinia dilecta</i> A. Adams, 1856	3	0.01	0.18	0.01	0.56	0.74
5.			<i>Marcia hiantina</i> (Lamarck, 1818)	10	0.04	0.60	0.03	1.30	1.90
6.			<i>Meretrix</i> sp.	112	0.41	6.72	0.24	12.22	18.94
7.			<i>Placamen isabellina</i> (R. A. Philippi, 1849)	22	0.08	1.32	0.07	3.70	5.02
8.		Mactridae	<i>Mactra incarnata</i> Reeve, 1854	3	0.01	0.18	0.01	0.56	0.74
9.			<i>Heterocardia gibbosula</i> Des- hayes, 1855	50	0.19	3.00	0.13	6.48	9.48
10.		Mesodesmatidae	<i>Amarilladesma</i> sp.	1	0.00	0.06	0.00	0.19	0.25
11.	Cardiida	Tellinidae	<i>Psammacoma gubernaculum</i> (Hanley, 1844)	62	0.23	3.72	0.16	7.78	11.50
12.			<i>Jitlada philippinarum</i> (Hanley, 1844)	50	0.19	3.00	0.11	5.37	8.37

Table 1. Contd.

No.	Order	Family	Species	Ni	Ni/A (ind m ⁻²)	DRi (%)	F	FRi (%)	IVI (%)
13.			<i>Tellinides timorensis</i> Lamarck, 1818	59	0.22	3.54	0.13	6.67	10.21
14.		Semelidae	<i>Semele cordiformis</i> (Holten, 1802)	1	0.00	0.06	0.00	0.19	0.25
15.	Pectinida	Placunidae	<i>Placuna placenta</i> (Linnaeus, 1758)	1	0.00	0.06	0.00	0.19	0.25
16.	Mytilida	Mytilidae	<i>Vignadula mangle</i> (Ockelmann, 1983)	246	0.91	14.77	0.03	1.30	16.06
17.			<i>Arcuatula senhousia</i> (W. H. Benson, 1842)	214	0.79	12.85	0.02	0.93	13.77
18.	Ostreida	Ostreidae	<i>Saccostrea</i> sp.	145	0.54	8.70	0.06	2.96	11.67
19.			<i>Crassostrea</i> sp.	8	0.03	0.48	0.02	0.93	1.41

Crassostrea sp. was discovered on Rongkang Beach due to the presence of a rock substrate. Generally, *Crassostrea* sp. prefers habitats in tidal and subtidal areas, which have fine or coarse substrates including rocks (Harris 2008).

Solen sp. was the species with the highest Important Value Index (IVI) (58.87 %), playing a significant role in the ecosystem. A species' significance in the habitat is generally influenced by compatibility with the substrate characteristics, as showed by IVI value (Rohmayani et al. 2021). *Solen* sp. such as sandy mud substrates (Dharma 2023; Kurniawan et al. 2024), and the southern beaches of Bangkalan was dominated by sandy mud substrates. Meanwhile, the species with the lowest IVI value were *Anadara rhomboidaliss*, *Amarilladesma* sp., *Semele cordiformis*, and *Placuna placenta* with an IVI value of 0.25 %. This showed that these species have little role in the community.

Ecological characteristics of bivalve

The habitat profile on the southern beaches of Bangkalan in each station has different values. Based on the results of substrate composition analysis, the southern beaches of Bangkalan were dominated by sandy mud substrate, while Rongkang Beach had a rocky substrate at upper intertidal area (Table 2).

The analysis of Bartlett's test of sphericity, KMO, MSA, and communal-

Table 2. Habitat profile of bivalve at the southern beach of Bangkalan.

Station	Zone	Substrate pH	Water pH	Salinity (ppt)	Temperature (°C)	Gravel of substrate (%)	The sand of substrate (%)	Clay of substrate (%)
Rongkang Beach	Upper	rocks	rocks	rocks	rocks	rocks	rocks	rocks
	Middle	6.4	7.9	30	29	1.38	9.88	88.74
	Lower	6.4	7.9	30	30	1.05	10.52	88.43
Kwanyar Beach	Upper	5.2	7.9	30	29	8.50	21.81	69.68
	Middle	4.6	7.3	29	24	7.10	23.85	69.05
	Lower	5.2	7.4	29	24	6	25.10	68.89
Modung Beach	Upper	6	7.9	30	28	2.81	19.18	79.01
	Middle	6.2	7.9	30	28	3.01	17.56	79.43
	Lower	6.2	7.9	30	29	2.10	18.20	79.70

ities analysis of environmental factors of bivalve habitats showed significant results. The communalities value of each variable of feasible environmental factors including substrate pH, water pH, salinity, temperature, sand, and clay were > 0.5 . This showed that each variable could explain more than 50 % of the variance of the factors formed. The variable with the highest communalities of 0.891 was temperature, showing the potential to explain the variance or diversity by 89.1 %. In this case, the temperature variable had the highest closeness to the factors formed. Meanwhile, the variable with the lowest communalities of 0.796 was sand of substrate. This showed that the sand of substrate variable was able to explain the variance or diversity of the factors formed by 79.6 %. In this case, the sand of the substrate variable had the lowest closeness. To determine the number of factors formed, the eigenvalue reference > 1 was used as in Figure 5.

Based on Figure 5 there is a very significant decrease in eigenvalue from components 1 to 2. Meanwhile, the decrease in the line on components 2, 3, 4, 5, and 6 is not significant. Component 1 is the only factor with an eigenvalue > 1 , showing the potential to explain all variables optimally without including other factors.

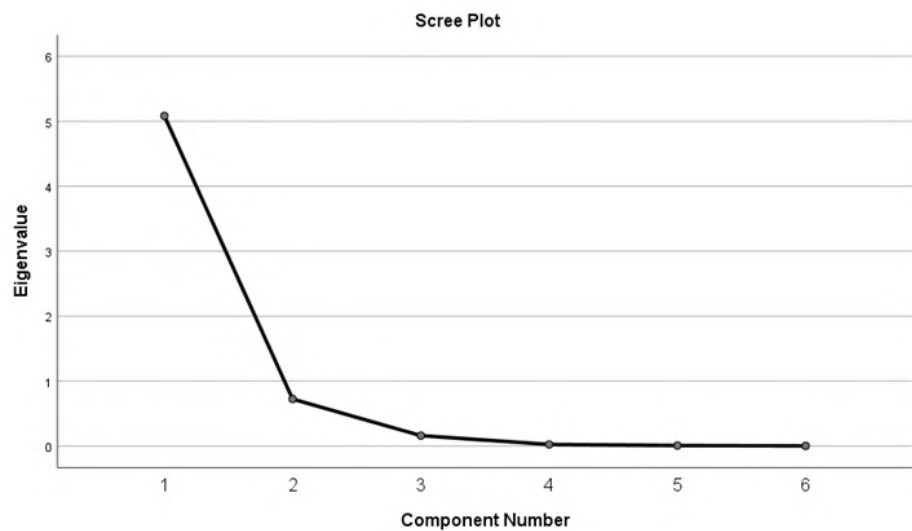


Figure 5. Scree plot of feasible environmental factors of bivalve habitat.

Based on Table 3, the eigenvalue of component 1 was $5.083 > 1$, while other factors had eigenvalues < 1 . Component 1 was able to explain the variance or diversity of all variables which was very high at 84.714 %. Therefore, six variables including substrate pH, water pH, salinity, temperature, sand, and clay could be summarised into 1 component. This showed that environmental characteristics affecting bivalve community with a total variance could be explained by 84.714 %. The results also showed the habitat characteristics importance for intertidal community (Dame 2012; de Fouw et al. 2020).

Table 3. Total variance explained by environmental factors of bivalve habitat.

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	5.083	84.714	84.714	5.083	84.714	84.714
2	0.723	12.053	96.767			
3	0.160	2.666	99.433			
4	0.024	0.408	99.841			
5	0.008	0.134	99.975			
6	0.001	0.025	100.000			

The composition of substrate types serving as a place to stick, crawl, or dig, plays a significant effect in the development of fauna in coastal environments (Setyono 2006). Mud substrates with a very small grain composition contain food and organic matter, which support water in the sediment, and remain intact as beach recedes (Cappenberg 2016; Atlanta et al. 2022).

The quality of the coastal area plays an important role for benthic animals including bivalve due to high tolerance, which serves as a bio-indicator of environmental changes (Kharisma et al. 2012). In this study, the difference in the pH value of each beach was due to the activities of community in fishing and the presence or absence of residential settlements. This is because community activity can generate waste along the shore, particularly from domestic waste causing significant changes in water pH (Hasibuan 2016). Ivani-na et al. (2020) stated that an optimum pH optimally supported bivalve metabolic process. Salinity is a supporting factor for bivalve and the values can be affected by the entry of freshwater into seawater (Lindawaty et al. 2016). Therefore, some bivalve species are found in estuarine areas where salinity fluctuates.

CONCLUSION

In conclusion, 19 bivalve species from seven orders and 10 families were found on the southern beaches of Bangkalan Madura Regency, namely Arcidae, Solenidae, Veneridae, Mactridae, Mesodesmatidae, Tellinidae, Semelidae, Placunidae, Mytilidae, and Ostreidae. Bivalve species found were in the medium category with a diversity index of 2.16, where the IVI of species was *Solen* sp. by 30.00 %. This beach had a favourable habitat profile for bivalve. The substrate pH, water pH, salinity, temperature, sand, and clay affected bivalve community. Therefore, the southern beaches of Bangkalan Madura had the potential for bivalve diversity.

AUTHORS CONTRIBUTION

N.N.R.W. designed the study, conducted sampling, and wrote manuscript. R.A. designed the study, identification, wrote manuscript and review.

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CONFLICT OF INTEREST

There is no conflict of interest.

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Research Article

Foraminifera Abundance in the Southern Waters of Sumbawa, Senunu Canyon, West Sumbawa, Sumbawa, West Nusa Tenggara

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ABSTRACT

The Southern Waters of Sumbawa, Senunu Canyon, West Nusa Tenggara, is influenced by the Indonesian Throughflow (ITF), crucial for regional marine dynamics. This area also faces impacts from mining tailings discharged into the seabed, raising concerns about their impacts on meiobenthic fauna, including foraminifera. This study examined changes in foraminiferal assemblages, abundance, and diversity using 20 gravity core samples from sites NM.021 and NM.023. The results revealed a diverse and abundant presence of foraminifera, with planktonic foraminifera dominating in terms of abundance compared to benthic foraminifera, as indicated by an average P/B ratio exceeding 90 %. Twelve species of planktonic foraminifera and 17 species of benthic foraminifera were identified, with distinct compositions across the sites. At site NM.021, the most abundant benthic foraminifera were *Bulimina* sp. (23.5 %), *Ceratobulimina pacifica* (18.7 %), and *Hoeglundina elegans* (13.3 %), while dominant planktonic foraminiferal taxa consisted of *Hastigerina pelagica* (21.6 %), *Pulleniatina obliquiloculata* (17.8 %), and *Neogloboquadrina dutertrei* (17.4 %). At site NM.023, the most abundant benthic foraminifera were *Bolivinita quadrilatera* (22.3 %) and *Ceratobulimina pacifica* (21.8 %), while the planktonic foraminiferal taxa showed similar dominance, with *Pulleniatina obliquiloculata* (20.8 %), *Hastigerina pelagica* (15 %), and *Neogloboquadrina dutertrei* (13.3 %) being the most abundant. The study highlights warm, eutrophic conditions with potential upwelling, indicated by planktonic species *Pulleniatina obliquiloculata* and *Neogloboquadrina dutertrei*. Additionally, these waters also have low oxygen levels (dysoxic), as evidenced by the presence of benthic species *Bulimina* sp. and *Ceratobulimina pacifica*.

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INTRODUCTION

Southern Waters of Sumbawa is one of the waterways traversed by the Indonesian Throughflow (ITF) pathway, making it compelling to study and important to understand due to its significant impact on both Indonesian and global climate conditions. One of the bioindicators that can be used to assess the condition of the waters is foraminifera. Foraminifera are single-celled microorganisms commonly found in brackish to deep-sea environments. While some foraminifera have adapted to a planktonic lifestyle, the majority are benthic. Their distribution is primarily affected by abiotic factors including temperature, salinity, oxygen levels, light, substrate type, turbidity, and nutrient availability. Various species assemblages are typical of different water depths, environments, and latitudinal zones (Pawlowski 2009). This makes foraminifera a potential indicator for understanding both ancient and modern marine environmental conditions.

Administratively, Southern Waters of Sumbawa are located in Sumbawa Regency, West Nusa Tenggara Province. The morphology of these waters consists of basins and ridges influenced by several factors including geological processes, submarine rock formations, sedimentation processes, and geodynamic activities, including the potential impact of mining activities in the area. Mining activities in this area produce tailings waste, while the tailings have been classified as non-hazardous to marine biota (Gwyther et al. 2009), the distribution of which affects the morphology around the tailings pipe outlet (Noviadi et al. 2011). Specifically, the seafloor morphology in this area consists of ridges with steep slopes, causing sediment movement that makes it unstable. This sediment instability can impact the ecosystem, affecting the ecology and the presence of organisms such as meiofauna (Susetiono et al. 2020). In general, the Southern Waters of Sumbawa have depths ranging from 0 to 4600 meters below the surface. Shallow depths are near coastal areas while the deepest parts are southwest of Sumbawa Island (LIPI 2018). Sea surface temperatures in Southern Waters of Sumbawa are relatively warm, ranging between 26–28 °C at a depth of 5 meters. The sampling locations for the research area can be seen in Figure 1.

Marine investigations in the Southern Waters of Sumbawa were conducted by the Oceanography Team of the Oceanographic Research Center Laboratory of the Indonesian Institute of Sciences (LIPI) in 2018. Additionally, there have been numerous studies conducted in the Southern Waters of Sumbawa, including research analyzing the relationship between meiofauna abundance and the presence of tailings in sediments by Susetiono et al. (2020). Other studies by Putra and Nugroho (2019), Zhang et al. (2019), and Ardi et al. (2021, 2023) provide additional insights into the marine environment of the region. There is still a lack of research specifically focusing on the abundance and distribution of foraminifera, particularly in areas influenced by the presence of tailings. This study aims to address this gap, contributing to a more comprehensive understanding of local marine ecosystems of the Southern Waters of Sumbawa.

MATERIALS AND METHODS

Materials

The data used in this research are the abundance and distribution data of foraminifera microfauna from 20 samples, collected from 2 gravity cores, each 50 cm in length. The samples were extracted at 5 cm intervals, with 10 samples taken from each core, from two sites, NM.021 at a depth of 2173 meters and NM.023 at a depth of 2018 meters, in the Southern Waters of Sumbawa. The selection of these sites was based on their proximity to the tailing pipeline, with NM.021 located along the path of the pipeline and NM.023 positioned outside its influence. This approach was intended to facilitate comparative analysis to assess the impact of tailings on the abundance and distribu-

tion of benthic foraminifera and to investigate differences in environmental conditions between the impacted and non-impacted sites.

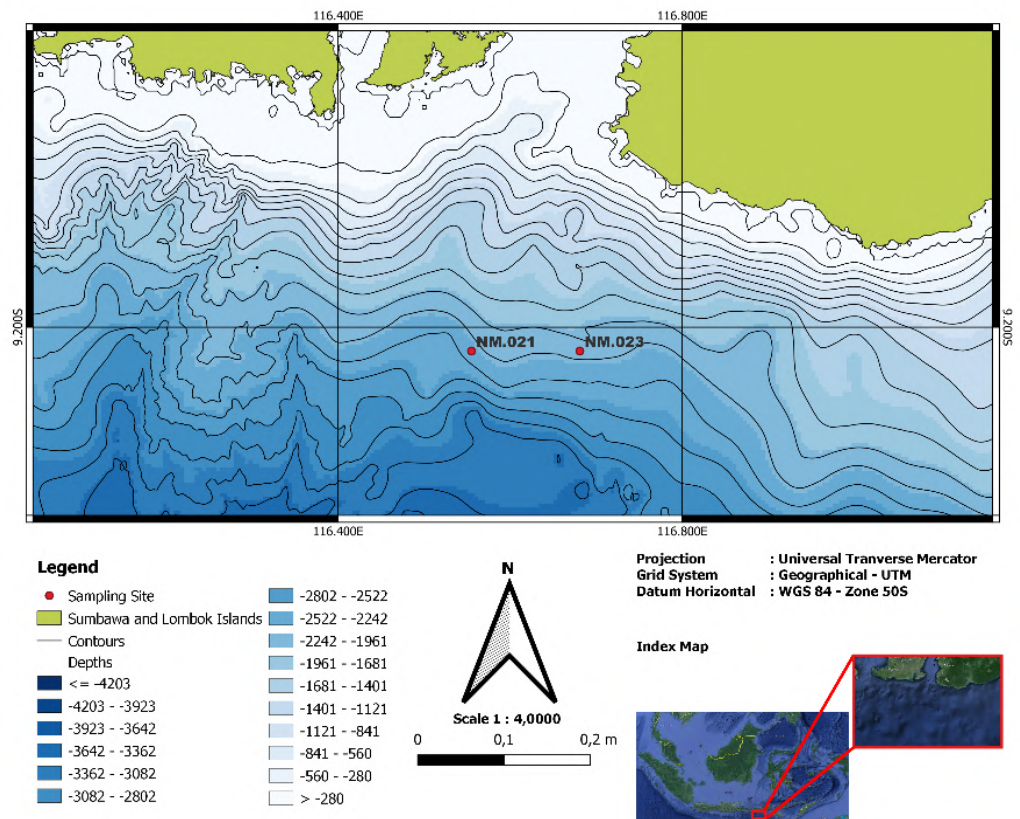


Figure 1. Location of research and sediment sampling in the Southern Waters of Sumbawa.

Methods

Sediment samples were prepared and analyzed at the Oceanographic Research Center Laboratory of the National Research and Innovation Agency of Indonesia (BRIN) located in Ancol, North Jakarta. The equipment used in the research included oven, sieve mesh, scale, splitter, binocular microscope, digital camera, and PAST software (Paleontology Statistical 3) to calculate diversity index. Samples were cut into 5 cm segments, dried at 80 °C for 24 hours, and weighed. They were then soaked in hot water for 2 hours to disaggregate the sediment and sieved using a granulometry mesh for grain size separation. The fractions were dried again, weighed, and stored in plastic bags by size. For foraminifera analysis, 1 gram of the 60 mesh (0.25 mm) size fraction was taken from the net weight obtained in the granulometric analysis. Subsequently, approximately 300 foraminifera shells were separated from the sediment material within each sample. In cases where the sample volume was large and contained abundant foraminifera shells, the sample was divided into several parts using a splitter. The identification of foraminifera was conducted using references from Postuma (1971), Bolli et al. (1985), Loeblich and Tappan (1994), and Holbourn et al. (2013). Determination was also referred to foraminiferal websites including <http://www.foraminifera.eu>, <http://www.marinespecies.org> (World Register of Marine Species, WoRMS), and <https://www.mikrotax.org>.

Quantitative Analysis

Relative Abundance

Relative abundance of foraminifera is determined through quantitative analysis involving the calculation of the percentage of each species relative to

the total number of individuals in each sediment sample, which will be presented in Table 1 and Table 2, using the formula (Murray 2006):

$$\text{Relative abundance} = \frac{N_i}{N} \times 100\%$$

With:

N_i = total number of individuals of species

N = total number of foraminifera in one sample

P/B ratio

The P/B ratio represents the percentage of planktonic foraminifera in the total foraminiferal community (Phleger 1951). The P/B ratio provides information about the depth of marine waters. Under normal marine conditions, the P/B ratio tends to be higher in deep-sea environments compared to shallow marine waters. The calculation of the P/B ratio is done using the formula:

$$\frac{P}{P+B} \text{ Ratio} = \frac{P}{(P+B)} \times 100\%$$

With:

P = number of planktonic individuals

B = number of benthic individuals

Diversity index calculation (H')

The Shannon-Wiener diversity index (H') is calculated to determine ecological conditions. The index ranges and their ecological implications are as follows (Bakus 1990):

- $H' < 1.0$: Low – The environment indicates high ecological pressure, low productivity, and a disturbed ecosystem.
- $1.0 < H' < 3.0$: Moderate – The environment indicates moderate ecological pressure, moderate productivity, and a slightly disturbed ecosystem.
- $H' > 3.0$: High – The environment indicates low ecological pressure, high productivity, and a stable ecosystem.

