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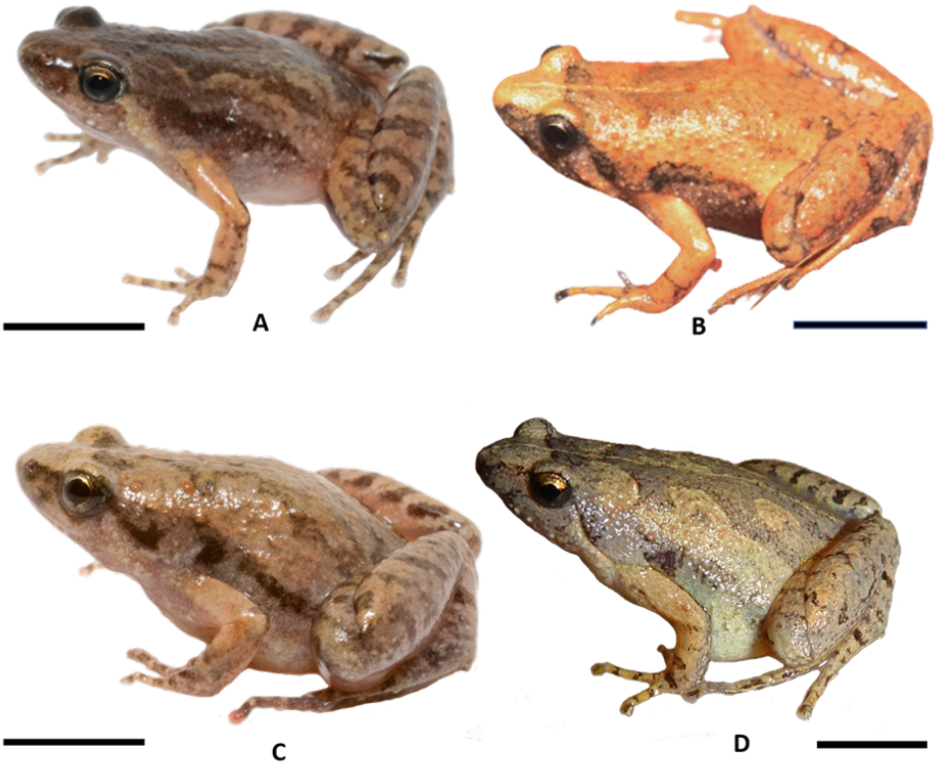

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Short Communication

Molecular Bird Sexing of Tanimbar Cockatoos (*Cacatua goffiniana*) by Using Polymerase Chain Reaction Method

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ABSTRACT

This study aimed to determine the sex of Tanimbar Cockatoo (*Cacatua goffiniana*) birds by amplifying Chromodomain Helicase DNA-binding-1 (CHD-1) gene on Z and W sex chromosomes as well as to compare the quality of DNA extraction and PCR amplification products from samples derived from peripheral blood and plucked feathers. This work used five *C. goffiniana* birds which were collected from the Wildlife Rescue Center (WRC) in Pengasih, Kulon Progo, Yogyakarta. From each *C. goffiniana*, feather samples were collected by plucking feathers on the ventral wing and peripheral blood samples were taken by cutting their nails and collecting the blood into microhematocrit tubes containing heparin. The next stage was DNA extraction and DNA amplification on the CHD-1 gene using the PCR method by NP, P2, and MP primer pairs. Then, products of DNA extraction and PCR amplification were electrophoresed on 1.5% agarose gel and visualized under a UV light transilluminator with a wavelength of 260 nm. The visualization showed that samples from peripheral blood generated clearer DNA fragments compared to plucked feathers. Two of the five samples were male *C. goffiniana* and the other three samples were females. In the male Tanimbar Cockatoo was amplified a single DNA fragment of the Z sex chromosome in size of 300 bp, whereas in the female *C. goffiniana* was amplified double DNA fragments of Z and W sex chromosomes in size of 300 bp and 400 bp respectively. The DNA quality showed that the DNA quality from peripheral blood samples were better in quality than the DNA collected from plucked feather samples.

Keywords: *Cacatua goffiniana,* CHD-1, molecular bird sexing, PCR, sex chromosome

Indonesia is known as a country with abundant biodiversity, both flora and fauna, especially various species of birds. Indonesia is the fourth country after Columbia, Peru and Brazil with the number of bird species. Indonesia has 1.598 species of birds, including 372 (23.28%) endemic bird species, 149 (9.32%) species of migratory birds and 118 (7.38%) species of endangered birds (Kurniawan et al. 2018). These birds need to be protected and preserved in order to avoid extinction. One species of endangered bird is *Cacatua goffiniana*, which is also called Tanimbar Cockatoo, Kakatua Tanimbar, or Corella Tanimbar because it is an endemic bird from the

Tanimbar island in Southeast Maluku. Its habitat is in dry tropical forests and humid deciduous forests (Mioduszewska et al. 2018). In 1992, Tanimbar Cockatoo was included in the Appendix 1 category by the Convention on International Trade in Endangered Species (CITES) because it was on the verge of extinction, so this bird was banned to be traded in all forms of international trading (Jepson et al. 2001). The declining population has raised the conservation status of *Cacatua goffiniana* to near threatened (BirdLife 2018). The threat was identified as a result of habitat loss and illegal hunting. They lost their habitat because of forest fires to create more agricultural fields and fewer nesting sites due to logging of Manilkara fasciculate and *Canarium indicum* trees (Mioduszewska et al. 2018).

Tanimbar Cockatoo has distinctive characteristics of white feathers and a white crest with red patches on the feathers around the beak. Its body size is about 320 mm and it weighs 350 grams (Zein et al. 2017). This bird is rather small compared to other cockatoo species. The uniqueness of the Tanimbar Cockatoo is its loud voice and its intelligence in combining image objects (Habl & Auersperg 2017). Its beauty and uniqueness have led to illegal hunting of this bird to be traded so that the population is endangered. Efforts in preserving the population of Tanimbar Cockatoo include improving the ecological environment, biological evolution, breeding, and conservation. Determining the sex of the bird quickly and accurately also supports these efforts. (Ravindran et al. 2019).

Tanimbar Cockatoo is a monomorphic bird, in which male and female birds have the same physical characteristics until they are mature so it is difficult to distinguish them morphologically. Determination of sex in monomorphic bird groups can be done using several methods such as laparoscopy, karyotyping and vent sexing. Laparoscopic and karyotyping methods have weaknesses; they are expensive and require a long time, while the weakness of the vent sexing method is that the results are less accurate and can only be done during the breeding season. Other methods that can be used are morphometric analysis and molecular bird sexing. Morphometric analysis has the advantages of being fast and inexpensive as well as applicable outside of the breeding season, but it has a low confidence level if there is no other supporting data. Molecular bird sexing has several advantages, namely it is accurate, applicable for any age of the bird, and relatively safe. This method has been widely applied to groups of parrots (Psittacidae). However, the weakness of this method is that it can only be done in a laboratory and is relatively expensive (Liu et al. 2011; Kurniawan & Arifianto 2017).

Molecular bird sexing is a DNA-based sex determination that is most commonly applied to wild birds. Samples used to obtain genetic material can derive from peripheral blood or plucked feathers. The blood of birds has nucleated erythrocytes that are rich in DNA. On the other hand, a plucked feather has less DNA and is easily degraded if it originates from dead cells such as mature feathers. However, the advantage of plucked feather samples is that they are easy to obtain, reduce the risk of contamination and avoid pain so that the costs required are also lower (Harvey et al. 2006). Determination of sex by molecular bird sexing involves sex chromosomes in the Chromodomain Helicase DNA-binding-1 (CHD-1) gene. The sex chromosomes of birds are ZW hemizygous chromosomes in female birds and homozygous ZZ chromosomes in male birds. The CHD-1 gene on the Z and W sex chromosomes have different intron sizes. This can be used as a basis for determining the sex of birds by amplifying the CHD-1 target gene by using the Polymerase Chain Reaction (PCR) method. The PCR method requires specific primer pairs that are designed to amplify the two exon areas in a sequence of nitrogenous bases that are similar in many bird species and are located between introns. The results of amplification in male birds

produce only a single DNA fragment from the Z sex chromosome, whereas in female birds the amplification produces double DNA fragments from the Z and W sex chromosomes (Liu et al. 2011; Nugroho & Zein 2015).

Primer pairs used for amplification of the CHD-1 gene intron segment have been extensively developed by researchers. The intron segment of the CHD1 gene is used as a marker of sex determination because it has significant size differences between the sexes (Nugroho & Zein 2015). The P2/P8 primers design which was developed by Griffiths et al. (1998) cannot distinguish between male and female birds. Fridolfsson and Ellegren (1999) have developed a 2550F/2718R primers design that is able to overcome problems of P2/P8 primary pairs. The latest development states that birds from the Psittaciformes order such as Conure, Parrot, and Macaw have been able to be tested for their sex by using NP, P2, and MP primers which result in clearer differences between male and female birds. NP primer is a forward primer that will attach to the CHD-1Z and CHD-1W genes, while a reverse primer consisting of two primers, P2 and MP, will attach to the CHD-1Z gene and the CHD-1W gene (Thammakam et al. 2007). This study aimed to determine the sex of the Tanimbar Cockatoo bird by amplifying the CHD-1 gene on the Z and W sex chromosomes, as well as comparing the quality of extracted DNA and PCR amplification products from samples derived from peripheral blood and samples derived from plucked feathers.

The research samples were plucked feathers and peripheral blood from five Tanimbar Cockatoo (*Cacatua goffiniana*) birds collections of the Wildlife Rescue Center (WRC), Pengasih, Kulon Progo, Daerah Istimewa Yogyakarta. The feather samples were taken from the ventral wing. The peripheral blood samples were taken by cutting the nails, and then the blood was kept in microhematocrit containing heparin anti-coagulant. The samples were sent to the Biochemistry Laboratory of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta. The samples were coded CD1, CD2, CD3, CD4, and CD5 for samples derived from peripheral blood and CB1, CB2, CB3, CB4, and CB5 codes for samples derived from plucked feathers.

The procedure used for DNA extraction from feather and peripheral blood samples was carried out following the standard procedure from Geneaid gSYNC™ DNA Extraction Kit Quick Protocol.

The results of DNA extraction are used as a template for the DNA amplification process using the PCR method. DNA fragments were amplified with the CHD-1 gene target on the Z and W sex chromosomes by using specific primer pairs, namely P2-Forward, NP-Reverse, and MP-Reverse primers. The composition of the nucleotide sequence, number of bases, references of the three primers are presented in Table 1.

The master mix composition for PCR amplification with a total volume of 25 µl consists of 12.5 µl MyTaq HS Red Mix DNA polymerase, 1 µl P2 primer, 1 µl MP primer, 1 µl NP primer, and 9.5 µl DNA template. The mixture was mixed until homogeneous and then transferred into a thermocycler with a process consisting of the pre-denaturation stage at a temperature of 94 °C for 2 minutes, denaturation at a temperature of 94 °C for 20 seconds, annealing at a temperature of 46 °C for 30 seconds, elongation at a temperature of 72 °C for 40 seconds and post-elongation at a

Table 1. Nucleotide sequence of P2, NP, and MP primers for amplification of CHD1 gene.

Primer	Nucleotide Sequence	Σ Base	Reference
P2	5'-TCTGCATCGCTAAATCCTTT-3'	20	Griffiths et al. 1998
MP	5'-AGTCACTATCAGATCCGGAA-3'	20	Ito et al. 2003
NP	5'-GAGAAACTGTGCAAAACAG-3'	19	Ito et al. 2003

temperature of 72 °C for 10 minutes. The denaturation, annealing, and elongation stages were repeated in 40 cycles.

DNA electrophoresis was carried out to visualize the results of DNA extraction and the results of PCR amplification with ultraviolet (UV) transilluminators. Agarose gel was used for electrophoresis at a concentration of 1.5% in a 1x TBE solution supplemented with 2 µl SYBRsafe DNA staining. Electrophoresis of all samples in 1.5% agarose gel was carried out at a voltage of 100 volts and an electric current of 80 mA for 45 minutes. Electrophoresis results were observed with an ultraviolet (UV) transilluminator with a wavelength of 260 nm in a dark room.

Electrophoresis of total DNA extraction and CHD-1 gene amplification results observed by using UV-Transilluminator in a dark room are presented in Figures 1 and 2.

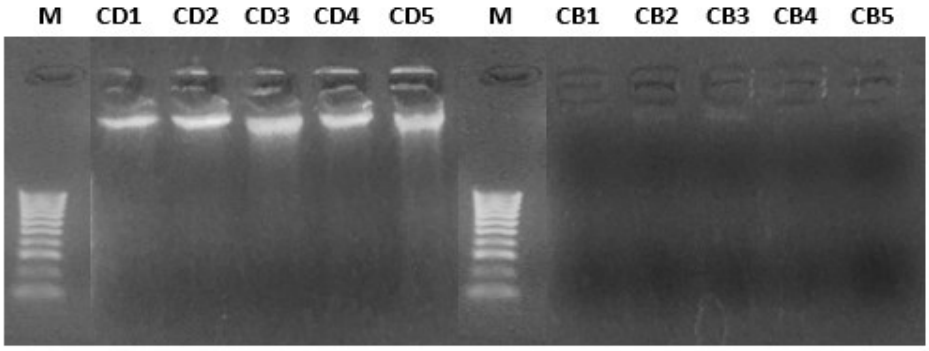

Figure 1. Electrophoresis of total DNA extraction. Note: M = marker (hyperladder 100 bp), CD1-CD5 = peripheral blood samples of Tanimbar Cockatoo, CB1-CB5 = feather samples of Tanimbar Cockatoo (*Cacatua goffiniana*).

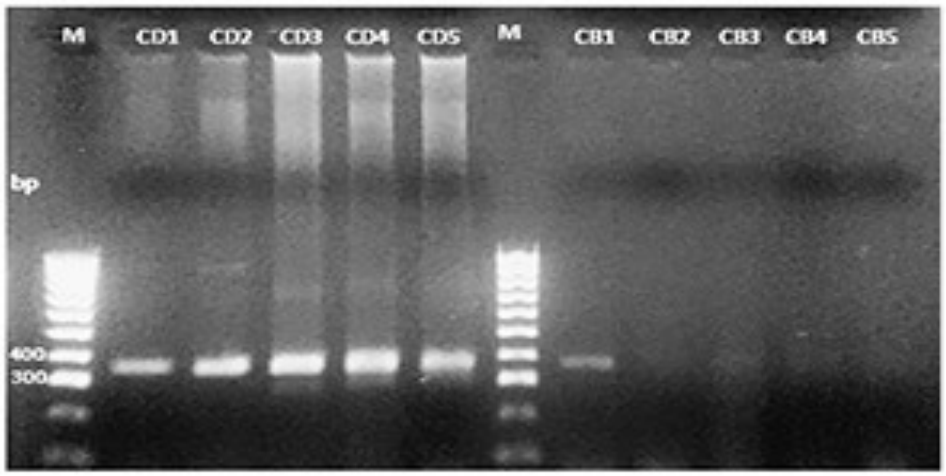

Figure 2. Electrophoresis of CHD-1 gene amplification of samples from Tanimbar Cockatoo (*Cacatua goffiniana*). Note: M = marker (hyperladder 100 bp), CD1-CD5 = *C. goffiniana* peripheral blood samples, CB1-CB5 = *C. goffiniana* feather samples.

Based on the electrophoresis results in Figures 1 and 2, a comparison of the quality of the DNA extraction results. Here, we have compared the purity of DNA extracted from peripheral blood and plucked feather samples did not perform quantitatively using a UV spectrophotometer, but performed descriptively based on Figures 1 and 2. These Figures showed that the extracted DNA and PCR products derived from peripheral blood samples appeared clearer, cleaner and there was no smear DNA band compared to the samples from plucked feathers. The products of PCR amplification of peripheral blood and plucked feather samples were more detailed presented in Table 2.

Table 2. Comparison of extraction and amplification results between *Cacatua goffiniana* blood and feather samples.

No	Bird Code	Extraction Results		Amplification Results	
		Blood	Feather	Blood	Feather
1.	*C. goffiniana* 1	+++	+	+++	++
2.	*C. goffiniana* 2	+++	+	+++	+
3.	*C. goffiniana* 3	+++	+	+++	+
4.	*C. goffiniana* 4	+++	+	+++	+
5.	*C. goffiniana* 5	+++	+	+++	+

Note: +: not really clear, ++: quite clear, +++: clear

Visualization of the results of DNA amplification by PCR method in the *C. goffiniana* CHD-1 gene was further interpreted with the results presented in Table 3.

Table 3. Interpretation of the visualization of PCR amplification.

No.	Sample Code	Electrophoresis Result	Interpretation
1.	CD1	Single band	Male
2.	CD2	Single band	Male
3.	CD3	Double bands	Female
4.	CD4	Double bands	Female
5.	CD5	Double bands	Female
6.	CB1	Single band	Male
7.	CB2	Not really clear	-
8.	CB3	Not really clear	-
9.	CB4	Not really clear	-
10.	CB5	Not really clear	-

The electrophoresis results in Figure 1 showed that DNA fragments are seen in peripheral blood samples with the sample code CD1, CD2, CD3, CD4, and CD5, while feather samples with the sample code CB1, CB2, CB3, CB4, and CB5 looked empty and do not display the DNA band. This indicates that the DNA in the feather samples was either very thin or non-existent, so it cannot be visualized properly in the agarose gel 1.5% by SYBRsafe staining. According to Harvey et al. (2006), DNA extracted from feather samples was less in quantity compared to DNA extracted from peripheral blood. The results of total DNA extraction in Figure 1, are then used as a template for PCR amplification with the CHD-1 gene target on the Z and W sex chromosomes using NP, P2, and MP primer pairs.

PCR is an enzymatic method for in vitro DNA amplification. This method is used to multiply DNA fragments millions of times in a short time with the use of the enzyme DNA polymerase and a pair of primers specific to the target DNA. Specific primers used in this study included 3 primers, namely NP, P2, and MP primers developed by Ito et al. (2003) as a substitute for P2/P8 primers that were not able to detect differences in CHD-1 genes intron on Z and W chromosomes so that they cannot distinguish between male and female birds. Out of 29 bird species in East Asia, NP, P2 and MP primers can identify 25 bird species belonging to seven different orders namely Passeriformes, Falconiformes, Columbiformes, Strigiformes, Gruiformes, Ciconiiformes, and Caprimulgiformes (Lee et al. 2008). According to Thammakam et al. (2007), the primers were also successfully used to determine the sex of birds in Psittaciformes order such as Conure,

Parrot, and Macaw. In addition, NP, P2, and MP primers were able to determine 100% of samples from 14 Rosy-faced Lovebirds (*Agapornis roseicollis*) (Nugraheni et al. 2019). Other similar studies also showed that the sex of 54 birds in captivity originating from 11 different families can be determined by using these primers (Purwaningrum et al. 2019) and Pamulang and Haryanto (2021) who successfully carried out a molecular bird sexing on kutilang (*Pycnonotus aurigaster* and *Pycnonotus melanicterus*) birds by using PCR amplification on CHD-Z and CHD-W targeted genes.

The PCR amplification results of the CHD-1 gene on Tanimbar Cockatoo (*Cacatua goffiniana*) in Figure 2 showed that male birds only generated a single DNA fragment from the Z sex chromosome, whereas female birds generated double DNA fragments from Z and W sex chromosomes (Nugroho & Zein 2015). NP, P2 and MP primers were used for amplification of introns in the CHD-1Z and CHD-1W gene segments. NP primer was a forward primer that attaches to the CHD-1Z and CHD-1W genes, while the reverse primer consists of two primers: P2 and MP primers. P2 primer will attach to the CHD-1Z gene and MP primer will attach to the CHD-1W gene (Thammakam et al. 2007). Electrophoresis results in Figure 2 showed that the peripheral blood samples produce good and clear DNA bands in both male and female *C. goffiniana*, whereas the feather samples produce thin DNA bands. The electrophoresis of DNA extraction results cannot be seen clearly, so the DNA cannot be visualized properly. In addition, DNA extraction results from feather samples appear to be less in quantity and more easily degraded compared to DNA extracted from peripheral blood samples (Harvey et al. 2006). Another factor that caused the thin DNA bands in the feather samples was the different amount of calamus collected in the five samples. According to Purwaningrum et al. (2019), feather samples with more number of calamus produce clearer DNA bands in the electrophoresis.

Comparison of DNA extraction results and PCR amplification results in Table 2 showed that peripheral blood samples were observed to be the better source of DNA template in molecular bird sexing by PCR method compared to feather samples. According to Harvey et al. (2006), bird blood has nucleated erythrocytes that are rich in DNA. Therefore, only a few drops of blood were needed for the DNA extraction process. However, the collection of blood samples was more difficult because birds have relatively small blood vessels that can cause stress that may lead to death. The blood samples in this study were collected by cutting one of the nails of the Tanimbar Cockatoo, which was then collected on a microhematocrit containing anti-coagulant heparin. This method was chosen to minimize the risk of stress in Tanimbar Cockatoo. Feather samples for determining the sex of Tanimbar Cockatoo were taken from the ventral wing by plucking the feather up to the base of the hair (calamus). Calamus is a source of DNA that contains many living epithelial cells or blood deposits in the feather shaft. According to Harvey et al. (2006), a feather sample produces less quantity of DNA compared to blood samples, and the DNA was easily degraded which makes the both the quality and quantity of DNA were lower. Ravindran et al. (2019) also state that blood samples were more accurate than feather samples as DNA sources. Extraction of feather samples produces a smaller amount of DNA because there was a keratin protein in the calamus that can act as an inhibiting protein in the DNA extraction process. (Hickman et al. 1984).

Based on the interpretations presented in Table 3, it can be seen that from the five Tanimbar Cockatoo (*Cacatua goffiniana*) blood samples coded as CD1, CD2, CD3, CD4, and CD5 there were two male Tanimbar Cockatoos (CD1 and CD2 samples), while the other three (CD3, CD4, and CD5 samples) were female Tanimbar Cockatoos. Male sex was characterized by

the production of a single DNA fragment of 400 bp from the Z sex chromosome, whereas females were characterized by the appearance of double DNA fragments of 300 bp and 400 bp from the W and Z sex chromosomes (Nugroho & Zein 2015). A single DNA fragment indicating male sex was produced in one feather sample (CB1 sample), while CB2, CB3, CB4, and CB5 feather samples produced thin and unclear bands that could not be interpreted.

Molecular bird sexing of five Tanimbar Cockatoo (*C. goffiniana*) birds showed that two samples were male *C. goffiniana* with an amplification product of a single DNA band in size of 400 bp from the Z sex chromosome, while three samples were female *C. goffiniana* because generated double DNA bands in the size of 300 bp from the Z sex chromosome and 400 bp from the W sex chromosome respectively. This finding was in line with other researchers (Savitri et al. 2021), who have successfully carried out molecular bird sexing on Sulphur-crested Cockatoo (*Cacatua galerita*) by PCR amplification. The quality of extracted and PCR amplified DNA showed that the DNA quality from peripheral blood samples were better in quality than the DNA collected from plucked feather samples.

AUTHOR CONTRIBUTION

RFKH carried out laboratory works (samples collection, DNA extraction, PCR amplification, agarose gel electrophoresis, data analysis, and drafted the manuscript). AH planned and designed the research work as well as translated and revised the final manuscript. DS got permission for field research conducting. IP and WPN handling and collected field samples. All authors contributed to this research and approved the final manuscript.

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CONFLICT OF INTEREST

The author declares that there is no competing interest

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Short Communication

Body Weight Gain and Carcass Quality of the Hybrid Chicken Derived from the Crossing between Female F_1 Kampung Super and Male F_1 Kampung-Broiler

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ABSTRACT
This research was conducted to observe the body weight gain and carcass quality of the hybrid chicken derived from the crossing between female F_1 Kampung Super and male F_1 Kampung-Broiler. The weekly weight gain for seven weeks and carcass quality, including breast yield, protein content, lipid content, moisture, and pH were measured with each group consists of six individuals. The chickens were slaughtered for meat quality measurements on the 7th week. The results showed that the hybrid chickens' weight at the 7th week (888.22±139.63) was higher than layer (467.06±85.7) and pelung (436.39±42.33). The hybrid chicken's breast yield (3.20%) was significantly higher than pelung's (1.66%). These characteristics of hybrid chicken breast muscle lead to the conclusion that hybrid chicken is a potential alternative meat-type chicken with local chicken meat characteristics and has relatively fast growth.

Keywords: breast yield, chicken breeding, hybrid chicken, meat quality, weekly weight gain

Growth performance and carcass traits are essential for poultry production, especially for supporting high production and lower market price (Anh et al. 2015). The continuous growing demand for poultry meat puts more insistence on producers to improve chicken production, including growth rate and feed conversion (Petracci et al. 2015). The demand for poultry meat production has led the development of the commercial breed, which is widely known as broiler, to very rapid growth.

The selection of meat-type chicken is primarily focused on the increased growth performances and improved body composition. To fulfil the demand of dietetic products, the producers are demanded to increase the breast meat yield and to lower fat content (Berri et al. 2001). Hybrid chicken is derived from crossing between different types of parental lines. The crossing between native chicken and commercial meat-type chicken results in some advantages, including higher growth rate, better feed conversion ratio,

and higher carcass yield compared to the native chicken (Sokolowicz et al. 2016).

Pelung is one of the Indonesian native chicken domesticated in Cianjur, West Java. As reported in Nataamijaya (2010), pelung has the highest weight of all Indonesian native chickens, which can reach 2.20 kg on week 20. It is also reported in Saragih et al. (2019) that pelung is a potential native breed for meat production. Several studies have been conducted to improve the growth performance and production of pelung by utilizing selective breeding, such as the crossing between female broiler and male pelung (Saragih et al. 2017) to increase the body weight and the crossing between female layer and male pelung (Puspita et al. 2017). But, there is still no observation on the offspring of both type of F1, especially on body weight and carcass characteristics.

Meat is one of the most nutritious foods that is considered essential to optimal human growth and development. As one of the animal-based food, poultry contains desirable characteristics, including low lipid content and high concentrations of polyunsaturated fatty acids, which are essential to meet the consumers' health considerations (Barbic et al. 2014). Poultry meat quality is governed by several factors including genetics, feeding, husbandry, pre-slaughter handling, stunning, slaughter procedures, chilling, processing and storage conditions that interacts each other, resulting in different conditions of the meat (Milicevic et al. 2015). Previous studies reported different meat quality among different breeds that strongly indicated the correlations with different genetic characteristics. Those meat qualities include breast muscle weight, moisture, protein content, lipid content, and pH value (Lonergan et al. 2003; Le Bihan-Duval et al. 2008).

This study aims to observe the effect of breeding of pelung with broiler and layer chickens on the body growth and meat characteristics of the hybrid chicken and its potentials as the new alternative for meat-type chicken.

Four chicken groups were involved in this study: broiler as the commercial meat-type (positive control), layer as the negative control, while pelung and the hybrid chicken derived from crossing between pelung and broiler and layer. The pedigree chart and the breeding results are presented in Figure 1. Six day-old-chicks (DOCs) from each chicken type were housed with a semi-intensive system and kept under standard management conditions. The access to water and feed were provided *ad libitum* along with the experimental term. The DOCs were fed with starter feed BR1 from Japfa Comfeed Indonesia Tbk (Table 1).

Table 1. Nutrition fact chicken feed used in this research (BR1 Japfa Comfeed from PT. Japfa Comfeed Indonesia Tbk).

Nutrients	Concentration
Crude Protein	21.5-22.5%
Water	Max. 12%
Fat	Min. 5 %
Crude Fiber	Max. 5%
Ash	Max. 7%
Calcium	0.8-1.1 %
Phosphor	Min. 0.5 %
Metabolic Energy	2950-3050 Kcal/Kg

Body weight was measured every week started directly post-hatch until week 7. The Research Ethics Commission, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Indonesia has approved all of the protocols

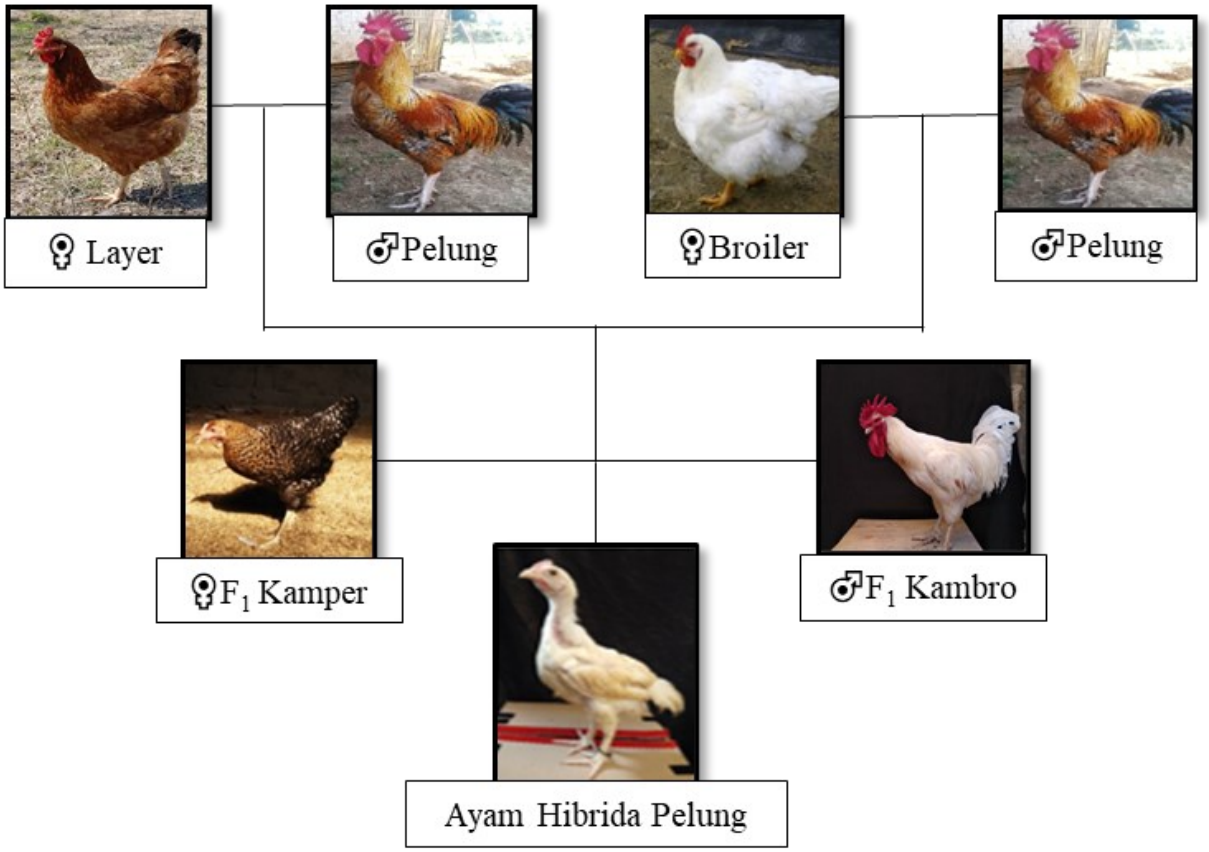

Figure 1. The hybrid chicken pedigree chart derived from the crossing between female F_1 Kamper and male F_1 Kambro.

conducted in this research (Ethical clearance No. 0105/EC-FKH/Eks./2019).

Six chickens from every type were randomly chosen for breast yield and carcass quality measurement. Every group consisted of three males and three females. Before slaughtered, chickens were weighed and then terminated via vena jugular cutting. The total chicken breasts (right and left, pectoralis major and minor) were collected and weighed. Approximately 30 g of each sample was used for carcass quality analysis.

Breast muscle samples of early post-hatch chicks: post-hatch day 1, day 3, and day 5 (then mentioned as P0, P3, and P5, respectively) were traced on a piece of plastic sheet and then traced on a paper to be scanned for measurement. The measurement was conducted using ImageJ software.

Fat, moisture and protein were measured using FOSS FoodScan™ Near-Infrared (NIR) Spectrophotometer (FOSS North America, 8091 Wallace Rd, Eden Prairie, MN 55344, USA) with AOAC Official Method 2007 (AOAC International 2007). pH value was measured using standardized pH meter HI 9811X Piccolo (Hanna Instrument, USA). The test was conducted at the Laboratory of Meat Science and Technology, Faculty of Animal Science, Universitas Gadjah Mada, Indonesia.

The data obtained from this study were analyzed using one-way ANOVA using SPSS 22.0 software (IBM Corporation, New York, USA) with the probability values $p<0.05$ to assume the statistical significance.

The hybrid chicken breeding was conducted to realize the breeding goals mentioned by Wang et al. (2012), such as increased growth rate, decreased abdominal fat, great skeletal system growth and development, and better health. We used broiler, layer, and pelung as parental types and control groups to meet the goals. The hybrid chicken used in this study was derived from a crossing between female F_1 Kampung Super derived from female layer and male pelung (Puspita et al. 2017) and male F_1 hybrid derived from

crossing between female broiler and male pelung (Saragih et al. 2017). The feed used in this study was the standard broiler feed as served in Table 1. The use of this diet is to eliminate the negative effect on body weight gain due to an unsuitable diet. Pauwels et al. (2015) reported that, unlike slow-growing chicken, the fast-growing broiler is low in diet flexibility, and a significant negative effect was found on the fast-growing broiler body weight that was given a scavenger diet.

The bodyweight growth measurement of all chickens in this study was started directly after hatching until week 7. The early growth of chickens was measured at day 0, 3 and 5 post-hatches (then mentioned as P0, P3, and P5, respectively) to observe the total body weight and breast yield as presented in Table 2. The broiler chicken had the highest body and breast weight among all chicken breeds at P0, P3, and P5. On the other hand, hybrid chicken showed higher body and breast weight than layer and pelung at P3 and P5 ($p > 0.05$). However, as shown in Table 3, hybrid chicken breast yield percentage did not show a significant difference with layer chicken at P5.

Dixon et al. (2016) reported that chick's early development might have an important effect throughout life. Several factors have been reported to play an important role in early body weight growth, such as genetic selection from different breeds (Buzala et al. 2015). On the other hand, Everaert et al. (2010) reported that protein nutrition plays a big role in the early body weight growth of broiler chicken. As shown in Table 3, all individuals observed in this study encountered breast area enlargement. Velleman (2007) explained that post-hatch muscle growth happens due to hypertrophy due to satellite cell nuclei recruitment.

The chickens then continued being kept and weighed weekly until week seven and slaughtered for meat quality analysis. The weekly growth of all groups starting from week one to week seven is presented in Table 4 and the weekly weight gain from week 0 to week seven is shown in Table 5. Weekly growth of broiler chicken was consistently the highest among the others for seven weeks. On the other hand, hybrid chicken followed in the

Table 2. Early weight and muscle growth comparison among chicken breeds (n=3).

Chicken Breeds	Total Weight (g)			Breast Muscle Weight (g)		
	P0	P3	P5	P0	P3	P5
Pelung	27.96±3.33 [a]	36.00±1.00 [a]	41.00±0.00 [a]	0.34±0.04 [a]	0.620±0.04 [b]	0.680±0.08 [a]
Broiler	40.83±2.48 [d]	85.00±13.53 [d]	87.33±9.50 [d]	0.50±0.02 [c]	2.607±0.52 [d]	4.700±1.44 [d]
Layer	38.93±0.62 [c]	39.00±3.46 [b]	54.33±5.69 [b]	0.50±0.06 [c]	0.613±0.11 [a]	1.727±0.29 [b]
Hibrida	35.06±3.15 [b]	45.00±3.00 [c]	60.00±4.00 [c]	0.41±0.03 [b]	0.830±0.11 [c]	1.920±0.11 [c]

[a-d]Means with the different superscripts in the same column are significantly different ($p < 0.05$).

Table 3. Individual breast muscle area in early post-hatch chicken (n=3).

Chicken Breeds	Breast Muscle Area (cm^2)			Breast Yield (%)		
	P0	P3	P5	P0	P3	P5
Pelung	0.878±0.06 [a]	1.500±0.18 [a]	2.191±0.07 [a]	1.22[a]	1.72[b]	1.66[a]
Broiler	1.685±0.21 [c]	5.752±0.29 [d]	8.440±0.68 [d]	1.22[a]	3.07[c]	5.38[c]
Layer	1.303±0.03 [b]	2.194±0.23 [b]	3.136±0.33 [b]	1.30[b]	1.57[a]	3.18[b]
Hibrida	1.373±0.28 [b]	2.324±0.08 [c]	3.241±0.22 [c]	1.17[a]	1.84[b]	3.20[b]

[a-d]Means with the different superscripts in the same column are significantly different ($p < 0.05$).

Table 4. Chicken weight gain for seven weeks (g; n=18).

Chicken Breeds	Weight Per Week (g; n=18)						
	1	2	3	4	5	6	7
Pelung	46.22±5.1[a]	92.56±10.6[b]	129.06±17.6[b]	189.44±23.0[b]	230.89±27.0[b]	325.33±45.7[b]	467.06±85.7[b]
Broiler	60.44±3.28[d]	123.06±23.19[c]	322.11±75.68[d]	521.11±88.62[d]	775.33±96.03[d]	1002.94±131.52[d]	1300.83±191.05[d]
Layer	51.33±1.91[c]	83.72±7.27[a]	118.94±17.04[a]	176.11±25.77[a]	224.17±25.68[a]	322.89±38.55[a]	436.39±42.33[a]
Hybrid	50.61±3.35[b]	122.39±3.35[c]	178.33±30.56[c]	279.06±40.51[c]	480.11±67.24[c]	651.61±99.88[c]	888.22±139.63[c]

[a-d] Means with the different superscripts in the same column are significantly different ($p < 0.05$).

Table 5. Weekly weight gain among chicken breeds (g; n=18).

Chicken Breeds	Weekly Weight Gain (g)						
	0 to 1	1 to 2	2 to 3	3 to 4	4 to 5	5 to 6	6 to 7
Pelung	11.72±3.83[a]	46.33±6.46[b]	36.50±9.95[a]	60.39±12.2[a]	41.44±15.04[a]	94.44±32.23[a]	141.72±47.67[b]
Broiler	40.89±9.64[c]	41.50±24.08[b]	198.17±59.37[c]	199.00±59.7[c]	254.22±44.51[c]	227.61±66.29[c]	297.89±79.51[d]
Layer	10.44±1.58[a]	32.39±6.06[a]	35.22±12.36[a]	57.17±18.54[a]	48.06±16.15[a]	98.72±44.20[a]	113.50±22.66[a]
Hybrid	19.78±6.92[b]	66.22±22.76[c]	55.94±18.68[b]	100.72±34.13[b]	201.06±42.53[b]	171.50±54.56[b]	236.61±77.32[c]

[a-d] Means with the different superscripts in the same column are significantly different ($p < 0.05$).

second place starting from week two until week seven. This data indicates that hybrid chicken had a better growth rate than layer and pelung and might be a better alternative for meat-type chicken.

The breast muscle yield and carcass quality of all chicken types at week seven are presented in Table 6. The breast yield and protein of broiler presented in Table 6 were significantly the highest compared to the other types. On the other hand, the layer showed the highest pH value and moisture. Pelung was observed to have the highest fat content and the lowest pH value among all chicken types. The hybrid type showed higher breast muscle weight than layer and pelung types and lower fat content than the broiler type. However, there was no significant difference in collagen among those four types ($p > 0.05$).

Breast muscle is frequently used in many research types as one of skeletal muscle development and poultry meat quality indicators. The breast yield measurement resulted in this research show that the broiler group has the highest breast yield percentage, followed by hybrid and layer groups. This finding agrees with Fanatico et al. (2005) reported that stated a fast-growing broiler obtains the highest breast yield percentage compared to the slow- and medium-growing broiler. Several genes affecting breast yield have been reported, such as insulin-like growth factor 1 (Sato et al. 2012), growth hormone receptor (Khaerunnisa et al. 2017), and chicken growth hormones (Anh et al. 2015). On the other hand, Berri et al. (2007) reported that high breast yield is associated with large fibre cross-sectional area and high pH post-mortem value, leading to better adaptation to further process of the meat.

One of the important factors determining chicken meat quality is its pH value (Le Bihan-Duval et al. 2008). After slaughter, oxygen availability is limited and causes the escalation of lactic acid production. Lactic acid accumulation in muscle causes the pH drops. It leads to protein denaturation, decreasing the protein solubility, and overall results in the decrease of the protein reactive sites, which play a role in water binding in the muscle protein. Low pH in meat causes the meat's protein to disperse, thus causing the light to bounce off and reflects pale colour (Mir et al. 2017).

The pH of the chicken meats observed in this research was diverse. Pelung had the lowest pH value among all types, while the layer had the highest pH. No significant difference was analysed in the pH of broiler and

Table 6. Breast muscle yield and carcass quality among chicken breeds on week 7 (n=6).

Chicken Breeds	Breast Muscle Weight (g)	Breast Yield (%)	pH	Collagen (%)	Fat (%)	Moisture (%)	Protein (%)
Pelung	30.833±3.03 [a]	5.66±1.84[a]	5.35±0.19[a]	1.43±0.33	4.27±0.73[a]	74.63±1.40[a]	21.58±0.35[b]
Broiler	283.500±31.73[c]	21.81±1.20[c]	5.61±0.03[b]	1.41±0.18	2.11±0.79[b]	73.22±0.34[b]	22.36±0.68[a]
Layer	39.640±3.94 [a]	9.083±0.95[b]	5.66±0.04 [c]	1.43±0.11	1.51±0.37[c]	74.12±0.46[a]	21.88±0.48[b]
Hybrid	90.500±6.18 [b]	10.71±3.12[b]	5.57±0.04 [b]	1.49±0.05	1.54±0.25[c]	73.94±0.84[b]	21.39±0.70[b]

[a-c] Means with the different superscripts in the same column are significantly different ($p< 0.05$).

hybrid chicken. These findings are aligned with the research conducted by Berri et al. (2007) that concluded that low post-mortem pH is associated with high post-mortem muscle glycolytic potential that causes high lactate content in muscle with small fibre cross-sectional area. The chicken, which has low pH, uses carbohydrate metabolism as the energy production process. On the other hand, chicken with high pH uses alternative catabolic pathways, compromising its muscle development and integrity (Beauclercq et al. 2017).

Another factor that determines the texture, and generally the meat quality of the chicken, is collagen. Collagen is the main component in intramuscular connective tissue that determines muscle hardness (Torrescano et al. 2003). Moreover, moisture plays a role in determining the meat tenderness, juiciness, firmness, and appearance that is important to the meat quality and economic value (Mir et al. 2017).

Intramuscular fat (IMF) definition refers to the fat that is deposited between myofibers. The IMF plays a crucial role in defining meat characteristics such as meat tenderness, juiciness, taste, and consumers' acceptability (Guo-Bin et al. 2010). Liu et al. (2017) reported that IMF deposition starts from incubation day 17 until post-hatch day one and is regulated by the fatty acid pathway's beta-oxidation. Several factors are reported affecting IMF content, such as sex (Guo-Bin et al. 2010), age (Liu et al. 2016; Decai et al. 2017), genetics (Ye et al. 2014), and muscle type (Hocquette et al. 2010). The results of this study showed that hybrid chicken contained lower fat content (1.54±0.25 %) compared to broiler and pelung, with no significant difference with layer (p>0.05) on the same age and the same feeding. This finding is in agreement with the research by Liu et al. (2019) that reported nine genes were related to differential IMF deposition between chicken breeds.

The body growth, breast yield, and meat characteristics of the hybrid chicken derived from crossing between pelung, broiler, and layer chickens show intermediate characteristics resulting from the combination of the elder chickens. The results from this study showed the pelung hybrid chicken had lower body weight on week 7 (888.22±139.63 gram) compared to broiler (1300.83±191.05 gram), but significantly higher than layer (436.39±42.33 gram) and pelung (467.06±85.7 gram; p<0.05). The pelung hybrid chicken meat contained 1.54±0.25% fat, which was lower than pelung (4.27±0.73%) and broiler (2.11±0.79%). Pelung hybrid had 5.57±0.04 pH value which was higher than the pelung had (5.35±0.19) as well. On the other hand, pelung hybrid had lower protein content (21.39±0.70%) than broiler (22.36±0.68%) significantly (p<0.05). Breast yield of the hybrid chicken was 10.71±3.12%, which was significantly higher than pelung (5.66±1.84%; p<0.05). These characteristics of hybrid chicken breast muscle led to the conclusion that hybrid chicken is a potential alternative meat-type chicken with local chicken meat characteristics and faster growth than slow-type chickens such as layer and pelung.

AUTHORS CONTRIBUTION

U.E.P conducted the research, data analysis and wrote the manuscript, H.T.S.S.G.S supervised the research and manuscript writing, T.H. and B.S.D. supervised and designed the research.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Short Communication

Sibling Indices as Comparisons in Personal Identification Process through Short Tandem Repeats [STR] Loci CSF1PO, THOI, TPOX, vWA of Maduranese Ethnic in Surabaya

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ABSTRACT
Sibling indices can be used as a comparison through alleles Short Tandem Repeats [STR] loci. This is an observational study among Maduranese with 4 STR loci (CSF1PO, THOI, TPOX, vWA) obtained from their blood samples. The percentage of alleles shared: 82.5% [33 times] with 2 allele sharing, 12.5% [5 times] with 1 allele sharing, and 5 % [2 times] with 0 sharing alleles. Sibling indices (SI) calculation results: 65% of sibling indices pairs have SI greater than 100 and 15% of them were between 10-100 (strong and very strong). Sibling indices interpretation is supported; therefore, the claimed sibling indices relationships were indeed true among Maduranese ethnic group in Surabaya.

Keywords: Allele sharing, Maduranese , sibling indices, STR DNA

The identification process through deoxyribonucleic acid [DNA] analysis is based on the allele comparison process, which is through a sample that is found and compared to another allele from another person in the family line, especially from the parents or children. The allele is known to use the Short Tandem Repeats [STR] loci is widespread through human genome and the largest source of polymorphic markers that can be acknowledged by Polymerase Chain Reaction. Determining STR is a strict process, the purpose of identification using STR is useful in various cases such as when determining a perpetrator of a violent act (murder and sexual assault) (Butler 2006; Butler & Hill 2012; Butler 2015).

In certain conditions, such as the absence of parents or children, comparison f rom a close family line is needed as one of the alternatives for forensic DNA analysis process, such as from sibling indices (Fung et al. 2004; Consentino et al. 2015; Abbas et al. 2018). Sibling indices that are involved in forensic identification process are also taken their alleles at STR loci which is located at nuclear DNA. However, the use of sibling indices as a comparison through STR is still unknown in Indonesia and other countries

despite demands from ethnic diversity, population, natural disasters, and many other events that require forensic preparedness (Consetino et al. 2015; Karbeyaz et al. 2016).

This identification process through STR loci is important for broader purposes such as determining sibling indices. Genomic variation occurs beyond geographic boundaries, individual loci, and population-based STR accuracy determination. Determination of inclusive STR in a country not only helps in database and reference point formations, but also a series of known core STRs is able to effectively determine a population (Untoro et al. 2009; Maeda et al. 2015; Abbas et al. 2018).

The reason behind the selection of Maduranese ethnicity in this study is that in general, Maduranese is generally known to be temperamental and has a distinctive accent. Most Maduranese people have high work ethic and adventurous spirit, which make them prefer to migrate from their place of origin, and in fact, 20-30% of Surabaya population is of Maduranese descent (Prastowo et al. 2018; Sosiawan et al. 2019).

This study aims to analyze allelic sharing of STR loci in sibling indices among the Maduranese ethnic group in Surabaya as an alternative source of forensic identification in the absence of their parents. This study also uses four kinds of STR loci (CSF1PO, THOI, TPOX, vWA) as the main STR loci. It is not the miniature of the previous study conducted by Sosiawan et al. (2019) that has been published because there are two things that strengthen this statement, such as: 1. The CODIS used in these two journals are different, 2. The populations used in these two journals are different. The previous journal talked about the Maduranese population lived in Madura Island. This journal talks about the Maduranese population lived in Surabaya, so the subject is very different. The uses of these four STR loci are based on the previous finding by Prastowo et al. (2018). His study mentioned that these four STR loci had the highest power discriminant in Maduranese, so it was suitable to use it as an alternative source of forensic identification in the absence of their parents (Prastowo et al. 2018).

Forensic identification means that identification must be done to prove someone's relationship (origin of the child, paternity cases, genealogical relation, or identifying unknown crime victims) therefore there should be no errors in its identification. One of the Forensic Identifications can be done using deoxyribonucleic acid (DNA) examination because it has achieved greater recognition in supporting Indonesia's law enforcement (Atmaja 2005; Karni et al. 2013; Yudianto & Setiawan 2020). This study also used Maduranese populations because these populations are close to the mobilization to Surabaya City.

The sample of the study was volunteers' DNA from families consisting of parents and children. This study has received ethical eligibility from the Dentistry Faculty of Airlangga University No: 275 / HRECC.FODM / VI / 2020. This research conducted by using a total of 40 blood samples taken from a total of 10 families. Each family consists of a biological father, mother, and two children who are not twins. All samples collected in blood collection tubes marked with the letters F [father], M [mother], and S [child] to represent samples from the biological father, mother, and child, and isolated DNA pellets in each pair contains 10 DNA pairs added with 50 µl of distilled water (Chomczynski et al. 1997).

DNA amplification process was carried out via PCR-STR process (PowerPlex® 21Systems, Promega, USA) which objected to characteristic DNA sequence area to make a number of the isolated DNA's copies. Multiplication process of all 40 samples used 4 autosomal STR locus (CSF1PO, TH01, TPOX, vWA) using primers CSF1PO: 5'-AACCTGAGTC TGCCAAGGACTAGC-3' and 5'-TTCCACACACCACTGGCCATCTTC-

3', THOI: 5'-CTGGGCACGTGAGGGCAGCGTCT-3' and 5'-TGCCGG AAGTCCATCCTCACAGTC-3', TPOX: 5'-ACTGGCACAGAACAGGC ATCTAGG-3' and 5'-GGAGGAACTGGGAACCACACAGGT-3', vWA: 5'-CCTAGTGGATGATAAGAATAATCAGTATG-3' and 5'-GGACAGA TGATAAATACATAGGATGGATGG-3'. The PCR settings loci CSF1PO, THOI, TPOX, and vWA were used as a manual procedure derived from Promega (Gene Amp^{r} PCR System 9700 Thermal Cycler, Promega Corp. 2001). After being amplified by PCR, the PCR results were electrophoresed vertically with polyacrylamide agarose gel [PAGE] 6% [Bio-Rad Mini-PROTEAN®] with Silver Nitrate staining.

Sample's Allele Profile through Allele Sharing The readings from electrophoresis gel were alleles for each locus with K562 as control. The analysis was based on allele sharing frequency from kinship analysis, where the sibling indices' STR loci were used to determine specific STR loci that have kinship among Maduranese in Surabaya. Furthermore, it is analyzed through allele sharing as shown in Figure 1. Furthermore, the Sibling indices Index/full sibling indices index/Sibship index (SI) was determined. SI is calculated based on kinship analysis in the equation (Slooten 2011). Allele probability is taken from the allele frequency of the Indonesian population (Untoro et al. 2009). Combined sibship indices (CSI) were determined by multiplying individual SI values of the selected locus and projected for each pair locus. (Wenk 1996) (Table 1).

Based on Figure 1, allele sharing percentage in the current study from 40 observations (10 pairs x 4 loci) in sibling indices were as followed: 82.5% [33 times] with 2 allele sharing, 12.5% [5 times] with 1 allele sharing and 5% [2 times] with 0 allele sharing.

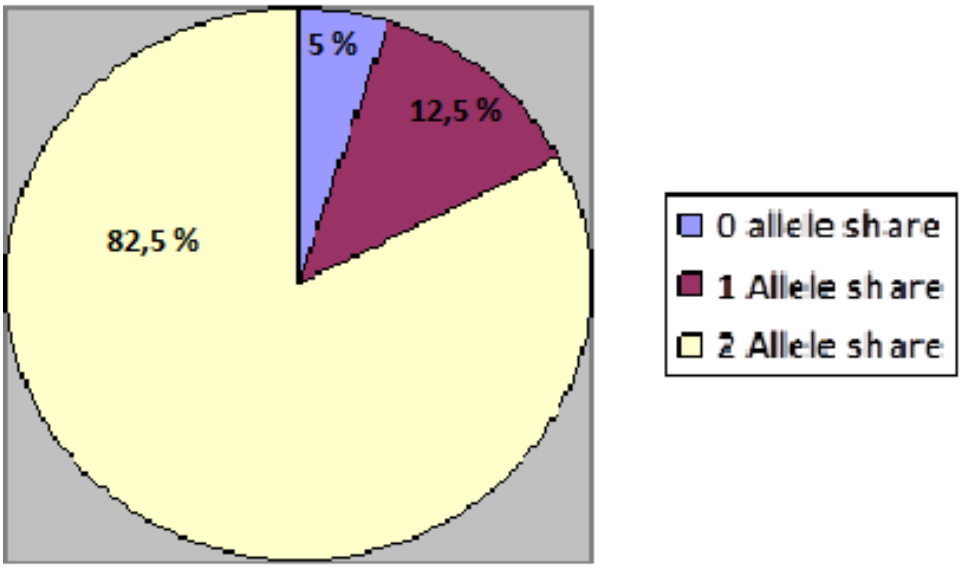

Figure 1. Allele sharing percentage in sibling indices.

Sibship Indices [SI] calculation in this study is shown in the Table below:

Table 1. Sibship Indices [SI] percentage in this study (Wenk 1996).

Probability ration in the form of *Sibship indices* (SI)	Strength	Percentage
<1	Weak	10%
1 – 10	Moderate	10%
10 -100	Strong	15%
>100	Very Strong	60%

Sibling Indices (SI) are shown in both table 1 and based on Figure 2 calculation, they showed that 65% of sibling pairs had SI greater than 100 and 15% of them had SI between 10-100. Its SI could be categorized as strong and very strong.

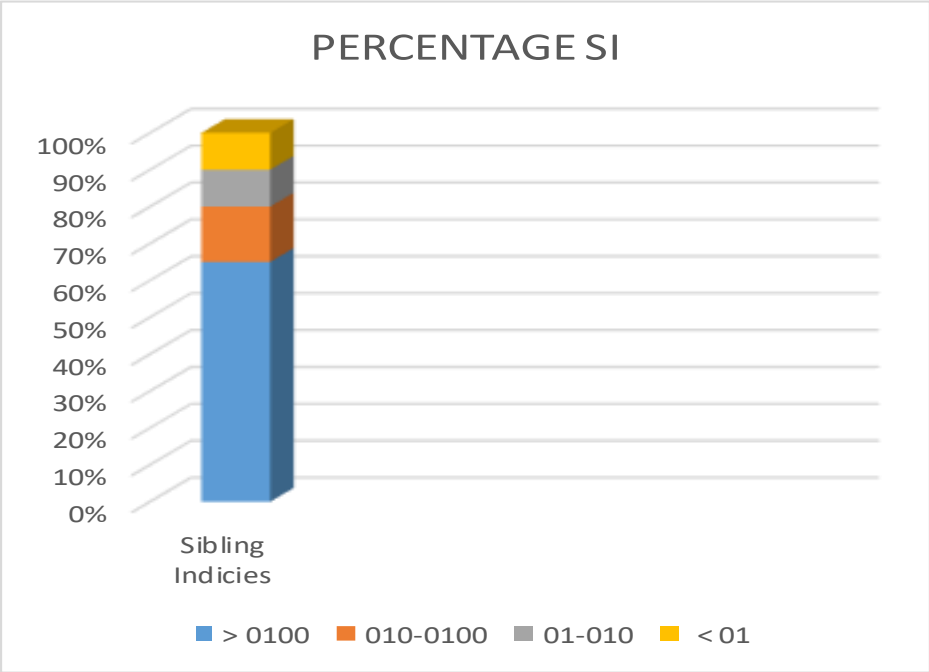

Figure 2. Percentage of Sibling Indicies (SI) in this study.

Areas containing repeating nucleotide sequences (eg: STR sequences) attract the attention of forensic experts because they contain a lot of information about variations and can be used for human identification. Repeating nucleotide sequences is used in identification, primarily in paternity tests. The paternity test is the use of a DNA profile to specify whether an individual is the biological parents of other individuals. A paternity test can be especially important when the rights and obligations of the father are at issue and the paternity of the child is in doubt. The test can also predict the probability of becoming a biological grandfather. There are older methods that also exist as a genetic testing to predict the probability of becoming a biological grandfather, such as ABO blood group, analysis of various proteins and enzymes (Human Leukocyte Antigen). (Omran et al. 2009; O'Connor 2011; Kido et al. 2003; Hares 2015; Marano et al. 2019).

STR has alleles for each locus. In allele examinations, allele frequencies are presented in the form of homozygosity, heterozygosity, the effective number of alleles (n), polymorphism information content (PIC), the power of discrimination (DP), and the power of exclusion (PE). A good index for the genetic polymorphism is based on the number of alleles because of the presence of characteristic alleles in the population. Therefore, it is necessary to carry out genetic screening before conducting a study. It is theorized that the larger the population size, the number of alleles observed will be also increased.

If the biological parents or children are not present in the paternity test, a comparison is needed from a close family line as an alternative for identification checks through DNA, such as from sibling indices. The use of sibling indices as a comparison is one of the identification methods (Reid et al. 2008: O'Connor 2011). Sibling indices will share zero or two identical alleles through offspring at certain loci with the same probability of 0.25 and share one allele with a probability of 0.5 (Wenk 1996; O'Connor 2011). In

the current study, sibling indices shared 5%, 12.5%, and 82.5% of zero, one, and two identical alleles, respectively, at CSF1PO, TH01, TPOX, and vWA loci. This is a different result from a study by Wenk (1996). This difference is likely caused by ethnicity that has different genetic contributions between populations. This can be attributed to historical and demographic processes that lead to genetic drift (Yamamoto et al. 2003).

In Sibling indices (SI) calculation, 65% of sibling indices pairs had SI greater than 100 and 15% of them had SI between 10-100 (strong and very strong), indicating that the use of STR loci CSF1PO, TH01, TPOX, and vWA might be very predictive to identify sibling indices from the Maduranese ethnicity in Surabaya.

If SI is below 10, then two alternative study options can be made. First, the study must use other loci that are still related to the selected locus. The second is that the study could use additional DNA testing from their uncles, aunts, and cousins. The first alternative is based on the Combined Sibling Indices (CIS) guideline for test results, where 90% of the test loci have a strong probability. The final alternative is in accordance with CIS Guidelines for evaluating sibling indices DNA testing (Wenk et al. 1996; Slooten et al. 2011).

This study indicates that two allele sharing is the strongest compared to one allele sharing and zero allele sharing. The value of 82.5% with two allele sharing indicated that the sibling indices's interpretation is fully supported, therefore the claimed relationship is true. Difficulties in the form of minimal DNA samples that were obtained in this study can be overcome by using other loci associated with the selected locus or additional DNA from uncles, aunts, and cousins.

AUTHORS CONTRIBUTION

A.Y. designed the research and supervised all of the processes, S.M.M.N. collected and analyzed the data, F.S. wrote and submit this manuscript.

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CONFLICT OF INTEREST

The authors declare that there are no conflict interests in publishing this article.

