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Review Article

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Short Communications

Genetic Variation of Butternut Squash (*Cucurbita moschata* Duchesne) based on Inter-Simple Sequence Repeat

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ABSTRACT
Butternut squash (*Cucurbita moschata*) is a Cucurbitaceae plant that has been widely cultivated in Indonesia. Butternut squash is known to have various cultivars. A new cultivar introduced by the Faculty of Biology UGM is named 'Citra Laga' which is expected to be able to compete with the imported cultivars. The number of cultivars within a species may indicate genetic variation. This research was conducted to observe genetic variation and the phenetic relationship between 'Citra Laga' and the imported butternut squash cultivars based on the molecular marker ISSR. The ISSR analysis between 'Citra Laga' and the imported cultivars showed an average low polymorphism rate by 18.61% with a high similarity percentage of 83.7%. Thus, it can be said that the genetic variation is low and 'Citra Laga' is not genetically much different from the imported cultivars.

Indonesia is high biodiversity country, especially in the horticulture field. Squash or *waluh* (genus *Cucurbita)* is one of the abundant horticultural crops in Indonesia and has the potential to be developed into a food source. *Cucurbita moschata* or butternut squash is one type of squash that has been widely cultivated. Butternut squash has various fruit shapes, namely globular, flattened, cylindrical, turbinate, dumbbell, elongated, pyriform, and crooked neck (Purnomo et al. 2015). Butternut squash as a vegetable plant has a lot of nutrients that are good for the human body. The nutritional content includes fiber, protein, vitamins, fats, iron, phosphorus, potassium, beta carotene, amino acids, antioxidants, minerals, phenolics, and flavonoids (Marie-Magdeleine et al. 2011; Dari & Yaro 2016; Nopianasanti & Daryono 2018).

Butternut squash is known to have various cultivars. Nopianasanti & Daryono (2018) stated that various cultivars in *Cucurbita* species are based on their mitochondrial gene. One cultivar introduced by the Faculty of Biology, Universitas Gadjah Mada recently is the 'Citra Laga' butternut squash which was developed by Nopianasanti and Daryono (2018). This new cultivar is a result from a cross between 'Labu Madu' F3 and 'Hannah' F2 (Figure 1). 'Citra Laga' is known to have a faster harvest period, contains high beta carotene, and has a higher resistance to Begomovirus. The fruit shape of this cultivar is diverse, namely pyriform,

dumbbell, and globular (Figure 2). The diverse fruit shape happens because there is still segregation gene that produces genotypic variations so that the fruit shape is still not stable. Some of the imported cultivars that have been widely commercialized are 'Tiana', 'Waltham', and 'Jacqueline'. The imported cultivars are known to have a stable fruit shape. 'Tiana' and 'Jacqueline' have a blocky fruit shape meanwhile 'Waltham' has a pyriform fruit shape (Figure 2). However, the imported cultivars are known to have a relatively high price because the seeds are gained from limited cultivation in Australia, the Netherlands, and others. Therefore, the breeding research of 'Citra Laga' butternut squash is very important so that this cultivar can be recognized as a native cultivar of Indonesia. This cultivar is also expected to have superior traits and good competitiveness with imported cultivars but at a relatively cheaper price.

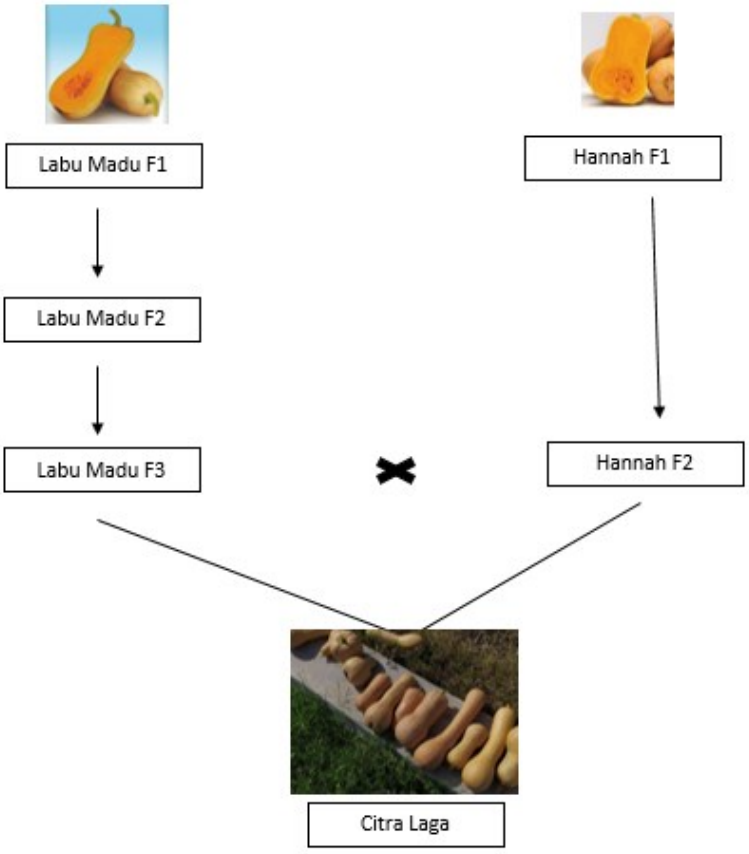

Figure 1. Parental history of 'Citra Laga' butternut squash.

A large number of cultivars in one species may indicate the presence of genetic variation within the species. Therefore, genetic variation research between 'Citra Laga' and imported cultivars specifically 'Tiana', 'Waltham', and 'Jacqueline' needs to be conducted. If the genetic variation level between 'Citra Laga' and imported cultivars is low, then it is likely that 'Citra Laga' is not genetically much different from imported cultivars. If the genetic variation level between 'Citra Laga' and imported cultivars is high, then it can be said that 'Citra Laga' has genetic differences as special characteristics for the 'Citra Laga' cultivar.

Genetic variation can be analyzed using DNA molecular markers. One of the molecular markers that can be used is the Inter-Simple Sequence Repeat (ISSR) (Abdein 2018). ISSR is a *Polymerase Chain Reaction* (PCR) based technique that uses microsatellite sequences for DNA amplification (Andriyani & Jadid 2021). The regions between these microsatellite sequences when amplified through PCR with a single primer will produce products that can be used as multilocal markers in the genetic

variation study (Ng & Tan 2015). The ISSR has the advantage of being simple and having a high degree of polymorphism. ISSR is also known to be reproducible for *fingerprinting*, genetic diversity studies, and kinship studies (Abdein 2018).

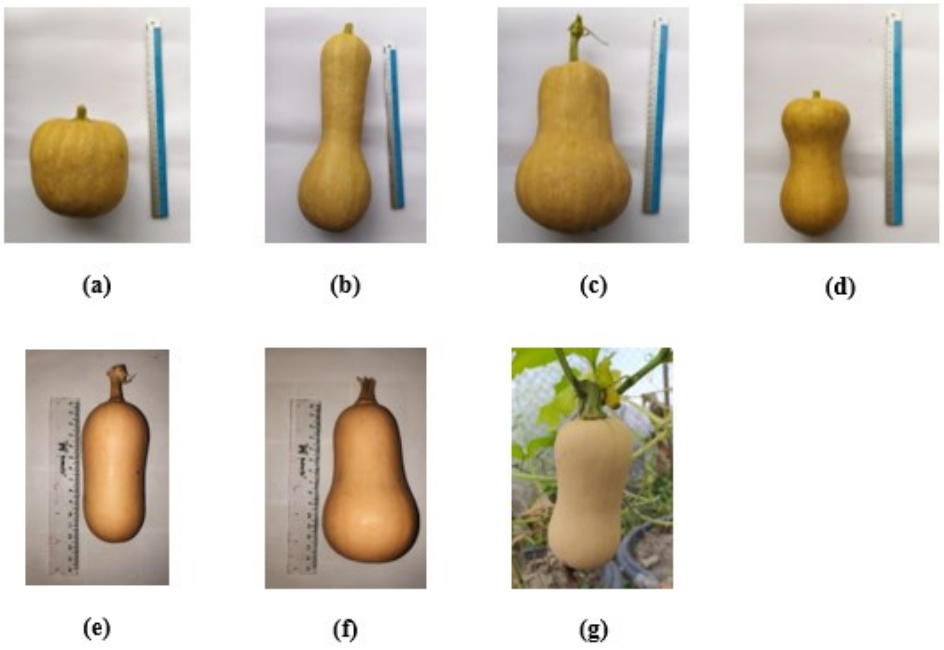

Figure 2. Fruit shape of (a-d) 'Citra Laga', (e) 'Tiana', (f) 'Waltham', and (g) 'Jacqueline'. ((a) globular, (b, c, f) pyriform, (d) dumbbell, and (e, g) blocky).

This study was done from April to July 2022 at the Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada. The sampling was carried out at Pusat Inovasi Agroteknologi of UGM Green House, Kalitirto, Berbah, Sleman, Yogyakarta. The samples were 3-4 weeks old leaves of 'Citra Laga', 'Tiana', 'Waltham', and 'Jacqueline' that were healthy and not infected with viruses to minimize contaminants. The too-young leaves generally contain high RNA, while the too-old leaves contain high secondary metabolites. DNA extraction as a first step in molecular analysis in plants was performed to separate DNA from various contaminants such as cell membranes, RNA protein, and other cell components so that pure DNA can be obtained (Gupta 2019). In general, the DNA extraction steps are the cell walls and membranes lysis, nucleolar lysis, and DNA precipitation (Sari et al. 2014). In this study, the DNA extraction method was done using Geneaid Genomic DNA mini kit for the plant according to Melani et al. (2018) with some modifications. The materials used were 100 mg leaves, 800 µL GP1 buffer, 100 µL GP2 buffer, 1,5X GP3 buffer, 400 µL W1 buffer, 600 µL wash buffer, and 50 µL elution buffer. The quality of the DNA extract obtained was then measured with Nanodrop UV-Vis Spectrophotometer.

Electrophoresis visualization of PCR products using ISSR primers can show the polymorphic and monomorphic DNA bands. If the primer is capable to amplify varied genome regions, then the DNA bands produced will also be polymorphic whereas if the primer did not amplify varied genome regions, it will produce monomorphic DNA bands. Thus, it can be said that polymorphic bands are DNA bands that do not always appear in all observed individuals. In contrast, monomorphic DNA bands are always found in all observed individuals (Munif et al. 2004). The ISSR primers used in this study were UBC 807, UBC 809, UBC 810, UBC 812, and UBC 841. The use of these 5 ISSR primers refers to the research of Inan et al. (2012) which examined the efficacy of the ISSR technique for

molecular characterization in squashes (*Cucurbita*). The UBC 807, UBC 809, UBC 810, UBC 812, and UBC 841 primers on Inan et al. (2012) research succeeded in producing 100% polymorphism level in *Cucurbita pepo*, *Cucurbita moschata*, and *Cucurbita maxima*. Therefore, those 5 primers were used to see whether UBC 807, UBC 809, UBC 810, UBC 812, and UBC 841 could also produce high levels of polymorphism for *the Cucurbita moschata* variety level.

The PCR composition for DNA amplification was 12,5 µL MyTaq HS Red Mix 2X Bioline PCR kit, 2 µL DNA template, 1 µL ISSR primer, and 9,5 µL ddH_2O. The protocols for DNA amplification were 3 minutes of pre-denaturation at 95°C, 1 minute of denaturation at 95°C, 1 minute and 30 seconds of annealing at 49,5-54,1°C (depend on primers in Table 1), 1 minute of extension at 72°C, 5 minutes of post-extension at 72°C, and infinity hold at 12°C. The denaturation, annealing, and extension processes were performed in 35 cycles. The amplified PCR-ISSR products were then separated by electrophoresis on 2% agarose gel with 1X TBE buffer for 60 minutes at 50V. DNA band patterns were visualized with gel doc UV-Transilluminator. The DNA band patterns were converted into binary data 0-1 based on the presence or the absence of the DNA band then they were analyzed using MVSP 3.1 program with the UPGMA method. The PCR visualization results are presented in Figure 3.

Table 1. ISSR primers.

Primer	Sequences (5'→3')	Annealing Temperature (°C)
UBC 807	AGAGAGAGAGAGAGAGT	52,3
UBC 809	AGAGAGAGAGAGAGAGG	54,1
UBC 810	GAGAGAGAGAGAGAGAT	45
UBC 812	GAGAGAGAGAGAGAGAC	47,5
UBC 841	GAGAGAGAGAGAGAGATC	49,5

The visualization using UBC 807 presented in Figure 3a shows a total of 9 DNA bands at size ±151 - ±1253 bp. The DNA bands consist of 2 polymorphic and 7 monomorphic DNA bands. Polymorphic band sizes were ±845 bp found in T and W, also 391 bp found in T, W, and J. Figure 3b shows a visualization using UBC 809 primer. The total DNA bands obtained were 12 bands with sizes of ±195 - ±1720 bp. The total polymorphic DNA bands were 4 bands with sizes of ±1191 bp found in CL, T, and W, ±1175 bp found in CL, W, and J, ±231 bp found in J, and ±195 bp found in CL. The visualization using UBC 810 primer in Figure 3c shows the total of DNA bands obtained were 8 with all of them being monomorphic. The size of the DNA bands was ±245 - ±1330 bp. The visualization using UBC 812 primer in Figure 3d shows 5 monomorphic bands in the size of ±271 bp - ±1290 bp without polymorphic bands. The absence of polymorphic DNA bands in both results means that the UBC 810 and UBC 812 primers produced low genetic variation. Figure 3e shows that the visualization using UBC 841 primer produced 8 DNA bands with sizes of ±357 bp - ±1861 bp that consist of 3 polymorphic bands and 5 monomorphic bands. The polymorphic bands sized ±1760 bp, ±539 bp, and ±359 bp were found in T, W, and J.

Table 2 shows that the total DNA bands amplified in this study were 42 bands with a total of 33 monomorphic bands and 9 polymorphic bands. The average number of polymorphic bands and the polymorphism percentage were 1.8 and 18.61%. Based on all the results above, it can be seen that the UBC 841 primer produced the highest genetic variation

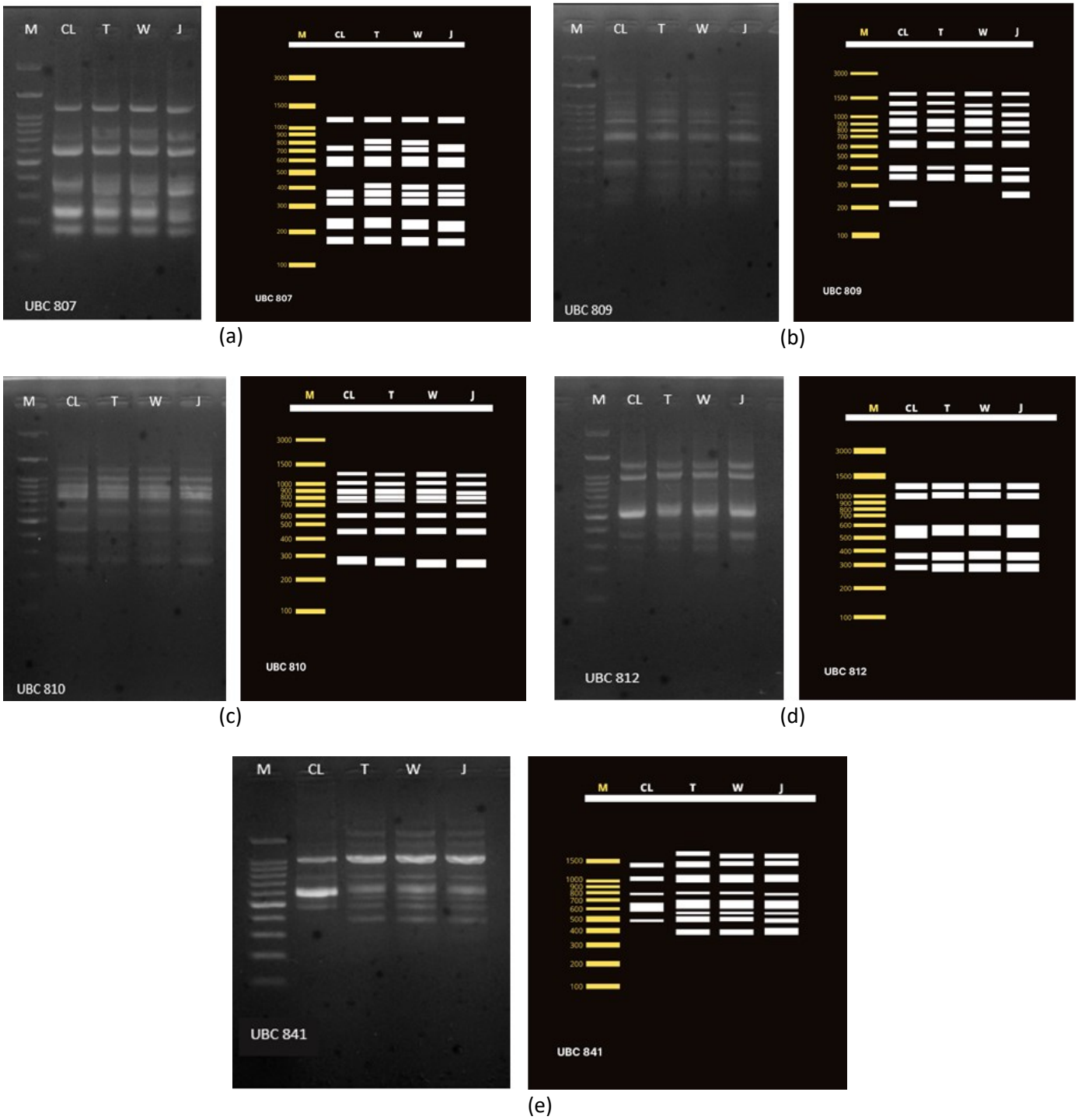

Figure 3. PCR products visualization of 4 butternut squash cultivars using (a) UBC 807 primer, (b) UBC 809 primer, (c) UBC 810 primer, (d) UBC 812 primer, and (e) UBC 841 primer. (M= Marker, CL= 'Citra Laga', T= Tiana, W= Waltham, and J= Jacqueline).

with a polymorphism percentage of 37.5%, followed by the UBC 809 primer with a percentage of 33.33%, and UBC 807 with a percentage of 22.22%. The UBC 810 and UBC 812 primers had the lowest genetic variation due to the absence of polymorphic DNA bands. Samiyarsih et al. (2020) stated that primers with a high level of polymorphism are those with percentage of polymorphism ≥50%. Therefore, it can be said that all primers used in this study had a low level of polymorphism.

The similarity index between the 4 cultivars of butternut squash may indicate their kinship. In phenetic analysis, the similarity of all existing characters will be compiled. If the similarity value is high, then the kinship relation will also be close (Rahayu & Jannah 2019). The results of

Table 2. Polymorphism percentage of 4 butternut squash cultivars based on 5 ISSR primers.

ISSR Primer	Total DNA Bands	Total Monomorphic Bands	Total Polymorphic Bands	Percentage of Polymorphism (%)	Size of DNA Bands (bp)
UBC 807	9	7	2	22.22	151-1253
UBC 809	12	8	4	33.33	195-1720
UBC 810	8	8	0	0	24-1330
UBC 812	5	5	0	0	271-1290
UBC 841	8	5	3	37.5	357-1861
Total	42	33	9	-	-
Average	8.4	6.6	1.8	18.61	-

the phenetic relationship analysis of 'Citra Laga', 'Tiana', 'Waltham', and 'Jacqueline' are presented in Figure 4.

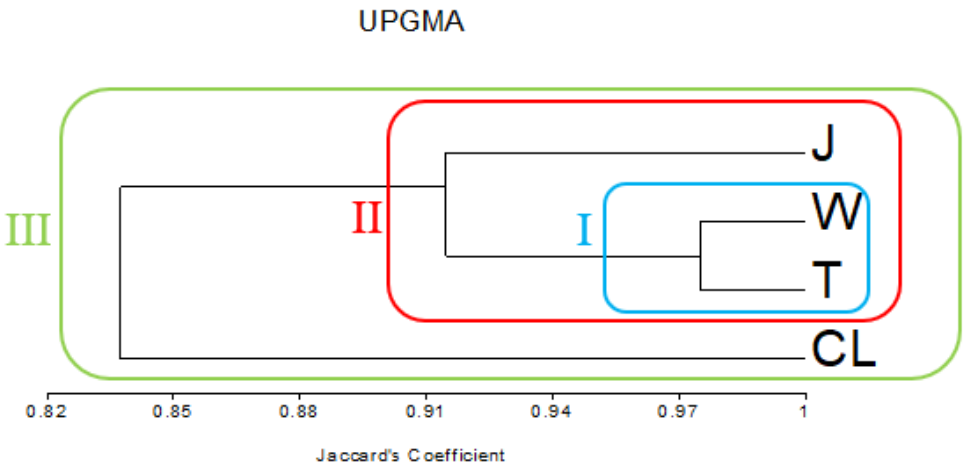

Figure 4. Dendrogram showing the clustering of 4 butternut squash cultivars based on the molecular character using the UPGMA methods with Jaccard's Coefficient (CL= 'Citra Laga', T= 'Tiana', W= 'Waltham', dan J= 'Jacqueline').

Figure 4 shows that the analysis of the phenetic relationship between 4 butternut squash cultivars resulted in a total of 3 clusters, namely I, II, and III. Cluster I consist of 'Waltham' and 'Tiana' with a similarity percentage of 97.5%. Furthermore ' Waltham' and 'Tiana' fused into one cluster with 'Jacqueline' forming cluster II with a similarity percentage of 91.5%. Next, 'Citra Laga' fused with 'Waltham', 'Tiana', and 'Jacqueline' forming cluster III with a similarity percentage of 83.7%.

Based on the clusters obtained, it can be seen that 'Tiana' had the closest phenetic kinship to 'Waltham', while 'Citra Laga' had the most distant phenetic kinship relationship compared to all the cultivars tested. Nonetheless, all cultivars have a high level of similarity because they are valued at ≥70% so it can be said that the phenetic kinship relationship between the 4 butternut squash cultivars tested is very close. The high similarity level is in line with the low polymorphism level and that means the genetic variation of 4 butternut squash cultivars in this study is molecularly low. The low polymorphism level and the high similarity level are due to the samples tested in this study and are still being included in one species, namely *Cucurbita moschata*. According to Hidzroh & Daryono (2021), the genetic components in the same species tend to be the same, so genetic variation may be low. The accuracy of the primers selection can also be the reason for the results obtained in this study because the number of polymorphic DNA bands depends on the ISSR primer and the sample used. Inan et al. (2012) in their study using the UBC 807, UBC 809, UBC 810, UBC 812, and UBC 841 primers resulted in high rate of poly-

morphism at the genus level of *Cucurbita*. Therefore, it can be said that the 5 primers used in this study are more precise to analyze polymorphism at the *Cucurbita* genus level, not for the *Cucurbita moschata* variety level. Despite this, some primers can still detect the presence of genetic variation between the 4 butternut squash cultivars. Genetic variations that appear in the same species can be caused due to mutations and recombination as a result of a long selection process (Daryono & Maryanto 2017).

This study concluded that 'Citra Laga' cultivar and 3 imported cultivars, specifically 'Tiana', 'Waltham', and 'Jacqueline', had a low polymorphism level and their phenetic kinship relationship was close. Thus, it can be said that their genetic variation is low. The results of this study are expected to be supporting data for the process of proposing 'Citra Laga' as an Indonesian local cultivar. The closeness genetic between 'Citra Laga' and the imported cultivars means that genetically 'Citra Laga' is not much different from the imported one, so the 'Citra Laga' will be able to compete as a local cultivar native to Indonesia.

AUTHORS CONTRIBUTION

N.R.A.P analyzed the data and wrote the manuscript. P.S.K. and B.F.A. collected the samples. D.S., P., and B.S.D. designed the research and supervised all research processes.

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CONFLICT OF INTEREST

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

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Short Communication

An Update on the Habitat Suitability Model of *Dacrycarpus imbricatus* (Blume) de Laub. and Its Conservation Status in Bali, Indonesia

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ABSTRACT
Dacrycarpus imbricatus provides essential ecosystem functions and various potential uses. Therefore, studying this distribution and conservation status in Bali Islands is crucial. The Habitat Suitability Model (HSM) and Geospatial Conservation Assessment Tool (GeoCAT) were used to predict this distribution and conservation status. The results showed changes in the predicted habitat suitability in 2050. Climate change conditions will impact the preferential habitat of the current location. The analysis classifies *D. imbricatus* as an endangered (EN) species in Bali. The model does not consider anthropogenic factors which change the land use/land cover. Therefore, more severe conservation efforts in Bali are needed for this species.

Dacrycarpus is a gymnosperm genus belonging to the Podocarpaceae family that appeared in Gondwanaland in the Paleogene period, then it became more widely distributed across continents by cross-equatorial migration (Wu et al. 2019). The cross-equatorial migration involved the island formation in Indonesia as a bridge between the Northern and Southern hemispheres. One species of *Dacrycarpus* that is widely distributed in Indonesia is *Dacrycarpus imbricatus* (Blume) de Laub., also known as Java podocarpus or Malayan yellowwood. Presently, more broadly, this species is native to China, Malaysia, Thailand, Vietnam, Laos, Cambodia, Philippines, Papua New Guinea, and Indonesia, including on the island of Bali (POWO 2012). The distribution of *D. imbricatus* in Indonesia is spread from Sumatra, Java, Kalimantan, Sulawesi, Bali, NTB, NTT

and Papua (Soerianegara & Lemmens 1993). *Dacrycarpus imbricatus* is commonly found in sub-montane to montane habitats at elevations of 800 -2,500 m asl. and can grow at elevations of 3,600 m asl. (Soerianegara & Lemmens 1993). *Dacrycarpus. imbricatus* is a typically a tall tree up to 50 m high with a trunk diameter of up to 2 m, a hard trunk, and a rough surface with lenticels spread across the trunk; in old trees, the bark peels off in the form of small thick slabs extending vertically (de Laubenfels 1988; Waskitaningtyas et al. 2018).

This species is used as a means of traditional ceremonies for the Hindu community in Bali (Sumantera 2004). Additionally, it has resin potential, and the wood is used for construction, furniture, and firewood. In traditional medicine, *D. imbricatus* leaves are used to treat bone fractures in North Sumatra (Silalahi et al. 2015). *Dacrycarpus imbricatus* tree may also be used in land rehabilitation following gold mining because it has a high survival capacity and is able to absorb lead (Dharmawan & Siregar 2014).

Dacrycarpus imbricatus is difficult to find in natural forests, although its official conservation status in the IUCN Red List is categorized as Least Concern (IUCN 2022). *Dacrycarpus imbricatus* is included in a experiencing vulnerability criteria in China (Su et al. 2010). The regeneration of *D. imbricatus* in one of the conservation forests on the island of Java has been poor due to presence of invasive plant species such as Kaliandar (*Caliandra* sp.) and Kirinyuh (*Chromolaena odorata* (L.) R.M.King & H.Rob.) (Waskitaningtyas et al. 2018). *Dacyrcarpus imbricatus* is often locally uncommon as reported for the species in Bromo Tengger Semeru National Park (Rahadiantoro et al. 2013) and in China (Li et al. 2014). Therefore, in many areas, appropriate and research-informed conservation strategies are needed in order to increase its population.

The patchy distribution of the species potentially increases its vulnerability at local scales, especially if major disturbances reduce the population size. Furthermore, *D. imbricatus* has a vital role in the ecosystem stability in mountain forests in several ways. Firstly, compared to angiosperm trees, the soil beneath the *D. imbricatus* has more abundant saprophytic fungi that correlated with high soil acid phosphatase activity (Kitayama et al. 2011). In other words, the existence of *D. imbricatus* was associated with enhanced efficiency of the phosphate acquisition from the soil and decomposing litter (Kitayama et al. 2011). In addition to its role in the nutrient cycling, *D. imbricatus* also contributes to forest stratification as being a shade-intolerant species, it is often an emergent or canopy tree reaching more than 40 m in height (Su et al. 2010). Forests with mature *D. imbricatus* typically have greater structural complexity which is associated with enhanced niche differentiation, which means such areas could provide suitable habitat for species with a particular microclimatic preference. Because of these important roles in the ecosystem, studies of *D. imbricatus* local distribution are warranted to improve our understanding of its habitat preference and potential threats.

Re-assessment of the conservation status of *D. imbricatus* at local scales, especially in Bali, has never been conducted previously. Population studies of *D. imbricatus* were conducted by Sutomo (2011) in Pohen Mountains, Bedugul, Bali. This current paper will be an update of Sutomo (2011), with additional emphasis on the use of Habitat Suitability Model (HSM) to predict its current and future potential distribution across Bali Island. Sutomo et al. (2018) also has mention Species Distribution Model (SDM, another term for HSM) of *D. imbricatus*. However, that SDM is applied to the whole Indonesian area for year 2050. In this current study, we focus only for Bali Island, comparing it distribution

between current climatic conditions and a future climate change projection. In addition, this paper also assesses the current conservation status of *D. imbricatus* specifically in Bali Island, to fill this gap in the literature.

A desktop study regarding the distribution history of *D. imbricatus* in Bali Island was conducted in Global Biodiversity and Information Facility database (http://www.gbif.org/) (GBIF Secretariat 2021). The HSM was constructed and analysed using the BCCVL (Biodiversity and Climate Change Virtual Laboratory) (Huijbers et al. 2016) online application (http://www.bccvl.org.au/), this now has become eco-commons (ecocommons.org.au). The GBIF (Global Biodiversity and Information Facility) database which is available as one of the biodiversity databases in the BCCVL provides data on species occurrence (GBIF.org 2023). A current climate layers option available in the BCCVL which was chosen was from Worldclim (1950-2000) 10 arcmin (~20 km) data. The climatic layers chosen were namely in table 1.

Table 1. The climatic variables use in the model.

Layer data	Content data
B01	Annual Mean Temperature
B02	Mean Diurnal Range (Mean of monthly (max temp - min temp)
B03	Iso-thermality
B04	Temperature Seasonality
B07	Temperature Annual Range
B12	Annual Precipitation
B13	Precipitation of Wettest Month
B14	Precipitation of Driest Month
B15	Precipitation Seasonality

These layers were selected as they provided a wide range of different climate variables likely to be important in this region. The Generalized Linear Model approach was used to analyse the SDM, both for current climate projection as well as for climate change experiment projection in the BCCVL (Huijbers et al. 2016). For the climate change experiment projection, we used the RCP8.5 emission scenario (which assumes continued high future greenhouse gas emissions), with the circulation model is MIROC-ESM for the year 2050 (Huijbers et al. 2016; Sutomo & van Etten 2017). The forecast is represented as a grid cell's fitness on a scale of 0 to 1, with 0 indicating extremely low habitat suitability and 1 indicating very high suitability. The main SDM output is a map depicting the expected distribution of *D. imbricatus* based on the previously input baseline data. The anticipated distribution refers to the distribution of suitable habitat as determined by the model's environmental factors, rather than the actual existence of the species (Huijbers et al. 2016). The AUC (Area Under the Curve) of the ROC (Receiver-Operating Characteristics) curve was used to assess model strength. The value for ROC is the area under the curve (AUC). AUC score is interpreted as follow: a value above 0.9 is excellent, good 0.9 > AUC > 0.8, fair 0.8 > AUC > 0.7, poor 0.7 > AUC > 0.6 and fail 0.6 > AUC > 0.5 (Crego et al. 2014; Sutomo et al. 2021).

Based on the results of personal experiences and previous field surveys in previous years in Bali by Sutomo and team (Sutomo 2009, 2011; Sutomo et al. 2012), the locations of *D. imbricatus* were plotted in the GeoCAT to obtain information about AOO (Area of Occupancy) and EOO (Extent of Occurrence). Field exploration-derived geographic and biolog-

ical data are significant sources of knowledge for EOO, AOO, and fragmentation. The area occupied by taxon in the more common EOO (IUCN 2022) is referred to as AOO. One of the most widely utilized parameters in IUCN Red List evaluations is species AOO. In assessing the IUCN Red List based on Criterion B, specific measurements of the geographic range of species (EOO) and AOO are utilized. The analysis' AOO is based on the IUCN's standards, with a grid cell width of 2 km (IUCN 2022). GeoCAT is used to determine AOO and EOO (Geospatial Conservation Assessment Tool). A web-based application tool called GeoCAT uses primary biological data to facilitate semi-automated IUCN Red List assessment and analysis. This free and open-source website tool enables quick geographic analysis to assist with Red List species self-evaluation (Bachman et al. 2011). https://www.kew.org/science/our-science/projects/geocat-geospatial-conservation-assessment-tool is the website address.

A desktop study of *D. imbricatus* based on the Global Biodiversity Information Facility (GBIF) databases shows an increase in its occurrences in Indonesia or the Nusantara Archipelago, from one to two occurrences in 1862-1888 around West Java, and then over the next two decades, another 11 occurrences ranging from west to east Java. In 1919, GBIF shows records of the species in Sumatra, Ambon and Papua Islands. Then it shows in Kalimantan and Sulawesi in 1928, and 1938 in Lombok and Buru. Only then in 1958, this species was recorded in Bali Island around the Bedugul Highlands based on preserved specimens from Royal Botanic Garden-Kew (GBIF Secretariat 2021). This increase is perhaps due to the increasing detection/survey efforts.

Results from HSM analysis show changes in the prediction of habitat suitability of *D. imbricatus* with Habitat Suitability Index (HSI) in Bali Island between the current condition to the year 2050 (Figure 1). Habitat Suitability Index (HSI) has range value from 0 to 1 where the higher index reflects the more suitable habitat and this is classified to ten classes (interval 0.1) of HSI. In the current climate projection (Figure 1A), *D. imbricatus* occurrences is predicted to be the most suitable in the eastern part of Bedugul with the index > 0.8. Whereas in the western part of Bedugul up to Pupuan, it has a index value IND of 0.5. The Batur Volcano and Kintamani areas in the Eastern part of the Island, it has HSI value of about similar to the HSI of the western part of the Bedugul. Bangli District is unsuitable for *D. imbricatus* with HSI only 0.2 based on the current climate projection. Based on future climate projection, the whole Bedugul area and Batur Volcano as well as near the Agung Volcano near Besakih becoming very suitable for the *D. imbricatus* to spread in these areas, with HSI of 0.8 or greater (Figure 1B).

Probability of species occurrence showed distinct relationships with several of the climate variables thus indicating they were important in influencing the suitability of *D. imbricatus* in the area, namely: Annual Mean Temperature (B01), Iso-thermality (B03), Temperature Seasonality (B04) and Precipitation of Wettest Month (B13) (Figure 2). *Dacrycarpus imbricatus* has an optimum suitability to inhabit areas which has mean annual temperature between 16 to 22°C with less seasonality in the temperature and with around 400 to 500 mm/month of precipitation in the wettest month. The results of the model performance evaluation are represented in the AUC value. Based on the AUC value, the *D. imbricatus* habitat suitability in Bali Island is modelled as 'very good' because it is in the value range 0.9–1 (Figure 3). Prediction model of habitat suitability for *D. imbricatus* could have significantly affecting its conservation efforts. Ex-situ conservation can be better conducted in sites which has high

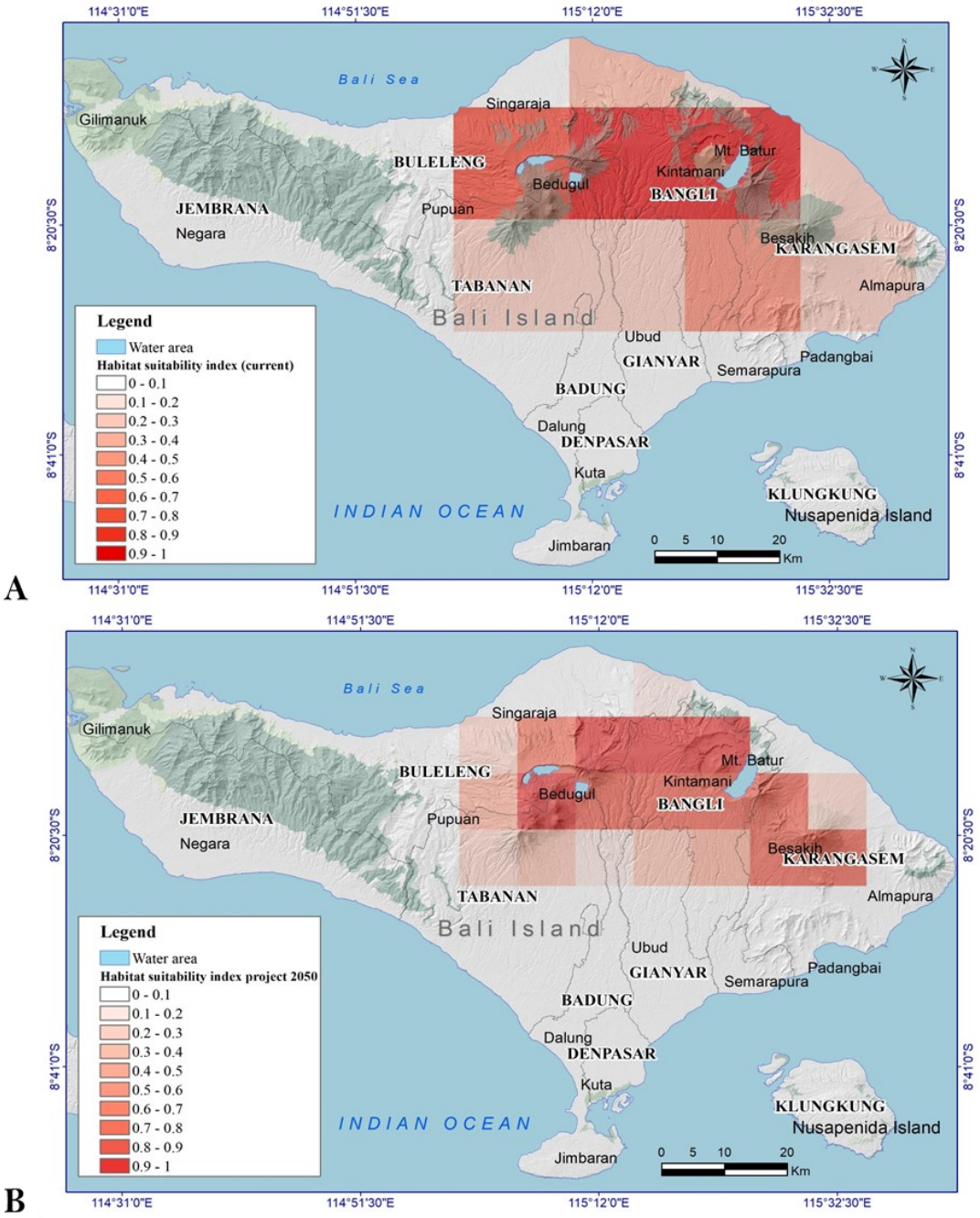

Figure 1. Habitat suitability model of *Dacrycarpus imbricatus* as resulted from the BCCVL web apps (bccvl.org.au). A: Current climate projection model; B: Climate change projection model in year 2050 based on RCP8.5 future emission scenario.

suitability index. Various stakeholders such as local government, botanical garden can use this as policy brief in better managing the conservation for *D. imbricatus*.

The estimated Extent of Occurrence (EOO) (Figure 4) *of D. imbricatus* in Bali is 680 km² according to the spatial analyses using GeoCAT. *Dacrycarpus imbricatus* is classified as endangered (EN) based on this estimation of EOO (Criteria B1). Based on the estimated Area of Occupancy (AOO; Category B2) of 120 km² (Figure 4), the species in Bali satisfies the requirement for "Endangered" (AOO less than 500 km²) (IUCN 2022). As a result, it is regarded to be in grave danger of extinction in the wild (EW) especially in Bali.

Conservation efforts of *D. imbricatus* has been conducted by the Bali Botanical Garden (BBG). Bali Botanic Garden as ex-situ conservation institution has living collection planted in the garden, herbarium speci-

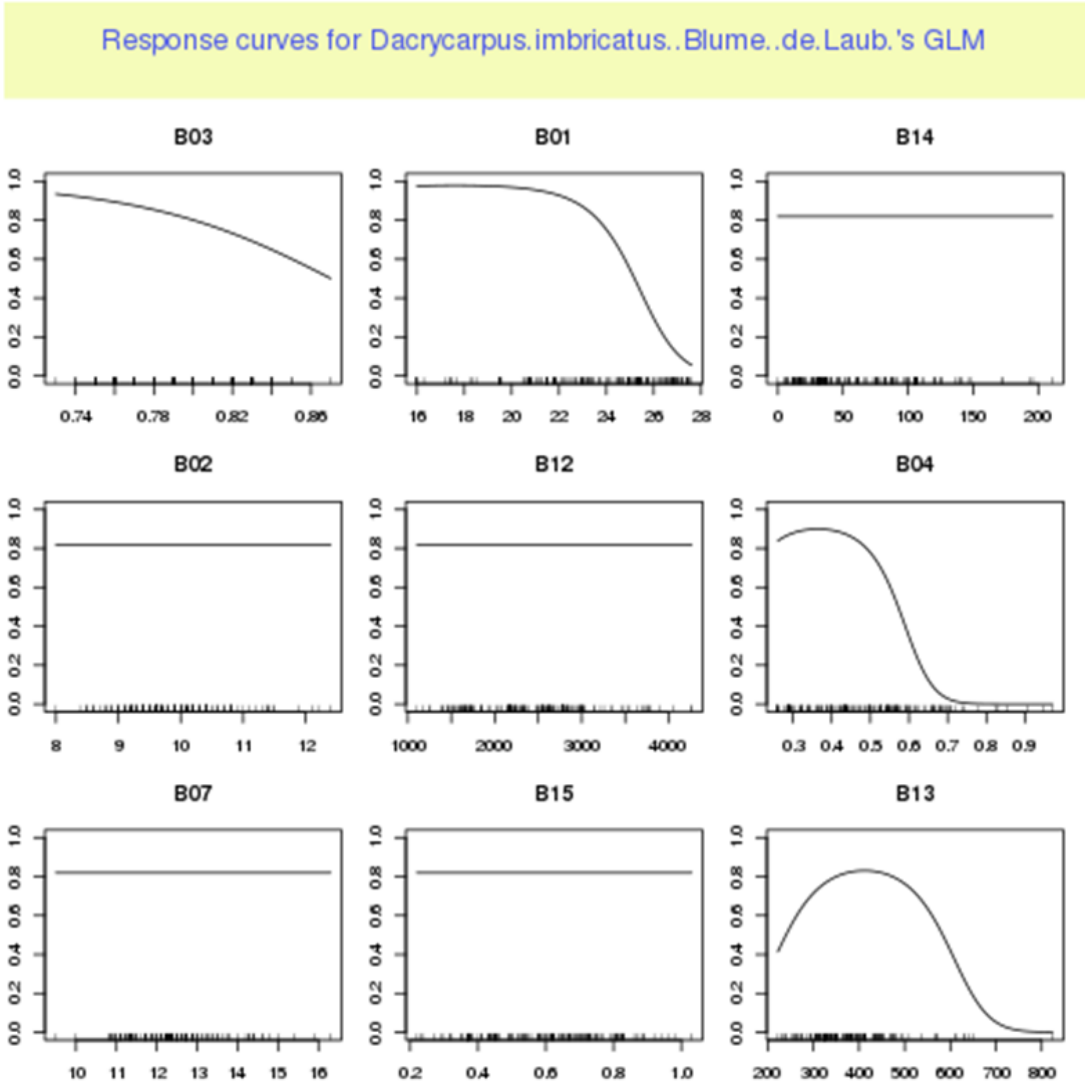

Figure 2. Species response curve of *D. imbricatus* toward environmental factors that were used in this study. X axis refers to the environmental factors and Y axis refers to occurrence index of the species. Annual Mean Temperature (B01); Mean Diurnal Range (Mean of monthly (max temp - min temp) (B02); Iso-thermality (B03); Temperature Seasonality (B04); Temperature Annual Range (B07); Annual Precipitation (B12); Precipitation of Wettest Month (B13); Precipitation of Driest Month (B14); Precipitation Seasonality (B15).

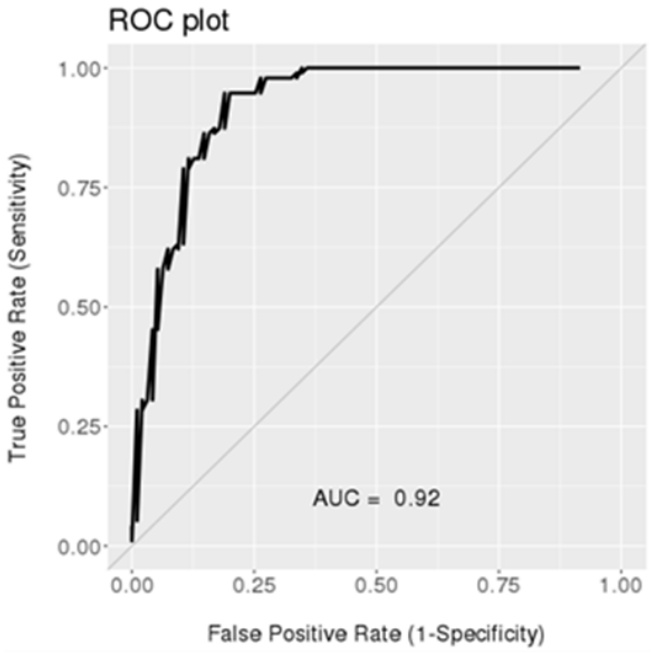

Figure 3. Robustness indicator of the model. ROC plot with AUC value of 0.92.

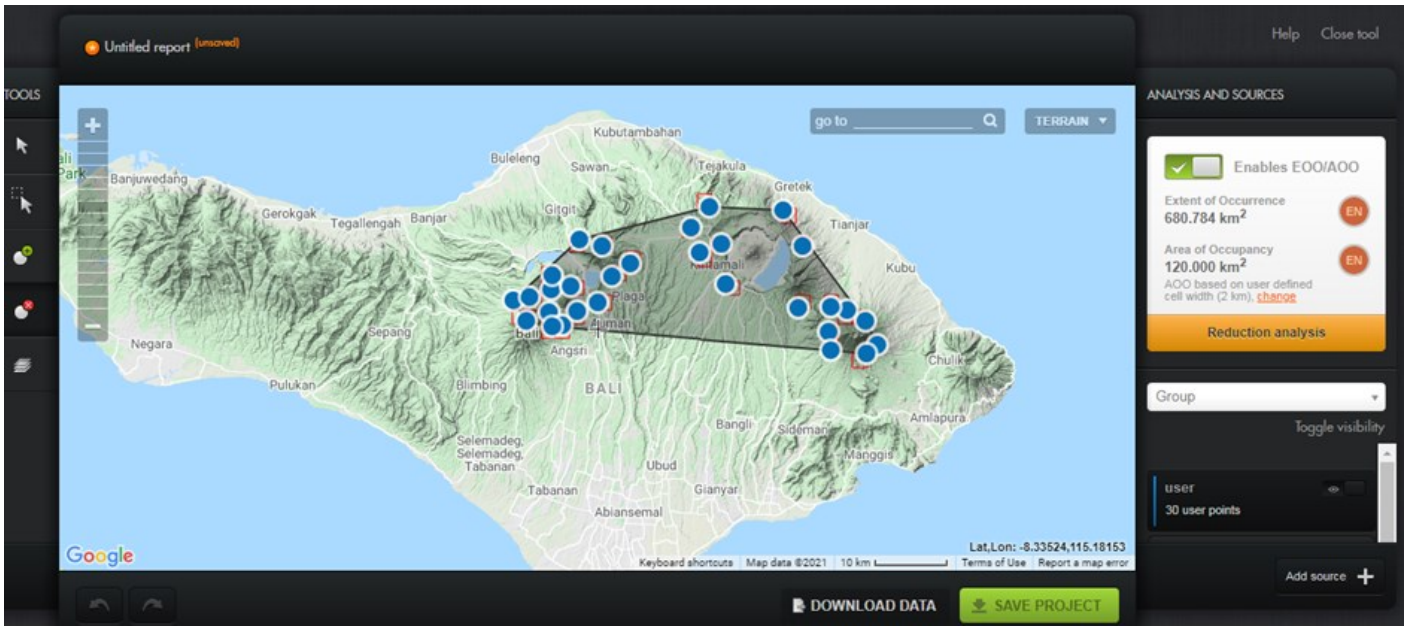

Figure 4. Results of assessment using the Geospatial Conservation Assessment Tool / GeoCAT http://geocat.kew.org/

men stored in the hortus botanicus Balinese and also has been successfully propagate the species. Several reintroduction efforts have also been initiated, especially on the Bukit Pohen in Bedugul, where the population of *D. imbricatus* is declining, mainly due to large forest fires that took place in 1994 (Sutomo 2009, 2011).

The distribution data of *D. imbricatus* and climate change modelling based on physical environmental variables can provide information regarding predictions of habitat preferences in the future taking into consideration anticipated climate change. Such predictions for the species in Bali for 2050 show a shifting of preferred habitat from Bedugul to the east towards Pupuan and Besakih, an area which has become more fragmented in recent decades. *Dacrycarpus imbricatus* has vulnerable (VU) status at global scale currently, but when the species' Area of Occupancy information approach is applied to the island landscape unit of Bali, its conservation status is endangered (EN) indicating potential extinction in the wild (EW).

AUTHORS CONTRIBUTION

S. is the main author who designed the research, analysed the data and supervised all the process, wrote parts of introduction, whole method and parts of results-discussion sections. M.B.A., re-formatted the draft based on the journal template, added part of the abstract section. I.D.P.D. helped in the discussion of the paper. R.I., wrote part of the abstract, introduction and conclusion sections. A.H., wrote part of the introduction section. I.M.S.W., wrote part of the introduction section. M.M.W., helped in the data collection and discussion of the paper. E.V.E. provide the English proofreading of the manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding research or funding in this paper.

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Short Communication

Diversity of *Fusarium* Endophytes Isolated from Wild Bananas in Pandenglang, Indonesia

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ABSTRACT

A group of *Fusarium* spp., in the *Fusarium oxysporum* species complex is known as pathogens on bananas, i.e., Fusarium wilt or Panama Disease. However, many *Fusarium* spp. are also known to be endophytes inside healthy banana plants and have been less explored and investigated. *Fusarium* endophytes have been demonstrated to be effective against the *Fusarium* pathogen that causes wilting in some crops such as tomatoes and watermelon. Thus, we explored endophytes *Fusarium* from local bananas in Pandenglang Banten for further use as biocontrol of Fusarium wilt. Four wild banana accessions were identified, from which 9 *Fusarium* isolates recovered from its pseudostems asymptomatic plants. All isolates were characterized based on their morphological characters and sequence of the Internal Transcribed Spacer (ITS) gene. These isolates belong to four complexes of *Fusarium* i.e. *Fusarium equiseti* species complex, *Fusarium oxysporum* species complex, *Fusarium sambucinum* species complex, and *Fusarium solani* species complex (currently described as *Neocosmospora*). Further study on molecular characterization of these isolates using specific genes and their potential antagonists of pathogens still needs to be discovered for other use as a biocontrol against Fusarium wilt.

Banana is one of the fruit commodities that plant pathologies have given many intentions for the past two decades because of the emerging destructive disease called Fusarium wilt, known as Panama Disease (Ploetz 2006; Ordonez et al. 2015; Westerhoven et al. 2022). However, in a country where banana is native such as Indonesia, the awareness of the disease lacks of attention. Moreover, the abundant diversity and luxury of choosing many different bananas to the table make the condition unheard for most people. The unawareness of farmers to isolate their banana plants that are infected with Fusarium wilt perhaps cause the pathogen, *Fusarium* spp., evolve with the host with different varieties of banana (Ploetz et al. 1999; O'donneal et al. 2008; Maryani 2018).

Fusarium wilt on a banana can be recognized by symptoms such as wilting and yellowing of the leaves, longitudinal splitting of the pseudostem, internal pseudostem showing reddish and brownish vascular lines (Maryani 2018). Once banana plants are infected with Fusarium

wilt, the soil becomes contaminated with the pathogen that can be survived for decades. This is because the chlamydospores of *Fusarium* spp. can survive more than 30 years in soil (Ploetz 2006). To date, there is no effective strategy to control the Fusarium wilt of bananas except to exclude the pathogen from non-infested areas, prevention by using pathogen-free tissue culture plants, and quarantine strategies (Kema et al. 2021). Applying disinfectant against soil pathogens could harm the microbial community in the soil, anaerobic soil disinfestation might be too costly, and the development of resistant cultivars is not foreseen in the short-term (Dale et al. 2017; Salacinas 2019; Ahmad et al. 2020). Therefore, biological control is considered a promising alternative to manage the disease and is environmentally friendly.

Endophytes are microbes that colonize plant tissue without causing disease. Many endophytes microorganism is known to decrease the disease susceptibility of their host upon infection. Thus, it is an attractive agent for organic farming. In the case of fungal *Fusarium* spp., despite its abundant species as a pathogen of many important crops, many of these species are endophytes (Leslie & Summerell 2006). Some *Fusarium* endophytes can protect the plants against Fusarium wilt caused by pathogenic *Fusarium* (Aimé et al. 2013). *Fusarium oxysporum* endophyte isolates Fo47 is well studied and known for reducing Fusarium wilt in many crops, including tomato, asparagus, cotton, and chickpea (Zhang et al. 2018; Constantine et al. 2020). However, the potential of fungal endophytes against Fusarium wilt on a banana is unknown. Therefore, studying *Fusarium* endophytes to fight the Fusarium wilt of bananas is crucial and will give insights into the possibility of managing this disease in a friendly-environmentally way. As a first step towards that goal, this study aimed to identify endophytic fungi from wild banana (*Musa* spp.) in Kabupaten Pandeglang Banten Province, Indonesia, for later use as a biocontrol against Fusarium wilt on banana.

Sampling collection was undertaken through exploration in six districts of Pandeglang Banten; Cadasari, Banjar, Menes, Cisata, Saketi, and Pandeglang. Wild banana was able to be identified based on the presence of abundant seed in their fruit (Figure 1). Pseudostem from symptomless plants was sampled and placed on filter paper to dry and packed in a paper envelope. Global Positioning System (GPS) coordinates were recorded and ecological parameters including soil pH, light intensity and vegetation around the sampling area, were noted at each site. Wild banana identification was based on morphological characters (Valmayor et al. 1999) and in-situ comparison with *Musa* collection at Kebun Plasma Nutfah, Pusat Biologi, Cibinong, Bogor, Indonesia (Poerba et al. 2018).

Samples were isolated using the direct plating method with surface sterilization (Maryani 2018). Dried pseudostem was cut into pieces, approximately 2 x 3 cm, and plated into potato dextrose agar (PDA) plates. After 2-3 days, colonies resembling fungi were transferred to new PDA plates. Monosporic culture was derived by streaking small amount of conidia, collected with the tip of inoculation needle on water agar (WA) plates, which allowed conidia to separate. After 24h incubation, plates were observed under stereo microscope and germinating conidia were collected and transferred to new PDA. Monosporic isolates were maintained on PDA for working culture and 20% (v/v) glycerol at -20^{0}C for long preservation. All isolates were deposited at the laboratory of Biology Education Culture Collection, Universitas Sultan Ageng Tirtayasa (UNTIRTA) Banten.

Fungal isolates were grown on carnation leaves agar (CLA) for sporodochium formation, synthetic nutrient agar (SNA) for chlamydo-

spore formation, and PDA for conidia formation. All cultures on each medium were incubated under continuous light at room temperature. Growth rates of isolates were determined on PDA plates, after 7-day incubation at room temperature in the dark. All isolates were identified based on their morphological characters (Seifert et al. 2011; Maryani 2018) as well as comparison with well-identified living culture from the collection of Biology Education Laboratory, UNTIRTA and Typed isolates from Indonesian Culture Collection (InaCC) BRIN, Cibinong. Molecular identification of the isolates was using pair of primers ITS4 (5`-TCC TCC GCT TAT TGA TAT GC- 3`) and ITS5 (5`-GGA AGT AAA AGT CGT AAC AAG G-3`) which amplified the ITS (Internal Transcribed Spacer) regions (White et al. 1990). Fungal DNA extraction following the protocol of Hidayat & Ramadhani (2019).

Both ancestors of cultivated bananas, *Musa acuminata* Colla dan *Musa balbisiana* Colla were found in this exploration (Nasution 1990). *M. acuminata* and *M. balbisiana* can be distinguished by their male flower, leaf petiole, and fruit (Ahmad 2021). The flower of *M. acumianata* has no pigmentation, usually white or creamy. In contrast, the flower of *M. balbisiana* has red or red-to-purple pigmentation. Opened brachtea of the male bud of *M. acuminata* is rolled up, but not for *M. balbisiana. M. acuminata* has opened petiole canal leaf, but the petiole canal leaf of *M. balbisiana* is closed or inward. The transverse of the fruit of *M. acuminata* is rounded, while that of *M. balbisiana* is pronounced ridges.

None of the wild bananas showed any symptoms of diseases. Leaves diseases commonly identified on cultivated bananas were absent, and pseudostem was clean from any internal symptoms of wilt disease of bananas. All identified wild bananas are seeded, less pulpous, and found in abandoned areas or near rivers (Figure 1). Three wild banana species were found in four locations at *Kabupaten Pandenglang*, of which only one of *Musa* species could be identified in variety levels, *Musa acuminata* var. *breviformis* (Table 1).

In total, 9 *Fusarium* isolates were recovered from the asymptomatic pseudostem of *M. acuminata* and *M. balbisiana* (Table 2). *Musa acuminata* identified beside a river at Desa Cisata Pandegang bared the highest number and diversity of *Fusarium. M. balbisiana* species from Saketi obtained only one isolate of *Fusarium* sp. The rest of the wild *Musa* discovered in this study were not contained *Fusarium* isolates in their pseudostem. Combining morphology and molecular identification using Internal Transcribed spacer (ITS) gene (Supplementing Table 1.), we can identify four complex members of *Fusarium* i.e., *Fusarium equiseti species complex* (Figure 2), *F. oxysporum species complex* (Figure 3), *F. sambucinum species complex* (Figure 4), *and F. solani species complex* (Figure 5).

Figure 1. Wild bananas collected in Pandeglang. A-B. *Musa acuminata*, C. *M. balbisiana.*

Table 1. Details Location, GPS, and Wild *Musa* Species Found in Pandeglang as a Source of Isolation Endophytic Fungi.

Location		GPS			Wild Musa	Genome
District	Village	Long.	Lat.	Alt. (m)		
Menes	Tegalwangi/ Kampung Cipancur	105.93	-6.39	34,5	*Musa acuminata*	AA
Cisata	Cisata	106.09	-6.32	59.4	*Musa acuminata*	AA
Pandeglang	Cikondang	105.93	-6.39	231	*M. acuminata* var. *breviformis*	AA
Saketi	Kadu Dampir	105.96	-6.39	129	*Musa. balbisiana*	BB

Table 2. List of the diversity of *Fusarium* isolates recovered from pseudostem of wild banana, based on morphology and molecular identification on ITS gene sequence.

Isolate code	Species name	*Fusarium* complexes	Host	Location
NMC273	*Fusarium* sp.	NA		
NMC274	*Fusarium solani*	*Fusarium solani species complex*		
NMC276	*Fusarium oxysporum*	*Fusarium oxysporum species complex*		
NMC277	*Fusarium solani*	*Fusarium solani species complex*		
NMC278	*Fusarium equiseti*	*Fusarium incarnatum-equiseti complex*	*M. acuminata*	Cisata
NMC279	*Fusarium longipes*	*Fusarium sambucinum species complex*		
NMC280	*Fusarium solani*	*Fusarium solani species complex*		
NMC281	*Fusarium* sp.	NA		
NMC293	*Fusarium* sp.	NA	*M. balbisiana*	Saketi

It is well-known, reported and studied that the primary pathogen of bananas is *Fusarium*. More in this research, *Fusarium* is categorized as endophytic fungi. Symptomless infections by *Fusarium* species have been reported in many plants, such as medicinal plants (Jia et al. 2016) and agricultural plants (Zakaria & Ning 2013; Nuraini et al. 2017). In bananas, *Fusarium* is reported to recover from symptomatic and asymptomatic plants with Fusarium wilt diseased (Maryani 2018; Maryani et al. 2019). *Fusarium oxysporum* was reported to recover from the roots of wild *Musa acuminata* from Malaysia but not in their pseudostem (Zakaria et al. 2011). Interestingly, the most devastated pathogen of bananas, Tropical Race 4, is also a member of the *Fusarium oxysporum* species complex (Ordonez et al. 2015; Maryani 2018). Comparative studies between these members of the complex will be beneficial in revealing why wild bananas are rarely found to be infected with fusarium wilt.

Fusarium equiseti is the most reported species isolated from each part of the banana plants, including leaves, fruit, roots, and pseudostem (Zheng et al. 2012; Zakaria & Wan Aziz 2018; Maryani et al. 2019). *Fusarium longipes* is never reported from the pseudostem of asymptomatic bananas since its first description by Reinking & Wollenweber (1927), who isolated from leaves *Musa sapientum*. *Fusarium solani*, currently recognized as a member of the genus *Neocosmospora*, is known to be an opportunistic pathogen on plants and animals (Sandoval et al. 2019). Thus, it is interesting that most of the isolates we discover in this study are members of this complex.

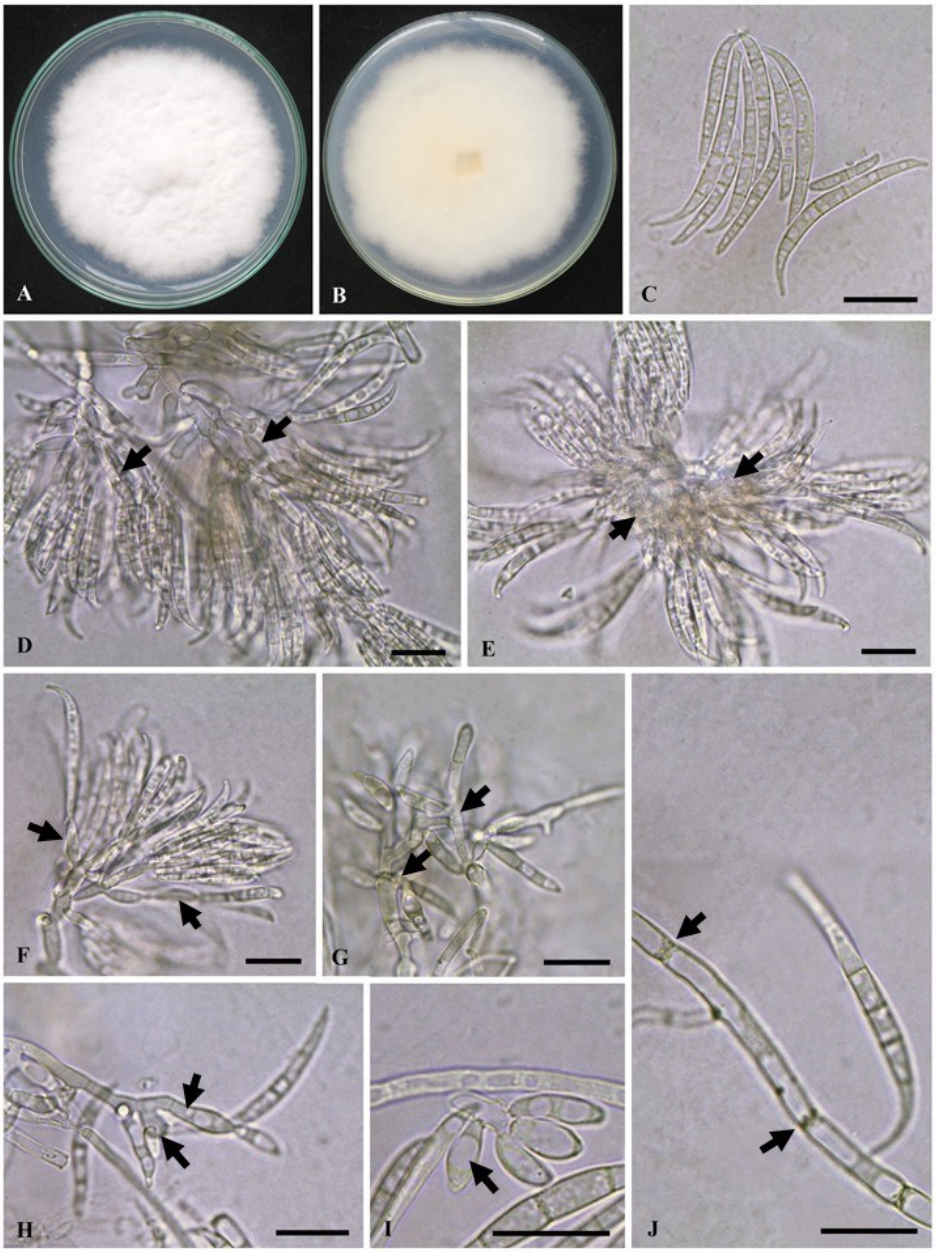

Figure 2. *Fusarium equiseti* NMC278. **A-B**. Culture grown on PDA after 7d incubation, **C**. Macroconidia, **D-G**. Aerial phialides, **H**. Polyphialid, **I**. Conidiogenous cell, **J**. Hyphae septate. Scale bars C-J = 10 µm.

The role of the *Fusarium* endophyte inside the banana plants is still to be discovered. However, some studies demonstrated that *Fusarium* could decrease disease incidence in some crops with wilt disease (Aimé et al. 2013; Zhang et al. 2018; Constantine et al. 2020). Future studies on the potential of *Fusarium* endophyte to control primary disease on bananas, i.e., Fusarium wilt, will be critical as both types of *Fusarium* colonies have the same host. Moreover, endophyte *Fusarium* isolated from the wild banana will be interesting as many wild bananas are resistant to Fusarium wilt (Ahmad et al. 2020). In this study, we expand the knowledge of the diversity of endophytic fungi from wild bananas. Further characterization of the isolates based on molecular data using specific genes in each genus is needed. The role of each of these fungi inside healthy bananas and their potential to be antagonists of pathogens are also to be discovered for further use as a biocontrol against Fusarium wilt.

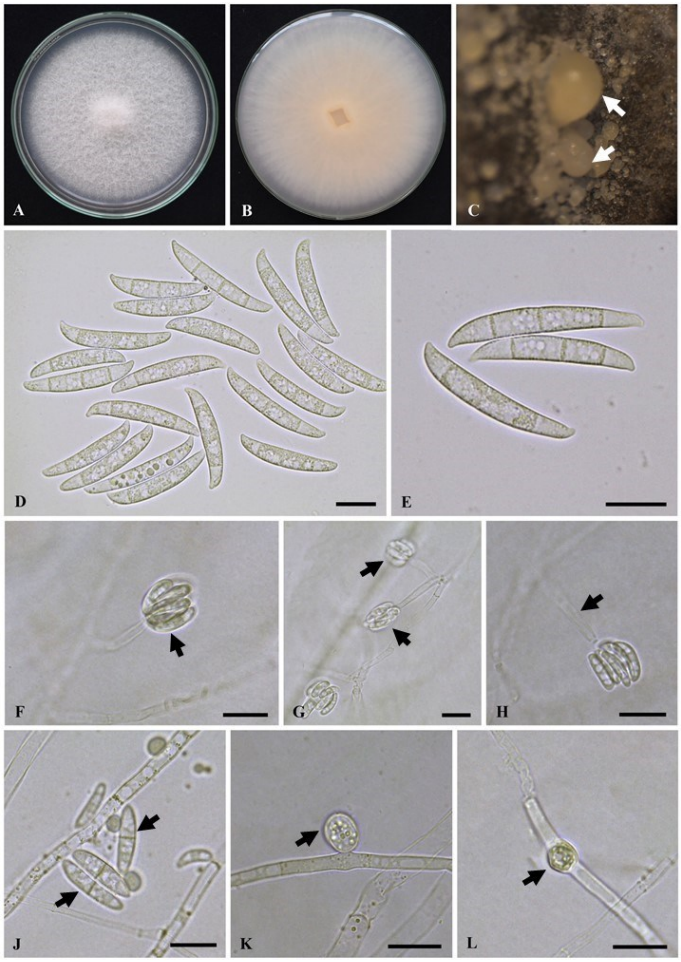

Figure 3. *Fusarium oxysporum* NMC276, **A-B.** Culture grown on PDA after 7d incubation, **C.** Sporodochia on CLA, **D-E.** Macroconidia, **F-G.** False head, **H.** Monophialide, **J.** Microconidia, **K-L.** Chlamydosphore. Scale Bares C-L = 10 µm.

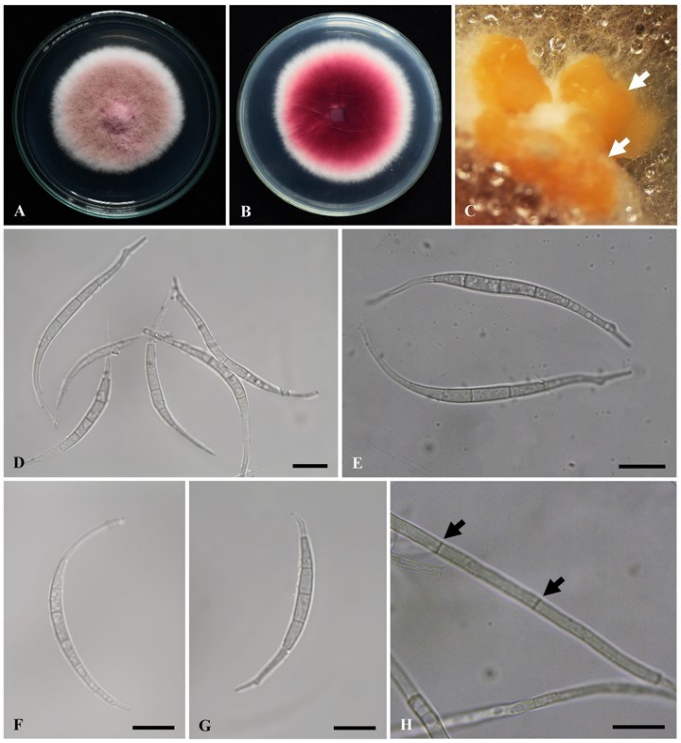

Figure 4. *Fusarium longipes* NMC 279 member of *Fusarium sambucinum* species complex. A-B. Culture grown on PDA after 7d incubation, C. Sporodochia on CLA, D-G. Macroconidia, H. Hyphae septate. Scale bars C-H= 10µm.

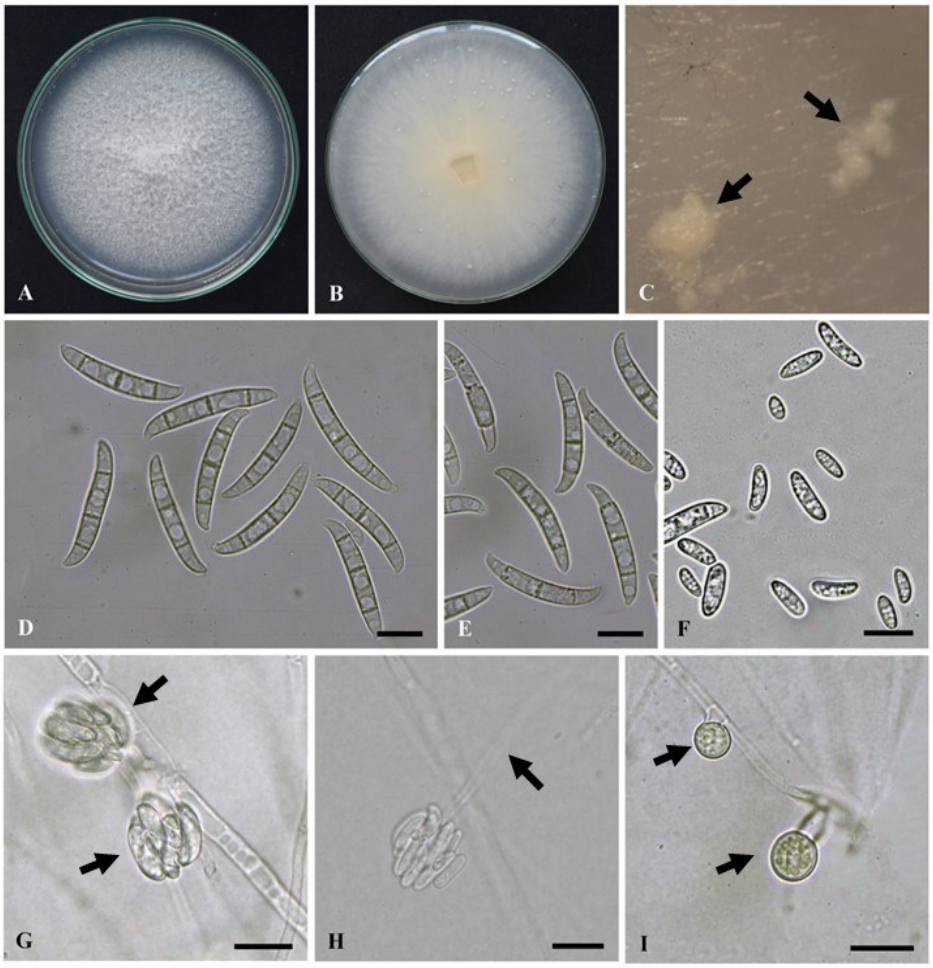

Figure 5. *Fusarium solani* NMC277. **A-B.** Culture grown on PDA after 7d incubation, **C.** Sporodochia on CLA, **D-E** Macroconidia, **F.** Microconidia, **G.** False head, **H**. Monophialid, **I.** Chlamydosphore. Scale bars C-I = 10 µm.

AUTHOR CONTRIBUTION

NM designed the research, collected and analysed the data, and wrote the manuscript, SY executed the research in the laboratory, IR sequenced *Fusarium* isolates, ROK and SML supervised SY.

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CONFLICT OF INTEREST

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

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Short Communications

Effectiveness of Liquid Organic Fertilizer Byproduct of Black Soldier Fly Maggot to the Growth of Mustard Plant (*Brassica juncea* L.)

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ABSTRACT

The need for consumption of mustard plant (*Brassica juncea* L.) has increased every year. One of the efforts to increase its production and quality by applications of inorganic and organic fertilizer. This study aimed to evaluate the productivity of mustard plants treated with liquid organic fertilizer, a by-product of black soldier fly (BSF), and inorganic fertilizer (NPK). Mustard plants were grown at Karanggayam Research Station, Caturtunggal, Depok, Sleman, Yogyakarta were treated with water as a control, NPK fertilizer, DoctoRS organic fertilizer at 0.05%, organic fertilizer A, and B at 0.1, 0.15, 0.20, and 0.25%. The effects of treatments to the phenotypic and the chlorophyll of the mustards were done after 2 weeks of treatments. The results showed that there were significant different on the stem height, number of leaves, leaf width, leaf length, and wet weight. These were in line with the total chlorophyll. Liquid organic fertilizer content analysis showed that DoctoRS liquid organic fertilizer and liquid organic fertilizer A were the most in accordance with the national standards for organic fertilizers on the parameters of pH, Mg, Ca, and TPC.

Indonesia is known as agricultural country. One of crops which mostly cultivated by the community is mustard. Mustard plant (*Brassica juncea* L.) is a crucifer originating from China and East Asia. It was cultivated or 2500 years ago in China then spread widely to the Philippines and Taiwan (Rukmana 2007). The consumption of mustard is increasing with the increase of human population. It has benefit for health because contains protein, fat, carbohydrates, Ca, P, Fe, vitamin A, vitamin B, and vitamin C (Fahrudin 2009). The production of mustard plants decreased by 5.08% to 117.68 quintals/ha from 123.98 quintals/ha in 2013 and the average productivity of mustard plants continues to decline every year in line with the decrease in land area (BPS 2020). Thus, we need to increase the quality and quantity of mustard plant production.

One of the efforts to increase mustard production is by fertilizer applications. There are two types of fertilizers mostly used, namely inorganic fertilizers or chemical fertilizers and organic fertilizers (Zamriyetti

et al. 2019). Farmers mostly depend on the use of inorganic fertilizers for their crops production. In the long term, the use of inorganic fertilizers can cause the decrease of organic matter levels in the soil. It also may damage the soil structure, and increase the environmental pollution. This fertilizer is also quite expensive for farmers (Dewanto et al. 2017). In contrast, organic fertilizers can be used by farmers and are very beneficial for increasing the productivity both in quality and quantity. It also has a role in reducing the pollution as well as improve land quality in a sustainable manner. The use of organic fertilizers for a long term is also able to increase soil fertility and to prevent land degradation (Prasetyo 2014).

Susilo (2009) revealed that pakcoy plant (*Brassica rapa* L.) with the treatment of liquid organic fertilizer (LOF) and watering interval has significant effect on the yields. The fertilizers at 20 ml/L and 6 days interval was the best treatment which produce weight of 190.67 g or equal to 47.5 tons/ha. One of the organic fertilizers that can be used by farmers on horticultural crops is organic fertilizer made from organic household waste. The organic waste can ferment by using BSF to produce liquid organic fertilizer which has been widely developed by rural communities, for example the village community at Gejayan, Condongcatur, Sleman, Yogyakarta, Indonesia. The use of BSF larvae as organic waste processor is very promising, because it accelerates organic waste break down, and the BSF also can be harvested as animal feed alternatives (Firmansyah & Noor 2020).

Furthermore, organic fertilizer has some advantages as cheaper, eco-friendly, and indirectly can help to reduce waste and to support sustainable agriculture (Hartatik et al. 2015). The used of maggot as a component for fertilizer productions are still new among farmers. This research is important to provide information on the effectiveness of the use of liquid organic fertilizer by product of BSF as a ZPT agent for plant, especially mustard plant production.

The research was conducted in September – October 2021 located at Karanggayam Research Station, Depok, Sleman, Yogyakarta Special Region, Indonesia. The LOF properties such as pH, Mg, S, Na, Ca, and bacteria contents were carried out at the Laboratory of Analysis at CV Chem-Mix Pratama, Yogyakarta in October 2021. The analysis of chlorophyll content of mustard leaves after grown under different fertilizer treatments were carried out at the Laboratory of Plant Physiology, Faculty of Biology, UGM. The 0.5 g of fresh leaves for every treatment was homogenized in a homogenizer with 10 mL of acetone 80%. The samples were centrifuged at 10,000 rpm for 15 minutes at 40°C. The 0.5 mL of supernatant was collected and mixed with 4.5 mL of acetone 80%. The mixture was analysed for chlorophyll-a content in a spectrophotometer (Parkin) at a wavelength of 663.2 nm (Sumanta et al. 2014). The chlorophyll content measurements were done triplicates.

There were several steps taken to produce liquid organic fertilizer. First, the rotten fruits or vegetables were periodically put in a digester (20 L volume). Then the BSF maggots were introduced in the digester. This will trigger faster microbial growth so that the produced volatile aroma will initiate wild BSF to lay eggs. Second, organic household waste can be added regularly. Third, after 4 weeks of fermentation, the leachate from the larvae's digestion was harvested then put into a clear bottle with a loosened cap. Fourth, the leachate in the bottle then where exposed under the sun light until it turned brown in colour and there no strong odour. The sunlight exposure of the leachate was done for 12 and 10 months for LOF A and B, respectively. These two LOFs were produced by Gejayan Condongcatur community, whereas, DoctoRS LOF was taken from DoctoRS organic fertilizer production house in Klaten,

Central Java.

Mustard seeds were sought at the Karanggayam Research Station, Depok, Sleman, Yogyakarta. The seeds were planted in a medium which was made from a mixture of soil: goat manure: husks in a ratio of 1:1:1. Planting medium was put on the pot tray for sowing mustard seeds for 14 days. After that, the seedlings were transferred into large polybags (15 cm x 15 cm) using the above-mentioned medium ratio. Mustard seedlings then placed in the field with full sunlight. LOF A and B at 5 different concentrations, namely 0.05, 0.1, 0.15, 0.2, and 0.25% (v/v), distilled H_2O as a control, NPK fertilizer (N:P:K = 16:16:16 Mutiara ®), and DoctoRS LOF at 0.05% as a positive control treatment for LOF were used to evaluate the LOFs quality. All the treatments were done for five replicates. The 14 days old mustards were treated with the weekly above-mentioned treatments up to 35 days after planting.

In this study, the plant height, number of leaves, leaf length, leaf width, plant gross weight, and chlorophyll content were observed after 35 days of planting. These phenotypic data were then analysed using One Way ANOVA which then continued with mean separation using Duncan Multiple Range Test (DMRT at α: 0.05). All the statistical procedures were done using SPSS version 24. The results of the average chemical content of macro and micro elements of DoctoRS, LOFs A, and B in the study were listed in Table 1.

In this study, synthetic fertilizers and organic fertilizer were used. The use of NPK, a synthetic fertilizer, was assumed to be a positive control. NPK fertilizers are commonly used by the community and are easy to find. DoctoRS, LOFs A, and B were made from household waste or fruit residue which is then fermented with the help of BSF larvae. BSF maggot consumes and degrades several organic materials contained in the waste up to 70% (Lalander et al. 2014). This fermentation not only produces liquid organic fertilizers (LOF) and compost but also maggots which can be used as animal feed and biofuels (Gao 2019).

The results of chemicals analysis on the three organic fertilizers showed the average of pH ranging from acidic to alkaline, respectively, namely LOFs A > LOFs DoctoRS > LOFs B. It showed that LOFs A has a higher pH than LOFs DoctoRS and LOFs B. Based on the Ministry of Agriculture Decree No.28/Permentan/SR.130/5/2009 about the organic fertilizer, biological fertilizer and soil rehabilitation, the standard pH for LOF is 4-9. It suggested that that the pH value in LOFs B exceeds the standard and it does not meet the requirements, but both LOFs DoctoRS and LOFs A have met the standard. At the beginning of the fermentation process, the acidity will convert organic matter into organic acids, after that, the changes that occur during the fermentation process will produce nitrogen and ammonia so that it will cause an increase in the pH value (Rukmayanti 2020). The longer the fermentation time does not mean the

Table 1. The content of macro and micro elements of DoctoRS, A, and B organic liquid fertilizers.

No	Parameters	Results		
		DoctoRS	A	B
1	pH	8.74 ± 0.005^{b}	5.3 ± 0.001^{a}	$9.29 \pm 0{,}005^{b}$
2	Magnesium (%)	0.641 ± 0.024^{c}	0.495 ± 0.021^{a}	0.514 ± 0.014^{b}
3	Sulphur (%)	1.133 ± 0.007^{a}	1.274 ± 0.042^{b}	1.574 ± 0.040^{c}
4	Potassium (%)	0.673 ± 0.190^{b}	0.553 ± 0.032^{a}	0.927 ± 0.010^{c}
5	Calcium (%)	1.370 ± 0.006^{a}	1.406 ± 0.027^{b}	1.313 ± 0.006^{a}
6	Bacteria (10^3 CFU/mL)	14 ± 1.00^{a}	20 ± 2.00^{b}	34 ± 1.00^{c}

Note: Numbers (mean ±SE) followed by different letters in the same row mean significant different at α:0.05.

pH value is also increased, because the fermentation process is directly related to micro-organisms (Kusumadewi et al. 2019). The high pH on LOFs B can occur due to various environmental factors that affect the growth of microorganisms such as unstable temperatures, nutrients, or the medium in which bacteria grow (Meriatna et al. 2019).