The Shannon-Wiener diversity index equation is calculated as follows (Bakus 1990):

$$H' = - \sum_{i=1}^n P_i \cdot \ln P_i$$

With:

H' = Shannon-Wiener index

P_i = Proportion of species i

N = Individual n

Evenness index (E')

The evenness index analysis uses the Buzas and Gibson (1969) equation:

$$E' = \frac{e^H}{S}$$

With:

E' = Evenness index

H' = Diversity index

S = Number of species

Index range :

$E' < 0,4$: Low evenness of population

$0,4 < E' < 0,6$: Moderate evenness of population

$E' > 0,6$: High evenness of population

Dominance index (D)

The dominance index value is calculated using the following formula (Gibson

& Buzas 1973):

$$D = \left(\frac{ni}{N} \right)^2$$

With:

D = Dominance index

ni = Total number of individuals of species i

N = Total number of individuals in the total sample

Index range :

$0 < D \leq 0.5$: Low of dominance

$0.5 < D \leq 0.75$: Moderate of dominance

$0.75 < D \leq 1$: High of dominance

RESULTS AND DISCUSSION

Based on the identification results, the foraminifera found at the two sites in the study area consist of 12 species of planktonic foraminifera (Figure 2a) and 17 species of benthic foraminifera (Figure 2b), with different foraminiferal contents at each site (Table 1 and Table 2). The abundance of foraminifera at both sites shows that the number of planktonic foraminifera is more dominant compared to benthic foraminifera. At site NM.021, the average P/B ratio percentage is 98.95 % based on 10 depth samples (Figure 3a), whereas at site NM.023, the average P/B ratio percentage is 98.77 % from 10 depth samples (Figure 3b). The high percentage of planktonic foraminifera may indicate that the study area is part of a bathyal environment. Planktonic foraminifera live in the water column, and the water depth of all sediment samples is more than 200 m.

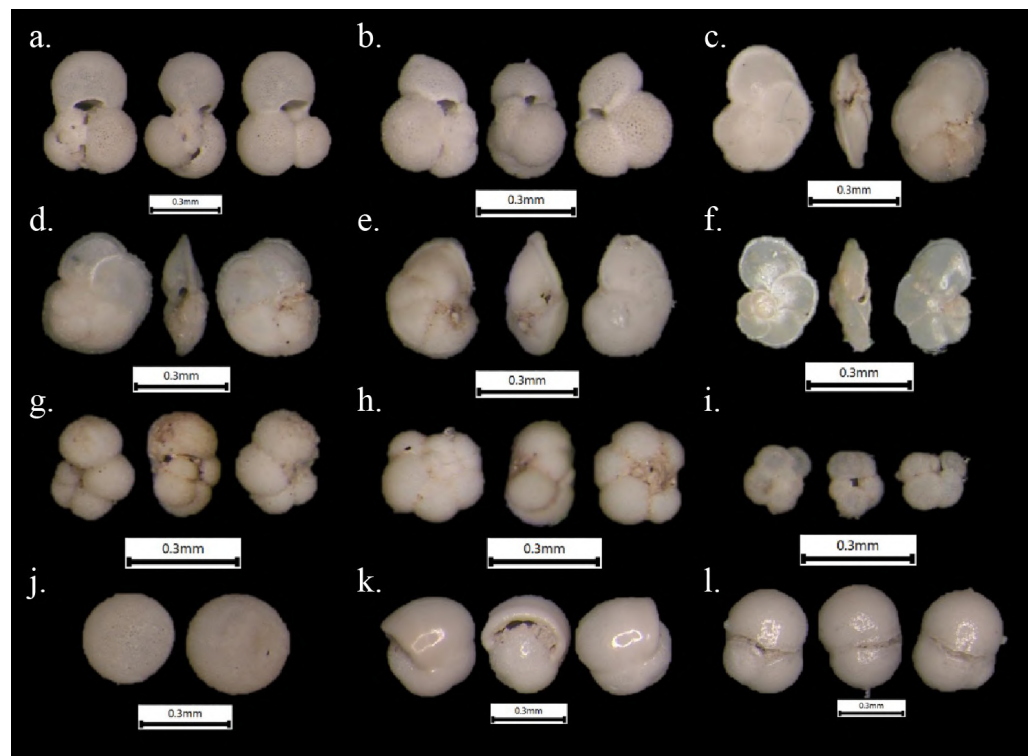


Figure 2a. Dorsal, peripheral, and ventral appearances of planktonic foraminifera. a. *Globigerinoides sacculifer*, b. *Globigerinoides sacculifer* (1), c. *Globorotalia cultrate*, d. *Globorotalia menardii*, e. *Globorotalia tumida*, f. *Globorotalia unguolata*, g. *Hastigerina*, h. *Neogloboquadrina dutertrei*, i. *Neogloboquadrina dutertrei* (1), j. *Orbulina universa*, k. *Pulleniatina obliquiloculata*, l. *Sphaeroidinella dehiscens*.

The diversity index values at both research sites have an average of 2.25 (Figure 4). The low dominance value, averaging 0.13, contrasts with the high evenness value, averaging 0.67. This indicates that the research area still

supports the growth of foraminifera (Buzas & Gibson 1969; Gibson & Buzas 1973). This indication is further supported by the abundant and diverse presence of foraminifera at both sites (Bakus 1990).

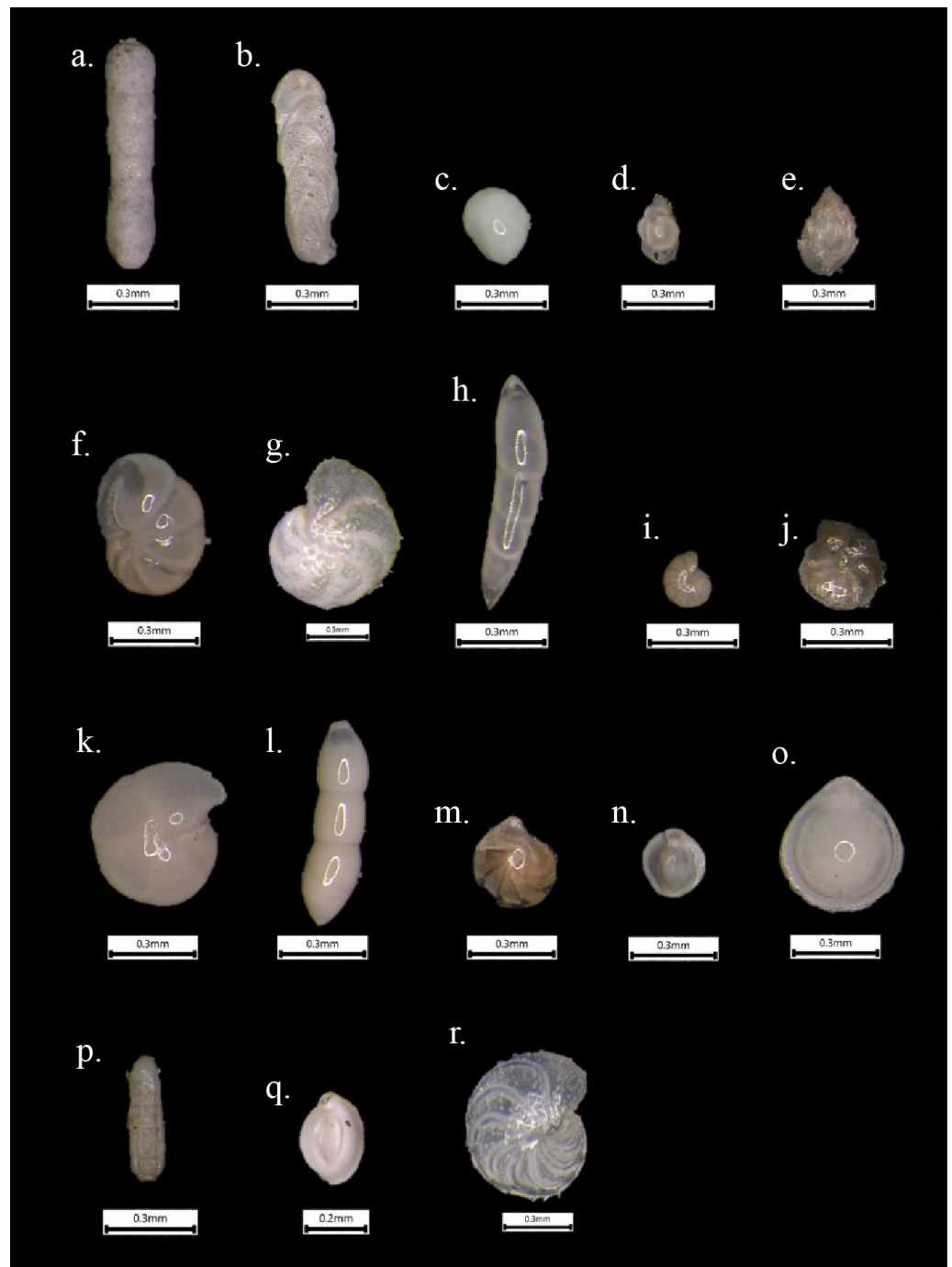


Figure 2b. Benthic foraminifera. a. *Ammobaculites* sp., b. *Bolivinita quadrilatera*, c. *Bolivina* sp., d. *Bulimina marginata*, e. *Bulimina* sp., f. *Ceratobulimina pacifica*, g. *Cibicides*, h. *Dentalina decepta*, i. *Gyroidina orbicularis*, j. *Heterolepa bradyi*, k. *Hoeglundina elegans*, l. *Laevidentalina*, m. *Lenticulina submamilligera*, n. *Pyrgo alata*, o. *Pyrgo lucernula*, p. *Siphogenerina raphanus*, q. *Triloculina* sp., r. *Rosalina bertheloti*.

Planktonic Foraminifera

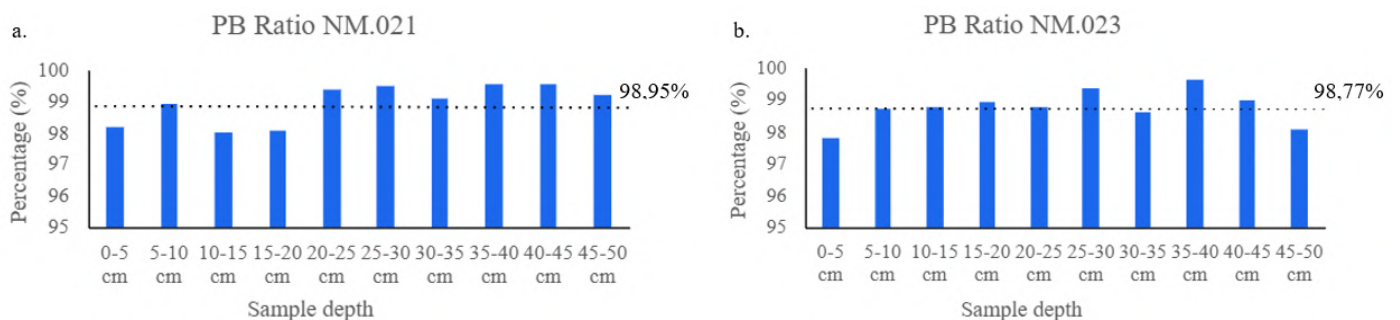
Planktonic foraminifera have a very high abundance in the study area. These planktonic foraminifera live in the water column, thus their presence is abundant in open deep-sea areas, while in shallow seas their abundance is relatively less. At site NM.021, 12 species of planktonic foraminifera were found with a total number of 30,316 individuals. The most abundant planktonic foraminifera found at this site were *Hastigerina pelagica* (21.6 %), *Pulleniatina obliquiloculata* (17.8 %), and *Neoglobobulimina dutertrei* (17.4 %) (Figure

Table 1. Distribution and number of foraminifera specimens (%) in marine sediment at Site NM.021.

Site	Sample depth	Benthic Foraminifera												Planktonic Foraminifera												
		<i>Ammobaculites</i> sp.	<i>Bolivina</i> sp.	<i>Bolivinita quadrilata</i>	<i>Bulimina marginata</i>	<i>Bulimina</i> sp.	<i>Ceratobulimina pacifica</i>	<i>Cibicides</i>	<i>Gyroidina orbicularis</i>	<i>Heterolepa bradyi</i>	<i>Hoeglundina elegans</i>	<i>Pyrgo alata</i>	<i>Pyrgo lucemula</i>	<i>Triloculina</i> sp.	<i>Globigerinoides sacculifer</i>	<i>Globigerinoides sacculifer</i> (1)	<i>Globorotalia cultrata</i>	<i>Globorotalia menardii</i>	<i>Globorotalia tumida</i>	<i>Globorotalia unguolata</i>	<i>Hastigerina pelagica</i>	<i>Neogloboquadrina dutertrei</i>	<i>Neogloboquadrina dutertrei</i> (1)	<i>Orbulina universa</i>	<i>Pulleniatina obliquiloculata</i>	<i>Sphaeroidinella dehiscentis</i>
NM.021	0-5 cm	0	0	0	0	37,5	25	0	12,5	0	25	0	0	8,3	3,7	5,5	8,6	0	1,1	15,5	17	15,1	0	24,7	2	
	5-10 cm	0	33,3	0	0	0	0	0	0	33,3	33,3	0	0	21,48	7,03	1,11	1,48	0	7,77	35,55	8,51	0	1,11	15,92	4,81	
	10-15 cm	42,9	0	0	0	14,3	28,5	0	0	0	0	14,3	0	13,78	1,75	11,14	5,57	2,34	1,17	24,63	21,4	0	0	15,18	3,51	
	15-20 cm	0	12,5	0	12,5	12,5	0	25	0	0	25	0	12,5	0	11,05	2,3	10,36	3,45	3,22	1,61	18,89	17,51	8,29	0	23,27	1,38
	20-25 cm	0	0	0	33,3	33,3	33,3	0	0	0	0	0	0	18,29	1,24	4,78	1,87	1,03	0,83	27,02	20,16	6,86	0,41	17,46	1,45	
	25-30 cm	0	0	0	0	50	50	0	0	0	0	0	0	13	4,25	4,25	5	1	0,75	16	24,5	12,5	0,75	18	0,75	
	30-35 cm	0	0	50	0	50	0	0	0	0	0	0	0	14,44	2,66	9,55	2,66	2,22	1,55	14,88	22,88	16	0	13,11	0,88	
	35-40 cm	0	0	0	0	0	0	0	0	0	50	0	50	11,02	2,85	8,57	3,26	1,42	1,02	27,75	11,02	11,42	0,61	18,97	2,04	
	40-45 cm	0	0	0	0	0	50	0	50	0	0	0	0	17,86	1,3	11,98	4,57	1,96	3,26	16,33	15,03	9,58	0,21	15,9	1,96	
45-50 cm	0	0	0	0	37,5	0	12,5	0	0	0	25	0	25	18,03	1,59	9,36	2,28	0,68	1,36	19,17	15,52	14,38	0,45	15,06	2,05	

Table 2. Distribution and number of foraminifera specimens (%) in marine sediment at Site NM.023.

Site	Sample depth	Benthic Foraminifera														Planktonic Foraminifera													
		<i>Ammobaculites</i> sp.	<i>Bolivina</i> sp.	<i>Bolivinita quadrilata</i>	<i>Bulimina</i> sp.	<i>Ceratobulimina pacifica</i>	<i>Dentalina decepta</i>	<i>Gyroidina orbicularis</i>	<i>Heterolepa bradyi</i>	<i>Hoeglundina elegans</i>	<i>Laevidentalina</i>	<i>Lenticulina submamilligera</i>	<i>Pyrgo alata</i>	<i>Pyrgo lucemula</i>	<i>Rosalina bertheloti</i>	<i>Triloculina</i> sp.	<i>Globigerinoides sacculifer</i>	<i>Globigerinoides sacculifer</i> (1)	<i>Globorotalia cultrata</i>	<i>Globorotalia menardii</i>	<i>Globorotalia tumida</i>	<i>Globorotalia unguolata</i>	<i>Hastigerina pelagica</i>	<i>Neogloboquadrina dutertrei</i>	<i>Neogloboquadrina dutertrei</i> (1)	<i>Orbulina universa</i>	<i>Pulleniatina obliquiloculata</i>	<i>Sphaeroidinella dehiscentis</i>	
NM.023	0-5 cm	0	0	44,44	0	11,11	0	0	0	11,11	0	0	0	22,22	11,11	10,18	3,19	7,61	9,09	9,33	3,68	10,31	11,54	11,05	0,49	20,88	1,96		
	5-10 cm	0	0	50	0	0	0	0	0	0	0	0	50	0	0	12,98	5,19	6,49	0	0	0	20,77	12,98	14,93	0	24,02	2,59		
	10-15 cm	0	0	0	0	25	25	0	0	25	0	0	25	0	0	9,36	2,41	9,66	2,41	6,64	5,43	13,59	17,82	6,34	0,9	21,75	3,62		
	15-20 cm	0	0	25	25	25	0	0	0	0	0	0	0	0	25	9,04	0	6,11	2,92	2,12	3,45	20,21	16,22	22,6	0	16,48	0,79		
	20-25 cm	0	0	0	0	20	0	0	0	20	0	0	0	0	60	9,85	2,95	0	9,35	3,44	3,44	15,27	12,31	17,24	0,24	23,15	2,7		
	25-30 cm	0	0	0	0	50	0	0	50	0	0	0	0	0	0	12,73	3,5	3,82	10,19	0	3,5	15,6	7,96	18,47	0,95	21,97	1,27		
	30-35 cm	16,67	0	33,33	0	16,67	0	0	16,67	0	16,67	0	0	0	0	11,29	5,76	5,29	5,06	5,76	2,99	22,58	11,05	9,9	0,46	16,82	2,99		
	35-40 cm	0	0	50	0	0	0	0	0	0	0	0	50	0	0	15,47	3,13	6,43	2,43	2,26	1,91	12,86	15,47	18,43	1,04	18,08	2,43		
	40-45 cm	0	50	0	25	0	0	25	0	0	0	0	0	0	0	11,03	2,64	3,97	7,28	2,42	0,66	10,59	11,92	10,81	1,1	24,06	13,46		
	45-50 cm	0	0	20	0	70	0	0	0	0	0	10	0	0	0	9,27	6,13	10,25	7,1	3,15	2,16	7,88	16,17	10,45	1,18	20,51	5,52		

**Figure 3.** P/B Ratio percentage at Site NM.021 (a) and Site NM.023 (b).

5). Meanwhile, at site NM.023, the planktonic foraminifera found consists of 11 species, with a total of 26,370 individuals. The most abundant planktonic foraminifera at this site are *Pulleniatina obliquiloculata* (20.8 %), *Hastigerina pelagica* (15 %), and *Neogloboquadrina dutertrei* (13.3 %) (Figure 6). In general, the abundant foraminifera found at site NM.023 are similar to those at site NM.021.

The high abundance of *Pulleniatina obliquiloculata* and *Neogloboquadrina dutertrei* indicates that the study area in the Southern Waters of Sumbawa has a high nutrient content, as these species are abundant in the thermocline layer and tend to thrive in warm, productive waters (Troelstra et al. 1989; Ding et

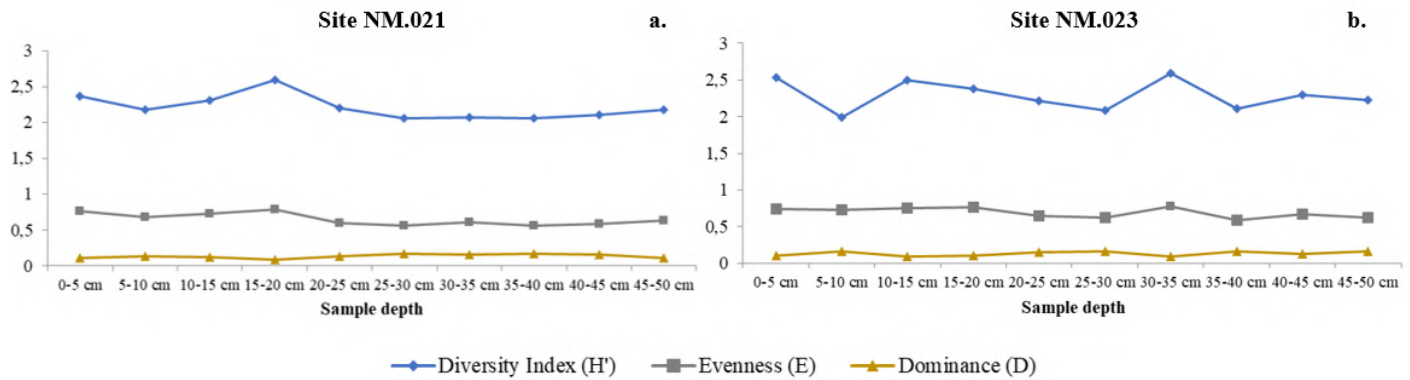


Figure 4. Foraminiferal indices chart in Southern Waters of Sumbawa at site NM.021 (a) and NM.023 (b).

al. 2006). *Pulleniatina obliquiloculata* is a species that lives and is widely distributed in tropical seas and is associated with the presence of abundant phytoplankton (Ravelo et al. 1990; Baohua et al. 1997; Pflaumann & Jian 1999). The consistent presence of *Neogloboquadrina dutertrei* at all sample depths also characterizes eutrophic environments with low salinity (Barmawidjaja et al. 1993; Ding et al. 2006). *Hastigerina pelagica*, a tropical-subtropical species, prefers warm waters with high salinity (Bé & Hutson 1977; Hull et al. 2011). Another planktonic foraminifera species found in abundance and present at all sample depths is *Globigerinoides sacculifer*, a tropical species tolerant of a wide range of temperatures but preferring relatively warm and oligotrophic waters (Pflaumann & Jian 1999; Ding et al. 2006).

