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Research Article

Understanding The Current Knowledge and Potential Research of Indonesia's Only Protected Amphibian: The Bleeding Toad (*Leptophryne cruentata*)

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ABSTRACT

Globally, amphibian populations are facing a substantial decline attributed to environmental degradation and lack of public attention. Indonesia, which one of countries with the high number of amphibian species in the world, paradoxically holds the record for the highest number of data-deficient amphibians. Indonesia currently has only one protected species, the bleeding toad (*Leptophryne cruentata*) also categorized as Critically Endangered. Considering this, our study undertakes a comprehensive review of bleeding toad research, highlighting research gaps and identifying potential topics for future investigations. In this study, we used an electronic database to acquire relevant studies aligned with our research objectives. The literature collection process involved the utilisation of the Publish or Perish (PoP) and manual internet searches. Our documentation reveals limited literature on bleeding toads, comprising only 20 reviews, with a notable prevalence of grey literature. This underscores the critical endangerment of bleeding toads, coupled with their neglect in research endeavours. Furthermore, our examination presents limited information on crucial aspects, such as taxonomy, morphology, geographical distribution, habitat characteristics, encounter records, behaviour, protection status, threats, and bioprospecting. The research gap is exceptionally high, with only two out of the 11 research topics attaining sufficient research status. Our findings underscore the urgent need for further research in this area. We identified at least 18 potential research areas that were essential for completing the baseline data. These findings serve as a valuable resource for researchers and policymakers seeking to address the critical endangerment of bleeding toads and to formulate effective conservation strategies.

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INTRODUCTION

Biodiversity loss has been ranked the third most threatening global risk in the last decade, following climate change and extreme weather (World Economic Forum 2022). The Anthropocene is a contributing factor to the high loss of biodiversity (Meng et al. 2021), which may indicate an ongoing mass extinction process attributable to factors such as habitat loss, overharvesting, climate change, fragmentation, forest and land fires, and invasive species (Singh et al. 2021). Amphibians, as a taxonomic group, have experienced significant population decline over the last decade for various reasons, signalling a biodiversity crisis (Luedtke et al. 2023). Currently, amphibians are confronted with an unprecedented extinction crisis (Catenazzi 2015). According to Luedtke et al. (2023), from a preliminary assessment in 2023, the global condition of amphibians is progressively worsening, with 40.7 % (2873 species) currently categorised as globally threatened under the IUCN Red List classifications of Critically Endangered (CR), Endangered (EN), and Vulnerable (VU). This represents an increase of 37.9 % (2681 species) in 1980 and 39.4 % (2788 species) in 2004. Luedtke et al. (2023) also mentioned the biggest factors contributing to amphibian extinction include agriculture (impacting 77 % of species), timber and plant harvesting (53 %), infrastructure development (40 %), climate change effects (29 %), and disease (29 %). Moreover, a lack of attention to amphibians has resulted in the significantly low popularity of amphibian conservation. Data from Evolutionarily Distinct and Globally Endangered (EDGE) Species indicates that amphibians and corals are the taxa receiving the least conservation attention, further emphasizing the urgency of addressing their conservation needs (Zoological Society of London 2020).

Indonesia, a globally significant Southeast Asian country for biodiversity, unfortunately possesses the highest number of species at serious risk of extinction (Duckworth et al. 2012; ASEAN Centre for Biodiversity & IUCN SSC Asian Species Action Partnership 2020). Indonesia ranks among the top ten countries globally, with the highest number of amphibian species, boasting a total of 418 species, including 224 endemic and 194 non-endemic species (Re:wild, Synchronicity Earth, and IUCN SSC Amphibian Specialist Group 2023). Habitat degradation and fragmentation are the primary factors that contribute to species extinction in Indonesia (Maskun et al. 2021). In addition to habitat destruction, Indonesia faces challenges in amphibian conservation due to low attention and research directed towards less charismatic amphibian species. This lack of focus contributes to the limited amount of research and funding dedicated to amphibian conservation efforts (Kusrini 2007a). Indonesia has the distinction of being the country with the highest number of amphibians, categorised as having a data-deficient status (Re:wild, Synchronicity Earth, and IUCN SSC Amphibian Specialist Group 2023). The low level of amphibian research is also exacerbated by numerous constraints; for example, the science community (bureaucratic processes at all project stages, including planning, visa procedures, fieldwork permits, scientific exchange, and project management issues, coupled with government budget cuts for basic research and limited access to international literature for Indonesian researchers) significantly impedes the internationalisation of biodiversity-related science (von Rintelen et al. 2017).

According to the ASEAN Centre for Biodiversity and IUCN SSC Asian Species Action Partnership (2020), Indonesia currently has three amphibian species on the IUCN Red List with the Critically Endangered (CR) category. There are bancet tompotika (*Occidozyga tompotika*), jacobson's bubble nest frog (*Philautus jacobsoni*) and bleeding toad (*Leptophryne cruentata*). The bleeding toad is a species that warrants special attention, as it is the sole amphibian species protected by Indonesian law under Permen-LHK P.106/2018. This species faces a high threat status owing to its limited distribution on Java Is-

land, declining population, and habitat destruction. Despite these challenges, attention given to this species is notably low. Consequently, we conducted a comprehensive review and analysis of the available data on the conservation status of the bleeding toad, aiming to (i) Analyse the extent of the research conducted on the bleeding toad. (ii) Present a comprehensive overview derived from existing literature on taxonomy, morphology, geographical distribution, habitat characteristics, encounter records, behavior, protection status, threats, and bioprospecting of the bleeding toad. (iii) Identify research gaps and potential topics for future investigations on bleeding toads. Therefore, our study represents a comprehensive synopsis of the largest online databases from which we highlight the extent of research on bleeding toads.

MATERIALS AND METHODS

Before initiating this study, we established the specific inclusion criteria (Table 1). These criteria play a pivotal role in the review process and serve as a precise framework for the selection of acquired publications. Furthermore, they contribute to the credibility of the review by allowing other researchers to employ the same protocol to replicate the study, thereby facilitating cross-validation and verification (Xiao & Watson 2019).

Table 1. Inclusion criteria for studies included in systematic review.

No.	Criteria	Rationale
1	Publications were gathered through the utilization of the *Publish or Perish* application and manual internet searches. The collected data encompassed publications indexed by Scopus and Google Scholar, as well as grey literature.	Ensure the quality and quantity of research. Grey literature included because many research is unpublished but valuable
2	Publication are written in English or Indonesian	Publications are not only written in English, but many publications are in Indonesian but have high value.
3	Publication titles and authors are complete and searchable	Publications must be traceable so that they can be analysed in more depth

In this study, we used an electronic database to acquire relevant studies aligned with our research objectives. The literature collection process involved the utilisation of the Publish or Perish (PoP) application (Harzing 2011) and manual Internet searches. Our primary research databases were Google Scholar and Scopus. The search procedure was carried out in the "title words" section, using the terms "bleeding toad," "kodok merah," and "*Leptophryne cruentata*." Subsequently, the search results were saved as *.csv* files, and later transformed into *.xls* format for further analysis. The data cleaning process was systematically executed to ensure the completeness of publication metadata and eliminate any instances of duplicated data, which may have originated from searches conducted on Google Scholar, Scopus, and through manual internet searches (Figure 1). The data search spanned from the earliest available year to 1 September 2023.

A total of 25 records were initially identified, but only 20 met the inclusion criteria for subsequent analyses (Figure 1). The selected literature for analysis encompassed various categories of publications, including those published in scientific journals, theses, unpublished reports, seminar proceedings, books, and other types of publications such as multimedia, posters, and magazines. Additionally, data collected through the *Publish or Perish* (PoP) application were saved in *.ris* format for subsequent analyses using *VOSviewer*.

VOSviewer is a software tool designed for generating network-based maps and visualising and exploring these maps. This application is primarily intended for analysing bibliometric networks (van Eck & Waltman 2022). In this study, we used *VOSviewer* to construct collaboration maps based on co-authorship and co-occurrence analyses, utilising the entire dataset. The analysis of research gaps and potential topics for future research was conducted using the data approach for the analysis of frog survival and population viability using Vortex by Davis et al. (2019) and The Amphibian Conservation Action Plan (Gascon et al. 2007; IUCN SSC Amphibian Specialist Group 2022).

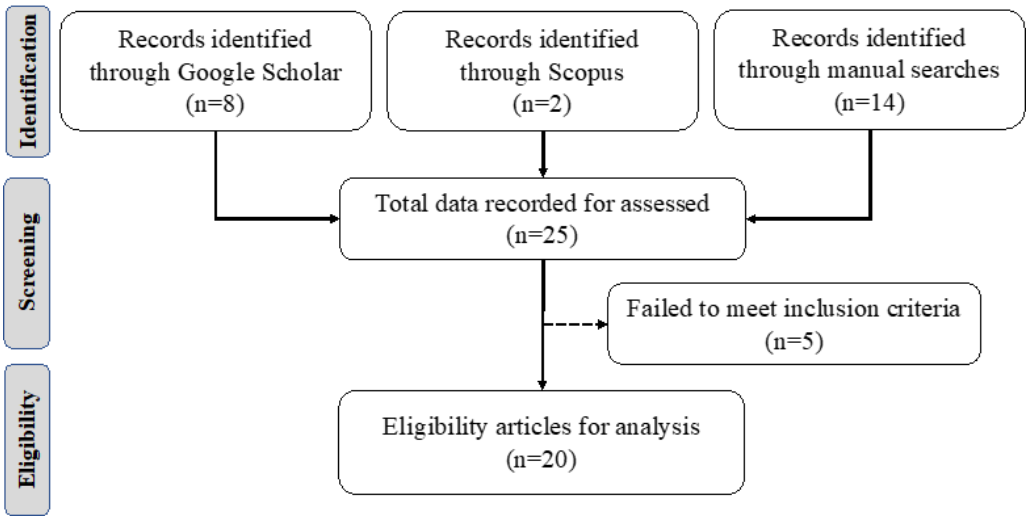


Figure 1. Data cleaning process.

RESULTS AND DISCUSSION

The current state of research conducted on the bleeding toad

As of 1 September 2023, 20 studies had been conducted on this amphibian species. However, despite the bleeding toad status as the only protected amphibian species in Indonesia, the quantity of research dedicated to this species remains relatively limited compared to other species. Literature searches on this species are still notably constrained, with only 25 % of the total literature having citations on Google Scholar and Scopus, whereas 75 % are uncited literature (manual searches) (Figure 2a). Literature that is not cited in Google Scholar and Scopus is typically referred to as grey literature. Grey literature encompasses output texts that are not formally commercially published by academic publishers. This includes, but is not limited to, webpages, patents, preprints, white papers, social media content, unpublished reports, theses, conference proceedings, and government documents (Schöpfel 2006). While grey literature may have shortcomings and often does not undergo the peer review process, its significance becomes evident when the availability of cited literature is limited (Kousha et al. 2022). The large amount of grey literature proves that this species is neglected. There are 40 % of bleeding toad literature published in journals, 40 % in identification manuals, and 20 % in magazines and unpublished theses (Figure 2b). Of the 40 % of the literature published in journals, around 15 % are not cited on Google Scholar or Scopus.

Research on the bleeding toad has shown an increasing trend, particularly following its designation as the only protected amphibian in Indonesia in 2018 (Figure 2d). The trajectory of research development on this species dates to 1838, when Tschudi initially described the species found in Cibodas, Cianjur, and West Java. Then in 1998, Iskandar (1998) stated that this species was no longer found in Cibodas. In 2005, Kusrini et al. (2005) embarked on a search and successfully found individual of bleeding toads. Due

to the precariousness of population of this species Kusrini (2006) proposed that this species should be protected. From 2006 to 2023, bleeding toad research gained momentum, with a total of seven studies conducted, notably led by research leader Mirza D. Kusrini (Kusrini 2007b; Yazid 2007; Oktalina 2010; Artika et al. 2015a; Artika et al. 2015b; Setiawan et al. 2021; Hasan 2022). Thus, bleeding toad researchers are predominantly Indonesian, comprising 75 %, with the remaining 25 % being foreign researchers (Figure 2c).

Only eight studies were cited by Scopus and Google Scholar and subsequently used as sources for *VOSviewer* mapping. The network visualization map of *Leptophryne cruentata*, as depicted in Figure 3a, highlights that the most influential and widely cited research pertains to the study of skin secretion (Artika et al. 2015a, 2015b), This is attributed to the fact that these two citations were published in reputable journals indexed by Scopus. The limited publication of bleeding toad research in reputable journals has resulted in unconnected network visualisation. Among the 20 available literature, bleeding toad research has covered seven distinct topics, including distribution, habitat, behavior, population, bioprospecting, taxonomy and morphology, and disease threats (Figure 3b). Distribution was the most discussed topic in 11 studies, followed by habitat, which was covered in nine studies. Behaviour was discussed in six studies, while the other topics had ≤5 occurrences, indicating a relatively low level of discussion on these subjects. Although the topic of distribution is the most discussed, the records of discovery locations tend to remain in the same locations. Consequently, some topics lacked sufficient data coverage. The most influential and widely cited author is IM. Artika and Mirza D. Kusrini became a frequent author in every bleeding toad publication (Figure 3c).

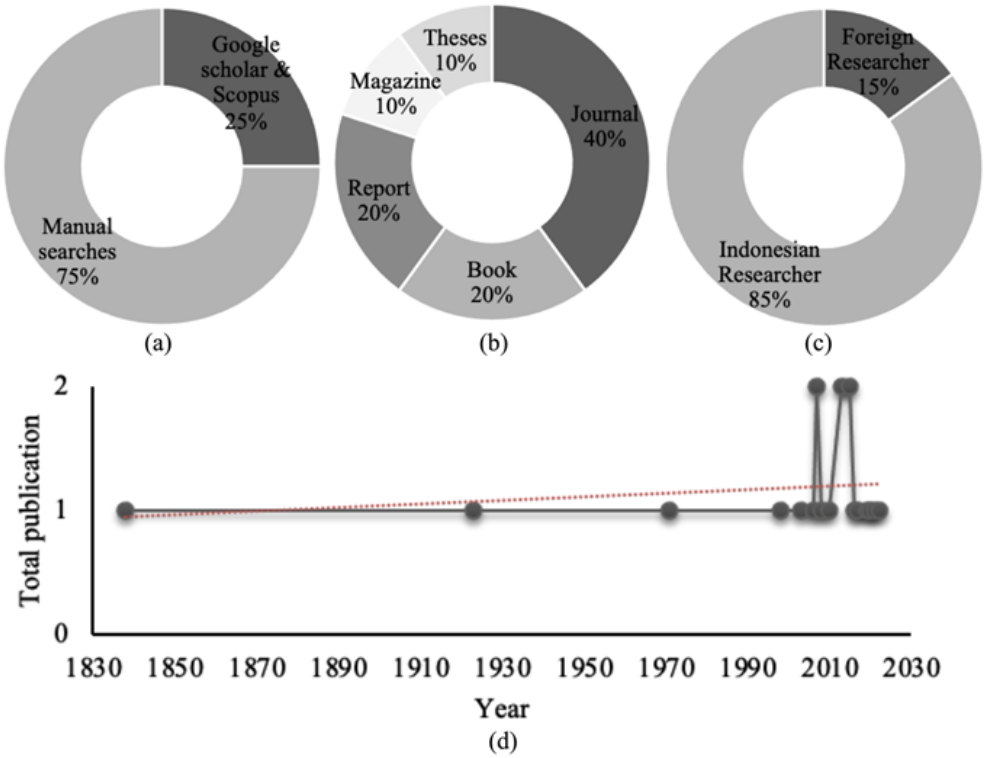
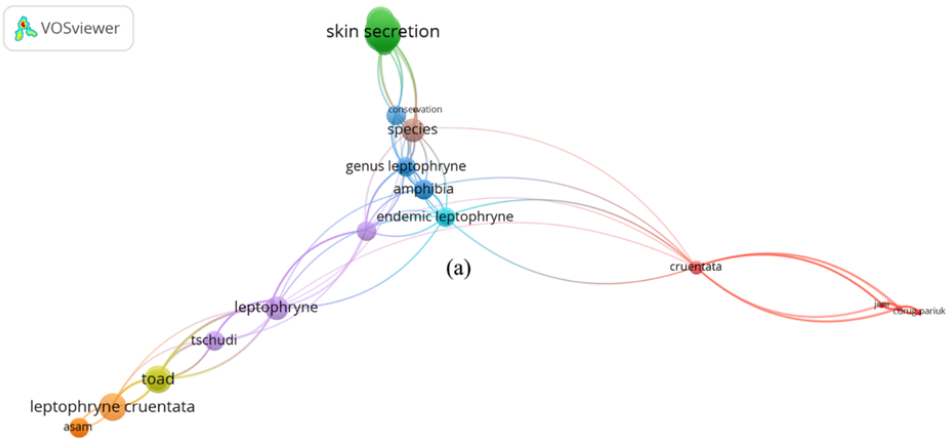


Figure 2. Basic findings of bleeding toad research. (a) literature search; (b) literature type; (c) researcher; (d) research trend.



Research topics	Number of studies that discuss
Distribution	11
Habitat	9
Behavior	6
Population	5
Bioprospecting	2
Taxonomy & morphology	2
Disease threat	1

(b)

Authors	Cites
IM Artika, S Pinontoan, MD Kusrini	5
IM Artika, S Pinontoan, MD Kusrini	3
H Kurniati	3
DT Iskandar, Mumpuni	2
MD Kusrini, M Yazid	2
GM Hasan, MD Kusrini	0
S Oktalina, MD Kusrini	0

(c)

Figure 3. (a) Research topics, (b) network-visualization map research, and (c) influential researcher.

Existing research information on bleeding toad

Taxonomy and morphology

The bleeding toad was first described in 1838 by Tschudi from two specimens, probably from Cibodas. *Leptophryne cruentata* is the type species for the genus *Leptophryne* which was described by Fitzinger in 1843, at which time the name was still *Bufo cruentatus*. The bleeding toad has the synonyms *Bufo cruentatus* and *Cacophryne cruentata*. The name "cruentata" refers to Latin which means bleeding (Iskandar 1998). The taxonomic classification of this species belongs to the class Amphibians, family Bufonidae, genus *Leptophryne*, and species *Leptophryne cruentata* (Iskandar 1998). Molecular descriptions of bleeding toad specimens have been conducted to distinguish between *Leptophryne cruentata* and *Leptophryne javanica*. The results, based on the percentage comparison of uncorrected *16S* rRNA differences, amounted to 5.1-5.6 % (Hamidy et al. 2018).

Morphologically, the bleeding toad has a small slender body with small parotoid glands, no bony prominences on the skull, slightly bent fingers and toes, 3rd and 5th fingers webbed to the last subarticular tubercle, and a skin texture filled with small granular tubercles (Figure 4a) (Iskandar 1998; Hamidy et al. 2018). This species is characterised by its striking body colour, which is black with red and yellow patches (Iskandar 1998; Yazid 2007). However, our field observations show that there is a wider variety of colours, with at least six-colour combinations of bleeding toad patterns (Figure 4b).

The eggs were small and black. Tadpoles are similar to *Bufonidae* tadpoles, but the lower lip is bordered by papillae (Iskandar 1998). Tadpoles are small and bluish black in colour and are thought to have a short lifespan to become juvenile toads (30 days). In an experiment to collect stage 42 (Gosner) specimens, it took only 4 days for the tail to disappear completely. Tadpoles were found in shallow waters with a slow streamflow. The body sizes of males and females are quite different (Yazid 2007). Females have a larger body size than males. Female body

size ranges from 24.51-46 mm with a body weight of 2.69-3.63 g, while male body size ranges from 20-30 mm with a body weight of 1.33-1.56 g (Iskandar 1998; Yazid 2007; Kusrini 2007b; Hasan 2022).

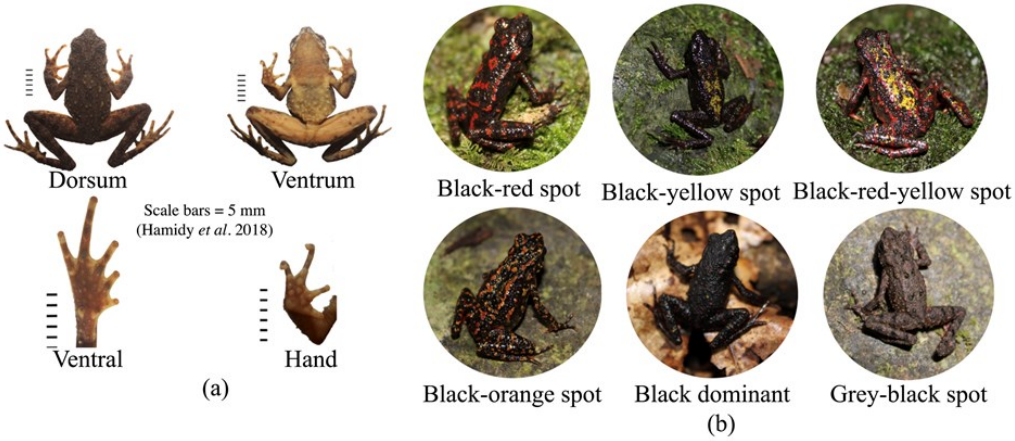


Figure 4. (a) Morphology of bleeding toad (Hamidy et al. 2018) and (b) showing various body pattern and color.

Geographical distribution

The bleeding toad is endemic to Java Island, and there are now 22 locations (Figure 5). In general, this species is primarily concentrated within two National Park regions, specifically in Gunung Gede Pangrango National Park (GGPNP) and Gunung Halimun Salak National Park (GHSNP). Within GGPNP, the bleeding toad can be found in the following areas: Curug Cibeureum, Rawa Denok, Selabintana, Ciapus, Lebak Saat, Curug Luhur, Cipelang, Perbawati, Curug Cikundul, Curug Pariuk, Curug Ceret, and Jajaway River (van Kampen 1923; Liem 1971; Iskandar 1998; Kusrini et al. 2005; Yazid 2007; Kusrini 2007b; Oktalina 2010; Ningsih et al. 2013; Kusrini et al. 2017; Permana et al. 2020; Setiawan et al. 2021; Kusrini et al. 2021; Hasan 2022). Meanwhile, its distribution within GHSNP includes Cikeris, Sagaranten, Gunung Botol, Curug Cibadak, Cikaniki, Lebak Banten, the eastern side of Kawah Ratu, and the Curug Heulang Hulu Ciliwung watershed (Kurniati 2003; Kurniati 2006; Kusrini et al. 2018; Kusrini et al. 2021). In addition to the national park areas, it is noteworthy that the bleeding toad has also been observed in the vicinity of the Waterfall at Taman Safari Cisarua-Bogor (Siregar 2016).

Habitat characteristics

This species lives in mountainous areas with clear streams and cold temperatures (Hasan 2022). Bleeding toads in the GGPNP can be found at altitude of 1370-2500 m asl. However, Kusrini et al. (2017) showed that the bleeding toad did not recover at an altitude of 2500 m asl (Lebak Saat), so the confirmed distribution started from 1370 to 2000 m asl. The bleeding toad in the GHSNP was found at an altitude of 1600-2200 m asl. (Kusrini et al. 2018).

The suitable temperature for the bleeding toad habitat is 10-25 °C and the humidity is 70-99 %. This species is mostly found along small rivers with slow currents of approximately 0.17-0.86 m sec^{-1}. River width ranges from 0.37-12.5 m, depth 30-50 cm, and with water pH 6. The river substrate was dominated by mossy rocks (*Sphagnum gedeanum*) and sand. The canopy density was 61-80 % dense–81-100 % very dense. Bleeding toads are often found on mossy rocks, river cliff wall holes and mossy weathered wood (Iskandar 1998; Yazid 2007; Oktalina 2010; Ningsih et al. 2013; Setiawan et al. 2021; Hasan 2022).

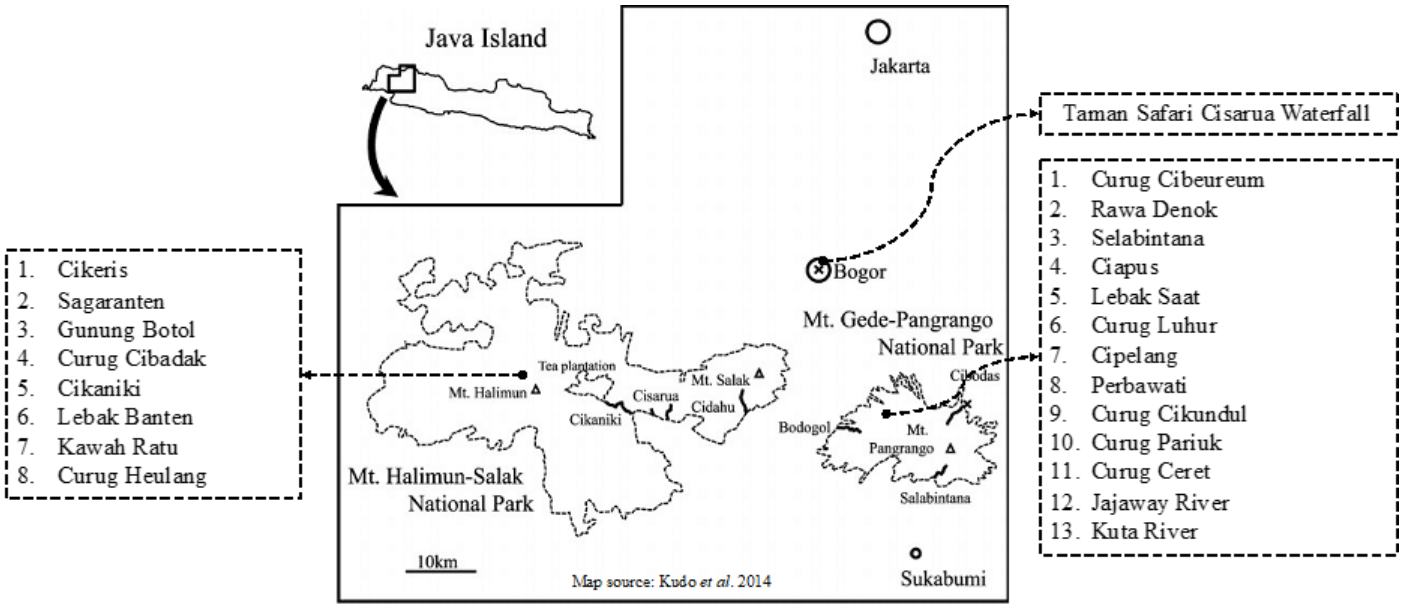


Figure 5. Bleeding toad geographical distribution (Map source: Kudo et al. 2014).

Encounter population record

After being described by Tschudi in 1838 from specimens from Cibodas, Liem (1971) found 149 individuals. Until 1998, bleeding toads were indicated to have experienced a population decline because they could not be found again around the Curug Cibeureum in GGPNP. (Iskandar 1998). This concern led Kusrini et al. (2005) to conduct another search for bleeding toad species in GGPNP and found four individuals. There are at least 7 literature mentions of bleeding toad encounters and the latest Hasan (2022) found 206 individuals in GGPNP area, with abundance ranging from 2.3-22.7 individuals/100 m and 410 tadpoles were found. Bleeding toad encounter records by year and distribution details from various sources (Table 2) (Liem 1971; Kusrini et al. 2005; Yazid 2007; Kusrini 2007b; Oktalina 2010; Setiawan et al. 2021; Hasan 2022).

Behaviour

Bleeding toads exhibit clustering behaviour in specific locations, predominantly demonstrating nocturnal activity but also being active during the day. Male individuals engage in calling activity at night in breeding sites. Bleeding toads are often associated with other toad species such as *Megophrys montana* and *Wijayarana masonii* (Yazid 2007; Hasan 2022). Bleeding toads mate throughout the year or season (Yazid 2007; Ningsih et al. 2013). Mating in bleeding toads occurs at night, and the eggs are affixed to rocks or plant roots in the form of mound-like structures that resemble grapes (Liem 1971). Tadpoles are found in small rivers (Iskandar 1998). According to Kusrini (2007b) from female specimens, bleeding toads have eggs that are uniform in size and are released simultaneously. Egg counts averaged 318.1 ± 59.9 (n=10) and could be up to 1200 eggs. The bleeding toad tadpoles in phases 24 -28 had a mean body length of 19.6 mm. (Ningsih et al. 2013). The dominant food of this species were *Hymenoptera* (60.38 %), *Optera* (7.55 %), *Orthoptera* (6.60 %), *Diptera* (6.60 %), *Lepidoptera* (4.72 %), *Hemiptera* (1.89 %), *Collembola* (1.89 %), and *Isopoda* (0.94 %) (Kusrini 2007b). Despite living in aquatic or riverine areas, this species forages on terrestrial habitats (Kusrini 2007b). In addition, according to Yazid (2007) Based on field observations, bleeding toads feed on winged ants, flies, and caterpillars. It is also known that bleeding toad tadpoles feed on organic materials such as food scraps from hikers in the river (Hasan 2022).

Protection status, threat, and bioprospecting

Bleeding toad is currently listed on the IUCN Red List as Critically Endangered (IUCN SSC Amphibian Specialist Group 2019). In 2006 Kusrini (2006) proposed the bleeding toad to be protected by Indonesian law. Therefore, the bleeding toad is currently the only protected amphibian in Indonesia. Bleeding toad has not been listed in the CITES (Convention on International Trade in Endangered Species) appendix. Bleeding toad threated by habitat disturbance by human activities or tourists who litter in several sites including Curug Cibeureum, Rawa Gayonggong, and Sungai Jajaway (Hasan 2022). Kusrini et al. (2008) found the infection of the fungus *Batrachochytrium dendrobatidis* (Bd) in bleeding toad although at low levels. This needs to be anticipated because Bd is one of the factors responsible for the decline in amphibian populations (De León et al. 2019). Two studies on the utilisation of bleeding toads have been conducted, with the potential of bleeding toad skin secretions as antibacterial and antifungal agents. Bleeding toad skin secretions have potential as anti-fungal *Trichophyton mentagrophytes* and antibacterial *Escherichia coli* and *Staphylococcus aureus* (Artika et al. 2015a, 2015b).

Research gaps and potential topics for future research

We were able to collect information on taxonomy and morphology, geographical distribution, habitat characteristics, encounter population records, behavior, protection status, threats, and bioprospecting. We used this limited information to conduct a research gap analysis. Identifying research gaps and potential areas for future research is crucial for advancing our understanding of the subject. By addressing these research gaps and pursuing these potential research avenues, we can contribute to a more comprehensive understanding of the bleeding toad ecology, conservation needs, and broader implications for biodiversity conservation in its habitat. Several fundamental

Table 2. Bleeding toad encounter record.

Year	Location												Total
	CCB	CCR	CP	RD	LS	RG	SJ	PB	CS	HL	SL	SKB	
1932									1				1
1959	5												5
1964	118				31								149
1972	1												1
1977	6							8	5		3		22
1978									1				1
1984												1	1
2003										2			2
2004	2												2
2005	4												4
2007	88												88
2007	15												15
2010	16			14		3							33
2021		26											26
2022	36	46	68	28		14	14						206
Total	291	72	68	42	31	17	14	8	7	2	3	1	

Curug Cibeureum (CCB); Curug Ceret (CCR); Curug Pariuk (CP); Rawa Denok (RD); Lebak Saat (LS); Rawa Gayonggong (RG); Sungai Jajaway (SJ); Perbawati (PB); Cisarua (CS); Halimun (HL); Salak (SL); Sukabumi (SKB). **Note**: The distribution locations are different from those in Figure 5 because there are several locations where bleeding toads can no longer be found.

research topics can serve to establish a solid scientific foundation for bleeding toad conservation, including ex-situ conservation (captive programs), reintroduction, habitat protection, survey and monitoring, trade, and sustainable use, as well as communication and education (Gascon et al. 2007; Davis et al. 2019; IUCN SSC Amphibian Specialist Group 2022). To fulfil the baseline information for bleeding toads, there were 11 essential topics, each with varying degrees of information sufficiency (sufficient, insufficient, and deficient). Identifying these sufficiency statuses can highlight data gaps and guide potential research topics for future investigation (Table 3).

Out of the 11 identified topics, only two currently hold a sufficient research status: taxonomy-morphology and protection status. This designation is attributed to the availability of ample data in these areas, particularly in relation to bleeding toad taxonomy and morphology, and the implementation of protective measures since 2006 (Kusrini 2006). An insufficient research status implies that research has been conducted, but there is still a lack of data. This inadequacy extends to topics such as geographical distribution, habitat characteristics, habitat suitability, bioprospecting, feeding behaviour, and population. Topics categorised as deficient indicate that there has been no prior study on bleeding toads in these areas, encompassing daily behaviour, reproductive behaviour, and threats. This paper also presents 18 potential research topics that could be conducted as part of fulfilling the bleeding toad baseline information. The substantial lack of fundamental data on the ecology and conservation of the bleeding toad poses challenges for formulating effective conservation policies. Consequently, the outcomes of this study can serve as a valuable guide for researchers and policymakers, aiding in prioritising research endeavours that are imperative for a more comprehensive understanding of bleeding toad ecology and facilitating effective conservation strategies.

CONCLUSIONS

Our findings indicate a scarcity of literature on bleeding toads, encompassing merely 20 reviews, predominantly sourced from grey literature. This highlights the precarious conservation status of bleeding toads and their relative neglect in scholarly investigations. Additionally, our scrutiny reveals gaps in essential domains including taxonomy, morphology, geographical distribution, habitat characteristics, encounter records, behaviour, protection status, threats, and bioprospecting. Notably, only two of the 11 thematic areas achieved adequate coverage, highlighting the significant research gap. Our insights emphasize the imperative for expanded research endeavours in this domain. We have pinpointed approximately 18 prospective research domains pivotal for augmenting the knowledge base on bleeding toads. Such revelations offer indispensable insights for academics and policymakers alike, aiming to mitigate the critical endangerment of bleeding toads and devise efficacious conservation measures.

AUTHOR CONTRIBUTION

R.K.T designed the research, collected, analysed, and wrote the manuscript, M.D.K, A.M, D.A.R wrote and review the manuscript.

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Table 3. Research gaps and potential topics for future research.

No.	Research topics	Research Status	Data deficiency and potential topics for research
1	Taxonomy and morphology	Sufficient	[1]Additional morphological data may include an analysis of the coloration patterns exhibited by bleeding toad and [2]population genetics in each habitat
2	Protection status	Sufficient	-
3	Geographical distribution	Insufficient	[3]Insufficient distribution data exists for the southern and western regions of GGPNP, and a notable paucity of data is evident in the case of GHSNP. [4]Species distribution modelling, [5]dispersality or local migration
4	Habitat characteristics	Insufficient	[6]It is imperative to conduct further validation of the utilization of waterfall habitats, fast and slow stream river, and lake
5	Habitat suitability	Insufficient	[7]A dedicated investigation of a specific habitat location utilizing high-resolution data has not been conducted
6	Bioprospecting	Insufficient	[8]Need to explore other potentials
7	Feeding behaviour	Insufficient	[9]This can be complemented by an exploration of the daily feeding requirements
8	Population	Insufficient	The current research is only bleeding toad encounter records. [10]Population estimation and carrying capacity have not yet been documented [11]Captive breeding
9	Daily behaviour	Deficient	[12]Comprehensive data regarding daily behaviour spanning a 24-hour period, [13]movement patterns, [14]bioacoustic characteristics,
10	Reproduction behaviour	Deficient	[15]Information pertaining to mating stages, [16]selection of mating and nesting sites, [17]reproductive system, egg clutch per year, first breeding age of males and females, maximum age, sex ratio at birth and mortality.
11	Threats/ Catastrophes	Deficient	[18]No research has been undertaken to assess the sensitivity of bleeding toad to human disturbance, biotic environmental alterations such as climate change, and volcanic eruption.

CONFLICT OF INTEREST

There are no conflict interests.

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Research Article

Antifeedant Activity of Limonoids from the Seeds of *Lansium domesticum* Corr. Against Subterranean Termite *Coptotermes curvignathus*

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ABSTRACT

Lansium domesticum is one of *Meliaceae* plants produces limonoids with various biological activities, except for anti termites. Seven limonoids, dukunolides A-D (DA-DD), F (DF), and langsatides A-B (LA and LB), each previously isolated from the seeds of *L. domesticum* and prepared at 5 %, together with methanol root extract (MRE) 5 % were evaluated for insecticidal activity against *Coptotermes curvignathus*. Fifty workers and five soldiers of *C. curvignathus* were tested in a No-Choice Test to determine which limonoid was the most active. Dukunolides A-D, F, langsatides A-B, and MRE showed weaker antifeedant activity than the regent 50sc (positive standart, 8.04 %), except for dukunolide B (DB) that was stronger antifeedant activity, with a 7.28 % paper weight loss and 33.3 % mortality against *C. curvignathus*. Conclusion, this study showed limonoid compounds that were isolated from the seeds of *L. domesticum* could be developed for antitermite drugs.

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INTRODUCTION

Limonoids are derived from tetracyclic triterpenoids by several oxidative changes, obtained mainly as secondary metabolites in plants of Rutaceae and Meliaceae (Roy & Saraf 2006; Nebo et al. 2015; Lin et al. 2022). These compounds exhibited various bioactivities such as antibacterial, antifungal, antiviral, anticancer, antimalarial, and insecticidal. Limonoids such as azadirachtins A and B are major natural products isolated from the seeds of *Azadirachta indica* (Meliaceae) showed insecticidal properties and exhibited very low toxicity to mammals and birds (McKenzie et al. 2010). Many insecticidal activities of limonoids from several genera of Meliaceae plants have been reported (Roy & Saraf 2006; Happi et al. 2018; Sun et al. 2018; Lin et al. 2022). However, scarce information about antifeedants has been reported, particularly anti-termite activity.

One of Meliaceae plants, *Lansium domesticum*, grows mostly in Southeast Asia, has a fruit used as a popular dessert. However, the peels are believe to be toxic to domestic animals. In the Philippines and Borneo, indigenous people control mosquitoes by burning leaves and bark of *L. domesticum* (Monzon et al. 1994; Leaman et al. 2015). The seeds were also reported to contain antimalarial constituents, namely domesticulides A-E (Saewan et al. 2006). Moreover, kokosanolide A, isolated from the seeds of *L. domesticum* cv Kokossan, showed an antifeedant activity against instar larvae of *Epilachna vigintioctopunctata* (Mayanti et al. 2011).

A scientific effort has been made to design drugs from medicinal plants; in contrast, investigation to develop insecticides has played a minor role (Nakayama & Osbrink 2010). A vast number of plant extracts has been explored and screened to search the insecticidal compounds, particularly for anti-termites; however, only a few phytochemical compounds have been used commercially to control them (Bourminta et al. 2013). The methanol extract of the heartwood of *Calophyllum inophyllum* revealed termiticidal activities (Kadir et al. 2015). Further, chemical constituents such as 5-phenyl-2-(1-propynyl)-thiophene and 1-phenylhepta-1,3,5-tryne that were isolated from stems of *Coreopsis lanceolata* showed antitermitic activity against the subterranean termite *C. curvignathus* (Pardede et al. 2018). In addition, leaf extracts from clove (*Syzigium aromaticum*) and cajuput (*Melaleuca cajuputi*) have been tested for paper weight loss and mortality against subterranean termite *C. curvignathus* (Indrayani et al. 2016).

Coptotermes genus contains approximately 28 species and they are known to cause serious problem for environment (Su & Scheffrahn 1998). *Coptotermes* sp. is the main species of subterranean termites and the most aggressive one. In continuation of our concern for insecticidal constituents, seven limonoids, dukunolides A-D (DA-DD) and F (DF), and langsatides A-B (LA-LB), which have been previously obtained from the seeds of *L. domesticum* Corr. (Rudiyansyah et al. 2018) and the methanol root extract (MRE) of *L. domesticum* Corr. were evaluated for anti-termite against *C. curvignathus*.

MATERIALS AND METHODS

Samples and Filter Papers Preparation

The root of *L. domesticum* Corr. was obtained from Pontianak, West Kalimantan, Indonesia. It was powdered (300 g) and macerated with methanol for 3 x 24 hours to give a dried methanol root extract (MRE) (12 g) by a rotary evaporator at 40 °C. Each limonoid, dukunolides A-D (DA-DD), F (DF), langsatides A-B (LA-LB) (Figure 1), and methanol root extract (MRE) was dissolved in chloroform and methanol, respectively to prepare a 5% (w v^{-1}) solution. Those limonoids were isolated from the seeds of *L. domesticum* (Rudiyansyah et al. 2018). Filter papers (Whatman No. 1, 50 mm diameter) were weighed and soaked with 5 % (w v^{-1}) of each tested sample and MRE for

approximately an hour, air-dried at room temperature for a day, and re-weighed before the test (Indrayani & Alkhadi 2021; Oramahi et al. 2023). The control papers were treated with distilled water (negative) and regent 50sc (positive).

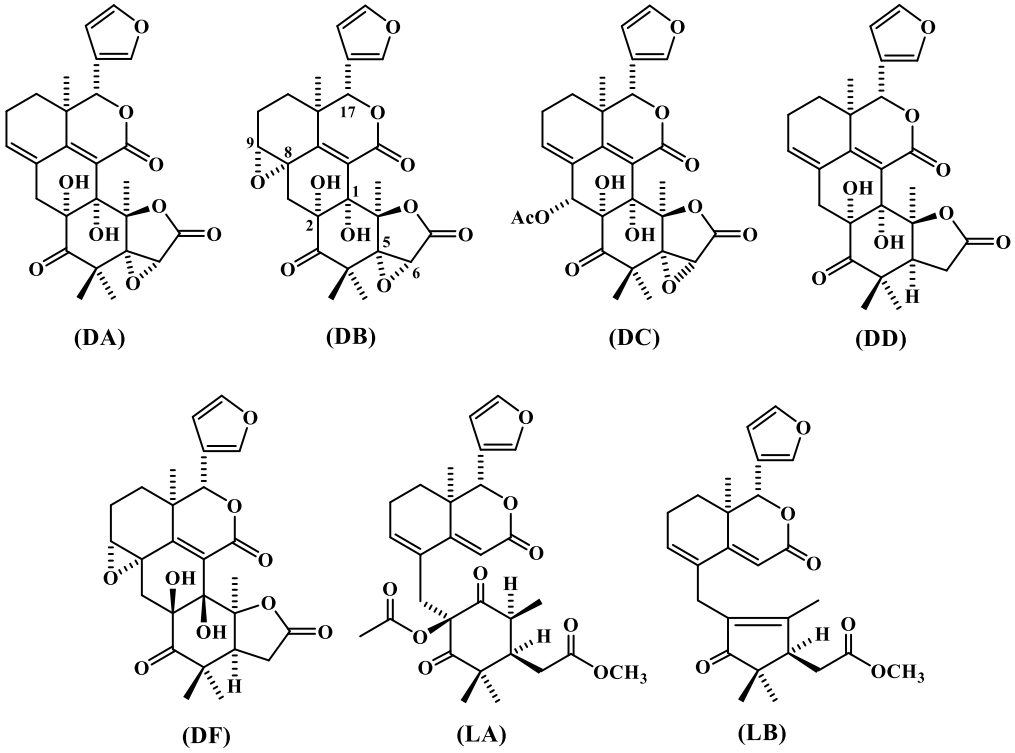

Figure 1. Dukunolides A-D (DA-DD), F (DF), and langsatides A-B (LA-LB) isolated from the seeds of *L. domesticum* Corr. (Rudiyansyah et al. 2018).

Termites Preparation

Subterranean termites *C. curvignathus* were collected from the local rubber forest, Pontianak, West Kalimantan, Indonesia. Termites and rubber wood (*Hevea brasiliensis*) were conditioned inside the perforated plastic containers at 28-30 °C (70-80 % humidity) for a month. Termites with good health conditions were selected (five soldiers and fifty workers), relocated into a new container, and they were not feeding for 24 hours before the bioassay.

Termite Bioassay

A no-choice test with some modification was carried out for anti-termite activity against *C. curvignathus* (Ohmura et al. 2000; Güzel et al. 2017; Quiroz et al. 2017; Liu et al. 2019). The tests were conducted in plastic cups (bottom diameter 6 cm, height 6 cm). Each cup was filled with sterilised sea sand (50 mesh, height ± 1 cm) on the bottom and moistened with 3 mL of distilled water. Each treated paper was placed on the plastic plate (40 mm diameter) and subsequently it was set on top of sea sand, then 55 termites were introduced. All treatment units were stored at 27-28 °C and 70-82 % humidity in the dark room for 3 weeks. Each treatment was maintained for five replicates, including the control papers (filter papers soaked in distilled water and regent 50sc, respectively). The weight loss of filter papers and termite mortality were measured when the test period ended.

RESULTS AND DISCUSSION

Paper Weight Loss and Termite Mortality

Paper weight loss is siqnificant to investigate in order to show the preference for termite to eat bait. All tested samples showed paper weight losses from 7.28 to 11.6 %, much smaller than the negative control paper, which was

80.85 % (Figure 2). Dukunolide B (DB) gave a paper weight loss of 7.28 % and other limonoids DA, DC-DD, and LA-LB including dukunolide F (DF) showed weight loss of 11.6 %. Furthermore, dukunolide B also exhibited a bit stronger paper weight loss than the positive control, that was 8.04 %. Although the paper weight loss values differed among all tested papers, however these were not significant difference except for the negative control paper. These paper weight losses were presumably because each limonoid consists of the furan ring and epoxide groups (Bentley et al. 1988; Roy & Saraf 2006; Nebo et al. 2015; Happi et al. 2018).

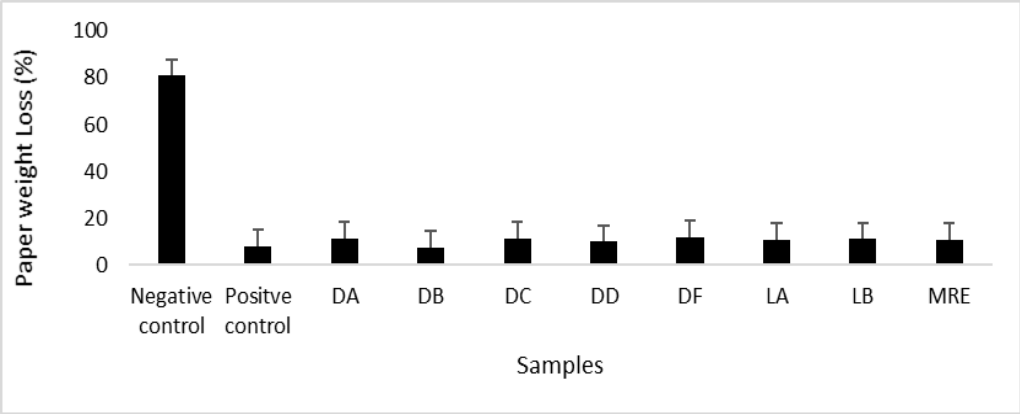

Figure 2. Paper weight loss of limonoids and MRE subjected for 3 weeks to *C. curvignathus*.

The efficacy of other limonoids could also be seen from the weight loss percentages of tested papers after being subjected to *C. curvignathus*. Except for DB, all treated papers displayed similar weight loss, 11.34, 11.29, and 11.66 % for DA, DC, and DF, respectively (Figure 2). Even though the epoxide position is different between structures DA and DC at C5/C6 and DF at C8/C9, the attachment of a furan ring at C-17 is the same (Matos et al. 2014; Shi et al. 2020). Further, limonoids DD, LA, and LB that only had a furan ring at C-17 resembled paper weight loss activity to limonoids contained both the furan ring and epoxide groups. As a positive control in this study, synthetic anti-termite (regent 50sc) was used with a concentration of 5 %. The value of paper weight loss in the positive control was smaller then other treatments, which was 8.04 % except for DB with a weight loss of 7.28 %. It can be said that DB is potential as an environmentally friendly termite repellent.

Additionally, anti-termite activity between MRE and all tested limonoids was not distinguishable, indicating that it contained limonoids or similar types of compounds. Based on these data, all limonoids DA-DD, DF, and LA-LB together with MRE had activity against subterranean termite *C. curvignathus*. Some literatures about structure-activity studies have discussed that limonoids with a furan ring and epoxides on their structures are associated with antifeedant activity (Bentley et al. 1988; Roy & Saraf 2006; Nebo et al. 2015). For example, the limonin which is the main chemical constituent from *Citrus* plants and contains a furan ring at C-17 and the epoxide on the structure exhibited antifeedant activity against beetle larvae (Bentley et al. 1988).

Similar to paper weight loss activity, the percentages of termite mortality for all tested samples were indistinguishable, around 30.00 %, except DB (21.09 %) (Figure 3). These values were two-fold higher than the negative control paper (14.54 %) and three-fold lower than the positive control paper (100 %). Compound DB was less toxic than regent 50sc, indicating that it should pose less risk to human and environmental health.

All limonoids DA-DD, DF, and LA-LB have the same sidechain furan ring attached at C-17. Again, this finding supported literature that a furan

ring at C-17 in limonoid structures has an important role in antifeedant activity (Bentley et al. 1988; Roy & Saraf, 2006; Matos et al. 2014; Nebo et al. 2015; Happi et al. 2018; Shi et al. 2020) including against subterranean termite *C. curvignathus*. In other words, in terms of mortality, there is no significant difference in the position of the epoxide, sidechain ring, and other groups in limonoid structures.

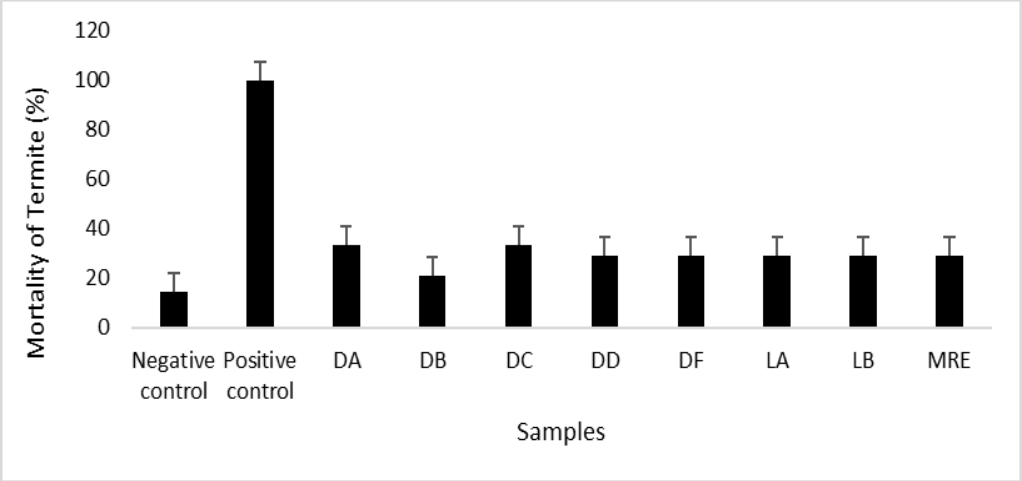

Figure 3. Percentages mortality of limonoids and MRE against termite of *C. curvignathus*.

CONCLUSIONS

These results proved that the tested limonoids from the seeds and methanol root extract of *L. domesticum* Corr. could be a potential eco-friendly pest management and probably developed into antitermite drugs.

AUTHOR CONTRIBUTION

R. and Y.I. designed, analysed the data, and wrote a manuscript, A.H.A. and A.S. supervised all stages of research, and E.P.M. run and collected the data.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Research Article

Enhanced Antibacterial Potential of Fractionated Bioactive Compounds Isolated from Endophytic *Nigrospora oryzae* UILRZ1 in *Ocimum gratissimum*

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ABSTRACT

The recurring global health challenges due to antimicrobial resistance require an impulsive response to search for alternative drugs with strong activities against multidrug-resistant pathogens. This study evaluated and screened endophytic fungi of strong antibacterial potential from *Moringa oleifera* and *Ocimum gratissimum*. Primer pairs of ribosomal DNA's internally transcribed spacer regions (ITS1 and ITS4) were used to determine their evolutionary relationships. A principal component analysis (PCA) biplot was used to identify the most effective endophyte at a 95 % confidence level ($P<0.05$). Improved culture conditions for the production of bioactive metabolites was done using the Taguchi design of experiment. Considering PCA biplot analysis, *Nigrospora oryzae* UILRZ1 from *Ocimum gratissimum* was most effective against selected pathogens. Production of metabolites was optimum at pH 5, 0.3 % (w v^{-1}) protein, 6-day inoculation time, and 4-plug inoculum, while variable of highest contribution was percentage of protein used. The column and thin layer chromatography were used to fractionate the extracts after optimization of production conditions while GCMS analysis was adopted to identify the chemical compounds. The crude extract's minimum inhibitory concentration (MIC) for chosen test microorganisms was 256 µg mL^{-1} prior optimization; while fractions of partially purified optimized extract of *Nigrospora oryzae* UILRZ1 showed enhanced antibacterial activity against *Staphylococcus aureus* with a MIC of 64 µg mL^{-1}. Efficient synthesis of bioactive metabolites was significant in the enhanced antibacterial activity against *S. aureus*.

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INTRODUCTION

Global threat to human and livestock's health due to antimicrobial resistance (AMR) is at an alarming rate. This is caused by misuse and overuse of antimicrobials, leading to a high level of morbidity and mortality, if proper attention is not paid, the death toll will increase to 10 million by 2050 with $1 trillion in economic loss (Zhang et al. 2020; Walsh et al. 2023). Meanwhile, a global action plan (GAP) expected to address innovation, research, and development (IR&D) to solve the existing problem was noted by Iwu and Patrick (2021) to have yet to be adequately implemented in the African region. To combat the current menace of AMR, researchers are constantly looking for novel antibiotics in natural products to combat resistant microorganisms (Breijyeh et al. 2020; Salam et al. 2023).

Medicinal plants as sources of novel alternative antimicrobials in drug discovery are being explored because of associated natural products. These products play a vital role in traditional medicine in various cultures around the globe. They are also identified as dominating 25 % of modern medicine (Hosseinzadeh et al. 2015). The selection of indigenous plants like *Moringa oleifera* and *Ocimum gratissimum* for antibacterial endophytes, which have several active compounds that have been used to treat and manage various diseases in local environments, was motivated by ethnobotanical history and subsistence (Yadav & Meena 2021). According to several studies, fungal endophytes connected to medicinal plants have chemicals in common with those of their host plants and may even benefit host plants more.

Furthermore, it has been reported that their symbiotic or mutualistic relationship confers resistance to invading pathogens and biotic and abiotic stress due to their symbiotic or mutualistic relationship (Kusari & Spitteller 2012; Naik et al. 2019). According to Schulz et al. (2002) and Khare et al. (2018), secondary metabolites of fungal endophytes are correlated with the biological activity of metabolites of their hosts, as shown by research on taxol which is present in endophytic *Taxomyces andreanae* of Yew plant which is being tested for anticancer properties. Despite this, exploring endophytes will contribute greatly to conserving and preserving plants' habitats, sustaining the environment, and improving the economy (Rao et al. 2015; Chen et al. 2016; Rausch et al. 2019). Bioprocessing technology has gained advocacy by using statistical optimization methods to develop experiments that optimize complicated physiochemical parameters desirable for bioactive metabolite synthesis (Navarrete-Bolaños et al. 2017). This study investigated *Moringa oleifera* and *Ocimum gratissimum* for endophytic fungi with antibacterial properties. A Taguchi experimentation design was also adopted using orthogonal arrays to reduce the number of experiments to a minimum level, thus ensuring that all required conditions are met for optimum production of bioactive metabolites.

METHODS

Plant Collection and Identification

In September 2017, we collected leaves and branches of *Moringa oleifera* and *Ocimum gratissimum* from a home garden and the University of Ilorin Moringa plantation in Ilorin, Kwara State, Nigeria. Submission of plants to the Department of Plant Biology, University of Ilorin for confirmation and voucher numbers: *Ocimum gratissimum*-UILH/001/019 and *Moringa oleifera*-UILH/002/559.

Isolation of Endophytic fungi

Following recommended procedures by Shen et al. (2014), young, healthy leaves and branches of *Moringa oleifera* and *Ocimum gratissimum* were washed in running water to remove soil particles, cut into pieces (0.5-1 cm), and

sequentially sterilized with 70 % ethanol (Fisher Chemicals, Belgium) for 1 min, 1 % sodium hypochlorite (Fisher Chemical, Belgium) for 1 min, and further cleaned by rinsing with sterile distilled water. Sterilized plant materials were blotted and dried under a laminar flow bench. Three pieces each imprinted into solidified PDA plates supplemented with 200 mg L^{-1} concentration of streptomycin to suppress bacterial contamination. Water washed after sequential sterilization was plated as a control to confirm the sterility of plants, and all Petri plates were incubated at 28 ± 2 °C for four weeks. All tests were done in triplicate.

Colonization Frequency of Endophytic fungi

According to De Padua et al. (2019) and Alsharari et al. (2022), diversity and colonization frequency (CF) of an endophytic isolate from various locations of chosen plants was determined by dividing the number of segments colonized by a single endophyte by a total number of the segment by 100. Hence,

$$\text{Percentage CF} = \frac{\text{Number of segment colonized endophytes}}{\text{Total number of segment}} \times 100$$

Identification of Isolated Endophytic Fungi

Cultural and morphological methods, as described by Watanabe (2002) were adopted to classify fungi into groups and molecularly identified by extraction of DNA, Amplification, and Sequencing.

DNA Extraction, Amplification, and Sequencing

DNA was extracted from fresh mycelium using a Quick-DNATM Fungal/ Bacteria Miniprep kit following the Manufacturer's instructions. Primer pairs, ITS 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) used for amplification by amplifying internally transcribed spacer region of total genomic DNA in Applied Biosystem Veriti Thermal cycler.

Preparation of PCR mixtures was done according to Rathod et al. (2015) with slight modifications by adding 12.5 µl of master mix (Ampli Taq Gold 360), 0.5 µl each of 10 µM both forward and reverse primers (ITS1 and ITS4), 1 µl of DNA template and 10.5 µl Nuclease free water. Programming of thermal cycler PCR reaction for 35 cycles (Table 1).

PCR products were sent to Inqaba Biotec for sequencing using the same forward and reverse primers. Seqtrace version 0.9.0 was used to obtain consensus sequences. Nucleic acid sequences were aligned and compared with those fungal isolates available in the database of NCBI (http://blast.ncbi.nlm.nih.gov) with the assistance of the Basic Local Alignment Search Tool (BLAST). The identified sequences were further deposited in the Genebank, and the accession numbers given are shown below: MT565285; MT565286; MT565287; MT565288; MT565289; MW020703; MW020704; MW020705; MW020706; MW020707; MW020708.

Table 1. Programming of Thermal Cycler PCR Reaction for 35 cycles.

Step	Temperature	Time
Initial Denaturation	95 °C	5 minutes
Denaturation	95 °C	30 seconds
Annealing	55 °C	30 seconds
Extension	72 °C	1 minute
Final Extension	72 °C	7 minutes
Hold	4 °C	∞

Phylogenetic analysis

Evolutionary relatedness was performed using maximum likelihood with bootstrap values calculated from 1000 replica runs using MEGA X version 10.0.2 (Kumar et al. 2018). Sequences of endophytes were deposited in the Gen bank database.

Test bacterial isolates

Test bacterial isolates were obtained from the Department of Microbiology and Parasitology at the University of Ilorin Teaching Hospital in Kwara State. They included *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Proteus mirabilis*. The test microorganisms were inoculated to sterile broth in suspension and adjusted to 0.5 McFarland standard, or 1×10^{8} CFU ml^{-1}.

Primary Screening

Tight streaking of a sterile Mueller Hinton plate with a standardized test microorganism; agar plug removed using a 6mm cork borer, and a 6mm plug of actively growing endophytic fungi was inoculated into the primary plate as described by Balouiri et al. (2016); Jayatilake and Munasinghe (2020), incubated for 18-24 hours at 35 °C. Subsequently, a zone of inhibition was taken using a millimeter scale.

Cultivation and Extraction (Secondary screening)

Methods of Merlin et al. (2013) adopted fermentation broth: 0.01 g Phenylalanine, 0.5 g Peptone, 40 g Glucose, 0.5 g Magnesium sulfate, 3.0 g Ammonium sulfate, 0.8 g Yeast extract (Oxoid, United Kingdom), 2.0 g KH_2PO_4, 24.1 g Potato Dextrose broth (Rapid Labs, Colchester, United Kingdom) dissolved in 1 L of distilled water and adjusted pH to 5.5, sterilized at 121 °C for 15 minutes. After cooling, inoculation of four plugs of actively growing endophytes in 100 mls of fermentation broth in a 250 ml Erlenmeyer flask, incubated in an orbital shaker incubator at 120 rpm for 14 days at 28 ± 2 °C. After incubation, the mycelial mat was crushed and filtered through a muslin cloth. The liquid-solvent extraction method was adopted by mixing 1:3 (fungal broth: solvent) for maximal extraction and shaking vigorously using a magnetic stirrer for 1 hour. After that, the solution was poured into a separating funnel to separate the filtrate from the broth. Filtrate was subsequently subjected to rotary evaporation and lyophilization to obtain crude extract.

Determination of Antibacterial Activity of Crude Extract of *Nigrospora oryzae* UILRZ1

The crude extracts were reconstituted in 3 % dimethyl sulfoxide (DMSO) for antibacterial bioassay. The agar well diffusion technique, as described by Zakariyah et al. (2017) was adopted by boring a tightly streaked sterile Mueller Hinton agar using a 6 mm cork borer and dispensing reconstituted extracts of 512 µg ml^{-1} into wells, allowed to diffuse and incubate for 18-24 hours. The reading of the zone of inhibition was taken to the nearest millimeter.

Optimization of Culture Conditions for Production of Secondary Metabolites

The growth condition of *Nigrospora oryzae* UILRZ1 was optimized by adopting the Taguchi design of experiment (DOE) described by El-Moslamy et al. (2017) using MINITAB 16 statistical software. Process parameters considered were protein source, pH, inoculation time, and inoculum size. L9

orthogonal array was generated (Table 2) with a combination of factors selected for three levels.

Table 2. Actual Design for Optimization of Bioactive Metabolite Production using L9 Orthogonal Array Taguchi Design of Experiment.

Inoculation time (days)	pH	Inoculum (disc mm^{-1})	Protein source (% w v^{-1})
6	5	4	2
6	6	8	3
6	7	12	4
12	5	8	4
12	6	12	2
12	7	4	3
18	5	12	3
18	6	4	4
18	7	8	2

Extraction of Bioactive Metabolites

Analytical grade ethanol (Fisher Chemicals, Belgium) was used for liquid-solvent extraction by mixing 1:3 (fungal broth: solvent) for maximal extraction and shaking vigorously using a magnetic stirrer for 1 hour. After that, the solution was poured into a separating funnel to separate the filtrate from the broth. The filtrate was subjected to rotary evaporation and, subsequently, to lyophilization using a freeze dryer (model LAB KITS FD-12 -MR) to obtain crude extract, as described by Abonyi et al. (2018).

Antibacterial Activity of Optimized Extract of *Nigrospora oryzae* UILRZ1

Antibacterial activity of optimized extracts of *Nigrospora oryzae* UILRZ1 against selected Gram +ve and Gram –ve organisms (*Staphylococcus aureus* and *Acinetobacter baumanni*) using agar well diffusion technique as described by standard methods of CLSI (2012). Disc diffusion technique was adopted to test selected microorganisms using Gentamicin (Oxoid, United Kingdom).

Determination of Minimum Inhibitory Concentration (MIC)

The micro broth dilution method, reported by Balouiri et al. (2016), was adopted to determine the minimum inhibitory concentration of crude, optimized, and fractions of *Nigrospora oryzae* UILRZ1 extracts. The procedure was by using round bottom 96-well microtiter plates with 50 µl of Mueller Hinton Broth (MHB) into wells 2-9; 256 µg ml^{-1} of gentamicin sulphate (Oxoid, United Kingdom) was added to 9th well (positive control) while 100 µl of MHB was added to 10th well to represent negative control. Fifty microliters (50 µl) of 256 µg ml^{-1} extract was dispensed into the first two wells, and a serial two-fold dilution was done by transferring 50 µl of suspension from well 2 to subsequent wells until eighth well and 50 µl discarded. Standardized inoculum was dispensed to wells 1 to 10th. MIC values were determined using a solution of ρ–iodonitrotetrazolium (INT) as an indicator compound. A colour change from yellow to purple indicated that a viable organism was still present. In contrast, maintenance of the yellow-indicated organism had been inhibited, thereby selecting the well with the lowest concentration as MIC.

Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration of the optimized extract was determined by pipetting 10 µl of MIC and higher concentrations into separate sterile Petri dishes; sterile nutrient agar was poured, swirled, and solidified. Petri plates were incubated at 37 °C for 18 hrs. MBC was the lowest extract concentration that eliminated the initial bacterial population, killing 99.9 % of

it and preventing bacterial growth from being seen on test plates.

Fractionation of Crude Extract and Thin Layer Chromatography (TLC)

An optimized extract of *Nigrospora oryzae* UILRZ1 was subjected to column chromatography using the standard method of Hamid et al. (2016) by making a slurry of extract with silica gel prepared with 100 % ethyl acetate. The extract of *Nigrospora oryzae* UILRZ1 was coated with silica gel (230–400 mesh, 60 Å) for loading onto the top of the column. Partial purification of extract using column chromatography over a silica gel in a stepwise gradient elution from ethylacetate: methanol (99:1 to 90:10) mixtures with increasing polarity was achieved. After column elution, all fractions were dried under reduced pressure in an evaporator at 45 °C. Using an optimum solvent system of ethyl acetate: methanol (4:1; v v^{-1}) and UV light with a wavelength of (280-315 nm), fractions were produced and then developed in TLC.

Direct TLC–bioautography Assay

Bioactivity of all fractions was conducted as described by Dewanjee et al. (2014) by using initially impregnated TLC plates with fractions of optimized extracts of *Nigrospora oryzae* UILRZ1 and sprayed with a standardized inoculum of selected bacterial isolates. Incubation was done for 18 hrs. TLC plates were overlaid with 10 µl of 2 mg mL^{-1} ρ–iodonitrotetrazolium (INT) to confirm the result, incubated for 1hr, and observed for colour change; purple colour indicated the presence of viable organism while maintenance of initial colour was an indication that organism had been inhibited.

Analysis of Chemical Components of extracts of Endophytic fungi

Fractions of optimized extract were subjected to GC-MS analysis using the method of Tonial et al. (2016). It was performed with QP2010 SE Shimadzu, Japan. The extract was solubilized in respective solvents (n-hexane, ethyl acetate, and methanol) to form a solution. GC–MS measurements were performed using a nonpolar capillary column Rtx-5MS (5 % diphenyl + 95 % dimethyl polysiloxane, 30 × 0.25 mm, 0.25 µm) operated under a temperature -programmed condition from 60 ºC to 250 ºC at 3 ºC per minute. The carrier gas was helium, with a 3.22 mL min^{-1} flow rate. The injection port was set at 250 ºC, with a volume of 1 µL in split mode (ratio 1:20). The Detection mass range was 45–700 m z^{-1}, ion source temperatures were 250 ºC, and interface temperature was 230 ºC. The electron impact ionization was 70 eV. Retention indices (RI) were calculated relative to a homologous series of n-alkanes (C9–C20). Identification was made by comparing retention indices (RI) and mass spectral patterns with those available in literature data and spectral library.

Data Analysis

Values expressed as mean ± SE and ANOVA performed with Tukey's multiple comparisons using SPSS 20. Principal component analysis biplot for most effective endophytes at a 95 % confidence interval ($P<0.05$) was performed using PAST software version 3.2.0. design of the experiment was analyzed using ANOVA of signal-to-noise ratio based on larger is better and model is given by $S/N = -10 * \log(10*\log(\Sigma(1/Y^2)/n)$

Where

Y = responses for given factor level combination, and

n = number of responses in factor level combination.

RESULTS

Colonization Frequency and Identification of Endophytic Fungi

Endophytic fungi colonized leaves and branches of plants to a different extent. Leaves and branches of *Ocimum gratissimum* and *Moringa oleifera* in

location B were highly colonized by endophytic fungi. There is no significant difference between the frequency of colonization of endophytic fungi on leaves of *O. gratissimum* in locations A and B. However, as shown in Table 3, there are notable variations in the frequency of endophytic fungus colonization on leaves and branches of *M. oleifera* in both locations, A and B. Figure 1 below shows the evolutionary relationship between endophytic fungi isolated from both plants with varying bootstrap values.

Table 3. Frequency of Colonization of Endophytic fungi on *Ocimum gratissimum* and *Moringa oleifera*.

Location	Plant	Mean	Std. Error
A	LO	4.40E+01	1.15E+00
	BO	5.53E+01	1.76E+00
	LM	2.20E+01	1.15E+00
	BM	5.60E+01	2.31E+00
B	LO	4.40E+01	1.15E+00
	BO	8.90E+01	1.73E+00
	LM	8.90E+01	1.73E+00
	BM	7.80E+01	1.15E+00

Key: LO; leaf of *Ocimum gratissimum*, BO; Branches of *Ocimum gratissimum*, LM; Leaf of *Moringa oleifera*, BM; branches of *Moringa oleifera*. A- Household garden and B- Farmland plantation (Values are means ± SE of three replicate).

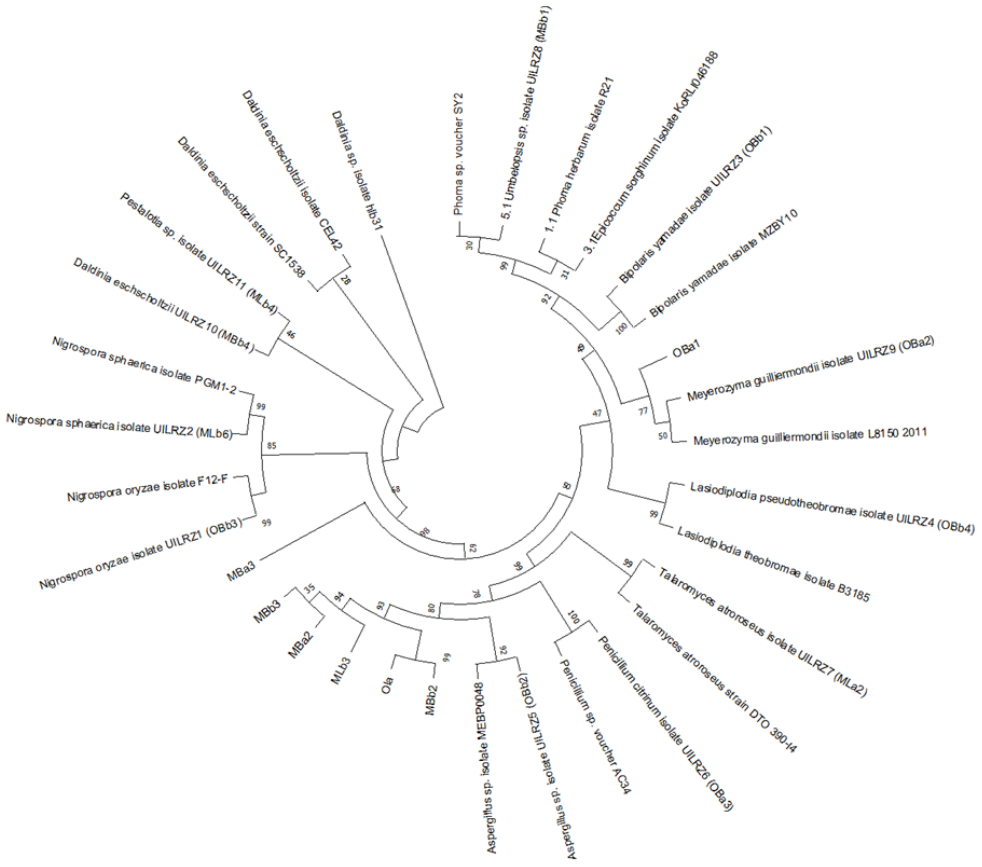

Figure 1. Evolutionary relationship of fungi from *Moringa oleifera* and *Ocimum gratissimum* with those on the database.
Key: ML-Moringa leaf, MB-Moringa branches, a- location 1, b- location 2, OB-Ocimum branch, OL-Ocimum leaf.

Antibacterial Properties of Isolated Endophytic Fungi

PCA biplot adopted in the screening of endophytic fungi against selected test microorganisms was for easy identification of endophytic fungi of significance by distributing them into four quadrants, having most effective farther from

the centroid, as seen in figures 2 and 3. The first quadrant consisted of OBa2, MBa3, MLb2, MBb1, MLb6, and OBa3, and the second quadrant had OBb1, OBb5 overlapping MBa1, MLa2, and OLa without inhibiting any of the test microorganisms. Quadrant 3 comprised eight endophytic fungi: MLb3, OBb4, OBa1, MLb5, OBb2, MBb2, and MBb3; overlapping MLb4 exhibited varying inhibition of three test microorganisms indicated in the quadrant's distribution. Similarly, quadrant 4 had four endophytic fungi: MBb4, OBb6, MBa2, and OBb3, with *A. baumanni*, *E. faecalis*, *K. pneumoniae*, *E coli*, and *P. aeruginosa* distributed in quadrant indicating similar antibacterial activity to test microorganisms.

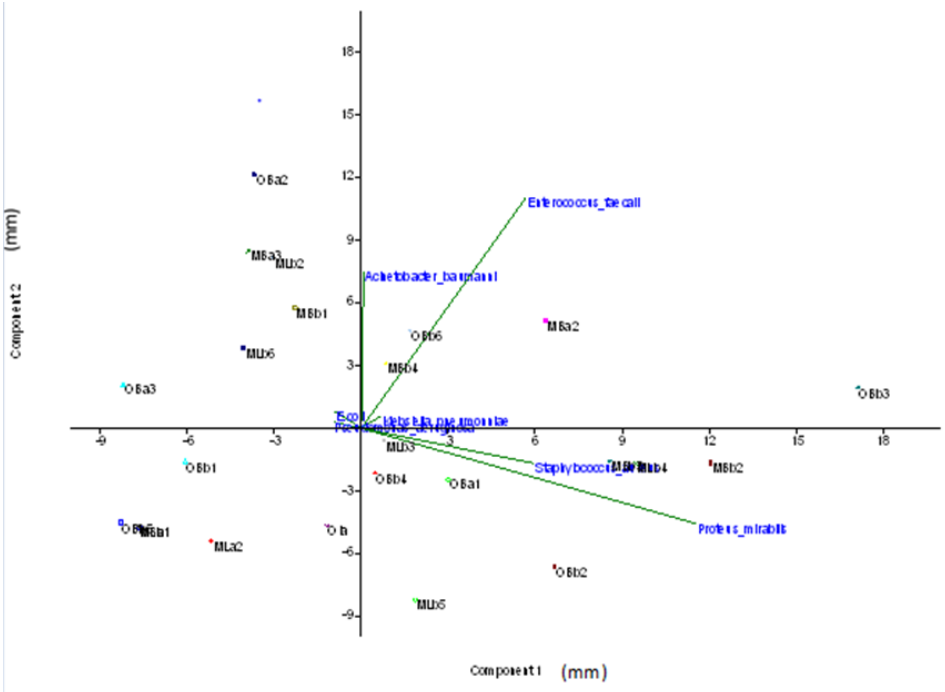

Figure 2. PCA Biplot of Antibacterial Potential oIsolated Endophytic fungi ($p<0.05$).
Key: ML-Moringa Leaf, MB-Moringa Branch, OL-Ocimum Leaf, OB-Ocimum Branch, a-Location 1, b-Location 2.

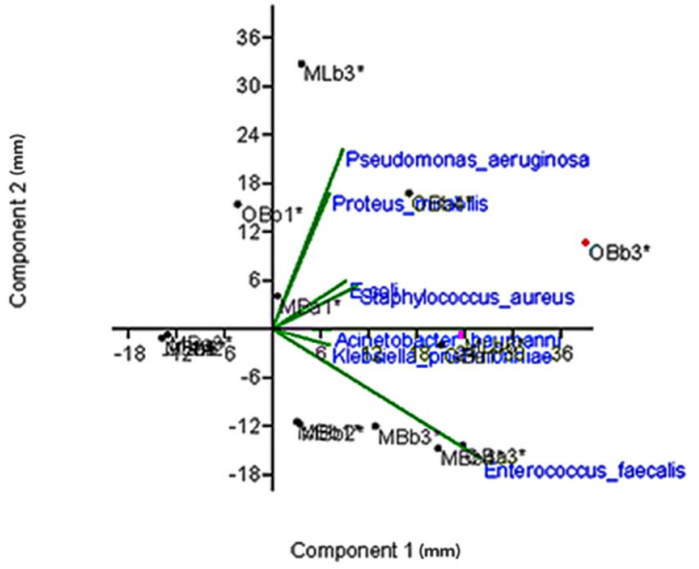

Figure 3. PCA Biplot of Antibacterial potential of extracts of Endophytic fungi ($p<0.05$).
Key: ML-Moringa Leaf, MB-Moringa Branch, OL-Ocimum Leaf, OB-Ocimum Leaf, a-Location 1, b-Location 2.

The lowest concentration of crude *Nigrospora oryzae* UILRZ1 extract inhibited all test microorganisms with MIC of 256 µg mL^{-1} except *Proteus mirabilis* at 512 µg mL^{-1}. At the same time, *K. pneumoniae* exhibited resistance to extract, as presented in Table 4.

Table 4. Minimum Inhibitory Concentration (MIC) of Crude extracts of *Nigrospora oryzae* UILRZ1.

Test Organisms	MIC (µg ml^{-1})
Escherichia coli	256
Staphylococcus aureus	256
Klebsiella pneumoniae	-
Acinetobacter baumanni	256
Pseudomonas aeruginosa	256
Enterococcus faecalis	256
Proteus mirabilis	512

-; not detected

Effect of Optimization on Production of Secondary Metabolites

Protein ranked first as most desirable in producing bioactive compounds that inhibited *A. baumannii* in Table S1 and *S. aureus* in Table S3 (Appendices). Figure S1 and S2 (Appendices) showed optimum conditions for the production of bioactive compounds using the mean of S/N ratio and levels considered in the design, with Days 6, pH 5, inoculum 4, and protein 3 being optimum conditions for the production of bioactive metabolites for both test bacteria. Table S2 (Appendices) shows an analysis of variance (ANOVA) of desirable factors, with pH having the highest percentage contribution against *A. baumannii*. In contrast, protein had the highest percentage contribution in producing bioactive metabolites against *S. aureus*, as indicated in Table S4 (Appendices).

Susceptibility patterns of test bacteria to optimized metabolites (Gentamicin as control) are presented in Table 5. *S. aureus* was more susceptible to optimized metabolite than control. The zone of inhibition of Gentamicin was higher for *A. baumannii* when compared with that of fungal extract.

Table 5. Comparison of Antimicrobial activities of Optimized *Nigrospora oryzae* UILRZ1 Metabolites with Conventional Antibiotics.

Test bacteria	Zone of Inhibition (mm) Optimized extract	Gentamicin (30µg)
Staphylococcus aureus	17.73±0.37	14.4±0.38
Acinetobacter baumannii	18.75±0.58	27.17±0.44

The predicted value for susceptibility of *Acinetobacter baumannii* to optimized *Nigrospora oryzae* UILRZ1 after analysis of Taguchi DOE was $22.83^{a} \pm 0.58^{a}$, while the experimental value after post-optimization was $18.75^{b} \pm 0.58^{b}$. the predicted value for susceptibility of *S. aureus* after analysis of Taguchi DOE was $18.58^{a} \pm 0.30^{a}$ while the experimental value after post-optimization was $17.73^{a} \pm 0.37^{a}$. two-sided test of equality for means reveals that values that do not share the same superscript differ significantly at $p < 0.05$.

The lowest concentration of optimized extract of *Nigrospora oryzae* UILRZ1 inhibited *Staphylococcus aureus* with MIC of 64 µg mL^{-1} while *A. baumannii* had its lowest at 128 µg mL^{-1}. The extract was bactericidal to both test organisms, as presented in Table 6.

Table 6. Minimum Inhibitory and Bactericidal Concentrations of Optimized *Nigrospora oryzae* UILRZ1 Extract.

Test bacteria	MIC (µg ml^{-1})	MBC (µg ml^{-1})
Staphylococcus aureus	64	128
Acinetobacter baumannii	128	128

Effect of Bioactivity of Fractions of *Nigrospora oryzae* UILRZ1 Extract

Test microorganisms, *S. aureus* and *A. baumannii*, were susceptible to fractions 4, 6, and 20, as shown in Table 7 and the inhibitory concentrations are shown in Table 8. Bioactivity was the basis for selecting three fractions for further study.

Chemical Constituent of Optimized extracts of *Nigrospora oryzae* UILRZ1

Tables 9-11 below are compounds obtained from GC-MS analysis of three fractions with the highest bioactivity.

DISCUSSIONS

This study shows that *Moringa oleifera* and *Ocimum gratissimum* were colonized by endophytic fungi at varying percentages. High colonization frequency in leaves from location B is similar to locations studied in works of Ramadhani et al. (2021). This similarity in location may be the reason for the high colonization frequency in leaves. In addition, Fontana et al. (2021) reported that microenvironments such as structural and chemical properties (thickness and nutrient composition) influence endophytic fungi's colonization rate. This assertion may be the reason for the high colonization frequency of leaves of *M. oleifera* in location B since the tree structure in this location had higher exposure to sunlight intensity than that of location A, which is canopy-like.

Meanwhile, the latter's thickness may also cause notable variation in the frequency of endophytic fungus colonization between *M. oleifera* and *O. gratissimum*. However, unlike findings from a study by De Padua et al. (2019) that identified that plants from the Catinga ecosystem with the least colonization rate had the best L-asparaginase activity, this study observed that plants with the highest colonization frequency had the best antibacterial activity, as seen in Figures 2 and 3. The presence of *Nigrospora* species in *M. oleifera* leaves agrees with the study of Abonyi et al. (2018), who have reported its isolation from leaves of *M.oleifera*. Likewise, the isolation of *Nigrospora* sp from *Ocimum gratissimum* in this study agrees with the finding of Atiphasaworn et al. (2017), who reported the isolation of *Nigrospora* sp from *Ocimum basilicum var. thyrsiflora*. In this study, branches of *Ocimum gratissimum* were colonized with *Lasiodiplodia pseudotheobromae*, which had earlier been reported from *Ocimum sanctum* Linn for its varying antimicrobial activities by Taufiq and Darah (2018).

Figure 1 shows the maximum likelihood of evolutionary relatedness of endophytic fungi from *Moringa oleifera and Ocimum gratisimmum*. The phylogenetic tree showed *Aspergillus* sp. isolate UILRZ5 (OBb2) and *Penicillium citrinum* isolate UILRZ6 (OBa3) clustered with existing species from the NCBI database at 95 and 100 % bootstrap values, respectively, indicating level of evolutionary relatedness. It also shows that seven of the amplified sequences of isolates had low-quality sequences even though all of them still had the same evolutionary root, except MBa3, which was a completely different entity as an outgroup. The strain of *Talaromyces* sp. isolated from a coffee plant by Sette et al. (2006) had a 99 % bootstrap value with *Talaromyces gossypii* (L14523), which exists in the NCBI database; this

aligns with what was obtainable in this study as *Talaromyces atroroseus* isolate UILRZ7 showed 99 % evolutionary relatedness with *Talaromyces atroroseus* strains DTO 390-I4.

Fernández-Pastor et al. (2021) reported a strain of a wheat endophytic *Nigrospora oryzae* to have 100 % similar sequence BLAST with *Nigrospora oryzae* ATCC 12,772 found in Genbank, which indicated they are genetically related. This study observed this similarity as *Nigrospora sphaerica* UILRZ2 had a 100% similar sequence BLAST in the Genbank. *Nigrospora oryzae* UILRZ1 had a 99 % similarity index indicating genetic relatedness with those in Genbank. Contrarily, Ramesha et al. (2020) reported *that Nigrospora sphaerica* isolated from *Adiantum philippense* L had a 45 % bootstrap value with previously deposited species in the Genbank database. *Nigrospora oryzae* UILRZ1 (OBb3) from the center axis indicated it was most effective against test microorganisms in primary and secondary screening. Meanwhile, dos Reis et al. (2022) reported that primary/qualitative screening is insufficient to select essential endophytic fungi.

Conversely, the outcome of fermentation and extraction of endophytes impacted bioactivity, as seen in Figure 3. Unsurprisingly, there was an

Table 7. Bioactivity of Fractions of Optimized *Nigrospora oryzae* UILRZ1 Extract.

Fractions																											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
S.aureus	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
A.baumannii	-	-	-	+	-	+	-	-	-	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	-	-

-; Not susceptible to fractions
+; susceptible to fractions

Table 8. Minimum Inhibitory Concentration of Fractions of *Nigrospora oryzae* UILRZ1 Metabolite (µg ml^{-1}).

Test bacteria	4	6	20
Staphylococcus aureus	64	64	128
Acinetobacter baumannii	128	256	128

Table 9. Chemical Constituent of Fraction 4 of *Nigrospora oryzae* UILRZI Metabolite.

Peaks	Retention time (Secs)	Compounds	Area (%)	Formula	Weight (µg)
1	10.480	n-Decanoic acid	0.75	$C_{10}H_{20}O_2$	172
2	12.049	Tetradecanoic acid, 12-methyl-, methyl ester	0.18	$C_{16}H_{32}O_2$	256
3	12.571	Dodecanoic acid	31.89	$C_{12}H_{24}O_2$	200
4	14.182	Tetradecanoic acid	12.20	$C_{14}H_{28}O_2$	228
5	15.358	Cyclopentanetridecanoic acid, methyl ester	0.38	$C_{19}H_{36}O_2$	296
6	15.602	n-Hexadecanoic acid	7.63	$C_{16}H_{32}O_2$	256
7	16.499	9-Octadecenoic acid (Z)-, methyl ester	0.86	$C_{19}H_{36}O_2$	296
8	16.623	Phytol	0.85	$C_{20}H_{40}O$	296
9	16.731	Oleic Acid	8.19	$C_{18}H_{34}O_2$	282
10	18.414	Heptadecanal	0.96	$C_{17}H_{34}O$	254

Table 9. Contd.

Peaks	Retention time (Secs)	Compounds	Area (%)	Formula	Weight (µg)
11	18.866	Octacosane	6.99	$C_{28}H_{58}$	394
12	19.938	Octacosane	10.75	$C_{28}H_{58}$	394
13	20.524	Di-n-decylsulfone	0.94	$C_{20}H_{42}O_2S$	346
14	21.183	Tetratetracontane	14.69	$C_{44}H_{90}$	618
15	22.218	.gamma.-Tocopherol	1.07	$C_{28}H_{48}O_2$	416
16	22.523	2,6-Lutidine 3,5-dichloro-4-dodecylthio-	0.99	$C_{19}H_{31}C_{12}NS$	375
17	22.755	Di-n-decylsulfone	0.68	$C_{20}H_{42}O_2S$	346

Table 10. Chemical Constituent of Fraction 6 of *Nigrospora oryzae* UILRZI Metabolite.

Peaks	Retention time (Secs)	Compounds	Area (%)	Formula	Weight (µg)
1	6.322	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-	0.14	$C_{10}H_{18}O$	154
2	6.747	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-	0.09	$C_{10}H_{18}O$	154
3	7.752	2,2-Dimethyl-3-vinyl-bicyclo[2.2.1] heptane	0.21	$C_{11}H_{18}$	150
4	7.940	Terpinen-4-ol	0.18	$C_{10}H_{18}O$	154
5	8.659	Benzene, 2-methoxy-4-methyl-1-(1-methylethyl)	0.06	$C_{11}H_{16}O$	164
6	9.174	4-[N'-(2-Methyl-benzoyl)-hydrazino]-4-oxo-butyric acid	0.14	$C_{12}H_{14}N_2O_4$	250
7	9.401	Thymol	29.55	$C_{10}H_{14}O$	150
8	9.543	Thymol	0.52	$C_{10}H_{14}O$	150
9	11.253	Caryophyllene	1.41	$C_{15}H_{24}$	204
10	11.603	Humulene	0.24	$C_{15}H_{24}$	204
11	11.682	Phenol, 3-(1,1-dimethylethyl)-4-methoxy-	2.58	$C_{11}H_{16}O_2$	180
12	11.931	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-	2.92	$C_{15}H_{24}$	204
13	12.023	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-	1.30	$C_{15}H_{24}$	204
14	12.234	Isoledene	0.62	$C_{15}H_{24}$	204
15	12.549	Dodecanoic acid	14.53	$C_{12}H_{24}O_2$	200
16	12.796	Caryophyllene oxide	1.59	$C_{15}H_{24}O$	220
17	13.016	17-Octadecynoic acid	0.19	$C_{18}H_{32}O_2$	280
18	13.516	cis-Z-.alpha.-Bisabolene epoxide	0.22	$C_{15}H_{24}O$	220
19	13.896	Cedran-diol, 8S,13-	0.30	$C_{15}H_{26}O_2$	238
20	14.175	Tetradecanoic acid	5.84	$C_{14}H_{28}O_2$	228
21	14.853	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.35	$C_{20}H_{40}O$	296
22	15.157	Decalin, 1-methoxymethyl-	0.25	$C_{12}H_{22}O$	182
23	15.269	Decalin, 1-methoxymethyl-	0.25	$C_{12}H_{22}O$	182

Table 10. Contd.

Peaks	Retention time (Secs)	Compounds	Area (%)	Formula	Weight (µg)
24	15.357	Cyclopentanetridecanoic acid, methyl ester	0.25	$C_{19}H_{36}O_2$	296
25	15.600	n-Hexadecanoic acid	3.71	$C_{16}H_{32}O_2$	256
26	16.492	Cyclopropanebutanoic acid, 2-[2-[2-[(2pentylcyclopropyl)methyl]cyclopropyl] methyl]cyclopropyl]methyl]-methyl ester	1.10	$C_{25}H_{42}O_2$	374
27	16.622	Phytol	0.41	$C_{20}H_{40}O$	296
28	16.678	5.alpha.-Pregn-16-en-20-one, 12.beta-hydroxy-acetate	0.46	$C_{23}H_{34}O_3$	358
29	16.733	Oleic Acid	3.74	$C_{18}H_{34}O_2$	282
30	17.226	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	1.06	$C_{20}H_{40}O$	296
31	17.723	3.beta.-Acetoxy-5-bisnorcholenic acid	1.18	$C_{24}H_{36}O_4$	388
32	18.838	.beta.-Sitosterol	2.58	$C_{29}H_{50}O$	414
33	19.017	Cholest-5-en-3-ol, 24-propylidene-, (3.beta.) -	4.38	$C_{30}H_{50}O$	426
34	19.318	.alpha.-Amyrin	1.95	$C_{30}H_{50}O$	426
35	19.767	1,2-Bis(trimethylsilyl)benzene	1.18	$C_{12}H_{22}Si_2$	222
36	19.931	Di-n-decylsulfone	2.09	$C_{20}H_{42}O_2S$	346
37	20.421	Cholan-24-oic acid, 3-(acetyloxy)-12-oxo-methyl ester, (3.alpha.,5.beta.)	0.99	$C_{27}H_{42}O_5$	446
38	20.651	Squalene	9.74	$C_{30}H_{50}$	410
39	21.172	Di-n-decylsulfone	1.69	$C_{20}H_{42}O_2S$	346

Table 11. Chemical Constituent of Fraction 20 of *Nigrospora oryzae* UILRZI Metabolite.