Magnesium (Mg) in the form of magnesium oxide (MgO) is one of the main minerals in the process for the formation of plant's chlorophyll. Magnesium plays an important role in the process of exchanging phosphate substances, participating in influencing the respiratory process and activating the enzymes transphosphorylase, dehydrogenase, and carboxylase (Amelia et al. 2017). The results showed that on average the content of MgO from the largest to the smallest was LOFs DoctoRS > LOFs A > LOFs B. Based on Ministry of Agriculture Decree, the standard value Mg in organic fertilizers is < 0.63%, which mean that the Mg content in the three liquid organic fertilizers meet the standard. The sulphur content showed that the average sulphur content of the three organic fertilizers from the largest to the smallest was LOFs B > LOFs A > LOFs DoctoRS. Sulphur that is applied to the soil will be converted into H_2SO_4 by microorganisms. S element is an important part of ferredoxin, which is a complex of Fe and S in chloroplasts that is used in carbohydrate catabolism for optimal photosynthate. Furthermore, photosynthate will be translocated to all parts of the plant (Hanifah et al. 2021).

In the potassium analysis, the result showed that the average potassium content from the largest to the smallest was LOFs B > LOFs DoctoRS > LOFs A. Based on the Ministry of Agriculture decree, the standard K_2O value in the organic fertilizer is 3-6%. It revealed that the K_2O content in all liquid organic fertilizers in this study has not met the standard. Potassium is one of the macro nutrients other than P- and C- which is needed by plants. Potassium is needed in increasing plant resistance in the process of osmotic regulation, enzyme catalysis of cellular pH regulation, and ion regulation in cells. During the optimal period of fermentation, generally, the potassium content in liquid fertilizer will increase, due to the activity of microorganisms in the decomposition of organic matter. The activity of microorganisms in the degradation process results in the breaking of the carbon chain in organic matter to be simpler so that there is an increase in the element of potassium in fertilizers. Bacteria produce potassium compounds and use K+ ions in fertilizer raw materials for the benefit of their metabolism so that potassium levels will increase along with the growing bacteria (Widyabudiningsih et al. 2021). It is possible that the decrease in potassium levels occurs due to the cleavage activity of microorganisms. When the microorganisms have reached the equilibrium phase, the longer the fermentation time does not mean the potassium content is also increasing. If the fermentation is continued, the microorganisms will die because the nutrients from the microbes have been reduced, so that in this phase the activity of microorganisms in breaking down organic compounds will decrease and the results will be less potassium (K) levels (Kusumadewi et al. 2019). The result indicated that fermentation period of the used fertilizers was not optimal enough to support the activity of microorganisms in the decomposition of organic compounds into potassium.

In the calcium oxide analysis, the results showed that the average of calcium oxide content in the three organic fertilizers from the largest to the smallest was LOFs A > LOFs DoctoRS > LOFs B. Based on Ministry of Agriculture decree, the standard value of Ca is < 25.49% so that the Ca content in the liquid organic fertilizers studied has met the requirements. Calcium oxide or lime is known as a source of ameliorant material used in improving soil fertility. Calcium oxide is a contributor to

Ca^{2+} which is an indispensable nutrient in plant growth as well as its function in neutralizing acidic compounds (Fauzi et al. 2020). Plants absorb calcium in the form of Ca^{2+}. Ca^{2+} plays a role in the formation of the structure and permeability of cell membranes. Deficiency of this element cause disruption of plant growth at the root growth points and storage networks (Tehubijuluw et al. 2014).

The presence of pathogenic bacteria in fertilizers also needs careful observation. This study used the total plate count (TPC) methods to detect the number of microbes in liquid organic fertilizer. Based on Ministry of Agriculture decree, the standard number of bacteria contained in LOFs is $< 10^2$ MPN/mL, while in this study, the number of bacteria found exceeds that standard so that this LOFs does not meet the standard. The high bacteria content can cause microbial foodborne disease. Therefore, it is necessary to carry out further assessment of these risk factor to identify the possible impact of contamination in the production area (Sarmiento et al. 2014).

The results in Table 2 and Figure 1 showed that the application of inorganic fertilizers and liquid organic fertilizers with different concentrations had significant effects on stem length, number of leaves, leaf length, leaf width, and plant wet weight. It gave different growth responses because plants obtained different amounts of macronutrients and micronutrients. In stem length, it was known that treatment with 0.25% LOF A had the best value compared to other treatments. In the number of leaves, it was known that treatment with LOF A at the concentration of 0.15% has the best value compared to other treatments. Meanwhile, on leaf length, leaf width, and wet weight, it was known that treatment with NPK fertilizer had the best value compared to other treatments (Makmur & Magfirah 2018).

The length and width of the leaves influence the metabolism of mustard plants, especially in the process of photosynthesis. The more levels of fertilizer the more stimulation of the metabolic processes of cells, especially in the meristematic tissue at the point of leaf growth. Components in fertilizers such as P, K, and N may stimulate the plant growth (Oviyanti et al. 2016). In addition, the wet weight data obtained in the NPK fertilizer treatment showed that it has the highest. The rapid

Table 2. The effects of organic liquid fertilizers to the growth of mustard plants.

Treatment	Stem Length (cm)	Number of Leaves (cm)	Leaf length (cm)	Leaf Width (cm)	Wet Weight (g)
Control	3.2 ± 0.25^{abc}	7.6 ± 0.50^{a}	11.8 ± 0.90^{ab}	7.72 ± 1.93^{a}	31.8 ± 1.77^{abc}
NPK fertilizer	2.7 ± 0.30^{ab}	9.6 ± 0.40^{bc}	13.1 ± 0.30^{b}	10.2 ± 0.33^{b}	44.2 ± 3.0^{abc}
DoctoRS	2.4 ± 0.36^{a}	9.0 ± 0.44^{abc}	10.92 ± 0.62^{a}	8.22 ± 0.50^{ab}	35.0 ± 3.98^{abc}
0.05% OLF A	3.9 ± 0.33^{c}	8.6 ± 0.67^{abc}	12.0 ± 0.41^{ab}	9.9 ± 0.30^{ab}	36.8 ± 3.67^{bc}
0.1% OLF A	2.9 ± 0.18^{abc}	10.0 ± 0.77^{c}	11.02 ± 0.40^{a}	8.86 ± 0.10^{ab}	34.2 ± 1.71^{abc}
0.15% OLF A	3.2 ± 0.43^{abc}	10.2 ± 0.48^{c}	11.64 ± 0.59^{ab}	9.24 ± 0.27^{ab}	38.0 ± 3.96^{c}
0.20% OLF A	3.02 ± 0.15^{abc}	10.0 ± 0.44^{c}	12.26 ± 0.67^{ab}	9.84 ± 0.49^{ab}	34.8 ± 1.46^{abc}
0.25% OLF A	3.9 ± 0.29^{c}	9.2 ± 0.37^{abc}	12.16 ± 0.68^{ab}	9.2 ± 0.38^{ab}	33.0 ± 2.19^{abc}
0.05% OLF B	3.3 ± 0.30^{abc}	7.8 ± 0.48^{ab}	11.1 ± 0.48^{a}	8.34 ± 0.49^{ab}	26.4 ± 2.89^{a}
0.1% OLF B	3.5 ± 0.44^{bc}	8.6 ± 0.50^{abc}	10.5 ± 0.45^{a}	8.4 ± 0.29^{ab}	31.0 ± 1.78^{abc}
0.15% OLF B	3.2 ± 0.25^{abc}	9.8 ± 0.66^{c}	10.84 ± 0.66^{a}	9.36 ± 0.46^{ab}	28.0 ± 3.56^{ab}
0.20% OLF B	3.3 ± 0.12^{abc}	9.2 ± 0.58^{abc}	10.5 ± 0.14^{a}	8.1 ± 0.13^{ab}	29.0 ± 1.30^{abc}
0.25% OLF B	3.1 ± 0.36^{abc}	10.0 ± 0.83^{c}	10.82 ± 0.49^{a}	9.06 ± 0.66^{ab}	31.4 ± 2.15^{abc}

Note: Numbers (mean ±SE) followed by different letters in the same column mean significant different at α:0.05.

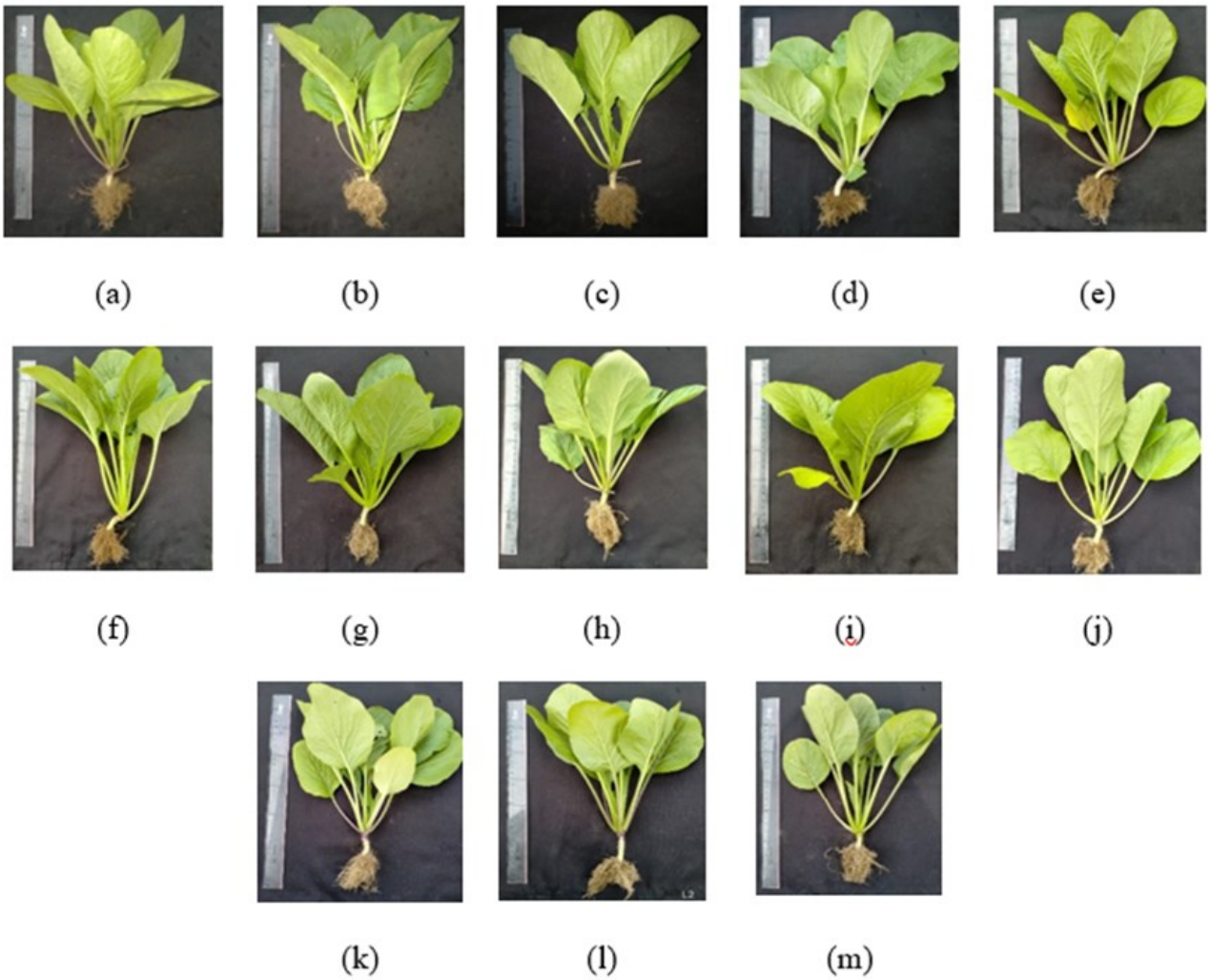

Figure 1. The phenotypes of mustard plants on treatments: (a) control; (b) NPK fertilizer; (c) DoctoRS; (d) 0.05% OLF A; (e) 0.1% OLF A; (f) 0.15% OLF A; (g) 0.20% OLF A; (h) 0.25% OLF A; (i) 0.05% OLF B; (j) 0.1% OLF B; (k) 0.15% OLF B; (l) 0.20% OLF B; (m) 0.25% OLF B.

growth of roots and leaves causes optimal absorption of nutrients, water, and light for a more optimal photosynthesis process. It resulting in high assimilate, thus showing fast growth. In addition, the higher plant height and leaf area, the higher the fresh weight of the plant (Prasetya 2009).

Figure 2 showed that the highest chlorophyll content of mustard plant to the lowest in a row, namely plants with a treatment of NPK fertilizer >0.25% LOF A >0.15% LOF A > DoctoRS LOF >control >0.05% LOF A >0.15% LOF B >0.05% LOF A >0.20% LOF A > 0.1% LOF B > 0.20% LOF B > 0.1% LOF A >0.25% LOF B. There were four treatments which had greater chlorophyll content (NPK, 0.15% LOF A, 0.25% LOF A, and DoctoRS LOF) than control. This is in line with the morphological data obtained, showing that these treatments had significant effect on the plant growth. The treatment of NPK fertilizer has the highest chlorophyll content of 18.46 mg/L. Furthermore, fertilizer treatment is thought to have an influence on the content of chlorophyll as a bio booster or growing regulatory substance or phytohormones. Other factors, that may affect the chlorophyll content are availability of nutrients in the media (Manurung et al. 2020). Photosynthesis occurs in chloroplasts where there is a chlorophyll pigment, especially in the thylakoid section. Photosynthesis occurs in 2 stages, namely light reactions and dark reactions (Urry et al. 2017). The process of photosynthesis can be influenced by several factors, both internal factors, such as chlorophyll

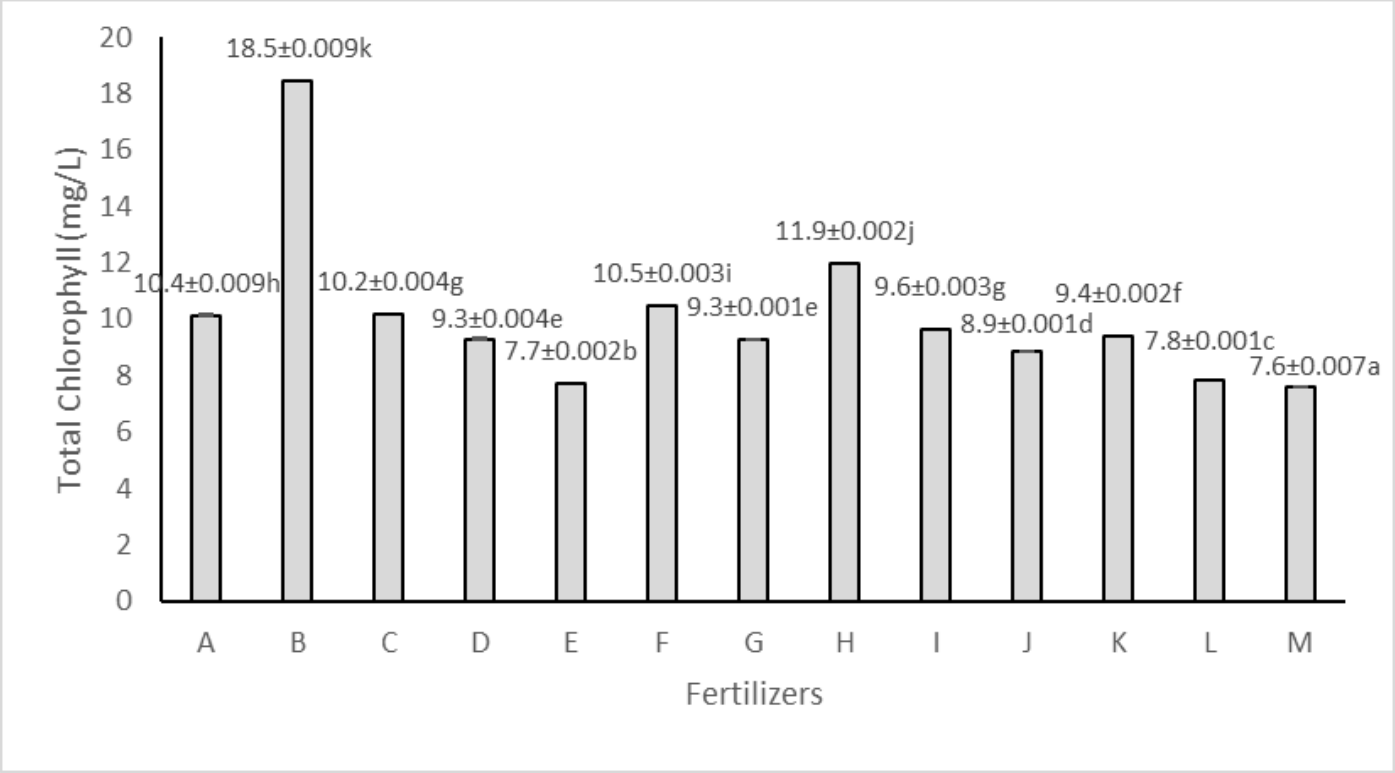

Figure 2. Chlorophyll content of mustard plant with treatment (a) control; (B) NPK fertilizer; (C) DoctoRS LOF; (D) (D) 0.05% LOF A; (E) 0.1% LOF A; (F) 0.15% LOF A; (G) 0.20% LOF A; (H) 0.25% LOF A; (I) 0.05% LOF B; (J) 0.1% LOF B; (K) 0.15% LOF B; (L) 0.20% LOF B; (M) 0.25% LOF B. (Numbers (mean ± SE) followed by different letters mean significant different at α:0.05).

contents and number of stomata, and external factors, such as temperature (Ferdous et al. 2017). In addition, the nitrogen is one of the main components of chlorophyll, which is about 60%. Nitrogen is one of the components in protein molecules, purines, pyrimidines, and porphyrins. Porphyrins are important in the formation of chlorophyll. Nitrogen is catalysed by the enzyme glutamine synthetase into glutamic acid which functions as a porphyrin ring precursor for the formation of chlorophyll (Fadilah et al. 2020).

The results of the study proved that the application of a combination of organic and inorganic fertilizers can improve physical, chemical, and biological properties. Madusari et al. (2021) revealed that the administration of LOF fermented maggot showed a positive increase in the growth of oil palm seedlings, especially in plant height and root length. But, it did not show significant differences on the morphological and physiological of oil palm plants. Although the application of LOF has potential to increase crop production, the application of inorganic fertilizers (N, P, and K) sometimes still needed (Wasito & Tedjasarwana 2003; Hanifah et al. 2021) to support the growth and production. These results conclusively showing that in order to have high crop productivity we should have balanced fertilizers application.

AUTHORS CONTRIBUTION

L.N.J. design the research and supervised the processes, A.A.N.A analysed the research data, A.N.H collected the data samples, V.A wrote the initial manuscript. S.S. manage the research funding, design and supervise the experiment analysis. SS and B.S.D. critically reviewed, revised, and proofread the final manuscript.

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CONFLICT OF INTEREST

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

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Short Communications

Diversity of Butterflies in Ledokombo Hillocks Jember, East Java, Indonesia

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ABSTRACT
Ledokombo hillocks are small hills located in Jember, East Java that have natural resources and face habitat alteration such as plantation and mining. However, a study of the diversity of butterflies has not been carried out in this area. We analysed the diversity of butterflies using the Shannon-Wiener diversity index (H') and Pielou evenness index (E). We identified 514 individuals from 34 species and demonstrated a moderate diversity of butterflies (H'= 1.907) in this area. Our study results could be used for sustainable ecological management of plantations in Ledokombo Hillocks, Jember, East Java.

Butterflies (Lepidoptera) play an essential role as pollinators, maintaining ecosystem balance, enriching biodiversity, and as bioindicators (Pe'er & Settele 2008; Septiana et al. 2019). Land changes in ecosystems and habitats can affect the diversity of butterflies (Blair & Launer 1997; Azahra et al. 2016; Nkongolo & Bapeamoni 2018; Putri et al. 2021). Moreover, butterflies can respond to environmental changes including vegetation changes, poor air, light conditions, climate change, also geographic coordinates (Vickery 2008; Wagner et al. 2013; Sulistyani et al. 2014; Diana et al. 2015; Nkongolo & Bapeamoni 2018; Rizki et al. 2022). Several studies showed that the diversity of butterflies decreases along with increasing environmental changes (Kyerematen et al. 2014; Azahra et al. 2016; Panjaitan et al. 2020), however other studies report otherwise (Hamer et al. 1997; Putri et al. 2021), or even had no significant effect (Wagner et al. 2013).

Hillocks or *Gumuk* is the small hill type with an elevation of fewer than 60 meters. These hillocks are a remnant of erosion from a devastating avalanche, and some of it is most likely due to the lava flooding of Mount Raung in East Java (Van Bemmelen 1949). Ledokombo hillocks are located in Ledokombo District, Jember East Java, and have many local potentials, including natural resources. Ledokombo hillocks have the potential to be a place of groundwater reserves that are beneficial to the surrounding environment (Priyantari et al. 2017; Prasetyo et al. 2021).

Nowadays, the Ledokombo hillocks are facing anthropological degradation such as rock and sand mining and habitat alteration becoming monoculture plantations. According to a survey study, several plants such as Asteraceae, Bombacaceae, Fabaceae, Melastomataceae, Euphorbiaceae, and Leguminosae were recorded in this location. These plants are known as host plants and nectar plants of butterflies (Rusman et al. 2016). However, the diversity of butterflies in this location has not been studied yet. Fauna research conducted in Ledokombo District is about bird diversity (Maisyaroh et al. 2021). Considering the rarity of faunal studies in the area, we aim to analyze the diversity of butterflies in Ledokombo hillocks, Jember, East Java. This study provides primary data for the sustainable management of Ledokombo Hillocks, Jember, East Java.

This study was conducted in six hillocks in Ledokombo district Jember East Java from January to February 2022. Moreover, six hillocks have the same characteristic of habitat type and different altitudes (Figure 1, Table 1). The habitat type was a mixed garden dominated by *Sengon* (*Albizia chinensis*) plantation. Most of the hillocks in the Ledokombo district have been converted into plantations. Therefore, we chose this habitat type as the first step for diversity research, especially for butterflies. The coordinates and altitude of each sampling location were measured using the Global Positioning System (GPS).

Sample collection was conducted using the scan sampling method (Rusman et al. 2016). The number of species and individuals were sur-

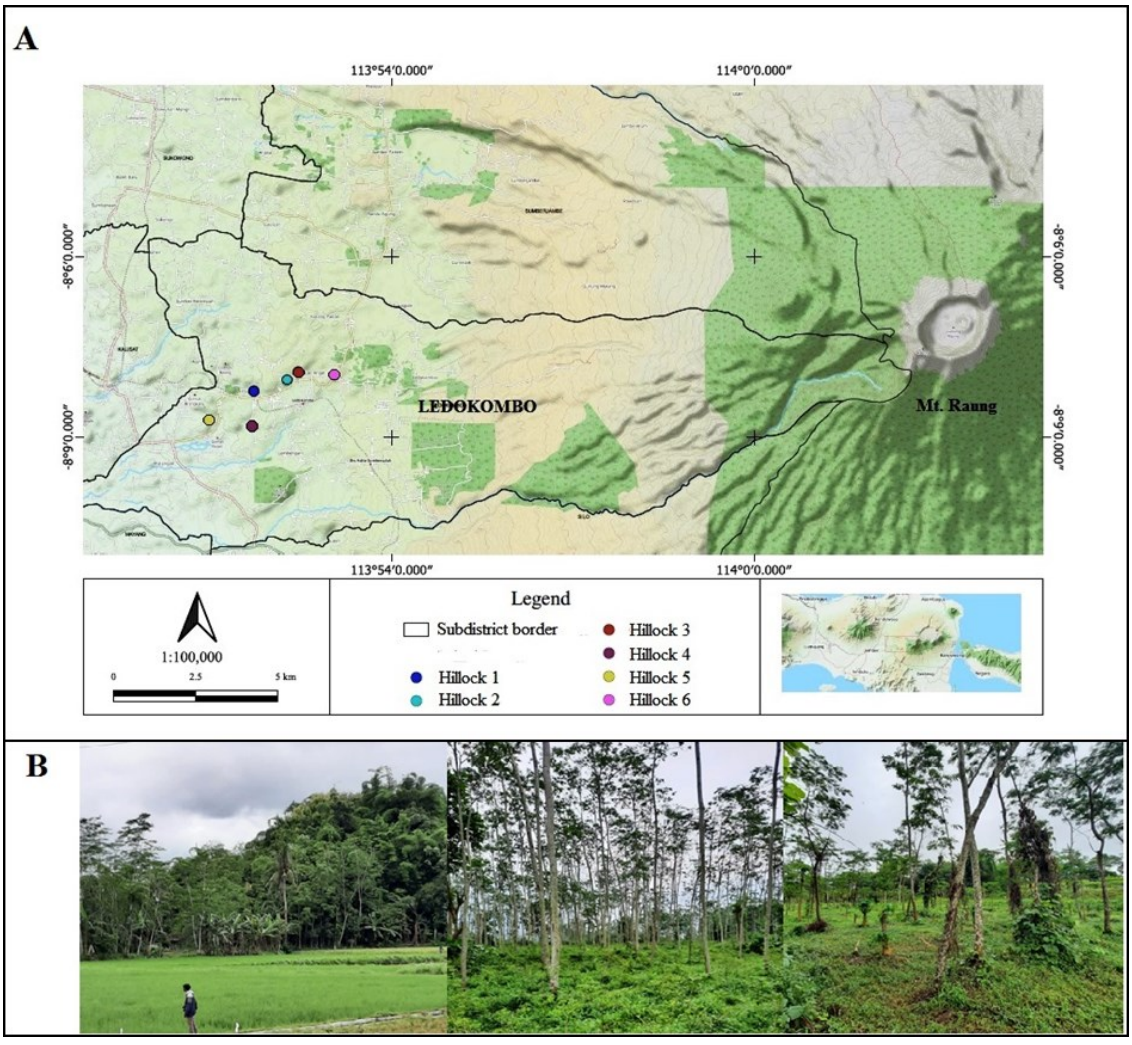

Figure 1. Location of this study. **A**. Sampling location, **B**. Habitat type of Ledokombo hillocks, dominated with *Albizia chinensis* plantation.

Table 1. Details of sampling location in this study.

Hillock-	Coordinate	Altitude (m)	Habitat Type
Hillock-1	-8°8'14"S 113°51'43"E	377	The mixed garden that dominated with *Sengon* (*Albizia chinensis*) plantation and herbs, near rice fields and an urban area
Hillock-2	-8°8'3"S113°52'16"E	430	Mixed garden, *Sengon* (*Albizia chinensis*) plantation and herbs, near rice fields and an urban area
Hillock-3	-8°7'55"S 113°52'27"E	408	Mixed garden, *Sengon* (*Albizia chinensis*) plantation and herbs, near rice fields and an urban area
Hillock-4	-8°8'49"S 113°51'41"E	337	Mixed garden, *Sengon* (*Albizia chinensis*) plantation and herbs, near rice fields and an urban area
Hillock-5	-8°8'43"S 113°50'59"E	294	Mixed garden, *Sengon* (*Albizia chinensis*) plantation and herbs, near rice fields and an urban area
Hillock-6	-8°7'58"S 113°53'3"E	417	Mixed garden, *Sengon* (*Albizia chinensis*) plantation and herbs, near rice fields and an urban area

veyed along the survey track for 45 minutes per hour from morning (08:00-11.00 am) to afternoon (01.00-05:00 pm) at an interval of five days per location. The butterfly samples were captured using a sweep net and kept in a triangular paper envelope. Afterward, specimens collected were transported to Laboratorium Terpadu, Universitas Islam Negeri Kiai Haji Achmad Siddiq Jember for identification. Specimen identification was conducted based on Aoki et al. (1982), Peggie and Amir (2006), Peggie (2011), Panjaitan et al. (2021), also expert validation. We also recorded biotic factors in this study (nectar plant species visited by butterflies) and abiotic factors such as air temperature, air humidity, and light intensity. Air temperature and humidity were measured using a thermo-hygrometer, whereas light intensity was measured using a Lux meter.

The number of individuals and species was tabulated. Diversity analysis such as the Shannon-Wiener diversity index (H') and Pielou evenness index (E) analysis and pairwise comparison based on Whittaker's beta diversity analysis among habitats was also carried out using the PAST-Paleontological statistics software ver. 4.09 (Hammer et al. 2001).

A total of 514 individuals from 34 species within four families were collected in this study, presented in Table 2. The highest number of species observed came from the family Nymphalidae (19 species). The butterfly species that was mostly found in Ledokombo hillocks were *Eurema* spp. (Pieridae), *Leptosia nina* (Pieridae), *Papilio memnon* (Papilionidae), *Hypolimnas bolina* (Nymphalidae), and *Mycalesis mineus* (Nymphalidae) (Table 2). Previous studies demonstrated that Nymphalidae and Pieridae were the most commonly found butterflies in different habitat types (Leo et al. 2016; Koneri et al. 2019; Winarni et al. 2020; Sukma et al. 2021). Moreover, these families were considered the effective bioindicator of environmental health in Bali Barat National Park (Winarni et al. 2020). Thus, the presence and abundance of Nymphalidae and Pieridae in Ledokombo hillocks may also indicate the environmental conditions of the hillocks.

Hillock-3 showed the highest number of individuals and species of butterflies compared to other hillocks. Furthermore, Hillock-6 showed the highest diversity of butterflies (H'= 2.401), and the lowest was Hillock-5 (H'= 1.323). Meanwhile, the highest evenness value was Hillock-4 (E= 0.905), and the lowest was Hillock-5 (E= 0.376) (Table 2). In general, the butterfly diversity in Ledokombo hillocks was medium level (H'= 1-3) and low evenness (0-1) according to Odum and Barrett (1971)

Table 2. List of butterfly species and diversity index in six Ledokombo hillocks, Jember, East Java.

Family and species	Number of individuals in hillock-						Total
	1	2	3	4	5	6	
Lycaenidae							
Curetis thetis			2				2
Jamides celeno						1	1
Lampides boeticus						1	1
Nymphalidae							
Amathusia phidippus	10						10
Doleschallia bisaltide					1		1
Elymnias hypermnestra	2		2		2	2	8
Euploea core	3		1				4
Hypolimnas bolina	5	3	14	1		2	25
Hypolimnas misippus	3		4				7
Ideopsis juventa			1			2	3
Junonia almana			1				1
Junonia atlites					1		1
Junonia hedonia						1	1
Junonia iphita			1			1	2
Junonia villida			4				4
Melanitis leda			1				1
Mycalesis horsfieldi			3				3
Mycalesis mineus	9	3	5	1	2	2	22
Neptis hylas	4	6	3			4	17
Orsotriaena medus			1				1
Pantoporia hardonia					1		1
Tanaecia palguna						2	2
Papilionidae							
Graphium doson			1				1
Graphium sarpedon	3		1			1	5
Papilio demolion	1						1
Papilio memnon	1	1	28		1	2	33
Papilio polytes			9				9
Pieridae							
Appias libythea			3		1	3	7
Catopsilia pomona	4	2	4				10
Eurema blanda	23	12	40	6	36	13	130
Eurema hecabe	28	10	80	5	28	14	165
Leptosia nina	6		11	5	1	12	35
Number of individuals	102	37	220	18	74	63	514
Number of species	14	7	23	5	10	16	34
Shannon diversity index (H')	2.244	1.758	2.208	1.510	1.323	2.401	1.907
Pielou's evenness (E)	0.674	0.828	0.396	0.905	0.376	0.690	0.645

criterion. The high diversity of species within a community would be followed by a high number of species found with an even abundance of species and vice versa (Sukma et al. 2021). However, the diversity of butterflies in Ledokombo hillocks was not correlated with the evenness value (H'= 1.907, E= 0.645). It occurred due to a higher number of several species, such as *Eurema hecabe* and *Eurema blanda* in Hillock-3, although the dominance level was moderate (0.50-0.75) according to Odum and Barrett's (1971) criterion. Furthermore, Koneri et al. (2019) showed that the member of the genus *Eurema* was the highest abundance of butterfly species in three different habitat types.

The pairwise comparison of Whittaker's beta diversity analysis

among habitats was carried out to see the variation in community composition between habitats. The results demonstrated that the highest Whittaker's beta diversity value was between hillock-3 and hillock-4 (0.643) (Table 3). This result is in line with the survey that hillock-3 had the highest number of individuals and butterfly species, while hillock-4 had the lowest.

The different number of butterfly species found in Ledokombo hillocks can be due to several factors. Differences in location, period, season, altitude, habitat type, the complexity of the vegetation structure, and environmental factors such as air humidity, air temperature, and light intensity impact the difference number of butterfly species in sampling locations (Koneri et al. 2019). Habitat type (mixed garden, plantation), period, season (rain season), and abiotic factors in this study were relatively the same. Based on the measurement of abiotic factors, the average value of air temperature in Ledokombo hillocks was 29.09 °C, and the average air humidity and light intensity were 78.58% and 5737.60 Lux, respectively. Therefore, altitude and the complexity of the vegetation structure (host and nectar plants) are suspected factors that impact the different number of butterfly species in this study. However, further analysis is needed.

The number of host plants and nectar plants strongly affects the presence and diversity of butterfly species. Moreover, it is also affected by water and minerals sources (Koneri et al. 2019). Higher plant diversity corresponds with higher butterfly diversity in the ecosystems (Panjaitan et al. 2020). Butterflies in Ledokombo hillocks were observed to visit flowering plants as their food resources, including family Asteraceae (*Tridax procumbens*, *Ageratum conyzoides*, and *Chromolaena odorata*), Verbenaceae (*Lantana camara* and *Stachytarpheta jamaicensis*), Compositae (*Emilia sonchifolia* and *Zinnia peruviana*), and also Commelinaceae (*Commelina erecta*). The presence of these plants was relatively abundant in this area (Figure 2). A previous study demonstrated that *Chromolaena odorata* and *Lantana camara* were common nectar plants that were most visited by butterflies, especially Pieridae and Nymphalidae families (Sukma et al. 2021). Ledokombo hillocks also contain essential materials, including sand (Maisyaroh et al. 2021). This sand may cause the hillock to become muddy after rain and it becomes a source of minerals for butterfly puddling.

The availability of nectar plants and mineral sources in this area was suspected as factors that may affect the diversity level of butterflies, considering the habitat type of this location was monoculture plantations. The monocultural plantation habitats such as oil palm and rubber plantations were reported to have low diversity of butterflies and were not supported the existence of butterflies as well as forest habitats (Rusman et al. 2016; Panjaitan et al. 2020). However, several studies on butterfly diversity in the Jember district showed only 13 species that can be found in forest areas in Meru Betiri National Park (Mustikawati 2016). Meanwhile, a total of 23 species from four families were observed

Table 3. Pairwise comparison based on Whittaker's beta diversity of six Ledokombo hillocks.

	Hillock 1	Hillock 2	Hillock 3	Hillock 4	Hillock 5	Hillock 6
Hillock 1	0	0.333	0.351	0.474	0.500	0.400
Hillock 2	0.333	0	0.533	0.333	0.529	0.478
Hillock 3	0.351	0.533	0	0.643	0.576	0.385
Hillock 4	0.474	0.333	0.643	0	0.467	0.524
Hillock 5	0.500	0.529	0.576	0.467	0	0.462
Hillock 6	0.400	0.478	0.385	0.524	0.462	0

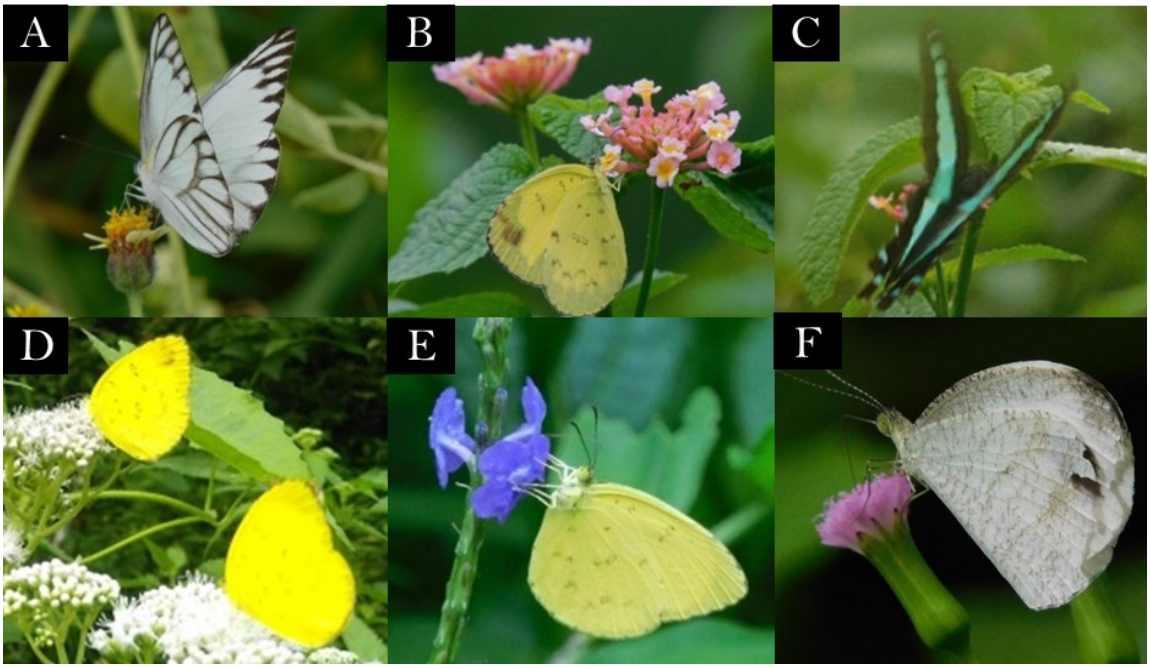

Figure 2. Butterflies in Ledokombo hillocks visited their flowering plants. *Appias libythea* (Pieridae) nectary feeding on *Tridax procumbens* (A); *Eurema* spp. (Pieridae) on *Lantana camara* (B), *Chromolaena odorata* (D), and *Stachytarpheta jamaicensis* (E); *Graphium sarpedon* (Papilionidae) on *Lantana camara* (C); *Leptosia nina* (Pieridae) on *Emilia sonchifolia* (F).

in the rehabilitation zone of Meru Betiri National Park (Setiawan et al. 2018). Furthermore, only 11 species from three butterfly families were found in the Savanna area of Meru Betiri National Park (Setiawan et al. 2019). According to those studies, Ledokombo hillocks have more butterfly species compared to other regions or habitats in the Jember district.

In conclusion, Ledokombo hillocks, with the characteristic habitat of monoculture plantations, have a moderate diversity of butterflies (H'= 1.907). Moreover, the butterfly family commonly found in Ledokombo hillocks was Nymphalidae, whereas the species were *E. hecabe* and *E. blanda*. The existence and abundance of flowering plants as food resources support the presence and diversity of butterflies in Ledokombo hillocks. In addition, it is necessary to record data on host plants in this area and data from different habitat types (such as habitats with sand and rock mining) for further research. Therefore, sustainable plantation management is needed.

AUTHORS CONTRIBUTION

All authors equally contributed to the manuscript writing. H.M., B.S., and W.M. designed, and supervised the study. H.M., A.E.D.C., B.S., and W.M. collected and analyzed the data. E.Y and A.Q. validated and analyzed the data and also wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Research Article

Attacus atlas (L.) sericin extract as an effective UV Protectant of *Bacillus thuringiensis* serotype *kurstaki* for controlling *Spodoptera litura* (Fab.)

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ABSTRACT

Bacillus thuringiensis serotype *kurstaki* is an entomopathogenic bacteria commonly used to control the cutworm *Spodoptera litura* (Fab.). However, *B. thuringiensis* has disadvantage of being easily degraded due to sunlight. The objective of this research was to determine the effectiveness of adding *A. atlas* (L.) cocoon extract as UV protectant *B. thuringiensis* to the mortality of *S. litura*. This research formulated 2.5% of the original substance of *A. atlas* cocoon extract and *B. thuringiensis* serotype *kurstaki* strain HD-7 applied from commercial product DiPel-WP®. The formulation was exposed to sunlight for 0, 1, 2, and 3 weeks. The suspension treated for 20 individuals of first instar larvae *S. litura* shifted into the artificial diet using 3-5 replicates. The scanning electron microscope (SEM) method began from a sample that was vacuumed, sample coated, and observed on SEM with the electron in a certain level probe. This research showed that the mortality of *S. litura* decreased with the growth of *S. litura*. The mortality of *S. litura* achieved 20-100% mortality after treatments. The *A. atlas* cocoon extract was effective as UV protectant *B. thuringiensis* for three weeks of exposure to sunlight. The SEM analysis represented that formulation of *B. thuringiensis* and *A. atlas* cocoon extract with sunlight exposure for one week has more rough surface than that of exposed during three weeks.

INTRODUCTION

Spodoptera litura (Fab.), known as armyworm is considered one of the major pests of crops. It attacks 27 species of crops from 24 genera, 14 families divided into several ranges of plants, comprising vegetables, weeds, fruits, and ornamental plants (Ahmad et al. 2013). For example, *S. litura* threatens the production of soybeans up to 80%, thus affecting cultivation. It may also attack tobacco and cause 57% loss of crop yields during the dry season (BPTD 2011). It considers a polyphagous insect, thus, they able to adapt to an unstable environment (Kennedy & Storer 2000).

The chemical pesticide commonly used to control *S. litura* led to insect resistance (Vengateswari et al. 2020) and toxic residues in the environment and crop products. For example, pyrethroid and organophosphate applied in insecticide treatments for *S. litura* reported the most sig-

nificant resistance (Ahmad et al. 2013). To overcome these problems, one of the biological agents commonly used for *S. litura* control is *B. thuringiensis* (Lacey et al. 2001) . *B. thuringiensis* has crystal protein that can be toxic for many larvae of insects (Baum et al. 1999), called as cry toxins (Glare et al. 2017). It can be used as a pest management component (Obeidat et al. 2004; Glare et al. 2017). It is known that *B. thuringiensis* is safe for non-target organisms. But, it deteriorated rapidly in an environment (Khetan 2001) due to ultraviolet exposure from sunlight (Cohen et al. 1991).

Attacus atlas cocoon extract has sericin and fibroin potential as UV protectants for *B. thuringiensis* (Roy et al. 2012). Sukirno et al. (2021) found that 2.5% of *A. atlas* cocoon extract effectively protects *B. thuringiensis* for up to 4 weeks under UV B treatment. The UV spectrophotometry analysis showed that *A. atlas* cocoon extract was able to absorb UV-C (200-280 nm), UV-B (280-320 nm), and UV-A (320-400 nm). Therefore, this research was conducted to study the effectiveness of sericin from *A. atlas* cocoon extract as UV protection for *B. thuringiensis* exposed to sunlight based on the mortality of *S. litura*. The formulation of sericin and *B. thuringiensis* was observed by SEM (Scanning Electron Microscope).

MATERIALS AND METHODS

Materials

Attacus atlas cocoon was collected from rearing at Entomology Laboratory and fed on baringtonia leave (*Baringtonia asiatica* Kurzt.). A total of 1,600 individuals' larvae first instar of *S. litura* were treated by *B. thuringiensis*. TRO (Turkish Red Oil) for making extract solution. *B. thuringiensis* DiPel-WP® serotype *kurstaki* strain HD-7. Pure honey (Madu Nusantara®, PT. Madu Murni Nusantara, IN) for adult feeding and the ingredient of artificial diet used in this study is presented in Table 1.

Table 1. The compositions of an artificial diet for larvae of *S. litura*

Ingredients	Total amount
White beans (g)	250
dH_2O (ml)	1,200
Agar powder (g)	50
Benzoic acid (g)	10
Yeast (g)	80
Ascorbic acid (g)	20

Methods

This research was conducted at Entomology Laboratory, Faculty of Biology Universitas Gadjah Mada from October 2021-February 2022.

Collecting and Rearing of the Insects

Spodoptera litura was collected from cabbage, onion, and cauliflower at Sengi, Dukun, Magelang Central Java (7°31'41.8"S 110°21'06.4"E). The rearing was conducted at the Entomology laboratory with temperatures of about 27 + 33°C and 70 + 75% relative humidity (r.h.). An artificial diet for *S. litura* was made based on Sukirno et al. (2021). The composition of artificial diet for larvae of *S. litura* is shown in Table 1. Firstly, larvae of *S. litura* were placed in a plastic cup (70 ml) containing 15 ml of artificial diet until pupae. Pupae were collected daily and kept until emerging in a glass jar. Ten percent of honey solution was used for moth feeding. The folded opaque paper was put in the middle of a jar for oviposition.

Extraction of *A. atlas* Cocoon

A. atlas extraction was using the alkaline lysis method. TRO (Turkish Red Oil) was used to make 5% sericin solution. As much as 15 g of *A. atlas* cocoon and 2 g TRO was added with up to 300 ml dH_2O and heated for 60 minutes at 100°C. The 2.5% solution of *A. atlas* cocoon made using 75 ml was taken from the stock solution, and dH_2O was added to 150 ml. The suspension was kept at 4°C until further used.

Preparation of *B. thuringiensis* suspension

Bacillus thuringiensis commercial formulation DiPel-WP® serotype *kurstaki* strain HD-7 (Abbot Co., IN) mixed with the cocoon extract solution (1 g/10 ml of dH_2O). Then, it homogenized using a vortex for several minutes.

SEM (Scanning Electron Microscope) analysis of *B. thuringiensis* formulation

The first procedure took the suspension using a micropipette on carbon tape in the specimen holder and put it into a castable vacuum until it dried. The second step is Au coated procedure. Then, the sample was put in an auto coater and waited for vacuum coater until the pressure was on +3.2 Pa, then it started to coat for +120 seconds. Thirdly, observation of sample used the SEM method. The sample from the suspension then put in SEM and vacuumed for +-60 seconds, the sample electron shot by certainly probe level. The last step observed the topography of the sample surface.

The effect of sunlight on the pathogenicity of *B. thuringiensis* against *S. litura*

The bioassay was carried out using four treatments with three to five replicates, and each replication used twenty larvae. In Table 2, the treatments were applied 1 ml formulation of *B. thuringiensis* and *A. atlas* cocoon extract into a disposable petri dish, the cover of the petri dish was wrapped tightly using parafilm, and exposed to sunlight. After exposure, the suspension was diluted with 10 ml autoclaved dH_2O. The diluted suspension (1 ml) was taken to an artificial diet and left air dried for 2 hours under lab conditions, then it was tested against 20 individuals' first instar larvae of *S. litura*.

Table 2. Treatments of the Bt. Formulations under sunlight exposures

Code	Category	Treatments
P1	Exposure	*B. thuringiensis* added with cocoon extract
P2	Exposure	*B. thuringiensis* without cocoon extract
P3	No Exposure	*B. thuringiensis* added with cocoon extract
P4	No Exposure	*B. thuringiensis* without cocoon extract

Notes; (-): no cocoon extract; (+): add cocoon extract

Experimental design and Statistical analysis

Mortality of *S. litura* was recorded on the first, third, and seventh days after treatment (DAT). The percentage of increased mortality of *S. litura* on the third day was analyzed using analysis of variance (ANOVA) at α: 0.05 and post hoc analysis using Tukey HSD. All the statistical procedures were using IBM SPSS 23. Analysis *B. thuringiensis* used SEM was analyzed descriptively.

RESULTS AND DISCUSSION

The effect of sunlight on the pathogenicity of *B. thuringiensis* against *S. litura*

This research was used to treat the first instar larvae of *S. litura*. as they were more sensitive than other instars. Blouch et al. (2020) and Hallad et al. (2011) reported that the increasing of *S. litura* age in larvae resulted in the decreasing of the larval mortality. The larvae growth was followed by physiological resistance to the toxin, thus declining the ability of the toxins to bind in the midgut epithelium.

The mortality of *S. litura* after treated with *Bt.* is shown in Figure 1. Exposure to W-1 to W-3 increased the mortality of *S. litura* from the first day until the seventh day. Moreover, exposure to W-0 percentage of mortality increased from the first day until the third day. On seventh-day mortality, it was not increased due to the mortality rate approaching 100%. *B. thuringiensis* toxins need few hours to dissolve and to interact with the epithelium of midgut (Song et al. 2016). It required 8 hours to activate the toxin at midgut and caused cell apoptosis (e Castro et al. 2019).

Table 3 present the R^2 and the regression equations. Statistical analysis showed that there was no significant correlation between variable of sunlight exposure periods and mortality on the first day of mortality $F_{2,57} = 0.97$; $P>0.005$. On the third day of observation, the mortality was a statistically significant difference between variable exposure and mortality $F_{2,57} = 45.14$, $P<0.005$, and on the seventh day of mortality, there was a statistically significant relationship between variable exposure and mortality $F_{2,57} = 19.51$, $P<0.005$. The highest value R^2 value is

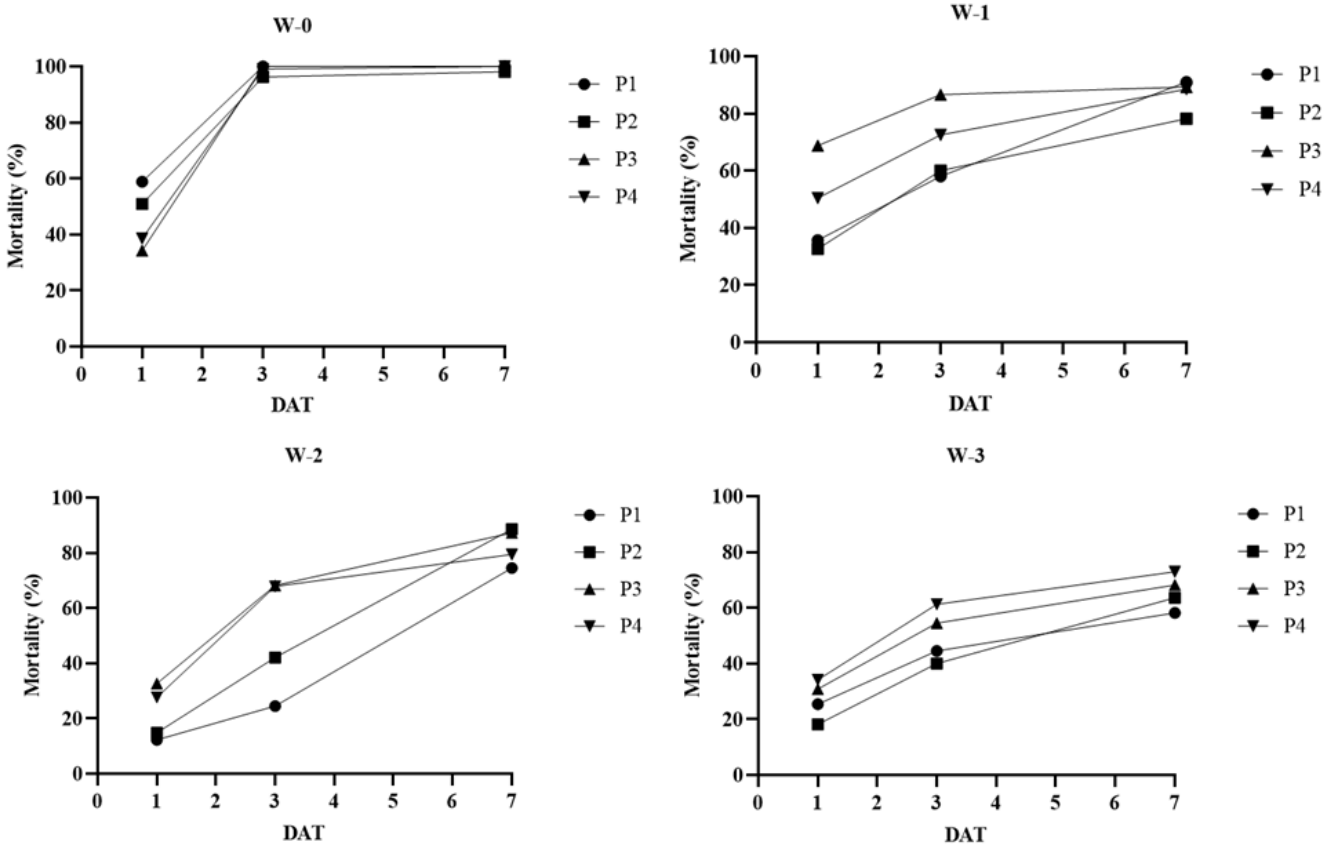

Figure 1. The effect of sunlight on the pathogenicity of *B. thuringiensis* against *S. litura*.
Note: DAT (day after treatment) W-0 (0 week); W-1(one week); W-2 (two weeks), and W-3 (three weeks). Mortality was recorded on the first, third, and seventh days after being treated with P1 (exposed and added cocoon extract); P2 (exposed and without cocoon extract); P3 (no exposed and added cocoon extract); P4 (no exposed and without cocoon extract).

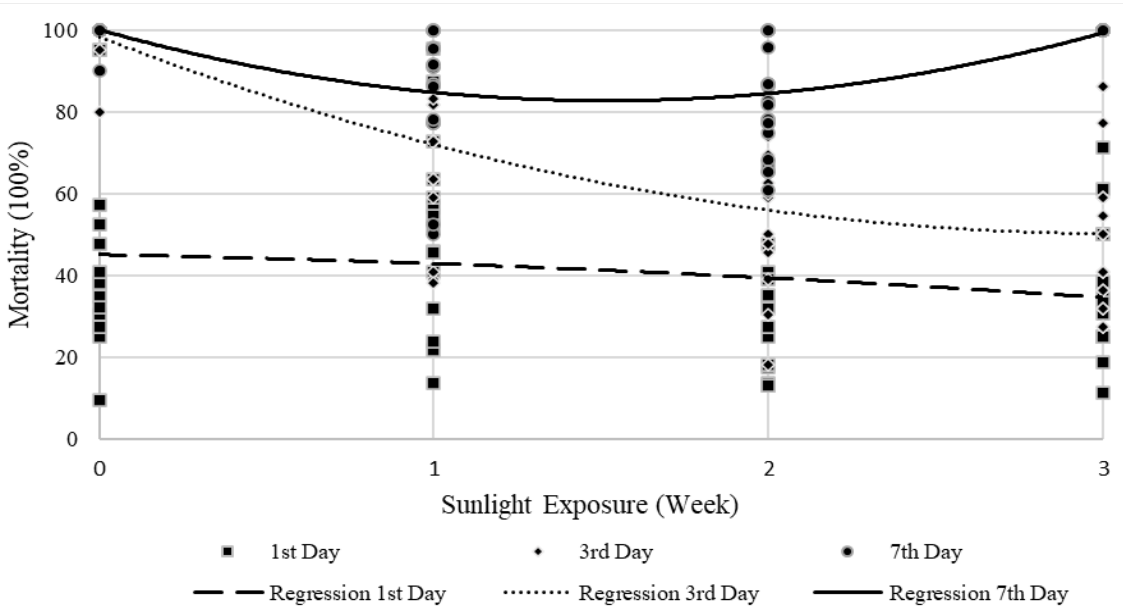

Figure 2. The interaction of *S. litura* mortality and sunlight exposure (week) at first, second, and third day after treatment of *B. thuringiensis*.

Table 3. Regression equation on different days of *S. litura* mortality after treated with *B. thuringiensis* at different mixtures.

Day of Mortality	R^2	Equation Regression (Quadratic Model)
1st Day	0.03	$y = -0.6611x^2 - 1.524x + 45.042$
3rd Day	0.58	$y = 5.1582x^2 - 31.52x + 98.357$
7th Day	0.39	$y = 7.5525x^2 - 22.908x + 100.13$

Note: y= mortality of *S. litura*; x = times of exposure (weeks).

0.58 on the third day. This finding suggested that the interaction pattern of sunlight exposure was as a quadratic model.

Cocoon extract of 2.5% *A. atlas* protected *B. thuringiensis* up to three weeks of sunlight exposure. The decreased of mortality was followed by the increased exposure time on the first, third, and seventh days after treatments showing quadratic model (Figure 2). The previous research reported that 2.5% of *A. atlas* cocoon extract protected *B. thuringiensis* up to the fourth week of exposure UV B (Sukirno et al. 2021). The cocoon of *A. atlas* containing sericin and protein fibroin (Fabiani et al. 1996) protected *B. thuringiensis* from sunlight. The mortality was decreased due to destruction of tryptophan and loss of biological activity (Cohen et al. 1991).

The comparison of *S. litura* mortality at the first day after treated with *B. thuringiensis* is showed in Table 4. The *S. litura* in *B. thuringiensis* formulations which were exposed to sunlight for two and three weeks, was no significant difference. However, at week-0 and week-1, the protection of *A. atlas* cocoon extract treatments and mortality showed significant difference in exposed *Bt.* with extract and unexposed *Bt.* with extract.

On the third day of mortality, the treatment of cocoon extract of *A. atlas* was not a significant difference. Nevertheless, the average mortality declined with the increasing time of exposure. The pathogenicity of *B. thuringiensis* against *S. litura* reached 100% at zero week, whereas, the lowest mortality was 32% at the third week of exposure. The *S. litura* mortality in the treatment of one week sunlight exposure was significant difference in exposed *Bt.* with extract, which was recorded on the 7th day.

Table 4. The average mortality (%) of first instar larvae of *S. litura* (mean ± SE) treated *B. thuringiensis* formulated of 2.5% *A. atlas* cocoon extract at different exposure under sunlight.

Treatments	Sunlight Exposure (Week)			
	0	1	2	3
Mortality of *S. litura* on the first day (%)				
Bt + extract + exposure	79.37 ± 15.87 Ab	27.03 ± 5.94 Aa	20.49 ± 7.15 Aa	23.94 ± 7.12 Aa
Bt - extract + exposure	41.24 ±7.95 Ba	51.02 ± 14.39 Aab	21.77 ± 4.41 Aa	51.59 ± 11.03 Aa
Bt + extract - exposure	34.33 ± 2.93 Ba	68.41 ± 9.75 Bb	38.89 ± 3.52 Aa	44.91 ± 6.13 Aab
Bt - extract - exposure	25.89 ± 5.67 Ba	50.51 ± 3.52 Aba	35.34 ± 11.59 Aa	24.84 ± 3.47 Aa
Mortality of *S. litura* on the third day (%)				
Bt + extract + exposure	100.00±0.00 Ab	50.69±7.72 Aa	35.05±8.80 Aa	39.39±5.46 ABa
Bt - extract + exposure	96.00±4.00 Ac	68.66±12.67 ABbc	47.10±6.62 ABab	32.95±3.41 Aa
Bt + extract - exposure	100.00±0.00 Ac	86.52±4.59 Bbc	67.97±3.65 Bab	54.55±7.33 ABab
Bt - extract - exposure	99.05±0.95 Ab	72.32±3.92 ABab	68.42±8.63 Bb	68.18±11.13Bb
Mortality of *S. litura* on the seventh day (%)				
Bt + extract + exposure	100.00 ± 0.00 Ab	91.03 ± 4.28 Aa	74.51 ± 6.42 Ab	100.00 ± 0.00 b
Bt - extract + exposure	98.00 ± 2.00 Aa	77.79 ± 10.87 Aa	88.75 ± 4.97 Aa	100.00 ± 0.00 a
Bt + extract - exposure	100.00 ± 0.00 Ab	89.24 ± 3.71 Aa	87.51 ± 3.62 Aa	100.00 ± 0.00 b
Bt - extract - exposure	100.00 ± 0.00 Ab	88.53 ± 4.59 Aab	79.80 ± 5.62 Aa	100.00 ± 0.00 b

Notes: The average mortality and SE within the same column followed by uppercase letters are not significantly different tested at $\alpha < 0.05$. The average mortality and SE within the same row followed by lowercase letters are not significantly different tested at $\alpha < 0.05$.

Furthermore, the mechanisms of action of *B. thuringiensis* were: (1) protoxin was solubilized to be activated in the midgut. Protoxin activated when dissolved in alkaline and highly acid insect midgut; (2) after activated, place toxin bound by receptors (specific protein) in the apical brush border membrane midgut; (3) toxin forms pores and losses cell development; (4) pores within the cell produce air, and other ions entered. The cells swell and eventually lyses. The cells were destroyed, lost growth, and caused death (Khetan 2001).

The SEM (Scanning Electron Microscope) analysis of *B. thuringiensis* formulation

The experiment was using a commercial *B. thuringiensis* DiPel-WP serotype *kurstaki* HD-7. DiPel-WP was commonly made with some ingredients that kept the quality of *B. thuringiensis* under natural conditions, rain, and exposure to sunlight (Ignoffo et al. 1977). Under natural conditions, *B. thuringiensis* commercially survived in 42-56% RH (Teera-Arunsiri et al. 2003). SEM was used to know the different surfaces of treatments. The *B. thuringiensis* which was formulated with the addition of *A. atlas* cocoon extract and *B. thuringiensis* alone were exposed under sunlight, then was observed using SEM with 3 different samples and with various magnifications.

Based on SEM, the surface of three samples is similar, because DiPel-WP was commonly made with some ingredients to be granule. So, that part of *B. thuringiensis* was not clearly visible in SEM. On SEM crystal and spores of unexposed *B. thuringiensis* from DiPel-WP suspension showed a coarse solid (Figure 3A), whereas *B. thuringiensis* alone showed smoother texture than the powder formulated on the surface (Teera-Arunsiri et al. 2003). DiPel suspension added of *A. atlas* cocoon extract with exposure to the sunlight for one week showed more rough surface (Figure 3B1.1) than *B. thuringiensis* alone. After being exposed for three

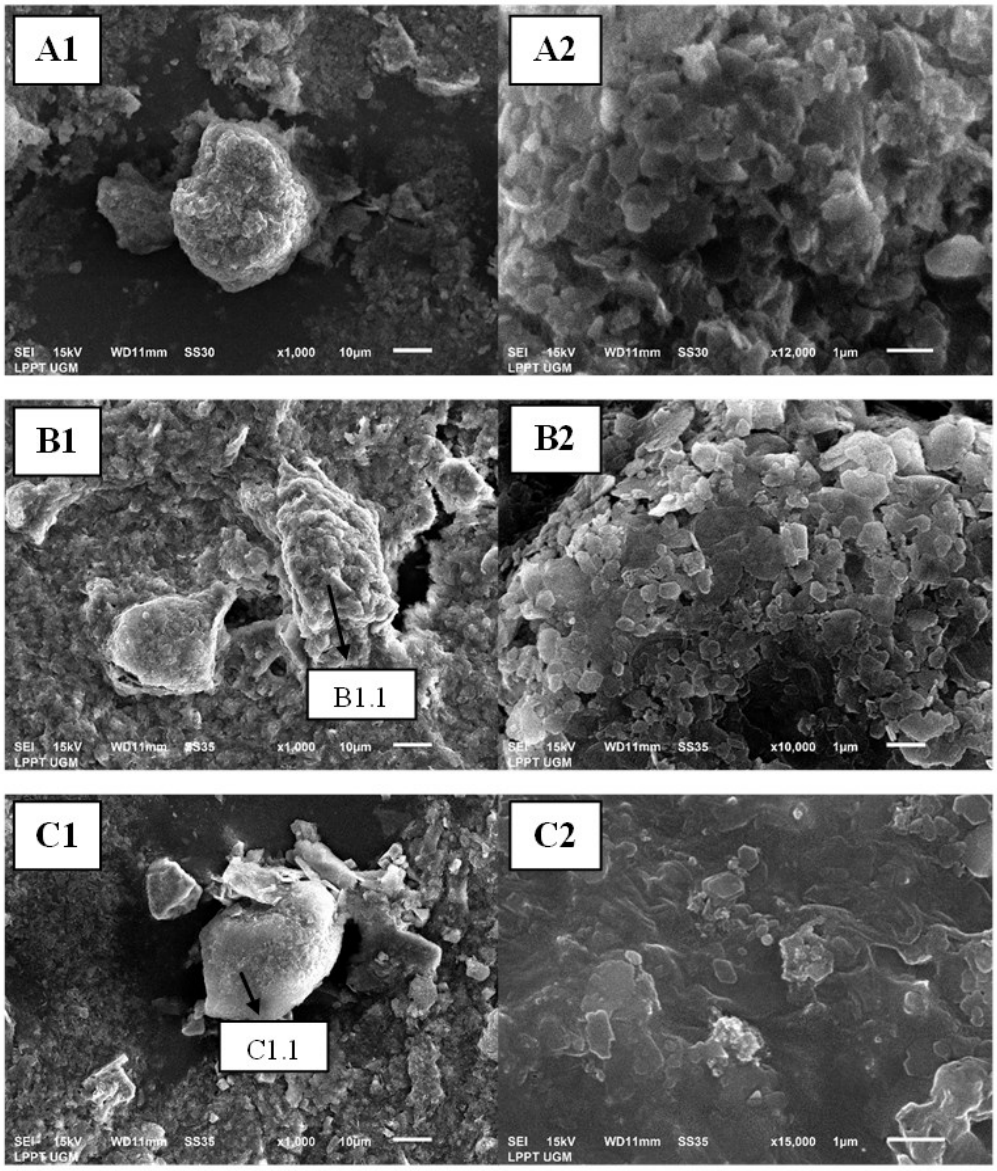

Figure 3. SEM analysis of *B. thuringiensis* formulations.
Note: (A1) Suspension of *B. thuringiensis* from DiPel-WP with 1,000 magnifications; (A2) Suspension of *B. thuringiensis* from DiPel-WP with 12,000 magnifications; (B1) Formulation of *B. thuringiensis* and 2.5% *A. atlas* cocoon extract exposed under the sunlight for one week with 1,000 magnifications; (B2) Formulation of *B. thuringiensis* and 2.5% *A. atlas* cocoon extract exposed under the sunlight for one week with 10,000 magnifications; (C1) Formulation of *B. thuringiensis* and 2.5% *A. atlas* cocoon extract exposed under the sunlight during three weeks with 1,000 magnifications; (C2) Formulation of *B. thuringiensis* and 2.5% *A. atlas* cocoon extract exposed under the sunlight during three weeks with 15,000 magnifications.

weeks, the particles showed a smooth surface (Figure 3C1; C2; C1.1). The addition of 2.5% *A. atlas* cocoon extract aimed to protect *B. thuringiensis* commercial from exposure to sunlight. Increased temperature to 100°C declined the survival of spores *B. thuringiensis* to 3% (Teera-Arunsiri et al. 2003).

CONCLUSION

This study showed that the mortality of *S. litura* decreases, followed by an increased time of exposure to sunlight. The result concluded that 2.5 % of *A. atlas* cocoon extract protects *B. thuringiensis* commercially for up to three weeks of exposure to sunlight. On the other hand, the mortality of *S. litura* was reaching maximum on the seven days after treatments. The suspension of *B. thuringiensis* and *A. atlas* cocoon extract presented differences in the surface at the other exposure times.

AUTHORS CONTRIBUTION

N.S.N. conceived, designed the experiments, wrote the manuscript, reared and collected *S. litura*. R.R. conceived, designed the experiments, reared and collected *S. litura*. S.S. planned, organized, supervised the experiments, made critical revisions, and approved the final version. A.S.P.W., N.S.S.S., H.A., A.A., T.P.S., and H.A. reared and collected *S. litura*.

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CONFLICT OF INTEREST

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

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Research Article

Assessing Indigenous Soil Ureolytic Bacteria as Potential Agents for Soil Stabilization

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ABSTRACT

Microbially induced carbonate precipitation by ureolysis is a biomineralization process that has been adapted by various microorganisms in different natural environments. This widespread natural phenomenon can be employed in numerous civil engineering and soil stabilization applications. In the present study, the potential of indigenous soil urease-producing bacteria as potential agents for soil stabilization method was investigated. Assessment of the eight active urease-producing bacterial species isolated from the farm soil samples has demonstrated that all the isolates were Gram-positive rod-shaped bacteria with promising characteristics such as the formation of endospore which is essential for bacterial survival in harsh conditions within the soil environment. The pH profile and growth profile of the isolates were studied and urease activity was measured by phenol hypochlorite assay method. Two isolates designated isolate O6w and isolate O3a were selected based on the highest urease activity recorded at 665 U/mL and 620 U/mL, respectively, and they were able to increase and sustain alkaline culture condition (pH 8.71 ± 0.01 and 8.55 ± 0.01) which was suitable for $CaCO_3$ precipitation. The isolates were identified based on 16S ribosomal RNA sequencing to be *Bacillus cereus* (O6w) and *Bacillus paramycoides* (O3a). This current study suggested that indigenous soil ureolytic bacteria are potential raw material for the biotreatment of soils stability.

INTRODUCTION

Geotechnical engineers termed a particular soil as problematic when it is observed to have inferior engineering characteristics and cannot be effectively utilised for relevant construction purposes, without the application of an improvement procedure (Rabenhorst & Buchanan 2020). Recently, urease-producing bacteria potential in the biotreatment of problematic soils via biocalcification has presented encouraging and impressive results in the literature (San Pabio et al. 2020; Miftah et al. 2020). Biocalcification also referred to as microbially induced calcite precipitation (MICP) is a biomineralization technique involving a biochemical process of precipitating calcium carbonate ($CaCO_3$) crystals induced by active ureolytic bacterial activity due to urea hydrolysis (ureolysis) occurring within the environment (Zamer et al. 2018; Chen et al. 2019; Yang et al. 2020). The success of the MICP process is promoted primarily by ureolytic bacterial species such as *Bacillus sphaericus*, *Pararhodobacter* sp., *Mor-*

ganella morgana, *Bacillus licheniformis* and *Bacillus cereus* which are capable of utilizing urea as a source of nitrogen by passively diffusing or actively transporting the urea into the cytoplasm of the cell and the bacterial cell wall acting as nucleation sites (Dardau et al. 2021). In search for alternative soil improvement technology with minimal environmental consequences, less adverse effect on the ecosystem and maintaining ecological balance (Khaliq & Ehsan 2016), over conventional methods (cement, chemical grouting & deep mixing technique) that varied in terms of environmental impact, cost, penetration depth, energy consumption and treatment uniformity which portrays their merits and demerits (Hiranya et al. 2018; Duo et al. 2018; Bui Truong et al. 2020), and advances in material and geotechnical research, led to the development of an innovative, novel bio-mediated soil improvement technique utilizing urease-producing bacteria as potential agents.

Several genera of ureolytic bacteria have been recognised as potential MICP agents, including *Clostridium*, *Bacillus*, *Desulfotomaculum*, *Sporolactobacillus* and *Sporosarcina* (Ivanov & Chu 2008) with *Sporosarcina pasteurii* widely utilised in most studies on MICP (Wen et al. 2018), due to tolerance to high pH and precipitation of large amounts of calcite due to high urease activity (Minto et al. 2018; Ruan et al. 2019). Noteworthy, ureolytic bacteria species with the potential of forming endospores, have the advantage of enduring harsh environmental conditions such as nutrient deficiencies, extreme temperature, absence of humidity, and exposure to radiation, disinfectants, antibiotics and chemicals (Badiee et al. 2019). Generally, the selection of desired MICP bacterial agent with high potential survival in an alkaline environment and tolerant to extreme conditions, endospore-forming urease-producing bacteria should be the first choice (Li et al. 2019). On the other hand, the ureolytic bacterial success during the MICP process is promoted primarily by *in situ* environmental conditions such as pH, soil particle size and distribution, competition, predation, osmotic pressure, water content and the conditions of treatment like cementation solution, concentrations of bacteria, availability of suitable nutrients, and temperature (Burbank et al. 2011; Dadda et al. 2018). For example, the rate of ureolysis is higher at 30°C while extreme temperatures may affect the microbial urease activity, nucleation rate and solubility. Further, microbial urease enzyme may be denatured irreversibly at a pH value lower than 5.0 (Ng et al. 2012). In addition, urea concentrations higher than 0.75mol/L may inhibit bacterial ureolytic activity due to too high transportation of urea molecule into the cell membrane which could inhibits other cellular processes (Wu et al. 2019).

Previous studies have documented the potential MICP technological application of urease-producing bacteria towards the improvement of soil (Ming-juan et al. 2017; Junjie et al. 2020), biotreatment of calcareous beach sand (Miftah et al. 2020), strengthening compressed interlocking earth blocks (Zamer et al. 2018), microbial restoration of degraded marble structures (Minto et al. 2018), biohealing of cracks in concrete (Ruan et al. 2019) and wind erosion control (Zomorodian et al. 2019) as an effective, economically engineered natural occurring green biotechnological process. Conversely, despite the numerous advances in MICP, most urease-producing bacteria utilised for various MICP applications are commercially procured from culture collection centres, which contribute to cost (Zomorodian et al. 2019). According to the present global market price, it cost approximately US$402.0 to procure the original patent of *S. pasteurii* ATCC 11859, which suggests the low-cost advantage of utilizing indigenous ureolytic bacteria for various MICP applications (Ezzat & Ewida 2021). Further, the procured microorganisms are often associated

with drawbacks regarding reduction in the population of the introduced bacteria into the soil due to competition, mechanical stress and predation arising from the non-adaptability of the organisms to the local environment (Burbank et al. 2011). In addition, the introduced bacteria can negatively influence the soil microbial communities by affecting the ubiquitous interactions among the soil microorganisms and altering the traits expressed by these microbial communities (Badiee et al. 2019).

Meanwhile, species of ureolytic bacteria documented in literature as promising MICP agents for various civil engineering applications include; *Micrococcus* sp., *Virgibacillus* sp., and *Pseudoalteromonas* sp. applied for coastal erosion protection (Al imran et al. 2019), *Bacillus sphaericus* employed to stabilize dispersive soils (Moravej et al. 2018) while *Pararhodobacter sp.* was utilized for coral sand solidification (Khan et al. 2016). A similar study on concrete healing with *Bacillus cereus* by Wu et al. (2019) reported a crack healing of 100 – 800 µm after 28 days of treatment with a decrease in rate of water permeability by about two orders of magnitude. On the other hand, the findings from the study of Zamani & Montoya (2019) on improvement in the cyclic strength of silty sand utilizing *Sporosarcina pasteurii* as MICP agent have shown a decrease in rate of excess pore water generation with a significant increase in cyclic resistance in comparison to their untreated state. Hence, increases the number of cycles essential to reach liquefaction at a constant cycle stress ratio value. A study by Tiwari et al. (2021), observed 205% increase in calcite content of bio-stimulated MICP treatment of expansive soil with indigenous urease-producing bacteria. An increase in split tensile strength and unconfined compressive strength as well as a decrease in swell strain and swelling pressure were also reported.

Noteworthy, indigenous microorganisms distributed within the soil environment can be enriched *in situ* (bio-stimulation) by modifying local environmental conditions which favour the diversity and distribution of existing bacterial community with required urease capabilities for various MICP applications (Gowthaman et al. 2019; Graddy et al. 2021). Thus, it confirms the promising nature of utilizing indigenous urease-producing bacteria as agents for MICP applications. Hence, research on the utilization of indigenous soil ureolytic bacteria with high urease activity as an alternative towards biotreatment of problematic soil becomes paramount and still a budding line of research. This study aimed to assess the potential of indigenous ureolytic bacteria towards biotreatment of problematic soils. Therefore, the objectives were to isolate and screen for *in situ* soil ureolytic bacteria for their urease activity and their potential for calcite production.

MATERIALS AND METHODS

Soil sampling

A total of ten soil samples were collected from the topsoil layer of Ladang 15, Faculty of Agriculture (2°36'05"N 102°42'11"E), Universiti Putra Malaysia in Selangor, Malaysia based on methods adapted from Kang et al. (2015). The soil samples type and texture were determined as described by Towner (1974) and Ritchey et al. (2015). Meanwhile, pH of soil samples was measured using a standard pH meter as described by Kalra (1995).

Isolation and preservation of ureolytic bacteria

The method described by Navneet et al. (2011) and Wei et al. (2015) was adapted for ureolytic bacteria isolation. Soil samples were suspended in a sterile physiological solution (8.5g/L of NaCl in distilled water). A serial

dilution of the soil sample suspensions (10-fold) was prepared and each serial dilution was plated on Calcium Carbonate Precipitation (CCP) agar (3g/L nutrient agar, 20g/L urea, 10g/L NH_4Cl, 20g/L agar, 2.12g/L $NaHCO_3$ and 25g/L $CaCl_2.2H_20$). Cultures were incubated at 28°C ± 0.5° C and assessed on daily basis within 7 days. The appearing colonies were selected and streaked on CCP Minimal Medium agar (3g/L nutrient agar, 20g/L urea, 10g/L NH_4Cl, 20g/L agar, 2.12g/L $NaHCO_3$) and used for urease screening.

Qualitative Screening for Urease Activity

All the pure isolates were qualitatively screened for urease activity on urea agar base (1.0gm/L peptone, 1.0gm/L glucose, 5.0gm/L sodium chloride, 1.2gm/L disodium phosphate, 0.8gm/L potassium dihydrogen phosphate, 0.012gm/L phenol red, 15.0gm/L agar). The pure isolates were streaked and incubated at 28°C ± 0.5°C for 120 hours and observed every 6 hours. A change in medium colouration from orange to pink was interpreted as positive urease production (Akyol et al. 2017).

Basic characterization of ureolytic bacteria as MICP

Colony morphology of the urease-producing bacteria was observed as a preliminary step towards their identification based on Algaifi et al. (2020). Gram's staining was performed based on the method by Smith & Hussey (2005). Meanwhile, Eosin Methylene Blue agar plates were conducted as a confirmatory test for Gram staining results as described by Leininger et al. (2001). The endospore staining technique was carried out as previously described by Kim et al. (2018).

Molecular identification

For the identification of the unknown ureolytic bacteria culture, a single pure colony on CCP agar was incubated at 28°C ± 0.5°C overnight and molecular identification base on 16S ribosomal RNA sequencing (Zhang et al. 2019). The 16S rDNA, full length 1.5 kb, were amplified using the universal primers 27F (5' - AGAGTTTGATCCTGGCTCAG- 3') and 1492R (5' - GGTTACCTTGTTACGACTT- 3') (Wu et al. 2014). The bi -directional sequencing of purified PCR products was done with universal sequencing primers 785F (5' -GGATTAGATACCCTGGTA- 3') (Manoharan et al. 2020) and 907R (5' -CCGTCAATTCCTTTRAGRTT - 3') (Reysenbach et al. 2000) using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Massachusetts, U.S.A). The forward and reverse sequencing results are edited and assembled into one full-length sequence. The genes were compared with the generated sequence of National Centre for Biotechnology Information (NCBI) nucleotide Basic Local Alignment Search Tool (BLAST), (https://www.ncbi.nlm.nih.gov/nuccore/NR_115714.1); (https://www.ncbi.nlm.nih.gov/nuccore/NR_157734.1).

Growth and pH profile

The CCP broth medium was used for the measurement of bacteria growth and pH profiles as adapted from Navneet et al. (2011). Meanwhile, the pH profile was studied by measuring the pH of the culture medium using pH meter every 24 hours throughout the incubation period under study (120 hours).

Quantification of urease activity

The quantification of bacterial urease activity was done in accordance with the method described by Navneet et al. (2011) based on the phenol

hypochlorite assay method. Approximately 2.5mL of 0.1M urea was mixed with 0.1M potassium phosphate buffer (pH 8.0) followed by 250 µl of the culture filtrates. The mixture was incubated at 37˚C ± 0.5˚C for 5 minutes. Then 1mL of alkaline hypochlorite (56g/L phenol, 0.25g/L sodium nitroprusside, 0.02g/L EDTA) and phenol nitroprusside (2.1g Sodium hypochlorite in 25g/L NaOH), respectively and further incubated for another 25 minutes at 37˚C ±.0.5˚C. Optical density was measured at 626 nm and one unit of urease is defined as the amount of enzyme hydrolysing 1µmol urea per minute. Ammonium chloride (50 to 100 µM) was used as standard.

Calcium carbonates precipitation

Bacterial isolates were grown individually in 100 mL of CCP broth and incubated for 48 h at 28˚C ± 0.5˚C, respectively and an uninoculated CCP medium was used as a control. Later, 1 mg/mL of lysozyme was added to the whole suspension and incubated further for another 60 min at 37˚C ± 0.5˚C. The cell debris was removed by centrifuging at 4,000 g for 4 min and the precipitates were thoroughly washed with distilled water (pH 8.5) (Wei et al. 2015) before the washed precipitates were viewed under the light microscope. The confirmatory test for the precipitated calcium carbonate was carried out using a quick acid test (Richardson et al. 2014). The precipitated calcium carbonate produced was poured into dried test tubes and a few drops of 10% (v/v) of hydrogen chloride was dropped into the test tube and rapid effervescence with bubble formation was interpreted as a positive response.

Statistical analysis

The statistical analysis for the results from this study was carried out using Microsoft Excel 2016. One-way Analysis of variance (ANOVA) was used to study the ureolytic bacterial isolates growth and urease enzyme production variations at 95% confidence level using Graph Pad Prism version 9.1.2.

RESULTS AND DISCUSSION

Isolation and screening for indigenous soil ureolytic bacteria

All soil samples designated as A – E were identified as brown sandy clay soil with a fine texture and a pH value ranging from 3.93 to 4.12 while soil samples designated as F – J were identified as black organic soil with a silky texture and a pH value ranging from 4.11 to 4.51. Soils from farms are known to be rich in urea due to frequent use of organic manure and synthetic urea fertilizers which improve microbial activity through stimulating *in situ* urease-producing bacteria already distributed within the soil pore spaces (Al-Thawadi & Cord-Ruwisch 2012). It is normal that organic soils and urea-rich soils favours the distribution and diversity of ureolytic bacteria which utilizes urea as a sole source of nitrogen and energy (Zhu & Dittrich 2016). Thus, enhancing the rapid growth of ureolytic bacteria within the soil environment (San Pabio et al. 2020; Svane et al. 2020). Previous literatures successfully reported the potential isolation of ureolytic bacteria from urea-rich soils (Phang et al. 2018; Noor et al. 2021).

The fact that ureolytic bacteria are common natural inhabitants of urea-rich soils, the CCP media was supplemented with urea (20g/L) as suggested by Wei et al. (2015) to selectively target active ureolytic bacteria that are tolerant to higher concentrations of ammonia and urea. This was observed by a pungent smell indicating the release of ammonia gas from the culture plates due to the degradation of urea by the bacterial

isolates. Thus, it is an indication of the bacterial isolates' suitability for calcite precipitation towards the soil stabilization process (Zomorodian et al. 2019). In the current study, a total of 16 pure cultures of bacterial isolates with distinct morphological colonies were isolated. Noteworthy, all the bacterial isolates were isolated from soil samples with pH values ranging from 3.73 to 4.51 indicating an acidic environment. In a similar study, Phang et al. (2018) isolated five ureolytic bacterial strains of the genus *Bacillus* from an acidic tropical peat soil of pH value 3.8 to 4.9 in Miri, Sarawak, Malaysia. Most studies on MICP applications reported the isolation of ureolytic bacteria from slightly neutral to alkaline soils (Gat et al. 2014; Dhami et al. 2017) because generally harsh acidic conditions might result in a total loss of bacterial urease activity. However, current findings suggest the adaptation of urease-producing bacteria to an acidic environment where for such bacteria to adapt to the acidic environment, a large amount of urease enzyme is secreted within the microenvironment. Hence, urea hydrolysis will neutralize the acidic condition which favours bacterial survival within the environment and thus favouring the MICP process (Gowthaman et al. 2019). The findings from these different studies implies that isolated strains of ureolytic bacteria vary between alkaline and acidic soil environment.