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Research Article

DNA Barcode of Barred Mudskipper (*Periophthalmus argentilineatus* Valenciennes, 1837) from Tekolok Estuary (West Nusa Tenggara, Indonesia) and Their Phylogenetic Relationship with Other Indonesian Barred Mudskippers

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ABSTRACT
Barred mudskipper (*Periophthalmus argentilineatus*) has a potency to be developed as protein for human consumption and ornamental fish. The fish also has an important role in mangrove ecosystems. Nevertheless, many barred mudskippers have been considered a cryptic species. Therefore, accurate identification is needed to clarify species identification of the barred mudskipper using DNA barcoding. This research aimed to identify barred mudskippers from Tekolok Estuary (East Lombok, West Nusa Tenggara, Indonesia) using *COI* mitochondrial gene as a DNA barcode and analyze genetic relationship with other barred mudskippers from several regions of Indonesia recorded in GenBank. This study used a PCR method with universal primers FishF2 and FishR2. The data was then analysed using DNASTAR, BLAST, Mesquite, MEGA, DnaSP, BEAST, GenAlEx, and NETWORK. The results revealed that barred mudskipper from Tekolok Estuary has been verified as *Periophthalmus argentilineatus*. The results also exhibited that *P. argentilineatus* from Tekolok Estuary has a close genetic relationship to *P.argentilineatus* from Tukad Bilukpoh (Jembrana, Bali). In addition, phylogenetic analysis showed that *P.argentilineatus* from Indonesia consisted of two clades with a genetic distance of approximately 6.64%. This analysis revealed evidence of the cryptic diversity of *P.argentilineatus* from Indonesia. Further detailed studies are needed to clarify whether Indonesian *P.argentilineatus* should be categorized into more than one species or single species with several subspecies.

Keywords: barred mudskipper, cryptic diversity, DNA barcode, polymorphism

INTRODUCTION
Barred mudskipper (*Periophthalmus argentilineatus*) belongs to suborder Gobioidei, family Gobiidae and subfamily Oxudercinae. Family Gobiidae consists of 130 genera with 1,866 species (Nelson 2016; Eschmeyer 2019). One of the most widely distributed of this family in the Indo-Pacific region is the genus *Periophthalmus* and according to Froese and Pauly (2004), the genus *Periophthalmus* consists of about 20 valid species.

Barred mudskipper is like an amphibian, so the fish is commonly known as an amphibious fish. The fish has a large-eyed feature and sticks out of its head, and the pectoral fins at the base are muscular and these fins

can be bent to function like arms that can be used to crawl or jump on mud and perch on mangrove roots (Elviana & Sunarni 2018). Barred mudskipper spends mostly outside of the water and prefers muddy habitat especially in mangrove areas (Vanhove et al. 2012). The mangrove habitat is a suitable habitat for barred mudskipper survival life due to lots of detritus which is used for the fish food. In addition, barred mudskipper is also a carnivorous opportunist feeder eating small crabs, small fish, shrimps, and other small arthropods that are commonly found in mangrove habitat. Barred mudskipper can also inhabit extreme conditions (Lee et al. 2005).

Previous studies have reported a common phenomenon of cryptic species in the members of the suborder Gobioidei including barred mudskippers which have a high similar morphology but genetically different. As a consequence of this phenomenon is taxonomic confusion. This is due to inaccurate identification of a species indicated by a cryptic species may lead to erroneous conclusions in naming species (Leys et al. 2016). This phenomenon can cause synonymous problems where there is a double name in the same species or cause an increase in cryptic diversity defined as two or more different species and classified as a single species due to morphological similarities (Tronteli & Fisher 2009). Double species name due to misidentification will have an impact on conservation efforts and sustainable use.

The discovery of cryptic species was reported from members of family Rhyacichthyidae, Odontobutidae, Xenisthmidae, Eleotridae Gobiidae, Kraemeriidae, Schindleriidae, Microdesmidae, and Pereleotridae using mitochondrial genes: *ND1, ND2* and *COI* (Thacker 2003; Thacker & Hardman 2005), genus *Schindleria* (family Schindleriidae) using *16S* mitochondrial gene (Kon et al. 2007), genus *Economidichthys*, genus *Knipowitschia*, and genus *Pomatoschistus* using mitochondrial genes *12S* and *16S* (Vanhove et al. 2012). Comprehensive research was conducted by Agorreta et al. (2013) in members of the suborder Gobioidae using four mitochondrial genes (*12S, 16S, COI, Cyt b*) and two nuclear genes (*Zicf* and *RAG-1*), but this study did not include members of the genus *Periophthalmus*. The cryptic diversity of *P. argentilineatus* and *P. kalolo* was also investigated by Polgar et al. (2014) using two mitochondrial genes namely *D-Loop* and *16S* as well as one nuclear gene namely *RAG-1*. Research on the molecular identification of mudskippers belonging to the genus *Periophthalmus* using the *COI* mitochondrial gene as a DNA barcoding marker in Indonesia is still very limited. Dahruddin et al. (2017) identified three species of members of the genus *Periophthalmus* namely *P. novemradiatus*, *P.kalolo*, and *P. argentilineatus* in Java (Pandeglang, Cilacap, Tulung Agung) and Bali (Jembrana). Furthermore, Arisuryanti et al. (2018) successfully identified two species namely *P. argentilineatus* and *P. kalolo* in Bogowonto Lagoon (Yogyakarta). This study also revealed that *P. argentilineatus* from Bogowonto Lagoon has a close genetic relationship with *P. argentilineatus* from Pandeglang. However, no genetic information of barred mudskipper has been conducted from Tekolok Estuary (West Nusa Tenggara). Therefore, this study used *COI* mitochondrial gene as a DNA barcoding marker to identify barred mudskippers from Tekolok Estuary and analyze their genetic relationship with other Indonesian barred mudskippers recorded in GenBank. The use of *COI* mtDNA as a DNA barcoding marker for a molecular-based approach to the taxonomic investigation such as identification of closely related species, revealing cryptic speciation and phylogeographic structures within a species has been conducted for over recent years (Healey et al. 2018; Shelley et al. 2018; Rathipriya et al. 2019). This marker is a molecular standard approach developed by Hebert et al. (2003) which is applied to identify species and support species delimitations.

MATERIALS AND METHODS

Sample collection, DNA extraction, amplification, and sequencing of COI mitochondrial gene

Ten barred mudskippers (code MSL) were collected from Tekolok Estuary, East Lombok, West Nusa Tenggara, Indonesia (8°20'30.0"S 116°42'31.0"E) (Figure 1). Before dissecting the tissue sample, the barred mudskipper was documented (Figure 2). The tissue sample contained 50-100 mg of muscle tissue. Each tissue sample was dissected with a sterilized surgical scissor, placed into a 1.5 ml tube, and preserved in 99% ethanol in the field, and stored at -20˚C in the laboratory for further analysis.

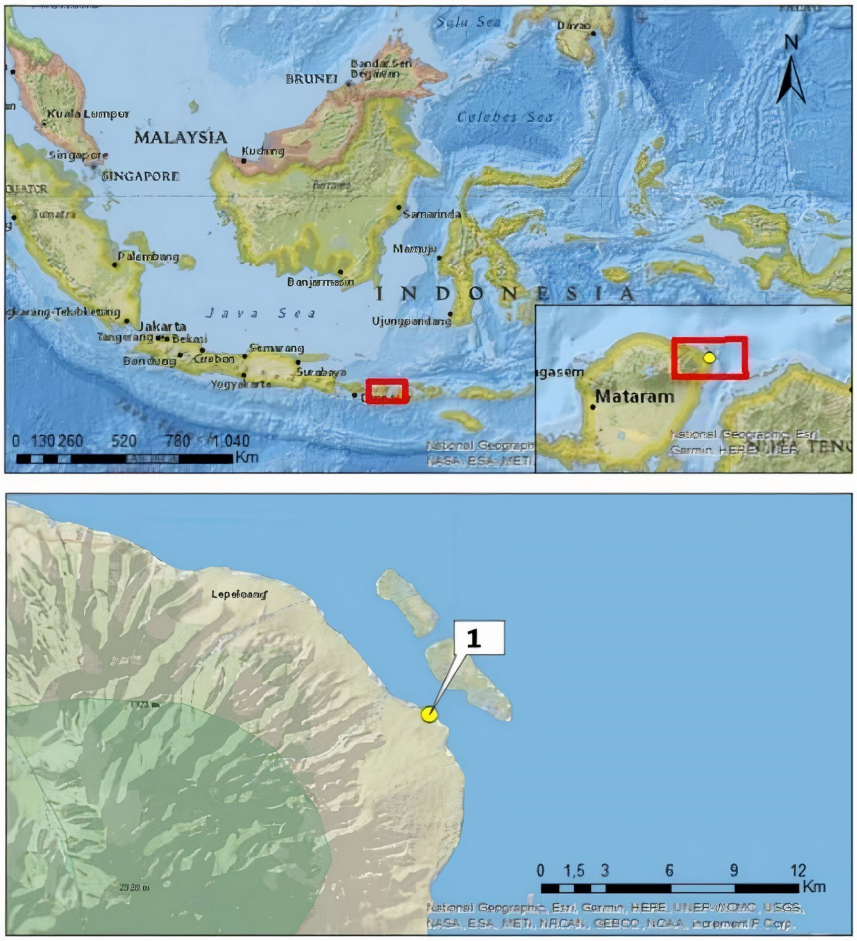

Figure 1. Map location for collecting barred mudskipper at Tekolok Estuary (West Nusa Tenggara, Indonesia).

Figure 2. Barred mudskipper collected from Tekolok Estuary. Bar = 1 cm.

Total Genomic DNA was extracted from muscle tissue of each specimen using DNeasy blood and tissue kit (QIAGEN, Valencia, CA, USA) according to the manufacture's protocols. The partial *COI* mitochondrial gene was amplified using primers FishF2 (5'-TCGACTAATCATAAAGATATCGGCAC-3') and FishR2 (5'-ACTTCAGGGTGACC GAAGAATCAGAA-3') (Ward et al. 2005). The MyTaq HS Red Mix PCR kit (Bioline) was used for the polymerase chain reaction (PCR) and PCR amplifications were conducted in 50µL reaction volumes containing 10-100 ng of genomic DNA, 25µL MyTaq HS Red Mix PCR, 1mM $MgCl_2$, 0.6µM of each primer and 11µL double distilled water (ddH2O). Negative control was set up by omitting template DNA from the reaction mixture to evaluate the reliability of the DNA amplification. The amplification process was conducted using the following cycling conditions as initially conducted by Arisuryanti et al. (2019). The PCR products were then run on 1% agarose gel buffered with Tris-acetate EDTA (TAE), stained with Gel Red (Bioline), and visualized under UV light. All amplification samples were then sent to First Base Sdn Bhd (Malaysia) through P.T. Genetika Science (Jakarta) for purification and sequencing in both forward and reverse directions using the Big Dye Terminator (Applied Biosystems) and the ABI 3730xl Genetic Analyzer (Applied Biosystems).

Genetic Analysis

Data obtained from DNA sequencing results were edited with SeqMan and EditSeq on the DNASTAR program (DNASTAR Inc. Madison, USA). For each individual, sequencing reactions were performed using both forward and reverse primers. Chromatograms were inspected manually to check ambiguous bases and stop codons. Next, the *COI* sequence data is converted to FASTA format through the Mesquite v.3.5 program (Maddison & Maddison 2018). *COI* sequences of barred mudskipper (*P. argentilineatus*) were then aligned using Opal in Mesquite v.3.5 (Maddison & Maddison 2018) and ClustalW on the MEGAX program (Kumar et al. 2018). In this study, 17 *COI* mitochondrial sequence data of *P. argentilineatus* (10 samples from Tekolok Estuary and 7 samples from several regions in Indonesia recorded in GenBank with accession number KU692743, KU692744, KU692745, KU692746, MT439598, MT439599, MT439600) were used to analyze the intraspecific genetic relationship and their phylogenetic relationship. The composition of the *COI* nucleotides was calculated through the EditSeq program in the DNA Statistics menu using DNASTAR software. The composition of C+G is validated using the DnaSP program v. 6 (Rozas et al. 2017). Intraspecific diversity was estimated as the number of haplotypes, the number of polymorphic sites, haplotype diversity, and nucleotide diversity using the software DnaSP v.6 (Rozas et al. 2017). Next, intraspecific genetic distance was analysed using the Kimura-2 Parameter (K2P) model and was summarised in a Neighbour-Joining (NJ) tree as this is the standard methodology used in barcoding studies with bootstrap 1,000 replicates (Hebert et al. 2003). Phylogenetic relationships were estimated using a Bayesian approach. The Akaike Information Criterion (AIC) implemented in jModelTest ver 2.1.10 (Darriba et al. 2012) was used to determine the best fit evolutionary model. Bayesian inference (BI) was performed with BEAST ver 1.10 (Suchard et al. 2018) under the best-fit model. Two simultaneous Markov chain analyses (MCMC) were run for 10^6 generations to estimate the posterior probabilities distribution with a sampling frequency set to every 1,000 generations. The analysis was done until the standard deviation of split frequencies was below 0.01. The analysis used a relative burn-in of 25% for diagnostics. Consensus trees were visualised in FigTree 1.4.4 (Rambaut 2019). *COI* sequences of eight other

species included in the genus *Periophthalmus* (accession number KU692751, KU692755, KU692756, KU692759, KP966189, KP966192, KT368097, and KT368106) and *Pseudapocryptes lanceolatus* (JX260938) were included in this analysis as an outgroup and to construct phylogeny tree. Patterns of divergence between haplotypes were analyzed using Principal Coordinate Analysis (PCoA) based on the genetic distance of *COI* mitochondrial gene conducted in GenAlEx version 6.51 (Peakall & Smouse 2012) and NETWORK version 10.1 (https://www.fluxus-engineering.com).

RESULTS AND DISCUSSION

PCR amplification result and DNA barcode of barred mudskipper from Tekolok Estuary

The results revealed that the amplification of *COI* mitochondrial gene from all 10 barred mudskipper fish from Tekolok Estuary produced fragment lengths between 600 and 700 bp (Figure 3). After editing on chromatograms, the resulting consensus sequence was 678 bp which can be translated into 226 amino acids. Furthermore, the results of the analysis using Nucleotide BLAST, 10 barred mudskipper samples from Tekolok Estuary investigated in this study had a similarity of 99.23-100% with *Periophthalmus argentilineatus* with accession number KU692743. Therefore, all of the ten barred mudskippers from Tekolok Estuary were verified as *Periophthalmus argentilineatus*. All of the *COI* mitochondrial genes of *P. argentilineatus* from Tekolok Estuary have been submitted to GenBank with accession number MW514015-MW514024.

Composition of mtDNA *COI* nucleotide

In this study, 17 samples of *P. argentilineatus* consisted of 10 samples of *P. argentilineatus* from Tekolok Estuary and 7 samples of *P. argentilineatus* from several regions in Indonesia recorded in GenBank were analyzed. After alignment on 17 samples of *P. argentilineatus* and obtained fragment length 519 bp. We can arrange the composition of mitochondrial COI nucleotide (Table 1). No similar value can be seen from all of the samples. From Table 1, the differentiation of the composition of nucleotide T, C, A, and G were

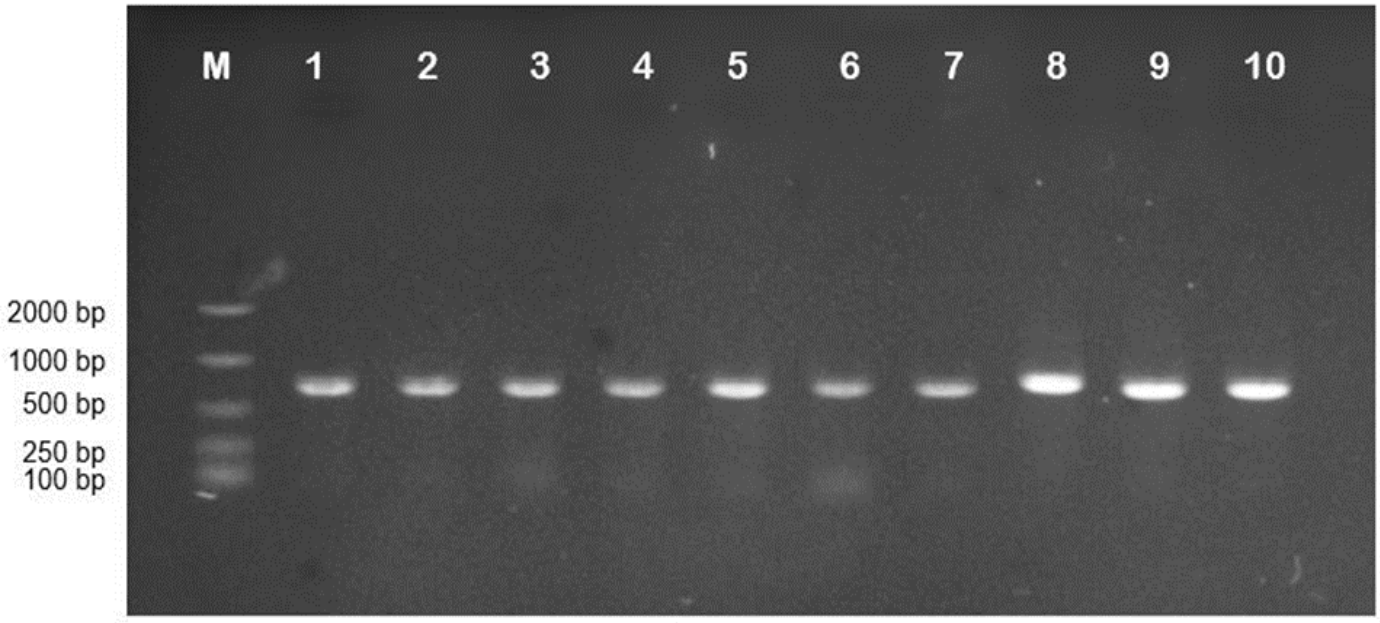

Figure 3. PCR amplification result of *COI* mtDNA of *P.argentilineatus* from Tekolok River migrated in 1% agarose gel electrophoresis. M is a marker visualised from DNA ladder 2 Kb (Bioline).

Table 1. Average composition of a 519 bp fragment of *COI* nucleotide of *P. argentilineatus* from Tekolok Estuary and other Indonesian regions recorded at GenBank database.

Sample Code	T(U)	C	A	G	A+T	C+G
MSL	33.43	25.70	25.49	15.38	58.92	41.08
KU692743*	33.53	25.63	25.63	15.22	59.15	40.85
KU692744*	33.33	25.63	25.43	15.61	58.77	41.23
KU692745*	33.53	25.43	25.43	15.61	58.96	41.04
KU692746*	33.53	25.43	25.43	15.61	58.96	41.04
MT439598*	33.72	25.24	25.43	15.61	58.15	41.85
MT439599*	33.53	25.43	25.43	15.61	58.96	41.04
MT439600*	33.53	25.43	25.43	15.61	58.96	41.04

* Samples were taken from GenBank.

0.0-0.20%, 0-0.46%, 0-0.20%, and 0-39% respectively. In addition, *P. argentilineatus* from Tekolok Estuary had the highest frequency of nucleotide C (25.70%) while *P. argentilineatus* from Pandeglang (KU692745 and KU692746) had similar nucleotide T, A, C, G with *P. argentilineatus* from Bogowonto Lagoon (MT439599 and MT439600). The result revealed the divergence of T, C, A, and G nucleotides in *COI* mitochondrial genes among *P. argentilineatus* samples analyzed in this study. This data revealed polymorphism in the *COI* mitochondrial gene of *P. argentilineatus* in Indonesia which indicated the intraspecific genetic variation of *P. argentilineatus*.

COI mitochondrial sequence variation and phylogenetic relationship

Seventeen *COI* sequences that were obtained from *P. argentilineatus* individuals from Tekolok Estuary and other Indonesia regions recorded at the GenBank database (Table 2) represented 10 distinct haplotypes. The 519-bp alignment contained 42 polymorphic sites (8.09%) with 34 being parsimony-informative (Figure 4) and no gaps and nonsense codon was found among the 17 sequences after the *COI* sequences were translated using the vertebrae mitochondrial code. From 42 polymorphic sites, 35 sites were detected transitions whereas 7 sites were transversions (Figure 4).

The optimal model of nucleotide sequence substitution for the *COI* gene including the outgroup samples was the HKY model with the gamma-distributed rate as inferred by jModelTest2 under the Akaike information criterion (AIC). The Bayesian analysis of *COI* mitochondrial data, together with additional sequences of eight species that belong to the genus *Periophthalmus* and *Pseudapocryptes lanceolatus* taken from GenBank, revealed that the barred mudskippers from Indonesia fall into two distinct clades (Clade A and B), with the associated nodes supported by posterior probability 1.00 (Figure 5). Similarly, the neighbour-joining (NJ) tree exhibited two distinct clades (Clade A and B) with a bootstrap value of 99% (Figure 6). The two clades or OTUs generally showed strong geographic patterns of differentiation. The clade A consisted *P. argentilineatus* from eastern part of Indonesian region (Tekolok Estuary, West Nusa Tenggara and Tukad Bilukpoh, Jembrana, Bali) while the clade B comprised *P. argentilineatus* from western (Pandeglang) and central part of Indonesian region (Bogowonto Lagoon, Yogyakarta). The geographic distribution within clades showed considerable overlap between haplotypes such as haplotype A_1 and B_2 (Figure 7).

The genetic distance based on Kimura-2 Parameter (K2P) model among all *COI* haplotypes within *P. argentilineatus* was quite variable ranging from 0.0 to 8.24% (mean= 3.43%) (Table 3). The average nucleotide sequence divergence between haplotypes within clade A and clade B is

Table 2. Grouping of the haplotype of *P. argentilineatus* based on phylogenetic analysis of the *COI* mitochondrial gene fragment.

Clade	Haplotype	Sample Number	Sample Code/Accession Number	Location
A	Hap-A_1	6	MSL-01	This study
			MSL-03	This study
			MSL-05	This study
			MSL-07	This study
			MSL-09	This study
			KU692743*	Tukad Bilukpoh, Jembrana, Bali
	Hap-A_2	1	MSL-02	This study
	Hap-A_3	1	MSL-04	This study
	Hap-A_4	1	MSL-06	This study
	Hap-A_5	1	MSL-08	This study
	Hap-A_6	1	MSL-10	This study
B	Hap-B_1	1	KU692744*	Pandeglang
	Hap-B_2	3	KU692745*	Pandeglang
			KU692746*	Pandeglang
			MT439599*	Bogowonto Lagoon, Kulon Progo,Yogyakarta
	Hap-B_3	1	MT439598*	Bogowonto Lagoon, Kulon Progo,Yogyakarta
	Hap-B_4	1	MT439600*	Bogowonto Lagoon, Kulon Progo,Yogyakarta

***** Samples were taken from GenBank.

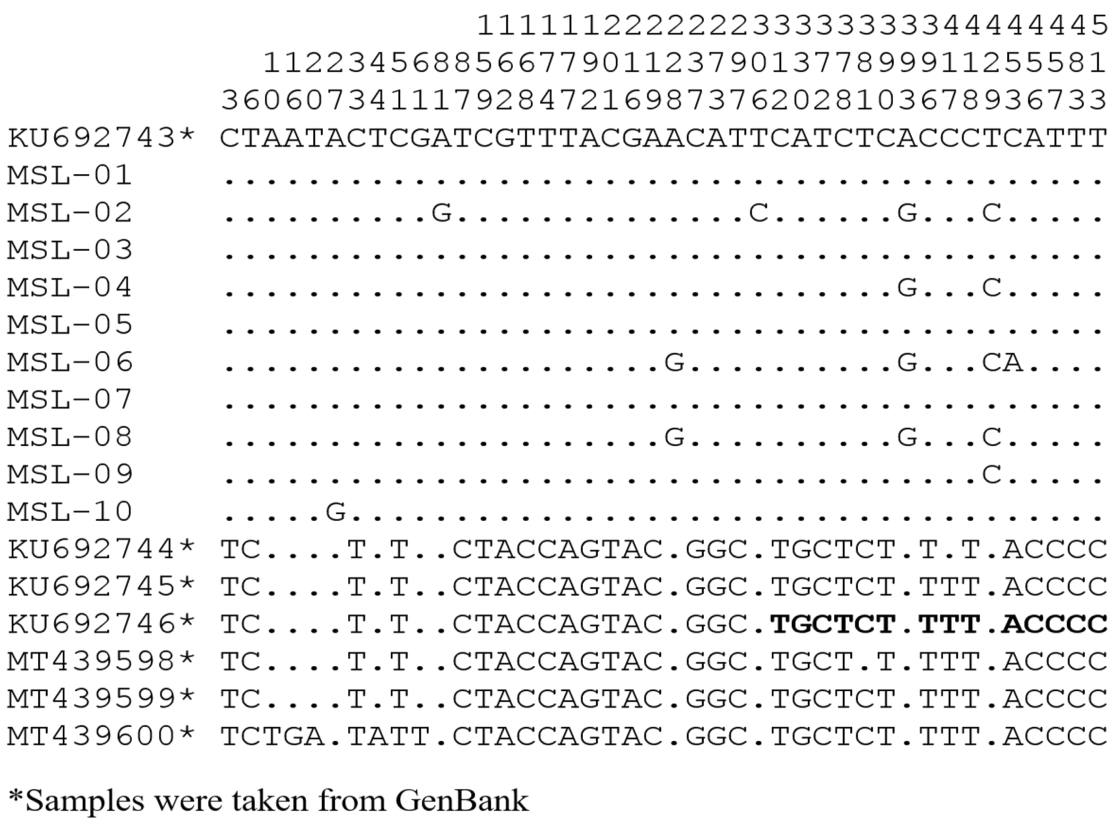

Figure 4. Summary of polymorphic sites in the partial *COI* mtDNA of *P. argentilineatus* analysed in this study. Only variable sites are show and dots indicate identity with *P. argentilineatus* (KU692743) sequence taken from GenBank. Number above corresponds to the nucleotide base pair position.

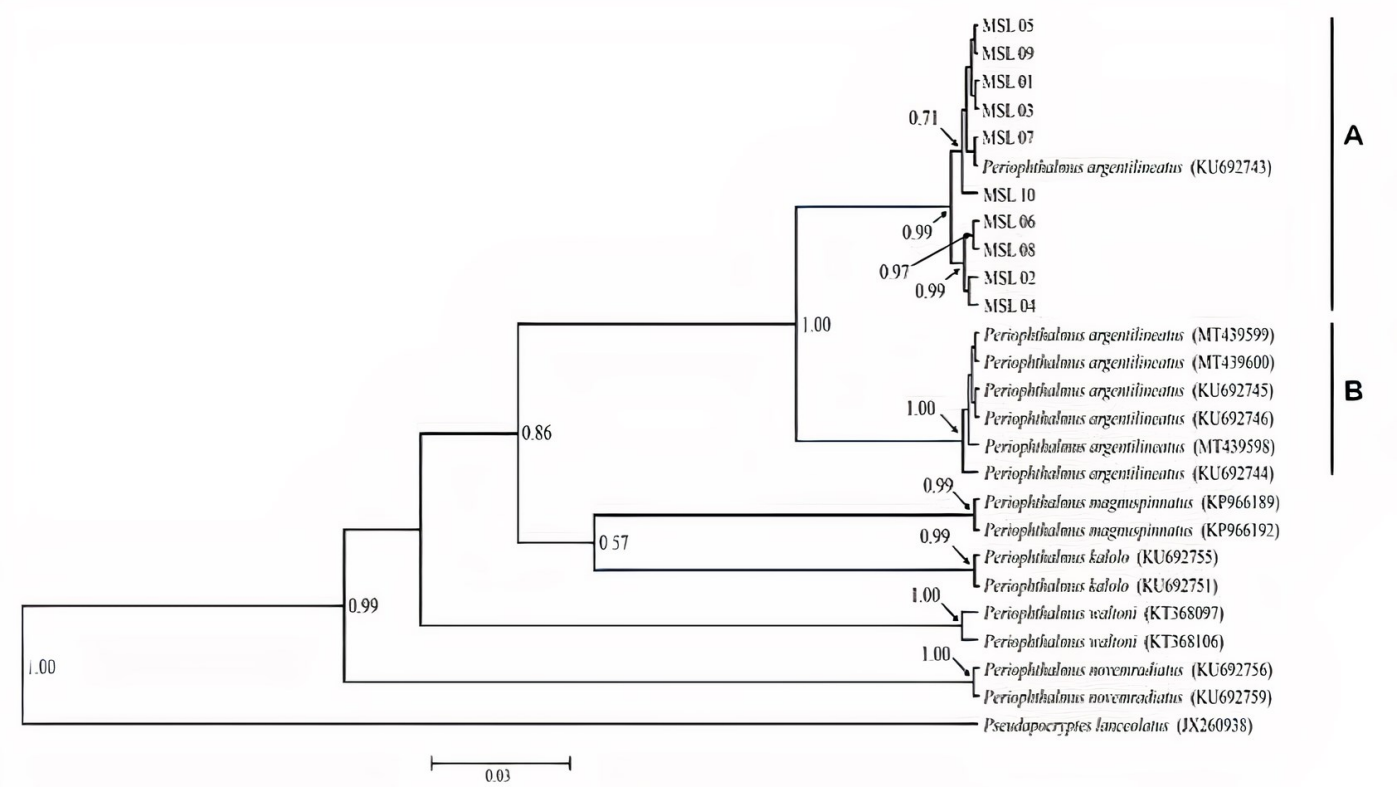

Figure 5. Bayesian tree inferred from *COI* mitochondrial gene sequences. Numbers of each node represent posterior probabilities and scale correspond to substitution/site.

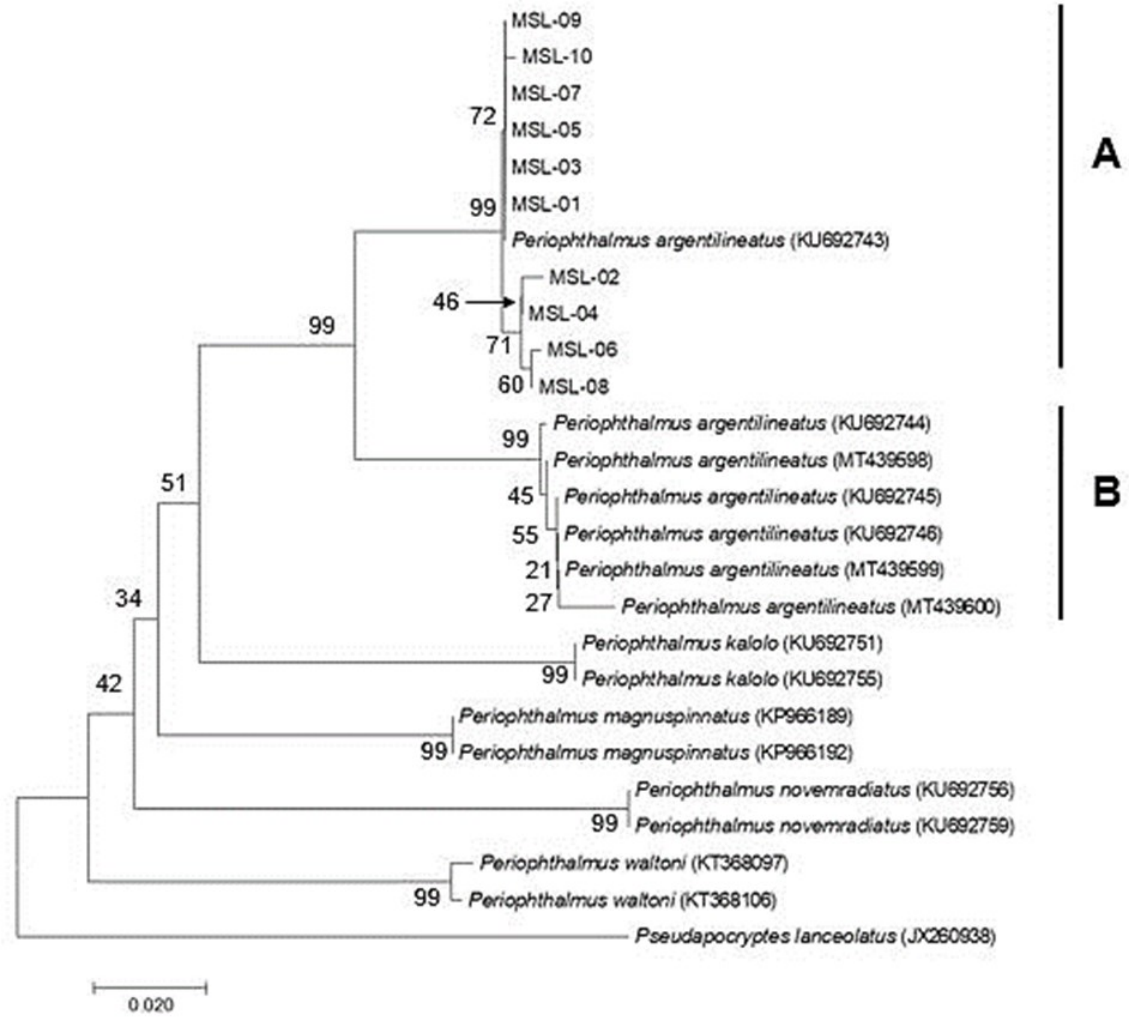

Figure 6. Neighbour-joining tree based on *COI* sequence data using Kimura-two parameter (K2P) substitution model. Number at each node represents bootstrap values and scale corresponds to substitution/site.

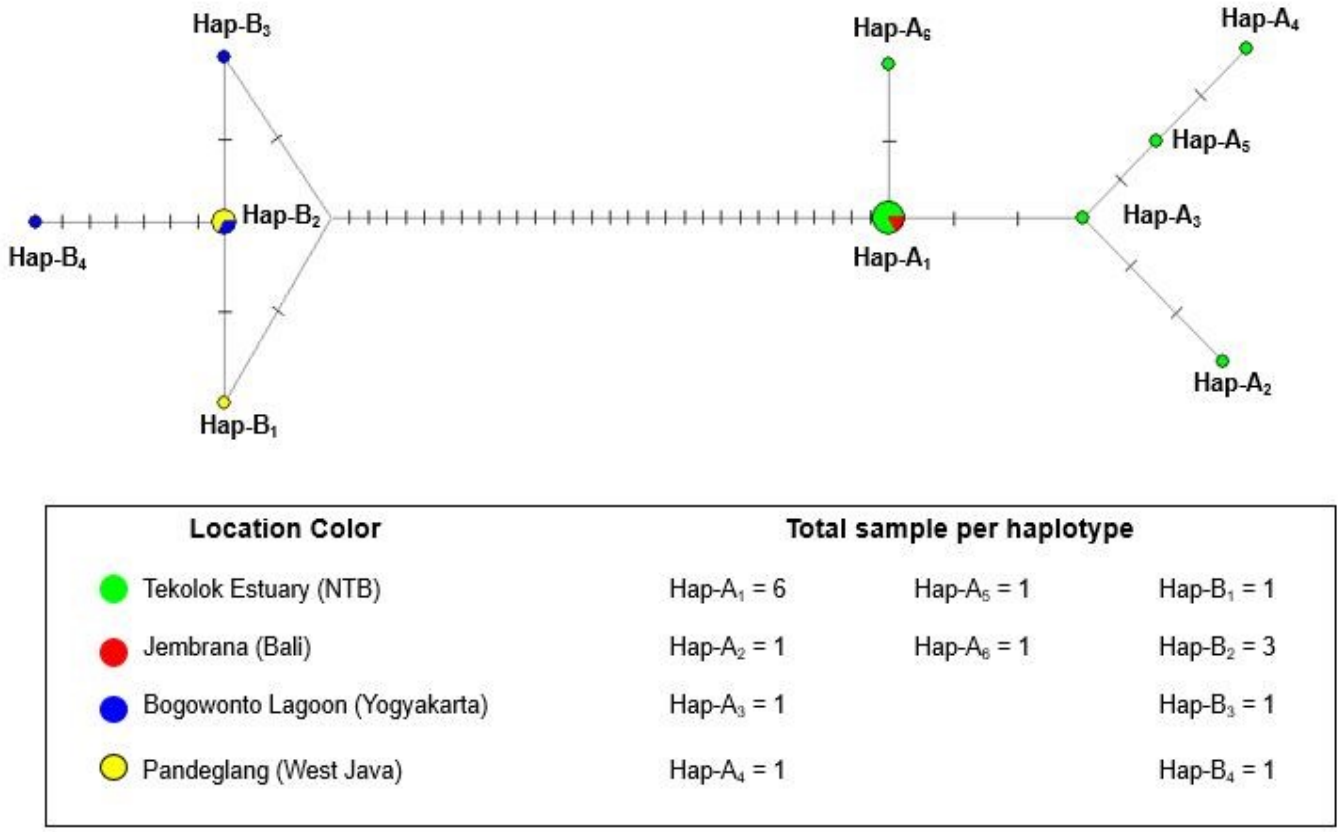

Figure 7. Construction of median joining network from 519 bp *COI* sequence of *P. argentilineatus* from several regions in

Table 3. Percentage nucleotide sequence divergence of a 519 bp fragment of the *COI* mitochondrial gene among samples of *P. argentilineatus* from Indonesia.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1.MSL-01	-																
2.MSL-02	0.78	-															
3.MSL-03	0.00	0.78	-														
4.MSL-04	0.39	0.39	0.39	-													
5.MSL-05	0.00	0.78	0.00	0.39	-												
6.MSL-06	0.78	0.78	0.78	0.39	0.78	-											
7.MSL-07	0.00	0.78	0.00	0.39	0.00	0.78	-										
8.MSL-08	0.58	0.58	0.58	0.19	0.58	0.19	0.57	-									
9.MSL-09	0.00	0.78	0.00	0.39	0.00	0.78	0.00	0.58	-								
10.MSL-10	0.19	0.97	0.19	0.58	0.19	0.97	0.19	0.78	0.19	-							
11.KU692743*	0.00	0.78	0.00	0.39	0.00	0.78	0.00	0.58	0.00	0.19	-						
12.KU692744*	6.09	6.96	6.09	6.52	6.09	6.53	6.09	6.74	6.09	6.30	6.09	-					
13.KU692745*	6.30	7.18	6.30	6.74	6.30	6.75	6.30	6.96	6.30	6.52	6.30	0.19	-				
14.KU692746*	6.30	7.18	6.30	6.74	6.30	6.75	6.30	6.96	6.30	6.52	6.30	0.19	0.00	-			
15.MT439598*	6.09	6.96	6.09	6.52	6.09	6.53	6.09	6.74	6.09	6.30	6.09	0.39	0.19	0.19	-		
16.MT439599*	6.30	7.18	6.30	6.74	6.30	6.75	6.30	6.96	6.30	6.52	6.30	0.19	0.00	0.00	0.19	-	
17.MT439600*	7.35	8.24	7.35	7.79	7.35	7.80	7.35	8.02	7.35	7.57	7.35	1.17	0.97	0.97	1.17	0.97	-

* Samples were taken from GenBank.

0.402% (range 0-0-0.97%) and 0.452% (0-1.17%) respectively. The genetic divergence between clade A and B is quite high ranging from 6.09 to 8.24% (mean=6.64%).

The two distinct clades of *P. argentilineatus* from Indonesia supported by genetic distance (6.64%) show that *P. argentilineatus* is a cryptic species. According to Zemlak et al. (2009), if the genetic distance between the two populations or clade exceeds 3.5% then both are considered different species. The phenomenon of cryptic species is commonly found in many freshwater invertebrates such as *Penaeus monodon* (Yudhistira & Arisuryanti 2019) and *Etheria elliptica* (Elderkin et al. 2018) as well as in freshwater fish such as *Monopterus albus* (Arisuryanti et al. 2016) and *Cirripectes alboapicalis* (Delrieu-Trottin et al. 2018). The discovery of cryptic species of *P. argentilineatus* in Indonesia with the use of DNA barcoding is a novel finding. Therefore, a more detailed taxonomic revision of *P. argentilineatus* in Indonesia needs to be justified whether it consists of different species or remains one species with two subspecies. The correct and clear taxonomic status of *P. argentilineatus* can be applied for the conservation of this fish species in its habitat.

Levels of differentiation within and between *P. argentilineatus* clades are summarised by Principal Coordinate Analysis (PCoA) in Figure 8. This analysis indicates that the degree of differentiation between clades is much greater than within clades. From Figure 8, the clades remain distinct over significant geographic space, which includes a zone of geographic overlap (Figure 7), provide support for speciation of *P. argentilineatus* in Indonesia.

Figure 8. Principal Coordinate Analysis (PCoA) of pairwise genetic distance for 10 haplotypes of *P. argentilineatus* based on *COI* mitochondrial sequences. This analysis included 7 samples taken from GenBank.

Variation within clades

Six *COI* haplotypes were found in clade A from 11 individuals and four haplotypes were detected in clade B from six individuals (Table 2). Within clade A the level divergence among haplotypes was between 1-4 bp. From 519 bp, seven polymorphic sites were detected with three parsimony informative sites. The seven polymorphic sites contained six transitions and one transversion. In addition, haplotype diversity and nucleotide diversity were 0.727 and 0.0040 respectively. Next, within clade B the level divergence among haplotypes was slightly higher than clade A which was between 1-6 bp consisted of seven variables sites without parsimony informative sites. The seven polymorphic sites comprised four transitions and three transversions. The haplotype diversity and nucleotide diversity were 0.800

and 0.0045 respectively. The finding of variation within clades exhibited polymorphism of the *COI* mitochondrial gene. This gene polymorphism indicates the intrapopulation genetic variation of *P. argentilineatus* within clades.

CONCLUSION

Barred mudskippers from Tekolok Estuary are successfully identified as a *Periophthalmus argentilineatus* using *COI* mtDNA as a DNA barcoding marker. In addition, *P. argentilineatus* from Tekolok Estuary (West Nusa Tenggara, Indonesia) has a close genetic relationship with *P. argentilineatus* from Tukad Bilukpoh (Jembrana, Bali). Furthermore, DNA barcoding using *COI* mitochondrial gene has provided strong evidence that *P. argentilineatus* from Indonesia contains cryptic species. In this analysis, the Indonesian *P. argentilineatus* fell into two distinct groups, which are genetically distinct from each other. Further investigations are needed to clarify the two genetically distinct groups of Indonesian *P. argentilineatus*.

AUTHORS CONTRIBUTION

T.A. designed the research and supervised all the processes, F.A.R collected and analyzed the data, D.J.A and L.H provided and documented the samples, and helped F.A.R. to analyze the data. All authors wrote and revised the manuscript. All authors read and approved the final paper.

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CONFLICT OF INTEREST

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

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Research Article

Macroinvertebrates Reveal Water Quality Differences in Various Agricultural Management

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ABSTRACT
Monitoring benthic communities under different agricultural practices and management could potentially become an important tool to evaluate ecosystem health and stability. Benthic macroinvertebrates have been widely used as water quality bioindicators. This study aims to analyze macroinvertebrates in rice field ecosystems affected by three types of management practices, including conventional, semi-organic, and organic. This study was conducted in Sumberjambe and Kemiri, Jember Regency. Macroinvertebrate samples were collected at three sampling stations for each type of rice field, giving out a total of nine stations. Through Ekman grab, samples were obtained and transferred into a jam jar filled with 70% ethanol using a brush. Six ecological indices were selected to describe the diversity of each station. The Principal Component Analysis (PCA) using PAST3 software provided the sample's preference towards the stations and the higher taxa (Class). We also analyzed the similarity of the macroinvertebrate communities between the sampling stations using the Jaccard Similarity Index (JSI). A total of 11 families and 4 classes of macroinvertebrates are recorded. The Shannon-Wiener index shows high diversity for stations with organic management practices (1.318), while the Evenness index shows the highest value for conventional stations (0.9449). The Jaccard similarity index value reports two stations with semi-organic stations as well as semi-organic and organic stations having the highest similarity (JSI = 76.47%), while the lowest similarity value is characterized for conventional and organic stations (JSI = 13.19%).

Keywords: agroecosystem, diversity, macroinvertebrates, water quality

INTRODUCTION
Macroinvertebrates have been commonly used as biological indicators to assess water quality. Previous studies have revealed that macroinvertebrates could detect various contaminants, such as heavy metals (Qu et al. 2010), crude oil (Vinson et al. 2008), and pesticides (Berenzen et al. 2005). Macroinvertebrates are considered better to monitor water quality than standard instruments since they have a broader detection range and a longer exposure time to pollution (Duran 2006; EPA 2012). Moreover, a study by Choe et al. (2013) revealed that the macroinvertebrate diversities show informative and accurate results to determine the ecological function of irrigation ponds in rice cultivation ecosystems.

In the agroecosystem, the application of pesticides and fertilizers affects water quality. Since the rice plants easily adsorb the water contaminant and store it in its grain, the application of pesticides and inorganic fertilizers might also determine the quality of rice products (Pingali & Roger 2012). However, the use of inorganic fertilizers and pesticides produces residues that contaminate the water and change the natural physical -chemical condition of the water. The changing physical-chemical conditions of water, hereby, change the surrounding communities of benthic macroinvertebrates (Rizo-Patrón V. et al. 2013). Monitoring the benthic macroinvertebrates as bioindicator communities, thus, plays a crucial role in indicating the water quality as the response of the additive additions.

Monitoring benthic communities under different agricultural practices and management could potentially become an important tool to evaluate ecosystem health and stability. Despite there has been an introduction of organic and semi-organic rice cultivation, only a few studies have conducted research on the macroinvertebrates in various agroecosystems in Indonesia (i.e. Furaidah & Retnaningdyah 2013). Therefore, this study was carried out using lentic waters in rice fields under three different management practices (i.e. conventional, semi-organic, and organic) to assess the impacts of chemicals from fertilizers and pesticides on the ecosystem. The conventional systems use both inorganic fertilizers and pesticides, while organic systems use only organic additions. The semi-organic systems most likely combine inorganic pesticides and organic fertilizers. This study aims to analyze the findings of macroinvertebrates in 3 types of rice field management.

MATERIALS AND METHODS

Study Sites

This study was conducted in Sumberjambe and Kemiri, Jember, Indonesia, on September 20-21, 2020 (Figure 1). The data collection was done in the dry season, with the average amount of rainfall about 34 mm^3 (BPS Jember 2021). Macroinvertebrate samples were collected at three sampling stations for each type of rice field (i.e. conventional, semi-organic, or organic), giving out a total of nine stations (Table 1). These three types of management were selected to provide comparable information on the different impacts of the management practices on the benthic macroinvertebrate communities inside the ecosystems.

All three types of rice fields in this study were observed to be in the vegetative phase when data was collected. The water was supplied from the surroundings river through irrigation channels. Conventional rice fields used both inorganic pesticides, fungicides, and inorganic fertilizers. It used 'Phonska' branded-fertilizers with the composition consists of Nitrogen (N) 15%, Phosphate (P) 15%, Kalium (K) 15%, Sulphur (S) 10%, max water 2%. In order to control the pests, 'Fenite' branded-pesticides was applied with the active ingredients of emamektin 75g/l and 'Zole' branded-fungicides with the active ingredients of difenocolazole 250 g/l. Semi-organic rice fields also used 'Zole' inorganic fungicides but combined with organic fertilizers made of cow manure. For full-organic rice fields, organic fertilizers were the fermentation of 70% cow manure, 10% banana stems, 10% bran (i.e *dedak*), corn roots, water, and bacterial starter 10%. The organic pesticides/ fungicides were made of cow urine, garlic, Kipahit (*Tithonia diversifolia*), and Mimba (*Azadirachta indica*).

Data collection

For each station, sampling was repeated at three points to ensure objectivity. Sampling was done using Ekman grab with dimensions of 15x15x18 cm. We

used Ekman grab to collect macroinvertebrate samples living submerged in the water and those living on the surface of sediments and water surface. Through Ekman grab, water and sediment samples were obtained and then poured into the layered strainer with different sizes ranging from 2.36 mm, 1.5 mm to 0.5 mm, in order to sift the sediments from the samples. A brightly colored plastic plate and a brush were also used to pick out macroinvertebrates from the litter or the sediments. The benthic macroinvertebrates were then transferred into a jam jar filled with 70% ethanol using a brush. The macroinvertebrates were sorted based on their Order. Then, identification was made using a light microscope by putting the samples in a disposable petri dish. The samples were classified and counted to the families of the taxon using Gooderham and Tsyrlin (2002) and Lehmkuhl (1979).

Table 1. The sampling stations and the comparison of treatments for each management practice type.

Stations	Types	Fertilizer	Pesticide/fungicide
S1-S3	Conventional	Phonska (1 kg/600 m²) every 2 weeks, week 5 to 9	Fenite (active ingredients: Emamektin), Zole (active ingredients: difenocolazole)
S4-S6	Semi-organic	Organic (10 kg/800 m²) every 2 weeks, week 3 to 9	Zole (active ingredients: difenocolazole)
S7-S9	Full Organic	Organic (20 kg/900 m2) every 2 weeks, week 3 to 9	Organic

Data analysis

We estimated family richness and its abundance for each management practice type. The graphs of family numbers and individual numbers with stations as x-axis were shown to illustrate the trend on management types. Six ecological indices were selected to describe the diversity of each station (i.e. individual and taxa number, Shannon-wiener, Margalef, Dominance Simpson, and Evenness index). Moreover, the Principal Component Analysis (PCA) using PAST3 software provided the sample's preference towards the stations and the higher taxa (Class).

We also analyzed the similarity of the macroinvertebrate communities between the sampling stations using the Jaccard Similarity Index (JSI). The JSI was used to determine the degree of similarity and dissimilarity ranging from 0-100% based on the number of species in each community (Hao et al. 2019; Oluyinka Christopher 2020).
Below was the formula used in this study:

a. Shannon-wiener (Odum & Barrett 1971)
$H = - \Sigma n_i . N^{-1} . \ln(ni.N^{-1})$
where H = Shannon-wiener index
n_i = individual number of particular species i
N = total individual number of all species found

b. Margalef (Clifford & Stephenson 1975)

$$Dmg = \frac{S - 1}{ln\ N}$$

where Dmg = Margalef index
S = total number of species
N = total number of individuals in the sample

c. Dominance Simpson (Brower et al. 1990)

$$Id = \frac{\Sigma n_i(n_i - 1)}{N(N-1)}$$

where Id = Dominance Simpson index
n_i = individual number of particular species i
N = total individual number of all species

d. Evenness (Krebs 1972)

$$E = \frac{H}{Hmax}$$

where E = Evenness index
H = Shannon-wiener index
Hmax = Log_2S
S = total number of species in the sample

e. Jaccard (Jaccard 1900)

$$J = \frac{B + C}{A + B + C}$$

where J = Jaccard Index
A = number of species shared by two communities
B, C = number of species unique to each of the two communities

RESULTS AND DISCUSSION

A total of 11 families in 4 classes were recorded and grouped as Gastropoda, Polychaeta, Clitelatta, and Insecta. The number of individuals was shown varied between S1-S9 (Figure 2). Among other stations, S9 had the highest number of Gastropods. This result was supported by the finding of *Pomacea canaliculata*, which could easily adapt to an organic ecosystem (de Brito & Joshi 2016). For Insecta, the number of individuals increased for S5 -S9, while the number of Clitellata decreased for S1-S9. Moreover, Polychaeta was present only at S4-S6.

Based on the average number of families, the results showed that the Gastropoda distributed more equitably to all stations compared to other groups. Despite these results, a noticeable result can be seen at S9 with the highest individual number of Gastropods. Both Clitellata and Polychaeta showed decreased family richness for S1-S9. On the contrary, the family richness of Insecta increased for S1-S9, with S8-S9 had the highest families. The individual number trend of Clitelatta, Polychaeta, and Insecta was apparently in agreement with the results of the family number. The individual number of Clitellata and Polychaeta were decreasing, while Insecta was increasing for S1-S9, respectively.

Most of the Insecta found in this study, including Philorheithridae, Hydrophilidae, and Chironomidae are indicators for clean waters (Gooderham & Tsyrlin 2002). Thus, it was completely understandable if these families were higher in the organic rice fields with minimum contaminants. On the other hand, the findings of Clitelatta and Polychaeta, such as Tubifex sp. and Nereidae showed that the water had been contaminated by organic pollutants as in conventional and semi-organic rice fields (Martins et al. 2008; Swari et al. 2014).

The macroinvertebrates were scientifically proven to be very important for maintaining ecosystems' balance in the waters and surrounding areas (Magbanua et al. 2010). Based on the results, organic rice fields have a higher number of aquatic macroinvertebrate families. In contrast, the conventional

rice fields (i.e. S1-S3) had a more simple composition of macroinvertebrates, with Naididae of Clitellata predominating the stations, indicating polluted water with organic contaminants (Martins et al. 2008).

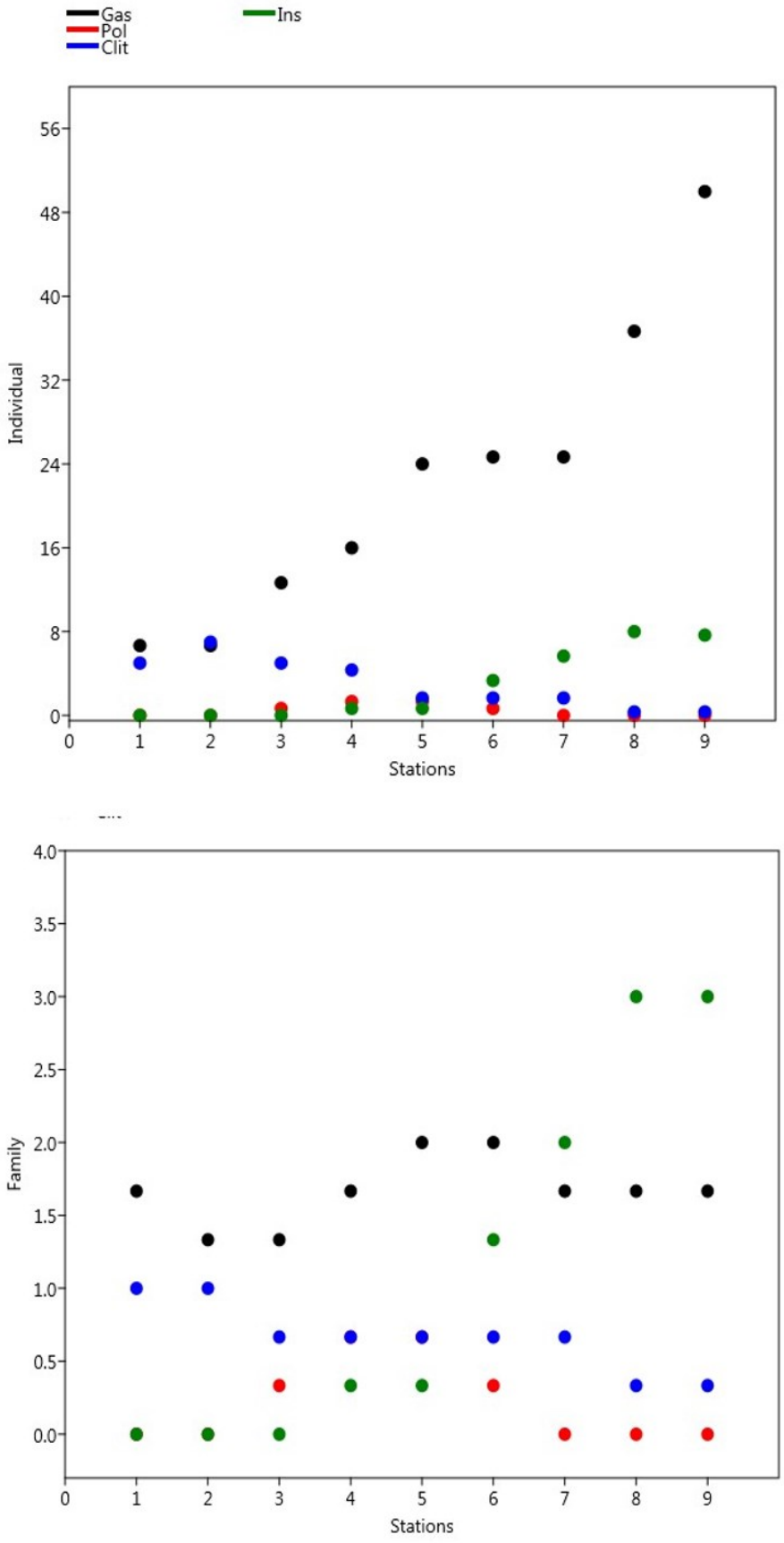

Figure 2. The average number of individuals and families at sampling stations. Keys: Class of macroinvertebrates described by color. Gastropoda (Gas): Black, Polychaeta (Pol): Red, Cliellata (Clit): Blue, and Insecta (Ins): Green.

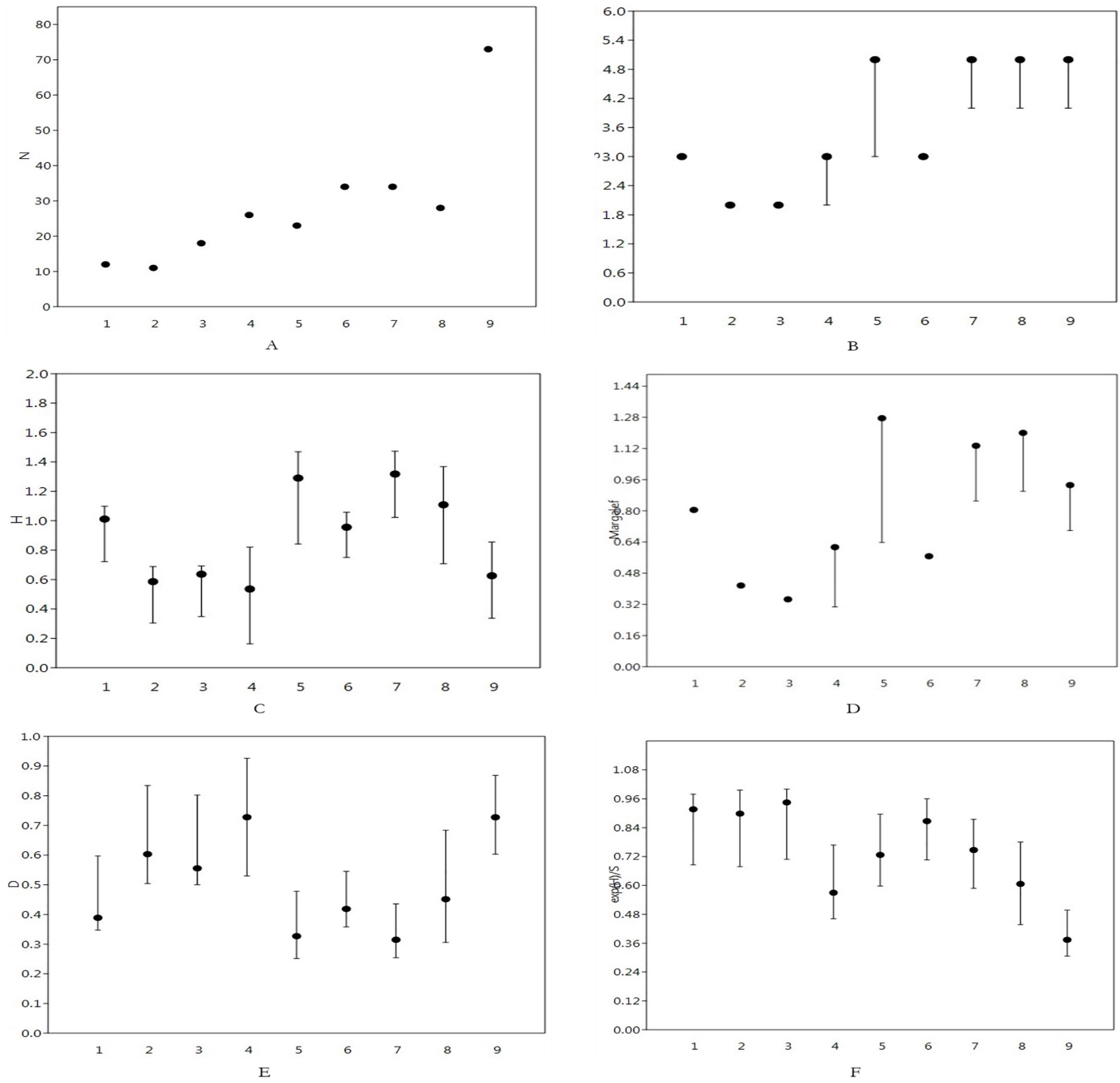

Figure 3. Statistical index based on PAST3 analysis (Alpha Diversity Indices) A) Total number of individuals (N); B) Total number of families (S); C) Shannon-wiener index (H'); D) Margalef Species Richness Index; E) Dominance Index (D); F) Species Evenness Index. All graphs used stations as the x-axis.