Peaks	Retention time (Secs)	Compounds	Area (%)	Formula	Weight (µg)
1	8.001	Octanoic acid	0.38	$C_8H_{16}O_2$	144
2	10.501	n-Decanoic acid	1.24	$C_{10}H_{20}O_2$	172
3	12.654	Dodecanoic acid	35.78	$C_{12}H_{24}O_2$	200
4	14.207	Tetradecanoic acid	12.00	$C_{14}H_{28}O_2$	228
5	15.613	n-Hexadecanoic acid	6.64	$C_{16}H_{32}O_2$	256
6	15.817	Ethyl 14-methyl-hexadecanoate	0.43	$C_{19}H_{38}O_2$	298
7	16.497	9-Octadecenoic acid (Z)-, methyl ester	0.37	$C_{19}H_{36}O_2$	296
8	16.735	Oleic Acid	6.46	$C_{18}H_{34}O_2$	282
9	16.900	9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione	2.64	$C_{11}H_{16}O_2$	212
10	17.781	2-methyltetracosane	0.25	$C_{25}H_{52}$	352
11	18.334	2-methyltetracosane	0.28	$C_{25}H_{52}$	352
12	18.412	14-Octadecenal	0.58	$C_{18}H_{34}O$	266

Table 11. Contd.

Peaks	Retention time (Secs)	Compounds	Area (%)	Formula	Weight (µg)
13	18.593	i-Propyl 9-tetradecenoate	0.29	$C_{17}H_{32}O_2$	268
14	18.866	Octacosane	3.00	$C_{28}H_{58}$	394
15	18.947	Bis(2-ethylhexyl) phthalate	1.67	$C_{24}H_{38}O_4$	390
16	19.127	Z-9-Pentadecenol	0.63	$C_{15}H_{30}O$	226
17	19.276	Ethyl 14-methyl-hexadecanoate	1.18	$C_{19}H_{38}O_2$	298
18	19.385	Sulfurous acid, pentadecyl 2-propyl ester	0.84	$C_{18}H_{38}O_3S$	334
19	19.940	Octacosane	6.52	$C_{28}H_{58}$	394
20	20.019	Tetracosanoic acid, methyl ester	1.90	$C_{25}H_{50}O_2$	382
21	20.402	Docosanoic acid, ethyl ester	1.35	$C_{24}H_{48}O_2$	368
22	20.524	Di-n-decylsulfone	1.37	$C_{20}H_{42}O_2S$	346
23	20.651	2H-3,9a-Methano-1-benzoxepin, octahydro-2	1.15	$C_{15}H_{26}O$	222
24	21.187	Tetratetracontane	9.85	$C_{44}H_{90}$	618
25	21.289	Hexacosanoic acid, methyl ester	1.35	$C_{27}H_{54}O_2$	410
26	21.760	Pentacosanoic acid, 2,10-dimethyl-, methyl	0.88	$C_{28}H_{56}O_2$	424
27	22.122	Z-6-Pentadecen-1-ol acetate	0.82	$C_{17}H_{32}O_2$	268
28	22.752	Di-n-decylsulfone	0.13	$C_{20}H_{42}O_2S$	346

increase in the bioactivity of extracts of endophytic fungi may be because Merlin et al. (2013) and studies by Pant et al. (2021) reported that the production of antimicrobial agents from endophytic microorganisms could be enhanced by fermentation, taking into consideration physical and chemical parameters. Likewise, Eid et al. (2019) reported that genetic and physicochemical parameters could influence the production of bioactive compounds. A recent study by Teimoori-Boghsani et al. (2020) also reported that endophytic fungi from the same plants could possess different bioactive metabolites due to differences in geographical location. Similarity or overlapping of different endophytic fungi from coordinates, as seen in Figures 2 and 3, confirms the spin-off hypothesis proposed by Kumara et al. (2014), Pang et al. (2021), Kumari et al. (2023) that piles of endophytic fungi produce same bioactive metabolites by employing genetic machinery of their host plant into its own irrespective of diverse taxonomy or phylogeny.

The nutritional components of the medium used to produce secondary metabolites are essential to increase their yield or activity (Singh et al. 2017; Méndez-Hernández et al. 2023). Yeast as a protein source used at varying concentrations for secondary metabolite production ranked as the most desirable factor that produced metabolites that inhibited *S. aureus* at 3 % (w v^{-1}) concentration as optimum. Comparatively, Verma et al. (2017) also reported that 3 g L^{-1} of yeast concentration in a medium yielded optimal bioactive metabolite from endophytic *Aspergillus* sp by classical optimization. Conversely, optimum bioactive metabolites were produced at a concentration of 2 g L^{-1} of seed oil cake as a nitrogen source in the work of Vellingiri et al. (2020). Against the findings in this study, which achieved optimum at day 6, optimum metabolite production was attained on day nine from a similar study

by Merlin et al. (2013). More so, the pH of optimum production of bioactive metabolites from this study was pH 5, which is contrary to the study by Vellingiri et al. (2020), which produced optimum bioactive metabolites at pH 7. The most influential conditions for optimum antibacterial activity were protein source and pH using L9 orthogonal array. These physicochemical parameters were earlier reported by Eid et al. (2019) to have influenced the synthesis of secondary metabolites. Statistical outcome of the design of experiment that exhibited a significant difference between predicted value and experimental value of susceptibility of *A. baumannii* may not be far from concerns of Geisinger et al. (2020) and Carcione et al. (2021) that urgent attention needs to be paid to pathogen as it is now a multidrug pathogen that needs to be considered in discovery and development of antibiotics while there was no significant difference in predicted and experimental value of susceptibility of *S. aureus* to optimized extract. Therefore, the Taguchi method DOE fits in discovering suitable compounds for inhibiting *S. aureus* but not for *A. baumannii*.

Partial purification of the optimized extract resulted in the detection of numerous compounds with antibacterial potential. Based on Taguchi's design of the experiment, interactions among physicochemical parameters influenced secondary metabolite production. Squalene detected as a chemical constituent in fraction 6 of *Nigrospora oryzae* UILRZ1 extract (Table 10) may have contributed to significant inhibition of *S. aureus* in this study. It has been reported by Martínez-Beamonte et al. (2020) to be effective in skin health as a moisturizer. Naphthalene derivatives, humulene, and caryophyllene oxide detected from fraction 6 of *N. oryzae* UILRZ1 extract (Table 10) have been reported to be detected as volatile bioactive compounds from endophytic *Phoma* sp. by Aamir et al. (2020) and Preethi et al. (2021). Detection of 4, 22-Stigmastadiene-3-one in fraction 20 of optimized extract (Table 11) can contribute significantly to the antibacterial activities exhibited by the extract. Singariya et al. (2013) reported that 4, 22-Stigmastadiene-3-one was detected among other bioactive components in ethyl acetate extract of *Cenchrus setigerus*.

Mishra et al. (2017) have reported that Tetradecane, pentadecane, and phthalic acid detected in fraction 20 of *N. oryzae* UILRZ1 extract (Table 11) have antimicrobial capability. Detection of tetradecanoic acid, an IUPAC name of myristic acid in all fractions, may also be the reason for the significant bioactivity of fractions against *Staphylococcus aureus*. Okukawa et al. (2021) reported that tetradecanoic acid is used in skincare. Also, hexadecanoic acid in three fractions is an IUPAC name for palmitic acid used for skin care. The abundance of two compounds in fractions can also be determined to be significant compounds responsible for the inhibition of *S. aureus*. A study by Okukawa et al. (2021) reported inhibition of *S. aureus* NBRC13276 by myristic and palmitic acid with MIC of 157 and 188 µg ml^{-1}, respectively. Likewise, Ji et al. (2021) reported the detection of myristic and palmitic acid in four species of *Hypericum* volatile oil to have antibacterial activities against *S. aureus* at MIC ranging between 118.32 ± 2.32 - 441.32 ± 2.75 µg ml^{-1}. Alpha amyrin detected from fraction 6 at 1.95 % area (Table 10) may also be presumed to contribute to the inhibition of *S. aureus* at MIC 64 µg ml^{-1} (Table 8). A similar result was also detected in an ethanolic extract of *Ocimum gratissimum* at 1.4 % area in a study by Chowdhury et al. (2021), which exhibited antibacterial activity against *Staphylococcus aureus* at MIC 50 mg ml^{-1}. Through optimization, *N. oryzae* UILRZ1 fractions contain a wide range of bioactive compounds, which may enhance the medium for secondary metabolites production.

CONCLUSIONS

This study established that endophytic fungi of antibacterial potential colonized two medicinal plants. Most importantly, *Nigrospora oryzae* UILRZ1 isolated from *O. gratissimum* was more effective against selected resistant pathogens. It further demonstrated that physicochemical interactions of process parameters in the classical optimization method, Taguchi DOE, allowed efficient synthesis of bioactive compounds that inhibited *Staphylococcus aureus*. Therefore, chemical constituents from the fractionated extract of *Nigrospora oryzae* UILRZ1 can be harnessed as antibacterial agents against infections caused by *S. aureus*.

LIST OF ABBREVIATIONS

UILRZ- University of Ilorin Rahmat Zakariyah

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AUTHOR'S CONTRIBUTIONS

R.F.Z., A.K.A., and R.N.A. designed the experiment. R.F.Z. did a literature survey. R.F.Z., A.K.A., R.N.A., and A.A.H. did the laboratory analysis. R.F.Z., R.N.A., and A.K.A. analyzed the results. R.F.Z. wrote the manuscript.

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APPENDICES

Table S1. Response for S/N Ratios for larger, better Optimized extract to *Acinetobacter baumannii.*

Source	DF	Seq SS	Contribution (%)	Adj SS	Adj MS	F-Value	P-Value
Inoculation Time(days)	2	40.006	11.42	40.006	20.003	6.83	0.128
pH	2	221.210	63.15	221.210	110.605	37.76	0.026
Protein	2	83.228	23.76	83.228	41.614	14.21	0.066
Error	2	5.858	1.67	5.858	2.929		
Total	8	350.302	100.00				

Table S2. ANOVA of the response of Susceptibility of *Acinetobacter baumannii* to Extract of *Nigrospora oryzae* UIL-RZ1.

Level	Inoculation Time (days)	pH	Inoculum (disc mm^{-1})	Protein (w v^{-1})
1	20.902	23.878	18.351	3.796
2	11.582	10.478	13.526	21.715
3	11.014	9.141	11.621	17.986
Delta	9.888	14.737	6.730	17.919
Rank	3	2	4	1

Table S3. Response for Signal to Noise Ratios to *Staphylococcus aureus.*

Level	Inoculation Time (days)	pH	Inoculum (disc mm^{-1})	Protein (w v^{-1})
1	19.449	21.804	16.276	1.722
2	11.582	10.157	13.205	21.395
3	11.014	10.083	12.562	18.927
Delta	8.435	11.721	3.714	19.673
Rank	3	2	4	1

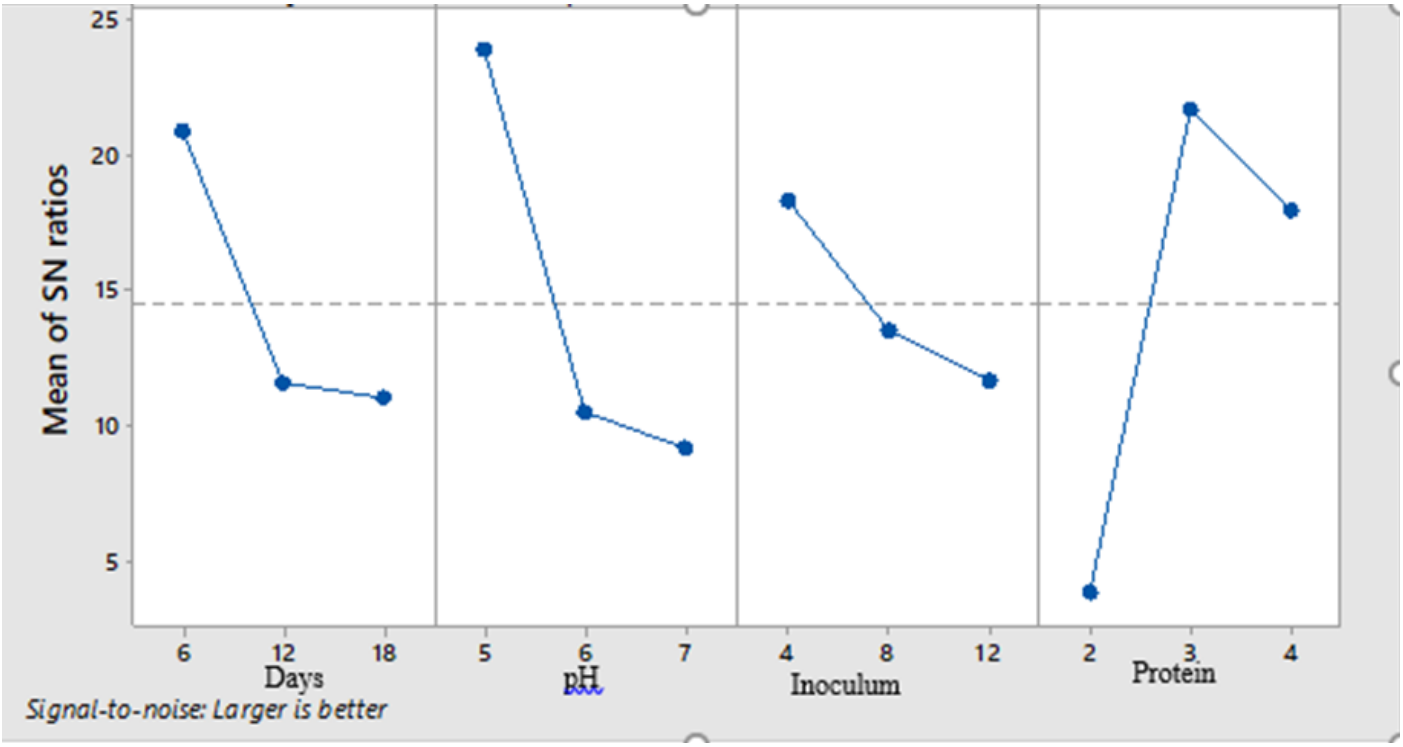

Figure S1. Response Susceptibility of *Acinetobacter baumannii* to Extract of *Nigrospora oryzae* UILRZ1.

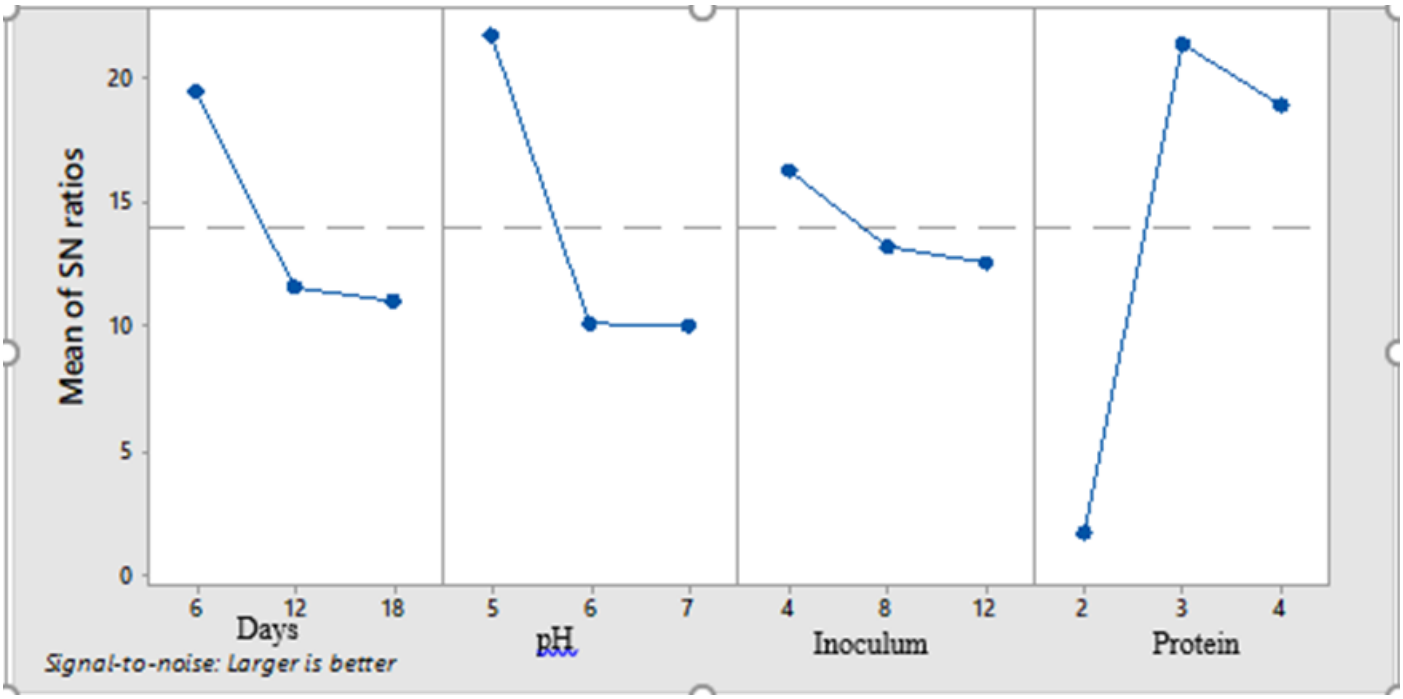

Figure S2. Response Susceptibility of *Staphylococcus aureus* to Extract of *Nigrospora oryzae* UILRZ1. Different levels of significant factors are on the horizontal axis.

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Research Article

The Distribution and Behaviour of Lesser Whistling-Duck (*Dendrocygna javanica)* at Lang Sen Ramsar site in Mekong Delta Vietnam

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ABSTRACT

This study aimed to investigate the distribution, behaviour, potential food, competitor, and potential predator of Lesser Whistling-Duck (*Dendrocygna javanica*) by direct observations at six sub-zones in the Lang Sen Ramsar site in the south of Vietnam, from September 2021 to August 2022. Two hundred sixty nine (269) individuals of the *D. javanica* were seen on site mainly in pairs and small flocks. The survey showed that Lesser Whistling-Duck often appears in areas with water lettuce and duckweed at sub-zone 9, wild rice and lotus fields at sub-zone 12, and low water level fluctuation at sub-zone 5 in the early morning and late afternoon. The food source of Lesser Whistling-Duck in the reserve Lang Sen are mainly plants (duckweed, water lettuce, young shoots or seeds of lotus, water lily, water hyacinth, and wild rice) and small animals (snail, worm, shrimp, fish, and insect). This bird species is relatively sensitive to environmental influences and their ability to perceive, reflect, and make sound depends on the size of flocks. The carnivores often damage the Lesser Whistling-Duck including black kite, greater coucal, lesser coucal, and python. They are also affected by competition for food and habitat of other waterbirds, activities of tourists, and people around the reserve. The result of observation of Lesser Whistling-Duck is a concern from new area, so it added the information on the distribution of the species, including behaviour, the potential food, competitors, predators, and human threats, which are important in managing the species.

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INTRODUCTION

Lang Sen Ramsar site, a wetland reserve, was recognized as the 2227th in the world and the 7th Ramsar site in Vietnam and has an area of 4,802 hectares in Tan Hung district, Long An province, which is situated in the Plain of Reeds of the Mekong Delta Vietnam (Hau & Tuyen 2017). Lang Sen is one of the eight important bird zones of Vietnam's freshwater wetlands, which regularly supports more than 20,000 waterbird individuals in the dry season (Ramsar 2016). The reserve has a high biodiversity with 68 zooplankton species, 261 plant species, 11 zoobenthos species, 87 fish species, 17 reptile species, 122 bird species, and 6 mammal species (Cuong 2015; Son et al. 2019). The biodiversity of this reserve has been affected by the hydrological regime of the Vam Co River, climate change (Triet et al. 2019), invasion of alien species, and human activities (Tho et al. 2018).

Lesser Whistling-Duck (*Dendrocygna javanica*) is a species of waterbirds in the genus Dendrocygna, order Anseriformes, family Anatidae (del Hoyo et al. 2014; Onwuka et al. 2020). Lesser Whistling-Duck is widely distributed throughout India, Nepal, Sri Lanka, Malaysia, Singapore, Indonesia, Myanmar, and Thailand (Rittiboon & Karntanut 2011; Chukwuemeka 2017; Zakaria et al. 2020). They mainly appear in flocks and live in freshwater wetlands such as ponds, lakes, and swamps with predominant vegetations and aquatic animals (Martins et al. 2017). This waterbird is an indicator organism for wetland ecosystems because they are more responsive to changes in plant composition and fluctuations in water levels than other animal species (Rajpar & Zakaria 2011).

Lesser Whistling-Duck is evaluated as the Least Concern (IUCN Red List 2022), so the monitoring and protection of this bird have not been focused, but the population of this waterbird is decreasing in trend (Bird Life International 2016; Martins et al. 2017). According to the annual bird monitoring results, the number of Lesser Whistling-Duck in Lang Sen Ramsar site decreased from about 4000 individuals in 2015 to 1600 individuals in 2019 and only about 300 individuals in March 2021 (Lang Sen Nature Reserve 2015, 2019, 2022). Although the species is not considered endangered, the information on the use of wetland is important for conservation efforts. Therefore, the study on distribution, behavior, and factors influencing *Dendrocygna javanica* populations in the Lang Sen Ramsar site was carried out to provide data for the management, conservation, and restoration of this waterbird.

MATERIALS AND METHODS

The survey was carried out from September 2021 to August 2022 at subzones 5, 6, 9, 10, 11, and 12, where *D. javanica* has appeared commonly in the last five years in Lang Sen Ramsar Site (Figure 1).

The distribution of Lesser Whistling-Duck in the reserve was surveyed using direct visual observation and point sampling method with binoculars (30 x 80) and photographed with a camera (Nikon-Coolpix P610). The survey was meticulously conducted twice daily, once in the morning (6:00-10:00 am) and once in the evening (4:00-6:30 pm), spanning four days each month. It was a comprehensive sub-zone, cross-sectional survey carried out through direct observation. The behaviours of waterbird were recorded including (1) activity and flock behavior, (2) feeding behaviours and food sources, (3) effect of other birds, and threats from human activities to this bird in the reserve, which were surveyed 4 days per month at the same time surveying of waterbird distribution.

The vegetation structure and water levels were diligently recorded once a month at the same sites where Lesser Whistling-Ducks had been sighted. The water level data was collected for the purpose of comprehending the im-

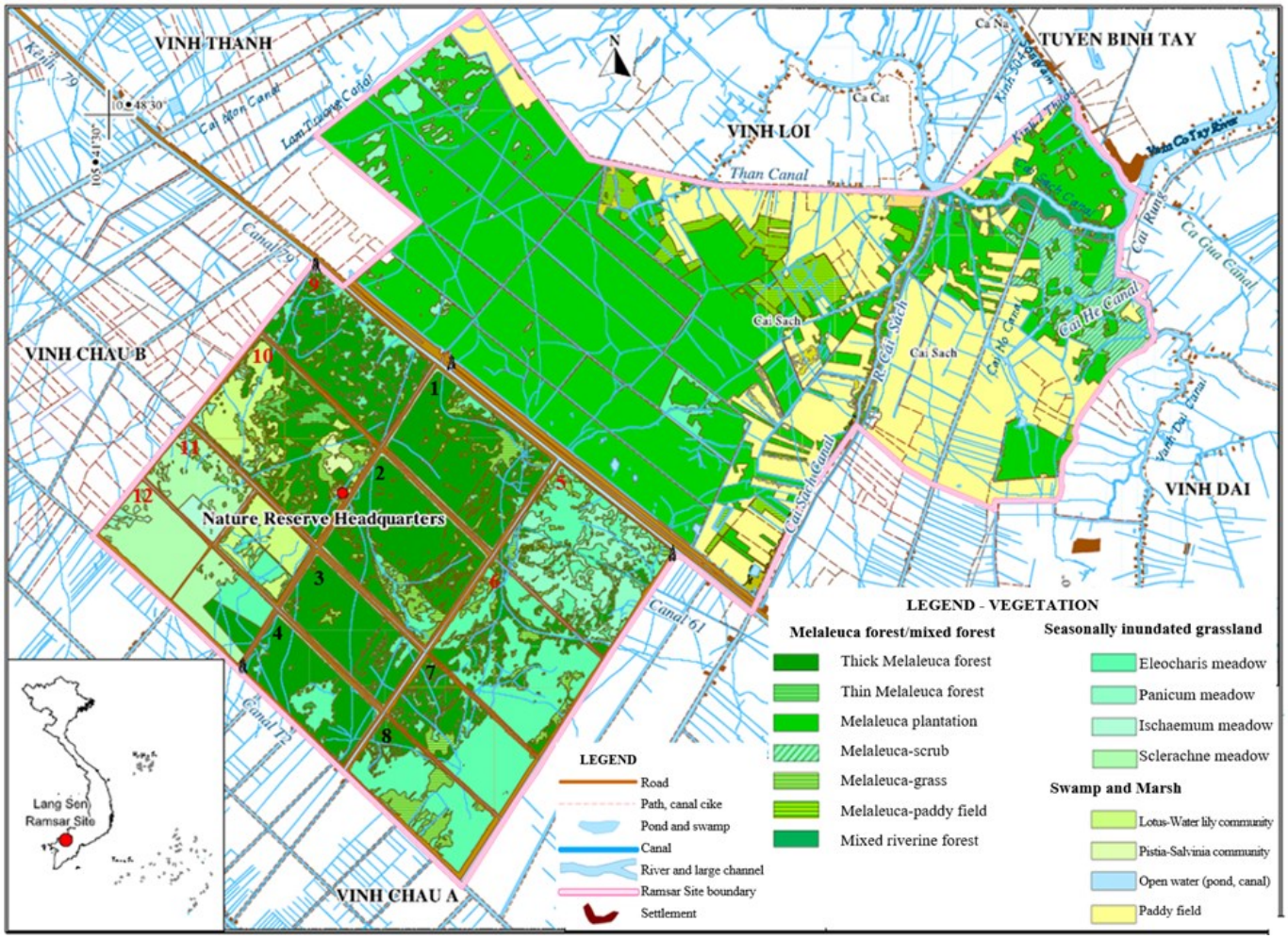

Figure 1. Sample point to survey Lesser Whistling-Duck in Lang Sen Ramsar site.

pact of water level fluctuations on the distribution of waterbirds and aquatic vegetation. The monitoring of water quality parameters such as temperature, pH, DO, EC (with AQUACOMBO HM 3070), TDS (with Hanna combo HI 98130); and climatic factors including temperature, air humidity (with ISO-Lab), and light intensity (with WalkLab Digital Lux meter) in the distribution areas of Lesser Whistling-Duck was conducted once a month at the same time surveying of waterbird distribution.

The data were summarized using Excel. The difference in an average number, the occurrence frequency of Lesser Whistling-Duck by time and location were examined using IBM SPSS statistics 22 software. Sigma Plot 12.5 software was used for making graphs.

RESULTS AND DISCUSSION

Distribution of Lesser Whistling-Duck in Lang Sen Ramsar site

The survey recorded a total of 269 individuals of Lesser Whistling-Duck from September 2021 to August 2022, at six sub-zones in the Lang Sen Ramsar site. The number of Lesser Whistling-Duck recorded has decreased approximately 15 times compared to 2015 and about 6 times compared to 2019 (Lang Sen Nature Reserve 2015, 2019). Lesser Whistling-Duck is also found in some wetlands in Vietnam such as U Minh Thuong National Park (Kien Giang Province), Tram Chim National Park (Dong Thap Province), Ca Mau Biosphere Reserve, and Cat Tien National Park (Dong Nai Province) (Thang 2011; Uyen et al. 2013; Duc & Dung 2020; Luong et al. 2022). The frequency of appearance Lesser Whistling-Duck during the survey period was about 46.2 %. They mainly appeared in pairs (2 individuals) with 18.8 %, followed by flocks of 3 individuals (5.0 %), and flocks of 15 to 57 individuals only from

1.3 % to 2.5 %. According to De Silva et al. (2015) and Salari et al. (2016), this bird species is commonly seen in large flocks often comprising several hundred individuals. However, these flocks tend to disperse into smaller groups consisting of mated pairs and families. The size of the flock and frequency of encountering the Lesser Whistling-Duck at Lang Sen Ramsar site are detailed in Figure 2.

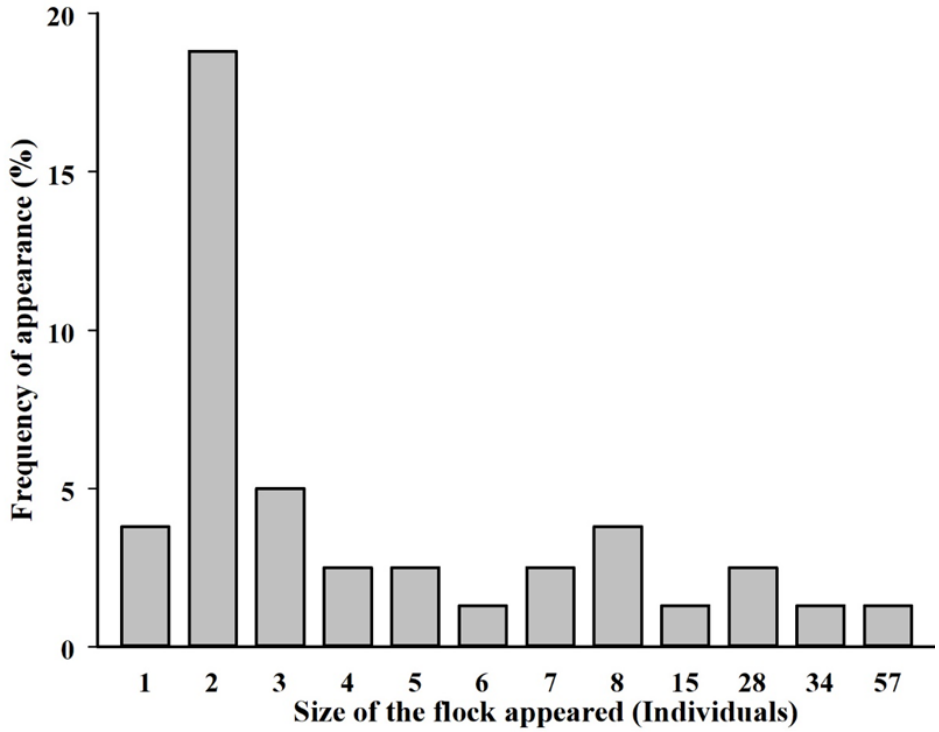

Figure 2. Size of the flock appeared and frequencies of appearance of Lesser Whistling-Duck at Lang Sen Ramsar Site.

The appearance of the waterbird species among the sub-zones is largely different. The frequencies of appearance of Lesser Whistling-Duck in sub-zones 9, 10, and 5 were 66.7; 58.3; and 52.9 %, respectively, which were higher than those in sub-zones 6, 11, and 12 with 33.3; 30.8; and 35.7 %, respectively. Besides, this spatial distribution also changed in the year, frequency of encountering Lesser Whistling-Duck in September 2021 was 83.3 %, followed by July at 66.7 %, November at 57.1 %, and in January, April, August, December at 16.7 % (Figure 3). In general, the frequencies of encountering and the number of Lesser Whistling-Duck in the sub-zones were correlated. There were 102 individuals in sub-zone 9 followed by sub-zone 12 with 56 individuals, sub-zone 5 with 43 individuals, and sub-zone 11 with 11 individuals (Table 1).

The survey showed that Lesser Whistling-Duck often appeared in areas with water lettuce (*Pistia stratiotes*) and duckweed (*Lemna minima*) at sub-zone 9 as well as wild rice (*Oryza rufipogon*) and lotus (*Nelumbo nucifera*) fields at sub-zone 12, besides water open area and water level fluctuation (sub-zone 5), because these areas will be favorable for them to swim, rest, feed, and hide. The remaining sub-zones are dominated by Melaleuca to obscure the view, the low open water area and high-water level fluctuation limited the distribution of the waterbird population. According to Salari et al. (2016) and Lazuardi et al. (2020), this waterbird preferred freshwater wetlands, which had abundant aquatic vegetation and aquatic invertebrates to feed. The distribution of Lesser Whistling-Duck was inversely proportional to the percentage coverage of the mixed species of spikerush (*Eleocharis dulcis*) and water lily (*Nympheae lotus*) as well as to the water level fluctuation (Salari et al. 2016). In

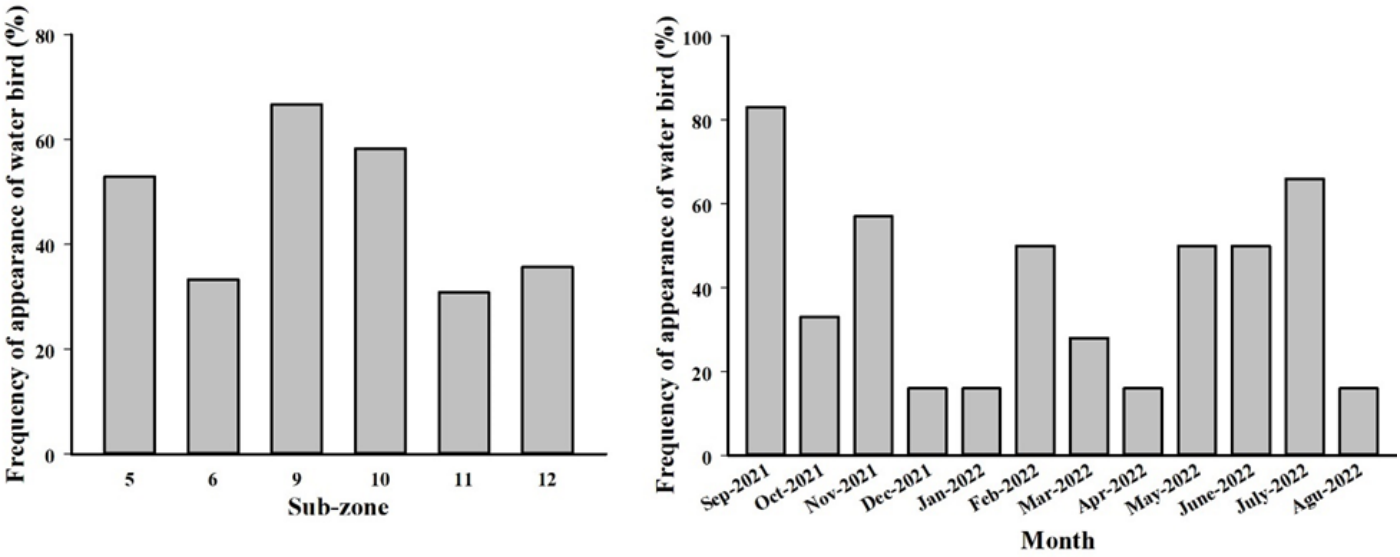

Figure 3. Frequencies of appearance of Lesser Whistling-Duck in sub-zones (left) and following months of the year (right) in Lang Sen Ramsar site.

cases of elevated water level, such as during heavy rain, the Lesser Whistling-Duck tends to leave (De Silva et al. 2015). Other studies have suggested that water depth is a significant factor influencing the selection of habitat for waterfowl. of habitat for waterfowl, so it directly affects the ability to approach prey (Koli et al. 2014; Sulaiman et al. 2018). Additionally, in polluted water bodies, high nutrient content, low dissolved oxygen content, and low pH contribute to changes in the habitat of waterfowl, leading to a decrease in their population (Kuruvilla 2016).

The distribution of Lesser Whistling-Duck also changed between times of day. Most of them were recorded from 8:30 a.m. to 9:30 a.m., mainly in sub-zone 9, with additional recording from 4:00 p.m. to 5:30 p.m. in sub-zone 6 and 10 (Table 2). Lesser Whistling-Duck foraged from 8-9 a.m, they often found shelter and avoided the hot weather but they were mainly observed in mild morning and afternoon sunshine (De Silva et al. 2015). The results of the study showed that 220 individuals of Lesser Whistling-Duck (accounting for more than 80 % of the total) were distributed and active in sunny conditions in the morning, while Lesser Whistling-Duck less appeared in the cool and cloudy weather in the afternoon. The influence of temperature on the distribution of Lesser Whistling-Duck was reported in the study of Mazumdar and Ghosh (2005) cited by Lazuardi et al. (2020), warm temperature will encourage this bird to fly and mate.

The survey results and correlation analysis of water quality parameters (temperature, pH, DO, EC, TDS); climatic factors (temperature, air humidity, light intensity), and the amount of Lesser Whistling-Duck showed that water quality and climatic factors also contributed to affecting the distribution of this waterbird. The number of Lesser Whistling-Duck appearing positively correlated to the pH of the water (correlation coefficient person of 0.457, $p < 0.05$), TDS (0.572, $p < 0.05$); but negatively correlated to water temperature (-0.739, $p < 0.01$) and water level fluctuation (-0.716, $p < 0.01$). The frequency of occurrence of Lesser Whistling-Duck also positively correlated to air temperature and light intensity with person correlation coefficients of -0.930 ($p < 0.01$) and -0.723 ($p < 0.01$), respectively. Therefore, water quality parameters such as dissolved oxygen, water depth, and salinity fluctuations can affect the presence of aquatic plants and animals in wetland ecosystems and limit the food sources for these birds (Hansson et al. 2010; Rajpar & Zakaria 2011).

Table 1. Appearance of Lesser Whistling-Duck in the sub-zones in Lang Sen Ramsar site.

Month	Sub-zone						Total of Lesser Whistling-Duck	
	5	6	9	10	11	12	Individuals	Ratio (%)
Sep-2021	26	2	2	2	2	45	79	29.4
Oct-2021	-	-	-	2	-	3	5	1.9
Nov-2021	4	5	-	-	4	-	13	4.8
Dec-2021	-	-	2	-	-	-	2	0.7
Jan-2022	-	-	2	-	-	-	2	0.7
Feb-2022	-	28	57	8	-	-	93	34.6
Mar-2022	-	-	28	3	-	-	31	11.5
Apr-2022	-	-	6	-	-	-	6	2.2
May-2022	7	-	3	1	-	-	11	4.1
June-2022	4	2	-	2	-	-	8	3.0
July-2022	-	-	2	2	5	8	17	6.3
Agu-2022	2	-	-	-	-	-	2	0.7
Total (Individuals)	43	37	102	20	11	56	269	
Ratio (%)	16.0	13.8	37.9	7.4	4.1	20.8		100

Table 2. Distribution of Lesser Whistling-Duck at time of day in sub-zones in Lang Sen Ramsar site.

Time	Sub-zone						Total of Lesser Whistling-Duck (individuals)
	5	6	9	10	11	12	
7-8h30 am	11	-	6	-	-	37	54
8h31-9h30 am	11	5	96	4	5	8	129
9h31-10h30 am	19	-	-	-	-	3	22
4h-5h30 pm	2	32	-	16	6	8	64
Total (individuals)	43	37	102	20	11	56	269

Characteristics of habitats Lesser Whistling-Duck in Ramsar Lang Sen site

The present study indicated that Lesser Whistling-Duck in Ramsar Lang Sen preferred wetlands habitats with open water surface with lotus (*Nelumbo nucifera*), wild rice (*Oryza rufipogon*), *Eleocharis dulcis*, *Hymenachne acutigluma*, others grass and shrubs, scattered melaleuca (*Melaleuca cajuputi*), and floating aquatic plants such as water lettuce (*Pistia stratiotes*), duckweed (*Lemna minor*). In these habitats, they can easily and quickly observe and detect threats from predators and humans. When Lesser Whistling-Duck detect danger, they often fly to other areas or swim and hide in emergent aquatic plants. Therefore, Lesser Whistling-Duck's activities in grassland and Melaleuca forest habitats were recorded flying and hiding (Table 3). Lesser Whistling-Duck is a gregarious waterbird, widely distributed in marshy swamps, freshwater wetlands with rich vegetation and animals (Zakaria et al. 2020) for their activities, so they are the most dominant species in open water (De Silva et al. 2015), accounting for 42.16 % of total species (Rajpar & Zakaria 2011). They are found resting on the banks, around lakes and water rice fields during the day (Baral et al. 2018). This species often builds nests in freshwater areas with dense vegetation to facilitate access to water and provide concealment and protection from predators for the young birds (Aarif & Babu 2010).

Lesser Whistling-Duck's *behaviors* in Ramsar Lang Sen site

Activity and flock behavior

Lesser Whistling-Duck in Lang Sen Natural Reserve is relatively sensitive to environmental impacts. During the 12 months of survey, most Lesser Whis-

tling-Duck was recorded flying up and flying to another area, or hiding in the grass, lotus field and shrub, and were recorded the Lesser Whistling-Duck foraging, swimming or resting in open water and grass habitats in the morning with 11 times. Because these birds mainly feed at night (De Silva et al. 2015; Baral et al. 2018); while they mainly preen, swim and flap their wings during the day (De Silva et al. 2015).

Lesser Whistling-Duck's responses to environmental influences depend on the number of individuals in the flocks. When seeing danger, the Lesser Whistling-Duck in large flocks often stops feeding and continuously observes surrounding and then they usually fly away, however in small flocks (1-8 individuals/flock), they often fly up or swim to hide (Table 4). Therefore, Lesser Whistling-Duck preferred to rest, swim, and feed in open water with grass, shrubs, and emergent plants around.

The survey results showed that the ability to detect the environmental impacts of Lesser Whistling-Duck also depends on the number of individuals in the flocks. In the large flocks, Lesser Whistling-Duck can detect humans at a distance of about 300 m. The results of Pearson correlation analysis showed that the number of individuals in the flocks had a very strong positive correlation with the distance of detecting human impact and flying away with a correlation coefficient r of 0.91 ($p < 0.01$). The reason may be that in large flocks, the members of the flock always take looking around when they are eating or resting, so they can detect threats easily and faster.

The results of the 12-month survey showed that the Lesser Whistling-Duck were mostly catching up from June to August, and their parents were looking for food with young birds in sub-zone 5 and sub-zone 10 from August to September. However, no nests were found except for one old nest (without eggs and juveniles) in the hollow of a melaleuca tree. According to the annual bird monitoring results in the Ramsar Lang Sen reserve, the natural breeding season of the Lesser Whistling-Duck mainly occurs from June to August. The number of their nests surveyed in the reserve has decreased in recent years. This may be due to a significant decrease in the number of Lesser Whistling-Duck in the reserve. The population of the Lesser Whistling-Duck may have been impacted by competitors, predators, and human activity, leading them to nest in more secluded areas far from their food sources.

Feeding behavior and food sources

The study also recorded that Lesser Whistling-Duck produces a wheezy sound while flying up, flying or feeding (Table 5), therefore they are called whistling duck. The behaviors of this waterbird in this study are similar to the study of Shaheer Ansari et al. (2017). The majority of Lesser Whistling-Duck makes vocalizations when flying up (54.5 % of times survey) and this behavior mainly occurs when they are active in pairs or small flocks of 1-8 individuals. They also make sound when flying, swimming, and feeding with large flocks (7-28 individuals/flock). In the survey, no instances were found of individuals shouting when feeding in pairs or small groups of 1-6 individuals (Table 5). The reason may be that they want to share information about food sources and danger to other individuals in flocks when operating in large herds, and silent to avoid threats from predators when foraging in small flocks.

Lesser Whistling-Duck is an omnivorous species, in Lang Sen natural reserve they feed in water habitats such as open swamps, water surfaces, floods, grass, and shrublands. Their food is mainly free-floating aquatic plants especially duckweed (*Lemna minor*), water lettuce (*Pistia stratiotes*), *Azolla pinnata*, *Wolffia schleidenii*, giant duckweed (*Spirodela polyrrhiza*), *Salvinia cucullata*; young shoots of water lily (*Nymphaea pubescens*, *Nymphaea indicum*), water hyacinth (*Eichhornia crassipes*), and young shoot and seed of emergent aquatic

Table 3. Distribution of Lesser Whistling-Duck in the studied habitats.

Habitat characteristics	Lesser Whistling-Duck's activities	Lesser Whistling -Duck (individuals)	Frequencies of Lesser Whistling-Duck (%)
Melaleuca forest, *Eleocharis dulcis* field	Fly across the survey area	112	43.3
Open water surface, lotus, *Eleocharis dulcis* field	Fly up	61	27.0
Open water surface with duckweed, dusty grass, *Eleocharis dulcis*, scattered melaleuca	Swim, stand, and rest	8	10.8
Open water surface, lotus, wild rice field	Feed	86	16.2
Grassland	Land and hide	2	2.7

Table 4. Reaction of Lesser Whistling-Duck when affected by humans and changes in environmental factors.

Lesser Whistling-Duck's activities	Size of flock (individuals)	Total Number of Lesser Whistling-Duck (individuals)	Frequency of total individuals (%)
Fly up	1-8	25	50.0
Swim, hide	2-8	12	21.4
Whistling, swimming, looking for a place to hide	2-34	36	14.3
Silence, constantly observing your surroundings	3-57	60	14.3

Table 5. Lesser Whistling-Duck made a wheezy sound.

Activities when made a wheezy sound	Size of flock (Individuals)	Total of Lesser Whistling-Duck (Individuals)	Ratio (%)
Flying up	1-8	19	54.5
Flying	2-28	37	27.3
Swim and feed	34-57	91	18.2

plants consist of lotus (*Nelumbium nucifera*), wild rice (*Oryza rufipogon*), *Hymenachne acutigluma*, *Pseudoraphis brunoniana*, *Eleocharis dulcis*, *Panicum repens*, and *Ischaemum hirtum*. The animal food sources include aquatic invertebrates such as mollusks (snails), worms, crustaceans (small shrimp), and aquatic vertebrates such as small fish and insects (Table 6). The number of Lesser Whistling-Duck had a positive correlation between species and genus diversity of aquatic plants with person correlation coefficients of 0.584 and 0.591, respectively ($p < 0.05$). However, the high density of emergent plants and invasive floating macrophytes (water hyacinth (*Eichhornia crassipes*), water lettuce (*Pistia stratiotes*)), affected the free water surface for these waterbird activities (swim, feed), so it also limited the appearance of the waterbird populations. In addition, the rapid decline of the Lesser Whistling-Duck population is also one of the reasons leading to the increase in density and area of two invasive aquatic plant species: water lettuce and water hyacinth in these areas. This expansion is particularly noticeable in the open water surface in sub-zones 9 and 10 of the Ramsar Lang Sen site. This highlights the crucial role of protecting the brown leek population in preserving biodiversity and maintaining the ecological balance of the reserve.

Table 6. Food sources of Lesser Whistling-Duck in Lang Sen Ramsar site.

Lesser Whistling-Duck's food at Lang Sen	Size of flock (individuals)	Ratio of Lesser Whistling-Duck /total number of Lesser Whistling-Duck foraging surveyed (%)
Water lettuce	1	0.7
Water lettuce, snail	10	7.2
Water lettuce, wild rice, snail	3	2.2
Lemna minor, water lettuce, *Panicum repens*, small fish, small shrimp, insect	2	1.4
Salvinia cucullata, seed of lotus, *Ischaemum hirtum*, water lily, small fish, Small shrimp	34	24.6
Lemna minor	5	3.6
Lemna minor, *Wolffia schleidenii*, water lettuce, snail	59	42.8
Lemna minor, snail	8	5.8
Small fish	2	1.4
Small fish, small shrimp	7	5.1
Small fish, snail	7	5.1
Total	138	100

Threats to the Lesser Whistling-Duck in Lang Sen Ramsar Site

In addition, environmental factors such as weather, food sources, and habitat structure, Lesser Whistling-Duck in Lang Sen natural reserve was also affected by carnivores such as black kite (*Milvus migrans*) and python (*Python molurus*), greater coucal (*Centropus sinensis*), lesser coucal (*Centropus bengalensis*), some snakes species, which can hunt and eat young and adult birds and their eggs. Morever, Lesser Whistling-Duck is also competed for habitat and food with waterbirds such as Indian spot-billed duck *(Anas poecilorhyncha)*, little egret *(Egretta garzetta)*, little cormorant (*Phalacrocorax niger)*, Asian openbill *(Anastomus oscitans)*, western swamphen *(Porphyrio porphyrio)*, oriental darter (*Anhinga melanogaster*), and pheasant-tailed jacana (*Hydrophasianus chirurgus*). Their populations in Lang Sen are large and active in flocks, making noises and competing for food with the Lesser Whistling- Duck. The Lesser Whistling-Duck often flies away or hides in shrubs and grass. The Lesser Whistling- Duck in Lang Sen Natural Reserve is also very sensitive to human impacts such as noise from travel, tourism, agricultural production (pesticides, equipment) and illegal hunting of people around the reserve. Therefore, it is essential to implement solutions to safeguard the environment and minimize the impact on the Lesser Whistling-Duck population at Ramsar Lang Sen.

There was no evidence found of residents encroaching on the conservation area to hunt *D. javanica*. However, nearby residents are using devices that mimic the calls of *D. javanica* to lure them into the paddy fields and then capture them using nets. This hunting activity typically takes place in the first month of the rice crop. This may be one of the main reasons leading to the rapid decline of the Lesser Whistling-Duck population in the Lang Sen Ramsar site in recent times. Therefore, there is a need for educational solutions to raise awareness among the local community about the conservation of this waterbird species.

CONCLUSION

In the study, 269 individuals of Lesser Whistling-Duck (Dendrocygna javanica) were recorded at Lang Sen Ramsar Site with mainly in pairs and small flocks. Most Lesser Whistling-Duck appeared in sub-zone 9 (102 individuals) and in February (93 individuals) mainly at light sunshine of the day. They often swim, feed and rest in open water surrounding lotus, grass, shrubs or scattered melaleuca where support food source and shelter. Their food source of Lesser Whistling-Duck in the reserve includes plants and animals. This bi-

rd is quite sensitive to environmental influences, their ability to perceive, reflectivity and make sound depends on the size of the flocks. Their carnivores are black kite, greater coucal, lesser coucal, and python. They were also affected by competition for food and habitat of other waterbirds, activities of tourists and people around the reserve. Therefore, protecting the living environment and limiting harmful species and human activities should be paid more attention.

AUTHOR CONTRIBUTION

L.D.K. designed the research and supervised all the process, analysed the data and wrote the manuscript; P.Q.N. collected and analysed the data and wrote the manuscript; N.T.L collected the data; N.T.G. collected and analysed the data.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Exploration the Potency of Copper and Dyes Multi-Resistant of Indigenous Bacteria Isolated from Cikijing River, West Java

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ABSTRACT

Various types of textile dye have been reported to contaminate the Cikijing River, West Java, Indonesia due to its location within the industrial region of Rancaekek District. It has been understood that certain bacterial species develop copper resistance and dye decolourisation as a mechanism of stress adaptation. The study aims at isolating and characterising copper and dye resistance as well as decolourisation ability of bacteria isolated from the Cikijing River. Copper-resistant bacteria were isolated using a series dilution method on Luria Bertani media supplemented with the addition of 1-10 mM $CuSO_4$. Purified bacterial isolates were then tested for copper resistance onto LB agar medium supplemented with $CuSO_4$ concentrations ranging from 0 mM to 20 mM and decolourisation of various dyes. A total of 59 copper-resistant bacteria were successfully isolated, nine of them showed the highest copper resistance with a MIC value from 11 mM up to 16 mM $CuSO_4$ and resistance to 4 types of dyes up to 700 ppm. The 16S rDNA analysis showed that the nine isolates were *Klebsiella* sp., *Klebsiella pneumoniae*, *Lysinibacillus boronitolerans*, *Lysinibacillus fusiformis*, *Bacillus proteoliticus*, *Pseudomonas stutzeri*, *Klebsiella variicola*, *Citrobacter freundii*, and *Klebsiella variicola*. Out of nine isolates, five were found resistant to 5 mM $CuSO_4$ and decolourise Methylene Blue, Congo Red, and Basic Fuchsine dyes at a maximum concentration of 700 ppm.

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INTRODUCTION

Cikijing River is a densely populated industrial area located in the Rancaekek District of West Java, Indonesia. Categorised as a tributary of Citarik and Citarum Rivers that flow through 13 cities, the Cikijing River is an important water source for residential and industrial regions, agriculture irrigation, and hydroelectric turbines (Cavelle 2013; Pantjawati et al. 2020; Riyadi et al. 2020). However, the Cijiking River has an alarmingly high level of pollution due to rapid population growth and anthropogenic activities such as farming and product manufacturing (Prananda et al. 2017). Since Rancaekek District is the main centre for textile industries, Cikijing River is known to be contaminated with a variety of heavy metals and synthetic dyes on the sediments (Fadhilah et al. 2018). Deposition and accumulation of heavy metals in river sediments may infiltrate the aquatic food chain, potentially leading to bioaccumulation and biomagnification. Heavy metals that are toxic may also disrupt the growth and survival rate of terrestrial plants. Several toxic heavy metals that have been found circulating Cikijing River include cadmium, chromium, copper, zinc, mercury, arsenic, and lead (Septiono et al. 2015). Cikijing River contains a significantly high copper concentration (0.0233 ppm) that surpasses the international limit (0.014 ppm) (Mahardika & Salami 2012). As a transition metal, copper is an essential micronutrient and cofactor for all living organisms at low concentrations, but detrimental at high concentrations (Zeng & Han 2020). Copper waste is commonly generated through industrial, agricultural, and aquacultural activities (Argudín et al. 2019). Due to its non-biodegradability, copper is a human and environmental health hazard. Extended exposure to copper may result in allergic rhinitis, hyper lacrimation, hypersalivation, and photophobia, while chronic exposure leads to Wilson's Disease which is characterised by a Kayser-Fleischer ring caused by copper accumulation inside the cornea. Nervous and gastrointestinal (especially liver and kidney) disorders may also result from copper toxicity (Karim et al. 2018).

Synthetic dye is a toxic and reactive compound that creates environmental imbalances. Textile, paper printing, colour photography, pharmaceutical, cosmetic, and food industries are among the most frequent synthetic dye users (Tkaczyk et al. 2020). Dye-contaminated wastewater is difficult to degrade and detoxify due to the complex chemical structure of dyes (Lu et al. 2009). The complexity of dyes is determined because of their diverse functional group and aromatic system, dyes typically contain multiple group functions that contribute to their chemical behaviour and interaction with substrates, while the aromatic system gives dye structure the ability to decolonize electrons which make the structure stable. Dye-covered water surfaces prevent sunlight from entering, causing the increase of biochemical oxygen demand, decreasing photosynthetic activity, and disrupting aquatic biota growth.

Cikijing River needs to be immediately treated to minimise its level of copper and dye contamination. Conventional physical and chemical treatment methods are ineffective as they are high costs and produce secondary waste (Ayangbenro & Babalola 2017). In contrast, bioremediation is an efficient, cost-effective, and eco-friendly biological method that utilises microorganisms to remove environmental contaminants (Palanivel et al. 2020). Bacteria are pervasive microorganisms that can be found in almost any setting, including copper- and dye-contaminated areas (Cocconcelli & Fontana 2014). Certain bacterial species are equipped with morphologically and metabolically advantageous features that allow them to develop copper resistance and dye decolourisation mechanisms as an adaptive response to cellular stress. Previous studies demonstrate that various types of bacterial species, such as *Siccibacter colletis*, *Acinetobacter baumanii*, *Bacillus cereus*, and *Escherichia coli* isolated

from Citarum River in Indonesia are capable of tolerating copper and dye toxicity (Irawati et al. 2023). Therefore, an investigation to discover bacterial species capable of resisting copper and dye, as well as decolorizing dye, is imperative. Accordingly, this study was aimed at: 1) Identifying of multi-dye resistant bacteria, 2) measuring the copper resistance of selected isolates, and 3) analysing the dye-resistance and dye-degrading abilities of selected isolates on 4 types of commonly used textile dye.

MATERIALS AND METHODS

Water Sampling

Water samples were obtained from Cikijing River in West Java, Indonesia, around the industrial area of PT. Kahatex. Random sampling was performed at three different location points close to the textile factory waste disposal site. The first sample was taken from under a metal bridge, the second sample was approximately 10 meters from the first sampling scene, and the third sample was approximately 5 meters from the second sampling location. Samples were stored inside a sterile bottle before being brought to laboratory for further research.

Bacterial isolation and purification

Copper-resistant bacteria were isolated by cultivating water sampling on Luria Bertani (LB) Agar supplemented with $CuSO_4$ with serial dilution. The stock of 1M $CuSO_4$ was added to an autoclaved medium with various concentrations. Approximately 100 µl of each water sample was spread onto LB agar (25 g L^{-1}) supplemented with the addition of 1-10 mM $CuSO_4$ and was incubated at 37 °C for 48 hours. Each sampling was done in duplicate, with a non-copper-supplemented medium prepared as a negative control.

Bacterial colonies that appeared after the incubation with unique morphological characteristics were purified, then sub-cultured on LB agar medium supplemented with the same $CuSO_4$ concentrations used during the initial culture. Cultures were incubated at 37 °C for 48 hours before undergoing next round of purification for preservation.

Copper-resistance determination assay

Minimum Inhibitory Concentration (MIC) was determined by streaking one full loop of bacterial isolate onto LB agar medium supplemented with $CuSO_4$ concentrations ranging from 0 mM to 20 mM (Irawati et al. 2023). Each assay was repeated four times and incubated at 37 °C for 48 hours. The highest copper concentrations with no observed bacterial growth were noted as the MIC of each isolate. Bacterial isolates that grew on the highest $CuSO_4$ concentration were selected for further investigation.

Dye resistance assay

Bacterial isolates were inoculated onto LB agar medium supplemented with various concentrations of textile dye. Twelve textile dye variants were used for the dye-resistance and decolourisation assay, namely Methylene Blue (MB), Malachite Green (MG), Congo Red (CR), Mordant Orange (MO), Reactive Black (RB), Direct Yellow (DY), Basic Fuchsine (BF), Reactive Orange (RO), Disperse Orange (DO), Remasol (R), Wantex Red (WR), and Wantex Yellow (WY). The stock solution of 10,000 ppm dyes was diluted in sterile aquadest and was sterilised with a filter membrane of 0.20 µM. Each dye was added to the autoclaved LB medium up to the appropriate concentration. The dye concentration used for dye resistance and decolourisation essays ranged from 100-1000 ppm (Irawati et al. 2023). Each assay was done in quadruple and incubated at 37 °C for 48 hours. The highest dye concentrations with no observed bacterial growth were noted as the MIC of each isolate. Bacterial

isolates that grew on the highest dye concentration were selected for further investigation. Decolourisation was observed based on the formation of clear zones around bacterial colonies.

Copper and dye multi-resistance assay

Copper and dye multi-resistance of the bacterial isolates were determined based on growth observations. Bacterial isolates were inoculated onto LB agar medium supplemented with 5 mM of $CuSO_4$ and either 200 ppm, 500 ppm, 600 ppm, or 700 ppm of Methylene Blue (MB), Malachite Green (MG), Congo Red (CR), or Basic Fuchsine (BF) dye (Irawati et al. 2023). Bacterial growth was observed after an incubation period of 37 °C for 48 hours.

Molecular identification of copper and dye-resistant bacteria

Selected bacterial isolates were first inoculated into Nutrient Broth agar medium (13 g L^{-1}), and then isolated using the TIANamp Genomic DNA Kit (Tiangen). DNA concentration and purity were confirmed using a Nanodrop™ 2000 Spectrophotometer (Thermo Fischer Scientific). Bacterial 16S rDNA amplification was performed by Polymerase Chain Reaction with a master mix of 25 µl volume consisted of the following: 12.5 µl of GoTaq® Green (Promega), 1 µl of forward primer (5'-CGCCTGTTTAACAAAAACAT-3'), 1 µl of reverse primer (5'CCGGTCTGAACCAGATCATGT-3'), 2 µl of DNA template, and 8.5 µl of nuclease-free water. Visualization of PCR product was done using gel electrophoresis. Results of the electrophoresis were observed under a UV transilluminator. DNA sequencing results were edited using the ChromasPro 2.6.2 (Technelysium) followed by homology search using the Basic Local Alignment Search Tool (BLAST) on http://www.ncbi.nlm.nih.gov.

RESULTS AND DISCUSSION

Copper-resistant bacteria isolated from Cikijing River

Fifty-nine copper-resistant bacteria have been successfully isolated from Cikijing River. Table 1 shows that all copper-resistant bacteria isolated have copper resistance with the MIC values from 1 until 16 mM $CuSO_4$. Irawati et al. (2020) reported that bacteria with a MIC value of more than 4.7 mM were categorized as very resistant. Among the 59 copper-resistant isolates, 58 isolates showed the highest MIC value of 6-16 mM and only 1 isolate CKJ 0.3.1 which has a MIC value of 1 mM, therefore it was concluded that most of the bacterial isolates had high resistance to copper. Four bacterial isolates (CKJ 300 1.2, CKJ 300 3.1, CKJ 500 2.1.2, and CKJ 500 2.2) had higher copper resistance than the previous studies on copper-resistant bacteria isolated from copper-polluted areas in Indonesia. Previously, it was reported that the MICs of bacterial isolates from Cikapundung and Cisadane River in West Java were up to 6-8 mM (Nurlaila et al. 2020), Kapuas River in Central Kalimantan up to 7 mM (Irawati et al. 2022), Citarum River in West Java up to 10 mM (Irawati et al. 2023), and Kemisan River in Banten Province tolerated up to 10 mM (Irawati et al. 2017).

Resistance to copper and dyes

Nine selected bacterial isolates demonstrated varying resistance to the four types of dyes, with some isolates showing resistance up to a maximum concentration of 700 ppm. These isolates are shown in Table 2.

Multi-resistance testing was conducted on selected copper-resistant bacteria previously cultured on LB agar media supplemented with 5 mM of $CuSO_4$ and various dyes (MB, MG, CR, and BF) with concentrations ranging from 200 ppm to 700 ppm. Out of the nine selected bacterial isolates, five isolates (CKJ 300 1.2, CKJ 500 2.1.2, CKJ 1000 2.2, CKJ 1000 3.1.1, and CKJ

Table 1. Determination results of copper-resistant bacterial isolates from Cikijing River.

No.	MIC value (mM)	Isolate Codes
1.	1	CKJ 0 3.1
2.	6	CKJ 0 1.1; CKJ 0 2.1; CKJ 0 2.2; CKJ 0 3.2; CKJ 200 1.2.2; CKJ 200 3.1; CKJ 400 1.2; CKJ 500 1.2; CKJ 500 2.1; CKJ 500 3.1
3.	7	CKJ 600 2.1; CKJ 600 3.1
4.	8	CKJ 700 1.1; CKJ 700 1.2; CKJ 700 2.1; CKJ 700 3.1; CKJ 700 3.2
5.	9	CKJ 200 2.2; CKJ 400 3.2; CKJ 800 1.1; CKJ 800 1.2; CKJ 800 3.1; CKJ 800 3.2
6.	10	CKJ 400 2.1; CKJ 900 1.1; CKJ 900 1.1.2; CKJ 900 1.2; CKJ 900 1.2.1; CKJ 900 2.1; CKJ 900 2.1.1; CKJ 900 2.2; CKJ 900 2.2.1; CKJ 900 3.1; CKJ 900 3.2
7.	11	CKJ 200 1.1; CKJ 200 1.2; CKJ 200 2.1; CKJ 200 3.2; CKJ 300 2.2; CKJ 300 3.2; CKJ 400 2.2; CKJ 500 1.1; CKJ 500 3.2; CKJ 1000 1.1; CKJ 1000 1.1.1; CKJ 1000 1.2; CKJ 1000 2.1; CKJ 1000 2.1.1; CKJ 1000 2.2; CKJ 1000 2.2.1; CKJ 1000 3.1; CKJ 1000 3.1.1; CKJ 1000 3.2; CKJ 1000 3.2.1
8.	16	CKJ 300 1.2; CKJ 300 3.1; CKJ 500 2.1.2; CKJ 500 2.2

1000 3.2.1) were found to be resistant to 5 mM $CuSO_4$ and capable of decolorizing dyes (MB, CR, and BF) at the highest concentration of 700 ppm.

Table 2. Resistance of bacterial isolates to copper and dyes.

No.	Isolate Codes	Maximum concentration of Dye Type (ppm)			
		MB	CR	BF	MG
1.	CKJ 300 1.2	700	700	700	-
2.	CKJ 500 2.1.2	700	700	700	-
3.	CKJ 500 2.2	700	500	500	-
4.	CKJ 1000 1.1	700	500	500	-
5.	CKJ 1000 1.2	-	200	-	-
6	CKJ 1000 2.2	700	700	700	-
7.	CKJ 1000 3.1	500	-	-	-
8.	CKJ 1000 3.1.1	700	700	700	500
9.	CKJ 1000 3.2.1	700	700	700	500

Although research on bacteria with multi-resistance to copper and various dyes remains limited, numerous studies have investigated bacteria resistant to dyes. Ren et al. (2006) found that *Aeromonas hydrophila* isolated from textile printing activated sludge in Guangzhou, China, displayed resistance to 50 mg L^{-1} of malachite green, basic fuchsine, and reactive black. Additionally, An et al. (2002) showed that *Citrobacter* sp. obtained from soil near a textile dyeing industrial effluent treatment plant in Korea exhibited resistance to both basic fuchsine and congo red dye. Bacterial isolates can be resistant to copper by accumulating copper into cells (Irawati el al. 2020; Irawati et al. 2021a, 2021b).

Decolourisation at high dye concentrations appeared to be less distinct compared to low concentrations due to different levels of toxicity (Jamee & Siddique 2019). Furthermore, each bacteria have different ability to adapt the toxicity for each dye. The result of each bacterium on 3 dyes can be seen in Figure 1.

Decolourisation is defined as the process of removing dyes from stained specimens through adsorption or degradation (Victor et al. 2020). Bacteria-mediated dye decolourisation is determined by several factors, including but not limited to dye structure, dye concentration, and bacterial metabolism. Decolorization mainly occurs due to the synthesis and collaboration of extracellular enzymes such as azoreductase, laccase, lignin peroxidase, and protease

(Misal et al. 2011). Specific enzymes cleave specific bonds and chromophore centres, contributing to the overall decolorization process (Jamee & Siddique 2019). Azoreductase cleaves azo bonds (–N=N–) under anaerobic conditions (Saratale et al. 2009). Laccase breaks up nitro functional groups $N(CH_3)_2$ (Zucca et al. 2015).

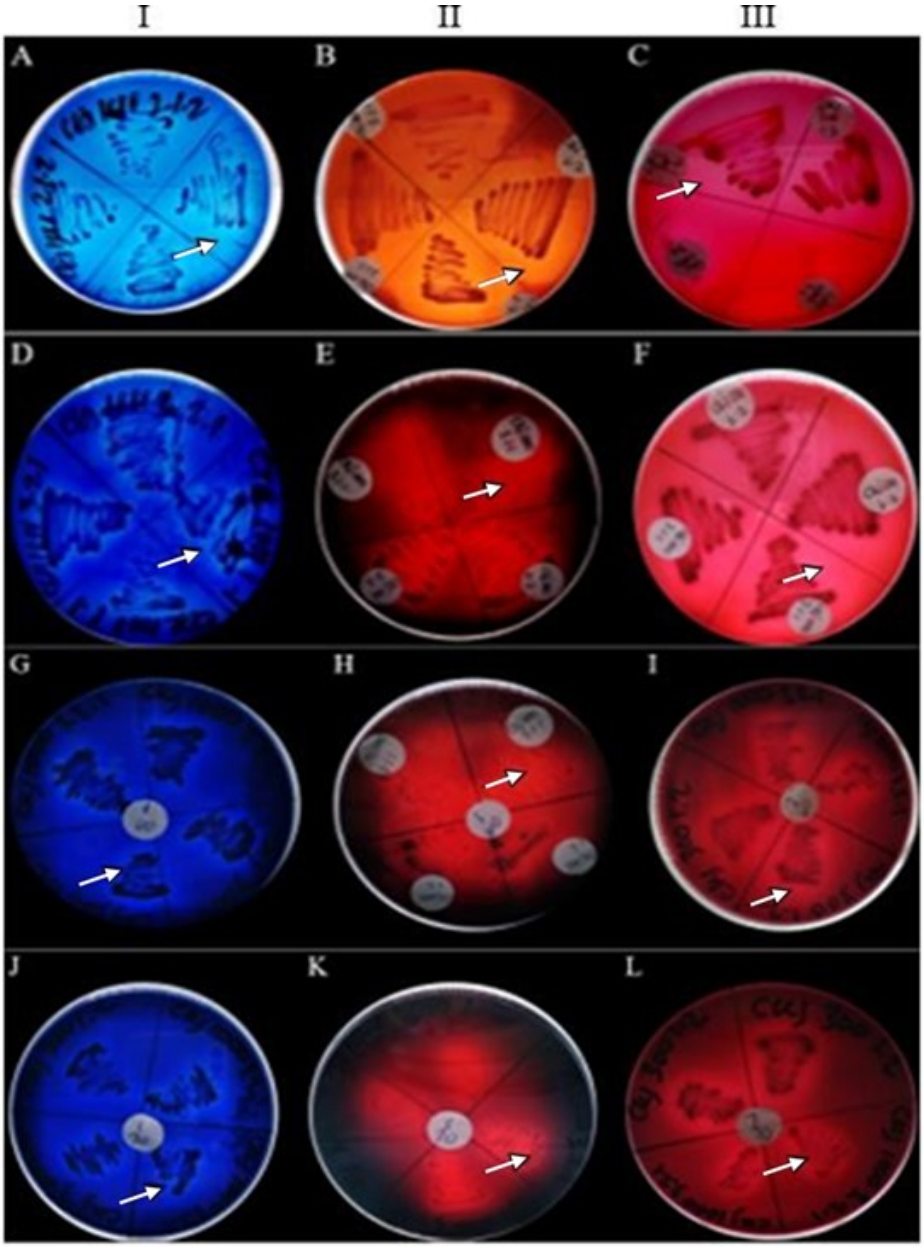

Figure 1. Decolourisation of copper and dye-resistant bacteria isolated from the Cikijing River on LB agar media supplemented with 5 mM $CuSO_4$ and 200-700 ppm of dyes: I. Methylene Blue, II. Congo Red, III. Basic Fuchsine. A. Isolate 2.1.2; B. Isolate 2.2; C. Isolate 1.2; D. Isolate 3.2.1; E. Isolate 3.1.1; F. Isolate 2.2 G. Isolate 3.2.1; H. Isolate 3.2.1; I. Isolate 1.2; J. Isolate 1.2, 3; K. Isolate 2.1; L. Isolate 2.2. Arrows show a clear zone surrounding the bacterial colonies.

Excluding CKJ 1000 1.2, each of the nine bacterial isolates demonstrated high resistance to at least one type of dye. Excluding CKJ 1000 3.1, eight isolates successfully decolorized at least one of the three selected dyes. However, only two out of nine isolates, CKJ 1000 3.1.1 and CKJ 1000 3.2.1, were capable of decolorizing dye including MG. According to Junqueira et al. (2010), MG is a triphenylmethane dye that exerts photodynamic antimicrobial effects on a variety of bacterial species. It is plausible that CKJ 1000 3.1.1 and CKJ 1000 3.2.1 were the only non-affected bacterial isolates in the selection.

Interestingly, CKJ 1000 1.2 was incapable of decolourising any type of dye in the absence of copper but successfully decolorized CR dye in the presence of copper. In contrast, CKJ 1000 3.1 was capable of decolorizing MB dye in the absence of copper but unable to decolorize any type of dye in the presence of copper. The first phenomenon supports the theory that copper is a cofactor for enzymatic reactions, while the second confirms that copper disrupts metabolic activity (Knop et al. 2017; Xue et al. 2023).

Identification of copper-resistance bacterial isolates

Based on morphological and molecular characterization (Table 3), four bacterial isolates belong to the *Klebsiella* genus. *Klebsiella* is a Gram-negative bacterium with a rod-shaped cell. Based on the determination data in this study, *Klebsiella* has the highest MIC value, which is 16 mM, except for CKJ 1000 3.1.1 which is 11 mM (Table 1). Bacteria belonging to this genus include *Klebsiella* sp. (CKJ 300 1.2) with 100 % sequence homology, *K. pneumoniae* (CKJ 500 2.1.2) with sequence homology above 98 %, *K. pneumoniae* (CKJ 500 2.2) with sequence homology above 98 %, and *K. variicola* (CKJ 1000 3.1.1) with sequence homology above 99 % (Table 3). The phylogenetic analysis indicated that CKJ 500 2.1.2 and CKJ 500 2.2 isolates are clustered into coherent groups with *K.pneumoniae* (Figure 2). These bacteria are common opportunistic pathogens for humans and animals as well as resident or temporary flora (especially in the digestive tract). Zulfiqar and Shakoori (2012) previously isolated *K. pneumoniae* that was resistant to copper with a range of MIC values of 5-6 mM. Furthermore, Mustafa et al. (2021) successfully cultivated *Klebsiella* that decolourised around 96 % of 200 ppm Disperse Blue dye within 24 hours.

Two bacterial isolates belong to the *Lysinibacillus* genus, a Gram-positive bacterium with a rod-shaped cell. Bacteria belonging to this genus include *L. boronitolerans* (CKJ 1000 1.1) with a sequence homology above 99 %, *L. fusiformis* (CKJ 1000 1.2) with 100 % sequence homology (Table 3). The phylogenetic analysis showed that CKJ 1000 1.1 and CKJ 1000 1.2 isolates are clustered into coherent groups with *L. boronitolerans* and *L. fusiformis*, respectively (Figure 2). *Lysinibacillus* has been known to have the potential as a heavy metal biosorption agent (Mathivanan et al. 2016). *Lysinibacillus* can decolourise azo dyes up to 96 % with the help of azoreductase, laccase, lignin, and peroxidase enzymes (Sari & Simarani 2019).

Isolate CKJ 1000 2.2 was identified as *B. proteoliticus* with 100 % sequence homology (Table 3) and based on phylogenetic analysis indicated the same coherent group with *B. thuringiensis* and *B. proteoliticus* (Figure 2). This bacterium is a Gram-positive bacterium with a rod-shaped cell. The MIC value for this bacterium is 11 mM. Research on *B. proteoliticus* as a copper bioaccumulation agent has been well known. According to Islam et al. (2020), *B. proteoliticus* was found in polluted environments and can also be a copper biosorption agent. *Bacillus* is effective in degrading azo dyes with the help of extracellular enzymes such as azoreductase and ligninase (Wu et al. 2022).

Isolate CKJ 1000 3.1 was identified as *P. stutzeri* with a sequence homology above 99 % (Table 3) and also confirmed by the results of phylogenetic analysis (Figure 2). This bacterium is a Gram-negative bacterium with a rod-shaped cell. The MIC value of the isolate was 11 mM (Table 1). According to Palanivel et al. (2020), *P. stutzeri* is a potential agent for copper bioremediation. *Pseudomonas* has also been reported to degrade up to 80 % of crystal violet dye at a concentration of 60 µM.

Isolate CKJ 1000 3.2.1 was identified as *C. freundii* with a sequence homology above 97 % (Table 3) and also confirmed with phylogenetic analysis that CKJ 1000 3.2.1 are in the same group as *C. freundii* (Figure 2). This bacterium is a Gram-negative bacterium with a rod-shaped cell. The MIC value

of the isolate was 11 mM (Table 1). This bacterium has great potential for the treatment of industrial waste containing copper under aerobic and anaerobic conditions (Wang et al. 2013). Benhalima et al. (2019) reported that *C. freundii* is a copper-resistant bacterium with an MIC value of 10 mM. *Citrobacter* is known to remove dyes through enzymatic degradation mechanisms (An et al. 2002).

For further research, Copper and dyes multi-resistance bacterial isolates will be determined for the ability to decolourise of dyes and to reduce copper concentration. The bacterial isolates which have multi resistance to copper and dyes will be applied in wastewater treatment plant using bioreactor.

CONCLUSION

Nine isolates from 59 copper resistant bacteria isolated from Cikijing River, that are *Klebsiella pneumoniae*, *Lysinibacillus boronitolerans*, *Lysinibacillus fusiformis*, *Bacillus proteoliticus*, *Pseudomonas stutzeri*, *Klebsiella variicola*, and *Citrobacter freundii*. Out of the nine selected bacterial isolates, five isolates (CKJ 300 1.2, CKJ 500 2.1.2, CKJ 1000 2.2, CKJ 1000 3.1.1, and CKJ 1000 3.2.1) were found to be resistant to 5 mM $CuSO_4$ and capable of decolorizing dyes (Methylene Blue, Congo Red, and Basic Fuchsine) at the highest concentration of 700 ppm. The selected bacterial isolates from the Cikijing River exhibit great potential to be further developed as bioremediation agents employed in biological processes for wastewater treatment.

AUTHORS CONTRIBUTION

W.I. and D.N.S designed the research and supervised all the processes. R.P. and T.Y designed the IS collected and analysed the data, and VL wrote the manuscript.

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CONFLICT OF INTEREST

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

Table 3. Top homology search of copper- and dye-resistant bacterial isolates based on 16S rDNA.

Isolate code	The closes taxon to BLAST results on NCBI	Max score	Query coverage (%)	Accession	Sequence Similarity (%)
CKJ 300 1.2	*Klebsiella* sp.	1410	100	HM462447.1	100
CKJ 500 2.1.2	*Klebsiella pneumoniae*	1400	100	LC455961.1	98.85
CKJ 500 2.2	*Klebsiella pneumoniae*	1351	100	LC455961.1	98.94
CKJ 1000 1.1	*Lysinibacillus boronitolerans*	1410	100	MH385002.1	99.87
CKJ 1000 1.2	*Lysinibacillus fusiformis*	1430	100	MT605500.1	100
CKJ 1000 2.2	*Bacillus proteolyticus*	1450	100	MT573794.1	100
CKJ 1000 3.1	*Pseudomonas stutzeri*	1432	100	MF125023.1	99.87
CKJ 1000 3.1.1	*Klebsiella variicola*	1395	100	MN725749.1	99.74
CKJ 1000 3.2.1	*Citrobacter freundii*	824	100	MH668092.1	97.69

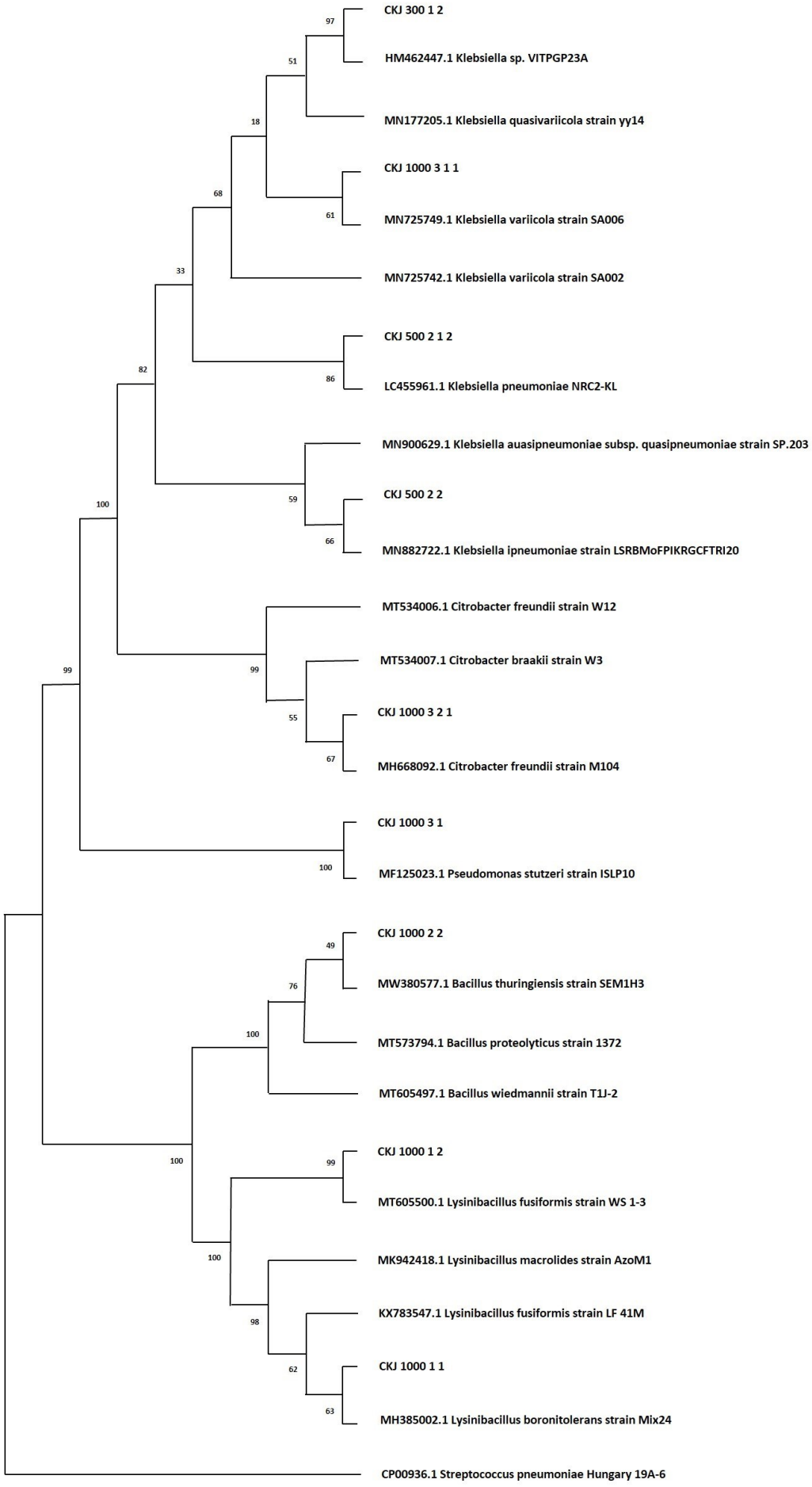

Figure 2. Phylogenetic tree based on 16S rDNA sequences of isolates CKJ 300 1.2, CKJ 500 2.1.2, CKJ 500 2.2, CKJ 1000 1.1, CKJ 1000 1.2, CKJ 1000 2.2, CKJ 1000 3.1, CKJ 1000 3.1.1, and CKJ 1000 3.2.1. This tree was made using the neighbour-joining method with Kimura two-parameters distances with the no-gap option. Number indicated the percentages of occurrence in 1000 boost traps trees.

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Research Article

Biodegradable Sheets from Dried Mycelia of Edible Mushrooms

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ABSTRACT
Due to its quick growth and biodegradability, mushroom mycelium has been used to create alternative materials. This study aimed to produce mycelium sheets from market-purchased edible mushrooms (*Lentinus* sp. and *Pleurotus* sp.) and to assess the mycelium sheet properties. They were isolated and cultured in various liquid media. The production of four mycelium sheets was successful. After drying, the mycelium sheets of *Pleurotus* sp. using potato dextrose broth had the largest water contact angle. With a tensile strength, the mycelium sheet of *Lentinus* sp. using malt extract broth obtained the highest value. The dried mycelium sheet from *Pleurotus* sp. cultured on yeast extract broth had the greatest hardness value in the microhardness testing. After 7 days, the residual dry weight of the mycelium sheets in different conditions—soil burying, soil surface exposure, and water immersion—was less than 50 % of the initial weight. This work has demonstrated the biodegradability of mycelium sheets.

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INTRODUCTION

Synthetic materials e.g., plastics are known to have a long period of degradation which is problematic to the environment including marine life, soil pollution, ecosystems, and natural resources. One of the causes of death to marine mammals, sea birds, and sea turtles is the ingestion of plastic debris. Thus there is a strong need for renewable and biodegradable alternatives to reduce environmental impact. One of them is mushrooms Click or tap here to enter text. (Bond et al. 2013; Zhu et al. 2019; Silverman et al. 2020). Ecologically, the mushroom is a decomposer that can grow rapidly in large quantities when environmental conditions are favorable. The mushroom species are different in their textures ranging from soft to hard, shapes, and properties such as waterproof ability, germicidal properties, and flexibility (Haneef et al. 2017). Interestingly, mushrooms, agricultural waste, and other biomasses containing fibers can be combined to create alternatives for biodegradable materials (Bayer et al. 2014; Mostafa et al. 2018).

Materials derived from mushrooms in combination with natural plant fibers have been increasingly studied such as bricks, soundproof walls, and green packaging, e.g., boxes including fibers for textile industries (Haneef et al. 2017; Islam et al. 2017; Girometta et al. 2019; Jones et al. 2018; Whabi et al. 2024). This involves the production of mycelium-based composites with plant fibers to enhance the physical properties e.g. tensile and compressive strength (Ziegler et al. 2016). Moreover, mycelium-based composites can be applied to various objects with different purposes – decoration, furniture and insulation panels (Appels et al. 2019). Environmentally, these materials are also biodegradable which can reduce the carbon footprint which is one of global sustainability goals (Elsacker et al. 2021). There are various types of mushrooms in Thailand. The edible mushrooms are now widely cultivated and can be found in local markets in northeastern Thailand such as *Lentinus* spp. and *Pleurotus* spp. Silverman et al. (2020) prepared composites of *Pleurotus ostreatus* (oyster), *Pleurotus citrinopileatus* (yellow oyster), *Pleurotus eryngii* (king oyster), and *Ganoderma lucidum* (reishi) with fabric mat and sawdust to make environmentally friendly footwear. Also, Nawawi et al. (2019) made chitin paper from crab shells, *Agaricus bisporus*, and some polypores mushrooms. However, the mycelium sheets and the physical properties of *Lentinus* sp., or log white mushroom as the mushroom local to the northeastern region of Thailand, have not largely been studied like *Pleurotus* which was therefore the objectives of this research.

MATERIALS AND METHODS

Source of mushroom and pure culture isolation

Two edible local mushrooms, *Lentinus* sp. (log white mushroom, Lw) and *Pleurotus* sp. (oyster mushroom, Oy), were purchased from a local market in Muang district, Khon Kaen province. To prepare the pure mycelium of each mushroom, tiny pieces (0.2×0.2 cm^2) were isolated from the inside mushroom stalk and placed at the center of a Petri dish containing potato dextrose agar (PDA). After an incubation period of 2-3 days, mycelia obtained from pieces of fresh basidiocarp were placed on PDA. Then, the edge of the grown mycelium (0.2×0.2 cm^2) was taken and placed onto new PDA dishes to obtain the pure mushroom mycelia for the next experiments.

Mycelium sheet production

Four different broth media were used, namely potato malt peptone broth (PMPB), potato dextrose broth (PDB), malt extract broth (MEB), and yeast extract broth (YEB) in static condition. Preliminary experiments indicated that PMPB and MEB were the most suitable media for the growth of Lw, but PDB and YEB were found optimal for Oy growth which was determined

based on the average distance of the mycelia on the media. An equal volume (50 mL) of the liquid medium was added to each flask and autoclaved at 121 ° C for 15 minutes. After the broth medium was cooled down, the mushroom mycelium plugs from the pure culture (1 cm diameter) were inoculated. The mycelium sheets were grown on the surface of the liquid media in static conditions at laboratory room temperature (20–35 °C). After 30 days, the mycelium sheets were taken out of the flasks and dried in the hot air oven for 3 hours at 60 °C to remove moisture and inhibit further growth. The dried mycelium sheets were packed in plastic bags and stored in desiccators containing silica beads to protect them from contamination and moisture.

Determination of mycelium sheet properties

The intact, dried mycelium sheets underwent various tests to assess their morphology, water protection, flexibility, hardness, and degradability.

Morphology

The mycelium sheet morphology was visualized under a Scanning Electron Microscope (SEM, Hitachi S–3000 N) to observe the fibrous structure. The sample was coated with gold using Emitech sputter coater K500X before SEM analysis.

Water protection

The water resistance of the mycelium sheets was assessed using a water contact angle (WCA) goniometer (OCA 15EC, Dataphysics). It was to determine the waterproof ability of the mycelium sheet surface. The samples were flattened using 2 pieces of cover glass, and 5 µL water was dropped on the dried mycelium sheets. After 10 sec, the side view images were captured at room temperature (29-35 °C). The WCA was then automatically calculated using SCA20 software) (Massa-Angkul et al. 2020). This experiment was done with 5 replicates.

Flexibility test

The flexibility test was performed using a universal testing machine (Lloyd Instruments LR30K). Each sample was cut into a strip shape (0.4 cm × 0.25 cm) and was fixed with clamps on the machine. The deformation rate was set at 2 mm min^{-1} until failure. The ultimate tensile strength (UTS) and test-load value (max-min) dwell time were calculated from NEXYGEN™ PLUS Materials Testing Software. The mechanical analysis of the mycelium sheet was referred by Appels et al. (2020). This experiment was done with 5 replicates.

Hardness test

The hardness of the mycelium sheets was evaluated using microhardness tester Future-Tech FM-800 machine and FT-ARS software version 1.15.13 (Future-Tech Corporation, Japan) which automatically calculated the surface Hardness values of Vickers (HV). The test machine was the Vickers indenter that pressed into a surface under a static load. The indenter was a spherical diamond-tipped cone. The mycelium sheets were fixed on a wooden cube before testing. The compression force value for the Lw sheet was applied at 25 gf/15s and 50 gf/15s for the Oy sheet. The software visualized microscopic images of the pyramid shape after the materials were pressed. This experiment was done in 5 replicates.

Biodegradability

Mycelium sheet samples (0.10 g) were put inside a nylon teabag with 3 replicates and exposed to 3 environments; 1) on the soil surface (SS), 2)

soaked in water (SW), and buried in the soil (BS). For conditions SS and BS, soil for planting was purchased from an agriculture shop and was mixed with rough sand in a ratio of 1:1. For condition SS, the bags were placed on the soil in the transparency box with holes at the bottom to drain excess water and circulate the air. The bags were kept in position by rocks around the mycelium sheet and watered every 3 days. In condition BS, the samples were buried in the soil mixture then watering water to make the soil moist up to 85 -90 %. The samples exposed to SW condition were immersed in 150 mL water collected from a natural reservoir. The experiment was conducted at room temperature (20-29 °C). The soil temperature was 22-29 °C and the water temperature was 21-29 °C with pH of 7.11-8.23. After 1-week, the samples were carefully rinsed with water and dried in the hot air oven at 60 ° C for 3 hours and weighed again by sensitive electronics (Ibrahim et al. 2014; Ounkaew et al. 2018).

Statistical analysis

Analysis of variance (ANOVA) was used to compare the values derived from the tests to compare the performances of the mycelium sheets ($p<0.05$).

RESULTS AND DISCUSSION

Mycelium sheet production

According to Figures 1A-1D, grown mycelium sheets were shown successfully on the liquid media after 30 days. When comparing the two mushroom species, Lw was able to rapidly produce the fibrous structure and covered the surface of both MEB (Figure 1A) and PMPB (Figure 1B). Meanwhile, Oy could grow and stay on the surface of both PDB (Figure 1C) and YEB (Figure 1D). After drying, the Lw fibers were formed into a thick layer and the texture was leathery and tough (Figures 1E and 1F). The Oy fibers were thick and soft like a sponge and the dried mycelium sheet was thin, crispy, and fragile (Figures 1G and 1H).

Material analysis of mycelium sheets

Morphology

The dry mycelium sheets were stored in a desiccator with silica gel before being investigated under the scanning electron microscope. The micrographs of the morphological characteristics of the dry mycelium sheets are illustrated in Figure 2 below. All mycelium sheets showed a similar microstructural morphology. The mycelium fibers of the sheets were flat because of dehydration and were woven together without a specific pattern (arrowheads).

In this study, intact mycelium sheets of *Lentinus* sp. and *Pleurotus* sp. were successfully grown in 30 days at room temperature. The toughness presented a major weakness because the mycelium sheet was composed of the filamentous structure of the mycelia. The dried mycelia of the mushroom became very fragile. The electron microscope images revealed the mycelium sheets were porous because of the mycelial crosslink.