All 16 bacteria isolates were qualitatively screened for urease activity on Urea Agar Base media containing a pH indicator, phenol red. The change in colouration of the medium was caused by an increase in pH due to the generation of hydroxyl ions from ammonium ions production as a result of urea degradation which is detected by the phenol red (DeJong et al. 2010). In addition, the glucose and peptone present in the medium enhance the rapid growth of diverse species of ureolytic bacteria (Dortey et al. 2020). A change in medium colouration from orange to pink was interpreted as positive urease production and all the isolates showed those responses at different time intervals (Table 1).

Table 1. Bacteria isolates' test for urease activity on urea agar base (UAB).

No	Isolates	(hours)
1	O6w	18
2	O5w, O3a	24
3	O6a,	30
4	O42, S73	36
5	O41	42
6	S70	48
7	O32, O31	78
8	S75, S74	84
9	S72, S76, S77, S71	90

Hence, indicates the different rates of urease enzyme production by the bacterial isolates. Subsequently, in order to target potential high urease producers, only isolates that result in colour change within 48 hours were selected, as isolates that results to change in urea agar base colouration within 48 to 72 hours are potential bacteria with urease activity (Akyol et al. 2017). Thus, justify the selection of 8 isolates (O6w, O42, O5w, O3a, O6a, O41, S73 and S70) utilized in subsequent analysis.

Characterization of ureolytic bacteria as MICP

The colony appearance of 8 selected urease-producing bacteria was studied through visual observation and recorded under standard protocols. There were notable morphological differences across the isolated ureolytic bacteria. Although all the isolates form circular colonies, the close morphological difference noticed might be due to enrichment methods fa-

vouring dominant species during isolation and cultivation periods (Stocks-Fischer et al. 1999). All isolates were Gram-positive, and having a thick layer of peptidoglycan gives an advantage for these isolated Gram-positive bacteria, as the peptidoglycan layer contains 60% teichoic acids which are negatively charged due to high phosphate groups in it (Swoboda et al. 2010; Rauch & Leigh 2015). Thus, it creates a dense network of negative charges on Gram-positive cell wall surfaces to attract positively charged ions such as calcium and this forms the fundamental principle behind the MICP potential of ureolytic bacteria (Wong 2015). Sharma et al. (2021) demonstrated soil biotreatment via MICP in Narmada sand, India using the following Gram-positive bacteria; *Sporosarcina pasteurii*, *B. subtilis* and *B. sphaericus*. In a similar study, Ali et al. (2020) reported all six urease-producing bacteria isolated from urea-rich soils with potential for calcification were Gram-positive. A similar tendency was reported by Gowthaman et al. (2019).

However, the Gram staining technique is subject to inherent limitations leading to technical variation, arising probably due to under decolourization, over decolourization and misinterpretation (Thairu et al. 2014). Thus, further confirmation was carried out by cultivation of the ureolytic bacterial isolates on EMB agar, as the EMB agar contained methylene blue and eosin Y that inhibits the growth of most Gram-positive bacteria (Leininger et al. 2001). None of the ureolytic bacterial isolates grew on the agar medium. Hence, confirming a Gram-positive result across all isolates.

The results from the Endospore staining procedure indicate all isolates under study are spore-forming ureolytic bacteria. Bacterial endospores are unique dormant structures formed especially within the cell, essential for bacterial survival in harsh environmental conditions such as extreme soil temperatures and chemical exposure (Algaifi et al. 2020). A significant characteristic feature preventing ureolytic bacterial death at extreme conditions such as mechanical stress and variations in temperature during the MICP application processes. This makes ureolytic bacterial isolates suitable for a wide variety of MICP applications, particularly stabilization of problematic soils and biocementation in concrete (Khadhim et al. 2019). To emphasize, it has been documented that dormant spore-forming ureolytic bacteria have potential survival at extreme pH (above pH 12) and remain viable in Portland cement-based concrete for a very long period of up to 200 years (Gavimath et al. 2012). It is significant to note that endospores contribute to the success of the MICP process with their capacity to survive desiccation, crosslinking, mixing of concrete and have experimentally been proven to have a positive effect on calcium carbonate precipitating potential on treated concretes (Nielsen et al. 2020). Generally, endospores prolong the survival of bacteria within concrete and soil environment by encasing the vegetative bacterial cells with a multi-layered protein complex structure referred to as coat, which provides two main functions toward the success of the MICP process; (i) it protects against bactericidal chemicals and enzymes such as chloroform and lysozyme, hence enhance spore's resistance viability and properties and (ii) it contributes to endospore's ability to monitor its microenvironment and response within minutes to germinate when exposed to appropriate nutrients (De Muynck et al. 2010; Erşan et al. 2015; Grabiec et al. 2017). Thus, contributing to a greater extent the endospore's calcium carbonate precipitation capacity for applications in MICP (Basha et al. 2018). Several other studies have reported endospore-forming ability of numerous ureolytic bacteria, mostly from the genus Bacillus and Sporosarcina (Harikrishnan et al. 2015; Kim & Youn 2016).

Ureolytic bacterial pH profile

Six out of eight isolates (O6w, O3a, O42, S73, O5w and O6a) sustain a steady rise in pH up to 96 hours (Figure 1). This steady rise in pH changes the microenvironmental conditions which inhibit all other competitive processes within the system. Thus, enhancing bacterial urea hydrolysis which favours permanent precipitation of more $CaCO_3$ crystals between soil grains (DeJong et al. 2010; Jiang et al. 2020). Urea decomposition by ureolytic bacteria generates ammonium ions which increase the pH of the culture medium (Al-Thawadi 2011). Several literatures stated the range of pH values of between 8.3 and 9.3 to be ideal for bacterial calcium carbonate precipitation for biotreatment of problematic soils (DeJong et al. 2010; Sidik et al. 2014). Therefore, the ability of the bacterial isolates to survive at the aforementioned alkaline pH demonstrated their potential to be utilized as agents for MICP towards soil stabilization. According to Hammes & Verstraete (2002) and Krajewska (2018), an increase in pH within the microenvironment is crucial in creating a physiological condition favourable for bacterial cell walls acting as the nucleation site for mass $CaCO_3$ precipitation. In addition, a rise in pH value played a significant role in bacterial adhesion and transportation on and in-between soil grains, to achieve improved homogeneous distribution of precipitated $CaCO_3$ across treated problematic soils (Al Imran et al. 2019).

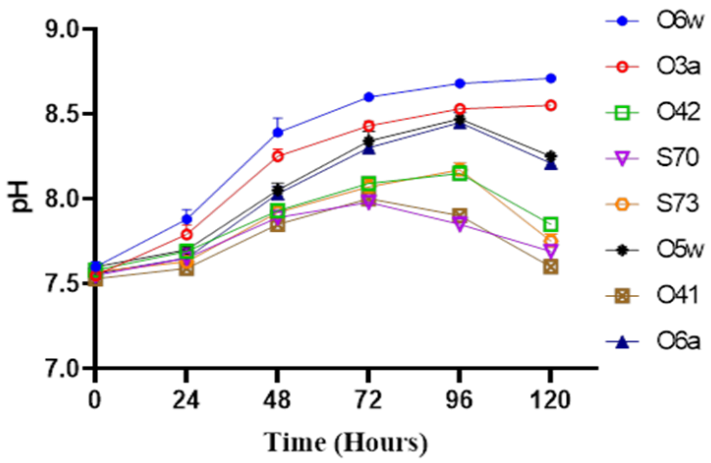


Figure 1. pH profile of selected bacterial isolates that result in colour change of urea agar base medium from orange to pink within 48 hours. Error bars represent standard deviation of the mean.

However, in the present study, the steady rise in pH was followed by a continuous decline after 96h. This might be due to exhaustion of urea within the culture medium due to hydrolysis by ureolytic bacteria leading to accumulation of ammonia as by-products (Whiffin & Paassen 2007). This by-product is usually accumulated either as ammonium salt (NH_3) or ionized (NH_4^+), while the former contributed mainly to the toxicity. Noteworthy, most of the ammonium produced during ureolysis are converted to ammonium salt when the medium pH exceeds 9.5 while bacterial denitrification converts the remaining fraction to nitrate (NO_3^-) (Soon et al. 2014). The ammonia gas is highly detrimental to human health when inhaled, particularly at high concentrations (Omoregie et al. 2016) and this is the major shortcoming of MICP.

Ureolytic bacterial growth profile

All isolates show a similar growth pattern with corresponding progressive cell growth in response to time (Figure 2). The logarithmic phase of growth was observed within the first 24 hours after incubation, which

was sustained up to 72 hours. This growth phase is characterised by an exponential increase in cell growth which favours precipitation of calcium carbonates due to two factors; (i) alteration of the environmental pH through the production of more urease enzyme and (ii) the new available bacterial cells provide surfaces that act as a heterogeneous nucleation site for $CaCO_3$ precipitation by attracting calcium ions rapidly on to its negatively charged cell wall (Phang et al. 2018). Isolate O5w recorded the highest bacterial growth with an optical density (OD) of 1.05 with other isolates maximum growth varied between 0.82 to 1.0 OD. Thus, demonstrating diversity in their metabolic requirements. The maximum optical density recorded can be influenced by the bacterial strain of choice, environmental and growth conditions (Richardson et al. 2014). However, after 96h, the stationary growth phase had started to show a negative rate of growth leading to the death phase, indicating continuous cell death due to limited transfer of nutrients and extremely high alkaline environment inhibiting cell growth (Li et al. 2019). Nevertheless, the growth profile demonstrated the potential of the isolates as promising agents for sustaining steady growth up to 96 hours, which is sufficient to favour mass precipitation of $CaCO_3$ (Kim et al. 2018).

Quantification of urease activity

All isolates recorded a steady rise in urease activity with an increase in incubation time up to 72 hours (Figure 3). The higher urease activity observed with time implies an increase in bacterial growth and an increase in bacterial production of urease enzyme due to the availability of urea as the sole nitrogen source within the medium (Omoregie et al. 2019a; Omoregie et al. 2019b). However, a continuous decrease in urease activity after 120 hours was observed due to exhaustion of nutrients, metabolism inhibition, cell death and enzyme degradation with time leading to an irreversible loss of urease activity (Jiang et al. 2016). Isolate O6w and isolate O3a recorded the highest urease activity of 665 U/mL and 620 U/mL respectively. Thus, an indication of their suitability for application as potential agents towards biotreatment of problematic soils via MICP. One-way ANOVA analysis with a 95% confidence level shows a significant difference in urease activity of all isolates across all the time intervals, which implies the different rate of urease enzyme produced by the individual bacterial isolates. Several studies have reported native ureolytic bacterial strains with different urease activity (Dhami et al. 2017; Jain & Arnepalli 2019; Ma et al. 2020).

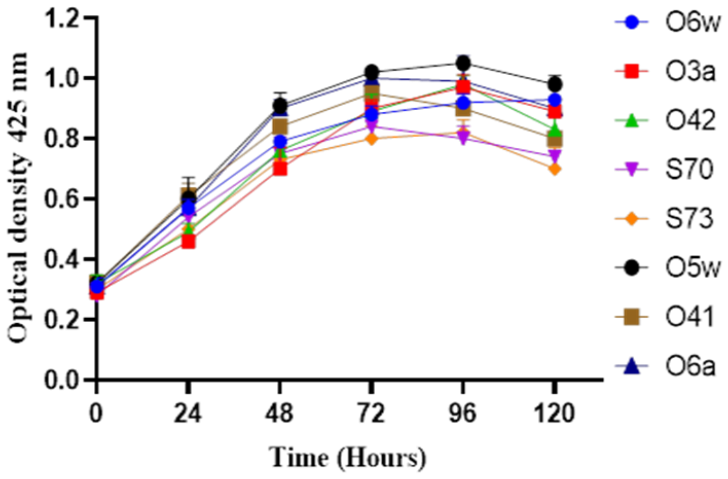


Figure 2. Growth profile (optical density 425 nm) of selected bacterial isolates that result in colour change of urea agar base medium from orange to pink within 48 hours. Error bars represent standard deviation of the mean.

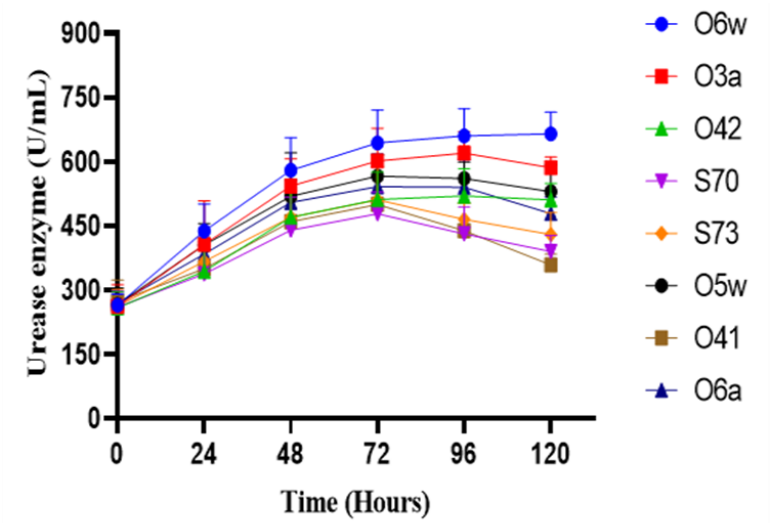


Figure 3. Urease activity (optical density 626 nm) of selected bacterial isolates that result in colour change of urea agar base medium from orange to pink within 48 hours. Error bars represent standard deviation of the mean.

Noteworthy, higher urease activity within the microenvironment, favours the production of more dissolved CO_2 in the form of HCO_3^- or CO_3^{2-} and ammonium ions are generated leading to an increase in pH (Zaghloul et al. 2021). The dissolved CO_2 does not only form part of the precipitated $CaCO_3$ but also act as a buffer within the system (Wu et al. 2017), while the ammonia produced is also advantageous to the ureolytic bacteria towards the generation of energy in the form of Adenosine triphosphate (ATP) (Cheng & Cord-Ruwisch 2013). However, higher concentrations of ammonia are detrimental to ureolytic bacterial growth and affect urease activity via biochemical reactions (Tang et al. 2020). Further, an ideal urea concentration is crucial for the survival and metabolic requirements of the ureolytic bacterial growth within the medium (Ng et al. 2012). Hence, in the current study, a urea concentration of 0.33 mol/L was utilized for bacterial growth as suggested by several earlier studies (Burbank et al. 2012; Wei et al. 2015; Akyol et al. 2017). Based on the experimental conditions on the study conducted by Okwadha & Li (2010) using *S. pasteurii* reported urea concentrations of 0.66 mol/L to be optimum for the bacterial growth and MICP processes. Higher urea concentrations can inhibit the ureolytic activity of even the bacteria with high urease activity due to too high transportation of urea molecule into the cell through the cell membrane which inhibits other cellular processes. Hence, urea concentrations in excess of 0.75mol/L are not recommended for applications in MICP (Wu et al. 2019).

Noteworthy, Bibi et al. (2018) reported that higher bacterial cell growth may not correspond to higher urease activity which does not necessarily translate to higher $CaCO_3$ yield. Rather, higher urease activity and the isolate potential to sustain and survive at a higher alkaline pH, other than bacterial growth are the basic parameters favouring the fundamental success of the MICP application process towards soil biostabilization (Wath & Pusadkar 2016; Bibi et al. 2018). Based on the aforementioned basic factors (urease activity and pH), isolate O6w and O3a were favoured as the bacterial candidates that sustained the optimum conditions correct for calcium carbonate precipitation activity towards biotreatment of problematic soils.

Identification of potential MICP isolates

BLAST results against NCBI 16S ribosomal RNA sequence using the neighbour joining method were used to construct a phylogenetic tree for

individual isolates as shown in Figure 4. The phylogenetic tree suggests the closest description of isolate O6w to be *Bacillus cereus* while isolate O3a closest description is *Bacillus paramycoides*. This might be due to the dominance of *Bacillus* spp. present within the soil environment and in comparison, to other genus by high degree favoured by their physiological adaptation to harsh environmental conditions (Elmanama & Alhour 2013). In addition, this coincided with previous investigations, which indicate most ureolytic bacteria from soil origin are Bacillus species. Bibi et al. (2018) in their study, isolated eighteen ureolytic bacterial isolates from Qatari soil and found all to be of the genus Bacillus. Another study by Phang et al. (2018) reported the isolation of five Bacillus species with calcifying potential from the tropical peat soil in Sarawak, Malaysia.

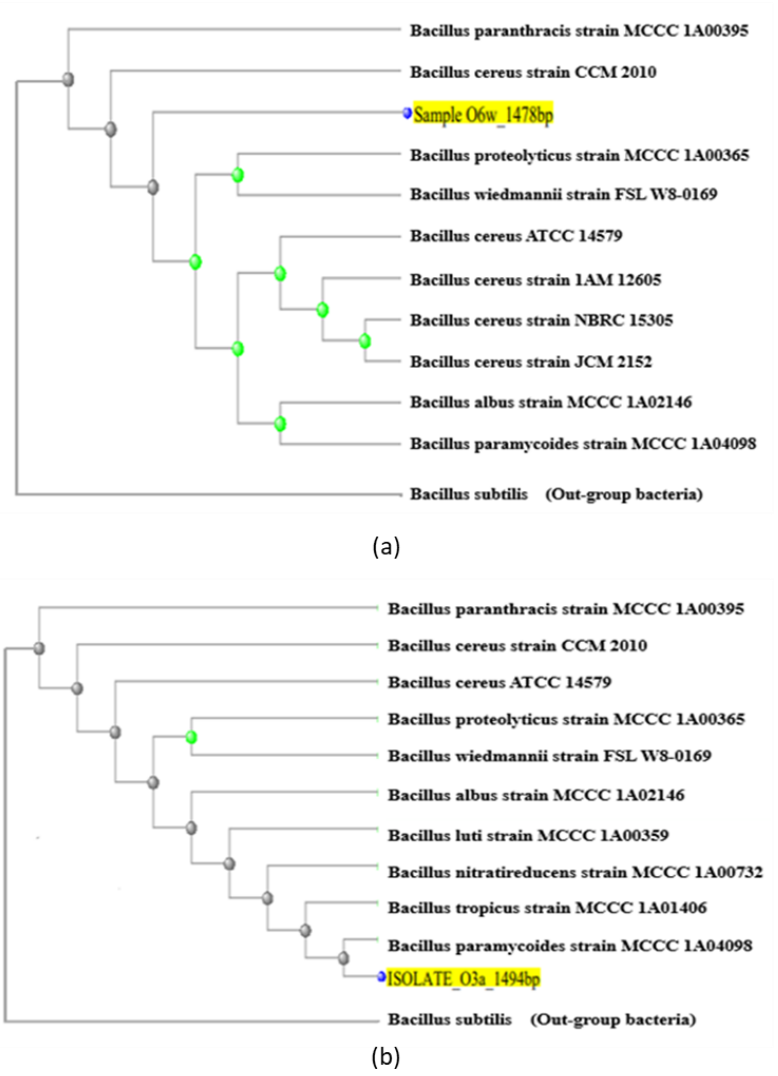


Figure 4. Phylogenetic Tree – Neighbour Joining (Unrooted Tree) by NCBI Blast Tree Method, as compared to known species (a) Isolate O6w and (b) Isolate O3a.

Isolate O3a has 99.93% similarity to *B. paramycoides* (Table 2) which was characterised as non-urease producing bacteria (Liu et al. 2017). However, this current study has shown that isolate O3a recorded high urease activity (Figure 3) thus, contrary to the Liu et al. (2017) claim. A further search had shown that several recent studies have reported bacterial isolate with 98.1% to 98.9% identification similarity with *B. paramycoides* and characterized the isolate as a urease producing bacteria (Mekonnen et al. 2019; Mekonnen et al. 2021; Caglayan 2021), which is consistent to the findings of this study.

Table 2. Molecular identification base on 16S rRNA sequencing data using NCBI nucleotide BLAST database.

No		Isolate O6w	Isolate O3a
1	Closest description	*Bacillus cereus* (16S ribosomal RNA, partial sequence)	*Bacillus paramycoides* (16S ribosomal RNA, partial sequence)
2	Maximum score	2666	2691
3	Total score	2666	2691
4	Query cover	100%	100%
5	Percentage identification	100%	99.93%
6	Accession number	NR115714.1	NR 157734.1
7	Base pair	1478	1494
8	Genus	*Bacillus*	*Bacillus*
9	Species	*Bacillus cereus*	*Bacillus paramycoides*

Ureolytic bacterial calcium carbonates precipitation

Both *B. cereus* and *B. paramycoides* precipitated calcium carbonates after 48 h incubation in CCP medium at 28°C ± 0.5°C, respectively. Precipitated $CaCO_3$ was confirmed by a quick acid test and rapid effervescence of carbon dioxide gas with a continuous formation of bubbles was observed. Hence, confirming the possible presence of an alkaline based material presumptively identified as $CaCO_3$ (Richardson et al. 2014). In the present study, the precipitated $CaCO_3$ was visualized under a light microscope, some of the precipitated crystals formed clusters of two or more, as such visualized as aggregates (Figure 5a), as also been observed in a study by Al-Thawadi and Cord-Ruwisch (2012), while no precipitates were formed in uninoculated medium (Figure 5b).

Further, scanning electron microscopic images (Figure 6) of the $CaCO_3$ precipitated by both *B. cereus* and *B. paramycoides* were morphologically visualized as agglomerated $CaCO_3$ crystals. By comparison, agglomerated $CaCO_3$ crystals precipitated by *B. paramycoides* were similar to the ones produced by *B. cereus* and were found to be in agreement with earlier similar observations (Kakelar et al. 2016). Noteworthy, these precipitated crystals were formed by supersaturation within the medium with availability of bacterial cell wall acting as sites for nucleation (Wang et al. 2017). In addition, the formation of agglomerated $CaCO_3$ crystals occurs due to nucleation and growth of existing $CaCO_3$ crystals, which leads to the precipitation of larger $CaCO_3$ crystals (Al-Thawadi & Cord-Ruwisch 2012; Mujah et al. 2017). Finally, based on survival at alkaline pH and the high urease activity achieved by both *B. cereus* and *B. paramycoides* and their capability to sustain the culture conditions which favours precipitation of calcium carbonate as confirmed by quick acid test and viewed under light microscope and SEM, demonstrated their potential utilization as agents toward biotreatment of problematic soils.

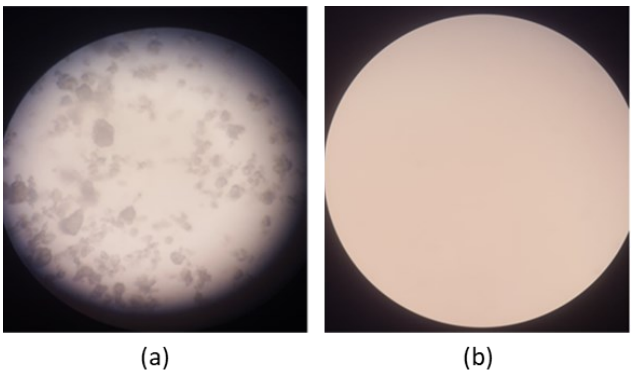
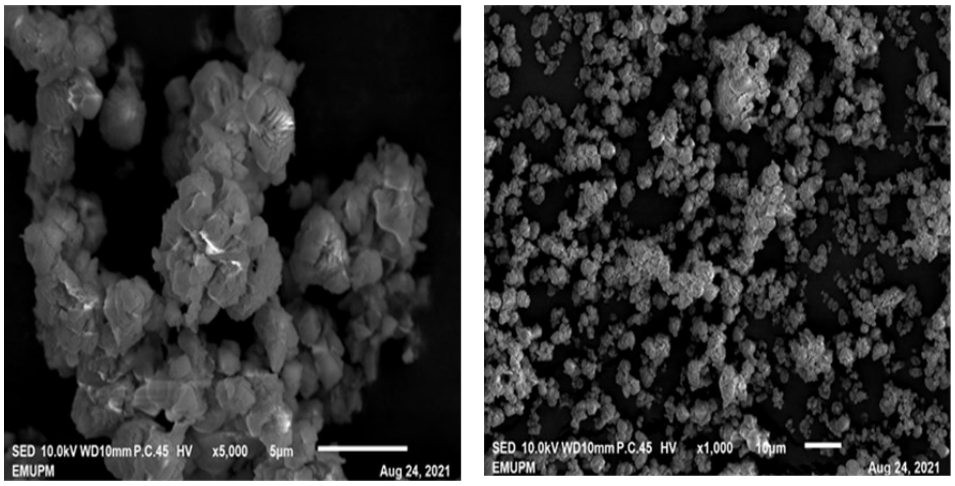


Figure 5. Microscopic images of precipitated calcium carbonates produced by *Bacillus cereus* viewed under light microscope (a) ×40 and (b) Uninoculated calcium carbonate precipitation medium.



(a) (b)

Figure 6. SEM micrographs of precipitated calcium carbonate crystals by (a) *Bacillus cereus* and (b) *Bacillus paramycoides*.

CONCLUSION

This study effectively established the presence of indigenous urease producing bacteria distributed within the farm soil environment. Among the sixteen easily isolated strains of ureolytic bacteria evaluated, *B. cereus* and *B. paramycoides* recorded the highest urease activity, survived a steady growth at alkaline pH and was able to sustain the activity for the precipitation of $CaCO_3$. This study also reported the presence of *B. paramycoides* (known non-urease producing bacteria) within the active indigenous urease $CaCO_3$ precipitating bacteria in the farm soil environment. Both *B. cereus* and *B. paramycoides* had a promising potential application as MICP agents for the soil stabilization method.

AUTHORS CONTRIBUTION

All the co-authors contributed to the research experimental design, data analysis, drafting of manuscript, editing and completing the revisions. All co-authors have agreed and read the final version of the submitted manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Alternative Strategy to Improve the Conservation of Javan Deer in Pangandaran Nature Reserve, West Java, Indonesia

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ABSTRACT
The habitat of Javan deer in Pangandaran Nature Reserve (PNR) faced natural changes, particularly due to the succession process of vegetation community in grazing areas, and inadequate infrastructures that affected the deer to roam outside PNR. This study aimed to formulate strategies for the conservation of Javan deer in PNR, focusing on ecological aspects and conservation management. The methods were encountering Javan deer individuals; scan sampling and continuous recording to observe the behaviour of Javan deer; calculating the productivity of grazing area by defoliation experiment and vegetation analysis; reviewing documents, reports and interviews; and analysing strategy using SWOT-QSPM. Results showed there were 43 Javan deer encountered roaming in PNR and outside the conservation area, and nine individuals gathered in Cikamal grassland. The productivity of the grazing areas (5.61 ha) was 93,826 kg of feed annually and was only sufficient for 23 individuals. The grazing areas were dominated by *Cynodon dactylon*. Javan deer spent their time feeding. Javan deer herd in Cikamal is more intolerant to humans compared to the herd in Pangandaran Nature Tourist Park (PNTP). This study recommends: considering the management status of Javan deer in the conservation management of PNR and PNTP; improving the conservation management of Javan deer and its habitat; improving facilities and the management system of those facilities and conservation-supporting infrastructures;collaboration with researchers to perform some research and innovations for Javan deer conservation; improving the capability of PNR staff theoretically and practically; and educating and empowering the local people in terms of Javan deer conservation.

INTRODUCTION
Javan deer (*Rusa timorensis*, de Blainville 1822) or also called Timor deer, is a species that belongs to the Cervidae and is native to Indonesian islands, particularly Java and Bali, and had been introduced to other Indonesian islands and overseas (Hedges et al. 2015; Ali et al. 2021). Categorised as Vulnerable by IUCN in 2015, the Javan deer is protected by the Indonesian Government through the Regulation of Minister of Environment and Forestry No. P.20/Menlhk/Setjen/Kum.1/6/2018 concerning Protected Species of Plants and Animals. Javan deer have significant roles in ecology, economics, and socio-cultural, specifically since the species domesticated for commercial meat and antlers managed to be a game

-hunting animal and being invasive feral populations outside Indonesia (Hedges et al. 2015; Ali et al. 2021). However, Javan deer deal with extermination in many highly human-populated areas, including poaching, habitat loss, livestock competition, predator threats, diseases, and natural disasters (Rahman et al. 2020). In Pangandaran Nature Reserve (hereafter 'PNR'), Javan deer roam outside the conservation area, foraging on trashes, destructing public infrastructures, polluting the Pangandaran Beach tourist destination, disturbing local people's activities, and blocking transportation access that harms the deer themselves. The most robust hypothesis about the causal factors related to Javan deer roaming outside PNR are the scarcity of natural feed inside PNR (Firdaus et al. 2022) and the behaviour pattern changes of the species (Withaningsih et al. 2020).

PNR once comprised roughly 57 ha of five separated grazing areas as habitat for Javan deer and banteng (*Bos javanicus*), where three of them (Badeto, Batu Meja, and Karang Pandan) were abandoned to be secondary forests, and the other two (Cikamal and Nanggorak) still exist although only Cikamal being managed more intensively (Rosleine & Suzuki 2012). The alteration of grazing areas limited the sources of food for Javan deer. On the other hand damaged boundary walls between PNR and non-conservation areas allowed Javan deer to traipse outside the conservation site. As late as this research was conducted, we did not find any scientific data regarding the encounter and behaviour of Javan deer in Cikamal as the basic data to adapt the deer to roam only in the PNR, mainly in Cikamal.

Animal behaviour research is one of the main factors for animal management planning (Singh & Kaumanns 2005; Caro 2007; Pairah et al. 2014). Animal behaviour research could be applied to manage both domesticated animals (e.g. Venter et al. 2019; Orihuela 2021; Herrera et al. 2022; Lardy et al. 2022) and wild animals (e.g. Koirala 2021; Laguna et al. 2021; Miglioli & Vasconcellos 2021; Lardy et al. 2022; Rose et al. 2022). Behavioural research was also performed to reduce human-animal conflict (Silk 2007). For free-ranging wild Javan deer, behavioural research was rarely conducted due to time-consuming of observing Javan deer in their home range, and most of the research was concentrated in captivity. Yet, observing Javan deer in their natural home range would give information to conserve the species in their natural habitat (Pairah et al. 2015). Additionally, behavioural research on Javan deer was usually run due to the conservation priority in the conservation area. In Wan Abdul Rachman Forest Park, Javan deer was bred to be a tourist attraction, and behavioural research was used to analyse the deer behaviour to improve tourist-based conservation (Sofyan & Setiawan 2018). However, in Komodo National Park, Javan deer behaviour research was aimed at sustaining the population of the komodo dragon (Ariefiandy et al. 2019).

Javan deer in PNR was a priority species to protect since it is the remaining large herbivore and is an iconic animal for a tourist attraction. Moreover, the conservation of Javan deer has been declared as a primary conservation management plan of PNR since 2015 through PNR's Strategic Planning 2015-2020, followed by the next period of year 2021-2025. Nevertheless, the 2015-2020 conservation efforts to conserve Javan deer have insignificant results.

This study aimed to recommend alternative strategies for Javan deer conservation in PNR based on ecological and management aspects. The ecological aspects covered Javan deer population, behaviour patterns, and the potency of their habitat. The management aspects included the current programs implemented by PNR. Strength-Weakness-Opportunity-Threat (SWOT) analysis and Quantitative Strategic Plan-

ning Matrix (QSPM) were applied in this study. The SWOT-QSPM has been used to solve issues in many fields, including conservation management as it was applied in Baluran National Park (Siswanto 2020). SWOT -QSPM is dynamically adapted following the condition of internal and external factors in a management.

MATERIALS AND METHODS

Materials

The research was carried out in PNR and PNTP (Pangandaran Nature Tourist Park). Behavioural research on Javan deer was conducted in Cikamal, PNR. Cikamal is the primary feeding ground for Javan deer in PNR (Figure 1).

Methods

Javan Deer Encounter

We observed Javan deer population and their behaviour patterns from 06.00 to 17.00 in seven days continuously, dated December 23-29, 2021, in Cikamal. Javan deer population data was acquired using manual counting according to Javan deer encountered at the site. We identified Javan deer individuals into: fawn, juvenile male, juvenile female, adult male, and adult female, based on visual characteristics. The age of Javan deer was classified according to the size of their physical appearance. Big-sized deer are assumed to be older (Yuliawati 2011; Pairah et al. 2014). Meanwhile, the sex was classified by antler characteristics which only male Javan deer have antlers on their heads whether they are juvenile or adult (Yudha et al. 2019).

Javan deer commonly lived in groups called herds (Ali et al. 2021). Therefore, the behavioural observation was conducted using the scan sampling method, which observed most individuals' animal behaviours in a herd of deer (Altmann 1974). The scan sampling was modified and combined with the continuous-time recording that simultaneously observed the object and stopped when they moved out of sight (Altmann 1974; Oliveira et al. 2018). We recorded Javan deer behaviour in seconds and limited minimum duration for the behaviour to be recorded as 10 minutes. This Javan deer behavioural observation was conducted by F.I.F. solely to use the method consistently (Lemos de Figueiredo et al.

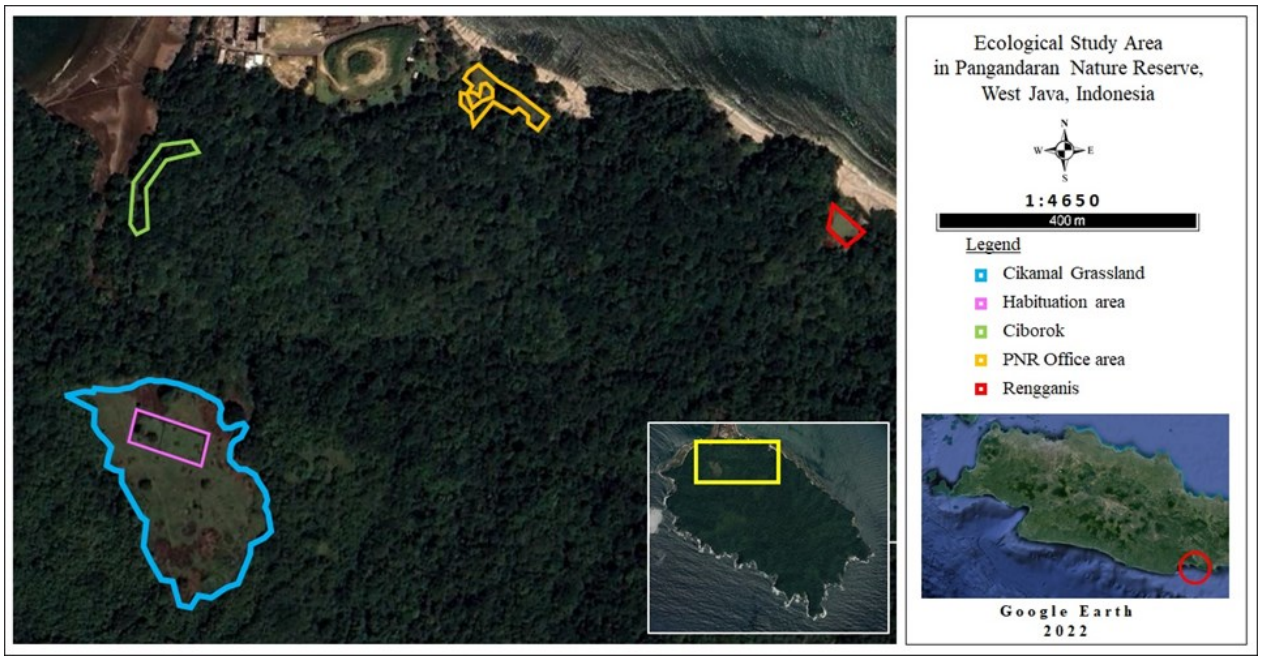


Figure 1. Study area in Cikamal.

2021). The Javan deer behaviour pattern was classified according to Pairah et al. (2014):

1. Feeding, including foraging, digesting, ruminating, drinking, and suckling (for adult females with fawn);
2. Locomoting, including moving, stampeding, and jumping;
3. Inactive, including standing still, resting, and wallowing in mud or pond;
4. Alerting, including anti-predator adaptations, detecting threats, and alarming to evade predators;
5. other activities, including interaction amongst individuals, fighting, rutting, grooming, urinating and defecating.

The Javan deer behavioural data was compiled in hour intervals (06.00-17.00). We calculated the mean of the seven-day observation data and transformed it into an hour-interval ethogram to illustrate the percentage of Javan deer daily time budget (Jakopin et al. 2021).

Potency of Javan Deer Habitat

This research uses plots for undergrowth measuring 1 m^2 (Kusmana 1997; Nurjaman et al. 2017) as many as 12 pieces, lawn mowers, and digital kitchen scales with an accuracy of 1 gram. We used PlantNet and Google Lens to identify the vegetation species encountered and asked PNR staff. The identification was then verified using website-based Plants of the World Online by Royal Botanic Garden Kew. Research on the potential of Javan deer habitat was primarily carried out in PNTP, totally from December 19, 2021 to February 8, 2022, in three locations: Rengganis, PNR Office area, and Ciborok

The 1 m^2 plot used to analyze the vegetation under the forage of the Javan deer was determined using a simple random sampling method to suit field conditions and regulations from PNR. Three plots were installed in Rengganis, five plots were installed at the PNR Office area, and four plots were installed in Ciborok. As a complementary, we used secondary data on feed plant productivity in Cikamal. Vegetation analysis classified the areas in the plots into areas of Javan deer feed plants, non-food vegetation areas of Javan deer, and areas without vegetation. Then, the data is compiled and averaged to obtain the percentage of the denseness. The reference of data on Javan deer understorey feed plants showed in Table 1.

Table 1. Javan deer feed plants.

No.	Species	Palatability	Source
1	*Axonopus compressus*	0.62	Purwanto (2013)
2	*Panicum repens*	0.41	Purwanto (2013)
3	*Fimbristylis aestivalis*	0.33	Purwanto (2013)
4	*Cyperus kyllingia*	0.33	Purwanto (2013)
5	*Fimbristylis dichotoma*	0.26	Purwanto (2013)
6	*Cynodon dactylon*	0.21	Purwanto (2013)
7	*Chrysopogon aciculatus*	0.17	Purwanto (2013)
8	*Grona triflora*	0.36	Kangiras (2009)

Vegetation analysis was followed by estimating the productivity of the Javan deer feed vegetation. The productivity of Javan deer feed was calculated based on field experiments in the form of defoliation refers to Azwar et al. (2019) by modification. The experiment was carried out in two phases comprising 20 days for the first phase and then 30 days for the second one. The annual productivity was summed from the dry season and rainy season productivity. The carrying capacity was estimated according to Susetyo (1980):

CC = P×A×X/C, where CC = carrying capacity; P = productivity of feed vegetation (kg/m²/year); A = area (m²); X = correction factor (0.7); and C = 6,725 kg/individual/day (Kangiras 2009).

Analysis of Alternative Strategy

Analysis of alternative strategies is used to choose the best strategy based on priorities (Alizadeh et al. 2021). Analysis of alternative strategies was analysed qualitatively and quantitatively through the SWOT-QSPM method. SWOT is used to determine the potential of internal (Strength and Weakness) and external (Opportunity and Threat) factors in Javan deer conservation management and to design a solution strategy. Meanwhile, the QSPM (Qualitative Strategic Planning Matrix) is applied to determine the priority of the strategy to be selected based on the quantification of the strategy resulting from the SWOT analysis. The steps in the SWOT-QSPM method are as follows (Wang et al. 2020; Budihardjo et al. 2021).

Step 1: Analysing internal and external factors in the management of Javan deer conservation based on ecological data, interviews with PNR staff, and information from files and scientific literature related to Javan deer in PNR;

Step 2: Evaluating internal and external factors using IFE (Internal Factors Evaluation) and EFE (External Factors Evaluation). In IFE-EFE, factors are quantified in the form of weighting and scoring. The weights are given in the range 1-4, with the number 4 given if the SWOT factor significantly influences the management situation (Alamanda et al. 2019). The weight figures are processed by dividing the weight value of each factor by the total weight for each factor classification, namely internal (Strength + Weakness) and external (Opportunity + Threat), to produce a decimal number with a total internal and external weight that is equal to 1 (Alizadeh et al. 2021). Meanwhile, the score is filled with provisions of Strength/Opportunity, given a score range of 3 or 4, while Weakness/Threat is given a score range of 1 or 2. Values on weights and scores are multiplied to obtain a Weighted Score. The Weighted Score of Strength and Weakness are summed to obtain the Total Weighted Score of internal factors, and the Weighted Score of Opportunity and Threat are summed to obtain the Total Weighted Score of external factors. The researcher and four staff of PNR conducted the weighting, yet the score was filled in only by the researcher to establish the conservation strategy specifically related to this study.

Step 3: Total Weighted Score on internal and external factors is interpreted in the IE matrix with cells I-IX to determine the direction of the SWOT strategy (Wibowo et al. 2021).

Step 4: Formulating the SWOT strategy on the SWOT matrix. The Strength factor was crossed with the Opportunity factor to produce an S-O strategy and then crossed with the Threat factor to produce an S-T strategy. Then, the Weakness factor was crossed with the Opportunity factor to produce a W-O strategy and the Threat factor to produce a W-T strategy.

Step 5: Quantifying strategy using QSPM. The SWOT strategy is given an Attractive Score (AS) assessment heading to the SWOT factors. Then, the AS value is multiplied by the weight of each SWOT factor to obtain the Total Attractive Score (TAS). TAS are summed to produce a total value which is the priority value of the strategy.

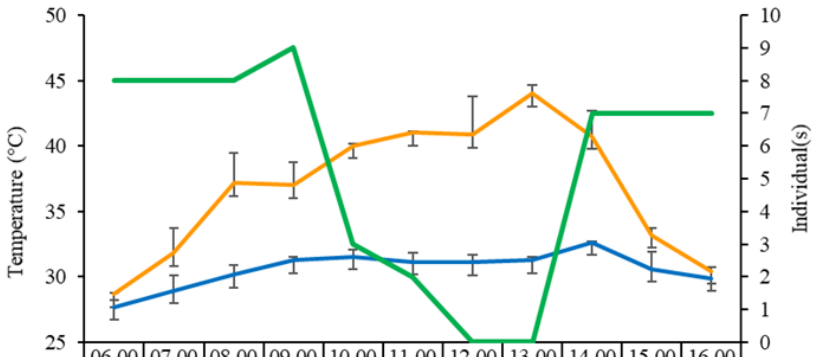
RESULTS AND DISCUSSION

Presence of Javan Deer

Observation showed that the highest number of Javan deer encountered in Cikamal was nine individuals, eight of them grouped, and another one was usually a solitary adult male. Eight individuals of the herd consisted of two adult females, a juvenile female, three juvenile males, and two fawns. The herd was less than 20% of the census which was 43 individuals with a substandard 17:14 ratio of male to female (Firdaus et al. 2022) and indicated that the majority of Javan deer present outside Cikamal were either still in the conservation area (forest area in PNR and PNTP) or roaming out of the conservation area border. The herd was often encountered in the morning (06.00-10.00), subsequently unseen as the Sun rose (10.00-14.00), and they appeared again in the afternoon (14.00-17.00). The presence of Javan deer was inversely proportional to temperature in Cikamal (Figure 2). The temperature in Cikamal was measured as having a higher mean than shaded vicinity, with the highest point reaching more than 40°C at 13.00-14.00. Javan deer and most other ungulates spend time wallowing in muddy waterholes or resting under the tree canopy when the temperature increases to avoid scorching Sun rays and to cool down the temperature of their bodies (Bismark et al. 2011; Arumugam & Buesching 2019; Selvarajah et al. 2022).

The presence of Javan deer in Cikamal was additionally influenced by human activities and feral dogs (*Canis familiaris*) intruding on the site. We found many tourists illegally came across Cikamal on the way to the waterfalls in the Southern PNR, and they intimidated the Javan deer herd. Nature reserve in Indonesia, as stated in the Regulation of the Government of the Republic of Indonesia No. 28 of 2011 concerning Management of Nature Protected Area and Nature Reserve Area, is a conservation area strictly aimed to preserve the pristine ecosystem without any kind of usage besides research and ecological monitoring. Meanwhile, feral dogs came into and trespass the conservation area, damaged the border wall and fence. As stated by PNR rangers in interviews, feral dogs continually preyed on deer and dominantly occupied Cikamal. Hence, the presence of the deer was unpredictable.

The Javan deer herd was together with a feral Balinese cow when they were in Cikamal or out of the site (Figure 3). The feral Balinese cow was the last surviving of its species since the introduction of several individuals in 2000-2010. The cow follows the deer herd wherever they browse, yet occasionally roamed solitary when feral dogs chased the Javan deer herd.



Hour interval	06.00 - 07.00	07.00 - 08.00	08.00 - 09.00	09.00 - 10.00	10.00 - 11.00	11.00 - 12.00	12.00 - 13.00	13.00 - 14.00	14.00 - 15.00	15.00 - 16.00	16.00 - 17.00
Cikamal Grassland (°C)	28.65	31.8	37.15	37	40.05	41.05	40.9	44	40.75	33.2	30.45
Forest area (°C)	27.7	28.95	30.15	31.25	31.55	31.15	31.1	31.25	32.65	30.6	29.9
Deer individual(s) in Cikamal	8	8	8	9	3	2	0	0	7	7	7

Figure 2. The presence of Javan deer and temperature in Cikamal.

Figure 3. Javan deer herd with a Balinese cow in Cikamal.

Daily Behaviour Patterns of Javan Deer

The result illustrated the daily activities of Javan deer in Cikamal without specifying the sex and ages. Data in Figure 4 indicated that Javan deer allocated their time in Cikamal mainly for feeding activities followed by alerting, inactive activities, locomoting, and other activities.

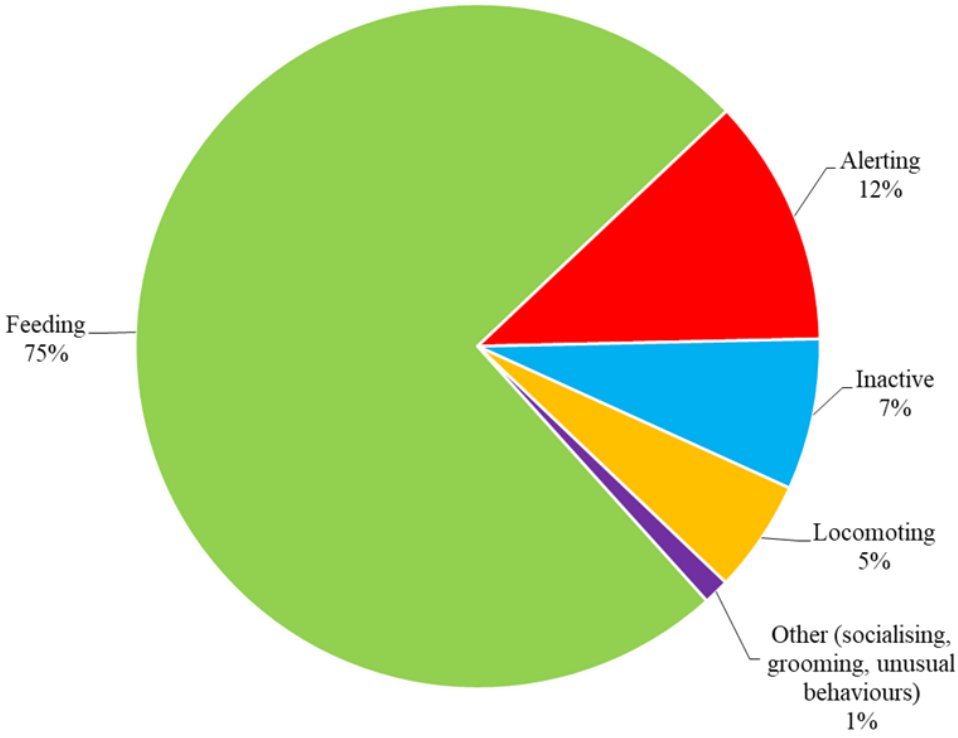


Figure 4. Activity-time budget of Javan deer in Cikamal.

Feeding activity mostly seen was picking up grass, chewing, foraging, and ruminating. It was similar to that in the outside area of Cikamal, particularly PNTP, Javan deer were spotted spending their time mostly for feeding (Withaningsih et al. 2020). In Panaitan Island of Ujung Kulon National Park, Javan deer spent time feeding (Pairah et al. 2014). The Javan deer herd in Cikamal also performed alerting primarily when the observer came to the site for the observation. The herd posed interrogat-

ing with eyes and ears focusing on observer movement and infrequently intimidated by stomping ground and vocalisation (Teichroeb et al. 2013). Instead of attacking the observer, they ran away to the dense forest if they were frightened. In contrast, the Javan deer herd in PNTP were more tolerant of humans and other distracting factors. Javan deer in PNTP tended to locomote slowly and rest after feeding (Withaningsih et al. 2020). Furthermore, Javan deer in Wan Abdul Rachman Forest Park had fed and rested respectively to spend their daytime, not to alert (Sofyan & Setiawan 2018). As confirmed by the PNR rangers and staff, the difference in behaviour between the Javan deer herd in Cikamal and PNTP classified them as inner and outer herds. The inner herd tended to be more intolerant than the outer ones. Therefore, the inner Javan deer herd roamed normally in Cikamal and dense forest in the southern part of PNR. Moreover, the inner herd comprised a smaller number of individuals and visually had slender bodies than the outer herd.

Adult females carried out more protectively aggressive behaviour concerning their fawns. They intimidated the observer dan the feral dogs if they came closer. During the lactating period, mother deer would be more vigilant to protect themselves and show their offspring how to confront predation threats (Grovenburg et al. 2009). Females with fawns dedicated plenty of time to look after them (Hunter & Skinner 1998). Therefore, adult female deer with fawn also needed more feed to provide nutrition for the young (Cook et al. 2004; Ma et al. 2011)

Javan deer and other species of Cervids are matriarchal grouping fauna (Hawkins & Klimstra 1970; van Buskirk et al. 2021). One of the adult females of the herd in Cikamal was seen as the leader, guiding the herd to what they must do and where they must go. The leader female would investigate the situation to ensure that the place was safe for the herd and might be signalling the herd earlier to anticipate a danger. The dominant female might also dominate their home range, as happened to white-tailed deer (*Odocoileus virginianus*) (van Buskirk et al. 2021), mule deer (*Odocoileus hemionus*) (Roerick et al. 2019), and roe deer (*Capreolus capreolus*) (Maublanc et al. 2012).

Inactive behaviour, locomoting, and other activities, including socialising, fighting, rutting, grooming, urinating, and defecating, were not seen as often as feeding and alerting. Javan deer herd in Cikamal showed those minor percentage behaviours roughly once in one-or-more hour interval monitoring. Moreover, the herd was observed doing minor percentage behaviours while feeding. Performing a behaviour while doing other behaviour also occurred in other species, as well as pampas deer (*Ozotoceros bezoarticus*) (Aniano & Ungerfeld 2020), musk deer (*Moschus berezovskii*) (Yang et al. 2020), and red deer (*Cervus elaphus*) (Churski et al. 2021). Javan deer also performed minor seasonal behaviours, for example, rutting. Rutting or mating behaviour would be fighting among adult males (Powell & Evans 2019; de la Peña et al. 2021).

The daily time budget of the inner Javan deer herd is distributed stably in the ethogram (Figure 5). In Figure 2, deer were seen once at 11.00-12.00 during observation, yet it was less than 10 minutes and thereby the behaviour was not qualified to be shown in the ethogram. At that time, the feral dogs run after the deer herd provoking chaos in Cikamal and breaking up the herd. Thus, the behaviour pattern at that time was not recorded. Feeding behaviours constantly dominated the ethogram with the green bar.

Meanwhile, alerting mode (red bar) towered up at 06.00-07.00, 10.00-11.00, and 14.00-15.00, pressing down the feeding-time budget as it was also often affected by the barking of feral dogs in the surrounding

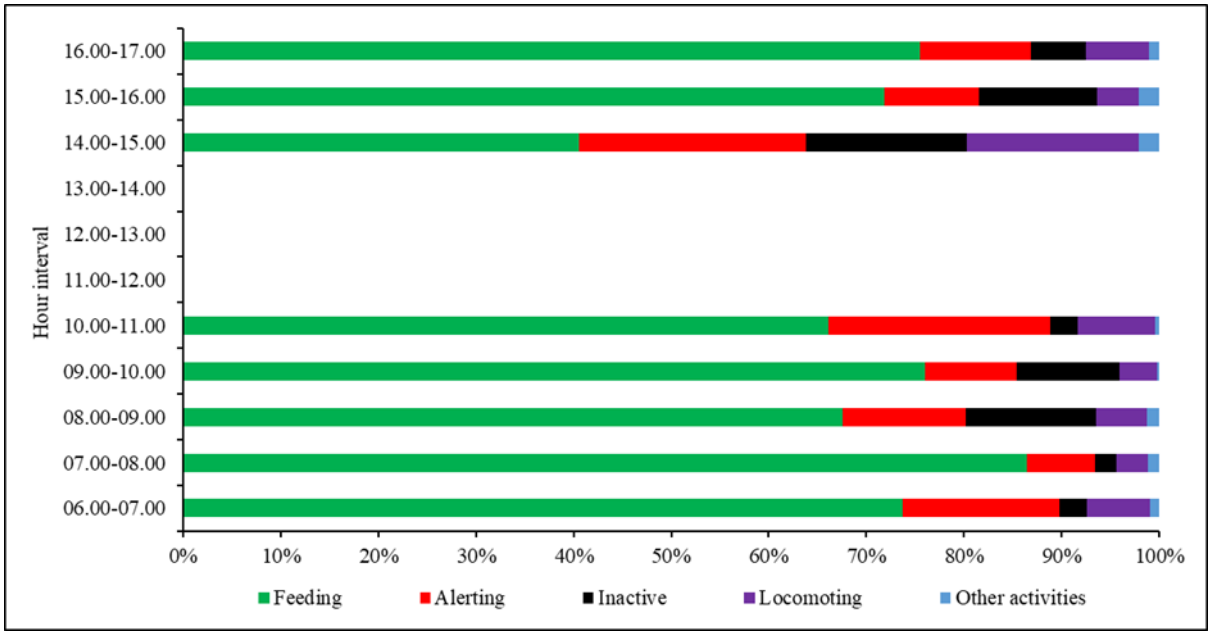


Figure 5. Daily time budget of Javan deer in Cikamal.

area. The increasing alerting behaviour in prey also happened to African Wildebeests when they heard a lion roar (Dannock et al. 2019). Naturally, Javan deer in PNR had no predator, neither dholes nor Javan leopard, yet feral dogs became the one. The intensity of the presence of predators in a territory could influence the prey's home range and diet palatability (Gower et al. 2008; Esparza-Carlos et al. 2016; Mumma et al. 2018; Churski et al. 2021; Gigliotti et al. 2021).

Potency of Javan Deer Habitat

The Javan deer grazing areas at Rengganis, PNR Office, Ciborok, and Cikamal have the same characteristics, especially understorey vegetation that dominates. Table 2 indicates *Cynodon dactylon* dominates the grazing area in PNTP and PNR. Meanwhile, the dominance of other species varies across the four research areas.

C. dactylon and *Imperata cylindrica* dominated Cikamal with a 33% difference, while *Axonopus compressus* was observed to dominate the PNR Office area (percentage difference around 58.5%) and Ciborok (percentage difference around 23.44%). Meanwhile, *Cyperus kyllingia* and *Zyphyranthes* sp mainly were found in the grassy area of Rengganis, although the per-

Table 2. Percentage of understorey plants in grazing areas of PNR.

Species	Category	Cikamal*	Rengganis	PNR Office area	Ciborok
Cynodon dactylon	Feed plant	53.00%	89.17%	78.50%	48.44%
Axonopus compressus	Feed plant	0.13%	-	20.00%	25.00%
Chrysopogon aciculatus	Feed plant	6.40%	-	-	10.94%
Grona triflora	Feed plant	0.60%	2.50%	0.50%	15.63%
Cyperus kyllingia	Feed plant	-	4.17%	0.50%	-
Abildgaardia ovata	Feed plant	8.20%	-	-	-
Zephyranthes sp.	Non-feed plant	-	4.17%	0.50%	-
Imperata cylindrica	Non-feed plant	20.00%	-	-	-
Cyperus rotundus	Non-feed plant	5.67%	-	-	-
Oldenlandia sp	Non-feed plant	3.00%	-	-	-
Chromolaena odorata	Non-feed plant	1.67%	-	-	-
Blumea balsamifera	Non-feed plant	0.20%	-	-	-
Unvegetated area	-	1.13%	-	-	-

*Source: Firdaus et al. (2022).

centage of coverage was significant, around 85%. In several other conservation areas in Indonesia, *C. dactylon*, *A. compressus*, and *I. cylindrica* also grow invasively. *C. dactylon* also dominates open areas in the Wan Abdul Rachman Forest Park, Lampung (Nurseba et al. 2020). *C. dactylon*, *A. compressus*, and *I. cylindrica* also dominate grassland areas in Wasur National Park (Hariadi & Sraun 2014), Ujung Kulon National Park (Sudibyo 2015), and Way Kambas National Park (Yanti et al. 2017).

The dominance of *C. dactylon* is caused by several factors, including resistance to disturbances, such as stamping and cutting, because it has rhizomes that can regenerate, providing more significant opportunities for *C. dactylon* to grow back spread to the surrounding area (Zwerts et al. 2015). Grasses and other creeping undergrowth, including *A. compressus*, *I. cylindrica*, *Abildgaardia ovata*, *Chrysopogon aciculatus*, *Cyperus kyllingia* and *Cyperus rotundus*, thrive because they have rhizome and light seed structure that can easily be carried by the wind and stick to the body surface. animals and humans (Simpson 2010; Setyawati et al. 2015; Schweingruber & Berger 2019). Other vegetation, namely *Grona triflora* and *Oldenlandia* sp, spread rapidly with vegetative propagation in the form of stolons (Setyawati et al. 2015; Silalahi & Mustaqim 2021). Meanwhile, *Blumea balsamifera* and *Chromolaena odorata* have light flower structures that are easily carried away by water currents and wind gusts, and both are tolerant of land damage (Setyawati et al. 2015; Guan et al. 2022).

Field experiments conducted in Cikamal, Rengganis, PNR Office area, and Ciborok Area resulted in total productivity data of 93,826 kg/year with a grazing area of 5.61 ha and an estimated habitat carrying capacity of 23 heads/year. The carrying capacity of the grazing area habitat in PNR/PNTP has not been able to support life of the population of Javan deer. The population of Javan deer that exceeds the carrying capacity of its habitat is caused by the activities of the Javan deer, which most of the population roam outside the PNR/PNTP area and obtain sufficient feed from various types of feed and places. Details of the plant productivity and the carrying capacity of their habitat are in Table 3.

Table 3. Feed plant productivity in grazing areas of PNR.

Location	Area (ha)	Productivity ± StD (kg/year)	Carrying capacity (head(s)/year)
Rengganis	0.15	5,976.25 ± 900.53	2
PNR Office	0.36	7,582.79 ± 497.90	2
Ciborok	0.67	16,859.19 ± 2,199.69	1
Cikamal Grassland*	4.43	63,407.93 ± 12,696.99	18

*Source: Firdaus et al. (2022).

The carrying capacity of the Javan deer habitat in PNR/PNTP was threatened to decrease along with land cover changes in the area. In 1970s, 18 ha in Cikamal, 8 ha in Nanggorak, and 15 ha in Badeto were all grazing areas (Sumardja & Kartawinata 1977), then in 2011 were 3 ha, 0 ha, and 0 ha, respectively (Rosleine & Suzuki 2012). In 2021, 4.4 ha in Cikamal was still grassland (Firdaus et al. 2022). The grazing areas in PNTP were threatened by the shade of the tree canopy around the grassy area and the distribution of forest vegetation seedlings (Kangiras 2009). Meanwhile, the carrying capacity of Cikamal was threatened by invasive vegetation growth, especially teak (*Tectona grandis*) planted in 1932 and 1936 (Nakazono 2012). Broad teak leaves prevent sunlight from reaching the forest floor, limiting shade-intolerant plants' growth and the grassland's dominant population (Behera et al. 2015). Changes in pasture land

cover by succession have also occurred in various conservation areas in Indonesia, including the Bekol Savana in Baluran National Park which was threatened by the invasion of *Acacia nilotica* (Istomo & Farida 2017; Muis et al. 2018) and the succession of the Cigenter Grassland in Ujung Kulon National Park *Arenga obtusifolia* (Febriana et al. 2019). Natural succession in forest ecology is a positive dynamic. Conversely, it is a threat when analysed from the perspective of grassland ecology where the climax phase is land fires, and the existence of grasslands and their reforestation will always occur as part of the dynamics of nature even though these dynamics differ in time and place (Oliveras & Malhi 2016).

Alternative Strategy to Improve Javan Deer Conservation

Internal Factor Analysis

In this section, besides showing the ecological aspects, we also showed the management implication of Javan deer and the habitat conservation performed by the PNR staff. Internal factor analysis is presented as an IFE table, as shown in Table 4. In this research, ten internal factors are based on the ecological aspects and management of Javan deer conservation in PNR. The strength factor consists of two factors, both of which are ecological aspects, while the weakness factor consists of eight factors consisting of three factors from the ecological aspect (W1, W2, W3) and five factors from the conservation management aspect (W4, W5, W6, W7, W8). The highest point for internal factors is S1, with a value of 0.44. The S1 factor was supported by the statement that Javan deer conservation in PNR has been ongoing since 1921 by the Dutch Colonial Government (Sumardja & Kartawinata 1977; Rosleine & Suzuki 2012), indicating that PNR conditions are suitable as a habitat for Javan deer. Meanwhile, the total IFE value for Javan deer conservation in PNR is 2.10.

External Factor Analysis

External factors were analysed using the EFE table as shown in Table 5. This study analysed ten external factors, three of which are opportunity factors and the other seven are threat factors. The opportunity factors collected are factors in the management aspect, while the threat factors consist of three factors in the ecological aspect (T1, T2, T3) and four in

Table 4. Internal Factor Evaluation.

Code	Factor	Weight	Score	Weighted Score
Strength				
S1	Geographical and climatic conditions in PNR/PNTP are suitable as a habitat for Javan deer	0.11	4	0.44
S2	There is no territorial competition between Javan deer and other herbivores	0.07	3	0.22
Weakness				
W1	The substandard ratio of male to female Javan deer	0.09	1	0.09
W2	The inadequate ecological carrying capacity of Javan deer habitat	0.11	2	0.22
W3	The changes in Javan deer behaviour patterns inside and outside PNR	0.11	1	0.11
W4	High cost for the conservation management	0.12	2	0.24
W5	Unskilled staff theoretically and practically	0.09	2	0.18
W6	Destruction of conservation-supporting facilities and infrastructures	0.11	2	0.22
W7	There was no agreement on the status of the Javan deer in conservation management between PNR and PNTP	0.11	2	0.22
W8	Undeveloped conservation management of Javan deer	0.08	2	0.16
	Total	1		2.10

the conservation management aspect (T4, T5, T6, T7). The external factor with the highest value is O1 with a total of 0.44 because the majority of the ecological data that forms the basis for Javan deer conservation in PNR is data obtained from academic research. Meanwhile, the total value of the analysed EFE is 2.31, which is greater than the total value on the internal aspect.

Analysis of SWOT Strategy

The IFE and EFE values are combined to obtain a point on the internal-external matrix as the direction of strategy formulation that must be planned. With an IFE value of 2.10 and EFE of 2.31, the position of the Javan deer conservation in PNR is in cell V (Figure 6). Therefore, the direction of the conservation strategy was to hold and maintain that could be in the form of integrative renewal or improvisation (intensification) of the existing management system (David 2011).

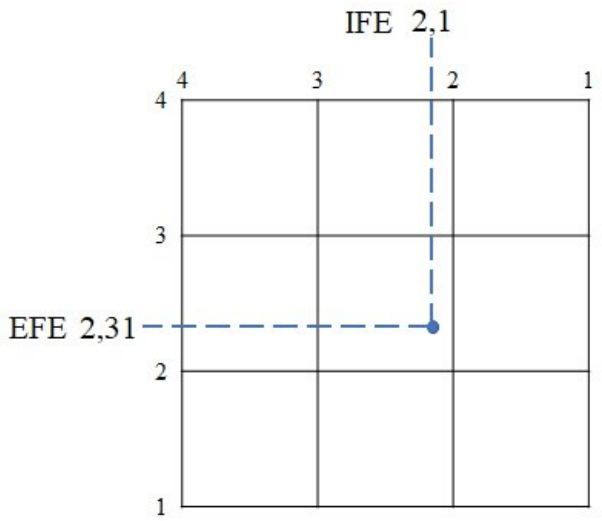


Figure 6. Internal-External matrix.

Table 5. External Factor Evaluation.

Code	Factor	Weight	Score	Weighted Score
Opportunity				
O1	The role of academics and researchers in the research and development of Javan deer and their habitat	0.11	4	0.44
O2	Local community participation in Javan deer conservation outside the PNR	0.10	3	0.30
O3	Availability of data and literature from Javan deer research in PNR as a guide for conservation management	0.10	4	0.40
Threat				
T1	Disturbance of wild dogs that prey on Javan deer	0.10	2	0.20
T2	Destruction of habitat, facilities, and conservation-supporting infrastructures by local communities and tourists	0.09	2	0.18
T3	Distribution of non-feed invasive vegetation species that can restrict the growth of the feed plant	0.13	1	0.13
T4	Delays in the process of applying for funding for Javan deer conservation	0.10	2	0.20
T5	Natural destruction of conservation-supporting infrastructures	0.10	1	0.10
T6	Priority conflict between conservation management of Javan deer and other species	0.07	2	0.14
T7	There is no contribution from external non-governmental stakeholders or institutions (NGO) to Javan deer conservation	0.11	2	0.22
	Total	1		2.31

The strategy formulated based on internal and external factors is displayed in the SWOT matrix. The number of alternative strategies obtained was six strategies. Each strategy has the possibility of a solution to several internal and external factors. The correlation between solutions and SWOT factors was shown by writing the factor code at the end of each strategy in Table 6.

The SWOT strategy matrix produced six alternative strategies for Javan deer conservation in PNR. Some of the strategies in Table 6 combined several internal and external factors, intending that alternative strategies can integrate all potentials to be more effective and efficient. Furthermore, the six strategies obtained were quantified in the QSPM matrix (Table 7) by analysing the correlation and influence of each strategy on internal and external factors. The correlation value of each strategy was displayed in the Attractive Score column and multiplied by the weight of each SWOT factor as contained in the IFE-EFE. The six alternative strategies were given a strategy code as follows:

A. improving the conservation management of Javan deer and its habitat;
B. improving facilities and the management system of those facilities and conservation-supporting infrastructures;
C. improving the capability of PNR staff theoretically dan practically;
D. collaboration with researchers to perform some research and innovations for the Javan deer conservation;

Table 6. SWOT analysis matrix for Javan deer conservation strategies.

Internal / External	Strength (S)	Weakness (W)
	S1, S2	W1, W2, W3, W4, W5, W6, W7, W8
Opportunity (O)	S-O Strategies	W-O Strategies
O1, O2, O3	• Educating and empowering the local people in terms of Javan deer conservation (S1, O1, O2) • Collaboration with researchers to perform some research and innovations for Javan deer conservation (S1, S2, O1, O2, O3)	• Improving the capability of PNR staff theoretically dan practically (W1, W2, W3, W4, W5, W8, O1, O2, O3) • Collaboration with researchers to perform some research and innovations for Javan deer conservation (W1, W2, W3, W4, W5, W7, W8, O1, O2, O3) • Improving the conservation management of Javan deer and its habitat (W1, W2, W3, W8, O1, O2, O3) • Improving facilities and the management system of those facilities and conservation-supporting infrastructures (W4, W5, W6, O1, O3) • Considering the management status of Javan deer in the conservation management of PNR and PNTP (W1, W2, W3, W4, W5, W6, W7, W8, O1, 03)
Threat (T)	S-T Strategies	W-T Strategies
T1, T2, T3, T4, T5, T6, T7	• Educating and empowering the local people in terms of Javan deer conservation (S1, T1, T2) • Improving the conservation management of Javan deer and its habitat (S1, S2, T1, T3, T4, T6, T7) • Improving facilities and the management system of those facilities and conservation-supporting infrastructures (S1, T1, T2, T3, T4, T5, T7)	• Improving the capability of PNR staff theoretically dan practically (W1, W2, W3, W4, W5, W7, W8, T1, T2, T3, T4, T5, T6, T7) • Improving the conservation management of Javan deer and its habitat (W1, W2, W3, W8, T1, T3, T4, T6, T7) • Improving facilities and the management system of those facilities and conservation-supporting infrastructures (W4, W5, W6, W7, T1, T2, T3, T4, T5, T7) • Considering the management status of Javan deer in the conservation management of PNR and PNTP (W1, W2, W3, W4, W5, W6, W7, W8, T2, T3, T4, T6, T7)

E. educating and empowering the local people in terms of the Javan deer conservation;
F. considering the management status of the Javan deer in the conservation management of PNR and PNTP.

The QSPM matrix sorted strategies based on the priority as quantified in TAS values.

1. Considering the management status of the Javan deer in the conservation management of PNR and PNTP.

Pangandaran Nature Conservation Agency (hereafter "PNCA") manages PNR, while Perum Perhutani manages PNTP. The difference between the managers indirectly abstracts the status of Javan deer conservation management. So far, the Javan deer is fully managed by the PNCA, even though the Javan deer often wander in the PNTP and become one of the tourist attractions in that place. Therefore, the Javan deer conservation management system must ensure whether the Javan deer is only a protected animal in the PNR or at the same time as part of a tourist attraction in PNTP. This strategy is essential to also determine the duties of internal stakeholders (PNCA and *Perum Perhutani*) in preserving the Javan deer and their habitat management.

1. Improving the conservation management of Javan deer and its habitat;

PNCA had activities to conserve Javan deer in PNR, mainly managing Javan deer which were outside the Javan deer area. However, improvement in the conservation management system is needed so that the problem of the Javan deer can be ecologically resolved. Two implementation steps in improvising Javan deer conservation management: 1) Javan deer population census periodically to ensure population growth and encounters of Javan deer; 2) intensification and extensification of the Javan deer habitat, mainly in Cikamal (Firdaus & Sakenia 2021).

Table 7. Quantitative Strategic Planning Matrix (QSPM).

Factor code & Weight		(A)		(B)		(C)		(D)		(E)		(F)	
		AS	TAS	AS	TAS	AS	TAS	AS	TAS	AS	TAS	AS	TAS
S1	0.11	4	0.44	4	0.44	1	0.11	4	0.44	1	0.11	4	0.440
S2	0.07	2	0.144	1	0.072	1	0.072	3	0.216	1	0.072	4	0.288
W1	0.09	4	0.36	1	0.09	2	0.18	4	0.36	1	0.09	3	0.270
W2	0.11	4	0.44	1	0.11	4	0.44	4	0.44	1	0.11	4	0.440
W3	0.11	4	0.44	4	0.44	4	0.44	4	0.44	4	0.44	4	0.440
W4	0.12	4	0.48	4	0.48	2	0.24	1	0.12	1	0.12	4	0.480
W5	0.09	4	0.36	4	0.36	4	0.36	4	0.36	2	0.18	4	0.360
W6	0.11	2	0.22	4	0.44	4	0.44	1	0.11	4	0.44	3	0.330
W7	0.11	4	0.44	4	0.44	2	0.22	3	0.33	4	0.44	4	0.440
W8	0.08	4	0.32	4	0.32	4	0.32	4	0.32	1	0.08	4	0.320
O1	0.11	4	0.43	3	0.323	3	0.323	4	0.43	4	0.43	4	0.430
O2	0.10	2	0.195	1	0.098	4	0.39	4	0.39	4	0.39	2	0.195
O3	0.10	4	0.387	3	0.29	4	0.387	4	0.387	3	0.29	4	0.387
T1	0.10	4	0.392	4	0.392	4	0.392	2	0.196	4	0.392	4	0.392
T2	0.09	4	0.366	4	0.366	4	0.366	1	0.092	4	0.366	2	0.183
T3	0.13	4	0.506	1	0.127	4	0.506	4	0.506	1	0.127	3	0.380
T4	0.10	4	0.403	4	0.403	3	0.302	3	0.302	1	0.101	4	0.403
T5	0.10	1	0.098	4	0.393	1	0.098	1	0.098	3	0.295	1	0.098
T6	0.07	4	0.265	4	0.265	4	0.265	4	0.265	1	0.066	4	0.265
T7	0.11	2	0.228	4	0.456	2	0.228	4	0.456	1	0.114	4	0.456
Total			6.915		6.305		6.08		6.259		4.654		6.998

3. Improving facilities and the management system of those facilities and conservation-supporting infrastructures

PNR's infrastructure and facilities significantly influence Javan deer conservation activities. It is not only the form of procurement of utilization and essential infrastructure and facilities but also their management. The proposed implementation based on this research is an investigation/ inventory of the condition of facilities and infrastructure, followed by the procurement of facilities and development and infrastructure planning.

4. Collaboration with researchers to perform some research and innovations for the Javan deer conservation

PNR has become a research location for various fields, especially conservation. Field research data from researchers and agencies from different years can be used as the basis for conservation development for PNR conservation as a whole and specifically for Javan deer. The popularity of PNR as a research area has excellent potential to collaborate with researchers to improve the quality of Javan deer conservation. The research process by academics and researchers also provides opportunities for PNR staff to participate. It possibly can increase their capabilities in collecting and processing field data.

5. Improving the capability of PNR staff theoretically dan practically

The availability of facilities, infrastructure, and research data cannot fully support Javan deer conservation activities in PNR. PNR staff's ability to use the latest technology tools, process field data, and interpret conservation management results is needed to optimize other strategies in Javan deer conservation management. Training and certification are needed to improve the ability of PNR staff to operate high-tech equipment, to be able to conduct field observations and process ecological data, conservation management, as well as data interpretation and reporting on conservation management.

6. Educating and empowering the local people in terms of the Javan deer conservation

The people who are the main targets of this strategy are residents around PNR and PNTP tourists. The surrounding community is PNR's closest partner in dealing with Javan deer that roam outside the PNR area. Technically, population empowerment will be minimized along with improving the Javan deer habitat and infrastructure in PNR that focus on the activities of the Javan deer in the conservation area. Meanwhile, the implementation of the tourist empowerment strategy in PNTP must still be carried out considering that PNTP is one of the sources of income for area conservation management in PNR/PNTP; alternative tourism for tourists; and a source of income for the surrounding population (Dhalyana & Adiwibowo 2013). Based on field data, the proposed implementation form for this strategy is the establishment of regulations that are more binding on tourists in relation to nature and Javan deer conservation and discussion accompanied by coordination with residents regarding the Javan deer that roam in tourist beach areas and residential areas.

CONCLUSION

Six alternative strategies were obtained based on the analysis of internal and external factors on the ecological aspects and conservation management, as well as quantification using the QSPM method. Strategies can be used as consideration for PNR in preparation of strategic conservation plans in PNR, identification of internal and external factors that affect conservation management, and determination of long-term vision and mission. Data updates must always be carried out based on the planning,

organizing, actuating, and evaluating points of each management period to develop the conservation management strategy in the future.

AUTHORS CONTRIBUTION

F.I.F. designed the research, collected and analysed data, and wrote the manuscript. R.R.I. and E.S. supervised the research and corrected the manuscript.

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CONFLICT OF INTEREST

The authors confirm no conflict of interest in this research.

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Research Article

Phytoplankton Diversity as a Bioindicator of Water Quality Mangrove Ecosystems in Clungup Mangrove Conservation, Kondang Merak and Sempu Island, Malang Regency

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ABSTRACT
Local community groups have handled damage to the mangrove ecosystem on the coast of South Malang by carrying out restoration. The purpose of this study was to evaluate water quality in the restoration mangrove ecosystem based on phytoplankton diversity as bioindicators. A water and phytoplankton sampling was repeated three times with a depth of about 10-15 cm (below the surface water) at each location consisting of 4 restored mangrove ecosystems in Clungup Mangrove Conservation (CMC) and Kondang Merak as well as one natural mangrove ecosystem in Teluk Semut, Sempu Island, Malang Regency. Water quality parameters include water temperature, air temperature, conductivity, pH, Dissolved Oxygen (DO), Biochemical Oxygen Demand (BOD), nitrate, and orthophosphate content. The biotic index includes the Trophic Diatom Index (TDI) as an indicator of water nutrient content and Percentage Pollution Tolerance Value (%PTV) as an indicator of organic pollution. The water quality in the five mangrove ecosystems of CMC, Kondang Merak, and Teluk Semut has met the water quality standard for marine biota except for DO, nitrate, and orthophosphate content in several locations. Water quality in five mangrove ecosystems CMC, Kondang Merak, and Teluk Semut based on phytoplankton indicators did not show any contamination with toxic materials (H'). Based on TDI, it is categorized as eutrophic – hypereutrophic, except at the reference site of Teluk Semut mangrove; based on PTV polluted with moderate to high organic matter except at the reference site locations, namely Teluk Semut, and CMC 2. Thus, a location that has good phytoplankton bioindicators is Teluk Semut.

INTRODUCTION
Mangrove vegetation combines coastal and lowland plant communities in tidal or brackish areas (Serosero et al. 2020). Mangrove vegetation is the most productive ecosystem and has high economic value as building materials, medicines, industrial raw materials, and food ingredients (Giri et al. 2008; Khairnar et al. 2013). Mangrove ecosystems also have an ecological function to protect the coast from abrasion, a source of germplasm, prevent seawater intrusion, and provide a place to live for aquatic, land, and air biota (Asuk et al. 2018; Saputra et al. 2020). Howev-

er, rampant human disturbances in the mangrove ecosystem, such as exploitation of biota, logging, industry, shrimp ponds, and agriculture, have reduced most of the mangrove forest area (Malik et al. 2015; Nichols et al. 2019; Bao et al. 2020).

Mangrove forest covered 18,253,871 hectares in East Java in 2013 (Saputro et al. 2009). 344 hectares located on the southern shore of Malang Regency (Imaduddien & Krisnadi 2020). Mangrove forests of south Malang are scattered on Kondang Merak, Balekambang, Clungup Mangrove Conservation (CMC), Sendang Biru, and Sempu Island. From 1998 to 2010, the mangrove forests that suffered the worst damage were in the Kondang Merak Beach, Sendang Biru, and Clungup Mangrove Conservation areas. The damage was caused by land conversions, such as forest fires, tourist attractions, plantations, and agriculture (Ridhoi et al. 2020; Rudianto et al. 2020). Continuous damage to mangrove ecosystem will cause a decrease in ecosystem services. The decline in ecosystem services reduces the biophysical quality of mangrove forest ecosystems and the surrounding environment, such as loss of habitat for biota, coastal abrasion, flooding, and decreased water productivity (Rahmania et al. 2019). Restoration is one of the best solutions to this problem (Amalia et al. 2018). Restoration is a program of planting or rearranging damaged ecosystems back to their original functions, such as ecosystems at the reference site (López-Portillo et al. 2017). One example of a beach implementing a mangrove restoration program is CMC Beach and Kondang Merak Beach. CMC Beach restoration began in 2005, while at Kondang Merak Beach, it began in 2008 (Hakim et al. 2017; Ridhoi et al. 2020). To find out whether the quality of the mangrove ecosystem is good or bad, an unspoiled comparison location is used, namely Teluk Semut mangrove ecosystem in Sempu Island. Sempu Island is an area that has a natural mangrove ecosystem and is a protected area as a Nature Reserve (Hakim et al. 2017). The success of mangrove ecosystem restoration can be evaluated by monitoring mangrove ecosystem services, one of which is using the assessment of supporting services. The assessment may include measurement of the water physicochemical, whereas the biological quality can be assessed using community structure and phytoplankton diversity as bioindicators.

Phytoplankton is microorganisms that live passively floating in the waters. Phytoplankton in aquatic ecosystems plays a role as the primary source of producers, regulating nutrient cycles, stabilizing marine sediments, and utilizing organic matter (Effendi et al. 2016; Hilmi et al. 2020; Inyang & Wang 2020). Phytoplankton can be a bioindicator because it has a short life cycle and can respond quickly to environmental changes (Hilmi et al. 2020; Febriansyah & Retnaningdyah 2021). Phytoplankton survival is supported by good and measurable physicochemical quality of water, including pH, DO (Dissolved Oxygen), BOD (Biochemical Oxygen Demand), conductivity, temperature, and turbidity (Singh et al. 2017). Therefore, it is necessary to evaluate the mangrove ecosystem's water quality based on the phytoplankton community's structure as a bioindicator in Clungup Mangrove Conservation, Kondang Merak, and Sempu Island, Malang Regency, East Java.

MATERIALS AND METHODS

Study area

The research was carried out from September to December 2021. The location of water and phytoplankton sampling was carried out in the mangrove ecosystem at Clungup Mangrove Conservation (CMC), Kondang Merak Beach, and Sempu Island, Malang Regency, East Java

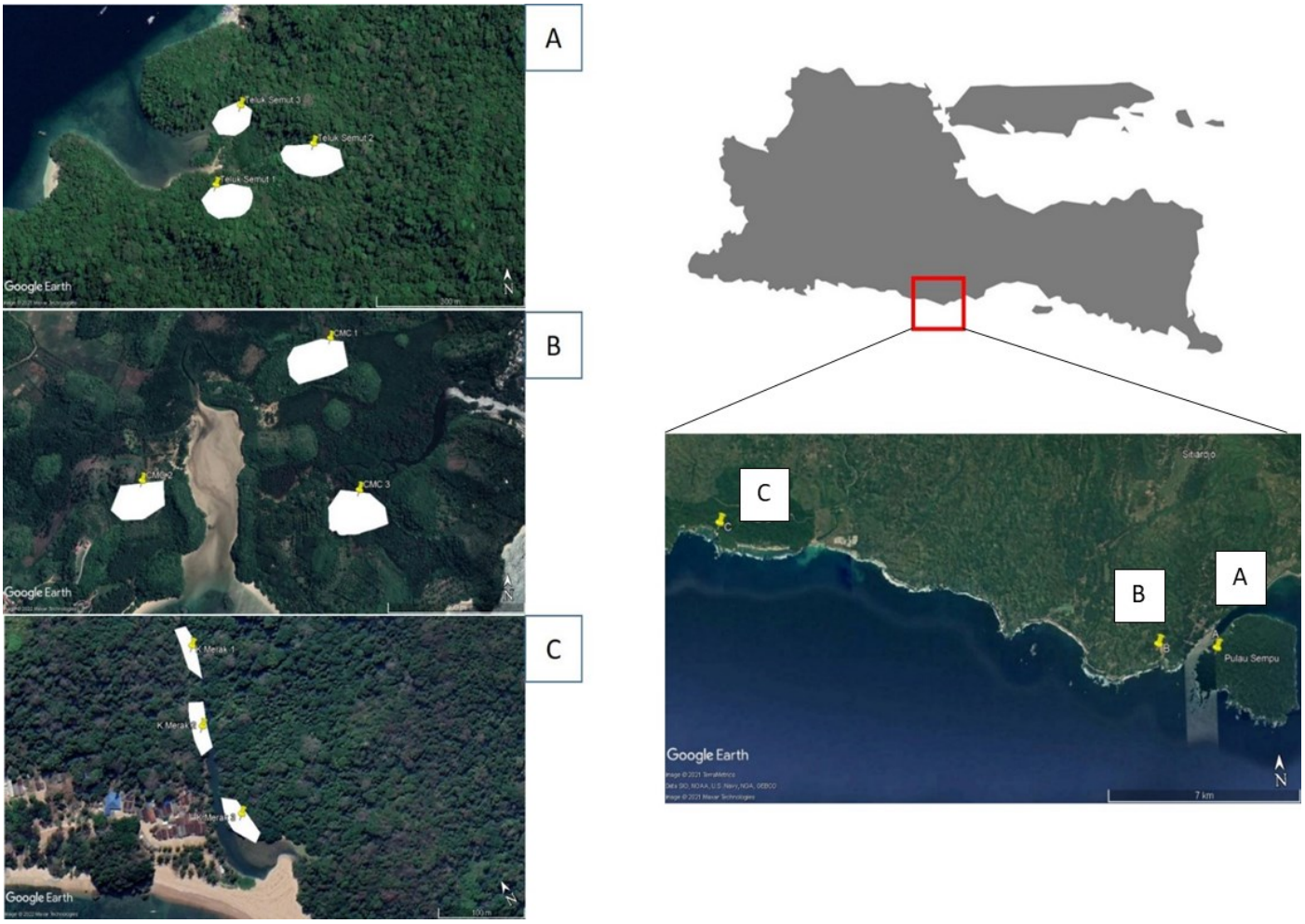

Figure 1. Sampling location at the coast of South Malang. (Note: A = Teluk Semut (Sempu Island); B = Clungup Mangrove Conservation; C = Kondang Merak).