Ecological indices

The number of individuals showed a steady increase for S1-S8, while the highest number was recorded for S9 (Figure 3A). At semi-organic rice fields (S4- S6), there was a high number of Ampullaridae and Pachychilidae. Moreover, Polychaeta (Family Naereidae) was also found. It was clear that S7-S9 had the highest taxa for the taxa richness compared to other stations (Figure 3B).

The Shannon-wiener index showed a high diversity of macroinvertebrate) at S5, S7, and S8, with S2 and S4 had the lowest (Figure 3C). The Margalef species richness index described similar conditions (Figure 3D), with the difference that station 6 had lower diversity than the results of the Shannon-wiener index.

The Simpson dominance index illustrated the opposite condition (Figure 3E), with S2-S4 showed the highest dominance. It was reasonable

since low diversity could also mean high dominance. Furthermore, the Evenness index also showed a value close to 1 at S1-S3, reflecting low diversity and populations (Figure 3F).

The number of families in organic rice fields was higher compared to other agroecosystems. Moreover, based on the Shannon-wiener index, S7 with organic management type showed the highest diversity index (H=1.318). Then, the second highest with a small difference with the first one was S5 with semi-organic management practices (H=1.291). We assumed the use of organic fertilizers used in semi-organic stations might accommodate the various types of macroinvertebrates to live. In addition, the high presence of Insecta in organic stations indicates the stability of the ecosystem even without the addition of chemicals from pesticides or inorganic fertilizers (Gooderham & Tsyrlin 2002). There were 6 insect families found in the organic stations (Caenidae, Philorheithridae, Tipulidae, Gerridae, Hydrophilidae, Chironomidae), but only Tipulidae was found in the semi-organic station (i.e. S5).

Philorheithridae is the indicator of clean water (Morse et al. 2019). They not only capture and utilize a wide variety of nutrients in many forms but also transforming them for use by other organisms in freshwaters and surrounding riparian areas (Morse et al. 2019). Caenidae is commonly used as the indicator of possible low-organic polluted water (Hilsenhoff 1988). Tipulidae and Gerridae are known as an indicator of water conditions with moderate water quality (Pritchard 2003; Priawandiputra et al. 2018), while Hydrophilidae, and Chironomidae are indicators for clean waters (Gooderham & Tsyrlin 2002).

Principal Component Analysis (PCA)

The PCA result defined three components. First, the family was a specific taxa group. Second, a Class described a more general classification of ecological roles. The third was the taxa preference for 9 stations based on the biplot analysis (Figure 4). Ampullaridae was considered as an outlier due to a large number of findings. Thus, Ampullaridae, together with Pachyllidae, hereby, were the reasons for the high numbers of Gastropods. Ampullaridae was family taxa found in all types of rice fields. This family most likely has high adaptability and high tolerance in any kind of environment, making this family highly found in both conventional and organic stations. We identified one of the Ampullaridae species found in the sites as *Pomacea canaliculata*. This species is known to have high resistance to various environmental conditions and even high adaptability to other areas as an invasive species (de Brito & Joshi 2016). This snail has been introduced in Indonesia around 1983, and currently, it can be easily found in various wetlands in Indonesia (Marwoto & Nur 2011). Since this species has an ecological role as a herbivore and a pest because of its rapid reproduction, it is reported to soon become one of the most devastating rice pests in Asia (Naylor 1996). These snails are distributed for various reasons, such as the aquarium trade, foodstuffs, fish pond cleaners, and ship introduction (de Brito & Joshi 2016).

Insects had a location preference towards the S7, S8, S9. Chironomidae and Hydrophilidae were clustered in the middle, and Philorheithidae and Gerridae approached the center. The composition of most insects found in this study indicated good water quality, therefore, affects their preference towards organic stations with no addition of inorganic pollutants from the fertilizers and pesticides.

Clitellata and Polychaeta were plotted closer to the center and have a preference for S1 and S2. The members of Clitellata and Polychaeta found in this study however revealed poor water quality. Therefore, the preference of *Tubifex* sp., as the member of Class Clitelatta and Family Nereida, on

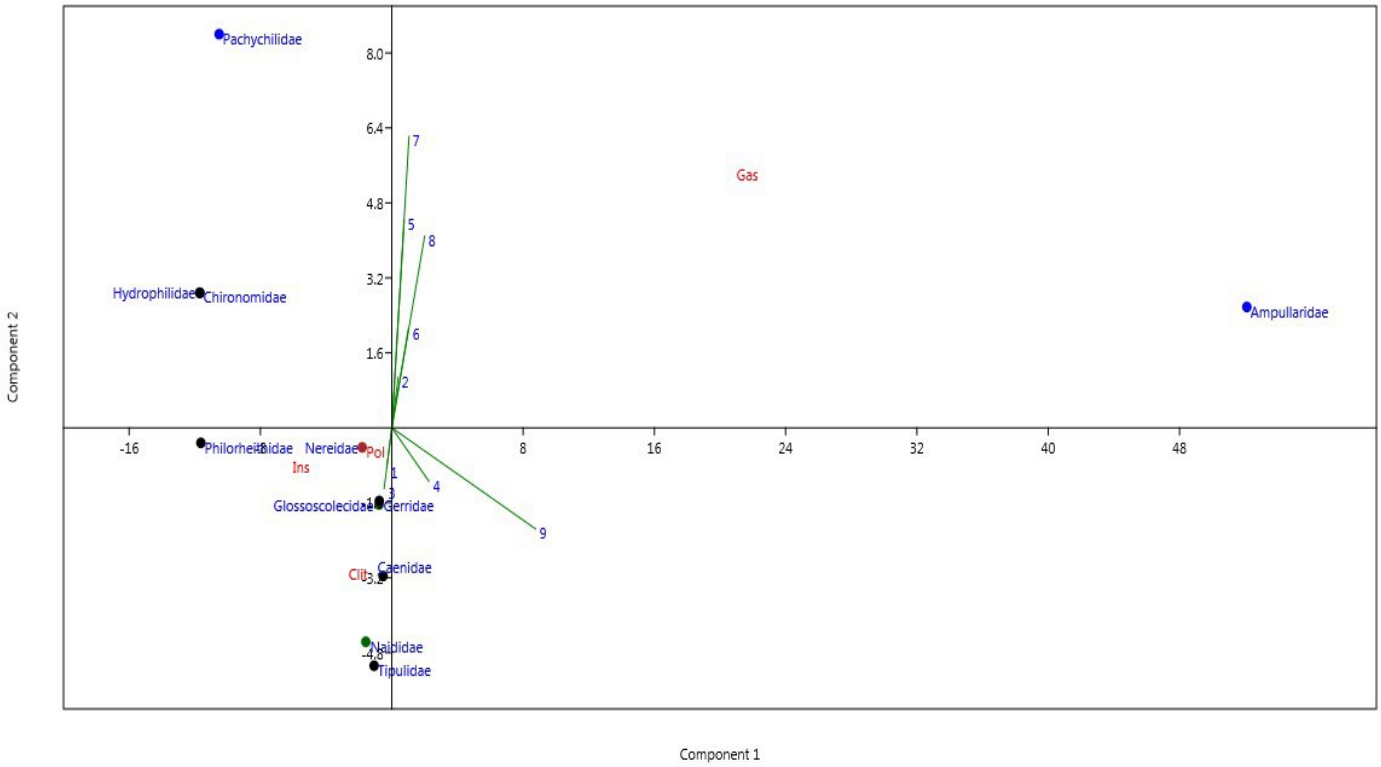

Figure 4. Biplot analysis on Principal Component Analysis (PCA) of collected macroinvertebrate families versus 9 sampling stations. Classes as groups are also provided. Keys: Gastropoda (Gas), Polychaeta (Pol), Cliellata (Clit), and Insecta (Ins).

conventional stations indicated highly polluted water by organic contaminants (Martins et al. 2008; Swari et al. 2014).

Jaccard Similarity Index (JSI)

We analyzed the similarity of the species between stations using the Jaccard similarity index (Table 2). The highest similarity was found for S5 and S6, as well as S6 and S7 (JSI = 76.47%). This value reflected the number of species of macroinvertebrates at the two stations had no significant difference.

S3 and S9 had the lowest similarity based on the JSI value (JSI = 13.19%), representing large differences in the number of species for conventional and organic rice fields. A similar value can also be seen for S1 and S9 (JSI = 14.12%) as well as S2 and S9 (JSI = 19.05%), reflecting different compositions in macroinvertebrates in conventional and organic rice fields, respectively.

Locally, in Jember, organic farming development often leaves treatment to maintain water quality since water is an important component to maintain the cycle and physiological processes of plants, especially rice. Another study shows that rice is categorized as a bioremediation plant that stores most of the chemical content (Ali et al. 2018). The chemical substances that are absorbed and stored in several parts of the plant will reduce the quality of the products and further affect the consumer's health. Monitoring water quality, thus also, is needed as a measurement model for assessing chemical-free products (Sivaranjani & Rakshit 2019).

Based on this study, we recommend continuous monitoring of macroinvertebrate communities to detect and avoid pollution early. Furthermore, we suggest that it is important to apply bioremediatory crops for organic farming as a barrier to avoid high exposure to chemical pollutions caused by surrounded conventional farming.

Tabel 2. Values of Jaccard Similarity Index (JSI) in 9 Stations (%).

Stations (S)	1	2	3	4	5	6	7	8	9
1		43,48	53,33	21,05	40,00	26,09	26,09	15,00	14,12
2	43,48		62,07	43,24	52,94	35,56	35,56	46,15	19,05
3	53,33			27,27	34,15	23,08	23,08	30,43	13,19
4	21,05				57,14	66,67	53,33	66,67	48,48
5	40,00					76,47	70,18	50,98	29,17
6	26,09						76,47	58,06	41,12
7	26,09							61,29	39,25
8	15,00								41,58
9	14,12								

CONCLUSION

The organic management practice for rice cultivation increases the diversity of aquatic macroinvertebrates compared to conventional and semi-organic management. The result of PCA analysis also showed that Gastropoda and Insecta have a high preference for organic rice fields with minimum organic pollutions. Furthermore, JSI analysis revealed the diversity and numbers of macroinvertebrates were significantly different in conventional and organic managed rice fields ensuring the objectivity of the selected stations.

AUTHORS CONTRIBUTION

A.S.K. collected, analyzed the data, and wrote the manuscript. R.N.B. analyzed the data and wrote the manuscript. H.P. designed the research and supervised all the process.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the content and there is no financial interest to report. We certifiy that the manuscript is original work, and is not under review at any other publication.

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Research Article

Stable Transformant of *Phalaenopsis amabilis* Somatic Embryo Carrying *35S::AtRKD4* Develops Into Normal Phenotype of Transgenic Plant

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ABSTRACT

Phalaenopsis amabilis (L.) Blume orchid is an Indonesian national flower. The number of these orchids in their natural habitat is very limited, therefore plant propagation efforts are needed. One of the promising methods is plant propagation by inserting embryo gene *AtRKD4* from a model plant *Arabidopsis thaliana* into the orchid genome to produce many somatic embryos. From previous research, we have obtained 28 plant *P. amabilis* transformants carrying the *AtRKD4* gene, however, it was unknown whether these plants have normal phenotypes and growth similar to their parents. Therefore, descriptions on growth and morphology are needed. This research aimed to evaluate the phenotype of *P. amabilis* carrying *35S::AtRKD4* the transformants grown in greenhouse. To achieve it, *AtRKD4* gene integration stability on transformants genome was analyzed. Morphology and cross-section anatomy structure on transformant and non-transformant plants were described. The stability of *AtRKD4* gene integration in the plant genome was confirmed by amplification of the *AtRKD4* gene from genomic DNA with Polymerase Chain Reaction (PCR) using a specific primer for *AtRKD4* and *ACTIN* genes as the internal control. The quantitative data from morphology and anatomy measurements were analyzed statistically using ANOVA. The results showed that *AtRKD4* was stably integrated into the genome of *P. amabilis* transformants and all transformant plants showed similar morphology and anatomy characteristics as non-transformant plants. The *AtRKD4* embryo gene was stably integrated into the orchid genome and the transformant plants grow normally without significant changes in phenotype.

Keywords: *Arabidopsis, AtRKD4,* Orchid, *Phalaenopsis amabilis* (L.) Blume, Somatic embryogenesis

INTRODUCTION

Orchid is one of the commercial plants in the field of floriculture (Pamarthi et al. 2019) and has high economic value (Palma et al. 2010). One of the important orchids is *Phalaenopsis amabilis* (L.) Blume, which is commonly called the Moth Orchid, which produces beautiful flowers shaped like a moth. The beauty of *P. amabilis* flowers has made it called "Puspa Pesona"

which is used as one of Indonesia's national flowers, along with jasmine and *Rafflesia arnoldii* (Schuiteman 2010; Semiarti et al. 2018). The uniqueness of this *P.amabilis* flower is the presence of an antenna-like structure at the end of its labellum, which always appears in the flower of this orchid's hybrid so that *P. amabilis* is commonly used as a parental species for breeding purposes to produce superior *Phalaenopsis* hybrid in the world (Semiarti et al. 2011).

Generative/sexual propagation depends on the success of the pollination process. It is ineffective because it takes a long time to produce a new plant (Shekarriz et al. 2014; Mursyanti et al. 2015; Saputro et al. 2018). Somatic embryogenesis is an effective method for orchid propagation. It can produce large numbers of uniform plants in a relatively short time (Feng & Chen 2014; Mose et al. 2017; Yang et al. 2020). The development of methods through genetic transformation could be beneficial for increasing crop production and conservation (Semiarti et al. 2007; Hsing et al. 2016; Zulwanis et al. 2020).

It is well known that plant development is controlled by gene regulation. The *RKD4* gene is a member of the RWP-RK protein family in a model plant *Arabidopsis thaliana* encoding transcription factors for gene expression in early plant development (Waki et al. 2011), promotes zygote extension (Jeong et al. 2011), induces somatic embryogenesis (Zimmerman 1993) and induces cell proliferation and expression of an egg cell (Kőszegi et al. 2011). The insertion of *AtRKD4* gene from *A. thaliana* succeeded in inducing somatic embryo in the *Phalaenopsis* "Sogo Vivien" orchid hybrid (Mursyanti et al. 2015) and *Dendrobium phalaenopsis* (Setiari et al. 2018). So far, there has never been comprehensive studies regarding the influence of ecological factors on the growth of transformant plants during the acclimatization period after being transferred from *in vitro* condition which is strictly controlled to *ex vitro* condition, in which various environmental factors highly influence it. In fact, the process of transformant plants going through the acclimatization period to be able to grow normally in *ex vitro* condition is crucial. Therefore, we need to ensure that the transformant plants produced by somatic embryo induction from *AtRKD4* expression can grow and develop similarly as non-transformant plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The plant materials used in this study were ten non-transformant of wild type *P. amabilis* (Yogyakarta Forms) plants age 8-10 months and 10 plants of *P. amabilis* transformants, carrying the 35S::*AtRKD4* gene, obtained from previous research results of the Orchid Research Team Faculty of Biology UGM. Each plant was planted in pots with a combination of fern and wood charcoal (1: 1), they were maintained in a greenhouse under natural sun lighting environment (28-31˚C) and relative humidity ranges 74-80%.

Detection of *AtRKD4* gene in the genome of *P. amabilis* transformants

Isolation of the whole genome DNA of wild type and transformants *P. amabilis* was performed by the standard method of plant genome DNA isolation as described by Semiarti et al. (2007) by using Cetyltrimethyl Ammonium Bromide (CTAB). Detection of the existence of *AtRKD4* gene in the transformants was performed using *Polymerase Chain Reaction* (PCR) with KOD FX NEO PCR Kit (Toyobo) and specific primers for *AtRKD4* gene and *Phalaenopsis* actin gene (*ACT4*) (Yuan et al. 2014) as the internal control (Table 1).

PCR reactions for both genes were performed in 3-step-cycles: an initial denaturation 94°C for 2 min, denaturation at 98°C for 10 seconds,

annealing at 58 °C for *AtRKD4* and 51°C for *ACT4* for 30 seconds, and extension at 68°C for 6 sec seconds using a Thermal Cycler (T100, Biorad). PCR steps were performed in 35 cycles. The amplified DNA fragments were checked with 0.8% agarose gel (Agarose Type II, SIGMA).

Table 1. Primers used in this study.

Primer name	Primer direction	Sequence (5' to 3')	Product size (bp)
AtRKD4	*forward*	GTTCATTTCATTTGGAGAGGACG	198
	reverse	CTTCCATATCTAGGAGAGAATCAAG	
ACT4	*forward*	GTATTCCCTAGCATTGTTGGT	114
	reverse	CAGAGTGAGAATACCTCGTTTG	

Morphological Analysis

Plant growth was observed every week for eight weeks to get detailed similarities and differences in growth between *P. amabilis* transformant and non-transformant plants, ten plants each for both non-transformant and transformant plants. Plant morphological data covering the total number of leaves, leaf length, leaf width, as well as the total number of roots, root length and root diameter were observed and measured weekly. The phenotypic analysis includes the shape and color of leaves, roots, and survival ability. The observations were documented using a digital camera (EOS M100, Canon), color characteristic was determined using RHS color chart (The Royal Horticultural Society, London).

Anatomical Structure Analysis of Roots and Leaves

Anatomical preparations of roots and leaves cross-section were conducted using the paraffin embedding method according to Sutikno (2016) and stained by 1% safranin in 70% alcohol, the sample was fixed with FAA solution (Formalin: glacial acetic acid: 70% alcohol = 5: 5: 90). Observations of the anatomy slides were performed by using a light microscope (CX22LED, Olympus) and documented using a digital microscope camera (Optilab Advance V2, Miconos).

Statistical analysis

Quantitative data is processed using Microsoft Excel 2013 applications and Raster 3 image, then statistically analyzed using IBM SPSS Statistics 26.0 to find out whether there are growth and phenotype differences. Analysis of Variance (ANOVA) was conducted to test the significance difference of the growth by both groups with significance level 0.05. Qualitative data was descriptively analyzed.

RESULTS AND DISCUSSION

P. amabilis transformants and non-transformants growth

Growth of *P. amabilis* transformant and non-transformant plants were observed for 8 weeks. The phenotypes of *P. amabilis* transformant and non-transformant plants showed very similar growth patterns (Figures 1 and 2). Observation was conducted as a first step to determine morphological characteristics of the two groups.

During acclimatization, *P. amabilis* non-transformant and transformant orchids at week 0 (Figures 1A and 2A) had compact green leaves (RHS Green group 138A) and compact green roots (RHS Green group 145A). At

week one (Figures 1B and 2B) the leaves began to undergo chlorosis at the tips. *P. amabilis* non-transformant orchids began to adapt in *ex vitro* environment at week 4 (Figure 1D), which marked by new root and leaf growth after 5-week acclimatization (Figure 1E). Whereas, *P. amabilis* (T) at week 2 (Figure 2C) have shown the ability to adapt in *ex vitro* environment characterized by the growth of new roots and leaves on week 4 (Figure 2E). Mirani et al. (2017) reported a similar phenomenon in *Dendrobium nobile* acclimatization. Maintenance of humidity and temperature around the plantlets during the initial days of acclimatization is of utmost importance. During *in vitro* culture, plantlets were grown in high humidity conditions in jars. Meanwhile, in greenhouse or field has much lower humidity than *in vitro* condition. Therefore, rapid changes in environmental conditions, especially in terms of temperature and humidity, could led to wilting and chlorosis. *P. amabilis* non-transformant and transformant have an obovate leaf shape, where the widest part at the tip of the leaf blade. The shape of the leaf tip is retuse and the leaf edge is integer (Figures 1 and 2).

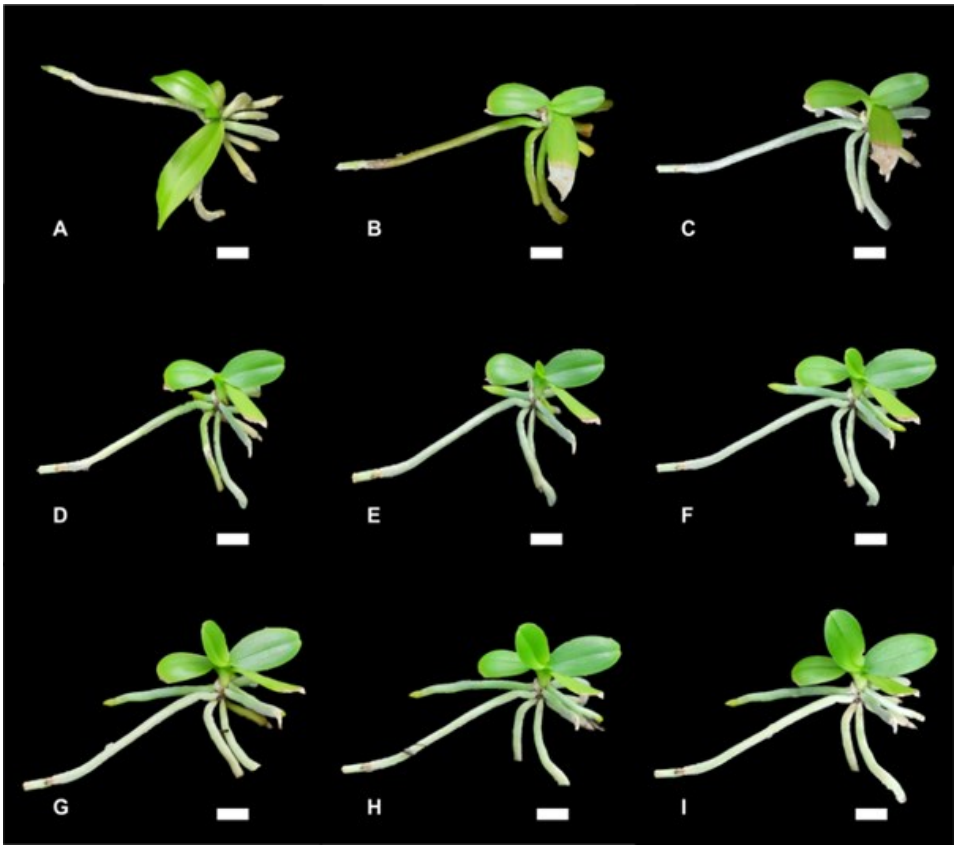

Figure 1. Non-transformant *P. amabilis* growth during acclimatization was observed each week from (A) week 0 to (I) week 8.

The growth of *P. amabilis* non-transformant and transformant plants was statistically analyzed to determine significant growth differences. The viability percentage of *P. amabilis* non-transformant and transformant plants from week 0 to 8 during acclimatization showed that both of them were successfully adapting in *ex vitro* environment. Total number of leaves of *P. amabilis* non-transformant and transformant plants decreased during week 1 of acclimatization but showed a positive trend starting from week 2 of acclimatization. The leaf length of *P. amabilis* non-transformant plants decreased until week 2 of acclimatization, then showed an increase in trend until week 8. Whereas the *P. amabilis* (T) plants decreased slightly at week 1 then showed an increase in trend until week 8 . Leaf width, the total number of roots, root length and diameter of the root of *P. amabilis* non-transformant

and transformant plants from week 0 to 8 of acclimatization showed an increase in trend.

Figure 2. Transformant *P. amabilis* carrying *AtRKD4* gene during acclimatization was observed each week from week 0 (A) to week 8 (I).

A significance level above 0.05 indicates no difference in growth between *P. amabilis* non-transformant and transformant plants. As shown in Table 2, *P. amabilis* non-transformant and transformant plants had

Table 2. Comparison of the growth rate between *P. amabilis* non-transformant and transformant plants using ANOVA.

		Sum of Squares	df	Mean Squares	F	Signifi-cance
Total Number of Leaf	Beetwen Groups	0,251	1	0,251	0,844	0,370
	Within Groups	5,352	18	0,297		
	Total	5,603	19			
Leaf Length	Beetwen Groups	4,990	1	4,990	3,812	0,067
	Within Groups	23,560	18	1,309		
	Total	28,550	19			
Leaf Width	Beetwen Groups	0,305	1	0,305	4,351	0,051
	Within Groups	1,262	18	0,070		
	Total	1,567	19			
Total Number of Roots	Beetwen Groups	4,901	1	4,901	4,382	0,051
	Within Groups	20,131	18	1,118		
	Total	25,031	19			
Root Length	Beetwen Groups	4,418	1	4,418	1,698	0,209
	Within Groups	46,834	18	2,602		
	Total	51,252	19			
Root Diameter	Beetwen Groups	0,006	1	0,006	1,199	0,288
	Within Groups	0,092	18	0,005		
	Total	0,098	19			

Note: significance level 0.05

significance levels above 0.05 in all parameters. The results of this study are in line with Semiarti et al. (2018) who compared the vegetative growth between *Phalaenopsis* "Sogo Vivien" carrying *35S::Gal4::AtRKD4::GR* and the non-transformant plants that showed no change in plant phenotype and growth condition.

Anatomic structure of roots and leaves of *P. amabilis* transformants and non-transformants

Cross-section of root anatomical structure (Figure 3) and leaf anatomic structure (Figure 4) shows high similarities in the shape and size of the leaf and root cells between transformant and non-transformant plants. There were no significant differences between transformant and non-transformant plants on the anatomic structure of roots and leaves.

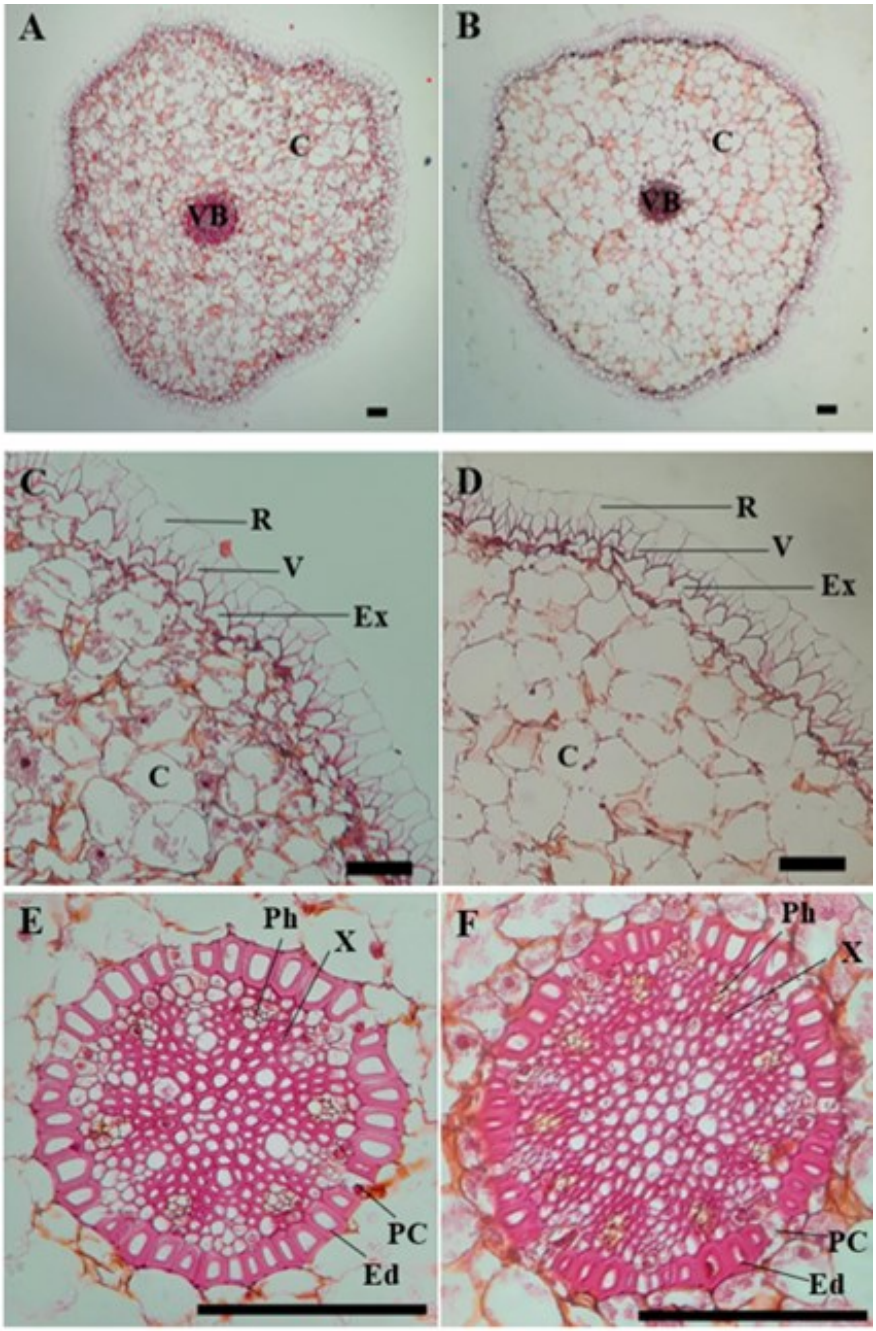

Figure 3. Anatomic structure of root cross-section of *P. amabilis*. (A, C, E) *P. amabilis* non transformant; (B, D, F) *P. amabilis* transformant. (A, B) Full cross-sectional view of the roots; (C, D) cortex and exodermis; and (E, F) stele. C, Cortex; Ed, Endodermis; Ex, Exodermis; PC, Passage Cells; Ph, Phloem; R, Rhizodermis; V, Velamen; VB, Vascular Bundle; X, Xylem. Bars: 50 μm.

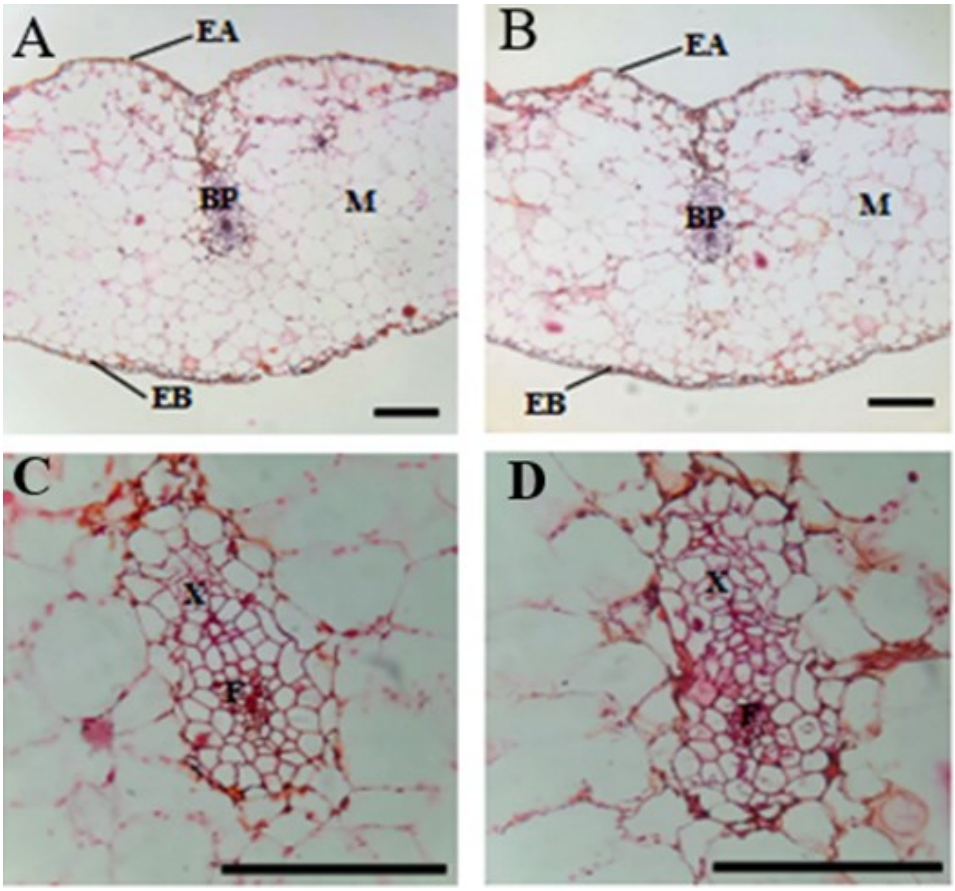

Figure 4. Anatomic structure of leaves cross-section of *P. amabilis*. (A,C) *P.amabilis* non transformant; (B,D) *P.amabilis* transformant. (A,B) Cross-section of the leaves; and (C,D) vascular bundle in the center part of the leaves. AbE, Abaxial Epidermis; AdE, Adaxial Epidermis; M, Mesophyll; Ph, Phloem; VB, Vascular Bundle; X, Xylem. Bars: 50 μm.

The cross-section of roots of *P.amabilis* orchid transformant and non-transformant showed a very similar anatomical structure consisting of Rhizome, Cortex and Stele (Figure 3). Table 3 shows that the size of the length and width of these cells did not show a significant difference of Rhizome, Cortex and Stele with ANOVA significance of 5%. The anatomical structure of the leaves (Figure 4) also did not show significant differences in both the structure and size of cells Epidermis, vascular bundle (Xylem and Phloem), and parenchyma (Table 4). This phenomenon indicated that the activity of the *AtRKD4* gene in somatic embryo initiation did not affect on the growth and further development of the orchid somatic embryos, only at an early stage for the initiation of somatic cells into embryos. The *AtRKD4* gene, therefore, has no impact on the activity of growth genes to continue the growth and development of embryos into complete plants. This fact is highly expected by genetic engineers for *P. amabilis* orchid's mass propagation as a conservation effort as well as for producing large number of plants for agribusiness purposes.

Detection of the integration of *AtRKD4* gene in the genome of *P. amabilis* transformant

Positive amplification results of genomic DNA with specific primers for *AtRKD4* gene and primers for housekeeping gene *ACTIN* as an internal control for PCR reaction showed that a 114 bp of *ACTIN* fragment was amplified from the genome of both *P. amabilis* non-transformant and transformant, whereas 198 bp of *AtRKD4* fragment was amplified only from the genome of *P. amabilis* transformants (Figure 5). This data indicated that

Table 3. Root anatomical parameters of *P.amabilis* non-transformant and transformant.

Parameters	*P. amabilis* orchid		Significance
	non-transformant	transformant	
Rhizodermis Length (µm)	13,85 ± 2,40	13,27 ± 2,13	0,575
Rhizodermis Width (µm)	17,82 ± 1,94	17,36 ± 1,55	0,571
Velamen Length (µm)	7,13 ± 1,77	7,29 ± 1,20	0,810
Velamen Width (µm)	9,57 ± 1,77	9,29 ± 1,40	0,704
Exodermis Length (µm)	14,78 ± 1,58	14,56 ± 2,19	0,805
Exodermis Width (µm)	12,70 ± 2,09	12,39 ± 1,41	0,695
Cortex Diameter (µm)	49,69 ± 8,04	52,95 ± 8,02	0,377
Endodermis Length (µm)	6,11 ± 0,64	6,15 ± 0,55	0,894
Endodermis Width (µm)	9,60 ± 1,01	9,12 ± 0,79	0,262
Passage Cell Length (µm)	5,41 ± 0,51	5,58 ± 0,51	0,463
Passage Cell Width (µm)	7,32 ± 0,77	7,32 ± 0,80	1,000
Xilem Diameter (µm)	2,93 ± 0,20	2,96 ± 0,13	0,761
Phloem Diameter (µm)	1,88 ± 0,34	1,86 ± 0,19	0,899

Significant level 0,05

Table 4. Leaf anatomical parameters of *P.amabilis* non-transformant and transformant.

Parameters	*P. amabilis* orchid		Significance
	non-transformant	transformant	
Adaxial Epidermis Length (µm)	18,00 ± 1,26	18,58 ± 1,20	0,307
Adaxial Epidermis Width (µm)	9,97 ± 1,22	9,92 ± 1,14	0,921
Mesophyll Diameter (µm)	35,89 ± 3,25	35,95 ± 2,38	0,961
Xilem Diameter (µm)	6,13 ± 0,44	6,13 ± 0,38	0,983
Phloem Diameter (µm)	1,97 ± 0,14	1,92 ± 0,09	0,460
Abaxial Epidermis Length (µm)	17,38 ± 1,50	17,28 ± 1,36	0,877
Abaxial Epidermis Width (µm)	9,94 ± 1,03	9,79 ± 0,83	0,716

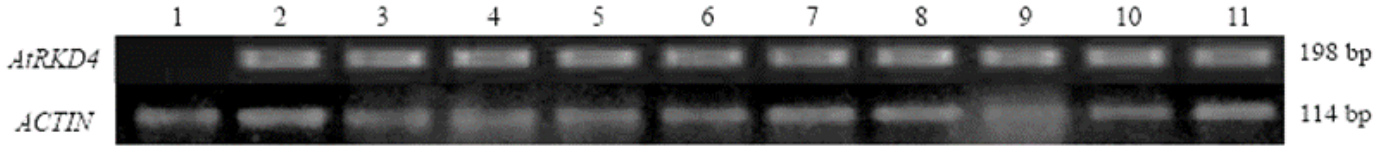

Figure 5. Detection of *ACTIN* and *AtRKD4* genes integration in the genome of non-transformant and transformant *P. amabilis* orchid lane 1: *P. amabilis* non-transformant; lane 2-11: *P. amabilis* transformant. Fragment of *AtRKD4* gene (198 bp) was amplified on *P. amabilis* transformant but not in non-transformant. Fragment of *ACTIN* gene 114 bp was amplified from genom of both *P. amabilis* non-transformant and transformant.

the *AtRKD4* gene is stably integrated in the genome of *P. amabilis* transformants while the transformant plants grow and develop similarly as non-transformant plants.

Waki et al. (2011) reported that in the *Arabidopsis thaliana* plant, the *AtRKD4* gene produced the RKD4 protein triggers gene expression and pattern formation in early embryogenesis. This is also supported by research data on somatic embryogenesis induction on *Phalaenopsis* 'Sogo Vivien' from Mursyanti et al. (2016) and research by Setiari et al. (2018) in somatic embryogenesis of *Dendrobium phalaenopsis*. This is also in accordance with Mose et al. (2020), who succeeded in inducing somatic embryogenesis by treating growth regulators thidiazuron and NAA from protocorms and *P. amabilis* leaf pieces as explants.

CONCLUSION

P. amabilis transformant plants remain stable on carrying *AtRKD4* gene and grew normally as the same as non-transformant plant. It has great potential to be used as an explant donor for the propagation of *P. amabilis* plants for various purposes, including production of seedlings for both conservation and agribusiness purposes. Overall, morphological data, anatomical data, and molecular data support the use of the *AtRKD4* gene for orchid propagation for the conservation of *P.amabilis* orchids whose population is decreasing in nature, as well as orchid production for economic purposes.

AUTHORS CONTRIBUTION

N.G.A.P collected and analyzed the data and wrote the manuscript, W.M idea of the experiment and wrote the manuscript. M.D.L revised and finalized the manuscript, J.G.M founder of T-DNA construct, E.S. designed the research and supervised all process.

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CONFLICT OF INTEREST

The authors declare there is no competing interest.

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Research Article

Detection of *AtRKD4* Protein During Induction of Somatic Embryogenesis in *Dendrobium lineale* Rolfe Transgenic Orchids Carrying *35S::GR::AtRKD4*

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ABSTRACT
Dendrobium lineale is an Indonesian native orchid from the Spatulata section in *Orchidaceae* Family. This orchid is important because it is usually used as a parental plant in orchid breeding and is predicted to have a potential phytochemistry compound. In addition, in their natural habitat, this orchid is threatened due to forest exploitation and natural disaster. Therefore the precision mass propagation techniques for this orchid need to be conducted. Biotechnological approaches through inserting embryo gene such as *AtRKD4* from *Arabidopsis thaliana* has already been successfully conducted. This study aims to check the integration stability of T-DNA harboring 35S::GR::*AtRKD4* from ten selection transformants and to detect the existence of AtRKD4 protein after induction by Dexamethasone and/ Thidiazuron. The result showed that T-DNA were stably integrated into the genome of *D. lineale* transformants and the AtRKD4 protein with a molecular weight of 28.53 kDa was detected in *D. lineale* transformant plants after being induced by 15 µM DEX and 3 mgL-1 TDZ for 5 days.

Keywords: *AtRKD4* gene, *At*RKD4 protein, *Dendrobium lineale,* dexamethasone, somatic embryo, thidiazuron

INTRODUCTION
Dendrobium lineale Rolfe is an endemic orchid of Papua that is threatened in its habitat due to over-exploitation. *D. lineale* is favored because it shows tall stems and produces many beautiful purplish-white flowers (Rolfe 1889; Pridgeon 1992). This orchid is named *D. lineale* because has lined sepals and petals (Pridgeon 1992). In addition, *D. lineale* can be used for medical purposes such as anti-cancer. It contains types of phytochemicals such as flavonoids, polysaccharides, bibenzyl, phenanthrene, alkaloids, sesquiterpenoids, and steroids. A common problem with *D. lineale* is that it is not easy to breed and grow naturally, so the population of this orchid is less abundant (Semiarti et al. 2020). Several types of orchids are increasingly rare to be found in nature due to overexploitation, domestication, and usage as parental orchids in cultivation (Ivakdalam & Pugesehan 2016). *In vitro* propagation is expected to be the right solution for *ex situ* conservation

because it's possible to produce a mass number of plants with similar characteristics to its parental (Setiari et al. 2018). Propagation through *in vitro* culture can increase the quantity and the number of tillers obtained in a relatively short time (Hartati et al. 2016). Optimization of *in vitro* propagation can be done by using genetic engineering, by inserting foreign genes through *Agrobacterium tumefaciens*. An embryo gene of *Arabidopsis thaliana*, *AtRKD4* gene in this study was inserted into the genome of *D. lineale* protocorms through *A. tumefaciens* strain EHA105 that harbor pTA7002 plasmid, therefore it can induce large numbers of somatic embryos in a relatively short time. The gene construction used an inducible promoter equipped with *Glucocorticoid Response Element* (GRE), requiring dexamethasone (DEX) which is a glucocorticoid compound as an inducer to induce transcription or activation of transgenes (Mursyanti et al. 2015). The insertion of the *AtRKD4* gene into the genome of orchids has been carried out and has successfully demonstrated the formation of somatic embryos in transformant orchid leaves after being induced with DEX on leaves of *Dendrobium phalaenopsis* (Zulwanis et al. 2020).

The formation of somatic embryos in orchid plants can be induced by the addition of growing regulatory substances in the medium of *in vitro* culture. Auxin and cytokinin are commonly used for the induction of somatic embryogenesis (Moradi et al. 2017). Thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea) is a cytokinin class growth regulator known to have potential activity in shoot regeneration and proliferation was effective in inducing somatic embryo formation (Ghosh et al. 2018). TDZ with 3 mgL^{-1} concentration is the best concentration used to induce the formation of somatic embryos in the roots, stems, leaves explants, and protocorm of *P. amabilis* orchid (Mose et al. 2017).

According to Semiarti et al. (2007 & 2011), plant genetic transformation is the right method to improve the quality of orchid plants because it can break barriers between species and insert beneficial traits from superior genes from other plant species. The success of the genetic transformation is shown by the detection and integration of *AtRKD4* transgenes in the orchid genome, and also the activation of these genes in orchid transformants (Setiari et al. 2018). Somatic embryogenesis (SE) is a promising technique used in the proliferation of plants. It is also a way to support *ex situ* conservation (Maruyama & Hosoi 2019). Protein detection of AtRKD4 needs to be done because the function of genes is biochemically carried out by proteins which is the result of gene expression and it determines the character of plants at each stage of their development (Utami et al. 2007). RKD4 Protein (*RWP-RK motif-containing 4 putative transcription factors domain*) is one of two sub-family of RWP-RK protein motifs (amino acid sequences consists of arginine (R), tryptophan (W), prolin (P), arginine (R), and lysine (K) proteins located inside the nucleus which are transcription factors based on similarities of form with basic leucine zipper protein and basic helix-loop-helix. The function of the RKD4 protein is to trigger the expression of genes needed for the initiation of the process of forming a divisional pattern in the zygote and the process of the early development of the embryo (Chardin et al. 2014)

The *AtRKD4* gene has been successfully inserted into the *D. lineale* orchid genome, but the stability of the *AtRKD4* gene integration and the expression of the gene in the orchid genome are not yet known. This research aims to analyze the stability level of *AtRKD4* gene integration in the genome of *D. lineale* transformant and to analyze the expression of the gene which can produce AtRKD4 protein in *D. lineale* transformants.

MATERIALS AND METHODS

Plant Materials and T-DNA Construct

Eighty plantlets of 1 year and 10 months-old *D. lineale* that consisted of 46 non-transformants and 34 transformants carrying T-DNA with the construct of 35S::GR::AtRKD4 were used as plant materials for this work. For protein analysis, 2 non-transformant plantlets and 10 candidates of transformant plantlets of *D. lineale* that carrying the 35S::GR::AtRKD4 construct were used. All plantlets were planted on New Phalaenopsis (NP) basal medium (Islam et al. 1998). The T-DNA contains an inducible system of GVG, which contains a synthetic protein fusion for the GAL4 DNA binding domain, the transcriptional activator VP16, and a portion of the rat Glucocorticoid Receptor (GR) (Figure 1). The expression of the GVG gene was driven by a strong promoter 35S promoter from the *cauliflower mosaic virus* (CAMV). To facilitate transformant selection, T-DNA is also filled with *Hygromycin phosphotransferase* (*HPT*) gene as a selection marker, which confers resistance to hygromycin on the selection medium (Figure 1).

Detection of *AtRKD4* integration in the genome of orchid transformant

DNA isolation of non-transformant and candidates of *D. lineale* transformant

Samples used for genome DNA isolation were 2 samples of non-transformant and 10 samples of transformant plantlets. Leaves of non-transformant plantlets and candidates of transformant plantlets weighing 20 –30 mg, were put in the mortar added 250 µl CTAB 3%, and crushed until smooth in the form of powder using a pestle and then added more 250 µl of CTAB solution. The mixture was homogenized and incubated in a water bath at 60°C for 30 min. The mixture was then added with 500 µL of chloroform solution, and mixed with shaking the tube 6 times inversely, following by incubation with a shaker for 30 min at room temperature. The sample was then centrifuged at 14.000 rpm for 10 min, the supernatant was moved into a new tube. An equal volume of isopropanol was added to the supernatant, mixed well, then incubated at room temperature for 10 min. The mixture was then subsequently centrifuged at 14.000 rpm for 10 min. The pellet of DNA and RNA appeared at the base of the tube, discarded the supernatant, pellets added 500 µl EtOH 70% into the tube, then centrifuged at 10.000 rpm for 5 min to wash the pellet from cell debris. The supernatant was then discarded, and dry up the DNA pellets by using an incubator at a temperature of 37°C for 30 min. The dried pellet of DNA was diluted with 30 µl TE, incubated in the water bath at 60°C for 5 min. Isolated genomic DNA was stored at a temperature of -20°C for subsequent analyses. An aliquot volume of genome DNA suspension was taken to check the concentration by using 0.7% agarose gel electrophoresis.

Detection of T-DNA that carrying *35S::GR::AtRKD4* integration in the genome of orchid transformants plants

Detection of transgene *AtRKD4* and *HPT* in the genome of *D. lineale* were performed by Polymerase Chain Reaction (PCR) method. DNA genome of

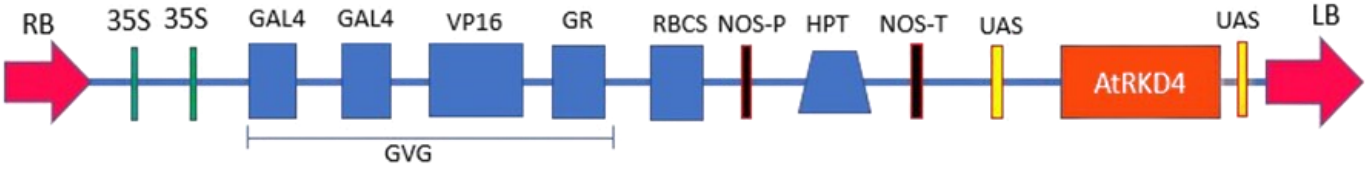

Figure 1. The structure of T-DNA carrying *35S*::GR/UAS::*AtRKD4* in plasmid pTA7002/EHA 105 (Mursyanti et al. 2015).

non-transformant and transformant plant candidates carrying *35S::GR:: AtRKD4* analyzed with PCR using a specific primer for *AtRKD4* and *HPT* genes. Specific primers for the *AtRKD4* gene are AtRKD4F1 (5'GTTCATT TCATTTGGAGAGGACG-3') and AtRKD4 R1 (5'CTTCCATATCTAGG AGAGAATCAAG-3') which produced a 382 bp band of DNA.

Specific primers for HPT genes are HygF (5'-TCGGACGATTGCGT CGCATC-3') and HygR (5'AGGCTATGGATGCGATCGCTG-3') that produced a 545 bp band of PCR product. The PCR process was carried out under the conditions according to the protocols of the Bioline MyTaq™ HS Red Mix 2x. The steps are pre-denaturation stage at 95°C temperature for 1 min, denaturation at 95°C temperature for 15 sec, the annealing stage at 58°C for *AtRKD4* and 61°C for the *HPT* for 15 sec, the extension stage at a temperature of 72°C for 10 sec, the final stage was cooling at 4°C temperature with 35 PCR cycles. Interspace fragment of chloroplast DNA trnL-F (1200 bp) as an internal control of the PCR process. The primers for trnL-F were trnL-F F1 (5' CGAAATCGGTAGACGCTACG-3') and trnL-F R1(5' ATTTGAACTGGTGACACGAG-3'). The results of the PCR were run in electrophoresis gel using 1% agarose gel in TAE 1X buffer with 100 volts for 20 min and observed with UV transilluminator (Extragene).

The PCR products were checked by 1% agarose gel electrophoresis in 1X TAE solution with 3µl of EtBr. The sample was running using electrophoresis with 100 volts for 20 minutes and the bands were observed by UV transilluminator.

AtRKD4 protein analysis on *D. lineale* transformant plantc

Protein analysis of transformant plant was carried out using the Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) method. The total protein isolation was carried out using transformant plant leaves which had been induced in DEX media 15 µM and TDZ 3 mgL-1 for 5 days. The isolation was carried out by crushing 200 mg leaves by mortar and pestle in 300 µl *Phosphate Buffer Saline* (PBS) with pH 7 as an extraction buffer until it becomes powder and homogenous. The sample was put in a 1.5 ml eppendorf tube. After that, the sample was centrifuged at a speed of 10,000 rpm for 10 minutes with a temperature of 4°C. The acquired supernatant was then transferred into a new tube. A volume of 200 µl supernatant was added with 50 µl 5X sample buffer (3.9 ml of aquabidest; 1.0 ml 0.5 M Tris pH 6.8; 0.8 ml glycerol; 1.6 ml SDS 10%; 0.4 ml 2-mercaptoethanol; 0.4 ml 1% Bromophenol Blue). The mixture was then heated in a water bath at 90°C temperature for 5 minutes to degrade the protein. Samples were stored at -20°C. Eight µl of supernatant was used to measure the total concentration of proteins by using spectrophotometry.

Determination of protein concentration was carried out by using 8 µl supernatant added with 200µl Bradford solution. The protein concentration was measured by spectrophotometry at a wavelength of 595 nm. Bovine Serum Albumin (BSA) proteins were used as a standard to calculate the protein concentrations. The steps of electrophoresis with SDS-PAGE have put a plate glass arranged with a frame from Bio-Rad and printed 12% lower gel. Then the 12% running gel mixed homogeneous (30% acrylamide 4.0 ml; 4X LGB (Lower Gel Buffer) 2.50 ml; distilled water 3.45 ml; TEMED 5 µl, APS 10% 50 µl) the mixture then inserted into a plate glass through its chamber up to one cm from the upper limit of the plate. The upper gel is made like a lower gel. The 5% upper gel mixed homogeneous (30% acrylamide 0.67 ml; 4X UGB (Upper Gel Buffer) 1.25 ml; distilled water 3.05 ml; TEMED 5 µl, APS 10% 50 µl) then the mixture inserted on top of the lower gel that has hardened to full. Electrophoresis was run in 50 volts for 50 minutes and 100 Volts for 60 minutes. The staining process was carried out

using 40% methanol, 10% glacial acetic acid, 50% distilled water, and 0.1% Coomasie Blue for 24 hours. The next process was the destaining stage using 40% methanol, 10% glacial acetic acid, and 50% distilled water.

The profile of the protein was formed, a weight analysis of protein band molecules was performed using the software of microsoft excel. The Linear equations were obtained by molecular weight log measurements and a migration band size of y = -0.1537X + 2.235 with R of 0.9581. The results of the analysis of protein molecular weight can be arranged in a Table.

RESULTS AND DISCUSSION

Integration stability of putative transformants

Semiarti et al. (2018), reported in transgenic *Phalaenopsis "Sogo vivien"* plant carrying T-DNA *35S::Gal4::AtRKD4::GR* is stably integrated with genome after one year of transformation. Zulwanis et al. (2020), reported that transgenic *Dendrobium phalaenopsis* carrying *35S::GR::AtRKD4* so far is stably integrated inside the genome. Despite various percentages.

The detection of *35S::GR/UAS:AtRKD4* integration stability in *D. lineale* was carried out using ten transgenic plantlets and two wild-type plantlets (Figure 2). In general phenotype of non-transfromant and transformant plantlets carrying T-DNA with construct 35S::GR::AtRKD4 there is no significant difference as seen in Figure 2. This is in accordance with the expected, unchanged phenotypes of plants from somatic embryos, the phenotype of transformant plants remains the same as plants derived from zygotic embryos.

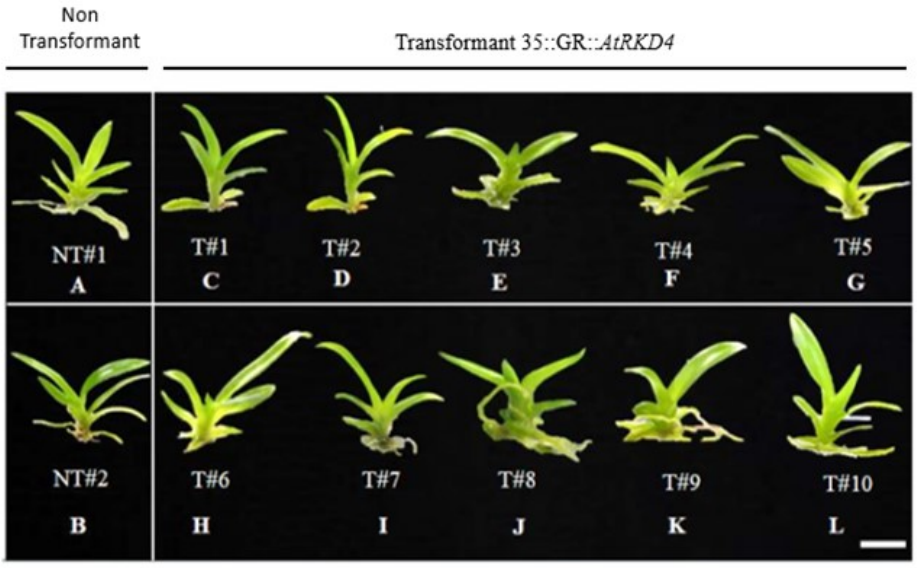

Figure 2. Phenotype of *D.lineale* Putative Transformant carrying T-DNA with 35S::GR::AtRKD4 and non-transformant. . A-B: Non-transformant plants (NT#1, NT#2) and C-L: 10 putative transformant (T#1-T#10), plant age 1 year 10 months. Bar = 1 cm.

Figure 3 shows detection of the stable integration of T-DNA carrying the gene *AtRKD4* in the *D. lineale* transformant orchid indicates that the *AtRKD4* gene could be amplified from all transformant plants, resulted in a DNA fragment of 382 bp in length. *AtRKD4* genes could not be detected in NT plants, indicated that all non-transformant plants did not contain the *AtRKD4* gene in their genomes. The presence of the *AtRKD4* genes in genome DNA of transformant plants indicates there were stable integration of the *35S::GR/UAS:AtRKD4* transgene in the *D. lineale* genomes and that

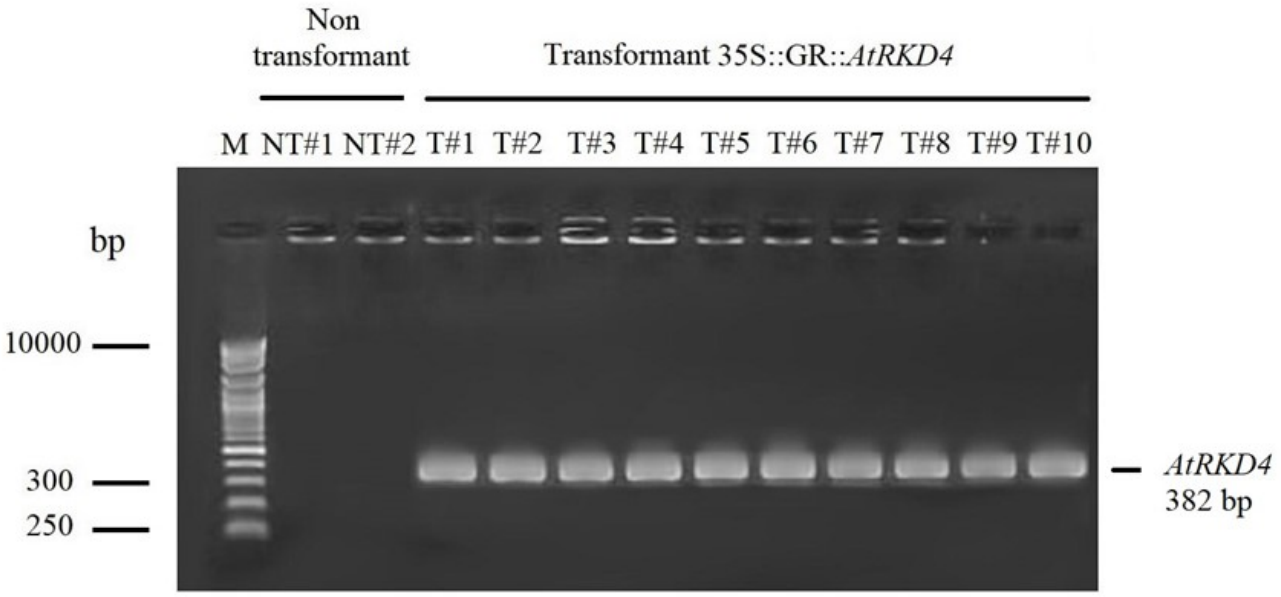

Figure 3. Detection of *AtRKD4* integration in the genome of *D.lineale* transformants carrying T-DNA with 35S::GR::AtRKD4. M: DNA marker. Lanes 2-3: non-transformant (NT); 4-13: transformant.