Benthic Foraminifera

Benthic foraminifera have a much lower abundance compared to planktonic foraminifera in the study area, but they exhibit higher diversity than planktonic foraminifera. Seventeen species have been identified, with some frequently occurring individuals such as *Bolivinita quadrilatera*, *Ceratobulimina pacifica*, *Bulimina* sp., and *Hoglundina elegans*. At site NM.021, benthic foraminifera found consisted of 13 species with a total of 336 individuals. The most abundant benthic foraminifera at this site were *Bulimina* sp. (23.5 %), *Ceratobulimina pacifica* (18.7 %), and *Hoglundina elegans* (13.3 %) (Figure 5). Meanwhile, at site NM.023, benthic foraminifera found consisted of 15 genera with a total of 344 individuals. The most abundant benthic foraminifera at site NM.023 were *Bolivinita quadrilatera* (22.3 %) and *Ceratobulimina pacifica* (21.8 %) (Figure 6).

The species *Bulimina* sp. and *Ceratobulimina pacifica*, which were found to be abundant at site NM.021, are indicative of environments with minimum oxygen content (dysoxic) and rich in organic carbon (Rathburn et al. 1996; Martins et al. 2015; Psheneva et al. 2017). Meanwhile, at site NM.023, the abundance of *Bulimina* sp. decreased, and *Bolivinita quadrilatera* was found to be the most abundant species. *Bolivinita quadrilatera* is a benthic foraminifera species that thrives in moderately oxygenated (suboxic) conditions (Kaiho 1991, in Aksu et al. 2002), suggesting a possible increase in oxygen levels at site NM.023.

The planktonic foraminifera, dominated by *Pulleniatina obliquiloculata*, *Neogloboquadrina dutertrei*, and *Hastigerina pelagica*, indicate that the waters of Sumbawa are warm, nutrient-rich conditions, productive waters, characterized by eutrophic environment and possible upwelling events (Troelstra et al. 1989; Barmawidjaja et al. 1993; Ding et al. 2006). Similarly, the study by Gustiantini et al. (2018) in the Arafura Sea also found *Pulleniatina obliquiloculata* and *Neogloboquadrina dutertrei*. The benthic foraminifera indicates that the environmental conditions in the Sumbawa waters generally have low oxygen

levels (dysoxic), as evidenced by the abundance of *Bulimina* sp. and *Ceratobulimina pacifica* (Rathburn et al. 1996; Martins et al. 2015; Psheneva et al. 2017). However, there are some changes in oxygen levels indicated by the presence of *Hoeglundina elegans*, which is a marker of oxic conditions and a species sensitive to low oxygen levels (Caulle et al. 2015). At sites NM.021 and NM.023, despite indications of oxygen fluctuations shown by benthic foraminifera, the high diversity index values indicate that the tailings do not affect the foraminifera. As mentioned in the study by Susetiono et al. (2020), the disposal of tailings in the waters of Sumbawa has been managed very well, with the tailing's disposal pipeline located below the thermocline layer. Therefore, the changes in the abundance of benthic foraminifera are more likely caused by sediment instability rather than the presence of tailings.

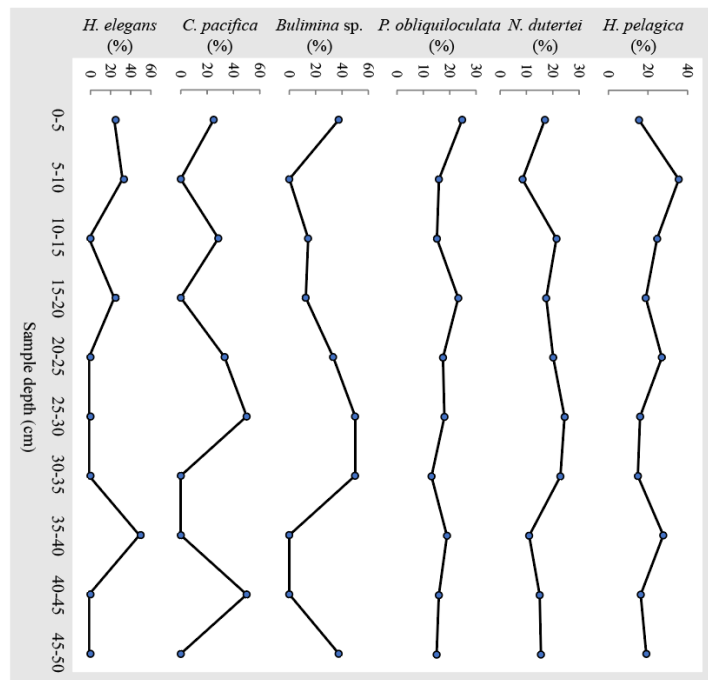


Figure 5. Dominant foraminiferal assemblages identified at site NM.021.

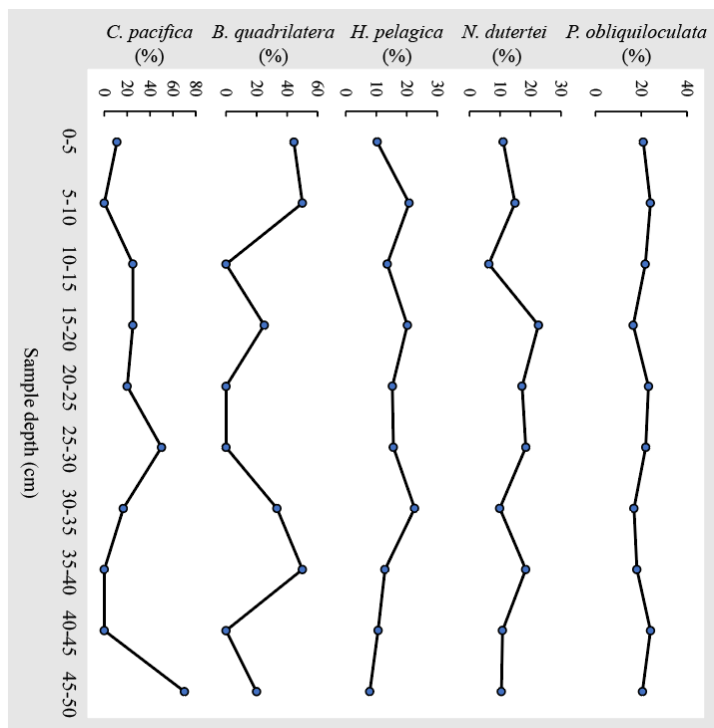


Figure 6. Dominant foraminiferal assemblages identified at site NM.023.

CONCLUSION

The observation of foraminiferal assemblages at sites NM.021 and NM.023 in the Sumbawa Waters revealed the presence of 12 planktonic foraminifera species and 17 benthic foraminifera species. The majority of the planktonic species identified include *Hastigerina pelagica*, *Pulleniatina obliquiloculata*, *Neogloboquadrina dutertrei*, and *Globigerinoides sacculifer*. Meanwhile, the dominant benthic species are *Bolivinita quadrilatera*, *Ceratobulimina pacifica*, *Bulimina* sp., and *Hoglundina elegans*.

The abundance of foraminifera at both sites indicates that the Sumbawa Waters are considered relatively stable for foraminiferal growth, as reflected by the high diversity index values. In general, the conditions in the Sumbawa Waters are characterized by warm and eutrophic waters, with the potential for upwelling events, as indicated by the presence of *Pulleniatina obliquiloculata* and *Neogloboquadrina dutertrei*. The presence of benthic species such as *Bulimina* sp. and *Ceratobulimina pacifica* suggests low oxygen (dysoxic) environment, although the presence of *Hoglundina elegans*—an indicator of oxygen-rich conditions—suggests occasional increases in oxygen levels. Despite these variations, the consistently high diversity index at both NM.021 and NM.023 suggests that tailing disposal activities do not significantly impact the foraminiferal communities. This observation aligns with Susetiono et al. (2020), who reported that tailing disposal in Sumbawa waters has not adversely affected the marine ecosystem.

AUTHORS CONTRIBUTION

R.S.J. designed, supervised the research process, and wrote the manuscript; G.A.M. and A.M. collected and analyzed the data; and Y.W. supervised the entire project.

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CONFLICT OF INTEREST

There are no conflicts of interest related to the research activities and funding.

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Research Article

Partially Purified L-asparaginase with Low Glutaminase and Urease Co-Activities of Bacteria from the Rancabuaya Coast, Indonesia

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ABSTRACT

L-asparaginase, enzyme that can hydrolyse L-asparagine into L-aspartate and ammonia is used as a therapeutic enzyme in treating Acute Lymphoblastic Leukemia (ALL). The use of this enzyme is restricted by the presence of dual substrate specificity towards asparagine and glutamine, which causes side effects. The objectives of this research were to isolate and to identify marine bacteria from Rancabuaya Coast that generate L-asparaginase with low glutaminase and urease co-activity and to produce and to measure the asparaginase activity using the Nessler reagent. Bacteria were isolated from seawater, and screened using Zobell Marine media containing either L-asparagine, glutamine or urea, with phenol red as an indicator. The bacterial isolate with the highest asparaginase activity and relatively lower glutaminase and urease activity, was identified by 16S rRNA gene sequence. This bacterial isolate, RB3, was identified as *Pseudoalteromonas tetraodonis* GFC with greater than 99 % homology. Enzyme specific activities of crude extracellular and intracellular enzymes were 72.30 U mg⁻¹ and 67.18 U mg⁻¹ respectively, while the highest enzymes specific activity from ammonium sulphate fractionation was found at 40-60% saturation (F3), which was 136.03 U mg⁻¹. SDS-PAGE of the enzyme solutions showed the presence of a 35 kDa band suspected to be the L-asparaginase protein.

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INTRODUCTION

L-asparaginase (E.C. 3.5.1.1) is an enzyme used extensively for the treatment of Acute Lymphoblastic Leukemia (ALL) (Shrivastava et al. 2016). The function of this enzyme is to hydrolyse asparagine, forming aspartic acid and ammonia (Zuo et al. 2014). Asparagine, a non-essential amino acid, is needed for protein synthesis and growth of cancer cells. L-asparaginase would reduce the availability of asparagine to the cancer cell, eventually resulting in cancer cell death (Nagarethinam et al. 2012). Even though L-asparaginase is found in higher organisms like animals and plants (Prihanto et al. 2018), bacterial sources are preferable for L-asparaginase production due to the ease in culturing, extraction, and large scale production (Prihanto et al. 2019).

Several types of L-asparaginase formulations have been produced commercially, such as *Escherichia coli* asparaginase, PEGylated *E. coli* asparaginase, and *Erwinia chrysanthemi* asparaginase (Erwinase) (Pieters et al. 2011). While these formulations were effective in treating leukemia, they had side effects which were probably due to the presence of by-products such as glutaminase and urease (Doriya & Kumar 2016). The side effects included mild to serious immune reactions (hypersensitivities, allergies such as skin rash, fever and anaphylactic shock), central nervous system toxicity and other toxicities such as pancreatic disfunction, liver disfunction, edema, diabetes, leukopenia, and bleeding (Batool et al. 2016).

To address these challenges, it is essential to produce L-asparaginase freely from glutaminase and urease activities. Urease breaks down urea in the bloodstream, generating ammonia and carbon dioxide, which can have toxic effects (Bano & Sivaramakrishnan 1980; Doriya & Kumar 2016; Ashok et al. 2019). Glutaminase- and urease-free L-asparaginase is considered beneficial for cancer therapy (Ashok et al. 2019). Examples include *Bacillus licheniformis*, isolated from the Red Sea, Saudi Arabia, produced L-asparaginase with no glutaminase activity (Alrumman et al. 2019), and from *Pseudomonas stutzeri* ATCC 17588 (T) isolated from medicinal plants (Sulistiyani & Kusumawati 2019). These findings suggested that marine and coastal environments are promising yet underexplored source of novel enzymes (Prihanto & Wakayama 2016). Additionally, glutaminase-free L-asparaginase also had been isolated from *Streptomyces exfoliates*, *Streptomyces cyaneus*, and *Streptomyces phaeochromogenes* (Saxena et al. 2015). Enzymes from bacteria in marine environments exhibit unique properties, such as tolerance to high salt concentrations, hyperthermostability, barophilicity and high adaptation to cold temperatures (Zilda et al. 2021). Screening of thermohalophilic bacteria from Wawolesea hot springs of Southeast Sulawesi identified 14 isolates capable of producing L-asparaginase. Among these, the AAT3.2 isolate exhibited the highest activity at 86.61 IU mL⁻¹, while the CAT1.1 isolate showed the lowest activity at 38.24 IU mL⁻¹. In terms of specific activity, CAT3.2 had the highest value at 6767.98 IU mg⁻¹, whereas CAT1.1 had the lowest at 684.54 IU mg⁻¹ (Muzuni et al. 2024).

Research on L-asparaginase-producing bacteria from Indonesian marine environments, such as the Rancabuaya Coast, remains limited. This area holds potential for discovering enzymes with novel characteristics. Therefore, this study focused on isolating bacteria from the seawater of Rancabuaya Coast with L-asparaginase activity that could potentially be used for ALL treatment.

MATERIALS AND METHODS

Sampling of seawater

A total of 1000 mL of seawater samples were collected from the Rancabuaya Coast area in West Java, Indonesia. Sampling took place at 08:00 AM, at a water depth of 30 cm and 5 meters from the shoreline. The samples were col-

lected along with algae and marine sediment. The seawater had a pH of 7.27 and a salinity of 3.5 ‰. It contained dissolved organic substances such as carbohydrates, amino acids, organic-rich particulates, and chemical elements like magnesium and bromine, which influence microbial activity (Byrne & Mackenzie 2024). The samples were placed in sterilised bottles and transported in an icebox during a six-hour journey to the Biotechnology Laboratory at Muhammadiyah University, Bandung, Indonesia.

Isolation and purification of marine bacteria

Isolation of bacteria was carried out by spreading 100 µL of seawater directly on Zobell Marine Agar (ZMA) in a petri dish using an L-shaped cell spreader and incubating at 30 °C for 16 hours. Bacterial colonies with different morphologies were then subcultured separately on ZMA medium using the four-quadrant-streak method and incubating for 16 hours at 30 °C, to obtain pure cultures. Subculturing was repeated until uniform single colonies were obtained in one petri dish. Single colonies were grown on ZMA slants and stored at 4°C or maintained and stored in 10 % glycerol at -20 °C.

Characterization of bacteria

The characteristics of the pure bacterial cultures were then determined based on the standard colony shape: 1) Colony shape: circular, irregular, and punctiform (in the form of dots); 2) Margin shape (colony edge shape): entire (smooth and flat), undulate (wavy), filamentous, rhizoid (branched like roots), and lobate (grooved edges); 3) Colony elevation: can be seen from the side in the form of flat, raised, convex, umbonate, plateau, raised, raised spreading edge, flat raised margin, and growth into; 4) The colour of the colonies formed can also be determined by colour appearance, such as dull (pale), shiny, translucent (slightly transparent), and opaque (Leboffe & Pierce 2012).

Screening for bacteria producing L-asparaginase low in glutaminase and urease

Screening for bacteria producing L-asparaginase low in glutaminase and urease was carried out by inoculating purified colonies onto Zobell Marine Agar (ZMA) with added 5 g L⁻¹ of amino acids of either asparagine, glutamine or urea and 0.009 % phenol red, together with *E. coli* ATCC 8739 as positive control and distilled water as negative control. The culture was then incubated at 30 °C for 16 hours. Observation of bacteria producing L-asparaginase low in glutaminase and urease was carried out visually, by observing the zone index of each bacterium. Colony diameter and diameter of red zone formed around each colony was measured using a caliper after 16 hours of incubation. The phenol red indicator would turn red due to the increase in pH caused by the appearance of ammonia as a result of the hydrolysis of asparagine by L-asparaginase (Qeshmi et al. 2018). The zone index was calculated as the ratio of the diameter of the red zone and the colony (Ashok et al. 2019).

$$\text{Zone Index} = \frac{\text{Diameter of red zone}}{\text{Diameter of colony}}$$

DNA isolation

Bacteria were cultured in 5 mL of Zobell Marine Broth (ZMB) medium with optimum growth conditions. A total of 1.5 mL of culture was centrifuged at 5000 x g for 10 minutes, the supernatant was discarded, 1.5 mL culture was added, recentrifuged at 5000 x g for 10 minutes, then the supernatant was discarded. DNA from the pellet was isolated using GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific) and performed in accordance to the instruction manual.

PCR amplification and electrophoresis

The template for the PCR reaction used 1 µL of isolated genomic DNA from samples, 1 µL of *E. coli* genomic DNA, and 1 µL of nuclease-free water (as a negative control). DNA was amplified with DreamTaq master mix with a pair of primers BactF1(5'-AGAGTTTTGATC(A/C)TGGCTCAG-3') and UniB1 (5'-GGTTAC(G/C)TTGTTACGACTT-3') to amplify a 1400 bp fragment of the 16S rRNA gene (Baker et al. 2001; Gaffar et al. 2014). Each tube consisted of 10 pmol BactF1 primer, 10 pmol UniB1 primer, 12.5 µL of DreamTaq master mix, 9.5 µL of nuclease-free water, and 1 µL of template DNA, resulting in a total reaction volume of 25 µL. Amplification was carried out under the following PCR conditions: initial denaturation 95 °C for 2 minutes, followed by 25 cycles consisting of denaturation 95 °C for 1 minute, annealing 50 °C for 1 minute, elongation 72 °C for 1 minute and ending with final elongation 72 °C for 10 minutes. The amplicons were analysed in agarose gel electrophoresis, using 1 % agarose gel in TAE buffer. To 5 µL of sample, 1 µL of loading dye and 1 µL of GelRed were added. Electrophoresis was carried out at a voltage of 75 mV for 45 minutes. The electrophoresis results were observed using a UV-transilluminator.

Sequencing 16S rRNA gene and phylogenetic analysis

The PCR products were sequenced at 1st BASE using the Sanger method, which relies on a DNA synthesis technique that selectively terminates the synthesis at specific nucleotide bases (Sanger et al. 1977; Lander et al. 2001; Moorcraft et al. 2015). Data were processed using BioEdit software (RRID:SCR_007361) (https://scicrunch.org/resolver/SCR_007361) and search for sequence similarity by performing BLAST (<https://blast.ncbi.nlm.nih.gov>) (Altschul et al. 1990). Phylogenetic tree analysis using the neighbor-joining (NJ) method with a 2-parameter Kimura model and Gamma distribution, implemented in MEGA 11 (Tamura et al. 2021) with a bootstrap value of 1000x.

Bacterial growth curve

The optimal isolate from the screening, which exhibited high asparaginase activity and low glutaminase and urease activities, was used as an inoculum to prepare a starter culture by inoculating 10 mL of Zobell Marine Broth containing 10 g L⁻¹ asparagine. This culture was incubated at 30 °C with shaking at 150 rpm for 24 hours. Subsequently, 100 mL of Zobell Marine Broth with 10 g L⁻¹ asparagine was inoculated with 1 mL of the starter culture and incubated on a shaker at 30 °C and 150 rpm for 50 hours. Samples (1 mL) were taken every two hours to measure optical density at 600 nm (Kumar et al. 2010a).

L-asparaginase production

The method for L-asparaginase production was based on (Kumar et al. 2010a) with modification. A preculture was made by inoculating 10 mL of Zobell Marine Broth with 10 g L⁻¹ asparagine, then incubating at 30 °C, 150 rpm until the logarithmic phase. This preculture was used as a 2 % v v⁻¹ inoculum to 500 mL Zobell Marine Broth with 10 g L⁻¹ asparagine, then the culture was incubated at 30 °C, 150 rpm until the logarithmic phase. After incubation, the culture was centrifuged at 4000 x g for 15 minutes at 4 °C. The cell-free supernatant contained crude extracellular L-asparaginase. Meanwhile, the cell pellet was suspended in sonication buffer (50 mM Tris-Cl and 10 mM EDTA pH 7.5), transferred to a Falcon tube, and sonicated on ice at 20 MHz, 35 % amplitude, 20 minutes (15 seconds on and 5 seconds off). The lysate was centrifuged at 20,000 x g for 10 minutes (4 °C). The supernatant contained intracellular L-asparaginase.

Partial Purification of the crude extracellular L-asparaginase

Partial purification was based on the method described by (Kumar et al. 2010b) with modification. Crude extracellular L-asparaginase was fractionated with ammonium sulphate to obtain five fractions with the following percent saturation: 0-20 % (F1), 20-40 % (F2), 40-60 % (F3), 60-80 % (F4) and 80-100 % (F5) (Scopes 1994). Ammonium sulphate was weighed according to the desired fraction to obtain the desired concentration, then was added to the supernatant solution little by little while stirring with a magnetic stirrer at 4 °C for 30 minutes. The mixture was then incubated at 4 °C overnight, centrifuged at 3000 x g for 30 minutes. The precipitate obtained was dissolved in 50 mM Tris-Cl pH 8.6. Cellophane membrane dialysis tubings (cutoff 12-14 kDa) were soaked in distilled water for 30 minutes at 40 °C, then rinsed with distilled water. One end of the tubes was tied with a thread. Then the tubes were filled with ammonium sulphate fractions of L-asparaginase, after which the other end of the tube was tied with a thread. The dialysis tubes were then soaked in 0.025 M Tris-Cl buffer pH 8.6 and stirred with a magnetic stirrer at 4 °C. Every two hours the buffer was changed to reduce the concentration of salt ions in the tubes. The presence of ammonium sulphate was tested with 0.1 M BaCl₂. Dialysis was stopped when testing with BaCl₂ did not result in a white precipitate of BaSO₄. The solution in the dialysis tube which contained the partially purified intracellular L-asparaginase of *Pseudoalteromonas tetradonis* RB3 was transferred into a new bottle.