(Figure 1). Phytoplankton identification was conducted at the Laboratory of Ecology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, East Java.

Sampling locations at the Clungup Mangrove Conservation (CMC) area was carried out at three different stations, namely CMC1 (the results of the restoration in 2015), CMC2 (the mangrove ecosystem remaining from the 2008 fires, which were restored periodically), and CMC3 (the natural mangrove ecosystem). The research location at Kondang Merak Beach consists of only one site, namely the mangrove ecosystem which was rehabilitated in 2019. A sampling at the Sempu Island mangrove ecosystem was carried out at Teluk Semut. Teluk Semut is the Reference site in this study because it has a natural mangrove ecosystem and is protected as a Natural Reserve. The water and phytoplankton samplings were carried out in triplicates at each location.

Phytoplankton Sampling, Identification, and Counting

Phytoplankton samples were taken by filtering 4 liters of water at a depth of ± 15 cm (below the water surface) using a plankton net. The phytoplankton sample was transferred to a sample bottle, then 1 mL of 4% formalin and 0.5 mL of $CuSO_4$ were added. Phytoplankton observations were done by dropping 1 mL of sample water into the Sedgewick-Rafter cell counting chamber. Next, the sample was observed under a light microscope with a magnification of ×200 (APHA 2005). Identification of phytoplankton by comparing the species observed with the images in the identification manual (Gell et al. 1999; Du Buf & Bayer 2002; Van

Vuuren et al. 2006; Bellinger & Sigee 2010). Phytoplankton samples were observed in 500 boxes in the Sedgewick Rafter Chamber which were counted at each station. Cell density was calculated according to the equation (Effendi et al. 2016):

$$K = \frac{1}{A} \times \frac{B}{C} \times \frac{V}{v} \times n$$

Notes:
K : phytoplankton abundance (ind/L);
A : volume of filtered water sample (L);
B : total area/container area of Sedgwick-Rafter Counting Cell (mm^2);
C : observation area (mm^2);
V : volume of filtered water (mL);
v : concentrate volume of Sedgwick Rafter Counting Cell (mL);
n : number of observed phytoplankton

Water Sampling and Measurement of Water Physicochemical's Parameters

Water physicochemical's parameters were measured at each specified location. 1.5 L of water samples were taken using a water sampler at a depth of ± 15 cm (below the water surface). The depth is only around the water surface because the water depth at each location is shallow. The parameters measured consisted of physical and chemical properties of water, namely water temperature, air temperature, pH, conductivity, Dissolved Oxygen (DO), Biochemical Oxygen Demand (BOD), nitrate, and orthophosphate content (Table 1).

Table 1. Physicochemical parameters of water with its measurement method.

Parameter	Unit	Tool/Method
Water temperature	°C	Thermometer
Air temperature	°C	Thermometer
pH	-	pH meter
Conductivity	Siemens/meter	Conductivity meter
DO	mg/L	DO meter
BOD	mg/L	Winkler Method
Nitrate	mg/L	Colorimetric
Orthophosphate	mg/L	Colorimetric

Data Analysis

Analysis of the profile of community structure and phytoplankton diversity included The Importance Value Index (IVI), Shannon-Wiener Diversity Index (H'), Dominance Index (Id) and Evenness Index (E) were done based on the formula from Wu et al. (2014). Analysis of physical, chemical, and biological parameter data at each location was carried out using descriptive analysis (minimum & maximum values). In addition, to determine the correlation between the physicochemical properties of water and the biotic index, a biplot analysis was performed using PAST 16.0 software.

Analysis related to community structure and phytoplankton diversity calculated, among others, Importance Value Index (IVI), Simpson Dominance Index (Id), Evenness Index (E), Shannon-Wiener Index and Diversity (H'). Analysis of phytoplankton data related to the level of pollution of organic matter in the waters uses the biotic index of phytoplankton, namely the Trophic Diatom Index (TDI), and % Pollution Tolerant Value (% PTV). Trophic Diatom Index (TDI) is an index to determine the level of nutrients in the waters. Species counted in TDI analysis, i.e. species included in diatoms only based on Kelly & Whitton (1995).

The equation used to determine the TDI value index (Wu et al. 2014):

$$TDI = (WMS \times 25) - 25$$

Where, WMS is the weighted average sensitivity and can be obtained from the following formula:

$$WMS = \sum_{i=1}^{n} (ai \times si \times vi) / \sum_{i=1}^{n} (ai \times vi)$$

Notes:
WMS: weighted mean sensitivity
a_i : proportion of all individuals in a sample that belong to species i
s_i : pollution sensitivity (1-5) of species i
v_i : indicator values (1-3) of species i
n : total number of species in a sample (based on Kelly & Whitton 1995)

PTV is an index to determine the level of organic matter pollution in the waters. The equation used to determine the PTV value index (Kelly & Whitton 1995):

$$\%PTV = \frac{Abundance\ of\ tolerant\ taxa}{total\ taxa\ abundance}$$

The %PTV value was calculated based on comparing the abundance of tolerant diatoms (*Gomphonema* sp., *Navicula* spp., *Sellaphora* spp., and *Nitzschia* spp.) with the number of diatoms obtained (Kelly & Whitton 1995).

RESULTS AND DISCUSSION

Water Quality Profile Based on Water Physicochemical Parameters in CMC Mangrove, Kondang Merak, and Teluk Semut

Measurement of water physicochemical's parameters in mangrove CMC, Kondang Merak, and Teluk Semut included of water temperature (°C), air temperature (°C), pH, conductivity (S/m), DO (mg/L), BOD (mg/L), nitrate (mg/L), and orthophosphate content (mg/L) (Table 2). Our results showed that the water temperature and air temperature at the 5 locations of sampling were met the water quality standards for marine biota in mangrove based on government rules (PP NO.22/MENLH/2021), namely 28-32°C. According to Pourafrasyabi & Ramezanpour (2012), the optimal temperature that can affect plankton growth ranges from 25 -30° C.

Table 2. The profile of physicochemical water quality in CMC, Kondang Merak, and Teluk Semut.

Location	Physicochemical Parameters (min-max)							
	WT (°C)	AT (°C)	pH	Cond (S/m)	DO (mg/L)	BOD (mg/L)	NL (mg/L)	OL (mg/L)
CMC 1	25-27	27-29	7.07-7.36	5.4-5.64	3.23-3.87	6.16-8.44	0.023-0.181	0-0.0101
CMC 2	25-26	27-29	7.6-7.67	2.27-4.24	3.6-4.6	3.72-6.08	0.128-0.137	0-0.0101
CMC 3	27-29	30-30	7.57-7.63	4.8-4.86	4.32-4.75	2.84-4.72	0.02-0.104	0.009-0.024
Kondang Merak	25-27	26-28	7.72-7.9	0.19-0.25	3.88-4.03	3.16-4.74	0.43-0.76	0.01-0.03
Teluk Semut	26-27	27-29	7.21-7.59	4.67-4.94	4.28-4.67	2.52-5.6	0.02-0.072	0.01-0.0115
Water Quality Standart (Indonesia Ministry of Environment Regulation No 22/2021)	**26-32**		**7-8.5**	**-**	**>5**	**20**	**0.008**	**0.015**

Notes: WT: Water Temperature; AT: Air Temperature; Cond: Conductivity; NL: Nitrate Content; OL: Orthophosphat Content.

The pH values showed no significant difference between the 5 locations (Table 2). Based on the water quality standard for marine biota in mangroves based on government rules (PP NO.22/MENLH/2021), the pH value still meets the optimal limit of 7-8.5. A good pH value to support the sustainability of aquatic life ranges from 6.5-8 (Wassie & Melese 2017). Changes in the degree of acidity of the water are also influenced by the metabolic activity of phytoplankton that utilizes organic matter content and light intensity (Gao & Zheng 2010).

The conductivity values obtained indicate a significant difference between locations (Table 2). The highest conductivity value was found in the mangrove CMC 1, which ranges from 5.4-5.64 S/m, while the lowest conductivity value was located in the Kondang Merak mangrove, which ranges from 0.19 to 0.25 S/m. In addition, the conductivity values of the 4 locations were compared with those in Teluk Semut, and the Kondang Merak mangrove location has a value that is much different from the Reference site. It is because the waters in the Kondang Merak mangrove are freshwater that comes from the seashore of the Kondang Merak river and are not connected to seawater. Water conductivity fluctuations are influenced by the content of inorganic materials, salts, pollutants, currents, and water turbidity (Hatzikos et al. 2008).

Based on the data in Table 2, the DO values obtained ranged from 3.23-4.75 mg/L. The highest DO value 4.75 mg/L was found in mangrove CMC 3, while the lowest DO value 3.23 mg/L was found in mangrove CMC 1 (Table 2). Based on the water quality standard for marine biota in mangrove based on government rules (PP NO.22/MENLH/2021), the DO value from 5 locations did not meet the quality standard value > 5 mg/L. However, the optimal DO value for aquatic microorganisms ranges from 4-6.5 mg/L (Onyema 2013). According to Pour et al. (2014), DO plays a vital role in reduction and oxidation of organic and inorganic materials. DO levels that are too low in aquatic ecosystems can interfere with the life of aquatic organisms, such as affecting cell respiration (Wirabumi 2017).

Our results showed that BOD values ranged from 2.52-8.44 mg/L (Table 2). The highest BOD value 8.44 mg/L was found in mangrove CMC 1, while the lowest BOD value 2.52 mg/L was found in Teluk Semut mangrove. Based on the water quality standard for marine biota in mangroves based on government rules (PP NO.22/MENLH/2021), the BOD value at five mangrove locations met the 20 mg/L standards. According to Anyanwu & Solomon (2015), BOD is the total dissolved oxygen consumed by microorganisms to degrade organic matter such as food waste and the remains of other living things, where the higher the BOD indicates a higher amount of DO reduction in the waters.

The nitrate levels obtained in CMC, Kondang Merak, and Teluk Semut mangroves ranged from 0.02-0.76 mg/L (Table 2). The highest nitrate level was found in the Kondang Merak mangrove at a the concentration of 0.76 mg/L, while the lowest nitrate level was found in the Teluk Semut mangrove at the concentration of 0.02 mg/L. Based on the water quality standard for marine biota in mangrove based on government rules (PP NO.22/MENLH/2021), the value of nitrate levels at 5 locations did not meet the optimal standard of 0.008 mg/L. It is because around the location, there are human activities that contribute to the in littering organic matter (Eddy et al. 2021). Remaining waste originating from agricultural, plantation and livestock activities will be carried by runoff water from rivers and accumulates in coastal and sea areas (Rohila et al. 2017).

The orthophosphate levels in CMC, Kondang Merak, and Teluk Semut mangroves ranged from 0 to 0.0235 mg/L (Table 2). The highest

value of orthophosphate content was found in mangrove CMC 3 at the concentration of 0.024 mg/L, while the lowest value of orthophosphate content was found in mangroves CMC 1 and CMC 2 at the concentration of 0 mg/L. Based on the water quality standard for marine biota in mangrove based on government rules (PP NO.22/MENLH/2021), the value of orthophosphate content met the standard (0.015 mg/L) in all mangrove locations except in CMC 3 was 0.024 mg/L. According to Saifullah et al. (2016), nitrate and phosphate are potential elements that affect the fertility of waters and the abundance of phytoplankton. Based on the measurement results, the physical and chemical parameters of the observed water all meet the quality standards except DO. From the water physicochemical data above, the growth of phytoplankton can be influenced by water quality conditions in an environment (Yuliana et al. 2012; Zhang et al. 2021).

Profile of Community Structure and Phytoplankton Diversity in CMC Mangroves, Kondang Merak, and Teluk Semut

Assessment of the success of mangrove ecosystem restoration is based on biological water quality parameters, including community structure and phytoplankton diversity. Diatoms are part of phytoplankton with limited mobility and are more sensitive to changes in water quality so they can be bioindicators (Suther & David 2009). Analysis related to the community structure of the phytoplankton diversity calculated, among others, The Importance Value Index (IVI), Simpson Dominance Index (Id), Evenness Index (E), Shannon-Wiener Diversity Index (H'), the phytoplankton biotic index Trophic Diatom Index (TDI), and %Pollution Tolerant Value (%PTV) (Figure 2-5 and Table 3). Based on the results of mangrove identification and data analysis, it was found that different species compositions at each location (CMC 1, CMC 2, CMC 3, Kondang Merak, and Teluk Semut) were found to be 13, 13, 11, 12, and 15 species, respectively.

The results of the IVI showed that phytoplankton species dominate at each location (Figure 2). At the mangrove locations in CMC 1, CMC 3, and Kondang Merak two codominant species were found., namely *Nitzschia* sp. dan *Navicula* sp. with IVI values of CMC 1 (35.09%,

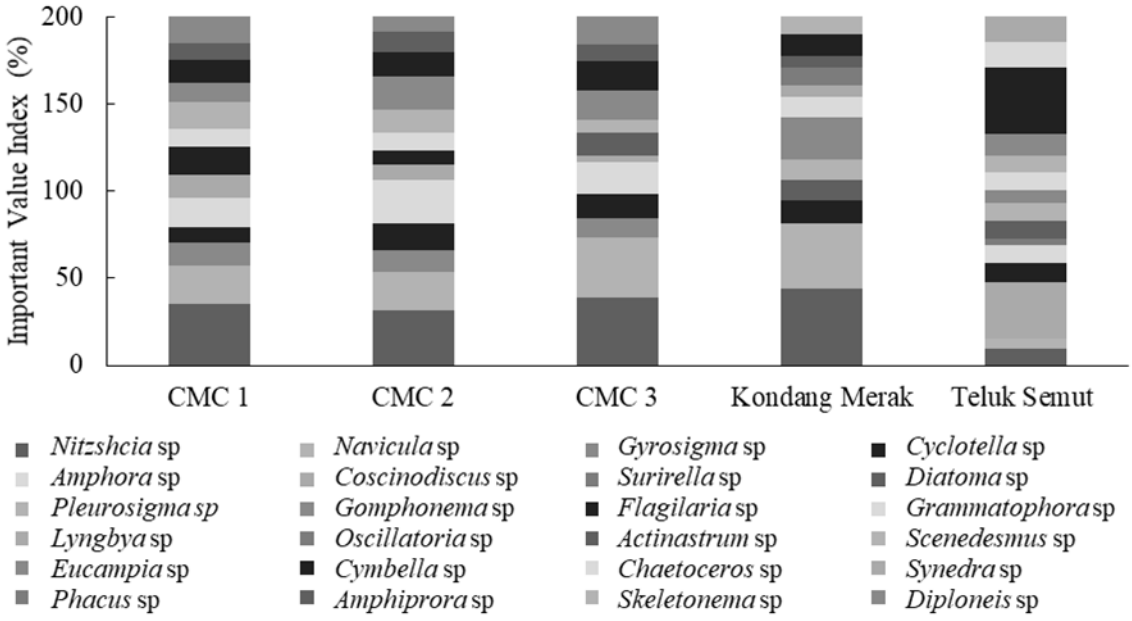

Figure 2. Spatial variation of IVI values in Clungup Mangrove Conservation (CMC), Kondang Merak, and Teluk Semut.

21.98%); CMC 3 (38.73%, 34.37%); and Kondang Merak (43.5%, 37.17%) respectively (Figure 3B and 3D). At the CMC 2 location, the dominant species was found *Nitzshia* sp., with IVI values of 31.32% (Figure 3D). At the Teluk Semut location, two codominant species were found, namely *Tabellaria* sp. and *Coscinodiscus* sp., with IVI values of 37.83% and 32.57%, respectively (Figure 3A and 3C). Dominant species found in an aquatic ecosystem indicate instability that causes the water quality to be categorized as poor (Inyang & Wang 2020). According to Onyema (2013), *Nitzschia sp.* is a diatom with a high level of adaptation and tolerance to organic matter pollution or in high nutrients water. It can be said that CMC 1, CMC 2, CMC 3 and Kondang Merak locations were exposed to organic matter pollution. According to Taylor et al. (2007), *Tabellaria* sp. is a phytoplankton species that can live in oligotrophic conditions and is sensitive to high organic matter. In addition, *Coscinodiscus* sp. is a cosmopolitan and phytoplankton species that usually lives in brackish and marine waters.

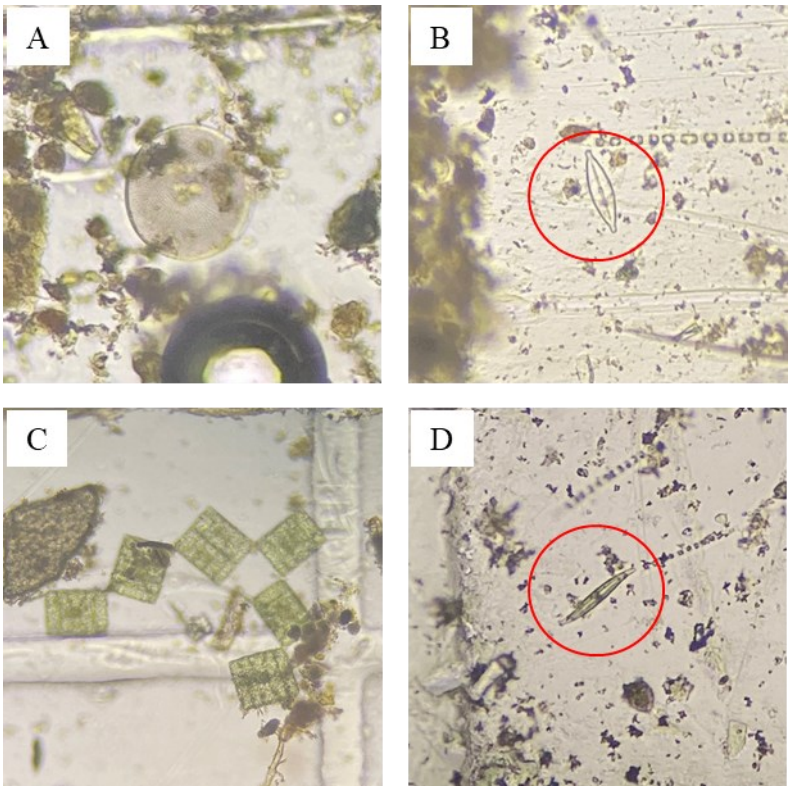

Figure 3. Images of the phytoplanktons found in Clungup Mangrove Conservation (CMC), Kondang Merak, and Teluk Semut. Notes: A. *Coscinodiscus* sp.; B. *Navicula* sp.; C. *Tabellaria* sp.; and D. *Nitzschia* sp. with a magnification of ×200.

Our results showed that the biotic index analysis, the Shannon-Wiener diversity index (H'), evenness index (E), and Simpson dominance index (Id) differ between locations (Table 3). The calculation results of H' show values ranging from 2.48 to 3.10, which means the five research sites were not contaminated with toxic substances. According to Wu et al. (2014) and Junaidi & Azhar (2018), the range of waters contaminated with toxic materials based on the Shannon-Wiener diversity index is divided into two categories, namely lightly polluted ($2 < H' < 3$), and moderately polluted ($1 < H < 2$).

The Simpson dominance index obtained at five mangrove forest locations ranged from 0.10 - 0.23, which means low partial dominance (Table 3). According to Febriansyah & Retnaningdyah (2021), the range of Id values ranges from 0-1. If the value < 0.4 includes low partial domi-

nance, 0.4-0.6 includes moderate partial dominance and > 0.6 includes high partial dominance. The Evenness index (E) values obtained at five mangrove locations ranged from 0.7 to 0.84 (Table 3). Based on the value of E obtained, it showed that the five mangrove forest locations was classified as evenly distributed with the E value > 0.6, including species evenly distributed (Wu et al. 2014). It is positively correlated with the results of the Importance Value Index, where it was assumed that each location did not have a dominant species, but rather species codominance.

Table 3. Spatial variation of phytoplankton diversity index in Clungup Mangrove Conservation (CMC), Kondang Merak, and Teluk Semut.

Location	Restoration Time	Biotic Index		
		E	Id	H'
CMC	CMC 1	0.70	0.11	2.61
	CMC 2	0.79	0.18	3.10
	CMC 3	0.72	0.20	2.48
Kondang Merak	Rehabilitated in 2019	0.72	0.23	2.57
Teluk Semut	Natural	0.84	0.10	3.07

Notes: Evenness Index (E); Simpson Dominance Index (Id); Shannon-Wiener Diversity Index (H').

The Trophic Diatom Index is a biotic index developed for monitoring the level of eutrophication by organic pollution from diatom groups (Kelly & Whitton 1995). According to Wu et al. (2014), the level of eutrophication is divided into four levels. Those are oligo-eutrophic (TDI < 24), which means the waters have low nutrients and primary productivity, meso-eutrophic (25 < TDI < 49), which means the waters have medium nutrients and primary productivity, eutrophic (50 < TDI < 74), which means the waters have high nutrient content and primary productivity, and hyper-eutrophic (75 < TDI < 100), which means the waters have very high nutrient content and primary productivity.

The TDI index results obtained from the five mangrove forest locations ranged from 26.25 – 76.07% (Figure 4). The location of the Kondang Merak mangrove was classified as poor (hyper-eutrophic) with a TDI value of 76.07%. This result was positively correlated with high nitrate levels in Kondang Merak mangroves. The existence of active anthropogenic activities causes the accumulation of organic matter such as nitrate and phosphate (Çulha et al. 2022). Moreover, the CMC 1, CMC 2, and CMC 3 mangrove locations were classified as moderate (eutrophic) with TDI values of 64.5%, 53.9%, and 70.03%, respectively. The location of the Teluk Semut mangrove was categorized as a meso-eutrophic location with a TDI value of 26.25%. It was because the mangrove location is within the Sempu Island Nature Reserve, which is conserved and minimally anthropogenic. The main cause of eutrophication is the presence of phytoplankton that can utilize organic matter as nutrients for metabolism (Bellinger & Sigee 2010). According to Adesuyi et al. (2015), the high content of nitrate and phosphate cause an increase in the abundance of diatoms. In addition, the accumulation of organic matter is caused by the environmental carrying capacity that exceeded the limit so that it cannot be absorbed and remediated (Zhang et al. 2021).

The %PTV index describes the level of organic pollution by comparing the abundance of tolerant diatoms (*Gomphonema* sp., *Navicula* spp., *Sellaphora* spp., and *Nitzschia* spp.) with the total number of diatoms obtained (Wu et al. 2014). Our results showed that the %PTV values at the five locations of mangrove forests ranged from 6.4 to 71.2 % (Figure 5). The Teluk Semut and CMC 2 mangroves obtained %PTV values of 6.4%

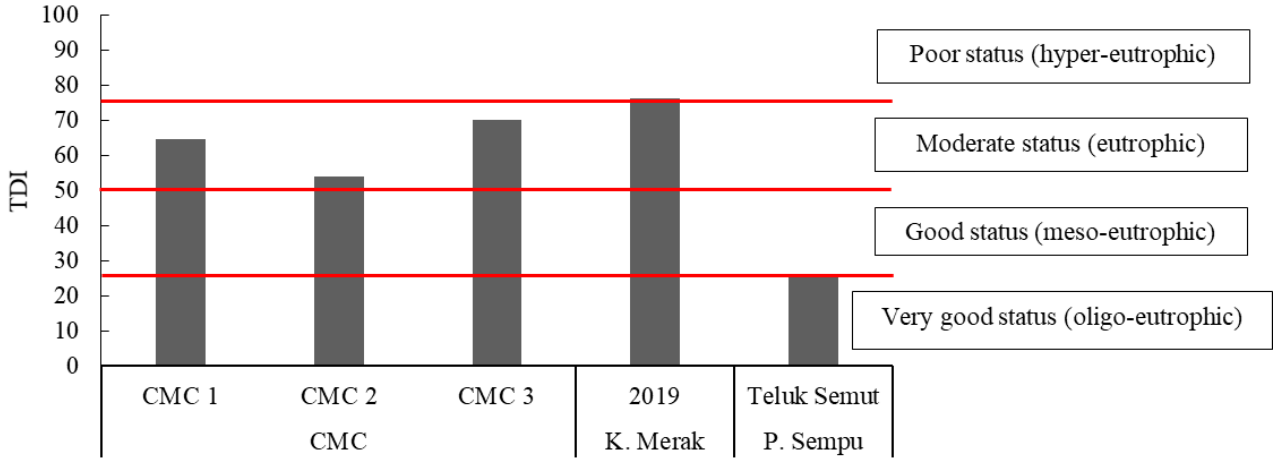

Figure 4. Spatial variation of Trophic Diatom Index values in Clungup Mangrove Conservation (CMC), Kondang Merak, and Teluk Semut. (Note: ——— = Value limit between categories)

and 17.9%, respectively, indicating that they were not polluted with organic matter. The location of CMC 1 obtained a %PTV value of 53.6%, classified as moderate organic pollution, which can contribute significantly to eutrophication. The location with the highest %PTV value was in the mangrove CMC 3 and Kondang Merak with 65.1% and 71.2%, respectively, indicating heavy organic matter pollution. These results showed a positive correlation with the TDI value, if the level of organic pollution is high, the level of eutrophication is also high. The influence of organic matter from anthropogenic activities is evidenced by the many types of diatoms found as indicators of organic matter pollution, such as *Nitzschia* sp. and *Navicula* sp. (Ferreira-Marinho et al. 2014; Han et al. 2016).

Correlation between water quality and plankton community structure in CMC, Kondang Merak, and Teluk Semut

The correlation between the physicochemical water parameters, the diverse community structure, and the plankton biotic index were shown in the principal component analysis (PCA) diagram in Figure 6. Mangrove ecosystems CMC 1 and CMC 2 have similar water quality, characterized by high E and H', and also low DO and orthophosphate content. Teluk Semut (Sempu Island) mangrove location was characterized by low TDI and %PTV values and high DO. The location of CMC 3 and Kondang

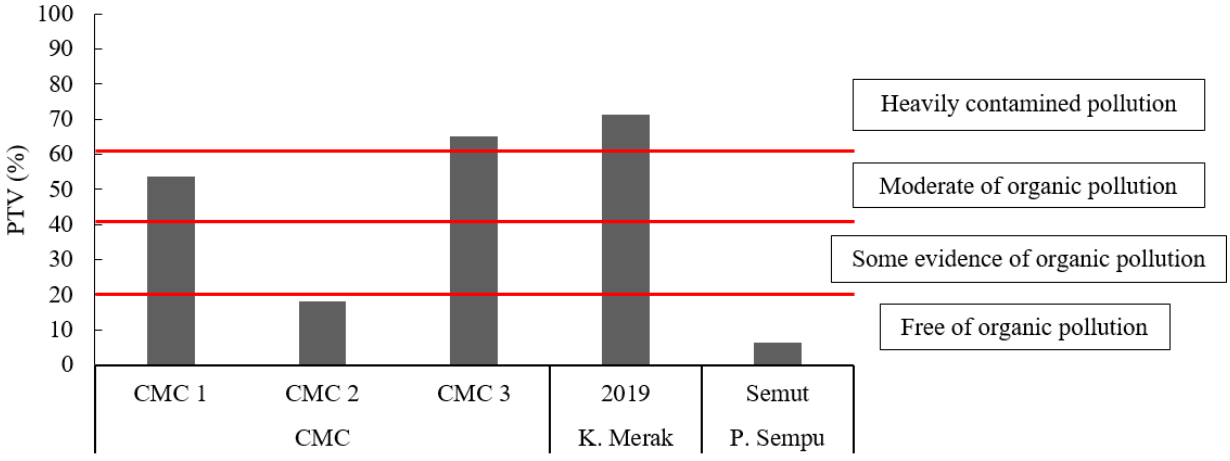

Figure 5. Spatial Variation of Pollution Tolerance Values in Clungup Mangrove Conservation (CMC), Kondang Merak, and Teluk Semut. (Note: ——— = Value limit between categories)

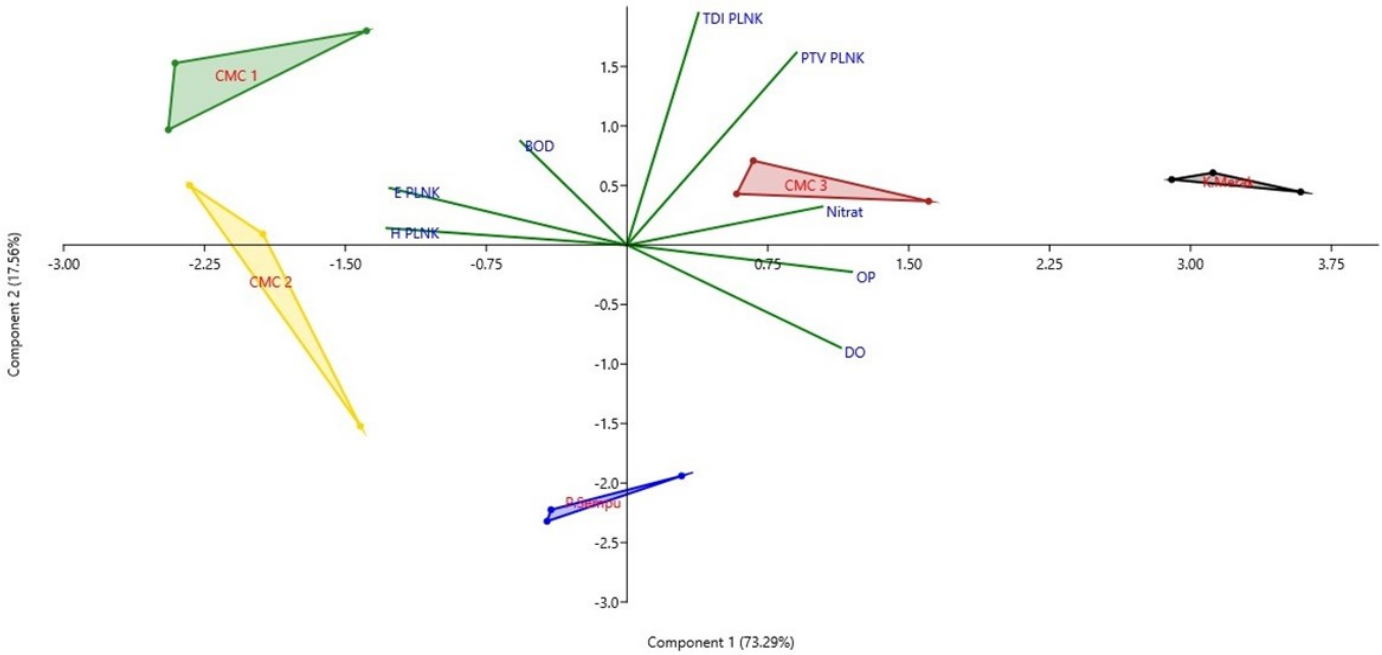

Figure 6. Correlation between water quality and phytoplankton community structure in Clungup Mangrove Conservation (CMC), Kondang Merak, and Teluk Semut using Biplot analysis. Notes: OP: Orthophospate, TDI PLNK: TDI Plankton; PTV PLNK: PTV Plankton; E PLNK: E Plankton; H PLNK: H' Plankton; Component 1 & 2 = variety of computational data.

Merak are characterized by high TDI, %PTV, nitrate, orthophosphate, and DO values. So, it can be concluded that the places that have successful mangrove restoration, which showed promising results, were the location of CMC 1 and CMC 2 because the water quality parameters were almost similar to those at Teluk Semut (Sempu Island). The location of CMC 3 and Kondang Merak is very different from Teluk Semut because there are still excessive human activities such as tourism, and settlements, beside the Kondang Merak mangrove adjacent to agricultural and plantation areas.

CONCLUSION

Water quality of the five mangrove ecosystems of CMC (2015, 2008, and natural), Kondang Merak, and Teluk Semut has met some of the water quality standards for marine biota. Water quality in five mangrove ecosystems Clungup Mangrove Conservation, Kondang Merak, and Teluk Semut based on phytoplankton indicators not contaminated with toxic materials (H'); based on TDI, it is categorized as eutrophic – hypereutrophic, except at the reference site of Teluk Semut mangrove; based on PTV polluted with moderate to high organic matter except at the reference site locations, namely Teluk Semut, and CMC 2. The conclusion is that the location with good water quality based on the diversity of phytoplankton is Teluk Semut.

AUTHORS CONTRIBUTION

All authors have contributed to completing this research. The contributions of each author were as follows, F; collecting data, analyzing data, compiling and writing manuscripts. R and H; compiling main conceptual ideas and critical revision of articles. S; granting research permits for mangrove locations and as a guide for research locations. All authors discussed the results and contributed to the final manuscript.

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CONFLICT OF INTEREST

The author confirms that there are no known conflicts of interest regarding this publication and there is no financial support for this work yet, which can affect the results.

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Research Article

The Evaluation of the Combination of Additives and Fungal Dyes to Produce Color for Textile Painting

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ABSTRACT
Fungal dyes are an eco-friendly alternative to synthetic dye. This study aims to evaluate additives to dye, using mixed fungi, to paint the picture on cloth. In the present study, the cloth was painted with mixed *Aspergillus* and *Paecilomyces* dye. The mixed fungi were grown on a mineral salt glucose medium. Five tests were conducted to evaluate additives to dye from mixed fungi that could be used to paint pictures on cloths to evaluate the effect of additives, a combination of additives producing tidy colors and other additives, the dye pH, mordant, and a variety of different mordants and the dye pH on color tidiness and hue. The additives used were alkali, acid, salts, glycerine, and urea. The Royal Horticultural Society (RHS) color chart was used to measure the color of filtrate and range developed on the painted color on the cloth. The results showed that the mixture of vinegar or lemon as additives and the dye pH of 3 produced tidy colors. The mordant application had a more significant effect on the color that appears than pH treatment. Colors formed on images can add variations to textile painting.

INTRODUCTION
The use of synthetic dyes in the textile industry is gradually receding not only due to the increase of environmental awareness for the harmful effects of either toxic degraded products or their non-biodegradable nature, but also the cause of cancer and allergic reactions (Teli et al. 2013). These problems motivate efforts to develop eco-friendly natural dyes derived from animals, microbes, and plants. Most of Indonesia's textile dyeing process use natural dyes from plants (Rini et al. 2011). Still, only few natural dyes are available in adequate quantities for use in textile dyeing (De Santis et al. 2005). Thus, it is necessary to explore the potential use of other biological sources, such as algae, bacteria, and fungi.

Dye production by microbes (including fungi) is very important to industries because microbes can proliferate, are highly productive, and are available throughout the year, regardless of the weather conditions (Méndez et al. 2011). Microbial dyes are often more soluble and stable than those obtained from animals or plants (Gunasekaran & Poorniammal 2008). Various dyes from fungi were informed, including yellow dyes by *Aspergillus sydowii* (CML 2967), two isolates of *A. aureolatus* (CML 2964 and E.4.1), and two isolates of *A. keveii* (CML 2964 and E.4.1) (Nirlane da Costa Souza et al. 2016); greyed-orange dyes by *Asper-*

gillus terreus and *Aspergillus* sp. strain 2 (Suciatmih & Hidayat 2017); greyed-purple dyes by *Paecilomyces lilacinus* (Thom) Samson and *Paecilomyces* sp. strain 542 (Suciatmih & Yuliar 2018); and red dyes by *Aspergillus* sp. strain 1 (Suciatmih & Yuliar 2018) and *Paecilomyces farinosus* (*Isaria farinosa*) (Velmurugan et al. 2010a).

Previous research reported that mixed *Aspergillus* and *Paecilomyces* produced 187A greyed-purple dye and could dye cloths (Suciatmih et al. 2018; Suciatmih 2019; Suciatmih 2020), but its ability to paint pictures was untested. Using mixed fungi is intended to maintain the fungi's ability to produce dye. Except for mixed conditions, *Aspergillus* and *Paecilomyces* grew in potato dextrose agar medium separately for one month. Then, each of these fungi was cultured in the production medium; each fungus was no longer able to produce dye or has lost its ability to produce dye. Bader et al. (2010) reported that in a habitat, different microorganisms may compete for substrates as well as act symbiotically. In this case, it may be a symbiosis among *Aspergillus* and *Paecilomyces* caused by synergies of their different enzymatic systems and metabolic pathways (Bader et al. 2010). Some higher activities produced by co-culture are reported. Cellulolytic fungal co-cultures were more effective in substrate saccharification, which ranged between 85~88% compared to the 62~67% saccharification shown by the monocultures (Eyini et al. 2002). The antibiotic activity produced in the co-cultures of *Rhizopus peka* P8 and *Bacillus subtilis* IFO3335 (inhibition zone of 25 mm) was higher than in each of the pure cultures (inhibition zones of 0-15 mm) (Fukuda et al. 2008).

According to Kurnia (2011), painting on the cloth called textile painting, is a technique for making motifs or decorating fabrics. The technique involves using special paints that are resistant to water and ironing. Cloth paint is generally a thick liquid with paste melted in a small amount of water. Rohandi and Listian (2015) stated that the paint component consists of additives, binders, pigments, and solvent, while Kumari and Singh (2017) informed that the main components contained in the paint are additives, pigments, solvents, and thickener.

Teli et al. (2013) reported that natural dyes with few expectations are non-substantive, hence, they must be used in conjunction with mordants. Mordant helps in binding dyes to the fiber by forming a chemical bridge between the dyes and fiber (Satyanarayana & Chandra 2013). Single dyes source added with different mordant types produced different colors and tones on the dyed cloth (Sangeetha et el. 2015; Suciatmih & Hidayat 2017; Suciatmih et al. 2019; Suciatmih 2020).

Rohandi and Listian (2015) stated that the paint quality was determined by selecting components, such as appropriate adhesives and additives. Some additives such as alkali (baking soda and soda ash), acid (cream of tartar, lemon, and vinegar), brown sugar, milk, glycerine, salts, and urea are also often added to the dyes to get the desired color. Different shades and tones were also obtained from a single dye source by application of different pH values (Wang et al. 2014; Ren et al. 2016; Suciatmih & Yuliar 2018; Suciatmih 2020).

The research objective is to evaluate the ability of various materials commonly used for dyeing cloth to help dyes from mixed fungi paint the picture on the cloth without any dye spreading beyond the boundaries of the motif of the design.

MATERIALS AND METHODS

Inoculation process

The inoculation process of mixing *Aspergillus* and *Paecilomyces* fungi

(Figure 1) was performed following previous studies (Suciatmih et al. 2018; Suciatmih 2019). Briefly, five mycelial prints were inoculated into an Erlenmeyer flask containing 200 ml of mineral salt glucose medium (Baker & Tatum 1998) with modification, and statically incubated at room temperature for one month in a dark place. Composition of the mineral salts-glucose medium is described as follows (in ppm): $NaNO_3$, 848; KCl, 300; $MgSO_4.7H_2O$, 165; NaH_2PO_4, 100; $CaC1_2.2H_2O$, 40; H_3BO_3, 5.7; $FeSO_4.7H_2O$, 5.0; $ZnSO_4.7H_2O$, 4.4; $MnSO_4.H_2O$ (monohydrate), 3.1; $Na_2MoO_4.2H_2O$, 2.5; $CuSO_4.5H_2O$, 0.4; and glucose, 20,000. After the incubation period, the culture was passed through five layers of cheesecloth, and the filtrate was centrifugated at 8500 rpm for 20 min as performed previously. The filtrate's optical density was determined spectrophotometrically (Shimadzu) at 530 nm for quantifying the dyes (Suciatmih et al. 2018; Suciatmih 2019). The dye yield was counted as OD units (UA530).

Figure 1. Mixing *Aspergillus* and *Paecilomyces* fungi in Potato Dextrose Agar medium.

Effect of additives

Primisima brand cotton cloth (4 cm × 4 cm or 0.24 g) was first drawn with a pencil and then painted with the dye added with additives 2% and dried at room temperature. We evaluated 11 treatments of additives (Table 1). Additives applied to the dye produced tidy colors (the dye did not break and only occupied the predetermined place) on the cloths and were used for further testing.

Table 1. Treatment of additives

No	Treatment
1.	The dye 0.2 ml (pH 8) (control)
2.	The dye 0.2 ml + backing soda (pH 11) 20 µl
3.	The dye 0.2 ml + brown sugar (pH 6) 20 µl
4.	The dye 0.2 ml + cream of tartar (pH 3) 20 µl
5.	The dye 0.2 ml + glycerin (pH 4) 20 µl
6.	The dye 0.2 ml + lemon (pH 3) 20 µl
7.	The dye 0.2 ml + milk (pH 7) 20 µl
8.	The dye 0.2 ml + salt (pH 6) 20 µl
9.	The dye 0.2 ml + soda ash (pH 11) 20 µl
10.	The dye 0.2 ml + urea (pH 7) 20 µl
11.	The dye 0.2 ml + vinegar (pH 2) 20 µl

Combination effect of additives producing tidy colors and other additives

Vinegar or lemon was applied to the dye in the previous test produced a tidy color, but the hue was different from the control color. To get a tidy color and the same hue as the control color, we tested 9 treatments of a

combination of vinegar or lemon and other additives (Tables 2 and 3). The primisima cotton cloth (4 cm x 4 cm or 0.24 g), drawn with a pencil, was painted with a mixture of dye, vinegar or lemon, and other additives (2%), and then dried at room temperature. The composition of materials produced a tidy color, with the same hue as the control color, which was used for further testing.

Table 2. Treatment of combination of vinegar and other additives

No	Treatment
1.	The dye 0.2 ml (pH 8) + vinegar (pH 2) 20 µl (control)
2.	The dye 0.2 ml + vinegar (pH 2) 20 µl + backing soda (pH 11) 20 µl
3.	The dye 0.2 ml + vinegar (pH 2) 20 µl + brown sugar (pH 6) 20 µl
4.	The dye 0.2 ml + vinegar (pH 2) 20 µl + cream of tartar (pH 3) 20 µl
5.	The dye 0.2 ml + vinegar (pH 2) 20 µl + glycerin (pH 4) 20 µl
6.	The dye 0.2 ml + vinegar (pH 2) 20 µl + milk (pH 7) 20 µl
7.	The dye 0.2 ml + vinegar (pH 2) 20 µl + salt (pH 6) 20 µl
8.	The dye 0.2 ml + vinegar (pH 2) 20 µl + soda ash (pH 11) 20 µl
9.	The dye 0.2 ml + vinegar (pH 2) 20 µl + urea (pH 7) 20 µl

Table 3. Treatment of combination of lemon and other additives

No	Treatment
1.	The dye 0.2 ml (pH 8) + lemon (pH 3) 20 µl (control)
2.	The dye 0.2 ml + lemon (pH 3) 20µl + backing soda (pH 11) 20 µl
3.	The dye 0.2 ml + lemon (pH 3) 20 µl + brown sugar (pH 6) 20 µl
4.	The dye 0.2 ml + lemon (pH 3) 20 µl + cream of tartar (pH 3) 20 µl
5.	The dye 0.2 ml + lemon (pH 3) 20 µl + glycerin (pH 4) 20 µl
6.	The dye 0.2 ml + lemon (pH 3) 20 µl + milk (pH 7) 20 µl
7.	The dye 0.2 ml + lemon (pH 3) 20 µl + salt (pH 6) 20 µl
8.	The dye 0.2 ml + lemon (pH 3) 20 µl + soda ash (pH 11) 20 µl
9.	The dye 0.2 ml + lemon (pH 3) 20 µl + urea (pH 7) 20µl

Effect of mordant

Natural dye has a low coloring power within itself so that it requires chemicals compounds (mordants) for dye fixation into the fiber. We evaluated different mordants with 2% (alum, ferrous sulfate, and lime) painted with the mixture of the dye, vinegar, and soda ash that produced tidy colors and its hue approached the control colors; on the color's tidiness and hue. Cotton cloth (4 cm × 4 cm or 0.24 g) was first drawn with a pencil, painted with the mixture, and dried at room temperature. There were four treatments studied (Table 4).

Table 4. Treatment of mordant

No	Treatment
1.	The dye 0.2 ml (pH 8) + vinegar (pH 2) 20 µl + soda ash (pH 11) 20 µl (control)
2.	The dye 0.2 ml + vinegar (pH 2) 20 µl + soda ash (pH 11) 20 µl + alum 20 µl
3.	The dye 0.2 ml + vinegar (pH 2) 20 µl + soda ash (pH 11) 20 µl + lime 20 µl
4.	The dye 0.2 ml + vinegar (pH 2) 20 µl + soda ash (pH 11) 20 µl + ferrous sulfate 20 µl

Effect of the dye pH

Painting pH can increase or decrease the dye's ability to bind the fiber.

The differentiation effect of the dye pH on the color's tidiness and hue was evaluated. Cotton cloth (4 cm × 4 cm or 0.24 g) was drawn with a pencil each was painted with the pH of the dye of 3, 7, and 11; and dried at room temperature.

Combination effect of different mordants and the dye pH

Mordant and pH can change the final color of the dyed cloth or painted picture on the cloth. We tested the combined effect of different mordants 2% (alum, lime, and ferrous sulfate) and dye pH (3, 7, and 11) on the color's tidiness and hue. Cotton cloth (4 cm × 4 cm or 0.24 g), drawn with a pencil, was painted with different mordant combinations and dye pHs, then dried at room temperature. Treatment of a combination of different mordants and pH of the fungal dye is presented in Table 5.

Table 5. Treatment of combination between different mordants and pH of the fungal dye

No	Treatment
1.	pH 3 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + alum 20 µl
2.	pH 3 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + ferrous sulfate 20 µl
3.	pH 3 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + lime 20 µl
4.	pH 7 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + alum 20 µl
5.	pH 7 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + ferrous sulfate 20 µl
6.	pH 7 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + lime 20 µl
7.	pH 11 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + alum 20 µl
8.	pH 11 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + ferrous sulfate 20 µl
9.	pH 11 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + lime 20 µl

Determination of color hue

We determined the dye and the color hues after being painted with the dye from each test by matching the dye and the color hues with the Royal Horticultural Society (RHS) RH color chart, repeating each treatment twice (The Royal Horticultural Society 1966).

RESULTS AND DISCUSSION

At high glucose stress (20g/L) contained on the Baker and Tatum medium (1998) with modification, a mixed *Aspergillus* dan *Paecilomyces* produced 187A greyed-purple pigment (Figure 2) with an absorbance of 3.67 UA/L that can be used to paint the image on the cotton cloth. The control's painted cotton cloth color (only the fungal dyes) was 65B red-purple (Table 6 and Figure 3). Similar results were reported by Suciatmih et al. (2018), Suciatmih (2019) and Suciatmih (2020) that the same mixed fungi produced the same color pigment that can be used to dye cloth. The dye is included in the substantive dyes group because the dye can directly paint textile fibers or give good color when used alone (Marie et al. 2015). The fungi are thus potential sources as alternative sources for environmentally safe natural pigment production. Fungi is found to be a promising ecological source of pigments, as several fungal species are rich in stable colourants such as anthraquinone, carboxylic acids, pre-anthraquinones (Poorniammal et al. 2013). Besides adding desired colours to foods and textiles, fungal pigments have other attractive qualities such as anthelmintic activity (Sreedevi and Pradeep 2016), antidiabetic (Shi et al. 2012), anti-inflammation (Hsu et al. 2012), antimicro-

bial and antimutagenic (Teixeira, 2012); anti-obesity and anti-tumor (Lee et al. 2013), and antioxidants (Li et al., 2009). Sarkar et al. (2017) reported that pigments from *Aspergillus* sp. and *Penicillium* sp. found non allergic to human skin.

Figure 2. Color filtrate from mixing *Aspergillus* and *Paecilomyces* fungi.

Cloth painted with the dye added with vinegar (pH 2) or lemon (pH 3) produced tidy colors; the dye did not break and only occupied the predetermined place, while those administered with the dye alone (control) and the dye added each with other additives, such as baking soda, brown sugar, cream of tartar, glycerine, milk, salt, soda ash, and urea, produced untidy colors (Table 6 and Figure 3 & 4). The dye with a pH of 3 also produced tidy colors (Figure 5). The effect of painting pH can be attributed to the correlation between the pigment structure and cotton cloth. The anion of the pigment has complex characters, and when it is bound on the fiber at pH 2–3, with ionic forces, the ionic attraction would increase the dye-ability of the cloth (Velmurugan et al. 2010b) so that the dye only occupied the predetermined place, producing a tidy color. Meira (2016) stated that glacial acetic acid has the property of absorbing water in a pure state, thereby; making the mixture of the dye and vinegar painted on the cloth produced tidy colors. Aliño (2014) informed that acetic acid also contributes to lowering the dye bath pH. Vinegar is a mixture of glacial acetic acid and water with a ratio of 1:3. Similar results using vinegar was reported by Pliny in Adamu et al. (2013) in making a paste to paint papyrus; and Zhang et al. (2015) on the mixing of white vinegar and ink used for hand-painting silk organza, while Kumari and Singh (2017) added acetic acid in printing paste. Ramelawati et al. (2017) reported that lemon has an acid content that can bind dyes absorbed into the fabric. A similar result was reported by Sarkar (2013) using lemon juice is mixed with dye to get the desired shade for a Mithila painting of Bihar. Cream of tartar had a pH of 3 but produced untidy colors when painted on cloth with the dye. It might be that cream of tartar is containing ingredients which are capable of making the color untidy.

In contrast, cloth painted with the dye pH of 7 and 11; and other additives with pH 4–11 each produced untidy colors (Table 6 and Figures 3, 4 & 5). Velmurugan et al. (2010b) and Tayade and Adivarekar (2013) stated that the ionic interaction between the pigment and cotton cloth at high pH decreased due to the decreasing number of protonated hydroxyl groups on the cellulose leading to the electrostatic repulsion between dye and fiber. Thus, lowering the dye-ability of the cloth at the predetermined place, so the dye broken, produced an untidy color.

Table 6 and Figure 3 and 4 also present the results of color hue on the cotton cloth painted with the dye added with 20 µl of additives (2%). The dye without additives or control (65B red-purple); and had additives added to it, such as baking soda, brown sugar, cream of tartar, glycerine, milk, salt, soda ash, and urea, each of which produced the same hue but with different intensity, namely 65A, C, and D red-purple. The dye added with vinegar or lemon each obtained 177C greyed-orange colors. The dye with a pH of 3 also produced 177C greyed-orange colors, while those with a pH of 7 and 11 each produced 65C red-purple colors (Figure 5). Even though vinegar with pH 2, lemon with pH 3 (each 177C greyed-orange), or a dye with pH 3 (177C greyed-orange) could block dye or was not break when it was painted on cloth, thereby producing tidy colors. However, the resulting colors were different from the control colors (65B red-purple). Our previous study (Suciatmih & Yuliar 2018; Suciatmih 2020) reported that changing the dye pH can change the dye color of mixed *Aspergillus* and *Paecilomyces*. The dyes' ionic nature enabled the changes of the molecule structures according to the prevailing pH values and results in different colors and hues at different pH values (Mishra et al. 2012). They further stated that, when the acidic condition (vinegar with pH 2 or lemon with pH 3 or the dye with pH 3) is added to the dye, hydrogen atoms from vinegar or lemon or the dye with pH 3 stick to the dye molecules in such a way that when dyed or painted on cloth, it produced different hues.

Table 6. Effect of 0.2 µl of additives 2% on the color tidiness and hue painted with the fungal dye.

No	Treatment	Color tidiness	Color hue
1.	The dye 0.2 ml (pH 8) (control)	Untidy	65B Red-purple
2.	The dye 0.2 ml + backing soda (pH 11) 20 µl	Untidy	65A Red-purple
3.	The dye 0.2 ml + brown sugar (pH 6) 20 µl	Untidy	65D Red-purple
4.	The dye 0.2 ml + cream of tartar (pH 3) 20 µl	Untidy	65C Red-purple
5.	The dye 0.2 ml + glycerin (pH 4) 20 µl	Untidy	65C Red-purple
6.	The dye 0.2 ml + lemon (pH 3) 20 µl	Tidy	177C Greyed-orange
7.	The dye 0.2 ml + milk (pH 7) 20 µl	Untidy	65D Red-purple
8.	The dye 0.2 ml + salt (pH 6) 20 µl	Untidy	65D Red-purple
9.	The dye 0.2 ml + soda ash (pH 11) 20 µl	Untidy	65A Red-purple
10.	The dye 0.2 ml + urea (pH 7) 20 µl	Untidy	65D Red-purple
11.	The dye 0.2 ml + vinegar (pH 2) 20 µl	Tidy	177C Greyed-orange

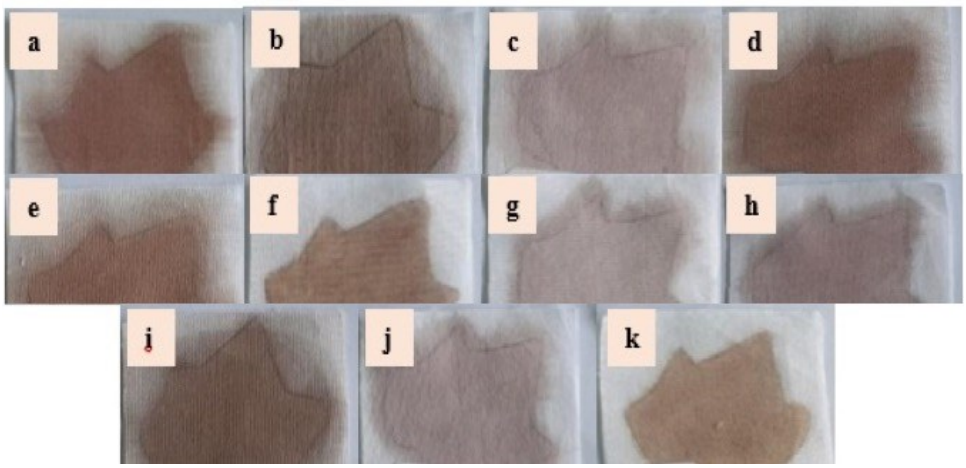

Figure 3. Color tidiness and hue painted with the dye added with additives. The dye (a), the dye + backing soda (b), the dye + brown sugar (c), the dye + cream

of tartar (d), the dye + glycerine (e), the dye + lemon (f), the dye + milk (g), the dye + salt (h), the dye + soda ash (i), the dye + urea (j), and the dye + vinegar (k).

In the previous test, vinegar or lemon applied to the dye produced the painted tidy colors, but the hue was different from the control color (the dye without additives). Therefore, it is necessary to use additives that produce tidy colors with their hue similar to the control when added to the dye. Tables 7 and 8 and Figure 6, 7, and 8 present the combination results between vinegar or lemon and other additives on hue and tidiness of the color on the cloth painted with the dye. Except for the combination of vinegar and soda ash (182C greyed-red, the color hue approaches the control, 65B red-purple) (Table 7 No 8, Figure. 4.a, Figure 6.h, and Figure 8.b), the dye was only added with vinegar (177C greyed-orange) and its combination with other additives each produced the same color, namely 173D greyed-orange (the colors are different from the control, 65B red -purple) (Table 7; Figure 3.a, Figure 4.a, Figure 6, and Figure 8.a). It could be because vinegar was acid (pH 2), while soda ash was alkaline (pH 11), and when both are mixed, it will change the pH of the dye so that changing the color of the dye. The baking soda also had a pH of 11, but when combined with vinegar and painted on the cloth with the dye, it produced a different color hue (173D greyed-orange) to the control (65B red-purple). It was possible that baking soda containing ingredients which are capable of making the color hue different from the control.

Figure 4. Color tidiness and hue painted with the dye added with additives. The dye (a), the dye + vinegar (b), and the dye + lemon (c).

Table 7. Combination effect between vinegar and other additives 2% on the color tidiness and hue painted with the dye.

No	Treatment	Color tidiness	Color hue
1.	The dye 0.2 ml (pH 8) + vinegar (pH 2) 20 µl (control)	Tidy	177C Greyed-orange
2.	The dye 0.2 ml + vinegar (pH 2) 20 µl + backing soda (pH 11) 20 µl	Tidy	173D Greyed-orange
3.	The dye 0.2 ml + vinegar (pH 2) 20 µl + brown sugar (pH 6) 20 µl	Tidy	173D Greyed-orange
4.	The dye 0.2 ml + vinegar (pH 2) 20 µl + cream of tartar (pH 3) 20 µl	Tidy	173D Greyed-orange
5.	The dye 0.2 ml + vinegar (pH 2) 20 µl + glycerin (pH 4) 20 µl	Tidy	173D Greyed-orange
6.	The dye 0.2 ml + vinegar (pH 2) 20 µl + milk (pH 7) 20 µl	Tidy	173D Greyed-orange
7.	The dye 0.2 ml + vinegar (pH 2) 20 µl + salt (pH 6) 20 µl	Tidy	173D Greyed-orange
8.	The dye 0.2 ml + vinegar (pH 2) 20 µl + soda ash (pH 11) 20 µl	Tidy	182C Greyed-red
9.	The dye 0.2 ml + vinegar (pH 2) 20 µl + urea (pH 7) 20 µl	Tidy	173D Greyed-orange

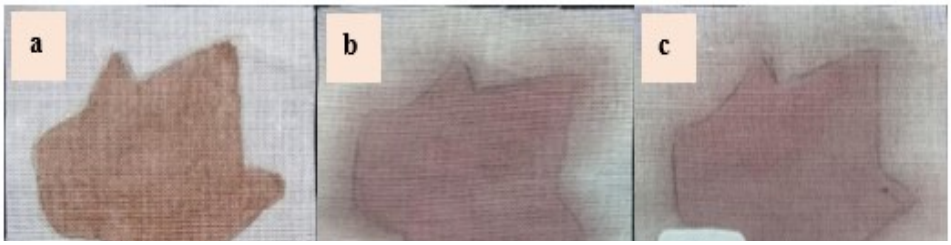

Figure 5. Color tidiness and hue painted with different the dye at pH 3 (a), pH 7 (b), and pH 11 (c).

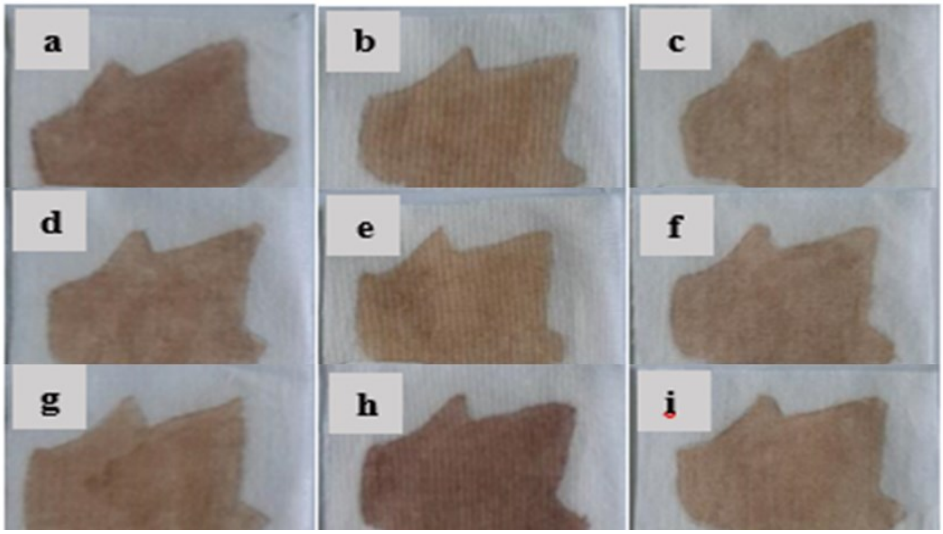

Figure 6. Color tidiness and hue painted with the dye added vinegar and other additives. The dye + vinegar (a), the dye + vinegar + backing soda (b), the dye + vinegar + brown sugar (c), the dye + vinegar + cream of tartar (d), the dye + vinegar + glycerin (e), the dye + vinegar + milk (f), the dye + vinegar + salt (g), the dye + vinegar + soda ash (h), the dye + vinegar + urea (i).

Table 8. Combination effect between lemon and other additives 2% on the color tidiness and hue painted with the dye.

No	Treatment	Color tidiness	Color hue
1.	The dye 0.2 ml (pH 8) + lemon (pH 3) 20 µl (control)	Tidy	177C Greyed-orange
2.	The dye 0.2 ml + lemon (pH 3) 20µl + backing soda (pH 11) 20 µl	Tidy	173D Greyed-orange
3.	The dye 0.2 ml + lemon (pH 3) 20 µl + brown sugar (pH 6) 20 µl	Tidy	173D Greyed-orange
4.	The dye 0.2 ml + lemon (pH 3) 20 µl + cream of tartar (pH 3) 20 µl	Tidy	173D Greyed-orange
5.	The dye 0.2 ml + lemon (pH 3) 20 µl + glycerin (pH 4) 20 µl	Tidy	173D Greyed-orange
6.	The dye 0.2 ml + lemon (pH 3) 20 µl + milk (pH 7) 20 µl	Tidy	173D Greyed-orange
7.	The dye 0.2 ml + lemon (pH 3) 20 µl + salt (pH 6) 20 µl	Tidy	173D Greyed-orange
8.	The dye 0.2 ml + lemon (pH 3) 20 µl + soda ash (pH 11) 20 µl	Tidy	177B Greyed-orange
9.	The dye 0.2 ml + lemon (pH 3) 20 µl + urea (pH 7) 20µl	Tidy	173D Greyed-orange

According to Suciatmih and Hidayat (2017), the characteristics of color shadings on the cloth depends on the mordant type used during the

dyeing process. A composition containing the dye, vinegar, and soda ash in the previous test produced the painted tidy colors that controlled the color hue. Thus, the composition is used to test different mordants (alum, ferrous sulfate, and lime) on the color tidiness and hue (Table 9 and Figure 9). Different mordants applied to the mixture of the dye, vinegar, and soda ash painted on the cotton cloth produced different color hues. The mixture, each added with mordant lime and without mordant (control), produced the same tidy and 182C greyed-red colors. When alum was added, it obtained tidy greyed-purple colors at 186 between C and B, and when ferrous sulfate was added, it produced tidy and 201 between B and A grey colors. The same results indicated that different mordants generated various colors on cotton cloth (Suciatmih & Hidayat 2017; Suciatmih 2020) and woolen yarn (Suciatmih & Yuliar 2018) each dyed with the fungal dyes. Satyanarayana and Chandra (2013) informed that mordant helps to tie the dye to the fiber by forming chemical bridges between the dye and the fiber. Pujilestari (2014) reported that the mordant serves to strengthen the color and change the dye's color according to the type of metal that binds it and locks the dye into the fiber. The mixture combined with ferrous sulfate resulted in darker color than alum and lime. Similar results showed that ferrous sulfate produced dark colors on silk and wool fabrics dyed with *Eucalyptus* leaf extract dye (Mongkholrattanasit et al. 2011), cotton cloth and polyester wool dyed with *Ficus cunia* dye (Kundal et al. 2016), and cotton cloth dyed with fungal dyes (Suciatmih & Hidayat 2017; Suciatmih 2019; Suciatmih 2020).

Except for lime, when alum or ferrous sulfate applied to the mixture of the dye, vinegar, and soda ash produced colored deposits. However, when the mixture was painted on the cloth, the colored deposits were destroyed, thereby enabling the dye to bind to the cloth, but the color result is uneven. This uneven result is due to the difference in pH of the mordant solution. Lime had a pH of 11.8, while alum and ferrous sulfate had pH of 3.4 and 3.1, respectively. Alum or ferrous sulfate causes very high acidity in the dye bath, resulting in colored deposits, which cause uneven color results on the painted cloth (Figure 9 and 10). Similar results were reported by Baig (2012) at pH values lower than 5.5–6 in the bath cause most of the leucovat acid is precipitated so that at the end of dyeing, a black dispersion is produced and, therefore, very little dye is absorbed into the fabric.

Our previous study (Suciatmih & Yuliar 2018; Suciatmih 2020) obtained that a single dye source added to the application of its bath's pH value generates different colors and tones. A composition containing the dye, vinegar, and soda ash is also used to test a combination of different mordants and varieties pH of the dye (3, 7, and 11) on tidy and hue of the color on the cloth (Table 10 and Figure 10). A combination between different mordants (alum, ferrous sulfate, and lime) and pH of the dye (3 and 7) added to the mixture of vinegar and soda ash painted on the cloth each produced the same hue of color (186C greyed-purple, 201B grey, and 182D greyed-red). Except for alum (177D greyed-orange), different mordants combined with the dye pH of 11 produced the same color hue as the previous treatments. The color hue intensity was one level below them (186D greyed-purple and 201C grey). The study indicated that at pH 11, the cotton cloth could not optimally absorb the dyes. Similar results were reported by Tayade and Adivarekar (2013) on *Cuminum cyminum* seeds extract dye uptake decreased by cotton cloth at pH 11, and Mukherjee and Kanakarajan (2017) on dyeing cotton yarn with *Aerva sanguinolenta* leaves extract dye in acidic pH showed better result than alkaline pH. Tayade and Adivarekar (2013) stated that the higher pH

causes cellulose to carry more and more negative charged, de-protonated hydroxyl groups, increasing negative charge on the cellulose, leading to an electrostatic repulsion between dye and fiber, which may cause lesser dye uptake. Wang et al. in Mukherjee and Kanakarajan (2017) reported that higher pH oxidizes natural dye's conjugate structure, decreasing the dye-ability of natural dye.

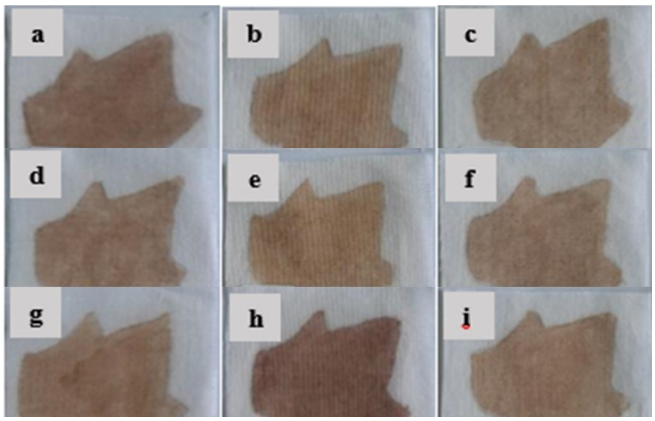

Figure 7. Color tidiness and hue painted with the dye added vinegar and other additives. The dye + lemon (a), the dye + lemon + backing soda (b), the dye + lemon + brown sugar (c), the dye + lemon + cream of tartar (d), the dye + lemon + glycerin (e), the dye + lemon + milk (f), the dye + lemon + salt (g), the dye + lemon + soda ash (h), the dye + lemon + urea (i).

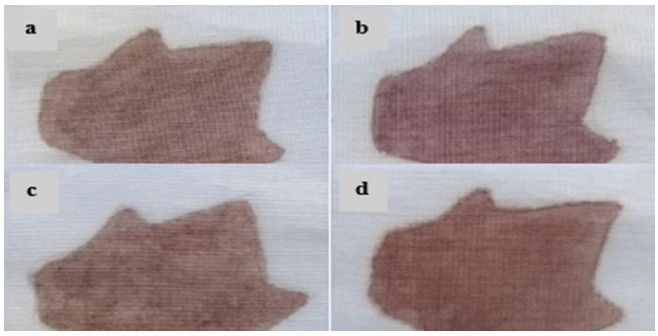

Figure 8. Color tidiness and hue painted with the dye added with additives. The dye + vinegar (a); the dye + vinegar + soda ash (b); the dye + lemon (c); and the dye + lemon + soda ash (d).

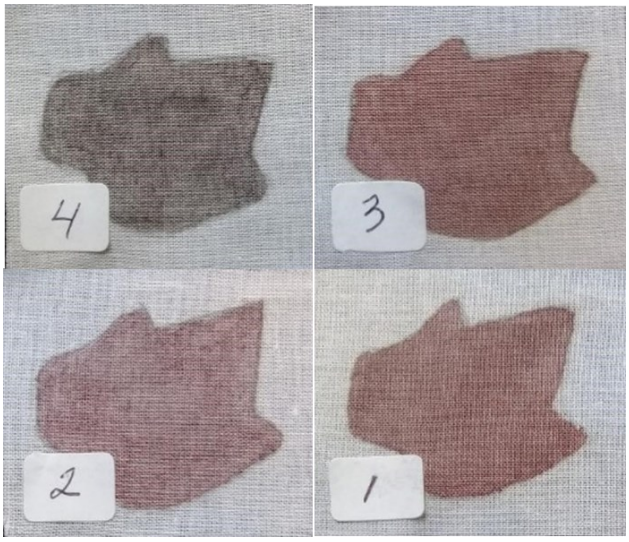

Figure 9. Color tidiness and hue painted with the mixture of the dye, vinegar, soda ash, and mordant, (1) The dye + vinegar + soda ash; (2) the dye + vinegar + soda ash + alum; (3) the dye + vinegar + soda ash + lime; and (4) the dye + vinegar + soda ash + ferrous sulfate.

Table 9. Effect of the mixture of the dye, vinegar, soda ash, and mordant on the painted color tidiness and hue.

No	Treatment	Color tidiness	Color hue
1.	The dye 0.2 ml (pH 8) + vinegar (pH 2) 20 µl + soda ash (pH 11) 20 µl (control)	Tidy	182C Greyed-red
2.	The dye 0.2 ml + vinegar (pH 2) 20 µl +soda ash (pH 11) 20 µl + alum (pH 3) 20 µl	Tidy	186 between Cand B Greyed-purple
3.	The dye 0.2 ml + vinegar (pH 2) 20 µl +soda ash (pH 11) 20 µl + lime (pH 12) 20 µl	Tidy	182C Greyed-red
4.	The dye 0.2 ml + vinegar (pH 2) 20 µl + soda ash (pH 11) 20 µl + ferrous sulfate(pH 3) 20 µl	Tidy	201 between Band A Grey

Table 10. Combination effect between different mordants and pH of the fungal dye on the painted color tidiness and hue.

No	Treatment	Color tidiness	Color hue
1.	pH 3 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + alum (pH 3) 20 µl	Tidy	186C Greyed-purple
2.	pH 3 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + ferrous sulfate (pH 3) 20 µl	Tidy	201B Grey
3.	pH 3 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + lime (pH 12) 20 µl	Tidy	182D Greyed-red
4.	pH 7 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + alum (pH 3) 20 µl	Tidy	186C Greyed-purple
5.	pH 7 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + ferrous sulfate (pH 3) 20 µl	Tidy	201B Grey
6.	pH 7 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + lime (pH 12) 20 µl	Tidy	182D Greyed-red
7.	pH 11 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + alum (pH 3) 20 µl	Tidy	186D Greyed-purple
8.	pH 11 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + ferrous sulfate (pH 3) 20 µl	Tidy	201C Grey
9.	pH 11 of the dye 0.2 ml + vinegar 20 µl + soda ash 20 µl + lime (pH 12) 20 µl	Tidy	177D Greyed-orange

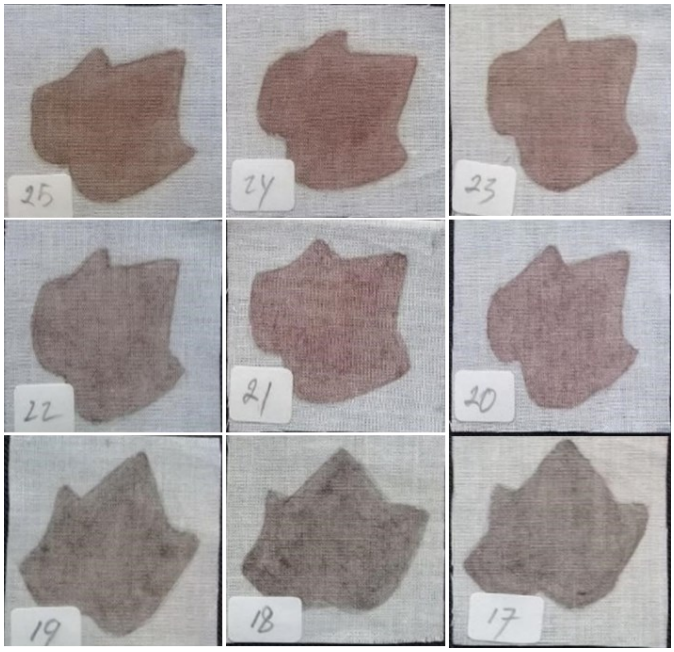

Figure 10. Color tidiness and hue painted with combination between different mordants and the dye pH (17) The dye pH 3 + vinegar + soda ash + ferrous sulfate; (18) The dye pH 7 + vinegar + soda ash + ferrous sulfate; (19) The dye pH 11 + vinegar + soda ash + ferrous sulfate; (20) The dye pH 3 + vinegar + soda ash + alum; (21) The dye pH 7 +vinegar + soda ash + alum; (22) The dye pH 11 + vinegar + soda ash + alum; (23) The dye pH 3 + vinegar + soda ash + lime; (24) The dye pH 7 + vinegar + soda ash + lime; and (25) The dye pH 11 + vinegar + soda ash + lime.

CONCLUSION

In conclusion, a mixed *Aspergillus* and *Paecilomyces* cultured on the modified medium of Baker and Tatum produced potential dyes that can be used in the paint industry. A mixture of the fungal dye, vinegar, and soda ash produced a tidy and good color hue so that it can be used to paint colors on cloth. Different mordants were applied to the mixture of the dye, vinegar, and soda ash produced varied hues of the painted color on the cloth. A combination between different mordants and pHs of the dye (3 and 7) was added to the mixture of vinegar and soda ash painted on the cloth each producing the same color hue. While except for alum, different mordants combined with the dye pH of 11 produced the same color as the treatments, but the intensity of color hue was one level below them. The present study proves the possibility of using the mixed *Aspergillus* and *Paecilomyces* as a source for the natural painting of colors on the cloth. The mixed fungi can be one of the substitute alternatives for many hazardous synthetic dyes for the painting of cloth.

AUTHORS CONTRIBUTION

Agung Adi Nugroho and Suciatmih conducted all the research and drafted manuscript.

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CONFLICT OF INTEREST

The author stated that there is no conflict interest.

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Research Article

The Diversity and Uniqueness of Avifauna in Erek-Erek Geoforest at Ijen Geopark, East Java, Indonesia

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ABSTRACT
Erek-Erek Geoforest (EEG) is one of the Biosites of Ijen Geopark located at the eastern slope of Mount Ijen. This location has unique topography of highland forests restricted by mountain ridges. This topography creates dense vegetation and humid ecosystem supporting microhabitats for endemic birds. This study aims to investigate the diversity and uniqueness of avifauna in EEG based on the existing value of birds. The method used is a point count at three potential station for the presence of birds. The data collected are the bird species, individual number of species, and species existence based on conservation status, distribution, and protection status. Data analysis includes the Shannon Wiener diversity index (H'), Evenness index (E), and existence factor (Ef) of bird community. The results show there are 57 species of birds belonging to 46 genera and 31 families. The diversity of birds in EEG Biosite has a high value (H'=3.40) and also a high evenness value (E=0.84). The Ef value of birds in this area is 51.35, which means the uniqueness value is a medium category. There are three bird species that have the highest Ef value, i.e *Arborophila orientalis* (Ef=80.00), *Pycnonotus bimaculatus* (Ef=73.33) and *Locustella montis* (Ef=73.33). The three species are endemic to Indonesia, especially *A. orientalis* whose distribution is limited to the highlands of East Java. Based on the composition, diversity, and uniqueness of avifauna in the EEG, it becomes valuable information for the government, Ijen Geopark Manager, and local communities to manage EEG Biosite conservatively by maintaining the existence of avifauna and their habitats.

INTRODUCTION
Mount Ijen is a complex highland area that stretches from the Bali Strait to the Bondowoso region (Caudron et al. 2015; Wirakusumah et al. 2019). In 2018, this area was designated as the Ijen National Geopark along with parts of the Meru Betiri National Park and Alas Purwo National Park. Then in 2020-2021, the East Java provincial government proposed the Ijen National Geopark to be a UNESCO Global Geopark (UGG) candidate (Geopark Ijen 2022). Geopark is a single or combined geographic area, which has a Geological Heritage Site (Geosite), Cultural Heritage Site (Culture-Site), and Biological Heritage Site (Biosite). One

of biosite covered in Ijen Geopark is Erek-Erek Geoforest (EEG). This area is a highland tropical rainforest ecosystem that has reached climax succession (Mulyana 2005). Geographically, EEG is located on the eastern slope of Mount Ijen and is the confluence of the valleys of Mount Merapi Ungup-ungup and Mount Rante (Siddiq 2015; Geopark Ijen 2022). This highland (1000-1800mdpl) has a high complexity stratification of plants. This condition provides important microhabitats and ecological niches specifically for bird communities (avifauna).

The basic information of birds community in the EEG is very important for ecotourism development supporting Ijen Geopark UGG. It is well known that a distinctive attraction for visitors in biosite is avitourism (Kuuder et al. 2015; Liu et al. 2021). According to Sitanggang et al. (2020), avitourism or bird-watching-based tours is one of the potential attractions by watching various kinds of birds with attractive colours and behaviours in their natural habitat. Furthermore, the characteristics of colours, sounds, shapes, or behaviours of the birds are attractive to bird-watchers (Moss 2004; Aditya et al. 2020). All the birds found in this area, specifically the endemic one, have potential value because of their unique characteristics and existences.

Birds can become an ecosystem, area, or even country icons because of their uniqueness, for example beautiful and endemic *Oreornis* (Papua), *Macrocephalon* (Sulawesi), and *Buettikoferella* (Nusa Tenggara) (Prawiradilaga 2019). Therefore, increasing knowledge and databases regarding the diversity of birds in EEG can be an additional reference for the development strategy in the Ijen Geopark UGG candidates' tourism site so that it becomes a distinctive attraction for visitors. One method for its development is to determine the diversity and unique value of the avifauna community. The uniqueness of flora and fauna can be determined by comparing the frequency of encounters, conservation status, and endemicity (Sulistiyowati & Buot 2015). In its development, the unique value approach, especially endemicity, can also be implemented for the bird community (Prawiradilaga 2019).

The existence of avifauna in the Ijen Mountains has not been clearly revealed. The East Java Natural Resources Conservation Center reported about 107 bird species were found in the Ijen Crater Nature Park, but the report is still in the form of field notes. Mittermeier et al. (2014) in their expedition on Mount Ijen, reported about 82 bird species occupying habitats at an altitude of 920-3000 masl. Mount Ijen is a habitat for bird species with limited distribution and endemic to highlands (Mackinnon et al. 2010). As stated by Pujolar et al. (2022), the unique topography of highland forests that are restricted by hills, valleys, or mountains will provide specific habitats for birds. So that these conditions affect the level of uniqueness of the bird community. Meanwhile, there is no scientific report on the existence of birds in the eastern part of Ijen, especially EEG. So this study aims to determine the diversity and uniqueness of avifauna in the EEG Ijen Geopark, East Java, Indonesia.

MATERIALS AND METHODS

Study Area

The research project was conducted in August–September 2021 at the Erek-erek Geoforest (EEG) of the Ijen Mountains, East Java (Figure 1). Observations were carried out in August, then data collection was carried out in September. The research area consists of forest area (1378 masl), forest edge area (1404 masl), and river bank forest area (1367 masl).

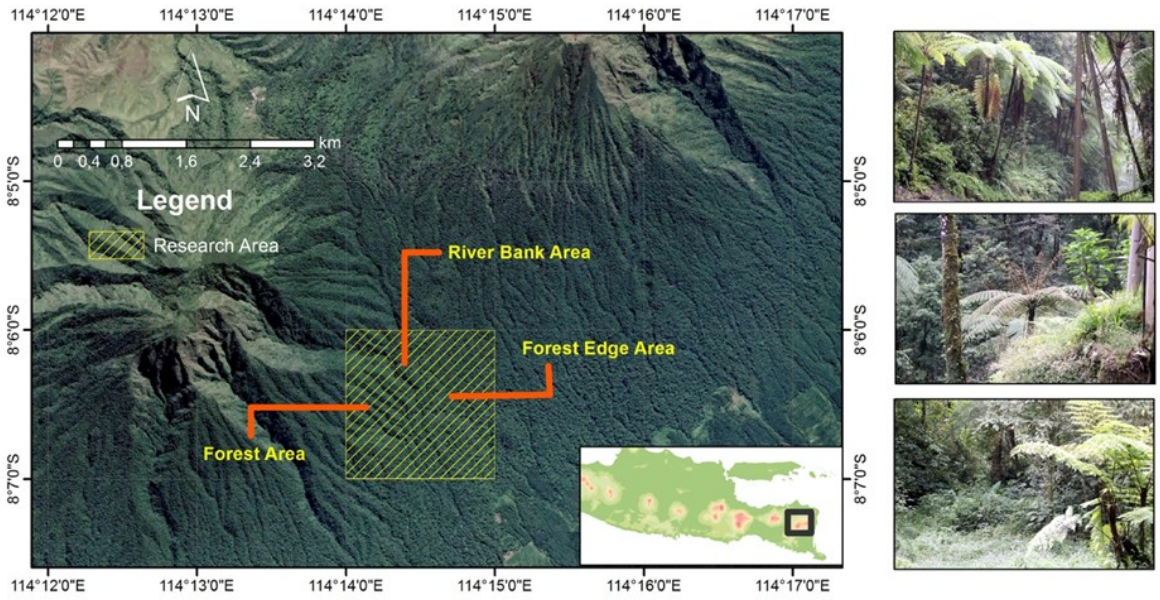

Figure 1. Study area in Erek-erek Geoforest, Ijen Geopark.