Water protection

The waterproofing ability of the mycelium sheets was tested by measuring the water contact angle (WCA). A higher water contact angle means a higher hydrophobicity and thus waterproofness. Here, the values of the water contact angle of the mycelium sheets were 103.3 ± 19.13° (LwMEB), 72.0 ± 32.9° (LwPMPB), 131.91 ± 9.70° (OyPDB), 113.14 ± 30.55° (OyYEB). The greatest WCA value was from the mycelium sheet of OyPDB, which was only significantly different from LwPMPB ($p < 0.05$) as shown in Table 1.

The hydrophobic performance of the mycelium sheets was determined from the water contact angle, i.e., a higher value implied a better waterproof

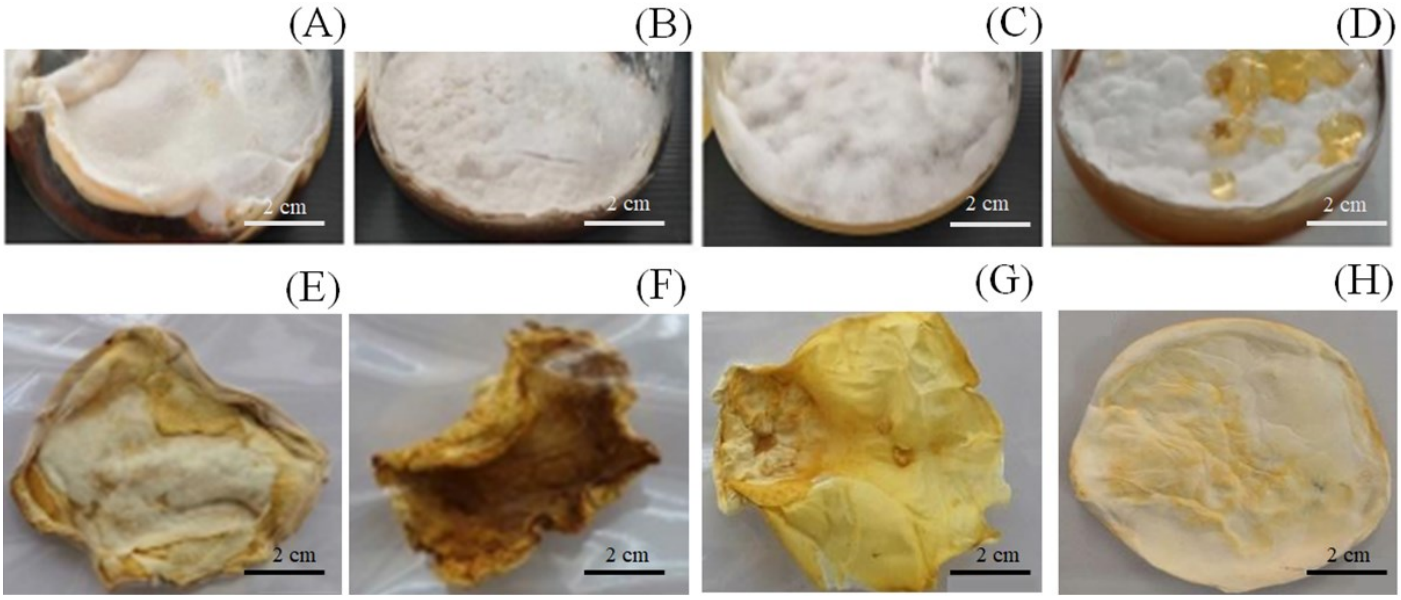

Figure 1. Freshly grown mushroom mycelia on the surface of the broth media after 30 days (A-D) and dried mycelium sheets (E-H). The following samples are displayed: LwMEB (A and E), LwPMPB (B and F), OyPDB (C and G), and OyYEB (D and H).

Table 1. Average values of water contact angles, ultimate tensile strength and average hardness values of the mycelium sheets. The different letters indicate significantly different ($p < 0.05$).

Mycelium Sheet	Water Contact Angle (° ± SD)	Ultimate Tensile Strength (MPa ± SD)	Hardness (HV ± SD)
LwMEB	103.3 ± 19.13a	15.88 ± 11.62a	5.81 ± 0.96b
LwPMPB	72.0 ± 32.9b	0.86 ± 0.54b	6.89 ± 1.76b
OyPDB	131.91 ± 9.70a	1.54 ± 1.71b	12.39 ± 1.72b
OyYEB	113.14 ± 30.55a	1.43 ± 1.14b	25.82 ± 6.05a

ability (Shen et al. 2024). The water contact angle was the highest on the OyPDB sheet at 131.9° and the lowest was found for the LwPMPB sheet (72.0°). The contact angle is the measurement to determine whether the material could lessen the moister absorption (Shen et al. 2024). This water protection property is derived from hydrophobins, proteins produced by fungi, leading to hydrophobicity on the mycelium surfaces. These proteins act as a protective layer that is resistant to water. Due to this property, the water absorption rate is lower on the mycelium sheet surface (Walter & Gürsoy 2022). Nawawi et al. (2019) produced paper from blue swimming crab shells, *A. bisporus*, and polypores mushrooms. The paper from these components gave the water contact angles at 65.6°, 24.2°, and 54.5° for the crab shell paper, *A. bisporus*, and polypores mushrooms respectively but the value of our mycelium sheets was relatively high at 131.9°. The WCA of *Schizophyllum commune* mycelium film from Appels et al. (2020) study was 129 ± 2° but when they added glycerol, the WCA was decreased depending on glycerol concentration. This implies the potential to prevent water on the material surface.

Flexibility test

The mycelium sheets were studied with a dual-column universal testing machine for their flexibilities, and the average tensile strengths were as follows. LwMEB (15.88 ± 11.62 MPa) had the highest value, which was significantly different from the others, OyPDB (1.54 ± 1.71), LwPMPB (0.86 ± 0.54 MPa), and OyYEB (1.43 ± 1.14 MPa) ($p < 0.05$) (Table 1). The highest tensile strength values were 15.88 ± 11.62 MPa, which was in the

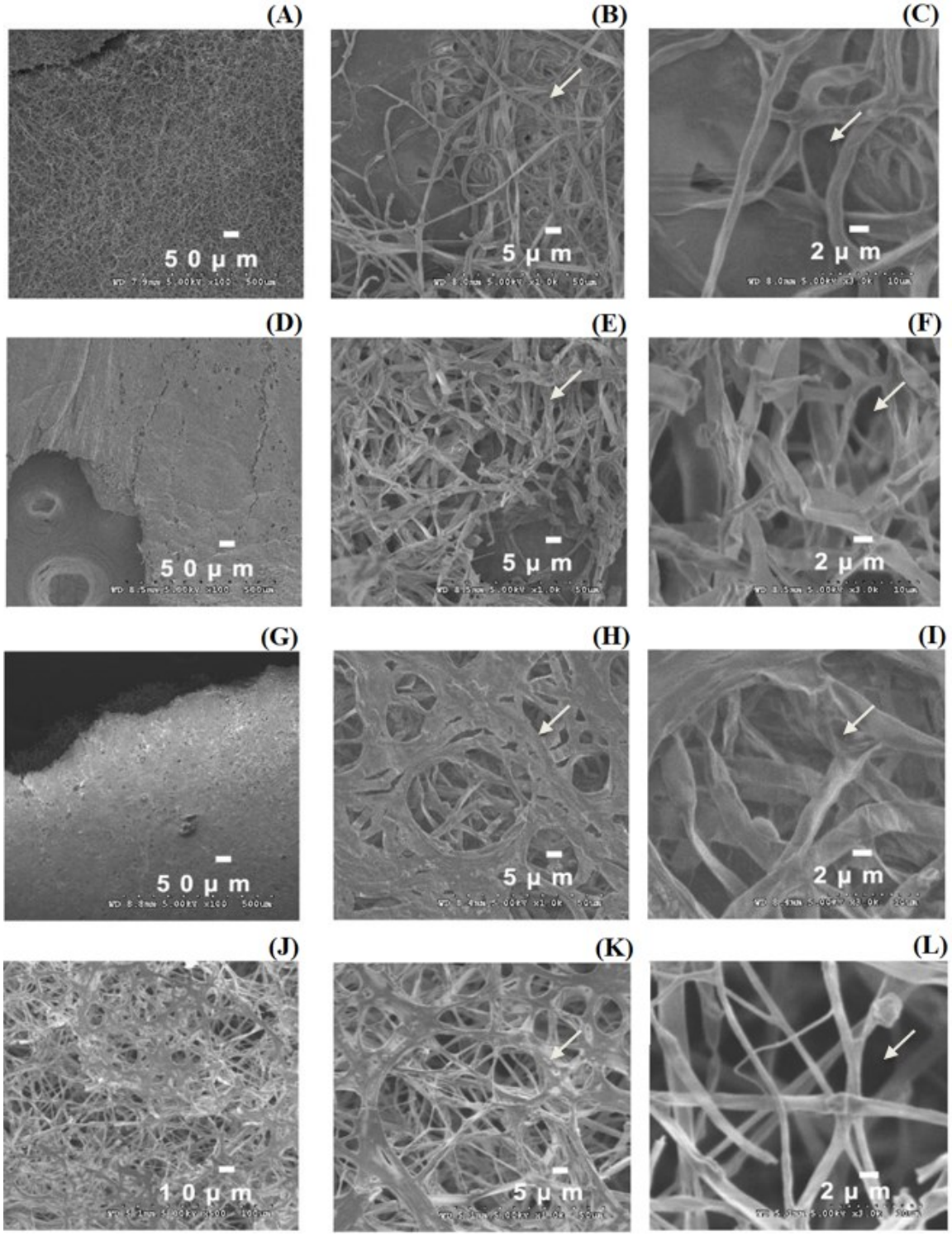

Figure 2. Electron micrographs of mycelium LwMEB sheet (A, B, and C), LwPMPB sheet (D, E, and F), OyPDB sheet (G, H, and I), and OyYEB sheet (J, K and L).

LwMEB sheet, while the minimum value was 0.86 ± 0.54 MPa in the LwPMPB sheet. In another research conducted by Appels et al. (2018), they used *S. commune* to make the mycelium-based composite and tested it for its tensile strength. The resulting test for this was 5.1-9.6 MPa which was around 4-10 times higher than the LwMEB and LwPMPB. The mushroom leather from *Phellinus ellipsoideus* by Bustillos et al. (2020) had a tensile strength of 0.34 MPa and the tensile strengths for the *A. bisporus*, crab shell, and polypore mushroom of Nawawi et al. (2020) were respectively 204.4, 65-204, and 65.3 Mpa, which were also higher than the MS with the highest strength (15.88 Mpa). Research by Bayer et al. (2014) showed bioplastics synthesized from microcrystalline cellulose and plant wastes (parsley and

spinach stems, rice hulls, and cocoa pod husks) powder mixed with trifluoroacetic acid. Our MS could be comparable to parsley (5 MPa) and spinach stems (1 MPa).

To improve the flexibility of the mycelium sheet, Appels et al. (2020) incorporated glycerol to enhance the flexibility of mycelium fiber, making it comparable to leather or rubber. The ultimate tensile strength of the mycelium sheet was 1.8 ± 0.1 MPa with 32 % glycerol, 12.3 ± 1.2 MPa with 2 % glycerol, and 5.0 ± 0.1 MPa for the sheet without the glycerol addition. Additionally, 20 % polyethylene was proved to increase the tensile strength of the mycelium leather from brown rot fungi comparable to the real leather (Raman et al. 2022). The interconnectivity of the mycelium leads to the uniform distribution of fungal mycelium throughout the material surface which could increase the capabilities to endure forces (Haneef et al. 2017). Shen et al. (2024) reported that their results could produce the mycelium material with a tensile strength of 0.72 MPa higher than the tensile strength of expanded polystyrene foam. Therefore, there should be other polymers to enhance this property of the mycelium sheets of this study.

Hardness tests

The compression force test or Vickers hardness test was performed and calculated. The hardness value of OyYEB was the highest (25.82 ± 6.05 HV) and significantly different from the others as shown in Table 1, OyPDB (12.39 ± 1.72 HV), LwPMB (6.89 ± 1.76 HV) and LwMEB (5.81 ± 0.96 HV) ($p < 0.05$).

It was found that OyYEB had the highest hardness of 25.83 HV, whereas LwMEB had the lowest hardness (5.81 HV). While the polymer composites obtained from kenaf fiber according to Abdullahi et al. (2018), the result showed a hardness value of 64.5-83 HRL based on the Rockwell hardness test (more than 600 HV) which is higher than the MS in this study. Meanwhile, the hardness value of the orange peel polymer composite with a combination of resin and natural fiber reported by Ojha et al. (2012) was 17.89-20.72 HV, which is comparable to the Oy and Lw sheets.

The Vickers hardness number (HV) is a unit to determine the hardness of the mycelium sheet derived from calculating the loaded force on the surface area (Wu et al. 2022). Materials with natural plant fiber e.g. lignin possess higher hardness because of the rigidity of lignin molecules (Lee & Choi 2021). In this study, the mycelium sheets reveal very low harness values because they comprised only the mushroom mycelium. Haneef et al. (2017) found the mycelium sheets showed different hardness depending on the mushroom species. The density and structure of the mycelium influence the hardness value. Moreover, the additional technique applied during the formation of mycelium sheets like pressing can improve the interconnectivity of the mushroom mycelium, contributing to the evenness of fungal mycelium throughout the sheets (Haneef et al. 2017). Therefore, this technique could be applied in further study to enhance the hardness of the mycelium sheet.

Biodegradability

The ability of the mycelium sheets to be degraded in nature was determined based on the weight loss of the mycelium sheet in 3 different conditions, i.e., being buried in the soil (BS), soaked in water (SW), and left on the soil surface (SS). The initial weight of each sheet was 0.10 g. In the first 3 days, mycelium sheets placed on the soil surface (SS) were found to be covered by other fungi. Every 7 days, all the mycelium sheets were taken, gently cleaned, dried, and weighed. The weight loss of all mycelium sheets in each condition was greater than 50 % after 7 days (Figure 3).

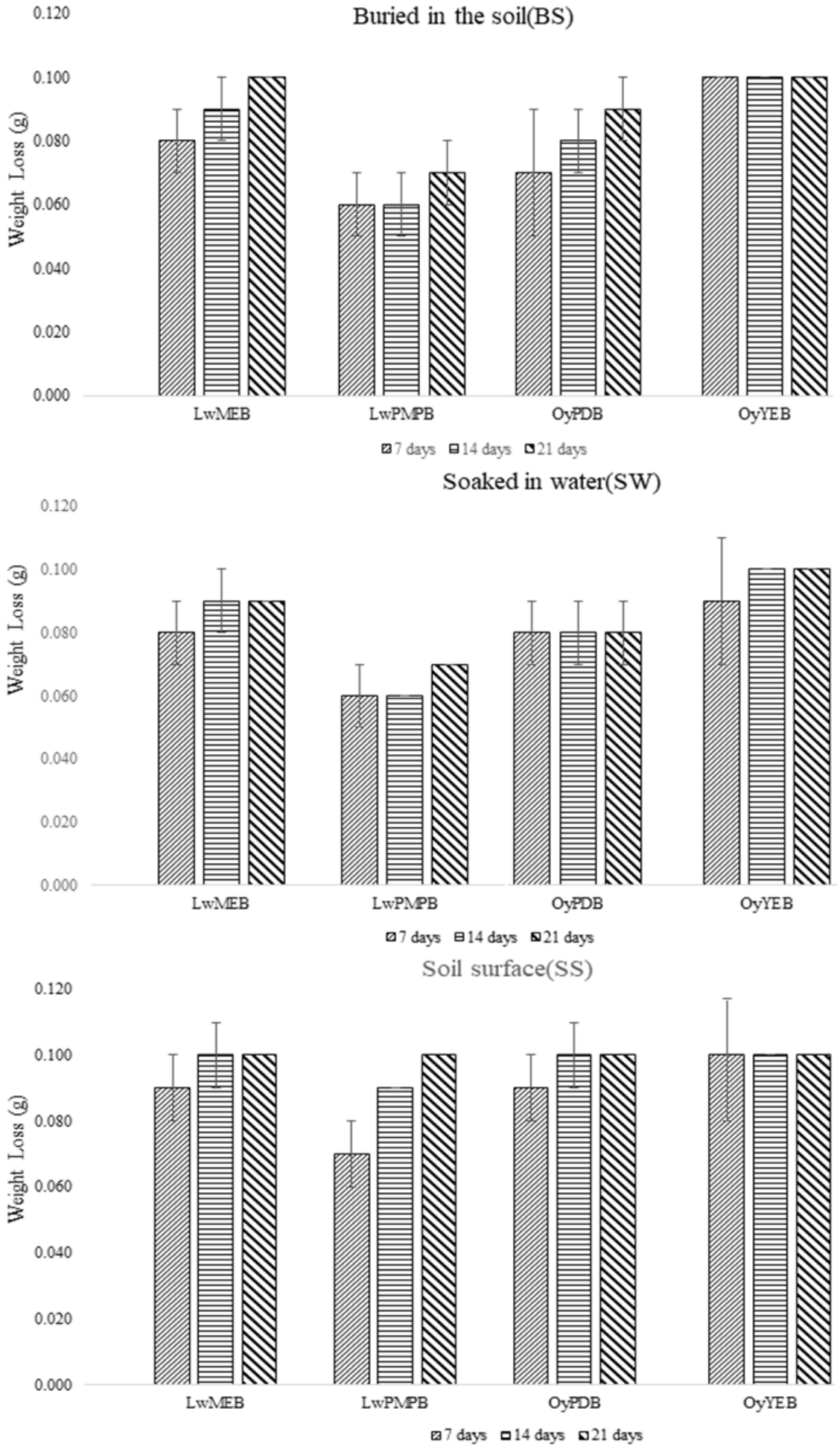

Figure 3. The potential biodegradability of the mycelium sheets in three different environments, being buried in the soil (BS), soaked in water (SW), and left on the soil surface (SS) for 7, 14, and 21 days. Error bars indicate ± SD.

The biodegradability of the mycelium sheets in all conditions was reported as follows. Firstly, in BS condition, at day 7, the OyYEB sheet had completely disappeared according to the weight loss (0.10 ± 0 g) followed by the OyPDB (0.07 ± 0.01g), LwMEB (0.08 ± 0.01 g) and LwPMPB (0.05 ± 0.01). At day 14, the weight loss of OyPDB and LwPMPB were 0.08 ± 0.01g and 0.05 ± 0.01g respectively. After 21 days, only some partial mass of the LwPMPB sheet (60 %, 0.06 ± 0.01 g) was left in this condition. Regarding the SW condition, at day 7, the weight reduction of the OyPDB sheet (0.07 ± 0.01 g) and OyYEB sheet (0.08 ± 01 g) was significantly different from the LwPMPB sheet (0.05 ± 0.01 g) ($p < 0.05$). After being soaked in water for 21 days, the OyPDB lost 0.083 g (83 %), LwMEB lost 0.090 g (90 %) and LwPMPB lost 0.070 g (70 %). In the last environment, which was SS condition, the LwMEB and OyPDB sheets had a weight loss of 0.9 ± 0.01 g, which was significantly different from the LwPMPB sheet (0.06 ± 0.01 g) but non-significantly different from OyYEB (0.10 ± 0) after 7 days of exposure to this condition. Seven days later, the mycelium sheets of OyYEB and LwMEB were completely biodegraded (0.10 ± 0 g) and were not significantly different from the LwPMPB sheet (0.09 ± 0.01 g) and OyPDB (0.09 ± 0.0 g). After 21 days, all samples disappeared, indicating their complete biodegradation.

The ability to decompose in this study, after 7 days of testing in all 3 conditions, the weight loss of every example was more than 50 % and decreased continuously until it could not be measured. We found that out of the four mycelium sheets, OyYEB was rapidly degraded and LwPMPB was the slowest. It demonstrated the MS could be decomposed in a very short period, 1-3 weeks. In congruence with Ounkaew et al. (2018), they invented the polyvinyl alcohol starch film for bio packaging and this film was able to be degraded naturally by 65.28–86.64 % in 30 days. Similarly, the biodegradability of the pectin/polyvinyl alcohol films prepared by Linn et al. (2022) was 50-75 % within 7 days. This common approach to determine the biodegradability of materials is the soil burial test and the loss weight of the material is periodically quantified. This method is very practical for biodegradability determination because it includes environmental factors from the soil or water such as temperature, natural microbes and moisture that have impacts on the rate of biodegradability activities (Vandelook et al. 2021). In another report related to this issue, the bioplastics synthesized by Bayer et al. (2014) took 1 week to fragment the film into smaller pieces and 1 month to completely disintegrate in water. However, no research solely reported the potential biodegradability of mycelium sheets from mycelia.

CONCLUSIONS

This research reports the simple method of mycelium sheet production but there are points to be improved. The fresh mycelium sheet collection from the flask should be more convenient but there was a limitation with the container used to make the mycelium sheet. Fibers grown in flat bottles could form the mycelium sheets more quickly because of the lower water level compared to using a high level of broth. In the flasks, after the mushroom plugs were inoculated, the plugs sunk to the bottom of the flask. Once the mycelia started to grow, they floated to the surface of the liquid medium and began to form the fiber covering the water surface as the mycelium sheets because of the inter-weaving of the mycelia, which was most evident in Lw and Oy. However, the method of harvesting the mycelium sheets from the flasks in this study was too difficult because of the narrow neck of the culture flasks. Specific culture bottles should be obtained to meet both the technical and biological protocols, being simple to harvest and able to prevent contamination. To improve its mechanical properties in the future, the mycelium sheet needs to be enhanced with additional materials. For example,

it could be co-cultured with other mushrooms or coated with polymers such as resin, rubber or other polymers to increase flexibility and provide more stiffness to the texture. However, since the mycelium sheets in this study were made purely from the mushroom mycelia that assured the friendliness to the environment. In conclusion, this study provides the first light to achieve the practical alternative material from mushroom mycelia.

AUTHOR CONTRIBUTION

C.P. designed, conducted the research, collected and analyzed data and drafted the manuscript. W.J. designed the research and supervised all the processes, proofread and reviewed the manuscript. J.T.N.K. designed the research, supervised the experiments, and reviewed the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Present and Future Distribution Model using MaxEnt: A Risk Map for Dengue Haemorrhagic Fever based on *Aedes aegypti* Mosquitoes Distribution in Malang Region, East Java, Indonesia

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ABSTRACT

The prevalence of Dengue Haemorrhagic Fever (DHF), a disease prevalent in countries with tropical and sub-tropical climates, including Indonesia, has exhibited a notable increase over the past two decades. A study case of a region experiencing this surge is Malang Region, which situated in East Java. The transmission of DHF within individual human is facilitated by the existence of *Ae. aegypti*, which serves as one of the intermediate vector mosquitoes. MaxEnt modelling was employed to analyse the niche and distribution of *Ae. aegypti*. The results of this study demonstrated that the integration of environmental and anthropogenic variables in a combination model provided more comprehensive approach for comprehending the niche and distribution patterns of *Ae. aegypti* compared to relying only regarding a climatic model. Areas characterised by higher temperatures, high population density, and limited vegetation cover possess the inherent capacity to serve as suitable habitats for *Ae. aegypti*. According to the modelling results, the distribution of *Ae. aegypti* in Malang region currently encompasses approximately 14.5 % (545.5 km^2) of the total area. It is projected that this distribution can potentially expand to 15.5 % (568.9 km^2) by the year 2040. Several sub-districts, namely Klojen, Blimbing, Sukun, Lowokwaru, Kedungkandang, Pakisaji, and Kepanjen, have been classified as high-risk areas that require special concern. The combination model of environmental variables and anthropogenic variables provide more comprehensive approach to understand the niche and the distribution patterns of *Ae. aegypti* in Malang Region compared to relying solely on climate models.

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INTRODUCTION

Dengue Hemorrhagic Fever (DHF) is a tropical and subtropical disease that is receiving increased attention in several regions of the world, including Indonesia (Capinha et al. 2014; Kraemer et al. 2015). The disease is caused by four types of dengue arbovirus (DENV-1, -2, -3, and -4), the family Flaviridae, the genus flavivirus, with the mosquito species *Aedes* aegypti serving as one of several vectors for transmitting the virus to humans (Tuiskunen & Lundkvist 2013; Swaidatul et al. 2022). According to Harapan et al. (2019) and the Indonesia Ministry of Health (2017), dengue case reports have increased in various regions of Indonesia over the past 2 decades. Although the implementation of the dengue preventive program initially led to a decrease in the number of cases, few years later there was a subsequent increase in the number of cases. This phenomenon is evident from the data compiled by Sulistyawati (2020) and The Indonesia Ministry of Health (2020), which reveals a significant increase in the number of cases since 2000, with fluctuations occurring between 2007 and 2018. In 2020, the number of individuals affected by DHF in this region increase significantly, with over 1700 people being afflicted (East Java Provincial Health Service 2021).

The Indonesia Ministry of Health (2017) implemented multiple strategies to counteract the spread of the dengue virus. Since the 1980s, vector control has heavily relied on active community participation, employing strategies such as larvicide use, fogging, mosquito nets, the 3M ("*Menguras, Menutup, Mendaur ulang barang bekas*") Program, larvae monitoring officers, elimination of mosquito nests, and the more recent "*Gerakan 1 Rumah 1 Jumantik*" (this movement is carried out by selecting a family member at home to monitor for larvae and using social media to report regularly) (Sulistyawati 2020). Nevertheless, according to Harapan et al. (2019), these strategies have not yet proven to be successful in significantly and optimally decreasing the number of individuals affected by dengue in Indonesia. The Indonesia Ministry of Health (2021) states that one of the challenges in dengue virus control is the lack of technology, so a quality and sustainable information system is expected to prioritise resource distribution to the most vulnerable regions. Tolinggi and Dengo (2019) suggested that utilising spatial niche analysis could serve as empirical evidence that is crucial for targeted interventions and can be used for designing programs aimed at preventing and controlling DHF.

Vector mosquitoes, such as *Ae. aegypti*, exhibit niches which provide a crucial role in determining their distribution. These niches are influenced by various factors, including environmental conditions (Lozano-Fuentes et al. 2012) and anthropogenic factor (Obenauer et al. 2017). The interaction of both factors in relation to the distribution of vector mosquito populations involves significant importance, particularly due to the anthropophilic nature of *Ae. aegypti* (Gomes et al. 2005). According to their nature, unique anthropogenic profile within a specified area, including population density and poverty also remain as spreading factor (Obenauer et al. 2017). Hence, the explanation of mosquito niches is not restricted to climatic factors, as this oversimplifies the dynamics of mosquito niche (Eisen & Moore 2013).

Climate change is a contributing factor to the distribution dynamics of *Ae. aegypti* populations, as these insects, like other poikilothermic species, are influenced by changes in temperature (Upshur et al. 2019). The IPCC (2021) has documented that global temperatures have, on average, risen by 1.07 °C between the years 1850-1900 and 2011-2019, with a range of 0.8 °C to 1.3 °C. The average land temperature is significantly higher, measuring 1.59 °C, with a variation between 1.34 °C and 1.83 °C. In addition, there has been an increase in both the intensity and frequency of rainfall as a climate variable since the 1950s (Wan et al. 2014; Knutson & Zeng 2018). Nevertheless, tem-

perature and rainfall fluctuations exhibit significant variation across different geographic regions (Liu & Allan 2013; Schurer et al. 2020; Susilawaty et al. 2021). Therefore, it is imperative to conduct adjusted modeling in order to obtain accurate and representative results. The Coupled Model Intercomparison Project (CMIP6) is a widely utilised Global Climate Model. It is currently utilized alongside Shared Socioeconomic Pathways (SSPs) to establish emission scenarios (Meinshausen et al. 2020).

A commonly employed method recent times is machine learning (Witten et al. 2016). MaxEnt (Maximum Entropy), has gained significant popularity since its introduction by Phillips et al. (2006) as a machine learning algorithm. MaxEnt is a common technique for utilising presence-only data. It is known for its capability to incorporate background data and spatial variables as a deliberate approach (Peterson et al. 2011). Furthermore, MaxEnt, a type of machine-learning algorithm, is considered an effective modelling technique due to its capacity to accurately represent intricate patterns of data (Elith et al. 2010). Several research studies (e.g., Kraemer et al. 2015; Santos & Meneses 2017; Dickens et al. 2018; Iwamura et al. 2020, etc.) have used geographical distribution or ecological niche modelling to explore *Ae. aegypti* as a disease vector on a worldwide scale. Sallam et al. (2017) asserted that the utilisation of the MaxEnt algorithm for modelling the *Aedes* genera can be considered a suitable technique.

Nevertheless, conducting a reassessment specifically in the Malang region area is important due to the potential discrepancies in modeling outcomes between global and regional scales (Hastie et al. 2009; Früh et al. 2018). The objective of this research was to generate a spatial model using MaxEnt algorithm to determine the potential niche and distribution of *Ae. aegypti* in the region of Malang region. In addtition, a future distribution model was conducted to project the potential distribution of *Ae. aegypti* in the coming decades.

MATERIALS AND METHODS

Study Area

Malang region is a metropolitan area situated in the province of East Java, Indonesia and predominantly characterized by its highland urban and suburban areas, with the exception of its southern part which is a lowland area. This region is adjacent by several mountains (e.g., Mt. Bromo-Tengger-Semeru on the south side, Mt. Arjuno-Welirang and Mt. Panderman-Kawi-Butak on the west side). This region covers a total area of 3882.44 km² and is consists of three distinct administrative regions: (i) Malang City (145.28 km²; 5 sub-districts), (ii) Malang Regency (3534.86 km²; 33 sub-districts), and (iii) Batu City (202.3 km²; 3 sub-districts) in (Figure 1).

Analysis

The modeling process involves utilising the occurrence data of encounter *Ae. aegypti*. In this study, two distinct models were utilised: (1) utilizing merely an environmental model, and (2) integrating both environmental and anthropogenic variable into a combined model. In addition, we projected future models involving two climate change scenarios using the present distribution model that performed the best. Furthermore, resampling was performed on all predictor variables. Multicollinearity tests are particularly necessary when examining environmental variables.

Data

A total of 35 occurrence data of *Ae. aegypti* in the Malang region was obtained through the collection of primary and secondary data sources from literatures (Gama et al. 2013; Gama & Salsabila 2021). Primary data collection was con-

ducted at the larval and imago stages, using a random selection of locations both indoors and outdoors. Both active and passive methods were used to obtain samples. The larval sample collection was conducted using the dipping method, whereas the imago specimens were collected using aspirators. The larvae are collected using the passive method called ovitrap, while the adult mosquitoes are collected using a UV mosquito trap. In order to validate the species of mosquitoes, we adhere to the identification guidelines provided by Becker et al. (2020). Each sampling location coordinate recorded using GPS Garmin 64s.

The predictor variables utilised in our study are associated with environmental factors, such as climate and topography, as well as population characteristics, including population density and poverty number. The climate variable data utilised in this study was acquired from the WorldClim v.2.1 dataset (~1 km²) (Fick & Hijmans 2017). Additionally, the topography data was obtained from the Shuttle Radar Topography Mission (SRTM) dataset (Jarvis et al. 2008). Furthermore, we obtained the Normalized Difference Vegetation Index (NDVI) variables (~30 m²) and tree canopy cover data for the year 2000 from composite datasets of Landsat cloud-free images (Hansen et al. 2013). The latest available anthropogenic data pertaining to population density and poverty was obtained from the BPS-Statistics Indonesia Batu Municipality (2022), BPS-Statistics Indonesia Malang Municipality (2023), and BPS-Statistics Indonesia Malang Regency (2021).

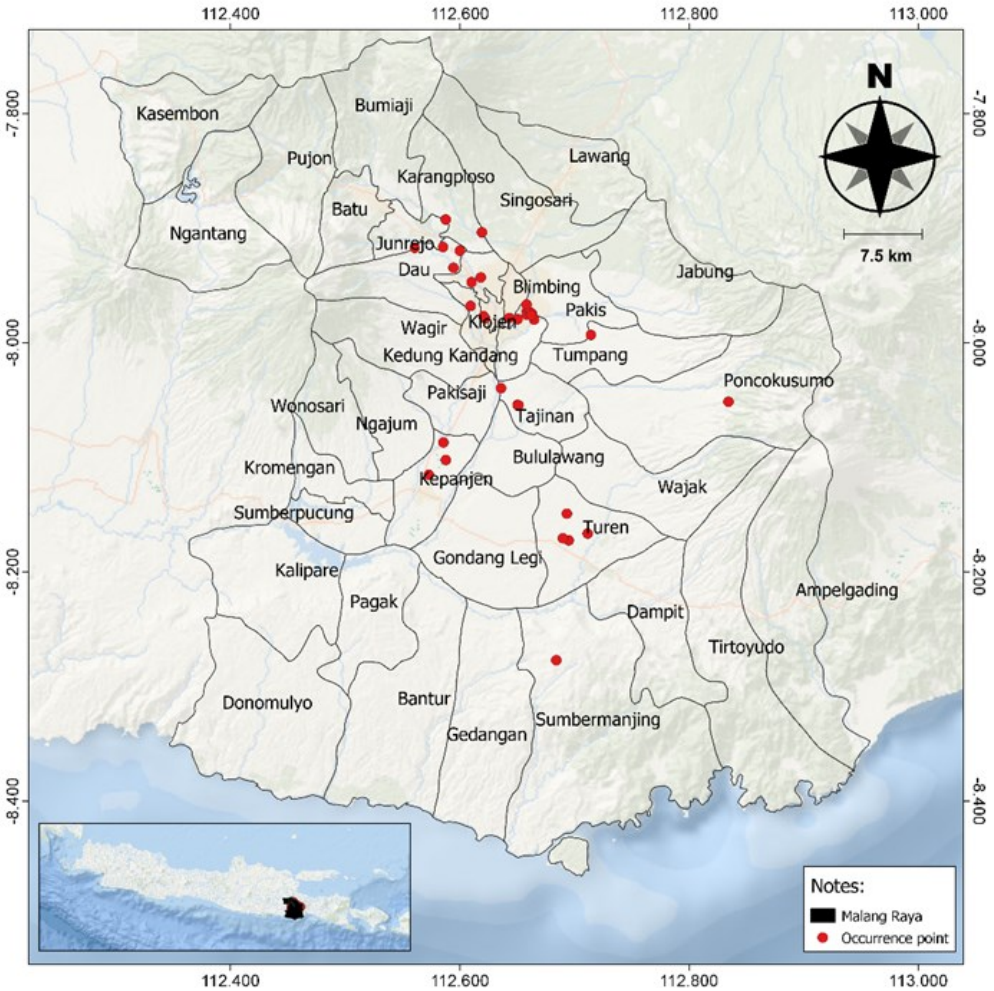

Figure 1. Occurrences of *Ae. aegypti* utilize in this study.

Preprocessing

The RStudio 2023.06.2+561 version software was utilised in order to carry out the preprocessing on each and every predictor variable. The raster repre-

sentations of all environmental predictor variables are obtained, though not necessarily in the same resolution or spatial projection across the board. Hence, we are going to have to perform resampling using the raster package first (Hijmans 2023). We decided on a resolution of 250 by 250 meters so that it would be better to give the biological rational following Wiese et al. (2019). We determine variables related to the anthropogenic profile by referring to Obenauer et al. (2017), which are population density and poverty.

Variable selection

It is essential to evaluate the presence of multicollinearity among the predictor variables to prevent overfitting, which can render the model unsuitable (Pradhan & Setyawan 2021). A multicollinearity test was performed on all environmental variables utilising the VIF (variance inflation factor) statistical approach. The *vifcor* function, which is part of the *usdm* package (Naimi et al. 2014), is employed to perform this step. The VIF analysis generated results indicating that there were 10 environmental predictor variables that exhibited no issues of multicollinearity (Table 1).

Table 1. The variance inflation factor (VIF) values of the environmental variables selected in the modeling process.

Code	Variable	VIF value
bio2	Mean Diurnal Range	1.91
bio3	Isothermality	1.78
bio4	Temperature Seasonality	2.53
bio13	Precipitation of Wettest Month	14.48
bio14	Precipitation of Driest Month	23.72
bio15	Precipitation Seasonality	25.98
bio18	Precipitation of Warmest Quarter	6.45
Elev	Elevation	6.48
ndvi	Normalized Difference Vegetation Index Landsat 8	2.08
treecov	Tree Cover	2.22

Species Distribution Modelling using MaxEnt

Several distribution modelling algorithms have been employed to represent mosquito niches and spatial distribution, including the maximum entropy algorithm. The utilisation of this algorithm, which is based on machine learning, is widespread due to its ability to operate effectively with a modest quantity of presence data (Elith et al. 2006). The software utilised in this study was MaxEnt version 3.4.4 (Phillips et al. 2023), employing a cross-validated approach with 10 replications. A training presence threshold at the 10th percentile was employed, which involved excluding areas with habitat suitability values below 10 % of the encounter point. In order to enhance the optimisation of the conducted modelling, we implemented various adjustment in experimental parameters. These parameters include the q2lqpt threshold set to 0, the l2lq threshold set to 0, the beta threshold set to 1.83, the beta categorical set to 0.1, the beta lqp set to 0.9, and the beta hinge set to 0.5. It is essential to establish appropriate measures to reduce the possibility of overfitting or underfitting, particularly in scenarios where the sample size is relatively limited (Radosavljevic & Anderson 2014). In addition to the implemented modifications, the default adjustments provided by the MaxEnt software were utilised. The evaluation of the model generated in each iteration is conducted

by evaluating the area under the curve (AUC) value revealed on the receiver operating characteristic (ROC) curve.

Data visualisation

We used QGIS 3.18 to visualize probability maps of *Ae. aegypti* distribution in each model, environmental and combination. After finding the model with the highest AUC value, which was assumed to be the best model, we mapped the area with the level of risk by adapting the HSC (Habitat Suitability Classification) classification concept carried out by Khan et al. (2022). HSC is categorised into five classes: p<0.2 (not suitable), 0.2-0.4 (least suitable), 0.4-0.6 (moderately suitable), 0.6-0.8 (highly suitable), and p>0.8 (very highly suitable). We also calculated the area of each class and made a ratio with the total area in each sub-district.

RESULTS

Environmental model

The Maxent model evaluation results indicated that a number of the selected environmental predictor variables (Table 1) comprised the most appropriate environmental model for estimating the presence of *Ae. aegypti* in the Malang region. The prediction reliability of the average value of the omission rate and predicted area was high. The replication resulted in an average AUC value of 0.877, with a standard deviation of 0.072. This value indicates that the predictive ability of the distribution modelling results is high.

According to the results of the jackknife analysis (Table 2), it is apparent that the variable with the highest percentage contribution was the mean diurnal range (bio2) variable. In addition, it was discovered that four additional variables exhibited a contribution percentage value exceeding 4 %. These variables include the normalised difference vegetation index (NDVI), isothermality (bio3), and tree canopy cover (treecov). Furthermore, the response curve is constructed as a probability estimation for a specific value of each variable (Figure 2). For instance, the variable bio2 exhibits an exponential increase within the temperature range of 7-10 °C, followed by a plateau once it surpasses 10 °C. Similarly, the variable NDVI demonstrates an exponential decrease within the range of 0-1, and then stabilizes after exceeding 1. Moreover, the variable bio3 displays an exponential increase within the range of 77-84, and then reaches a stagnation after surpassing 84. Furthermore, the variable treecov demonstrated a decreasing trend between 0-10 % and 50-100 %, while continuing to reach a stable state between 10-50 %.

Combination model (Environment + Anthropogenic)

The evaluation results of the MaxEnt model indicated that it exhibited robust predictive capabilities, as demonstrated by its performance when considering selected environmental predictor variables (refer to Table 1) in combination with population variables such as population density (PopDensity) and poverty (Poverty). The mean value of the omission rate and the predicted area demonstrated a high level of reliability in prediction. The mean area under the curve (AUC) value for the replication was found to be 0.890, accompanied by a standard deviation of 0.201. This finding suggests that incorporating both environmental and population variables in the modelling of *Ae. aegypti* distribution results in a more accurate and comprehensive model, as compared to utilising exclusively on environmental variables.

The findings of the jackknife analysis conducted on the combined variables (Table 3) regarding the relationship between environment and population exhibit that they congruent with the environmental model. This is evident as the variables bio2 (52.9 %) and ndvi (10.2 %) continue to demonstrate a substantial contribution percentage, despite the addition of the PopDensity

variable (27.2 %) in the analysis. Despite the aforementioned, the response curve was generated to represent the probability estimation at specific values of each variable within the combination model (see Figure 3). Specifically, the variable "bio2" exhibited an increase within the temperature range of 7.0-10.25 °C, followed by a decrease beyond 10.25 °C. Similarly, the variable "PopDensity" demonstrated a decrease within the ranges of 0-500 and 2500-3500 families per subdistrict, while the probability increased within the range of 500-2500 household per subdistrict.

Table 2. Percentage contribution of environmental predictor variables based on jacknife analysis.

Predictor variable	Percentage contribution
bio2	63
ndvi	18.6
bio3	5.7
treecov	5.2
bio4	3.2
elev	1.4
bio13	1
bio14	0.9
bio18	0.9
bio15	0.1

Table 3. Percentage contribution of predictor variable involve in combination model based on jackknife analysis.

Predictor variable	Percentage contribution
bio2	52.9
PopDensity	27.2
ndvi	10.2
treecov	2.5
bio4	2.2
Poverty	2.1
bio3	1.5
bio13	0.6
elev	0.2
bio14	0.2
bio18	0.2
bio15	0.1

Future distribution model

Considering that a combination of models (based on AUC values) can precisely represent *Ae. aegyptis* niche and distribution, we tend to utilise this model to categorised risk level based on HSC (Habitat Suitability Classification). According to Figure 4, Malang City and several areas of Malang district have a high probability of encountering *Ae. aegypti*, while

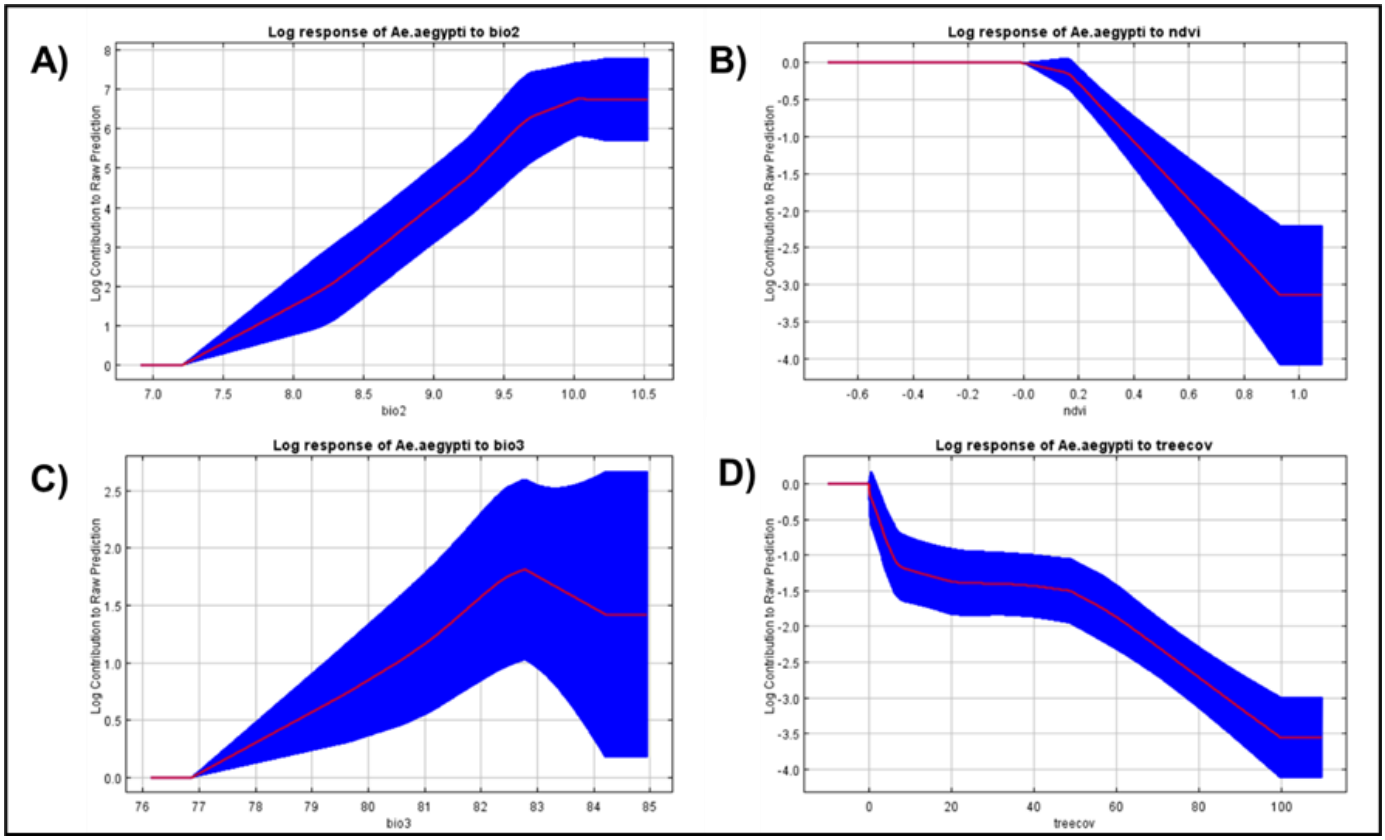

Figure 2. Variable response curve (contribution >4 %) according to environmental model.

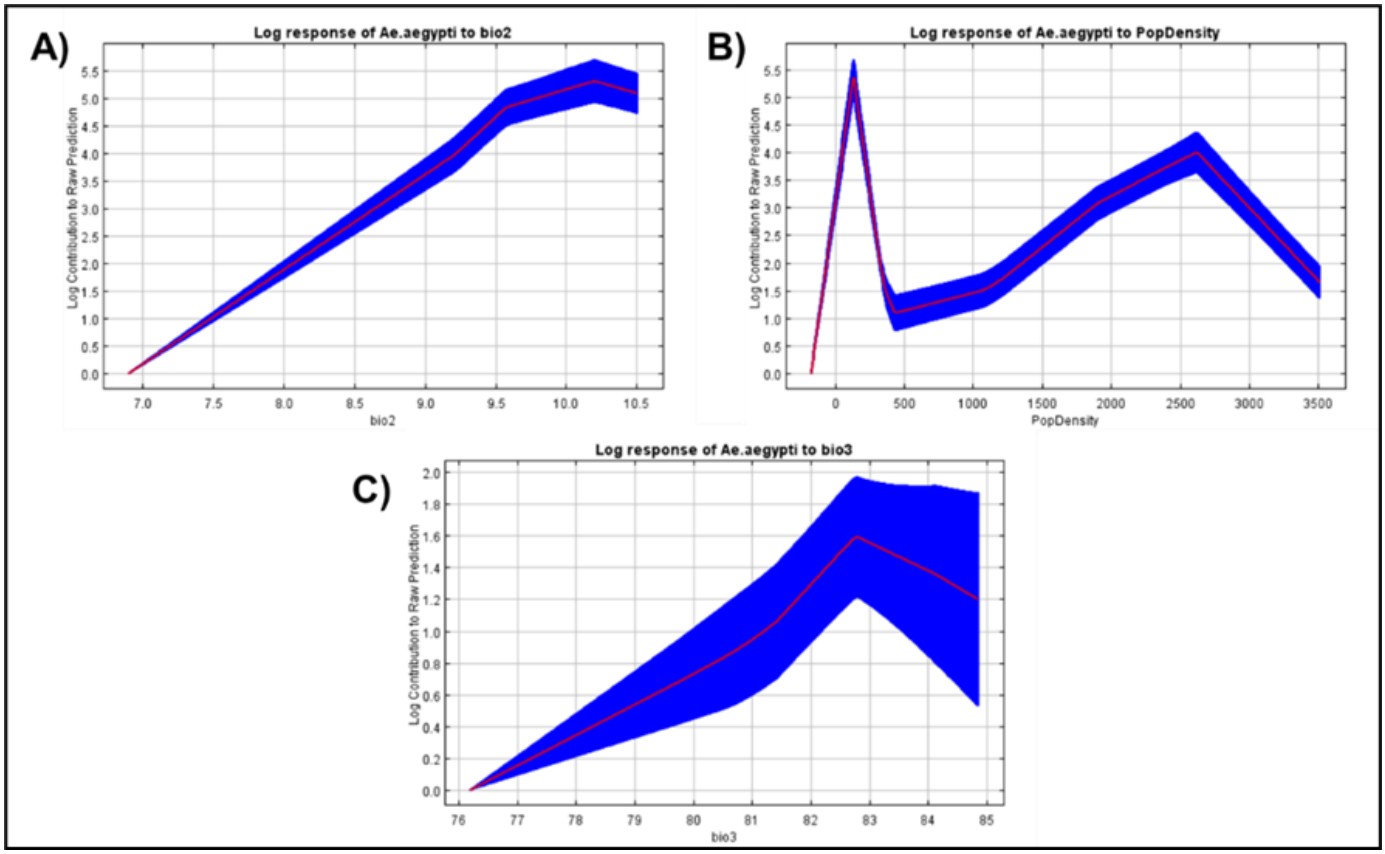

Figure 3. Variable response curve (contribution >4 %) in combination model (environment + population).

Batu City has a low probability. Malang Region has a potential distribution area for *Ae. aegypti* (HSC-2, HSC-3, HSC-4, and HSC-5) of approximately 545.5 km². The findings of our future projection analysis suggest a projected expansion in the suitable habitat for *Ae. aegypti* in 2040. The total area of this expansion encompasses a best-case scenario that involves an approximate

expansion of 0.63 % (23.4 km²). In comparison, the worst-case scenario entails a 0.56 % expansion (21 km²) (Table 4).

The prioritisation of recommended locations for mosquito control in Malang Region is determined by calculating the ratio between the area in each HSC category and the total area in a sub-district. Several sub-districts within the Malang region exhibit the potential for dengue fever transmission, albeit occupying only 14.5 % of the total area. This likelihood is determined by the presence of areas with a probability exceeding 0.2, specifically within the HSC 2 to HSC 5 range. The districts in the Malang region that exhibit a habitat suitability percentage of over 50 % and highest HSC-5 ratio for the transmission of dengue fever in present distribution model, in sequential order, are as follows: Klojen, Blimbing, Sukun, Lowokwaru, Kedungkandang, and Pakisaji. In the projection of the *Ae. aegypti* distribution model for the year 2040, it is observed that several sub-districts continue to be classified as high-priority areas. The proportion of these sub-districts in the risk list is significantly greater when compared to the present distribution model. Furthermore, it has been observed that a specific sub-district, namely Kepanjen, which was previously excluded from the priority area, has indeed witnessed a rise in the proportion of suitable *Ae. aegypti* distribution (Figure 5).

Table 4. Current and future habitat suitability of *Ae. aegypti* in Malang region.

Habitat Suitability Classification	Current		2040			
			ssp126		ssp585	
	km²	%	km²	%	km²	%
HSC-1	3215.7	85.5	3194.3	84.9	3218.5	84.9
HSC-2	295.3	7.85	306.3	8.14	286.9	7.63
HSC-3	107.8	2.87	108.1	2.88	134.9	3.59
HSC-4	74.1	1.97	71.3	1.90	73.4	1.95
HSC-5	68.3	1.82	83.2	2.21	71.1	1.89
Total suitable area (HSC-2 – HSC-5)	545.5	14.50	568.9	15.13	566.5	15.06

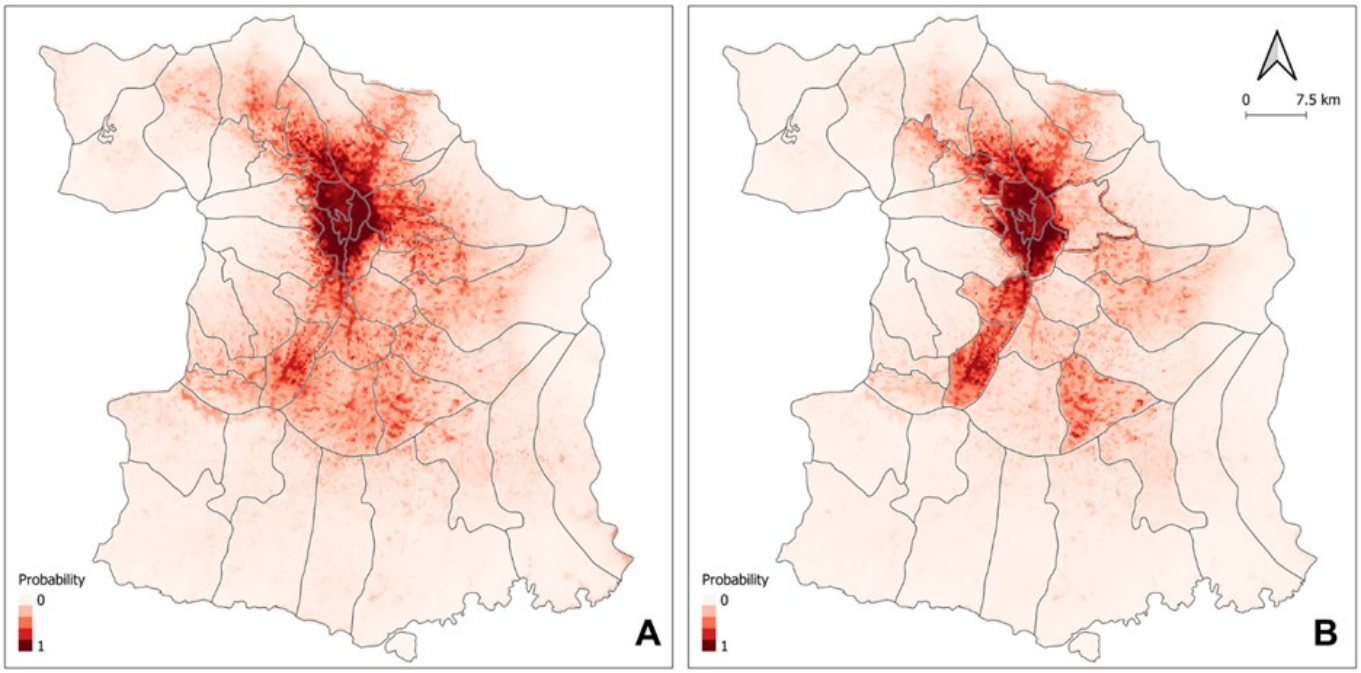

Figure 4. Possible extant of *Ae. aegypti* based on (A) environmental and (B) combination (environment + population) models.

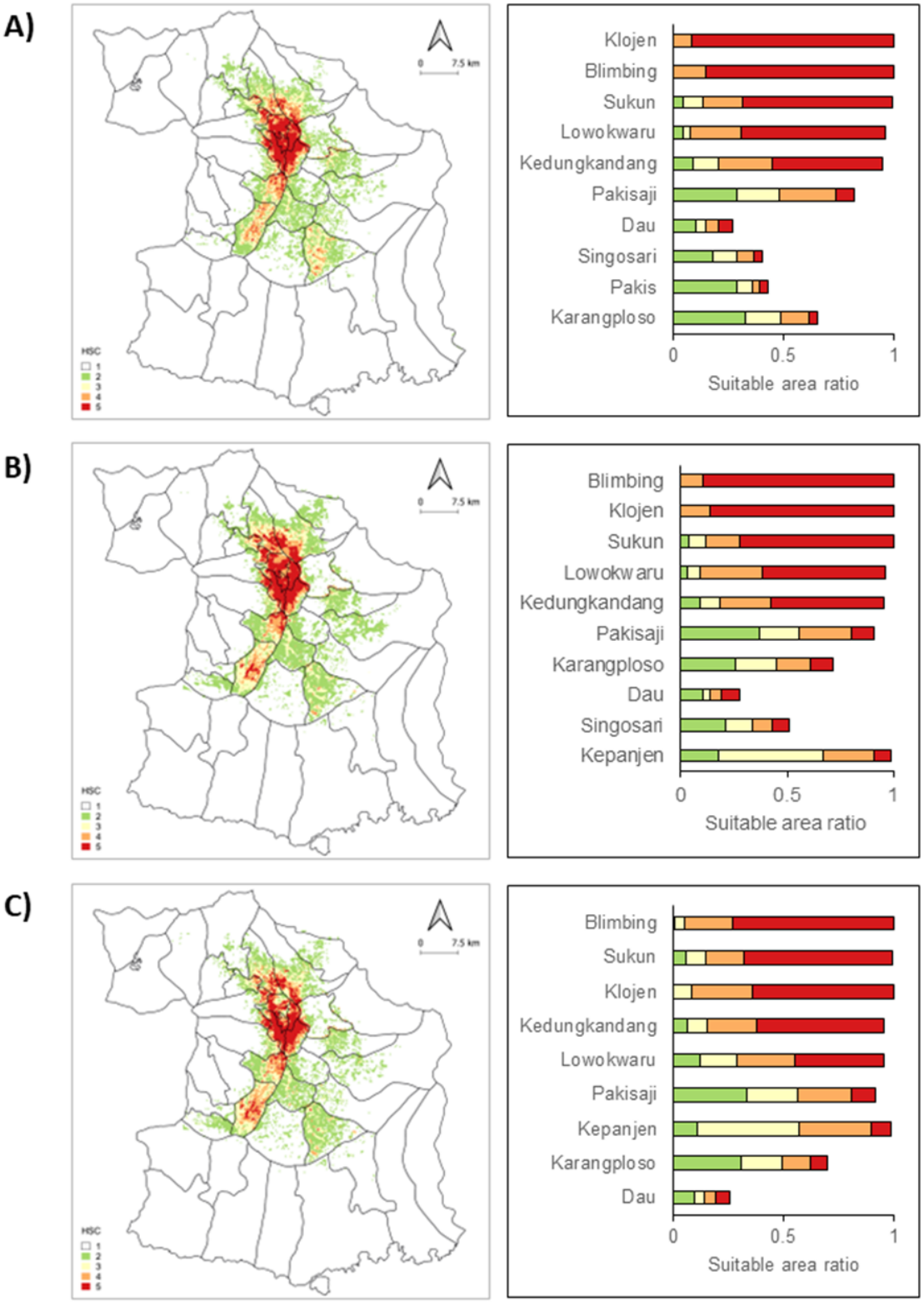

Figure 5. Spatial distribution of *Ae. aegypti* in each sub-district assessed based on HSC in (A) the present and the next 20 years ((B) best scenario and (C) worse scenario).

DISCUSSION

According to the outcomes of our modeling analysis, encompassing both environmental and combination models, it is evident that the prevalence of *Ae. aegypti* is notably higher in regions characterized by relatively warmer temperatures. Multiple prior studies have also reported similar findings (Lozano-Fuentes et al. 2012). The reason for this behavior can be attributed to *Ae. aegypti* poikilothermic nature (Upshur et al. 2019). Moreover, it is important to understand that elevated temperatures typically have an impact on the rela-

tive humidity as a result of evaporation phenomena (Thani et al. 2017). Consequently, under such circumstances, *Ae. aegypti* mosquitoes are able to fly without encountering any hindrance caused by the increased relative humidity present in the surrounding environment. Valdez et al. (2018) demonstrated that there exists a negative correlation between the abundance of *Ae. aegypti* and precipitation levels. The influence of climatic factors, such as temperature and precipitation, on mosquitoes or insects in general is well-recorded. However, it is noteworthy that vegetation conditions exhibit a distinct relationship with the habitat preferences of *Ae. aegypti*. Specifically, these mosquitoes tend to select areas characterized by low vegetation density.

Despite the fact that the environmental model demonstrates concordance with numerous studies and exhibits favourable model evaluation outcomes, the best model derived from this study was determined to be a combination of models integrating both environmental and anthropogenic variables. According to this model, it is hypothesized that, *Ae. aegypti* exhibits a preference for densely populated residential regions, with a particular preference for indoor habitats (Samson et al. 2015; Martin et al. 2019). This preference is associated with an attraction for human blood (Gomes et al. 2005; Mengko & Tuda 2016). Ratnasari et al. (2020) have also indicated that, *Ae. aegypti* commonly engages in oviposition within diverse categories of artificial receptacles that hold stagnant water, such as water drums, flowerpots, plastic cups, and discarded tires. Currently, the management of *Ae. aegypti* existence has proven to be challenging due to the diverse range of containers found in human settlements, which has led to uncertainty regarding their preferred sites for egg-laying (Hribar et al. 2004; Barrera et al. 2008; Arana-Guardia et al. 2014). The containers in question are frequently linked to disadvantaged areas of poverty (Obenauer et al. 2017; Martin et al. 2019; Souza et al. 2023). However, our model indicates that in the Malang region, poverty does not provide a significant contribution to our model. We hypothesise that, *Ae. aegypti* favours reproduction in various forms of standing water (Agustin et al. 2017), which is not exclusive to poor neighborhoods but can also occur in more affluent areas. Furthermore, urban areas that lack efficient waste management and drainage systems can serve as favourable environment for the development of *Ae. aegypti* mosquitoes (Banerjee et al. 2015), even though the socioeconomic condition of the subdistrict. Furthermore, the validity of the model's demonstration requires additional empirical research to verify the data in Malang region.

Our future projection model suggests that, *Ae. aegypti* may expand in 2040 within scenarios. In the best-case scenario, CO_2 emissions are reduced and dispersed more widely than in the worst-case scenario. This is unusual because insects prefer high CO_2 and temperatures (Menéndez et al. 2007; Menéndez 2007). The rise in temperature caused by CO_2 emissions may also contribute to this condition. High temperatures (20–30 °C) accelerate *Aedes* spp. metabolism, speeding up its life cycle. The rising temperatures in Malang region may lead to the spread of *Ae. aegypti*. In addition to in consequently, could result in an increase in reported cases of dengue virus, as indicated in previous research conducted by Stephenson et al. (2022). However, extreme high temperatures (>30 °C) directly affect the hatching percentage of *Ae. aegypti*, with higher temperatures resulting in fewer hatchlings and vice versa (Mohammed & Chadee 2011). *Aedes* spp. larvae and pupae develop in puddles of water, and relative humidity indirectly affects evaporation (Steinhoff et al. 2016). The presence of exceedingly elevated temperatures leads to a reduction in relative humidity and an increase in evaporation rates, thereby instigating competition among larvae. Increasing the evaporation rate reduces the standing water where *Aedes* spp. larvae develop, potentially reducing their density (Alto et al. 2015; Bara et al. 2015).

In general, the utilisation of MaxEnt as a distribution modeling method for the distribution of *Ae. aegypti* in the Malang Region demonstrates promising outcomes and can serve as initial data for subsequent investigations. Nevertheless, further investigation is required to validate the optimal ecological niche for *Ae. aegypti* and to effectively mitigate the transmission of DHF in Malang Region. This entails employing alternative algorithms (e.g., GLM or GAM), utilising predictor variables derived from locally accurate climate data. This research is particularly crucial in sub-districts with higher risk levels. In addition, it would be advantageous to acquire over time case data and involve direct observations to authenticate the congruity between the model constructed in this study and actual situations across different years.

CONCLUSIONS

The combination model of environmental variables and anthropogenic variables provide more comprehensive approach to understand the niche and distribution patterns of *Ae. aegypti* compared to relying solely on climate models. Areas with higher temperatures, high population densities, and limited vegetation cover could become suitable habitats for *Ae. aegypti*. Based on modeling results, the distribution of *Ae. aegypti* in Malang Region currently covers around 14.5 % (545.5 km^2) of the total area. It is projected that this distribution has the potential to expand to 15.5 % (568.9 km^2) in 2040. Several subdistricts, namely Klojen, Blimbing, Sukun, Lowokwaru, Kedungkandang, Pakisaji, and Kepanjen are classified as high-risk areas that require special concern.

AUTHOR CONTRIBUTION

Z.P.G., B.Y., and N.K. were responsible for the design of the study. M.F.A., M.A.R., and P.R both participated in the collection of data. Z.P.G., M.A.R., and R.J.K. participated in the analysis and interpretation of the data. M.A.R initially drafted the manuscript. Z.P.G., B.Y., and N.K. supervised the entire research project as well as reviewed, edited, also proofread the final draft.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research Article

Stand Structure Characteristics of Fragmented and Primary Forests and Their Correlation to Carbon Stocks

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ABSTRACT
Stand structure contributes to forest biodiversity and productivity. The disparity of stand structure between fragmented and primary forests and how they affect carbon storage are poorly understood. This study determined differences among some stand parameters in fragmented and primary forests and the correlation between forest stand structure and carbon stock. Twenty-five replicate quadrats were established in Bukit Durang and Division 5, representing the fragmented forests, and Lambir Hills National Park and Kubah National constitute the primary forests. All trees with diameter at breast height of 10 cm and above were measured, and the tree species were recorded. Aboveground biomass was calculated and converted to carbon stock. Statistical analyses showed that tree density is comparable among the forests. However, species abundance, species dominance, basal area aboveground biomass, and carbon stocks are different. Large-diameter trees significantly contribute to carbon storage. Principal component analyses revealed basal area, tree diameter and carbon stock were positively intercorrelated and associated. Species dominance and tree density are intercorrelated and strongly associated. Conversely, the number of species is negatively correlated to species dominance and tree density. This study showed the significance of tree diameter in impacting carbon stock.

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INTRODUCTION

Recognising the association between forest stand structure and productivity in natural communities is essential to sustaining ecosystem services for humanity (Guo et al. 2021; Wang et al. 2024). Ecosystems regulate the Earth's climate by taking out carbon dioxide (CO_2) from the environment, with photosynthesis sequestering carbon dioxide and depositing it in natural reservoirs like forests, oceans and soil (Friedlingstein et al. 2019; Anjum et al. 2022; McLaughlin et al. 2023). These reservoirs retain carbon for extended periods, preventing its contribution to global warming (Deemer et al. 2016; Li et al. 2018). Terrestrial forests are particularly effective in sequestering excessive CO_2, aiding its recovery into ecosystem pools (Jeyanny et al. 2014). According to Lal (2018), approximately 80 % of the Earth's terrestrial carbon accumulates within 1 m of the soil globally. Living tree biomass contributes to carbon sequestration until the tree's demise (Nave et al. 2019). Forests in different regions exhibit varying C stock rates, influenced by factors such as forest and tree structure attributes, plant diversity and tree aboveground biomass (AGB) (Dossa et al. 2013; Jeyanny et al. 2014). The distribution of C stocks across regions depends on vegetation type, climate and land-use history. Tropical rainforests, which cover 30 % of the Earth's tree-fill areas and 45 % of the total worldwide forest areas (Abbas et al. 2020), stock 50 % of the global carbon in trees (Global Resource Institute 2023). Intact tropical forests, characterised by massive old trees and diverse plant species, significantly contribute to carbon sequestration and storage (Watson et al. 2018).

However, deforestation, forest degradation and fragmentation threaten tropical forests' carbon stocks (C stocks) (Chaplin-Kramer et al. 2015; Maxwell et al. 2019). Selective logging activities are responsible for 50 % of emissions from forest degradation, deplete tree species with high carbon content, and increase the risk of biodiversity loss (Ellis et al. 2019). Although richness, diversity and biomass may recover to unlogged levels within 35 years after logging, community composition differs from nearby primary forests (Hayward et al. 2021). The disturbance of the mature forests can significantly impact aboveground biomass in forests where areas with high disturbance intensity typically have lower aboveground biomass (Temesgen et al. 2015; Wulder et al. 2020).

The status of the logged-over forest after some period in terms of carbon storage and forest structure is essential to know, especially the one surrounded by an oil palm plantation. Deforestation can decrease species diversity (Priatna et al. 2012). The forest structure in forest fragments differs from the primary forests due to the changes in microclimatic conditions, such as higher temperatures, light prevalence, and lack of moisture (Camargo & Kapos 1995; Oliveira et al. 2008). Forest fragmentation significantly affects diameter at breast height (DBH), crown diameter and tree density (Hending et al. 2023). Many forest fragments are degraded, with poor habitat quality and low ecological integrity (de Paula et al. 2011). However, degraded forests can still store and sequester carbon; consequently, protecting damaged habitats alleviates greenhouse effects by reducing carbon footprint and enhancing carbon sequestration (Gross 2020).

Estimating biomass and carbon is crucial for forest conservation (Matthew et al. 2018). Estimating C stocks in forests needs an accurate aboveground biomass estimation (Yeboah et al. 2014). The basal area of a tree is a valuable indicator of its biomass and carbon (Power et al. 2019). Thus, a relationship between these variables is anticipated since the basal area and biomass are correlated to the stem diameter (Torres & Lovett 2013). The correlation between carbon stock and DBH in tropical forests varies depending on the forest type (Banoho et al. 2020). Intact primary forest tends

to have larger trees, which dominate the forest's carbon storage and are common across almost all types of forests, regardless of their niches or locations (Mildrexler et al. 2020; Bordin et al. 2021; Johnston et al. 2021). However, the relationship between C stock and the basal area varies depending on forest type and management practices (Dignac et al. 2017).

This study investigated the variations in selected stand parameters between fragmented and primary forests. We also evaluated the intercorrelation among tree stand parameters. Studies (Ellis et al. 2019; Hayward et al. 2021) have shown differences in vegetation assemblage and forest structure between unlogged and logged forests throughout all groups of tree size. Moreover, fragmented forests are exposed to unfavourable environments such as temperature, humidity, wind and light intensity, which typically impact the ecosystem structure and function in tropical forests, causing habitat isolation, loss of biodiversity, and decreased soil fertility (Wade et al. 2003; Abdullah 2016). Thus, it highlights the importance of exploring the differences in stand characteristics between fragmented and primary forests. Many ecological studies have focused on undisturbed tropical forests; however, vast areas of tropical fragmented forests are still inadequately studied regarding their composition and structure (Feeley & Silman 2011; Baynes et al. 2016). The main objectives of the present study were to assess the differences in stand structure of fragmented and primary forests and the correlation between forest stand structure and C stock. The findings can provide valuable baseline data for forest managers, aiding in decision-making processes to achieve best management forest practices. Furthermore, the study contributes to understanding disturbed forests' health status and addresses the critical need for sustainable forest management procedures.

MATERIALS AND METHODS

Study Area

The locations of selected study areas are illustrated in Figure 1. The selected fragmented forests in this study were located at Bukit Durang (N3° 29' 3.10", E113° 49' 15.60") and Division 5 (Div 5) (N3°34'3.45", E113°46'3.45"). The fragmented forests were located within an oil palm plantation owned by Saremas Sdn Bhd, a PPB Oil Palms Berhad subsidiary. Both forests are lowland mixed dipterocarp forests (MDF) with the historical selectively logged that ended in 1996. The fragmented forests in this study have been preserved as high conservation value (HCV) forests. The larger HCV area is Bukit Durang, approximately 989.9 ha, which is linear-shaped, while Div 5 forest is approximately 604 ha and is almost
heart-shaped. Bukit Durang and Div 5 forest areas are approximately 10.06 km apart. The average terrain of the forests is within 45 to 75 m a.s.l and receives 1,700–5,800 mm of precipitation around the year (Malaysian Meteorological Department 2020).

This study examined the primary forests of Lambir Hills National Park (N4°12'9.44", E 114° 2'33.03") and Kubah National Park (N1°36'44.16", E 110°9'51.37"). Lambir Hills National Park (NP) is approximately 6,949 ha and consists of lowland MDF with an average annual rainfall of about 1700–5800 (Malaysian Meteorological Department 2020). Kubah NP is 2230 ha, mainly covered with mixed dipterocarp forest receives between 2000 and 3000 mm (Khan et al. 2017). Kubah NP had a historical selective logging activity decades ago and stopped in the 1960's when the forest area was left to regrow (Hazebroek & Abang Kashim 2001).

Field Sampling

Vegetation surveys in all study areas were conducted to understand forest stand characteristics. The quadrat plot sampling method was employed.

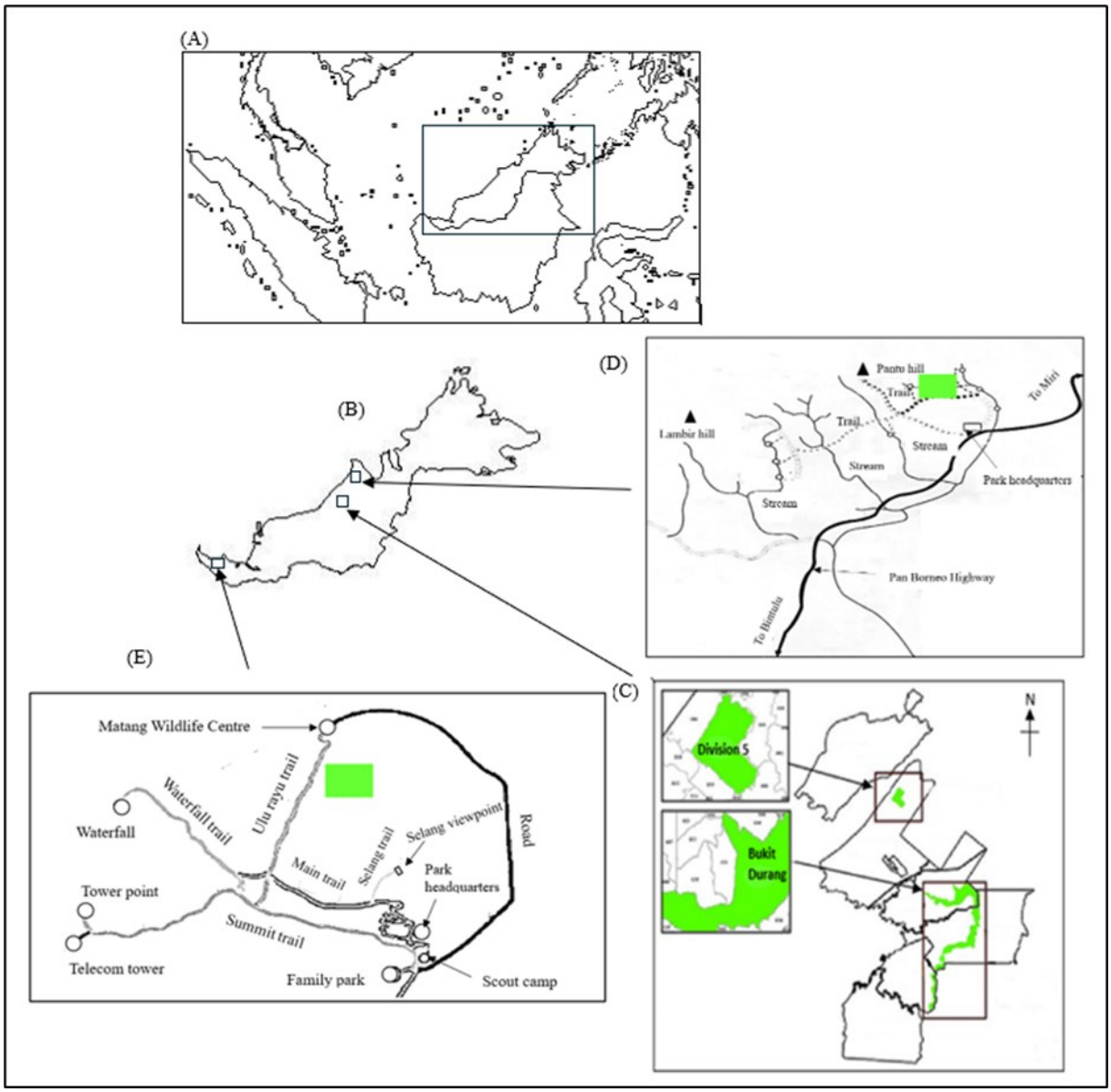

Figure 1. (A) Outline map parts of Malaysia and Indonesia. (B) Outline map of the Malaysian states of Sarawak and Sabah showing the study area. (C), (D) and (E) Location of the sampling area (shaded) of fragmented forests within the oil palm plantation, Lambir Hills National Park and Kubah National Park, respectively.

Quadrats were established and spaced at 100 m intervals on five parallel transect lines which were 100 m apart. This method provides a convenient method of enumerating the tree population as more ground can be covered with modest resources (Buckland et al. 2015). Twenty-five 20 m x 20 m replicate quadrats were created on the transect line in all study sites. Trees with DBH ≥10 cm were enumerated, and preliminary identifications of the tree species were performed according to their bark, slash, and leaf characteristics. Unidentified tree species were recorded at the genus level. Leave samples and fruits (if any) were collected for detailed species identification, which was conducted at the Universiti Malaysia Sarawak (UNIMAS) and Forest Department Sarawak herbarium.

Determination of forest stand parameters

The forest stand parameters refer to tree DBH, basal area, tree density, and number of species. Each tree DBH was measured at the breast height level (1.30 m above ground) using a tape diameter of the nearest cm. The total basal area of a species was calculated by multiplying the species tree density to obtain the species combined basal area in terms of m^2 ha^{-1}.

The calculation of a tree basal area is as follows:

$$\text{Basal area (BA)} = \frac{\pi D^2}{4}$$

Where π = Constant pi (approximately 3.14), D = tree diameter measured at breast height (DBH). The tree density (d) was computed by dividing the total number of trees (n) by the area of study site (m^2);

$$d = \frac{\text{Total number of trees (n)}}{\text{Area of study site } (m^2)}$$

Tree frequency (f) was determined by dividing the number of subplots the species present by the total number of subplots and multiple with 100;

$$f = \frac{\text{Number of subplot the species present}}{\text{total number of subplot}} \text{ x } 100$$

The importance value index (IVI) index describes and compares species dominance within each study area. It was determined by summing the total relative density, relative frequency and relative coverage (Gebeyehu et al. 2019). The IVI values vary from 0 to 300 and reflect each species dominance and abundance. A larger value indicates the more dominant a species is.

$$\text{Relative density (RD)} = \frac{\text{Number of individual species}}{\text{Total number of individual}} \text{ x } 100$$

$$\text{Relative frequency (RF)} = \frac{\text{Frequency of a species}}{\text{Sum of frequency of all species}} \text{ x } 100$$

$$\text{Relative dominance } (RD_o) = \frac{\text{Total basal area of a species}}{\text{Total basal area of all species}} \text{ x } 100$$

Estimation of Carbon Stock

Tree biomass and C stock were estimated using nondestructive methods. The AGB of all enumerated trees with a DBH of 10 cm and greater was computed by applying an allometric equation provided by Chave et al. (2005). The equation for the estimation of AGB is as follows:

$$AGB = p \text{ x } \exp(-1.499 + 2.148 \ln(D) + 0.207 (\ln(D))^2 - 0.0281 (\ln(D))^3$$

Where AGB is the dry weight of AGB (kg $tree^{-1}$), D is the diameter at breast height in centimetres (cm), and p is the basic density (g cm^{-3}) of the tree. Wood density values were acquired from the wood density database on the International Council for Research in Agroforestry (ICRAF 2007) website and PROSEA (1994, 1995, 1998). Air-dry wood density (10–18 % moisture content) was transformed to basic wood density by multiplying it by 0.861 (Vieilledent et al. 2018). Chave et al. (2005) equation is frequently used in forest inventory studies to approximate the aboveground biomass of trees in tropical forests. This equation is based on a large dataset of trees from different tropical forest sites worldwide. It incorporates the region's forest data, including Pasoh (Malaysia), Sumatra and Kalimantan (Indonesia), and provides a relatively simple and accurate way to estimate AGB. It uses a logarithmic transformation of D to consider the non-linear function between tree size and biomass. The equation was developed using a rigorous statistical approach and has been demonstrated to provide accurate aboveground biomass values throughout a broad range of tropical forest types and sizes. The equation is appropriate for determining the AGB of the different classes of forest locations included in this study. The C stock of all enumerated trees was estimated. A conversion coefficient of 0.5 was used to transform AGB (dry weight) to carbon equivalents (C) (ton) (Petersson et al. 2012). The AGB values were converted to C stock using the equation below.

Total Carbon Stock (Mg C ha^{-1}) = Total AGB x 0.50

Data Analysis

Differences in stand parameters (DBH, basal area, species abundance), AGB and C stocks among the study areas, were made using Analysis of variance (ANOVA). The parameter means of the study areas were compared using Tukey's HSD tests. Principal component analysis (PCA) with varimax rotation was performed to determine the variables responsible for the most variations in the datasets. The association between C stock and tree structure parameters was established by performing the PCA. The PCA also identified the principal components (PCs) that are dominant factors in determining the correlative effect among tree structure parameters. Principal components with eigenvalues of 1.0 or more that explained 70 % of the variation were selected as the dominant factors. These datasets included stand structure parameters, IVI, and C stock across the combined datasets in the overall study areas. Statistical analyses were done using IBM Statistical Software Statistical Product and Service Solution (SPSS) 2.0 for Windows Version 9.1.

RESULTS AND DISCUSSION

Stand Structure

Generally, the trend of tree density with DBH≥10 cm recorded in fragmented forest areas is comparable to the primary forests (Table 1). The statistical analyses show that the tree density in the study areas is comparable.

Nevertheless, Bukit Durang (723 trees ha^{-1}) showed a relatively higher tree density than other study areas. The past recorded data reported that the number of trees per ha in other tropical primary forests found in southern regions of Sarawak was relatively comparable to this study, such as in Semenggoh Nature Reserve (710 trees ha^{-1}) (Diway et al. 2009), Bako National Park (792 trees ha^{-1}) and Batang Ai National Park (813 trees ha^{-1}) (Ling & Julia 2012). The tree density in other disturbed forests was reported to be comparable, as in the fragmented forest of Bukit Jugam, Bintulu logged-over forest (728 trees ha^{-1}) (Demies et al. 2019). The higher tree density in fragmented forests suggests that the study areas might be experiencing favourable conditions for regrowth or could have a different composition of tree species that are more resilient to the disturbance factors present. Species resilient to forest disturbance are important in understanding the potential for forest recovery in fragmented areas and can provide insight into strategies for promoting regrowth and restoration in deteriorated forests (Nunes et al. 2021; Mills et al. 2023).

The number of species differed between study areas. Div 5 and Kubah NP recorded more species than the other two study areas. According to MacKinnon et al. (1997), an intact rainforest typically can have up to 240 different plant species within one ha. Comparatively, the present study recorded a higher species number per hectare in the lowland mixed dipterocarp forests in Semenggoh Arboretum, Sarawak (61 species ha^{-1}) (Ling & Julia 2012), Batang Ai NP (45 species ha^{-1}) and Pasir Tengkorak Forest Reserve, Langkawi Island (120 species ha^{-1}) (Hayat & Abd Kudus 2010). However, the number of species observed within the study area was relatively low compared to Kuala Keniam, Pahang NP (371–450 species ha^{-1}) (Suratman 2012).

The comparatively lower species number in Lambir Hills NP than in other study areas could be attributed to the forest's high basal area, which implies that the forest is in an advanced stage of maturity with larger, older trees, promoting increased competition for resources among tree species. Competition among trees in closed-canopy forests like mature tropical ones usually reduces individual tree growth and mortality, primarily due to the limited light levels beneath the canopy (Lasky et al. 2014; Rozendaal et al. 2020). The intense competition also diminishes tree species' adaptive

Table 1. Structural characteristics of Bukit Durang, Division 5, Lambir Hills National Park and Kubah National Park of trees with DBH ≥ 10 cm.

	Study area			
	Fragmented forest		Primary forest	
Variable	Bukit Durang	Division 5	Lambir Hills National Park	Kubah National Park
Tree density (trees ha^{-1})	723 (± 39)^{a*}	785 (± 47)^{a}	747 (± 31)^{a}	684 (± 37)^{a}
Number of species (ha^{-1})	161 (± 22)^{b}	218 (± 28)^{c}	131 (± 27)^{a}	213(± 18)^{c}
Basal area (m^{2} ha^{-1})	29 (± 3)^{a}	30 (± 2)^{a}	43 (± 3)^{b}	29(± 2)^{a}
Aboveground biomass (Mg ha^{-1})	355(± 36)^{b}	299 (± 26)^{a}	501 (± 46)^{c}	290 (± 24)^{ab}
Aboveground carbon stock (Mg C ha^{-1})	178(± 18)^{b}	150 (± 13)^{a}	251 (± 23)^{c}	145 (± 12)^{ab}

*Mean (±standard error) values within a row followed by different letters show significant differences at $P < 0.05$ using Tukey's test.

capability, elevating forest ecosystems' susceptibility to environmental stress (Magalhães et al. 2021). Intense competition might constrain the establishment and survival of certain species, ultimately leading to a decreased species count in the Lambir Hills NP study area.

Lambir Hills National Park has a significantly higher basal area than the other study areas because the forest area is intact without major disturbances, which suggests that Lambir Hills NP might have a more established and mature ecosystem than the other three study areas. An undisturbed forest with minimal disturbance can maintain its ecological balance (Shumba et al. 2020).

The variability in spatial and temporal disturbances in historically disturbed forests significantly impacts the regeneration, dominance and long-term survival of woody species within a forest ecosystem (Altman et al. 2016). The primary cause for the reduction in basal area, affecting tree growth and establishment, could be attributed to past anthropogenic disturbances in the fragmented forests (Bukit Durang and Div 5) and Kubah NP.

This study found varied C stocks in fragmented (Bukit Durang and Div 5) and primary forests (Lambir Hills National Park and Kubah National Park). Carbon stock values of fragmented forests and Kubah NP study areas were 145–149 Mg C ha^{-1}, which is lower than the typical C stock for tropical forests, estimated to be around 209 Mg C ha^{-1} (Slik et al. 2013). The primary forests of Lambir Hills NP have remarkably higher C stocks (251 Mg C ha^{-1}) in comparison to the other study areas, surpassing the typical C stock levels found in tropical forests. The results are expected as Shen et al. (2021) stated that fragmented forests had lower aboveground biomass than intact forests and suggested that this is due to the disturbance and reduced habitat quality. Previous studies reported that disturbed forests that have experienced selective logging have lower C stocks than natural forests (Berenguer et al. 2014; Rutishauser et al. 2015; Longo et al. 2016) since selective logging removes the large trees, leaving behind smaller and younger trees with lower carbon storage values.

The notably higher C stock in Bukit Durang than in Div 5 suggests that these forest areas have different carbon sequestration rates due to distinct contributing factors such as forest structure and species composition. Key elements contributing to the differences in diversity and vegetation carbon storage patterns in tropical forests include factors such as climatic variations and edaphic influences (Hofhansl et al. 2020). The post-logging impact on C stocks remains evident despite two decades since selective logging in both the fragmented forests and six decades in Kubah NP. This study indicates that forest recovery occurs relatively slowly after several decades of disturbance.

Lambir Hills National Park study area showed a remarkably high C stock value since the big and old trees dominated the forest. The intact

primary forest tends to contain considerable amount of AGB since it retains trees with greater diameter (Ngo et al. 2013). The old-growth forests generally have more AGB than logged forests, even after 20 years of logging. Rozendaal et al. (2022) reported in their meta-analysis study that the AGB values of pristine forests were significantly greater than those of logged forests. This was because the old-growth forests have more time to accumulate C stocks by retaining large trees, resulting in greater AGB values.

Species Composition

Table 2 shows the top five dominant tree species in fragmented and primary forests. In this study, fragmented forests had high abundance of fast-growing species. The abundance of *Macaranga triloba* in Bukit Durang and Div 5 is evident. *Macaranga* spp. is a major feature of disturbed vegetation in Malaysian forests (Demies et al. 2019; Takeshige et al. 2023). Fast-growing tree species are commonly found to be dominant in disturbed forest (Demies et al. 2019). Their study in a logged-over area of Anap Muput FMU, Bintulu, Sarawak recorded that pioneer species such as *Macaranga* spp., *Nauclea* spp., *Porterandia* spp., *Glochidion* spp. and *Gironniera* spp. dominate the area.

Lambir Hills National Park had high IVI values of *Dryobalanops aromatica*, with 21.4. The other species, such as *Xanthophyllum velutinum*, *Vatica nitens*, *Shorea falcifera*, and *D. globosus* have IVI ranging from 11.1 to 18.6. Kubah National Park was observed to have a high value of IVI of *Syzygium havilandii*, at 6.9. Other species' IVI represented by *Santiria tomentosa*, *Santiria rubiginosa* and *Shorea macroptera* ranged from 2.1 to 2.7. Generally, the study areas are dominated by the genera *Shorea*. This observation is typical because *Shorea* is highly distributed in Bornean dipterocarp forests (Purwaningsih & Kintamani 2018). All study areas showed a prominent presence of tree family belonging to the Dipterocarpaceae family. The dominance of Dipterocarpaceae in the study areas is in the order of Lambir Hills NP (33 %), Bukit Durang (30 %), Div 5 (27 %) and Kubah NP (23 %). It is well-known that Dipterocarpaceae predominates the lowland tropical forest of Borneo. A comprehensive research by Slik et al. (2005) reported that 21.9 % of the trees recorded in the lowland dipterocarp forest of Borneo were prevalently Dipterocarpaceae. This family also had the highest basal area contribution in the tropical forest (Gobilik 2008). Some of the trees in this family grow large and tall and often reach emergent or upper canopy heights. Their ability to attain substantial sizes over long periods contributes to their higher representation in the basal area of the forest area.

Carbon stock distribution among diameter classes

The distribution of C stocks by diameter class in fragmented and primary forests is shown in Table 3. Generally, the variation of C stock in the mid-diameter class (15–19.9 to 45–49.9 cm) is small. Although tree density decreased with diameter size, the C stock appears constant among these diameter classes. Lambir Hills National Park and Bukit Durang exhibited high C stock for DBH ≥ 60 cm. There were 20 and 15 trees with DBH ≥ 60 cm in Lambir Hills NP and Bukit Durang, respectively. In contrast, Div 5 and Kubah NP exhibit lower C stocks within the same diameter range, with only seven and three trees, respectively, indicating a substantial contribution to carbon storage in these forests comes from the presence of trees with larger diameters. Smaller trees in the diameter range of 10–14.9 cm DBH appear to have a relatively smaller contribution to C stocks despite their higher abundance. This finding concurs with early research by Chave et al. (2005) and Qie et al. (2017). The limited presence of large tree populations in forest

Table 2. Top five dominant species according to the Importance Value Index in fragmented and primary forests with DBH ≥ 10 cm.

Bukit Durang					
No.	Species	RD (%)	RF (%)	RD_o (%)	IVI
1	*Macaranga triloba* Müll.Arg.	7.1	3.6	6.1	5.6
2	*Elateriospermum tapos* Blume	4.8	3.2	5.5	4.5
3	*Shorea parvifolia* Dyer	3.9	1.8	4.5	3.4
4	*Shorea pubistyla* P.S.Ashton	3.2	2.0	4.9	3.4
5	*Shorea subcylindrica* Slooten	4.2	2.0	2.6	2.9
Division 5					
No.	Species	RD (%)	RF (%)	RD_o (%)	IVI
1	*Shorea macroptera* Dyer	5.6	2.7	5.4	4.5
2	*Elateriospermum tapos* Blume	1.5	1.7	1.8	1.7
3	*Shorea beccariana* Burck	1.7	1.7	1.8	1.6
4	*Shorea amplexicaulis* P.S.Ashton	1.7	0.8	2.0	1.5
5	*Palaquium pseudorostratum* H.J.Lam	1.6	1.8	1.0	1.5
Lambir Hills National Park					
No.	Species	RD (%)	RF (%)	RD_o (%)	IVI
1	*Dryobalanops aromatica* C.F.Gaertn.	5.4	3.5	12.6	21.4
2	*Xanthophyllum velutinum* Chodat	8.6	4.7	5.3	18.6
3	*Vatica nitens* King	8.2	4.5	4.3	17.0
4	*Shorea falcifera* Dyer ex Brandis	3.6	3.1	4.8	11.5
5	*Dipterocarpus globosus* Vesque	3.6	3.5	4.0	11.1
Kubah National Park					
No.	Species	RD (%)	RF (%)	RD_o (%)	IVI
1	*Syzygium havilandii* (Merr.) Merr. & L.M.Perry	8.0	3.6	8.9	6.9
2	*Santiria tomentosa* Blume	2.5	2.1	3.5	2.7
3	*Santiria rubiginosa* Blume	1.5	1.5	3.4	2.1
4	*Shorea macroptera* Dyer	2.9	1.7	1.8	2.1
5	*Castanopsis evansii* Elmer	2.1	1.7	2.0	1.9

Table 3. Tree density and carbon stock distribution by diameter class.