Determination of L-asparaginase activity

L-asparaginase assay was carried out using the Nessler method with modifications from (Kumar et al. 2010a). Nessler's reagent (K₂HgI₄) reacted with ammonia in a basic solution to form a yellow-brown colloidal dispersion. This reaction produced a yellow-brown solution which followed the Lambert-Beer law (Vogel 1951). The intensity of the colour in the sample was measured by absorbance using a UV-Vis spectrophotometer at a wavelength of 420 nm, which was chosen based on the highest absorbance. This wavelength was chosen to minimise errors, because the greater the absorbance, the measurement will tend to be constant for repeated measurements (Kresnadipayana & Lestari 2017). The assay was performed on samples *Pseudoalteromonas tetradonis* RB3 enzymes, negative control (distilled water), and positive control. To 100 µL samples, 900 µL substrate L (10 mM asparagine in 50 mM Tris-Cl pH 8.6) was added. The reaction mixture was incubated at 37 °C for 30 min and the reaction was stopped by adding 100 µL of 1.5 M trichloroacetic acid (TCA) solution. The reaction mixture was centrifuged at 10,000 rpm for 5 min at 4 °C. The ammonia released in the supernatant was determined by taking 100 µL of the supernatant and adding 100 µL of Nessler's reagent and 3.8 mL of distilled water. This mixture was vortexed and incubated at room temperature for 20 minutes. Then the absorbance of the solution was measured with a UV-Vis spectrophotometer at the optimum wavelength with a blank (asparaginase substrate in Tris-Cl + TCA buffer). The ammonium ions produced was determined based on the linear regression equation of ammonium chloride standard curve. One IU of L-asparaginase was defined as the amount of enzyme required to generate 1 µmol of ammonia per minute at the experimental conditions. The enzyme activity was calculated using the following formula:

$$\text{Enzyme Activity (IU mL}^{-1}\text{)} = \frac{(y - b)}{a} \times \frac{V_{\text{total}}}{V_{\text{analysis}}} \times \frac{1}{V_{\text{enzyme}}} \times \frac{1}{T_{\text{incubation}}}$$

where:

y	: Absorbance
a	: Slope

b	: Intercept
V. Total	: Volume of enzyme + substrat + buffer + TCA
V. Analysis	: Analyzed volume
V. Enzyme	: Volume of enzyme
T. Incubation	: Incubation time

Determination of protein concentration

Protein concentrations of standard solutions, crude extracellular, partial purified, intracellular L-asparaginase of *Pseudoalteromonas tetraodonis* RB3, and positive control (*E. coli* ATCC 8739) were measured by the Lowry method. The blank consisted of 50 mM Tris-Cl buffer. Lowry B reagent (2 mL) was added to 0.25 mL samples, then the mixture was homogenised and incubated for 10 minutes. Then 0.25 mL Lowry A reagent was added and incubated at room temperature for 20 minutes. The absorbance of the solution was measured using a UV-Vis spectrophotometer at the optimum wavelength relative to the blank solution (Lowry et al. 1951; Waterborg 2009). Protein concentration was determined based on the linear regression equation of the Bovine Serum Albumin (BSA) standard curve, using the following formula:

$$\text{Protein concentration (mg mL}^{-1}\text{)} = \frac{(y-b)}{a}$$

y : Absorbance of sample
 a : Slope of the linear regression equation of the BSA standard
 b : Intercept of the linear regression equation of the BSA standard

Analysis of L-asparaginase enzyme by SDS-PAGE

Proteins in the enzyme solutions were separated by SDS-PAGE on 12 % acrylamide gel and stained with Coomassie Brilliant Blue stain (Sambrook et al. 1989). Samples were prepared by adding 5 µL sample buffer (60 mM Tris-Cl pH 6.8, 25 % glycerol (v v⁻¹), 2 % SDS (w v⁻¹), 0.1% (w v⁻¹) bromophenol blue) to 10 µL sample, heated in a waterbath at 95 °C for 15 minutes, centrifuged at 10,000 x g, 4 °C, for 2 minutes. Samples (15 µL) and protein molecular weight markers were placed inside the wells of the gel. Electrophoresis was performed at 100 volts for 100 minutes. After that, staining was carried out using a staining solution for 30 minutes, and color removal was carried out using a destaining solution for 60 minutes. Protein bands would appear and the molecular mass was calculated using GelAnalyzer 23.1.1 (Lazar Jr. et al. 2023).

RESULTS AND DISCUSSION

Isolates of bacteria from sea water at Rancabuaya Coast

From the resulting colonies, four colonies were taken that had differences in shape, margin, elevation, and colour (Table 1). These four bacterial colonies were subcultured and purified, and were named RB1, RB2, RB3 and RB4.

L-asparaginase producing bacteria low in glutaminase and urease

The bacterial isolates were screened using a semi-quantitative rapid plate test method by means of direct visualization (Figure 1). Enzyme activity could be measured by calculating the zone index (Ashok et al. 2019; Chakraborty & Shivakumar 2021). Rancabuaya Coast seawater bacteria had higher L asparaginase activities compared to the positive control. Isolate RB3 had the highest L-asparaginase activity as indicated by a zone index value of 2.5 (Table 2).

Molecular identification of RB3

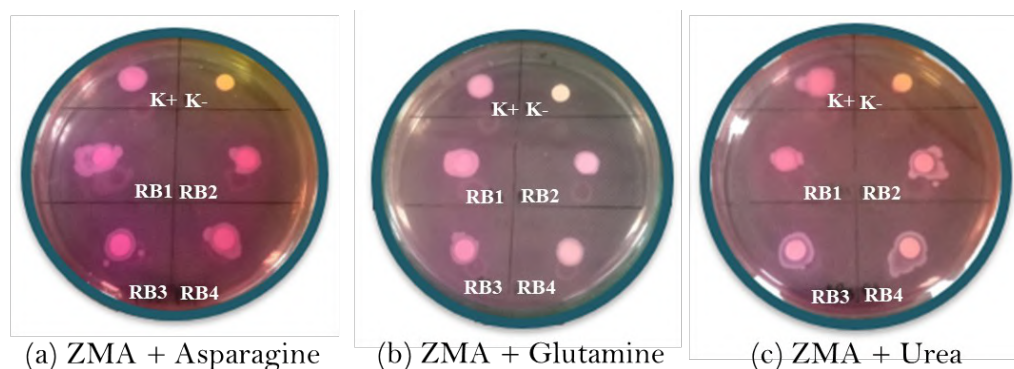
Molecular identification was carried out on RB3 which had the highest L-asparaginase activity. Amplification of the 16S rRNA gene from the genomic DNA of RB3 resulted in a 1.5 kb fragment (Figure 2).

Table 1. Colony Morphology of Rancabuaya Coast Seawater Bacterial Isolates.

No.	Bacterial Isolate	Colony shape	Colony Margin	Colony Elevation	Colony color
1.	RB1	Undulate	Entire	Convex	white
2.	RB2	Circular	Irregular	Convex	white
3.	RB3	Circular	Entire	Convex	white
4.	RB4	Undulate	Irregular	Convex	white

Table 2. Screening for bacteria producing L-asparaginase low in glutaminase and urease on ZMA medium given different substrates (L-asparagine, glutamine and urea).

Bacterial Isolates	ZMA + L-asparagin + <i>Phenol red</i>	ZMA + glutamin + <i>Phenol red</i>	ZMA + urea + <i>Phenol red</i>
K-	1.0	1.0	1.0
K+	1.7	1.3	1.7
RB1	1.7	1.7	2.0
RB2	1.7	0.8	1.7
RB3	2.5	1.3	2.2
RB4	1.7	0.8	1.7

**Figure 1.** Screening for bacteria producing L-asparaginase low in glutaminase and urease.

Sequencing and phylogenetics

Sequencing of the 16S rRNA gene of RB3 produced a DNA sequence with a length of 1491 bp. BLAST (Basic Local Alignment Search Tools) analysis of the sequence was carried out online on the NCBI website (<http://www.ncbi.nlm.nih.gov>) to obtain a list of sequences and information on sequence alignments of RB3 16S rRNA gene with sequences in the data bank (Table 3).

Pseudoalteromonas tetraodonis GFC strain KMM 458 (NR_114547.1) had a maximum Score of 2577, total score of 2577, query coverage of 98 %, E value equals 0.0 and homology of 99.51 %. Isolates with a 16S rRNA sequence identity of more than 97 % would represent the same species. Meanwhile, sequence identities between 93-97 % would represent the same genus, but different species. A 16S rRNA sequence similarity of 97 % has been proposed as delimiter for bacterial species (Hagström et al. 2002). To state species similarity, 16S rRNA gene sequences must have at least 97 % nucleotide base similarity (Stackebrandt & Goebel 1994).

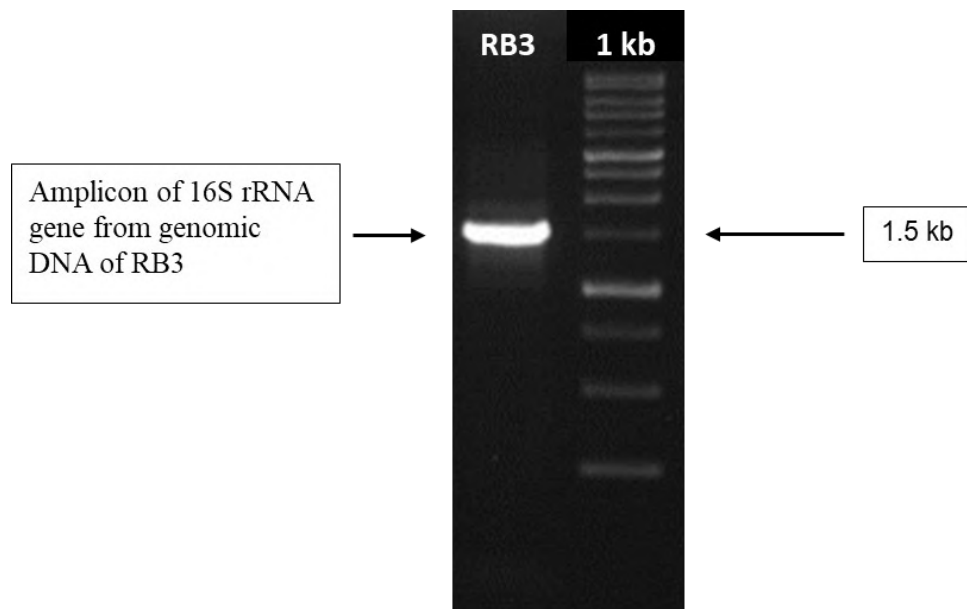


Figure 2. Electropherogram of the 16S rRNA gene amplicon from RB3.

Eight sequences were used for the construction of a phylogenetic tree for RB3 and the resulting phylogenetic tree (Figure 3) showed that the RB3 had the closest relationship to *Pseudoalteromonas tetraodonis* GFC strain KMM 458 with a bootstrap value of 100. A bootstrap value between 70–100 indicated that the branches were robust and difficult to change. However, if the bootstrap value was less than 70, then the possibility of branching changes being formed would be very high (Apollon et al. 2017). Since RB3 sequence has a high identity with all of the *Pseudoalteromonas tetraodonis* species (>99.29 %), RB3 is now named *Pseudoalteromonas tetraodonis* RB3.

Pseudoalteromonas tetraodonis, classified as gram-negative bacteria, are aerobic and have the form of bacilli (rods) measuring 1.0 μm 2.4 μm . Generally, these bacteria are halophilic organisms, namely bacteria that can grow optimally in environments with high salt concentrations and can move using flagella. Halophilic bacteria can be divided into three groups based on their

Table 3. Bacterial strains with the highest 16S rRNA partial sequence homology with RB3 16S rRNA gene.

Species	Max Score	Total Score	Query Cover	E Value	Homology	Acc. Len	Accession
<i>Pseudoalteromonas tetraodonis</i> GFC strain KMM 458	2577	2577	98 %	0.0	99.51 %	1491	NR_11457.1
<i>Pseudoalteromonas tetraodonis</i> GFC strain NBRC 103034	2573	2573	98 %	0.0	99.44 %	1459	NR_114187.1
<i>Pseudoalteromonas tetraodonis</i> GFC strain IAM 14160	2545	2545	98 %	0.0	99.29 %	1428	NR_119142.1
<i>Pseudoalteromonas spiralis</i> strain Te-2-2	2510	2510	98 %	0.0	98.73 %	1468	NR_114801.1
<i>Pseudoalteromonas carrageenovora</i> strain NBRC 12985	2507	2507	98 %	0.0	98.59 %	1461	NR_113605.1
<i>Pseudoalteromonas distincta</i> strain KMM 3548	2507	2507	98 %	0.0	98.59 %	1506	NR_025654.1
<i>Pseudoalteromonas agarivarons</i> DSM 14585	2501	2501	98 %	0.0	98.52 %	1505	NR_025509.1
<i>Pseudoalteromonas atlantica</i> strain NBRC 103033	2497	2497	98 %	0.0	98.38 %	1461	NR_114186.1
<i>Pseudoalteromonas espejiana</i> strain NBRC 102222	2497	2497	98 %	0.0	98.45 %	1461	NR_114049.1

ability to live, namely low halophilic (slight halophiles) grow optimally in the range of 2-5 % NaCl content, moderate halophiles in the range of 5-20 % NaCl content, and extreme halophilic (extreme halophiles) range of 20-30 % NaCl content. *P. tetraodonis* grows between 4-35 °C. This bacterium was capable of producing tetrodotoxin, gelatinase and lipase enzymes (Simidu et al. 1990; Ivanova et al. 2001; Hassanshahian & Jafar 2011). Halotolerant microorganisms grow both at high concentration of salt and without salt, while halophilic microorganisms are salt-loving microorganisms (Kaneekar & Kaneekar 2022). The type strain *P. tetraodonis* GFC can grow in 2-10 % NaCl and cannot grow in 0 % NaCl (Reimer et al. 2022; BacDive 2024). Therefore, it is halophilic, not halotolerant. *Pseudoalteromonas* had been characterized and identified as L-asparaginase producing bacteria, such as *Pseudoalteromonas arabiensis* that produced L-asparaginase from marine sources (Lakshmi et al. 2020).

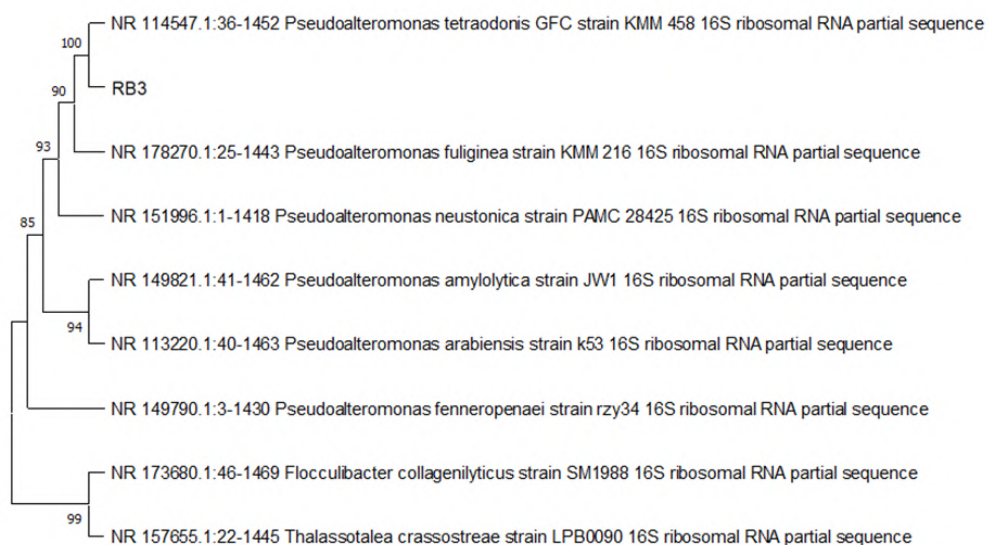


Figure 3. Phylogenetic tree based on 16S rRNA gene sequence of RB3 using the neighbour-joining method, 2-parameter Kimura model and Gamma distribution with a bootstrap value of 1000 replicates.

P. tetraodonis belonged to the phylum Proteobacteria, where several species from this phylum have been tested to produce L-asparaginase. *Pseudomonas aeruginosa* was obtained from fish intestines, belonging to the phylum Proteobacteria, which produced L-asparaginase (Qeshmi et al. 2022). *Bacillus licheniformis* of the Proteobacteria phylum was isolated from the Red Sea, Saudi Arabia which was reported to produce L-asparaginase (Alrumman et al. 2019). *Pseudomonas stutzeri* ATCC 17588(T) from the phylum Proteobacteria, could produce glutaminase-free L-asparaginase, was isolated from Indonesian medicinal plants (Sulistiyan & Kusumawati 2019). Four strains of bacteria belonging to Gammaproteobacter, a subdivision of the Proteobacteria phylum, namely *Pseudomonas* sp., *Bacillus* sp., *Zobellella* sp. and *Oceanimonas* sp. which could produce L-asparaginase were obtained from the Persian Gulf (Qeshmi et al. 2014).

Growth curve

The growth of *Pseudoalteromonas tetraodonis* RB3 that was isolated from Rancabuaya Coast is characterised by an increase in density or turbidity in the ZMB medium according to (Kumar et al. 2010a). Determining the culture time used to measure enzyme activity was based on the growth curve (Kurniawati et al. 2019). Figure 4 shows the growth curve of *Pseudoalteromonas tetraodonis* RB3. The growth curve of *Pseudoalteromonas tetraodonis*

RB3 was needed to determine the right time to produce L-asparaginase, because from the growth curve, there is a bacterial logarithmic phase that actively produces primary metabolites such as enzymes (Alrumman et al. 2019). From two to eighteen hours, the exponential phase occurred which was characterised by significant bacterial cell growth. Logarithmic phase of *Pseudoalteromonas tetraodonis* RB3 begins to end at hour 18. In this phase, the cells metabolism was constant and growth was quite balanced. The logarithmic phase was the best time to use the cells, because the bacteria had an optimal growth rate so they could be used as inoculum in a treatment (Wijanarka et al. 2016; Mahjani & Dwi 2020).

In addition, because in the logarithmic phase there was an increase in the number of bacterial cells, this would increase the synthesis of enzymes for metabolic processes in cells (Saropah et al. 2012). Metabolite began to form when they entered the middle of the exponential phase and decreased in production in the stationary phase (Judoamidjojo et al. 1990; Ruiz et al. 2010; Hidayatulloh et al. 2019). The samples used for testing enzyme activity were crude extract samples of enzymes produced during the logarithmic phase, where during this phase bacteria produced primary metabolites in the form of enzymes (Kurniawati et al. 2019).

From the explanation above, it showed that primary metabolite compounds were produced optimally in the logarithmic phase. Therefore, L-asparaginase activity of the *Pseudoalteromonas tetraodonis* RB3 culture was tested at 18th hour, just before the end of the logarithmic phase and before entering the stationary phase.

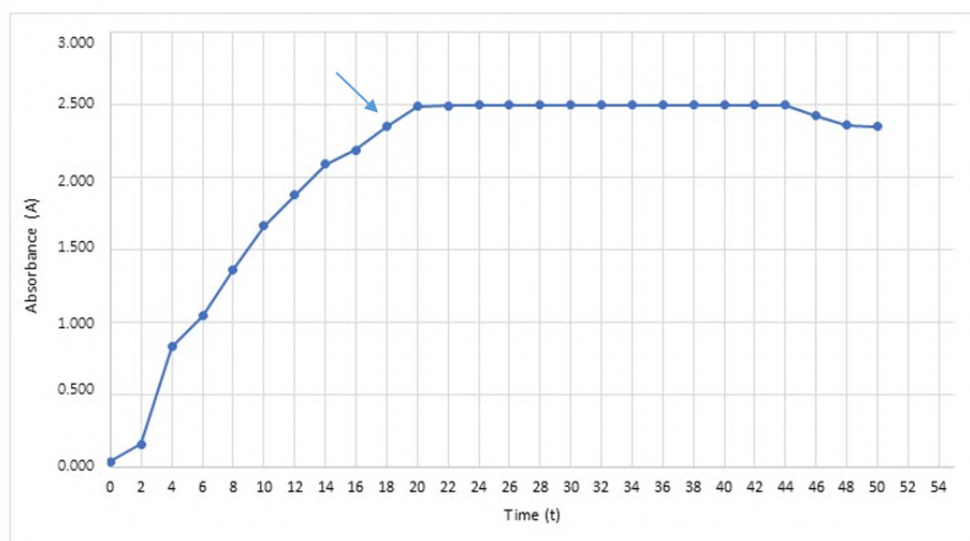


Figure 4. Growth curve of *Pseudoalteromonas tetraodonis* RB3. Arrow showed growth at the 18th hour where L-asparaginase activity was tested.