Data Collection

The point count method was used to collect data on avifauna and detect the presence of birds through physical sightings carried out in a fixed position for a predetermined duration (Bibby et al. 2000). There are three observation points set up based on vegetation characteristics. The range used in the observation (r) is 20 meters around the observation point. Observation time was ± 20 minutes at each point. Physicals or sounds of the birds were used to identify the presence of bird species (Sumaila et al. 2020). Data were collected on three consecutive days every week, then divided into two sessions in one day, morning (diurnal) (Sutherland et al. 2004) at 05.00-09.00 WIB and night (nocturnal) (Bartolommei et al. 2013) at 19.00-23.00 WIB. The visual morphology characteristics of birds were observed using the Binoculars (Bushnell Powerview 10x50), DSLR Camera (EOS 60D Cannon), Telephoto Lens (75-300mm), and field stationery. The sound of the birds was recorded using a Sony ICD-PX240. The process of identification and verification of physical avifauna species uses morphological characteristics referred to MacKinnon et al. (2010) and Taufiqurrahman et al. (2022), while that of sound-recorded confirmation using https://xeno-canto.org//. Furthermore, the number of individual birds for each species and type of habitat occupied is also counted.

Data Analysis

Data analysis through three approaches, i.e determining species composition, species diversity, and species qualification. The species composition is analysed based on bird taxonomy, conservation status, and protection status. The conservation status and endemicity of avifauna were referred to the International Union for Conservation of Nature (IUCN) Redlist (https://www.iucnredlist.org/). Meanwhile, the protection status was referred to the Regulation of the Minister of Environment and Forestry No. P.106 of 2018 and international trade regulation (https://cites.org/eng/app/index.php. Bird species diversity was determined by the Shannon-Wiener index (H') and species evenness (E) (Magurran 1988). Furthermore, the determination of the species qualification value (Ef) of birds

in the EEG refers to Sulistiyowati and Buot (2015) by considering three variables, i.e frequency of encounters, endemicity, and conservation status. Finally, the Ef value is converted into the weight and status of uniqueness which is the result of the revised formula by Tim Studi Keunikan Flora dan Fauna Universitas Indonesia (1995) and Sulistiyowati (2008).

RESULTS AND DISCUSSION

The composition of avifauna in EEG Biosite, Ijen Geopark

We identified 57 bird species belonging to 46 genera and 31 families from EEG Biosites of Ijen Geopark (Table 1). Ten species are protected by the Indonesia Government, including Crested Serpent-eagle (*Spilornis sheela*), Black eagle (*Ictinaetus malaiensis*), Javan kingfisher (*Halcyon cyanoventris*), Wreathed hornbill (*Rhyticeros undulatus*), Flame-fronted barbet (*Psilopogon armillaris*), Javan banded pitta (*Hydrornis guajanus*), White-bellied fantail (*Rhipidura euryura*), Streaky-breasted spiderhunter (*Arachnothera affinis*), White-flanked sunbird (*Aethopyga eximia*), and Javan Grey-throated White-eye (*Heleia javanica*). Based on the data above, there are two species that are endemic to Java (*R. euryura*; *Ae. eximia*) and four endemic to Java-Bali (*H. cyanoventris*; *P. armillaris*; *H. guajanus*; *H. javanica*) (Table 1). The species *R. euryura* and *Ae. eximia* is commonly found in mountainous areas. Furthermore, *H. javanica* also has a distribution in the highlands, which is above 1500 masl, as well as *P. armillaris* which has a higher elevation range of up to 2500 masl (MacKinnon et al. 2010; Mittermeier et al. 2014). Meanwhile, *H. cyanoventris* and *H. guajanus* are distributed in lowland to highland forests at 1000-1500 masl, especially *H. guajanus* prefer near rivers (MacKinnon et al. 2010; Iskandar et al. 2021).

The family with the highest species richness was Muscicapidae (8 species). This family is a very large and diverse in the old world. In the great Sunda, there are about 43 species and some of which are wintering migrants (MacKinnon et al. 2010). This study also confirmed–records from previous expeditions in the Ijen mountains (Mittermeier et al. 2014), such as Black eagle (*I. malaiensis*), Red Junglefowl (*Gallus gallus*), Grey-cheeked green pigeon (*Treron griseicauda*), Oriental cuckoo (*Cuculus saturates*), Barred Eagle-owl (*Bubo sumatranus*), Common flameback (*Dinopium javanense*), Javan banded pitta (*H. guajanus*), Velvet-fronted nuthatch (*Sitta frontalis*), Siberian Thrush (*Zoothera aurea*), White's thrush (*Geokichla citrina*), Arctic warbler (*Phylloscopus borealis*), Blue whistling thrush (*Cyanoptila cyanomelana*), and Javan Grey-throated White-eye (*H. javanica*).

According to the IUCN Red List, birds in the EEG have four conservation statuses (LC: 54 species (95%); NT: one species (2%); VU: two species (3%)) (Figure 2). It shows that EEG is one of the important habitats for near-threatened and vulnerable birds in East Java. One of which is *Rhyticeros undulatus* which has a limited distribution in the primary forest of the Greater Sunda region (Sukmantoro et al. 2007; MacKinnon et al. 2010). It is due to the very specific selection of feed and nests (Poonswad 1993; Rahayuningsih et al. 2017). The EEG area is also suspected to be one of the nesting or foraging areas of this species in Ijen. Meanwhile, based on the Convention on International Trade in Endangered Species (CITES), 53 species (93%) were non-appendix and four species (7%) were Appendix II (Figure 2). Appendix II is not included in the endangered category but has the possibility to be threatened with extinction if the trade is not regulated, so a licensing mechanism is needed through the management authority. Another important piece of infor-

Table 1. Species composition of avifauna in the EEG. Abbreviation as follows: Least Concern (LC), Near Threatened (NT), Vulnerable (VU), Not Appendix (NA), Protected (P), Not Protected (NP).

Family: Species		Common Name	Status		
			IUCN	CITES	National Status
Accipitridae					
	Spilornis cheela	Crested Serpent-eagle	LC	II	P
	Ictinaetus malaiensis	Black eagle	LC	II	P
Alcedinidae					
	*Halcyon cyanoventris**	Javan kingfisher	LC	NA	P
Bucerotidae					
	Rhyticeros undulatus	Wreathed hornbill	VU	II	P
Campephagidae					
	Coracina larvata	Sunda cuckooshrike	LC	NA	NP
	Pericrocotus miniatus	Sunda minivet	LC	NA	NP
Caprimulgidae					
	Caprimulgus macrurus	Large-tailed nightjar	LC	NA	NP
Cisticolidae					
	*Orthotomus sepium**	Olive-backed tailorbird	LC	NA	NP
	Phyllergates cucullatus	Mountain tailorbird	LC	NA	NP
Columbidae					
	Treron griseicauda	Grey-cheeked green pigeon	LC	NA	NP
	Ptilinopus porphyreus	Pink-headed fruit dove	LC	NA	NP
	Macropygia ruficeps	Little Cuckoo-dove	LC	NA	NP
	Macropygia emiliana	Ruddy Cuckoo-dove	LC	NA	NP
	Ducula lacernulata	Dark-backed imperial pigeon	LC	NA	NP
Cuculidae					
	Phaenicophaeus curvirostris	Chestnut-breasted malkoha	LC	NA	NP
	Cuculus saturates	Oriental cuckoo	LC	NA	NP
Dicruridae					
	Dicrurus leucophaeus	Ashy drongo	LC	NA	NP
	Dicrurus remifer	Lesser racket-tailed drongo	LC	NA	NP
	Dicrurus paradiseus	Greater racquet-tailed drongo	LC	NA	NP
Locustellidae					
	*Locustella montis**	Sunda Grasshopper-warbler	LC	NA	NP
Megalamidae					
	*Psilopogon armillaris**	Flame-fronted barbet	LC	NA	P
Muscicapidae					
	Brachypteryx leucophrys	Lesser shortwing	LC	NA	NP
	Cyanoptila cyanomelana	Blue-and-white flycatcher	LC	NA	NP
	Enicurus velatus	Sunda forktail	LC	NA	NP
	Eumyias indigo	Indigo flycatcher	LC	NA	NP
	Ficedula westermanni	Little pied flycatcher	LC	NA	NP
	Ficedula hyperythra	Snowy-browed flycatcher	LC	NA	NP
	*Myophonus glaucinus**	Javan whistling-thrush	LC	NA	NP
	Myophonus caeruleus	Bluewhistling-thrush	LC	NA	NP
Nectariniidae					
	Arachnothera affinis	Streaky-breasted spiderhunter	LC	NA	P
	*Aethopyga eximia***	White-flanked sunbird	LC	NA	P
Paridae					
	Parus major	Great Tit	LC	NA	NP
Pellornidae					
	Malacocincla sepiaria	Horsfield's babbler	LC	NA	NP
	Trichastoma pyrrogenys	Temminck's babbler	LC	NA	NP

Table 1. Contd.

Family: Species		Common Name	Status		
			IUCN	CITES	National Status
Phasianidae					
	*Arborophila orientalis****	Grey-breasted partridge	VU	NA	NP
	Gallus gallus	Red Junglefowl	LC	NA	NP
Phylloscopidae					
	*Phylloscopus grammiceps**	Javan warbler	LC	NA	NP
	Phylloscopus trivirgatus	Mountain warbler	LC	NA	NP
	Phylloscopus borealis	Arctic warbler	LC	NA	NP
Picidae					
	Dinopium javanense	Common flameback	LC	NA	NP
Pittidae					
	*Hydrornis guajanus**	Javan banded pitta	LC	NA	P
Pnoepygidae					
	Pnoepyga pusilla	Pygmy Wren-babbler	LC	NA	NP
Podargidae					
	Batrachostomus javensis	Javan frogmouth	LC	NA	NP
Pycnonotidae					
	Pycnonotus bimaculatus	Orange-spotted bulbul	NT	NA	NP
	Ixos virescens	Sunda bulbul	LC	NA	NP
Rhipiduridae					
	*Rhipidura euryura***	White-bellied fantail	LC	NA	P
Sittidae					
	Sitta azurea	Blue nuthatch	LC	NA	NP
	Sitta frontalis	Velvet-fronted nuthatch	LC	NA	NP
Strigidae					
	Bubo sumatranus	Barred Eagle-owl	LC	II	NP
Timaliidae					
	*Cyanoderma melanothorax**	Crescent-chested babbler	LC	NA	NP
Turdidae					
	Geokichla citrina	White's thrush	LC	NA	NP
	Zoothera aurea	Siberian Thrush	LC	NA	NP
	Zoothera sibirica	Orange-headed thrush	LC	NA	NP
Vangidae					
	Hemipus hirundinaceus	Black-winged flycatcher-shrike	LC	NA	NP
Vireonidae					
	Pteruthius aenobarbus	Triling shrike-babbler	LC	NA	NP
	Pteruthius flaviscapis	Pied Shrike-babbler	LC	NA	NP
Zosteropidae					
	*Heleia javanica**	Javan Grey-throated White-eye	LC	NA	P

Notes: *endemic to Java and Bali; ** endemic to Java; *** endemic to East Java highland.

mation, there are also 18% protected birds in the EEG. Expectedly, this protected status will certainly limit hunting and trade in Indonesia. However, awareness is also needed to maintain the birds that have not been protected and their natural habitats.

The EEG that are not conservation areas must be of particular concern in the monitoring of birds, especially those that are endemic and threatened. The potential for habitat degradation and illegal poaching in Indonesia is still high. As reported by Nijman et al. (2022), the threat of poaching and illegal trade, especially raptors on Java, Bali, and Lombok still exists. So that proposing this EEG area to be Ijen Geopark Biosites is a very appropriate step for in-situ conservation efforts.

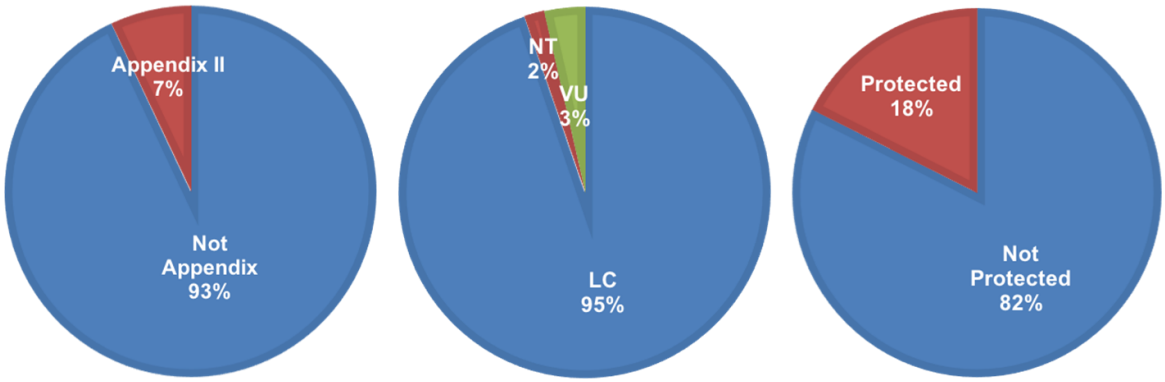

Figure 2. The CITES Appendices (left); Conservation status (middle); and National status (right) of Avifauna in EEG.

Furthermore, the discovery of Javan banded pitta (*H. guajanus*) in this study is valuable information for government and conservationists because this species is endemic and has a decreasing population (Birdlife International 2016). The *H. guajanus* species is endemic to Java and Bali which prefers closed primary and secondary forests at 1500 masl (Rheindt & Eaton 2010; Haryono & Pramono 2019). This species is found in the Ciletuh-Pelabuhan Ratu Geopark, West Java (Iskandar et al. 2021) and the natural forest of Mount Salak (Husodo et al. 2020). In this study, *H. guajanus* is found in forest area and occupied in the forest floor with dense tree canopy cover. This species was observed to be active during the day and more often observed sound than physically visible. The EEG is also a habitat for nocturnal birds, one of which is the Javan frogmouth (*B. javensis*). This species is a nocturnal species that was distributed in Southeast Asia, Palawan, and the Greater Sunda (MacKinnon et al. 2010; Puan et al. 2015). In the EEG area, this species occupies more of the riverbank forest area.

The diversity of avifauna in EEG Biosite, Ijen Geopark

According to the Shannon Wiener index, the diversity value of birds in the EEG Biosite was included in the high category (H'=3.40). This value is higher than other Java highlands, such as Telaga Warna Bogor (Ekowati et al. 2016), the land around mount Argopuro (Aryanti et al. 2018), and Promasan hiking trail Mount Ungaran (Purnamaningrum et al. 2021). This shows that EEG is one of the preferred habitats for birds in the highlands of Java. Based on this, further research is also needed on the characteristics and suitability of the avifauna habitat in the EEG. The high diversity value is also influenced by the abundance of each species. This species diversity consists of two primary components, i.e species richness and evenness (Ludwig & Reynolds 1998). In this study, it was

Figure 3. From left: *Arborophila orientalis*, *Hydrornis guajanus*, *Batrachostomus javensis* (Captured by Samsuri).

found that the average value was quite high (E=0.84). This value indicates that the avifauna in the EEG Biosite tends to be evenly distributed and no species dominates. In addition, this condition indicates the complexity of the interactions that occur in various species (Soegianto 1994). It means that the EEG is suitable habitat for 57 species because of foods and nesting availability supported evenly. Geopark Ijen (2022) states that the EEG area has a complete vegetation structure, i.e. herbs, shrubs, and trees such as *Cyathea contaminans*, *Mallotus* sp, *Annona* sp, *Toona sureni*, *Casuarina junghuhniana*, *Pterospermum diversifolium*, and *Ficus* sp. Further research is also needed on the presence of nests, availability of food, and preferences of vegetation as habitat. The availability of food resources and nests is an important factor that affects the abundance of bird species in a habitat (Martin 1995; Jara et al. 2020).

The qualification value (Ef) of the bird community at the Ijen Geopark EEG Biosite is 51.35, so it means the uniqueness of the bird community in this area is a medium category. The determination of this category is based on Tim Studi Keunikan Flora dan Fauna UI (1995) and Sulistiyowati (2008). This category is mainly influenced by each bird in this area having variations in frequency, endemicity, and conservation status values. There are three species of birds that have the highest Ef values, i.e *A. orientalis* (80.00), *P. bimaculatus* (73.33) and *L. montis* (73.33) (Figure 4). These three species are limited distribution in highland forests. Species *A. orientalis* is limited to the mountains Iyang and Ijen at an altitude of 500-2000 masl (MacKinnon et al. 2010). Meanwhile, *P. bimaculatus* has a more extreme altitude distribution (800-3000 masl). This species was found in the mountains of Sumatra, Java, and Bali. (MacKinnon et al. 2010; Mittermeier et al. 2014). Species *L. montis* prefer in open areas with dense bushes and shrubs at the edge of the forest or crater slopes above 1500-2100 masl (Madge 2016). This species also has a very limited distribution, i.e Mount of Central Java, East Java, and Bali (Taufiqurrahman et al. 2022).

The bird species composition, diversity and existence found in EEG is valuable information for the government and Ijen Geopark Managers for further conservation action specifically as an avitourism destination. This area has a unique species composition and high avifauna diversity. The information is also can be used as a conservation-based educational area for all elements of society, whether local communities, students, or researchers in developing their research. The development of avitourism in collaboration with local communities is an indispensable conservation strategy. Hereinafter, further research on avifauna ecology in EEG is also very needed, such as population or habitats of endemic birds. One of which is to estimate the population of the Grey-breasted partridge (*A. orientalis*) and assess their habitat characteristics. In addition, it can also determine the threat level of avifauna in the EEG. Accordingly, the data of avifauna, both ecological and threats in the EEG are more complete.

CONCLUSION

In this study, 57 bird species were found in the EEG Ijen Geopark Biosite as their habitat. There are 10 birds that are protected under government regulations, then 54 birds categorized as LC, one NT species, and two VU species. The Ijen Geopark EEG Biosite area has a high diversity of bird species that indicates the avifauna in the EEG Biosite tends to be evenly distributed and no species dominates. The unique value of the bird community in this area is in the medium category. There are three bird species that have the highest Ef values, i.e *A. orientalis*, *P. bimaculatus*, and *L. montis*.

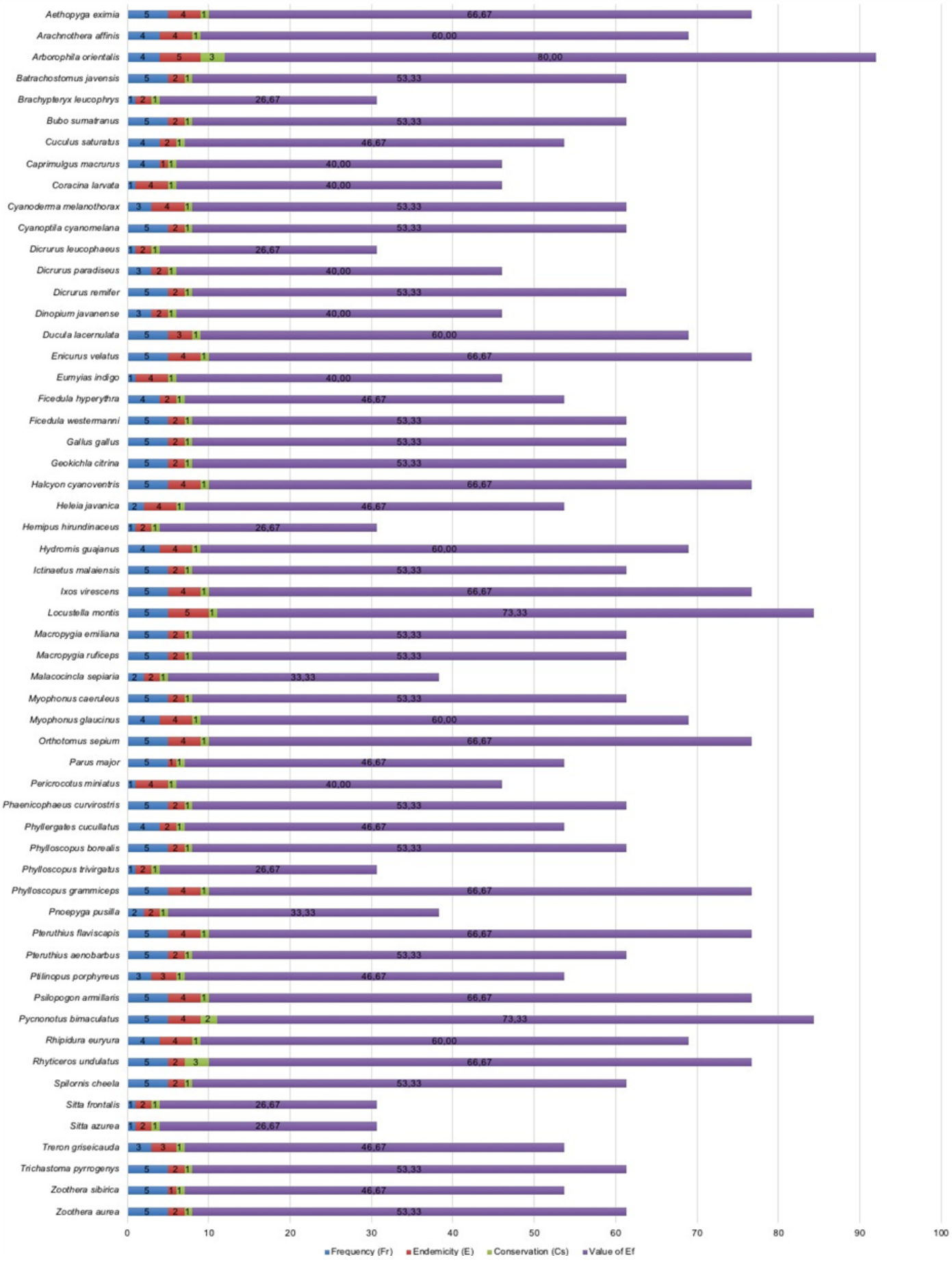

Figure 4. The Ef Value of Birds Community in EEG Ijen Geopark.

AUTHORS CONTRIBUTION

A.M.S designed the research, collected the data, species documentation, analysed the data, and wrote the initial manuscript. A.S.K., A.A., and S.

contributed to collecting the data, species documentation, and verification of birds species. H.S. and A.S.K. reviewed, revised, and proofread the final manuscript.

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CONFLICT OF INTEREST

The authors confirm that there are no known conflicts of interest associated with this publication.

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Research Article

The Formula media *in vitro* Propagation and Conservation of *Ludwigia* sp.

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ABSTRACT
The aquatic plant "Red Malang" (*Ludwigia* sp.) has a fairly high economic value as an ornamental aquatic plant, so it has the potential to be developed. The growth of *in vitro* cultures in culture bottles is high-speed, so it is necessary to find a formula media to inhibit growth so that the frequency of subcultures is reduced. The current research aims to produce a formula media for shoot multiplication and *in vitro* culture conservation. The research was carried out at the ICABIOGRAD tissue culture laboratory from April 2020 to June 2021. Research activities included plant propagation, conservation, and regeneration after conservation. Plant material was using in the form of a culture collection in the ICABIOGRAD tissue culture laboratory, treatment media for propagation were BA (0; 0.1; 0.3; 0.5; 0.7 and 0.9 mg/L) + thidiazuron (TDZ) (0 and 0.1mg/L). For conservation were MS + BA medium (0 and 0.1 mg/L) + paclobutrazol (0; 0.1; 0.3; 0.5; 0.7 mg/L) and for shoot regeneration after conservation using MS medium without Plant Growth Regulator (PGR). Data analysis using the Anova SAS version 9.0 test program. Further test using DMRT test with alpha level 5%. There was no difference in the mean value between levels of TDZ treatment on the number of shoots and leaves. The difference in the mean value between levels of TDZ treatment was very significant on shoot height, the number of roots, and root length. BA treatment with a concentration of 0.7 mg/L is better because it gives higher results for each observation variable. For conservation, treatment with paclobutrazol 0.5 mg/L inhibited shoot and leaf count, and 0.3 mg/L inhibited shoot formation. Cultures stored for six months grew normally after being regenerated. The highest shoots and the highest number of leaves were obtained from the treatment of paclobutrazol without BA. This study indicated that the propagation media of aquatic plants *Ludwigia* sp. did not require high concentrations of BA. Cultures could be stored for over six months using paclobutrazol with 0.3-0.6 mg/L.

INTRODUCTION
Red Malang (*Ludwigia* sp.) is an aquatic water plant that has been widely developed, and its uses include beautifying aquariums. *Ludwigia* sp. aquatic plants are attractive for commercialization because they have economic value and are unique because of their bright red leaves. The benefits of aquatic ornamental plants are an addition to beautifying aquariums or fish ponds. It also protects fish from sunlight and improves water quality because they can produce oxygen and absorb toxins such as ammonia in the water and as a place for fish to lay eggs (Nugraha et al. 2018). Types of ornamental plants currently being developed include *Bacopa* genus, *B. australensis*, *B. caroliniana*, *B. ianigera*. *B. myriophylloides*. *B.*

monnieri, *B. rotundifolia*. *B. chamaedryoides* (Kunth) Wettst *(Syn. Herpestis chamaedryoides Kunth)*. *B. australis* (Nugraha et al. 2017).

Aquatic plants' micro-propagation is still conventional (Yunita et al. 2018). Constraints from conventional propagation for mass production of aquatic ornamental plants include non-uniform and non-sterile seeds produced. Developing tissue culture techniques to obtain significant and sterile seedlings is necessary to solve the problem. Tissue culture technology currently being applied has many benefits, including mass propagation of high-yield seedlings to assemble new varieties and preserve germplasm (Siddique et al. 2015). The formula media for the propagation of aquatic plants *B. australis* has been produced by Yunita et al. (2018), Nugraha et al. (2017), and Nugraha et al. (2018), using MS media + 0.5 mg/L Benzyl Adenine (BA)+ 0.5 mg/L kinetin and 0.5 mg/L BA + 0.1 mg/L TDZ.

BA, a growth regulator from the cytokinin group, has been widely used for shoot induction and propagation *in vitro* culture because BA has a vital activity for cell division and is more stable than kinetin and zeatin (Lestari 2011; M. et al. 2017). PGR, such as BA has a significant role in cell division during plant metabolism in shoot induction and multiplication (Ashraf et al. 2014). The exact concentration of BA for each plant is not the same depending on the type of plant, physiological conditions of the explant, type of primary media, environmental conditions of growth, and genetic factors (Lestari 2015). Propagation of ginger using ZPT 4.5 mg/L BA is the best concentration to stimulate shoot multiplication (Abbas et al. 2011). Besides PGR, minerals are essential elements in media culture (Kumar & Reddy 2011). TDZ is a compound belonging to diphenyl urea and has almost the same activity as cytokinins, often used to stimulate cell division in shoot proliferation, especially in woody plants (Lestari 2015). Using a combination of TDZ with BA has succeeded in increasing the ability of cell proliferation in various plants, for example, *Plumbago zeylanica* (Syahid & Kristina 2008; Lestari et al. 2013).

In vitro culture conservation for active collection usually uses a formula media for propagation to only last for 2-3 months because the nutrients have run out (Dewi et al. 2016). For culture growth to be extended, it needs to be inhibited to prolong the time of sub-culture. Reducing the frequency of sub-cultures will lower maintenance costs and reduce the risk of contamination (Dewi et al. 2016; Mendes et al. 2021). Conservation of aquatic ornamental plants *B. australis* and *Alternatia reinecki* using MS media + 0.7 mg/L paclobutrazol showed inhibition until the 6th on shoot height, number of shoots, number of roots, root length, and number of leaves. In this conservation medium, the culture remains green and looks fresh (Lestari et al. 2021). In culture conservation, through *in vitro* culture, several techniques can be applied, including cryopreservation, simple conservation, and slow growth conservation (Dewi et al. 2016).

Conservation media techniques with slow growth techniques generally use chemical compounds to inhibit the growth of cultures, including paclobutrazol, cycocel (CCC), and osmotic compounds such as sorbitol and mannitol (Huang et al. 2014; Silva et al. 2019). Paclobutrazol is an active compound inhibiting the oxidation of kaurene to ent-kaurene as a precursor of the growth regulator of gibberellic acid in the apical meristem, causing inhibition of cell division at the growth point (Negi et al. 2017; Bisht et al. 2018). In slow-growth conservation, growth and cell division are conditioned to occur very slowly, or metabolism is stopped so that culture development stops and does not change the genetic nature of the plant (Lestari et al. 2021). The benefits of *in vitro* culture conservation include keeping germplasm accessions/collections from becoming extinct (Huang et al. 2014) by inhibiting cell growth and division from

becoming very slow (Indrayanti et al. 2018).

The advantages of *in vitro* culture for conservation include that it does not require a large/wide place/container, accessions, or germplasm stored in thousands and can be stored for more than five years (Dewi et al. 2016). To maintain accession, which is very valuable germplasm has a high economic value. In contrast, if it is stored conventionally, there is a risk of damage due to natural disasters or drought (Silva et al. 2019). The collection of germplasm accessions is also beneficial as genetic material in the assembly of new varieties (Arrigoni-Blank et al. 2014). Formulas media for the propagation of aquatic ornamental plants are still limited, namely *B. australis* (Nugraha et al. 2017; Yunita et al. 2018), as well as formula media for *in vitro* culture conservation.

Lestari et al. (2021) conducted a conservation study through *in vitro* culture of *B. australis* and *A. reinecki* using MS + paclobutrazol media. So many aquatic plants are currently being developed that research is needed to obtain formula media for *in vitro* propagation and conservation. The study aimed to produce the best formula media for the propagation and conservation of ornamental plant cultures.

MATERIALS AND METHODS

Materials

Plant material is a sterile culture of aquatic plants aged two months and collected of the Tissue Culture Laboratory.

Methods

The research was carried out at the tissue Culture Laboratory, the Indonesian Centre for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), at Cimanggu street No. 3 Bogor from April 2020 to July 2021.

The research consisted of 3 activities, namely (1) Testing the formula media for propagation, (2) Testing the formula media for conservation (3) Testing the regeneration ability of the culture after conservation. The media used were MS basic media plus macro salt, micro salt, vitamins from group B (thiamine, pyridoxine, nicotinamide acid), and myo inositol, two grams of agar gel were added as a compactor, and 30 g of sucrose as a carbon source.

The media's pH was 5.7 by adding 0.1 N HCl or NaOH solution. The media was sterilized using an autoclave with a pressure of 121 Psi for 15 minutes. Sterile culture bottles filled with 25 ml of treatment media

Test the media formula for propagation.

The ex-plant was used as a single node with a size of ± 1 cm. The experimental design was factorial and completely randomized. consisting of two factors. the first factor was the concentration of BA (0; 0.1; 0.3; 0.5; 0.7 and 0.9 mg/L). Moreover, the second factor was the concentration of TDZ (0 and 0.1 mg/L). Each treatment was repeated in ten bottles. The observed variables were shoot height, number of shoots, number of leaves, number of roots, and root length. The basic medium commonly used for shoot induction and shoot multiplication is MS base media (Murashige & Skoog 1962).

Test the formula media for conservation.

Ex-plants are stem from sterile single-node culture ± 1 cm. The experimental design was factorial and completely randomized, consisting of two factors: two levels of BA concentration (0 and 0.1 mg/L), and the second factor was five levels of paclobutrazol concentration (0; 1; 0.3; 0.5;

0.7 and 0.9 mg/L). Bottles planted with ex-plants are placed on the culture rack under irradiation using a TL lamp. Illumination intensity 1500 lux for 16 hours in 1 day. The variables observed were shoot height, number of shoots, number of leaves, root length, and number of roots.

Culture regeneration after conservation.

Cultures stored for ± six months were transferred to MS 0 media (without PGR). Ex-plants in the form of stems of single nodes, one explant per bottle. The number of ex-plants from each conservation medium was planted in as many as ten bottles. The observed variables included shoot height, number of shoots, and number of leaves.

Data Analysis

The data were analysed using the Anova SAS version 9.0 test program, further testing using DMRT with an alpha level of 5%.

RESULTS AND DISCUSSION

Results

Induction of shoot multiplication

Analysis of variance on the variables of shoot height, number of shoots, number of leaves, and number of roots and interactions between TDZ and BA treatments are presented in Tables 1 and 2. Analysis of variance on all observed variables showed an interaction between TDZ treatment and BA.

Table 1. The variance of each observation variable on shoot proliferation.

Variable	F-Calculate treatment			CV (%)
	Thidiazuron (TDZ)	Benzil Adenin (BA)	TDZ*BA	
Shoot height	107.88**	8.30**	6.39**	27.90
Number of shoots	0.05ns	2.57*	3.51**	46.19
Number of leaves	3.55ns	1.59ns	1.81ns	36.82
Number of roots	175.06**	3.25**	17.89**	68.35
Root length	137.28**	5.16**	10.52**	79.07

Note: ns) not significantly different at = 5% based on F-test results. *) significantly different at = 5% based on F-test results. **) very significant difference at = 1% based on F-test results. tdz*BA Interaction between this treatment and BA. CV) Coefficient of Diversity.

Test formula media for conservation

Analysis of variance and Interaction between BA treatment and paclobutrazol on the variables of shoot height, number of leaves, number of roots, root length and number of shoots are presented in Tables 3 and 4.

Shoot regeneration after conservation

Analysis of the various effects of BA with paclobutrazol treatments on conservation media showed an interaction, and the results were significantly different for all observed variables (Table 5). The effect of interaction between BA and paclobutrazol during conservation on shoot regeneration ability is presented in Table 6.

The Interaction between TDZ and BA for shoot propagation showed that BA treatment of 0.7 mg/L without TDZ produced the highest shoots at 5.93 cm. However, for the number of shoots, the number of roots and root length were not significantly different (Table 2).

It is suspected that the *Ludwigia* sp. contains high levels of PGR, both cytokinins and auxins, so in the media, without BA and TDZ quite a lot of shoots were produced (Table 1). The results prove that the activity

Table 2. Interaction of Thidiazuron and Benzyl Adenine. on shoot height, number of shoots, number of roots, and root length five weeks after planting (MST).

Thidiazuron (mg/L)	Benzil Adenin (mg/L)						Mean
	0.0	0.1	0.3	0.5	0.7	0.9	
shoot height (cm)							
0.0	3.91^{b}	3.80^{b}	3.20^{bc}	3.10^{bc}	5.40^{a}	3.80^{b}	3.86
0.1	3.41^{b}	1.83^{d}	1.83^{d}	1.86^{d}	2.06^{d}	2.51^{cd}	2.25
Mean	3.66	2.81	2.51	2.48	3.73	3.15	
CV (%)	7.90						
Number of shoots							
0.0	4.60^{abc}	5.50^{ab}	5.80^{ab}	6.80^{a}	3.80^{bc}	5.40^{ab}	4.35
0.1	7.00^{a}	2.60^{c}	6.20^{ab}	5.70^{ab}	5.70^{ab}	4.10^{bc}	5.21
Mean	5.8	4.05	6	6.25	4.75	4.75	
CV (%)	46.19						
Number of roots							
0.0	7.50^{a}	0.00^{c}	8.02^{a}	7.00^{a}	0.00^{c}	7.90^{a}	5.1
0.1	3.20^{b}	0.30^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.58
Mean	5.35	0.15	4.01	3.5	0	3.95	
CV (%)	8.35						
Root length (cm)							
0.0	0.00^{c}	1.65^{a}	1.05^{b}	1.37^{ab}	1.43^{ab}	1.32^{ab}	0.94
0.1	0.37^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.37
Mean	0.18	0.85	0.5	0.68	0.7	0.66	
CV (%)	79.07						

Note: Data followed by the same letter in the same variable are not significantly different based on DMRT test level = 5%.

Table 3. Analysis of the variance of each variable in the treatment of BA and paclobutrazol Six months after planting.

Variable	F calculate			CV (%)
	Benzil Adenin (BA)	Paklobutrazol	BA*paclobutrazol	
Shoot height	17.9**	11.93**	29.8**	26.56
Number of leaves	8.99**	10.74**	6.55**	30.14
Number of roots	3.31^{ns}	3.50*	5.21**	35.23
Root length	22.62**	kon11.35**	0.52^{ns}	55.39
Number of shoots	7.12**	3.52*	11.48**	32.92

Note: ns) is not significantly different at = 5% based on the F-test results. *) is significantly different at = 5% based on the F-test results. **) is significantly different at = 1% based on the F-test results. BA*paclobutrazol) interaction between BA and paclobutrazol treatments. CV) Coefficient of diversity.

of growth regulators depends on the type of chemicals, chemical structure, and chemical concentration, plant genotype and physiological phase (Satyavathi et al. 2004).

TDZ can be given together with other growth regulators, such as cytokinins or auxins. At the initiation of mangosteen shoots, using BA plus TDZ is the best treatment for shoot formation (Lestari et al. 2015). TDZ stimulates shoot multiplication in several plants, including breadfruit (Supriati et al. 2005) and aromatic ginger, by adding 0.1 mg/L TDZ to MS + BA 5 mg/L (Lestari & Hutami 2005). However, not all plants responded to shoot multiplication in starfruit plants, TDZ only increased shoot height and number of leaves (Supriati et al. 2005).

Paclobutrazol is a growth inhibitory compound that has been applied to inhibit the growth of cultures in *in vitro* culture. and in various plants, including fruit trees (Hasan et al. 2013; Mendes et al. 2021). The

Table 4. Effect of Interaction between BA and paclobutrazol on the growth of shoot height number of leaves, number of roots, and number of shoots.

Benzil Adenin (mg/L)	Paclobutrazol(mg/L)				Mean
	0.1	0.3	0.5	0.7	
Shoot height (cm)					
0.0	2.50^{d}	4.67^{b}	2.95^{cd}	2.88^{cd}	3.25
0.1	6.80^{a}	3.30^{cd}	3.78^{c}	2.90^{cd}	4.19
Mean	4.65	3.98	3.36	2.89	
CV (%)	26.56				
Number of leaves					
0.0	29.30^{bc}	3.70^{b}	20.10^{d}	23.10^{cd}	26.3
0.1	47.20^{a}	25.90^{bcd}	25.67^{bcd}	30.30^{bc}	32.26
Mean	38.25	29.3	282.88	26.7	
CV (%)	30.14				
Number of roots					
0.0	18.30^{bc}	21.40^{bc}	15.00^{c}	21.50^{bc}	19.05
0.1	29.30^{a}	14.80^{c}	20.56^{bc}	23.40^{ab}	22.01
Mean	23.8	18.1	17.78	22.45	
CV (%)	35.23				
Number of shoots					
0.0	1.40^{b}	1.60^{b}	1.10^{b}	1.50^{b}	1.4
0.1	2.20^{a}	1.10^{b}	2.22^{a}	1.30^{b}	1.7
Mean	1.8	1.35	1.66	1.45	
CV (%)	32.92				

Note: Data followed by the same letter on the same variable are not significantly different based on Duncan's test with a level of = 5%.

Table 5. The variance of each variable of the effect of BA and paclobutrazol treatment media on shoot regeneration after conservation.

Variable	F-Calculate			
	Benzil Adenin (BA)	Paclobutrazol	BA*Paclobutrazol	CV (%)
Shoot height (cm)	6.59 *	1.74 ns	8.37 **	33.81
Number of shoots	3.32 ns	7.54 **	14.24 **	46.77
Number of leaves	9.97 **	4.76 **	8.62 **	38.40

Note: ns) is not significantly different at = 5% based on the results of the F-test. *) is significantly different at = 5% based on the results of the F-test. **) significantly different at = 1% based on the results of the F-test. BA*paclobutrazol interaction between the treatment of BA and paclobutrazol. CV=Coefficient of diversity.

effectiveness of paclobutrazol concentrate in inhibiting plant growth depends on the physiological conditions of each plant and environmental conditions (Mog et al. 2019). Its inhibitory effect is through the regulation of physiological processes such as inhibiting plant size from shortening, namely the presence of short internodes, and reduction of leaf size (Muengkaew & Chaiprasart 2016).

The research of Roostika et al. (2009) showed that *Pimpinella pruatjan* cultures conservation *in vitro* using paclobutrazol inhibitors also caused robust inhibition, so the conservation period could not be extended more than four months from the culture during recovery a rosette appears. In addition to using paclobutrazol inhibitors to inhibit culture, it can be done by diluting the primary medium to 50 and 75% in combination with the mannitol osmoregulator 20 g/L, as was done for conservation on *Carica papaya* Dieng. The culture can be stored in that media for 16 weeks (Rahayu et al. 2015).

Table 6. Effect of Interaction of Benzyl Adenine and Paclobutrazol on Culture Growth after.

Benzil Adenin (mg/L)	Paclobutrazol (mg/L)			
	0.1	0.3	0.5	0.7
Shoot height (cm)				
0.0	3.41^{e}	4.97^{abc}	5.23^{ab}	5.93^{a}
0.1	4.73^{bcd}	4.47^{bcde}	3.85^{cde}	3.78^{de}
Mean	4.07	4.72	4.54	4.85
CV (%)	33.81			
Number of shoots				
0.0	1.72^{c}	1.80^{c}	1.73^{c}	3.80^{a}
0.1	1.93^{c}	2.67^{b}	1.60^{c}	1.63^{c}
Mean	1.82	2.2	1.66	2.71
CV (%)	46.77			
Number of leaves				
0.0	21.95^{cd}	22.20^{cd}	25.93^{bc}	35.73^{a}
0.1	19.23^{cd}	29.20^{b}	17.20^{d}	20.13^{cd}
Mean	20.59	25.7	21.56	27.93
CV (%)	38.40			

Note: Data followed by the same letter on the same variable are not significantly different based on Duncan's test with a level of = 5%.

Cultures grown on media with paclobutrazol without BA showed lower yields on all observed variables other than the number of roots. Inhibition of shoot height growth and the number of leaves obtained from treatment with paclobutrazol of 0.5 mg/L and paclobutrazol of 0.3 mg/L. It is inhibited the formation of the number of roots, root length, and the number of shoots (Table 4).

The results showed that the paclobutrazol concentration up to 0.7 mg/L did not inhibit the culture when regenerated after conservation. The average shoot height was 4.85 cm, the number of shoots was 2.71, and the number of leaves was 27.9, but this concentration influenced growth inhibition during Conservation (Table 4). Shoots regenerated from the treatment of BA 0 + paclobutrazol 0.7 mg/L produced higher shoots than those from treatment paclobutrazol 0.1-0.5 mg/L, as well as for variables number of shoots and number of leaves except in the treatment paclobutrazol of 0.5 mg/L (Table 6). Shoots from paclobutrazol 0.7 mg/L + BA 0.1 mg/L treatment shorter shoots were obtained and fewer leaves. The study results by Mendes et al. (2021) showed that the citrus cultures stored for 12 months using a paclobutrazol growth inhibitor did not undergo genetic changes based on analysis using SSR markers.

CONCLUSIONS

Based on the results of this research, it can be concluded that the best media for the propagation of *Ludwigia* sp. is MS primary media without PGR. The best medium for culture conservation was MS + 0.5-0.7 mg/L paclobutrazol. Cultures stored in 0.7 mg/L paclobutrazol for six months produced the best response to growth after conservation.

AUTHOR CONTRIBUTION

All authors in this article have the same contribution as the main contributors both in research and in the preparation of the paper

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CONFLICT OF INTEREST

The Authors declare that there is no conflict of interest.

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Research Article

Relationships Among Biomass, Carbon, and Microfibril Angle in Young *Shorea* spp. (Dipterocarpaceae) in Indonesia

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ABSTRACT
Indonesia, particularly the island of Java, is dominated by a number of *Shorea* tree species (Dipterocarpaceae). Trees of the genus have been utilized for various practices, and they play a fundamental role in managing the stability of tropical forests. This study was carried out to understand the relationships between biomass and microfibril angle in *Shorea* spp. growing in West Java, where *Shorea* spp., are abundant. A total of 35 young trees belonging to 5 species were studied. The average age of these trees was 9 years, but in general there was a wide variation in tree diameter and total height. On average, biomass was the highest in *S. leprosura* and the lowest in *S. palembanica*. The lowest average microfibril angles (MFAs) were found in *S. leprosura* and *S. mecistopteryx*. The regression relationship between biomass and diameter was strong with an R^2 value of 0.85, while the strength of the relationship between MFA and diameter was weaker (R^2 = 0.195). In general, the MFA degree decreased with increased biomass accumulation *Shorea* species, which affects tree resistance to environmental variables and competitiveness in Indonesian tropical forests.

INTRODUCTION
Management and calculating forest biomass are crucial in addressing the issue of global climate (Mbow et al. 2014). Measuring the carbon stored in tree biomass has been widely applied in order to accurately and inclusively determine the capacity of forests to absorb and store carbon from the atmosphere (Wegiel & Polowy 2020; Rozendaal et al. 2021; Sufrayoga & Mardiatmoko 2022).

Tree biomass can be defined as the mass of organic materials that underpin all physiological and mechanical functions during the life of a tree. As a plant-based material, the biomass contained in trees is important because it contains a myriad of valuable components including carbon dioxide sequestration (Mildrexler et al. 2020; Ameray et al. 2021). Through photosynthesis, energy and other products are then transported to all parts of trees, leading to the accumulation of cellulose, hemicellulose, lignin, and a number of extractive compounds (Ng et al. 2015; Ma et al. 2018) in the wood stem tissue. Cellulose and hemicelluloses are long-

chain polysaccharide compounds that function as robust structural components in tree woody tissues (Berglund 2018; Donev et al. 2018). Both are involved in the construction of cell walls, help to maintain vertical tree growth, and buffer the effect of extreme environmental conditions alongside lignin compounds (Gibson 2012; Gril et al. 2017; Rongpipi et al. 2019).

Microfibril angle (MFA) is one of the key variables used to evaluate tree growth mechanisms as it describes the orientation of cellulose microfibril in the secondary layer of the cell wall, which significantly influences the mechanical properties of the whole tree (Auty et al. 2017; Xu et al. 2012). However, MFA varies greatly among tree species, locations, and climatic conditions. Even within the same tree, dramatic variation in MFA can be found among the basal, middle, and top parts of the tree (Groover 2016). In general, MFA is high when trees are in the juvenile growing phase, mostly on account of the effects of environmental factors. When trees are growing, the rate of photosynthesis is increased which leads to the accumulation of biomass and carbon sequestration (Kohl et al. 2017; Huang et al. 2021). Consequently, when biomass production is accelerated in young trees, the biomass will be slightly more stable, and this can lessen the impacts of environmental factors such as wind and storms. Craine & Dybzinski (2013), indicated that the frequency of photosynthesis and biomass accumulation is dependent on the surrounding environment, soil nutrients, micro-climate, light availability, and competition.

Shorea is a species-rich tree genus in the Dipterocarpaceae family, which is currently spreading across Southeast Asia. Classified as dense tropical hardwood, the genus has been commercially exploited for construction and housing across Indonesian forests as an economically-important woody product (Gaveau et al. 2013; Widiyanto et al. 2020). Owing to this high rate of usage, species of the genus were also planted and grown in some locations for either merchantable or silvicultural purposes. The decision to undertake this study was based on the wide-ranging benefits of the *Shorea* species in Indonesia. The general objective of this study was to understand the relationships between biomass, carbon sequestration, and MFA angle in some young *Shorea* trees planted in the West Java province of Indonesia. Furthermore, additional analyses were conducted to examine interactions with other variables such as diameter, height, volume, and wood density.

MATERIALS AND METHODS

Site

This research was conducted in Gunung Walat educational forest belonging to the Bogor Agricultural University (Institut Pertanian Bogor) in West Java Province, Indonesia, in 2013. The forest was established in 1968 and is primarily used for educational activities, research, endemic tree collection, and recreational purposes. The total area of the forest is 359 hectares, and it is divided into three blocks: an eastern block (Cikatomas) with a total area of 120 hectares, a western block (Cimenyan) of 125 hectares, and a central block (Tangkalak) of 114 hectares. Overall, the forest contains more than 44 tree species, which provide habitats for endemic bird, lizard, and insect species (Kusmana & Susanti 2015).

This forest lies between 460 m – 726 m above sea level. The typical soil is dominated by red-yellow latosols, brown latosols, and some podzols (Wibowo & Alby 2020). The area is relatively humid, with annual precipitation between 1700 mm and 4400 mm, while the temperature ranges from 18 °C to 30 °C (Kusmana & Susanti 2015). The present re-

search was conducted in an *ex situ* conservation plot, which was established in 2004 and was fully planted with *Shorea* spp. The plot measures around 0.6 hectares in area, with the topography relatively even and sloping < 35°.

Biomass & Carbon Estimates

Trees of the following five *Shorea* species planted in 2004 were included in the present study: *Shorea leprosula*, *Shorea mecistopteryx*, *Shorea pinanga*, *Shorea stenoptera*, and *Shorea palembanica*. Seven trees from each species were measured, giving a total of 35 sampled trees. The criteria for tree selection included good physical appearance and distribution within the plot. Then, the non-destructive biomass measurements of circumference at breast height (1.3 m) (DBH) and total height were taken for each tree. The circumference of each stem was measured using a diameter tape measure wrapped around the tree. For the total height measurements, a laser hypsometer was used (clinometer suunto PM–5). The volume was determined using the following allometric equation (Husch et al. 2003):

$$V = ¼ (\mu \times d^2 \times t \times a)$$

where: V = tree volume in m^3; d = diameter at breast height (DBH); t = total height of tree sample measurements; a = form factor (0.6) which is a term describing the ratio of tree volume to specified geometric solid volume based trees with similar diameter and height; and μ = phi which is the circumference measurement to diameter.

To obtain carbon accumulation in each sample tree, wood density (g/cm^3) was calculated first, which is the ratio of wood mass to volume under particular moisture conditions for each species. Samples were taken from one side of the trunk at breast height using an increment borer (Haglöf) with a 12 mm core size. In order to obtain more accurate wood density data, the corer was then kept in a green condition (refrigerator). After the cores were collected wood densities were calculated in the laboratory using ASTM standard. The cylindrical core samples were cut from the pith to the outer xylem rings into segments of approximately 1 cm, and these were weighted again until a constant value was achieved (Ricker et al. 2020). This procedure was used to obtain an estimate of average wood density, which was calculated using the following formula:

$$X = \frac{c \cdot \rho}{(b-a)}$$

where: X is the sample density obtained from core measurements, a is the balance obtained when the weighing basket was empty, b is the balance obtained when the weighing basket contained the sample, c is the mass of the dry cores, and ρ is the density of water equal to 1000 kg per cubic meter (Scandinavian Pulp, Paper and Board Testing Committee 1995).

Carbon sequestration measurements were based on the methodology of Krisnawati et al. (2012) because it was assumed this formula was more representative of the specific forest conditions in Indonesia's tropical forest, where the present study had taken place.

$$W = Vt \times BEF \times SG$$

where: W = total tree biomass in kilogram; Vt = total volume of tree in m^3; BEF = biomass expansion factor for tree (0.3; the ratio of the total above ground biomass to the biomass of the merchantable wood)), and SG = wood specific gravity (kg/m^3).

MFA and Fiber Length Measure

The MFA was measured in 35 *Shorea* trees. An increment borer was used to obtain core samples from one side of the tree trunk at a height of 1.3

m. These cores were then stored inside straws to maintain freshness. Visually, the collected cores showed no clear signs of tree ring boundaries. The cores were then sliced into a 1 cm sections to measure MFA and fiber length. Core pieces were first treated using HNO_3, and then KCl was added within minutes until the fibers were separated and the middle lamella portions were dissolved. A total of 30 fibril pieces were taken per sample, and these were placed onto a microscope slide for observations using a microscope interfaced with a digital camera. Images were then taken using the Motic imaging software to obtain fibril length measurements. To observe the fibril orientation angle, thin slices of core samples (± 30 µm) were placed under a microscope with a 100× magnification lens, and 10 images were taken of each sample slide. Three images were selected for further analysis of the microfibril angle, which was measured in degrees in Motic.

Data Analysis

A non-linear model was derived to analyze the relationships between biomass, carbon sequestration, and MFA orientation as well as the correlations among diameter, height, volume, and density in young *Shorea* trees. Fang and Bailey (1998) indicated that non-linear models are more adaptable when studying dense tropical forests. Given that certain uncontrollable factors affected this study, the power non-linear function for two parameters was used as a regression analysis tool. In addition, a preanalysis was carried out using some existing models, and this revealed that the non-linear power model was the best fit for the measured variables. Nonlinear models can be used to make biological interpretations and are less sensitive to individual data points, making them better and more reliable for data extrapolation (Archontoulis & Miguez 2015). This model has also been widely used to understand tree allometric relationships, and particularly to model height-diameter relationships (Scaranello et al. 2012). The power model had the following form (Fang & Bailey 1998):

$$Y = aX^b$$

where, a is the scaling coefficient (constant), and b is the scaling exponent derived from the regression fit to the empirical data. The variables included diameter, MFA (°), height (m), volume (m^3), total biomass (kg), total carbon (kg), and wood density (gr/cm^3). The chi square test was used to estimate the significance of differences among parameters (biomass accumulation, carbon accumulation, MFA, diameter, and height). In addition to nonlinear power models, statistical analysis of parameters was also undertaken. All statistical analyses were performed using SPSS version 23.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Biomass & MFA Variation

Biomass accumulation varied across the five measured *Shorea* species, with *S. leprosura* (15.728 kg) producing the greatest biomass, and *S. palembanica* (1.842 kg) producing the lowest biomass. Such variation within the genus is due to the number of tree species and size, particularly tree diameter and height. On the other hand, MFA only differed slightly among the *Shorea* species, with the highest average degree found in *S. mecistopteryx* (41.67°) and the lowest average degree observed in *S. leprosura* (35.94°).

Biomass accumulation differed significantly among species, while MFA did not differ significantly among species (Figure 1). The chi-square analysis showed significantly different biomass accumulation in both *S. leprosura* and *S. stenoptera* with P values of 0.038 and 0.014, re-

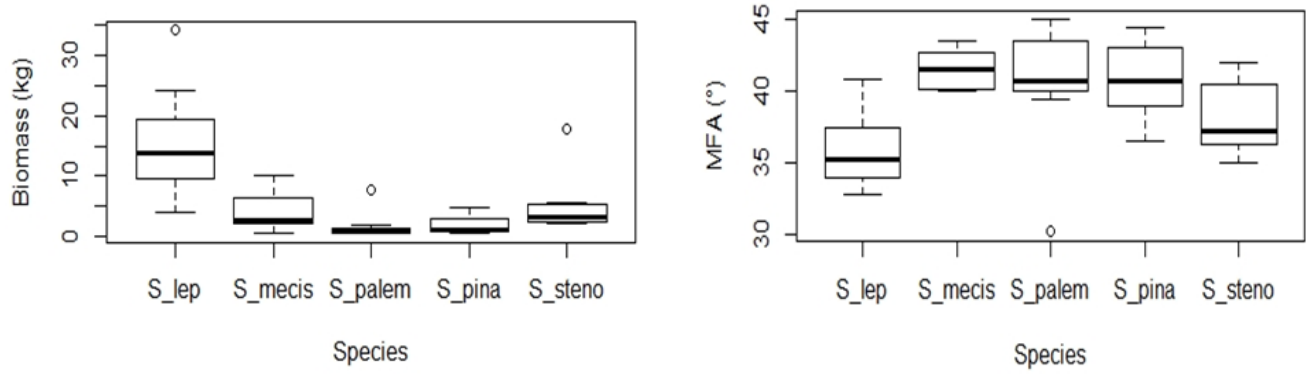

Figure 1. (A) Boxplots show the total number of biomass accumulation among five *Shorea* species with a significant difference as indicated by p-value of 0.00034 (< 0.05 of significance level); **(B)** Boxplot shows the average of MFA value from five species of Shorea with the significant difference as indicated by p-value of 0.017 (< 0.05 of significance level) in Gunung Walat. *Asterisk* (*) and *null* (○) information in both figures indicated the statistical significance among each measured species.

spectively.

Generally, there was a direct relationship between the diameter and the dependent variables (biomass, carbon, volume, and tree height) in the five *Shorea* species (Table 2). Biomass and carbon had a tendency to increase with increasing diameter (kg year^{-1}). Figure 2a and 2b indicate these relationships within the five *Shorea* species, with an R^2 of 0.85 and mean ± standard deviation of 5.54 for the relationship between biomass and diameter and an R^2 of 0.85 and mean ± standard deviation of 5.47 for the relationship between carbon and diameter. Other positive relationships were observed between volume and diameter as well as between height and diameter (Figure 2c and d). At 0.88, the greatest R^2 was observed for volume and diameter, while the least strong positive relationship was observed between height and diameter relationship, with an R^2 of 0.77.

S. leprosura produced the greatest biomass accumulation and carbon content of the five species, with average annual accumulations of 1747 kg and 0.803 respectively (Table 1). Moreover, *S. leprosura* also shows produced the greatest tree volume, with an average annual increase of 0.0101 g/cm^3. The lowest of biomass, carbon, and volume accumulations were observed in *S. palembanica* with average annual increases of 0.2047 kg, 0.0941 kg, and 0.0014 g/cm^3, respectively. With regard to tree height, *S. leprosura* had the greatest average annual growth of the *Shorea* species, with an average annual growth of 1.4746 m year^{-1}. On the contrary, *S. palembanica* had an annual primary growth of just 0.6063 m year^{-1}, which was the lowest of the *Shorea* species.

Table 1. Growth of Wood Characteristic of the Five *Shorea* Tree Species.

Species	Age (year)	Density (g/cm^3)	MFA (°)	Volume (m^3)	Biomass (kg)	Carbon (kg)	Annual biomass accumulation (Kg year^{-1})	Annual carbon accumulation (Kg year^{-1})
S. leprosura	9	0.575	35.94	0.0912	15.728	7.235	1.747	0.803
S. mecistopteryx	9	0.476	41.67	0.0297	4.266	1.962	0.474	0.218
S. pinanga	9	0.53	40.82	0.0123	1.959	0.901	0.217	0.100
S. stenoptera	9	0.491	38.24	0.0376	5.534	2.546	0.614	0.282
S. palembanica	9	0.478	40.41	0.0129	1.842	847	0.204	0.094

Table 2. Statistical Parameters for the Non-linear Regression Equations based on the Power Model for Five *Shorea* Species.

Species	Dependent variable	Independent variable	Parameter a coefficient	Parameter a SE	Parameter b coefficient	Parameter b SE	Model R^2	Model SE	Mean ± Standard Deviation	Model P Value
S. leprosura	Biomass	Diameter	-25.6185	10.2770	4.2563	1.0366	0.725	5.4290	9.9387	0.0093
	Carbon	Diameter	-11.78396	4.7275	1.9578	0.4768	0.725	2.4974	3.7676	0.0093
	MFA	Biomass	37.9519	2.1024	-0.1277	0.1141	0.040	2.8965	23.0575	0.3138
	MFA	Carbon	37.9519	2.1024	-0.2776	0.2480	0.040	2.8965	29.3582	0.3138
S. mecistopteryx	Biomass	Diameter	-5.7778	2.1948	1.2122	0.2546	0.783	1.5990	4.2699	0.0050
	Carbon	Diameter	-2.6577	1.0096	0.5576	0.1171	0.783	0.7355	6.4396	0.0050
	MFA	Biomass	42.3062	0.9363	-0.1822	0.1759	0.011	1.4800	37.4717	0.3477
	MFA	Carbon	42.3062	0.9363	-0.3962	0.3825	0.011	1.4800	39.6382	0.3477
S. palembanica	Biomass	Diameter	-4.9656	0.8709	1.1005	0.1330	0.918	0.7554	4.3956	0.0004
	Carbon	Diameter	-2.2841	0.4006	0.5062	0.0611	0.918	0.3474	5.4504	0.0004
	MFA	Biomass	40.8541	2.5404	-0.2395	0.8294	0.000	5.3699	38.9552	0.7842
	MFA	Carbon	40.8542	2.5404	-0.5208	1.8031	0.000	5.3699	39.8617	0.7842
S. pinata	Biomass	Diameter	-2.6323	0.4311	0.7616	0.0678	0.954	0.3593	4.1081	0.0000
	Carbon	Diameter	-1.2108	0.1983	0.3503	0.0312	0.954	0.1653	5.2912	0.0000
	MFA	Biomass	43.1069	1.3825	-1.1663	0.5527	0.365	2.2730	39.0547	0.0886
	MFA	Carbon	43.1069	1.3825	-2.5356	1.2017	0.365	2.273	40.0461	0.088
S. stenoptera	Biomass	Diameter	-21.6231	4.9624	3.0662	0.5518	0.832	2.2658	4.9986	0.0025
	Carbon	Diameter	-9.9466	2.2827	1.4104	0.2538	0.832	1.0422	6.4041	0.0025
	MFA	Biomass	39.6830	1.4395	-0.2602	0.1907	0.125	2.5887	33.4124	0.2307
	MFA	Carbon	39.6830	1.4394	-0.5656	0.4147	0.125	2.5887	35.9541	0.2307

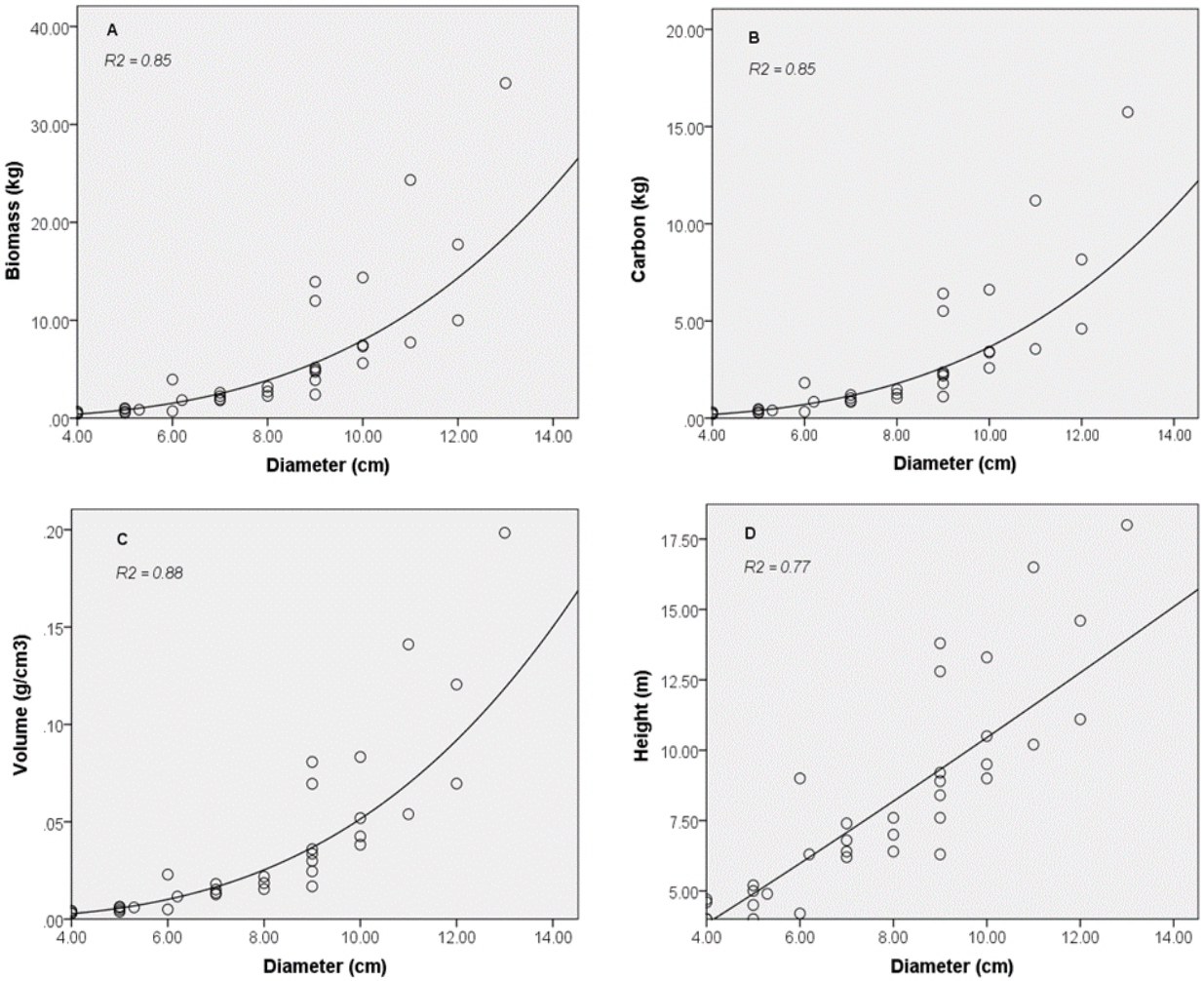

Figure 2. **(A)** The relationship between diameter (cm) as an independent variable and biomass (kg) as in the dependent variable; **(B)** The relationship between diameter (cm) as a dependent variable and carbon (kg) as an independent variable; **(C)** The relationship between diameter (cm) as a dependent variable and tree volume (g/cm^3) as an independent variable; and **(D)** is the relationship between diameter (cm) as a dependent variable and height (m) as an independent variable.

In general, MFA declined with increasing biomass, carbon, diameter, and wood density across the five *Shorea* species (Figure 3a and b). The relationships between MFA and diameter and between MFA and tree density (Figure 3c and d) were slightly positive, with R^2 value of 0.95 and 0.78, respectively.

DISCUSSION

Biomass accumulation varied among the five *Shorea* species (Figure 1a), and there are numerous factors that could have been an effective catalyst for such variations. It is likely that ecological variables, such as water and soil nutrient distribution, light availability, CO_2 availability, competition among trees, slope of the growing area, rate of photosynthesis, as well as intrinsic properties of the wood, significantly contributed to the huge disparity among species (Raich et al. 2014). A number of hypotheses (Brancalion et al. 2019; Phan et al. 2019) have suggested that increases in tree biomass accumulation make trees more robust to various environmental factors that affect growth, automatically reducing tree microfibril orientation angle.

The relationships between MFA and diameter as well as between MFA and density were not found to be significant in the present study. However, in reality, these variables are impactful during the tree grow-

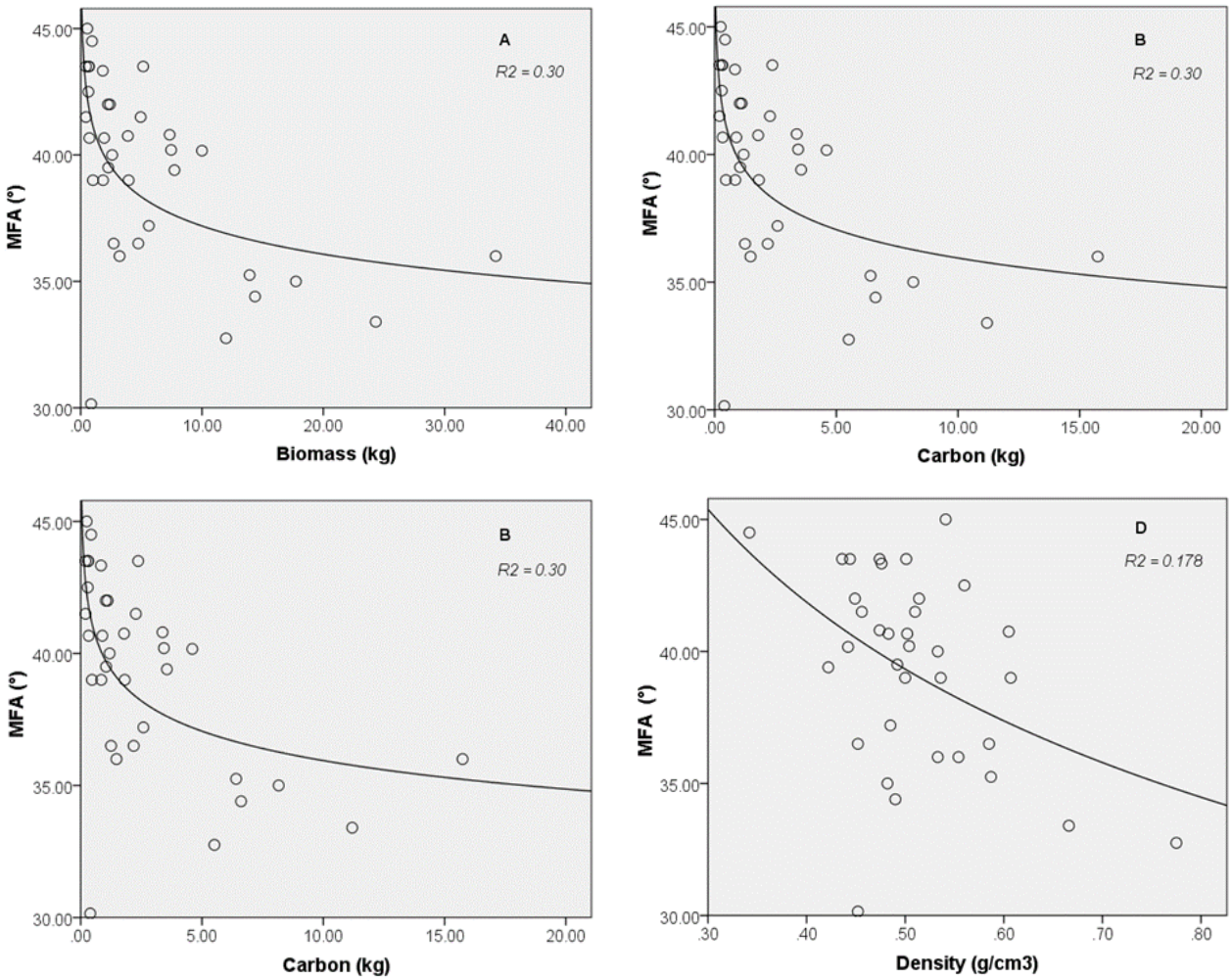

Figure 3. (A) The relationship between MFA (°) as an independent variable and biomass (kg) as in the dependent variable; **(B)** The relationship between MFA (°) as a dependent variable and carbon (kg) as an independent variable; **(C)** The relationship between MFA (°) as a dependent variable and tree diameter (cm) as an independent variable; and **(D)** is the relationship between MFA (°) as a dependent variable and wood density (g/cm^3) as an independent variable.

ing process. The increasing tree diameter (cm year^{-1}) is a key element in forest dynamics which also determines competitiveness among young trees of various species. Tree density is also an important indicator in measuring tree existence. It is also one of the upholding mechanical characteristics which keeps trees growing correctly. Higher wood density provides essential resistance and causes trees to be more robust toward other environmental disturbances.

Overall, the MFA degrees measured in this study are considered high when compared to other tropical tree genera. This is likely due to the fact that all measured trees were still young (< 10 years old) and were therefore very susceptible to a wide range of environmental factors such as wind, slope of the growing area, and anthropogenic activities (Minamino & Taneko 2014).

The soil in the study area consisted of red-yellow latosols, brown latosols, and some podzols. However, because the study area was not large (< 1 hectare), this factor is unlikely to be a key driving factor of biomass distribution. In fact, the study site was primarily composed of latosol soils (> 75%) but podzols were found in some areas, which contain huge concentrations of soil nutrients from tropical forest decomposition. Based on the obtained biomass accumulation results, it is likely that *S. leprosura* has higher total N, P, and K contents than other *Shorea* species.

Lira-Martins et al. (2019) noted positive correlation between tree trunk growth and the accumulation of N, P, K, and Ca in young tropical trees of tropical tree species in Amazonia and Australia. Jiang et al. (2018) showed that the amount of nitrogen in the trunk has a significant effect on total biomass and other biomass components in young tree. In addition, *S. leprosura* is more productive in terms of nutrient-use efficiency. Zhang et al. (2021) observed that some tropical trees could have different nutrient use efficiencies even among similar growing areas. Water availability likely had no significant influence on biomass accumulation because the location experienced similar precipitation levels throughout, and it can be assumed that each of the *Shorea* trees in the location received sufficient water to support their life. With the annual precipitation from 1700 mm to 4400 mm, water availability was high enough for trees using a variety of ecophysiological mechanisms.

Competition among young *Shorea* trees was also an important factor in this study. In general, *Shorea* species, classified as a shade tolerant, need enough shade during growing phase (Pamoengkas & Prasetia 2014; Hussein 2015). In this study, most of the young *Shorea* species were shaded by the bigger *Agathis* trees, which possibly lessened the amount of sunlight reaching the ground that could be used in photosynthesis. Lack of light at early life stages has a negative impact on the growth rates of young trees (Tripathi et al. 2020; Bianchi et al. 2021). In the present study, there was variation in the locations of trees growing simultaneously. In fact, the study site had slopes (between 10° to 45°) that may have affected the ability of young trees to absorb light during the middle of the day (Lekitoo et al. 2017). Various mechanisms are employed by trees to obtain enough sunlight which effectively contributes to their productivity (Darko et al. 2014; Li et al. 2020).

However, the variation in biomass accumulation may come from leaf differentiation and leaf numbers in each young tree in the context of impacting upon ecophysiological effectiveness. It was observed that young *S. leprosura* tree generally had good overall health compared to other species. Visual observation revealed that the species looked greener with many regular leaves, longer branches, and a healthy trunk. In contrast, around a third of *S. palembanica* leaves were unhealthy due to disease and insect pests. Lelana et al. (2022) specified several leaf predators such as *Mucanum* spp., *Pteroma plagiophelps*, *Valanga nigricornis*, and *Pestalotia* spp., which have been found to effectively cause reduced leaf quality in *Shorea* species in Indonesia. Leaf size, shape, and canopy arrangement can influence photosynthesis rate and total biomass production in many tree species (Falster et al. 2018; West 2020). Given that these factors are important for young trees, and based on visual observation of each young tree, it can be assumed that *S. leprosura* retains higher biomass through a higher rate of photosynthesis compared with other species.

Measuring MFA can provide important insight into various tree mechanisms and how they contribute toward tree productivity (Xu et al. 2012; Auty et al. 2013). Here, MFA varied among *Shorea* tree species, and there was a negative relationship between biomass content and MFA (Figure 3). These results indicate that there is a tendency for MFA to decline when both biomass accumulation and carbon content increase. Negative relationships were also present between MFA, wood density, and tree diameter (table 2). Microfibril angle has been reported to be high when trees are in the growing phases (seedling and sapling periods), and it continues to decline gradually when trees grow in both vertical and lateral directions. Since most of the trees in this study were considered juvenile, they were not really composed of solid wood structures and

mainly comprised young cell walls, which in turn caused the negative relationships with the mechanical functions of wood properties (Xi 2018; Rocha et al. 2019; Eder et al. 2020).

The present study found that the MFA was high among the 35 young trees, with the average ranging from 35.94° to 41.67°. This wide range of MFAs is likely due to vulnerability to various environmental disturbances. It is likely that external factors, such as regular gales and the slope of the growing area (between 10° to 45°), have contributed to high MFA variation in these young trees. With a regular windy period during the rainy and dry seasons, wind is a key factor in determining MFA orientation in most young trees in Indonesia (Ishigura et al. 2012; Alteyrac 2015). In addition, the slope in the study area has forced the young trees to retain their growing orientation over a period of time since they were planted. Hein et al. (2015) indicated that the effects of slope and wind are positively associated with MFA orientation in young trees. Variation in MFA was also found among *Shorea* species (table 1), which indicates differences in wood stiffness and durability against various environmental disturbances such as wind, rain, and flood. In many cases, MFA is negatively correlated with wood stiffness, meaning that when MFA decreases, wood stiffness increases as a consequence of biomass accumulation and wood productivity over a growing period. Although several studies have failed to find a positive correlation between wood density and MFA, a positive tendency between these was noted in the present study (Figure 3d). Overall, MFA declined when wood density increased. In conclusion, *S. leprosura* seems to have a stiffer trunk than other species. Tree wood tends to be denser when the rate of photosynthesis is higher which leads to biomass accumulation throughout the year. In contrast, *S. palembanica* showed the lowest rate of biomass accumulation and the lowest wood density and tended to be unhealthy. This may be because the species has not fully adapted to the existing growing area, as it normally grows along riverbanks, in swampy forests, and in low-lands with Inundated areas (Erizilina et al. 2019).

CONCLUSIONS

The relationship among carbon, biomass, and MFA for the five young-tree species of *Shorea* were tested through quantitative data analysis. A total of 35 tree species were considered with good physical appearances and distributing along the observed plot. Non-destructive biomass measurement was carried out using diameter tape and a laser hypsometer to determine allometric equation. Carbon accumulation was estimated through laboratory analysis of wood core sample with the ASTM standard. MFA was measured using core samples that were sliced every centimeter then combined with HNO_3 and KCl to separate fibers. Fiber angle orientation was observed under a microscope with 100 time magnification lens Motic Image Software. In order to identify the best fit correlation between carbon, biomass and MFA, non-linear Power models were performed. *S. leprosura* turned out to be the highest biomass and carbon accumulation, volume and annual increase, while *S. palembanica* was the lowest among the five young *Shorea* species. It is obvious that MFA was declining with the increase in biomass, carbon, diameter, and wood density among the five *Shorea* species. The relationship between biomass and diameter was strong with an R^2 value of 0.85, while the strength of the relationship between MFA and diameter was weaker with R^2 value of 0.195. MFA degree decreased with increased biomass accumulation in in *Shorea* species.

AUTHOR CONTRIBUTION

R.L.C., D.W., D.A.J. and S.C. have equal contributions to this work as the main contributor. R.L.C., and D.W. designed the project and collected the data. R.L.C. & S.C. performed the analyses. R.L.C., D.W., D.A.J., and S.C. wrote, revised, and approved the manuscript

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Research Article

Orang-utans' (*Pongo pygmaeus wurmbii*) Activity Pattern in Camp Release and Feeding Site of Lamandau Wildlife Sanctuary, Central Borneo, Indonesia

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ABSTRACT
The Bornean orang-utan (*Pongo pygmaeus wurmbii*) release process at Lamandau Wildlife Reserve applies the soft-release method. In this method, there is still provisioning additional food on the feeding site by human. The existence of camp release and feeding sites in the vicinity of release forests is assumed to have an impact on orang-utans' activity patterns. This assumption is related to the principle of releasing them into their natural forest habitat, namely by reducing direct interaction between humans and orang-utans. The aims of this research were: (i) to measure the intensities of orang-utan presence on camp and feeding site, (ii) to analyze the correlation between phenology and orang-utan's presence on camp and feeding site, and (iii) to assess orang-utan's activity budgets, diet composition, and vertical used on camp, feeding site, and forest. Activity budgets of five group orang-utans with different categories based on age and sex were compared using the focal animal sampling method and instantaneous records. The analysis showed no correlation between the intensities of orang-utan presence and phenology. However, there were significant differences in activity patterns between adult and adolescent orang-utans. The findings revealed that adult orang-utans activity pattern tended to be high in resting at all observation locations while adolescents spending more their activity for feeding. Orang-utans at 0-10 m of height classes tend to do more activity. Almost all orang-utans feed on a non-forest diet (45-67%) in the camp release, feeding site, and surrounding. We assumed that the existence of a camp release and feeding site near the release point are affecting factors in a successful reintroduction of ex-rehabilitate orang-utans.

INTRODUCTION
Orang-utans are the only member of great apes lived in Asia. Three species of orang-utans in the world are Bornean orang-utan (*Pongo pygmaeus*), Sumatran orang-utan (*Pongo abelii*), and Tapanuli orang-utan (*Pongo tapanuliensis*) (Roos et al. 2014; Nater et al. 2017). Bornean orang-utan is divided into three subspecies based on their distribution, including *Pongo pygmaeus wurmbii*, which spreads from the southwest of the Kapuas River in West Kalimantan to South Kalimantan (Hulu Utara River and Taba-

long) (Warren et al. 2001; Utami-Atmoko et al. 2017). Orang-utans are a critically endangered species by IUCN. The main threats to orang-utans' decreasing population include habitat loss in the wild, habitat fragmentation, habitat conversion, and poaching. Approximately 78% of the wild orang-utan population in Kalimantan (*Pongo pygmaeus*) is found outside of conservation area that is fragmented and degraded. This finding indicates that the Bornean orang-utan faces a high risk of extinction in the wild (Wich et al. 2012). Deforestation and the illegal pet trade have resulted many orang-utans being orphaned. Each immatures individual orang-utan in illegal custody represents at least one individual mother killed by poachers (Rijksen & Meijaard 1999; Basalamah 2016).

Orang-utans that have been confiscated and rescued from illegal custody must be immediately rehabilitated and released back into their natural habitat (reintroduction). The reintroduction process involves a rehabilitation process to help animals learn the ecological and behavioral necessary aspects for survival in their natural habitat (Beck et al. 2007). Ecological skills consist of ranging skills, nest building skills, food selection, foraging techniques, roaming skills, and social skills (Beck et al. 2007; van Noordjwik et al. 2009; Russon et al. 2015). Each rehabilitation center has different steps; some have orang-utan school programs or merely quarantine (Basalamah 2016). Orang-utan school is one of the conservation projects aimed to restore the wild instincts and ecological skills of ex-captive orang-utans.

Ex-captive animals that are released into natural habitats may take longer to acquire these skills (Russon 2006). The adaptability of orang-utans, such as the ability to recognize and consume forest food types and to make sleeping nests are also some of the ecological factors in determining the success of reintroduction as a conservation effort (Beck et al. 2007; van Noordjwik et al. 2009; Russon et al. 2015). The importance of adaptability is related to the important principle of reintroduction, which is done by reducing direct interactions between orang-utans and humans as well as increasing interactions between orang-utans.

The rehabilitation process managed by Orang-utan Foundation International (OFI) does not involve the orang-utan school program. The release process at Lamandau WS applies the soft-released method. This method requires post-release support for released individuals such as additional food. In contrast to soft-released, hard-released does not provide additional food support. The additional food in Lamandau WS is provided routinely once a day.

Orang-utans can live independently in the forest if they have passed at least two fruit seasons and two low/scarcity seasons without human support (Zweifel 2009). This assumption could become invalid based on this case from the history of Sumatran orang-utans at the reintroduction Center in Jambi (PROS) (Soft-released). Despite having been released and underwent an adaption period of more than two years, Siregar et al. (2018) discovered that some orang-utans released in PROS still need humans for feeding. Prior research at Lamandau WS by Nawangsari et al. (2016) found that some individuals have a high level of intensity towards additional food at the feeding site. Furthermore, the results of Basalamah et al. (2018) study in the Kehje Sewen forest also showed that there was one ex-captive individual who had a high human orientation. In other words, individual activity in approaching and initiating interaction with humans is still high even though it has long been released.

Providing additional food from technicians is an alternative to support physical fitness for orang-utans in the forest, yet this can also make ex-captive orang-utans dependent on technicians and cannot live inde-

pendently in the forest (Siregar et al. 2018). Human activities in the release camp of forest, as well as the availability of additional food at the feeding site, are thought to be inhibiting the effective reintroduction of orang-utans, based on the findings of prior studies (Nawangsari et al. 2016; Basalamah et al. 2018; Siregar et al. 2018). As a result, it is important to study the behavior patterns of orang-utans that are influenced by the existence of the release camps and feeding sites in release sites. Lamandau WS has four camp release locations and four active feeding sites, making it appropriate to serve as research sites.

