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Review Article

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Short Communications

Seed Conservation of *Anaxagorea luzonensis* A. Gray (Annonaceae) Through Storage Behaviour and Morphology

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ABSTRACT
Fruits and seeds morphological traits and seed storage behaviour of *Anaxagorea luzonensis* are important for seed and plant conservation. Fruits and seeds characterization using a digital microscope and morphometry method. Seed storage behaviour was determined using 100-seed test method. The findings revealed that the colour of *A. luzonensis* fruit did not change and *A. luzonensis* seed was glossy, brittle, black in colour, 9.05 x 5.89 x 2.93 mm sized and oval. The seeds are desiccation-tolerant and are categorized as orthodox seeds. These results can become the findings of *A. luzonensis* seed storage behaviour, because information about it never existed before. Knowing the seed storage behaviour and seed morphology of *A. luzonensis* is essential so that the recommendation of conservation efforts at PBG in the future are covering the fruit, modifying the environment, breaking dormant seeds, vegetative propagation, and preserving seeds.

Anaxagorea is one of the genus from Annonaceae family (the soursop group), which has 43 species that are generally found in Indo-China, Borneo, Java, Sumatra, Philippines, Thailand, Burma, Ceylon, and tropical America (Husain et al. 2012). *Anaxagorea luzonensis* A. Gray is one of the plant collections from Annonaceae family where conserved in Purwodadi Botanic Garden (PBG), East Java, and become a new plant collection since 2014 (Lestari 2014). This species has shrub habitus, brown cylindrical stems with single and alternate leaves. Based on the information from PBG Registration Unit (2022), there is one specimen of *A. luzonensis* in PBG and categorized as critical plant collection. According to the IUCN Red List, *A. luzonensis* is categorized as Least Concern (LC) for conservation status (Verspagen & Erkens 2020). Barks of *A. luzonensis* have been used as blood tonic, stomachic, antipyretic, and treat of muscle pain. Stems of this species also contain xanthones and flavonoids, both of which have antioxidant properties (Gonda et al. 2000). The leaves can be used as medicine to treat articular rheumatism and can be used for postpartum care (Aziz et al. 2016).

Considering its conservation status and the utilization of *A. lu-*

zonensis, ex-situ conservation activities are required through a study of seed morphology and storage behaviour which is included in reproductive biology research. Reproductive biology research can be used to propagate plants for conservation purposes. Monitoring and research about reproductive traits of living collection in botanic garden is one of the factors that encourage prolific breeding and long-term maintenance. Fruit, seed, and germination traits are connected to plant reproduction which are crucial for plant dispersal (Xu & Zang 2023). Fruit and seed characteristics may be used as a potential tool to forecast dispersal mechanisms for conservation and restoration of biodiversity. In addition, other plant characteristics such as size of fruits and seeds, might influence seed dispersal. Size variation can have an impact on dispersal distance, because small seeds are often dispersed farther by water or wind but large seeds are dispersed by animals (Leslie et al. 2017). de Jager et al. (2019) reveal that in warm lowlands, large seeds generally spread more widely than small seeds when carried by flowing water. Besides that, large seeds that come from open-growth environments will be more adaptable than small seeds (Smith et al. 2022). Small seeds are preferred by rodents as dispersal agent in the zoochory mechanism (Lang & Wang 2016). Seed dispersal techniques are positively associated with size, mass, and form of seed (Liu et al. 2014). So far, research or study about fruit and seed characteristics, seed storage character and germination of *A. luzonensis* has never been reported, especially in PBG.

Proper seed storage is essential and will be able to protect germplasm in the form of seeds. The seeds storage condition also relies on several variables, including oxygen pressure, moisture content, storage temperature, and storage period. However, the most important factor when storing seeds is the tolerance level of seed towards to desiccation or drying. This factor aims to know the seed storage character. Based on the drying tolerance, seeds are categorized into three groups, i.e., orthodox seeds, recalcitrant seeds, and intermediate seeds. Orthodox seeds are tolerant-drying seeds, can be stored for a long time at -10°C and can endure drying to a moisture content below 7%. In contrast to orthodox seeds, recalcitrant seeds are extremely sensitive to drying and freezing because they can quickly lose their viability when kept in conventional method. Metabolic functions of recalcitrant seeds will be active during fruit development until harvesting. But intermediate seeds lose their viability rather quickly than orthodox seeds (Kijak & Ratajczak 2020). Recalcitrant seeds do not experience water content loss during the ripening process, so that its very sensitive to the seed desiccation. Therefore, the distribution of recalcitrant seeds is limited to the tropical forests. The differences of desiccation tolerance in orthodox, intermediate and recalcitrant seeds are crucial for long-term seed storage, biodiversity protection, and seed conservation (Smolikova et al. 2021).

Aims of this study are to know the morphological traits of *A. luzonensis* fruit and seeds, to determine the storage behaviour of *A. luzonensis* seeds, and to recommend the conservation efforts in PBG. This is required to ensure the long-term viability of seeds and plant collections in PBG. The findings of this study are recommended as a fundamental guide for managing plant collection, especially for *A. luzonensis*, in supporting seed and plant conservation at PBG.

Fruit of *A. luzonensis* was harvested from plant collection in block XVIII.E.26 at PBG, East Java (Figure 1). Fruit of *A. luzonensis* is categorized as dehiscent fruit, when fruits that open up at maturity in order to release their content (Figure 2). This is because *A. luzonensis* seeds are dispersed by explosion (ballistically), at a maximum distance of 5-7 meters from the mother plant (Gottsberger 2016).

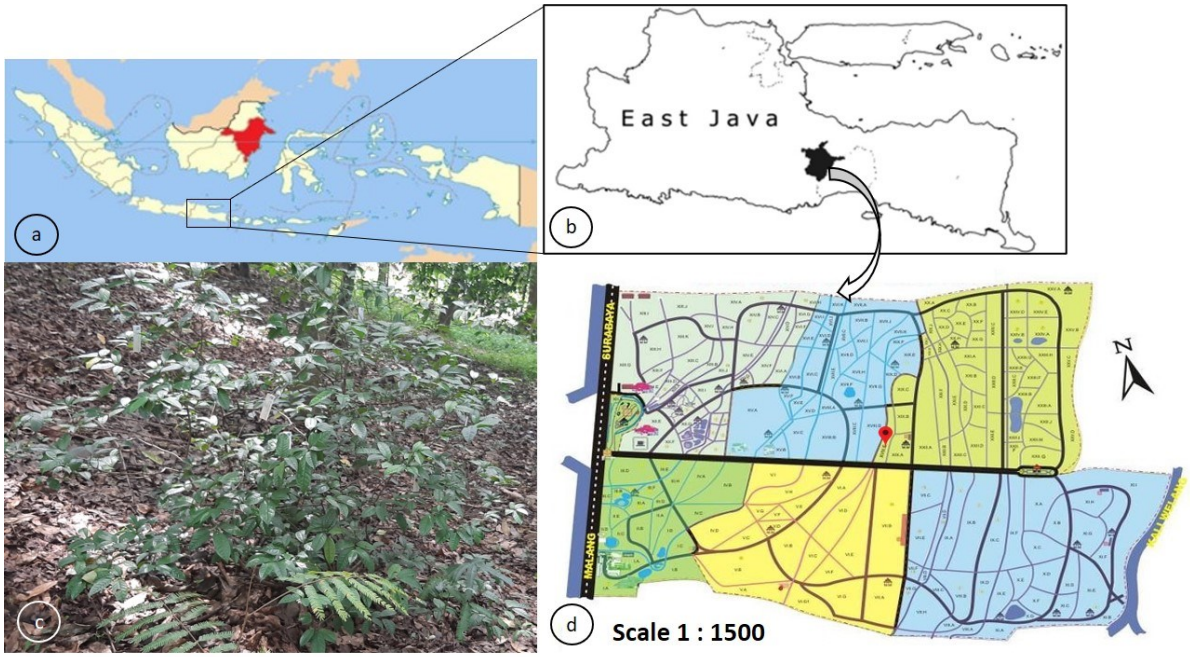

Figure 1. Study site of *Anaxagorea luzonensis* in Purwodadi Botanic Garden; a. map of Indonesia, b. map of East Java, c. habitus of *Anaxagorea luzonensis* in block XVIII.E.26, and d. map of Purwodadi Botanic Garden (location symbol).

Figure 2. Peels of *Anaxagorea luzonensis* fruit at maturity and ready to harvest.

Morphological characterization of *A. luzonensis* fruits and seeds was done in laboratory of PBG in March 2022. The morphological characteristics of fifty fruits were determined, including size (length, width, and thickness of fruit), colour, texture and shape of fruit, and the quantity of seeds in each fruit. Seed extraction is done manually with hand, by separating seeds from peel and allowing them to air-dry at ambient temperature. According to Lestari (2013), 100 seeds are taken randomly and characterized using morphometry method. The morphological characteristics were observed include size (length, width, and thickness), colour, texture, shape, and condition of the seed being observed (the whole or part of the seed). The morphology of internal seeds was examined using the Dino lite AM-311 digital microscope, by cutting cross-sections of the seeds using scalpel. The detail of internal seed was described in the figure from seed coat to the deepest part of *A. luzonensis*.

Seed storage behaviour study of *A. luzonensis* was done in laboratory and greenhouse in PBG from March until October 2022. The defined *A. luzonensis* seeds were sown using the 100-seed test method to determine seed storage behaviour (Pritchard et al. 2004). A total of 100 seeds

are divided into four categories, i.e., 10 seeds for initial moisture content, 26 seeds (13 seeds per replication) for initial germination, 32 seeds for desiccation with silica gel (6 seeds for moisture content after desiccation and 26 seeds for germination after desiccation), and 32 seeds for controlled humidity storage treatment. Desiccated *A. luzonensis* seeds were packed in a calico bag and placed in a desiccator with silica gel, with the weight of the silica gel being equal to the weight of the seeds to be inserted into the desiccator. The seeds were weighed once every three days, and the process was ended when the weight was steady. The *A. luzonensis* seeds, on the other hand, were enclosed in black fabric bags and placed in a desiccator filled with vermiculite with storage treatment in controlled humidity. The percentage of humidity is kept at 8-10%. The desiccator was opened once every three days to maintain air aeration in the desiccator. After assessing the moisture content of the seeds destructively using the oven method at 108 °C for 18 hours, *A. luzonensis* seeds for initial germination were sowed (ISTA 2015) (Formula 1). *A. luzonensis* seeds with desiccation and controlled humidity treatment are sowed simultaneously whereas after weight of seeds in desiccation treatment are stable. Seeds were sown in straw paper medium. Straw paper is the medium testing for seed germination. When the paper straw medium appears dry during germination period, the seeds are watered with aquadest. The germination parameters observed include the initial seed count for germination and the total number of seeds germinating for each treatment. Calculation of seed germination percentage using (Sutopo 2010) (Formula 2). All data is processed and analysed using Microsoft Excel 2017 with mean and standard deviation analysis. The resultant curve from seed germination result is adjusted the graph according to (Pritchard et al. 2004).

$$seed\ water\ content\ (\%) = \frac{M2 - M3}{M2 - M1} x\ 100\%$$ ..... Formula 1

Where M1 = the weight of petridish, M2 = the weight of seed and petridish before the oven and M3 = the weight of seed and petridish after the oven

$$germination\ percentage = \frac{\text{number of germinated seeds}}{\text{number of seeds sown}}\ x\ 100\%$$ .... Formula 2

Plant collection of an *A. luzonensis* in PBG produce fruit twice a year in 2022, at the beginning of the year (February–March) and at the end of the year (August–September), resulting in a twice-yearly fruiting period. This is consistent with Lestari (2019) who reported that *A. luzonensis* fruiting twice a year throughout the year in a sub-annual cycle. A pattern in the phenological period of blooming and fruiting that happens more than once a year is known as a sub-annual pattern (Newstrom et al. 1994; Barbosa et al. 2018). When *A. luzonensis* reaches fruiting stage, the colour of fruit does not change until maturity (dehiscent fruit) and the fruit is yellowish-green in colour (Table 1; Figure 3a). Seed of *A. luzonensis* is forced out by the fruit through a ballistic process, ripe fruit is distinguished by the shattering of peel. Seed morphology of *A. luzonensis* is characterized by seed coat colour is shiny black, ovate, which has mean seed size of 9.05 x 5.89 x 2.93 mm (Table 1; Figure 3b). According to Lestari (2014), ripe fruit of *A. luzonensis* is dark green and ranges in size from 2.5 to 4.4 cm, seeds are shiny black with a little white hilum at the tip of the seed. Scharaschkin and Doyle (2006) claimed that seeds from the *Anaxagorea* group have two seeds linked by having seeds that are

Table 1. Morphological character of *Anaxagorea luzonensis* fruits and seeds.

Morphological character	Fruits	Seeds
Quantitative character		
Length (mm)	22.44 ± 2.76	9.05 ± 0.52
Width (mm)	7.27 ± 0.66	5.89 ± 0.41
Thickness (mm)	6.83 ± 0.75	2.93 ± 0.51
Sum of seeds in one fruit	1.72 ± 0.45	-
Qualitative character		
Colour	green yellowish	black
Surface	glabrous	glabrous, shiny
Shape	spatulate	ovate
Condition	rind is broken	a flat surface facing one other in one fruit

Figure 3. Fruits and seeds of *Anaxagorea luzonensis*; a. ripe fruit, b. seeds, and c. seed cross section with Dinolite (scale bar = 50 mm).

asymmetrical and have a flat surface facing one other in one fruit. As with plants species from *Hura crepitans* or *Hevea* spp., which feature an active burst mechanism in which the fruit will release its seeds at a substantial distance from the parent plant, *A. luzonensis* seeds are categorized as an active autochory dispersal type (Parolin et al. 2013).

Cross-sectional images of *A. luzonensis* seeds were taken with Dinolite digital microscope revealed ruminate endosperm, which is a feature of seeds from the Annonaceae family (Figure 3c-ii). Ruminate endosperm is a distinguishing feature of Annonaceae seeds and can be used to identify them (Gottsberger 2016; Johnson & Murray 2018; Lestari & Pratiwi 2022). The seed coat is fragile, thin and shiny black colour (Figure 3c-i), and an embryo and a microphyllar plug are also present (Figure 3c-iv). An embryo of *A. luzonensis* has white colour (Figure 3c-iii).

The amount of water in a seed that determines its viability and ability to be stored for a certain period is known as seed water content. Initial water content of *A. luzonensis* seeds is 7.91% and decline to 5.65% after desiccation treatment. However, after being stored in controlled humidity treatment, seed water content of *A. luzonensis* seeds increased to 8.88% (Table 2). This happens as a result of the seeds absorbing water from the substrate. The water content of *Mauritia flexuosa* seeds reduced by up to 25% after desiccation (de Almeida et al. 2018). The amount of *Coccoloba gigantifolia* seeds decreased after 6 days of desiccation (Ferreira et al. 2021). Seed water content value declined and will be followed by a decrease in seed germination percentage, both seeds were treated with desiccation or at controlled humidity up to range of 38-46% (Table 2). When seeds are dried in a desiccator using silica gel, their water content will decline, and therefore their chance of germinating will decrease (Asomaning & Sacande 2019).

Table 2. Water content and germination percentage of *Anaxagorea luzonensis* seeds based on 100-seed test method.

Treatment	Seed water content (%)	Seed germination percentage (%)
Initial	7.91 ± 0.08	50 ± 5.44
Desiccation	5.65 ± 0.29	12 ± 5.40
Storage with controlled	8.88 ± 0.53	3.85 ± 5.44

The 100-seed test method can be used as an initial screening method for seeds storage behaviour or characteristics of various species of seeds. It is used to determine the seed storage characteristics with a small number of seed lots i.e., minimum of 100 seeds (Pritchard et al. 2004; Wardani & Mimin 2020). Figure 4 demonstrates that *A. luzonensis* seeds are categorized as orthodox seeds. This is demonstrated by the curve of the percentage of germination during desiccation treatment, which can still germinate because the seeds are desiccation-tolerant. The seed is categorized as a recalcitrant seed if the desiccation treatment's germination curve is unable to demonstrate germination. Seed storage behaviour of *A. luzonensis* seeds is likewise not accessible, according to Seed Information Database from Royal Botanic Gardens, Kew.

After being desiccated and stored in controlled humidity treatment, the seed germination of *A. luzonensis* seeds decreased. The percentage of seeds that germinate will decline when kept in vermiculite at ambient temperature with controlled humidity (Yuan et al. 2019). González-Morales et al. (2016) reported that following desiccation and storage for a week, seeds will lose their viability and decline by at least 5%.

Based on Figure 4, initial germination of *A. luzonensis* seed occurs in 8 weeks after sown. This shows that this seed has slow germination rate and has physical dormancy. Germination can be accelerated through seed breaking dormancy with physical dormancy treatment using physical or mechanical scarification and immersion in the chemical solution. Seed germination of *A. luzonensis* categorized as–epigeal type germination (Figure 5), the cotyledons are raised above the soil surface for the first time during germination. Some species from Annonaceae family have epigeal germination type, such as *Annona montana*, *Artabotrys hexapetalus*, *Cananga odorata*, *Fissistigma latifolium*, *Uvaria purpurea*, *U. micranthum* etc. (Handayani 2019; Pratiwi et al. 2022).

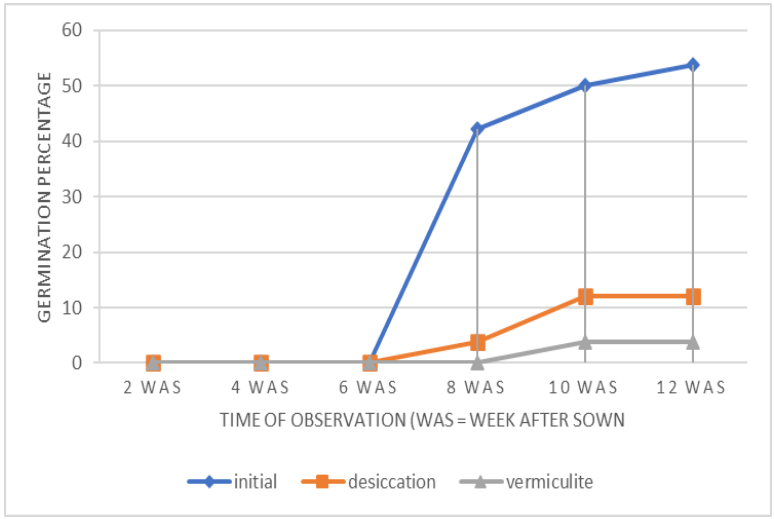

Figure 4. Curve of seed germination percentage of *Anaxagorea luzonensis* with 100-seed test method.

Figure 5. Epigeal germination type of *Anaxagorea luzonensis*

Based on this study, some of conservation efforts can be carried out with point of view from seed dispersal mechanism, fruit and seed morphology, and seed storage behaviour. These conservation efforts can support plant collection management scientifically in PBG, especially for *A. luzonensis*. Some of conservation efforts that can be taken are covering the fruit, modifying area around *A. luzonensis* plant collection, further research about breaking dormant seeds, vegetative propagation, and preserving seeds for long-term conservation.

Fruit of *A. luzonensis* is covered when fruit is in immature phase. This endeavour was made because *A. luzonensis* seed has the ballistic (explosive) type in seed dispersal, so that seeds would be accommodated in the fruit cover if the fruit had been shattered and ripe. The mesh fruit cover is one of the options for use to cover fruit, which aims to improve plant growth in the form of fruit quality and to reduce pests and other environmental factors that could lead to fruit abortion before the fruit is dispersed (Setiawan 2018).

Besides covering the fruit, other conservation efforts that can be done are modifing an environmental area around plant collections through reducing the shade plants around *A. luzonensis* plant collection, fertilizing with growth hormones to promote flowers and fruits development, and pruning to rejuvenate plant collection thus stimulating the growth of shoots. Reducing amount of shade plants around *A. luzonensis* plant collection is required because the canopy cover around *A. luzonensis* is lush and require full sun to support plant growth and development.

Manure or organic fertilizers and growth regulators including auxins, gibberellins, and cytokinin, which can be sprayed on plants frequently, can all be used to fertilize plants. To improve the quality and quantity of flower and fruit production, natural growth regulator such as such as coconut water can also be employed as a natural plant hormone (Njoku & Okorie 2021). Fruits are abundant with high quality will produce more seeds, which can be saved for future use in seed conservation.

Considering the low seed production of *A. luzonensis* (<500 seeds per harvest) in PBG and the fact that there is only one plant specimen of *A. luzonensis* in PBG, it is necessary to propagate this plant through vegetative propagation. Several methods can be used to propagate are stem cuttings or tissue culture. Tissue culture will increase the number of seedlings that can be reproduced and ensure that they inherit their parents' genetic traits (Tefera 2019; Rather et al. 2022).

A. luzonensis seeds had very slow germination rate and required very long time to germinate. This suggests that further study of *A. luzonensis* seeds is required to break seed dormancy so that seeds can germinate more quickly and simultaneously. The findings of this study indicate that *A. luzonensis* seeds are orthodox seeds, which typically have dormant phase. Therefore, further research can be conducted in breaking seed dormancy research with a variety of scarification techniques such as mechanical, chemical, and physical treatment. Orthodox seeds can be scarified using a variety of techniques (Side et al. 2021), likewise with *A. luzonensis* seeds.

AUTHORS CONTRIBUTION

D.A.L. designed and conceptualization the research, conducted field observation, collected and analysed data, wrote and review the original manuscript. A.K.N.F. collected data, wrote and review the original manuscript.

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CONFLICT OF INTEREST

The authors state no conflict of interest from this manuscript.

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Short Communications

The First Report of the Occurrence of the Root Mealybug *Ripersiella multiporifera* Jansen (2008) (Hemiptera: Coccoidea: Rhizoecidae) in Indonesia

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ABSTRACT

Ripersiella multiporifera is a root mealybug species within the family Rhizoecidae that has distinctive bitubular pores on the dorsal and ventral. This species was first discovered by Jansen (2008) in the Netherlands during an import interception of *Sansevieria* sp. from Indonesia and *Hoya kerrii* from Thailand. This species was also found during inspections in Sicily (Italy) on the roots of *Sansevieria trifasciata*. In Indonesia, there are no reports of the existence of *R. multiporifera*, emphasising the need for research on its presence. This work was conducted the morphological method based on modified determination key and the molecular method based on MtCOI gene. The identified species was *R. multiporifera*, and this finding represents the first evidence of *R. multiporifera*'s presence in Bali (Indonesia) which can be used as a reference for future research, especially in population control approaches.

Mealybug is a species of fauna that has a propensity for inhabiting and proliferating inside soil environments, often establishing colonies on plant roots. Awareness regarding the presence of root mealybugs within plant roots remains limited due to their concealed habitat and the constraints imposed by existing research approaches. Root mealybugs are known to infest both monocot and dicot plants, causing various symptoms, including leaf wilting, alterations in leaf colour, inhibited flower growth, and even the death of the host plant when the infestation is severe (Jansen 1995).

In Indonesia, a total of 370 species of mealybugs have been identified, 30 species of which are recognized as root mealybugs that inhabit plant roots, including family of Rhizoecidae (sixteen species), Pseudococcidae (eight species), and Xenococcidae (six species). *R. multiporifera* is a root mealybug species from the Rhizoecidae family which was initially documented as a novel species (Jansen 2008). The first report of this species was the import interceptions of the Dutch Plant Protection Service on *Sansevieria* sp. from Indonesia and *Hoya kerrii* from Thailand. Other data pertaining to this species was also found during inspections in com-

mercial nurseries by the Phytosanitary Service in Sicily (Italy) on the roots of *Sansevieria trifasciata* Prain (Mazzeo et al. 2023). Before being reported by Jansen (2008), the existence of *R. multiporifera* in Indonesia had not been documented, including "The Pests of Crops in Indonesia" (Kalshoven 1981). The existence of the root mealybug *R. multiporifera* species in Indonesia appears highly probable. Nevertheless, no official reports have proven its presence in this country. Therefore, this work is really important to conduct as an initial report on the species and its relationship with mealybug from other countries.

This research was carried out from March to May 2023, starting with a purposive sampling method on the roots of *Adenium* sp. plants in Denpasar City, Bali Province, Indonesia. Regarding Indonesia's tropical climate, the development of the mealybug occurs throughout the year. However, it tends to be higher during the dry season than rainy season with an estimated eight to eleven generations during February to November and two to three generations from November to January (Mani & Shivaraju 2016). The selection of *Adenium* sp. as the host plant is based on personal communication in late 2022 with several farmers and enthusiasts of *Adenium* sp. plants in Denpasar regarding the occurrence of mealybug infestations on the plant roots. The purposive sampling method was employed by obtaining 20-25 female imago mealybugs using a brush on *Adenium* sp. that exhibited symptoms characterized by white wax filaments around the root plants. The female imago mealybug root specimen was taken to the Plant Quarantine Entomology and Biomolecular Laboratory of the Denpasar Class I Agricultural Quarantine Centre and then slide-mounted for morphological identification. Ten specimens were successfully slide-mounted and identified using an Olympus CX21 compound microscope at the Plant Pest and Disease Laboratory, Faculty of Agriculture, Udayana University. Subsequently, the morphological characteristics of the specimen were compared with the determination key based on Mazzeo et al. (2023), adapted from Marotta (1992), Russo & Mazzeo (1992), Jansen (2008), and Jansen & Westernberg (2015). Molecular identification initiated by isolating female mealybug root DNA (Doyle & Doyle 1987), then continued with the PCR method with mitochondrial cytochrome oxidase subunit I (MtCOI). The MtCOI sequence's ability to successfully identify specific species in taxonomic groups in the Animalia kingdom has been verified; thus, it is possible to utilize it as a basis for a DNA barcoding marker (Hebert et al. 2003; Rahayuwati et al. 2016).

Based on the results of matching mealybug preparations with the determination key provided by Jansen (2008) (Table 1), there was one type of mealybug found on the roots of *Adenium* sp. plants, namely *R. multiporifera*. The general characteristics of *R. multiporifera* were an elongated to slightly oval body, white to yellowish white covered in white wax powder with brown antennae and legs. Morphological observations were carried out after slide mounting of female mealybug imago based on modified method work instructions published by Agricultural Quarantine Centre Test Laboratory of Indonesia in Tanjung Priok in 2016. In this method, specimens were cleared using chloroform and Essig's solution in the Syracuse dish, then heated and stained with acid fuchsin. Subsequently, the specimens were transferred to an object glass treated with heinz solution and then secured with a cover glass.

The identification results showed that the specimen has an average body length of 1.4 mm with an average width of 0.6 mm (Figure 1B). The female *R. multiporifera* imago described by Jansen (2008) has a length of 1.3 mm and a width of 0.7 mm (Figure 1A), had no eyes on the head

(Figure 1D), had an antenna 250–267 µm long, five-segmented and biceps (Figure 1E). There were two pairs of spiracles with a length of 41 µm with a peritreme width of 28 µm (Figure 1I). It has many trilocular pores spread across the dorsal and ventral parts with a size of 3.5–4 µm and 40-45 multilocular pores with an average diameter of 13 µm (Figure 1C). The labium with three joined segments is 100–127 µm long, and the clypeolabral shield is 145–167 µm long. It has two circles in the ventral abdominal segments II and III with a diameter of 43–52 µm (Figure 1G). Legs fully develop with hind trochanter + femur length 51–69 µm; hind tibia + tarsus 50–70 µm; and hind claw 9–11 µm long (Figure 1J). The anal ring is 63–71 µm wide with six setae 112-127 µm long (Figure 1H), a general characteristic of the *Rhizoecini*, such as the *Rhizoecus* and *Ripersiella*. The unique characteristic of mealybug root identification was the presence of small bitubular pores on the ventral and dorsal parts measuring 4.3–4.8 µm (Figure 1F). Based on Kozár & Benedicty (2004), the presence of bitubular pores on mealybug roots was the main characteristic in distinguishing the genus *Rhizoecus* and *Ripersiella*, which still come from the same family, namely *Rhizoecini*. The presence of multilocular pores on the ventral and dorsal parts of the head, as well as several bitubular pores on the ventral side, were also found in the identified specimen. Additionally, Jansen (2008) reported that *R. multiporifera* closely resembles to *R. saintpauliae* (Williams), but distinguishes itself by featuring up to 45 multilocular disc pores arranged in one to occasionally two rows on the posterior edges and single ones on the rest of segment. In contrast, *R. saintpauliae* has multilocular disc pores in small numbers, up to ten per segment, on the thorax and sixth abdominal segments. Furthermore, the distribution of multilocular disc pores in *R. multiporifera* apart from *R. hibisci*. *R. multiporifera* set out these pores on the head and single rows on the thorax and the initial two abdominal segments, accompanied by two circuli, whilst *R. hibisci*, potentially having 0-2 circuli, lacks multilocular disc pores on the head and shows single pores on the thorax and the first two abdominal segments. All of those characteristics were also found in the identified specimen and closely resemble those observed in the species *R. multiporifera*. However, morphological characters are complicated to ascertain, prompting the need for additional investigation to gather information about molecular characteristics.

The isolated DNA was then multiplied using Veriti™ Thermal Cycler by the PCR method using one pair of MtCOI primers that successfully amplified mealybugs DNA at 649 bp, namely the forward primer PcoF1 5'CCTTCAACTAATCATAAAAATATYAG3' and the reverse primer LepR1 5'TAAACTTCTGGATGTCCAAAAAAATCA3' (Park et al. 2011). The composition of PCR products in molecular identification was carried out in a total volume of 20 µL consisting of 10 µL PCR master mix, 1 µL forward primer PcoF1, 1 µL reverse primer LepR1, 7 µL nuclease-free water, and 1 µL DNA template. Meanwhile, 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 the final stage at 72°C for 7 minutes. The PCR products were then visualized by electrophoresis using a 2% agarose gel made by mixing 60 ml of TAE 1X buffer, 1.2 g of agarose gel, and 5 µL of gel stain. Electrophoresis was carried out for 1 hour at 80 volts (Figure 2).

The PCR product that was successfully amplified was then sequenced to obtain the base sequence of the mealybug root species found in the roots of the *Adenium* sp.. The base sequences obtained were then analysed for homology in GenBank using BLAST. BLAST results with similar homology to the root mealybug species from Bali were collected

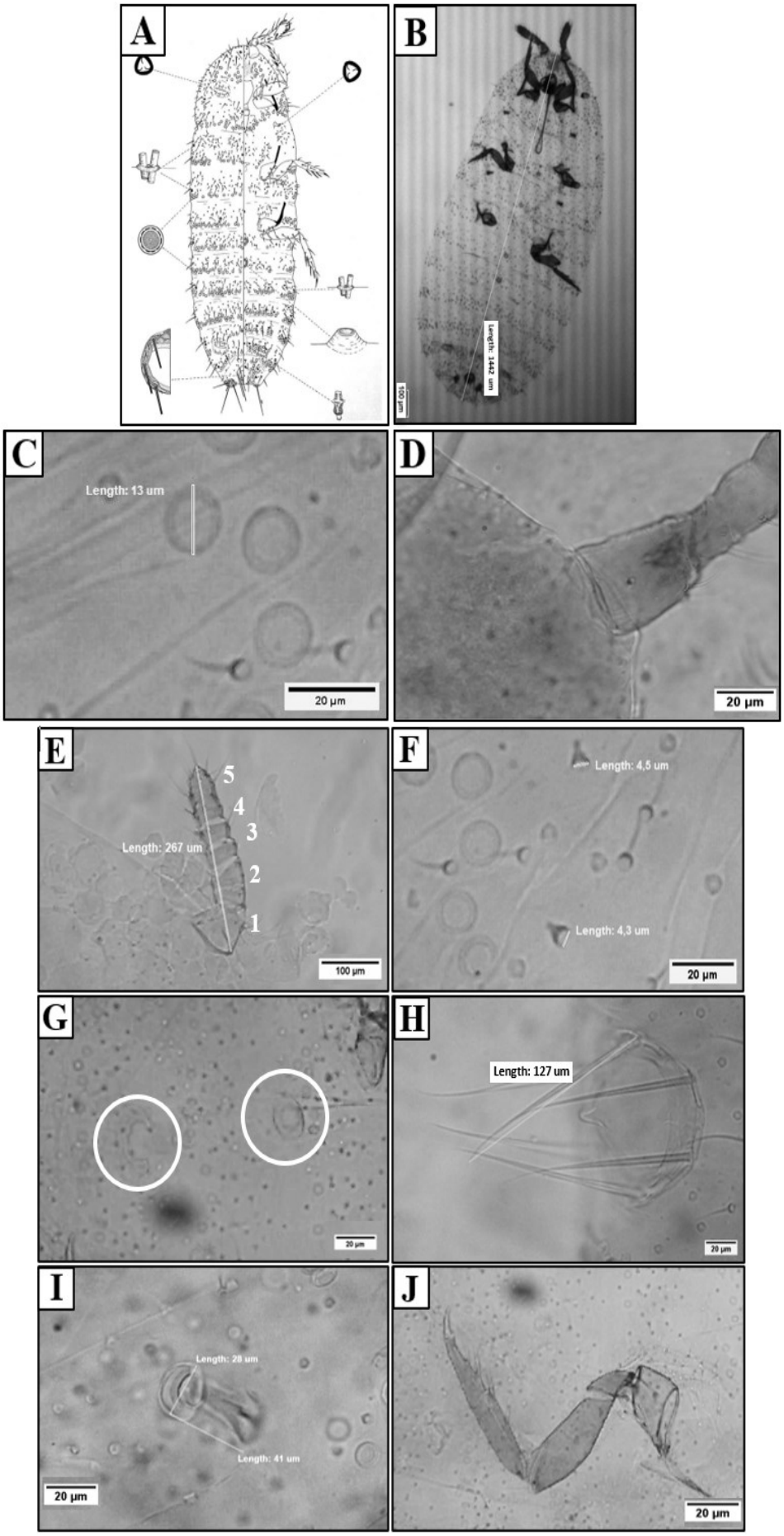

Figure 1. Morphological identification of *R. multiporifera* specimens. **A**. Detailed image. **B**. Female imago. **C**. Multilocular pores on the ventral side. **D**. Do not have eyes. **E**. Five-segment antenna. **F**. Bitubular pores on the ventral side. **G**. Two circulii in the second and third abdominal segments on the ventral. **H**. Six setae on the anal ring. **I**. Size of ventral spiracles on ventral. **J**. Limbs. Images were taken using an Olympus CX21 compound microscope at 4 x 10, 10 x 10, and 40 x 10 magnification.

Table 1. Determination key of mealybugs based on morphological identification (Mazzeo et al. 2023).

No.	Description	
1	Presence of bitubular or tritubular pores	2
	Absence of bitubular and tritubular pores ***Ripersiella potavae***	
2	Presence of bitubular pores and absence of tritubular pores	3
	Absence of bitubular pores and presence of tritubular pores	8
3	Multilocular disc pores present only on venter, with one circulus	4
	Multilocular disc pores present on venter and dorsum, with 0-2 circulus	6
4	With tubular ducts; bitubular pores short or long	5
	Without tubular ducts; bitubular pores of one size, scattered on dorsum ***Ripersiella maasbachi***	
5	Bitubular pores short, wide 2-3 times longer than wider ***Ripersiella periolana***	
	Bitubular pores long, narrow, 3-6 times longer than wide ***Ripersiella vidanoi***	
6	Circulus present, multilocular disc pores present on venter and dorsum, scattered on head and thorax, in rows across all abdominal segments ***Ripersiella lelloi***	
	Circulus present or absents, multilocular disc pores not with this combination of characters	7
7	Multilocular disc pores absent on head. Small type bitubular pores 5 µm wide present on venter and dorsum ***Ripersiella hibisci***	
	Multilocular disc pores present on head. Small type bitubular pores about 4 µm wide confined to venter ***Ripersiella multiporifera***	
8	Circulus present, multilocular disc pores absent	9
	Circulus absent, multilocular disc pores present	10
9	Labium 60-70 µm long ***Rhizoecus albidus***	
	Labium 75-90 µm long ***Rhizoecus cacticans***	
10	Antennae five-segmented ***Rhizoecus falcifer***	
	Antennae six-segmented	11
11	Tritubular pores of one size ***Rhizoecus dianthi***	
	Tritubular pores of 2-3 sizes	12
12	Tritubular pores of two sizes ***Rhizoecus latus***	
	Tritubular pores of three sizes ***Rhizoecus americanus***	

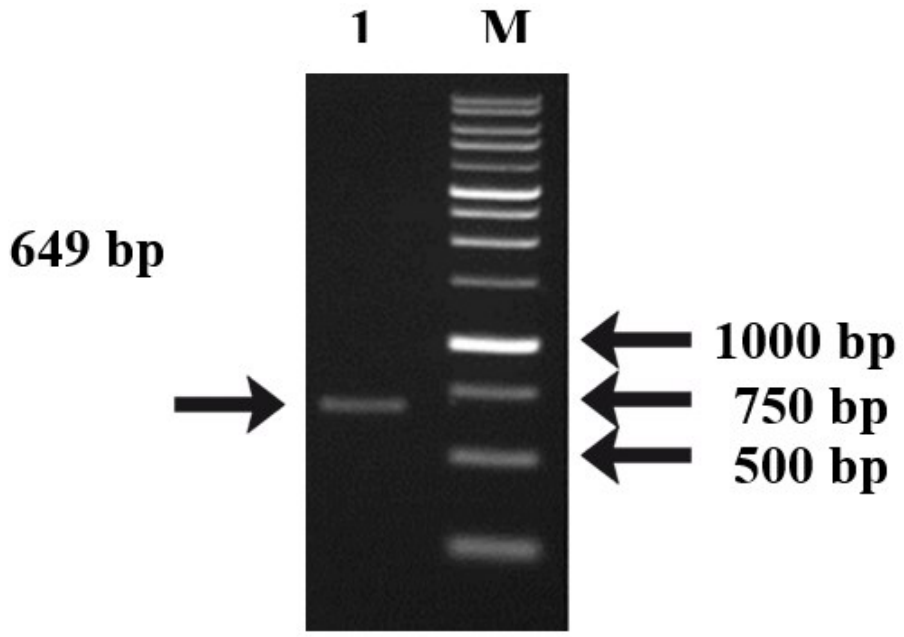

Figure 2. Results of DNA amplification of *R. multiporifera* using the PCR method with primers PcoF1 and LepR1. Columns 1 is the band of mealybug root DNA amplified at 649 bp. M = 1 kb.

for further alignment using BioEdit software with the ClustalW program. The obtained outcomes were subsequently transformed into an identity matrix displayed in Table 2.

The alignment of mealybug sequences with the MtCOI target gene obtained from Indonesia revealed 100% similarity with *R. multiporifera* Italy (OQ833547) and *R. multiporifera* Netherlands (KM453216). A phylogenetic tree was constructed based on sequencing data obtained through the utilization of MEGA 11 and BioEdit version 7.5.2, employing a bootstrap repetition of 1000 iterations using Maximum Parsimony method. According to the phylogenetic tree in Figure 3, the mealybug sequence found on the roots of *Adenium* sp. in Bali (Indonesia) is *R. multiporifera* based on GenBank. The findings of the identification matrix analysis indicate a significant degree of homology between *R. multiporifera* specimens from Bali (Indonesia) and those from Italy (OQ833547) and the Netherlands (KM453216). This further supports Jansen (2008) initial report regarding the finding of *R. multiporifera* in imported *Sansevieria* sp. from Indonesia in the Netherlands, as well as *R. multiporifera* found in Italy (Mazzeo et al. 2023) showing a homology percentage of 100% with *R. multiporifera* from Netherlands. Therefore, it is very likely that the root mealybug Italy specimen also came from Indonesia by imported plant since it was found in commercials nurseries (Mazzeo et al. 2023). Based on Ptaszynska et al. (2012) the MtCOI gene fragment indicated to have a similarity of species ranging from 95.1 to 100%. It can be concluded that the root mealybug found in Indonesia is *R. multiporifera*, which is the same as *R. multiporifera* found in the Netherlands and Italy. Therefore, this also clarify the low similarity value of *R. multiporifera* from Indonesia with *R. emarai* from Netherlands (82.1%) because they were different species.

The findings of the phylogenetic analysis indicate the presence of distinct group variations between *R. multiporifera* Bali and *R. hibisci* specimens collected from the Netherlands (KM453214), specifically 86.7%. Additionally, a lower degree of homology is observed, specifically 82.4%, between *R. multiporifera* Bali (Indonesia) and *R. dianthi* Netherlands (KM453217), as determined by sequence data obtained from GenBank. The percentage of similarity between *R. multiporifera* Indonesia and the genus *Rhizoecus* in Table 2 indicates higher results compared to *R. emarai* Netherlands (KM453217). This is attributed to discussion within Rhizoecidae, the genera *Ripersiella* and *Rhizoecus* have often been referred to synonymously for several times, making it highly likely that the genus *Rhizoecus* in the GenBank data used is either *Ripersiella* or vice versa (Mazzeo et al. 2023). However, currently both genera have been separated as a distinct genus in accordance with Choi & Lee (2022), who stated that the genus *Ripersiella* and the genus *Rhizoecus* form different groups because they are non-monophyletic or do not originate from the same ancestor. Meanwhile, the lowest percentage of homology and being an outgroup was shown by *R. multiporifera* from Bali with *B. tabaci* from Nigeria (MN164777) taken from GenBank, amounting to 46,6%.

The similarity of *R. multiporifera* from Bali (Indonesia) with *R. multiporifera* from Italy and the Netherlands is also proven by the pairwise distance analysis shown in Table 3. The results of the pairwise distance analysis show that *R. multiporifera* from Bali (Indonesia) has the smallest genetic distance value, namely 0,000 with *R. multiporifera* from Italy (OQ833547) and Netherlands (KM453216). Meanwhile, *R. multiporifera* from Bali (Indonesia) showed the largest genetic distance value with *Bemisia tabaci* from Nigeria (MN164777), namely, 0.961. The pairwise distance value indicates the genetic distance or level of similarity between

Table 2. Percentage of homology levels of *R. multiporifera* found in Bali (Indonesia) with other countries found in GenBank based on the MtCOI gene.

No.	Sequence	Homology (%)						
		1	2	3	4	5	6	7
1	Adenium root mealybug Bali Indonesia							
2	KM453216 *Ripersiella multiporifera* Netherlands	100						
3	OQ833547 *Ripersiella multiporifera* Italy	100	100					
4	KM453213 *Ripersiella emarai* Netherlands	82.1	82.1	82.1				
5	KM453215 *Rhizoecus hibisci* Netherlands	86.7	86.7	86.7	81.9			
6	KM453217 *Rhizoecus dianthi* Netherlands	82.4	82.4	82.4	85.1	81.9		
7	MN164777 *Bemisia tabaci* Nigeria	47.1	47.1	47.1	44.1	45.6	45.3	

Table 3. Pairwise distance value of *R. multiporifera* found in Bali (Indonesia) with other countries found in GenBank based on the MtCOI gene.

No.	Sequence	1	2	3	4	5	6	7
1	Adenium root mealybug Bali Indonesia							
2	KM453216 *Ripersiella multiporifera* Netherlands	0.000						
3	OQ833547 *Ripersiella multiporifera* Italy	0.000	0.000					
4	KM453213 *Ripersiella emarai* Netherlands	0.148	0.148	0.148				
5	KM453215 *Rhizoecus hibisci* Netherlands	0.210	0.210	0.210	0.220			
6	KM453217 *Rhizoecus dianthi* Netherlands	0.198	0.198	0.198	0.204	0.164		
7	MN164777 *Bemisia tabaci* Nigeria	0.961	0.961	0.961	1.026	1.127	1.054	

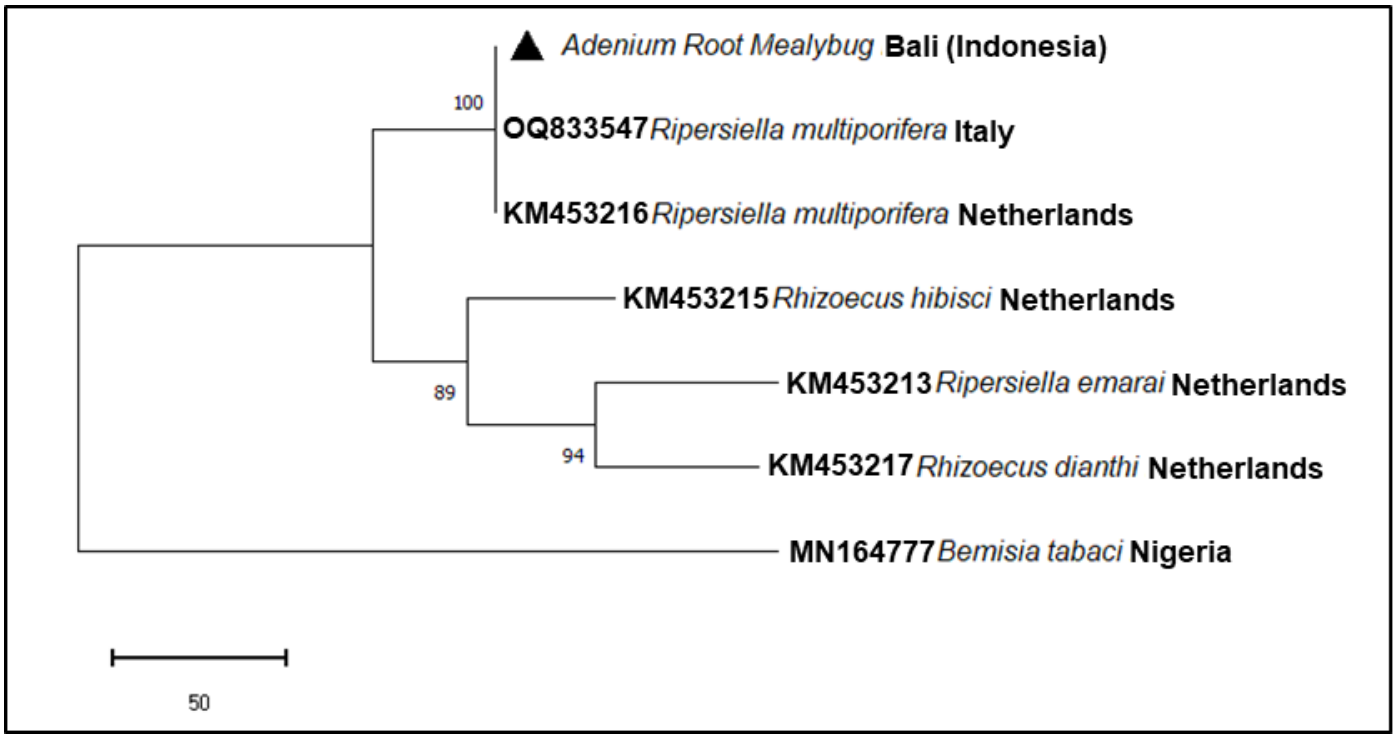

Figure 3. *R. multiporifera* Bali phylogeny tree is marked with symbols compared with several similar sequences taken from GenBank based on the MtCOI marker gene. ▲ *R. multiporifera* Bali (Indonesia); *R. multiporifera* Italy (OQ833547); *R. multiporifera* Netherlands (KM453216); *R. hibisci* Netherlands (KM453215); *R. emarai* Netherlands (KM453213); *R. dianthi* Netherlands (KM453217); *B. tabaci* Nigeria (MN164777).

sequences. A smaller genetic distance value indicates higher similarity between sequences. This was also stated by Hebert et al. (2003), who stated that the sequences with a genetic distance value less than 0.03 are considered to be the same species. This statement further strengthens that the mealybugs found on the roots of *Adenium* sp. in Bali (Indonesia) is *R. multiporifera*.

AUTHOR CONTRIBUTIONS

K.S.D., I.P.S., A.A.A.A.S.S., and G.N.A.S.W. contributed equally to the writing of the article. K.S.D. and I.P.S. collected samples from the field and finalisation of the manuscript. K.S.D and P.S.D contributed to the morphological identification stage. K.S.D and F.E.W. contributed from molecular identification to bioinformatics analysis.

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CONFLICT OF INTEREST

Authors declare that there is no competing interest regarding the publication of manuscripts.

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Short Communications

The Complete Chloroplast Genome of *Medinilla tapete-magicum* (Melastomataceae) from Sulawesi, Indonesia

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ABSTRACT
In this study, the genome of an endemic Sulawesi's plant, *Medinilla tapete-magicum* was sequenced using Illumina NextSeq 500 and assembled the whole chloroplast genome. Results showed that the cpGenome is 155,602 bp in size with typical quadripartite structure of a large single copy (LSC) region (85,409 bp), a short single copy (SSC) region (16,629 bp), and a pair of inverted repeats (IRs) regions (26,782 bp). The cpGenome is composed of 132 genes, which consists of 87 protein coding genes, 37 tRNAs, and 8 rRNAs. The sliding window analyses showed that *psbB-psbH* and *ndhF-rpl32* can potentially be used as markers. Microsatellite motifs of mononucleotide A and T dominated in the cpGenome. The phylogenetic trees from the concatenated 76 shared protein coding gene sequences showed the *Medinilla* clade was monophyletic and *M. tapete-magicum* is a sister species in the SE Asian clade which contain *M. magnifica* and *M. speciosa*.

Medinilla Gaudich. is an epiphytic, climber, or terrestrial shrubs in the family Melastomaceae (Kartonegoro 2022) that consist of 380 species (POWO 2023). Its habitat ranges from tropical Africa to Madagascar, India, Sri Lanka, Southeast Asia, Taiwan, Melanesia, northern Australia, and the Pacific Islands (Kartonegoro 2022).

Medinilla belongs to the Sonerileae/Dissochaeteae clade in the Melastomaceae family. However, the understanding of the generic boundaries and phylogenetic relationships within this clade is limited, and there is uncertainty regarding the taxonomic validity of many of the genera (Zhou et al. 2019). The limited sampling of genera or species in most analyses causes the discussion of generic limits to be hindered (Zhou et al. 2022), especially in its molecular aspect. The utilization of multiple markers in nuclear and chloroplast DNA barcoding has not yet resolved the phylogenetic issues in *Medinilla*, as observed in Kartonegoro et al. (2021). Furthermore, Kartonegoro et al. (2021) and Veranso-Libalah et al. (2022) reported that *Medinilla* was polyphyletic, thus requiring further intensive molecular research. The complete sequences of chloroplast ge-

nomes offer a high resolution in reconstructing phylogenetic between species (Li et al. 2022) and proven effective in addressing challenging phylogenetic inquiries, as an example the genus *Pseudodissochaeta* (Zhou et al. 2019). *Pseudodissochaeta* was then suggested to be kept as a synonym of *Medinilla* (Chen & Renner 2007; Zhou et al. 2019). Kartonegoro et al. (2021) have reclassified *Pseudodissochaeta* to a distinctive genus from *Medinilla* based on molecular, anatomical, and morphological evidence.

Out of 380 species of *Medinilla* sequences, only 9 species with complete plastome are now available in the GenBank, indicating that chloroplast genome data for *Medinilla* is still limited (Veranso-Libalah et al. 2022). *Medinilla tapete-magicum* Cámara & Leret & Veldk. is a terrestrial shrub that is endemic to Sulawesi, Indonesia. This species has a unique characteristic that its flowers are supported on many leafless branches incrassate both on and above the ground surface forming a dense mat around the plant's base (Cámara-Leret & Veldkamp 2011). This study aims to characterise the complete chloroplast genome of *M. tapete-magicum* and assess its phylogenetic position based on the chloroplast genome sequence. The results may be used as a foundation for further studies of this species, including but not limited to phylogeny, biogeography, conservation genetics, bioprospecting, and may serve as the super-barcode for this endemic species.

Leaf sample was collected from a living collection (Collection number NS. 191, plot no XV.B.319) in Bali Botanic Garden which originated from Batusetan Hill, Enrekang, South Sulawesi, Indonesia. This plant was propagated via stem-cutting from XV.B.242 described in Cámara-Leret & Veldkamp (2011) in which the holotype collection was stored at the Naturalis Biodiversity Center, Leiden (van Balgooy 7557). The leaf was silica-gel dried and transported for DNA extraction.

The genomic DNA was extracted from the silica-gel dried leaf sample by using Quick-DNA HMW MagBead Kit Zymo D6060 (Zymo Research, CA, USA). The quality control procedures were DNA visualization in Agarose Gel Electrophoresis, quantification, and purity assessment by Nanodrop 2000 (Thermo Scientific, MA, USA), Qubit dsDNA HS Assay (Thermo Scientific, MA, USA), and DNA integrity Quality checked by TapeStation (Agilent, CA, USA). The gDNA was enzymatic sheared to match insert size of 350 bp. The sequencing library was prepared by PCR free xGen™ DNA Library Preparation (IDT, IA, USA). The sequencing was performed using an Illumina NextSeq 500 (PT Genetika Science, Tangerang, Indonesia) to generate approximately 10 Gb total output of 150 bp paired-end (PE) raw reads.

The raw reads were subjected to FastQC v. 0.11.9 analysis (Andrews 2010). Low quality reads and those with sequencing adapter residues were trimmed by Trimmomatic v. 0.39 (Bolger et al. 2014), by these following settings: ILLUMINACLIP:TruSeq3-PE-2.fa:2:26:10 SLIDINGWINDOW:5:26 LEADING:26 TRAILING:26 HEADCROP:5 MINLEN:36 AVGQUAL:26. The complete chloroplast genome sequence was *de novo* assembled from the trimmed raw reads by NOVOPlasty v. 4.3.1 (Dierckxsens et al. 2016) with default settings. The full cpGenome sequence of *M. speciosa* NC_068172 was used as the reference and the extracted *rbcL* sequence as the seed. To assess the coverage, trimmed raw reads were mapped back to the sequence of final assembly by Bowtie2 v.2.4.5 (Langmead & Salzberg 2012). The SAM output was converted to BAM with Samtools v.1.9 (Danecek et al. 2021) and visualized in Unipro UGENE v.45.1 (Okonechnikov et al. 2012) for exporting the coverage table. Automatic annotation of the circularized assembly was performed in CPGAVAS2 (Shi et al. 2019) followed by manual checks using Unipro

UGENE v. 45.1 (Okonechnikov et al. 2012) and NCBI Genomic Workbench v. 3.8.2 (Kuznetsov & Bollin 2021). CPGView was used (Liu et al. 2023) to check the annotation completeness. The sequence was deposited in the genbank with accession no. OQ831429. The raw reads supporting this study were deposited in the NCBI Sequence Read Archive (SRA) with BioProject accession number PRJNA979173, BioSample SAMN35566527, and SRA SRR24805950 respectively. OGDRAW v. 1.3.1 was used (Greiner et al. 2019) to visualize the cpGenome.

Twenty-six complete cpGenome sequences of Melastomataceae and those 3 of Myrtaceae as the outgroup were downloaded from the genbank. From these 29 sequences, as many as 76 of protein coding genes loci were extracted for the phylogenetic analyses. Prior to the analysis, multiple sequence alignment (MSA) was processed for each locus by Muscle implemented in Mega 11 (Tamura et al. 2021). The aligned sequences were concatenated (63,674 in length), then subjected to model test-ng (Darriba et al. 2019) and showed the best model of TVM+G4. The maximum likelihood (ML) phylogenetic analysis was performed using IQTree2 v. 2.2.0 (Minh et al. 2020) with 1000 ultrafast bootstrap option (Hoang et al. 2018) and the Bayesian inference (BI) analysis in MrBayes v. 3.2.7 (Ronquist et al. 2012). The generated trees were edited in FigTree v. 1.4.4 (Rambaut 2009). To explore regions the newly assembled chloroplast genome for molecular markers, four sequences (NC_068172, MK994885, NC_049130, and OQ831429) were selected for whole genome alignment (WGA) in Mauve (Darling et al. 2004). The MSA was subjected to DNA Polymorphism analysis (windows length 400, step size 100) in DNASP v.6.12.3 (Rozas et al. 2017). The nucleotide diversity (pi) threshold of 0.14 was determined to select the hotspot regions. The sequence of OQ831429 was also subjected to MISA-web v.2.1 (Beier et al. 2017) analysis with its default settings to characterise the microsatellite/simple sequence repeats (SSR) loci. The reported di- and tri-nucleotides motifs were checked in the previous 4 sequences MSA for polymorphism.

The trimmed data consisted of 75.6 million reads and 2.8 million reads assembled into the cpGenome sequence of 155,602 bp. The coverage analysis showed the assembly depth of 3,562 ± 843. The cpGenome shows typical quadripartite structure of a large single copy (LSC) 85,409 bp, short single copy (SSC) 16,629 bp, and a pair of inverted repeats (IRs) 26,782 bp (Figure 1). The GC content of the plastome was 36.99% which was slightly higher than the LSC (34.77%), while in the SSC was 30.78% and the IRs 42.47%. The annotations showed 132 genes (111 unique), which consisted of 87 protein coding genes (79 unique), 37 tRNA (28 unique), and 8 rRNA (4 unique) (Table 1). Of these 132 genes, 20 genes contained an intron and 2 genes with two introns. The cpGenome size of *M. tapete-magicum* is similar to those reported *M. speciosa*, *M. beamanii*, *M. amplectens*, *M. petelotii*, and *M. fengii* (Zhou et al. 2019), which ranged from 155,084 –155,841 bp. Longer figures of cpGenome's size ranged from 156,420-156,790 bp observed for *Pseudodissochaeta assamica*, *P. septentrionalis*, and *P. lanceata* which in their report, Zhou et al. (2019) listed as *M. assamica*, *M. septentrionalis*, and *M. lanceata*. This was because Zhou et al. (2019) followed a taxonomic treatment by Chen & Renner (2007) which then was revised (Kartonegoro et al. 2021; Zhou et al. 2022).

The DNASP analysis showed 2 (two) regions: *psbB-psbH* and *ndhF-rpl32* (Figure 2), potentially used as markers (nucleotide diversity value above 0.14). Microsatellite motifs of mononucleotide A and T dominated in the cp Genome, followed by dinucleotides AT and TA, and trinucleotides ATT (Table 2). Among the dinucleotides motifs, $(AT)_6$ in base

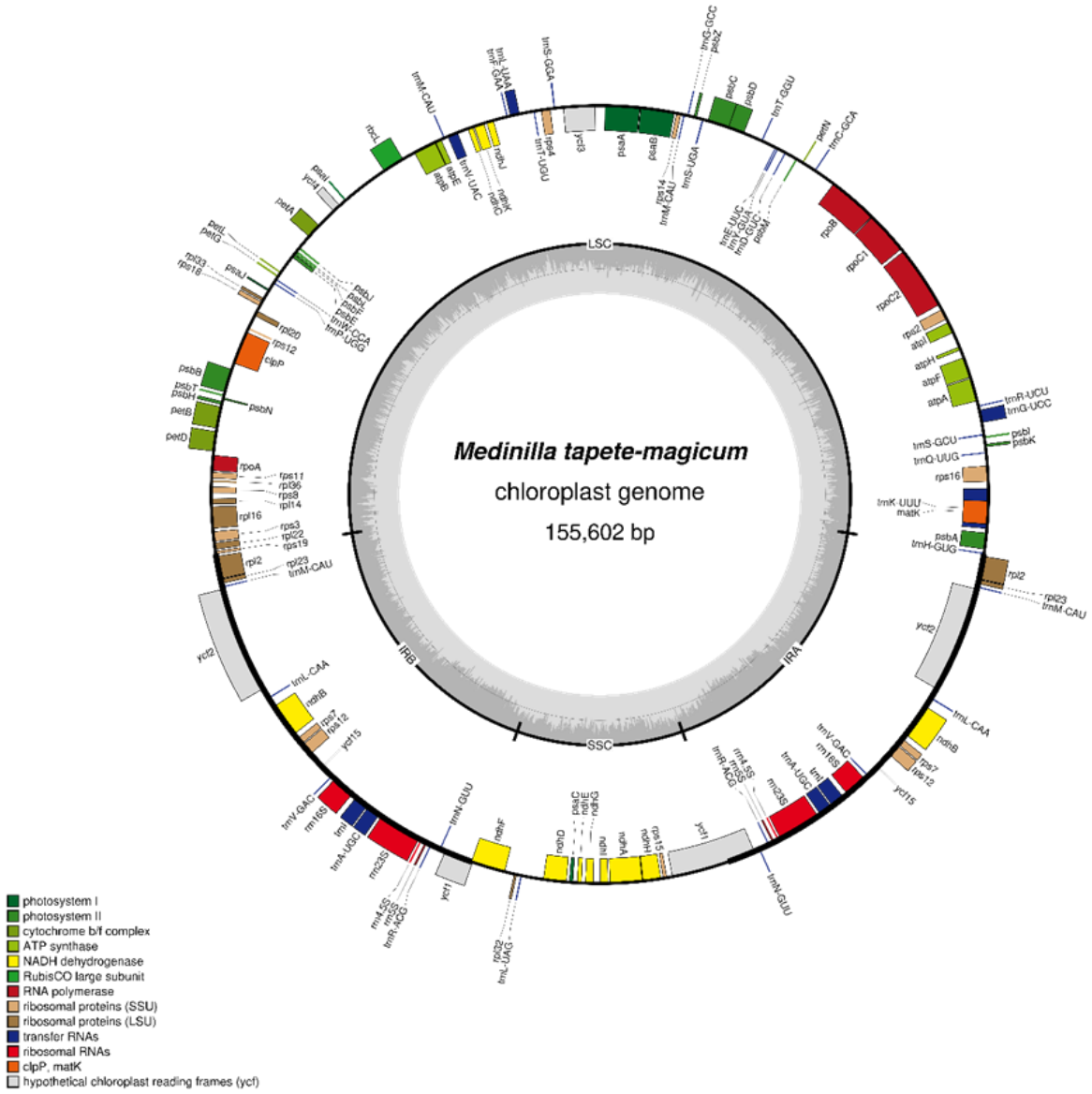

Figure 1. The complete chloroplast genome *of Medinilla tapete-magicum.*

31,931-31,942, $(AT)_7$ in base 51,618-51,631, and $(TA)_9$ in base 51,913-51,930 were polymorphic where as the solely trinucleotide motif $(ATT)_5$ was monomorphic.

The phylogenetic trees produced by the Maximum Likelihood (ML) and Bayesian Inference (BI) Methods had an identical topology and clades with strong bootstrap support (Figure 3). In this study, the *Medinilla* clade is monophyletic as a sister clade that contains *Blastus* and *Phyllagathis*. This result agrees with those previously reported by Zhou et al. (2019), Zhou et al. (2022) and Kartonegoro et al. (2021). *M. tapete-magicum* is a sister species of a clade contains *M. speciosa* and *M. magnifica*. This species is grouped within the South-East Asian clade and is separated from the Continental Asian counterpart (*M. fengii* and *M. petelotii*). Since *M. tapete-magicum* is the first sample of *Medinilla* genus from Indonesia with a cpGenome resource, other data of the congeneric species sampled from the region is needed to confirm their phylogenetic relationship. Additionally, Figure 3 shows that *M. assamica*, *M. septentrionalis*, and *M. lanceata* are renamed as *Pseudodissochaeta assamica*, *P. septentrionalis*, dan *P. lanceata* as reported by Zhou et al. (2022). This complete cpGenome sequence of *M. tapete-magicum* may serve as the super-barcode for this endemic plant species, as well as a source for DNA barcodes.

Table 1. Gene composition of *Medinilla tapete-magicum* chloroplast genome.

Group of genes	Name of genes	Number
tRNA	*trnA-UGC** (2x), *trnC-GCA*, *trnD-GUC*, *trnE-UUC*, *trnF-GAA*, *trnG-GCC*, *trnH-GUG*, *trnK-UUU**, *trnL-CAA* (2x), *trnL-UAA**, *trnL-UAG*, *trnM-CAU* (4x), *trnN-GUU* (2x), *trnP-UGG*, *trnQ-UUG*, *trnR-ACG* (2x), *trnR-UCU*, *trnS-GCU*, *trnS-GGA*, *trnS-UGA*, *trnT-GGU*, *trnT-UGU*, *trnV-GAC* (2x), *trnW-CCA*, *trnY-GUA*, *trnG-UCC**, *trnV-UAC**, *trnI** (2x)	37
rRNA	*rrn16S* (2x), *rrn23S* (2x), *rrn5S* (2x), *rrn4.5S* (2x)	8
Subunits of ATP synthase	*atpA*, *atpB*, *atpE*, *atpF**, *atpH*, *atpI*	6
Subunits of photosystem II	*psbA*, *psbB*, *psbC*, *psbD*, *psbE*, *psbF*, *psbH*, *psbI*, *psbJ*, *psbK*, *psbL*, *psbM*, *psbN*, *psbT*, *psbZ*, *ycf3***	16
Subunits of NADH-dehydrogenase	*ndhA**, *ndhB** (2x), *ndhC*, *ndhD*, *ndhE*, *ndhF*, *ndhG*, *ndhH*, *ndhI*, *ndhJ*, *ndhK*	12
Subunits of cytochrome b/f complex	*petA*, *petB**, *petD**, *petG*, *petL*, *petN*	6
Subunits of photosystem I	*psaA*, *psaB*, *psaC*, *psaI*, *psaJ*	5
Subunit of rubisco	*rbcL*	1
Large subunit of ribosome	*rpl14*, *rpl16**, *rpl2** (2x), *rpl20*, *rpl22*, *rpl23* (2x), *rpl32*, *rpl33*, *rpl36*	11
DNA dependent RNA polymerase	*rpoA*, *rpoB*, *rpoC1**, *rpoC2*	4
Small subunit of ribosome	*rps11*, *rps12*** (2x), *rps14*, *rps15*, *rps16**, *rps18*, *rps19*, *rps2*, *rps3*, *rps4*, *rps7* (2x), *rps8*	14
Subunit of Acetyl-CoA-carboxylase	*accD**	1
c-type cytochrome synthesis gene	*ccsA*	1
Envelope membrane protein	*cemA*	1
Protease	*clpP***	1
Maturase	*matK*	1
Conserved open reading frames	*ycf1 (2x)*, *ycf15 (2x)*, *ycf2 (2x)*, *ycf4*	7
Total		**132**

2x: presents in the IRs, * gene with an intron, ** gene with two introns

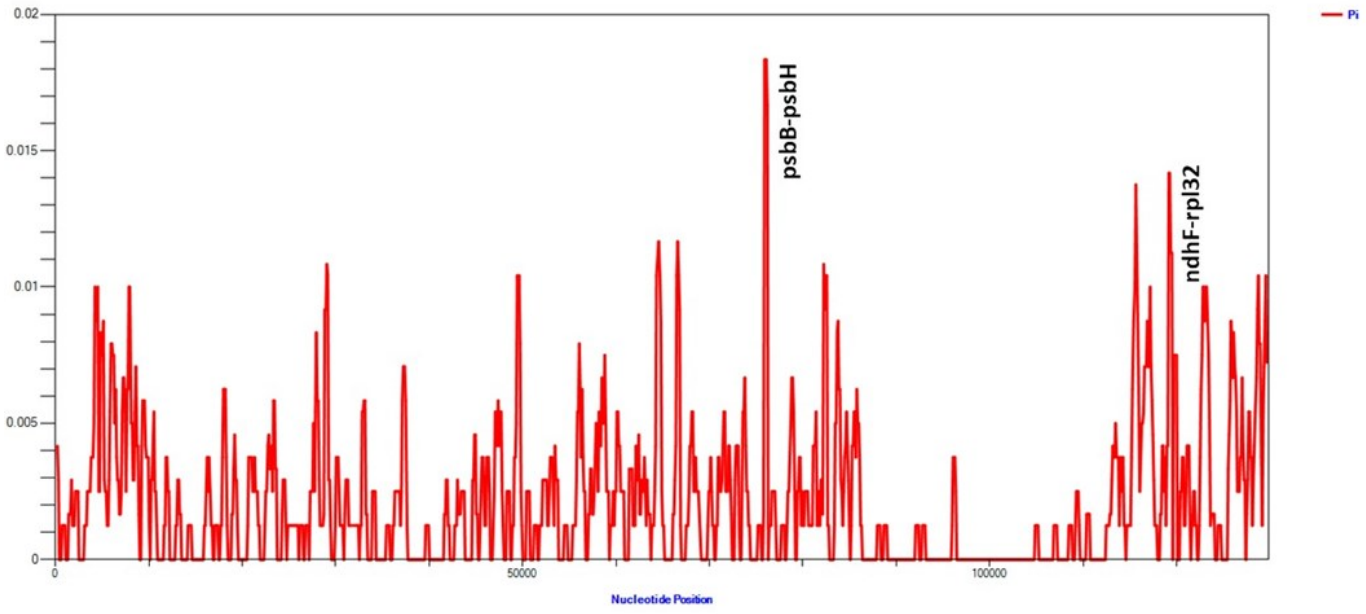

Figure 2. Nucleotide diversity value (pi) in *M. tapete-magicum.*

Table 2. Frequency distribution of the SSR repeat motif in the chloroplast genome of *M. tapete-magicum*.

Repeat motif	Number of repeats of the motif													
	5	6	7	8	9	10	11	12	13	14	15	16	17	total
A	-	-	-	-	-	12	7	-	2	-	-	1	-	22
T	-	-	-	-	-	12	7	4	-	2	1	-	1	27
AT	-	2	1	2	-	-	-	-	-	-	-	-	-	5
TA	-	-	-	-	1	-	-	-	-	-	-	-	-	1
ATT	1	-	-	-	-	-	-	-	-	-	-	-	-	1

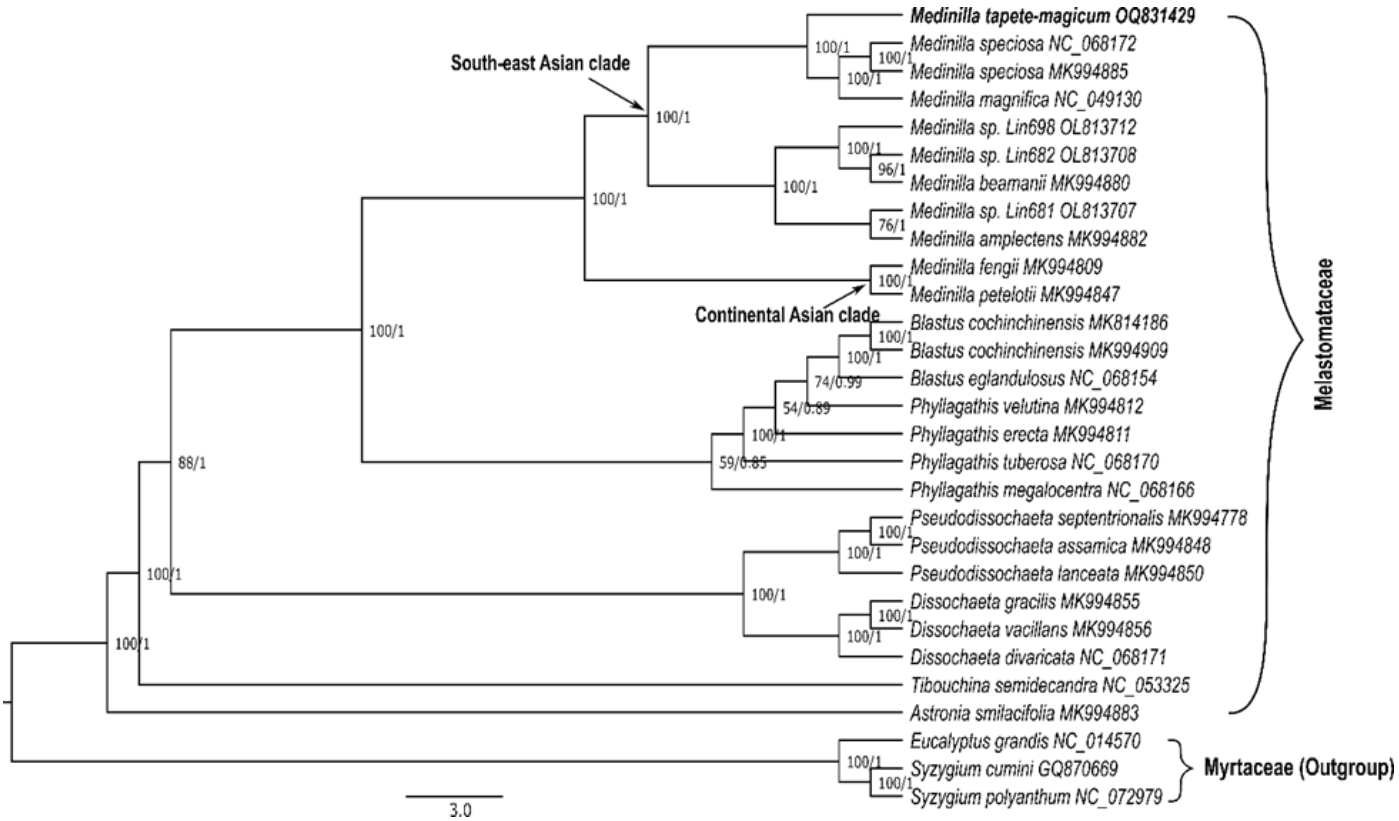

Figure 3. The Maximum Likelihood and Bayesian Inference Phylogenetic Tree of 26 species of Melastomataceae and 3 species of Myrtaceae (outgroup). Numbers in each node are the bootstrap value/posterior probability.

AUTHORS CONTRIBUTION

A.P. collected samples, analysed the data and supervised the manuscript, N.P.S.A. and R.A.P. wrote the manuscript, P.C.K. and E.Y. performed critical review and revision. A.P. is the main contributor, N.P.S.A., R.A.P., P.C.K. and E.Y. are the member contributors.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

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Research Article

Composition and Conservation Status of Avifauna in Urban Non-protected Important Bird Area (IBA) Site of Western India

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ABSTRACT
Kumbharwada wetland, an Important Bird Area (IBA) component in Bhavnagar, Gujarat, India, is a crucial wintering ground for migratory birds. Long-term monitoring of the avian community over a period of long time-frame is an excellent way to examine the health of this IBA site and thus provide an important ground to foster the conservation of birds in the region and management of this wetland. Given this consideration, field surveys were carried out from December 2020 to May 2023, following point count method to study the avian species richness in the study area. A total of 204 bird species belonging to 20 orders and 56 families are recorded, of which 85 species are migratory and 119 are resident. Anatidae is the most species-rich avian family (16 species). Highest number of species was recorded in the month of January (165 species in 2021 and 163 species in 2022). This wetland supports 107 (52.45%) wetland-associated species and 97 (47.55%) terrestrial species of birds. Twelve species are considered as Near Threatened, four species as Vulnerable and one species (*Aquila nipalensis*) as Endangered in IUCN Red List of Threatened Species. Four species (*Sterna aurantia*, *Mycteria leucocephala*, *Phoeniconaias minor*, and *Threskiornis melanocephalus*) with globally declining trend, are commonly seen in the study area, which shows that the wetland is a crucial habitat for bird species with high conservation priorities. Industrialization, encroachment, discharge of sewage water and chemical effluents, high-tension powerlines, stray dogs, and expansion of exotic vegetation remained the major threats to the habitat and avian community.

INTRODUCTION
Wetlands are highly productive and dynamic ecosystems that play a crucial role in our natural environment (Ghermandi et al. 2010; Hu et al. 2017). Wetlands are referred to as land transitional between the terrestrial and aquatic habitats (Mitsch & Gosselink 2000). Wetlands are critical habitat for avian diversity. Out of 1353 bird species with 38 endemic species recorded from India (Praveen & Jayapal 2023), 310 species are found to be wetland birds (Kumar et al. 2005). Birds are widely considered as an important taxon for wetlands. Their presence and activities have profound positive impacts on the health of these ecosystems. They play a vital role in maintenance of aquatic biodiversity, insect pest con-

trol, nutrient cycling as well as ecosystem functioning (Green & Elmberg 2014; Kumar et al. 2016). Furthermore, birds show a high sensitivity to the structural changes in their habitats, making them an important bio-indicators for examining the quality, productivity, and stability of wetland habitats (Morrison 1986; Bhat et al. 2009).

Being located along the Central Asian Flyway, wetlands of India are crucial wintering grounds for migratory birds (Kumar et al. 2016). The semi-arid parts of the western India have biodiverse wetland habitats. Approximately 17.56% geographical area of the Gujarat is comprised of wetlands (SAC 2010) with 4 Ramsar sites (Gujarat Ecology Commission 2022) and 19 Important Bird Areas (IBA) (Rahmani et al. 2016). The Kumbharwada wetland is part of one such IBA (Salt pans of Bhavnagar, Site code: IN093) situated in the Bhavnagar city of the Gujarat. It is currently taken over by extensive network of salt pans which have created an important habitat for waterbirds and are now being utilized by various avian species as a wintering ground (Rahmani et al. 2016), which were once occupied by a few waterfowl (Dharmakumarsinhji 1973). Formerly, some studies have been conducted in the Bhavnagar region pertaining to avian diversity (Dodia & Chavda 2012; Makwana & Dodia 2022; Khatsuriya et al. 2023). Particularly, 71 species of birds have been recorded (Parekh & Gadhvi 2013) from the Kumbharwada wetland and 161 species from the Saltpans of Bhavnagar (Rahmani et al. 2016). However, Kumbharwada wetland is facing increasing threats of industrialization, encroachment due to urbanization, pollution of water attributed to discharge of untreated domestic sewage and chemical effluents and a network of high-tension powerlines. These anthropogenic activities not only deteriorate the water quality but also exert severe consequences on avian populations, leading to alterations in the structure and composition of the avian community and a decline in their abundance (Reginald et al. 2007; Kumar & Sharma 2019).

The species richness and abundance of wetland birds have been found to be affected by several wetland features such as topography, size, water depth, water quality, availability of food, suitable roosting and nesting sites, as well as the presence of predators and inter-species competitors (Mukherjee et al. 2002; Ma et al. 2010). Therefore, the monitoring of bird assemblages holds substantial importance in evaluating the integrity and functions of wetland ecosystems and plays a crucial role in designing appropriate conservation and management implications to ensure sustainable biodiversity conservation (Lee et al. 2004; Sundar & Kittur 2013). Obtaining information on the composition and abundance of avian communities is essential for understanding the importance of regional or local landscapes in conservation of avian diversity (Kattan & Franco 2004; Bibi & Ali 2013). Despite being a heaven for birds, the information about the status of avian assemblages in this wetland is still limited and sporadic. The primary objective of the present study lies in assessing the species diversity, with a particular focus on threats associated with the habitat as well as globally threatened bird species within the study area. This region holds utmost significance as one of the key Important Bird Areas (IBAs) in Gujarat.

MATERIALS AND METHODS

Study area

The present study was conducted in Bhavnagar, Gujarat, India, where the Kumbharwada wetland (21°46'54.84"N, 72° 6'10.34"E) is located on the northern-western outskirts of the city (Figure 1). It is a component of Important Bird Area (IBA) site IN093 comprising variety of habitats for birds including marshland with hydrophytic vegetation, open waters,

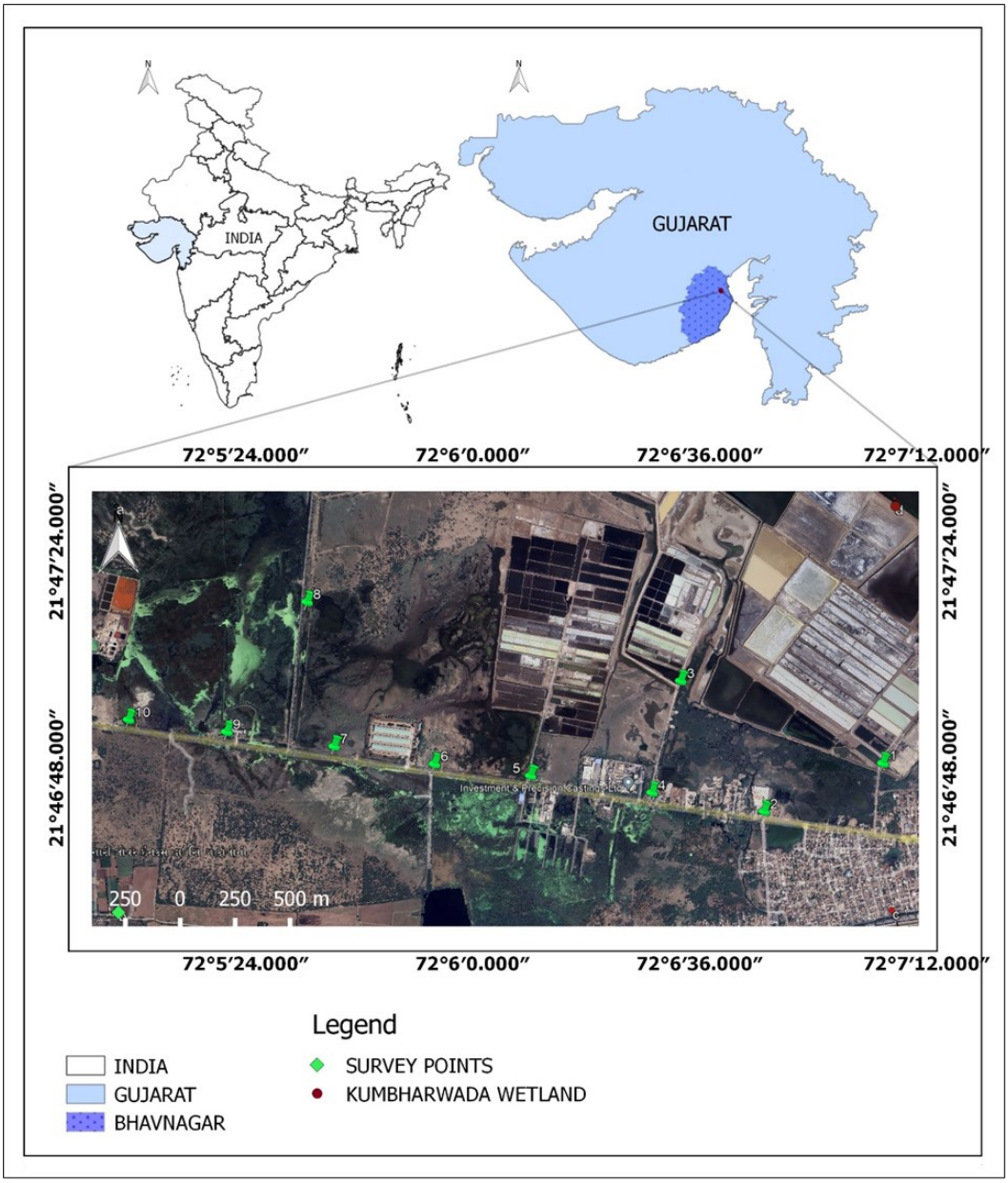

Figure 1. Map of study area in Kumbharwada wetland, Bhavnagar, Gujarat, India.

grassy patches, muddy areas, dry saline parts, thorny vegetation, and network of saltpans. Hydrophytic vegetation includes submerged (*Hydrilla* sp., *Potamogeton* sp.) and emergent macrophytes (*Typha* sp., *Fimbrystylis* sp.). Thorny vegetation is dominated by *Prosopis juliflora*, some sparsely distributed species like *Salvadora* sp. and *Zizyphus* sp. are also found at the study area. Some other important plant species include *Suaeda* sp., *Sporobolus* sp., *Chloris* sp. The map of the study area was prepared with the help of QGIS (version 2.18.14) open-source software.

Data Collection

The point count method was used to detect the bird species present in the study area for a fixed duration of time as it is suitable for the species-rich habitat with higher population density (Sutherland et al. 2005). The study area was divided into ten survey points, which were at minimum of 300 m apart from each other (Figure 1). Surveys were carried out twice a month between December 2020 to May 2023 comprising 60 observations during the study period. All these points were surveyed to record the species richness and assemblage of birds in the study area between 6:00 to 10:00 AM and 03:30 to 07:30 PM. Birds were observed unaidedly or

with a binocular (Nikon ACULON A211 10x50), a spotting scope (Nikon 20-60 x 80) and Nikon B700 point-and-shoot camera. Identification as well as the residential status (Resident or Migratory) and habitat use (Wetland associated or Terrestrial) were done using the field guide and available scientific resources (del Hoyo et al. 2014; Grimmett et al. 2016; Birds of the World 2022). Taxonomic classification and Names of the birds were followed from Praveen & Jayapal (2023).

Data Analysis

The occurrence of the species was studied and classified them into four categories namely Common (C-species recorded in 80-100% of field visits), Uncommon (U-species recorded in 50-79.9% of field visits), Occasional (O-species recorded in 20-49.9% of field visits) and Rare (R-species recorded in <19.9 % of field visits) following Mazumdar (2019). The IUCN status of birds and its global population trend was also noted to compare with the regional and local status (IUCN 2023). During the surveys, information related to the threats associated with birds and habitat along with conservation management was noted by direct observations. Threats are defined as the factors causing adverse impacts on the avian species richness and the overall wetland habitat identified from similar previous studies (Kumbhar & Mhaske 2023). Study period was divided into four seasons in a year viz. Winter (Dec-Feb), Summer (Mar-May), Monsoon (Jun-Aug), and Post monsoon (Sep-Nov) and the species richness data corresponding to these seasons were also recorded. Year-1 represents December 2020 to November 2021 and Year-2 represents December 2021 to November 2022. Results comprising a comprehensive analysis based on two years of data (December 2020 to November 2022) for consistency, with a final checklist compiled from 30 Months of study period starting from December 2020 to May 2023. Charts and Graphs were prepared using the MS Excel 2019.

RESULTS & DISCUSSION

Species Richness

The present study recorded 204 species of birds belonging to 20 orders and 56 families from the Kumbharwada wetland, Bhavnagar (Table 1/ Appendix 1). During this study, highest number of species belonged to order Passeriformes (63 species), followed by Charadriiformes (43 species), Pelecaniformes (17 species), Anseriformes (16 species), Accipitriformes (13 species), and others. Almost three-fourth (74.5%) of the species recorded during the study belonged to these five orders. Out of which three orders (viz. Charadriiformes, Pelecaniformes and Anseriformes) comprise the wetland-associated species. Family Anatidae was the most diverse avian family comprising 16 species. There were 17 families found to have only single species in the study area (Appendix 1).

Habitat utilization, Migratory status and Seasonal variation in avian assemblage

Highest number of species was reported during January (165 species in 2021, 163 species in 2022) and least during May (68 species in 2021, 65 species in 2022) as shown in Figure 2. Out of the total species recorded during the study period, 85 species (41.67%) were migratory and 119 species (58.33%) were resident (Figure 3a). Migratory birds utilize this habitat as their wintering ground. During winter season, number of migratory species recorded was 76 in year-1 and 75 species in year-2. The birds start departing for return migration from Kumbharwada wetland during the last week of March. The migratory species of birds varied in

Table 1. Status of avifauna recorded at Kumbharwada wetland, India.

Order	Family	Genus	Species	Residential Status		Occurrence				IUCN Status			
				Re	M	C	U	O	R	EN	VU	NT	LC
Anseriformes	1	9	16	4	12	3	11	0	2	0	1	0	15
Galliformes	1	3	4	3	1	1	0	2	1	0	0	0	4
Phoenicopteriformes	1	2	2	2	0	2	0	0	0	0	0	1	1
Podicipediformes	1	2	2	1	1	1	0	0	1	0	0	0	2
Columbiformes	1	3	5	5	0	3	0	1	1	0	0	0	5
Cuculiformes	1	4	4	3	1	2	1	1	0	0	0	0	4
Caprimulgiformes	2	2	2	2	0	1	1	0	0	0	0	0	2
Gruiformes	2	7	9	5	4	4	1	2	2	0	0	0	9
Charadriiformes	9	26	43	15	28	12	8	12	11	0	1	4	38
Ciconiiformes	1	2	2	2	0	1	0	1	0	0	0	1	1
Suliformes	2	3	4	4	0	0	2	2	0	0	0	1	3
Pelecaniformes	3	12	17	15	2	9	5	1	2	0	0	2	15
Accipitriformes	2	10	13	5	8	0	3	9	1	1	2	1	9
Strigiformes	2	2	2	2	0	1	0	0	1	0	0	0	2
Coraciiformes	3	5	8	6	2	3	1	1	3	0	0	0	8
Piciformes	2	2	2	1	1	0	1	0	1	0	0	0	2
Falconiformes	1	1	3	1	2	0	0	3	0	0	0	1	2
Psittaciformes	1	1	2	2	0	1	0	1	0	0	0	1	1
Passeriformes	19	38	63	41	22	16	20	16	11	0	0	0	63
Bucerotiformes	1	1	1	0	1	0	0	1	0	0	0	0	1
Total 20	56	135	204	119	85	60	54	53	37	1	4	12	187

Note: Re-Residential, M-Migratory; C-Common, U-Uncommon, O-Occasional, R-Rare
IUCN-International Union for Conservation of Nature and Natural Resources,
EN-Endangered, VU-Vulnerable, NT-Near Threatened, LC-Least Concern

different seasons and their richness shown an increase from post-monsoon, reaching its peak in winter, and subsequently declined in summer and was lowest in monsoon seasons (Figure 5). The species richness of the resident birds, exhibited no discernible seasonal fluctuation and remained similar over the course of the study period (Figure 5).

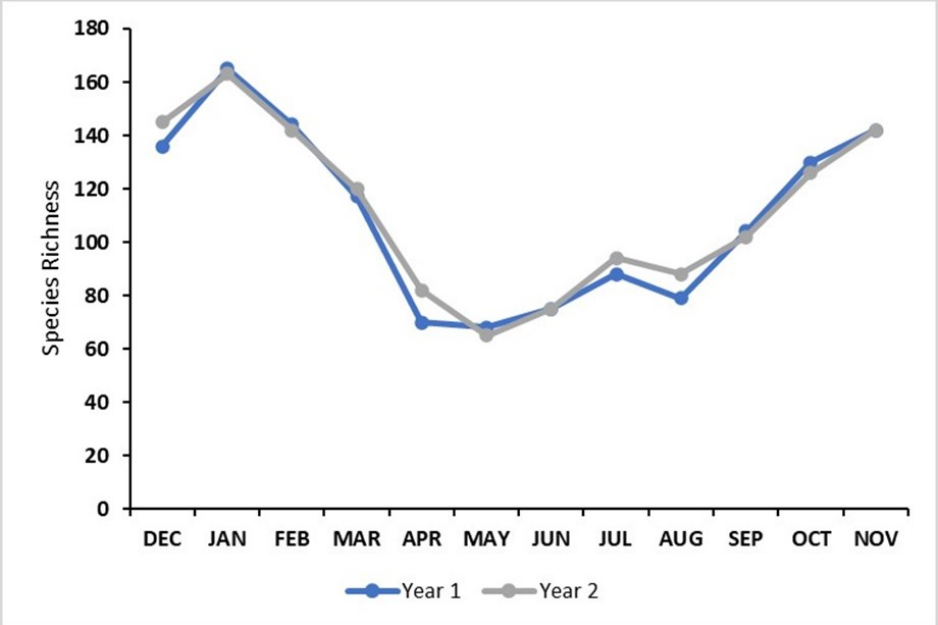

Figure 2. Species Richness of avifauna at Kumbharwada wetland, India.

Kumbharwada wetland is one of the most productive wetlands of Bhavnagar city (Parekh & Gadhvi 2013). More than half (52.45%) of the bird species found in Kumbharwada are wetland-associated, which sur-

vive in the marshland, open water, and saltpans of this wetland habitat. Besides, 47.55% of terrestrial avian species reside in the bushes, thorny scrubland dominated by *Prosopis juliflora*, some of the trees and dry saline parts. Anatidae and Scolopacidae are two of the most diverse families in Kumbharwada wetland similar to other studies in wetland habitats (Parekh & Gadhvi 2013; Mazumdar 2019; Makwana & Dodia 2022; Khatsuriya et al. 2023) and IBAs (Changder et. al. 2015; Rai et. al. 2017; Mahanta et. al. 2019). Wetland birds like geese, ducks, swans, and some resident aquatic avifauna rely on wetland plants for food, consuming various plant materials like roots, shoots, foliage, fruits, and seeds of floating, submerged or emergent vegetation (Ali & Ripley 1987; Rahmani & Islam 2008). In Kumbharwada wetland, variety of different habitats within the wetland area is probably one of the important factors responsible for high species richness of birds.

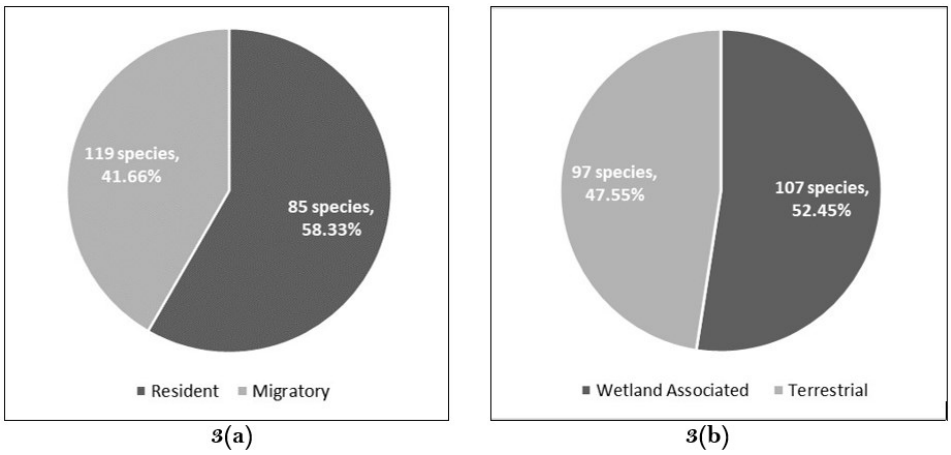

Figure 3. Proportion of **(a)** Resident and Migratory, **(b)** Wetland-associated and Terrestrial avifauna at Kumbharwada wetland, India.

Out of 204 species, 107 species (52.45%) were wetland-associated and 97 species (47.55%) were terrestrial (Figure 3b). Seasonal variation in wetland-associated species was clearly depicted (Figure 4). The highest count was recorded during the winter seasons, mainly attributed to the arrival of migratory waders and waterfowls. The numbers gradually increased from the post-monsoon season, reaching their peak during winter, and then declined during the summer, reaching their lowest during the monsoon seasons.

Kumbharwada wetland is crucial wintering ground for the migratory birds. In winter season, waterfowls and waders are distributed throughout the habitat as water was available in most of the parts. Winter visitors start appearing in mid-September, subsequently increasing in October, reaching to its peak in January, then steadily decrease and depart from the wetland by early April. Therefore, with the onset of the winter season, many migratory waterfowls and waders arrive at the Kumbharwada wetland, which in result shows significant seasonal variation in species richness of wetland-associated species. Many parts of the Kumbharwada wetland had dried up in the summer seasons and the availability of water become scarce and patchy, resulting in clumped dispersal of the birds (Figure 4). In June, all other migratory birds leave the Kumbharwada wetland except four species in year-1 and five species during year-2.

Apart from the migratory avifauna, Kumbharwada wetland serves as a crucial habitat for the resident aquatic as well as terrestrial species. Seasonal variation in species richness of resident terrestrial and wetland-associated avifauna did not show any significant variation, which indi-

cates that these species are dependent on this habitat throughout the year to survive. The species richness of any wetland habitat depends on the water quality and surrounding vegetation for the survival of any species (Buckton 2007). Therefore, appropriate management of water availability and quality is very critical for the protection and conservation of the avian community inhabiting this wetland.

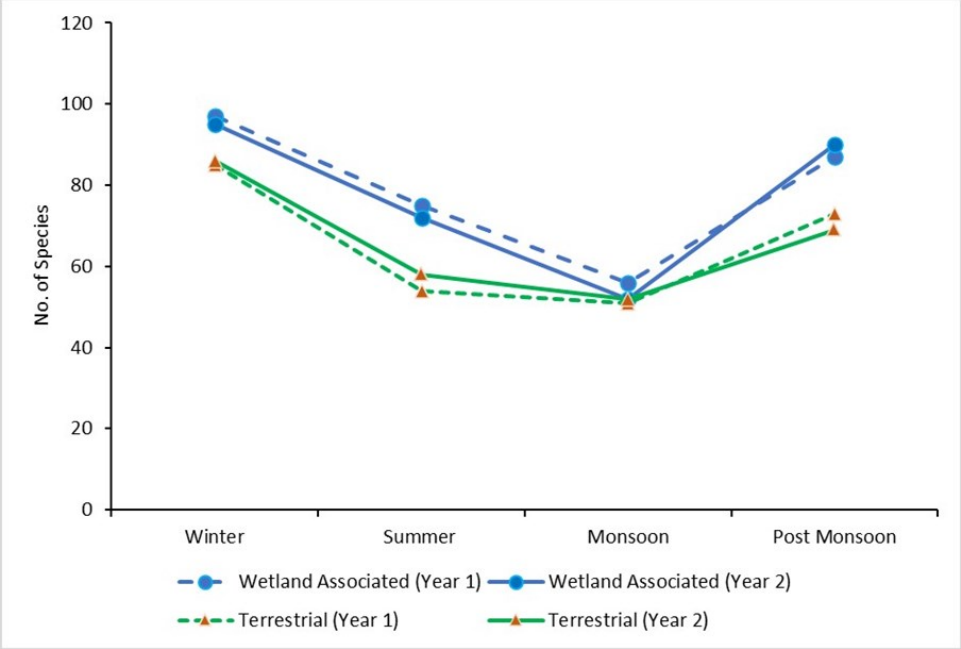

Figure 4. Seasonal occurrence of wetland-associated and terrestrial avifuauna at Kumbharwada wetland, India.

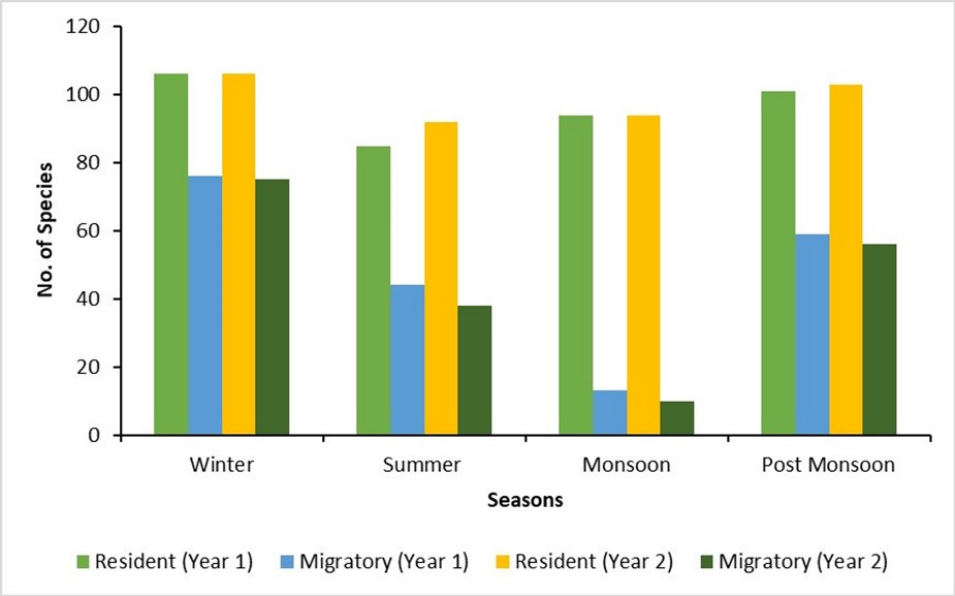

Figure 5. Seasonal Species Richness of Resident and Migratory avifauna at Kumbharwada wetland, India.