The stationary phase of *Pseudoalteromonas tetraodonis* RB3 occurred from the 20th hour to the 44th hour. Several factors could cause the number of living cells to remain constant and form a straight or constant line in the stationary phase of the growth curve. In this phase, there was a state of balance between the rate of cell growth (cell division) and the rate of cell death, which meant that the number of living cells was the same as the number of dead cells (Mardalena 2016). The availability of growth nutrients in the medium had been depleted and the population had limited space to grow, called the 'biological space'. Another factor could be the accumulation of metabolic end products which may be toxic to the growth of the bacteria themselves (Umniyati et al. 2009; Kusumaningati et al. 2013). From the 46th hour to 50th

hour, the death phase occurred, which was characterised by a decrease in the number of bacteria due to a depletion of nutrients in the medium (Risna et al. 2022). In addition, the products of microbial metabolism themselves may be toxic, so they could cause cell death (Suharyono et al. 2012).

Production of crude extract of L-asparaginase

Production of L-asparaginase from *Pseudoalteromonas tetraodonis* RB3 was carried at 18 hours of culture with an OD of 2.3, namely near the end of the logarithmic phase (Figure 4). Isolation of the *E. coli* enzyme as a positive control was also carried out near the end of the logarithmic phase of the culture, namely at 16 hours. This had the largest population, so it was hoped that maximum enzyme activity would be obtained (Gunawan 2017; Ratnadewi et al. 2017).

Table 4 showed that the crude extracellular and intracellular L-asparaginase activities were different between positive control and *Pseudoalteromonas tetraodonis* RB3. The crude extracellular and intracellular L-asparaginase activities from *Pseudoalteromonas tetraodonis* RB3 were 28.34 U mL⁻¹ and 25.46 U mL⁻¹, while crude extracellular and intracellular L-asparaginase activity of positive control were 25.92 U mL⁻¹ and 24.20 U mL⁻¹ respectively. These results showed that crude extracellular and intracellular L-asparaginase from *Pseudoalteromonas tetraodonis* RB3 had a higher activity value compared to positive control.

Table 4. Crude extracellular and intracellular L-asparaginase activity.

Source	Sample	Enzyme Activity (U mL ⁻¹)	Volume (mL)	Total Activity (Units)
<i>Pseudoalteromonas tetraodonis</i> RB3	Crude Extracellular	28.34	500	1.417 × 10 ⁴
	Intracellular	25.46	6	1.528 × 10 ²
Positive control	Crude Extracellular	25.92	500	1.296 × 10 ⁴
	Intracellular	24.20	6	1.452 × 10 ²

Several gram-negative bacteria that produced extracellular L-asparaginase were from the *Pseudomonas* genus, including *Pseudomonas ovalis* IAM 1153 which had activity of 16 U mL⁻¹, *Pseudomonas fluorescens* had activity of 20 U mL⁻¹, and *Pseudomonas dacunhae* IAM 1668 which had an L-asparaginase activity of 33 U mL⁻¹ (Arima et al. 1972).

In Figure 5, the L-asparaginase activities of *Pseudoalteromonas tetraodonis* RB3 ammonium sulphate fraction was different in each fraction. The activities increased from saturation level of 0-20 % (F1), 20-40 % (F2), to up to 40-60 % (F3), then decreased again from saturation level 60-80 % (F4) to 80-100 % (F5). The highest L-asparaginase enzyme activity was at a saturation level of 40-60 % (F3), namely 27.75 U mL⁻¹.

In contrast in positive control, the L-asparaginase activity of ammonium sulphate fractions increased from saturation level of 0-20 % (F1) to 60-80 % (F4), then decreased at 80-100 % saturation (F5). So, the highest L-asparaginase activity was highest at saturation level of 60-80 % (F4), namely 9.514 U mL⁻¹. The L-asparaginase activity in the F3 fraction of *Pseudoalteromonas tetraodonis* RB3 was greater than the F4 fraction of *E. coli*.

The protein content of each enzyme solution was measured using the Lowry method. The absorbance was measured 655 nm, which was chosen based on the highest absorbance. Figure 6 showed that the specific activity of the L-asparaginase in each sample was different. Fractions with large activi-

ties did not necessarily have large specific activities, nor do enzyme fractions with small protein concentrations necessarily had large specific activities. Because specific activities were determined by comparison of activity to protein levels (Arpintasari et al. 2008).

Crude extracellular and intracellular enzymes obtained from centrifugation still contained many impurities. The crude extracellular enzyme of *Pseudoalteromonas tetraodonis* RB3 had a specific activity of 72.30 U mg⁻¹, while the intracellular enzyme had a specific activity of 67.18 U mg⁻¹. Likewise, in the positive control, namely the *E. coli* crude extracellular enzyme had a specific activity of 97.80 U mg⁻¹, which was greater than the intracellular enzyme specific activity of 90.97 U mg⁻¹. *E. coli* still had higher specific activities for the L-asparaginase compared to *Pseudoalteromonas tetraodonis* RB3.

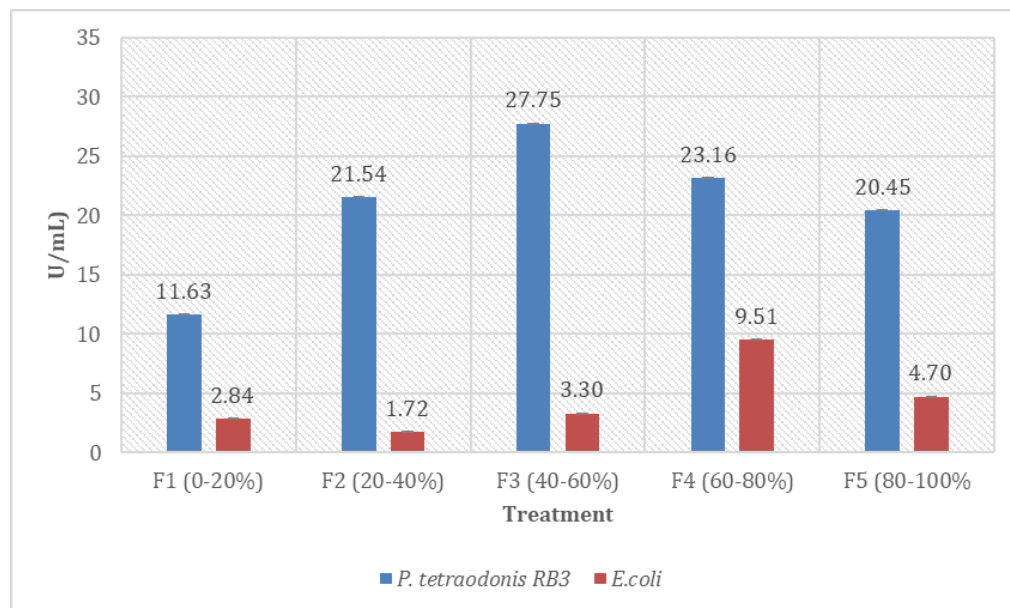


Figure 5. L-asparaginase activity in the ammonium sulphate fractions.

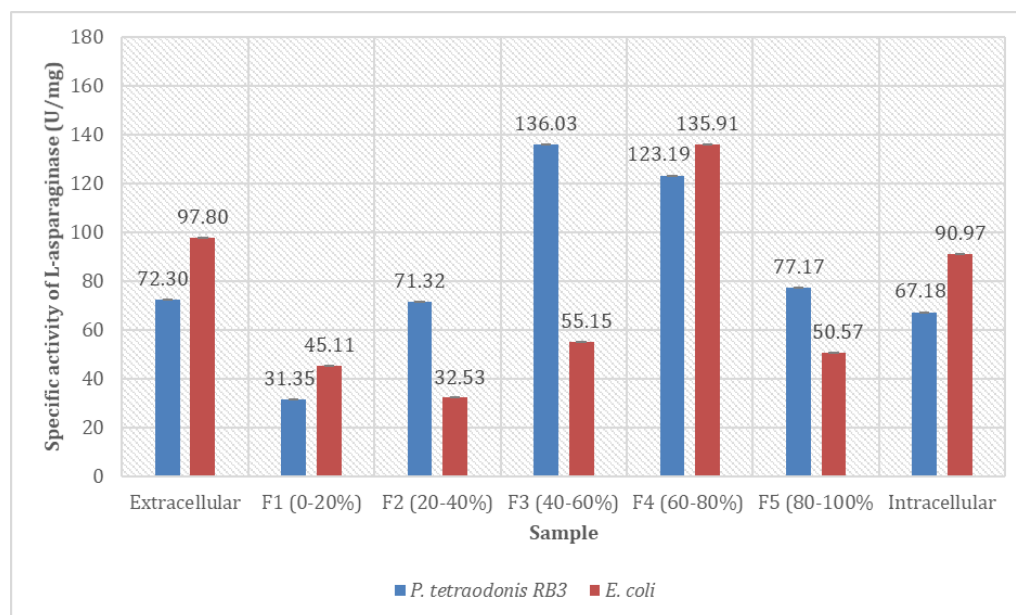


Figure 6. Specific activity of L-asparaginase.

The specific activities of L-asparaginase from each *Pseudoalteromonas tetraodonis* RB3 fractions were different and increased from ammonium sulphate saturation levels of 0-20 % (F1), 20-40 % (F2), to 40-60 % (F3) then decreased again on saturation levels of 60-80 % (F4) to 80-100 % (F5). The spe-

cific activity value of the *Pseudoalteromonas tetraodonis* RB3 L-asparaginase enzyme was highest at a saturation level of 40-60 % (F3), namely 136.03 U mg⁻¹. There was a fold purification of 1.9-fold in fraction F3. However, the specific activity between F3 and F4 was not much different, the specific activity of F4 being 123.19 U mg⁻¹. This meant that to decrease loss of enzyme, fractionation could be performed at 40-80 % saturation in the enzyme purification process and a higher fold purification could be expected to be achieved.

In contrast, the specific activities of the L-asparaginase *E. coli* ammonium sulphate fractions decreased from saturation level of 0-20 % (F1) to 20-40 % (F2), then increased from 40-60 % (F3) to 60-80 % (F4) then decreased again at saturation level 80-100 % (F5). So, the highest specific activity of *E. coli* L-asparaginase was at a saturation level of 60-80 % (F4), namely 135.91 U mg⁻¹. The fold purification in F4 was 1.4-fold. When comparing the highest L-asparaginase specific activity of the fractions between *Pseudoalteromonas tetraodonis* RB3 and *E. coli*, the *Pseudoalteromonas tetraodonis* RB3 had a higher L-asparaginase specific activity compared to *E. coli*. The L-asparaginase of *Pseudoalteromonas tetraodonis* RB3 samples was mostly found in F3 and the L-asparaginase of *E. coli* samples was mostly found in F4.

The difference in the level of ammonium sulphate saturation required to achieve the highest L-asparaginase enzyme activity in *Pseudoalteromonas tetraodonis* RB3 samples (in F3) and *E. coli* (in F4) could be caused by several factors such as species differences between *Pseudoalteromonas tetraodonis* RB3 with *E. coli*. Each species has unique genetic characteristics, including for certain enzymes such as L-asparaginase. These differences could affect the expression, structure, and stability of the enzyme, as well as its interactions with the ammonium sulphate ions. When the amino acid composition of the same enzyme from different species was different, it could affect the interaction with ammonium sulphate ions, the stability of the enzyme in ammonium sulphate solutions, as well as affecting the isoelectric point. The isoelectric point of a protein depended on the composition of amino acids in the polypeptide chain. Each amino acid has a different isoelectric point due to the differences in their functional groups, such as hydrophobic and ionic groups. The interaction between these groups and the ammonium sulphate ion influences the ability of the enzyme to precipitate at different levels of saturation (Scopes 1994).

Hydrophobic interactions between protein molecules in a high ionic atmosphere will cause protein precipitation, which is called salting out (Duong-Ly & Gabelli 2014). Proteins with high hydrophobicity will precipitate first, while proteins with polar residues will dissolve even at high salt concentrations. The isoelectric point is the pH of the solution at which the net charge of the protein becomes zero and at this isoelectric point the solubility of the protein decreases and the protein will eventually precipitate and clump. Thus, the difference in the level of saturation of ammonium sulphate to precipitate the L-asparaginase enzyme between *Pseudoalteromonas tetraodonis* RB3 and *E. coli* could be due to the factors above which were influenced by the enzyme properties of each species. Table 5 showed the enzyme activity and specific enzyme activity of crude extracellular, F3 (40-60 %), and intracellular enzyme from *Pseudoalteromonas tetraodonis* RB3 and positive control respectively.

SDS PAGE

In Figure 7, it could be seen that the SDS-PAGE results of *E. coli* fraction 4 (F4) there was a 35 kDa and 45 kDa band, and in the intracellular enzymes of *E. coli* the thickest band was at 35 kDa and in the 60-70 kDa range. Based on the literature, the molecular mass of *E. coli* L-asparaginase was 35 kDa (Dantas et al. 2019). F3 contained bands with sizes around 35 kDa, 45 kDa and 90 kDa. Meanwhile, in the intracellular enzyme of *Pseudoalteromonas*

tetraodonis RB3 (sample) protein bands appeared from 15 kDa to > 250 kDa. There were still many protein bands that appeared because the intracellular samples did not undergo any purification process. However, the thickest bands were at 35 kDa and 50 kDa. Most type II L-asparaginases from mesophilic sources were tetramers of identical subunits with molecular masses in the range 140-160 kDa or 35-40 kDa per subunit, and marine bacterial L-asparaginases were mostly in the range of 25-41.1 kDa (Qeshmi et al. 2018). The 35 kDa was suspected to be the L-asparaginase protein.

Table 5. Enzyme activity and specific enzyme activity of *Pseudoalteromonas tetraodonis* RB3.

Source	Sample	Enzyme Activity (U mL ⁻¹)	Specific Enzyme Activity (U mg ⁻¹)
<i>P. tetraodonis</i> RB3	Crude Extracellular	28.34	72.30
	F3 (40-60 %)	27.75	136.03
	Intracellular	25.46	67.18
Positive control	Crude Extracellular	25.92	97.80
	F4 (60-80 %)	9.51	135.91
	Intracellular	24.20	90.97

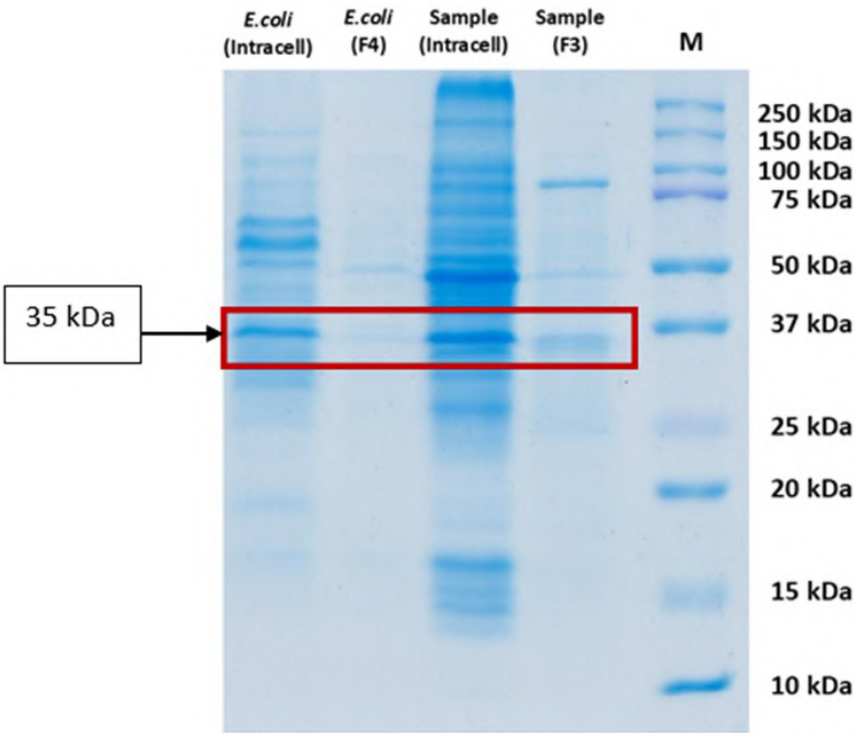


Figure 7. Electropherogram of intracellular and fractionation result of *Pseudoalteromonas tetraodonis* RB3 and *E. coli* as positive control.

CONCLUSION

Bacteria producing L-asparaginase with low glutaminase and urease activity had been isolated from seawater on the Rancabuaya Coast Indonesia. There were four different colonies named RB1, RB2, RB3 and RB4. RB3 colonies had the highest L-asparaginase activity with a zone index of 2.5 on ZMA+L-asparagine, zone index of 1.3 on ZMA+glutamine, and zone index of 2.2 on ZMA+urea. Based on the 16S rRNA gene sequence, RB3 was identified to have the closest phylogenetic relationship to *Pseudoalteromonas tetraodonis* GFC strain KMM 458, with a 16S rRNA gene homology of 99.51 % and

bootstrap of 100. Enzyme specific activity of crude extracellular and intracellular enzyme were 72.30 U mg⁻¹ and 67.18 U mg⁻¹ respectively, while the highest enzyme specific activity from ammonium sulphate fractionation was found at 40-60% saturation (F3), which was 136.03 U mg⁻¹. SDS-PAGE of the enzyme solutions showed the presence of a 35 kDa band suspected to be the L-asparaginase protein.

AUTHORS CONTRIBUTION

W.P. designed and supervised the research and wrote the manuscript, I.N. collected and analysed the data and wrote the manuscript, A.J.N. collected and analysed the data, M.R.M. supervised the research and wrote the manuscript, M.F. collecting sample and data, T.S. supervised the research.

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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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Research Article

Early Event at *In Vitro* Propagation of PGL-15 Tea Clone

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ABSTRACT

In vitro propagation is one solution to maintain or even to increase tea's productivity in Indonesia. This study aimed to obtain early-event information related to *in vitro* tea propagation using embryo axis and cotyledon of PGL-15 clone. This research was carried out by culturing the explants on MS medium with half strength ($\frac{1}{2}$ MS) (A medium); $\frac{1}{2}$ MS + BAP 2 mg L⁻¹ (B medium); $\frac{1}{2}$ MS + BAP 2 mg L⁻¹ + GA3 0.5 mg L⁻¹ (C medium); and $\frac{1}{2}$ MS + BAP 2 mg L⁻¹ + GA3 0.5 mg L⁻¹ + eco enzyme 0.1 % v v⁻¹ (D medium), with five explants per bottle and each treatment was repeated three times. The result showed that the D medium was the best medium in inducing further development of embryo axis and cotyledon explants. Morphological analysis showed that embryo axis explants cultured on the B, C, and D medium seemed better vigour, indicated by the increasing size and blooming of leaf primordial(s). Surface ultrastructure analysis showed that speed development of leaf primordial(s) on embryo axis explant varied among tested explants, depending on the type of the culture medium. For cotyledon *in vitro* propagation, it showed that at 7 days after culture (7-DACs), the D medium gave the best results in inducing explants for further development. This successful induction of further development of these explants is expected to open possibility of its multiplication in large quantity and its application for further research on tea plant.

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INTRODUCTION

Tea is known as the most widely drink consumed by people, especially in Indonesia. Apart from being known as a thirst-quenching drink, tea is also known as a healthy drink many benefits because of its content, i.e. active compounds such as polyphenol Gramza et al. (2005). This polyphenol compounds and other antioxidant substances are one reason for almost people in the world consumed it as healthy drinks.

The uniqueness of tea makes it to be one of the most widely consumed healthy drinks in Indonesia. This is very important for Indonesia to ensure the availability of tea plant for domestic consumption. Apart from that, Indonesia is known as one of the largest tea exporting countries in the world, next to Kenya, Sri Lanka, India, and Vietnam (Bolton 2015). These two things must be maintained and even increased by Indonesia for tea production both in quantity and quality.

Tea is an annual plant that is usually propagated vegetative through cuttings. However, in the field, obstacles are often found in efforts to increase tea production, for example lack of cuttings as planting material. Difficulty of rooting and obtaining the materials are the main problems for the use of cutting to be used as planting materials (Liu et al. 2019). The solution to this problem is through *in-vitro* propagation using seeds. One of the advantages of propagation through seeds is that the resulting plants have strong roots in addition to normal shoots because of the completeness of the two axis poles in the seed (El-Maarouf-bouteau 2022). Several research reports have shown the success of *in-vitro* tea propagation with specific clones, culture medium, or explant types (Gonbad et al. 2014; Eskundari et al. 2018; Widhianata & Taryono 2019).