Basically, animals have an instinct to forage (Kaiser et al. 2015). Similarly, orang-utans will forage in places where food sources are abundant, following the season of fruit availability and the distribution pattern of fruit in the forest (Saputra et al. 2017). As a result, the presence of a feeding site area is assumed to hinder orang-utans' capacity to forage organically, limiting their daily range. The research's results can be used as basic information to perform strategies for further orang-utan conservation efforts by taking into account aspects of orang-utan behavior.

Summarizing the previous explanation, then the objectives of this study are to (i) measure the intensity of orang-utan presence at camps and feeding sites, (ii) analyze the phenological correlation with the presence of orang-utans at camps and feeding sites, and (iii) assess activity budget of orang-utans, diet composition, and vertical used in camps, feeding sites, and forests.

MATERIALS AND METHODS

Study site

This research was conducted on July to November 2019 in the Lamandau Wildlife Sanctuary. Lamandau WS is former area of permanent production forest (HPH) located in Central Borneo, Indonesia and roughly ± 76.110 Ha in size. Survey in 2016 by Nawangsari found that there is a high abundance of orang-utan food species (at least 101 tree species). Each vegetation has a different level of vegetation growth, i.e; seedlings, saplings, poles, and trees. The seedling vegetation type dominates, the vegetation type is the lowest. The ecosystem area of the Lamandau WS is a lowland forest and swamp forest ecosystem affected by tides and periodically submerged. Both of these habitats are orang-utan release habitats.

Lamandau WS has several post-release monitoring camps, i.e; Gemini camp, Siswoyo camp, JL camp, Rasak camp, and Buluh camp (Figure 1). Each camp completed with the feeding sites. The observation sites covered all of those camps except Camp Siswoyo. Orang-utan release camps and feeding sites were built to help orang-utans survive after they were released, as well as to monitor reintroduced orang-utans.

Subjects

The research subjects observed were ex-captive orang-utans released in the Lamandau WS. Orang-utan Foundation UK (OF-UK) has released 56 orang-utans who have been monitored since 1999 to date. Observations of activity patterns at three locations (release locations, feeding sites, and forests) were conducted on 23 orang-utans from 56 individuals who are still being monitored. The orang-utans observed were differentiated based on age classes (adolescents and adults) and sex. Female adults group was divided into two groups based on the presence of offspring. The mother, in this case, should be an adult female with an offspring (Table 1). Most of the orang-utan population in Lamandau WS is

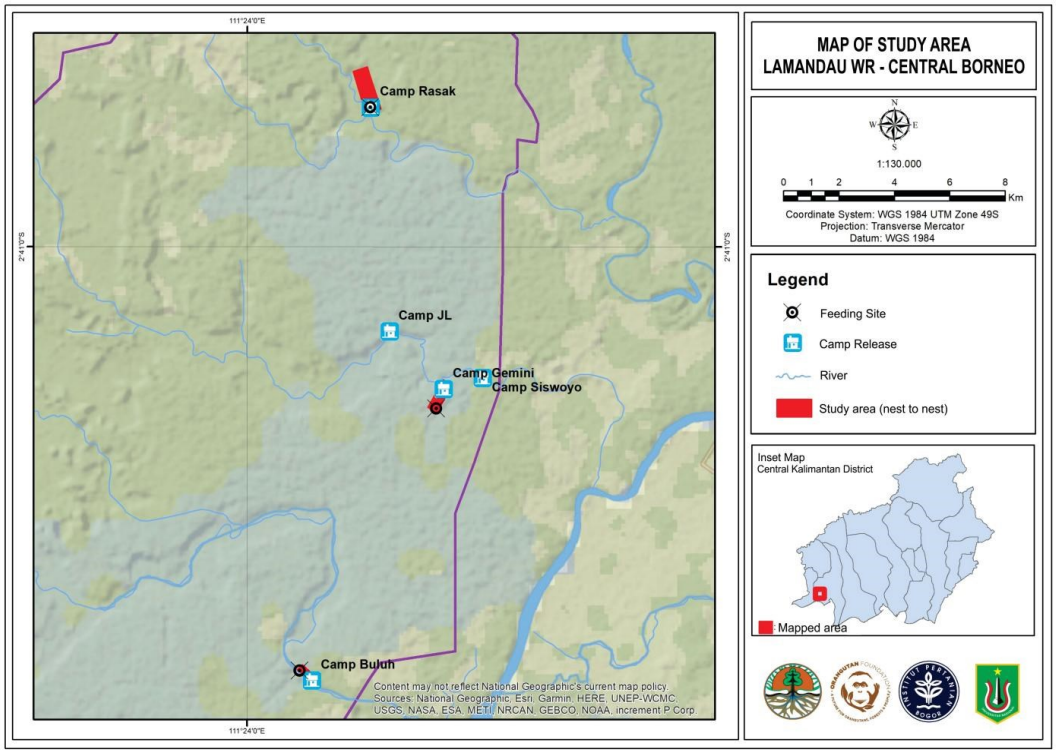


Figure 1. Map of Lamandau Wildlife Sanctuary, Central Borneo, Indonesia.

ex-rehabilitate from OCCQ (Orang-utan Care Center and Quarantine) before released in Lamandau WS (Table 2). OCCQ is a Bornean orang-utan rehabilitation center managed by Orang-utan Foundation International (OFI) located in Pangkalan Bun, Central Kalimantan. Almost all adult female individuals observed in this study were OCCQ ex-rehabilitate orang-utans, while some individuals in the adolescent group were newly reproduced individuals from ex-rehabilitate individuals.

Table 1. Categories, total observed individuals, total observation days and age of subjects.

Categories	(N) Individuals	∑Day	Age (yo)	∑Hours
Adult Male	2	12	20-30	84
Adult Female	2	9	20-30	40
Adolescent Male	2	17	6-15	100
Adolescent Female	6	54	8-12	237
Mother	11	68	15-29	397

Data collection

Intensities of orang-utan presence

Observation data includes the frequency of orang-utan presence at the camp and feeding site. The data is a combination of secondary data (data on the presence of orang-utan per month in the camp obtained from the camp staffs' note) and primary data (data during the study period). Every month during the study, observations were conducted in collaboration with camp staff.

Orang-utan's daily activities

The study included the difference of daily activity patterns based on the observation sites (camp, feeding site, and forest). At the forest site, individuals were followed for three consecutive days whenever encountered. Upon finding an animal, they were followed all day until they built their evening nest, and no activities recorded. The next day, start the follow

Table 2. Focal (individuals), history, released/birth year, and locations of camp release.

Focal	Categories	Age (yo)	History	Released/Birth (yr)	Camps
Yoko	Adult Male	23	Rehabilitation	2004	Buluh
Carlos	Adult Male	?	?	?	Gemini
Ekon	Adolescent Male	6	Ebony's offspring	2014	Gemini
Ewet	Adolescent Male	15	Rehabilitation	2006	Rasak
Kotim	Adolescent Female	10	Rehabilitation*	2014	Rasak
Suwita	Adolescent Female	11	Sawit's offspring	2009	Rasak
La betti	Adolescent Female	12	Ladidi's offspring	2007	Rasak
Sakura	Adolescent Female	11	Sela's offspring	2008	Gemini
Pauline	Adolescent Female	12	Paula's offspring	2007	Gemini
Sugi	Adolescent Female	8	Rehabilitation*	2016	Buluh
Queen	Adult Female	23	Rehabilitation	2003	Buluh
Ilik	Adult Female	20	Translcationi	2007	Gemini
Vania	Mother	26	Translocation	2015	Buluh
Morres	Mother	20	Rehabilitation	2003	JL
Dedek	Mother	21	Rehabilitation	2002	JL
Acuy	Mother	22	Rehabilitation	2006	Rasak
Amina	Mother	12	Acuy's offspring	2007	Rasak
Sela	Mother	20	Rehabilitation	2004	Gemini
Passion	Mother	20	Rehabilitation	2005	Gemini
Camelia	Mother	19	Rehabiitation	2006	Gemini
Max	Mother	16	Mantra's offspring	2003	Gemini
Hola honolulu	Mother	15	Huber's offspring	2004	Gemini
Maya	Mother	22	Rehabilitation	2003	Gemini

*Rehabilitation at Lamandau WS.

after they wake up and leave their (morning) nest, whereas observation at camp and feeding site began recorded when orang-utans are seen/come to the camp and feeding site. Orang-utans are considered to be out of sight if the observed orang-utan moves more than the radius specified at camp and feeding site, which is 20 m from each side of camp and feeding site.

Behavioral data was recorded using standard ectograms provided by OF-UK, except for other activity categories. Behavioral observations were made using the focal animal sampling method (Altmann 1974) during orang-utan observations at release sites, foraging areas, and forest areas. The ad libitum method was also used to observe rare activities that occur outside the observation time which were then recorded using the instantaneous recording method with an interval of 2 minutes.

Orang-utan's diet composition

We recorded the type of food consumed: flowers, fruit, leaves, bark, insects, vegetation, water, and others such as; soil, additional food from staff, and human trash food leftovers around the camp. Fruit phenology observations at each camp were conducted monthly with staff during the study. The level of food availability category for fruiting trees in the Lamandau WS refers to Harrison (2009), with the following information: 1) Low < 4%, 2) Moderate 4% -5.9%, 3) Moderate-high 6-7.9%, and 4) High ≥ 8%).

Non-forest food including soil, human food leftovers that are thrown away around the camp/garbage, and other food that is in the river around the camp. Sometimes orang-utans also eat additional food waste around the acclimatization cages around the camp, while non-forest categories at the feeding site are fruit (additional food) provided by the staff at the feeding site.

Vertical used and daily ranges

Vertical use refers to orang-utans carrying out their activities at various height categories, which are divided into four levels: 1-10 m (lower level), 10-20 m (medium level), 20-30 m (upper level), and > 30 m (Top). Individuals were tracked for three days in a row for mapping purposes. Because of the time and energy constraints, only 10 orang-utans out of a total of 23 were followed from nest to nest in this study employing focal animal sampling. The orang-utans in this study were sampled from five different groups of orang-utans. Table 3 show the individuals who were followed. Every 30 minutes, the geographic position of the focal animal was recorded. Data on orang-utan intensity at camp and feeding site, as well as phenology, were collected in collaboration with OF-UK staffs. These staffs were taught and rehearsed the data collection process to standardize data gathering and maximize data comparability.

Data analysis

The data analysis of collected orang-utan activity patterns was done quantitatively and presented in relevant tables and graphs. Non-parametric test of Kruskal-Wallis was used to test differences in frequency of activity patterns in each orang-utan group. The Pearson-chi-square test was used to examine the correlation between phenology and intensity of orang-utan presence at camps and feeding sites. Significance was set at the p-value level ≤ 0.05. Data analysis was performed using SPSS 20.0 and R software. Orang-utan's daily range maps were created based on the waypoints from Garmin 64s GPS while following orang-utans and analyzed using ARcGIS 10.2 software.

RESULTS AND DISCUSSION

Result

Orang-utan's intensity presence at camp and feeding site

The observed orang-utan divided into mother, adult male, adult females (without offspring), adolescent females, and adolescent males. Intensity of orang-utans' presence at camp and feeding site is dominated by mother with the highest intensity level in July (Figure 2). Some individuals didn't appear at the feeding site but appeared in the camp, and vice versa. Individuals who come to the feeding site almost every day are Hola and Maya, while individuals who frequently come to the camp are Acuy, Sela, and Max. Those individuals were found visiting the camp almost every day. Severe orang-utans are individuals who often come to the camp, but rarely visit and do activities at the feeding site and vice versa. Camelia and Sugi are orang-utans who rarely travel to the feeding site but come to camp almost every day. Maya and Hola rarely travel and do activities around the camp, but often come to the feeding site.

The average amount of fruitful tree availability in almost all camps was low (< 4%), except for Buluh camp which was high (> 8%). However, the results of the phenological correlation test and the intensity of the presence of orang-utans at the camp and feeding sites showed no correlation (P-value: Camp = 0.32 > 0.05; feeding site = 0.44 > 0.05) between the two. Therefore, camp release and feeding sites is one of the factors that can affect the frequency of orang-utan's presence intensity.

Orang-utans' activity pattern

The daily activities of reintroduction of the orang-utan in Lamandau WS are presented in Figure 3. The daily activities of orang-utans are determined by their capacity after years of release, as well as their age-sex classes. In general, the activity patterns of all observed ex-rehabilitate

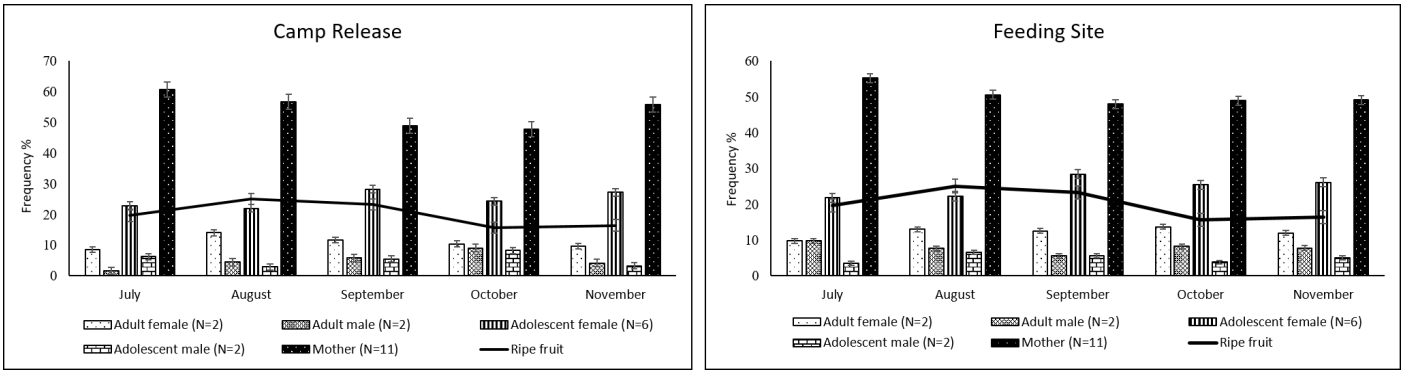


Figure 2. Phenology per months and intensities of orang-utan's presence at A) camp, and B) feeding site.

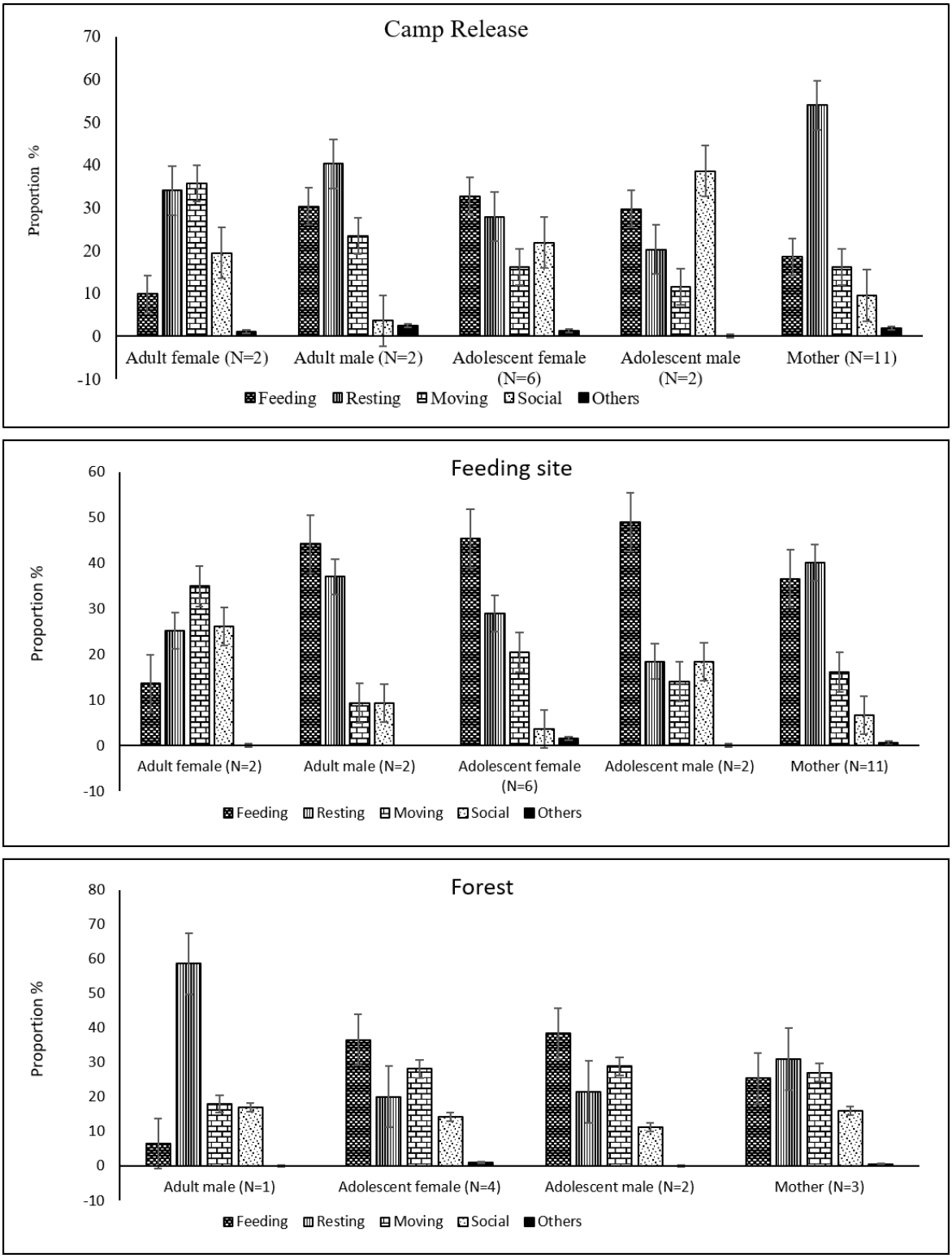


Figure 3. Orang-utans' activity pattern based on different sites.

orang-utan showed a similar pattern but differed from those found among wild counterparts. Resting time accounted for the largest part of the released orang-utans' activity budgets, especially the adult group. Feeding and travelling time was correspondingly reduced.

All individual orang-utans in the adult group, especially those in adult male and mother had the highest allocation of resting activities, while the highest of adolescent female and adolescent male activities was feeding. Feeding and resting time accounted for the largest part of the released orang-utans' activity budget while at camp and feeding site (P-value < 0,05), whereas in forest there is no significant difference between activities. Travelling and social time were correspondingly reduced. Adolescent female spent most time of feeding (32.6%), and Adolescent male spent most time of social (38.5%) while around camp. Adolescent male and adolescent female feeding proportion at feeding site and in forest was similar. Mother spent most time by resting consistently at three observation sites (31-53%).

In addition, almost all observed individuals have an other activities time (1-3%) such as; trying to enter the camp building, trying to damage the camp facilities, trying to take objects/diet related to humans, picking up fruit in the acclimatization cages for orang-utans juvenile around camp, and interacting with the camp staffs. These activities tend to occur more around camp. Based on observation, it results that the other activities were done by almost all individuals. Yoko had the most other activities time reached 3.3%, but this can occur due to the limitations of adult male individuals as observed subjects for long period. Yoko was the only individual that representative the adult male sample, if Yoko not included in the assessment, then the individual who has the highest time in other activity is Acuy (2.4%) from mother group.

Other activity categories are created based on observation results of activities outside main activity that lead to an interest in humans. Other activities are classified into four categories 1) Break in, when orang-utans try/are entering camp buildings such as stockrooms, staffrooms, and kitchens; 2) Steal, when orang-utans try/are taking object/food related to humans such as fruit stock/food from the staff in the stockrooms or kitchen, or also fruit provided for baby orang-utans in acclimatization cages around the camp; 3) Interact, when orang-utans initiate interactions/respond to staff/humans around the observation site, and; 4) Botch, when orang-utans are trying to/are destroying camp facilities.

Sela, Sakura, Hola, and Max are the same individual orang-utans observed by Nawangsari et al. (2016). Hola and Max were adolescents (no offspring) when the study was conducted. Max and Sakura are individuals who actively explore and forage sources in the forest to fulfill their daily necessities. Sakura's average daily movement distance is the farthest among other adolescent individuals. The results of the current study also showed that the proportion of Sakura spent exploring was higher (37.9%) than all the individual orang-utans observed. The feeding proportion of Max was found to be decreased from the previous study, namely from 45.7 to 23% while the feeding proportion of Sakura was increased from 25.8 to 28.6%. Max is one of Mother individuals who often come to the vicinity of the camp and spends most of their time resting (52.3%) at the camp while the resting proportion in the forest is lower (29.4%).

The intensity of Sela's presence at the feeding site was less than the intensity of her presence around the camp during the study. Most of Sela's daily activities were spent by resting (55.9%) and playing (14.5%) around the camp. Sela showed her interest several times in activities/ objects used by humans such as washing her face using water in the

camp staff's bathroom and trying to put on staff's socks. These activities are included in the category of other activities, with a total proportion of Sela's being 2.1% of all daily activities.

Orang-utan's diet composition

The proportions of the food types eaten by orang-utans are shown in Figure 4. The Diet composition of orang-utans in the camp is almost the same as when they are around the feeding sites. Non-forest food have the highest proportion (Camp; 39-75% and feeding site; 50-98%). The category of non-forest food in camp site such as soil, human food scraps that are disposed of around the camp/garbage, and other food in the rivers near camp.

Furthermore, orang-utans also eat additional leftover food around the acclimatized cages located surrounding the camp, whereas non-forest food at the feeding site is fruit (additional food) provided by staff at the feeding sites. The total percentage value in the adult male group reached 97% due to the limited number of samples, only one adult male (Yoko) was observed eating non-forest food.

Adolescents tend to be more active in exploring the food forest around the camp compared to adult orang-utan groups. Fruit, leaves, bark, vegetation, and insects are among the food forest kinds consumed by orang-utans when they visit the camp, however fruit consumption is quite low (3-5%). The location of the camp near the river allows orang-utans to explore the river and its surroundings during low tide. From September to November, the condition of the river water around the research camp receded. In this condition, it was observed that orang-utans mostly eat tubers from alternating vegetation such as selingsing (*Hypolytrumnemorum*) and rasau (*Pandanus helicopus Kurz ex Miq*) around the river (7-12%).

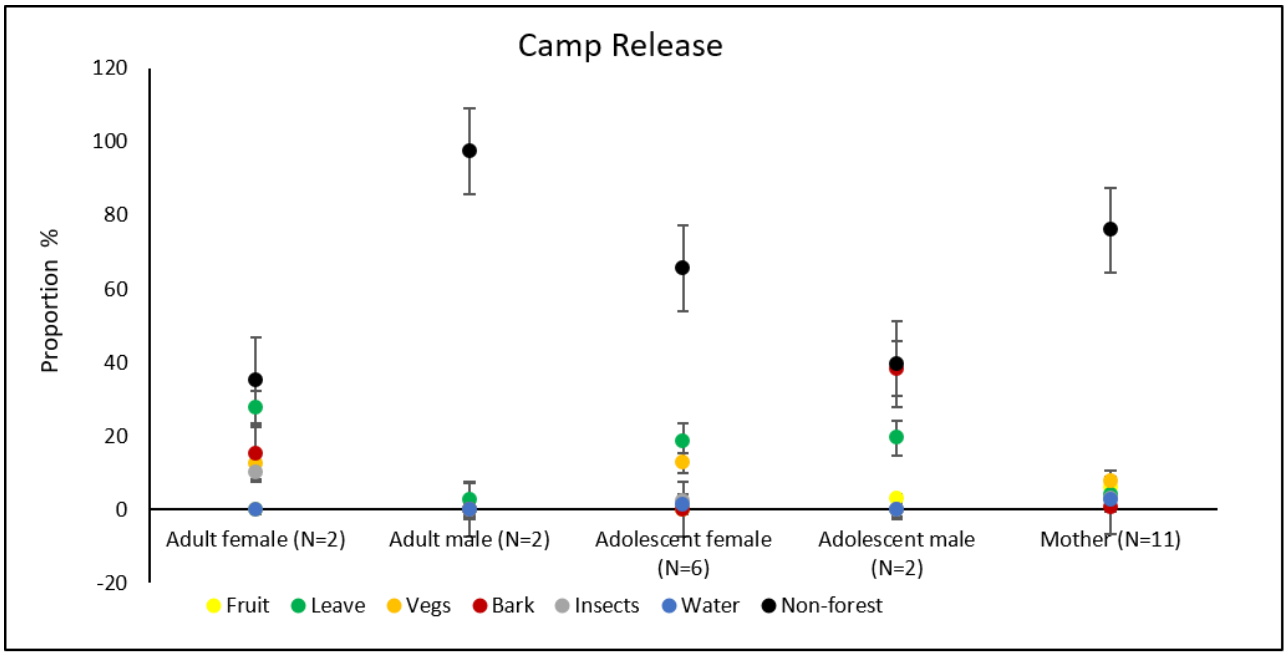
Various forests food that orang-utans eat when at the feeding site and surroundings are fruit, leaves, bark, and insects. Forest-food mostly eaten by orang-utans are leaves (14-30%) and fruit (9-17%). Adolescents mostly eat leaves and fruit, while adult orang-utans, especially Mother, eat more insects (14.6%). The activity of eating insects is done after eating additional food ends, with a long enough duration.

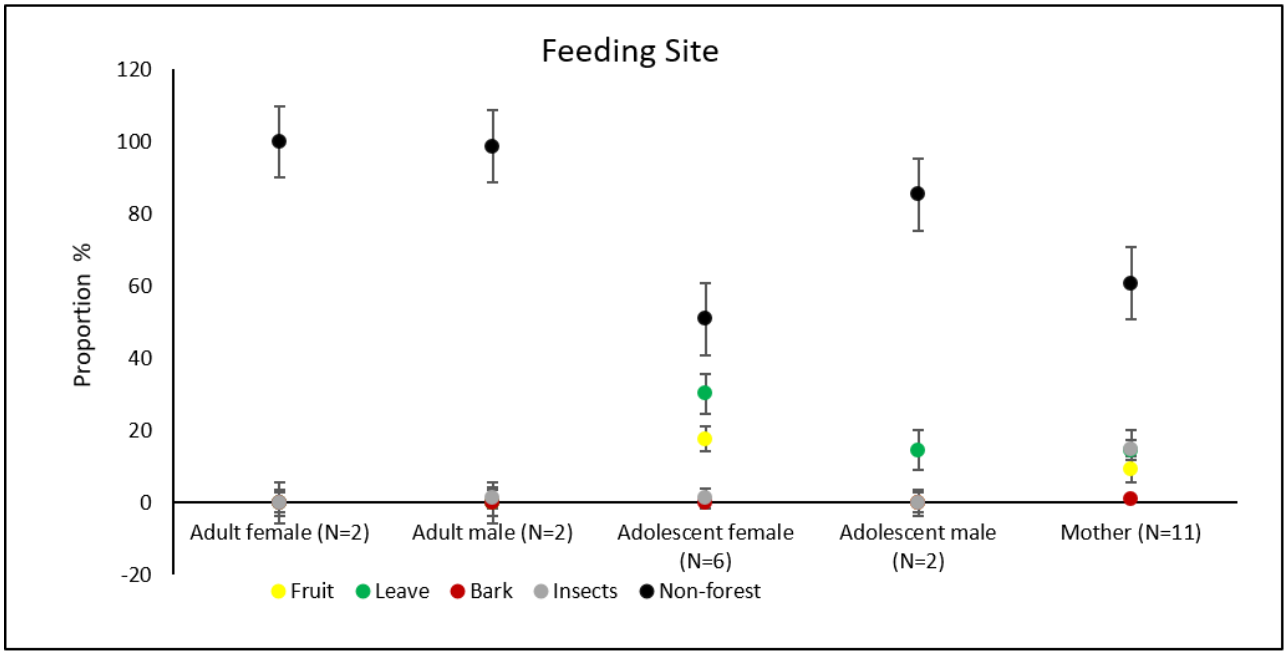
Mother orang-utans consistently prefer non-forest food over forest food types with the highest percentage value of 25.1% while in the forest. Female and male adolescent groups chose to eat more fruit than other types of food, with the percentage of 41.8% for female adolescents and 61.4% for male adolescents. The types of fruit that orang-utans eat are puak (*Artocarpus anisophyllus*), pemponing (*Quercus bennettii*), jejambu (*Eugenia cuprea*), and bekunyit (*Diospyro spolita*).

Vertical used

Height usage by orang-utans is shown in Figure 5. Orang-utans are more active on the ground and at low altitudes at the camp and feeding site, although adult orang-utans spend more time on the ground. All orang-utan groups use the maximum height in the middle category at the camp and the feeding site, while in the forest the maximum height is at upper level. The adolescent female and adolescent male groups mostly utilized lower and middle levels, whereas adult orang-utans were at the lower height only.

Mothers do most of their activities on the ground while at the camp (84.7%) and the feeding site (53.8%). When in the forest, orang-utans tend to take advantage of the low-medium altitude category. Because the restricted number of samples was only represented by one person, Yoko,





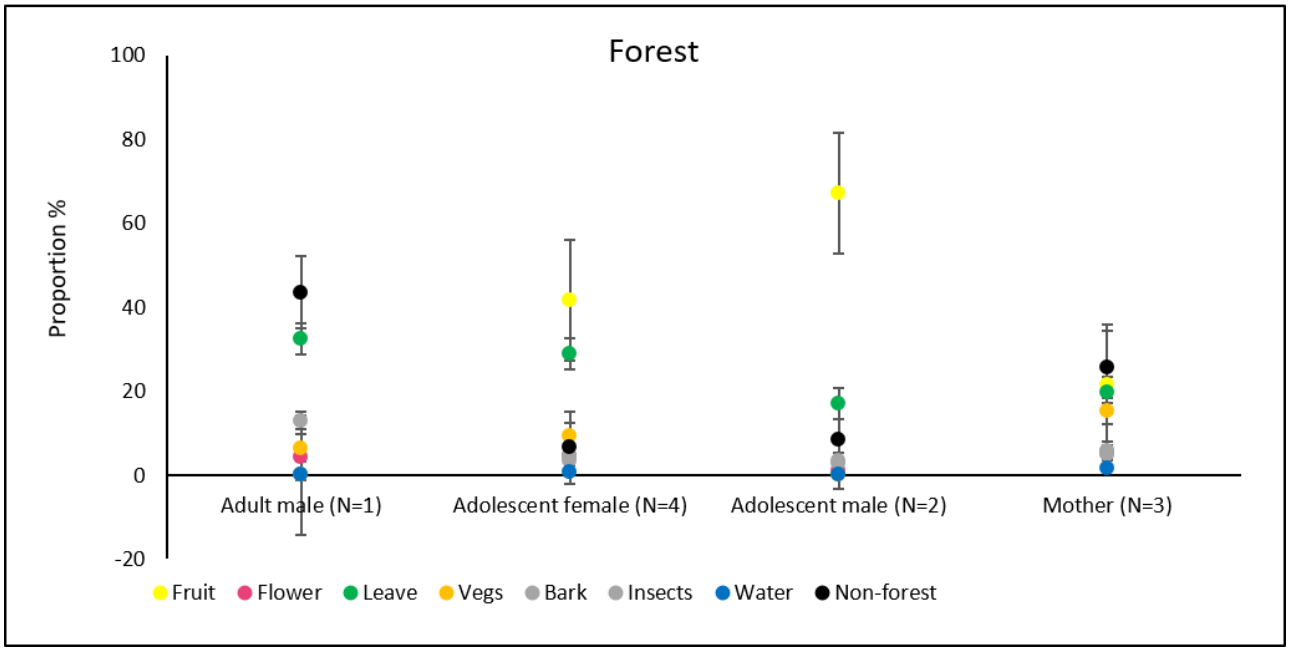
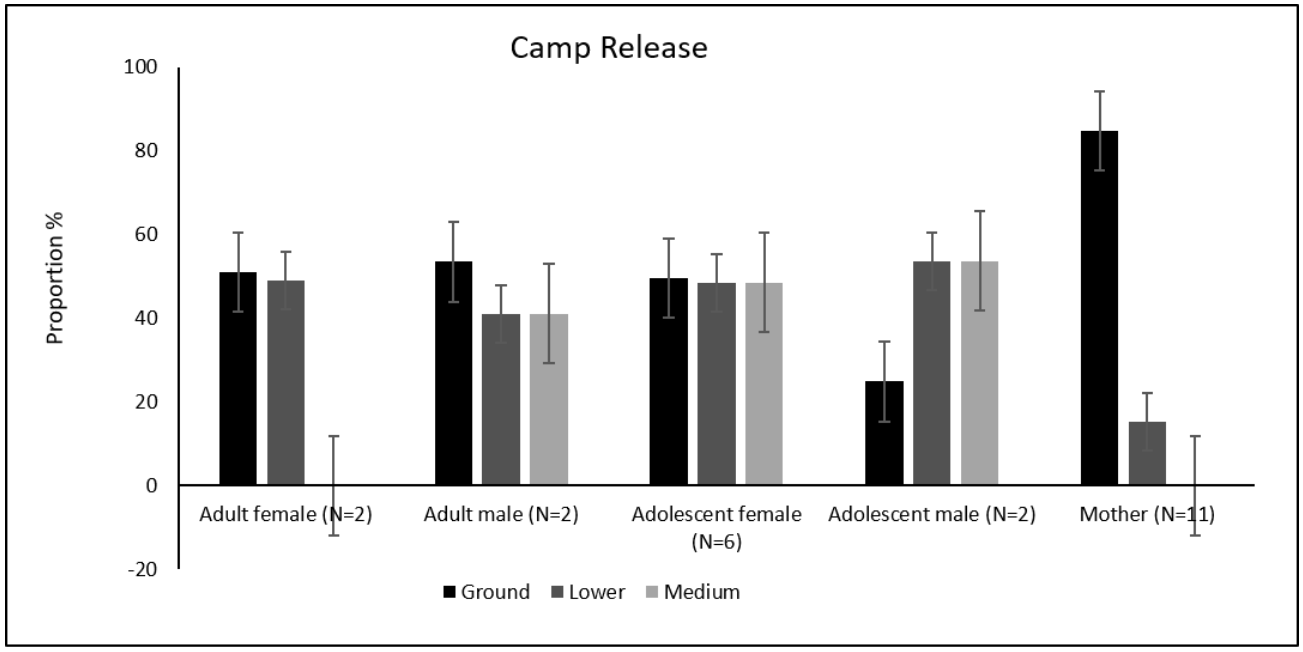
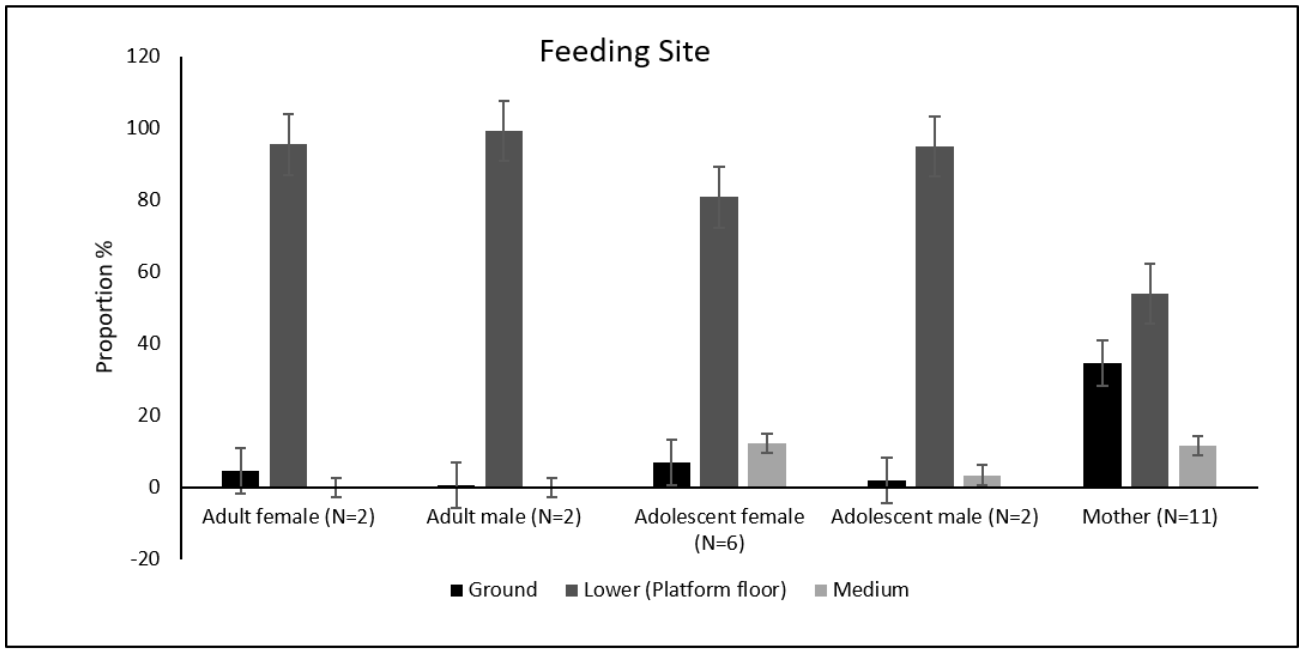


Figure 4. Orang-utans' diet composition at different sites.





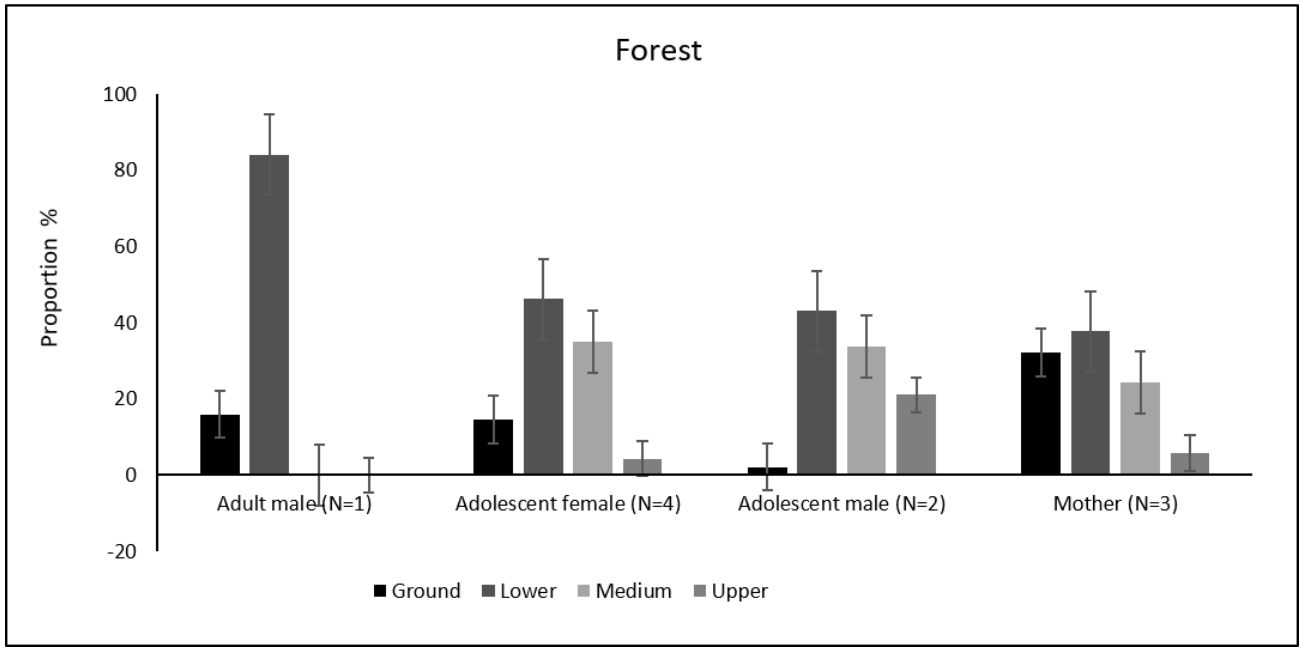


Figure 5. Orang-utan's vertical used at camp, feeding site, and forest.

the number of percentages in the adult male group that reached (> 80%) was negligible. Individuals that use the upper level are Suwita, Acuy, Kotim, Amina, Ekon, and Ewet while in the forest.

Daily range

Some orang-utans still spend their time for activities around the camp and feeding sites during nest to nest observation periods (Table 3). These individuals remain stuck at a lower diet breadth from the staff to fulfill their nutritional intake and tend to spend most time just around the camp and feeding sites. Orang-utan even comes around the camp before the staff departs for the feeding site to provide additional food.

Table 3. Duration of orang-utans in the camp, feeding site, and forest during the nest-to-nest observation period.

Focals	Categories	Duration (%)			Daily ranges (km)
		Camp	Feeding site	Forest	
Yoko	Adult male	8.2	27.8	64	1.7
Ewet	Adolescent male	0	8	92	6.2
Ekon	Adolescent male	14.8	0	85.1	4.7
Sakura	Adolescent female	23.4	10	66.7	2.2
Kotim	Adolescent female	18	14	68	4.9
Suwita	Adolescent female	0	18.4	81.6	5.5
Sugi	Adolescent female	18.1	0	81.8	1.5
Acuy	Mother	43.7	0	56.2	1.8
Amina	Mother	0	14.4	85.6	5.6
Max	Mother	40	0	60	1.5

The ranges of followed orang-utans are shown in Figure 6. The longest daily range was dominated by adolescent groups with an average movement which is 6.2 km. Adolescents tend to be more active than adult orang-utans in roaming into the forest. Adult orang-utans, especially mother, are still comfortable and doing activities around camp, so they have less time to roam into the forest.

Discussion

This study analyzed the effect of the release camp and feeding site on orang-utan activity patterns in Lamandau WS by measuring two parameters; the intensity of orang-utans' presence at the camp and feeding site as well as the activity patterns of orang-utans (also including the diet composition and vertical used). Female orang-utans were found to be the ones who visited and spent the most time at the camps and feeding places, according to the findings. Mother and adolescent female had a high intensity of presence at camp and feeding sites. Enhancement of orang-utans' presence at camp and feeding site did not correlate with the phenological in Lamandau WS.

These results implied that female individuals have a higher interest in camp and feeding sites than male individuals. One possible explanation for this difference is closely related to the philopatry character of female orang-utans (van Noordwijk et al. 2012). Female orang-utans tend to stay in their natal range. They begin to explore extensively around their natal range and increase the size of their daily range when they enter the independent immature phase (Wartmann et al. 2010; van Noordwijk et al. 2012). Exploration continues into the sexually active phase, in which adolescent females associate with flanged/unflanged males (Ashbury et al. 2020). The philopatry nature of females will have a serious impact on population stability when in disturbed habitats (habitat fragmentation and degradation occurs) (Ashbury et al. 2020). In this case,

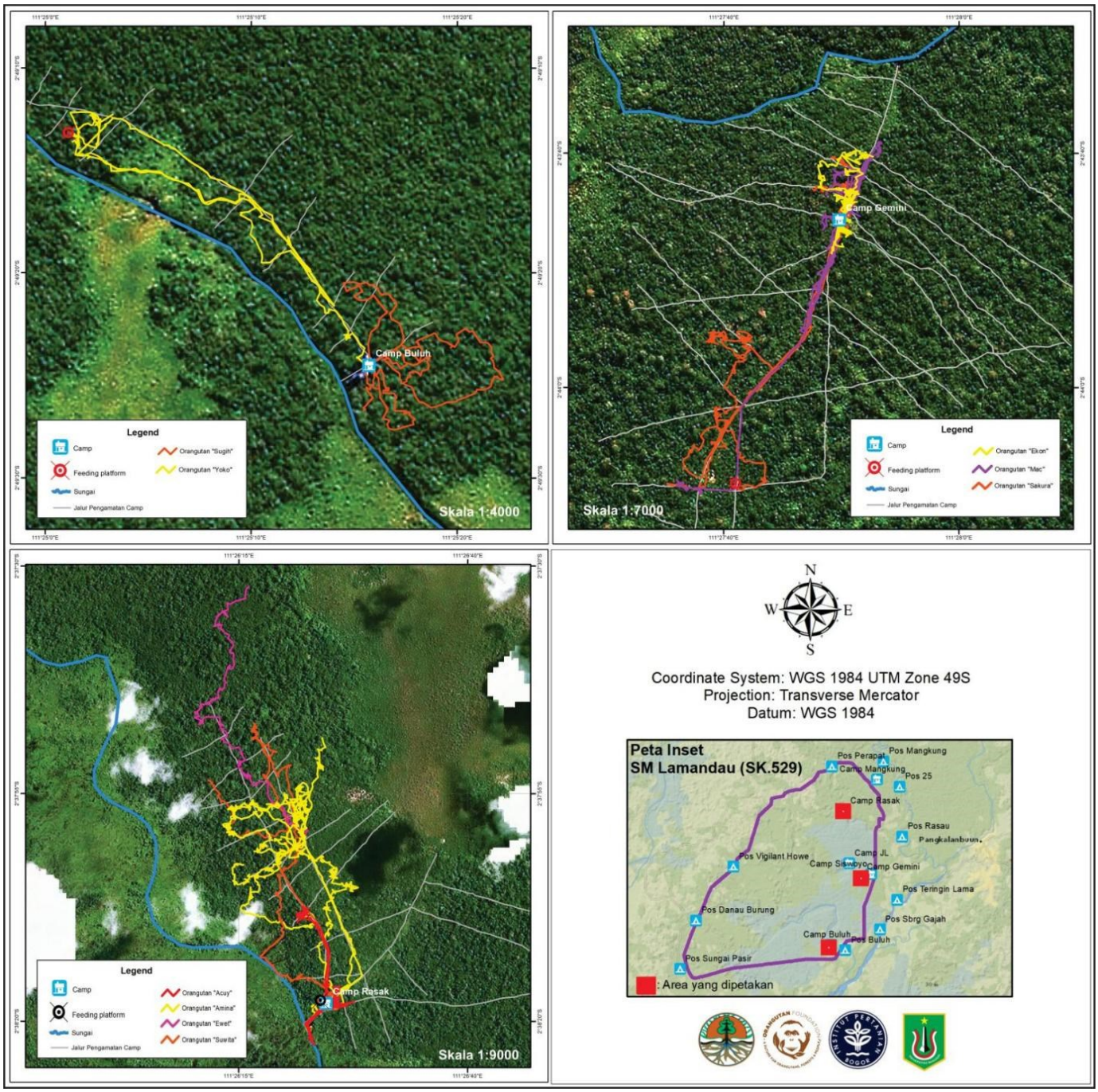


Figure 6. Orang-utan's daily ranges at A) Camp Buluh: Yoko dan Sugi, B) Camp Gemini: Sakura, Ekon, dan Max, C) Camp Rasak: Acuy, Amina, Suwita, dan Ewet.

the philopatry of females may influence the independence of orang-utans which leads to high inactivity and decreasing the quality of each individual.

The presence of orang-utans at the camp and feeding site was then continued by starting activities around. All age-sex classes of orang-utans show different activity patterns. Adult orang-utans, especially mothers, spend most of their time resting and playing on the ground. Almost all individuals of the mother are ex-rehabilitate orang-utans and oriented toward human activity around the camp. Human interest can be a factor in the high inactivity of adult orang-utans. The different experiences of each orang-utan with humans cause a difference of each individual curiosity (Damerius et al. 2017).

Adolescent orang-utans spend time feeding while at the feeding site. However, there are differences in their activity patterns when at

camp where the male adolescent is remarkably more gregarious. Adolescent male was represented by Ekon and Ewet individuals. Both are quite different individuals. Ekon often visits the camp, while Ewet rarely visits and ranges around the camp, except during the consortship period.

According to information from the camp staff, Ekon often followed Max, precisely after Ekon's mother died (Ebony) shortly before this research began. Ekon is known to be in frequent contact with Max and other individuals who often visit the camp. The study conducted by Schuppli et al. (2016) showed that observational social learning occurs at an immature age. Social learning outcomes, when combined with individual socially selective practices over several years, will be an important component in acquiring important skills in orang-utans. In this case, Ekon was 6 years old at the time of observation and was still in the semi-independent stage (Schuppli et al. 2016) when his mother died. For this reason, social learning through adult individuals around them becomes crucial.

Comparison of activity pattern on reintroduced orang-utans have similarities in adolescent with the Kehje Sewen forest, ex-captive orang-utans spend most of their time eating fruit (Basalamah et al. 2018). Some ex-captive orang-utans in Jambi's PROS forest (Sumatran Orang-utan Reintroduction Center) do not exhibit the same activity patterns as wild orang-utans, with longer rest time (Siregar et al. 2018). Ex-captive orang -utans, on average, spend more time resting than feeding and moving (Riedler et al. 2010).

The differences in activity patterns can be attributed to adaptability. In wild orang-utans, the adaptability is acquired from their mother from an early age, while in ex-rehabilitate orang-utans the role of the mother in the Rehabilitation Center is replaced by humans as mother-care (Russon & Galdikas 1995; Morrogh-Bernard et al. 2009). The diet composition of orang-utans in Lamandau WS tends to be similar. There is also a fairly high tendency to eat non-forest diet types. This tendency, where orang-utans rely on additional food obtained from humans, can be a deterrent to successful reintroduction. The selection and composition of broad and good quality of forest-diet depend on the experience and ability of each individual in recognizing and processing diet. Ex-captive orang-utans may gradually expand their diet, but the quality of their diet remains low when compared to wild orang-utans (Russon 2002).

There are different results of daily ranges between adult and adolescent orang-utans. Adolescent orang-utans were more active than adult orang-utans in exploring forest diets. Adult orang-utans, especially mother, are still comfortable and doing activities around camp, which make their forage skill may have less. The ranging movement of reintroduced orang-utans is influenced by the abundance of diet sources (Nayasilana et al. 2017). Orang-utans used the central area/near the main camp area if it is supported by the availability of a patch diet. The presence of orang-utans around the camp also shows that released orang-utans are still comfortable with humans.

Adolescent orang-utans will expand their home range when fruit availability is abundant, with monthly home ranges distance referring to areas with high to medium levels of fruit abundance (Saputra et al. 2017). They will reduce their monthly range to a narrower range and tend to explore areas with moderate to high liana abundance when fruit scarcity. This foraging strategy is an effort to optimize low-quality habitat for orang-utans to survive (Morrogh-Bernard 2009; Saputra et al. 2017).

Ex-rehabilitate orang-utans released through different pre-release stages and release methods generally have different activity patterns. Re-

leased orang-utans through a pre-release stage such as forest school suggest higher feeding and exploring time (Basalamah et al. 2018; Bani et al. 2018) than those who did not (Nawangsari et al. 2016; Siregar et al. 2018). Then orang-utans released by the hard-release method suggesting to have a higher feeding and exploring time in the forest (Basalamah et al. 2018; Bani et al. 2018) compared to orang-utans released by the soft-release (Nawangsari et al. 2016; Siregar et al. 2018).

CONCLUSION

In conclusion, the intensity of the presence of orang-utans at camps and feeding sites is dominated by the Mother group. There is no correlation between the intensity level of orang-utan presence at the camp and the feeding site with the phenological conditions. Factors that influence the pattern of orang-utan activity are the existence of the release camp and the feeding site in the release forest area. Interest in humans may also be another factor affecting the presence of orang-utans around camps and at feeding sites. So, further efforts are needed to reconsider the placement of the camps' release and feeding sites around the orang-utan release points especially those built without barriers.

AUTHORS CONTRIBUTION

D.P.F and S.S.U designed the research and supervised all the process, N.A. collected and analyzed the data and wrote the manuscript, A.P supports in collecting data.

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CONFLICT OF INTEREST

Authors do not have any conflict of interest regarding the research or the research funding.

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Research Article

Genetic Variation Within Four Captive Chital (*Axis axis*) Populations in Indonesia

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ABSTRACT
Chital is a native animal from South Asia. Chital had been introduced to many countries, including Indonesia. Chital was first introduced to Indonesia in 1811 at Bogor Palace and since had been kept captive around Indonesia. Currently, no research had been done concerning the genetic variation of Indonesian chital. Therefore, the purpose of this research was to analyze genetic variation and phylogenetic relationship of chital from Pusat Inovasi Agroteknologi Universitas Gadjah Mada (PIAT UGM), Prambanan Temple, Gembira Loka Zoo, and Bogor Palace, based on mitochondrial *D-loop* fragment. This study used a Polymerase Chain Reaction (PCR) method. DNA was extracted from faecal samples and amplified with L15995 and H16498 primers. The analysis used for this research were genetic variations, haplotype networking, and phylogenetic relationships between populations. This study detected 5 haplotypes out of 20 sequences with 10 polymorphic sites and 2 indels. The haplotype diversity and the nucleotide diversity were 0.443 and 0.002 respectively, and the genetic distance was between 0 and 2.03% (average 0.55%). This research also showed one main haplotype, labelled as haplotype 1, which consisted of all individuals from PIAT and Prambanan Temple, four individuals from Bogor Palace, and one individual from Gembira Loka. This grouping proves that the majority of chital population in Indonesia came from Bogor Palace. One individual from Gembira Loka has a considerable genetic divergence from the rest of the samples, which might indicate it originated from a different source population.

INTRODUCTION
Chital or also called spotted deer (*Axis axis*) is a native animal in South Asia. Even so, this animal has been introduced to many countries (Sankar & Acharya 2004). Chital in Indonesia was originally introduced by the British colonials to Bogor Palace in 1811 (Garsetiasih & Herlina 2005). In the present, chital can be seen around Indonesia, kept in zoos or as livestock (Suharto et al. 2019; Gembira Loka Zoo 2022)

As time goes by, a lot of changes occurred to the policies for captive animals. Animals were kept in captivity originally as a fulfilment of human needs, such as for recreational purposes or kept as livestock. Nowadays, animals are also kept in captivity as a conservation effort, including the animals in zoos (Keulartz 2015). Some animals are deliberately kept

to increase their population size to then be released into the wild. Even though this action is a good effort, the release of unfit animals into the wild could cause some problems. Without proper preparation, released animals cannot survive in the wild. This low survivability is caused by several factors, which could be different across species (Farquharson et al. 2021). The release of individuals from a captive population with high homozygosity can also reduce the genetic variation of the species in the wild. Therefore, genetic variation of captive animals should also be concerned (Purohit et al. 2021). Reduction in genetic diversity has been associated with an increased risk of extinction (Saccheri et al. 1998). To minimize loss of genetic diversity, several zoos have strategies and different methods for genetic conservations. Some of those are to prevent inbreeding, maintaining a high and constant population size in all generations, and population fragmentations, while also keeping track of the DNA of captive animals (Leus et al. 2011).

Genetic variation of chital using Mitochondrial DNA control region (*D-loop)* fragment target has been investigated in Pakistan, Australia, and Croatia, which they were also introduced (Abbas et al. 2016; Hill et al. 2019; Šprem et al. 2021). Meanwhile, no research has been done concerning the genetic variation of chital population in Indonesia. In addition, some captive areas in Indonesia often transfer their animals to and from other places, which can cause uncertainty about the population origin of those animals. Therefore, genetic characterization and genetic variation research need to be done for Indonesian chital to understand the genetic structure of those populations and to determine the origin of the animals. This study aimed to analyze the genetic variation and phylogenetic relationship of chital from Bogor Palace, Gembira Loka Zoo, PIAT UGM, and Prambanan Temple, based on mitochondrial *D-loop* fragment.

MATERIALS AND METHODS

Sample collection

Faecal samples from five individuals were collected each from Bogor Palace (BP2D, BP3D, BP4D, BP7D, and BP8D), Gembira Loka Zoo (GL3D, GL4D, GL6D, GL7D, GL11D), PIAT UGM (PI2D, PI3D, PI4D, PI5D, PI7D), and Prambanan Temple (PT1D, PT2D, PT4D, PT5D, PT10D), with the total of 20 samples. Each of these locations held its own captive chital in a closed enclosure. Three fresh faecal pellets were collected and stored in a stool collection tube with 96% ethanol as the preservative. The tubes were then stored inside a cooler box for transportation. Samples were then transported to the Laboratory of Genetics and Breeding (Faculty of Biology, Universitas Gadjah Mada) and kept inside a box at room temperature until the following process.

DNA extraction

Each faecal sample was removed from the stool collection tube and the surface was scraped using a sterile scalpel. The scraps were then collected in a total of about 160-240mg of materials for DNA extraction. The DNA extraction was done using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol, with a modification of using 100 µL instead of 200 µL ATE buffer.

D-loop Amplification

The partial *D-loop* fragment was amplified using primers: L15995 (5'-CTCCACTATCAGCACCCAAAG-3') (Taberlet & Bouvet 1994) and H16498 (5'-CCTGAAGTAAGAACCAGATG-3') (Fumagalli et al.

1996), which are universal *D-loop* primers used for mammals (Harsini et al. 2017). PCR amplifications were performed using T100 Thermal Cycler (Biorad) with 25µL reaction volume consisting of 12.5 µL of MyTaq™ HS Red Mix (Bioline), 1 mM $MgCl_2$, 0.4 µM each of forward and reverse primer, 4.5 µL ddH_2O, and 5 µL template DNA (11.09-27.34 ng/ µL). The DNA amplification PCR profile following Arisuryanti et al. (2020) consisted of pre-denaturation of the template at 95°C for 1 minute, denaturation at 95°C for 15 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, repeated for 35 cycles, and final extension at 72°C for 10 minutes. PCR product yields 410-455bp of amplicons.

Electrophoresis and Sequencing

Electrophoresis of the PCR products was run on 1% agarose gel stained with FloroSafe (Bioline) and buffered with Tris-Acetate EDTA (TAE) buffer at 100 volts for 25 minutes. Visualization was conducted under UV light. All amplified amplicons were sent to Apical Scientific Sdn. Bhd. (1st BASE, Malaysia) via P.T. Genetika Science Indonesia (Jakarta) for purification and sequencing using the Big Dye Terminator (Applied Biosystems) and the ABI 3730xl Genetic Analyzer (Applied Biosystems). Amplicons were sequenced through bi-direction using primers used for PCR amplification.

Sequence Editing

Sequences obtained were edited and the consensus sequences were made with GeneStudio program and validated with SeqMan and EditSeq on DNASTAR program (DNASTAR Inc., Madison, USA). Chromatograms were inspected manually to check ambiguous nucleotides.

Sequence Alignment

The consensus sequences were then analysed using Opal package (Wheeler & Kececioglu 2007) in MESQUITE ver. 3.6.1 program (Maddison & Maddison 2019) and ClustalW in MEGAX (Kumar et al. 2018).

Substitution Model Selection

An analysis of the most fitting substitution model was done using jModelTest2.1.10 (Darriba et al. 2012) based on the Bayesian Information Criterion (BIC).

Nucleotide Composition and Genetic Distance

Nucleotide composition and genetic distance were analysed using an already integrated function in MEGAX. Genetic distance was analysed using Tamura 3-Parameter with Gamma Distribution model (T92+G) with 1,000 bootstrap replicates.

Genetic Variation Analysis

Genetic variation analysis was done using DnaSP ver.6 program (Rozas et al. 2017). Parameters analysed in this research include haplotype number, number of polymorphic sites, haplotype diversity, and nucleotide diversity.

Haplotype Network and Principal Coordinate Analysis (PCoA)

Haplotype network and PCoA analysis were done using additional sequences from GenBank. The sequences used are shown in Table 1. Haplotype network was constructed using median joining network method in NETWORK ver 10.2. Principal Coordinat Analysis (PCoA) was done

using GenAlEx ver. 6.51b2 plugin for Microsoft Excel (Peakall & Smouse 2012).

Phylogenetic Analysis

Phylogenetic analyses were done using additional sequences from GenBank. The sequences used are shown in Table 1. The phylogenetic tree was constructed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. Phylogenetic analysis with Maximum Likelihood was done in MEGAX program using Tamura 3-Parameter + Gamma Distribution with invariable sites (T92+G+1) with 1,000 bootstrap. Analysis with Bayesian Inference was done using BEAST v1.10.4 program (Suchard et al. 2018). Bayesian Inference analysis was done using Hasegawa-Kishino-Yano + Gamma Distribution model (HKY+G). The Markov Chain Monte Carlo (MCMC) was run for 10^7 generations with a sampling frequency set to every 1,000 generations. Phylogenetic tree visualization was done using FigTree v.1.4.4 program (Rambaut 2019).

RESULTS AND DISCUSSION

Genetic Variability

Analysis was done using 411 bp of *D-loop* fragment. The average nucleotide composition (Table 2) revealed a relatively similar composition between each population. This indicates no major mutation had occurred. Based on the genetic distance (Table 3), the chital from PIAT UGM and Prambanan Temple have identic sequences. Those populations had a relatively lower genetic distance compared to the population from Bogor Palace. Chital was first introduced to Indonesia in the Bogor Palace. Chital population in Bogor Palace had grown significantly and became difficult to be sustained inside the Bogor Palace area. Since then, some individuals had been transferred to several places in Indonesia to maintain the population size in Bogor Palace (Garsetiasih & Herlina 2005). This low genetic distance between population in Bogor Palace and both PIAT UGM and Prambanan Temple could indicate that the population in PIAT UGM and Prambanan Temple originated from Bogor Palace.

Based on the genetic variation analysis (Table 4), chital population in Bogor Palace has 2 haplotypes with 1 variable site, which is an indel. This population has a low haplotype diversity (Hd) value of 0.400. This finding is in line with previous research done in Australia (Hill et al. 2019) and Croatia (Šprem et al. 2021). Analysis using 576 bp of *D-loop* fragment on 35 individuals from Queensland, Australia found 2 haplotypes with 4 polymorphic sites, with Hd of 0.461±0.07 and nucleotide

Table 1. Sequences obtained from GenBank.

No	Accession Number	Species	Location	Author
1	MN226865	*Axis axis*	Australia	Hill et al. 2019
2	MN226866	*Axis axis*	Australia	Hill et al. 2020
3	MZ421332	*Axis axis*	Croatia	Šprem et al. 2021
4	MZ421333	*Axis axis*	Croatia	Šprem et al. 2021
5	JN596141	*Axis axis*	India	Kumar et al. (direct submission)
6	JN596142	*Axis axis*	India	Kumar et al. (direct submission)
7	MT998906	*Axis axis*	Island of Lanai, Hawaii	Buchholz et al. (unpublished)
8	MT998894	*Axis axis*	Texas	Buchholz et al. (unpublished)
9	MW348981*	*Rucervus duvaucelii*	India	Kumar et al. (direct submission)
10	MH392156*	*Axis porcinus*	India	Gupta et al. (direct submission

Note: *Used only for phylogenetic analysis as outgroup

diversity (π) value of 0.0023±0.0004 (Hill et al. 2019). Analysis using 576bp of *D-loop* fragment on 32 individuals from the Island of Rab, Croatia found 2 haplotypes with 7 polymorphic sites, with Hd value of 0.514 and π value of 0.006. Meanwhile, the analysis of 7 individuals from Dugi Otok, Croatia found 2 haplotypes with 7 polymorphic sites, with Hd value of 0.286 and π value of 0.004. Both of these populations from Croatia shared the same haplotypes (Šprem et al. 2021).

Table 2. Average nucleotide composition of 411 bp *D-loop* fragment of chital from Bogor Palace, Gembira Loka, PIAT UGM, and Prambanan Temple.

Population	T	C	A	G	A+T	G+C
Bogor Palace (n=5)	29.52	24.64	32.17	13.67	61.69	38.31
Gembira Loka (n=5)	29.45	24.62	32.28	13.65	61.73	38.27
PIAT UGM (n=5)	29.51	24.63	32.20	13.66	61.71	38.29
Prambanan Temple (n=5)	29.51	24.63	32.20	13.66	61.71	38.29

Table 3. Average genetic distance of chital from Bogor Palace, Gembira Loka, PIAT UGM, and Prambanan Temple.

	Bogor Palace	Gembira Loka	PIAT	Prambanan Temple
Bogor Palace				
Gembira Loka	0.55% (0-2.03%)			
PIAT UGM	0.05% (0-0.24%)	0.50% (0-1.77%)		
Prambanan Temple	0.05% (0-0.24%)	0.50% (0-1.77%)	0.00%	

Notes: The number inside the bracket represent the range of each samples genetic distance.

According to an article in Trubus magazine in 1996 (as cited in Garsetiasih & Herlina 2005), chital was introduced to Bogor Palace, Indonesia in 1811 with 6 pairs of individuals. This could cause a founder effect which could significantly reduce the genetic variability of the population. Those individuals were then allowed to breed with each other freely, and for 210 years, no additional individual was brought in from other populations (R.Y. Andini, personal communication, November 11, 2021). This process could then further reduce the genetic variability of the population, which led to the low diversity in the present. Bogor Palace has a relatively small area for the present chital population, which strictly limits the carrying capacity. According to Garsetiasih and Herlina (2005), Bogor Palace could ideally only support 169-286 individuals of chital, but the carrying capacity was fluctuating. Individuals from Bogor Palace were from time to time needed to be translocated to other places because the low carrying capacity could not support the population. The population is deliberately kept being around 400 to 600 individuals. Sometimes, a reduction of hundreds of individuals happened to establish this constant number (R.Y. Andini, personal communication, November 11, 2021). This could potentially become a genetic drift which further reduces the already low genetic variation. As most chitals from around Indonesia were translocated from Bogor Palace (R.Y. Andini, personal communication, November 11, 2021), the low genetic variation of this source population could further cause the low genetic variation of chital in Indonesia.

Compared to the population from Australia and Croatia, the Bogor

Palace population have a lower genetic variability. Chital in Indonesia shared a similar introduction history with the population from Australia and Croatia. Chital was introduced at two different times to Queensland in the 19th century (Hill et al. 2019). As Queensland received two founder populations two different times, this original population in the past might had more genetic diversity with more haplotypes compared to the population in Bogor Palace. As the founder population could have lower genetic diversity, this could explain why the same condition can be applied to the present. In Croatia, chital was first introduced to Brijuni Island in 1911. Eight individuals from Brijuni Island were then translocated to Rab in 1974. In 2012, 13 individuals from Brijuni Island escaped and were established on the island of Dugi Otok (Šprem et al. 2021). Since the population established in Croatia was introduced a century later than the one in Queensland and Bogor Palace, fewer generations had passed which means fewer genetic diversity loss probability. This could explain why the genetic diversity of Rab Island chital population is higher than the one from Bogor Palace and Queensland.

Based on the genetic diversity analysis (Table 4), the chital population in Gembira Loka Zoo has 4 haplotypes out of 5 samples with 9 variable sites. This population has the Hd value of 0.9 and π value of 0.00829. This Hd value is high, especially compared to the value from other populations in this study. Gembira Loka Zoo often received animals from BKSDA (Indonesian Natural Resources Conservation Center), which were confiscated from illegal keepers. Gembira Loka had received new chital individuals from BKSDA every couple of years for the past several years (B.R. Samuels, personal communications, April 21, 2022). These confiscated individuals were from unknown origins, hence it could be possible that some of these individuals did not originate from Bogor Palace population. This varied source of individuals could cause the high genetic diversity of this population.

Based on the genetic variability analysis (Table 4), the chital population in both PIAT UGM and Prambanan Temple shared similar haplotypes (haplotype 1) and were the only haplotype observed in both populations. This haplotype was also shared with the Bogor Palace population. PIAT UGM and Prambanan Temple both received the first few individuals from Bogor Palace around the late 2000s to early 2010s. Both populations then grew to around 50 to 60 individuals around mid 2020 to early 2021 (Adji & Astuti 2020; D. Sutanto, personal communication, November 1, 2021). The chital population from Bogor Palace already have a low genetic variation. Since Bogor Palace is the source population and the transfer was relatively recent, the few individuals which were transferred to Prambanan Temple and PIAT UGM might already have a very low genetic variability. This could explain why only 1 haplotype exists in Prambanan Temple and PIAT UGM in the present and how it shares the

Table 4. Genetic diversity indices based on 411 bp *D-loop* fragment of chital populations from Bogor Palace, Gembira Loka, PIAT UGM, and Prambanan Temple.

Population	Bogor Palace	Gembira Loka	PIAT UGM	Prambanan Temple	All Samples
n	5	5	5	5	20
Nh	2	4	1	1	5
S	1	9	0	0	10
Hd	0.400	0.900	0.000	0.000	0.442
π	0.000	0.008	0.000	0.000	0.002

Notes: n. number of samples; Nh. Number of haplotypes; S. number of polymorphic sites; Hd. Haplotype diversity; π. Nucleotide diversity.

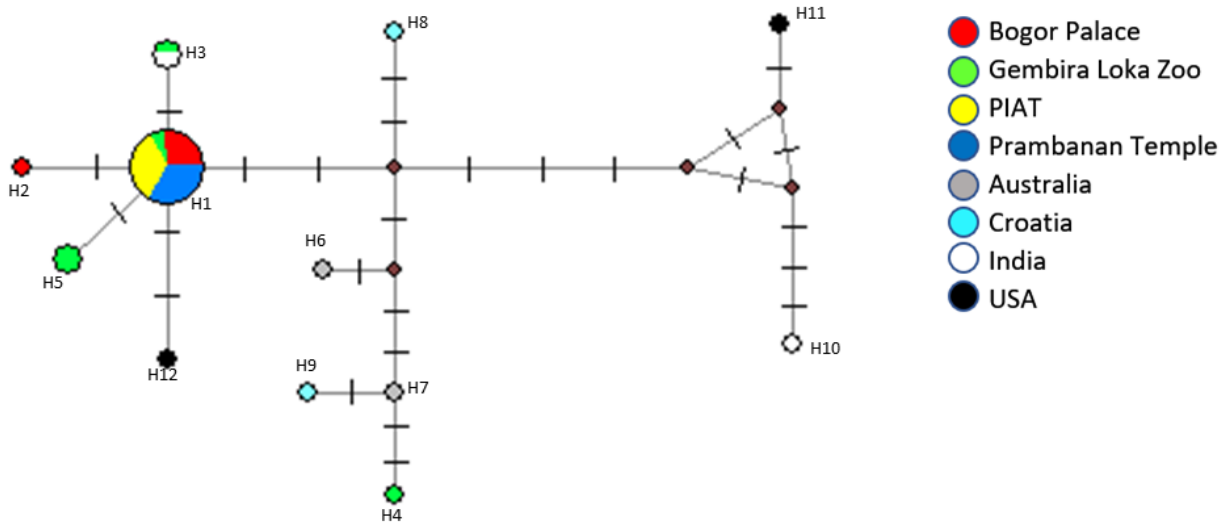

Figure 1. Haplotype network based on 401bp *D-loop* fragment of chital from this study and sequences obtained from NCBI GenBank.

haplotype with Bogor Palace population. Furthermore, both of these populations had undergone mass mortality events. The population from PIAT UGM recently underwent mass mortality of more than 30 individuals which was caused by the disease. A large number of fawns from Prambanan Temple often died by drowning in the pool. Both of these events could become a bottleneck effect that eliminates several haplotypes from these populations if ever existed.

Phylogenetic Relationship

Phylogenetic relationships were analysed using 401 bp of *D-loop* fragment. The haplotype network (Figure 1) reveals the main haplogroup which consists of most samples from this study. Haplotype 1 is shared between all study populations. Haplotype 2, 3, and 5 only have 1 mutation step from haplotype 1. Haplotype 3 is shared between population from Gembira Loka Zoo and from India. Haplotype 4 which consists of 1 individual from Gembira Loka Zoo (GL6D) is relatively far from the main group, where it is closer to a population from Australia. Principal Coordinate Analysis (Figure 2) shows a similar result with the haplotype network.

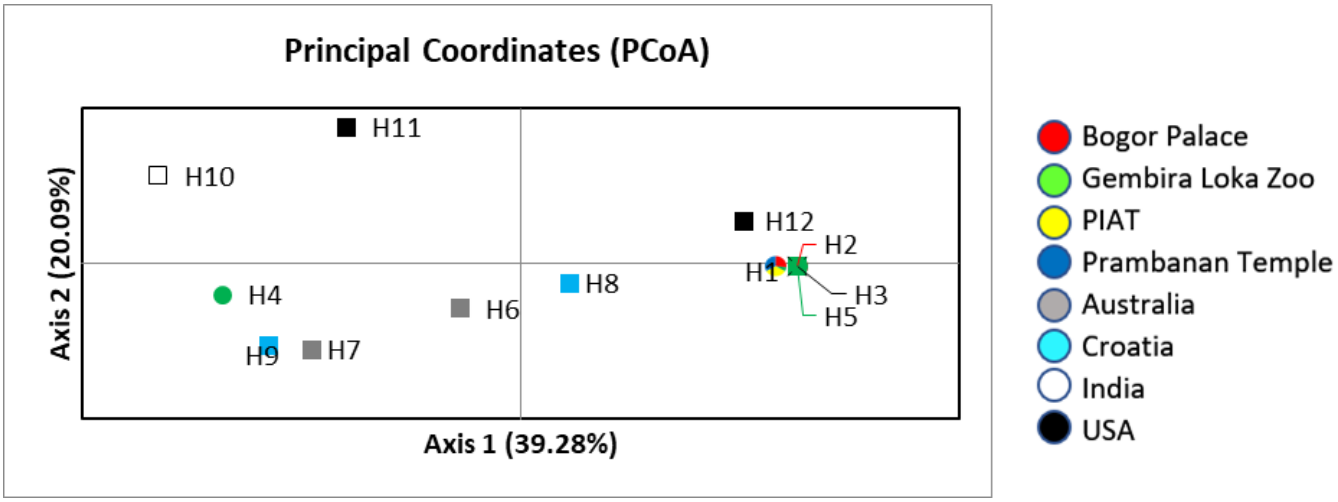

Figure 2. PCoA between haplotypes based on 401 bp *D-loop* fragment of chital population from this study and sequences obtained from NCBI GenBank.

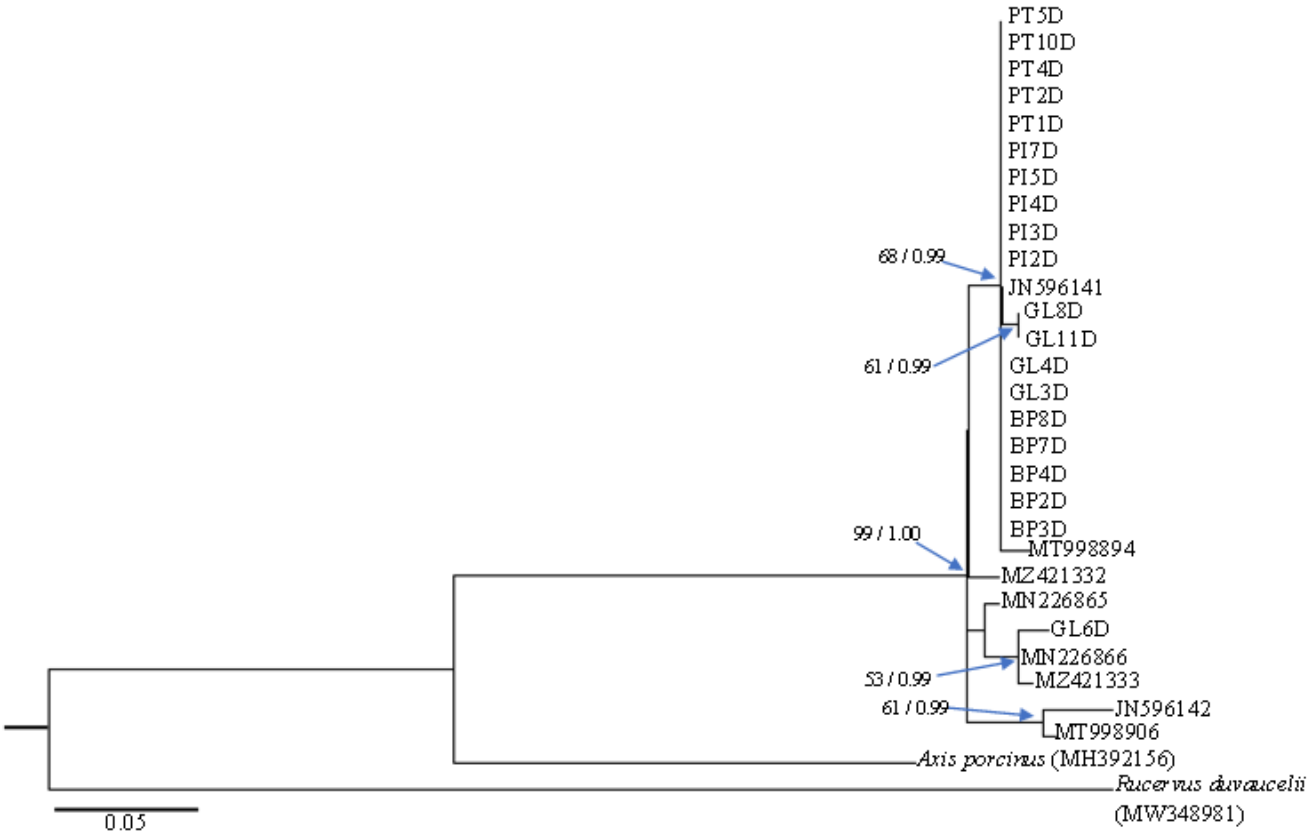

Figure 3. Phylogenetic tree of chital based on 401bp *D-loop* fragment of chital population from this study and sequences obtained from NCBI GenBank. Number on the node shows bootstrap value (ML) and posterior probability (BI) respectively

The phylogenetic tree (Figure 3) shows a grouping of most samples from this study with one sequence from India (JN596141). A sequence of chital from Texas, USA is also closely related to this group. This result is consistent with the haplotype network (Figure 1) and PCoA (Figure 2) results. As the population from India is native, this close relationship could indicate that the population which was first introduced to Indonesia originated from India. Nevertheless, a certain claim could not be made considering the few numbers of *D-loop* sequence data of chital available in the present. Sample GL6D, which is the haplotype 4, formed a clade with a sample from Australia (MN226866) and Croatia (MZ421333). This result is also consistent with the haplotype network (Figure 1) and PCoA (Figure 2) results.

As sample GL6D is closely related to the population from Queensland, Australia, a possible explanation would be that this individual originated from Australia. As it is also closely related to the population from Croatia, another possible explanation would be that these three haplotypes originated from closely related populations. The population from Queensland, Australia originated from Sri Lanka (Hill et al. 2019), while the origin of the population from Croatia is unknown (Šprem et al. 2021). This GL6D individual could also be originated from Sri Lanka, or another population closely related to the population in Sri Lanka. Nevertheless, this possibility could not be proven in the present as no chital *D-loop* sequence data from Sri Lanka is available as of now.

Both theories could be considered as a possibility considering the increasing trend of animal import and smuggling in Indonesia. These include exotic and wild animals and are often will be kept as a pet or a collection. A lot of animals kept in Gembira Loka are obtained from confiscated animals by BKSDA. The GL6D individual might be imported

from outside Indonesia quite recently by smugglers or certified traders and was kept by an Indonesian citizen, which was then confiscated by BKSDA and donated to Gembira Loka Zoo.

CONCLUSION

From this research, two haplotypes (haplotype 1&2) were found in Bogor Palace with 0.400 haplotype diversity value, one haplotype (haplotype 1) was found in both PIAT UGM and Prambanan Temple with 0 haplotype diversity value, and four haplotypes (haplotype 1,3,4,5) were found in Gembira Loka Zoo with 0.900 haplotype diversity value. Haplotype 1,2,3, and 5 were closely related to each other and one haplotype from India, while haplotype 4 was more closely related to Australian and Croatian populations.

AUTHORS CONTRIBUTION

M.Z.M.P. performed sample collection, laboratory work, data analysis, and writing of manuscript. T.A. designed the data analyses and supervised all the processes. Z.R. gave input on the research planning and co-supervised the research.

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CONFLICT OF INTEREST

The authors state that they do not have any conflicts of interest. The authors are responsible for the article's content and writing.

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Research Article

Medicinal Plants Diversity Used by Balinese in Buleleng Regency, Bali

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ABSTRACT
The Lontar Usada Bali is a collection of the science of traditional medicine recorded in the lontar leaves. It contains information about the diversity of medicinal plants and treatment procedures used by Balinese for generations. However, most of the information stored in the lontars is only known by the Balians (Traditional Healers). The aim of the study was to investigate and document the diversity of medicinal plants known by Balians in Buleleng Regency, Bali Province, Indonesia. Direct interview with Balians, combined with purposive sampling (for the usada plants), was used in this study and conducted in August-September 2022. The data obtained comprised plant species, habitat, habitus, local names, plant parts used, and how they were used. The data were analysed qualitatively and quantitatively using diagrams, graphs, and tables and measured by the use-value index. Sixty-five species and 37 families of plants were recorded as a medicinal plant. The most widely used plant families by Balians were Zingiberaceae, Poaceae, Rutaceae, Euphorbiaceae, Lamiaceae, Lauraceae, and Malvaceae. In most cases, leaves were used, followed by tubers, fruits, and other parts. Maceration and powder or mushy were the primary modes of making herbal medicine, and external application was the most common method of drug administration. Most medicinal plants were obtained from the home garden, taken from nature, or bought in the market. Several diseases often treated by Balians were convulsions, itching, cramps, headache, black magic, stroke, herpes and tumor/cancer. This research is important to complete the ethnobotanical data on the diversity of medicinal plants in Bali. This data is important information for the development of new drugs and must be maintained for sustainability.

INTRODUCTION
In recent years, modern society's attention and interest in "back to natural products" has increased along with awareness of the side effects of using chemical-based drugs (Rahayu & Rosleine 2020). In addition, the results of modern research also support that herbal medicinal products are generally sourced from the ethnobotanical knowledge of the local community. Furthermore, this herbal product has been proven to have various unique biochemical components and potential as a drug source (Hiben et al. 2019; Belgica et al. 2021). Therefore, knowledge about the ethnobotany of medicinal plants in a traditional society is an essential source of information for developing and discovering new compounds in

the pharmacology industry.

Until recently, alternative medicine using traditional medicines sourced from ethnobotanical studies passed down from generation to generation is still developing and used by traditional communities in various ethnic groups in Indonesia. So ethnobotanical information is an essential source of information that must be continuously recorded and developed (Nguyen et al. 2019; Kachmar et al. 2021).

Indonesia has high ethnic diversity and biodiversity, including the diversity of medicinal plants used by these ethnic communities. They have used various compositions of medicinal plants to treat various diseases in humans from generation to generation (Pandiangan et al. 2019). However, in the last few decades, traditional communities living in rural areas in Indonesia, especially in Bali, continue to face increasing cultural erosion due to interactions with foreign cultures over a long period, along with ecotourism activities. Evidence of this cultural erosion is marked by the decreasing knowledge of the ethnobotany of the local community, especially about the use of plants for traditional medicine, whereas knowledge about traditional medicinal plants is an essential asset for Indonesia (Oktavia et al. 2017).

The Balinese tribe has a source of information about the diversity of medicinal plant species and their treatment procedures, known as Lontar Usada Bali. Based on the legacy of the inscription, Lontar Usada Bali is estimated to have originated from medical knowledge in India and developed along with the development of Hinduism on the island of Bali. *Lontar Usada* is an ancient manuscript that contains information about traditional medicine systems passed down from generation to generation. This information is generally written in palm or siwalan leaves (*Borassus flabellifer* plant). It is estimated that 55,000 lontars are stored by the Balinese themselves, Pedanda, Balian, and several local institutions in Bali (Oktavia et al. 2017). Some ethnobotanical information on the Usada Bali lontar has been recorded by Tengah (Tengah 1995), but most of it is still stored in the lontar records or in the knowledge of the Balians found in Bali. This study aims to explore information on the diversity of medicinal plants stored or known by balians (traditional healers) in Buleleng Regency, Bali Province.