Conservation Status

Occurrence of birds showed that 60 species were common (29.41%), 54 species were uncommon (26.47%), 53 species were occasional (25.98%) and 37 species were rare (18.14%) at Kumbharwada wetland (Appendix 1). Among the bird species recorded during the study period, Common Pochard (*Aythya ferina*), River Tern (*Sterna aurantia*), Greater spotted Eagle (*Clanga clanga*), and Eastern Imperial Eagle (*Aquila heliaca*) are vulnerable under category of the IUCN Red List of Threatened Species. Ad-

ditionally, Steppe Eagle (*Aquila nipalensis*), falls under the Endangered category, while 12 species fall under the Near Threatened category (viz. Lesser Flamingo *Phoeniconaias minor*, Great Thick-knee *Esacus recurvirostris*, Eurasian Curlew *Numenius arquata*, Black-tailed Godwit *Limosa limosa*, Curlew Sandpiper *Calidris ferruginea*, Painted Stork *Mycteria leucocephala*, Oriental Darter *Anhinga melanogaster*, Dalmatian Pelican *Pelecanus crispus*, Black-headed Ibis *Threskiornis melanocephalus*, Pallid Harrier *Circus macrourus*, Red-necked Falcon *Falco chicquera* and Alexandrine Parakeet *Psittacula eupatria*) (IUCN 2023). When comparing the occurence of these avian species with its global population trend, a noteworthy finding emerged that certain species with the declining population trend globally, were frequently encountered during the study period at Kumbharwada wetland (Figure 6). The study area holds multiple species having been globally threatened, which serve as one of the qualifying criteions (A1) as Important Bird Area (IBA).

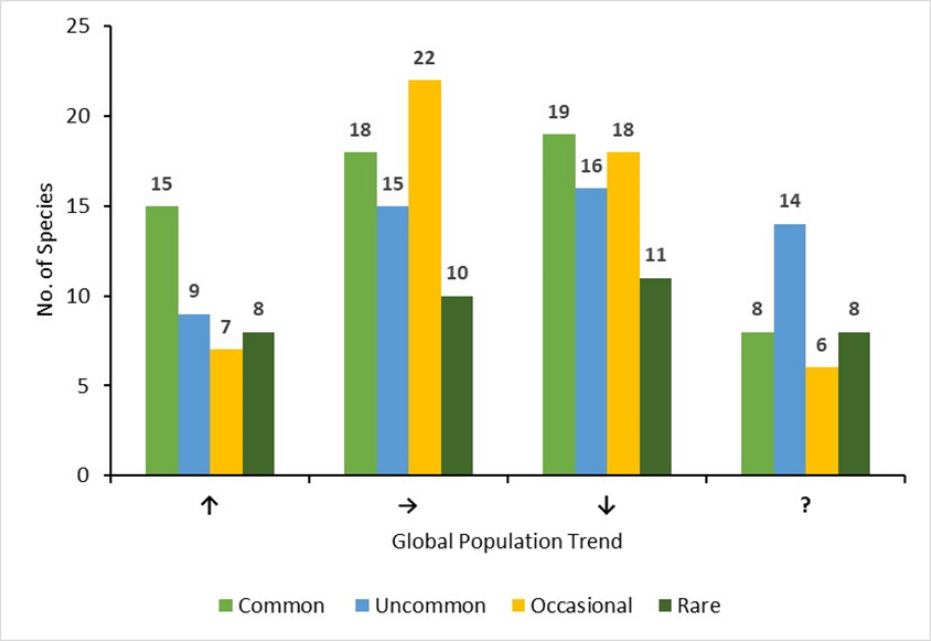

Figure 6. Comparision of occurrence of avifauna at Kumbharwada wetland with its global population trend ↑ Increasing, → Stable, ↓ Decreasing, ? Unknown

As per the State of India's Birds (SoIB 2020), 7 species (viz. Cotton Pygmy Goose *Nettapus coromandelianus*, Common Greenshank *Tringa nebularia*, Gull-billed Tern *Gelochelidon nilotica*, Pacific Golden Plover *Pluvialis fulva*, Short-toed Snake Eagle *Circaetus gallicus*, Red-necked Falcon *Falco chicquera*, and Steppe Eagle *Aquila nipalensis*) found during the study period are under the High Conservation Concern category for the country and 4 species recorded (viz. Gull-billed Tern *Gelochelidon nilotica*, Eastern Imperial Eagle *Aquila heliaca*, Greater-spotted Eagle *Clanga clanga*, and Common Pochard *Aythya ferina*) are listed as a key species for the Gujarat state. Many globally threatened species such as Lesser Flamingo *Phoeniconaias minor*, Black-headed Ibis *Threskiornis melanocephalus*, River Tern *Sterna aurantia*, Painted Stork *Mycteria leucocephala* with declining population trend are commonly found at Kumbharwada wetland, which indicate that the favourable resources were available for these species during the study period.

Two vagrant species (viz. Greater White-fronted Goose *Anser albifrons*, and Long-billed Dowitcher *Limnodromus scolopaceus*) were observed during the study period, which were very rare for the region. Grazing species such as Ruddy Shelduck *Tadorna ferruginea*, Bar-headed Goose

Anser indicus, and Greylag Goose *Anser anser* were found in high numbers during the study period indicating the availability of favourable resources during their wintering period at the Kumbharwada wetland. The study area serves as a critical foraging habitat for Greater Flamingo *Phoenicopterus roseus* and Lesser Flamingo *Phoeniconaias minor* throughout the year and they are found in large numbers (Tere & Parasharya 2011). Therefore, from the conservation perspective of such globally threatened species, it is required to prioritise the regular monitoring and long-term population studies of these species along with other threatened taxa inhabiting the Kumbharwada wetland. The study area holds multiple species which are globally threatened and serve the qualifying criterion as an Important Bird Area (IBA).

Threats

During the study period, it has been observed that the habitat is facing many threats (Figure 7). Anthropogenic pressure leads to the deterioration of water quality. Many parts of the wetland have been facing encroachment issue and remaining areas are also under severe threats such as pollution and habitat degradation. The growth and expansion of invasive *P. juliflora* in the habitat is also a concerning factor due to its adverse effects on the native vegetation. Untreated water, solid waste and chemical effluents are discharged in the wetland area which is one of the major factors contributing to the water pollution in this wetland. Extensive network of saltpans may alter the water quality and salinity levels. Polluted water can be toxic to birds, affecting their health and potentially leading to reduced reproductive capacities and survival rates (Richard et al. 2021; Makwana et al. 2023). The presence of polluted water and excessive growth of exotic vegetation can adversely impact the bird com-

Figure 7. Different types of threats to the avifauna and the Kumbharwada wetland.

munity of this Important Bird Area site. The high-voltage power lines passing over the wetland area is a severe threat to the large-sized birds residing in the habitat. The frequent cases of collision and mortality of birds especially both the species of flamingos have been reported during the study period and documented previously as well (Gadhvi 2011; Tere & Parasharya 2011; Parekh & Gadhvi 2013). Free-ranging dogs and cattle are also a significant factor for disturbance to the residential as well as migratory species residing in this wetland. Therefore, it is very important to mitigate the impacts of these anthropogenic threats and to maintain the healthy ecosystem in the region.

CONCLUSION

The present study recorded 204 species of birds belonging to 20 orders and 56 families from the Kumbharwada wetland, which is approximately one-third (33.33%) avian diversity of the entire Gujarat state. High species richness of avifauna at Kumbharwada wetland warrants for long-term population studies to understand the possible impacts of threats. Apart from that, more detailed research on the resource utilization by birds in Kumbharwada wetland are necessary to understand the significance of this Important Bird Area site. Maintaining the ecological integrity and conserving avian diversity at the Kumbharwada requires reduction in contamination of water, controlling invasive plants and weeds, preventing habitat destruction, minimizing bird-powerline collisions, and managing proper water levels. These crucial measures will ensure a healthy ecosystem for various bird species and sustain the overall biodiversity of the area. Local awareness and community participation can also play a vital role in conservation of this habitat. These combinations of efforts are imperative to foster the overall biodiversity of the entire region.

AUTHOR'S CONTRIBUTION

P.P.D. designed and supervised the study, V.M.M., A.G.M. and P.A.K. collected the data and verified the identification of species. VMM wrote the manuscript, P.P.D., P.A.K. and A.G.M. proofread, reviewed, and revised the final manuscript.

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CONFLICT OF INTEREST

The author confirms that there are no known conflicts of interest regarding this publication.

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APPENDICES

Appendix 1. Checklist of Avifaunal diversity at Kumbharwada wetland, Bhavnagar, Gujarat, India

Sr. No.	Family	English Name	Scientific Name	Habitat	Residential Status	Occurrence	Global Trend	IUCN Status
			Order: Anseriformes					
1	Anatidae	Lesser Whistling Duck	*Dendrocygna javanica (Horsfield, 1821)*	WA	Re	C	↓	LC
2	Anatidae	Bar-headed Goose	*Anser indicus (Latham, 1790)*	WA	M	U	↓	LC
3	Anatidae	Greylag Goose	*Anser anser (Linnaeus, 1758)*	WA	M	U	↑	LC
4	Anatidae	Greater White-fronted Goose	*Anser albifrons (Scopoli, 1769)*	WA	M	R	?	LC
5	Anatidae	Knob-billed Duck	*Sarkidiornis melanotos (Pennant, 1769)*	WA	Re	C	↓	LC
6	Anatidae	Ruddy Shelduck	*Tadorna ferruginea (Pallas, 1764)*	WA	M	U	?	LC
7	Anatidae	Cotton Pygmy Goose	*Nettapus coromandelianus (J.F. Gmelin, 1789)*	WA	Re	U	→	LC
8	Anatidae	Garganey	*Spatula querquedula (Linnaeus, 1758)*	WA	M	U	↓	LC
9	Anatidae	Northern Shoveler	*Spatula clypeata (Linnaeus, 1758)*	WA	M	U	↓	LC
10	Anatidae	Gadwall	*Mareca strepera (Linnaeus, 1758)*	WA	M	U	↑	LC
11	Anatidae	Eurasian Wigeon	*Mareca penelope (Linnaeus, 1758)*	WA	M	U	↓	LC
12	Anatidae	Indian Spot-billed Duck	*Anas poecilorhyncha (J.R. Forster, 1781)*	WA	Re	C	↓	LC
13	Anatidae	Mallard	*Anas platyrhynchos (Linnaeus, 1758)*	WA	M	R	↑	LC
14	Anatidae	Northern Pintail	*Anas acuta (Linnaeus, 1758)*	WA	M	U	↓	LC
15	Anatidae	Common Teal	*Anas crecca (Linnaeus, 1758)*	WA	M	U	?	LC
16	Anatidae	Common Pochard	*Aythya ferina (Linnaeus, 1758)*	WA	M	U	↓	VU
			Order: Galliformes					
17	Phasianidae	Indian Peafowl	*Pavo cristatus (Linnaeus, 1758)*	TR	Re	O	→	LC
18	Phasianidae	Rain Quail	*Coturnix coromandelica (J.F. Gmelin, 1789)*	TR	M	O	→	LC
19	Phasianidae	Painted Francolin	*Francolinus pictus (Jardine & Selby, 1828)*	TR	Re	R	↓	LC
20	Phasianidae	Grey Francolin	*Ortygornis pondicerianus (J.F. Gmelin, 1789)*	TR	Re	C	→	LC
			Order: Phoenicopteriformes					
21	Phoenicopteridae	Greater Flamingo	*Phoenicopterus roseus (Pallas, 1811)*	WA	Re	C	↑	LC
22	Phoenicopteridae	Lesser Flamingo	*Phoeniconaias minor (E. Geoffroy Saint-Hilaire, 1798)*	WA	Re	C	↓	NT
			Order: Podicipediformes					
23	Podicipedidae	Little Grebe	*Tachybaptus ruficollis (Pallas, 1764)*	WA	Re	C	↓	LC
24	Podicipedidae	Black-necked Grebe	*Podiceps nigricollis (C.L. Brehm, 1831)*	WA	M	R	?	LC
			Order: Columbiformes					
25	Columbidae	Rock Pigeon	*Columba livia (J.F. Gmelin, 1789)*	TR	Re	C	↓	LC

Appendix 1. Contd.

Sr. No.	Family	English Name	Scientific Name	Habitat	Residential Status	Occurrence	Global Trend	IUCN Status
26	Columbidae	Eurasian Collared Dove	*Streptopelia decaocto (Frivaldszky, 1838)*	TR	Re	C	↑	LC
27	Columbidae	Red Collared Dove	*Streptopelia tranquebarica (Hermann, 1804)*	TR	Re	O	↓	LC
28	Columbidae	Laughing Dove	*Streptopelia senegalensis (Linnaeus, 1766)*	TR	Re	C	→	LC
29	Columbidae	Yellow-footed Green Pigeon	*Treron phoenicopterus (Latham, 1790)*	TR	Re	R	↑	LC
			Order: Cuculiformes					
30	Cuculidae	Greater Coucal	*Centropus sinensis (Stephens, 1815)*	TR	Re	C	→	LC
31	Cuculidae	Pied Cuckoo	*Clamator jacobinus (Boddaert, 1783)*	TR	M	O	→	LC
32	Cuculidae	Asian Koel	*Eudynamys scolopaceus (Linnaeus, 1758)*	TR	Re	C	→	LC
33	Cuculidae	Common Hawk Cuckoo	*Hierococcyx varius (Vahl, 1797)*	TR	Re	U	→	LC
			Order: Caprimulgiformes					
34	Caprimulgidae	Indian Nightjar	*Caprimulgus asiaticus (Latham, 1790)*	TR	Re	U	→	LC
35	Apodidae	Indian House Swift	*Apus affinis (J.E. Gray, 1830)*	TR	Re	C	↑	LC
			Order: Gruiformes					
36	Rallidae	Common Moorhen	*Gallinula chloropus (Linnaeus, 1758)*	WA	Re	C	→	LC
37	Rallidae	Eurasian Coot	*Fulica atra (Linnaeus, 1758)*	WA	Re	C	↑	LC
38	Rallidae	Grey-headed Swamphen	*Porphyrio poliocephalus (Latham, 1801)*	WA	Re	C	?	LC
39	Rallidae	Watercock	*Gallicrex cinerea (J.F. Gmelin, 1789)*	WA	M	O	↓	LC
40	Rallidae	White-breasted Waterhen	*Amaurornis phoenicurus (Pennant, 1769)*	WA	Re	C	?	LC
41	Rallidae	Ruddy-breasted Crake	*Zapornia fusca (Linnaeus, 1766)*	WA	Re	R	↓	LC
42	Rallidae	Baillon's Crake	*Zapornia pusilla (Pallas, 1776)*	WA	M	R	?	LC
43	Gruidae	Demoiselle Crane	*Grus virgo (Linnaeus, 1758)*	WA	M	U	↑	LC
44	Gruidae	Common Crane	*Grus grus (Linnaeus, 1758)*	WA	M	O	↑	LC
			Order: Charadriiformes					
45	Burhinidae	Indian Thick-knee	*Burhinus indicus (Salvadori, 1865)*	TR	Re	O	↓	LC
46	Burhinidae	Great Thick-knee	*Esacus recurvirostris (Cuvier, 1829)*	TR	Re	R	↓	NT
47	Recurvirostridae	Black-winged Stilt	*Himantopus himantopus (Linnaeus, 1758)*	WA	Re	C	↑	LC
48	Recurvirostridae	Pied Avocet	*Recurvirostra avosetta (Linnaeus, 1758)*	WA	M	U	?	LC
49	Charadriidae	Pacific Golden Plover	*Pluvialis fulva (J.F. Gmelin, 1789)*	WA	M	O	↓	LC
50	Charadriidae	Yellow-wattled Lapwing	*Vanellus malabaricus (Boddaert, 1783)*	WA	Re	R	→	LC
51	Charadriidae	Red-wattled Lapwing	*Vanellus indicus (Boddaert, 1783)*	WA	Re	C	?	LC
52	Charadriidae	White-tailed Lapwing	*Vanellus leucurus (M.H.C. Lichtenstein, 1823)*	WA	M	O	?	LC
53	Charadriidae	Kentish Plover	*Charadrius alexandrinus (Linnaeus, 1758)*	WA	Re	C	↓	LC
54	Charadriidae	Lesser Sand Plover	*Charadrius mongolus (Pallas, 1776)*	WA	M	R	?	LC
55	Charadriidae	Little Ringed Plover	*Charadrius dubius (Scopoli, 1786)*	WA	Re	C	→	LC

Appendix 1. Contd.

Sr. No.	Family	English Name	Scientific Name	Habitat	Residential Status	Occurrence	Global Trend	IUCN Status
56	Rostratulidae	Greater Painted-snipe	*Rostratula benghalensis (Linnaeus, 1758)*	WA	Re	R	↓	LC
57	Jacanidae	Pheasant-tailed Jacana	*Hydrophasianus chirurgus (Scopoli, 1786)*	WA	Re	C	↓	LC
58	Scolopacidae	Eurasian Curlew	*Numenius arquata (Linnaeus, 1758)*	WA	M	R	↓	NT
59	Scolopacidae	Black-tailed Godwit	*Limosa limosa (Linnaeus, 1758)*	WA	M	U	↓	NT
60	Scolopacidae	Ruff	*Calidris pugnax (Linnaeus, 1758)*	WA	M	C	↓	LC
61	Scolopacidae	Curlew Sandpiper	*Calidris ferruginea (Pontoppidan, 1763)*	WA	M	O	↓	NT
62	Scolopacidae	Temminck's Stint	*Calidris temminckii (Leisler, 1812)*	WA	M	U	?	LC
63	Scolopacidae	Little Stint	*Calidris minuta (Leisler, 1812)*	WA	M	C	↑	LC
64	Scolopacidae	Long-billed Dowitcher	*Limnodromus scolopaceus (Say, 1822)*	WA	M	R	?	LC
65	Scolopacidae	Common Snipe	*Gallinago gallinago (Linnaeus, 1758)*	WA	M	U	↓	LC
66	Scolopacidae	Common Sandpiper	*Actitis hypoleucos (Linnaeus, 1758)*	WA	M	C	↓	LC
67	Scolopacidae	Green Sandpiper	*Tringa ochropus (Linnaeus, 1758)*	WA	M	U	↑	LC
68	Scolopacidae	Spotted Redshank	*Tringa erythropus (Pallas, 1764)*	WA	M	O	→	LC
69	Scolopacidae	Common Greenshank	*Tringa nebularia (Gunnerus, 1767)*	WA	M	O	→	LC
70	Scolopacidae	Marsh Sandpiper	*Tringa stagnatilis (Bechstein, 1803)*	WA	M	U	↓	LC
71	Scolopacidae	Wood Sandpiper	*Tringa glareola (Linnaeus, 1758)*	WA	M	C	→	LC
72	Scolopacidae	Common Redshank	*Tringa totanus (Linnaeus, 1758)*	WA	M	U	?	LC
73	Turnicidae	Yellow-legged Buttonquail	*Turnix tanki (Blyth, 1843)*	TR	M	R	→	LC
74	Turnicidae	Barred Buttonquail	*Turnix suscitator (J.F. Gmelin, 1789)*	TR	Re	R	↑	LC
75	Glareolidae	Collared Pratincole	*Glareola pratincola (Linnaeus, 1766)*	WA	Re	O	↓	LC
76	Glareolidae	Small Pratincole	*Glareola lactea (Temminck, 1820)*	WA	Re	O	?	LC
77	Laridae	Slender-billed Gull	*Chroicocephalus genei (Breme, 1839)*	WA	M	O	?	LC
78	Laridae	Black-headed Gull	*Chroicocephalus ridibundus (Linnaeus, 1766)*	WA	M	O	?	LC
79	Laridae	Brown-headed Gull	*Chroicocephalus brunnicephalus (Jerdon, 1840)*	WA	M	O	→	LC
80	Laridae	Pallas's Gull	*Ichthyaetus ichthyaetus (Pallas, 1773)*	WA	M	R	↓	LC
81	Laridae	Lesser Black-backed Gull	*Larus fuscus (Linnaeus, 1758)*	WA	M	R	↑	LC
82	Laridae	Little Tern	*Sternula albifrons (Pallas, 1764)*	WA	Re	O	↓	LC
83	Laridae	Gull-billed Tern	*Gelochelidon nilotica (J.F. Gmelin, 1789)*	WA	M	U	↓	LC
84	Laridae	Caspian Tern	*Hydroprogne caspia (Pallas, 1770)*	WA	Re	C	↑	LC
85	Laridae	Whiskered Tern	*Chlidonias hybrida (Pallas, 1811)*	WA	M	C	→	LC
86	Laridae	Common Tern	*Sterna hirundo (Linnaeus, 1758)*	WA	M	R	?	LC
87	Laridae	River Tern	*Sterna aurantia (J.E. Gray, 1831)*	WA	Re	C	↓	VU
			Order: Ciconiiformes					

Appendix 1. Contd.

Sr. No.	Family	English Name	Scientific Name	Habitat	Residential Status	Occurrence	Global Trend	IUCN Status
88	Ciconiidae	Asian Openbill	*Anastomus oscitans (Boddaert, 1783)*	WA	Re	O	?	LC
89	Ciconiidae	Painted Stork	*Mycteria leucocephala (Pennant, 1769)*	WA	Re	C	↓	NT
			Order: Suliformes					
90	Anhingidae	Oriental Darter	*Anhinga melanogaster (Pennant, 1769)*	WA	Re	O	↓	NT
91	Phalacrocoracidae	Little Cormorant	*Microcarbo niger (Vieillot, 1817)*	WA	Re	U	?	LC
92	Phalacrocoracidae	Great Cormorant	*Phalacrocorax carbo (Linnaeus, 1758)*	WA	Re	O	↑	LC
93	Phalacrocoracidae	Indian Cormorant	*Phalacrocorax fuscicollis (Stephens, 1826)*	WA	Re	U	?	LC
			Order: Pelecaniformes					
94	Pelecanidae	Great White Pelican	*Pelecanus onocrotalus (Linnaeus, 1758)*	WA	M	U	?	LC
95	Pelecanidae	Dalmatian Pelican	*Pelecanus crispus (Bruch, 1832)*	WA	M	O	↓	NT
96	Ardeidae	Yellow Bittern	*Ixobrychus sinensis (J.F. Gmelin, 1789)*	WA	Re	R	?	LC
97	Ardeidae	Grey Heron	*Ardea cinerea (Linnaeus, 1758)*	WA	Re	U	?	LC
98	Ardeidae	Purple Heron	*Ardea purpurea (Linnaeus, 1766)*	WA	Re	C	↓	LC
99	Ardeidae	Great Egret	*Ardea alba (Linnaeus, 1758)*	WA	Re	U	?	LC
100	Ardeidae	Intermediate Egret	*Ardea intermedia (Wagler, 1829)*	WA	Re	C	↓	LC
101	Ardeidae	Little Egret	*Egretta garzetta (Linnaeus, 1766)*	WA	Re	C	↑	LC
102	Ardeidae	Western Reef Egret	*Egretta gularis (Bosc, 1792)*	WA	Re	U	→	LC
103	Ardeidae	Cattle Egret	*Bubulcus ibis (Linnaeus, 1758)*	WA	Re	C	↑	LC
104	Ardeidae	Indian Pond Heron	*Ardeola grayii (Sykes, 1832)*	WA	Re	C	?	LC
105	Ardeidae	Striated Heron	*Butorides striata (Linnaeus, 1758)*	WA	Re	R	→	LC
106	Ardeidae	Black-crowned Night Heron	*Nycticorax nycticorax (Linnaeus, 1758)*	WA	Re	U	↓	LC
107	Threskiornithidae	Glossy Ibis	*Plegadis falcinellus (Linnaeus, 1766)*	WA	Re	C	↓	LC
108	Threskiornithidae	Black-headed Ibis	*Threskiornis melanocephalus (Latham, 1790)*	WA	Re	C	↓	NT
109	Threskiornithidae	Red-naped Ibis	*Pseudibis papillosa (Temminck, 1824)*	WA	Re	C	↓	LC
110	Threskiornithidae	Eurasian Spoonbill	*Platalea leucorodia (Linnaeus, 1758)*	WA	Re	C	?	LC
			Order: Accipitriformes					
111	Pandionidae	Osprey	*Pandion haliaetus (Linnaeus, 1758)*	WA	M	O	↑	LC
112	Accipitridae	Black-winged Kite	*Elanus caeruleus (Desfontaines, 1789)*	TR	Re	U	→	LC
113	Accipitridae	Oriental Honey Buzzard	*Pernis ptilorhynchus (Temminck, 1821)*	TR	Re	O	↓	LC
114	Accipitridae	Short-toed Snake Eagle	*Circaetus gallicus (J.F. Gmelin, 1788)*	TR	Re	O	→	LC
115	Accipitridae	Greater Spotted Eagle	*Clanga clanga (Pallas, 1811)*	TR	M	O	↓	VU
116	Accipitridae	Booted Eagle	*Hieraaetus pennatus (J.F. Gmelin, 1788)*	TR	M	O	→	LC
117	Accipitridae	Steppe Eagle	*Aquila nipalensis (Hodgson, 1833)*	TR	M	O	↓	EN
118	Accipitridae	Eastern Imperial Eagle	*Aquila heliaca (Savigny, 1809)*	TR	M	O	↓	VU
119	Accipitridae	Western Marsh Harrier	*Circus aeruginosus (Linnaeus, 1758)*	WA	M	U	→	LC
120	Accipitridae	Pallid Harrier	*Circus macrourus (S.G. Gmelin, 1770)*	TR	M	R	↓	NT
121	Accipitridae	Montagu's Harrier	*Circus pygargus (Linnaeus, 1758)*	TR	M	O	↓	LC

Appendix 1. Contd.

Sr. No.	Family	English Name	Scientific Name	Habitat	Residential Status	Occurrence	Global Trend	IUCN Status
122	Accipitridae	Shikra	*Accipiter badius (J.F. Gmelin, 1788)*	TR	Re	U	→	LC
123	Accipitridae	Black Kite	*Milvus migrans (Boddaert, 1783)*	TR	Re	O	→	LC
			Order: Strigiformes					
124	Tytonidae	Common Barn Owl	*Tyto alba (Scopoli, 1769)*	TR	Re	R	→	LC
125	Strigidae	Spotted Owlet	*Athene brama (Temminck, 1821)*	TR	Re	C	→	LC
			Order: Coraciiformes					
126	Alcedinidae	Common Kingfisher	*Alcedo atthis (Linnaeus, 1758)*	WA	Re	C	?	LC
127	Alcedinidae	White-throated Kingfisher	*Halcyon smyrnensis (Linnaeus, 1758)*	WA	Re	C	↑	LC
128	Alcedinidae	Pied Kingfisher	*Ceryle rudis (Linnaeus, 1758)*	WA	Re	U	?	LC
129	Meropidae	Green Bee-eater	*Merops orientalis (Latham, 1801)*	TR	Re	C	↑	LC
130	Meropidae	Blue-cheeked Bee-eater	*Merops persicus (Pallas, 1773)*	TR	M	R	→	LC
131	Meropidae	Blue-tailed Bee-eater	*Merops philippinus (Linnaeus, 1767)*	TR	Re	R	→	LC
132	Coraciidae	European Roller	*Coracias garrulus (Linnaeus, 1758)*	TR	M	R	↓	LC
133	Coraciidae	Indian Roller	*Coracias benghalensis (Linnaeus, 1758)*	TR	Re	O	↑	LC
			Order: Piciformes					
134	Megalaimidae	Coppersmith Barbet	*Psilopogon haemacephalus (Statius Muller, 1776)*	TR	Re	U	↑	LC
135	Picidae	Eurasian Wryneck	*Jynx torquilla (Linnaeus, 1758)*	TR	M	R	↓	LC
			Order: Falconiformes					
136	Falconidae	Common Kestrel	*Falco tinnunculus (Linnaeus, 1758)*	TR	M	O	↓	LC
137	Falconidae	Red-necked Falcon	*Falco chicquera (Daudin, 1800)*	TR	Re	O	↓	NT
138	Falconidae	Peregrine Falcon	*Falco peregrinus (Tunstall, 1771)*	TR	M	O	↑	LC
			Order: Psittaciformes					
139	Psittaculidae	Alexandrine Parakeet	*Psittacula eupatria (Linnaeus, 1766)*	TR	Re	O	↓	NT
140	Psittaculidae	Rose-ringed Parakeet	*Psittacula krameri (Scopoli, 1769)*	TR	Re	C	↑	LC
			Order: Passeriformes					
141	Dicruridae	Black Drongo	*Dicrurus macrocercus (Vieillot, 1817)*	TR	Re	C	?	LC
142	Laniidae	Isabelline Shrike	*Lanius isabellinus (Hemprich & Ehrenberg, 1833)*	TR	M	O	→	LC
143	Laniidae	Long-tailed Shrike	*Lanius schach (Linnaeus, 1758)*	TR	Re	C	?	LC
144	Laniidae	Bay-backed Shrike	*Lanius vittatus (Valenciennes, 1826)*	TR	Re	O	→	LC

Appendix 1. Contd.

Sr. No.	Family	English Name	Scientific Name	Habitat	Residential Status	Occurrence	Global Trend	IUCN Status
145	Corvidae	Rufous Treepie	*Dendrocitta vagabunda (Latham, 1790)*	TR	Re	U	↓	LC
146	Corvidae	Large-billed Crow	*Corvus macrorhynchos (Wagler, 1827)*	TR	Re	C	→	LC
147	Corvidae	House Crow	*Corvus splendens (Vieillot, 1817)*	TR	Re	C	→	LC
148	Alaudidae	Rufous-tailed Lark	*Ammomanes phoenicura (Franklin, 1831)*	TR	Re	U	→	LC
149	Alaudidae	Ashy-crowned Sparrow Lark	*Eremopterix griseus (Scopoli, 1786)*	TR	Re	U	→	LC
150	Alaudidae	Sand Lark	*Alaudala raytal (Blyth, 1845)*	TR	Re	C	→	LC
151	Alaudidae	Crested Lark	*Galerida cristata (Linnaeus, 1758)*	TR	Re	C	↓	LC
152	Alaudidae	Sykes's Lark	*Galerida deva (Sykes, 1832)*	TR	Re	C	→	LC
153	Cisticolidae	Common Tailorbird	*Orthotomus sutorius (Pennant, 1769)*	TR	Re	U	→	LC
154	Cisticolidae	Grey-breasted Prinia	*Prinia hodgsonii (Blyth, 1844)*	TR	Re	O	→	LC
155	Cisticolidae	Delicate Prinia	*Prinia lepida (Blyth, 1844)*	TR	Re	R	?	
156	Cisticolidae	Jungle Prinia	*Prinia sylvatica (Jerdon, 1840)*	TR	Re	R	↓	LC
157	Cisticolidae	Ashy Prinia	*Prinia socialis (Sykes, 1832)*	TR	Re	U	→	LC
158	Cisticolidae	Plain Prinia	*Prinia inornata (Sykes, 1832)*	TR	Re	C	→	LC
159	Cisticolidae	Zitting Cisticola	*Cisticola juncidis (Rafinesque, 1810)*	WA	Re	U	↑	LC
160	Acrocephalidae	Booted Warbler	*Iduna caligata (M.H.C. Lichtenstein, 1823)*	TR	M	O	↑	LC
161	Acrocephalidae	Sykes's Warbler	*Iduna rama (Sykes, 1832)*	TR	M	O	→	LC
162	Acrocephalidae	Paddyfield Warbler	*Acrocephalus agricola (Jerdon, 1845)*	TR	M	R	↓	LC
163	Acrocephalidae	Blyth's Reed Warbler	*Acrocephalus dumetorum (Blyth, 1849)*	WA	M	R	↑	LC
164	Acrocephalidae	Clamorous Reed Warbler	*Acrocephalus stentoreus (Hemprich & Ehrenberg, 1833)*	WA	Re	U	→	LC
165	Hirundinidae	Sand Martin	*Riparia riparia (Linnaeus, 1758)*	WA	M	O	?	LC
166	Hirundinidae	Dusky Crag Martin	*Ptyonoprogne concolor (Sykes, 1832)*	TR	Re	U	↑	LC
167	Hirundinidae	Barn Swallow	*Hirundo rustica (Linnaeus, 1758)*	TR	M	U	↓	LC
168	Hirundinidae	Wire-tailed Swallow	*Hirundo smithii (Leach, 1818)*	TR	Re	U	↑	LC
169	Hirundinidae	Red-rumped Swallow	*Cecropis daurica (Laxmann, 1769)*	TR	Re	O	→	LC
170	Hirundinidae	Streak-throated Swallow	*Petrochelidon fluvicola (Blyth, 1855)*	TR	Re	O	↑	LC
171	Pycnonotidae	Red-vented Bulbul	*Pycnonotus cafer (Linnaeus, 1766)*	TR	Re	C	↑	LC
172	Pycnonotidae	White-eared Bulbul	*Pycnonotus leucotis (Gould, 1836)*	TR	Re	U	↓	LC
173	Phylloscopidae	Common Chiffchaff	*Phylloscopus collybita (Vieillot, 1817)*	TR	M	R	↑	LC
174	Sylviidae	Lesser Whitethroat	*Curruca curruca (Linnaeus, 1758)*	TR	M	R	→	LC
175	Sylviidae	Eastern Orphean Warbler	*Curruca crassirostris (Cretzschmar, 1830)*	TR	M	R	↑	LC
176	Zosteropidae	Indian White-eye	*Zosterops palpebrosus (Temminck, 1824)*	TR	Re	U	↓	LC
177	Leiothrichidae	Common Babbler	*Argya caudata (Dumont, 1823)*	TR	Re	C	→	LC
178	Leiothrichidae	Large Grey Babbler	*Argya malcolmi (Sykes, 1832)*	TR	Re	U	→	LC
179	Sturnidae	Rosy Starling	*Pastor roseus (Linnaeus, 1758)*	TR	M	U	?	LC
180	Sturnidae	Brahminy Starling	*Sturnia pagodarum (J.F. Gmelin, 1789)*	TR	Re	U	?	LC
181	Sturnidae	Common Myna	*Acridotheres tristis (Linnaeus, 1766)*	TR	Re	C	↑	LC

Appendix 1. Contd.

Sr. No.	Family	English Name	Scientific Name	Habitat	Residential Status	Occurrence	Global Trend	IUCN Status
182	Sturnidae	Bank Myna	*Acridotheres ginginianus (Latham, 1790)*	WA	Re	C	↑	LC
183	Muscicapidae	Indian Robin	*Copsychus fulicatus (Linnaeus, 1766)*	TR	Re	C	→	LC
184	Muscicapidae	Oriental Magpie Robin	*Copsychus saularis (Linnaeus, 1758)*	TR	Re	U	→	LC
185	Muscicapidae	Bluethroat	*Luscinia svecica (Linnaeus, 1758)*	WA	M	O	→	LC
186	Muscicapidae	Black Redstart	*Phoenicurus ochruros (S.G. Gmelin, 1774)*	TR	M	R	↑	LC
187	Muscicapidae	Siberian Stonechat	*Saxicola maurus (Pallas, 1773)*	TR	M	U	?	LC
188	Muscicapidae	Pied Bushchat	*Saxicola caprata (Linnaeus, 1766)*	TR	Re	O	→	LC
189	Muscicapidae	Isabelline Wheatear	*Oenanthe isabellina (Temminck, 1829)*	TR	M	O	→	LC
190	Muscicapidae	Desert Wheatear	*Oenanthe deserti (Temminck, 1825)*	TR	M	O	→	LC
191	Nectariniidae	Purple Sunbird	*Cinnyris asiaticus (Latham, 1790)*	TR	Re	C	→	LC
192	Ploceidae	Baya Weaver	*Ploceus philippinus (Linnaeus, 1766)*	TR	Re	O	→	LC
193	Estrildidae	Indian Silverbill	*Euodice malabarica (Linnaeus, 1758)*	TR	Re	U	→	LC
194	Passeridae	House Sparrow	*Passer domesticus (Linnaeus, 1758)*	TR	Re	C	↓	LC
195	Passeridae	Chestnut-shouldered Petronia	*Gymnoris xanthocollis (E. Burton, 1838)*	TR	Re	R	→	LC
196	Motacillidae	Grey Wagtail	*Motacilla cinerea (Tunstall, 1771)*	WA	M	O	→	LC
197	Motacillidae	Western Yellow Wagtail	*Motacilla flava (Linnaeus, 1758)*	WA	M	U	↓	LC
198	Motacillidae	Citrine Wagtail	*Motacilla citreola (Pallas, 1776)*	WA	M	U	↑	LC
199	Motacillidae	White-browed Wagtail	*Motacilla maderaspatensis (J.F. Gmelin, 1789)*	WA	Re	R	→	LC
200	Motacillidae	White Wagtail	*Motacilla alba (Linnaeus, 1758)*	WA	M	O	→	LC
201	Motacillidae	Tawny Pipit	*Anthus campestris (Linnaeus, 1758)*	TR	M	O	→	LC
202	Motacillidae	Paddyfield Pipit	*Anthus rufulus (Vieillot, 1818)*	TR	Re	C	→	LC
203	Motacillidae	Long-billed Pipit	*Anthus similis (Jerdon, 1840)*	TR	M	R	→	LC
			Order: Bucerotiformes					
204	Upupidae	Eurasian Hoopoe	*Upupa epops (Linnaeus, 1758)*	TR	M	O	↓	LC

Note: ↑ Increasing, → Stable, ↓ Decreasing, ? Unknown; Re-Residential, M-Migratory; C-Common, U-Uncommon, O-Occasional, R-Rare; IUCN-International Union for Conservation of Nature and Natural Resources; EN-Endangered, VU-Vulnerable, NT-Near Threatened, LC-Least Concern

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Research Article

Major Royal Jelly Protein 2 (*mrjp2*) Gene Detection in *Apis dorsata* Fabricius, 1793, *Apis dorsata binghami* Cockerell, 1906, *Apis florea* Fabricius, 1787, and *Apis nigrocincta* Smith, 1860

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ABSTRACT
Indonesian people's interest in honey, the product from honey bees, is quite high. It caused many cases of honey fraud such as mislabelling the entomological origin of honey. The Major Royal Jelly Protein 2 (*mrjp2*) gene, which encodes MRJP, can be used to determine the entomological origin of honey. The *mrjp2* gene, for example, can be detected in honey from *A. mellifera* and *A. cerana* using species-specific primers for *A. mellifera* (MF-MR) and *A. cerana* (CF-CR). This study aims to detect the *mrjp2* gene in several honey bee species native to Indonesia, namely *A. dorsata*, *A. dorsata binghami*, *A. florea*, *A. nigrocincta*, *A. mellifera*, and *A. cerana* as well as analyse the feasibility of MF-MR and CF-CR primers in determining the entomological origin of honey. The results showed that the MF-MR primers can amplify the DNA of A. *dorsata binghami*, *A. florea*, and *A. mellifera*, while CF-CR primers can amplify the DNA of both *A. nigrocincta* and *A. cerana*. The amplicons were subsequently sequenced. The phylogenetic tree and the genetic distance showed that there were differences and variation between each species of honey bee samples with the honey bee database. The data obtained from this research indicated that both primers could not determine the entomological origin of honey directly up to species level. The species level determination will only be possible using sequences information. However, in certain situations, the MF-MR and CF-CR primers were able to differentiate the honey bee species by including the information of the geographical origin of honey sample and the distribution area of each species of honey bees in Indonesia.

INTRODUCTION
Honey bees are insects that are members of the order Hymenoptera, the family Apidae, the subfamily Apinae, the tribe Apini, and the genus *Apis* (ITIS 2020). Honey bees are divided into two groups based on the type of hive in where they live: open nesting honey bees and cavity nesting honey bees (Hadisoesilo 2001). There are numerous species of honey bees found across Indonesia. In Indonesia, there are at least seven species of honey bees: *A. andreniformis*, *A. florea*, *A. dorsata*, *A. cerana*, *A. koschevni-*

kovi, *A. nigrocincta*, and *A. mellifera*. *A. andreniformis* is distributed throughout the western part of Indonesia as far as Makassar Strait in Sumbawa and westernmost Flores (Engel 2012). *A. dorsata* has a wide distribution and is commonly found in Indonesia, except Maluku and Papua (Hadisoesilo 2001). *A. dorsata* has three subspecies with two subspecies distributed in Indonesia, namely *A. dorsata dorsata* that is distributed in west part of Wallacea line and *A. dorsata binghami* that is distributed only in Sulawesi Island (Sakagami et al. 1980; Nagir et al. 2016). *A. cerana* is a honey bee that is widely distributed in Asia. One subspecies of *A. cerana*, *A. cerana nuluensis* can be found in Sabah, Kalimantan. *A. koschevnikovi* can be found on the Malay Peninsula, and throughout Kalimantan, Sumatra, and Java. *A. nigrocincta* is an endemic honey bee found on Sangihe Island, and Sulawesi. *A. mellifera* is a honey bee species introduced to Indonesia (Hadisoesilo 2001; Engel 2012).

Honey has been used by people from ancient times to serve nutritional needs and as a therapy. Honey is proven to be beneficial for health since it functions as an antioxidant, anti-inflammatory, antibacterial, antidiabetic, and overcomes many health problems. The macro and micronutrient of honey affected by various factors, such as bee type, flower source, and environmental and processing factors (Samarghandian et al. 2017; Ranneh et al. 2021).

Honey is commonly used by people all over the world as a natural product with numerous benefits. Because of the strong demand for honey, there have been examples of honey fraud involving modifying the composition and mislabelling the entomological origin of honey. Honey fraud is defined by Apimondia (International Federation of Beekeepers' Associations) as an illegal and purposeful act committed to gain an unfair economic advantage by manipulating and selling honey that do not match global honey standards. Honey derived from *A. mellifera* and *A. cerana* honey bees is often adulterated by mislabelling *A. mellifera* honey as *A. cerana* honey or modifying the composition of honey by mixing *A. mellifera* honey with *A. cerana* honey. Due to the limited production of *A. cerana* honey bees and consumer interest, honey generated from *A. cerana* honey bees sells for up to five times the price of *A. mellifera* honey on the market (Zhang et al. 2019).

Such a case of honey fraud is also suspected to occur in Indonesia, which is adulteration of *A. mellifera* honey claimed to be forest honey. According to SNI 8664 (2018), forest honey is a natural liquid with a sweet taste produced by *A. dorsata* and/or *Apis* spp. from the juice of forest plant flowers (floral nectar) or other sections of forest plants (extra floral). Meanwhile, cultured honey is defined as a natural liquid with a sweet taste produced by cultivated bees *A. mellifera* or *A. cerana* from plant flower juice (floral nectar) or other plant components (extra floral).

Various methods have been used to determine the entomological origin of honey such as analysing the differences in molecular weight and surface structure of *mrjp1* (Won et al. 2008), analysis of honey bee protein profile using SDS-PAGE (Ramón-Sierra et al. 2015), and using *mrjp2* gene (Zhang et al. 2019). Major Royal Jelly Protein (MRJP) is the primary protein component in royal jelly, which represents approximately 90% of the total protein content (Drapeau et al. 2006). Major Royal Jelly Protein (MRJP) consists of nine types of proteins (MRJP1-MRJP9) with molecular weights of about 49 to 87 kDa and was encoded by 9 gene namely *mrjp1-mrjp9* gene (Drapeau et al. 2006; Buttstedt et al. 2013). Zhang et al. (2019) could determine the entomological origin of honey using species-specific primers of *A. mellifera* (MF-MR) and *A. cerana* (CF-CR) based on differences in *mrjp2* gene sequences in *A. mellifera* and *A. cerana*. Raffiudin

et al. (2023) have conducted research on the *mrjp2* gene in *A. mellifera* and *A. c. javana* from Indonesia and showed that CF and CR primers previously used in Zhang et al. (2019) research can amplify honey bee species *A. c. javana*.

In this study, following the previous study conducted by (Raffiudin et al. 2023; Zhang et al. 2019) this research will provide new information about the *mrjp2* gene present in honey bee species found in Indonesia such as *A. dorsata*, *A. dorsata binghami*, *A. florea*, and *A. nigrocincta*. New information provided by this research can be used to determine the entomological origin of honey sold in Indonesia as one way to avoid honey adulteration.

MATERIALS AND METHODS

Materials

All of the honey bee samples that used in this study were the collection of Laboratory of Entomology, Faculty of Biology, Universitas Gadjah Mada and were collected from Jambi, Central Java, East Java, and Central Sulawesi. All species samples that used in this research were identified molecularly based on 16S rRNA gene to ensure that it was identified correctly (unpublisihed data). In this study, *A. cerana* and *A. mellifera* were used as positive controls, and four species of honey bees namely, *A. dorsata*, *A. dorsata binghami*, *A. florea*, and *A. nigrocincta* were used as shown in Table 1. From each species, one honey bee samples were used for the analysis. In this study, species-specific primers of *mrjp2* gene for *A. mellifera* (MF-MR) and *A. cerana* (CF-CR) as shown in Table 2 were used.

Methods

The research method is designed based on Zhang et al. (2019) and Raffiudin et al. (2023) with some modification. This research was conducted from August 2022 to February 2023 in Laboratory of Genetic Engineering, Inter-University Centre, Universitas Gadjah Mada which included DNA extraction and isolation, DNA amplification and visualisation, and data analysis.

DNA extraction and isolation

DNA extraction and isolation was performed using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit according to the given protocol. One honey bee sample from each species were used to be extracted. The extraction procedure was carried out by cutting all the honey bee legs and grounding it in microcentrifuge tube using micropestle. The honey bee

Table 1. Honey bee samples' location.

Sample Code	Location	Species
AM	Kota Baru, Geragai, East Tanjung Jabung Regency, Jambi	*Apis mellifera*
AC	Petungkriyono, Pekalongan, Central Java	*Apis cerana*
AD	Petungkriyono, Pekalongan, Central Java	*Apis dorsata*
DB	Rogo Village, Central Sulawesi	*Apis dorsata binghami*
AF	Baluran National Park, East Java	*Apis florea*
AN	Bakubakulu Village, Central Sulawesi	*Apis nigrocincta*

Table 2. Species-specific primers of *mrjp2* gene for *A. mellifera* (MF-MR) and *A. cerana* (CF-CR) (Zhang et al. 2019)

Species	Primer	Sequence	Product size (bp)
Apis cerana	C-F	TTTAACAATAAAAATAATCAGAAGA	212
	C-R	TTACATCCTAATTGATTTTAATGCG	
Apis mellifera	M-F	GCCATCCCTTGAAATTGTCACTCGT	560
	M-R	TCTGCAAACGACCAATCAGGATAT	

samples were then added 200 mL FATG1 Buffer and homogenised with vortex. Next, 20 µL Proteinase K (10 mg/mL) was added to the samples and then the samples were incubated at 60°C for 3 hours and inverted 10X every 30 minutes. Following the incubation period, 200 µL of FATG2 buffer was added, vortexed, and incubated at 70°C for 10 minutes. The sample was then added with 200 µL of cold 96% ethanol drop by drop, then inverted 10X and transferred to the FATG Mini Column. The sample was then centrifuged for 1 minute at 12,000 rpm at 4°C. After transferring the sample to a new Collection Tube, 400 µL of buffer W1 was added and centrifuged for 1 minute at 12,000 rpm at 4°C. The solution collected in the collection tube was discarded and 750 µL of Wash Buffer was added before centrifuging at 12,000 rpm for 3 minutes at 4°C. The preheated elution buffer was then added 100 µL to the FATG Mini Column membrane and left for 3 minutes for complete absorption. The sample was then centrifuged at 12,000 rpm at 4°C for 2 minutes. The DNA extraction results were then quantified using nanodrops and stored in a refrigerator at 4°C.

DNA amplification and visualisation

DNA samples and control of honey bees extracted were amplified using the Polymerase Chain Reaction (PCR) method. PCR is carried out by making a 25 µL PCR cocktail consisting of 12.5 µL PCR Promega GoTaq Green Master Mix, 9.5 µL Nuclease Free Water, 1.5 µL primer, and 1.5 µL DNA template. The *mrjp2* gene was amplified with an initial pre-denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C (for samples with *A. mellifera* primer) and 47°C (for samples with *A. cerana* primer) for 30 s, extension at 72°C for 30 seconds, and final extension at 72°C for 5 minutes (Zhang et al. 2019; Raffiudin et al. 2023). After going through the PCR process, 1.2% agarose gel electrophoresis was used to separate the amplicon, which was then visualised with a UV transilluminator.

Data analysis

The amplified DNA samples were sent to the Integrated Research and Testing Laboratory, Universitas Gadjah Mada for sequencing. The forward and reverse sequences from sequencing process were then edited using MEGA11 software. The consensus sequences were then analysed with BLAST-N on the NCBI website (http://blast.ncbi.nlm.nih.gov/) to find sequence similarities. The sequence results were also processed for phylogenetic tree analysis after being analysed with BLAST-N. The sequence results and the database sequence from GenBank were analysed using MEGA11 software and were aligned using ClustalW. The alignment result was then used in phylogenetic tree construction. The phylogenetic tree was constructed using the Maximum Likelihood (ML) algorithm with a bootstrap value of 1000.

RESULTS AND DISCUSSION

DNA amplification

DNA derived from four honey bee samples (*A. dorsata*, *A. dorsata binghami*, *A. florea*, and *A. nigrocincta*) and two control honey bees (*A. mellifera* and *A. cerana*) were successfully amplified using two primers, namely species-specific primers for *A. mellifera* (MF-MR) and species-specific primers for *A. cerana* (CF-CR) The concentration of extracted DNA used for amplification in this study ranged from 29.67-237.14 ng/µL with purity ranging from 1.914-2.195 (A260/230) and 1.703-2.079 (A260/280) as

shown in Table 3. The purity of the DNA that used in this research were good based on Abdel-Latif & Osman (2017) which is the range of good DNA purity 1.8 – 2 (A260/A280) and 2 – 2,2 (A260/230).

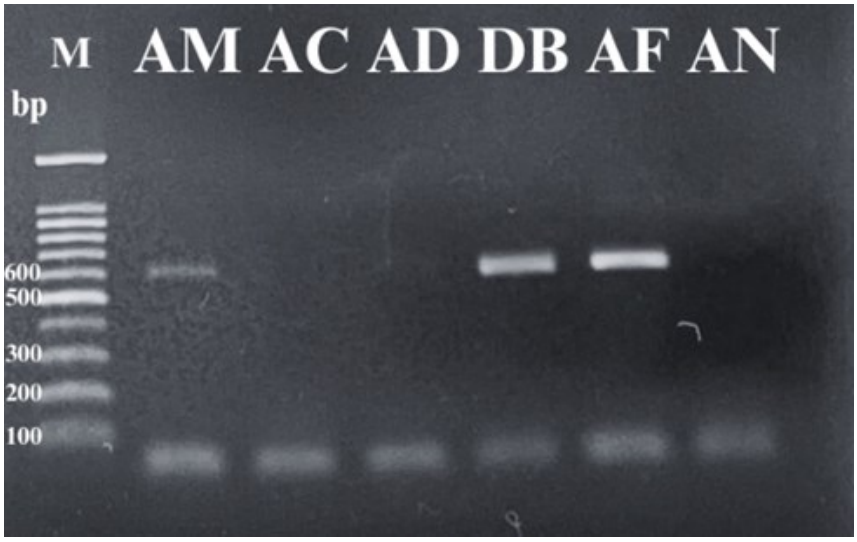

Figure 1. Results of DNA amplification in honey bees with MF-MR primer at annealing temperature 53°C. M: Marker, AM: *A. mellifera*, AC: *A. cerana*, AD: *A. dorsata*, DB: *A. dorsata binghami*, AF: *A. florea*, AN: *A. nigrocincta*

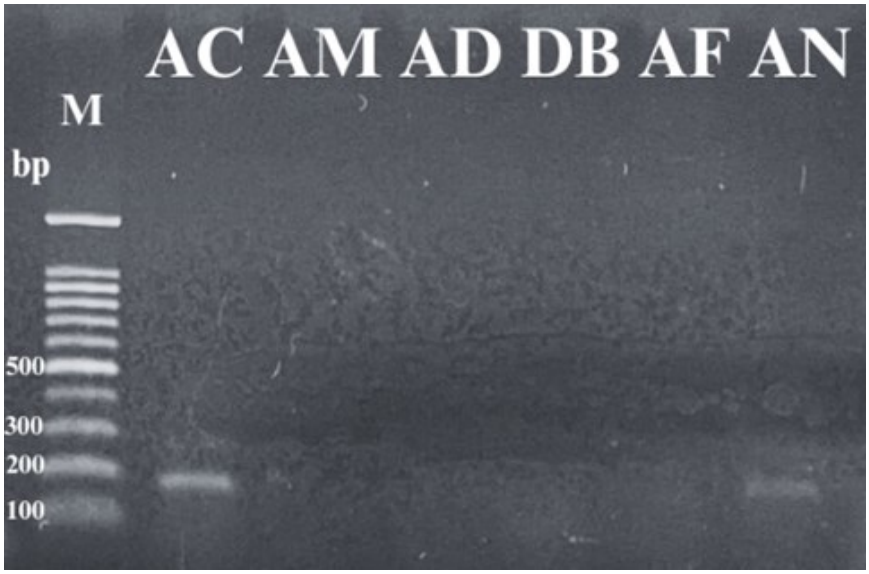

Figure 2. Results of DNA amplification in honey bees with CF-CR primer at annealing temperature 47°C. M: Marker, AC: *A. cerana*: AM: *A. mellifera*, AD: *A. dorsata*, DB: *A. dorsata binghami*, AF: *A. florea*, AN: *A. nigrocincta*

From four honey bee samples and two honey bee sample as control, this research succeeds in yielding DNA from three honey bee species, which are *A. dorsata binghami*, *A. florea*, and *A. mellifera* using species-specific primer of *mrjp2* gene for *A. mellifera* (MF-MR) at the annealing

Table 3. DNA extraction results of honey bee samples.

Species	Sample Code	A260/230	A260/280	Concentration (ng/µL)
Apis mellifera	AM	2,193	1,829	237,14
Apis cerana	AC	2,027	1,972	38,53
Apis dorsata	AD	2,049	2,079	105,60
Apis dorsata binghami	DB	2,195	1,982	187,88
Apis florea	AF	2,035	1,851	29,67
Apis nigrocincta	AN	1,914	1,703	53,25

temperature 53°C (Figure 1). This research also succeeded in yielding DNA from two honey bee species, which are *A. nigrocincta* and *A. cerana* using species-specific primers of *mrjp2* gene for *A. cerana* (CF-CR) at the annealing temperature 47°C (Figure 2). Raffiudin et al. (2023) was successfully amplified the DNA of *A. mellifera* using the MF-MR primer at the annealing temperature 50, 53, 55, 57, and 59°C, while the DNA of *A. cerana* was successfully amplified at the annealing temperature 47, 50, 53, and 55°C. The amplicon of the *mrjp2* gene in MF-MR primers was obtained at a size about 600 bp, while in CF-CR primers it was obtained at a size about 200 bp. The amplicon size of the results was as predicted as the product size of MF-MR primers and CF-CR primers which is 560 bp and 212 bp (Zhang et al. 2019). Visualisation of amplicon obtained in both MF-MR and CF-CR primers can be seen that the amplicon band is clearly visible and is a single band.

Based on these results, it can be concluded that the MF-MR primers can amplify not only *A. mellifera* DNA but also *A. dorsata binghami* and *A. florea* DNA, whereas CF-CR primers can amplify *A. cerana* and A. *nigrocincta* DNA from the cerana honey bee group. The results of this study agreed with Zhang et al. (2019) and Raffiudin et al. (2023) that the DNA from *A. cerana* could not be amplified using the MF-MR primers and the DNA from *A. mellifera* could not be amplified using the CF-CR primers. Consequently, the MF-MR primers are also species-specific for *A. dorsata binghami* and *A. florea* while the CF-CR primers are also species -specific for *A. nigrocincta*.

Based on the BLAST-N result of honey bee samples, it was found that the sequence of the controlled honey bees which are *A. mellifera* and *A. cerana*, were confirmed to be the sequence of the *mrjp2* gene from *A. mellifera* and *A. cerana* available in database, as well as the sequence of honey bee samples *A. florea* which was confirmed to be the sequence of the *mrjp2* gene from *A. florea* available in database. The sequence of the *mrjp2* gene from *A. mellifera* sample was showing homology with the *A. mellifera mrjp2* sequence from Korea (GQ160519.1) with 96.58% percent identity. The sequence of the *mrjp2* gene from *A. dorsata binghami* sample was showing homology with the *A. dorsata mrjp5-like* sequence from Indonesia and Thailand (XM_031510778.1) with 98.67% percent identity. The sequence from *A. dorsata binghami* sample was showing homology with the *A. florea mrjp2* sequence from Korea (XM_003695113.3) with 95.45% percent identity. The sequence of the *mrjp2* gene from *A. florea* sample was showing homology with the *A. florea mrjp2* sequence from Korea (XM_003695113.3) with 98.34% percent identity. The sequence of the *mrjp2* gene from *A. cerana* sample was showing homology with the *A. cerana cerana mrjp2* sequence from China (AY392758.1) with 95.48% percent identity as well as *A. nigrocincta* sample showing 93.14% percent identity with the *A. cerana cerana* mrjp2 sequence from China (AY392758.1).

The only DNA that was unable to be amplified with two primers was *A. dorsata*. Meanwhile, the subspecies of *A. dorsata*, which is *A. dorsata binghami* was amplified by MF-MR primers. To ensure that the DNA of *A. dorsata* could not be amplified using MF-MR primer, we used the other DNA template of *A. dorsata* (AD1) with DNA concentration about 1.83 ng/µL and purity 2.031 (A260/230) and 1.970 (A260/280). The result showed that the DNA of *A. dorsata* (AD1) was also not amplified using MF-MR primers. In the results of the MF-MR Primer-BLAST, it was found that the sequence of MF-MR primers did not attach to any *A. dorsata* sequence and only attached to the *mrjp2* gene sequence of *A. mellifera* and *A. florea*. Meanwhile, the CF-CR Primer-BLAST only attached

to the *mrjp2* gene sequence of *A. cerana cerana*. The alignment results of MF-MR primers and CF-CR primers with the *mrjp2* gene sequence of *A. dorsata* (KY087957.1) showed that the primer sequences were not completely attached to *A. dorsata* sequence so that it can be confirmed that the MF-MR and CF-CR primers could not amplified the DNA of *A. dorsata*.

Sequences Differences Analysis Between Honey Bee Samples and Reference Sequences

Amplicons of *A. dorsata binghami*, *A. florea*, and *A. nigrocincta* produced final product size in 586, 600, and 187 bp respectively. Meanwhile, *A. mellifera* and *A. cerana* produced final product size in 619 and 186 bp respectively. The sequence results of this research were shown in Table 5. The samples and controls sequence obtained were aligned with the database sequence of *mrjp1-mrjp9* of *A. mellifera* and *mrjp2* of *A. cerana cerana* available in NCBI using ClustalW in MEGA11 software. The phylogenetic tree was then constructed using Maximum Likelihood (ML) algorithm with a bootstrap value of 1000 and a sequence from *Bombus ignitus* (EU391535.1) as the outgroup. The Maximum Likelihood (ML) algorithm is a phylogenetic construction method that looks for the model with the highest likelihood of producing the input sequence under a certain evolutionary model (Munjal et al. 2019).

Based on the phylogenetic tree between honey bee samples and honey bee database (Figure 3), the three honey bee samples amplified using MF-MR were incorporated into three different clades, which are clade 1, clade 2, and clade 4. The *mrjp2* gene of honey bee sample *A. florea* were incorporated in clade 1 with the *mrjp2* gene of *A. florea* (XM_003695113.3) with genetic distance 0.017 or 1.7% and showing high similiarities which value 99%. Also, the *mrjp2* gene of honey bee sample *A. florea* showing similiarities with *A. florea* (XM_031917464.1) with genetic distance 0.066 or 6.6%. The honey bee sample *A. mellifera* were incorporated in clade 2 with the *mrjp2* gene of *A. mellifera* (NM_001011580.1), showing high similarities which value 97% and genetic distance 0.039 or 3.9%. Meanwhile, the *mrjp2* gene of honey bee sample *A. dorsata binghami* were incorporated with the *mrjp5-like* gene of *A. dorsata* (XM_031510778.1) in clade 4. The *mrjp2* gene of *A. dorsata binghami* and the *mrjp5-like* gene of *A. dorsata* showing high similiarities with which value 99% and low genetic distance 0.013 or 1.3%.

The two honey bee samples that amplified using CF-CR primers were incorporated in clade 6 with the *mrjp3* gene of *A. cerana* (AY663105.1) and the *mrjp3-like* gene of *A. cerana cerana* (AY394726.1). The *mrjp2* gene of honey bee sample *A. cerana* showing genetic distance 1.625 to the *mrjp3* gene of *A. cerana* (AY663105.1) and 2.056 to the *mrjp3 -like* gene of *A. cerana cerana* (AY394726.1). Meanwhile, the *mrjp2* gene of honey bee sample *A. nigrocincta* showing genetic distance 1.503 to the *mrjp3* gene of *A. cerana* (AY663105.1) and 2.230 to the *mrjp3-like* gene of *A. cerana cerana* (AY394726.1). The honey bee samples *A. cerana* and *A. nigrocincta* showing high similiarities which value 98% with genetic distance 0.195 or 19.5%.

Feasibility Analysis of *Apis mellifera* Species-Specific Primers (MF-MR) and *Apis cerana* Species-Specific Primers (CF-CR) in Determining the Entomological Origin of Honey

In this study, the MF-MR primers can amplify the DNA of other honey bee species besides *A. mellifera*, which are *A. dorsata binghami* and *A. florea*. In this case it can be said that the MF-MR primers cannot differenti-

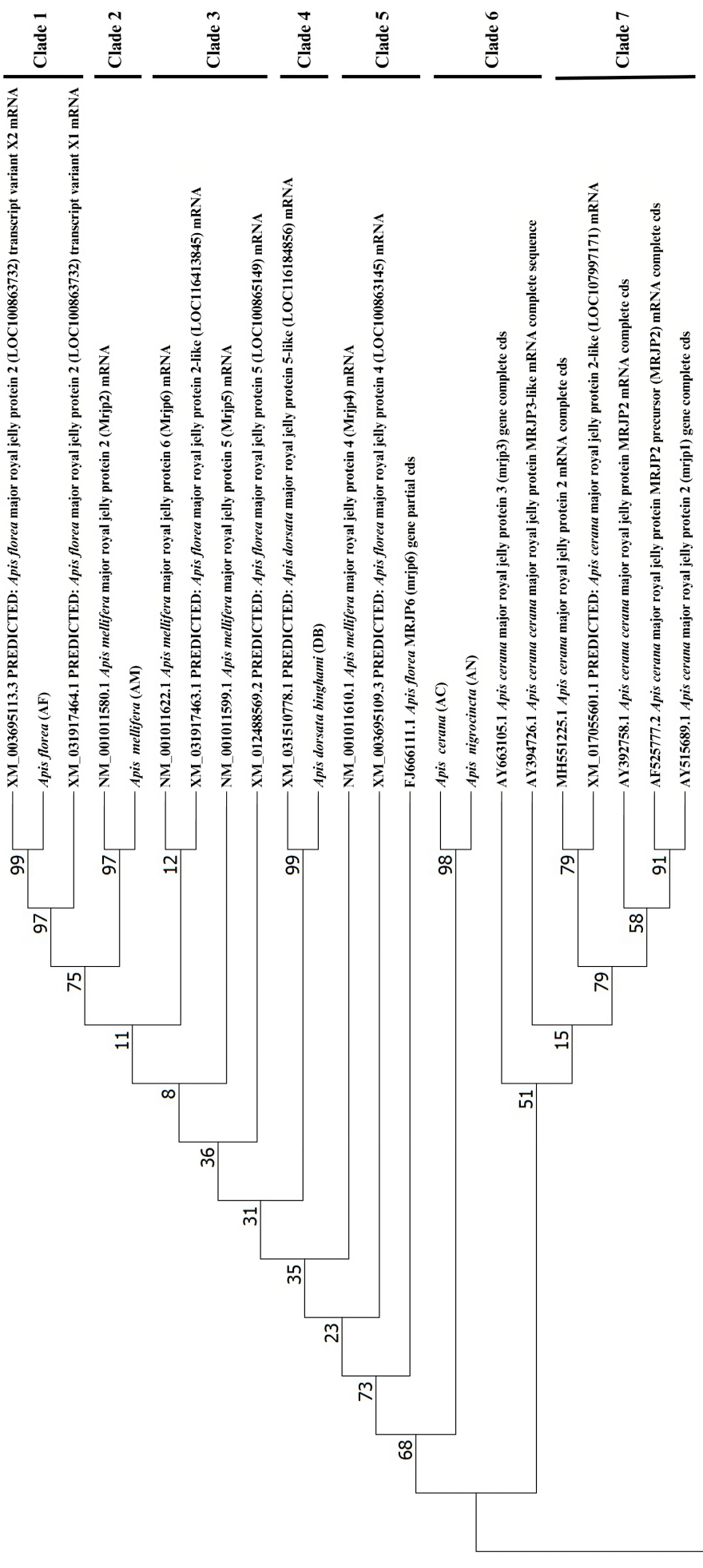

Figure 3. Phylogenetic tree of *mrjp2* gene of honey bee samples and *mrjp* gene database based on Maximum Likelihood (ML) algorithm with 1000 bootstrap value.

Table 4. The *mrjp2* gene sequence results of honey bee samples.

Apis mellifera (AM)
TGCCATCCCTTGAAATTGTCACTCGTAAAATATTCGCAGTATA- TAAGTAAGTGTTTCTAATTGTGTCGGTTGCAGCATTTATTTGCAATCTTTCATTTATCTGCATAC AAAAAAGATATTTTATTTAAGAAAAATGACAAGGTGGTTGTTCATGGTGGCATGCCTCGGCATAG CTTGTCAAGGCGCCATTGTTCTAGAAAATTCTCCAATAAACTTGGAAAAATCCTTGAACGTAATTC ACGAATGGATTTATTTTGATTATGACTTCGGTAGCGAAGAAAGAAGACAAGCTGCGATTCAATCT GGCGAATATGACCATACGAAAAATTATCCCTTCGACGTCGATCAATGGCGTGGTAAAATTTTCTTA TTTTAAATTATTAATCCATTTCCAATCGTCGAAACACTTAATATTCAATAATTTTCGTCGCTCATA TTTCTTCATTTTTGAATAACTAAAAGGATATTTCACGTTTTGTATGTCTTGTTTAAAATAAGACT TTTGTCACCATACTAAGATACGATGGTGTTCCTTCTACTTTGAACGTGATATCTGGTAAAACTGG TAATGCTGTACGTCAGACGATTGCATATCCTGATTGGACGTTTTGCAGAATCT
Apis cerana (AC)
CTTTTACATCCTAATTGATTTTAATGCGATTTTGAAGAACGACGAACTTGAT- TATCATTCTGATTGTTAGGATTCTGATTGTTATTTTTCTGATTGTTATTCTTCTGATTGTTATTC TTCTGATTGTTATTGTTCTGATTGTTATTCTTCTGATTGTTATTCTTCTGATTATTTTTATTGTT AAAA
Apis dorsata binghami (DB)
TGCCATCCCTTGAAATTGTCACTCGTAAAATATTTGCAGCATCCTACGTAAGTGTTTCTA- TATATCTTAATTGTATTTATTTGCAATCTTTCATTTGTCTAACACCCATTTAATATTTTATAGAAA AATGACAAACTGGTTACTATTGATAGTGTGTCTTGGCATAGCTTGTCAAGGTATCACAGGCGCTG TTGTTCGAGAAAATTCCTCGAGAAAAAACTTGACAAATTCGAAGAACGTGATTTACGAATGGAAG TATCTTGAATATGACTTCAGTAACGCGATTCAATCTGACGAATATGACCACACAAAAAATTATCCT TTCGATATCGATCAATGGCATGGTAAAATATTCGCATTTCATTTAATATTACATTTTATTTATCAA ATCAATTTAATATTCAATTATTTTTGTCATTCAAATTTTTTTATTTTTGTATCGTTATAAAATATC AAACATTTTGCATTTCTTCAAGATAAGATTTTTGTCGCCGTACCAATAAATTAAGGTGTTACCTTC CTCTTTAAATATGATATCTGACAAAACTGGGTAAGGTAGACGCTCTTCAGGAAATCCAGGGTGAA G
Apis florea (AF)
GCCATCCCTTGAAATTGTCACTCGTAAAATATCTGCCGCACACCCCCCTAACGTCCTTAA- TATATTTCAATTGTAATATTTATTTTCATTCTTTTAATCTATCAAAACGAAATATTTTATAGAAAA ATGATAAAGTGGGTGTTTATGGTGGCATGCCTTGGCATAACTTGTCAAGGCGCAACTGTTCGAGA AAATTCTGCAAAAAACTTGGAAAATTCTTTGAACATAATTCACGAATGGAAATATATTGATTATGA TTTCGGTAGCGACGAAAGAAGACAAGCTGCGATTCAATCTGGCGAATATGACTATAGAAGAAATT ATCCCTTCGATGTCGATCAATGGCGTGGTAAAATTTTCGTATTTTAAATTATTATTGCATTCTGA TCGTCGAAACATTTAATATTCAATAATTTTCATCATTCATATTCCTTCATTTTTGAATCATTAAAA ATGATATTCCACTTCTTGTTTAAGATAAGACTTTTGTCACCGTATTAAGATACGATGGTGTGCCT TCTTCTTTGAACCGTGATATCTGAGAAAAATGGTAAGAGAGCGAGAAAGCCACAACCATATCCTGA TTGGTCGTTTGCATAA
Apis nigrocincta (AN)
CATTTTACATCGACTTGATTATCATTCTGATTGATTAGTATTCTGATTGTTAG- TATTCTGAACTTTGTTATTCTTCTGATTGTTATTCTTCTGATTGTTATTCTTCTGATTGTTATTT TTCTGATTGTTATTTTTCTGATTGTTATTCTTCTGATTGTTATTTTTCTGATTGTTTTTATTGTT AAAA

ate whether a honey bee specimen is an *A. mellifera*, *A. dorsata binghami*, or *A. florea*. However, MF-MR primers can differentiate honey bees that belong to the cerana group, *A. cerana* and *A. nigrocincta* because the DNA of the two honey bees are not amplified using MF-MR primers. Meanwhile, the CF-CR primers themselves could not differentiate honey bees from the Cerana Group, which are *A. cerana* and *A. nigrocincta*. The MF-MR primers can amplify the DNA of other honey bee species beside the *A. mellifera* species, same with the CF-CR primers can amplify the DNA of other honey bee species beside the *A. cerana* species. The two primers showed that the primers were not species-specificied to one species because of the amplification process producing amplicons in the same size and after sequencing process results all amplicon produces sequence are

different. Based on this study, it can be said that the two primers cannot be used to differentiate honey bee species directly, and a sequencing process is needed to confirm the species of the honey bee sample.

The used of the MF-MR primer and CF-CR primer as a method to determine the entomological origin of honey can be compared with other primer such as the LR13107-F and LR12647-R primer (Thummajitsakul et al. 2013). The LR13107-F and LR12647-R primer were primers that can be used to identify honey bee species based on the 16S rRNA gene. In terms of determining the entomological origin of honey, we suggest that it is better to use the LR13107-F and LR12647-R primers because of the MF-MR and CF-CR primer, based on this study, could not determining the entomological origin of honey directly. To be able to find out the entomological origin of honey using MF-MR primers and CF-CR primers, after going through the PCR, it must go further through the sequencing process. If the sequencing process is required to distinguish the honey bee species, the used of the LR13107-F and LR12647-R primer would be an accurate method to determine the entomological origin of honey. Furthermore, if we're sequencing the 16S rRNA gene of a honey bee sample, we can discover the precise sequence on the database because of the 16S rRNA gene data is already available, as opposed to the *mrjp2* gene which is rarely utilised and hence more difficult to find on the database.

Indonesia has numerous honey bee species that are found all over the islands. Indonesia has at least seven species of honey bees that are found throughout the country, namely *A. andreniformis*, *A. florea*, *A. dorsata*, *A. cerana*, *A. koschevnikovi*, *A. nigrocincta*, and *A. mellifera* (Engel 2012). The large number of honey bee species distributed in Indonesia makes the determination of the entomological origin of honey from Indonesia more difficult. However, since each species of honey bee in Indonesia has a distinctive distribution area, by observing the information of the distribution area and geographical origin of honey, it is still possible to determine the entomological origin of honey.

Based on the research that has been done, it can be concluded that the feasibility of using MF-MR primer and CF-CR primer in determining the entomological origin of honey is feasible provided that the geographic origin of the honey is known. Because of the MF-MR primers and CF-CR primers could amplified the DNA of other honey bee species, the use of the two primers in determining the entomological origin of honey need to be evaluated. In the research of Zhang et al. (2019), MF-MR primers and CF-CR primers were able to determine the entomological origin of honey derived from *A. mellifera* and *A. cerana* because they only concern on the two species of honey bees, namely *A. mellifera* and *A. cerana*. This is different from Indonesia which has a variety of honey bee species so that MF-MR primers and CF-CR primers did not show the same results as in the previous study.

CONCLUSIONS

Based on the research that has been carried out, it is concluded that the species-specificied primers of *mrjp2* gene for *A. mellifera* (MF-MR) can detect the *mrjp2* gene not only in *A. mellifera* but also in *A. dorsata binghami* and *A. florea*. Meanwhile, the species-specific primers of *mrjp2* gene for *A. cerana* (CF-CR) can detect the *mrjp2* gene not only in *A. cerana* but also in *A. nigrocincta*. The MF-MR primers and CF-CR primers are feasible in determining the entomological origin of honey sold in the Indonesian market as long as the information of the geographical origin of honey sample and the distribution area of each species of honey bees in Indonesia are provided.

AUTHOR CONTRIBUTION

Y.D. collected and analysed the data and wrote the manuscript, H.P. designed the research and supervised all the processes.

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CONFLICT OF INTEREST

The authors declared there are no conflicts of interest regarding the research.

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Research Article

Mangosteen (*Garcinia mangostana* L.) Peel Decoction Effect on Embryological Development of Wader Pari Fish *Rasbora lateristriata* (Bleeker, 1854)

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ABSTRACT
Mangosteen (*Garcinia mangostana* L.) is a tropical fruit that has become a sought-after commodity by enthusiasts from various countries, including Indonesia. The active components found in mangosteen peel primarily consist of active xanthone compounds, such as mangostenol, mangostin, mangostino A, mangostino B, tvophylin B, trapezifolixanthone, alpha mangostin, beta mangostin, garcinon B, mangostano, as well as flavonoids epicatechin and gartanin. These compounds exhibit a range of beneficial properties, including anti-inflammatory, antibacterial, antifungal, antihistamine, antidiabetic, anticancer, and more. Consequently, there is significant potential in developing mangosteen peel extract as a valuable ingredient in herbal medicine. However, there is currently no available data on the effects of exposure to mangosteen peel decoction on fish animal models. Therefore, it is essential to investigate the impact of mangosteen peel decoction on wader pari fish *(Rasbora lateristriata)* embryos. In this study, wader pari embryos were subjected to various concentrations of mangosteen peel decoction (0.5, 1, 5, and 25 µg/mL). The effects on egg hatchability, survival rate (SR), heart rate frequency, and heart morphology of the larvae were meticulously examined using a Leica microscope. The data obtained were subjected to statistical analysis using one-way ANOVA. The findings demonstrated that exposure to mangosteen peel decoction resulted in lower hatching rates and embryonic survival, alongside an increased heart rate frequency. Additionally, the exposed embryos displayed cardiac edema and cardiac bending, particularly at the concentration of 25 µg/mL. In conclusion, the exposure of wader pari fish embryos to mangosteen peel decoction at the concentrations of 25 µg/mL and higher significantly affected the hatching rate, survival rate, and heart rate of *R. lateristriata* fish larvae.

INTRODUCTION
Natural treatment using herbal medicine is widely practiced in Indonesia. Herbal medicines consist of active ingredients derived from various parts of plants, which are processed into forms such as powders, pills, and liquids. The production of herbal remedies strictly avoids the use of synthetic chemicals, which is believed to contribute to the minimal side effects associated with herbal medicine (Pane et al. 2021).

The mangosteen fruit originates from a tropical evergreen plant and is extensively distributed in countries like Malaysia, Indonesia, India, Thailand, Singapore, and Sri Lanka. Mangosteen is known for its array of

beneficial properties, including anti-inflammatory, antibacterial, antifungal, antihistamine, antidiabetic, anticancer effects, among others (Kalick et al. 2023). Its active content primarily comprises xanthone compounds like mangostenol, mangostin, mangostino A, mangostino B, tvophylin B, trapezifolixanthone, alpha mangostin, beta mangostin, garcinon B, mangostano, as well as flavonoids such as epicatechin and gartanin (Rohman et al. 2019). Previous research has demonstrated significant potential of mangosteen fruit peel extract for use in herbal medicine formulations (Suttirak & Manurakchinakorn 2014).

Phytochemical screening tests conducted on a 95% ethanol extract of mangosteen (*Garcinia mangostana* L.) peel revealed the presence of flavonoid compounds, saponins, alkaloids, triterpenoids, tannins, and polyphenols. However, herbal medicines cannot be directly applied to humans without undergoing prior screening tests. The World Health Organization (WHO) (2005) stipulateds that substances used for medicinal purposes must undergo preclinical and clinical trials. This is consistent with the Regulations of the Minister of Health of the Republic of Indonesia Number 760/Menkes/Per/IX/1992, which mandated efficacy and safety examinations of plant ingredients before their application. Therefore, the utilisation of mangosteen peel for herbal purposes necessitates a toxicity test (Mustapa et al. 2018).

Suwignyo (2014) reported that mangosteen peel caused pericardial and brain abnormalities in zebrafish embryos, which could lead to lethality. *Rasbora lateristriata* is an Indonesian native fish that can adapt to and resist changes in extreme environmental conditions (Retnoaji et al. 2016). This freshwater fish is commonly used as a bioindicator of environmental pollution (Zakeyudin et al. 2012). Wader fish are prolific animals with the potential to be used as animal models in research (Zahro et al. 2022) because wader pari fish genetically exhibit faster embryo development than Danio rerio (Raharjeng & Retnoaji 2021). Thus, this study aimed to determine the toxic effects of mangosteen peel extract at various concentrations on wader pari fish embryos.

MATERIALS AND METHODS

Materials

The materials were wader pari fish from the Faculty of Biology wader pari cultivation, mangosteen (*Garcinia mangostana* L.) peel decoction, egg water media (1,000 mL water, 1.5 mL salt water, and 1–2 drops methylene blue), and distilled water (aquadest-Genera Labora).

Methods

The fish embryos were treated with the ingredients at blastula stage (Raharjeng et al. 2022). The following are the experiment methods, which consist of fish reproduction and egg collection, range finding test, egg treatment, egg hatchability rate, larval survivability, heart rate, heart morphology examination, and data analysis.

Fish Reproduction and Egg Collection

Female and male fish with mature gonads were selected and maintained in standard condition and feed with fish commercial food as well as controlled environmental conditions of 14 = 10 photoperiod cycle (Pratama et al. 2021), 28-29^{o}C of temperature, and dissolved oxygen levels around 6-8 ppm. Eggs were obtained by spawning male and female fish in a spawning aquarium, with a ratio of two males and one female. Eggs were collected at around 07:00 am, cleaned and maintained on egg water for further treatment at 24-72 hpf.

LC_{50}-48h Test of Wader Pari's Egg Fish in High Concentration

The LC_{50}-48-hour preliminary toxicity test were conducted to determine the lethal concentration (LC_{50}) of mangosteen peel decoction based on a 48-hour mortality effects study that caused the death of 50% of eggs with the concentrations of 0; 250; 500 and 750 µg/mL. following Widiastuti et al. (2018).

Egg Treatment with Mangosteen Peel Decoction Exposure

Mangosteen peel at the weight of 0.5, 1, 5, and 25 mg was dissolved and boiled at 90°C for 30 minutes in 500 mL distilled water. A total of 120 pre-gastrulation embryonic stage embryos (3.5–4 hpf) were chosen for the treatment, for the reason of, it's an initial stage for organogenesis to perform; 10 wader eggs were placed at each well plate containing egg water and mangosteen peel decoction at the concentrations of 0.5, 1, 5, and 25 µg/mL, respectively. Exposure of mangosteen peel decoction was conducted for the period of 72 hours, with three replications for each treatment, following Raharjeng & Retnoaji (2021).

Water Quality Monitoring for Hatchability Rate Wader Pari Fish

Water properties were similar on control and treatment, with overall water quality parameters during experiment period were in optimal quality for the fish embryo to develop and hatch, as presented in Table 1.

Table 1. The water parameters values measured during the experiment period.

Parameter	1st 24 hours value	
	control	0.5, 1, 5, and 25 µg/mL
pH	7.5	8
Temperature (°C)	28	28
DO (ppm)	8	8

The optimal water quality assures that only treatment factors contribute to the experiment result. The optimum temperature for egg hatching and larval development in *Rasbora lateristriata* is at the range of 27-30 °C and the pH ranges from 7-8 and the good DO range for hatching eggs is around 7 to 8 (Abida et al. 2021)

Egg Hatchability Rate of Wader Pari Fish (*Rasbora lateristriata*)

Egg hatchability is an indicator of good quality egg with normal cleavage and development processes (Zahro et al. 2021). The eggs hatchability rate was measured by the number of egg hatch at the period of 24-72 hours after the treatment started. The number of hatched eggs were recorded in the concentrations of 0.5, 1, 5, and 25 µg/mL, respectively.

Heart Rate and Heart Morphology of Wader Pari Fish (*Rasbora lateristriata*)

Embryonic heart rate was measured at 48 hpf stage. Larvae were randomly picked and placed in a petri dish at the position of latero-lateral and were observed by using a Leica light microscope. the heart rate was counted for one minute period.

Heart morphology observations were conducted on 72 hpf larvae. The larvae of each treatment were placed in a petri dish and photographed for ventral and lateral embryonic side. The cardiac morphology, the atrium and ventricles position and looping, the presence barrier in the atrioventricular canal (AVC), as well as the distance between the sinus venosus (SV) and the bulbous arteriosus (BA) were observed. The SV -BA distance was measured with Image-J software following Pratama et al. (2022).

Data Analysis

Quantitative data such as percent of eggs hatchability, survival rates, and heart rate, as well as, the mean differences of SV-BA lengths between treatments, were statistical analyzes with one way ANOVA to determine the significance between treatments ($p < 0.05$). Cumulative data on wader fish mortality in the LC_{50} test was analyzed using probit analysis to determine the 48-hour LC_{50} value, with SPSS probit statistical analysis.

RESULTS AND DISCUSSION

Preliminary Test (Range Finding Test) of Wader Pari's Eggs at High Concentrations

The results of the range finding test conducted at concentrations of 250, 500, and 750 µg/mL revealed lethality in all the embryos within 48 hours, with none of the embryos successfully hatching. This contrasted sharply with the control group treated with 0 µg/mL, which exhibited an 85% hatching rate and a 67.5% embryo survival rate. The results suggest that concentrations of 250 µg/mL and higher were lethal to embryos prior to the hatching period. Therefore, a lower concentration was selected for the sublethal treatment, with concentrations as follows: 0.5, 1, 5, and 25 µg/mL.

LC_{50} Toxicity Test 48 Hours of Wader Pari's Egg at Chosen Concentration

The results indicate that the mortality rate of fish embryos over the 48-hour period was not significantly different across all treatment groups. Lethality of the embryos was evident when the eggs exhibited cloudiness and a change in color, followed by drifting and sinking to the treatment media's bottom. The LC_{50}-48hours value, representing the median lethal concentration, was determined through probit regression analysis, yielding a result of 24.305 µg/mL.

However, when examining the effects of mangosteen peel decoction, it is notable that the highest concentration of 25 µg/mL demonstrated a higher average larval mortality rate compared to the other concentrations (refer to Figure 1).

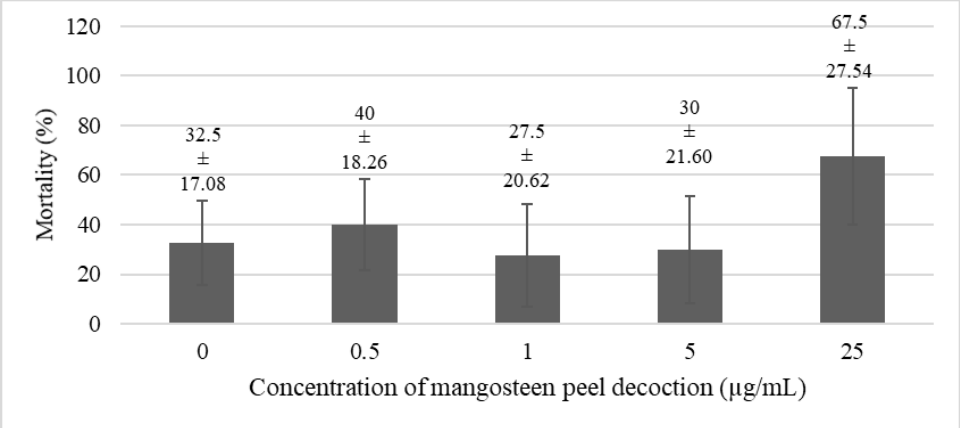

Figure 1. Embryo mortality percentage on each treatment, showed significant difference in treatment concentrations of 0, 1, and 5 µg/mL compared to 25 µg/mL ($p < 0.05$).

The mortality percentage of embryos in the control and all treatments showed a high mortality rate of 67.5% at the highest concentration of mangosteen peel decoction, which was 25 µg/mL, and the lowest percentage of 27.5% at a concentration of 1 µg/mL of mangosteen peel de-

coction, respectively. The statistical analysis ($p < 0.05$) revealed a significant difference in treatment concentrations of 0, 1, and 5 µg/mL compared to 25 µg/mL.

Exposure to high concentrations of mangosteen peel decoction caused lethality in fish embryos, possibly due to cellular damage or physiological abnormalities during the early stages of embryonic cleavage and development. Cell death is one of the cellular responses to disturbances occurring in embryos (Miller & Zachary 2017).

Fish Egg Hatchability Rate of Wader Pari Fish (*Rasbora lateristriata*)

The hatching rate of wader pari fish eggs, treated with various concentrations of mangosteen peel decoction, is presented below (Figure 2). The hatching rates for treatments at 0, 0.5, 1, 5, and 25 µg/mL ranged from 52.5 to 85%. The lowest hatching percentage was observed at a concentration of 25 µg/mL (52.5%), while the highest survival rate of 85% was recorded in both the control and the 1 µg/mL concentration treatments. Statistical analysis ($p < 0.05$) revealed significant differences in the average egg hatching rates between treatments.

Egg hatchability could be used as an indicator to determine the normal process of fish development and the potential influence of external factors. Fish eggs are known to possess an acellular envelope composed of multiple layers of materials, including a protein called chorion, which functions as a protective layer for embryos (Rothenbucher et al. 2019). However, the results (Figure 2) demonstrate a decrease in hatchability at higher concentration treatments. Certain substances are believed to modify the structure of the chorion, resulting in shrinkage and damage to the protective coating. This, in turn, leads to disruptions in embryonic development and ultimately embryonic lethality (Pelka et al. 2017).

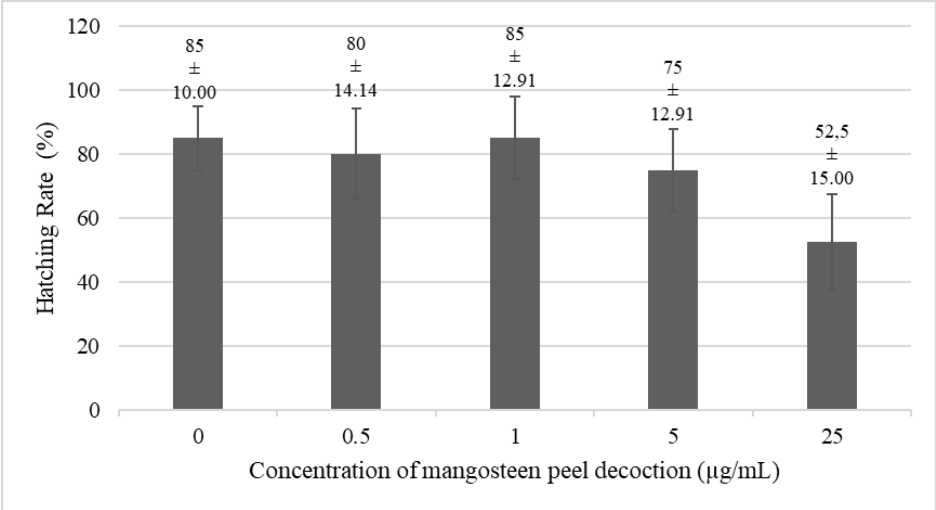

Figure 2. The percentage of egg hatching rate in the control group and all treatment exposure at concentrations of 0.5, 1, 5, and 25 µg/mL showed significant differences between treatments ($p < 0.05$).

Survival Rates of the Fish Larvae Wader Pari (*Rasbora lateristriata*)

The treatment during the pre-gastrulation egg stage resulted in a decreased larvae survival rate. The control and treatments with 0.5, 1, and 5 µg/mL mangosteen peel decoction exhibited similar survival rates of 67.5, 60, 72.5, and 70%, respectively. In contrast, the mangosteen peel decoction at a concentration of 25 µg/mL showed a lower survival rate of 32.5% (Figure 3). Statistical analysis ($p < 0.05$) revealed no significant differences in survival rates among the fish larvae treatments.

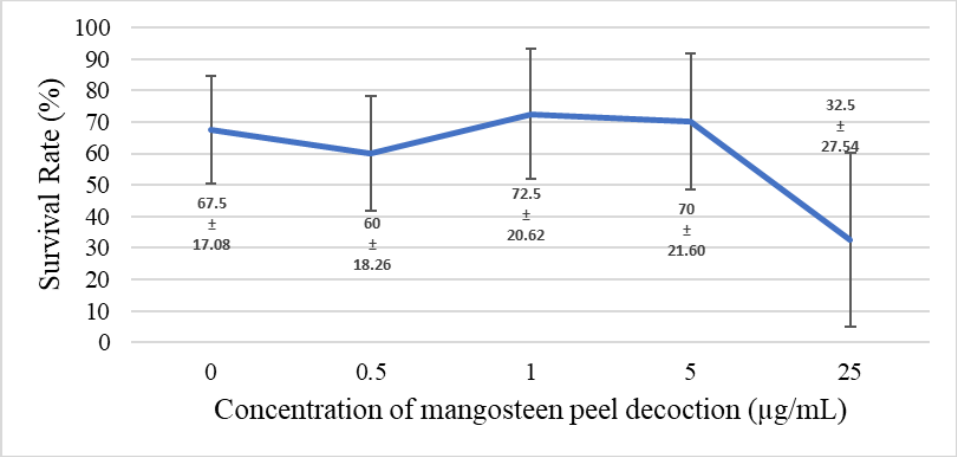

Figure 3. The percentage of survival rate of treated fish larvae showed no significant differences between treatments ($p < 0.05$).

The larvae survival rate in all treatments displayed a tendency of high survivability at the initial stage, followed by a consistent reduction as the treatment period progressed. The survival rate represents the fish's ability to withstand exposure during the research period. A total of 120 embryos were exposed during the experiment. Figure 3. illustrates the number of larvae that survived the treatment period from 24 to 72 hpf. The control and treatments with 0.5, 1, and 5 µg/mL of mangosteen peel decoction exhibited survival rates of 67.5, 60, 72.5, and 70% respectively. On the other hand, the mangosteen peel decoction at a concentration of 25 µg/mL exhibited a survival rate of 32.5%. These findings suggest that higher concentrations of mangosteen peel decoction (25 µg/mL or more) led to a reduction in larvae development and survivability.

Mangosteen peel contains tannins, which can potentially inhibit cellular metabolic activities and lead to metabolic abnormalities. These abnormalities could result in fish fatality and an inability to survive. Additionally, it contains compounds that can physiologically hinder sterol carrier protein-2 within the larvae's body, which is crucial for lipid metabolism. This disruption can lead to impaired integrity and disability. This situation triggers lipid degradation and disrupts the fish's body metabolism, ultimately causing death (Masduki 1996).

Heart Rate of the Fish Larvae Wader Pari (*Rasbora lateristriata*)

The heart rate of fish larvae was measured at 48 hpf (Wan-Mohtar et al. 2021) to examine the physiological responses of the larvae to the treatment (Figure 4). Control larvae showed a heart rate of 165.5 beats/minute, while higher heart rates were observed in the treatment group, ranging from 166.5 to186 beats/minute.

Exposure of fish larvae to mangosteen peel decoction significantly affected the average heart rate of the larvae between treatments ($p < 0.05$). It has been reported that heart physiological function is closely related to heart rate and blood flow (Männer et al. 2010). Abnormalities in the heart muscle could lead to a decrease in heart rate and reduced blood flow, potentially resulting in heart failure. Zhu et al. (2007) stated that the normal heart rate for a 48 hpf fish embryo ranges from 165 to 186 beats/minute. Therefore, the results showed a heart rate of 186 beats/minute in embryos at 48 hpf when exposed to a treatment concentration of 25 µg/mL, which is considered higher than the normal rate. This indicates that mangosteen peel decoction exposure could alter heart physiological activity.

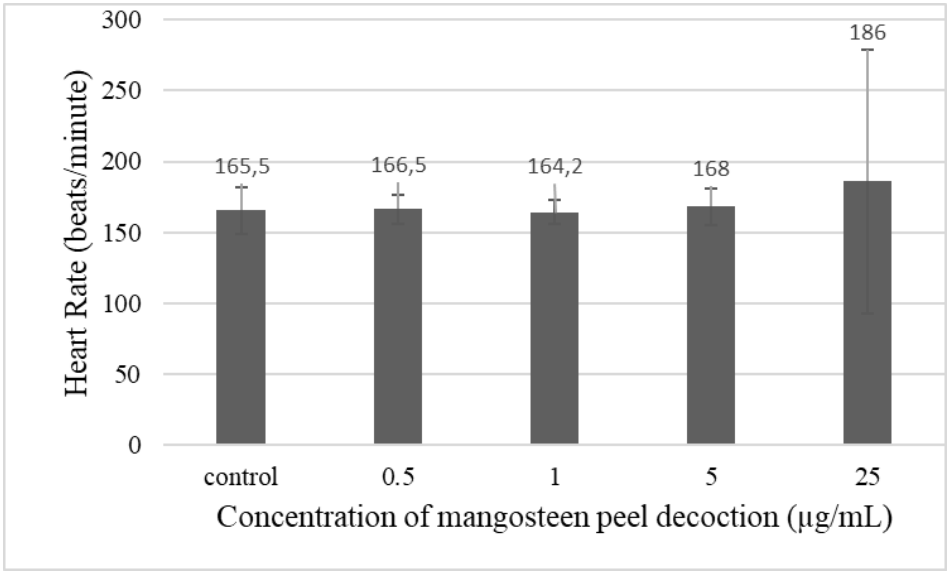

Figure 4. The average heart rate of treated fish larvae showed significant differences between treatments ($p < 0.05$).

Heart Morphology of the Fish Wader Pari (*Rasbora lateristriata*)

Heart Morphology of the Fish Wader Pari (*Rasbora lateristriata*) The observation of heart morphology (Figure 5) revealed the occurrence of pericardial edema, as well as the presence of indentations or obstructions in the atrioventricular canal (AVC) within the mangosteen treatment group. In contrast, the control group showed no signs of edema or indentations in the AVC.

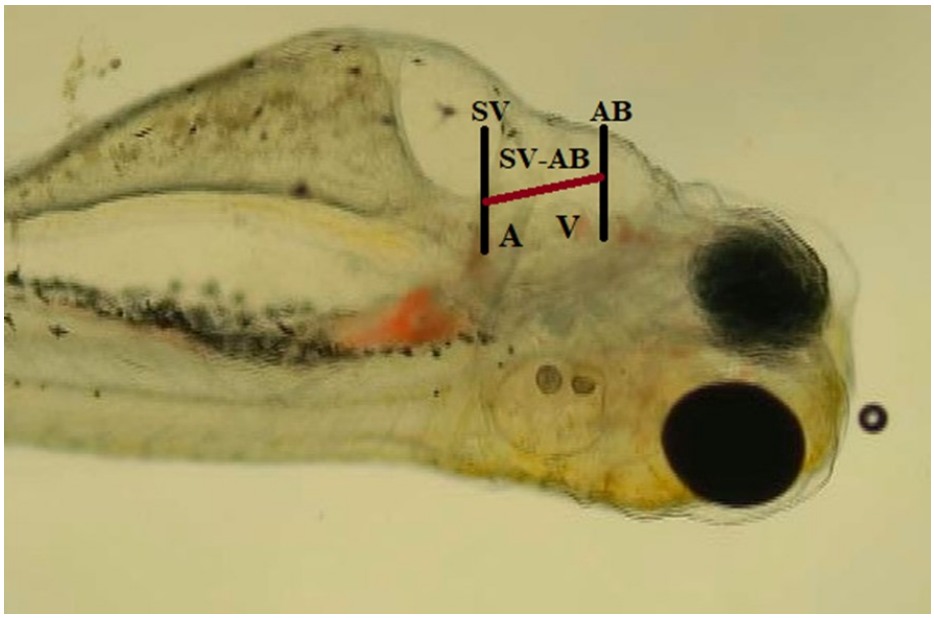

Figure 5. Measurement of sinus venosus and bulbous arteriosus distances of wader fish (*Rasbora lateristriata*). (A) atrium, (V) ventricle, (BA) bulbous arteriosus, (SV) sinus venosus.