Diameter class (cm)	Fragmented forest				Primary forest			
	Bukit Durang		Division 5		Lambir Hills National Park		Kubah National Park	
	No. of tree	C stock (Mg C ha^{-1})	No. of tree	C stock (Mg C ha^{-1})	No. of tree	C stock (Mg C ha^{-1})	No. of tree	C stock (Mg C ha^{-1})
10–14.9	262	9	322	11	265	10	263	9
15–19.9	179	13	186	14	150	12	155	13
20–24.9	97	14	107	16	108	18	103	16
25–29.1	61	15	55	14	59	16	54	14
30 –34.9	37	15	36	14	48	20	33	13
35–39.9	27	16	31	18	27	16	25	16
40–44.9	17	14	19	16	26	22	20	17
45–49.9	10	10	13	14	25	30	13	13
50–54.9	8	12	7	9	11	15	5	7
55–59.9	10	18	2	4	8	15	10	17
≥ 60	15	42	7	20	20	77	3	10

areas reduces carbon sequestration potential in forest ecosystems (Lutz et al. 2018).

Carbon Stock Distribution Among Tree Species

The top five cumulative C stocks for tree species in fragmented and primary forests are shown in Table 4 and Table 5, respectively. Generally, shade-

Table 4. Top five cumulative carbon stocks for tree species in fragmented forests.

Bukit Durang			Division 5	
No.	Species	C stock (Mg C ha^{-1})	Species	Carbon (Mg C ha^{-1})
1	*Elasteriospermum tapos*	13	*Shorea macroptera*	8
2	*Shorea pubistyla*	8	*Ternstroemia citrina*	5
3	*Glochidion obscurum*	7	*Elateriospermum tapos*	4
4	*Shorea parvifolia*	7	*Lithocarpus bennettii*	4
5	*Macaranga triloba*	6	*Dipterocarpus sublamellatus*	4

Table 5. Top five cumulative carbon stocks for tree species in primary forests.

	Lambil Hill National Park		Kubah National Park	
No	Species	Carbon (Mg C ha^{-1})	Species	Carbon (Mg C ha^{-1})
1	*Dryobalanops aromatica*	41	*Syzygium havilandii*	13
2	*Shorea falcifera*	17	*Santiria rubiginosa*	6
3	*Shorea parvifolia*	16	*Santiria tomentosa*	5
4	*Xanthophyllum velutinum*	12	*Shorea havilandii*	5
5	*Elateriospermum tapos*	12	*Koompassia malaccensis*	5

tolerant species are slow-growing and comprise dipterocarp and non-dipterocarp tree species that dominate the carbon values in these study areas. Slow-grower trees with greater DBH, height, wood density and basal area typically showed higher AGB and C stock (Yeboah et al. 2014). Slow-growing tree species were consistently found to contribute significantly to high C stocks in all study areas. Tree species *Elasteriospermum tapos* (13 Mg C ha^{-1}) in Bukit Durang, *Shorea macroptera* (8 Mg C ha^{-1}) in Div 5, *Dryobalanops aromatica* (41 Mg C ha^{-1}) in Lambir Hills NP, and *Syzygium havilandii* (13 Mg C ha^{-1}) in Kubah NP were the primary contributors to the high levels of C stock in their respective study areas.

The high population of tree species of *D. aromatica* accounted for the high contribution of C stocks (41 Mg C ha^{-1}) in Lambir Hills NP. In this study, *D. aromatica* was also recorded to possess a high IVI (21.4). In contrast, the species IVI in other investigation regions fell within the range of 4.5–6.9. For instance, in the mixed dipterocarp forest of Endau Rompin National Park, Johor, the non-dipterocarp trees such as *Litsea costata*, *Dillenia reticulata* and *Syzygium* sp. were recorded as major contributors to the total forest carbon with the values of more than 12 Mg C ha^{-1} (Matthew et al. 2018). These non-dipterocarp tree species were recorded to have high numbers, large DBH, tall tree height and high wood density, contributing to the considerably high cumulative carbon content.

Correlation of Carbon Stock with Stand Parameters

The principal component analysis showed the association of C stock with some stand structure characteristics (Table 6 and Figure 2). It shows two principal components described 49 % of the variation. The first component explains 31.1 % of the total variation, and the second explains 17.8 %. Basal area, DBH and C stock were positively intercorrelated and associated with PC1 (Table 6). Species IVI and tree density are intercorrelated and strongly associated with PC2. In contrast, species number is negatively correlated to species IVI and tree density with PC2.

Kauppi et al. (2014) recorded that trees with large diameters significantly impact the carbon density in forests, suggesting that tree populations with large diameters are responsible for storing high amounts of carbon within the forests. These groups of trees also provide significant

weight as the foundation structures in forest ecosystems. They are also crucial in forest ecosystems as a food provider, shelter, microclimate modulation and hydrological system (Lindenmayer et al. 2014; Bradford & Murphy 2019). A study in Gunung Leuser National Park, Indonesia, recorded that carbon storage highly depends on the variation of tree diameters (Onrizal & Auliah 2020). Van Do et al. (2020) stated that basal area and AGB are correlated to trees of more than 30 DBH in old-growth forests of Vietnam, suggesting that forest areas with a higher concentration of huge trees will have a higher C stock.

Table 6. Variations from principal component analysis of stand structure across all the study areas explained by principal component axes.

Parameters	Component*	
	1	2
Basal area	**0.94**	0.02
Diameter at breast height	**0.91**	0.03
Carbon stock	**0.65**	0.09
Importance value index	0.11	**0.92**
Number of trees	-0.01	**0.73**
Number of species	-0.14	**-0.65**
Wood density	-0.02	0.41
Eigenvalues	2.18	1.25
% Variance	31.09	17.84
Cumulative explanation %	31.09	48.93

*Loading scores of stand characteristics in the first two principal component axes. Loading scores in bold are considered significant.

The PCA showed the correlation of C stocks with DBH, and the basal area is the strongest among the variables considered, suggesting that the higher C stock will be found in the forested region having the larger basal area. The result indicated that the basal area is possibly one of the significant elements that characterise the carbon content of living trees in a forest. The finding parallels the studies done by Dossa et al. (2013), Stephenson et al. (2014), Jeyanny et al. (2014) and Lutz et al. (2018). The basal area affects primary forests' C stock (Mensah et al. 2016). Wassihun et al. (2019) pointed out that increased basal area increases AGB and C stock. Joshi and Dhyani (2021) recorded a strong positive correlation between carbon stock and basal area in the forest that is dominated by *Shorea robusta* in Dhangadhi, Nepal. Over time, old trees with larger trunks and more extensive root systems contribute to higher biomass stocking (Köhl et al. 2017).

Ma et al. (2023) stated that basal area becomes more influential in determining AGB when forests are developed. The basal area is commonly associated with the diameter increment of stands in the forest (Chave et al. 2005). This study's results suggest that the size of trees and the total cross-sectional area of trees have a more substantial impact on C stocks. Trees with higher wood densities generally contain more carbon per unit volume, contributing to the forest carbon stocks. However, this study observed no significant correlation between wood density and C stock across the study areas based on the PCA analysis, which agrees with Francis et al. (2017), who reported that biomass is not related to wood density. However, the results contradicted the results of Dossa et al. (2013) and Jeyanny et al. (2014). They stated that the wood density significantly influences C stocks in forests. The ecological complexity within the study areas might be attributed to uncorrelated wood density with C stock. The wood density of trees within the study areas could be influenced by geographical area and species-specific

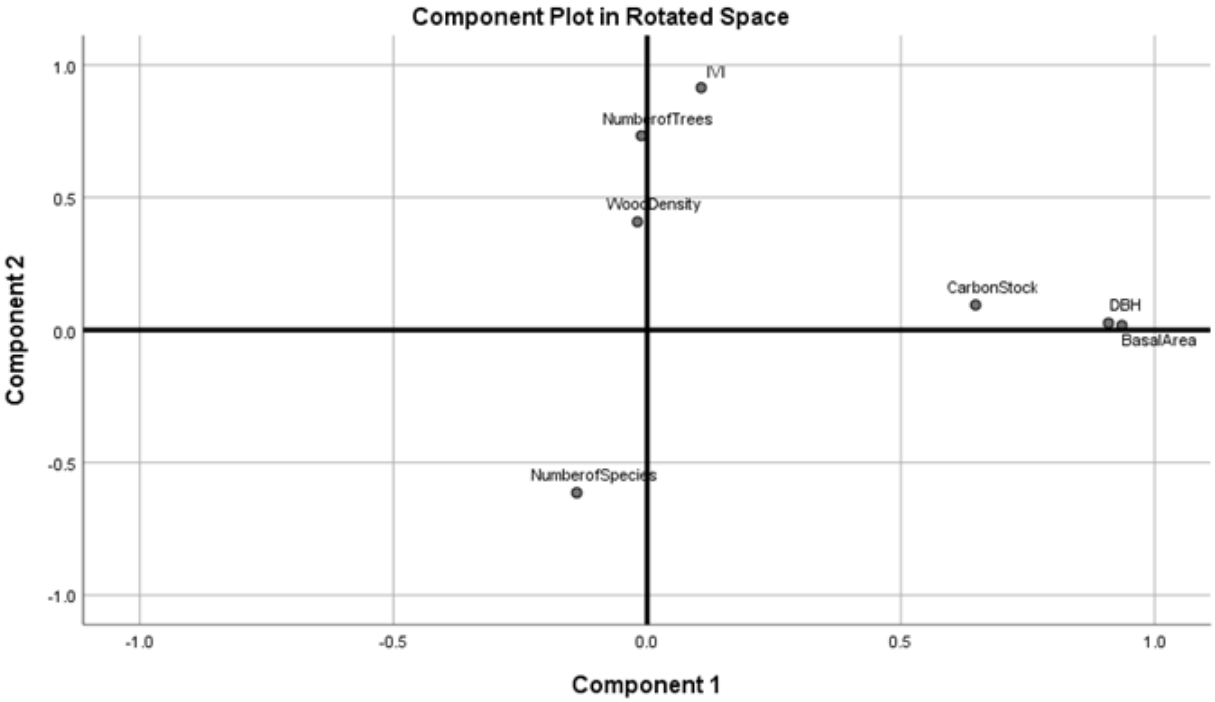

Figure 2. Distribution of stand structure attributes, importance value index and carbon stock in relation to axes of combined data of all the study areas.

factors because different tree species had varying carbon sequestration rates and tree sizes (Pérez-Cruzado et al. 2012). Wood density varies greatly across the tree species in tropical forests (Djomo et al. 2017), and tree diameter does not influence the variation of wood density (Ramananantoandro et al. 2016), implying that large trees do not necessarily have high wood density, which explains why wood density is not correlated with C stocks in this study. Species composition, soil properties and climatic influences have higher dynamic influence on carbon (Saimun et al. 2021). Thus, the forests dominated by low carbon sequestration and smaller trees have low C stock. Regarding species abundance, a negative association with species dominance (IVI) is shown, concurring with results from Shirima et al. (2015). It has been reported that greater stem density and basal area can reduce the species richness of an area (Djuikouo et al. 2014).

CONCLUSIONS

This study has demonstrated that tree density with DBH ≥ 10 cm between fragmented and primary forests is comparable. Fragmented forests were shown to have higher number of species. Generally, tree basal area and AGB are greater in the primary forest. The DBH and basal area significantly correlated with forest carbon stock. The findings highlight the vital role of large trees in storing carbon in forest ecosystems. Preserving large trees will keep carbon stored in the forest for a long time. Thus, removing large-diameter trees will result in major carbon emissions to the environment. Disturbed forests, such as fragmented forests and Kubah NP, have declined certain characteristics typical of undisturbed virgin forests. This includes alterations in the entire composition of forest species and a reduction in large tree diameters, thus decreasing C stocks. Generally, historically disturbed forests possess the potential for recovery, exhibiting the capability to develop stand structures comparable to primary forests, provided no human-caused disturbances occur. The present study has provided valuable insights into the correlation between tree DBH and C stock. The findings emphasise the necessity of conservation initiatives to deal with the issues related to forest

degradation and fragmentation. The potential future forest fragmentation and disturbances could negatively impact the presence of large trees and species diversity, which is particularly crucial for preserving C stocks and biodiversity in tropical forests.

AUTHOR CONTRIBUTION

A.N. conducted data collection, performed data analyses and wrote manuscript drafts. I.J. and M.H.B. were involved in the design and implementation of the research, results analyses and manuscript review.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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Research Article

Utilising Plant Extracts as Lures to Capture Ambrosia Beetles (Coleoptera: Curculionidae) in Cocoa Plantation

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ABSTRACT

The ambrosia beetle (Coleoptera: Curculionidae) is a significant pest affecting cocoa plants in South Sulawesi. The high intensity of their attacks poses a serious threat to cocoa production, causing plants to wilt and die. This study developed traps baited with active ingredient compounds from various plant species, including coffee, carrot leaves, fermented cocoa wood, and eucalyptus oil, and compared their efficacy with ethanol. Beetles collected in these traps were identified based on morphological characteristics using a stereo microscope. The attraction test results indicated that all treatments successfully attracted ambrosia beetles, with ethanol capturing the highest number of individuals (1391). The results showed that ethanol and other extracts could capture ambrosia beetles of various kinds. The highest number of captures was found in ethanol and carrot leaf extract treatments. Additionally, eleven species were identified: *Coccotrypes* sp., *Diuncus quadrispinulosus*, *Eccoptopterus spinosus*, *Hypothenemus* sp. 1, *Hypothenemus* sp. 2, *Hypothenemus* sp. 3, *Xyleborus affinis*, *Xylosandru s mancus*, *Xylosandrus crassiusculus*, *Xylosandrus eupatorii*, and *Xylosandrus morigerus*. Traps baited with carrot leaf extract were most effective in capturing *Hypothenemus* sp. 3. These findings underscore the importance of developing various attractant traps utilising plant chemical compounds to detect and identify ambrosia beetle species and mitigate severe crop damage.

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INTRODUCTION

Cocoa is one of Indonesia's staple plantation commodities (Bhattacharjee & Akoroda 2018). Indonesia is the world's largest cocoa producer, ranking third in 2019 after Cote d'Ivoire and Ghana. Indonesia contributed 1.6 million tons or 44 % of the world's cocoa production. However, this position could not be maintained, and Indonesia currently ranks sixth in global cocoa production (Yemima & Novianti 2020; Ministry of Agriculture 2022). South Sulawesi is one of the provinces that produces the most cocoa, with the Luwu region being the largest cocoa fruit-producing center, accounting for up to 35 % of production (Ministry of Agriculture 2022). The development of cocoa production has declined over years due to pest and disease attacks, one of which is ambrosia beetle infestation.

The ambrosia beetles belong to the order Coleoptera, specifically within the family Curculionidae and its subfamilies, Scolytinae and Platypodinae (Kirkendall et al. 2015). These beetles cause significant plant damage, leading to wilting and death by boring into plant stems, entering the tissue, and causing structural damage to the woody stems. This activity results in cracks in the stem, causing the cocoa bark to dry out and peel. Ambrosia beetles feed on the juicy phloem tissue and dead plant parts, and some species can infect healthy trees (Triplehorn et al. 2005; Hulcr et al. 2007). In addition to direct damage, ambrosia beetles can transmit various pathogens, such as fungi and bacteria, which can accelerate plant damage and death. In East Luwu Regency, several fungi were isolated from cocoa plants attacked by ambrosia beetles, including Fusarium, Lasiodiplodia, Ceratocystis, and Diaporthe colonies (Asman et al. 2021).

More information is needed regarding ambrosia beetles infesting cocoa plants in South Sulawesi. Research results indicate a high number of ambrosia beetles in cocoa plants. To control this, farmers generally use chemical insecticide spraying technology to reduce the population of ambrosia beetles. However, this method is ineffective because the beetles attack and develop inside plant tissues (stems and twigs). Therefore, an alternative technology that can be offered is the use of compounds that facilitate communication between insects and plants (Norin 2001). One effective approach is to use lures to attract ambrosia beetles, which are attractants derived from plant extracts. Previous studies have tested attractants using coffee and carrot extracts against the Cocoa Pod Borer pest and found that ambrosia beetles were also caught (Witzgall et al. 2010; Rivay et al. 2023).

Ambrosia beetle attacks on stems occur because the bark layer of plant stems contains aromatic volatile compounds that insects use to find suitable host plants (Rohman 2020). Ethanol plays a crucial role in attracting ambrosia beetles as a kairomone, enabling them to detect suitable hosts, such as stressed or dying trees (Graham 1968; Ranger et al. 2015). Ethanol is not only present in healthy trees but also found in the xylem and phloem, with its concentration increasing when the tree is stressed (Kimmerer & Stringer 1988; Kelsey et al. 2014; Lehenberger et al. 2021). In addition to ethanol, semiochemicals, such as attractant kairomones derived from plant chemical compounds, are widely used to monitor insect pest populations and keep them below threshold levels (Komala et al. 2021).

The behaviour of ambrosia beetles favors the scent emitted by woody plants, making fermented extracts from the decaying wood of the host plant highly effective. Newly emerged female beetles are particularly attracted to volatile compounds from damaged trees, especially ethanol (Cavaletto et al. 2023). Eucalyptus oil, a key compound in attractant mixtures, can also test the attractiveness of beetles (Kuhns et al. 2014). Adult beetles can be more easily captured during their dispersal phase by luring them into traps that emit volatile compounds mimicking the plant odors to which they are naturally attracted (Mazon & Gaviria 2013). Based on the problem caused by am-

brosia beetles attacking cocoa plantations, traps were set using bait-containing attractants to capture the presence of ambrosia beetles and reduce the attack.

MATERIALS AND METHODS

Study Site

This research was conducted in Tarengge Village, Wotu Subdistrict, East Luwu District, South Sulawesi Province, Indonesia is located between 2° 31' 58" – 2° 39' 57" South latitude and 120° 45' 20" - 120° 55' 38" east longitude (Figure 1) from March to August 2023. The study site is situated at an altitude of 15-68 m.a.s.l., with an average temperature, humidity, and rainfall of 26.97 °C, 82.39 %, and 15.57 mm, respectively. Observations were carried out in cocoa plantations featuring 10-year-old cocoa clone 45 trees infested with ambrosia beetles, as identified in a previous preliminary study.

Plant Extracts

The extraction of robusta coffee leaves and carrot leaves by performing a maceration process involves chopping the leaves until smooth then soaking them in a methanol solvent at a ratio of 1 kg of leaves to 3 liters of methanol. After three days, the extract is filtered to obtain a liquid extract, which was then evaporated using a rotary evaporator at 55 °C with a rotation speed of 100 rpm to separate the insoluble parts in methanol. The resulting extract was then placed in a water bath to obtain a 100 % plant extract. Furthermore, dilutions were made using the formula V1.M1 = V2.M2 to achieve a 10 % concentration by adding 10 g of extract into 90 mL of 70 % ethanol for carrot leaf extract. Dilution is done to reach a concentration of 15 % by adding 15 g of extract into 85 mL of 70 % ethanol for coffee leaf extract, so that the extract is ready to be used as an attractant.

Fermented cocoa wood is prepared from rotten cocoa wood, which is ground into a powder. Approximately 300 mg of this powder is weighed and placed into a container, then immersed in a water and molasses mixture at a 1:1 ratio. The container is tightly sealed to facilitate the fermentation process. After 14 days, the container is opened, the fermented liquid is filtered and used as an attractant. The eucalyptus oil utilised has been formulated and acquired from Cap Lang products of 100 % concentration, sourced from PT Eagle Indo Pharma, located in Tangerang City, Banten, Indonesia. The ethanol

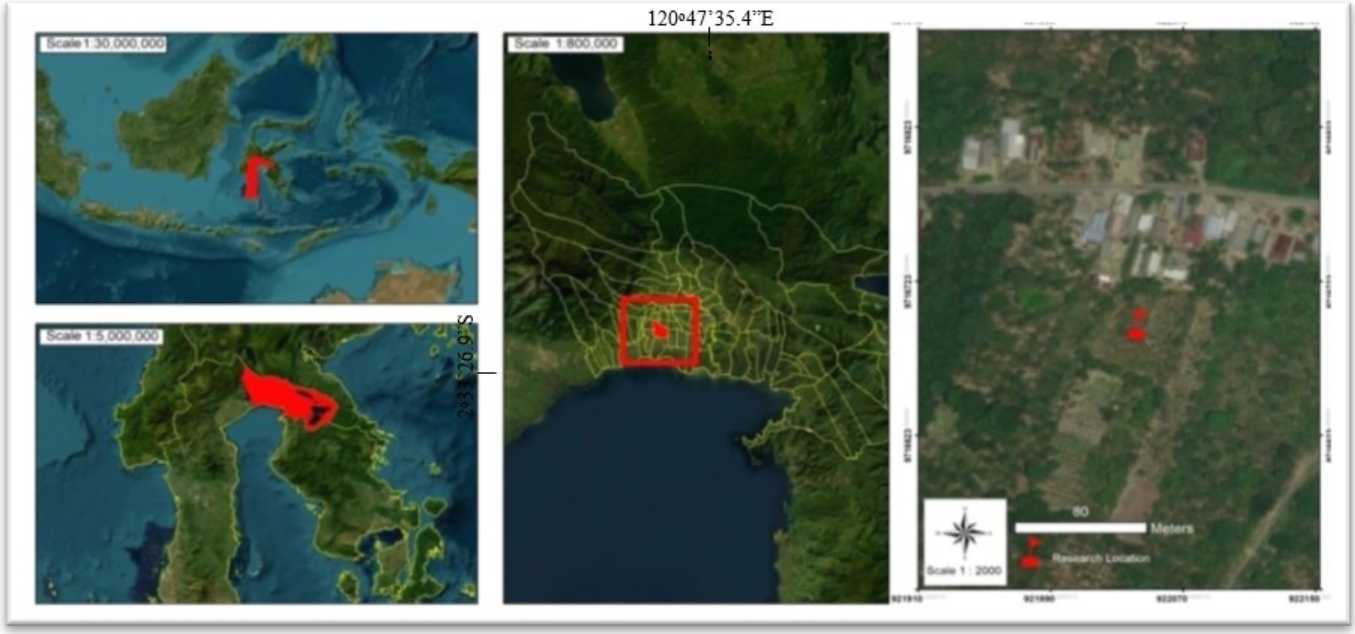

Figure 1. Study site in East Luwu District, South Sulawesi Province, Indonesia.

employed is sourced from One Med Alcohol 70 %, a PT Jaya Mas Medica Industri Product in East Java, Indonesia.

Attractant Trap

The traps utilised were created from plastic bottles measuring 32.2 cm in height and 8.2 cm in diameter, with one side of the bottle cut to form a 7×12 cm window. To attract insects, 0.5 g of cotton was hung inside the bottle, onto which 2 mL of attractant was sprayed. Additionally, an adhesive tube containing 5 mL of 70 % ethanol was placed at the bottom of the bottle to capture insects entering the trap. To protect against rainwater, a plastic plate was placed on top of the trap as a roof (Figure 2). The traps were then fastened to cocoa plants using plastic rope, positioned 50 cm above the ground. The treatments consisted of four types of plant extracts and ethanol. Each treatment involved two trees, with one trap set on each tree, and this was repeated three times, resulting in 30 traps set in each plot arranged in a randomized block design. The traps were positioned diagonally with 12 m spacing between them.

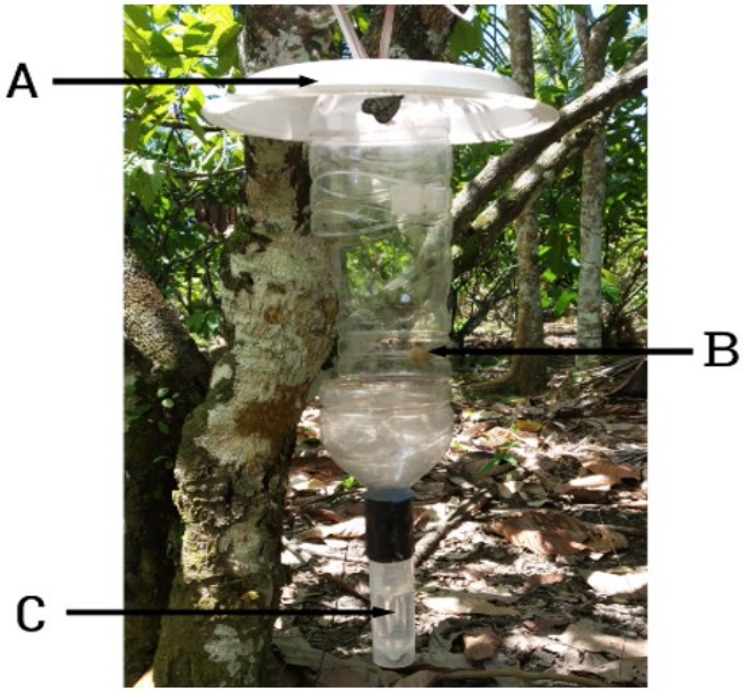

Figure 2. Bottle trap model. A) trap lid, B) cotton swab containing attractant, and C) tube containing soap/alcohol solution.

Insect Collection and Identification

The beetle population count was determined through the observation of ambrosia beetles trapped in the study. Observations were conducted 14 times, with 3-day intervals between each observation. Simultaneous observations were made on all sample trees. The collected samples were stored in specimen bottles, each labeled with the respective treatment and date. Ambrosia beetles were identified using an Olympus SZ microscope, following the guidance provided in the book "Bark and Ambrosia Beetles of South America (Coleoptera: Scolytidae)", which outlines morphological characteristics such as body size, pron otal shape, and elytral shape.

Data Analysis

The collected data underwent statistical analysis using analysis of variance (ANOVA) followed by a post hoc test, specifically the Fisher's Least Significant Difference (LSD) test at a significance level of 5 %.

RESULTS AND DISCUSSION

Populations of Ambrosia Beetles

The results showed the population of ambrosia beetles caught in the trap bottle. Analysis of the population of each species revealed that *Hypothenemus* sp. 3 was the most abundant species, with significantly different results compared to other species. Conversely, *Hypothenemus* sp. 2, X. *crassiusculus*, and X. *morigerus* showed no significant differences in their populations (Figure 3).

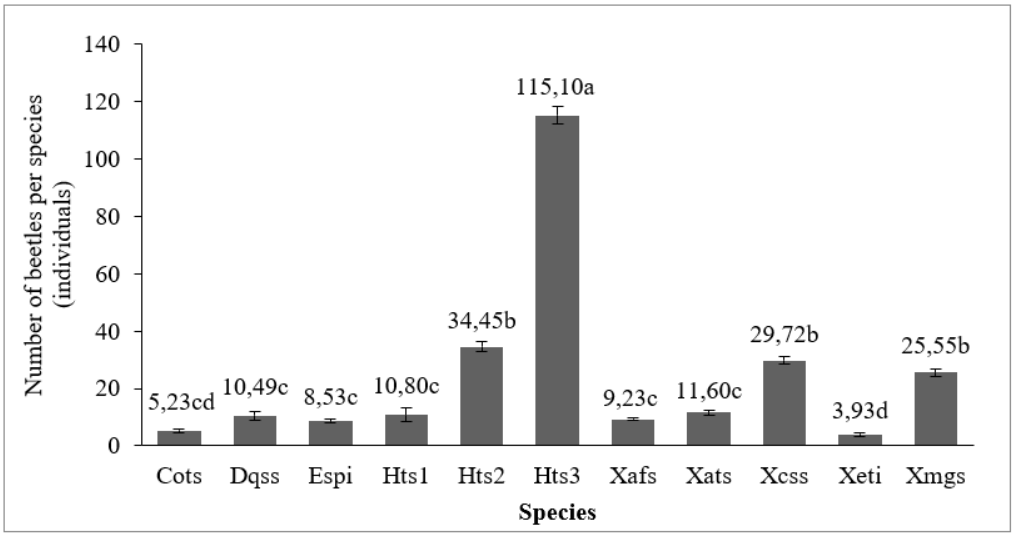

Figure 3. The number of individual ambrosia beetles captured, categorized by species, is as follows: Cots (*Coccotrypes* sp.), Dqss (*Diuncus quadrispinulosus*), Espi (*Eccoptopterus spinosus*), Hts1 *(Hypothenemus* sp. 1), Hts2 (*Hypothenemus* sp. 2), Hts3 (*Hypothenemus* sp. 3), Xafs (*Xyleborus affinis*), Xats (*Xylosandrus mancus*), Xcss (*Xylosandrus crassiusculus*), Xeti (*Xylosandrus eupatorii*), and Xmgs (*Xylosandrus morigerus*). The means ± standard deviation is presented and species denoted by the same lowercase letters at the top of the bars were not significantly different (LSD test at 0.05 significance level).

Hypothenemus emerged as the dominant species attracted to the baited traps, accounting for 82 % of the total identification results. This ambrosia beetle species is recognised for its ability to reproduce on a wide range of host plants. *Hypothenemus* is highly polyphagous, capable of colonising various plant families, including vines, fruits, live seeds, bark, and twigs, even those that are dead and nutrient-poor (Vega et al. 2015). Population diversity of these beetles is influenced by several factors, including high plant diversity, which in turn impacts the diversity of ambrosia beetle species (Haddad et al. 2001)

Based on the five treatments tested, ethanol captured the highest number of ambrosia beetles, with 1391 individuals, followed by carrot leaf extract with 1313 individuals, fermented cocoa wood with 1179 individuals, eucalyptus oil with 991 individuals, and coffee leaf extract with 964 individuals. Statistical analysis indicated a significant difference among the treatments involving ethanol, coffee leaf extract, and eucalyptus oil. However, no significant difference was observed between carrot leaf extract and fermented cocoa wood treatments, as depicted in Figure 4.

After conducting observations at the research site, 11 species of ambrosia beetles were identified, with varying occurrences in each treatment. All species belonged to the Scolytidae family, exhibiting distinct population distributions across the treatments. The species with the highest population was *Hypothenemus* sp. 3, particularly prevalent in the carrot leaf extract treatment (Table 1). The trend of beetle capture during 14 observations can be seen in Figure 5.

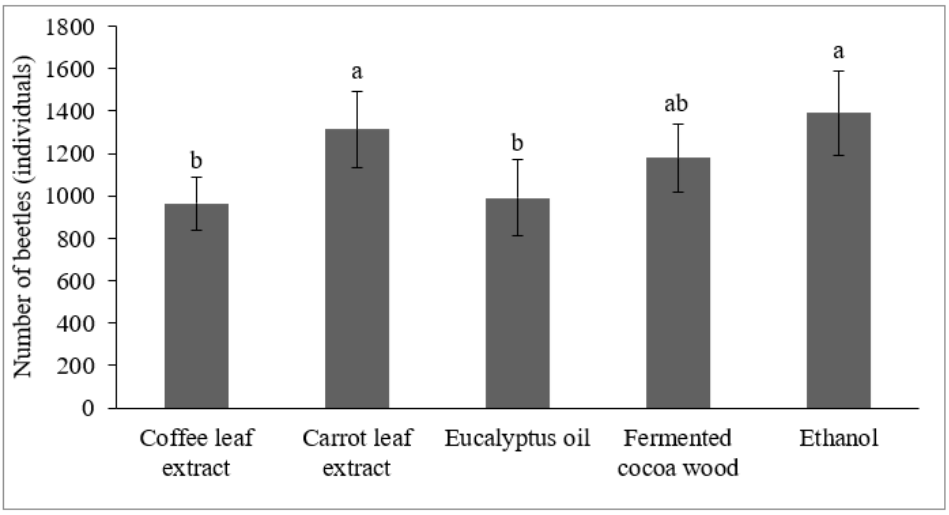

Figure 4. The number of beetles trapped from each treatment was recorded over 14 observations, with a 3-day interval between each observation. The means ± standard deviation was presented, and treatments sharing the same lowercase letters at the top of the bars were not significantly different (LSD test at 0.05 significance level).

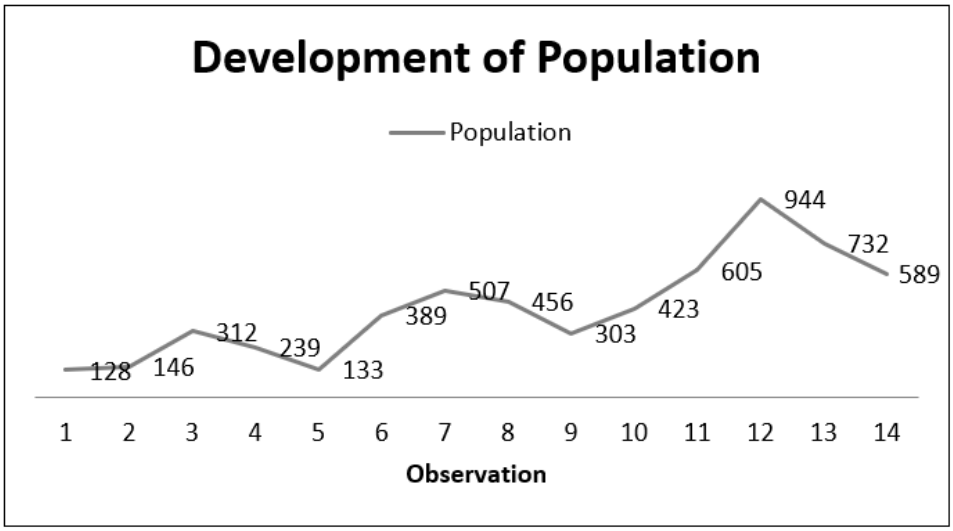

Figure 5. Population development of beetles caught during 14 observations.

The utilisation of traps baited with plant extract successfully captured ambrosia beetles, yielding significant results across all treatments with notably high catch rates. Increased plant species diversity is known to have a posi-

Table 1. Populations of several of ambrosia beetles trapped on each attractant trap.

Species	Population of each treatment				
	Coffee leaf extract	Carrot leaf extract	Eucalyptus oil	Fermented cocoa wood	Ethanol
Coccotrypes sp.	1	3	4	0	0
Diuncus quadrispinulosus	8	0	2	9	29
Eccoptopterus spinosus	1	1	11	5	6
Hypothenemus sp1	17	45	2	0	0
Hypothenemus sp2	89	43	36	74	107
Hypothenemus sp3	767	1150	801	943	1075
Xyleborus affinis	5	2	4	6	3
Xylosandrus mancus	5	13	6	3	8
Xylosandrus crassiusculus	51	30	73	70	94
Xylosandrus eupatorii	2	1	1	0	0
Xylosandrus morigerus	18	25	51	69	69

tive impact on insect abundance, particularly herbivorous species (Dinnage et al. 2012). Among the treatments, ethanol and carrot leaf extract yielded the highest number of captures from the post hoc test results, declared not significantly different with carrot leaf extract. This is attributed to the fact that ethanol, used as bait, mimics the ethanol emitted by stressed trees, serving as an olfactory signal for ambrosia beetles seeking susceptible host plants for colonization (Galko et al. 2014; Cavaletto et al. 2021). Furthermore, ambrosia beetles rely on ethanol for the development of fungal symbionts, essential for the production of their offspring (Ranger et al. 2018).

In addition to ethanol, plant-derived compounds with active ingredients can serve as effective attractants for detecting ambrosia beetles in cocoa plantations. These compounds have been evaluated for their attractiveness to insects and exhibit varying levels of efficacy as insect attractants (Maner et al. 2013; Kendra et al. 2014). Ethanol captured the most beetle populations, while carrot leaf extract captured the most *Hypothenemus* species. Chromatographic characterisation of these extracts has revealed that carrot extracts contain primary phenolic acids, with chlorogenic acid being quantitatively identified (Blando et al. 2021). Research on the preference of cocoa pod borers using carrot and coffee extracts as bait has shown that the traps can also capture other insects, particularly those from the Coleoptera order (Rivay et al. 2023).

Fermented extracts offer a cost-effective and environmentally friendly method for managing large pest populations. Fermented extracts have been discovered to also function as attractants, effectively luring insects. This is exemplified by cocoa wood extracts, which exhibit a high attraction potential for ambrosia beetles. The primary ingredient in this fermentation process is cocoa wood that has undergone decay due to ambrosia beetle infestation. The compounds believed to be present in the extract include ethanol, which is naturally found in the xylem and phloem of trees and increases in concentration during periods of tree stress (Lehenberger et al. 2021).

The results further demonstrate that eucalyptus oil can serve as effective trap baits for capturing ambrosia beetles and are comparably effective to coffee leaf extracts. Eucalyptol, the primary compound in eucalyptus oil has been identified as the key component responsible for beetle attraction. Eucalyptol is known for its lower volatility compared to other attractants, making it particularly suitable for capturing larger numbers of beetles. Previous research has shown that eucalyptol can attract beetles from the Coleoptera order, including *Xyloborus glabratus*, on avocado plants. Additionally, eucalyptus oil can aid in identifying specific beetle varieties that are attracted to it (Kuhns et al. 2014).

On the other hand, coffee leaf extract can also act as an effective attractant due to its chlorogenic acid content. This compound, classified as a secondary metabolite in the phenol class, has the ability to stimulate or attract adult insects to lay eggs (Siregar 2016) reported that chlorogenic acid attractants derived from coffee leaves and fruit peels effectively control *Hypothenemus hampei* Ferr.

From the results of the development of the ambrosia beetle population carried out during 14 observations with a span of 3 days, the results of the development of the trend show that at the beginning of the observation, which is still tiny, then at the seventh and 12th observations is the highest population peak with before that there is a decrease in population in the fifth and ninth observations, it shows a decrease in population which then increases due to the exposure period on the attractant plants used which can only maintain the aroma for 12-15 days so that when the population has decreased then replenish the attractant in the trap so that the population caught again increases as in the following observation.

Morphology Identification

Based on their morphological characteristics, 11 species of ambrosia beetles were identified specific morphological characteristics of each species were detailed in (Figure 6).

The results of identifying the morphological characteristics of the ambrosia beetles show that the dominant characteristics are body size, body color, pronotum, and the slope of the elytra, which are different for each ambrosia beetle obtained. A 1.2-2.5 mm length characterizes Genus *Coccotrypes*. The pronotum curves anteriorly from weak to strong, with a smooth to rough surface (Wood 2007). The pronotum looks flattened and somewhat flat when viewed from the lateral side. On the elytra, there are blackish spots. The elytra slope is convex and not equipped with additional tools (Figure 6.1a).

Diuncus quadrispinulosus found in this study has a yellowish brown body, medium body size, and length of 1.8 mm. The pronotum viewed from the lateral side is rounded and firm. The protibia are obliquely triangular (Figure 6.2a). From the dorsal side, the pronotum appears rounded. The scutellum is visible and flush with the elytra (Figure 6.2b). *Eccoptopterus spinosus* has a blackish brown body, a body measuring 2.8 mm, and a sturdy pronotum shape almost as large or more significant than the abdomen; at the pronotal base, there are dense setae. When viewed laterally, the slope of the elytra extends almost to the base of the elytra, conc ave with spines at the edges (Figure 6.3a).

Genus *Hypothenemus generally* has a length of 1.0–2.0 mm, and its color ranges from pale yellowish brown to black, with a broader pronotum and convex elytra slopes (Wood 2007). *Hypothenemus* beetles can differentiate based on vesture details, frontal sculpture, and surface texture (Vega et al. 2015). *Hypothenemus* sp. 1, when viewed from the lateral side, the elytra are convex, have emarginated compound eyes, and have a prominent lump on the pronotum (Figure 6.4a). The vestiure consists of erect, brightly colored setae that cover the entire body when viewed from the dorsal side. At the same time, *Hypothenemus* sp. 2 has morphological characteristics in the form of a body measuring 1.9 mm with a dark brown color. When viewed from the lateral side, the elytra are convex, have emarginated compound eyes, and have a conspicuous lump on the pronotum (Figure 6.5a). The vestiture consists of erect, brightly colored setae that cover the entire body when viewed from the dorsal side (Figure 6.5b) (Wood 2007).

Hypothenemus sp. 3 on the lateral side (Figure 6.6a), it is clear that the whole body is covered with setae, the tip of the pronotum is hairy and pointed downwards, and the slope of the elytra is convex, and di is covered with setae and spots (Hulcr et al. 2015). This indicates that the dominant characteristics of ambrosia beetles were small bark beetles with a black coloration that did not exceed 1.2 mm in size. When observed laterally (Figure 6.6a), the body is densely covered with setae, the tip of the pronotum is hairy and points downwards, and the elytral slope is convex, also covered with setae and spots. Unlike other ambrosia beetle species, the wings of *Hypothenemus* sp. do not extend to cover the tip of the abdomen (Hulcr et al. 2015; Dzurenko & Hulcr 2022).

Xyleborus affinis is characterised by a slender body of dark red to brown (Figure 6.7). The oblique elytra are curved at the end of the eel, there are fine hairs on the pronotum, and the slanting eel on all parts of the body has short setae, the head is directed downwards and curved, and the mouth is pointed (Figure 6.7a). The *Xylosandrus mancus* determined in this research has a yellowish-brown body. The elytra looks like it has been cut off, and the slope of the elytra suddenly separates from the disc (Figure 6.8a). The pronotum is the same length and width, and when viewed from the dorsal side, the pronotum appears rounded, and the base is shiny (Figure 6.8b) (Wood 2007).

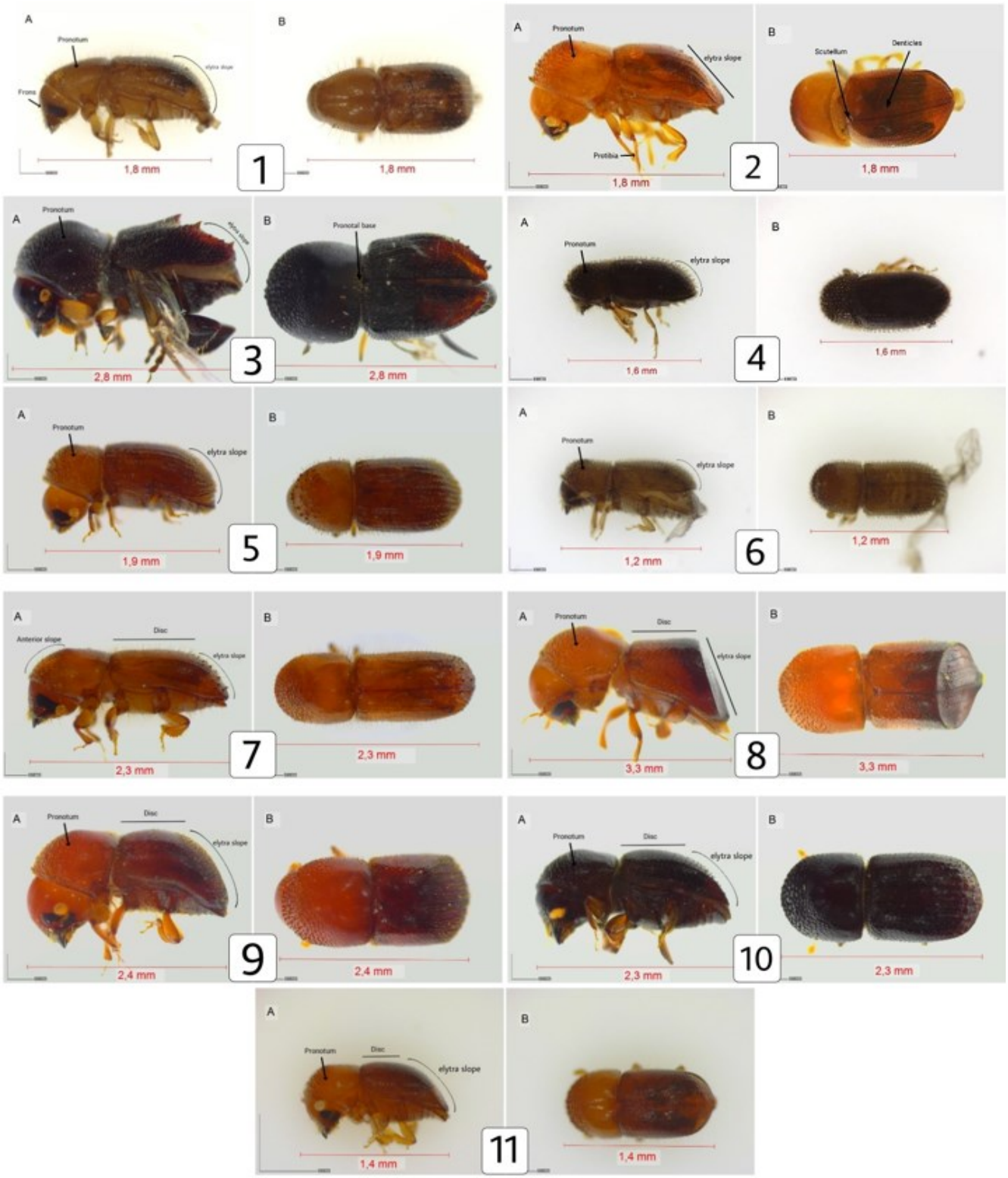

Figure 6. Ambrosia beetles trapped in attractant trap. (1) *Coccotrypes* sp., (2) *Diuncus quadrispinulosus*, (3) *Eccoptopterus spinosus*, (4) *Hypothenemus* sp. 1, (5) *Hypothenemus* sp. 2, (6) *Hypothenemus* sp. 3, (7) *Xyleborus affinis*, (8) *Xylosandrus mancus*, (9) *Xylosandrus crassiusculus*, (10) *Xylosandrus eupatorii*, (11) *Xylosandrus morigerus*. (A) Lateral view, (B) Dorsal view.

Xylosandrus crassiusculus, characterised based on the pronotum, is round when viewed from the lateral side. The granulate ambrosia beetles have a "granulated" region located on the front portion of the downward-facing head and setae on the back end of the elytra (Figure 6.9a) (Poudel et al. 2023). The slope of the elytra is very convex and has setae all over the body surface evenly (Wood 2007). In the *Xylosandrus eupatorii*, when viewed from the dorsal side, the pronotum appears rounded, has the same length and width, and the

basal part is smooth and shiny (Figure 6.10b). The slope of the elytra is convex and appears rounded, with the elytra disc gradually curving towards the slope (Smith et al. 2020). The *Xylosandrus morigerus* is characterized by a length of 1.4-1.7 mm, yellowish to reddish brown color, convex, steep elytra slopes with a rounded base (Wood 2007).

Hypothenemus sp. 3 is the most dominant species, reaching 82 % of the total species obtained. Due to the large population of ambrosia beetles found on cocoa plants, the attacks are also getting worse. This can be the basis for controlling this population and minimizing the damage and loss of yield.

CONCLUSION

The study indicated the efficacy of utilising plant extracts as lures to capture ambrosia beetles in cocoa plantation. Ethanol, carrot leaf extract, and fermented cocoa wood emerged as promoting attractants, offering effective means of monitoring beetle population. Ethanol captured the most beetle populations, while carrot leaf extract captured the most *Hypothenemus* species. Chromatographic characterisation of these extracts has revealed that carrot extracts contain primary phenolic acids, with chlorogenic acid being quantitatively identified. These findings underscore the importance of developing alternative pest management strategies to safeguard cocoa production.

AUTHOR CONTRIBUTION

The authors SS and AR contributed to the conceptualisation, implementation, and preparation of the scientific article. AA contributed to the preparation of the scientific article, MBM contributed to the identification and preparation of the scientific article, ES and AA contributed to the research design, S and N contributed to the implementation of the research, NAF contributed to the preparation of the article, and AS contributed to the implementation of the research and identification. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declared that the present study was conducted in the absence of any conflict of interest or competing interests.

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Research Article

Biodiversity and Ecosystem Services Analysis to Develop a University Botanical Garden: A Case Study in the University of Palangka Raya, Central Kalimantan

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ABSTRACT

Information on existing site characteristics both biotic and abiotic factors is mandatory to provide an initial picture for a baseline to develop a botanical garden. This study aimed to analyse the biodiversity and ecosystem services in the candidate botanical garden area as an existing site, a case study in the University of Palangka Raya (UPR) to prepare the university botanical garden development. The fieldwork was conducted in 5 transects consisting of 100 plots with a plot size of 20x20 m^2. The results showed that the site is categorized as a Sundaland peat swamp forest ecoregion. The peatlands thickness varies from shallow to medium and deep, with the remaining area reaching 75 % of the total campus. The floristic condition is categorized as an early stage of succession after fires, consisting of 26 plant species belonging to 25 genera and 18 families, with various potential uses. Wildlife comprised 42 species including amphibians, reptiles, birds, fishes and prawns, also insects. Three high conservation value plants and two wildlife were documented. The stand carbon storage reached 14.33 tons ha^{-1}. A botanical garden consists of both natural and artificial ecosystems, thus it is important to strategically plan in setting the plant collections layout and species enrichment efforts. The UPR botanical garden will provide the conservation of native and endemic plants of Kalimantan, with high conservation value, potentials, and local wisdom value; and provide ecosystem services for storing carbon, improving hydrological services, habitat and protection for various existing and incoming wildlife.

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INTRODUCTION

Many universities in the world today have botanical gardens for student teaching and academic research, such as the University of Padua Botanical Garden, Italy; University of Cambridge Botanical Garden, UK; Hortus Botanicus Leiden, Netherlands; University of California Botanical Garden at Berkeley, USA; the Bonn University Botanical Garden, Germany; and many more. Furthermore, the University of Padua Botanical Garden was awarded UNESCO World Heritage status in 1997 as the world's first botanical garden, created in 1545 (UNESCO 1997). Until today, it continues to serve its original purpose as a center for scientific research of medicinal plants (UNESCO 2024). Meanwhile, the Sumatera Institute of Technology Botanical Garden, Lampung, is the first university botanical garden that has been developed in Indonesia (Purnomo et al. 2020), followed by the University of Halu Oleo Botanical Garden, Kendari, Southeast Sulawesi, which is still in progress; and several other universities will follow.

University of Palangka Raya (UPR) is a state-governed university strategically located at Palangka Raya, the provincial capital of Central Kalimantan, which has a strong commitment to biodiversity conservation. The main UPR campus in Tunjung Nyaho, with an area of about 365 ha, is considered a green campus as only ± 5 % of the area is used for building academics, research, and student affairs, and the remaining ± 95 % area is green space (UPR 2018). Interestingly, the UPR campus is located very close to Sebangau National Park one of the largest tropical peat swamp and *in-situ* conservation forest areas in Indonesia and Southeast Asia. Thus, it is a strategic and valuable factor supporting UPR as a research and development center for science and technology, including biodiversity and local wisdom related to swamp peatland forests. Moreover, as mentioned in the long-term UPR Master Plan (2018-2034), UPR plans to develop a university botanical garden (UPRBG) in their green spaces with a sustainability concept to harmonize people and plants.

A comprehensive master plan is necessary to establish a botanical garden. It is a dynamic long-term planning document that provides a conceptual layout to guide current and future growth/development. It is based on public inputs, surveys, planning initiatives, existing development, physical characteristics, and social and economic conditions (Purnomo et al. 2020). In particular, existing site characteristics, including both biotic and abiotic factors, are mandatory since they give an initial picture for a baseline to maintain and develop the *ex-situ* conservation area and also for infrastructure and plant collections strategic planning (Siregar et al. 2020). The principle of developing a botanical garden is to the greatest extent to keep the existing natural landscape to ensure the sustainability of the existing ecosystem. One of the conservation functions of a botanical garden is the protection of existing ecological services. Nevertheless, establishing a botanical garden also means creating value-added new ecosystems and services due to ecological system development. Meanwhile, the development of botanical gardens (landscape and infrastructure) has the potential to disrupt the habitat of native plants, so it needs to be planned carefully (Witono et al. 2020).

The existing site characteristics of a botanical garden candidate can be identified through ecoregion and vegetation analyses. The ecoregion is a geographical area with similar characteristics of climate, soil, water, native plants, wildlife, and patterns of human interaction with nature, which describe the integrity of natural systems and environment (Olson & Dinerstein 2002). Vegetation analysis, also known as phytosociological analysis, is the method to study species composition and structure of plant communities. Hence, this study aimed to analyse the biodiversity and ecosystem services in the UPRBG candidate area as the existing site. The biodiversity analysis was

approached from both a floristic and wildlife perspective; in addition, their conservation status was also assessed. At the same time, the ecoregion and habitat characteristics were identified and its ecosystem services were approached from the regulatory function based on carbon storage, provisioning function through bioprospecting potential utilization of plant and wildlife; supporting function through discussing habitat for plants and wildlife and hydrological aspects; and cultural function through discussing the development of botanical gardens as means of recreational, education, and culture. The result of this study will provide essential information as the basis for developing UPRBG particularly. It can also serve as a reference and give insight to other universities to initiate and develop a university botanical garden, also for regional and international policymakers in general.

MATERIALS AND METHODS

Study area, ecoregion, and habitat characteristics identification

This study was conducted in the UPR campus at Tunjung Nyaho, Palangka Raya, Central Kalimantan, Indonesia, specifically at the candidate botanical garden area. Geographically, the study area is located at the coordinates of 02°12'52.43" S to 02°13'40.77" S and 113°53.23.67" E to 113°52'15.52" E (Figure 1).

Characteristics of landscape, soil, water, and vegetation were observed in the study area. Peat soil thickness was measured manually using a measuring tape after digging the hole in the peat soil. The soil and water pH were measured using a pH meter. Furthermore, the ecoregion and vegetation habitat analyses were carried out by identifying the characteristics of the ecosystem in the area according to Olson and Dinerstein (2002) and the Indonesian Institute of Sciences's head regulation no. 1 of 2017.

Vegetation analysis

Fieldwork was undertaken in October 2020. The sampling method of vegetation analysis used the transect method adjusted to the characteristics of the area's landscape by making observation plots (20x20 m^2) at every 20 m distance on the transect line alternately (Figure 1). The transect location was chosen because it is an area that is planned to be a candidate botanical garden according to the UPR master plan. The nested sampling plots were established to record four vegetation layers including understory (including tree seedlings, herbaceous, and shrubs) with a plot size of 2x2 m^2, saplings (trees with a diameter at breast height/DBH of less than 7 cm) with a plot size of 5x5 m^2, poles (trees with a DBH of 7-22 cm) with a plot size of 10x10 m^2, and trees (trees with minimum DBH 23 cm) with a plot size of 20x20 m^2 (Indriyanto 2010). For each transect, 5 plots were laid out with 4 layers, so 100 plots were observed in this study. The plant species name, number of species, and number of individuals for each species were recorded.

The data obtained from the field were then tabulated in an Excel format table for further analysis using vegetation analysis formulas. The parameters of floristic diversity indices were calculated, including the Importance Value Index (IVI), the Shannon-Wiener diversity index, the species richness index, and the species evenness index (Indriyanto 2010).

The IVI was calculated using the formula as follows:

$$IVI\ (\%) = RDe + RF + RDo$$

in which,

RDe (Relative Density) = (density of species-i/ total density of all species) x 100

RF (Relative Frequency) = (frequency of species-i total frequency of all species) x 100

Rdo (Relative Dominance) = (dominance of species-i/ total dominance

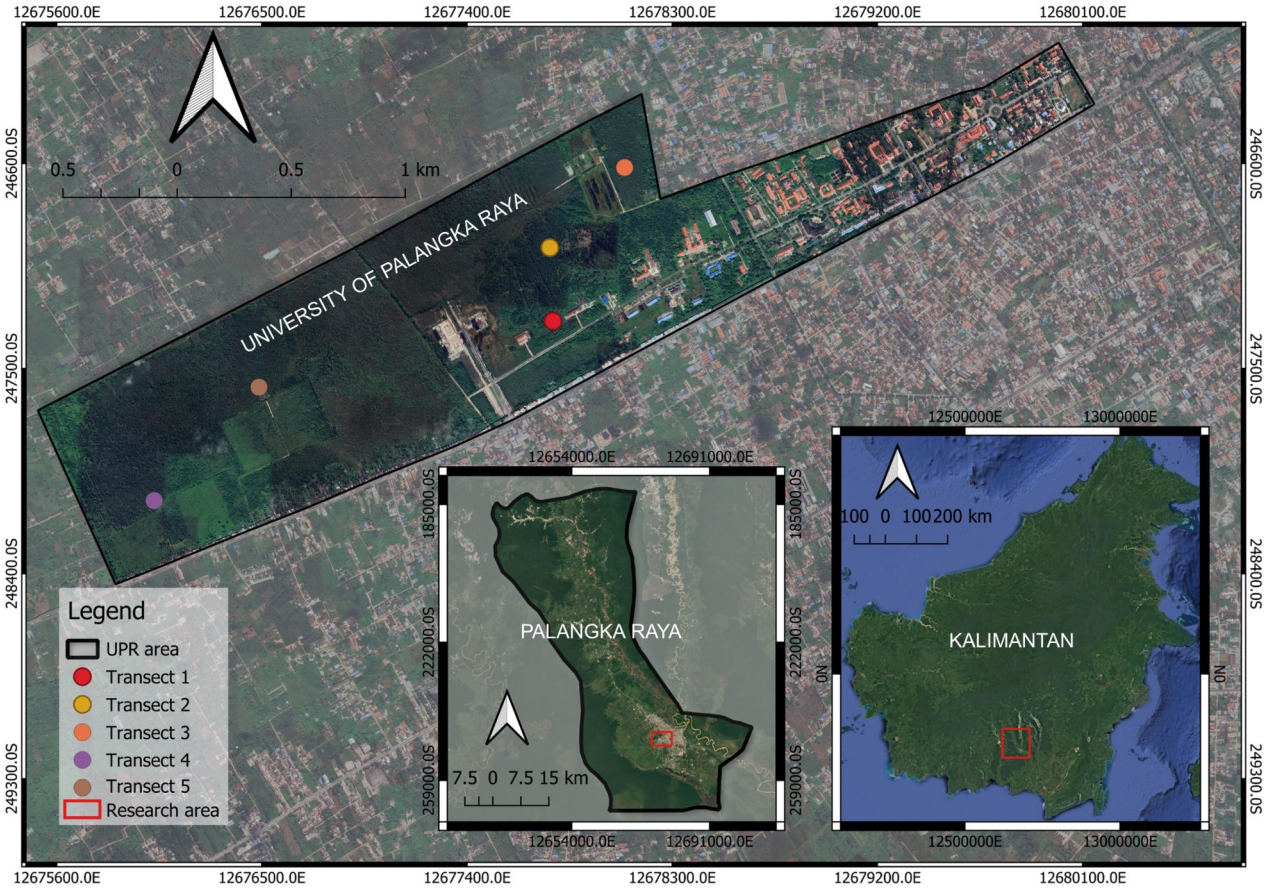

Figure 1. Map of study area in UPR, Central Kalimantan, Indonesia.

of all species) x 100

The Shannon-Wiener Diversity Index (H') was calculated using the equation:

$$H' = -S\, pi \times \ln pi; \text{ and } pi = ni / N$$

in which,

ni = number of individuals of species I, N = total individuals of all species. The diversity level (H') can be classified into three classes, i.e., low if H'<1; moderate if 1≤H'≤3, and high if H'>3 (Indriyanto 2010).

The species richness index (R) was calculated using the formula:

$$R = (S\text{-}1) \div \ln (N)$$

in which,

S = total number of species, N= total number of individuals in the community. The species richness is low if R<3.5, moderate if 3.5 ≤R≤ 5.0, and high if R> 5.

The evenness index (E) was calculated as:

$$E = H \div \ln (S)$$

in which,

E = evenness index, H = diversity index, S = number of species. The evenness is small (the community has low distribution among species) if 0< E≤0.4, moderate if 0.4<E≤0.6, and high (the community has equal distribution among species) if 0.6<E≤1.0 (Indriyanto 2010).

Wildlife inventory

The occurrence of wildlife was recorded using the Visual Encounter Survey (VES) method with a time-constrained search (Doan 2003). The VES method was used to capture species of wildlife based on direct encounters on a transect in terrestrial and aquatic areas. Wildlife inventoried comprised of amphibians, reptiles, birds, fishes, and insects. Several previous studies were also compiled to enrich the results of this study.

Plant and wildlife conservation status assesment and potential uses

The conservation status of plant and wildlife species was evaluated using the application of the International Union for Conservation of Nature (IUCN): Conservation Categories and Criteria at http://iucnredlist.org/search and also checked in the document of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) at https://cites.org/. In addition, interviews with local people were conducted to gather information on the potential uses of the plant and wildlife species; also verified through scientific literature study. Specifically for plants, we also checked the database of Plant Resources of Southeast Asia (PROSEA) at https://www.prota4u.org/prosea/search.aspx,

Carbon storage estimation

The estimation of stand carbon storage was carried out on sapling, pole, and tree layers using allometric equations. Growth parameters related to plant biomass measured include trunk diameter, plant height, and wood density. The diameter of the stand was employed by measuring the circumference of the trunk at DBH (approximately 1.30 m from the ground). The wood density was obtained from the global wood density database (http://www.globallometree.org/). The calculation of standing carbon storage using the equation as follows (Chave et al. 2005):

$$C = \text{Tree volume} \times \text{wood density} \times 0.5$$

Tree volume was calculated using the formula: $V = ¼\ \pi\ D2 \times T \times FF$,
in which,

C = carbon storage (ton/ha), π = 3.14, D = Tree diameter at breast height (1.3 m), T = Tree height, and FF = Form factor, the constant value of tree geometric shape of 0.6.

Development strategy planning of UPRBG

Based on characteristics of the study area gathered from this fieldwork including ecoregion, habitat, biodiversity (floristic and wildlife), and ecosystem services; conceptual frameworks and strategy planning for developing a botanical garden in UPR can be formulated. The development strategy planning of UPRBG was conducted following the regulation of the Indonesian Institute of Sciences no. 4 of 2019 concerning a botanical garden development. The conceptual frameworks developed and discussed in this study include vision, mission, icon or flagship species, plant collection zone, species collection priority, recreational zone, research, and education functions.

RESULTS AND DISCUSSION

Ecoregion and habitat characteristics

The ecoregion and forest ecosystem based on vegetation habitat in the UPRBG candidate area is categorized as a Sundaland peatswamp forest. It has the unique characteristics of saturated organic peat soil, which grows in waterlogged areas under acidic conditions with a low pH of 3.5-4.0. The peat soil thickness varies from shallow (10-40 cm) to medium (100-200 cm) and deep (>200 cm). Approximately 75 % of the UPR campus area is still an empty expanse, which is dominated by shallow to medium thickness of peat soil (Figure 2). The peat soil is formed by the accumulation of organic matter derived from the remains of plant tissue/natural vegetation in the past, which prevents it from fully decomposing due to frequent flooding (Posa et al. 2011).

Furthermore, in the intact Sundaland peatswamp forests particularly in Borneo (Kalimantan) are habitats for a large number of rare, specialized, and threatened species. This includes numerous endemic plants like various rattan

species and unique dipterocarps, as well as a rich array of wildlife, such as the Bornean orangutan and pygmy elephants. The ecosystem's adaptation to waterlogged conditions has led to specialized plant and animal interactions, making it a critical area for biodiversity conservation (Olson & Dinerstein 2002; Posa et al. 2011).

Peat swamp forests are typically surrounded by lowland rain forests on better-drained soils and brackish or salt-water mangrove forests near the coast. Peat swamp forest has a high conservation value, which supports many important services in the ecosystem, such as the protection and preservation of unique plant and wildlife diversity, hydrological service, climate regulation, carbon storage, nutrient cycling, and other ecological services, therefore need to be managed wisely and sustainably (Kalima & Denny 2019). Meanwhile, peat swamp forest is considered a fragile ecosystem that is easily disturbed and damaged, making it difficult to return to its original state. Peat swamp forests are vulnerable to fire hazards during the dry season (Yulianti et al. 2020). Several patches of peat swamp forests in UPR have experienced reccurent fires in 1997, 2002, 2015, and 2019, causing damage to natural ecosystems. The plant succession process is currently underway. However, restoring the damage will take a very long time and result in changes to species composition.

Floristic community structure, conservation status, and potential uses

The floristic community structure had a high number of species and individual abundance in the understory layer and decreased in the sapling, pole, and tree layers. The number of species and individual abundance at the pole and tree layers were low. Likewise, the species diversity and richness indices were categorized as moderate at the understory layer and low at the sapling, pole, and tree layers. Meanwhile, the species evenness index is considered high at understory and tree layers but low at sapling and pole layers (Figure 3). Hence, the floristic community structure of the UPRBG candidate area is categorized as an early stage, characterized by a high number of species and individual abundance in the understory layer and decreasing in the sapling, pole, and tree layers (Trimanto et al. 2021). It is also recognized that the vegetation succession is still in the early stage, which is indicated by the abundance growth of understory that accumulates biomass and covers the site

Figure 2. The peatswamp forest landscapes in the UPRBG candidate area.

with a large leaf surface area and the presence of saplings that face competitive challenges (Hapsari et al. 2020).

Within 100 observation plots, there were recorded 26 plant species belonging to 25 genera and 18 families (Table 1). The dominant families that have the most species are Myrtaceae (4 species), Cyperaceae (3 species), Rubiaceae (3 species), and Blechnaceae (2 species). The complete plant species list found is presented in Table 1. There were 7 plant life forms recorded. Trees are the most common life form found (11 species), followed by grasses (4 species), terrestrial ferns (3 species), lianas (3 species), shrubs (3 species), terrestrial orchids (1 species), and rhizomatous herb (1 species). The plant species numbers recorded in the UPRBG candidate area were considered very low compared to those found in the Sebangau National Park (NP), which is the nearby natural peat swamp forest adjacent to the UPR campus. Several previous plant inventory studies in some areas of Sebangau NP reported that there were at least 310 species (78 families) (Simbolon 2008); 133 species (34 families) (Mirmanto 2010) and 99 plant species comprised 77 genera and 42 families (Kalima & Denny 2019). Although they cannot be compared equally due to different study area extent, those previous studies can provide an overview of the estimated number of plant species in the nearest conservation area that probably once existed in UPR.

Importance value index analysis showed that terrestrial ferns from the species of *Stenochlaena palustris* and *Blechnum* sp. dominated the understory layer. Whilst, seedlings from *Cratoxylum glaucum*, *Melaleuca cajuputi*, *Ploiarium elegans*, and *Melastoma malabathricum* dominated in almost all transects. The sapling and pole layers were dominated by *Melaleuca cajuputi*, *Cratoxylum glaucum*, *Combretocarpus rotundatus*, *Acacia mangium*, and *Rubroshorea balangeran*. *Melicope lunu-ankenda* and *Ploiarium elegans* species were only found in the sapling layer; neither species was found in the pole layer. The tree layer was dominated by the same species, with only three species recorded: *Combretocarpus rotundatus*, *Acacia mangium*, and *Cratoxylum glaucum* (Figure 4 & 5).

Therefore, plant species composition with a high IVI in all layers is relatively homogeneous (Figure 4). Interestingly, most species with high importance values are considered pioneers typical of post-fire peatlands and tolerant species in acidic soil habitats, such as *Stenochlaena palustris*, *Cratoxylum glaucum*, *Melaleuca cajuputi*, *Ploiarium elegans*, *Melastoma malabathricum*, *Combretocarpus rotundatus*, and *Acacia mangium* (Davies & Semuit 2006; Graham 2009).

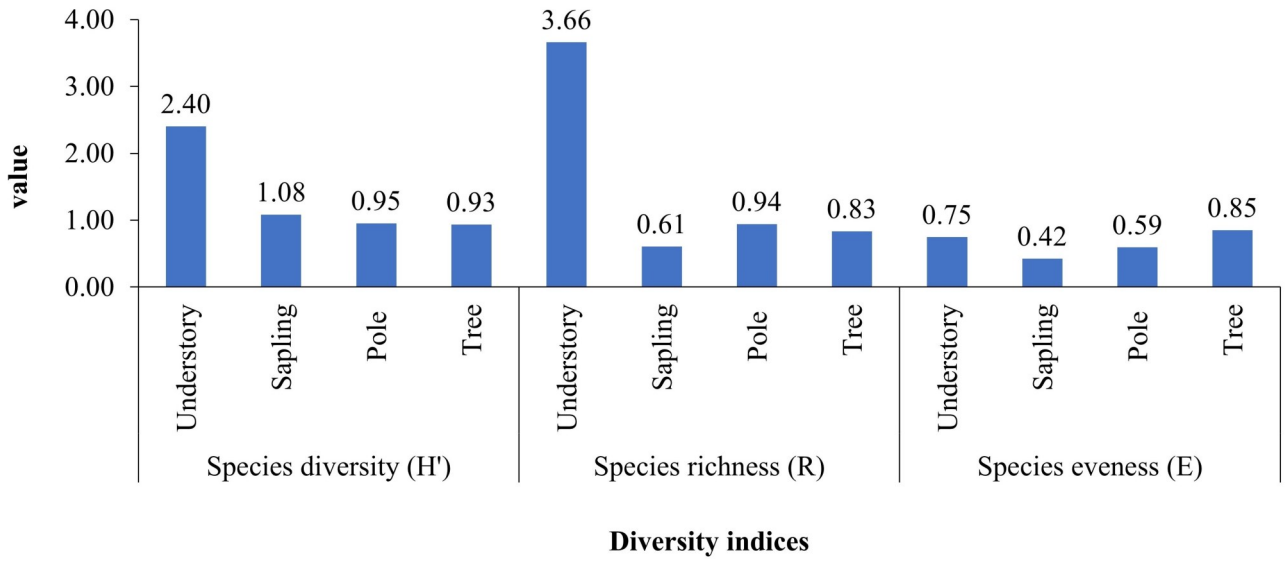

Figure 3. Vegetation diversity indices in the UPRBG candidate area.

Furthermore, two IUCN red listed plant species were found, i.e., a rhizomatous herb, *Etlingera balikpapanensis* (Endangered), and a tree species, *Rubroshorea balangeran* (Vulnerable) (Table 2; Figure 5A-B). Nevertheless, *Rubroshorea balangeran* is not a spontaneous plant that grows naturally in the UPRBG candidate area but is the result of species enrichment planting. In addition, there is one species of Kalimantan orchid whose trade is restricted, listed in CITES Appendix II, i.e., *Dipodium paludosum* (Table 2; Figure 5C).

Table 1. List of plant species recorded in the area of candidate UPRBG.

No	Species name	Vernacular name	Family	Life form	IUCN/ CITES	Potential uses & prospects
1	*Acacia mangium* Willd.	Akasia	Fabaceae	Tree	LC	Timber, industry
2	*Blechnum* sp.		Blechnaceae	Fern	-	Ornamental fern, fiber
3	*Combretocarpus rotundatus* (Miq.) Danser	Tumih	Anisophyllaceae	Tree	LC	Timber, industry
4	*Cratoxylum glaucum* Korth.	Gerunggang	Hyperaceae	Tree	NE	Timber
5	*Cyperus* sp.	Hiring	Cyperaceae	Grass	-	Fiber
6	*Dicranopteris linearis* (Burm.f.) Underw.	Paku ata	Gleicheniaceae	Fern	LC	Fiber, handicraft, medicinal, ornamental fern
7	*Dipodium paludosum* (Griff.) Rchb.f.	Anggrek	Orchidaceae	Orchid	CITES	Ornamental orchid
8	*Fimbristylis* sp.	Purun	Cyperaceae	Grass		Fiber, handicraft
9	*Etlingera balikpapanensis A.D.Poulsen*	Jahe raksasa	Zingiberaceae	Herb	EN	Medicinal, ornamental ginger
10	*Ficus oleifolia* King	Nunuk nahi	Moraceae	Shrub	LC	Ecological service
16	*Gynochthodes umbellata* (L.) Razafim. & B.Bremer	Mengkudu akar	Rubiaceae	Liana	NE	Medicinal
11	*Imperata cylindrica* (L.) Raeusch.	Ilalang	Poaceae	Grass	NE	Fiber, handicraft, medicinal
12	*Lepironia articulata* (Retz.) Domin	Purun	Cyperaceae	Grass	NE	Fiber
13	*Melaleuca cajuputi* Maton & Sm. ex R.Powell	Galam	Myrtaceae	Tree	LC	Medicinal, essential oil
14	*Melastoma malabathricum* L.	Karamunting	Melastomataceae	Shrub	NE	Medicinal
15	*Melicope lunu-ankenda* (Gaertn.) T.G.Hartley	Sempayang	Rutaceae	Tree	LC	Timber, industry
17	*Nepenthes rafflesiana* Jack	Kantong semar	Nepenthaceae	Liana	LC	Ornamental carnivorous
18	*Ploiarium elegans* Korth.	Masam-masam, beriang	Bonnetiaceae	Tree	LC	Timber
19	*Rubroshorea balangeran* (Korth.) P.S.Ashton & J.Heck.	Balangeram, balau merah,	Dipterocarpaceae	Tree	VU	Timber
20	*Stemonurus secundiflorus* Blume	Mempasir	Stemonuraceae	Tree	NE	Timber
21	*Stenochlaena palustris* (Burm.f.) Bedd.	Kelakai	Blechnaceae	Fern	NE	Edible vegetable, handicraft, medicinal, ornamental fern
22	*Syzygium acuminatissimum* (Blume) DC.	Ubah samak	Myrtaceae	Tree	LC	Edible fruit for wildlife
23	*Syzygium incarnatum* (Elmer) Merr. & L.M.Perry	Jambu-jambu	Myrtaceae	Tree	NE	Edible fruit for wildlife, roadside and garden tree
24	*Syzygium pycnanthum* Merr. & L.M.Perry	Jambu-jambu	Myrtaceae	Tree	NE	Edible fruit, natural dye
25	*Timonius flavescens* (Jack) Baker	Kaum kopi	Rubiaceae	Shrub	NE	Timber
26	*Uncaria attenuata* Korth.	Gambir	Rubiaceae	Liana	NE	Medicinal

Notes: NE = not evaluated, LC = least concern, VU = vulnerable, EN = endangered

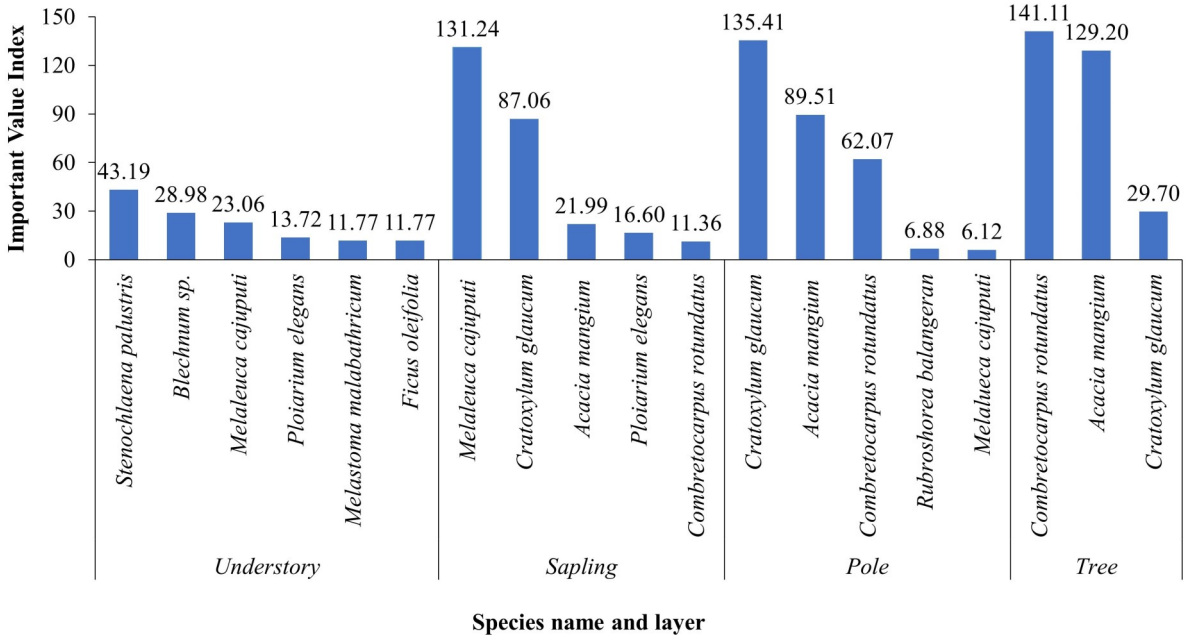

Figure 4. Plant species with high IVI per layer in the UPRBG candidate area.

Hence, those three plant species are highly prioritized for conservation in the botanical garden.

Plants provide provisioning services in the ecosystem. Uses evaluation showed that the plant species recorded have many potential prospects such as for timber, fiber, medicinal, ornamentals, food, etc. (Table 1, Figure 5). Some of the tree species, such as *Rubroshorea balangeran*, *Acacia mangium*, *Cratoxylum glaucum*, *Combretocarpus rotundatus*, and *Melicope lunu-ankenda*, prospect for timber and industry (pulp, particleboard, panel, etc.). Most fern, orchid, and ginger species are potential for ornamental plants. Ferns and grasses are potentially prospected as fiber and handicraft materials. As for medicinal purposes including *Melaleuca cajuputi*, *Gynochthodes umbellata*, *Uncaria attenuata*, etc. The information on existing plant species becomes a reference for plan-setting recommendations for plant collections and species enrichment efforts in developing the botanical garden (Usmadi et al. 2018).

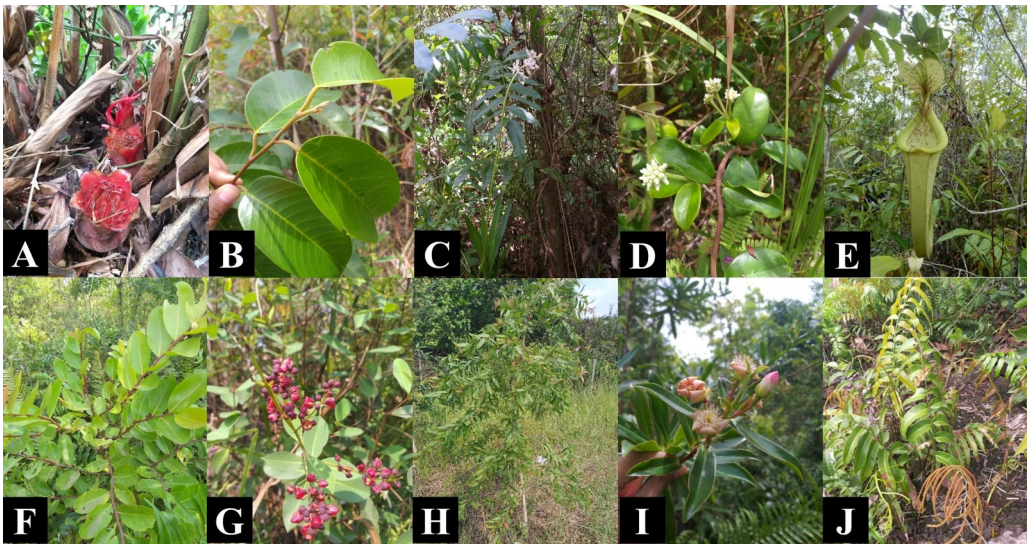

Figure 5. Some plant species documented in the UPRBG candidate area. IUCN red-listed: A. *Etlingera balikpapanensis* (EN) and B. *Rubroshorea balangeran* (VU); CITES App. II: C. *Dipodium paludosum*; Potential species: D. *Gynochthodes umbellata* and E. *Nepenthes rafflesiana*; Dominant species: F. *Combretocarpus rotundatus*, G. *Cratoxylum glaucum*, H. *Melaleuca cajuputi*, I. *Ploiarium elegans*, and J. *Stenochlaena palustris*.

Wildlife species, conservation status, and potential uses

There were at least 14 species of herpetofauna (amphibians and reptiles) found in the area of candidate UPRBG, including frogs, toads, snakes, lizards, and turtles. Four local fish species were found in the reservoir or surrounding waters, and one prawn species. Three bird species were identified, comprised of scarlet-backed flowerpecker, sooty-headed bulbul, and little egrets. In addition, the diversity of insects is also quite high, consisting of 12 species of butterflies, 5 species of dragonflies, 2 species of grasshoppers, and 1 leafhopper (Table 2, Figure 6).

The remnant peat swamp forest in the area of candidate UPRBG is a habitat for various types of wildlife with high diversity and endemicity. Two of the reptile species are included in the IUCN red list, i.e., endangered Southeast Asian box turtle (*Cuora amboinensis*) and vulnerable king cobra snake (*Ophiophagus hannah*) (Figure 6E- F; Maulidi et al. 2020), so they need to be of conservation priority. However, it was identified an invasive alien species of prawn *Macrobrachium lanchesteri* in the surrounding waters (Figure 6J). It has become a precaution for conservation management to monitor its population so that it does not invade the local fish and prawn populations (Maulina et al. 2020).

The diversity of wildlife, both permanent and migratory, plays an important role in provisioning and supporting services in the plant life cycle and maintains the balance of the forest ecosystem. They also have a positive relationship between habitat environmental factors such as water availability, temperature and humidity; and diversity of vegetation as a source of food and shelter (Gonggoli et al. 2021). Wildlife such as mammals, amphibians, reptiles, insects, birds, etc. are crucial as pollinators, seed dispersal agents, and predators to control the populations of certain plants and other wildlife species in the forest ecosystem (Brockerhoff et al. 2017).

Carbon storage in the UPRBG candidate area

The peatland has an important role in climate regulation through carbon storage, which involves above-ground vegetation and peatland biomass. There are carbon balance processes in the peatlands related to carbon, including the absorption of CO_2 in the atmosphere, the emission of CH_4, and the production and export of dissolved organic carbon (Harenda et al. 2018). However, this study focused solely on measuring the carbon storage contained in the standing biomass of plants, including saplings, poles, and trees. Meanwhile, the carbon contribution from the peat itself was not evaluated in this study.

Results from this study showed that the total stand carbon storage value in the UPRBG candidate area reached 14.33 tons/ha, with the sapling layer as the most carbon contributor up to 8.32 tons/ha, followed by the pole layer at 5.18 tons/ha and tree layer at 0.83 tons/ha (Table 3). Trees are generally much larger than saplings and possess greater biomass, which means they can store more carbon. As trees mature, they accumulate more mass in their trunks, branches, and leaves, leading to higher carbon stocks (Chave et al. 2005). However, since the UPRBG candidate area is a secondary peatforest that experienced frequent fires. The trees are very sparse, while the number and density of saplings and poles are very high, resulting in a carbon stock stand from sapplings and poles higher than trees.

The last fire incident in the area UPRBG candidate area occurred in 2019, about two years ago from this study. The stand carbon storage value in the UPRBG candidate area is considered to be approximately the same as reported in previous study by Dharmawan et al. (2013) on three years post-burned peat forest in Central Kalimantan, i.e., 13.64 tons/ha. Meanwhile, according to Jaya et al. (2007), the stand carbon storage in ten years post-

Table 2. List of wildlife species recorded in UPRBG candidate area.

Group	Species	Vernacular name	Family	IUCN
Amphibia	*Hylarana erythraea*	Common green frog	Ranidae	LC
	Pulchrana baramica	Baram river frog	Ranidae	LC
	Fejervarya cancrivora	Crab-eating frog	Dicroglossidae	LC
	Polypedates leucomystax	Common tree frog	Rhacophoridae	LC
	Duttaphrynus melanostictus	Southeast Asian toad	Bufonidae	LC
	Ingerophrynus biporcatus	Crested frog	Bufonidae	LC
	Eutropis multifasciata	Common mabuya lizard	Scincidae	LC
Reptile	*Cuora amboinensis*	Southeast Asian box turtle	Geoemydidae	EN
	Ophiophagus hannah	King cobra snake	Elapidae	VU
	Dendrelaphis pictus	Painted bronzeback snake	Colubridae	NE
	Pareas carinatus	Keeled slug-eating snake	Pareidae	LC
	Xenochrophis trianguligerus	Red-sided keelback water snake	Natricidae	LC
	Rhabdophis flaviceps	Orangeneck keelback snake	Natricidae	LC
	Phytolopsis punctata	Blackwater mud snake	Homalopsidae	DD
Fish	*Rasbora laticlavia*	Clown rasbora	Danionidae	LC
	Belontia hasselti	Kapar fish, Malay combtail	Osphronemidae	LC
	Trichogaster trichopterus	Three spot gourami	Osphronemidae	LC
	Osteochilus hasseltii	Nilem fish, Bonylip barb	Cyprinidae	LC
Prawn	*Macrobrachium lanchesteri*	Riceland prawn	Palaemonidae	LC
Bird	*Dicaeum cruentatum*	Scarlet-backed flowerpecker	Dicaeidae	LC
	Pycnonotus aurigaster	Sooty-headed bulbul	Pycnonotidae	LC
	Egretta garzetta	Little egret	Ardeidae	LC
Butterfly	*Hypolimnas bolina*	Brush-footed butterfly	Nymphalidae	NE
	Junonia atlites	Grey pansy	Nymphalidae	NE
	Junonia orityha	Blue pansy	Nymphalidae	NE
	Junonia coenia	Common buckeye	Nymphalidae	NE
	Acraea terpsicore	Tawny coster	Nymphalidae	NE
	Catopsilia pumona	Lemon emigrant	Pleridae	NE
	Catopsilia pyranthe	Mottled emigrant	Pleridae	NE
	Appias olferna	Striped albatross	Pleridae	NE
	Appias libythea	Striped albatross	Pleridae	NE
	Eurema blanda	Three-spot grass-yellow	Pleridae	NE
	Papilio demoleus	Lime swallowtail	Papilionidae	NE
	Udara placidula	Glassy butterfly	Lycaenidae	NE
Dragonfly	*Cariagrion cerinorubellum*	Orange-tailed marsh dart	Coenagrionidae	NE
	Rhyothemis phyllis	Yellow-barred flutterer	Libellulidae	LC
	Neurothemis fluctuans	Red grasshawk	Libellulidae	LC
	Brachydiplax chalybea	Blue dasher	Libellulidae	LC
	Trithemis aurora	Crimson marsh glider	Libellulidae	LC
Grasshopper	*Acrida* sp.	Silent slant-faced grasshoppers	Acrididae	NE
	Phlaeoba sp.	Short-horned grasshopper	Acrididae	NE
Leafhopper	*Zelus* sp.	Assassin bugs leafhopper	Reduviidae	NE

Notes: NE = not evaluated, DD = data deficient, LC = least concern, VU = vulnerable, EN = endangered

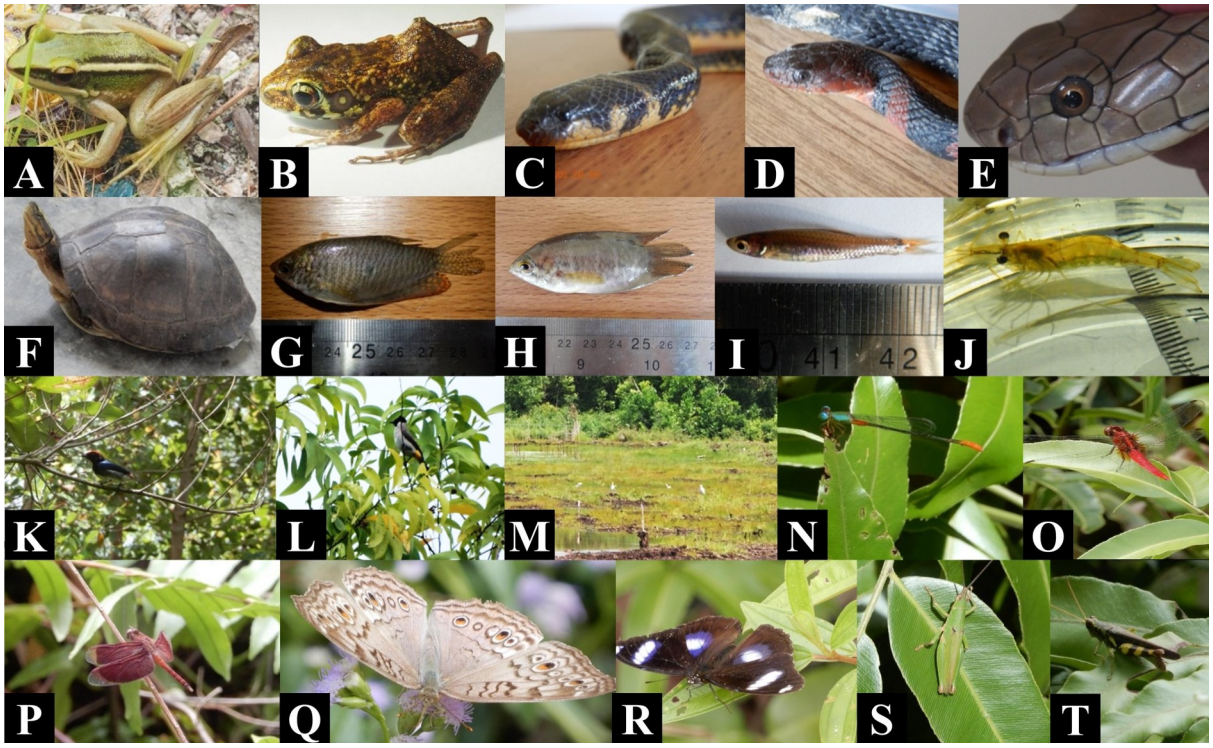

Figure 6. Some wildlife species documented in the UPRBG candidate area. Amphibians: A. *Hylarana erythraea*, and B. *Pulchrana baramica*. Reptiles: C. *Phytolopsis punctata*, D. *Rhabdophis flaviceps*, E. *Ophiophagus hannah* (VU), and F. *Cuora amboinensis* (EN). Fishes and prawn: G. *Trichogaster trichopterus*, H. *Belontia hasselti*, I. *Rasbora laticlavia*, and J. *Macrobrachium lanchesteri*; Birds: K. *Dicaeum cruentatum*, L. *Pycnonotus aurigaster*, and M. *Egretta garzetta*. Insects: N. *Cariagrion cerinorubellum*, O. *Trithemis aurora*, P. *Neurothemis fluctuans*, Q. *Junonia atlites*, R. *Hypolimnas bolina*, S. *Acrida* sp., and T. *Phlaeoba* sp.

burned peat forests (burned twice) is 15-21 tons/ha. However, it is much lower than eight years post-burned peat forest, i.e., 26.13 tons/ha; and is quite higher than stand carbon storage ini peat forest which burns repeatedly each year, i.e., 4.94 tons ha^{-1} (Dharmawan et al. 2013). The stand carbon storage in post-burned peat forests must be lower than in intact peat forests, but recovery can occur over time as succesion occurs and vegetation regrows.

The findings of this study reflect only the carbon storage potential of the stand carbon storage, leaving out a potentially significant component of the overall carbon dynamics in the ecosystem. This could lead to an incomplete understanding of the total carbon storage in the area of candidate UPRBG, as peat soils can store large amounts of carbon. Therefore further study to measure the carbon contribution on the peatland biomass is suggested.