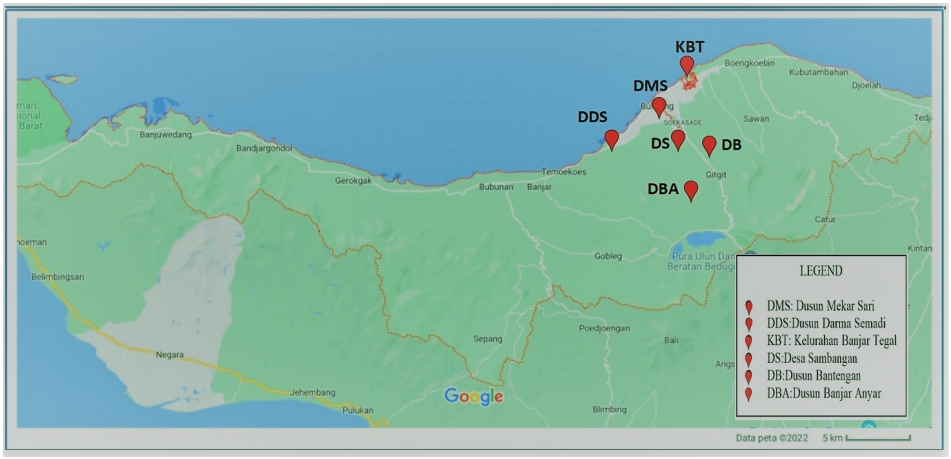
Buleleng Regency is an area on the north coast of the island of Bali with the capital city Singaraja. This area has an area of about 1,365. 88 km2 or 24.25% of the total area of Bali Province. The Balinese people in Buleleng Regency still carry out their traditional life, including in traditional medicine. They have many Balian's (traditional healers) who pass on knowledge about the diversity of medicinal plants used in the Buleleng area from generation to generation. Although some of this information has been documented in *Lontar Usada*, in fact there is still much information about the diversity of these medicinal plants that are only known by Balian and has not been well documented. This makes the topic of Balian's knowledge about the diversity of medicinal plants in Buleleng district very interesting to study.

STUDY AREA AND METHODS

Study Area

The research was located in the villages in Buleleng Regency, Bali Province (Figure 1). The selected locations were based on the presence of Balians (traditional healers) as an informant to be interviewed in this study. They were Kelurahan Banjar Tegal, Buleleng subdistrict (KBT); Dusun Bantengan, Ambengan village, Sukasada subdistrict (DB); Dusun Mekar Sari, Panji Village, Sukasada subdistrict (DMS); Dusun Darma

Semadi, Desa Tukad Mungga, Bulleleng Subdistrict (DDS), Desa Sambangan, Sukasada subdistrict (DS), and Dusun Banjar Anyar, Sambangan village, Sukasada subdistrict (DBA).



KBT: 8° 12' 25" S, 115° 08' 73" E DB : 8° 16' 66" S, 115° 12' 04" E
DMS: 8° 18' 35" S,115° 10' 13" E DDS: 8° 14' 36" S,115° 05' 86" E
DS : 8° 14' 86" S,115° 09' 82" E DBA: 8° 15' 21" S,115° 09' 96" E

Note: The research location: Kelurahan Banjar Tegal, Buleleng District (KBT); Dusun Bantengan, Ambengan Village, Sukasada District (DB); Dusun Mekar Sari, Panji Village, Sukasada District (DMS); Dusun Darma Semadi, Tukad Mungga Village, Bulleleng District (DDS), Desa Sambangan, Sukasada District (DS), and Dusun Banjar Anyar, Desa Sambangan, Sambangan Village, Sukasada District (DBA).

Figure 1. The study area of Medicinal Plants Diversity Used by Balinese In Buleleng Regency, Bali, Indonesia.

Ethnobotanical Data Collection

This research aims to record the diversity of medicinal plant species used by traditional healers (Balian) in Buleleng Regency, Bali Province. This research was conducted with an interview method with purposive sampling and using a semi-open questionnaire (Yudiyanto et al. 2022). Nine Balians from 6 villages in Buleleng Regency were interviewed as informants. Some non-numeric data on ethnobotany of medicinal plants were collected include the diversity of medicinal plant species, local names, scientific names, families, habitat, habitus, including data on plant parts used, processing methods, usage methods, types of diseases that can be treated, and the origin of obtaining medicinal plants (Budiarti et al. 2020). Photos and vouchers for medicinal plants were recorded and collected, then sent to the Bali "Eka Karya" Botanical Gardens to be identified by taxonomists. Species identification is made by comparing the morphological characteristics of medicinal plant vouchers with herbarium collections and live plant collections at the Eka Karya Bali Botanical Gardens, as well as comparisons with several literatures in the form of books Flora of Java (Backer & Bakhuizen van den Brink Jr. 1965); Flora Pegunungan Jawa (Steenis 2010) or online taxonomy sites. The scientific name was determined and verified using online database of scientific plant names The Plant List, 2018 and The International Plant Names Index, 2018 (Andila et al. 2021). In addition, some critical information was also collected, including socio-economic conditions, community culture, education level, income sources, livelihoods, informants' views on forests, and threats related to diversity (Mwangi et al. 2021).

Data Analysis

The interview data were analysed qualitatively and quantitatively. Dia-

grams, graphs, and tables were utilized to depict qualitative analysis. During the quantitative analysis, the use-value index (UV) of a species, Family Use Value (FUV) and Plant Part Value (PPV) were calculated. Some descriptions of efficacy and how to mix medicine are also explained.

a) The Use Value index (UV)

The Use Value index (UV) of a species aims to measure the relative usefulness of a medicinal plant species quantitatively (Zenderland et al. 2019; Jadid et al. 2020; Damayanti et al. 2021; Merouane et al. 2022). the UV was measured using the following formula:

$$UV = \sum Ui / N$$

UV: use value index, Ui: number of uses by informants, N: Number of respondents.

b) Family Use Value (FUV)

Family Use Value (FUV) was calculated as described by Jadid et al. (2020) and Merouane et al. (2022). FUV is defined as the use value of a plant family as an ingredient of traditional medicine by an ethnic group.

$$FUV = \Sigma UV / n$$

ΣUV is the UV sum of all species belonging to the same plant family. n: is the number of species belonging to the same plant family.

c) Plant Part Value (PPV) (%)

Plant part value (PPV) is the percentage of plant parts (stems, leaves, roots, fruit, bark, and flowers) used by traditional healers as a source of traditional medicine. PPV is calculated by the formula according to Chaachouay et al. (2019), Jadid et al. (2020) and Najem et al. (2020) as follows:

$$PPV = (\sum RU\ (plant\ part) / \sum RU) \times 100$$

Where $\sum RU\ (plant\ part)$ is the number of uses per plant part and $\sum RU$ represents the sum of uses reported for all plant parts.

RESULTS AND DISCUSSION

This study documented the diversity of medicinal plants used by Balians in Buleleng Regency, Bali Province. All informants in this study work as experts in traditional medicine in Buleleng Regency. All informants (100%) answered all the questions in the questionnaire list using the semi-interview method. Socio-demographic information of the informants (traditional healers or Balians) was shown in table 1. Overall, 87.5% of informants were male and 12.5% female, with a female/male sex ratio of 0.14. A survey of traditional healers based on gender shows that men are more dominant than women. These results are the same as those reported by the same study in other areas (Bachiri et al. 2015; Harouak et al. 2018; Najem et al. 2020). Traditional medicine practitioners in Buleleng Regency, Bali, Indonesia, have a range of age groups that vary, with the most dominant age range being 61-70 years (37.5%), then followed by an age range of 51-60 years (25%), and 31-40 years old and 71-80 years old with a percentage of 12.5% each. Based on the age range, traditional healers in Buleleng Regency, Bali, are dominant between the age range of 51-70 years, indicating that knowledge of traditional medicine and the diversity of medicinal plants requires years of experience (Najem et al. 2020). Analysis of the data based on the formal educational background of the informants showed that the most dominant formal education of the informants was a primary school (37.5%) and senior high school (37.5%), followed by informants with non-formal education (12.5%) and informants with high education in university (12.5%). Transmission of medical

knowledge by traditional healers in Buleleng Bali from generation to generation is carried out through various methods, including orally from the older generation to the younger generation and in writing through traditional medical records in *Lontar Usada*. Inheritance of medical knowledge through oral was what needs to be appropriately recorded scientifically because treatment information passed down orally is easier to experience a reduction in information (Najem et al. 2020).

Based on the results of this study (shown in table 3), it was recorded 66 species and 37 families of plants used as medicinal plants, including their scientific name, local name, family, the origin of the plant, the parts plant used, the type of disease that can be treated, location of the informant, the method of concocting the drug, the method of application of the drug, and the Useful Value index. The list of the most important plant families in terms of species used as medicinal plants by Balians in the Buleleng Regency, Bali, Indonesia was calculated following the method reported by Luziatelli et al. (2010) and shown in Figure 2. From the highest to the lowest percentage, it consisted of Zingiberaceae (18.97%), Poaceae (8.62%), Rutaceae (6.90%), Euphorbiaceae, Lamiaceae, Lauraceae, Malvaceae (5.17% each), Amaryllidaceae, Apocynaceae, Leguminosae, Loranthaceae, Magnoliaceae, Myristicaceae, Oxalidaceae (3.45% each). While the Family Use Value (FUV) is shown in Table 2. This study revealed that the plant families with high FUV were Zingiberaceae (1.71), Euphorbiaceae (1.89), Arecaceae (1.5), and Amaryllidaceae (1.5), Piperaceae (1.2), Poaceae (1.1). However, the magnitude of the FUV value is not represented by the more significant number of species members. This means that the use value of a plant family ethnobotanically does not depend on the richness and diversity of species but rather on the use value and importance of the species (Najem et al. 2019). The high value of FUV can also be caused by the high content of active compounds in those plant family groups that play a role in various biological activities such as antibacterial, anti-inflammatory, antiviral and antioxidant (Najem et al. 2020). This study showed that Zingiberaceae had the highest FUV, meaning that members of the Zigiberaceae family were most widely used

Table 1. Socio-demographic information of the informants (traditional healers or Balians).

Variables	Category	Percentage (%)	Respondent's location (number of respondents)
Age	31-40 years	12.5	DMS (1)
	41-50 years	0	-
	51-60 years	25	DS (2)
	61-70 years	37.5	KBT (1), DMS (2)
	71-80 years	12.5	DDS (1)
	81-91 years	12.5	DBA (1)
Sex	Male	87.5	DMS (3), DS (1), KBT (1), DDS (1), DBA (1)
	Female	12.5	DS (1)
Education level	no formal education	12.5	DBA (1)
	Primary School	37.5	DMS (1), DS (1), DDS (1)
	Junior high school	0	-
	Senior high school	37.5	DS (1), KBT (1), DMS (1)
	university	12.5	DMS (1)

Note: The research location: Kelurahan Banjar Tegal, Buleleng District (KBT); Dusun Bantengan, Ambengan Village, Sukasada District (DB); Dusun Mekar Sari, Panji Village, Sukasada District (DMS); Dusun Darma Semadi, Tukad Mungga Village, Bulleleng District (DDS), Desa Sambangan, Sukasada District (DS), and Dusun Banjar Anyar, Desa Sambangan, Sambangan Village, Sukasada District (DBA).

as medicinal plants. Zingiberaceae, also known as the ginger family, is a group of flowering plants and herbs that contain aromatic compounds in all parts of the plant. This plant contains a variety of secondary metabolites that exhibit a variety of significant biological and pharmacological activities. For example, Zingiber officinale species that are widely used as traditional medicine for various treatments illness include as analgesics, sedatives, antioxidants, antipyretics, antimicrobials, anticancer, anti-inflammatory and anticonvulsant drugs (Tamokou et al. 2017).

Grass et al. (2021) suggested that botanical studies have a close relationship with the selection of plant families that play a role in determining the medicinal plants used in ethnobotany. This comparative study revealed that ethnobotany's most widely used medicinal plant families were Lamiaceae, Asteraceae, Rosaceae, Malvaceae, Adoxaceae, Apiaceae, Amaryllidaceae, Oleaceae, Pinaceae, and Rutaceae. Four families, including Apiaceae, Oleaceae, Pinaceae, and Rutaceae, were grouped as important plant taxa groups. Based on the study results on the diversity of

Table 2. Family Use Value of medicinal plant families in Buleleng Regency, Bali, Indonesia.

NO.	Family	ΣUV	Number of Species n	FUV
1	Acanthaceae	1	1	1
2	Amaryllidaceae	3	2	1.5
3	Annonceae	1	1	1
4	Apiaceae	1	1	1
5	Apocynaceae	2	2	1
6	Araceae	1	1	1
7	Arecaceae	1.5	1	1.5
8	Casuarinaceae	1	1	1
9	Clusiaceae	1	1	1
10	Compositae	2	2	1
11	Crassulaceae	1	1	1
12	Cucurbitaceae	1	1	1
13	Euphorbiaceae	5.67	3	1.89
14	Lamiaceae	3	3	1
15	Lauraceae	1	1	1
16	Leguminosae	2	2	1
17	Loranthaceae	2	2	1
18	Lythraceae	3	1	3
19	Magnoliaceae	2	2	1
20	Malvaceae	3	3	1
21	Menispermaceae	1	1	1
22	Moraceae	1	1	1
23	Moringaceae	1	1	1
24	Myristicaceae	2	2	1
25	Myrtaceae	1	1	1
26	Nymphaeaceae	1	1	1
27	Oxalidaceae	2	2	1
28	Pandanaceae	1	1	1
29	Phyllanthaceae	1	1	1
30	Piperaceae	1.2	1	1.2
31	Poaceae	5.49	5	1.1
32	Polypodiaceae	1	1	1
33	Rosaceae	1	1	1
34	Rutaceae	4	4	1
35	Santalaceae	1	1	1
36	Talinaceae	1	1	1
37	Zingiberaceae	18.84	11	1.71

medicinal plants in Buleleng Regency, Bali, the most widely plant families used by Balians were Zingiberaceae (18.97%), Poaceae (8.62%), Rutaceae (6.90%), Euphorbiaceae, Lamiaceae, Lauraceae, Malvaceae (5.17 each). Therefore, three families of them (Rutaceae, Lamiaceae, and Malvaceae) were included in the theory proposed by Grass et al. (2021).

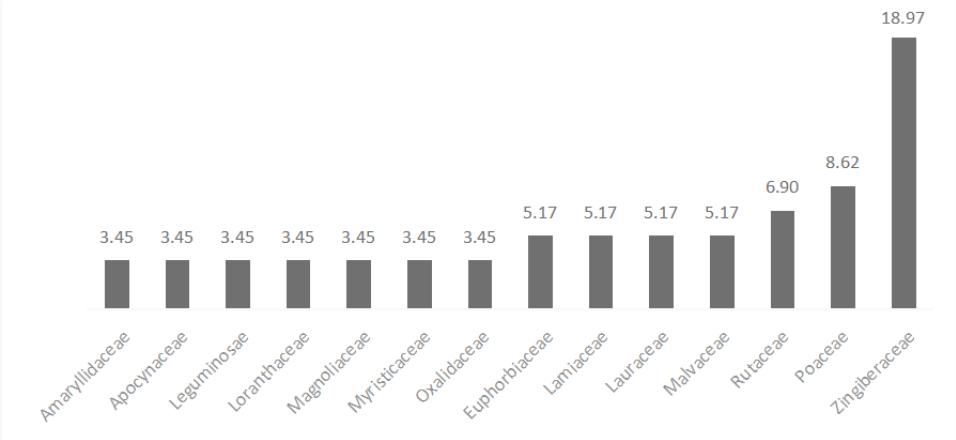


Figure 2. The list of the most important plant families in terms of species used as medicinal plants by Balians in the Buleleng Regency, Bali, Indonesia (% used).

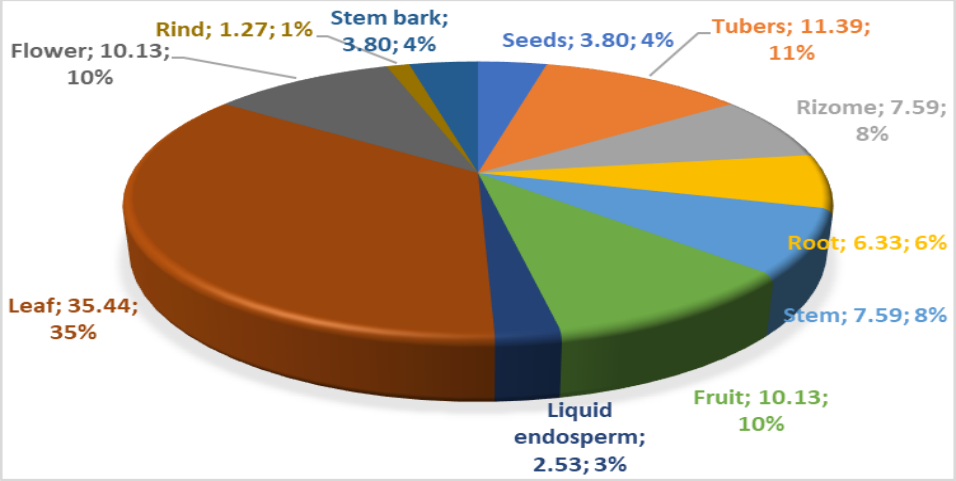


Figure 3. Percentage use of plant parts used as medicinal plants by Balians in the Buleleng Regency, Bali Province, Indonesia.

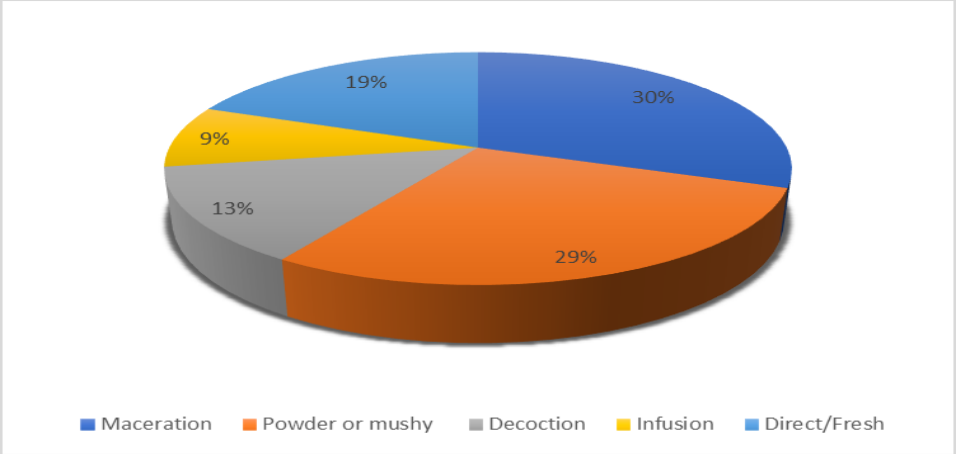


Figure 4. Method of preparing recipes as medicinal plants by Balians in the Buleleng Regency, Bali Province, Indonesia.

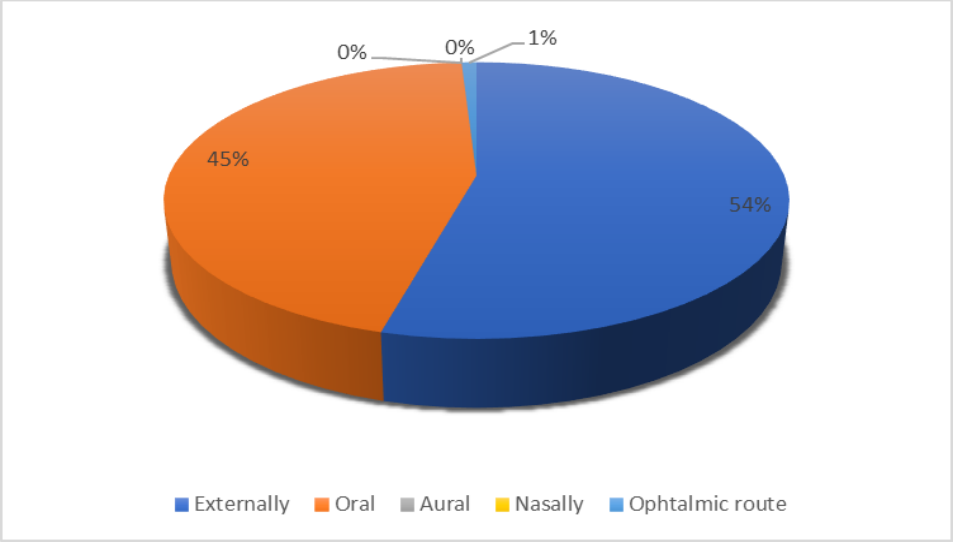


Figure 5. Modes of administration as medicinal plants by Balians in the Buleleng Regency, Bali Province, Indonesia.

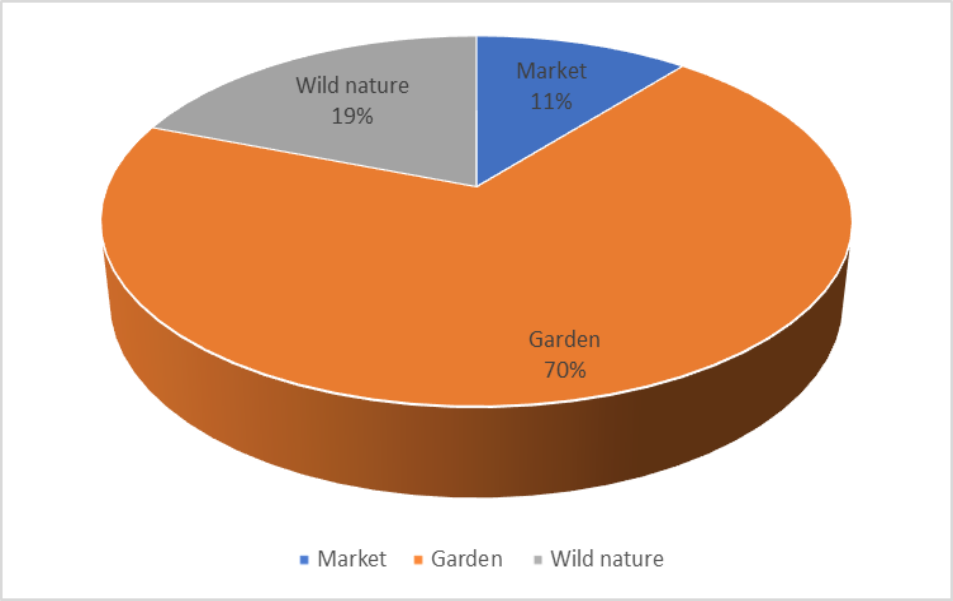


Figure 6. The origin of the medicinal plants used by Balians in the Buleleng Regency, Bali Province.

In Figure 3 was recorded that some parts of the plant were used as ingredients for traditional medicine by Balians in Buleleng Regency, Bali. In most cases, leaves were used (35.44 %) followed by tubers (11.39%), fruits (10.13 %), flowers (10.13 %), rhizomes (7.59 %), stems (7.59 %), roots (6.33 %), seeds (3.80%), stem barks (3.80%), liquid endosperm (2.53%) and rinds (1.27%). The percentage of use of each plant part as herbal medicine by traditional ethnicities varies in various places. However, generally, the leaves are the most widely used part. Elfrida et al. (2021) reported that local communities in Jambur Labu Village, East Aceh, Indonesia, mostly used leaves in herbal medicine ingredients (38%), then followed by fruit (22%), rhizome (8%), roots (7%), sap (3%), stem and tuber (2% each), and seeds (1%). A similar study was reported by Damayanti et al. (2021) on the study of the diversity of medicinal plants on the island of Lombok, which explains that the leaf is the organ that is most widely used compared to other parts such as roots, stems, flowers, fruit or seeds. Leaves are plant organs that are easily obtained and contain many secondary metabolites that are useful for health treatment,

such as flavonoids, phenols, terpenoids, and alkaloids (Tungmunnithum et al. 2018).

Meanwhile the diagram in figure 4 explained about methods of making herbal medicine by Balinese people in Buleleng Regency. According to this study, maceration (30%) and powder or mushy (29%) are the most widely used method. Other preparation methods, such as Direct/ Fresh (20%), Decoction (13%), and Infusion (9%), were also used successively. According to Arsana (2019), in traditional Balinese medicine there are 6 ways of processing drugs, namely; loloh, boreh, sembar or simbuh, tutuh or pepeh, tampel or tempel and Ses. Traditional medicine processing by maceration has a definition similar to "Loloh" which is in the form of concentrated starch liquid obtained by squeezing or grinding and adding a predetermined liquid and its use is drunk. Powder or mushy can be defined as making "boreh" and "tampel". Boreh is a concoction obtained by mashing a mixture of ingredients and in use mixed with liquids such as water, vinegar, or wine. While tampel is a concoction obtained by mashing a mixture of ingredients and in use is attached to the treated part, usually in the centre of the pulse.

Comparative studies on traditional medicinal processing methods that have been carried out by other researchers have shown varying results. Husaain et al. (2018) reported that communities residing in Koh-e-Safaid Range, northern Pakistani-Afghan borders used Decoction as the powerful method of drug preparation, followed by vegetable, powder, infusion, ash, and roast. While people in Lombok Island used Decoction (47%) as the primary mode of herbal medicine preparation (68%). It was followed by other preparation methods such as mashed (20%), chewed (15%), direct application (8%), and etch (Damayanti et al. 2021). A similar study reported by Kachmar et al. (2021) revealed local community in the Northeastern Part of Morocco used Decoction (29.11% and infusion (27.84%) as the most used methods of sample preparation, followed by fresh material (20.25%), powder (17.72%) and etc.

Interestingly, the results of this study revealed that the most common method of drug administration by Balians in Buleleng Regency, Bali Province was the external application (54%), followed by the oral mode (45%) (Figure 5). External application of treatment was carried out by various methods, such as sprayed onto certain parts of the body, applied to the affected area, used as a scrub and applied to the affected part, bathed all over the body or attached to certain body parts. While the application of the drug orally is by drinking it regularly. Higher frequency of traditional medicine external application than orally is not common. Hussain et al. (2018) reported that The administration of traditional medicine that is most often used is the oral intake method.

Curcuma viridiflora (Zingiberaceae) had the highest usability index (UV=4). Then followed by *Z. officinale* (Zingiberaceae), Jatropha gossypiifolia (Euphorbiaceae), Punica granatum (Lythraceae) (each UV= 3), *C. massoy* (Lauraceae) (UV= 2.5), Aleurites moluccanus (Euphorbiaceae), Z. montanum (Zingibercaeae), Michelia alba DC (Magnoliaceae) and A. cepa (Amaryllidaceae) (each UV= 2) (Showed in table 3). Value in use (UV) is an index or measurement widely used in ethnobotany to measure a plant species' relative importance or usefulness. UV is often used to mark the most widely used or most useful plant species. The higher the UV value of a species, it will usually tend to be cultivated more often than wild plants (Assefa et al. 2019).

The interview results revealed that most of the medicinal plants used by Balian in Buleleng Regency (Bali) came from medicinal plants planted in the home garden (70%). While some were taken directly from

nature (wild) (19%), and some were bought in the market (11%) (as shown in Figure 6). Sujarwo & Caneva (2015) reported that tropical home gardens were important sites and had a long tradition for Balinese ethnicity to maintain plant diversity. It had been recorded that more than 20 families and 29 genera were planted in Balinese home gardens. The Zingiberaceae family was the most widely planted, followed by *Poaceae*, *Fabaceae*, *Anacardiaceae*, *Cucurbitaceae*, *Asteraceae*, and *Euphorbiaceae*. The most commonly used were leaves, fruits, tuberous roots, young leaves, and young shoots.

The tradition of planting valuable plants in the home garden from generation to generation is a plant conservation effort that needs to be preserved. The conservation and sustainability of medicinal plants have been studied extensively for decades. However, the use of medicinal plants massively to produce herbal products globally has caused the availability of medicinal plants in nature to decrease significantly from time to time (Hilongan et al. 2018; Posthouwer et al. 2018). Studies reveal that currently the world is losing 100 to 1000 times more plant species than naturally occurring extinctions. This causes the earth to lose at least one crucial medicinal plant every two years (Chen et al. 2016). Therefore, it is highly recommended to carry out plant conservation efforts in-situ (Sun et al. 2022), ex-situ (Kovács et al. 2021), or in cultivation gardens so that the availability of plants in a sustainable manner can be obtained (Shao et al. 2021).

Some of the human diseases and medicinal ingredients used by Balian in Buleleng Regency Bali were described as follows:

a) Convulsions
Formula: Lulur (loloh) is made from *Centella asiatica* (L.) Urb. and *Moringa oleifera* Lam. leaves, chopped and mashed, and rubbed on the patient's feet. If the patient is not conscious, then the patient's lips are rubbed with saliva mixed with *Kaempferia galanga* L. and the front head is sprayed with *Cryptocarya massoy* (Oken) Kosterm. Usually, 1-2 times, the sickness is healed.

b) Itching
Formula: Three pieces of *Piper betle* L. leaves are rolled up and added with coconut oil, salt, "Kesuna Tunggal" (*Allium sativum* L.) and insect house called by local name "Kalisasoan". All ingredients are kneaded and crushed, then applied to the itchy part. During illness, you should not eat eggs and salted fish. Healing happens usually after three days of use.

c) Cramps or tingling
Formula: Lulur (boreh) is made of isen (*Alpinia galangal* (L.) Willd., mesuwi (*Cryptocarya massoy* (Oken) Kosterm), jebug arum (*Myristica fragrans* Houtt.), vinegar or arak, brown rice and singrong. All ingredients are mashed and rubbed on the affected area. Usually, after two days of use, it is healed.

d) Headache
Formula: sprayed water made of mesuwi (*C. massoy*), bangle (*Zingiber purpureum* Roscoe) burned, added with salt, charcoal and water. Use by spraying on the forehead and nape of the patient. Usually, once use the headache is healed. There is a ritual of praying to God (Hyang Widhi) using Banten worship facilities.

e) Black magic (Mental Disorder)
Formula: Three leaves of *Ocimum tenuiflorum* L. mixed with water, allowed standing for 8 minutes and then drunk. There is a ritual by saying 9x Om Nama Ciwaya.

f) Stroke

Formula 1: Lulur (boreh) was made of three-piece *Piper betle* leaves, added a little table salt and lengkuas (*A. galanga*). All these ingredients are mashed and applied all over the patient's body, from the neck to the feet. Most patients recover after 1-3 times of treatment.

Formula 2: Jamu (loloh) is made of ginger (*Z. officinale*), cekuh (*K. galanga*), salam leaves (*Syzygium polyanthum*) and soaked rice. All ingredients are mashed Herbal medicine is drunk in the morning and afternoon as much as half a glass.

Formula 3: Spray water consists of mesuwi (*C. massoy*), isen (*A. galanga*), Ginger (*Z. officinale*), 5 bunga jepun (*Plumeria rubra*) and soaked rice. These materials are crushed (chewed) and then sprayed on the patient's neck. Usually, 3-5 times treatments needed to be healed.

g) Herpes

Formula: Spray water is made from banyan leaves (*Ficus benjamina* L.) and 1 tablespoon of injin (rice naturally black glutinous rice). All the ingredients are mashed or chewed and then sprayed on the affected part of herpes.

h) Tumor/cancer

Formula 1: Herbal medicine (loloh) is made of Belimbing wuluh besi leaves (*Averrhoa carambola* L.), katuk leaves (*Sauropus androgynous* (L.) Merr.), red union (*A. cepa*) and added a little salt. All ingredients are mashed and the herbal medicine is drunk in the morning and afternoon as much as half a glass.

Formula 2: herbal medicine (loloh) made from kepasilan (Lorantaceae), and jeruk kinkit (*Triphasia trifolia* (Burm.f.) P. Wilson, taken 2 times a day until healed.

i) Diabetes

Formula 1: herbal medicine (loloh) is made of Talas Gajah (*Anthurium crystallinum* Linden & Andre) mashed and then filtered. Herbal medicine is taken once a day until healed.

Formula 2: Jamu (loloh) is made of 9 fruitss of *Averrhoa bilimbi* L. and 1 bulb of red onion (*A. cepa*). These materials are boiled in 3 cups of water to 1 cup. Half a glass of herbal medicine is drunk in the morning and evening and continues to be drunk until it heals.

j) Nephropathy

Formula: herbal medicine (loloh) is made from Kecibeling (*Strobilanthes crispa*) leaves (3,7,11). Herbal medicine is drunk once a day until healed.

k) Toothache

Formula 1: three peaces of leaves add a little table salt and pangolin (klesih) oil. All materials were given warm water and used for mouthwash. Most patients recover once get the treatment.

Formula 2: For cavities, put the oil into the cavities using a cotton swab. Usually, twice of use will heal.

l) Broken bones or swollen bones

Formula: herbal medicine (loloh) is made from a mixture of mesuwi (*C. massoy*), turmeric (*Curcuma viridiflora*), onion bulbs (*A. cepa*) and rice that has been soaked in water. This material is mashed and added with the water continues to be filtered. Previously, the patient was massaged using coconut oil.

m) Vomiting blood or bleeding

Formula: Jamu (loloh) is made from a mixture of undis leaves (*Cajanus cajan*), ginger (*Z. officinale*), Cekuh (*K. galanga*) and soaked rice. All ingredients are mashed, continue to be filtered. The fil-

tered results are used as herbal medicine and drunk by the patient. While the dregs are used as a scrub. The scrub is rubbed on the patient's chest and abdomen.

n) Asthma

Formula 1: Jamu (loloh) is made of roasted coconut (*Cocos nucifera*) Ginger (*Z. officinale*), Cekuh (*K. galanga*) roasted, Salam leaves (*Syzygium polyanthum*) 5 pieces and soaked rice. All ingredients are mashed. Herbal medicine is drunk in the morning and afternoon as much as half a glass.

Formula 2: Spraying Water: Isen (*A. galanga*), Ginger (*Z. officinale*), 3 Japanese flowers (*Plumeria rubra*) and soaked rice. All materials were crushed (chewed) and then sprayed on the patient's chest. Usually three times of use the diseases is healed.

o) White vaginal discharge

Formula: Jamu (loloh) consists of Isep nanah/ meniran putih (*Euphorbia thymifolia*) and Isep getih/ meniran merah *(Euphorbia thymifolia*) and red onion (*A. cepa*). These materials are boiled from 3 cups of water to 1 cup. Drank half a glass of herbal medicine in the morning and evening. Usually, 1-3 times of treatment would heal.

Table 3. Medicinal plant species used among the local community of Buleleng Regency, Bali, Indonesia.

No	Scientific name	Origin of Plants	Local name	Part of plant used	usefulness	Location	UV
1	*Aleurites moluccanus* (L.) Willd. (Euphorbiaceae)	garden	Kenari	seed	Tumor, cancer, liver	KBT	2.00
2	*Allium cepa* L. (Amaryllidaceae)	market	Bawang Merah	tubers	Swollen genitals; broken bones or swelling, low appetite, vaginal discharge, diabetes, cancer and tumor	KBT, DSM, DBA	2.00
3	Allium sativum L. (Amaryllidaceae)	garden	Kesuma Tunggal	tubers	Itching / Boils	DMS	1.00
4	*Alpinia galanga* (L.) Willd. (Zingiberaceae)	garden	Isen	rhizome	shortness of breath (asthma), stroke, pinched nerve, vertigo, liver disease, jaundice, poisoning, eye pain, cramps, stroke	KBT, DDS, DMS	1.67
5	*Anthurium Crystallinum (Araceae)*	garden	Keladi Gajah	tubers	Diabetes	DS	1.00
6	*Arcangelisia flava* (L.) Merr. (Menispermaceae)	wild nature	Kayu Kuning	root and stem	hepatitis	DMS	1.00
7	*Areca catechu* L. (Arecaceae)	wild nature	Pinang	fruits	Wound, vertigo	DMS	1.50
8	*Averrhoa bilimbi L. (Oxalidaceae)*	garden	Belimbing Buluh	fruits	diabetes	KBT	1.00
9	Averrhoa carambola L. (Oxalidaceae)	garden	Belimbing Besi	leaves	Tumor and cancer	KBT	1.00
10	*Blumea balsamifera* (L.) DC. (Compositae)	garden	Daun sembung	leaves	Liver	KBT	1.00
11	*Bryophyllum pinnatum* (Lam.) Oken (Crassulaceae)	garden	Cocor Bebek	leaves	Diabetes	DDS	1.00

Table 3. Contd.

No	Scientific name	Origin of Plants	Local name	Part of plant used	usefulness	Location	UV
12	*Cajanus cajan* (L.) Millsp. (Leguminosae)	garden	Daun Undis	leaves	Blooding	KBT	1.00
13	*Calotropis gigantea* (L.) Dryand. *(Apocynaceae)*	market	Bunga Meruri Putih	seed	Mental disorder and stroke	DDS	0.50
14	*Cananga odorata* (Lam.) Hook.f. & Thomson (*Annonceae*)	garden	Sandat Bali	flower	Breast cancer	DB, DDS	1.00
15	*Casuarina junghuhniana* Miq. (Casuarinaceae)	garden	Cemara Angin	leaves	Mental disorder		1.00
16	*Centella asiatica (L.) Urb. (Apiaceae)*	Wild nature	Pegagan	leaves	bladder disorders and convulsions	DMA, DBA	1.00
17	*Citrus aurantiifolia* (Christm.) Swingle (Rutaceae)	garden	Jeruk Nipis	fruits	Kidney stones	DDS	1.00
18	*Citrus medica* L. (Rutaceae)	garden	Jeruk Lengis	fruits	Stroke	DDS	1.00
19	*Cocos nucifera* L. (Poaceae)	garden and market	Kuud	fruits	Shortness of breath (asthma), teeth, haemorrhoids, mental disorder, diabetes, itching, ulcers, breast cancer, broken bones, swelling, bladder disorder	KBT, DMS, DDS,DS, DB,DBA	1.09
20	*Cryptocarya massoy (Oken) Kosterm. (Lauraceae)*	garden	Mesuwi	stem and leaves	fractures or swelling, cramping and headache	DBA	2.50
21	*Curcuma aeruginosa* Roxb. (Zingiberaceae)	garden	Temu Ireng	rhizome	Stroke	DDS	1.00
22	*Curcuma longa* L. (Zingiberaceae)	garden	Warangan	rhizome	Stroke	DDS	1.00
23	*Curcuma purpurascens* Blume (Zingiberaceae)	garden	Temu Tis	rhizome	Liver disease, hepatitis, poisoning	DDS	3.00
24	*Curcuma viridiflora* Roxb. (Zingibercaeae)	garden	Kunyit	rhizome	Swollen genitals; broken bones or swelling, pain in the navel or black magic	KBT, DBA	4.00
25	*Curcuma zedoaria* (Christm.) Roscoe (Zingiberaceae)	garden	Temu Putih	rhizome	Stroke	DDS	1.00
26	*Cymbopogon citratus* (DC.) Stapf (Poaceae)	garden	Sereh	stem	Diabetes	DDS	1.00
27	*Drymoglossum piloselloides* (L.) M.G. Price. *(Polypodiaceae)*	wild nature	Paku Naga	leaves	Herpes	DBA	1.00
28	*Erythrina variegata* L. (Luguminocae)	garden	Daun Dadap Sakti	leaves	stroke	DDS	1.00
29	*Euphorbia thymifolia* L. *(Euphorbiaceae)*	garden	Isep Getih	leaves	Fluor albus/ white discharge	KBT, DMS	0.67

Table 3. Contd.

No	Scientific name	Origin of Plants	Local name	Part of plant used	usefulness	Location	UV
30	*Ficus benjamina L. (Moraceae)*	garden	Beringin	leaves	itchy	DS	1.00
31	*Garcinia × mangostana* L. (Clusiaceae)	garden and market	Manggis	rind	haemorrhoid	DMS	1.00
32	*Imperata cylindrica* (L.) Raeusch. (Poaceae)	wild nature	Pucuk Ilalang	leaves	Swollen genitals	DBA	1.00
33	Jatropha gossypiifolia L. (Euphorbiaceae)	wild nature	Jarak keliki	leaves	Liver disease, hepatitis, poisoning	DDS	3.00
34	Kaempferia galanga L. (Zingibercaeae)	garden	Cekuh	rhizome	vomiting blood, asthma, stroke, pinched nerves and convulsions	DMS	1.67
35	*Kleinhovia hospita* L. (Malvaceae)	garden	Ketiman	leaves	Mental disorder	DDS	1.00
36	Knema cinerea Warb. (Myristicaceae)	wild nature	Jelema	stem bark	stroke	DDS	1.00
37	*Kaempferia galanga* L. (Zingibercaeae)	garden	Cekuh	rhizome	vomiting blood, asthma, stroke, pinched nerves and convulsions	DMS	1.67
38	*Magnolia champaca* (L.) Baill. ex *(Magnoliaceae)*	garden	Bunga Cempaka Merah	flower	Breast cancer	DB	1.00
39	*Michelia alba* DC (Magnoliaceae)	garden	Cempaka Putih	flower	Breast cancer	DB, DDS	2.00
40	*Momordica balsamina* L. (Cucurbitaceae)	garden	Paye Puuh	leaves	Mental disorder	DMS, DDS	1.00
41	*Moringa oleifera* Lam. (Moringaceae)	garden	Daun Kelor	leaves	Mental disorder, convulsions	DMS	1.00
42	*Murraya paniculata* (L.) Jack (Rutaceae)	Wild nature	Kemuning	leaves	Impotent	DDS	1.00
43	*Myristica fragrans Houtt. (Myristicaceae*	market	Jebug Arum	fruits	cramps and tingling	DMS	1.00
44	*Nymphaea alba* L. (Nymphaeaceae)	garden	Bunga Tunjung Putih	flower	Mental disorder	DDS	1.00
45	*Ocimum tenuiflorum L. (Lamiaceae)*	garden	Daun Tulasi	leaves	Black magic	DMS	1.00
46	*Oryza sativa* L. (Poaceae)	market	Padi	seed	Mental disorder, Vomiting blood or bleeding, Asthma, Cramping/tingling, Breast cancer, fractures or swelling	DBA	1.40
47	*Pandanus amaryllifolius* Roxb. (Pandanaceae)	garden	Pandan harum	leaves	Liver disease	KBT	1.00
48	*Piper betle* L. (Piperaceae)	garden	Sirih	leaves	insomnia, sores, itching, boils, stroke and herpes, toothache	DSM, DS, DBA	1.20
49	Plectranthus amboinicus (Lour.) Spreng. (Lamiaceae)	wild nature	Don ginten	leaves	Heat or fever, backache	DBA	1.00

Table 3. Contd.

No	Scientific name	Origin of Plants	Local name	Part of plant used	usefulness	Location	UV
50	*Plumeria rubra L. (Apocynaceae)*	garden	Bunga Jepun	flower	shortness of breath (asthma), stroke, pinched nerve	KBT	1.50
51	*Punica granatum* L. (Lythraceae)	garden	Dalima Wanta	leaves	Liver disease, hepatitis, poisoning	DDS	3.00
52	*Rosa chinensis* Jacq. (Rosaceae)	garden	Mawar Merah	flower	Mental disorder	DDS	1.00
53	*Saccharum spontaneum* L. (Poaceae)	Wild nature	Agar Gelagah	root	Diabetes	DDS	1.00
54	*Bombax ceiba* L. (Malvaceae)	Wild nature	Randu Alas	stem bark	Stroke	DDS	1.00
55	*Santalum album* L. (Santalaceae)	garden	Asaban Cendana	fruits	Mental disorder	DDS	1.00
56	*Sauropus androgynus* (L.) Merr. (Phyllanthaceae)	garden	Daun Katuk	leaves	Tumor and cancer	KBT	1.00
57	*Sterculia foetida* L. (Malvaceae)	garden	Kayu Kepuh	stem bark	Stroke	DDS	1.00
58	*Strobilanthes crispa* Blume (Acanthaceae)	garden	Kecibeling	leaves	Kidney stones	DS	1.00
59	*Syzygium polyanthum* (Wight) Walp. (Myrtaceae)	garden	Daun Salam	leaves	shortness of breath (asthma)	KBT	1.00
60	*Talinum paniculatum* (Jacq.) Gaertn. (Talinaceae).)	garden	Temu Ginseng	tubers	Stroke	DDS	1.00
61	*Tagetes erecta* L. (Compositae)	garden	Bunga Mitir	flower	Tumor or cancer	KBT	1.00
62	*Triphasia trifolia (Burm.f.) P.Wilson (Rutaceae)*	garden	Jeruk Kinkit	fruits	Cancer	DS	1.00
63	*Vitex trifolia L. (Lamiaceae)*	wild nature	Liligundi	root, stem, leaves,fruits, flower	Impotent	DDS	1.00
64	*Zingiber montanum (J.Koenig) Link ex A.Dietr (Zingibercaeae)*	garden	Bangle	rhizome	Headache and chills	DMS	2.00
65	Zingiber officinale Roscoe (Zingiberaceae)	garden	Jahe	rhizome	Vomiting blood, asthma, stroke or pinched nerves	KBT	3.00
66	Zingiber purpureum Roscoe *(Zingiberaceae*)	garden	Gamongan	rhizome	Tumor, cancer, breast cancer	KBT, DB	1.50

Note: Kelurahan Banjar Tegal, Buleleng subdistrict (KBT); Dusun Bantengan, Ambengan village, Sukasada subdistrict (DB); Dusun Mekar Sari, Panji Village, Sukasada subdistrict (DMS); Dusun Darma Semadi, Desa Tukad Mungga, Bulleleng Subdistrict (DDS), Desa Sambangan, Sukasada subdistrict (DS), Dusun Banjar Anyar, Sambangan village, Sukasada subdistrict (DBA).

CONCLUSIONS

Zingiberaceae, Poaceae, Rutaceae, Euphorbiaceae, Lamiaceae, Lauraceae, Malvaceae are the most widely used plant families by the Balinese as traditional medicinal ingredients. The leaves were the most widely used, followed by tubers, fruits and other parts. While maceration and powder or mushy were the main ways of making herbal medicine and external application is the most common way of administering drugs. Various types of diseases could be treated, including: convulsions, itching, cramps, headaches, black magic, stroke, herpes, tumours/cancer, etc. Thus, ethnobotanical data on medicinal plants is important information for the development of new drugs and needs to be preserved. Balinese people have had conservation awareness from generation to generation where most of these medicinal plants are planted in the home gardens.

AUTHOR CONTRIBUTION

P.S.A.: Research designing, writing the first draft of the manuscript, data analysis, conceiving and supervising the research. I.G.T.: Interviewing informants, plant collection. T.W.: Voucher species preparation, literature research. S.: data analysis, literature research. All authors approved the final version of the manuscript.

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CONFLICT OF INTEREST

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Research Article

Food Preference of Bullet Tuna (*Auxis rochei* Risso, 1810) in Prigi Coast of Trenggalek Regency, East Java

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ABSTRACT
As a commercial fish, bullet tuna is constantly caught in purse seine fisheries to provide economic benefits for coastal communities. Biological information such as food preference has to be known due to their sustainability in the future. This research aims to figure out the food composition and preference of bullet tuna (*Auxis rochei* Risso, 1810) in Prigi Coast, Trenggalek Regency East Java. A total of 294 fish landed by purse seine fishermen have been collected from March up to May 2018. Each individual was measured in its total length and weight, then was dissected for sex determination, measured the length of intestine, and analyse the stomach content. The data analysis encompasses frequency distribution of total length, relative gut length, frequency of occurrence, index of preponderance, trophic level, niche breadth, and food overlapping. The research result indicates that the main foods of *Auxis rochei* are fish and crustacean, while the complementary and additional foods were copepod, mollusc, annelid, and debris. Bullet tuna was a carnivorous fish with the trophic level of 3.7 and shows the existence of competition for food resources. Overlapping of feeding occurred in the 19-20 cm long group against the 23-24 cm long group in male fish and the 19-20 cm size group against the 21-22 cm length group in female fish. Bullet tuna use the same feed resources among the size groups of fish, where females use feed over a wider area than males.

INTRODUCTION
Tunas belonging to the genus Auxis has a wide distribution in tropical and oceanic sub-tropic oceans (Uchida 1981). Bullet tuna (*Auxis rochei*, Risso 1810) is one of the epipelagic neritic tuna found in the equator ocean and migrate as schooling (Pillai & Satheeshkumar 2012; IOTC 2017; Herath et al. 2019). Bullet tunas are commonly caught by gillnet, handline, and trolling, but it is a potential purse seine commodity (IOTC-WPNT09 2019) and also usually by-cached in pelagic fishery using danish seine, purse seine, drifting gillnet, lift net, pole, and line, as well as hand lines (Widodo et al. 2014). Prigi fishing port reported total catch of purse seine fishermen dominated by bullet tuna reached 500 tons in a day with a production value of about 2 billion to 3.5 billion rupiahs (Prigi Fishing Port 2022).

Fishery activities in Prigi bay are known as one of the main capture fisheries dominated by purse seine fishery. Fishery capture data from

Ministry of Marine Affair and Fisheries of Indonesia (MOMAF 2018) showed that almost 93.9% of purse seine had dominated the total fish catch landing in Prigi bay. At the Prigi Coast, the fishes caught using purse seine and dominated by small and big pelagic fish, followed by demersal fish. In 2018, fish capture using purse seine was dominated by bullet tuna and shortfin scad with 18,317 tons (66.08%) and 6,001 tons (21.65%), respectively (MOMAF 2018). Bullet tuna is one of the essential and potential pelagic fish resources in Prigi bay and has a valuable economic contribution to the local community.

Bullet tuna has not been massively exploited yet in several fishing areas because of its relatively small size. Nevertheless, frigate tuna species decline will increase bullet tuna exploitation (Collette et al. 2011). Bullet tuna resources on the west coast of Sumatera (Noegroho & Chodrijah 2015) and Prigi bay, East Java (Agustina & Rachman 2019) have been actively exploited. Intensive and continuous fishing could potentially endanger the sustainability of fish resources in the future. Therefore, appropriate management of bullet tuna is needed to ensure its sustainable fish stock and population. Good fisheries management should be based on the bio-ecological information of the fish; one of them is related to the food and feeding habits. Food preference can be used to determine the fish's natural nutrition and the interaction between the fish as well as its environments such as feeding habits, forms of predation, competition, and trophic level through the food chain (Effendi 2002). A few numbers of stomach contents analyses of bullet tuna were collected from different waters, namely Philippine waters, Tunisian waters, Indian waters and Makassar strait (Jasmine et al. 2013; Baeck et al. 2014; Hajjej et al. 2018; Kantun et al. 2019). These studies found that Bullet tuna commonly consume crustaceans, small fishes, and molluscs (Jasmine et al. 2013). Bullet tuna caught in Makassar Strait feed on crustaceans and cephalopods (Kantun et al. 2019), whereas on the west coast of Sumatera, the bullet tuna feed on anchovies (*Stolephorus* sp.) (Noegroho et al. 2013). Previous studies indicate that there are different types of food consumed by fish that might be controlled by food availability in the habitat and feeding behaviour. Up to now, there is still a scarcity of information on the food preferences and feeding habits of bullet tuna in the Prigi Coast. Therefore, this research aims to determine the food composition and preference of bullet tuna in Prigi Coast, Trenggalek Regency, East Java.

MATERIALS AND METHODS

In total, 294 samples of bullet tuna were collected once a month using the catch of purse seine fisherman from March up to May 2018 in Prigi Coast, Trenggalek Regency East Java (Figure 1). Each individual collected was measured in its total length (± 0.1 cm) and weighed using electric balancing (± 0.1 g). The specimens were dissected for visual inspection of their gonad for sex determination.

The digestive tract of fish is separated from the intestine and stomach. The length of the fish intestine was measured, while the fish stomach was separated and preserved using solution of formalin 4% (Berg 1979) in bottles labeled with the time of sampling. Samples of stomach contents only came from fish stomachs. Stomachs were dissected and the contents were added to the graduated test tube filled with distilled water. The volume of the sample of stomach contents was taken using a dropper and placed in Sedgwick rafter counting chamber containing a small of 2 x 2 x 1 mm^3 box. Sedgwick rafter is used to measure the volume of each food items. The larger food items were identified visually, whereas the small-sized food items were identified using dissecting microscope (Olympus CX 21, magnification 4× and 10×). The frequency of occurrence method

analyzed each type of food items found in the stomach, and the volume of each food items was analyzed by the volumetric method (Effendie 2002). The obtained data were analyzed based on the following formulas:

Relative gut length describes the type of fish food based on the ratio of the length of the intestine to the length of the fish's body. It was calculated as (relative gut length = total gut length (cm)/total length (cm)). Relative gut length is categorized as follows: carnivore < 1, omnivore 1-3, herbivore > 3

The frequency of food occurrence determined the presence of each type of feed contained in the stomach of the fish containing the food in their stomach. The frequency of occurrence was calculated as (% Oi = Ji/ p x 100),

Where:
Ji = number of fish containing food items
p = number of fish with food in their stomach

Volumetric method (% Vi), the percentage volume of the prey component i was calculated as:

$$\%V_i = \frac{\text{Number of points allocated to component } i}{\text{Total points allocated to subsample}} \times 100$$

Index of Preponderance describes the relative abundance of different organisms in the fish diet and is calculated as: (Biswas 1993)

$$IP = \frac{VixOi}{\sum_{i=1}^{n} VixOi} x100\%$$

where:
IP = index of preponderance
Vi = percentage of fish food volume type i
Oi = percentage of occurence frequency of food type i
N = number of fish food organism (i = 1, 2, 3,.....n)
Index of preponderance is classified into three categories as follows: main food = IP > 25%, complementary food = 5% ≤ IP ≥ 25%, additional food = IP < 5%.

Trophic Level describes whether the fish species is classified as carnivore, omnivore or herbivore, and calculated as:

$$Tp = 1 + \sum \left\{ \frac{Ttp x Ip}{100} \right\}$$

where:
Tp = trophic level
Ttp = trophic level of food type p
IP = index of preponderance of food type p
Trophic level is categorized as follows: trophic level 2 = herbivore; trophic level 2.5 = omnivore; trophic level 3 = carnivore.

Niche Breadth shows the adaptation of fish species to the food availability in the habitat, and is calculated as:

$$BA = \frac{1}{n-1}\left[\frac{1}{\sum Pij^2} - 1\right]$$

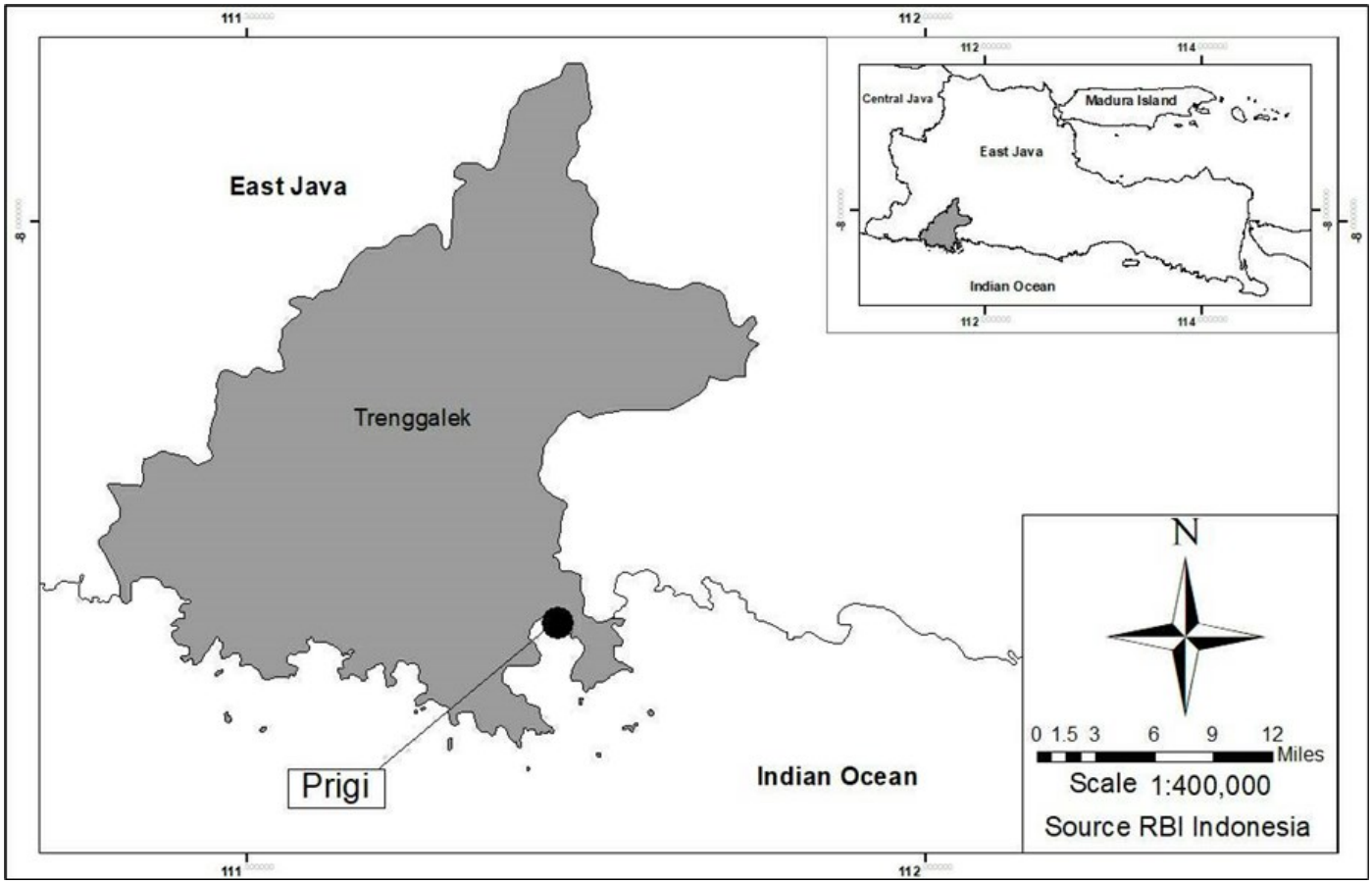

Figure 1. Landing site of bullet tuna (*Auxis rochei*) in Prigi Coast Trenggalek Regency East Java.

where:
BA = niche breadth of fish size i toward food resource j
Pij = proportion of fish size I that is related to food resource j
n = number of food of fish size (i=1, 2, 3,.....n)
Niche breadth values are considered as: high when BA > 0.6, moderate when 0.6 < BA > 0.4, low when BA < 0.4.

Food Overlapping describes multiple shared food sources, and is calculated as follows:

$$Ch = \frac{2\sum PijxPik}{\sum P^2ij + P^2ik}$$

where:
Ch = simplified Morisita Index between fish size j and size k
Pij, Pik = proportion of resource I from total resource used by fish size j and k (i = 1, 2, 3,.....n)

RESULTS AND DISCUSSION

Results

Food composition and preference of bullet tuna

The total of bullet tuna collected in Prigi Coast were 294 fishes and filled -stomach samples were 271 fishes (92.17%). Specimens of bullet tuna ranged between 17.3 - 27.4 cm in total length, which were consisted by male 52.38% and female 47.62%. Male and female fish were commonly found in the 19 - 20 cm length group. Most male fish were found in the length group of ≤ 18 cm, 23 - 24 cm, and 25 - 26 cm , while the least is ≥ 27cm. Most female fish were found in the length group of 23-24 cm and ≤ 18 cm, while ≥ 27 cm were not found.

The relative gut length of fish showed in Table 1 is used to describe the type of food fish ate. The intestine of bullet tuna is about 7 - 12 cm, with a relative gut length of about 0.37 - 0.43. Based on the relative gut length of the intestine, the bullet tuna is a carnivorous fish. The food composition of bullet tuna consists of fishes (39%), shrimps (25%), copepods (6%), molluscs (4%), annelids (1%), and debris (25%). Only fish pieces, fish spines, and fish scales were found in the fish food item. The food composition of bullet tuna in Prigi Coast showed in Figure 2.

Table 1. Gut length size of bullet tuna in Prigi Coast.

Length Size Group (cm)	Q (species)	Mean of Gut Length	Relative Gut Length
≤ 18	39	7.86	0.43
19-20	145	8.35	0.42
21-22	26	9.09	0.42
23-24	42	8.81	0.37
25-26	40	10	0.39
≥ 27	2	11.85	0.43

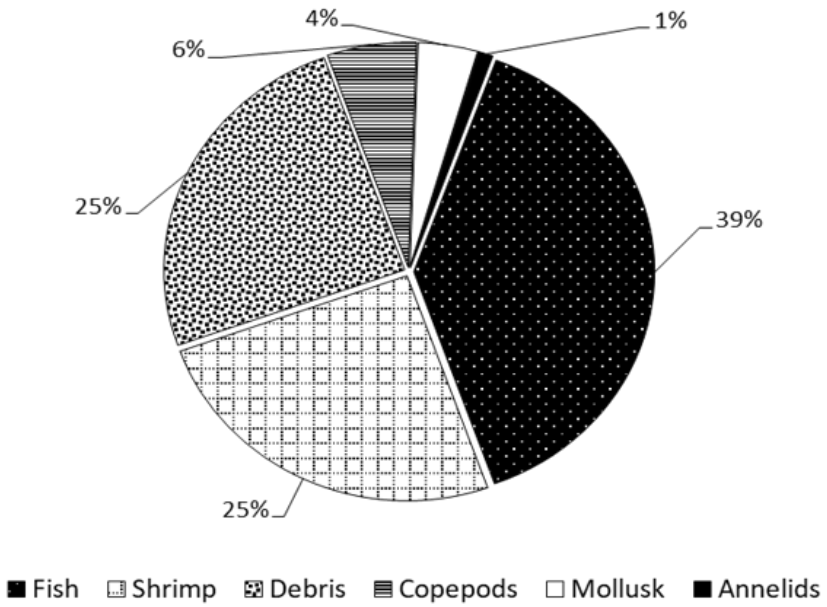

Figure 2. Food composition of bullet tuna in Prigi Coast.

The frequency of fish was mainly found in bullet tuna stomachs. The most common types of fish (87.80%) were found in male fish, while shrimp (83.33%) and fish (72.22%) were most commonly found in female fish. The frequency of occurrence of debris (53.66%) and shrimp (23.17%) is rarely found in male fish. Copepods and mollusks were also found in fish stomachs with less frequency. Annelids are most rarely found in male and female fish.

Food composition indicates bullet tuna as a carnivore. Index of preponderance (IP) shows the percentage of the greatest amount of food contained inside fish's stomach. Bullet tuna's index of preponderance based on their sex can be seen in Table 2 and 3 as follows.

Table 2. Index of preponderance (IP) of male bullet tuna (*Auxis rochei)* in Prigi Coast.

Type	IP	Category
Fish	39.66	Main food
Crustacean	14.7	Complementary food
Mollusca	0.79	Additional food
Annelids	1.58	Additional food
Copepods	3.21	Additional food
	40.05	-

Table 3. Index of preponderance (IP) of female bullet tuna (*Auxis rochei*) in Prigi Coast.

Type	IP	Category
Fish	26.51	Main food
Crustacean	29.93	Main food
Mollusca	2.68	Additional food
Annelids	0.29	Additional food
Copepods	6.58	Complementary food
Debris	40.05	-

Fish was the main food of male bullet tuna (39.66%), while crustacean was the complementary food (14.7%). A similar result was presented in female bullet tuna. The main food of female bullet tuna is fish (26.51%) and crustacean (29.93%), while copepods become the complementary food (6.58%).

Trophic level indicates the position of organisms in a food chain. In Prigi Coast, bullet tuna is categorized as a carnivore (3.6 - 3.8) (Table 4). Small to adult bullet fish do not experience any shift of food type.

Table 4. Trophic level of bullet tuna (*Auxis rochei*) in Prigi Coast.

Length (cm)	Trophic level	Description
≤ 18	3.8	Carnivore
19 - 20	3.8	Carnivore
21 - 22	3.6	Carnivore
23 - 24	3.7	Carnivore
25 - 26	3.6	Carnivore
≥ 27	3.7	Carnivore

Niche breadth indicates the selectivity of intraspecies fish from a certain size class toward the availability of food resources. Female bullet tuna has a broader range of niche breadth than the male one. Table 5 shows the detailed niche breadth of male and female bullet tuna in the Prigi Coast.

The niche breadth of male bullet tuna is ranged between 2.26 -3.28, with a range of standardization between 0.27 - 0.46. Male bullet tuna which sized ≥ 27 cm, consumes more various food than the others of smaller size. The niche breadth of female bullet tuna ranged between 2.78 - 4.63, with standardization ranging between 0.35 - 0.73. The broadest niche breadth of female bullet tuna ranged between 25 - 26 cm.

Food overlapping refers to the similarity of food type consumed by male and female bullet tuna, and also by bullet tuna of several length classes. The food overlapping of male and female bullet tuna can be seen in Table 6 and 7.

Table 5. Niche breadth of bullet tuna.

Length Size Class (cm)	Male		Female	
	Niche Breadth	Standardization	Niche Breadth	Standardization
≤ 18	2.66	0.33	2.78	0.35
19 - 20	2.35	0.27	2.84	0.37
21 - 22	3.26	0.45	3.05	0.41
23 - 24	2.82	0.36	4.05	0.61
25 - 26	2.82	0.36	4.63	0.73
≥ 27	3.28	0.46	-	-
Average	2.86	0.37	3.47	0.49

Table 6. Food overlapping of male bullet tuna (*Auxis rochei*) in Prigi Coast.

Length Class (cm)	≤ 18	19 - 20	21 - 22	23 - 24	25 - 26	≥ 27
≤ 18	1	0.97554	0.91612	0.96799	0.9788	0.94536
19 - 20		1	0.93175	0.99098	0.98122	0.91237
21 - 22			1	0.95718	0.9669	0.94046
23 - 24				1	0.98435	0.92223
25 - 26					1	0.96959
≥ 27						1

Food overlapping of male bullet tuna is ranged between 0.91612 - 1. The highest number of food overlapping was in length class 19 - 20 cm toward length class 23 - 24 cm. Thus there is a similarity of food resources consumed by bullet tuna of both length classes.

Table 7. Food overlapping of female bullet tuna (*Auxis rochei*) in Prigi Coast.

Class (cm)	≤ 18	19 - 20	21 - 22	23 - 24	25 - 26
≤ 18	1	0.91932	0.89838	0.88799	0.8462
19 - 20		1	0.99148	0.5513	0.86972
21 - 22			1	0.94833	0.89655
23 - 24				1	0.98653
25 - 26					1

Food overlapping of female bullet tuna is ranged between 0.5513 - 1. The highest number of food overlapping is indicative in length class 19 - 20 cm toward length class 21 - 22 cm. Both of these length classes consumed the same food resources.

Discussion

Bullet tuna in this study has a smaller length than in the South Tyrrhenian (Mostarda et al. 2007) and the Mediterranean (Morote et al. 2008). Lisong tuna has a total length of up to 50 cm with a length of 35 cm at the first maturity of the gonads (Fishbase 1993). The relative gut length is used to see the natural food consumed by fish. The length of the digestive tract of fish depends on the natural food consumed (Biswas 1993). Bullet tuna is categorized as a carnivorous fish based on the relative gut length. The relative gut length varies from one species to another and in the same species at different life stages.

In the present study, the bullet tuna in Prigi Coast preferred to eat fish and crustacean as main foods with additional foods ítems were copepods, molluscs, and annelids. Our results are similar to those mentioned by Hejjej et al. (2018), where *Sardinella aurita* are the most important prey species found in the majority of bullet tuna's stomachs, while crustaceans and molluscs are secondary food types. Feeding habit study of *A. thazard* by Mariyasingarayan et al. (2018) collected from Southeast coast of India reported the presence of fishes and crustacean (88% and 12%, respectively) with the dominant fish species were *Anchoviella* spp. and *Leiognathus* spp. Based on the food preference, the bullet tuna was a non-selective feeder, which mainly prefers the crustaceans, small fishes, and molluscs (Jasmine et al. 2013). Another study reported that the food type of bullet tuna in Makassar Strait consisted of unidentified food, cephalopods, crustaceans, and small fishes (Kantun et al. 2019). Bullet tuna on the west coast of Sumatera preferred the anchovies' group as the main food (Noegroho et al. 2013). As a comparison, the bullet tuna in the Southern Tyrrhenian Sea mostly consumes crustacea, fish, mollusc, polychaeta, siphonophora, chaetognatha, and urochordate (Mostarda et al. 2007), while the Indian Sea, the main food of bullet tuna is fish (Clupeidae), crustacea, dan mollusc (Kumaran 1964). Plandri et al. (2009) recorded that the dominant prey of bullet tuna from the Ligurian sea were fish and euphasiid crustaceans, while Jasmine et al. (2013) reported the common food of bullet tuna collected from Indian waters were fish, crustaceans and zooplankton. Bullet tuna in Philippines waters are epipelagic feeder that ate fish as dominant food and prey for others like crustacean, copepods, crab larvae, amphipods, and cephalopods (Baeck et al. 2014).

The food type of bullet tuna can easily change. Besides, due to the preference of a particular prey, the main food of fish seems to depend on the availability of prey in the habitat where the fish live. The prey items of *A. rochei* studied from Prigi Coast are in accordance with the feeding patterns of this species from different coastal areas. The tendency of fish to consume a certain type of food is highly influenced by several factors such as food size, color, taste, texture, and appetite, and also the availability of food in a particular sea (Effendie 2002). Bullet tuna consume prey based on their availability in the environment and geographic abundance (Baeck et al. 2014). The variation of the food type of bullet tuna is used as the optimal quality water indicator and food availability in the fishing ground of bullet tuna (Kantun et al. 2019).

The **trophic level** estimated of bullet tuna in Prigi Coast is 3.7, positioning it as a carnivore that opportunistically eats various food types and preferred ate fish and cructacean. Fatah and Adjie (2015) state that fish catching is capable of altering the spatial distribution and fish abundance, which will affect species interaction and trophic structure in general. The **trophic level** of a particular fish can be affected by life expectancy (size), gonads, ecomorphology, behavior, intraspecies and interspecies competition, as well as distribution of resources and parasites (Elliot & Hemingway 2002).

Female bullet tuna has a broader range of **niche breadth** than the male one. Therefore, female bullet tuna consumes many kinds of food. The difference in niche breadth can be caused by food availability, food abundance, and habitat of the fish. The organism that consumes several kinds of food resources, its niche breadth will increase even though the availability of food resources is low (Anakotta 2002). The niche breadth of bullet tuna is different in each class. Body length and a great variety of food do not guarantee a broad niche because niche breadth is also deter-

mined by the fish's capability of using the available resources. Fish capability to use the food variation in the water is not determined by fish length (Ariasari et al. 2018). Bullet tuna in Philippine waters has narrow niche and as a specialized feeder which dominantly ate fish as dominant prey (Baeck et al. 2014). The niche breadth of bullet tuna in Prigi Coast is ranged between 2.35 - 4.63. The total and food type consumed by a particular fish species generally depends on age, place, and time (Effendie 2002).

Food overlap reveals the occurrence of similar foods that fish consume. The highest proportion of foods overlapped by male 19 - 20 cm bullet tuna is 0.99098. The largest value of foods overlapped by female 21 - 22 cm bullet tuna is 0.99148. These findings demonstrate that certain length classes of bullet tuna have a similar type of diet. Thus there is a competition in obtaining food resources between these two length classes of female bullet tuna. Thus it induces competition among length classes. The high number of competitions is affected by the high number of similarities in using niche and the same space. In general, fish will undergo food type shifting along with the increase in fish length. It is influenced by various factors such as competition of obtaining food resources, food abundance, and tendency level toward the prey.

CONCLUSIONS

Fish and crustacean are the main food of *Auxis rochei* in Prigi Coast (39% and 25%, respectively), while the complementary and additional foods are copepod, mollusc, annelid, and debris. Bullet tuna was a carnivorous fish with the trophic level of 3.7, and shows existence of competition for food resources, especially between different fish sizes. Bullet tuna female has a wide area of consumed food resources than male.

AUTHOR CONTRIBUTION

B.P.A collecting data and analysis, A.A. analysed the data and wrote the manuscript, T.B.S. wrote the manuscript, E.S. designed the research, wrote the manuscript and supervised all the process.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Research Article

Analyses of Vegetation Used by Long-tailed Macaque (*Macaca fascicularis* Raffles 1821) in Tinjil Island

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ABSTRACT

Tinjil Island is a semi-natural breeding facility for long-tailed macaques (*Macaca fascicularis*) managed by Primate Research Center, IPB University, located at the southern of Java Island and surrounded by the Indian Ocean. Long-tailed macaques are considered frugivorous even though they are well-known for their flexible diet. This study aims to analyse the vegetation supporting the population of long-tailed macaques. Data were collected from six tracks using square sampling plots with the size of 20 m x 20 m for trees as the main plot, inside the main plot were square subplots consisting of 10 m x 10 m for poles, 5 m x 5 m for saplings, and 2 m x 2 m for seedlings. The Important Value Index (IVI) was calculated for each level of vegetation. Hanjuang (*Dracaena elliptica*) dominated the seedlings with 29.35%, followed by Kampis (*Hernandia peltata*) with 18.73%, and Kalapari (*Pongamia pinnata*) with 13.73%. Hanjuang (*Dracaena elliptica*) also dominated the saplings with 26.83%, followed by Pancal (*Syzygium antisepticum*) with 19.19%, and Laban (*Vitex pubescens*) with 12.30%. The poles were dominated by Ki Cau (*Dolichandrone spathacea*) as high as 59.28%, while Waru (*Thespesia populnea*) and Ki Ciat (*Ficus septica*) dominated at 40.47% and 36.15%, respectively. Kampis (*Hernandia peltata*) dominated the trees with 39.28%, followed by Ki Ara (*Ficus glomerata*) with 35.56%, and Ki Langir (*Dysoxylum amooroides*) with 28.70%. Species found on Tinjil Island are mostly Moraceae (9.84%) and Fabaceae (9.84%), followed by Malvaceae (8.20%), Euphorbiaceae (4.92%), Myrtaceae (4.92%), and Anacardiaceae (4.92%). The vegetation in Tinjil Island supports the livelihood of long-tailed macaques on the island because they have an abundance of food and staple food such as figs to help them fulfil the energy needed to survive and reproduce.

INTRODUCTION

Long-tailed macaques (*Macaca fascicularis* Raffles 1821) are primates from the Cercopithecoidea family and are widely distributed in Asia, including Indonesia (Hansen et al. 2022). Based on the IUCN Red List of Threat-

ened Species, long-tailed macaques are considered Endangered (EN) species because of concerns regarding excessive hunting and persecution that have led to the trend of the species' population being decreased (Eudey et al. 2021; Hansen et al. 2022). The distribution of long-tailed macaques includes Laos, Vietnam, Cambodia, Thailand, Indonesia, the Philippines, and Vietnam (Roos et al. 2014). Long-tailed macaques are known to live in a wide range of habitats, including lowland and beach forests (Matsumura 2001), such as Tinjil Island. Tinjil Island is a semi-natural breeding facility managed by the Primate Research Center IPB University, located at the southern of Java Island and surrounded by the Indian Ocean. Since its introduction in 1988, long-tailed macaques have been bred multiple times and, since then, have been harvested regularly.

Long-tailed macaques are considered frugivorous, even though they can feed on insects, young stems, mature leaves, flowers, seeds, grass, mushrooms, lichens, invertebrates, bird eggs, clay, and bark (Thierry 2007; Tsuji et al. 2013; Kassim et al. 2017). Even though long-tailed macaques are well-known for their flexible diet (Aldrich-Blake 1980; Chivers & Raemakers 1980; MacKinnon & MacKinnon 1980), the availability of food in their habitat is an essential factor for their survival. Research regarding vegetation on the island has been done multiple times in 1992 (Santoso 1996), 2001 (Fadilah 2003), and 2009 (Yusuf 2010). Vegetation structure and composition in Tinjil Island is the association of *Dysoxylum amorooides* - *Intsia amboinensis* and influences the population distribution of long-tailed macaques (Santoso 1996). Numerous amounts of species have been used by long-tailed macaques as part of their diets, such as *Antidesma montanum*, *Melanorrhoea wallichii*, and *Barringtonia asiatica* (Santoso 1996).

The current vegetation status in Tinjil Island has to be determined, and further action is needed to support the long-tailed macaque population on the island. Thus, current data regarding vegetation on the island and its potential food is needed to assess the island's current condition. The information obtained from this study will be further used by the government and Primate Research Center IPB University to manage the semi-natural breeding facility.

MATERIALS AND METHODS

Study Area

The study was conducted on Tinjil Island, Banten Province, Indonesia. Located at 6°57′44″S and 105°47′0″E, the island is surrounded by the Indian Ocean with an area of approximately 5.65 km^2. The topography of the island is mostly flat, with no extreme height difference. Comprised of beach and lowland forest, the island is mainly covered by vegetation.

Long-tailed macaque was first introduced to the island in 1988, then the island became a semi-natural breeding facility managed by Primate Research Center IPB University. To simplify the process of observing the long-tailed macaques, multiple tracks have been made throughout the island. Each track was named after the acronym of a significant person who has contributed largely to the well-being of the island.

Procedures

This study has been approved by the Animal Care and Use Committee Protocol Assessment (IPB PRC-21-E006). The observation of vegetation was conducted on the tracks on Tinjil Island. Due to extreme obstacles and time-constraint, only six tracks could be observed (DS, HW, JK, RK, OS, and KO). Data were collected using square sampling plots (Figure 1) with the size of 20 m x 20 m for trees as the main plot, inside the main plot were square subplots consisting of 10 m x 10 m for poles, 5 m x 5 m

for saplings, and 2 m x 2 m for seedlings.

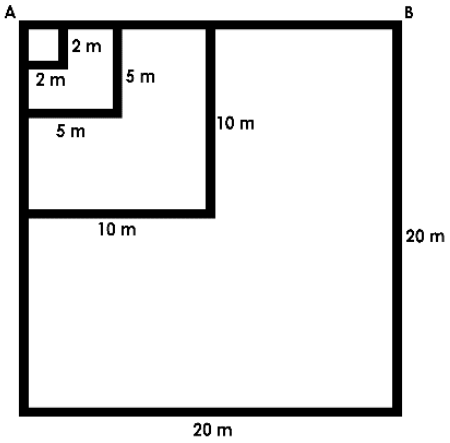

Figure 1. Square sampling plot of vegetation analysis, where A is the initial point, and A-B is parallel to the tracks.

Three main plots were placed on each track, resulting in 18 plots in total (Figure 2). The vegetation was classified into four levels: 1) Tree, woody species with a diameter above 20 cm, 2) Poles, woody species with a diameter between 10 cm-20 cm, 3) Saplings, small trees taller than 1.5 m and diameter less than 10 cm, and 4) Seedlings, small trees with height less than 1.5 m. The vegetation parameters, such as species, number of species, number of individuals per species, and the diameter of trees and poles, were collected. The name of the species was determined by identifying the characteristics of the vegetation and the local name, and then we cross-checked them using literature. Tree and pole heights were measured using a haga hypsometer. The crown width of trees and poles was measured with a meter tape using vertical crown projection to ground level. Tree and pole positions were written down by referring to the X and Y-axis of the main plot pointing to the same azimuth. Based on the notes taken on the field, the final forest profile diagram was constructed to scale. Observation of the environment and special findings are written when needed to be used as additional observation data.

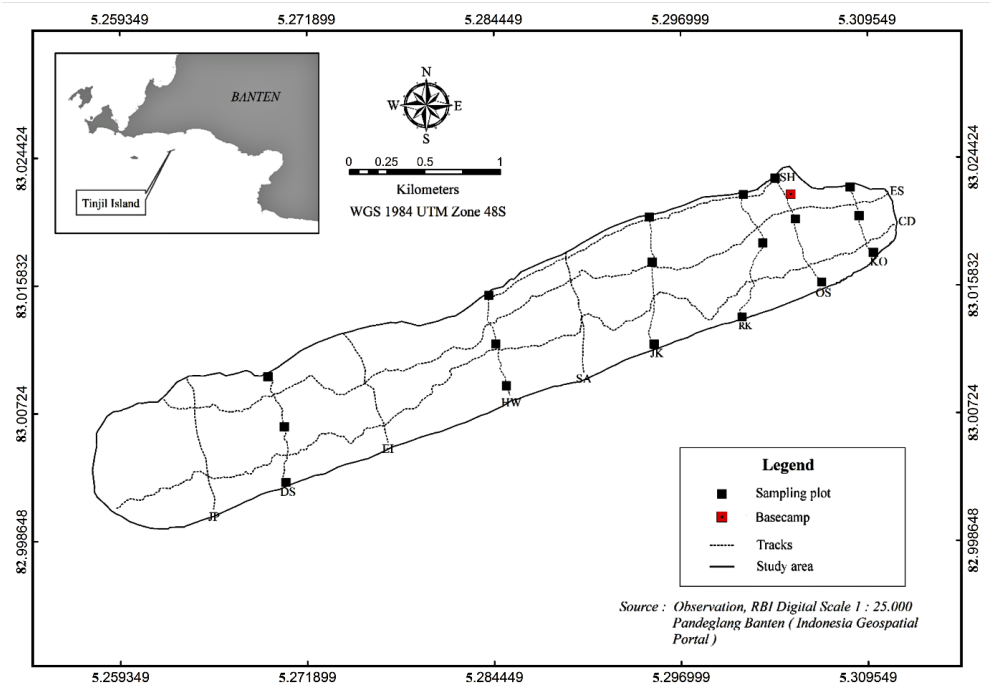

Figure 2. Sampling plot locations for vegetation analysis.

Data Analysis

Vegetation analysis was done to identify the composition of species on Tinjil Island. The dominancy of species was calculated using the Important Value Index (IVI). The following are the equations used to acquire the data (Kusmana 2017):

Species Density = Number of individuals per species/Area of plot
Relative Density = (Species Density/Total Species Density) x 100%
Species Frequency = Number of plots where the species was found/ Total number of plots
Relative Frequency = (Species Frequency/Total Species Frequency) x 100%
Species Dominance = Basal area of a species/Area of plot
Relative Dominance = (Species Dominance/Total Species Dominance) x 100%
Important Value Index (IVI) for trees and poles = Relative Density + Relative Frequency + Relative Dominance
Important Value Index (IVI) for seedlings and saplings = Relative Density + Relative Frequency

RESULTS AND DISCUSSION

Vegetation Composition

The Important Value Index (IVI) for each level of vegetation was calculated, resulting in different percentages of IVI. Hanjuang (*Dracaena elliptica*) dominated the seedlings with 29.35%, followed by Kampis (*Hernandia peltata*) with 18.73% and Kalapari (*Pongamia pinnata*) with 13.73%. Hanjuang (*Dracaena elliptica*) also dominated the saplings with 26.83%, followed by Pancal (*Syzygium antisepticum*) with 19.19% and Laban (*Vitex pubescens*) with 12.30%. The poles were dominated by Ki Cau (*Dolichandrone spathacea*) as high as 59.28%, while Waru (*Thespesia populnea*) and Ki Ciat (*Ficus septica*) dominated at 40.47% and 36.15%, respectively. Kampis (*Hernandia peltata*) dominated the trees with 39.28%, followed by Ki Ara (*Ficus glomerata*) with 35.56% and Ki Langir (*Dysoxylum amooroides*) with 28.70%.