The heart and blood vessels of fish embryos constitute two systems that are commonly studied in toxicity tests. They often manifest as changes in heart rate rhythm and reductions in blood flow, as observed in studies such as Zoupa & Machera (2017). Furthermore, studying these systems offers the potential to identify additional toxicological effects on the morphology and function of the heart. The present study presents the morphological defects of the heart in Table 2.

Table 2. Morphological abnormalities of wader pari fish embryo's heart which were exposed to mangosteen peel decoction.

Heart Defects	Concentration (µg/mL)				
	0	0.5	1	5	25
Heart edema	+	+	+	-	-
Bending of the heart, abnormal heart	+	+	+	-	-
Heart rate disorders	+	+	-	+	+
Heart tube	+	+	+	+	+

+ = Normal
- = Abnormal

One of the toxicity parameters involves the treatment's effect on the heart of fish embryos, categorised based on the measurement of the distance between the venous sinus and the arterial bulb (SV-BA interval) as described by Li et al. (2018). The Venous Sinus-Arterial Bulb (SV-BA) distance varied from 61.6 to 64.3 µm (Figure 6).

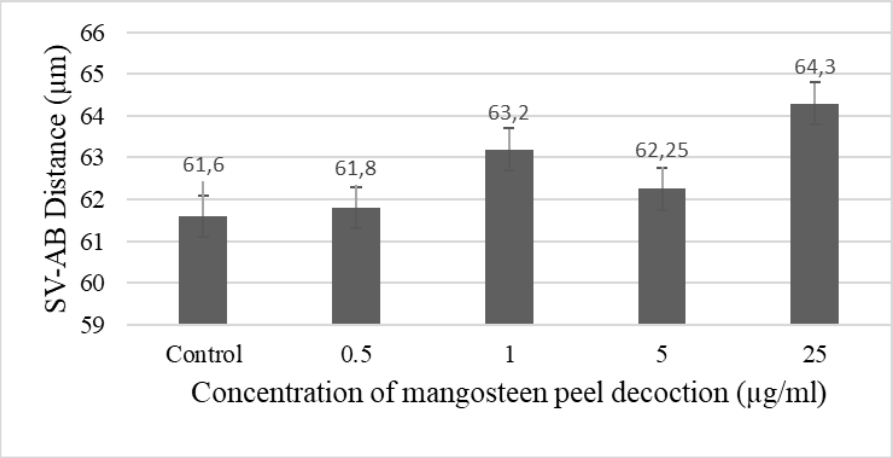

Figure 6. The Venous Sinus-Arterial Bulb (SV-BA) distance graph of 72 hpf larvae in the control group and treatment exposure at concentrations of 0.5, 1, 5, and 25 µg/mL showed no significant differences between treatments ($p < 0.05$).

Cardiac development is a parameter used to assess the toxic effects of certain substances on fish embryonic development. The heart is the first organ to form during embryonic development. Consequently, exposure to toxic chemicals at this early stage can result in malformations in the heart's morphology (Antkiewicz et al. 2005). The results of cardiac morphology observations indicated that the embryonic heart developed normally in both the control group and the lower concentrations of the treatment. However, higher concentrations of the treatment led to heart malformations or abnormalities, such as cardiac edema.

Notably, marked cardiac or pericardial edema was observed at the distance between the attachment points of the heart to the inlet and outlet channels (SV-AB). The formation of pericardial edema can separate the points of attachment for inflow and outflow, causing an elongated cardiac morphology (Antkiewicz et al. 2005).

The assessment of the venous sinus-arterial bulb (SV-BA) distance was conducted to determine the treatment's effect on the hearts of wader fish larvae. The SV-BA distance essentially serves as an index of cardiac loops that can be utilised to investigate cardiac malformations (Antkiewicz et al. 2005). The results indicated that the SV-BA distance (Figure 6) did not show a significant difference between the control group and all treatment groups. This suggests that exposure to mango-

steen peel decoction did not impact the development of cardiac looping in the fish embryo.

CONCLUSION

Exposure to mangosteen peel decoction at a concentration of 25 µg/mL and higher proved to be toxic to wader fish embryos, leading to reduced hatchability, survival rates, and influencing heart rate as well as heart and body morphology.

AUTHOR CONTRIBUTIONS

P.P. and L.U.K. designed and conducted experiments, conducted statistical analysis, visualised data, and compiled papers. B.R. assisted in designing experiments, visualising data, and compiling the papers. All the authors have read and approved the last manuscript.

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CONFLICT OF INTEREST

The authors declare that there are no competing interests.

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Research Article

Evaluation of Temperature Stress Under Different Hydroponic Systems on Growth and Saponin Content of *Talinum paniculatum* Gaertn. Cuttings

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ABSTRACT
Increases in the temperature of nutrient solutions have restricted the use of hydroponic cultivation in the tropics, predominantly due to plant stress. This study aimed to evaluate the effects of temperature stress under different hydroponic systems on the growth and saponin content of *Talinum paniculatum* cuttings. Three hydroponic systems, i.e., deep flow technique (DFT), nutrient film technique (NFT), and aeroponic, were tested. The temperature of the nutrient solution was set for each system, i.e., under ambient temperature (UAT) and with controlled temperature (WCT) at 26° C. The cultivation period was 60 days. The result showed peroxidation activity and proline accumulation for the adventitious roots of *T. paniculatum* cuttings with UAT and WCT, alongside various levels of plasma membrane damage. Levels of Malondialdehyde (MDA) and proline were analyzed by spectrophotometer. Membrane damage was analyzed with Evans blue dye. The results indicated that the levels of MDA and proline accumulation under the three hydroponic systems were higher for the WCT than for the UAT treatment. In contrast, vegetative growth was higher in UAT than in WCT. The saponin content of the adventitious root correlated with the MDA level. Saponin production was triggered by oxidative stress during cultivation, while the adventitious roots had a higher saponin content in all three hydroponic systems with the WCT treatment compared to the UAT treatment. Among the systems, aeroponic was superior for biomass and saponin. Root growth was promoted in the nutrient solution under ambient temperature whereas the production of saponins was stimulated under the controlled temperature. In the aeroponic system, root biomass values of 1.17 and 0.478 g dry weight were obtained under ambient and controlled temperatures, respectively. The total saponin contents differed slightly, namely 189.83 and 195.61 mg/g, respectively.

INTRODUCTION
Indonesia, as a tropical country, is rich in medicinal plants. One such plant is *Talinum paniculatum* (Jacq.), which has the local name Ginseng Jawa. *Talinum paniculatum* roots are rich in saponins, which have medicinal properties. The root is known for its stamina enhancers and reproductive tonics such as Korean and Chinese ginseng. Traditional farming methods have been insufficient to satisfy market demand for *T. panicula-*

tum roots in terms of both quantity and quality. However, cultivation *in vitro* may offer a solution. Previous studies have reported success in cultivating adventitious roots of *T. paniculatum* in a balloon-type bubble bioreactor (BTBB) (Manuhara et al. 2015). Elicitation using methyl jasmonate and methyl salicylate has been found to increase saponin while reducing adventitious root weight (Ahmad & Anggita 2019). Slow growth, natural contamination by endogenous microbes, and low saponin content are among the problems posed by *T. paniculatum* adventitious root culture in BTBB, which to date have remained unresolved. The production of secondary metabolites from cell or organ culture in a bioreactor has been characterized by low productivity, high cost, and high process technology to control the culture parameters and create sterile conditions (Bourgaud et al. 2001). Furthermore, for large-scale production, the use of adventitious roots as inoculums has proven challenging with regard to transferring (Nguyen et al. 2003). Therefore, alternative *T. paniculatum* cultivation methods are required for root biomass production and saponin content. Many prior studies have demonstrated the positive impact of saponins on health (Sharma et al. 2023; Wang et al. 2023).

Recently, the potency of hydroponic cultures for secondary metabolites of medicinal plants has been investigated. Hydroponics is an alternative method of growing plants that are difficult to cultivate on land or in closed bioreactors with axenic conditions (Nguyen et al. 2003). There is no high technology or high cost involved in the construction of hydroponic systems or in controlling the parameters of culture. The hydroponic nutrient solution is sugar-free and requires no sterilization. These conditions result in inexpensive nutrient preparation and save energy. Nevertheless, the physical and chemical parameters of hydroponic culture can still be controlled to produce biomass with a high content of secondary metabolites (Giurgiu et al. 2014). The successful production of plant secondary metabolites using hydroponic techniques has been reported from various medicinal plants, including the production of flavonoid from *Acmella oleraceae* (Abeysinghe et al. 2014), tropane alkaloid from *Datura innoxia*, and taxanes from *Taxus baccata* (Gontier et al. 2002).

To date, the hydroponic method has not been tested for *T. paniculatum* cuttings. High air temperatures act as a barrier to hydroponic cultivation in tropical regions. In the tropics, with average daily temperatures of around 30–32° C, plastic or metal containers are heated, thereby increasing the temperature of the nutrient solution in the system (Gur et al. 1972). Heat stress in plants can reduce their growth, number, and root mass (Huang et al. 2012) before impacting the growth of tissue above ground (Giri et al. 2017). It also triggers the accumulation of Reactive Oxygen Species (ROS), causing lipid peroxidation followed by membrane instability or damage (Taratima et al. 2022). The presence of lipid peroxidation products (such as MDA) and electrolyte leakage were identified as indicators of heat stress in rice (Taratima et al. 2022). Malondialdehyde (MDA) is widely used as a marker of free radical formation due to oxidative stress (El-Aal 2012). Excessive proline production in plants is also associated with membrane damage owing to its ability to stabilize cell membranes and prevent electrolyte leakage (Hayat et al. 2012).

In the tropics and countries and regions with high air temperatures, such as Indonesia, it is vital to investigate the appropriate type of hydroponic systems with or without controlled nutrient solution temperatures. Each system type has a specific pattern of nutrient delivery that affects the capacity of the nutrient solution to act as a buffer against temperature changes. This research aims to evaluate the effect of nutrient solution temperature as a consequence of the type of hydroponic system

on the stress, growth, and saponin content of *T. paniculatum* cuttings. The stress indicators comprise the MDA content, proline level, and the results of staining and analysis of absorbed Evans blue dye.

MATERIALS AND METHODS

Materials

Talinum paniculatum cuttings were cultivated in the greenhouse of the Biology Department, Faculty of Science and Technology, Airlangga University, Indonesia. The cultivation period lasted for 60 days, between September and November 2018. The cuttings were sourced from 1-year-old mother plants from the collection of the Biology Department. The cuttings were selected from the straight branch with a length of 10–12 cm from the tip and a diameter of 0.7–1 cm. A sterile blade was used to cut the chosen branch. The base of the cuttings was cut at a slant, at approximately a 45° angle. Each cutting was set in a net pot with circular foam as the holder and immediately placed in a planting hole within the hydroponic system to begin cultivation.

Methods

Preparation of nutrient solution

The hydroponic nutrient solution used Murashige and Skoog medium: NH_4NO_3 (1650 mg), KNO_3 (1900 mg), $CaCl_2.2H_2O$ (440 mg), $MgSO_4.7H_2O$ (370 mg), KH_2PO_4 (170 mg), $FeSO_4.7H_2O$ (27.8 mg), $MnSO_4.H_2O$ (22.3 mg), $ZnSO_4.4H_2O$ (8.6 mg), H_3BO_3 (6.2 mg), KI (8.3 mg), $NaMoO_4.2H_2O$ (0.25 mg), $CuSO_4.5H_2O$ (25 mg), $CoCl_2.6H_2O$ (25 mg) (Murashige & Skoog 1962). All materials were diluted in 1000 mL distilled water to make 100% MS. The acidity level of the nutrient solution was maintained within a pH range of 6.0–6.5 using HCl 37% or KOH 25% (Yachya et al. 2020).

Cultivation condition

This study used three types of hydroponic systems, i.e., the deep flow technique (DFT), nutrient film technique (NFT), and aeroponic. The three systems have different patterns of nutrient solution delivery. All systems (DFT, NFT, and aeroponic) were located in a greenhouse with similar environmental conditions, i.e., air temperature, humidity, and sunlight intensity. Each system had ten planting holes. In the aeroponic chamber, five micro sprayers (360° pattern) with a spraying capacity of 1 L/min were located below the planting holes. The volume of the nutrient solution in each system was 30 L. Circulation of the nutrient solution was conducted using a submersible pump with a maximum flow rate of 2400 L/h. Two temperature treatments were applied to the nutrient solutions in all systems, i.e., without controlled temperature or under ambient temperature (UAT) and with controlled temperature (WCT) or under a cooling treatment at 26° C using a chiller. The following nutrient solution strength levels were used during cultivation: 1st to 6th day of cultivation – 0% MS; 7th to 20th day of cultivation – 12.5% MS; 21st to 34th day of cultivation – 25% MS; 35th to 48th day of cultivation – 25%; 49th to 60th day of cultivation – 50% MS. The nutrient solution was replaced every two weeks. During cultivation, the air and nutrient solution temperatures were measured at midday. The acidity level of the nutrient solution was maintained within a pH range of 6.0–6.5. HCl 37% or KOH 25% solution was used to increase or reduce the pH of a nutrient solution if it moved outside the target pH range. The volume of the nutrient solution was also maintained using distilled water.

Measurement of MDA level

Reaction with thiobarbituric acid (TBA) was used to measure the MDA level (Zhang & Huang 2013). Fresh adventitious roots (500 mg) and 4.5 mL 1% sulfuric acid were homogenized using a mortar and pestle. The homogenate was centrifugated at 6000 rpm for 10 min. A volume of 0.5 mL supernatant was collected in a test tube and reacted with 0.5 mL 0.1% trichloroacetic acid (TCA) and 2 mL 0.5 % TBA. The test tube was heated in a water bath at 95° C for 30 min to induce a reaction. After this, the test tube was immediately cooled in ice for 30 min to stop the reaction. A red color will appear in the acid buffer as TBA reacts with MDA. The reaction mixture was then centrifuged at 6000 rpm for 5 min. The supernatant was collected for absorbance reading at λ 532 nm. The absorbance value was then converted to MDA using a regression equation of the standard curve. Construction of the standard curve used a serial concentration of tetra ethoxy propane (TEP): 0.625, 1.25, 2.5, 5, 10, 20, and 40 µM. The regression equation was $y = 80.433x - 0.0392$ with a correlation coefficient $R^2 = 0.9999$. The MDA concentration (µM) was plotted on the y-axis, while the x-axis showed the absorbance of the sample.

Measurement of proline level

This research also measured the level of proline and Evans blue uptake to determine damage in the root plasma membrane due to lipid peroxidation. The proline level in adventitious roots was measured spectrometrically (Bates et al. 1973). Fresh adventitious roots (500 mg) were ground using a mortar and pestle with 3% sulfosalicylic acid (4.5 mL) added to the mortar during preparation. The homogenate was centrifuged (6000 rpm) for 15 min or filtered with Whatman #2 filter paper. The supernatant (0.5 mL) was then placed in a test tube and reacted with ninhydrin reagent (1 mL) and glacial acetic acid (1 mL). The test tube was boiled in a water bath at 100° C for 1 h after which it was immediately soaked in ice water for 30 min to stop the reaction. Next, toluene (1.5 mL) was added to the test tube for extraction. The reaction mixture was shaken using a vortex for 1 min or until the chromophore appeared. The chromophore, which was one phase with toluene, was taken for absorbance reading at λ 520 nm. A regression equation of the standard curve was used to convert the absorbance value to proline. The construction of the standard curve used a serial concentration of proline: 1, 10, 50, 100, 150, 200, and 300 mM. The regression equation was $y = 465.43x - 8.3411$ with a correlation coefficient $R^2 = 0.9921$. The y-axis showed proline concentration (µM), with the sample absorbance plotted on the x-axis.

Quantification of membrane damage

Evans blue dye was used to quantify the membrane damage in adventitious roots (Preethi et al. 2017). The tips of adventitious roots (weight 0.1 g and length 1 cm) were collected and then transferred to 2 mL Eppendorf tubes. Evans blue solution (2 mL) was added and the Eppendorf tubes were shaken using an orbital shaker at 50 rpm for 20 min. The roots were then washed using distilled water until no further color emerged. Next, a selected root was drained for observation and documentation under a transmitted light microscope. This was followed by quantification of the membrane damage. Evans blue dye, which was absorbed by the root tissue, was extracted using 1 % sodium dodecyl sulfate. Stained roots (0.1 g) and 1 mL SDS were homogenized using a mortar and pestle and then transferred to a centrifuge tube. Calcium dichloride 0.1 M pH 5.6 was added to the extract to produce an extract volume of 5

mL and then centrifuged at 6000 rpm for 10 min. The collected supernatant was taken for absorbance reading at λ 600 nm. The absorbance value was converted to Evans blue uptake using a regression equation of the standard curve. The standard curve was composed of a serial concentration of Evans blue solution: 100, 200, 300, 400, 600, 700, 800, 900, and 1000 ng/mL. The dissolution of Evans blue dye used 1 M $CaCl_2$ pH 5.6. The regression equation was $y = 1342x$ -8.5442 with a correlation coefficient $R^2 = 0.9982$. Evans blue concentration (µM) was plotted on the *y*-axis, while the *x*-axis showed the sample absorbance.

Measurement of growth parameters

The growth parameters in this study, i.e., leaf area, plant height, and the fresh and dry weight of adventitious root, were measured at the end of the cultivation period. A scanner and Image J software were used to measure the leaf area. Plant height was measured manually using a ruler. The fresh and dry weights of adventitious root were measured using an analytical balance and moisture analyzer, respectively. Adventitious roots were separated from the stem using a blade and then blotted for 10 min on tissue paper to absorb excess water (Yu et al. 2005). The roots were then weighed using an analytical balance to obtain the fresh weight. Dry weight analysis was performed with a moisture analyzer (Mettler Toledo HB43) at 105ºC for 20 min.

Measurement of saponin content

The extraction and analysis of saponin in adventitious roots was conducted based on Manuhara et al. 2015. The adventitious root (1 g dry weight) was first ground using a mortar and pestle. Absolute ethanol (10 mL) was then added and left overnight. The saponin content in the filtrate was measured using a Shimadzu HPLC with a 5 µm (150 × 14.6 mm) HRC ODS Shim pack column. The column temperature was 40° C with acetonitrile and water as the mobile phase. The saponin analysis results showed no repetitions due to sample limitations; therefore, statistical analysis was not carried out.

Data analysis

Data analysis was performed using the ANOVA test. If an F-value in ANOVA was significant, the next test was the Duncan or Games-Howell test with a significance level of 5%. The statistical tests were analyzed using IBM SPSS Statistics 23.

RESULTS AND DISCUSSION

Nutrient solution temperature

Figure 1 shows the daily temperature profile of the air and nutrient solutions in the three hydroponic systems with the UAT and WCT treatments during cultivation. The pattern of change in the nutrient solution temperature with the UAT treatment was relatively similar to that of the air temperature. In contrast, the nutrient solution temperature with the WCT treatment was categorically different from the air temperature. Due to the chiller, it remained relatively stable at 26°C. The average air temperature during cultivation was 36.22°C. This was a higher and significantly different temperature compared to the average nutrient solution temperature in the three hydroponic systems with the UAT and WCT treatments (Table 1).

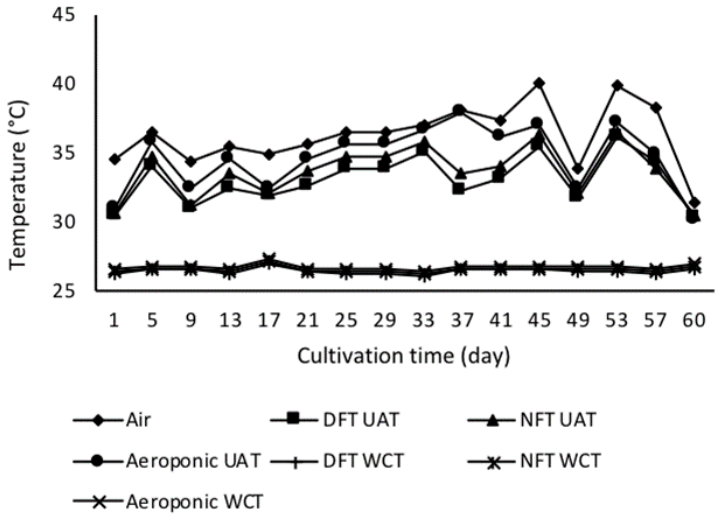

Figure 1. Profile of the air and nutrient solution temperatures of three hydroponic systems (DFT, NFT, and aeroponic) in two temperature treatments: under ambient temperature (UAT) and with controlled temperature (WCT) during 60 days of cultivation. Measurements were taken around midday, between 12:00 AM – 01:00 PM.

The similarity of the change patterns in the nutrient solution temperatures across the three hydroponic systems with the UAT treatment against the air temperature (Figure 1) indicates that the air temperature influenced the nutrient solution temperatures. However, different levels of influence on the nutrient solution temperature were evident (Table 1). The nutrient solution temperature in the DFT system was lower than in the NFT and aeroponic systems. In DFT, a high volume of nutrient solution was circulated via a pipe, which helped to stabilize the temperature of the solution (Park et al. 2001). In the NFT system, the nutrient solution flowed thinly inside a gully, which did not impede any temperature change (Van Os et al. 2008). Meanwhile, the nutrient solution temperature was highest in the aeroponic system and differed significantly from that of the DFT and NFT systems.

Table 1. Nutrient solution temperatures of three hydroponic systems under ambient temperature (UAT) treatment during 60 days of cultivation. Temperatures were measured around midday, between 12:00 AM – 01:00 PM.

Hydroponic System	Temperature (° C)
DFT	32.82 ± 1.7483^{a}
NFT	33.33 ± 1.8370^{a}
Aeroponic	34.56 ± 2.4083^{b}

Average values in the same column followed by different letters are significantly different, according to Duncan's multiple comparison tests at $p<0.05$.

In the aeroponic system, the nutrient solution was sprayed around the root zone for 20 seconds every 1 min. This method encouraged faster heating of the nutrient solution compared to the DFT and NFT systems (Table 1). The patterns of temperature change in the nutrient solutions among the three systems with the UAT treatment prove that the method of nutrient solution delivery affected the temperature of the solution during cultivation.

Oxidative stress and membrane damage on adventitious root

The MDA and proline levels, along with the Evans blue uptake and Evans blue staining images for adventitious roots in the three hydroponic

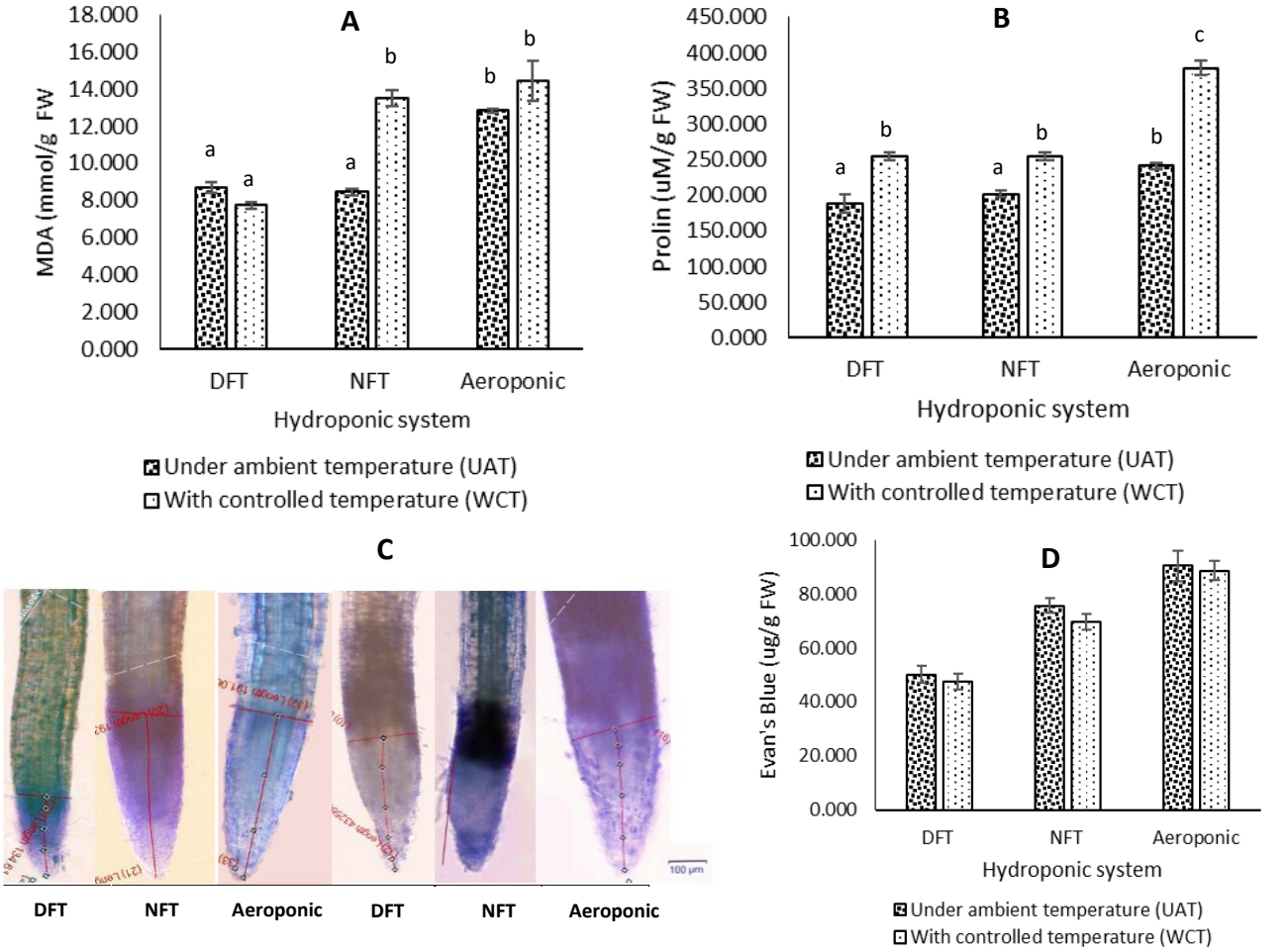

Figure 2. The content of MDA **(A)** and proline **(B)**, the result of Evans blue staining **(C)**, and the level of Evans blue uptake **(D)** in *T. paniculatum* adventitious roots after 60 days of cultivation in three hydroponic systems (DFT, NFT, and aeroponic) with two temperature treatments on nutrient solution: under ambient temperature (UAT) and with controlled temperature (WCT). The Games-Howell test at $p<0.05$ was used to calculate the significant difference in MDA, while Duncan's test at $p<0.05$ was used for proline and Evans blue.

systems with the UAT and WCT treatments were observed at 60 days of cultivation. The levels of MDA (Figure 2A) and proline (Figure 2B) in the three hydroponic systems were relatively higher with the WCT treatment compared to the UAT treatment. Relatively similar uptake levels of Evans blue were recorded for both the UAT and WCT treatments (Figure 2D). Among the three hydroponic systems in either group (UAT or WCT), the highest levels of MDA, proline, and Evans blue uptake were observed for adventitious roots in the aeroponic system. Within the aeroponic system, however, the MDA and proline levels (Figures 2A, 2B) of adventitious roots were higher for the WCT treatment compared to the UAT treatment. In contrast, there was no difference in the levels of Evans blue uptake between the UAT and WCT treatments in the aeroponic system (Figure 2D).

The lipid peroxidation analysis result (Figure 2A) indicated various levels of oxidation stress on *T. paniculatum* adventitious root. Accumulation of MDA, which is a by-product of lipid peroxidation, correlates with plant stress levels (Wang et al. 2015; Chen & Zhang 2016). With the UAT treatment, the aeroponic system had the highest MDA level, which was also significantly different from that observed with DFT and NFT, while no significant difference was found between the latter two systems (DFT and NFT). The result of the MDA analysis in Figure 2A was in line with the average nutrient solution temperature of the three hydro-

ponic systems with UAT treatments (Table 1). This implies that oxidative stress on the adventitious roots in the UAT treatment was linked to the change in the nutrient solution temperature during the cultivation period. The increase in the nutrient solution temperature to 32.83–34.56° C (Table 1) in the three hydroponic systems with the UAT treatment may have proven relatively hot to the adventitious roots of *T. paniculatum* cuttings, thereby triggering heat stress. For the WCT treatment, the lowest MDA level was recorded for the DFT system and was very similar to the respective levels for the DFT and NFT systems with the UAT treatment. This result indicated that the adventitious roots of *T. paniculatum* cultivated using DFT with the WCT treatment also underwent abiotic stress, particularly cold stress. Meanwhile, the MDA levels for NFT and aeroponics with the WCT treatment were relatively similar and higher than for both NFT and DFT with the UAT treatment. These results indicated that the adventitious roots of *T. paniculatum* cultivated using NFT and DFT with the WCT treatment underwent more abiotic stress than when using NFT and DFT with the UAT treatment.

In addition to cold stress, the adventitious roots in both the NFT and aeroponic systems with the WCT treatment underwent heat stress. In both the NFT and aeroponic systems, the adventitious roots did not sit entirely within the nutrient solution. In the NFT system, the lower roots, which were exposed to the flow of nutrient solution, were in a relatively cold condition (26°C), which may have triggered cold stress. The upper roots, which were exposed to the air, were in a relatively hot condition (36.22°C), which could have triggered heat stress. Among the various types of abiotic stress, cold is one of the most significant barriers to growth and also influences the genetics, physiology, and biochemical responses in a plant (Soydam Aydin et al. 2013). Cold stress will increase protein transcription and the activity of various enzymes that break down ROS in plants (Suzuki & Mittler 2006). Accumulation of hydrogen peroxide, which was also followed by lipid peroxidation, was observed in the leaves of red lettuce (*Lactuca sativa*) when plants were cultivated in the DFT system with cooling treatment in the root zone (Sakamoto & Suzuki 2015). Similar to the NFT system, adventitious roots in the aeroponic system were subjected periodically to both temperature conditions, i.e., cold and heat. When the pump was turned on, the cold nutrient solution was sprayed over all parts of the adventitious roots for 20 seconds. When the pump was subsequently turned off for 1 min, all parts of the adventitious roots were exposed to the air and were in a relatively hot condition. Finally, adventitious roots in both the NFT and aeroponic systems displayed overactivity of lipid peroxidation compared to DFT.

The accumulation of MDA in the adventitious roots of the *T. paniculatum* cuttings was followed by an accumulation of proline (Figure 2B). This indicated that proline accumulation was a response by the *T. paniculatum* cuttings to lipid peroxidation activity. The accumulation was triggered by temperature stresses (heat or cold stress) as proline accumulates when plants are subjected to various kinds of abiotic stress (Anwar Hossain et al. 2014). Previous studies have reported that proline concentration usually increases when plants experience abiotic stress (Yue et al. 2019). A rise in proline level during cold and heat stresses was reported for two chrysanthemum species and *Petunia* seedlings (Cheng et al. 2014; Yue et al. 2019). The trend for proline (Figure 2B) was relatively similar to that of MDA (Figure 2A). This indicated that *T. paniculatum* cuttings were able to maintain oxidative homeostasis by producing proline to reduce the MDA level since proline is among the non-enzymatic antioxidants that act as ROS scavengers (Hayat et al. 2012). Proline's ability to reduce

MDA has been reported for several plants, including chickpea (RSG 44) (Singh et al. 2010), melon (Yan et al. 2011), and barley (Reza et al. 2006). Proline also plays a role as a stabilizer of cellular and membrane structures to prevent electrolyte leakage (Hayat et al. 2012). The instability of the plasma membrane is due to lipid peroxidation, which can alter the composition, formation, structure, and dynamics of lipid membranes (Gaschler & Stockwell 2017). In contrast, it has also been reported that proline can protect the integrity of the callus membrane of *Solanum nigrum* treated with cadmium stress (Xu et al. 2012). Therefore, proline accumulation in all root tip samples was an indirect indicator of the instability of the plasma membrane in the adventitious roots of *T. paniculatum* cuttings.

It was possible to confirm disorder or damage in the plasma membrane of adventitious roots in the UAT and WCT treatments using an Evans blue assay. The results of the Evans blue staining (Figure 2C) showed that all samples were positive, thus confirming that the plasma membranes of the adventitious roots cultivated in the three hydroponic systems in conditions of UAT and WCT did not function normally or had damage. Normal plasma membranes are impermeable to Evans blue dye, while damaged plasma membranes are permeable and absorb Evans blue dye, staining the cells (Roy et al. 2019). Evans blue dye can only pass through cell membranes that lose their semi-permeability, or in which the plasma membranes do not function normally (Gaff & Okong'o-Ogola 1971). The accumulation of Evans blue in cells correlates with membrane damage (Preethi et al. 2017). The level of damage to the plasma membrane determines the volume of Evans blue that enters the cell to bind to many proteins. According to Alves et al. (2018), Evans blue can bind to many proteins and is not metabolized. Finally, the absorbance of extracted Evans blue rises with increasing membrane damage (Roy et al. 2019). Additionally, adventitious root cells that react positively with Evans blue may also have viability disorder. Loss of plasma membrane integrity affects cell viability, and Evans blue staining could determine this disorder (Tamás et al. 2006; Vemanna et al. 2017). Meanwhile, the uptake of Evans blue (Figure 2D) can determine the extent of plasma membrane damage and cell viability disorder in each treatment. The level of Evans blue uptake showed a similar trend to that of both MDA and proline. These results confirmed that lipid peroxidation activity caused membrane damage and viability disorders. Therefore, Evans blue dye is used to determine the presence of dead cells and damage in the plasma membrane, especially at the lipid bilayer (Tistama et al. 2012; Vemanna et al. 2017). Plasma membrane damage or cell viability disorder occurred in all of the adventitious roots cultivated in the three hydroponic systems with UAT and WCT treatments, although the *T. paniculatum* cuttings remained alive throughout the cultivation period.

Growth of cuttings

Table 2 and Figure 3 show the growth profiles of the *T. paniculatum* cuttings at the end of the cultivation period. All growth parameters (leaf area, plant height, fresh root, and dry weight) in the three hydroponic systems with the UAT treatment were higher compared to the same systems with the WCT treatment. In terms of each group (UAT or WCT), the highest to lowest growth parameter levels were obtained, respectively, by the aeroponic, NFT, and DFT systems. However, the growth level of the *T. paniculatum* cuttings in the aeroponic system with UAT treatment was significantly different and higher than the other systems with the UAT and WCT treatments.

Table 2. Growth level of *T. paniculatum* cuttings after 60 days of cultivation in three hydroponic systems (DFT, NFT, and aeroponic) with two temperature treatments on the nutrient solution: under ambient temperature (UAT) and with controlled temperature (WCT).

Hydroponic system - temperature treatment	Leaf area (cm^2)	Plant height (cm)	Adventitious root	
			Fresh weight (g)	Dry weight (g)
DFT – UAT	1714.91±41.81^d	70.00±2.00^c	2.49±0.17^a	0.24±0.02^a
NFT – UAT	2391.49±41.66^e	68.67±3.22^c	3.59±0.24^b	0.36±0.02^b
Aeroponic –UAT	2984.43±30.02^f	80.33±0.587^d	15.19±0.29^d	1.17±0.08^d
DFT – WCT	451.68±30.75^a	42.33±2.52^a	2.45±0.24^a	0.24±0.03^a
NFT – WCT	583.85±46.39^b	53.67±1.53^b	2.21±0.15^a	0.21±0.01^a
Aeroponic – WCT	1028.78±49.02^c	67.00±2.65^c	6.89±0.09^c	0.48±0.02^c

Average values in the same column followed by different letters are significantly different, according to Duncan's multiple comparison tests at $p<0.05$.

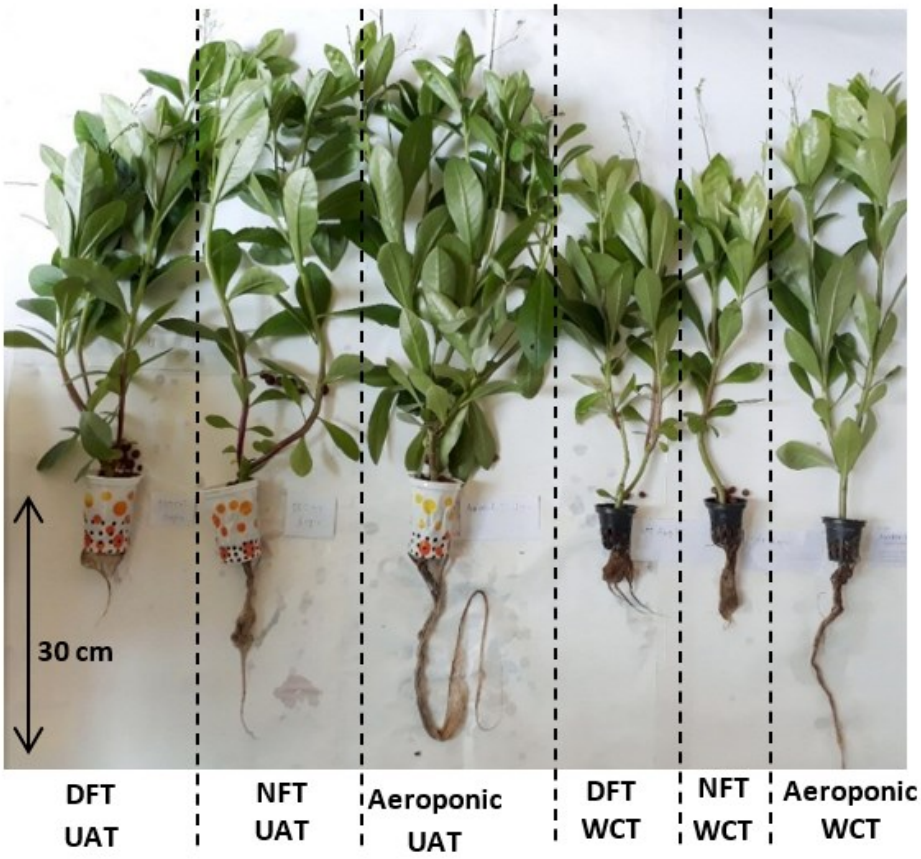

Figure 3. Morphology of *T. paniculatum* cuttings after 60 days of cultivation in three hydroponic systems (DFT, NFT, and aeroponic) with two temperature treatments on the nutrient solution: under ambient temperature (UAT) and with controlled temperature (WCT).

Among the three hydroponic systems with either UAT or WCT, while the aeroponic system produced the highest level of *T. paniculatum* cutting growth, the adventitious roots grown in this system also showed the highest levels of MDA (Figure 2A) and plasma membrane damage (Figure 2D). This indicated that oxidative stress and plasma membrane damage did not affect the vegetative growth of *T. paniculatum* cuttings in this study. Wang et al. (2022) reported different results, namely that damage to the plasma membrane of *Cinnamomum camphora* inhibited nutrient absorption and reduced biomass. In general, temperature stress can damage the plasma membrane and disrupt nutrient absorption, which is followed by a reduction in the growth, number, and

mass of roots and tissue above the ground (Huang et al. 2012; Giri et al. 2017; Wang et al. 2022). This can be caused by a level of oxidative stress or MDA (Figure 2A) below the tolerance limit, or by improving the adaptability through proline accumulation (Figure 2B). It was assumed that the nutrient solution temperature and oxygen availability played an essential role in the vegetative growth of *T. paniculatum* cuttings. Water temperature is another important growth factor that can affect plant development in hydroponic systems (Nxawe et al. 2010).

The cold nutrient solution in the WCT treatment could reduce the absorption of nutrients by the roots, while a warm solution in the UAT treatment could increase nutrient absorption (Morgan et al. 1980; Calatayud et al. 2008). Therefore, the increase in the three growth parameters in the three hydroponic systems was higher with the UAT than with the WCT treatment. The effect of nutrient solution temperature on nutrient uptake has also been demonstrated in various commercial plants. The rise in the nutrient solution temperature was found to accelerate the growth and increase the crop yields of *Cucumis sativus* (Daskalaki & Burrage 1998), *Simmondsia chinensis* (Reyes et al. 1977), and pine (Vapaavuori et al. 1992). In addition to the temperature of the nutrient solution, the availability of oxygen at the root zone area can support the vegetative growth of *T. paniculatum* cuttings. In the DFT system, all parts of the adventitious roots were immersed in the nutrient solution. However, due to a lack of oxygen, this position was unfavorable for the root. In the NFT system, only the lower parts of the adventitious roots were placed within the nutrient solution, while in the aeroponic system, all adventitious parts roots were in the air. Adventitious roots that were not fully immersed in the nutrient solution benefited from an increase in oxygen and gas exchange within all root structures. This condition is beneficial for growth and has been scientifically proven to increase yields by 10 times compared to conventional planting yields (Roberto 2003). Therefore, the result of this study showed that *T. paniculatum* cuttings cultivated in the aeroponic system and UAT showed the best results in terms of vegetative growth. A similar result was found for *Anemopsis californica* cultivated for eight months in an aeroponic system, namely increased production of its medicinal root, whereas NFT was found to reduce root production (Hayden 2006).

Saponin content of adventitious roots

The adventitious roots cultivated for 60 days in the three hydroponic systems with WCT treatment showed a higher saponin content than roots grown in the three systems with UAT treatment (Table 3). Similar to the growth level, for either group (UAT or WCT), the aeroponic, NFT, and DFT systems showed the highest to lowest saponin contents, respectively. Relatively similar saponin contents were obtained in the aeroponic system with both the UAT and WCT treatments. In comparison, *T. paniculatum* cuttings cultivated conventionally in soil for 120 days showed a lower saponin content than those cultivated in the three hydroponic systems.

Generally, secondary metabolites are produced under stress conditions (Dixon 2001). This study found that saponin production (Table 3) was aligned with the MDA level (Figure 2A). This result implies that the *T. paniculatum* cuttings cultivated in the three hydroponic systems with UAT and WCT treatments produced saponin under stress conditions. Figure 2A and Table 3 show that the high saponin content corresponded with a high level of MDA in adventitious roots in the aeroponic system with the UAT and WCT treatments. While in general, the production of

Table 3. Saponin content of the adventitious roots of *T. paniculatum* cuttings cultivated in three different hydroponic systems with two temperature treatments on the nutrient solution for 60 days and conventionally in soil for 120 days.

Cultivation method	Saponin content (mg/g DW)	
	Under ambient temperature (UAT)	With controlled temperature (WCT)
Hydroponic – DFT	131.76	126.46
Hydroponic – NFT	135.05	177.15
Aeroponic	189.83	195.61
Conventional (Soil)	56.61	

secondary metabolites in plants is not associated with cell growth (Malik et al. 2013), this study found otherwise. Figure 2A and Table 2 indicate that MDA levels did not affect vegetative growth. However, in this study, vegetative growth aligned with saponin production. This phenomenon was beneficial because under stress conditions, the cells, especially root cells, could grow and produce biomass and saponin. *Talinum paniculatum* root with a high saponin content will have a higher commercial value as both the pharmaceutical industry and local citizens will use it as a substitute for Korean or Chinese ginseng root.

The hydroponic cultivation of *T. paniculatum* cuttings showed greater potential in terms of obtaining root biomass with a high saponin content compared to conventional cultivation using soil as a medium. *Talinum paniculatum* cuttings cultivated conventionally for four months produce an adventitious root biomass of 0.014 g (DW) with saponin levels of 56.6 mg/g (DW). In this study, the best harvest was obtained by cultivating *T. paniculatum* cuttings for two months in an aeroponic system with UAT treatment. Compared with conventional methods, the use of an aeroponic system reduced the cultivation period by two months, produced a 30-fold increase in weight (1.175 g DW), and increased the saponin content by 3.5 times (195.61 mg/g DW). Finally, in the tropics, an aeroponic system with the nutrient solution under ambient temperature was identified as the appropriate system in which to cultivate *T. paniculatum* cuttings. These results are consistent with the previous assertion that aeroponic systems are most effective for growing medicinal and aromatic herbal plants in a controlled environment, where the plants and roots are harvested (Hayden 2006).

CONCLUSIONS

T. paniculatum cuttings were successfully cultivated in the tropics using three hydroponic systems, i.e., DFT, NFT, and aeroponic. Differences in the nutrient solution flow patterns in the three test systems affected the temperature of the nutrient solutions, which in turn promoted different levels of stress that affected the accumulation of biomass and saponins of *T. paniculatum* adventitious roots.

AUTHOR CONTRIBUTION

A.Y and Y.S.W.M conceptualized the research study. A.Y and A.N.K designed the research methodology. A.Y performed the research and analyzed the data. A.Y, Y.S.W.M, and A.N.K wrote the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Species Composition and Inshore Migration of the Tropical Glass Eels (*Anguilla* spp.) Recruiting to the Jali River, Purworejo Regency

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ABSTRACT

Purworejo Regency is located in the south coast of Java where rivers flow into the Indian Ocean. The Jali River in Purworejo becomes a migration route for eels where juvenile eels (glass eels) move from the ocean to freshwater passing through the estuary. During migration, glass eels will make efforts to survive, one of which is to manage their osmotic pressure (osmolarity). With regards to those, a study on the upstream migration of glass eels in Jali River was carried out. Glass eels samplings were conducted using hand-held lift nets at the new moon phase, from 20:00 pm to 4:00 am. Sampling stations included the estuary, a branch river, and below the dam. The osmolarity of both glass eels and media (river water) were measured. Furthermore, the chemical and physical parameters of the waters as the natural habitat for glass eels were also measured. Glass eel samples were then identified to species level. The results showed that glass eels entering the Jali River consisted of *Anguilla bicolor bicolor* and *Anguilla nebulosa nebulosa* with the former being the most common. Total length of glass eels ranged between 44.10 to 69.70 mm with total weights 0.08 to 0.54 g. Glass eel migration has a close relation with surface current and tide. The osmotic work level (OWL) of glass eels approaches 0.34 mOsm/l H_2O which is close to iso-osmotic. This happened at salinity 0 $^{o}/_{oo}$ so that the glass eels migrate to fresh water which has a lower salinity to achieve iso-osmotic condition.

INTRODUCTION

Eels (*Anguilla* spp.) have unique characteristics in a way that they adapt to multiple water conditions including fresh water, estuary and sea. Anguillid eels are catadromous species; they spawn at ocean, then the larvae migrate to rivers, and reach adulthood in fresh waters. By the time of spawning, the eels swim downstream and return to the ocean (Muthmainnah et al. 2016). The life cycle of eels consists of five stages, namely *leptocephalus*, *glass eel*, *elver*, *yellow eel* and *silver eel* (McKinnon 2006; Milosevic et al. 2022). Leptocephalus is a larval stage with its planktonic behaviour in open sea. This larva then metamorphoses into

glass eel (juvenile stage) which actively migrate to estuary (Tabeta & Mochioka 2003; Cresci 2020; Wichelen et al. 2022).

The migration of eels was influenced by environmental and physiological factors (Edeline et al. 2006). During migration, eels make efforts to enhance their survival, one of which involves regulating their osmotic pressure (osmolarity). The migrating eels have a wide tolerance for changes in salinity. Migration in fish is usually caused by physiological response to both internal and external inputs received. Migration is an important part of the fish life cycle for the continuity of the regeneration process (Lucas et al. 2001; Cao et al. 2021).

Distribution of eels in Indonesia starts from the coast of Sumatra, the south coast of Java, Bali, NTB, NTT, east coast of Kalimantan, Sulawesi waters, Maluku to Papua (Fahmi 2015). About 19 species of *Anguilla* have been reported worldwide (Arai 2020), two-thirds of eel species inhabit Indonesian waters (Sugeha et al. 2008a). However, there are three species were present in Java Islands, e.g. *A. bicolor bicolor*, *A. nebulosa nebulosa* and *A. marmorata* according to research in Palabuhanratu, Sukabumi (Hakim et al. 2015); Progo River, Yogyakarta (Budiharjo 2010); Segara Anakan, Cilacap (Sukardi et al. 2022).

In Java Island, the estuary of Jali River in Purworejo Regency is one of the migration routes for juvenile eel (glass eel). Geographically, this estuary is located on the south coast of Java, right next to the Indian Ocean. The estuary becomes a migration route for tropical eel species that exist in the waters of the Indian Ocean. Little is known about glass eels recruiting in Purworejo rivers.

The migrating glass eels are usually caught by local fishermen in the Jali River below the dam using hand-held lift nets. Therefore, most glass eels do not have the opportunity to grow up and reach gonadal maturity (silver eel stage) which hamper the addition process of new individuals to the population (recruitment). The removal of most glass eels will accelerate the decline of eel population in Jali River. Until now, to meet the high market demand, eels have been supplied from the catch of glass eels in open waters. The availability of this fish in the market depends on the success of the fishing in nature. Information about the life of tropical eels including the migration is needed for the basis of conservation and management efforts (Aoyama et al. 2014; Righton et al. 2021). Therefore, this study was conducted to investigate the migrating glass eels in the Jali River, consisting of biodiversity (identification of glass eel species entering the estuary of Jali River and their sizes), ecology aspect (abundance and distribution in Jali River and the influence of environmental factors to the abundance), as well as physiological aspect (the osmotic work level).

MATERIALS AND METHODS

Study Area

Glass eels were sampled in three stations shown in Table 1 and Figure 1. The sampling stations were chosen with the following considerations. Station 1 (ST 1) is the estuary which becomes the entrance for eel juveniles to fresh waters. After glass eels enter the Jali River, they migrate upstream. Glass eels migrate by swimming close to the banks of the river. Therefore, to know the distribution of glass eels, samplings should also be conducted in the river branches. At a distance of 3.5 km from the river mouth, Jali River branches into Kali Lereng. Therefore, this branch was set as sampling station 2 (ST 2) so that it can be found whether or not some glass eels migrate to the branch. At a distance of 7.5 km from the river mouth, there is a dam building. Samples of glass eels were taken

Table 1. The locations of sampling stations for glass eels.

Location	Coordinate		Distance from the river mouth	Information
	Latitude	Longitude		
Station 1	S7° 51' 11.73	E109° 54' 37.76"	0 km	River mouth
Station 2	S7° 49'25.53"	E109° 55'10.39"	3.5 km	A branch of Jali River
Station 3	S7° 47'30.38"	E109° 55'13.21"	7.5 km	Below the dam

below the dam, namely Station 3 (ST 3). In each sampling station (ST 1, ST 2 and ST 3), the physical and chemical parameters of waters were measured. This was to see the influence of the environmental factors to the abundance of migrating glass eels.

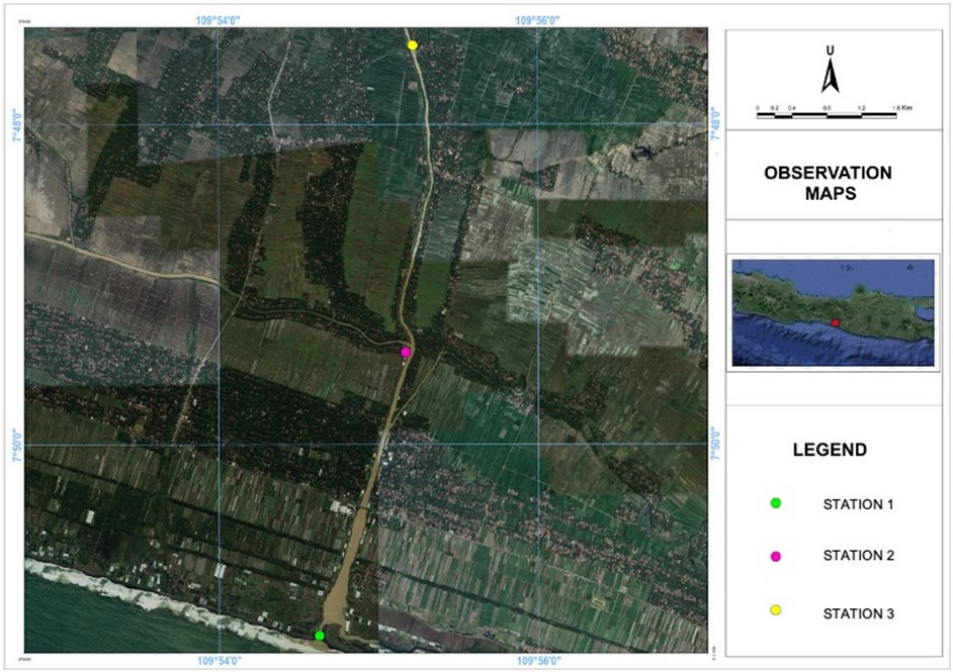

Figure 1. The locations of sampling stations for glass eels in Jali River, Purworejo Regency.

Procedures

Glass eels recruiting to the Jali River were sampled monthly from November 2015 to January 2016, in total of 9 times at 3 stations. As the migration season of glass eels in not year-round in Jali River, this research conducted in the three months of rainy season. The water discharge of Jali River was periodic and influenced by rainfall. The estuary of Jali River was closed by sand dunes during the dry season and followed by its opening in the rainy season. The information from local fisherman is also needed to conduct this field research. Fish collections were performed using hand-held lift nets of size 1 x 1 m (square shape with the area 1 m^2) with the mesh size of the net 1 mm^2. Glass eels were caught during one night from 20.00 to 04.00 Western Indonesian Time of the new moon phase. The time interval between two consecutive samplings was 2 hours. The transect size was 2 m length and 1 m wide, following the bank of the estuary. The bulk of fish captured were divided into two groups, one for species identification and another one for analysis of osmotic work levels. Fish samples for species identification were put into plastic containers filled with oxygen and brought to the laboratory alive. Meanwhile, the ones for osmotic level analysis were put in a freezer. The physical and chemical parameters of water measured included salinity ($^o/_{oo}$), temperature (^oC), pH, dissolved oxygen (ppm), river current velocity (m/s) and turbidity (NTU). At the time the eel samples were collected,

the water sample was also taken which was used to measure the osmolarity of the media (habitat) of eel juveniles. The measurement used Automatic Micro-Osmometer Roebling. Daily rainfall and sea tide data needed as supporting data were obtained from the Meteorological, Climatological, and Geophysical Agency (BMKG) of Central Java Province.

Data Analysis

Species identification

Identification of glass eels to species levels used morphological criteria and caudal pigmentation. Moreover, morphological characters including external morphology with morphometric measurements (ano-dorsal length) and internal morphology by counting the ano-dorsal vertebrae. In addition, the caudal pigmentation patterns which appear during the glass eel pigmentation process, were also used for species identification. To prepare the samples for species identification, live glass eels were put into clove oil solution (0.001%) for 30 seconds for anesthesia. Each glass eel was placed on an object glass for observation under the stereo microscope. To analyse the morphology, some morphometrics including dorsal fin length (L_D), anal fin length (L_A) and total length (L_T) were measured (Figure 2). The results of morphometric measurements were then analysed using an equation which could be used to recognize the species (Tabeta et al. 1976; Elie 1982; Reveillac et al. 2009; Leander et al. 2012).

$$A/D\ \% = \frac{(LD-LA)}{LT} x\ 100$$

A particular range of A/D% indicates a particular eel species. The value of A/D% was compared with the description of morphological characteristics of each species shown in Table 2.

Internal morphological analysis was carried out by counting the number of ano- dorsal vertebrae. The vertebrae counted were those right between the dorsal and anal fin tips (Ege 1939). If there was a symmetrical vertebrae to the tip of the dorsal fin, anal fin or both, then that vertebrae was not counted, as can be seen in Figure 2 (Ndobe 2010). The number of ano-dorsal vertebrae was then analysed by looking at the table of morphological characteristics (Table 2).

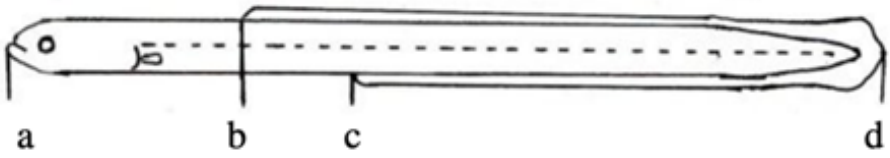

Figure 2. Analysis of external and internal morphology. Morphometric measurements: a-d total length (L_T), b-d dorsal fin length (L_D), c-d anal fin length (L_A). Counting of vertebrae: b-c ano-dorsal vertebrae (Ndobe 2010).

Table 2. Identification of glass eels based on external and internal morphology.

No	The species were found in the Java Island waters	A/D (%)	Number of ano-dorsal vertebras (vertebrae)	Initial positions of dorsal and ventral fins
1	*A. bicolor bicolor*	0.8	1 – 2	DFS
2.	*A. nebulosa nebulosa*	9.0	6 – 13	DFL
3.	*A. marmorata*	16.3	14 - 18	DFL

Note: DFL = *dorsal fin long*; DFS = *dorsal fin short*.

Length and weight frequencies of glass eel

Measurements of length (cm) and weight (gram) were conducted to all eel juveniles caught. The resulted data were analysed using frequency histogram. To find the average total length and body weight for each species in each month, the mean and standard deviation were computed using the following (Rice 2007; Wackerly et al. 2008):

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n} \quad \text{and} \quad Sd = \sqrt{\frac{\sum_{i=1}^{n} (X_i - \bar{X})^2}{n-1}}$$

Description:

Sd = Standard deviation (each species)
X_i = Length or weight of each individual
n = Total number of individuals
$\bar{X}$ = The mean length or weight each month

Osmotic

Osmotic work level (OWL, mOsm/l H2O) was obtained by measuring the osmotic pressure (osmolarity) of the environment (media/river water) and osmolarity of glass eels using *Automatic Micro-Osmometer Roebling*. To measure the osmolarity of glass eels, the glass eel was put in a microtube. OWL (mOsm/l H_2O) was then computed using the following formula (Anggoro & Nakamura 1996; Anggoro 2000; Anggoro et al. 2018):

OWL = ⌈P.Osm eel – P.Osm media⌉

Description:

OWL = Osmotic work level (mOsm/l H_2O)
P.Osm eel = Osmotic pressure of glass eel (mOsm/l H_2O)
P.Osm media = Osmotic pressure of river water (mOsm/l H_2O)
⌈ ⌉ = Absolute value

Note:

OWL > 0, means hyperosmotic osmoregulation pattern
OWL = 0, means iso-osmotic osmoregulation pattern
OWL < 0, means hypoosmotic osmoregulation pattern

The relationship between environmental factors and abundance of migrating glass eels

Multiple linear regression analysis was implemented to determine the relationship between various water physical and chemical parameters and the abundance of migrating glass eels. The physical and chemical parameters considered included temperature, pH, DO, current, salinity, turbidity, rainfall and tides. However, prior to regression analysis, these candidate independent variables were subjected to multicollinearity test to diagnose the inter-relations between two or more independent variables. In addition, the correlation level between independent and dependent variables for each significant covariate was computed using Pearson correlation analysis.

RESULTS AND DISCUSSION

Species Diversity

Glass eels that entered Jali River consisted of two species of eel juveniles, namely *Anguilla bicolor bicolor* and *A. nebulosa nebulosa*. This finding was confirmed after identifying 169 individuals of glass eels (Table 3). Morphological characters differed among glass eel species shown in Table 4, the mean ± standard deviation total length of 153 *A. bicolor bicolor* was 56,17 ± 4,48, and 50,40 ± 4,78 in 16 *A. nebulosa nebulosa*. Meanwhile, the difference of ano-dorsal length and the caudal pigmentation patterns of

Table 3. The species of glass eels migrating through Jali River.

Station	Month	The number sampled	*A. bicolor bicolor*	*A. nebulosa nebulosa*
1 (Estuary)	Nov	6	6	-
	Dec	4	4	-
	Jan	0	-	-
2 (River branch)	Nov	2	2	-
	Dec	11	11	-
	Jan	0	-	-
3 (Below dam)	Nov	83	77	6
	Dec	9	7	2
	Jan	54	46	8
Total		169	153	16

Table 4. Morphological characters of glass eels collected from Jali River.

Species	Mean ± S.D.						
	Weight (g)	Total length (mm)	Dorsal fin length (mm)	Anal fin Length (mm)	Ano-dorsal Length (mm)	A/D%	Ano-dorsal vertebrae
A. bicolor bicolor	0,17 ± 0,09	56,17 ± 4,48	34,84 ± 3,42	34,33 ± 3,38	0,51 ± 0,24	0,90 ± 0,41	1,45 ± 0,65
A. nebulosa nebulosa	0,12 ± 0,03	50,40 ± 4,78	33,98 ± 2,17	29,73 ± 2,06	4,25 ± 0,41	8,44 ± 0,43	7,00 ± 0,76

two glass eels species: *Anguilla bicolor bicolor* and *A. nebulosa nebulosa* in Figure 3.

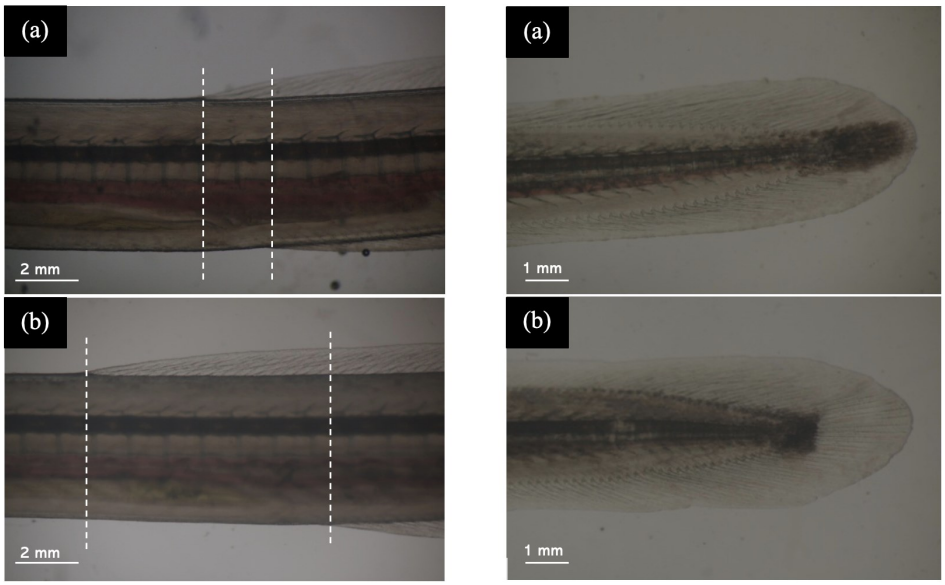

Figure 3. The distance between the dorsal and anal fin, as well as caudal pigmentation of the species (a) *Anguilla bicolor bicolor*, (b) *Anguilla nebulosa nebulosa.*

The percentages of glass eels of *A. bicolor bicolor* entering the Jali River over three consecutive months were 93, 92, and 85%, respectively, while those of *A. nebulosa nebulosa* were 7, 8, and 15%, respectively. The composition of glass eels found at all sampling stations is summarized in Figure 4. *A. bicolor bicolor* emerged as the most prevalent species. The species composition in the Jali River exhibited similarities with those of the Cimandiri River in Sukabumi (Fahmi et al. 2010; Annida et al. 2021; Triyanto et al. 2021) and the Progo River in Yogyakarta (Budiharjo 2010). In these locations, the species observed included *A. bicolor bicolor*, *A. marmorata, and A. nebulosa nebulosa.* Although this study exclusively encountered two distinct species, specifically *A. bicolor bicolor* and *A. nebulosa nebulosa*, migrating into the Jali River, Purworejo Regency. The spe-

cies A. *bicolor bicolor* is indeed a species commonly found in the waters of Java Island that are directly connected to the Indian Ocean (Annida et al. 2021), in significant numbers. On the other hand, *A. nebulosa nebulosa* is a type often found in the waters of Indian Ocean: East Africa to Sumatera, and its presence in the Jali River is believed to be due to the phenomenon of counter-currents in the eastern part of the Indian Ocean, known as the "Wyrkty Jet" or "equatorial jet," which transports the larvae of this species to the waters of Java Island.

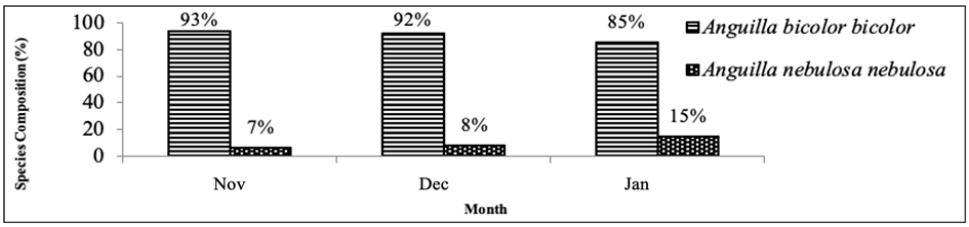

Figure 4. Species composition of glass eels entering the Jali River.

The presence of tropical anguillid eels within Indonesian waters indicated a diverse array of nine distinct species and subspecies inhabiting the region. These encompass *A. celebesensis*, *A. marmorata*, *A. borneensis*, *A. interioris*, *A. obscura*, *A. bicolor bicolor*, *A. bicolor pacifica*, *A. nebulosa nebulosa*, and *A. megastoma* (Sugeha et al. 2008a; Sugeha et al. 2008b). The central Indonesian waters demonstrated the highest level of diversity, where five species were found including four long-finned eel species (*A. marmorata*, *A. celebesensis*, *A. borneensis*, *A. interioris)* and one short-finned eel species (*A. bicolor pacifica*). Meanwhile, the waters of western Indonesia are predominantly inhabited by two species (*A. nebulosa nebulosa* and *A. bicolor bicolor*), and the waters of eastern Indonesia comprise *A. obscura* and *A. megastoma* (Sugeha et al. 2008a; Sugeha & Suharti 2009). This study found two species *A. nebulosa nebulosa* and *A. bicolor bicolor*, in the Jali River, Purworejo Regency, located in western Indonesia, consistent with the previous studies.

According to Minegisihi et al. (2012), *A. bicolor* consists of two subspecies, namely *A. bicolor bicolor* in the Indian Ocean (this study) and *A. pacifica* that is distributed in Pacific Ocean. However, previous molecular studied showed there are four distinct populations of this species, namely the ones in North Pacific, South Pacific, Indian Ocean and Mariana. Glass eels in Jali river are part of the population in the Indian Ocean. Both species of eel, namely *A. bicolor* are of interest because of its distribution and abundance in the center of economic position and these species are also known to have a relatively wide geographical distribution (more than 18,000 km east-west) (Fahmi et al. 2012). The IUCN listed five commercially exploited eel species in Indonesia in its Red List, including *A. bicolor* and *A. nebulosa nebulosa* classified as Near Threatened ; therefore, close monitoring of intensive exploitation of this eel is necessary (Nijman 2015; Righton et al. 2021).

Length and weights of glass eels in the Jali River

A total of 169 glass eels with a minimum length of 44.1 mm and a maximum length of 69.7 mm were collected during the study (Fig. 5). There seems to be differences in size distributions of glass eels for different months (November, December and January). In November, the length ranged from 48.0 to 60.0 mm, with a mean and median 54.36 and 54.0 mm, respectively. In December, the smallest glass eel found was 44.1 mm TL, and the largest was 66.4 mm, with a mean and median 5.1 and 53.8 mm, respectively. In January, the smallest glass eel found was 46.7 mm

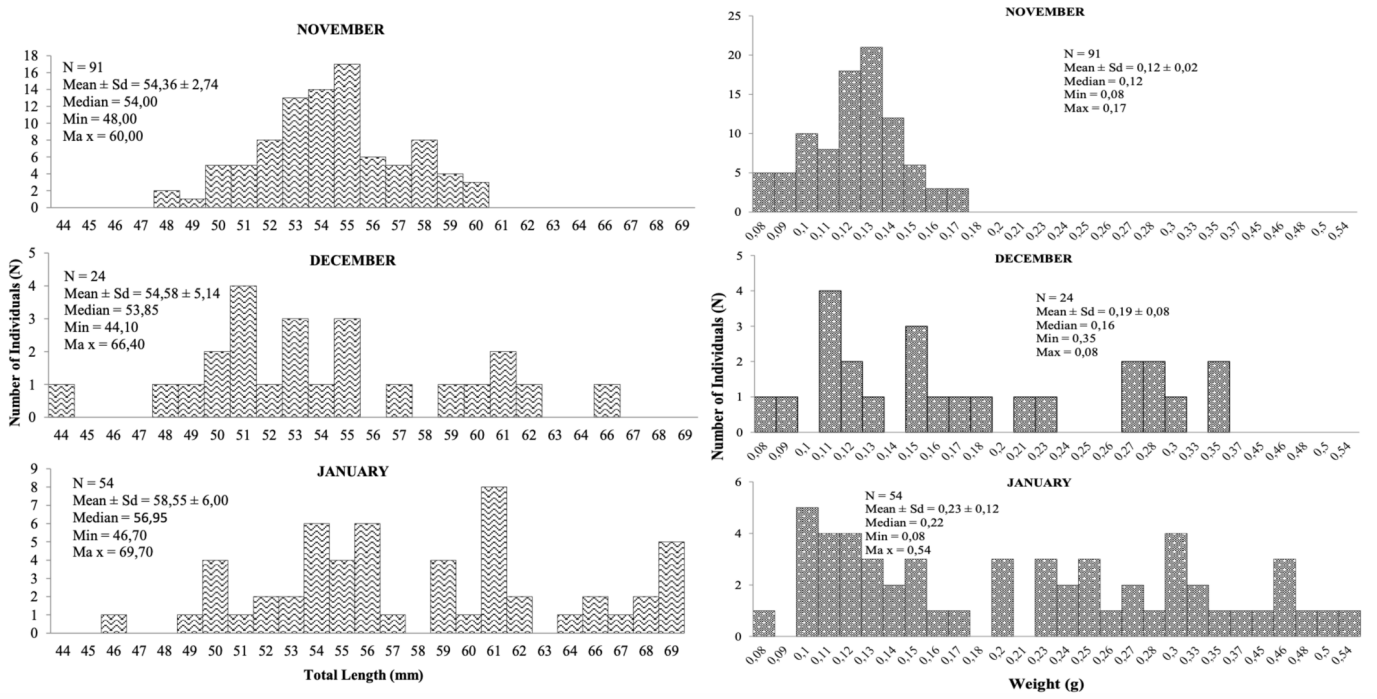

Figure 5. Frequencies of total lengths (left) and weights (right) of glass eels based on sampling time.

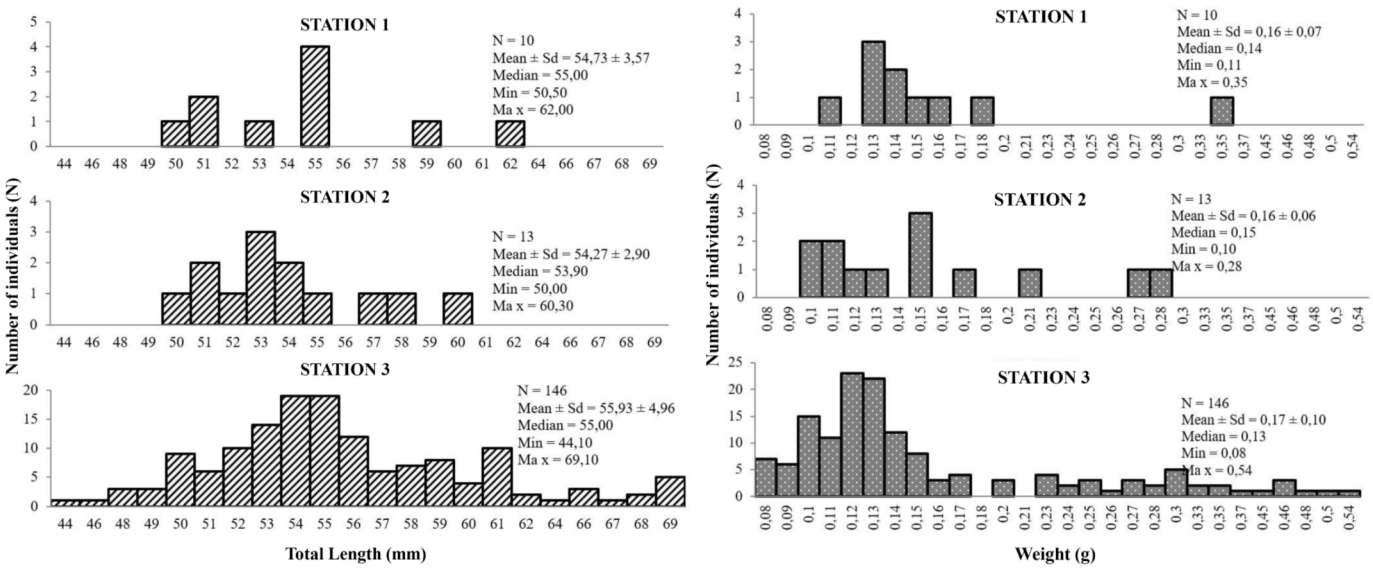

Figure 6. Frequencies of total lengths (left) and weights (right) of glass eels based on sampling stations.

and the largest was 69.7 mm, with a mean and median 6.0 and 56.9 mm, respectively. The weights of glass eels found during the study were 0.08 – 0.54 g. In November to January, the glass eels weight ranges were 0.08 -0.17, 0.08-0.35 and 0.08-0.54 g, respectively.

The size distribution of glass eels found in this study was similar with other studies. Sriati (2003) reported total lengths of glass eels in the Cimandiri River ranging from 51.57 to 53.27 mm. Glass eels of species *A. marmorata* caught in the Palu River estuary, Central Sulawesi, ranged in length from 41 to 50 mm (Ndobe 2010). According to Setiawan et al. (2003), the total length of tropical glass eels appears to be lower than that of some temperate species, such as the European eel *A. anguilla* (68 mm), and Japanese eel *A. japonica* (57 mm).

According to the findings of this study, glass eels in November have a smaller size range than December and January. At the same time, glass eel with the highest size was found in January (69.7 mm and 0.54

g). This observation aligns with the results reported by Triyanto et al. (2020). January showed the highest variations in glass eel lengths and weights. These variations are probably influenced by factors such as differences in spawning time, migration duration, and broodstock quality during reproduction, as previously suggested by Triyanto et al. (2020). In addition, the variations in eel length can be attributed to the slow growth rate of eels during the leptocephalus stage, leading to smaller glass eels during recruitment, as suggested by Arai et al. (1999) and confirmed by Marui et al. (2001). The differences also likely related to the duration of larval development, as emphasised by Triyanto et al. (2020).

The distribution of length and weight of eels varied considerably among sites (Fig. 6). In station 1 (estuary), length ranged from 50.5 to 62.0 mm and weight from 0.11 to 0.35 g; Station 2 (river branch) has a length of 50.0–60.3 mm and weight of 0.1-0.28 g, and Station 3 (below the dam) 44.1 – 69.1 mm and 0.08-0.54 g. The glass eels at station 3 had longer bodies than the ones at Station 1 and 2. This study found a pattern of increasing body length of eel with the increase of distance from the sea. Kume et al. (2020) also found the similar pattern on Japanese eels. This suggests that recruitment to the upper sections of the river begins at a larger size, mainly as a consequence of the presence of the weir. The weir restricts upstream migration. In addition, at the time of sampling below the dam (Station 3), in December and January there were young eels that already had perfect pigmentation. The length of the young eel (elver) caught during the study in the Jali River ranged from 71–231 mm with the body weight ranged between 0.53–15.70 g. Kwak et al. (2019) reported that eels can spread within a river at the glass and elver stages, and then they will stay there until the onset of maturation when they move out to the sea.

The relationship between environmental factors and abundance of glass eels

The number of migrating glass eels caught in relation to sampling time was shown in Figure 7. Measurements of environmental parameters during this study resulted in the data shown in Table 5. The daily rainfall and sea tide data from Meteorological, Climatological, and Geophysical Agency were shown in Table 6.

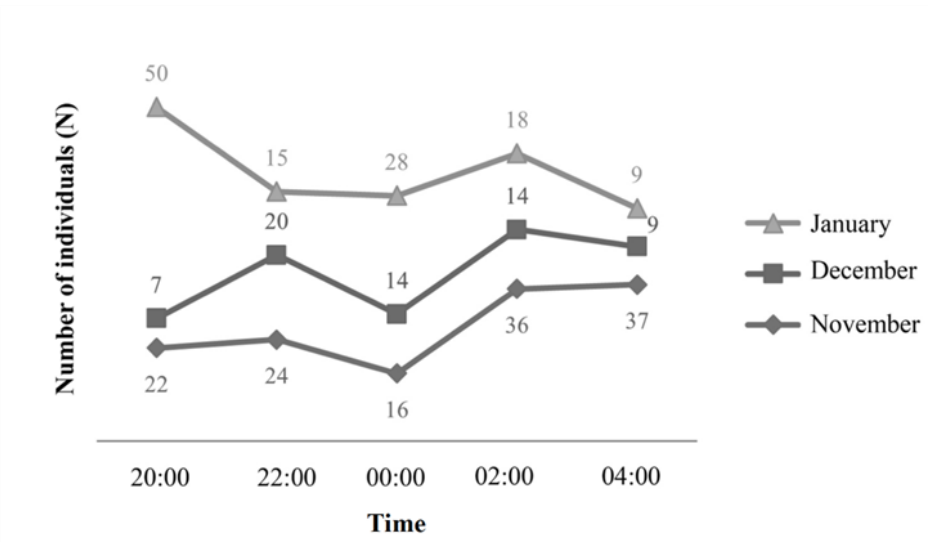

Figure 7. Daily migration of glass eels.

Multicollinearity test showed that variables temperature, pH, DO, salinity, and rainfall are multicollinear. Therefore, of all eight environ-

Table 5. The measurement results of water physical and chemical parameters in Jali River.

Month	Station	Temp. (°C)	pH	DO (mg/l)	Current(m/s)	Salinity (°/₀₀)	Turbidity (NTU)
Nov	ST 1	28.44	6.49	7.59	(-)0.25 - (-)0.50	36 - 20	1.23
	ST 2	30.10	7.14	6.34	(+)0.10 - (+)0.25	0	2.68
	ST 3	30.16	7.08	6.40	(-)0.01 - (+)0.03	1 - 0	1.69
Dec	ST 1	28.70	6.98	7.23	(-)0.30 - (+)0.50	16 - 0	78.59
	ST 2	26.12	7.46	7.15	(-)0.10 - (-)0.33	5	352.08
	ST 3	28.46	7.21	8.68	(+)0.33 - (+)0.50	0	45.90
Jan	ST 1	28.68	7.62	7.77	(-)0.50 - (-)1.50	34 - 12	10.47
	ST 2	30.05	7.11	5.79	(+)0.10 - (+)0.20	0	27.56
	ST 3	29.00	7.93	7.45	(-)0.01 - (+)0.08	0	20.75

Note:
Positive current (+): The direction of the current was from upstream to downstream
Negative current (-): The direction of the current was from downstream to upstream

Table 6. The rainfall and tide data.

Month	Rainfall (millimetre/day)	The number of rainy days	Tide (m)
November	6 – 47	6	0.3 - 1
December	3 – 115	14	0.3 – 0.9
January	2 – 52	9	0.4 – 2.1

Source: (Meteorological, Climatological, and Geophysical Agency 2015, 2016)

mental parameters, only turbidity, current and tide were used as independent variables in the multiple linear regression. Furthermore, variable turbidity was eventually removed because it was not found to have a significant effect on the dependent variable. Therefore, the resulting relationship between environmental parameters (current and tide) and the abundance of migrating glass eels is as follow:

$Y = 2.507 - 34.921\ X_1 + 18.173\ X_2$

Description:

Y = Dependent variable (the number of glass eels)
X_1 = Independent variable (current)
X_2 = Independent variable (tide)

Linear regression analysis showed that the daily migration of glass eels is closely related to both current and tide $R^2 = 0.56$. The regression coefficient associated with current has a t-value of -4.463, while the one associated with tide has a t-value of 3.784. The p-values for both t-values are <0.05, implying that both coefficients are significantly greater than 0. These indicate significant effects of both independent variables to the abundance of migrating eel juveniles. From the values of regression coefficients, it is clear that the relationship between juvenile abundance and current is negatively correlated (r = -0.585), while with tide is positively correlated (r = 0.508).

Current and tide are two variables affecting the abundance of migrating eel juveniles in Jali River. The eel juveniles caught, migrated through the estuary in November to January at the start of the rainy season. The lowest abundance was in December. Heavy rainfall in December around 3-115 mm/day with 14 rainy days caused strong current in Jali River. The velocity of the current in the estuary affected the time the eel juveniles entered the estuary. Glass eels would enter the river which had only moderate waves and current. This occured when there was no rain, as heavy rain resulted in a significant in water discharge and strong currents.

The sampling of glass eels in the estuary of Jali River were done at night during the new moon. The migration peaked when the seawater was rising (high tide) during which a substantial amount of seawater flows into the estuary. In that condition, eel juveniles migrated by making use of the tidal currents. They passively rode on the moving water pushed into the estuary in order to streamline the energy use (Tesch 2003; Zompola et al. 2008; Cresci et al. 2020).

Osmotic work level (OWL)

Osmotic work level (OWL) represents the difference between the osmolarity of glass eels and the media (water) (Anggoro 2000). Results of OWL analysis from various salinities of the media show that water with salinity 0 ‰ led to the lowest OWL, with the mean 0.34 mOsm/l H_2O. Meanwhile, water with salinity 27 ‰ led to the highest OWL, i.e. 327.86 mOsm/l H_2O (Table 7). The relationship between OWL and salinity was presented in Figure 8. The measurement of osmolarity of eel juveniles, its media as well as OWL resulted in values shown in Table 7.

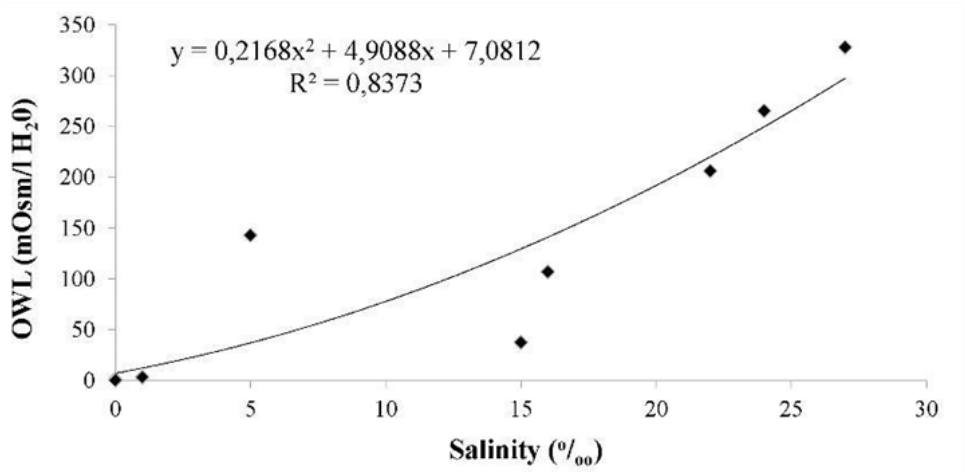

Figure 8. The relationship between salinity and osmotic work level (OWL).

The regression analysis showed that the relationship between salinity and OWL satisfies the following equation:

$Y = 0.216x^2 + 4.908x + 7.081$

Description:

Y = Osmotic work level

X = Salinity

The estuary (ST 1) had a wider range of salinity than those in other sampling stations. The salinity in the estuary ranged from 0 to 34 ‰

Table 7. Data on salinity of media, mean osmolarity of media and glass eels as well as the osmotic work level (OWL) (mOsm/l H_2O).

Salinity (‰)	Osmolarity of media (mOsm/l H_2O)	Osmolarity of eel juvenile (mOsm/l H_2O)	Osmotic work level (mOsm/l H_2O)
0	2.92	3.26	0.34
1	29.19	26.05	3.13
5	145.915	3.14	142.77
15	437.72	400.35	37.37
16	466.91	360.00	106.91
22	641.97	436.02	205.95
24	700.32	435.01	265.32
27	787.90	460.04	327.86

where the high salinity occurred when the seawater flew into and the low salinity occurred during low tide when the water of the river flew to the sea. The osmolarities of the media (water as the habitat for eel juvenile) in three sampling stations ranged between 2.92 - 787.90 mOsm/l H_2O. Meanwhile, the osmolarity of eel juveniles in the same three sampling locations ranged between 3.14 - 460.04 mOsm/l H_2O.

Osmotic work levels (OWL) resulted from the differences of osmolarities of the media and eel juveniles were between 0.34 - 327.86 mOsm/l H_2O. The OWL 0.34 mOsm/l H_2O was the closest to iso-osmotic condition and occurred at salinity 0 $^{o}/_{oo}$. Therefore, to reach iso-osmotic condition, eel juveniles need to migrate to freshwater which has lower salinities than the sea. According to Anggoro (1992) and Anggoro et al. (2018), salinity is a very important environmental parameter for aquatic organisms, especially in maintaining the osmotic balance between the protoplasm of the organism and its environment.

Glass eels have an osmoconformer pattern, that is its osmotic pressure adjusts to the water they inhabit. Therefore, when the salinity increases, the osmotic pressure of glass eels increases too. In contrast, eels at elver stage live and develop in freshwater and have an osmoregulator pattern, i.e. the osmotic pressure of the elvers is not affected by the water salinity. Based on salinity data and the calculation of OWL, some glass eels migrating to Jali River were in the transition from osmoconformer to osmoregulator. Those glass eels were close to the elver stage which has a perfect pigmentation already.

CONCLUSIONS

Eel juveniles entering and migrating through to Jali River consist of *A. bicolor bicolor* and *A. nebulosa nebulosa*. Species *A. bicolor bicolor* was the most abundant. The migration of eel juveniles is closely related to current and tide. Eel juveniles enter the river with a low salinity to pursue an osmotic work level close to zero, hence reaching an iso-osmotic condition. Considering that glass eels are a crucial phase in the eel life cycle, catching them should take into account the sustainability of these glass eels. In the future, management strategy using e.g. catch quota of glass eels should be implemented in Jali River. Therefore, further study should aim to identify the abundance of glass eels migrating in Jali River to enable the formulating of this quota.

AUTHOR CONTRIBUTION

A.I. designed the research, collected, analysed data and wrote manuscript, P.S.I. analysed data and wrote manuscript, F.Y.Y analysed data and wrote manuscript, W.K. analysed data and wrote manuscript, S.Ai. analysed data and wrote manuscript, S.A. designed, supervised the research and wrote manuscript, S.W.S. designed, supervised the research and wrote manuscript.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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Research Article

Habitat Suitability Modelling of *Rhacophorus reinwardtii* (Schlegel, 1840) in Java, with Notes on Habitat Characteristics from Malang Region-East Java

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ABSTRACT
The distribution of the Reinwardt's Gliding Frog, *Rhacophorus reinwardtii* (Schlegel, 1840), was recently thought to be restricted in Java. Furthermore, the constant and rapid deforestation in Java highlights the significance of the frog's habitat requirements in establishing the accurate species' conservation status. This study aims to predict the suitable habitat for this species in Java and to identify the habitat characteristics in Malang Region-East Java. Using the Maximum Entropy (MaxEnt) approach, we modelled the distribution of species and examined numerous types of breeding sites in Malang Region. Our findings revealed that the species inhabits a much more restricted and fragmented habitat in Java, where it inhabits both forest and agroforestry areas.

INTRODUCTION
Reinwardt's Gliding Frog, *Rhacophorus reinwardtii* (Schlegel, 1840) was previously thought to be widely distributed in Java, Sumatra, the Malay Peninsula, and Borneo (Ohler & Delorme 2006). Research has found that populations outside of Java Island are classified as other species: population from the Malay Peninsula and Southern Thailand are classified as *R. norhayatiae* Onn and Grismer, 2010; population from southern Vietnam are classified as *R. helenae* Rowley, Tran, Hoang, and Le, 2012; population from Sabah and Sarawak are classified as *R. borneensis* Matsui, Shimada, and Sudin, 2013. Several discovery of new species indicates that R. reinwardtii is restricted to Java (Frost 2021).

R. reinwardtii is found at elevations ranging from 20 to 1800 m asl (above sea level) on Java Island (Kurniati 2008; Yazid 2009; Ratna & Wijaya 2013; Wening 2017; Septiadi et al. 2018; Amrullah 2019; Setiawan et al. 2019; Kadafi et al. 2019; Malik 2019; Deslina 2021). It was discovered in a previous study that *R. reinwardtii* can live in both primary and secondary tropical rainforests (IUCN Red List of Threatened Species 2022). In addition, there are few reports of *R. reinwardtii* in production forest areas or residential areas near forest boundaries (Iskandar 1998; Ohler & Delorme 2006). Aside from that, the rare occurrence of *R. reinwardtii* at low-to-moderate altitudes is also questionable, thereby, it is difficult to determine if this phenomenon is due to a lack of data or a shift in the suit-

able altitudinal range. Between the 19th and 20th centuries, the state of the forests in Java Island changed dramatically (Peluso 1991). Currently, this has potentially and negatively affect *R. reinwardtii* survival in its natural habitat in Java.

The assessment of the conservation status based on The IUCN Red List of Threatened Species (2022) classifies *R. reinwardtii* as a species of least concern due to its widespread distribution, presumed large population, and lack of data indicating a significant population decline. Nonetheless, it is essential to analyze and reevaluate the distribution using alternative methods, such as species distribution modelling. In addition, information on the habitat requirements of *R. reinwardtii* is lacking. In this study, we aim to predict the suitable habitat for this species in Java and to identify the habitat characteristics for this species in Malang Region. This will hopefully help to shed a light on the current status of *R. reinwardtii* and support its action plan and conservation strategy in the future.

MATERIALS AND METHODS

Study area

Java is a 12829.7 ha–island that serves as the social, economic, political, and cultural center of Indonesia (Whitten et al. 1996; BPS 2015). The island has a variety of land cover types, including forested areas, lowland cultivation, regrowth, grassland, savanna grassland, unvegetated land, and land used primarily for the settled garden, arable, or tree crop cultivation (Whitten et al. 1996). Since 2001, Java has concerningly experienced up to 264.7 ha of deforestation (150 ha in East Java; 75.9 ha in Central Java; 38 ha in West Java) (Global Forest Watch 2021).

East Java, recognised for its concerning deforestation rate, specifically in the Malang Region, presents a convincing case study. The Malang Region, including Malang City, Malang Regency, and Batu City, is a metropolitan area located in the province of East Java, Indonesia. It is primarily known for its urban and suburban areas situated in highland scenery, although its southern area is characterised by lowland topography. The geographical area in concern is in close proximity to a number of mountain ranges, including but not limited to Mt. Bromo-Tengger-Semeru to the east, and Mt. Arjuno-Welirang-Anjasmoro and Mt. Panderman-Kawi-Butak to the west.

Species distribution modelling

The Species Distribution Model (SDM) uses maximum entropy (MaxEnt) to produce relevant and robust result even with limited occurrence records (Proosdij et al. 2016). The minimum distance between two closely occurring points should be more than 1.5 km since the predictor variables resolution is 30 arc-second (~1 km^2). A total of 23 occurrence points of *R. reinwardtii* (datum: WGS 84) from western to eastern Java were utilised after the filtering process. These points were acquired through direct observation (6 occurrences) and literatures survey (17 occurrences) (Kurniati 2002; Kurniati 2003; Yazid 2009; Yanuarefa et al. 2012; Ratna & Wijaya 2013; Mumpuni 2014; Wening 2017; Amrullah 2019; Kadafi et al. 2019; Malik 2019; Setiawan et al. 2019; Idrus 2020).

The predictor variables was selected based on the species biology and ecology which included climatic, topographic, and biophysics predictors. A total of 24 independent predictor variables with 30 arc-second resolution were employed, these include 19 bioclimatic variables, representing seasonality, extremity, and annual trend from Worldclim v2.1 datasets (https://worldclim.org/data/worldclim21) (Fick & Hijmans 2017);

digital elevation modeling data datasets (http://srtm.csi.cgiar.org) (Jarvis et al. 2008), which were derived into slope data and roughness data; Landsat cloud-free image datasets composite (https://glad.earthengine.app/view/global-forest-change) (Hansen et al. 2013) which were extracted for Normalised Difference Vegetation Index (NDVI); and tree canopy cover data for the year 2000 datasets (Hansen et al. 2013) (Table 1).

These predictor variables were reprojected, resampled, and clipped accordingly to the study area. The preparation of predictor variables was carried out in RStudio v.3.4.1 (R Core Team, 2013), using various packages including *rgdal* (Bivand et al. 2022), *rgeos* (Bivand & Rundel 2022), *sp* (Pebesma & Bivand 2005), *raster* (Hijmans 2022), and *tidyr* (Wickham & Girlich 2022).

Prior to the SDM analysis utilising MaxEnt, it is crucial to correct for multicollinearity and exclude highly correlated predictor variables to prevent overfitting prediction (Merow et al. 2013). Consequently, we utilised a multicollinearity test with a variance inflation factor (VIF) to detect collinearity between variables using a stepwise procedure. The multicollinearity test for predictor variables was conducted in RStudio v.3.4.1 utilising multiple packages, i.e., *usdm* (Naimi et al. 2014) and *terra* (Hijmans 2022). This procedure eliminated 13 predictor variables due to collinearity issues.

The maximum entropy technique utilising the MaxEnt v. 3.4.1 software (https://biodiversityinformatics.amnh.org/open_source/maxent/) was utilized (Phillips et al. 2006). We jackknifed our model to determine the best predictor variables for the distribution of *R. reinwardtii*, used clog-log output format, and examined response curves. We used 10% of the data for testing and 90% of the data for training; a total of 15,000 background points were used, ten replications were performed

Table 1. Details of predictor variables employed in MaxEnt of *R. reinwardtii* in Java, using 30 arc-second resolutions.

Code	Predictor variables	Source	Unit
bio1	Annual mean temperature	WorldClim	°C
bio2	Mean diurnal range	WorldClim	°C
bio3	Isothermality	WorldClim	%
bio4	Temperature seasonality	WorldClim	°C
bio5	Max. temperature of warmest month	WorldClim	°C
bio6	Min. temperature of coldest month	WorldClim	°C
bio7	Temperature annual range	WorldClim	°C
bio8	Mean temperature of wettest quarter	WorldClim	°C
bio9	Mean temperature of driest quarter	WorldClim	°C
bio10	Mean temperature of warmest quarter	WorldClim	°C
bio11	Mean temperature of coldest quarter	WorldClim	°C
bio12	Annual precipitation	WorldClim	mm
bio13	Precipitation of wettest month	WorldClim	mm
bio14	Precipitation of driest month	WorldClim	mm
bio15	Precipitation seasonality	WorldClim	%
bio16	Precipitation of wettest quarter	WorldClim	mm
bio17	Precipitation of driest quarter	WorldClim	mm
bio18	Precipitation of warmest quarter	WorldClim	mm
bio19	Precipitation of coldest quarter	WorldClim	mm
trecov	Tree canopy cover	Hansen et al. 2013	%
ndv	Normalized difference vegetation index	Hansen et al. 2013	Nil
ele	Elevation	Jarvis et al. 2008	m
slo	Slope	derived from ele	°
rou	Roughness	derived from ele	°

using subsampling replication run type and 500 iterations were maintained (Khan et al. 2022); we used regularisation of 3 because it performs better and reduces the over-fitting of the prediction results. We reported the receiver operating characteristic (ROC) area, which indicated the area under curve value (AUC value). If the AUC value is greater than 0.70, it indicated that the predictive model is performing well (Araújo et al. 2005). We reported the average AUC value under the ROC curve and calculated the probability of presence based on the average of ten replicate models.

For the binary presence/absence map, we used the 10th percentile training threshold, which excluded all regions with habitat suitability lower than the suitability values for the lowest 10% of occurrence records. The binary presence/absence map was derived from the previous probability of presence output. This analysis was conducted in RStudio version 3.4.1, following Hu and Jiang (2018).

Using the Geospatial Conservation Assessment Tool (GeoCAT) (http://www.kew.org), we also determined the area of occupancy (AOO) and extent of occurrence (EOO) polygons from *R. reinwardtii* occurrence points.

We visualised the results with the QGIS 3.18 software and arranged three outputs: i) the species occurrence overlaid with tree canopy cover, ii) the probability of presence map, and iii) the binary presence/absence map.

Field observation

To illustrate the general habitat characteristics of *R. reinwardtii*, we investigated the previously documented species occurrence and breeding sites in Malang Region (Figure 1), as representative of Java. In addition, the occurrence in East Java may represent the actual threats to their populations, as East Java is the province with the highest extent of forest degradation (Global Forest Watch 2021).

From August 2021 to February 2022, we conducted the Visual Encounter Survey in conjunction with the Breeding Site Survey (Heyer et al. 1994), and collected data on habitat characteristics. Each of these activities is conducted on every month throughout the observation period. Observations were conducted during the time period of 18:00 to 22:00 to account for the peak activity exhibited by amphibians. After documenting the individuals encountered and the breeding site, measuring the microclimate and abiotic factors and conducting a comprehensive examination of the individuals and breeding sites, all captured individuals were released back into the habitat where they had been found. To avoid the degradation risk of breeding sites, the suffering of animals, and the transmission of animal disease (e.g., chytridiomycosis), we strictly adhere to the biosecurity aspect during the surveys (wearing gloves, disinfected the tools, be aware of the surroundings).

RESULTS

Species distribution modelling

Based on the selected remaining predictor variables (11 predictor variables: bio2, bio3, bio4, bio13, bio14, bio18, bio19, trecov, ndvi, ele, and slo), the MaxEnt models evaluation revealed that the selected predictor variables produced the best models for estimating the probability of *R. reinwardtii* presence. The averaged test omission rate and predicted area performed better than random when tested for omission and demonstrated good reliability of prediction (Appendix 1). The average AUC of the replicate tests is 0.878, and the standard deviation is 0.079, indicating that the SDM has a strong predictive performance (Appendix 2).

