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Short Communication

Local Adaptation of Invasive Plant, *Synedrella nodiflora*, in Urban Tropical Lowland Landscape, Universitas Indonesia

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ABSTRACT
Synedrella nodiflora is an invasive species that originated from tropical America and now has spread throughout Indonesia. We analysed the ability of *Synedrella nodiflora* from the level of HSP70 gene expression at different heat stress in urban tropical lowland landscape Universitas Indonesia. We used the qPCR to quantify the level of HSP70 gene expression and analysed using the Pfaffl model. We found the level of HSP70 gene expression got higher related to elevated temperature from 29°C to 39°C with a range of fold from 123.1 to 1676.9. This ability reflects the adaptive plasticity of *Synedrella nodiflora* in the course of the invasion process.

Keywords: *Synedrella nodiflora*, adaptation, HSP70, qPCR, Pfaffl model

Phenotypic plasticity is defined as the capability of a certain genotype to express a phenotype under different environmental conditions. The variation of genotype support on the different development stage of plants. Therefore, it is important for the adaptation of plants (Bradshaw 2006). In the case of plant invasion, the variation of genotype should not be measured as a critical factor for invasion. The plasticity should be measured at the phenotypic and molecular level because it is likely to persist in changing environments (Gratani 2014). As an example, *Synedrella nodiflora*, which has a wide distribution in Java, showed low genetic differences (Susanto et al. 2018). The fixation of genetic variation caused by the evolution of plasticity mechanism as a result of local adaptation. These mechanisms allowing to produce different phenotypes from a single genotype (Schlichting & Smith 2002).

The remaining tropical lowland urban forest in Indonesia is limited. One of the representatives is Universitas Indonesia Campus Depok, located in two cities, Jakarta and Depok. The university landscape has six reservoirs and an urban forest representing a green and sustainable campus (Sheherazade et al. 2017; Anis et al. 2018). The campus also has well-documented data about plant communities, included family Asteraceae. Many studies have been conducted about Asteraceae, such as species diversity (Oktarina & Salamah 2017), chromosome variations (Salamah et al. 2018), and pollen morphology (Salamah et al. 2019).

Synedrella nodiflora is a member of the family Asteraceae that originated from tropical America. Found firstly in Java in 1888 and now has spread throughout Indonesia (Kostermans et al. 1986). *Synedrella nodiflora* is known as the dominant invasive weed in the urban ecosystem. The allelopathic activity from this species reduced the chance of other species in the surrounding to survive (Ghayal et al. 2010). Humans' continuous disturbance also promotes many plants weeds' response to a new environment and becomes invasive (Mooney & Cleland 2001). Plant invasion depends on the response with the flexibility to changing environment. Phenotypic plasticity can be advantageous for plants because it may increase adaptive response (Liu et al. 2016).

Climate changes influence the distribution of plants with thermotolerance because it allows for photosynthesis during periods of high temperatures (Godoy et al. 2011). Plants have evolved by regulating thermotolerance protein that responds to an elevated temperature that minimizes damage and ensures protection of cellular homeostatic (Kotak et al. 2007). Heat shock proteins (HSPs) level is different in accumulation in organisms adapted to heat and can reflect the local adaptation or acclimatization (Moseley 1997). The increased level of heat shock protein 70 (HSP70) showed better survival in invasive species (Hammann et al. 2016).

The present study was undertaken to address how the local adaptation of *Synedrella odiflora* in urban tropical lowland landscape Universitas Indonesia regarding different temperature stress by measuring the level of Hsp70 gene expression.

Young leaves from *Synedrella nodiflora* with ± 2 cm in length were collected from several locations at UI Depok Campus from September to November 2018. The samples were collected from 11:00 to 13:00 pm. The temperature was recorded to find the minimum, maximum, and average temperatures. We also planted the *Synedrella nodiflora* at room temperature as a control. The leaf samples were placed in a 50 ml centrifuge tube with 15 ml of NAP buffer (Camacho-Sanchez et al. 2013) before RNAs extraction.

Total RNA isolation was carried out using the modified cetyltrimethylammonium bromide (CTAB) method based on Zeng & Yang (2002). The concentration and purity of total RNA obtained were measured with a BioDrop spectrophotometer, and the total RNA was visualized with 1% agarose gel electrophoresis to check the RNA integrity. Total RNA was treated with RNase-Free DNase to remove DNA contaminants. The RNA obtained was then stored in -86°C or can be used directly in the next step.

Total RNA converted to cDNA using the RevertAid First Strand cDNA Synthesis Kit [Thermo Scientific™]. Furthermore, cDNA was amplified using QuantiNova SYBR® Green PCR Kit [Qiagen] and Hsp70 *Arabidopsis thaliana* primer based on Sung et al. (2001) with forward sequence 5'-TCAAGCGGATAAGAGTCACT-'3 (CG258F) and reverse sequence 5'-CTCGTCCGGGTTAATGCT-3' (CG259R) with targeted Hsp70 at cytosol. We performed qPCR in a total volume of 20ul comprising 1-5ul cDNA template, 10ul 2x QuantiNova SYBR Green PCR Master Mix, 1.4ul each primer (0.7uM), and RNase-Free water up to 20ul. This reaction mixture was subjected to a qPCR condition as follows: pre-denaturation at 95° C for 2 mins, 40 reaction cycles consisting of denaturation at 95o C for 5 secs, primer annealing at 55° C for 10 secs. We also put GAPDH as an internal reference gene for relative quantification with forward sequence 5'-TGAGAAGGCAGCCACCTATG -3' and reverse sequence 5'-TGCTGTCACCCTGGAAGTCA -3' (Yang et al. 2015). All the qPCR reactions ran at eco 48 qPCR machine [PCR Max].

Relative quantification of HSP70 gene expression was calculated with Pfaffl (2001) model using the equation:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}}(\text{control} - \text{sample})}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}}(\text{control} - \text{sample})}$$

The average temperature during observation from September to November 2018 at 11.00-13.00 was 34°C with a minimum of 29°C and a maximum of 39°C. We collected the young leaf of *Synedrella nodiflora* at three different heat stress that we recorded during the observation. Total we collected 134 samples of young leave and successfully extracted the RNA and got the cDNA template for qPCR analysis (Figure 1).

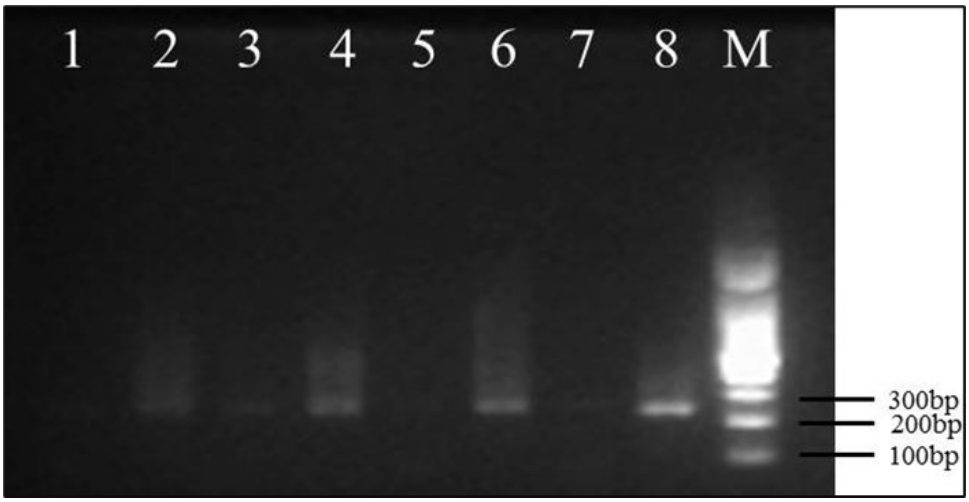

Figure 1. The electrophoresis visualization of Hsp70 amplification on cDNA template of *Synedrella nodiflora*. Line M is a 100bp marker of DNA, lines 1-8 are the representative samples.

Expression of HSP70 in *Synedrella nodiflora* has different levels related to heat stress. We found that the relative expression of HSP70 to GPADH as an internal reference gene got higher along with the temperature. At 29°C, the expression of HSP70 was 123,1 fold to the control and up to the highest at 39°C with 1676.9 fold (Figure 2).

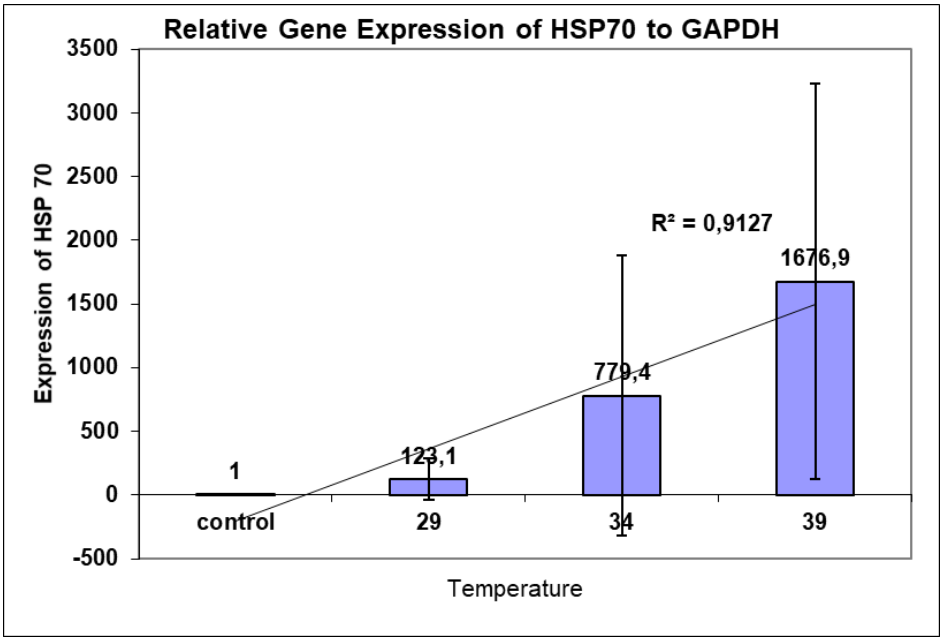

Figure 2. Expression of HSP70 in *Synedrella nodiflora* with different heat stress in urban tropical lowland landscape Universitas Indonesia.

On the basis of our result, the population of *Synedrella nodiflora* had developed local adaptation or acclimatization in urban tropical lowland landscape Universitas Indonesia. The resistance to heat shock could have evolved in the course of the invasion process—either during the transport or in the new area (Hamman et al. 2016). As we know, *Synedrella nodiflora* is a native of tropical America and now has spread throughout Indonesia (Kostermans et al. 1986). From the molecular level, heat shock proteins are the molecular chaperon that aids the refolding of macromolecules due to heat and other stressors. Induction of HSP is to be greater for invasive species relative to native (Kelley 2014).

Response to and survival of heat stress is a complex phenomenon in plants (Kotak et al. 2007). Only aware of few examples of elevated chaperon expression in non-native species or populations of terrestrial non-native organisms, based on the light heat-shock protein expression may be a more relevant determinant of invasion success in aquatic organisms than in terrestrial organisms (Hammann et al. 2016).

From our result, we provided a clear observation about the local adaptation of terrestrial plant, *Synedrella nodiflora*, in urban tropical lowland landscape with the elevated of HSP70 gene expression due to the different heat stress.

AUTHORS CONTRIBUTION

A.E.M and A.S. designed the research, analysed data, wrote the manuscript, and supervised all the process, C.K.A. and M.S. collected and running the samples.

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CONFLICT OF INTEREST

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

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Short Communication

Intergeneric Hybridization between *Phalaenopsis* 2166 and *Vanda* 'saint valentine': Characterization of Parents Using *ndhE* cpDNA Partial Sequence

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ABSTRACT
An intergeneric cross between *Phalaenopsis* 2166 and *Vanda* 'saint valentine' has successfully produced protocorms that will be grown further to form seedlings. The present study aims to genetically characterize both parents by using *ndhE* partial gene as its sequence is shown polymorphic among five orchid genera of the subtribe Oncidiinae. The results reveal that the *ndhE* partial sequences of *Phalaenopsis* 2166 and *Vanda* 'saint valentine' are considerably homologous with those of *Oncidium*. However, alignment of *ndhE* partial sequences between both parents shows only 58% similarity, leading to the conclusion that a relatively high genetic difference between them may occur.

Keywords: intergeneric hybridization, *Phalaenopsis* 2166, *Vanda* 'saint valentine', *ndhE* partial gene

Parent selection is an important initial step that determines the success of hybridization in orchids. This must involve knowledge of parental traits, particularly those of economic values such as flower size and shape of the hybrids will be produced (Widyastoety et al. 2010). For successful hybridization, maternal parents of strong and long-lasting flower buds with short gynostemium enabling pollen tubes to reach embryo sacs more readily should be selected (Hartati 2010).

Intergeneric hybridization between genus *Phalaenopsis* and *Vanda* has been reported to successfully produce seedlings with a number of characteristics seemingly showing maternal inheritance (Hartati 2010). Several species of *Phalaenopsis* and *Vanda* are commercially exploited to produce hybrids of high economic and aesthetic values (Sharma et al. 2013). *Phalaenopsis* is an epiphytic monopodial orchid with long leaves and various flower patterns, while *Vanda* is also epiphytic monopodial with flowers of sharp colour. Some *Phalaenopsis* species show long-lasting flowers with a blooming period of up to three months. On the other hand, *Vanda* flowers blossom commonly for only three weeks.

Monopodial orchids have stems of continual apical growth with little or no axillary sprouting (Johnson & Kane 2007). From an evolutionary point of view, monopodials are the most diverse developing orchids by means of

anagenesis. The members of this group show potentials in horticultural practices because they have vigorous stems and long-lasting flowers blooming for 15 – 30 days (Sharma et al. 2013).

More specifically, a cross between *Phalaenopsis* 2166 of pink flower with red spots as the female parent and *Vanda* 'saint valentine' of plain red as the male parent has also been performed resulting in hybrid fruits brought in *Phalaenopsis*. The seeds of this intergeneric cross have been grown *in vitro* and successfully produced protocorms or zygotic embryos. Then, these intergeneric protocorms will be grown to form seedlings using several *in vitro* culture media.

To predict whether the phenotypical traits of the hybrid seedlings are prone to correspond to those of *Phalaenopsis* 2166 as their female parent or not, characterization of both *Phalaenopsis* 2166 and *Vanda* 'saint valentine' genotypes using a particular marker from cpDNA is required. The *ndhE* gene is one of the sequences in the cpDNA suitable to be used as a molecular marker in orchids since the sequence is polymorphic among five orchid genera of the subtribe Oncidiinae (Wu et al. 2010). Hence, it is assumed that the sequence also shows polymorphism between *Phalaenopsis* 2166 and *Vanda* 'saint valentine', so that both genera can be genetically characterized using *ndhE* as a molecular marker. This study aims to characterize *Phalaenopsis* 2166 and *Vanda* 'saint valentine' as parents of the intergeneric hybridization using *ndhE* partial sequence.

In our study genomic DNAs were isolated from the completely developed second leaves of *Phalaenopsis* 2166 and *Vanda* 'saint valentine' following the CTAB method (Doyle & Doyle 1990). We used *Phalaenopsis* 2166 of four years old and *Vanda* 'saint valentine' of six years old. The quality and quantity of the isolated DNAs were measured using a genequant. Amplification of *ndhE* partial sequences was performed using universal primers, i.e. 5' – GCTAGCCCAATAGCTGCTTC – 3' as forward primer and 5' – TCGAAGCATGGTTAGAGCAC – 3' as reverse primer. This pair of primers have been designed using Primer 3 on the basis of *ndhE* conserved areas of three *Oncidium* hybrid cultivars available at the NCBI database, i.e. Grower Ramsey (acc. no. GU175400.1), Grower Ramsey 'Sunkist' (acc. no. GU175389.1) and Sweet Sugar 'Million Coin' (acc. no. GU175397.1). A total volume of 10 µl PCR mixture containing 2.5 µl template DNA, 5 µl Gotaq green, 2.25 µl nuclease-free water (NFW), and 0.25 µl of individual primer was subjected to PCR condition as follows: pre-denaturation at 94˚C for 3 mins, proceeded by 35 cycles of denaturation at 94˚C for 30 secs, primer annealing at 50˚C for 30 secs, primer elongation at 72˚C for 90 secs, and terminated by final elongation at 72˚C for 10 mins prior to storage at 4˚C. The PCR products were visualized in a 1.5% agarose gel using TBE buffer. These were then sent to Firstbase Malaysia for sequencing after (Sanger & Nicklen 1977) automated with terminator labelling.

Data of sequences were edited using Bioedit version 7.0.4.1 (Hall 1999) and were checked manually. The edited sequences were then examined for similarity with *ndhE* sequences available at the NCBI database using BLAST analysis. Sequence alignment was carried out with ClustalW (Thompson et al. 1994), which was also implemented in Bioedit version 7.0.4.1 (Hall 1999).

An amplicon resulting from *Vanda* 'saint valentine' sample is well visualized as an electrophoretic band of approximately 160 bp. BLAST analysis on the sequence of the amplicon shows 91% similarity with that of cpDNA of an orchid species, i.e., *Neofinetia falcata* (acc. no. KT726909.1). In addition, they also show 90% similarity with *ndhE* gene sequences of three *Oncidium* hybrid cultivars, i.e., Grower Ramsey, Grower Ramsey 'Sunkist' and Sweet Sugar 'Million Coin'. This certainly makes sense, because the *ndhE* sequences of the *Oncidium* hybrid cultivars are from which the PCR primers

used in this study have been designed. Thus, the amplicon obtained from *Vanda* 'saint valentine' is undoubtedly a *ndhE* partial sequence.

As in the case of *Vanda* 'saint valentine', the amplicon obtained from *Phalaenopsis* 2166 has also about 160 bp in length. Its sequence shows 92% similarity with *ndhE* gene sequences of the three *Oncidium* hybrid cultivars. The similarity to these sequences is even slightly higher than that of *Vanda* 'saint valentine' (i.e., 90% similarity). Thus, the amplicon of *Phalaenopsis* 2166 sample is also obviously a *ndhE* partial sequence like that of *Vanda* 'saint valentine'.

The partial *ndhE* sequences of both *Phalaenopsis* 2166 and *Vanda* 'saint valentine' have now available at the NCBI database with accession numbers of MH646649 and MH646650 respectively. The alignment of both sequences, however, shows only 58% similarity (Figure 1).

Intergeneric hybridization in plants, either naturally or artificially, are very important to evolution and speciation. It has been proven to provide beneficial significance including increased genetic diversity, improved environmental adaptation, and reproductive isolation breaking. On the other hand, it has also some detrimental effects such as genetic domination which may result in the potential extinction of original species. To assess intergeneric hybridization in plants, molecular markers could be employed along with morphological and chemical properties (Akita et al. 2021).

CpDNA constitutes a source of molecular markers commonly used to assess intergeneric hybridization in plants. Some of them are microsatellites or simple sequence repeats (SSR), the application of which in the pine family has been reviewed revealing their potentials as molecular markers in evolutionary biology of the plant family (Filiz & Koc 2014; Niu et al. 2017). Another marker widely used is *ndhE* gene, which is a member of *ndh* genes. The sequences have been proven to be polymorphic among five orchid genera of the family Oncidiinae, i.e. *Oncidium*, *Beallara*, *Odontoglossum*, *Odontocidium,* and *Zelenkocidium* (Wu et al. 2010).

The *ndh* genes are assumed to increase photosynthetic performance in fluctuating terrestrial conditions (Peredo et al. 2013). As many as 11 subunits of *ndh* genes, i.e., from *ndhA* to *ndhK*, are encoded in cpDNA of higher plants. In addition, three cyanobacterial nuclear-encoded subunits genes, i.e., *ndhM*, *ndhN,* and *ndhO*, are found in cpDNA. This indicates that nuclear *ndh* genes originated in cyanobacteria and were transferred from cpDNA to the nuclear genome during evolution. In *Oncidium* hybrid cultivar Grower Ramsey, however, all the 11 subunits of *ndh* genes are encoded in cpDNA, and it is merely *ndhE* that is translated into a functional protein. Genes that

```
P2166new        TTTCGAAGCATGGTTAGAGCACTTATG-GGTGTCTTGAACTTATACTCAA---TTCGGTT
Vanda           TTTCGAAGCATGGTTAGAGCACCAATGTGATCCCTGGAGTTTATACTGGGAATTTCGGGT
                **********************  *** * *  ** **  *******      ***** *

P2166new        AATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAATTAATTAA
Vanda           GGAATCTAAATTCGTCGCTCACAATTTTCCAATCTATGTTGATAGTCAACAATTAAAAAA
                   *** **  ****  *     ******  ** *** *********  *******  **

P2166new        AAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGA
Vanda           A----GAAATGTTATCAATCTTTGT----TATAGCC-ATTCCTGCTGTTG----------
                *    ** ** ** **********    ** ***   **   *******

P2166new        AGCAGCTATTGGGCTAGC
Vanda           ------------------
```

Figure 1. Sequence alignment of *ndh*E partial genes of *Phalaenopsis* 2166 and *Vanda* 'saint valentine'.

do not encode functional protein have usually a high evolution rate causing high polymorphism. This is also the case in *ndh* genes of *Phalaenopsis aphrodite* (Wu et al. 2010; Kim et al. 2020). It is reported that *ndhE* genes in *P. aphrodite* and *P. equestris* are not in complete condition, while *ndhA*, *ndhE,* and *ndhI* genes in *Erycina* and two varieties of *Oncidium* have the same pattern (Luo et al. 2014; Smidt et al. 2020).

Complex *ndh* genes are responsible for encoding NADH dehydrogenase, which serves as transferring electrons from NADH to plastoquinone in the cyclic electron cycle. NADH should be reduced into NADPH in the non-cyclic electron cycle in photosystem I, but when NADPH is not needed, electron is not caught by NADH. In this case, cyclic electron cycle occurs, where electron from photosystem I is caught by pheredoxin, and then is passed to cytochrome B6 complex, cytochrome f, and back to photosystem I. From this cyclic electron cycle, only ATP is produced. All the other photosynthetic elements are encoded by cpDNA genes (Wu et al. 2010; Peredo et al. 2013; Wang et al. 2013; Kim & Chase 2017; Dong et al. 2018; Zhu et al. 2019). The loss of *ndh* genes or the presence of pseudogenes has been elucidated in the genome of monocotyl plants. Degenerative genes in cpDNA of photosynthetic orchids result from the change in *ndh* gene structure. Non-functional *ndh* genes controlled by cpDNA are observed in C3 and CAM plants (Pan et al. 2012; Kim et al. 2020). The loss of genes encoded in cpDNA has no effect on the plant life cycle (Luo et al. 2014).

The absence of *ndh* genes is found in monocotyledon plants other than orchid species of *Najas flexilis* and *Petrosavia stellaris*. This loss of *ndh* genes is caused by adaptation to submerge environments. In orchids, particular types of *ndh* genes sometimes disappear, but *ndhE* can be found in some orchid species of *Oncidium*, *Cymbidium,* and *Cattleya*. Specifically, in *Dendrobium*, *ndhE* gene is found but with a deletion of 21 bp (Kim et al. 2015).

In *Phalaenopsis aphrodite ndhA*, *ndhF,* and *ndhH* genes are not found in the cpDNA, while *ndhB*, *ndhC*, *ndhD*, *ndhE*, *ndhG*, *ndh I*, *ndhJ,* and *ndhK* genes show variation (Wu et al. 2010; Sui et al. 2018; Kim et al. 2020). Then, in *P. aphrodite*, which is the ancestor of all orchids, a change in *ndh* gene control into the nuclear genome occurs. It is confirmed that *ndhE* gene is observed in *P. aphrodite* (Kim et al. 2015). In *Oncidium*, *ndhE* gene is subjected to truncation and becomes pseudogene, while in *P. equestris*, the loss of *ndhA*, *ndhE*, *ndhF,* and *ndhH* genes is reported (Jheng et al. 2012; Kim et al. 2020).

Vanda and *Phalaenopsis* are of two different genera despite the same subtribe, i.e. Aeridinae. *Vanda* is, however, known capable of hybridization with other monopodial genera, e.g. *Rhyncostylis*, *Aerides*, *Ascocentrum,* and *Phalaenopsis* (Sharma et al. 2013; Johnson & Kane 2007). Nevertheless, some intergeneric hybridizations show reciprocally different results. For instance, the relative success of intergeneric hybridization between *Renanthera imschootiana* as the female parent and *V. coerulea* as the male parent is reported, while the reciprocal crosses remain failed although both genera are monopodials (Kishor & Sharma 2009). The use of *V. testacea* as the female parent instead of *V. coerulea* shows better results (Kishor & Sharma 2008). Similarly, the percentage of pods ready to harvest is relatively higher when *Phalaenopsis* sp. are used as male parents in the intergeneric crosses of *Phalaenopsis* x *Vanda* rather than in the case of the reciprocal combinations (Hartati 2010). On the contrary, we found in our study that no pod at all was produced when *Vanda* 'saint valentine' was used as the female parent and *Phalaenopsis* 2166 served as the male parent. The occurrence of maternal inheritance was demonstrated in the case between *Phalaenopsis* and *Vanda* previously reported (Hartati 2010) so that it will be very interesting to further study the *ndhE* partial sequences of the intergeneric hybrid seedlings that will

be produced.

The relatively low similarity between *ndhE* partial sequences of *Vanda* 'saint valentine' and *Phalaenopsis* 2166 leads to the conclusion that a relatively long genetic distance between both genera may exist. This supports their taxonomical status as belonging to two different genera, implying the possibility of a heterosis phenomenon in the intergeneric hybrids that will be produced.

AUTHORS CONTRIBUTION

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

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CONFLICT OF INTEREST

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

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Short Communication

Vocalization of Western Tarsier (*Cephalopachus bancanus* Horsfield, 1821) in Bangka Island, Indonesia

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ABSTRACT

Every tarsier species performs different vocalization behaviour. *Cephalopachus bancanus* as one of the tarsier species listed as vulnerable in the IUCN red list has limited and different information about their vocalization. This research was designed to explore the species vocalization in the vicinity of Petaling Village, District of Bangka, Bangka Island, Indonesia. Tarsier vocalization inside temporary enclosures was recorded using a handy recorder and analysed using bioacoustics software Audacity 2.3.3 and Raven Pro 1.6.1. We described seven vocalization types with different functions and spectrogram patterns. One type of vocalization, squeak, is produced only by the infant. Two types of vocalizations (whistle and cheeps) were produced by the infant and adult, and four vocalization types were performed by adults. Those types of vocalizations can be heard within human hearing. Some types of vocalizations have peak frequencies at the ultrasonic level, i.e.: agonistic scream, alarm call, distress call, and hysteresis.

Keywords: Bangka, *Cephalopachus bancanus*, vocalization, western tarsier

Primates are social creatures, which communicate in several ways such as agonistic (Perreira & Kappeler 1997), grooming (Dunbar 1991), scent marking (Heyman 2006), and vocalization (Waser & Brown 1984; Cheney & Seyfrath 1996). Primates, especially nocturnal primates, use vocalization to interact with the conspecific, announce territorial boundaries or potential threats, spacing, and group coordination (Bearder 1999; Braune et al. 2005; McComb & Semple 2005; Rasoloharijaona et al. 2006; Zuberbuehler 2005). The knowledge of primate vocal behavior can be used for primate populations survey, one of which is tarsiers (Nietsch 1999; Rehakova-Petru et al. 2012).

Tarsiers are small nocturnal faunivorous native to the south-eastern part of Asia. Currently, there are three genera of tarsiers (Groves & Shekelle 2010; Shekelle et al. 2017, 2019), which was distributed in differents biogeographic region. Eastern tarsier or *Tarsius* spp. inhabit Sulawesi and its surrounding small islands, the Philippine Tarsier *Carlito* in the Mindanao Shelf, and Western tarsier *Cephalopachus* in the Sunda Shelf (Borneo, Natuna, Belitung, Bangka, and southern part of Sumatra) (Groves & Shekelle 2010).

Western tarsier *Cephalopachus bancanus* is categorized as vulnerable by the IUCN. *Cephalopachus bancanus* has four subspecies, *C.b. saltator* in Belitung Island, *C.b. borneanus* in Borneo, *C.b. natunensis* in Natuna island, and *C.b. bancanus* which inhabit Bangka Island and southern part of Sumatra. The last subspecies are categorized as endangered by IUCN (Shekelle & Yustian 2008). This intriguing nocturnal primate, including all species and subspecies, is also protected by the Indonesian Minister of Environment and Forestry Regulation No. P.106/2018, strengthening the Indonesia Government Regulatory No. 7 of 1999.

The difference in vocalization characteristics and behavior can be used as a basis for confirming the taxonomy of tarsier species (Nietsch & Kopp 1998; Burton & Nietsch 2010; Shekelle et al. 2017, 2019). Tarsiers in Sulawesi perform duet calls behavior, with each species exhibits different duet calls behavior (MacKinnon & MacKinnon 1980; Nietsch 1999; Nietsch 2003; Merker & Groves 2006; Shekelle et al. 2008). In contrast to Eastern tarsier, neither Philippine tarsier *Carlito syrichta* nor Western tarsier *Cephalopachus bancanus* show a duet calls behavior (Yustian 2007; Rehakova-Petru et al. 2012). The acoustic characterization of ultrasonic vocalization of *Tarsius syrichta* and *Tarsius spectrum* has been described by Gursky (2013, 2015). In addition, Rehakova-Petru et al. (2012) also explained the acoustic behavior and individual variation of long-distance calls of the Philippine tarsier.

So far, there are no data of Western tarsier vocalization in Sumatra or Bangka Island. Crompton and Andau (1987) heard numerous clear vocalization of Western tarsier at Sepilok Forest Reserve, Sabah, Borneo (*C. b. borneanus*). They proposed that, even though the sound frequency is high, three types of *C. b. borneanus* vocalizations are relatively easy to be distinguished. Niemitz (1979) described at least seven different types of sounds produced by *C.b. borneanus* in Semongok Forest Reserve, Sarawak, Borneo. Nevertheless, Niemitz (1979) also reported that Western tarsier is "ordinarily silent" and cannot often be heard in the wild. Yustian (2007) did not obtain any audible vocalization from *C. b. saltator* in Belitung Island. Niemitz (1979) and Gursky (2015) proposed that Western tarsier might also communicate via ultrasonic sound. This study was designed to explore the vocalization and determine the spectrographic description of Western tarsier *C. b. bancanus* in Bangka Island.