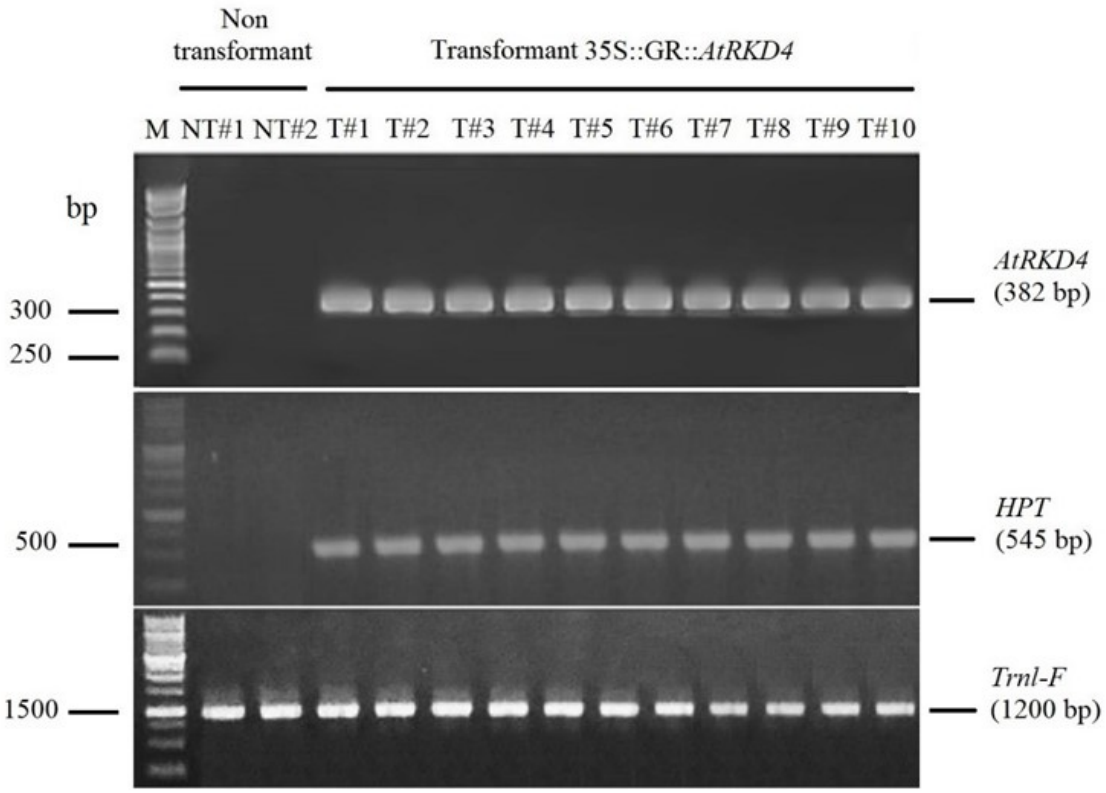

Figure 4. Detection integration of T-DNA harboring 35S::GR::AtRKD4 in the transformant genome of *D. lineale*. A. PCR product of *At*RKD4 gene (382 bp); B. *HPT* gene (545 bp), b.; and C. Interspace fragment of chloroplast DNA *trnL -F* (1200 bp) as an internal control of the PCR process. Lanes 2-3 Non-transformant plants; lanes 4-13: candidates of transformant plants.

during growth and development of transformant the transgene was somatically maintained.

Detection of *D. lineale* transformant lines was carried out by using *HPT* primers that amplified a 545 bp DNA fragment (Figure 4). HPT is an enzyme produced by the *Streptomyces hygroscopicus* bacteria that is resistant to hygromycin. HPT was used as a selection marker found in T-DNA of *A. tumefaciens* induce into the genome of orchids that were used to prove positive

transformant plants. Detection of the *HPT* gene in the plant genomes proved that transformant plants were resistant to hygromycin antibiotics and plantlets can grow well on a medium containing hygromycin (Setiari et al. 2018).

Molecular markers role as control is an important part of plant biotechnology development related to gene regions both in DNA and genomes (Yeşiltaş et al. 2019). The interspace fragment trnL-F of chloroplast genome was used as an internal control of PCR analysis. Chloroplasts have their genome which contains conserved genes and encodes many specific components. It is usually passed down maternally in most angiosperms (Filiz et al. 2018)

Evaluation of T-DNA integration stability was examined by PCR method, which used two inserted genes: HPT (545 bp), and *AtRKD4*(382bp) as indicators to confirm the integration stability of *AtRKD4* transgene into *D. lineale genome* (Figure 4). It is proven that T-DNA transgene carrying 35S::GR::*AtRKD4* is stably integrated into the genome of *D. lineale*. This is reinforced by the amplification of the interspace fragments of trnL-F chloroplast DNA (1200 bp), which indicates that the plant DNA genome has good quality and the PCR reactions have been well underway, then the results can be accounted for the truth.

Induction and Detection of AtRKD4 protein in orchid transformant plants after being Induced by Using DEX and TDZ

Detection of *AtRKD4* gene expression carried out after leaves of transformant plants induced on NP media containing 15 µM DEX or 3 mgL^{-1} TDZ for 1, 3, 5, and 7 days. T-DNA in the pTA7002 plasmid construct used to insert the *AtRKD4* gene contains a GRE activated by steroid hormone such as DEX in the orchid genome. In this study, TDZ played a role as an alternative to activate somatic embryos. TDZ is a powerful synthetic growth regulation exhibiting both auxin-cytokinin-like effects in plants, the indirect effect of TDZ is considered to be its ability to inhibit the enzyme cytokinin oxidase/dehydrogenase which degrades cytokinin (Nisler 2018). TDZ uses in inducing SE were reported by Gow et. al (2018) to induce SE in *Phalaenopsis aphrodite*. Mose et al. (2020) reported that direct somatic embryogenesis in *P. amabilis* orchid can be effectively induced from various explants by using a combination of auxin and cytokinin, with the best combination being 3.0 mg L-1 TDZ and 1.0 mg L-1 NAA in dark condition. Zulwanis (2020) found that SE could be induced by TDZ in leaf explant of transformant plant of *Dendrobium phalaenopsis* orchid that carrying AtRKD4 transgene, therefore it assumed that the existence of TDZ could trigger plant endogenous steroid hormones to drive the expression of *AtRKD4* transgene. The most optimal expression of the *AtRKD4* gene was detected after 5 days with DEX or TDZ. Based on Zulwanis (2020), DNA bands that amplified from the explants were induced using DEX and TDZ during 1 to 9th day, the highest transgene expression was found .in day 5th of induction as the highest DNA band density. These results correspond to the results of gene expression analysed by Febryanti et al. (2020) showing that AtRKD4 mRNA appeared in *D.lineale* transformant samples induced with medium NP +15 µM DEX or NP + 3 mgL-1 TDZ. This proves that the translation process goes well judging by the formation of *AtRKD4* gene expression products in the form of proteins. One of the growth regulators widely used in *in vitro* propagation systems is TDZ (Mahendran & Narmatha Bai 2016). The formation of somatic embryos in *Phalaenopsis* 'Sogo Vivien' could successfully be induced by using 10 mg/L TDZ supplemented into NP medium (Kasi & Semiarti 2016). However, the direct mechanism of TDZ for activating gene expression in SE is still unclear (Modi & Kumar 2018).

DEX works across plasma membrane diffusion and bonded with *glucocorticoid receptors* (GR), which are in the unprotected state, as a cytoplasmic complex with 90 kDa *Heat Shock Protein* (HSP90). The separation of HSP90 was due to the absence of ligands that localized the transcription factor into the nucleus. Inside the nucleus, GR binds to specific DNA sequences and activated the expression of the *AtRKD4* gene (Schena et al. 1991).

AtRKD4 transgene expression at the translation level was known in the appearance of protein formation in the *D. lineale* carried *35S::GR/UAS::AtRKD4* after treated by DEX or TDZ. Fellers et al. (1997) and Tchorbadjieva (2005) identified two proteins with molecular weights of 43 and 27 kDa which were used as embryogenic cell markers in wheat callus. Analysis of protein molecules can be carried out using the SDS-PAGE method. This method was used to detect predicted AtRKD4 protein bands with a molecular weight of ~28.53 kDa by electrophoresis separation (Heda et al. 2016). The isolated total protein from leaves of transformant plant carrying the construct of *35S::GR/UAS::AtRKD4* after 5 days treated by 15 µM DEX, and 3 mgL^{-1} TDZ showed 7 protein bands with various molecular weight. One of the protein band was 28.53 kDa in molecular weight, that can be predicted as AtRKD4 protein (Figure 5). This is supported by data on the absence of protein bands with a size of 28.3 kDa in non-transformant plants.

It is also supported by data showing that in transformant plants that planted on the NP basic medium without additional DEX or TDZ the protein band formation with a size of 28.3 kDa were not detected. Thus, it can be assumed that transgene *AtRKD4* supposed to be well expressed in *D. lineale* transformant plants that carrying 35S::GR::AtRKD4 after being induced by DEX and/TDZ.

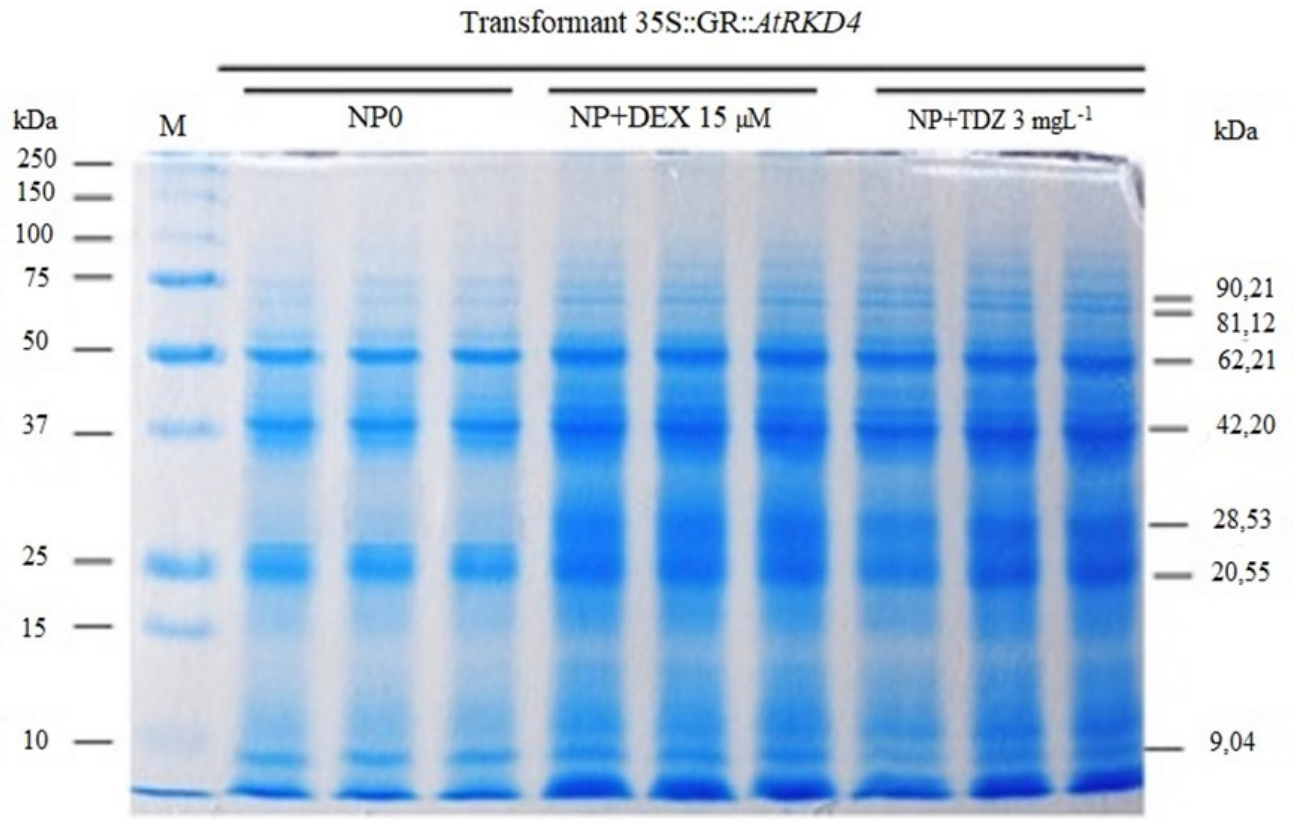

Figure 5. Protein profile of transformant plant *D. lineale* carrying T-DNA with *35S::GR/UAS::AtRKD4*. Lane 1: protein marker 250 kDa (Biorad); Lane2-4: SE induction on NP Basic Medium. Lanes 5-7: SE induction with 15 µM DEX, Lanes 8 -10: induction with 3 mgL^{-1}TDZ.

The molecular size of *D.lineale* proteins induced by DEX and TDZ is determined based on linear equations of molecular weight log measurement and migration of protein band size in SDS-PAGE presented in Table 1.

Table 1. Protein products in the transformant plant *D. lineale* that carry the *AtRKD4* gene after being induced with DEX and TDZ for somatic embryogenesis.

Number of Bands	Protein products of *D. lineale* transformant carrying 35S::GR::AtRKD4 planted on various mediums		
	NP0 (kDa)	NP+DEX 15 µM (kDa)	NP+TDZ 3mgL^{-1} (kDa)
1	90.21	90.21	90.21
2	81.12	81.12	81.12
3	62.21	62.21	62.21
4	43.20	43.20	43.20
5	-	28.53	28.53
6	20.55	20.55	20.55
7	9.04	9.04	9.04

Table 1 showed that there were 7 kinds of protein products that could be detected after 5 days treated by both DEX and TDZ, and only 6 proteins can be detected from in NP0 treatment. Protein with 90,21 kDa was the largest protein detected in all treatments, and protein with a size of 9.04 kDa was the smallest one. AtRKD4 protein with 28.3 kDa can be detected as the fifth band in *D. lineale* transformant that planted on NP medium supplemented by both 15 µM DEX, or 3 mgL^{-1}TDZ. A thick protein band weighing 20.55 kDa was found, thought to be the cycline protein responsible for cell division. This protein is present in all developing cells and plays a role in regulating the transition of phase G1 to phase S and phase G2 to phase M. Cycline proteins have a molecular weight range of 16-25 kDa. While the protein band measuring 9.04 kDa is thought to be a protein that encodes the SAUR (Small Auxin-Up RNA) gene that encodes proteins measuring 9-10 kDa. The SAUR gene is known to be responsible for cell division and lengthening and serves as a promoter for activating genes in protein syntheses such as cycline and expansion proteins (Li et al. 1994).

In orchids, Utami et al. (2007) found a protein measuring 44 kDa during the induction of somatic embryogenesis of the orchid *P. amabilis in vitro* culture. The successful formation of the AtRKD4 protein from the *Arabidopsis thaliana* model plant in the *D.lineale* orchid transformant plant suggests that the embryonic gene can work well in orchids, even though the gene is derived from dicot plants. This will open up opportunities in the future that the *AtRKD4* gene can be used for a wide variety of plants both dicots and monocots for plant micropropagation through somatic embryogenesis.

CONCLUSION

The *AtRKD4* genes are stably integrated into *D. lineale* transformant orchid genome, expressed and function for inducing somatic embryogenesis. All ten transformant plants stably carrying the T-DNA with 35S::GR::AtRKD4 constructs. The AtRKD4 protein was detected in all ten transformants after being induced for 5 days by both15 µM DEX or 3 mgL^{-1} TDZ, which showed the predicted AtRKD protein band in 28.53 kDa molecular weight. The direct role of TDZ in the induction of somatic embryos in this study is

still unclear. Therefore, more research is needed on the function of TDZ in embryo formation in plants, mainly in orchids.

AUTHORS CONTRIBUTION

G.C.W.: data collection, analysis and interpretation data, preparation and writing of the article. N.L.P.K.F.: idea of the experiment, data collection, preparation, and writing of the article. F.P.: data collection, analysis and interpretation data, preparation and writing of the article. D.S.: data collection, analysis and interpretation data, preparation and writing of the article. J.G.M.: founder of T-DNA construct. E.S.: adviser of work, data collection, and analysis, interpretation, critical review.

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CONFLICT OF INTEREST

The authors declare there is no competing interest.

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Research Article

The Increase of Sumatran Tiger's Prey Following Eradication of *Melastoma malabatrichum* in Way Kambas National Park, Indonesia

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ABSTRACT
The invasion of the planter's rhododendron (*Melastoma malabatrichum*) in Way Kambas National Park caused the loss of the sumatran tiger preys feeding ground, therefore efforts were made to eradicate the plant. This study aimed to compare the presence of sumatran tiger preys between *M. malabatrichum*-invaded location and eradicated location. Eradication was carried out by removing *M. malabatrichum* on a plot measuring 80 x 60 m^2. To record the animal visit, the camera traps were placed at the eradicated and invaded location of *M. malabatrichum* for comparison. The results showed that the *M. malabatrichum* eradicated location was more frequently visited by sumatran tiger preys. At the *M. malabatrichum* eradicated location, camera traps recorded 19 species of wild boar having the highest encounter rate (55.23) followed by sambar deer (33.24), and long-tailed macaque (17.43). Meanwhile, at the *M. malabatrichum* invaded location, camera traps recorded 13 species with wild boar having the highest encounter rate (30.56), followed by sambar deer (14.75), and long-tailed macaque (14.48). Thus, the eradication of *M. malabatrichum* had a good impact on increasing the number of sumatran tiger preys due to the availability of feed after being free from *M. malabatrichum* invasion.

Keywords: feeding ground, invasive species, Melastoma malabatrichum, sumatran tiger, tiger preys

INTRODUCTION
The sumatran tiger (*Panthera tigris sumatrae*) is a highly endangered carnivore whose distribution is limited on the Sumatra island, Indonesia. These animal conditions are threatened by poaching, both tigers and their prey animals. Other threats are caused by habitat fragmentation and degradation, as well as being killed due to conflicts that frequently occur between humans and tigers in border areas of tiger habitat and human settlements (Wibisono & Pusparini 2010; Linkie et al. 2018). Sumatran tiger population continues to decline, in its natural habitat only about 500 animals remain and some occupy

fragmented forests (Kenney et al. 2014; Goodrich et al. 2015; Joshi et al. 2016; Harihar et al. 2017). This condition becomes a considerable challenge for the conservation efforts of these animals.

Nowadays, the remaining population of sumatran tiger is partly in conservation areas, for example, Way Kambas National Park (WKNP), Lampung Province, Indonesia (Wibisono et al. 2011). Like other national parks, WKNP is also not immune to a variety of threats. One of the threats that recently happening is *Melastoma malabatrichum* invasion. This problem causes the loss of feeding grounds for sumatran tiger preys, such as sambar deer (*Cervus unicolor*), barking deer (*Muntiacus muntjak*), and java mouse-deer (*Tragulus javanicus*) (Master et al. 2020). One of the locations that invaded quite badly is the Kali Biru swamp. Previous research (Subagyo 2000) revealed that *M. malabatrichum* is not dominant in Kali Biru. However, recently the plant has covered 88% of this swamp area with a density of 30 individuals/m^2 (Master et al. 2018).

Kali Biru swamp in WKNP is a swamp formed by a tidal run-off of a river that runs in the middle. This location is important for animals in WKNP, especially the ungulates because the availability of grass is always green in this location (Master et al. 2020). The gathering of various animals in this location attracts sumatran tiger as predator and an important location for prey hunting. Tigers are very dependent on the abundant of prey animals (Thapa & Kelly 2016). With the invasion of *M. malabatrichum* which covers the location, the grass as ungulate's food cannot grow, this cause ungulate population decline (Master et al. 2020). This certainly has an impact on sumatran tiger which utilizes the location as a hunting area.

The conservation of prey species is an important factor for sumatran tiger survival (Carbone & Gittleman 2002). As a carnivore, tigers entirely depend on the existence of their prey as food source. Although tigers can inhabit various forest types, they need suitable prey bases to survive (Harihar & Pandav 2012; Sunarto et al. 2012; Hebblewhite et al. 2014).

Plant invasion has become a global concern. This is due to the negative impacts of the uncontrolled development of these plants in the conservation area causing biodiversity loss (Master et al. 2013). Indonesian Ministry of Environment reported that there were at least 1936 invasive plant species in Indonesia (S. Tjitrosoedirdjo et al. 2016), and *M. malabatrichum* is one of the most 75 threatening invasive species (S. S. Tjitrosoedirdjo et al. 2016).

The invasion of *M. malabatrichum* has been suggested to cause grazing areas to change into shrubs which are not preferred by tiger preys. These changes will affect the populations of prey species and will have a direct influence on tiger populations (Karanth et al. 2011). This study aimed to compare the presence of sumatran tiger preys between *M. malabatrichum*-invaded location and eradicated location.

METHODS

Study Site

The study was conducted in Kali Biru tidal swamp, Way Kambas National Park, Lampung Province, Indonesia (105°47'44"E, 4 °59'50"S) on December 2018 - December 2019. This tidal swamp area is included in the Tiger Elephant Rhino Monitoring Area (TERMA) location, which is an intensive monitoring location of priority animals in WKNP (sumatran tiger, sumatran elephant, and sumatran rhino) determined based on the decree of the Head of WKNP Hall No. 13/BTN.WK-1/2015. The topography of the WKNP area is relatively flat and bumpy with a height between 0 - 50 meters above sea level (Balai Taman Nasional Way Kambas 2012). WKNP area has five vegetation types namely mangrove forest, coastal forest, riparian forest, tidal

swamp, and lowland forest. Based on the land cover, most of the lowland forest is dryland forest followed by reeds and shrubs (Amalina et al. 2016).

Eradication of *M. malabathricum*

Kali Biru swamp has an area of approximately 140 ha, of which about 88% is currently covered by *M. malabatrichum*. In this study, the experiment was carried out to eradicate the invasion of these plants on a plot measuring 80 x 60 m^2. Eradication was performed manually by pulling out to the roots by hand. The eradication of *M. malabatrichum* was conducted in December 2018. Plots measuring 2 x 2 m^2 as many as 6 sub-plots were placed randomly in the *M. malabatrichum* plot to count the number of individuals of each species that grew after the withdrawal of *M. malabatrichum*. Post-revocation monitoring was performed 6 times, in January, February, March, May, June, and September 2019 by recording all types and number of plants contained in the sub-plot (Figure 1).

Based on the Schmidt and Ferguson classification, WKNP and its surroundings are included in type B climate (Balai Taman Nasional Way Kambas 2012). This study was carried out in the rainy season (January to April 2019) and the dry season (May to September 2019). Rainfall conditions also affect the condition of the Kali Biru swamp where the swamp is inundated during the rainy season and is dried in the dry season (Master et al. 2020).

Wildlife Species Monitoring

Camera traps were placed in two different habitat conditions, the first location was the location invaded by *M. malabatrichum*. Both locations are in Kalibiru Swamp known as the sumatran tiger home range in WKNP. At the first location, a camera trap was placed on the edge of the forest bordering the invaded location. The second location was in the grazing area of the eradicated plot from *M. malabatrichum*. At the edge of the eradication plot

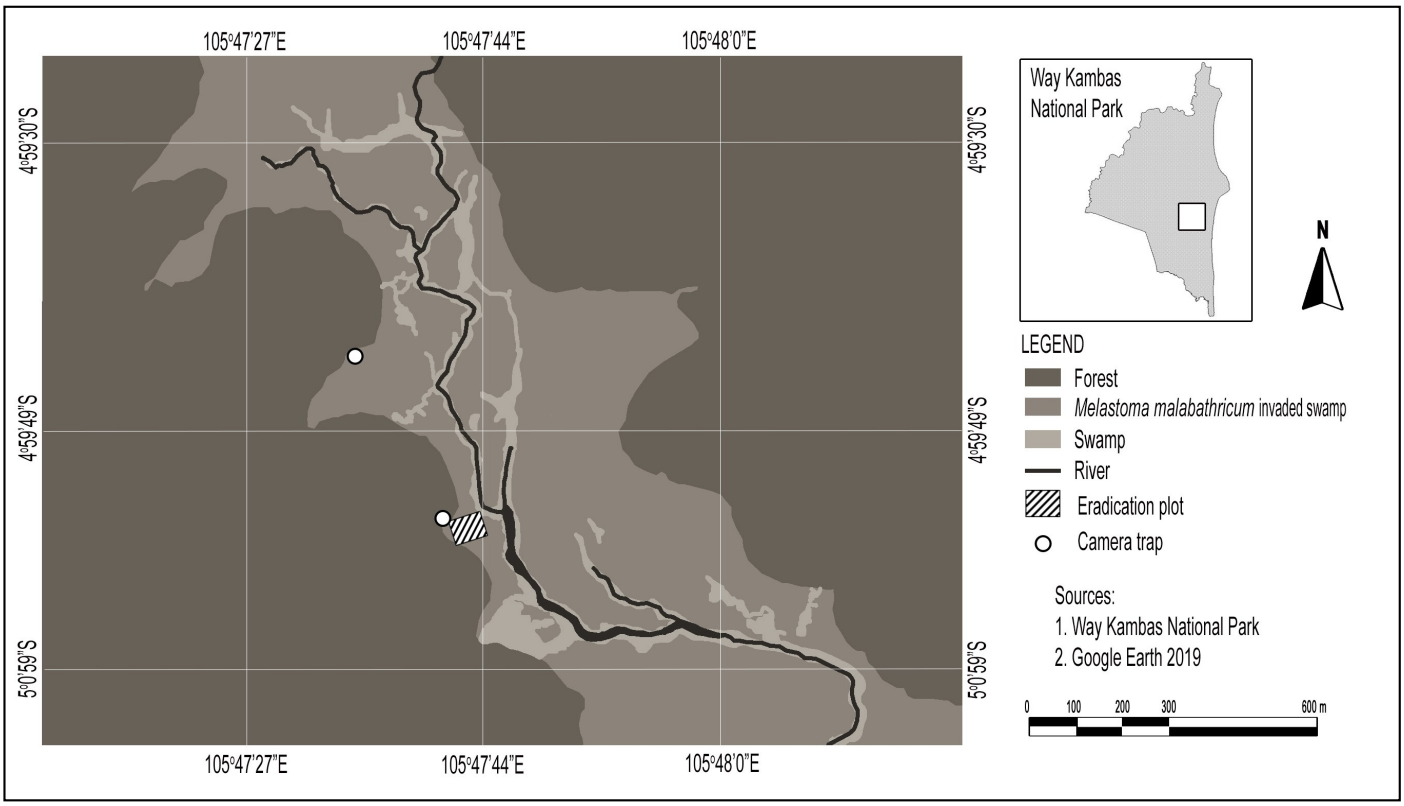

Figure 1. Research location and placement design of eradication plot and camera trap in the Kali Biru Swamp, Way Kambas National Park (WKNP), Lampung Province, Indonesia.

were camera traps that were placed at transitional locations. It is because tigers and their prey species were more easily found in transitional areas between forests and grasslands (Siswomartono et al. 1994).

Each location had a camera trap attached to the tree. The camera trap was installed 40 cm from the ground facing the animal crossing. Camera traps were installed for 382 days (14 December 2018 - 25 December 2019) with a checking period once a month. The value of the encounter rate (ER), which is the rate of encounters of animals against camera traps or the frequency of animals recorded by cameras, was calculated based on the formula (O'Brien et al. 2003): $\Sigma ER = \Sigma f/\Sigma d \times 100$, where ER = encounter rate; Σf = Total number of photos obtained; Σd = Total number of camera operation days.

The assumption used to identify individuals using independent videos was videos sequentially recorded on one file in a memory card that has been filtered based on time. Videos were said to be independent if (1) videos of different species or different individuals on a memory card, (2) sequential videos of the same individual (same species) in a video file with a span time of more than 30 minutes, or sequential videos of different individuals if it can be clearly distinguished, and (3) videos of the same individual or the same species that are not consecutive on one memory card file (Kelly et al. 2008; O'Brien et al. 2003).

RESULTS AND DISCUSSION

Habitat Conditions After Eradication of *M. malabathricum*

The control of *M. malabathricum* invasion in conservation areas has not been widely studied. Most of the controls on cultivated land use systemic herbicides for woody weeds. The use of herbicides in the Kali Biru swamp has a high risk of biodiversity in that location because the area is waterlogged so that the herbicides spread quickly around the treatment area and pollute the waters.

Manual weed control by pulling out has also been carried out to control the invasion of *Acacia nilotica* in Baluran National Park because control by cutting and burning of *A. nilotica* has not shown optimal results (Basari 2012). *M. malabathricum* has a shrub habitus, so it is easier to do it manually than *A. nilotica* which has tree habitus.

Before controlling, the plot was overgrown by *M. malabathricum* with a density of 30 individuals/m^2 and a height of up to 2.1 m. Under the *M. malabathricum* stand, there were only two species of grass namely *Cyperus* sp. and *Cynodon* sp. with a density of 5 individuals/m^2 and 1 individual/m^2 respectively. These plants cannot compete with *M. malabathricum* because the density of *M. malabathricum* causes other plants to not get enough sunlight so that the growth is stunted (Figure 2). In addition, *M. malabathricum* has allelopathic compounds to reduce germination rates and lengthening of grassroots and shoots (Faravani et al. 2008; Sari & Prakusya 2020).

The *M. malabathricum* control was performed manually by pulling out to the roots by hand. *M. malabathricum* control aimed to restore the function of the invaded ecosystem to grazing areas. After *M. malabathricum* was removed, grass for herbivores began to grow. There were 12 types of plants grown after the treatment namely *Fimbristylis* sp., *Limnophila* sp., *Cyperus* sp., *Cynodon* sp., *M. malabathricum*, and several unidentified species. Post-eradication, forage crop was originated from the seed bank and seed dispersal from around the plot. The seeds can be scattered into the plot assisted by wind, water, and animal activity, especially herbivores such as the Sambar deer (*Cervus unicolor*).

Figure 2. Invasion of *Melastoma malabathricum* in the Kali Biru Swamp, Way Kambas National Park (WKNP), Lampung Province, Indonesia. *Melastoma malabathricum* a. flower, b. fruit.

During the observation period, there was a fluctuation in the number of individual plants in each plot, this was caused by the growth and activity of herbivorous animals eating these plants. Not only grass, but *M. malabathricum* returned to grow with the second-highest amount after *Fimbristylis* sp. Post-control *M. malabathricum* was originated from the seed bank in the soil or the seeds fell during the *M. malabathricum* pull out or from the seeds being spread from locations around the plot where *M. malabathricum* was grown. In addition to seeds, *M. malabathricum* shoots were grown from broken stumps and remain in the ground when uprooted. Based on the graph in Figure 3, only *M. malabathricum* did not experience a decrease in the number of individuals during January – July. This is possible because *M. malabathricum* is not eaten by animals and has a better competitive ability than other plants. From July to September, the number of individuals of each plant species decreased due to drought (Figure 4).

The Presence of Sumatran Tiger Preys (*Panthera tigris sumatrae*)

The wildlife species encounter (ER) especially mammals is used to compare habitat use by wildlife species in two different locations. Videos of camera traps recorded at the two locations showed that more animals were found at the eradicated sites (Table 1) (Figure 5 and 6). Camera traps at eradicated locations recorded 19 species of wild boar as the animals with the highest ER values (55.23) followed by sambar deer (33.24) and long-tailed macaque (17.43), whereas in the location invaded by *M. malabatrichum* recorded 13 species with wild boar as animals that have the highest ER value (30.56) followed by sambar deer (14.75) and long-tailed macaque (14.48). These species are the main prey for sumatran tigers.

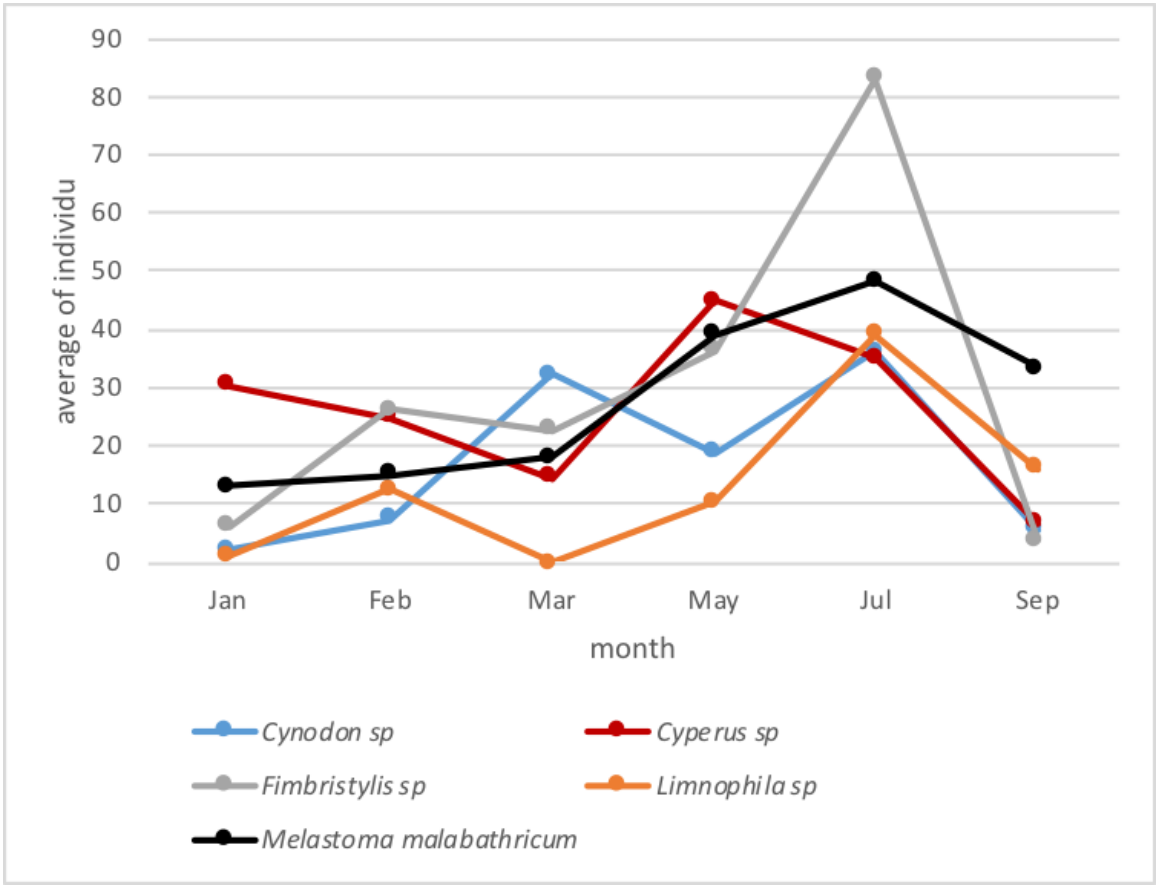

Figure 3. Plant composition and average from 6 monitoring plots after eradication of *Melastoma malabathricum,* January –September 2019.

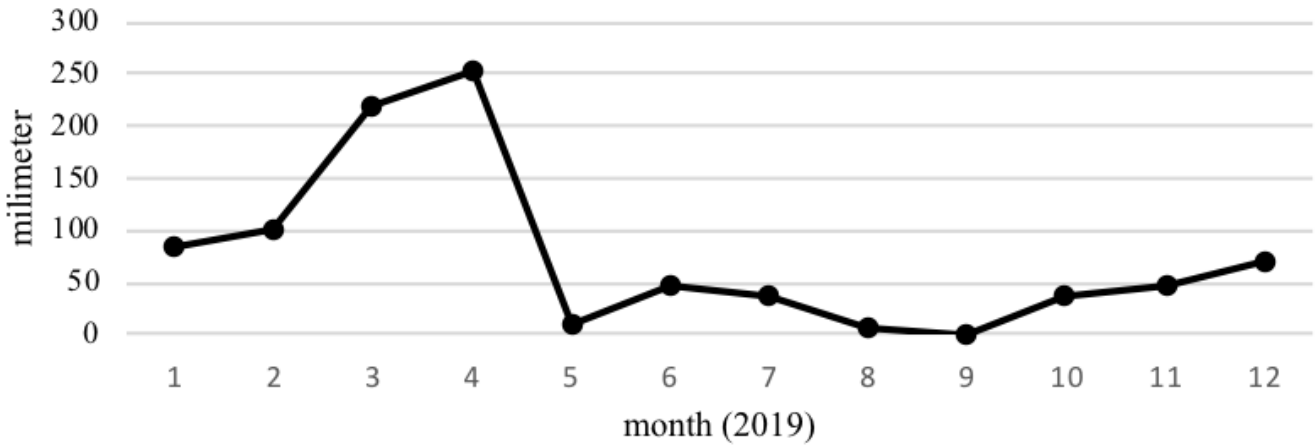

Figure 4. Total monthly rainfall of Lampung Province in 2019 (Master et al. 2020).

The eradicated habitat provided resources needed by herbivorous animals. These animals respond to these habitats adaptively because herbivores depend on the abundance and distribution of plant species as food (Hale & Swearer 2017). A large number of herbivorous encounters which are tiger preys, in eradicated locations are caused by abundant food (grasses) after the eradication of *M. malabatrichum*. The abundant presence of prey animals in the location eradicated attracts the attention of sumatran tigers and other predators to come, such as clouded leopards and leopard cats.

The density of tiger population in a location is influenced by the quality of habitat and the availability of tiger preys because prey animals are one of

Table 1. Comparison of encounter rates using camera traps at the invaded location of *Melastoma malabathricum* and controlled location.

No	Species	IUCN Status	Potential Prey for Sumatran Tiger	Encounter Rate (Photos/100 days)	
				Eradicated location	Invaded location
1.	Wild boar (*Sus scrofa*)	Least Concern	√	55.23	30.56
2.	Sambar deer (*Cervus unicolor*)	Vulnerable	√	33.24	14.75
3.	Long-tailed macaque (*Macaca fascicularis*)	Least Concern	√	17.43	14.48
4.	Greater mouse-deer (*Tragulus napu*)	Least Concern	√	4.02	10.46
5.	Muntjac (*Muntiacus muntjak*)	Least Concern	√	3.75	2.95
6.	Lesser mouse-deer (*Tragulus javanicus*)	Data Deficient	√	2.68	5.36
7.	Asian Palm Civet (*Paradoxurus hermaphroditus*)	Least Concern	√	2.68	2.41
8.	Red Junglefowl (*Gallus gallus*)	Least Concern	√	1.88	0.54
9.	Malayan sun bear (*Helarctos malayanus*)	Vulnerable	√	1.61	0
10.	Leopard cat (*Prionailurus bengalensis*)	Least Concern		0.80	0.27
11.	Sumatran elephant (*Elephas maximus sumatranus*)	Critically Endangered		0.80	0
12.	Javan mongoose (*Herpestes javanicus*)	Least Concern	√	0.80	0
13.	Banded palm civet (*Hemigalus derbyanus*)	Near Threatened	√	0.80	0
14.	Asian water monitor (*Varanus salvator*)	Least Concern		0.27	0.27
15.	Sumatran tiger (*Panthera tigris sumatrae*)	Critically Endangered		0.27	0
16.	Clouded Leopard (*Neofelis diardi*)	Vulnerable		0.27	0
17.	Malayan Tapir (*Tapirus indicus*)	Endangered	√	0.27	0
18.	Malayan civet (*Viverra tangalunga*)	Least Concern	√	0.27	0
19.	Asian small-clawed Otter (*Aonyx cinerea*)	Vulnerable	√	0.27	0
20.	Pigtail macaque (*Macaca nemestrina*)	Vulnerable	√	0	0.80
21.	Crested Fireback (*Lophura ignita rufa*)	Near Threatened	√	0	0.80
22.	Masked palm civet (*Paguma larvata*)	Least Concern	√	0	0.27
Number of species				**19**	**13**
Total Individuals				**905**	**465**

the factors that determine the size of tiger territories (Sherpa & Maskey 1998). Based on the results of hair analysis found in sumatran tiger feces, the main animals of tiger prey in WKNP were wild boar, macaque, sambar deer, and sun bear (Franklin et al. 1999; Sriyanto 2003).

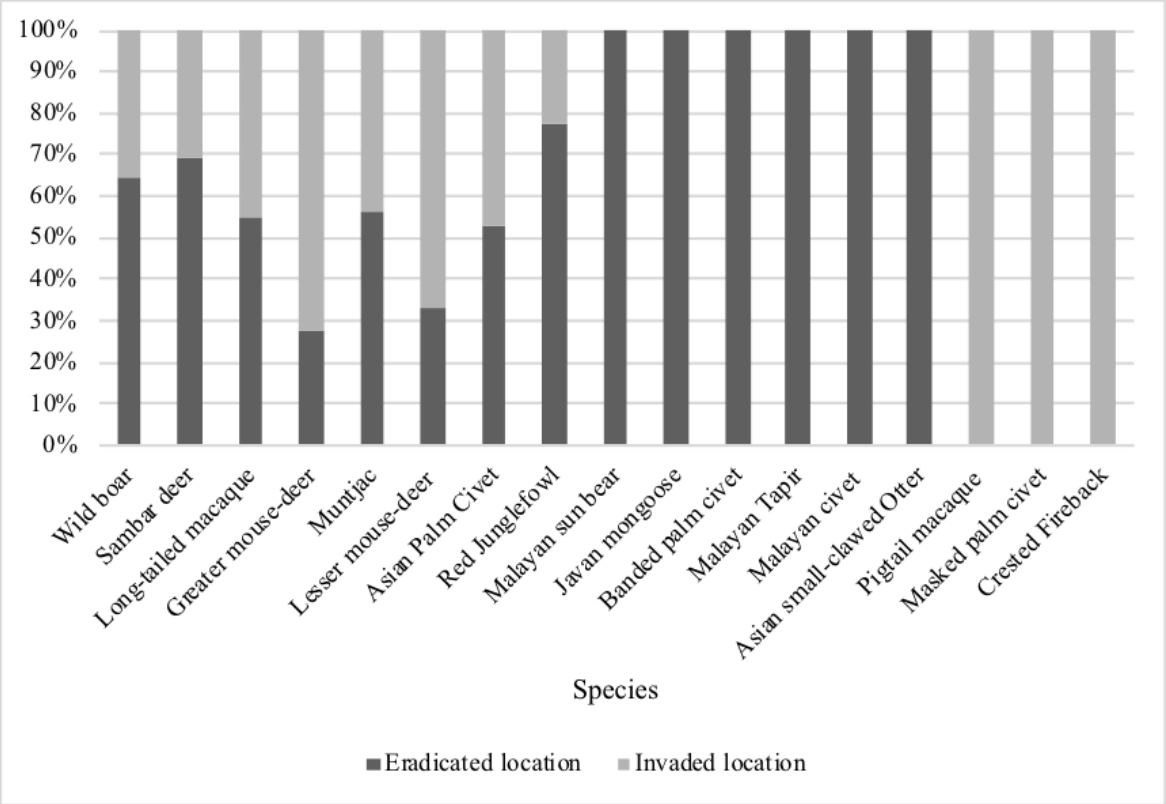

Figure 5. The comparison of prey animal numbers at eradicated and invaded locations.

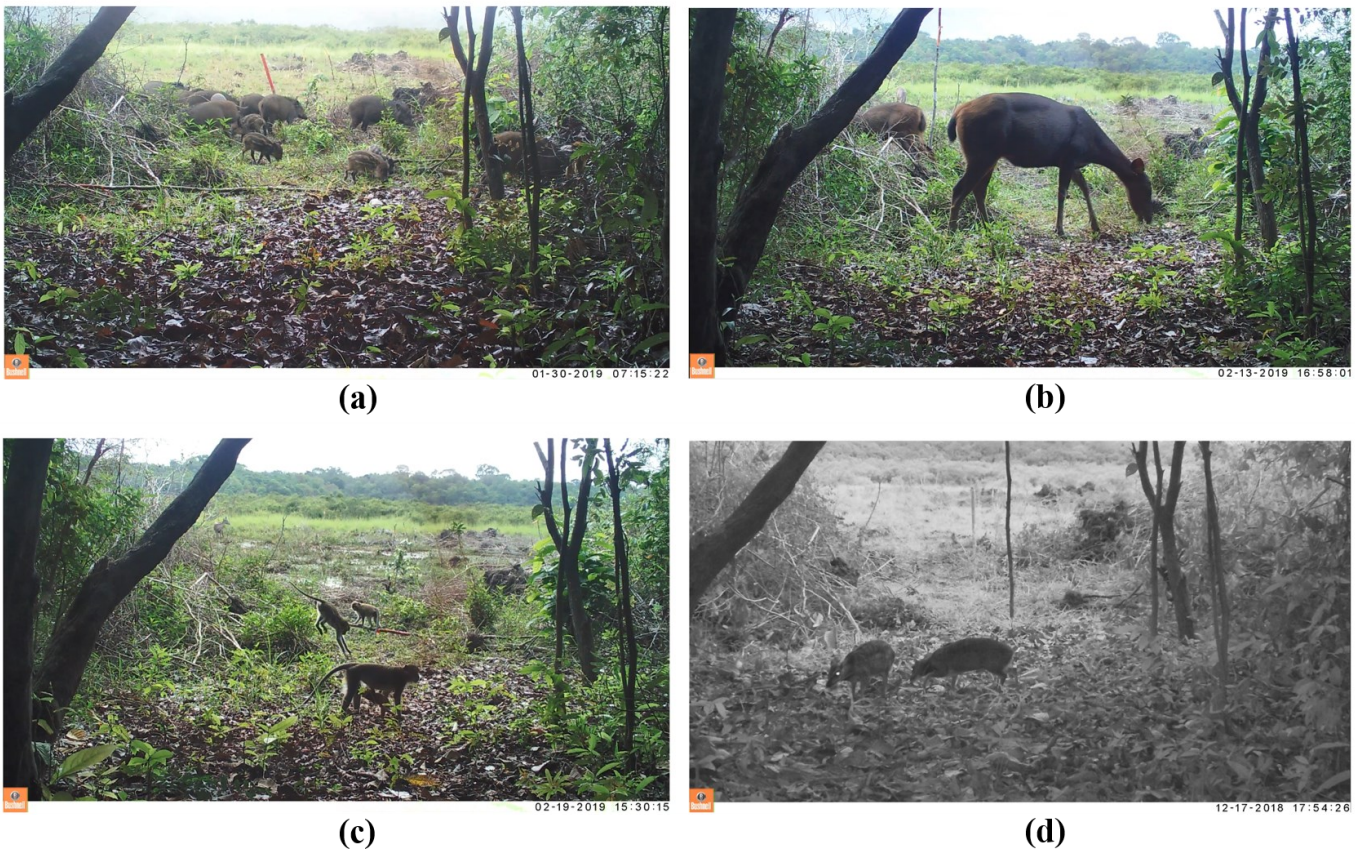

Figure 6. Sumatran tiger preys in eradicated area: (a) wild boar, (b) sambar dear, (c) long-tailed macaque, and (d) greater mouse-deer.

Based on the results of camera traps, wild boar (*Sus scrofa*) was the most recorded prey animals both at the invaded and eradicated locations.

However, the rate of encounter of wild boar in the eradicated location was much higher than the invaded one. These animals were usually recorded in groups with a number ranging from 2-18 individuals. Wild boars have a wide range of areas and are active at night and daytime, so the level of encounter is high. The availability of food sources is one of the factors affecting the presence of wild boar in a location because most of its activities are used for foraging (Azhima & Vincent 2001). Wild boars are omnivores, these animals eat grass, roots, worms, and insects (Chapman & Trani 2007). High levels of wild boar encounter at locations eradicated due to the availability of grass and on the open ground due to *M. malabatrichum* revocation processes.

The next animal that has a high level of encounter was sambar deer (*Cervus unicolor*). Sambar deer is a dominant prey for tigers (Biswas & Sankar 2002; Joseph et al. 2007; Kumaraguru et al. 2011; Hayward et al. 2012), this animal occupies a variety of habitats, ranging from coastal forests, secondary forests, swamp forests, reed fields and in mountain areas. However, sambar deer habitat will never be far from water sources (Bagchi et al. 2003; Simcharoen et al. 2014). The availability of nourishment plants is also important for the existence of deer (Forsyth & Davis 2011; Ginantra et al. 2018). Kali Biru Swamp of WKNP is a good habitat for sambar deer because it provides grasslands as a source of food, swamps, and rivers as a source of water, and forests around the swamp as a shelter. The flat, open topography of Kali Biru Swamp is preferred by sambar deer because it makes it easy to detect the presence of predators (Simcharoen et al. 2014; Valeix et al. 2009).

Sambar is included in the intermediate feeder group because it can be both a grazer (grass eater) and a browser (bush eater) (Priyono 2007). Grasses are the most preferred and important diets for sambar deer (Maksudi et al. 2010; Mustari et al. 2016). The invasion of *M. malabatrichum* causes grasses as the source of food to decrease, while the palatability of sambar deer to *M. malabatrichum* is quite low (Ismail & Jiwan 2015). This is due to the invasion of *M. malabatrichum* blocking the light intensity received under the plants. The lower intensity of the light will negatively influence the growth of grass as a deer diet (Masy'ud et al. 2008; Kunarso & Azwar 2013; Araujo et al. 2018). After being eradicated, the grass grew back and this caused the rate of encounter of sambar deer at the eradicated location to be twice as high as that of at the invaded location.

Based on the level of predation, animals mentioned above were among the favorite / main prey animals of sumatran tigers because the meat composition is under what is needed by tigers. A tiger needs 5-6 kg of meat a day for its survival (Sunquist 1981). A tiger can kill a muntjac weighing 20 kg every 2-3 days or kill a deer and muntjac weighing 200 kg every few weeks (Sunquist 1981). Other prey animals recorded by camera traps at eradicated locations were a long-tailed macaque, greater mouse-deer, deer, asian palm civet, red junglefowl, bear, javan mongoose, striped weasel, tapir, and malayan civet. Some animals such as bear, javan mongoose, banded palm civet, tapir, and malayan civet were not found at invaded locations but Southern pig-tailed macaque, masked palm civet, and crested fireback were. Different types of animals that inhabit a particular habitat are caused by factors and the carrying capacity of its habitat following animal needs.

The availability of adequate food is a major factor in the abundance of tiger prey animals which are herbivores. Rivers, swamps, and grasslands surrounded by forests are suitable locations for ungulates. The loss of grass due to the invasion of *M. malabatrichum* causes the density of herbivores to decrease, especially grazer species. Sumatran tigers can occupy a variety of habitats, but the most preferred habitat is border areas between the forest and grassland which is usually inhabited by predominant species such as wild boar, deer, barking deer, and greater mouse-deer. Sumatran tigers prefer

places that have high prey biomass and meet their daily needs. Therefore, sumatran tigers usually inhabit habitats related to forests near the river, swamp forests, and grasslands, but are very difficult to find in bush vegetation areas that are too dense (Sastrapradja et al. 1992). Kali Biru Swamp in WKNP has habitat criteria favored by sumatran tigers. Hence, the growth of *M. malabatrichum* in that location needs to be controlled, because the invasion of *M. malabatrichum* causes the grass not to grow and the grasslands turn into dense shrubs. One of the main problems that are closely related to the survival of tigers is the unequal distribution of plant species which are the source of food for tiger prey animals. Management actions that need to be taken to maintain the preservation of sumatran tigers are to increase the carrying capacity of their habitat through habitat improvement activities.

The eradicated location is not only preferred by sumatran tiger preys, but also by several other threatened animals. Besides sumatran tigers, animals that have a Critically Endangered status based on IUCN caught on cameras at the eradicated location are sumatran elephants (Figure 7). Several other animals caught on cameras at the eradicated location have a status of Vulnerable to Endangered. This indicated that this location was an important habitat for animals and required immediate treatments from the invasion of *M. malabatrichum*.

CONCLUSION

The eradication of *M. malabatrichum* had a good impact on increasing the number of sumatran tiger preys due to the availability of food after being free from *M. malabatrichum* invasion. Several animals recorded by camera traps at the location which has been eradicated were higher (19 species) than those at invaded locations (13 species). The eradicated location also had a higher value of tiger prey encounters than the invaded location. Therefore, it is necessary to carry out habitat management to support sumatran tiger preys

AUTHORS CONTRIBUTION

J.M., I.Q., D.S., and N.S. designed the research and supervised all the processes, J.M. collected and analyzed the data and wrote the manuscript.

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Figure 7. Critically endangered species in eradicated area: (a) sumatran tiger and (b) sumatran elephants.

CONFLICT OF INTEREST

The authors state no conflict of interest from this manuscript.

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Research Article

Endophytes and Rhizosphere Fungi from Galam (*Melaleuca cajuputi* Powell.) which has the Potential to Produce Indole Acetic Acid (IAA)

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ABSTRACT
Some types of fungi are known to have the ability to produce Indole Acetic Acid (IAA). Fungi can be isolated from the rhizosphere and tissues of various plants, including from the rhizosphere and the root "Galam" (*Melaleuca cajuputi* Powell.), which grow predominantly in peatlands. Therefore, the purposes of this study were: (a) to isolate and measure the potential of fungi from endophytic and rhizospheric of "Galam"(*M. cajuputi*) as a producer of IAA hormone, (b) determine the types of fungal interaction that occur and their potential to increase the total IAA hormone produced. This research begins with isolation, purification, isolate screening, analysis of IAA hormone production, data analysis, seed germination test and isolates identification. The result showed that the concentration of IAA produced by *Penicillium* sp. IRZ15 was 5.86 ± 0.47 µg.mL^{-1} to 8.46 ± 0.26 µg.mL^{-1} and *Syncephalastrum* sp. AG15 is 4.77 ± 0.44 µg.mL^{-1} to 8.77 ± 0.25 µg.mL^{-1}. Meanwhile, the combination of rhizospheric fungi *Penicillium* sp. IRZ15 and endophytic fungi *Syncephalastrum* sp. AG15 does not produce significantly different IAA concentrations (6.42 ± 0.34 µg.mL^{-1} to 9.19 ± 0.50 µg.mL^{-1}) compared to fungi used alone without combinations.

Keywords: endophytic fungi, Galam, *Melaleuca cajuputi* Powell., Indole Acetic Acid, peatland, rhizospheric fungi

INTRODUCTION
Indole acetic acid (IAA) is a hormone included in the auxin group (Wojciech Szajdak & Maryganova 2007). The IAA hormone plays an important role in triggering the extension and division of plant cells. Auxin is a hormone composed of indole rings and can trigger the growth and development of plants (Setyowati et al. 2017). The IAA hormone can support root extension and increase the absorption of nutrients from the soil (Suebrasri et al. 2020). The presence of endogenous IAA hormones (produced by plants) and the addition of exogenous IAAs can affect plant growth rates.

There have been many studies that show the presence of soil fungi and endophytic fungi capable of producing the IAA hormone (Larosa et al. 2013). Endophytic fungi can be isolated from various types of plants, one of which is "Galam" plants. "Galam" (*M. cajuputi*) is a plant that grows predominantly on peatlands. These plants are distributed in the peatlands of Central Kalimantan, South Kalimantan, and the coastal part of South Sumatra. "Galam" wood is classified as a pioneer plant that can grow in burnt areas and is tolerant of flooded areas (Supriyati et al. 2014), and generally can survive in acid sulfate swamp conditions with humus and peat soils. Therefore, it is assumed that "Galam" plants might be colonized by endophytic fungi that have the potential to help the host plants to survive under conditions of environmental stress by secreting secondary metabolites that are profitable, especially IAA.

Waqas et al. (2012), reported that endophytic fungi isolated from the roots of plants can secrete gibberellin, auxin, and cytokinin. Endophytic fungi such as *Penicillium* and *Phoma* which are isolated from cucumber plant tissue are known to have the ability to produce IAA hormones (Subowo 2016; Waqas et al. 2012). In addition to endophytic fungi, several types of fungi originated from the soil rhizosphere are known to have the ability to produce hormones (Unyayar 2000; Maor et al. 2004; Astriani et al. 2014). *Phanerochaete chrysosporium* (Unyayar 2000), *Aeschynomene*, and *Colletotrichum gloeosporioides* isolated from the rhizosphere are known to have the ability to produce hormones, such as IAA (Robinson et al. 1998; Maor et al. 2004; Astriani et al. 2014).

The interaction between fungi in the plant tissue (endophytes) and fungi around the rooting area (rhizosphere) can certainly give a diverse effect on plant growth, either positive or negative. One form of positive interaction is known as synergism. This synergism can be utilized both to support the growth of host plants and between species of fungus that interact with each other (Hestrin et al. 2019). Research by (Suebrasri et al. 2020) shows that endophytic fungi *Diaporthe phaseolorum* and *Macrophomina phaseolina* isolated from siam weed and ginger can produce the IAA hormone and increase the growth of sunchoke plant.

The production of IAA hormone by fungi can be optimized to perform combinations between fungi from various origins. However, there is less data on the research of rhizosphere fungi and endophytic fungi growing in peatlands in South Kalimantan and the forms of their interactions that have the potential to produce the IAA hormone. Therefore, it is necessary to perform research related to the exploration of rhizosphere and endophytic fungi from the "Galam" (*M. cajuputi*) and its interactions that have the potential to produce the IAA hormone. So that the information can be used for further studies on the utilization of fungi from peatland trees to support peatland farming.

MATERIALS AND METHODS

Materials

Materials used are plant roots of "Galam" (*M. cajuputi*), peat soil (10 gr), potato dextrose agar (PDA), and broth (PDB) media added with 1% chloramphenicol, NaOCl 5.3%, ethanol 70%, tryptophan, methanol, synthetic AIA solution, and Salkowski reagent (50 mL, 35% sulfuric acid, 1 mL of 0.5 mol $FeCl_3$ solution).

Methods

"Galam" (*M. cajuputi*) Rhizosphere Fungi Isolation

Soil samples (10 gr) were collected from the area around the plant roots

(rhizosphere) *M. cajuputi* with a depth of 10-20 cm. The isolation fungi according to Larekeng et al. (2019) method with modification, that was the pour plate method with multilevel dilution from 10^{-1} until 10^{-5} . Cultures were incubated at 22°C - 25°C for 5-7 days.

"Galam" (*M. cajuputi*) Roots Endophytic Fungi Isolation

Isolation of endophyte fungi was carried out by direct method. Endophyte fungi were isolated from "Galam" plants with a height of 3 meters and a diameter of 10 cm. The root used was the lateral root part close to the root hair. The roots were rinsed with water. Furthermore, sterilized the surface by soaking for 1 minute in ethanol 70%, 3 minutes in a solution of NaOCl 5.3%, and another 30 seconds in ethanol 70%. Then dried and cut into 1 cm sizes. Each root piece (3 pieces) was placed on PDA media with chloramphenicol on Petri dish, incubated at 22°C - 25°C for 5-7 days (Yunaedi et al. 2016).