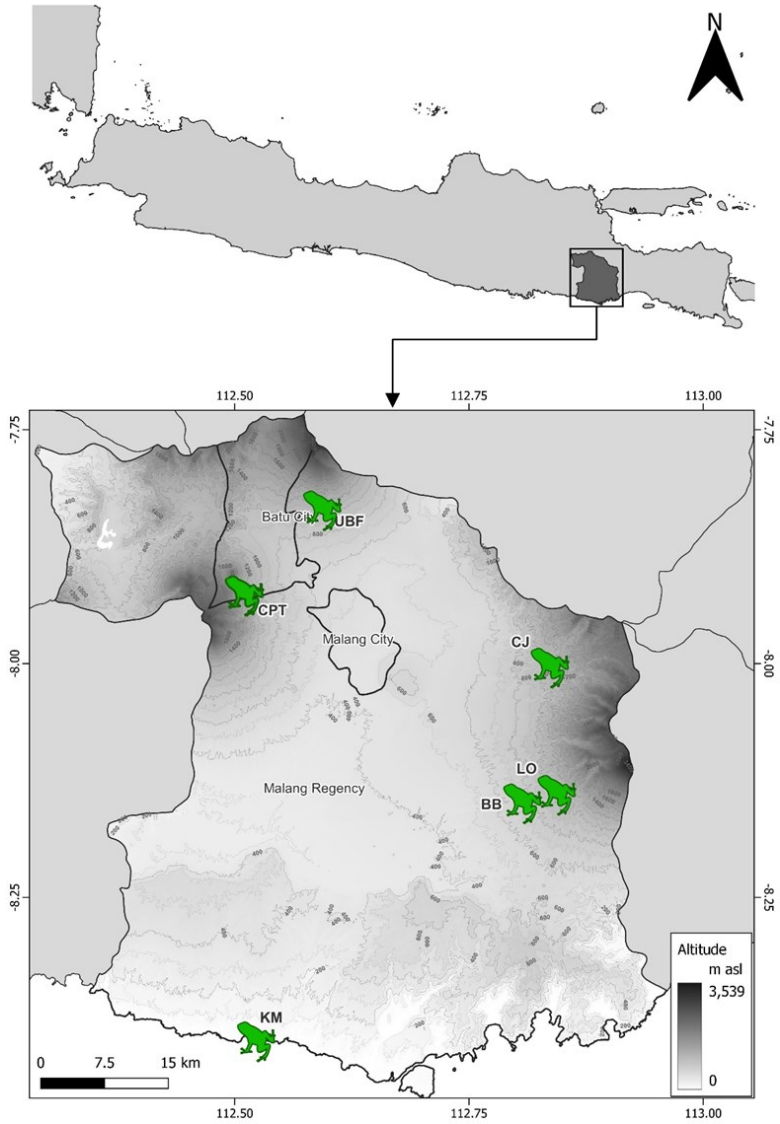

Figure 1. Field observation study area conducted in Malang Region-East Java. Note: **KM:** Kondang Merak, **BB:** Bambang village, **UBF:** Universitas Brawijaya Forest, **CPT:** Coban Parang Tejo, **CJ:** Coban Jodo, **LO**: Ledok Ombo.

The percent contribution and permutational importance of the six variables that contributed the most to the model are shown in Table 2. Based on percentage contribution, the order of the six predictor variables is as follows: tree canopy cover (46.5%), elevation (38%), precipitation of warmest quarter (13.5%), precipitation of coldest quarter (0.8%), temperature seasonality (0.7%), and slope (0.6%). In the species distribution modeling, tree canopy cover, elevation, and precipitation of the warmest quarter were found to be the most significant predictor variables

Table 2. Variable contribution (in %) of the leading six predictor variables in species distribution modelling of *R. reinwardtii* in Java.

Code	Predictor variables	Percent contribution	Permutation importance
trecov	Tree canopy cover	46.5	49.5
ele	Elevation	38	14.7
bio18	Precipitation of warmest quarter	13.5	26.8
bio19	Precipitation of coldest quarter	0.8	4
bio4	Temperature seasonality	0.7	4.9
slo	Slope	0.6	0

(Appendix 3). Moreover, the tree canopy cover was the predictor with the highest gain when used in isolation.

On the basis of the optimal predictor conditions that best represented the presence probability, a response curve composed of six leading predictor variables is generated (Appendix 4). The optimal detected predictor variables included tree canopy cover (exponentially increased after 45%), elevation (exponentially increased after 0 to 1500 m, remains stationary after 1500 m), and precipitation of warmest quarter (exponentially increased after 200 to 1200 mm, remain stationary after 1200 mm). Due to the high value of standard deviation, the other three predictor variables (precipitation of the coldest quarter, temperature seasonality, and slope) were difficult to interpret due to bias.

The results (Figures 2B-C) depicted that several large continuous forests with small forest corridors and interconnected forest patches, particularly in Western and Central Java, have the highest probability of presence (>0.75). Eastern Java is less suitable for *R. reinwardtii* because its habitat suitability is mostly in mountainous highlands with fragmented to medium-unconnected forest patches. A binary presence/absence map illustrated that only 27% (3500 out of 12829.7 ha) of Java Island is suitable for *R. reinwardtii* habitat. According to an IUCN-recommended method, *R. reinwardtii*'s extent of occurrence (EOO) is 6500 ha and its estimated area of occupancy (AOO) is 9200 ha. Our findings revealed that *R. reinwardtii* has a much smaller and fragmented habitat in Java.

Observation of habitat characteristics in Malang Region

In Malang Region our survey uncovered six cluster occurrences (Coban Parang Tejo, Coban Jodo, Ledok Ombo, Kondang Merak, Bambang village, and Universitas Brawijaya Forest), of which three were categorized as breeding sites—based on the presence eggs that accumulated in the foam nest or tadpole in nearby water bodies. We discovered that *R. reinwardtii* is arboreal and can be found perched in the tree canopy between 2.5 and 5.5 meters above the forest floor.

We also discovered that *R. reinwardtii* prefers dense vegetation, as it was observed perched in a variety of plant habitats, including herbs, shrubs, and trees, with broadleaf plants (e.g., *Colocasia* spp., *Musa* spp., *Coffea* spp., and *Persea americana*) being the most preferred. We found that they lived both solitary and communally. Males were approximately three times more common than females (male: female ratio approximately 3:1).

Based on the measured abiotic parameters (Table 3), the breeding pool's water temperature ranges from 20.5 to 24.6 ℃, with a pH of 6.86 to 7.13, dissolved oxygen of 1.24 to 4.28 mg/L, and conductivity of 0.1 to 0.7 mS/cm, while the air temperature ranges from 18.5 to 25.3 ℃ and relative humidity ranges from 82 to 100%. Several species were found to be sympatric with *R. reinwardtii*, including *Polypedates leucomystax*, *Microhyla achatina*, *Hylarana chalconota*, and *Fejervarya limnocharis*.

Our observation reveals that it utilised a variety of habitat types (Table 4), with the majority of occurrences in agricultural areas at high

Table 3. Abiotic parameters measured on the six cluster occurrences of *R. reinwardtii* in Malang Region.

Parameter		KM	BB	UBF	CPT	CJ	LO
Microclimate	Temperature (°C)	25.3	18.7	19.7	18.5	23	22
	Relative Humidity (%)	96.1	100	100	93	86	82
Water	Temperature (°C)	24.6	22.8	20.5	-	-	-
	pH	6.90	7.13	6.86	-	-	-
	Dissolved Oxygen (mg/L)	4.28	1.24	2.21	-	-	-
	Conductivity (mS/cm)	0.7	0.1	0.7	-	-	-

altitudes (660–1094 m asl), such as the fields of Universitas Brawijaya forest and plantations in Bambang village, Coban Parang Tejo, Coban Jodo, and Ledok Ombo; the exception being Kondang Merak, which recorded a single occurrence in remaining Malang's lowland forest. However, some of these locations share similar characteristics, such as proximity to a forest and low or high tree cover density.

Based on our observations of the three breeding sites (Kondang Merak, Bambang village, and Universitas Brawijaya Forest), we determined that the spawning area was characterised by densely vegetated waters (Figures 3–5). It produces eggs and foam nests at the base of leaves and wraps eggs in the leaves of trees near bodies of water. The pools include permanent ponds, temporary water reservoirs in plantation areas, and puddles that form on roadsides.

DISCUSSION

Based on SDM (Figure 2C), *R. reinwardtii* may not be as widespread as previously thought (IUCN SSC Amphibian Specialist Group 2022). This frog is only found in a small portion of the remaining lowland forest in eastern Java, where habitat loss is highest. Sjörgen (1991) also recognised habitat loss and fragmentation as the important causes of post-metamorphic dispersion failure against anuran species. According to the IUCN SSC Amphibian Specialist Group (2022), it is assumed that the species has a large population, but data are lacking to support this. We argue that the presumption of a large population might be based on local-

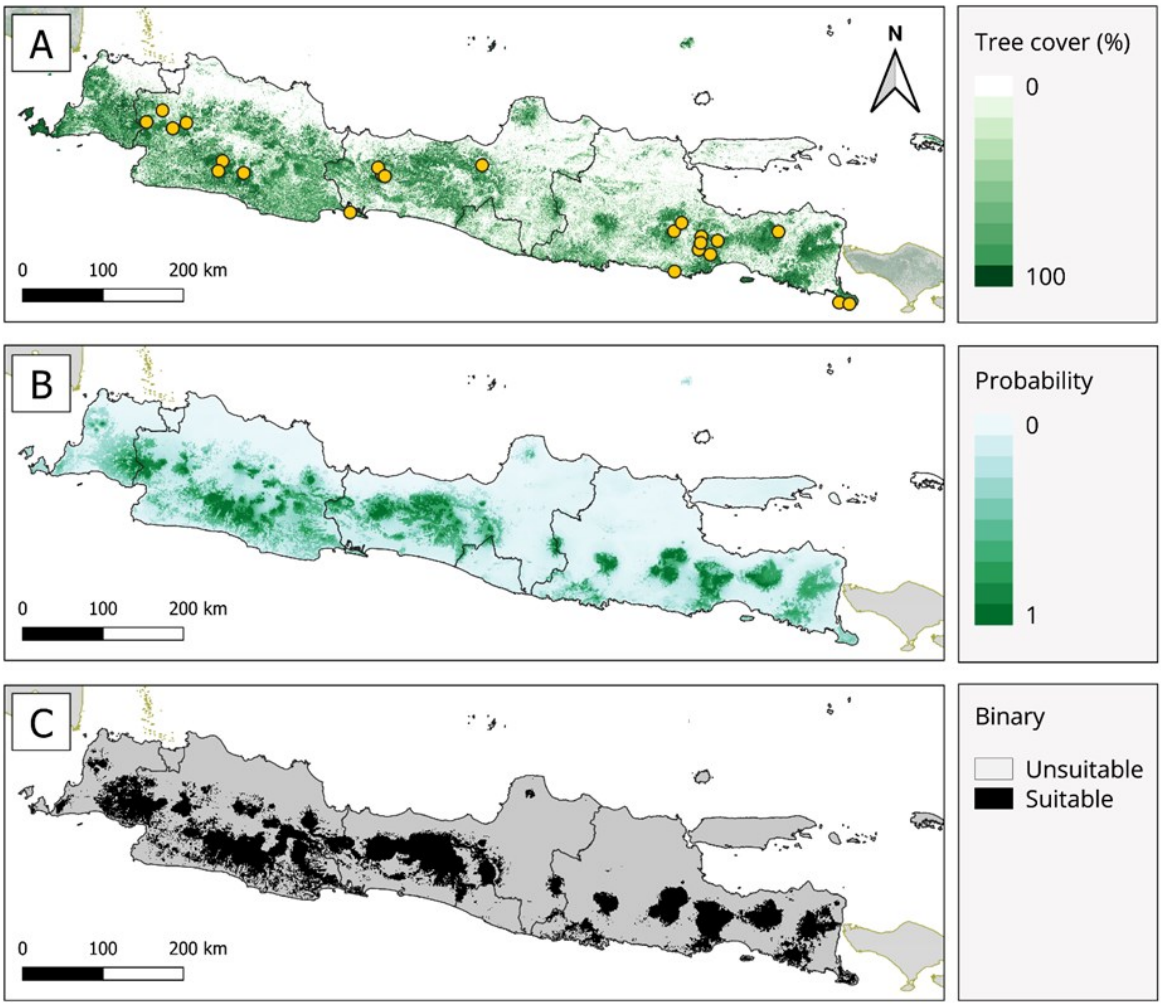

Figure 2. Geographical map showing the A) species occurrence overlayed with tree canopy cover, B) probability of presence, and C) binary habitat suitbaility of *R. reinwardtii* in Java.

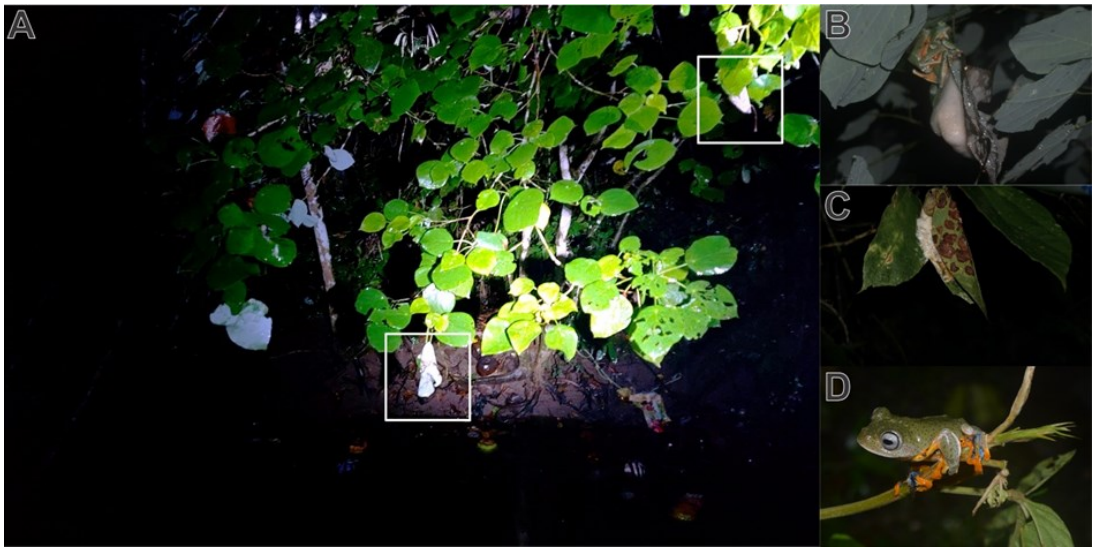

Figure 3. Observation of *R. reinwardtii* in Kondang Merak, revealing A) breeding sites in natural ponds, the squares indicate foam nests; B) male-female ovipositioning; C) remaining foam nests; and D) adult male individuals.

Figure 4. Observation of *R. reinwardtii* in Bambang village, revealing A) breeding sites in semi-permanent water storage ponds, the circle shows two male individuals perching in *Ricinnus communis*, the square shows a foam nest; B) male-female individuals in amplexus mode; C) adult male individual; and D) foam nest covered with leaves.

Figure 5. Observation of *R. reinwardtii* in Universitas Brawijaya Forest, revealing A) breeding sites in puddles that form on roadsides; B) tadpoles; C) froglets; and D) adult female individuals.

Table 4. Detailed habitat characteristics of *R. reinwardtii* observed in Malang Region.

Locality	Habitat Type	Altitude (m asl)	Breeding sites	Notes
Kondang Merak	Lowland forest	20	v	Natural ponds located near roads in primary lowland forests with dense vegetation.
Bambang	Agroforest (plantation)	660	v	Plantation areas with rainwater reservoirs around secondary high land forests with low vegetation density.
UB Forest	Agroforest (field)	1094	v	Puddles formed by vehicle paths in pine forests and coffee plantations
Coban Parang Tejo	Agroforest (plantation)	1238	-	Plantation areas around secondary highland forests with low vegetation density and close to river flows
Coban Jodo	Agroforest (plantation)	1041	-	Plantation area surrounded by secondary highland forest with low vegetation density
Ledok Ombo	Agroforest (plantation)	900	-	Plantation area surrounded by secondary highland forest with low vegetation density

ly abundant encounters, where the species descend from upper forest strata to mate and gathers, which could include up to ten to fifteen individuals during the breeding season (explosive breeder), concentrated only at those sites. Although there is no evidence that *R. reinwardtii* is experiencing a significant population decline (IUCN SSC Amphibian Specialist Group 2022), it is evident that this specialist frog relies primarily on dense canopy cover and water bodies. Consequently, it is essential to reevaluate the threat, population trend, and current distribution in light of differing viewpoints and multiple pieces of evidence.

According to numerous sources, *R. reinwardtii* is typically found at elevations ranging from 20 to 2000 m asl (IUCN SSC Amphibian Specialist Group 2022). However, the state of the forests from low-to-moderate altitude has been drastically reduced (Global Forest Watch 2021), presumably as a result of the rapid urbanization and conversion of land use (Christie 2007), leaving only the preserved highland forest and a few remaining lowland forests in the southern part of Java. This is in accordance with our habitat suitability modelling, revealing that *R. reinwardtii* is more suitable in the highlands than in the lowland forest of eastern Java (Figures 2B-C), except for western-central Java, where our prediction indicated a different result. Given the presence of intact lowland and highland forests in western Java, *R. reinwardtii* is likely to occur. In addition, Western Java has the highest proportion of vegetation and the highest density canopy; as a result, precipitation is higher and air temperatures are lower, making it an ideal habitat for *R. reinwardtii*. In comparison to eastern Java, the proportion of dense vegetation is considerably lower, and the climate is drier (PVMBG 2022). Therefore, prioritization is needed to protect Java's remaining lowland forest.

According to previous observations, *R. reinwardtii* can also hatch in vegetation near slower-flowing lotic waters (McDiarmid & Altig 1999; Kurniati 2008; Malik 2019), and it is evident from this study that *R. reinwardtii* requires artificial or natural ponds for the development of its tadpoles. Nonetheless, our observation in Malang Region, revealed an alarming threat. According to our cluster occurrence of the species breeding sites (mostly agroforestry areas), the farmers near the area frequently used agrochemicals, e.g., pesticides and herbicides, which could be harmful to the frog population (Ramadani et al. 2022). Given that a

larger proportion of Java's occupants were farmers, resolving this issue could become more challenging. Therefore, an action plan and conservation strategy are necessary for the future.

We suggest that *in-situ* and *ex-situ* conservations be carried out as follows: i) establishing artificial water reservoirs at various points around natural habitats can facilitate *in-situ* conservation, particularly in agroforestry lands. The temporary reservoir could serve as a breeding site for *R. reinwardtii*, and broadleaf shade plants could be planted around the pond area to facilitate their oviposition; ii) *ex-situ* conservation—by means of reintroduction of the breed individual to the natural population, can be achieved through a captive breeding program, as in other Rhacophorid species (e.g., Tapley & Girgin 2015; Vassilieva et al. 2016; Galunder & Rodder 2018). In addition, since captive breeding programs require understanding regarding the establishment of enclosure systems (i.e.,. vivariums) and breeding protocols, it is important that we have a comprehensive understanding of a species (e.g., natural behavior, ecology, and habitat).

CONCLUSIONS

This research leads us to the following conclusions: (1) The suitable habitat for *R. reinwardtii* exists only in a small portion of fragmented habitat, which comprises only 27% of Java Island's total area. As an additional notes, (2) they inhabit areas containing or adjacent to tree cover, such as forests and agroforests in Malang Region. Nonetheless, a future action plan and conservation strategy will be required to mitigate the negative effects of a number of potential factors in these regions.

AUTHOR CONTRIBUTION

N.K., M.F., and M.A.R. were responsible for the design of the study. M.F.A. and M.F. both participated in the collection of data. M.A.R. and L.S. participated in the analysis and interpretation of the data. M.A.R. initially drafted the manuscript 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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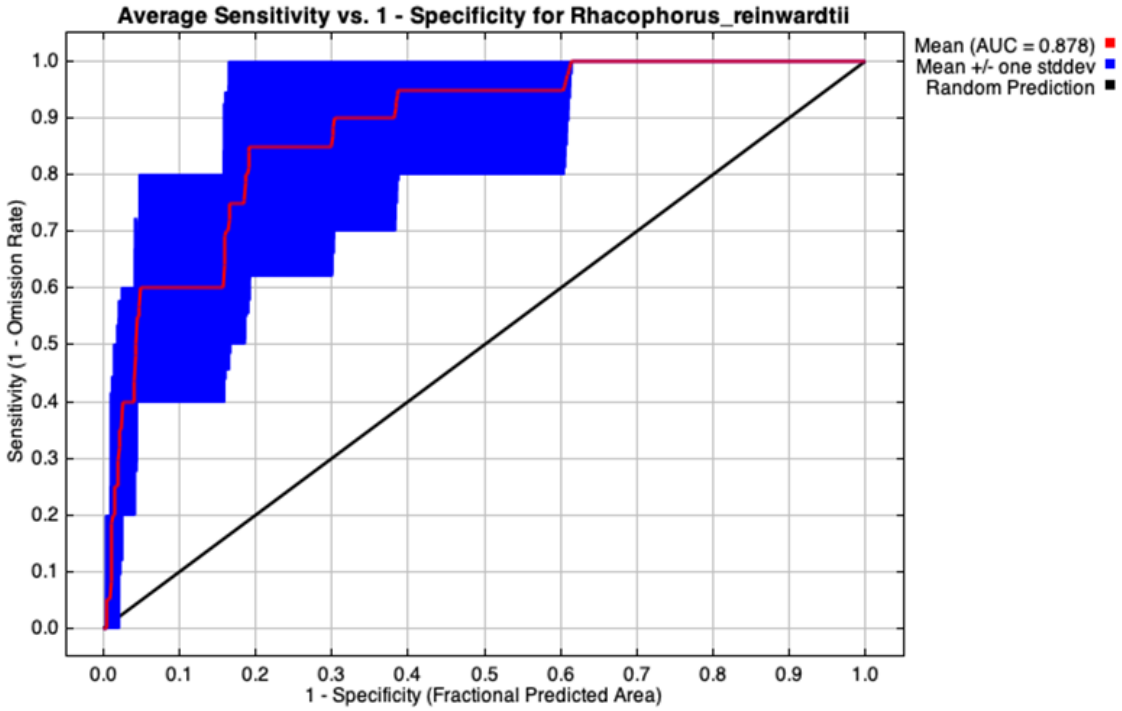
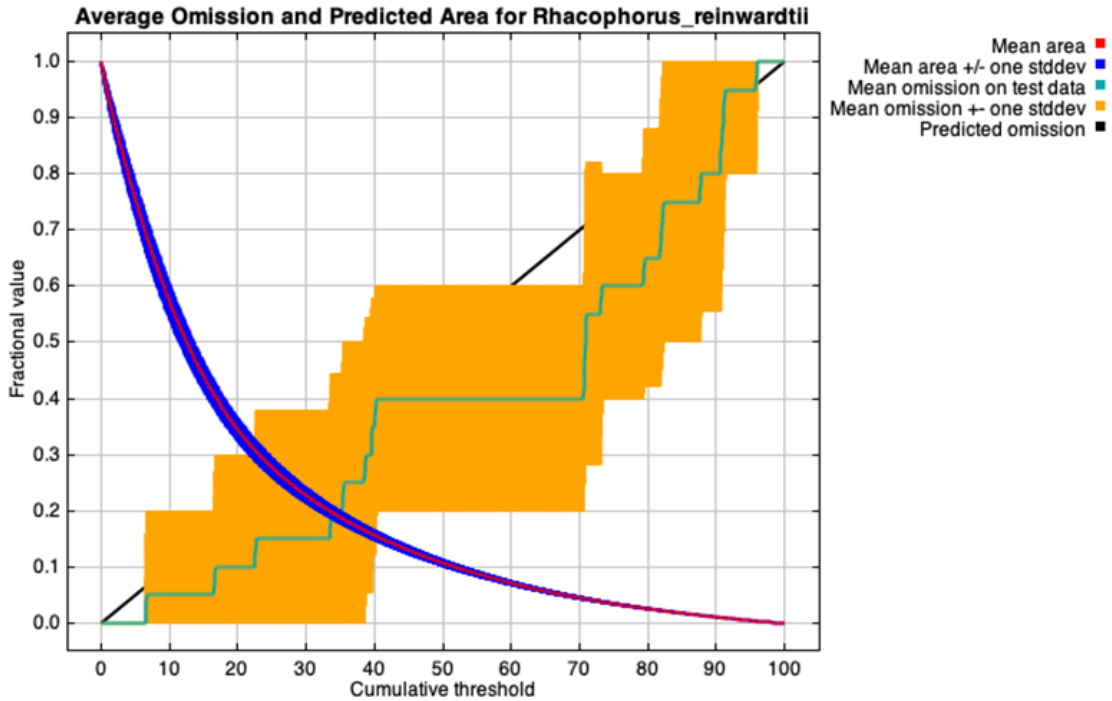
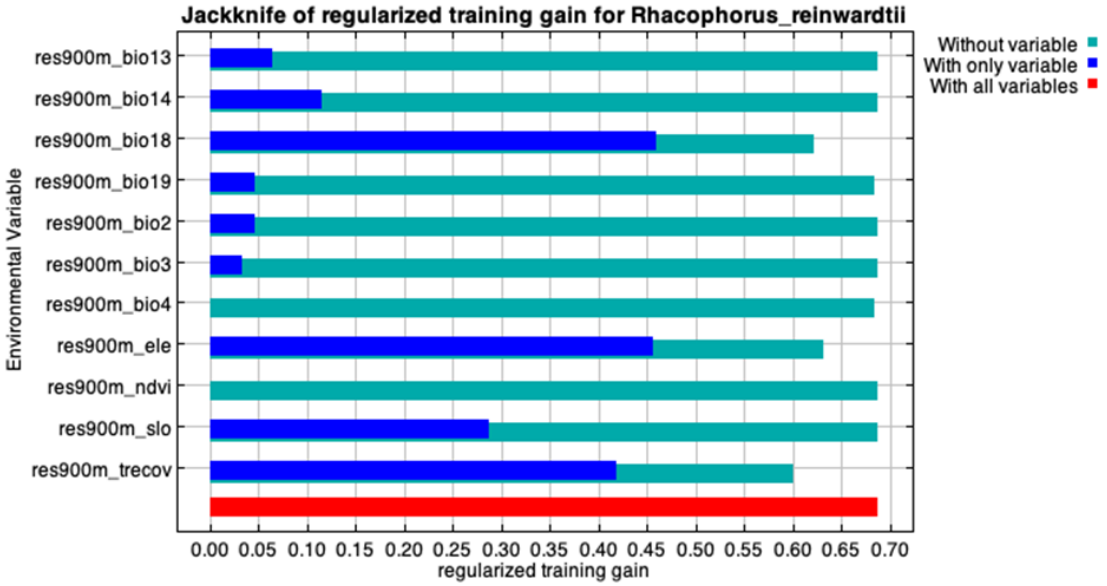
APPENDICES



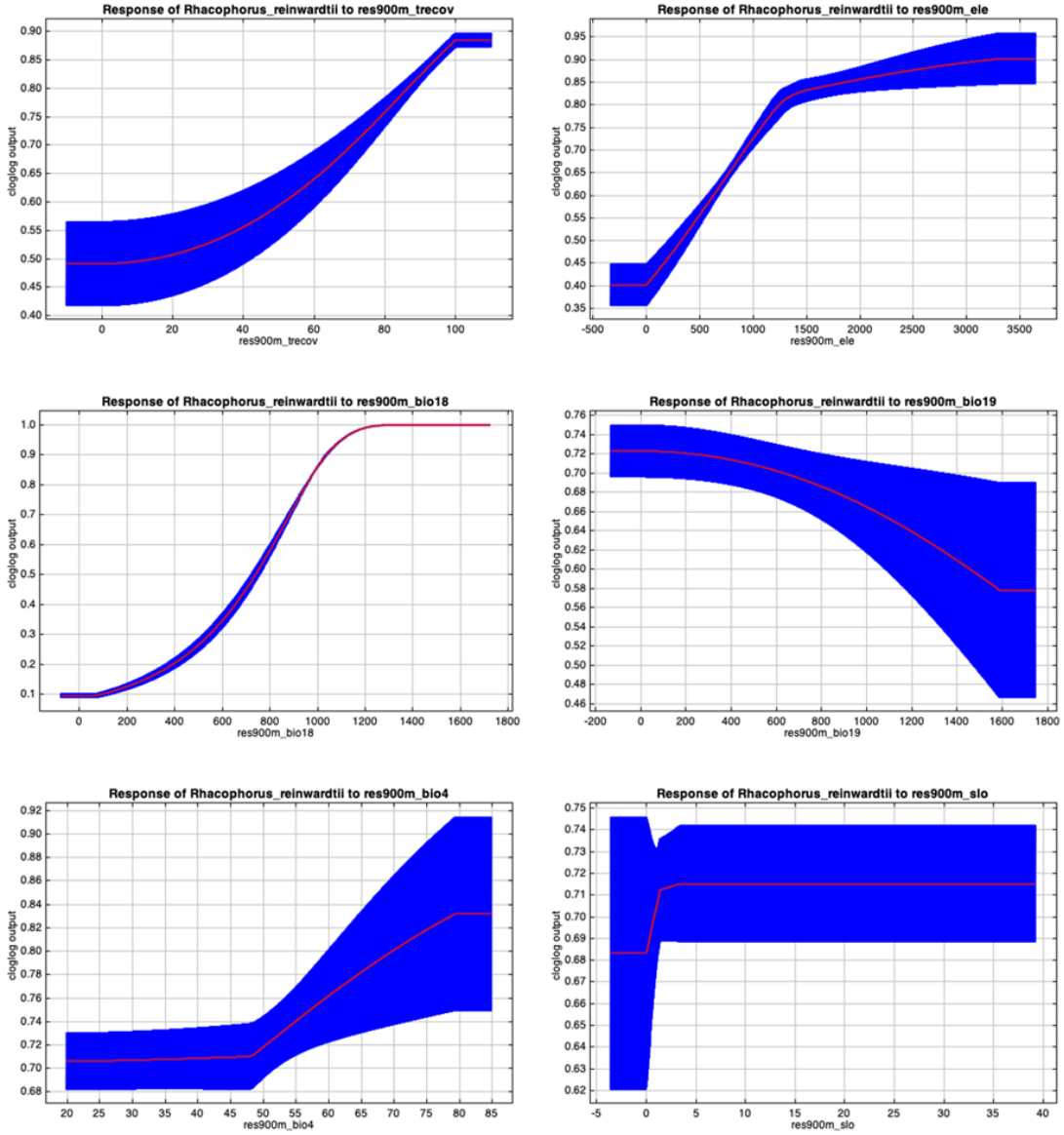
Appendix 1. Average omission and predicted area averaged for replicated runs in species distribution modelling of *R. reinwardtii* in Java.



Appendix 2. Receiver operating characteristic (ROC) curve averaged for replicated runs in species distribution modelling of *R. reinwardtii* in Java.



Appendix 3. Jacknife test of regularized training gain contribution of the predictor variables and their importance for habitat suitability in species distribution modelling of *R. reinwardtii* in Java.



Appendix 4. The response curve of the leading six predictor variables used in species distribution modelling of *R. reinwardtii* in Java.

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Research Article

Diversity of Actinomycetes Isolated from Peat Soil of Undistrubed Forest and Pineapple Plantation in Sessang, Sarawak

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ABSTRACT
Peatland plays an important role not just as a carbon store but also in facilitating the flux of greenhouse gasses into the atmosphere. Apart from that, peatland is also home to a diverse population of microorganisms such as bacteria, fungi, and actinomycetes. Actinomycetes were known to be one of the most ubiquitous microbes that can be found in most of the soil types including peat soil. In this study, seventy isolates of actinomycetes were isolated from the peat soil using the soil dilution method. The 70 isolates of actinomycetes were later screened for their ability to produce secondary metabolites and antimicrobial activities using the agar diffusion method before the selected potential isolates were identified by targeting their 16S rRNA region. The results obtained showed 34.3% produce cellulase followed by, 12.8, 31.7, 80.0, and 51.4% for mannanase, xylanase, lipase, and protease respectively. The percentage of actinomycetes producing antimicrobial activity was 27.1 and 21.4% for *Ralstonia solanacearum* and *Colletotrichum gleosporioides* respectively. All the selected isolates of actinomycetes were identified as belonging to the genus of Streptomycetes spp. The potential actinomycetes were stored in freeze-dried form for future usage. This study showed that more diverse population of actinomycetes was obtained from the undisturbed forested peat soil area ecosystem compared to the agricultural peat soil area.

INTRODUCTION
Malaysian peatlands were estimated to be about 2.6 Mha, with approximately 70% of these peatlands located in Sabah and Sarawak (Meilling 2016). These peat soils contained microbes that supported the formation of the peat and the carbon and nutrient cycling which is important to the ecosystem (Meilling 2016). One of the most studied peat soil microbes is *Streptomyces* sp which belongs to the family of Actinomycetes.

Streptomyces sp. has been well known to researchers for its ability to produce various beneficial activities such as bioactive compounds producer, decomposer, and plant growth promoter (Abdulla & El-Shatoury 2007; Umi et al. 2019; Sapkota et al. 2020). *Streptomyces* spp. has been known to produce more than 7600 compounds which makes them the largest producers of bioactive compounds for microorganisms (Bérdy 2005). Secondary metabolites obtained from actinomycetes are of special

interest to many researchers due to their diverse biological activities such as antibacterial, antifungal, antioxidant, antitumor, and antiviral (Niyasom et al. 2015). Many studies have been conducted using *Streptomyces* spp. isolated from soil samples for their bioactive compounds. Gopal and Thripathi (2020), noted that *Streptomyces* spp. isolated gave promising bioactive activity towards the plant pathogen *Pseudomonas aeruginosa*. Sapkota et al. (2020), noted that actinomycetes isolated from soil samples from different altitudes in Nepal were found to produce not just enzymatic activities but also antimicrobial activities against *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli*.

Several studies on the isolation and activities of soil actinomycetes isolated from peat soil have been documented (Lestari et al. 2019; Lisa et al. 2022). Peat soil actinomycetes have been known to show good activities in producing bioactive properties. Lestari et al. (2019), observed that actinomycetes isolated from a peat soil sample collected at Tajok Kayong Village, Ketapang Regency a potential antimicrobial producing candidate towards pathogenic microbes such as *Escherichia coli* and *Staphylococcus aureus*. Lisa et al. (2022), also indicated that actinomycetes isolated from soil had the potential to be developed into a biopesticide for *Ralstonia solanacearum*. Apart from that Jeffrey et al. (2011), it was indicated that actinomycetes isolated previously from forested soil at MARDI Peat Land Research Station at Sessang, Sarawak were capable of inhibiting *Ralstonia solanacearum* under *in vitro* screening.

In this study, the authors described the diversity and characteristics of actinomycetes isolated from peat soil obtained from forested and agricultural area at MARDI Peat Land Research Station at Sessang, Sarawak

MATERIALS AND METHODS

Collection of soil samples

Soil samples were collected from the MARDI Peat Land Research Station at Sessang, Sarawak. Two types of soil samples that were collected were the undisturbed forest soil (1.91890, 111.23657) where the peat soil has not been used for any agricultural activity, and pineapple-planted soil (1.92500, 111.23762) where it is currently being used for pineapple planting. Soil was collected by digging a hole approximately 10 cm deep using a spade after removing approximately 3 cm of the soil surface. Samples were kept in different sterile zip-lock polyethylene bags during transportation from MARDI Peat Land Research Station at Sessang, Sarawak to MARDI Headquarters at Serdang, Selangor.

Isolation and enumeration of actinomycetes

Soil samples were air-dried for about a week to reduce the growth of gram-negative bacteria. The dried soil samples were then ground to powder form using a pestle and mortar. The ground samples were added to the sterilised distilled water (sH_2O) at the ratio of 10 g of soil in 100 ml of sH_2O. The suspensions were later agitated at 280 rpm for 1 h. Serial dilution of 10^{-4} to 10^{-7} was later prepared for each soil sample collected. Hundred and fifty microliters of each suspension were later pipetted onto the Starch Casein Agar plate (SCA) containing soluble starch, 10.0 g; Casein (vitamin free), 0.3 g, KNO3, 2.0 g, MgSO4.7H2O, 0.05 g, K2HPO4, 2.0 g, NaCl, 2.0 g; CaCO3, 0.02 g; FeSO4.&H2O, 0.01 g and agar, 18.0 g in 1000 ml of distilled water with pH adjusted to 7 and lawned using a spreader stick (Zareenkousar et al. 2022). The agar plates were later incubated at room temperature (28 ± 2°C) for 10-14 days for the actinomycetes to emerge. Total plate counts were calculated from average counts of three replicates and expressed as colony-forming units (c.f.u.) per gram

of dry soil. The emerging colonies of actinomycetes were later transferred onto a fresh SCA plate and used as working cultures (Jeffrey et al. 2011).

Screening of extracellular enzyme activity

Screening for extracellular hydrolysis enzyme activities (mannanase, xylanase and cellulase) were carried using minimal medium agar containing bacteriological peptone, 1.0 g; yeast extract, 1.0 g; KH_2PO_4, 0.5 g; $(NH_4)_2HPO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; agar, 15.0 g; and substrate, 1.0 g which contained Megazyme: AZO-CM-Cellulose, AZO-Carob-Galactomannan and AZO-Xylan (oat) in 1000 ml of distilled water with pH adjusted to 7 (Jeffrey et al. 2011). The protease test was conducted using gelatin hydrolysis assay (Jing et al. 2020) while the lipase test was conducted using the method used by Sinha et al. (2014). Plug of pure culture of Actinomycetes isolates were later inoculated onto these substrate mediums. Results were obtained on day 5 after inoculation (Figure 1).

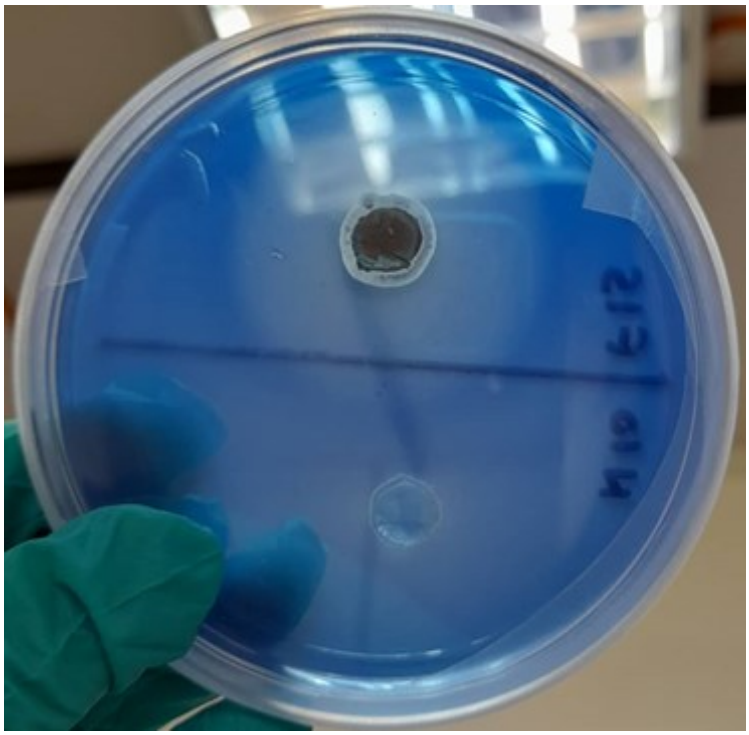

Figure 1. Clear zone forming indicating actinomycetes with the ability to produce enzyme to degrade cellulose.

Screening of antimicrobial activity

Plate diffusion method as propoesed by Bauer et al. (1966) with the sverals modification suggested by Barakate et al. (2000) was used in screening of antimicrobial activities. Under this protocol, Actinomycetes isolates were removed using a sterile cork bore (5 mm in diameter) to make agar stabs and placed onto tested pathogenic bacteria lawn plates. Formation of clearing zone was observed and measured after 5 days of incubation. Inhibition zone was measured from diameter of clear zone as observed from the antagonistic reaction (Figure 2). For anti-fungal activity screening, the fungal plug was placed on the middle of Potato Dextrose Agar plate and the actinomycetes agar plug was placed at two opposite side of the agar plate. Halo zone formed indicates antifungal activity between actinomycetes and the fungal test strain (Figure 3). Pathogenic microbes strains used for in the screening were *Ralstonia solanacearum* and *Colletotrichum gleosporioides*.

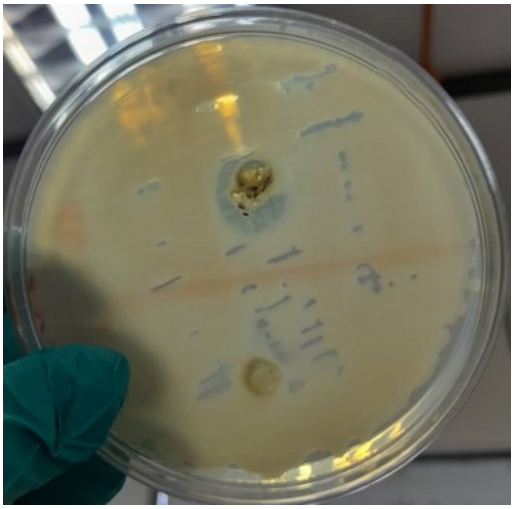

Figure 2. Halo zone indicating antibacterial activity between actinomycetes and *Ralstonia solanacearum*.

Figure 3. Halo zone forming indicating antifungal activity between actinomycetes and *Colletotrichum gleosporioides*.

Isolation of genomic DNA

Genomic DNA (gDNA) was performed for 5 potential isolates of actinomycetes using MagAttract Microbial DNA Kit. Protocol used was as indicated by the manufacturer (Qiagen 2022a).

Polymerase chain reaction (PCR) amplifications

Five of the best producing actinomycetes selected were identified using primers flanking at their 16S rRNA region. Polymerase chain reaction was carried out using 15.5 µl sterile distilled H_2O, 2.5 µl 10X PCR buffer, 2.0 µl 25 Mm MgCl, 0.6 µl dntps, 0.4 µl Taq polymerase, 1.0 µl of 20 pmol of each primers COM1 (5′-CAGCAGCCGCGGTAATAC-3′) and COM2 (5′-CCGTCAATTCCTTTGAGTTT-3) (Congestri et al. 2020) which were used to identify the variable region of V4 and V5 of the ribosomal DNA and 2.0 µl of DNA per single reaction. Protocol for the thermal cycler was as follows; initial denaturing at 94°C for 3 mins, followed by 35 cycles of denaturing at 94°C for 45 s, annealing at 62°C for 45 s, and elongation at 72 °C for 2 mins and a final elongation of 72 °C for 10 mins. PCR products obtained were loaded into the wells of a 1.0 % agarose gel and ran at 80 V for 50 mins. The gel was later viewed using Gel Documentation System from Biorad. PCR products were later purified using QIAquick PCR & Gel Cleanup kit according to the protocol suggested by the manufacturer (Qiagen 2022b).

Sequencing of PCR products

Purified PCR products were later sent for sequencing, at the facilities of Apical Scientific Sdn. Bhd., Selangor using ABI PRISM ® 377 DNA Sequencer (Applied Biosystems). The obtained sequences were then compared to sequences available in the National Centre for Biotechnology Information (NCBI) genebank database using the Basic Alignment Search Tool (BLAST) (Altschul et al. 1990). The phylogenetic tree was then constructed using the sequences obtained using Clustral X software (Thompson et al. 2002). Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm was used for the construction of the phylogenetic tree.

Conservation of potential isolates

All the potential microbes were kept in freeze dry form (powder form) for longer term safekeeping at MARDI Microbial Culture Collection (MMCC), MARDI Serdang, Selangor.

RESULTS AND DISCUSSIONS

Isolation and enumeration of actinomycetes

A total of 70 isolates of actinomycetes were isolated from the peat soil. The average colony forming unit of the actinomycetes from undisturbed forest soil was 4.7 x 10^5 however the pineapple planted soil showed only 7.3 x 10^3. In a previous study conducted by Jeffrey et al. (2011), it was reported that cfu/g of actinomycetes obtained from the forest soil was 3.0 x 10^4. There was a slight increase in the cfu of actinomycetes from the same location 10 years ago. This increase of actinomycetes cfu/g of soil indicates that microbial population tends to build up if it is left undisturbed from any development. However, research done on the population of actinomycetes at Sarawak wetland gave a lower cfu count on wetland versus agriculture soil at 6.84 x 10^5 cfu/g and 1.61 x10^6 cfu/g respectively (Ann et al. 2020) which justifies that actinomycetes preferred dry area rather than wet area.

It was observed that approximately 75.7% of the isolated actinomycetes produce grey spores, followed by 20.0% with brown spores and only 4.3% of the actinomycetes produce black spores (Table 1). This finding was almost the same as 10 years ago when Jeffrey et al. (2011) observed that grey spore producer was the highest followed by brown and white spores. However, it was not in line with the observation obtained from Sapkota et al. (2020), where the researchers indicated that 10% of the actinomycetes they isolated produced grey spores and only 3% produced brown spores. Sapkota et al. (2020) noted that 45% of their isolates produce yellow spores, however, there is no yellow spore formation was observed from actinomycetes isolated in this study. It was observed that actinomycetes isolated from the undisturbed forest and pineapple-planted soil gave the highest reading of 37/51 (72.5%) and 16/19 (84.2%) respectively for the grey spore formation (Table 1).

In this study, 65.7% and 28.6% of actinomycetes produce diffusible yellow and brown pigmentation respectively (Table 1). This shows that actinomycetes isolated from Sessang peat soil produce dominantly only yellow and brown diffusible pigment. This is in agreement with Fernandes et al. (2021), where the authors noted that the production of diffusible pigments ia prevalent in soil actinomycetes. According to Gupta et al. (2022), actinomycetes are potent producers of dark-brown coloured melanin pigments however in this study it was observed yellow coloured pigment was the dominant. Both actinomycetes isolated from the undisturbed forest and pineapple planted soil showed that brown pigment pro-

Table 1. Spores colour formation and pigmentation produced by 70 isolates of actinomycetes isolated.

Sampel	Actinomycetes isolates					
	Spore colour			Pigmentation		
	Grey	Brown	Black	Yellow	Brown	No pigment produce
Actinomycetes isolated from undisturbed forest (n=51)	37	11	3	30	18	3
Actinomycetes isolated from pineapple planted soil (n=19)	16	3	-	16	2	1
Total (n=70)	53 (75.7%)	14 (20.0%)	3 (4.3%)	46 (65.7%)	20 (28.6%)	4 (5.7%)

duction was low in comparison to yellow pigment (Table 1). This might be due to the conditions of the environment these actinomycetes grow in (Celedón & Díaz 2021).

Screening of extra-cellular enzyme and anti-microbial activity

Approximately 80.0% of the actinomycetes isolated produce enzyme lipase and 51.4% produce enzyme protease. This is in accordance with the results that were observed 10 years ago at MARDI Sessang. In the previous study, it was observed that lipase (32.5%) was also produced more by actinomycetes compared to protease (12.5%) (Jeffrey et al. 2011). It also observed that the affinity of actinomycetes producing cellulase is higher than xylanase and mannanase (Table 2). This might be due to the reason that peat soil contained generally lignin, cellulose, hemicellulose, and protein thus higher cellulose present required more or better cellulose producer microbes in the surroundings. This is supported by the observation of Veloo et al. (2014), where the authors observed that tropical peat soil contains wood materials in the soil solum which explained why the cellulase producing actinomycetes is more compared to xylanase and mannase producers. It was also noted in this study that *Streptomyces griseus* strain PS42 can be considered the most potent producer of enzymes screened (Table 3). This statement was supported by Talib Saleh et al. (2023), where the researchers observed that *S. griseus* has the ability to produce multiple enzymes such as celllulase, lipase and protease.

Antimicrobial test conducted showed that 27.1% and 21.4% of the actinomycetes has the ability to inhibit the growth of *Ralsonia solanacearum* and *Colletotrichum gleosporioides* respectively (Table 2). *Streptomyces* sp. strain 33 showed good antagonistic activity towards both *Ralsonia solanacearum* and *Colletotrichum gleosporioides* with the average zone size of 1.3 cm and 1.2 cm for both pathogens respectively (Table 3). This showed that actinomycetes isolated from MARDI Sessang, is good at producing antimicrobial activity against *R. solanacearum* because previous study done by Jeffrey et. al. (2011) also indicated that actinomycetes isolated from MARDI Sessang area showed the ability to produce antimicrobial activity against *R. solanacearum* with the average halo zone of 2 cm was produced by *Streptomyces* sp. strain TN06. In a study done by Zhao et al. (2019) *Streptomyces sporangiiformans* was observed to produce antimicrobial activity towards *R. solanacearum*. In a recent study by Lisa et al. (2022), the authors showed that actinomycetes were capable of increasing the fruit yield and decreased *R. solanacearum* the causal agent for bacterial wilt infection on tomato. Bhat et al. (2022) also indicated the potential of actinomycetes as a potential biocontrol agent for *Colletotrichum* spp.

Table 2. Bioactivity produced by actinomycetes isolated.

Samples	Enzyme activity					Antimicrobila activity	
	Cellulase	Mannanases	Xylanase	Lipase	Protease	*Ralstonia solalacearum*	*Colletotrichum gloeosporioides*
Actinomycetes isolated from undisturbed forest	18/70	9/70	17/70	46/70	26/70	16/70	10/70
Actinomycetes isolated from pineapple planted soil	6/70	0/70	9/70	10/70	10/70	3/70	4/70
Total	24/70 (34.3%)	9/70 (12.8%)	26/70 (31.7%)	56/70 (80.0%)	36/70 (51.4%)	19/70 (27.1%)	14/70 (21.4%)

Table 3. Bioactivity of selected 4 actinomycetes isolates.

Isolate no.	Enzyme screening (average ± SD cm)					Antagonistic activity (average ± SD cm)	
	Lipase	Protease	Cellulase	Mannanase	Xylanase	*Ralstonia solanacearum*	*Colletotrichum gloeosporioides*
PS1	1.4± 0.100	1.9± 0.153	1.7± 0.153	1.7± 0.231	4.9± 0.058	1.0± 0.058	1.2± 0.132
PS10	1.9± 0.153	2.3± 0.115	3.1± 0.200	1.1± 0.580	3.9± 0.321	1.2± 0.058	1.0± 0.208
PS42	2.2± 0.200	2.4± 0.200	2.6± 0.153	1.7± 0.200	3.9± 0.173	0	0.5± 0.115
PS46	3.9± 0.300	0	3.0± 0.208	2.3± 0.173	2.8± 0.058	1.0± 0.161	0

It was also observed that actinomycetes collected from forested area showed that undeveloped forest land has higher ability to produce extracellular enzymes and also antimicrobial activities (Table 2). It is believed that microbes in the soil were very sensitive to changes in the soil land use (Romaniuk et al. 2017). Several studies suggested that the function of microbial communities in soil are strongly affected by types of tress and the composition of the soil (Li et al. 2014). This supports well the reason why actinomycetes isolated from forested soil have better bioactivity compared to pineapple plantation soil.

Identification of actinomycetes

Streptomyces spp. had been well known as the dominant actinomycetes in the soil. The morphology of the 4 strains was shown in Figure 4. *Streptomyces griseus* strain PS1 was observed to be having black spore colour while *Streptomyces hyproscopicus* strain PS10 *Streptomyces griseus* strain PS41 and *Streptomyces olivaceus* strain PS46 having grey spore colour. Research done by Ann et al. (2020) indicated that out of 578 strain of actinomycetes isolated from Sarawak wetland, 120 isolates belongs to the *Streptomyces* genus (Ann et al. 2020) this showed that *Streptomyces* spp. were prevalent in soil. In this study, it was obesrved that *Streptomyces griseus* accounted for 2 strains of the potential actinomycetes, followed by 1 strain each for *Streptomyces hyproscopicus* and *Streptomyces olivaceus*. The sequence and relatedness of each strain of *Streptomyces* are shown in Table 4 and Figure 5 respectively.

Table 4: Sequence and identification of 4 selected *Streptomyces* strains.

Isolate No.	Sequence	ID	E value	Percentage of identity
PS1	gcctcccgcg acctgggctt cgactcgctc accgccgtcg acctccgtaa ccgcctcaag gccgccaccg gggagcggct gtccgcgacc gtcgtcttcg accacccgac ccccgccgag ctggccgccc acctccacga ccgttgcttc ccggacgccg acggccggcc gcagcggtcg gtcccggccg tgacggctgt cgccgcgctc cacgacgagc cggtcgcgat cgtcggcatg gcctgccggc tgccgggcgg cgtcaccacc ccggaggagc tgtggcagct cctccgggac ggcggagacg cgatcaccgg cttcccggag aaccgcggct gggacctgga cggtctctac gaccccgatc ccgccacccc cggtaagacc tatgcccgcg acggcggatt cctccacgac gcggcggagt tcgacgcggg gttcttcggg atctcgccgc gtgaggcgct ggcgatggac ccgcagcagc ggctgctgct ggagacgtcc tgggaagcga tcgagcacgc cggcatcgac cccacggtcc tcaagggcac ccgcaccggt accttcatcg gcgccaaccc gtcggactac cgggcggcca tgggacaggc gccggtgggc tacgagggcc acctcgtcac cggaggccac aacagcgtcg tctccggccg gatcgcctac accttcggcc tcgaaggccc ggccgtcacc gtcgacaccg cctgctcctc	*Streptomyces griseus*	0.0	100%
PS10	cctggtagtc cacgccgtaa acggtgggaa ctaggtgttg gcgacattcc acgtcgtcgg tgccgcagct aacgcattaa gttccccgcc tggggagtac ggccgcaagg ctaaaactca aaggaattga cgggggcccg cacaagcagc ggagcatgtg gcttaattcg acgcaacgcg aagaacctta ccaaggcttg acatacaccg gaaaaccctg gagacagggt ccccttgtg gtcggtgtac aggtggtgca tggctgtcgt cagctcgtgt cgtgagatgt tgggttaagt cccgcaacga gcgcaaccct tgtcctgtgt tgccagcatg cccttcgggg tgatggggac tcacaggaga ccgccggggt caactcggag gaaggtgggg acgacgtcaa gtcatcatgc cccttatgtc ttgggctgca cacgtgctac aatggccggt acaaagagct gcgataccgt gaggtggagc gaatctcaaa aagccg	*Streptomyces hygroscopicus*	0.0	100%
PS42	cttaacacat gcaagtcgaa cgatgaagcc tttcggggtg gattagtggc gaacgggtga gtaacacgtg ggcaatctgc ccttcactct gggacaagcc ctggaaacgg ggtctaatac cggataacac tctgtcccgc atgggacggg gttaaaagct ccggcggtga aggatgagcc cgcggcctat cagcttgttg gtggggtgat ggcctaccaa ggcgacgacg ggtagccggc ctgagagggc gaccggccac actgggactg acacacggcc cagactccta cgggaggcag cagtggggaa tattgcacaa tgggcgaaag cctgatgcag cgacgccgcg tgagggatga cggccttcgg gttgtaaacc tctttcagca gggaagaagc gaaagtgacg gtacctgcag aagaa	*Streptomyces griseus*	0.0	100%
PS46	gagcatgtgg cttaattcga cgcaacgcga agaaccttac caaggcttga catacaccgg aaacggccag agatggtcgc ccccttgtgg tcggtgtaca ggtggtgcat ggctgtcgtc agctcgtgtc gtgagatgtt gggttaagtc ccgcaacgag cgcaaccctt gtcccgtgtt gccagcaagc tccttcgggg gtgttgggga ctcacgggag accgccgggg tcaactcgga ggaaggtggg gacgacgtca agtcatcatg ccccttatgt cttgggctgc acacgtgcta caatggccgg tacaatgagc tgcgataccg caaggtggag cgaatctcaa aaagccggtc tcagttcgga ttggggtctg caactcgacc ccatgaagtc ggagtcgcta gtaatcgcag atcagcattg ctgcggtgaa tacgttcccg ggccttgtac acaccgcccg tcacgtcacg aaagtcggta acacccgaag ccggtggccc aacccttgt gggagggagc tgtcgaaggt gggactggcg	*Streptomyces olivaceus*	0.0	100%

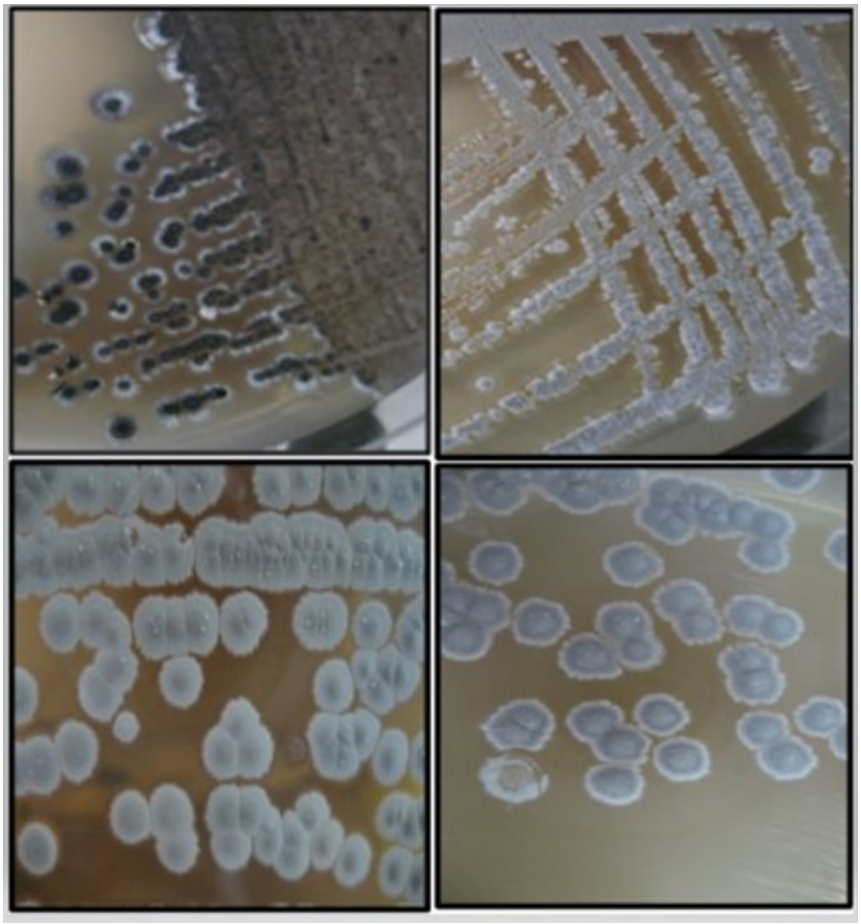

Figure 4: Morphology of 4 selected *Streptomyces* strain, (a) *Streptomyces griseus* strain PS1; (b) *Streptomyces hyproscopicus* strain PS10; (c) *Streptomyces griseus* strain PS42 and (d) *Streptomyces olivaceus* strain PS46.

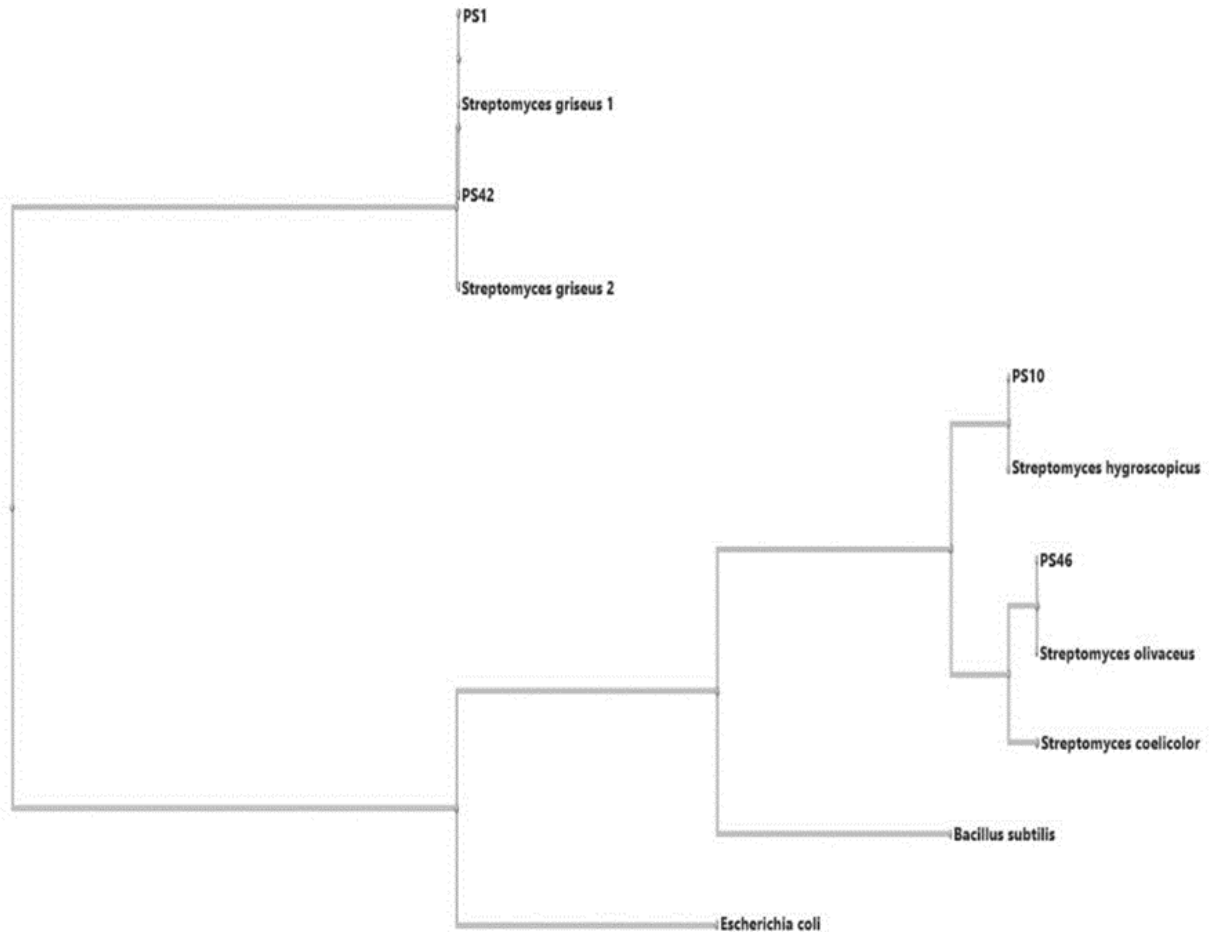

Figure 5: Phylogenetic tree for 4 selected *Streptomyces* strain.

CONCLUSION

Morphological diversity of the actinomycetes was observed from the spore forming colour of grey, brown, and black. The characteristic of the actinomycetes was observed when 34.3%, 12.8%, 31.7%, 80.0%, and 51.4% of the actinomycetes produced cellulase, mannanase, xylanase, lipase, and protease activity respectively. While 27.1% and 21.4% produced antagonistic activity towards *Ralstonia solanacearum* and *Colletotrichum gleosporioides* respectively. All the eight potential actinomycetes isolated were later identified to originate from the genus of *Streptomyces* sp. This study showed the potential usage of peat soil actinomycetes as well as the vast diversity of the actinomycetes in peat soil.

AUTHORS CONTRIBUTION

J.L.S.H. designed and conducted experiment, analysed data and wrote the article. H.H. conducted experiment, collected data and wrote material and methods section. N.A.N. conducted experiment, collected data and wrote introduction section.

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CONFLICT OF INTEREST

There is no conflict of interest regarding to this research.

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Research Article

First Report of *Cladosporium dominicanum Zalar*, de Hoog & Gunde-Cim. Infecting Whitefly on Ornamental Plants in Bali, Indonesia

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ABSTRACT

As natural enemies, entomopathogenic fungi are essential for controlling certain plant pests, such as whitefly. Many types of entomopathogenic fungi can infect whiteflies, including those from the genera *Aschersonia, Metarhizium, Beauveria, and Cladosporium.* Currently, there is great interest in using entomopathogenic fungi as an environmentally friendly pest control in organic and sustainable agricultural systems. This study aimed to identify entomopathogenic fungus associated with whiteflies. Identification of entomopathogenic fungus was performed morphologically and molecularly using DNA barcoding with ITS-1 and ITS-4 as primers, in addition to sequencing and phylogenetic tree analysis. Whiteflies infected with entomopathogenic fungus were discovered on ornamental plants (*Premna serratifolia, Ficus religiosa, and Ficus rumphii*) in Denpasar, Bali, Indonesia from January to March 2023. We found that samples from the field had mycelium that was unbranched or sparingly branched with solitary conidiophores arising terminally from ascending hyphae or laterally from plagiotropous hyphae. Samples from PDA media had ramoconidia with darkened septa, straight and conidia in long branched chains, branching in all directions, conidia were obovoid, ovoid to limoniform. The fungal colonies had the characteristics of being grey-olivaceous, olivaceous-black, and the margins were grey-livaceous to white, gray olivaceous in colony center due to abundant sporulation. In addition, based on the BLAST nucleotide sequence from NCBI GenBank, the insect pathogen phylogenetic tree found in Denpasar, Bali, is in the same clade as *Cladosporium dominicanum* strain SCAU014, accession number KY827344.1. Based on morphological and molecular analysis the fungus that infects whiteflies on ornamental plants is *C. dominicanum.*

INTRODUCTION

Whiteflies are significant crop pests that cause direct and indirect damage (Lee et al. 2014). There are many species of whiteflies, (Hidayat et al. 2018) reported a dichotomous identification key of whiteflies, completed

based on morphological characteristics of 35 collected species in Bogor, Indonesia. Whiteflies are also reported to be vectors of several plant viruses in tomatoes (Ghanim 2014) and chili peppers (Ayu et al. 2021; Temaja et al. 2022; Selangga et al. 2023). Whiteflies are reported to be polyphagous, attacking multiple kinds of vegetables, fruits, and ornamental plants (Syafitri et al. 2017; Hidayat et al. 2018). However, according to Mrosso et al. (2023), farmers' knowledge to control whitefly on tomato plants varies widely, so practical research is needed to determine the best management practices to control this pest. To control whiteflies the majority of farmers continue to use synthetic chemical insecticides. However, the application of these insecticides on a regular basis and without proper adherence to label requirements is detrimental to the environment and humans (Bajwa & Sandhu 2014). Consequently, more eco-friendly control measures are required.

One of the environmentally friendly controls is the application of natural enemies such as insect pathogens. Bacteria, fungi, and viruses play an important role as insect pathogens (Lovett & St. Leger 2017). There are many genera of fungi infecting insects such as whitefly. *Aschersonia, Metarhizium, Beauveria, and Cladosporium* are the common genera of fungi that infect whiteflies. The entomopathogenic fungi of the genus *Aschersonia* play a crucial role in controlling whitefly populations in subtropical and tropical regions worldwide (Sudiarta et al. 2019; Bhosale et al. 2020; Prayogo & Bayu 2020). In addition, the *Aschersonia placenta* was reported to infect the whitefly in citrus plants in Bali, Indonesia (Sudiarta et al. 2019; Suputra et al. 2019). An in vitro study was carried out to assess the pathogenicity of an *A. aleyrodis* isolate against blackfly and whitefly (Bhosale et al. 2020). On the other hand, *Metarhizium* has reportedly been highly effective in controlling whiteflies (Paradza et al. 2021, 2022). The pathogenicity of dry conidia and fungal suspensions of ten strains *Metarhizium anisopliae* and six strains *Beauveria bassiana* strains were evaluated against adults and second instar nymphs of the greenhouse whitefly (Paradza et al. 2021). In addition, *B. bassiana* was reported as a biological control of whiteflies. The effectiveness of *B. bassiana* against cotton whitefly (*Bemisia tabaci*) was analysed. *B. bassiana* isolate (Bb-01) was found to be the most effective with an LC50 value of (2.4×107 spores/ml) which caused the highest mortality of eggs (65.30%) and nymphs (88.82%) (Zafar et al. 2016). At the same time, the *Cladosporium* genus has also been reported to be able to infect whiteflies in several countries such as Bangladesh and Egypt (Abdel-Baky & Abdel-Salam 2003; Islam et al. 2019; Islam 2022). According to Islam et al. (2019), *Cladosporium cladosporioides* isolated from rice brown planthopper can control whitefly (*B. tabaci*). However, the information of *Cladosporium* infecting whiteflies in Indonesia is still limited, especially on ornamental plants. Therefore, additional research must be conducted on exploring, identifying, and applying *Cladosporium* as a biological control for whitefllies The aim of this study is in order to identify the entomopathogenic fungi *Cladosporium*, which was found on infected whitefly on ornamental plants (*Premna serratifolia, Ficus religiosa, and Ficus rumphii*). The identification was based on morphological and molecular characteristics using general DNA barcoding with primers ITS-1 and ITS-4. According to Sudiarta et al. (2019) the DNA barcoding has been used successfully to confirm the species of entomopathogenic fungi.

MATERIALS AND METHODS

Study location

Research exploring entomopathogenic fungus on whiteflies was conduct-

ed at the Experimental Garden of the Faculty of Agriculture, Udayana University, and bonsai hobbyists in Denpasar City, Bali, Indonesia from January to March 2023. Denpasar City, Bali, Indonesia is located in the lowlands with an altitude of ±100 meters above sea level. The observed host plants of whitefly were ornamentals (*Premna serratifolia*, *Ficus religiosa*, *and Ficus rumphii*). Samples of insects infected with the fungus were observed, photographed, and then taken to the Laboratory of Plant Diseases, Faculty of Agriculture, Udayana University.

Morphological identification

Photos of living individuals of whitefly in the host plant and nature infection of *Cladosporium* in whitefly were taken with an Olympus OM-D camera, E-M 1, 50 mm Macro lens. Infected insect samples were observed under an Olympus CX21 Microscope, 40X/0.25, Optilab before isolating the entomopathogenic fungus on artificial media. The suspected entomopathogenic fungus of whitefly was isolated by washing the sample using sterile water and 70% alcohol and rinsing again with sterile water before planting on Potato Dextrose Agar (PDA) media. After 1-2 weeks, the fungus was sub-cultured to obtain pure cultures. Microscopic examination was conducted to describe the morphological characteristics of the fungal isolate (Bensch et al. 2018).

Koch's postulate test

Furthermore, Koch's postulate test was carried out to ensure the fungus was an insect pathogen. The *Cladosporium* Bali isolate was cultured in Liquid Potato Dextrose media and then the conidia were examined under a microscope. The conidia were sprayed on the whitefly nymphs on the *Ficus religiosa* plants. While the control was sprayed with sterile water containing 0.02% tween 80. Whiteflies that have been treated were incubated for 20 days at 25OC. After 20 days of whitefly nymphs being observed, whitefly nymphs infected with the fungus were observed under a microscope to ensure the true presence of the *Cladosporium.*

Molecular identification

After the fungus was confirmed to be an insect pathogen, molecular characterization was carried out. First, DNA extraction was completed before the fungal DNA was amplified by PCR. Extraction of total DNA was conducted on a single colony of fungal growth on a PDA medium on a modified method performed using CTAB Doyle and Doyle method (Sudiarta et al. 2019). Total DNA was used as a template for the Polymerase Chain Reaction (PCR). DNA amplification used primer set ITS-1 Forward (5'TCCGTAGGTGAACCTGCGG-3') and ITS-4 Reverse (5'-TCCCTCCGCTTATTGATATGC-3') targeting ± 560 bp of the ITS rDNA of Fungi (Sudiarta et al. 2019). The 1 µl of PCR product (plus 2 µl of loading dye) was electrophoresed in 1% TBE agarose gel. Electrophoresis was carried out for 30 minutes at 100 volts. The DNA that has been electrophoresed was then visualized with a UV transluminator. PCR products were then sequenced at 1st BASE Laboratories (Malaysia). Phylogenetic analysis was conducted by comparing the sample sequences and entomopathogenic fungi sequences and an outgroup comparison from GenBank. A phylogenetic tree of the entomopathogenic fungi was constructed using the Clustal X program (Thompson et al. 1997), Bio Edit 7.2.5 (Genious), and MEGA 6.06 (Tamura et al. 2013). The phylogenetic tree was constructed using Mega 6.06 (Algorithm Neighbor Joining with 1.000 bootstraps replicates) (Tamura et al. 2013; Nurbaya et al. 2022).

RESULTS AND DISCUSSION

Natural incidence of Cladosporium dominicanum in whitefly

This study was conducted on ornamental plants (*Premna serratifolia*, *Ficus religiosa*, *and Ficus rumphii*) attacked by whitefly. Based on the insect morphology, it can be ascertained that the observed insect was a whitefly (Figure 1). According to Hidayat et al. (2018), whiteflies (Hemiptera: Aleyrodidae) are a group of small insects with a white color and soft body. In Bahasa Indonesia, this insect is called "kutukebul" because when flying in groups it looks like smoke ("kebul" in Javanese means smoke). The size of the whitefly nymph in the final instar was ±0.8-1 mm, while that of the imago was ±1-1.2 mm. Ornamental plants attacked by whiteflies were *Premna serratifolia*, *Ficus religiosa*, *and Ficus rumphii*. Those ornamental plants are a popular material for bonsai. The whitefly attacked the leaves of ornamental plants on the lower leaf surface (Figure 1), with symptoms of yellowing, mosaic and curling leaves. These whiteflies were not identified using the determination key or DNA barcoding. Further research is needed to confirm the identity of this whitefly species. However, evidence shows whiteflies are polyphagous, feeding on food crops, vegetables, fruit, and ornamental plants (Hidayat et al. 2018; Syafitri et al. 2017). After knowing that the insect attacking the leaves of the ornamental plant is a whitefly, observations were made of the fungus infecting the whitefly.

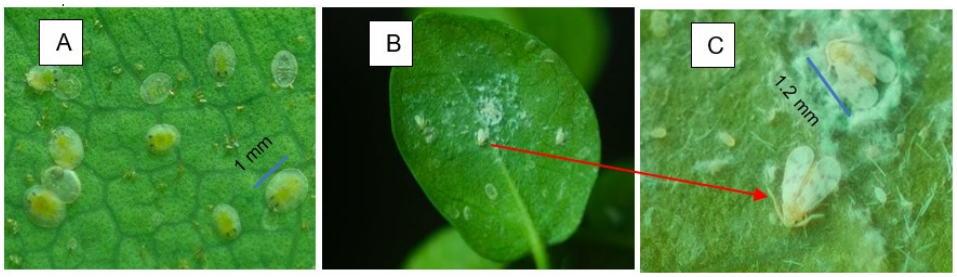

Figure 1. Living individuals of whitefly in the host plant (*Premna serratifolia*, *Ficus religiosa*, *and Ficus rumphii*). A) Whitefly nymphs; B,C) Whitefly imago.

Infected whiteflies were covered with a bluish-gray stroma with varying degree of mycosis (Figure 2) wherein some infected nymphs were mummified with light fungal growth (Figure 2a) and profuse uniform growth of grey-black stroma containing spores at a later stage of infection (Figure 2b,c) in comparison with healthy whiteflies (Figure 2d). Observations were made under a light microscope to clarify the presence of mycelia on whitefly collected from the field. The symptoms of fungal infection are clearly visible outside the nymph's body (Figure 3A,B), the appearance of these symptoms after the pathogenic fungal hyphal comes out of the insect cuticle. The mechanisms are highly complex involving gene interactions between insect pathogenic fungi and insect hosts. Wang & Wang (2017) reported by using species in the *Beauveria* and *Metarhizium* genera as models, molecular biology studies have revealed the genes that function in fungus-insect interactions and thereby contribute to fungal virulence. The observations revealed that black-green mycelia and conidia could be seen emanating from the body of the whitefly. Unlike entomopathogenic bacteria and viruses that mode of entry to cause diseases after oral ingestion, entomopathogenic fungi infect insects via direct penetration of the surface of the insect (cuticle) and enter the body of the insect, multiplying in the body cavity (hemocoel). The contact mode of entry of entomopathogenic fungi is dependent on a biochemical mechanism using enzymes such as chitinase (Wang & Feng 2014; Lovett & St. Leger 2017).

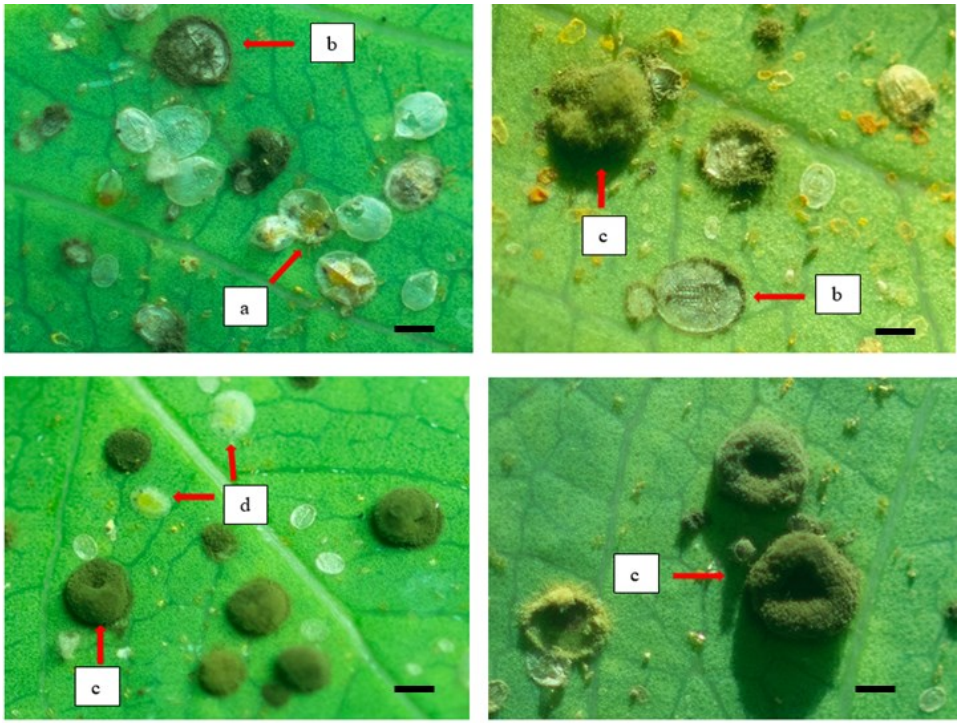

Figure 2. The variation of nature infection of *Cladosporium dominicanum* in whitefly. a) nymph death, nymphs were infected in the early phase; b) mycelia with spores have begun to appear in nymphs; c) infected nymphs in the late phase (stroma); d) healthy nymph, scale bars 0.5 mm.

Morphological characteristics of pathogenic fungi on whitefly

Morphological identification was carried out by observing the shape and colour of the fungal colonies on PDA media, conidial development and branching patterns of conidial chains, and general morphology and size of conidiophores and conidia (Bensch et al. 2018). The morphological observation was carried out on the fungal-infected whiteflies upon field collection and on the isolated fungus grown on PDA. Samples taken directly from the field had mycelium that was unbranched or sparingly branched with solitary conidiophores arising terminally from ascending hyphae or laterally from plagiotropous hyphae (Figure 3D, E). Samples from PDA media had ramoconidia with darkened septa, straight (Figure 3F, Arrow) and conidia in long branched chains, branching in all directions (Figure 3C); conidia were obovoid, ovoid to limoniform (Figure 3F). In addition, the results of observations showed that the fungal colonies that attacked whiteflies on ornamental plants in Denpasar, Bali, which were cultured on PDA media, had the characteristics of being grey-olivaceous, olivaceous-black, and the margins were grey-livaceous to white, gray olivaceous in colony center due to abundant sporulation (Figure 4A,B). On PDA media, the size of the fungal colony after 14 days after planting was around 35 mm. The hyphae observed from PDA media were the same as hyphae from field collection (Figure 4C,D). Based on these morphological characteristics, the fungus that attack the whitefly is predicted as *Cladosporium* (Bensch et al. 2018; Islam et al. 2019). The similarity of conidiophores and conidia both in shape and size in the genus *Cladosporium* was so high that it was difficult to distinguish between species. There are three *Cladosporium* species complexes: *C. dominicanum*, *C. herbarum*, and *C. sphaerospermum* species complexes (Bensch et al. 2018). It is necessary to confirm identification with a DNA barcoding approach using ITS with design primers.

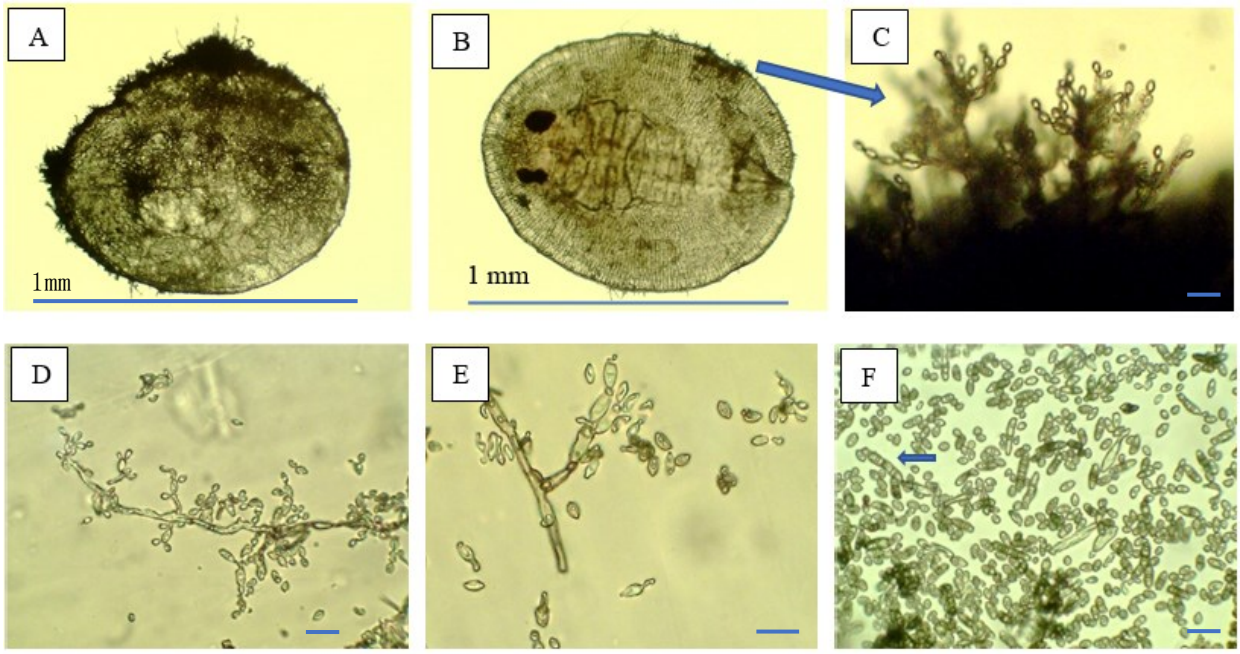

Figure 3. Microscopic observation of the fungus *Cladosporium* attacking whiteflies isolated directly from the field. A-B) Nymphs infected with *Cladosporium* fungus; C-F) Macronematous conidiophores and conidial chains. Arrow is ramoconidia.

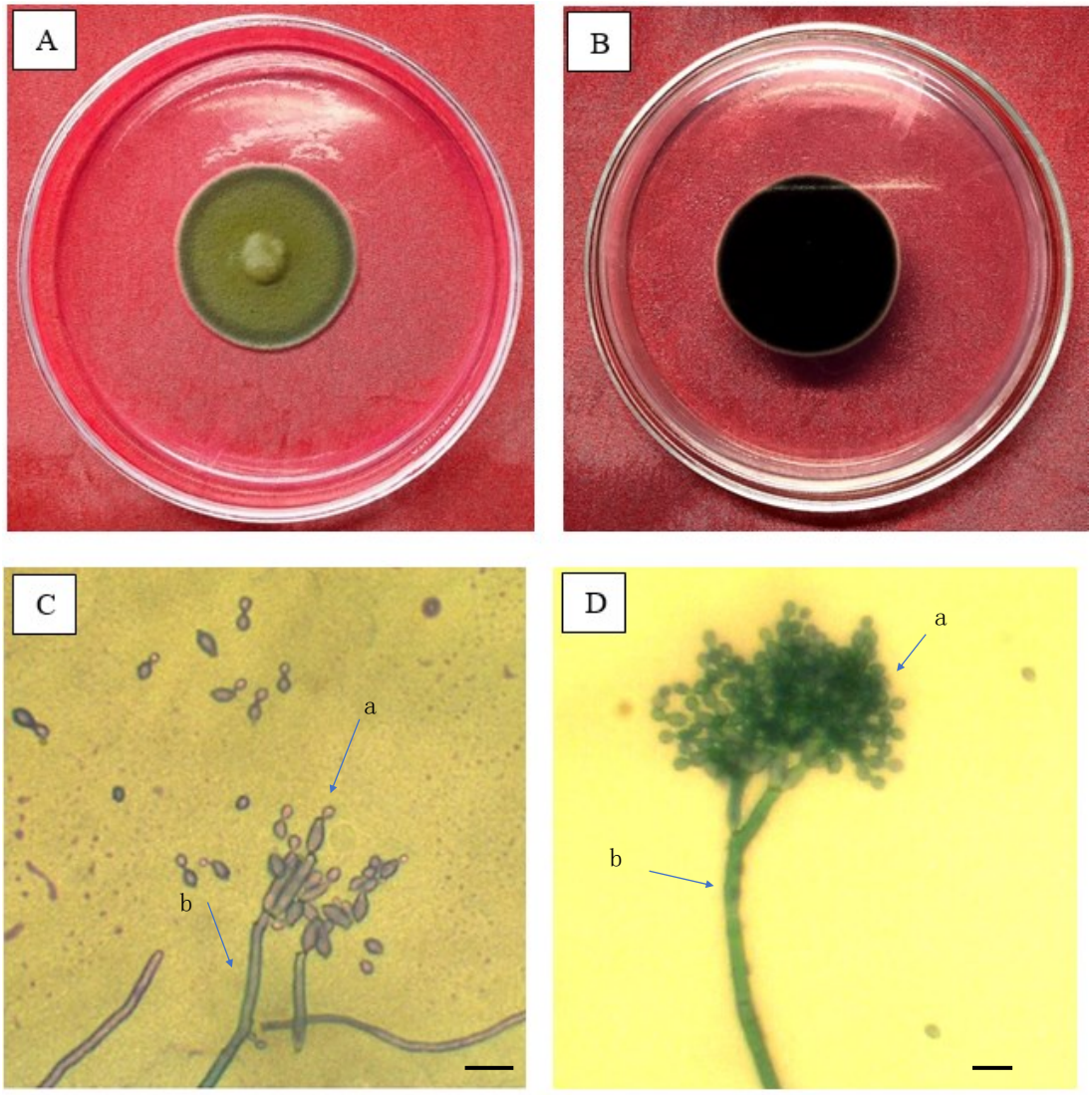

Figure 4. Culture of the fungus *Cladosporium* Bali isolate on PDA media 14 days after inoculation. A) Front view of fungal colony on PDA media; B) Rear view of fungal colony on PDA media; C,D) Conidia (a) and conidiophore (b), scale bars 10 µm.

Koch's postulate assay

The observation was carried out after 20 days the insects treated with the *Cladosporium* showed symptoms similar to the field observation. The symptom was observed, some infected nymphs were mummified with light fungal growth and profuse uniform growth of grey-black stroma containing spores at a later stage of infection (Figure 5A) in comparison with healthy whiteflies (Figure 5B). Uninfected nymphs were emerged as adults, marked by the remaining pupae shown in Figure 5B. The data indicated the control was not infected by *Cladosporium*.

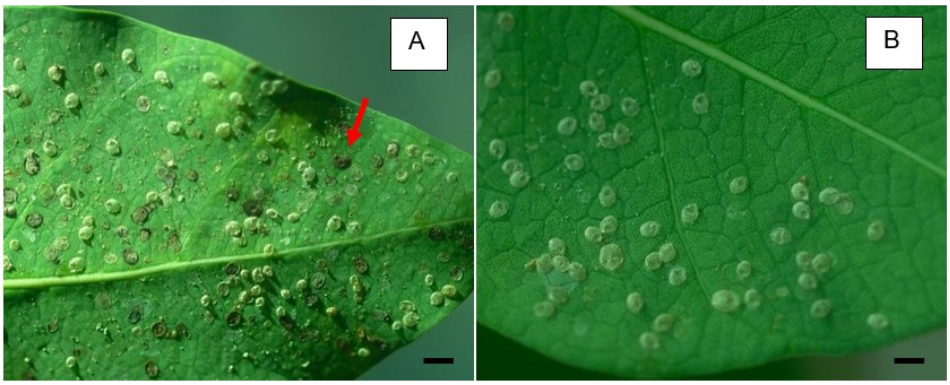

Figure 5. Koch's postulate assay result. A) The infected nymphs symptom (arrow); B) Healthy nymph, was emerging to imago, scale bars 2 mm.

Molecular characters of pathogenic fungi on whitefly

PCR was performed with the Infinigen PCR machine, the ±560 bp ITS target gene was successfully amplified. PCR product results were visualized with an agarose gel size of 560 bp (Figure 6A). These PCR product was then used for sequence and phylogenetic trees analysis. From the results of the BLAST search, the entomopathogenic fungus found in Denpasar, Bali, has an affinity with *Cladosporium dominicanum* strain SCAU014, accession number KY827344.1 (Per. Ident and Query Cover 100%) and *C. dominicanum* strain SCAU099, accession number MF061761.1. The insect pathogen phylogenetic tree found in Denpasar, Bali, is in the same clade as *C. dominicanum* strain SCAU014, accession number KY827344.1 (Figure 6B). Based on those information the entomopathogenic fungus species associated with the whitefly from Denpasar, Bali is same to *C. dominicanum*. Barcoding DNA using ITS to confirm the morphological character of entomopathogenic fungi was commonly used, our previous study succeeded in identifying how the entomopathogenic fungi *Aschersonia placenta* infects the citrus whitefly using primers ITS-1 and ITS-4 (Sudiarta et al. 2019). According to Alfiky (2022) based on ITS sequence analysis and phylogeny, the entomopathogenic fungi were identified as *B. bassiana* (four isolates), *M. anisopliae* (two isolates), and one isolate of *Cordyceps javanica*. In addition, using ITS for identification, the *Cladosporium* species was reported. Ghiaie et al. (2017), reported four species of *Cladosporium* successfully, using ITS barcoding. The morphological methods are not specific enough to differentiate species of the genus, Therefore, the DNA barcoding approach is very helpful in increasing the accuracy of identification.

C. dominicanum is a species of fungus that belongs to the *C. sphaerospermum* species complex, found in the Dominican Republic, Iran, Philippines, USA, Aruba, Taiwan, and Bermuda (Zalar et al. 2007; Bensch et al. 2018). Information about *C. dominicanum* that infects whiteflies has not been widely reported in Indonesia, although in several countries, the genus *Cladosporium* has been reported to infect insects, including whiteflies

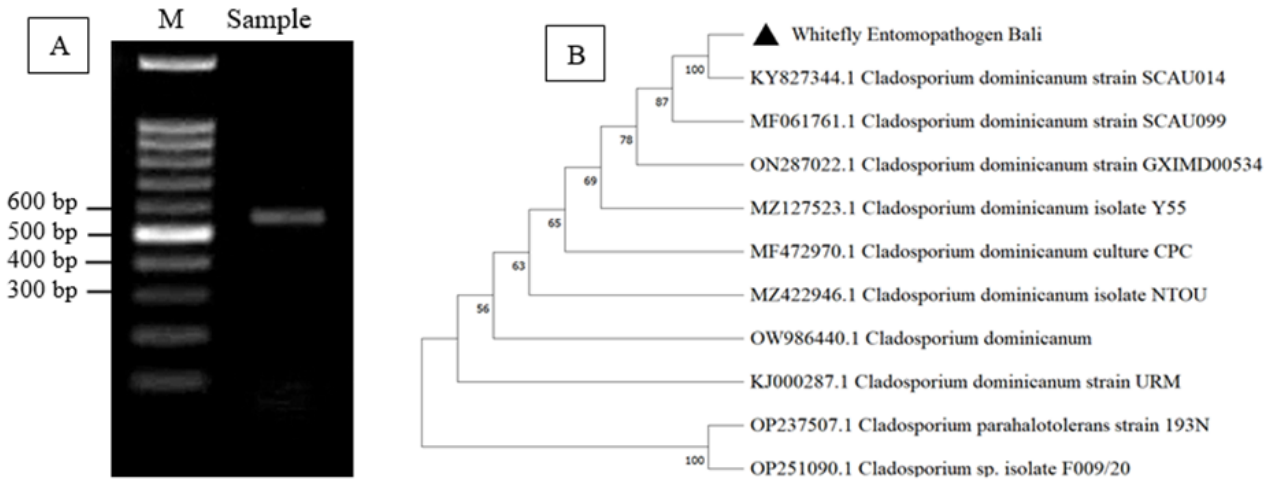

Figure 6. The molecular characterization of the fungus *Cladosporium dominicanum*. A) Agarose gel electrophoresis of PCR amplified products (size about 560 bp) using ITS1 and ITS4 PCR primer sets (volume of sample loaded per lane 1 µl). The marker was a 100 bp DNA ladder (M). B) Phylogenetic tree arranged based on DNA composition of *C. dominicanum* with the Maximum Parsimony method. The number in the branch was the percentage of the level of trust in the group.

(Abdel-Baky & Abdel-Salam 2003; T. Islam et al. 2019; M. T. Islam 2022). Compared with other entomopathogenic fungi (*B. bassiana* and *M. anisopliae*), *C. dominicanum* have not been utilized as a biological control in Indonesia. *B. bassiana* and *M. anisopliae* are common entomopathogenic fungi, and have been utilized as biological controls of insect pests (McGuire & Northfield 2020). This is the first report of *C. dominicanum* infecting whiteflies in Bali and Indonesia on an ornamental plants (*Premna serratifolia*, *Ficus religiosa*, *and Ficus rumphii*). Based on this, the opportunity to use *C. dominicanum* as a bioinsecticide in the support of sustainable agriculture is extremely promising. The biotechnological application of *Cladosporium* as a microbial biopesticide is likely to be further developed (Kumar et al. 2019; Tripathi et al. 2019). Currently, there is limited information available to utilize the entomopathogenic fungus *Cladosporium* as a biological control, and this research will be essential information for future development including mass production, selecting an appropriate medium, and developing an application formulation.

CONCLUSIONS

Based on morphological and molecular characteristics that *C. dominicanum* was identified as the entomopathogenic fungus that infected whiteflies on ornamental plants (*Premna serratifolia*, *Ficus religiosa*, *and Ficus rumphii*) in Denpasar, Bali, Indonesia.

AUTHOR CONTRIBUTIONS

IP.S., NN.P.S.W., D.S. and D.G.W.S. contributed to the article equally. IP.S. and G.N.A.S.W. conceptualized the research and collected the samples from the field. IP.S. carried out the morphological identification, D.G.W.S. performed the molecular analysis. IP.S. and D.G.W.S. prepared the manuscript and final editing by IW.D.G. and K.K.. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no competing interest regarding the publication of manuscripts.

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Research Article

Morphological Variation of *Diospyros* spp. Native to Sulawesi Based on Vegetative Organ Characters

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ABSTRACT
Diospyros spp. is a tree or shrub species belonging to the family Ebenaceae. Researchers have not extensively conducted studies on the morphological characteristics of *Diospyros* spp. to examine their diversity or enhance information for taxonomic hierarchy purposes. *Diospyros* spp. in Indonesia has great potential for in-depth research due to its unique characteristics, particularly in Sulawesi. This research aims to fill the knowledge gap regarding investigating phenotypic variations in *Diospyros* spp., specifically in the Sulawesi region. Nine species of *Diospyros* spp. from the Bogor Botanical Gardens collection were observed for their morphological characteristics and described according to their character traits. The results indicate 25 variations in morphological characteristics out of the 50 characters used. These 14 characteristics are key characteristics that influence the grouping of *Diospyros* spp. from Sulawesi. Phenetic analysis generates three clades of 9 accessions *Diospyros* spp. analyzed.

INTRODUCTION
Diospyros spp. is a flowering plant species classified in the family Ebenaceae. This species generally has a tree or shrub habit (Singh 2005). The number of accepted species of *Diospyros* spp. has reached 780 individuals. Initially, *Diospyros* spp. was only found in Asia, Australia, Africa, and the Americas, making it a native species. However, it has now spread to Europe, particularly in Italy, Switzerland, France, Greece, and Yugoslavia. The presence of *Diospyros* spp. is commonly found in Indonesia, on all of its islands, including Sulawesi and Sumatra which are part of the Malesian group (POWO 2023). Malesia is known for its tropical rainforest region, which supports the growth of this plant species (Kinho 2014). In Indonesia, the available number of *Diospyros* spp. is 32 species (Ariati et al. 2019).

Diospyros spp. has morphological characteristics such as being a medium- to large-sized tree with an upright stem reaching a height of 40 meters (Gunawan et al. 2019). The stem of *Diospyros* is black, although some species have a green color. The branch exhibits grooves, lacks latex, and features a swollen tip. The leaves are single and have an entire edge, an elliptical or elongated shape, a pointed-blunt tip, and an alter-

nate arrangement. The lower surface of the leaves frequently bears fine hairs. When young, the leaves are greenish-yellow and will change to green or dark green as they develop. *Diospyros* flowers are *dioecious*, with more male flowers than female flowers. The flowers are located in the leaf axils and belong to the cauliflorous type, where the flowers and fruits grow on the main stem. The fruit is fleshy with a fibrous pericarp and contains about 1–16 seeds per fruit (Wallnöfer 2001; Singh 2005).

Researchers have not extensively conducted morphological studies of *Diospyros* spp. based on vegetative and generative characteristics. This study is crucial to examine as it can ensure the accuracy of each taxonomic hierarchy of *Diospyros* spp., which often changes nomenclature due to limited morphological information (Rideng 1989; Puglisi et al. 2022). Another challenge frequently encountered in the morphological study of *Diospyros* spp. is that its generative organs have a short period, with flowering occurring only once a year (Yuniastuti et al. 2021). However, flower organs will not undergo phenotypic changes due to external factors. Therefore, an alternative solution is to utilize vegetative organs such as leaves, stems, or roots, as they can serve as alternative sources of information for *Diospyros* spp. plants instead of relying solely on flower organs. The science of morphology is crucial in taxonomy as it enables a comprehensive understanding of various plant organs, including their genetic variations and environmental influences (Stuessy 2009). Gnonlonfin's research (2022), which focused on phenotypic variations in *Diospyros mespiliformis*, demonstrates that leaf and flower organs continue to adapt due to environmental and geographical influences.

Several morphological studies have been conducted, such as the research by Putri and Chikmawati (2015), which revealed variations among the eight *Diospyros* spp. species based on their vegetative characteristics, precisely leaf flushing. Meanwhile, Wanda et al. (2022) successfully uncovered morphological variations within the species, albeit restricted to various locations by their morphometric analysis of the *Diospyros discolor* leaf organ. Morphological variation studies have also revealed relationships based on phenetic analysis (Rindyastuti et al. 2021) and identified new *Diospyros* species that have not been previously discovered (Puglisi et al. 2022).