Study Site and Animals

The research was conducted in the agroforest area of Petaling Village in Bangka Regency, Province of Bangka Belitung Islands ($2^{0}16'$ S, $105^{0}57'$ E), located about 22 km west of Pangkal Pinang, the capital city of the province (Figure 1). The area consists of secondary forests and plantations such as rubber, pepper, banana, and palm oil. The rubber plantations are more than 10 years old, and other vegetation grows around the plantation, forming dense secondary vegetation.

The study took place from February to April 2020 and was initiated by conducting a preliminary survey to confirm the presence of tarsiers at the research site through their movement, vocalization, and recent urine marks on the bark of trees. Once we located the animal, followed by attempts to hand-capture the tarsiers. We captured one female and two males, which were then used for the first recording process. After the first recording was completed and all the individuals were released, we conducted other attempts to capture tarsiers in other parts of the forest. Again, we managed to catch three individuals (one pregnant female and two males). In total, we captured six individuals, i.e. four adult males and two adult females. The adult females gave birth during the study, bringing the total to seven individuals. Two temporary wire-net enclosures of 2´2´2 m^3 size, with natural vegetation

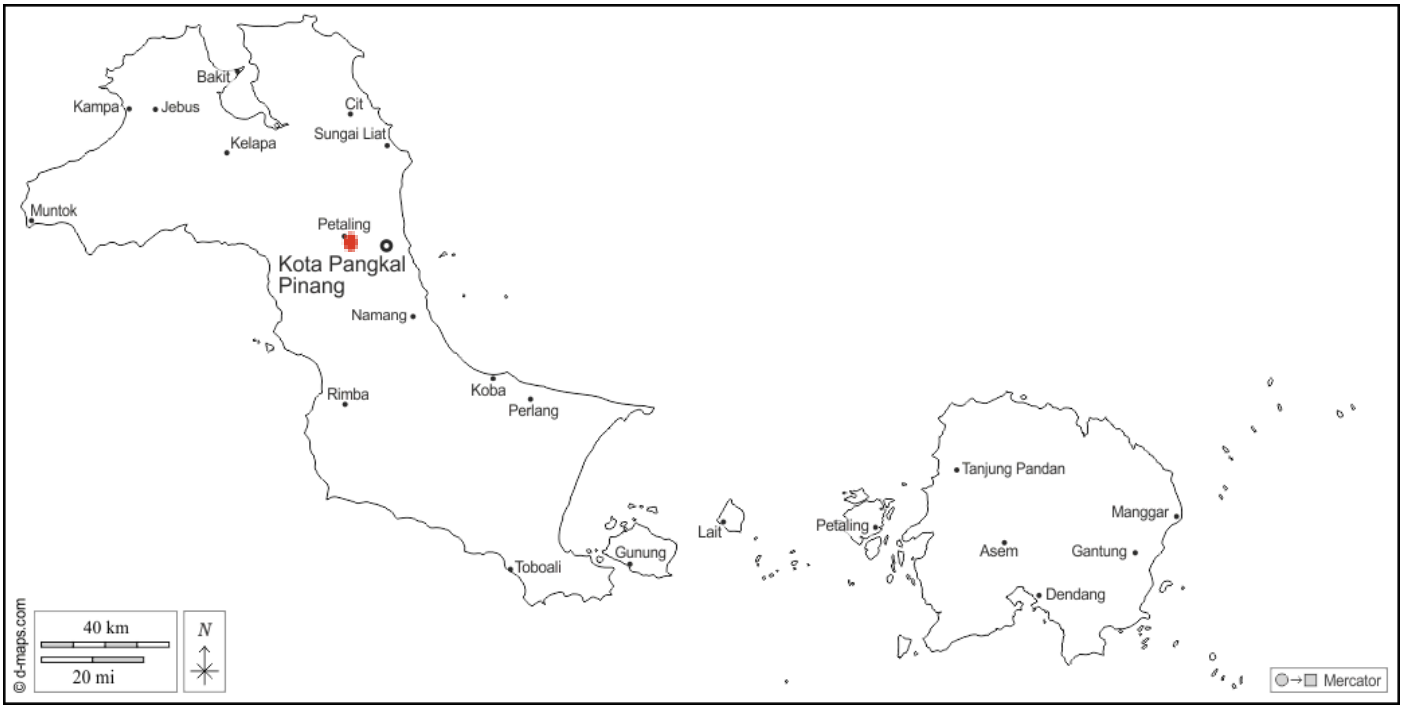

Figure 1. Site location (shown in red dote) in Petaling Village, Bangka Regency. (Map source: d-maps.com).

inside, were made within their habitat of captured, each with a distance of ± 30 m apart. In each of the enclosures, the tarsiers were fed twice daily with live crickets or grasshoppers. The difficulties in finding, capturing, and maintaining tarsiers in the enclosures had been a limitation in this research. After data collection, all tarsiers were released right or to close the place when they are being captured. The study was carried out with the permission of the Indonesian Government through Bangka Regency Government Permit No. 070/04/Bankesbangpol/2020.

Data collection and processing

The recording process was conducted after the animals were habituated for five days. We conducted two periods of recording. In the First period of recording, one enclosure consists of one adult male and one female, whilst in other enclosures is consists of only one male. A period of recording was done in three consecutive nights. In the second period of recording, as mentioned above, one of the females gave birth on the second night of the recording process.

Tarsier vocalizations were recorded from 17.00 PM to 07.00 AM using Zoom H1 Handy Recorder (Zoom Co. Japan), built-in microphone, with 96 kHz sample rate and 16-bit recording quality (Gursky 2015; Rehakova-Petru et al. 2012). The recorder device is placed two meters outside the enclosure. Replacement of the recorder battery was carried out every 10 hours. Additionally, animals were recorded using a handphone (Redmi 7, Xiaomi Co.) microphone with a 44,1 kHz capacity while being handled for release. The use of this handphone device is not standardized and validated method.

During the recording process, the time and the animal activities were also observed to explain the context of the produced vocalization. We refer to Niemitz (1979, 1984), Crompton and Andau (1987), Nietsch (2003), and Rehakova-Petru et al. (2012) to interpreting the acoustic structure and the context of each type of vocalization.

Recorded vocalizations were extracted and analysed using Audacity 2.3.3 (Audacity(R): Free Audio Editor and Recorder from https://audacityteam.org/) and spectrogram visualization produced by Raven Pro

1.6.1 Bioacoustics Analysis Software (The Cornell Lab Center for Conservation Bioacoustic) (Rehakova-Petru et al. 2012; Kulander 2018). The spectrogram was made with FFT parameters 512, Hann window, and overlap of 87.5% (Gursky 2013).

Data Analysis

The analysis was carried out to determine the initial frequency (start frequency of the selection in kHz), lowest frequency (the lowest frequency within a selection in kHz), highest frequency (the highest frequency within a selection in kHz), duration of one vocalization, and the number of vocalization (adapted from Charif et al. 2010; Kulander 2018). Only samples with the good recording quality and low background noise were analyzed. The small number of the vocalization produced by the observed animals, as well as the small number of good recording quality, became the limitations in this study. Data were discussed with a descriptive analysis approach.

Cephalopachus bancanus Vocalization Types and Characteristics

We identified seven types of Western tarsier vocalizations with different patterns (Table 1). One type of vocalization, squeak, is produced only by the infant. Two types of vocalizations (whistle and cheeps) were produced by both infants and adults, and four vocalization types were performed only by adults.

Infant squeak

Infant squeaks (Figure 2) are types of single-beat tone vocalization produced by an infant. This type of vocalization was audible, starting from 11.1 kHz to 18 kHz, but could reach the lowest frequency at 9.6 kHz. Infant squeak is thought to be a form of summons to the mother. Infant squeak sounds more

Table 1. *Cephalopachus bancanus* vocalization characteristics in Bangka Island (mean ± SD).

Type of Vocalization	Initial Frequency (kHz)	Lowest Frequency (kHz)	Highest Frequency (kHz)	Range of Frequency (kHz)	Duration (ms)	Number of vocalizations
Squeak (Infant)	11.1 ± 3.4	9.6 ± 3.4	18 ± 1.8	8.4	102 ± 30	83
Whistle						
Infant	11.7 ± 0.95	9.1 ± 0.73	12.1 ± 0.84	3	104 ± 30	51
Adult	13.5 ± 1.8	9.5 ± 0.3	14.7 ± 0.88	5.2	96 ± 6	3
Cheep						
Infant	16.8 ± 1	16.1 ± 1.2	18.2 ± 0.8	2.1	138 ± 40	15
Adult	12.8 ± 1.3	12.1 ± 1.4	13.5 ± 2.0	1.4	102 ± 20	5
Agonistic Scream						
Adult	4.8 ± 0.56	4.4 ± 0.46	22.7 ± 2.7*	18.3	654 ± 276	27
Alarm Call						
Adult	6.0 ± 1.6	5.5 ± 1.4	22.8 ± 3.6*	17.3	163 ± 98	23
Distress Call						
Adult	5.9 ± 0.83	3.2 ± 0.79	20.4 ± 1.7*	17.0	82 ± 21	5
Hysteresis						
Adult	5.3 ± 0.3	0.22 ± 0.2	22.0 ± 0*	16.7	384 ± 151	5

* reached ultrasonic level

evident and has a higher frequency range in comparison to infant whistle. This vocalization is presumed to have the function which is related to communication with the mother. Infant tarsiers make a sound when they have a feeling of loneliness, anxious, feel sick, hungry, or cold (Niemitz 1979; Nietsch 2003).

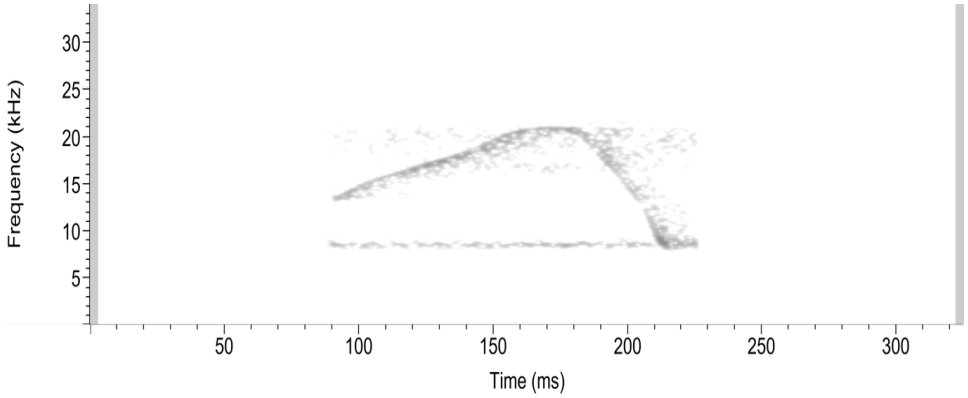

Figure 2. Spectrogram of infant squeak of *Cephalopachus bancanus* in Bangka Island.

Infant whistle

Infant whistles (Figure 3) are also types of single-beat tone vocalization produced by tarsier's infant and can be heard by humans. Infant whistle is often encountered just before dawn, or when the tarsier is going back to the sleeping site. As the second frequent call observed, this whistle was also thought to be a form of summons to the mother tarsier. These types of vocalizations are presumed to have the same function as infant squeak, which is related to communicate with the mother.

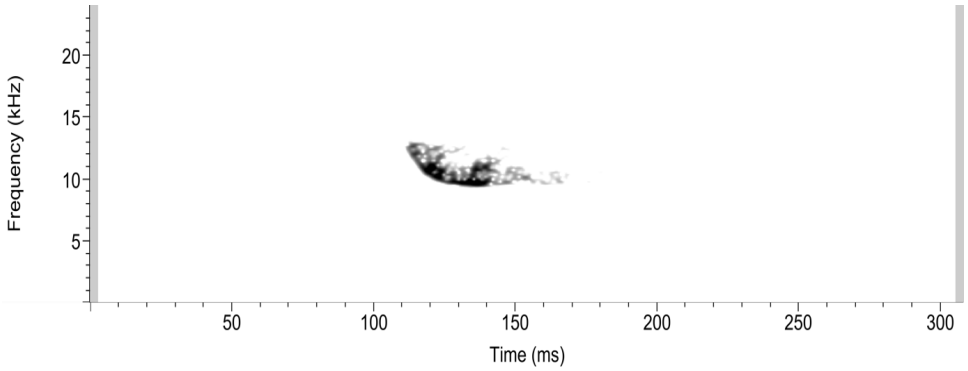

Figure 3. Spectrogram of infant whistle of *Cephalopachus bancanus*.

Adult Whistle

The whistle is thought to provide information on the presence of nearby tarsiers related to territorial function in the adult. Whilst in the infant the vocalization intensity was the second act after squeak, this audible single-beat tone vocalization is the fewest vocalization type. The Western tarsier whistle has the same visual spectrogram form as *Carlito syrichta's* whistle (forming the letter U, Figure 4), which is associated with territorial functions and attracting the attention of the opposite sex (Rehakova-Petru et al. 2012).

Infant Cheep

Infant cheeps (Figure 5) is a type of audible vocalization performed by the infant. This type of vocalization can be heard clearly. In comparison to the other types of vocalizations produced by the infant, this type is the fewest number. Nevertheless, infant cheeps are also presumed to have the same function as infant squeak and whistle, to communicate with the mother.

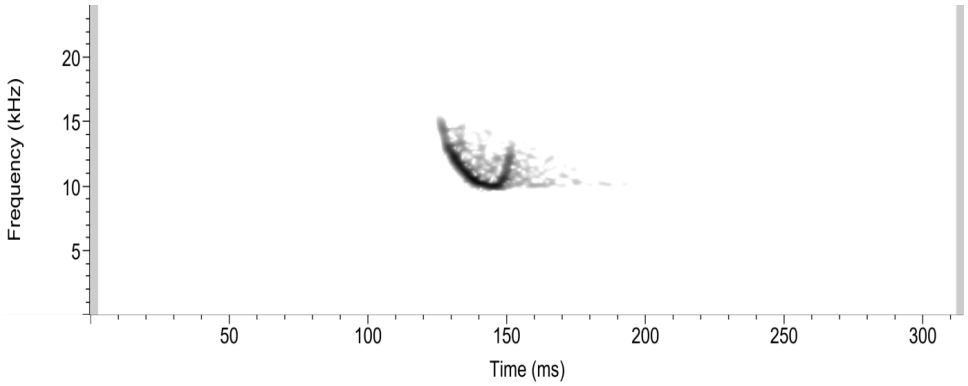

Figure 4. Spectrogram of whistle vocalization of *Cephalopachus bancanus*.

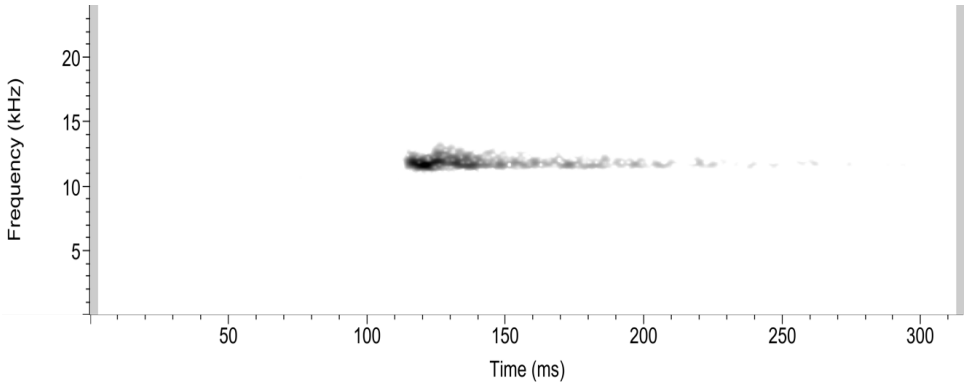

Figure 5. Spectrogram of infant cheep vocalization of *Cephalopachus bancanus*.

Adult Cheep

Adult cheeps (Figure 6) is a type of audible one-beat vocalization found in mothers with her infant. The same type of vocalization is also found in *Carlito syrichta* (Rehakova-Petru et al. 2012). In *Cephalopachus bancanus*, this type of vocalization can appear along with other types of vocalizations, such as agonistic scream, whistle, infant squeak, and mother call.

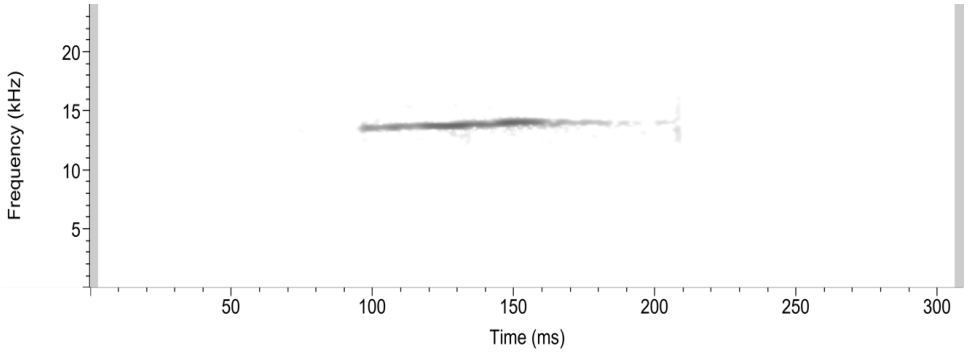

Figure 6. Spectrogram of cheeps vocalization of *Cephalopachus bancanus*.

Agonistic Scream

This vocalization pattern (Figure 7) appears when there was accidental nearly physical contact between two adult male tarsiers. One individual or both will make sounds related to agonistic functions or pressure other individuals to move away. After making this sound, which could reach an ultrasonic level (more than 20 kHz), the two individuals would stay apart from each other. This is related to the tarsier's solitary nature who feels disturbed when there are other individuals at the territory. This type of vocalization was performed by tarsier in Borneo (Niemitz 1979), however, there is no report from the Philippine tarsier group (Rehakova-Petru et al. 2012).

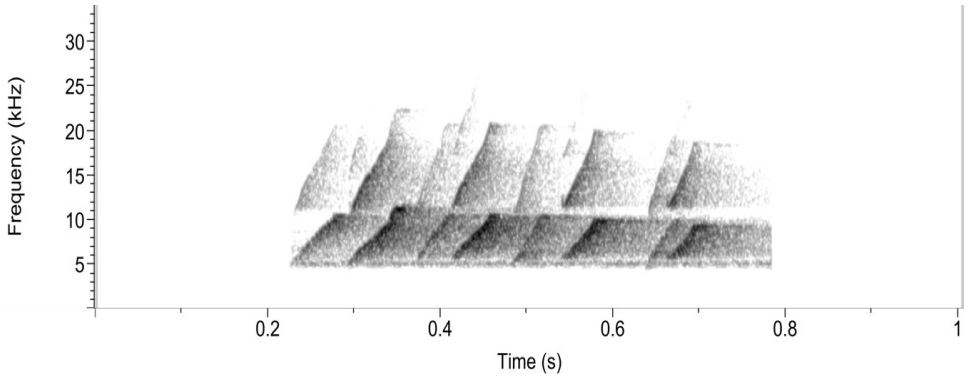

Figure 7. Spectrogram of agonistic scream vocalization of *Cephalopachus bancanus*.

Alarm Call

Alarm vocalization (Figure 8 a, b & c) were generated as a response to the threats around. This type of vocalization is followed by a very active movement behaviour. Nietsch (2003) states that when they sense a threat, tarsier (*Tarsius spectrum*) will produce a loud vocalization, which is called a whistle alarm and alarm call. In Western tarsier, there are two other types of alarm: short alarm call (Figure 8b) and long alarm call (Figure 8c). Short alarm and long alarm calls have a nearly similar frequency, but they are different in the length of the vocalization duration as well as the visual form of the spectrogram. These types of vocalizations have the highest peak frequency, more than 22 kHz (Table 1), which indicates that *Cephalopachus bancanus* can also communicate at the ultrasonic level, although no pure ultrasound sounds were found.

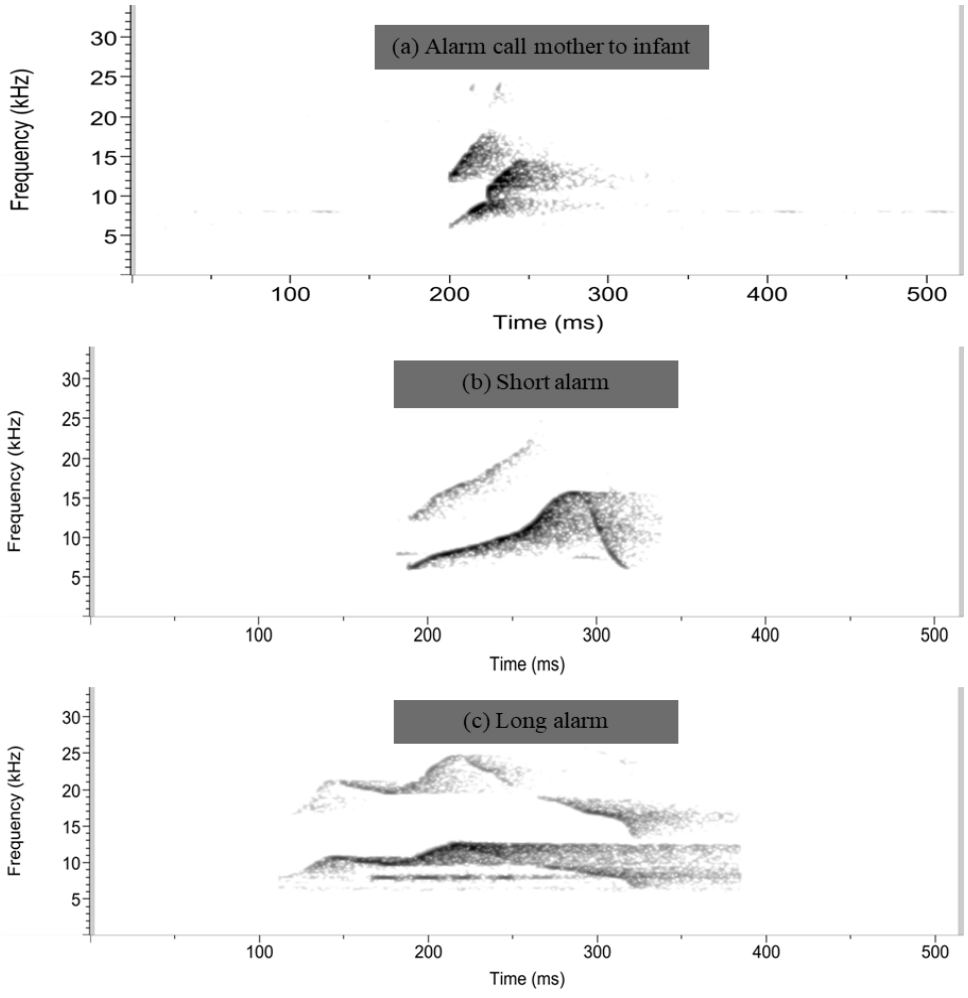

Figure 8. Spectrogram of alarm calls of *Cephalopachus bancanus*. Alarm call mother to infant (a), short alarm (b), and long alarm (c).

Distress call and Hysteresis

Cephalopachus bancanus can produce very loud, shrill, repetitive sounds, followed by rebellious behaviour during the capture and handling process. This behaviour occurs because the tarsier feels depressed and panic. These types of vocalizations (Figure 9a & b) may also appear when tarsiers become prey for predators. In spectrogram visualization, these two types of vocalizations appeared complexly and difficult to be analysed. This vocalization appears when we handled the animals to release, and the recording process is only done using a handphone microphone with a sample rate of 44.1 kHz. If a higher sample rate is used, it is possible that the frequency of this type of vocalization exceeds the frequency seen in the current spectrogram. Distress calls and hysteresis are often encountered simultaneously at one vocalization. However, distress calls can also appear without hysteresis being followed. Distress calls always appear in one beat, while hysteresis occurs in several beats.

By means of sonographic diagrams, Niemitz (1979) described at least seven different types of sounds produced by *Cephalopachus bancanus*. This study also described seven types of vocalizations, although perhaps in a slightly different designation and context. For example, one twittering type of vocalization produced by an infant, Niemitz designated as "patches", and we defined it as "squeak" and "infant whistle". While Niemitz also described the distress call of a tarsier infant when left alone as "colon and apostrophe", we assumed that was a "cheep" call. In another example, Niemitz designated "hysteresis" as vocalization with agonistic correlation, while we could distinguishably two types of vocalizations as "agonistic scream" and "hysteresis". We agree with Niemitz, that vocalization in this species is much more complex than previously described.

Through this research, we concluded that all types of vocalizations of Western tarsier *Cephalopachus bancanus*, which was observed and recorded in Bangka Island, can be heard within human hearing and some of the vocalizations, such as alarm call, agonistic scream, distress call, and hysteresis have peak frequencies at the ultrasonic level (> 20 kHz). We recommend further research to compare vocalization within subspecies (Western tarsier in Bangka and southern Sumatra) to prove at least the ultrasonic vocalization of this species.

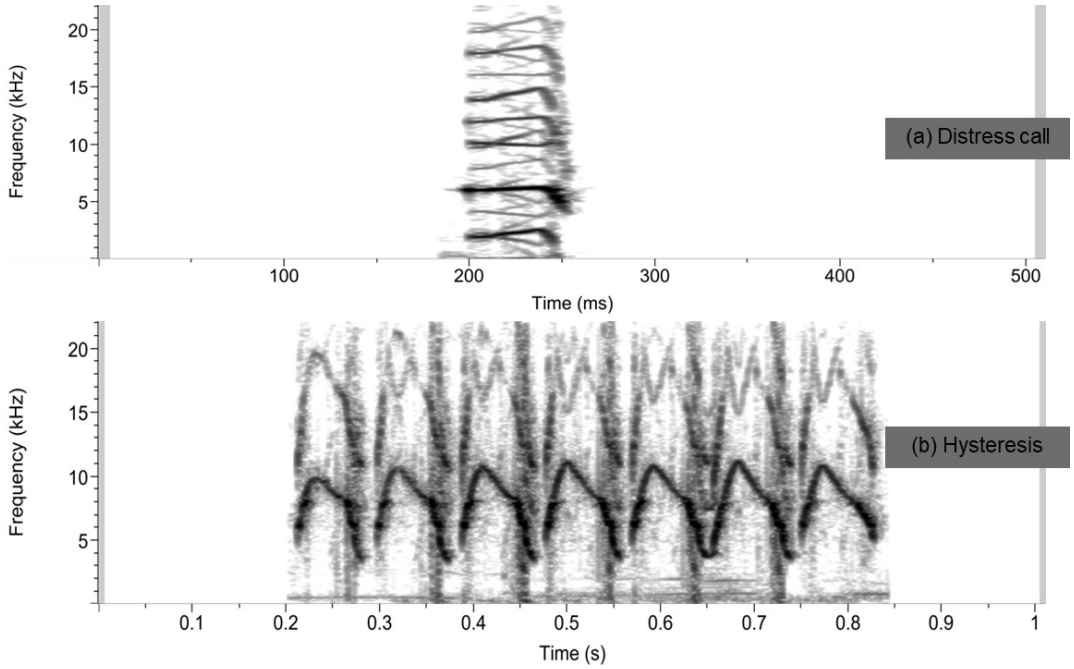

Figure 9. Spectrogram of distress call and hysteresis of *Cephalopachus bancanus*. Distress call (a) and Hysteresis (b).

AUTHORS CONTRIBUTION

I.Y. and A.S. designed the research and supervised all the studies. D.K. collected and analysed the data. I.Y. and D.K. wrote the initial manuscript. Z.E. contributed to data collection. D.S., E.P., and L.H. reviewed, revised, and proofread the final manuscript.

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CONFLICT OF INTEREST

The authors confirm that there is no conflict of interest regarding the research and/or financial support for this work.

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Short Communications

Identification of ISSR-based Molecular Markers Associated with Ploidy Level of Orange Watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai)

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ABSTRACT
Indonesia is an agrarian country whose territory is partly used as an agricultural sector, has especially watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai). At present, the development of watermelons in Indonesia still requires a variety of colors, superior watermelons need to be developed by making triploid orange watermelons. The new watermelon cultivar (F_1) is the result of crossing 'Jelita' (♀) and 'BallyBall' (♂) which are expected to provide a combination of characters that can produce large and sweet fruits. The purpose of this study was to identify ISSR markers that were associated with ploidy levels of horticultural crops, particularly watermelon. The watermelons were growns on the agricultural land of Kebondalem Hamlet, Madurejo Village, Prambanan District, D.I.Y. and analyzed at the Laboratory of Biotechnology PAU, UGM and Laboratory of Genetics and Breeding, Faculty of Biology UGM. Analysis of genetic variation was carried out using the PCR-ISSR method and the DNA bandwidth was calculated using *Paint Apps*, *Microsoft Excel* 2013, and *Microsoft Word* 2013. The results of crossing 'Jelita' and 'King Quality 'is sterile. The result of 'Jelita' with 'BallyBall' produces tillers until sterile harvest time. The results of the analysis of genetic variation using the PCR-ISSR method indicate that the ISSR BI, B3, B5, and CBTC 1 molecular markers cannot be used to distinguish ploidy from the tested watermelons.

Keywords: ISSR, Marker Assisted Breeding, orange watermelon, ploidy, triploid

INTRODUCTION
Watermelon is a fruit that is in great demand by the public. The peculiarity of the sweet, crunchy, and juicy taste of watermelon makes it attractive for consumption and cultivation. Moreover, watermelon contains high and complete nutrition (Khomsan 2009). In the health sector, watermelon has benefits as an antioxidant, anti-inflammatory, and vasodilating agent (Poduri et al. 2013) because of the vitamins, proteins, and minerals found in fruit skins, pulp, and seeds. Rapid technological advances make the quality and production power of superior watermelons continue to increase. Various shapes, levels of sweetness, color, and size of genetically engineered watermelons are cultivated rapidly both domestically and abroad. Unfortunately, watermelon, which has an attractive appearance and high economic value, comes from imported seeds. Therefore, it is necessary to make efforts to improve the quality of local watermelons. Faculty of Biology

UGM tries to contribute by crossing yellow watermelon 'maduri' with red watermelon 'princess pomegranate' to produce superior local melons with orange flesh fruit. Furthermore, this hybrid is being developed into a tetraploid orange watermelon by crossing several watermelon varieties.