In Vitro Screening of Rhizospheric and Endophytic Fungi

Screening rhizosphere and endophytic fungi isolate was performed by several methods which were: observation and measurement of the growth rate of fungi, pathogenicity test, and the test of antagonism.

a. Fungi Growth Rate

The growth rate of a single isolate of rhizospheric and endophytic fungi that have been isolated and purified was measured. The growth of fungi isolates was measured by the colony surface area every day after inoculation for 4 days. Measurement of growth rate was done according to (Miyashira et al. 2010) with modification by calculating the difference of the fungi's surface area divided by the time difference in the measurement of fungi.

b. Pathogenicity Test

The pathogenicity test of fungi was carried out using the healthy orchid (*Phalaenopsis* sp.) leaves. First, orchid leaves were washed using water and cut into 3 x 3 $Cm^{2.}$ Furthermore, surface-sterilized orchid leaf surface with 10% Clorox, 10% alcohol, and distilled water. Leaf samples were then placed in a Petri dish with an inverted surface. Orchid leaf pierced by 3 punctures per leaf using a sterile needle. Next, the fungi colonies to be tested were affixed to the puncture section that has been made. The presence or absence of disease progression was calculated based on the score disease rating (Chung et al. 2011).

c. In Vitro Antagonistic Test

Each non-pathogenic rhizospheric fungi isolate was interacted with non-pathogenic endophytic fungi isolates using multiple culture test techniques. Rhizospheric fungi isolates were grown in pairs with endophytic fungi. The pieces isolate were placed in pairs at a distance of 3 cm from the edge of the media PDA on the Petri dish and were incubated at 26°C - 28°C. The diameter colony of both fungi isolates was measured every day for one week. The inhibition percentage was calculated using the formula according to (Skidmore & Dickinson 1976). The interaction between the two fungi isolates was also observed microscopically by the slide culture method (Rabha et al. 2014). A pair of isolates that showed the lowest percentage of inhibition would be analyzed for the production of the IAA hormone.

Analysis of Indole Acetic Acid Production

Analysis of IAA hormone production was carried out by the method of Gordon and Weber (1951). Rhizospheric fungi isolates, endophytic fungi, and a combination of both fungi were inoculated into a 5 mL potato dextrose broth (PDB) medium which was enriched with 100 $\mu g.mL^{-1}$

tryptophan and potato dextrose broth (PDB) medium without tryptophan. Next, both media were incubated for 3 days at a speed of 150 rpm in a non-light condition and centrifuged for 15 minutes at a speed of 6000 rpm. 1 mL of the supernatant was transferred to a sterile test tube and 4 mL of Salkowski reagent was added. The suspension was then incubated in a non-light condition for 60 minutes at room temperature. If the color of the solution turned pink, it means that the solutions contained the IAA hormone. Next, absorbance measurements were carried out using a spectrophotometer with a wavelength of 530 nm. The determination of IAA concentration was done by comparing the absorbance value obtained with the IAA standard curve through regression analysis (Astriani et al. 2014).

Germination Test Using Chilli Seeds

The fungi which have the potential of IAA production were tested directly on the chili seeds. The first step was to surface sterilized the seed with 1% NaOCl. The chili seeds were then soaked for 1 day in sterile distilled water as a control and in the media which contain fungi isolates. The chili seeds were then placed on a Petri dish which contained 2 sheets of moist filter paper. The number of germination seeds, hypocotyl length, and epicotyl length measured every day in one week (Syamsia et al. 2015).

Data Analysis

The data of IAA hormone production that was obtained were presented in figures, tables, and descriptive analysis. The data also would be analyzed to the middle-value test (Astriani et al. 2014) and Duncan's Multiple Range Test (DMRT).

Identification of Rhizospheric and Endophytic Fungi

Rhizospheric and endophytic fungi that have the smallest inhibitory and proven potential to produce hormones IAA were identified. Identification was carried out by observing its macroscopic and microscopic morphological character based on the identification book *Illustrated Genera of Imperfect Fungi* (Barnett & Hunter 1987).

RESULTS AND DISCUSSION

Results

The Growth Rate and Patogenecity of "Galam" Root Rhizospheric and Endophytic Fungi

In total, there were 16 isolates of rhizospheric (IRZ) and 22 endophytic (AG) fungi successfully isolated during this study. The average rhizospheric and endophytic fungi growth rate is presented in Table 1 and Table 2.

The high value of the growth rate average is shown on IRZ7, IRZ8, IRZ10, IRZ13, IRZ15, AG10, AG11, AG15, AG18, and AG20 isolate.

The pathogenicity test showed no symptoms of the disease in control, IRZ7, IRZ8, IRZ10, IRZ15, and AG15 (Table 3). Meanwhile, the necrosis on AG18 isolate appeared from the first day with a score of disease rating of 2. The necrosis on the IRZ13, AG10, and AG11 appeared on the second day with a score of disease rating of 2. However, on isolates AG10 and AG11 consecutively on the fifth and fourth day, the symptoms of the disease were accompanied by the appearance of soft rot on the leaves so that the score of the disease rises to 3. Therefore, only isolate rhizospheric fungi IRZ7, IRZ8, IRZ10, IRZ15, and AG15 endophytic fungi were used in antagonism tests because they were classified as non-pathogenic based on disease scores arising from leaves.

Table 1. The rhizospheric fungal growth rate.

Isolate	Colony Growth Rate (Cm^2/day)			
	1th-DAI	2th-DAI	3th-DAI	4th-DAI
IRZ1	2	2	2	4
IRZ2	3	2	1	3
IRZ3	1	2	4	4
IRZ4	3	1	0	7
IRZ5	2	0	0	0
IRZ6	1	2	0	1
IRZ7T	2	4	4	5
IRZ8T	2	2	4	4
IRZ9	1	3	3	3
IRZ10T	2	3	3	3
IRZ11	1	2	2	4
IRZ12	1	0	0	0
IRZ13T	10	12	15	4
IRZ14	3	0	2	3
IRZ15T	2	5	46	0
IRZ16	1	0	0	0

Note: T selected isolate (the five isolates with the high colony growth rate), DAI (Day After Incubation).

Table 2. The endophytic fungal growth rate.

Isolate	Colony Growth Rate (Cm^2/day)			
	1th-DAI	2th-DAI	3th-DAI	4th-DAI
AG1	2	4	1	0
AG2	1	0	0	0
AG3	1	5	1	0
AG4	1	4	2	5
AG5	1	4	2	4
AG6	3	13	0	0
AG7	1	3	1	1
AG8	43	24	1	0
AG9	2	3	0	0
AG10T	5	18	26	0
AG11T	4	18	20	15
AG12	4	12	17	0
AG13	4	0	1	0
AG14	1	0	0	0
AG15T	5	9	11	9
AG16	5	8	10	0
AG17	2	0	0	0
AG18T	13	39	1	5
AG19	2	5	4	3
AG20T	4	14	17	1
AG21	1	0	0	0
AG22	4	0	0	0

Note: T selected isolate (the five isolates with the high colony growth rate), DAI (Day After Incubation).

Table 3. Pathogenicity test in vitro on the leaves of *P. amabilis* for 5 DAI.

No	Isolat	*Score Disease Rating*				
		1th-DAI	2th-DAI	3th-DAI	4th-DAI	5th-DAI
1	Leaf without fungal infection	0	0	0	0	0
2	IRZ7	0	0	0	0	0
3	IRZ8	0	0	0	0	0
4	IRZ10	0	0	0	0	0
5	IRZ13	0	2	2	2	2
6	IRZ15	0	0	0	0	0
7	AG10	0	2	2	2	3
8	AG11	0	2	3	3	3
9	AG15	0	0	0	0	0
10	AG18	2	2	2	2	2
11	AG20	0	0	0	2	2

Note: 0 = no symptoms of leaf disease, 1 = necrosis diameter ≤ 2 mm, 2 = necrosis diameter > 2 mm, 3 = necrosis diameter > 2 mm, and soft rot on the leaves. DAI (Day After Incubation).

In Vitro Antagonistic Test

Figure 1 showed that there was inhibition between the interaction of each isolate. The percentage of inhibition continued to increase until the seventh day. The interaction of AG15 and IRZ15 has a relatively slow increase in inhibition percentage and has a smaller value compared to other isolate combinations, so this combination was chosen for the analysis of IAA hormone production.

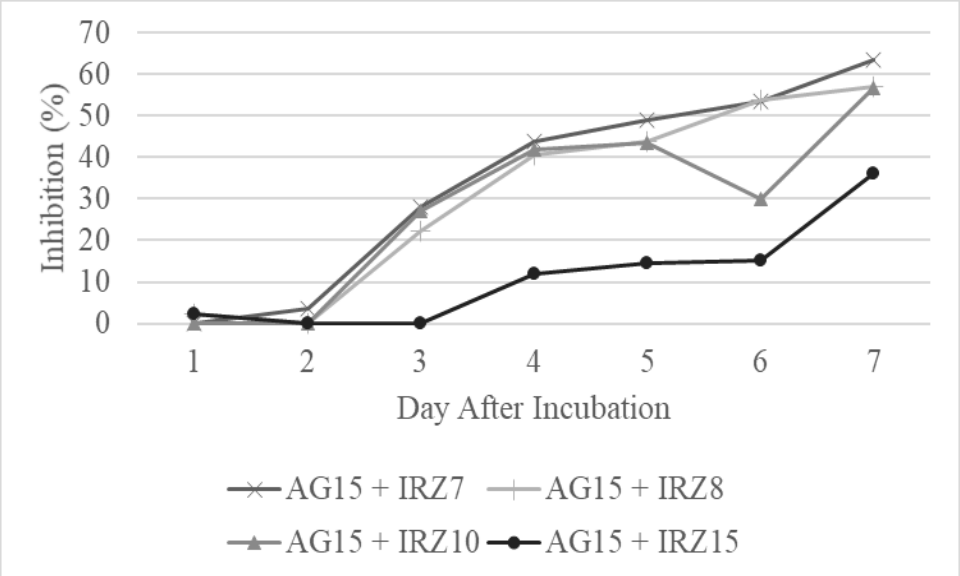

Figure 1. Inhibition rate between endophytic fungi and rhizospheric fungi tested by dual culture method, AG15 (the endophytic fungi selected isolate), IRZ7,8,10,15 (the rhizospheric fungi selected isolates).

The endophytic fungi AG15 was tested for interaction with the four selected rhizospheric fungi isolates through the dual culture method. The microscopic data (Figure 3) showed that the hyphae of AG15 attached to hyphae of IRZ7, IRZ8, IRZ10, and IRZ15. This interaction is suspected as the mycoparasitic interaction scheme. The AG15 breaks out the walls and

structure of the IRZ7, IRZ8, and IRZ10. Furthermore, the macroscopic data showed that AG15 isolate competes with IRZ7, IRZ8, and IRZ10. AG15 isolate grows faster than rhizospheric fungi isolates that cause the inhibition of rhizospheric fungi growth. Meanwhile, interactions between AG15 and IRZ15 showed an inhibitory zone (Figure 2, d). This indicates that there was an antibiotic mechanism that occurred between the fields.

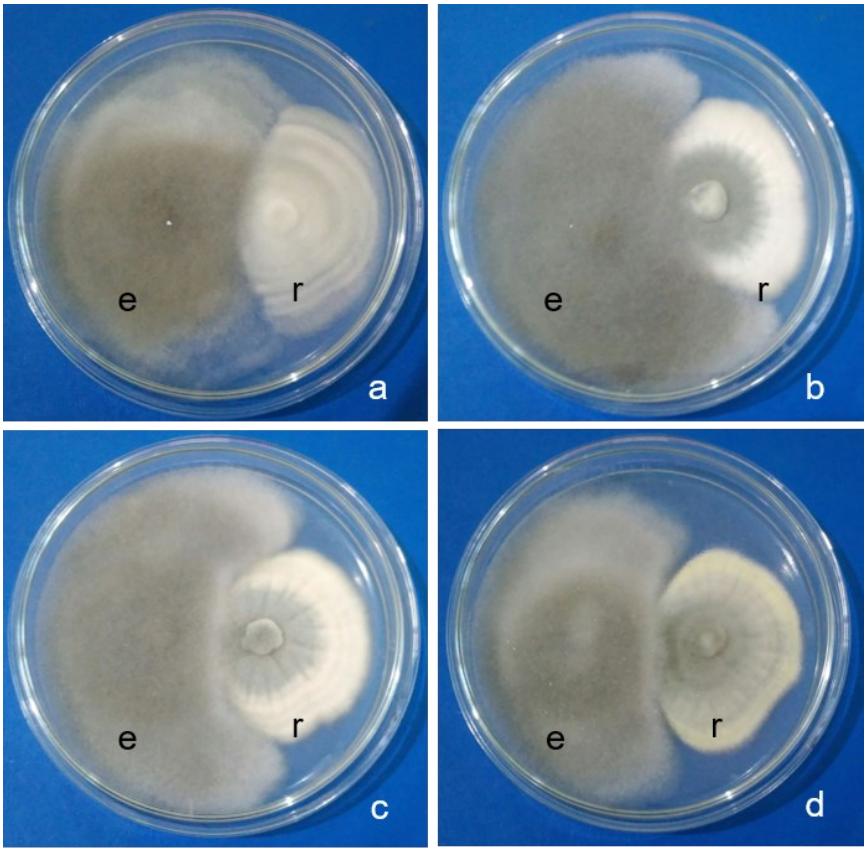

Figure 2. The interaction between (a) AG15 and IRZ7, (b) AG15 and IRZ8, (c) AG15 and IRZ10, (d) AG15 and IRZ15; e = endophytic fungi; r = rhizospheric fungi.

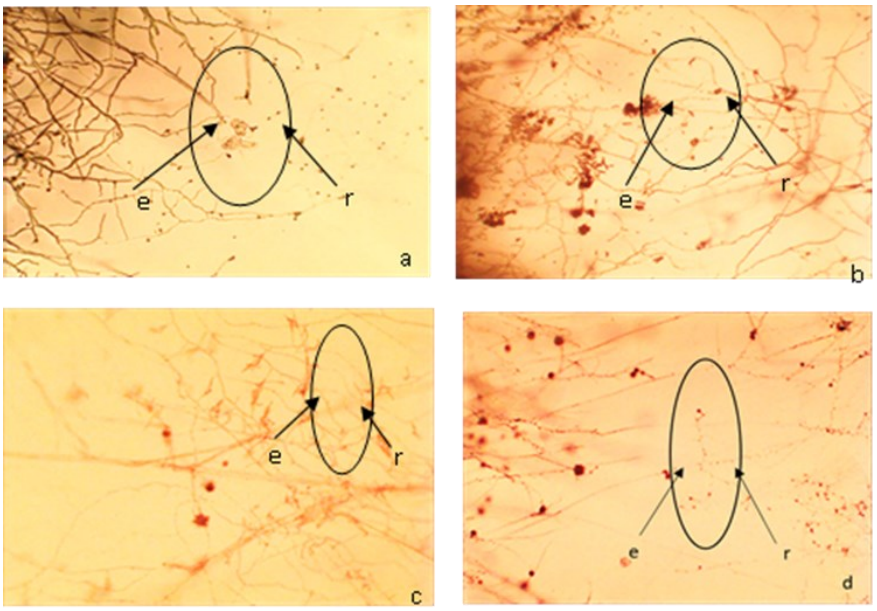

Figure 3. The mycoparasitic interaction between endophytic and rhizosphere fungi at 40X magnification (a) AG15 and IRZ7, (b) AG15 and IRZ8, (c) AG15 and IRZ10, and antibiosis (d) AG15 and IRZ15; e = endophytic fungi; r = rhizospheric fungi.

Indole Acetic Acid Production

Table 4 showed the concentration of IAA produced has varying values. However, the fungi isolates with tryptophan enriched media showed higher concentration values and significantly different than IAA concentrations produced by isolates on media that were not enriched with tryptophan, which was 8.77 ± 0.25 µg.mL^{-1} (from endophytic fungi AG15), 8.46 ± 0.26 µg.mL^{-1} (from rhizosphere fungi IRZ15), and 9.19 ± 0.50 µg.mL^{-1} (from a combination of rhizosphere and endophytic fungi). Besides, the combination of rhizospheric fungi and endophytes can increase the total amount of IAA concentration produced by rhizospheric fungi and endophytic fungi. However, the result of the concentration measurement was not significantly different from the use of fungal isolate in a single.

Table 4. The concentration of IAA hormones produced by selected isolates.

Code	Concentration (µg.mL^{-1})
AG15T	8.77 ± 0.30^{c}
IRZ15T	8.46 ± 0.32^{c}
KART	9.19 ± 0.61^{c}
AG15	4.77 ± 0.53^{a}
IRZ15	5.86 ± 0.58^{b}
KAR	6.42 ± 0.46^{b}

Note: AG15T: endophytic fungi isolate with tryptophan enriched media; IRZ15T: rhizospheric fungi isolate with tryptophan enriched media; KART: Combination of endophytic and rhizospheric fungi isolate with tryptophan enriched media; AG15: endophytic fungi isolate without tryptophan enriched media; IRZ15: rhizospheric fungi isolate without tryptophan enriched media; KAR: Combination of endophytic and rhizospheric fungi isolate without tryptophan enriched media.

Seed Germination

The treatment with AG15 isolate had a significant effect on increasing the number of germination and epicotyl lengths compared to other treatments and controls. However, hypocotyl length measurements did not show significantly different results from control (Table 5).

Identification of genus

Penicillium sp. IRZ15 obtained in this study has a greyish white color with a smooth colony surface (such as velvet). The microscopic morphology fungi showed a conidium that is spherical in shape, metula, conidiophores, and

Table 5. The effect of fungi extract treatment on germination number, hypocotyl length, and epicotyl length on chili sprouts.

Treatment	Number of germinated seed	Hypocotyl Length (mm)	Epicotyl Length (mm)
Control	3.9 ± 3.03^{ab}	4.80 ± 0.00^{b}	3.80 ± 0.00^{b}
AG15	5.05 ± 3.50^{c}	5.73 ± 2.26^{b}	7.77 ± 2.02^{c}
IRZ15	3.62 ± 3.33^{bc}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
KAR	2.71 ± 3.24^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
AG15T	1.90 ± 2.44^{a}	0.23 ± 0.40^{a}	0.37 ± 0.64^{a}
IRZ15T	3.43 ± 3.12^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
KART	1.95 ± 2.25^{a}	0.46 ± 1.30^{a}	1.37 ± 2.88^{a}

hyphae (Figure 4). Macroscopically, *Syncephalastrum* sp. AG15 has blackish-grey color morphology with a cotton-like surface. Microscopic observations showed that the *Syncephalastrum* sp. AG15 has wide hyphae with septa and a large sporangiophores tip. Macroscopic and microscopic morphological character based on the identification book *Illustrated Genera of Imperfect Fungi* (Barnett & Hunter 1987).

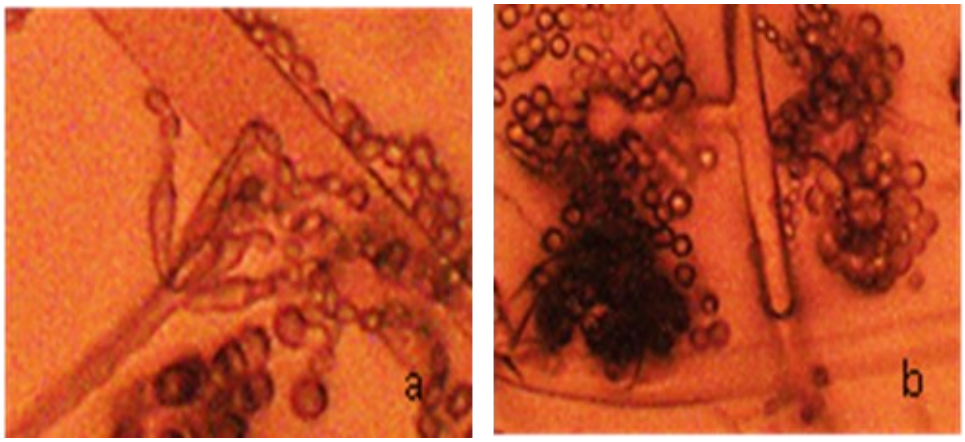

Figure 4. The microscopic morphology of *Penicillium* sp IRZ15 (a) and *Syncephalastrum* sp AG15 (b) at 100X magnification.

Discussion

A total of 16 isolates of fungi from the rhizosphere and 22 isolates of "Galam" plant were successfully isolated. Each isolate has a different growth rate character on the PDA medium (Tables 1 and 2). Growth area ranges from 0.25 to13.25 Cm^2/day for rhizospheric isolates and from 0.25 to 14.25 cm^2/day for endophytic isolates, respectively. Five rhizospheric fungi isolates (IRZ7, IRZ8, IRZ10, IRZ13, IRZ15) and five endophytic fungi isolates (AG10, AG11, AG15, AG18, and AG20) have the best growth rate and selected for further testing. The disadvantage of this test is that it is done without repetition, so isolate is selected based on only one data.

Each type of fungi certainly has a different rate of growth. This is because each type of fungi has optimal conditions to grow. Several factors that can affect the isolation process were nutrients, humidity, oxygen availability, temperature, acidity, and the content of organic matter in the soil (Alexander 1978). Besides, the diversity of endophytic fungi obtained in the isolation process can be affected by the sensitivity of the host plant to environmental factors, such as light intensity, soil temperature, humidity, nutrients, and soil pH (Manaroinsong & Lolong 2016).

The ten isolates were then tested for pathogenicity, from ten isolates obtained four isolate rhizospheric fungi (IRZ7, IRZ8, IRZ10, IRZ15) and one isolate endophytic fungi AG15 that are not pathogens (Table 3). Based on in vitro antagonist test, rhizospheric fungi isolate and endophytic fungi isolate, IRZ15 and AG15 isolate were the lowest pair showing antagonism, so these two isolates are tested for the ability to produce IAA (figure 2).

Both isolates were based on identification known as *Syncephalastrum* sp. and *Penicillium* sp. *Syncephalastrum* is known as a non-pathogenic saprofit fungi (Richardson & Rautemaa-Richardson 2020) and *Penicillium* is also reported as fungi supporting plant growth (Hossain et al. 2014) although also some species are reported as pathogens in garlic (Valdez et al. 2009). The results obtained are similar to Huda et al. (2019) that isolated *Syncephalastrum* sp. on "Galam" root. Penicillium was also found in peatland (Omar et al. 2012). This is because tropical peatlands generally consist of lignin consisting mostly of wood, so many species of fungi can be found.

In this study the IAA concentrations produced by *Penicillium* sp. IRZ15 (5.86 ± 0.47 µg.mL-1 to 8.46 ± 0.26 µg.mL-1) after a three-day incubation period is higher than reported by Syamsia et al. (2015) 0.635 to 2.651 µg.mL-1 obtained from rice endophytic fungi. The concentration of the IAA produced by each isolate could be influenced by the enriched media of tryptophan as a precursor. The presence of tryptophan could increase the biosynthesis of IAA up to 2.7 times compared without tryptophan addition. Tryptophan increases enzymatic activity in fungi so that IAA could be produced (Tudzynski & Sharon 2002). The more concentrations of tryptophan produced are directly proportional to the increasing concentration of IAA produced (Suebrasri et al. 2020). However, the results obtained also showed that the isolate of *Syncephalastrum* sp. AG15 and *Penicillium* sp. IRZ15 were able to produce the IAA hormone in media conditions without tryptophan enriched. This was because fungi were able to maintain enzymes to synthesize IAA at low concentrations even though there is no addition of tryptophan (Astriani et al. 2014; Maor et al. 2004).

On the contrary, the combination of rhizospheric fungi and endophytic fungi was able to increase the total amount of IAA concentration. However, the result of concentration measurement was not significantly different from the use of fungi isolates singly. The interactions conducted by *Syncephalastrum* sp. AG15 and *Penicillium* sp. IRZ15 was suspected of antibiosis. The antibiosis mechanism that occurred in this study is in line with the mechanism reported in the study of Mendoza et al. (2015) which reports antibiosis among some species of Trichoderma with *Macrophomina phaseolina*. *Penicillium* sp. known to produce antifungal compounds to inhibit the growth of other fungi. It is also following the results of microscopic observations that show that the end ophytic hyphae *Syncephalastrum* sp. AG15 grows away from the fungi *Penicillium* sp. IRZ15.

The treatment by *Penicillium* sp. IRZ15, a combination of *Syncephalastrum* sp. AG15. and *Penicillium* sp. IRZ15 without the addition of tryptophan and the treatment of *Syncephalastrum* sp. AG15, *Penicillium* sp. IRZ15 and the combination of both with the addition of tryptophan in the analysis of previous IAA levels showed higher results compared with IAA levels produced by *Syncephalastrum* sp. AG15 without tryptophan, which ranges from 5.86 ± 0.47 µg.mL^{-1} (*Penicillium* sp. IRZ15 without tryptophan) to 9.19 ± 0.50 µg.mL^{-1} (a combination of *Penicillium* sp. IRZ15, and *Syncephalastrum* sp. AG15 with tryptophan) so that it could inhibit the growth of hypocotyl and epicotyl lengths in seeds. According to Ludwig-Müller (2011), auxin concentrations that exceed the needs of each plant can be an inhibitor of plant root growth. Meanwhile, *Syncephalastrum* sp. AG15 produces IAA without tryptophan addition with the lowest concentration compared to other treatments, which was 4.77 ± 0.44 µg.mL^{-1}. This result is lower when compared to auxin produced by endophyte fungi from some medicinal plants which are 4 to 55 µg.mL-1 with the addition of 0.2% tryptophan (Suebrasri et al. 2020). This was because fungi are able to maintain enzymes to synthesize AIA at low concentrations even though there is no addition of tryptophan (Astriani et al. 2014). These results are in accordance with statements from Woodward and Bartel (2005) and Astriani et al. (2014), that there are two paths to the formation of IAA, namely tryptophan dependent and tryptophan independent.

Exogenous auxins such as IAA can influence the metabolism of endogenous auxins which will result in reverse inhibition of IAA biosynthesis (Ribnicky et al. 1996; Imaningsih et al. 2019). Based on the results, the direct treatment of a single isolate *Syncephalastrum* sp. AG15 gives the best results to accelerate germination, hypocotyl, and epicotyl length compared to the combination of *Syncephalastrum* sp. AG15 with *Penicillium* sp. IRZ15.

CONCLUSION

The result showed that the concentration of IAA produced by *Penicillium* sp. IRZ15 was 5.86 ± 0.47 µg.mL^{-1} to 8.46 ± 0.26 µg.mL^{-1} and *Syncephalastrum* sp. AG15 was 4.77 ± 0.44 µg.mL^{-1} to 8.77 ± 0.25 µg.mL^{-1}. Meanwhile, a combination of rhizospheric fungus *Penicillium* sp. IRZ15 and endophytic fungus *Syncephalastrum* sp. AG15 does not show significantly different concentration values with fungal isolates without combinations. Interaction between rhizospheric fungi *Penicillium* sp. IRZ15 and endophytic fungi *Syncephalastrum* sp. AG15 able to produced IAA hormones ranging from 6.42 ± 0.34 µg.mL^{-1} to 9.19 ± 0.50 µg.mL^{-1}. Interactions that occur between the *Penicillium* sp. IRZ15 and *Syncephalastrum* sp. AG15 was suspected of antibiosis.

AUTHORS CONTRIBUTION

W.I designed the research and supervised all the process, analyzed the data and wrote manuscript, S.S.H designed the research and supervised all the process, N.D.R collected and analyzed the data and wrote the manuscript, S.S.H designed the research and supervised all the process.

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CONFLICT OF INTEREST

The author declare that there is no conflict of interest.

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Research Article

Butterfly Pea (*Clitoria ternatea* L.: Fabaceae) and Its Morphological Variations in Bali

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ABSTRACT
Butterfly pea (*Clitoria ternatea* L.) is an important perennial herbaceous plant with a range of uses as ornamental plants, fodder crops, medicine, and sources of natural food colorant and antioxidants. The leaves and pods are commonly used as a source of protein in fodder, while the flowers are usually dried and processed as a high antioxidant-containing tea. The blue variant of butterfly pea was the most commonly used variety, although there are quite diverse butterfly pea varieties. The present study aimed to observe the morphological variations among the 26 butterfly pea accessions that originated from a wide range of areas in Bali. The explorative method was used to obtain diverse specimens (accessions) of butterfly pea in Bali, and subsequently, morphological characterization of the accessions was performed. The primary data of morphological traits that were recorded included stems, leaves, flower structures, flower colors, pods, and seeds. The data were analyzed descriptively to determine the morphological variations between accessions. The results showed three major morphological variations: (i) the colour of the flower (corolla), (ii) the corolla structure, and (iii) the stamen structure. The colour of corolla has four variations: white, mauve, light blue, and dark blue; while the corolla structure has two variations: normal and multiple layered corollas. The stamen character showed a correlation with the structure of the corolla. The normal corolla has diadelphous stamens, while the multiple layered corollas have solitary stamens. These morphological variations are the genetic richness of Indonesia's biodiversity and should be protected and conserved.

Keywords: butterfly pea, corolla abnormality, *CYC* gene interaction, diadelphous, papilionoid

INTRODUCTION
Butterfly pea (*Clitoria ternatea* L.) is a perennial herbaceous creeping plant that belongs to the Fabaceae family. This plant is mainly distributed in a tropical region that requires high light intensity and is relatively persistent with abiotic stress (Jamil et al. 2018; Oguis et al. 2019). As a typical legume plant, its roots form nodules that play a major role in nitrogen fixation in soil and act as a natural fertilizer in agricultural land (Oguis et al. 2019). Butterfly pea was first cultivated as an ornamental plant and then shifted as fodder for cattle

because of its high protein content (Mahmad et al. 2016; Jamil et al. 2018; Oguis et al. 2019). As fodder, butterfly pea is also used as a short to middle pasture plant (Hall 1985; Mahmad et al. 2016) with high-quality nutrition for cattle and other ruminant livestock (Abreu et al. 2014). Although popular as an ornamental plant and fodder, the latest research of butterfly pea is on the exploration of its bioactive compound, such as the antioxidant and protein-derived bioactive compounds in butterfly pea (Oguis et al. 2019). Cyclotide was an example of a protein-derived bioactive compound that was found almost in every part of butterfly pea and used as a natural pesticide (Nguyen et al. 2011; Oguis et al. 2019).

The flower of the butterfly pea is the most recognizable part of the plant with very attractive colors. The blue colour is the most widespread variation, so the butterfly pea is also known as the blue pea, although there are other colour variations such as mauve and white. The blue butterfly pea contains a specific anthocyanin called delphinidin 3,5',5'-triglucoside which is also known as ternatins (Saito et al. 1985). The anthocyanin content made the flower has been used as a source of natural antioxidant and natural dyes for food and cosmetic (Oguis et al. 2019). Flower extract of butterfly pea shows better colorant properties among other popular natural colorants such as *Garcinia mangostana* peels, *Ardisia colorata* fruits, and *Syzygium cumini* fruits (Azima et al. 2017). For its utilization in food, Pasukamonset et al. (2018) used the extract of blue-flowered butterfly pea to increase polyphenol content in sponge cake without showing any significant physicochemical differences.

Anthocyanin is not the only antioxidant compound in butterfly pea, it also contains other powerful antioxidants, such as flavonoid, phenolic acid, procyanidin, and flavonol glycoside (Jamil et al. 2018). Although it is popular for its antioxidant content, butterfly pea also contains many bioactive compounds, such as tannin, resin, steroid, saponin, triterpenoid, and xanthene (Manjula et al. 2013). Jamil and Pa'ee (2018) listed every bioactive compound in the roots, stems, leaves, and flowers of butterfly pea that are potential as anti-microbial agents.

In Indonesia, butterfly pea is widely distributed. The variability within species is valuable germplasm and should be well-recorded. Those variabilities can be preserved through sustainable use that combined aspects of utilization and conservation for their long-term utilization and persistence. Butterfly pea is much used as ornamental plants and worshipping materials in Indonesia. In Bali, the demand for flowers as worshipping materials in Hindu ceremonies was high. The people's preference to use local flowers as worshipping materials might unpremeditatedly conserve the existence of butterfly pea variations. The present study aimed to document and record the variability of butterfly pea in Bali based on morphological characterization.

MATERIALS AND METHODS

Study area

The explorative method was used to observe the morphological variation of butterfly pea in Bali. The research was conducted from April to November 2019 in ten areas in Bali that were marked with GPS as shown in Figure 1. The locations and coordinates of each area are listed in Table 1.

Study Species

Butterfly pea grows at 1–700 m above sea level, especially at the dry area on the edge of the road and bush forest (Backer & van den Brink 1963). In this research, the range of its habitat was wider, from dry to wet area (edge of rice field). Most of the butterfly peas were found in the residential area as an

Table 1. Locations of butterfly pea in Bali.

Number	Locations	District, Regency	Latitude
1.	Pekutatan	Jembrana	S: 08°28′32,3″ E: 114°50′24,8″
2.	Kayu Putih	Buleleng	S: 08°15′33,1″ E: 115°01′20,4″
3.	Angseri	Tabanan	S: 08°21′49,6″ E: 115°10′11,2″
4.	Buahan Kelod	Gianyar	S: 08°24′10,0″ E: 115°14′13,1″
5.	Rendang	Karangasem	S: 08°25′58,3″ E: 115°25′38,9″
6.	Seraya	Karangasem	S: 08°27′31,8″ E: 115°38′11,7″
7.	Kenderan	Gianyar	S: 08°28′14,3″ E: 115°17′08,8″
8.	Ubud	Gianyar	S: 08°31′22,1″ E: 115°16′04,4″
9.	Masceti	Gianyar	S: 08°35′30,5″ E: 115°20′46,8″
10.	Jimbaran	Badung	S: 08°47′30,1″ E: 115°10′45,2″

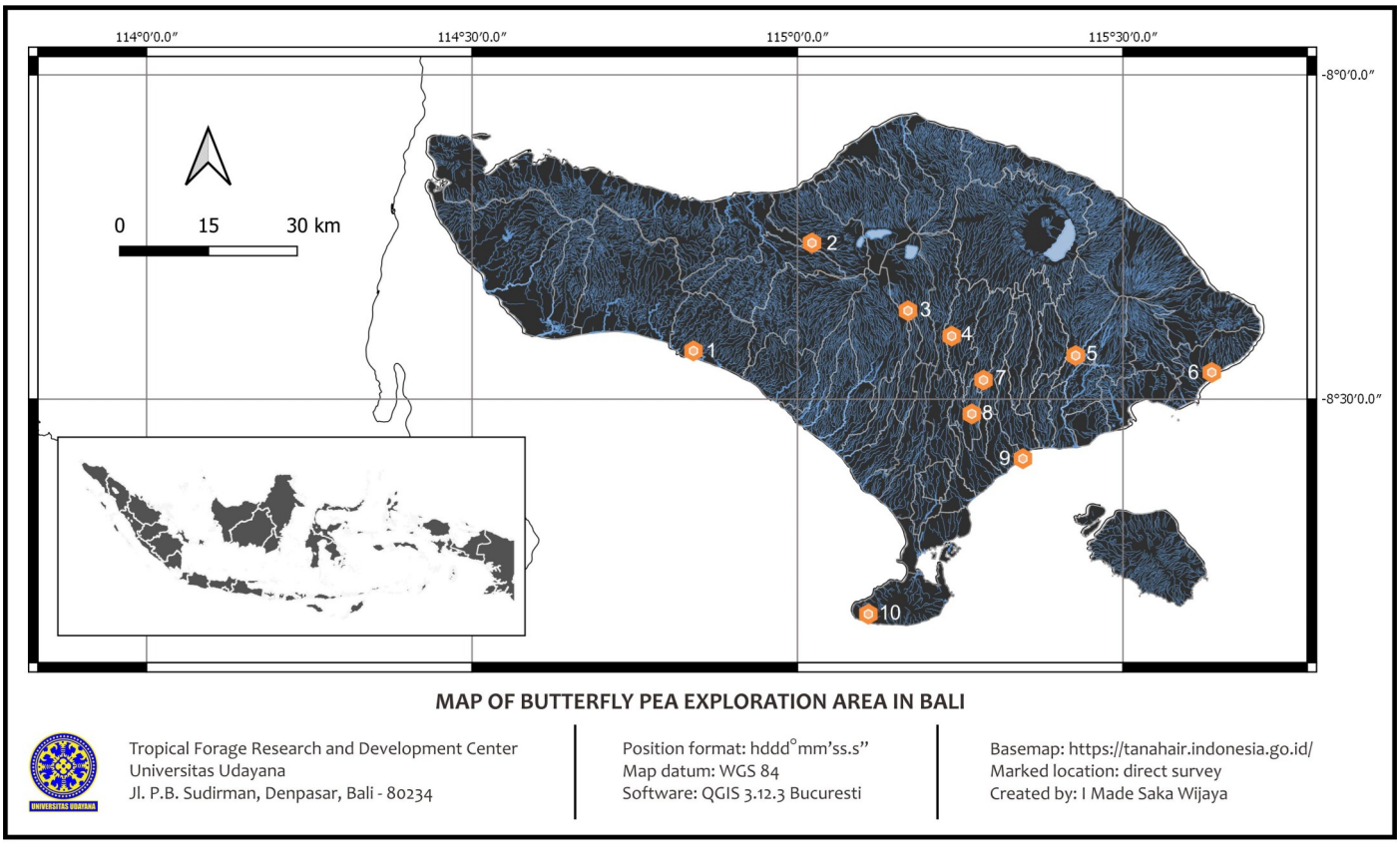

Figure 1. The study site of butterfly pea observation. The numbers indicate the study site: (1) Pekutatan; (2) Kayu Putih; (3) Angseri; (4) Buahan Kelod; (5) Rendang; (6) Seraya; (7) Kenderan; (8) Ubud; (9) Masceti; (10) Jimbaran.

ornamental plant (Figure 2a) or as a worshiping material in Hindu ceremonies (Figure 2b). The adult butterfly pea (>1.5 m in height) produces many flowers that bloom every day. It makes this plant suitable both as an ornamental plant and as a source of worshiping material.

(a) (b)

Figure 2. The use of butterfly pea (a) as an ornamental plant on sidewalks; and (b) white butterfly pea as a praying material.

In the scope of taxonomy, butterfly pea was grouped in Leguminosae. Leguminosae is a *nomina conservanda* of the Fabaceae family that has three subfamilies based on its flower morphology: Caesalpinioideae (peacock flower), Mimosoideae (sensitive plant), and Papilionoideae/Faboideae (pea or butterfly flower). Among those three, Papilionoideae was the subfamily showing more advanced evolutionary characteristics (Ojeda et al. 2009). The complete classification of butterfly pea was shown below:

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Fabales
Family : Fabaceae
Subfamily : Papilionoideae
Genus : *Clitoria*
Species : *Clitoria ternatea* L.
Synonym :
Clitoria albiflora Mattei; *C. bracteata* Poir.; *C. coelestris* Siebert & Voss; *C. parviflora* Raf.; *C. philippensis* Perr.; *C. pilosula* Benth.; *C. ternatensium* Crantz; *Lathyrus spectabilis* Forssk.; *Ternatea ternatea* (L.) Kuntze; *T. vulgaris* Kunth (all derived from The Plant List website at http://www.theplantlist.org/tpl1.1/record/ild-2539)
Common name :
Butterfly pea, blue pea (English); kembang telang (Indonesia); bunga telang, bunga celeng (Bali)

Methods

The in-site observation was performed to record the coordinates, habitats, sources (wild or planted), utilizations, and morphological variations. Purposive exploration was conducted around the marked coordinates. The coordinates were marked using GPS Garmin 64csx, then visualized in software QGIS 3.12.3 with base map derived from tanahair.indonesia.go.id.

Twenty-six specimens (accessions) of butterfly pea were characterized directly in the field. The morphological characterization was conducted through the description and measurement of vegetative organs (roots, stems, and leaves) and generative organs (flowers, fruits, and seeds) (Tjitrosoepomo 1985; Rugayah et al. 2004; Bishoyi & Geetha 2013). The description was compared with the main reference *Flora of Java vol. I* (Backer & van den Brink 1963). The morphological characterization was completed with the specimen's documentation by taking the photographs.

The root sampling was performed selectively to get the characteristic of nodules. The roots were sampled from eight plants. The root nodules were sectioned using a cutter knife to expose the infection region, then observed using a magnifier glass. The data of morphological variations were analyzed descriptively as a single description. To preserve the germplasm, the seeds of mature pods were collected. Those seed collections were then deposited at Tropical Forage Research and Development Center in Universitas Udayana, Bali.

RESULTS AND DISCUSSION

Morphological variability of the vegetative organs in butterfly pea (*Clitoria ternatea* L.)

Results of the present study showed there was no morphological variation between the 26 butterfly pea accessions. It appears that roots (including root nodules), stems, and leaves of the 26 accessions of butterfly pea had similar morphology. The 26 accessions had root nodules that were composed of large and small nodules with a round to irregular shape with a size that ranged from 0.5–1.5 cm in diameter, cortex thickness ranged from 0.05–0.1 cm, and infection region up to 90% of the size of the nodule (Figure 3a-c). The distribution of the large and small nodules accords with the research by Skerman et al. (1988). The large nodules distributed sparsely, while the small nodules more clumped or close to each other.

Root nodule is an important specialised organ as the place of the symbiotic association between butterfly pea and nitrogen-fixing bacteria such as *Rhizobium*. At the inner part of the nodule, there is the red part that contains leghemoglobin, a hemeprotein in legume which is responsible to provide oxygen for *Rhizobium* and protect the nitrogenase enzyme complex from irreversible inactivation if exposed to the atmospheric level of oxygen (Singh & Varma 2017). The symbiosis and activity of nitrogen-fixing bacteria in butterfly pea roots made this plant able to grow in an area with low nutrients and at the same time recover the soil fertility in that area (Hall 1985).

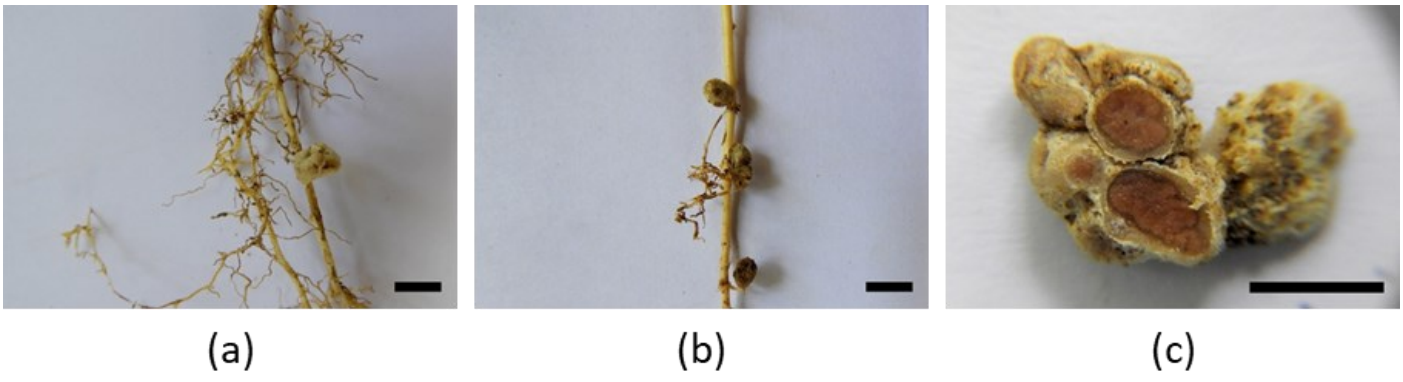

Figure 3. Characteristics of root nodules in *Clitoria ternatea* (a) large-sized nodule; (b) small-sized nodule; and (c) section of nodule showing cortex (pale brown) and medulla or infection regions (brown). Scale bar: 0.5 cm.

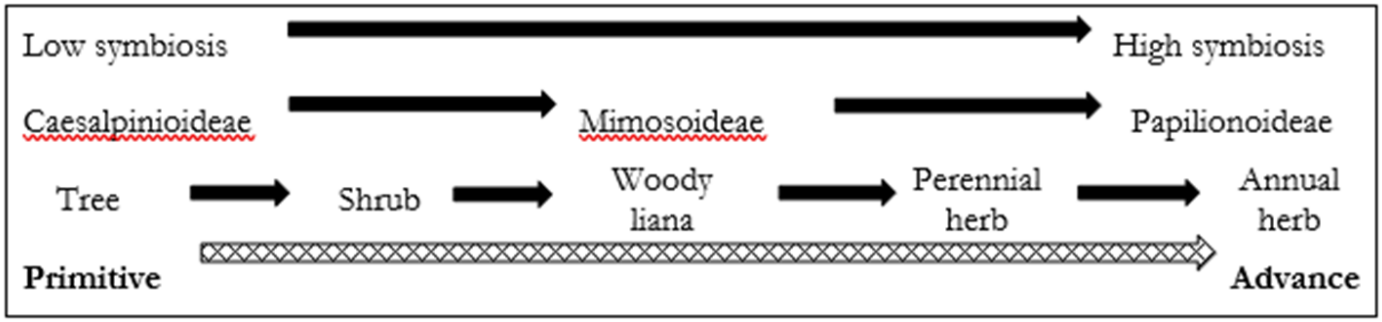

Figure 4. Scheme of evolutionary theory in Fabaceae (Skerman et al. 1988).

Based on the root-nodule symbiosis, butterfly pea (*Clitoria ternatea*) that belongs to Papilionoideae can be classified as a more advanced species in evolution compared to other species from other subfamilies within Fabaceae family (Caesalpinioideae and Mimosoideae). In the evolutionary approach of Fabaceae based on the symbiotic relationship, the subfamilies that have a more complex symbiosis with *Rhizobium* are classified as more advanced subfamilies as they usually develop more advanced structures. Skerman et al. (1988) constructed the evolution of Fabaceae as a scheme in Figure 4 and stated that Papilionoideae was the subfamily that was more advanced in evolution based on its high symbiotic relationship.

The stem of the 26 accessions of the butterfly pea in the present study did not show any differences, all was creeping to other plants in the competition of sunlight. The length of the stem reached 5 m, matching with the description from Backer & van den Brink (1963) who stated the length of stems ranged between 1 to 5 m. The stem was woody and green with lignified brown at the base. The green part has pubescent white along the stem.

Butterfly pea accessions in the present study had alternate compound leaves, length of peduncle 6–10.5 cm, 5 and 7 leaflets, and opposite phyllotaxis. Leaf-blade ovate–oval, sized 1.5–4.7 x 1–3.6 cm, entire margin, rounded at the base, retuse apex, green at the adaxial surface and paler at abaxial, linear trichomes at both sides of the blade. The terminal leaflet is usually bigger, while the base leaflet is smaller (Figure 5). Peduncle, pedicle, and vein had trichomes as same as stem. Leaflets had 2 linear stipules lengths of 0.2–0.3 cm. Each stem node had a spear-like stipule, 0.2–0.4 x 0.1 cm in size.

Figure 5. Compound leaves of butterfly pea with a bigger terminal leaflet for 5-leaflet and smaller terminal leaflet for 7-leaflet. Scale bar: 3 cm.

Morphological variability of the generative organs in butterfly pea (*Clitoria ternatea* L.)

The generative organs of butterfly pea consist of flowers, fruits, and seeds. The present study showed the flower of the butterfly pea was the most variable organ and easily recognized. The variable parts were the colour and structure of corollas (Figure 6a–h) and stamens (Figure 8), while the other parts such as pistils, fruits/pods, and seeds did not show any qualitative variability (Figure 7 and 9).

The flower parts of the butterfly pea consist of bractea, corolla, calyx, pistil, and stamens. Bractea rounded–ovate, 0.5–0.7 x 0.5–0.6 cm in size, entire margin, apex rounded–acute, light green color, pubescent white at both surfaces.

The corolla of butterfly pea is a typical Papilionoideae corolla. Five petals of corolla consist of one petal (the biggest) at anterior called standard or vexillum, two petals at lateral called wings or alae, and two petals at posterior called keels or carina. Vexillum ovoid, 3.5–4 x 4–5 cm, margin entire, acute at the base, apex retuse. Alae semi-circular, 2–3 x 1.1–1.5 cm, elongated at the base, from the middle to terminal overlapped one another. Carina semi-circular, 1–2 x 0.3–0.5 cm, elongated at the base and attached to the receptacle, concave at the middle to protect the pistil and stamens. In some accessions, half part of the carina is attached to the alae.

Variation in the structure of corolla in the present study is distinguished as (i) normal corolla and (ii) multiple layered corollas (Figure 6). The normal corolla was the typical Papilionoideae corolla described above, while the multiple layered corollas were the mutant's petal variation. The wings and keels in mutants were bigger and even attaining the size of a vexillum (Bishoyi & Geetha 2013). It leads to the shift of the flower symmetry from bilateral symmetry that is typical in papilinoid flower to be radial symmetry (Bishoyi & Geetha 2013).

Fu et al. (2005) stated that the character of corolla was determined by two genes *CYC2* (*CYCLOIDEA 2*) which act as a transcription factor for TCP gene complex to code vexillum and *CYC3* (*CYCLOIDEA 3*) that influence the character of alae. The appearance of multiple corollas in butterfly pea could be caused by mutation or crossing over that involved in

Figure 6. Variation in the corolla structure of the butterfly pea (a) the comparison of the whole corolla in normal (left) and multiple layered corollas (right); (b) Normal corolla (left), multiple layered corollas (right). The parts of normal corolla are carinas or keels (i), wings (ii), and vexillum (iii). The difference between the normal and multiple layered corollas is in the size of keels and wings relative to the size of the vexillum. The carinas, wings, and vexillum in multiple layered corollas also had recurved to revoluted margin and base. Scale bar: 3 cm.

over-expression of *CYC2* gene and induce all of the petals shaped and sized as vexillum (Figure 7). Wang et al. (2008) also reported the ortholog gene of *CYC2* and *CYC3* also played a vital role in the zigomorphism of the *Pisum sativum* flower. Those gene interactions indicate that the genetic regulation was responsible to determine flower characteristics in Papilionoideae.

The colour of corolla of the butterfly pea accessions in the present study has four variations: white, mauve, light blue, and dark blue, which were found in both normal and multiple layered corollas (Figure 7a–h). The variations of butterfly pea corolla colour in the present study were similar to Backer & van den Brink (1963), Reid & Sinclair (1980), Morris (2009), and Backer & van den Brink (1963) that stated that the corolla of butterfly pea was dark blue, mauve, or white with yellow in the middle and white spot at the edge. The differentiation of colour in corolla is caused by the secondary metabolites anthocyanin. In butterfly pea, the mauve and blue colour are caused by anthocyanin content such as delphinidins, while the white colour did not contain anthocyanin (Kazuma et al. 2003).

The variation in corolla and colour structure of butterfly pea in the present study might be related to genetic factors. Cronk (2006) stated that gene interactions can lead to the variation in shape, color, and epidermal type of corolla.

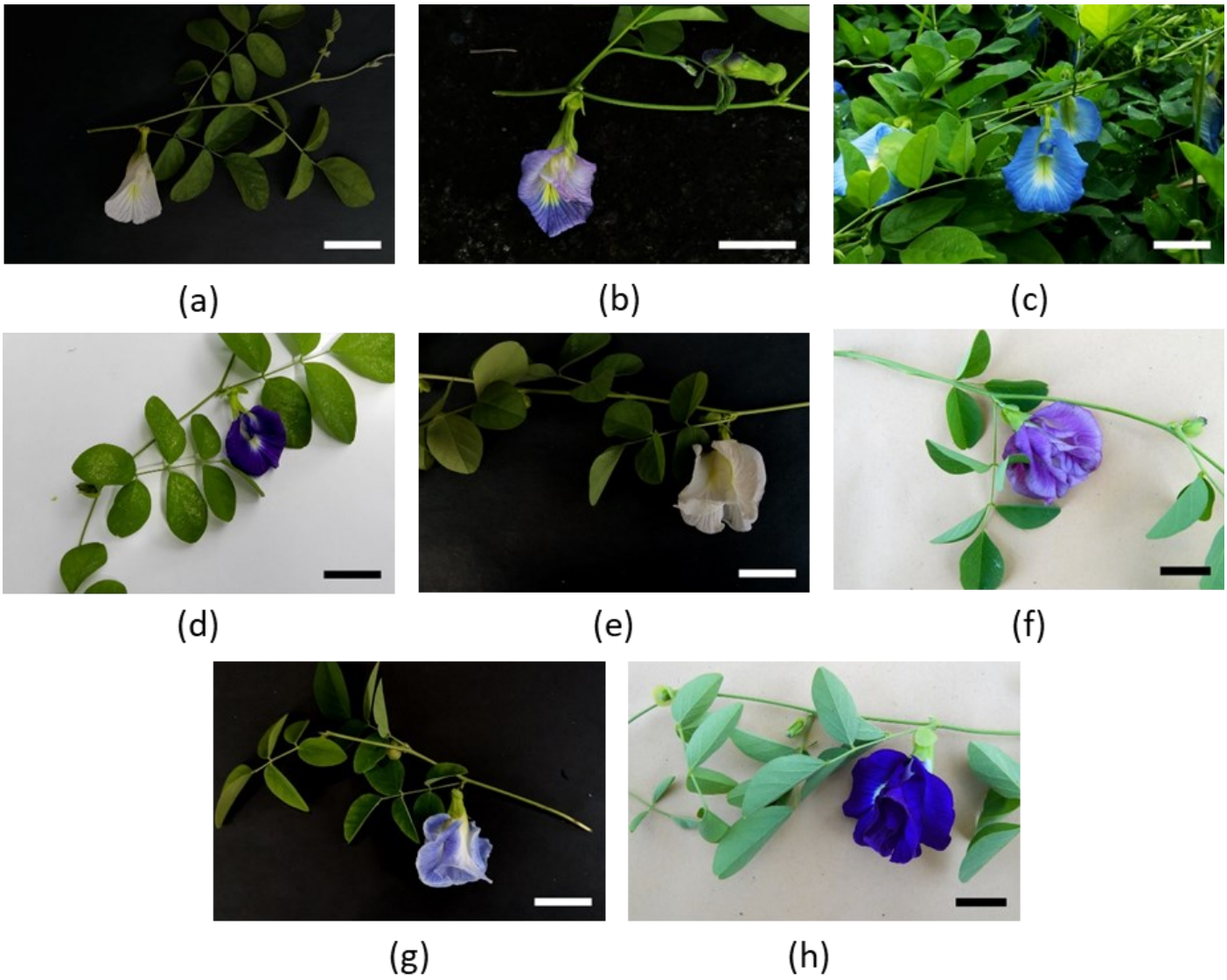

Figure 7. Characteristic of structure and colour of butterfly pea corolla (a) normal white corolla; (b) normal mauve corolla; (c normal light blue corolla; (d) normal dark blue corolla; (e) multiple layered white flower; (f) multiple layered mauve flower; (g) multiple layered light blue corolla; and (h) multiple layered dark blue corolla. Scale bar: 3 cm.

The calyx of the butterfly pea in the present study did not show any variation. The calyx consisted of five sepals that adnate from the base to the middle (partitus). Sepal was green, pubescent white, and forming calyx-tube with length 1.5–1.7 cm. In dry areas, the colour of sepals tends to purplish or reddish.

The present study also showed variation in stamen structure which was related to the variation of corolla structure (normal and multiple layered corollas). Although both normal and multiple layered corollas had 10 stamens (Figure 8), the stamens in normal corolla were arranged in a diadelphous structure with 9 adnate stamens and 1 solitary stamen below the pistil as same as recorded by Backer & van den Brink (1963). Adnate stamen length 1.3 cm continued with 0.5 cm filament that supports each anther. In multiple layered corollas, all those 10 stamens were arranged solitarily around the pistil with straight or curly filaments 1.8–2.0 cm in length.

Figure 8. Stamen variation of butterfly pea. Diadelphous stamens (left) and solitary stamens (right). Scale bar: 0.5 cm.

In diadelphous, all of the stamens were located around the pistil, while in solitary stamen was not. The revoluted base of the keels and wings in multiple layered corollas isolated 2–4 solitary stamens from the pistil. It affected the quantity of the anthers around the stigma. If compared with diadelphous with 10 anthers around the stigma, the solitary stamens only had 6-8 anthers around the stigma. For pistil, there was no qualitative variation between the 26 accessions of the butterfly pea in the present study. The stylus was green and thick, 2–2.2 cm in length, the stigma square-shaped, green, and covered with dense white trichomes that have a role to catch the pollens.

Pollination occurs when pollen was attached to stigma, then continued to form pods. The pods of the 26 accessions of butterfly pea in the present study had an elongated oval shape, 7–10 x 1 cm, dried calyx, and bractea at the base, apex caudate with 1–1.5 cm length, green when young and turn brown at maturity, pod cracked at the middle and then rolled up (Figure 9a). The seeds were brown-black with white elaiosome, cylindrical-square or reniform, 1 x 0.4 cm (Figure 9b). Each pod contains 3–10 seeds.

Bali has eight morphological varieties of butterfly pea. In annotations, the normal-light blue and the normal mauve were the rarest variety in Bali. Morris (2009) stated that morphological variations in butterfly pea are related

to its diverse geographic origins. The eight morphological variabilities can be used to compose the identification key of the butterfly pea at the level of intraspecific as shown below.

1. a. The corolla is composed of five petals which are modified as one vexillum at the posterior, two wings at lateral, and two keels at anterior. Ten stamens are arranged in diadelphous: 1 solitary stamen at posterior and nine adnate stamens at anterior ……..……. 2
   b. The corolla is composed of five petals that are not modified; all petals have vexillum-shaped. Ten stamens are solitary, arranged around the pistil ……...…….. 3
2. a. The corolla colour is white, rarely has a purple spot at the edge ……...…… normal-white variant
   b. The corolla colour is mauve or light mauve …… normal-mauve variant
   c. The corolla colour is light blue …… normal-light blue variant
   d. The corolla colour is dark blue ……. normal-dark blue variant
3. a. The corolla colour is white, rarely has a purple spot at the edge …….. multiple layered-white variant
   b. The corolla colour is mauve …… multiple layered-mauve variant
   c. The corolla colour is light blue …… multiple layered-light blue variant
   d. The corolla colour is dark blue ……. multiple layered-dark blue variant

Based on its potential and variability, butterfly pea has a high value as important germplasm in Indonesia that can be utilized as fodder, natural dye for clothes and food, and as a source of bioactive compounds. In Australia, butterfly pea has been developed as a superior fodder by cultivar selection that is known as 'Milgarra' (Conway & Doughton 2005). The use of butterfly pea in Indonesia is not as massive as in Australia, even though the climate in Indonesia is suitable for butterfly pea cultivation.

A Conservation approach is required in the utilization of butterfly pea in the scheme of sustainable use combining aspects of conservation and utilization in a balance for the long-term survival and long-term utilization of the butterfly pea. Furthermore, research in a wide scope on butterfly pea is required for the sustainable use of the butterfly pea. Mahmad et al. (2016) developed an artificial seed of butterfly pea from its callus to decrease the exploitation in the wild. The research in the scope of ecology, physiology, biochemistry, cytology, and molecular is required to support the conservation and sustainable use of butterfly pea.