Diospyros species that are native to Indonesia, specifically in the Sulawesi region, have a distinctive feature, which is their unique wood pattern. This species is *Diospyros celebica*, or ebony (Mustari 2021). Efforts to rescue ebony from extinction have been carried out by various methods, including forestry with soil repairs and various shading treatments to boost its growth optimally (Kurniawan 2013; Rauf et al. 2016). The study of data morphological variations is also one of the approaches used in plant conservation (Santos et al. 2011). Previous studies on the morphological variation of *D. celebica* have shown differences in leaf and stem characteristics across several regions in Sulawesi (Wahyuningsih et al. 2014). However, researchers have not studied the morphological variations of other distinctive *Diospyros* species from Sulawesi. Therefore, this research aims to fill the knowledge gap regarding the study of phenotypic variations in *Diospyros* spp., specifically in the Sulawesi region.

MATERIALS AND METHODS

Materials

The research utilized *Diospyros* spp. sourced from the Bogor Botanical Gardens (BBG) collection, which resulted from explorations in various regions of Sulawesi (Table 1). The age of the trees ranged from 10 to 28 years.

Table 1. The *Diospyros* spp from the Sulawesi region that used in the research.

No.	Bed number in BBG	Registration number	Species name	Origin
1.	X.G.130b	IRF 706	*D. buxifolia* (Blume) Hiern	Central Sulawesi
2.	XIX.B.41	IRF 719	*D. ridleyi* Bakh.	North Sulawesi
3.	XIX.B.37	IRF 721	*D. sumatrana* Miq.	Gorontalo
4.	IV.D.190a	IRF 722	*D. celebica* Bakh.	South Sulawesi
5.	XIII.F.16	IRF 727	*D. andamanica* (Kurz) Bakh.	Central Sulawesi
6.	IV.C.II4	IRF 729	*D. malabarica* (Desr.) Kostel.	SE. Sulawesi
7.	XXIV.A.202-202a	IRF 740	*D. maritima* Blume	North Sulawesi
8.	XXIV.A.270	IRF 714	*Diospyros sp.* 1	SE. Sulawesi
9.	IV.D.205	IRF 738	*Diospyros sp.* 2	South Sulawesi

Methods

The determination of morphological characters refers to the books and journals authored by Indriyanto (2012), Tjitrosoepomo (2009), Ellis et al. (2009), and Ghazalli et al. (2017). Fifty morphological characters, quantitative and qualitative, were used (Table 2). Leaf sampling took ten leaf blades (Alcántara-Ayala et al. 2020) from the third order of mature leaves per species (Semagn 2014). We took stem bark samples using a knife with a thickness of approximately 0.5 cm. The pieces were placed in plastic clips and labeled with species identification (British Columbia Ministry of Forest 1996). We directly observed each morphological character using a microscope 1.3 MP Dino-Lite edge plus 3.0. We utilized the Royal Horticultural Society (RHS) color chart to keep the color characters (Asih et al. 2022), while quantitative characters were measured using a ruler with a precision of 1 mm (Handayani 2013).

Table 2. Morphological characteristics observed.

Plant parts	Morphological character
Stem	Habit, canopy shape, stem development, stem base, stem surface texture, shape of stem, outer bark color, inner bark color, stem color, types of stem fiber, branch pattern, branch growth direction
Leaf	Leaf stalk shape, leaf stalk position, leaf stalk length (cm), leaf stalk type, leaf shape, leaf apex shape, leaf base shape, leaf margin type, type, leaf shape, leaf apex shape, leaf base shape, leaf margin type, leaf symmetry, leaf length (cm), leaf width (cm), laminar ratio, apex angle, base angle, young leaf color of an upper surface, young leaf color of a lower surface, mature leaf color (upper surface), mature leaf color (lower surface), leaf texture, upper leaf surface, lower leaf surface, leaf bones, variation of major secondary angle to midvein, number of secondary leaf vein, leaf venation, areolation, marginal ultimate (venation), areolar venation, lateral venation pattern, anastomosing of lateral venation, leaf glands, placement of leaf glands, number of glands per leaf, trichomes on leaf stalk

Data Analysis

The observational data on *Diospyros* spp. morphology consists of documentation of each character's traits. We analyzed it descriptively, quantitatively, and qualitatively to determine the morphological differences among *Diospyros* spp. The morphological character data were arranged in an n × t using Microsoft Excel software (Prasgi et al. 2022). Each descriptive morphological character was transformed into quantitive data by assigning score based on the observed characterictics. The binary data

from the quantitive were then scored, standardized, rescored and were further processed using MVSP 3.22 software to construct a phenogram using the UPGMA algorithm (Sneath & Sokal 1973). The similarity index was calculated based on the Simple Matching Coefficient (Sokal & Meichener 1958). Additionally, Principal Component Analysist was performed to clarify the pattern of character grouping that influenced the variations (Haekal et al. 2020).

RESULTS AND DISCUSSION

The results of observations on 50 morphological characters within the nine *Diospyros* species in the Sulawesi region indicate that only 25 characters exhibit variations representing 50% polymorphism, while the remaining characters show similarities. The morphological similarities of *Diospyros* spp. include having a tree habit, a pyramid-shaped canopy, and a monopodial type of central stem development. The stem is cylindrical, and the stem fibers are fine. The branching pattern of *Diospyros* spp. is continuous, with horizontal branch growth. The leaves of *Diospyros* spp. are simple, with an *alternate* leaf arrangement and a *semi-terete* leaf stalk positioned at the leaf margin. The leaves are symmetrical and have an *acute* leaf apex. The color of *Diospyros* spp. leaves is generally greenish-yellow, and the type of leaf bones is *penninervis*. Leaf venation is *pinnate*, areolar venation shows simple and uni-veinlets, and the lateral venation pattern is ascending, with anastomosis positioned near the leaf edge. The leaves of *Diospyros* spp. also exhibit glandular characteristics located on the leaf surface in small dots.

Leaves

Variations in leaf morphology are evident in the leaflet character, with variations in shape including *elliptical*, *ovate*, *oblongata*, and *lanceolate*. The *oblongata* shape dominates in species such as *D. malabarica*, *D. celebica*, *D. ridleyi*, *D. sumatrana*, and *Diospyros* sp.1. The *elliptical* shape is found in *D. buxifolia* and *Diospyros* sp.2. *Diospyros maritima* exhibits a unique shape, unlike other species, with an *ovate* form, while *Diospyros andamanica* has a *lanceolate* shape (Figure 1. I).

The leaflets of *Diospyros* spp. exhibit variations in leaf flesh, either *papyraceous* or *perkamenteus*, with a thin and stiffly fine texture. The upper and lower surfaces of the leaves differ but are predominantly *glabrous* (without hairs or scales), except for *D. buxifolia* and *Diospyros* sp.2. The upper surface of the leaves of *D. buxifolia* and *Diospyros* sp.2 has a smooth texture (*laevis*). The lower surface of *D. buxifolia* is *pilosus* (covered with short and fine hairs) (Putri & Chikmawati 2015), while *Diospyros* sp.2 has a smooth texture (*laevis*). The lower surface of *D. celebica* leaves is *pilosus*, or covered with fine hairs (Wahyuningsih et al. 2014) (Figure 1. II).

Leaf margin characteristics show two variations: wavy and entire. *D. andamanica* is the only species with wavy leaf margins (Figure 1. III), but Rindyastuti et al. (2021) found that the leaf margins of *D. andamanica* are entire. Regarding the leaf apex, there are two variations: *acutus* and *acuminatus*. Species like *D. buxifolia*, *D. maritima*, and *D. malabarica* have an *acutus* leaf apex. In contrast, *D. celebica*, *D. ridleyi*, *D. sumatrana*, *D. andamanica*, *Diospyros* sp.1, and *Diospyros* sp.2 have an *acuminatus* leaf apexes (Figure 1. IV). The shape of the leaf base in *Diospyros* spp. shows three variations: *acutus*, *obtusus*, and *rotundatus*. *Acutus* shapes are found in species such as *D. buxifolia*, *D. malabarica*, *D. celebica*, *D. andamanica*, and *Diospyros* sp.2. In contrast, *D. ridleyi*, *D. sumatrana*, and *Diospyros* sp.1 exhibit *obtusus* leaf bases. *D. maritima* is the only species with a *rotundatus* leaf base. Silalahi & Mustaqim (2020) also described *D. maritima* as having

rotundatus leaf bases (Figure 1. V). Variations in qualitative leaf characteristics indicate that each species undergoes adaptation to its environment, leading to the conditioning of organ shapes (Anatov & Mallaliev 2022). Similarly, trichomes on leaf stalk show variations in their abundance, ranging from few to many, in response to environmental conditions (Figure 2. I).

Diospyros spp. generally have leaf glands located on the upper or lower surface of the leaf. The number of leaf glands found in *Diospyros* spp. in the Sulawesi region ranges from approximately 1-14, depending on the leaf size for each species. Leaf bones play a crucial role as a character in identifying species (Setiaji et al. 2016). The secondary leaf veins of *Diospyros* spp. show two variations: uniform angle and inconsistent angle, with the number of veins ranging from 5 to 16 in each species.

The characters of leaf length, leaf width, leaf ratio, leaf base angle, and leaf stalk length exhibit quantitative variations. The size and width of leaves in *Diospyros* spp. vary greatly, ranging from approximately 2-32.3 cm in length and 0.7 to 7.3 cm in width. *D. celebica* has the largest leaf size, while *D. buxifolia* has the smallest. Based on the leaf length and width measurements, the resulting leaf ratios are 1:2.5, 1:3.5, and 1:4. Regarding the leaf base angle, *D. maritima*, which has a *rotundatus* leaf base, produces *obtuse* leaf base angles. Other species have *acute* leaf base angles. *Diospyros* spp. also vary in size, ranging from 0.1-1.1 cm. *D. buxifolia* has the shortest leaf stalk, measuring 0.1 cm. The most extended leaf stalk, measuring 1.1 cm, is found in *D. celebica* and *D. ridleyi*.

Variations in leaf color can often be difficult to identify accurately due to subjective interpretation. Therefore, the use of an RHS *color chart* helps determine leaf colors. The variations in color on the upper surface of mature leaves include green and greenish-yellow. The leaves have the same color but differ in contrast levels (Figure 2. II). The leaf stalk and leaf buds also exhibit color variations. The leaf stalk of *Diospyros* spp. shows various color variations, including grayish-brown, green, and greenish-yellow. *D. celebica* is the only species that has a different color variation compared to the other species, which is grayish-brown (Figure 2. III). Leaf buds are essential in leaf organs as they represent the initial stages of leaf formation. Color variations in leaf buds include greenish-yellow, brown, grayish-brown, and grayish-yellow. *D. buxifolia* and *Diospyros* sp.2 have different colors for their leaf buds compared to other species, with greenish-yellow and grayish-yellow colors, respectively. *D. maritima*, *D. malabarica*, *D. ridleyi*, *D. sumatrana*, and *D. andamanica* exhibit brown-colored buds. In contrast, grayish-brown buds are found in *D. celebica* and *Diospyros* sp.1 (Figure 2. IV).

Stem

The characteristics of outer bark color, inner bark color, stem color, stem surface texture, and stem base indicate the variations in morphology found in the stem organ. Generally, the outer bark color of *Diospyros* spp. is black (Singh 2005), but this research shows color variations such as brown, grayish-brown, and black (Figure 2 V). The color of the inner bark is predominantly grayish-orange, while the stem color is dominated by yellow. The stem base of *Diospyros* spp. usually does not have buttresses, but occasionally buttresses are found, as in the case of *D. celebica* and *D. maritima* species (Figure 2 VI). Some stems of *Diospyros* spp. have a smooth stem texture, while others have soft and rough grooves. *D. celebica* is the only species with a rough, grooved texture, unlike other species (Figure 2 VII). Gunawan et al. (2019) stated that *D. celebica* has a fluted stem. Rindyastuti et al. (2021) also revealed that the stem surface

Table 3. Variations in leaf morphology of *Diospyros* spp.

No.	Morphological Character	*D. buxifolia*	*D. maritima*	*D. malabarica*	*D. celebica*	*D. ridleyi*	*D. sumatrana*	*D. andamanica*	*Diospyros* sp.1	*Diospyros* sp.2
1.	Leaf stalk length (cm)	0-0.1	0.6-0.9	0.5-0.8	0.8-1.1	0.7-1.1	0.5-0.9	0.2-0.4	0.6-1	0.2-0.4
2.	Leaf stalk color	Greenish-yellow	Greenish-yellow	Greenish-yellow	Grayish-brown	Green	Greenish-yellow	Greenish-yellow	Greenish-yellow	Greenish-yellow
3.	Leaf bud color	Greenish-yellow	Brown	Brown	Grayish-brown	Brown	Brown	Brown	Grayish-brown	Grayish-yellow
4.	Leaf flesh type	*Papyraceus*	*Perkamenteus*	*Perkamenteus*	*Papyraceus*	*Perkamenteus*	*Perkamenteus*	*Papyraceus*	*Perkamenteus*	*Papyraceus*
5.	Laef shape	*Elliptical*	*Ovate*	*Oblongata*	*Oblongata*	*Oblongata*	*Oblongata*	*Lanceolate*	*Oblongata*	*Elliptical*
6.	Leaf apex shape	*Acutus*	*Acutus*	*Acutus*	*Acuminatus*	*Acuminatus*	*Acuminatus*	*Acuminatus*	*Acuminatus*	*Acuminatus*
7.	Leaf base shape	*Acutus*	*Rotundatus*	*Acutus*	*Acutus*	*Obtusus*	*Obtusus*	*Acutus*	*Obtusus*	*Acutus*
8.	Leaf margin type	Entire	Entire	Entire	Entire	Entire	Entire	Wavy	Entire	Entire
9.	Leaf length (cm)	2-3.8	12.5-18.2	12.1-18.5	25.5-32.3	16.2-22.8	15.5-22	7.4-10.9	15.5 – 23	4.6-7.3
10.	Leaf width (cm)	0.7-1.5	5.1-7.2	3.2-5	6-7.3	3.5-6.2	3.1-5.2	2-2.6	3.5 – 5.5	1.4-2.1
11.	Laminar ratio	1:2.5	1:2.5	1:3.5	1:4	1:4	1:4	1:4	1:4	1:3.5
12.	Base angle	*Acute*	*Obtuse*	*Acute*	*Acute*	*Acute*	*Acute*	*Acute*	*Acute*	*Acute*
13.	Mature leaf color (upper surface)	Greenish-yellow	Greenish-yellow	Greenish-yellow	Green	Green	Greenish-yellow	Greenish-yellow	Green	Greenish-yellow
14.	Leaf texture	Thin-fine	Stiff-fine	Stiff-fine	Stiff-fine	Stiff-fine	Stiff-fine	Thin-fine	Stiff-fine	Thin-fine
15.	Upper leaf surface	*Laevis*	*Glaber*	*Glaber*	*Glaber*	*Glaber*	*Glaber*	*Glaber*	*Glaber*	*Laevis*
16.	Lower leaf surface	*Pilosus*	*Glaber*	*Glaber*	*Pilosus*	*Glaber*	*Glaber*	*Glaber*	*Glaber*	*Laevis*
17.	Variation of major secondary angle	Secondary angle uniform	Secondary angle uniform	Secondary angle uniform	Secondary angle inconsistent	Secondary angle uniform	Secondary angle uniform	Secondary angle uniform	Secondary angle uniform	Secondary angle uniform
18.	Number of secondary leaf vein	5-8	7-8	5-10	9-16	5-8	8-10	5-8	7-10	7-10
19.	Number of glands per leaf	5-8	5-10	5-12	5-8	5-9	5-9	1-4	8-14	1-2
20.	Trichomes on leaf stalk	Many	Few	Few	Many	Few	Few	Few	Few	Many

Table 4. Contd.

No.	Morphological Character	*D. buxifolia*	*D. maritima*	*D. malabarica*	*D. celebica*	*D. ridleyi*	*D. sumatrana*	*D. andamanica*	*Diospyros* sp.1	*Diospyros* sp.2
1.	Stem base	Non-buttress	Buttress	Non-buttress	Buttress	Non-buttress	Non-buttress	Non-buttress	Non-buttress	Non-buttress
2.	Stem surface texture	Smooth	Smooth	Smooth grooved	Rough grooved	Smooth grooved	Smooth grooved	Smooth	Smooth grooved	Smooth
3.	Outer bark color	Grayish-brown	Brown	Black	Black	Brown	Brown	Black	Brown	Black
4.	Inner bark color	Grayish-orange	Grayish-orange	Orange-white	Orange-white	Grayish-orange	Grayish-orange	Grayish-orange	Grayish-orange	Yellow-orange
5.	Stem color	Yellow	Yellow-white	Orange-white	Yellow-white	Orange-white	Yellow-white	Yellow-white	Yellow-white	Yellow-white

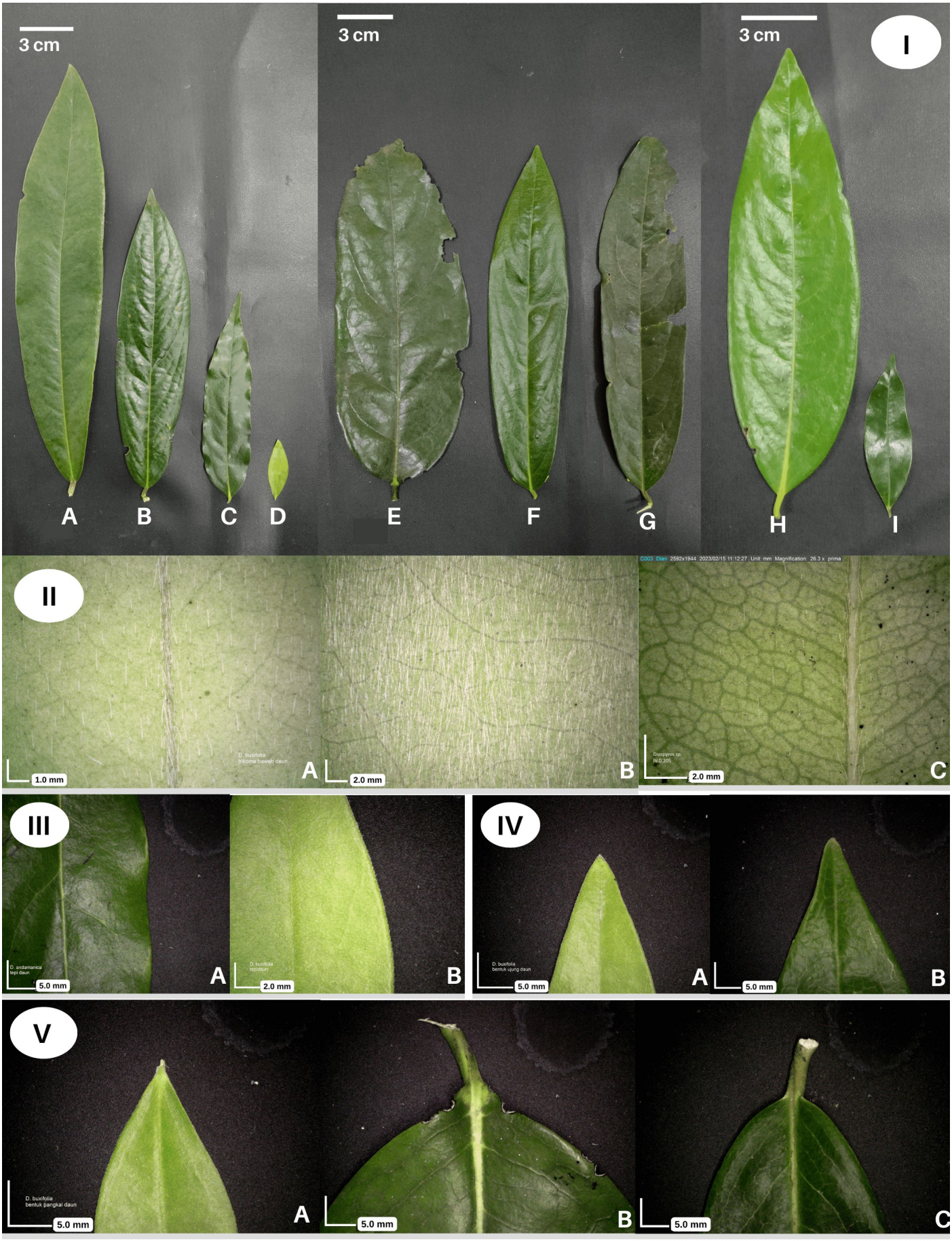

Figure 1. I) Leaf variations of *Diospyros* spp. in Sulawesi region. Description: (A) *D. celebica*, (B) *D. malabarica*, (C) *D. andamanica*, (D) *D. buxifolia*, (E) *D. maritima*, (F) *D. sumatrana*, (G) *D. ridleyi*, (H) *Diospyros* sp.1, (I) *Diospyros* sp.2.; II.) Lower leaf surface. Description (A). *D. buxifolia* (B). *D. celebica* (*pilosus*), (C). *Diospyros* sp.2 (*laevis*); III.) Types of leaf margins. (A). wavy, (B). entire.; IV.) Shape of leaf apex. (A). *acutus*, (B). *acuminatus*; V.) Shape of leaf base. (A). *acutus*, (B). *rotundatus*, (C). *obtusus*

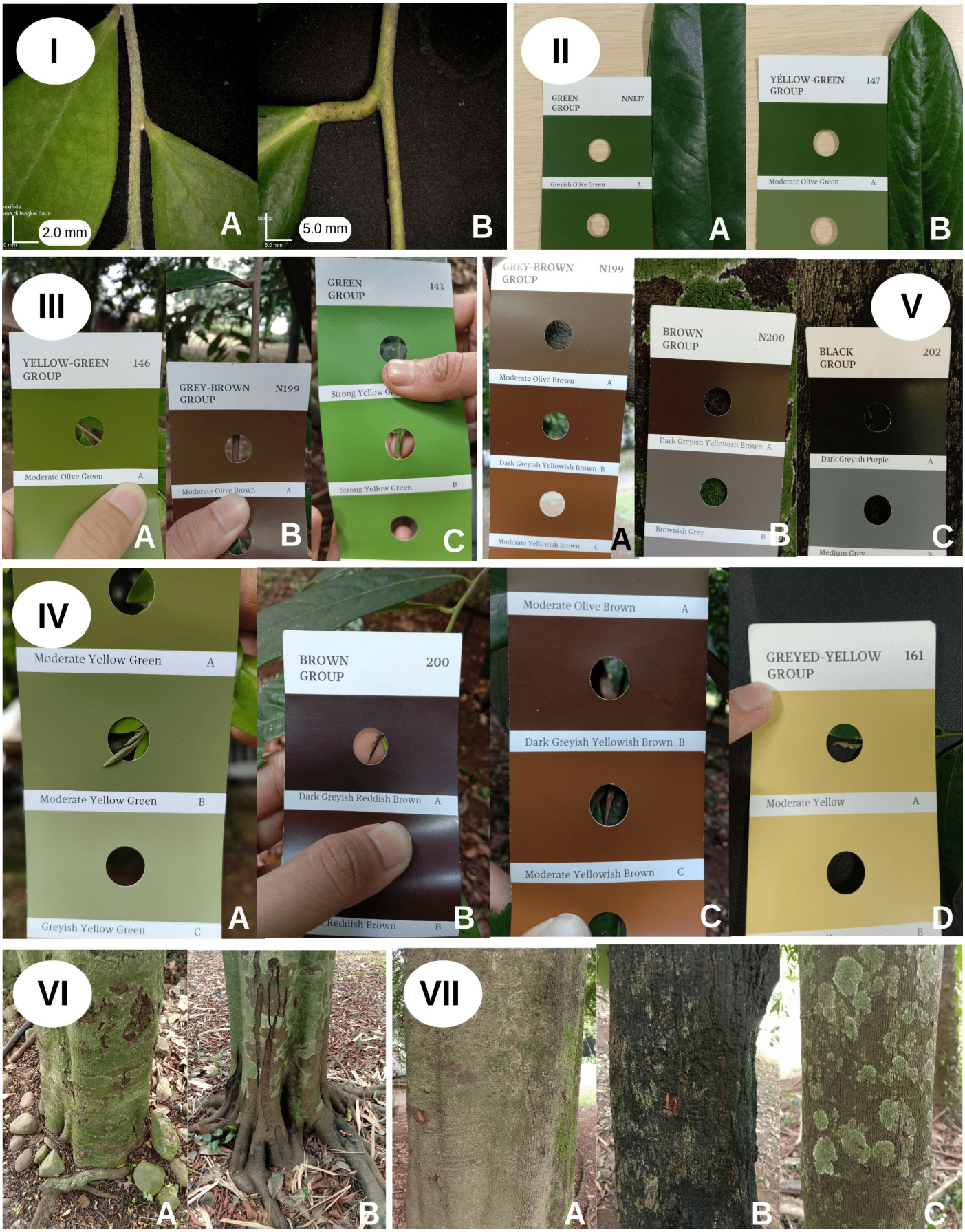

Figure 2. I) Number of trichomes on the leaf . (A). Many, (B). Few; II.) Color of mature leaves on the upper surface. (A). Greenish-yellow, (B). Green; III.) Leaf stalk color. (A). Greenish-yellow, (B). Grayish-brown, (C). Green; IV.) Leaf buds color. (A). Greenish-yellow, (B). Brown, (C). Grayish-brown, (D). Grayish-yellow; V.) Outer bark color. (A). Grayish-brown, (B). Brown, (C). Black; VI.) Types of stem base. (A). Non-buttress, (B). Buttress; VII.) Surface texture of stem. (A). Smooth, (B). Rough grooved, (C). Smooth grooved

texture of *D. celebica* is rough and grooved, while *D. andamanica* and *D. malabarica* have smooth stem surfaces.

Identification key for *Diospyros* spp. native to Sulawesi

1) a. The variation of the major secondary angle is inconsistent, number of secondary leaf veins ranges ≥10 ..... *D. celebica* Bakh.
   b. The variation of the major secondary angle is uniform, number of secondary leaf veins ranges ≤10 2
2) a. The stem base is a buttress, with an *obtuse* leaf base angle ..... *D. maritima* Blume
   b. The stem base is non-buttress, with an *acute* leaf base angle 3
3) a. Smooth stem surface texture, leaf stalk length is <5 cm, leaf texture is thin-fine 4
   b. Smooth-grooved stem surface texture, leaf stalk length is ≥5 cm, leaf texture is stiff-fine 6
4) a. The shape of the leaf apex is *acutus*, outer bark color is grayish-brown ..... *D. buxifolia* (Blume) Hiern
   b. The shape of the leaf apex is *acuminatus*, outer bark color is black 5
5) a. Wavy leaf margin, *glaber* upper leaf surface, *glaber* lower leaf surface ..... *D. andamanica* (Kurz) Bakh.
   b. Entire leaf margin, *laevis* upper leaf surface, *laevis* lower leaf surface ..... *Diospyros* sp.2
6) a. The shape of leaf base is *acutus*, inner bark color is orange-white ..... *D. malabarica* (Desr.) Kostel.
   b. The shape of leaf base is *obtusus*, inner bark color is grayish-orange 7
7) a. The stem color is orange-white ..... *D. ridleyi* Bakh.
   b. The stem color is yellow-white 8
8) a. Brown leaf bud color, greenish-yellow upper mature leaf color ..... *D. sumatrana* Miq.
   b. Grayish-brown leaf bud color, green upper mature leaf color ..... *Diospyros* sp.1

Based on the morphological similarities data of *Diospyros* that has been shown the key identification, the next step is phenetic analysis to reveal more definitely the relationship. Phenetic techniques systematically classify species based on morphological similarities and establish phylogenetic correlations. The phenetic method encompasses two analytical techniques, namely cluster and PCA analysis (Kovach 2007; Singh 2010). The phenogram analysis yielded two primary groups, Cluster I and Cluster II which had a similarity index of 0.83 as depicted in Figure 1. The morphological disparities seen between *D. celebica* and other *Diospyros* species result in their classification into two primary groupings. *Diospyros celebica* is classified as a separate species (Cluster I) based on its distinct morphological characteristics, including variances in the texture of the stem surface, the color of the leaf stalk, variation in the angle between the major secondary vein and the midvein, and the number of secondary leaf veins. Cluster II can be subdivided into two distinct subclusters, B1 and B2, with a similarity index value of 0.907. The primary distinguishing features differentiating subclusters B1 and B2 from the center cluster are the leaf shape variations and the upper leaf surface characteristics.

Within subcluster B2 are *Diospyros* sp.1 and *D. sumatrana*, which have a similarity index 1.00. Due to the considerable physical resemblances between these two species, they can be deemed comparable and closely affiliated. The homogeneity of physical characteristics can be in-

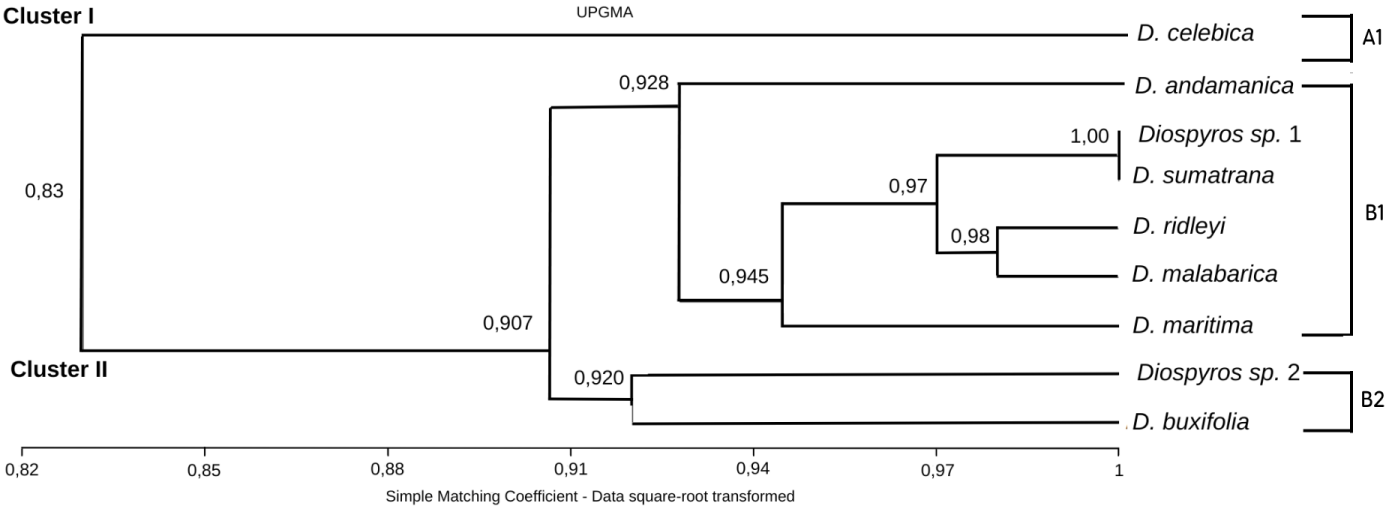

Figure 3. *Diospyros* phenogram of Sulawesi region based on 50 morphological characters.

fluenced by both genetic and environmental variables (Bramasto et al. 2015). Nevertheless, subtle variations in coloration exist between the physical attributes of leaf buds and fully developed upper leaves.

The analysis of similarity index values reveals that *Diospyros* sp.1 and *D. sumatrana* exhibit the highest resemblance, as indicated by a similarity index of 1.00. On the other hand, *D. celebica* and *D. ridleyi* show the most divergent relationship, as noted in a similarity value of 0.80 (Table 4). According to Singh (2010), when the similarity values between operational taxonomic units (OTUs) surpass 85%, they are still considered to belong to the same species. The utilization of vegetative traits in the biosystematic investigation of *Diospyros* is of utmost importance due to its role in species identification and the elucidation of relationships (Rindyastuti et al. 2021).

The grouping of *Diospyros* spp. is influenced by various morphological characteristics, including stem surface texture, the color of the outer bark of the stem, stem color, leaf bud color, leaf stalk color, leaf flesh type, leaf shape, leaf base shape, leaf width, leaf ratio, lower leaf surface, number of secondary leaf veins, and the number of trichomes on the leaf stalk (refer to Table 5 and Figure 2). The grouping is highly influenced by Principal Component 1 (PC1) due to its greatest eigenvalue of 42.287%, surpassing that of other principal components. According to Ningrum and Chasani (2021), there is a positive relationship between the value of the major component and its impact on the grouping of species.

The scatter plot analysis of OTU grouping using cluster analysis demonstrates consistent findings, revealing the existence of two distinct clusters. The first cluster, labeled A1 and corresponding to *D. celebica*, remains unchanged. The second cluster, denoted as B, can be further divided into two subclusters: B1, consisting of *D. andamanica*, *D. ridleyi*, *D. sumatrana*, *Diospyros* sp.1, *D. malabarica*, and *D. maritima*; and B2, comprising *D. buxifolia* and *Diospyros* sp.2. According to Setiawati et al. (2013), the proximity of clusters B1 and B2 within the same quadrant suggests a high degree of relatedness among the species belonging to these groupings.

CONCLUSIONS

This research reveals the presence of morphological variations in *Diospyros* spp. based on the characteristics of their vegetative organs. The observations of 50 morphological characters indicate the presence of 25 var-

Table 4. Similarity index between *Diospyros* species based on morphological characters.

No.	Species Name	A	B	C	D	E	F	G	H	I
A	*D. buxifolia*	1,00								
B	*D. maritima*	0,90	1,00							
C	*D. malabarica*	0,90	0,92	1,00						
D	*D. celebica*	0,88	0,82	0,82	1,00					
E	*D. ridleyi*	0,92	0,94	0,98	0,80	1,00				
F	*D. sumatrana*	0,94	0,96	0,96	0,82	0,98	1,00			
G	*D. andamanica*	0,92	0,90	0,94	0,84	0,92	0,94	1,00		
H	*Diospyros* sp.1	0,94	0,96	0,96	0,82	0,98	1,00	0,94	1,00	
I	*Diospyros* sp.2	0,92	0,86	0,90	0,84	0,88	0,90	0,92	0,90	1,00

Table 5. Morphological characters that affect PC1, PC2, PC3, and PC4.

No.	Morphological character	PC1	PC2	PC3	PC4
1	Stem surface texture	0,280	-0,219	0,162	-0,019
2	Outer bark color	0,295	0,337	0,629	0,242
3	Inner bark color	0,084	0,352	-0,116	0,190
4	Stem color	-0,189	-0,021	0,484	-0,406
5	Leaf stalk color	0,280	-0,219	0,162	-0,019
6	Leaf bud color	0,084	0,352	-0,116	0,190
7	Leaf flesh	0,440	0,338	-0,119	0,003
8	Leaf shape	0,007	0,170	0,208	0,207
9	Leaf base shape	-0,072	-0,219	-0,174	0,491
10	Leaf width (cm)	0,280	-0,219	0,162	-0,019
11	Leaf ratio	0,208	-0,438	-0,012	0,473
12	Lower leaf surface	0,349	-0,185	-0,211	-0,393
13	Number of secondary leaf vein	0,280	-0,219	0,162	-0,019
14	Trichomes on leaf stalk	0,433	0,168	-0,327	-0,203

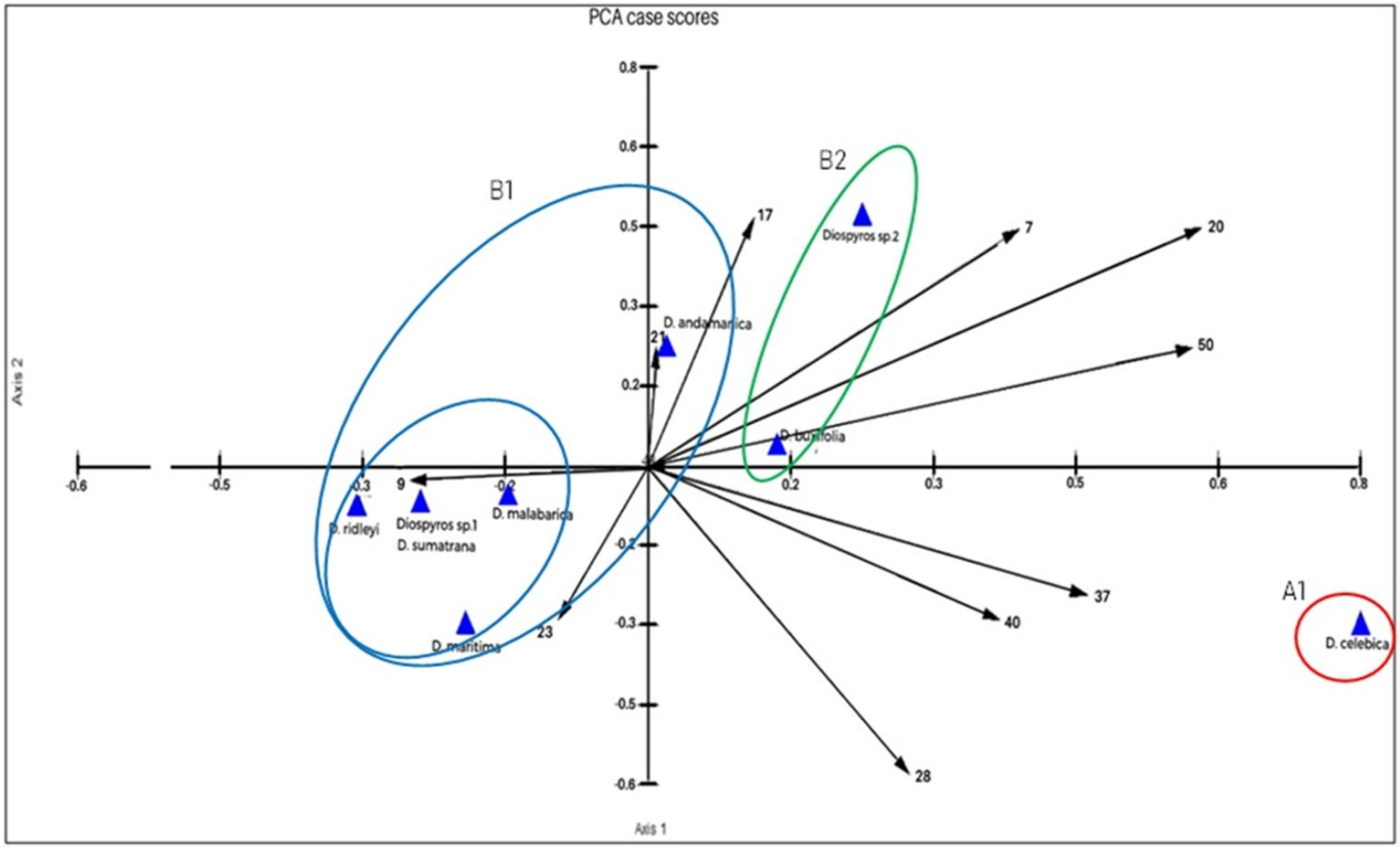

Figure 4. Scatter plot of *Diospyros* Sulawesi region.

iations in both qualitative and quantitative traits. These 14 characteristics are key characters that influence the grouping of *Diospyros* spp. from Sulawesi. The vegetative organs depict the diversity of morphological variations within *Diospyros* spp. While the vegetative organs can represent the diversity of morphological characters, the generative organs are still necessary to show other variations in the morphological traits of *Diospyros* spp.

AUTHOR CONTRIBUTION

The author's contribution: AAW designed the research, collected and examined the data, and wrote the manuscript. LK designed the research, collected, analyzed the data, and improved the manuscript. IFW developed the study, collected, analyzed the data, and improved the manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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Research Article

Intraspecific SSR Marker Screening for Detection of *Dendrobium crumenatum* Mutants Generated from *In Vitro* Gamma Irradiation

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ABSTRACT

Determination of *D. crumenatum* mutant obtained from *in vitro* mutation breeding needs a long time due to its long-life cycle. SSR molecular markers can be used for early mutant detection. Specific SSR markers developed in *D. crumenatum* are not yet available. Alternative published SSR markers were developed from *D. catenatum*. The aims of this study are to screen the most informative SSR markers generated from *D. catenatum* tested in irradiated *D. crumenatum* population and to determine the gamma irradiation dose resulting the most mutants. Ten SSR markers were randomly selected and tested in 25 individuals of *D. crumenatum* plantlets irradiated with several doses (0, 5, 10, 15, and 20 Gy; 5 plantlets each dose). The result showed 7 of 10 primers were polymorphic and the other three were monomorphic. All seven polymorphic primers can be used to identified intraspecific variation in the *D. crumenatum* mutant population. Markers *dnsr28* and *dnsr98* were the most informative, with the highest polymorphic information content (PIC) value of 0.5. Irradiation *D. crumenatum* protocorms using 10-15 gray doses were detected as the highest mutant percentage obtained up to 100% in the sample tested. This resulting marker information can be used to screen wider mutant population to decrease the non-mutant individuals in the population for maintenance and cost efficiency. The 10-15 Gy can be used as reference doses for gamma irradiation in 3 months old *D. crumenatum* protocorm materials.

INTRODUCTION

Mutation breeding through several doses of gamma irradiation has been conducted to increase *Dendrobium crumenatum* diversity. Gamma Irradiation is one of many mutagens in mutation breeding techniques that can randomly induce variability of plant materials (Udage 2021). Many reports showed that gamma irradiation treatment has successfully changed the vegetative, generative, and metabolite performance of the plant species (Simanjuntak et al. 2020; Fathin et al. 2021; Aisyah et al. 2022a). The goal was to obtain novel *D. crumenatum* mutants with longer inflorescence bloom periods and/or novel flower morphology phenotypes. However, flower phenotypes require a long time to be observed due to

long-life cycle especially in the orchid genus. In general, it takes 3-4 years for orchid to complete one life cycle from seed to bloom (Hsu et al. 2018). Early screening of putative mutants will greatly help researchers to reduce the number of plants maintained, costs, and labor. Early screening based on visual or phenotypic plants is difficult to do because not many variances can be observed in vegetative *in vitro* stage and plantlets that do not differ much between controls and those that have been irradiated (except for the plantlet sizes).

Early putative mutant selection can be done molecularly to determine individuals whose genetics have changed post mutation treatment. Among many molecular markers, SSR marker is a codominant marker based on specific simple sequence repeat (SSR) motifs that highly polymorphic and spread in the whole genome possible to differentiate inter- or intraspecific variations (Zhao et al. 2019; Li et al. 2021) that are the best option for diversity analysis in mutant population.

The SSR markers are mainly generated from whole genome (Bhattarai et al. 2021) or RNAseq (Tsai et al. 2015) data. Despite the fact that next-generation sequencing (NGS) has been well developed, the research in the field is relatively pricy. The data analysis can only be performed by bioinformatic specialist supported with super computer. Luckily, some of the SSR motives are conserved in cross species (Zhao et al. 2019) that mean the SSR generated from one species genome can also be used in other species. The SSR markers of *Dendrobium* species have been published and generated from *D. catenatum* genome scaffolds and have not been tested in *D. crumenatum*. The *D. crumenatum* intraspecific diversity, specifically in the mutant population study using informative primer, has not been reported. This study aims to screen the most informative SSR primer generated from *D. catenatum* tested in irradiated *D. crumenatum* population and determine the gamma irradiation dose resulting in the most mutants.

MATERIALS AND METHODS

Materials

Plant material, *in vitro* gamma irradiation mutation, and protocorm maintenances

Plant material used in this experiment are 3 months old *Dendrobium crumenatum* protocorm that have been gamma irradiated in several doses, the main different phenotypic between the materials is the plantlet sizes (Figure 1). The seeds were extracted from seedpod resulting from self-pollinated wild *D. crumenatum* obtained in Bogor, West Java. The extracted seeds then cultured in *in vitro* culture following Sanjaya et al. (2022) germination medium. Briefly half-strength Murashige and Skoog medium supplemented with 15% coconut water, 30 g L^{-1} sugar, 7 g L^{-1} agar. The pH of the medium was adjusted to 5.6-5.8. The protocorms were subcultured to germination medium in the disposable petri dish (50 protocroms/petri) for 1 week and then irradiated in Gamma cell 220 with several doses consisting of 0, 5, 10, 15, and 20 gray with 3 replicates. The protocorms were then directly transferred to grown in fresh growing

Figure 1. *D. crumenatum* gamma irradiated plantlet materials used in this experiment.

orchid medium (Lubis et al. 2020). Briefly 2 gr L^{-1} hyponex 20:20:20, 50 gr L^{-1} ripe banana, 50 gr L^{-1} potato, 2 gr L^{-1} active charcoal, MS vitamins, 20 g L^{-1} sugar, and 7 gr L^{-1} agar, and the pH of the medium was adjusted to 5.6-5.8. The protocroms were maintain in the media for 6 months.

Methods

DNA isolation

The DNA were isolated from five individuals from each treatment with the total of 25 samples following modified Putri et al. (2021). Each plantlet (±0.2 g) was taken and ground in 1.5 mL microtubes manually using mini pestle then 700 µL of CTAB buffer was added and vortexed until homogeneous. The samples were then incubated in a water bath at 65^{O}C for 30 minutes (vortexed every 10 minutes). The samples were then centrifuged at a speed of 8500 rpm for 15 minutes. The upper phase (supernatant) was transferred to a 1.5 mL microtube, taken carefully using a micropipette. CIA (chloroform : isoamyl alcohol, 24 : 1) was added in 1x volume of supernatant and inverted for 10 minutes then centrifuged again at 8500 rpm for 15 minutes. The upper phase was carefully taken using a micropipette and transferred to a new 1.5 mL microtube. A total of 1x the volume of cold absolute ethanol was added to the sample and homogenised by turning it back and forth manually. The sample was then precipitated by incubating in a freezer (-40^{O}C) overnight (16-18 hours). After precipitation, the sample was centrifuged at 8500 rpm for 30 minutes. The supernatant was discarded and the pellet was washed by adding 400 µL of cold 70% ethanol, homogenised by vortexing and centrifuged at 8500 rpm for 5 minutes. Samples were washed two times. The supernatant was discarded and the resulting DNA pellet was air-dried until no visible liquid trace or alcohol odor can be smelled. The DNA pellet was diluted by adding 100 µL ddH_2O. The resulting stock DNA is then stored in a -20^{O}C freezer until use.

Primer screening

Ten SSR Primers were selected from Zhao et al. (2019), Table 1. From 72 of the published primers, the primer selection criteria in this research was the primer that can amplify and resulting PCR product in other species (*D. catenatum*, *D. denneanum*, and *D. nobile*) as the indicator that primers are universal for species in *Dendrobium* genus. The primers were tested by PCR amplifying in 25 *D. crumenatum* irradiated plantlets (5 plantlets each dose; 0, 5, 10, 15, and 20 gy) with the setting of pre-

Table 1. Selected primers from SSR mining from *Dendrobium catenatum* genomic scaffolds generated by Zhao et al. (2019) tested in gamma-irradiated *Dendrobium crumenatum* population.

Primer ID	Forward Sequence (5′-3′)	Reverse Sequence (5′-3′)	Target Size (bp)	Repeat Unit
dnsr9	CCATTTATGTTGCTTGCGAGTT	GCTCCGACGTAAGATTCACAA	181	$AG_{(51)}$
dnsr17	GCCGCCTTTCTTATGTTAGTTG	GCGTAATGCTGTGACACCAA	285	$TAA_{(39)}$
dnsr24	TTCGAGGCAACGGAGTCAG	TCCACCAGCAAAGCACACT	451	$TTA_{(57)}$
dnsr25	GCATCATAAGCAGTAGGTAAAC	CCACTAGACTTGTTGATAGCAT	329	$AC_{(50)}$
dnsr28	TGGAGCAAGACTTGTCTAAGC	ACTTGAGATTAGCAAACAGCAC	217	$TACA_{(11)}$
dnsr55	GTCCTAAGATTCTACCGCATCA	AAGGTGAAGCCTAAGGTCTACT	473	$TG_{(91)}$
dnsr58	GGTAGGTTGAGTAGCTGAGAC	TCCCTAACAACAAACAGACATG	159	$TTTC_{(9)}$
dnsr92	CCAACAGAACTTGCAGGACTAG	CGACTCCACGGGACTACTTT	319	$AT_{(46)}$
dnsr95	CTTCTTCTCCTGAGCCTGTGA	TGCTGCTGCCCTTACTAAGT	344	$ATT_{(73)}$
dnsr98	TTTGTGCTCAGTTTGTGTTTCC	GAATCTCACGCCATCTCTGC	408	$TTA_{(77)}$

denaturation in 94°C for 5 minutes, denaturation in 94°C for 30 seconds, annealing in 55°C for 30 seconds, extension in 72°C for 25 seconds, and final extension in 72°C for 5 minutes. The PCR products were then separated in 1% agarose gel, electrophoresis setting of 100 volts for 30 minutes, and visualised under UV-transilluminator.

Data analysis

The agarose gel visualisations were scored manually. The scoring data were used to calculate the allele number, main allele frequency, and PIC. The PIC formula:

$$PIC=1-\sum_{i=1}^{l} P_i^2 - \sum_{i=1}^{l-1}\sum_{j=i+1}^{l} 2P_i^2 P_j^2$$

P*i* and P*j* are the population frequency of the *i*th and *j*th allele.

The dendrogram was constructed using NTSYS 2.1 software with the command of Sequential Agglomerative Hierarchical and Nested-Unweighted Pair-Group Method with Arithmetic (SAHN-UPGMA) (Rohlf 2000).

RESULTS AND DISCUSSION

Results

The PCR using 10 SSR-selected primers successfully amplified all the *D. crumenatum* sample tested. The primers detected as many as 45 alleles, ranging from 1 to 9 alleles with an average of 4.5 alleles (Table 2). Primer *dnsr95* and *dnsr98* showed the highest number of alleles (9 alleles) and highest number of polymorphic alleles (9 alleles; 100% polymorphic) (Figure 2), while primer *dnsr24*, *dnsr25*, and *dnsr55* were the lowest containing only one allele (0% polymorphic) and monomorphic. The average allele frequency was 0.67, the lowest was 0.51 in *dnsr28* and the highest were 1.00 in *dnsr24*, *dnsr25*, and *dnsr55*. The SSR used are dominant markers because the obtained alleles are more than 2, which is perfect for diversity analysis but may be inappropriate in progeny-parentage analysis.

The alleles from polymorphic SSR primers were used as a base for manual genotyping and phylogenetic tree construction using NTSYS 2.1 software. Based on the phylogenetic tree, the seventh primers can differentiate the samples into two big group (A and B) and totals of 4 sub-

Table 2. Polymorphism of 10 SSR primer pairs in gamma-irradiated *Dendrobium crumenatum* population.

Primer ID	No. of allele	No. of polymorphic allele	% allele polymorphic	Allele Freq.	PIC
dnsr9	7.00	7.00	100.00	0.79	0.33
dnsr17	6.00	6.00	100.00	0.59	0.48
dnsr24	1.00	0.00	0.00	1.00	0.00
dnsr25	1.00	0.00	0.00	1.00	0.00
dnsr28	3.00	3.00	100.00	0.51	0.50
dnsr55	1.00	0.00	0.00	1.00	0.00
dnsr58	3.00	3.00	100.00	0.96	0.08
dnsr92	5.00	5.00	100.00	0.57	0.49
dnsr95	9.00	9.00	100.00	0.76	0.36
dnsr98	9.00	9.00	100.00	0.55	0.50
Total	45.00	42.00			
Average	4.50	4.20		0.67	0.39

Note: PIC= polymorphic information content.

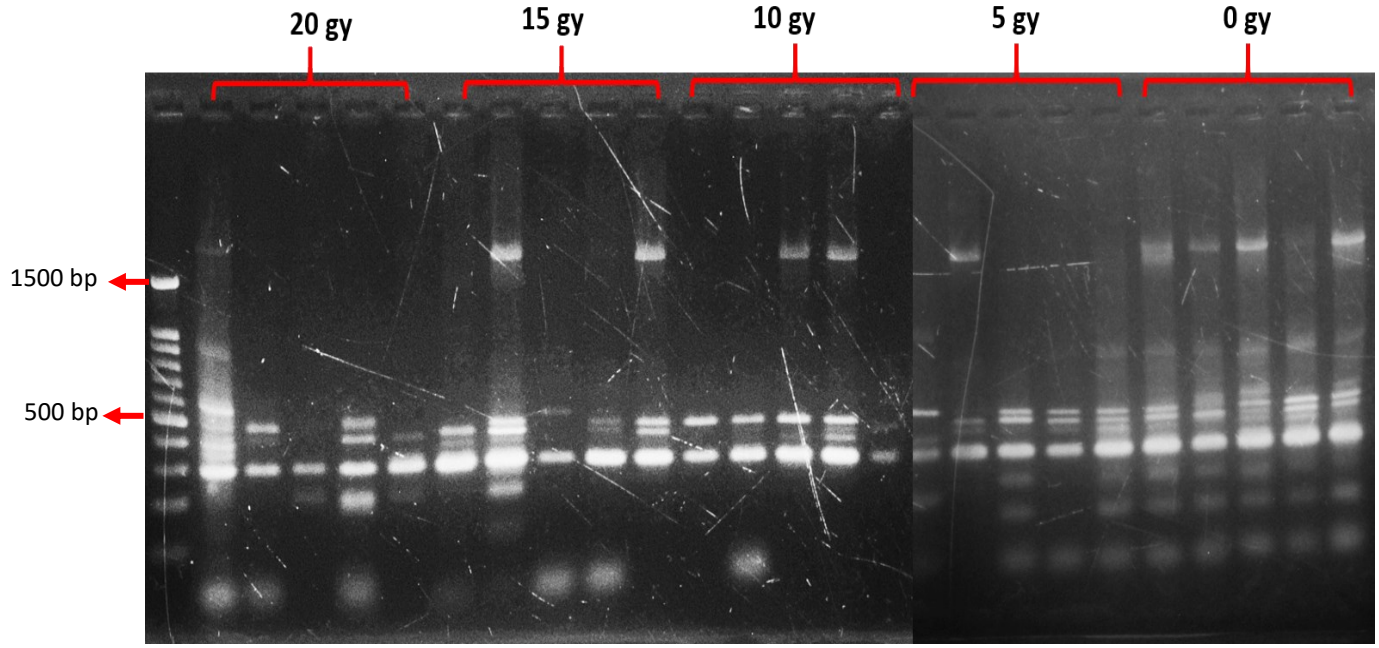

Figure 2. Gel visualisation of amplified PCR products from 25 gamma-irradiated *D. crumenatum* samples using primer *dnsr98* showed the highest polymorphism.

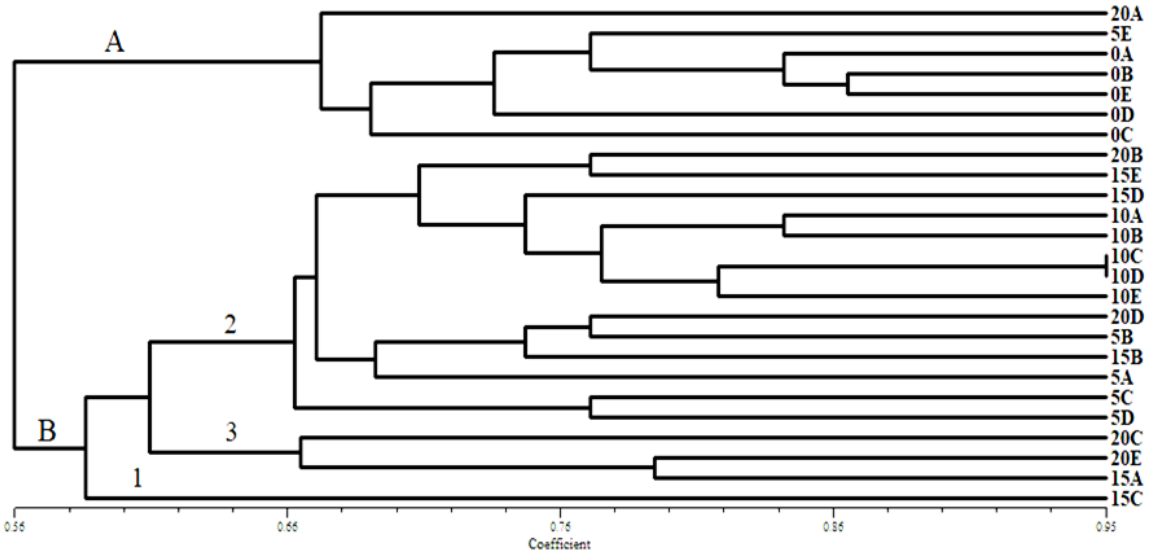

Figure 3. Phylogenetic from 25 individuals *D. crumenatum* Irradiated with five different doses differentiated with 7 SSR primers generated from *D. catenatum* genome sequence.

groups (A1, B1, B2, and B3) (Figure 3). The A1 subgroup consist of all control samples (0Gy; 0A-E), and two irradiated samples (5Gy; 5E and 20Gy; 20A), while the rest samples are scattered into group B1-3. The result indicates that the SSR motives of the samples in subgroup A1 having high similarity means that irradiated samples 5E and 20A are not putative mutants, while the other subgroups have more different SSR motives that resulting from the gamma irradiation means the other samples in subgroup B1-3 are mutants.

We also found that 18 of the 25 samples (72%) are mutants from the result of seven SSR primers screening in the gamma-irradiated samples. The highest mutant percentage, up to 100% were obtained in 10 and 15 Gy samples, while the 5 and 20 Gy samples showed 80% of the materials are mutants (Table 3). The data indicates that 10-15 Gy are effective in generating mutants from 3 months-old *D. crumenatum* protocroms.

Table 3. Mutant percentage determined using SSR primers.

Gama irradiation dose	Mutant with 7 primers
0 gray	0%
5 gray	80%
10 gray	100%
15 gray	100%
20 gray	80%

Discussion

D. crumenatum is an orchid species that is commonly found in Sumatra (Melinda et al. 2022), Yogyakarta (Semiarti et al. 2020), Bali (Darmawati et al. 2018) and spreads in many other areas in Indonesia. The characteristics of *D. crumenatum* are very small (< 20cm) plant height (from base to the tip of the flowering pseudobulb) and small flower (width < 3 cm) (De et al. 2015). Despite the species having potential traits like strong fragrance, multiple flowers, and ease of propagate, the flower has low or no economic value due to the small flower size and short blooming time (overnight) (Figure 4). Some traits modifications of the species may able to improve the species phenotype that may increase the economic value of the species. The long flowering time of the species is the main target to be improved.

Figure 4. *D. crumenatum* plant (left) and flower (right).

Breeding can improve the traits of the species. Mutation breeding through gamma irradiation considered as faster and efficient breeding method compared to cross-breeding or genetic engineering. Mutation breeding is considered more effective and time efficient (Udage 2021). Mutation using gamma irradiation was able to increase the flower phenotype variability in *Portulaca grandiflora* (Aisyah et al. 2022a), *Celosia cristata* (Simanjuntak et al. 2020), *Celosia argentea* var. *plumosa* (Aisyah et al. 2022b) *Echinacea purpurea* (Cahyaningsih et al. 2022), *Chrysanthemum morifolium* 'Donglinruixue' (Wang et al. 2020) and many plant species. Mutation breeding is known as a random induced mutation that can change one or several plant traits. Due to random mutation, it is also possible to obtain other potential mutant of *D. crumenatum* that may attract market interest.

Despite several advantages of mutation breeding, there is a limitation: mutation breeding always works with a vast population that needs to be maintained and selected. Early selection will narrow up the population number. In our chase, the only phenotypic change that we can observed 6 six months post-mutation was only the plant size. The plant size

may be caused by the treatment successfully changed the material genetics or just the epigenetic effect. The most effective method for early mutant determination is molecular screening. SSR is the common molecular marker used for mutant detection (Mansyur et al. 2019; Asadi et al. 2020; Arrufitasari et al. 2022; Vighneswaran et al. 2022). SSR markers are generated from repeat sequences that are primarily found in microsatellites of the chromosome that are relatively more conserved compared to p or q chromosome arms (Mason 2015) and can differentiate plants at inter-and intraspecific level (Zhao et al. 2019; Li et al. 2021; Guerrero et al. 2022; Pauldasan et al. 2022).

Published SSR markers generated from *D. catenatum* genome (Zhao et al. 2019) were used in this study but further optimisation is needed to be applied in the different species population. The screening result showed as many as seven markers are polymorphic and clustering the control material in one subgroup A1 (Figure 2) showed high sensitivity of the marker in mutant detection. Interestingly, two gamma-irradiated materials in dose of 5 (5E) and 20 Gy (20A) also grouped in subgroup A1 indicating both samples may not mutant. The SSR primers developed from *D. catenatum* genome scaffold has been published and tested in *D. catenatum*, *D. denneanum*, and *D. nobile* intraspecific population and showed highest PIC of 0.751, 0.681, and 0.626, respectively (Zhao et al. 2019). But lower PIC (the highest obtained in this research were only 0.5) obtained in this research possibly due to different sequence repeat between *D. crumenatum* and *D. catenatum* from Zhao et al. (2019) report. The material possibly has low diversity even after mutated.

Not many morphological characters can be observed in *in vitro* plantlets even six months post-mutation (nine months old *in vitro* culture from seed). No phenotypic trait was measured due to no distinct phenotype can be observed visually. In this stage, we focused on SSR genotyping result to determine the best primers in differentiating putative mutant. But, the most striking difference is the size of the plantlets which looks more dwarf when treated with higher gamma irradiation doses at 15-20 Gy (Figure 1). The most putative mutants from phenotypic observations based on plantlet size were obtained at 15-20 Gy. However, this is slightly different from what is obtained from SSR-genotyping data. That most mutants were obtained in 10-15 Gy.

Information from this research is important to us to verify that our gamma irradiation treatments were able to increase the variability of the *D. crumenatum* to molecular level and possibly to important commercial traits. The phenotypic of the obtained mutant materials needs to be further evaluated to determine the potential use of the materials. Besides the ornamental values, *D. crumenatum* was reported to have pharmacological effect in dermatological problems like boils and pimples (Wang 2021) also antimicrobial properties and several potential metabolites like saponin, terpenoid, and alkaloid compounds (Sandrasagaran et al. 2014). The strong flower fragrance can also be developed as raw material in perfumery.

CONCLUSION

The SSR marker generated from different species (*D. catenatum*) can differentiate intraspecific *D. crumenatum* population generated from gamma irradiation. Out of the 10 markers tested, three were monomorphic while the rest were polymorphic. All polymorphic markers were able in differentiating individual in the population but the *dnsr28* and *dnsr98* were more informative with the highest PIC value of 0.5. Diversity analysis by the similarity using the polymorphic markers

showed that the tested population is grouped into 2 big groups (A and B) and 4 subgroups (A1, B1, B2, and B3). All of the control samples (0 Gy) were clustered in A1 together with 5E and 20A, while the rest of the samples were scattered in group B. The polymorphic markers were informative to differentiate mutants and non-mutant material. The highest mutant percentage was obtained in 10-15 Gy gamma irradiation doses.

AUTHORS CONTRIBUTION

R.D. conducted the research and wrote the manuscript., I.P.W.S. supervised the manuscript., H.Y. and I.A.D.P., conducted data analysis and provided photographs., I.K.S. and P.K.M. recapitulated data, created tables, and formatted the manuscript., Y.F. conducted the research.

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CONFLICT OF INTEREST

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

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Research Article

Composition and Diversity of Dragonflies (Odonata) in Several Habitat Types in Lumajang Regency, East Java Province, Indonesia

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ABSTRACT
Lumajang is one of the regency in East Java Province that has various types of freshwater ecosystems and have great potential as habitats for various insects, especially dragonflies. Dragonflies are insects that mostly live aquatically, so their existence is highly dependent on the condition of aquatic ecosystems. This study aims to compare the composition and diversity of dragonfly species in various habitat types in Lumajang. The study was conducted in lentic and lotic ecosystems in Lumajang. The method used was the Visual Encounter Survey (VES) technique adapted from the sweeping net. The data analysis used to determine differences in dragonfly species composition was the Bray-Curtis similarity analysis, while diversity analysis was conducted using the Shannon-Wiener index. This study recorded 29 species from seven families, including seven endemic dragonfly species found only on several islands in Indonesia. In the analysis of the Shannon-Wiener diversity index, the results show that in all research locations have a value of H'= 1.07–2.11, where the Rice Field habitat is the location with the highest value among other locations, with a value of H' = 2.11. The similarity analysis of dragonfly species composition using Bray Curtis similarity showed that it was divided into three groups. The composition of dragonflies found in several habitats in Lumajang is different, which can be influenced by many factors such as site elevation, habitat type (lentic or lotic), and habitat condition, as well as several other factors such as microclimate and vegetation (related to food availability).

INTRODUCTION
Dragonflies are insects that belong to the order Odonata and experience incomplete metamorphosis with three stages, namely eggs, nymphs, and adults (O'Malley et al. 2020). Adult dragonflies can be found in terrestrial ecosystems (Choong et al. 2020), while eggs and nymphs can be found in aquatic ecosystems, especially freshwater ecosystems (Kietzka et al. 2021). Dragonflies spend most of their lives in the nymph stage in freshwater ecosystems (Choong et al. 2020), so their existence is highly dependent on water conditions (Tang et al. 2010; Susanto et al. 2023). Therefore, adult dragonflies can be found around freshwater ecosystems such as rivers, ponds, and lakes (Choong et al. 2020). The ideal habitat for adult dragonflies is in around aquatic ecosystems with good water quality and the presence of riparian and aquatic vegetation (Vilenica et al. 2020; Worthen et al. 2021; Cheri & Finn 2023).

Dragonflies are one of the insects that have sensitivity to change in environmental quality, so dragonflies (certain types) can be used as indicators of water quality. Some dragonfly species are very sensitive to environmental changes (Buczyński et al. 2020), thus dragonflies are suitable as bioindicator agents to measure the quality of an ecosystem (Aziz & Mohamed 2018). This is because the presence of dragonflies is disrupted if their natural habitat is disturbed or damaged (Buczyński et al. 2020; Susanto et al. 2023). Therefore, it is important to monitor dragonfly composition and diversity as early indicators in the assessment of changes in water quality (Koneri et al. 2022).

Research on dragonfly diversity in several habitat types in East Java Province has been conducted in several areas, including the diversity of Odonata along an altitudinal gradient in East Java Province (Leksono et al. 2017), Diversity of dragonflies on the natural reserve areas of Mt. Sigogor and Mt. Picis, Ponorogo Regency, which was carried out in different habitat types, namely the forest-dominated area and open area (Pranoto et al. 2019), diversity of dragonflies (Odonata) in various types of habitat, namely in ponds, reservoirs, rivers, and rice fields in Lakarsantri district, Surabaya (Susanto et al. 2023), Odonata diversity and composition using the ArcGIS in springs, waterfalls, and river habitats in Malang and Batu, East Java (Albab et al. 2019), and diversity and community structure of Odonata in large river and small stream habitats in the Selorejo waterfall area, Ponorogo (Susanto & Zulaikha 2021). Research on dragonflies in Lumajang has been conducted by Abdillah (2020), on several rivers in the Pasrujambe district, and Susanto and Abdillah (2019), who conducted research on Zygoptera diversity in lakes and rivers in Bromo Tengger Semeru National Park (TNBTS). Research on dragonflies in the Lumajang regency is still very limited, so it is necessary to conduct research on dragonflies in a wider area that includes several habitats and regions.

Lumajang is a regency in East Java Province, with land elevations ranging from 0 to >2000 m above sea level and various ecosystems. Lumajang is known to have many freshwater ecosystems such as lakes, rivers, streams, swamps, ponds, fish ponds, and dams (Lumajang Regional Government 2018). The Lumajang Regional Government (2018) states that the freshwater ecosystems in Lumajang are utilized by the surrounding communities to support life. Such as domestic water sources, irrigation of the agricultural sector, and industry, and can even be used as a source of income for tourism or cultivation. Human activities pose a significant threat to aquatic ecosystems (Williams et al. 2016), thus it is necessary to research the composition and diversity of dragonfly species in various habitat types in Lumajang to prevent ecosystem damage. Studies on the latest information on dragonfly biodiversity are also indispensable due to ecological dynamics (Hastomo et al. 2022). The study was conducted in various habitat types to determine the composition and diversity of dragonflies found in various habitats in Lumajang, East Java.

MATERIALS AND METHODS

Time and Location Study

The study was conducted from July to September 2022, with one repetition each month. In July, we conducted research on the 20th–29th, then in August on the 1st–10th, and finally in September on the 1st–10th. The research was conducted in lentic and lotic aquatic ecosystems at eight different locations in Lumajang regency, East Java Province (Figure 1). Rice field, pond, pond city park, and lake were lentic waters. While in lotic waters, among others, waterfalls, agriculture rivers, village rivers, and

forest rivers (Table 1). Sampling was carried out every time in sunny conditions, with observation time from 08.00 WIB to 16.00 WIB with a break from 12.00 to 13.30 WIB.

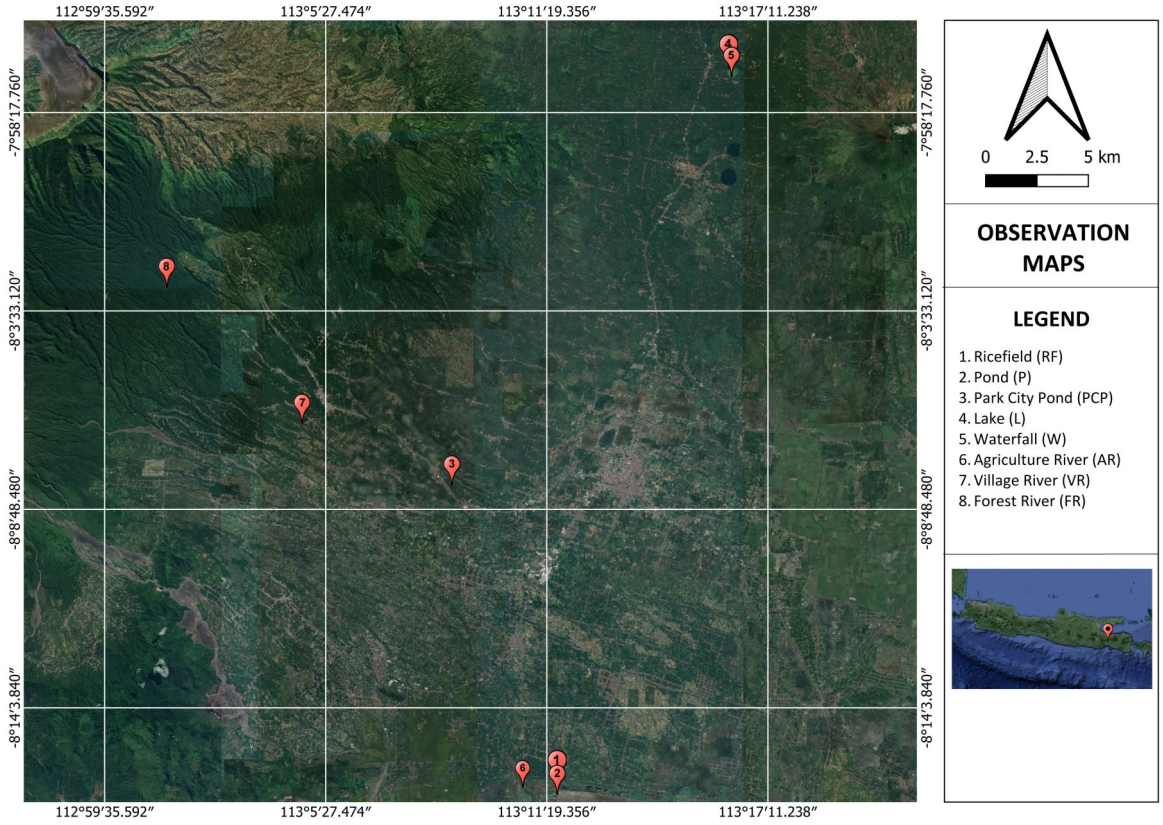

Figure 1. Map of the eight research sites in Lumajang.

Table 1. Description of the eight research sites in Lumajang.

Location	Coordinate Point		Elevation (masl)	Description
	Latitude	Longitude		
Ricefield (RF)	S8° 16' 6,4"	E113° 11' 37,1"	4–12	Lentic aquatic habitats in rice fields
Pond (P)	S8° 16' 22,5"	E113° 11' 36,5"	4–12	Lentic water habitats are utilized by the community as fishing grounds and irrigation sources
Park City Pond (PCP)	S8° 8' 5,8"	E113° 13' 25,1"	55–58	Lentic water habitats are used by the community as a place to travel
Lake (L)	S7° 56' 57,7"	E113° 16' 10,0"	167	Lentic water habitats are used by the community as tourist attractions and for fishing
Waterfall (W)	S7° 57' 9,9"	E113° 16' 14,7"	167–280	Lotic water habitats are utilized by the community as tourist attractions
Agriculture River (AR)	S8° 16' 11,0"	E113° 10' 41,3"	4–12	Lotic water habitat that is used by the community as a fishing spot and irrigation source
Village River (VR)	S8° 6' 26,9"	E113° 4' 49,2"	432	Lotic water habitats near residential areas
Forest River (FR)	S8° 2' 48,3"	E113° 1' 13,2"	1103–1420	Lotic water habitats are located in conservation forests that are utilized by the community as a water source.

Sampling Methods

Determination of the research path using the Transect method (Oppel 2006) and Belt Transect (Haritonov & Popova 2011). The Transect method was used to observe dragonflies by following a predetermined straight line. Meanwhile, the Belt Transect method is carried out by walking along a predetermined circular line. We used transection lines and transection belts with a size of 250 m in length and 4 m in width, with a total area of 1000 m^2. Data collection of adult dragonflies was conducted through direct observation using the Visual Encounter Survey (VES) technique adapted to the sweeping net. Observations began with capturing individuals of each species first using a sweeping net, and then individuals of the same species were observed visually. Then, to minimize potential bias in individual counts, we captured certain species that had high flight rates and then released them again at a later time after observation. Adult dragonfly data was collected by recording and counting the number of individuals of each species found. In addition, unidentified species were captured using insect nets, and each individual was documented in detail using a camera. Also, dragonflies found in the wild were documented for aesthetic purposes. Each individual collected or documented is identified at the species level. Dragonfly species identification is done by observing individual morphological characteristics such as body size, colour, pattern, wing venation, and umbilical shape. Identification was carried out using manual identification guides (Orr 2005; Orr & Kalkman 2015; Setiyono et al. 2017).

The research data collection also considered microclimate factors such as light intensity, air humidity, and temperature. Temperature and humidity factors were measured using a thermohygrometre, while light intensity was measured using a light meter. Vegetation data were also documented by identifying the dominant plant species present at the study site.

Data Analysis

The qualitative analysis was conducted by describing dragonflies to generate an interpretation of dragonflies and its habitat (Hastomo et al. 2022). The quantitative analysis was conducted through the analysis of obtained data for diversity using the Shannon-Wiener index (Metcalfe 1989), dominance index (Magurran 2004), relative abundance, and presence frequency. With the following formula:

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

Information:
H' = Shannon-Wiener diversity index
ni = Number of individuals of type i
N = Number of individuals of all types

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

Information:
D = Dominance index
ni = Number of individuals of type i
N = Number of individuals of all types

$$RA = \frac{ni}{N} \times 100\%$$

Information:
RA = Relative abundance
ni = Number of individuals of type i

N = Number of individuals of all types

$$PF = \frac{Total\ post\ found\ species\ i}{the\ total\ of\ posts} \times 100\%$$

Information:
PF = Presence frequency

Furthermore, a principal component analysis (PCA) and Canonical Correspondence Analysis (CCA) were conducted to understand the relationship between observation locations and abiotic factors (environment) as well as biotic indices (Koneri et al. 2022). To assess the similarity of dragonfly composition, the UPGMA clustering analysis method was employed using the Bray-Curtis index. The correlation between biotic and abiotic factors was analysed using Pearson's correlation. All analyses were performed using PAST (paleontological statistics) 4.03 software (Hammer 2001). The data on the composition and abundance of dragonflies is also used to calculate the important value index (IVI), which is used to assess the importance of species present at each location (Nguyen et al. 2014). The formula for calculating the important value index (IVI) is as follows IVI = RA + FR, where RA is relative abundance (%), FR is the relative frequency (%) (Febriansyah et al. 2022).

RESULTS AND DISCUSSION

The results of dragonfly observations at the eight research sites showed 29 species of dragonflies belonging to seven families (Table 2). In this study, the family with the highest number of species was the Libellulidae, with 14 species. The Libellulidae family is a family from the suborder Anisoptera that is commonly found. The Libellulidae family was found to have the highest number of species, which can be because this family has many members of species that have generalist properties or can be found in various habitat types. Koneri et al. (2022) are also mentioned that the Libellulidae family is a family that has excellent adaptability, so this family is very common in lentic and lotic water habitats. The Libellulidae family is a family that is very common in various lentic and lotic water habitats (Orr & Kalkman 2015). In this study, six species of the Libellulidae family were found in three or more habitat types, namely *Crocothemis servilia*, *Diplacodes trivialis*, *Orthetrum sabina*, *Pantala flavescens*, *Potamarcha congener*, and *Rhodothemis rufa* (Table 2).

The species with the highest presence frequency in this study were *Orthetrum sabina* with 87.5%, which was found in seven study sites and *Rhodothemis rufa* with 75%, which was found in six study sites (Table 2). *O. sabina* was found perched on aquatic vegetation or woody branches at the water's edge. In addition, *O. sabina* is also found in grass and shrub vegetation near or far from the water, thus *O. sabina* species include generalist species or those that can be found in various habitat types. This is in accordance with the research of Leksono et al. (2017) conducted at various altitudes in East Java Province, which found that *O. sabina* is the most dominant species in various locations and is very common. Another study from Susanto et al. (2023), who conducted research on various types of aquatic habitats in Lakarsantri district, Surabaya, found that *O. sabina* species were found in all observed aquatic habitats, namely rice fields, rivers, reservoirs, and ponds.

R. rufa was found perched on aquatic vegetation in the water, but also found on grass and shrub vegetation near the water. This is in accordance with Susanto et al. (2023), who reported that *R. rufa* was found perched on aquatic vegetation in aquatic habitats of ponds, reservoirs, and rice fields. In another study conducted by Chaudhry et al. (2015),

they also found *R. rufa* in grass vegetation around rivers, ponds, and swamps.

At the study site, seven endemic dragonfly species were found that are only found on several islands in Indonesia, or called multiple island endemics (Figure 2). The encounter of several endemic species in Lumajang is an important finding to discuss. This is because endemic species

Table 2. List of species found in the eight research sites in Lumajang.

No	Species	Family	Conservation status	PF (%)	Lentic				Lotic			
					RF	P	PCP	La	W	AR	VR	FR
1	*Paragomphus reinwardtii* (Selys, 1854)*	Gomphidae	LC	25.0	-	-	+	-	-	+	-	-
2	*Ictinogomphus decoratus* (Selys, 1854)	Gomphidae	LC	37.5	+	-	-	+	+	-	-	-
3	*Acisoma panorpoides* (Rambur, 1842)	Libellulidae	LC	12.5	+	-	-	-	-	-	-	-
4	*Brachythemis contaminata* (Fabricius, 1793)	Libellulidae	LC	37.5	+	+	-	+	-	-	-	-
5	*Crocothemis servilia* (Drury, 1770)	Libellulidae	LC	50.0	+	+	-	+	-	+	-	-
6	*Diplacodes trivialis* (Rambur, 1842)	Libellulidae	LC	37.5	+	-	+	+	-	-	-	-
7	*Neurothemis ramburii* (Brauer, 1866)	Libellulidae	LC	25.0	-	-	-	+	+	-	-	-
8	*Neurothemis terminata* (Ris, 1911)	Libellulidae	LC	25.0	-	-	-	+	+	-	-	-
9	*Orthetrum glaucum* (Brauer, 1865)	Libellulidae	LC	12.5	-	-	-	-	+	-	-	-
10	*Orthetrum pruinosum* (Burmeister, 1839)	Libellulidae	LC	25.0	-	-	-	-	+	-	-	+
11	*Orthetrum sabina* (Drury, 1770)	Libellulidae	LC	87.5	+	+	+	+	+	+	+	-
12	*Orthetrum testaceum* (Burmeister, 1839)	Libellulidae	LC	25.0	-	-	-	+	+	-	-	-
13	*Pantala flavescens* (Fabricius, 1798)	Libellulidae	LC	50.0	+	-	+	+	-	+	-	-
14	*Potamarcha congener* (Rambur, 1842)	Libellulidae	LC	37.5	+	+	+	-	-	-	-	-
15	*Rhodothemis rufa* (Rambur, 1842)	Libellulidae	LC	75.0	+	+	+	+	+	+	-	-
16	*Rhyothemis phyllis* (Sulzer, 1776)	Libellulidae	LC	12.5	-	+	-	-	-	-	-	-
17	*Vestalis luctuosa* (Burmeister, 1839)*	Calopterygidae	LC	25.0	-	-	-	-	+	-	+	-
18	*Rhinocypha anisoptera* (Selys, 1879)*	Chlorocyphidae	LC	25.0	-	-	-	-	+	-	-	+
19	*Heliocypha fenestrata* (Burmeister, 1839)*	Chlorocyphidae	LC	12.5	-	-	-	-	-	-	+	-
20	*Agriocnemis femina* (Brauer, 1868)	Coenagrionidae	LC	25.0	+	+	-	-	-	-	-	-
21	*Agriocnemis pygmaea* (Rambur, 1842)	Coenagrionidae	LC	12.5	+	-	-	-	-	-	-	-
22	*Ischnura senegalensis* (Rambur, 1842)	Coenagrionidae	LC	37.5	+	+	-	+	-	-	-	-
23	*Pseudagrion microcephalum* (Rambur, 1842)	Coenagrionidae	LC	25.0	+	-	-	+	-	-	-	-
24	*Pseudagrion pruinosum* (Burmeister, 1839)	Coenagrionidae	LC	25.0	-	-	-	-	-	+	+	-
25	*Pseudagrion rubriceps* (Selys, 1876)	Coenagrionidae	LC	12.5	-	-	-	-	-	+	-	-
26	*Euphaea variegata* (Rambur, 1842)*	Euphaeidae	LC	25.0	-	-	-	-	+	-	+	-
27	*Coeliccia membranipes* (Rambur, 1842)*	Platycnemididae	LC	25.0	-	-	-	-	-	-	+	+
28	*Copera marginipes* (Rambur, 1842)	Platycnemididae	LC	25.0	-	-	-	-	-	+	+	-
29	*Nosoticta insignis* (Selys, 1886)*	Platycnemididae	LC	25.0	-	-	-	-	+	-	+	-

Note: (*) Endemic species, (+) Present, (-) Absence. PF= Presence frequency. Status conservation: LC= Least Concern (IUCN 2023)

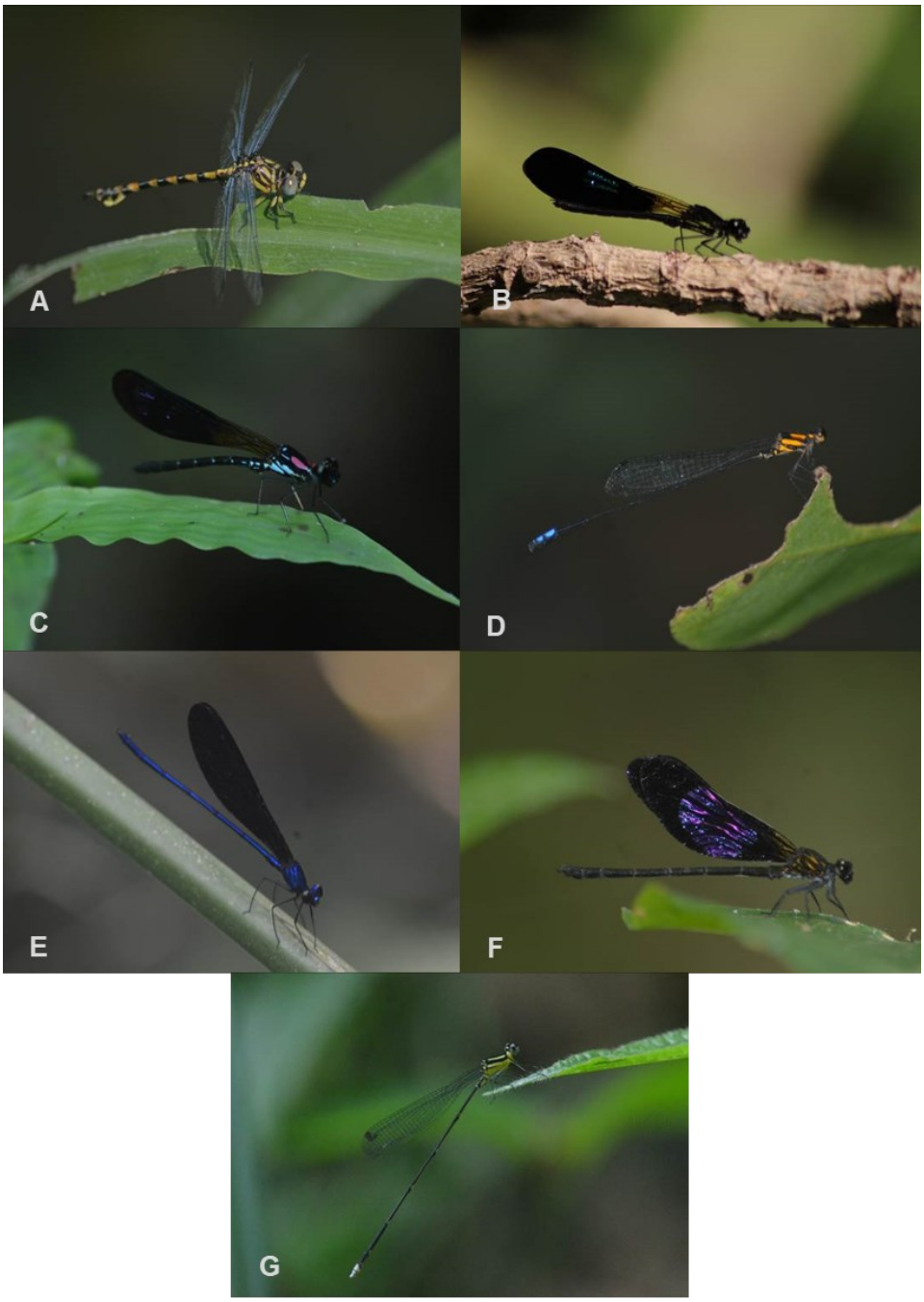

Figure 2. Several of the endemic species in the eight research sites in Lumajang, East Java Province. A. *Paragomphus reinwardtii*, B. *Rhinocypha anisoptera*, C. *Heliocypha fenestrata*, D. *Nosoticta insignis* E. *Vestalis luctuosa*, F. *Euphaea variegata*, G. *Coeliccia membranipes*. (Photo: M. Azmi Dwi Susanto 2022).

can only be found on certain islands or regions, so meeting endemic species in their natural habitat can be useful in knowing suitable habitats that can be useful in future conservation efforts.

Paragomphus reinwardtii (Figure 2-A) is a species that can only be found in Java and Bali (Dow et al. 2022). *P. reinwardtii* species were found roosting in riparian vegetation with open to slightly closed canopy conditions and many trees in urban park ponds and agricultural rivers. *P. reinwardtii* found in agricultural rivers are teneral individuals, individuals that have just molted from the naiad to adult phase, so agricultural rivers are likely habitats for *P. reinwardtii* to lay eggs and become nymphs. This is consistent with Dow et al. (2022) who reported that *P. reinwardtii* species can be found in waters in agricultural areas or rice fields, and this species has a tolerance to low levels of environmental pollution.

Rhinocypha anisoptera (Figure 2-B) and *Heliocypha fenestrata* (Figure

2-C) are members of the Chlorocyphidae family and are endemic species. *R. anisoptera* can only be found in Java and Sumatra (Günther 2019b), while *H. fenestrata* can only be found in Java and Bali (Günther 2019a) In this study, *R. anisoptera* was found perched on twigs and tree branches at the edge of forest waters, namely at the location of waterfalls and forest rivers. This is in accordance with Abdillah & Lupiyaningdyah (2020), who reported that *R. anisoptera* can be found perched on twigs or tree branches on the banks of forest rivers. Meanwhile, *H. fenestrata* was found perched on riparian vegetation and rocks on the banks or middle of the river in open canopy conditions at the village river location. Susanto & Bahri (2021), are also mentioned that *H. fenestrata* can be found in forest rivers with open canopies.

Nosoticta insignis (Figure 2-D) is a species that has a distribution in Java, Sumatra, and Bali (Dow 2019c). *N. insignis* was found perched on slightly dense vegetation with a slightly closed canopy near the water at the waterfall location. This is in accordance with the research of Dharmawan et al. (2022), which reported that *N. insignis* was found in the location of Alas Purwo National Park with a fairly high humidity of 72% and a fairly low light intensity of 596 Lux. In addition, the research of Rachman & Rohman (2016) also found *N. insignis* in the waterfall area in secondary forest.

Based on the conservation status assessment from IUCN (2023), three endemic dragonfly species found in this study have declining populations, namely *V. luctuosa* (Figure 2-E), *E. variegata* (Figure 2-F), and *C. membranipes* (Figure 2-G). *V. luctuosa* and *E. variegata* are species that can only be found in Java, Sumatra, and Bali (Dow 2019b; Dow 2019d). *V. luctuosa* was found to spend more time perching on riparian vegetation and tree branches on riverbanks with slightly closed to open canopy conditions. In addition, *V. luctuosa* was also found perched on rocks in the middle or on the banks of the river. This is consistent with Aswari (2004), who reported that *V. luctuosa* is not very active in flight and more often perches on vegetation with closed to open canopy conditions in flowing waters. *V. luctuosa* species are often found perched on vegetation on riverbanks with open or closed canopy conditions (Susanto & Bahri 2021; Nafisah & Soesilohadi 2021).

E. variegata and *V. luctuosa* were found in almost the same habitat conditions, and also both species were only found in the same two places, namely the waterfall and village river locations. *E. variegata* has a similar habitat to *V. luctuosa* (Abdillah & Lupiyaningdyah 2020; Nafisah & Soesilohadi 2021; Susanto & Bahri 2021). *E. variegata* is found perched on riparian vegetation, tree branches, and rocks on riverbanks. There were the same results on Susanto and Zulaikha (2021), who found *E. variegata* in streams with open and closed canopy conditions. *E. variegata* is more often found perched on rocks in the middle or banks of rivers under open canopy conditions (Nafisah & Soesilohadi 2021). *C. membranipes* is a species that can only be found in Java and Sumatra (Dow 2019a). *C. membranipes* was found perched on fairly dense riparian vegetation with closed to slightly open canopy conditions. Susanto and Zulaikha (2021), are also founded *C. membranipes* in waters with fairly dense riparian vegetation and closed canopy conditions.

The results of the analysis of the species richness value at the study site showed that the Rice Field location had the highest species richness value, with 13 species (Figure 3). The high species richness value at the Rice Field location can be attributed to the fact that this location is stagnant water with various types of vegetation, and there are various types of abundant small insects that have the potential to become a food source

for dragonflies. In addition, the rice field location is in the lowlands, so it can be a habitat for various types of dragonflies, especially generalist dragonflies. In rice fields dominated by rice plants, there are many small insects that damage agricultural crops or are called pests (Oo et al. 2020; Wakhid et al. 2020). These pest insects have great potential to be a source of food for various types of dragonflies. This is also supported by Suroto et al. (2021), who reported that dragonflies are one of the insects that are often found and have an important role in the rice field ecosystem, namely acting as predators of insect pests such as leafhoppers.

The results of the abundance analysis at the research site showed that the lake location had the highest abundance of 338 individuals (Figure 3). The high abundance of dragonflies at the lake location could be due to the fact that this location is surrounded by lowland community forests, so at the lake location there is minimal human activity and there are not many changes in environmental quality. At the lake location, there are two species that have high abundance, namely *N. ramburii* with 109 individuals and *O. sabina* with 70 individuals.

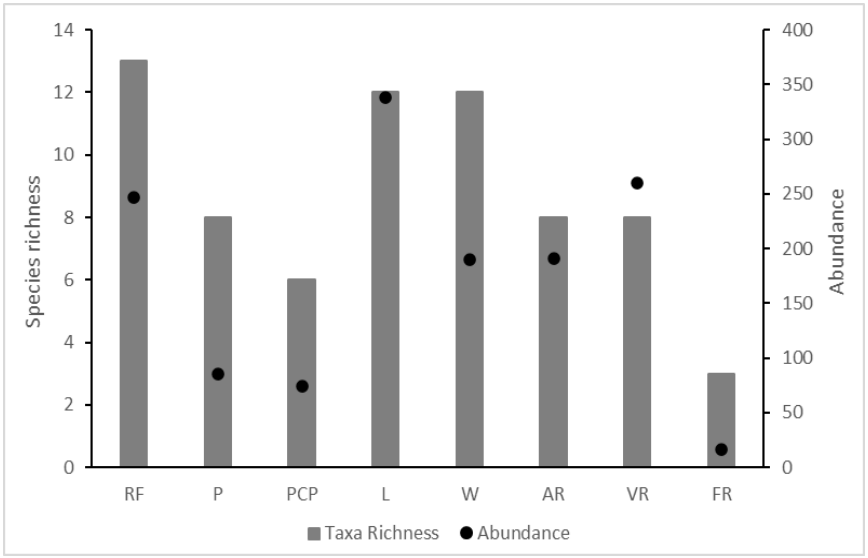

Figure 3. Species richness and abundance of dragonflies in the eight research sites in Lumajang, East Java Province. Note RF: Ricefield, P: Pond, PCP: Park City Pond, L: Lake, W: Waterfall, AR: Agriculture River, VR: Village River, & FR: Forest River.

Forest river is the location with the lowest species richness and abundance, with only three species, namely *Orthetrum pruinosum*, *Rhinocypha anisoptera*, and *Coeliccia membranipes* with a total of 16 individuals. The research location in the highlands, with dense vegetation and closed canopy conditions, can be one of the factors that cause low diversity values at this location. This is supported by Kinvig & Samways (2000), who reported that a dense canopy (especially invasive vegetation) can be a factor in reducing dragonfly diversity. The study by Briggs et al. (2019) also assumed that canopy cover could be the cause of low dragonfly diversity. If the canopy conditions are closed, the sunlight intensity will be blocked and the humidity will be higher, so it is not optimal for dragonflies to use it for basking. Humidity measurement results showed that the forest river location has the highest average humidity of all observation locations.

Grouping locations based on the similarity of dragonfly composition can be useful in analysing the similarity of habitat conditions so that it becomes a suitable habitat for the life of certain dragonfly species. In addition, it can also be useful in determining the preferences of dragonfly

species in choosing habitat types. The analysis of the Bray-Curtis similarity index showed that the composition of dragonfly species between research locations is in three groups (Figure 4).

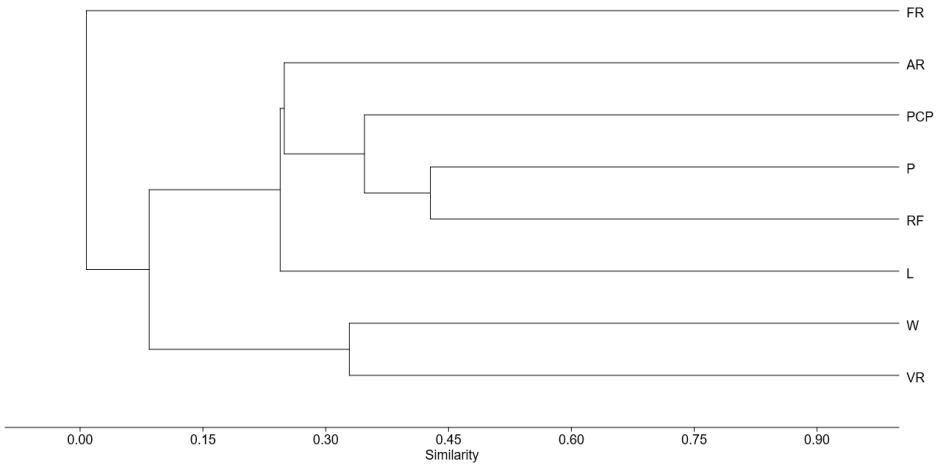

Figure 4. Similarity of dragonfly composition of dragonflies in the eight research sites in Lumajang, East Java Province. Note: RF: ricefield, P: pond, PCP: park city pond, L: lake, W: waterfall, AR: agriculture river, VR: village river, & FR: forest river.

In group one, the waterfall and village river locations have similar water types, namely lotic waters. In this location, there were four species found in both locations, namely *Orthetrum sabina*, *Vestalis luctuosa*, *Euphaea variegata*, and *Nososticta insignis*. In group two, namely rice field, pond, park city pond, agriculture river, and lake, most of which are lentic waters (only the agriculture river location is lotic) located in the lowlands, the composition of these five locations is mostly generalist species. The dragonfly composition at the five sites in this group is most likely also due to the elevation factor, where all sites are in lowlands, with four sites below 100 msal and one site, Lake, below 200 msal. In group three, forest river has the lowest similarity value, which indicates a different composition from other locations. This can be due to the forest river location in the highlands and in secondary forests, so the composition of dragonfly species in this location has the least number of three species.

The results of the dominance index analysis showed that six locations have a dominance index value in the low category, which has a value below 0.40, namely the location of the rice field, pond, park city pond, lake, waterfall, and village river. And there are two locations that have a medium category, with a value of D = 0.40–0.60, namely the location of the forest river D = 0.49, and the agriculture river D = 0.47 (Figure 6). The high dominance value at the forest river location can be explained by the fact that at this location only three species were found, each of which had a high IVI value, namely *Rhinocypha anisoptera* 111.61%, *Orthetrum pruinosum* 47.32%, and *Coeliccia membranipes* 41.07% (Figure 5). Whereas in the agriculture river location, which has the second highest dominance index value after the forest river, there are two codominant species with high IVI values, namely *Pseudagrion pruinosum* (83.35%) and *Orthetrum sabina* (46.18%).