As known, the selection of hybrids in conventional crosses takes a lot of time, money, and effort because it must wait until a certain plant age when the desired phenotype appears. Therefore, it is necessary to develop a molecular hybrid selection technique developed from the ISSR marker. Molecular selection in the breeding process is known to increase the success of breeding programs. Phenotype expression is closely related to the genetic diversity of an organism. The loci of genes of an agronomic trait of interest can be related to molecular marker loci so that they can be used for genotype selection. The use of molecular markers allows the selection of indirect traits at the seedling stage thereby accelerating the breeding process and facilitating the increase in difficult traits that cannot be easily selected using morphological markers (Varshney et al. 2009). Molecular markers can be used to eliminate inferior genotypes early so that breeders can only focus on the genotypes that have the greatest potential (Moose & Mumm 2008). Several previous studies have also demonstrated the successful use of molecular markers for genotype selection in the breeding process, such as breeding for resistance to soybean cyst nematodes (Cregan et al. 1999), resistance to cereal diseases (Varshney et al. 2006), and drought tolerance in maize (Ribaut & Ragot 2007).

Although possible, the development of molecular markers for ploidy selection has not been widely used. Research by Feng et al. (2018) attempted to develop EST-SSR markers for ploidy identification in *Misgurnus anguillicaudatus*. Schie et al. (2014) used a combination of several molecular markers for polyploid identification in garden dahlia. Therefore, the aims of this study were to identify ISSR markers that were associated with ploidy level of horticultural crops, particularly watermelon.

The materials used in this study were triploid watermelon leaves 'Jelita' and 'King Quality' and diploid red watermelon 'BallyBall' which had been grown in greenhouses and open planting areas, Prambanan, DI Yogyakarta, ice gel, Phytopure DNA isolation kit 1 chloroform, isopropanol, 70% ethanol, TE buffer 1x, PCR Kit (Taq HS Red Mix 2X Bioline), ISSR Primer (Table 1), sterile ddH2O, MgCl2, agarose, TBE 1X, fluorosafe DNA staining, aquades, and 100 bp Geneaid DNA marker.

Table 1. The sequence of ISSR markers used in this research (Vinoth & Ravindhran 2016).

Primer	Primer Sequence 5' – 3'	The DNA band length (bp)
CBTC 1	(AG)8-T	200 - 2000
B1	(AG)8-C	400 - 2200
B3	(GA)8-A	400 - 1900
B5	(GA)8-T	400 - 1600

Watermelon samples were grown in four beds (mounds). Leaf samples were taken from 15 plants for each bed randomly. A total of 45 leaf samples were used in the molecular analysis of this study. On average, 3 to 5 leaf samples were taken for each watermelon cultivar. Leaves taken were eaves fifth to ninth from the base of the leaves with a healthy appearance (green, smooth, and do not have infection symptoms). The leaf samples were then put into plastics with a certain code in the icebox. After arriving at the laboratory, the sample was then put in a freezer or cooler so that the DNA on the leaves is not damaged.

As much as 0.3 g leaf samples were used for DNA isolation as described by Daryono and Natsuaki (2002) with little modifications. The leaf samples (without leaf bones) were crushed with a pestle and mortar. The crushed samples were transferred into a 1.5 mL tube then added with 500 µL of Phyto pure reagents I and 200 µL of reagent II, continued with incubation at 65 ℃ for 20 minutes. Then, the samples were incubated at room temperature for 5 minutes followed by incubation in the freezer for 5 minutes. After that, the samples were added with 400 µL of cold chloroform and 20 µL of resin then shake for 30 minutes. Furthermore, the sample solution was centrifuged at 1,300 rpm for 10 minutes. The supernatant obtained was transferred to a new tube and added with cold isopropanol through the tube wall as much as the volume of the supernatant. The sample solution was shaken gently by hand for 10 minutes and centrifuged at 10,000 rpm for 10 minutes. DNA pellets were washed and purified with 100 µL of 70% ethanol 3 times. As a storage buffer, 50 µL 1X TE buffer was added. The isolated DNA was stored at -20 ºC and was checked for purity using Nanovue Plus Nanodrop spectrophotometer at absorbance 260/280 nm.

For PCR analysis, we only selected three DNA isolates of the highest quality from each cultivar. In this research, DNA samples were analysed using four ISSR primers as mentioned in Table 1. The targeted DNA bands were varying from 200 – 2200 bp. As much as 25 µL PCR solution containing 12.5 µL PCR kit Bioline 2x MyTaq™ HS RedMix (Bioline, United Kingdom), 1.5 µL primer (10 pmol), 2 µL DNA samples (200 ng), 1 µL $MgCl_2$, and 8 µL ddH_2O was used. The PCR reaction was conducted with an initial denaturation at 95 ˚C for 5 min; continued with 35 cycles of 95 ˚C for 45 s, 47.9 ˚C for 45 s, and 72 ˚C for 60 s; and then ended with a final extension at 72 ˚C for 5 min. The PCR results were analyzed using 60 µL of 2% agarose gel stained with a 6 µL floroSafe DNA stain (First BASE, Singapore).

Genetic markers are molecular markers that can describe certain traits so that they can be used to assist the plant selection process in the plant breeding process. This research was conducted to obtain specific DNA band candidates that can distinguish triploid and haploid watermelon plants. The method that was used in this research was the PCR-ISSR method because it does not require genome sequence information (Arifiyanti 2015). ISSR is a multi-locus molecular that amplified regions between both ends of microsatellite. ISSR was suitable for asses genetic diversity because of its high genetic variability and ability to generate multi-locus data even though there was limited sequence information. Moreover, ISSRs are more reproducible than RAPDs and cheaper dan faster than AFLP (Ng & Tan 2015).

In some PCR visualization results, smears or bands that look faint are seen, so an electropherogram was made to facilitate analysis (Figure 1b – 4b). According to Azizah (2009), poor amplification results can be caused by mis-priming, efficiency, and optimization of the PCR process. In practice, mis-priming can occur because primers may anneal at incorrect sites. The occurrence may depend on the difference between the melting rates of primers at correct and incorrect sites. Failure in the PCR process can produce many non-specific DNA products of various sizes that appear as ladders or smears on agarose gels and sometimes even no product at all. Apart from mis-priming, mutations that are accidentally introduced into the amplicon can lead to heterogeneous or non-target PCR products. resulting in a heterogeneous population of PCR products (Mamedov et al. 2008).

Based on Table 2, B1 has the highest polymorphic bands than other primers. The percentage of polymorphisms that were obtained from this primer was 80%. From Figure 1, it was known that there was a DNA band measuring 770 bp which was only amplified in the triploid sample. These

Table 2. Primer used in molecular analysis with corresponding bands scored with polymorphic bands, observed in samples.

Primer	DNA bands size	Amplified bands	Monomorphic bands	Polymorphic bands	Percent of Polymorphism (%)
B1	335-1407 bp	10	2	8	80
B3	170-943 bp	9	6	3	33.3
B5	222-985 bp	7	4	3	42.8
CBTC1	228-1472 bp	10	7	3	30

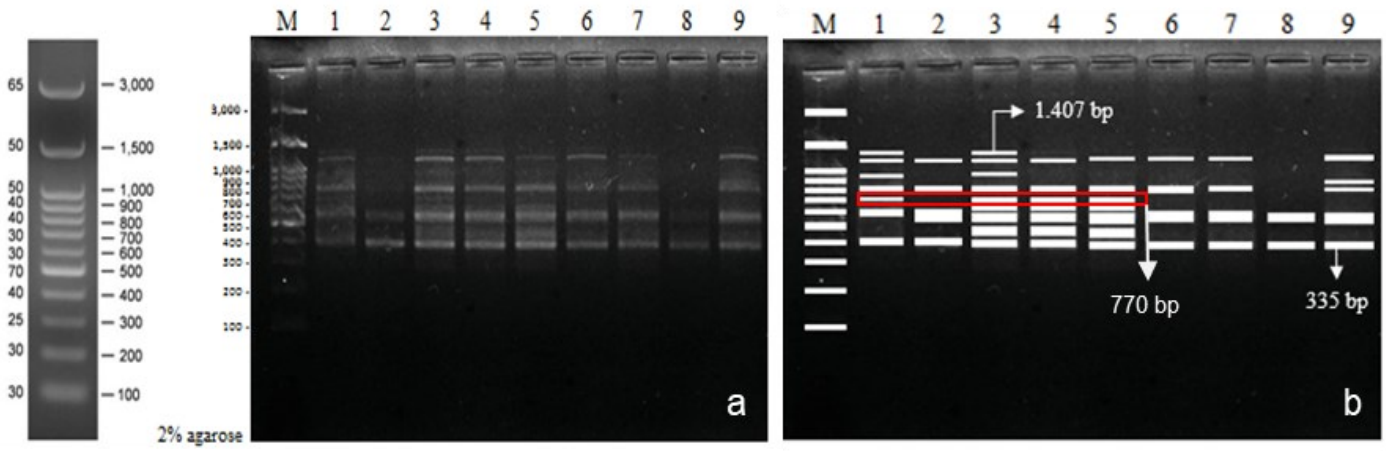

Figure 1. The PCR-ISSR molecular test results of watermelon using B1 primer (a) visualization of gel electrophoresis (b) electrophoregram (Note: M = DNA ladder; 1 - 3 = Triploid watermelon 'King Quality'; 4 - 6 = Triploid watermelon 'Jelita'; 7 - 9 = Diploid watermelon 'Bally Ball').

DNA bands could be used as candidates for the development of molecular markers linked to ploidy levels on watermelons. Safaei-Chaeikar & Rahimi (2017) reported that ISSR marker can be used to develop molecular markers that associated with agronomical traits in the medicinal plant lemon balm. The ISSR marker had high polymorphic bands indicating its high efficiency in differentiating targeted traits.

Based on Table 2, it is known that B3 primer produces only 33% of polymorphic bands. The existence of 6 identical DNA fragment patterns in all samples showed the genetic stability of watermelon plants tested at a certain length of DNA fragments. From Figure 2, we also know that the primer B-3 produces fewer polymorphic bands than other primers.

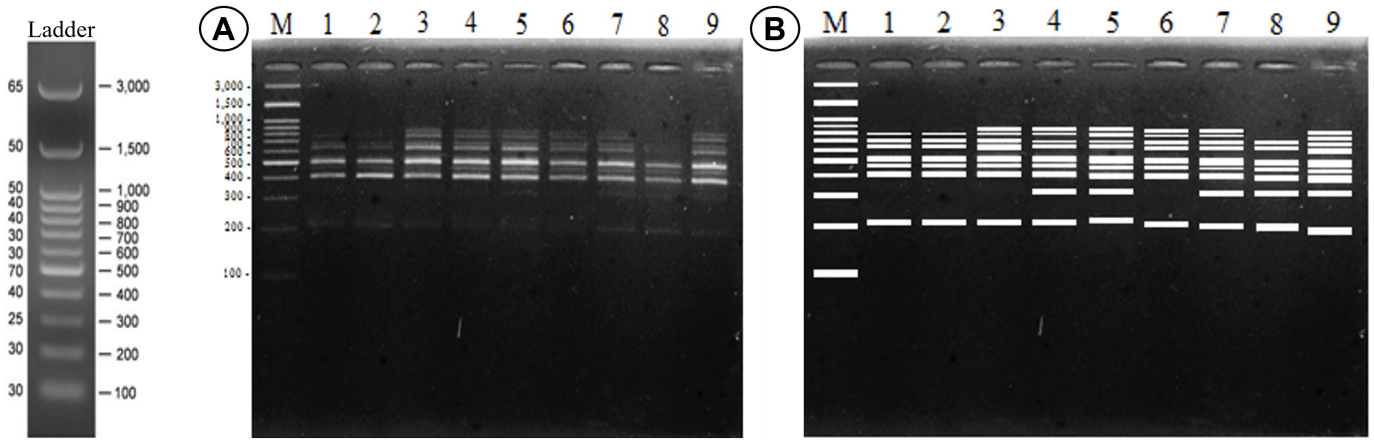

Figure 2. The PCR-ISSR molecular test results of watermelon using B3 primer (a) visualization of gel electrophoresis (b) electrophoreram (Note: M = DNA ladder; 1 - 3 = Triploid watermelon 'King Quality'; 4 - 6 = Triploid watermelon 'Jelita'; 7 - 9 = Diploid watermelon 'Bally Ball').

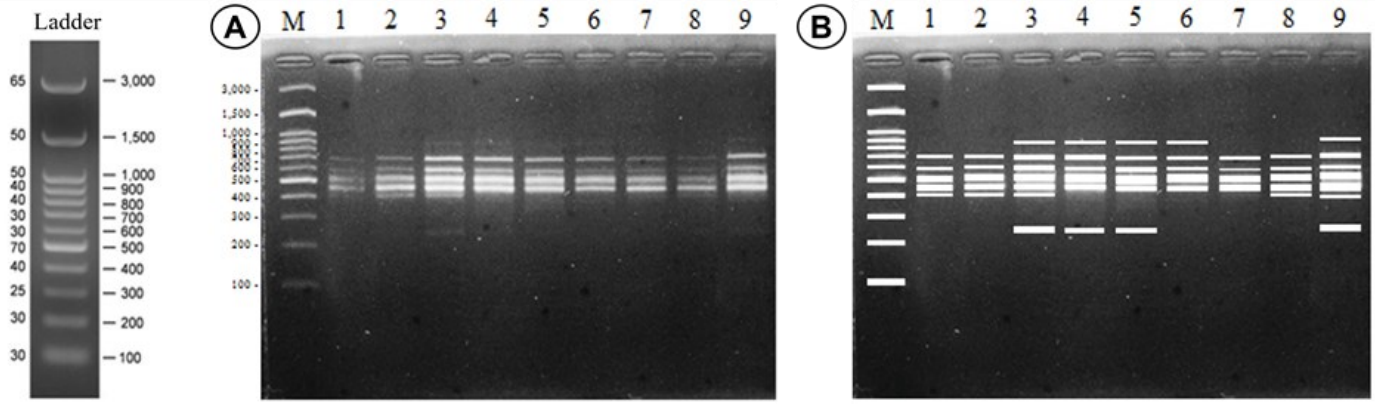

Figure 3. The PCR-ISSR molecular test results of watermelon using B5 primer (a) visualization of gel electrophoresis (b) electrophoregram (Note: M = DNA ladder; 1 - 3 = Triploid watermelon 'King Quality'; 4 - 6 = Triploid watermelon 'Jelita'; 7 - 9 = Diploid watermelon 'Bally Ball').

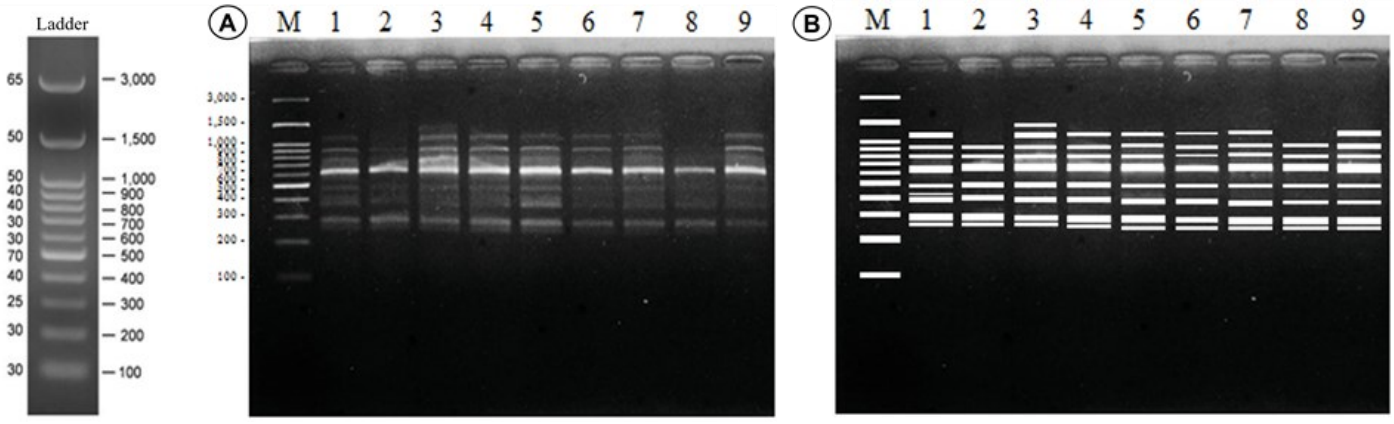

Figure 4. The PCR-ISSR molecular test results of watermelon using CBTC1 primer (a) visualization of gel electrophoresis (b) electrophoregram (Note: M = DNA ladder; 1 - 3 = Triploid watermelon 'King Quality'; 4 - 6 = Triploid watermelon 'Jelita'; 7 - 9 = Diploid watermelon 'Bally Ball').

PCR-ISSR using primer B5 (Figure 3) produced 7 DNA fragments measuring from 222 - 985 bp. There were four monomorphic DNA fragments and three polymorphic DNA fragments. The pattern of obtained DNA fragments showed genetic variation with a percentage of 42%. There was a similar pattern of DNA fragments in wells 1 - 6 because these plants are non-seed or triploid plants with the same number of ploidies so they have many similarities even though the colour of the flesh fruit is different. Based on this result, it was known that primer B1 (AG) 8 cannot be used as a molecular marker to distinguish watermelon ploidy.

PCR-ISSR results using CBTC 1 primer (Figure 4) produced the lowest polymorphic band among the used primer. A similar pattern was found on the three plant varieties, both diploid and triploid watermelon samples. It is also known that CBTC 1 primer did not produce specific DNA bands that were different in diploid and triploid watermelons so that it cannot be developed further as a molecular marker linked to the ploidy level of watermelon.

Based on the results of the PCR-ISSR study, the four primers used produced 36 DNA fragments consisting of 17 polymorphic DNA and 19 monomorphic DNA. The highest polymorphism was obtained using primer B1, namely 80%. Dje et.al. (2009) reported that primers BI (AG) 8-C, B3 (GA) 8-A, and B5 (GA) 8-T when used for genetic analysis of watermelons, produced a polymorphism percentage of 100% while the CBTC 1 (AG) primers 8 -T is 92.3%. The difference in the percentage of this polymorphism occurred in previous studies using different watermelon

varieties grown in planting media with different rainfall so that they had higher genetic variations. The low percentage of polymorphisms causes the possibility to obtain molecular markers that are linked to certain properties to become smaller. This is because the more polymorphic fragments are produced, the greater the chances of finding variations. The high number of polymorphic bands indicates that the used primers amplified genomic regions that have high genetic variation between samples (Sivaprakash et al. 2004; Jena & Chand 2021). The research of Vinoth and Ravindhran (2016) was known to use the same type of ISSR primer as this study to analyse the genetic diversity of watermelons resulting from somatic embryogenesis. The four primers were reported to produce DNA bands of 2-11, were reproducible, and were able to show genetic stability. These results are like those obtained in this study which the amount of obtained DNA bands ranged from 7-10 bands. Based on this, it could be said that this primer amplifies the relatively conserved regions in the watermelon genome.

The molecular markers expected because of this study were specific DNA fragments that can only be found in triploid individuals but cannot be found in diploid individuals and vice versa. These fragments can be used as the basis for molecular selection of diploid and triploid watermelons without having to wait for phenotypic expressions to appear at a certain plant age. The Marker Assisted Selection (MAS) also makes the phenotypic ploidy hybrid selection more effective because it does not have to use other analyzes such as flow cytometry or karyotyping. MAS development for ploidy analysis has been carried out several times, such as in Dahlia (Schie et al. 2014) and blueberries (Oliviera et al. 2020). The use of MAS for plant ploidy selection has not been widely used. MAS is generally used for the selection of disease resistance, resistance to environmental stress, color, size, and the number of fruit or flowers (Ibitoye & Akin-Idowu 2011).

The results of this study, a DNA band measuring 770 bp amplified only on triploid watermelons (Figure 1), need to be developed further before being used as molecular markers in watermelon ploidy selection. This is because the ISSR primer was still universal and random, so if it produces a lot of DNA bands when used in a selection, thus it will become less specific. The molecular markers for genetic selection must be reproducible and robust enough to tag specific traits not only in one progeny line of the crop. Generally, molecular links to specific traits can be SCAR markers, allele-specific markers, or SNP associated markers (Collard & Mackill 2008; Ibitoye & Akin-Idowu 2011). The success of MAS development was influenced by three main factors: (1) Genetic map with a number of polymorphic markers with sufficient uniform space for marking quantitative trait locus (QTL) or major genes accurately, (2) A close relationship between adjacent markers with QTL or the targeted main genes, and (3) Adequate recombination between markers and the rest of the genome (Ibitoye & Akin-Idowu 2011).

The ISSR primers B1 (AG) 8-C, have the potential to be developed as a molecular marker to differentiate ploidy in watermelon plants to increase the effectiveness of conventional plant breeding. Development can be done by constructing a SCAR marker from a specific 770 bp DNA band sequence amplified from primer B1.

AUTHORS CONTRIBUTION

A.J.S. carried out laboratory works (samples collection, DNA extraction, PCR amplification, agarose gel electrophoresis, data analysis, and drafted the manuscript). A.S.S. translated and revised the final manuscript as well as supervised the laboratory works and publication process. B.S.D. designed the research and supervised all of the processes from laboratory works until

publication. All authors contributed to this research and approved the final manuscript.

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CONFLICT OF INTEREST

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock 0wnership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Short Communications

Community Structure of Dragonfly (Ordo: Odonata) in Natural Forest and Tourist Sites Petungkriyono Forest, Central Java, Indonesia

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ABSTRACT
Petungkriyono forest is a tropical rainforest with high biodiversity. The increasing tourism activities in Petungkriyono lead to land conversion. Dragonfly (order Odonata) is a good bioindicator for aquatic and terrestrial. This study aimed to compare the community structure of Odonata in natural forests and tourist sites. The method of collecting imago Odonata was done by direct searching, samples were captured using sweep netting. The results showed that the dragonflies found in all locations consisted of the same family, 2 families (Gomphidae and Libellulidae) from the suborder Anisoptera and 6 families (Calopterygidae, Chlorocyphidae, Coenagrionidae, Euphaidae Platycnemididae, and Platystictidae) from the suborder Zygoptera. The total species of dragonflies found in Sokokembang were 15 species with a total of 293 individuals, Tirta Muncar 13 species of 287 individuals, Karanggondang 17 species of 276 individuals, and Curug Lawe 14 species of 242 individuals. The highest relative abundance of individuals was in the natural forest of Sokokembang is *Drepanosticta spatulifera* (26.28%) and in Karanggondang *Vestalis luctuosa* (24.64%), while in the tourist forests of Tirta Muncar and Curug Lawe were *Euphaea variegata* (34.84% and 28.51 %). The structure of the Odonata community is based on the Shannon-Wiener diversity index in the natural forests of Sokokembang (2.18) and Karanggondang (2.21) at the tourist sites of Tirta Muncar (1.84) and Curug Lawe (2.11). The results showed that the structure of the Odonata community based on the level of the diversity index value, evenness index, and dominance index in natural forests and tourist sites in Petungkriyono forest was not significantly different.

Keywords: community structure, dragonfly, Odonata, Petungkriyono

Indonesia is the world's third largest area of tropical rainforest with high biodiversity terrestrial and a high level of endemism (Cleary & Devantier 2011). The main threat to biodiversity is the practice of deforestation due to land conversion. More than 83% of the world's forest region has been used by humans for various land conversion practices. Land conversion reduces biodiversity and the effectiveness of forests as conservation areas (Hamilton et al. 2013).

Petungkriyono forest is tropical rainforest managed by the Perum Perhutani East Pekalongan, which has high biodiversity. Various rare and endemic flora and fauna can be found there, including the Javan endemic

species that belong to Genus *Drepanosticta* (Nugrahaningrum & Soesilohadi 2018; Damayanti et al. 2018). The increasing of agroforestry and tourism activities in Petungkriyono lead land conversion in Petungkriyono. Some examples of activities that have the potential land conversion in the Petungkriyono forest are planting coffee in the core zone, opening waterfall tours, water rides, agro techno forestry-park, and opening coffee shops or cafes in buffer and transition zones (Mubarok et al. 2019)

Land management based on monitoring indicators needs to assess the function and quality of the Petungkriyono. Community Structure and species richness of invertebrates are trends that can represent an assessment of habitat quality in a location (Samways et al. 2010). Dragonfly (Odonata) is a good bioindicator for aquatic and terrestrial areas (Simaika & Samways 2009; Suhling et al. 2005). Odonata community can be efficiently used as indicators of habitat status through a study of community structure (Simaika & Samways 2011; de Moor 2017). This is related to the function of Odonata in the ecosystem. Odonata are predatory insects, both when they are as nymphs and as adults. The existence of Odonata as predators is important because it restricts the population of herbivorous insects, types of pests, insect vector larvae, and their natural populations, so as to maintain the balance of the community in the ecosystem (May 2019). Ecosystems can be disturbed if the population of the Odonata community decreases.

Recent research has shown that disturbances from human activities in tropical developing countries are a major factor in reducing diversity, community and local species extinctions of various arthropods (Lawton et al. 1998; Clausnitzer et al. 2009; Seidu et al. 2017). The decline in dragonfly diversity occurs in tropical (Clausnitzer 2003) and subtropical (Stewart & Samways 1998) areas from intensive land use. Human activities can disrupt the abiotic conditions of the environment. That condition will only support the existence of tolerant species (Calvão et al. 2018). Intensive land use changes species composition, where specialist species tend to be replaced by generalist species due to habitat disturbance caused by human activities (Rouquette & Thompson 2007). The practice of monitoring an area using dragonflies has been developed in monitoring the quality of conservation areas such as in South Africa called the Dragonfly Biotic Index (DBI) (Simaika & Samways 2011). The purpose of this study was to compare the community structure of Odonata found in natural forests and tourist sites in the Petungkriyono forest.

This research was conducted in September 2020, November 2020, and January 2021 at 4 locations in Petungkriyono, Pekalongan Regency, Central Java Province (Sokokembang, Tirta Muncar, Karanggondang, and Curug Lawe). The selection of locations includes natural forest (Sokokembang and Karanggondang) and tourist sites (Tirta Muncar and Curug Lawe). Map of Odonata data collection and habitat condition in the Petungkriyono forest is shown in Figures 1 and 2.

The tools used in this study include a thermo hygrometer, lux meter, anemometer, sweep net, specimen box, Garmin GPS, camera (CANON 700D), and digital endoscope A005+ 500x. Data collection was done by the purposive sampling method. Sampling was carried out using the line transect method which has a length of 100 m, a width of 5 m on the right, and 5 m on the left. Each data collection location consists of 5 line transects. The method of collecting imago Odonata was done by direct searching method (Sutherland 2006). Imago Odonata samples were captured using the sweep netting technique (Samways et al. 2010).

Observations were made at 08.00 am-16.00 pm. Community structure was analyzed from Shannon-Wienner Diversity Index, Shannon-Evenness Index, Simpson's Dominance Index, and Relative Abundance (Samways et al.

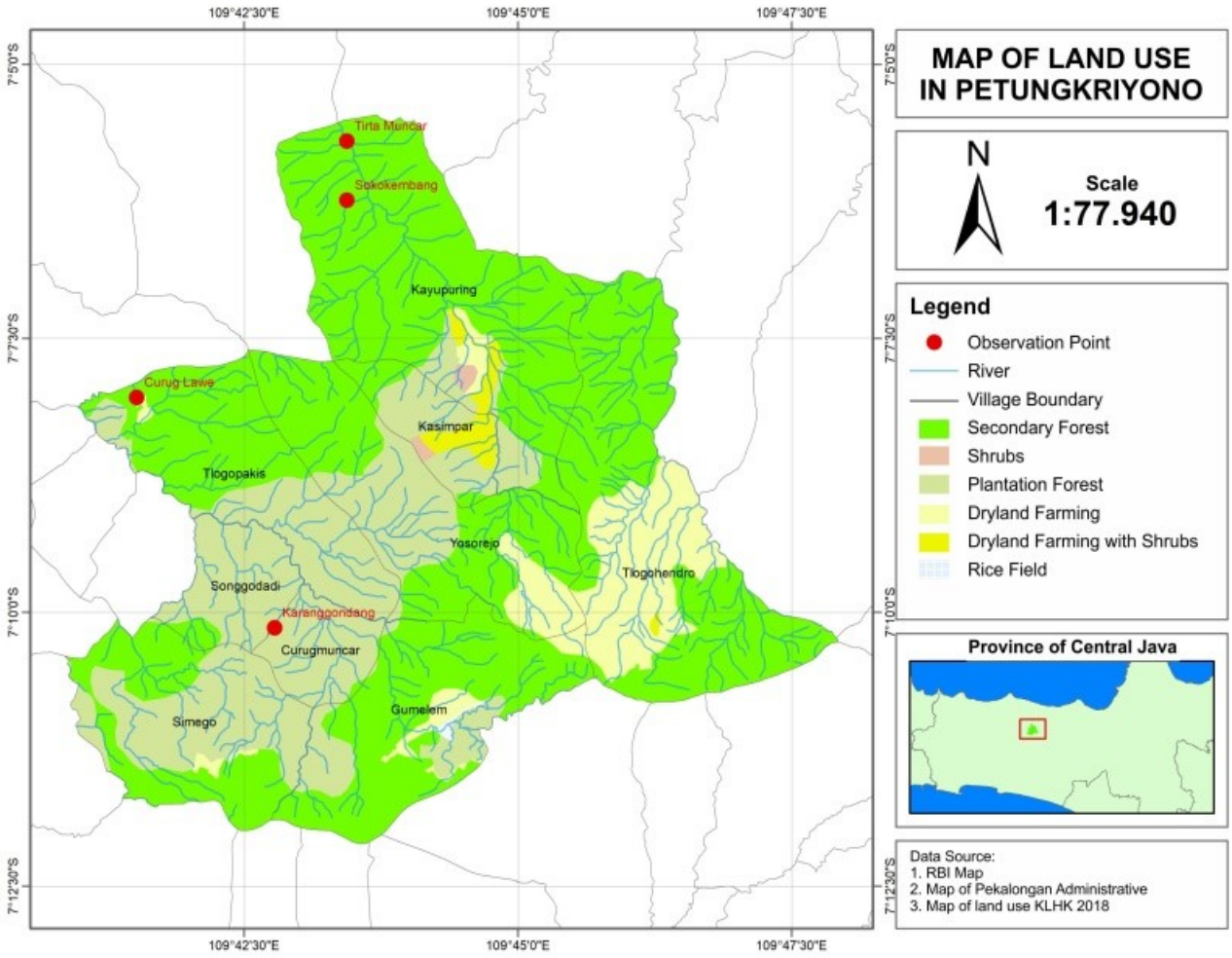

Figure 1. Map of Odonata data collection in the Petungkriyono forest.