Figure 9. The characteristics of pods and seeds (a) development of the pods from the green pods (left) to the brown pods (right). The mature pods are usually hidden under the newly sprout leaves; and (b) seeds. Scale bar: 1 cm (a) and 0.5 cm (b).

CONCLUSION

Butterfly pea (*Clitoria ternatea* L.) has three major variations (i) the corolla color, (ii) the corolla structure, and (iii) the stamen structure. The colour of

corolla has four variations: white, mauve, light blue, and dark blue, while the corolla structure has two variations: normal and multiple layered corolla. The stamen morphology is related to the corolla structure. The normal corolla has diadelphous stamens, while the multiple layered corollas has 10 solitary stamens. These variations were the potential germplasm that should be protected and conserved.

AUTHORS CONTRIBUTION

I.W.S. designed the research, collected the data, and wrote the manuscript. I.M.S.W. collected and analyzed the data and wrote the manuscript.

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CONFLICT OF INTEREST

We have no conflict of interest regarding the research or the research funding.

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Research Article

The Oriental Tiny Frog of the Genus *Microhyla* Tschudi, 1839 (Amphibia: Anura: Microhylidae) Revealed across Geographical Barriers of the Wallace Line

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ABSTRACT

The frog genus *Microhyla* was considered as the South, East, and Southeast Asian frog species. *Microhyla orientalis* was described in 2013, distributed in Java and Bali, Indonesia. Thenceforth, it was known as the easternmost distribution of this genus within the oriental region, but recently this species was recorded from the Timor Island and Sulawesi on the Wallace regions. We applied molecular analysis to evaluate the taxonomic status and the origin of the Wallacean population. Phylogenetic analysis using the partial 16S mitochondrial gene demonstrated that the Java, Timor and Sulawesi populations were not significantly different from the Bali population. This Wallacean population of *M. orientalis* was originated from Java and possibly it is accidentally distributed by humans through the expansion of agricultural activity.

Keywords: introduced species, *microhyla*, molecular markers, species confirmation, identification

INTRODUCTION

Microhyla Tschudi, 1838, a genus of tiny narrow-mouthed frogs that consisted of 46 species which were widely spread from Japan (Ryukyu Islands), southern China across India to Sri Lanka, and through Southeast Asia region into the Indonesian archipelago (Matsui et al. 2005; Kurabayashi et al. 2011; Frost 2021; Garg et al. 2019; Gorin et al. 2020; Poyarkov et al. 2020). *Microhyla* were distributed along to Indonesia through the Sunda shelf region. It was spread from Sumatera, Kalimantan, Java, Bali, and its adjacent islands, Timor and Sulawesi (van Kampen 1923; Iskandar 1998; Inger & Frogner 1979; Inger & Stuebing 2005; McKay 2006). Previously, *M. orientalis* from Bali was known as the easternmost distribution of the genus *Microhyla* within the oriental region (Matsui et al. 2013). Recently, this frog was recorded from Java (Yudha et al. 2019) and across the Wallace line in Timor Island (Reilly et

al. 2020) and Sulawesi (identified as *Microhyla* sp. in Wiantoro et al. 2019) based on morphological characters.

The disjunct distribution of *Microhyla orientalis* from the western and eastern parts of Wallace line raised a question about the origin of this species. By using a molecular technique, we evaluated the species identification of the Sulawesi and Timor populations then reconstructed its phylogenetic relationships. The estimation on the phylogenetic relationship using molecular technique has succeeded to prove the origin of certain vertebrates (e.g. Moritz et al. 1993; Suzuki et al. 2011; Suzuki et al. 2014; Johnson et al. 2011, Hadi et al. 2020) as well as in the frogs (Kuraishi et al. 2009; Wogan et al. 2016; Reilly et al. 2017; Vences et al. 2017).

Here, we provide the distribution history encompassing the entire populations of *M. orientalis* in Indonesia. The presumption of *M. orientalis* genetic variation in the Wallace region, which is possibly associated with natural or human-mediated distribution, was appraised.

MATERIALS AND METHODS

Materials

We examined specimens of *Microhyla orientalis* stored in Museum Zoologicum Bogoriense (MZB), Research Center for Biology, Indonesian Institute of Sciences. A number of 25 partial 16S mtDNA sequences of the *M. orientalis* were analysed (Table 1). We compared several populations *viz.* Java, Bali, Timor (specimen from Reilly et al. (2020)), and Sulawesi Island (specimens from Wiantoro et al. (2019) previously identified as *Microhyla* sp.) (Figure 1 and Table 1).

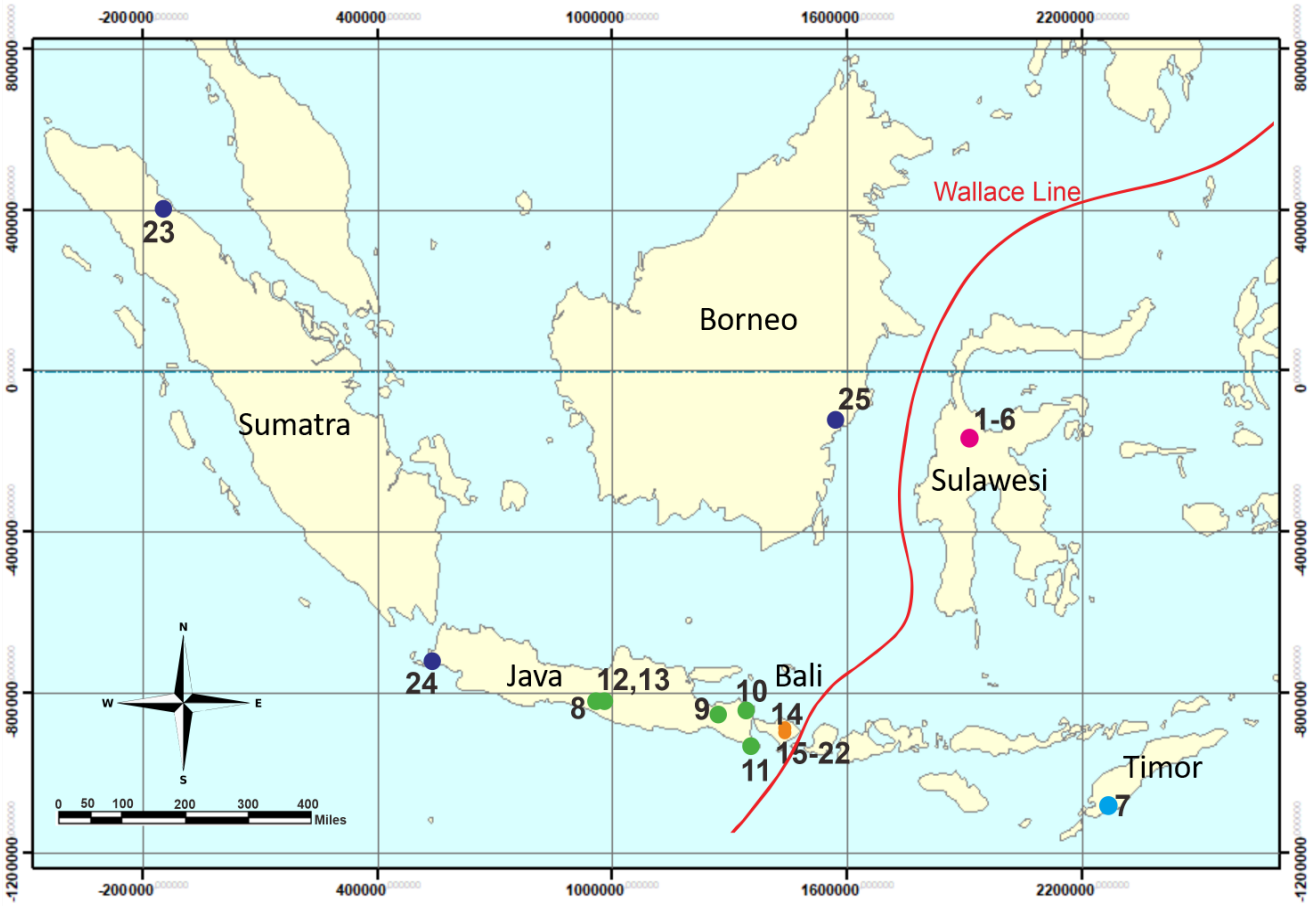

Figure 1. Indonesian Archipelago map of the localities of *Microhyla orientalis* specimens in Sulawesi (**purple**), Timor Islands (**cyan**), Java (**green**), and type locality, Bali (**orange**). Outgroups are shown by the **dark blue** circle. The specimen numbers represent those listed in Table 1. (Map modified from ArcMap 10.7.1, February 5th, 2021).

Table 1. Samples of Indonesian *Microhyla orientalis* and outgroups generated for 16S mtDNA analysis in this work with detailed information on Museum number, locality, GenBank accession numbers, and sources.

No	Species	Museum Number	Locality	Accession Number	Sources
1	*Microhyla orientalis*	MZB Amph 26972	Sulawesi, Sigi, Saluki	MW683205	This study
2	*Microhyla orientalis*	MZB Amph 26973	Sulawesi, Sigi, Saluki	MW683206	This study
3	*Microhyla orientalis*	MZB Amph 26974	Sulawesi, Sigi, Saluki	MW683207	This study
4	*Microhyla orientalis*	MZB Amph 26975	Sulawesi, Sigi, Saluki	MW683208	This study
5	*Microhyla orientalis*	MZB Amph 26976	Sulawesi, Sigi, Saluki	MW683209	This study
6	*Microhyla orientalis*	MZB Amph 26977	Sulawesi, Sigi, Saluki	MW683210	This study
7	*Microhyla orientalis*	MZB Amph 21091	Timor, Kupang	MW683211	This study
8	*Microhyla orientalis*	MZB Amph 28435	Java, Kulon Progo	MW683212	This study
9	*Microhyla orientalis*	MZB Amph 26914	Java, East Java Mt. Argopuro	MW683213	This study
10	*Microhyla orientalis*	MZB Amph 12989	Java, East Java, Baluran NP	MW683214	This study
11	*Microhyla orientalis*	MZB Amph 12986	Java, East Java, Alas Purwo NP	MW683215	This study
12	*Microhyla orientalis*	ZMMUA 5067-1	Java, Yogyakarta	MN534556	Gorin et al. 2020
13	*Microhyla orientalis*	ZMMUA 5067-2	Java, Yogyakarta	MN534557	Gorin et al. 2020
14	*Microhyla orientalis*	MZB Amph 16259	Bali, Batu Karu	AB634679	Matsui et al. 2011
15	*Microhyla orientalis*	KUHE 55048	Bali, Wongaya Gede	AB781465	Matsui et al. 2013
16	*Microhyla orientalis*	KUHE 55049	Bali, Wongaya Gede	AB781466	Matsui et al. 2013
17	*Microhyla orientalis*	KUHE 55050	Bali, Wongaya Gede	AB781467	Matsui et al. 2013
18	*Microhyla orientalis*	KUHE 55072	Bali, Wongaya Gede	AB781468	Matsui et al. 2013
19	*Microhyla orientalis*	KUHE 55073	Bali, Wongaya Gede	AB781469	Matsui et al. 2013
20	*Microhyla orientalis*	KUHE 55074	Bali, Wongaya Gede	AB781470	Matsui et al. 2013
21	*Microhyla orientalis*	KUHE 55076	Bali, Wongaya Gede	AB781471	Matsui et al. 2013
22	*Microhyla orientalis*	KUHE UL-M11	Bali, Wongaya Gede	AB781472	Matsui et al. 2013
	Outgroup				
23	*Micryletta inornata*	MZB Amph 23947	Sumatra, Medan	LC208136	Alhadi et al. 2019
24	*Microhyla achatina*	ZMMUA 5054-2	Java, Banten, Ujung Kulon	MN534565	Gorin et al. 2020
25	*Microhyla malang*	MZB Amph 16364	Kalimantan, Balikpapan	AB634677	Matsui et al. 2011

Methods

Fragments of the 16S mtDNA (ca. 462) were employing a method as delineated by Matsui et al. (2011, 2013). DNA sequences obtained in this study were checked and edited using the ChromasPro software (Technelysium Pty Ltd., Tewantin, Queensland, Australia). The newly *M. orientalis* sequences were deposited in GenBank with accession numbers MW683205—MW683215 together with those from GenBank (Table 1) aligned applying Clustal W in MEGA X (Kumar et al. 2018). Phylogenetic trees were reconstructed using Neighbor-Joining (NJ), Maximum Likelihood (ML), and Bayesian Inference (BI) analyses. The NJ tree was administered in MEGA X using p-distances with 1000 bootstrap replicates. The Akaike Information Criterion (AIC) performed using Kakusan 4 were used to identify the models of rate evolution for ML and BI analyses (Tanabe 2011). ML analysis was performed using Treefinder ver. March 2011 (Jobb et al. 2004) with general time-reversible (GTR) and a gamma shape parameter (G). The Bayesian analysis was performed in MrBayes 3.2.7 (Ronquist et al. 2012)

with general time-reversible (GTR) and a gamma shape parameter (G). Analysis of the Markov Chain Monte Carlo (MCMC) for the dataset was run for 5 million generations and every 100 cycles, trees were sampled. The convergence of the runs was determined by a split frequency of < 0.01 standard deviations and potential scale reduction factors of ~1.0. We discarded the first 20% of the sampled trees as burn-in and generate a majority-rule consensus tree using the remaining samples. Strong supports of tree nodes were considered when possessing bootstrap values of 70% or more for ML and NJ analyses (Hillis & Bull 1993). The genetic distances of the 16S mtDNA gene were computed using uncorrected p-distances with MEGA X. We regarded tree nodes with BI posterior probabilities values over 0.95 as strongly supported; values between 0.90 and 0.95 were considered as moderately supported; while the lower values were regarded to have no nodal support (Huelsenbeck & Hillis 1993).

Genetic Diversity Analysis

Twenty-two sequences of 462 bp 16S mtDNA gene from three sampling locations: Java, Timor (Reilly et al. 2020), and Sulawesi (Wiantoro et al. 2019) with additional sequences of the type specimen of *M. orientalis* from Bali were included in the generating process of 14 haplotype variations. Genetic diversity parameters including the haplotypes diversity (Hd) and nucleotide diversity (π) were computed using the DNASp v6.12.03 (Rozas et al. 2017) and Arlequin v.3.5.2.2 software (Excoffier & Lischer 2010).

Analysis on the structure of populations

Molecular variance (AMOVA) and fixation index (F_{ST}) (Wright 1951) analysis was performed for the group within intraspecific populations of *M. orientalis* using the Arlequin v.3.5 programs (set up, 1000 permutations; significance level threshold, $\alpha = 0.05$). The analyses allowed the approximation of the overall extent of the genetic variation and differentiation level within *M. orientalis* population. Furthermore, population differentiation and its significance between sampling locations were also calculated using pair-wise estimates (Weir & Cockerham 1984; Excoffier et al. 1992; Weir 1996).

Genetic haplotypes analysis

The median-joining method was generated to build the haplotype network using Network v10.2.0.0 program (Bandelt et al. 1999). Haplotypes distribution for each location was presented in the informative map (Figure 1) to show the recent genetic connectivity and distributions among populations.

RESULTS AND DISCUSSION

Morphologically, Timor and Sulawesi populations of *M. orientalis* showed high similarity in appearance with Java and Bali populations. The diagnostic character such as bright orange to pinkish brown on the upper forearm, a vertebral line at the dorsal of the body, and the web formulae of toes confirmed the species identification as *M. orientalis* (Figure 2).

We obtained 462 bp 16S mtDNA fragments of 25 samples including outgroups, 371 nucleotide sites were conserved, 87 site variables, and 28 sites parsimoniously informative. A topology with the highest log likelihood -1223.5300, gamma shape parameter 0.1407, and frequencies of the nucleotides: A=0.333, T=0.251, G=0.197, and C=0.217 resulted from the ML analysis. BI analysis with nucleotide frequencies: A=0.335, T=0.248, G=0.202, C=0.215, and a gamma shape parameter 0.3701. All analyses produced similar topologies, which differed only in several low-supported nodes. Thus, only the ML tree is shown (Figure 3).

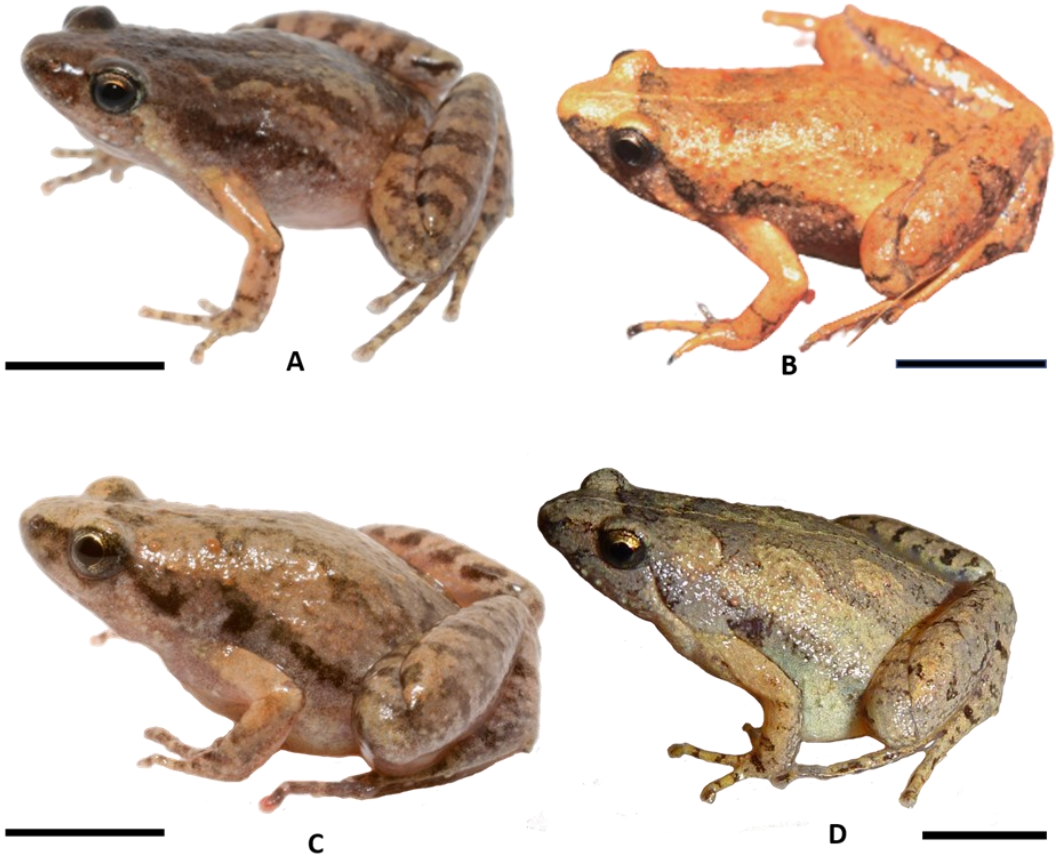

Figure 2. Color pattern variation on *Microhyla orientalis*. **A.** Bali (reproduced from Matsui et al. 2013), **B.** Java (Yogyakarta, Photographed by Misbahul Munir), **C.** Sulawesi (Central Sulawesi, Photographed by Farits Alhadi), **D.** Timor (reproduced from Reilly et al. 2020). Scale bar = 5 mm.

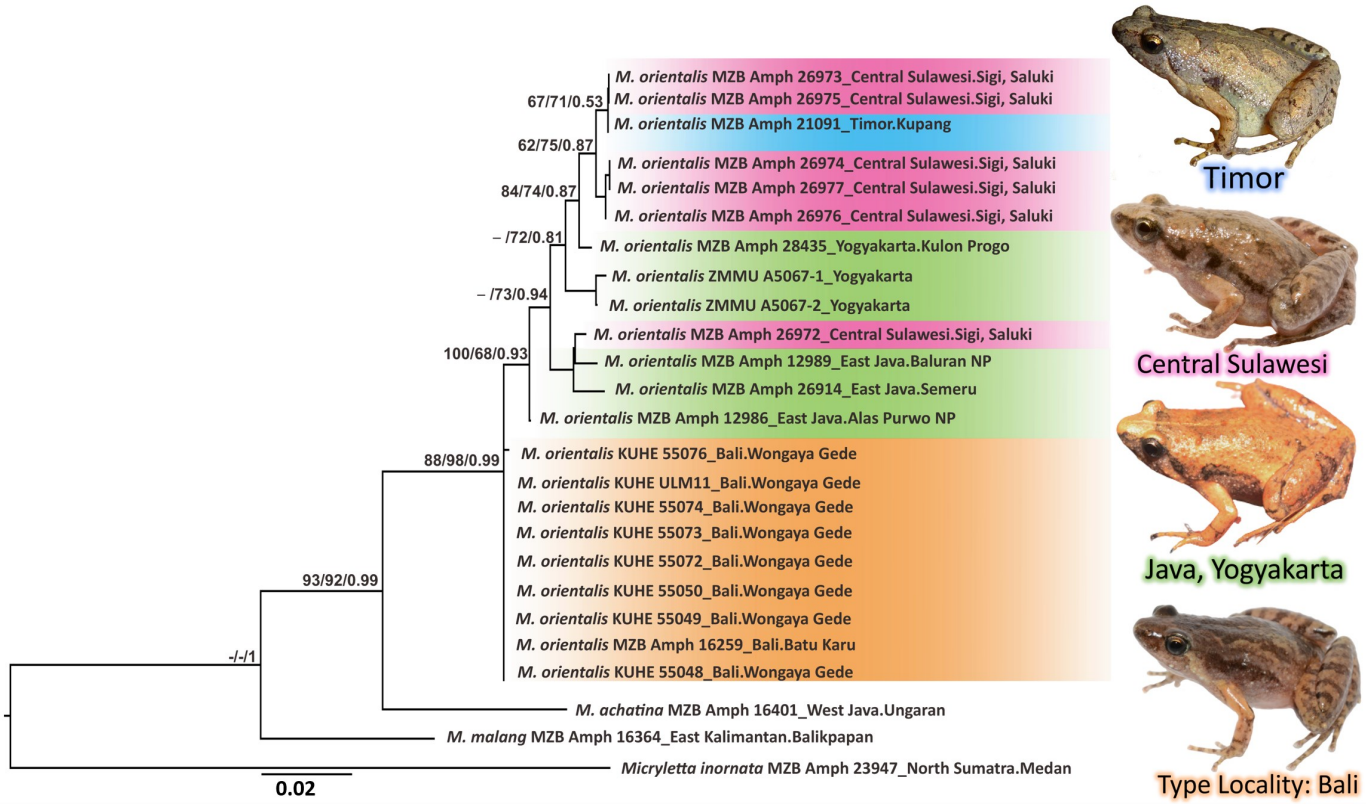

Figure 3. Maximum likelihood phylogeny tree of *Microhyla orientalis* samples and outgroups based on 462 bp of the 16S mtDNA gene. The Numbers shown above the branches represent bootstrap support for NJ/ML/and BI.

The population of *Microhyla* from Sulawesi and Timor examined here formed a well-supported monophyletic with *M. orientalis* from Java and Bali (NJ: 93; ML: 92; BPP: 99). Population from Timor and Sulawesi are closely similar to the population from Java with high significant supports (NJ: 88; ML: 98; BPP: 99). The tree showed the consistent clade of *M. orientalis* as the sister taxa of *M. malang* and *M. achatina*, although their relationships were not fully resolved.

Genetic Diversity

Sequences (n= 22) from four localities (Java, Timor, Bali, and Sulawesi) resulting in 462 bp 16S mtDNA was gained. All sequences, including samples from Bali as type locality of *M. orientalis* resulted in 14 haplotype variations with 17 polymorphic sites (Table 2).

The contrast of the genetic diversity among person tests of *M. orientalis* uncovered the nearness of variety (Table 3). Among the samples, it was obtained that the haplotype diversity (Hd) ranged from 0.222 to 1.0 and the nucleotide diversity (π) ranged from 0.000 to 0.015. Samples from Java were observed as the highest genetic diversity (Hd = 1.0; π = 0.015), the second is Timor, which shows a single haplotype and nucleotide diversity (Hd = 1.0; π = 0.000). The *M. orientalis* population from Sulawesi revealed a fairly high genetic diversity (Hd = 0.933; π = 0.008) and the lowest was from Bali (Hd = 0.222 and π = 0.011).

Table 2. Seventeen polymorphic sites of 14 haplotypes derived from 22 samples of the 16S mitochondrial DNA of *M. orientalis* from four localities.

Nucleotide position*	10	11	21	76	114	176	180	187	200	238	240	252	264	270	285	296	339
Haplotype 1–Bali	A	C	T	C	G	C	T	G	T	T	A	C	C	A	T	A	A
Haplotype 2–Bali	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
Haplotype 3–Sulawesi	G	T	.	.	A	.	.	A	.	.	.	T	.	.	.	.	G
Haplotype 4–Sulawesi	G	T	.	.	A	.	C	A	.	C	.	T	.	G	.	.	.
Haplotype 5–Sulawesi	G	T	.	.	A	.	C	A	.	C	.	T	.	G	.	.	.
Haplotype 6–Sulawesi	G	T	.	.	A	.	C	A	.	C	.	T	.	G	.	.	.
Haplotype 7–Sulawesi	G	T	.	.	A	.	C	A	.	C	.	T	.	G	.	.	.
Haplotype 8–Java	G	T	.	T	A	T	.	A	.	.	G	T	.	.	.	.	.
Haplotype 9–Java	G	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
Haplotype 10–Java	G	T	G	.	A	.	.	A	.	.	.	T	.	.	.	G	.
Haplotype 11–Timor	G	T	.	.	A	.	C	A	.	C	.	T	.	G	.	.	.
Haplotype 12–Java	.	.	.	T	A	.	.	A	C	C	.	T	.	.	.	.	.
Haplotype 13–Java	.	.	.	.	A	.	.	A	C	C	.	T	.	.	.	.	.
Haplotype 14–Java	G	T	.	.	A	.	C	A	.	C	.	T	.	.	C	.	.

Notes:
*Nucleobase at each position is for Haplotype 1-Bali while those differences are written for all other haplotypes. Nucleobase identical to Haplotype 1-Bali are shown with dots (.).

Table 3. Genetic diversity samples of *Microhyla orientalis* derived from sample size (n), haplotype number (Hn), Haplotype diversity (Hd), and nucleotide diversity (π).

Population	bp	Number of samples (n)	Haplotype number (Hn)	Haplotype Diversity (Hd)	Nucleotide Diversity (π)
Bali	457	9	2	0.222 ± 0.166	0.011 ± 0.001
Java	458	6	6	1.000 ± 0.096	0.015 ± 0.009
Timor	457	1	1	1.000 ± 0.000	0.000 ± 0.000
Sulawesi	458	6	5	0.933 ± 0.122	0.008 ± 0.005
Overall	**462**	**22**	**14**	**0.814 ± 0.064**	**0.011 ± 0.001**

Populations of *Microhyla orientalis*

The fixation index (F_{ST}) and *P*-values analysis between and within *M. orientalis* populations (Java, Bali, Timor, and Sulawesi) are presented in Table 4. The comprehensive F_{ST} value within four populations were highly significant (F_{ST} = 0.605; $P < 0.001$). This was predicted due to multiple subdivisions.

Table 4. The percentage of variation (%), F_{ST} value, and significance level (*P*-value) using the molecular variance (AMOVA) analysis for *M. orientalis* specimens.

Source of Variation	df (degrees of freedom)	Percentage of Variation (%)	F_{ST} value	*P*-value
Interpopulation	3	60.53	0.605	0.000
Intrapopulation	18	39.47		
Total	21			

Values of the pair-wise F_{ST} between the populations of *M. orientalis* are provided in Table 5. Significant differentiation between Bali and the other three populations (Java, Sulawesi, and Timor) was detected through the overall pair-wise analysis using the distance method.

Table 5. Pairwise F_{ST} values (**black**) and *P*-values (**blue**) between *M. orientalis* populations from Java, Bali, Timor, and Sulawesi.

Sample sites	Bali	Sulawesi	Java	Timor
Bali	-	0.000	0.009	0.990
Sulawesi	0.848	-	0.018	0.990
Java	0.573	0.231	-	0.990
Timor	0.972	-0.275	-0.211	-

Genetic Connectivity of *M. orientalis*

The haplotype network analysis recognized a complex ancestor or origin of Wallacean *M. orientalis* population (Timor and Sulawesi) (Fig 4). Two major groups of haplotypes are named clade Bali and Java-Timor-Sulawesi. Bali as the type locality of *M. orientalis* shows two different haplotypes H1 and H2. Timor (one haplotype) and Sulawesi (five haplotypes) have mixed connectivity with each other, several probabilities of genetic connection of various ancestor points evolved from the Java population (six haplotypes).

Two confined haplotype groups between Bali and Java-Timor-Sulawesi *M. orientalis* were differentiated by 6 discrete nucleotide bases due to the mutations. Concerning the significant value of F_{ST} among populations (F_{ST} = 0.605; $P < 0.001$) as well as haplotype network (Figure 4) and phylogenetic tree (Figure 3).

Discussion

Here, we confirmed the species identification of Yudha et al. (2019), Wiantoro et al. (2019) and Reilly et al. (2020) as *Microhyla orientalis* genetically . Genetic variation has not been significantly detected among the sundaic and wallacean population of *M. orientalis*. This assumed that the Wallacean *M. orientalis* were genetically mixed, feasibly limited in number, and accidentally introduced, as stated earlier by Reilly et al. (2020). It is predicted that oceanic dispersal to adjacent islands must also have occurred in association with human activities, such as the condition on some frogs species in Japan (Ota et al. 2004; Kuraishi et al. 2009).

This introduction of *Microhyla orientalis* to Timor and Sulawesi was

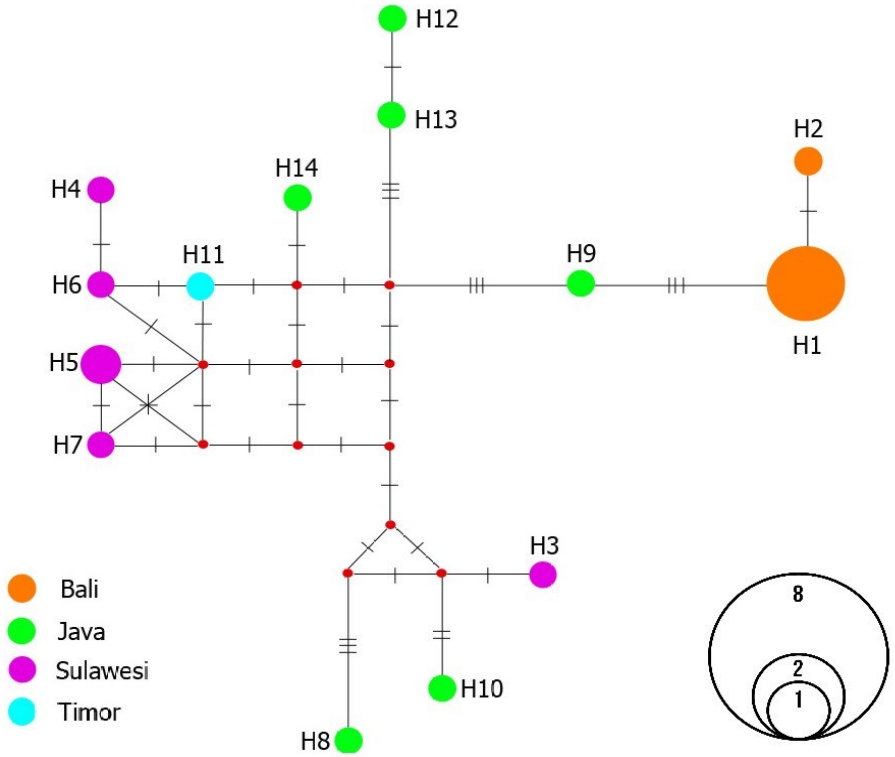

Figure 4. Results of median-joining methods for *M. orientalis* population (n = 22) haplotype network from Indonesia: Java, Sulawesi, Timor, and type locality, Bali.

similar to the tracing result of the origin of *Duttaphrynus melanostictus* in Madagascar (Wogan et al. 2016; Vences et al. 2017) and Wallacean region (Reilly et al. 2017). Madagascar *D. melanostictus* was found to be a single origin from the Southeast Asian lineage. The Southeast Asia mainland population shows high haplotypes diversity than the island's population, 51 haplotypes in the mainland, two and four in the island's population (Wogan et al. 2016). As well as, Wallacean *D. melanostictus* were shared single haplotype originated from Sundaic region of Sumatra and Java (Reilly et al. 2017).

Microhyla orientalis from Java showed higher haplotypes diversity than Bali, Timor and Sulawesi. The haplotype network of *M. orientalis* showed two major haplotype groups, Bali and Java-Timor-Sulawesi, with low genetic diversity. Since the Wallacean population of *M. orientalis* is closest to Java than Bali, we assumed that this Wallacean population is originated from Java similar to Reilly et al. (2017) finding.

M. orientalis were suspected as an accidentally introduced species by the colonization event back to The Dutch East Indies era in 1905 (Kementerian Desa, Pengembangan Daerah Tertinggal dan Transmigrasi RI 2015a). One of the major sources of people who move to other areas of Indonesia is Java. It was mentioned that Timor and Central Sulawesi were the destinations of the colonization called "*transmigrasi*" program of the Indonesian government (Kementerian Desa, Pengembangan Daerah Tertinggal dan Transmigrasi RI 2015a; Kementerian Desa, Pengembangan Daerah Tertinggal dan Transmigrasi RI 2015b). Colonization is usually also involved with agriculture and fisheries for living. Possibly , people from Java also brought their cultural agriculture and fisheries to fulfill their basic needs in the new land. It is likely that the species was recently introduced by human activity, and genetic analysis has proven the human interfere of *M. orientalis* distribution. Moreover, the paddy field is the suitable habitat of *M. orientalis* (Matsui et al. 2013), the expansion of agricultural activity in the Wallace region might play an important role in distributing this species out from Java.

CONCLUSIONS

Both molecular analysis and morphological identification on the population of *M. orientalis* from Java, Timor and Sulawesi show similarities to Bali population. Two major groups were detected molecularly within the *M. orientalis* population, Bali and Java-Sulawesi-Timor. The Javan population of *M. orientalis* assumed accidentally introduced to the Wallacean region.

AUTHORS CONTRIBUTION

R.E. and V.Y.A. contributed equally to this work, formulated and designed the assessment, conducted the research, analysed the data, generated figures and tables, authored and reviewed drafts and approved the final draft; M.M. contributed to data collection, scrutinized the data, authored and reviewed drafts of this paper and approved the final draft; A.H. supervised all the process of this work, designed the experiments, recorded and scrutinized the data, authored and reviewed drafts of this paper and approved the final draft; T.A. and R.U. contributed to examining the data, authored and reviewed drafts of this paper and approved the final draft.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Research Article

Newly Recorded Alien Species of *Ficus* L. (Moraceae) in Java, Indonesia

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ABSTRACT

Ficus is the largest genus in the Moraceae family, with the syconium inflorescence as its characteristic. This genus has a wide distribution worldwide, and about 367 species were found in the Malesia region. There are 74 species have been recorded on Java, and seven species are known as introduced plants. Based on our recent observations in West Java, Central Java, Yogyakarta, and East Java, three newly recorded *Ficus* species were found, namely *F. auriculata* Lour., *F. maclellandii* King, and *F. natalensis* subsp. *leprieurii* (Miq.) C.C. Berg. The species were introduced to Java as an ornamental plant. Descriptions, photographs, and a brief discussion of the three newly recorded species are presented in this manuscript.

Keywords: Alien Flora, *Ficus*, Java, Moraceae

INTRODUCTION

Ficus L. is the largest genus in the Moraceae and it separated taxonomically from other genera into the Ficeae Gaudich tribe. Morphologically, the genus is characterized by its figs. The flowers are both unisexual and bisexual and are located in the receptacle base (Backer & Bakhuizen van den Brink 1965; Berg & Corner 2005). *Ficus* has a wide distribution area in the world, especially in tropical and subtropical regions. The genus consists of 735 species and 367 species found in the Malesia region (Berg & Corner 2005). The information on the diversity of *Ficus* species in Java has been compiled by Backer & Bakhuizen van den Brink (1965) and Berg & Corner (2005). As many as 74 species of *Ficus* are found on the island and seven of them are introduced plants, namely *F. benghalensis* L., *F. callosa* Willd., *F. carica* L., *F. lyrata* Warb., *F. pumila* L., *F. religiosa* L., and *F. rumphii* Blume (Backer &

Bakhuizen van den Brink 1965; Berg & Corner 2005). Since then, the updated information on the genus *Ficus* in Java, including the newly introduced species, has not been provided yet.

In recent years, many non-native species were reported from Java by many authors (*e.g.* Girmansyah 2014; Mustaqim & Nisyawati 2016; Irsyam & Mountara 2018; Hariri et al. 2019; Irsyam et al. 2019; Anshori et al. 2020; Hariri et al. 2020; Effendi & Mustaqim 2021). It shows that the number of non-native plant species in Java will continue to increase. In this study, we report the occurrence of three newly recorded species, namely *F. auriculata* Lour., *F. maclellandii* King, and *F. natalensis* subsp. *leprieurii* (Miq.) C.C. Berg, for Java. The study was also conducted to provide an updated taxonomic data on the genus *Ficus* in Java.

MATERIALS AND METHODS

Materials

The study was carried out in several locations in West Java (Bandung, Bogor, Sumedang, Subang), Central Java (Kebumen), Jogjakarta, and East Java (Malang, Surabaya, Banyuwangi, Madura Island) from September 2019 to December 2020. The materials used in this study were specimens stored in the Herbarium Bogoriense (BO), field-collected specimens, and non-collected specimens grown in Bogor Botanic Gardens (BBG).

Methods

The field observations used exploring method following Rugayah et al. (2004), while the sampling method following van Balgooy (1987). The syconia were also collected as wet specimens preserved in 70% ethanol. The other data recorded were the locations, names of collectors, collection numbers, coordinate points, morphological features that could be lost during the preservation process (the color of latex, indumentum, leaves, and syconia), vernacular names, and their utilization.

The specimens collected from the field were taken to the Bandungense Herbarium (FIPIA), School of Life Sciences and Technology, ITB, to be processed into herbarium specimens following Djarwaningsih et al. (2000). The samples were also re-identified using Flora Cochinchinensis 2 (Loureiro 1790), Checklist of *Ficus* in Asia and Australasia with keys to identification (Corner 1965), Flora Malesiana Ser. I Vol 17 (2) (Berg & Corner 2005), New Taxa and Combinations in *Ficus* (Moraceae) of Africa (Berg 1988), and Taxonomic treatment of the *Ficus auriculata* complex (Moraceae) and typification of some related names (Zhang et al. 2019).

RESULTS AND DISCUSSION

Field observations reveal the presence of three newly recorded *Ficus* in Java (Figure 1), namely *F. auriculata* Lour., which belongs to *Ficus* subg. *Sycomorus* (Figure 2). Two other species were grouped into *Ficus* subg. *Urostigma*, namely *F. maclellandii* King (Figure 3) and *F. natalensis* subsp. *leprieurii* (Miq.) C.C. Berg (Figure 4).

Taxonomic treatment

Ficus auriculata Lour., Fl. Coch. 2: 660. 1790; Corner, Gard. Bull. Singapore 19: 395. 1962; 21: 82. 1965; Berg & Corner, Fl. Males. Ser I, 17(2): 341-343, t. 56. 2005.

Ficus macrophylla Roxb. & Buch.-Ham. ex Sm. in Rees, Cycl. 14, Ficus, no. 32. 1810, non Desf. ex Pers. 1807; Roxb., Fl. Ind., ed. Carey 3: 556. 1832; Wight, Ic. 2 t. 673. 1843. — *Ficus roxburghii* Wall. ex Steud., Nomencl. Bot. ed. 2, 1: 637. 1840; Burkill, Dict. Econ. Prod. Malay Penins. 1014. 1935; Rehder, J.

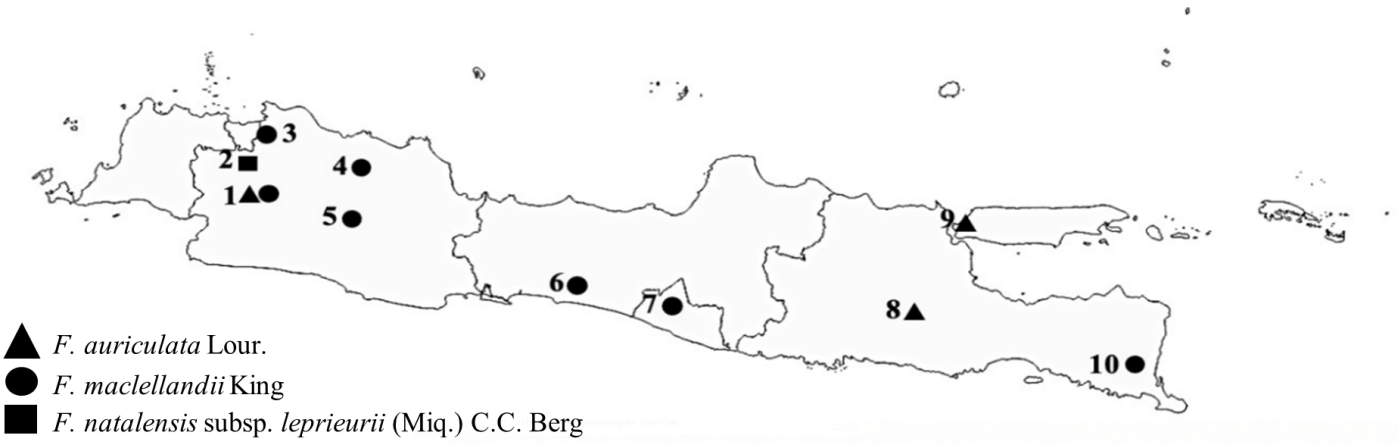

Figure 1. The distributional map of the newly recorded *Ficus* in Java. 1. Bogor; 2. Depok; 3. Bekasi; 4. Subang; 5. Bandung; 6. Kebumen; 7. Jogyakarta; 8. Batu; 9. Bangkalan; 10. Banyuwangi

Arnold Arbor. 17: 81. 1936. — *Covellia macrophylla* (Roxb. ex Sm.) Miq., London J. Bot. 7: 465. 1848.
Ficus sclerocarpa Griff., Notul. Pl. Asiat. 4: 397. 1854; Ic. Pl. Asiat. 4, t. 558. 1854, '*scleroptera*'.
Ficus regia Miq., Ann. Mus. Bot. Lugduno-Batavi 3: 230, 296. 1867.
Ficus oligodon Miq., Ann. Mus. Bot. Lugduno-Batavi 3: 234, 297. 1867; Corner, Gard. Bull. Singapore 18: 43. 1960; 19: 395. 1962; 21: 82. 1965; Kochummen, Tree Fl. Malaya 3: 152. 1978.
Ficus pomifera Wall. ex King, Ann. Roy. Bot. Gard. (Calcutta) 1(2): 171, t. 215. 1888, non Kurz 1873; Fl. Malay Penins. 3: 350. 1924; Corner, J. Malayan Branch Roy. Asiat. Soc. 11: 46, f. 24. 1933.
Ficus macrocarpa H. Lév. & Vaniot, Mem. Real Acad. Ci. Barcelona 3: 152. 1907, non Blume 1823; Rehder, J. Arnold Arbor. 17: 81. 1936.
F. hainanensis Merr. & Chun, Sunyatsenia 2(3-4): 215, pl. 40. 1935.
F. beipeiensis S. S. Chang, Acta Phytotax. Sin. 22(1): 69, pl. 11. 1984.
Tree, up to 20 m tall, with milky latex. **Bark** greyish brown, lenticelled; leafy twigs puberulous to subtomentose or glabrous, internodes hollow or solid. **Stipules** 1.5–2.5 cm long, puberulous, caducous. **Leaves** simple, spirally arranged to subdistichous; petioles 4.5–12 cm long, with brownish or whitish hairs or glabrous; lamina cordate to suborbicular, 14.5–28 × 10.5–25.5 cm, base cordate to rounded, margin subentire or coarsely dentate to denticulate, apex acute to acuminate, upper surface glabrous, lower surface puberulous, lateral veins 3–7 pairs, tertiary venation scalariform, waxy glands in the axils of the basal lateral veins. **Figs** cauliflorous or stoloniflorous; peduncle 4–8 cm long, hairy; basal bracts 3, deltoid, 7–8 mm long, persistent; receptacle pyriform, globose to depressed-globose, 4.8–4.9 cm diam., velutinous, reddish-brown, with white or greenish blotches, wall 6–7 mm thick; ostiole 6 mm diam., with a prominent rosette of ostiolar bracts; flowers unisexual; tepals connate. **Staminate flowers**: ± 2 mm long, stamens 2, connate at base. **Pistillate flowers**: dimorphic, ± 2–5 mm long; ovary superior, the ovary of the short-styled flowers differentiated into a gall, style 1.
Distribution: The species native to India (Himalayas), Bhutan, Nepal, Sikkim, Myanmar, Thailand, and Southern China (Berg & Corner 2005; Lim 2012). In the Malesia Region, *F. auriculata* was only recorded from Selangor and Pahang, Malaysia (Corner 1965; Berg & Corner 2005; Zhang et al. 2018). However, in this study, this species has been planted in Java and Madura.
Habitat: Mixed forest and secondary forest at an altitude of 100–1,700 m (Berg & Corner 2005; Lim 2012). *F. auriculata* is planted at the observation site in gardens, open places, and public spaces at an altitude of 5–271 m.
Examined specimens: **WEST JAVA**. Cult. in Bogor Botanic Gardens, VI

I.H.9A, 13-3-1956, *L.L. Forman 59* (BO); Budi Mulia, Paledang, Bogor Tengah District, Bogor, 2 January 2020, *Peniwidiyanti & MR Hariri 05* (FIPIA); Jl. Malabar Park, Babakan, Bogor Tengah District, Bogor, 2 January 2020, *Peniwidiyanti & MR Hariri 06* (FIPIA); Bogor Botanical Gardens, 2 January 2020, *Peniwidiyanti & MR Hariri 07* (FIPIA). **EAST JAVA**. Batu, Malang, 16 December 2019, Peniwidiyanti, *PNW 30* (FIPIA); Telang Asri, Kamal, Bangkalan Regency, Madura Island, 26 December 2019, *ASD Irsyam & MR Hariri 27* (FIPIA).
Vernacular name: *Tin afrika* (Indonesian).
Uses: The ripe syconia have a sweet taste and it can be eaten fresh or processed into sweets, jams, juices, and curry ingredients (Jansen et al. 1991; Lim 2012). The species is also planted as a shade tree and ornamental plant.

Ficus maclellandii King, Sp. Ficus 1: 52, t. 64. 1887; Fl. Brit. India 5: 512. 1888; Corner, Gard. Bull. Singapore 21: 19. 1965; Kochummen, Tree Fl. Malaya 3: 150. 1978; Berg & Corner, Fl. Males. Ser. I, 17(2): 665. 2005; Berg, Pattharahirantricin, & Chantarasuwan, Fl. Thai. 10(4): 360. 2011.
Ficus thorelii Gagnep., Notul. Syst. (Paris) 4: 97. 1927; Fl. Indo-Chine 5: 781. 1928.
Urostigma rhododendrifolium Miq., London J. Bot. 6 (1847) 579. — *Ficus rhododendrifolia* (Miq.) Miq., Ann. Mus. Bot. Lugd.-Bat. 3: 286. 1867, non Kunth & C.D. Bouché 1847; King, Sp. Ficus 1: 57, t. 58. 1887 — *Ficus maclellandii* King var. *rhododendrifolia* (Miq.) Corner, Gard. Bull. Singapore 17: 392. 1960.
Tree, up to 25 m tall, hemi-epiphytic or terrestrial, with milky latex. **Bark** greyish, lenticelled; leafy twigs glabrous or hairy on the upper scars of the stipules, angular to subterete, internodes solid. **Stipules** 5–10 mm long, with yellowish hairs, caducous. **Leaves** simple, spirally arranged; petioles 2–2.5 cm long, glabrous, drying blackish; lamina lanceolate to oblong, 8–20 × 2.1–5.2 cm, base cuneate to rounded, margin entire, apex acuminate to caudate, upper and lower surfaces glabrous, tertiary venation parallel to the lateral veins, lateral veins 8–14 pairs, waxy gland at the base of the midrib. **Figs** axillary, paired or solitary; sessile; basal bracts 3, 1–3 mm long, persistent; receptacle subglobose, 1.5–2 cm diam., glabrous, yellow; ostiole 1.5 mm wide, with ostiolar bracts; flowers unisexual; tepals connate, reddish.
Staminate flowers: ± 1 mm long, stamens 2, connate at base. **Pistillate flowers**: dimorphic, ± 1 mm long; ovary superior, the ovary of the short-styled flowers differentiated into a gall, style 1.
Distribution: The species is distributed from India, Southern China (Yunnan), Laos, Thailand to Peninsular Malaysia (Berg & Corner 2005).
Habitat: *F. maclellandii* is naturally found in lowland forests (Berg & Corner 2005). In our study, the species was cultivated in open areas at an altitude of 74 to 269 m.
Examined specimens: WEST JAVA. Jl. P.H.H. Mustofa, Bandung, 22 Februari 2020, *ASD Irsyam 347* (FIPIA); Dawuan, Subang, 9 Maret 2020, *Peniwidiyanti, PNW 32* (FIPIA); Taman Ade Irma Suryani, Bogor, 22 September 2020, *Peniwidiyanti, PNW 44* (FIPIA); PT PJB UP Muara Tawar, Bekasi, 6°05'18.6"S 106°59'49.3"E, 28 September 2019, *Zakaria Al Anshori, PJBMTW0919-005* (FIPIA); **CENTRAL JAVA.** Alun-alun Prembun, Jl. Nasional 3, Pejaten, Bagung, Kecamatan Prembun, Kebumen, 27 Desember 2020, *Peniwidiyanti, PNW 224* (FIPIA); **JOGJAKARTA.** Areal taman masjid kampus UGM, 23 Oktober 2020, *A Nugroho 23* (FIPIA); **EAST JAVA**. Resto Pondok Indah, Jl Raya Ijen Km 8.7, Desa Glagah, Banyuwangi, 8 Januari 2020, *Peniwidiyanti*, *PNW 31* (FIPIA).
Vernacular name: *Beringin jepang* (Indonesian).
Uses: The beautiful crown shape makes *F. maclellandii* used as an ornamental

plant (Berg 2007). It has also been cultivated as a shade tree, and its leaves can be used for party decorations by the Javanese people. The previous pharmacological studies reveal that the leaves, twigs, and fruit extracts of *F. maclellandii* can be a natural source of antioxidants (Tamuly et al. 2015; Raza et al. 2016).

Ficus natalensis* subsp. *leprieurii (Miq.) C.C. Berg, Kew Bull. 43(1): 88. 1988. — *Ficus leprieurii* Miq., Ann. Mus. Bot. Lugd. - Bat. 3: 219. 1867.
Ficus chrysocerasus Warb. in Bot. Jahrb. 20: 167. 1894.
Ficus excentrica Warb. in tom. cit. 168.
Ficus furcata Warb. in tom. cit. 173.
Ficus triangularis Warb. in tom. cit. 174.
Ficus leprieurii var. *intermedia* Hutch. in Prain, Fl. Trop. Afr. 6 (2): 159. 1916.
Ficus brevipedicellata De Wild. in Ann. Soc. Sci. Brux. 40: 279. 1921.
Small tree or shrub, with milky latex. **Bark** brownish, greenish or greyish, lenticelled. **Stipules** 4–6 mm long, dark purplish. **Leaves** simple, spirally arranged; petioles 4–11 mm long, brownish-green to purplish; lamina obdeltoid, 3.7–6.7 × 2.9–5.2 cm, base cuneate, margin entire, apex truncate to emarginate, upper surface green to variegated, glabrous, lower surface green to white, midrib on the lower surface purplish brown to brownish, lateral veins 4–8 pairs. **Figs** axillary, paired; peduncle 0.6–0.7 cm long; receptacle globose, 7.5–8.4 cm diam., yellow to orange, wall 0.4–0.8 mm thick; ostiole 0.5–0.7 mm diam.; tepals connate, reddish. **Staminate flowers**: ± 1.5 mm long, stamens 2, connate at base **Pistillate flowers**: dimorphic, ± 1.5 mm long; ovary superior, the ovary of the short-styled flowers differentiated into a gall, style 1.
Distribution: The species is naturally distributed from Senegal, Southern Sudan, Zaire to Northern Angola (Berg 1988). The presence of *F. natalensis* subsp. *leprieurii* was also recorded in Pakistan (Ajaib & Khan 2012).
Habitat: In its native distributional area, *F. natalensis* subsp. *leprieurii* grows in forests and around streams at an altitude of 0–1,200 m asl.
Examined specimens: WEST JAVA. Depok, 7 September 2020, *Peniwidiyanti, PNW 33* (FIPIA); Bogor, 7 April 2021, *Peniwidiyanti, PNW 324* (FIPIA).
Vernacular name: Natal fig.
Uses: The species is currently cultivated as a bonsai plant in Java. The previous study revealed the potential of *F. natalensis* subsp. *leprieurii* as a source of natural antibacterial compounds (Tkachenko et al. 2019).

Taxonomically, *Ficus* is divided into 6 subgenera, namely *Ficus*, *Pharmacosycea* (Miq.) Miq., *Sycidium* (Miq.) Mildbr. & Burret, *Sycomorus* (Gasp.) Miq., *Synoecia* (Miq.) Miq., and *Urostigma* (Gasp.) Miq (Berg 2003). Based on its morphological characteristics, *F. auriculata* belongs to the subg. *Sycomorus*, while *F. maclellandii* and *F. natalensis* subsp. *leprieurii* comes from subg. *Urostigma*. These three species are commonly found on the island of Java as ornamental plants because they have beautiful stature and canopy shapes.

F. auriculata has a gynesic flowering system (Zhang et al. 2019), without adventitious roots, syconium grows cauliflory or on specialized branches and is also found in the root (stoloniflory), 2 stamens, and 1 the stretching of the pistil (Figure 2). Previous research has shown that subg. *Sycomorus* found in Java consisted of 8 species, namely *F. fistulosa* Reinw. ex Blume, *F. hispida* L.f., *F. lepicarpa* Blume, *F. racemosa* L., *F. ribes* Reinw. ex Blume, *F. septica* Burm.f., *F. variegata* Blume, and *F. vrieseana* Miq. (Backer & Bakhuizen van den Brink 1965; Berg & Corner 2005). Field observations have been carried out at several locations in Java, and however, *F. auriculata* was only found from Bogor, Malang, and Madura Island. The existence of this species in Java has

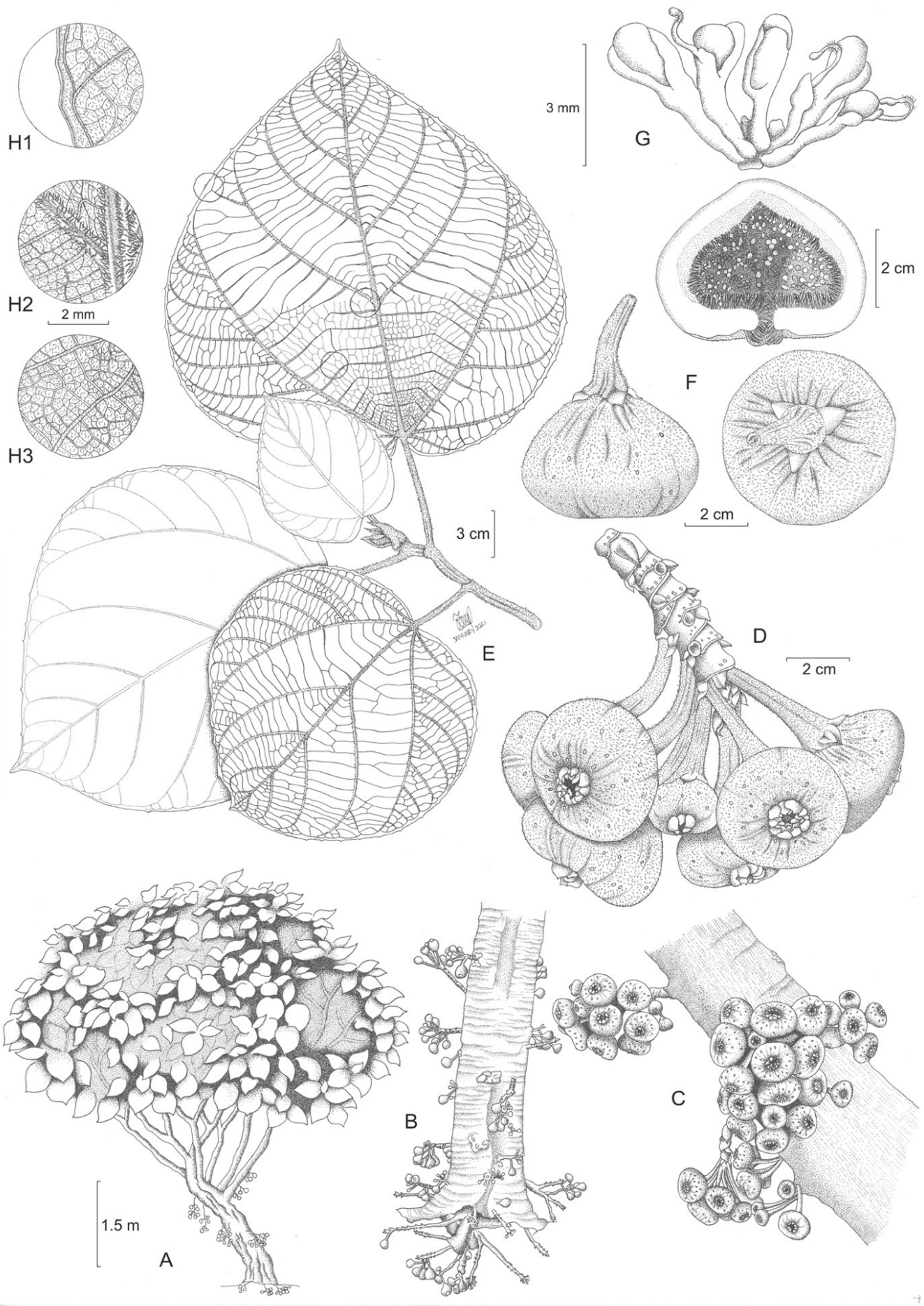

Figure 2. *Ficus auriculata* Lour.: A. Habit, B & C. Syconia emerged from the main stem, D. Syconia, E. Leaf, F. Side view, top view showing the inflorescence stalk and transversal section of Syconium, G. Female flowers and gals (showing style), H. Abaxial leaf (H1 side, H2 angles on the primary and secondary leaf vein, H3 middle part of the plate showing secondary and tertiary leaf veins). Illustration by Zakaria Al Anshori.

never been registered before. Therefore, this finding will increase the number of *Ficus* subg. *Sycomorus* members for Javanese flora information to be nine species. *Ficus auriculata* is an introduced plant originating from India and mainland Southeast Asia, while the remaining eight species are naturally distributed in the Malesia region (Berg & Corner 2005).