High dominance at the forest river site does not necessarily indicate a degraded habitat, but it can indicate a shift in habitat structure and composition due to environmental conditions and quality. Although the forest river had the highest dominance index value, two of the three species found were endemic. This can be due to the condition of the forest river location which is located at an altitude of 1103–1420 masl with

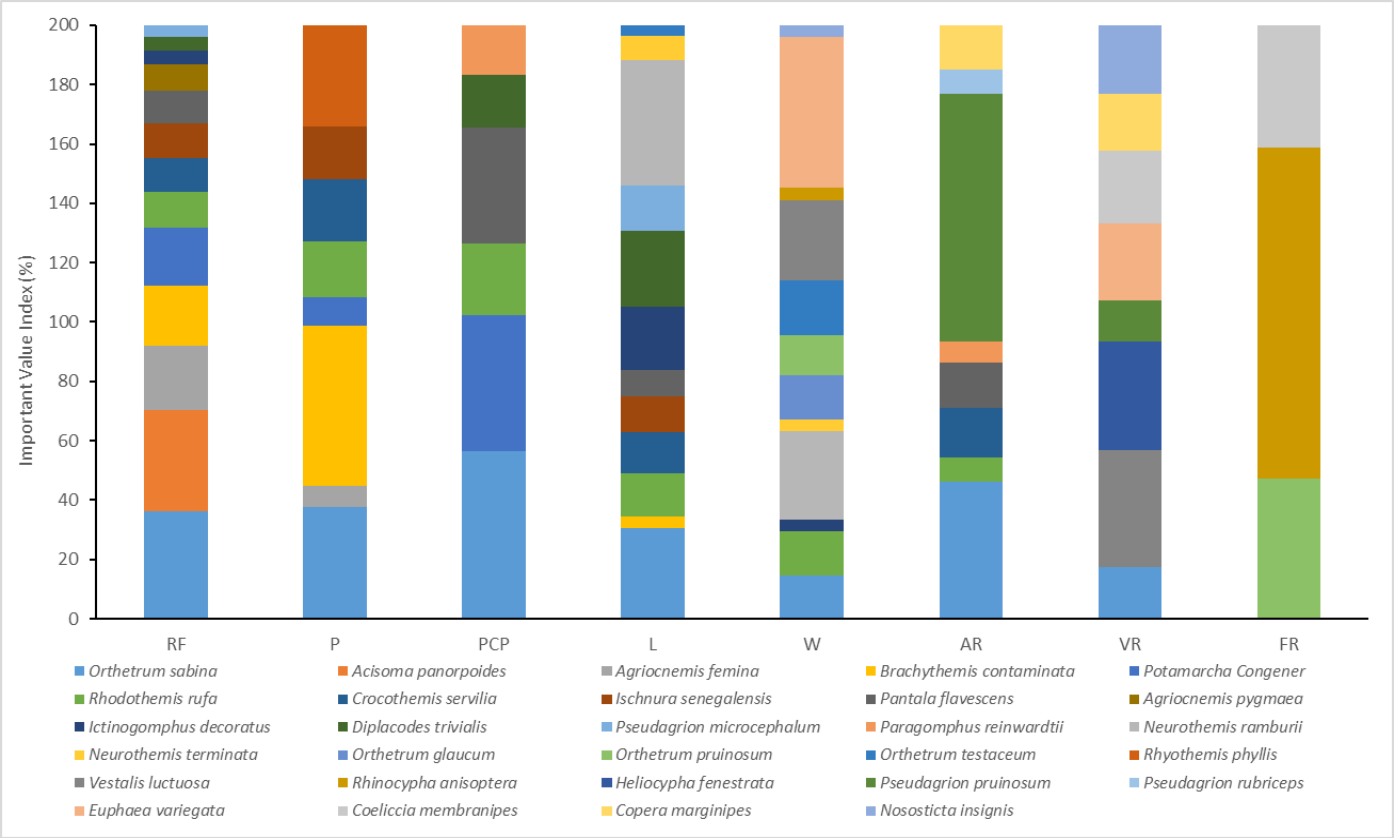

Figure 5. Important Value Index (IVI) of dragonflies in the eight research sites in Lumajang, East Java Province. Note: RF: ricefield, P: pond, PCP: park city pond, L: lake, W: waterfall, AR: agriculture river, VR: village river, & FR: forest river

closed canopy conditions and has the highest humidity in this study, so that only a few species can live in such conditions.

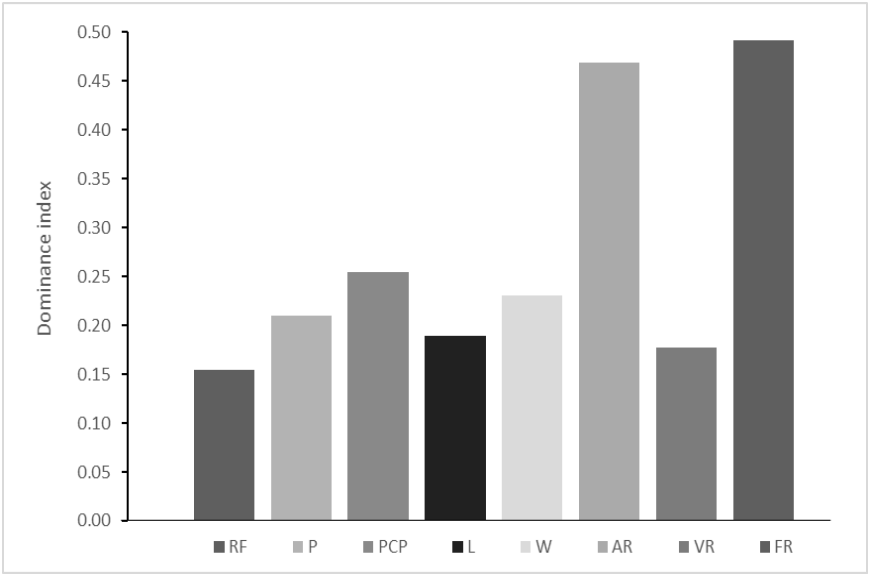

Figure 6. Value of dominance index of dragonflies in the eight research sites in Lumajang, East Java Province. Note: RF: Ricefield, P: Pond, PCP: Park City Pond, L: Lake, W: Waterfall, AR: Agriculture River, VR: Village River, & FR: Forest.

Based on the results of the Shannon-Wiener diversity index analysis, there are two locations that have a low category value, with a value below 1.50, namely the location of the agricultural river and the forest river. And there are six locations that have a value in the medium catego-

ry, namely with a value of H'= 1.50–3.00, namely the location of the rice field, pond, park city pond, lake, waterfall, and village river (Figure 7). The rice field is the location with the highest diversity value in this study (H = 2.11), but not an endemic species was found in this location. Most of the species found in this location are generalist species, namely species that have tolerance to various environmental conditions so that they can be found in various habitat types. The high value of diversity in the rice field location may also be due to the presence of various insects (especially pests) in the agricultural location. The presence of dragonflies in rice field locations can be useful as natural predators of various pest insects (Wakhid et al. 2020).

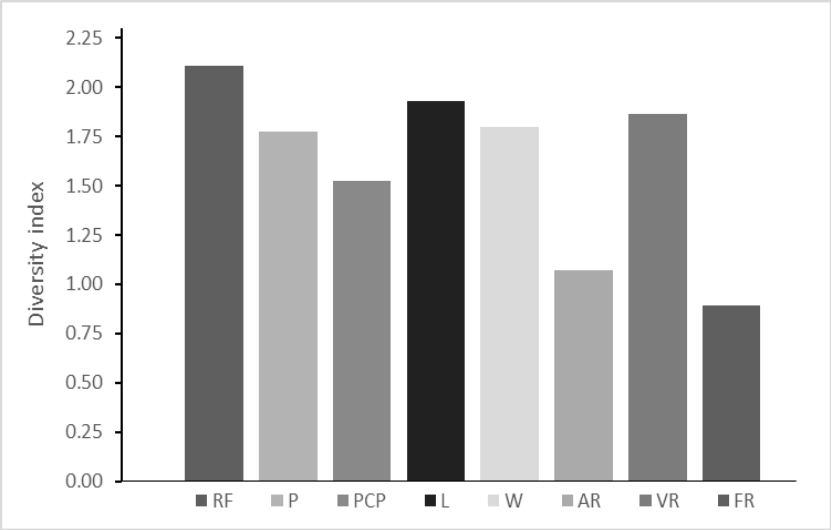

Figure 7. Value of Shannon-Wiener Diversity Index of dragonflies in the eight research sites in Lumajang, East Java Province. RF: Ricefield, P: Pond, PCP: Park City Pond, L: Lake, W: Waterfall, AR: Agriculture River, VR: Village River, & FR: Forest.

The difference in diversity index values at each research location can be due to each location having different habitat types (Table 1), elevations (Table 1), and vegetation composition (Table 3). In addition, differences in microclimate at each location (Table 4), can also be a factor that affects the value of the dragonfly species diversity index at each research location.

Water habitat type is one of the main factors that cause differences in dragonfly diversity (Figure 7). The presence of water in a location is the most important factor in the presence and abundance of dragonflies (Luke et al. 2020). Dragonflies are highly dependent on water for egg-laying and larval development (Clausnitzer et al. 2009). In the larval stage, the presence of water is a very important factor, when water discharge decreases or experiences scarcity, larvae will also decrease (Luke et al. 2020). The results showed that the two locations that had the highest diversity value were in lentic water habitats (rice fields and lakes), while the lowest diversity value was in lotic habitats (agriculture rivers and forest rivers) (Figure 7). Lentic water types tend to have more stable fluctuations in dragonfly composition because lentic waters do not experience extreme changes due to water currents. Meanwhile, lotic water types tend to have a more volatile dragonfly species composition because they are highly affected by water currents.

Differences in elevation at the study site can also be the cause of the different composition and diversity of dragonfly species found. The results showed that the location with the highest diversity value was in the lowlands, namely with an elevation of 4–12 msal. This can be because

each elevation has specific environmental conditions. The composition and diversity of dragonfly species in an area can be influenced by landscape conditions such as elevation (Simaika et al. 2016). Lowland sites tend to be more diverse, but many generalist species can live in various habitat types. According to Mafuwe & Moyo (2020) lowland areas generally have higher dragonfly species diversity than highland locations.

Differences in vegetation composition at each study site (Table 3) may be one of the main factors influencing dragonfly diversity. The presence of riparian vegetation in the form of herbs or trees at the study sites can be a component that supports adult dragonfly activities such as roosting, basking, and protecting from predators. The presence of vegetation on the water's edge has a positive correlation with dragonfly abundance and diversity (Deacon et al. 2019; Worthen et al. 2021). In addition, riparian vegetation is also a habitat for small insects that have the potential to become dragonfly food. This is because vegetation is one of the main habitat components of small insects that become dragonfly food resources (Knuff et al. 2020; New et al. 2021). Therefore, the absence of vegetation is also one of the factors that limits the presence of dragonflies (Luke et al. 2020). The presence of aquatic vegetation in aquatic sites can be a component that supports the activities of adult dragonflies (perching

Table 3. List of plant species present in the eight research sites in Lumajang, East Java Province.

No.	Type	Plant Species	RF	P	PCP	L	W	AR	VR	FR
1	Herb	*Pennisetum purpureum*	++	+	-	++	+	++	+	-
2	Herb	*Digitaria ciliaris*	++	+	-	-	-	+	-	-
3	Herb	*Colocasia esculenta*	++	+	-	-	-	+	-	-
4	Herb	*Eupatorium* sp.	-	-	++	-	-	-	-	-
5	Herb	*Ruellia* sp.	-	-	+	-	-	-	-	-
6	Herb	*Axonopus* sp.	-	-	++	-	-	-	-	-
7	Herb	*Alternanthera* sp.	-	-	-	+	++	-	-	-
8	Herb	*Synedrella* sp.	-	-	-	+	+	-	-	-
9	Herb	*Ageratina riparia*	-	-	-	+	++	-	+	++
10	Herb	*Tridax* sp.	+	+	-	++	+	+	-	-
11	Herb	*Impatiens* sp.	-	-	-	-	-	-	-	++
12	Herb	*Laportea* sp.	-	-	-	-	-	-	-	+
13	Herb	*Colocasia* sp.	-	-	-	-	-	-	++	-
14	Herb	Bambusoideae	-	-	-	+	+	-	++	+
15	Tree	*Muntingia calabura*	+	+	-	+	+	++	-	-
16	Tree	*Albizia chinensis*	+	+	-	-	++	+	-	+
17	Tree	*Cocos* sp.	++	+	-	-	-	+	-	-
18	Tree	*Hibiscus tiliaceus*	+	+	-	+	+	+	-	-
19	Tree	*Ficus* sp.	-	-	+	+	+	-	-	-
20	Tree	*Syzygium oleina*	-	-	+	-	-	-	-	-
21	Tree	*Cupressus* sp.	-	-	+	-	-	-	-	-
22	Tree	*Pterocarpus indicus*	-	-	++	-	-	-	-	-
23	Tree	*Manilkara* sp.	-	-	+	-	-	-	-	-
24	Tree	*Toona sureni*	-	-	-	-	-	-	-	+
25	Tree	*Ficus racemosa*	-	-	-	-	+	-	-	++
26	Tree	*Swietenia mahagoni*	-	-	-	-	-	-	-	++
27	Tree	*Ficus retusa*	-	-	-	-	-	-	-	+
28	Aquatic Plant	*Limnocharis flava*	++	-	-	-	-	+	-	-
29	Aquatic Plant	*Pistia stratiotes*	-	+	-	+	-	-	-	-
30	Aquatic Plant	*Eichhornia crassipes*	-	+	-	-	-	-	-	-
31	Aquatic Plant	*Ipomea aquatica*	++	+	-	+	-	-	-	-

Note: (+) Present, (-) Absence, (++) Dominance. Note: RF: ricefield, P: pond, PCP: park city pond, L: lake, W: waterfall, AR: agriculture river, VR: village river, & FR: forest river

Table 4. Microclimate values and PCA scores in the eight research sites in Lumajang. East Java Province.

Location	Microclimate				PCA Score	
	Temperature (°C)	Humidity (%)	Wind (m/s)	Light (lx)	PC 1	PC 2
RF1	35	52	0.7	44200	1.712	2.111
RF2	32.7	55	0.1	41000	1.066	0.602
RF3	28.1	54	0.1	38100	1.109	-1.014
P1	32.7	50	0.9	52100	-1.296	1.250
P2	30.6	54	1.4	48600	-0.344	1.053
P3	31.6	53	0.7	46600	-0.096	0.763
PCP1	34.2	54	2.8	51600	-2.175	3.249
PCP2	32.5	50	0.1	32800	-0.272	-0.014
PCP3	31.1	56	0.5	39200	-0.142	0.154
L1	32.2	54	1.2	32300	2.149	1.363
L2	32.1	56	0.1	34100	1.292	0.165
L3	32.4	55	0.1	33100	2.008	0.407
W1	30.1	57	0.1	5400	1.691	-1.356
W2	31.9	56	0.1	1900	1.603	-0.909
W3	30.8	56	0.1	3200	1.075	-1.294
AR1	30.5	57	1.1	44000	-2.344	0.173
AR2	30.7	51	0.1	29900	-1.074	-0.909
AR3	32.8	51	0.7	55600	-2.092	0.965
VR1	30.2	56	0.1	7400	1.599	-1.216
VR2	32.4	57	0.1	8700	1.726	-0.426
VR3	31	55	0.1	6500	1.816	-0.933
FR1	27.1	57	0.1	11400	-2.516	-2.936
FR2	29.7	56	0.1	34500	-3.983	-1.688
FR3	33.7	67	0.1	48600	-2.512	0.437

Note: RF: Ricefield, P: Pond, PCP: Park City Pond, L: Lake, W: Waterfall, AR: Agriculture River, VR: Village River, & FR: Forest.

and basking) and dragonfly nymphs (shelter from predators). Deacon et al. (2019) are also reported that dragonfly nymphs utilise aquatic vegetation to hide from predators and hunt prey.

PCA ordination analysis showed the variation of environmental factors in the eight study sites with different aquatic habitats (Figure 8). The results of the analysis obtained showed that there are four distinct groups, namely the first group of lakes and rice fields, which are characterized by high values of abundance, diversity index, and species richness. The second group includes waterfall and village river locations that have adjacent and overlapping plots. According to Koneri et al. (2022), adjacent and overlapping plots indicate that the environmental characteristics between locations have many similarities. The third group of pond and park city pond locations have adjacent and overlapping plots, which are characterized by high values of light intensity and wind speed factors. The fourth group is the agriculture river and forest river locations, which are characterised by high dominance values.

The results of the PCA analysis showed that the first and second axes of the data accounted for 47.949 and 26.114% of the variation in the data, respectively. The environmental variables that contribute most to the diversity and dominance indices are light intensity and temperature. The light intensity variable has a negative correlation with the diversity index, dominance, and species richness, while temperature has a negative correlation with the dominance index.

CCA analysis showed that each dragonfly species had different responses to the microclimate (Figure 9). There are 10 species that have high abundance in locations with high temperatures and low humidity,

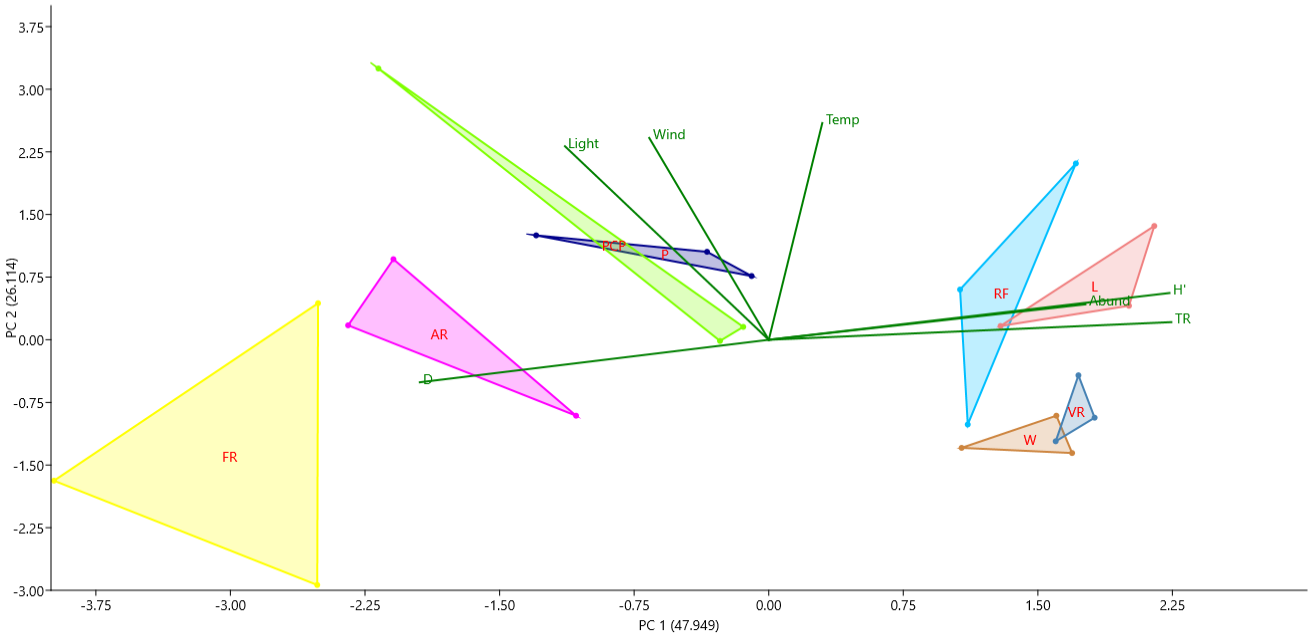

Figure 8. Ordination diagram of the Principal Component Analysis (PCA) in the eight research sites in Lumajang, East Java Province. Note: Hum: humidity, Temp: temperature, Light: light intensity. Location: RF: Ricefield, P: Pond, PCP: Park City Pond, L: Lake, W: Waterfall, AR: Agriculture River, VR: Village River, & FR: Forest.

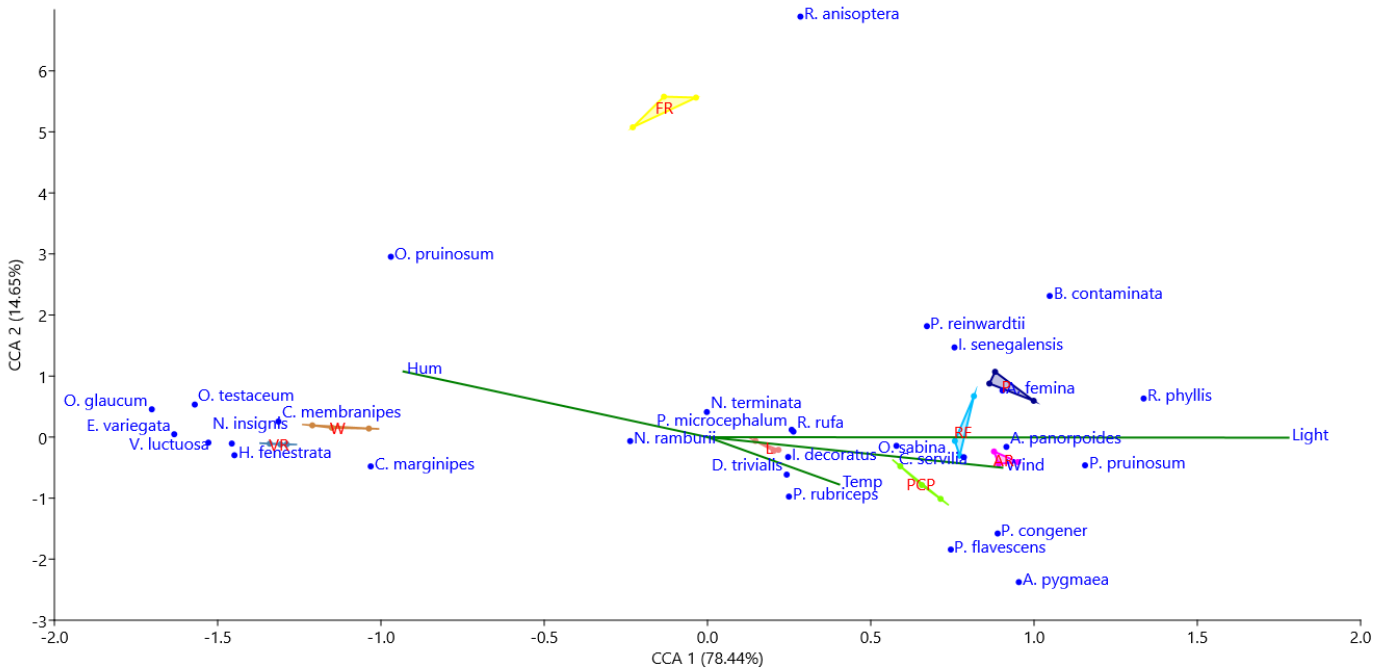

Figure 9. Ordination diagram of the Canonical Correspondence Analysis (CCA) in the eight research sites in Lumajang. Note: Hum: Humidity, Temp: Temperature, Light: Light intensity. Location: RF: Ricefield, P: Pond, PCP: Park City Pond, L: Lake, W: Waterfall, AR: Agriculture River, VR: Village River, & FR: Forest River

namely *A. pymaea*, *P. congener*, *P. flavescens*, *P. rubriceps*, *I. decoratus*, *D. trivialis*, *C. servilia*, *O. sabina*, *A. panorpoides*, and *P. pruinosum*. This is supported by the research of Susanto et al. (2023), who in their research also found 9 of the 10 species (except *P. pruinosum*) in urban areas that have a fairly high temperature of 32.40 to 35.61 °C.

In a different distribution, there were nine species that responded positively to locations with high humidity and low light intensity, namely *O. glaucum*, *O. testaceum*, *O. pruinosum*, *C. membranipes*, *C. marginipes*, *N.*

insignis, *H. fenestrata*, *E. variegata*, and *V. luctuosa*. This is in accordance with the research of Susanto & Zulaikha (2021) which reported that *O. glaucum*, *O. pruinosum*, *C. membranipes*, *E. variegata*, and *V. luctuosa* species were found in locations with high humidity of 74.6 to 75%. However, these results differ from the research of Nafisah and Soesilohadi (2021) who reported that CCA analysis of odonate communities and environmental factors in Petungkriyono forest, Central Java, showed that *O. pruinosum*, *O. glaucum*, and *C. membranipes* had a negative response to humidity and responded positively to high light intensity. This could be due to the different habitat conditions in the two locations, so it can be assumed that *O. pruinosum*, *O. glaucum*, and *C. membranipes* species can be found in areas with low to high humidity and light intensity values. The CCA analysis also showed that *E. variegata* and *V. luctuosa* had similar responses to microclimate, suggesting that these two species have similar habitat preferences.

Revision of species identification

In a study by Susanto et al. (2023), in Lakarsanti District, Surabaya, East Java Province, the damselfly species was identified as *Pseudagrion nigrofasciatum* (Lieftinck, 1934). The species *P. nigrofasciatum* was found in pond habitats near settlements. This is still in accordance with the report from Setiyono et al. (2017) who reported that *P. nigrofasciatum* can be found in residential habitats, fish ponds, rivers, and rice fields. However, the unclear documentation of *P. nigrofasciatum* and not taking specimens make identification complicated. Therefore, the species identification of *P. nigrofasciatum* in by Susanto et al. (2023) needs to be corrected and revised. Therefore, we conducted repeat observations to confirm the validity of the species identification and found two species of the genus *Pseudagrion*, namely *Pseudagrion microcephalum* (Rambur, 1842) (Figure 10) and *Pseudagrion rubriceps* (Selys, 1876).

Figure 10. Documentation of male *P. microcephalum* species during re-observation in Lakarsanti, Surabaya.

The species *P. nigrofasciatum* has a very similar morphology to *P. microcephalum* (Setiyono et al. 2017). Both species have differences in the dorsal part of the 2nd segment; *P. microcephalum* has a narrower black pattern compared to *P. nigrofasciatum*, which has a broad black pattern on the dorsal (Setiyono et al. 2017). After re-observing dragonflies in Lak-

arsanti district and surrounding areas that still have similar habitats as in Karangpilang and Gununganyar Districts, Surabaya City, the identification results showed that only *P. microcephalum* species were found. Therefore, we revised the species identification of *P. nigrofasciatum* in by Susanto et al. (2023) to the species *P. microcephalum*.

CONCLUSIONS

The dragonfly species in several habitat types in Lumajang recorded 29 species from seven families. The composition and diversity of dragonfly species in some types of habitats in Lumajang are different, especially in lotic and lentic water habitats. The composition of species based on the Bray-Curtis similarity index shows that lotic and lentic waters have different dragonfly compositions (except for the agricultural river, which is included in the lentic group). Most of the study sites had a moderate Shannon-Wiener diversity index value (H' = 1.50–3.00), except for two sites: agricultural rivers and forest rivers, which have low values (H'<1.50). Based on the CCA analysis, five out of a total of seven endemic species responded positively to high humidity and negatively to too-high light intensity. Therefore, human activities that have the potential to change humidity and light intensity, such as the illegal logging of trees around the water ecosystem, can potentially damage one of the factors that make up the natural habitat of endemic dragonfly species. The research is a preliminary study of the composition and diversity of dragonflies in various habitat types, with the limitations of research locations that do not represent all types of dragonfly habitats in their natural habitat, so the results of this study are expected to be understood carefully. Therefore, future research can be conducted in more research locations so that it is expected to represent all dragonfly habitats.

AUTHOR CONTRIBUTION

M.A.D.S designed the research, collected and analysed the data, and wrote the manuscript, N.M. collected the data, and wrote the manuscript, A.S.L. assisted in manuscript revision and supervised all the process, and Z.P.G. assisted in manuscript revision and supervised all the process.
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CONFLICT OF INTEREST

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

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Research Article

Diversity and Phenetic Relationship of Mountain Papaya (*Vasconcellea Pubescens*) in Dieng Plateau Based on Morphological Marker

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ABSTRACT
Vasconcellea pubescens A. DC., commonly referred to as mountain papaya, belongs to the Caricaceae family and is native to the Andean highlands. In Indonesia, mountain papaya can be found on the Dieng Plateau and has become one of the typical processed products from the Dieng area. The aim of the study is to explore the diversity and phenetic relationship of mountain papaya from the Dieng Plateau based on morphological markers. This study is important to provide information in guiding future conservation efforts. 18 samples were collected from three areas with different altitudes in the Dieng Plateau. In addition, it proved valuable results by elucidating patterns of variation, enabling the identification of distinct groups. A total of 18 samples were collected from three areas with varying altitudes in the Dieng Plateau. Morphological analysis used 22 characters with The Clustering Analysis Method, Principal Component Analysis (PCA), and Diversity Analysis using Multivariate Statistical Package (MVSP) software version 3.1A. The results of cluster analysis showed that mountain papaya accessions were grouped into two main clusters and five sub-clusters. Cluster grouping based on sex distribution characters, flower stalk length, and inflorescence density; there is no grouping based on geographical location or altitude. The Shannon's Index Value (H') for mountain papaya shows moderate phenetic diversity. This suggests that the mountain papaya community remains stable within its substrate and its environmental parameters.

INTRODUCTION
Vasconcellea pubescens A. DC., also known as mountain papaya, is a species of the genus *Vasconcellea* of the family Caricaceae. This plant flowers and bears fruit throughout the year. Mountain papaya usually grows well at an altitude of 1500 meters above sea level (Prabhukumar et al. 2018). Since mountain papaya originates from the highlands and has similarities with the species *Carica papaya* L., it is often referred to as "highland papaya". This species originating from the Andean Highlands that has an untapped potential that can be developed. For instance, the use of mountain papaya, which has a good taste and high quality and can be reproduced and commercialised. In addition, the genetic heritage of mountain papaya that has resistance to cold temperatures can be transferred to ordinary

papaya to add to the adaptability of ordinary papaya in subtropical regions (National Research Council 1989).

Mountain papaya in Indonesia can be found in the Dieng Plateau area, which is included in the Wonosobo and Banjarnegara Districts, Central Java. In addition, mountain papaya can also be found in the Bromo and Cangar areas of East Java (Laily et al. 2021). The people of the Dieng Plateau area call the mountain papaya tree "Carica". In the Dieng area, mountain papaya fruit has become one of the leading consumption products by being processed into candied carica, and it is now a typical souvenir product from the Dieng Plateau area (Sarno 2018).

Research on morphological characterisation is one of the most important basic rules in the identification of phenetic and genetic diversity in plants, especially in mountain papaya (Martiwi et al. 2020). Morphological characterisation of plant accessions provides several advantages, including revealing the environmental factors underlying changes in plant conditions, providing an indication of physiological status, and providing predictions of future plant population status (Buckley et al. 1997). Previous research conducted by Kyndt et al. (2005) on relationships in the genus *Vasconcellea* (mountain papaya) based on molecular and morphological evidence resulted in relationships based on morphological characters that only show external similarities and are not directly related to genetic relationships or taxon validity. Phenetic inter compatibility and plasticity among mountain papaya species tend to confuse morphological boundaries in each taxon.

Morphological markers have been the primary markers for classification for centuries. Early classifications were based on macroscopic morphological characters. Over the past two centuries, more and more microscopic morphological characters have been included. Although flower morphology has been the main material for classification, other morphological characters also contribute to specific plant groups (Singh 2019). A study conducted by Laily et al. (2022) on macromorphological characters in three sex types of mountain papaya in Java showed varied leaf and flower characters. Likewise, the micromorphological characteristics of mountain papaya show great differences in the trichomes of female and monoecious plants. As a foundation for determining the phenotypic and genotypic diversity within a species, research on morphological characterisation in plants with great morphological variation is crucial. In cultivated plants like mountain papaya, information on phenotypic and genotypic variety is particularly important (Ayana & Bekele 2000).

Phenetic classification groups species into higher taxa based on the overall similarity of observable characters, regardless of their phylogeny or evolutionary relationships. Similarity coefficients are used to create similarity matrices and generate phenograms, which are tree-like networks that express phenetic relationships (Chouduri 2014). Phenetic analysis methods can generally be classified into methods that produce tree-like diagrams (clustering) and methods that produce scatter diagrams on two or more axes (Nixon 2013). In this study, the method used for clustering is the UPGMA (Unweighted Pair Group Method with Arithmetic Average) or average linkage method, which is a direct approach to reconstructing phylogenetic trees using distance matrices (WeiB & Goker 2010). The UPGMA method gives equal weight to each OTU (Operational Taxonomic Unit) and calculates the average similarity or difference between OTUs in cluster formation (Singh 2019).

The morphological characteristics of mountain papaya used in this study included qualitative and quantitative characters. These morphological characters are represented in the form of nominal, ordinal, or binary data. Samples were taken from the Dieng plateau area, which is divided

into two districts, which are the east Dieng and the west Dieng. East Dieng is administratively included in the Wonosobo District area. While West Dieng is administratively included in the Banjarnegara District. Research on diversity and relationships of mountain papaya in the Dieng Plateau area is important to provide information for future conservation. In addition, information on phenetic diversity of mountain papaya is useful in providing patterns of variation so that it can make it possible to identify different groups that can be classified as species. Therefore, the objective of this study is to explore and to uncover phenetic diversity and relationships of mountain papaya from the Dieng Plateau based on morphological markers.

MATERIALS AND METHODS

Mountain papaya

In this study, from August to October 2022, a total of 18 mountain papaya accessions were collected from three locations at different altitudes (Table 1). The sampling locations were carried out in three villages: Kejajar, with an altitude of 1400 masl; Sembungan, with an altitude of 2200 masl; both areas are in Wonosobo District; and Kepakisan, with an altitude of 1800 masl, in Banjarnegara District (Figure 1). Sampling was conducted by purposive sampling using the Explore Method (Rugayah et al. 2004).

Methods

Morphological analysis of mountain papaya was carried out by observing 22 characters (Table 2) using references from the Descriptor of Papaya

Table 1. Location, altitude, and sex distribution of mountain papaya accessions.

Code	Collection Site			Altitude (masl)	Sex Distribution
	Sub-district	Regency	Province		
KJ1	Kejajar	Wonosobo	Central Java	1579	Female
KJ2	Kejajar	Wonosobo	Central Java	1567	Female
KJ3	Kejajar	Wonosobo	Central Java	1567	Female
KJ4	Kejajar	Wonosobo	Central Java	1376	Hermaphrodite
KJ5	Kejajar	Wonosobo	Central Java	1425	Hermaphrodite
KJ7	Kejajar	Wonosobo	Central Java	1579	Hermaphrodite
SB1	Sembungan	Wonosobo	Central Java	2227	Female
SB2	Sembungan	Wonosobo	Central Java	2223	Female
SB3	Sembungan	Wonosobo	Central Java	2224	Female
SB6	Sembungan	Wonosobo	Central Java	2185	Hermaphrodite
SB7	Sembungan	Wonosobo	Central Java	2224	Hermaphrodite
SB8	Sembungan	Wonosobo	Central Java	2223	Hermaphrodite
PK1	Kepakisan	Banjarnegara	Central Java	1887	Hermaphrodite
PK3	Kepakisan	Banjarnegara	Central Java	1899	Female
PK4	Kepakisan	Banjarnegara	Central Java	1921	Female
PK6	Kepakisan	Banjarnegara	Central Java	1921	Hermaphrodite
PK7	Kepakisan	Banjarnegara	Central Java	1884	Female
PK8	Kepakisan	Banjarnegara	Central Java	1899	Hermaphrodite

(IBPGR 1988) and data from UPOV (Union for the Protection of New Varieties of Plants) (2014). Morphological characters analyzed include the vegetative and generative organs of mountain papaya. Morphological character analysis was carried out by analysing the data obtained qualitatively and descriptively. Quantitative analysis was also carried out using the cluster analysis method.

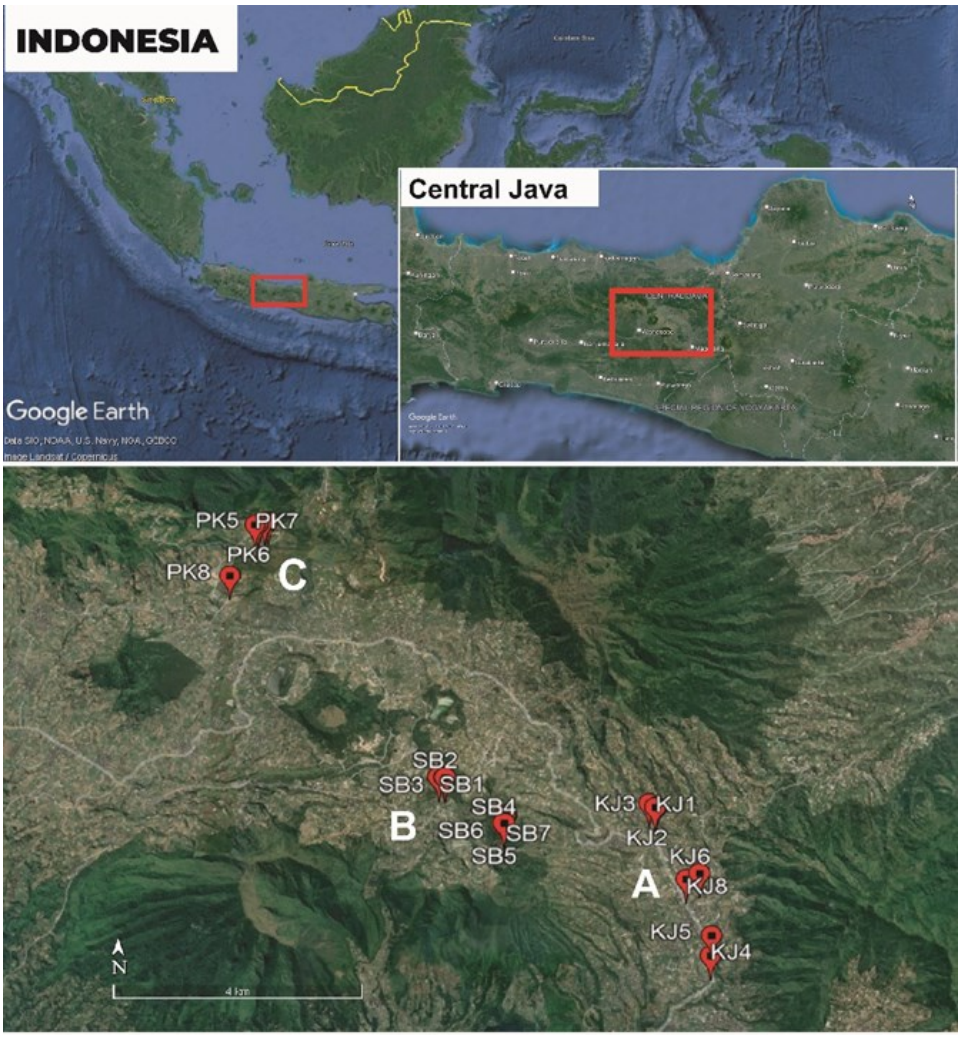

Figure 1. Mountain papaya's sampling location in the Dieng Plateau. **A**. Kejajar, Kejajar, Wonosobo. **B**. Kepakisan, Batur, Banjarnegara. **C**. Sembungan, Kejajar, Wonosobo (Google Earth 2023).

Each mountain papaya sample found is considered a separate operational taxonomic unit, or OTU. Morphological character data totaling 22 characters, both in the form of qualitative and quantitative data, were converted into numerical data in the form of two-state and multi-state data. Then the data were transformed to approach a normal distribution, the transformation was carried out using the standardisation method. Based on the transformed data, the distance index is calculated. The distance index between OTUs is calculated using the Gower General Similarity Coefficient (Sneath & Sokal 1973), which is (1):

$$Sm = \frac{m}{(m+v)} \quad (1)$$

Description:
m= a character shared by a pair of OTUs
v = a character that belongs to only one of the pair of OTUs

Index values between OTUs based on morphological character data were then used to reconstruct dendrograms using UPGMA (Unweighted Pair Group with Arithmetic Average) group analysis (Sneath & Sokal 1973) with the Multivariate Statistical Package (MVSP) version 3.1A program. The resulting dendrogram based on cluster analysis is used to present phenotypic similarity in a group. Cluster analysis is a multivariate statistical technique that has been developed for biological classification. Cluster analysis can be used to efficiently classify data sets of relevant organism characteristics (Saracli et al. 2013). Cluster analysis in this study was conducted using the UPGMA or average linkage method, which is a direct approach to reconstructing phylogenetic trees using distance matrices (WeiB & Goker 2010). Principal component analysis (PCA) was used to determine the role of each morphological character in grouping accessions. PCA will map the distribution of characters into two principal component axes. PCA analysis of data was previously carried out using log transformation and then standardised. Cluster analysis and PCA were conducted using MVSP version 3.1A program (Kovach 2007) to see the relationship between mountain papaya OTUs.
Determination of the diversity level of mountain papaya utilizing the Shannon-Wiener Diversity Index (Fachrul 2008), which is (2):

$$H' = -\sum_{i=s}^{s} pi \ln pi \quad (2)$$

Description:
H' = Shannon-Wiener Diversity Index
Pi = $\frac{ni}{N}$
ni = Total individuals of each i-th character
s = Total number of observed plant samples

There are three classifications of diversity levels based on the Shannon Wiener Diversity Index: (i) H'<1, indicating low diversity, (ii) 1< H'<3, indicating medium diversity, and (iii) H'>3, indicating high diversity.

RESULTS AND DISCUSSION

Morphological character observations of mountain papaya on the Dieng Plateau were carried out using 22 morphological markers. These morphological characters include quantitative and qualitative characters (Table 2). Based on these morphological characters, observations were made on 18 samples of mountain papaya in the Dieng area (Table 1). Mountain papaya in the Dieng Plateau has an overall medium (>1 m) to high (>2 m) stem height with single and multiple stem branching (Figure 2). In their description, Prabhukumar et al. (2018) mentioned that the stem length of mountain papaya ranges from 3.5–5 m, while Paniagua-Zambrana et al. (2020) mentioned that mountain papaya has a height of only up to 4 m. This shows that mountain papaya has a wide range of stem heights. However, compared to the stem height of carica papaya species, which reaches 2–10 meters (Wadekar et al. 2021), the height of mountain papaya is lower.

Mountain papaya leaves are palmate compound-type leaves with 5 to 6 lobes (Prabhukumar et al. 2018). On the abaxial side of the leaf there are pubescens, or fine hairs, and palinactinodromous leaf veins. The tooth shape of mature leaves is convex, with a low to medium ratio of leaf length and width. The sinus shape of the petiole is open, slightly open,

Table 2. List of morphological characters of mountain papaya.

Character	Code	Character States
Tree habit	THB	0=single stem, 1= multiple stems
Sex distribution	SD	0=female, 1=hermaphrodite, 2=male
Color of stem (young plant)	CS	0=only green, 1=yellowish green, 2=brown, 3=green and purple, 4=only purple
Height to first fruit	HF	0=low bearing (<1.0 m), 1=intermediate, 2=height bearing (>1.5 m)
Stem colour (adult trees)	STC	0=greenish or light grey, 1=greyish brown, 2=green and shades of red purple (pink), 3=red purple (pink), 4=other (specify)
Leaf length/width ratio	LR	0=low (3:2), 1=medium (1:1), 2=height (3:4)
General shape of petiole sinus	SPS	0=open, 1=slightly open, 2=slightly closed, 3=strongly closed, 4=other
Inflorescence density	ID	0=sparse (few flowers), 1=intermediate, 2=dense (many flowers)
Colour of corolla	CC	0=white, 1=cream, 2=yellow, 3=green, 4=purple
Colour of inflorescence stalk	CIS	0=greenish, 1=purplish/pinkish, 2=dark red purple/pink, 3=other
Flower stalk length	FSL	0=short (<3 cm), 1=medium, 2=long (>5 cm)
Stalk end fruit shape	SFS	0 = depressed, 1 = flattened, 2 = inflated, 3 = pointed
Size of blossom end scar	SBS	0=small (<0.5 cm), 1=intermediate, 2=large (>1 cm)
Fruit skin texture when ripe	FST	0=smooth, 1=intermediate, 2=rough (ridged)
Ridging on fruit surface	RFS	0=absent or very weak, 1=weak, 2=moderate, 3=strong
Shape of central cavity	SCC	0=irregular, 1=round, 2=angular, 3=slightly star shaped, 4=star shaped, 5=other
Fruit shape	FS	0=ovate, 1=elliptic, 2=obovate, 3=pyriform, 4=oblong, 5=obovate waisted
Number of seeds per fruit	NSF	0=absent or very few (>50), 1=few (51-100), 2=medium (101-150), 3=many (151-200), 4=very many (<200)
Seed colour	SC	0=grey yellow, 1=grey, 2=medium brown, 3=dark brown, 4=black
Seed ratio length/width	SR	0=low (3:2), 1=medium (1:1), 2=high (2:3)
Seed position of broadest part	SPB	0=at middle, 1=slightly towards base, 2=strongly towards base
Seed shape	SS	0=generally round, 1=generally spherical or ovoid, 2=other

slightly closed, and very closed. The petiole is green with red-purple shades; there is anthocyanin coloration on the petiole (Figure 3).

a **b**

Figure 2. Stem appearance of mountain papaya: **a**. Mountain papaya with multiple stem branching; **b**. Mountain papaya with single stem branching.

Mountain papaya (*Vasconcellea pubescens*, A.DC.) is a Trioecious plant where female, male (dioecious), and hermaphrodite (monoecious) plants coexist in one species (Salvatierra-González & Jana-Ayala 2016). Flowers on mountain papaya are panicle-type flowers, which are compound flowers in the form of multiple clusters. Flowers on mountain papaya in the highlands have variations in inflorescence stalk length ranging from short (< 3 cm) to long (> 5 cm). Flower density also shows variations ranging from sparse to dense. The colour of the corolla of mountain papaya flowers is generally yellow, green, and cream (Figure 4).

The variation in fruit shape of mountain papaya found in the Dieng plateau area shows fruits with obovate, pyriform, and elliptic shapes. The fruit surface has a slightly prominent (moderate) and prominent (strong) ridge. The flesh of the fruit, when ripe, is yellow in colour with a dense texture (crunchy). The shape of the central cavity of the fruit has variations of angular, slightly starry, and rounded shapes (Figure 5). Letelier et al. (2020) mentioned in their paper that ripe mountain papaya fruits are about 8–15 cm long, 5–6 cm in diameter, and weigh an average of about 200 grams.

Variations in the shape of mountain papaya seeds in the Dieng plateau area show ovoid or spherical seeds and oval seeds. Whereas in Prabhukumar et al. (2018), the shape of the mountain papaya seeds is elliptic, oval, or fusiform. The colour of mountain papaya seeds in the Dieng plateau area is generally dark brown or light brown, with a thick layer of mucus covering the seeds. The seed surface is opaque and has no luster. The number of seeds in one fruit varies, ranging from 73 seeds per fruit to 168 seeds per fruit (Figure 6).

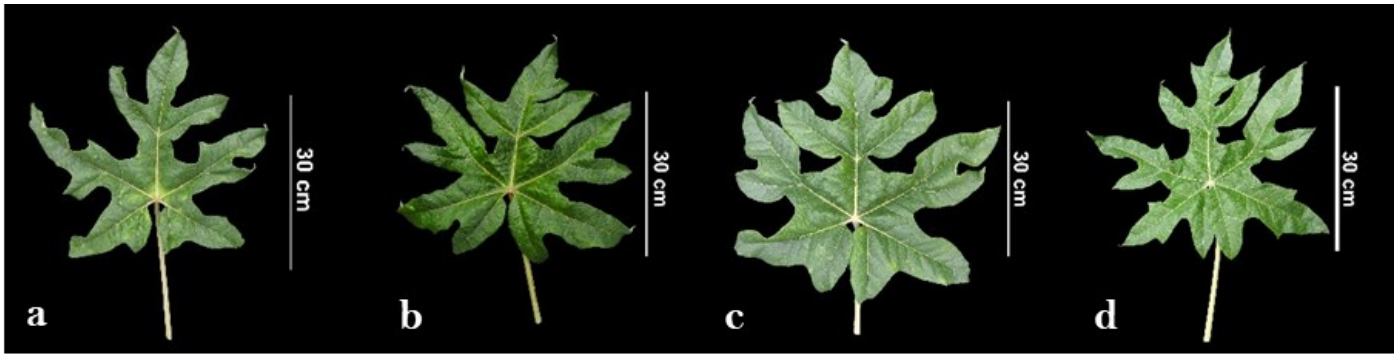

Figure 3. Mountain papaya leaf variation: **a.** Medium ratio/open stalk sinuses; **b.** Low ratio/slightly open stalk sinuses; **c.** Low ratio/slightly closed stalk sinuses; **d.** Medium ratio/closed stalk sinus.

Figure 4. Flower variations of the mountain papaya plant: **a**. Hermaphrodite flowers; **b**. Male flowers; **c**. Female flowers.

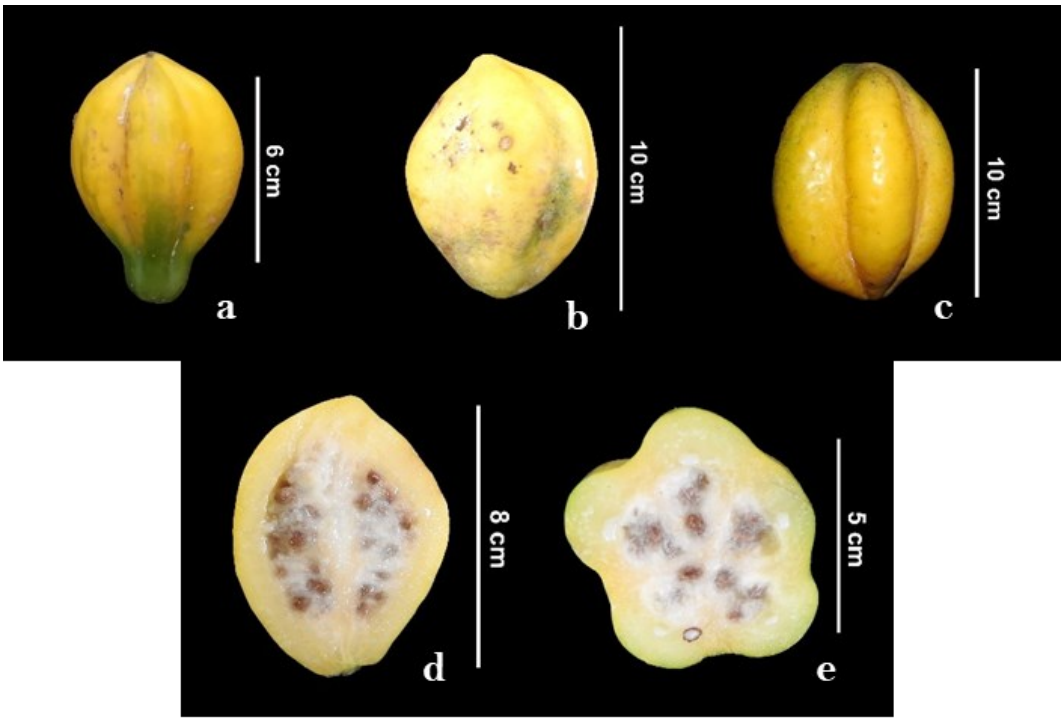

Figure 5. Variations of Mountain Papaya Fruit: **a.** Pyriform-shaped fruit; **b.** Obovate-shaped fruit; **c.** Ellyptic-shaped fruit; **d.** Longitudinal cross-section of ellyptic-shaped fruit slices; **e.** Cross-section of ellyptic-shaped fruit slices.

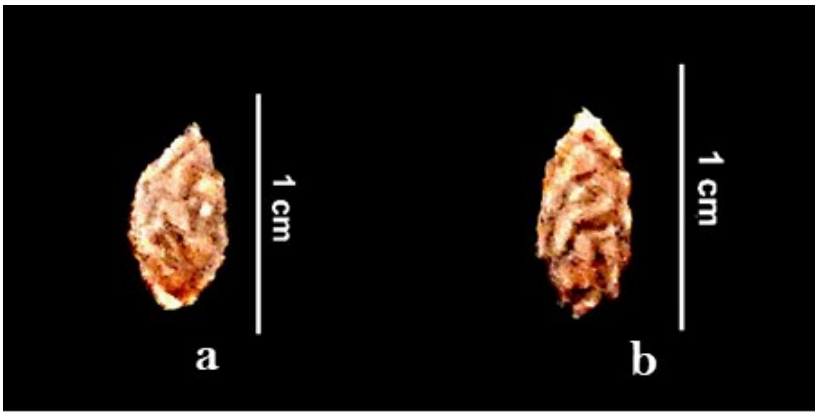

Figure 6. Seed shape of mountain papaya: **a.** ovoid or spherical; **b.** Oval.

The morphological relationship analysis of mountain papaya from the Dieng Plateau used two types of analysis methods, namely cluster analysis and principal component analysis (PCA). Kettenring (2006) states in his article that there are three main methods in multivariate analysis, which are cluster analysis (CA), principal components analysis (PCA), and discriminant analysis (DA). The results of cluster analysis on mountain papaya based on morphological characters using the UPGMA method showed similarity indices ranging from 0.631–0.945 (63.1–94.5%).

The dendrogram of the cluster analysis showed two main clusters with five sub-clusters (Figure 7). Cluster I consist of accessions that have hermaphrodite sex distribution, moderate to dense flower density, and a flower stalk length of more than 5 cm. Cluster II consists of accessions that have female sex distribution, sparse to medium flower density, and short (< 3 cm) to medium (< 5 cm) flower stalk length. Cluster I have two sub-clusters, namely sub-cluster Ia and sub-cluster Ib, with a similarity index of 78.7%. Subcluster Ia consists of accessions PK6, SB6, PK8, and PK1, which have similar characteristics, such as medium texture of

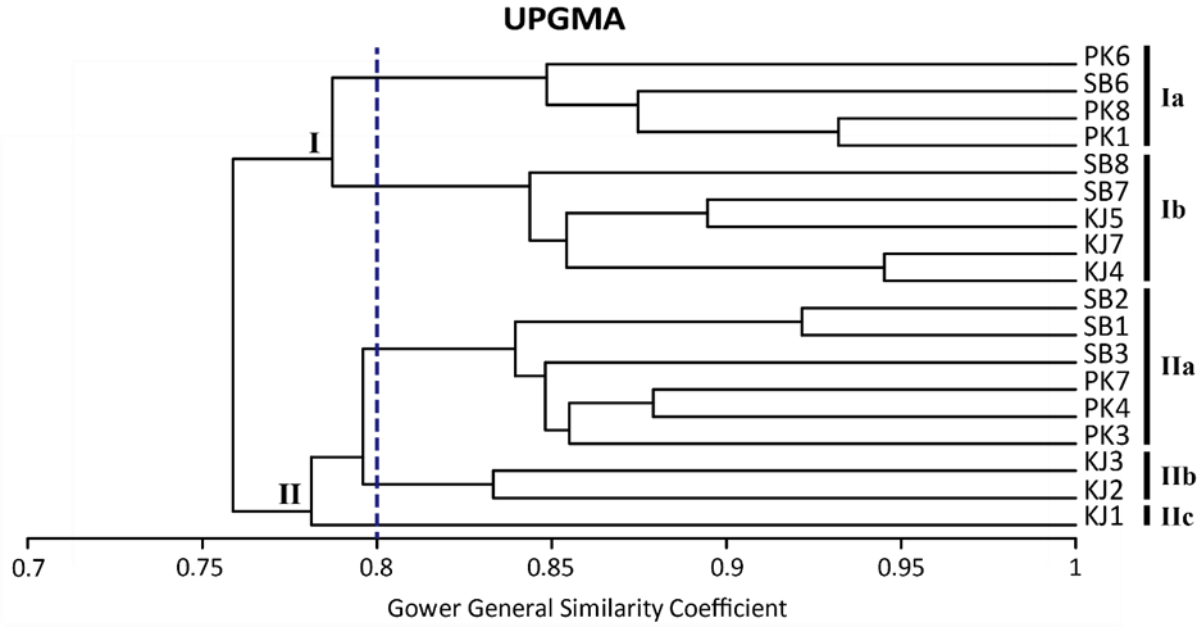

Figure 7. Dendrogram of cluster analysis results based on morphological characters of Mountain Papaya using the UPGMA method. **KJ.** Accession from Kejajar Sub-District, Wonosobo Regency; **SB.** Accession from Sembungan Sub-District, Wonosobo Regency; **PK.** Accession from Kepakisan Sub-District, Banjarnegara Regency.

the fruit skin at maturity, low seed ratio, and the position of the widest part of the seed in the centre. Subcluster Ib consists of 5 accessions, including SB8, SB7, KJ5, KJ7, and KJ4. The common characteristics of subcluster Ib are rough or prominent fruit skin texture, a high seed ratio, and the position of the widest part of the seed slightly towards the bottom.

Cluster II has sub-clusters IIa and IIb that cluster at a similarity index of 79.6%. It then regroups with sub-cluster IIc at a similarity index of 78.1%. Sub-cluster IIa consists of six accessions (SB1, SB2, SB3, PK3, PK4, and PK7) with multiple stem characters, a low seed ratio, and the position of the widest part of the seed in the middle. While sub-cluster IIb consists of two accessions, KJ2 and KJ3, which have single stem characters. While cluster IIc, which consists of one accession, KJ1, has a moderate ridge character on the fruit surface, the other sub-clusters have a strong ridge on the fruit surface.

The results of cluster analysis on mountain papaya in the Dieng Plateau area based on morphological characters showed no grouping based on geographical location or altitude. In contrast to the mountain papaya research conducted by Carrasco et al. (2008) in Chile, which clustered based on the location of growth. Cluster grouping of mountain papaya in the Dieng Plateau is mainly influenced by the characters of sex distribution, stalk length, and flower density. Moore (2013) mentioned that the most distinctive and significant phenotypic traits of papaya varieties are related to flower and fruit characteristics. The form of morphological variation in flowers and inflorescences in papaya varies based on the sex of the tree.

Principal component analysis (PCA) is used to determine the source and structure of variation and the contribution of the observed characteristics to total variability (Glogovac et al. 2012). The results of principal component analysis of morphological characters of mountain papaya in the Dieng plateau showed an eigenvalue of 5.22 on the first axis to 1.80 on the 6th axis (Table 3).

According to Jeffers (1967) in his article, there is a practical and useful role in determining components that have a significant value,

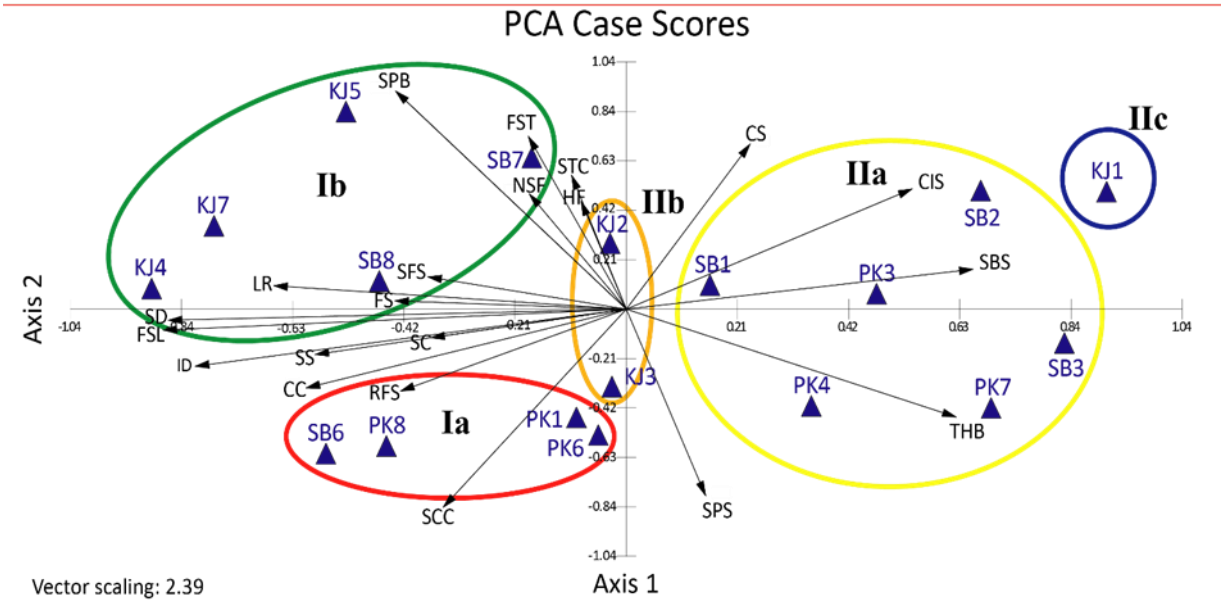

Figure 8. Scatter plot of mountain papaya grouping based on 22 morphological characters.

which is to only consider components with an eigenvalue equal to 1.000 or greater. The grouping of mountain papaya accessions based on principal component analysis is shown in the scatter plot image (Figure 8); the length of the arrow indicates the role of the character in the grouping.

Table 3. Eigenvalue and variable loading of mountain papaya PCA analysis.

Eigenvalues		
	Axis 1	Axis 2
Eigenvalues	**5.22**	**3.51**
Percentage	23.73	15.98
Cum. Percentage	23.73	39.70

PCA *variable loadings*		
	Axis 1	Axis 2
Tree habit	-0.26	-0.19
Sex distribution	**0.36**	-0.02
Colour of stem (young plant)	-0.10	0.29
Height to first fruit	0.04	0.19
Stem colour (adult trees)	0.04	0.24
Leaf length/width ratio	0.28	0.04
General shape of petiole sinus	-0.06	**-0.33**
Inflorescence density	**0.34**	-0.10
Colour of corolla	0.25	-0.14
Colour of inflorescence stalk	-0.23	0.21
Flower stalk length	**0.37**	-0.04
Stalk end fruit shape	0.16	0.06
Size of blossom end scar	-0.27	0.07
Fruit skin texture when ripe	0.08	**0.31**
Ridging on fruit surface	0.18	-0.15
Shape of central cavity	0.14	**-0.35**
Fruit shape	0.18	0.02
Number of seeds per fruit	0.08	0.20
Seed colour	0.15	-0.05
Seed ratio length/width	0.18	**0.39**
Seed position of broadest part	0.18	**0.39**
Seed shape	0.25	-0.08

The results of the principal component analysis of 22 qualitative and quantitative characters of mountain papaya are shown in Table 3. The first six components accounted for 78.15% of the total variation in the data sheet. The first component (axis 1) accounts for 23.73% of the total percentage, indicating that the components on axis 1 have the most important role in clustering the other components. Variable loading in PCA relates to the correlation coefficient between the character and the derived component (Su et al. 2007). Characters with high loading are sex distribution, flower stalk length, and flower density, with loading values of more than 0.3.

The diversity analysis of mountain papaya using the Shannon-Weiner Diversity Index (H') is shown in Table 4. The average Shannon's Index in mountain papaya shows moderate genetic diversity (2.514). The lowest H' value is shown in the inflorescence stalk colour character of 0.693 and is included in the low diversity category. While the highest H' value on the ridge character on the surface of the fruit amounted to 2.889 and fell into the category of moderate diversity. According to Ortiz-Burgos (2015), the main purpose of the Shannon-Wiener Diversity Index is to obtain a quantitative estimate of biological variability that can be used to compare biological entities in space or time. This index considers two different aspects that contribute to the concept of diversity in a communit: species richness and evenness.

Table 4. Morphological diversity of mountain papaya based on Shannon-Weiner index (H').

Character	Shannon's Index
Tree habit	2,485
Sex distribution	2,197
Colour of stem (young plant)	2,073
Height to first fruit	2,821
Stem colour (adult trees)	2,565
Leaf length/width ratio	2,398
General shape of petiole sinus	2,802
Inflorescence density	2,277
Colour of corolla	2,865
Colour of inflorescence stalk	0,693
Flower stalk length	2,468
Stalk end fruit shape	2,804
Size of blossom end scar	2,872
Fruit skin texture when ripe	2,865
Ridging on fruit surface	2,889
Shape of central cavity	2,864
Fruit shape	2,878
Number of seeds per fruit	2,856
Seed colour	2,885
Seed ratio length/width	1,946
Seed position of broadest part	1,946
Seed shape	2,869
Average	2,514

In Mountain Papaya stem characters, the diversity index value ranges from 2.073–2.821 and is included in the classification of moderate diversity index. The value of the diversity index in leaf characters ranges from 2.398 to 2.802 and is included in the medium category. Flower characters show an index ranging from 0.693 to 2.865 and are in the low to medium category. The value of the diversity index in fruit characters ranges from 2.804 to 2.889 in the medium diversity index category. In

seed characters, the diversity index value ranges from 1.946–2.885 and is in the medium category. The diversity of mountain papaya in the Dieng Plateau is based on morphological characters in the moderate category. However, based on molecular characters (internal transcribed spacer sequence), the diversity of mountain papaya is high (Rifqi & Chasani 2023). This indicates that the condition of the plant community is stable on the proponent substrate and environmental parameters (Naniu et al. 2021).

CONCLUSION

The relationship between mountain papaya accessions in the Dieng Plateau based on morphological markers is grouped based on sex distribution characters, flower stalk length, and inflorescence density; there is no grouping based on geographical location or altitude. Meanwhile, the diversity analysis of mountain papaya is classified as moderate. This is shown by the average value of the Shannon-Weiner Diversity Index (H') on mountain papaya in the medium category, which indicates that the condition of the plant community is stable. Other character studies that support identification in mountain papaya need to be carried out, especially by using more conserved character such as molecular markers.

AUTHORS CONTRIBUTION

M.S.R. designed the research, collected and analysed data, and wrote the manuscript. A.R.C. supervised all the processes.

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CONFLICT OF INTEREST

No competing interests were disclosed.

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Research Article

Microbial Count and *AvBD10* Expressions in Ovaries and Oviducts of Kampung Unggul Balitbangtan (KUB)-1 Chickens Following Intravaginally CpG-ODN and *S.* Enteritidis

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ABSTRACT

Indonesia boasts diverse native chickens (*Gallus gallus domesticus*) known for more disease resistance in comparison to broiler chicken, and Kampung Unggul Balitbangtan (KUB)-1 is designated as Indonesia's superior breed. *Salmonella* Enteritidis (SE) is associated with salmonellosis, a foodborne illness that can be transmitted by transovarial, wherein colonisation in the oviduct ascends to the ovaries. However, studies mimicking transovarial salmonellosis via intravaginal treatment of chicken have been limited. Meanwhile, Cytosine-phosphate-guanine oligodeoxynucleotide (CpG-ODN) stimulation has been known to induce avian β-defensins (*AvBDs*). This *in vivo* study aimed to determine the effects of intravaginal CpG-ODN treatment and SE challenged on microbial count and *AvBD10* expression regarding the potential of intravaginally CpG-ODN to enhance innate immunity as an alternative approach against transovarial Salmonellosis. A total of 39 KUB-1 chickens were divided into four groups: T1 (CpG-ODN treatment), T2 (SE treatment), T3 (CpG-ODN treatment + challenged with SE), and C (Control). Observation was carried out from day 1 to day 4 post-intravaginal (PI). We found a significant increase in ovarian microbial count ($p \leq 0.05$). Notably, ovaries and oviducts remained uncontaminated post-SE challenge. Intravaginal CpG-ODN treatment significantly upregulated *AvBD10* in both ovaries ($p=0.016$) and oviducts ($p=0.023$). Therefore, KUB-1 chickens exhibit SE immunity, and intravaginal CpG-ODN administration holds promise for preventing transovarial Salmonellosis in laying hens.

INTRODUCTION

In several countries, *Salmonella enterica* subsp. *enterica* serovar Enteritidis (SE) is a major cause of foodborne illnesses worldwide, often associated with chicken meat and egg consumption (Antunes et al. 2016; Midorikawa et al. 2020). Nonetheless, chicken egg is a popular and economical protein source with high biological values (Lopez et al. 2018). Chicken products, on average 1.11 million tons are consumed annually,

with Indonesia producing 1.48 million tons, are widely consumed (Wardhana et al. 2021). In the USA, 1.4 million annual cases of foodborne diseases cause are attributed to *Salmonella*, originating from chickens (Midorikawa et al. 2020). SE infections impact the poultry industry as infected chickens can spread the pathogen and pose a zoonotic risk through contaminated eggs. Notably, over half a billion eggs were withdrawn in the USA due to an SE contamination outbreak (Mon et al. 2020).

The Centers for Disease Control and Prevention (CDC) reported that *Salmonella* is a significant public health concern, causing an estimated 1.35 million infections, 26,500 hospitalisations, and 420 deaths annually in the USA. Most of these infections stem from contaminated food, with chicken being a major source, as roughly 1 in 25 packages at grocery stores are tainted with *Salmonella*. Gong et al. (2022) reported that in a global context, non-typhoidal *Salmonella* (NTS) leads to 93.8 million cases of gastroenteritis each year, resulting in 155,000 deaths. Investigation in Indonesia found high *Salmonella* contamination rates in chicken meat (85%), intestine contents (57.5%), and rinse water (52.5%) at traditional markets in Surabaya as reported by Yulistiani et al. (2019), meanwhile Walyani et al. (2019) reported in Subang, 75% isolates of *Salmonella* spp. which infects chickens were resistant to antibiotics with a 95% confidence interval (0%-35.4%), one of which was SE.

SE contamination on eggshells can occur horisontally from bacteria that originally inhabited the gut or from fecal contact during or after egglaying. Vertical transmission happens through direct contamination of yolk, albumen, or shell before laying, from reproductive organs infected with SE (Gantois et al. 2008). The interaction between pathogens like *Salmonella* and their host relies on a complex microbiota. Understanding the association between chicken microbiota and *Salmonella* is crucial to reducing its harm (Foley et al. 2013). Vertical transmission of SE contamination in eggs and the passage of chicken microbiota to chicks occur during egg formation in the reproductive tract (Ding et al. 2017). Microbiota from the parental oviduct, including bacterial species, is conveyed to the embryo through albumen, suggesting the potential for microbiota transfer during egg formation (Lee et al. 2019).

Cytosine-phosphate-guanine oligodeoxynucleotide (CpG-ODN) is an ODN with an unmethylated CpG motif found in bacterial DNA which is read as a "danger signal" by the immune system, so it can stimulate innate and adaptive immune responses in humans and various animal species (Li et al. 2017). In response to SE infection, oviductal epithelial cells constitutively expressed *AvBDs* (Ebers et al. 2009), and following stimulation with CpG-ODN, the expression of *AvBDs* is upregulated (Abdel-Mageed et al. 2011; Sonoda et al. 2013). The ability of reproductive tissues to synthesise *AvBDs* and their regulation in response to pathogens may play an essential role in innate immunity in the ovary and oviduct, establishing localised defenses within the host. Synthesised *AvBDs* notably fortify the defense mechanism of reproductive organs (Yoshimura et al. 2014) and offer protection against infection (Yoshimura 2015).

This study focuses on analysing partially and temporally microbial counts and *AvBD10* expressions in the ovaries and oviducts of Kampung Unggul Balitbangtan (KUB)-1 chickens, a superior Indonesian native strain. These chickens exhibit improved egg production and disease resistance due to six generations of selective breeding (Sartika 2014). The research investigates innate immune responses in ovaries and

oviducts using an intravaginal CpG-ODN treatment and SE challenge test with observations occurring at four-time points (day 1 to day 4) post -intravaginal (PI) treatment.

MATERIALS AND METHODS

Experimental design and animal groups

Experiments were performed under the ethical guidelines and regulations set forth by the Ethics Commission of Institut Teknologi Bandung, Indonesia, with the issuance of Decree No. 01/KEPHP-ITB/3-2019 by the provisions internationally accepted principles for laboratory animal use and care. The study involved 39 KUB-1 females, 60 weeks old, *Salmonella*-free, and divided into four groups: T1 (CpG-ODN treatment, n=12), T2 (SE treatment, n=12), T3 (CpG-ODN treatment + challenged with SE, n=12), and C (Control, n=3). CpG-ODN (5 µg/500 µl) from ODN 2007 Class B CpG oligonucleotide 5′-TCGTCGTTGTCGTTTTGTCGTT-3′ (InvivoGen) and SE $5x10^7$ CFU (Culti-Loops SE ATCC 13076™) were used. Ovary and oviduct samples were aseptically in a BSL2+ room from day 1 to day 4 PI and stored separately. *Salmonella* isolation and TPC followed *Office International des Epizooties* (OIE) protocols. *AvBD10* relative expression gene (RGE) was assessed through real-time qPCR using the Livak & Schmittgen (2001) $2^{-\Delta\Delta Ct}$ formula.

Salmonella isolation test and Total Plate Count (TPC) analysis

A *Salmonella* isolation test was conducted according to OIE (2018). The procedure involved pre-enrichment and enrichment, followed by selective medium culturing and *Salmonella* identification using biochemical assays, accompanied by a positive control. During pre-enrichment, a 25 g sample was homogenized with 225 ml of 0.1% Buffer Peptone Water (BPW). The mixture was incubated at 35°C for 24 hours (h). In the enrichment step, the pre-enrichment culture was inoculated into Tetra Thionate Broth (TTB) medium and Rappaport Vassiliadis (RV) medium, then incubated at specific temperatures for 24 h. From the enrichments, selected colonies were inoculated onto Xylose Lysine Deoxycholate Agar (XLDA), Hektoen Enteric Agar (HEA), and Bismuth Sulfite Agar (BSA), then incubated at 35°C for 24 h. *Salmonella* identification was performed by streaking suspected colonies onto Triple Sugar Iron Agar (TSIA) and Lysine Iron Agar (LIA), followed by 24 h incubation. Biochemical assays include urease, indole, Methyl Red-Voges Proskauer (MR-VP), citrate, and malonate tests.

A TPC analysis, following the OIE 2018 guidelines, involved homogenising a 25 g sample with 225 ml of 0.1% BPW. The resulting suspension was transferred to a sterile tube for a 10^{-1} dilution, followed by a 1 ml sample transfer into 9 ml of 0.1% BPW for further dilutions up to 10^{-6}. From each dilution, 1 ml of suspension was plated in Duplo on Petri dishes. Subsequently, 15-20 ml of 45°C plate count agar (PCA) was added to each plate and mixed. The PCA was allowed to solidify and incubated upside down at 36°C for 24-48 h. Colonies were counted with a colony counter for each dilution series. Petri dishes containing 25-250 colonies were selected for measurement and interpretation of results.

Real-time qPCR analysis of *AvBD10* expressions

RNA extraction from the ovary and oviduct tissue utilised the Rneasy Mini Kit (Qiagen) according to the manufacturer's instructions. Subsequent cDNA synthesis employed the Cyclescript RT Premix Synthesis Kit (Bioneer) following the manufacturer's protocols. The

resulting cDNA was mixed with primers and SYBR Green in the SensiFAST SYBR Lo-Rox Kit (Bioline), according to the manufacturer's guidelines. Applied Biosystems™ 7500 Fast Real-Time PCR Systems for qPCR and used NFW without cDNA as a negative control in the qPCR reaction to ensure that there is no contamination or error in the reaction. Total reaction volume in qPCR = 20 µl (10 µl SYBR, 0.8 µl forward primer, 0.8R µl reverse primer, and 4.4 µl NFW). Real-time qPCR condition involved an initial polymerase activation step at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 5 seconds (s), and annealing/extension at 58°C for 30 s. The treatment groups (T1, T2, and T3) were compared to the Control (C) group. Cycle Threshold (CT) values were determined in six replicates and quantifications were normalised using Beta-actin (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as housekeeping genes. Table 1 for primer details.

Data analysis and statistics

The *Salmonella* isolation test involves a series of culture steps to analyse results. Tissue samples are subjected to TPC analysis to quantify microbial presence, with a contamination threshold of $1x10^6$ CFU/gram, based on OIE. The *Salmonella* contamination threshold value, set by the Indonesian Ministry of Agriculture, is non-detectable in 25 g samples. For statistical analysis, One-way ANOVA and Duncan's test were employed in SPSS25 with significance $p \leq 0.05$. Data normality was confirmed through the Shapiro-Wilk test (Sig.>0.05). Graphical representation was created using GraphPad Prism 9.4.0. The data set comprised the mean ± SEM from day 1 to day 4 PI.

RESULTS AND DISCUSSION

Intravaginal SE significantly increased microbial count, but there was no microbial contamination in the ovaries of KUB-1 chickens

On day 4 PI, intravaginal SE did not induce clinical signs in KUB-1 chickens. This could be attributed to age, as adult poultry show milder Salmonellosis symptoms per OIE 2018. Host-pathogen interactions, age, stress, and health, emphasised by Foley et al. (2013), play key roles, while Wessels et al. (2021), note morbidity/mortality influenced by age, breed, nutrition, and flock management. We chose 60-week-old chickens known for their prolific egg-laying abilities and Lee et al. (2019) findings that bacterial species significantly increased after sexual maturation, and the microbiota in adult chicken oviducts remained consistent throughout the reproductive tract. Additionally, Wigley et al. (2014) reported that *Salmonella* infection in the reproductive tract primarily occurs after chickens reach reproductive maturity, making them more susceptible to SE challenges during egg-laying.

Observations were limited to 4 days PI as Rehman et al. (2021) noted a four-day duration for the innate immune response. Meanwhile,

Table 1. Primer of *AvBD10* and housekeeping genes.

Identity	Primer	Length	Accession No.
AvBD10	Forward: TGTTAAACTGCTGTGCCAAGATTC Reverse: TGTTGCTGGTACAAGGGCAAT	98 bp	NM_001001609.1
GAPDH	Forward: AGCCATTCCTCCACCTTTGA Reverse: CAACAAAGGGTCCTGCTTCC	190 bp	NM_204305.1
ACTB	Forward: ATGAAGCCCAGAGCAAAAGA Reverse: GGGGTGTTGAAGGTCTCAAA	244 bp	NM_205518.1

Miyamoto et al. (1998) studied SE levels in the cloaca at 7 days PI due to intravaginal SE, while Pudjiatmoko et al. (2014) highlighted 4–5 days *Salmonella* incubation period with 3-5 weeks of symptoms in chickens. Despite SE treatment (T2, T3), *Salmonella* was not present in the ovaries and oviducts of KUB-1 chickens, but positive controls showed *Salmonella* presence. Oludairo et al. (2022) described *Salmonella* as a Gram-negative rod-shaped bacterium, non-spore-forming, oxidase, indole, urease, lactose, sucrose negative, facultative anaerobic, and motile. In our study, based on OIE for the *Salmonella* test, SE exhibited characteristic features, including pink/black colonies on XLDA, green/blue on HEA, and black/gray on BSA, negative *Salmonella* for indole, urease, MR-VP, citrate, malonate, and a motile bacteria. Day 1 PI *Salmonella* isolation and biochemical assays did not reveal colonies in ovaries and oviducts. Okamura et al. (2001) found no *Salmonella* in ovary cultures on day 2 PI and day 3 PI. Ojima et al. (2021) observed no *Salmonella* colonies in ovaries/oviducts on day 4 PI.

In this study, we investigated the impact of SE $5x10^7$ CFU intravaginally inoculation in T2 and T3 groups on microbial contamination in ovaries and oviducts. Previous research (Chatterjee & Abraham 2018) established the concept of microbial contamination, encompassing unwanted microbes such as *Salmonella* sp., *Pseudomonas*, *Listeria monocytogenes*, *Shigella flexneri*, *Vibrio cholerae*, *Bacillus* sp., and *Campylobacter jejuni*. Such contamination could arise from clinical infections caused by these microbes. Microbial contamination assessment involved TPC derived from cultures on NA medium, quantifying colony numbers. Tissue was deemed contaminated if TPC exceeded $1x10^6$ CFU/g. Observing TPC across T1, T2, T3, and C groups from day 1 to day 4 PI revealed that 2.56% ovaries and 12.8% oviducts surpassed the threshold value (data not shown).

A partial analysis of TPC values was visualised in Figure 1. The results indicated an increase in microbial counts in T1, T2, and T3. However, only the ovaries in T2 showed a significant increase ($p \leq 0.05$). Interestingly, despite being the first site encountered by SE after intravaginal inoculation, the oviduct did not exhibit a significant increase. This phenomenon aligns with Kaspers et al. (2014), who proposed that in birds, lacking lymph nodes, epithelial cells of the oviduct can detect pathogens and mount an active immune response to fend off invasion. Furthermore, Wigley (2014) elucidated that *Salmonella* infection elicits a local innate immune reaction, notably the secretion of the antimicrobial peptide AvBD throughout the reproductive tract, particularly in the vagina and uterus.

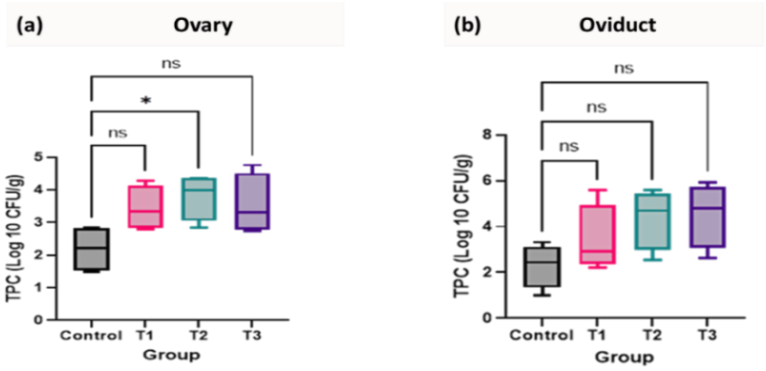

Figure 1 . Microbial count in the ovary (a) and oviduct (b) of KUB-1 chickens at day 1 to day 4 PI through TPC, in partial analysis, and each bar represents the mean ± SEM.
Sign * $p \leq 0.05$, ns = not significant.

The absence of significant microbial contamination in the ovaries and oviducts of KUB-1 chickens, as indicated by the TPC value below the threshold, suggests the potential involvement of non-harmful microbes in pathogen control. El-Saadony et al. (2022) highlighted the intricate association between chicken microbes and *Salmonella*, emphasising their role in pathogen limitation. Intravaginally introduced *Salmonella* encounters epithelial and immune cell protection and competes with local microbes, as noted by Foley et al. (2013). *Pseudomonas veronii*, per Montes et al. (2016) and Canchignia et al. (2017), safeguards chicken oviducts against bacterial infections. Lee et al. (2019) identified dominant bacteria like *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* in the chicken oviduct. Among these, *Pseudomonas*, *Lactobacillus*, *Megamonas*, and *Bacteroides* prevail in adult chickens' oviducts, with similar abundance and diversity in purebred and SPF chickens, with the phylogenetic diversity values representing species richness, and the Shannon index representing alpha diversity, were similar between the two chicken breeds, with H = 0.001923, p-value = 0.965022, and q-value = 0.965022. The TPC results of microbial count in our study indicate good sanitation and hygienic practices, aligning with Mpundu et al. (2019), who attributed bacterial contamination to unhygienic practices and exposure risks.

Figure 2 illustrates the temporal analysis of microbial count fluctuations from day 1 to day 4 PI. Notably, T2 exhibited a decreasing trend in both ovaries and oviducts. This suggests the presence of commensal microbes that may compete with SE. Kogut & Arsenault (2017) highlighted that SE competes with commensals for colonisation and survival, while Lopez et al. (2018) identified significant poultry product contaminants like *Pseudomonas*, *Brochothrix*, *Salmonella*, and others. Investigation of contaminating microbes was limited to *Salmonella*, as it was to determine whether intravaginally SE had contaminated the ovaries and oviducts; and whether it increased the number of microbes in T2 and T3. While the result did not observe colony growth of *Salmonella* in T2 and T3, TPC analysis did reveal a slight increase in microbial counts, albeit below the contamination threshold. Therefore, non-pathogenic microbes and host defense molecules may have influenced the results.

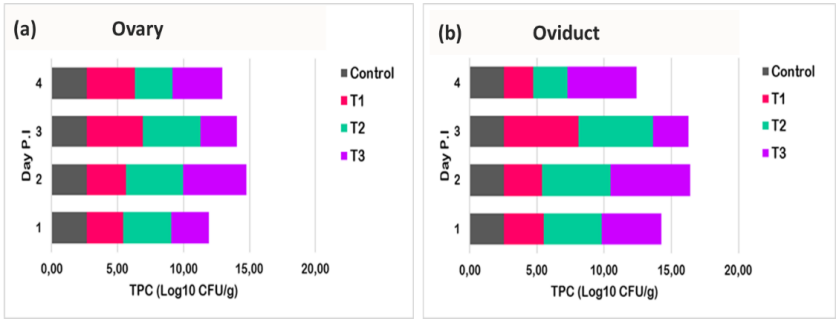

Figure 2. Microbial count in the ovary (a) and oviduct (b) of KUB-1 chickens through TPC, in temporal analysis, from day 1 to day 4 PI.

The host and its microbiota collaborate to prevent pathogen invasion as reported by Rogers et al. (2021), a defense mechanism against SE invasion. Microbiota-produced short-chain fatty acids selectively filter out bacteria unable to maintain pH balance. This, combined with epithelial hypoxia restricting respiratory electron acceptors, hinders *Salmonella*'s resource access. Facultative anaerobic microbes like *Escherichia coli*, *Streptococcus danieliae*, and *Staphylococcus xylosus* compete to strengthen this defense against *Salmonella* infections. Although

Salmonella contamination does not alter egg content, color, smell, and consistency, vigilance for human health remains crucial. Meanwhile, Ahmed et al. (2010) and Li et al. (2023) reported that the effect of the ovary and oviduct dysfunction disorders if exposed to SE for a long is oophoritis and salpingitis. In laying hens, salpingitis is characterized by a decrease in egg production, no or short peak laying time, poor quality of egg shells, and increased production of thin, soft, and sandy eggshells. This poses economic losses as the poultry industry relies heavily on bird reproductive health.

Johnston et al. (2012) reported cases of salpingitis and oophoritis resulting from SE contamination of eggs, wherein bacteria from the cloaca can migrate to the oviduct and ovaries. This process is facilitated by SE fimbriae adhesins that aid in mucosal adhesion, and immune alterations may play a role in infection. Microbial counts were observed in the ovaries and oviducts in the control group, revealing a TPC of ≤ 5 log10 CFU/g. In contrast to the previous study (Miyamoto et al. 1998) intravaginal SE inoculation in adult White Leghorn resulted in a microbial count of 5.7 log10 CFU/g in the control group before SE inoculation. This indicates that the initial microbial count in KUB-1 chicken oviducts was lower than in White Leghorn chickens.

This research was initiated due to a government program providing low-income households with local chickens (KUB-1) for food production. According to Oludairo et al. (2022), Salmonellosis in chickens results in significant public health concerns. The disease is primarily transmitted through animal-derived foods and direct contact with animals, contributing to a wide range of health issues, including enteritis, septicemia, abortion, and meningitis, leading to global morbidity and mortality. Most *Salmonella* strains are pathogenic and can invade and survive within host cells, posing a significant health risk. Additionally, a study by Sartika & Iskandar (2019) demonstrated that KUB-1 chickens exhibit better resistance to avian influenza (AI) than broiler chickens, as indicated by the *Mx* gene. As a zoonotic disease, Salmonellosis control in Indonesia is given priority based on the Decree of the Minister of Agriculture of the Republic of Indonesia No. 237/Kpts/PK.400/M/3/2019. The internationally recognized strategy for preventing foodborne illnesses involves controlling *Salmonella* in poultry farming (Pulido-Landínez 2019), therefore it is essential to explore alternative approaches to combat salmonellosis. Moreover, considering colonisation of *Salmonella* is also influenced by the immune system (Foley et al. 2013), we analysed *AvBD10* expression levels to explore innate immunity variations.

Intravaginal CpG-ODN treatment significantly increased *AvBD10* RGE in the ovaries and oviducts of KUB-1 chickens

Partial analysis of *AvBD10* levels from day 1 to day 4 PI, shown in Figure 3, *AvBD10* RGE increased in T1 oviducts ($p \leq 0.05$), while not significantly in ovaries. There was a significant upregulated *AvBD10* in both ovaries ($p \leq 0.01$) and oviducts ($p \leq 0.05$) of T3. This is consistent with a previous study by Abdel-Mageed et al. (2011). However, a study by Sonoda et al. (2013) using 400-day-old White Leghorn chickens for vaginal cell culture found that *AvBD10* expression was detectable even without stimulation and could be regulated by LPS. In addition, CpG-ODN from microbes, through interaction with TLR21, upregulated *IL1B* and *IL6*, which in turn, induced *AvBD1* and *AvBD3* to combat vaginal infections. This study also revealed upregulated *AvBD10* in the ovaries and oviducts following CpG-ODN stimulation and SE challenge, suggesting activation of *AvBD10* in reproductive tissues, as one of 14

AvBDs that serve as antimicrobial molecules in the body's defense against SE, particularly those caused by LPS in Gram-negative bacteria.

In T2, upregulated *AvBD10* levels in both ovaries and oviducts, although it is not significant. Notably, the findings contrast with a previous study (Abdel-Mageed et al. 2014) that downregulated *AvBD10* in the oviduct cell cultures after CpG-ODN stimulation and challenged with LPS. However, their use of 0.1-10 µg/ml CpG-ODN in adult White Leghorn chickens and our study of 5 µg/0.5 ml intravaginal CpG-ODN in adult KUB-1 chickens might account for these differences. Upregulated *AvBD10* after SE infection in KUB-1 chicken aligns with previous research (Anastasiadou et al. 2013) which observed increased *AvBD10* expression in the vagina post-infection. While the upregulated *AvBD10* was not significant in the T2 group, it was noteworthy.

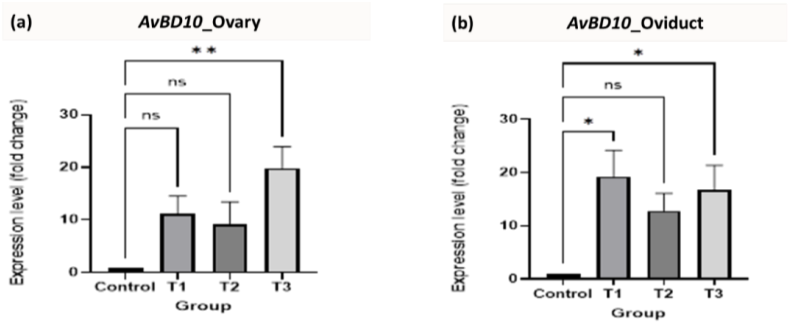

Figure 3. The relative expression of *AvBD10* levels in the ovary (a) and oviduct (b) of KUB-1 chickens at day 1 to day 4 PI in partial analysis through real-time qPCR normalised by *ACTB* and *GAPDH*, analyzed with One-way ANOVA and each bar represents the mean ± SEM. Sign * $p \leq 0.05$; ** $p \leq 0.01$.

In contrast, the T3 group displayed significant upregulated *AvBD10* in both ovaries ($p \leq 0.01$) and oviducts ($p \leq 0.05$). Meanwhile, in a study by Mowbray et al. (2018), AvBD10 with a charge of ≤+4, displayed less potent antimicrobial activity in broiler chicks 21 days old. Bacterial time-kill testing with 0.5 – 12µM recombinant peptide (r) AvBD10 revealed its strong antimicrobial efficacy against fecal *Enterococcus*. This finding supports the evidence of transovarial transmission of Salmonellosis, starting from fecal contamination in the cloaca and progressing to the oviducts and ovaries, as previously reported by Gantois et al. (2009), and this study mimics the transovarial transmission. In another investigation by Goonewardene et al. (2021), CpG-ODN was administered to day-old broiler chicks, challenged with a lethal dose of *E. Coli* altered the metabolic landscape, leading to enhanced antimicrobial immunity in neonatal chicks. Those receiving CpG-ODN had significantly lower bacterial counts ($p<0.05$) compared to the control group. In our investigation involving 60-week-old chickens, also observed enhanced antimicrobial immunity, particularly *AvBD10*. Given the differences in age and microbial populations in the ovaries and oviducts of older chickens compared to day-old chicks, we sought to determine whether the microbial count in these reproductive organs could be categorized as microbial contamination.

The increase in *AvBD10* levels suggests potential immunological memory from previous exposure, reflecting an effective immune response, which aligns with an earlier report (Bultman 2017). Additionally, Van der Mer et al. (2015) highlighted that trained immunity can confer protection against subsequent infection, enhancing immune defense. Moreover, Rusek et al. (2018) outlined that innate immunity exhibits immunological memory akin to adaptive immunity,

bolstering its resistance to subsequent infections. This memory is manifested through trained innate immunity, induced by exposure to infectious or non-infectious agents. This concept holds promise for proactive disease prevention. The study focused on stimulating innate immune memory in chickens using SE bacteria as an infectious agent and CpG-ODN as a non-infectious trigger for *AvBD10* modulation.

Temporal analysis of *AvBD10* levels from day 1 to day 4 PI, shown in Figure 4, revealed noteworthy patterns. In T1, an increase of 21-fold and 26-fold occurred on day 3 PI in the ovary and oviduct respectively, persisting till day 4 PI with a peak increase of 28.3-fold. This suggests that intravaginal CpG-ODN induced *AvBD10* in KUB-1 chicken's ovaries and oviducts. Divergent fluctuations were observed in T2 between the ovary and oviduct. In the oviduct, levels ascended from day 1 to day 4 PI, while the opposite was seen in the ovary. Specifically, the ovary of T2 exhibited a 19-fold increase by day 4 PI, and the oviduct showed a 20-fold increase by day 1 PI. This indicates the involvement of *AvBD10* in the innate immune system against SE, which might differ from its interaction with commensal microbes. Notably, as reported in previous studies (Michailidis et al. 2012) *AvBD* expression in ovaries, and Yoshimura (2015) demonstrated gradually upregulated *AvBD10* in ovaries. In T3, *AvBD10* levels peaked significantly, increasing by 29.5-fold in the ovary and 25-fold in the oviduct on day 3PI. Subsequent fluctuations on day 4 PI suggested decreasing *AvBD10* post-microbial clearance, consistent with a previous report (Van der Meer et al. 2015) indicating a rapid decline of innate immunity after infection resolution. Additionally, Sonoda et al. (2013) supported the role of *AvBD* in eliminating microbes in vaginal tissue during infections. In the oviduct of T3, an increase of 21-fold followed by a decline to 11-fold could be attributed modulation of *AvBD10* by CpG-ODN, promoting an immune response against SE. This aligns with a previous report (Yoshimura 2015), that *Salmonella* infection upregulates *AvBDs* in the oviduct.

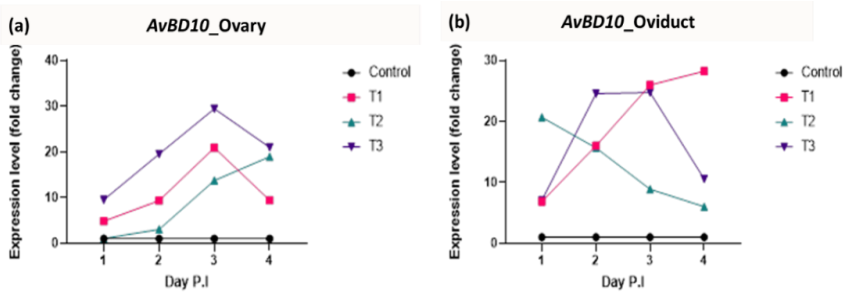

Figure 4. The relative expression of *AvBD10* levels in the ovary (a) and oviduct (b) of KUB-1 chickens from day 1 to day 4 PI in temporal analysis through real-time qPCR normalised by *ACTB* and *GAPDH*. Each plot represents the mean ± SEM.

Animal experiment with three representative chickens from the Control (C) group from day 1 to day 4 PI, each with a gene expression level of 1, as a valid reference for being compared with the 12 chickens in each Treatment (T) group. Reduction in the number of productive laying hens just in the C group as recommended by the Ethics Commission in line with the 3R principle of animal welfare. Additionally, Rieu et al. (2009) suggest conducting qPCR experiments with a minimum of three independent biological replicates for each treatment and at least two technical replicates for each qPCR biological replicate.

This study investigated the effects of CpG-ODN on productive-laying hens, both when administered alone and in combination with an

SE challenge. In the group stimulated by CpG-ODN and challenged with SE, notable upregulated *AvBD10*, supporting its role as a host defense molecule. This is consistent with Sonoda et al. (2013), indicating that *AvBD10* promotes an immune response through activation of its receptor, TLR21. As emphasised by Grzymajlo (2022), the *in vivo* experiment provides more robust insights into host protection factors, thereby this study can provide a basis for further investigation exploring interactions between CpG-ODN and the other 13 *AvBDs* in chickens, contributing to the development of host protection molecules.

CONCLUSIONS

The *AvBD10* was significantly induced by intravaginally CpG-ODN ($p \leq 0.05$) and there was no microbial contamination in the ovaries and oviducts of KUB-1 chickens after being challenged with *S.* Enteritidis. This indicates its ability to induce *AvBD10*, which can be developed as an alternative to preventing foodborne disease caused by transovarial salmonellosis. Further, these *in vivo* studies can be developed on other *AvBDs* in chickens.

AUTHOR CONTRIBUTION

Conceptualisation, S.H.S., E.A.G.R. and R.R.B.S.; methodology, R.R.B.S.; software, R.R.B.S.; validation, R.R.B.S.; formal analysis, R.R.B.S., S.E.P., R.S., and D.N.W.; investigation, R.R.B.S.; resources, R.R.B.S.; data curation, R.R.B.S.; writing—original draft preparation, R.R.B.S.; writing—review and editing, R.R.B.S.; supervision, S.H.S. and E.A.G.R.; project administration, R.R.B.S.; funding acquisition, R.R.B.S.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Research Article

Growth and Development of Winged Bean (*Psophocarpus tetragonolobus* (L.) DC.) Treated with Paclobutrazol

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ABSTRACT
Winged bean (*Psophocarpus tetragonolobus* (L.) DC.) is one of the tropical legumes commonly grown for vegetable in Indonesia. Winged bean is a kind of plants that growth on vine so that for cultivation it requires stakes or awnings. It is known that paclobutrazol is a growth retardant that acts by inhibiting gibberellin biosynthesis and application of paclobutrazol could make plant become semidwarf or even dwarf. This study was aimed to evaluate the effect of paclobutrazol on growth, development, some phytochemicals content and yield of winged bean plants. This study used a Completely Randomized Design (CRD) with one factor, namely paclobutrazol, which was applied at four different concentrations, namely 0 ppm (control), 25 ppm, 50 ppm, 75 ppm or 100 ppm. Three replicates were made for each treatment. The results showed that paclobutrazol significantly decreased plant height, number of leaves, leaf area, number of pods per plant, pod length, fresh weight of fruit, levels of vitamin C and protein in the pods, but increased the leaf chlorophyll content and stomata density on the abaxial (lower) leaf surfaces. Paclobutrazol showed its effect on accelerating flowering time at a concentration of 50 ppm.