PGL-15 tea clone is one of the superior tea clones in Indonesia. This clone is known for its high yield and pest resistance (Wagiman & Trimman 2011). However, no method for *in vitro* propagation of PGL-15 clones has been reported so far. Thus, researchers consider that it is necessary to conduct research regarding the method of propagating PGL-15 clones, especially using seeds as explants. Thus, it is hoped that the current research results will be useful in efforts to increase tea planting materials to meet tea needs both domestic and foreign demands.

MATERIALS AND METHODS

Materials

The PGL-15 clone tea seeds used as explants were obtained from Kayulandak Garden-PT. Pagilaran, Batang, Central Java, Indonesia (109°40'19"-110°03'06" E and 6°51'46"-7°11'47" S) at 1000-1500 m above sea level during September 2023. For culture media, the used materials include MS medium (Phytotech), benzylaminopurin (BAP; Himedia), gibberellic acid (or GA3; Himedia), and gelzan (Phytotech). The eco-enzyme solution which will then be added to the culture media was made by fermenting molasses, fruit skins (melon, mango, and banana), and water with comparison 1:3:10 for about 6 months. These fruits were collected from Gunung Kidul Region, Yogyakarta, Indonesia (110°21'-110°50' E and 7°46'-8°09' S) at altitude 206 m above mean sea level during January 2023 and these fruits were verified by botanist from Universitas Veteran Bangun Nusantara, Indonesia.

Methods

Preparation for culture medium

Culture medium that used in this study were MS medium with half strength ($\frac{1}{2}$ MS) (A medium); $\frac{1}{2}$ MS + BAP 2 mg L⁻¹ (B medium); $\frac{1}{2}$ MS + BAP 2 mg L⁻¹ + GA3 0.5 mg L⁻¹ (C medium); and $\frac{1}{2}$ MS + BAP 2 mg L⁻¹ + GA3 0.5 mg L⁻¹ + eco-enzyme 0.1 % v v⁻¹ (D media). To all medium, 3 % (w v⁻¹) sucrose

and 0.8 % (w v⁻¹) gelzan were added. All medium was adjusted to 5.6-5.8 for its pH before autoclaving at 121 °C for 15 minutes.

Preparation seed of PGL-15 as explant

PGL-15 tea seeds with age of around 8 months after seed-set formation were sterilized using antibacterial (Agrept 20WP, Streptomycin sulphate 20 %) for about 10 minutes, followed by rinsing using running water three times, sterilized using antifungal (Antracol 70WP; Propineb 70 % and Sulticob 93WP; copper oxysulphate) for 5-10 minutes, and rinsed using running water. The sterilisation process was continued in laminar air flow by soaking the seeds in a 96 % alcohol solution for 1 minute, then the seeds were burned and the seed shell was opened. Axis and cotyledon embryos were separated and then cultured on tested medium respectively.

Seed *in-vitro* propagation

Embryo axis and cotyledon were then cultured on tested medium with five explants on a bottle with three replications. Culture conditions were set at temperature of 22 ± 2 °C and light illumination intensity of 3250 lm, 90 lm W⁻¹ (Philips TLD 3684 cool daylight).

Morphological analysis

Explant was observed at the morphological indicators that occur (i.e. the emergence of germination) and captured by digital camera (CanonTM).

Data Analysis

This research was conducted in a completely randomised design with three repetitions and five explants in each bottle. The data was analysed statistically using F-test and the means among each treatment were analysed separately using Tukey test and $P < 0.05$ was considered as statistically significant.

Surface ultrastructure analysis

Explant at 21-DAC was treated using low vacuum at 70 KPa for about 20 seconds until stability was achieved, and then the explant was observed its morphology using scanning electron microscope (SEM Quanta 250).

RESULTS AND DISCUSSION

Embryo Axis Culture

The observation results showed that almost all embryo axis explants cultured on tested medium had grown that characterised by development of root and shoot pole at 14-DAC (Table 1). The A medium provided the least explant that responded to lead further development compared to other three culture mediums. The D medium, which was half strength of MS medium with the addition of BAP, GA3, and eco-enzyme, was succeeded in promoting further development of axis embryo.

The success of D medium in inducing further development may be due to the PGR's content on D medium. PGRs on D medium consist of BAP, GA3, and eco-enzymes and they were possible reasons for axis embryo to develop further. Cytokinin is known as a PGR which plays a role in plant development, such as the initiation and development of the shoot apical meristem (SAM) (Hnatuszko-Konka et al. 2021). Cytokinin are reported to not only play role in rating cell division but also in regulating the transition from undifferentiated cells to primordial differentiated cells, via one of the regulations of *Knotted-like homeobox1* (*Knotted 1*) gene so it can be said that these two things are related to each other (Uzelac et al. 2012). Furthermore, cytokinin will attach to the Arabidopsis Histidine Kinase (AHK) receptor which will then result in phosphorylation cascade marked by the activation of Arabidop-

sis Histidine Phosphotransfer Proteins (AHPs). This protein will then activate the Arabidopsis Cytokinin Response Regulators gene (type-A and -B ARR) so that the ARR protein will activate genes related to SAM formation such as *WUSCHEL* (*WUS*) and inactivate the *YUCCA* gene (*YUC*) (Pokimica et al. 2024).

Cytokinin is known as one of the PGRs that plays role in shoot germination. According to Villalobos and Martin (1992), cytokinin is found at embryo axis, whereas conjugated cytokinin is found at epicotyl and hypocotyl with its highest concentration is in the apical area. Therefore, the D medium provided further developmental response for embryogenic axis explants, and this matter might be due to the presence of cytokinin added to the D medium.

The D medium also contained gibberellic acids in the form of GA3. Several studies reported that GA3 plays a role in seed germination, such as on coffee's embryo axis (Bojórquez-Quintal et al. 2011). GA3 actually plays a role in germination because of the degradation of mannose in the endosperm cell walls so that it will have an impact on the breakdown of food reserves and finally it can support germination (Subandi et al. 2015).

The success of germination may also be influenced by addition of fruit-based eco-enzyme solution on culture medium. Eco-enzyme is reported to contain phytochemical compounds such as alkaloids (Eskundari et al. 2022) so it is suspected that these chemical compounds can induce the embryonic axis response to germination. Alkaloids are compounds that contain nitrogen with one or more nitrogen atoms (Debnath et al. 2018) so they are beneficial for plant growth (Bakri 2020). Thus, once again, the addition of fruit-derived eco-enzyme was thought to stimulate further development of tea embryonic axis explants.

Morphological analysis showed that at 0-DAC, the embryo axis was yellowish and no germination was visible (Figure 1). After 21-DAC on tested medium, almost of the explants progressed to further development, i.e. germination (Figure 2). The cultured explants seemed fresh, indicated by the change in colour of the explants from greenish to dark green. Apart from changing colour, explants also experienced germination which is characterized by the development of shoots (leaves) and roots.

After 35 days of culture, most of the explants proceed to further development (Figure 3.). Explants cultured on the B, C, and D medium grew well that can be seen in increasing of size and blooming of leaf primordial(s). Explants cultured on the A medium also experienced further development although the development pattern was not similar to explants cultured on the B, C and D medium. Root development on explants cultured on the A medium appeared to be more developed than shoot development. This phenomenon was possibly due to the absence of PGR added to the A medium so that the A medium is more suitable for root development. In contrast, shoot development in explants cultured on the B, C, and D medium was more dominant than root development. This phenomenon was most likely due to the addition of cytokinin to culture medium. Cytokinin plays a role in shoot organogenesis (Šmeringai et al. 2023), and there are many reports regarding the *in-vitro* propagation of tea that used cytokinin on culture medium to induce shoots, such as clones TRI2025 (Eskundari et al. 2018), Kiara-8 (Widhianata & Taryono 2019), and Iran-100 (Gonbad et al. 2014).

Table 1. Mean number of embryonic axis explants that experienced further development at 14-DAC

Type of medium	A	B	C	D
Mean of developing explants	3.35 ^a	4 ^a	4.35 ^a	4.65 ^a

* Means followed by the same letter are not significantly different based on Tukey's test ($p > 0.05$)



Figure 1. Axis embryos a moment after culture (0-DAC). Black arrow showed shoot apical meristem; blue arrow showed root apical meristem.

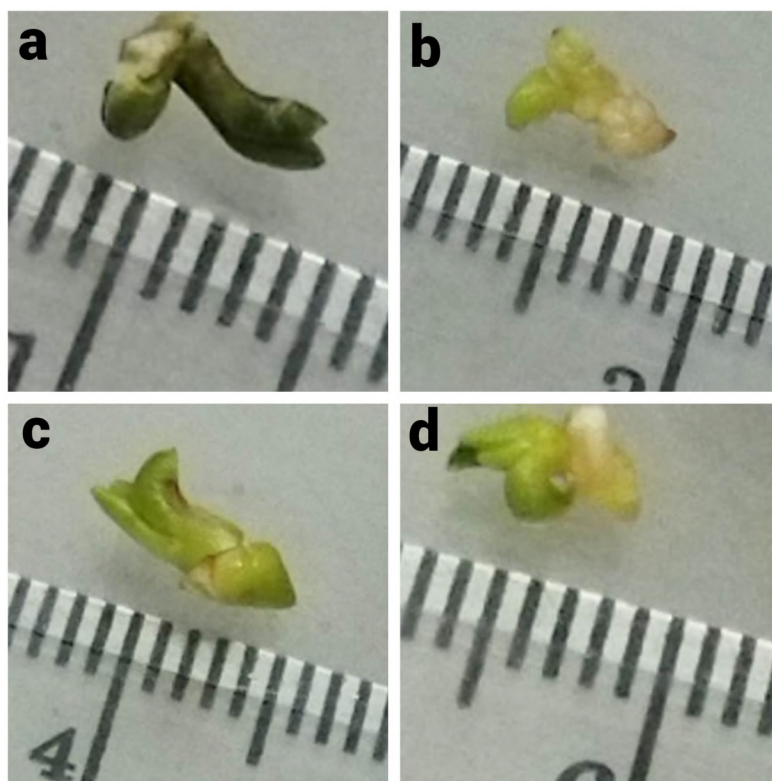


Figure 2. Morphological analysis of embryo axis explant at 21-DAC. Various explants were cultured on: A medium (a); B medium (b); C medium (c); and D medium (d).

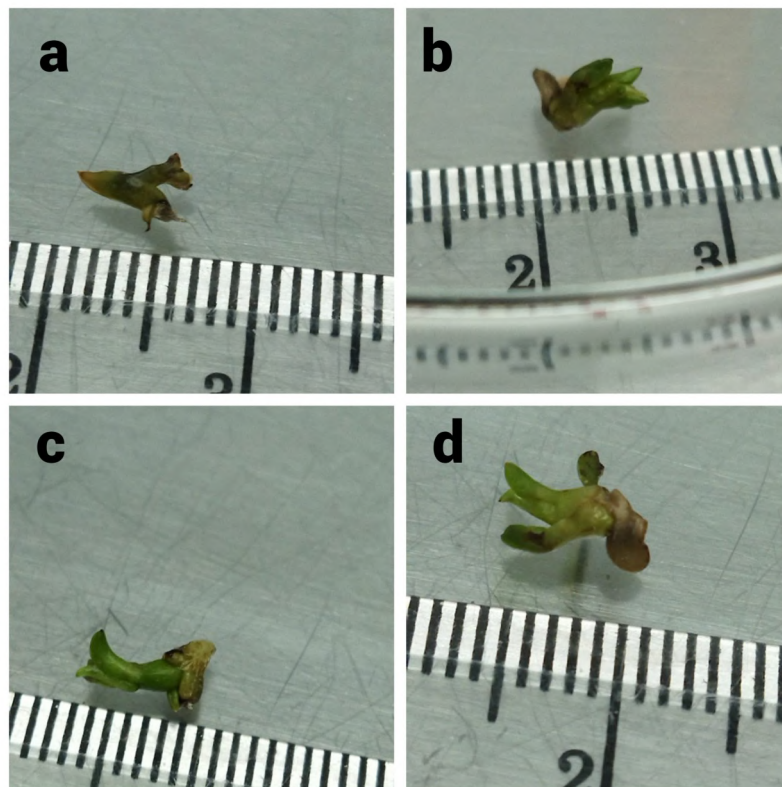


Figure 3. Morphological analysis of embryo axis explant at 35-DAC. Various explants were cultured on: A medium (a); B medium (b); C medium (c); and D medium (d).

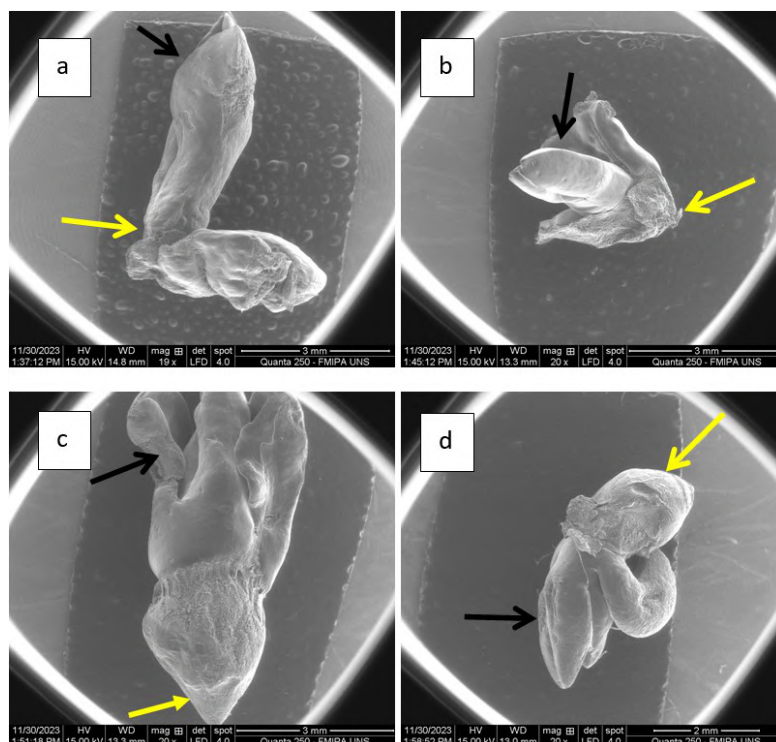


Figure 4. Surface ultrastructure analysis of embryo axis explant at 21-DAC cultured on: A medium (a); B medium (b); C medium (c); and D medium (d). Black arrows showed leaf primordial development; yellow arrow showed root development.

Surface ultrastructure analysis showed that at 21-DAC explants have had grown to germination phase, one of which was marked by the development of shoots (leaves). The speed of development of leaf primordial(s) varied at tested explants, depending on the culture medium type (Figure 4). It can be seen that explants cultured on B, C and D medium, the leaf primordial(s) has

further developed than explants cultured on A medium. This matter was most likely due to the suitability of the PGR(s) added to the medium so that the explants responded faster and finally the leaf primordium(s) developed.

Surface ultrastructure analysis showed the response of root organogenesis occurred at upper basal end of embryo axis. This phenomenon was indicated by changes in root size and the development of taproot. According to Zobel and Waisel (2010), taproot develops from a central root pole called the radicle, forming a central root axis system and Mackay et al. (2020) reported that this type of root allows plants to more easily absorb water found at the deep of the soil, and finally this is one way for plants to deal with drought stress.

Cotyledon culture

Tea propagation in this study was also carried out using cotyledons as explants. Almost all of the cotyledon explants responded when cultured on tested medium and observation at 7-DAC showed that the D medium gave the best results in inducing explants to further development (Table 2; Figure 5). On the other hand, the A medium which did not receive any additional PGR(s) has the lowest results in terms of the average number of explants that experienced further development.

Table 2. Mean number of cotyledon explants that experienced further development at 14-DAC.

	A medium	B medium	C medium	D medium
Mean of developing explant	3 ^a	4 ^a	3.65 ^a	4.34 ^a

* Means followed by the same letter are not significantly different based on Tukey's test ($p > 0.05$)

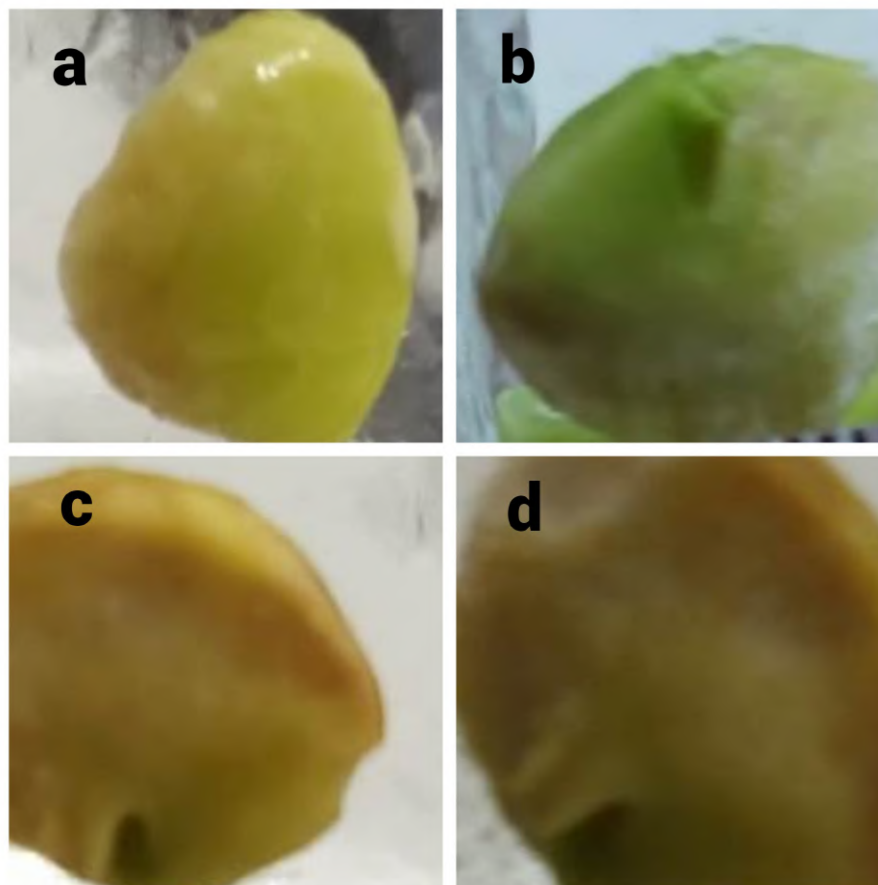


Figure 5. Morphological analysis of cotyledon explant on 7-DAC. Explants were cultured on: A medium (a); B medium (b); C medium (c); and D medium (d).

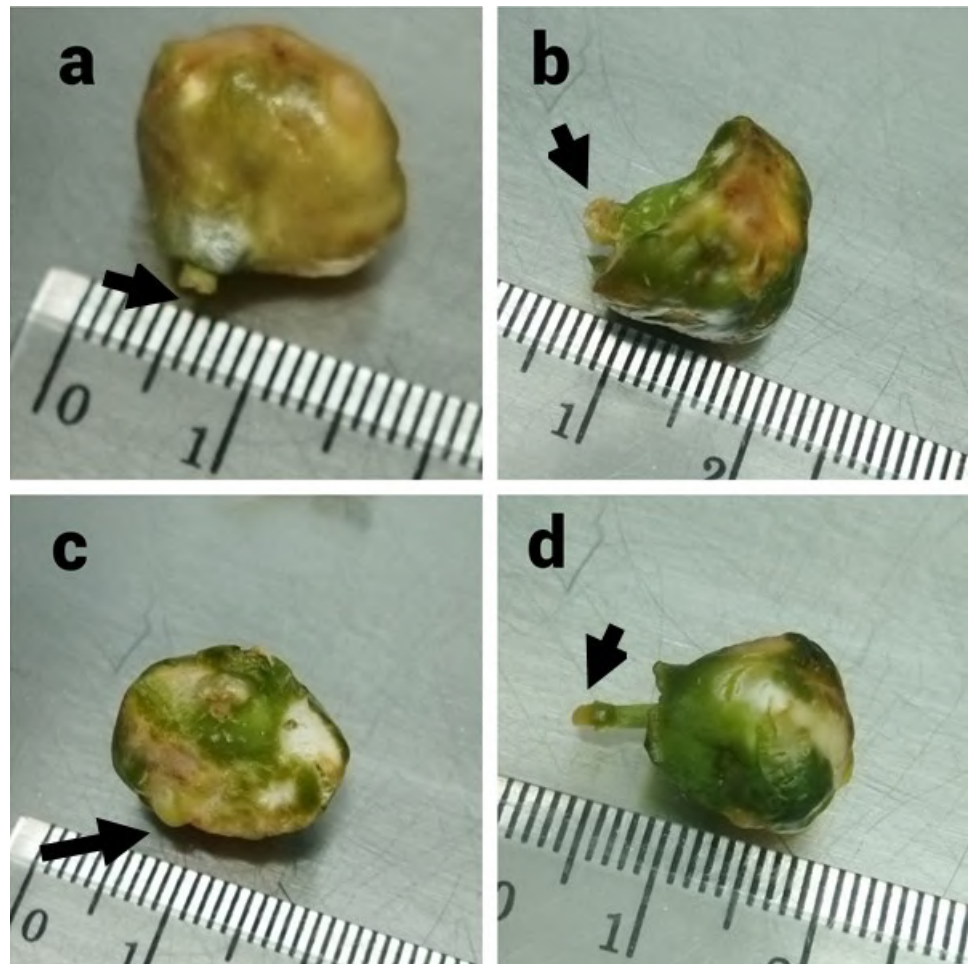


Figure 6. Morphological analysis of cotyledon explant on 35-DAC. Explants were cultured on: A medium (a); B medium (b); C medium (c); and D medium (d). Black arrow showed cotyledon petiole.