Figure 2. Habitat condition of data collection in the Petungkriyono forest.

2010). Environmental parameter variables were analyzed by Canonical Correspondence Analysis (CCA) using the PAST3 software.

The result of this research is an approach that could describe the structure of a community is species diversity. The diversity of species in an area depends on species richness and evenness in the community (Scholwalter 2006). The results show that the total species from all locations were 19 species. The Odonata species found consisted of 2 families (Gomphidae and Libellulidae) of the suborder Anisoptera and 6 families (Calopterygidae, Chlorocyphidae, Coenagrionidae, Euphaidae Platycnemididae, and Platystictidae) of the suborder Zygoptera with a total of 1.098 individuals found. The total species of dragonflies found in Sokokembang were 15 species with a total of 293 individuals, Tirta Muncar 13 species of 287 individuals, Karanggondang 17 species of 276 individuals, and Curug Lawe 14 species of 242 individuals.

The dragonflies found in Sokokembang were higher (15 species) than the previous study which only found 8 species in Sokokembang (Utari 2018). The difference in the results may be due to various factors, such as the location of the sampled area and the time of the study. Based on Table 1, the highest species abundance was different for each location. The highest relative abundance in Sokokembang was *Drepanosticta spatulifera* (26.28%), in Karanggondang *Vestalis luctuosa* (24.64%), in Tirta Muncar and Curug Lawe were *Euphaea variegata* (34.84% and 28.51%).

Table 1. Diversity and relative abundance of Odonata in Petungkriyono forest.

	TAXA	Relative Abundance (%)			
Suborder & Family	Species	SK[a]	KR[b]	TM[a]	CL[b]
		382-583 m asl		777-1236 m asl	
Suborder Anisoptera					
Gomphidae	*Heliogomphus drescheri*	-	-	1.05	-
	Leptogomphus lansbergei	1.71	3.99	-	1.65
Libellulidae	*Neurothemis ramburii*	-	2.17	-	-
	Neurothemis terminata	1.02	1.81	-	1.24
	Orthetrum glaucum	1.02	1.45	-	4.96
	Orthetrum pruinosum	1.37	1.45	-	3.72
	Orthetrum sabina	9.22	1.45	1.74	2.07
	Pantala flavescens	12.97	1.09	2.44	-
	Potamarcha congener	-	1.09	-	-
	Zygonix ida	7.17	14.13	5.23	2.48
Suborder Zygoptera					
Calopterygidae	*Vestalis luctuosa*	16.72	24.64	22.65	11.98
Chlorocyphidae	*Heliocypha fenestrata**	1.02	-	1.39	-
	Rhinocypha heterostigma	2.05	3.62	1.74	6.20
Coenagrionidae	*Pericnemis stictica*	-	1.09	1.05	1.24
Platycnemididae	*Coeliccia membranipes*	2.05	2.54	3.14	24.38
Platysticidae	*Drepanosticta gazella**	2.39	2.90	1.39	2.89
	*Drepanosticta spatulifera**	26.28	15.94	20.21	6.61
	*Drepanosticta sundana**	1.71	1.09	3.14	2.07
Euphaeidae	*Euphaea variegata*	13.31	19.57	34.84	28.51

Note: Sokokembang (SK), Tirta Muncar (TM), Karanggondang (KR), Curug Lawe (CL), natural forest(a), tourist sites (b), Javan endemic species (*).

Figure 3. Species with highest relative abundance (A) *Drepanosticta spatulifera* male, (B) *Vestalis luctuosa* male, (C) *Euphaea variegata* male.

The difference in species abundance in each of these locations can be influenced by several factors that limit the abundance of these species, including the abundance of food sources, mortality caused by predators (including cannibalism), and stress responses to the presence of predators in each location. In addition to biotic factors as already mentioned, abiotic factors of environmental physical conditions such as temperature, light intensity, air humidity also affect the abundance of a species population. This is because different species have different ecological requirements to maintain a population abundance of more than zero (McPeek 2008).

Species with high abundance in Sokokembang there are *Drepanosticta spatulifera* male, *Vestalis luctuosa* male, *Euphaea variegata* male (Figure 3). *Drepanosticta spatulifera* relatively high abundance in Sokokembang is related to the environmental conditions at the Sokokembang location that support the existence of this species. *D. spatulifera* is quite often found around rivers with small flows and covered by a canopy. They perch under shady riparian vegetation. Based on data on environmental factors, the air temperature in Sokokembang ranges from 23.8-31.8˚C with a humidity of 60-96% and a light intensity of 875-53500 lux (Table 2). Sokokembang is an unspoiled forest covered with a canopy and little human activity. *D. spatulifera* is one of the endemic Javan dragonflies that has a specific microhabitat and is dominantly found in canopied rivers (Diniarsih 2016; Irawan et al. 2017). *Vestalis luctuosa* and *Euphaea variegata* have the same habitat type, both species are often found together in forest rivers with fast and small flows. However, although the two species are often found together, their roosting sites are different, *V. luctuosa* prefers to perch on shady riparian vegetation, and *E. variegata* is more often found perching on river rocks with high light intensity. The high abundance of *E. variegata* in Tirta Muncar and Curug Lawe could be caused by the nearly same (forest) condition in both locations, which are tourist sites that have waterfalls with rocky rivers. *E. variegata* is species of Odonata that is quite common in forest river habitats with rocky streams and breeds in slightly open to dense vegetation (Lieftinck 1954). The air

Table 2. The environmental factors during September 2020 – January 2021.

Location	Air temperature (˚C)	Humidity (%)	Light intensity (lux)	Wind speed (m/s)	Elevation (mdpl)
Sokokembang [a]	23.8-31.8	60 - 96	875-46.600	0-1.5	458-553
Tirta Muncar [b]	23-30.4	65-91	285-55.000	0-2.1	382-583
Karanggondang [a]	22-34.6	50-90	432-34.370	0-1.2	777-848
Curug Lawe [b]	21.9-35	40-83	466-48.700	0-2.2	591-1236

temperature range in Tirta Muncar is 23-30.4 °C with a humidity of 65-91% and a light intensity of 285-55.000 lux, while in Curug Lawe the air temperature ranges from 21.9-35 with a humidity of 40-83% and a light intensity of 466-48.700 lux.

Species richness in tourist sites (Tirta Muncar and Curug Lawe) is lower than in natural forests, this can occur because of the probability of the presence of more diverse species being found in unspoiled locations compared to locations that have changed, such as urban or rural areas. tourist sites (Ball-Damerow et al. 2014). Assemblages of species found in pristine forest areas will be able to stabilize extinctions and have a positive effect on changes in species composition (Koch et al. 2014).

The most common species from the suborder Anisoptera are the family Libellulidae and from the suborder, Zygoptera is the family Platysticidae. The Libellulidae family is a family that has the most members of the species group in the suborder Anisoptera and its distribution is widespread throughout the world. While most of the family Plastycidae (Zygoptera)'s members have a specific microhabitat. Several species of Zygoptera dragonflies found were endemic to Java, including *Heliocypha fenestrata*, *Drepanosticta sundana*, *Drepanosticta gazella*, and *Drepanosticta spatulifera* (Setiyono 2014). *H. fenestrata* species were only found in Sokokembang and Tirta Muncar. The three species belonging to the genus *Drepanosticta* were found throughout the site.

The level of diversity index values, evenness index, and dominance index in the 4 research sites (Figure 4) shows values that are not much different between locations. The Shannon-Wiener diversity index in Sokokembang, Tirta Muncar, Karanggondang, and Curug Lawe ranged from 1.84 - 2.21. Evenness index values in the four locations also have values that are not much different (0.72 – 0.80). Likewise with the dominance index value, where the four locations have values ranging from 0.15 - 0.22.

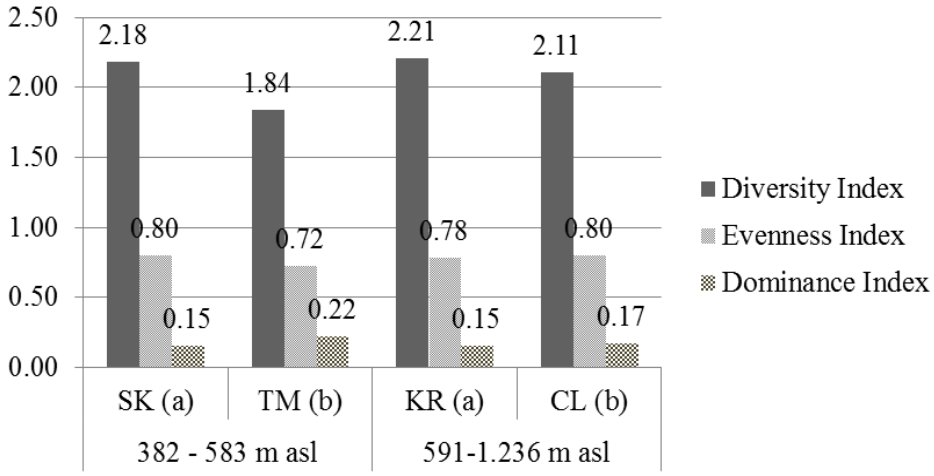

Figure 4. Index of diversity, evenness, and dominance at Sokokembang (SK), Tirta Muncar (TM), Karanggondang (KR), Curug Lawe (CL), natural forest(a), tourist sites (b).

The diversity, evenness, and dominance index values indicate that the diversity and abundance of species in the four locations are still in good condition, this is supported by the relative abundance values between species in each location where there is only slight variation (Table 1) so that each species in the Odonata community is evenly distributed (Nasiruddin & Barua 2018).

Environmental parameters were analyzed using Canonical Correspondance Analysis (CCA) to determine the effect of environmental

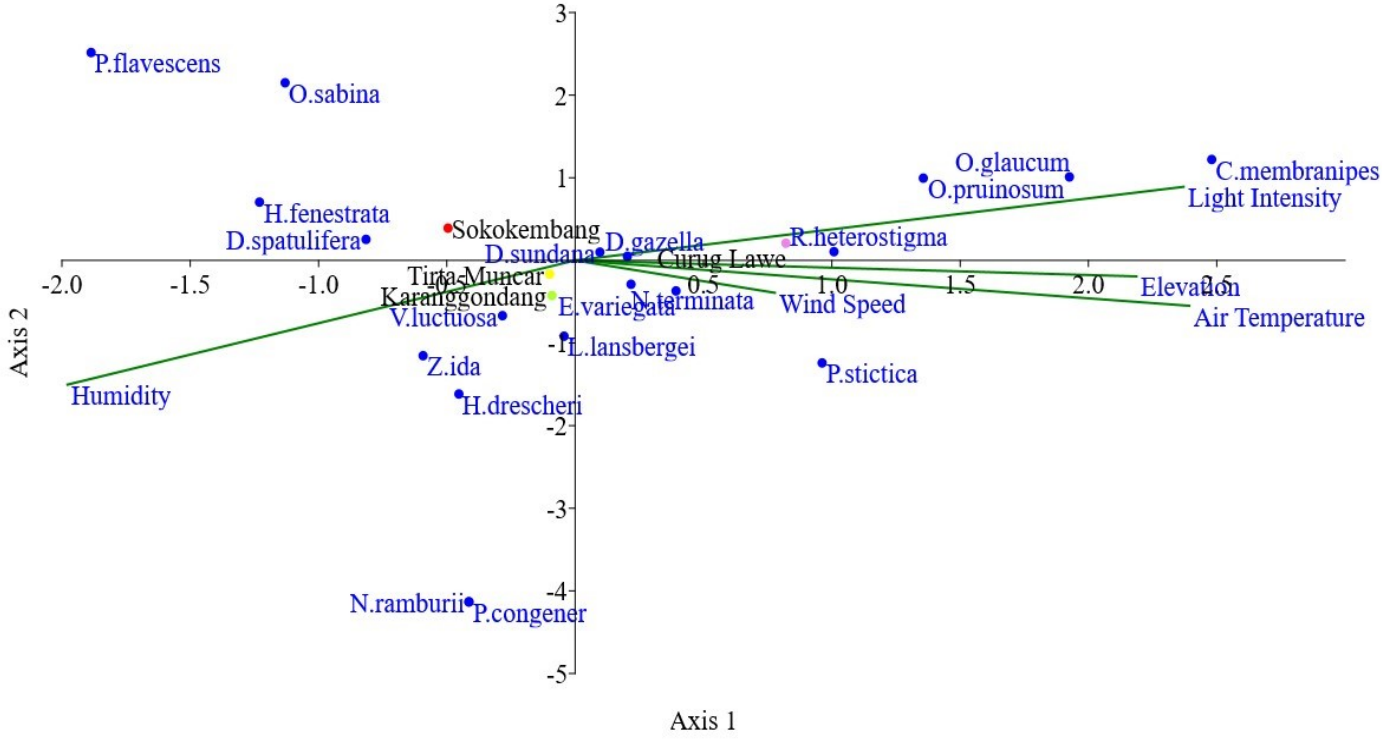

Figure 5. CCA triplot of environmental factors and Odonata community.

parameters on community structure found in 4 locations of Petungkriyono forest. Environmental factors affect the presence of dragonflies The results of the CCA analysis show that light intensity contributes to the structure of the Odonata community. CCA ordination map at the second axis showed that more of Odonata distributed a long gradient light intensity (Figure 5).

Light intensity strongly influences the distribution and abundance of *D.sundana*, *D.gazella*. *R.heterostigma*, *O.pruinosum*, *O.glaucum*, and *C.membranipes* (Figure 5). Species *D. sundana* and *D. gazella* in Curug Lawe have a strong correlation with light intensity, the abundance of both species is influenced by fluctuations in light intensity because species from the Genus *Drepanosticta* are a group of dragonflies found around rivers with low light intensity canopies (Diniarsih 2016). Zygoptera groups such as those from the genus *Drepanosticta* tend to be more commonly found in areas with low light intensity than Anisoptera such as those from the genus *Orthetrum*. The light intensity can affect air temperature fluctuations related to thermoregulation and the importance of dragonfly flying. Dragonflies need sunny weather and warm temperatures to be able to flap their wings (fly). This is supported by reports from (Lutz & Pittman 1970) that when there is a sudden change in light intensity, the number of species and individuals of dragonflies observed tends to change. such as during rainy weather the number of species and individuals of dragonflies observed will move to areas covered by vegetation.

AUTHORS CONTRIBUTION

N.A.N. and H.S. designed the research, N.A.N. collects, analyzed the data, and wrote the initial manuscript. H.S. reviewed and proofread the final manuscript.

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CONFLICT OF INTEREST

The authors confirm that no conflict of interest. The sponsors had no role in the design of the study, collection data, analysis, and writing the script.

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Research Article

Variations of Movement, Dispersal, and Morphometrics among Subpopulations of Javan Endemic Damselfly, *Drepanosticta spatulifera* (Odonata: Platystictidae) in Petungkriyono Forest

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ABSTRACT
Drepanosticta spatulifera is a Javan endemic damselfly. The population is spread unevenly in the Petungkriyono Forest and is threatened due to environmental pressure. The aims of this research are to know the variation of the movement, dispersal, and morphometric among subpopulations of *D. spatulifera*. Movement and dispersal variation data collection was done using Mark Release Recapture (MRR) for six weeks from early August until mid-September 2020. The collection of morphometric samples was done during the last week of the MRR survey and 46 individuals were measured with 12 continuous characters. During the MRR survey, 596 males of *D. spatulifera* were marked and 302 were recaptured. *D. spatulifera* had short movement and dispersal thus no individuals were found across the subpopulations. The distance moved of successive capture and net lifetime movement were dominantly less or equal to five meters. The duration of the MRR survey had a low correlation with the dispersal distance of *D. spatulifera*. In the morphometric variations, closer subpopulations tended to have a similar cluster of morphometric characters. Variation of distance moved between successive capture and wing size from Mangli Stream was significantly different from other sites. The subpopulation of Mangli, the farthest and higher altitude of the sites, had the highest distance move, more disperse, and the largest wing size. Our study showed that *D. spatulifera* was extremely sedentary damselfly. It will enhance inbreeding and vulnerability to extinction. Therefore, the interaction between the subpopulations of *D. spatulifera* in the Petungkriyono Forest needs to be done more.

Keywords: *Drepanosticta*, damselfly, dispersal, morphometrics, variation

INTRODUCTION
Variation is a crucial case for adaptation and evolution. Variation within species is referred to as intraspecific variation (O'Dell & Rajakaruna 2011). Intraspecific variation emerges from the local population or metapopulation (Hanski 2008). Intraspecific variation will maintain the resilience and persistence level of the population. Several forms of intraspecific variation can be explained with movement, dispersal, and morphometric studies (Gyulavári et al. 2011; Dolný et al. 2014; Gibert 2016).

Movement and dispersal variation contribute to rescue effect, colonization, population turnover, and extinction (Hanski 2008). Movement and dispersal give an impact on gene flow then influence the genetic variation and phenotype appearances. Morphometry is a form of phenotype appearance. Previous studies examining the flight ability of Odonata suggested that morphometrics such as wings size and thorax size are potential to affect their flight performance. Flight performance will affect the movement and dispersal behaviour (McCauley 2013).

Drepanosticta spatulifera is one of the Javan endemic damselflies from the Zygoptera Order (Lieftinck 1929). The distribution of Drepanosticta is restricted in Asian tropical forests (van Tol 2009). Species of *Drepanosticta* are usually endemic to the island and have poor flight ability (Kalkman & Orr 2013) likewise *D. spatulifera* which is endemic to Central Java. *D. spatulifera* is only distributed in Slamet Mountain, Ungaran Mountain, and Petungkriyono Forest. Based on previous studies, *D. spatulifera* is a sensitive species, inhabits a small niche, and small population (Diniarsih 2016; Nugrahaningrum 2018; Zaman et al. 2019). These characters are population characteristics that are vulnerable to extinction (Suhonen et al. 2010).

D. spatulifera is more abundant in the Petungkriyono Forest than Slamet Mountain and Ungaran Mountain (Diniarsih 2016; Nugrahaningrum 2018; Zaman et al. 2019). Based on the observation, adult *D. spatulifera* in Petungkriyono Forest appeared throughout the year. Although *D. spatulifera* in Petungkriyono Forest is easier to find, their habitat is uneven and threatened by land conversion. Therefore, it is important to study the intraspecific variation of *D. spatulifera* in Petungkriyono Forest. The effort will inform their survival in patchy habitat and help the conservation management of *D. spatulifera*. In line with that, this study aims to study the variation of movement, dispersal, and morphometrics of *D. spatulifera* subpopulation in Petungkriyono Forest.

MATERIALS AND METHODS

Study Species

D. spatulifera is a member of the family Platystictidae (Lieftinck 1929). The members of the family Platystictidae inhabit deep forests with dense vegetation near a stream. The species is well known as the percher damselfly (van Tol 2009) and the information about it is limited. Based on the IUCN Red List, the status of *D. spatulifera* is Data Deficient (Dow 2009).

D. spatulifera is an endemic Java damselfly. Recently, *D. spatulifera* is only found on the slopes of Mount Slamet, Ungaran Mountain, and Petungkriyono Protected Forest (Diniarsih 2016; Nugrahaningrum 2018; Zaman et al. 2019). Its body size is smaller than other Genera Drepanosticta in Java. The length of the male abdomen is about 29 mm and the length of the hindwing is approximately 20.5 mm. Male *D. spatulifera* have a completely black synthorax, brownish-black abdomen with small yellowish-brown rings on segments 3 to 7, the distal segment 8 is sky-blue, similar to segments 9 and 10. Female *D. spatulifera* have the same synthorax and abdomen segments 1-7 as that of the male, while segment 8 on the dorsum is blue, and segments 9 and 10 are black (Lieftinck 1929). Figure 1 shows the picture of *D. spatulifera.*

Study Sites

The study area was located in Petungkriyono Forest, Pekalongan, Central Java. *D. spatulifera* was found mainly on small streams with dense vegetation in the site. Based on the field survey in Januari, 2020, *D. spatulifera* were found in nine streams; namely Bido (BD), Bancet (BC), Cakrawakti (CK),

Figure 1. (A) Male and (B) Female *D. spatulifera* in Petungkriyono Forest © Amelia Nugrahaningrum.

Mangli (MG), Sokokembang 1 (SK.1), Sokokembang 2 (SK.2), Sokokembang 3 (SK.3), Sepingit (SP), and Srenggi (SR) (Figure 2). Environmental factors at the nine locations were measured (Table 1). However, MRR studies and morphometric sampling of *D. spatulifera* could be performed only in Bido, Sokokembang 1, Sokokembang 2, Sokokembang 3, and Mangli stream because of the high number of *D. spatulifera* in these locations during August-September 2020.

Mark Release Recapture (MRR) Survey

Mark Release Recapture (Rouquette & Thompson 2007; Hassall & Thompson 2012) was conducted from August 8, 2020, to September 19, 2020. All sites were sampled from approximately 9 A.M to 4 P.M for six weeks. The capture and marking of *D. spatulifera* were done on each river with a length of 100m and a width of 4m. Recapturing was done with a length of 300m and a width of 4m for each stream except in Sokokembang 1 where the data was only collected along 50m length and 4m width due to the steep stream.

The aerial net was used to catch *D. spatulifera*. The captured individuals were marked on the thorax using V-Tec paint-based oil and V-Tec round

Table 1. Coordinate locations and environmental factors of the nine sampling sites during August-September 2020.

Loc.	Coordinate Loc.	Alt. (m asl)	Herbaceous Vegetation Cover (%)	Total Vegetation Cover (%)	Air Temp. (℃)		Humidity (%)	
					mean	sd	mean	sd
BD	S 07°04.433' E 109°43.377'	528	51.14	89.42	26.16	3.30	90.57	6.79
SP	S 07°05.064' E 109°43.411'	509	33.83	78.30	24.59	1.36	94.40	4.05
SK.3	S 07°05.160' E 109°43.380'	517	49.76	94.26	24.97	2.46	94.64	4.29
SK.2	S 07°05.338' E 109°43.412'	522	51.35	92.89	27.28	3.28	86.86	7.46
SK.1	S 07°05.721' E 109°43.481'	532	51.03	92.09	25.20	1.34	96.44	3.77
SR	S 07°08.854' E 109°42.369'	817	23.80	72.68	26.86	3.03	80.00	10.18
BC	S 07°08.749' E 109°42.229’	831	58.90	93.20	26.72	1.64	80.39	7.33
MG	S 07°08.739' E 109°42.177'	859	45.28	92.94	24.71	1.54	86.59	6.74
CK	S 07°08.119' E 109°43.934'	1190	22.35	87.80	26.21	1.95	73.99	10.48

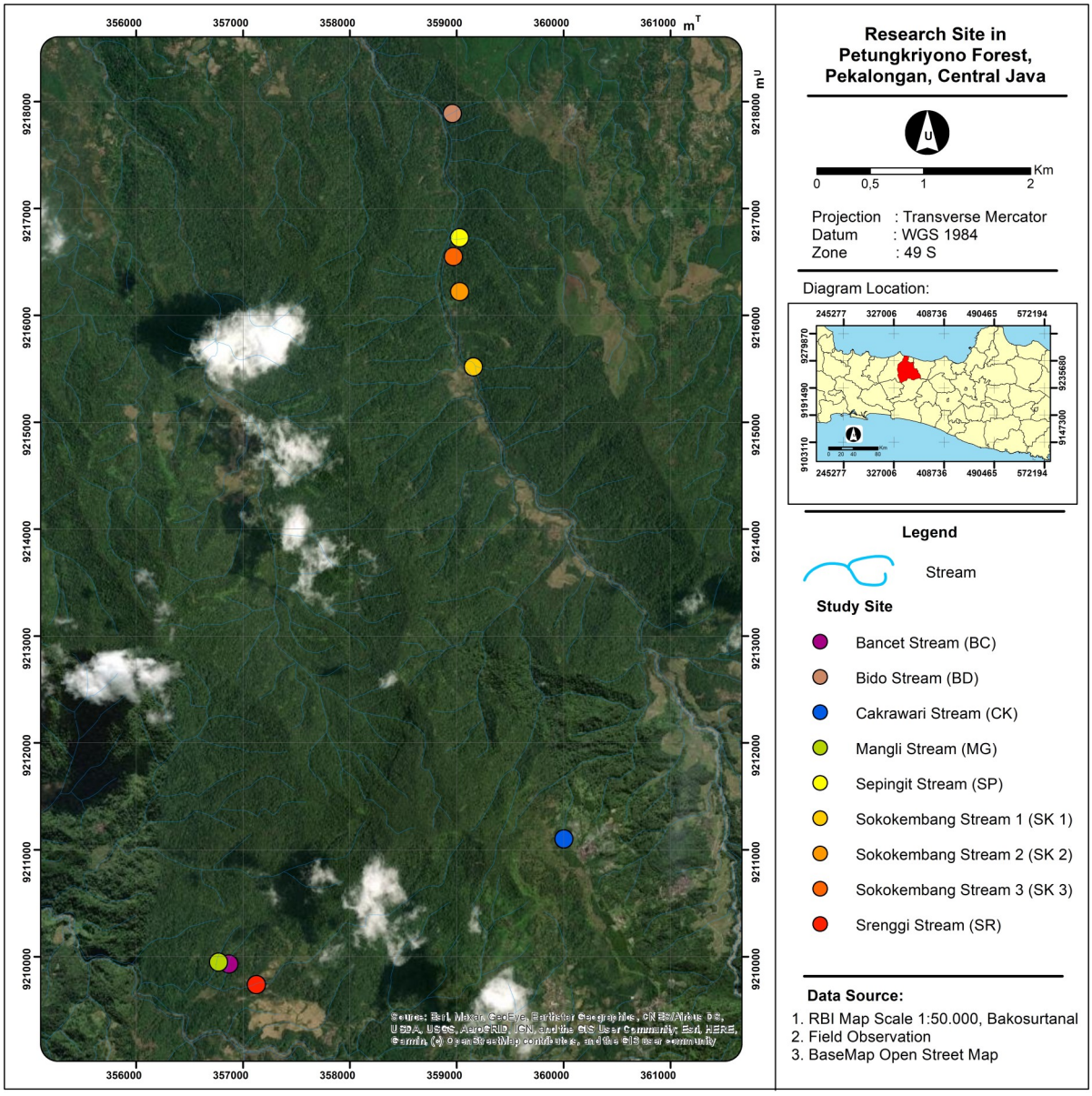

Figure 2. The Map of Study Site of *D. spatulifera* in Petungkriyono, Pekalongan.

brush with a unique code. Unique codes were drawn by colour and shape variation (dot and line). Afterwards, the perch points of the captured individuals were tagged by a ribbon and paper tape. Information about the date, time, and unique code had been written on the paper tape. Furthermore, the captured individuals were released. The process of capturing and marking individuals was done at weeks 0 to 5.

Individual recaptures were done from the 1st to the 6th week. The perch points of the recaptured individuals were tagged by a ribbon and paper tape. Information about the date, time, recapture frequency, and unique code was written on the paper tape. The Euclidean distance between the perch points of the recaptured and previous capture of each individual was measured by roll meter and recorded. The distance value was called as d value. On the 6th week, the Euclidean distance between the first and last capture of each individual was measured as a net lifetime movement or L value.

Morphometric Measurement

Morphometric samples of individuals were randomly selected from the MRR study in the 6th week (13th-19th September 2020). Specimens from the field

were preserved in ethanol 96%. In total, 46 individuals of *D. spatulifera* were measured. Individual details are presented in Table 2. Twelve continuous characters were measured using *Supereyes B011*. These characters consist of the total length of the body (BL), abdomen (AL), right of fore and hind wings (RFw, RHw), width of the head (HW), labrum (LW), distance between the compound eyes (CED), distance between scapes (ASD), distance between nodus and pterostigma on the right wings (RFwNP, rHwNP) (Gyulavári et al. 2011), length of cerci (CL), and length of paraproct (PL). Measurement activities were carried out in November 2020 at the Entomology Laboratory of Biology Faculty, Universitas Gadjah Mada.

Table 2. Number of morphometric individuals of *D. spatulifera*.

Site	n
BD	10
MG	10
SK.1	6
SK.2	10
SK.3	10
All Site	46

Statistical Analysis

The total number of marked individuals, the total number of recaptured individuals, and the percentage of moved and non-moved individuals were calculated. Due to the low movement and dispersal, its value was categorized every five meters. All calculations were done using Microsoft Excel 2019. Parameters information about the data movement and dispersal were arranged in Table 3. This study chose the Kruskal-Wallis and Pairwise Wilcox Test with the Benjamini-Hochberg method to analyse the parameters of movement and dispersal variation. Boxplot was used to compare d values and L values between the sites. A scatterplot was used to visualize the mean of d value and the cumulative distance moved (D) between weeks and sites. We used One Way ANOVA and Post Hoc Duncan Multiple Rank Test to calculate the level of significant differences of morphometric variations between subpopulations. Semi-supervised Local Fischer Discriminant Analysis (SELF) was used to visualize the group of morphometric variation between the sites. Kendall correlation was used to measure the correlation between wing size and dispersal. All analyses were performed using R Studio version 4.0.3.

Table 3. Parameter of movement and dispersal variation (Rouquette & Thompson 2007).

Parameter	Definition
d	Distance moved between successive capture for each individual (m)
D	Cumulative distance moved (m); sum of *d* for each individual
L	Net lifetime movement (m); distance between the first and last capture

RESULTS AND DISCUSSION

Number of Marks and Recaptures

Mark Release Recapture (MRR) was only done in five sites or subpopulations considering the abundance of *D. spatulifera*. They were Bido, Mangli,

Sokokembang 1, Sokokembang 2, and Sokokembang 3. On the other side, only one individual of *D. spatulifera* existed at Sepingit Stream and Bancet Stream on the first week and sixth week. Afterwards, *D. spatulifera* was absent at Srenggi Stream dan Cakrawati Stream due to the lowest canopy cover and the lowest humidity (Table 1).

Table 4. Number of Marked and Recaptured Individuals of *D. spatulifera* during MRR Survey.