INTRODUCTION
Winged bean (*Psophocarpus tetragonolobus* (L.) DC.) is a tropical legume plant belonging to the Fabaceae family. Winged beans grow as herbaceous vines, it has root tubers and pods resembling wings. This plant is widely spread in tropical countries including Indonesia, Malaysia, Thailand, Philippines, India, Bangladesh, Myanmar and Sri Lanka (Lepcha et al. 2017). Winged bean has good prospects and market potentials. The potential yield of winged bean pods varies from approximately 35.5 to 40.0 tonnes/ha, higher than the yard-long bean pods of approximately 20 tonnes/ha (Rukmana 1995; Rukmana 2000). Winged beans can reach a potential yield of approximately 4.5 tonnes/ha of dry seeds, which exceeds the average potential yield of all soybean cultivars ranging from approximately 1.8 to 2.5 tonnes/ha of dry seeds (Rukmana 2000; Kusumowarno 2015). It has been reported that the content of protein in yard long bean pods is 2.8 g/100g, which is comparable to winged bean pods, whereas vitamin C content in yard long bean pods is 18.8 mg/100g, which is less than winged bean pods (U.S. Department of Agriculture 2018b). Furthermore, the protein content of winged bean seeds is 32.8 g/100 g, which is not much different from soybean seeds

(36.5 g/100 g) (Rukmana 2000; U.S. Department of Agriculture 2018a). Winged bean pods contain variety of nutrients that are crucial as a food plant such as crude protein (1.9 g/100 g - 3.0 g/100 g) and vitamin C (21 mg/100 g - 37 mg/100 g). In addition, winged bean pods also contain carbohydrates, fiber, fat, calcium, phosphorus, sodium, potassium, iron, vitamin A, vitamin B1, vitamin B2, and niacin (Kadam et al. 1984). The winged bean parts that can be consumed as food are its green pods, young seeds, root tubers, leaves and mature seeds (Mohanty et al. 2020).

Cultivation problems, however, often occur in vines such as difficulties in caring for creeping plants so that efforts to limit the growth of climbing stems are necessary. Growth inhibition can be applied to shorten the stem but still maintains the quality of the winged bean organs that are used such as seeds, fruit or young leaves. With semi-dwarf or dwarf plants, it will be easier to take care of the plants and do the harvest. Paclobutrazol is one of the compounds from the triazole group which has been known as a growth retardant. This compound has a ring structure containing three nitrogen atoms, chlorophenyl and a carbon side chain (Desta & Amare 2021). Paclobutrazol works by inhibiting the process of *ent*-kaurene oxidation to *ent*-kaurenoic acid through the inactivation of cytochrome P450-dependent oxygenase so that endogenous GA3 (gibberellin) levels decrease. Paclobutrazol causes morphological side effects, namely reducing plant height, inhibiting stem internodal elongation, and reducing the number of leaves (Soumya et al. 2017). Nevertheless, paclobutrazol is one of the compounds that can make the leaf color darker. This is caused by increased levels of chlorophyll or compaction of chlorophyll in the smaller parts of the leaves (Fletcher et al. 2000). In addition, it has been reported that in mangoes plant paclobutrazol can accelerate flowering time, total fruit per tree and average fruit weight (Yeshitela et al. 2004). The application of paclobutrazol can alter the partitioning of assimilate and nutrient supply in plants (Desta & Amare 2021). Paclobutrazol was reported to be able to increase vitamin C levels in *Curcuma alismatifolia* leaves at a concentration of 1500 ppm (Jungklang et al. 2017), whereas paclobutrazol application at a dose of 125 mg/L can increase protein levels in *Camelina sativa* (Kumar et al. 2012). Paclobutrazol can increase stomatal density by 15% - 17% on the abaxial and adaxial leaf surfaces of Quinoa (*Chenopodium quinoa*) at a dose of 20 mg/L (Waqas et al. 2017).

This research was conducted to evaluate the effect of paclobutrazol application on the growth, development, some phytochemical contents from young pods, and pod yields of winged bean (*Psophocarpus tetragonolobus* (L.) DC.). Furthermore, the purpose of this study was aimed to determine the appropriate concentration of paclobutrazol for reducing vine growth but yet still maintaining the yield quality of winged beans.

MATERIALS AND METHODS

This experiment was carried out at the Sawitsari Research Station, Faculty of Biology, Universitas Gadjah Mada (UGM), Condongcatur, Depok District, Sleman Regency, Special Region of Yogyakarta. Measurement for physiological and anatomical parameters, chlorophyll and vitamin C content were carried out at the Plant Physiology Laboratory, Faculty of Biology, Universitas Gadjah Mada. Testing for protein levels in winged bean pods was carried out at the Department of Food Technology and Agricultural Products, Faculty of Agricultural Technology, Universitas Gadjah Mada. This study used Completely Randomized Design (CRD) with one factor, namely paclobutrazol, which was applied at four different concentrations, namely 0 ppm (control), 25 ppm, 50 ppm, 75 ppm or 100

ppm. Three replicates were prepared for each treatment.

Winged bean seeds were soaked in warm water for 12 hours. Seeds were then sown in a plastic germination tray filled with planting medium. The planting medium contains a mixture of manure, soil, husk charcoal, cocopeat and bamboo leaf humus. Water (as a control) or paclobutrazol was sprinkled on the media as much as 5 mL. Winged bean seedlings were then transferred to polybags that had been filled with a mixture of soil, compost, and husk charcoal at a ratio of 2:1:1 per polybag when they reached the age of 2 weeks after seed sowing. Total weight of planting medium is 5 kg per polybag and one seedlings was planted in each polybag. Bamboo stick of 1.5 meters high was prepared and plugged into the soil as a place for the vine to grow and climb. Paclobutrazol was applied again every two weeks until 8 weeks after planting (WAP) and each plant received 50 mL. Watering was carried out regularly every two days. Additional NPK fertiliser at a ratio of 15:15:15 was applied with 10 grams doses per polybag. Pests and diseases were controlled with pesticides. The parameters observed were plant height, leaf number, leaf area, flowering time, pod length, total pods per plant, fresh weight of fruit, stomatal density, chlorophyll content, vitamin C content, and protein content in fruit.

Measurement of Growth and Development Parameters

Plant height and leaf number were measured manually from 2 to 7 WAP. Plant height was measured with tape measure, from the surface of growth medium to the highest shoot tip. Flowering time was calculated from time of seed sowing to the first flower emerged. The length of the fruit (pod) was measured by marking the raffia string according to the indentation of the fruit and the length of the rope is measured with a tape measure. The number of pods was counted manually for each plant. The fruit (pod) fresh weight was determined by weighing on an analytical balance. Leaf area was measured using the gravimetric method (Irwan & Wicaksono 2017). The formula for calculating leaf area is as follows:

$$\text{Leaf area} = \frac{\text{weight of leaf replica (g)}}{\text{weight of paper cut 10 cm x 10 cm (g)}} \text{ x } 100 \text{ cm}^2$$

Stomatal Density Measurement

The 3rd leaf from the shoot was used in making this preparation. Epidermal incisions were made on the lower surface of the leaves and fixed in 70% alcohol. Preparation for sample's dyeing was carried out using safranin reagent for one minute. The sample was placed on top of the object glass and covered with a cover glass. Observations were made under a microscope with a magnification of 40 x 10. Pictures of sample 's stomata were taken with Optilab v.3.0 software. Calculation of the number of stomata and the view area was carried out with the ImageRaster v.3.0 application. Stomatal density was calculated based on Lestari (2006) with the following formula:

$$\text{Stomatal Density} = \frac{\text{Number of stomata}}{\text{Unit of view area}}$$

Measurement of Chlorophyll Levels

Winged bean leaves were weighed as much as 0.1 gram and then mashed using a mortar and pestle and added with 10 mL of 80% acetone. Then the solution was filtered using Whatmann filter paper No. 3 and the volume was adjusted to 10 mL with 80% acetone. The absorbance value of the leaf extract was measured at a wavelength of 663 nm and 646 nm using a Spectrophotometer. Chlorophyll levels were calculated based on

Harborne (1998) with the following formula:

Total chlorophyll (mg/L) = $17.3A_{646} + 7.18A_{663}$

Chlorophyll *a* (mg/L) = $12.21A_{663} - 2.81A_{646}$

Chlorophyll *b* (mg/L) = $20.13A_{646} - 5.03A_{663}$

Conversion to (mg/g) = $\frac{10/1000 \; x \; Chlorophyll \; Levels}{0.1 \; mg/g}$

Measurement of Vitamin C Levels

Vitamin C levels were measured using the iodometric titration method based on Sudarmadji et al. (1984). A young fruit sample of 10 grams was mashed and diluted by adding distilled water to a volume of 100 mL. The sample solution was filtered using Whatmann filter paper No. 3 and then 5 mL of the filtrate was taken. A 125 mL Erlemeyer flask was filled with the filtrate from the sample and 2 mL of 1% starch solution was added. The titration was carried out by the iodometric method with a standard 0.01 N iodine solution. The titration was ended when the color of the solution change to a blue color constantly. Vitamin C levels were calculated based on Sudarmadji et al. (1984) with the following formula:

$$\text{Vit. C level (mg/100 g)} = \frac{\text{Viod x 0.88 mg x fp x 100}}{\text{sample weight(w)}}$$

Notes:

V Iod = volume iodin (mL)

1 mL 0.01 N Iod = 0.88 mg ascorbic acid

w = sample weight (gram)

fp = dilution factor

Measurement of Protein Levels

The young winged bean pods were mashed using a blender. After that, the sample was weighed as much as 100 mg. Furthermore, the protein content of the sample was measured based on the micro Kjeldhal method as described in AOAC 981.10 (Latimer 2016). Protein levels were calculated based on Sudarmadji et al. (1984) with the following formula:

$$\% \text{ N} = \frac{(\text{mL NaOH blank} - \text{mL NaOH sample})}{\text{sample weight (g)x 1000}} \text{ x 100 x 14.008}$$

$$\% \text{ protein} = \% \text{ N x factor}$$

Notes:

w = sample weight (gram)

% N = nitrogen percentage

Factor = 6.25

Data Analysis

Quantitative data were analysed using Microsoft Excel and SPSS version 25 software, with a significance of 5%. If the One Way ANOVA results are significantly different (F count > F table), then the analysis was continued with the DMRT test (Duncan Multiple Range Test) at the 95% confidence level.

RESULTS

The Effect of Paclobutrazol Application on the Growth of Winged Bean (*Psophocarpus tetragonolobus* (L.) DC.)

Paclobutrazol treatment reduced plant height and leaf number of winged bean. The higher the concentration of paclobutrazol applied, the greater the reduction in plant height and leaf number were observed (Table 1).

Table 1. The effect of paclobutrazol on plant height and leaf number of winged bean (*Psophocarpus tetragonolobus* (L.) DC.) at 7 WAP.

Treatment	Plant height (cm)	Leaf number (sheet)
P0 (control)	251.17 ± 17.39^{d}	70.67 ± 4.04^{b}
P1 (25 ppm)	215.07 ± 9.86^{c}	57.33 ± 10.69^{ab}
P2 (50 ppm)	143.67 ± 29.91^{b}	54.33 ± 11.72^{a}
P3 (75 ppm)	9.53 ± 3.08^{a}	51.67 ± 3.79^{a}
P4 (100 ppm)	8.60 ± 0.53^{a}	49.67 ± 3.06^{a}

Note: The same letter shows results that are not significantly different in the DMRT test with a significance of 5%.

Figure 1. Morphology of winged bean leaves treated with paclobutrazol at various concentration: (a) 0 ppm (b) 25 ppm (c) 50 ppm (d) 75 ppm (e) 100 ppm.

From Figure 1 it is clear that the trifoliate leaf of winged bean treated with paclobutrazol also become smaller compared to control. The measurement of leaf area showed that the higher concentration of paclobutrazol applied, the greater reduction in leaf area were observed (Table 2).

Table 2. Effect of paclobutrazol on winged bean (*Psophocarpus tetragonolobus* (L.) DC.) leaf area.

Treatment	Leaf Area (cm^2)
P0 (control)	116.56 ± 6.71^{c}
P1 (25 ppm)	95.40 ± 11.60^{b}
P2 (50 ppm)	89.81 ± 11.15^{b}
P3 (75 ppm)	70.70 ± 8.09^{a}
P4 (100 ppm)	68.66 ± 7.00^{a}

Note: The same letter shows results that are not significantly different in the DMRT test with a significance of 5%.

Further ANOVA and DMRT tests, showed that there were significant effect due to the application of various paclobutrazol concentrations on reducing plant height and leaf number of winged bean plants (α<0.05) (Table 1). The leaf area of plants treated with paclobutrazol were significantly smaller compared to control (α<0.05) (Table 2).

Effect of Paclobutrazol on the Development of Winged Bean (*Psophocarpus tetragonolobus* (L.) DC.)

Based on ANOVA and DMRT tests, there was a significant effect of paclobutrazol on the initiation of flowering ($\alpha<0.05$) (Table 3). The fastest flowering time occurred in those plants treated with 50 ppm paclobutrazol, which was 45.33 days (Table 3).

Table 3. Effect of paclobutrazol on winged bean (*Psophocarpus tetragonolobus* (L.) DC.) flowering time.

Treatment	Flowering Time (DAP)
P0 (control)	50.00 ± 1.00^{b}
P1 (25 ppm)	51.33 ± 1.53^{bc}
P2 (50 ppm)	45.33 ± 0.58^{a}
P3 (75 ppm)	52.00 ± 1.73^{bc}
P4 (100 ppm)	52.33 ± 0.58^{c}

Note: The same letter shows results that are not significantly different in the DMRT test with a significance of 5%

The application of various concentrations of paclobutrazol gave a significant decrease on the number of pods per plant according to statistical tests, ANOVA and DMRT tests ($\alpha<0.05$) (Table 4).

Table 4. Effect of paclobutrazol on the number of winged bean (*Psophocarpus tetragonolobus* (L.) DC.) pods per plant.

Treatment	Number of Pod (fruit)
P0 (control)	15.00 ± 5.69^{c}
P1 (25 ppm)	9.00 ± 4.00^{b}
P2 (50 ppm)	8.33 ± 1.53^{b}
P3 (75 ppm)	7.33 ± 2.52^{b}
P4 (100 ppm)	2.33 ± 0.58^{a}

Note: The same letter shows results that are not significantly different in the DMRT test with a significance of 5%.

From Figure 2, it can be seen that the application of paclobutrazol of 25 ppm, 50 ppm or 75 ppm only slightly affected pod length but paclobutrazol of 100 ppm decreased pod length. It is shown that pod length of those plants treated with paclobutrazol up to 75 ppm are similar to control but pod length of plants treated with 100 ppm decreased significantly compared to control (Table 5). However, the fresh weight of pod decreased according to the increase in the concentration of paclobutrazol applied (Table 5).

Table 5. Effect of paclobutrazol on winged bean (*Psophocarpus tetragonolobus* (L.) DC.) pod size.

Treatment	Length of pod (cm)	Fresh weight per fruit (gram)
P0 (control)	17.83 ± 1.48^{b}	21.47 ± 1.13^{c}
P1 (25 ppm)	16.80 ± 1.22^{b}	14.13 ± 1.01^{ab}
P2 (50 ppm)	17.50 ± 0.36^{b}	16.33 ± 2.10^{b}
P3 (75 ppm)	15.20 ± 3.15^{ab}	9.58 ± 5.08^{a}
P4 (100 ppm)	12.47 ± 1.27^{a}	9.26 ± 1.38^{a}

Note: The same letter shows results that are not significantly different in the DMRT test with a significance of 5%.

Figure 2. Morphology of winged bean pods treated with paclobutrazol at various concentration: (a) 0 ppm (b) 25 ppm (c) 50 ppm (d) 75 ppm (e) 100 ppm.

Pod length and fruit fresh weight of winged bean plants decreased significantly with increasing application of paclobutrazol concentration according to ANOVA and DMRT test results ($\alpha<0.05$) (Table 5).

Effect of Paclobutrazol on Stomatal Density of Winged Bean (*Psophocarpus tetragonolobus* (L.) DC.)

Using epidermal incisions on the lower surface (abaxial) of the treated winged bean leaves at different concentrations of paclobutrazol, Figure 3 shows the observed illustrations of the stomata structure. From Figure 3, it could be observed that the treated leaf with the higher concentration of paclobutrazol (50 ppm, 75 ppm, and 100 ppm) had a higher number of stomata than the treated leaf with a lower concentration of paclobutrazol (25 ppm) and the control. Higher stomatal density is directly proportional to the higher number of stomata. The application of various concentrations of paclobutrazol had a significant effect on increasing the stomata density on the abaxial (lower) leaf surfaces of winged bean leaves ($\alpha<0.05$) (Table 6). The highest density of stomata was found in those plants treated with 50 ppm paclobutrazol, namely 30.111 Σ/mm^2 whereas paclobutrazol of 75 ppm or 100 ppm also increased stomata density of winged bean leaf compared to the control (0 ppm) or those plants treated with 25 ppm paclobutrazol (Table 6).

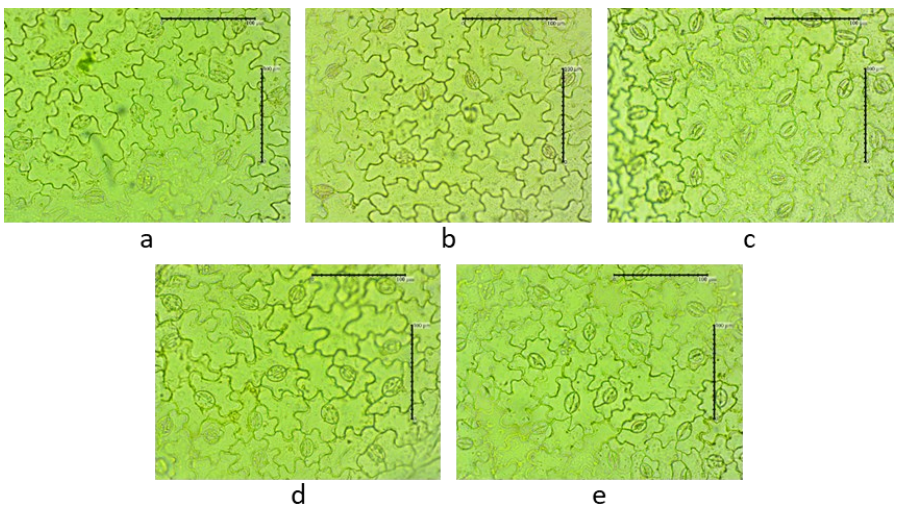

Figure 3. Anatomy of the stomata of winged bean leaves treated with

paclobutrazol at various concentration: (a) 0 ppm (b) 25 ppm (c) 50 ppm (d) 75 ppm (e) 100 ppm.

Table 6. Effect of paclobutrazol on stomatal density on the abaxial (lower) surface of winged bean leaves (*Psophocarpus tetragonolobus* (L.) DC.).

Treatment	Stomatal Density(Σ/mm²)
P0 (control)	15.526 ± 1.411[a]
P1 (25 ppm)	18.349 ± 3.293[a]
P2 (50 ppm)	30.111 ± 6.587[b]
P3 (75 ppm)	26.504 ± 2.757[b]
P4 (100 ppm)	29.954 ± 3.338[b]

Note: The same letter shows results that are not significantly different in the DMRT test with a significance of 5%.

Effect of Paclobutrazol on some Phytochemicals Levels of Winged Bean (*Psophocarpus tetragonolobus* (L.) DC.) Pods

ANOVA and DMRT test results proved that the application of paclobutrazol had a significant effect on increasing chlorophyll levels in winged bean pods ($\alpha<0.05$) (Table 7). The highest total chlorophyll was 1.144 mg/g found in plants treated with 100 ppm paclobutrazol. On the contrary, the application of various concentration of paclobutrazol had a significant effect on reducing vitamin C levels as well as protein levels in winged bean pods ($\alpha<0.05$) (Table 8).

Table 7. Effect of paclobutrazol on chlorophyll content of winged bean (*Psophocarpus tetragonolobus* (L.) DC.) leaves.

Treatment	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total of Chlorophyll (mg/g)
P0 (control)	0.049 ± 0.045	0.027 ± 0.026	0.076 ± 0.072[a]
P1 (25 ppm)	0.090 ± 0.016	0.059 ± 0.004	0.149 ± 0.020[a]
P2 (50 ppm)	0.286 ± 0.010	0.141 ± 0.006	0.426 ± 0.004[b]
P3 (75 ppm)	0.363 ± 0.067	0.179 ± 0.023	0.541 ± 0.090[c]
P4 (100 ppm)	0.727 ± 0.009	0.418 ± 0.017	1.144 ± 0.018[d]

Note: The same letter shows results that are not significantly different in the DMRT test with a significance of 5%.

Table 8. Effect of paclobutrazol on vitamin C and protein levels in winged bean (*Psophocarpus tetragonolobus* (L.) DC.) pods.

Treatment	Level of Vitamin C (mg/100 g)	Level of Protein (%)
P0 (control)	91.91 ± 17.92[b]	2.70 ± 0.02[d]
P1 (25 ppm)	56.71 ± 8.96[a]	2.40 ± 0.03[c]
P2 (50 ppm)	48.89 ± 6.77[a]	2.34 ± 0.02[b]
P3 (75 ppm)	54.76 ± 3.39[a]	-
P4 (100 ppm)	54.77 ± 12.21[a]	2.00 ± 0.04[a]

Note: The same letter shows results that are not significantly different in the DMRT test with a significance of 5%, no sample was available at harvest for protein level test in those plants treated with 75 ppm paclobutrazol.

DISCUSSION

The Effect of Paclobutrazol on the Growth of Winged Bean (*Psophocarpus tetragonolobus* (L.) DC.)

The results of this study proved that the higher the concentration of paclobutrazol applied to the winged bean (*Psophocarpus tetragonolobus* (L.)

DC.), the plant height declined in those plants applied with 75 ppm or 100 ppm paclobutrazol (Table 1). This is caused by the action of paclobutrazol which inhibits the activity of enzyme that catalyzed oxidation of *ent*-kaurene to *ent*-kaurenoic acid, namely the cytochrome p450-dependent oxygenase enzyme, often called *ent*-kaurene oxidase (Soumya et al. 2017). Consequently, the KO enzyme (*ent*-kaurene oxidase) cannot catalyze the conversion of *ent*-kaurene to *ent*-kaurenol, the conversion of *ent*-kaurenol to *ent*-kaurenal and the conversion of *ent*-kaurenal to *ent*-kaurenoic acid (Olszewski et al. 2002; Soumya et al. 2017). In plants treated with paclobutrazol, the cells may continue to divide even though the level of endogenous gibberellins was reduced. However, cell elongation was hampered due to inhibition of gibberellin biosynthesis in the subapical meristem. This can reduce the rate of cell division and cell elongation. As it was reported in other species, plants treated with paclobutrazol produced stems with shorter internodes and become stunted (Meena et al. 2014).

The results of this study showed that the higher the concentration of paclobutrazol, the fewer the number of leaves produced by the winged bean (Table 1). This is probably caused by the reduction in gibberellin as well as auxin biosynthesis in those plants treated with paclobutrazol and it slow down the leaf growth. It has been reported that application of paclobutrazol may increases cytokinin levels that normally play a role in leaf morphogenesis (Fletcher et al. 2000). Cytokinins play a role in triggering the maintenance of SAM size, structure, and activity to do proliferation and growth. It contributes to the control of leaf primordia development by preventing stem cell differentiation. However, cytokinins also affecting the establishment of the auxin gradient by regulating auxin biosynthesis and transport (Wu et al. 2021). As paclobutrazol was applied at the early seedling stage by soil drenching, it seems that severe reduction in both gibberellin and auxin leads to the reduction of leaf number especially in those plants treated with paclobutrazol of 50 ppm, 75 ppm or 100 ppm. The increase in cytokinin seems manifested in the increase of chlorophyll content and delaying the leaf senescence in those plants treated with paclobutrazol.

In this study the leaf number of those plants treated with either 25 ppm, 50 ppm, 75 ppm or 100 ppm paclobutrazol was not significantly different statistically even though the value tends to decrease as the concentration of paclobutrazol applied to the plants increased. This is because the number of leaves is influenced by genetic and environmental factors (Tumewu et al. 2012).

The results of this study indicated that the higher the concentration of paclobutrazol applied, the smaller leaf area of winged bean observed (Table 2). Application of paclobutrazol reduces gibberellin levels, thus reducing the intensity of proliferation and expansion of leaf plate meristem cells (Du et al. 2018). The plate meristem is one of the leaf meristem tissues, which is located at the base of leaf blade primordia, long-lived, consisting of parallel layers of anticlinal dividing cells. It has been implied that paclobutrazol plays a major role in inhibiting leaf morphogenesis, including growth of leaf size until the mature phase (Yeshitela et al. 2004; Du et al. 2018).

Effect of Paclobutrazol Application on the Development of Winged Bean (*Psophocarpus tetragonolobus* (L.) DC.)

The results of this study show that the paclobutrazol treatment of 25 ppm, 75 ppm or 100 ppm leads to the initiation of flowering in winged bean that was not significantly different compared to control, the fastest

flowering time was found in plants treated with paclobutrazol of 50 ppm (Table 3). This is probably caused by paclobutrazol which already limits vegetative growth due to inhibition of gibberellin biosynthesis, but there is an accumulation of photosynthate in the stroma of the chlorophyll. The photosynthate will be used to support vegetative growth initially, but as vegetative growth of those plants treated with 50 ppm paclobutrazol was inhibited then photosynthate is allocated to induce shoots to enter generative growth (Wardani et al. 2020). In the winged bean treated with 50 ppm paclobutrazol, flowering was earlier because plant height can be reduced by about 42.8% compared to the control. The photoassimilate is sufficient for vegetative growth and then it was seems to be allocated for the formation of flowers.

However, in winged bean applied with higher concentration of paclobutrazol (75 ppm or 100 ppm) flowering time was slightly delayed compared to control. This is probably caused by limited levels of gibberellins that found in those plants. Besides that, flowering initiation is influenced by genetic and enviromental factors that regulate flowering patterns. When the winged bean has not yet reached the mature phase, delayed flowering initiation may occured because of paclobutrazol treatment (Harpitaningrum et al. 2014). In addition, each plant possibly has a different levels of sensitivity to paclobutrazol treatment (Ardigusa & Sukma 2015). Plant height in those winged bean treated with 75 ppm and 100 ppm paclobutrazol was significantly reduced by about 96.2% and 96.6%, respectively. It indicated that the plants were severely stunted. In addition, the number of leaves was also reduced in those plants. Consequently, the amount of photosynthate produced was drastically reduced and it is still be used to support vegetative growth rather than generative growth. The application of paclobutrazol in this study resulted in the number of pods per plant of winged bean appearing less (Table 4). This is probably due to the paclobutrazol that also make assimilate transport ineffective because the phloem cells may become smaller or denser. Application of paclobutrazol shortens the distance between internodes or stem internodes. The leaves emerge from the nodes of the stems, resulting in densely growing leaves that narrow the leaf's light-receiving area. There is a possibility of reduced photosynthetic activity so that the supply of carbohydrates for flower and fruit formation is reduced. The number of fruit that is successfully formed depends on the amount of available photosynthetic products, namely carbohydrates. Carbohydrates have a role as a food supply which is stored in winged bean pods. Competition for photosynthetic assimilation also occurs in flowers and fruit in the same plant. The imbalance between supply and demand makes flowers more at risk of falling on stunted plants treated with paclobutrazol as it was reported in *Cucumis sativus* (Harpitaningrum et al. 2014). This also results in less pod production in dwarf winged bean plants.

The results of the study showed that the higher the concentration of paclobutrazol, the shorter the length of the winged bean fruit (Table 5). The decrease in winged fruit length was not significantly different at various concentration of paclobutrazol, namely 0 ppm, 25 ppm, 50 ppm and 75 ppm. It can be caused by several factors, namely genetic factors, environmental habitat, and treatment at harvest. Genetic factors may be very dominant in influencing fruit length growth compared to environmental factors and growth inhibitory substances which help to increase assimilate translocation to sink organs, such as those found in cucumber (*Cucumis sativus* L.) venus cultivars that were treated with paclobutrazol (Harpitaningrum et al. 2014). Treatment of 100 ppm

paclobutrazol reduced fruit length because of paclobutrazol concentration that were too high could reduce the rate of cell division, weight gain, and cell size. As a result, fruit growth becomes stunted so they don't grow bigger. Similar finding has been reported in water melon treated with paclobutrazol (Jasmine et al. 2014).

The results also showed that the higher the concentration of paclobutrazol, the fresh weight per fruit of winged bean was lighter (Table 5). This may be due to the influence of paclobutrazol which already acting since growth of seedlings. Paclobutrazol inhibits gibberellins biosynthesis, especially in the *ent*-kaurene oxidation phase. Paclobutrazol can also increase ABA biosynthesis due to the accumulation of precursors in the terpenoid pathway which triggers its biosynthesis (Soumya et al. 2017). The increased concentration of ABA causes stomatal guard cells to lose ions, followed by loss of turgor and water which ends with stomata closing (Hopkins & Hüner 2009). According to Chaves et al. (2009) in Wardani et al. (2022), closing stomata causes photosynthesis to be hampered due to decreased stomatal conductance. As a result, the amount of assimilates (carbohydrates) that will be translocated to sinks decreases, so that the fresh weight of sink organ (fruit) produced is less.

Effect of Paclobutrazol on Stomatal Density of Winged Bean (*Psophocarpus tetragonolobus* (L.) DC.)

The stomata of winged bean leaves which are located on the abaxial side of the leaves have a parasitic type (Figure 3). Parasitic stomata are stomata that have a single neighboring cell with a parallel arrangement of the connecting walls to the guard cells (Hostettmann 2014). The results of this study show that the higher the concentration of paclobutrazol, the greater the number of stomatal density of winged bean leaves (Table 6). The application of paclobutrazol at 50 ppm is best for increasing stomatal density. However, the application of paclobutrazol at 75 ppm and 100 ppm produced stomatal density that was not significantly different from the 50 ppm paclobutrazol treatment. This may be caused by the application of paclobutrazol that can increase cytokinin biosynthesis. Paclobutrazol is one of the compounds from the triazole group with the performance of inhibiting the cytochrome P-450 dependent monooxygenase enzyme which plays a role in gibberellin biosynthesis. This resulted in the inhibition of the catalytic reaction in a stepwise oxidation reaction involving the conversion of *ent*-kaurene to *ent*-kaurenoic acid. This inhibition results that one of the precursor for isoprenoid pathway, namely isopentenyl diphosphate, is not used for gibberellin biosynthesis. Consequently, isopentenyl diphosphate is diverted for cytokinin biosynthesis (isopentyl adenosine) so cytokinin levels increase (Fletcher et al. 2000). Cytokinin is one of the phytohormones that has several roles in regulating cell division and differentiation by influencing the control of the cell division cycle (Taiz & Zeiger 2010). It has been reported that paclobutrazol causes the stimulation of division and differentiation of leaf epidermal cells to become stomata (Willmer & Fricker 1996; Hostettmann 2014).

Stomatal density is the number of stomata per unit area of the leaf surface. Stomatal density affects the amount of CO_2 that enters or is fixed into the leaf for photosynthesis and regulates transpiration, in which the water vapor will diffuse out of the leaf. Both of the processes affect plant productivity. CO_2 acts as the reactant on the Calvin cycle or dark reaction of photosynthesis. The Calvin cycle begins with the fixation of CO_2 with the acceptor that called RuBP (ribulose-1,5-bisphosphate) and pro-

duces phosphorylated carbon intermediates (glyceraldehyde-3-phosphate) which will become photo-assimilate. This photo-assimilate will be converted to sucrose in the mesophyll vacuole or starch in the chloroplast stroma. Sucrose and starch will be translocated to non-photosynthetic organs and tissues. Excessive transpiration causes productivity decline. This is due to the function of water as a temperature regulator in plants cells, a suitable medium for absorbing and distributing soluble compounds, and a reactant or product in the biochemical reactions (Hopkins & Hüner 2009). The results of the study proved that enhancement of stomata density may support plant growth and development, especially at the initiation of flowering.

Effect of Paclobutrazol on Phytochemical Levels of Winged Bean (*Psophocarpus tetragonolobus* (L.) DC.) Pods

The results of this study show that the higher the concentration of paclobutrazol, the higher the chlorophyll content of winged bean leaves (Table 7). Winged bean leaves that were applied with paclobutrazol became darker in color. This is due to the performance of paclobutrazol which inhibits gibberellin biosynthesis by inhibiting the cytochrome p450-dependent oxygenase enzyme. The geranylgeranyl pyrophosphate precursor accumulates from the terpenoid pathway so that it can be used for the synthesis of phytol, which is a precursor compound for the chlorophyll component (Chaney 2005; Soumya et al. 2017).

In addition, the results in Table 8 show that the higher the concentration of paclobutrazol, the lower the vitamin C content in winged bean young pods. This may be caused by the application of paclobutrazol which shortens the internodal distance. The effect of paclobutrazol on vitamin C is probably derived from the amount of photosynthate produced. Photosynthesis plays a role in producing soluble carbohydrates. Soluble carbohydrates are needed as precursors of vitamin C biosynthesis such as D-glucose-6-phosphate (Paciolla et al. 2019). Extreme dwarfing of plants results in a drastically reduced amount of photosynthate produced. As a result, the vitamin C content of young winged bean pods is reduced due to lack of precursors for biosynthesis.

The results also show that the higher the concentration of paclobutrazol, the lower the protein content in winged bean young pods (Table 8). The application of paclobutrazol on winged bean causes an increase in leaf chlorophyll content. The increase in chlorophyll accumulation supposedly causes the rate of photosynthesis to increase thereby affecting assimilate translocation (Sambeka et al. 2012). Nitrogen is one of the elements that make up the chlorophyll and protein components (Hopkins & Hüner 2009). The availability of N in plant organs is obtained from absorption of N from the soil or remobilisation of N from source organs to sink organs (Tekalign & Hammes 2005). Photo-assimilate competition for nitrogen supply occurs between chlorophyll synthesis for leaves and protein synthesis for pods. The supply of photo-assimilates and nitrogen may be more focused on increasing leaf chlorophyll synthesis in those plants treated with paclobutrazol. The imbalance between supply and demand causes protein levels in winged bean pods to decrease.

CONCLUSIONS

Paclobutrazol has a significant effect on the growth of winged beans, which reduces the plant height, leaf number, leaf area. Paclobutrazol affects on the yield of winged bean pods significantly, which reduces number of pods per plant, pod length and fresh weight per fruit. Paclobutrazol has a significant effect on reducing the levels of winged bean pod phytochemicals, namely levels of vitamin C and protein levels.

Paclobutrazol application gave the best results in increasing the stomatal density, enhancing chlorophyll content and accelerating flower initiation. The flowering time of winged beans can be accelerated by the application of paclobutrazol at 50 ppm. The chlorophyll content of winged bean leaves tend to increase by paclobutrazol application. The stomatal density of the abaxial surface of winged bean leaves was highest with paclobutrazol application of 50 ppm.

AUTHORS CONTRIBUTION

J.S.W. collected and analysed the data, as well as wrote and revised the manuscript. K.D. designed and supervised the research processes, critically proofread the manuscript writing and participated in revising the manuscript.

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CONFLICT OF INTEREST

There is no conflict of interest regarding research and research funding.

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Research Article

Metal Bioaccumulation in Albino Rat Tissues Treated with Decontaminated Sea Lettuce (*Ulva lactuca* L.)

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ABSTRACT

Ulva lactuca is a macroalgae that contains high nutritional values. The heavy metal contaminants in natural *Ulva lactuca* needs to be eliminated or decreased using natural agent. The aim of this research was to determine the bioaccumulation of Pb, Cd, Hg, and the impact on liver and gastrointestinal function. Parameters of this research were Hepatosomatic Index (HSI), SGPT levels, SGOT levels, bioaccumulation Pb, Cd, Hg, and histological structure of liver and small intestine. Besides that, the progression of body weight was observed. Twelve female Wistar rats (*Rattus norvegicus* Berkenhout, 1769) were randomly assigned to three groups: Control, NU (treated with natural *Ulva lactuca*), and DU (treated with heavy metal decontaminated *Ulva lactuca* using *Averrhoa bilimbi* juice).Treatment was carried out orally at a dose of 1000 mg/Kg BW/day for 30 days. Histological structure of rat's liver and small intestine were prepared after necropsy at the end of this research. Based on results, it can be concluded that there were no significant differences observed in HSI, SGPT, and SGOT levels among the groups. However, there was a tendency for an increase in total bilirubin levels in the decontaminated *Ulva lactuca* group. Both natural and heavy metal decontaminated *Ulva lactuca* showed histological damage on liver and small intestine. Bioaccumulation of Cd and Hg in the liver and gastrointestinal tract of rats after consuming decontaminated *Ulva lactuca* was lower than the natural *Ulva lactuca* group, but need more observations.

INTRODUCTION

Ulva lactuca or sea lettuce is a species of green algae that has been widely used by humans. *Ulva lactuca* has been used in agriculture, cosmetics, bioremediation, food, and biomedical sector (Rasyid 2017). It contains many vitamins, proteins, carbohydrates, and minerals that are beneficial to the body. *Ulva lactuca* also has the potential as a nutraceutical candidate such as anticoagulant, anticancer, and antibacterial, because it has high levels of antioxidants. The biochemical composition in *Ulva lactuca* is strongly influenced by climatic conditions, geographical location, and the local environment (Yaich et al. 2011).

Ulva lactuca is a bioindicator of marine pollution because it is widely distributed along the coast which is susceptible to water pollution and accumulation of heavy metals (Ardiyansyah et al. 2019). *Ulva lactuca* fits numerous features that make it one of the best bioindicators of marine

pollution. Previous research by our teams since 2019 from Ngrumput and Sepanjang Beach, Gunungkidul, Yogyakarta proved that *Ulva lactuca* was contaminated with heavy metals such as Pb, Cd, and Hg. The Cd content is known to be 0.59 mg/Kg dry weight, exceeding the *Badan Pengawas Obat dan Makanan* (BPOM)'s threshold for Cd contamination in seaweed, which is 0.2 mg/Kg.

The investigation persisted in 2021, focusing on the decontamination of heavy metals such as Pb, Cd, and Hg in *Ulva lactuca* using a 15% filtrate of bilimbi (*Averrhoa bilimbi*) for a duration of 60 minutes. Heavy metals can be reduced using bilimbi filtrate as a decontaminant because bilimbi contains citric acid as a sequestrant which binds with polyvalent metal ions, forming a complex compound. This enhances the stability and quality of food.

The functional groups -OH and COOH of citric acid play a role in the binding of citrate ion with metal ions that results in the accumulation of citrate complexes (Nurhayati & Navianti 2017). In the *Ulva lactuca* group subjected to washing with distilled water, the Pb content measured 0.1857 mg/Kg dry weight, Cd content was <0.008 mg/Kg dry weight, and Hg content was 0.4 mg/Kg dry weight. Contrastingly, in the decontaminated *Ulva lactuca* group, Pb was recorded at 0.1369 mg/Kg dry weight, Cd at 0.008 mg/Kg dry weight, and Hg at 0.26 mg/Kg dry weight. The assessment of liver function involved monitoring levels of SGPT, SGOT, and bilirubin. The liver, an important organ in heavy metal detoxification, uses enzymes such as SGPT, SGOT, and bilirubin. Liver damage can be discerned through alterations in SGPT, SGOT, and bilirubin levels, serving as indicative parameters. Elevated levels of these three parameters may be used as indicators of liver damage.

Heavy metals can be accumulated and distributed at a higher trophic level through the food web. Consumption of *Ulva lactuca* that has been contaminated with heavy metals can lead to accumulation within the organs of organisms at higher trophic levels, particularly in the kidneys, liver, and digestive tract, resulting in adverse health effects (Ardiyansyah et al. 2019). Heavy metals are substances that are indigestible by the digestive system so they accumulate in the long term.

Lead can be distributed through blood and accumulates in the liver. Bioaccumulation of lead in rat livers causes liver cell damage degeneration and necrosis). Cadmium (Cd) is a heavy metal with high toxicity. Accumulation of cadmium in the body triggers an increase in reactive oxygen species (ROS) which causes damage to the liver and kidneys (Koyu et al. 2006). Cadmium exposure causes vacuolar, granular degeneration, and necrosis in rat livers (Yu et al. 2021).

Mercury (Hg) is the most toxic metal in the natural environment. Microbial activity in water has the capability to transform mercury into methylmercury (CH_3-Hg), a substance known for its toxic properties (Aba 2016). The bioaccumulation of mercury in rat livers can result in adverse effects such as cell swelling, hydropic degeneration, fatty degeneration, and necrosis. Mercury exposure causes edema and necrosis in the fish tilapia intestine (*Oreochromis niloticus*) (Kaoud & El-Dahshan 2010).

The objectives of this investigation were to assess the impact of the consumption of decontaminated *Ulva lactuca* on (i) liver function parameters, encompassing hepatosomatic index (HSI), as well as the levels of SGPT, SGOT, and bilirubin; (ii) the bioaccumulation of Pb, Cd, and Hg in the liver and gastrointestinal tract; and (iii) the histopathological changes in the liver and small intestines of female Wistar albino rats (*Rattus norvegicus* Berkenhout, 1769).

MATERIALS AND METHODS

Materials

Twelve female Wistar rats, aged 8 weeks and weighing between 150 and 200 grams, were procured from the Faculty of Pharmacy, Universitas Gadjah Mada, to serve as experimental subjects. The materials administered to the animals included *Ulva lactuca* sourced from Ngrumput Beach, Gunungkidul, D.I. Yogyakarta, and filtrate derived from bilimbi (*Averrhoa bilimbi*).

Methods

This research was conducted under the Ethical Clearance certificate number 00020/04/LPPT/VII/2022, issued by the Institutional Committee of Animal Use and Care (ICAUC) of The Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada.

Ulva lactuca Preparation

Ulva lactuca L. samples were collected from Ngrumput Beach, Gunungkidul, Yogyakarta, by the preceding research team in 2021. The acquired *Ulva lactuca* samples were subsequently categorized into two groups. The natural *Ulva lactuca* (NU) group comprised specimens that underwent washing with distilled water. The decontaminated *Ulva lactuca* (DU) group encompassed *Ulva lactuca* subjected to washing with distilled water and immersion in bilimbi (*Averrhoa bilimbi*) filtrate at a 15% concentration for 60 minutes. The samples were incubated for 3–7 days at a temperature range of 40–45°C until a consistent dry weight was achieved. The desiccated *Ulva lactuca* was then pulverized using a blender and filtered through a 60-mesh strainer to obtain a finer powder.

Animal Treatment

Female Wistar albino rats (*Rattus norvegicus* Berkenhout, 1769) were used as animal models and acclimatized for 14 days. Animals were kept in cages (44 x 35 x 50 cm^3) with shaved wood bedding, where each cage contains four rats. The animals' rooms were maintained at temperature range of 22–26°C and humidity range of 60–70% in the Animal House facility, Faculty of Biology, Universitas Gadjah Mada. Body weight of animals was measured once a week using a digital balance. This data was used to calculate the amount of *Ulva lactuca* and ketamine-xylazine cocktail needed for administration and anaesthesia, respectively. Body weight was also used to calculate Hepatosomatic Index (HSI).

Twelve female Wistar rats were randomly divided into 3 groups: Control (administered with 1.5 mL distilled water/day), NU (Natural *Ulva lactuca* at a dose of 1000 mg/Kg BW/day), and DU (Decontaminated *Ulva lactuca* with a dose of 1000 mg/Kg BW/day). The treatment involved the administration of a suspension containing *Ulva lactuca* powder at a dose of 1000 mg/Kg BW/day in 1.5 mL of distilled water via oral gavage. After 30 days of treatment, rats blood was taken and the rats were euthanized to collect their liver and gastrointestinal tract including stomach, small intestine, large intestine, and rectum for undergoing the histopathological preparations.

Measurement of Hepatosomatic Index (HSI)

The Hepatosomatic Index was calculated at the end of the study by weighing body weight and liver weight. The Hepatosomatic Index (HSI) was calculated using the formula (Albalat et al. 2019) as follows:

$$\text{HSI} = \frac{Liver\ weight}{Body\ Weight} x100\%$$

Liver Function Evaluation

Liver function evaluation was conducted by measuring Serum Glutamic Pyruvic Transaminase (SGPT), Serum Glutamic Oxaloacetic Transaminase (SGOT), and bilirubin levels in the blood serum. Blood samples were collected from the sinus orbitalis of rats on Day 0 (D_0) and Day 30 (D_{30}) after a fasting period of 6–8 hours. Animals were anesthetized using Ketamine (KTM-HCl®) and Xylazine (Holland®) in a cocktail (0.1 mL/100 g BW) by intramuscular administration. Blood samples were analyzed in The Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada using enzymatic photometric methods to obtain the SGPT, SGOT, and bilirubin levels.

Bioaccumulation of Lead, Cadmium, and Mercury

The animals were euthanized using double dosage of ketamine-xylazine cocktail on D_{30}. Liver and gastrointestinal organs including stomach, small intestine, large intestine, and rectum were collected and washed using NaCl 0.9% solution. Samples of 5 grams each were put into a 100 mL Erlenmeyer then added 15 mL HNO_3 70% and 5 mL $HClO_4$ 70%. After that, it was digested on a heating plate until dissolved and clear, continued until it was almost dry, then added 10 mL of distilled water and homogenized. The solution was then filtered in a 25 mL measuring flask, and distilled water was added up to the mark. The solution was used to determine the cadmium and lead levels using Atomic Absorption Spectrophotometer (AAS) and to determine the mercury level using Mercury Analyzer by the Integrated Research and Testing Laboratory (LPPT) Unit II, Universitas Gadjah Mada .

Histopathological Examination

Histological preparation was carried out using the paraffin method after 30 days of treatment. The liver and gastrointestinal organs, including the stomach, small intestine, large intestine, and rectum, were collected and washed using a 0.9% NaCl solution. Subsequently, they were fixed in 10% neutral buffered formalin (NBF) for 24 hours. Following fixation, the organs underwent dehydration using graded alcohol and were embedded in paraffin blocks. The right lobe of the liver and the distal part of the small intestine were sliced into 1 cm sections. The blocks were then sectioned at 4 µm and stained with hematoxylin and eosin (HE). The sections were observed under a light microscope (Leica Microsystem®) and photographed using a Leica LAS EZ (Leica Microsystem®).

Data Analysis

The research data obtained were Hepatosomatic Index (HSI), Serum Glutamic Pyruvic Transaminase (SGPT), Serum Glutamic Oxaloacetic Transaminase (SGOT), and bilirubin levels, the Pb, Cd, and Hg heavy metal levels in the liver and gastrointestinal tract of rats, as well as histopathological examination of the liver and small intestine. The rats body weight, HSI, SGOT, SGPT, dan bilirubin data were analyzed with One-Way ANOVA (Analysis of Variance) statistical test and Duncan's post hoc test with alpha = 5%. Paired-samples T-test conducted for D_0 and D_{30} of SGOT, SGPT, and bilirubin levels. Statistical test conducted using Statistical Package for the Social Sciences (SPSS) version 16. Heavy metal levels and histopathological examination were analyzed descriptively with reference to similar research journals.

RESULTS AND DISCUSSION

Body Weight

The result showed an increase in body weight in the Control, NU, and DU groups on the D_7 and D_{14}, further, there is a slight decrease in body weight in the Control group on D_{21} (Table 1). *Ulva lactuca* was proven to contain high vitamins, fats, carbohydrates (Rasyid 2017) so it can influence the body weight increase of the rats during treatment period. Consumption of *Ulva lactuca* that is high in Fe content 873.72 mg/kg (Mulyati et al. 2020) has potency to increase body weight.

Table 1 showed that there were no significant differences in body weight between groups on D_0 and D_7. However, there were significant differences between the Control and DU groups on D_{14}. There were no significant differences for the all group of the treatment period ($p>0,5$).

Hepatosomatic Index

The Hepatosomatic Index (HSI) is a value that can be used to describe the amount of toxic compounds that enter the body and gives the status of energy availability in the body (Yuneldi et al. 2018) (Table 2). This study showed that the HSI after the treatment period was not significantly different between treatments ($p>0.5$). However, the result showed that the NU and DU groups tend to have a higher hepatosomatic index than the Control group. A higher hepatosomatic index can be influenced by hepatic metabolic activity and consumption activity in rats so that the ratio increases.

Based on Table 2, this study shows that the HSI after the treatment period was not significantly different between treatments. However, the table shows that the NU and DU groups tend to have a higher hepatosomatic index than the Control group. This study used *Ulva lactuca* that have been decontaminated using bilimbi (*Averrhoa bilimbi*) filtrate. This decontamination process has reduced the level of lead contamination from 0.186 mg/Kg of *Ulva* powder to 0.1367 mg/Kg of *Ulva* powder (26.3%) and reduced the level of mercury from 0.40 mg/Kg of *Ulva* powder to 0.26 mg/Kg of *Ulva* powder (35%). Even though the levels of heavy metals have decreased, heavy metals are still found in the NU and DU groups within safe limits according to BSN (2009).

Serum Glutamic Pyruvic Transaminase (SGPT)

Serum Glutamic Pyruvic Transaminase (SGPT) or also known as Alanine Aminotransferase (ALT) is an enzyme found in hepatocytes that converts amino groups ($-NH_2$) from glutamate to pyruvate, and vice versa. Transamination is the removal of amino groups as a combination of amination and deamination (Ninkov et al. 2015). The increase or decrease of this enzyme's activity in a serum acts as an indicator of hepatocyte damage. The results of SGPT levels analysis are presented in Table 3.

Table 1. Body weight of female Wistar albino rats (*Rattus norvegicus* Berkenhout, 1769) after 30 days treatment.

Group	Body Weight (g)				
	D_0	D_7	D_{14}	D_{21}	D_{28}
Control	197.50 ± 22.06^a	207.75 ± 23.68^a	207.50 ± 22.90^b	206.00 ± 22.85^a	210.75 ± 23.31^a
NU	177.25 ± 15.58^a	183.25 ± 18.25^a	185.25 ± 18.68^{ab}	185.75 ± 17.73^a	188.25 ± 18.96^a
DU	165.25 ± 20.35^a	176.50 ± 19.49^a	176.00 ± 11.80^a	182.75 ± 21.30^a	187.00 ± 21.23^a

Data were presented as Mean ± Standard Deviation. The different superscript letters on the same column note a significant difference at $p < 0.05$ between groups within the same day. NU: treatment with natural *Ulva lactuca*, DU: treatment with decontaminated *Ulva lactuca*. D_0: Day 0, D_7: Day 7, D_{14}: Day 14, D_{21}: Day 21, and D_{28}: Day 28.

Based on Table 3, SGPT level in D_0 does not show a significant difference between each treatment group. This means that all the animal models used in this research have the same health status with no anomalies. According to Vigneshwar et al. (2021), the reference value of SGPT levels is 29.34 – 72.16 UI/L for female Wistar albino rats.

In the D_{30} treatment, an increase of SGPT level was found in each of the treatment groups, without any significant difference ($p > 0.05$). SGPT levels in the blood is one of the sensitive indicators of hepatic damage, because this enzyme resides in the cytoplasm. Furthermore, when hepatocyte damages happen, those hepatocytes release SGPT.

Table 2. Hepatosomatic Index (HSI) of female Wistar rats (*Rattus norvegicus* Berkenhout, 1769) after treatment with *Ulva lactuca* L.

No	Group	HIS (Mean ± SD)
1	Control	$3.89 \pm 0.19_a$
2	Natural *Ulva lactuca* (NU)	$4.22 \pm 1.24_a$
3	Decontaminated *Ulva lactuca* (DU)	$4.34 \pm 0.44_a$

The different superscript letters note a significant difference at $p < 0.05$ between groups. NU: treatment with natural *Ulva lactuca*, DU: treatment with decontaminated *Ulva lactuca*.

Table 3. Serum glutamic pyruvic transaminase (SGPT) levels of female Wistar albino rats (*Rattus norvegicus* Berkenhout, 1769) before (D_0) and after (D_{30}) treatment with *Ulva lactuca* L.

No	Group	D_0 (U/I)	D_{30} (U/I)
		Mean ± SD	Mean ± SD
1	Control	45.40 ± 6.51^a	51.18 ± 4.73^a
2	Natural *Ulva lactuca* (NU)	40.83 ± 6.45^a	41.45 ± 8.87^a
3	Decontaminated *Ulva lactuca* (DU)	44.80 ± 5.38^a	48.38 ± 11.01^a

The different superscript letters note a significant difference at $p < 0.05$ between groups within the same day. There is no significant different at SGPT levels before and after treatment with *Ulva lactuca* L. for each group. NU: treatment with natural *Ulva lactuca*, DU: treatment with decontaminated *Ulva lactuca*. D_0: Day 0, D_{30}: Day 30.

Serum Glutamic Oxaloacetic Transaminase (SGOT)

Serum Glutamic Oxaloacetic Transaminase (SGOT) or also known as the Aspartate Transaminase (AST) is an enzyme to repair hepatocytes damage. When liver cell damage occurs, hepatocytes will produce SGOT which is released into the bloodstream. SGOT levels can be used as a parameter to determine liver damage as indicated by increased SGOT levels (Yuneldi et al. 2018). The results of SGOT levels analysis are presented in Table 4.

Table 4 shows that SGOT levels were not significantly different among groups before and after treatment. This shows that all the groups were in similar condition and were within the normal range. Normal range of SGOT level of rats was 74 – 143 UI/L (Sarapultsev et al. 2012). There was a decrease in SGOT levels in D_{30} of the Control group, whereas in the NU and DU groups there was an increase but not significantly different ($p > 0.05$). The highest increase in SGOT levels occurred in the DU group. This could be due to the presence of heavy metal contaminants in DU, even though heavy metals contaminations in DU have already decreased.

Table 4. Serum glutamic oxaloacetic transaminase (SGOT) levels of female Wistar albino rats (*Rattus norvegicus* Berkenhout, 1769) before (D_0) and after (D_{30}) treatment with *Ulva lactuca* L.

No	Group	D_0 (U/I)	D_{30} (U/I)
		Mean ± SD	Mean ± SD
1	Control	131.23 ± 30.82[a]	92.08 ± 49.90[a]
2	Natural *Ulva lactuca* (NU)	107.10 ± 68.76[a]	125.13 ± 23.54[a]
3	Decontaminated *Ulva lactuca* (DU)	104.98 ± 63.92[a]	183.25 ± 105.44[a]

The different superscript letters note a significant difference at $p < 0.05$ between groups within the same day. NU: treatment with natural *Ulva lactuca*, DU: treatment with decontaminated *Ulva lactuca*. D_0: Day 0, D_{30}: Day 30.

Bilirubin

Bilirubin is a breakdown product of old red blood cells (erythrocytes) by macrophages in the phagocyte mononuclear system, especially in the liver. The first breakdown of the RES (Reticuloendothelial System) begins with the capture of iron and peptide globin chains. Bilirubin comes from a RES derivative product, namely biliverdin. Bilirubin in plasma is cooled by albumin and undergoes a conjugation process in the liver before being excreted through the intestine (Erlinger et al. 2014). According to Mulyati et al. (2020) normal serum bilirubin levels in white rats ranged from 0.3–0.8 mg/dL. Increased total bilirubin levels above normal can be used as an indicator of liver damage, especially those affected by the process of glucuronidation and hemolysis. The results of bilirubin levels analysis are presented in Table 5.

Table 5. Blood bilirubin levels of female Wistar albino rats (*Rattus norvegicus* Berkenhout, 1769) before (D_0) and after (D_{30}) treatment with *Ulva lactuca* L.

No	Group	D_0 (mg/dL)	D_{30} (mg/dL)
		Mean ± SD	Mean ± SD
1	Control	0.84 ± 0.27[a]	0.79 ± 0.32[ab]
2	Natural *Ulva lactuca* (NU)	0.63 ± 0.10[a]	0.58 ± 0.20[a]
3	Decontaminated *Ulva lactuca* (DU)	0.69 ± 0.22[a]	1.06 ± 0.25[b]

The different superscript letters note a significant difference at $p < 0.05$ between groups within the same day. NU: treatment with natural *Ulva lactuca*, DU: treatment with decontaminated *Ulva lactuca*. D_0: Day 0, D_{30}: Day 30.

Table 5. showed that all groups in D_0 were still within the normal range of bilirubin levels and there is no significant difference between treatment groups. This result showed that the animals used were normal and healthy. After 30 days of treatment, there was a trend of decreasing bilirubin levels in the Control group and the natural *Ulva lactuca* treatment, but the group treated with heavy metal decontaminated *Ulva lactuca* experienced an increasing trend.

According to Andjelkovic et al. (2019), exposure to heavy metals affects the reduction of red blood cells significantly due to intravascular hemolysis which causes oxidative stress. Significantly different results were shown in the NU and DU groups. Although the levels of heavy metals contained in the DU group have decreased, the presence of heavy metals can still affect the physiology of rats. In addition, *Ulva lactuca* is a macroalgae that contains high Fe (Rasyid 2017). The high Fe content in the *Ulva lactuca* may affects the formation of red blood cells and their conversion to bilirubin in the liver so that the level can increase.

Bioaccumulation of Pb, Cd, and Hg

The bioaccumulation profile of the studied heavy metals including lead (Pb), cadmium (Cd), and mercury (Hg) in the liver and gastrointestinal tract was analyzed. Lead accumulation in the control groups was lower compared to the NU (Natural *Ulva lactuca*) and DU (Decontaminated *Ulva lactuca*) (Figure 1a). This could happen because both groups were given the *Ulva lactuca* which was used in previous research in 2021. The research in 2021 showed that *Ulva lactuca* was proven to contain lead of 0.186 mg/Kg dry weight in the natural *Ulva lactuca* and 0.137 mg/Kg dry weight in the decontaminated *Ulva lactuca*.

The highest levels of lead were found in the DU group in the gastrointestinal tract (Figure 1a). It can be associated with interactions between dietary fiber in *Ulva lactuca* with lead. *Ulva lactuca* contains 54.9% food fiber that can bind lead metal, and reduce absorption of lead in other tissues (Qi et al. 2019). Binding of lead by dietary fiber can reduce accumulation in the liver, heart, kidneys, and bones, through increased gastrointestinal motility (Mehrandish et al. 2019). In addition, the high level of lead in both the NU and DU groups could also be caused by the antagonism of heavy metals. The presence of lead can interfere with the absorption of other metals such as Zn, Fe, Cd, and Hg in the body (Mariadi et al. 2018).

Furthermore, Pb levels in the liver of the DU group were higher than those in the NU group. It can be indicated an antagonistic interaction between Pb and other metals especially Cd (Okon et al. 2020). The high levels of Pb in the liver of the DU group could be associated with the lower levels of Cd in the liver of the DU group (Figure 1b). The opposite also applies, in which low levels of Pb in the liver of the NU group can be attributed to higher levels of Cd in the liver of the NU group (Figure 1b). Divalent metals are absorbed in the body through similar mechanisms and accumulation in the same tissues. Once in the body, they can alter each other's absorption, distribution, and accumulation. Smith et al. (2012) observed that Pb antagonizes Cd accumulation in the liver and kidney.

The accumulation of cadmium in the liver of the control group showed much lower levels than the NU and DU groups (Figure 1b). This accordance with Winiarska-Mieczan and Kwiecień (2016) that consumption of foods contaminated with cadmium, 60% accumulates in the liver, 30% accumulates in the kidneys, and the remaining 10% accumulates in the bile organs, brain, lungs, and heart. Based on research Andjelkovic et al. (2019) bioaccumulation of cadmium in rats exposed to $CdCl_2$ 30 mg/Kg BW was highest in the liver affected by high synthesis of metallothionein (MT) in the liver. Metallothionein synthesis was able to detect the presence of the toxic metal cadmium. Metallothionein will bind to cadmium and form Cd-MT complexes that protect tissues (Matović et al. 2011).

Cadmium levels in the liver of the DU group were lower when compared to the NU group. This was influenced by the process of soaking *Ulva lactuca* with 15% bilimbi solution for 60 minutes. Bilimbi (*Averrhoa bilimbi*) contains citric acid which acts as a heavy metal chelating agent. Citric acid is able to bind heavy metals in the form of complex bonds and reduce the side effects of heavy metals in foodstuffs (Mariadi et al. 2018). The interaction between citric acid and heavy metals can be enhanced by soaking (Ulfah et al. 2014). Therefore, soaking *Ulva lactuca* in bilimbi solution can reduce cadmium levels in *Ulva lactuca* and its accumulation in the liver.

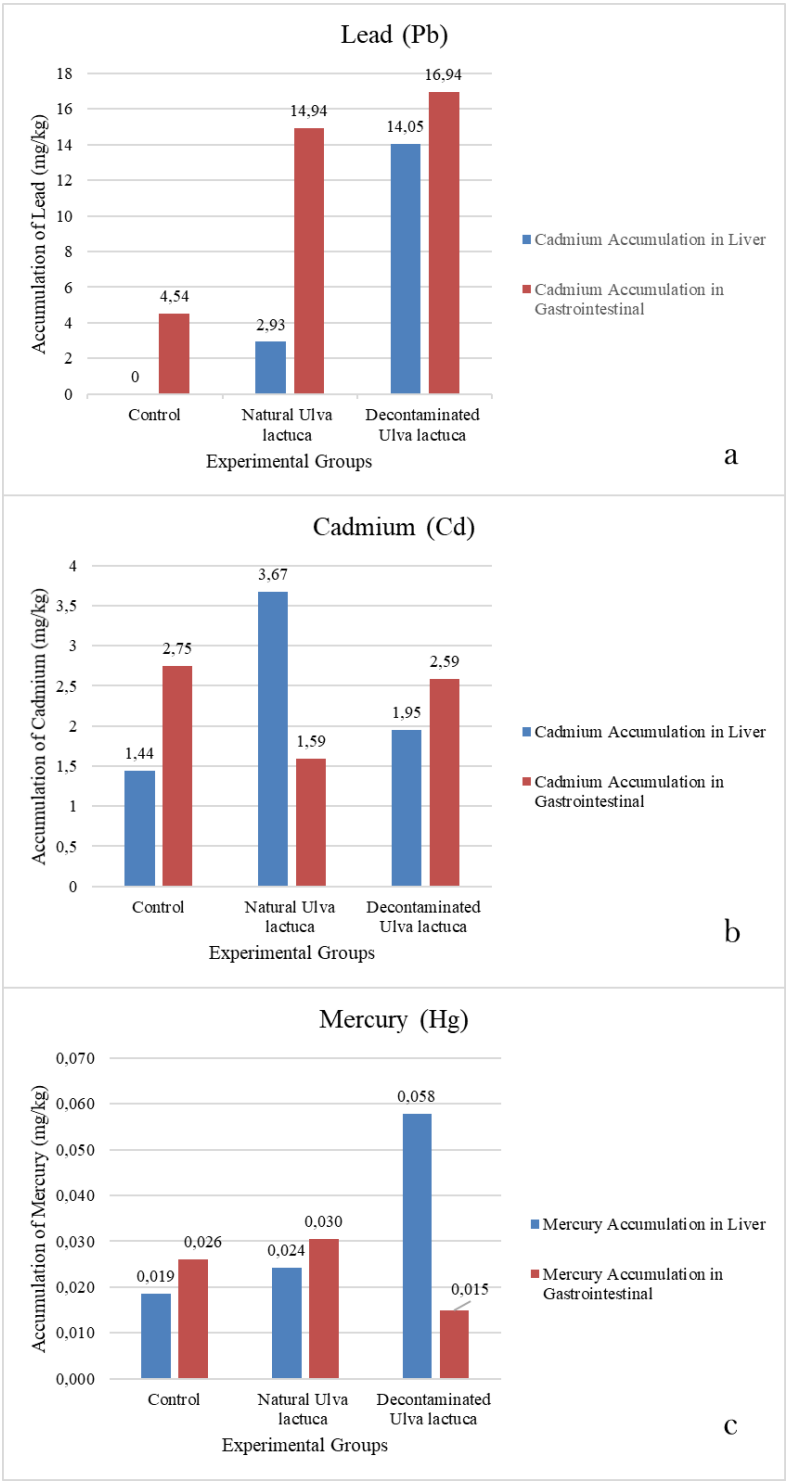

Figure 1. Metals accumulation of (a) Lead (Pb), (b) Cadmium (Cd), (c) Mercury (Hg) in liver and gastrointestinal tract of female Wistar albino rats (*Rattus norvegicus* Berkenhout, 1769) treated with *Ulva lactuca* L.

Furthermore, cadmium levels in the GIT organs of the control and DU groups showed higher values than NU groups. It can be related to interaction between cadmium and dietary fiber in *Ulva lactuca*. Dietary fiber has been reported to bind Pb and Cd in animal models thus promoting their excretion. *Ulva lactuca* contains cellulose as a dietary fiber that noticeable capacity of binding Pb and Cd in gastrointestinal. Mechanism underlying dietary fibers protective effect against heavy metals accumulation in the reduction in active transport sites in the intestine. It has been suggested that time reduction in intestinal transit could be responsible for the increased rate of heavy metals excretion in body (Guo et al. 2022).

The highest accumulation of mercury was in the DU group in the liver organ (Figure 1c). This can happen because of the synergistic effect between lead, cadmium, and mercury. Synergism interactions occur when two or more chemicals that have the same toxicity are combined so that they have greater toxic properties and accumulation. The absorption of lead, cadmium, and mercury metals is equally high in the DU group.

The highest mercury levels were found in the gastrointestinal organs, except for the DU (*Ulva lactuca* decontaminated) group (Figure 1c). Mercury is a substance that is easily absorbed by the tissues in the intestine. Based on research by Aba (2016) it shows that organic mercury is easily absorbed by the gastrointestinal (GI) tract by 95% and then distributed throughout the body. Mercury levels in the gastrointestinal organs of the DU group were lower than those of the NU group. This was influenced by the process of soaking *Ulva lactuca* with 15% bilimbi solution for 60 minutes. Soaking *Ulva lactuca* in bilimbi solution in more effective in reducing mercury levels in *Ulva lactuca* and its accumulation in the gastrointestinal organs than lead and cadmium levels.

Histopathological Effect of *Ulva lactuca* treatment on The Liver

The liver was chosen as the histological object to be observed because the liver is an organ exposed to heavy metals and plays a role in its detoxification process. The observations made were qualitative observations based on hepatocyte damage including cell swelling, hydropic degeneration, fatty degeneration, and necrosis.

The histological structure of liver on the control group was still normal, with the presence of many Kupffer cells and cell regeneration (Figure 2A-2B). Cell damage that occurs in the control group is because of the liver role as a place for detoxification of harmful substances. The hepatocytes are often damaged, but this is accompanied by a high rate of regeneration (Mulyati et al. 2020).

The histological condition of the liver in the NU group also experienced cell damage, including cell swelling or hydropic degeneration, fatty degeneration, and necrosis (Figure 2C-2D). This can be indicated to the accumulation of heavy metals lead, cadmium, and mercury in the liver. Accumulation of heavy metals in rats' bodies forms free radicals, causing oxidative stress that triggers liver histological damage in NU group rats. Based on Dardouri et al. (2016) exposure to $CdCl_2$ (100 mg/l) and $HgCl_2$ of 25 mg/l in rat's drinking water for 10 weeks caused hepatocyte degeneration, vacuolization, sinusoidal dilatation, central vein dilatation, and Kupffer cell proliferation.

In the DU group, there were cell damages including cell swelling or hydropic degeneration, fat degeneration, and necrosis (Figure 2E-2F). The DU group was the group that was treated with decontaminated *Ulva lactuca*. Cell damage still occurred in the DU group because it was proven previously that the decontaminated *Ulva lactuca* group still contained heavy metals of Pb 0.1369 mg/Kg, dry weight Cd 0.008 mg/Kg, dry weight and Hg 0.26 mg/Kg dry weight, although at lower levels than natural *Ulva lactuca*. Cell swelling or hydropic degeneration occurs due to an imbalance in the Na^+ and K^+ ion pumps in the cell membrane. These results are in line with the research of Andjelkovic et al. (2019) exposure to cadmium and lead causes inflammation that triggers hydropic degeneration in hepatocytes.

In addition to hydropic degeneration, fatty degeneration was also found in the DU group. The necrosis that occurs is the pyknosis stage indicated by the N symbol in Figure 2F. The cell nucleus undergoes pyknosis or nuclear shrinkage due to cytoplasmic homogenization and in-

creased eosinophilicity. After pyknosis occurs, the cell nucleus will disintegrate and leave chromatin fragments and spread in the cell which is called the karyorexis stage. Furthermore, the cell nucleus will undergo karyolysis, namely death which is characterized by loss of the ability to be colored.

Figure 2F also shows the presence of sinusoidal dilatation which is indicated by the star symbol. Dilatation or widening of the sinusoids occurs due to contact between heavy metals which are toxic to endothelial cells lining the sinusoid. If the concentration of heavy metals is high and the exposure is long, it can cause widening of the sinusoids (Wadaan 2009). However, besides the cell damage that occurred in the DU group, was also followed by cell regeneration that was indicated by the circle symbol (Figure 2F). This shows that the exposure to heavy metals in the DU group was qualitatively lower than NU groups. Cell damage in the DU group could still be accompanied by a high rate of cell regeneration.

Histopathological Effect of *Ulva lactuca* treatment on the Small Intestine

In each group, the histological structure of the small intestine was visible the vili, lumen, and muscle layer. The histological structure of the small intestine in the control group showed a uniform structure, with intact and elongated villi (Figure 3A). Whereas in the NU (Figure 3B) and DU (Figure 3C) groups, the structure is relatively uniform. However, there is also degeneration of the villi in the portion leading to the luminal area.

Figure 3D showed the histological structure of the small intestine of the control group which did not show any cell damage to the villi. Based on Figure 3E, it shows the histological structure of the intestines of the NU group which suffered damage, namely villous erosion (VE) and bleeding (H). The intestine is an organ that plays a role in absorption, protection, and hormonal processes. The NU group was a group that was

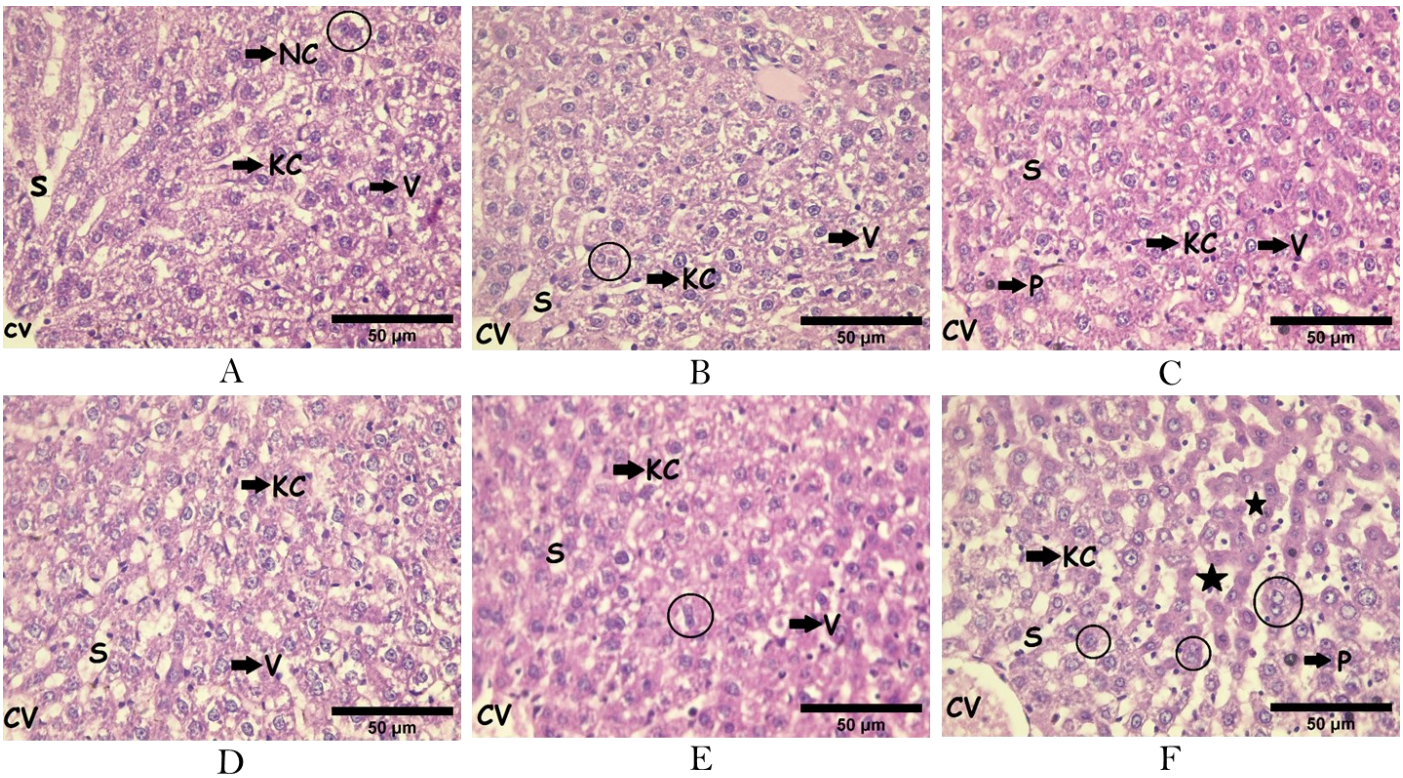

Figure 2. Histological strcuture of liver of rats (A-B) Control, (C-D) Natural *Ulva lactuca* (NU), (E-F) Decontaminated *Ulva lactuca* (DU). CV: Central Vein, NC: Normal cells, KC: Kuppfer Cell, S: Sinusoids, V: Vacuolization/fat degeneration, P: Pyknosis, Circle: Cell regeneration, Star: Dilated sinusoids. H&E stain. 40 x 10. Scale bar: 50 µm.

given natural *Ulva lactuca* treatment which was proven to contain the heavy metals lead, cadmium and mercury. Intake of food ingredients that contain heavy metals will affect the organs in the body. These results are in line with the study of Bais and Lokhande (2012) which showed that exposure to cadmium for four days caused hydropic degeneration, villi erosion, epithelium degeneration, cell swelling, and necrosis in the intestinal villi of snakehead fish (*Ophiocephalus striatus*).

Based on Figure 3F, it shows the histological structure of the small intestine in the DU group which was damaged, namely bleeding of the lamina propria and erosion of the villi. Even though decontamination had been carried out, *Ulva lactuca* soaked using bilimbi solution proved to still contain the heavy metals lead, cadmium and mercury at lower levels than the NU group. The presence of these heavy metals can induce free radicals through the formation of reactive oxygen species (ROS). This causes a decrease in the ability of antioxidants in the body and trigger inflammation and damage to the histological structure of the intestines of the DU group rats. Exposure to cadmium of 25 – 100 µg/L in zebrafish *Danio rerio* caused bleeding in the lamina propia, cell swelling, and increased proliferation of goblet cells (Motta et al. 2022).

Based on Figure 3F, it was also seen that there was dilatation of the villi in the intestines of the DU group. Villi dilatation is the widening of the villi due to the induction of toxic compounds in the intestine. These results are in line with the research by Adigüzel and Kalender (2015) that lead (Pb) 22.5 mg/Kg BW in rats causes villous dilatation (D), necrosis, and epithelium degeneration. The process of villous dilatation triggers villous shortening. The shorter the size of the villi will interfere with the process of absorption of nutrients in the lumen. The villous dilation that occurred in the DU group could be associated with high levels of lead bioaccumulation in the liver and gastrointestinal tract of rats (Figure 1a). Lead is mostly absorbed by the intestinal cell walls and is responded to by the histological structure of the intestine with the formation of villous dilatation.

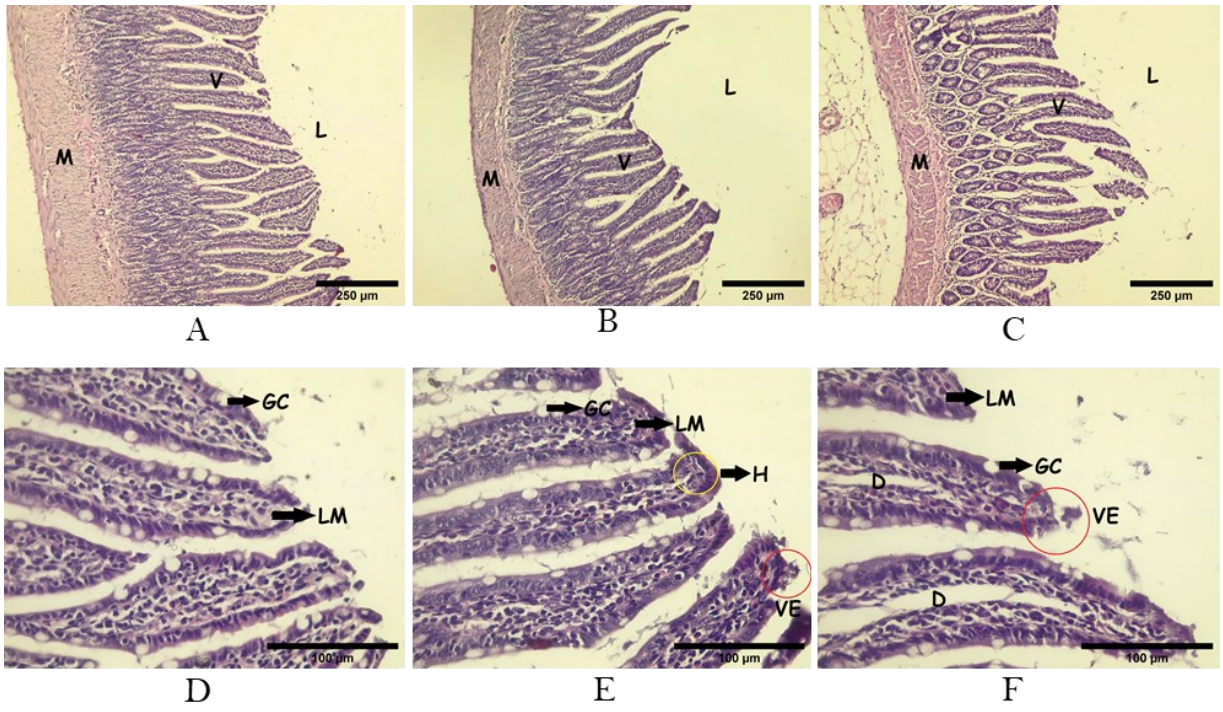

Figure 3. Histological structure of small intestine of rats (A) Control 10 x10, (B) Natural *Ulva lactuca* (NU) 10 x10, (C) Decontaminated *Ulva lactuca* (DU) 10 x10, (D) Control 40 x10, (E) Natural *Ulva lactuca* (NU) 40 x10, (F) Decontaminated *Ulva lactuca* (DU) 40x10. V: Villi, L: Lumen, M: Muscularis, GC: Goblets cell, LM: Lamina propia, H: Haemorrhage/bleeding, VE: Villi errosion, D: Dilatation villi. H&E stain. Scale bar: 50 µm.

Based on Figure 3D-3F, qualitatively, the damage to the histological structure of the intestine in all treatment groups in this study was almost the same, namely experiencing villous erosion or degeneration of the epithelium, bleeding, Goblet cell vacuolization and villous dilatation. Qualitative observation of histological findings showed that the most serious damage was found in the NU groups. However, these cannot be compared because there is no absolute value. Based on the histological picture of the intestine, it can be stated that the DU still had damage accompanied by cell regeneration. This research show that qualitatively histological structure liver and small intestine of DU groups has a better condition than the NU groups.

CONCLUSION

The administration of heavy metal decontaminated *Ulva lactuca* at a dosage of 1000 mg/kg BW/day over a 30-day treatment did not significantly alter HSI, SGPT, and SGOT levels. Despite a discernible rise in total bilirubin levels attributable to elevated iron content in *Ulva lactuca*, likely influencing an increase in red blood cells, the observed changes did not reach statistical significance. Nevertheless, the histological examination revealed damage to the liver and small intestine cells, coupled with a commendable regenerative response. These findings contribute valuable insights the effects of *Ulva lactuca* on hepatic function and heavy metal detoxification, paving the way for future research in the field of marine-based interventions for metal toxicity mitigation.

AUTHORS CONTRIBUTION

S.W., A.R.S.S., A.H.H., A.N.I., and S.R.D.S. conducted the research, data collection, data analysis, and wrote the manuscript, while M. designed the research, supervised, and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publishing of the article.

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Research Article

In Vitro Seed Germination and Shoot Growth of *Nepenthes jamban* Chi. C. Lee, Hernawati & Akhriadi, A Unique Pitcher Plant from Indonesia

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ABSTRACT

The study to optimize *in vitro* propagation of the Indonesian native and critically endangered species, *Nepenthes jamban*, in order to support the *ex situ* conservation efforts has been done. Using Murashige and Skoog (MS) as a basal medium, disinfected seeds of *N. jamban* were germinated on five types of germination media, viz. ¼ MS, ½ MS, MS, ¼ MS+benzyl adenine (BA)+Biotin and MS+BA+Biotin. Afterwards, *in vitro* shoots with 6-7 leaves were inoculated on growing media, i.e., ¼ MS, ¼ MS 60 (3:1) (MS modification with a higher concentration of nitrogen), and ¼ MS+naphtalene acetic acid (NAA)+BA. The results showed that the germination of *N. jamban* seeds was slow, indicated by the percentage of germination being less than 20% after six months of being planted on germination media. The highest percentage of germination at the sixth month and the greatest pitcher development at the tenth month were obtained on ¼ MS medium. Furthermore, shoot growth and pitchers development consistently increased for twelve months in ¼ MS 60 (3:1) medium while other media resulted in a decrease in pitcher formation. It seemed that low concentrations of nutrients in the medium proved to be more effective to induce *in vitro* seed germination and enhance shoot growth which was also supported by a higher nitrogen (nitrate) concentration in the medium. This study provides information that supports *ex situ* conservation action of native and critically endangered *Nepenthes* species from Indonesia.

INTRODUCTION

Nepenthes spp. are locally known in Indonesia as "Kantong Semar" due to the modification of the leaf tips into a pitcher structure that resembles Semar's belly, a Javanese traditional puppet character (Dinarti et al. 2010). More than 180 species of *Nepenthes* are distributed from Madagascar to South China and New Caledonia, where Indonesia, Malaysia, and the Philippines are considered the centres of *Nepenthes* diversity (Cross et

al. 2020; Mansur et al. 2023). In Indonesia, Sumatra Island has the most *Nepenthes* species by 39 species and 34 among them are endemic (Hernawati et al. 2022; Mansur et al. 2023).

Although *Nepenthes* is a carnivorous plant, it can also be a prospective commercial ornamental plant because of the attractive variation in the shape, size, and colour of the pitcher (Handayani 2021). It also has ethnobotanical utilisation by some local people as a potential herb plant since it contains phytochemical and phytopharmacological activity from its extracts (Sanusi et al. 2017).

Nepenthes jamban is one of nine new species of *Nepenthes* spp. (Nepenthaceae) reported from Sumatra between 2002 to 2022 (Hernawati et al. 2022) and one of the new species found by Lee et al. (2006) in North Sumatra. Lee et al. (2006) reported that *N. jamban* was distributed around Bukit Barisan in North Sumatra with the habitat of mossy forest in the upper mountainous area and scrub vegetation on the top of the mountain. The word "jamban" refers to a toilet in Bahasa Indonesia due to the resemblance to the pitcher (Lee et al. 2006).

According to the International Union for Conservation of Nature (IUCN) report, *N. jamban* is not listed on the red list (IUCN 2023). However, a study on the conservation of carnivorous plants classified this species as critically endangered (Cross et al. 2020). Furthermore, the documentations about conservation attempts, plant propagation, or other potential conservation approaches have not been reported. On the other hand, the majority of natural habitats inhabited by carnivorous plants, such as *Nepenthes* species are confined to areas that have been significantly disturbed and degraded (Cross et al. 2020). Certain species have come perilously close to extinction as a result of the constant conflict between development and conservation, which includes industries and housing. Therefore, it is critical to establish a dependable tissue culture technique in order to prevent the extinction of these native and endemic species (Siti-Suhaila & Norwati 2021).

The propagation of *Nepenthes* spp. can be done using seed, stem cutting, stem grafting (air layering), and ground layering, however, stem cutting and grafting methods are time laborious with low yields and difficult to carry out (Sukamto et al. 2011; Meinaswati et al. 2022). Moreover, according to Dwiati et al. (2023), performing stem cutting without *in vitro* techniques resulted in just two shoots in the propagation of *N. gymnamphora* and *N. adrianii*. *In vitro* techniques have been used in various species of *Nepenthes* spp. by shoot tip culture (Sukamto et al. 2011), callus induction (Novitasari & Isnaini 2021), micro-cutting technique from the shoot (Yelli 2013; Budisantoso et al. 2018), and seed culture (Isnaini & Handidi 2007; Khuraijam & Roy 2015; Meinaswati et al. 2022; Joshi et al. 2022). *In vitro* seed culture is an appropriate biotechnological approach for the massive propagation and conservation of many scarce and endangered species. Moreover, this method can maintain the genetic diversity as the seed is heterozygous (Nongrum et al. 2009).

As there is no previous report about *in vitro* propagation of *N. jamban*, a study to understand the effectiveness of various media to support the optimal germination and growth is required. This study is expected to add more knowledge about *in vitro* propagation of one native and critically endangered *Nepenthes* species from Sumatra, Indonesia.

MATERIALS AND METHODS

Seed Observation

The plant material used in this study was *Nepenthes jamban* seeds collected from Pasaman Regency (1200 masl), West Sumatra, Indonesia. Natu-

rally pollinated and mature seeds of *N. jamban* were collected from unopened seed pods in their natural habitat. The seed pods were stored in an envelope at room temperature until they naturally opened. These seeds were then utilised as the material for this study after being stored for one month.

Air-dried mature seeds were observed under a stereo microscope (Nikon SMZ 645) with 5× magnification. For further observation, observing the seeds under a scanning electron microscope (SEM) (JSM-IT200 InTouchScope™) proceeded after the middle cut of the seeds were placed on the stub and coated with gold using an EIKO IB-3 ion coater.

Seed Germination

Planting the seeds on solid MS basal media (Murashige & Skoog 1962) proceeded in a non-factorial completely randomised design with five replicates to observe their viability and growth. Containing MS macro-nutrient (NH_4NO_3, KNO_3, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, KH_2PO_4) and micro-nutrient ($MnSO_4.4H_2O$, $ZnSO_4.5H_2O$, $Na_2MoO_2.2H_2O$, $CoCl_2.6H_2O$, H_3BO_3), MS vitamins (nicotinic acid, thiamine HCl, pyridoxine HCl, glycine), iron source (NaEDTA, $FeSO_4.7H_2O$), 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, and 5 g L^{-1} agar as gelling agent, germination media consisted of five treatments as follows: i) quarter strength of MS macro-nutrient and full strength concentration of other components (¼ MS); ii) half strength of MS macro-nutrient and full strength concentration of other components (½ MS); iii) full strength of all MS components (MS); iv) ¼ MS supplemented with 0.5 mg L^{-1} benzyl adenine (BA) and 1 mg L^{-1} biotin (¼ MS+BA+Biotin); v) MS supplemented with 0.5 mg L^{-1} BA and 1 mg L^{-1} biotin (MS+BA+Biotin). The acidity was set at pH 5.7-5.8 by adding several drops of 1 N NaOH or HCl before adding the gelling agent. Sterilising the media proceeded at 121 °C for 20 min using an autoclave, then 25 mL sterile media was poured into petri dishes with a diameter of 10 cm. In this study, adding BA as a plant growth regulator and biotin, known as vitamin B7, in the germination media was expected to boost the germination rate of *N. jamban in vitro*.

The seeds were sterilised in laminar air flow cabinet by soaking in 0.25% NaClO (Bayclin®, active ingredient 5.25% NaClO) with a few drops of Tween 20 for 20 min, followed by rinsing with sterile distilled water three times. The planting of 20 sterile seeds on the germination media was repeated five times for each treatment so that the total number of seeds planted was 500. Seed cultures of *N. jamban* were then incubated in a culture room under dim conditions (without direct lighting from lamps) at a room temperature of 25±2 °C and 40% humidity. Observations of seed germination were carried out periodically until the embryos grew and formed leaves and pitchers. Non-germinated seeds and dead sprouts were observed as well. Transferring the explants to the same medium proceeded three times after the seeds germinated. The best medium was then used for shoot growing basal media.

Shoot Growth and Pitcher Formation

Designed using a non-factorial completely randomised design with ten replicates, the experiment used ¼ MS as basal media for growing media treatments including: i) ¼ MS; ii) ¼ MS 60 (3:1); iii) ¼ MS supplemented with 0.5 mg L^{-1} naphthalene acetic acid (NAA) and 0.5 mg L^{-1} BA (1/4 MS+NAA+BA). MS 60 (3:1) medium was a modification of MS medium using a total nitrogen concentration (N) of 60 mM with a nitrate and ammonium (NO_3^-:NH_4^+) ratio of 3:1, which was adjusted using the compounds KNO_3, NH_4NO_3 and $(NH_4)_2SO_4$ as sources of NO_3^- and NH_4^+ in

the medium (Handayani et al. 2021). The acidity level was set at pH 5.7-5.8 by adding several drops of 1 N NaOH or HCl before adding the gelling agent. As much as 25 mL sterile media was poured into a 300 mL jar prior to autoclaving at 121 °C for 20 min.

Fourteen-month-old sterile shoots at the 6-7 leaf stage were planted in each medium treatment and incubated in a culture room for twelve months under a photoperiod condition of 16/8 h (light/dark) with a light intensity of 46 µmol m^{-2} s^{-1}, room temperature at 25±2 °C, and humidity of 40%. Transferring the shoots on the same media was carried out once every three months, as well as observing growth parameters including number of pitchers, number of leaves, and shoot height. The number of pitchers was counted on the leaves that formed pitchers as shown in Figure 2C. The height of the shoots was measured by taking out the shoots from the bottle and then measuring them on sterile millimetre block paper in the laminar air flow cabinet.

Data Analysis

Data processing and presentation were performed using Microsoft Excel and analysed using ANOVA and continued with the Least Significant Difference (LSD) test at $p \leq 0.05$ using R 4.1.0 software.

RESULTS AND DISCUSSION

Morphology of *Nepenthes jamban* seeds

Resembling orchid seeds (Orchidaceae), *Nepenthes* seeds have an embryo (dark brown) located at the centre and are coated with several layers of seed coat cells (testa) extending from either end of the embryo. *Nepenthes* seed is larger than that of dust-like orchid seed and 8-30 mm long, with the seed coat about six times longer than the embryo. With a distinct characteristic in the shape of a pitcher resembling a toilet (Figure 1A), *N. jamban* produces filiform and 14-20 mm long seeds that are transversely wrinkled at the centre (Figure 1B-E). The seed coat is light brown with a lighter structure than the embryo which is useful in the process of dispersal of seeds assisted by wind or water (Watson & Dallwitz 1992; Cheek & Jebb 2001; Barclay 2015). The seeds of *Nepenthes* from the humid tropics seem to be recalcitrant (Ellison & Adamec 2017). This kind of seed cannot tolerate dehydration during storage at the low water content or subzero temperatures. Therefore, the storage of recalcitrant seeds cannot use standard gene bank approaches (Pammenter & Berjak 2014).

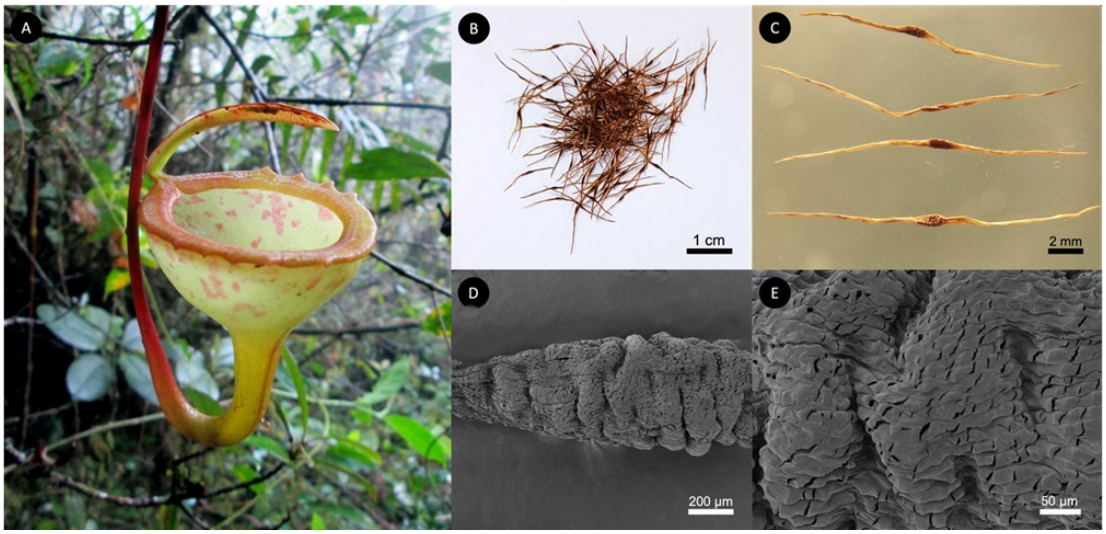

Figure 1. A) The pitcher of *N. jamban* in nature; B) Mature seeds; C) Seeds observed under a stereo microscope; D-E) The centre part of the seed observed under a scanning electron microscope.

In Vitro Seed Germination

Germination is a vital part of plant development. It is also a complicated physiological process that is driven by intrinsic factors (i.e., seed dormancy and food availability) and extrinsic factors (i.e., water, temperature, oxygen, and relative humidity) (Bhardwaj et al. 2014; Makena et al. 2018; Savaedi et al. 2019). From the research about seed germination in *Nepenthes*, we can assume that the characteristics and requirements of nutrient content to get the best germination rate are genetic-dependent. In this study, *N. jamban* seed coat became transparent after sowing on the germination medium so that the embryo in the middle part of the seed was clearly seen, however, there were also germinated seeds that failed to grow and eventually died (Figure 2A). Dead sprouts were indicated by the blackening of cotyledon leaves due to their inability to grow to the next stage of the growth. Viable seeds began to germinate indicated by a change in colour to green in the centre part of the seed, followed by the emergence of two cotyledon leaves. This stage is considered the sprouting seed stage, as shown in Figure 2B. The shoot grew and started to develop a pitcher on the fourth leaf (Figure 2B-C).

Nepenthes jamban seeds began to germinate six months after planting on *in vitro* media, except for MS and MS+BA+Biotin media (Figure 3). Figure 3A shows the seeds planted on ¼ MS and ¼ MS+BA+Biotin germinated with the same germination percentage, which was 15±4.4%. Seeds planted at ½ MS also germinated, but with a lower percentage, which was only 1±1%. Water is the main regulator of germination, as it is imbibed into the seed and initiates the germination process (Luna & Chamorro 2016). Moreover, the shortage of water availability will restrain seed germination. Since the lower concentration of MS medium indicates greater water availability in the medium, we suggest that this is why the germination rate on ¼ MS medium is higher than on ½ MS.

Due to the very small size of the endosperm in *Nepenthes* seeds, germination is extremely low. The conventional germination period for *Nepenthes* seeds is approximately two months (45-65 days), which is considered a long period of time (Meinaswati et al. 2022). Previous studies indicated there were variations in germination time on various *Nepenthes* species *in vitro*, i.e., 4 weeks after planting (WAP) for *N. gymnamphora* (Meinaswati et al. 2022), 5 WAP for *N. khasiana* and *N. mirabilis* (Nongrum et al. 2009; Dinarti et al. 2010), and 13 WAP for *N. distillatoria* (Siriwardana et al. 2013). These results were different from this study where *N. jamban* needed a longer time to germinate, around 6 months or

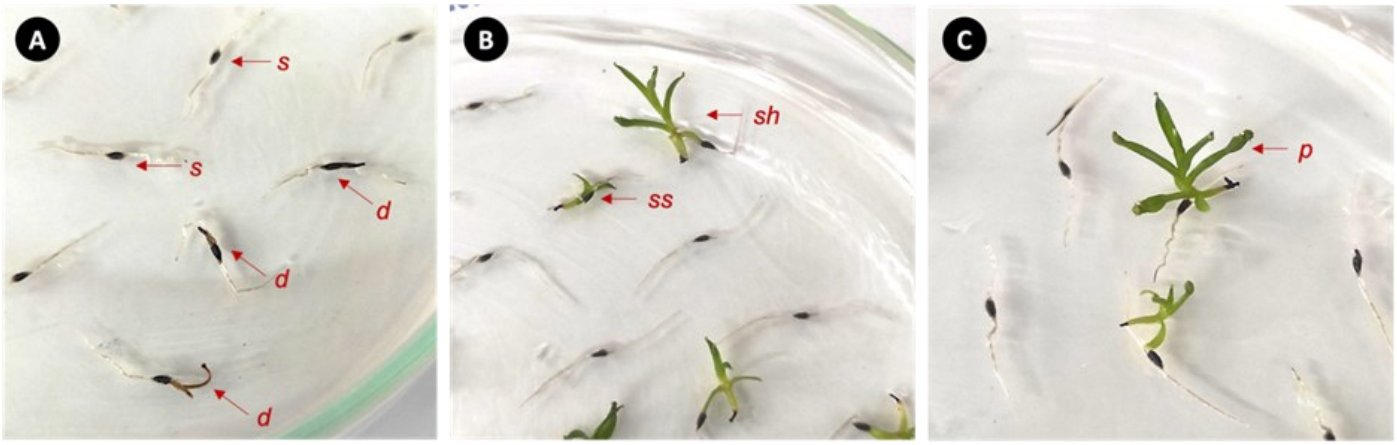

Figure 2. The growth and development of *N. jamban* from seeds. A) Non-germinated seed (*s*) and dead sprout (*d*); B) Sprouting seed (*ss*) and the shoot (*sh*) growing and starting to develop very young pitchers; C) The shoot with well-developed young pitchers (*p*).

26 WAP. The seeds used in this study were stored at room temperature after being harvested until they were used for *in vitro* germination experiments. The storage method was considered one of the factors causing the long germination time, as in the result from (Mao & Ranyaphi 2007) which stated that the seed of *N. khasiana* rapidly lost its viability after one year of storage. The germination time of *N. jamban in vitro* was longer than other *Nepenthes* species which germinated in *ex vitro* medium. For instance, the seed of *N. khasiana* planted in compost medium (Mao & Ranyaphi 2007) and coir media without fertiliser (Khuraijam & Roy 2015) started to germinate in 4 WAP. Additionally, the low germination rate of *N. jamban* seeds may be a contributing factor to its endangerment in natural habitat, a phenomenon observed commonly in rare and endemic species such as *Betula humilis* (Bona et al. 2022), *Manglietia crassipes* (Wang et al. 2021), and *Boswellia* spp. (Hamdiah et al. 2022).