Ficus auriculata was introduced to Java through the Bogor Botanical Gardens in 1823, under the name *F. macrocarpa* H.Lév. & Vaniot. Data on the dynamics of *F. auriculata* in the Bogor Botanical Gardens are shown in Table 1. Traceable collections of *F. auriculata* in the Bogor Botanical Gardens, namely collections in plot VII.G.139 collection. The collection originated from the Himalayas, India, and was planted from grafts from June 1956 to 1985. Currently, the collection is dead. However, field observations indicate the existence of one non-collected *F. auriculata* individual in plot VII.G. This non-collected plant species is thought to have grown spontaneously from the dead vegetative part (stem) of the previous collection.

Table 1. The dynamics of *F. auriculata* collection in Bogor Botanical Gardens based on catalogs.

No.	Year	Species Name	Collection Locations
1	1823	*Ficus macrocarpa* H. Lév. & Vaniot	Unknown
2	1855; 1866	*Covellia macrophylla* Miq.	Unknown
3	1914; 1916; 1930	*Ficus roxburghii* Steud.	VII.H.9
4	1930	*Ficus roxburghii* Steud.	VII.H.9a
5	1957; 1973; 1985	*Ficus auriculata* Lour.	VII.G.139

Source: Blume (1823); Binnendijk & Teysmann (1855); Binnendijk & Teysmann (1866); Boldingh (1914); Boldingh (1916); Dakkus (1930); Setyodiwiryo (1957); Sastrapradja (1973); Danimihardja & Notodihardjo (1985).

In general, *F. auriculata* is known by the community as the African tin trade name. However, this species is different from the true tin, namely *F. carica* L. Both species are taxonomically assigned to two different subgenera. *Ficus auriculata* is a member of the subg. *Sycomorus*, while *F. carica* belongs to the subg. *Ficus*. Morphologically, both have distinguishable characteristics (Table 2).

Table 2. Morphological characters comparison of *F. auriculata* and *F. carica*.

Morphological characters	***Ficus auriculata***	***Ficus carica***
Habit	Tree	Shrub
Bark	Greyish brown	Grey
Leaf sheet	With no lobe or palmatifid	3–5 lobed to palmatifid
Leaf margin	Dentate, serrate, or rather flat	Crenate to serrate
Adaxial leaf surface	Smooth	Scabrous
Young leaf color	Brownish red	Yellowish green
The location of syconium grows	Stem or specialized branch	Axilar or on the leaf marks (scar)
Inflorescence stalk	1–8 cm	0.2–1.5 cm
Shape of flower base	Pyriform to appressed round	Pyriform
Outer surface of flower base	Puberulous, subtomentose, velutinous, or subglabrous	Glabrous
Flower base color when ripe	Orange to brownish red	Red to dark purple
Number of stamen	2	1 or 3(–5)

Ficus maclellandii and *F. natalensis* subsp. *leprieurii* are a monoecious plant (Zhang et al. 2019), which include to subg. *Urostigma*. However, they both from different sections, where *F. maclellandii* in the sect. *Urostigma*, which distributed from Africa to the Pacific, while *F. natalensis* subsp. *leprieurii* in the sect. *Galoglyphic*, which is indeed the endemic section of Africa (Berg & Corner 2005).

Currently, *F. maclellandii* is widely cultivated and planted as a road-shading tree. The tree canopy is rounded and a thick canopy, so, it is potential as a noise damper and pollution absorber. Recent studies show that *F. maclellandii* have important ecological roles, such as providing habitat and nesting sites of birds in urban areas (Sulaiman et al. 2013). Naturally, this species is only distributed in India, China, and mainland Southeast Asia but it has been introduced to other tropical regions, including Indonesia. Most of the *F. maclellandii* specimens collected in Java did not produce generative organs, except for the PJBMTW0919-005 specimens from Bekasi. Following previous research, the species is sterile and rarely produces flowers, so, propagation is prepared through the stem cutting method (Berg 2007). *Ficus maclellandii* belongs to *Ficus* subg. *Urostigma*, because of air roots on its branches (Figure 3), its closely related to *F. binnendijkii* (Miq.) Miq. (Berg & Corner 2005; Berg 2007). All of the *F. maclellandii* specimens collected from Java hairy on its leaf base and have pinnate venation. These two are the main different characteristics between *F. maclellandii* and *F. binnendijkii* (Berg & Corner 2005; Berg 2007).

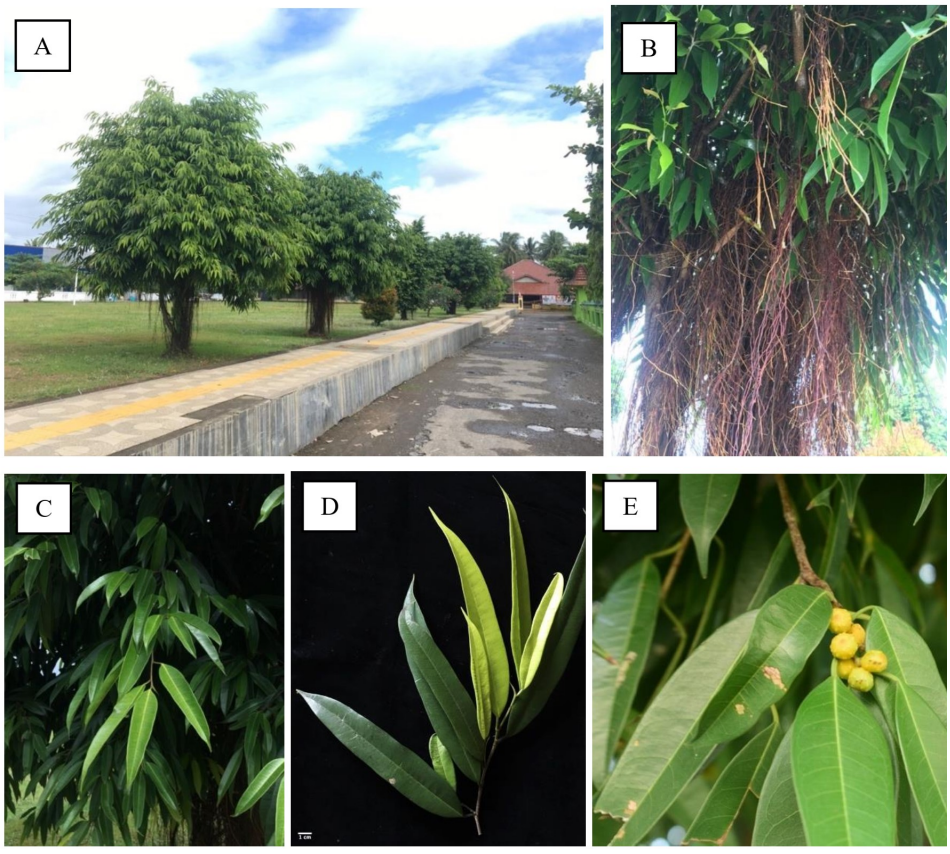

Figure 3. *Ficus maclellandii* King: A. Habit; B. Air roots; C & D. Variation of young leaves; and E. Syconia in the axillary leaf.

Three species of exotic *Ficus* originating from Africa, namely *F. cyathistipula* Warb., *F. lyrata* Warb., and *F. natalensis* Hochst., have been introduced to the Malesia region as ornamental plants (Berg & Corner 2005). However, records of herbarium specimens of these three species are not available yet (Berg & Corner 2005). During the study specimens of

F. natalensis subsp. *leprieurii* was not found at the Bogorinese (BO) Herbarium. Therefore, *F. natalensis* subsp. *leprieurii* was collected from Java for the first time. Morphologically, this species has flattened and shiny leaflets and is similar to *F. deltoidea* Jack. The two species can be distinguished based on the presence of wax glands on the leaves. The abaxial surface of *F. deltoidea* leaves has black wax glands in the axillary part of the vein (Figure 4-I). Meanwhile, the wax gland structure was not found in the leaves of *F. natalensis* subsp. *leprieurii* (Figure 4-H).

CONCLUSION

The additional notes, three species of the *Ficus* as introduced plants in Java can increase the number of species diversity of *Ficus* spp. Besides having potential as ornamental plants, *F. auriculata, F. maclellandii*, and *F. natalensis* subsp. *leprieurii* have other prospects that can be studied further in the future.

AUTHORS CONTRIBUTION

All authors had an equal contribution to this manuscript since they designed the research concept, collected data, analyzed it until they wrote all the manuscript sections. All authors read and approved the final manuscript.

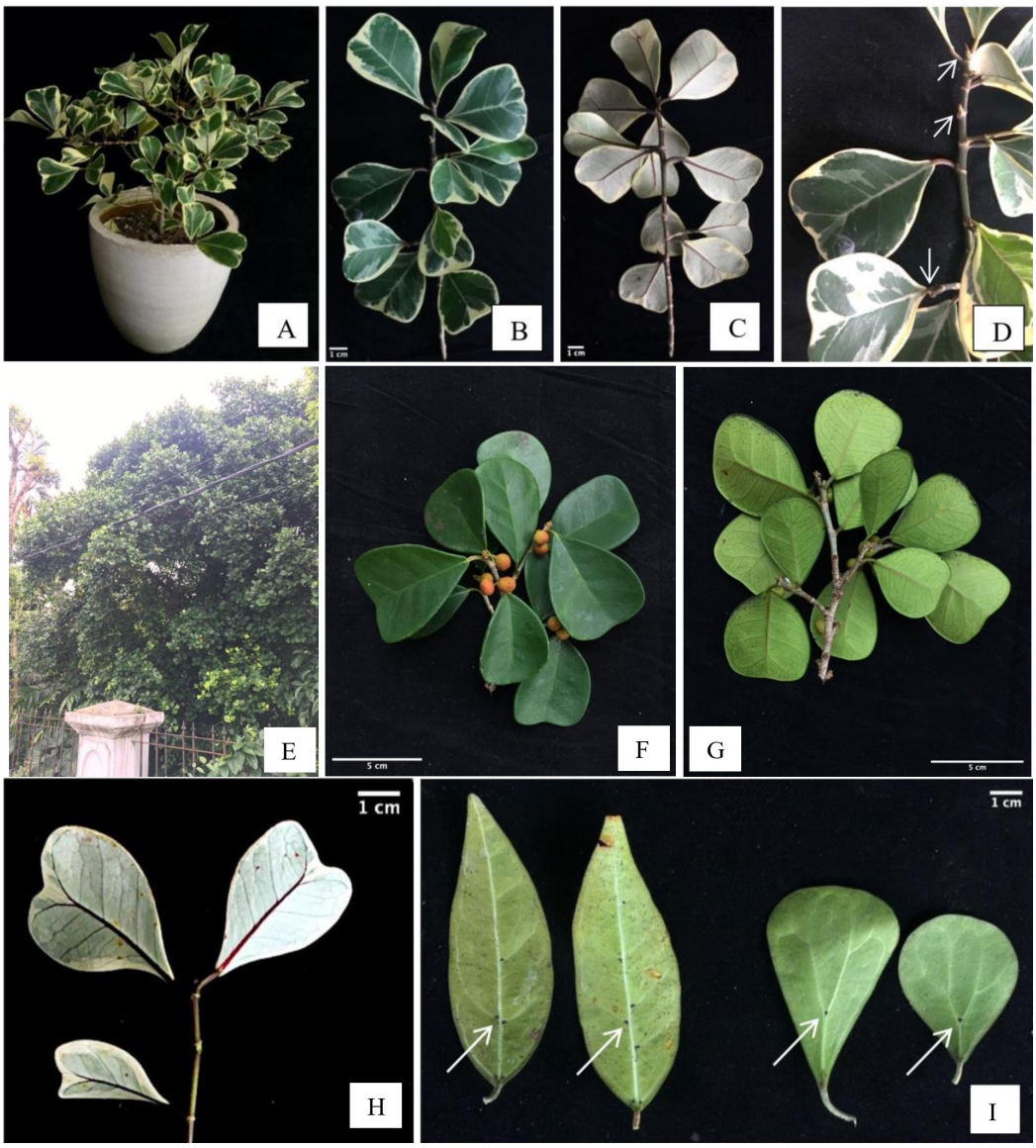

Figure 4. Variegated form of *F. natalensis* subsp. *leprieurii* (Miq.) C.C. Berg: A. Habit; B. Adaxial surface of the leaf; C. Abaxial surface of the leaf; D. Stipules position (arrows). Common form of *F. natalensis* subsp. *leprieurii*: E. Habit; F. Adaxial surface of the leaf; G. Abaxial surface of the leaf; H. Abaxial leaf surface of *F. natalensis* subsp. *leprieurii* without wax gland; I. Abaxial leaf surface of *F. deltoidea* showing blacks wax glands (arrow).

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CONFLICT OF INTEREST

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

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Research Article

Food Preference, Fecundity, Proximate Analysis on Eggs and Meat of *Turbo crassus* (W. Wood 1828) and *Turbo setosus* (Gmelin 1791) in Sepanjang Beach, Gunungkidul, Special Region of Yogyakarta

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ABSTRACT
Turbo snails have important roles in the ecosystem as prey for predators, and decomposition of dead objects, and it is starting to decrease. This is quite worrying so that conservation efforts must be carried out immediately. Turbo snail species found in Sepanjang Beach, Gunungkidul are *Turbo crassus* and *Turbo setosus*. The aims of this study were to determine food preferences, the relationship between body size and fecundity, analyze egg quality based on proximate levels of the two species, and meat proximate of *T. crassus* and *T. setosus*. Samples were taken from Sepanjang Beach, Gunungkidul. Food preference was observed using the sedgwick rafter, fecundity was counted using the gravimetric method. Moisture content, protein content, fat content, ash, and carbohydrate content of both species eggs and meat were determined with proximate analysis at the UGM Central Laboratory for Food and Nutrition Studies. The results showed that the dominant food for *T. setosus* was Phaeophyta, whereas the dominant food for *T. crassus* was *Microcoleus* sp. and Phaeophyta. The fecundity of both Turbo species is exponentially positively correlated with all body sizes. Proximate analysis showed that the levels of fat and protein in the eggs of both species were quite high, which meant that the eggs are in good quality. The conclusion of this study is both species have slightly different food preferences. The fecundity of both species will increase exponentially with increasing body size and the broodstock of *T. crassus* and *T. setosus* Sepanjang Beach has good quality.

Keywords: Fecundity, Food Preference, Proximate Analysis, *Turbo crassus*, *Turbo setosus*

INTRODUCTION
Turbo snails have been sold as food since 1995 and have started to be in great demand since 1998 at Kukup Beach (Azizah 2019). Based on a preliminary study conducted through interviews with local residents, Turbo snails in Gunungkidul were abundant in certain seasons, especially the rainy season, around February to April. In this season, people hunt every size of Turbo snail. This activity causes decrease in Turbo snail's availability in nature. Thus, conservation efforts like breeding must be carried out immediately.

Breeding efforts of Turbo snails have been successfully carried out on *T. marmoratus* (Dwiono et al. 2001) in Ambon and *T. chrysostomus* (Setyono et al. 2013) in Lombok by taking wild sires from nature to be spawned in the laboratory, while their reproductive biology has not been studied extensively. Biological aspects that are important to study as breeding efforts include fecundity, food preferences, and egg quality. Robinson (1992) evaluated that, the fecundity of individual females *Turbo smaragdus* increases along with shell size. This pattern relates to the allocation of energy in organisms, younger snails need more energy to grow while the older can use more energy to reproduce. Egg quality is important to know because the early development of the embryo is strongly influenced by the quality of the eggs, which is related to the adequacy of energy reserves and raw materials for organogenesis. Larvae that hatch from eggs with the highest protein and fat content survive the longest without food. Proximate analysis of the gonads can be carried out to determine the quality of eggs based on their nutrient composition (Fukazawa et al. 2005).

Gastropods use the radula to scrape their food from the substrate. Turbo snails have a type of radula known as rhipidoglossan commonly possessed by Ordo Archaeogastropoda. Members of the Order Archaeogastropods eat filamentous algae and other algae that grow on rocks. Kikutani et al. (2002) studied that *Turbo marmoratus* has different food preferences in different habitats. Those found in the intertidal area prefer algae while those found in the tidal area prefer foraminifera, copepod, hydrozoa, nematode, bryozoa, and even snail juvenile for their food. Another research shows that *Megastraea undosa*'s diet consists of several macroalgae groups such as Rhodophyta, Chlorophyta, and even from Cnidaria groups such as *Dynamena* sp., Mollusca such as *Capulus* sp and *Petricola* sp., Arthropods such as *Idotea* sp and Amphipoda, and Chordata such as *Metandrocarpa* sp. (Mazariegos-villarreal et al. 2017).

Based on our preliminary study, there are two species of Turbo snails found in Sepanjang Beach, namely *Turbo crassus* and *Turbo setosus*. The aims of this study were to determine food preferences, the relationship between body size and fecundity, analyze egg quality based on proximate levels of the two species, and meat proximate of *Turbo crassus* and *Turbo setosus*.

MATERIALS AND METHODS

Materials

The materials used in this study were *T. crassus* and *T. setosus* snails; aquades, 4% formalin, and 70% alcohol, Gilson's solution (consisting of 100 mL 60% alcohol, 800 mL distilled water 15 mL 80% nitric acid, 18 mL glacial acetic acid, and 20 g mercuric chloride). The tools used in this study were analytical and semi-analytic scale, coolboxes and freezers, tweezers, pipettes, optilab and microscopes (Boeco, Germany), hand counters, calipers, millimeter blocks, and rulers, knives and scissors, flacons glass, microtubes, beaker glass, sedgewick rafter, and measuring cup.

Methods

Data collection was divided into 4 steps, namely sample collection and morphometry data collection, fecundity counting, gut content analysis, and proximate analysis.

Sample Collection

Sample collection was conducted at Sepanjang Beach, Gunungkidul, at night, 21 July, 15-16 August, and 26-27 September 2020 using the purposive random sampling method. The snail samples that have been caught were

immediately put in the freezer or icebox. The total weight of the snail, length, and width of the shell were measured in the laboratory, then the shell was carefully broken with a hammer so that the visceral mass was not damaged. The visceral mass was separated from the shell fragments and the operculum, weighed, then put in a container containing 4% formalin for 24 hours. Formalin was then replaced with alcohol 70%, sample can be stored until the time of observation. The sex of the samples was also observed based on the color of the gonads (female gonads are olive green and the males are yellowish cream). The gonad weight was measured using a semi-analytic scale.

Gut Content Analysis

To determine the food preferences of both species, dissection was performed and the stomach contents were observed using the sedgewick rafter. The stomach content was diluted in 10 mL aquadest. The solution of the stomach contents is then taken using a 1 mL dropper and placed on top of the sedgewick rafter. The contents of the stomach were observed under a microscope, each type of food was counted using the sedgewick rafters and calculated as follows to evaluate the food preferences of *T. crassus* and *T. setosus*.

$$\text{Relative Frequency (RF)} = \frac{\text{Frequency of species X}}{\text{Total frequency}} \text{x100\%}$$

$$\text{Relative Abundance (RA)} = \frac{\text{Abundance of species X}}{\text{Total abundance}} \text{x100\%}$$

$$\text{Important Value Index (IVI)} = \text{RF} + \text{RA (Islamiyah et al. 2013)}$$

Observations were made in 3 repetitions.

Fecundity Counting

The number of eggs was count using the gravimetric method. Whole gonads were separated from other organs for weighing. A little piece of the whole gonad (sub-samples) was taken as much as 0.001-0.002 g from the posterior, middle, and anterior parts. Sub-samples of gonad were then placed in Gilson's solution for 48 hours or more, to separate the eggs from the connective tissue. Eggs were counted using a hand counter tool. The results of the sub-sample counting were used to obtain an estimate of the total number of eggs. Fecundity was estimated using the formula as follows:

$$\text{Fecundity} = \frac{\text{total gonad weight}}{\text{average of sub-samples weight}} \text{x average of the number of eggs in sub} - \text{samples}$$

Note: The average of sub-samples (weight and the number of eggs) were from 3 parts of each gonad.

Proximate Analysis

The samples of *T. crassus* eggs analyzed were 4.9 g from 11 samples, while the samples of *T. setosus* eggs analyzed were 6 g. Samples of meat or foot from *T. crassus* and *T. setosus* were taken as much as ± 30 g. The proximate analysis was done using the Kjeldahl method for protein content, Soxhlet for fat content, Thermogravimetry for moisture content, Muffle Furnace for ash content, and carbohydrate by difference for carbohydrate content (Ratna 2019).

RESULTS AND DISCUSSION

Gut Content Analysis

The stomach contents of *Turbo setosus* indicate a variety of natural food. *Turbo setosus* samples used in this observation were 24 individuals and divided

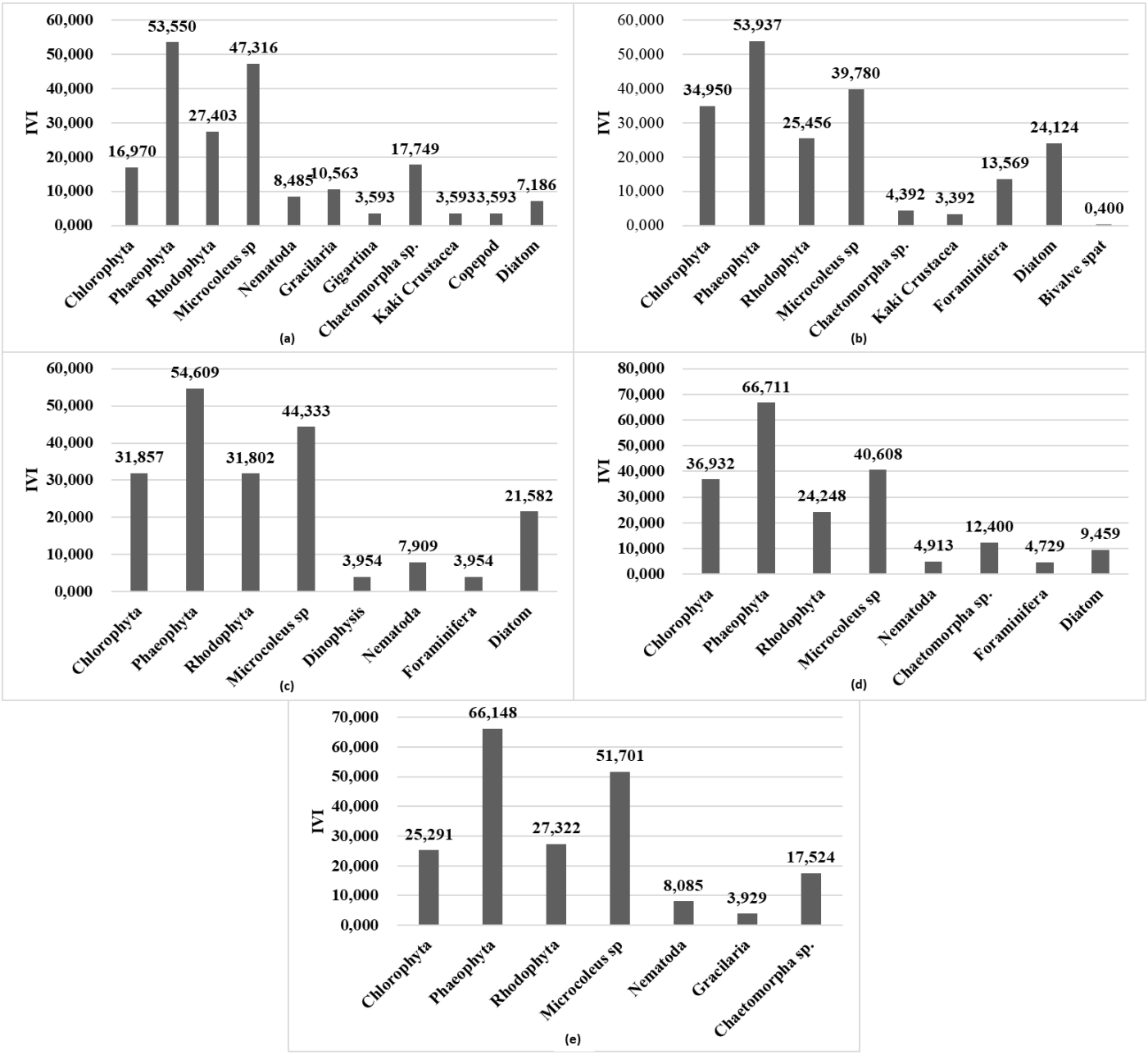

Figure 1. Importance value index (IVI) of *T.setosus* gut content (a) group 3,5-4 cm (b) group 4-4,5 cm (c) group 4,5-5 cm (d) group 5-5,5 cm (e) group 5,5-6 cm. n=1.

into 5 size groups based on shell length with each class having 5 samples. The first group consisted of 3.5-4 cm shell lengths. The second group consisted of 4-4.5 cm shell lengths. The third group consisted of 4.5-5 cm shell lengths. The fourth consisted of 5-5.5 cm shell lengths. The fifth group consisted of 5.5-6 cm shell lengths. Group size 5-5.5 cm in length has only 4 samples because of the inadequate sample quantity.

The IVI diagram in Figure 1 showed that Phaeophyta or brown algae have the highest Importance Value Index, followed by *Microcoleus* sp. and Rhodophyta or Chlorophyta in the third position. Importance Value Index (IVI) is used to analyze the dominance of a species in a particular community (Pamungkas & Zamzam 2017). The high IVI in Phaeophyta indicates the dominance of Phaeophyta in the stomach contents of *Turbo setosus* snails.

Based on the diagram in Figure 2, it is found that in the 3.5-4 cm size group *Microcoleus* sp. has the highest IVI, and Phaeophyta has the second-highest IVI. Phaeophyta has the highest IVI in class sizes 4-4.5 cm and 4.5-5 cm, and *Microcoleus* sp was in the second position.

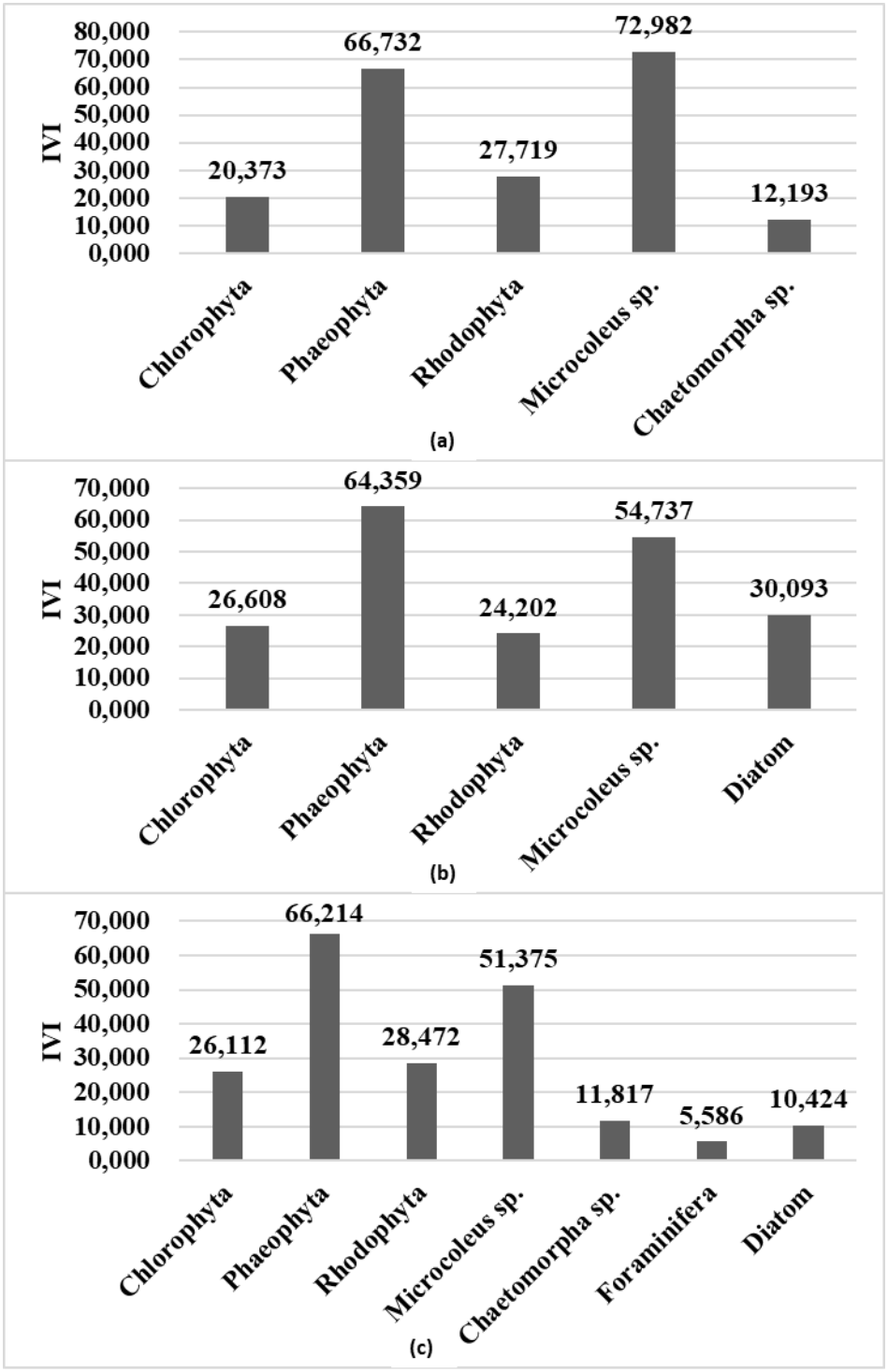

Figure 2. Importance value index of *T.crassus* gut content (a) group 3,5-4 cm (b) group 4-4,5 cm (c) group 4,5-5 cm. n=1.

Figure 3 shows the conditions of Phaeophyta that can be found in the stomach of the *Turbo setosus* and *Turbo crassus*. Brown algae talus that can be found in the stomach indicates a tear that is thought to be due to the radula. The algae cell wall is also damaged due to the grinding process on the gizzard. The brown algae found in the stomach of *Turbo crassus* are shaped like filaments, while the Phaeophyta found in *Turbo setosus* is more sheet-like.

Rhodophyta in Figure 4 shows conditions that are not much different from Phaeophyta. Tearing is also estimated by the snail's radula, but it can be seen that the cell wall is still relatively better than the Phaeophyta found. The relatively better cell wall is estimated because the lag between taking and preserving the samples obtained and the time for the snails to feed is not too long so that the algae wall in the stomach is still relatively intact.

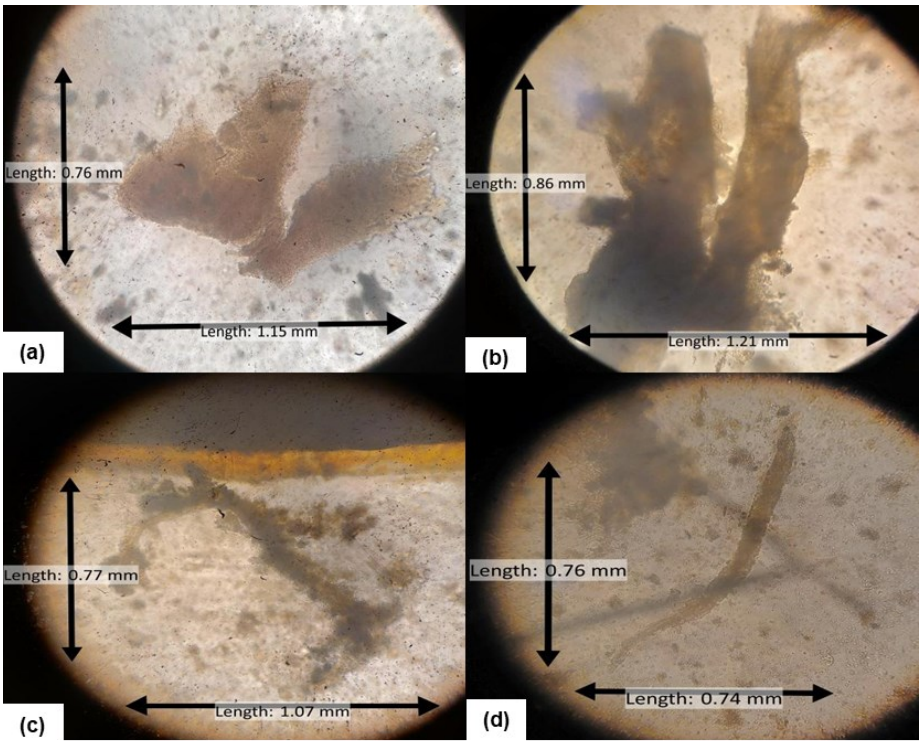

Figure 3. Phaeophyta found in *T. setosus'* stomach (a,b) and in *T. crassus'* stomach (c,d), magnification of ; 10x10.

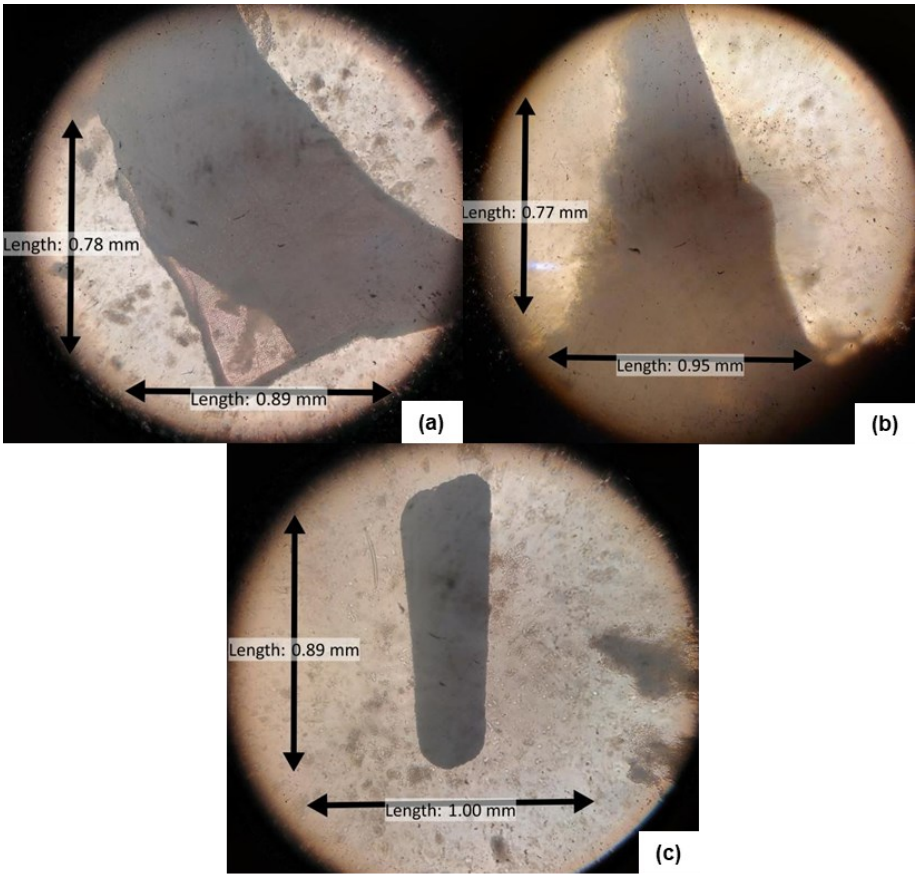

Figure 4. Rhodophyta found in *T. setosus'* stomach (a,b) and in *T. crassus'* stomach (c), magnification of 10x10.

Figure 5 showed the condition of Chlorophyta which has sheet-shaped talus and is overlapped by brown algae. The tearing of the talus is thought to be due to the snail's radula tearing the green algae talus. The cell wall is still in a relatively intact condition.

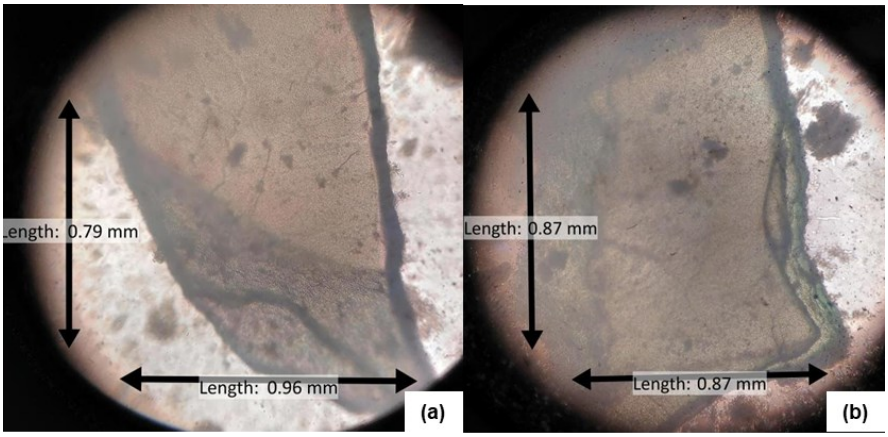

Figure 5. Chlorophyta found in *T. setosus* stomach (a,b) magnification of 10x10.

The condition of *Microcoleus* sp inside *Turbo crassus'* stomach is not much different from the condition of *Microcoleus* sp in *Turbo setosus'* stomach (Figure 6). The characteristic conditions seen in *Microcoleus* sp. are in the form of filaments, filaments that join together into a thin layer, arranged in parallel, and are colorless (Guiry 2020).

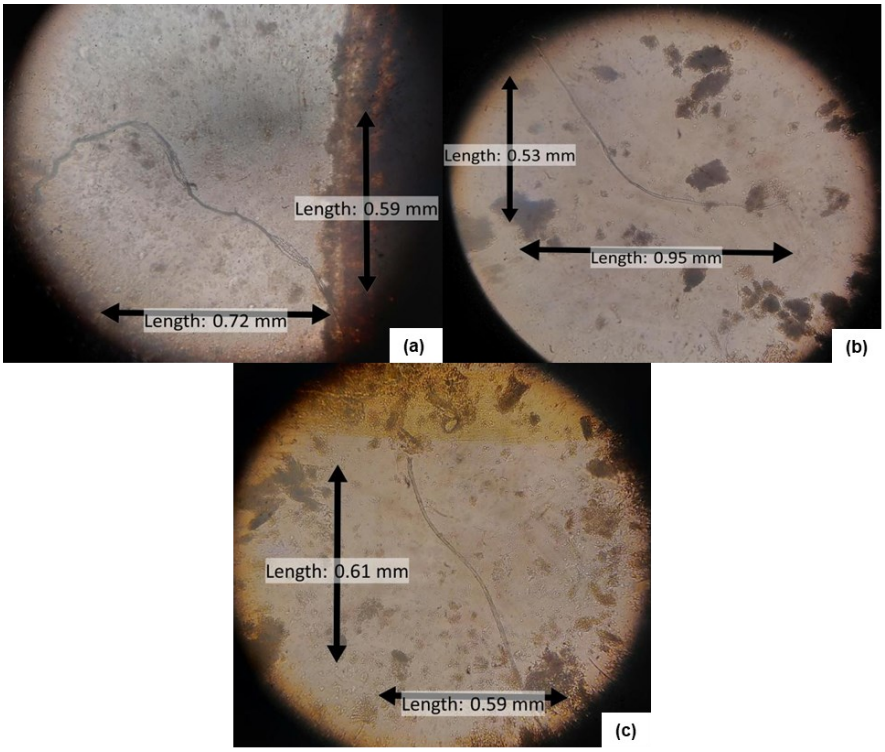

Figure 6. *Microcoleus* sp found in *T. setosus* stomach (a,b) and in *T. crassus'* stomach (c) magnification of 10x10.

In another study in the Tuamotu Islands, *Turbo setosus'* stomach contents could be found several types from various kinds of algae groups. The members of the Chlorophyta group that can be found were *Cladophora* sp. The members of the Phaeophyta group found were *Lobophora variegata* and *Sphacelaria* sp. The Rhodophyta group members found were *Jania* sp. and *Laurencia* sp. Members of the Cyanobacteria group that could be found were *Calothrix confervicola*, *Microcoleus lyngbyaceus*, *Schizothrix calciola*, and *Spirulina subsalsa* (Tsuda & Randall 1971).

Gastropods have chemoreceptor organs, one of which is osphradium. Osphradium is a primitive chemoreceptor organ that is connected to the visceral arch of the central nervous system (Kamardin et al. 2001). Studies have been taken to prove that Gastropods do have preferences on their food.

Osphradium stimulation with L-aspartate in *Lymnaea stagnalis* causes an increase in osphradial nerve activity (Kamardin et al. 2001). Rhinophores also have an active role in finding habitats for nudibranch larvae based on the presence of suitable food (Bornancin et al. 2017). Metamorphosis in *Aplysia californica* also occurs with several chemical signals from several algae such as *Rhodymenia california, Corallina officinalis, Dyctyopteris undulata, Pachydictyon coriaceum, Pterocladia capillacea, Centroceras clavulatum*, and *Chondria californica* (Bornancin et al. 2017).

The result from different shell length groups has only slight differences, all shell length sizes have Phaeophyta as their most dominant food, followed by *Microcoleus* sp. as the second most dominant food in their stomach. There is no different food preference based solely on shell length. This shows that their diet is roughly the same even among different sizes. Further research in captivity or cultivation may be needed for the confirmation of this result.

Based on the observations in this study, Turbo snails seem to have a preference for their food. Both Turbo species have shown some slight differences in their food preferences. *Turbo setosus* food preferences consist of 3 types of macroalgae, *Microcoleus* sp, 1 type of nematode, crustacean, and diatoms. *Turbo crassus* food preferences consist of 3 types of macroalgae, *Microcoleus* sp, foraminifera, and diatoms. The results showed that both species seem to be able to differentiate their food.

Fecundity

Decapoda and Bivalvia's carapace or shell capacity is the limit of gamete productivity. A hard, closed-shell will limit the space available for the development of the gonads. In other words, carapace or shell size has a positive correlation with fecundity. The more space available, the more eggs that can be produced. The growth of the organism is also greatly influenced by the food available in its environment, if there is an increase in the amount of food, the rate of growth will increase and the egg production becomes more abundant. The energy possessed by living things will be allocated to growth and reproduction, and the distribution will adjust to the age or phase of their life. In some Bivalvia, there is massive growth in the early years of its life, meaning that the energy is focused on growth. The growth rate will begin to decline as gamete production increases. The increase in *Mytilus edulis* and *Yoldia notabilis* fecundity was more closely related to the body size of the broodstock than age (Llodra 2002). The fecundities of *T. crassus* and *T. setosus* per size group mean can be seen in Figure 7.

Based on Figure 7, of the 11 individual *T. crassus* species studied, the increase in shell length had a positive exponential correlation with the increase in egg number or fecundity. Likewise with the increase in shell width and total weight. The highest R^2 value is 0.6683 in the ratio of the number of eggs to shell length (Figure 7a). The lowest R^2 value is 0.3967 in the ratio of the number of eggs to shell width (Figure 7b).

Based on Figure 8, of the 18 individual *T. setosus* species studied, the increase in shell length had a positive exponential correlation with the increase in egg number or fecundity. Likewise with the increase in shell width and total weight. The highest R^2 value is 0.5252 in the ratio of the number of eggs to shell width (Figure 8b). The lowest R^2 value is 0.3964 in the ratio of the number of eggs to the total weight (Figure 8c).

The value of R^2 is a marker of how much data can be explained by a regression formula. In this fecundity data, the exponential regression that has the best R^2 value means that an increase in body size will increase the number of eggs exponentially. The body size parameter that describes fecundity well is the one with the highest R^2 value. *T. crassus* had the highest

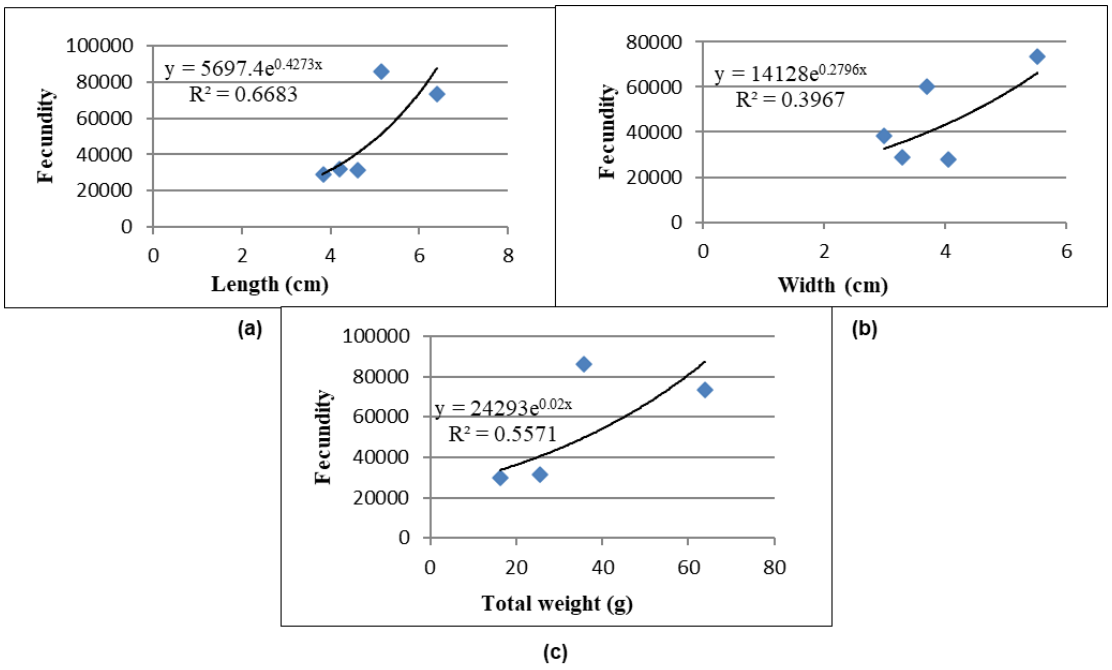

Figure 7. Comparison of the shell length (a), shell width (b), and total weight (c) of *T. crassus* to fecundity.

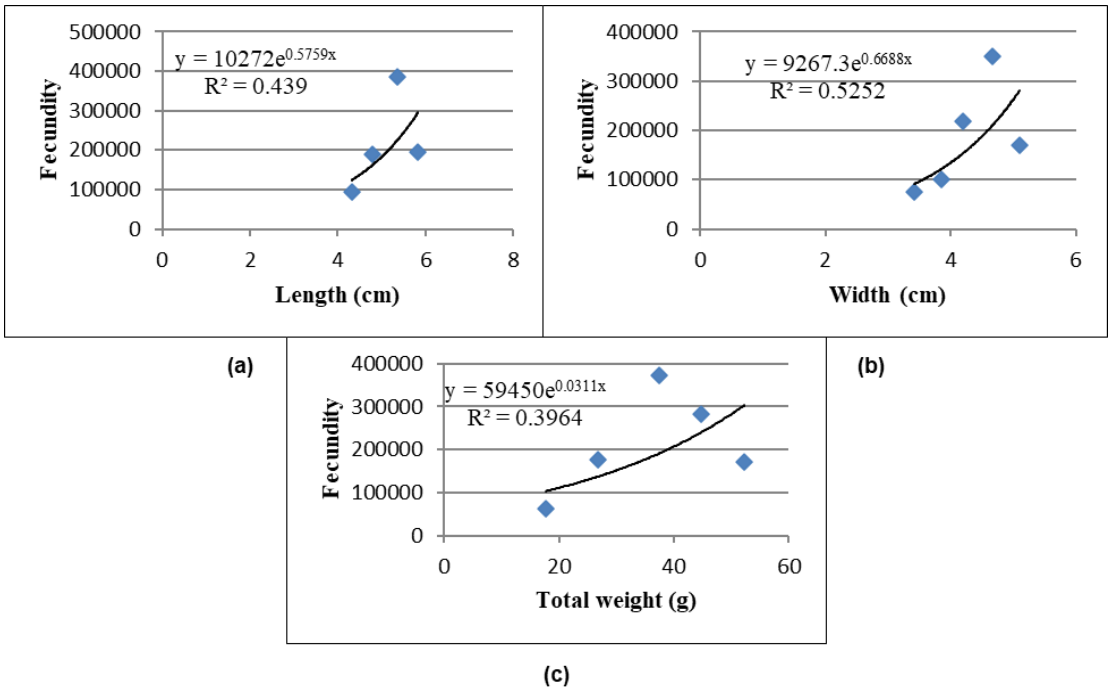

Figure 8. Comparison of shell length (a), shell width (b), and total weight (c) of *T. setosus* to fecundity.

R^2 value for shell length parameters, while *T. setosus* had the highest value for shell width. This difference can be attributed to the shell morphology of *T. crassus*, which has a longer spire than *T. setosus* (Poutiers 1998). The ratio between the length and width of the shells of the two species did not differ much, that is 1: 0.82 in *T. crassus* and 1: 0.85 in *T. setosus*.

Based on the description above, body size will determine the number of eggs that can be produced, in relation to the availability of space for gonad development in the shell. Larger snails have a higher number of eggs, so the comparison of the number of eggs to the length and width of the shell shows a positive correlation. The complete weight of the snail also correlates with its fecundity, as the number of eggs will also have an effect on its total body weight. The positive correlation between fecundity and body size also occurs in other gastropod members such as *Conus pennaceus* (Perron 1983); Limpet

species *Notoacmea petterdi*, *Patelloida alticostata*, and *P. latistrigata* (Creese 1980); Invasive gastropod *Crepidula fornicata* (Pechenik et al. 2017).

If a Turbo snail breeding efforts is carried out such as *T. chrysostomus* (Setyono et al. 2013) and *T. marmoratus* (Dwiono et al. 2001), the quality of broodstock must be considered so that the resulting spawns are also of good quality. One of the considerations to determine the quality of broodstock is their fecundity. The results of this study indicate that the first maturity period of *T. crassus* and *T. setosus* gonads were found in the shell length of 3-4.5 cm. The least gonads are produced at that size, so it is better to use broodstock at a shell length of at least 4-5 cm to increase the number of offspring. Turbo snails take a long time to grow. The government can create a program to release the seeds to help restore the population in the wild and create limits on their catch. The availability of nutrients also has an important effect, such as *Haliotis laevigata* and *H. cyclobates* experiencing the highest gonad growth when food is abundant (Robinson 1992).

In *T. smaragdus* (Robinson 1992) which has a certain period for spawning, fecundity observations were made in January where the gonads were in the immediate pre-spawn phase when the ovaries were filled with mature oocytes. In this study, there was no information regarding the reproductive cycle throughout the year. It is possible that *T. crassus* and *T. setosus* fecundities could reach higher numbers. The highest number of eggs in *T. crassus* individuals was counted is 85,876 eggs, while the least number of eggs was 8,881 items. The *T. setosus* individual counted at most 567,719 eggs, and at least 35,563 eggs. The number of *T. crassus* eggs that are less can occur due to different reproductive cycles, or indeed the reproductive ability is lower than that of *T. setosus*. This can be confirmed by observing fecundity every month, for a full year.

Proximate Analysis

Proximate analysis is performed to determine the moisture, ash, fat, protein, and carbohydrate content in the sample being tested. In this study, testing was carried out on meat and egg samples of *T. crassus* and *T. setosus* at the Center for Food and Nutrition Studies Laboratory UGM. The results of 2 repetitions were averaged and shown as a percentage, it can be seen in Table 1.

Table 1. shows that overall, the highest composition of all samples is moisture content, where it is known that water is the main constituent of living bodies, and also because the samples analyzed are wet samples. In dry samples, the composition will be very different. The next highest level is protein, which is the building block for the structure and maintenance of body tissues. The highest water content measured in *T. crassus* meat was 77.11 ± 0.21%, while the lowest was in *T. setosus* eggs of 69.75 ± 0.06%.

As a comparison, previously researched proximate content of *T. setosus* meat in Ujung Genteng Waters, Sukabumi, wet sample had a water content of 74.8%, 0.8% ash, 0.02% fat, 16% protein, and 6.8% carbohydrates. The dry sample has a moisture content of 10.15 ± 0.69%, ash 6.87 ± 0.12%, fat 2.20 ± 0.01%, protein 70.34 ± 0.13%, and carbohydrates 10.06 ± 0.04 (Merdekawati et al. 2017). The proximate analysis of *T. crassus* has not been studied, while the proximate of other Turbo species such as *T. militaris* has a water content of 73.1%, 2.1% ash, 5.6% fat, 16.2% protein, and 3% carbohydrates (Santhanam 2019). Ash content describes the mineral content in an organism's body, the regulation of which can be different for each organism (Merdekawati et al. 2017).

Egg fat content is much higher than meat fat content. Egg protein content is lower than meat, while egg carbohydrate content is higher. In Mollusca, most of the energy sources are stored in the leg muscles and

Table 1. Proximate analysis of *T. crassus* and *T. setosus*.

Proximate content (%)	*T. Setosus*		*T. crassus*	
	Eggs (n=2)	Meat (n=2)	Eggs (n=2)	Meat (n=2)
Moisture	69.75±0.06	75.89±0.55	75.60±0.19	77.11±0.21
Ash	1.02±0.01	0.825±0.05	1.04±0.24	0.925±0.13
Lipid	9.37±0.14	0.38±0.00	6.06±0.18	0.39±0.01
Protein	15.27±0.22	20.04±0.13	14.23±0.66	18.59±0.17
Carbohydrate	4.60±0.29	2.77±0.49	3.08±0.91	2.99±0.23

digestive glands (Najmudeen 2007), therefore the highest protein levels are in *T. crassus* and *T. setosus* measured in the foot. In gastropods, protein is an important component in egg yolk in the form of ferritin, which is derived from the hepatopancreas (Najmudeen 2007). The protein content of meat is influenced by growth and metabolic stages, as well as nutrient factors and environmental conditions (Najmudeen 2007). Research by Litaay & De Silva (2003) on the *Haliotis rubra* abalone stated that the main energy in reproduction is fat, as evidenced by the significant reduction in fat levels in the gonads after spawning. In a study on *Pecten maximus*, lipid reserves in the body will be used in gametogenesis and lipid levels will differ according to the reproductive cycle with higher levels in more mature gonads (Litaay & De Silva 2003). Lipids in eggs are very important because they are used in the formation of membranes and the main energy source for embryogenesis and early larval development (Litaay & De Silva 2003). The difference in fat content in the eggs of the two species in this study may be related to the phase of gametogenesis, wherein *T. setosus* the gonads are much thicker than *T. crassus* so that the fat content is also higher.

There are several instances where carbohydrates are converted into fat during gametogenesis (Najmudeen 2007). Carbohydrates in ordinary Mollusca tissue are in the form of glycogen and their levels can differ according to their utilization in the organism's body (Merdekawati 2017). The high carbohydrate content in *T. crassus* and *T. setosus* eggs may be related to the glycogen component in mature oocytes which becomes energy reserves for further development. Figures 3. and 4. showed that both species have a variety of natural food. This may result in a more balanced diet as compared to having only a single type of food. Variation in food types may lead to a better growth rate (Naidoo et al. 2006).

In marine invertebrates, the quality of female broodstock will affect the size and quality of eggs, which is related to the survival and growth of spawns (Fukazawa et al. 2005). Egg nutrient reserves are not only important in the process of embryogenesis, for example, abalone larvae and postlarva will also depend on nutrient reserves in eggs. Eggs with the highest levels of fat and protein will produce spawns that can last longer without food. Fat content is also directly proportional to protein content, both of which are important sources of energy for larval and postlarvae development. In *Haliotis discus hannai* which has multiple spawning types, it was found that protein and egg fat levels would be higher in eggs that were spawned at the last spawning period in one period (Fukazawa et al. 2005). From this study, it was found that the levels of fat and protein in the eggs of *T. crassus* and *T. setosus* were quite high, which meant that they had good quality and were expected to produce offspring with good growth and survival. In breeding or conservation efforts, the availability of suitable feed will be able to support the productivity of Turbo snails. If the nutritional needs of the broodstock are met, the quality of eggs or offspring will increase.

CONCLUSION

From this study, we concluded that *Turbo crassus* and *Turbo setosus* have a slightly different diet. There is also a slightly different diet between different sizes. The most dominant food in *Turbo setosus* was Phaeophyta, *Microcoleus* sp, and Chlorophyta or Rhodophyta. The most dominant food in *Turbo crassus* is *Microcoleus* sp, Phaeophyta, and diatoms or Rhodophyta. The meat proximate of *T. setosus* has the highest protein content. The meat proximate of *T. crassus* showed higher water, fat, carbohydrate, and ash content than *T. setosus* meat. The fecundity of *T. crassus* and *T. setosus* will increase with increasing body size, where *T. crassus* has the highest R^2 value in the shell length parameter, while *T. setosus* on the shell width. The broodstock of *T. crassus* and *T. setosus* in Sepanjang Beach have good quality, because of the high levels of fat and protein in eggs and meat.

AUTHORS CONTRIBUTION

All three authors conceived and designed the research. Trijoko was the supervisor for this research, provided critical revision, and also approved the final version to be published. Rijal R and Izzatul Auliya' both collected the samples for this research, analyzed the data and wrote the article.

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CONFLICT OF INTEREST

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

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Research Article

Neither Coral- nor Symbiont- Genetic Diversity may Explain the Resistance of the Coral *Echinopora lamellosa* to Bleaching

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ABSTRACT
Genetic diversity has an important role in the stability of coral populations in coping with disturbances. In the last three bleaching events, the coral *Echinopora lamellosa* survived better in the eastern- than the western- Lombok waters that are not related to algal symbiont diversity. The present study aimed to assess the genetic diversity of *E. lamellosa* from the two locations in the Lombok waters. The ITS1-5.8S-ITS2 (whole ITS region) marker was used to identify and to determine the genetic structure, genetic variation, and demographic pattern of *E. lamellosa*. The results showed that *E. lamellosa* of the two locations are two different populations. The haplotype diversity was very high indicating a predominance of sexual reproduction mode for both eastern and western populations. The phylogenetic topology suggests there is possible connectivity between populations, whereas the haplotype network exhibits a restricted gene flow between the two populations. The results suggest that the present *E. lamellosa* populations were from both surviving colonies and new recruitment of long-distance larvae. Both populations likely share the same larvae supply brought from source-reefs in the Flores Sea or Makassar Strait by the Indonesian Throughflow. The present and previous studies revealed that genetic diversity alone yet to explain the resistance of *E. lamellosa* in eastern and western Lombok waters.

Keywords: bleaching, larval dispersal, population, re-colonization, survivorship

INTRODUCTION
Genetic diversity has been recognized as one of the most important factors for any community in coping with disturbance. So far, studies on coral population genetic related to coral bleaching mostly focused on the diversity of coral's symbionts (Swain et al. 2021). Many studies showed that symbionts play an active role in determining the resistance of corals during bleaching events (e.g. Wall et al. 2020), however, the link between the resistant symbiont and the coral genetic diversity is obscure.

Genetic diversity is responsible for differences in the physiological responses of a coral population to environmental changes (Cooke et al. 2020). A coral population with a more diverse genotype will have more

chances to survive from coral bleaching (Morikawa & Palumbi 2019). Coral genetic diversity may support coral survivability from bleaching, by determining coral's capability in sorting the right symbiont (Peixoto et al. 2017), production of photopigment (Zamani et al. 2019; Wall et al. 2020), or production of heat shock protein (Fuller et al. 2020). Biological legacies that survive in the population after disturbance will secure population recovery from both sexual and asexual reproductions. Surviving corals can increase their chances by shifting symbiont to cope with the next bleaching event, a mechanism commonly known as the adaptive bleaching hypothesis (Buddemeier & Fautin 1993).