Table 3. Stand carbon storage estimation in the UPRBG candidate area

Site	C-storage (tons/ha)		
	Tree	Pole	Sapling
Transect 1	1.24	9.61	11.72
Transect 2	0.73	11.58	12.88
Transect 3	0	0.43	5.80
Transect 4	0.91	1.45	5.32
Transect 5	1.26	2.83	5.90
Total	**4.14**	**25.90**	**41.61**
Average	**0.83**	**5.18**	**8.32**

Development strategy planning of UPRBG

The UPR area will be developed into a botanical garden with a landscape consisting of several types of natural and artificial ecosystems, which provide

some ecosystem services. The UPRBG will provide the service of conservation of Kalimantan plant in the form of various plant collections, prioritized to native, endemic, high conservation value, high potentials, high value of local wisdom, etc. However, in the development process, species prioritization is needed to allocate limited resources for *ex-situ* conservation planning and action effectively (Purnomo et al. 2015).

The development of a new botanical garden is prioritized to conserve the richness of local plant species based on their habitat suitability. Hence, based on the type of ecosystem that characterizes the UPRBG candidate area from this study, the recommended plant collection theme for the UPRBG is an *ex-situ* conservation of peat swamp plants. In the context of peatlands, water is considered the first-level factor that determines its emergence, growth, and development (Harenda et al. 2018). The development of UPRBG will improve hydrological services by protecting and providing water reserve balance. Peatlands serve as a buffer for the water landscape through the rapid absorption of rainwater to reduce the impact of flooding. Therefore, the existing water reservoir in UPRBG needs to be maintained by conserving the above vegetation integrity and species enrichment with water-storing plants around the riparians and water catchment area.

The vision of the proposed UPRBG is to be the best botanical garden in the world in the fields of conservation, research, and education based on Kalimantan peat swamp plants for sustainable use. With several considerations, including local species and high conservation value species, well adapted to peat swamp habitat and have high economic values for the community. The proposed iconic or flagship species for UPRBG is *Gonystylus bancanus* (Miq.) Kurz (vernacular name: Ramin, family: Thymelaeaceae). According to this study, this species is no longer found in the UPRBG candidate area. However, it is found in abundance and is a key species in Sebangau NP, yet it is at a high risk of extinction with an IUCN status of critically endangered (Karni et al. 2021).

Due to the low vegetation diversity index and species richness in the UPRBG candidate area, it is important to strategically plan the layout of plant collections and species enrichment efforts. Plant collection block planning or lay-outing of collection zones is one of the important formulations of a botanical garden master plan (Purnomo et al. 2020). Management of some existing valuable species, such as *Etlingera balikpapanensis*, *Rubroshorea balangeran*, *Dipodium paludosum*, *Nephenthes rafflesiana*, and *Gynochthodes umbellata* (Figure 5A-E) in the UPRBG candidate area, are prioritized to be recorded as spontaneous collections. Their populations must be maintained for their best performance, survive for a long time, and be well-reproduced.

The proposed green layout concept (collection zone) can be in taxonomic classification patterns and thematic gardens. The recommended taxonomic classification gardens in UPRBG include Dipterocarpaceae, Apocynaceae, Myrtaceae, Arecaceae, Thymelaeaceae, Pandanaceae, Zingiberaceae, Moraceae, Anacardiaceae, Gymnospermae, Dicotyl, and Monocots, meanwhile, for the thematic gardens including medicinal and spices, ornamentals, aromatic, aquatic, fiber, natural dyes, timber, local fruit, carnivorous, industry, wildlife fodder, honorary, and peat swamp.

UPRBG will provide habitat and protection for various existing wildlife and invite more of them (Figure 6). This service can be maintained by providing a jungle zone or forested area. The jungle zone is characterized by high canopy density, many species of fruit-producing trees, and water source areas. Guided tours with birdwatching routes can be developed in the jungle zone. Artificial lakes and riparian zones will protect habitats, especially for freshwater fishes, prawns, amphibians, reptiles, and peat swamp water birds.

The plant collections, ecosystem sevices, and facilities of the UPRBG

combined with university research facilities at UPR may become valuable assets as teaching and learning media to strengthen the education functions of the academic community. The valuable plant collections may serve as materials to support various research and teaching projects. Furthermore, it can be used for teaching purposes by various related departments in many subjects, such as Integrative Biology, Botany, Zoology, Molecular Systematics, Ecology and Evolution, Medicinal and Therapeutics, Landscape Architecture, etc. In addition, to promote and engage public understanding of the importance of plants and the environment, UPRBG may develop various educational curricula and seasonal thematic courses for wider audiences, community, school students, children, and the general public.

In addition, to accelerate the development of a new botanical garden, it is encouraged to collaborate with some strategic parties, namely academic, business/private, community, and government. The collaboration can be in infrastructure development support, collection enrichment through exploration, collection maintenance, internship, research, etc. (Purnomo et al. 2020). In particular, the UPRBG needs to collaborate with the management of the Sebangau NP as a partner in the context of species enrichment and maintenance (sources of plant collection materials) and further develop a research center in various disciplines, especially in the fields of Botany, Ethnobotany, Ecology, and Forestry about the conservation of peat swamp forest.

CONCLUSIONS

The UPRBG candidate area is categorized as the Sundaland peat swamp forest ecoregion. The plant community structure was identified as an early stage with low vegetation diversity and species richness. Most plant species are typical pioneers of post-fire peatlands. At least 26 species of plant and 42 species of wildlife have been recorded. Five species of plant and wildlife of high conservation value have been identified. Standing carbon stocks reached 14.33 tons/ha, indicating the contribution value of two years post-burning peat forests. Therefore, in developing it into a university botanical garden, it is necessary to plan the layout of plant collections and species enrichment, especially with native and endemic species, well adapted to peat swamp habitats; have high conservation value, utilization potential, local wisdom value, and other thematic issues. UPRBG will provide ecosystem services to store carbon, improve hydrological services, and provide habitat and protection for various wildlife. The richness of biodiversity, ecosystem services, and botanical garden facilities combined with university research facilities at UPR can become a valuable asset as a teaching and learning medium to strengthen education and research functions, also recreational for general public.

AUTHOR CONTRIBUTION

L.H., M.R., A.M., J.R.W., I.P.A, designed the research, collected and analysed the research data, D.S., W, E.S., Y.E.G, H.S., S.G. supervised the entire research process. All authors were involved in writing and revising the manuscript.

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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

The Effect of *Thidiazuron* and *Naphtalene Acetic Acid* on *In Vitro* Development of *Eria hyacinthoides* (Blume) Lindl Orchid

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ABSTRACT
Eria hyacinthoides (Blume) Lindl. is an Indonesian orchid species found in Sumatra, Java, and Bali. This orchid is a sympodial orchid with flowers that has fragrant aroma, suspected containing phytochemicals for herbal medicines, so mass plant propagation is necessary. The aim of this research is to obtain the best *in vitro* conditions for this orchid through somatic embryos using growth regulators and analysing the structure of the *Dendrobium Orchid Homeobox 1* (*DOH1*) homologous gene in *E. hyacinthoides* to *Dendrobium* 'Madame Thong-In' which is known to induce bud formation. The method used in this study: (1) the leaves of the plant spread about 20 - 30 days from shoots measuring about 6.3-6.7cm on the mother plant aged ± 8 years, stored in an incubation room with picture of 16 hours of light and 8 hours of darkness in the heat. 25 ± 1 °C , (2) compared Murashige and Skoog, Vacin and Went, Knudson C and New Phalaenopsis growth to get the best medium, (3) added PGR to medium (Thidiazuron (TDZ) 1 - 3 mg L^{-1} and Naphthalene-1-acetic acid (NAA) 1-3 mg L^{-1}), (4) isolate partial gen *DOH1* homologous by using primer of *DOH1*, (5) analyse sequence of PCR products. Optimal medium for callus embryogenesis production from leave was Knudson C + TDZ 1 mg L^{-1} + NAA 1 mg L^{-1}. Amplification of DNA fragments using degenerate primers of *DOH1* resulted 175 bp, indicating similarity about 88.64 % with between the *DOH1* gene structure in *E. hyacinthoides* and in *Dendrobium* 'Madame Thong-In'.

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INTRODUCTION

Orchids belong to the family Orchidaceae, one of the largest families of flowering plants with about 30,000 species worldwide, of which 5000 are distributed across almost all the islands of Indonesia (Semiarti 2018). One of them is the natural orchid *Eria hyacinthoides* (Blume) Lindl. *E. hyacinthoides* is a sympodial orchid with beautiful flowers. Fragrant flowers; therefore, they are thought to contain potential metabolites. The phytochemical content has been reported in *Eria stricta* (*phenanthrenes*) (Wang et al. 2012), and *Eria marginata* (*flavonone*) (Sun et al. 2014). Therefore, *E. hyacinthoides* orchids may contain chemical compounds that are useful for medicine, so it is necessary to propagate them in bulk and obtain uniform results by means of *in vitro* culture.

The population of *E. hyacinthoides* is not well known in its habitat, so its alleged high potential as a source of medicine needs to be known by propagating this orchid *in vitro* to provide plants. Therefore, it is important to identify the most suitable medium and PGR for *in vitro* propagation of this orchid. The combination of auxins and cytokinins plays an important role in the success of *in vitro* plant cultures. According to Mose et al. (2017), TDZ 3 mg L^{-1} was the most effective PGR for inducing direct somatic embryogenesis from various *Phalaenopsis amabilis* (L.) explants. Kasi and Semiarti (2016) reported that TDZ 10 mg L^{-1} combined with NAA 1 mg L^{-1} and NP + 15 % coconut water medium was the best treatment to induce somatic embryogenesis in *Phalaenopsis* 'Sogo Vivien' leaf explants.

The KNOX family is a well-defined homeobox transcription factor that is part of the TALE (three-amino acid loop extension) subclass. It is essential for leaf morphogenesis and apical meristem (SAM) formation (Gan et al. 2023). According to Ruben et al. (2022), shoot growth begins with the activation of homeobox genes in the *Shoot Apical Meristem* (SAM) which activates associated genes that control plant organ growth. *Homeobox* genes in orchids have been reported in *Dendrobium* and *Phalaenopsis*. *Dendrobium* 'Madame Thong-In' has *DOH1*, a *KNOTTED1-like homeobox* gene (Yu et al. 2000). The homologous of the *DOH1* gene in *P. amabilis* orchid was isolated by Semiarti and named *Phalaenopsis Orchid Homeobox 1* (*POH1*) (Semiarti et al. 2008). As a result, it is assumed that *Eria* has *homeobox* gene homology with *DOH1*, so it is necessary to isolate and characterize the *DOH1* gene homologous in the *E. hyacinthoides* orchid.

MATERIALS AND METHODS

Plant Material, Medium Preparation and Growth Conditions

The explants utilised in this study were sourced from quality, disease-free in orchid *E. hyacinthoides* parent ± 8 years old. The research object used was the second leaf from inside the bud of an *E. hyacinthoides* orchid, which is about 20 - 30 days old and measures 6.3 - 6.7 cm in height shot in the Karangayam Research Station, Faculty of Biology, UGM, Yogyakarta. The orchid shoots of *E. hyacinthoides* were first cleaned with soap under running water before being dried. To sterilise the shoots, they were soaked in fungicide for 10 minutes and rinsed thrice using sterile distilled water in a laminar airflow (LAF) environment. They were then soaked in a bactericide for another 10 minutes, rinsed thrice with sterile distilled water, treated with a solution of 5.25 % NaOCl combined with Tween 20 for 3 minutes, and finally rinsed three times with sterile distilled water. Shoots were immersed in 70 % alcohol for 30 s and washed three times in deionized water 3 times. The shoots are immersed in a PPM solution for 15 min. The 2nd leaf from the tip was selected as an explant for *in vitro* culture. Explants were cut using a 1 × 1 cm scalpel and planted on a growth medium. Explants were grown on basic mediums such as MS (Murashige & Skoog), NP (New Phalaenopsis), KC (Knudson C), and VW (Vacin and Went) medium. The best medium was treated with the addition of

growth regulators (TDZ 1 - 3 mg L^{-1} + NAA 1 - 3 mg L^{-1}) (Table 1). Explants were derived from the leaf bases. Explants were cultivated in petridish (16 x 2.5 cm) with five repetitions. Subculture was once a month. The culture was placed in an incubation room maintained at a temperature of 25 ± 1 °C, featuring 16 hours of light and 8 hours of darkness. Growth and development of explants were observed for 12 weeks.

Table 1. The treatment of NAA and TDZ into medium for somatic embryo induction of *E. hyacinthoides*.

Treatments*	T0	T1	T2	T3
N0	N0T0	N0T1	N0T2	N0T3
N1	N1T0	N1T1	N1T2	N1T3
N2	N2T0	N2T1	N2T2	N2T3
N3	N3T0	N3T1	N3T2	N3T3

Anatomical preparation

A paraffin embedding method with a single staining method of safranin was used by Ruzin (Schmid 1999) to confirm the anatomical structure of the cross leaf and nodular masses of *in vitro* cultured leaf explants. The preparation was observed under a light microscope (Olympus, Jepang) using an Optilab 2.2 (MICONOS, Indonesia).

Detection of *DOH1* homologous in the genome of *E. hyacinthoides*

The genome DNA was isolated by using Murray and Thompson's (1980) method, with modification by adding 1 % PVP to the CTAB 3 %. The gene was amplified by using Polmerase Chain Reaction (PCR) with *DOH1* and *ACTIN* primers (Table 2). PCR amplifications were performed using MyTaq™ Red Mix protocols (Bioline). The results were seen by using a UV transilluminator and 1 % agarose gel. The confirmed PCR results containing the *DOH1* target gene were sequenced. Sanger sequencing was conducted. The samples were analyzed using an Applied Biosystems 3500 Genetic Analyzer 2550 equipment (Hitachi, Japan) at the Integrated Research and Testing Laboratory of Universitas Gadjah Mada (LPPT, UGM).

Table 2. List of primers used in this study.

Primer	Sequence		Product length
DOH1	F	5'- CACCAACGATGGATGAGATG -3'	
			175bp
	R	5'- CGAGAAGATGGGGATAACG -3'	
ACTIN	F	5'- GTATTCCCTAGCATTGTTGGT -3'	
			114bp
	R	5'- CAGAGTGAGAATACCTCGTTTG -3'	

Data Analysis

In vitro culture data obtained from explant growth and development for 12 weeks were analysed using Microsoft Excel 2010. Furthermore, the mean values of the parameters were analyzed by ANOVA 95 % degree of confidence, followed by Duncan's multiple range test was carried out whenever there were significant differences between the treatments. Molecular analysis of the sequencing results used BioEdit to help edit and prepare the target sequences and analysed it using the Basic Local Alignment Search Tool (BLAST). Nucleotide sequences were translated into amino acids using The Expasy-translate website (https://web.expasy.org/). Protein motifs were searched on the MotifFinder website (https://www.genome.jp/tools/). Sequence align-

ment was done using the MultAlin website (http://multalin.toulouse.inra.fr/). Exploration of reference sequences were obtained from NCBI (National Center for Biotechnology Information) and OrchidBase 6.0 websites.

RESULTS AND DISCUSSION

Orchid *E. hyacinthoides* has sympodial growth type with pseudobulb, from one pseudobulb there are 2 - 3 leaves attached to the end of *pseudobulb*. The average pseudobulb height of *E. hyacinthoides* is ± 8 cm with a width of ± 2.5 cm. The leaves of *E. hyacinthoides* are lanceolate with fingered leaf veins and unsymmetrical pointed leaf tips, with an average leaf length of ± 26.6 cm and leaf width of ± 4.5 cm. *E. hyacinthoides* flowers grow from the leaf axils, clustered white and contain ± 28 flowers with a diameter of ± 1.5 cm with a flower stalk length of ± 23.4 cm. *E. hyacinthoides* flowers bloom for ± 7 days. According to Nursanti et al. (2020) *Eria* orchids like shady and humid places, usually grows well at an altitude of 250 - 1000 m above sea level. Buds are the parts for somatic embryo induction in this study (Figure 1). Leaf number 2 from the inside is used as an explant measuring 1 x 1 cm.

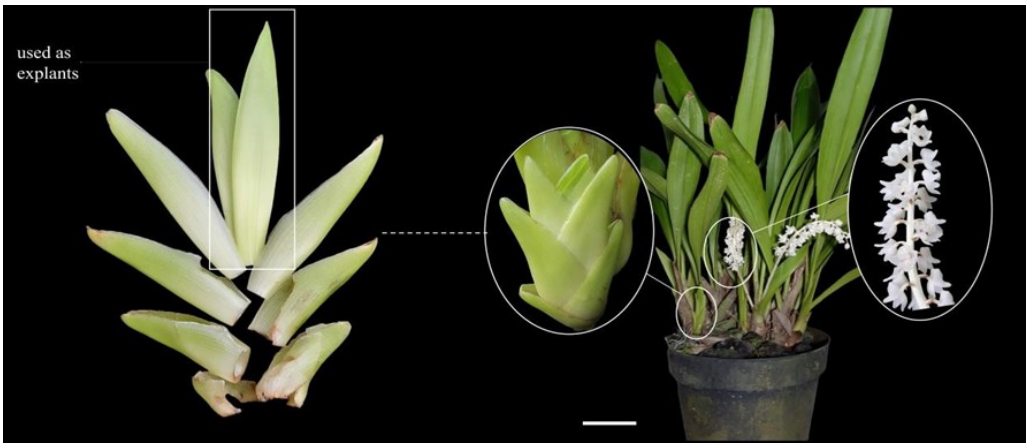

Figure 1. Morphology of *E. hyacinthoides*. Leaf number 2 from the inside of the shoots was used as an explant with a size 1 x 1 cm. (Scale bar 1 cm)

The Best Medium for *In vitro* Growth and Development of *E. hyacinthoides* cultures

Based on the results, after 15 days of explant inoculation on basal medium, the best medium for *in vitro* explant growth of *E. hyacinthoides* is a KC medium as shown in Figure 2. KC medium shows the best medium because it has reached phase 2. The development of *E. hyacinthoides* orchid *in vitro* explants is categorised into 4 phases modified from the research of Dewi et al. (2012), including: phase 1 the explants responded by showing indentations on the wounded part; phase 2 the indentations on the explants thickened (bubbling); phase 3 the thickening on the explants is getting bigger or there is a sign that there will be a protrusion; phase 4 the appearance of nodular masses protruding from the surface of the epidermis.

Finding a suitable culture medium is the most important aspect of culture conditions. In plant tissue culture, a good culture medium will determine the results that will be obtained. Good medium is not only able to support the life of explants but also can increase the growth of explants optimally (Semiarti et al. 2020). Leaf number 2 from the inside of *E. hyacinthoides* orchid buds were used as explants in *in vitro* culture on KC medium with pH 5.8. Explants were maintained in an incubation room with photoperiod of 16 hours of light and 8 hours of dark light and temperature set at 25 ± 1 °C. Growth was observed for 3 months. Based on the observations in Table 3, it is known that the combination treatment of TDZ 1 mg L^{-1} + NAA 1 mg L^{-1} stimulated the

formation of nodular mass by 20 % at the tip of the explant after three months of inoculation.

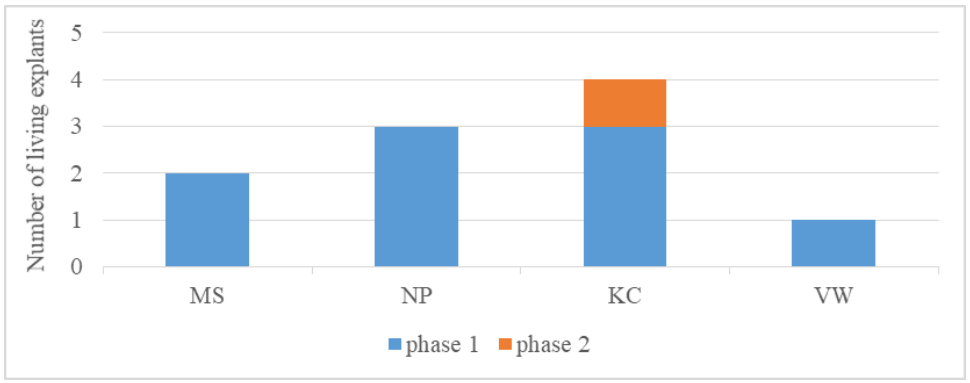

Figure 2. Growth frequency of explants on basal medium assessed after 15 days in culture. MS = Murashige and Skoog; VW = Vacin and Went; KC = Knudson C; NP = New Phalaenopsis.

The optimum amount of TDZ plays an important role in the translocation and distribution of NAA, so that it will accumulate in cells that have high totipotency (Ningrum et al. 2017). When plants are grown in a medium containing auxin without cytokinin, their size increases and the cells do not divide. Adding cytokinin to the medium causes cell division and differentiation. The presence of auxin and cytokinin in the same quantity causes the formation of callus without growth (Bhatla & Lal 2018). Several previous studies have found that leaf blowers respond well to somatic embryo initiation in several orchid species, including *P. amabilis* (Mose et al. 2017) and *Tolumnia* (Chookoh et al. 2019). In general, it is thought that somatic embryogenesis occurs in response to changes in PGR levels, especially auxin and cytokinin in tissue culture systems. Embryonic tumors appear as nodular masses appearing on the surface of the injured tissue (Mose et al. 2017).

The application of TDZ in the *in vitro* culture of *E. hyacinthoides* produced a positive response at 1 mg L^{-1} TDZ, with surviving explant growth reaching 80 %. Adding hormones into the medium is an important step in *in*

Table 3. The Growth percentage of *in vitro* explants of orchid *E. hyacinthoides* on KC medium with various PGR and their concentrations.

Treatments	Percentage of living explants	Percentage of embryogenic explants	Percentage of explants that formed embryogenic callus
N0T0	80%ab	60%ab	0%a
N1T0	60%ab	60%ab	0%a
N2T0	0%a	0%a	0%a
N3T0	0%a	0%a	0%a
N0T1	20%ab	20%ab	0%a
N0T2	20%ab	20%ab	0%a
N0T3	0%a	0%a	0%a
N1T1	80%b	60 %ab	**20%b**
N2T1	80%b	80%b	0%a
N3T1	80%b	80%b	0%a
N1T2	40%ab	20%ab	0%a
N2T2	60%ab	20%ab	0%a
N3T2	60%ab	40%ab	0%a
N1T3	40%ab	40%ab	0%a
N2T3	60%ab	60%ab	0%a
N3T3	60%ab	60%ab	0%a

Description: The frequency of explants forming embryogenic callus was assessed after 84 days in culture. N = NAA; T = TDZ; 0 = Single; 1 = 1 mg L^{-1}; 2 = 2 mg L^{-1}; 3 = 3 mg L^{-1}, n = 5

vitro propagation to increase explant growth, which the amount and type of PGR used must be in accordance with the objectives to be achieved. TDZ is often used alone or in combination with other PGRs to induce somatic embryogenesis in orchids (Balilashaki & Ghehsareh 2016). Cytokinins are a class of plant growth hormones that promote cell division (Bhatla & Lal 2018). According to Feng and Chen (2014), *Phalaenopsis aphrodite* orchid plants can produce somatic embryos when TDZ is used alone. TDZ is an important PGR factor in the *in vitro* micropropagation of some orchids such as *Dendrobium aqueum* Lindl. (Parthibhan et al. 2015), *Cypripedium lentiginosum* (Jiang et al. 2017), *Gastrochilus japonicus* (Kim et al. 2019a), *Ansellia africana* Lindl. (Bhattacharyya et al. 2019), and *Pecteilis* radiate to produce protocorms (Kim et al. 2019b).

The use of a single 1 mg L^{-1} NAA is the best single NAA concentration which the percentage of surviving explants reaches 60%. One characteristic of cell walls in growing cells is that they stretch faster at acidic pH than at neutral pH. This phenomenon is called acid taste. Cleland and Rayle proposed a hypothesis to explain auxin-stimulated cell elongation. According to them, auxin causes acidification of the cell wall through the release of protons in the cell. The low pH of apoplastic (acidic) produced causes the process of loosening the cell wall through enzymatic activity. Another German scientist named Hager also suggested the role of plasma membrane-bound H+-ATPases in auxin-stimulated proton release. These two strategies, formerly known as the Cleland-Hager strategy, are now known as the acid growth factor for cell proliferation (Bhatla & Lal 2018).

Based on anatomical analysis of 3-month-old *E. hyacinthoides in vitro* culture explants, it is known that meristematic cells were still actively dividing, indicating that secondary somatic cells continued to grow (Figure 3e-f) in contrast to explant cells at the age of 0 days (Figure 3d). Staining with safranin reacted well to the part of the cell that had thickened lignin. Lignin was thought to be a tissue containing phenolic compounds. Nodular mass formation began with the development of embryogenic cells at the wound site. The visible stages show the formation of early globular embryos characterised by a rounded structure at the explant wounding site (Figure 3b-c). Multiple layers of embryogenic cells are characterised by densely arranged cells with dense cytoplasm and clearly visible nuclei. Explants on day 0 of inoculation are shown in Figure 3a.

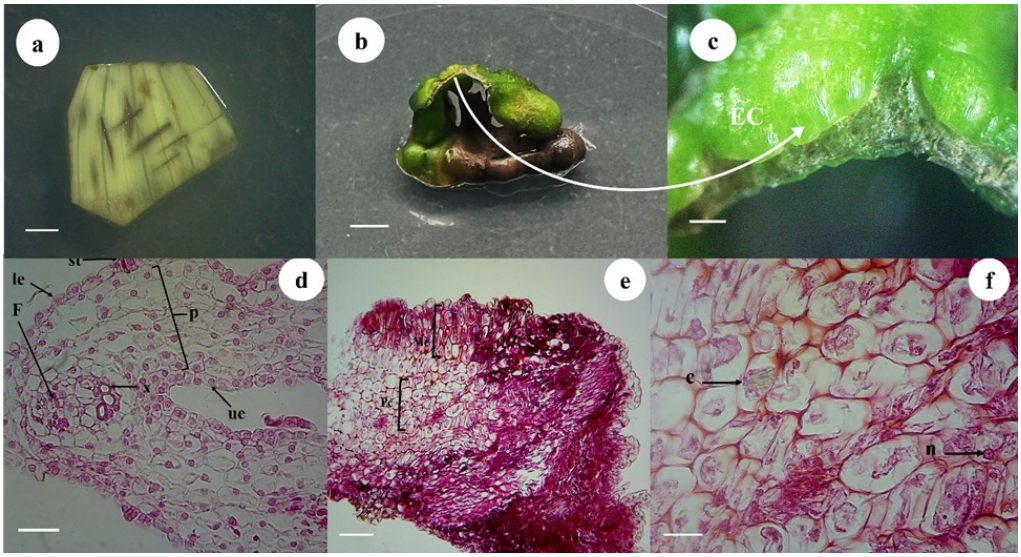

Figure 3. Development of nodular mass (arrowheads) in *in vitro* culture of *E. hyacinthoides*. (a) Leaf spreading and shoot of *E. hyacinthoides* aged 0 days; (b-c) embryonic cells formed in leaf explants in *E. hyacinthoides* shoots treated with TDZ 1 mg L^{-1} and NAA 1 mg L^{-1} after 3 months of inoculation, the explants showed early globular-like

structures derived from the epidermal cell layer. Cross section of (d) 0-month-old explants (e) nodular mass (arrow) Mc indicates meristematic part, Pc stands for parenchyma cells and the darker colored part is dead sclerenchyma tissue. (f) polarized embryogenic suspensor mass. Abbreviations: ec (embryonic cells); ue (upper epidermis); le (lower epidermis); st (stomata); p (parenchymal cells); f (phloem); x (xylem); c (oxalate crystals); n (cell nucleus). Barres a-b = 100 µm; c = 400 µm; d-e = 200 µm; f = 300 µm.

In general, it is thought that somatic embryogenesis will occur in response to changes in PGR levels, especially auxin and cytokinin in tissue culture systems. The first embryo appears as a nodular figure emerging from the surface of the infected material (Mose et al. 2017). The effectiveness of plant tissue culture is greatly influenced by the interaction between auxin and cytokinin. In this study, NAA alone did not induce morphogenetic reactions in explants. The inhibitory activity of exogenous auxin on leaf regeneration was also observed in *Dendrobium* cv. Chiengmai Pink (Chung et al. 2007).

Analysis of the sequence *DOH1* homologous gene in *E. hyacinthoides*

The results of genomic DNA isolation of *E. hyacinthoides* are shown in Figure 4. The amplification of the *ACTIN* gene in the *E. hyacinthoides* genome of 114 bp, this is indicating that the genomic DNA is in good condition and can be utilised to control the amplified gene. *ACTIN* is a housekeeping gene and the most stable reference gene for all plant tissues and leaves, therefore it might be utilised as an internal/positive control for further amplification (Yuan et al. 2014).

The amplification product of the *homeobox* gene in *E. hyacinthoides* using the *DOH1* primer was found to have a length of 175 bp (Figure 5). The genomic sample obtained was then amplified using the *DOH1* gene to see its homology with *E. hyacinthoides* and the *ACTIN* gene which is a housekeeping gene used as a positive control.

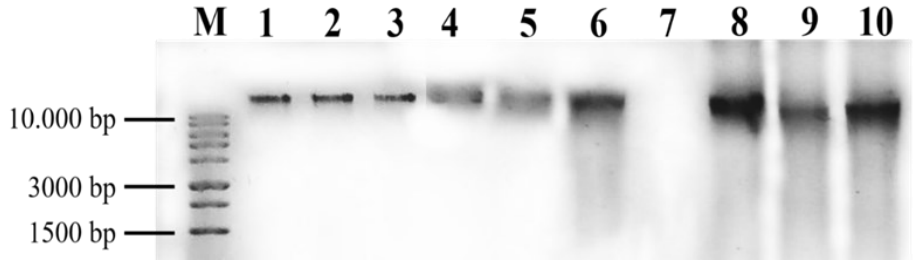

Figure 4. Visualisation of genomic DNA of *E. hyacinthoides*. Lane descriptions: M = Markers; 1-3 = shoots; 4-5 = roots; 6-8 = leaves; 9-10 = flowers.

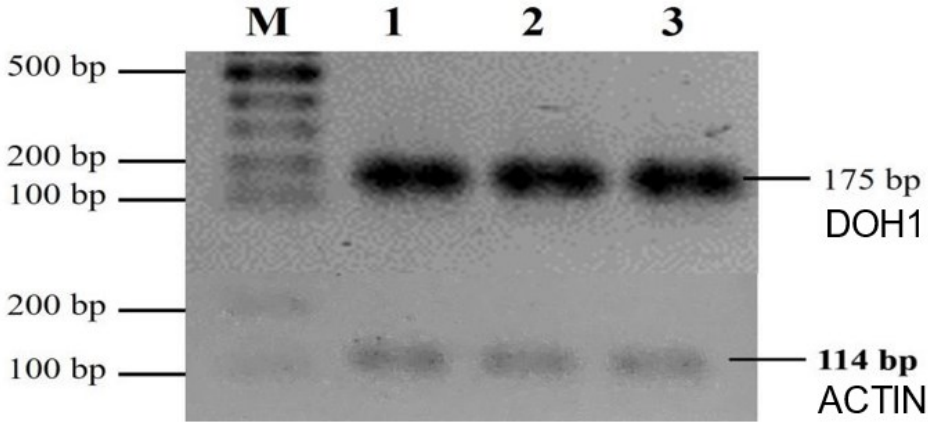

Figure 5. Detection of *DOH1* gene in *E. hyacinthoides* genomic DNA samples. A) Bands of 175 bp amplification of the *DOH1* gene. B) Bands of 114 bp amplification of the *ACTIN* gene. Lane descriptions: M = Markers 100bp; 1-3 = sample 1-3.

Homology analysis between the amplified DNA fragment of the *DOH1* gene in *E. hyacinthoides* and the database using BLAST NCBI database found that the *DOH1* gene in *E. hyacinthoides* revealed similarities with *DOH1* gene in *D.* 'Madame Thong-In' in 88.64 %. Therefore, based on the similarity results of the homologous *DOH1* gene in *E. hyacinthoides*, it may be stated to be *Eria Orchid Homeobox1 (EOH1)*. The sequence of *EOH1* also showed similarity with other *KNOX* genes in orchid species, including *Homeobox Protein Knotted-1-Like 6* in *Dendrobium catenatum* with 90.34 % similarity and *Class 1 KNOX* in *Dendrobium nobile* with similarity of 90.34 %. This similarity needs to be followed up for longer amplification.

The alignment results (Figure 6) showed the location of the *DOH1* gene in *E. hyacinthoides* with *DOH1* gene in *D.* 'Madame Thong-In' in the exon region. The encoded amino acids indicated the *KNOX1* protein motif. The function of the *KNOX1* gene was associated with tissue proliferation and the preservation of the meristematic potential of moss sporophytes and blooming plants. The diversity of leaf shape observed in flowering plants were partly attributed to the control of *KNOX1* activity (Furumizu et al. 2015). Based on motif analysis conducted between *E. hyacinthoides* and other orchids (Figure 7), it showed a representation of motif images that was not full. This was be-

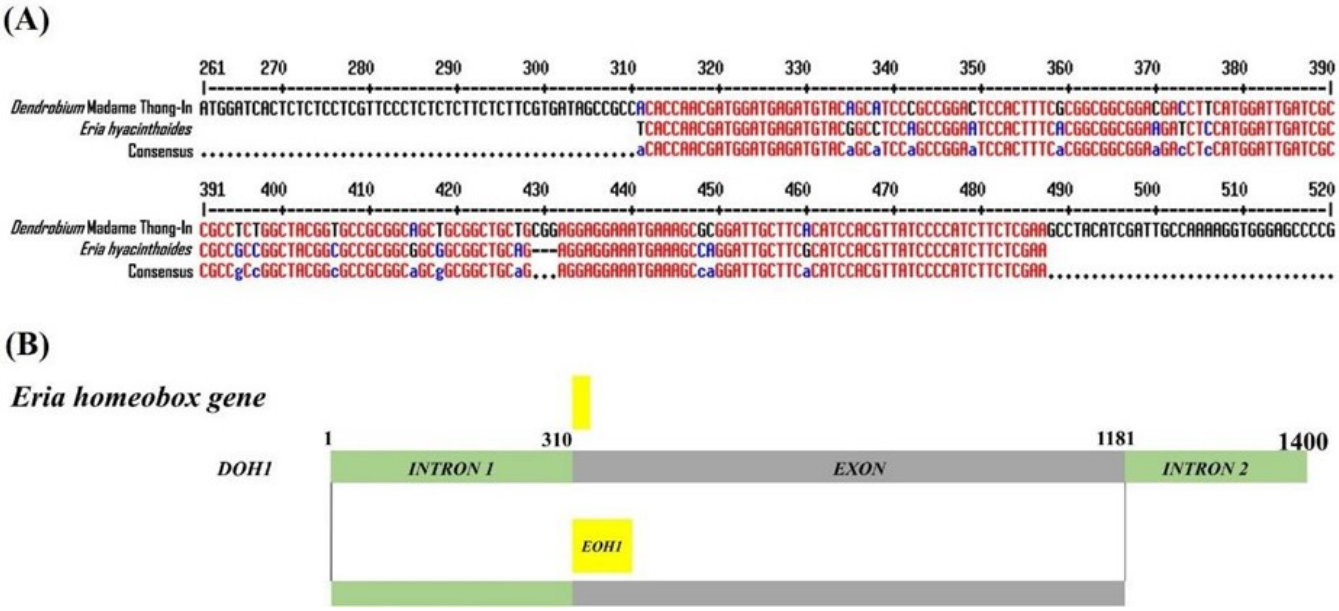

Figure 6. Sequence analysis of *DOH1* homologous genes in *E. hyacinthoides* with *DOH1* in *D.* 'Madame Thong-In' (A) Sequence alignment between *DOH1* and *EOH1*, (B) Schematic representation of *EOH1* fragment aligned with *DOH1*.

Figure 7. Schematic representation of *DOH1* protein motifs homologous to *E. hyacinthoides* and other orchids. Color description: Green = *KNOX1*; Blue = *KNOX2*; Purple = *ELK*; Red = *Homeobox KN*.

cause the amplification of the *DOH1* gene in *E. hyacinthoides* obtained only 175bp.

The functions of most *homeobox* genes acted as transcriptional regulators, especially of developmental processes (Viola & Gonzalez 2016). The activation of the *KNOX1* homeobox gene which caused tissue proliferation, may be linked to nodular masses with constantly dividing meristematic cells in *in vitro* cultures of *E. hyacinthoides* orchids.

CONCLUSIONS

The conclusion obtained from this study is the best explant for the growth of *in vitro* culture of *E. hyacinthoides* orchids is the youngest leaf from shoot. The best medium for the *in vitro* culture of the orchid *E. hyacinthoides* is KC medium, and the optimal concentration of PGR to induce the development of somatic embryos in *E. hyacinthoides* is TDZ 1 mg L^{-1} + NAA 1 mg L^{-1}. The *EOH1 homeobox* gene structure in *E. hyacinthoides* showed 88.64 % homology to the *DOH1* gene of *D.* 'Madame Thong-In'.

AUTHOR'S CONTRIBUTION

ES designed and managed the research process. RF performed the analysis and data analysis. KNP maintained the mother plant for *in vitro* culture.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this research.

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Research Article

Phenol and Tannin Contents of Fresh Phyllodes and Leaf Litter Materials from Three *Acacia* Species in Brunei Darussalam

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ABSTRACT

Invasive *Acacia* species have been increasingly encroaching into degraded forests across Brunei Darussalam since their initial introduction in the 1990s. Understanding the secondary metabolites in these invasive species is crucial to assess their potential impact on leaf litter decomposition in invaded forest areas. This research focused on analysing the pH, total phenolic, and tannin levels of three invasive *Acacia* species (*Acacia auriculiformis* A. Cunn. ex Benth., *Acacia holosericea* A. Cunn. Ex G. Don, and *Acacia mangium* Willd.) and a native heath forest tree (*Buchanania arborescens* (Blume) Blume) found in Brunei. Measurements of pH, total phenolics, and tannins were taken and compared across these four species and between different leaf types (fresh phyllodes vs. leaf litter). Results showed that all invasive *Acacia* species exhibited higher pH, phenolic, and tannin contents than the native species. Fresh phyllodes generally had higher pH and phenolic content than leaf litter in all species, although tannin levels did not vary between fresh phyllodes and leaf litter samples. The elevated pH, phenolic, and tannin contents in the invasive *Acacia* species could make their leaves less palatable to herbivores and decomposers, potentially slowing decomposition compared to the native species, which could, in turn, affect decomposition rates in *Acacia*-invaded heath forests. Overall, these findings on species-specific and leaf type-specific variations in secondary compounds provide valuable insights into the decomposition patterns of *Acacia* species relative to native tree species.

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INTRODUCTION

In the early 1990s, invasive *Acacia*, originally from Australia, was introduced to Brunei Darussalam, Northwest Borneo. The purpose was to support the local timber and furniture industry and to help mitigate soil erosion caused by road constructions (Osunkoya et al. 2005). Four *Acacia* species are known in Brunei Darussalam: *Acacia mangium* Willd., *Acacia auriculiformis* A.Cunn. ex Benth., *Acacia cincinnata* F. Muell. and *Acacia holosericea* A.Cunn. ex G.Don (Sukri et al. 2017; Yusoff et al. 2019). Their ability to rapidly grow in nutrient -poor soils allows these *Acacia* species to effectively establish in disturbed and fire-affected coastal heath forests (Din et al. 2015; Tuah et al. 2020), further spreading inland into disturbed forests and displacing native vegetation (Osunkoya & Damit 2005).

Leaf secondary compounds are widely recognised as a key driver of plant leaf litter decomposability (Cornwell et al. 2008; Perez-Harguindeguy et al. 2013; de Oliveira et al. 2023), playing a major role in leaf litter decomposition and nutrient cycling (Chomel et al. 2016; Jaafar et al. 2022). They influence leaf litter decomposition directly through toxic effects limiting the growth and activity of decomposers (Lin et al. 2018). Phenolic compounds and tannins function as herbivory- and pathogen-defence for plants (Abdulrazak et al. 2000) and can negatively affect microbial decomposers (Rahman & Motiur 2012). Leaf tissue pH is an essential indicator of leaf degradation processes that correlates with tannins, as high tannin concentrations lower the pH in leaf tissue, reducing leaf digestibility to herbivores and detritivores (Perez-Harguindeguy et al. 2013).

Acacia phyllodes, barks, and fruits are the main sources of phenols and tannins for *Acacia* species (Rubanza et al. 2004; Sathya & Siddhuraju 2012; Anand & Mohan 2014; Elgailani & Ishak 2014, 2016; Amoussa et al. 2015; Arasaretnam & Venujah 2017; Ogawa & Yazaki 2018). In tropical Brunei Darussalam, several studies have attempted to quantify the antioxidant contents (including phenols; Sharifulazar 2017), fresh phyllodes and leaf litter pH of invasive *Acacia* species (Yusoff 2015; Jaafar et al. 2022), but investigations of total tannin content remain limited. Analysing phenols, tannins, and pH in invasive *Acacia* species' fresh phyllodes and leaf litter is crucial for understanding their decomposition in Brunei Darussalam's forests. These factors affect decomposition by influencing microbial activity and nutrient cycling. Thus, studying them offers insights into plant decomposition dynamics and ecological impacts. As such, this study aimed to quantify fresh phyllodes and leaf litter pH, total phenolic content (TPC), and total tannin content (TTC) in three invasive *Acacia* species (*A. auriculiformis*, *A. mangium* and *A. holosericea*) and one native heath forest species (*Buchanania arborescens* (Blume) Blume.) in Brunei Darussalam. Our research provides a unique contribution by examining the qualitative aspects, specifically TPC and TTC, of invasive *Acacia* species in Brunei. This novel perspective complements existing studies focused on environmental impacts, offering insight into the ecological implications of these species. Two hypotheses were formulated: (1) *Acacia* species fresh phyllodes and leaf litter will show higher pH, TPC and TTC than the native species due to their distinct physical properties and chemical composition, and (2) Fresh phyllode samples will exhibit higher pH, TPC, and TTC compared to leaf litter samples, due to the ongoing degradation process in leaf litter samples.

MATERIALS AND METHODS

Description of study site

The study site selected was located within the Universiti Brunei Darussalam (UBD) campus in the Brunei Muara district of Brunei Darussalam (4° 58'29.8"N, 114°53'36.6"E; Figure 1). This site was chosen due to the abun-

dance of invasive *Acacia* species, with all four *Acacia* species (*A. auriculiformis*, *A. cincinnata*, *A. mangium* and *A. holosericea*) present around the campus area (Yusoff et al. 2019). UBD campus is located on the sandy soils of tropical heath or "Kerangas" forests (Tuah et al. 2020), which are acidic with low nutrient content (Sidiyasa 2001; Jaafar et al. 2016).

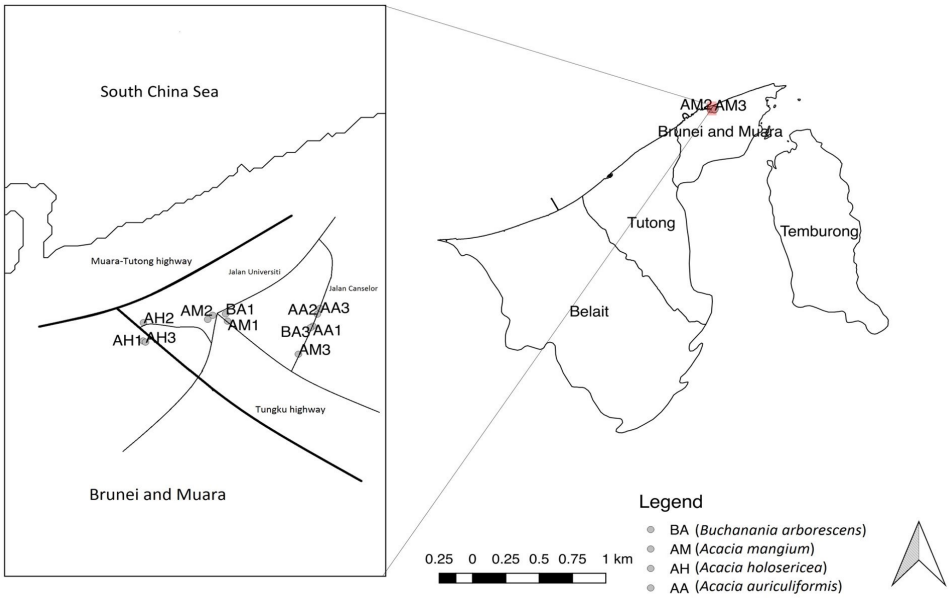

Figure 1. Location of the study site in remnant heath forests on Universiti Brunei Darussalam campus in Brunei Muara district. Three *Acacia* species (*A. mangium*, *A. holosericea*, and *A. auriculiformis*) and one native heath forest tree species (*B. arborescens*) were sampled at least 50 m apart from the same tree species. For each species, three trees were sampled.

Study species

Acacia mangium Willd.

In their natural habitat, *A. mangium* trees range from 7 to 30 m with a straight bole up to 4.5 m in height and a maximum diameter of 50 cm. The mature trees of *A. mangium* have pale grey-brown to brown coloured bark with rough and hard textures and fissures near the base, while the smooth dark green phyllodes are large and the length can be up to 27 cm and width of 3 to 10 cm with 3 to 5 main prominent nerves connecting at the base of the phyllodes near the lower margin (Krisnawati et al. 2011). *Acacia mangium* is closely related to *A. auriculiformis* and *A. holosericea* and is planted mainly for site rehabilitation due to its ability to grow well on infertile soils, particularly soils low in phosphorus (Francis 2002). It is a well-known plantation species due to its adaptability to grow on acidic soils with low nutrient availability, ability to shade out weeds efficiently, relative resilience towards diseases, and its desirable wood properties for use in various industries such as timber, furniture, and paper production (Krisnawati et al. 2011).

Acacia auriculiformis A. cunn ex Benth.

Trees of *A. auriculiformis* can reach a maximum height of 35 m, with long straight bole but most commonly are 8 to 10 m in height with a short, heavily branched trunk (Pinyopusarerk et al. 2018). Mature phyllodes are 8 to 20 cm long, with 3 to 6 pronounced parallel longitudinal nerves and a distinct node at the base of the phyllode (Pinyopusarerk et al. 2018). *Acacia auriculiformis* can withstand various conditions outside its natural habitat, such as coastal sands and waterlogged soils (Turnbull et al. 1998), highly alkaline, acidic, and saline soils, and a wider range of temperatures (Pinyopusarerk et al. 2018).

Acacia holosericea A. Cunn. Ex G. Don

Mature trees of *A. holosericea* can grow to an average height of less than 8 m with smooth bark (Orwa et al. 2009). The length of the thick phyllodes ranges between 10 to 25 cm long and typically 1.5 to 10 cm wide with three to five longitudinal veins (Orwa et al. 2009). In comparison to *A. mangium* and *A. auriculiformis*, *A. holosericea* tree can be distinguished by its phyllodes that are densely covered by fine hairs, giving an appearance of silvery green colour to the foliage (Liu et al. 2012; Yusoff 2015).

Buchanania arborescens (Blume) Blume

Buchanania arborescens is a native tropical tree species of the family Anacardiaceae that is commonly found on gentle slopes, terraces, raised beaches, or near seawater at 150 m altitude and also inhabits secondary forest as well as degraded and remnant heath forests of Brunei Darussalam (Coode et al. 1996). *Buchanania arborescens* tree can be recognised by its distinct, creamy white coloured flowers and shiny leaves that are oblong and narrowly obovate with upturned and wavy sides (Pesiu et al. 2016). This species was selected as a comparison to the three *Acacia* species due to its abundance and co-occurrence with *Acacia* species within the remnant heath forests in and around the UBD campus area (Tuah et al. 2020).

Leaf Sampling

Sampling was carried out in March 2018. Three individual trees from each species were randomly selected and sampled for fresh phyllodes (for *Acacia* species), fresh leaves (for *B. arborescens*), and leaf litter. For each tree, three replicates were collected, resulting in a total of 72 samples (*Acacia* species fresh phyllodes, n = 27 and native species fresh leaves, n = 9; *Acacia* species leaf litter, n = 27 and native species leaf litter, n = 9). Individual trees selected were at least 50 m apart from co-occurring individuals of the same species. The diameter breast height (dbh) and height for the *Acacia* trees sampled ranged from 28.04 to 167.64 cm and 1.68 to 10.67 m respectively, while dbh and height for *B. arborescens* trees sampled ranged from 27.43 to 28.96 cm and 6.10 to 7.65 m respectively. During the leaf sampling, branches and twigs were cut from the trees using secateurs and telescopic pruning shears. Leaves that were fully exposed to sunlight with little or no damage by herbivores were collected as fresh phyllodes samples. For leaf litter samples, only the freshly fallen leaf litter was collected below each sampled tree and replicated three times per tree.

Immediately after sampling, both fresh phyllodes and leaf litter samples were air-dried for three weeks at room temperature. Air-dried samples were then manually crushed by hand and further ground using a ball mill (Model MM400, Retsch, Germany). Ground samples were transferred into sealed zip-lock bags for the analysis of pH, TPC, and TTC.

Determination of pH, Total Phenolic and Total Tannin Content.

The pH of each ground fresh phyllodes and leaf litter samples were measured following Perez-Harguindeguy et al. (2013). Ground leaf powder was dissolved in 1:8 volume ratio of distilled water and thoroughly mixed in a rotary shaker for 1 hour. pH was then determined using calibrated pH meter (Hanna Instrument Ltd, UK). Total phenolic content (TPC) and total tannin content (TTC) were analysed for each sample using a modified Carnegie protocol (2011) by Ainsworth and Gillespie (2007) for phenols, and by Toth and Pavia (2001), and Makkar et al. (2007) for tannins. Ground samples of 0.20 g were extracted and homogenised using 95 % methanol and polyvinylpyrrolidone (PVP). The absorbance was measured at 750 nm of triplicate samples on a microplate reader (Biotek ELx800, USA). The calibration curve of gallic acid

was plotted in the range of 25-200 mg L^{-1}. The TPC and TTC values were calculated from the linear regression of the calibration curve. Thus, TPC and TTC were expressed as gallic acid equivalents (GAE) in mg per g dry mass of sample (mg g^{-1}).

Statistical Analysis

All statistical analyses were conducted using R version 3.4.4 (R Core Team 2018). Variables of pH, TPC, and TTC were initially subjected to separate Two-Way Analysis of Variance (ANOVA) tests to determine differences between the four study species, between leaf types (fresh phyllodes or leaf litter samples), and species by leaf type interaction. The two-way ANOVA for all variables did not record any significant interactions between species and leaf type. Therefore, separate one-way ANOVA tests were conducted for each variable to determine between-species differences for pH, TPC, and TTC. Between-species differences were analysed using one-way ANOVA for fresh phyllodes samples only, and separately for leaf litter samples only. Assumptions of normality and equal variances were tested and were not violated. Differences were considered significant when $P < 0.05$. A pair-wise multiple comparison of means between species and between leaf types was conducted using Tukey's honest significant difference (Tukey's HSD) tests when the one-way ANOVA test revealed significant differences.

RESULTS

The two-way ANOVA revealed significant differences in mean pH, TPC and TTC ($P < 0.001$) between the four study species and significant differences between the fresh phyllodes and leaf litter sample types for pH and TPC ($P < 0.001$; Table 1). However, differences in TTC between fresh phyllodes and leaf litter samples were not significant ($P > 0.05$). No significant interactions were detected between species and leaf types for any of the three variables measured ($P > 0.05$; Table 1).

All *Acacia* species recorded significantly higher mean pH, TPC and TTC compared to the native species regardless of leaf type ($P < 0.05$; Figure 2; Table S1), and fresh phyllodes recorded significantly higher mean pH, TPC and TTC compared to the leaf litter samples regardless of species ($P < 0.05$; Figure 2; Table S2). For fresh phyllodes samples, the highest mean pH recorded was for *A. auriculiformis* (5.62 ± 0.12), while the lowest mean pH recorded was for *B. arborescens* (4.91 ± 0.08). For leaf litter samples, the highest mean pH was for *A. holosericea* (4.94 ± 0.07), while the lowest mean pH was for *B. arborescens* (4.31 ± 0.08; Figure 2).

All three *Acacia* species consistently recorded significantly higher mean TPC than the native species for fresh phyllodes samples ($P < 0.01$; Figure 2), with the highest mean TPC recorded in *A. holosericea* (25.33 ± 0.61 mg g^{-1}), while the lowest mean TPC was recorded in native species, *B. arborescens* (14.69 ± 2.00 mg g^{-1}). For leaf litter samples, mean TPC was significantly higher in all three *Acacia* species than native species, *B. arborescens* ($P < 0.05$; Figure 2). The highest mean TPC was detected in leaf litter samples of *A. holosericea* (19.80 ± 0.90 mg g^{-1}), while the lowest mean TPC was recorded in leaf litter samples of native species *B. arborescens* (9.80 ± 2.98 mg g^{-1}).

DISCUSSION

Variation in pH, Total Phenol Content (TPC) and Total Tannin Content (TTC) Between Species

This study recorded significant differences in the mean pH, TPC and TTC between the four species investigated. Regardless of the leaf types (either fresh phyllodes or leaf litter samples), all three *Acacia* species (*A. auriculiformis*, *A. holosericea*, and *A. mangium*) consistently showed significantly higher

Table 1. A two-way analysis of variance (ANOVA) for differences between species (*Acacia auriculiformis*, *Acacia holosericea*, *Acacia mangium* and *Buchanania arborescens*), and between leaf type (fresh phyllodes or leaf litter samples), for the following variables: (a) pH (b) total phenolic content (mg g^{-1}) and (c) total tannin content (mg g^{-1}). The degree of significance is indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

	(a) pH				(b) Total phenolic content (mg g^{-1})				(c) Total tannin content (mg g^{-1})			
Factors	df	MS	F-value	P	df	MS	F-value	P	df	MS	F-value	P
Species	3	0.508	13.391	<0.001 ***	3	112.380	18.639	<0.001 ***	3	10.539	21.625	<0.001 ***
Type	1	1.808	47.692	<0.001 ***	1	267.020	44.288	<0.001 ***	1	0.250	0.514	0.484
Species x Type	3	0.033	0.865	0.492	3	4.720	0.782	0.521	3	0.538	1.105	0.376

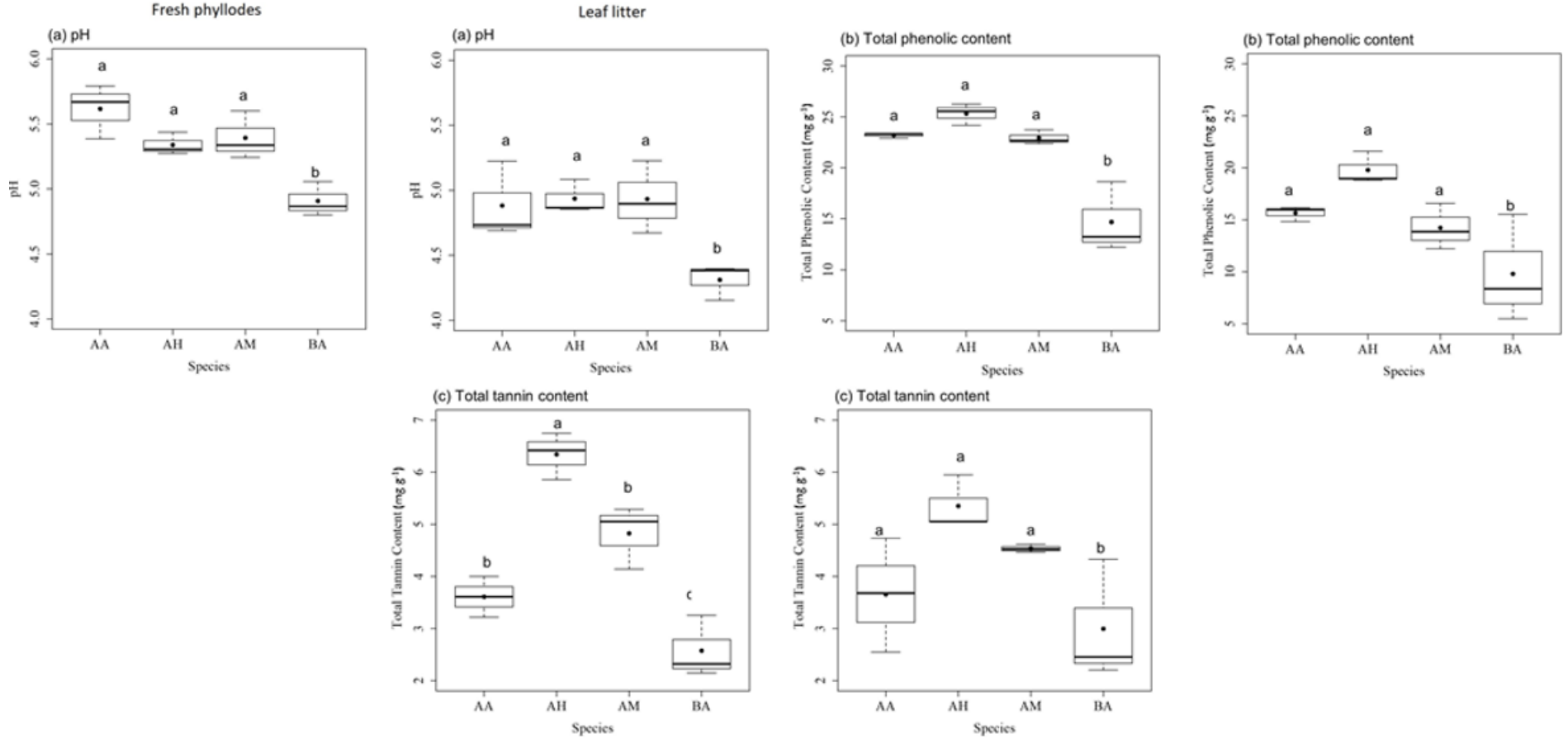

Figure 2. Variation in pH, total phenolic content (mg g^{-1}), and total tannin content (mg g^{-1}) between three *Acacia* species (*Acacia auriculiformis*, AA; *Acacia holosericea*, AH; *Acacia mangium*, AM) and native species (*Buchanania arborescens*, BA). Data values for pH (both fresh phyllodes and leaf litter) and total phenol content (fresh phyllodes) were $\log_{10}$ transformed due to a violation of its normality, but untransformed data were used in the presentation. Different letters within a panel indicate significant differences at P < 0.05 as obtained from Tukey's HSD after one-way analysis of variance (ANOVA). Closed circles represent mean values within each species.

mean pH, TPC and TTC than the native heath forest tree species, *B. arborescens*. This indicates that the invasive *Acacia* species studied contained higher concentrations of secondary compounds in their phyllodes compared to the leaves of this native heath forest species. This finding is consistent with higher initial leaf litter quality (N or C/N, lignin/N, tannin and phenols) of nitrogen-fixing trees than non-nitrogen-fixing trees in this case, *B. arborescens* (Gholami & Sayad 2022). Further, our results are consistent with previous studies on other *Acacia* species such as *Acacia ataxacantha*, *Acacia brevispica*, *Acacia confusa*, *Acacia nilotica*, *Acacia nubica*, *Acacia reficiens*, *Acacia senegal*, *Acacia seyal*, *Acacia sinuate* and *Acacia tortilis* that concluded these *Acacia* species were rich sources of phenols and tannins (Abdulrazak et al. 2000; Sathya & Siddhuraju 2012; Anand & Mohan 2014; Elgailani & Ishak 2014; Amoussa et al. 2015; Gupta & Bhat 2016; Arasaretnam & Venujah 2017).

Variation in TPC between all studied species may be largely due to genotypic factors that control the accumulation of polyphenolic compounds (Rubanza et al. 2004). Differences in leaf morphology can also influence TPC and TTC, for example, leaf thickness, leaf mass area, leaf age and environmental factors have been shown to positively correlate with TPC and TTC in providing leaf defense (Kitajima et al. 2012; Rahman et al. 2013). Despite being in the same genus, *A. holosericea* has a lower leaf mass area and leaf thickness than *A. mangium* and *A. auriculiformis* (Yusoff 2015). The lower leaf mass area and high leaf dry matter content of *B. arborescens* (Yusoff 2015) may influence their TPC and TTC levels.

Other studies outside Brunei Darussalam, using different plant parts of *Acacia* species, also reported inconsistent values of TPC (e.g. Abdulrazak et al. 2000; Sathya & Siddhuraju 2012; Anand & Mohan 2014; Amoussa et al. 2015). Low TPC value was only recorded by Anand and Mohan (2014) in methanol and aqueous extracts of leaves of *Acacia sinuate*. The TTC of *Acacia* species in this study was within the TTC range for fruits of *Acacia nubica*, *Acacia tortilis*, *Acacia brevispica* and leaves of *Acacia reficiens* and *Acacia senegal* (Abdulrazak et al. 2000). The difference in TPC and TTC of *Acacia* species between this study and other studies may be caused by the differences in climatic conditions and other environmental factors. Higher concentrations of polyphenols have been detected in the tissues of plants growing in the tropics compared to those in temperate regions (Coley 1983). Additionally, variations in extraction methods and solvent used could explain some differences in the TPC and TTC measured (Medini et al. 2014). The use of different *Acacia* plant parts at different maturity could further result in different values as TPC and TTC vary among plants, from one part to another, and in any one organ (Elgailani & Ishak 2014).

We found that *Acacia* leaves, irrespective of species, had higher pH than leaves of our native species, *B. arborescens*. Similarly, Yusoff (2015) reported higher mean pH values for leaves of invasive *Acacia* species (*A. auriculiformis*, *A. holosericea* and *A. mangium*) than native pioneer species (*Dillenia suffruticosa* (Griff. ex Hook. f. & Thompson) Martelli, *Ploiarium alternifolium* (Vahl) Melch. and *Melastoma malabathricum* L.) and tree species (*Symplocos polyandra* Brand, *B. arborescens*) and *Callophyllum soulatrri* Burm. f.) from disturbed coastal heath forests in Brunei. However, Jaafar et al. (2022) recorded a lower pH of *A. mangium* leaf litter in mixed-dipterocarp and heath forests, respectively, when compared to *Acacia* species in this study. Fresh phyllodes pH or leaf litter pH can vary greatly even among different species growing in the same soils (Perez-Harguindeguy et al. 2013), due to differences in the concentrations, movement, and interaction of various chemical compounds (Cornelissen et al. 2006). The low pH of *B. arborescens* leaves may act as a defence against herbivores' attack because low fresh phyllodes pH corresponds with poor digestibility and palatability (Cornelissen et al. 2006, 2011).

Low pH in leaves may result from high amounts of TPC (Khoo et al. 2014), organic acids and chemical defence compounds, such as tannins (Perez-Harguindeguy 2013), indicating that pH and secondary compounds TPC and TTC are inversely proportional. However, this contrasts with the present study where all three *Acacia* species recorded high pH values as well as high TPC and TTC in comparison to the native species. A possible reason for variation in pH, TPC, and TTC between *Acacia* species and native species may be due to differences in the growing stage of plants (Medini et al. 2014), leading to changes in the distribution or dynamics of secondary compounds during plant development and life cycle (Bano et al. 2003).

A previous study by Yusoff (2015) recorded significantly higher mean pH and specific leaf area (SLA) in the fresh phyllodes of *Acacia* species (*A. mangium* and *A. auriculifomis*) than in pioneer species (*D. suffruticosa*, *P. alternifolium* and *M. malabathricum*) and native species (*S. polyandra*, *B. arborescens* and *C. soulatrri*). While we did not directly measure the SLA of fresh phyllodes and leaf litter among different species in our research, our results imply a potential relationship between pH and SLA. Drawing a comparison with the findings of Yusoff (2015), our study suggests that this pH-SLA association might contribute to the variations in pH observed between fresh phyllodes and leaf litter samples of *Acacia* species and native species.

Variation in pH, Total Phenolic Content (TPC) and Total Tannin Content (TTC) Between Fresh Phyllodes and Leaf Litter Samples

This study recorded significantly higher mean pH and mean TPC in fresh phyllodes samples compared to leaf litter samples but did not find any significant differences in mean TTC between fresh phyllodes and leaf litter samples. Higher mean TPC in fresh phyllodes samples compared to leaf litter samples may be caused by the decomposition process in which organic matter is degraded and nutrients are released in inorganic forms (Rahman et al. 2013) through rapid leaching from leaf litter (Kuiters 1990). The rate of leaching of TPC is further enhanced by the presence of leaf-decomposing organisms on the leaf material (Kuiters 1990).

The higher mean pH in fresh phyllodes samples compared to leaf litter samples is comparable to those recorded by Yusoff (2015) for green leaves of *A. auriculiformis*, *A. holosericea*, and *A. mangium* and by Jaafar et al. (2022) on the leaf litter of *A. mangium*. Taken together, these studies consistently recorded higher mean pH values for all fresh phyllodes samples than leaf litter samples of *Acacia* species. The observed low pH values in leaf litter samples might be attributed to the leaching of acidic substances from vacuoles (Swift et al. 1979). Low leaf litter pH could potentially impact litter decomposition rates as highly acidic leaf litter has been linked to reduced digestibility by detritivores (Cornelissen et al. 2006, 2011).

The absence of significant variations in the mean TTC between fresh phyllodes and leaf litter samples, across all studied species, may indicate an adaptation by *Acacia* species for nutrient-poor soils. Plant communities rich in tannin often occur on soils that are acidic and poor in nutrients (Rahman et al. 2013). We suggest that TTC in both fresh phyllodes and leaf litter samples of all study species were similar as a response to the acidic and low-nutrient soil conditions of heath forests (Sidiyasa 2001; Din et al. 2015). Tannin has a mechanism that forms stable complexes with a plant protein to decrease its digestibility and to act as a toxin (Zucker 1983; Schofield et al. 2001; Rahman & Motiur 2012). Additionally, leaves with high initial TTC tend to have a slow decomposition (Valachovic et al. 2004; Rahman & Motiur 2012). Therefore, higher mean TTC in *Acacia* species could reduce the decomposition rate in leaf litter by forming protein-tannin complexes that slow down the activities of detritivores.

Significance of Study Findings to Acacia Invasion in Brunei's Heath Forests

The lowest TPC and TTC levels recorded in the native heath forest species (*B. arborescens*) compared to the three *Acacia* species may reflect different resource conservation strategies between native and invasive species. By having high TPC and TTC, *Acacia* species can invest more in tissue protection than growth (Reich et al. 1997; Leishman et al. 2007; Yusoff 2015). In contrast, the lower TPC and TTC for *B. arborescens* suggest that this native species may prefer a different approach than *Acacia* species through conserving resources. Differences in the investment of secondary compounds can give an advantage to invasive *Acacia* species over the native species as the former may possess either allelopathic, defensive, or antimicrobial compounds that are not present in native species (Cappuccino & Arnason 2006; Callaway et al. 2008; Weidenhamer & Callaway 2010).

Fresh phyllode's pH has the potential to influence carbon cycling processes such as herbivory, leaf litter decomposition, and mycorrhizal symbiosis (Cornelissen et al. 2006) because fresh phyllode pH may influence leaf litter acidity that could modify the pH of the soil organic matter (Grubb et al. 1969; Finzi et al. 1998). Low pH, however, may cause detrimental effects on plants (Long et al. 2017) by inhibiting the assimilation of CO_2 in some plant species and inducing oxidative stress in plants (Long et al. 2017). The higher mean pH recorded in *Acacia* fresh phyllodes and leaf litter samples compared to those in *B. arborescens* may indicate an adaptation of invasive *Acacia* species to maintain their survival in acidic environments (Ehrenfeld et al. 2001; Ehrenfeld 2004).

High lignin, TPC, and TTC in invasive *Acacia* species have led to slower decomposition rates when compared to native leaf litter (Devi & Prasad 1991; Drenovsky & Batten 2007; Godoy et al. 2010). This contrasts with Ehrenfeld (2003), Ashton et al. (2005), and Zhang et al. (2014) who found that exotic species exhibit faster decomposition rates and produce leaf litter that decomposes faster than native species. Suhaili (2017) also observed faster decomposition rates of *Acacia* leaf litter than mixed-heath leaf litter, regardless of forest type, while Jaafar et al. (2022) showed that leaf litter in non-invaded heath forests decomposed faster than in *Acacia*-invaded forest but leaf litter of *A. mangium* decomposed faster than mixed-native leaf litter. The presence of TPC in the leaf litter may affect the population of microbial decomposers (Harrison 1971) and delay microbial decomposition of plant leaf litter (Salusso 2000). Thus, low TPC in the leaf litter of three *Acacia* species and native species compared to the TPC in fresh phyllodes was probably caused by the release of phenolic compounds to the soil through leaching (Kuiters 1990) might increase the decomposition rate of the leaf litter. Condensed tannin serves as a deterrent to detritivores functioning as a toxin, rather than inhibiting digestion through protein precipitation (Rahman et al. 2013). Tannins can decrease the decomposition of plant leaf litter by forming complexes with proteins during leaf senescence (Kuiters 1990). Leaf litter decomposition is correlated to tannin concentration (Coq et al. 2010), where high tannin concentration in leaves resulted in slow leaf litter decomposability (Wantzen et al. 2002; Valachovic et al. 2004).

The higher TPC, TTC, and pH observed in *Acacia* species, the less potential leaf palatability to herbivores and detritivores. Hence *Acacia* leaf litter could have slower decomposition rates than native trees. By having slow decomposition rates, high TPC and TTC in *Acacia* species could inhibit the growth of native trees by forming thick layers of leaf litter, altering the habitat (Meira-Neto et al. 2017). Other than that, a thick accumulation of *Acacia* leaf litter could also affect the growth and germination of seeds of native species (Vaccaro et al. 2009).

CONCLUSION

This study has shown that pH, TPC and TTC were significantly higher in all three studied *Acacia* species than in native species. This is indicative of differences in strategies for survival, resource acquisition and defence between the invasive *Acacia* species and native species when they co-occur in the same heath forest. Our assessment of pH, TPC and TTC across different species and leaf types offers valuable insights into the decomposition rates of various *Acacia* species. This comprehensive understanding of nutrient cycling processes in *Acacia*-invaded heath forests not only enhances our ability to develop targeted strategies and management plans for *Acacia* invasion in Brunei Darussalam but also offers insights into the resilience of native species. By elucidating the interactions between invasive *Acacia* species and native flora, we can better conserve biodiversity and ecosystem functions while mitigating the ecological impacts of invasion.

AUTHOR CONTRIBUTION

S.J. undertook data collection, analysis, and manuscript composition. R.S.S. oversaw manuscript refinement, project supervision, and funding acquisition. F.M. contributed to manuscript enhancement and provided supervision. S.M.J. led research design, project supervision, and manuscript writing.

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CONFLICT OF INTEREST

All authors declare that no financial/personal interest or belief could affect their objectivity and that there exists no conflict.

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APPECNDICES

Table S1. Mean values of pH, total phenolic content (mg g^{-1}) and total tannin content (mg g^{-1}) between species, regardless of leaf type. Values are means ± standard error, SE. The mean values presented in this table were calculated averaging nine samples per species.

	Acacia auriculiformis	*Acacia mangium*	*Acacia holosericea*	*Buchanania arborescens*
pH	5.25 ± 0.10	5.16 ± 0.07	5.14 ± 0.06	4.61 ± 0.11
TPC (mg g^{-1})	17.4 ± 0.95	16.9 ± 1.03	20.4 ± 0.82	10.8 ± 1.21
TTC (mg g^{-1})	3.28 ± 0.23	4.27 ± 0.19	5.47 ± 0.17	2.79 ± 0.26

Table S2. Mean values of pH, total phenolic content (mg g^{-1}) and total tannin content (mg g^{-1}) between leaf type, regardless of species. Values are means ± standard error, SE. The mean values presented in this table were calculated averaging 36 samples per leaf type.

	Fresh phyllodes	Leaf litter
pH	5.31 ± 0.05	4.77 ± 0.07
TPC (mg g^{-1})	19.4 ± 0.73	13.4 ± 0.81
TTC (mg g^{-1})	4.04 ± 0.25	3.86 ± 0.20

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Research Article

The Complete Mitochondrial Genome of Critically Endangered Painted Terrapin, *Batagur borneoensis* (Testudines: Geoemydidae)

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ABSTRACT

Characterising mitochondrial genomes is a key to studying evolution in vertebrates including turtles. This study employed Next-Generation Sequencing (NGS) to characterise mitochondrial DNA sequences in *Batagur borneoensis* (Schlegel & Muller, 1844). We reported the nearly complete mitogenome to clearly characterise the gene sequence of *B. borneoensis* which has been deposited in GenBank under the accession number PP228865. Phylogenetic analyses using Maximum Likelihood (ML) on the 13 protein-coding genes were conducted with MEGA X Version 11 software. This study presents the second in-depth analysis of the *B. borneoensis* mitochondrial genome, spanning 16,397 base pairs and containing 13 protein-coding genes, 22 transfer RNAs (tRNAs), two ribosomal RNAs (rRNAs), and a major non-coding region, two non-coding regions: L-strand origin replication (OL) and control region (OH). The sequence length and organisation of this species' mitochondrial genome fall within the typical range and gene arrangement found in vertebrate species. Most genes, except for seven tRNAs and *nad6*, were encoded on the primary DNA strand. All protein-coding genes (PCGs) began with an ATG initiation codon, except for *cox1* and *trnF* which started with GTG codon, and *nad3_0*, started with a TTA codon. These findings enhanced our understanding of nucleotide composition and molecular evolution in the genus *Batagur*. Phylogenetic analyses identified vulnerable and ecologically important species, aiding biodiversity and ecosystem protection. They also expanded the dataset for comparative studies within the Geoemydidae family. Additionally, this research may help develop primers and conservation strategies for future studies.

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INTRODUCTION

The Painted Terrapin *(Batagur borneoensis)* is an aquatic turtle characterised by its hard shell. Other than Malaysia (Peninsular and Borneo), the species also can be found inhabiting Indonesia in Sumatra and southern Thailand. Notably, significant populations of this species are identified in a few rivers in Terengganu, Melaka and Negeri Sembilan (Kolandaiveloo et al. 2020). As a result of live trade, painted terrapins are now distributed worldwide. The Turtle Survival Coalition lists the painted terrapin (*B. borneoensis*) as Critically Endangered and among the 25 most imperilled tortoises and freshwater turtles globally. Population in Indonesia and Malaysia are uncertain, with Malaysia seeing a severe decline, justifying its status on the IUCN Red List and CITES Appendix II. The species faces rapid decline due to habitat degradation, coastal development, local consumption, and international trade (Toomey 2016; Kolandaiveloo et al. 2020). In Indonesia, it is also categorised as a Critically Endangered under IUCN 2010 and CITES Appendix II according to Hernawan et al. (2019). Previous studies produced and analysed molecular data to clarify the identity and evolutionary relationship of Geomydidae groupings (Kundu et al. 2020). The ongoing evolution of living organisms defies quantification through a singular speciation theory. Multiple biological and environmental factors intricately contribute to genetic modifications across generations, resulting in the transformation of genes in descendant species from those of their ancestral populations (Kundu et al. 2020). Aside from natural selection, genetic traits within a population undergo frequent alterations at random in response to a number of biotic and abiotic events, resulting in a species' evolutionary dynamics. The utilisation of gene sequences has been pivotal in elucidating the phylogenetic relationships and evolutionary mechanisms inherent within earth's biota, with a particular focus on reptiles. Among these taxa, Testudines, comprising turtles, tortoises, and terrapins, stand out as among the most ancient extant lineages, characterised by an extensive evolutionary chronicle (Lourenço et al. 2012). Meanwhile, a next-generation sequencing (NGS) is a powerful tool in genomics research that can simultaneously sequence millions of DNA fragments, offering detailed insights into genome structure, genetic variations, gene activity, and changes in gene behaviour (Satam et al. 2023). Mitochondrial DNA (mtDNA) barcoding has significantly simplified species identification and assessment of phylogenetic relationships, especially for morphologically similar, closely related taxa. This technique uses a specific portion of the mitochondrial genome to distinguish species with wide-ranging distributions (Sokefun 2022). As a result, a complete mitogenome from this study will be helpful in understanding the genus *Batagur's* deep evolutionary branching and primer design in the future. To date, despite the numerous species in the *Batagur* genus, only mitogenomes of six species in which; *B. borneoensis*, *Batagur affinis affinis* (Cantor, 1847), *Batagur affinis edwardmolli* Praschag, Holloway, Georges, Päckert, Hundsdörfer & Fritz 2009, *Batagur kachuga* (Gray, 1831), *Batagur dhongoka* (Gray, 1834) and *Batagur trivittata* (Duméril & Bibron, 1835) have been reported in GenBank (OQ808844, OQ409915, OQ645446, NC 069558, NC069559, NC032300 and KX817298).

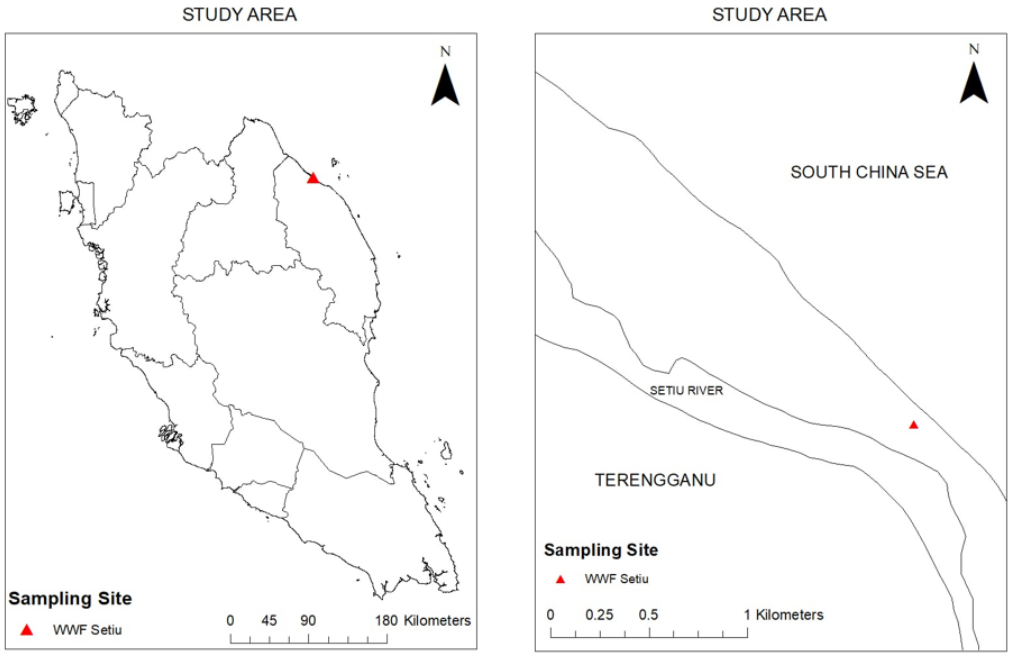
Despite a previous mitochondrial genome sequence of *B. borneoensis* available in GenBank (OQ808844), the sequence reported in this study originates from a distinct geographic location and different life stage, providing new insights into mitochondrial variability and adaptation within the species. Therefore, the objective of this study is to characterise the complete mitochondrial genome of critically endangered *B. borneoensis* specifically from the Setiu River, Terengganu. Thus, this study is the first publication on the complete mitochondrial genome of the critically endangered Painted Terrapin, *B. borneoensis*.

MATERIALS AND METHODS

Sample collection and mitochondrial DNA Extraction

In this study, we employed the genome skimming method to obtain the near-complete mitochondrial genome (Figure 1). The tissue sample was acquired from the World Wide Fund for Nature (WWF-Malaysia) Hatchery in Kampung Penarik Setiu, Terengganu (Figure 2a and 2b). On November 15th 2020, the deceased sample was taken from a recently hatched *Batagur's* egg reared at the WWF Hatchery (5.641482°N, 102.75159°E) and deposited at the Universiti Sultan Zainal Abidin (UniSZA) repository under the voucher number UniSZA/Reptile/01/2022. The survey was carried out with the previous approval of the Wildlife Authority granted to the World Wide Fund for Nature (WWF-Malaysia) and Universiti Sultan Zainal Abidin (UniSZA) Animal and Plant Research Ethics Committee, UAPREC (Letter No: UAPREC/06/009). All experiments were carried out in conformity with the relevant standards and legislation. A tissue sample weighing 20-50 mg was obtained from the carcass of a dead body juvenile terrapin and utilised for DNA extraction using the WizPrep gDNA Mini Kit (WizBio, Korea), following the manufacturer's guidelines.

Figure 1. A juvenile Painted Terrapin (*B. borneoensis*) from Setiu River, Kg. Penarik Setiu, Terengganu (own photo).



(a) (b)

Figure 2. (a) Map of Peninsular Malaysia, red triangle represents the location of Setiu, Terengganu on the east coast of Malaysia. (b) Map showing the sampling site where a juvenile terrapin carcass was collected by WWF Setiu, located at the Setiu River in Kg. Penarik, Setiu, Terengganu.

RNAse treatment and DNA Measurement

Approximately 2 mL of the DNA blood samples underwent treatment with 1 µL of 10 mg mL^{-1} RNAse at room temperature for 30 minutes, then purified with a 1x volume of SPIR bead (Oberacker et al. 2019). Then, 2 µL of pure DNA was examined with a Denovix high-sensitivity kit (Denovix, Wilmington, Delaware).

Illumina library preparation and partial genome sequencing

About 100 mg of DNA were fragmented into 350 bp fragments using a Bioruptor, followed by NEB Ultra II library preparation (NEB, Ipswich, MA) as per the manufacturer's instructions. Sequencing was conducted on a NovaSEQ6000 (Illumina, San Diego, CA) with a run configuration of 2 x 150 bp, resulting in approximately 1 GB of data per sample.

Assembly and Annotation

The total 16,397 base pairs of raw reads underwent trimming to remove low-quality bases and Illumina adaptor sequences using FASTP v0.21 (Chen et al. 2018). Trimmed readings were used for de novo assembly in MegaHIT-default settings (Li et al. 2015). MitoZ was used to identify and circularise the contigs originating from mitochondria. The circularised fish mitogenome was uploaded to MitoAnnotator (http://mitofish.aori.u-tokyo.ac.jp/annotation/input.html) for mitogenome re-orientation and annotation (Iwasaki et al. 2013). The circularised mitogenome of non-fish was re-oriented using a reference genome and annotated with MITOS (Bernt et al. 2013).

Phylogenetic Reconstruction

The complete mitogenome of *B. borneoensis* specimens obtained in this present study was analysed for a phylogenetic relationship with six complete mitogenomes of turtle species under the *Batagur* genus available from GenBank with three out group from other genera namely *Pangshura sylhetensis* Jerdon, 1870, *Pangshura tentoria* (Gray, 1834) *and Mauremys mutica* (Cantor, 1842). The

ClustalW tool in MEGA X version 11 was used to compare and align voucher sequences from GenBank with consensus sequences derived from this investigation for each species (Kumar et al. 2018). A Maximum Likehood (ML) trees were constructed using IQ-TREE multicore version 1.6.12 for Linux 64-bit with the TIM2+F+G4 as the best-fit substitution model selected by ModelFinder (Kalyaanamoorthy et al. 2017) chosen according to Bayesian Information Criterion (BIC) and visualized using software FigTree v1.4.4 (Rambaut 2018)

RESULTS AND DISCUSSION

Sequence Variation

The secondary intact mitochondrial genome of *B. borneoensis* (16,397 bp) was subjected to analysis and annotation. The assembly encompassed 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes, and two non-coding regions: L-strand origin replication (OL) and control region (OH). Aside from NADH dehydrogenase subunit 6 (nad6) and seven tRNA, the majority of genes were found to be encoded on the sense (majority) strand.

The mitochondrial genome (16,397 bp) of the endangered painted terrapin, *B. borneoensis* was submitted to GenBank and assigned an accession number PP228865 in the current research. The mitogenome contains 37 genes, comprising 13 mitochondrial PCGs *in B. borneoensis; the* standard start codon (ATG) and six stop codons (AGG, TAA, AGC, GAA, TAG, and AGA) are utilised for translation initiation and termination (Figure 3). The start codon ATG is employed in all PCGs except for cox1 (GTG) and *nad3_0* (TTA), while 22 PCGs use anticodons, including *trnS2*, *trnD*, *TmK*, *trnG*, *trnR*, *trnH*, *trnS1*, *trnL1*, *trnE*, *trnT*, *trnP*, *trnV*, *trnL2*, *trnL*, *trnI*, *trnQ*, *trnM*, *trnW*, *trnA*, *trnN*, *trnC*, and *trnY*. Six PCGs (cox2, atp8, atp6, cox3, nad4, and cob) use the stop codon TAA, while cox1 employs AGG, *nad3_1* uses AGC, *nad3_0* utilises GAA, *nad6* employs AGA, and TAG is only present in *nad5*, *nad1*, and *nad2*.

Control region (CR)

Non-coding regions in the metazoan mitogenome consist of the origin of replication (OR), intergenic spacers, and the control region (CR). The mitochondrial OL of *B. borneoensis* is 27 base pairs located between 5145-5306 regions, which consist of a cluster of trnN (73bp) and trnC (66bp) genes with the CR comprising 883 base pairs situated between 15515-16397 (Figure 3).

The overall A + T content of the mitogenome was higher (58.0 %) than the G + C content (42.0 %) which is typical for a mitogenome sequence. The protein-coding genes *COX1*, *trnF*, and *nad3* start with codons other than ATG. Most protein-coding genes end with the stop codon TAA. Twelve protein-coding genes terminate with complete stop codons (AGG, AGC, AGA, TAA, and TAG), while the remaining three end with TA as partial stop codons, which are assumed to be completed to TAA by post-transcriptional polyadenylation (Anderson et al. 1981). Most mitochondrial genes in *B. borneoensis* are encoded on the H-strand, except for the ND6 gene and eight tRNA genes. The trnC(gca) gene is the shortest among the mitochondrial protein-coding genes, while the ND5 gene is the longest. The 12S and 16S ribosomal RNAs are 965 and 1601 base pairs long, respectively.

The mitochondrial replication origin (OR) plays a crucial role in mitogenome replication. Non-coding regions within the metazoan mitogenome are indispensable for the processes of DNA replication and preservation (Fernandez-Silva et al. 2003), encompassing the origin of replication (OR), diverse intergenic spacers, and the control region (CR). The process of polyadenylation at the 3'-end of mRNA, occurring after transcription, typically

serves to complete incomplete stop codons (Boore 1999). However, our study did not uncover evidence of the polyadenylation process, suggesting that the absence of stop codons or overlaps between protein-coding genes (PCGs) may have arisen due to selective pressures aimed at reducing the size of the mitochondrial genome.

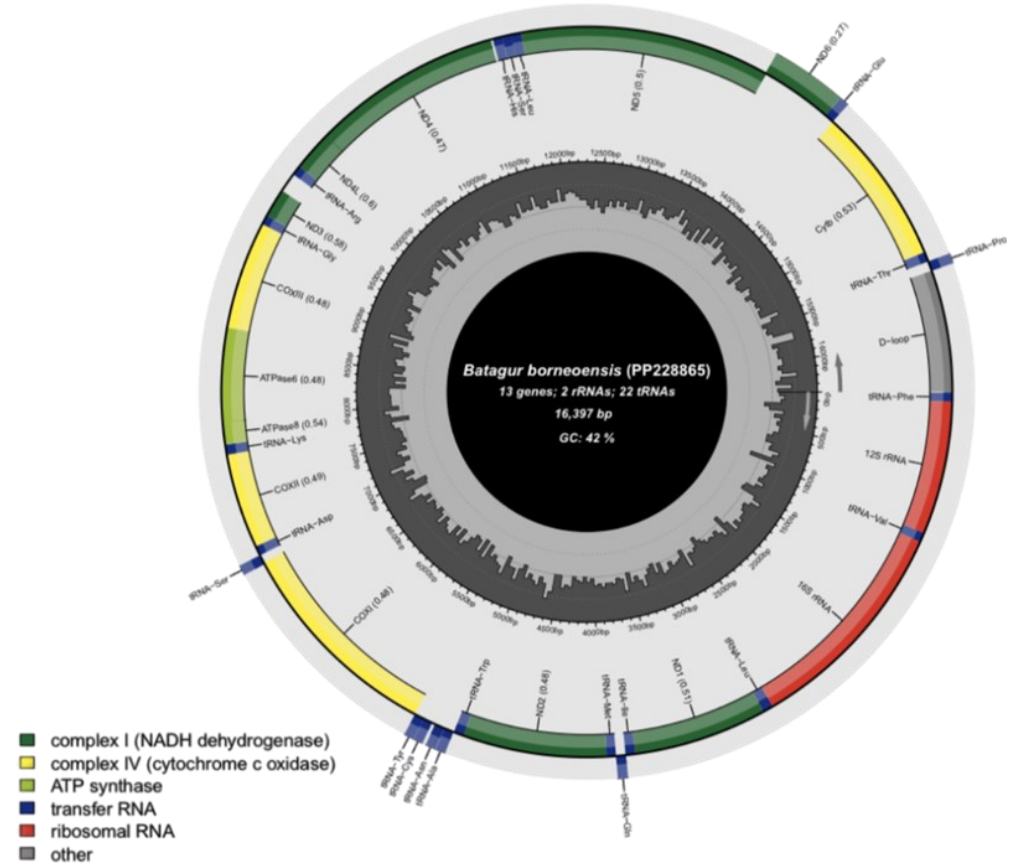


Figure 3. A novel genome map of the Malaysian *B. borneoensis* mitochondrial genome.

Phylogenetic Tree Analysis

There was a total of 22381 positions in the final dataset. The sequences used in this study including seven ingroups species, *B. borneoensis*, *B. trivittata*, *B. dhongoka*, *B. kachuga*, *B. affinis* and three outgroups, *P. sylhetensis*, *P. tentoria* and *M. mutica*.

The ML phylogenetic tree (Figure 4) offers insights into the evolutionary relationships among *Batagur* species, resulting in a well-resolved tree with all species supported with high bootstrap values. Figure 4 illustrates the construction of a TIM2+F+G4 as the best-fit substitution model selected by ModelFinder (Kalyaanamoorthy et al. 2017) using the dataset and IQ-TREE (Nguyen et al. 2015). According to the clustering in the phylogenetic tree, *B. borneoensis* forms a distinct group, with *B. trivittata* is the sister species, suggesting a close evolutionary relationship between these two species but relatively distant from other *Batagur* species in terms of evolutionary distance. Meanwhile, *B. affinis affinis* and *B. kachuga* share the closest evolutionary relationship. Unlike the conventional CO1 or CytB partial gene sequences, the full mitogenome provides a significantly higher number of variable sites, which will be valuable for delineating the evolutionary relationships of *Batagur* species in the future.

This research utilises comprehensive mitochondrial DNA data and bioinformatic analysis to investigate the evolutionary position and taxonomic classification of *B. borneoensis*. Mitochondrial genome has proven to be a valu-

able avenue for conducting phylogenetic and evolutionary investigations in vertebrates, including turtles (Kundu et al. 2019). Several studies have shown that phylogenies derived from mitogenome provide more robust insight than to those obtained from a single gene (Zhang et al. 2021). In addition, public Whole-Genome Sequencing (WGS) datasets which include numerous mitogenome sequences, are highly valuable across various field, including population research, disease association studies, and conservation genetics. Furthermore, the data supplied by WGS may also be relevant in other mitochondrial DNA (mtDNA) studies (Sturk-Andreaggi et al. 2022).