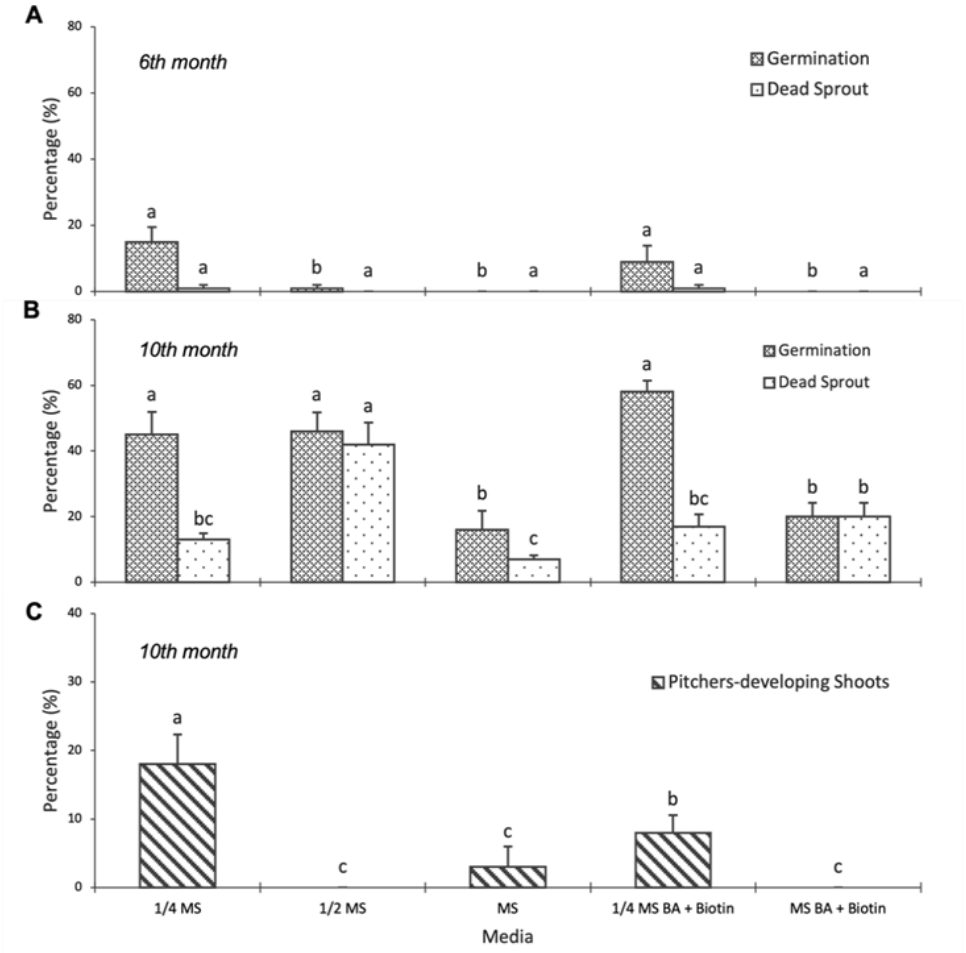

Figure 3. *In vitro N. jamban* seeds germination on five types of media: A-B) Germination percentage and death of sprouts at six and ten months after culture; C) The percentage of pitchers developing shoots at ten months after culture. The same letter on the bar for each parameter indicates no significant difference by LSD test at $p \leq 0.05$.

As we assumed that the germination rate in *Nepenthes* is species-and genetic-dependent, this also applies to germination swiftness. A review by Carrera-Castaño et al. (2020) clearly explained that germination swiftness, one of the important traits of germination, is modulated by a continuous interaction between the plant genetic makeup and the environment from dormancy to germination stages. These can be achieved through regulation of metabolism and hormone signalling (Carrera-Castaño et al. 2020). Variations in germination time of *Nepenthes* seeds *in*

vitro was affected by the type of media used. In this study, the seed of *N. jamban* germinated faster in the non-full-strength media, i.e., ¼ MS, ¼ MS+BA+Biotin and ½ MS compared to other media. This result was in line with a previous study where the ¼ MS and ½ MS were the best media for *N. mirabilis* germination (Dinarti et al. 2010) and the ½ MS was best for *in vitro* germination of *N. gymnamphora* (Meinaswati et al. 2022), *N. khasiana* (Nongrum et al. 2009), and *N. ampullaria* (Sani et al. 2000) seeds. The addition of hormone (BA) and vitamin (biotin) apparently did not affect *N. jamban* germination in this study. Furthermore, the result indicated that *N. jamban* seeds tend to thrive in media containing low nutrients. This observation is consistent with the general habitat conditions of *Nepenthes*, which predominantly grow in areas that are poor in nutrients (Mansur et al. 2022).

The result in Figure 3A showed that not all the germinated seeds survived. Some of them failed to grow and then died. About 1±1% of dead sprouts were found in ¼ MS and ¼ MS+BA+Biotin media, while there were no dead sprouts in ½ MS, MS and MS+Biotin media at six months after planting.

Ten months after planting, seeds on MS and MS+BA+Biotin showed a low germination rate by 16±5.8% and 20±4.2%, respectively (Figure 3B). The same figure showed an increasing germination rate in ¼ MS and ¼ MS+BA+Biotin media by 30% and 49%, respectively, from the germination rate at six months after planting. The significantly increasing germination rate was also occurred in ½ MS medium by 45%, however the percentage of dead sprouts in this treatment also considerably elevated compared to other treatments, by around 40%. The sprout death in other media treatments was lower and significantly different from ½ MS medium treatment.

Nepenthes jamban pitchers started to develop on the fourth leaf, on average. Figure 3C indicated that even though all the seeds had already germinated in 10 months, the shoot in the ½ MS and MS+BA+Biotin had not developed the pitcher while the others did. The most pitchers developed in ¼ MS medium (18±4.4%) while on the ¼ MS+BA+Biotin and MS media were only 8±2.5% and 3±3%, respectively. In line with this result, *N. khasiana* seeds grew in MS and MS+BAP did not develop any pitchers in four months after planting (Nongrum et al. 2009). In general, *in vitro* shoot performance of *N. jamban* 10 months after planting was better in ¼ MS and ¼ MS+BA+Biotin media, which supported the shoot growth and the pitcher development (Figure 4A, D). Other media exhibited lower performance, with only a few sprouts were able to regenerate and more dead sprouts found (Figure 4B, C, E).

In Vitro Shoot Growth and Pitcher Formation

Germinated seeds on the germination media were transplanted to the growing media to observe further growth for twelve months. The results showed that ¼ MS 60 (3:1) medium produced the greatest number of pitchers whereas ¼ MS+NAA+BA yielded the least. *N. jamban* plantlets on ¼ MS 60 (3:1) kept forming pitchers for twelve months (12 pitchers) after transplanting, while those on ¼ MS only formed 8,6±0.7 and 10.8±0.5 pitchers for six and nine months, respectively, and those on ¼ MS+NAA+BA formed 9 pitchers followed by a decrease in number by the twelfth month due to senescence. Pitcher formation on *in vitro* media varied for different species. Previous studies reported that *N. ampullaria* and *N. mirabilis* on ¼ MS medium formed 4 pitchers by the twelfth month (Yelli 2013), while *N. khasiana* formed 3 pitchers on MS NAA medium and no pitchers on MS BAP medium in the third month after plant-

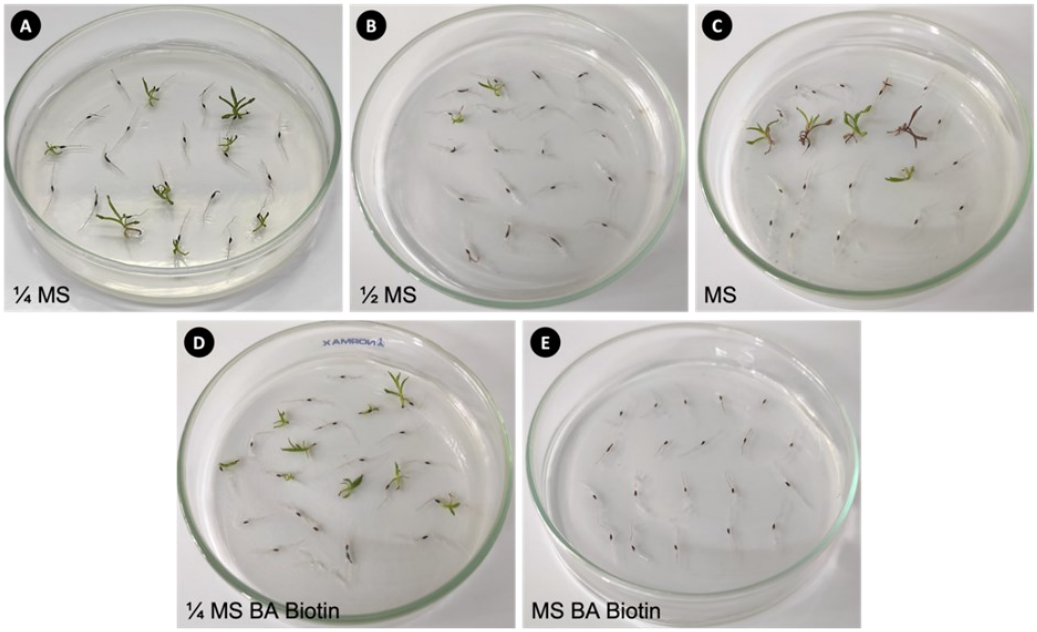

Figure 4. Shoot performance of *N. jamban* at ten months after sowing on germination media.

ing (Nongrum et al. 2009).

Number of leaves on the ¼ MS and ¼ MS 60 (3:1) increased for twelve months of culture (13.1±0.6 leaves), while that on ¼ MS+NAA+BA was increased only up to the ninth month (9.5±1.4 leaves), followed by a decrease in number (Figure 5B). A significant difference in number of leaves was only shown in the twelfth month after planting. Other *Nepenthes* species, *N. ampullaria* and *N. mirabilis*, grown in ¼ MS medium showed a different response compared to *N. jamban*, both of which developed 12 leaves by the twelfth month (Yelli 2013). The others were grown on ½ MS supplemented with cytokinin and auxin also showed different responses. An increase in the number of leaves in *N. ampullaria* occurred after twelve months of subculture (micro-cutting) in ½ MS supplemented with 0.5 mg L^{-1} BAP (Budisantoso et al. 2018). Another previous study also reported an increase in the number of leaves in *N. gymnamphora* (5 leaves) eight months after subculturing in ½ MS supplemented with 1 mg L^{-1} thidiazuron (Meinaswati et al. 2022).

The shoot height of *N. jamban* increased for twelve months in culture in all media treatments where the tallest shoots showed in ¼ MS medium with an average shoot height of about 2.57±0.1 cm (Figure 5C). However, the shoot height of another *Nepenthes* species, *N. khasiana*, grown on ¼ MS + 2.20-44.40 µM BAP medium reached 0.45-0.67 cm in the fourth month (Nongrum et al. 2009), 1.95 cm on ½ MS + 0.5 mg L^{-1} BAP medium in the twelfth month (Budisantoso et al. 2018), and 2.1 cm on ½ MS + 1 mg L^{-1} BAP medium in the eighth month of culture (Devi et al. 2013).

In general, the best growing medium for the three parameters observed in this study (number of pitchers, number of leaves, and plant height) was ¼ MS 60 (3:1) which was ¼ MS medium with a ratio of nitrogen sources of 3:1 (nitrate:ammonium). It showed that nitrogen sourced from nitrate (NO_3^-) gave a positive effect on the vegetative growth of *N. jamban in vitro*. Another previous study also reported that the combination of two nitrate sources (ammonium nitrate and calcium nitrate) as nitrogen sources was better for *N. khasiana*, *N. pervillei*, and *N. vieillardii* growth compared to adding only single source of nitrogen in the media (Mao et al. 2007). Schulze et al. (1999) also explained that there is a

transporter gene for ammonium localising ions in the *N. alata* pitcher glands associated with nitrogen uptake. Nitrogen is one of the most important minerals in plants as it is a part of amino acids and proteins. Moreover, it also regulates enzyme activities for energy metabolism (Hussain et al. 2016). In general, a high nutrient medium will significantly affect seed germination. However, excess levels of nitrogen and other nutrients will result in plant death as the plant experiences toxicity. On the other hand, improper composition and concentration of the basal medium can delay the germination rate (Jakovljević et al. 2017; Noorhosseini et al. 2018).

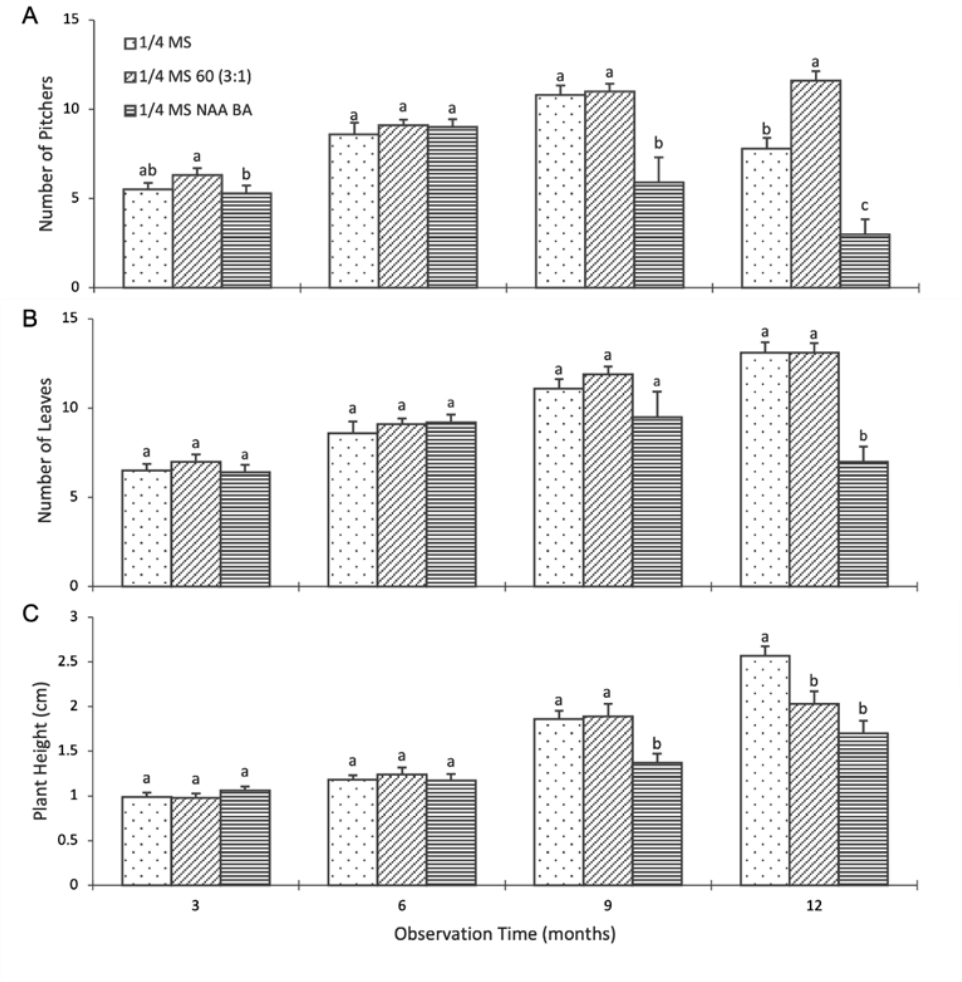

Figure 5. Shoot growth of *N. jamban* for twelve months observed on three types of growing media. The same letter on the bar in each time group showed no significant difference by LSD test at $p \leq 0.05$.

CONCLUSION

The concentration of nutrients in the medium proved to be more effective for inducing *in vitro* seed germination and enhancing shoot growth which is also supported by a higher nitrogen (nitrate) concentration in the medium. In this study, ¼ MS and ¼ MS 60 (3:1) media were the most suitable for *in vitro* seed germination and vegetative growth of *N. jamban*. This study provides information that supports the *ex situ* conservation action of native *Nepenthes* species from Indonesia. However, the germination started late at the sixth months after planting and attempts to make the germination faster should be done. The study about more effective and efficient culture media and its correlation to the seed preservation and dormancy might be advantageous for the conservation of *N. jamban*.

AUTHORS CONTRIBUTION

All authors have reviewed the final version of the manuscript and approved it for publication. ADP and RSR designed and performed the research and analyzed the data. All authors wrote and reviewed the manuscript.

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CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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Review Article

Bioactivity and Metabolites Compounds of Medicinal Plants Endophytic Fungi in Indonesia

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ABSTRACT

Indonesia is rich in diversity of medicinal plants, vital in traditional medicine and the pharmaceutical industry. However, overharvesting, along with population growth, land use changes, deforestation, and climate change, endanger these plants. This review investigates the potential of endophytic endosymbiont as an alternative. These endosymbionts can synthesise bioactive compounds similar to those found in medicinal plants. This study compiled data from various sources on endophytic fungi and their bioactivity. The review aims to categorise Indonesian medicinal plants, to identify their associated endophytic fungi from different plant parts, and to assess their bioactivity. The results revealed numerous medicinal plant families and a variety of endophytic fungi isolated from fruits, leaves, twigs, bark, roots, and rhizomes. These fungi exhibited bioactivities, including antioxidant, anticancer, antidiabetic, and antimicrobial effects, with metabolites such as alkaloids, flavonoids, peptides, phenols, polyketides, quinones, steroids, and terpenoids. *Fusarium* and *Colletotrichum* were the most common endophytic fungi found. Notably, the biological activity was consistent among endophytic fungi from various host organs, but variations were observed according to the host's geographical origin. This suggests that Indonesia's diverse geography influences metabolite production and activity. However, the same host plant may harbour different species in distinct organs. These findings indicate that endophytic fungi within medicinal plants represent a promising source of bioactive compounds for future Indonesian medicine production. Future research should explore metabolite compounds and bioactivity across different geographical regions.

INTRODUCTION

As the demand for herbal medicines, natural health products, and the production of secondary metabolites expands, the usage of medicinal plants is growing quickly throughout the world (Chen et al. 2016). In

developing nations, medicinal plants and their derivatives continue to be the primary sources for treating a variety of diseases due to their affordability and accessibility (Verma & Singh 2008). The distribution of medicinal plants worldwide encompasses over 50,000 plant species that find application in cosmetic and medicinal products, constituting more than a tenth of all plant species (Kopaei 2012). Even though conventional medication is readily available in many Asian nations, traditional medicine is still commonly used. Traditional medicine, also known as indigenous medicine or folk medicine, is a community-based technique of treatment that predates the invention of modern medicine (Gunjan et al. 2012). Extraction and cultivation of medicinal plants are essential activities in many Asian nations, such as Bangladesh, China, India, Nepal, Pakistan, Myanmar, and Indonesia (Astutik el al. 2019). An estimated half a million plant species in India have been examined phytochemically for their biological or medicinal properties (Bamola et al. 2018). China has employed 8000 types of medicinal herbs since the Paleolithic Age (Huang 2011). Approximately 472 species plants have been used as medicinal plants in Myanmar (DeFilipps & Krupnick 2018). Over 2187 plant species in Thailand have been identified as having therapeutic value (Phumthum et al. 2019). As a megadiverse nation, Indonesia possesses between 2500 and 7500 kinds of medicinal plants that are widely used as medications and cosmetics throughout the country (Cahyaningsih et al. 2021a).

In Indonesia, where a large quantity of medicinal herbs thrives, a significant portion of the population, particularly those residing in rural areas, traditionally depend on "jamu" or herbal medicines for disease treatment. This practice has led to the development of jamu production on an industrial scale (Elfahmi et al. 2014). However, this increased demand has the potential to cause overharvesting for the medicinal trade, resulting in the depletion of medicinal plant resources. Coupled with human population growth, land conversion, deforestation, and the impacts of climate change, the conservation of Indonesian plant species becomes both challenging and costly (Cahyaningsih et al. 2021b). Endophytic endosymbiont microorganisms present an alternative strategy. They possess the capability to synthesise bioactive compounds similar that produced by their host plants (Alnweiri 2021). Endophytes, which include microorganisms like fungi and bacteria, establish a unique symbiotic relationship with their host plants, residing within the internal plant parts (Pimentel et al. 2011). These microorganisms remain inconspicuous, causing no visible signs of infection or disease (Zheng et al. 2021). Consequently, they represent a hidden microbial world within plants, often overlooked compared to their more pathogenic counterparts. Yet, they constitute an underutilised resource for the discovery of novel therapeutic compounds (Kharwar et al. 2011). Endophytes are a valuable source of natural compounds due to their remarkable ability to produce a diverse range of biologically active substances. Compared to other endosymbiotic microorganisms, they generate a wide array of secondary metabolites. These compounds are the subject of ongoing research for various medicinal and industrial applications (Sudha et al. 2016). Endophytes are known to synthesise various classes of metabolites, including alkaloids, cytochalasines, furandiones, glycosides, isocoumarins, isoprenoids, lipids, perylene derivatives, phenols, polyketides, peptides, proteins, steroids, terpenoids, shikimates, and xanthones (Kharwar et al. 2011; Joseph & Priya 2011; Jalgaonwala et al. 2017; Rajamanikyam et al. 2017).

Endophytic fungus produces similar bioactive compounds from their host plants and therefore it is hypothesised that the complexities of fungal endophytes and host plant relationships vary across microorganisms and hosts (Verma et al. 2009). Ludwig-Müller (2015) delineates the

multifaceted interactions between plant and endophytes at various levels: (a) endophytes stimulate host metabolism, (b) the host reciprocates by stimulating endophyte metabolism, (c) the host and endophyte collaborate in distinct metabolic pathways, (d) both the host and endophyte may engage in the metabolism of endophyte products, and (e) secondary chemicals from the host can be metabolised by the endophyte. These options can apply to one or many enzymatic stages in the biochemical transformation process.

Numerous host plants have been explored for their endophytic fungi, with most of them found to harbour diverse fungal endophyte populations. The richness and diversity of these fungal endophytes are intricately linked to environmental factors such as rainfall and air humidity where the host plants thrive (Gigantea et al. 2011).

Endophytic fungi have proven to be prolific sources of novel bioactive compounds, demonstrating antibacterial, cytotoxic, anticancer, and insecticidal properties (Zheng et al. 2021). As highlighted by Newman & Cragg (2020) over 70% of anticancer and antibacterial medications derived from endophytic fungus contain natural bioactive compounds or their derivatives. Additionally, a wide range of endophytes hold potential for producing compounds that enhance plant growth and stimulate plant hormones. They are capable of generating chemicals with applications in agriculture, such as nematocidal and insecticidal properties, iron-chelating agents, and resilience to abiotic stress (Sharma et al. 2021).

Based on that information, it is necessary to determine the pharmacological potential of endophytic fungi isolated from Indonesian medicinal plants as natural bioactive source. This comprehensive review aims to elucidate the utilisation of endophytic fungi as primary resources for the pharmaceutical industry. According to Suhel (2022), Indonesia is still importing raw materials for medicines to meet the increasing demand for the medicines industry. Utilising endophyte fungi from Indonesian medicinal plants is a solution to the availability of raw materials obtained domestically, preventing the extinction of plants which are Indonesia's rich biodiversity and preventing ecological damage due to exploration of perennial plants which take a long time to grow. This paper review provides a thorough exploration the bioactive metabolite from Indonesia medicinal plants diversity from across the Indonesia archipelago.

INDONESIA MEDICINAL PLANTS

Indonesia has a great diversity in natural resources, including abundance of medicinal plants. It is the second largest tropical country after Brazil with a total of 40 thousand plants species are found Indonesia (Zakariya et al. 2020). The application of medicinal plants is a knowledge passed down from generation to generation, and still maintained as local wisdom in many tribes in Java, Sumatra, Kalimantan, Sulawesi, and Papua Island. Batak karo tribe in North Sumatera uses *Hibiscus rosa-sinensis* (kembang sepatu), *Ceiba pentandra* (kapuk), *Etlingera elatior* (kecombrang) as medicine to cure fever (Silalahi & Nisyawati 2018). Sundanese tribe in Java still use herbs as medicinal treats such as *Ageratum conyzoides* (babandotan), *Pluchea indica* (baluntas), *Indian jujube* (daun binara), *Lagerstroemis speciosa* (bungur), *Physalis Angulata* (cecendet), *Syzygium aromaticum* (cengkeh), *Phyllanthus acidus* (cereme), *Cogon grass* (eurih), *Graptophyllum pictum* (handeuleum,), *Bidens pilosa* (hareuga), and *Plumbago zeylanica* (ki encok) (Suganda et al. 2018). Keladi (*Colocasia esculenta* L) appears to be the most useful plant as medicine in Dayak Tribe, West Kalimantan (Supiandi et al. 2019), whereas Dayak Tribes in North Kalimantan used plants from *Asteraceae* family as the highest proportion me-

dicinal herb for treating pain (Novrianti et al. 2020). In South Sulawesi, *Allium cepa* is the most used plant at Rongkong community in North Luwu District (Mustofa et al. 2020). Papua has a diverse biodiversity, and there are various native plants which utilised as traditional medicine by Papua people, such as *Myrmecodia pendans* Merr, *Asteromyrtus symphyocarpa* Linn. Craven (Winara et al. 2015), and *Allophylus cobbe* (L.) Raeusch (Ibo & Arifa 2021).These plants are solely utilised as traditional medicine by the Papuan tribe. Since malaria is an endemic disease in Papua, they use *Andrographis paniculata* (Burm. f.) Nees, *Carica papaya* L., *Alstonia scholaris* (L.) R. Br., and *Physalis minima* L. as antimalaria traditionally Other common medicinal plants in Indonesia community listed in Table 1.

ENDOPHYTIC FUNGI AND THEIR BIOACTIVE POTENTIAL

Endophytic Fungi Ecological and Diversity from Indonesia Medicinal Plants

Endophytic fungi, unlike mycorrhizal fungi that mainly inhabit the root systems of plants, reside within various plant tissues, including roots, stems, and leaves. They establish a symbiotic relationship with their host plants. Unlike mycorrhizal fungi, which mainly reside in the root systems of plants, endophytic fungi colonize the entire plant, both intercellularly and intracellularly (Rodriguez et al. 2009; Schulz & Boyle 2014). The structural changes in endophytic root fungus colonization indicate varying degrees of colonization. This colonization pattern is often observed in intercellular sections, the outer cortical regions, and the epidermis of plant roots. Endophytes have been detected both adhering to the root

Table 1. Common Medicinal Plants in Indonesia.

No	Local Name	Scientific Name	Location	Family	References
1	Mahoni	*Swietenia mahagoni* (L.) Jacq.	North Sumatra and Papua Island	Meliaceae	Situmorang et al. 2015; Rahmawaty et al. 2019; Budiarti et al. 2020
2	Kemiri	*Aleurites moluccana* (L.) Willd.	South Aceh	Euphorbiaceae	Suwardi et al. 2020
3	Kelapa	*Cocos nucifera* L.	Southeast Sulawesi, West Kalimantan, and East Java	Arecaceae	Supiandi et al. 2019; Jadid et al. 2020; Fachruddin et al. 2021
4	Jeruk nipis	*Citrus aurantifolia* (Christm.) Swingle, orth.	East Java	Rutaceae	Shalas et al. 2021
5	Jambu Biji	*Psidium guajava* L.	South Sumatra	Myrtaceae	Kurniati et al. 2019
6	Jamblang	*Syzygium cumini* L.	Aceh		Gemsih et al. 2017
7	Jahe	*Zingiber officinale* Rosc.	Indonesia	Zingiberaceae	Ginting et al. 2013; Harwoko et al. 2021
8	Lengkuas	*Alpinia galanga* L.	Bali		Raniningsih & Sandy 2018
9	Pegagan	*Centella asiatica* (L.) Urban.	Bengkulu	Apiaceae	Radiastuti et al. 2019
10	Kelor	*Moringa oleifera* Lam.	South Sulawesi	Moringaceae	Rohadi et al. 2019
11	Alang-alang	*Imperata cylindrica* (L.) Beauv.	Indonesia	Poaceae	Jamilatun & Shufiyani 2019
12	Dewadaru	*Mesua ferrea* L.	Banyumas, Indonesia	Callophylaceae	Hartanti 2015
13	Merica	*Piper nigrum* Linn	Kutai Kartanegara, East Kalimantan	Piperaceae	Sopialena et al. 2018
14	Srikaya	*Annona squamosa* L.	Indonesia	Annonaceae	Yunianto et al. 2012
15	Kayu secang	*Caesalpinia sappan* L.	South Sulawesi	Caesalpiniaceae	Hafsan et al. 2018

epidermis and within the cell walls (Pal et al. 2020). Plant-fungal partnerships are highly symbiotic, a result of the close adaptation of host plants and their fungal companions, developed through coevolution and cohabitation, strengthening their genetic compatibility (Mengistu 2020). Successful endophyte colonisation depends on the compatibility of interactions between plant and microbe. Upon an endophyte's invasion, the host plant recognises it and initiates a signaling molecule exchange (Khare et al. 2018). Fungal endophytes, with a diverse range of host interactions, may employ various strategies to penetrate the host's internal systems. These strategies include altering plant elicitors, producing toxic metabolites, and suppressing the host's immune response (Selim et al. 2012).

Endophytic fungi must overcome the plant's initial defense mechanisms to establish a successful infection. They achieve this by manipulating host cells to form structures that enable them to penetrate plant tissues while preserving host cell integrity for a long-lasting association (Kogel et al. 2006). The first stage of plant-microbe interaction, which includes endophytes, involves the detection of plant exudates in the soil. There is a theory that plants can interact with microorganisms by releasing exudates, which are a mixture of substances such as carbohydrates, organic acids, and amino acids. The composition of exudates can vary depending on the plant species and its environmental conditions, whether they are biotic or abiotic (Haldar & Sengupta 2015). Mehmood et al. (2018) discovered that the IAA hormone and flavonoids are crucial participants in the intricate chemical conversation between plant roots and endophytic fungus. The use of IAA as an aerial spray to corn seedlings enhanced the roots' ability to interact with fungi and their effective colonisation of the roots. IAA application increased the concentration of flavonoids in endophyte-infected root exudates.

Fungal endophytes have been isolated from a wide range of plant biological environments, from dry, arid regions to arctic areas, and from temperate forests to tropical woodlands, savannahs, grasslands, and croplands. They have been discovered in a diverse range of plants, ranging from Angiospermae to Gymnospermae, mosses to ferns, and from simple to complex plants (Rashmi et al. 2019). Temperature, humidity, and soil nutrition levels are the key variables in determining secondary metabolite types and amounts produced by the plants, which in turn affected the population structure of the fungal endophytic (Jia et al. 2016). Endophytic assemblages vary in composition depending on region and host species. Endophytic communities have been discovered in various host lineages (Weig et al. 2013). This might be due to the actions of antifungal metabolites such flavonoids (Unterseher et al. 2016) and suitable populations of host and fungus are necessary for a successful infection (Guerreiro et al. 2022). Plant species adjust their biological systems under competitive and unfavourable ecological situations by creating diverse defense responses, which are mostly evident in the creation of protective secondary metabolites. These metabolites also help to create host defensive mechanisms, which improves the host's ability to adapt to a wide range of biogeographical habitats (Alam et al. 2021). Several endophytic fungi in different ecosystem are presented in Table 2.

Isolation of bioactive compounds directly from medicinal plants requires a lot of biomass which has an impact on the destruction of biological resources due to limited raw materials for herbal medicines (Godlewska et al. 2021). Therefore, one way of using biotechnology approach to increase the production of secondary metabolites from medicinal plants is by using endophytic microbes, namely endophytic fungi. Ogbe et al. (2020) elucidated the capacity of endophytic fungi to synthesise

Table 2. Endophytic fungi isolated from various plant in different ecosystem.

Most dominant Fungal endophytes	Host plants	Environmental condition	References
Alternaria sp., *Asperigillus* sp., and *Penicillium* sp.	Desert medicinal plants (*Artemesia sieberii* L., *Citrillus colocynthis* (L.) Schrad., and *Moringa peregrina* (Forssk.) Fiori)	Arid environment	Rehman et al. 2019
Neocamarosporium, *Preussia*, *Alternaria*, *Ascochyta*, *Phoma*, *Comoclathris*, *Neomicrosphaeropsis*, *Aureobasidium*, *Pleospora*, and *Fusarium*	Arid plant of Andalusia (*Limonium majus* (Boiss.) Erben or *Moricandia foetida* Bourg. ex Coss., *Helianthemum almeriense* Pau, *Centaurea dracunculifolia* Dufour., and *Euzomodendron bourgeanum* Cosson.)	Arid environment	González-Menéndez et al. 2018
Aspergillus sp., *Botryosphaeria sp.*, *Preussia sp.*, *Phoma sp.*, *Aureobasidium sp.*, *Penicilliumc sp.*, *and Chaetomium sp.*	*Simmondsia chinensis (Link) C.K. Schneid.*, *Phoradendron californicum Nutt.*, *Parkinsonia microphylla Torr.*, and *Larrea tridentata (de Candolle) Coville.*	Arid environment	Massimo et al. 2015
Aeopora sp., *Sebacina* sp., *Knufi* sp., *Tomentella* sp., and *Penicillium* sp.	*Argania spinosa* (Linnaeus) Skeels. root	Arid environment	Noui et al. 2019
Pezizomycotina sp., *Monosporascus ibericus*, and *Alternaria eichhorniae*	*Kalidium foliatum* (Pall.), *Salsola nitraria* Pall., *Seriphidium santolinum (Schrenk) Poljakov*, *Reaumuria songarica (Pall.) Maxim.*, *Ceratocarpus arenarius* L., *Suaeda acuminata (C.A.Mey.) Moq.*, *Suaeda salsa* (L.) Pall., *Eragrostis minor* Host, *Peganum harmala* (L.), and *Bassia dasyphylla (Fisch. & C.A.Mey.) Kuntze.*	Arid environment	Li et al. 2020
Cladosporium cladosporioides, *Fusarium oxysporum*, *Acremonium implicatum*, *Aureobasidium pullulans*, *Trichoderma viride*, *Chrysonilia sitophila*, and *Aspergillus flavus*	Cactus *Cereus jamacaru* DC.	Tropical forest	Bezerra et al. 2013
Acrocalymma sp., *Fusarium* sp., *Tolypocladium* sp., *Penicillium* sp, *Talaromyces* sp., *Exophiala* sp., *Dictyosporium* sp., *Pseudochaetosphaeronema* sp., *Mariannaea* sp., *Trichoderma* sp., *Mycoleptodiscus* sp., *Acrocalymma* sp., *Tolypocladium* sp., *Penicillium* sp., *Exophiala* sp., *Pseudochaetosphaeronema* sp., *Mariannaea* sp., and *Mycoleptodiscus* spp.	*Paraserianthes falcataria* (L.) Nielsen.	Tropical forest	Maulana et al. 2018
Talaromyces, *Phyllosticta*, *Diaporthe*, and *Colletotrichum.*	*Myracrodruon urundeuva Fr. Allem.*	Tropical dry forest	de Pádua et al. 2019

Table 2. Contd.

Most dominant Fungal endophytes	Host plants	Environmental condition	References
Aspergillus flavus, *Altenaria* sp., *Bionectria ochroleuca*, *Colletotrichum acutatum*, *Cladosporium* sp., *Cochliobolus sativus*, *Diaporthe amygdali Fusarium Oxysporum*, *Guignardia mangiferae*, *Phomopsis* sp., *Phyllosticta gardeniicola*, *Trichoderma harzianum*, *Tricharina gilva*, and *Nigrospora oryzae*.	*Warburgia ugandensis* Sprague	Tropical forest	Mbilu et al. 2018
Chrysosporium sp., *Curvularia lunata*, *Penicillium* sp., *Geotrichum candidum*, and *Trichoderma* sp.	*Caesalpinia sappan* L.	Tropical forest	Hafsan et al. 2018
Alternaria sp, *Pestalotiopsis* sp., *Fusarium* sp., *Nigrospora* sp., *Phoma* sp., and *Xylaria* sp.	*Rhizophora mucronate* Lam.	Mangrove forest	Hamzah et al. 2018
Auriculibuller, *Yamadazyma*, *Pseudoplectania*, and *Simplicillium*	Mangrove trees	Mangrove ecosystem	Yao et al. 2019
Genera *Pestalotiopsis* and *Phomopsis*	Rhizophoraceae mangrove plant	Mangrove ecosystem	Xing & Guo 2011
Aspergillus sp., *Phoma* sp., and *Penicillium* sp.	*Avicennia marina* (Forssk.) Vierh.	Mangrove ecosystem	Selvakumar et al. 2014
Guignardia sp. and *Colletotrichum gloeosporioides*	*Rhizophora mangle* L., *Avicennia schaueriana* Stapf & Leechm. ex Moldenke., and *Laguncularia racemose* (L.) C.F. Gaertn.	Mangrove ecosystem	Costa et al. 2012
Aspergillus niger, *Acremonium* sp., *Chaetomium* sp., *Cladosporium* sp., *Fusarium* sp., Phomopsis sp., and *Xylaria* sp.	Seaweeds	Marine ecosystem	Ahamed & Murugan 2019
Trichoderma sp.	*Padina* sp.	Marine ecosystem	Handayani et al. 2019
Aspergillus sp., *Fusarium* sp. and *Penicillium* sp,	*Hypnea musciformis (Wulfen) J.V.Lamouroux*, *Sargassum crassifolium* J. Agardh., *Dictyota dichotoma (Hudson) J.V.Lamouroux*, and *Caulerpa peltata J.V.Lamouroux*.	Marine Ecosystem	Officer & Road 2020

metabolites influenced by their host plants, rendering them a promising and dependable source of secondary metabolites. The isolation of endophytic fungi from diverse plant organs serves the purpose of identifying bioactive agents that produce secondary metabolites, often found in specific plant organs, with crucial medicinal potential. Plants produce secondary metabolite compositions in various parts, such as fruits, leaves, stems, and roots (Astuti & Respatie 2022). Endophytic fungi have been discovered from a diverse range of medicinal plants. These endophytic fungus generated a diverse spectrum of industrially significant bioactive compounds (Rana et al. 2020). Indonesia has abundant medicinal plants that used as traditional medicine for a long time. The exploration of their

endophytic fungi also become the focus of several researchers. Fungi, genus of *Fusarium* and *Colletotrichum* appears as the most dominant endophytes found in Indonesia medicinal plants. However, endophytic fungi do not show host specifications for certain plant hosts or certain organs. Diverse endophytic fungi that have successfully isolated from Indonesia medicinal fungi are listed in Table 3.

Table 3. Endophytic Fungi Isolated from Indonesian Medicinal Plants.

Plant Species	Organ	Endophytic Fungi	References
Calopogonium mucunoides Desv.	Leaves and stems	*Phomopsis* sp., *Corynespora* sp., *Rhizoctonia* sp., *Helicosporium* sp., *Curvularia* sp., *Acremonium* sp., *Torulomyces* sp., *Gliocladium* sp., *Humicola* sp., *Gloeosporium* sp, *Phoma* sp, *Tripospermum* sp., *Aureobasidium* sp., *Colletotrichum* sp., *Fusarium* sp., and *Sclerotium* sp.	Fitriarni & Kasiamdari 2018
Zingiber officinale Rosc.	Leaves, rhizome, roots, and stem	*Acremonium macroclavatum*, *Lecanicillium kalimantanense*, *Myrothecium verrucaria*, *Trichoderma harzianum*, *Beltraniella* sp., *Cochliobolus geniculatus*, *Colletotrichum gloeosporoides*, *Neonectria punicea*, *Periconia macrospinosa*, *Rhizopycnis vagum*, *Glomerella cingulate*, and *Talaromyces assiutensis*	Ginting et al. 2013; Harwoko et al. 2021
Centella asiatica (L.) Urban.	Stolons, leaves, roots, and petioles	*Acrocalymma vagum*, *Aspergillus oryzae*, *Aspergillus austroafricanus*, *Peroneutypa scoparia*, *Chaetomium globosum*, *Ceratobasidium* sp., *Phoma multirostrata*, *Fusarium* sp., *Colletotrichum karstii*, *C. gigasporium*, *C. tabaci*, *Colletotrichum siamense*, *Eutypella* sp., *F. oxysporum*, *F. falciforme*, *Penicillium capsulatum*, *F. keratoplasticum*, *F. striatum*, *Ceratobasidium cornigerum*, *Earliella scabrosa*, *Perenniporia tephropora*, *Perenniporia* sp., *Phanerochaete chrysosporium*, *Phanerochaete stereoides*, *Phyllosticta capitalensis*, *Phomopsis asparagi*, *Mycochaetophora gentinae*, *Phialemoniopsis* sp., *Talaromyces* sp., and *Trichaptum* sp.	Radiastuti et al. 2019
Moringa oleifera Lam.	Lamina, petiole, and stem	*Cladosporium* sp., *Colletotrichum* sp., *Fusarium* sp., *Phomopsis* sp., and *Phyllosticta* sp.	Rohadi et al. 2019
Syzygium cumini L.	Leaves	*Colleotrichum* sp., *Acremonium* sp., *Fusarium* sp., *Macrophopmina* sp., *Dactylella* sp., *Paecilomyces* sp., and *Nigrospora* sp.	Gemsih et al. 2017
Imperata cylindrica (L.) Beauv	Flowers, stem, and leaves	*Fusarium* sp. and *Mucor* sp.	Jamilatun & Shufiyani 2019
Alpinia galanga L	Rhizome	*Trichoderma viridae*, *Trichoderma harzianum*, and *Fusarium oxyforum*	Raniningsih & Sandy 2018
Mesua ferrea L.	Leaves and twigs	*Penicillium* sp. and *Aspergillus* sp.	Hartanti 2015
Piper nigrum Linn	Leaves and roots	*Aspergillus* sp., *Fusarium* sp., *Nigrospora* sp., and *Trichoderma* sp.	Sopialena et al. 2018
Guazuma ulmifolia Lam.	Leaves	*Curvularia affinis*, *Lasiodiplodia mahajangana*, *Talaromyces trachyspermus*, *Diaporthe tectonae*, and *Parengyodontium album.*	Sukarno et al. 2021
Hydrocotyle verticillata Thunb.	Leaves	*Muyocopron laterale* and *Didymella coffeae-arabicae*	Sukarno et al. 2021
Cinchona calisaya Wedd.	Flowers, leaves, petioles, stems, bark, and roots	*Fusarium oxysporum. Diaporthe* sp., *Neofussicoccum* sp., *Guignardia mangiferae*, *Colletotrichum gloeosporioides*, and *C. Brasiliense.*	Hidayat et al. 2019

Table 3. Contd.

Plant Species	Organ	Endophytic Fungi	References
Annona squamosa L.	Seeds	*Fusarium* sp. and *Nectria rigidiuscula*	Yunianto et al. 2012
Curcuma longa L.	All plant organs	*Phaeosphaeria ammophilae*, *Phanerochaete chrysosporium*, *Fusarium verticillioides*, *Fusarium solani*, *Fusarium proliferatum*, *Fusarium oxysporum*, *Arthrobotrys foliicola*, *Daldinia eschscholzii*, and *Cochliobolus kusanoi*.	Septiana et al. 2017
Morinda citrifolia Linn.	Fruits and leaves	*Stemphylium solani*, *Leptosphaerulina australis*, and *Xylaria* sp.	Wu et al. 2015
Caesalpinia sappan L.	Stem	*Geotrichum candidum*, *Trichoderma* sp., *Penicillium* sp., *Chrysosporium* sp., and *Curvularia lunata*.	Hafsan et al. 2018

Indonesia Medicinal Plant Endophytic Fungi Produced Antioxidant

Antioxidants are chemicals that, in very low quantities, dramatically block the role of oxidation in the targets (Rasheed & Azeez 2019). The antioxidant properties of certain polyphenolic compounds, like flavonoids, are often linked to the presence of aromatic phenolic rings. These rings have the ability to donate electrons and to transfer hydrogen atoms to free radicals, acting as scavengers of free radicals, inhibitors of the formation of single oxygen, and reducing agents (Wu et al. 2018).

Endophytic fungi are known to produce a variety of antioxidant chemicals, that are responsible for host plant stress tolerance (Manganyi & Ateba 2020). Many studies have been conducted to investigate the different fungal species found in medicinal plants, and the results have been promising (Toghueo & Boyom 2019). This section discusses the antioxidant effects of crude extracts and compounds obtained from endophytic fungi associated with several Indonesia medicinal plants.

Antioxidants activity have been observed from endophytic fungi that isolated from turmeric. Turmeric has been used as traditional medicine in Indonesia to treat many diseases include related to free radical diseases (Subositi & Wahyono 2019; Permatananda et al. 2021; Rahmat et al. 2021). Septiana et al. (2020) have been discovered that the ethyl acetate extracts of endophytic fungi from turmeric flowers have antioxidant activity. Moreover, the extracts of single and mixed cultures demonstrated antioxidant activity, with IC_{50} values ranging from 247.90 to 634.64 g/mL. Endophytic fungi isolated from various tissues of turmeric have also been shown to have antioxidant properties. Endophytic fungi extracts from turmeric rhizome, root, leaves, and also turmeric stem, exhibited antioxidant activity in ethyl acetate (Widowati et al. 2016; Eris & Simanjuntak 2017; Septiana et al. 2019; Septiana et al. 2020). *Chrisonilia sitophila*, *Acremonium* sp., and *Penicillium* sp. were isolated from Kandis Gajah twigs, a traditional medicine in south Sumatera, as endophytic fungi. The antioxidant bioactive molecule generated by *Acremonium* sp was sesquiterpene 3,5-dihydroxy-2,5-dimethyltrideca-2,9,11-triene-4,8-dione, according to NMR studies (Elfita et al. 2012). Other endophytic fungi that produced antioxidant are listed in Table 4.

Endophytic Fungi Produced Anticancer

Endophytic fungi have emerged as a consistent and abundant source of potential anticancer agents, marking a significant contribution for anticancer drug development (Uzma et al. 2018). These fungi are at the forefront of the search for novel bioactive secondary metabolites with cytotoxic properties, significantly contributing to the quest for new plant-derived anticancer medications. Research has identified approximately 19

Table 4. Antioxidant Compounds Produced by Endophytic Fungi from Medicinal Plants in Indonesia.

Endophytic Fungi	Host	Compound	References
Cladosporium tenuissimum	*Swietenia mahagoni* (L.) Jacq.	5-hydroxy-2-oxo-2H-Piran-4-yl methyl acetate	Fadhillah et al. 2019
Lasiodiplodia venezuelensis	*Syzygium samarangense* L.	5,7-dihydroxy-6,8-dimethyl flavanone	Budiono et al. 2019
Fusarium sp. and *Colletotrichum* sp.	*Physalis angulata* L.	Not identified yet	Palupi et al. 2021
Apodus oryzae and *Diaporthe* sp.	*Aquilaria malaccensis* Lam.	Not identified yet	Hidayat et al. 2019
Aspergillus minisclerotigens and *Aspergillus oryzae*	*Mangifera casturi* Kosterm.	Dihydropyran and 4H-Pyran-4-one,5-hydroxy-2-(hydroxymethyl-(CAS) Kojic acid	Nuraini et al. 2019
Trichordema reecei	*Syzygium aqueum* (Burm.f.) Alston.	(4-hydroxy-3-(4-hydroxyphenyl)-5 oxo-tetrahydrofuran-2-yl) methyl acetate	Habisukan et al. 2021
Geotrichum candidum, *Trichoderma* sp., *Chrysosporium* sp., *Curvularia lunata* and, *Penicillium* sp.	*Caesalpinia sappan* L.	Not identified yet	Hafsan et al. 2018
Neofusicoccum parvum	*Cinnamomum burmanni (Nees &Th. Nees).*	Not identified yet	Rachman et al. 2018
Fusarium oxysporum	*Dahlia variabilis* Willd.	Not identified yet	Marlinda et al. 2019
Phyllosticta sp., *Colletotrichum* sp., *Phomopsis* sp., *Phoma* sp., and *Lasiodiplodia* sp.	*Vernonia amygdalina* Del.	Not identified yet	Hidayat et al. 2019
Acremonium sp.	*Garcinia griffithii* T. Anderson	3,5-dihydroxy-2,5-dimethyltrideca-2,9,11-triene-4,8-dione	Elfita et al. 2012

chemical families of fungal secondary metabolites known to exhibit anticancer effects against 45 different cell lines (Bano et al. 2016). Among these bioactive compounds, figures such as paclitaxel, podophyllotoxin, camptothecin, ergoflavin, swainsonine, sclerotiorin, flavone chrysin, torreyic acid, vincristine, and vinblastine have been isolated from endophytic fungi, offering promising prospects in anticancer drug development (Ejaz et al. 2020) (Figure 1).

In Indonesia, endophytic fungi within medicinal plants have shown great promise as sources of anticancer compounds. Notably, endophytic fungi from soursop leaves, isolated in West Java, demonstrated anticancer potential against the MCF-7 cell line with an IC_{50} of 19.20 (Minarni et al. 2017). *Hypomontagnella monticulosa* Zg15SU, originating from *Zingiber griffithii* Baker in North Sumatera, exhibited cytotoxicity against cancer cell lines HCT116, NBT-T2, and Panc-1, as determined through in vitro MTT proliferation experiments (Lutfia et al. 2021). Furthermore, endophytic fungi *Eutypa linearis* from *Coleus amboinicus* (Lour.) were found to produce compounds such as benzenemethanol, 4-nitro-(p-nitrobenzyl alcohol), 2-pentadecanone, (1R*,6S*,10R*)-5,5-dimethyl-11,12-dioxatricyclo[8.2.1.0(1,6)] tridecan-10-ol, Methyl linoleate, and 3-furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-2,5-dioxo-2,5-dioxo-2,5-dioxo-2,5-dioxo-2,5-dioxo-2 (Gemantari et al. 2021). Remarkably, 2-carboxymethyl-3-N-hexyl-maleic anhydride exhibited the highest cytotoxicity against the Hela cell line, with an IC_{50} of 301.53 ± 11.34 g/mL, surpassing its effects on other cell lines.

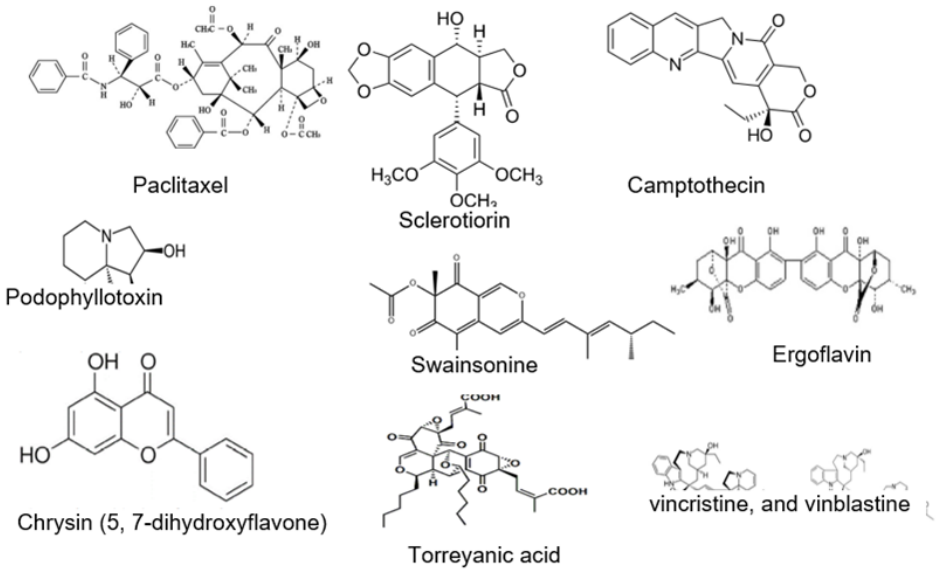

Figure 1. Anticancer Compounds Isolated from Endophytic Fungi (Ejaz et al. 2020).

Fungi Endophytes Produced Antidiabetic

Over the last decade, there has been a significant increase in diabetes prevalence, and will continue to rise in significance and quantity in the next future. As a result, screening for anti-diabetic benefits the novel and economical sources such as entophytes is required (Napitupulu 2018). Purified coumarone compound of 8-hydroxy-6,7-dimethoxy-3-methylisocoumarine obtained from the stem of *Quercus gilva* by endophytic fungi *Xylariaceae* sp. has considerable inhibitory effect against α-glycosidase (Indrianingsih & Tachibana 2017). Fungi *Colletotrichum* sp. from *Taxes sumatrana* cultivated in Indonesia produced the α-glycosidase inhibitor substances, include unsaturated fatty acids, specifically linolenic acids, linoleum, and oleic (Artanti et al. 2012). Others compounds that showed antidiabetic activity are quinadoline A (Strobel et al. 2004), scequinadoline J (Radjasa 2015), octadecanoic acid (Murugan et al. 2017), scequinadoline D (Čakar et al. 2018), peptide paecilodepsipeptide A (Glumac et al. 2017), asperpyridone A (Ancheeva et al. 2020), herbarin (Al-Hosni et al. 2020), aspergiamide A, and brevianamide K (Ye et al. 2021).

The diabetes has seen a significant increase over the past decade and is expected to continue rising in the future. Consequently, the search for novel and cost-effective sources with potential anti-diabetic properties, such as endophytes, is imperative (Napitupulu 2018). A purified coumarone compound, 8-hydroxy-6,7-dimethoxy-3-methylisocoumarine, derived from the stems of *Quercus gilva* via endophytic fungi *Xylariaceae* sp., has demonstrated a notable inhibitory effect against α-glycosidase (Indrianingsih & Tachibana 2017). *Colletotrichum* sp. from *Taxes sumatrana* cultivated in Indonesia has been found to produce α-glycosidase inhibitor substances, including unsaturated fatty acids such as linolenic acids, linoleic acid, and oleic acid (Artanti et al. 2012). Other compounds exhibiting anti-diabetic potential include quinadoline A (Strobel et al. 2004), scequinadoline J (Radjasa 2015), octadecanoic acid (Murugan et al. 2017), scequinadoline D (Čakar et al. 2018), peptide paecilodepsipeptide A (Glumac et al. 2017), asperpyridone A (Ancheeva et al. 2020), herbarin (Al-Hosni et al. 2020), aspergiamide A, and brevianamide K (Ye et al. 2021).

Antimicrobial Compounds Produced by Fungal Endophytes

Numerous endophytic fungi have been observed to synthesise alkaloids, flavonoids, peptides, phenols, polyketides, quinones, steroids, and terpenoids, which possess antibacterial properties (Mousa & Raizada 2013). Flavonoids are compounds generated by plants in response to microbial infections and exhibit hydroxylated phenolic characteristics. These antibacterial flavonoids display a diverse spectrum of activity against various cellular targets, facilitated through interactions such as hydrogen bonding, hydrophobic effects, and covalent bonding with proteins. Consequently, their antimicrobial activity is associated with the disruption of microbial adhesins, cell envelope transport proteins, enzymes, and other vital proteins. Furthermore, lipophilic flavonoids can influence microbial membranes (Cowan 1999; Mishra et al. 2009).

Endophytic fungi, specifically *Lasiodiplodia theobromae* and *Aspergillus oryzae* isolated from *Pometia pinnata* in Western Papua, have demonstrated inhibitory effects against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (Setyaningsih et al. 2020). Endophytic fungi residing in the endemic plants of South Kalimantan's *Mangifera casturi* have produced antibacterial compounds, including di-n-octyl phthalate, benzyl alcohol, high-oleic safflower oil, benzene acetonitrile, and benzotriazole, which exhibited inhibitory effects on Methicillin Resistant *Staphylococcus aureus* (MRSA) with an MIC value of 1.56% (Nuraini 2023). A molecular docking study has indicated that endophytic fungi *Trichoderma* sp., isolated from *Caesalpinia sappan* wood in South Sulawesi, effectively inhibited *Mellasezia* sp. (Susanti et al. 2021). A compound named 3-acetyl -2,5,7-trihydroxy-1,4-naphtalenedione produced by endophytic fungi isolated from *Tinospora crispa* displayed superior inhibitory capabilities compared to commercial antimicrobials (chloramphenicol, cabisidin, and nystatin) against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* (Praptiwi et al. 2013). Indonesian medicinal plants' endophytic fungi exhibiting potential against pathogenic bacteria are summarised in Table 5.

Table 5. Endophytic Fungi from Indonesian Medicinal Plants with Antimicrobial Potential.

Endophytic fungi	Host plants	Microorganism test	References
Trichoderma harzianum	*Zingiber officinale* Rosc.	*Staphylococcus aureus*	Harwoko et al. 2021
Neopestalotiopsis sp. and *Peniophora lycii*	*Rhizophora mucronata* Lamk.	*Eshcerichia coli* and *Staphylococcus aureus*	Fareza et al. 2018
Colletotrichum sp. and *Fusarium* sp.	*Physalis angulata* L.	*Eshcerichia coli* and *Staphylococcus aureus*	Palupi et al. 2021
Penicillium sp.	*Rhizophora apiculata* BI and *Bruguiera gymnorrhiza* (L.) Lamk.	*Salmonella typhi*	Rossiana et al. 2016
Cladosporium oxysporum	*Aglaia odorata* Lour	*Escherichia coli*, *Candida albicans*, and *Staphylococcus aureus*,	Sugijanto et al. 2016
Colletotrichum sp., *Penicillium* sp., *Trichophyton* sp., *Botrytis* sp., and *Trichophyton* sp.	*Medinilla speciosa* Blume	*Bacillus subtilis* and *Shigella dysenteriae*	Amelia et al. 2021
Culvularia lunata and Diaporthe phaseolorum	*Acanthus ilicifolius L.*	*Escherichia coli*, *Candida albicans*, *Staphylococcus aureus*, and *Shigella dysenteriae*	Widayanti et al. 2019

Table 5. Contd.

Endophytic fungi	Host plants	Microorganism test	References
Torulla sp., *Fusarium* sp., and *Drechcera* sp.	*Kaempferia galanga* L.	*Staphylococcus aureus*, *Vibrio cholera*, *Eschericia coli*, *and Bacillus subtilis*	Efendi et al. 2020
Aspergillus fumigatiaffnis	*Tribulus terestris* L.	*Streptococcus pneumoniae* and *Enterococcus faecalis*	Ola et al. 2018
Fusarium sp. and *Aspergillus* sp.	*Zingiber officinale* var. Roscoe	Methicillin Resistant *Staphylococcus aureus* (MRSA)	Sari et al. 2020

Antimicrobial peptides (AMPs) represent a class of broad-spectrum antimicrobials effective against bacteria, fungi, and viruses (Bahar & Ren 2013). These peptides play a crucial role in safeguarding various organisms, including humans, animals, and plants, by serving as the primary defense mechanism in the innate immune system (Lei et al. 2019). AMPs can be categorized based on their amino acid composition, structure, and function. They are generally classified into two groups: (1) linear, helical peptides devoid of cysteine, containing amino acids like glycine, proline, tryptophan, arginine, and histidine, exemplified by magainin and cecropin; and (2) cysteine-containing polypeptides characterized by disulfide bridge(s) formation, such as insect defensin (Bahar & Ren 2013; Moravej et al. 2018; Haney et al. 2019). Several AMP compounds produced by fungal endophytes, along with their chemical structures, are illustrated in Figure 2.

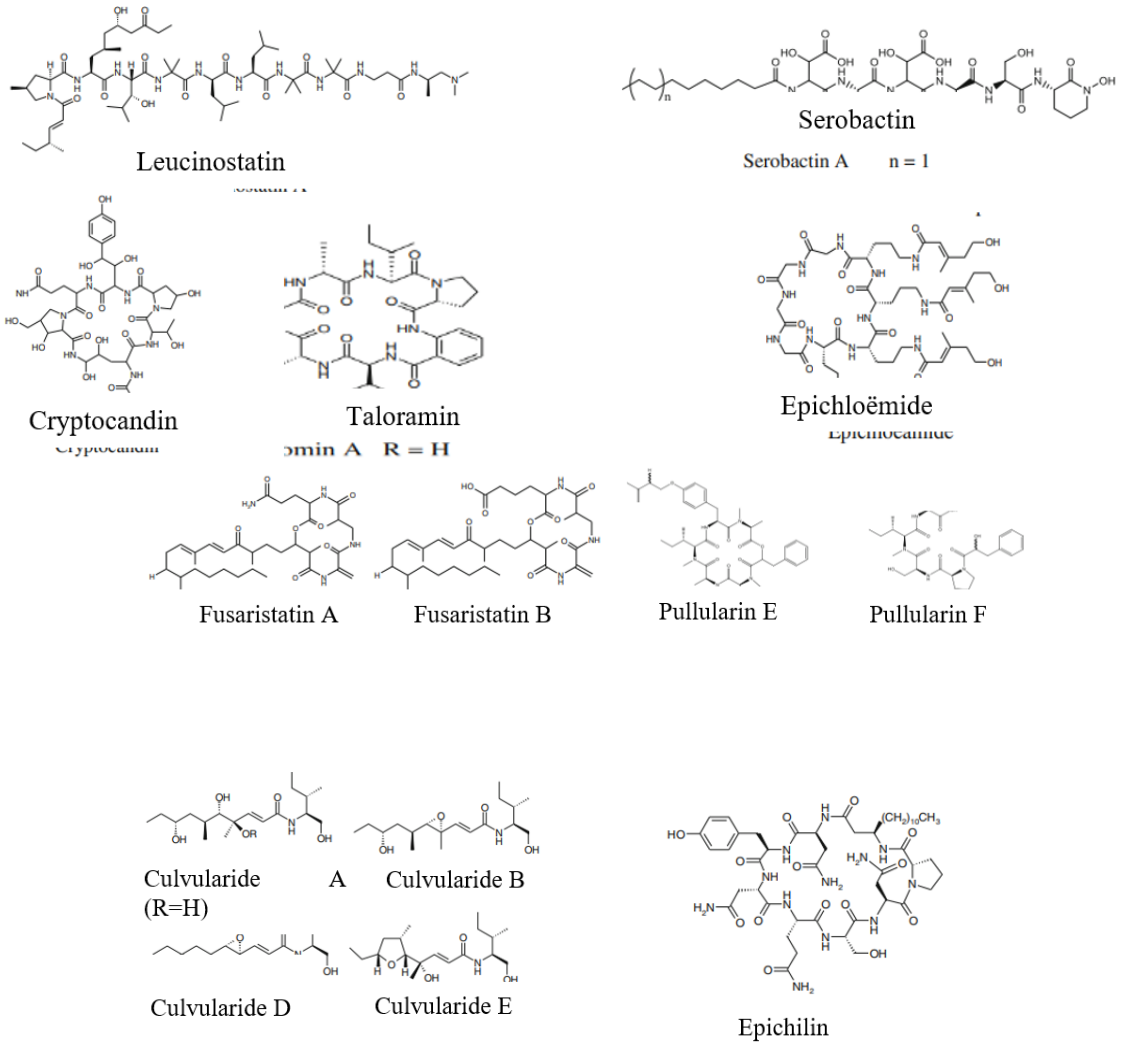

Figure 2. Antimicrobial peptides (AMPs) synthesised by endophytic fungi with antimicrobial properties.

CONCLUSION

Traditional plant-based medicine has been an integral part of Indonesian culture, across the diverse archipelago. Exploring their potential medicinal properties through the use of endophytic microorganisms, particularly fungi in this context, emerges as a promising strategy to safeguard the preservation of these valuable plant species. Endophytic fungi hold great promise due to their ability to synthesize compounds similar to those produced by their host plants, alongside a spectrum of bioactivities relevant to various therapeutic applications. Many of Indonesian medicinal plants have been investigated, yielding endophytic fungi with demonstrated antioxidant properties, anticancer potential, antidiabetic compounds, and antibacterial activities. Consequently, these endophytic fungi associated with medicinal plants represent a wellspring of bioactive compounds with the potential to shape the future of Indonesian pharmaceuticals.

AUTHOR CONTRIBUTION

E.S., searched the literatures and wrote the manuscript, A.K., Z.D., H.N., H.K., A.A., and S.H.L supervised the processes.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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Review Article

A Brief Review of Efficacious Plants in the World: *Tagetes* (Marigold)

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ABSTRACT
Tagetes is a genus consisting of several efficacious plant species known as marigold, which is native to Mexico and has spread worldwide due to its ability to adapt to different soil conditions. This genus is very popular among gardeners because it's easy to cultivate, adaptable, produces flowers all year round, has a free flowering habit, short duration, and the flower has an attractive color and shape. It has many flowers of varying colors such as red, orange, yellow, mixed red-orange or red-yellow, but the yellow and orange color are the most popular. The flowers contain many carotenoids, which act as antioxidants, antibacterial, anti-inflammatory, anti-carcinogen, nematicide, and cosmetics. The carotenoid can also cure fever, epileptic fits, astringent, scabies, liver complaints, stomachache, sore throat, and can be used as a natural moisturizer, and natural mosquito repellent Besides its use as a medicinal plant, *Tagetes* is also used as ornamental plants and edible plants. This manuscript's endeavour is to include some important investigations and studies about the general description, phytochemicals compounds and essential oils, medicinal uses, natural pesticides, and food and beverage uses of the marigolds.

INTRODUCTION
Tagetes is a genus consisting of several species known as marigolds, native to Mexico (Gupta & Vasudeva 2012; Adam 2017). Marigold is applied to several genera in Asteraceae (Compositae) with yellow or golden flowers (Mir et al. 2019). There are 53 species in this genus (Singh et al. 2020), while another publication mentioned that there are 33 species of *Tagetes* and only two species that are commonly known which are *Tagetes patula* L. (*T. patula* L.) known as French marigold and *T. erecta* L. (African marigold) (Ashritha et al. 2022). Besides its use as a medicinal plant, marigold is also a potential ornamental plant used in the culinary arts. The flower can be used as a natural dye and decoration. In the agricultural sector, this genus functions as a biological agent to deter pests and as garden decoration. The flowers contain many carotenoids, which

can act as antioxidants, anti-bacterial, anti-inflammatory, anti-carcinogen, nematicide, as well as can be used in cosmetics. The carotenoid properties also can also alleviate fever, epileptic fits, astringent, scabies, liver complaints, stomachache, sore throat, and can be used as a natural moisturizer, and natural mosquito repellent (Ampai et al. 2013; Priyanka et al. 2013; Munif et al. 2021; Kurniati 2021).

In many parts of the world, *Tagetes* has been used as an ornamental, edible and medicinal plant but has not been massively cultivated, especially in some areas that regularly use it for religious ceremonies and flower boards (Kurniati 2021). The province of Bali is the center of *Tagetes* cultivation in Indonesia, yielding about 100-200 billion IDR annually and meeting the demand of 8 tons per day (Beti 2020). In Indonesia, this plant is known as "bunga tahi ayam", "kenikir", "tahi kotok", "gumitir" and "randa kendana" and belongs to the *Asteraceae* family related to chrysanthemum and sunflower. The color is typically white, orange and yellow (Fauziana & Susandarini 2019; Zanovello et al. 2021; Lenawaty 2022). In Spain and France, *Tagetes erecta* L. (*T. erecta* L.) is mostly used for herbal medicine as an external detersive, resolutive and vesicant (Gras et al. 2017), while in Madagascar this plant is used for its antimalarial properties. The people of Rodrigues Island use this plant as a fever reducer by consuming the flower in liquid form (Joshi & Barbalho 2022).

The World Health Organization (WHO) mentioned that approximately 80% of the world population uses traditional medicine products, especially plant products, to maintain their health (Priyanka et al. 2013). Herbal medicine has been used in Indonesia since ancient times and has even become a part of the culture. Each region or ethnicity has its characteristics of traditional medicine due to natural conditions, especially the availability of some medicinal plants (Son et al. 2019; Az-Zahra et al. 2021). Indonesia has more than 400 ethnicities and sub-ethnics, spreading throughout the territories of Indonesia, such as Sumatra, Java, Kalimantan, and Sulawesi (Adiyasa & Meiyanti 2021). This country is rich in natural resources, and people use plants to cure some diseases because they are easy to grow and find, easy to prepare, contain safe ingredients, natural and have less side effects (Reid et al. 2016; Welz et al. 2018).

The development of traditional medicine has grown significantly in recent years, especially herbal medicine or plant-based medicine. Herbal medicine products have been produced on an industrial scale in some developing countries (Mir et al. 2019). Plant products have been long used for therapeutics, aromatherapy, medicine, food, and beverages, depending on their phytochemical properties and bioactivity (Mir et al. 2019). Herbal medicines, both individually and collectively, may cure some diseases. Consuming natural products either as food or medicine with minimal processing has become the habit of society in the world right now (Roman et al. 2017). The therapeutic value of marigold to treat some human ailments has been known for some time (Singh et al. 2020).

Studies on Marigold have been conducted by many researchers worldwide due to the carotenoid contents (alpha and beta carotene) and xanthophylls (lutein and zeaxanthin) (Buscemi et al. 2018; Manivannan et al. 2021). Other studies revealed that some parts of marigold could also be used as raw materials to make green organic fertilizer (Stroze et al. 2019). Secondary metabolites obtained from *Tagetes* are steroids, alkaloids, triterpenoids, carbon skeletons, derivates of thiophene, derivates of benzofuran, etc. (Verma & Verma 2012; Shetty et al. 2015; Munif et al. 2021). The primary objective of writing a review article on marigold plants is to comprehensively examine and consolidate existing literature,

thereby providing a comprehensive overview of the current knowledge regarding marigold's botanical characteristics, cultivation techniques, medicinal properties, and ecological significance. This review aims to contribute to the academic discourse on marigolds. It offers a valuable resource for researchers, horticulturists, and enthusiasts seeking to expand their understanding of this versatile and culturally significant plant species.

BOTANICAL CHARACTERISTICS

Marigold (Figure 1) belongs to *the Asteraceae* family and consists of 53 different species (Table 1). It originated from Mexico and Central America and can survive and grow even in drought conditions (Gupta & Vasudeva 2012; Khulbe 2015; Adam 2017). This plant is one of the ornamental herbs commonly used as a hedge plant and commercially as cut flowers because of the unique shape and flashy color of the flowers. *Asteraceae*, also called the Daisy family or Compositae is an important flowering plant family with more than 16200 genera and 23600 species. The species includes valuable medicinal plants that are not only used for medicinal purposes but also for ornamental, cosmetics, meditation, and food (Jan et al. 2009; Kashif et al. 2015; Politi et al. 2017), this genus is the second largest member in the Plantae Kingdom.

Tagetes is a genus consisting of annual or perennial species; most are herbaceous plants belonging to the sunflower family Asteraceae. The word *Tagetes* originated from the name of *Etruscan tagetes* (Shetty et al. 2015). This genus is very popular among gardeners due to the ease of which it is cultivated, wide adaptability, it produces the flower all year

Table 1. Accepted species of *Tagetes* (The Plant List 2013).

	Accepted species of *Tagetes*		
1	*Tagetes apetala* Posada-Ar.	28	*Tagetes micrantha* Cav.
2	*Tagetes arenicola* Panero & Villaseñor	29	*Tagetes microglossa* Benth.
3	*Tagetes argentina* Cabrera	30	*Tagetes minima* L.
4	*Tagetes biflora* Cabrera	31	*Tagetes minuta* L.
5	*Tagetes campanulata* Griseb.	32	*Tagetes moorei* H. Rob.
6	*Tagetes caracasana* Humb. ex Willd.	33	*Tagetes mulleri* S.F.Blake
7	*Tagetes congesta* Hook. & Arn.	34	*Tagetes multiflora* Kunth
8	*Tagetes coronopifolia* Willd.	35	*Tagetes nelsonii* Greenm.
9	*Tagetes daucoides* Schrad.	36	*Tagetes oaxacana* B.L.Turner
10	*Tagetes elliptica* Sm.	37	*Tagetes osteni* Hicken
11	*Tagetes elongata* Willd.	38	*Tagetes palmeri* A.Gray
12	*Tagetes epapposa* B.L.Turner	39	*Tagetes parryi* A.Gray
13	*Tagetes erecta* L.	40	*Tagetes perezi* Cabrera
14	*Tagetes filifolia* Lag.	41	*Tagetes praetermissa (Strother)* H.Rob.
15	*Tagetes foeniculacea* Desf.	42	*Tagetes pringlei* S. Watson
16	*Tagetes foetidissima* Hort. ex DC.	43	*Tagetes pusilla* Kunth
17	*Tagetes hartwegii* Greenm.	44	*Tagetes riojana* M.Ferraro
18	*Tagetes iltisiana* H. Rob.	45	*Tagetes rupestris* Cabrera
19	*Tagetes inclusa* Muschl.	46	*Tagetes stenophylla* B.L. Rob.
20	*Tagetes lacera* Brandegee	47	*Tagetes subulata* Cerv.
21	*Tagetes laxa* Cabrera	48	*Tagetes subvillosa* Lag.
22	*Tagetes lemmonii* A. Gray	49	*Tagetes tenuifolia* Cav.
23	*Tagetes linifolia* Seaton	50	*Tagetes terniflora* Kunth
24	*Tagetes lucida* Cav.	51	*Tagetes triradiata* Greenm.
25	*Tagetes lunulata* Ortega	52	*Tagetes verticillata* Lag. & Rodr.
26	*Tagetes mandonii* Sch.Bip. ex Klatt	53	*Tagetes zypaquirensis* Bonpl.
27	*Tagetes mendocina* Phil.		

round, free flowering habit, short duration, has attractive flower colors and shapes (Jain & Sing 2021: Awasthi et al. 2022).

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Subclass : Asteridae
Order : Asterales
Family : Asteraceae
Genus : *Tagetes*
Species : *Tagetes* sp.

This plant grows upright and reaches up to 1.3 m in height; the leaves are dark green pinnate-shaped and have a taproot. The diameter of the flower is between 7 cm to 10 cm, tubular, single, or collected in panicles; the flowers are white, yellow, orange, golden yellow, golden orange, cadmium orange, deep orange and bright orange (Shetty et al. 2009, Kumar et al. 2019; Kurniati 2021). It has a unique color of flower, but the yellow and orange color are more popular, and it could be cultivated any time of the year and in every season (Sheoran et al. 2022). The literature mentions that the leaves of marigolds are arranged in the opposite position, pinnate and complex kinds, oblong, and the edge is dentate. The leaf length reaches less than 2 inches, and the leaves are green. The leaves are curved, with sharp teeth on the edge (Shetty et al. 2009).

Marigolds need a mild climate (14.5 ° C – 28.6 ° C) for better growth to improve the flowering, while higher temperatures will affect the flower production. This plant can be located in a place that is exposed to full sun and some leaves must be pruned to induce the flower. Fertilizer can be applied, either organic or inorganic and needs to be watered twice a day (Chaurasiya 2020). Marigolds will have a better vegetative growth in a sunny location with high temperatures, while a mild climate can profuse the flower (Sheoran et al. 2022).

Figure 1. *Tagetes* sp. that is mostly grown in Indonesia.

Locating a sunny spot with at least six hours of sunlight per day, and well-drained soil are the first steps in growing marigolds. When there is no longer a chance of frost, move the seedlings into the garden. Plant the seeds indoors approximately four to six weeks before the date when they are frost-free. To improve the soil's fertility and consistency,

ensure it dries well and add compost as needed. Plant seeds directly in the garden, spaced one inch apart, or in seed trays if you plan to transfer them. Water the plants regularly, allowing the soil to dry out in between treatments. The process of deadhead to promote more flowers and pinching out tall variety branches to encourage bushier growth offer additional advantages of marigolds (Daycho Khaenamkaew 2021).

Marigolds' various uses contribute to their significant economic value. Their beautiful yellow and gold blossoms are utilized frequently in the food industry due to the essential oils and phytochemicals they contain which have a variety of uses (Mlcek & Rop 2011; Rop et al. 2012; Lee et al. 2013; Padalia & Chanda 2015; Wang et al. 2018; Moczkowska-Wyrwisz et al. 2022). In addition, marigolds are edible flowers whose petals are added to salads and other meals to add colour, increasing their market worth in the food business.

Marigolds are mostly used medicinally to treat skin ailments such as inflammation, bruising, varicose veins, minor cuts, and contusions. Marigold ointment helps sunburns and wounds recover (Salehi et al. 2018; Riaz et al. 2013). Marigold are a natural and efficient pest control alternative that are placed between vegetables in the agricultural sector because they provide an odour that deters insects (Hamaguchi et al. 2019; Fabrick et al. 2020). Because of these characteristics, they are beneficial for sustainable and organic farming practices, which lessen the need for chemical pesticides. As a result, marigolds are a valuable and adaptable plant in many different industries, with applications that include food and agriculture to medicine.

PHYTOCHEMICAL COMPOUNDS

There are two important basic species of *Tagetes*, they are: *T. erecta* L. (American marigold) and *T. patula* L. (French marigold). The flower of *T. erecta* is larger than *T. patula*, and the color is yellow, orange, golden, or bicolored (Shetty et al. 2015). Many studies have been conducted on those plants, especially observing and isolating the components for phytochemical compounds (Table 2) (Tripathi et al. 2012; Shetty et al. 2015).

Some important chemical agents are found in the marigold extract, such as phenylpropanoids, carotenoids, flavonoids, thiophenes, quercetagetin, 6-hydroxykaempferol, quercetin, patuletin, quercetagetin-7-O-glucoside and others (Bhave et al. 2020; Ratananikom et al 2021; Devrnja et al. 2022). The compound produced by plants could vary depending on some factors: part of plant extracted, harvesting seasons, plant development stage, and geographical conditions (Salehi et al. 2018). Other research reported that some chemicals isolated from *Tagetes* species mainly belong to the classes of thiophenes, flavonoids, carotenoids, and phenolic compounds (Gupta & Vasudeva 2012). So far, only 15 species from the *Tagetes* group have been extracted to isolate its chemical compounds, especially the essential oils in the aerial parts, capitula, and leaves (Salehi et al. 2018).

Essential oil is one of the most important phytochemical agents in Marigolds. Gupta and Vasudeva (2012) found that the essential oils in the leaves of *T. erecta* are d-limonene, α-pinene, β-pinene, dipentene, ocimene, β-phellandrene, linalool, geraniol, menthol, tagetone, nonanal and linalyl acetate. While the essential oil in the flower is d-limonene, ocimene, 1-1linalyl acetate, 1-linalool, tagetone and n-nnonyl aldehyde, aromadendrene, phenylethyl alcohol, salicylaldehyde, phenylacetaldehyde, 2-hexen-1-al, eudesmol, tagetone, ocimene, linalyl acetate, etc. Other researchers also concluded the essential oil content from different parts of the plant (Table 3).

Table 2. The phytochemical compound isolated from *Tagetes erecta* Linn.

No	Phytochemicals compound	Part of plant
1	Quinic acid (Tripathi et al. 2012; Llorent-Martínez et al. 2015)	Leaves
2	Dihexoside (Tripathi et al. 2012; Burlec et al. 2021)	Flower
3	Shikimic acid hexoside (Tripathi et al. 2012; Burlec et al. 2021)	Flower
4	Theogallin (Dou et al. 2007; Tripathi et al. 2012)	Flower
5	Phenylalanine (Tripathi et al. 2012)	Flower
6	Methyl-gallic acid (Tripathi et al. 2012; Burlec et al. 2021)	Flower
7	Tryptophan (Tripathi et al. 2012; Garcia et al. 2016).	Flower
8	Syringic acid-hexoside II (Parejo et al. 2004; Tripathi et al. 2012)	Flower
9	Syringic acid (Parejo et al. 2004; Tripathi et al. 2012; Burlec et al. 2021)	Leaves
10	Trigalloyl-hexoside (Meyers et al. 2006; Tripathi et al. 2012;	Flower
11	Ellagic acid-hexoside II (Tripathi et al. 2012; Fracassetti et al. 2013)	Flower
12	Quercetagitrin (Parejo et al. 2004; Tripathi et al. 2012)	Flower
14	Di-syringic acid hexoside I (Parejo et al. 2004; Tripathi et al. 2012)	Leaves
15	Di-syringic acid hexoside II (Parejo et al. 2004; Tripathi et al. 2012)	Leaves
16	Patulitrin (Parejo et al. 2004; Tripathi et al. 2012)	Leaves
17	8-Hydroxyquercetagetin (Parejo et al. 2004; Tripathi et al. 2012)	Leaves

The essential oil, also known as aromatic oil or volatile oil, is produced by the plant from metabolic processes, which are formed due to the reaction between various chemical compounds and air. This oil is synthesized in the gland cell of the tissue, and some are formed in resin vessels. It can also be produced from the degradation of triglycerides by enzymes, triglycerides indirectly contribute to volatile oil production in plants by releasing fatty acids upon enzymatic hydrolysis, and these fatty acids can serve as precursors for the synthesis of some volatile compounds. This oil has been used for many purposes for a long time and serves as a source of natural antimicrobials today (Herman et al. 2019).

T. patula L. that has been investigated by some researchers has shown some presence of secondary metabolites such as alkaloids, tannins, phenolic compounds and steroids, fatty acids and resins (Munhoz 2014; Kafaltiya et al. 2019). The concentration of essential oils in marigold depends on the species and environmental conditions. Some researchers found different essential oil concentrations in different countries where marigold is cultivated, such as five species collected in Venezuela which are *T. caracasana*, *T. filifolia*, *T. subulata*, *T. patula*, and *T. erecta* and show different concentrations with *T. erecta* L. In Indonesia and *T. erecta* L in India (Armas et al. 2012).

Another research study mentioned that marigold is also a natural source of xanthophyll which can be used as a natural food additive to lighten egg yolks and poultry skin. Marigold also can be used as a fabric dye because it contains ethanol (Saputri et al. 2021). Carotenoids are the most important chemical agent found in the marigold. Carotenoids and polyphenols are mostly found in the flower petals of marigold, where the major components are esters that account for more than 75% of the total carotenoids (Manzoor et al. 2017).

THE USE of *Tagetes* as HERBAL MEDICINE

Tagetes sp is well-known as traditional medicine, and some people use it to cure some diseases, including respiratory infections, as an anti-inflammatory, cough, wound healing, stomachache, anemia, and irregular menstruation (Aziz et al. 2020). These genera have cultural significance in traditional medicine systems and are integrated into various remedies, showcasing their broad relevance in herbal medicine practices across different regions worldwide, some species that commonly uses for herbal

Table 3. Essential oil components from *Tagetes erecta* Linn in various part of plant.

No	Essential oil components	Part of plant
1	d-limonene (Maciel et al. 2002; Marotti et al. 2004; Tripathi et al. 2012; Gupta & Vasudeva 2012)	Aerial, Capitula/flower, leaves
2	(E)-β-farnesene (Tripathi et al. 2012; Gupta & Vasudeva 2012)	Leaves, flower
3	(E)-β-ocimene (Tripathi et al. 2012; Gupta & Vasudeva 2012; Sharifi-Rad et al. 2018a)	Aerial, Capitula/flower and leaves
4	(Z)-myroxide (Salehi et al. 2018)	Capitula/flower
5	(Z)-β-ocimene (Tripathi et al. 2012; Gupta & Vasudeva 2012; Sing et al. 2015)	Aerial, Capitula/flower, leaves
6	1,8-cineole (Gupta & Vasudeva 2012; Sing et al. 2015; Salehi et al. 2018)	Aerial
7	2-hexen-1-al (Gupta & Vasudeva 2012; Sing et al. 2015; Salehi et al. 2018)	Capitula/flower
8	Aromadendrene (Gupta & Vasudeva 2012; Sing et al. 2015; Salehi et al. 2018)	Capitula/flower
9	Camphene (Gupta & Vasudeva 2012; Sing et al. 2015; Salehi et al. 2018)	Leaves
10	Carvacrol (Gupta & Vasudeva 2012; Sing et al. 2015; Salehi et al. 2018)	Aerial, Leaves
11	Cyperene (Gupta & Vasudeva 2012; Sing et al. 2015; Salehi et al. 2018)	Leaves
12	d-carvone (Gupta & Vasudeva 2012; Sing et al. 2015; Salehi et al. 2018)	Capitula/flower
13	dihydrotagetone (Sharifi-Rad et al. 2017)	Aerial
14	Dipentene (Gupta & Vasudeva 2012; Sing et al. 2015; Salehi et al. 2018)	Aerial, Leaves
15	Eudesmol (Gillij et al. 2008)	Capitula/flower
16	eugenol (Marotti et al. 2004; Sharifi-Rad et al. 2018b)	Capitula/flower

medicine are *T. erecta*, *T patula*, and *T. Minuta*, (Riaz et al. 2013; Salehi et al. 2018).

Various parts of the plant's components are used as herbal medicine (Table 4). The leaves are usually used to cure stomachache, especially in newborns, babies and toddlers in Indonesia. The leaves can also cure kidney problems, wounds, muscular soreness, ulcers, and boils (Shetty et al. 2009). The flower of *Tagetes* is usually used to reduce fever, epileptic fits, stomachache, liver and eye diseases, and scabies. The flower could be used to purify the blood, and the flower juice is used to treat bleeding, rheumatism, fever and bronchitis (Rhama & Mardhavan 2011). Furthermore, the flower juice has some functions in different countries. Indians use it as a blood purifier and to treat piles. Brazilians use leaf infusions as a vermifuge, while Mexicans use them to cure diuretics and carminatives (Chaudhary et al. 2022).

As mentioned previously, Marigolds contain yellow carotenoid pigments, such as carotene (alpha and beta carotene) and xanthophyll (luten and zeaxanthin). The yellow flower is caused by the carotenoid and flavonoid content. Some publications mentioned that flavonoids are the secondary metabolites of polyphenols, found widely in plants with various bioactive effects, including anti-viral, anti-inflammatory, anti-aging, antioxidant, cardioprotective, antidiabetic, and anti-cancer properties (Qinghu et al. 2016). The utilization of the marigold plant as herbal medicine is concluded in Table 4. In a tropical country like Indonesia, many species of marigold are found in nature without any special treatment as it is easy to grow.

Marigold attracted the attention of researchers due to the presence of some bioactive compounds and its therapeutic potential especially in

Table 4. The use of *Tagetes* as herbal medicine in various part of the plant.

No	Species	Uses	Part of plant	Preparation	References
1	*T. erecta L*, *T. filifolia L.*, *T. lucida L.*	Stomachache, dysentry, diarrhea	Leaves and flowers	crushed the leaves or flowers and applied on the stomach	Gupta & Vasudeva 2012; Ali et al. 2013; Joshi & Barbalho 2022
2	*T. erecta L*	cough, sore throat	Flowers	boils the flower and drink the water	Blanco & Thiagarajan 2017
3	*T. erecta L.*, *T. lucida L.*	Dental problem	Flowers	boils the flower and gargle the water	Joshi & Barbalho 2022
4	*T. erecta L.*, *T. minuta*	Wound healing	Leaves	boils the leaves to wash affected area	Jayavant 2018; Sharma & Kumari 2021; Chaudary et al. 2022
5	*T. erecta L.*,	Skin problems	Leaves	boils the leaves to relieve the rash and ichiness on the skin	Gupta & Vasudeva 2012; Kafaltiya et al. 2019
6	*T. erecta L.*,	Fever	Flowers	boils the flower and drink the water	Gupta & Vasudeva 2012; Kafaltiya et al. 2019
7	*T. erecta L.*	Diuretic problems	Leaves and flowers	boil the flowers or leaves and drink the water	Ali et al. 2013; Singh et al. 2015

reducing macular degeneration (Manzoor et al. 2022). The presence of some phytochemical agents and nutraceuticals in marigold can be used to cure eye-related diseases such as cataracts and age-related macular degeneration (AMD), and cardiovascular diseases. Lutein is one type of carotenoid and is very important for the health of the human eye (Nam et al. 2021). This chemical is the major pigment in the macular region of the retina, and it has been found to prevent damage to the retina cells due to an antioxidant effect which can remove free radicals, lutein also was able to block blue light and positively affects the immune response and inflammation (Kijlstra et al. 2012)

NATURAL PESTICIDE

The use of marigold in controlling pests has been researched by some researchers (Table 5). This plant produces a lot of bioactive compounds that can be used as natural pesticides, such as α-therthienyl which is known as one the most toxic chemical agents against pests. The chemical α-therthienyl is found in abundance in *Tagetes* especially in the roots and it is toxic for some number of insects (Hamaguchi et al. 2019; Fabrick et al. 2020). Other researchers also mentioned that this chemical is also used as a fish poison (Cunha et al. 2016). The chemicalα-therthienyl generates oxygen radical species and has the capacity to inhibit some activities of the important enzymes in insects (Nivsarkar et al. 2001).

In some tropical countries where a number of infectious diseases are carried through a vector, are to be increasing due to climate change. The flowers of Marigold have many functions due to their attractive colors. It has a strong smell, which is important as an insect repellent (Ponkiya et al. 2018). This flower contains a lot of chemical agents, such as eugenol compounds, flavonoids, alkaloids, saponins, tannins, and triterpenoids which provide a repellent effect against *Aedes aegypti* (Shinta 2020). The essential oils containing eugenol can repel mosquitos by interfering with their sense of the smell (Mossa 2016).

The chemical α-terthienyl is an allelochemical agent which is used to suppress plant parasitic nematodes such as the model organism *Caenorhabditis elegans* and the root-knot nematode, *Meloidogyne incognita* (Ponkiya et al. 2018; Hamaguchi et al. 2019). The essential oils that are available in *Tagetes minuta* L. shows some antimicrobial activities against

Table 5. The benefits of Marigolds as natural pesticides.

No	Species	Benefits	References
1	*T. erecta* L.	Mosquitoes' repellent	Ponkiya et al. 2018
2	*T. minuta* L	Against *Rhipicephalus microplus*, *Rhipicephalus sanguineus*, *Amblyomma cajennense* and *Argas miniatus*	Notes et al. 2013; Wanzala & Ogama 2013; Amaral et al. 2015; Cunha et al. 2016; Zulfikar et al. 2019
		Againts *Anopheles gambiae*	Kyarimpa et al. 2014
3	*T. patula* L	Against *Lygus hesperus* and *Bemisia tabaci*	Fabrick et al. 2020
4	*T. filifolia L.*	Againts *Tribolium castaneum*	Olmedo et al. 2015
5	*T. lucida* L.	Bio-nemacides	Omer et al. 2014

bacteria such as *Staphylococcus aureus* and *Enterococcus faecium*, those chemicals presented acaricidal activities against ticks *Rhipicephalus microplus*, *Rhipicephalus sanguineus*, *Amblyomma cajennense* and *Argas miniatus*, larvicidal activity against *Aedes aegypti* (Notes et al. 2013; Wanzala & Ogama 2013; Amaral et al. 2015; Cunha et al. 2016; Zulfikar et al. 2019). The extract of *Tagetes erecta* L. also has antimicrobial activities against *Enterococcus faecalis*, *Escherichia coli and Lactobacillus acidophilus bacteria and Candida albicans* (Tuna et al. 2021). The effects of the marigold against mosquitos have also been investigated, and the ethanolic extracts of *Tagetes erecta's* flower together with its solvent fraction have been shown to repel different instars of *Culex quinquefasciatus* (Nikkon et al. 2011). The use of 8 ml marigold leaf extract could kill the larvae of *Aedes aegypti* by 50% (Zulfikar et al. 2019).

Tagetes patula L. (French marigold) is an aromatic plant species that consists of many secondary metabolites and has the potential for pesticidal activities (Devrnja et al. 2022). The use of extracted leaf from *Tagetes patula* L. using water as a solvent has also been effectively used as bio-controlling agent against various diseases in tomato plants. Besides reducing the percentages of disease levels, the leaf extract has also been shown to increase the growth and yield of tomato plants (Nahak & Sahu 2017), due to the fact that it contains saponin and polyphenols (Andresen & Cedergreen 2010).

Tagetes erecta L. has been shown to have properties against arthropods and certain species of predatory insects (Silveira et al. 2009). Some research conducted by planting marigold plants in between rows of onion crops showed that marigold reduced the numbers of aphid, nematode, and whitefly populations and viruses in diseased plants (Silveira et al. 2009). However, the use of marigold flower as refugia in rice cultivation was also found to significantly affect the populations of pests (Wardani & Leksono 2013; Wardana et al. 2017; Erdiansyah et al. 2019).

Some species from the *Tagetes* genera are well-known as a natural pesticide because they contain some chemical agents with pesticidal properties. Many studies that have been conducted have shown insecticidal activity of *Tagetes erecta* L. or African marigold (Nikkon et al. 2009; Salinas-sanchez 2012; E Santos et al. 2022) against mosquitos (Nikkon et al. 2011), T*agetes minuta* L. (Mexican marigold) or *Tagetes patula* L. against mosquitos (Notes et al. 2013; Marini et al. 2017), sand flies (Kimutai et al. 2017), termites, activity as ascarides (Andreotti et al. 2013; Politi et al. 2013). Some chemical agents that are contained in *Tagetes* which are important for insecticides are; monoterpenoids, carotenoids, and flavonoids which are the major biocidal constituents, but unfortunately many of those chemicals have limited practical uses due to volatile and poor persistence under field conditions (Marahatta et al. 2012; Palacios-Landín et al. 2015; Fabrick et al. 2020)

FOOD AND BEVERAGE

Edible plants are innocuous and non-toxic plants that could be consumed with health benefits for humans (Chitrakar et al. 2019; Santos & Reis 2021). People usually eat flowers either as a fresh salad or used as food ingredients since ancient times. These flowers have also been used as a material for making jam, cakes, etc. (Mlcek & Rop 2011; Rop et al. 2012). For many years, humans have been using plants as sources of food and nutrients, and many of them are beneficial for human health (Lee et al. 2013; Padalia & Chanda 2015; Wang et al. 2018; Moczkowska-Wyrwisz et al. 2022). The use of marigold as a natural coloring for food and beverages has already been known worldwide, and there has been some researcher conducted in this area already (Navarro-González et al. 2015; Alim-un-Nisa et al. 2018; Yanti et al. 2019; Casella et al. 2021).

These plants are a rich source of lutein which is very important for the human eyes and immunity, thus its already commercially used and produced as a nutritional supplement in capsule or tablet form (Cannavale et al. 2019; Wu et al. 2023). Since the human body cannot synthesize those chemicals, it must be obtained from the diet, however, it is known lutein is also contained naturally in fruits, cereals and some vegetables (Gupta et al. 2022; Chauhan et al. 2022). High levels of lutein (oxygenated carotenoid xanthophyll) are contained in the petals of marigold and can be used for food coloring agents as well as for antioxidants (Ingkasupart et al. 2015; Cezare-Gomes et al. 2019; Casella et al. 2021; Gupta et al. 2022).

Noodles prepared using marigold powder contained a high content of lutein (Nam et al. 2021). In some countries, the fresh flowers of marigold added to the food such as salads or as an edible garnish, the taste is found to be bitter and balances out an overly sweet dish (Singh et al. 2015). The orange-yellow carotenoid lutein compound found in marigold's flowers had been identified, isolated, and approved by the European Union (EU) as a food coloring and nutrient supplement (food additive). It is widely used for baked foods and baking mixes, beverages and beverage bases, cereals for breakfast, and as an additional compound in chewing gum, egg products, fats and oil, desserts, gravies and sauces, candy, toddler foods, milk products, fruit juices, soup mixes, biscuits, etc. (Navarro-González et al. 2015; Tiwari et al. 2016; Rajvanshi & Dwivedi 2017; Alim-un-Nisa et al. 2018; Sathyanarayana et al. 2018; Toliba et al. 2018).

One study found that the partial replacement of flour with marigold extract to make a sponge cake was a suitable alternative. Marigold could be seen as more desirable to customers when compared to other alternatives in terms of its taste, smell, porosity and color (Moczkowska-Wyrwisz et al. 2022). In Chile and Argentina, marigold is a well-known ingredient in traditional foods such as in stews, due to its flavor, and in some other countries its flowers are used as a soup condiment (Cornelius & Wycliffe 2016; Paniagua-Zambrana et al. 2020). While in other parts of the world, *Tagetes* are commonly used for making hot tea and also cold beverages, as well as herbal teas (Kusuma et al. 2020).

Tagetes erecta L. is one of the most popular edible flowers in the world and is commonly used as an ingredient in salads and as a natural coloring agent for food (Table 6) (Navarro-González et al. 2015). Its flowers are rich in lutein and are grown many parts of the world such as in Mexico, Peru, Ecuador, Spain, India and China. The essential oil of *Tagetes minuta* L. is also used for food production and beverage preparation such as alcoholic drinks and cola, as well as for dairy desserts, some sweet things, jellies and spices (Vazquez et al. 2011). *Tagetes minuta* L. is

Table 6. The benefits of Marigolds for consumption.

No	Species	Part of plant	Consumed as	References
1	*T. erecta* L.	Flowers	Fresh salad	Mlcek & Rop 2011; Rop et al. 2012
			Food ingredients	Mlcek & Rop 2011; Rop et al. 2012
			Food coloring	Navarro-González et al. 2015; Alim-un-Nisa et al. 2018; Yanti et al. 2019; Casella et al. 2021
			Beverage (tea)	Kusuma et al. 2020
2	*T. minuta* L	Leaves and flower	Food production	Vazquez et al. 2011
			Beverage	Vazquez et al. 2011
3	*T. patula L.*	Flowers	Food additives	Szarka et al. 2007
			Marigold powder for bread, noodle and sponge cake	Nam et al. 2021

one of the more important species forms *Tagetes* genera that has been used for a long time as food, perfumes, medicines, ornamentals and for religious ceremonies (Gakuubi et al. 2016; Cornelius & Wycliffe 2016; Kumar et al. 2020).

CONCLUSION

Marigold is an important plant in the world and has been used as an ornamental, medicinal, and also as an edible plant. This genus is belonging to the *Asteraceae* family, and is easy to grow but is still not cultivated massively in some countries. Some important chemical agents are found in the marigold extract, such as phenylpropanoids, carotenoids, flavonoids, thiophenes, quercetagetin, 6-hydroxykaempferol, quercetin, patuletin, quercetagetin-7-O-glucoside and others. The essential oils of marigold leaves are d-limonene, α-pinene, β-pinene, dipentene, ocimene, β-phellandrene, linalool, geraniol, menthol, tagetone, nonanal and linalyl acetate. While the essential oils in the flower are d-limonene, ocimene, 1-1linalyl acetate, 1-linalool, tagetone and n-nnonyl aldehyde, aromadendrene, phenylethyl alcohol, salicylaldehyde, phenylacetaldehyde, 2-hexen-1-al, eudesmol, tagetone, ocimene, linalyl acetate, etc. The chemical agents in all parts of the plant can be used as medicine, such as to cure Stomachache, dysentery, diarrhea, wound healing, and fever. Some species from *Tagetes* genera are widely used as natural pesticides because they contain some chemical agents with pesticidal properties. As edible plants, they are used in many forms of food preparation and beverages can also be made from the marigold. Considering all the benefits of this plant, the cultivation of Marigold should be more widely practiced.

AUTHOR CONTRIBUTION

M.Z. designed the research and wrote the manuscript. V.N.A & S.F.H search for the informations and the literatures.

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CONFLICT of INTEREST

The authors stated that there is no conflict of interest regarding to this manuscript.

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