Site	Marked	Recaptured		
		Individuals	Percentage	Events
BD	176	92	52.27	150
MG	57	34	59.65	51
SK.1	31	19	61.29	35
SK.2	145	74	51.04	105
SK.3	187	83	44.39	133
All Sites	596	302	50.67	474

In total, 596 *D. spatulifera* were marked. Of these, 302 individuals were recaptured at least once through 474 recapture events. The percentage of recaptured was high, namely 50.67% (Table 4). Short movement behaviour is one of the factors of high recapture. Compared to another study on damselflies dispersal, such as *Coenagrion mercuriale*, the percentage of recapture during MRR only achieved 29% (Rouquette & Thompson 2007), 48% (Keller & Holderegger 2013), and 24.7% (La Porta & Goretti 2020). The maximum recaptured frequency was five times. The interval times between the first and last capture varied. The longest time between the first and last capture was six weeks or 42 days. The highest marked and recaptured individuals were at Bido Streams and the lowest was at Sokokembang 1.

All individuals of MRR were male. Male *D. spatulifera* were easier to observe than females. Most of the time, male *D. spatulifera* merely perched under a canopy. No female individuals were marked during the MRR survey due to the low encounters. Female *D. spatulifera* perched far from the stream or breeding site. Sometimes, female *D. spatulifera* were observed outside the transect and perched on herbaceous trees or tall branches. Dolný et al. (2014) and Zebsa et al. (2015) mentioned that female damselflies will close the stream or breeding site when they are ready to mate and lay eggs. Teneral of *D. spatulifera* was also not observed during the MRR survey since it was still fragile and very risky to handle. Rouquette & Thompson (2007) explained that there was no significant difference between the movement behavior of teneral and adult Odonata.

Variation of Movement and Dispersal

The variation of movement and dispersal were shown by the value of distance moved between successive capture for each individual (d), cumulative distance moved (D), and net lifetime movement (L). The mean distance moved between successive capture for each individual (mean d) was low. The farthest mean d was only reached 85.11m. The distance of mean d dominantly was less or equal to 5m (81.12%). The mean d that occurs more than 10m seems like outlier data (Figure 3).

There was no single individual of *D. spatulifera* who migrated to another stream during the MRR survey. Individual movements were only observed along the water body of the stream. This pattern is similar to *C. mercuriale* in Clitunno River, Italia (La Porta & Goretti 2020) and is classified as a short-

distance movement. They only explored or traveled through the surrounding habitat (Ramakrishnan 2018). Probably, *D. spatulifera* has long-distance behavior. However, long-distance movement of damselflies is hardly observed directly in the field (Keller & Holderegger 2013).

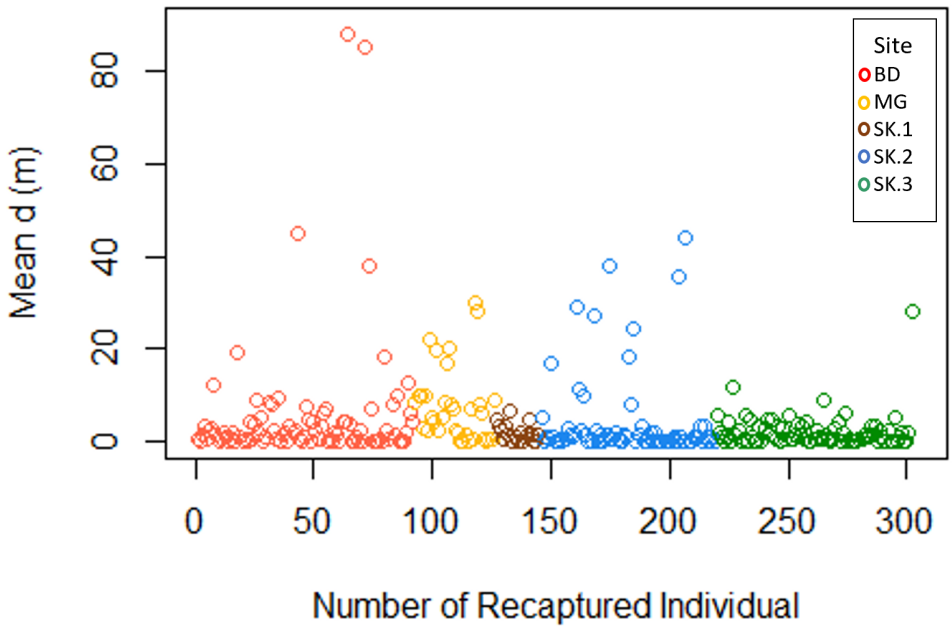

Figure 3. Variation mean d value of *D. spatulifera* individual recaptured between five subpopulations, BD (Bido), MG (Mangli), SK.1 (Sokokembang 1), SK.2 (Sokokembang 2), and SK.3 (Sokembang 3). In total 81,12% individuals had mean d ≤ 5 m; 10,60% individuals had 5<mean d≤10 m; and 8,28% had mean d > 10 m.

Drepanosticta genera are commonly known to have poor flight behavior. Its behavior encourages *Drepanosticta* to become an endemic species to the island (van Tol 2009). *D. spatulifera* in Petungkriyono Forest was an extremely sedentary species. Commonly, it perches at the same twig, branch, or leaf between successive capture. Based on the total number of recapture events (474), 66.03% moved and 33.97% stayed at the same perch point as the previous week.

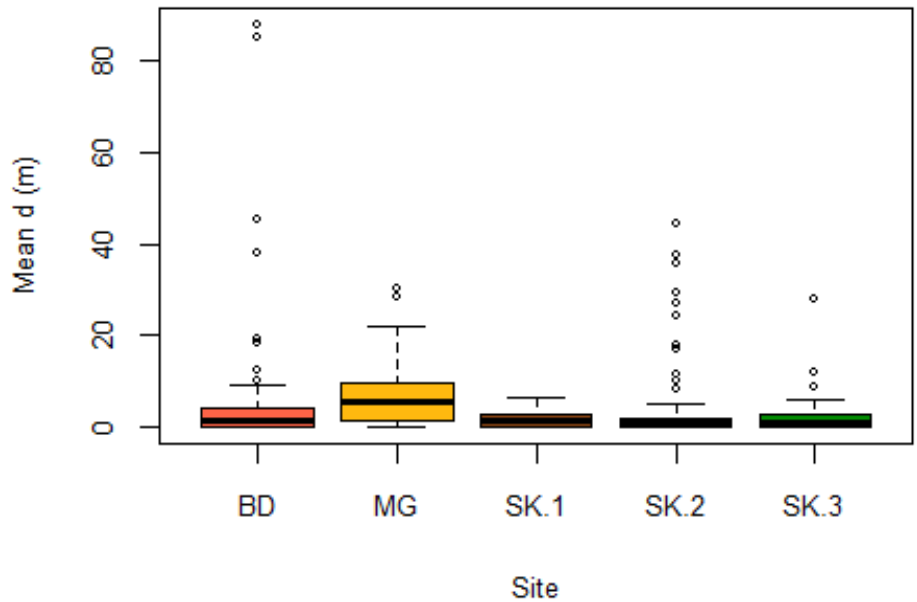

Figure 4. Comparison of d value means of *D. spatulifera* among five subpopulations, Bido (BD), Mangli (MG), Sokokembang 1 (SK.1), Sokokembang 2 (SK.2), and

Sokembang 3 (SK.3). Based on the Kruskal Wallis analysis, the mean d value of subpopulations among the sites was different significantly (X^2=19.757, df= 4, p value< 0.001). Post Hoc Pairwise Wilcoxon Test shows that Mangli Subpopulation is significantly different from other subpopulations (P<0.05).

Figure 4 shows that the Subpopulation of Mangli Stream has the highest mean d and significantly different from other subpopulations. The mean d value of Mangli was 7.51m. The inner-quartile of mean d at Bido, Sokokembang 1, Sokokembang 2, and Sokokembang 3 was less than 5m. The distance movement of Bido, Sokokembang 1, Sokokembang 2, and Sokokembang 3 was quite similar.

The cumulative distance moved of *D. spatulifera* was short. Out of a total of 302 individuals, there were no single individuals who moved more than 100 meters. Four individuals observed for six weeks seemed to be immobile (Figure 5). Net lifetime movement or the distance between the first and last capture also had a short distance. Dominantly, the distance of net

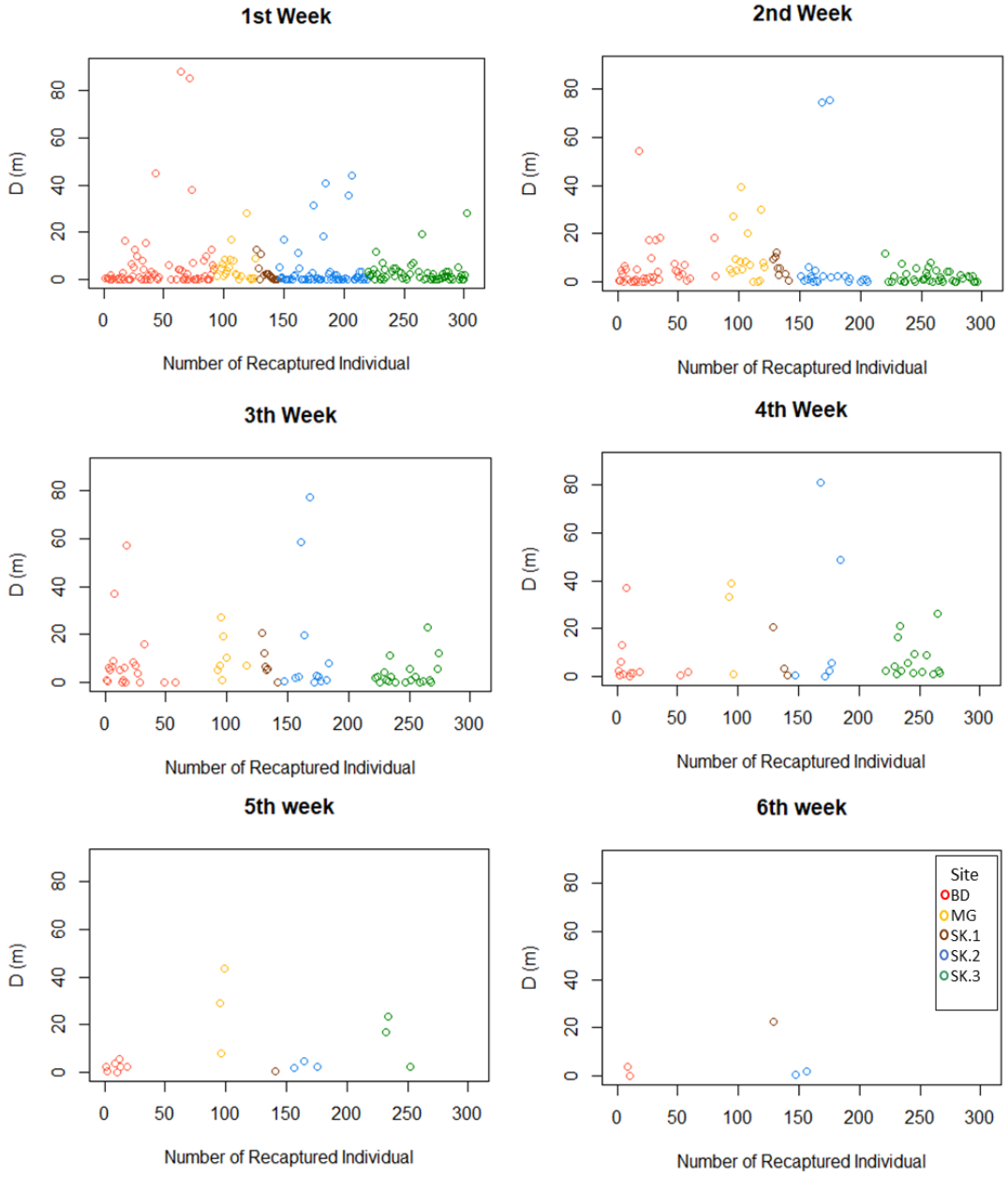

Figure 5. Cumulative Distance Moved (D) of *D. spatulifera* among the five sites, BD (Bido), MG (Mangli), SK.1 (Sokokembang 1), SK.2 (Sokokembang 2), and SK.3 (Sokembang 3), from the 1st week until 6th week. Kruskal Wallis shows that the D value is significantly different (X^2=19.264, df = 4, p-value< 0.001). Pairwise Wilcoxon Test shows that the D value of Mangli is significantly different from Bido, Sokokembang 1, and Sokokembang 3 (p value < 0.05).

lifetime movement was less than 5m (74.83%). A subpopulation of Mangli had the highest distance of net lifetime movement. The median of net lifetime movement at Bido, Sokokembang 1, Sokokembang 2, and Sokokembang 3 was quite similar (Figure 6). Although the time's duration between the first and last capture varied, the Kendall Correlation score showed that net lifetime movement had a low correlation with the duration of the recapture (R=0,21 p-value< 0.05). An increase in the duration of the MRR survey was not followed by an increase in the net age movement.

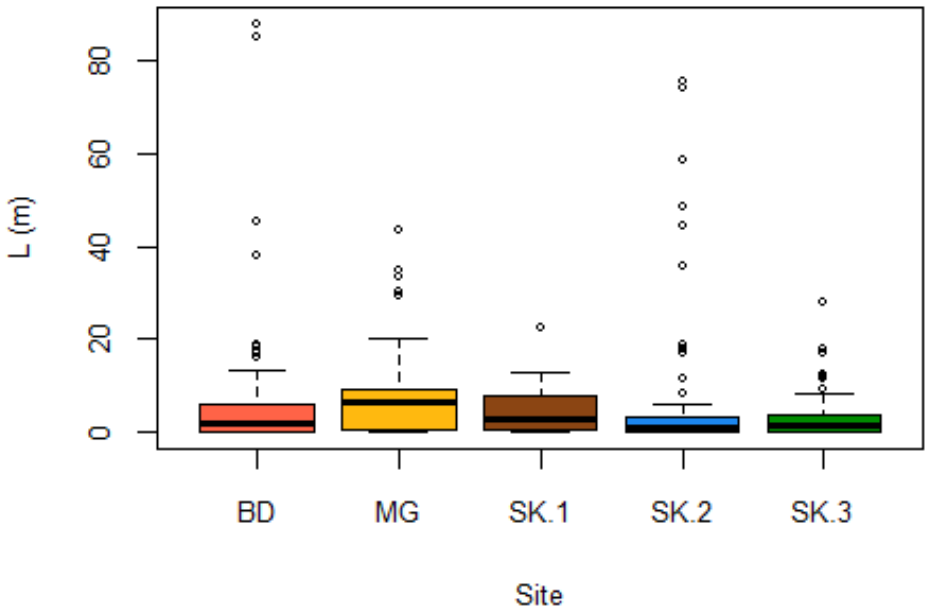

Figure 6. Comparison of net lifetime movement (L) of *D. spatulifera* among the subpopulations of BD (Bido), MG (Mangli), SK.1 (Sokokembang 1), SK.2 (Sokokembang 2), and SK.3 (Sokembang 3). Kruskal Wallis shows that the net lifetime movement among the subpopulations is significantly different (X^2=13.566, df= 4, p-value< 0.05). Pairwise Wilcoxon Test score shows that Mangli is significantly different from Sokokembang 2 and Sokokembang 3 then Bido is significantly different from Sokokembang 3 (p-value < 0.05).

The subpopulation of Mangli had the farthest dispersal than other subpopulations. Its density was lower than and significantly different from Bido, Sokokembang 2, and Sokokembang 3, indicating the potential of inverse density-dependent (Hixon & Johnson 2009). Male damselfly encourages further dispersal distances to find female damselfly to mate (Rouquette & Thompson 2007). However, the inverse density-dependent and dispersal relationship was not significant based on the regression correlation score (r^2=0,094, F= 2,902; P= 0,09957).

D. spatulifera was found in the dense riparian vegetation dominated by the herbaceous canopy (Table 1). The high density of riparian canopy reflects a good and stable habitat (Samways & Sharratt 2010). The stable habitat territory of male damselflies is followed by short dispersal behavior (Dolný et al. 2014). The dense riparian cover protect them from predators and direct sunlight (Palacino–Rodríguez et al. 2020). Damselflies avoid direct sunlight due to their body thermoregulation (Heinrich & Casey 1978; da Silva Monteiro Júnior et al. 2013). It is hard for them to decrease body temperature when the sun's intensity is high (Corbet & May 2008). Stable habitat territory also provides enough prey for damselflies (Shaw 2020). Therefore, the dispersal of *D. spatulifera* was tended to be stable in each stream. Even more, the density of riparian vegetation in Bido, Mangli, Sokokembang 1, Sokembang 2, and Sokokembang 3 was not significantly different.

The absence of migration or emigration of *D. spatulifera* among the subpopulations or streams will decrease the cost of dispersal. Short dispersal uses less energy than long dispersal, thereby lowering the probability of death due to migration (Zboralski et al. 2016; Ramakrishnan 2018). Short dispersal distance might be beneficial as bioindicator species. The presence of *D. spatulifera* in a forest stream represents the quality of stream condition.

Dispersal among subpopulations will influence their persistence and the rate of colonization of vacant patches (Conrad et al. 1999). Nevertheless, there was no strong evidence for the dispersal of *D. spatulifera* among the subpopulations although the streams among the subpopulations were connected by forest. Therefore, there was no evidence of a metapopulation (Allen & Thompson 2010) thus colonization and species turnover at vacant patches will not happen (Hanski 2008). In addition, *D. spatulifera* tends to mate with the same subpopulation. This phenomenon will be followed by the alee effect gradually (Pires & Duarte Queirós 2019) and increase the potential of extinction (Hanski 2008).

Morphometric Variation of *D. spatulifera*

Morphometric variations are the result of the development of organism processes and environmental factors (Hoffmann et al. 2002). The size of the thorax, body, and wings affected the dispersal behavior of damselfly (May 1976; Samejima & Tsubaki 2010). Therefore, we measured the morphometric variation to support the result of dispersal behavior of *D. spatulifera* among the subpopulations.

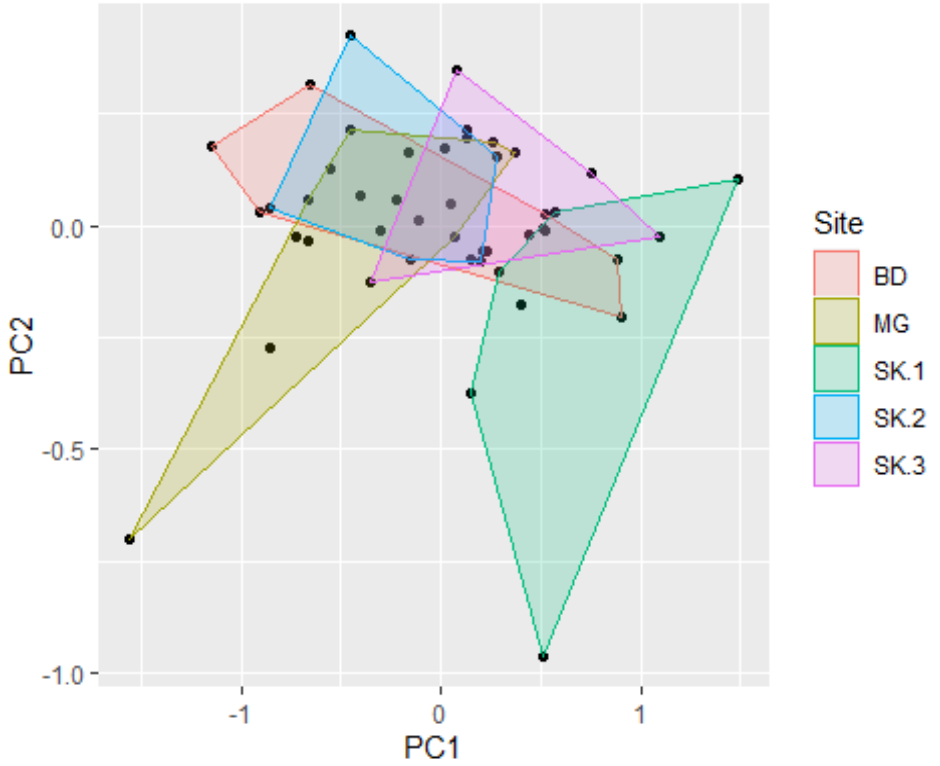

Figure 7. SELF diagram of morphometric variation of *D. spatulifera* among the five sites, namely BD (Bido), MG (Mangli), SK.1 (Sokokembang 1), SK.2 (Sokokembang 2), and SK.3 (Sokembang 3).

Figure 7 shows that morphometric variations among the subpopulations still overlap. Closer subpopulations seem to have quite similar morphometric. The wider overlaps are in Bido, Sokokembang 2, and Sokokembang 3. The distance among the subpopulations affects the degree of variations (Oliveira-Junior et al. 2019). The subpopulations of Bido,

Sokokembang 3, and Sokokembang 2 were closer than Mangli and Sokokembang 1. It is possible that gene flow is still occurring between these subpopulations although distribution across sites is not observed. On the other hand, Mangli and Sokokembang 1 have their own large and non-overlapping territory. SELF results confirmed by ANOVA and DMRT are presented in Table 5.

D. spatulifera is a small damselfly. The abdomen and hind wings of *D. spatulifera* are the shortest compared to other members of the *Drepanosticta* in Java. The range of body length was only 36.629-37.557 mm and the abdomen length was 29,813-31.450 mm, slightly larger than the holotype of *D. spatulifera* from Slamet Mountain (Lieftinck 1929). Based on the 12 continuous morphometric characters, six characters showed significant differences. The subpopulation of Mangli had the longest wings (RFw, RFwNP, RHw, RHwNP) and was significantly different from others. The thorax size (HW, CED, ASD) of Mangli was also greater than others (Table 5).

Mangli is far from other subpopulations (Figure 2) since it lies at a higher altitude. The mean temperature of Mangli (24.71°C) is also lower than Bido, Sokokembang 1, Sokembang 2, and Sokokembang 3 (Table 1). An increase in altitude and a decrease in climate temperature affect an increase in body size (Hagen 2017). Horne et al. (2018) proved that the body size of insects increases along with the additional altitude gradient. Hassall et al., (2008) showed *Coenagrion puella* (damselfly species) experienced an increase in body size and length of the wing along with the climate temperature gradient.

The microhabitat of Mangli had the lowest herbaceous canopy compared to Bido, Sokokembang 1, Sokokembang 2, and Sokokembang 3. Morphological plasticity was seen in response to habitat. Damselfly that inhabits a more open canopy tended to have larger wing morphology (Taylor & Merriam 1995). It must have flown farther to reach the herbaceous canopy cover to rest, hide from predators, and avoid direct sunlight (da Silva Monteiro Júnior et al. 2013; Palacino–Rodríguez et al. 2020).

The length of the wing correlates with dispersal capacity (Sacchi & Hardersen 2013) and occurrence (Rundle et al. 2007). The optimal wings will adjust flight behavior such as foraging and migration (Sacchi & Hardersen

Table 5. Comparison of the mean and standard deviation of the 12 parameters of *D. spatulifera* morphometric among the five subpopulations.

Parameter	BD		MG		SK.1		SK.2		SK.3	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
BL	37.152	1.789	37.221	0.899	37.577	1.008	36.629	1.324	37.509	2.218
AL**	29.813^{b}	1.320	31.450^{a}	0.942	31.817^{a}	0.885	30.755^{ab}	1.081	30.835^{ab}	1.091
RFw***	19.756^{b}	0.678	21.323^{a}	0.547	20.362^{ab}	0.616	20.566^{ab}	0.565	20.09^{b}	0.681
RFwNP***	11.903^{b}	0.502	12.882^{a}	0.374	12.370^{ab}	0.601	12.375^{ab}	0.305	12.083^{b}	0.424
RHw***	19.548^{b}	0.604	21.101^{a}	0.533	20.460^{ab}	0.505	20.429^{ab}	0.417	19.859^{b}	0.688
RHwNP***	11.240^{c}	0.400	12.216^{a}	0.380	11.928^{ab}	0.423	11.644^{abc}	0.245	11.416^{bc}	0.422
HW	3.512	0.115	3.610	0.050	3.576	0.085	3.478	0.123	3.431	0.155
LW	0.859	0.034	0.844	0.031	0.833	0.013	0.831	0.043	0.808	0.059
CED	1.647	0.061	1.674	0.042	1.663	0.078	1.635	0.043	1.602	0.059
ASD	1.028	0.030	1.080	0.077	1.030	0.019	1.022	0.062	0.998	0.052
CL	0.837	0.049	0.854	0.074	0.807	0.056	0.903	0.080	0.863	0.062
PL*	0.462^{b}	0.045	0.486^{b}	0.047	0.487^{b}	0.050	0.489^{b}	0.058	0.538^{a}	0.040

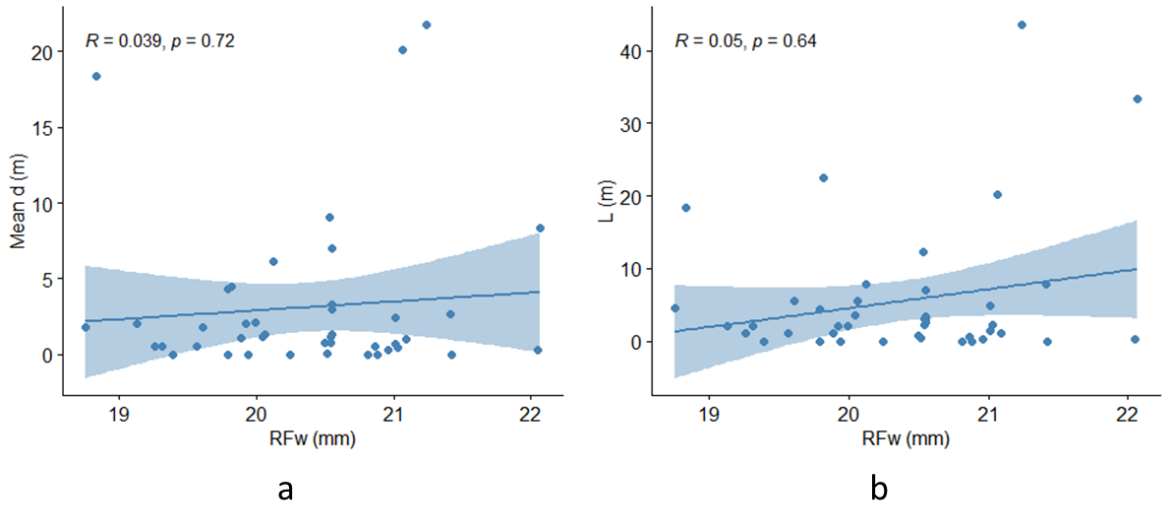

Figure 8. Kendal Correlation of Wing Size (the length of right forewing-RFw) with (a) Mean of Distance Moved between Successive Capture (d) and (b) Net Lifetime Movement (L). The relation between wing size and mean d and L is not significant (p-value >0.05).

2013). Larger male damselflies have more stamina, flight duration, and wing loadings (May 1976; Samejima & Tsubaki 2010). Larger heads of damselflies have greater thermoregulation and thus have better flight performance (Samejima & Tsubaki 2010). This case is similar to *Enallagma*. The larger *Enallagma,* the more distribution points in North America (Rundle et al. 2007). Individuals of *D. spatulifera* in Mangli had larger wings and farther distance movement than other sites. However, based on statistical analysis, the correlation of wing size with the distance moved between successive captures and net lifetime movement was still not significant (Figure 8). Possibly, to obtain a significant and strong correlation between wings and individual dispersal, more morphometric samples are needed.

The paraproct parameter of the *D. spatulifera* subpopulation also shows a different pattern (Table 5). The longest length of paraproct is in Sokokembang 3. In Zygoptera, paraproct is one of the determination keys for identifying the *Drepanosticta* genus (Dow et al. 2018). Paraproct lies at the tip of the abdomen and is paired with cerci. It has trichoid sensilla to hold female prothorax during mating. However, it is still unclear whether the large or small paraproct is more profitable (Suhling et al. 2015).

CONCLUSION

D. spatulifera has a short movement and low dispersal distance. The variation of distance moved between successive capture and wing size from Mangli's subpopulation was significantly different from the other sites. In the Mangli subpopulation, the farther and higher the altitude of the site, the farther the movement, the more dispersed, and the larger the wing size of *D. spatulifera*. Closer subpopulations were less likely to show significant differences in the dispersal and morphometric variations. Our study shows that *D. spatulifera* is an extremely sedentary species. It means that *D. spatulifera* is a good flagship species to represent the forest stream condition. However, it has been threatened due to its high potential of inbreeding, thereby decreasing its fitness and vulnerability to extinction. Furthermore, it is necessary to study the interaction among the subpopulations of *D. spatulifera* in Petungkriyono Forest. This will provide information on the rescue effect and turnover phenomenon of the *D. spatulifera* population. It can also classify the group of subpopulations as local population or metapopulation. In the end, it can show the conservation ways for *D. spatulifera* and the forest habitat.

AUTHORS CONTRIBUTION

A.N. and R.C.H.S designed the research. AN collected and analysed the data, and wrote the manuscript. R.C.H.S reviewed, revised, proofread the final manuscript, and supervised the process.

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CONFLICT OF INTEREST

We confirm that there is no conflict of interest associated with this publication.

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Research Article

Predicting Species Distribution for True Indigo (*Indigofera tinctoria* L.) in Citarum Watershed, West Java, Indonesia

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ABSTRACT

Citarum watershed is a region of approximately 6,610 km² in West Java, Indonesia. Citarum watershed has been degraded through historical land use and vegetation clearing. Rehabilitation of Citarum watershed uses *Indigofera tinctoria* L. that has value as a source of natural blue dye and is considered suitable for the region. Species distribution modelling and Habitat suitability index (SDM/HSI) were undertaken for *I. tinctoria.* The occurrence and environment data (bioclimatic, topography, and soil type) were input to HSI. Results of the *Indigofera tinctoria* habitat suitability model in Citarum watershed are very good (0.9–1) for some parts of the Citarum watershed. The medium and high suitability areas were respectively 4.49% and 4.37% of the area were located in the lowlands (Bekasi Regency and Karawang Regency). Prediction based on climate modelling for 2050 and 2070 estimated that the medium-high suitability area of *Indigofera tinctoria* will be reduced relative to the present.