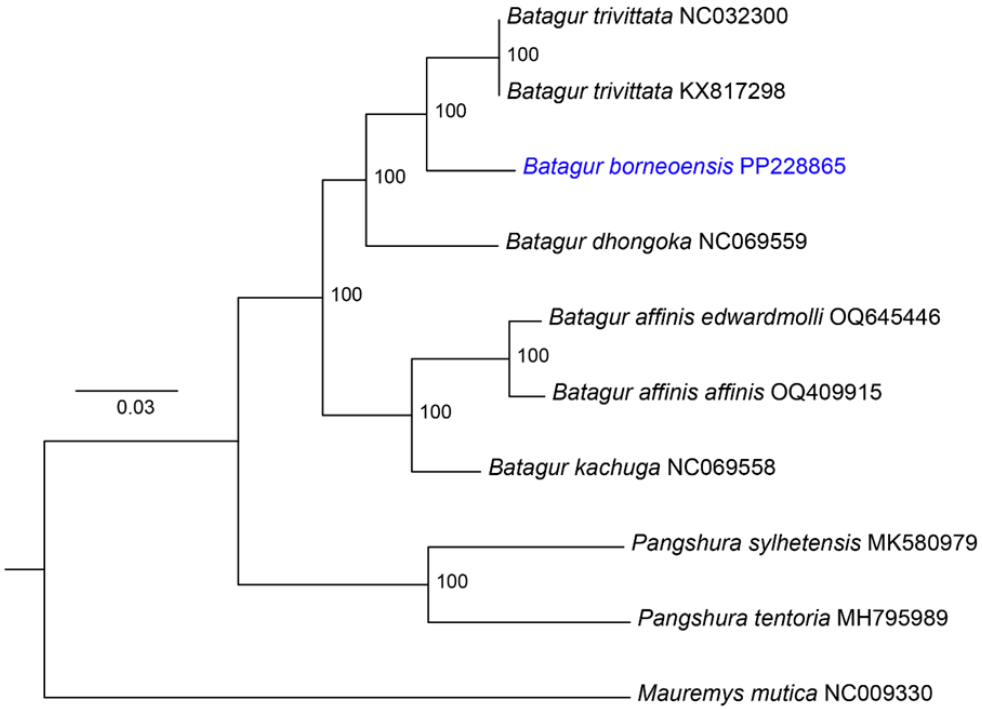


Figure 4. A Maximum Likehood phylogenetic tree from ten complete mitogenome of species in the Geomydidae family.

CONCLUSIONS

In conclusion, our study unveiled the gene organisation and features of the complete mitochondrial genome of *B. borneoensis*, marking the second characterisation of this species' entire mitogenome. Spanning 16,397 bp, the *B. borneoensis* mitogenome forms a circular molecule encompassing a regulatory region and the standard complement of 37 vertebrate mitochondrial genes. Its genetic arrangement and structural organisation closely resemble standard configurations found in other turtles mitogenome. These insights significantly contributed to our understanding of nucleotide composition and molecular evolution within *B. borneoensis* mitogenomes, providing essential data for comparative mitogenomics and advancing phylogenetic analyses within the Geomydidae family.

AUTHOR CONTRIBUTION

Conceptualisation, H.M.G, A.S.K, N.A.M; writing and laboratory work, N.A.M and M.S.A.M.N and A.G.K.; sampling and providing sample collection, M.Z.N and C.M.O; supervised and drafting, A.S.K and N.I; project administration, M.Z.M.D and S.M.S. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Research Article

From Bean to Biosphere: Vegetation Dynamics and Biodiversity in Arabica Coffee Agroforestry at Ijen Geopark

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ABSTRACT

This study evaluates vegetation biodiversity and ecological conditions at the Bondowoso Biosite of *Kebun Kopi*, Ijen Geopark, to support sustainable Arabica coffee production. Three agroforestry plots (KR1-3) and a plantation plot (PN) were analyzed for species composition. In KR1, *Acacia auriculiformis* (IVI=110) and *Trema orientalis* (IVI=190) are prominent. KR2 is dominated by *A. auriculiformis* (IVI=160) and *Ricinus communis* (IVI=80), while *Casuarina equisetifolia* is significant in KR3. The PN plot shows a more even IVI distribution between *Falcataria mollucana* and *Grevillea robusta*. Tukey's test reveals significant differences between KR1 and PN, and KR2 and PN plots (p-adj=0.001). The highest existence values are Anisoptera marginata (66.67 %) in KR1, *T. orientalis* (53.33 %) in KR2, *C. equisetifolia* (46.67 %) in KR3, and *Toona sureni* (26.67 %) in PN. Results indicate significant variations in species diversity, evenness, and dominance across plots, influenced by ecological, geographical, and anthropogenic factors. Higher Shannon-Wiener and Evenness indices in KR1 and KR2 suggest diverse species compositions are vital for ecosystem health, while KR3's dominance of few species highlights the need for biodiversity conservation. This research recommends regenerative Arabica coffee farming practices and sustainable conservation strategies at the Bondowoso Biosite of *Kebun Kopi*.

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INTRODUCTION

Ijen Geopark (7°45'18.0756"S–8°46'49.296"S and 113°47'28.3632"E–114°36'18.1548"E.), a UNESCO world heritage site, has 21 Geosites, 6 Biosites, and 9 Cultursites, with cultural, biological, and geological significance (Hasan & Ningrum 2023; Permanadewi et al. 2024). Bondowoso Biosite in Ijen Geopark produces high-quality Arabica coffee. The area's diverse flora, including Chinese Mahogany (*Toona sinensis*), Dadap (*Erythrina variegata*), Cinnamon (*Cinnamomum verum*), Pine (*Pinus* spp.), and White Paperbark (*Melaleuca leucadendra*), supports coffee agroforestry as shade plants. Studies by Anhar et al. (2021) show that high shade intensity supports optimal productivity for Arabica coffee, while Andika and Wicaksono (2020) found that 50 % light intensity increases chlorophyll content in coffee leaves compared to plants exposed to full light (100 %). These facts indicate a strong potential relationship between the presence of native vegetation and coffee agroforestry at the Bondowoso Biosite of *Kebun Kopi*.

Biodiversity not only reflects the health of ecosystems and their resilience to environmental changes but also determines the quality and uniqueness of the Arabica coffee produced (Sandifer et al. 2015; Carvalho et al. 2016). Ecological conditions, including soil quality, water availability, and climate, directly affect the growth of coffee plants and the sustainability of habitats for other species interacting within the coffee agroforestry ecosystem (Cerda et al. 2017; Sauvadet et al. 2019). This study aims to evaluate vegetation diversity at Bondowoso Biosite of *Kebun Kopi*, providing data and recommendations to support Ijen Geopark's conservation efforts and the sustainability of Arabica coffee agroforestry.

MATERIALS AND METHODS

Methods

The research at Bondowoso Biosite of *Kebun Kopi*, Ijen Geopark, was conducted from July to December 2023. Using a single plot method (20x20 m), vegetation analysis focused on agroforestry plots owned by the Community (KR) and PT Perkebunan Nusantara (PN). Three KR plots were selected for diverse vegetation, while PN was represented by one plot due to uniform agroforestry. Each plot had three repetitions to ensure comprehensive analysis, with criteria based on management diversity (Figure 1).

The primary data collected include species names, number of individuals, diameter at breast height (DBH), and total height for trees and poles, as well as stakes and seedlings. Horizontal structure data (diameter, density, and number of individuals) were also collected. Vegetation analysis results show plant diversity, Important Value Index (IVI), and specific valuable species. Further analyses include Shannon-Wiener, Margalef, and Evenness indices to determine diversity.

Important Value Index

The importance and position of a species within the observation area can be determined through the Importance Value Index (IVI). The formula that can be used to calculate the IVI (Khan 2016) is:

$$\text{Density (D)} = \frac{\text{Number of individuals of each species}}{\text{Area of Plot}}$$

$$\text{Relative Density (RD)} = \frac{\text{K}}{\text{Total number of all species}} \text{ x } 100\%$$

$$\text{Frequency (F)} = \frac{\text{Number of type a species found in a plot}}{\text{Total frequency of all species}}$$

$$\text{Relative Frequency (RF)} = \frac{\text{F}}{\text{Total frequency of all species}} \text{ x } 100\%$$

$$\text{IVI} = \text{Relative Density (RD)} + \text{Relative Frequency (RF)}$$

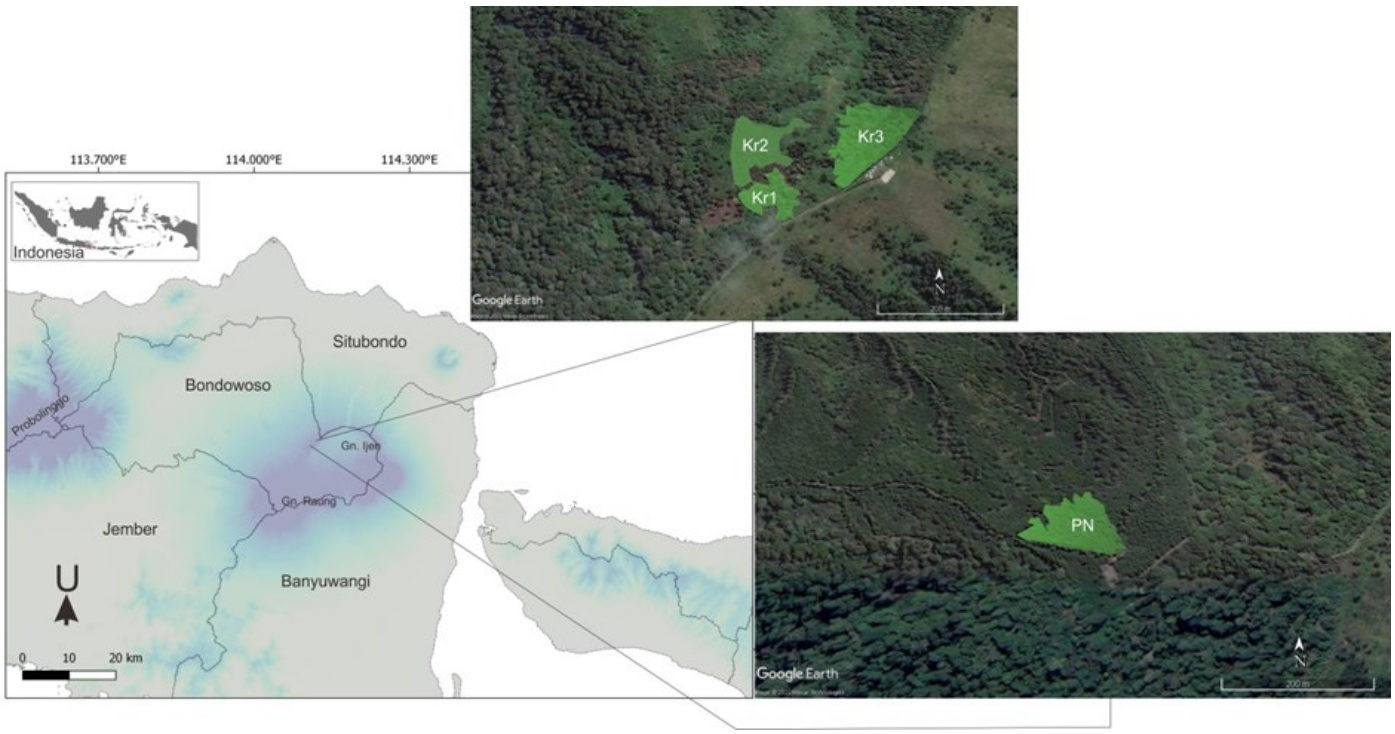

Figure 1. Map of the study area with area codes. KR is the agroforestry owned by the Community and PN is location owned by PT Perkebunan Nusantara, see methods for the details.

Shannon-Wiener Species Diversity Index (H')

The diversity of species in a region within an agroecosystem area can be determined through the Shannon-Wiener index. The formula used to calculate the Shannon-Wiener index value (Strong 2016) is as follows:

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

Keys:
H'= The Shannon-Wiener index; ni= The number of individuals of the i-th species; N= Total number of individuals of all species.
The assessment criteria for the Shannon-Wiener diversity index are:
H' < 2 (Low diversity); 2 < H' < 3 (Moderate diversity); H' > 3 (High diversity)

The Evenness Index (E)

The evenness index can be used to calculate the evenness of the abundance of individuals for each species. The formula used to calculate the evenness index (Kvålseth 2015) is as follows:

$$E = \frac{H'}{\ln S}$$

Keys:
E = Evenness Index; H' =Shannon-Wiener Diversity Index; S= The number of identified species
The assessment criteria for the Evenness Index are:
E > 0.6 (High evenness of species); 0.3 < E < 0.6 (Moderate evenness of species); E < 0.3 (Low evenness of species).

The Margalef Species Richness Index (DMg)

The species richness index can determine the species richness within an ecosystem compared to the total number of individuals. The formula used to calculate the Margalef species richness index (van Loon et al. 2018) is as follows:

$$DMg = \frac{(s-1)}{\ln N}$$

Keys:
DMg=Margalef Species Richness Index; S=The number of identified species; N = The total number of individuals of all species.
The assessment criteria for the Margalef species richness index are:
Dmg < 3.5 (Low level of species richness); 3.5 < Dmg < 5 (Moderate level of species richness); Dmg > 5 (High level of species richness).

Ecological Status Assessment

Subsequently, an ecological status assessment is conducted to examine the existence factors of species within the agroecosystem. The formula used is (Sulistyowati 2008):

$$Ex = \left(\frac{Fr\ status + Cs\ status\ + Gd\ status}{3 \times 5}\right) \times 100$$

Where:
Ex = Existence value; Fr status = Frequency status level of the species; Cs status = Conservation status level of the species; Gd status = Geographic distribution status level.

Fr status is obtained from analysis of vegetation results. Cs and Gd status were obtained from IUCN (https://www.iucnredlist.org/_). Below are some conversion tables for Fr, Cs, and Gd status (Table 1-3, Sulistyowati 2008):

Table 1. Frequency status level (Fr, Sulistyowati 2008)

Percentage	Fr Status
81-100	1
61-80	2
41-60	3
21-40	4
0-20	5

Table 2. Conservation status level (Cs, Sulistyowati 2008)

Cs	Cs Status
CR = *Critically Endangered*	5
EN = *Endangered*	4
VU = *Vulnerable*	3
NT = *Near Threatened*	2
LC = *Least Concern*	1

Table 3. Geographic distribution status level (Gd, Sulistyowati 2008)

Geographic Distribution (Area)	Gd status
Distributes in certain local area (dl)	5
Distributed in region/island within country (dr)	4
Distributed in Indonesia country (di)	3
Distributed in continental Asia (da)	2
Distributed throughout the world (dw)	1

Existence value differences were analyzed using ANOVA and Tukey's HSD test, visualized with PCA graphs using matplotlib, Seaborn, and KDE, and described descriptively.

RESULTS AND DISCUSSION

Results

Important Value Index

The Important Value Index (IVI) visualization highlights key species roles in KR1, KR2, KR3, and PN plots (Figure 2). In KR1, *Acacia auriculiformis* (IVI=110) and *Trema orientalis* (IVI=190) show high ecological significance. KR2 is dominated by *Acacia auriculiformis* (IVI=160) and *Ricinus communis* (IVI=80, Figure 6C). KR3's dominant species is *Casuarina equisetifolia*. In the PN plot, *Falcataria mollucana* and *Grevillea robusta* show a more balanced IVI distribution, indicating greater ecological diversity and balance.

Shannon Diversity Index, Evenness, and Simpson Dominance

The Shannon index shows KR1 and KR2 have higher species diversity and more even distribution than KR3 (Figure 3). High Evenness in KR2 indicates balanced distribution. The Simpson dominance index reveals KR1 and KR2 have lower dominance, while KR3 has higher dominance, needing more biodiversity conservation and management.

Biodiversity Value

The Analysis of Variance (ANOVA) on Ex values (%) shows a significant difference between locations (p-adj = 0.00036), below the 0.05 threshold. This indicates substantial variation in species existence due to ecological, geographical, or anthropogenic factors. Post-hoc Tukey's HSD test identifies significant differences between certain pairs, notably KR1 and PN, and KR2 and PN (p-adj=0.001), marked by double asterisks (**) for high significance (Table 4).

Table 4. Tukey test analysis result (p-adj) indicating vegetation differences between locations. See methods for detailed abbreviation codes.

Location	KR2	KR3	PN	KR1
KR1	0.9	0.4498	0.001**	-
KR 2		0.4297	0.001**	0.9
KR 3			0.1355	0.4498
PN				

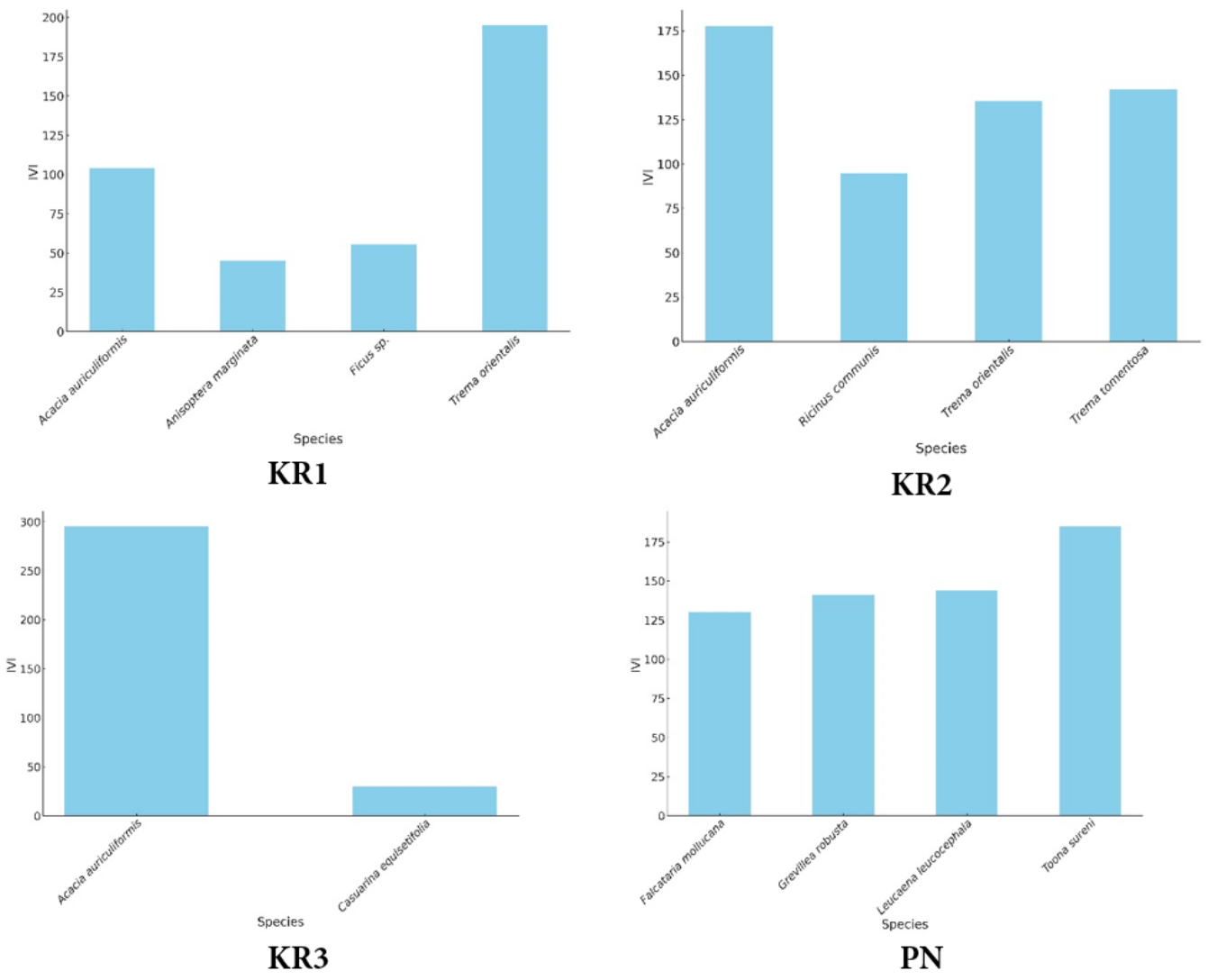

Figure 2. Important Value Index (IVI) graphs at 4 locations. See methods for detailed abbreviation codes.

The boxplot of Ex values (%) (Figure 4) shows significant variation in species existence across locations. Each location has species with the highest Ex values, indicating their prevalence, such as *T. orientalis*. PN and KR2 feature species with the lowest Ex values, indicating lesser dominance.

At KR1, *A. marginata* has the highest Ex value (66.67 %), while *A. auriculiformis* (Figure 6A) has the lowest (40 %, Figure 5). In KR2, the highest Ex value is 53.33 %, and *T. orientalis* (Figure 6B) the lowest (40 %). For KR3, *C. equisetifolia* is highest (46.67 %) and *A. auriculiformis* lowest (26.67 %). In PN, *Toona sureni* is highest (26.67 %) and *Leucaena leucocephala* lowest (20 %).

The PCA visualization displays a dataset that encompasses various locations (focusing on Ex (%) values). Each location is represented by a different color and plotted in a two-dimensional space generated by PCA. The size of each point on the graph is proportional, providing an overview of the biodiversity value at each location. Specifically, for the KR2 location, the more contrasting color highlights its extreme difference from other locations, facilitating the identification of its data distribution in PCA space, and indicating its distribution is much wider and larger compared to other locations.

The biplot lines in the graph, emanating from the center point, represent the features 'FR Status', 'Cs Status', and 'Gds' (Figure 7). Each arrow indicates the influence and contribution of these features to the variability in the dataset. The length and direction of the arrows show how significantly

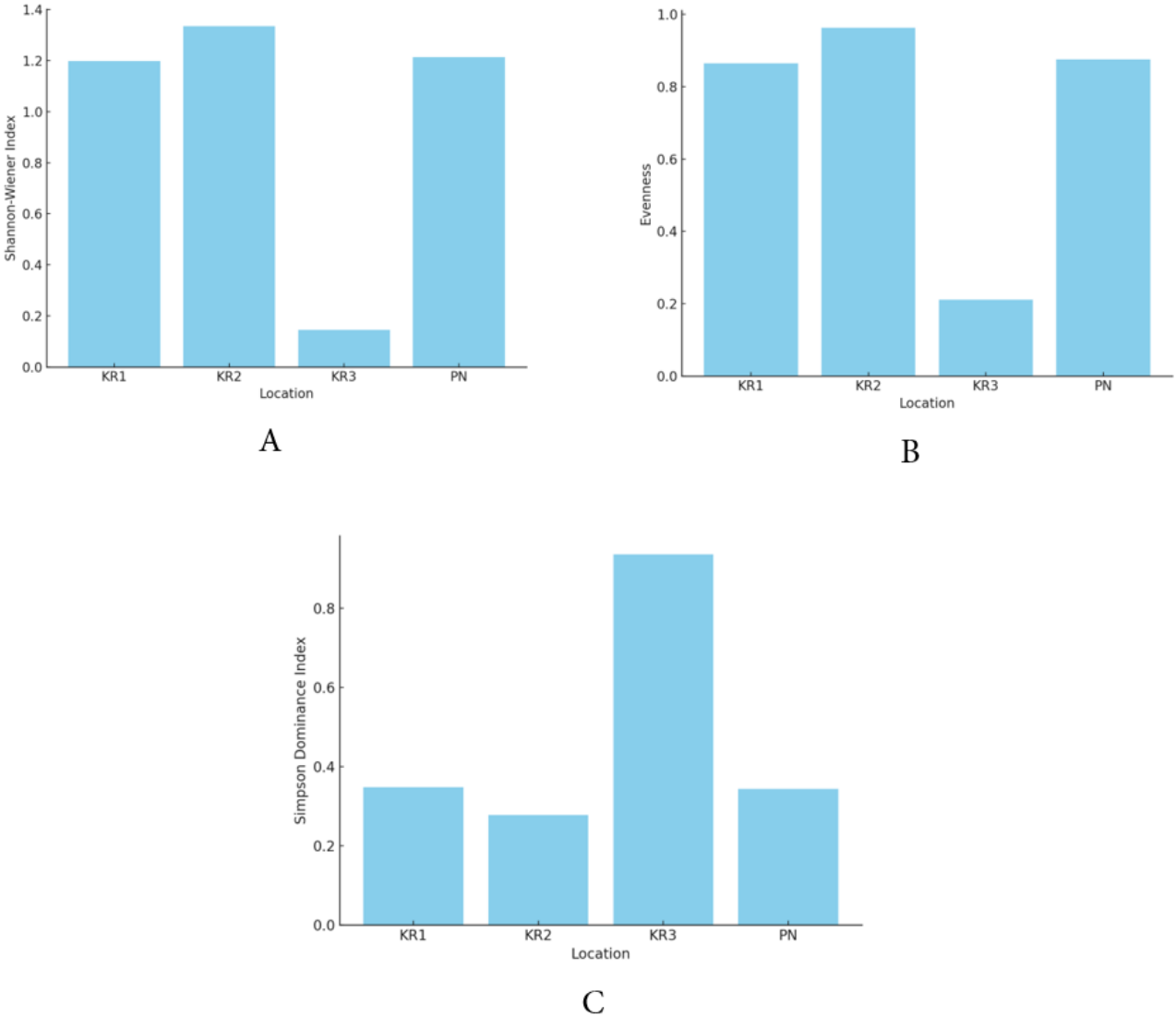

Figure 3. Ecological indexes of location. Keys=A. Shannon-Wiener; B=Evennes; C. Simpson Dominance. See methods for detailed abbreviation codes.

these features influence the formation of the principal components. The biplot helps to understand the relationship among all parameters: FR, Cs, and Gds, which interact with the overall KR2 distribution, and Cs and FR have a weak interaction with KR1.

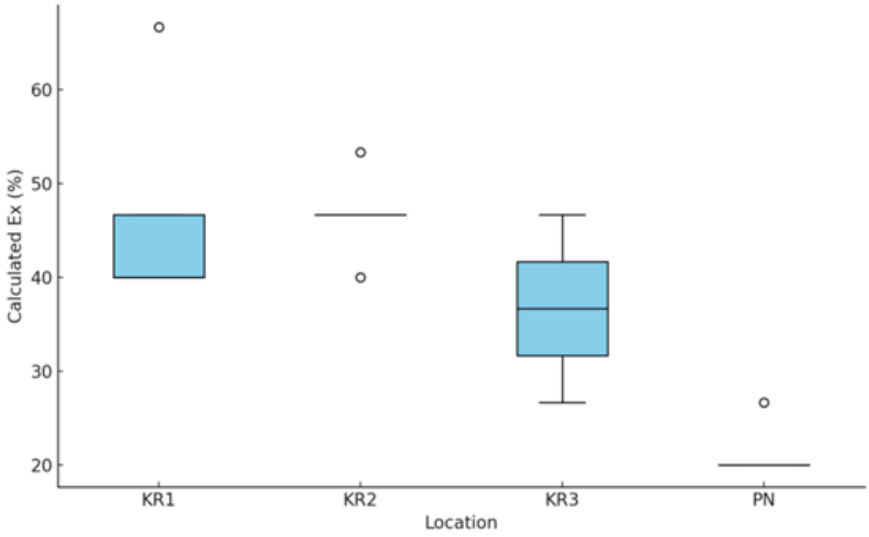

Figure 4. Boxplot graph of ex (%) across locations. This boxplot illustrates the different mean values and deviations across locations. See methods for detailed abbreviation codes.

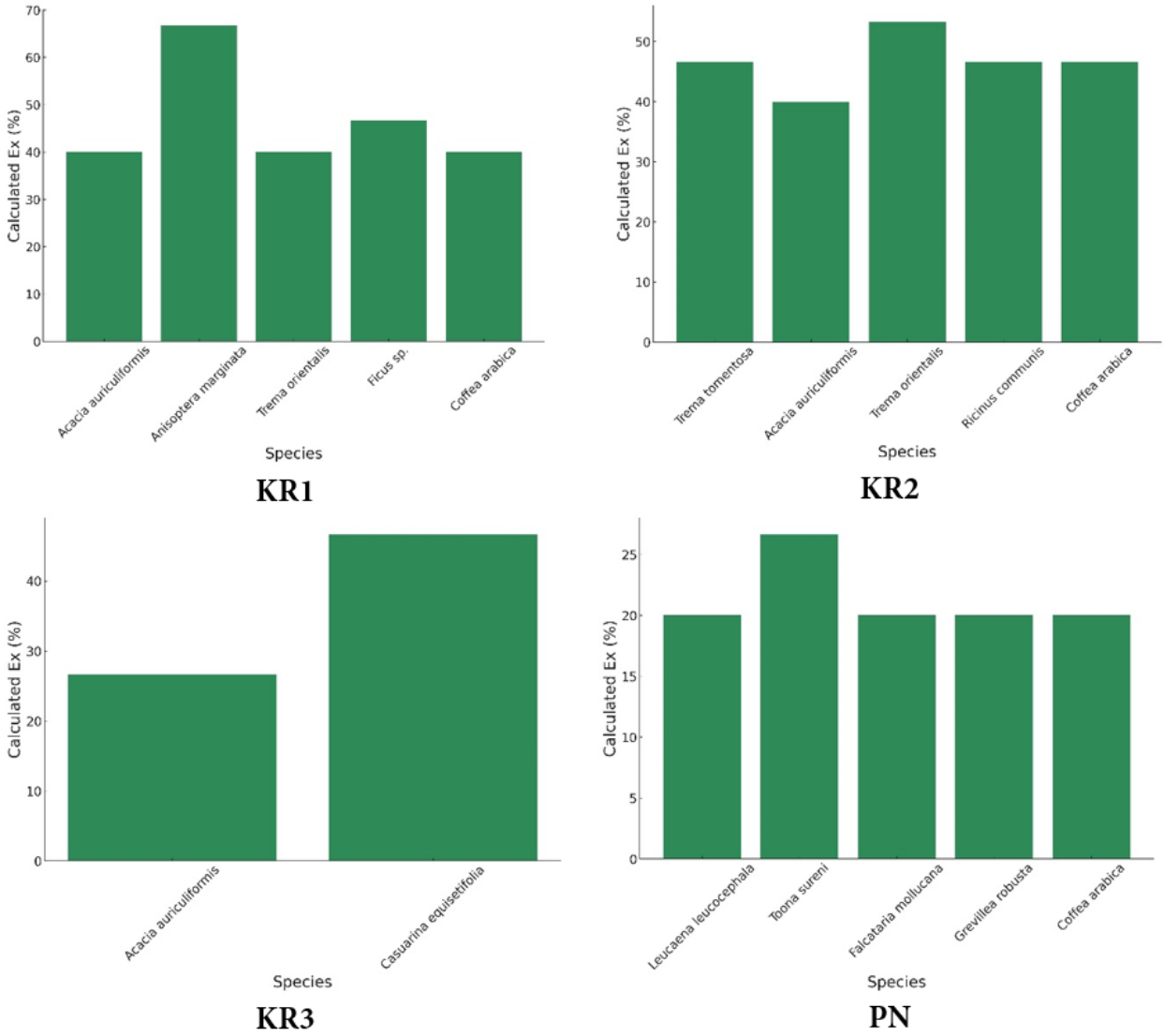

Figure 5. Comparison of Ex values at each location with different plant types.

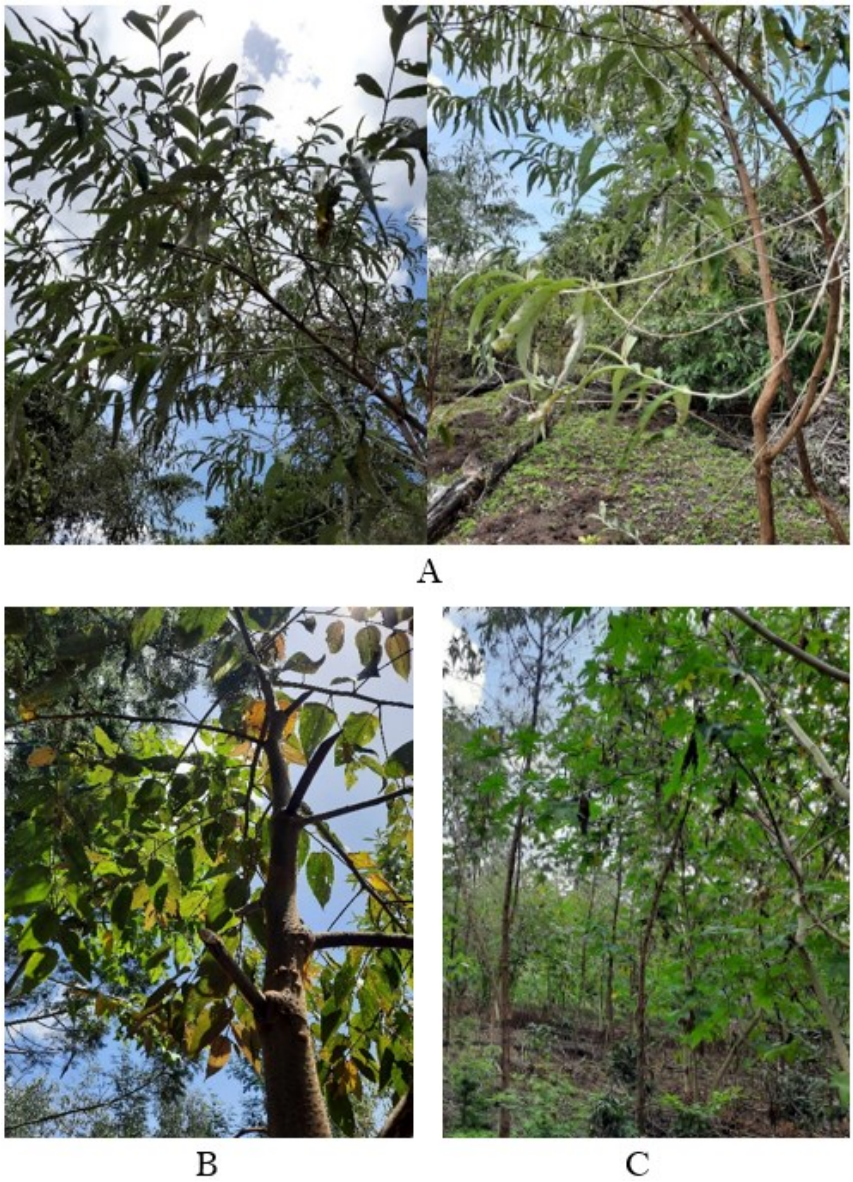

Figure 6. various vegetations at The Ijen Coffee Biosite. Keys=A. *Acacia auriculiformis*; B. *Trema orientalis*; C. *Ricinnus communis*.

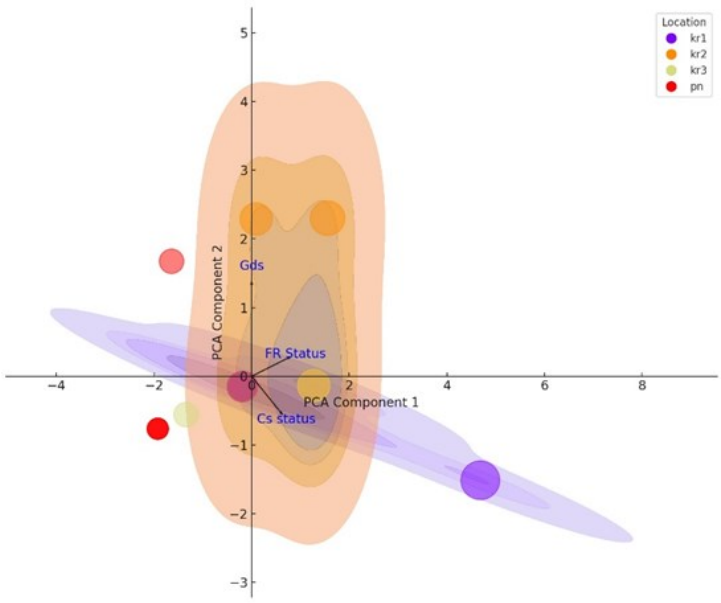

Figure 7. PCA graph of biodiversity value preference found at each location, linked to supporting components Gds, FR, and Cs Status. The size of the circles indicates the data density at each location.

Discussion

The IVI analysis reveals dominant species in each plot. In KR1, *A. auriculiformis* (IVI=110) and *T. orientalis* (IVI=190) are most dominant, indicating their ecological importance. KR2 shows high IVI scores for *A. auriculiformis* (IVI=160) and *R. communis* (IVI=80), reflecting their competitive advantages. *A. auriculiformis* dominates the vertical and horizontal structure, influencing other species' composition due to intense competition. (Azad & Sumon 2016; Ngoan et al. 2023; Sikuzani et al. 2024). Additionally, *A. auriculiformis* is a nitrogen-fixing plant that can increase soil nitrogen content. Furthermore, its dominance provides suitable habitats for many birds, small mammals, and insects. Its canopy also creates a favorable microclimate, particularly beneficial for coffee.

In KR3, *C. equisetifolia* holds a dominant role, possibly reflecting its unique adaptation to the plot's conditions. This plant has an extensive root system and needle-like leaves, providing considerable biomass and covering large areas, which can fundamentally influence the microclimate, such as light, water, and nutrients. *C. equisetifolia* has the ability to enhance soil fertility through nitrogen fixation (Atangana et al. 2014). The PN plot shows a balanced IVI distribution among species like *F. mollucana* and *G. robusta*, indicating greater ecological diversity. *F. moluccana*, known for rapid growth and nitrogen fixation, improves soil fertility and supports other plants. Its extensive canopy moderates light, temperature, and humidity, benefiting the ecosystem (Ambas et al. 2024). *G. robusta* is valued for its resilience, versatile canopy providing shade, maintaining soil moisture, attracting pollinators, and is used for timber, windbreaks, and shade in coffee plantation (Nesper et al. 2019; Sharma et al. 2023).

R. communis is capable of thriving at high densities due to its remarkable competition with other plants, a result of its production of allelopathic toxins that inhibit the growth of other plants (Saddiqe et al. 2020). It is also planted as a boundary marker (Boğan et al. 2020). Consequently, it becomes a new dominant regime, highlighting its importance in a location. The differences in diversity values indicate that the factors influencing species presence at these locations differ substantially, providing important insights for further research on the management and conservation of biodiversity in these areas (Udawatta et al. 2019). Locations KR1 and KR2 exhibit high diversity and significant biodiversity value, with *T. orientalis* and *A. auriculiformis* being key species. Variations in diversity, evenness, and dominance are influenced by species-specific interactions. Nitrogen-fixing *A. auriculiformis* in KR1 and KR2 enhances soil fertility, supporting higher diversity and evenness (Azad & Sumon 2016; Sikuzani et al. 2024). Conversely, the dominance of *C. equisetifolia* in KR3, with its extensive root system and needle-like leaves, may create a microenvironment that suppresses other species, resulting in lower diversity and higher dominance. Soil quality and fertility, microclimatic conditions such as light intensity and humidity, and human management practices, including land use intensity and agroforestry techniques, also contribute to these ecological differences (Atangana et al. 2014). The balanced IVI distribution in the PN plot, with species like *F. moluccana* and *G. robusta*, reflects greater ecological diversity and balance, likely due to effective management and favorable environmental conditions (Nesper et al. 2019; Sharma et al. 2023; Ambas et al. 2024).

The ANOVA results demonstrate substantial differences in species presence across various locations, likely resulting from a combination of ecological, geographical, and human-induced factors that influence species distribution and prevalence (Aronson et al. 2014; Ellis 2015). High Ex (%) values at KR1 indicate species play critical roles in ecosystem structure and function, such as providing habitats or food sources. Low Ex (%) values suggest species

face high competition or are unsuited to the environment, requiring special conservation attention if important for biodiversity. PN shows strong human -plant interaction. On the other hand, plantations are managed more for economic gain than for conservation (Haggar et al. 2017). Overall, Ex (%) values help identify key species in ecosystems and provide important insights for habitat management and conservation strategies (Sulistyowati 2008). Again, this highlights the importance of community-developed agroforestry for the well-being of communities around the Bondowoso Biosite of *Kebun Kopi*, while also serving as a protector in its conservation strength. This dual role makes agroforestry a promising sustainable agricultural system in the modern era (Wilson & Lovell 2016).

High Ex (%) values, such as for *A. marginata* in KR1 and *T. orientalis* in KR2, indicate the dominance or greater presence of these species at those locations, as well as their critical role in the ecosystem. *A. marginata*, a member of the Dipterocarpaceae family, which is characteristic of the Indonesian tropical region, signifies areas with good forest conservation (Brearley et al. 2016). However, many members of the Dipterocarpaceae are now severely damaged due to forest land conversion and the demand for high-quality building wood (Widiyono 2021). *T. orientalis*, or locally known as 'Mengkirai', is a species with high intolerance levels but capable of coping with high heavy metal content, making it suitable for restoration of former mining lands with high Ni content (Rodrigues & Rodrigues 2014). Its significant presence in the Ijen Biosite of *Kebun Kopi* highlights the importance of preserving species with high environmental sensitivities.

Conversely, species with low Ex values, like *L. leucocephala* in PN, may face higher competition or be less suited to the environmental conditions, indicating the importance of more specific conservation or management strategies for these species. The competition arises because this plant is intentionally provided as the primary shade in coffee agroforestry. This reflects how Ex values can provide insights into the ecological and conservation importance of various species within their local contexts.

CONCLUSIONS

The IVI highlights dominant species in each plot: *A. auriculiformis* (IVI=110) and T. *orientalis* (IVI=190) in KR1; *A. auriculiformis* (IVI=160) and *R. communis* (IVI=80) in KR2; *C. equisetifolia* in KR3. PN shows a balanced IVI among species like *F. mollucana* and *G. robusta*. Shannon Diversity, Evenness, and Simpson Dominance indices reveal KR1 (H'=1.20, E=0.86, D=0.35) and KR2 (H'=1.33, E=0.96, D=0.28) have higher diversity than KR3 (H'=0.15, E=0.21, D=0.94). This research affirms the comprehension of biological diversity and natural ecological conditions at the Bondowoso Biosite of *Kebun Kopi* with the sustainability of Arabica coffee production.

AUTHOR CONTRIBUTION

N.D. designed the research and was responsible for overseeing the entire research process. A.S.K. collected data and wrote the manuscript.

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CONFLICT OF INTEREST

All authors declare that there is no conflict of interest with any party, concerning the data collection, writing, and the forthcoming publication of this

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Research Article

Phytosociology of Trees in Siranggas Wildlife Sanctuary Area, North Sumatra, Indonesia

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ABSTRACT
The lower mountainous region's Siranggas Wildlife Sanctuary is a conservation area with a variety of tree species, however it is susceptible to exploitation-related harm. Despite the area's ecological advantages, little is known about the diversity of tree species and their associations. Therefore, the purpose of this study was to ascertain the Siranggas Wildlife Sanctuary Area's tree species diversity and associations. Using the purposive sampling approach, the study was carried out from March to April 2022. Five plots, each measuring 30 by 60 meters, were set up at various heights. The Importance Value Index (IVI) and the Shannon-Wiener Diversity Index (H') were used to examine the vegetation data. Associations between tree species were identified by considering two species with the highest IVI in the observation plot. The results showed that there were 119 tree species, 38 families, and 79 genera in the Siranggas Wildlife Sanctuary Area. The diversity index across the plot was relatively high with an H' value > 3. Trees with a height of 10 to 15 meters were the prevalent canopy height profile in the plot, while the dominant trunk diameter across the whole plot was 10 to 20 cm. The association that occurred in the study area was the *Schima wallichii* – *Syzygium cerasiforme* based on two of the greatest IVI and their distribution throughout all plots from varying altitudes. Future forest management and restoration initiatives in the Siranggas Wildlife Sanctuary area can benefit from the presence of both of these species.

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INTRODUCTION

Siranggas Wildlife Sanctuary is a montane forest area that is estimated to have high flora diversity in North Sumatra. This region is considered a conservation area that is highly protected from exploitation activities. The Siranggas Wildlife Sanctuary Forest is a transitional area from lowland to lower montane forests due to its location at an altitude of approximately 800 to 1273 meters above sea level (masl). The lower montane forest ecosystem is a priority area for conservation because of its high biodiversity and is vulnerable to forest conversion activities (Shoo & Catterall 2013). Therefore, for effective management and empowerment of this area, it is crucial to obtain data on the presence of species and important components contained in the ecosystem.

One of the key components of the foundation for the construction and management of conservation areas is an understanding of the diversity of plant species. Additionally, existing forest vegetation patterns that can be known through phytosociology studies also provide important information in the context of the conservation of these areas.

A subfield of vegetation science named phytosociology is concerned with the sociology or societies of plants. This scientific component is crucial for examining the composition and structure of plant communities, which helps to clarify how tree communities relate to other plant communities in a given area (Dengler 2017). Because they store vast amounts of biomass and carbon, trees are a vital component of the global biosphere. Only 12–15 % of the earth's terrestrial area is covered by tropical forests, which are home to approximately 40.000 tree species (Slik et al. 2015) and account for over 60 % of all forest biomass worldwide (Pan et al. 2011). Additionally, tropical forests are crucial to the earth's carbon, water, and other biogeochemical cycles. Due to their significant role in maintaining the sustainability of tropical rainforest ecosystems, trees were selected as the main focus of this study.

Phytosociology studies, specifically in mountainous areas in Indonesia are still very limited. Several studies have been conducted, such as Mirmanto (2013) on Mount Salak West Java, Purwaningsih et al. (2017) on Mount Wilis, East Java, as well as Mansur and Kartawinata (2017) in the Mount Batulanteh area, in Sumbawa, West Nusa Tenggara. In North Sumatra, where Siranggas Wildlife Sanctuary is located, study on tree phytosociology is also still neglected. Thus, the purpose of this study was to ascertain the Siranggas Wildlife Sanctuary Area's tree species diversity and associations. The results are expected to provide tree vegetation information to help forest management and further study related to plant communities. This important information can be scientifically documented, specifically in the Siranggas Wildlife Sanctuary Area.

MATERIALS AND METHODS

Study Site

This study was conducted from March to April 2022 in the Siranggas Wildlife Sanctuary Area, Pakpak Bharat Regency, North Sumatra. Geographically, the area is located between 02°33'48.6"- 02°38'11.3" N and 98°7'22.7"- 98°8'37.3"E with an area of ± 5,657 ha. Administratively, Siranggas is located in 5 districts, namely Kerajaan, Salak, Sitellu Talu Urang Jehe, Tinada, and Pargetteng-getteng Sengkut Districts, Pakpak Bharat Regency, North Sumatra, as shown in Figure 1. The climate is tropical with a relatively high average rainfall, ranging from 1900 to 3000 mm per year, an average temperature of 19.64 °C, and an air humidity of 83.28 %. The topography is very sloppy and steep, with altitudes between 800 – 1275 masl. The lowest area is adjacent to limited-production forests managed by residents. Furthermore, there is a primary forest, which serves as a conservation area from an altitude of 800 masl to the top.

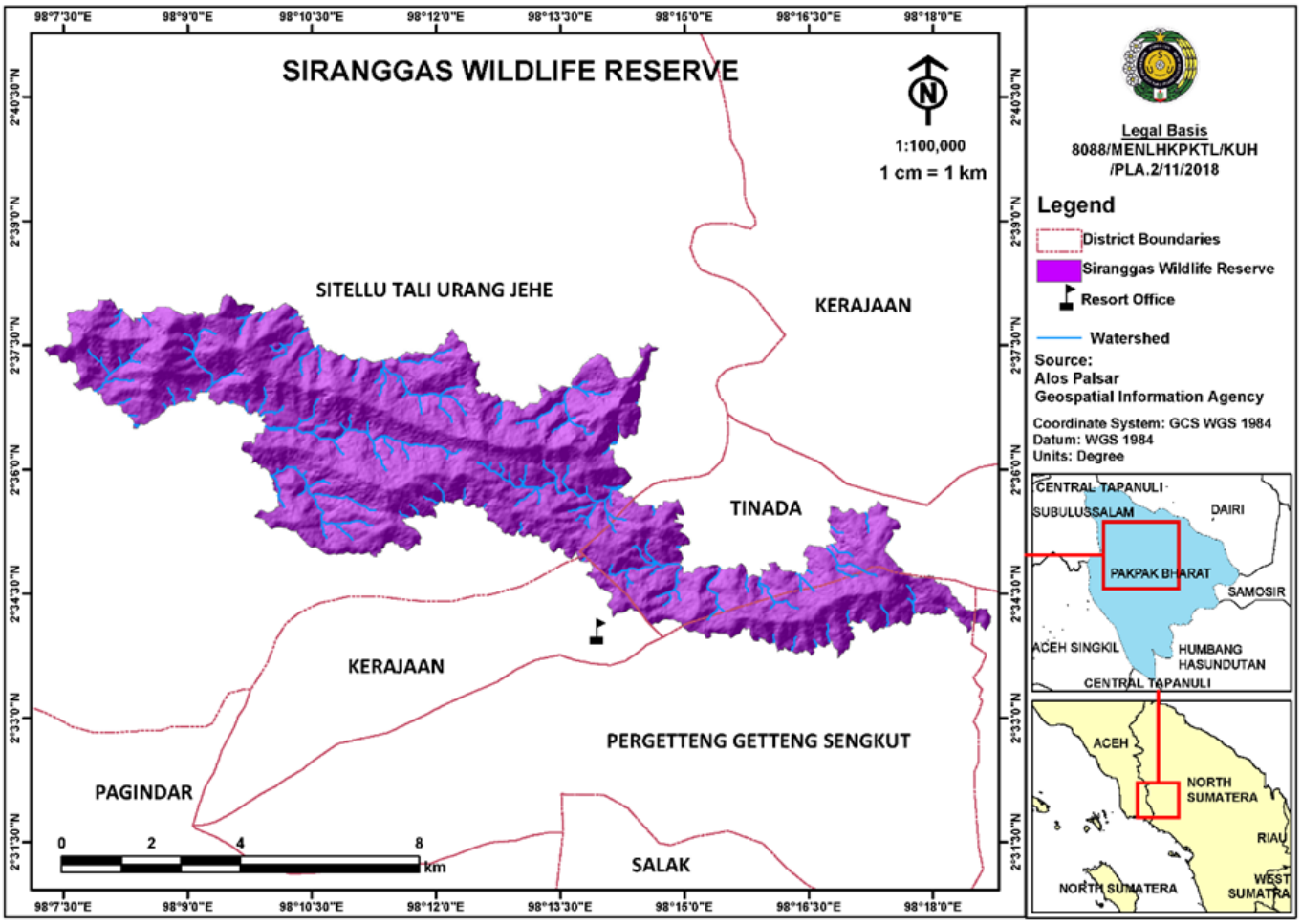

Figure 1. Map of Siranggas Wild Life Sanctuary.

Methods

This study was carried out using the purposive sampling method for plants with tree categories that were considered representative of the forest. Data collection was carried out starting from an altitude of 800 to 1100 masl. As shown in Figure 2, five plots with a size of 60 x 30 meters and 18 subplots measuring 10 x 10 meters in each plot, resulting in 90 subplots with a total area of 9000 m². The location of the plot was divided into 5 height zones, namely plot 1 (800 masl), plot 2 (900 masl), plot 3 (1000 masl), plot 4 (1100 masl), and plot 5 (1200 masl). In each subplot, all trees with a Diameter at Breast Height (DBH) ≥ 10 cm were measured using the clinometer. At each location of the plot, environmental parameters were measured as supporting data, including air temperature and humidity using a hygrometer, light intensity using a lux meter, soil temperature and pH with a soil tester, altitude with an altimeter, and coordinate points using the GPS on each plot. Plant species were identified using several identification methods as stated in previous reports (Soepadmo & van Steenis 1972; Geesink et al. 1981; Soerianegara & Lemmens 1994; Lemmens et al. 1995; Keller 1996; Sosef et al. 1998; Soepadmo & Wong 2005; Van Steenis 2006; Soepadmo & Wong 2007).

Data Analysis

The data obtained in the field were calculated as Relative Density (RD), Relative Frequency (RF), Basal Area (BA), Relative Dominance (RD), Important Value Index (IVI), and Diversity Index (H'). Calculations of density, frequency, and dominance values as well as their relative values were based on standard methods (Krebs 2014).

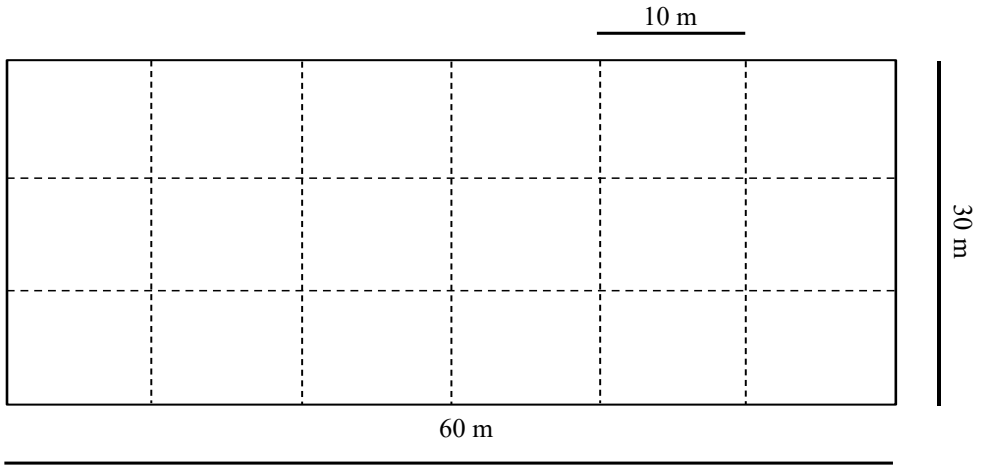

Figure 2. Plot layout and its subplots.

Species Composition

$$\text{Density (D)} = \frac{\text{Number of individuals of a species}}{\text{Total Area}}$$

$$\text{Reative Density (RD)} = \frac{\text{Density of a species}}{\text{Density of all species}} \times 100\ \%$$

$$\text{Frequency (F)} = \frac{\text{Number of individuals}}{\text{Total number of plots}}$$

$$\text{Relative Frequency (RF)} = \frac{\text{Frequency of a species}}{\text{Frequency of all species}} \times 100\ \%$$

$$\text{Dominance (D)} = \frac{\text{Basal area of a species}}{\text{Total area}}\ m^2\ Ha^{-1}$$

$$\text{Relative Dominance (RD)} = \frac{\text{Dominance of a species}}{\text{Total dominance of all species}} \times 100\ \%$$

Important Value Index (IVI)= RD+RF+RD

Species Diversity

$$H' = -\sum_{i=1}^{S} p_i \ln p_i \quad ; \text{pi} = \frac{\text{ni}}{\text{N}}$$

Description:
ni : Number of individuals of each species
N : The total number of individuals of all species
H' : Shannon-Wiener Diversity Index
ln : Natural logarithm
The Shannon-Wiener Diversity Index is divided into 3 categories:
H' < 1 = Low diversity
1 < H' < 3 = Moderate Diversity
H' > 3 = High diversity

Association and Subassociation

According to Willner (2006), an association is a unit of vegetation in a plant community that has a clear floristic composition and can describe the dominant community. Based on the Braun-Blanquet method (Mueller-Dumbois & Ellenberg 1974), an association can be determined using the distribution of species and their associations. In this method, two species with the highest Important Value Index or those exhibiting distribution most

evenly across the study sites are indicative of the associations that occur throughout the observed vegetation. Moreover, sub-association describes associations that occur on a smaller scale and are derived from two species with the highest dominance value of each plot.

RESULTS AND DISCUSSION

Vegetation Characteristics

The results presented in Table 1 showed that the diversity of trees in the Siranggas Wildlife Sanctuary Area, Pakpak Bharat Regency, consisted of 38 families, 79 genera, and 119 species with a total of 268 individuals.

Table 1. Vegetation Characteristics of the Entire Study Plot in Siranggas Wildlife Sanctuary, Pakpak Bharat District.

Vegetation Characteristics	All Plot	Plot 800 masl	Plot 900 masl	Plot 1000 masl	Plot 1100 masl	Plot 1200 masl
Plot size (Ha)	0.9	0.18	0.18	0.18	0.18	0.18
Number of species	119	48	38	39	24	23
Number of genera	79	39	33	32	18	18
Number of families	38	25	24	23	12	15
Total Individuals	268	72	52	64	45	35
Tree density/ hectare	298	400	288	356	250	194
Shannon-Wiener Index (H')	5.04	3.76	3.54	3.50	3.03	3.05

Several characteristics were observed in each vegetation with different altitudes in each plot created. The total area of the plot was 0.9 hectares with the size of each plot measuring 0.18 hectares. Among the 5 plots, the plot at 800 masl was the lowest with the highest richness of species, genera, family, many individuals, tree/hectare density, and the Shannon-Wiener Index. The diversity index between the plots of 800 masl, 900 masl, and 1000 masl was almost the same but dropped significantly from an altitude of 1100 masl and 1200 masl. Based on the Shannon-Wiener diversity index, the entire plot had a high diversity due to the H' > value of 3, the plot elevation of 1100 masl had the lowest value with an H' value of 3.03. This decrease in the value of diversity can be related to the altitude of the area, as studies conducted in the mountain rainforests on Sumatra Island showed a decrease in species richness with increasing altitude above sea level (Ashton 2003; Nishimura et al. 2006). Similarly, a decrease in the richness of tree species was also reported in the mountains of the Java region at varying altitudes ranging from 600 to 2000 masl (Helmi et al. 2009).

Based on environment parameter data obtained from this study, the 1200 masl plot had the lowest air temperature, specifically the soil pH compared to plots at other altitudes. This affected the diversity of tree species that grew on the plot. According to Cheng et al. (2020), soil pH was found to be one of the factors that significantly determined the type of vegetation and the diversity of plant species living in an environment. The diversity of tree species found in the Siranggas Wildlife Sanctuary decreased as the altitude increased, due to the low pH of the soil at altitudes more than 1100 masl.

Canopy Height Class

The measurement of the canopy height carried out on each tree obtained a profile chart as presented below.

The canopy height data in Figure 3 showed that the dominant trees had a height ranging from 10.1 to 15 meters, with the largest number of individu-

als. The fewest individuals were within the height category of > 30 meters, consisting of 5 individuals from 5 species, namely *Garcinia penangiana*, *Shorea curtisii*, *Santiria apiculata*, *Schima wallichii*, and *Castanopsis argentea*. The tallest tree found was *Shorea curtisii* with a height of 38 meters. Individuals with canopy heights above 30 meters were found only on plots of 800 masl. The study by (Adrah et al. 2021) on variations in canopy height in secondary forests also showed that the average canopy height in lowland areas was higher than that of highlands. This indicated that the height of the tree canopy decreases with increasing elevation due to several factors such as temperature, availability of nutrients, and extremely steep land conditions that can limit the growth of the tree canopy. According to Körner (2012), height was a major obstacle to tree growth in mountainous areas.

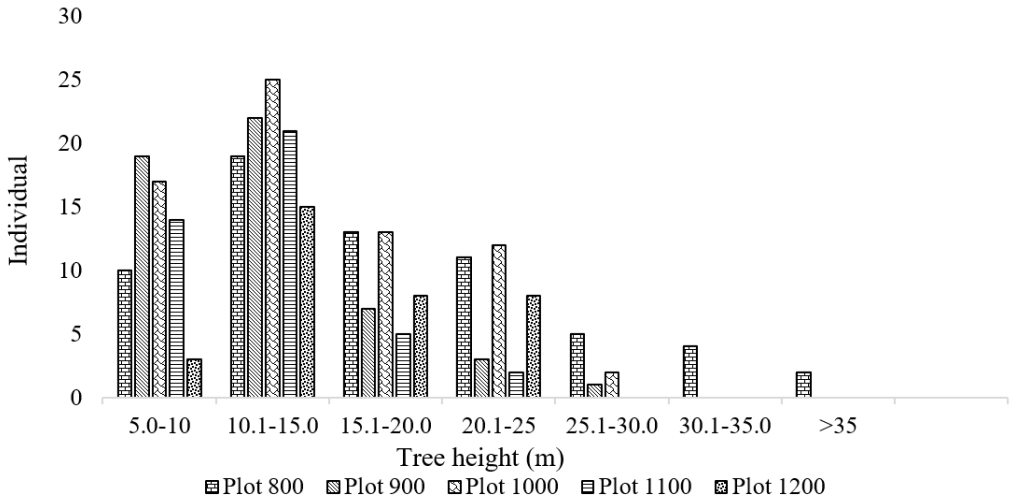

Figure 3. Canopy height profile chart.

The results showed that the tree with the highest canopy was only found in the lowest plot, which was 800 masl, classified as a lowland area. According to Jones et al. (2013), canopy height was a collection of individual tree height data in an area, which was one of the most important forest metrics. Forest canopy elevation played an important role in determining aboveground biomass, forest productivity and restoration, carbon sequestration and reserves, biodiversity, forest resilience to disturbances, climatic extremities such as drought, and tree mortality (Xu et al. 2018; Marselis et al. 2019).

Stem Diameter Class

The profile chart of the tree diameter measurements made on each tree is presented below.

Figure 4 showed that the diameter of the trunk in the range of 10–30 cm had the greatest number of individuals, namely 64.15 %. This value was greater than other diameter classes, consisting of 35.85 % of the diameter class 30.1–40 cm, 40.1–50 cm, and 40.1–50 cm. The lowest number of diameter classes was >70 cm in diameter with only 3 individuals from the entire plot. Based on the results, the size of the tree diameter in the Siranggas Wildlife Sanctuary did not show a decreasing trend. This was indicated by the average tree height which continued to decrease along with the increase in height at the study site. According to Coomes and Allen (2007), the diameter of trees did decrease or increase significantly to an altitude above 1000 masl. This was because individual trees with a diameter above 70 cm could still be found at this elevation.

The average size of the tree diameter did not decrease because the Siranggas Wildlife Sanctuary area was a lower mountainous area and a transition from lowland rainforest to highland forest. Therefore, the size of the

trees that grew in the area was not significantly different between altitudes. A study conducted in Gunung Ciremai National Park (Rozak & Gunawan 2015) showed that both the individual density and basal area did not change until the altitude above 2000 masl.

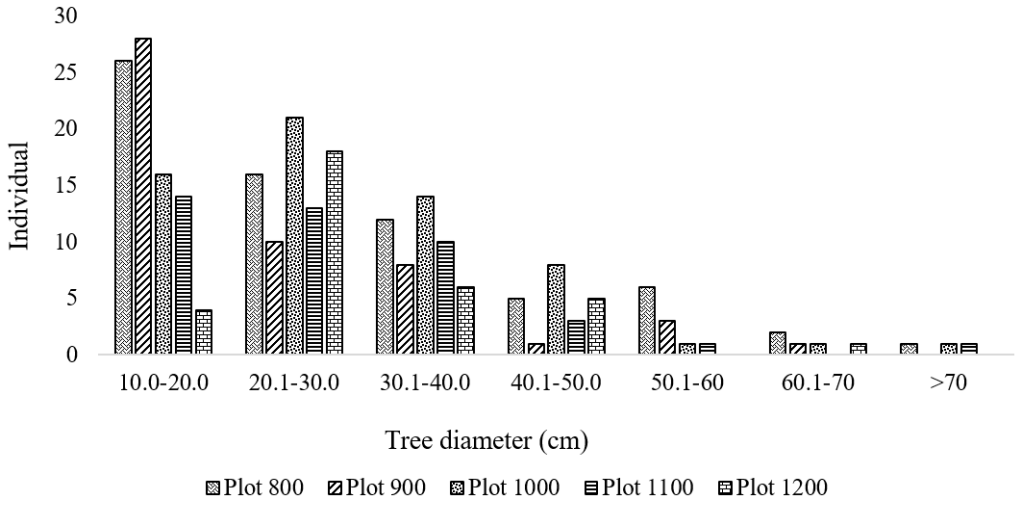

Figure 4. Tree diameter profile chart.

According to Moles et al. (2009), high vertical growth rates allowed trees to physically dominate other plant growth forms when in a suitable environment. Ecological studies relating to the horizontal components of forest structures, such as trunk density and basalt areas, also showed large-scale variations along vast environmental and edaphic gradients (Paoli et al. 2008). However, there was limited information on variations in the vertical components of forest structures and their causes. Several available evidence suggested that the height of the tree, H, for a given diameter (D), might vary significantly among species and across regions (Nogueira et al. 2008). These differences could significantly affect the carbon storage potential of tropical forests. This was because above-ground tropical tree biomass and carbon flux were usually estimated by applying allometric equations only for diameter measurements.

Vegetation Compositions

The vegetation composition of a region could be determined by examining the presence of species at each study site. In this study, the overall vegetation composition of trees from all plots with different heights obtained 79 genera and 38 families, with 10 families having the highest composition, as presented in Figure 5.

Siranggas Wildlife Sanctuary was dominated by the Myrtaceae family at 13.4 %, followed by Lauraceae, Fagaceae, and Myristicaceae with proportions of 10.4 %, 7.8 %, and 6.7 % respectively. The Myrtaceae obtained consisted of 8 species from one genus, namely *Syzygium*, with 36 individuals majorly found within the 1100-meter plot. Montane rainforest in the islands of Sumatra and Kalimantan at altitudes between 800 to 1400 masl were generally dominated by plants from the family of Myrtaceae, Lauraceae, Moraceae, and Meliaceae (Kueh et al. 2017).

According to Saw (2010), species diversity in the lower montane rainforests of Peninsular Malaysia was obtained from more than 900 species of seeded plants. The differences in dominant vegetation became apparent when comparing with lowland forests which were generally dominated by Dipterocarpaceae, Fagaceae family of the genus *Castanopsis*, *Lithocarpus*, *Quercus*, and the species of the Lauraceae family. Therefore, the lower mountain forest was often also called the Oak-Laurel montane forest.

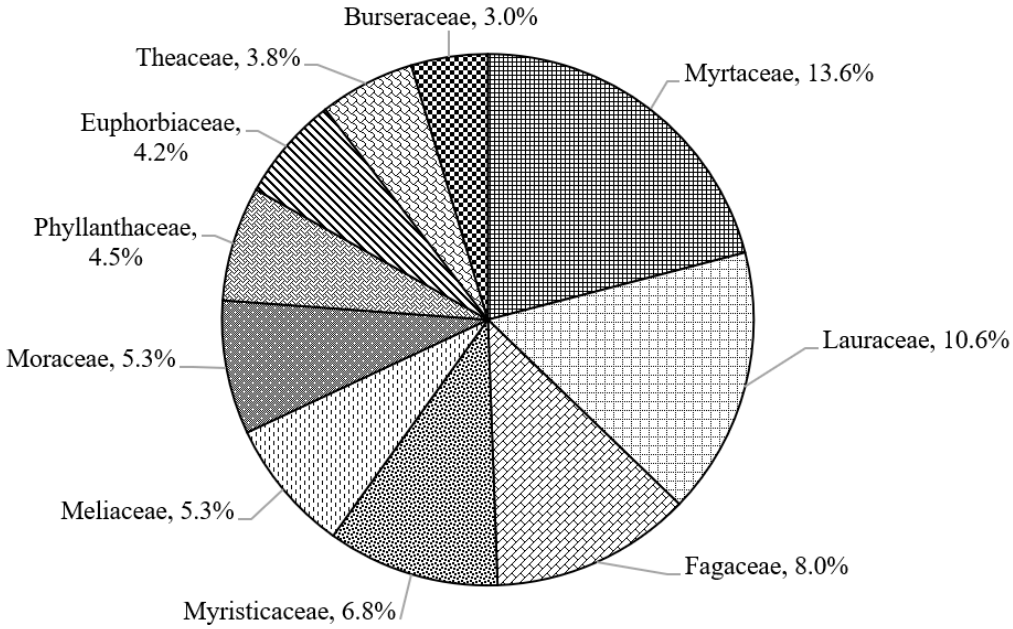

Figure 5. Families with the highest composition found on the study sites.

Association and Subassociation

The grouping of species by the number of plots of the specimen found was used as the basis of the association. A table grouping of all tree species from 5 plots created contained the base field area (m^2) and Important Value Index of each species. Subsequently, the 5 groups of tree species were determined based on the number of plots found.

Plant species that were found throughout the heights were *Schima wallichii*, *Syzygium cerasiforme*, and *Syzygium zeylanicum*. These species were included in group 1, while those found in 4 plots were included in group 2. Furthermore, the species found in 3 plots were included in group 3, those in 2 plots are in group 4, and the species found in only 1 plot were assigned to group 5, as shown in Table 2.

According to Kumaran et al. (2011), the rainforest area of the West Malesia mountains such as Borneo and Sumatra consisted of several important families, namely Fagaceae, Lauraceae, and Myrtaceae. The dominant species of the family Myrtaceae found in the Malesia region was the genus *Syzygium*. This study also observed a high number of individuals in the Myrtaceae family. After the identification process, most of the plant species identified at each height belonged to the Myrtaceae family with a high number of individuals, particularly from the genus *Syzygium*. This genus played a role in the rainforests of the Sumatra region because its flowers were one of the main sources of nectar and fruit for the fauna in the forest (Parnell et al. 2006). Consequently, *Syzygium* had achieved a wide distribution due to the large number of faunas involved in the pollination and seed dispersal of various species in the Syzygium genus in the Siranggas Wildlife Sanctuary.

Grouping types by the degree of consistency (Mueller-Dombois & Ellenberg 1974) obtained 100 % consistency from *Schima wallichii*, *Syzygium cerasiforme*, and *Syzygium zeylanicum*. Based on the consistency and index of the highest importance values of these three species, the tree community from the Siranggas Wildlife Sanctuary Area at an altitude of 800 to 1200 masl could be classified as the *Schima wallichii* – *Syzygium cerasiforme* or the *Schima* – *Syzygium* association. This association exhibited the highest Importance Value Index of 83.65 % and 74.93 %, respectively at the study location.

On the plot elevation of 800 masl, the *Styrax benzoin* – *Syzygium cerasiforme* subassociation could be written as the *Styrax* – *Syzygium* subassociation with important value indices of 19.51 % and 16.92 %, respectively. Mean-

Table 2. Total Basal Area (TBA) and Important Value Index (IVI) of tree species recorded in five plots in the Siranggas Wildlife Sanctuary.

No	Species	TBA (m^2)	IVI (%)
Association			
1.	*Schima wallichii*	2.19	83.65
2.	*Syzygium cerasiforme*	0.72	74.93
Subassociation at 800 masl			
3.	*Styrax benzoin*	0.49	19.51
4.	*Syzygium cerasiforme*	0.34	16.92
Subassociation at 900 masl			
5.	*Syzygiym cerasiforme*	0.11	19.22
6.	*Schima wallichii*	0.35	15.89
Subassociation at 1000 masl			
7.	*Castanopsis tunggurut*	1.20	45.05
8.	*Gironniera nervosa*	0.50	22.25
Subassociation at 1100 masl			
9.	*Myristica maxima*	0.71	27.80
10.	*Syzygium cloranthum*	0.07	15.36
Subassociation at 1200 masl			
11.	*Dysoxylum cauliflorum*	0.41	26.29
12.	*Baccaurea sumatrana*	0.19	24.25

while, on the 900-meter plot, the *Syzygium cerasiforme – Schima wallichii* or *Syzgygium – Schima* subassociation had important value indices of 19.32 % and 16.21 %. On the 1.000-meter plot, the *Castanopsis tunggurut– Gironniera nervosa* or *Castanopsis – Gironniera* subassociation had an important value index of 45.05 % and 22.25 %. The 1.100-meter plot obtained the *Myristica maxima – Syzygium cloranthum* or *Myristica – Syzygium* subassociation, with important value indices of 41.63 % and 17.36 %. On 1.200-meter plot, *Dysoxylum cauliflorum – Baccaurea sumatrana* or the *Dysoxylum – Baccaurea* subassociation was obtained, with an important value index of 26.29 % and 24.25 %, respectively.

Schima wallichii was a plant species widely distributed in the southern regions of East Asia to Southeast Asia. Furthermore, this species could grow well in highland areas up to an altitude of 3900 masl. The dominance of this species at almost every altitude was due to its excellent adaptability in extreme environments (Tang et al. 2020). Based on (Bussmann & Paniagua-Zambrana 2021), *Schima wallichii* flowering occurred in the year and its light seeds facilitated easy wind dispersal. These factors allowed a wider distribution of individuals in the Siranggas Wildlife Sanctuary Area at different altitudes compared to other species in this area. This species' resilience to harsh environmental conditions can help future conservation efforts to further understand how this tree can help restore damaged forests from human activities. Given how *Schima wallichii* is also a tree with great economic value, further research about the tree's conservation and cultivation can be done in the future reach even peoples of the surrounding areas to help with forest conservation efforts.

CONCLUSIONS

The diversity of trees in the Siranggas Wildlife Sanctuary Area was categorized as high, consisting of 38 families, 79 genera, and 119 species with a total of 268 individual trees from all plots. The height class of canopies with the highest number of individuals was the class of 10.0 – 15.0 meters with 102 individuals. The tree with the diameter class of 10 – 30 cm had the highest number, comprising 170 or 64.15 % of all individuals found in the plot.

Furthermore, the tree with the highest composition was the Myrtaceae family with an Important Value Index of 13.4 %. The forest communities across 800-1.200 meters altitude had high species richness and were designated as *Schima wallichii – Syzygium cerasiforme* association. This phytosociological data of the two species throughout the research site showed how they can be used to help future forest management to monitor the changes in Siranggas' vegetation and planning for future restoration efforts.

AUTHOR CONTRIBUTION

ESS designed the study, supervised all the process, and edited the manuscript. AFT collected and analysed the data and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Effect of Benzyl-Adenine and Thidiazuron on *In Vitro* Multiplication of Ginger (*Zingiber officinale* Rosc.) Shoots

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ABSTRACT

The wilt disease caused by *Ralstonia solanacearum* and the leaf spot disease caused by *Phyllosticta* sp. are significant co*n*straints in ginger cultivation as they can lead to crop failure. One approach to eliminating these diseases is to use disease-free ginger plantlets obtained through tissue culture propagation. This study investigated the influence of plant growth regulators, i.e., Benzyl Adenine (BA) and Thidiazuron (TDZ), on the in vitro multiplication of large white ginger shoots. The tested treatments included combinations of BA (0, 1, 2, 3 mg L^{-1}) and TDZ (0, 0.1, and 0.2 mg L^{-1}), with ten replicates each. A complete randomised factorial experimental design was employed. The observed variables were shoot height, number of shoots, number of leaves, and number and length of roots at 2, 4, 6, and 8 weeks of age. The results indicated an interaction between TDZ and BA for shoot number and root length. The highest numbers of shoots were obtained after eight weeks using 0.1 mg L^{-1} TDZ alone without BA. Meanwhile, the longest roots were obtained after eight weeks using a specific combination of TDZ and BA concentrations. Based on this study, we proposed a strategy to implement this protocol to induce the formation of shoots, leaves, and roots in a multistep tissue culture propagation.

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INTRODUCTION

Ginger (*Zingiber officinale* Rosc.) is an important medicinal plant belonging to the *Zingiberaceae* family (Shahrajabian et al. 2019a). In Indonesia, there are three types of ginger known based on size, shape, and color: large white ginger, red ginger, and small white ginger (also known as *emprit* ginger) (Sabulal et al. 2006; Guji et al. 2019). Ginger has been widely used in traditional medicine and is now utilised for producing herbal medicine standards (OHT) and phytopharmaceuticals (Sabulal et al. 2006; Azhari et al. 2017; Sharifi-Rad et al. 2017; Shahrajabian et al. 2019b). Large white ginger is primarily used as a culinary spice, while red ginger is more commonly used in traditional medicine (Shahrajabian et al. 2019a). Ginger rhizome is also widely used as a spice or condiment (El Sayed et al. 2016).

Ginger contains a diverse range of bioactive compounds, such as gingerol, shogaol, and zingerone (Sharifi-Rad et al. 2017; Shahrajabian et al. 2019b; Shahzad et al. 2023). Ginger has various nutritional and medicinal importance (Shahzad et al. 2023). These compounds have been shown to exert various pharmacological activities, including antioxidant, anti-inflammatory, analgesic, and anti-carcinogenic effects (Sharifi-Rad et al. 2017). For instance, gingerol and shogaol have antioxidant activities scavenging reactive oxygen species (Sharifi-Rad et al. 2017). Ginger extracts have exhibited anti-inflammatory effects by inhibiting pro-inflammatory cytokines (Sharifi-Rad et al. 2017). The analgesic activity of ginger may be attributed to modulation of neurotransmitters and suppression of prostaglandin synthesis. Gingerols play an important role in the alleviation of arthritis and pain (Shahzad et al. 2023). Further research into the pharmacological mechanisms and clinical efficacy of these ginger compounds is warranted. Overall, the presence of these bioactives contributes to the wide use of ginger as both a versatile culinary ingredient and medicinal plant (Semwal et al. 2015).

However, bacterial wilt caused by *Ralstonia solanacearum* and leaf spot caused by *Phyllosticta* sp. are major diseases that constrain ginger cultivation (Chaidir et al. 2019). These diseases pose a significant threat, as severe infestations can result in plant death and crop failure (Sitinjak 2010; Adriani et al. 2012). Both plant pathogens affect all types of ginger, including large white ginger, red ginger, and small white ginger, decreasing national ginger productivity (Fauzia & Nurcahyanti 2020).

One approach to mitigating these diseases in ginger is to use healthy ginger seeds from tissue culture propagation. This technology offers the advantage of obtaining disease-free seedlings (Kumar & Reddy 2011; Seran 2013; Lestari et al. 2023). Tissue culture propagation using disease-free ginger plantlets is one potentially effective way to obtain healthy planting material that can help ginger farmers combat disease pressures and improve productivity, offering increased yields, better quality, cost savings, and new market opportunities (Azhari et al. 2017; Sharifi-Rad et al. 2017; Shahrajabian et al. 2019a, 2019b).

Tissue culture technology has been widely applied to support agricultural development, including mass seed production, development of superior varieties, germplasm preservation, and secondary metabolite production (Kumar & Reddy 2011; Lestari 2016; Tegen & Mohammed 2016). The success of plant propagation through tissue culture is influenced by various factors, including the growth media composition, the source of explants, and the types of plant growth regulators (PGRs) used (Gupta et al. 2020; Zahid et al. 2021). Among the PGRs, cytokinin, particularly benzyl-adenine (BA), is commonly used to stimulate shoot proliferation (Lestari 2011; Mehaboob et al. 2019). BA is a cytokinin with strong activity compared to kinetin (Lestari 2011; Grąbkowska et al. 2014).

The aim of this study was to investigate the effects of different concen-

trations of benzyl-adenine (BA) and thidiazuron (TDZ) on *in vitro* propagation of ginger.

MATERIALS AND METHODS

Materials

The plant material used as explants was shoot buds obtained from rhizomes of the 'Cimanggu 1' variety of large white ginger (*Zingiber officinale*). This variety is susceptible to *Ralstonia solanacearum* (Ministry of Agriculture 2001). This ginger variety was obtained from a farmer's plantation in Sumedang, West Java, Indonesia. The collected ginger rhizomes were maintained in a greenhouse to provide a ready source of explants for experiments.

Methods

The research was conducted from March to December 2021 at the Tissue Culture Laboratory, Indonesia Centre for Agricultural Biotechnology and Genetic Resources (ICABIOGRAD), Cimanggu 3, Bogor, West Java, Indonesia. The collected ginger rhizomes were washed thoroughly with soap and water to remove debris. The cleaned rhizomes were then placed in 20 cm x 40 cm plastic containers and incubated in darkness for 48 hours. Before culturing in the medium, the explants were sterilised by soaking and rubbing them in 70 % alcohol for 5 minutes, followed by 20 % Clorox treatment for 10 minutes, and finally rinsing them three times with sterile distilled water. The sterilised explants were then planted on MS basal medium (Murashige & Skoog 1962) without plant growth regulators (PGRs) to obtain sterile shoot buds. To initiate and proliferate new axillary shoots from the rhizome explants, rhizome segments were cultured in vitro on Murashige and Skoog medium supplemented with 1.0 mg L^{-1} of the benzyl-adenine (BA) under low light for 4 weeks.

The basal medium used consisted of MS composition supplemented with 30 mg L^{-1} sucrose, 100 mg L^{-1} myoinositol + vitamin B group (thiamin, glycine, pyridoxine, and nicotinic acid), and 2 mg L^{-1} of gelrite as a gelling agent. The pH of the medium was adjusted to 5.8 using 1 N NaOH or HCl during pH measurement.

The media were sterilised using an autoclave at 121 °C. The explants were planted in laminar flow cabinets, with one bottle containing one explant of one cm-sized shoot bud. The plant explants were cultured in glass bottles that were arranged on racks inside a growth room dedicated for tissue culture. The room was equipped with neon lamps connected to an automated timer system, which provided 16 hours of light exposure per day at an intensity of 1500 lux. The room temperature ranged from 21 °C to 23 °C, with around 90 % humidity.

Shoots that resulted from the initial stage were subcultured on MS medium supplemented with 1.0 mg L^{-1} BA to obtain sufficient shoots for the treatment. The test media compositions were BA (0, 1, 2, 3 and 5 mg L^{-1}) and TDZ (0, 0.1, and 0.2 mg L^{-1}). Each treatment consisted of 10 bottles, resulting in a total of 120 bottles with 14 treatments, including BA (four concentrations) and TDZ (three concentrations).

Data Analysis

A completely randomised design with a factorial pattern was used for the experimental design. The variables observed were shoot height measured from the base to the tip of the shoot (cm), number of shoots, number of leaves, and number and length of roots at 2, 4, 6, and 8 weeks after planting (WAP). The data were analysed using SAS software. An analysis of variance was performed to determine the best treatment, and regression testing was used to determine the optimum dosage of PGRs.

RESULTS AND DISCUSSION

Influence of TDZ and BA treatment upon the height of ginger shoots

The analysis of variance for the shoot height variable indicated no interaction between TDZ and BA (Table 1). The use of 0.0 mg L^{-1} TDZ and 0.0 mg L^{-1} BA showed the highest shoots on week 8 (Tables 2 and 3).

Shoot elongation of ginger was faster in explants treated by the addition of TDZ compared to BA, indicated by the differences in the average height of ginger shoot during the 8-week culture duration. TDZ at 0.2 mg L^{-1} resulted in a significantly taller shoot versus other treatments from 2 to 8 weeks. Shoots treated with 0.2 mg L^{-1} TDZ were approximately 2–3 times longer than shoot treated with BA alone. The superior effect of TDZ on shoot height stimulation was likely due to its higher cytokinin activity, which promoted cell division and elongation. In contrast, BA had a weaker influence on these growth parameters. Overall, TDZ was more effective than BA for enhancing shoot elongation of ginger *in vitro*.

Table 1. Result of the analysis of variance for the height of ginger shoots.

Week After Planting (WAP)	Middle Square				CV (%)
	Replication	TDZ	BA	TDZ*BA	
2	0.201*ns*	1.276**	0.529*	0.172*ns*	28.82
4	0.506*	5.027**	0.358*ns*	0.222*ns*	28.57
6	0.288*ns*	10.005**	1.034*ns*	0.310*ns*	31.15
8	0.483*ns*	15.845**	11.925**	0.487*ns*	35.54

Note: statistical significance based on F-test $\alpha = 0.05$ - *ns* is not significantly different; (*) significantly different; (**) highly significantly different; CV = coefficient of variation. BA = Benzyl-adenine. TDZ = Thidiazuron. WAP = Weeks After Planting.

Table 2. The influence of TDZ on the height of ginger shoots.

Concentration of TDZ (mg L^{-1})	Average Shoot Height (cm)			
	2 WAP	4 WAP	6 WAP	8 WAP
0	1.47a	2.01a	2.57a	3.59a
0.1	1.20b	1.41b	1.70b	2.52b
0.2	1.14b	1.38b	1.71b	2.42b
Mean	1.27	1.6	1.99	2.85
F-Value of TDZ	9.58**	24.03**	25.97**	16.22**

Note: statistical analysis result based on Duncan's post-hoc test, number followed by the same letter in the same column considered not significant. (*) significantly different at $\alpha = 0.05$; (**) highly significant at $\alpha = 0.01$. WAP = Weeks After Planting.

Table 3. The influence of BA on the height of ginger shoots.

Concentration of BA (mg L^{-1})	Average Shoot Height			
	2 WAP	4 WAP	6 WAP	8 WAP
0	1.12c	1.63	2.03	3.62a
1	1.20bc	1.73	2.23	2.98b
3	1.42a	1.57	1.9	2.67b
5	1.32ab	1.47	1.81	2.07c
Mean	1.27	1.6	1.99	2.83
F-Value of BA	3.97*	1.71*ns*	2.68*ns*	11.71**

Note: statistical analysis result based on Duncan's post-hoc test, number followed by the same letter in the same column considered not significant. (*) significantly different at $\alpha = 0.05$; (**) highly significant at $\alpha = 0.01$. WAP = Weeks After Planting.

Influence of TDZ and BA treatment upon the number of ginger shoots

Analysis of variance showed a significant interaction between TDZ and BA on the number of ginger shoots (Table 4). Further analysis of this interaction at 8 weeks revealed the combination of 0.1 mg L^{-1} TDZ with 0 mg L^{-1} BA gave the highest shoots multiplication rate, resulting in an average of 6.3 shoots per explant (Figure 1). There was no significant difference in shoot number between 0.1 mg L^{-1} TDZ + 1.0 mg L^{-1} BA, 0.2 mg L^{-1} TDZ + 1.0 mg L^{-1} BA, and 0.2 TDZ + 0 mg L^{-1} BA after 8 weeks (Table 5).

Using TDZ at 0.1 mg L^{-1} without any BA supplementation gave the highest number of ginger shoots after 8 weeks. This indicates that 0.1 mg L^{-1} TDZ was optimal for shoot multiplication. Thidiazuron (TDZ) is a plant growth regulator that has shown both auxin and cytokinin-like effects despite chemically different from commonly used auxins and cytokinins. TDZ has been found to induce a wide array of physiological and biochemical events in cells (Zahid et al. 2021). At low concentrations (0.1-0.5 mg L^{-1}), TDZ plays a role in stimulating cell division and can be used alone or in combination with cytokinin (Gupta et al. 2020). TDZ has been utilised to promote shoot multiplication in several perennial plants (Mehaboob 2019).

Table 4. Result on the analysis of variance for the number of ginger shoots.

Week of observation	Middle Square				CV (%)
	Replication	TDZ	BA	TDZ*BA	
2	0.411*ns*	0.775*ns*	1.511**	0.519*ns*	42.95
4	2.352*ns*	19.658**	10.764**	4.481*	53.65
6	3.689*ns*	18.808**	18.355**	6.864*	58.56
8	2.87*ns*	19.560*	37.705**	11.657*	55.47

Note: statistical significance based on F-test $\alpha = 0.05$ - *ns* is not significantly different; (*) significantly different; (**) highly significantly different; CV = coefficient of variation. BA = Benzyl-adenine. TDZ = Thidiazuron. WAP = Weeks After Planting.

Table 5. Interaction of TDZ and BA upon addition to the number of ginger shoots.

Concentration of TDZ (mg L^{-1})	Concentration of BA (mg L^{-1})			
	0	1	3	5
	Average Number of Shoots (4 WAP)			
0	1.40f	1.80def	1.50f	2.00cdef
0.1	2.90bcde	3.80ab	1.60ef	2.10cdef
0.2	3.20abc	4.20a	3.00abcd	1.80def
	Average Number of Shoots (6 WAP)			
0	1.60d	2.50bcd	2.10cd	2.50bcd
0.1	4.10ab	4.60a	1.90d	2.90bcd
0.2	3.70abc	4.90a	2.80bcd	2.00cd
	Average Number of Shoots (8 WAP)			
0	2.50e	3.90bcde	2.90e	3.00de
0.1	6.30a	5.90ab	2.40e	3.20cde
0.2	5.20abc	5.10abcd	3.70cde	2.20e

Note: statistical analysis result based on Duncan's post-hoc test, number followed by the same letter in the same column considered not significant. (*) significantly different at $\alpha = 0.05$; (**) highly significant at $\alpha = 0.01$. WAP = Weeks After Planting.

Figure 1. In vitro culture of *Zingiber officinale* Rosc. Cimanggu 1 Variety.

Lestari (2011) reported that a combination treatment of BA with TDZ increased the number of shoots compared to treatments without PGRs in cassava propagation in vitro. The proliferation of *Harpagophytum procumbens* plants (Grąbkowska et al. 2014) showed similar results: soaking using 25 mol L^{-1} TDZ for 6 hours produced more vigorous shoots when the plantlets were acclimatised. The cultures originating from TDZ treatments exhibited better growth in shoot length, leaf size, flowering phase, and faster root formation (Tegen & Mohammed 2016). Another study by Grąbkowska et al. (2014) revealed that *in vivo* shoot multiplication rates and sucker growth of banana cv. Mzuzu, Bukoba, and Mtwike can be increased by dipping de-sheathed corms in a TDZ solution at 2.0 mg L^{-1} for 12 hours. TDZ was also more effective than BA on shoot proliferation (Shaheen 2020).

Influence of TDZ and BA treatment on the number of Ginger Leaves

The analysis of variance for the number of leaves variable indicated no interaction between TDZ and BA (Table 6). The use of thidiazuron (TDZ) showed significantly different results on week 4, while BA treatment showed significant differences on weeks 2, 4, 6, and 8 (Tables 7 and 8).

Influence of TDZ and BA treatment on the number of ginger roots

The analysis of variance for the number of root variables indicated no interaction between the use of TDZ and BA (Table 9). The application of TDZ significantly affected the number of roots at 2, 4, and 6 weeks after planting (WAP), while the BA treatment significantly affected the respo*n*se of the number of roots at 6 and 8 WAP (Table 10, 11).

The treatment without BA resulted in a response in the number of roots that was not significantly different from BA concentrations of 1.0 mg L^{-1} and 3.0 mg L^{-1} at 6 WAP and 8 WAP. It was not significantly different from BA 1.0 mg L^{-1} and significantly higher than BA concentrations of 3.0 mg L^{-1} and 5 mg L^{-1}. For the variable of the number of roots, the application of BA was more effective at low concentrations (1-3 mg L^{-1}). Increasing the concentration up to 5 mg L^{-1} tended to decrease the number of roots. In this experiment, rooting in vitro revealed the greatest roots by application of MS + BA 0 mg L^{-1} (13.07 roots). The addition of BA and TDZ to the medium had no significant effect on the number of roots produced. The cultural response to the addition of in vitro growth regulators is different for each plant including ginger.

Table 6. Result of the analysis of variance of the number of ginger leaves.

Week After Planting (WAP)	Middle Square				CV (%)
	Replication	TDZ	BA	TDZ*BA	
2	0.348*ns*	1.225*ns*	2.156*	1.114*ns*	75.38
4	1.297*ns*	12.700*	10.719*	3.878*ns*	55.24
6	12.018*ns*	15.808*ns*	51.178*	16.419*ns*	61.6
8	28.045*ns*	8.081*ns*	311.386**	49.995*ns*	58.56

Note: The statistical significance based on F-test $\alpha = 0.05$ - *ns* was not significantly different; (*) significantly different; (**) highly significantly different; CV = coefficient of variation. BA = Benzyl-adenine. TDZ = Thidiazuron. WAP = Weeks After Planting.

Table 7. The effect of TDZ concentration on the number of ginger leaves.