The D medium gave the best results in inducing organogenesis of PGL-15 tea clone in the form of cotyledon explants. This phenomenon was likely due to the suitability between the composition and ratio of PGR contained at culture medium and the explant's needs for further development. The D medium contained BAP 2 mg L⁻¹, GA3 0.5 mg L⁻¹, and eco-enzyme 0.1 % (v v⁻¹) and this composition was thought to be a reason of explants developed more quickly through the organogenesis pathway.

Explant that cultured on all tested medium showed the further development with different speed. The D medium was the fastest inducer for further development of explants and this medium successfully induced further explant development in the form of cotyledon petioles (Figure 6). This phenomenon was in line with the cotyledon petiole structure reported by Giovannelli et al. (2004) in chestnuts *in-vitro* propagation and Sari et al. (2021) in *ex-vitro* propagation of sugar palm. This structure had the unique characteristic on its first appearance in the area of embryo's axis former region.

Root development was seen at explants cultured on D medium in the form of cotyledon petiole. It was characterized by the presence of a long structure with a yellowish white tip. Later, this structure underwent to adventitious root. Similar thing has been reported in walnuts's histological analysis that showed cell division activity at the petiole's tip globular protein bodies in the central vacuole and starch grains in the cytoplasm (Gutmann et al. 1996).

This cotyledon petiole structure was a unique structure, and related to this structure, Giovannelli et al. (2004) stated that there were several chestnut explants that experienced to root organogenesis and the experienced to

shoot organogenesis. This phenomenon characterised by the presence of a nodular structure at the end of the petiole. Our previous study also showed the same phenomenon using TRI2025 cotyledon explants cultured on $\frac{1}{2}$ MS media with the addition of BAP 2 mg L⁻¹ (unpublished result; Figure 7).



Figure 7. Cotyledon of TRI2025 tea clone culture. Black arrow showed root development and yellow arrow showed shoot organogenesis.

The quantity of cotyledon explants that responded to culture medium were not as much as embryo axis explants that responded to further development after being cultured on tested medium. This matter was probably because the embryogenic axis has two growth poles, i.e. shoot and root pole, so that with just little induction, the explant would respond to grow. On the other hand, not all of explants experienced to further development and this phenomenon might be due to the presence of a small part of the embryonic axis remaining in the cotyledon area, resulting in response such as cotyledon petiole response in certain explants. This phenomenon was in line with the research results of Gutmann et al. (1996) that showed not all walnut cotyledon explants underwent to organogenesis initiation.

Cotyledon development occurred on all tested medium, with the D medium providing the best response for the development. The D medium contained cytokinin, gibberellic acid, and eco-enzyme on $\frac{1}{2}$ MS media. Among cytokinin, gibberellic acid, and eco-enzyme are strongly suspected to play positive role in initiating further development of tea cotyledons of the PGL-15 tea clone.

In this study, the cotyledon explants especially that cultured on the D medium appeared to regenerate, i.e. in the form of cotyledon petiole. Although none of the tested media contained auxin, Plant Growth Regulator (PGR) known for regulating root development, the compatibility between the PGR and explants for further development may play a role. Additionally, there is the possibility that a small portion of the embryogenic axis remained in the cotyledon.

Since MS medium contains macronutrients, micronutrients, and vitamin, changing the strength or concentration of MS medium could change the

nutrients component. Therefore, the effect of different medium strength could also affect the growth and development of plant. The amount of nutrients in medium for successful *in-vitro propagation* might vary in genotype of plant (Rezali et al. 2017). In other word, the suitability of the culture medium might influence the success in inducing further development of cultured explant.

Murashige & Skoog (MS) medium at half strength was used in this study, and the use of it at this study referred to the successful of *in-vitro propagation* of TRI2025 tea clone with axis embryo explants (Eskundari et al. 2018, 2019, 2021). Apart from that, the use of ½ MS media was also reported to be successful in inducing an explant response in other tea clones such as the Tambi, Jingga, Cinyuruan-143, and Kiara-8 clones (Widhianata & Taryono 2019). The use of ½ MS was also reported to provide a higher germination success rate than the use of full strength MS as in *Nothapodytes foetida* *in-vitro propagation* (Kaveri & Rao 2015).

In addition, in this study, eco-enzyme that also be added on to the D medium might be has an effect to initiate further development of tested explant. This result might be correlated with the content of eco-enzyme solution such as alkaloid that has one or more nitrogen ring that useful for plant growth development (Ain et al. 2016). Alkaloids are known as one kind of antioxidant that will play role in controlling the amount of Reactive Oxygen Species (ROS) through the ascorbate-reduced glutathione (AsA-GSH) pathway (Hasanuzzaman et al. 2020), so that it can increase the plant defence to stress oxidation. Furthermore, one type of alkaloid; purine alkaloid (Dey et al. 2020), can be broken down to allantoin and this compound compensates for soil nitrogen deficiencies, thereby maintaining nitrogen homeostasis and promoting plant growth and development (Kaur et al. 2023). This organic solution is reported has powerful inducer to initiate plant growth via *ex-vitro propagation*, such as in cassava (Faoji et al. 2021), chili (Wahyuni et al. 2023), and *Impatiens balsamina* (Eskundari et al. 2023).

In this study, no segregation test and percentage of phenotype changes that occur due to the use of seeds as explants were carried out. This is because this study aims to obtain a method for propagating PGL-15 clone tea using seeds. The use of seeds as planting material can almost certainly cause segregation and phenotype changes. In addition, according to (Chen et al. 2012), tea is a plant that cross-pollinates due to self-incompatibility, so it is certain that segregation will definitely occur in its propagation through seeds.

Based on the results of this research, it can be concluded that embryo axis explants promise a higher percentage of growth response compared to cotyledon explants. Embryo axis explants offer perfect plant development with two growth poles, i.e. roots and shoot meristem, so that the embryo axis is suitable as an explant for genetic engineering (Paes de Melo et al. 2020) although its number is limited compared to cotyledon explants. From this, we know that there were differences in *in-vitro propagation* of PGL-15 clone tea using axis embryo and cotyledon explants, thus it can be said that the success of *in vitro* propagation is influenced by many factors, such as the specificity of explant's type or culture medium. Certainly, specific research in order to find established methods for *in-vitro propagation* for other types of plants needs to be carried out. With the discovery of an established *in-vitro propagation* method, it is hoped that it will facilitate further research such as its application for genetic engineering or genome editing in certain plants.

CONCLUSIONS

The D medium was the best in initiating further development for both embryonic axis and cotyledon explants. Morphological analysis showed that embryo axis cultured on the B, C, and D medium seemed fresher and healthier, as noted by the increasing size and blooming of leaf primordia(s). Surface ul-

trastructure analysis showed that development's leaf primordial(s) speed varied among tested embryo axis explants, depending on the kind of culture medium. For cotyledon in-vitro propagation, it showed that the D medium gave the best results in inducing explants to further development i.e. "cotyledon petiole". This successful development induction with embryo axis and cotyledon explants of PGL-15 tea clone is expected to open the possibility for large scale multiplication or for further tea's research such as genetic engineering or genome editing.

AUTHOR CONTRIBUTION

R.D.E, T.T., and T.A. designed the research, R.D.E., T.T., T.A., and D.O. collected and analysed the data and wrote the manuscript, R.D.E and T.T. supervised all the processes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in preparing this research article.

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Research Article

Investigation on Nutritional Values of Three Different *Ananas comosus* (L.) Merr. Species Planted in Vietnam

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ABSTRACT

This study aimed to evaluate the nutritional values, phytochemicals, antioxidant activity, and anti-nutritional factors of three different pineapple cultivars (Cayenne, Queen, and MD2) cultivated in various provinces of Vietnam. The pineapple samples were collected from many farms ranging from the Central to the South of Vietnam. The analyses were performed as per standard test methods (the European standard and Association of Official Agricultural Chemists - AOAC methods). In general, there were no significant differences in energy-yielding nutrients (carbohydrates, fats, and protein). However, significant differences were observed in vitamin and mineral content. The MD2 cultivar had a high level of vitamin B5 and a low concentration of calcium, whereas both Cayenne and Queen were rich in vitamin C and chlorine. All three studied pineapple varieties exhibited moderate levels of phenolics and flavonoids, low concentrations of bromelain, and weak antioxidants. All three pineapple species studied contained low concentrations of oxalates and a void of tannins. These findings scientifically contribute valuable information to the food database system and can be considered in diet planning.

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INTRODUCTION

Ananas comosus (L.) Merr. (*A. comosus*), commonly known as pineapple, belongs to the genus *Ananas* of the Bromeliaceae family. The plant is native to the tropical and subtropical regions of South America, such as Brazil and Paraguay. However, this tropical plant is now extensively cultivated for its fruit in many regions worldwide, including Costa Rica, Hawaii, India, Philippines, Thailand, and Vietnam. Pineapple is locally known as paina (Hawaiian), abacaxi (Portuguese), dứa or thơm (Vietnamese) (Wali et al. 2019).

In Vietnam, pineapple is extensively planted on large scales in several provinces from the North to the South of Vietnam; however, approximately 90 % of output is in the South, provincially in Tien Giang, Kien Giang, Long An, Ca Mau, Binh Dinh, Dak Lak, Dong Thap, Quang Nam, and Phu Yen. The total pineapple cultivation area in Vietnam is estimated at approximately 40,000 hectares, producing around 500,000 tons annually for domestic and international markets.

Three different pineapple cultivars are commonly grown and consumed: MD2-Golden yellow, Smooth Cayenne, and Queen (Figure 1). These cultivars are distinguished based on the leaf and fruit characteristics. *Ananas comosus* var. queen (ACQ) is commonly known as Queen pineapple (Joy & Anjana 2017). This cultivar, best known as the world's sweetest pineapple, has dark yellow flesh, a crispy texture, distinct aroma and flavour. The leaf is recognizably characterized by a tiny spine on the edge of the leaf. *Ananas comosus* var. cayenne (CAC) is commonly known as Cayenne pineapple. The long, curved leaves have only a few spines at the tips. The fruit is large, dark green when unripe, and turns bronze when ripe. The fruit is juicy and has yellow flesh, large and shallow pineapple eyes, and thin skin. *Ananas comosus* var. MD2 (MD2), named after Millie Dillard, the wife of the former general manager of Del Monte, is a pineapple variety bred from two pineapple varieties Cayenne and Queen, with thin skin, succulent, bright yellow, fragrant, and extremely delicious flavour. The fruit is light green when unripe and bright yellow when fully ripe. The fruit's eyes are large and the eye pits are very shallow (Redwan et al. 2018).

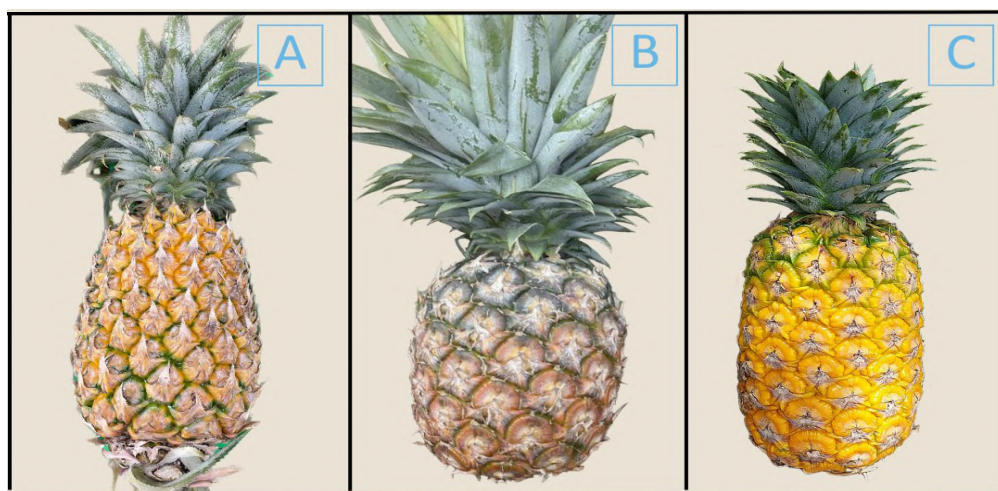


Figure 1. Three pineapple cultivars (A): *Ananas comosus* var. queen; (B): *Ananas comosus* var. cayenne; (C): *Ananas comosus* var. MD2.

Pineapple is commonly used as a popular food in Vietnamese people's daily meals. Pineapple is an ideal fruit for weight loss because it is a great source of fiber and low in calories and carbohydrates. Bromelain, a cysteine protease found in pineapple flesh, is probably the main ingredient contributing to medical applications. Pineapple has numerous medicinal properties, including the ability to aid digestion, heal wounds, and prevent platelet ag-

gregation. Pineapple exhibits anti-inflammatory and fibrinolytic effects, regulating cytokinin activity and immune response (Joy 2010). The nutritional values and phytochemicals of different pineapple cultivars may vary significantly depending on various factors, including species, genotypes, climatic conditions, geographical origin, cultivation practices, and harvesting periods (De Ancos et al. 2017). Furthermore, the biological activities of pineapple are not always correlated with the variation of components present in pineapple. Therefore, this study focused on investigating the variation of nutrients and phytochemical components across pineapple of different cultivars, namely Queen pineapple, Cayenne pineapple, and MD2, to empower consumers to make informed dietary choices and support further development in nutraceuticals and cosmeceuticals. Additionally, this study aims to provide data with significant potential to inform evidence-based policy formulation for governmental entities involved in sustainable agricultural development. Specifically, these findings offer actionable insights into enhancing production efficiency in targeted material sectors, facilitating the fulfilment of domestic demand, and the expansion of export markets.

MATERIALS AND METHODS

Materials

All chemicals and reagents were purchased from Sigma-Aldrich and provided by the Pharmaceutical Chemistry Laboratory and Applied Biochemistry Laboratory of the Applied Biochemistry Department at International University HCMC.

Selection and preparation of samples

Fresh pineapples of three varieties were randomly selected from various farms in Kien Giang (10°0'N 105°10'E), Dak Lak (12°40'N 108°3'E), Phu Yen (13°10'N 109°10'E), Tien Giang (10°25'N 106°10'E), and Dong Thap provinces (10°40'N 105°40'E) (Figure 2). Transportation was carried out in ice boxes and stored at 4 °C. The fruits were washed with distilled water, and the pineapple flesh was continuously dried at 48 °C for 18 hours (Ojike et al. 2020). The dried samples were then ground into a fine powder and stored in a sealed jar at 4 °C prior to further analysis.

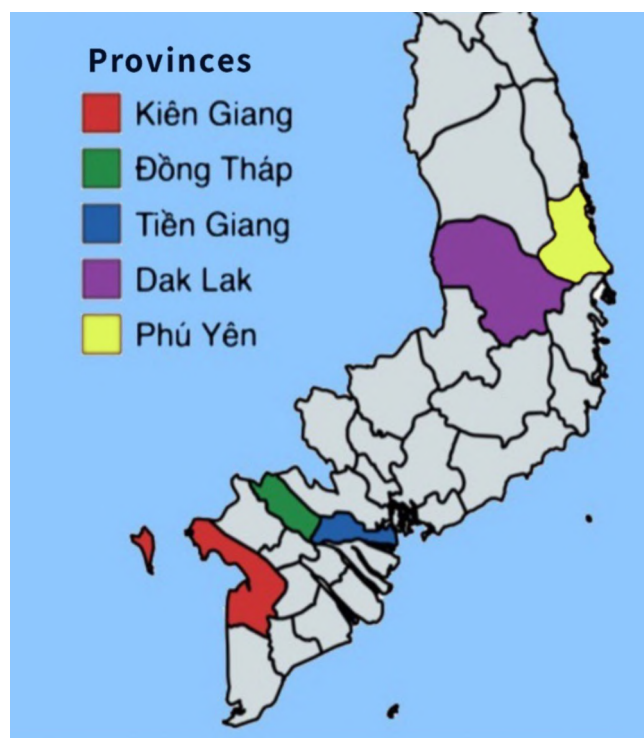


Figure 2. Sampling sites as highlighted.

Nutritional values

Total carbohydrate content

The total carbohydrate content of the DFS sample was determined using a phenol-sulfuric acid colorimetric assay (AOAC 988.12 1990); (Nielsen 2010). Briefly, 5 g of the pineapple sample was extracted with 45 mL of 80 % (v v⁻¹) ethanol under gentle agitation for 15 minutes. The resulting suspension was quantitatively transferred to a 50 mL volumetric flask and brought to volume with 80 % (v v⁻¹) ethanol. The mixture was subsequently filtered, and 10 mL of the filtrate was retained for analysis. For the colorimetric assay, 1 mL of the diluted filtrate was combined with 1 mL of 5 % (w v⁻¹) phenol solution and 5 mL of concentrated sulfuric acid. The reaction mixture was incubated in a water bath for 10 minutes, followed by a 5-minute cooling period at room temperature. The absorbance of the resulting coloured complex was measured spectrophotometrically at 490 nm against a reagent blank. Total carbohydrate content was quantified using a glucose standard curve and expressed as milligrams of glucose equivalents per gram of dry sample (mg GE/g) and as a percentage of the sample's dry weight.

Protein content

The determination of the protein content of pineapple samples was carried out using Kjeldahl method (AOAC 2001.11 2005; AOAC 991.20 2005). A Kjeldahl digestion and titration procedure was implemented. One gram of the sample was subjected to acid digestion within a Kjeldahl flask, utilizing 10 mL of concentrated sulfuric acid and 1 gram of a catalyst mixture. This catalyst was formulated from potassium sulfate and copper sulfate in a 9:1 mass ratio. The digestion process was carried out under heat until a clear solution was achieved. The resulting digestate was then diluted to a final volume of 100 mL using deionized water in a volumetric flask. Subsequently, the diluted digestate was subjected to steam distillation following the addition of 25 mL of 45 % sodium hydroxide solution to the distillation apparatus. The distillation continued until 100 mL of distillate was collected. This distillate was collected in a conical flask containing 10 mL of 4 % boric acid solution, along with a few drops of bromocresol green indicator. The nitrogen content was determined by titrating the distillate with 0.1 N hydrochloric acid until a pink endpoint was observed. The protein content of the sample was then calculated as a percentage using a standard nitrogen-to-protein conversion factor of 6.25.

Total crude fat content

The total crude fat content of the pineapple samples was determined using the Randall extraction-submersion method (AOAC 2003.05 2005; AOAC 2003.06 2005). A 4.5 g sample was precisely measured and transferred into a cellulose thimble suitable for Soxhlet extraction. This thimble was subsequently installed within the Soxhlet apparatus system (TT-SOX606), and 50 mL of hexane was introduced as the extraction solvent. The system was then activated, initiating a continuous reflux of the solvent through the sample material. After approximately 6 hours, ensuring comprehensive lipid removal, the heating element was deactivated, and the solvent was allowed to cool to ambient temperature. The collection flask, containing the extracted lipids, was then removed from the apparatus. To eliminate residual moisture, the flask was subjected to a drying process, followed by cooling in a desiccator, prior to final gravimetric analysis.

Crude fibre content

The Weeden method (AOAC 978.10 2000) was used to determine crude fibre content. A 2 g portion of each dried sample was weighed into a conical flask with 200 mL sulfuric acid (0.128 M) and incubated for 30 minutes in

periodically shaking. The filtrate was then washed with hot water to remove acid residues. The filtrate was then boiled and washed again with 200 mL of sodium hydroxide (0.313 M) in a separate conical flask. The filtrate was collected in a dried crucible, and the excess water was evaporated in the oven at 105 °C for 2 hours and cooled down in the desiccator for 20 minutes. The crucible containing fibre was weighed as W1. The crucible set was then placed in a muffle furnace and heated up to 500 °C for 2 hours. Once the crucible was removed from the furnace and cooled in a desiccator for 20 minutes, it was reweighed as W2. The crude fibre percentage was calculated by the formula:

$$\% \text{ crude fibre} = \frac{W_1 - W_2}{2} \times 100\%$$

Moisture content

The experiment was performed using the method of measuring the loss of volatile substances under temperature (AOAC 931.04 2000). A 10 g of samples were weighed in dried petri dishes and recorded as W₀. The sample was kept in a 70 °C-oven overnight until its mass no longer changed as W₁. The moisture formula was calculated as follows:

$$\% \text{ Moisture} = \frac{W_0 - W_1}{W_0} \times 100\%$$

Ash content

The ash content was determined using the gravimetric method AOAC 940.26, 2000 (Thiex et al. 2012). The empty crucibles were dried by the muffle furnace (Model LEF-230P) at 100 °C for 20 minutes and weighed to be W1. A 20 g of each wet sample was placed into those dried crucibles and weighed to obtain W2. The crucibles holding samples were kept in the furnace at 500 °C for 2 hours and then weighed for the W3 values. The percentage of ash content was calculated by the formula:

$$\% \text{ Ash content} = \frac{W_3 - W_1}{W_2} \times 100\%$$

Vitamin and mineral contents

The vitamin contents in pineapple samples were quantified primarily based on various AOAC methods. Vitamin A, E, D, K1, C, and vitamins group B including B1, B2, B3, B6, and B12 were analyzed by the high-performance liquid chromatography (HPLC) method (Vries et al. 1979; AOAC 961.14 1989; European Standard EN 14122-2003 2003; Mann et al. 2005; AOAC 2001.13 2011; AOAC 2011.10, 2011; AOAC 2012.21 2013; Delmonte et al. 2013; Hossain et al. 2019). The levels of vitamins B5 and B9 within the pineapple samples were quantified using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), following previously established protocols (AOAC 2011.06 2011; Andrieux et al. 2013). Vitamin content was expressed as per 100 grams of the pineapple sample.