Coral bleaching is one of the most threatening disturbances for coral reefs in the Anthropocene Epoch. During the last four decades, there have been four major bleaching events (severe bleaching), that severely caused global coral mortality in 1983, 1998, 2010, and 2016 (Brown & Suharsono 1990; Hughes et al. 2018). The bleaching events occurred at increasingly shorter intervals, leaving an insufficient time for corals to recover. The shorter time interval between each bleaching phenomenon potentially causes successive effects that may lead to an accelerated mass extinction of marine life.

In the Lombok waters, coral communities experienced at least three major bleaching episodes, in 1998, 2010, and 2016 (Bachtiar & Hadi 2019). During the three bleaching events, coral communities in the eastern population always survive, while the western population experienced severe mass coral mortality in many genera (Bachtiar 2001a; Bachtiar & Hadi 2019). The differential bleaching impacts between coral communities in western and eastern Lombok waters yet to be explained solely from the symbiont diversity. The coral *E. lamellosa* in the eastern population host multiple clades B and C, whereas those in the western population host only clade C (Bachtiar et al. 2019). Neither B nor C clades are the thermally tolerant symbiont. Surviving corals of *E. lamellosa* in both western and eastern populations provide opportunities to test a hypothesis that there is an association between coral genetic diversity and its bleaching resistance. If resistance to coral bleaching is associated with coral genetic diversity, coral populations of eastern Lombok waters should have more diverse DNA than the western population. The present work is the first study on the coral genetic population of *E. lamellosa* in Indonesian waters.

MATERIALS AND METHODS

Sample collection

Colony samples of the coral *Echinopora lamellosa* were collected from a key of Gili Petagan in the north-west of Alas Strait (eastern Lombok waters, 8°26'1" S and 116°44'33" E) and a key of Gili Anyaran in the south-east of Lombok Strait (western Lombok waters, 8°43'16" S and 115°54'50" E), as shown in Figure 1. Samples of *E. lamellosa* fragments at sizes of 2-3 cm^2 were collected in a SCUBA diving at 5-7 meters depth. To prevent the possibility of sampling clones, only colonies of *E. lamellosa* with a distance of at least 3 m apart were sampled. Samples were then preserved in 95% ethanol and stored at room temperature. The samples collected from the northwest Alas Strait are referred to as 'eastern populations' labeled with the 'GPE', while samples originated from Sekotong Bay (Lombok Strait) are referred to as 'western populations' labeled with 'SEL'.

DNA extraction

The genomic DNA was isolated from pulverized and sterilized *E. lamellose* samples by using the DNeasyTM Blood and Tissue Kit (Qiagen®) as per

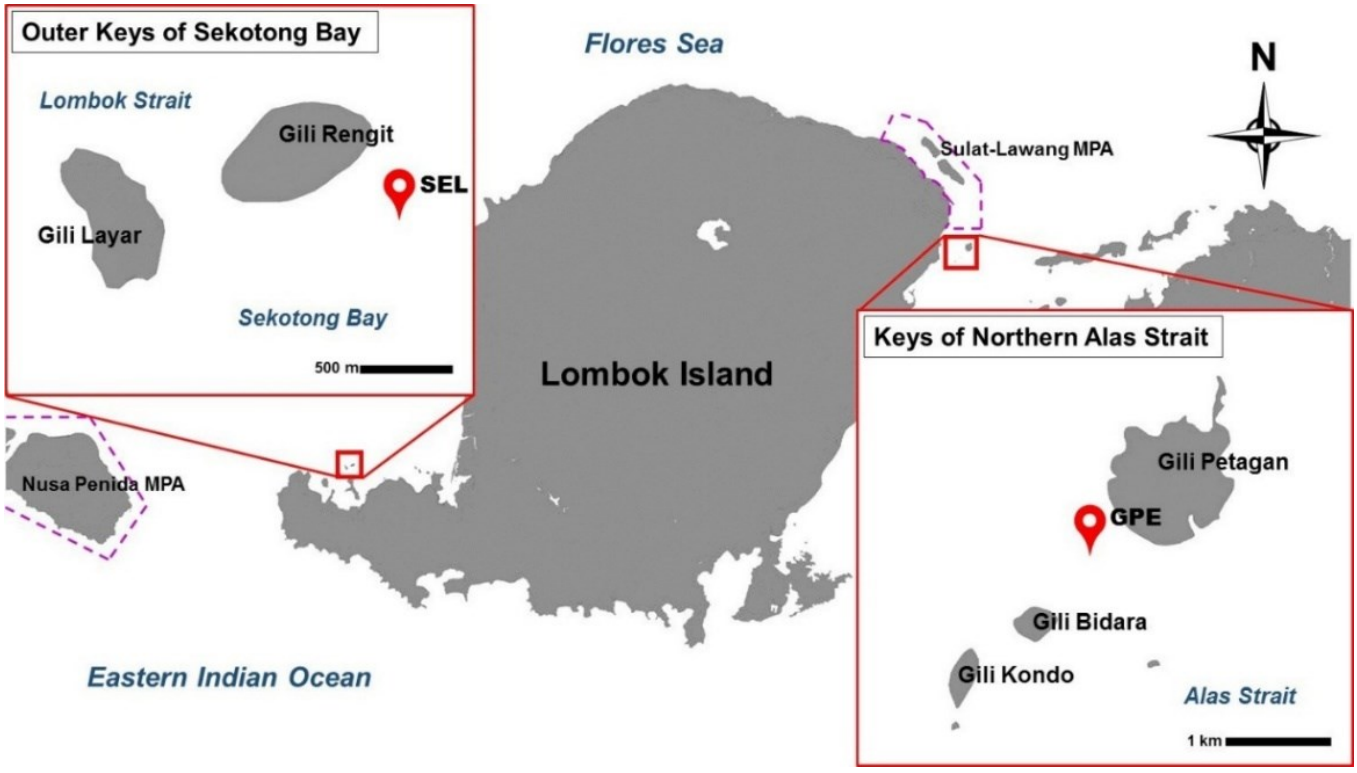

Figure 1. Sampling locations (SEL and GPE) of the study. The distance between the two locations is approximately 97 km.

manufacturer instruction (Qiagen® manufacturer protocol for extracting DNA of tissue and blood) with 12 hours pre-incubation. The DNA of coral was separated from the DNA of the symbiont through multilevel centrifugation steps as described in Kenkel & Bay (2018). A check for the quality of the extracted DNA was performed on a 1% agarose gel. Re-extraction of DNA was carried out on samples with low DNA quality, which is indicated by a faded band, or no band pattern appears on the agarose gel.

This study utilizes the whole ITS region (ITS1-5.8S-ITS2 region) of *E. lamellosa* rDNA to be amplified as a marker. A previous study conducted by Wijayanti et al. (2018) using a mtCOI marker on *Acropora hyacinthus* populations resulted in accurate genetic identification, but less sensitivity to build a phylogenetic tree. A single copy of ITS (Internal Transcribed Spacer) region has been extensively used for population genetic and phylogenetic studies of corals and symbionts (see Takabayashi et al. 2003; Terrana et al. 2021). It has been reported that the rDNA ITS region has potentially become an accurate and sensitive marker to reveal the genetic diversity in the coral population, even with a small sample size (Afiq-Rosli et al. 2019). Amplification of the whole ITS region was carried out using forward primer ITS1F (5'-CTTGTTCATTTAGAGGAAGTAA-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes & Bruns 1993) by Integrated DNA Technology™. The reaction mixture contained 2 µL of each primer, 12.5 µL of Taq DNA Polymerase (MyTaqTM HS Red Mix, Bioline®), 9 µLof ddH2O (SuperPure nuclease-free water, BioScience®), and 2 µL of DNA template. The amplification was processed in the peqSTART Thermal Cycler (PEQLAB®) with the program set as follows: 1 cycle at 95°C (2 min); 35 cycles at 94°C (45 s), 47°C (30 s), and 72°C (1 min). The amplicon quality was checked through electrophoresis on a 1% agarose gel and then visualized under a transilluminator. The high-quality amplicon appears as a clear and thick band. Only high-quality amplicons were sent for further sequencing to the 1st Base-Asia, Malaysia (http://www.base-asia.com).

Data analysis

All sequences were analyzed further to confirm species identification of the coral samples genetically using BLAST (Basic Local Alignment Search Tool) features on NCBI (http://ncbi.nlm.nih.gov). The chromatograph of each sequence was checked to verify and determine the base calling. ClustalW was used for sequence alignment. Sequences alignment and trimming were carried out in MEGA V.06 to generate a pairwise distance matrix and specific "fasta" formatted file (.fas) for downstream analysis. Molecular genetic diversity indices such as haplotype diversity and polymorphic site were obtained using DNAsp V5.10 (Librado & Rozas 2009), with defaulted exclude gap or missing data sets. The population genetic diversity value range from 0 to 1. Measurement of genetic differentiation was carried out using ARLEQUIN V3.5 (Excoffier & Lischer 2010) with AMOVA (Analysis of Molecular Variance) test, as well as the genetic difference between the two populations (Fst and P-value). The significance covariance components were tested with 10,000 permutations.

The phylogenetic tree, genetic distance matrix, and population genetic network were constructed to determine the connectivity and relationship between samples. The Neighbor-joining tree (NJ) was generated using MEGA V6.0 (Tamura et al. 2013) with Kimura 2-parameter and a bootstrap value of 1000× replication. The genetic pairwise distance was visualized in a similarity matrix using HeatMapper (https://heatmapper.ca) based on the Euclidean distance method. The POPart V1.7 (Leigh & Bryant 2015) was used to implement automatically the unrooted minimum spanning network (MSN), while Network V1.0 was used for haplotype network analysis using the median-joining method (Bandelt et al. 1999).

RESULTS

Sequence identification and genetic structure

A total of 30 samples of *E. lamellosa* were successfully sequenced from both eastern- and western- populations with the length of sequences ranging from 701 to 728 bp. All sequences were genetically identified as *E. lamellosa* using BLAST features. The query cover value for all sequences is above 90% indicating all the nucleotides sequence in the samples is very similar to the GenBank data. The value of identification percentage for all sequences ranging from 88.87% to 98.68%. The wide range of percentages may indicate a high genetic variation and the possible presence of unique sequences within populations that have not been registered in GenBank.

The topology of the Neighbor-Joining (NJ) tree divides the samples into 3 (three) clades (Figure 2). The NJ tree also shows a pattern of shared clades between the eastern and western groups. This indicated that there is close genetic connectivity between the two groups. Although both sequencing of GPE 33 and GPE 35 have the lowest identification percentage (88.87% and 88.81%, respectively), they still have very high query cover (96% and 95%, respectively). It suggests that these colonies were genetically unique. The phylogenetic tree reconstruction suggests to outgroup the GPE33 as the Clade C, while GPE35 remains within the Clade A (Figure 2). The phylogenetic tree was constructed based on NJ methods, including the downloaded GenBank sequence of *Seriatopora hystrix* was put as an outgroup. The use of the whole ITS region is feasible and very sensitive in representing small differences and mutations in *E. lamellosa* population. The ITS region also provides more robust data than any other genetic markers for coral, such as the mtCOI markers (Wijayanti et al. 2018). The Euclidean distance matrix also supports that colonies GPE 33 and GPE 35 are very distinct from other samples in both western and eastern groups (Figure 3).

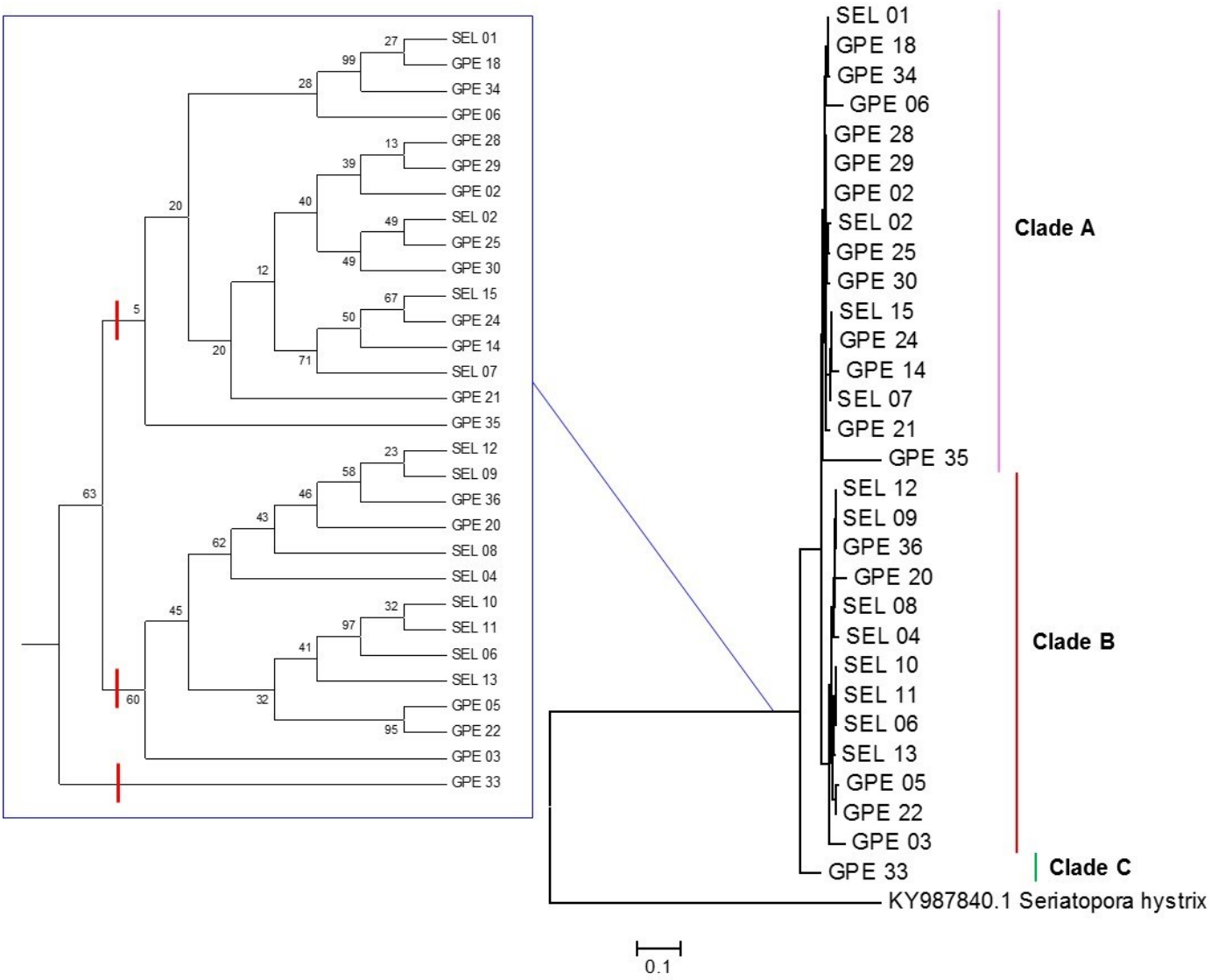

Figure 2. The phylogenetic tree represents the genetic structure of *E. lamellosa* from Lombok waters. This phylogenetic tree is detailed by a typology tree (inserted picture), showing 3 clades within the 2 geographic regions. Reconstruction of the phylogenetic tree only shows an indicative of coral clade variation based on ITS region, rather than a definitive means due to a lack of study on this species.

The heatmap distance matrix shows that there is a constrained flux of genes among the two populations. The genetic distance was lower than 0.144 within groups but moderate to high between groups (>0.144 to 0.288) (Figure 3). However, there is an exception for both GPE 33 and GPE 35, which exhibits a very high difference value that close to 0.5759 against all colonies.

Genetic variation and demographic pattern

Both the eastern and western groups were in the same high haplotype diversity category. The eastern group had a slightly higher haplotype diversity value (0.9804; n=18) than the western group (0.9394; n=12). The trimming and cutting were carried out for all data set, resulting in the similar-sized 581 bp segment of the 5' end of the obtained sequence from all individual samples. The comparison of those similar-sized sequences revealed 193 polymorphic sites. These polymorphic sites defined 23 haplotypes and 2 (two) of those haplotypes were shared between the eastern and western groups. A total of 19 haplotypes (63.33% of the total sample) were unique and each consisted of only a single sample. The haplotype diversity for the

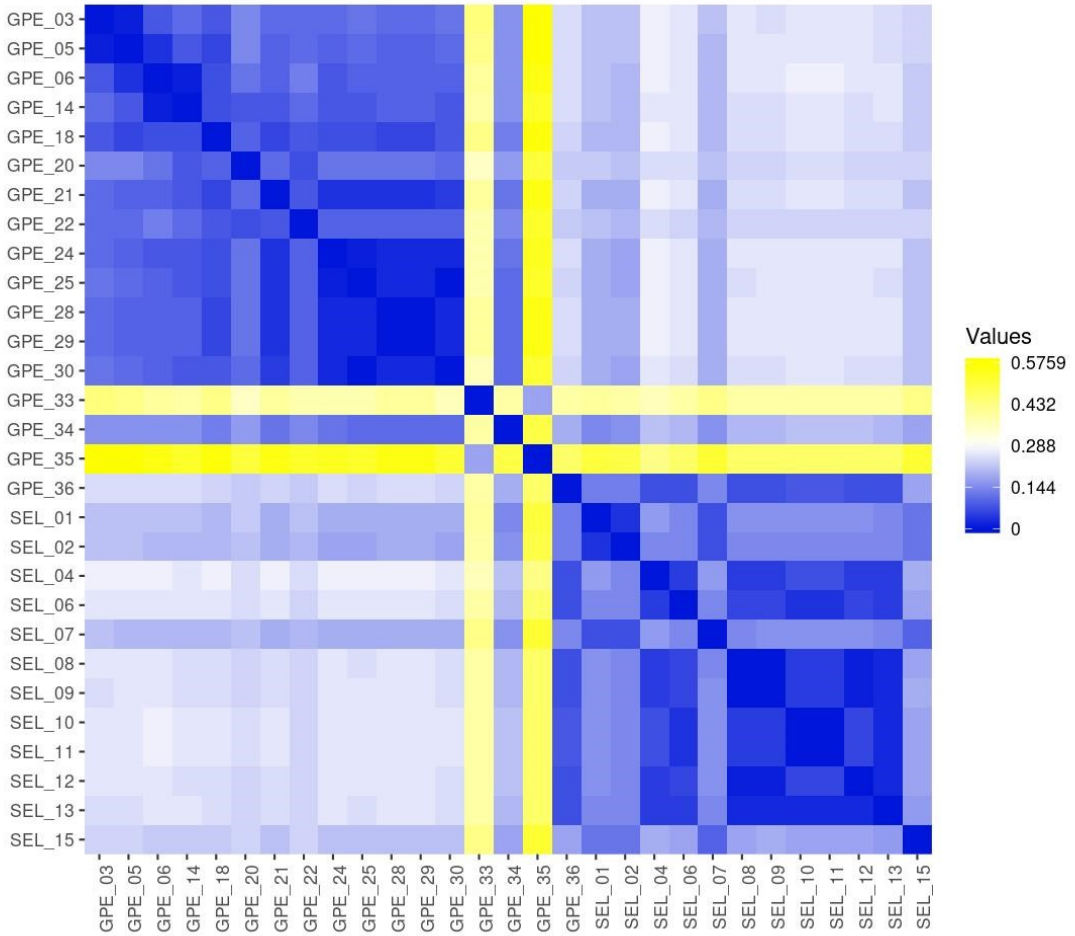

Figure 3. Pairwise distance matrix of *Echinopora lamellosa* colony visualized in a heatmap. Heatmap values show the differentiation scale.

entire *E. lamellosa* of Lombok waters (0.9770) were classified as very high diversity.

The network pattern analysis shows an indication of demographic expansion forming a large coral population. Both major clades (A and B) comprise both eastern and western colonies. This may suggest no obvious geographical differences in the distribution of haplotypes. Clade A consists of 13 haplotypes, clade B consists of 9 (nine) haplotypes, and clade C comprises only a single haplotype belong to the eastern population. This population genetic structure is also supported by the minimum spanning network, which indicates a very close relationship among the three clades (Figure 4A). Minimum spanning network shows that the GPE02, which belongs to clade A and the eastern group, formed the center of a star-like network. Haplotype 1, which belongs to the eastern group, was put at the center of the haplotype network (Figure 4B). It suggests that the *E. lamellosa* from Lombok waters had experienced a demographic expansion. All network analysis confirms a possibility of genetic connectivity among all clades and haplotypes.

Despite the close genetic connectivity among all clades and haplotypes, AMOVA revealed a significant difference in population genetic between the two locations (Table 1). Genetic variation among groups was 7.76%, but variation within a group was very high, reach up to 92.33%. The fixation index (Fst) was 0.07667, which is classified in a low category (see Excoffier et al. 1992) and indicates a weak genetic relationship between the two populations.

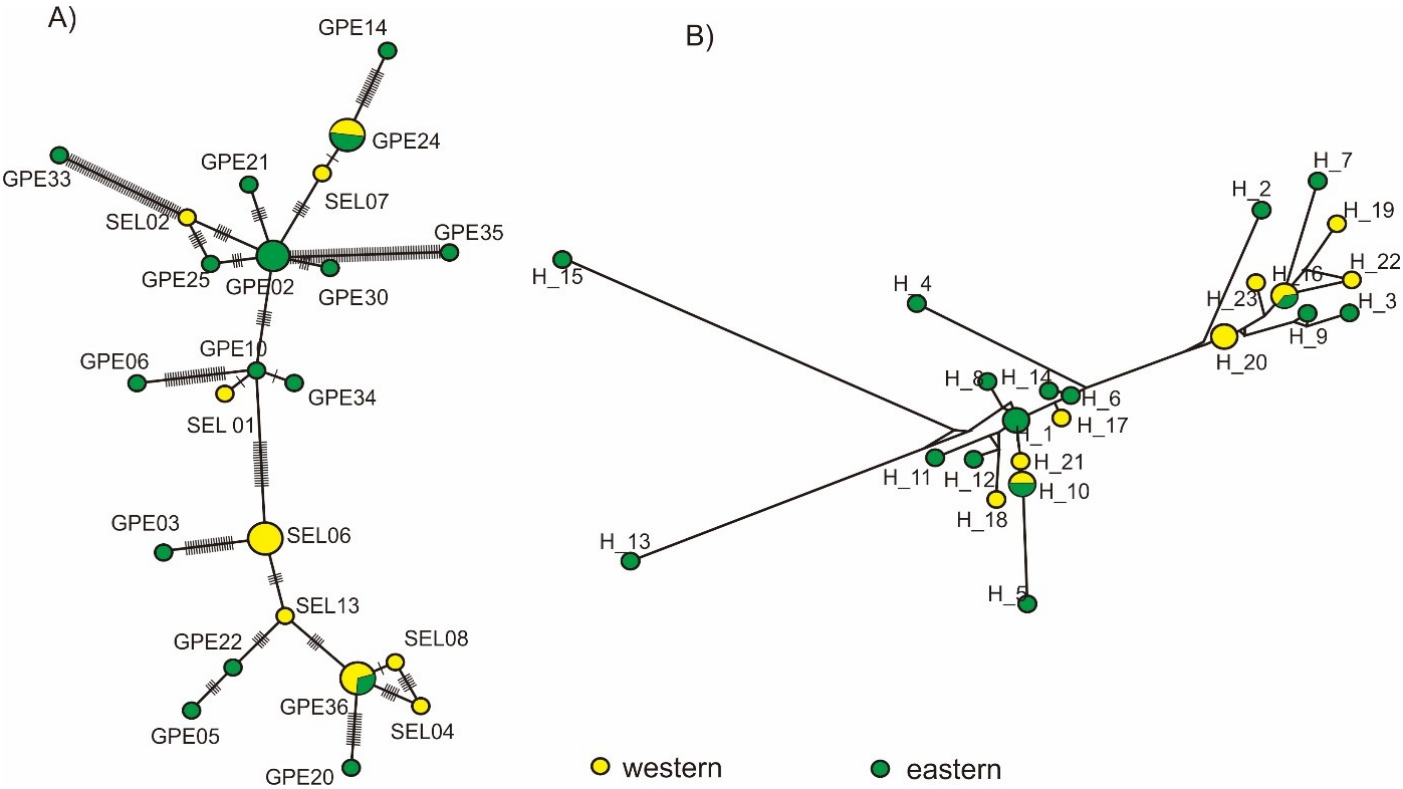

Figure 4. A) Unrooted minimum spanning network shows the genetic relationship among the population of *E. lamellosa*. B) Haplotype network illustrates the haplotype relationship within the *E. lamellosa* population.

Table 1. The AMOVA table revealed two different populations of the coral *E. lamellose*.

Source of Variation	d.f.	Sum of Squares	Variation Components	Variation Percentage
Among group	1	35.644	1.34794 Va	7.67%
Within-group	28	454.556	16.23413 Vb	92.33%
Total	29	490.200	17.58207	100%
			Fst	0.07667
			P-value	0.04985±0.00696

DISCUSSION

The present study revealed that the coral *E. lamellosa* in the western and eastern of Lombok waters are two different populations. This indicates that the geographical features separating the western and eastern waters of Lombok Island are a sufficient key barrier to their gene flow. This result supports a previous study by Nakajima et al. (2010), that corals within very close proximity could have small but significant genetic differences. Several studies suggest that in some cases, the geographical distance may not be correlated with the degree of genetic variation or divergence (Miller & Ayre 2004; Magalon et al. 2005). Oceanographic conditions and coral reproduction type seem to be more crucial in determining the genetic diversity and connectivity (Hemond & Vollmer 2010; Richards & van Oppen 2012; Peluso et al. 2018), rather than geographical distance and spatial factors.

Both western and eastern populations of *E. lamellosa* have high haplotype diversity. Although there is no haplotype diversity data on the pre-bleaching population, such high haplotype diversity indicates that there are no selection processes that recently worked in both western and eastern coral populations. The present population of *E. lamellosa* is a mixture of old colonies and post-bleaching re-colonization. The coral *E. lamellosa* was

recorded as one of the surviving species in the western population during the 2016 bleaching (Bachtiar et al. 2016). In the eastern population, coral bleaching in 2016 did not leave any noticeable impact when authors (IB and MIAG) dived to collect samples in 2018.

The present study also revealed that both western and eastern populations shared clades indicating the existence of gene flow between the two populations. Gene flow plays a major portion in determining the genetic distance of both intra- and inter-population. The Heatmap distance matrix shows that there is a restricted flux of genes between the two populations. Following genetic distance data, the AMOVA suggested a weak genetic relationship between the two populations. The genetic relationship between the two populations likely only occurs in the source reefs that supply planula larvae to both populations. The oceanographic condition of the two populations, which only have surface current from the Indonesian Throughflow (ITF), suggests that there are no source-sink interactions between the two populations. All ITF flow southward all year long in both the Lombok Strait (western population) and the Alas Strait (eastern population) (Sprintall et al. 2009).

Both coral populations are likely to be the sink of coral planula larvae drifted by the ITF from northern reefs in the Flores Sea or Makassar Strait. Previous studies suggest that the ITF contributed as the main transporter for larval dispersal across the coral triangle area. Bachtiar (2001b) reported similarity in spawning times between acroporid corals in Lombok Strait and Dampier Archipelago of Western Australia (Babcock et al. 1994) and hypothesized genetic connectivity from larval flow carried away in the ITF. More recent studies supported the hypothesis that acroporid corals in the Spermonde Islands (Makassar Strait) and Bolinao-Andara reef of the Western Philippines spawn at the same months as corals in the Lombok Strait (Yusuf et al. 2013; Gomez et al. 2018). Coral spawning in the Dampier Archipelago suggested that spawning synchrony is inherited from its ancestor in the tropical north (Babcock et al. 1994). Monismith et al. (2018) revealed that long-competence coral larvae may travel up to 1700 km in 100 days. Looking at the potential larval flow from northern reef sources, the recovery of both eastern and western groups was probably depending on the recolonization of larvae brought on by the ITF from common larval sources. This should explain why there were some common haplotypes (haplogroup) and clade variation shared between the two populations, with an expanded demographic trend. The post-settled corals then develop locally, resulting in restricted gene flow and differentiated populations geographically with the low genetic relationship among them.

The present study suggests that the two coral populations are mainly established from larval recolonization instead of the fragmentation of the surviving corals. The high genetic diversity values indicate the absence of the bottleneck effect. Such cases have been reported in the coral population of *Seriatopora hystrix* in the Flinders Reef (Noreen et al. 2009) and *Porites lutea* in the South China Sea (Huang et al. 2018). Both populations of *E. lamellosa* show higher genetic diversity values than other coral populations from Sulawesi waters and the eastern Lesser Sunda Islands (Jompa et al. 2020; Rosser et al. 2020). Several studies suggest that recolonization from long-distance source reefs contributes to relatively greater genetic diversity value in a recently established coral population (Starger et al. 2010; Aurelle et al. 2020; Smallhorn-West et al. 2020). The long-distance dispersal in corals can only be achieved by sexual reproduction with broadcast spawning mode.

Sexual reproduction improves genetic variation in a population through gene recombination between participating gametes. *E. lamellosa* is a hermaphrodite broadcast spawner (Petersen et al. 2007), but it has also a high

potential to have asexual reproduction through clonal fragmentation. The coral *E. lamellosa* has a thin plate-form colony that can easily break off by physical forces from wave, anchors, or divers. It was expected therefore that many colonies of the population possibly generated from clonal fragmentation. This study revealed a very high overall haplotype diversity score that indicates a sexual reproduction mode was predominant in the recruitment mechanism (Boulay et al. 2012; Schweinsberg et al. 2017). It is also found that *E. lamellosa* from western and eastern populations were mostly consisting of unique genotypes with no clone identified in our dataset, which supports the prevalence contribution of sexual reproduction mode for both populations.

The present study rejected the hypothesis stated in the introduction that there is an association between coral genetic diversity and its bleaching resistance. Coral genetic diversity cannot explain the resistance of the coral *E. lamellosa* in Lombok waters. The haplotype diversity between the two populations is only slightly different (0.9804 versus 0.9394). It is not very convincing therefore that such a small difference solely results in the ecological difference in coping with thermal stress. In a previous study, the algal symbiont of the two populations of *E. lamellosa* neither provide any explanation about the coral resistance to bleaching (Bachtiar et al. 2019), although algal symbiont has been reported by many authors to affect coral resistance to bleaching (Wall et al. 2020). Bachtiar et al. (2019) found that the algal symbiont of the corals in the eastern population consists of clade B and C, while the western population only has clade C symbiont. Both clades B and C symbionts are non-resistant to bleaching. Loram et al. (2007) reported that the clade B symbiont only provides minor physiological benefits that may aid in their host survival. Neither diversity of coral nor its symbiont has an association with the resistance of *E. lamellosa* to coral bleaching. Likely, the survival of coral *E. lamellosa* in Lombok may not depend on the existence of adaptive symbionts but rather depends on the other environmental factors.

The environmental setting may explain the resistance of *E. lamellosa* in the eastern population, but not in the western population. Both populations have different environmental complexities with the eastern population have an advantage, that likely serves as coral refugia. Gili Petagan in Eastern Lombok waters is a key entirely covered by mangroves and surrounded by two other mangrove-covered keys, Gili Sulat and Gili Lawang. High survivability of corals are often found within or close to coastal vegetation, such as mangrove forest or vast seagrass bed (Camp et al. 2016; Lord et al. 2020). Mangrove vegetation not only exports litter to its adjacent ecosystems but also disperse dissolved tannin (López-Portillo et al. 2017; Stewart et al. 2021). The dissolved tannin and other chromophoric dissolved organic matter (CDOM) derived from marine plants may protect corals under the stress of water temperature from excessive ultraviolet radiation (UV-R) exposure and act as a pH buffer during the bleaching (Kellogg et al. 2020). Such a mechanism may provide coral refugia that increase coral survival from bleaching. In contrast, coral in the Gili Anyaran of the western population is about 7 km away from the mangrove area, and 5 km from seagrass beds.

The resistance to bleaching of the coral *E. lamellosa* is likely related to its holobiont and trophic strategy. Coral *E. lamellosa* has been reported as moderately susceptible to bleaching, in the Great Barrier Reefs (Marshall & Baird 2000). Recent studies are accumulating to see the importance of the coral holobiont to the physiology of the coral, including its thermal stress tolerance (Boilard et al. 2020). Holobiont is a consortium of coral, algal symbiont, and microbiota. The microbiota is composed of prokaryotes, eukaryotes, and viruses. Coral susceptibility to bleaching is dependent on the robustness of the complex mutual relationship within the coral holobiont

(Bourne et al. 2016; Boilard et al. 2020). Coral trophic strategy may also explain the resistance of *E. lamellosa*. Corals may be autotrophic, heterotrophic, or mixotrophic. Conti-Jerpe et al. (2020) showed that autotrophic corals are more vulnerable to bleaching than heterotrophic corals. So far, there is no information about the trophic strategy of the coral *E. lamellosa*. Future studies should address the trophic strategy to obtain a more practical assessment method to determine the resistance of coral taxa to bleaching.

CONCLUSION

In conclusion, neither coral- nor symbiont- genetic diversity may explain the resistance of the coral *E. lamellosa* to bleaching. The genetic of *E. lamellosa* showed high diversity at either western or eastern Lombok waters. Population genetic connectivity between them may occur at the larval source. The two populations both survived well from the 2016 coral bleaching, regardless they have B and C clades symbiont. Both populations likely achieve their resistance to bleaching by adopting a robust holobiont or suitable trophic strategy.

AUTHORS CONTRIBUTION

I.B. designed the study. M.I.A.G. carried out the laboratory work. I.R. and B.H.A. analyzed the data. I.B. and M.I.A.G. collected samples and wrote the manuscript. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that they do not competing for interest to submit the manuscript to the *Journal of Tropical Biodiversity and Biotechnology (JTBB)*.

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Review Article

Important Role of Mycorrhiza for Seed Germination and Growth of *Dendrobium* Orchids

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ABSTRACT
Indonesia is a tropical country that has natural forests and is suitable for orchid species habitat, leading to more than 5,000 species of orchids grow. The tropical area is the main distribution centre for epiphytic orchids, one of which is *Dendrobium*, which grows more than 1,000 species throughout the world. Orchid seeds are very small and do not have an endosperm, making germination difficult in their natural habitat. Mycorrhizal association with orchids plays a role in the survival of orchids in nature through seed germination and growth. This study aims to provide a deeper understanding about the important role of mycorrhiza in seed germination and growth of *Dendrobium*. The mechanism of mycorrhizal association with orchids begins with the initial contact of the fungus with the orchid, hyphae enter the cortex cells to form peloton, peloton lysis, and exchange of nutrients occurs. Orchid mycorrhiza that mostly found groups in *Dendrobium* are *Rhizoctonia* (*Epulorhiza*, *Tulasnella*, *Rhizoctonia*). Mycorrhiza plays a role in increased secretion of phytohormone and enzyme activity which supports seed germination and growth of orchids. Specific mycorrhizal data on orchids can be used as an effort for in-situ and ex-situ conservation of Indonesian orchids, including *Dendrobium*.

Keywords: conservation, *Dendrobium*, orchid mycorrhiza, seed germination

INTRODUCTION
Orchidaceae is one of the largest family of flowering plants including more than 28,000 species in 880 genera (Fochiet al. 2017; Xing et al. 2017). Orchids are distributed worldwide both in tropical and subtropical areas with different habitats. *Dendrobium* is one of the epiphytic orchids commonly found in Indonesia and there are more than 275 species (Xing et al. 2013; Juswara et al. 2016).

Several species of *Dendrobium* in Indonesia are *Dendrobium lineale* (from Papua), *Dendrobium phalaenopsis* (from Larat Island, Southeast West Maluku), and *Dendrobium nobile* which widely distributed in Indonesia. The flowers are beautiful and can withstand flowering for a long time, thus have high economic value and often taken freely from nature causing population decrease of orchid species, even *D. lineale* categorized as endangered orchid species (Chadburn & Schuiteman 2018). The diversity of *Dendrobium* orchid continued to decrease in nature as a result of overexploitation and was not balanced with conservation effort (Kusmana & Hikmat 2015).

The unique trait of orchid is the seeds are very small (microscopic) so inside one fruit there are millions of seeds. Although one fruit contains a large number of seeds, only 0,2-0,3% could be germinated in nature, and the rest died. Besides that, orchid seeds usually do not have an endosperm and only contain a small amount of complex carbohydrates as nutrient sources for seed germination. Thus, germination is difficult to occur in their natural habitat. Orchid seeds require symbiosis with mycorrhiza in order to change complex carbohydrates into small molecules that initiate germination and growth (Chen et al. 2014; Yeh et al. 2019; Kaur 2020).

All orchid species are myco-heterotrophic at certain stages during their life cycle. Several specific mycorrhizas were symbiosis with orchid roots or seeds. Mycorrhizal associations were formed during the entire life cycle of orchids, provide nutrients, sugar, and minerals, but some orchids species are also known to form mycorrhizal associations only when plants under stress (Kaur 2020). Furthermore, the study about mycorrhizal identification is important in order to obtain specific mycorrhizal species in orchids that increase germination and growth either in the natural habitat or in vitro culture, as conservation effort for Indonesian orchid species including *Dendrobium*.

Epiphytic orchids are the largest group of class in Orchidaceae, mainly *Dendrobium,* however, there are not many kinds of researches focused on orchid mycorrhizal association (Dearnaley et al. 2012). This review aims to provide a deeper understanding of the mechanism of mycorrhizal association with *Dendrobium* orchids, identify mycorrhiza on *Dendrobium*, and the role of mycorrhiza in seed germination and growth of *Dendrobium*.

MYCORRHIZAL ASSOCIATION MECHANISM WITH ORCHIDS

The symbiosis of mycorrhiza and orchids has a mutual relationship. Mycorrhiza provides minerals, such as nitrogen, phosphor, and potassium for plants, and plants provide carbon (photosynthetic products) to mycorrhiza (Garcia & Zimmermann 2014; Hijri & Bâ 2018). Availability of more mineral sources will increase growth. In general, fungi also play a role in increasing tolerance to abiotic stresses, such as inundation, salinity, temperature, and biotic stresses, including pathogens and insects (Yeh et al. 2019).

Orchid seeds are very small and do not have an endosperm (Fochi et al. 2017; Xing et al. 2017), hence seed germination carries out a symbiosis process with mycorrhizal fungi (Xing et al. 2013, 2017; Qin et al. 2020; Wu et al. 2020). Orchids germination requires mycorrhiza to get nutrients and organic carbon (Fochi et al. 2017). Orchid seedlings that have not yet carried out the photosynthetic process get a supply of carbohydrates and nutrients from mycorrhiza.

Association between *Dendrobium* seeds and their symbionts begins with fungal invasion in the first week during the imbibition stage of water throughout the testa (tissue of death cell layer which encapsulated embryo). Hyphae enter through the posterior end of the embryo suspensor cell then invaded cortex cells and form the peloton. Peloton is formed in phases 1-3. Peloton is intracellular hyphae coiled inside cortex cells that contain accumulated organic materials such as protein, glycogen, and lipid because of nutrient absorption from the soil. The plant will absorb the organic materials and peloton degraded. Hyphae degenerate after protocorm formation, usually in phases 4-5, leaving the empty hyphae (Chen et al. 2014; Soelistijono et al. 2020).

Mycorrhizas enter embryo cells through the posterior end and then invaded several layers of basal cells during stage 1. Not all embryo cells can be colonized by fungi especially the meristem area. Along with embryo

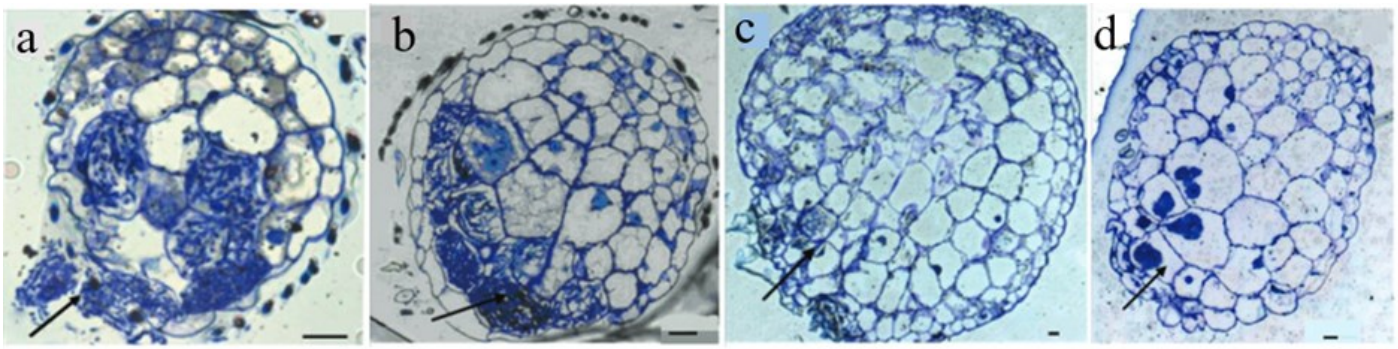

Figure 1. Mycorrhizal association during seed germination of *D. officinale*. a. Stage 1, fungal invasion at embryo basal cell. b. Stage 2, peloton formation. c. Stage 3, peloton degeneration along with protocorm formation. d. Stage 4, hyphae remaining forms clumps and peloton fully degraded. Bar: 20 μm (Chen et al. 2014).

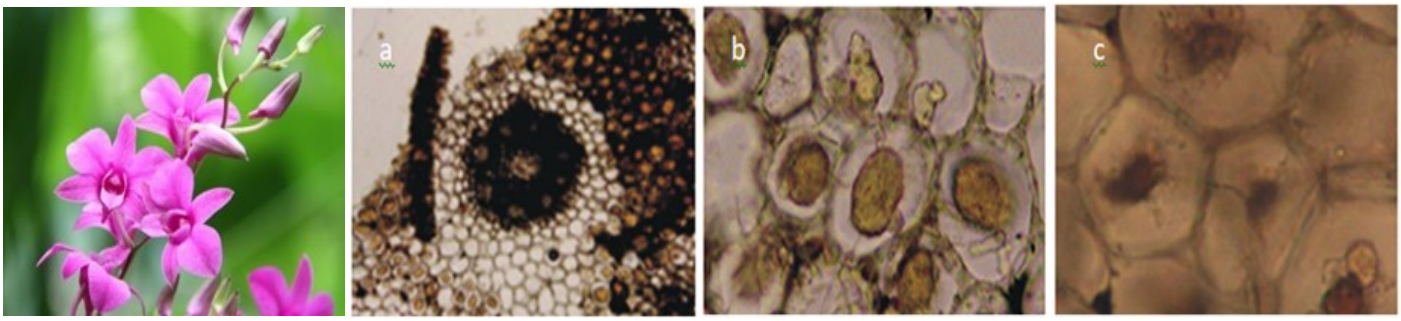

Figure 2. The *D. phalaenopsis* orchid (Hoeve 2020) and transverse slices of orchid roots. a. Fungal invasion in the parenchyma cells of the cortex. b. The peloton is connected to each other with the peloton in another cell. c. Lysed peloton (Kaur 2020).

development, fungi spreads from cell to cell until the basal the area is fully colonized. Fungal hyphae form pelotons in the outer cortical cell on entrance into the embryo cell (stage 2). Pelotons have degenerated in the inner cortical cell (stage 3). Mostly digested pelotons form clumps in stage 4 as shown in Figure 1 (Chen et al. 2014).

Initial contact of fungi mycorrhiza in adult orchid plants occurs during penetration through root hairs. Fungi mycorrhiza enters parenchymal cortex cells and hyphae coiled forms peloton. Intact peloton activity provided nutrients and mediated the transport between the orchid and mycorrhiza. The peloton is connected to each other with the peloton in another cell. Peloton then lysis and nutrient released (Figure 2) (Kaur 2020).

The mycorrhizal invasion caused biochemical and cytological changes in cells. The newly colonized cells by mycorrhiza have a large amount of starch in contrast, cells with degraded peloton only have a small amount. Peloton degradation caused changes in structural and functional cells. Nucleus cells with degraded peloton observed have differentiation into bigger size with increase of DNA content inside (Kaur 2020).

Mycorrhizal association in orchid can be distinguished into two types of the host cell as digestive cells and host cells. Digestive cells play a role in forming intact peloton then continued to degrade and again reinvasion. Host cells have hyphae that active in nutrient exchange and peloton not yet degraded or were not ready to degrade yet. This model of nutrient exchanges is called phytophagy. This model involves fungal lysis inside the cells. The fungal part that degraded is the tip area (growing end), then released the cell amount into interspace area between plant cells and hyphal membrane which later would be used by plant (Kaur 2020).

Fungi enter through suspensors or rhizoids, depending on the type of orchid and associated mycorrhizal (Yeh et al. 2019). Hyphae enter through

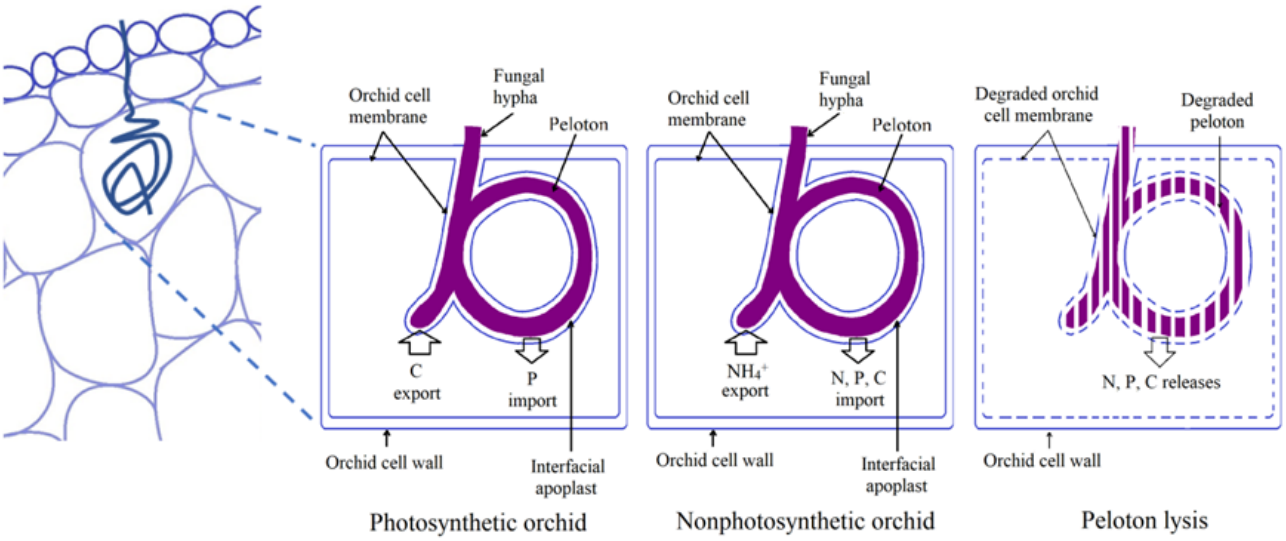

Figure 3. Nutrient exchange model of mycorrhizal association on photosynthetic orchid, non-photosynthetic orchid, and peloton degradation (Dearnaley & Cameron 2017; Yeh et al. 2019).

the posterior end of the embryo in relation to the structure of the suspensor cell which consists of elongation of cells and dead cells, thereby reducing colonization resistance (Chen et al. 2014). The incoming hyphae will form a peloton. The plant-fungus symbiosis occurs where the fungus provides a source of minerals, and the plant provides a carbon source for the fungus. The orchid-fungus symbiosis is unique since the nutrient transfer does not occur bidirectionally at the early stage of growth and in some adult plant species. Most of the orchids have photosynthetic abilities, whereas more than 100 species are myco-heterotrophic, which do not have chlorophyll during their life cycle. Partial mycoheterotrophy has two growth stages, namely chlorophyllous and achlorophyllous, thus it can survive in a shaded environment where the ability of photosynthesis is reduced (Yeh et al. 2019).

Nutrient exchange on photosynthetic orchid is different from the non-photosynthetic one (Figure 3). In photosynthetic orchid, the plant gives carbon to mycorrhiza and in exchange, the plant receives phosphor from the symbionts. Amino acid transport from mycorrhiza to plant stopped because plants need only inorganic material source. This action is regulated by fungal amino acid transporters/permeases (TcAAT1, TcAAT2, TcAAT6). Mycorrhiza lysis releases nitrogen (N), phosphor (P), carbon (C) which absorb by plants however in non-photosynthetic orchid, a small amount of ammonium (NH_4^+) transported from plant to fungal symbionts. NH_4^+ produce to attract mycorrhiza so that colonized orchid seed and initiated germination. A study on protocorm *Serapias vomeracea* shows orchid received nitrogen from fungi because fungal arginase and urease genes stimulated amino acid breakdown (Dearnaley & Cameron 2017; Yeh et al. 2019).

Orchids receive N, P, C through membrane. N and C are amino acids from fungal symbionts. Mycorrhizae provide inorganic and organic material for orchids because of their inability to doing photosynthesis. Mineral N, P, C released from peloton lysis also would be absorbed by the plant (Yeh et al. 2019). Thus, explain that symbiosis mutualism of mycorrhizal association with orchid occurs in all growth either photosynthetic or non-photosynthetic stage (Dearnaley & Cameron 2017).

MYCORRHIZA ON *Dendrobium*

The association of orchids with mycorrhiza is interesting to study. Since the embryo, orchids need the help of mycorrhiza to obtain nutrients during

germination in nature (Chen et al. 2014). The mycorrhizal identification studies obtained various types of specific mycorrhiza associated with orchids as described in Table 1.

Orchidaceae considered to interact largely with group *Rhizoctonia*. The form of genus *Rhizoctonia* is a polyphyletic group of filamentous fungi which have many similarities in their anamorph phase (asexual). *Rhizoctonia-like* disparate based on the number of nucleus in hyphae, anastomosis ability (hyphae fusion), and morphological character including colony colour, concentric pattern, sclerotia (joined to the mycelium), and hyphae size. Based on the number of nucleus in hyphae, *Rhizoctonia* disparate into uninucleate (one nucleus) such as *Ceratobasidium bicorne* strain, binucleate (number of nucleus 1-3) such as *Ceratobasidium*, and multinucleate (number of nucleus more than 2) such as *Rhizoctonia* sp. (Dearnaley et al. 2012; Suryantini et al. 2015).

Mycorrhizas that are commonly found in *Dendrobium* are *Epulorhiza* (11 species), *Tulasnella* (8 species), *Rhizoctonia* (6 species), and *Mycena* (5 species) (Table 1). *Epulorhiza* is the asexual form of genus *Tulasnella* which belongs to *Rhizoctonia* group (Dearnaley et al. 2012) so that the most found mycorrhiza

Table 1. Identified Mycorrhizal orchids in *Dendrobium* sp.

Orchid species	Isolated part	Mycorrhiza	Reference
Dendrobium sp.	root	*Tulasnella pinicola*	Sathiyadash et al. 2020
D. aphyllum	seed	*Tulasnella* sp.	Zi et al. 2014
		Rhizoctonia sp.	Soelistijono et al. 2020
D. candidum	root	*Mycena dendrobii*	Kaur 2020
D. crepidatum	seed	*Epulorhiza* sp.	Swangmaneecharern et al. 2012
D. chrysanthum	protocorm	Sebacinales	Chen et al. 2012
	root	*Epulorhiza* sp., *Mycena*	Kaur 2020
D. chrystallinum	root	*Tulasnella calospora*	Sathiyadash et al. 2020
	seed	*Epulorhiza* sp.	Swangmaneecharern et al. 2012
D. crumenatum	root	*Epulorhiza* sp.	Sathiyadash et al. 2020
D. dicuphum	root	*Tulasnella irregularis*	Sathiyadash et al. 2020
		Tulasnella deliquescens	
D. fimbriatum	root	*Epulorhiza* sp.	Xing et al. 2013
		Rhizoctonia sp.	Mala et al. 2017
		Tulasnella calospora	Soelistijono et al. 2020
		Tulasnella deliquescens	
D. findlayanum	seed	*Tulasnella deliquescens*	Mala et al. 2017
		Epulorhiza sp.	
D. friedericksianum	root	*Epulorhiza* sp.	Sathiyadash et al. 2020
		Tulasnella calospora	
D. hancockii	protocorm	*Epulorhiza anaticula*	Liu et al. 2010
D. lasianthera	root	*Rhizoctonia* sp.	Soelistijono et al. 2020
D. lindenii	root	*Tulasnella* sp.	Soelistijono et al. 2020
D. lineale	root	*Rhizoctonia* sp.	Soelistijono et al. 2020
D. nobile	protocorm	*Epulorhiza* sp.	Chen et al. 2012
	root	*Epulorhiza* sp., *Mycena* sp.,	Zhang et al. 2012; Xing et al. 2013;
		Rhizoctonia sp.,	Kaur 2020
		Mycena orchidicola	
D. officinale	root	*Mycena dendrobii*	Liu et al. 2010; Zhang et al. 2012;
		Mycena sp.	Sathiyadash et al. 2020
		Epulorhiza sp.	
D. sinense	root	*Mycena orchidicola*	Xing et al. 2013
D. speciosum	root	*Tulasnella* sp.	Sathiyadash et al. 2020
D. phalaenopsis	root	*Rhizoctonia* sp.	Soelistijono et al. 2020
D. pulchellum	seed	*Epulorhiza* sp.	Swangmaneecharern et al. 2012

belongs to genus *Rhizoctonia*.

Based on anastomosis ability, *Rhizoctonia* multinucleate diverse into 14 anastomosis groups (AG), *Waitea circinata, Rhizoctonia globulis,* and *Tulasnella* sp. (orchid mycorrhiza). *Rhizoctonia-like* binucleat dispersate into AG 1- AG 13, *W. circinata* var. *zeae* and var. *circinata*, member of AG A – AG except for AG BI, *Ceratobasidium cerealis, C. ramicola*, *Epulorhiza repens,* and *E. calendulina* (Dearnaley et al. 2012; Suryantini et al. 2015).

Rhizoctonia-like associated with orchids are from taxa Sebacinales, Ceratobasidiaceae, and Tulasnellaceae (class Agaricomycetes). Fungi can distinguish in anamorph or teleomorph form. Anamorph phase is an asexual form that appeals when fungi formed spores. Teleomorph phase is a sexual form that appeals when fungi using nuclei fusion to reproduce. Species anamorph *Epulorhiza* sp. (also known as *Rhizoctonia repens*) have teleomorph form *Tulasnella* sp. (Dearnaley et al. 2012; Suryantini et al. 2015).

MYCORRHIZA ROLES ON SEED GERMINATION AND GROWTH OF *Dendrobium*

Many studies observed the influence of mycorrhiza in seed germination and growth of orchids. Mycorrhiza that identified from *Dendrobium* orchid known as their role to initiate seed germination and growth of orchid including *Rhizoctonia*, *Tulasnella, Epulorhiza*, and *Mycena*.

Rhizoctonia produced niacin that increased germination and growth (Soelistijono et al. 2020). *Rhizoctonia* also increased seed germination up to 44-91% and advanced protocorm growth (Jiang et al. 2015). *Tulasnella* increased seed germination, protocorm formation, and orchid seedling development (Zi et al. 2014; Zhang et al. 2020; Freitas et al. 2020). *Tulasnella* has a role to stimulated growth and metabolism in *Dendrobium* (Wu et al. 2020). Roots with the addition of *Mycena* inoculum show change in morphology and has a bigger size. Mycorrhiza was isolated back and showed the same traits as the original inoculated strain (Zhang et al. 2012). *Epulorhiza* also increased the growth of *D. aphyllum* seedling. This shows that mycorrhiza and orchid have a specific relation at different growth phases.

Orchid mycorrhiza colonization increased both vegetative and generative growth in orchid, increased plant defense, initiate early flowering, increased flower quality, and reduced seed abnormality. Colonization of *Tulasnella repens* increased plant biomass. Colonization of *Epulorhiza* sp., *M. dendrobii*, and *M. anoectochila* on *D. nobile* increased height and plant biomass. Colonization of *D. officinale* roots by *Mycena* sp. increased plant height, biomass, and the number of shoots (Sathiyadash et al. 2020). *Epulorhiza* sp., *Mycena* sp., Tulasnellales, Sebacinales, Cantharellales increased nutrient intake in plants and increased seed germination on *D. nobile* and *D. chrysanthum*. Colonization of *M. dendrobii* on *D. candidum* helps growth with phytohormone secretion (Kaur 2020).

Mycorrhiza as symbionts has a role in widening surface area and increased intake ability of water and minerals in epiphytic orchid which has limited source access (Dearnaley et al. 2012). Mechanism of symbiont in seed germination and plant growth are provided nitrogen, phosphor, potassium in orchid also with secreting phytohormone indole-acetic acid (IAA), indole-3-acetonitrile, gibberellic acid (GA), zeatin, zeatin riboside and naphthalene acetic acid (NAA) and increased enzyme activity such as chitinase, β-1,3-glucase, phenylalanine ammonia-lyase, and polyphenol oxidase (Liu et al. 2010; Sathiyadash et al. 2020; Kaur 2020).

Seed germination of orchids needs mycorrhizal symbiosis. Orchid seeds are very small and do not have endosperm, thus germination is difficult to occur in their natural habitat. Orchid seeds required symbiosis with mycorrhiza to initiate germination (Chen et al. 2014; Yeh et al. 2019). The

nutrients from the peloton helped the embryo development process since orchid seeds did not have an endosperm. Embryonic development would form sprouts and produced new plants (Yeh et al. 2019). The symbiosis of mycorrhiza and orchids for seed germination was key for seed-based orchid conservation. It is very important to conserve over-taken orchids, for example, many of *Dendrobium* species (Wu et al. 2020).

Dendrobium is the largest epiphytic orchid genus in the world and Indonesia. Mycorrhiza is important for the survival of orchids in nature for seed germination and growth. Specific mycorrhizal data on orchids can be used as a basis for increasing seed germination and growth of *Dendrobium* in their natural habitat as well as in vitro culture. It can be an effort to conserve Indonesian orchids and alternative information for the public regarding the handling of problems in orchid cultivation, especially *Dendrobium* in order to encourage the progress of orchid species cultivation quickly with good quality.

CONCLUSION

Orchidaceae naturally depend on mycorrhizae during their lifecycle. Fungi and orchid form symbiosis in several developmental stages including both non-photosynthetic and photosynthetic phases. The mycorrhizal association plays a role in orchids survival through seed germination since its seed lacks endosperm and improves plant development. The initiative begins with contact between fungi and orchid followed by hyphae entering cortex cells. Hyphae coils and form peloton in cells thus initiate the exchange of nutrients between fungi and plant cells. Mycorrhiza provides nutrients, sugar, and minerals for plants, and as exchange photosynthetic orchids would give carbon, however, non-photosynthetic orchids transported small amounts of NH_4^+ to fungal symbionts due to their inability in photosynthesis. Peloton lysis and other hyphae would enter cells for reinvasion. Largely interact with group *Rhizoctonia*, mycorrhizal colonization influences seed germination, plant height, plant biomass by widening surface area and increased ability of water and minerals intake, also secreting phytohormone and increased enzyme activity.

AUTHORS CONTRIBUTION

B.T., T.F., and C.L.S. searched the literatures and wrote manuscript, E.S. supervised all the process.

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CONFLICT OF INTEREST

There is no conflict interest regarding this research and research funding.

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