Keywords: bioclimatic, modeling, natural, suitability

INTRODUCTION

True indigo (*Indigofera tinctoria* L.) belongs to the Fabaceae family. True indigo is known as the world's oldest source of natural blue color (Lemmens & Wulijarni-Soetjipto 1999). Based on Lemmens & Wessel-Riemens (1992), true indigo originates from Asia, but it is now pantropical due to cultivation. True indigo was widely cultivated in India and Southeast Asia in the 16th century, but its blue dye products have been recorded in Sanskrit data from 4,000 years ago (Lemmens & Wessel-Riemens 1992). True indigo is an upright and straight small shrub whose height can reach 1.5 m, branching well from the base of the plant. The leaves are imparipinnate and divide like opposite leaflets, are elongated, pubescent, the apex is micronous and the base tapered. Recemes are axillary and multiple, to 5 cm long and persistent. The petals are pea-form, 1 cm long and the sepals are hairy. The corolla is tementose and elliptical, and pale pink. The pods are about 3 cm long, curved in the apical area, otherwise cylindrical, glabrous and the seeds are 2 mm long. Each cylindrical pod contains about 10 seeds which are greenish gray in

color (Kumar et al. 2020). The other uses of *Indigofera* species include cover crops or green manure.

True indigo has been utilised especially livestock feed. The leaves of true indigo have been used for epilepsy and nervous disorders and to heal sores and ulcers in traditional medicine (Lemmens & Wessel-Riemens 1992).

True indigo is known as tarum in Indonesian. The name tarum comes from the Sundanese language and is known as the "*mangsi-mangsian*" plant (Ariyanti & Asbur 2018). The Javanese call it *tom*. In Bali, it is known as *Taum*, and was once dominant on the island of Nusa Penida Bali where the center of weaving craftsmen is located. The use of clothing dyes is mainly carried out in making *batik* or traditional *ikat* weaving from the archipelago. Apart from being a producer of blue dye, indigo or tarum is also used to produce a green color by combining it with other natural yellow dyes (Ariyanti & Asbur 2018). In the past, tarum was an inseparable part of Sundanese life. Many places in the West Java region are named after this plant, including Tarumanagara, Banjar Pataruman, Tarumajaya, and the Citarum watershed (Prasetyo 2019).

One of the largest and famous watersheds in West Java is the Citarum. The Citarum watershed has an area of approximately 6,610 km^2 with the area covering Bandung, Cimahi, Cianjur, Purwakarta, part of Bogor, part of Bekasi and Karawang (KLHK 2019). Citarum consists of two syllable components in Sundanese which means Ci = water and Tarum = indigo tree (*Indigofera tinctoria*) (Prasetyo 2019). The fact of this name indicates that since ancient times (perhaps the 4th century AD) there has been harmonization between rivers (water) and plants/vegetation or in this case the Citarum watershed is the possibility of domination of the species *Indigofera tinctoria*. Naturally, the quality of the watershed is influenced by soil-forming biophysical factors, namely relief, topography, physiography, climate, soil, water, and vegetation. However, due to various anthropogenic disturbances, the Citarum watershed is currently known as one of the most polluted watersheds in the world.

It is ironic when we see the fact that most of the pollution of Citarum watershed came from a textile producer which uses synthethic colouring. Since 1915, the use of tarum plants as a producer of dyes has been declining and no one is trying to process tarum more easily. Even until now, in the original area of tarum plant itself, namely in Pataruman, the local people no longer recognise the plant. This situation is due to the fact that *batik* entrepreneurs who initially used natural dyes from tarum plants to dye their *batik* fabrics switched to synthetic dyes that are considered more practical in their use (Ariyanti & Asbur 2018). This condition provides a warning for us to be able to act immediately to improve the ecosystem. Pollution is already so severe on agricultural lands that it is feared that it will disrupt food stability or food security. What is most often ignored is the prevalence of land use change and deforestation in the upstream area which exacerbates the sedimentation rate in the downstream and estuary areas, damaging the habitat of flora and can result in loss of species.

This paper analysed the potential distribution of *Indigofera tinctoria* in Citarum watershed areas specifically and in West Java Province in general to map areas that are suitable (have high habitat suitability index) and can be used to re-introduce *Indigofera tinctoria* in Citarum watershed and West Java Province as part of its speedy recovery program (*Program Percepatan Pengendalian Kerusakan*/P3K) as mandated in Presidential Regulation No. 15 of 2018 emphasize the Acceleration of Pollution and Damage Control (P3K) in the Citarum watershed.

MATERIALS AND METHODS

This study was located on Citarum watershed, West Java – Indonesia. It has approximately 6,610 km², of an area with the area covering Bandung, Cimahi, Cianjur, Purwakarta, part of Bogor, part of Bekasi, and Karawang (KLHK 2019) where has the geography location on 5.9107º- 7.2440º S and 106.9640º -107.9475º E (Figure 1). As a large watershed, Citarum area has large three dams: Jatiluhur, Cirata, and Saguling.

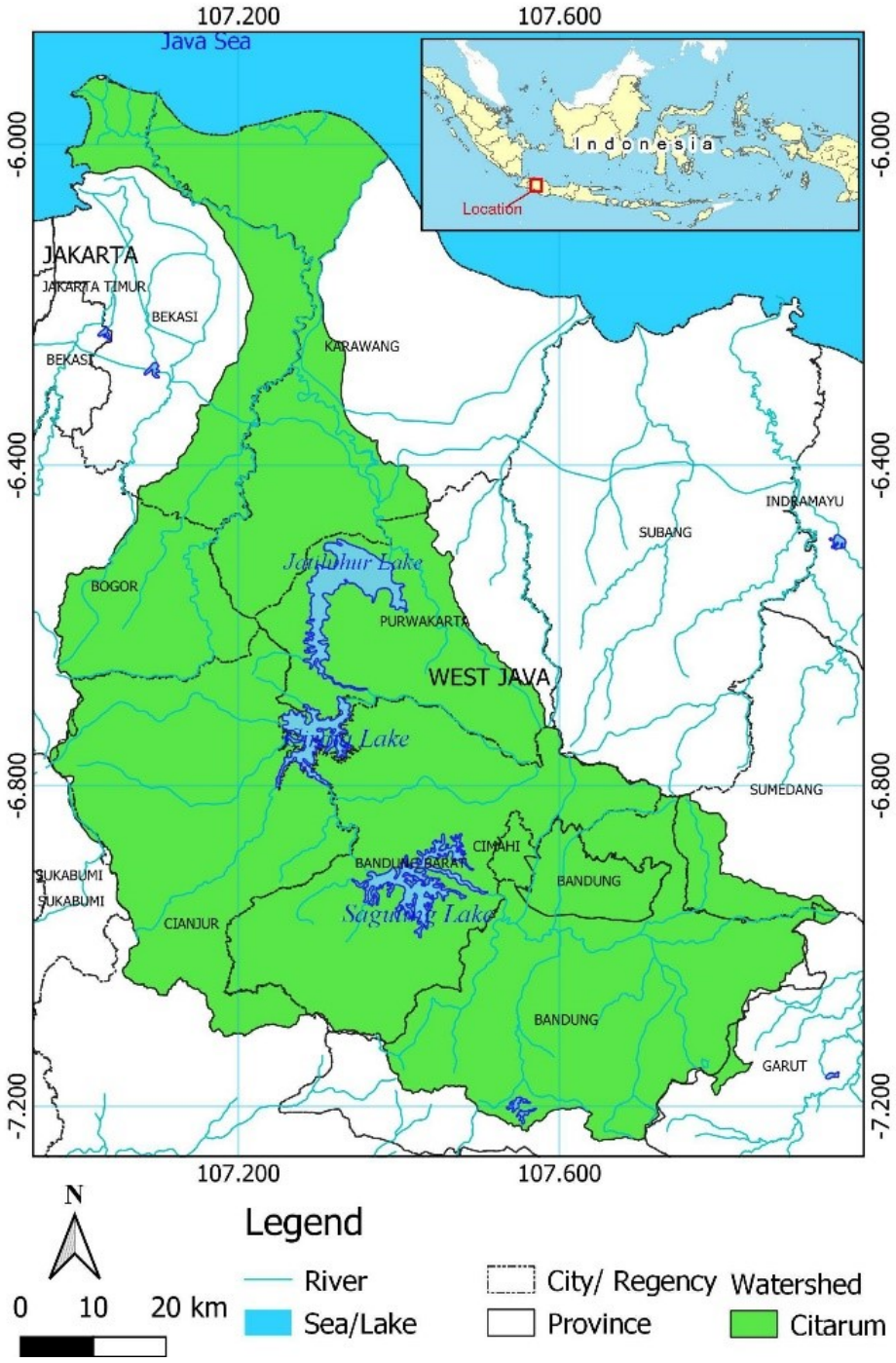

Figure 1. The study area of Citarum watershed, West Java – Indonesia.

Species distribution modelling/Habitat suitability index (SDM/HSI) was done for the targeted species true indigo. Occurrence data of the species obtained from the field as well as its geographic coordinates available in the Global Biodiversity Information Facility (GBIF/www.gbif.org) were used as input layers for the model. There are 1,405 geo-referenced records (worldwide) of this species in the GBIF database record. GBIF records show (based on preserved specimens, observations, and material samples, and

living specimen) that there was a declining number of records of the species. The occurrence data used in the species distribution model of true indigo is 40 occurrence data in Indonesia.

Environmental variables used to compile the species distribution model of true indigo are bioclimatic, topography, and soil. The bioclimatic variables used WorldClim version 2.1 which is the current data for 1970–2000. There are 19 bioclimatic variables data with id bio1 to bio19 (Table 1).

Table 1. Details of data obtained from WorldClim Version 2.1. https://www.worldclim.org/data/worldclim21.html

No	Layer data	Content data
1	bio1	Annual mean temperature
2	bio2	Mean diurnal range
3	bio3	Isothermality
4	bio4	Temperature seasonality
5	bio5	Maximum temperature of warmest month
6	bio6	Minimum temperature of coldest month
7	bio7	Temperature annual range
8	bio8	Mean temperature of wettest quarter
9	bio9	Mean temperature of driest quarter
10	bio10	Mean temperature of warmest quarter
11	bio11	Mean temperature of coldest quarter
12	bio12	Annual precipitation
13	bio13	Precipitation of wettest month
14	bio14	Precipitation of driest month
15	bio15	Precipitation seasonality
16	bio16	Precipitation of wettest quarter
17	bio17	Precipitation of driest quarter
18	bio18	Precipitation of warmest quarter
19	bio19	Precipitation of coldest quarter

Topography variables use SRTM (Shuttle Radar Topography Mission) data. The topographic variables used were elevation and slope which were downloaded from https://www.earthenv.org/topography. Soil variables used in constructing the model are soil types which were downloaded from http://www.fao.org/soils-portal. All environmental variables have a spatial resolution of 30 arsec (~ 1 km). Environmental variables that will be used in compiling the species distribution model were tested for collinearity in the form of paired Pearson correlations between variables. Environmental variables that have a paired Pearson correlation value (r) of more than 0.85 or less than -0.85, then one of the paired variables must be eliminated (Syfert 2013).

Analysis of the species distribution model using the maximum entropy method (Maxent) using maxent software version 3.4.4. Maxent can model the distribution of species using presence-only datasets, where the target probability distribution is identified based on the optimal convergence at maximum entropy (Phillips et al. 2006). Maxent can combine environmental variables in the form of continuous and categorical data in model analysis (Phillips & Dudík 2008). The data occurrence species used to build the model (train) is randomly selected 75% of the data, while 25% of the data is used for the performance model (test). Model robustness was evaluated using

the AUC (Area Under the Curve) (Crego et al. 2014). The model performance evaluation uses the Receiver Operating Characteristic (ROC) method. ROC works by comparing sensitivity and 1-specificity, sensitivity shows how well the model predicts presence, while specificity shows how well the model predicts absence (Phillips et al. 2006), the evaluation results are described in the AUC value (Table 2) (Crego et al. 2014; Sutomo & van Etten 2017; Ellen et al. 2020).

Table 2. Model performance value.

AUC Score	Model performance
0.9 – 1	Very good
0.8 – 0.9	Good
0.7 – 0.8	Moderate
0.6 – 0.7	Not good

The prediction is visualised as the suitability of a grid cell on a scale from 0 to 1, where 0 refers to very low suitability and 1 refers to very high suitability (Sutomo & van Etten 2017; Sutomo et al. 2018). The thresholds used to categorize the suitability area of species are: 0–0.2, non-suitable area; 0.2–0.4, low-suitability area; 0.4–0.6, general-suitability area; 0.6–0.8, medium -suitability area; and 0.8–1, high-suitability area (Li et al. 2020).

The primary output of a SDM is a map that shows the predicted distribution of *I. tinctoria* under the baseline conditions. The resulting raster format file was then processed at QGIS 3.16. A baseline map of West Java was added and the Citarum watershed map was also overlaid. It is important to note that this is not a prediction of where the species occurs, but rather the distribution of suitable habitat as defined by the environmental variables (in this case, current climate conditions) included in the model (Hallgren et al. 2016). Future distribution of species using MIROC5 data with scenario Representative Concentration Pathways (RCP) 8.5, projection year is 2050 (average for 2041–2060) and 2070 (average for 2061–2080).

RESULTS AND DISCUSSION

Variable selection

Examination of true indigo (*Indigofera tinctoria*) distribution around the Citarum watershed using Global Biodiversity Information Facility (GBIF) records (based on preserved specimens, observations, and material sample and living specimen) shows a total of 27 recorded *Indigofera tinctoria* in Java island that there was the declining number of records of the species (GBIF Secretariat 2021). Based on record data on these periodically, in 1829–1940 in total there are twenty-two records, in 1848–1950 four records, and since 1959 up to 2020 there was one record of this species.

Environmental variable selection is only performed on continuous environmental data (bioclimatic and topography), while soil data is not selected because it is categorical data. The results of the collinearity analysis show that several pairs of environmental variables have a Pearson correlation that is more than 0.85 or less than -0.85 (Table 3), so that some of these environmental variables are eliminated. The results of the analysis show that the environmental variables that will be used in constructing the species distribution model are 12 variables, namely: 2 topographic variables (elevation and slope), 1 soil variable (soil type), and 9 bioclimatic variables (number 2,3,7,12,13,14,15,18 and 19) (Tabel 1.)

Table 3. Pairwise Pearson correlations between environmental variables.

	Slope	Elevation	bio1	bio2	bio3	bio4	bio5	bio6	bio7	bio8	bio9	bio10	bio11	bio12	bio13	bio14	bio15	bio16	bio17	bio18	bio19
Slope	1.00																				
Elevation	0.49	1.00																			
bio1	-0.49	-0.93*	1.00																		
bio2	-0.14	0.16	0.03	1.00																	
bio3	-0.14	0.20	-0.14	0.27	1.00																
bio4	0.25	-0.15	0.09	-0.15	-0.90*	1.00															
bio5	-0.44	-0.84	0.95*	0.25	-0.24	0.21	1.00														
bio6	-0.49	-0.91*	0.96*	-0.09	0.02	-0.08	0.87*	1.00													
bio7	0.01	-0.01	0.13	0.68	-0.53	0.56	0.40	-0.10	1.00												
bio8	-0.43	-0.93*	0.98*	-0.07	-0.28	0.23	0.92*	0.92*	0.15	1.00											
bio9	-0.46	-0.88*	0.97*	0.10	0.01	-0.06	0.93*	0.97*	0.08	0.91*	1.00										
bio10	-0.44	-0.93*	0.99*	0.04	-0.27	0.23	0.97*	0.93*	0.24	0.98*	0.95*	1.00									
bio11	-0.53	-0.91*	0.98*	0.09	0.00	-0.07	0.93*	0.97*	0.08	0.93*	0.99*	0.95*	1.00								
bio12	0.06	0.17	-0.19	-0.19	0.49	-0.62	-0.31	-0.05	-0.53	-0.24	-0.10	-0.27	-0.09	1.00							
bio13	-0.15	-0.09	0.14	0.07	0.37	-0.49	0.08	0.20	-0.22	0.04	0.20	0.06	0.22	0.55	1.00						
bio14	0.02	0.09	-0.06	-0.22	0.54	-0.60	-0.19	0.12	-0.59	-0.12	0.05	-0.14	0.03	0.84	0.28	1.00					
bio15	-0.18	-0.19	0.18	0.12	-0.58	0.53	0.29	0.02	0.54	0.21	0.06	0.25	0.10	-0.69	0.01	-0.85	1.00				
bio16	-0.09	0.03	-0.04	-0.06	0.29	-0.48	-0.12	0.02	-0.27	-0.11	-0.01	-0.11	0.03	0.72	0.90*	0.36	-0.07	1.00			
bio17	0.04	0.13	-0.11	-0.20	0.56	-0.61	-0.23	0.06	-0.59	-0.17	0.00	-0.19	-0.02	0.85	0.25	0.99*	-0.88*	0.34	1.00		
bio18	0.07	0.07	-0.17	-0.45	0.13	-0.17	-0.34	-0.11	-0.48	-0.08	-0.22	-0.22	-0.18	0.66	0.07	0.57	-0.58	0.30	0.60	1.00	
bio19	-0.03	-0.04	0.14	-0.01	0.50	-0.52	0.09	0.30	-0.38	-0.01	0.29	0.07	0.23	0.60	0.57	0.65	-0.53	0.44	0.62	0.05	1.00

*collinearity occurs in pairs of variables.

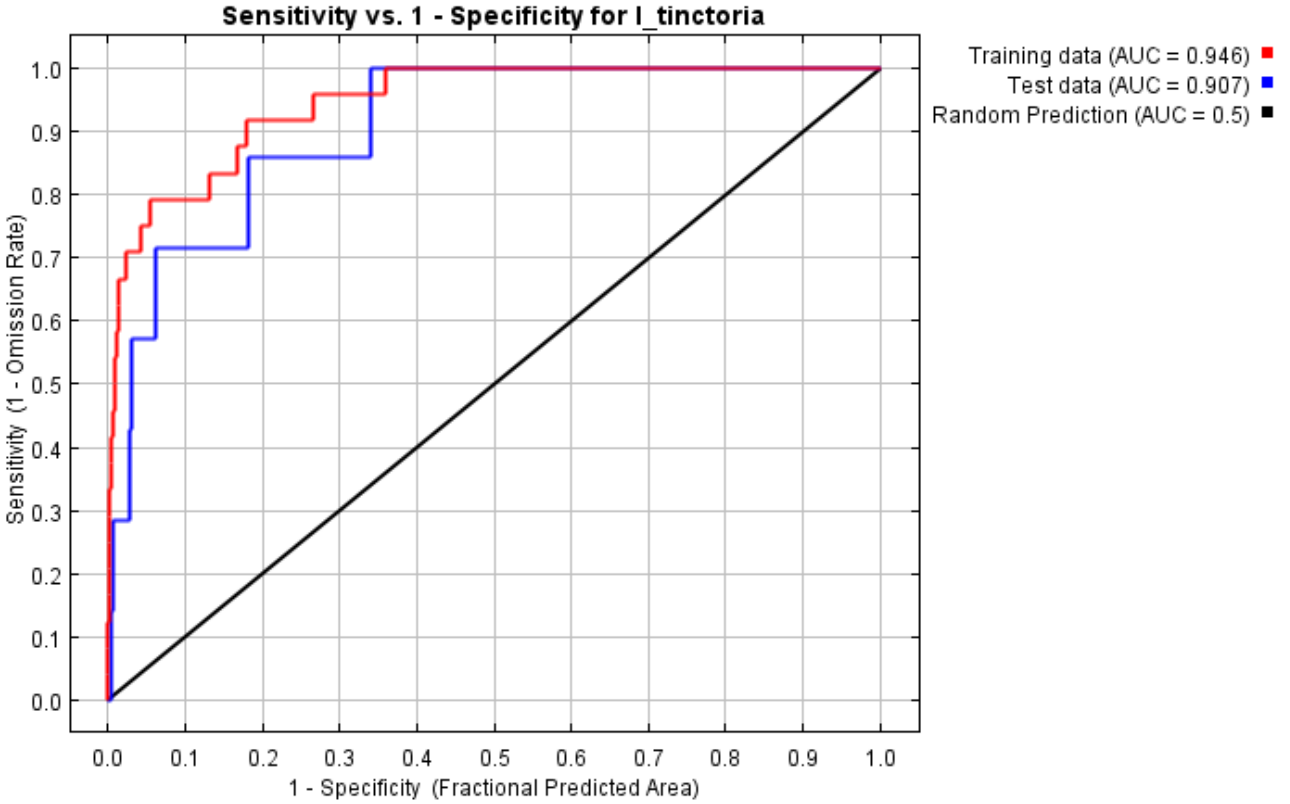

Figure 2. Receiver Operating Characteristic Curve plot habitat suitability model for *Indigofera tinctoria* in Citarum watershed.

Performance Model

The results of the model performance evaluation are represented in the AUC value. Based on the AUC value, the *Indigofera tinctoria* habitat suitability in DAS Citarum is modelled as 'very good' because it is in the value range 0.9–1 (AUC training data = 0.946 and AUC test data = 0.907) (Figure 2). The thin black line on the ROC curve shows the random model AUC, over the grey line, which indicates the better the model's performance is in predicting the presence of samples in the data.

Variable contribution and Importance

The maxent analysis shows that the highest relative contribution of environmental variables in the making of maxent model is bio14 (precipitation of driest month) with a contribution value of 30.73%. Other environmental variables that have a high contribution are the precipitation seasonality, the precipitation of the warmest quarter, and slope. The variables of precipitation of the wettest month, the precipitation of the coldest quarter, and the temperature annual range have a very low contribution with a contribution value of less than 1% (Table 4).

Variables that affect the distribution of species can be seen from the value of training gain and AUC with only the following variables. The variables of annual precipitation, the precipitation of the driest month, precipitation seasonality, and the precipitation of the warmest quarter have high training gain and AUC with only these variables compared to all other variables (Table 4). This indicates that these variables are important in modeling to predict these species distributions. The results of the model analysis show that the bioclimatic variables have a major role to predict the species distribution when compared to topography and soil factors. However, it can be understood that the elevation factor also has an influence on the climatic conditions of a region.

Table 4. Variable contribution and Importance.

variables	Contribution (%)	permutation importance	Training gain with only	AUC with only
Soil	6.50	2.27	0.44	0.79
Elevation	7.39	9.08	0.31	0.77
Slope	15.12	28.39	0.34	0.77
Bio2	1.10	0.00	0.02	0.53
Bio3	3.42	6.29	0.45	0.71
Bio7	0.00	0.00	0.33	0.67
Bio12	3.59	48.55	0.80	0.79
Bio13	0.65	4.39	0.01	0.67
Bio14	30.73	0.00	0.91	0.84
Bio15	18.31	0.24	1.00	0.84
Bio18	13.00	0.08	0.95	0.76
Bio19	0.19	0.69	0.22	0.73

The characteristics of true indigo habitat can be seen through the variable response curve from the modeling results. The response curve describes the response to the presence of true indigo to environmental variables (Figure 3). Based on the results in the response curve, true indigo generally grows in habitats with a mean diurnal range/mean of monthly temperature (bio2) between 5.8–11.6^0 C. This means that the higher the mean diurnal range, the higher the presence of true indigo in that habitat. True indigo grew in habitats with isothermality index value (bio3) of 64–95 with a mean value of 86. True indigo is generally found in the lowlands and mostly grows in areas with an altitude of less than 50 m asl with a slope of 0–18^0. This means that the higher the altitude and slope, the lower the presence of true indigo in these areas.

True indigo seems to prefer habitat with annual precipitation (bio12) between 800 –5600 mm per year with precipitation of driest month (bio14) ranging from 0–300 mm per month. The suitable habitat for true indigo with a logistic output value ≥ of 0.6 is a location with a mean diurnal range of more than 7.8^{0}C, isothermality index of 77–90, elevation less than 50 m asl, slopes less than 5^0, annual precipitation less than 1800 mm per year, and precipitation of driest month of less than 30 mm per month.

It is rare to find literature on the natural habitat environmental conditions for the growth and distribution of true indigo. However, several literatures found do match our model results. True indigo grows from lowland up to 300–400 m above sea level (Kumar et al. 2020). Hariri et al. (2017) report a collection of true indigo samples from locations from 7–343 m asl in Java and Madura Island. True indigo is intolerant to high variability in precipitation rate (Ariyanti & Asbur 2018) and waterlogging (Lemmens & Wessel-Riemens 1992). True indigo also can be found naturally on riverbanks which can explain the presence and abundance of this plant in the past on Citarum watershed.

Current Distribution Species

The result of true indigo habitat suitability modeling result in Citarum watershed can be seen in figure 4. These results indicate that many parts of Citarum watershed are suitable for true indigo, but with different suitability indexes. Most of the Citarum watershed area is non-suitable (an area of 4,645 km^2 or 70.27% of the Citarum watershed) area and low suitability area of 1,024 km^2 (or 15.49% of the Citarum watershed) (Table 5). Locations with non-suitable and low suitability areas are generally located in locations with

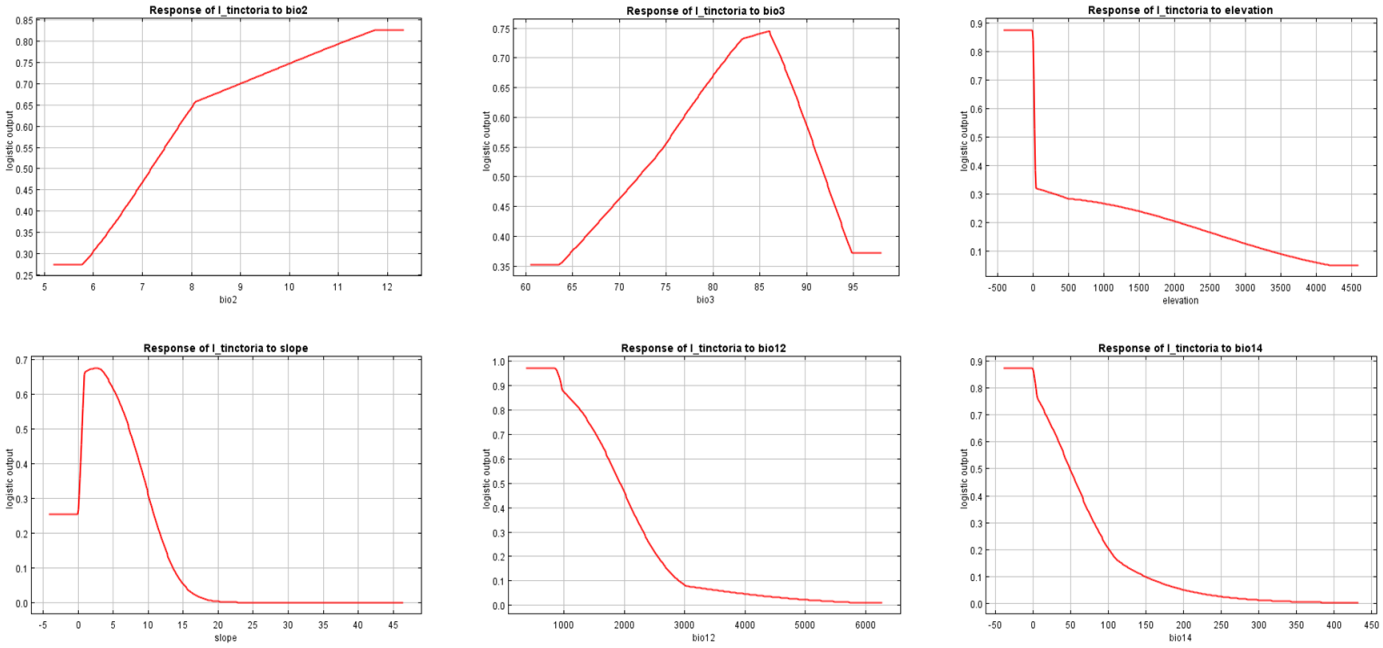

Figure 3. Response curve of the environmental variables that were included in the model.

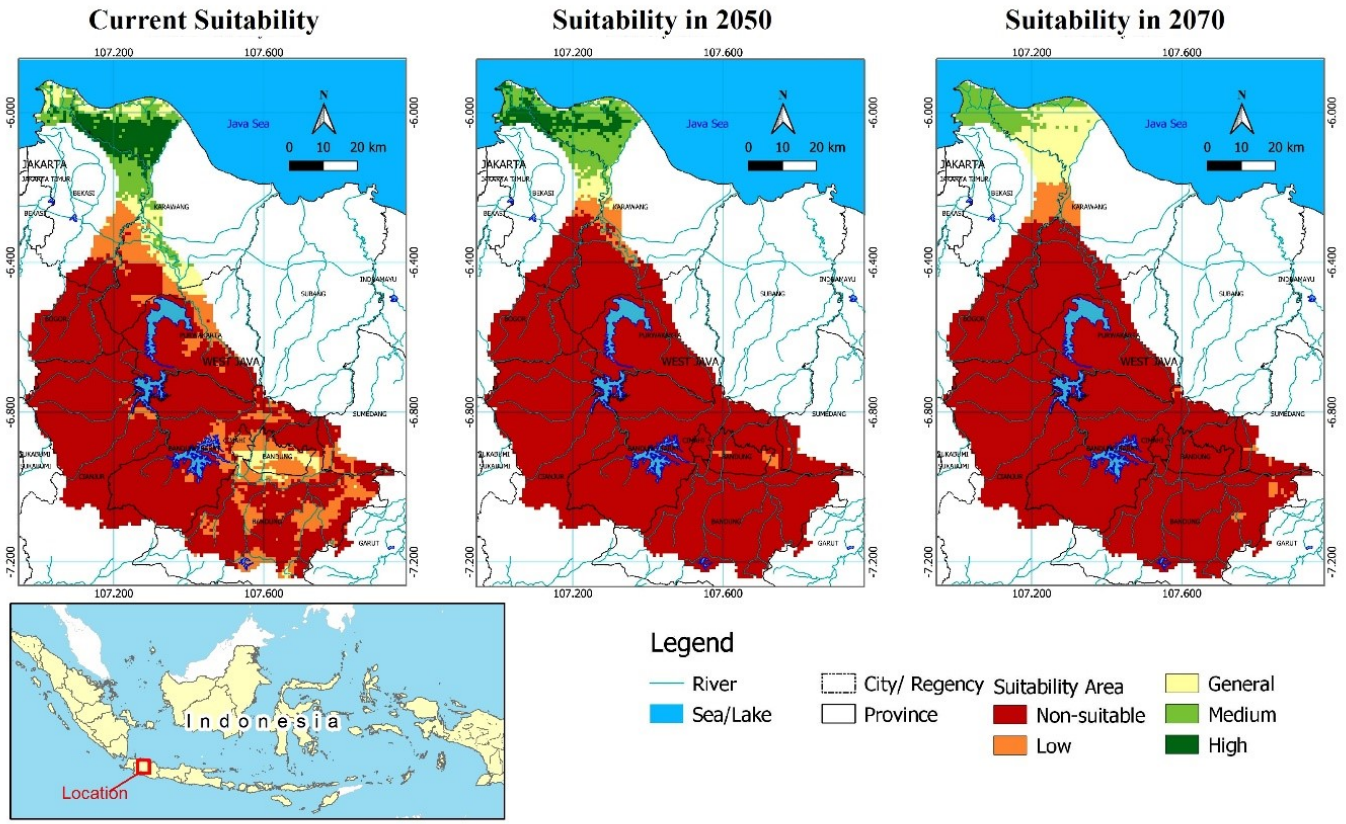

Figure 4. Habitat Suitability Index (HIS) maps show current and future species distribution maps of true indigo in Citarum watershed West Java.

high altitudes, mountains, and hills. These habitats are located in Bandung, West Bandung Regency, Sumedang Regency, Cianjur Regency, Purwakarta Regency, Bogor Regency, part of Bekasi Regency and part of Karawang Regency. The medium and high suitability areas were respectively 4.49% and 4.37% of the Citarum watershed area. Generally, this area is located in the lowlands which include part of Bekasi Regency and part of Karawang Regency.