Concentration of TDZ (mg L^{-1})	Number of Leaf Count			
	2 WAP	4 WAP	6 WAP	8 WAP
0	1.3	2.68b	5.63	10.62
0.1	1.03	2.73b	4.45	9.75
0.2	0.98	3.68a	5.43	9.8
Mean	1.1	3.03	5.17	10.06
F-Value of TDZ	1.78*ns*	4.55*	1.56*ns*	0.23*ns*

Note: The statistical analysis result was based on Duncan's post-hoc test, and the number followed by the same letter in the same column was considered not significant. (*) significantly different at $\alpha = 0.05$; (**) highly significant at $\alpha = 0.01$. WAP = Weeks After Planting.

Table 8. The influence of BA concentration on numbers of ginger leaves.

Concentration of BA (mg L^{-1})	Numbers of Leaves			
	2 WAP	4 WAP	6 WAP	8 WAP
0	0.97b	3.43a	6.43a	12.93a
1	1.50a	3.57a	6.10a	12.57a
3	1.00b	2.83ab	4.40b	8.37b
5	0.93b	2.27b	3.73b	6.21b
Mean	1.1	3.03	5.17	10.02
F-Value of BA	3.14*	3.84*	5.05**	8.99**

Note: The statistical analysis result was based on Duncan's post-hoc test, and the number followed by the same letter in the same column was considered not significant. (*) significantly different at $\alpha = 0.05$; (**) highly significant at $\alpha = 0.01$. WAP = Weeks After Planting.

Table 9. Analysis of variance for the number of ginger roots.

Week After Planting (WAP)	Middle Square				CV (%)
	Replication	TDZ	BA	TDZ*BA	
2	1.752**	2.844*	0.882*ns*	0.830*ns*	81.33
4	2.160*ns*	46.087**	3.903*ns*	1.870*ns*	68.34
6	8.218*ns*	187.534**	34.932*	5.744*ns*	72.22
8	22.327*ns*	105.125*ns*	305.189**	32.850*ns*	62.92

Note: statistical significance based on F-test $\alpha = 0.05$ - *ns* was not significantly different; (*) significantly different; (**) highly significantly different; CV = coefficient of variation. BA = Benzyl-adenine. TDZ = Thidiazuron. WAP = Weeks After Planting.

Table 10. Effect of TDZ on the number of ginger roots.

Concentration of TDZ (g L^{-1})	Number of Roots			
	2 WAP	4 WAP	6 WAP	8 WAP
0	1.30a	3.43a	6.83a	11.48
0.1	0.95ab	1.82b	3.33b	9.7
0.2	0.78b	1.38b	2.85b	8.26
Mean	1.01	2.21	4.33	9.81
F-Value of TDZ	4.23*	19.98**	18.72**	2.74ns

Note: The statistical analysis result was based on Duncan's post-hoc test, and the number followed by the same letter in the same column was considered not significant. (*) significantly different at $\alpha = 0.05$; (**) highly significant at $\alpha = 0.01$. WAP = Weeks After Planting.

Table 11. Effect of BA on the number of ginger roots.

Concentration of BA (g L^{-1})	Number of Roots			
	2 WAP	4 WAP	6 WAP	8 WAP
0	1.07	2.38	5.07a	13.07a
1	1.17	2.53	5.27a	11.41ab
3	1.03	2.29	4.30ab	9.04b
5	0.77	1.7	2.86b	5.69c
Mean	1.01	2.22	4.37	9.8
F-Value of BA	1.31ns	1.69ns	3.49*	7.96**

Note: statistical analysis result based on Duncan's post-hoc test, number followed by the same letter in the same column considered not significant. (*) significantly different at $\alpha = 0.05$; (**) highly significant at $\alpha = 0.01$. WAP = Weeks After Planting.

Influence of TDZ and BA treatment on the length of ginger roots

The analysis of variance for the root length variable indicated an interaction between the use of TDZ and BA at 4 and 8 weeks of age (Table 12). TDZ significantly influenced the response of ginger root length at 2, 4, 6, and 8 WAP, while the BA treatment significantly affected the response of root length at 4, 6, and 8 WAP (Table 13,14).

Table 13 shows that for the variable root length at 4 and 8 weeks after planting, the treatment without TDZ and BA gave responses that were not significantly different from the treatment with TDZ and BA at higher concentrations.

This is an in-depth look at the data on the influence of TDZ and BA on the height of ginger shoots. The highest growth was achieved after 8 weeks. The addition of TDZ (0.1 and 0.2 mg L^{-1}) and BA (1, 3, and 5 mg L^{-1}) resulted in lower height of ginger shoots compared to the results without TDZ and BA supplementation. Based on this result, neither more TDZ nor BA is required to induce ginger shoots' length in vitro.

Based on the results, the highest number of ginger shoots was achieved after week 8 using a combination of TDZ (0.1 mg L^{-1}) and BA (0.0 mg L^{-1}). However, the increase in the number of shoots was an average of one shoot compared to the medium without TDZ (0 mg L^{-1}) and less than one shoot compared to the media without BA (0 mg L^{-1}). The highest number of leaves and the number and length of ginger roots were also achieved without TDZ or BA, as was the highest number and length of ginger roots.

PGR treatments for the ginger rhizome, explant initial propagation, and explant subculture by adding 1 mg L^{-1} BA are considered sufficient for the optimal propagation of ginger. Therefore, there was no additional requirement for supplementation of TDZ and BA afterward. Endogenous phytohormones play important roles in ginger shoot formation and elongation (Lestari 2011). The extra addition of TDZ and BA probably activates negative feedback, thus inhibiting shoots and root formation and elongation instead.

Table 12. Analysis of variance for the variable of root length.

Root length (WAP)	Middle Square				CV(%)
	Replication	TDZ	BA	TDZ*BA	
2	0.356*ns*	3.290**	0.387*ns*	0.619*ns*	88.55
4	1.745**	13.751**	3.952**	1.576*	53.51
6	1.539*ns*	22.131**	9.925**	1.475*ns*	50.44
8	1.015*ns*	14.405**	20.371**	2.061*	33.92

Note: statistical significance based on F-test α = 0.05 - ns was not significantly different; (*) significantly different; (**) highly significantly different; CV = coefficient of variation. BA = Benzyl-adenine. TDZ = Thidiazuron. WAP = Weeks After Planting.

Table 13. Effect of interaction between TDZ concentration and BA on root length

THO Concentration (g L^{-1})	BA Concentration (g L^{-1})			
	0.0	1.0	3.0	5.0
	Root length 4 WAP (cm)			
0.0	1.95abc	2.17ab	2.52a	1.98abc
0.1	1.90abc	1.43bcd	1.03def	0.57ef
0.2	1.21cde	1.73abcd	1.01def	0.34f
	Root length 8 WAP (cm)			
0.0	3.87a	3.64ab	3.68ab	2.79bcd
0.1	3.62ab	3.10abc	2.48cd	1.52ef
0.2	3.03abc	3.48ab	1.91de	0.80f

Note: Data followed by the same letter on the same observation variable is not significantly different based on further tests Duncan α = 0.05.

CONCLUSIONS

The application of 0.1 mg L^{-1} TDZ achieved the highest number of shoots (6 shoots per-explants). The highest number of leaves and shoots height was also achieved without TDZ or BA, as well as the number and length of ginger roots. Efficient propagation requires no additional TDZ and BA afterward to achieve optimal height, number of shoots, number of leaves, and root number and length. Increasing the concentration of BA led to a decrease in the number of shoots and root length. Furthermore, there was no interaction between TDZ and BA treatments concerning plant height or the number of leaves.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the writing of the initial draft of the manuscript, as well as the conception and design of the study, material processing, and data collection. All authors reviewed and approved the final submitted version of the manuscript.

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CONFLICT OF INTEREST

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

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Research Article

Genotyping and Phytochemical Analysis of Kayu Pule Plant as Local Bali Medicinal Plant

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ABSTRACT
The bark of Kayu Pule plants in Bali is empirically known as a traditional medicinal ingredient and has been developed as a cosmetic and other health ingredient; however, scientific research has yet to be conducted on the profiles of the plant. This study aimed to determine the plant species, examine the scientific function of the compounds, and the antioxidant activity of the plant's ethanolic extract. This study performed a DNA analysis of the plant using matK primer, and the amplified DNA sequences were used to determine the phylogenetic tree. Based on the molecular analysis, the Kayu Pule plant bark from Bali, which was used as medicine, was *Alstonia scholaris*. The main compounds in Kayu Pule bark, such as ergost-5-en-3-ol and 12-oleanen -3-yl acetate, had anti-inflammatory, antioxidant, and antimicrobial properties. The antioxidant strength of the Kayu Pule plant was measured with IC50 of 3.7 µg mL^{-1} with a very strong category. This research showed the potential of Kayu Pule for developing medicinal and cosmetic products.

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INTRODUCTION

The greater public attention to the dangers of chemical products, the more organic products in food and herbal medicines are increasingly in demand by the public (Rahayu et al. 2020). Various types of medicinal plants have been developed and used traditionally in various regions of Indonesia. The medicinal properties of plants in Bali are listed in Lontar *Taru Pramana*, so their efficacy is believed to be hereditary. Some medicinal plants in the lontar include *Moringa oleifera* (Vergara-Jimenez et al. 2017; Tenri & Rivai 2020; Qadir et al. 2022). Several plants, including *Syzygium polyanthum*, *Andrographis paniculata*, *Clitoria ternatea*, tangi wood (Kayu Tangi), and pule wood (Kayu Pule) (Andila et al. 2023), are used to cure various diseases. One of the plants the community has widely used is the Kayu Pule plant. The Kayu Pule plant traditionally used its bark (*babakan)* for traditional scrubs (*boreh*), tea, and relatively modern a simple flour (simplisia) preparations used as scrubs or cosmetics without scientific proof. Therefore, we conducted this research by focusing on genotyping, and analysing phytochemical content (bioactive compounds) and antioxidant power.

Plants used in the community for generations generally lack scientific proof. Therefore, scientific studies are essential to support the development of traditional herbal medicine, mainly to correctly identify species information of plants, in particular for species with similar morphological features. Identification can be done by molecular analysis through DNA sequences and comparing them with the genetics present in Genbank. This study aims to genetically analyse to determine plant species based on MatK primers, determine the content and function of chemical compounds, and the strength of plant antioxidants. Therefore, the urgency of this research is very strategic for identifying which Balinese plant materials that can be used for health purposes, particularly as anti-inflammatory, antioxidant, and antimicrobial compounds.

MATERIALS AND METHODS

Genotyping

This study was conducted by isolating total DNA from the leaves of Kayu Pule plants with Quick DNA Plant/Seieid Miniprep Kit (Zymo Research), following the kit procedure. The isolated DNA was followed by polymerase chain reaction (PCR) using matK primer with Biorad RtPCR thermal cycler. The amplified DNA fragments were sequenced at Genetics Science Indonesia Jakarta using Next Generation Sequencing. The DNA fragment sequence of matK was compared (homology analysis) with DNA sequence data in the GenBank using the Blast method. Phylogenetic tree analysis was carried out to determine the molecular position of species or variety of Kayu Pule plants (Wirawan et al. 2020; Wirawan et al. 2022; Ariati et al. 2022).

Plant extraction

The extraction method refers to (Sarada et al. 2006; Azwanida 2015; Molino et al. 2018). The dried sample powder was extracted using maceration in 96 % ethanol for three days. The filtrate was then vacuumed to obtain crude extract.

Active compound identification

The ethanol extract of the Kayu Pule bark was analysed by the GC-MS method (HP-5MS Ultra Inert column, 30 cm in length, a diameter of 0.25 mm, and a column thickness of 0.25 µm). The sample of 1 µL was injected at a temperature of 50 °C for the first 5 minutes; then, for 2 minutes, the temperature was held until it reached 100 °C. The temperature was raised by 7 °C every minute to 300 °C, and in the last 3 minutes, the column was heated to 325 °C,

which was the final temperature. Phytochemical compounds were identified using Willey database version 7.0 by comparing the mass spectrum and fragmentation patterns of reference compounds stored in Willey's library.

Antioxidant activity assay using DPPH (2,2-diphenyl-1-picrylhydrazyl) method.

The DPPH technique was applied to perform an antioxidant activity assessment. A DPPH solution was prepared by dissolving 6 mg of DPPH in 50 mL of methanol. A combination of extracts at varying concentrations (0; 0.114; 0.170; 0.227; 0.284 µg mL^{-1}) was combined with 2.5 mL of DPPH solution and then stored in the dark at ambient temperature for 30 minutes. The absorbance was measured with a UV-Vis spectrophotometer at a wavelength of 517 nm. The percentage of radical inhibition was calculated using the subsequent formula:

Inhibition percentage = (A control - A sample) / A control x 100 %

The control absorbance (A control) denotes the absorbance of the DPPH solution without any extract, while sample absorbance (A sample) indicates the absorbance measurement of the sample. A linear regression model was constructed for each extract. The IC_{50}, or half-maximal inhibitory concentration, denotes the antioxidants required to diminish the initial DPPH concentration by 50 %. Each sample was analysed in triplicate.

RESULTS AND DISCUSSION

Genotyping of Kayu Pule Plant

Genotyping was used to molecularly identify the species of the sample. This research was conducted using matK primer. DNA amplification (Figure 1) showed a clear amplification at 500 kb. The size of the amplified PCR corresponds to the expected target size. Clear bands of the expected size in this study indicate that the PCR successfully reproduced the target DNA fragment well and was specific to the desired target, without producing non-specific amplification. The DNA obtained was then sequenced (Figure 1) and used for phylogenetic tree construction (Figure 2).

Alignment using BLAST (https://blast.ncbi.nlm.nih.gov/) showed the proximity of the sample to *Alstonia scholaris* MK9825941. Pairwise distance analysis was used to show the proximity of the Kayu Pule samples compared to the sequences generated from the blast analysis. Pairwise distance is a measure of the difference between two molecular sequences. Pairwise distance is used to estimate how far two organisms or species have diverged from each other from a common ancestor. Pairwise distance between species is shown in Figure 2. The value of 0.00 resulting from the comparison of nu-

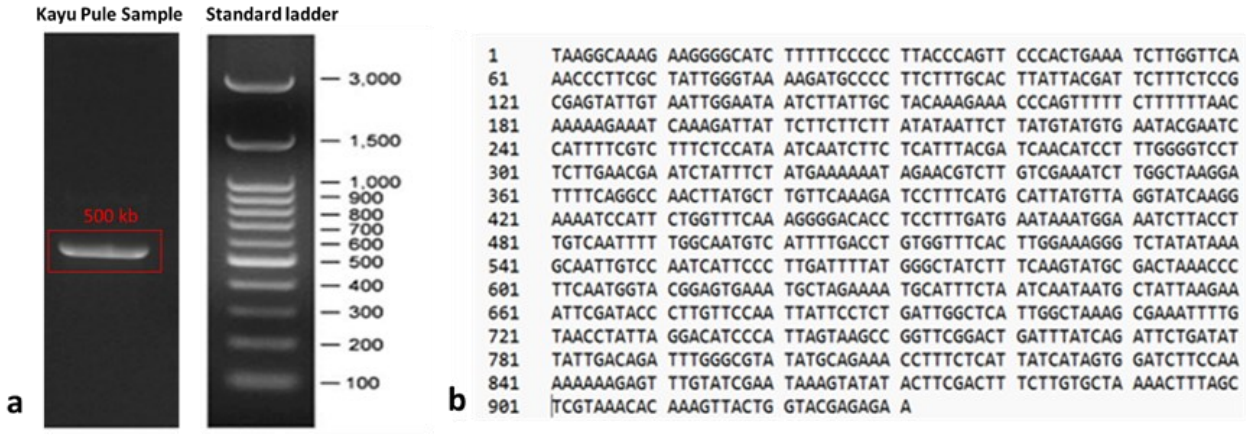

Figure 1. Visualisation of PCR products of Kayu Pule by electrophoresis 1 % TBE agarose (a) and DNA sequence (b) of Kayu Pule plant.

Pairwise distance	Pule	MK9825941_Alstonia scholaris	DQ6605151_Dyera costulata	KT9553461_Ochrosia kilneri	KT9553311_Catharanthus longifolius	DQ6605131_Craspidospermu verticillatuml	DQ6605251_Melodinus cochinchinensis	EF4563721_Carissa spinarum	DQ6605051_Carissa macrocarpa	KT9553501_Ochrosia poweri	DQ6605181_Gonioma kamassi	HQ3845531_Acokanthera oblongifolia	MN3701951_Hunteria simii	MT5941381_Mitragyna speciosa
Pule		0.00	0.01	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.10
MK9825941_Alstonia scholaris	0.00		0.01	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.09
DQ6605151_Dyera costulata	0.01	0.01		0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.09
KT9553461_Ochrosia kilneri	0.03	0.03	0.03		0.01	0.02	0.02	0.03	0.02	0.00	0.03	0.03	0.02	0.09
KT9553311_Catharanthus longifolius	0.03	0.03	0.03	0.01		0.03	0.03	0.03	0.03	0.01	0.03	0.03	0.03	0.10
DQ6605131_Craspidospermu verticillatuml	0.03	0.03	0.03	0.02	0.03		0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.09
DQ6605251_Melodinus cochinchinensis	0.03	0.03	0.03	0.02	0.03	0.01		0.02	0.02	0.02	0.02	0.02	0.02	0.10
EF4563721_Carissa spinarum	0.03	0.03	0.03	0.03	0.03	0.02	0.02		0.01	0.03	0.02	0.01	0.02	0.09
DQ6605051_Carissa macrocarpa	0.03	0.03	0.03	0.02	0.03	0.02	0.02	0.01		0.02	0.02	0.01	0.02	0.09
KT9553501_Ochrosia poweri	0.03	0.03	0.03	0.00	0.01	0.02	0.02	0.03	0.02		0.03	0.03	0.02	0.09
DQ6605181_Gonioma kamassi	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.03		0.02	0.01	0.09
HQ3845531_Acokanthera oblongifolia	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.01	0.01	0.03	0.02		0.02	0.10
MN3701951_Hunteria simii	0.03	0.03	0.03	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.01	0.02		0.10
MT5941381_Mitragyna speciosa	0.10	0.09	0.09	0.09	0.10	0.09	0.10	0.09	0.09	0.09	0.09	0.10	0.10	

Figure 2. Pairwise distance between Kayu Pule sample and other species sequences collected from blast analysis.

cleotides in Kayu Pule and *Alstonia scholaris* samples indicates that both sequences are identical. Alignment of sample sequence compared to *Alstonia scholaris* MK9825941 is shown in Figure 3.

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Query  1    GAAATCTTGGTTCAAACCCTTCGCTATTGGGTAAAAGATGCCCCTTCTTTGCACTTATTA  60
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Sbjct  1    GAAATCTTGGTTCAAACCCTTCGCTATTGGGTAAAAGATGCCCCTTCTTTGCACTTATTA  60

Query  61   CGATTCTTTCTCCGCGAGTATTGTAATTGGAATAATCTTATTGCTACAAAGAAACCCAGt  120
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Query  361  GTTAGGTATCAAGGAAAATCCATTCTGGTTTCAAAGGGGACACCTCCTTTGATGAATAAA  420
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Sbjct  481  AGGGTCTATATAAAGCAATTGTCCAATCATTCCCTTGATTTTATGGGCTATCTTTCAAGT  540

Query  541  ATGCGACTAAACCCTTCAATGGTACGGAGTGAAATGCTAGAAAATGCATTTCTAATCAAT  600
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Query  601  AATGCTATTAAGAAATTCGATACCCTTGTTCCAATTATTCCTCTGATTGGCTCATTGGCT  660
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Query  661  AAAGCGAAATTTTGTAACCTATTAGGACATCCCATTAGTAAGCCGGTTCGGACTGATTTA  720
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Query  841  GCTAAAACTTTAGCTCGTAAACACAAA  867
            |||||||||||||||||||||||||||
Sbjct  841  GCTAAAACTTTAGCTCGTAAACACAAA  867
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Figure 3. Nucleotide base alignment of Kayu Pule sample (sbjct) with *Alstonia scholaris* MK9825941 (query).

This result is supported by phylogenetic analysis (Figure 4), which shows the kinship between Kayu Pule samples and *A. scholaris* in the same cluster. The phylogenetic tree shows that Kayu Pule and *Alstonia scholaris* are in the same clade, indicating that both species originated from a common ancestor. This is also confirmed because both species have the same nodes. The

bootstrap number shows a high value (99 %) which indicates that the evolutionary relationship between the two species has a high level of confidence in its accuracy and truth.

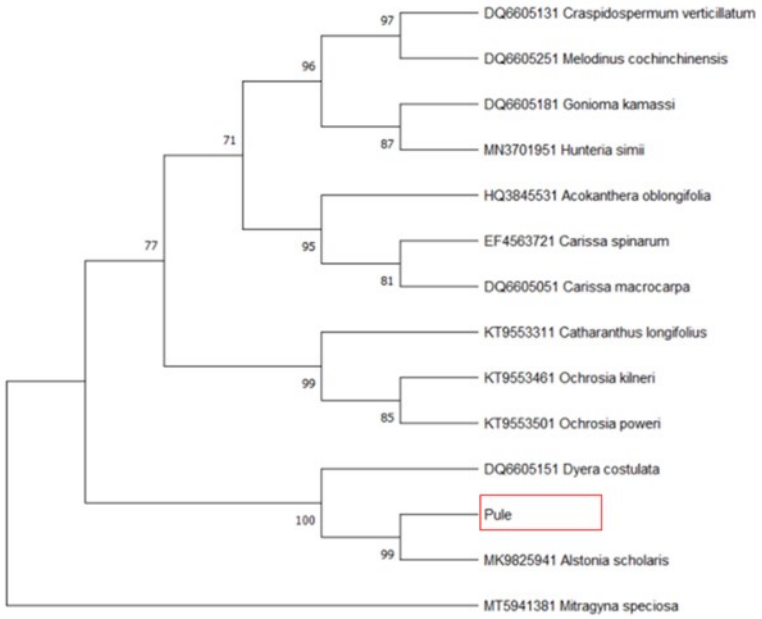

Figure 4. Phylogenetic tree of Kayu Pule plant based on matK primer.

The matK gene has emerged as a significant marker in molecular species identification, particularly in the context of DNA barcoding. Its utility stems from its high variability and substitution rates, enhancing its ability to distinguish between closely related species. MatK has shown remarkable discrimination power in specific taxonomic groups, such as the Orchidaceae, where it can identify over 90 % of species (Parveen et al. 2011; Saddhe et al. 2016). A study on conifer species in Vietnam reported a species identification rate of 98 % when combining matK with rbcL (Pham et al. 2021). Similarly, in identifying medicinal plants, the combination of matK and other loci, such as Internal Transcribed Spacer (ITS), has proven effective, highlighting matK's role in enhancing the accuracy of species identification (Thanh Pham et al. 2021). However, the effectiveness of matK is not uniform across all plant families. The matK gene is a valuable tool for identifying molecular species, mainly when used with other genetic markers. Its high variability and substitution rates contribute to its effectiveness, although its performance can vary significantly across different plant families.

Analysis of Antioxidant

Antioxidant activity assay showed the Inhibitory Consentration 50 % (IC_{50}) value of 3.7 µg mL^{-1} (regression equation y=14,91x-5,79) which showed that 3.7 µg mL^{-1} of Kayu Pule ethanol extract could act as an antioxidant to inhibit DPPH (free radicals) by 50 %. According to Molyneux (2004) and Kusumawati et al. (2021), this IC_{50} value belongs to the very strong category. The inhibitory power categories are IC_{50} of >250 µg mL^{-1} classified as inactive antioxidant compounds, IC_{50} of 100-250 µg mL^{-1} as weak, IC_{50} of 50-100 µg mL^{-1} as medium, IC_{50} of 10-50 µg mL^{-1} as strong antioxidant compounds, and IC_{50} of <10 µg mL^{-1} as very strong antioxidant compounds.

The antioxidant activity of Kayu Pule has been the subject of various studies, highlighting its potential as a source of natural antioxidants. The presence of bioactive compounds such as flavonoids, alkaloids, and phenolic acids in Kayu Pule contributes significantly to its antioxidant properties (Kanase & Mane 2018; Islamc 2020). As a comparison, a study reported that

the methanolic extract of *A. scholaris* leaves exhibited strong antioxidant activity with an IC_{50} value of approximately 69.50 µg mL^{-1} (Pratiwi 2023). Another study focusing on the chloroform extracts of *A. scholaris* found the IC_{50} values ranging from 45.77 to 62.03 µg mL^{-1} (Khanum 2014). The aqueous and ethanolic extracts of the bark of *A. scholaris* were evaluated for their cytotoxic and antioxidant properties, yielding IC_{50} values of 13.38 and 14.21 µg mL^{-1}, respectively (Jayashree et al. 2020). These values highlight the potential of *A. scholaris* extracts as an effective natural antioxidant, particularly in preventing oxidative stress-related diseases.

Phytochemical identification using GC-MS

Based on the results of gas chromatographic analysis of crude Kayu Pule extract, 103 compounds were obtained, as shown in chromatogram peaks in Figure 5 indicated the total chemical compounds detected in extract using GC-MS containing of secondary and primary metabolites especially fatty acid. Compounds were detected based on peaks in the chromatogram corresponding to each compound's retention time (RT). Compounds with a quality of >90 percent were selected 68 compounds (Table 1). The percentage of quality refers to the degree of match or similarity between the mass spectra of the detected compounds and the mass spectra of reference compounds in the GC-MS library database. The higher this quality number (>90 %) shows the greater confidence of the identified compound to the database.

Based on several publications, the compounds found in Kayu Pule ethanol extract showed various pharmacological activities, including anti-inflammatory (21 compounds, total AUC 17.85 %), antioxidant (23 compounds, total AUC 16.21 %), antimicrobial (16 compounds, total AUC 9.77 %), antibacterial (9 compounds, total AUC 8.97 %), antifungal (5 compounds, total AUC 3.07 %), and anticancer (7 compounds, total AUC 6.64 %). The primary function of extracts based on percent abundance was anti-inflammatory and antioxidant, with a total AUC of 17.85 and 16.21 %, respectively, supported by 21 and 23 compounds.

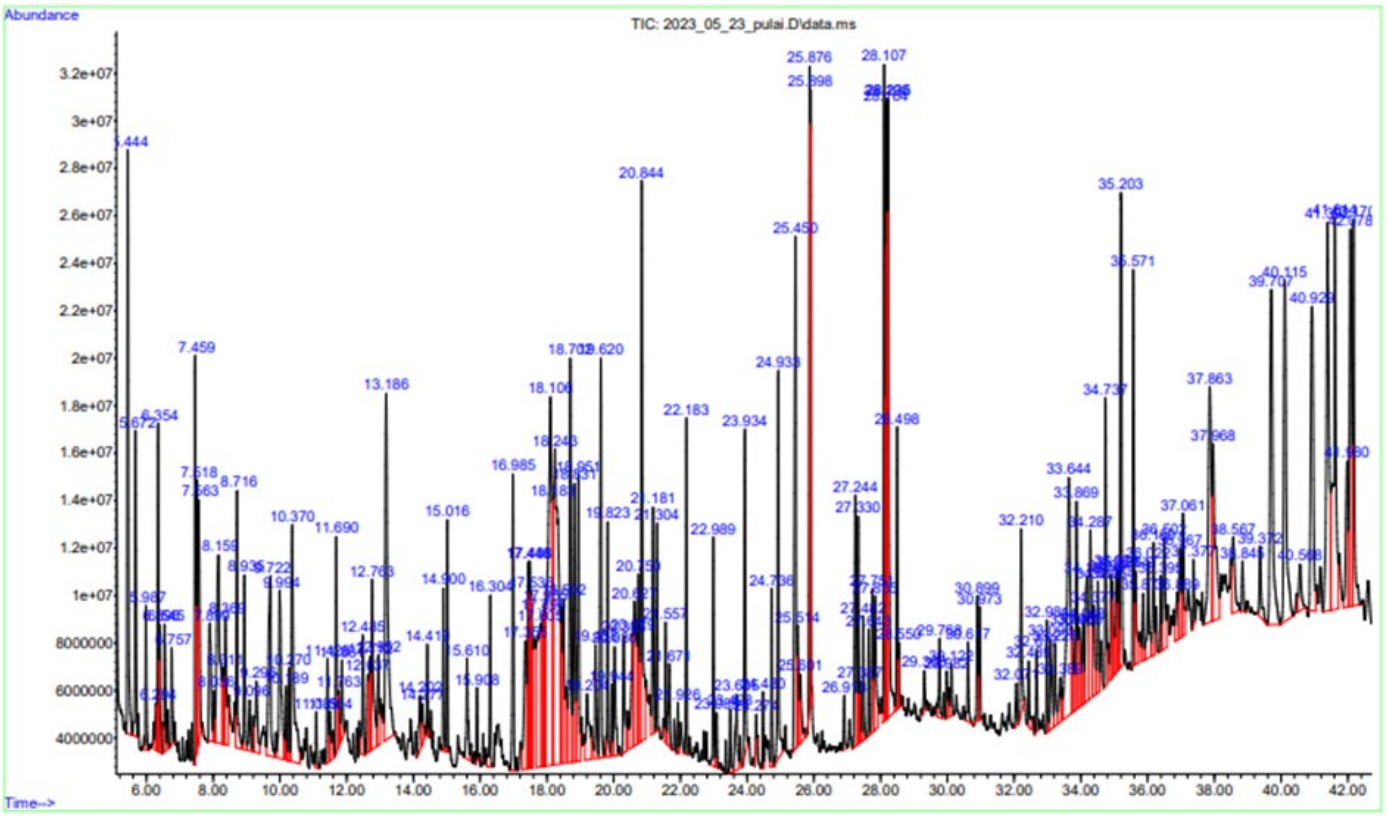

Figure 5. GC-MS chromatogram of ethanol extract of Kayu Pule.

Table 1. Compounds detected in ethanol extracts of Kayu Pule plants on GCMS machine.

No.	Compound name	Chemical formulas	RT	AUC (%)	Potential biological activity
1.	2-Furanmethanol (alcohol)	$C_5H_6O_2$	5.444	1.56	Antibacterial (Franko et al. 2017)
2.	3-Ethylpyridine (alkaloids groups)	C_7H_9N	7.459	0.79	Anticancer (Ji et al. 2002)
3.	5-Methylfurfural (5-methylfuran -2-carbaldehyde) (aldehydes)	$C_6H_6O_2$	7.563	0.83	Anticancer (Wang et al. 2023)
4.	Phenol (basic constituents of polyphenol groups)	C_6H_6O	7.899	0.53	Antioxidants, antimicrobial, and antiseptics (Diniyah et al. 2020)
5.	3-Methylcyclopentane-1,2-dione (diketone)	$C_6H_8O_2$	8.935	0.40	Flavoring agents (PubChem 2023a)
6.	4-Hydroxy-2,5-dimethyl-3(2H)-furanone (=strawberry furanone)	$C_6H_8O_3$	9.722	0.97	As a food and beverage additive (Xiao et al. 2021)
7.	Guaiacol (2-Methoxyphenol) (constituent groups of polyphenols)	$C_7H_8O_2$	10.270	0.21	Antioxidants and antimicrobial (Orlo et al. 2021)
8.	2,3-Dihydro-3,5-dihydroxy-6-methylpyran (organic compounds)	$C_6H_{10}O_3$	11.428	0.29	Antioxidants (Yu et al. 2013)
9.	Benzenemethanol (alcohol)	$C_8H_{10}O$	12.485	0.30	There has been no supporting research related to the function of these compounds
10.	1,2-Benzenediol catechol (constituent groups of polyphenols)	$C_6H_6O_2$	12.637	0.30	Precursors such as parfume and pharmaceuitical (Fiege et al. 2000)
11.	Furfuraldazine (2-furancarboxaldehyde, (2-furanyl methylene) hydrazone)	$C_{10}H_8N_2O_2$	13.186	1.85	Antimicrobial (Kapusterynska et al. 2023)
12.	Isobornyl acetate (1,7,7-trimethyl-2-bicyclo [2.2.1] heptanyl] acetate)	$C_{12}H_{20}O_2$	14.419	0.16	Anti-inflammatory (Wang et al. 2022)
13.	2-Methoxy-4-vinylphenol (constituent groups of polyphenols)	$C_9H_{10}O_2$	14.900	0.37	Antimicrobial (Rubab et al. 2020) and anticancer (Kim et al. 2019)
14.	Benzeneethanol (alcohol)	$C_8H_{10}O$	15.016	0.45	There has been no supporting research related to these compounds
15.	Phenol, 2,6-dimethoxy	$C_8H_{10}O_3$	15.610	0.35	Antimicrobial and fragrance (Dionisio et al. 2018)
16.	Benzoic acid, 4-formyl-, methyl ester	$C_9H_8O_3$	15.908	0.15	Antibacterial and antifungal (Farooq & Ngaini 2018)
17.	1-Tetradecene (alkenas)	$C_{14}H_{28}$	16.304	0.40	There has been no supporting research on the specific function of this compound
18.	Tricyclo[5.2.1.0 (2,6)] dec-4-en-8-yl acetate (acetic groups)	$C_{12}H_{16}O_2$	16.985	0.80	There has been no supporting research on the specific function of this compound
19.	4-Cyclopropyl-2-methoxyphenol (constituent groups of polyphenols)	$C_{10}H_{12}O_2$	17.446	0.56	Antibacterial (Rathinavel et al. 2023)

Table 1. Contd.

No.	Compound name	Chemical formulas	RT	AUC (%)	Potential biological activity
20.	1,2-Benzenedicarboxylic acid (phthalic acid)	$C_8H_6O_4$	17.536	0.50	Larvicide and antibacterial (Pachaiyappan et al. 2021)
21.	Phenol, 2,4-bis(1,1-dimethylethyl) (constituent groups of polyphenols)	$C_{14}H_{22}O$	18.502	0.50	Antifungal defense compound (Teresa et al. 2014)
22.	Lily aldehyde (lilial)	$C_{14}H_{20}O$	18.831	1.02	Lotions and cosmetics (Scherer et al. 2017)
23.	Benzoic acid (carboxylic acid group)	$C_7H_6O_2$	18.951	0.98	Antioxidant (Velika & Kron 2012), antibacterial, antifungal, and antioxidants (Liu et al. 2020)
24.	2,6-Dimethyl-3-methoxymethyl-p-benzoquinone	$C_{10}H_{12}O_3$	19.458	0.38	Detected but its use is unknown
25.	Benzoic acid (carboxylic acid group)	$C_7H_6O_2$	19.620	1.16	Antioxidant (Velika & Kron 2012), antibacterial, antifungal, and antioxidants (Liu et al. 2020)
26.	Hexadecene (alkenas)	$C_{16}H_{32}$	19.823	0.48	Antimicrobial and antioxidants (Mou et al. 2013)
27.	Hexadecene (alkenas)	$C_{16}H_{32}$	19.944	0.18	Antimicrobial and antioxidants (Mou et al. 2013)
28.	2-Pentyl-3-phenyl-2-propenal (cinnamaldehyde groups)	$C_{14}H_{18}O$	20.844	1.82	Flavouring agents (PubChem 2024a)
29.	1-(4-Isopropylphenyl)-2-methylpropyl acetate	$C_{15}H_{22}O_2$	21.181	0.68	There has been no supporting research related to these compounds
30.	1-Hexyl salicylate (benzoat ester)	$C_{13}H_{18}O_3$	21.304	0.48	Active ingredients cosmetics compound (PubChem 2024b)
31.	Heptadecane (alkanas)	$C_{17}H_{36}$	21. 557	0.28	Antiinflammatory (Kim et al. 2013), antibacterial, anticancer (Popović-Djordjević et al. 2016), antifungal, antioxidants, and antiseptic (Chehregani et al. 2010)
32.	E-15-heptadecenal (fats with aldehyde functional groups)	$C_{17}H_{32}O$	22.989	0.51	Anti-inflammatory and antioxidants (Chansiw et al. 2018)
33.	Octadecane (alkanas)	$C_{18}H_{38}$	23.089	0.13	Antidepressant (Guo et al. 2021), and antitumor (Tang et al. 2020)
34.	Neophytadiene (terpenoid)	$C_{20}H_{38}$	23.685	0.33	Antidepressant (Gonzalez-Rivera et al. 2023) and Anti-inflammatory (Bhardwaj et al. 2020)
35.	1,3,7-Trimethylpurine-2,6-dione (caffeine, alkaloids groups)	$C_8H_{10}N_4O_2$	23.934	0.90	Anti-inflammatory and antioxidants (Herman & Herman 2012)

Table 1. Contd.

No.	Compound name	Chemical formulas	RT	AUC (%)	Potential biological activity
36.	Hexadecene (alkenas)	$C_{16}H_{32}$	24.274	0.04	Antimicrobial and antioxidants (Mou et al. 2013)
37.	Pentadecanoic acid (fatty acid group)	$C_{15}H_{30}O_2$	24.480	0.15	Protecting cardiometabolic, immune, and liver health (Venn-Watson & Schork 2023)
38.	5,8-Dimethoxy-2,2-dimethyl-2h-chromene (basic constituents of flavonoids)	$C_{13}H_{16}O_3$	24.736	0.38	Formation of drugs (PubChem 2023b)
39.	Palmitic acid (fatty acid group)	$C_{16}H_{32}O_2$	24.933	1.03	Anti-inflammatory, antioxidant, and antimicrobial (Carta et al. 2017)
40.	Hexadecanoic acid or palmitic acid (fatty acid group)	$C_{16}H_{32}O_2$	25.450	1.66	Anti-inflammatory, antioxidant, and antimicrobial (Carta et al. 2017)
41.	Thiosulfuric acid (strong acids)	$H_2S_2O_3$	25.601	0.26	There has been no supporting research related to these compounds
42.	Hexadecanoic acid or palmitic acid (fatty acid group)	$C_{16}H_{32}O_2$	25.876	1.28	Anti-inflammatory, antioxidant, and antimicrobial (Carta et al. 2017)
43.	Hexadecanoic acid or palmitic acid (fatty acid group)	$C_{16}H_{32}O_2$	25.898	0.79	Anti-inflammatory, antioxidant, and antimicrobial (Carta et al. 2017)
44.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	27.244	0.21	Anti-inflammatory and antioxidants (El-Ashmawy et al. 2024)
45.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	27.330	0.60	Anti-inflammatory and antioxidants (El-Ashmawy et al. 2024)
46.	Hexadecene (alkanas)	$C_{16}H_{32}$	27.482	0.29	Antimicrobial and antioxidants (Mou et al. 2013)
47.	Octadecadienoic acid (alkenes, unsaturated fatty acids)	$C_{19}H_{34}O_2$	27.643	0.21	Anti-inflammatory and antioxidants (El-Ashmawy et al. 2024)
48.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (unsaturated fatty acids)	$C_{19}H_{34}O_2$	27.751	0.42	Anti-inflammatory and antioxidants (El-Ashmawy et al. 2024)
49.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (unsaturated fatty acids)	$C_{19}H_{34}O_2$	27.834	0.56	Anti-inflammatory and antioxidants (El-Ashmawy et al. 2024)
50.	Linoleic acid ethyl ester (unsaturated fatty acids, omega-6)	$C_{20}H_{36}O_2$	28.107	1.74	Anti-inflammatory (PubChem 2023b)
51.	Linoleic acid ethyl ester (unsaturated fatty acids, omega-6)	$C_{20}H_{36}O_2$	28.164	0.78	Anti-inflammatory (PubChem 2023b)
52.	Ethyl oleic acid (fatty acid group)	$C_{20}H_{38}O_2$	28.190	1.19	The role of nonoxidative alcohol metabolism in liver disease (Song et al. 2015)
53.	Ethyl oleic acid (fatty acid group)	$C_{20}H_{38}O_2$	28.235	1.88	The role of nonoxidative alcohol metabolism in liver disease (Song et al. 2015)

Table 1. Contd.

No.	Compound name	Chemical formulas	RT	AUC (%)	Potential biological activity
54.	Methyl 17-methyloctadecanoate (fatty acid group)	$C_{20}H_{40}O_2$	28.498	0.69	Anticancer, antitumor, and anti-inflammatory (Tang et al. 2020)
55.	Decosane (hydrocarbons)	$C_{22}H_{46}$	28.550	0.13	Antimicrobial (Lammers et al. 2021)
56.	Tricosane (hydrocarbons)	$C_{23}H_{48}$	29.786	0.22	Components of female sex pheromone of the bee (Francke & Schulz 2010)
57.	Acetic acid (13-tetradecenyl) ester (organic ester compounds)	$C_{16}H_{30}O_2$	30.144	0.14	There has been no supporting research related to these compounds
58.	2,5-Furandione,3-(dodecenyl) dihydro- (terpenoids)	$C_{16}H_{26}O_3$	30.617	0.23	It has a role as a plant metabolite (PubChem 2024c)
59.	Adipic acid (dicarboxilic aid) (organic acid)	$C_6H_{10}O_4$	30.973	0.30	Anti-inflammatory, anticancer, and antimicrobial (Liao et al. 2020)
60.	n-Eicosane (saturated organic compounds)	$C_{20}H_{42}$	32.071	0.13	There has been no supporting research related to these compounds
61.	2,6,10,14,1,22-Tetracosahexaene (terpenoids)	$C_{24}H_{38}$	35.571	1.02	There has been no supporting research related to these compounds
62.	17,24-Dihydroxy-3-oxopregn-4-en-21-al (steroid)	$C_{21}H_{30}O_4$	37.061	0.45	There has been no supporting research related to these compounds
63.	Ergost-5-en-3-ol (steroid)	$C_{28}H_{48}O$	39.707	2.09	Antidiabetic, antirheumatic, anthelmintic, antipsoriatic, antioxidants, antiepileptic, and antigonorrhea (Qadir et al. 2022)
64.	Stigmasterol (steroid)	$C_{29}H_{48}O$	40.115	1.86	Antioxidants and anticancer (Wang et al. 2022)
65.	Stigmast-5-en-3-ol (steroid)	$C_{47}H_{84}O_2$	40.929	1.86	Antioxidants and anticancer (Wang et al. 2022)
66.	12-Oleanen-3-yl acetate (steroid)	$C_{32}H_{52}O_2$	41.392	2.24	Antibacterial, antiprotozoal, and anti-inflammatory (Pérez-González, A. et al. 2017)
67.	Olean-12-ene, 3-methoxy- (terpenoid)	$C_{30}H_{50}$	42.087	1.54	Antibacterial (Muhammad et al. 2000)
68.	Amyrin (terpenoid)	$C_{30}H_{50}$	42.170	1.31	Anti-inflammatory (Okoye et al. 2014)

RT, retention time; AUC, area under curve

Compounds that act as anti-inflammatory in Kayu Pule extracts include unsaturated fatty acids such as *Octadecadienoic acid* (*linoleic acid ethyl ester*) and flavonoid group compounds. *Octadecadienoic acid* or *linoleic acid* is an essential fatty acid that is anti-inflammatory. Cytokine compounds as proinflammatory compounds, such as interleukin-1 beta and tumor necrosis factor-alpha, can be inhibited by linoleic acid. In addition, the linoleic acid present in Kayu Pule plants can reduce the expression of the cyclooxygenase-2 (COX-2) enzyme,

which synthesizes prostaglandin hormones that spur inflammation (Kusumawati 2002).

The anti-inflammatory properties of Kayu Pule have been the focus of several studies, revealing its potential therapeutic benefits in managing inflammation-related conditions. One significant study by Subraya et al. (2012) demonstrated that the stem bark extract of *A. scholaris* exhibited substantial anti-inflammatory activity comparable to that of indomethacin, a well-known anti-inflammatory drug. This study utilised models such as carrageenan-induced paw edema, dextran-induced edema, and cotton pellet-induced granuloma to assess the anti-inflammatory effects. The results indicated that *A. scholaris* effectively reduced edema, suggesting its potential as a natural anti-inflammatory agent. In another investigation, the bark extract exhibited significant inhibition of inflammatory mediators in a rat model. The study highlighted the extract's ability to restore antioxidant enzyme levels and prevent the rise of inflammatory mediators, indicating a dual action of antioxidant and anti-inflammatory effects (Zehra & Sanaye 2021). This aligns with the traditional use of the plant in folk medicine for treating inflammatory conditions. Moreover, Shang et al. (2010) conducted pharmacological evaluations that confirmed the anti-inflammatory and analgesic properties of *A. scholaris*. The study noted that the extract could inhibit the production of pro-inflammatory mediators, which are crucial in the inflammatory response (Shang et al. 2010). The inhibition of cyclooxygenase (COX) and lipoxygenase (LOX) pathways was also discussed, suggesting that the extract could modulate these pathways to exert anti-inflammatory effects. Additionally, the latex of *A. scholaris* has been evaluated for its anti-inflammatory properties. A recent study by Banik and Das (2023) highlighted the latex's effectiveness in various in vitro assays, demonstrating its potential in managing inflammatory diseases. The study focused on protein denaturation and membrane stabilization mechanisms, which are critical in the inflammatory process. Several studies showed that *Alstonia scholaris* exhibits significant anti-inflammatory activity through various mechanisms, including the modulation of inflammatory mediators and pathways.

The grouping of phytochemical compounds in the 68 compounds identified in the GC-MS analysis was also carried out mainly on alkaloids, phenolic compounds (tannins and flavonoids), steroids, terpenoids, and fatty acids, as shown in Table 2. These compounds are secondary metabolites that function to defend themselves from biotic and abiotic stress and are not directly involved in plant growth. Plants produce different metabolites, even a compound produced by only one plant species (Verpoorte & Alfermann 2000). Secondary metabolites such as salicylic acid generally have pharmacological solid effects, so they can be used as drugs or as new drug models. Phenolics (tannins and flavonoids) have antioxidants to prevent cancer-preventing free radicals. Flavonoid compounds have essential properties in the body, namely as anti-inflammatory, antioxidants, and anticancer, and belong to the largest class of natural phenol compounds and are easily obtained in various types of plants, including Kayu Pule plants (Panche et al. 2016; Ademiluyi et al. 2018; Tungmunnithum et al. 2018).

Steroids are among the dominant compounds in Kayu Pule extracts with a total AUC of 8.5 % (Table 2) consisting of 5 compounds such as 17.24-dihydroxy-3-oxopregn-4-en-21-al (AUC 0.45 %), ergost-5-en-3-ol (AUC 2.09 %), stigmasterol (1.86 %), stigmast-5-en-3-ol (1.86 %), and 12-oleanen-3-yl acetate (AUC 2.24 %) (Table 1). Steroids are anti-inflammatory, regulate body metabolism, as well as affect body growth and development (Patadiya 2020).

The compound 12-oleanen-3-yl acetate (Figure 6) was the most abundant compound with an AUC of 2.24 % and has properties as antibacterial,

Table 2. Group of compounds contained in Kayu Pule extract based on AUC value and number of function support compounds.

No.	Group of compounds	AUC value of all compounds (%)	No. of supporting compounds
1	Alkaloids	1.69	2
2	Phenolics (tannins and flavanoids)	3.2	8
3	Steroids	8.5	5
4	Terpenoids	4.10	4
5	Fatty acids	11.97	14
6	There have been no reports/studies related to the compound	3.46	8

Compound function based on Willey data base version 7, AUC=Area Under Curve in percent, the number of supporting compounds were compounds with the same group.

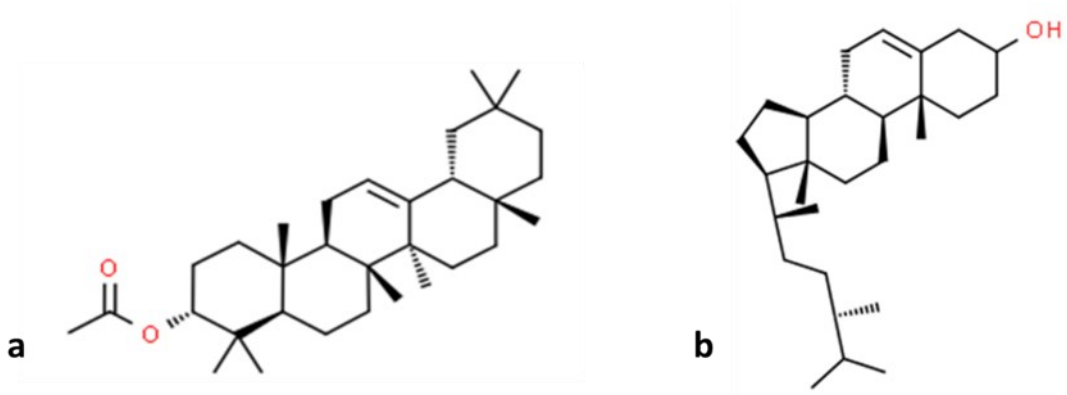

Figure 6. Chemical structure of 12-oleanen-3-yl acetate (a) and ergost-5-en-3-ol (b).

antiprotozoal, and anti-inflammatory (Pérez-González, M.Z. et al. 2017) and is cytotoxic with the molecular formula $C_{32}H_{52}O_2$ which appeared in RT 41,392, average mass 468,754 Da, and ChemSpider ID8727640.

Ergost-5-en-3-ol (Figure 6) is the second most abundant steroid compound (AUC 2.09 %) appearing at RT 39.707 with the molecular formula $C_{28}H_{48}O$, having a mass of 400.370514 Da, and ChemSpider ID18902059. Ergost-5-en-3-ol is antidiabetic, antirheumatic, anthelmintic, anti-psoriatic, antioxidants, antiepileptic, and anti-gonorrhea (Qadir et al. 2022).

Groups of steroid compounds can be cyclic or acyclic and often have aldehyde groups, carboxylic acids, or alcohols. Steroids have significant bioactivity, such as hormone formation, cell membrane parts, and vitamin D formation. Steroids can also act as an attractor or insect repellent and antimicrobial. Based on the results of GCMS, the bark extract of the Kayu Pule plant contains many compounds that have the potential for various pharmacological activities, including relieving convulsions, thinning phlegm, lowering blood sugar, curing malaria, and lowering blood pressure (Candrasari et al. 2018). Kayu Pule bark extract is proven to contain alkaloids, tannins, saponins, and steroids. However, terpenoids and flavonoids are found only in the leaves.

The presence of steroid groups in *Alstonia scholaris* has been documented in various studies focusing on the phytochemical composition of the plant. The plant contains various bioactive compounds, including alkaloids, flavonoids, saponins, steroids, and triterpenoids. One significant study by Islam et al. (2020) reported that the ethanolic extracts of *A. scholaris* revealed the presence of steroids among other phytochemicals such as tannins, glycosides, and alkaloids. This finding underscores the importance of *A. scholaris* as a source of steroid compounds known for their various biological activities, including anti-inflammatory and antioxidant effects. Additionally, a review by Verma et

al. (2015) highlighted that *A. scholaris* is rich in flavonoidal glycosides, indole alkaloids, and steroids, indicating a diverse phytochemical profile contributing to its medicinal properties. The presence of these compounds suggests potential therapeutic applications, particularly in traditional medicine systems where steroids are often utilized for their anti-inflammatory and immunomodulatory effects. Moreover, the study by Raju et al. (2022) identified several phytochemical constituents in the bark extract of *A. scholaris*, including steroids, saponins, flavonoids, and triterpenoids. Furthermore, the isolation of specific triterpenoids such as lupeol and betulin from *A. scholaris* has been reported, reinforcing the notion that steroidal compounds are indeed present in this plant (Zehra & Sanaye 2021). *Alstonia scholaris* contains various phytochemicals, including steroid groups, specifically in the form of triterpenoids and other steroidal compounds. These phytochemicals contribute to the plant's pharmacological activities, including anti-inflammatory effects, making it a valuable resource in traditional medicine and potential therapeutic applications.

CONCLUSIONS

Molecular identification with the matK marker showed that Kayu Pule is close to the species *Alstonia scholaris* (MK9825941). The compounds contained in the Kayu Pule plant have several pharmacological activities including anti-inflammatory, antioxidant, and antimicrobial properties. The antioxidant strength of Kayu Pule was measured with IC_{50} of 3.7 µg mL^{-1}, including the very strong category. Due to the solvent used in this research being polar (ethanol), further extraction using a non-polar solvent is necessary to identify the whole chemical in Kayu Pule.

AUTHOR CONTRIBUTION

I.K.S. organised the research, wrote the manuscript's draft; I.G.P.W. contributed substantially to the writing of the paper, I.N.W. data analysis and created the tables and the figures, validated the final version of the paper; A.A.S.I.G. data analysis and created the tables and the figures; G.A.P.T.A.H. provided a number of references; M.M.V.S. produced the final version of this manuscript; P.K.K. did a summary of relevant literature; I.M.O.P. provided a number of references. All authors approved the final version of the manuscript.

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CONFLICT OF INTEREST

We have no conflict of interest.

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Research Article

Exploring the Diversity of Arbuscular Mycorrhizal Fungi in Zingiberaceae Family Plants at the Tukung Gede Mountain Natural Reserve

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ABSTRACT
Arbuscular mycorrhizal fungi (AMF) are essential in improving soil quality and facilitating plant nutrient and water uptake through mutualistic associations. However, limited research exists on the diversity and distribution of AMF associated with plants in the Zingiberaceae family, especially in unique ecological habitats such as the Tukung Gede Mountain Natural Reserve. This study aims to assess and document the diversity of AMF linked to Zingiberaceae plants in this reserve. Sampling was performed at three locations with distinct plant compositions to explore the diversity of AMF genera. Soil samples were processed using a wet sieve technique, while root samples were chemically stained to evaluate AMF colonization. Key parameters studied included diversity indices, spore density, genus-level identification, and root colonization rates. The findings revealed the presence of eight AMF genera: *Sclerocystis*, *Septoglomus*, *Acaulospora*, *Gigaspora*, *Glomus*, *Scutellospora*, *Racocetra*, and *Rhizophagus*, identified based on spore morphology. Root staining revealed structural AMF infections, including vesicles, internal hyphae, and arbuscules. *Zingiber officinale* exhibited the highest AMF colonization rate (88 %) among the Zingiberaceae plants studied, whereas *Zingiber zerumbet* had the lowest (56 %). *Etlingera Solaris* and *Zingiber officinale* showed the highest spore densities at 172 and 254 spores per 100 g of soil, respectively. AMF diversity indices varied across locations, with values of 0.78 (Station I), 0.95 (Station II), and 0.84 (Station III). This research emphasizes the extensive AMF diversity within Zingiberaceae plants and its potential importance for conservation and ecological sustainability.

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INTRODUCTION

Protecting biodiversity has developed a universal importance in the twenty-first century, especially concerning agriculture. Global issues are severe because of the destabilizing consequences of climate change, economic instability, and biodiversity loss (FAO 2020; Kuila & Ghosh 2022). The Zingiberaceae family of plants is very adaptive, so protecting agricultural variety in this complex environment is imperative. Liang and Chen (2021) assert that these plants are essential for promoting a healthy and ecologically sustainable lifestyle. They are highly valued for their nutritional content and therapeutic potential because of their abundance of essential oils and polyphenols (Guerrini et al. 2023). Zingiberaceae are vital crops utilized in traditional medicine, the culinary arts, and cosmetic formulations; their significance extends beyond one's health.

The Zingiberaceae plant development and health are intimately connected to the activity of mycorrhiza, particularly arbuscular mycorrhiza. Mycorrhiza refers to the symbiotic association between fungi and plant roots, which is a common and critical component of natural ecosystems particularly arbuscular mycorrhiza. This relationship plays a vital role in plant health and soil fertility, with mycorrhizal fungi colonizing root systems and forming structures such as arbuscules, vesicles, and hyphal networks. In natural environments, mycorrhizae enhance nutrient cycling (Han et al. 2023) by increasing plant access to essential nutrients like phosphorus (Chiu & Paszkowski 2019), aid in water absorption (Qiao et al 2023) through extensive hyphal networks, improve soil structure by binding soil particles, and bolster plant health by forming protective barriers against pathogens (Kaur & Suseela 2020).

The characteristic growth of arbuscular, tree-like structures at the fungal hyphae terminals sets AMF apart. This fungus is surprisingly linked to about 80 % of terrestrial plants; in higher plant species that can grow in a wide range of environments, this percentage increases to 90 % (Alrejhei et al. 2021; Suharno et al. 2022). The mycorrhiza found in this habitat belongs to the Zingiberaceae family, which is found in tropical rainforests (Peng et al. 2022).

Specifically, one of these tropical rainforests is the Gunung Tukung Gede Nature Reserve in Banten Province. Indonesia is a global hotspot for biodiversity (Pironon et al. 2020). This verdant region spans 1519.50 hectares and is part of the A-type climate, distinguished by heavy annual rainfall with an average of 2151 mm (Banten Provincial Environmental and Forestry Service 2018). This climate causes this area to have a high level of biodiversity. Research related to AMF symbiosis in plants of the Zingiberaceae family has been conducted by Santos et al (2010) for a herbs spesies of *Zingiber officinale* ROSCOE. Additionaly Pandey et al. (2020) conducted a study for mycorrhiza in India on two types of ginger plants, *Zingiber montanum* and *Zingiber officinale* species. However, information on the diversity of arbuscular mycorrhizal fungi in plants of the Zingiberaceae family is still minimal. There has yet to be research related to AMF in the Mount Tukung Gede Nature Reserve.

Because these plants maintain the delicate balance of ecosystems, there is a solid connection between environmental protection and the Zingiberaceae family. Zingiberaceae plants are essential to biodiversity conservation methods because of their adaptability, particularly in natural reserves. Within these areas, sustainable agriculture methods that put the cultivation and protection of the Zingiberaceae family first can help create a harmonious relationship between human activity and the environment, which is in line with the overarching goal of responsible land management. Therefore, by emphasizing the relationship between biodiversity, agriculture, and environmental sustainability we can promote practices that support ecological balance and

the well-being of both natural ecosystems and human communities. The study's findings can serve as a scientific foundation for future investigations into various arbuscular mycorrhizal fungi that affect Zingiberaceae plants, which are more prevalent in the Mount Tukung Gede Nature Reserve. Understanding the diversity of Arbuscular Mycorrhizal Fungi (AMF) in this specific habitat is essential to enhancing our comprehension of the intricate relationships affecting ecological dynamics and plant health. Moreover, the increasing threats to biodiversity and global ecosystems emphasize the pressing need for these investigations. Examining the nuances of AMF in Zingiberaceae plants may offer fresh perspectives on long-term conservation tactics and essential data to the broader scientific community. Consequently, this expands our understanding of plant-fungal interactions and their impact on ecosystem resilience

MATERIALS AND METHODS

Materials

The materials and tools used in this study are as follows: digital camera (Canon EOS 1100 D), essential GPS, soil meter, thermometer, lux meter, Binocular Compound Microscope (Leica DM500), Stereo Microscope (Meiji Techno), preparation glass, petri dish, root samples of Zingiberaceae family from Gunung Tukung Gede Nature Reserve, shallots (Allium cepa), large plastic clips, 10 % KOH, 2 % HCl, glycerol, immersion oil, Trypan Blue, zeolite, hyponex 25-20-5 and distilled water.

Methods

Study Area

The research was conducted between July 2022 and February 2023. Zingiberaceae plant roots and soil samples have been collected from the Gunung Tukung Gede Nature Reserve in the Mancak District of Serang Regency, Banten Province (Figure 1). This region has a type of mountain forest, with June-August being the dry months and September-May being the wet months. The exploration method was employed in this study.

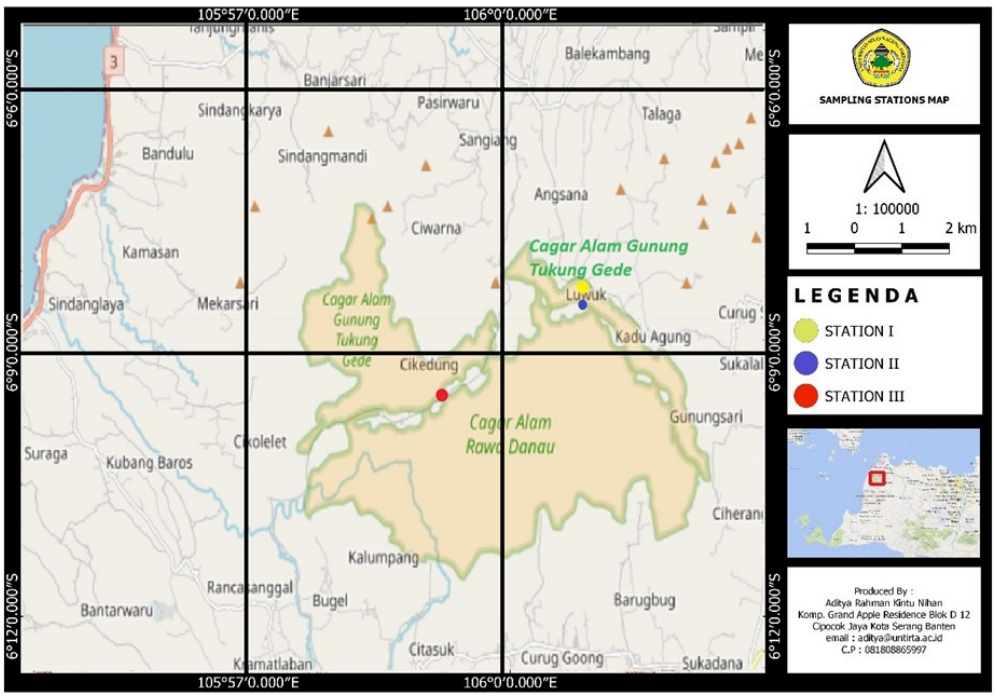

Figure 1. Sampling Stations in Tukung Gede Mountain Nature Reserves.

About three stations were established along the exploration path, each measured 100 m in length and 20 m in width. The selection of research station was based on the abudance of plant species of Zingiberaceae family. Sampling site condition can be seen in Figure 2. The environmental vegetation of Gunung Tukung Gede Nature Reserve was considered when separating the stations. Three stations were established along the exploration path, each measuring 100 meters in length and 20 meters in width, with five plots per station. The selection of research locations was based on the abundance of plant species of the Zingiberaceae family. The environmental vegetation of Tukung Gede Mountain Nature Reserve was considered when separating the stations. Station I and II are located in the eastern part of Mount Tukung Gede Nature Reserve. Station I with closed vegetation conditions at coordinates 6°8.1370'S 106°0.2460'E, while station II with open vegetation conditions at coordinates 6°8.2590'S 106°0.2460'E. Station III is located in the western part of Tukung Gede Mountain Nature Reserve with conditions near residential areas at coordinates 6°8.5220'S 105°59.0700'E. Environmental parameters measured were soil moisture, air temperature, soil pH, soil type, and light intensity.

Figure 2. Sampling site condition: a. Station I, b. Station II, c. Station III.

Sampling Methods

In the location where Zingiberaceae plants were found, designated sample points were established. Five sampling points were taken diagonally across a 5x10 meter plot. Soil and plant roots from the Zingiberaceae family were carefully gathered in samples ranging from 0 to 20 cm deep. With the help of a shovel, around 1 kg of soil was collected, and root samples were put in big plastic clips with openings for air and labeled separately. After that, these samples were sent to the lab for additional processing and analysis. The soil and root samples were stored at room temperature (25 °C) until the isolation began. While root samples were closely examined, soil samples were essential for setting up trapping cultures and assessing spore density.

Mycorrhiza bioassay methods

The root staining approach used in this study follows Brundrett et al. (1966) methodology. Root segments that are between one and two centimeters in length are removed in order to make staining and observation processes easier. Plant root samples are carefully washed under running water to remove any remaining dirt particles and put into test tubes. Immersion of the roots in a 10 % potassium hydroxide (KOH) solution lasts for around 24 hours or until the roots take on a pale or transparent yellow color. After being treated with KOH, roots are rinsed twice or three times with distilled water and then submerged in a 2 % hydrochloric acid (HCl) solution for a further twenty-four hours. This acid treatment is necessary to improve Trypan Blue's absorption capability during the ensuing staining procedure. After staining, the roots are put through a destaining step in which the color is lightened with a 50 % glycerol solution. Arbuscular Mycorrhizal Fungi (AMF)-infected roots are identified and designated for further examination. Brundrett et al. (1996) developed the colonized root length technique to calculate the proportion of col-

onized roots. One or more AMF structures, including hyphae, vesicles, and arbuscules, are markers of colonization. The thorough staining and analytical procedure offered crucial new information on the kind and degree of AMF root colonization in the plant samples under investigation.

$$Root\ Colonization\ Percentage\ (\%) = \frac{\sum marked\ root\ preparations}{\sum number\ of\ root\ preparations}\ x\ 100$$

The centrifugation method (Brundrett et al. 1996) will be employed in conjunction with the wet filter pour technique (Pacioni 1992) as the spore isolation method to extract AMF spores. About 100 g of soil are weighed for each plant sample as the first step in the soil sample separation procedure. Then the soil sample is put in a beaker and 500 ml of distilled water is added, then soaked for ± 5 minutes. Then filtered in a set of stratified mesh sieves with sizes 0.2 mm, 0.15 mm, and 0.075 mm with running water. The soil remaining on the 0.15 mm and 0.75 mm sieves was transferred into a centrifuge tube. Add 60 % glucose solution into the centrifuge tube and start centrifuge Hitachi (Himac CT15RE) at 1200 g rpm for 5 minutes. The centrifuge produced supernatant solution which was transferred to a petri dish. The density of spores was observed on a stereo microscope (Meiji Techno) and a Binocular Coumpound Microscope (Leica DM500) to clearly see the morphology of the spores. After the process of isolating the spores, spore preparations were made and placed on the preparation glass and carefully broken by pressing the glass cover of the preparation using a pen needle. AMF identification is based on the morphological characteristics of its spores (spore arrangement, hyphal shape, spore color, spore shape and spore ornamentation). Microscopic characteristics of spores found were then matched with identification guidelines used by the International Culture Collection of Vesicular Asbuscular Mycorrhizal-INVAM (2022), Brundrett et al. (1996), and other reliable journals.

$$Spore\ Density = \frac{\text{Spore count}}{\text{100 g soil}}$$

Trapping spora AMF

This study also used a trapping culture technique. Trapping culture is a method of capturing Arbuscular Mycorrhizal Fungi originating from the rhizosphere of plants in culture pots to obtain the number and other types of spores and then identified based on the genus level and density (Sefrila et al. 2021). This trapping culture uses the method of Brundrett et al. (1966). The trapping culture used *Allium cepa* host maintained for three months with zeolite media. Plastic pots contain 50 g of zeolite at the bottom, then the middle part contains 100g of soil and put a host of 2-week-old shallot plants, and then the top contains 50 g of zeolite again. Maintain the spores for 2 months with regular fertilizer watering (Hyponex Red nutrient solution 0.05 %) diluted in water. The nutrient solution was applied every 2 weeks, approximately 20 ml per pot after which a stressing process was carried out to stimulate the spores due to the grip of drought.

Diversity Indices

The results of the diversity index data analysis were obtained from the type of AMF genus successfully identified in the soil samples of each plant of the Zingiberaceae family using quantitative analysis. The data obtained from identification is then calculated using the Shannon Weiner formulation, using the following formula (Parwi et al. 2018):

$$H' = -\Sigma Pi\ (Ln.Pi)$$

Description:
H' : Diversity index
Pi : ni/N

(the ratio of the number of individuals of the i-th species to the total number)
ni : Number of individuals of the i-th species
N : Total number of individuals
The results obtained are seen based on the criteria:
H' < 1 = Low Diversity
1< H' < 3 = Medium Diversity
H'> 3 = High Diversity

RESULTS AND DISCUSSION

Environmental condition of Study Site

Observational findings reveal different AMF infections are present in Zingiberaceae plant family root samples. About nine plants belonging to the Zingiberaceae family were found in three different locations. Station I, with closed vegetation, found plant species are *Amomum hochreutineri*, *Etlingera solaris*, and *Zingiber zerumbet*. Dusty, loamy soil characterizes the soil texture at station I, and the distance between plants scattered along the cruising path is not close together. These plants grow wild and fertile in the Tukung Gede Mountain Nature Reserve area. Plants at station I are mostly under tall trees so that little sunlight enters. Station II with open vegetation found plant species such as *Curcuma longa*, *Alpinia galanga*, *Amomum hochreutineri*, and *Coctus speciosus*. The soil texture at station II is relatively coarse soil with fine sand. Each plant species was found not close to each other and scattered along the observation path, which is 0-100 m. With vegetation near residential areas, Station III found plant species such as *Zingiber officinale*, *Curcuma longa*, *Curcuma xanthorrhiza*, *Alpinia galanga*, and *Etlingera elatior*. The soil texture at station III is fine sandy loam soil. Plants found at station III grow close to other plants maintained by residents around the Tukung Gede Mountain Nature Reserve area, such as *Musa paradisiaca*, *Manihot esculenta*, etc. Even some plants from the Zingiberaceae family are cultivated by the surrounding community to meet their daily needs.

The average values for every site were combined to assess the environmental factors (Table 1). Arbuscular mycorrhizal fungi (AMF) grow best at a temperature range between 29 °C and 30 °C, as shown by the reported temperatures at each station. Interestingly, there were notable differences in the soil pH at each station: Station I was 4.8, station II was 6, and station III was 5.7, indicating acidic soil conditions. Spore density was found to be greater in somewhat acidic soil. Station I recorded the maximum soil moisture level at 68 %, while stations II and III recorded 40 %. The station values varied. It is essential in dry soil conditions to reduce soil moisture since it might promote AMF spore formation. Station I showed lower levels (211) of light intensity than Stations II (461.5) and III (547). The varying kinds of host plants found at each site were the reason for the discrepancy in light intensity readings. The investigation results showed that stations II and III had more significant concentrations of AMF spores, explained by their slightly acidic soil pH and lower soil moisture levels. The pH and moisture content of the soil were measured with a Soil meter, and the amount of light was measured with a Lux meter. The study focused on the effects of soil properties on spore density, colonization rate, and the overall capacity of AMF to operate in the ecosystems studied.

AMF Infections in Zingiberaceae Plant Family Root Samples

Roots were classified as colonized or infected based on the observation of certain characteristics, such as vesicles, internal hyphae, spores, external hyphae, and arbuscular structures associated with arbuscular mycorrhizal fungus (AMF). Hyphae vesicles, and arbuscules were seen in the root samples. AMF hyphae have a variety of morphologies, including branching, straight,

Table 1. Measurement Results of Environmental Parameters at each station

Environmental Parameters	Station I	Station II	Station III
Coordinates	6°8.1370'S 106°0.2460'E	6°8.2590'S 106°0.2460'E	6°9.295'S 105°58.232'E
Temperature	29 °C	30 °C	30 °C
Soil pH	4.8	6	5.7
Soil Type	Regosol	Regosol	Latosol
Soil Moisture	68 %	40 %	40 %
Light Intensity	211	461.5	547

and H-shaped forms. Determining root colonization or AMF infection required identifying vesicles, arbuscular structures, internal hyphae, spores, and external hyphae in the examined root preparations. An indication of a possible AMF infection in the host plant was the presence of one or more AMF formations in root preparations. Hyphae were the most common AMF structures found in Zingiberaceae plant root preparations. Conversely, vesicles considered uncommon AMF structures were seldom seen in some root preparations and only in particular kinds of roots. Figure 3 presents in-depth observations of root samples.

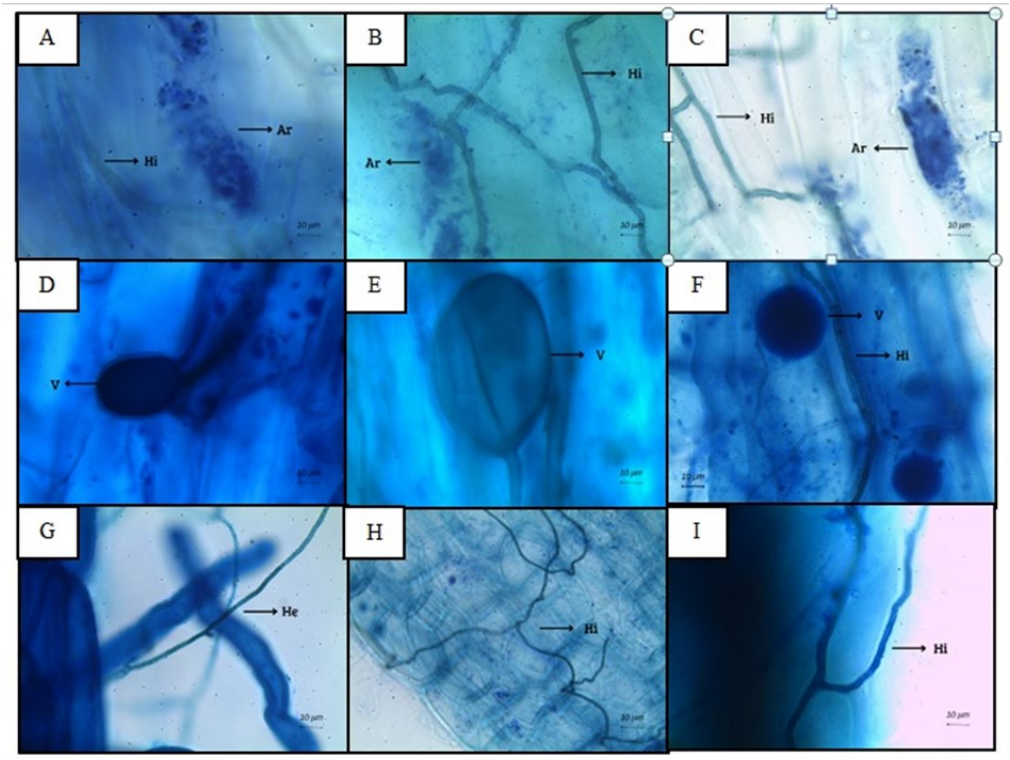

Figure 3. Structure of the Arbuscular Mycorrhizal Fungus; type: V: Vesicle, Hi: Internal hyphae, He: External hyphae, Ar: Arbuscular. A: *Zingiber officinale* roots, B & E*: Etlingera solaris* roots, C, D & H: Roots *of Amomum hochreutineri*, D: F root: *Alpinia galanga* root, G: *Curcuma longa* root, I: *Etlingera elatior* root.

The figure 4 presents a collection of various species zingiberace plant in Mount tukung Gede Nature reserves. Each of these species is not only significant within the Zingiberaceae family but also shows a distinct relationship with AMF, contributing to the understanding of plant-fungi interactions in this group.

As shown in Table 2, the examination of spore densities and AMF (arbuscular mycorrhizal fungi) colonization percentages among various stations and plant species provides important new information on the symbiotic interactions found in the Tukung Gede Mountain Natural Reserve. At Station I, we find that AMF root colonization percentages range from 56 to 66

Figure 4. Collection of Zingiberaceae Plants Associated with AMF: a. *Curcuma longa*, b. *Amomum hochreutineri*, c. *Zingiber officinale*, d. *Etlingera solaris*, e. *Etlingera elatior*, f. *Curcuma zanthorrhiza*, g. *Alpinia galanga*, h. *Zingiber zerumbet*, i. *Costus speciosus*.

%, percentage of colonization showed range 56-66 % and spore numbers from trapping methods range from 172 to 184 spores/100g of soil. In comparison to *Etlingera solaris* and *Zingiber zerumbet* at this station, *Amomum hochreutineri* exhibits the highest colonization rate, suggesting a possibly greater symbiotic interaction with AMF. This shows a somewhat steady but variable AMF presence. Spore densities in the nature (210–242 spores/100g of soil) and col-

onization percentages (70–80 %) are much higher at Station II, where the highest rates of colonization are seen in Alpinia *galanga* and *Amomum hochreutineri*. Information on AMF colonization percentage and spore densities also available in Station III with high AMF root colonization percentage of 88 % and 254 spores/100g in *Zingiber officinale*. *Etlingera solaris* showed the lowes spores (172/100g).

Identification of AMF Spore Genus

Based on the study's results, eight genera were identified in all soil samples. The genus identified were *Glomus* sp., *Septoglomus* sp., *Acaulospora* sp., *Gigaspor*a sp., *Scutellospora* sp., *Sclerocystis* sp., *Rhizophagus* sp., and *Racocetra* sp. Each type of soil sample of Zingiberaceae Family plants had different spore genus results and numbers. The types of genus *Glomus* sp. and *Acaulospor*a sp. were found in all soil samples of plants of the Zingiberaceae family, while the other six types of spores were not all found in each plant of the Zingiberaceae family The type of spores most commonly found in all soil samples of Zigiberaceae plants is the genus *Glomus* sp. The genus *Racocetra* sp. was mostly found in *Curcuma zanthorrhiza* plants. Figure 5 shows the genus data found at each station. Station I, II and III have the same genus of spores obtained are *Glomus* and *Acaulospora*. Station I, II and III have one type of spore in common which is *Gigaspora*. In contrast, stations II and III have five types in common which are *Gigaspora*, *Septoglomus*, *Scutellospora*, *Rhizophagus*, and *Sclerocystis*. *Racocetra* spores were only found at station III.

Examining each species of spores under a microscope reveals distinctive characteristics which can be seen in Figure 6. The germinal walls and spore decorations of *Acaulospora* sp. are characteristic of the genus. Subtending hyphae are a kind of hyphal holder seen in *Glomus* sp. Except for the germinal shield within, the bulbous suspensor of the genus *Gigaspora* sp. is found at the base of the hyphae and shares traits with *Scuttelospora* sp. The genus *Sclerocystis* sp. is characterized by its hyphal plexus and is found in clusters. The *Rhizophagus* genus is distinguished by its spores, which range in color from pale yellow to light brown and have broad subtending hyphae. The spores are round to subglobose and feature a thick, three-layered wall, a pattern consistent with other *Glomus* species (INVAM 2022). In this study we observed 2 species of *Rhizophagus*. *Rhizophagus* sp.1 has very thick, pale yellow-brown spores with cylindrical subtending hyphae, while *Rhizophagus* sp.2 has darker yellow-brown spores and broader subtending hyphae. Both species are found solitarily at Stations II and III, with distinct structural features (INVAM 2022). Among arbuscular mycorrhizal fungi (AMF),

Table 2. Calculation of AMF Colonization Percentage and Spore Density.

Station	Plant Spesies	Vernacular name	Sampel Code	Spores/100g soil		AMF root colonization (%)
				Nature	Trapping Methods	
I	*Amomum hochreutineri*	Kapulaga	KP_T	120	184	66 ± 2.38
	Etlingera solaris	Tepus	TP_T	109	172	62 ± 1.63
	Zingiber zerumbet	Lempunyang	LE_T	143	178	56 ± 1.63
II	*Curcuma longa*	Kunyit	KN_B	189	224	70 ± 1.63
	Alpinia galanga	Lengkuas	LA_B	178	236	78 ± 5.89
	Amomum hochreutineri	Kapulaga	KP_B	147	210	80 ± 2.83
	Coctus speciosus	Pacing	PA_B	224	242	72 ± 2.83
III	*Zingiber officinale*	Jahe	JH_W	153	254	88 ± 1.63
	Curcuma longa	Kunyit	KN_W	160	206	78 ± 1.63
	Alpinia galanga	Lengkuas	LA_W	138	210	82 ± 4.90
	Etlingera elatior	Honje	HJ_W	215	230	74 ± 2.83
	Curcuma zanthorrhiza	Temulawak	TM_W	262	212	70 ± 3.27

Septoglomus is a distinct genus that stands out for having characteristics that set it apart from other genera. In contrast to the clustered spores of many AMF taxa, its spores are usually colored and develop solitarily in the soil, making them easier to recognize. Unlike the more homogeneous architecture of taxa such as *Glomus* or *Rhizophagus*, the spores have a unique multi-layered wall with a hyaline, semi-flexible outer covering that peels with maturity. Moreover, *Septoglomus* spores have cylindrical or funnel-shaped bases with subtending hyphae that complement the color of the spore wall, in contrast to the less uniform hyphal characteristics of taxa such as *Acaulospora*. In contrast to other AMF taxa, the spores are often subglobose and have a more hard inner coating. The *Racocetra* genus is characterized by spores that range from pale yellow to light cream, with a round to subglobose shape and sporogenous cells. These spores possess two adherent layers, providing uniform thickness, with the second layer thickening as the spore grows and the wall differentiates (INVAM 2022). *Racocetra* spp. from research and literature, revealing that immature spores are pale cream to brownish. This genus was found exclusively on Temulawak plants at Station III, making it quite rare. *Racocetra* sp.1 exhibits slightly darker cream-colored, subglobose spores with two thin wall layers and sporogenous cell walls. The sporogenous cell walls consist of two hyaline layers, matching the spore's surface color, and are oval-shaped at the top. These spores are observed in solitary conditions.

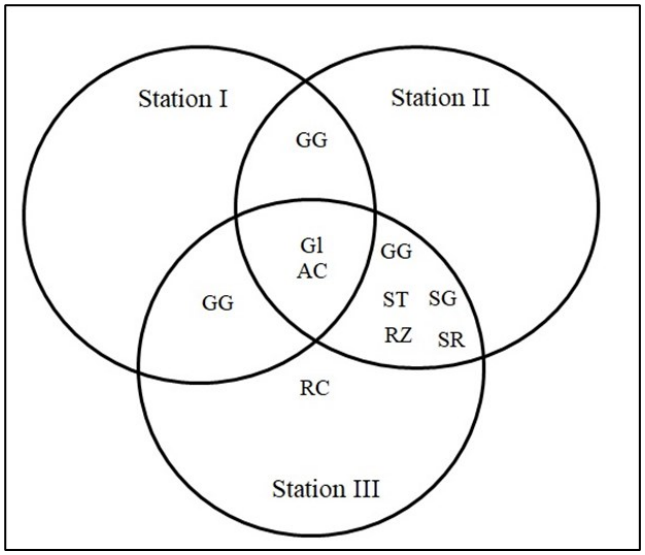

Figure 5. Types of spores found at each station. Description; Gl: *Glomus*, AC: *Acaulospora*, GG: *Gigaspora*, SG: *Septoglomus*, ST: *Scutellospora*, RZ: *Rhizophagus*, SR: *Sclerocystis*, RC: *Racocetra*.

There are differences in the number of spore genera across stations. Three genera were on display at Station I, seven genera at Station II, and eight genera were spotted at Station III. These differences in spore diversity and features draw attention to the unique qualities connected to each species in the studied settings.

Diversity Index

As shown in Figure 7, the AMF diversity index study shows that station II has the most significant diversity index value at 0.9571, while station I has the lowest value at 0.7823. H' < 1 indicates that the Shanon-Wiener AMF diversity index values at each station are in the low category. Notably, the reported genera are not evenly distributed among all soil samples. Few species were found during soil isolation, including *Gigaspora* sp., *Scutellospora* sp., *Sclerocystis* sp.,*Racocetra* sp., *Rhizophagus* sp., and *Septoglomus* sp. As

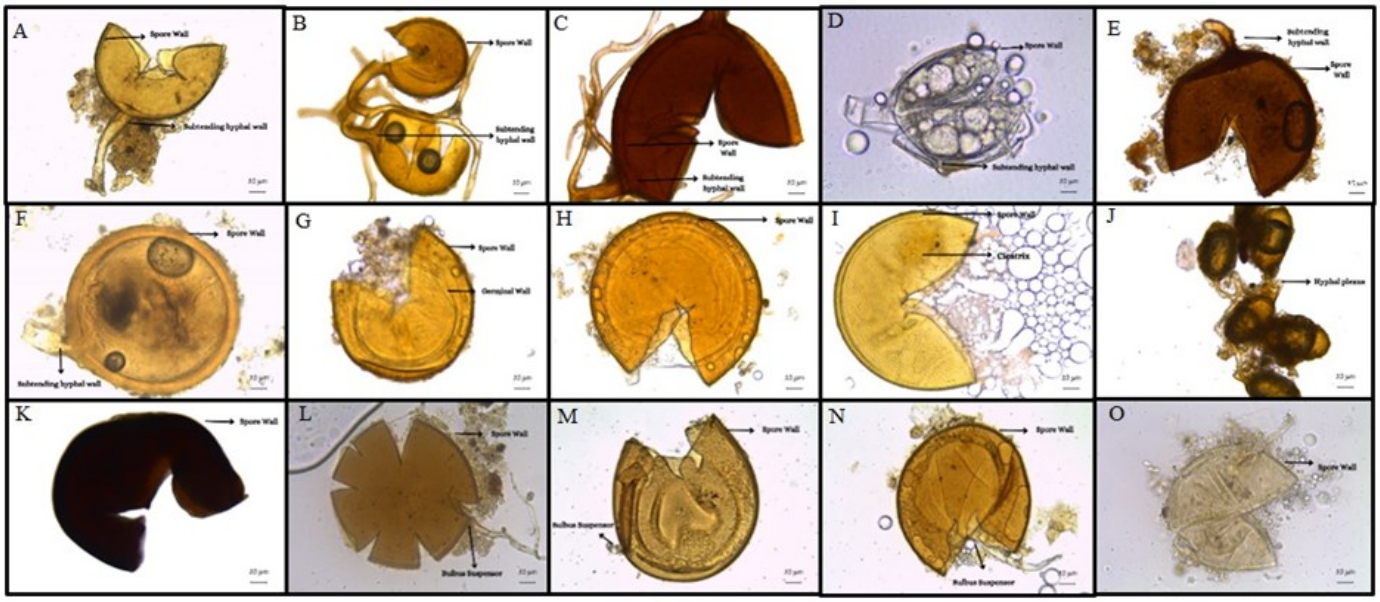

Figure 6. Microscopic images of AMF genus found in soil samples of Zingiberaceae family plants,, A: *Glomus* sp. 1, B: *Glomus* sp. 2, C: *Glomus* sp. 3, D: *Septoglomus* sp. 1, E: *Septoglomus* sp. 2, F: *Rhizophagus* sp., G: *Acaulospora* sp. 1, H: *Acaulospora* sp. 2, I: *Acaulospora* sp. 3, J: *Sclerocystis* sp., K: *Gigaspora* sp. 1, L: *Gigaspora* sp. 2, M: *Scutellospora* sp. 1, N: *Scutellospora* sp. 2 , O: *Racocetra* sp.

opposed to the other six recognized genera, *Glomus* sp. and *Acaulospora* sp. showed larger numbers. These results highlight the differential distribution patterns across various genera and offer insights into the varying AMF diversity throughout the tested stations.

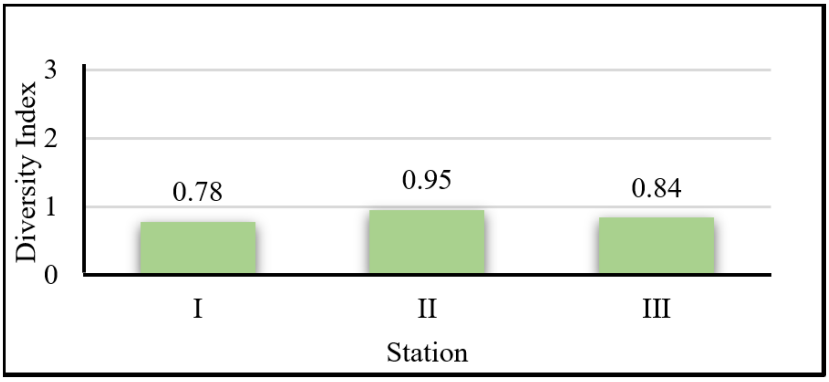

Figure 7. AMF diversity index at each station.

Discussion

The analysis reveals that the mycorrhiza colonization rate and spore density found at Station 3 indicate distinct ecological dynamics from those of Stations 1 and 2. Station 3 in the western half of Tukung Gede Mountain Nature Reserve has unique difficulties due to its proximity to residential areas. While Station 2 has open vegetation and Station 1 has closed vegetation, Station 3's environment encourages the growth of mycorrhizal fungi. The diversity index contradicts these findings, indicating that Stations 2 have greater values than Station 3. Numerous studies indicate that complex interactions between ecological parameters, such as soil type, moisture content, and temperature, may account for the observed changes in the diversity index (Sangwan & Prasanna 2021; Chikoti et al. 2022). Mycorrhizal colonization, spore density, and diversity index have a complex connection that demonstrates the complexity of the ecosystem and the need for a complete understanding of the linked elements impacting fungal symbiosis under different environmental conditions.

In this study, soil pH measurements at each station ranged from 4.8-6, this pH value indicates acidic soil. Not only soil pH, soil moisture and temperature also affect the growth of AMF spores. This study demonstrates that spore abundance is highly dependent on the characteristics of the soil. According to Alayya and Prasetya (2022), soils with a pH of 3.8–8 are suitable for the growth of AMF spores. Because AMF spores are arcidophylis, or comfortable in acidic soil conditions, they can exploit soil with an acidic pH to adapt to their surroundings and encourage the development of additional AMF spores (Mathurin et al. 2022). Environmental temperature in this study showed an optimal temperature ranging from 29-30 °C for AMF growth. This temperature measurement is in line with research conducted by Nugroho and Prasetya (2023), the best temperature for AMF development is 30 °C and the best mycelia development is at 28-34 °C. Soil moisture in this study ranged from 40-68 %. The number on the Soil meter shows that the more acidic the soil pH value is, the greater the soil moisture.

The presence of AMF spores is also influenced by soil type, some studies report that soil types with dusty loam fractions are predominantly abundant and good for the development of spores of the *Glomus* genus, while for sandy soil types, the spores found are *Gigaspora* and *Acaulospora* genus (Salim et al. 2019; Yuwati et al. 2020; Mahulette et al. 2021). This is in line with this study that station I has a loamy soil texture, at this station the types of genus found are fewer, only three genus were identified and dominated by the *Glomus* genus. Station I and II have a sandy soil texture, this study shows the number of spores at this station is more and the types of genus identified are also more varied. Station II has seven genus identified and for station III has eight genus identified.

Arbuscular Mycorrhizal Fungi (AMF) indicates a symbiotic relationship between AMF and plants in the root (Abdullahi et al. 2021). This study found AMF colonization in all root samples with varying percentage levels. Stations II and III showed higher AMF colonization than station I. According to Bernaola et al. (2018) AMF can be found in almost all soils and generally does not have a specific plant host. The level of colonization is influenced by soil fertility, soil type, and environmental conditions such as temperature and soil moisture. AMF is an obligate symbiont fungus, where AMF can work after infecting the host plant. AMF can infect plant roots quickly in dry land conditions that lack water and nutrients (Silva et al. 2022). The study results align with Pandey et al. (2020) that AMF spores and root colonization were found at slightly acidic soil pH. Soils with slightly acidic pH comparatively have a higher spore density than acidic soils. A high percentage of AMF colonies does not always indicate a high spore density, it is suspected that AMF infection in the roots has yet to reach the stage of spores. There is no close correlation between root infection and the number of spores produced, so a high number of spores does not necessarily mean that the percentage level of root infection is also high (Bohacz et al. 2022; Husein et al. 2022)

The different values of AMF colony percentage can be caused by environmental factors such as soil pH, soil moisture, and temperature and plant factors such as root length, root density and root infection (Rajpurohit & Jaiswal 2022) at each station. Soil depth can affect the number of spores studied by Samal et al. (2023). The results obtained are that the highest number of spores is at a depth of 10-20, this is due to the large number of roots that are still there. At a depth of 0-20 cm is also part of the top soil, which is part of the AMF spores. The deeper the soil layer, the fewer spores that will be found because it is further away from the root zone. INVAM (2022) stated that the genus *Glomus* Sp. has the most species and high frequency of presence compared to other genera. The abundance of the

Glomus sp. genus is because it is generally suitable for most of its natural habitats. *Glomus* sp. has a better resistance and adaptation level than other AMF spore types. *Glomus* sp. can adapt to nutrient-poor environments and environments polluted with hazardous waste and at acidic or neutral pH conditions.