Pineapple samples underwent analysis to determine their mineral composition, following established analytical procedures. Specifically, sodium and potassium levels were ascertained through flame photometry, adhering to AOAC 969.23, 2005. The calcium (Ca), magnesium (Mg), zinc (Zn), copper (Cu), and iron (Fe) were achieved using atomic absorption spectrophotometry (AOAC 968.08 2000). For manganese (Mn) and selenium (Se), inductively coupled plasma-mass spectrometry (ICP-MS) was employed, in accordance with AOAC 2015.01 (2015) and Nelson et al. (2019). Phosphorus content was determined via spectrophotometric analysis (AOAC 995.11 2000). The chlo-

rine concentrations were measured using the Volhard titration technique, as per the Volhard method (Nordtest method 1996).

Phytochemicals analysis and antioxidant activity

Determination of total phenolic content

According to the Folin-Ciocalteu method (Siddiqui et al. 2017), the total phenolic content (TPC) was measured and expressed as mg GAE/g, relative to gallic acid. The assay involved combining 150 μ L of the diluted supernatant with 375 μ L of Folin-Ciocalteu reagent and 375 μ L of a 7.5 % sodium carbonate (Na_2CO_3) solution. After a 5-minute vortex, the mixture was incubated in darkness at ambient temperature for 30 minutes. Absorbance was then recorded at 765 nm using a Biotek Synergy HT 96-well plate reader (USA).

Determination of total flavonoid content

The aluminium chloride method, as described in previous studies (Chang et al. 2020; Shraim et al. 2021), was used to determine the total flavonoid content (TFC) of pineapple samples. The supernatant, resulting from sample preparation, served as the basis for the TFC analysis. Specifically, 100 μ L of diluted supernatant was combined with 560 μ L of distilled water, 300 μ L of 80 % methanol, 20 μ L of 10 % AlCl_3 , and 20 μ L of 1M CH_3COOK . This mixture was then vortexed for 5 minutes and incubated for 30 minutes in the dark at room temperature. Quercetin (QE) was used as a standard to generate a calibration curve. Absorbance was measured at 430 nm using a Biotek Synergy HT 96-well plate reader (USA), with a blank as the reference. The TFC was reported as mg QE/g.

Estimation of bromelain

100 g of pineapple flesh was blended with 100 mL of phosphate buffer (0.05 M, pH = 7.0) prior to filtering. The yellowish precipitate was suspended in phosphate buffer (0.2 M, pH = 7.0) followed by re-fractionating with ethanol. The filtrate was centrifuged at 10,000 rpm at 4 °C for 20 minutes, and the supernatant was collected as “crude extract”. The crude extract was then subjected to ammonium sulfate precipitation by adding ammonium sulfate (60 %) (More et al. 2019) with gentle stirring on the ice for 30 minutes and then centrifuged at 10,000 rpm at 4 °C for 20 minutes. The pellets of their fractions were then collected and resolubilized with a phosphate buffer of pH 8.0 (Sari et al. 2018).

A 100 μ L aliquot of the soluble pellet was combined with 1.8 mL of 1 % casein and 2 mL of phosphate buffer (pH 8) in a test tube. Following a 5-minute incubation at 37 °C, the reaction was terminated by the addition of 10 % trichloroacetic acid (TCA), and the resulting mixture was centrifuged at 6000 rpm for 5 to 10 minutes. Subsequently, 2 to 3 mL of the supernatant was transferred to a tube containing 5 mL of 37 % formaldehyde solution. After thorough mixing and incubation at room temperature for 10 minutes, 1 mL of Folin reagent was added. Finally, absorbance was a 30-minute dark incubation prior to spectrophotometrically determined at 600 nm.

Determination of antioxidant activity

The ability to scavenge free radicals was evaluated using the DPPH assay, as previously described (Baliyan et al. 2022). The prepared supernatant served as the source material for antioxidant activity analysis. First, 750 μ L of a 0.1 mM DPPH solution was mixed with 250 μ L of the diluted sample or standard. After thorough agitation, the reaction mixtures were held in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm using a Biotek Synergy HT 96-well plate reader (USA) in a 96 well plate. The IC_{50} , defined as the sample concentration needed to achieve 50 % DPPH free radical inhibition, was used to quantify antioxidant activity.

Determination of anti-nutritional factors

Determination of oxalate content

The oxalate content of the pineapple samples was quantitatively evaluated by titration method (Karamad et al. 2019). An amount of 0.25 g of each pineapple sample was placed into a 250 mL Erlenmeyer flask containing 50 mL of de-ionized water. Subsequently, 5 mL of concentrated sulfuric acid was introduced, and the resulting mixture was heated in an 80 °C water bath. Before titrating the samples, a 0.1 N potassium permanganate (KMnO₄) solution was standardized against a 0.1 N oxalic acid standard. Following filtration of the treated sample through Whatman No. 1 filter paper, the filtrate was titrated with the standardized 0.1 N KMnO₄ solution until a persistent pale pink endpoint was observed, lasting a minimum of 30 seconds.

Determination of total tannin content

The determination of total tannin content was conducted using the colorimetric method (Siqueira et al. 2012). A calibration curve was generated using serial dilutions of a 0.1 mg mL⁻¹ tannic acid stock. The analytical procedure involved combining 0.1 mL of the pineapple sample with 500 µL of Folin-Ciocalteu reagent and 1 mL of 35 % sodium carbonate (Na₂CO₃) solution. Subsequently, deionized water was added to achieve a final volume of 10 mL. After a 5-minute vortex, the mixture was held in the dark at ambient temperature for 30 minutes. Absorbance was then recorded spectrophotometrically at 725 nm against a blank using a Biotek Synergy HT 96-well plate reader (USA). The total tannin content was reported as milligrams of tannic acid equivalent (TAE) per gram of sample.

Statistical analysis

All experiments were conducted in triplicate for statistical analysis. The data were statistically analysed using SPSS (version 22.0) software by the Kruskal-Wallis test and Dunn's test with the threshold of statistical significance at $p < 0.05$ and expressed as the MEAN $\pm$ STDEV.

RESULTS AND DISCUSSION

Essential nutrients

Carbohydrate, fibre, and moisture indices of pineapple varieties are presented in Table 1 whereas fat was absent in all studied species. The analyses of variance revealed significant differences ($p < 0.05$) in all nutrient values, except for carbohydrate and protein contents ($p = 0.72$).

The carbohydrate values were derived from the calibration curve of glucose, $y = 0.008x + 0.129$ ($R^2 = 0.997$), with the highest content in QAC, fol-

Table 1. Nutritional values of three different pineapple varieties.

Parameters	Quantity (g/100 g)		
	Cayenne pineapple	Queen pineapple	MD2 pineapple
Carbohydrate	9.96 $\pm$ 0.67 ^a	11.02 $\pm$ 2.56 ^a	10.74 $\pm$ 0.87 ^a
Fat	-ND-	-ND-	-ND-
Protein	0.86 $\pm$ 0.21 ^a	0.72 $\pm$ 0.03 ^a	0.64 $\pm$ 0.07 ^a
Fiber	1.05 $\pm$ 0.08 ^{ab}	1.20 $\pm$ 0.05 ^a	0.91 $\pm$ 0.05 ^b
Moisture	86.26 $\pm$ 0.40 ^a	87.60 $\pm$ 0.61 ^{ab}	93.15 $\pm$ 0.93 ^b
Energy (kcal/100 g)	44.00	47.00	48.50

-ND-: not detected.

^{a,b} Different letters in the same row indicate significant differences in means ($p \leq 0.05$)

lowed by MD2 and CAC. The amount of carbohydrates found in pineapples from Bangladesh was about 13.7 g/100 g in a previous study (Farid Hossain 2015), slightly higher than the carbohydrate content in the three pineapple species planted in Vietnam.

There was no significant difference in protein among the tested samples. It should be noted that the pineapples are generally not rich in protein, with a typical content of approximately 0.54 g/100 g as reported by the US Department of Agriculture (USDA). However, the lowest protein index found in MD2 was greater than that of the USDA document. The highest protein content was recorded in CAC (0.86 ± 0.21 g/100g).

The fibre content in all three pineapple species was significantly different ($p \geq 0.005$). The USDA reported that pineapple has a dietary fibre content of 1.4 g/100 g, slightly higher than that of QAC- the highest fibre content in this study (1.20 ± 0.05 g/100 g), followed by CAC and MD2.

The moisture content was higher than 80 % in all studied samples, while non-fat and low-calorie (44.00 - 48.50 kcal/100 g) were recorded, which is suitable for people aiming to control weight.

In a previous study A P & P M 2020, the MD2 pineapple species from Kerala revealed that those samples contained approximately equal amounts of carbohydrate and protein as this study at 10.873 ± 0.194 g/100g and 0.66 ± 0.02 g/100g, respectively, while indicating much higher fibre content and additionally documented the presence of crude fat content.

Vitamins and minerals

Overall, all fat-soluble vitamins and certain B-group vitamins, including B2, B3, B9, and B12, were not detected in all tested species (Table 2). These results were relatively similar to the data of the USDA pineapple record.

Vitamins B1 and B6 were only found in MD2 sample with the values of $0.20 \pm (1 \times 10^{-3})$ mg/100 g and 0.19 ± 0.06 mg/100 g, which respectively were 2.5 and 1.5 times higher than that of the USDA recorded (0.079 and 0.112 mg/100 g, respectively). Various studies have indicated that these vita-

Table 2. Vitamin contents of three different pineapple species.

Vitamins	Quantity (mg/100 g)		
	Cayenne pineapple	Queen pineapple	MD2 pineapple
A	-ND-	-ND-	-ND-
B1	-ND-	-ND-	$0.20 \pm (1 \times 10^{-3})$
B2	-ND-	-ND-	-ND-
B3	-ND-	-ND-	-ND-
B5	0.24 ± 0.08^a	0.23 ± 0.05^a	0.60 ± 0.04^b
B6	-ND-	-ND-	0.19 ± 0.06
B9	-ND-	-ND-	-ND-
B12	-ND-	-ND-	-ND-
C	31.10 ± 0.20^a	30.20 ± 0.20^b	12.56 ± 0.30^c
D	-ND-	-ND-	-ND-
E	-ND-	-ND-	-ND-
K	-ND-	-ND-	-ND-

-ND-: not detected ($LOD \leq 0.01$)

^{a,b,c} Different letters in the same row indicate significant differences in means ($p \leq 0.05$)

mins play essential roles in maintaining the central nervous system (CNS) and the peripheral nervous system (PNS). Notably, the combination of vitamins B1, B6, and B12 synergistically improves neuropathy, nociceptive, and neuropathic pain (Calderón-Ospina & Nava-Mesa 2020).

Vitamin B5, found in all three pineapple varieties, ranged from 0.23 mg per 100 g (QAC) to 0.60 mg per 100 g (MD2), which were nearly thrice as much as that of the USDA documented (0.21 mg/100 g). Vitamin B5 is a precursor of coenzyme A (CoA), which plays a crucial role in energy metabolism and fatty acid oxidation (Bourgin et al. 2022).

Vitamin C in MD2 (12.56 ± 0.30 mg/100 g) was recorded at the lowest value, which was less than a half of CAC (31.10 ± 0.20 mg/100 g) and QAC (30.20 ± 0.20 mg/100 g). These documented vitamin C contents were generally lower than that of the USDA recorded (average valued at 47.80 mg/100 g). Nevertheless, these results were significantly higher than the data conducted by previous research on two species of Bangladesh pineapple, which reported the vitamin C contents ranged from 9.62 ± 0.50 to 12.69 ± 0.20 mg/100 g (Kader et al. 2013); and from 6.45 ± 0.68 to 18.88 ± 0.03 mg/100 g for two pineapple species from Bangkok, Thailand (Kongsuwan et al. 2009).

Half of the studied essential minerals were detected (Table 3). The analysis of variance revealed significant differences ($p < 0.05$) among the three pineapple varieties. Three minerals (including Ca, K, and Mg) were identified in all pineapple varieties. QAC was found to be rich in calcium, with a value of 37.90 ± 0.20 mg/100 g, followed by CAC (36.30 ± 0.20 mg/100 g), significantly greater than the USDA record (6–19 mg/100 g). These species potentially provide rich sources of calcium compared to some other well-known calcium-rich fruits, such as tangerines (37 mg/100 g), oranges (40 mg/100 g), and pears (56 mg/100 g). Calcium is essential for healthy bones, blood clotting, nerve signalling, muscle contraction, and hormone release. Additionally, it lowers the risk of some chronic diseases and helps control blood pressure

Table 3. Mineral contents in three different pineapple species.

Minerals	Quantity (mg/100 g)		
	Cayenne pineapple	Queen pineapple	MD2 pineapple
Calcium (Ca)	36.30 ± 0.20^a	37.90 ± 0.20^b	4.87 ± 0.40^c
Chlorine (Cl)	340.00 ± 3.27	300.00 ± 3.72	-ND-
Potassium (K)	126.00 ± 0.20^a	120.00 ± 0.40^b	128.00 ± 0.20^c
Magnesium (Mg)	13.50 ± 0.01^a	12.80 ± 0.03^b	11.80 ± 0.01^c
Manganese (Mn)	-ND-	-ND-	-ND-
Sodium (Na)	1.06 ± 0.02	1.53 ± 0.04	-ND-
Phosphorus (P)	-ND-	-ND-	-ND-
Selenium (Se)	-ND-	-ND-	-ND-
Zinc (Zn)	-ND-	-ND-	-ND-
Copper (Cu)	-ND-	-ND-	-ND-
Iron (Fe)	0.24 ± 0.01	-ND-	0.26 ± 0.01
Iodine (I)	-ND-	-ND-	-ND-

-ND-: not detected ($LOD \leq 0.01$)

^{a,b,c} Different letters in the same row indicate significant differences in means ($p \leq 0.05$)

(Institute of Medicine 1998).

Both chlorine and sodium were found in QAC and CAC, but not MD2. Notably, both these species had low quantities of sodium, but extremely high concentrations of chlorine (300.00 ± 3.72 and 340.00 ± 3.27 mg/100 g, respectively). In addition to sodium's functions, chlorine, the most abundant electrolyte in blood serum, plays a vital role in the regulation of body fluids, electrolytes, electrical neutrality, acid-base balance, and participation in many other pathways (Berend et al. 2012). Meanwhile, the analysis of three tested pineapple species revealed moderate concentrations of potassium with the value of 120.00 ± 0.40 mg/100 g for QAC to 128.00 ± 0.20 mg/100 g for MD2 which is consistent with the USDA record ranging from 81- 162 mg/100 g.

Phytochemicals and antioxidant activity

Regarding their anti-inflammatory and antioxidant properties, polyphenols and flavonoids play a crucial role in mitigating the risk of inflammation and chronic diseases, including cardiovascular disorders. Using gallic acid and quercetin as standards for calibration in TPC and TFC quantification, the result was observed that the pineapple varieties exhibited moderate levels of both phenolic and flavonoid content. However, significant differences in concentration were noted for TPC ($\rho = 0.002$) but not TFC ($\rho = 0.012$), typically CAC pineapple had the highest value of TPC (21.93 ± 2.72 mg GEA/g) while MD2 showed the highest TFC (6.15 ± 0.77 mg QE/g) whereas QAC exerted the lowest amount of both TPC (12.75 ± 1.08 mg GEA/g) and TFC (3.26 ± 0.71 mg QE/g) (Table 4).

Bromelain is a natural enzyme that is believed to have many health benefits, including the ability to help digest protein, reduce inflammation and pain, and aid in the body's cleansing process and digestive issues (Rathnavelu et al. 2016). Studies in Vietnam and around the world have also shown that the enzyme bromelain has anti-inflammatory properties and is used effectively to reduce symptoms of arthritis, including rheumatoid arthritis. In this study, the QAC sample possessed the highest bromelain value of 1.56 ± 0.03 UI/g, whereas MD2 had the lowest bromelain concentration with a value of 0.92 ± 0.14 UI/g. In general, a person can consume up to 12 g of bromelain daily without any major side effects (Pavan et al. 2012).

The IC₅₀ value, indicative of the sample concentration required to inhibit 50 % of DPPH free radicals, was used to quantify the antioxidant activity of three pineapple species. Consistent with published literature, which indicates a relationship between phenolic and flavonoid content and antioxidant activity, the tested species demonstrated a moderate level of antioxidant activity when compared to ascorbic acid (Table 5). However, there was a significant difference in antioxidant activity among the three pineapple species ($\rho < 0.05$). MD2 sample had the strongest antioxidant activity (IC₅₀ = 99.17 ± 0.44 µg mL⁻¹) whereas the QAC sample exerted the weakest IC₅₀ of 162.70 ± 0.24 µg mL⁻¹.

Table 4. Phytochemicals and antioxidant activity of three different pineapple species.

	Calibration curve (R ²)	Cayenne pineapple	Queen pineapple	MD2 pineapple
Total phenolic (mg GEA/g)	y = 0.003x + 0.194 R ² = 0.971	21.93 ± 2.72	12.75 ± 1.08	16.96 ± 0.94
Total flavonoid (mg QE/g)	y = 0.004x + 0.067 R ² = 0.998	5.05 ± 0.91	3.26 ± 0.71	6.15 ± 0.77
Bromelain (UI/g) (ρ = 0.0004)		1.56 ± 0.03	1.36 ± 0.07	0.92 ± 0.14

Anti-nutritional factors

There were extremely low amounts of oxalate and no signals showing the presence of tannin in all tested species (Table 6). The American Academy of Nutrition and Dietetics advises patients with kidney stones to limit their daily oxalate intake to below 50 mg (Bernardino & Parmar 2017). The oxalate values recorded in the three species were approximately 130-206 times lower than the standard and also confirmed no significant differences among them ($p = 0.165$). These pineapple species are generally considered safe for human consumption, even for people with kidney problems or abnormal iron absorption.

CONCLUSIONS

In conclusion, no significant differences in energy-yielding nutrients were observed among the three pineapple species; however, significant variations were recorded in their vitamin and mineral content. All three studied pineapple species generally possessed moderate values of phenolics, flavonoids, low concentrations of bromelain, and weak antioxidants. The contents of tannin and oxalate were not significant and thus generally considered safe for daily consumption. These findings provide well-informed options for nutritionists/dietitians, food manufacturers, and consumers. Moreover, this study aligns with a strategic focus on maximizing the utility of pineapple resources through a two-pronged approach. By simultaneously increasing production for broader market penetration and meticulously analysing bromelain enzyme content for innovative product development, this research contributes to both economic expansion and biotechnological advancement. The integration of agricultural intensification with targeted biochemical research exemplifies a commitment to value-added processing, ultimately enhancing the overall contribution of pineapples to both domestic and international markets within the food and pharmaceutical sectors.

Table 5. Antioxidant activity in three pineapple species.

Sample/ Reference	Linear regression equation	IC ₅₀ (µg mL ⁻¹)
Ascorbic acid	$y = 9.677x + 26.578$ (R ² = 0.995)	2.45 ± 0.04
Cayenne pineapple	$y = 0.280x + 12.547$ (R ² = 0.995)	133.40 ± 0.46
Queen pineapple	$y = 0.259x + 7.742$ (R ² = 0.994)	162.70 ± 0.24
MD2 pineapple	$y = 0.217x + 28.420$ (R ² = 0.996)	99.17 ± 0.44

Table 6. Anti-nutrient contents in three pineapple species.

	Quantity (g/100 g)		
	Cayenne pineapple	Queen pineapple	MD2 pineapple
Oxalate	0.24 ± 0.09	0.32 ± 0.09	0.38 ± 0.05
Tannin	-ND-	-ND-	-ND-

-ND-: not detected.

AUTHOR CONTRIBUTION

SLH and TNKL conceived, formulated, supervised, designed the research, as well as wrote the manuscript. TNKL and NBH performed and analysed the data. All authors have read and approved this manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest, and this study received no funding from any individuals or organizations.

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