The results of this study cannot yet concluded if a location with low habitat suitability means that there is no true indigo presence in that location, this is because the modeling only uses species presence data to build the model. In addition, field survey efforts are needed to determine the existence of the species. Location of additional populations would allow for refinement of the model specific to the Citarum watershed. However, the results of this study could be important for the conservation and reintroduction program of the species in the framework of the speedy recovery of the Citarum watershed. Reintroduction of true indigo species could benefit from the habitat suitability map (HIS) map which acts as an indicator of locations that have suitable environmental conditions for the growth of true indigo.

Future Distribution Species

Changes in the suitability habitat of true indigo geographically in the RPC 8.5 scenario in 2050 and 2070 are presented in figure 4 while the change in area is presented the Table 5. In 2050, high suitability area is estimated to decline by 1.3% while medium suitability area is estimated to increase by 0.85%. The increase of medium suitability areas is due to the reduction in the suitability area from high to medium. In 2050 the total area of low suitability and non-suitable area is estimated to increase by 4.6% over the current year.

In 2070 the medium suitability area of true indigo is estimated to decline by 1.01%, while high suitability areas of true indigo is estimated to decline by 4.35% over the current year. In 2070 it is estimated that medium and high suitability areas of true indigo will only be in a small part of the Bekasi Regency and Karawang Regency. The reduction in suitability area indicates that the true indigo population is predicted to decrease in the future due to climate change.

CONCLUSION

In conclusion, currently, true indigo or tarum or *I. tinctoria* has small areas of suitability index across the Citarum watershed. Sadly, the future projection of the high suitability areas for true indigo also declining from 3.07% out of the Citarum watershed in 2050 to only 0.02% in 2070. We suggest that the results of this study can be useful for the conservation of the Citarum watershed's vegetation especially in the reintroduction efforts of the true indigo (*I. tinctoria*). Areas with high suitability index from the model can be used as priority areas for establishing plots for reintroduction studies and areas with low suitability might be improved through restoring ecological functions.

Table 5. Current and future suitability habitat of true indigo in Citarum watershed.

Suitability area	Current		2050		2070	
	km²	%	km²	%	km²	%
non-suitable	4,645	70.27	5,831	88.21	5,855	88.58
low	1,024	15.49	142	2.15	174	2.63
general	356	5.39	82	1.23	350	5.29
medium	297	4.49	353	5.34	230	3.48
high	289	4.37	203	3.07	1	0.02
Total	6,610	100	6,610	100	6,610	100

AUTHORS CONTRIBUTION

We declare that S., D.U., and R.I. are the main contributor whereas S.F.H., I.D.P.D., and I.P.A.H.W. are member contributors of this paper. S. designed the research, wrote down in almost all section of the paper. D.U. designed and wrote the method and results sections. R.I. analyses the data and added in the discussion. S.F.H. added in the discussion. I.D.P.D. added in the introduction and discussion. I.P.A.H.W. added in the introduction.

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CONFLICT OF INTEREST

We declare that there are no conflicts of interest regarding this research or the research funding.

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Research Article

Seedling Diversity Considerably Changes Near Localities in Three Salinity Zones of Sundarbans Mangrove Forest, Bangladesh

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ABSTRACT
The status of natural seedlings near localities in the Sundarbans Mangrove Forest was assessed through a stratified random sampling method to observe seedling composition and diversity, importance value index, family importance value, and species evenness. A total of 63 sample plots of 3,990 m² area were surveyed in three natural and anthropogenic influenced salinity zones of Sundarbans. A total of 16,166 seedlings of 15 species under 12 families were found. Family Euphorbiaceae showed the highest (59.59%) family relative density and Rhizophoraceae presented the highest (20%) family relative diversity Index (FRDI). The maximum family importance value (FIV) showed by Euphorbiaceae (66.26) and the maximum importance value index (IVI) of species has been observed in *Excoecaria agallocha* (114.74). *Excoecaria agallocha* showed the highest relative density (59.6%) and relative abundance (39.87%) where the highest relative frequency (15.27%) was observed by *Heritiera fomes*. The mean stem density and species diversity index in the whole survey area was 2701 stem ha^{-1} and 0.0009 correspondingly. The Shannon-Wienner's diversity index was 1.52 where the maximum Shannon-Wienner's diversity index was 2.708. The Simpson's diversity index and Dominance of Simpson index were 0.38 and 0.62 with Simpson's reciprocal index 2.632. The Species evenness index, Menhinick's, and Margalef's indices were 0.561, 0.118, and 1.445 respectively. The Species diversity index of the three salinity zones were 0.0017, 0.0029, and 0.0035 respectively. The Shannon-Wienner's diversity index of Low Salinity Zone (LSZ), Moderate Salinity Zone (MSZ), and Strong Salinity Zone (SSZ) were 0.887, 1.369, and 1.845 correspondingly where LSZ (0.632) showed the highest Simpson's diversity index follow ed by MSZ (0.394) and SSZ (0.21). The Species evenness index for LSZ, MSZ, and SSZ were 0.346, 0.505, and 0.742 where Menhinick's Index were 0.148, 0.210, and 0.207 respectively. The analysis showed poor diversity indices and the area was dominated by few species with few families. The status is also reduced with increasing salinity.

Keywords: diversity, evenness, family, importance value, richness, salinity, seedling

INTRODUCTION
The flora of the Bangladesh tropical forest is one of the ten global hot spot

zones for biodiversity (Mittermeier et al. 1998) and retains rich biological diversity due to its exceptional geophysical location (Chowdhury 2001; Hossain 2001; Nishat et al. 2002). The forest land and natural forest cover of this country are 1.442 million ha and 1.204 million ha respectively which are managed by the forest department, land ministry, and other individuals (Sobuj & Rahman 2011). Tropical forests are ecologically and economically important for the livelihood of local communities. For the last few decades, the natural forests of the country are rapidly decreasing at an alarming rate by 1- 4% of their current land area (Laurance et al. 1998) due to overpopulation, land use alterations, unsuitable and poor management practices (Khan 2008). The net forest loss in South and Southeast Asia, from 2010 to 2015 was about 25% higher compared to 1990 (Keenan et al. 2015) and Bangladesh is also situated in this zone. Agricultural extension, deforestation, extreme removal of woody and non-woody resources, urbanization, and applying unfitted management tools are the major causes of forest degradation in Bangladesh (Hasan & Alam 2006).

Mangrove forest is one of the most productive vital ecosystems in tropical countries. It provides valuable ecological and economic resources to accelerate sustainable livelihood like nursery grounds, breeding sites for birds, fish, crustaceans, shellfish, reptiles, and mammals with medicinal and aesthetic values (Helal Siddiqui & Islam 2019). It is also a renewable source of wood, accumulation sites for sediments, contaminants, carbon, and nutrients, while also protect against coastal erosion, cyclones, and tsunami (Paolini & Sánchez-Arias 2008; Danielsen et al. 2005; Kathiresan & Rajendran 2005; Badola & Hussain 2005; Alongi 2002; Mazda et al. 1997). Sundarbans mangrove forest is rich in both floral and faunal diversity compared to other mangroves of the world and it is a suitable place for collecting various minor forest products (Islam et al. 2020) and developing tourism (Dey et al. 2020). Prain (1903) and (Seidensticker & Hai 1983) recorded 334 species of plants belonging to 245 genera and 75 families where Field (1995) and Tomlinson (1986) included approximately 16-24 families and 54-75 species respectively. In addition, (Karim 1994) reported 123 plant species belonging to 22 families representing 30 genera, and (Helal Siddiqui 2009) reported about 230 species including non-mangroves. Unfortunately, plant species are decreasing gradually due to climatic change, edaphic factors, and various natural and anthropogenic causes. The diminution of native species is hastening at an alarming rate through the quick loss and degradation of forests in Bangladesh (Rahman et al. 2000; Hossain 2001).

Tree diversity, a portray of the natural forest community is fundamental as they offer resources and homes for almost all other forest species (Huston 1994; Richards 1963; Cannon et al. 1998; Hall & Swaine 1976). For understanding the actions and dynamics of forest ecosystems, the knowledge of the floristic arrangement, their quantitative structure, and diversity are vital (Hossain et al. 2015). Effective conservation measures for sustainable management of forests need an appreciation of phytosociological features of tree species diversity (Feroz et al. 2014; Biswas & Misbahuzzaman 2008). Natural regeneration of forest tree species should be enhanced by the appropriate artificial and natural process for defending forest flora and continuing sustainability of yield, goods, and services (Haque & Alam 1988). Potential information for many native tree species is received from various studies that focused on natural regeneration status in different natural forests of Bangladesh (Hossain et al. 2004; Hossain 1999; Hossain et al. 2013; Miah 1999; Motaleb & Hossain 2007; Rahman et al. 2011; Rahaman et al. 2020).

For formulating a conservation strategy of the forest, quantitative information like regeneration, species composition, and distribution is important (Malik et al. 2014; Malik & Bhatt 2016; Sharma et al. 2014). The

range of biodiversity loss in the Sundarbans mangrove forest of Bangladesh is not exactly known due to a very poor database and limited information (Hossain et al. 2004). So, the study of biodiversity is a crucial requisite which is the main objective of this study to effectively protect and manage the existing natural forests for sustainable livelihoods (Hossain et al. 2018; Hossain et al. 2020) for future generations. Some studies were done before in the total area of Sundarbans (Prain 1903; Heining 1892; Helal Siddiqui 2009; Chaffey et al. 1985; Hossain 2003; Rashid et al. 2008; Saiful et al. 2014; Saiful et al. 2015), but this study focused only those areas where various natural and anthropogenic influenced were present. The study was designed to provide quantitative information of the seedling composition, diversity, importance value index, family importance value, species evenness, and compares the diversity indices of three natural and anthropogenic influenced salinity zones in Sundarbans mangrove forest.

MATERIALS AND METHODS

Study area

In this study, a total of 21 of 55 compartments were selected randomly which represented the three salinity zones of Sundarbans. The Low Salinity Zone (LSZ) is situated between latitude 22°11'08" and 22°80´00"and longitude 89° 04´44" and 89°20'31" and the surveyed compartments were 1, 2, 3, 4, 5, 7, 24, 28 (Figure 1). For the Moderate Salinity Zone (MSZ) the latitude and longitude are between 22°45'13" and 21°15'22" and 89°57'10" and 89°19'59" respectively and the examined compartments were 15, 21, 30, 31, 32, 35, 36, 37, and 38. Also, Strong Salinity Zone (SSZ) are located between latitude 22° 11'08" and 22°80'00" and longitude 89°04'44"-89°20'31" and the measured compartments were 46 and 47. All the compartments are located outer part of Sundarbans and near to the localities where natural and anthropogenic influenced are present (Figure 1). The study areas are situated in the warm, humid tropical region. The salinity range of the three zones were <5 ppt for LSZ, 5−10 ppt for MSZ, and >10 ppt for SSZ. The minimum and maximum mean annual temperatures are 21°C and 30 °C, respectively. The mean annual relative humidity differs from 70% to 80% where annual rainfall varies from 1640 mm and 2000 mm (Forest Department 2010).

Methods

A total of 63 sample plots of 3990 m^2 (21 sample plots of 1330 m^2 area for each salinity zone) area were surveyed followed by a stratified random sampling method in April and May 2019 because most of the phonological behavior (flowering, fruiting, seed dispersal, etc) occurs in April and start to accelerate seed dispersal with seedling recruitment gradually which become more stable gradually to affect seedling diversity. The relative abundance (RA), relative density (RD), relative frequency (RF), and Important Value Index (IVI) were calculated by (Dogra et al. 2009; Nebel et al. 2001; Shukla & Chandal 2000). Family relative density (FRD) and family relative diversity (FRDI) were calculated by following (Rahman et al. 2011). Various diversity and richness indices were scrutinized by following, (Clifford & Stephenson 1975; Kent & Coker 1992; Margalef 1958; Michael 1990; Odum & Barrett 1971; Pielou 1995; Shannon 1948; Simpson 1949; Whittaker 1977). All the empirical data were calculated with the help of Microsoft Excel 2016. The equations were listed below:

a) Density of a species (D) = (Total no. of individual of a species in all quadrats / Total no. of quadrats studied)
b) Relative density (RD) (%) = (Total no. of individual of the species / Total no. of individual of all the species) x 100

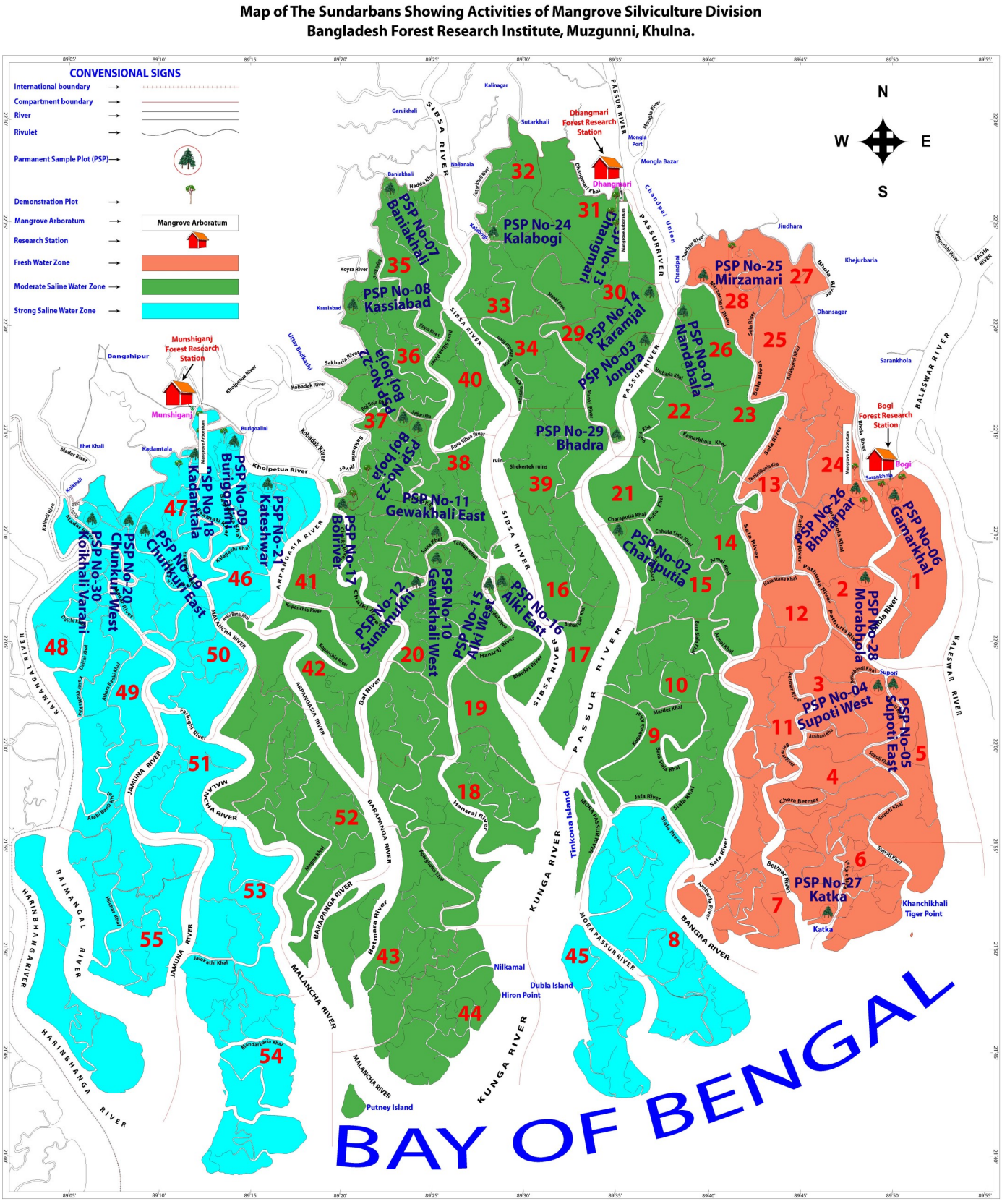

Figure 1. Maps of Sundarbans.

c) Frequency of a species = (Total no. of individual of quadrats in which the species occurs / Total no. of quadrats studied)

d) Relative frequency (%) = (Frequency of one species / Total frequency) x 100

e) Abundance of a species (A) = (Total no. of individual of a species in all the quadrats / Total no. of quadrats in which the species occurred)

f) Relative Abundance (RA) (%) = (Abundance of one species / Total abundance) x 100
g) Importance Value Index (IVI) = Relative Density + Relative Frequency + Relative Abundance
h) Species diversity index (Sd_i) = S/N
i) Margalef's/Species richness index (R) = (S-1)/ Ln (N)
j) Shannon-Wienner's diversity index (H) = - *Pi* In *Pi*
k) Shannon's maximum diversity index ($H_{max)}$ = Ln(S)
l) Simpson's diversity index (D) = $(Pi)^2$
m) Dominance of simpson index (D´) = 1-D
n) Simpson's reciprocal index (D_r) = 1/D
o) Species evenness index (E) = H/ ln(S)
p) Menhinick's Index ($D_{mn)}$ = S/ √N
q) Family relative density, FRD (%)= (N_f/T_i) X 100
r) Family relative diversity index, FRDI (%)= ($Ns/T_{s)}$ X 100
s) Family importance value (FIV) = FRD + FRDI

Where, S = Total number of species; H = Shannon-Wiener's diversity index; N_f = No. of individual in a family; N = Total no. of individuals of all the species; P_i = (Number of individuals of one species / Total number of individuals); T_i = Total number of individuals; N_s = No. of species; T_s = Total number of species.

RESULTS AND DISCUSSION

Seedling status

A total of 16,166 seedlings of 15 species under 11 families were found in a 3990m² area within three salinity zones of Sundarbans. A total of 7723 seedlings of 13 species under 9 families were found in the Low Salinity Zone (LSZ) where *Aegiceras corniculatum* and *Sonneratia apetala* was not found. Besides, 5075 seedlings of 15 species under 11 families were found in the Moderate Salinity Zone (MSZ). In addition, a total of 3368 seedlings of 12 species under 9 families were found in the Strong Salinity Zone (SSZ) where *Cynometra ramiflora*, *Rhizophora mucronata,* and *Sonneratia apetala* was not found in the Strong Salinity Zone (SSZ).

About 59% were represented by one family (Euphorbiaceae) and about 26% by two families (Rhizophoraceae and Sterculiaceae). Maximum of three species were found in family Rhizophoraceae and two species in Arecaceae and Meliaceae where other families had one species (Table 1). Family Euphorbiaceae showed the highest (59.59%) family relative density followed

Table 1. Family composition, number of species, number of seedlings under each Family, Family relative density (FRD), Familyrelative diversity Index (FRDI), and Family importance value (FIV) index of the seedling in Sundarbans.

Family	No of Species	No of Seedling	FRD (%)	FRDI (%)	FIV
Euphorbiaceae	1	9634	59.59	6.67	66.26
Rhizophoraceae	3	2565	15.87	20	35.87
Sterculiaceae	1	1677	10.37	6.67	17.04
Meliaceae	2	460	2.86	13.32	16.17
Arecaceae	2	151	0.93	13.32	14.27
Avicenniaceae	1	519	3.21	6.67	9.88
Myrsinaceae	1	495	3.06	6.67	9.73
Fabaceae	1	289	1.79	6.67	8.45
Acanthaceae	1	224	1.39	6.67	8.05
Pteridaceae	1	149	0.92	6.67	7.59
Lythraceae	1	3	0.019	6.67	6.69
Total	**15**	**16166**	**100**	**100**	**200**

by Rhizophoraceae (15.87%), Sterculiaceae (10.37%), Avicenniaceae (3.21%), and Myrsinaceae (3.06%) (Table 1). Family Rhizophoraceae presented the highest (20%) family relative diversity Index (FRDI) followed by Arecaceae (13.32%), Meliaceae (13.32%) and 6.67% showed by other families. Maximum (66.26) family importance value (FIV) showed by Euphorbiaceae followed by Rhizophoraceae (35.87), Sterculiaceae (17.04), Meliaceae (16.17), Arecaceae (14.27), and Avicenniaceae (9.88) (Table 1).

Quantitative characters of Seedling

Among 15 species, one species *Excoecaria agallocha* embodied 59% and a total of 85% was embodied by four species *(Excoecaria agallocha* 59.60%; *Heritiera fomes* 10.37%; *Ceriops decandra* 8.70% and *Bruguiera sexangula* 6.85%) (Table 2). The Highest (15.273%) relative frequency (RF) were exposed by *Excoecaria agallocha* trailed by *Heritiera fomes* (16.36%), *Ceriops decandra* (11.64%), and *Xylocarpus mekongensis* (10.182%) (Table 2). Again *Excoecaria agallocha* showed the highest (39.87%) relative abundance (RA) followed by *Aegiceras corniculatum* (12.29%), *Bruguiera sexangula* (7.70%), *Ceriops decandra* (7.64%), and *Heritiera fomes* (6.48%) (Table 2). The maximum importance value index (IVI) has been observed in *Excoecaria agallocha* (114.74) trailed by *Heritiera fomes* (33.22), *Ceriops decandra* (27.98), *Bruguiera sexangula* (23.64) and *Aegiceras corniculatum* (17.90) (Table 2). It was found that most of the species were least concert list of IUCN conservation status where *Ceriops decandra* and *Phoenix paludosa* were near threatened and *Heritiera fomes* was listed endangered.

Biological diversity indices for seedling

The mean stem density and species diversity index in the whole survey area was 2701 stem ha^{-1} and 0.0009 correspondingly (Table 3). The Shannon-Wienner's diversity index was 1.52 where the maximum Shannon-Wienner's diversity index was 2.708. The Simpson's diversity index and Dominance of simpson index were 0.38 and 0.62 with Simpson's reciprocal index 2.632 (Table 3). The Species evenness index/Pielou evenness index, Menhinick's Index, and Margalef's/Species richness index were 0.561, 0.118, and 1.445 respectively. The values of Shannon-Wienner's, Menhinick's, and Margalef's indices were specified inadequate plant diversity. The Simpson's index revealed that the species were not uniformly distributed and dominated by 3 or 4 tree species (Table 3).

Table 2. Relative Density (RD), Relative Frequency (RF), Relative Abundance (RA), Importance Value Index (IVI), and IUCN (CS) conservation status of the seedling in Sundarbans.

Scientific name	Local name	Family	NoS	RD (%)	RF (%)	RA (%)	IVI	IUCN CS
Excoecaria agallocha	Gewa	Euphorbiaceae	9634	59.60	15.27	39.87	114.7	LC
Heritiera fomes	Sundri	Sterculiaceae	1677	10.37	16.36	6.48	33.22	EN
Ceriops decandra	Goran	Rhizophoraceae	1407	8.70	11.64	7.64	27.98	NT
Bruguiera sexangula	kakra	Rhizophoraceae	1107	6.85	9.09	7.70	23.64	LC
Aegiceras corniculatum	Khalshi	Myrsinaceae	495	3.06	2.55	12.29	17.90	LC
Avicennia officinalis	Baen	Avicenniaceae	519	3.21	6.55	5.01	14.77	LC
Xylocarpus mekongensis	Passur	Meliaceae	296	1.83	10.182	1.84	13.85	LC
Cynometra ramiflora	Singra	Fabaceae	289	1.79	5.82	3.14	10.75	LC
Amoora cucullata	Amur	Meliaceae	164	1.01	7.64	1.36	10.01	DD
Acanthus ilicifolius	Hargoza	Acanthaceae	224	1.39	4.00	3.54	8.93	LC
Acrostichum aureum	Tigerfurn	Pteridaceae	149	0.92	1.82	5.18	7.92	LC
Phoenix paludosa	Hantal	Arecaceae	92	0.57	4.36	1.33	6.27	NT
Nypa fruticans	Golpata	Arecaceae	59	0.36	3.27	1.14	4.78	LC
Rhizophora mucronata	Jhana	Rhizophoraceae	51	0.32	1.09	2.96	4.36	LC
Sonneratia apetala	Keora	Lythraceae	3	0.019	0.36	0.52	0.91	LC
		Total	16,166	100	100	100	300	

Table 3. Different biological diversity indices for seedlings in Sundarbans.

Parameter	Total
Total Area (m^2)	3990
Mean Density (stem ha^{-1})	2701
Species diversity index	0.0009
Shannon-Wienner's diversity index	1.52
Shannon's maximum diversity index	2.708
Simpson's diversity index	0.38
Dominance of simpson index	0.62
Simpson's reciprocal index	2.632
Species evenness index/Pielou evenness index	0.561
Menhinick's Index	0.118
Margalef's/Species richness index	1.445

Biological diversity indices of three salinity zones

The total number of seedlings and mean density for Low Salinity Zone (LSZ), Moderate Salinity Zone (MSZ), and Strong Salinity Zone (SSZ) were 7723 and 4466 stem ha^{-1}, 5075 and 2543 stem ha^{-1} and 3368 and 2110 stem ha^{-1} separately (Table 4). The Species diversity index of the three salinity zones were 0.0017, 0.0029, and 0.0035 respectively. The Shannon-Wienner's diversity index of LSZ, MSZ, and SSZ were 0.887, 1.369, and 1.845 with Shannon's maximum diversity index were 2.565, 2.708, and 2.485 correspondingly (Table 4). LSZ (0.632) showed the highest Simpson's diversity index followed by MSZ (0.394) and SSZ (0.21). The Dominance of Simpson's index and Simpson's reciprocal index were LSZ (0.368 and 1.582), MSZ (0.606 and 2.538), and SSZ (0.79 and 4.762) separately. The Species evenness index/Pielou evenness index for LSZ, MSZ, and SSZ were 0.346, 0.505 and 0.742 where Menhinick's Index were 0.148, 0.210, and 0.207 respectively (Table 4). The Margalef's/Species richness index for LSZ, MSZ, and SSZ were 1.452, 1.758, and 1.477 correspondingly. The mean density explored a higher number of individuals but diversity indices indicated lower diversity in all three salinity areas near localities. That's mean the forest was converted and dominated by only three or four species.

Table 4. Different biological diversity indices for three salinity zones of Sundarbans.

Parameter	LSZ	MSZ	SSZ
Total Area (m^2)	1330	1330	1330
Compartment No	1,2,3,4,5,7,24,28	15,21,30,31,32,35,36,37,38	46, 47
Total Number of Seedlings	7723	5075	3368
Mean Density (stem ha^{-1})	4466	2543	2110
Species diversity index	0.0017	0.0029	0.0035
Shannon-Wienner's diversity index	0.887	1.369	1.845
Shannon's maximum diversity index	2.565	2.708	2.485
Simpson's diversity index	0.632	0.394	0.21
Dominance of simpson index	0.368	0.606	0.79
Simpson's reciprocal index	1.582	2.538	4.762
Species evenness index	0.346	0.505	0.742
Menhinick's Index	0.148	0.210	0.207
Margalef's/Species richness index	1.452	1.758	1.477

Discussion

Sundarbans has very rich floristic diversity compared to other mangrove forests of the world where half of the total number of mangrove species found in the world occurs in the Sundarbans (Bangladesh and Indian Sundarbans) (Patil 1962). But this study revealed the poor forest condition of Sundarbans Mangrove Forest (SMF) and about 59% comprised of one species (*Excoecaria agallocha*) where about 85% comprised of four species (*Excoecaria agallocha, Heritiera fomes, Ceriops decandra,* and *Bruguiera sexangula*). *Excoecaria agallocha* was increased in a highly disturbed area of Sundarbans because its leaves were poisonous and not suitable for grazing. A total of 15 species were found which was higher than the mangrove swamp forest in southern Nigeria (Asuk et al. 2018) and lower than Puerto Princesa Bay (Dangan-Galon et al. 2016) and some other studies carried out in whole Sundarbans (Hossain 2003, Rashid et al. 2008, Saiful et al. 2014, Saiful et al. 2015). The SMF was predominated by Euphorbiaceae and Rhizophoraceae and the dominancy of Rhizophoraceae was also found by (Asuk et al. 2018; Igu et al. 2017; Ogar & Asuk 2015). The total number of species found in this study was lesser than other forests of Bangladesh (Hossen et al. 2021; Malaker et al. 2010; Motaleb & Hossain 2011; Rahaman et al. 2020). This poor forest condition may be the effect of natural and anthropogenic disturbance whereas most of the people are dependent on Sundarbans for their livelihood directly or indirectly (Islam 2019). They entered these areas for collecting fuel, fodder, fruits, golpata, and so on through legal permission of the authorities but they are doing this hamper in their subconscious mind or intentionally. Islam et al. (2020) stated that most of the villagers adjacent to Sundarbans collect fish, honey, golpata, fuel wood, hogla, prawn, hantal, crab, nall, keora fruit, malia, goran stick, shrimp fry, and medicinal plants from Sundarbans but only 31.11% respondents had equipment facilities. Besides this, the government has declared officially the Sundarbans as a pirate-free area in 2018 which improved the destination's image perception to tourists (Haque et al. 2016) which affects diversity negatively. Besides, *Sonneratia apetala* was found only Moderate Salinity Zone (MSZ) because of high palatability and collection of *Sonneratia apetala* seeds for making pickles, it was declined drastically.