The previous study conducted by Fadilah (2003) in 2001 concluded that there were no significant differences in vegetation dominancy for seedlings, saplings, and poles from 1991 until 2001, while there was a difference in vegetation dominancy at tree level from 1991 until 2001. Seventy-two species were found in 2001 (Fadilah 2003), while Santoso (1996) stated that the structure and composition of vegetation on Tinjil Island is an association of *Dysoxylum amooroides-Intsia amboinensis* (Table 1). Based on both previous studies, there is a significant difference in vegetation dominancy with the result obtained from this study.

The factor that may influence the result is the time difference of each study. The contributing factor that may influence the difference in results, from year 1996, 2001, and 2021 besides the time difference is 1) The survey area of each research may be different because there's no permanent area in which an inter-yearly study is conducted to observe the vegetation composition difference each year, 2) The skill of the researchers in every research is different and no training has been done before to make sure that the standard skillsets needed to conduct this research successfully is acquired by every person involved in the research, 3) The succession of forest in Tinjil Island that is influenced by many elements, such as weather, soil, and seed dispersal by wild animals living in the island. Since the interval from the previous study is 20 years, dynamism in vegetation is inevitable. Succession contributes to the change and promotes the progressive dominance of the most competitive species (Loidi

Table 1. Vegetation dominancy based on previous research.

Vegetation Level	2001*		1992**	
	Vegetation Level	IVI (%)	Vegetation Level	IVI (%)
Seedlings	*Piper retrofractum*	39.47	*Piper retrofractum*	37.85
	Dracaena elliptica	38.34	*Dracaena elliptica*	29.04
Saplings	*Antidesma montanum*	21.26	*Antidesma montanum*	35.24
			Alphania montana	20.63
Poles	Bamboo	27.91	*Gnetum gnemon*	38.13
	Gnetum gnemon	27.30	*Dysoxylum amooroides*	24.43
Trees	Cotton	30.14	*Dysoxylum amooroides*	49.32
			Intsia amboinensis	37.15
			Ficus glomerata	28.46

* Fadilah 2003; ** Santoso 1996

2017).

Potential Food

The number of species found on Tinjil Island (Table 2) is 61 species, comprised of mostly Moraceae (9.84%) and Fabaceae (9.84%), followed by Malvaceae (8.20%), Euphorbiaceae (4.92%), Myrtaceae (4.92%), and Anacardiaceae (4.92%). Moraceae is known to be the family of figs, breadfruit, and mulberry (Naira et al. 2013), and most of the species found in the Moraceae family were figs. Figs are considered keystone species because of their important role in frugivorous tropical vertebrates, especially primates and birds (Kinnaird & O'Brien 2005). The availability of figs becomes essential when there are limited fruits available because they provide enough energy for frugivores (Foster 1982; Leighton & Leighton 1983; Terborgh 1983; Lambert 1991; Lambert & Marshall 1991). The fact that Tinjil Island is 5.65 km^2 and is surrounded by the Indian Ocean means that there is a limited foraging area for the long-tailed macaques. The presence of figs can provide the energy for long-tailed macaques to survive when other fruits are scarce.

Fabaceae is the third-largest land plant family, with 730 genera and over 19,400 species (Rahman & Parvin 2015). Both families have vital roles in the diet of primates, where Moraceae was mainly consumed as fruit and Fabaceae as non-fruit parts (Lim et al. 2021), which shows the diversity of plant species that could provide necessary nutrients for the long-tailed macaques, which could sustain the livelihood of long-tailed macaques. Macaques are known to be able to survive anywhere and can exploit a variety of food sources (Karuppannan et al. 2014). The ability of long-tailed macaques to adapt and the availability of food resources can support the chance for population increase in the future.

Studies regarding potential food for long-tailed macaques in Tinjil Island were conducted in 1992 (Santoso 1996) and 2001 (Fadilah 2003). Based on previous studies, the number of species that are considered potential food for long-tailed macaques is presented in Table 3. Twenty-three species are considered as potential food where fruits, leaves, and flowers are the potentially eaten parts of the plant. Long-tailed macaques in Telaga Warna showed similar preferences where they consumed flowers, stems, fruits, and seeds (Nila et al. 2014), while long-tailed macaques in Cikakak Monkey Park ate plant parts such as leaves, fruits, seeds, and flowers (Hadi et al. 2007).

Vegetation Density

The diameter breast high (DBH) of poles and trees ranges from 0.11 m to 2.04 m while the height ranges from 1-50 m (Figure 3). The spatial area

Table 2. Plant species found on Tinjil Island.

No.	Family	Species	Local Name
1	Amaryllidaceae	*Crinum asiaticum* L.	Bakung
2	Anacardiaceae	*Gluta renghas* L.	Renghas
3	Anacardiaceae	Undetermined	Renghas Kuning
4	Anacardiaceae	*Semecarpus heterophylla* Blume	Renghas Putih
5	Apocynaceae	*Cerbera manghas* Boiteau, Pierre L	Bintaro
6	Apocynaceae	*Alstonia angustiloba* Miq.	Lame
7	Araceae	*Cocos nucifera* Linn.	Kelapa
8	Asparagaceae	*Dracaena elliptica* Thunb. & Dalm.	Hanjuang
9	Bignoniaceae	*Dolichandrone spathacea* (L.f.) Seem.	Ki Cau
10	Boraginaceae	*Heliotropium arboreum* (Blanco) Mabb.	Babakoan
11	Calophyllaceae	*Calophyllum inophyllum* Linn.	Nyamplung
12	Clusiaceae	*Gracinia celebica* Linn.	Manggu
13	Combretaceae	*Terminalia catappa* L.	Ketapang
14	Commelinaceae	*Commelina oblique* Ham.	Ki Sepet
15	Dilleniaceae	*Dillenia indica*	Simpeureum
16	Dipterocarpaceae	*Shorea javanica*	Meranti Putih
17	Euphorbiaceae	*Bridelia glauca* Blume.	Ki Hoe
18	Euphorbiaceae	*Macaranga tanarius*L.	Mara
19	Euphorbiaceae	*Drypetes sumatrana*	Taritih
20	Fabaceae	*Pongamia pinnata* (L.) Pierre.	Kalapari
21	Fabaceae	*Millettia sericea* (Vent.)Benth.	Kawao
22	Fabaceae	*Cynometra ramiflora* L.	Ki Batok
23	Fabaceae	*Leucaena leucocephala* (Lam.) de W.	Lamtoro
24	Fabaceae	*Intsia bijuga* (Colebr.) O.K.	Merbau
25	Fabaceae	*Albizia chinensis* (Osbeck.) Merr.	Sengon
26	Gnetaceae	*Gnetum gnemon* Linn.	Melinjo
27	Hernandiaceae	*Hernandia peltata* Meisn.	Kampis
28	Lamiaceae	*Vitex pubescens* Vahl.	Laban
29	Lauraceae	*Litsea cordata* (Jack) Hook.f.	Huru
30	Lauraceae	*Cinnamomum iners* Reinw. Ex Bl.	Ki Teja
31	Lecythidaceae	*Barringtonia asiatica* (L.) Kurz.	Butun
32	Lecythidaceae	*Barringtonia macrocarpa* Hassk.	Songgom
33	Malvaceae	*Pterospermum javanicum* Jungh.	Bayur
34	Malvaceae	*Heritiera littoralis* Aiton	Carlang
35	Malvaceae	*Microcos tomentosa* Sm.	Darewak
36	Malvaceae	*Ceiba pentandra* L.Gaertn.	Kapas
37	Malvaceae	*Thespesia populnea* L.	Waru
38	Marantaceae	*Donax canniformis* K.Schum.	Bangban (Bemban)
39	Melastomataceae	*Pternandra azurea* (DC.) Burkill.	Ki Besi/Tulang
40	Meliaceae	*Dysoxylum amooroides* Miq.	Ki Langir
41	Meliaceae	*Swietenia macrophylla* King.	Mahoni
42	Moraceae	*Ficus hispida* L.f.	Bisoro
43	Moraceae	*Ficus glomerata* Roxb.	Ki Ara
44	Moraceae	*Ficus septica* Burm. F.	Ki Ciat
45	Moraceae	*Ficus ampelas* Burm. F.	Ki Hampelas
46	Moraceae	*Ficus variegata* Blume.	Kopeng
47	Moraceae	*Artocarpus elasticus* Reinw. ex Blume.	Teureub
48	Myrtaceae	*Eugenia cymosa* Lamk.	Jambu Kopo
49	Myrtaceae	*Eugenia* sp.	Jambu Lalai
50	Myrtaceae	*Syzygium antisepticum* (Blume.) Merr.	Pancal
51	Pandanaceae	*Pandanus odorifer* (Forssk.) Kuntze.	Pandan laut
52	Phyllanthaceae	*Bischofia javanica* Blume.	Gadog
53	Phyllanthaceae	*Antidesma montanum* Blume.	Peuris
54	Primulaceae	*Ardisia humilis* Vahl.	Lampeni
55	Rubiaceae	*Morinda citrifolia* L.	Mengkudu
56	Rubiaceae	*Randia patula* (Horsf. ex Schult.)Miq.	Wareng
57	Sapindaceae	*Lepisanthes tetraphylla* (Vahl.) Radik.	Ki Lalayu
58	Sapotaceae	*Manilkara kauki* (L.) Dubard.	Sawo Kecik

Table 2. Contd.

No.	Family	Species	Local Name
59	Undetermined	Undetermined	Pangku
60	Undetermined	Undetermined	Paranje
61	Undetermined	Undetermined	Pitako

Table 3. Potential food and eaten parts.

No.	Family	Species	Local Name	Potentially Eaten Parts
1	Asparagaceae	*Dracaena elliptica* Thunb. & Dalm.	Hanjuang	Leaves, fruit
2	Bignoniaceae	*Dolichandrone spathacea* (L.f.) Seem.	Ki Cau	Fruit
3	Combretaceae	*Terminalia catappa* L.	Ketapang	Leaves, fruit
4	Fabaceae	*Pongamia pinnata* (L.) Pierre.	Kalapari	Fruit
5	Fabaceae	*Intsia bijuga* (Colebr.) O.K.	Merbau	Fruit
6	Gnetaceae	*Gnetum gnemon* Linn.	Melinjo	Leaves, fruit
7	Hernandiaceae	*Hernandia peltata* Meisn.	Kampis	Fruit
8	Lecythidaceae	*Barringtonia asiatica* (L.) Kurz.	Butun	Leaves
9	Lecythidaceae	*Barringtonia macrocarpa* Hassk.	Songgom	Fruit
10	Malvaceae	*Pterospermum javanicum* Jungh.	Bayur	Fruit
11	Malvaceae	*Thespesia populnea* L.	Waru	Leaves, flowers
12	Meliaceae	*Dysoxylum amooroides* Miq.	Ki Langir	Fruit
13	Moraceae	*Ficus hispida* L.f.	Bisoro	Fruit, leaves
14	Moraceae	*Ficus glomerata* Roxb.	Ki Ara	Fruit, leaves
15	Moraceae	*Ficus septica* Burm. F.	Ki Ciat	Leaves, fruit
16	Moraceae	*Ficus ampelas* Burm. F.	Ki Hampelas	Leaves, fruit
17	Moraceae	*Ficus variegata* Blume.	Kopeng	Fruit, leaves
18	Myrtaceae	*Eugenia cymosa* Lamk.	Jambu Kopo	Leaves, fruit
19	Myrtaceae	*Eugenia* sp.	Jambu Lalai	Fruit, leaves
20	Phyllanthaceae	*Antidesma montanum* Blume.	Peuris	Fruit
21	Primulaceae	*Ardisia humilis* Vahl.	Lampeni	Leaves
22	Rubiaceae	*Morinda citrifolia* L.	Mengkudu	Fruit
23	Sapotaceae	*Manilkara kauki* (L.) Dubard.	Sawo Kecik	Fruit

of trees usually used by long-tailed macaques on the island is between 4-20 m (Santoso 1996). The forest profile diagram was sketched roughly, showing that the vegetation is quite dense. The density of poles for some species from Santoso (1996) was 20.83 plants/ha (*Antidesma montanum*), 16.67 plants/ha (*Ficus ampelas*), 12.50 plants/ha (*Terminalia catappa*), 12.50 plants/ha (*Ficus variegata*), 8.33 plants/ha (*Ficus glomerata*), and 4.17 plants/ha (*Barringtonia asiatica*). The density of trees in 1992 (Santoso 1996) for some species were 9.38 plants/ha (*Ficus glomerata*), 3.13 plants/ha (*Ficus variegata*), 3.13 plants/ha (*Terminalia catappa*), 3.13 plants/ha (*Barringtonia macrocarpa*), 2.08 plants/ha (*Ficus ampelas*), and 1.04 plants/ha (*Barringtonia asiatica* and *Eugenia cymosa*).

Additional Observation

Besides the four levels of vegetation mentioned earlier, there were other plants found on the island that weren't included in the calculation. Those plants were pandan laut (*Pandanus odorifer* (Forssk.) Kuntze.), bakung (*Crinum asiaticum* L.), and kelapa (*Cocos nucifera* Linn.). Ninety-seven *Pandanus odorifer* were found during the observation, while 64 individuals of *Crinum asiaticum* were found and only one individual of *Cocos nucifera* was found during the sampling. Those plants were found near the beach. Goeltenboth et al. (2006) stated that the species diversity in beach forests is usually low with only scarce representatives of conifers, lianas, and plants showing cauliflory, with abundant Pandanus sp. as well as epiphytes. *Cocos nucifera* develops a fruit or nut that can withstand seawater

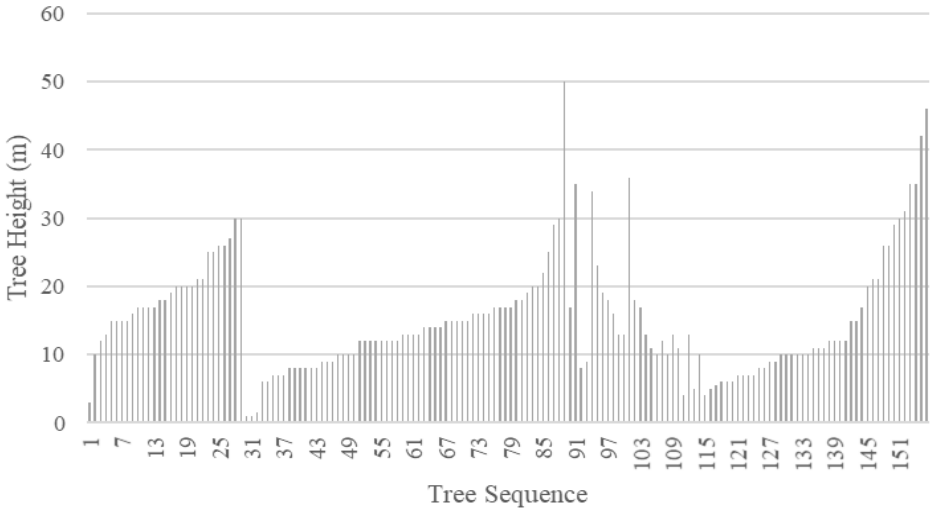

Figure 3. Tree height in meters (Y-axis) and tree sequence based on the observation (X-axis).

for months (Goeltenboth et al. 2006) which explains why they are only found on the beach and not in the lowland forest. Based on the information obtained from the local guides, long-tailed macaques have been spotted consuming young *Pandanus odorifer* when other food might seem unavailable. Long-tailed macaques also drink water from the tips of the plants, where water from the rain was unintentionally collected by the plants. The flexible drinking behavior of long-tailed macaques has been spotted in the Northeast of Thailand (Schurer et al. 2019), in Malaysia (Hambali et al. 2012; Hassim et al. 2018), and in Indonesia (Nila et al. 2014).

CONCLUSION

Tinjil Island can be considered an isolated location for long-tailed macaques where the potential for them to naturally move outside the island is almost impossible, implicating the limited foraging area for the long-tailed macaques. Based on the results of this research, the presence of figs can provide the energy for long-tailed macaques to survive when other fruits are scarce. The result shows the diversity of plant species in Tinjil Island that could provide necessary nutrients for the long-tailed macaques, which could sustain their livelihood by supporting their ability to survive and reproduce. Considering that macaques can survive anywhere and exploit various food sources, the ability of long-tailed macaques to adapt and the availability of food resources can support the chance for population increase in the future. Future studies regarding the population and habitat use of long-tailed macaques in Tinjil island might be necessary as part of a bi-annual monitoring plan between the stakeholders involved in the maintenance of the island.

AUTHORS CONTRIBUTION

D.P. reviewed the manuscript, H.I.S. collected and analysed the data and wrote the manuscript, S.T. collected the data, T.LA. created the map, E.I. reviewed the manuscript, H.S.D. supported the research.

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CONFLICT OF INTEREST

There's no conflict of interest regarding the research or the research funding.

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Research Article

Forest Structure and Tree Species Diversity of the Abasumba Globally Significant Biodiversity Area, Ghana

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ABSTRACT
We studied the forest structure and tree species diversity with diameter-at-breast-height (dbh) ≥ 10 cm in the Abasumba Globally Significant Biodiversity Area Ghana. Sixteen 25 m * 25 m plots were demarcated and trees with dbh ≥ 10 cm were inventoried following International Plant Nomenclature Index. The characteristic three–storey structure of tropical forests was shown, 68.7% of trees were in the lower 4.5–18 m and middle 18–30 m storeys. A majority 91.4% of 342 trees was in the dbh of 10–30 cm and a least 8.6% of 32 trees in 31–60 cm had dbh > 60 cm. Total of 46 species, 38 genera and 17 families, with mean Alpha, Shannon and Simpson's Diversity indices of 13.9, 1.44 and 0.07 and importance value index of 300.0 for 374 trees ha^{-1} was recorded. Plant families Sterculiaceae, Meliaceae, Leguminosae, Ulmaceae and Bombacaceae was the majority encountered while *Triplochiton* scleroxylon, *Cola millenii*, *Trichilia monadelpha*, *Hymenostegia afzelii*, *Celtis* mildbraedii, *Ceiba pentandra* and *Ficus sur* was the most occurring species in 54.0% of the plots accounting for 52.0% of the IVI for all trees. *Blighia sapida*, *Bridelia grandis*, *Dialium guineense*, *Draceana arborea*, *Ficus sur*, *Holarrhena floribunda*, *Holoptelea grandis*, *Margaritaria discoidea*, *Rauvolfia vomitoria*, *Trilepisium madagascariense*, *Vitex ferruginea*, *Ximenia americana* and *Xylia evansii* had one individual in the 10,000 m^2 area indicated that they are rare and should be given conservation priority in the forest reserve.

INTRODUCTION
Dry tropical forests are the utmost vulnerable of most dry forest ecosystems and they are contended to warrant significance for protection because they are fragments of formerly extended ecosystems which are now nonexistent (Ocón et al. 2021). They hold varied and exceptional biological communities which their logging backs the loss of the planets biodiversity (Cabin et al. 2000; DryFlor et al. 2016). A large part of these tropical forest ecosystems are found in Africa and other dry islands, accounting for ca. 40–70% forested areas in the world (Riggio et al. 2020). Presently, few intact dry forests exist which offer the ecological services required to aid millions of sustenance farmers in the world's deprived communities, with most of them been disturbed or fragmented through human activities as they can easily be cleared with fire for new grazing grounds or converted into agriculture land use (Jacobson et al. 2019; Vogt et al. 2019; Siyum 2020). Population growth, urbanization and food

production are the lead in the need for land giving rise to a greater logging within dry forests than in moist forests (FAO 2020).

The predominant view is that Ghana's forest ecosystem and its biodiversity are susceptible to anthropogenic disturbances viz. encroaching agriculture, and mining is one of the highest deforestation rates in the West African sub-region (Owusu et al. 2018; Bentsi-Enchill et al. 2022). Currently, annual logging at 0.70%, 0.50%, 0.40%, and 0.60% for the periods of 1990–2000, 2000–2005, 2005–2010 and 2010–2015 were reported within Ghana (Keenan et al. 2015; Acheampong et al. 2019). Logging within forest reserves has increased substantially, with regards to exhaustion of lands, Hawthorne & Musah (1993) previously conveyed that close to half 50.0% of conserved forestry were depleted or in bad state and with other current studies confirming similar trends (Acheampong et al. 2019; Brobbey et al. 2020).

According to Hall & Swaine (1981) in Ghana, the southern marginal dry forest is amongst the greatest threatened ecologies. It is classified amongst the driest forest ecosystems which occur as small-scattered patches about (ca. 20 km^2) characterized with low floral diversity and tree canopies, few commercial timber species with an annual rainfall of 750-1275 mm (Asase & Adeniyi 2021). In Ghana, there are two types of southern marginal dry forests, but the one located in the Cape Coast-Winneba area within the coastal savanna ecosystem is the most susceptible (Asase & Adeniyi 2021).

Abasumba Globally Significant Biodiversity Area (GSBA) is one of the outstanding remains of the Awutu Forest classified as southern marginal forest zone (Hall & Swaine 1981). It is an important GSBA for the conservation of plant biodiversity as it represents rare stands of forest which serve as home to forest-based species with special biological interest (Hawthorne & Abu-Juam 1995). However, there are no existing data on the diversity of *flora* or *fauna* in the Abasumba, GSBA after several years of demarcation as a forest reserve. Understanding the forest structure, plant species diversity and forms of tree distribution can offer the foundation for effective protection and superintendence of the biodiversity of the forest reserve (Asase & Adeniyi 2021; Thammanu et al. 2021). Knowledge about forest structure and plant biodiversity is important for conservation purposes, also the plants offer resources and serve as homes of other *fauna* (Huang et al. 2003; Lelli et al. 2019). Apart from the national forest inventory of economic timber species in the dry forests by the Forestry Commission of Ghana (Ghartey 1989), there is no other former research on the tree species diversity of the Abasumba, GSBA. The goal of the research was to (i) identify as well as enumerate all tree species at dbh ≥ 10 cm distribution across tree stands; (ii) analyze floristic constitution and tree species biodiversity; and (iii) determine tree species dispersion patterns, basal area distributions and importance value index (IVI) of the Abasumba, GSBA. The results of this research are expected to facilitate decisions regarding choice of species in afforestation programs for the restoration of the southern marginal dry forests in Ghana.

MATERIALS AND METHODS

Study Location

Abasumba GSBA is a portion of Southern Dry Forest Zone, in the Awutu –Effutu–Senya political district and it was first reserved as a protected area in 1927 (Baker & Willis 2014). It is situated between Cape Coast and Winneba Forest districts in Central Region, Ghana and lies approximately at Latitudes 5°37'37" and 5°38'38" N and Longitudes 0°31'32"and 0° 32'33" W (Figure 1). The altitude ranges between 160 and 200 m with

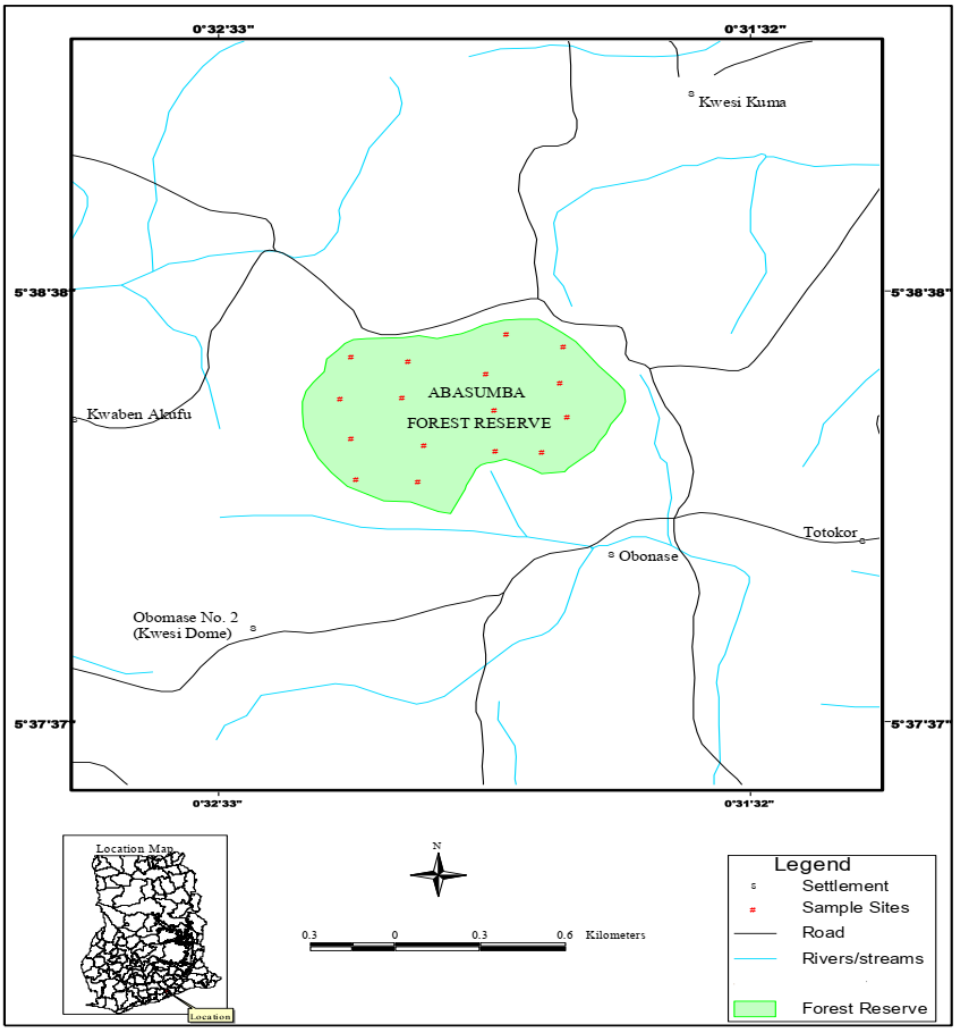

Figure 1. Topographical Map of Abasumba GSBA showing sampling sites.

isolated hills, reaching about 300 m above sea level, with a land area of 1.5 km² and a perimeter of about 3.48 km. The relief is generally hilly, undulating landform interrupted by gorges and valleys; the parent rock is quartzite/shales and granite. Rainfall pattern is even with two peaks; a major in May to June, and a minor in October, however, rainy seasons are preceded by two dry seasons occurring in November to February and August to September, respectively.

Data Collection

After the initial investigation, the forest reserve was divided into four compartments based on the cardinal points, South–West (Compartment 1), North–West (Compartment 2), North–East (compartment 3) and South–East (Compartment 4). Four sampling sites of (25 m * 25 m) 0.0625 hectares were randomly delineated within each compartment with the aid of Garmin's GPS 12 Personal Navigator. The choice of (25 m * 25 m) 0.0625 ha plots was based on successful usage in the sampling of vegetation types in Ghana so that this study would be comparable. The number of quadrats required to assess the biodiversity were determined using the species area curve (Sahu et al. 2007). To study the state of the forest and tree species variety of Abasumba, GSBA all trees within the sites were catalogued viz. species name, elevation, and diameter at breast height dbh ≥ 10 cm (1.3 meters). Tree species having many trunks were taken as one, as long as there was a visible linkage amongst the trunks and with the thickest trunk measured. All living trees were identified us-

ing tree spotters based on Flora of West Tropical Africa 1927 (Hutchinson & Dalziel 1927), Field guide of the forest trees of Ghana (Hawthorne 1990), Woody plants of Western African forests (Hawthorne & Jongkind 2006) and Photo guide for the Forest Trees of Ghana (Hawthorne 2006), all naming were based on the International Plant Nomenclature index (IPNI 2008).

Data analyses

The obtained forest information was evaluated for tree species diversity indices, basal area, relative density, relative frequency and relative dominance (Phillips 1959). Importance value index (IVI) for each tree was calculated as the sum of the relative frequency, relative density and relative dominance (Cottam & Curtis 1956). Local names were documented and evidence about the local uses of the trees identified was attained by accessing relevant literature (Hawthorne 2006).

Formulae used include:
Tree species variety of the Abasumba, GSBA was quantified using Shannon and Weiner indices, (Shannon & Weiner 1963),

$$H' = -\sum \left[\left(\frac{ni}{N}\right) log2 \left(\frac{ni}{N}\right)\right]$$

Simpson's (*D*) index of diversity (Simpson 1949),

$$D = \sum \left(\frac{ni(ni-1)}{N[N-1]}\right)$$

was calculated for each compartment and the complete Abasumba GSBA.
Where:
(*ni*) = Total number of individuals of species
(*i* and *N*) = Total number of all individual species within a vegetation.
Basal area (m^2) = Area occupied at breast height (1.3 m) = $[p * (dbh/2)^2]$
Where dbh = Diameter of a tree at Breast Height, approximately 1.3 m above ground level
Relative density = (Density of the species/Total density of all species) *100
Relative frequency = (Frequency of the species/Total frequency of all species) *100
Relative dominance = (Basal area of the species/Total basal area for all species) *100
Importance Value Index (IVI) = Sum of relative density + Relative frequency + Relative dominance.
Spatial distribution patterns of individual trees were determined using Biodiversity Professional (2022), Version 2.0 software. According to Kershaw (Green 1974), entities are haphazardly dispersed once their mean of variance ratio is 1 but gathered if the ratio is bigger than 1, but if the ratio is less than 1, then the entities are regularly dispersed.

RESULTS AND DISCUSSION

Results

Forest structure

The dbh measurement for trees indicated that majority of trees 62.0%, were small stemmed in dbh 10–30 cm. These sizes were recorded for 54.3% of the trees identified while, 29.4% exhibited dbh 31–60 cm but, 8.6% obtained dbh above 60 cm. *Ceiba pentandra* and *Triplochiton scleroxylon* trees represented the highest dbh of 136 and 134 cm respectively while the other species had dbh values below 120 cm Table 1.

Table 1. Tree species (dbh ≥ 10 cm) class interval and distribution in Abasumba GSBA.

DBH Class (Cm)	No. of Species	% of Species	No. of Individuals	% of Individuals
10 – 30	25	54.3	232	62.0
31 – 60	13	28.3	110	29.4
61 – 90	4	8.7	17	4.6
91 – 120	2	4.4	10	2.7
121 – 150	2	4.4	5	1.3
Total	**46**	**100.0**	**374**	**100.0**

Tree height distribution

The distribution of trees by height intervals revealed 34.2% of individual trees were in the heights of 15–20 m; 10–15 m forming 21.7% and 20–25 m forming 12.8% making up the second and third major groups respectively. Tree heights in the ranges of 30–35 m and 25–30 m constituted 10.4% and 9.9% respectively. The mean elevation of trees stood at 19.0 m in the elevation of 5–40 m as shown in Figure 2. There were few trees in the height range of 5–10 m and 35–40 m making up only 6.2% and 4.0% respectively, while trees with height ≥ 40 m constituted only 0.9% (Figure 2). The tallest individual trees in the Abasumba, GSBA were *Triplochiton scleroxylon* 48.3 m, *Celtis mildbraedii* 42.5 m, *Ceiba pentandra* 35.1 m and *Nesogordonia papaverifera* 32.6 m.

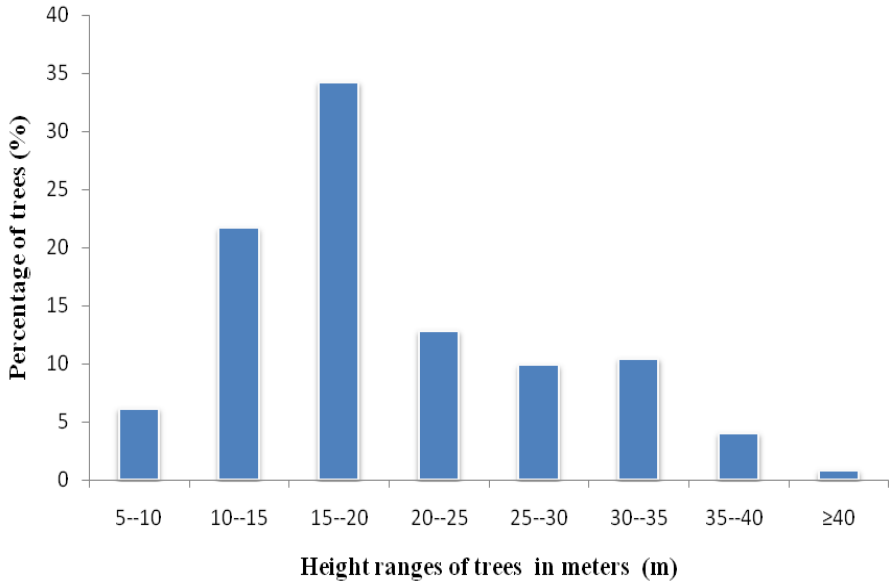

Figure 2. Height class wise distrubution of tree species in the Abasumba GSBA.

Species Diversity indices

Species diversity for the sampled compartments was determined Table 2. The highest Alpha species diversity 20.70 and Shannon diversity 1.56 indices were recorded in compartment 3, whiles compartment 1 had the highest Simpson index of 0.11. But, least value of Shannon 1.32 and Simpson 0.07 indices were in compartments 1 and 3, respectively. The Alpha diversity index were 8.70 to 20.70, Shannon diversity of 1.32 to 1.56 and Simpson's index of dominance measure were 0.04 to 0.11. The mean of Alpha, Shannon and Simpson's Diversity indices for the total Abasumba, GSBA was 13.9, 1.44 and 0.07 respectively.

Table 2. Species diversity indices of the sampled sites in Abasumba GSBA.

Diversity Indices	Compartment 1 (South–West)	Compartment 2 (North–West)	Compartment 3 (North–East)	Compartment 4 (South–East)	Mean
Alpha (α)	8.70	14.00	20.70	12.00	13.9
Shannon (H')	1.32	1.48	1.56	1.40	1.44
Simpson's (D)	0.11	0.06	0.04	0.05	0.07

Floristics composition

In total, 46 trees having dbh ≥ 10 cm within 38 genera and 17 families were recognized and enumerated in our recent research. The commonly encountered families were Sterculiaceae, Leguminosae and Moraceae, the remaining families were few between 1 to 2 species. Species belonging to Sterculiaceae which frequently occurred viz. *Cola millenii*, *Triplochiton scleroxylon*, *Nesogordonia papaverifera*, *Cola gigantea*, *Mansonia altissima*, *Hildegardia barteri*. Leguminosae, *Albizia adianthifolia*, *Albizia zygia*, *Baphia nitida*, *Dialium guineense*, *Erythrina senegalensis*, *Hymenostegia afzelii* and Moraceae, *Antiaris toxicaria*, *Ficus sur*, *Morus mesozygia* and *Trilepisium madagascariense* Table 3.

Basal area, Tree species dispersion patterns and Importance value index (IVI)

Generally, basal area was 2–35.3 m^2 ha^{-1} and a total stem density of 374 stems ha^{-1}. Basal area value per species was 2.0 m^2 ha^{-1} for *Dacryodes klaineana*, *Trichilia megalantha* and *Ficus sur* to 35.3 m^2 ha^{-1} for *Albizia zygia*, whiles the relative density varied from 0.3 in *Draceana perrottetii* to 12.7 in *Cola millenii*, respectively. Total tree species varied from 1 to 46 individuals, 38 genera and 17 families. Species dispersion patterns showed 8 (17.4%) exhibited clustering, while 38 (82.6%) were randomly dispersed with no uniform dispersion. IVI is vital for comparing biological importance of trees within forests, as it shows the extent of dominance of species within forest stands (Asigbaase et al. 2019). Trees which have the highest importance value indices are the foremost dominant (Kacholi 2019). The dominant trees were *Triplochiton scleroxylon*-37.6, *Cola millenii*-28.9, *Trichilia monadelpha*-28.1, *Hymenostegia afzelii*-26.3, *Celtis* mildbraedi-23.9 and *Ceiba pentandra*-15.6, respectively. Conversely, the least dominant trees were *Ficus sur*-0.6, *Vitex ferruginea*-0.6, *Ximenia americana*-0.6 and *Draceana perrottetii*-0.6. Tree families Sterculiaceae, Meliaceae and Leguminosae were the most dominant and *Triplochiton scleroxylon* was the leading dominant tree Table 4.

Discussion

The current study is the foremost report about tree species diversity within the Abasumba, GSBA. This work affirmed the number of trees enumerated fall within the arrays stated earlier for other dry forests. Klitgaard et al. (1999) reported 49 trees per ha^{-1} in a dry forest in Ecuador which compared well with 46 tree species identified in the Abasumba, GSBA. Conversely, Padalia et al. (2004) recorded 58 trees ha^{-1} belonging to 176 genera and 81 families in a tropical evergreen Andaman Islands forest, India. Also, Suratman (2012) listed tree stem density of 315-510, tree species of 280-450, and a mean genus of 340-435 per ha^{-1}, in dipterocarp Kuala Keniam national park Malaysia which were all higher than those recorded in the Abasumba, GSBA. But the number of 46 species, 17 families and 38 genera reported per hectare here is lesser than those recorded for the dry forests in Central America of 53 species and 22 families (Gentry 1982). However, it is higher than 21 trees per ha^{-1} recently enu-

Table 3. Summary of trees in the Abasumba GSBA according to families, local names and use.

Species Name	Family	Local Name	Uses
Albizia adianthifolia (Schumach.) W.Wight	Leguminosae–Mim.	Pampena	Medicinal
Albizia zygia (DC.) Macbr	Leguminosae–Mim.	Okoro	Timber, Fuel wood
Alstonia boonei De Wild	Apocynaceae	Nyame dua	Stoolwood, Timber
Antiaris toxicaria Lesch	Moraceae	Kyenkyen	Timber for Canoes
Baphia nitida Lodd.	Leguminosae–Pap.	Odwen	Walking stick
Blighia sapida K.D. Koenig	Sapindaceae	Ankye	Medicinal
Bridelia grandis Pierre ex. Huth.	Euphorbiaceae	Opamkotokrodu	medicinal
Ceiba pentandra L.	Bombacaceae	Onyina	Kapok, Timber
Celtis zenkeri Engl.	Ulmaceae	Esa–koko	Fuel wood
Celtis mildbraedii Engler	Ulmaceae	Esa	Fuel wood
Cola gigantean A. Chev.	Sterculiaceae	Watapuo/Owataku	Medicinal
Cola millenii K Shum.	Sterculiaceae	Mofra Cocoa	Medicinal
Dacryodes klaineana (Pierre) H.J.Lam	Burseraceae	Adwea	Paper making
Dialium guineense Willd.	Leguminosae–Caes.	Yoye (Asenaa)	Food
Dracaena perrottetii Baker	Dracaenaceae		Medicinal
Draceana arborea (Willd.) Link	Dracaenaceae		Medicinal
Erythrina senegalensis DC.	Leguminosae–Pap.		Medicinal
Ficus sur Forssk.	Moraceae	Odoma	Ornamental
Hildegardia barteri (Mast.) Kosterm.	Sterculiaceae	Akere–Kyewewa	Agri–horticulture
Holarrhena floribunda (G. Don) & Schinz	Apocynaceae	Sese	Carving
Holoptelea grandis (Hutch.) M.	Ulmaceae	Nakwa/Anakwa	firewood
Hymenostegia afzelii (Oliv.) H.	Leguminosae–Caes.	Takrowa	Timber, Medicine
Lannea acida A. Rich.	Anacardiaceae		Furniture
Lannea welwitschii (Hiern) Engl.	Anacardiaceae	Kum-Anini	Medicines
Lecaniodiscus cupanoides Planch.	Sapindaceae	Ankye (Dwindwera)	Food dye
Mansonia altissima (A.Chev.) A.Chev.	Sterculiaceae	Oprono	Valuable timber
Margaritaria discoidea (Baill.) Webster	Euphorbiaceae	Pepea/Duabo	Construction
Monodora myristica (Gaertn.)	Annonnaceae	Wedeaba	Spice, Medicine
Morinda lucda Benth.	Rubiaceae	Konkroma	Medicinal
Morus mesozygia Stapf	Moraceae	Woton	Food, Medicine
Napoleonaea vogelii Hook. & Planch	Lecythidaceae	Obua	Medicinal
Nesogordonia papaverifera (A. Chev.)R,Capuron	Sterculiaceae	Danta	Timber
Pterygota bequaertii de Wild.	Sterculiaceae	Kyere–bere	Bark fiber
Pterygota macrocarpa K.Schum.	Sterculiaceae	Kyerere	Medicinal
Rauvolfia vomitoria Afzel.	Apocynaceae	Kakapenpen	Medicine
Ricinodendron heudelotii (Baill.)	Euphorbiaceae	Wama	Pulp and paper
Sterculia rhinopetala K.Schum	Sterculiaceae	Wawabima	Medicinal
Sterculia tragacantha Lindl	Sterculiaceae	Sofo	Gun powder
Trichilia megalantha Harms	Meliaceae	Tanuro-kese	Medicinal
Trichilia monadelpha (Thonn.) J.J.de Wilde	Meliaceae	Kaka adukro	Fuel, Lighting
Trilepisium madagascariense DC.	Moraceae	Okure	Medicinal
Triplochiton scleroxylon K.Schum.	Sterculiaceae	Wawa	Timber, Plywood
Turraeanthus africanus (Wel.) Pel.	Meliaceae	Appapaye/Avodire	Timber
Vitex ferruginea Schu. & Thonn.	Verbenaceae	Otwentorowa	Firewood
Ximenia americana L.var. Amer.	Olacaeae		Medicinal
Xylia evansii Hutch.	Leguminosae–Mim.	Samantawa	Chewing sticks

Table 4. Tree Species diversity, distribution patterns and Importance value index (IVI) of Abasumba, GSBA.

Species Name	Family	Basal area	Relative Frequency	(IVI)	Distribution Patterns
Albizia adianthifolia (Schum.) W.F Wight	Leguminosae–Mimosoideae	9.2	0.5	1.10	Random
Albizia zygia (DC.) J.F. Macbr.	Leguminosae–Mimosoideae	35.3	0.8	3.30	Random
Alstonia boonei De Wildeman.	Apocynaceae	2.5	0.8	1.80	Random
Antiaris toxicaria (Rumph. Ex Pers.) Leschen.	Moraceae	4.2	4.3	10.68	Random
Baphia nitida Lodd.	Leguminosae–Papilionoideae	5.3	0.8	1.73	Random
Blighia sapida K. D. Konig.	Sapindaceae	12.6	0.3	0.65	Random
Bridelia grandis Pierre ex Hutchinson.	Euphorbiaceae	21.9	0.3	0.82	Random
Ceiba pentandra (Linnne') Gaertn	Bombacaceae	3.7	3.0	15.56	Random
Celtis zenkeri Engl.	Ulmaceae	7.8	4.8	12.33	Random
Celtis mildbraedii Engl.	Ulmaceae	5.0	8.0	23.90	Aggregated
Cola gigantea A. Chevalier.	Sterculiaceae	2.7	0.8	2.15	Random
Cola millenii K. Schum.	Sterculiaceae	2.0	12.6	28.88	Random
Dacryodes klaineana (Pierre) H. J. Lam.	Burseraceae	11.3	0.5	1.39	Random
Dialium guineense Willd.	Leguminosae–Caesalpinioideae	8.9	0.3	0.79	Random
Dracaena arborea (Willd.) Link.	Dracaenaceae	3.1	2.4	7.53	Aggregated
Dracaena perrottetii Baker.	Dracaenaceae	4.4	0.3	0.63	Random
Erythrina senegalensis DC.	Leguminosae–Papilionoideae	2.0	2.0	2.23	Random
Ficus sur Forsskal.	Moraceae	3.2	3.2	0.60	Random
Hildegardia barteri (Mast.) Kosterm.	Sterculiaceae	13.2	13.2	8.11	Aggregated
Holarrhena floribunda (G. Don) Dur. and Schi.	Apocynaceae	34.2	34.2	0.83	Random
Holoptelea grandis (Hutch.) Mildbr.	Ulmaceae	4.1	4.1	1.24	Random
Hymenostegia afzelii (Oliv.) Harms	Leguminosae–Caesalpinioideae	4.6	10.9	26.30	Aggregated
Lannea acida A. Rich.	Anacardiaceae	6.8	1.1	2.99	Random
Lannea welwitschii (Hiern.) Engler.	Anacardiaceae	15.7	1.3	6.53	Random
Lecaniodiscus cupanoides Planchon ex Bentham.	Sapindaceae	3.2	1.7	4.46	Random

Table 4. Contd.

Species Name	Family	Basal area	Relative Frequency	(IVI)	Distribution Patterns
Mansonia altissima (A.Chevalier) A. Chevalier	Sterculiaceae	13.2	0.5	1.99	Random
Margaritaria discoidea (Baillon) Webster	Euphorbiaceae	10.6	0.4	0.83	Random
Monodora myristica (Gaertn.) Dunal.	Annonaceae	4.0	1.2	2.19	Random
Morinda lucida Bentham	Rubiaceae	3.5	1.7	4.01	Random
Morus mesozygia Stapf.	Moraceae	4.3	1.5	4.79	Random
Napoleonaea vogelii Hooker and Planch.	Lecythidaceae	7.4	2.8	6.02	Aggregated
Nesogordonia papaverifera (A. Chevalier) R. Capuron	Sterculiaceae	6.0	6.1	15.22	Random
Pterygota bequaertii de Wild.	Sterculiaceae	3.3	1.6	4.20	Aggregated
Pterygota macrocarpa K.Schum.	Sterculiaceae	8.3	0.8	2.06	Random
Rauvolfia vomitoria Afzelius.	Apocynaceae	3.3	0.3	0.74	Random
Ricinodendron heudelotii (Baillon) pierre ex Pax.	Euphorbiaceae	10.5	2.7	6.63	Random
Sterculia rhinopetala K. Schum.	Sterculiaceae	2.3	0.5	1.29	Random
Sterculia tragacantha Lindley	Sterculiaceae	4.1	2.1	4.75	Random
Trichilia megalantha Harms.	Meliaceae	2.0	1.6	3.93	Random
Trichilia monadelpha (Thonning) J.J.de Wilde.	Meliaceae	3.1	4.0	28.10	Random
Trilepisium madagascariense DC.	Moraceae	19.6	0.3	0.86	Random
Triplochiton scleroxylon K. Schum.	Sterculiaceae	12.9	7.1	37.61	Random
Turraeanthus africanus (Welwitsch ex C.D.C) Pellegrin	Meliaceae	3.3	2.7	6.37	Aggregated
Vitex ferruginea Schum. and Thonning	Verbenaceae	2.8	0.3	0.61	Random
Ximenia Americana L.var. Americana	Olacaceae	2.5	0.3	0.62	Aggregated
Xylia evansii Hutchinson	Leguminosae–Papilionoideae	3.8	0.3	0.65	Random
Total 46 Species		**357.7**	**100.0**	**300.0**	

merated by Asase & Adeniyi (2021) in Ghana at the Apra Hills Sacred Grove within same southern marginal dry forest ecological zone.

Trees having dbh ≥ 10 cm ranged from 3–28 per ha^{-1} in Vindhyan tropical dry forest, India (Sagar & Singh 2005). Stem density of 374 ha^{-1} in Abasumba, GSBA was in the array of stems 276–905 ha^{-1} reported for tropical forests by (Pandey & Shukla 2001). White & Hood (2004) recorded a basal area of 20.7 m^2 ha^{-1} and 28.4 m^2 ha^{-1} in the humid dry forest of North Central Yucatan while, Gillespie & Jaffré (2003) recorded basal area of 32.7, 32.3 m^2 ha^{-1} for Ouen–Toro and Pindai in the dry humid forest of New Caledonia. We recorded basal area of 2 to 35.3 m^2 ha^{-1} and it compared well to the basal area for other tropical dry forests. The comparable basal area can be attributed to the existence of huge number of lower diameter class species in Abasumba, GSBA. As Abasumba is characterized by abundant small trees with dbh below 60 cm (as 62.0% of all individual trees sampled were in the ranges of 10–30 cm). This pattern is not unusual for primary forests, which are not or are only weakly affected by human exploitation showing a high potential of regeneration (Whitmore 1984). The relatively small number of trees with dbh ≥ 60 cm might be due to a limited number of species that naturally grow up to such diameters (Hartshorn 1990). As seedlings need to meet optimal conditions or locations for growth, in order to out-compete other fast-growing species (Khurana & Singh 2001). Although, the floristics composition is relatively low in the southern marginal dry forests of Ghana (Asase & Adeniyi 2021) they are usually made up of important species of management interest. In our present research, one of the enlisted tree species *Nesogordonia papaverifera* is enumerated as an endangered species in the IUCN Red List (www.iucnredlist.org) for been vulnerable or scarce tree. The findings of this study show that Abasumba, GSBA is a shelter for the protection of endangered tree species and essential for the maintenance of species biodiversity.

According to designation of trees that belong to storeys in tropical dry forests, three storeys are distinguished viz. uppermost (40–60 m), middle (18–30 m) and lower (4.5–18 m) storeys (Cloudsley-Thompson 1967; Lamprecht 1989). The tallest trees were *T. scleroxylon*, *C. mildbraedii*, *C. pentandra*, and *N. papaverifera*. Linares-Palomino & Alvarez (2005) discovered in the dry humid forests of Cerros de Amotape Cordillera, Peru that majority of the trees inventoried was in the (5–10 m) stratum. The second (10–15 m) covered trees and the third comprised of emergent trees above 15 m with few greater than 20 m. The majority of trees measured in Abasumba, GSBA fell in the middle and lower storeys whilst few were in the upper storey, this pattern is characteristic of tropical dry forests where most trees do not grow into the upper storey Swaine et al. (1990).

Trees with highest IVI were *T. scleroxylon*, *C. millenii*, *H. afzelii*, and *C. mildbraedii* belonging to Sterculiaceae, Meliaceae and Leguminosae families, respectively. This is consistent with (Cloudsley-Thompson 1967) description of Leguminosae, Sterculiaceae, Rubiaceae and Meliaceae as it has been the dominant family's composition in West African forests. Sterculiaceae, Leguminosae and Moraceae were the frequently occurring families as in the 10 most species–rich families in Africa, Asian and Neotropical forests (Huston 1994). Bombacaceae, Burseraceae, Annonaceae, Rubiaceae, Lecythidaceae, Verbenaceae and Olacaceae were the least frequent tree families within the plots. This is in contrast to Gentry (1995) who stated that the families Bombacaceae and Fabaceae were the dominant family in the Peruvian and the Neotropical dry forest ecosystem. The frequently encountered tree species were *C. millenii*, *T. scleroxy-*

lon, *N. papaverifera*, *C. gigantea*, *M. altissima*, *H. barteri*. *A. adianthifolia*, *A. zygia*, *B. nitida*, *D. guineense*, *E. senegalensis*, *H. afzelii*, *A. toxicaria*, *F. sur*, *M. mesozygia* and *T. madagascariense*. This is consistent with Asase & Adeniyi (2021), that frequently encountered *C. millenii*, *H. barteri*, *C. pentandra* and *D. aborea* trees in the Apra Hills Sacred Grove, Ghana.

The mean Shannon diversity index for the Abasumba GSBA was 1.44 this is below 1.67 recorded by Swaine et al. (1990) in Shai Hills, a humid dry forest in South–East Ghana but it is comparable to values obtained for other humid dry forests. In Kumaun dry forest, Himalaya a Shannon index of 0.8–2.3 was obtained by Garkoti (1992), while Srivastava (2002) reported 0.2-0.9 for the central Himalayan dry forests and Linares-Palomino and Alvarez (2005) reported 1.08–2.55 for the humid forests of Cerros de Amotape Cordillera, Peru. The mean Simpson's Diversity index of dominance 0.07 in this study is lower indicating low dominance of occurring species. Srivastava (2002) reported 0.20–0.90 in the central Himalayan forests India, additionally Linares-Palomino and Alvarez (2005) reported 0.51–0.91 for the humid forests of Cerros de Amotape Cordillera, in Tumbes, Peru.

Previous studies have revealed that uniform spreading of species is seen in limited species whiles, spreading of species is haphazard or gathered in nature. In a study of the highly diverse (87 tree species) of dry forest of Guanacaste Province in Costa Rica, Hubbell (1979) discovered that, trees were aggregated or haphazardly dispersed, with intermittent trees been aggregated compared to the frequent trees. In our research, uniform distribution was not exhibited, however 17.4% of trees showed aggregation whiles 82.6% displayed random dispersion. These indicates the environment in which the trees grow was homogeneous with many related factors acting on the population (Ewusie 1980) whiles rejuvenation near seed origin, asexual propagation, presence of 'sheltered sites' or anthropogenic disturbances caused aggregation (Augspurger 1984; Beatty 1984; Menaut et al. 1990).

CONCLUSION

The Abasumba GSBA is a tropical dry forest and remnants of the Awutu forest within the costal-–savanna–ecological–zone of Ghana. The forest structure and the species diversity indices coupled with the presence of a rare tree species shows the uniqueness for the conservation of plant biodiversity hence the demarcation as a Globally Significant Biodiversity Area. Some of the sampled plots were rich in tree species as they had many species, with a few individual trees. An active management regime including the restoration of species with low Importance Value Index is required from the plant conservationist view because they are the least dormant and rare species. The incorporation of local knowledge, practices and skills in the administration of the Abasumba, GSBA will enhance development of sustainable conservation methods. Further work on the effects of land and human caused factors on tree species diversity and distribution patterns will enhance our understanding of the location, spread and abundance of tress species for the purposes of biodiversity conservation. This study would be a starting point for the characterization of all the GSBA's in the Bawjiase district in particular and the southern dry forest reserves as a whole.

AUTHORS CONTRIBUTION

F.T.K. considered the ideas, and designed the sampling methods; F.T.K. and D.W. gathered the data and analysed; F.T.K. and D.W. contributed to the writing of the manuscript, and preparation for submission. All the

writers contributed equally to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest to declare, financial or otherwise, that could have influenced this paper.

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Review Article

Phytochemical and Pharmacological Activities of *Curcuma purpurascens* Blume, A Review

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ABSTRACT
Curcuma sp. is generally used for medicine, starch sources, preservatives, dyes and cosmetics. The use of *Curcuma* spp. for medical has increased because there have been many studies related to its active ingredients, such as flavonoids, essential oils, tannins, quinones, and terpenoids, as well as pharmacological activities, including wound healing, antioxidants, antifungal, anticancer, gastroprotective, and hepatoprotective. *Curcuma purpurascens* Blume is a species of *Curcuma* from family Zingiberaceae and used for traditional medicine. This article focuses on reviewing the literatures on *C. purpurascens* and discussing its morphology, phytochemical content, and pharmacological aspects. The method used to review this article was by exploring several databases such as Scopus, Pub Med, and Google Scholar to identify and download original articles and research journals related to the morphology, phytochemical content, and biological activity of *Curcuma purpurascens* Blume. The result of this review will later provide information about the uses and presence of *Curcuma purpurascens* Blume which is still rarely studied so further study related to its pharmacological activity tests and active compound as natural medicines can be explored.

INTRODUCTION
The genus Curcuma is a member of the Zingiberaceae family which is widely distributed and thrives in tropical and sub-tropical areas in Asia, Africa and Australia, and it has more than 93 species (Sasikumar 2005; Ayati et al. 2019). *Curcuma* is rich in medicinal properties, nutritional content and has a high economic value or used as an ornamental plant (Charon 2006; Li 2011; Rajkumari & Sanatombi 2018). Phytochemical content of the genus *Curcuma* includes phenolic compounds, diarylpentanoids (Sihanat et al. 2020), and essential oils (Hong et al. 2014), as well as curcumin (Li 2011; Ayati et al. 2019). The rhizome of *Curcuma* is commonly used as spices, herb, preservative, food flavoring, cosmetics, and a source of yellow dye for coloring agents as well as a source of starch. This part of the plant can cure various diseases such as

urinary tract infections, liver disease, chickenpox, wound healing, rheumatoid arthritis, cancer and digestive disorders. Also, it can reduce abdominal pain and minimize menstrual pain (Al-Reza et al. 2010; Xiang et al. 2011; Mahalakshmi et al. 2019).

The position of the inflorescences, the color of the rhizome and flowers, the shape of the protective leaves, as well as the flower parts are the primary determining characteristics of the species (Skornickova et al. 2007). The flowers have a variety of colors and blades of leaves tapering to the petiole (Larsen & Larsen 2006; Saensouk et al. 2015). *Curcuma* is an edible plant that can be eaten either raw or cooked as a vegetable (Larsen et al. 2000; Charon 2006; Saensouk et al. 2015).

Naturally and traditionally, *Curcuma* plant is used for treatment such as wound healing, gastric ulcers (Mishra et al. 2018), rheumatism, chest pain, ulcers, liver and spleen disorders, diabetes, cough, skin diseases and to clean the blood (Abas et al. 2005; Saikia & Borthakur 2010; Devi et al. 2014; Rana et al. 2015; Rajkumari & Sanatombi 2018).

Several studies have been conducted on the pharmacological activity of *Curcuma* (Rouhollahi 2016). *Curcuma* species plants such as *Curcuma xanthorizae*, *Curcuma aeroginosa*, *Curcuma soloensis*, *Curcuma domestica*, *Curuma amada*, *Curcuma aromatica* have several biological activities including antidiabetic, anticancer, antiviral, antifungal, anti-inflammatory, antihepatotoxic, gastroprotective, antiproliferative, antimicrobial, antirheumatic, hypochromatic, antifibrotic , antivenomous, antinociceptive (Srivastava et al. 2006; Policegoudra et al. 2010; Padalia et al. 2014; Jeon et al. 2015; Diastuti et al. 2019).

Several species of *curcuma* have been widely researched regarding pharmacological activity, phytochemical content, utilization of ethnobotany, and isolation of active compounds. However, there are still many species diversity of *Curcuma* which has not been explored, incluiding *Curcuma purpurascens* Blume. Thus we will study the plant morphology, characteristics, phytochemical content and pharmacological activity of *Curcuma puprpurascens* Blume.

GENERAL DESCRIPTION *Curcuma purpurascens* Blume

Curcuma purpurascens Blume is a plant species of *Curcuma* that is still rarely known and studied (Babu et al. 2016). *C. purpurascens* grows as a shurb with regional names, Temu Blenyeh, Temu glenyeh, Temu Tis, Koneng Tinggang with family Zingiberaceae (Chan et al. 2007; Koller 2009; Prasad & Aggarwal 2011; Rouhollahi et al. 2015c; Rouhollahi et al. 2015a). This plant can quickly grow in teak forest reaching 1.75 cm of height and growing branching rhizomes and flowers from October until February, it also grows well in temperatures of 20-30 ˚C (Koller 2009; Hong et al. 2014; Rouhollahi et al. 2015d; Rouhollahi 2016). The rhizome appearance of *C. purpurascens* is almost the same as the rhizome of *C. Longa*. However, it is larger and has a lighter or pale yellow color compared to *C. Longa* (Hong et al. 2014; Rouhollahi 2016). Genetic relationship and chemotaxonomy of *C. purpurascens* and *C. longa* have a similarity index of 75 % (Setyawan 2003).

MORPHOLOGY OF *C. purpurascens*

C. purpurascens has a branched rhizome with a yellow-orange color inside and outside to a whitish tip. It has terminal inflorescences on the leaf axils, the leaves of which are white at the base and predominantly pale green, also slightly pale brown and mottled above. The *C. purpurascens* flower has a white corolla up to 5cm long, a pale creamy yellow labellum measuring about 17 mm x 17 mm, a dark yellow median, pale creamy

Figure 1. Plants and rhizomes of *C. purpurascens* Blume (Photo by OP).

yellow staminodes, and long spurs. The leaves form an elliptical, slightly purple leaf blade along the upper leaf midrib (Koller 2009; Rouhollahi 2016). Rhizome stem of *C. purpurascens* can reach 1 m tall from branching rhizome. Each stem has several leaves with lengths ranging from 19-23 cm and 55-77cm (Rajkumari & Sanatombi 2018). Taxonomy and shape of *C. purpurascens* plants and rhizome can be seen in figure 1.

According to (Rouhollahi 2016), the taxonomy of *C. purpurascens* as follows;

Domain : Eukaryotes
Kingdom : Plants
Division : Fanerogamer
Class : Monocot flowering plants
Family : Zingiberaceae
Genus : Curcuma
Spesies : *Curcuma purpurascens* Blume

PHYTHOCHEMICAL CONTENT OF *C. purpurascens*

A number of studies have been carried out on several plants of the curcuma genus including chemical content, essential oils and pharmacological activities. *C. purpurascens* consists of various secondary metabolites, including essential oils, tannins, alkaloids, flavonoids, and terpenoids (Hong et al. 2014; Rouhollahi 2016). Based on a research by Sinaga et al. (2018), *C. purpurascens* has a group of secondary metabolites including flavonoids, saponins, quinones, and triterpenoids.

The active compounds of *C. purpurascens* have been reported by some research groups including arturmerone, 3,7-cyclodecadien-1-one, c-Elemene, curlone, 3,7-dimethyl-10-(1-methylethylidene), a-Elementone, 6-etheny;-4,5,6,7-tetrahydro-3,6-dimethyl-5-isopropenyl, turmerone, and benzofuran (Rouhollahi et al. 2014; Rouhollahi et al. 2015d; Rouhollahi 2016). As reported Hong et al. (2014) and Hamdi (2015) the largest *C. purpurascens* essential oil content was germacrone, arturmerone, germacrene-B, and curlon. In comparison, research from Hong et al. (2014) reported that the main components of essential oils are turmerone and ar-turmerone, isofurano-germacrene (curzerene) and xanthorrhizol. The rhizome of *C. purpurascens* contains 2-3% essential oil (Setyawan 2003). The volatile oil components identified from *C.purpurascens* are monoterpenoids 9.7%, sesquiterpenes 22.2% and sesquiterpenoids 56.2% (Hong et al. 2014). The monoterpenoid groups found in this study

include thymol, p-cymen-8-o;, 1,8-cineole, camphor, terpinen-4-ol, piperitone, terpineol and borneol, while the sesquiterpenoid group include ar-curcumene, elemen, germacrene-B, elemente, cis-bergamotene, selina-3,7- diene, trans-caryophyllene, elemente, aromadendrene, humulene, trans-farnesene, muurolene, humulene, selinene, bisabolene, sesquiphellandrene, and selinene (Hong et al. 2014; Rajkumari & Sanatombi 2018). On the other hand, the sesquiterpenoid groups identified by Hong et al. (2014) include turmerone, atractylone, curzerene, artumerol, trans-elementone, eudesmole, guaiol, arturmerone, curlone, and germacrone. Meanwhile, Hamdi (2015) in his research succeeded in isolating the active compounds of *C. purpurascens*, namely bisabolane (arturmernone) and one guaine (zedoalactone B) sesquiterpene, curcuminoids curcumin, bisdemethoxycurcumin and demethoxycurcumin. Meanwhile, Rouhollahi et al. (2015d) stated that turmerone is the main compound in *C. purpurascens*.

Apart from the above-mentioned reports, further research should be carried out on the effects of pure compounds, essential oils and fractions on their pharmacological activities. Besides, it is expected to isolate further, characterize and study the active compounds at the molecular level to explore the mechanism of action and pharmacological properties of *C. purpurascens*.

PHARMACOLOGICAL ACTIVITY

The ethnomedicinal of medicinal plants is history of ancient disease treatment which has been widely proven by research with various pharmacological activities (Hajiaghaalipour et al. 2013; Moghadamtousi et al. 2014). *C. purpurascens* is widely used by the community to treat various diseases. People traditionally use it as spices, treatment of boils, scabies, itching, fever, wounds and stomach aches, and externally to reduce fever (Koller 2009; Benzie & Galor 2011; Rouhollahi et al. 2014; Rouhollahi et al. 2015d; Rouhollahi et al. 2015a; Sinaga et al. 2018). In addition, *C. purpurascens* is also used by the community to treat coughs, burns, skin infections, dermatological disorders, and other skin diseases (Koller 2009; Rouhollahi et al. 2014; Rouhollahi et al. 2015a; Hong et al. 2014). To get rid of postpartum dizziness, pregnant women usually mix *C. purpurascens* with *Alyxia stellate* in a poultices formulation (Koller 2009; Rouhollahi et al. 2014).

Studies show that extracts and isolated compounds from the rhizome of *C. purpurascens* Blume have various pharmacological activities. Several activities of *C. purpurascens* have been reported including wound healing, gastroprotective (Rouhollahi et al. 2014; Rouhollahi 2016; Suprihatin et al. 2020), hepatoprotective (Sinaga et al. 2018), and antifungal (Rouhollahi et al. 2015b). Other studies mention that *C. purpurascens* provides anticancer activity (Rouhollahi et al. 2014; Rouhollahi 2016; Suprihatin et al. 2020), angiogenesis (Rouhollahi et al. 2015a), apoptogenic (Rouhollahi et al. 2015d; Hamdi 2015; Rajkumari & Sanatombi 2018; Sinaga et al. 2018), antioxidant (Jalip et al. 2013; Hamdi 2015; Sinaga et al. 2018; Suprihatin et al. 2020) cytotoxicity and antiproliferative (Hong et al. 2014; Rouhollahi et al. 2015e; Suprihatin et al. 2020).

The dichloromethane extract of *C. purpurascens* Blume rhizome has a cytotoxic effect, suppresses the proliferation of HT-29 colon cancer cells and triggers the induction of apoptosis through a mitochondria-dependent pathway with an IC_{50} value of 7.79 ± 0.54 g/mL (Rouhollahi et al. 2015b). The dichlormethane extract fraction of *C. purpurascens* has hepatoprotective activity by reducing the toxic effects of thioacetamide-containing cells, inhibiting cell proliferation, inducing HepG2 cell

apoptosis, and normalizing ROS (Rouhollahi et al. 2015e). The dichlormethane extract of *C. purpurascens* was experimentally able to exert a chemopreventive effect on the development of colon cancer cells by inducing apoptosis, reducing Bcl2 and PCNA and increasing Bax protein expression (Rouhollahi et al. 2015d). A toxicity test conducted by Rouhollahi et al. (2014), showed that *C. purpurascens* is safe to be used as a medicine from natural ingredients.

C. purpurascens Blume rhizome essential oil was reported to be selective as antiproliferative with strong cytotoxicity against HT29 cells with IC_{50} values of 4.9 ± 0.4 g/mL, and weak cytotoxicity against A549 cells, Ca Ski and HCT cells with IC_{50} values respectively of 46.3 ± 0.7; 32.5 ± 1.1, and 35.0 ± 0.3 g/mL, and it did not show any inhibitory effect on MCF7 cells. Meanwhile, the essential oil of *C. purpurascens* rhizome also had mild cytotoxicity against the non-cancerous human lung fibroblast cell line (MRC5), with an IC_{50} value of 25.2 ± 2.7 g/mL (Hong et al. 2014). Research conducted by Hong et al. (2014) stated that *C. purpurascens* essential oil extracted by hydrodistillation had a cytotoxic effect on breast cancer cells.

Based on research from Rouhollahi et al. (2014), hexane extract of *C. purpurascens* rhizome exerts a gastroprotective effect on ethanol-induced gastric ulcers. The gastroprotective effect is achieved by increasing the levels of NO and SOD, so gastric acid can be suppressed - to prevent it damage to the gastric mucosal wall. Hexane extract of *C. purpurascens* also affects wound healing through antioxidant, anti-inflammatory and angiogenesis mechanisms (Rouhollahi et al. 2015a). According to Jalip et al. (2013), antioxidant activity of 98% methanol extract of *C. purpurascens* Blume has a solid category with an IC_{50} value of 36.30 ppm and a flavonoid content of 14.27%. Meanwhile the ethanolic extract of *C. purpurascens* has moderate antioxidant power with an IC_{50} value of 112.93 ppm with a total flavonoid content of 4.77% (Sinaga et al. 2018). Hepatoprotective power of *C. purpurascens* ethanol extract inhibited the increase in GPT and GOT in mice induced by paracetamol (Sinaga et al. 2018). Chloroform and hexane extracts from the rhizome of *C. purpurascens* were reported to have intense inhibitory activity against *Candida albicans* (Vibrianti 2005; Hong et al. 2014). The ethanolic extract of *C. purpurascens* Blume rhizome was reported does not cause toxicity and death in acute and subchronic toxicity tests where the toxic level is 5 or non-toxic according to the Globally Harmonized System of Classification (GHS) classification (Suprihatin et al. 2020). The phytochemical content and pharmacological activity of *C. purpurascens* Blume can be seen in Table 1.

Here in, we documented the existing phytochemistry and pharmacological properties of *C. purpurascens*. The amount of experimental data evidenced vast biological active substance in *C. purpurascens*. It has potential for multiple pharmacological activities such as cancer, wound healing, hepatoprotective, anti-bacteria, anti-inflammatory, antioxidant, which can be explained by the presence of various essential oils, flavonoids, sesquiterpenoids, triterpenoids, steroids, tannins and alkaloids in the herb. Most of the mentioned pharmacological studies have provided some scientific evidence for its traditional medicine in wound healing, fever and anti-bacteria. Therefore, it is vital to conduct future research on the composition and pharmacological significance of the extract.

Wound Healing Activities of *C. purpurascens*

Wound healing is a complex process that restores the morphology and function of damaged tissues. The wound healing process will be deter-

Table 1. Phytochemical content and pharmacological activity *C. purpurascens* Blume.

Extraction	Solvent	Phytochemical content	Activity pharmacology	Empirical use	References
Maceration	Dichloromethane	γ-elemene, a-elemenone, turmerone, curlone and ar -turmerone	Chemopreventive, antioxidants	Itching, scabies, skin infections, boils, coughs, fever, wounds, poultices after childbirth	(Rouhollahi et al. 2015d)
Remaceration	n-hexane. Dichloromethane	c-elemene, a-elemenone, ar-turmerone, turmerone curlone	Anticancer	Fever, coughing sores, boils, hives, scabies	(Rouhollahi et al. 2015b)
Maceration	n-hexane	Sesquiterpenoids, ar-turmerone, turmerone, germacrone	Antioxidants, anti-inflammatory, angiogenesis	Dermatological disorders, wounds, burns, wounds, skin diseases	(Rouhollahi et al. 2015a)
Maceration	n-hexane	Benzofuran, 6-etenil-4,5,6,7-tetrahidro-3,6-dimetil-5-isopropenil, curlone, 3,7-dimethyl-10-(1-methylethylidene) turmerone, c-elemene, 3,7-cyclodecadien-1-one	Gastroprotective	Poultices, wounds, scabies, ulcers, itching, coughing, fever	(Rouhollahi et al. 2014)
Distillation	aqudest	Monoterpenoids; 1,8-cineole, piperiton, borneol, timol, kamper, terpinen-4-ol, p- cymen-8-ol, sesquiterpene; trans-caryophyllene, humulene, δ-elemene, germacrene-B, cis-α-bergamotene, aromadendrene, ar-curcumene, α-terpineol, trans-farnesene, β-- sesquiphellandrene, γ-muurolene, α-Selinene, γ-eudesmol β-bisabolene, selina-3,7(II)-diene, turmerone, curzerene, ar-turmerol, trans-elemenone, germacrone, guaiol, atractylone, ar-turmerone, and curlone	Antiproliferative, antifungal	Skin infection, cough antiproliferative, antifungal	(Hong et al. 2014)
Maceration	Methanol 98%	Flavonoids	Antioxidants	-	(Jalip et al. 2013)
Maceration	Ethanol 96 %	Quinones, triterpenoids, saponins and flavonoids	Antioxidants, hepatoprotector	Ulcers, itching of the skin, abdominal pain, external medicine for fever	(Sinaga et al. 2018)
Distillation	Aquadest	29 components of essential oil components	-	Cosmetics, food coloring and flavoring	(Setyawan 2003)
Maceration	Dichloromethane	-	Hepatoprotector	-	(Rouhollahi et al. 2015e)
Hydrodestilation	Aquadest	Sesquiterpene: guaiane, (zedoalactone) bisabalone (ar-turmerone), curcuminoids: curcumin, bidesmethoxycurcumin, demethoxycurcumin	Antioxidants, cytotoxic, neuroprotective		(Hamdi 2015)
Maceration	Ethanol	Steroids, terpenoids, flavonoids, triterpenoids, and essential oils	Antioxidants, anticancer, toxicity	Skin infections, coughs, and stomach pain	(Suprihatin et al. 2020)
Maceration	Ethanol 96%	Alkaloids, flavanoids, steroids, terpenoids, saponins, and tanins	Antioxidants,	-	(Pramiastuti & Murti 2022)

mined by angiogenesis, collagen synthesis, re-epithelialization and reduction of inflammatory cells (Longo et al. 2011; Rouhollahi et al. 2015a). The inflammatory process in wound healing involves neutrophils and other cells that produce ROS, where if there is excess ROS production, there will be induction of cell apoptosis, one of which is by activating the pro-apoptotic protein Bax (Buemi et al. 2004; Rouhollahi et al. 2015a). Various studies have been conducted on the potential of natural ingredients with anti-inflammatory activity, antibacterial antioxidants and procollagen synthesis as wound healing agents. Its medicinal properties come from the content of bioactive phytochemicals such as essential oils, saponins, alkaloids, tannins, flavonoids and phenolic compounds found in natural products (Ibrahim et al. 2018).

The n-hexane extract of *C. purpurascens* showed wound healing activity after excision in rats topically. Wound healing was evident after administering n-hexane extract of *C. purpurascens* at doses of 100 and 200 mg/kg for 20 days (Rouhollahi et al. 2015a). The number of inflammatory cells in the granulation tissue of mice decreased causing the scar width in mice also to decrease. The content of collagen and fibrotic cells increased after administration of the n-hexane extract of *C. purpurascens* which was characterized by the formation of new blood vessels at the wound site by intracystic cells (Rouhollahi et al. 2015a). The wounding process can also cause an increase in the expression of Hsp 70 protein. Wound healing is also accelerated by a decrease in the induction of Hsp70 protein expression by maintaining protein synthesis and conformation (Lamore et al. 2010; Rouhollahi et al. 2015a). The n-hexane extract of *C. purpurascens* at 100 and 200 mg/kg doses was able to reduce Bax protein expression and induce Hsp70 protein expression (Rouhollahi et al. 2015a).

The wound healing activity also involves the inflammatory process. From the data submitted there are still no research results on anti-inflammatory activity. Adequate studies have not been performed on the inflammatory cells, proinflammatory enzymes (COX and LOX, PLA_2, PGE_2), proinflammatory cytokines and reactive oxygen play a vital part in the pathogenesis inflammation. So it will be interesting to assess its anti-inflammatory effect from pure compounds and fractions of *C. purpurascens*.

Cytotoxic activity of *C. purpurascens*

The cytotoxic activity of *C. purpurascens* rhizomes was assessed against various human cancer cell lines such as MCF7, Ca Ski, A549, HT29, and HCT116 (Rouhollahi et al. 2015a). Essential oil from the rhizome of *C. purpurascens* exerts a strong antiproliferative effect against human carcinoma cells (Hong et al. 2014; Rouhollahi et al. 2015a). The mechanism of essential oils in inhibiting the growth of HT 29 cells are by suppressing COX-2 expression, blocking NF-Pathways B, PI3K/Akt, and ERK1/2 as well as the synergistic effect of other essential oil ingredients such as germacrone, germacrone -B, curlone, turmerone and Ar-turmerone (Hong et al. 2014).

The dichloromethane extract of *C. purpurascens* inhibited the proliferation of HT-29 colon cancer cells by activating the mitochondrial death pathway through the involvement of Bcl-2/Bax/Bcl-xl and ROS production, thereby inhibiting the growth of HT-29 cells. In vivo acute toxicity effect of *C. purpurascens* dichloromethane extracts provides a safe dose of 5 g/kg. The structure of the kidneys and liver remained normal according to histological results after administration of dichloromethane extract at a dose of 2 and 5 g/kg. The antiproliferative

effect of the dichloromethane extract of *C. purpurascens* was assessed by measuring the level of LDH released by HT-29 cells in the MTT assay. After treatment with dichloromethane extract of *C. purpurascens* for 24 hours, HT-29 cells underwent DNA fragmentation and shrinkage, with nuclei turning blue and phalloidin turning red. Administration of dichloromethane extract *C. purpurascens* can increase oxidative stress in HT-29 cells at ROS level in the presence of dihydroethidium oxidation to fluorescent ethidium in DNA due to ROS formation. Dichloromethane extracts *C. Purpurascens* at concentrations between 6.25 and 50 g/ml significantly increased cell membrane permeability and cytochrome c (cyt.c) release (Rouhollahi et al. 2015a). Dichlormethane extract *C. purpurascens* at doses of 250 mg/kg and 500 mg/kg was able to inhibit the formation of aberrant crypt foci (ACF) in azoxymethane-induced mice, followed by a decrease in (Proliferating cell nuclear antigen) PCNA protein expression (Rouhollahi et al. 2015d).

Most of the curcuma species studied had similar chemical components or the same isolates but the pharmacological properties were different and some showed opposite responses. The active constituents and underlying mechanisms responsible for anticancer properties of *C. purpurascens* still need to be discovered, for example studies of its cytotoxic activity against various human cancer cell lines like Hela, Coav-3, HepG2, HL-60 and Hep3B.

Hepatotoxic activity of *C. purpurascens*

The hepatotoxic effect of the ethanolic extract of *C. purpurascens* was tested by measuring the levels of SGOT and SGPT through the presence or absence of liver failure in test animals. The administration of *C. purpurascens* extract to increase SGOT levels in white rats can also be influenced by the number of doses and duration of treatment. An increase in SGPT or SGOT due to changes in permeability or damage to the hepatocyte wall is used as a marker of hepatocellular development (Rosida 2016). If there is an increase in SGOT levels, damaged body cells will be replaced with new cells. The increase in SGPT levels that occurred in the *C. purpurascens* extract was still within normal limits, and was not an indication of impaired liver function (Suprihatin et al. 2020).

Acute toxicity studies revealed that the ethanolic extract of *C. purpurascens* was relatively safe and did not cause symptoms of poisoning or death of animals at a dose of 5000 mg/kg BW. In addition, administration of *C. purpurascens* rhizome ethanol extract at a doses of 1250-5000 mg/Kg BW did not cause qualitative acute toxic symptoms such as changes in skin and hair, behavior, excretory system, respiration, eye injury, and coma and it did not interfere the growth or body weight of the tested animals (Suprihatin et al. 2020). Increased levels of SGPT or SGOT *C. purpurascens* extract had therapeutic potential effect for treatment pf liver dysfunction. However, the above study results were insufficient to draw meaningful conclusions. Therefore, mechanistic and up-to-date studies were needed to understand the hepatotoxic activity better.

Antioxidant activity of *C. purpurascens*

Based on the DPPH test, Temu Blenyeh extract has high antioxidant activity with an antioxidant capacity equivalent to vitamin C (Jalip et al. 2013; Pramiastuti et al. 2021). Antioxidant test conducted by Jalip et al. (2013) showed that the methanol extract of temu blenyeh rhizome had the highest antioxidant activity which was determined by measuring the total of flavonoid content (measured as quercetin) and free radical scav-

enging activity of DPPH. The total of flavonoid content with antioxidant activity of the extract described that the antioxidant activity of the extract might be due, at least in part, to the presence of polyphenols and flavonoids (Nahak & Sahu 2011; Khan 2012; Phuyal et al. 2020). The antioxidant activity of rhizome extract was assessed based on the free radical effect of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical activity (Pal et al. 2011). Antioxidant activity can be an important alternative therapy in preventing and reducing oxidative stress in several diseases including inflammation, cancer and cytotoxicity (Phuyal et al. 2020). Plants with high flavonoid content will have high antioxidant activity which generally has a high therapeutic potential (Sinaga et al. 2018; Widyastuti et al. 2020). The antioxidant potential of *C. purpurascens* needs further research using other methods to study their mechanism of action. Tests for antioxidant activity can use FRAP, TBA, CUPRAC, ABTS, ORAC and others.

Antimicrobial Activity of *C. purpurascens*

Antimicrobial agents are substances that have the ability to inhibit or kill the microbial growth. Many herbal plants can play a role as antimicrobial agents. *C. purpurascens* rhizome extract has antibacterial and antifungal activity. Bacterial growth that can be inhibited by *C. purpurascens* can be in the form of gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). *Candida albicans* is a type of fungus whose growth is inhibited by *C. purpurascens* extract.

The results of fractionation of n-hexane extract showed a minimum inhibitory concentration (MIC) and antibacterial activity against ampicillin of 2508.15 ppm for *Staphylococcus aureus* and 849.37 ppm for *Bacillus subtilis* bacteria. Meanwhile, its antibacterial activity against gram-negative bacteria was 1549.59 ppm for *Escherichia coli*; 2014,65 ppm for *Pseudomonas aeruginosa* (Andriyani & Udin 2006). Thus *C. purpurascens* can be said to have weak antibacterial activity due to the relatively high concentration of extracts that inhibit bacterial growth (Andriyani & Udin 2006). A study conducted by Vibrianti (2005) stated that the extract of *C. purpurascens* can inhibit the growth of the fungus *Candida albicans*, where the extraction using solvents n hexane, ethyl acetate, methanol and water (Vibrianti 2005; Suprihatin et al. 2020).

The inhibition of bacterial growth's production may be influenced by the content of active compounds contained in the rhizome of *C. purpurascens* such as flavonoids, essential oils, alkaloids, terpenoids, and tannins. Flavonoid compounds can damage cell walls and cause cell death (Dermawaty 2015). Tannin compounds can damage the formation of fungal conidia. The content of other compounds such as alkaloids in the rhizome of *C. purpurascens* can denature proteins to damage enzyme activity and cause cell death (Dermawaty 2015). The antibacterial activity of *C. purpurascens* needs to be extensively studied and the mechanisms involved in the antibacterial activity should be further explored.

CONCLUSIONS AND FUTURE PERSPECTIVES

C. purpurascens Blume is one of medicinal plants of the genus *Curcuma* which is used empirically for the treatment of wounds, skin diseases, itching scabies, ulcers and fever. *C. purpurascens* plant has a morphology almost the same as *C. longa* but the shape of the rhizome is larger and light yellow in color. The phytochemical content of *C. purpurascens* includes germacron, turmeron, curcumin, bisabalone, curlone, sesquiterpenes, ar-turmeron, flavonoids, quinones, tannins, terpenoids,

alkaloids and essential oils. These secondary metabolites are able to exhibit pharmacological activities including antioxidants, anticancer, hepatoprotective, gastroprotective, apoptogenic, anti-fungal, antimicrobial, cytotoxis, and anti-proliperative. However, all of these pharmacological activities have only been obtained from the activity of the rhizome extract. Meanwhile, research on essential oils mostly only identified the constituent components and levels of essential oils. *C. purpurascens* has the potential to be explored for pharmacological testing and its active compounds as natural medicine. Thus, a broader and more in-depth research needs to be done to determine its therapeutic potential or pharmacological activity.

AUTHORS CONTRIBUTION

O.P., searched the literatures and wrote the manuscript, S.W., N.F., and P.A. supervised the processes.

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CONFLICT OF INTEREST

There is no conflict interest regarding this research and research funding.

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