Plant photosynthesis depends on light intensity, which also affects the availability of nutrients and energy needed for symbiotic partnerships with arbuscular mycorrhizal fungus (AMF). Increased photosynthesis from higher light levels boosts the synthesis of carbohydrates, which is beneficial to AMF colonization and function. On the other hand, low light levels decrease photosynthetic productivity, which restricts AMF's resources and could decrease the effectiveness of the symbiotic relationship (Guisande-Collazo et al 2022). Changes in light levels can impact the development of mycorrhizal structures and plant growth, which can impact nutrient intake and general health (de la Hoz et al. 2021). Knowing how light intensity functions contributes to the optimization of plant and AMF performance and provides information on how to enhance plant-fungal interactions (Graham & Eissenstat 1998).

The number of spores after trapping increased at each station. Differences in the number of spores can also be caused by many interrelated factors such as salinity, temperature, season and environment. This factor can affect the community structure of the genera obtained (Pace et al. 2019). Alrejhei et al. (2021) reported no correlation between the number of spores and the level of root colonization, the number of spores in natural habitats is not always related to the percentage of AMF colonies. It is explained that AMF spores in certain species sometimes take a long time to germinate. Soil salinity can also affect AMF spore germination, hyphal growth rate and colonization percentage (Danesh et al. 2022). Mukhongo et al. (2023) findings, which showed that mycorrhiza sporulation was higher in acidic soils, are consistent with the observation that mildly acidic soils had a higher spore density than acidic soils. The diversity index value determines the diversity of spore types or genus at each station. This study's spore diversity index value shows that it is classified as low. This is thought to be because not all genus of AMF spores are evenly distributed and the richness of the spore genus also has a different amount (Liu et al. 2021). In line with the research of Sefrila et al. (2021) if in a community it is composed of species with uneven abundance or certain types of AMF dominate, then the diversity is low. A community's high and low diversity index depends on the number of species and individuals of each species (species richness).

At station III the number of spores and root colonization was higher than at stations I and II. In contrast to the diversity index value, the highest value was found at station II. The factor of difference in the number of spores occurs because the number of plants found at station III is more, namely five samples found. The number of spores is also caused by differences in soil factors. At station III the soil pH shows a value of 5.8, meaning that the pH of this soil is slightly acidic with little soil moisture compared to station I. The type of host plant also affects the infection of the host plant. The type of host plant also affects spore infection.

In our current study, we identified eight genera of arbuscular mycorrhizal fungi (AMF)—*Sclerocystis*, *Septoglomus*, *Acaulospora*, *Gigaspora*, *Glomus*, *Scutellospora*, *Racocetra*, and *Rhizophagus*—based on morphological characteristics. While this method allowed us to categorize the AMF genera observed, we acknowledge that morphological identification has limitations and can be challenging due to overlapping features among genera. Morphological methods may not always provide the most precise differentiation, as many AMF genera share similar spore structures and characteristics that can complicate

accurate identification (Noreen et al. 2023). Although our study did not include molecular analysis, we recognize that molecular techniques, such as DNA sequencing and polymerase chain reaction (PCR), offer enhanced accuracy and resolution for AMF identification as classification and systematics of AM fungi have substantially changed in recent years (Stürmer 2012).

Zingiber officinale is known to be a host plant that supports a particularly high diversity of arbuscular mycorrhizal fungi (AMF) spores, exhibiting the largest variety of spore types, the highest number of spores, and significant root infections. This plant was found exclusively at Station III, which is characterized by dry, sandy soil. The dry and sandy texture of the soil at Station III may contribute to the increased AMF diversity and abundance observed. Research has shown that soil texture and moisture levels can significantly impact the distribution and diversity of AMF communities (Cardoso & Kuyper 2006; Smith & Smith 2011). Sandy soils, for instance, often exhibit different AMF community structures compared to clayey or loamy soils due to variations in nutrient availability and water-holding capacity (Becerra et al. 2014).

Furthermore, the diversity index of the AMF community at each station is significantly influenced by a range of environmental and ecological factors, such as soil texture, moisture, and nutrient content (Reynolds et al. 2003). These factors collectively shape the AMF colonization patterns and species diversity. Studies have indicated that variations in soil conditions, including pH and organic matter content, play crucial roles in determining AMF community composition and their effectiveness in symbiotic relationships with host plants (Brundrett et al. 1996; Treseder 2004).

The exploration of arbuscular mycorrhizal fungi (AMF) associations in Zingiberaceae plants is crucial for enhancing our understanding of plant-fungal interactions and their implications for example, in agricultural practices. Marsh et al. (2021) highlighted the synergistic effects of AMF in *Zingiber officinale* Rosc. significantly boost growth, nutrient uptake, and the quality of ginger rhizomes, demonstrating improvements in secondary metabolites such as phenolic compounds, flavonoids, and essential oils. These findings underscore the vital role of AMF symbiosis in promoting plant health and biochemical production. Complementing these insights, Azizah and Hariyono (2022) further confirm the positive influence of AMF on ginger, revealing that the application of AMF fertilizer leads to significant enhancements in growth metrics, including plant height, wet and dry weight, and secondary metabolite content, particularly essential oils.

Considering the ecological implications of mycorrhizal colonization, it is essential to recognize how various environmental and anthropogenic factors shape the distribution and diversity of AMF. These fungi are vital for maintaining soil health and facilitating nutrient exchanges between plants and soil. By studying AMF communities across different habitats, we gain valuable insights into these interactions' ecological dynamics. Previous research has demonstrated that habitat type, vegetation structure, and human activities are key determinants influencing the diversity and functionality of AMF communities in diverse ecosystems. Marinho et al. (2019) investigated arbuscular mycorrhizal fungi (AMF) in the tropical semi-arid region of Caatinga, Brazil, revealing that AMF diversity and distribution are significantly influenced by habitat type, vegetation, and anthropogenic activities. Their findings highlighted notable differences in AMF communities between natural and anthropized areas, with distinct habitat types playing a pivotal role in shaping the structure of AMF communities. Our observation aligns with the finding that mycorrhizal colonization rates and spore density vary between stations in the Gunung Tukung Gede Mountain Nature Reserve, where proximity to residential areas (Station 3) and differences in vegetation cover (Stations 1

and 2) likely contribute to the observed dynamics.

In a related study, an evaluation of AM fungal diversity in agricultural fields in Estonia (Martinez et al. 2024) demonstrated how the availability of natural habitat, both current and historical, influences the diversity of AM fungi. This study found that the richness of AM fungal species was positively correlated with the amount of natural habitat available, even extending back 130 years. This historical legacy effect of past land use on current soil biodiversity underscores the long-lasting impact of habitat availability on AM fungal communities, particularly in disturbed or anthropized areas (Sangwan & Prasanna 2021). Similarly, our study shows how proximity to anthropogenic activity (as seen at Station 3) alters fungal colonization and community structure in the Gunung Tukung Gede reserve. These studies together suggest that maintaining natural habitats, whether in nature reserves or surrounding agricultural fields, can have lasting benefits for soil biodiversity, including AM fungal diversity. The comparison of these ecosystems highlights how AM fungi mediate nutrient exchanges between plants and soil across a wide variety of habitats, both natural and disturbed. Integrating these insights into conservation and land management strategies may enhance the sustainability and ecological resilience of such environments.

Bioengineering, particularly in bioremediation, offers immense potential for restoring degraded environments and enhancing soil health. Our findings highlight the synergistic role that bioengineering, specifically through the application of Arbuscular Mycorrhizal Fungi (AMF), can play in bioremediation. AMF, known for enhancing nutrient uptake, improving plant tolerance to stress, and facilitating soil structure stability, could be pivotal in restoring contaminated soils by improving their nutrient cycling and resilience (Gou et al. 2023; Zhu et al. 2024). By integrating AMF into bioengineering frameworks, bioremediation efforts can benefit from enhanced soil fertility and structure, ultimately leading to sustainable agricultural practices. This integration supports the regeneration of ecosystems, particularly in soils with degraded structures or contamination, fostering crop growth and improving food security in impacted areas (Barea et al. 2011). Future research should optimize AMF strains to improve their efficiency in specific environmental conditions and explore their combined use with other bioremediation technologies to expand their application in sustainable agriculture.

CONCLUSION

In the Tukung Gede Mountain Natural Reserve, Zingiberaceae plants are associated with Arbuscular Mycorrhizal Fungi (AMF). This study identifies eight different AMF genera such as *Sclerocystis*, *Septoglomus*, *Acaulospora*, *Gigaspora*, *Glomus*, *Scutellospora*, *Racocetra*, and *Rhizophagus* that are critical to the growth and well-being of these plants. Among the zingiberaceae growth in the area, *Zingiber officinale* exhibiting the most significant colonization and spore density revealed notable differences in AMF colonization rates and spore densities between species. The impact of regional environmental conditions on fungal dispersal was reflected in the diversity index for AMF spores, which likewise varied by location. By addressing a significant knowledge gap about AMF diversity in this biodiverse region of Indonesia, this study provides valuable information for conservation initiatives and sustainable farming methods.

AUTHOR CONTRIBUTION

R.O.K. led the conceptualization of the study, provided guidance throughout the research process, and contributed to writing and revising the manuscript. I.D.L. coordinated the fieldwork, collected soil and root samples, and performed data analysis related to A.M.F. colonization rates and spore density.

I.J.S. was responsible for identifying the A.M.F. genera through spore morphology and root staining techniques and contributed to analyzing diversity indices. N.S. assisted in statistical analysis and contributed to writing the manuscript. All authors participated in the interpretation of results and provided critical feedback, approving the final manuscript for submission.

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CONFLICT OF INTEREST

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

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Research Article

New Report of *Dysmicoccus brevipes* Cockerell (1893) (Hemiptera: Pseudococcidae) on *Heliconia* sp., *Lagenaria* sp., and *Zea mays* L. Root in Bali Indonesia

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ABSTRACT

The pink pineapple mealybug, scientifically known as *Dysmicoccus brevipes* (Cockerell) (Hemiptera: Pseudococcidae), can associate with all part of the plant, including the roots. Additionally, it plays crucial role as a carrier of plant viruses, highlighting its importance in relation to host plants. Reports of *D. brevipes* infestations on above-ground plant parts in Indonesia have been documented since 1917. However, there is a lack of data on the infestation of subterranean plant parts or roots by this organism which highlights the significance of this research. This article presents the identification of mealybugs on the roots of *Heliconia* sp., *Lagenaria* sp., and *Zea mays* L. from Bali, Indonesia, using a morphological method based on determination keys by Williams (2004) and a molecular method based on the MtCOI gene. The findings of this study suggested that the species observed on all three host plants was *D. brevipes*, making it the most recent record of *D. brevipes* presence on *Heliconia* sp., *Lagenaria* sp., and *Z. mays* roots in Bali, Indonesia.

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INTRODUCTION

Dysmicoccus is a genus of mealybugs belonging to the Pseudococcidae, the family with the second most numerous species after Diaspididae in the Coccoidea (Morales et al. 2016). Most species of mealybugs from the genus *Dysmicoccus* are polyphagous and have the potential to cause economic damage to various host plants. Mealybugs can reside and reproduce on every component of plants, including the roots (Sartiami 2006). They survive by sucking plant nutrients, thereby disrupting the absorption of water and other nutrients. The presence of mealybugs on plants indirectly contributes to developing sooty mold. Sooty mold grows on the excretions of mealybugs, known as honeydew, and is a carrier for different plant viruses (Franco et al. 2009; Daane et al. 2012). Mealybugs can cause further symptoms such as leaf withering, leaf browning, hindered flower formation, and even the death of the host plant when the infestation is severe (Jansen 1995).

Dysmicoccus brevipes in Indonesia were first documented in 1917 through plant quarantine interceptions in Washington DC, USA, involving commodities from Bogor (Sartiami et al. 2016). This species has a widespread reputation for its ability to infest host plants, targeting various plant components such as leaves, stems, fruit, and even concealed plant structures. Kalshoven (1981) reported that *D. brevipes* found in Java live in colonies at the base of the fruit, leaf folds, and underground. Zarkani et al. (2023) reported the species commonly attacks watery rose apple, *Syzygium aqueum* Alston (Myrtaceae) in Bengkulu, Sumatera. This is supported by the statement by Williams and Watson (1988) that *D. brevipes* which belongs to the Pseudococcidae family is capable of inhabiting both above-ground and below-ground plant portions.

In late 2022, several farmers in Badung Regency, Bali, Indonesia, reported the occurrence of mealybugs on the root systems of crops, specifically *Heliconia* sp., *Lagenaria* sp., and *Zea mays* L. However, the presence of subterranean mealybugs complicates identifying the specific mealybug species. There is currently a lack of reports on mealybug attacks on plant roots in Indonesia. Although there have been various reports on the level of damage caused by mealybug on plant roots in Indonesia as reported by Adus and Pu'u (2021) level of damage caused by mealybug on ginger plant (*Zingiber officinale* Rosc) reached a severe intensity of 66-84 %. Additionally, Akhsan and Dewi (2024); Lisnawita et al. (2023) reported that Indonesia as a pineapple exporter with an export value of US$102.67 million (80.06 thousand tons) is experiencing disruptions from mealybug which are known to be vectors of the Pineapple Mealybug Wilt-Associated Virus (PMWaV) shows a very high level of damage ranging from 73-92.3 % across various plant ages. This emphasises the significance of conducting research to determine the diversity of mealybug species in Indonesia and develop population control technology is crucial due to the potential economic losses it can cause.

MATERIALS AND METHODS

Sample collection

Specimens were collected from Badung Regency, Bali, Indonesia (8.82867°S 115.21582°E), in December 2022 after conducting personal interviews with farmers. Mealybugs sampling was carried out using the purposive sampling method. The brush was rubbed on the roots of *Heliconia* sp., *Lagenaria* sp., and *Zea mays* L., which showed symptoms of white wax filaments around the roots. Twenty to twenty-five female mealybug adults were collected from each host plant and preserved in 95 % alcohol.

Morphological identification

The collected specimens of female mealybug imago were then taken to the Entomology and Biomolecular Laboratory of the Bali Fish, Animal, and Plant

Quarantine Centre to be prepared to be used as observation preparations. A total of 10 female mealybug from each host plant were successfully slide-mounted using modifications to the work instructions by the Agricultural Quarantine Center Test Laboratory of Indonesia (2016). This technique used chloroform and Essig's solution to remove wax and fat from the specimen, followed by heating and staining with acid fuchsin. The specimen was then placed on an object glass given Heinz solution and covered with a cover glass. Sample slides were then identified using an Olympus CX21 compound microscope at the Plant Pest and Disease Laboratory, Faculty of Agriculture, Udayana University.

Morphological characters that were used for parameters in this research included body shape and size, number of antennae, presence of eyes, shape and size of various types of pores, description of limbs, size of mouthparts, anal lobe, as well as a description of seta, circulus, ostioles, and cerarii. The morphological characteristics of the specimens obtained were then compared with the key determination of "Mealybugs of Southern Asia" by Williams (2004).

Molecular Identification

One female imago from each host plant was selected for molecular identification conducted at the Plant Disease Laboratory, Faculty of Agriculture, Udayana University. The identification process started by isolating the total DNA using cetyltrimethylammonium bromide (CTAB), following the method established by Doyle and Doyle (1987). In this protocol, CTAB and β-mercaptoethanol solutions were prepared for each sample, and the mealybug sample was ground and powdered using liquid nitrogen. The CTAB buffer was added, and the sample was incubated for 60 minutes. Chloroform: isoamyl alcohol was added to separate fat, protein, and polysaccharides. The supernatant was transferred to a new tube, and sodium acetate, isopropanol, or absolute ethanol were added. The sample was then centrifuged at 12,000 rpm for 10 minutes, washed with 70 % ethanol, centrifuged at 8000 rpm for 5 minutes, and dried. DNA was resuspended using TE buffer or nuclease-free water and stored at -20 °C.

The process of DNA amplification was conducted in the mitochondrial cytochrome c oxidase subunit I (MtCOI) gene, which has proven to be effective in the identification of different insect species (Nurbaya et al. 2022; Sudiarta et al. 2023). The pair of primers used in amplification were designed by Park et al. (2011), namely PcoF1 5'CCTTCAACTAATCATAAAAATATYAG3' and LepR1 5'TAAACTTCTGGATGTCCAAAAAAATCA3' which amplified at 649 bp.

The PCR reactions were conducted using a Veriti™ Thermal Cycler. The total reaction volume was 20 µL, comprising of 10 µL of 2X Vivantis RedTaq PCR master mix, 1 µL of 10 pmol of each primer, 7 µL of nuclease-free water, and 1 µL of DNA template. The PCR program used was 94 °C for 5 minutes, 30 cycles at 94 °C for 1 minute, 52 °C for 35 seconds, 72 °C for 90 seconds, and continued with the final stage at 72 °C for 7 minutes. The PCR products were then visualized by electrophoresis using a 2 % agarose gel and 5 µL of SMOBIO FluoroVue™ gel stain (10,000X) for 1 hour at 80 volts. The PCR product that was successfully amplified was then sent to FirstBase sequencing service (Malaysia) for purification and sequencing to obtain the nucleotide sequence of mealybugs found on the roots of *Heliconia* sp., *Lagenaria* sp., and *Zea mays* plants. The nucleotide sequences were further edited to remove regions with low-quality nucleotides using MEGA 11 software (Tamura et al. 2021). Next, the sample sequence was aligned with several sequences obtained from the National Center for Biotechnology Information (NCBI) GenBank® using the Basic Local Alignment Search Tool (BLAST). This process resulted in many reference sequences that exhibited similarity to

the sample sequence.

Phylogenetic Analysis

The identification of mealybug species was conducted by comparing the nucleotide sequence of the sample with multiple reference sequences from GenBank. The proximity between the sample and the reference sequences was then calculated using a phylogenetic tree. Ten sequences of mealybugs of the genus *Dysmicoccus* and one sequence of *Bemisia tabaci* from Nigeria (MN164777) were obtained from GenBank and then selected to create a phylogenetic tree. Subsequently, all sequences were aligned using the ClustalW program in the MEGA 11 software. The Maximum Parsimony (MP) technique was used for constructing a phylogenetic tree due to its suitability for examining a limited number of sequences. MP employs a straightforward algorithm to identify the optimal tree (Dharmayanti 2011). Furthermore, Saitou and Imanishi (1989) asserted that the MP approach is highly effective in evaluating sequences with a base length of approximately 600 bp.

RESULTS AND DISCUSSION

Sign and Symptoms of Mealybug

When collecting samples, a consistent sign was the presence of white wax filaments around the roots of *Heliconia* sp., *Lagenaria* sp., and *Zea mays* L. These filaments could be found both in the soil and on the surface of the soil, as shown in Figure 1. The observable symptom during sample collection was the wilting of infected host plants and the yellowing leaves. Other symptoms that have been reported to appear due to *D. brevipes* attacks on plant roots are the decline in ginger production in East Nusa Tenggara, wilting in several plants such as pineapple, coffee, sugar cane, and bananas in Costa Rica (Williams & Granara de Willink 1992; Adus & Pu'u 2021). However, the sign and symptoms of attacks on plants caused by mealybugs are similar to those of other insects, such as white wax filaments and leaf chlorosis caused by whiteflies (De Barro 1995; Setiawati et al. 2007). Hence, the symptoms exhibited by the host plant during an attack do not provide conclusive evidence for detecting the presence of mealybugs on the roots. This is because mealybugs tend to target inaccessible plant parts, necessitating additional research to verify the symptoms caused by mealybug infestations on plant roots and their impact on reducing plant yields.

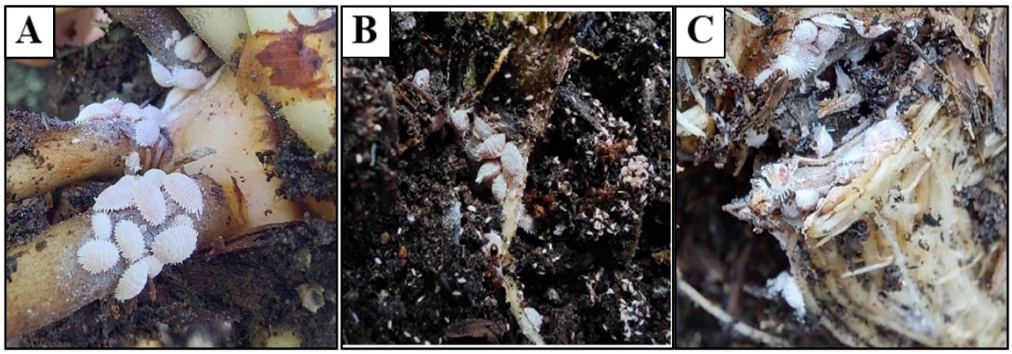

Figure 1. Living individuals of mealybugs on the host plant roots in Bali, Indonesia. **A.** *Heliconia* sp.; **B.** *Lagenaria* sp.; **C.** *Zea mays* L.

Taxonomic Identification

Preparations of root mealybugs found on the roots of *Heliconia* sp., *Lagenaria* sp., and *Z. mays* from Bali, Indonesia, are presented in Figures 2B, 2C, and 2D. The morphological characteristics of the mealybug samples showed an average body length of 2.1 mm and were very similar to *D. brevipes*. Therefore, an illustration of *D. brevipes* by Williams (2004) is also presented in Figure 2A.

All samples showed the presence of 17 pairs of cerarii on the edge of the body (Figure 2B). The anal lobe was moderately developed and has setae on the ventral surface with an average length of 151 µm. The anal ring had six setae with an average length of 151 µm (Figure 2E); one pair of antennas, each with eight segments with a length of 435-465 µm (Figure 2F); had eyes with 1-4 discoidal pores in each eye. The limbs were fully developed; the length of the hind trochanter + femur was around 306 µm, and the hind tibia + tarsus was around 263 µm (Figure 2G), and the claw was around 30 µm (Figure 2H). Translucent pores were absent in the trochanter but very numerous in the hind femur and hind tibia (Figures 2I and 2J). It had a labium of about 290 µm, which was longer than the clypeolabral shield. The circulus measured 96-131 µm and was divided by intersegmenta lines (Figure 2K). Ostioles were fully developed. The cerarii of the anal lobe had two pointed setae 25 µm long and 7.5 µm wide at the base, six or seven auxiliary setae, and a group of trilocular pores. The anterior cerarii were smaller in size than the cerarii in the anal lobe, namely 17 µm long with a basal width of 4.8 µm. It had many long setae on both the dorsal and ventral parts of segment VIII, 40-80 µm long. There were no multilocular pores on the dorsal side, but trilocular pores were evenly distributed. Discoidal pores had two sizes, large size with a diameter of 5 µm in the subdominal area of segments V-VIII. The small size was spread out but not too much. On the ventral surface, there were multilocular pores with a diameter of 8 µm on the posterior vulva to the edge of the abdominal segments VI and VII; sometimes 1-4 were found on the anterior abdominal segment VII. The trilocular pores were evenly distributed but were fewer in number than the dorsum.

The species *D. brevipes* is often discussed among entomologists because its morphological characteristics are similar to *D. neobrevipes*. Based on Williams and Granara de Willink (1992), several morphological characters are very similar in these two species, namely discoidal pores near the eyes, multilocular pores limited to the ventral segments VI, VII, and VIII, and the absence of oral rim tubular ducts throughout the body. The main difference between *D. brevipes* and *D. neobrevipes* is the body color of the adult female in real life. *D. brevipes* has a body color that tends to be pink, while *D. neobrevipes* has a gray body color. Beardsley (1992) stated that the main difference between *D. brevipes* and *D. neobrevipes* lies in the size of the dorsal abdominal seta in segment VII, which is longer than conical cerarian setae at around 45-80 µm (Figure 2L and 2M), was also found in sample specimens of mealybugs from Bali, Indonesia, further strengthening the identification results. In addition, Yan-Biao et al. (2014) stated that *D. neobrevipes* tends to have sclerotisation or thickening on the ventral part, and the anal lobe tends to be elongated, which is not shown in the morphological characteristics of mealybug samples found in Bali, Indonesia.

Molecular Analysis

The accuracy of the morphological identification results was subsequently verified using molecular identification. The three mealybug DNA samples from Bali, Indonesia, were successfully amplified at a fragment length of 649 bp. The results of electrophoresis visualization are presented in Figure 3. The amplified DNA was then sequenced to obtain the nucleotide base sequence, which was then searched for species proximity using BLAST in GenBank. Based on BLAST results, other sequences close to the sample sequence were then used as reference sequences for phylogenetic analysis.

Phylogenetic analysis began with the alignment of root mealybug sequences from Bali and several comparative sequences using MEGA 11 software with the ClustalW program. The results of this alignment were then seen at the homology level presented in Table 1. It presents the genetic dis-

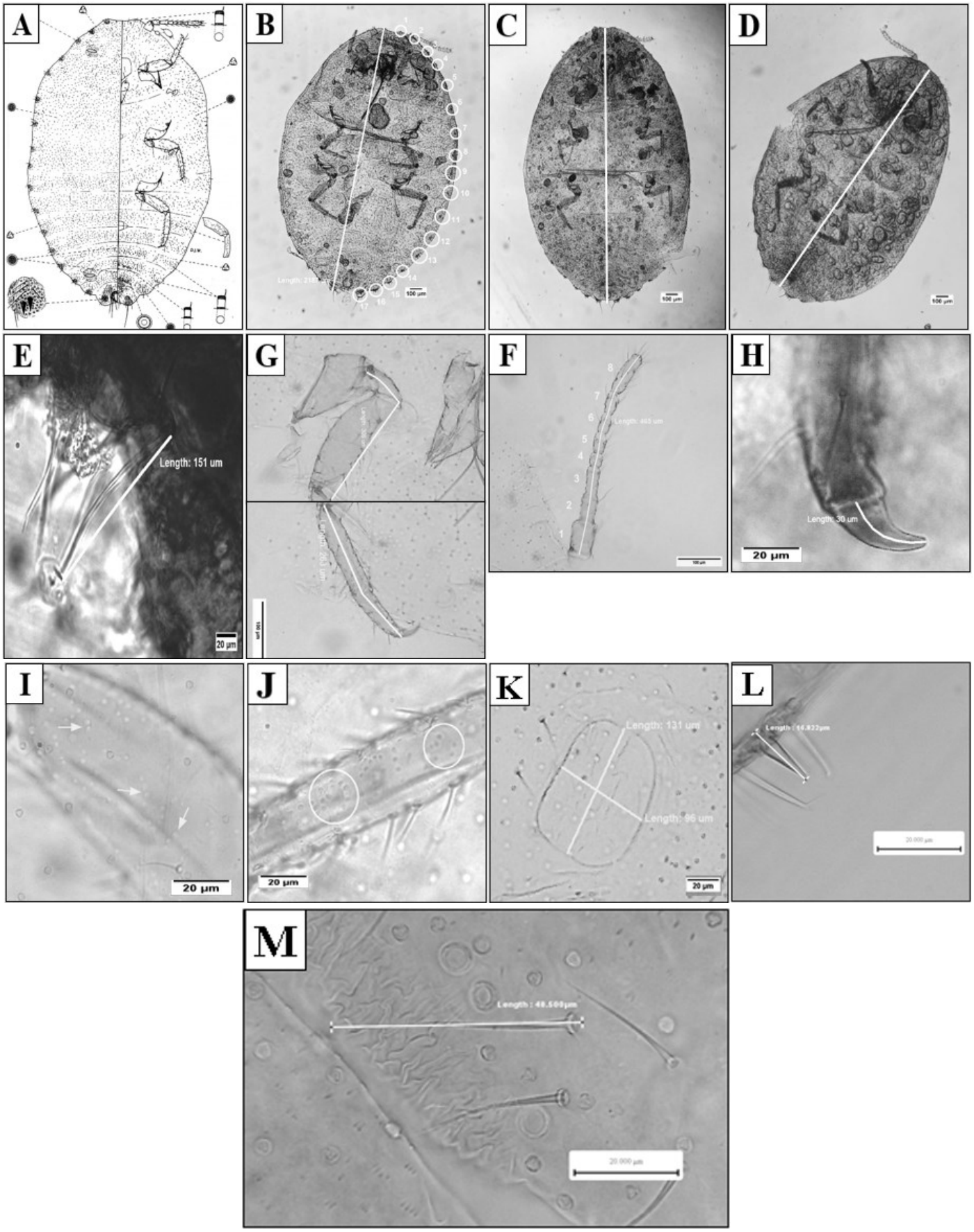

Figure 2. Morphological identification of *D. brevipes* specimens. (A) Illustration of *D. brevipes* by Williams (2004), (B) Samples of mealybugs on the roots of *Heliconia* sp., (C) Sample of mealybugs on the roots of *Lagenaria* sp., (D) Sample of mealybugs on *Zea mays* roots, (E) Seta on the anal ring, (F) Eight segment antenna, (G) Legs, (H) Claws, (I) Translucent pore on the hind femur, (J) Translucent pore on the hind tibia, (K) Circulus which was divided by intersegmental lines, (L) Conical cerarian setae, (M) Dorsal abdominal seta in segment VII and VIII. 4x, 10x, and 40x magnification

tances created through pairwise distance analysis between mealybug samples from Bali, Indonesia, and mealybug sequences from other countries. Based on this table, it can be seen that mealybugs from Bali, Indonesia, which were obtained from the roots of *Heliconia* sp., *Lagenaria* sp., and *Zea mays*, show a high similarity to *D. brevipes* with a range of homology percentages, namely 99.3 % - 100 % with a genetic distance of 0.000 - 0.002. Therefore, it was verified that the species of mealybug discovered in Bali was *D. brevipes*. These results are supported by the statement of Ptaszyńska et al. (2012) that using MtCOI fragments in molecular identification will indicate the same species in the 95.1 % - 100 % range. A statement by Hebert et al. (2003) also supports the correctness of the identification results, namely that genetic distances between sequences with a value of less than 0.03 can be declared as the same species. On the other hand, *D. neobrevipes*, which has morphological characters very similar to mealybug samples from Bali, Indonesia, shows a homology percentage range of 92.5 % - 92.7 %. Thus, it can be ascertained that the sample mealybugs were not *D. neobrevipes*.

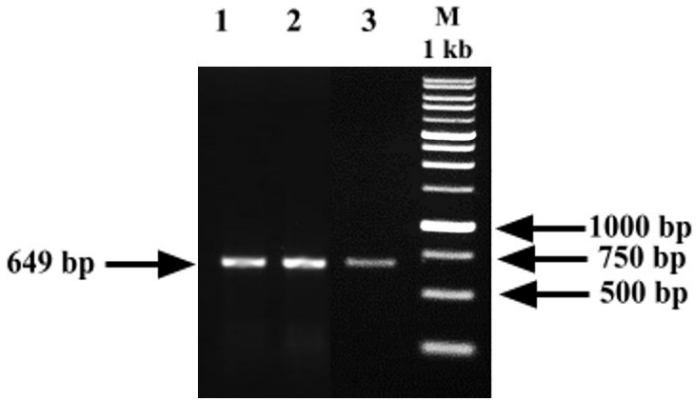

Figure 3. Results of *D. brevipes* DNA amplification using primers PcoF1 and LepR1 which were successfully amplified at 649 bp. Column 1 was a band of root mealybugs *Heliconia* sp., column 2 was a band of root mealybugs *Lagenaria* sp., and column 3 was a band of root mealybugs *Zea mays* L.

Molecular identification was continued with phylogeny analysis as a cladogram (Figure 4), created using the Maximum Parsimony (MP) method with bootstrap repetitions 1000 times. The results of the phylogenetic tree construction showed two main clades, namely the clade consisting of the sequence groups *D. boninsis*, *D. lepelleyi*, and *D. neobrevipes*. The other clade con-

Table 1. Level of homology and pairwise distance analysis of root mealybug sequences from Bali, Indonesia with mealybug sequences from other countries. *RM = Root Mealybug

Sequence	Homology (%); Pairwise Distance			Accession Number
	Heliconia sp. RM	*Lagenaria* sp. RM	*Zea mays* L. RM	
Dysmicoccus brevipes India	99.3; 0.002	99.5; 0.000	99.5; 0.000	OQ955830
Dysmicoccus brevipes Brazil	99.7; 0.002	100; 0.000	100; 0.000	OP450829
Dysmicoccus neobrevipes India	92.7; 0.081	92.5; 0.083	92.5; 0.083	OQ942202
Dysmicoccus lepelleyi Thailand	90.4; 0.110	90.4; 0.111	90.4; 0.111	HM474152
Dysmicoccus lepelleyi Vietnam	90.4; 0.110	90.4; 0.111	90.4; 0.111	KX015112
Dysmicoccus boninsis Brazil	87.4; 0.142	87.4; 0.142	87.4; 0.142	OP450830
Dysmicoccus boninsis China	87.8; 0.136	87.8; 0.137	87.8; 0.137	KP692714
Bemisia tabaci Nigeria	45.4; 2.012	45.2; 2.042	45.2; 2.042	MN164777

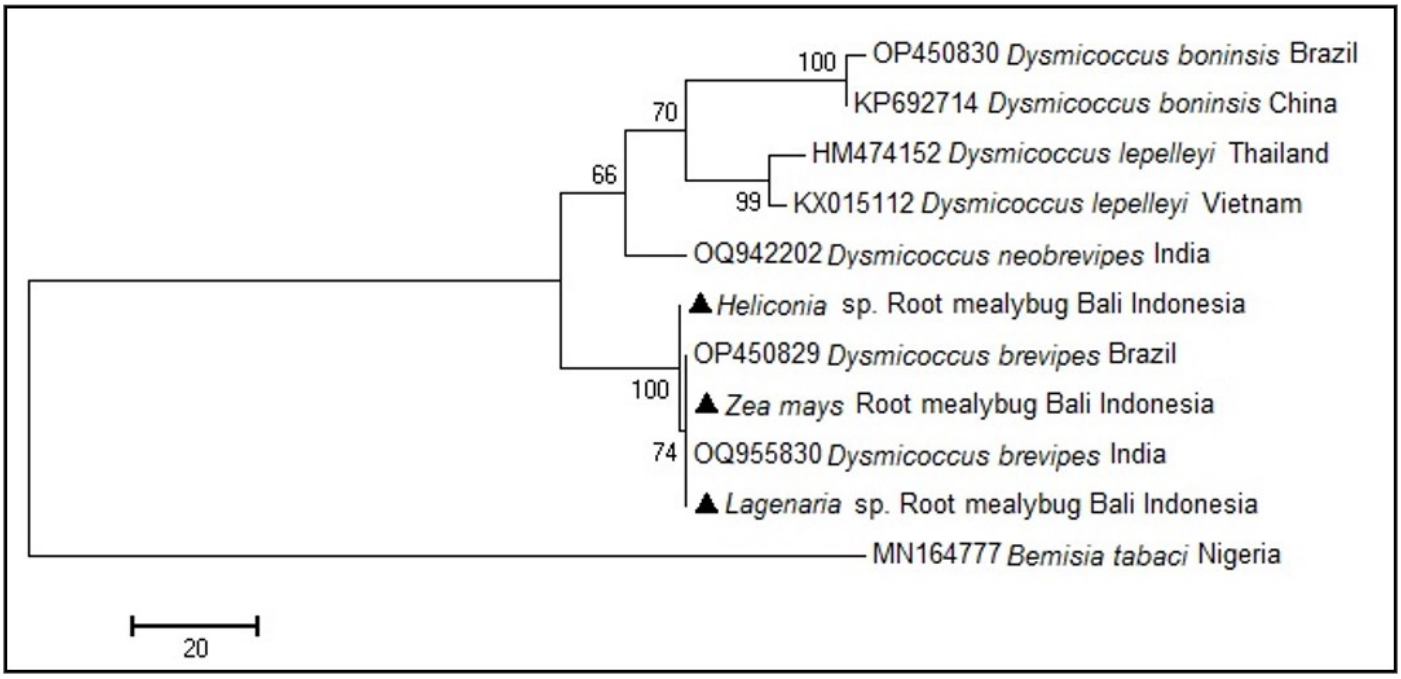

Figure 4. Phylogenetic tree of root mealybugs *Heliconia* sp., *Lagenaria* sp., and *Zea mays* from Bali, Indonesia, compared with mealybug sequences from other countries based on the MtCOI gene with a bootstrap repetition value of 1000 times.

sisted of sequences associated with *D. brevipes*. From the cladogram construction, it is evident that the two clades have a common ancestor in comparison to the outgroup sequence. It was verified that the root mealybug species found in Bali, Indonesia, was the *D. brevipes* species. *D. brevipes* attacked *Heliconia* sp. *Lagenaria* sp. and *Zea mays* have been reported worldwide, but mealybug samples did not come from plant roots (Graham 1983; Williams 2004; Matile-Ferrero & Étienne 2006). This also shows that this research is the first report of *D. brevipes* on plant roots in Indonesia.

CONCLUSIONS

The mealybug presence on *Heliconia* sp., *Lagenaria* sp., and *Zea mays* root specimen from Bali, Indonesia, has been confirmed as *D. brevipes* based on morphological and molecular analysis. This is the first report of the finding of *D. brevipes* presence on *Heliconia* sp., *Lagenaria* sp., and *Zea mays* root in Indonesia. It can be used as an initial reference for future study in order to further comprehend the distribution and level of damaged of *D. brevipes* on these three host plants.

AUTHOR CONTRIBUTION

I.P.S. and G.N.A.S.W. contributed to the conception of the study. K.S.D. and M.G.P.W. collected samples and obtained data. K.S.D and P.S.D. contributed to the morphological identification. K.S.D. and F.E.W. contributed to molecular and phylogenetic analysis. I.P.S. contributed to the final editing. All authors read, critically revised, and approved the final manuscript.

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CONFLICT OF INTEREST

Authors declare that there is no competing interest regarding the publication of manuscripts.

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Review Article

Impact of Global Climate Shifts on the Biodiversity and Functionality of Marine Zooplankton Communities

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ABSTRACT

Climate change represents one of the biggest crises confronting humanity today. Its effects are not limited to human populations but extend to the marine environment, including zooplankton communities, which are critical components of the marine food chain. This study provides a review of the impacts of global climate shifts on the biodiversity and functioning of marine zooplankton communities. Specifically, it examines how changes in temperature, ocean acidification, and other environmental stressors affect zooplankton populations. The study also includes an analysis of case studies and regional variations in the impacts of climate change on these communities, alongside a discussion of methodologies used in studying these effects. Furthermore, the research evaluates existing knowledge gaps and identifies future research directions that are necessary to enhance our understanding of these impacts. Through this latest evaluation, the study underscores the importance of continuous monitoring and the adoption of a multi-stressor research approach. It also highlights the need for designing effective adaptation strategies for marine zooplankton communities, which are crucial for the development of sustainable marine conservation policies in the future. The findings of this study emphasise the urgency of further research to preserve the integrity of marine ecosystems in the face of global climate change challenges.

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INTRODUCTION

Background Related to Climate Change

Definition and Causes of Climate Change

The climate is generally understood as a synthesis of atmospheric parameters such as temperature, precipitation, humidity, and wind patterns over significant periods (Ching-Ruey 2020; Kalkuhl & Wenz 2020). This broader concept encompasses both short-term weather fluctuations and long-term climate variations that span geological timescales (Safia et al. 2023). The Earth's climate system is a complex, interconnected network involving the atmosphere, oceans, ice masses, land surfaces, and vegetation, each influencing the others in intricate ways (Li et al. 2016; Fyke et al. 2018). Oceans, covering approximately 70 % of the Earth's surface, play a critical role in climate regulation through heat absorption and release, as well as the distribution of atmospheric moisture (Zardi 2024). Ocean circulation patterns, such as major ocean currents, impact regional and global climates by transporting warm water to polar regions and influencing humidity levels. Additionally, oceans serve as a significant carbon sink, affecting atmospheric CO_2 levels and, consequently, global climate (Doney et al. 2009). Vegetation interacts with climate by reflecting radiant energy, transferring water and latent heat, and influencing air movement, thereby forming a complex cycle of interactions within the Earth system that governs the dynamics and balance of the global environment (López-Pacheco et al. 2021; Wunderling et al. 2024).

Climate change represents one of the most pressing crises facing humanity today (Cornell & Gupta 2019). It encompasses variations in weather conditions, such as changes in precipitation levels, deviations from normal temperature ranges, and shifts in global temperatures (López-Pacheco et al. 2021). Riebeek (2011) defines climate change as a global phenomenon primarily driven by fossil fuel combustion, leading to increased greenhouse gas concentrations in the atmosphere. This results in global warming, rising sea levels, melting ice masses, altered plant and flower blooming patterns, and more frequent extreme weather events. The causes of climate change are twofold: natural and anthropogenic. Natural factors include variations in solar output, changes in Earth's orbit, volcanic activity, shifts in ocean currents, and continental drift (Sigl et al. 2015). In contrast, human activities such as greenhouse gas emissions from burning fossil fuels, land conversion, deforestation, and industrial activities significantly contribute to climate change by enhancing the greenhouse effect and increasing atmospheric CO_2 concentrations. Additionally, urbanisation and infrastructure development further exacerbate CO_2 emissions. Public awareness of climate variations spans short-term seasonal and annual changes to long-term decadal shifts (Smith et al. 2014). Therefore, understanding these intricate climate dynamics is essential for developing effective strategies to mitigate climate change impacts and adapt to future environmental conditions, ensuring the sustainability of ecosystems and human societies alike.

An Overview of Global Warming and Ocean Acidification

Global warming, driven by increased greenhouse gas emissions from human activities, is leading to increased atmospheric and ocean temperatures. This warming trend manifests in observable phenomena such as rising global temperatures, melting polar ice, and an increase in extreme weather events (Smith et al. 2014). Ocean acidification is another critical issue associated with global warming. It results from the absorption of CO_2 by seawater through diffusion, where it reacts to form carbonic acid. This acid then dissociates into bicarbonate and hydrogen ions, lowering the pH of seawater and increasing its acidity. Additionally, phytoplankton also absorbs CO_2 through photosynthesis, but their contribution to ocean acidification is much smaller than diffusion (Houghton 2012). Future projections indicate that under worst-case sce-

narios, ocean pH could drop by an additional 0.3 to 0.5 units by 2100, which would severely impact marine biodiversity, leading to the collapse of coral reef ecosystems and disrupting marine food webs (Caldeira & Wickett 2005). Ocean acidification negatively impacts marine organisms that rely on calcium carbonate to form their shells and skeletons, including corals and mollusks. The interaction between global warming and ocean acidification exacerbates both issues: higher sea temperatures reduce the ocean's capacity to absorb CO_2 due to decreased gas solubility. Meanwhile, phytoplankton is important for CO_2 absorption through photosynthesis, however, warming and ocean acidification disrupt their productivity and the global carbon cycle in complex ways. A decline in nutrient availability and changes in the ecosystem also reduce phytoplankton's ability to absorb CO_2, even though they still require CO_2 for photosynthesis (Tai et al. 2021). Together, these phenomena pose significant threats to marine ecosystems and overall planetary health (Figure 1). Addressing these interconnected challenges requires comprehensive mitigation strategies that involve both local and global efforts. These strategies include reducing CO_2 emissions through the transition to renewable energy sources, enhancing carbon sequestration in marine ecosystems such as seagrasses and mangroves, and implementing marine protected areas to support biodiversity resilience (Gattuso et al. 2018). What equally important is the role of international cooperation in tackling the root causes of climate change and ocean acidification, highlighting the necessity of collective action to safeguard marine ecosystems for future generations (IPCC 2019).

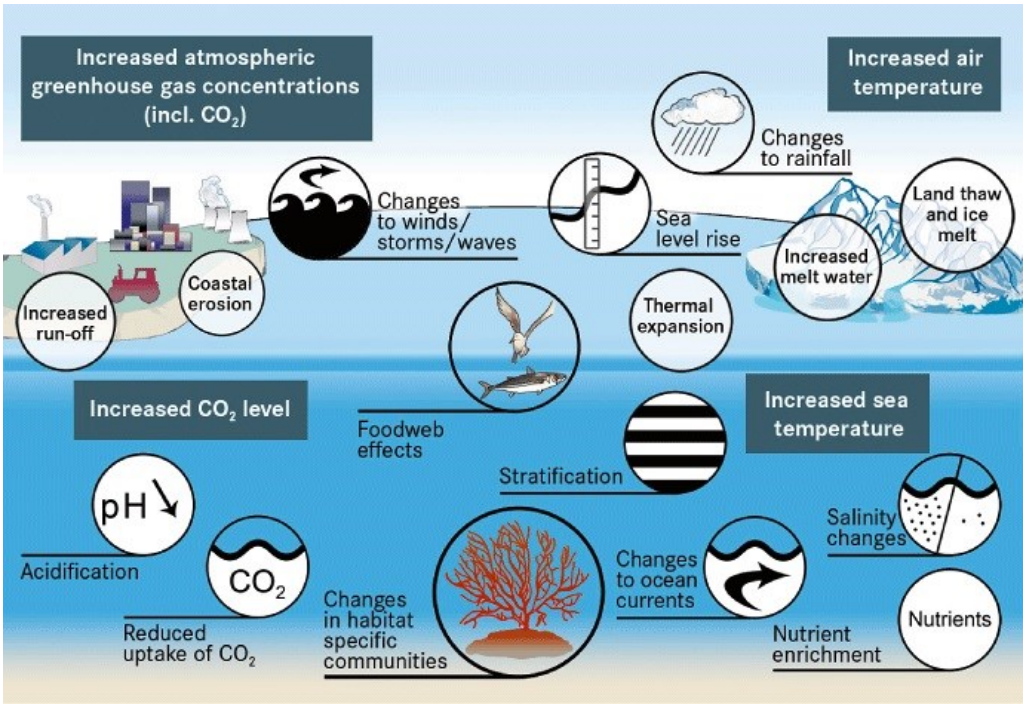

Figure 1. Overview of the effects caused by climate change and ocean acidification. (Source: Ospar Commission 2010).

The Importance of Zooplankton in Marine Ecosystems

Zooplankton are essential for food web stability, serving as a primary food source for many marine organisms, as well as a crucial link between phytoplankton and higher-level consumers such as fish and marine mammals. They are also essential for energy transfer and ecosystem sustainability. By regulating phytoplankton populations, zooplankton help to prevent harmful algal blooms that can degrade water quality and negatively impact marine life (Ratnarajah et al. 2023). Additionally, zooplankton contribute significantly to biogeochemical cycles, including the carbon and nitrogen cycles, through processes such as biological pumping and nutrient regeneration (Steinberg &

Landry 2017). With an estimated 28,000 marine zooplankton species, they are diverse and vital for maintaining nutrient balances that support primary productivity (Bucklin et al. 2021). However, they face significant challenges from rapid environmental changes, including shifts in temperature and acidity, which may disrupt their life cycles and availability as prey, ultimately destabilising marine ecosystems (Richardson 2008).

Study Objectives

This research review aims to achieve four core objectives. *First*, it seeks to analyse the impacts of temperature changes, ocean acidification, and other stressors on marine zooplankton communities, identifying shifts in biodiversity and ecosystem function that are crucial for ocean sustainability. *Second*, it incorporates case studies and explores regional variations to understand how climate change affects zooplankton across different marine ecosystems. This approach is vital for developing effective, region-specific adaptation strategies. *Third*, the review details the methodologies used to assess climate impacts on zooplankton, aiming to enhance the accuracy and reliability of data. Improved research methods are expected to yield more valid data, supporting better conservation policies and strategies. *Fourth*, it identifies existing knowledge gaps and outlines future research directions necessary to address these gaps. Addressing these gaps is essential for developing a comprehensive understanding of zooplankton-climate interactions and for crafting effective mitigation and adaptation strategies. Overall, this study provides insights into the effects of climate change on zooplankton, offers guidance on improving research methodologies, and supplies region-specific information for local policy development. It is anticipated to contribute significantly to global efforts to protect marine biodiversity and sustain ecosystem functions that are essential for life on Earth.

IMPACT OF TEMPERATURE INCREASE ON ZOOPLANKTON

Climate variability impacts zooplankton distribution and abundance in complex ways, with patterns differing across ocean basins (Ratnarajah et al. 2023). Deep waters have experienced warming ranging from 0.3 °C to 0.6 °C under different emissions scenarios, with the Southern Ocean projected to see the most significant warming (Ciais et al. 2013) (Figure 2). Rising global temperatures have led to shifts in zooplankton distribution, such as the northward migration of North Atlantic species like *Calanus finmarchicus* due to warming waters, while *Calanus glacialis* has declined in the western Barents Sea (Beaugrand et al. 2002). These shifts, driven by changes in sea ice conditions, impact predator-prey dynamics and disrupt marine food webs, potentially leading to imbalances in ecosystem dynamics (Møller & Nielsen 2020). The arrival of new zooplankton species can also alter predator-prey relationships and impact marine biodiversity. In addition, increased sea temperatures accelerate zooplankton metabolism and reproduction rates but may reduce larval survival and overall population density, affecting fisheries productivity and global climate dynamics through changes in carbon sequestration (Hays et al. 2005; Richardson 2008).

Rising ocean temperatures disrupt zooplankton reproduction and life cycles by altering spawning times and reducing larval survival, as reproductive cycles are closely tied to water temperature and phytoplankton availability (Edwards & Richardson 2004). Misalignment with food availability can lead to declines in predator populations, causing cascading effects throughout marine ecosystems (Durant et al. 2007). Elevated temperatures also induce thermal stress, reducing egg production and increasing embryo mortality, as observed in copepods (Byrne et al. 2009). Accelerated development from egg to adult may decrease longevity and body size, leading to shorter life cycles

and increased generational turnover, which can affect population stability and predator-prey interactions (Møller & Nielsen 2020). Changes in diet, such as copepods shifting to protozoa when phytoplankton are scarce and furtherly influence primary production and predator-prey relationships (Atkinson 1996).

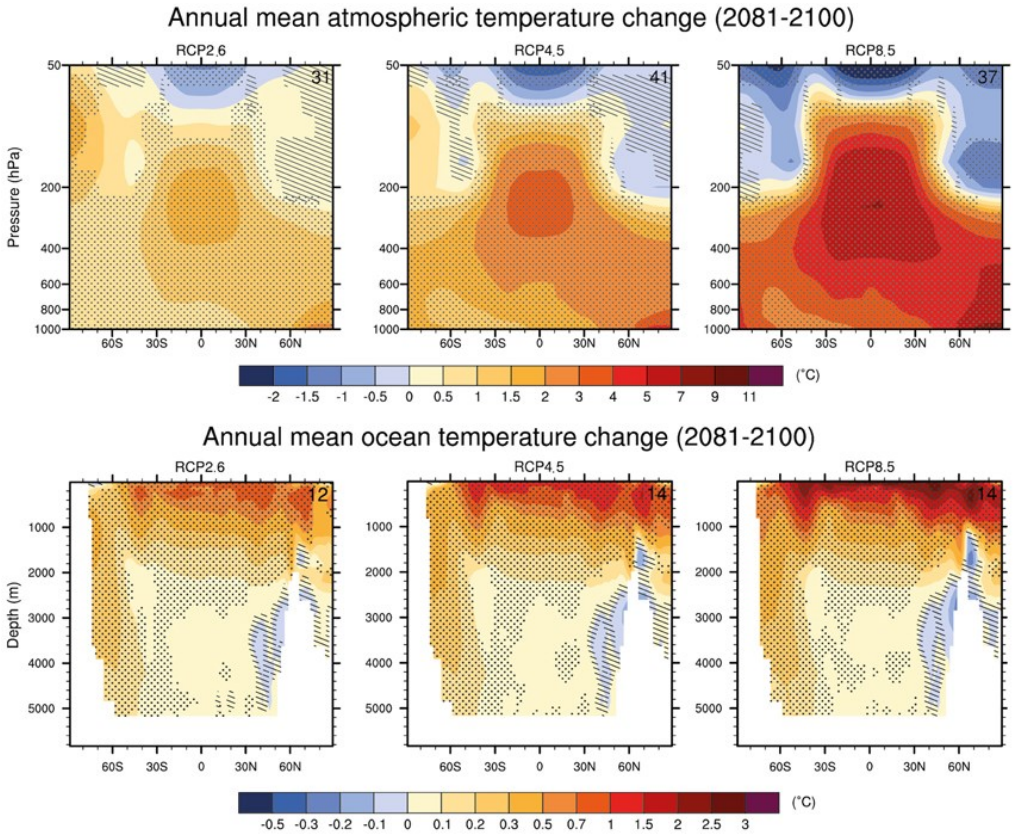

Figure 2. Changes in zonal annual mean temperature in the atmosphere and oceans, as projected by the Multi-Model Coupled Model Intercomparison Project Phase 5 (CMIP5), compared to the period 1986-2005 for the years 2081-2100. Projections are presented for the Representative Concentration Pathways (RCP) scenarios RCP2.6 (left), RCP4.5 (middle), and RCP8.5 (right). RCPs represent different greenhouse gas concentration trajectories. Shaded areas indicate regions where the multi-model averages fall below one standard deviation of internal variability. The dotted area highlights regions where the multi-model average exceeds two standard deviations of internal variability and where 90 % of the models exhibit consistent directional changes. (Source: Collins et al. 2013).

Zooplankton exhibit genetic adaptation to rising temperatures through changes in gene expression related to metabolism, reproduction, and stress tolerance. While rapid adaptation can occur in some species, the ability to adapt varies and may be limited by the rate of temperature change and other selective pressures (Geerts et al. 2014). Rapid or extreme temperature changes can exceed the genetic adaptability of zooplankton, potentially leading to population declines or local extinctions. Furthermore, adaptation to one stressor may impair the ability to cope with others, such as salinity changes or food scarcity (Bell & Collins 2008). In addition to genetic adaptation, zooplankton exhibit behavioural and physiological responses that depend on food availability, such as phytoplankton, which serve as a primary food source. They alter their vertical migration patterns to avoid warmer surface waters and forage in cooler depths. Furthermore, they produce heat shock proteins to enhance their thermal tolerance and resilience which is crucial in the face of temperature changes (Record et al. 2014).

IMPACT OF OCEAN ACIDIFICATION ON ZOOPLANKTON

Ocean acidification imposes significant physiological stress on zooplankton, particularly those with calcium carbonate structures such as pteropods and foraminifera. As seawater's pH declines, the availability of carbonate ions necessary for shell formation decreases, resulting in more brittle and vulnerable shells. For instance, the pteropod *Clio pyramidata* has shown a 35 % reduction in shell thickness under lower pH conditions, increasing its susceptibility to predation and environmental damage (Comeau et al. 2009). This weakening of shells compromises the ecological function of these zooplankton in contributing calcium carbonate to marine sediments and disrupts metabolic processes critical for survival, such as respiration and excretion (Fabry et al. 2008). Consequently, increased mortality and decreased reproductive success, such as the 20 % reduction observed in the copepod *Acartia tonsa*, can impact population dynamics and lead to significant ecological consequences, including potential long-term genetic adaptations or changes in community composition (Cripps et al. 2014).

Apart from inducing physiological stress, ocean acidification also impacts the feeding behaviour and growth of zooplankton. The decreased pH disrupts sensory mechanisms crucial for detecting and capturing food, leading to reduced feeding efficiency and slower growth rates. For example, copepods like *C. finmarchicus* experience up to a 15 % reduction in feeding efficiency under acidic conditions (Cripps et al. 2014). This reduction in feeding efficiency results in lower zooplankton biomass, which is critical for sustaining marine food webs. Furthermore, acidification diminishes the nutritional quality of zooplankton, including essential fatty acids such as omega-3 in species like *A. tonsa*, impacting their predators and disrupting marine ecosystems (Bairagi et al. 2019). At the ecosystem level, the consequences of ocean acidification are profound. Declines in zooplankton populations and their nutritional quality lead to reduced food availability for predators, including fish, crustaceans, seabirds, and mammals such as whales. This disruption can affect commercially important fish species such as herring and salmon, which rely on zooplankton during their early life stages (Orr et al. 2005). As a specific example, reducing copepod populations can decrease the biomass of small pelagic fish, impacting larger predatory fish and seabirds. Alterations in zooplankton community structure can disturb marine food webs, affecting ecosystem stability and energy transfer, ultimately diminishing marine biodiversity and ecosystem resilience (Fabry et al. 2008; Doney et al. 2009). Moreover, the economic impacts of ocean acidification are substantial, as declining zooplankton populations can lead to reduced fish stocks, adversely affecting fisheries and coastal communities that depend on them for livelihoods (Mangi et al. 2018). Mitigation strategies should focus on reducing CO_2 emissions through policy changes and transitioning to sustainable energy sources, while further research is crucial to understand the long-term effects of acidification on marine ecosystems and to develop adaptive management strategies (Doney et al. 2009)

INTERACTIVE EFFECTS OF VARIOUS STRESSORS

The Combined Impact of Warming and Acidification

Global warming and ocean acidification present significant synergistic stressors for zooplankton, with their combined effects often intensifying physiological stress beyond the impact of each stressor individually. Key stressors include elevated sea temperatures which accelerate zooplankton metabolism and increase energy demands, also ocean acidification which disrupts calcification and metabolic functions. Together, these factors lead to heightened oxidative stress, reduced feeding efficiency, and impaired reproductive health (Byrne & Przeslawski 2013). The interactions between these stressors can

vary significantly among zooplankton species. Typically, the copepod *C. finmarchicus* demonstrates significant declines in survival and reproduction under simultaneous high temperatures and low pH conditions, while other species may show differing levels of tolerance (Lewis et al. 2013). The compounded effects of these stressors can manifest as altered behaviour, such as reduced swimming ability in pteropods and disrupted migration patterns, impacting predator-prey interactions and marine food webs (Bednaršek et al. 2012).

Furthermore, measuring the impact of these stressors on zooplankton diversity involves assessing various physiological and ecological responses, such as changes in biomass, reproductive success, and community composition across different environmental conditions (Pörtner et al. 2014). Techniques like controlled laboratory experiments and long-term field studies can provide valuable insights into how these stressors affect zooplankton populations (Richardson et al. 2009). Over time, the interactions between warming and acidification can alter marine ecosystem structure and function by reducing zooplankton biomass and disrupting marine food chains, which affects higher trophic levels such as fish and marine mammals (Bednaršek et al. 2012). Therefore, understanding the synergistic impacts of these stressors is crucial for accurately predicting the effects of climate change on marine ecosystems (Byrne & Przeslawski 2013). Comprehensive research is essential to fully understand the long-term effects of these synergistic stressors on zooplankton health and ecological dynamics (Figure 3).

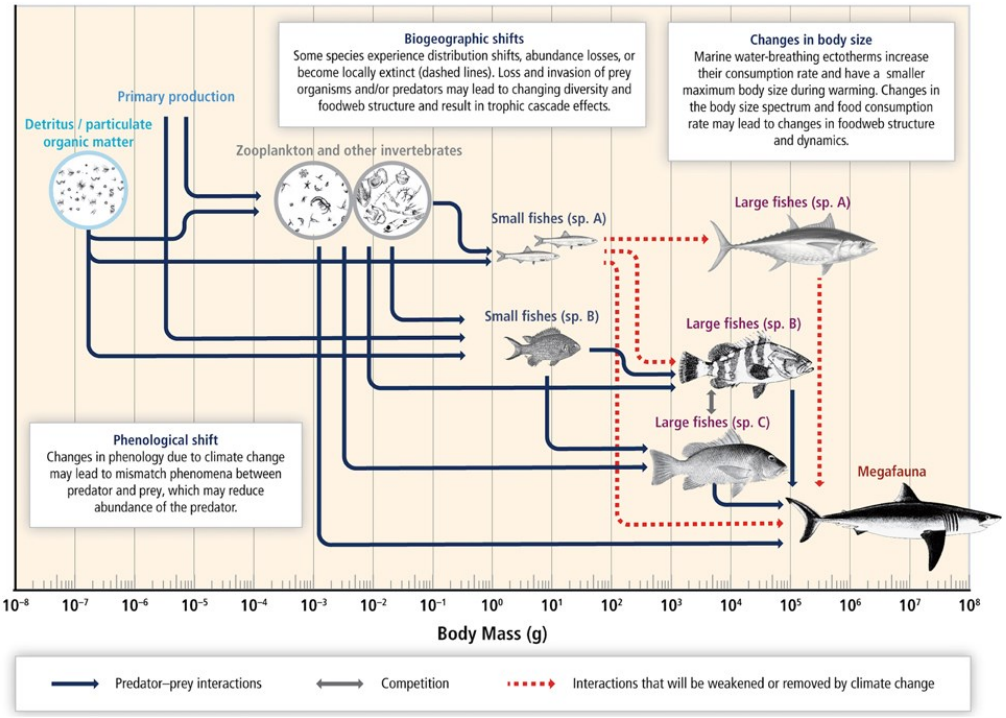

Figure 3. Schematic estimated response to climate change in seafood webs. This framework illustrates how interconnected pelagic and benthic food webs, organised by body size spectrum, respond to climate change. Warming, hypoxia, and ocean acidification cause reductions in body size, shifts in biogeography, changes in species composition and abundance, and alterations in trophic relationships. Fishing further reduces large-sized species, narrowing the body size spectrum and complicating the detection of climate change impacts on food webs. Arrows denote species interactions, such as predation and competition, while the dotted line represents potential losses in population and trophic relationships due to climate change. (Source: Pörtner et al. 2014).

Effects of Additional Stressors (e.g., pollution and hypoxia)

Anthropogenic pollution, including heavy metals, pesticides, and microplastics, compounds the effects of ocean warming and acidification on zooplankton, exacerbating physiological stress and reducing their adaptability to environmental changes (Gobler & Talmage 2013). Pollutants can accumulate in zooplankton, leading to cellular damage, reproductive issues, and increased mortality, while also impairing detoxification processes and increasing susceptibility to toxicity (Wang et al. 2022). Furthermore, pollutants like microplastics can disrupt nutrient digestion and absorption, impacting growth and development (Richon et al. 2022). In addition to pollution, declining oxygen levels or hypoxia, which are often linked to eutrophication and nutrient runoff, further stress zooplankton by reducing oxygen availability, increasing metabolic strain, and altering vertical distribution. This can enhance predation risks and disrupt feeding behaviors (Gobler & Baumann 2016). These cumulative stressors can lead to significant declines in zooplankton populations, affecting marine food webs and ecosystem dynamics. The combined effects of these stressors suggest that managing and protecting marine ecosystems requires a comprehensive understanding of their interactive impacts (Pörtner et al. 2014) (Figure 3).

CASE STUDIES AND REGIONAL VARIATIONS

Various studies underscore the significant impact of climate change on zooplankton communities. In the North Sea, rising ocean temperatures have led to shifts in zooplankton distribution and abundance, affecting local marine ecosystems and commercial fisheries (Beaugrand et al. 2002). In the Barents Sea, melting sea ice and temperature changes have altered zooplankton community composition, impacting predators such as Arctic cod and seabirds (Dalpadado et al. 2012). The Continuous Plankton Recorder (CPR) data from the Atlantic Ocean reveal long-term changes in zooplankton communities over recent decades, indicating that climate change disrupts zooplankton timing and spatial distribution, which can affect predator-prey interactions and energy transfer efficiency in marine ecosystems (Richardson et al. 2006). Research in the Southern Ocean showed that ocean acidification negatively affects pteropods, crucial zooplankton in the region, by impairing their shell formation and thus reducing their survival and reproduction (Bednaršek et al. 2012).

Polar Region

Zooplankton in polar regions are confronting distinct challenges due to rapid climate change. In the Arctic, rising temperatures accelerate sea ice melt, disrupting the habitat and life cycles of ice-dependent species such as *C. glacialis*. This disruption extends to algal phenology and food quality, negatively impacting zooplankton reproduction and growth. Additionally, ocean acidification impairs calcium carbonate exoskeleton formation, further increasing mortality rates among these organisms (Søreide et al. 2010; Bednaršek et al. 2012; Hatlebakk et al. 2022). In Antarctica, warming temperatures and reduced sea ice are affecting Antarctic krill (*Euphausia superba*), a vital food source for many predators, potentially altering the entire ecosystem's dynamics. These changes also impact krill larvae's reproduction and survival (Atkinson et al. 2004; Flores et al. 2012). Despite some species showing resilience, the combined effects of warming and acidification present significant challenges, necessitating further research to understand the broader impacts on polar ecosystems (Kebir et al. 2023). Case studies highlight these issues: in the Arctic Chukchi Sea, the research indicated that warming and acidification have led to declines in sea ice-dependent zooplankton and an increase in species tolerant to warmer conditions, affecting marine food webs and ecosystem

sustainability (Questel et al. 2013; Brower et al. 2018). In Antarctica, warming has shifted Antarctic krill distribution southward and negatively affected larval survival due to acidification, while studies in the Weddell Sea show how hydrographic variability influences zooplankton migration patterns and alters the behaviour of large predators such as elephant seals (Biuw et al. 2010; Flores et al. 2012).

Tropical Seas and Temperate Temperatures

Zooplankton in tropical and temperate regions demonstrate notable variability in their responses to environmental changes. Tropical regions, characterised by less seasonal variation and higher water temperatures, face distinct challenges. Notably, in the Indian Ocean, the El Niño phenomenon induces significant fluctuations in zooplankton abundance and distribution, necessitating rapid adaptation to shifts in temperature and food availability (Hays et al. 2005). In temperate regions such as the North Atlantic, rising temperatures have prompted shifts in zooplankton species distributions towards the poles, with cold-water species like *C. finmarchicus* being replaced by heat-tolerant species such as *Calanus helgolandicus*. This change disrupts marine food webs and ecosystems, as the latter thrives under higher temperatures while the former struggles (Falkenhaug et al. 2022). Additionally, ocean acidification impacts zooplankton differently across these regions; in tropical seas, it hinders calcium carbonate exoskeleton formation, whereas, in temperate seas, effects vary by species and local conditions, with some showing greater resilience than others (Fabry et al. 2008). Regional case studies offer further insight into these impacts. In the Caribbean Sea, ocean warming and acidification have caused declines in calcium carbonate-dependent zooplankton, highlighting the need to understand cumulative environmental stressors on tropical marine ecosystems (Howes et al. 2015). Similarly, in the Mediterranean, rising temperatures have led to shifts in zooplankton distributions, with warmer species displacing colder ones. This affects both zooplankton and their predators, such as fish and seabirds, thereby impacting temperate marine ecosystems (Raitsos et al. 2010). Furthermore, ocean acidification has been shown to reduce zooplankton abundance and diversity, particularly among calcifying species, which affects food chains and coastal ecosystem dynamics (Hall-Spencer & Harvey 2019).

METHODOLOGY FOR STUDYING THE CLIMATE CHANGE IMPACT ON ZOOPLANKTON

Experimental Approach

Laboratory Experiments and Mesocosms

Laboratory experiments and mesocosm studies are crucial for elucidating the effects of climate change on zooplankton. In controlled laboratory settings, researchers can precisely manipulate environmental variables, such as temperature, pH, and salinity, to isolate their impacts on zooplankton physiology and behaviour (Choi et al. 2021). Evidence indicates that elevated temperatures can accelerate the metabolic rates of zooplankton, subsequently influencing their developmental and reproductive processes (Richardson 2008). Furthermore, molecular techniques, including DNA and RNA analyses, facilitate the investigation of the physiological and genetic responses of zooplankton to environmental stressors, as demonstrated by Voznesensky et al. (2004). Mesocosm experiments, which are conducted in semi-controlled environments that simulate natural conditions, provide insights into the interactions between zooplankton and other components of aquatic ecosystems (Sharma et al. 2021). In mesocosm studies, zooplankton are housed in large tanks containing seawater, phytoplankton, and natural predators, enabling researchers to examine predator-prey dynamics and alterations in food webs

due to environmental changes (Boyd et al. 2018). These studies are also instrumental in assessing the long-term impacts of environmental stress on zooplankton populations (Algueró-Muñiz et al. 2017). Despite their value, laboratory and mesocosm experiments have limitations. The controlled conditions of these experiments may not fully capture the complexity and variability inherent in natural marine environments (Boyd et al. 2018). Consequently, integrating findings from these experimental approaches with field data is essential for achieving a comprehensive understanding of zooplankton responses to climate change (Heneghan et al. 2023).

Field Studies and In-Situ Observations

Field studies and in situ observations are essential for investigating the impacts of climate change on zooplankton. Researchers employ a range of tools, including plankton nets and automated sensors, to collect data on zooplankton distribution, abundance, and activity across diverse marine environments (Figures 4 and 5). Specifically, CPR has played a critical role in tracking long-term shifts in North Atlantic zooplankton communities (Richardson et al. 2006). In situ observations facilitate the direct measurement of environmental parameters such as temperature, pH, and oxygen, which are subsequently correlated with zooplankton responses to identify their preferred habitats (Mackas & Beaugrand 2010). Field studies also provide insights into how zooplankton populations react to seasonal and interannual variations, as well as extreme events such as ocean heatwaves. Research conducted in the subtropical waters of Brazil, for instance, revealed notable variations in zooplankton biomass associated with cold-water intrusions (Marcolin et al. 2015). Similarly, Xu et al. (2024) investigated the effects of marine heatwaves on zooplankton in the East China Sea using both in situ data and reanalysis techniques. Advanced technologies, including Autonomous Underwater Vehicles (AUVs) and Remotely Operated Vehicles (ROVs), have enhanced the capacity for observing zooplankton in challenging environments such as the deep ocean and polar regions. These innovations address the limitations of traditional methods and provide more detailed ecological insights (Wiebe & Benfield 2003).

Modelling and Predictive Tools

Using Climate Models to Project Future Impacts

Climate models are essential tools for forecasting the impacts of climate change on zooplankton populations. These models utilise historical data and future climate scenarios to simulate the effects of changes in temperature, pH, and other environmental variables on zooplankton distribution and abundance (Stock et al. 2014). Distinctly, biogeochemical models integrate data on biological and chemical processes within the ocean to project how increased atmospheric CO_2 and ocean acidification may influence marine food webs (Shi & Li 2024). By employing climate models, researchers can identify regions most susceptible to climate-induced changes and develop targeted mitigation strategies (Cheung et al. 2011). An illustrative case is provided by models that simulate ocean warming effects in the Bering Sea, which predict potential shifts in zooplankton distribution that could impact commercial fish populations (Whitehouse et al. 2021). Such predictions are crucial for marine resource management and ecosystem conservation (Stock et al. 2014). However, climate models have inherent limitations due to the complexity of marine ecosystems and uncertainties in future climate projections (Boyd et al. 2018). Enhancing model accuracy requires the continuous integration of empirical data and the advancement of modelling techniques (Cheung et al. 2011). Integrating climate models with field data is anticipated to yield more reliable and precise predictions.

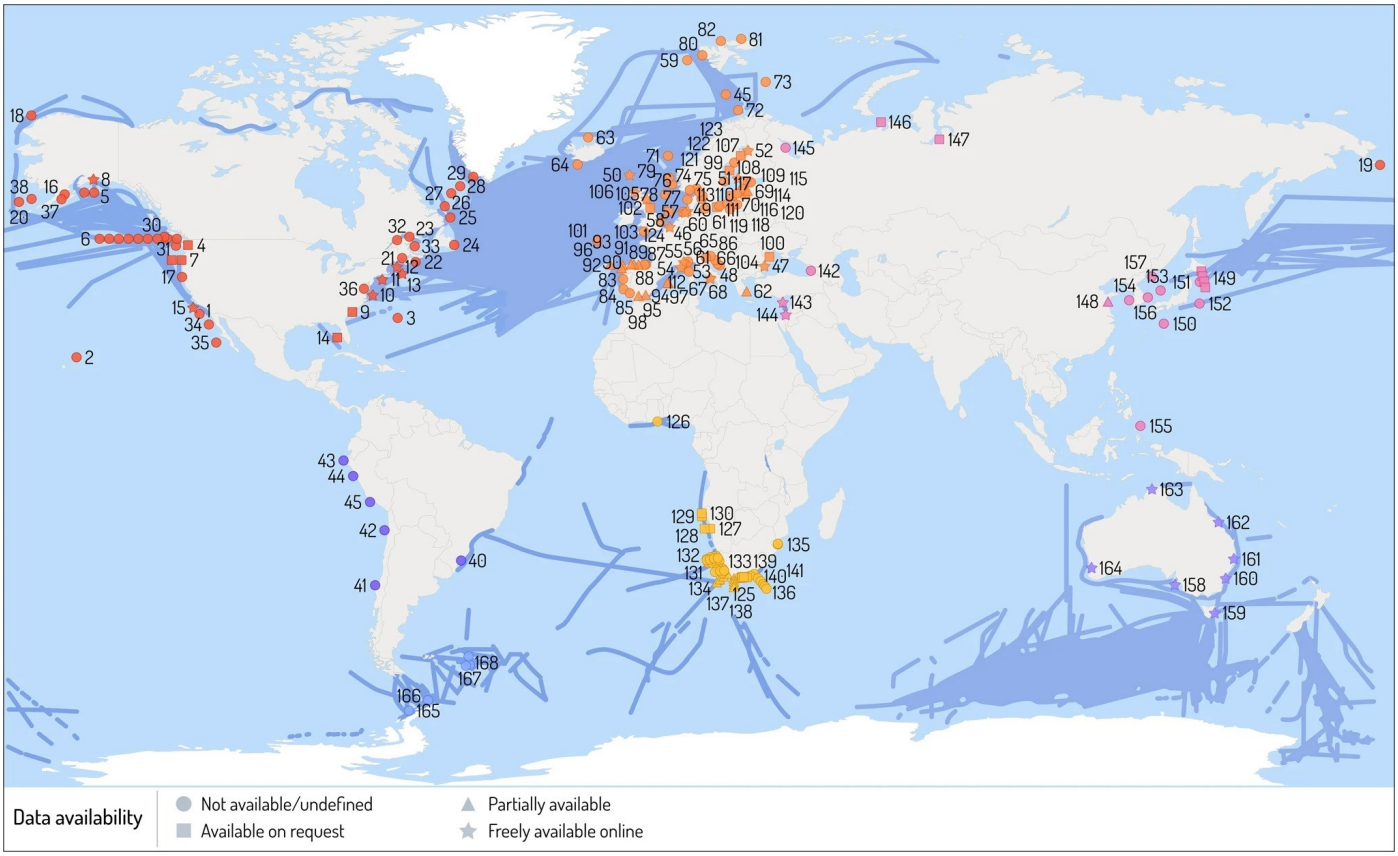

Figure 4. Map of long-term monitoring programs for zooplankton in the global ocean. The blue line denotes the trajectory of CPR survey, while the symbols represent the locations of specific long-term monitoring programs, with numbered locations detailed according to Ratnarajah et al. (2023). Star symbol: Indicates programs where data is available for free download. Box symbol: Represents programs with data accessible on demand. Triangle symbol: Denotes programs with partially available data. Circle symbol: Signifies programs where data is either not available or its availability is unclear. Only programs with documented coordinates are included on the map. Data sources include the Marine Ecological Time Series Database, the EuroSea survey, and additional surveys referenced in Ratnarajah et al. (2023). Detailed information and coordinates are provided in the supplementary materials of Ratnarajah et al. (2023). The map was designed by Dr. Stacey McCormack of Visual Knowledge. (Source: Ratnarajah et al. 2023).

Integration of Empirical Data into Predictive Frameworks and Artificial Intelligence (AI)

Integrating empirical data into predictive frameworks significantly enhances the accuracy of climate models. Millette et al. (2024) underscored the importance of incorporating empirical data from both laboratory and field studies within trait-based approaches to predict zooplankton ecology. This methodology involves synthesising in situ measurements and controlled experiments to ascertain zooplankton traits, which are subsequently utilised in ecosystem models to forecast their biogeographic distribution and biogeochemical impacts. Moreover, incorporating data on zooplankton growth and reproductive rates under diverse conditions further refines model predictions (Reygondeau & Beaugrand 2011). The integration of genetic, physiological, and ecological data facilitates deeper understanding of zooplankton responses to environmental changes (Stock et al. 2014), including insights into evolutionary adaptations (Bucklin et al. 2018) and thermal tolerance limits (Alma et al. 2020). This multidisciplinary approach contributes to the development of more comprehensive models (Reusch & Boyd 2013), which are crucial for effective marine resource management (Cheung et al. 2011). Additionally, Jain et al. (2023) highlight the potential of artificial intelligence (AI) in climate change adaptation, demonstrating how AI can leverage extensive data sources to inform decision-making. However, ethical considerations must en-

sure that AI solutions are transparent and equitable. Advancements in AI-driven climate adaptation strategies have the potential to foster a more resilient and equitable future. Anticipated research advancements are expected to further enhance the understanding of zooplankton dynamics in the context of climate change.

Technological Advancements

Remote Sensing and Automated Sampling Technology

Advancements in automated sampling and remote sensing technologies, including satellites and sea surface sensors, facilitate large-scale and continuous monitoring of maritime environmental conditions (Ma et al. 2023). Environmental sensors deployed on AUVs and the SilCam imaging tool enable sustained spatial and temporal observations, thereby enhancing our comprehension of marine ecosystems and zooplankton responses to climate change (Nøland 2022). Although these techniques necessitate further refinement in taxonomic resolution and the integration of data from diverse sensors, they offer considerable promise for advancing plankton research and climate change monitoring (Wiebe & Benfield 2003). Moreover, these technologies are particularly effective in monitoring remote locations, such as the deep ocean and polar regions, which are critical for understanding global marine ecosystem dynamics (Robison 2004).

Advances in Genetic and Molecular Engineering

Advances in genetic and molecular techniques have markedly improved our capacity to investigate the effects of climate change on zooplankton. Techniques such as DNA and RNA sequencing, particularly Next-Generation Sequencing (NGS), enable researchers to identify genetic modifications and gene expression changes in response to environmental stressors (Satam et al. 2023). Research indicates that zooplankton can modulate gene expression related to metabolism, reproduction, and stress responses under fluctuating environmental conditions (DeBiasse & Kelly 2016). Genetic analyses can uncover patterns of genetic variation and potential natural adaptations across different zooplankton populations (Reusch & Boyd 2013). Additionally, molecular techniques facilitate the examination of interactions between zooplankton and microorganisms, offering insights into the health and functionality of marine ecosystems (Suttle 2005). Techniques such as RNAlater® preservation enable the collection and analysis of RNA in both experimental and field settings, providing valuable information about the physiological state of zooplankton populations (Voznesensky et al. 2004) (Figure 5).

The application of DNA metabarcoding has significantly advanced the study of marine zooplankton diversity by utilising marker gene regions such as COI and 18S nuclear rRNA (Yang et al. 2017; Bucklin et al. 2019; Blanco-Bercial 2020). Databases such as the MetaZooGene Barcode Atlas and Database (MZGdb) offer comprehensive reference sequences for marine zooplankton, thereby facilitating the identification and assessment of biodiversity (Bucklin et al. 2021). MZGdb is interconnected with NCBI GenBank and BOLD data repositories, enhancing quality control, statistical analysis, and the visualisation of genetic data. Through the MZGdb portal, over 150,000 COI sequences for approximately 5,600 documented marine metazoan plankton species—including holoplankton and meroplankton—are readily accessible.

Interestingly, Bucklin et al. (2021) employed MZGdb as a repository for COI barcode data summaries and related information for specific regions, including the North Atlantic, Arctic, North Pacific, and Southern Ocean, as well as for major taxonomic families of marine zooplankton. To assess marine ecosystems and facilitate the rapid identification of climate change impacts,

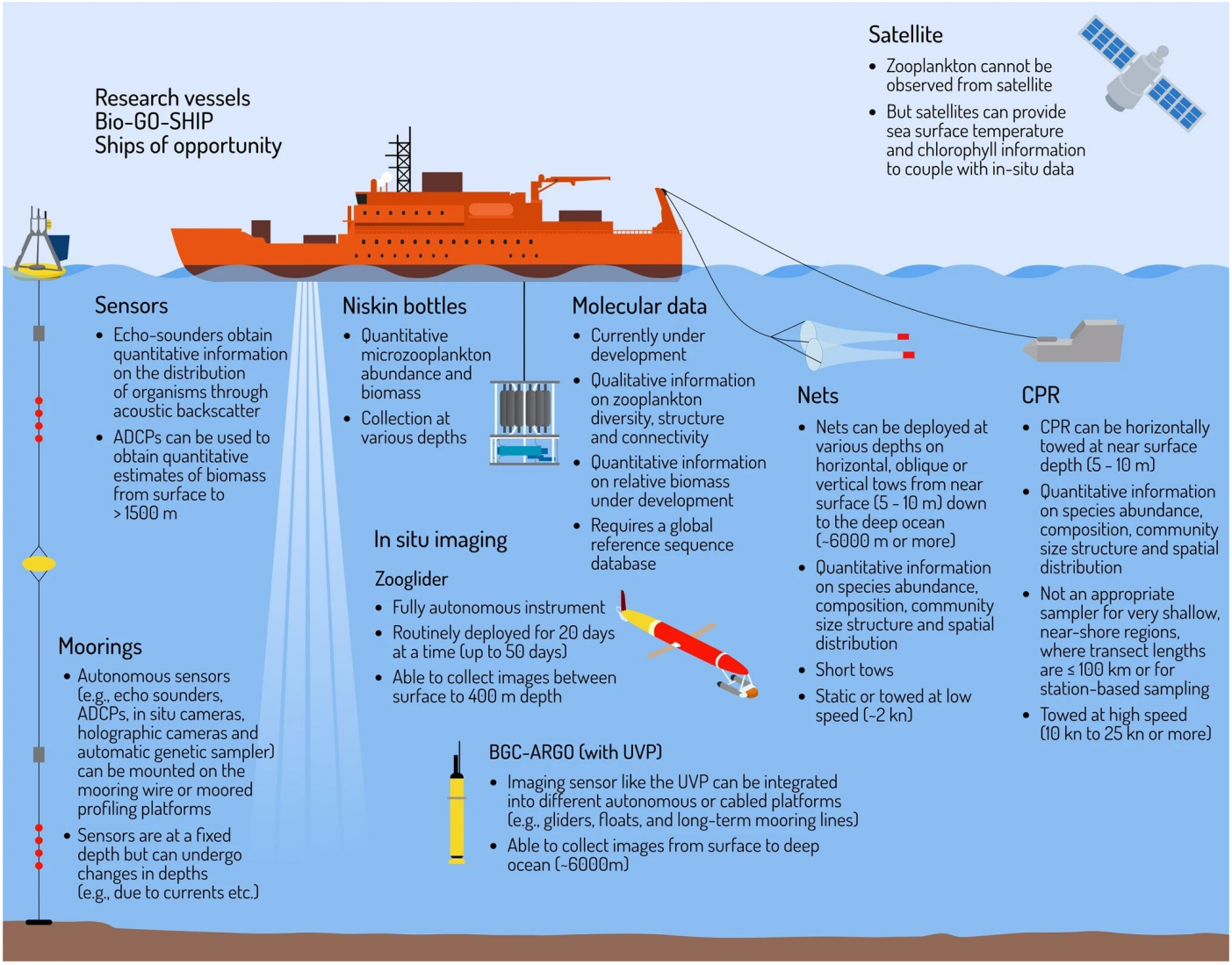

Figure 5. This illustration demonstrates the integration of traditional and modern methodologies in zooplankton studies. Conventional techniques, such as plankton nets, Niskin bottles, and the CPR, have been long-standing tools for zooplankton monitoring. However, the combination of these established methods with contemporary approaches—such as satellite observations, advanced sensors, in situ imaging techniques, and molecular analyses (including proteins, DNA, and RNA)—can significantly enhance geographic coverage, particularly in undersampled regions, and provide more profound insights into the effects of climate change on zooplankton communities. While traditional methods like nets, CPR, and Niskin bottles are typically employed independently, their integration with modern technologies offers more comprehensive understanding of zooplankton dynamics. This integration allows for a broader spatial and temporal resolution, improving our ability to monitor and analyze zooplankton populations effectively. The image illustrating this integration was created by Dr. Stacey McCormack (Visual Knowledge) and is sourced from Ratnarajah et al. (2023).

MZGdb is designed to serve as a foundational resource for studying the diversity of marine zooplankton species through DNA barcodes and metabarcoding. The integration of metabarcoding with morphological analyses enables effective characterisation of zooplankton community structure and biomass, offering essential tools for the research, assessment, and management of marine biodiversity (Matthews et al. 2021). These genetic and molecular advancements are crucial for developing strategies to understand and mitigate the effects of climate change on marine ecosystems.

In their work, "Call to Action: A COI Reference Library for Marine Zooplankton," Bucklin et al. (2021) issued a critical call for the development of a comprehensive COI reference sequence database for marine zooplankton. This database is essential for the widespread implementation of DNA barcoding and metabarcoding techniques, which are fundamental for effective fisheries management, environmental conservation, and assessing climate change

impacts on marine ecosystems. The diversity of marine environments and the taxonomic complexity of zooplankton pose significant challenges to accurate species identification. To address these challenges, it is imperative to provide public access to accurately identified DNA barcode sequence records and tools for constructing custom databases tailored to specific taxonomic groups and geographic regions. Such resources are crucial for maintaining quality control and detecting errors, thereby enhancing species-level diversity analyses for management, monitoring, and research purposes. Prioritising the completion of a global, taxonomically comprehensive zooplankton COI reference database is vital. Key priorities include focusing on ecologically significant species, ensuring high accuracy in species-level identification, georeferencing collection sites, and analysing intra-specific variations in COI barcodes to identify errors and detect cryptic species.

KNOWLEDGE GAPS AND FUTURE RESEARCH DIRECTIONS

Gaps Identified in Current Research

Areas with Limited Data and Understanding

Despite extensive research on climate change's impact on zooplankton, significant gaps remain in understanding their responses to multi-stressor variations in complex natural environments (Boyd et al. 2018). Current data often focus on individual stressors such as rising temperatures or acidification, but information on combined effects is lacking (Todgham & Stillman 2013). Additionally, there is limited knowledge about the genetic adaptation and evolution of zooplankton in response to environmental changes (Bucklin et al. 2021). Geographic variation in zooplankton responses is also under-studied, with most research concentrated in accessible regions like the North Atlantic and Eastern Pacific, while remote areas like the Indian Ocean and the Southern Ocean are poorly studied (Richardson et al. 2006). Understanding zooplankton responses in diverse habitats is crucial for predicting global climate change impacts (Morgado & Vieira 2020). Furthermore, much research is conducted in controlled laboratory settings, leading to a lack of understanding of zooplankton responses in dynamic natural ecosystems (Pörtner & Farrell 2008). Comprehensive field studies and long-term data are needed to fill this gap and provide a more accurate picture of zooplankton's response to climate change (Ducklow et al. 2007).

The Need for Long-Term Monitoring and Studies

Long-term monitoring and studies are essential for understanding trends and changes in zooplankton populations due to climate change (Ducklow et al. 2007). These studies enable scientists to identify temporal patterns and correlate changes in zooplankton with environmental variables such as ocean temperature, pH, and food availability (Zhou et al. 2020). They are also crucial for assessing the cumulative effects of various environmental stressors on zooplankton (Mackas & Beaugrand 2010). However, such monitoring demands substantial resources and coordination among countries and research institutions (Ducklow et al. 2007). New technologies like remote sensing and automated sensors can enhance monitoring efficiency and coverage (Behrenfeld & Falkowski 1997). Expanding monitoring efforts in underrepresented regions such as the tropics, poles, and deep seas is also vital for a comprehensive understanding of climate change impacts on zooplankton (Richardson et al. 2006). A collaborative approach and advanced technologies are key to addressing these challenges.

Future Research Advice

Future research on zooplankton should prioritise multi-stressor experiments to elucidate the complex interactions between various environmental factors,

such as elevated temperatures, ocean acidification, hypoxia, and pollution, which more accurately reflect realistic conditions (Todgham & Stillman 2013). This approach will enhance our understanding of how zooplankton adapt and survive under multifactorial stress, considering variations in stress responses across different species and life stages (Pörtner & Farrell 2008). Additionally, these studies could reveal broader ecosystem impacts, including alterations in predator-prey dynamics (Boyd et al. 2018). To capture comprehensive trends, it is essential to conduct these experiments across diverse temporal and spatial scales, supported by long-term studies and extensive field observations beyond short-term experiments (Ducklow et al. 2007). Concurrently, integrating genetic, physiological, and ecological data will deepen our understanding of zooplankton responses to environmental changes. This integrative approach can link genetic variation with physiological and ecological responses, can identify stress tolerance genes, and can develop accurate models for predicting climate change impacts on zooplankton (Sunday et al. 2014). Advanced technologies, such as genomic and metagenomic analyses, will further elucidate evolutionary adaptations and physiological plasticity, aiding in the development of effective climate adaptation strategies (Weydmann et al. 2017). Moreover, the development of comprehensive models that incorporate interactions between environmental conditions and zooplankton responses is crucial for forecasting climate change impacts. These models should integrate field observations, laboratory experiments, and advanced technologies like remote sensing, accounting for temporal and spatial variability and interactions with other marine organisms (Stock et al. 2014). Continuous evaluation and validation through field data and international collaboration among research institutions will enhance the accuracy and reliability of these models, providing critical insights into marine ecosystem dynamics and climate change challenges (Boyd et al. 2018). To emphasize, the development of research models as outlined above is crucial for understanding the complexities of zooplankton responses to environmental changes, as these models can provide insights into effective conservation strategies aimed at mitigating the impacts of climate change on marine ecosystems (Orr et al. 2005; Pörtner & Farrell 2008). Incorporating these models into conservation planning will enable targeted interventions that enhance the resilience of zooplankton populations and, consequently, the broader marine food web (Hoffmann & Sgrò 2011).

CONCLUSION

Climate change profoundly impacts zooplankton biodiversity and ecosystem functioning through key stressors such as rising ocean temperatures, acidification, and hypoxia. These stressors affect zooplankton physiology, behaviour, and distribution, leading to decreased survival and reproduction for some species, particularly those with calcium carbonate structures like pteropods, whose shell formation is impaired by acidification. Changes in zooplankton populations, in turn, influence their predators, including commercial fish, marine mammals, and seabirds that rely on zooplankton as a primary food source. Variability in responses to climate change is observed across species, locations, and ecosystem types. Polar zooplankton face challenges from melting sea ice and warming temperatures, while tropical and temperate species may shift to higher latitudes or greater depths. To address these challenges, future research should focus on multi-stressor experiments and improved integration of data across genetic, physiological, and ecological dimensions to better predict and mitigate impacts on marine ecosystems. Extensive long-term monitoring programs are essential for tracking shifts in zooplankton populations and their responses to environmental changes, particularly in underrepresented regions such as the deep ocean and polar areas.

Research should also explore zooplankton interactions with microorganisms and their roles in nutrient cycles and ecosystem health. Developing comprehensive models that incorporate various environmental conditions and biological responses, supported by international and interdisciplinary collaboration, will enhance our ability to forecast climate impacts. Mitigation efforts, including greenhouse gas reduction and habitat protection, are crucial for easing pressure on marine ecosystems and improving their resilience. Addressing these issues requires raising awareness among the public and policymakers about the importance of zooplankton for marine ecosystem health and fisheries economies to drive effective conservation and research initiatives.

AUTHORS CONTRIBUTION

All authors contributed to the formulation and design of this study. A.P. served as the lead researcher in this study. A.P. and S.A. were also responsible for the investigation and writing of the research, including conducting information and literature searches, conceptualizing the study, drafting the initial manuscript, and performing subsequent review and editing. H.H.1, C.M., L.A.J.Q., M.R., H.H.2, M.A., R.A.S., and T.T. handled the information and literature explorations, data curation, review, editing, and financial aspects of the research. All authors read and approved the final version of the manuscript and participated in its writing and revision.

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CONFLICT OF INTEREST

The authors declare that there are no financial conflicts of interest or personal relationships that could be perceived as influencing the content or findings of this paper.

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