The average mean density was 2701 stem ha^{-1} where a maximum of 4466 stem ha^{-1} for LSZ and a minimum of 2110 stem ha^{-1} for SSZ. The same rich density was found in Puerto Princesa Bay, Palawan Island, Philippines (Dangan-Galon et al. 2016). The density of SMF was higher than other forest parts of Bangladesh (Motaleb & Hossain 2011; Rahaman et al. 2020).

The Shannon-Wienner's diversity index (1.52), Simpson's diversity index (0.38), Margalef's value (1.445), and Species evenness index (0.561) were lower than Akpabuyo mangrove and the mangrove swamp forest in southern Nigeria (Olowokudejo & Oyebanji 2016; Asuk et al. 2018). But the Simpson's diversity index (0.38) in this study was higher than (Dey & Akther 2020; Hossen et al. 2019; Hossen et al. 2021) which indicated the lower diversity in SMF than other parts of Bangladesh. The values of Menhinick's (0.118) and Margalef's (1.445) indices designated the limited plant diversity in SMF.

The species richness of this study advocated that many plant species represented by few families may lead to the extinction of many of the species in SMF. The opposite result was found by (Olowokudejo & Oyebanji 2016) in southeastern Nigeria. Species evenness index (0.561) was lower than natural sal forest (0.8) (Rahaman et al. 2020), South Eastern Bangladesh (0.613) (Dey & Akther 2020), Himchari National Park (HNP) (0.853) (Hossen et al. 2019) that indicated the less equitable distribution of understory vegetation in SMF.

In this analysis, it was found that the number of seedlings and mean density gradually decreased with the increase of salinity. The highest number (7723 stem) and mean density (4466 stem ha^{-1}) were found in LSZ in the same area of 1330 m^2 followed by MSZ (5075 stem, 2543 stem ha^{-1}) and SSZ (3368 stem, 2110 stem ha^{-1}). The Shannon-Wienner's diversity index (0.887, 1.369, and 1.845) was steadily increased and Simpson's diversity index (0.632, 0.394, and 0.21) was gradually decreased from LSZ to MSZ and SSZ. Simpson's diversity index indicated that the diversity was higher in SSZ than in the other two zones. The highest species evenness was found in SSZ (0.742) where LSZ showed the lowest (0.346). The Menhinick's Index and Margalef's/Species richness index of all three zones indicated the lower diversity but the value was various with the location. It may be the effect of salinity in water and soil, inundation type, time and duration, grazing, erosion, etc. that is observed during field data collection. But the suggested hypothesis of Ball (1998) was the opposite of this observation; he denied the effect of salinity (water and soil) on diversity declination. All these variables may affect the germination and survival rate of species. Further research needs to identify the degree of effect of these factors in SMF.

It was found that most of the species were on the least concerned list of IUCN conservation status where *Ceriops decandra* and *Phoenix paludosa* were near threatened and *Heritiera fomes* was listed endangered. It indicated that this area only consisted of abundant least concern species like *Excoecaria agallocha*, *Bruguiera sexangula*, *Aegiceras corniculatum*, *Avicennia officinalis,* and *Xylocarpus mekongensis*. Anthropogenic factors, grazing, tourism, increased number of water vehicles, and excessive extraction of food, fodder, and fuel wood with various natural calamities may affect the diversity and richness of species. Helal Siddiqui and Islam (2019) were recommended that the sustainable enrichment plantation will be carried out which showed satisfactory growth and survivability to improve the species richness of these areas. This study had many limitations like differences in methods of study, the intensity of sampling, area covered, habitat types, and plot size used which may affect species richness obtained by other studies (Hossain 2003, Rashid et al. 2008, Saiful et al. 2014, Saiful et al. 2015).

CONCLUSION

The natural regeneration status of forests is very vital for the sustainable management of forests and forest resources. Bangladesh is still lucky to have a high percentage of forest cover in the Sundarbans Reserve Forest (SRF). However, this rich forest is decreasing with time particularly near human habitation to meet the basic needs of fuel, fodder, and small timber where the degree of causes is still unknown. The study will provide quantitative information about the seedling composition which may be very helpful for understanding the Sundarbans Mangrove Forest. The analysis showed poor diversity indices and the forest was dominated by few species with few families. It was recommended that the sustainable enrichment plantation will be carried out to improve the species richness of these areas. The Forest Department is concerned and to lessen this situation has designed co-management committees as well as providing alternative livelihoods to the Sundarbans user groups in cooperation with local NGOs. The study may be supportive and pave the way for advanced research on regeneration potentials of the mangrove species for safeguarding and heightening forests in the future.

AUTHORS CONTRIBUTION

A.S.M.H.S. and M.N.S.P. conceived the study, A.S.M.H.S., M.N.S.P., M.M.R., M.A.I., and S.M.M.H. participated in field survey, M.N.S.P. analyzed data and wrote the first draft, and all authors revised the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicting interests.

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Research Article

Shallow-water Sponges from a High-sedimentation Estuarine Bay (Brunei, Northwest Borneo, Southeast Asia)

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ABSTRACT
Tropical estuaries are important habitats for invertebrates including sponges, a group of marine organisms that fulfill significant ecological roles and provide ecosystem services. Here, we describe the sponge fauna from Pulau Bedukang, a small island in a turbid, variable salinity, acidified and eutrophic estuarine bay (Brunei Darussalam, northwest Borneo). We present records for 14 morphological species (OTUs). Six of these species belong to the Haplosclerida, an order of shallow-water sponges that usually tolerate more variable and extreme physical conditions. Our baseline data contribute to the regional biogeography of sponges and present a reference source for ecological studies on marine animals inhabiting variable estuarine environments. This is the first known record of sponges from the northwest Bornean region of the South China Sea that are not associated with a coral ecosystem; other studies have concerned Singapore, peninsular Malaysia, Thailand, Cambodia, Vietnam, southern China, and Taiwan.

Keywords: Brunei Darussalam, diversity, Extreme Aquatic Environment, Porifera

INTRODUCTION
Sponges are categorized as benthic filter-feeding metazoans that possess a range of size, shape, and color features. According to the World Porifera Database (WPD), more than 24 thousand species have been recorded and approximately more than nine thousand have been accepted as valid species worldwide (van Soest et al. 2018). Furthermore, sponges are a significant component of biodiversity in the Indo-Malayan region, with more than 1,500 described species (van Soest et al. 2012). The region's undisturbed natural substrata such as coral rubbles, rocks, and abandoned shells, provide for settlement and growth of a diverse fauna that dominates in a vast array of aquatic niches. Given the widespread diversity, distribution, and abundance, it is unsurprising that sponges are of ecological and biopharmaceutical importance. Ecologically, sponges impact the substratum through bioerosion and reef stabilization, but also potentially alleviate marine biofouling by filtering the water. Their sensitivity to environmental stress makes them suitable bioindicator species (Bell 2008). They are also of interest for medical and biochemical research, due to the remarkable range of bioactive

compounds they possess (Belarbi et al. 2003; Proksch et al. 2002). Also, in line with the growing preference for natural products in the cosmetic industry, sponges have consolidated their market position as defoliating 'bath sponges'.

Situated on Southeast Asia's Borneo Island with Indonesia and Malaysia, Brunei borders the South China Sea, having extensive sandy beaches and pristine estuarine mangrove and mudflat ecosystems. Fragmented into a Sundaland element just outside the westernmost boundary of a global biodiversity hotspot, the Western Coral Triangle province, Brunei harbors a vast species richness (Silvestre 1992; Spalding et al. 2007; Mustapha et al. 2021) in relation to the spatially-limited habitat. Despite this high degree of biogeographical significance, our knowledge of the sponge fauna along the northwest Bornean coastline is underexplored compared to that of other marine invertebrates, like Cnidarians (Chua et al. 1987; Hoeksema & Lane 2014), and other regions of the South China Sea (Manconi et al. 2013; Lim et al. 2016; Mustapha et al. 2021). The only recent study on sponges near Brunei is that of Yong et al. (2018), but this focused on the biotechnology of coral-associated taxa, rather than specifically investigating sponge diversity. Since much of the sponge fauna remains unknown, the numerous functional roles they play in the environment are underappreciated. The limited surveys and inventories, both spatially and taxonomically, of these invertebrates hamper scientific endeavors and research initiates to evaluate and use their components. Furthermore, their poor representation undermines conservation efforts. Hence, alpha taxonomic work in the region remains a prerequisite for any scientific program involving sponges, because alpha-taxonomy facilitates the preliminary mapping of biodiversity and provides universal species names (Mayo et al. 2008).

The inner Brunei Bay of the Brunei Estuarine System (BES) was selected for this study following observations of well-developed sponge communities at Pulau Bedukang (PB; Marshall et al. 2016). These communities become established on rocky outcrops and mangroves roots and trunks, in the otherwise heavily-sedimented bay (Hossain et al. 2014). Although devoid of wave action, the water is variable in salinity, pH, and suspended-sediment loads (Proum et al. 2018), representing extreme circumstances for typical marine invertebrate animals. For example, the salinity at PB may vary between 19.6 and 31.2 psu and the pH between 7.7 and 8.3 units (Hossain et al. 2019). The rate of sedimentation at the outer bay can exceed 70 $mg.cm^{-1}.day^{-1}$ (Lane & Lim 2013). The BES serves as an extensive nursery ground for fishes, though benthic animals are specifically adapted towards the variability in environmental conditions (Proum et al. 2017). In recent years, there has been a focus on understanding how the estuarine conditions and the extraordinary acidification of the BES affect communities and organisms (Marshall et al. 2021; Bolhuis et al. 2014; Marshall et al. 2016; Hossain et al. 2019). The aim of the present study was to improve understanding of local and regional sponge diversity, and of the sponge taxa associated with highly turbid tropical Asian estuaries.

MATERIALS AND METHODS

Study area

Pulau Bedukang (PB; 4.9784° N 115.0622° E) is a 20-ha island in the Inner Brunei Bay (Figure 1) and is fringed with *Avicennia* sp. and *Sonneratia* sp. dominated mangroves. Extensive mudflats project from the southwest boundary of the island towards a rocky outcrop, informally named as Oyster rocks. This outcrop comprises a substratum of mixed mud and coral and rock rubble that is exposed during spring low tides (Figure 1). The mudflat

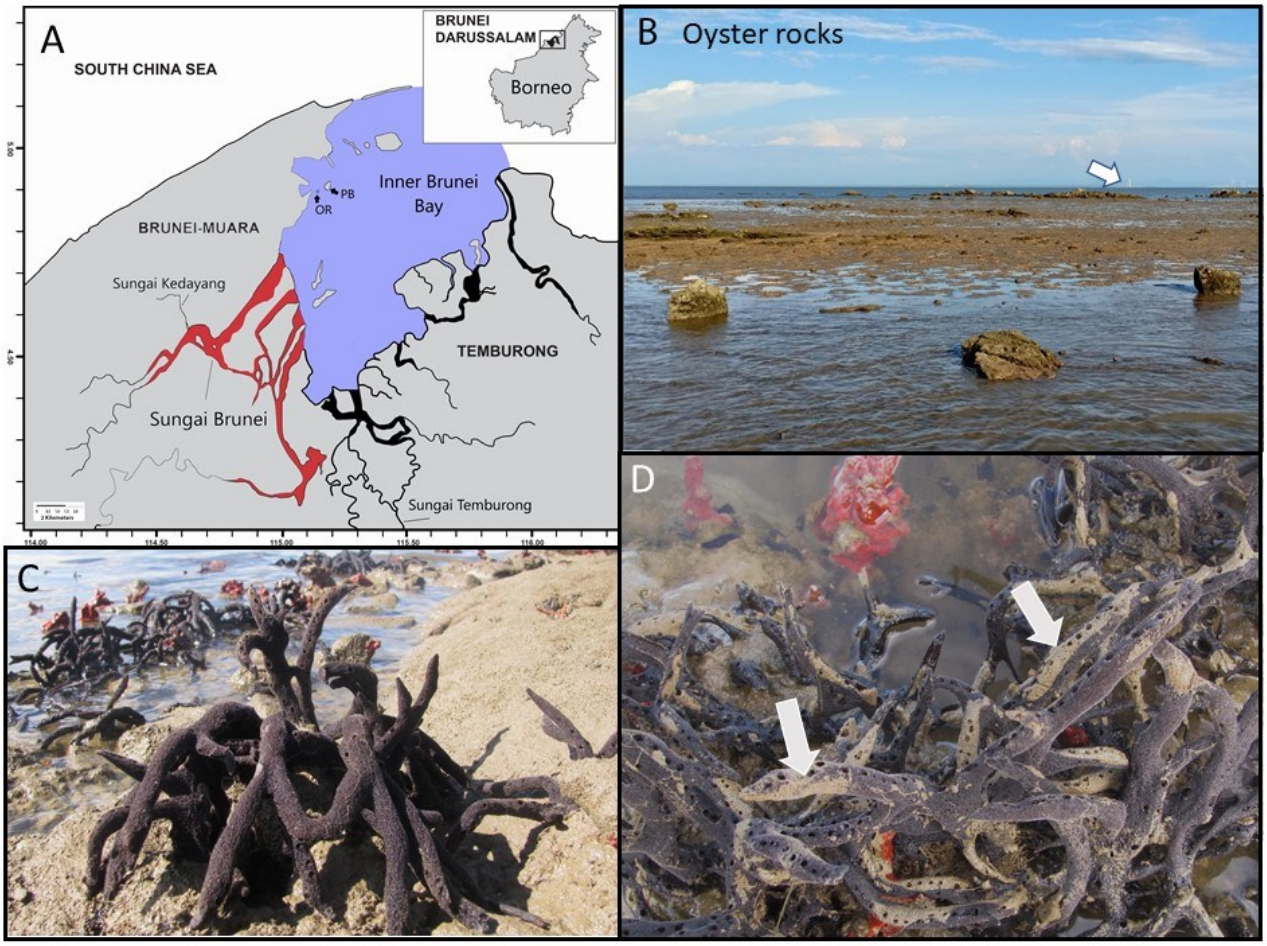

Figure 1. A. Map of the Inner Brunei Bay, showing locations of study sites at Pulau Bedukang (PB) and Oyster rocks (OR); **B.** Oyster rocks during spring low tide (0.2 m CD) revealing the mixed coral gravel, mud, and boulder substratum. Brunei Temburong Bridge construction on the south of PB (block arrow); **C & D.** Massive form of *Haliclona* sp. 1 exposed in air during low tide at Oyster rocks. *Haliclona* sp. 1 and *Niphates* sp. during low tide, arrows indicate sediment deposited on the sponge surfaces. Colour coding of the waterbodies in (A) refers to different salinity and pH regimes (Hossain et al. 2019). Fig C was taken from Marshall et al. 2016.

between the mangroves and Oyster rocks supports prominent communities of juvenile horseshoe crabs, edible mangrove crabs, penaeid shrimps, and gastropod and bivalve mollusks. Generally, the intertidal soft benthic system of Brunei Bay experiences a tropical climate with temperatures averaging around 27°C and an annual rainfall of 2880 mm per year (1966 – 2006). Its semi-diurnal tide ranges to 2.5 m Chart Datum (Marshall et al. 2016).

Sampling

Sponge specimens were sampled between February and June 2018, the dry season, during low tide from Oyster rocks and mangroves at Pulau Bedukang. Sponges in the mangroves were collected from pneumatophores and trunks. More importantly, we avoided collecting cryptic and sciophilous specimens. An area of ~ 100 m^2 was surveyed at each site, and duplicate specimens were collected, with the aim of representing the conspicuous sponge species in the area. Sampling was undertaken on two separate days.

Identification

Morphological features were first recorded in live specimens, including color, form, and size. Specimens were then preserved in 99% ethanol and transported to the laboratory at University Brunei Darussalam (UBD). Identification followed (Hooper & van Soest 2002) and the Thesaurus of

Sponge Morphology (Boury-Esnault & Rützler 1997). The examination required two kinds of histological preparation. The first was spicule preparation, to determine the kinds of spicules in the skeleton. Bleach digestion was used for this preparation. Small fragments of 'tissue', including from both the surface and deeper parts of the sponge were placed in sample bottles, into which a small quantity of active bleach (sodium hypochlorite) was added. After a short time, the organic components had dissolved leaving only the skeletons. This was followed by three washes of distilled water and a final wash of 99% ethanol. Spicule suspensions were allowed to settle for about 10 to 15 mins between each wash to avoid accidental decanting of smaller spicules. Clean spicule suspensions were then pipetted onto a glass slide and topped with a cover slide. Spicule type and size data are given as minimum–mean–maximum for 25 spicules unless stated otherwise. The second preparation involved cutting a thick section through the sponge tissue to determine the structure of the skeleton, the structure of the water-canal system, and other aspects of histology. Thick tangential and perpendicular hand-cut sections of 1.0 – 1.5 mm were procured from a preserved fragment to examine their skeletal arrangement. Sections were placed onto a glass slide and left to dry, while stacked with weight. After the sections were completely dried, they were mounted on glass slides with a cover slip using DPX mountant (06522 Sigma-Aldrich) to adhere both the spicules and the skeleton to the slides. Finally, both slides were observed using an Olympus SZX10 microscope and camera (DP-75) and digitized using cellSens software (OLYMPUS).

RESULTS AND DISCUSSION

Results

The specimens collected from the mangroves at PB and Oyster rocks consisted of 14 OTUs, constituting seven genera (Table 1). Species richness at both sites was represented predominantly by the *Hippospongia* and *Haliclona* (three species each), from the orders Dictyoceratida and Haplosclerida, respectively.

Species (OTU) descriptions based on morphological characters are given below. *Hippospongia* (three OTUs). *Hippospongia* sp. 1 (Figure 2A) has a

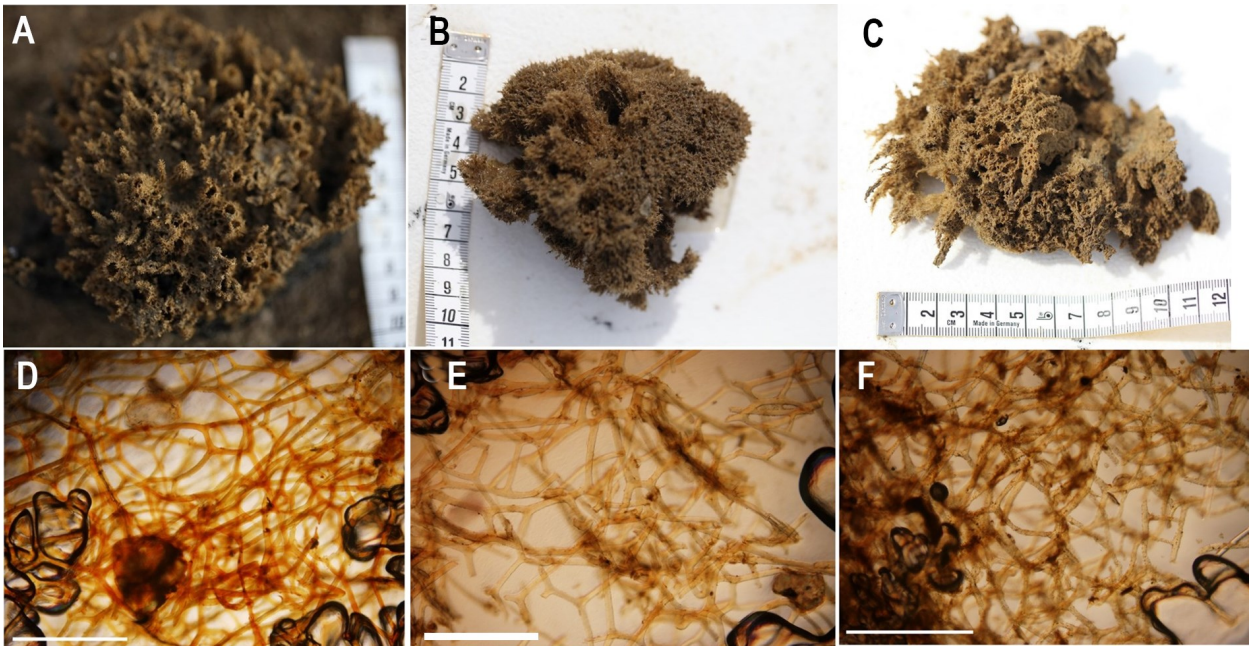

Figure 2. A. Specimen *Hippospongia* sp. 1; B. *Hippospongia* sp. 2; C. *Hippospongia* sp. 3. D. Spongin fiber reticulation for *Hippospongia* sp. 1; E. *Hippospongia* sp. 2; F. *Hippospongia* sp. 3. Scale Bar D - E, 1= 500 µm.

Table 1. Identified sponge specimens based on macroscopic and microscopic examinations. Haploscleriids sponges were the dominant OTUs, followed by sponges from the Dictyoceratiid order.

Class	Subclass	Order	Family	Genus	Species
Demospongiae (Sollas 1885)	Keratosa (Grant 1861)	Dictyoceratida, (Minchin 1900)	Spongiidae (Gray 1867)	Hippospongia (Schulze 1879)	*Hippospongia sp. 1*
					Hippospongia sp. 2
					Hippospongia sp. 3
	Heterosclero morpha (Lévi 1953)	Axinellida (Lévi 1953)	Axinellidae (Carter 1875)	Axinella (Schmidt 1862)	*Axinella sp. 1*
					Axinella sp. 2
		Clionaida, (Morrow & Cárdenas 2015)	Family Clionidae (Orbigny 1851)	Spheciospongia (Marshall 1892)	*Spheciospongia sp. 1*
					Spheciospongia sp. 2
		Haplosclerida (Topsent 1928)	Chalinidae (Gray 1867)	Halicona (Grant 1836)	*Haliclona sp. 1*
					Haliclona sp. 2
					Haliclona sp. 3
				Chalinula (Schmidt 1868)	*Chalinula sp. 1*
					Chalinula sp. 2
			Niphatidae (van Soest 1980)	Niphates (Duchassaing &Michelotti 1864)	*Niphates sp*
		Poecilosclerida (Topsent 1928)	Tedaniidae (Ridley & Dendy 1886)	Tedania (Gray 1867)	*Tedania sp*

massive encrusting to microbenthic form, attached directly to the substratum. Brown when alive and olive green to rust when preserved. Numerous oscules, large with an almost consistent diameter at the apex of turrets. Consistency is firm and compressible. It is also elastic and not easily torn even after preservation. Skeleton has a fiber that protrudes from ostia circumference, producing a porous reticulated surface (Figure 2D). *Hippospongia* sp. 2 (Figure 2B) has a massive, thickly encrusting cushion on hard substrata with microbenthic shape. Color is brown when alive and turns to drab brown when preserved. Oscules are discrete with a slight fistula border, random. Ostia are dispersed over the entire surface. Consistency is soft, spongy, compressible, and difficult to tear. Skeleton fibers protrude from ostia circumference, producing porous reticulated surface (Figure 2E). *Hippospongia* sp 3 (Figure 2C) possesses an encrusting form with irregular folding shape, insinuating over the substratum. It is brown when alive and drab when preserved. Oscules are not visible. Texture is spongy and compressible. Skeleton also possesses fibers protruding from the ostium circumference, producing a porous reticulated surface (Figure 2F).

Haliclona (three OTUs). *Haliclona* sp.1 possesses a branching form which is apically Y-ended. It is grayish-black when alive and turns brown in ethanol. Oscules are recessed along the inside of finger-like projections, whereas ostia are not visible. Consistency is brittle and easily crumbled (Figure 3A). *Haliclona* sp. 2 possesses massive form, ended with a rosette structure at its apex. Oscules are inconspicuous with turquoise color when alive, turning whitish in ethanol. Has brittle and crumbly consistency (Figure 3B). *Haliclona* sp. 3 possesses massive to encrusting forms with a light to dark color when alive that changes to drab when preserved. Oscules are conspicuous, discrete, and often with a raised membranous lip; scattered over

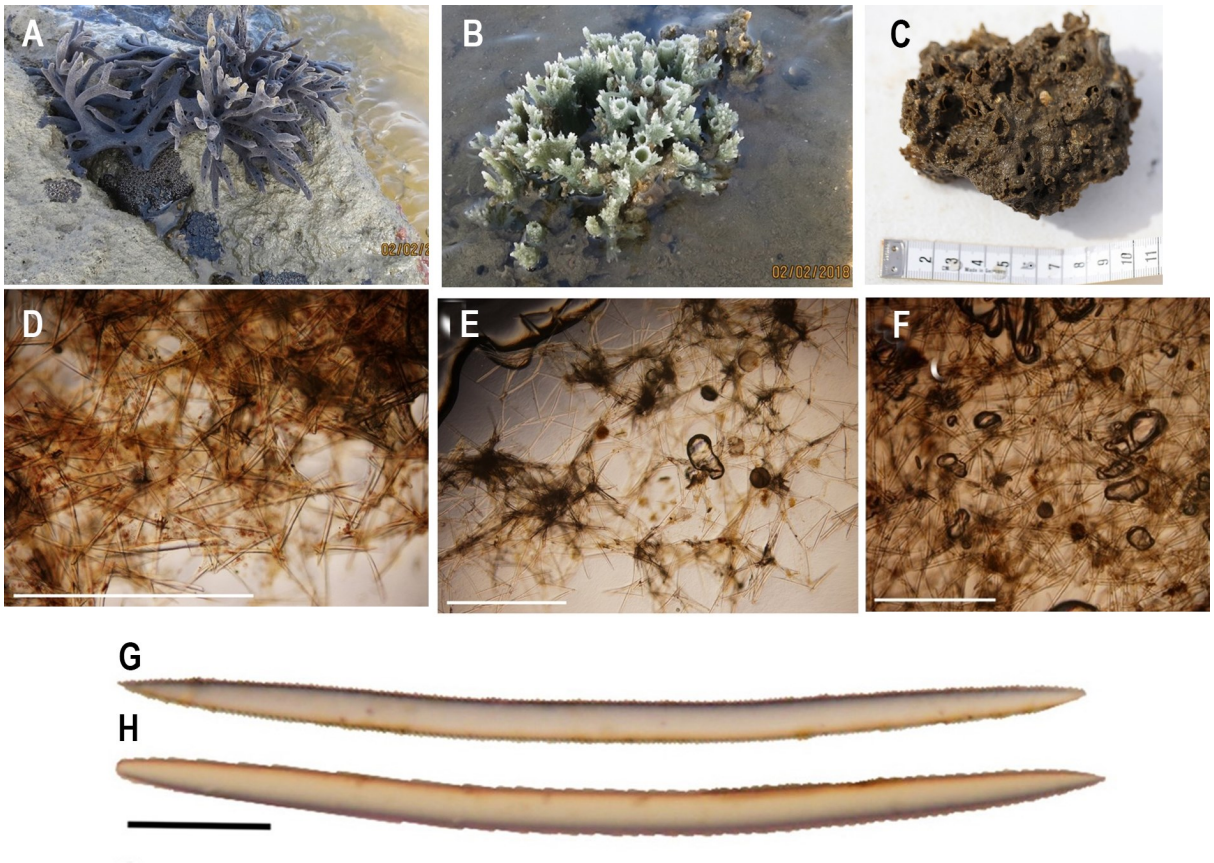

Figure 3. A. Specimen *Haliclona* sp. 1; B. *Haliclona* sp. 2; C. *Haliclona* sp. 3. D. Isodictyal reticulation *Haliclona* sp. 1; E. *Haliclona* sp. 2; F. *Haliclona* sp. 3; G. Oxeas; H. Style spicules. Scale Bar: D - F, 1=500 μm; G & H, 1=50 μm.

the surface. Flushed ostia are dispersed over lateral/external surfaces. The texture is unsubstantial, brittle, and easily crumbled. Surface uneven with dominant ridges of oscula 'lips'. All *Haliclona* spp. possess a skeleton with isodictyal reticulation (Figure 3D, E, F). Oxeas and style spicules are shown in Figure 3G, H & Table 2.

Chalinula (two OTUs). *Chalinula* sp. 1 possesses a microbenthic shape and enlarged basal portion. Oscula possess 'chimney-like' lips protruding through the benthic substratum. Color is brown when alive and drab when preserved. Oscula are conspicuous, but discrete with raised membranous lips. Ostia are not visible. The surface is uneven and unornamented, predominanted with oscula lips, unsubstantial, brittle and easily crumbled (Figure 4A). Isodictyal skeletal reticulation (Figure 4C). Spicules are styles and oxeas (Figure 4F, G, Table 3).

Chalinula sp.2 (Figure 4B) is cushion-shaped, with brown coloration when alive and drab when preserved. Oscules are inconspicuous and the sponge has a brittle and easily crumbled consistency. Skeleton is isotropic (Figure 4D) with secondary lines longer than one long spicule; spicules consist of strongyles, styles, and oxeas (Figure 4E, 4F, 4G, Table 3).

Niphates (one OTU) has a stipitate form, a spheroidal body with a basal stalk approximately the size of the body diameter. Surface uneven and with tubercules. Stalk attaches directly to crevices of rock substratum. Color is bright orange when alive, but beige in alcohol. Oscules are not visible but minute ostia are observed on lateral surface. The texture is firm but is friable and non-elastic in consistency, due to the lack of spongin (Figure 5A). Skeleton is plumoreticulate (Figure 5B), showing a radiate megasclere tract mainly consisting of styles and oxeas spicules (Figure 5C, D & Table 4).

Table 2. Spicule type and size of *Haliclona* spp. specimens based on minimum–**mean**–maximum for 25 spicules.

Specimen	Oxeas	Styles
Haliclona sp. 1	142.1–**159.2**–180.3 µm × 5.7–**7.7**–9.8 µm	130.3–**147.5**–180.3 µm × 6.9–**8.1**–9.6 µm
Haliclona sp. 2	167.9–**198.8**–221.6 µm × 5.1–**8.9**–10.7 µm	–
Haliclona sp. 3	297.9–**345.7**–374.7 µm × 11.8–**14.4**–16.2 µm	315.4–**334.2**–355.1 µm × 14.23–**15.7**–16.4 µm
Haliclona (Haliclona) oculata (Linnaeus 1759) type species of *Haliclona* subgenus *Haliclona* (van Soest et al. 2018)	80–250 µm × 5–10 µm up to 370 × 15 µm	–

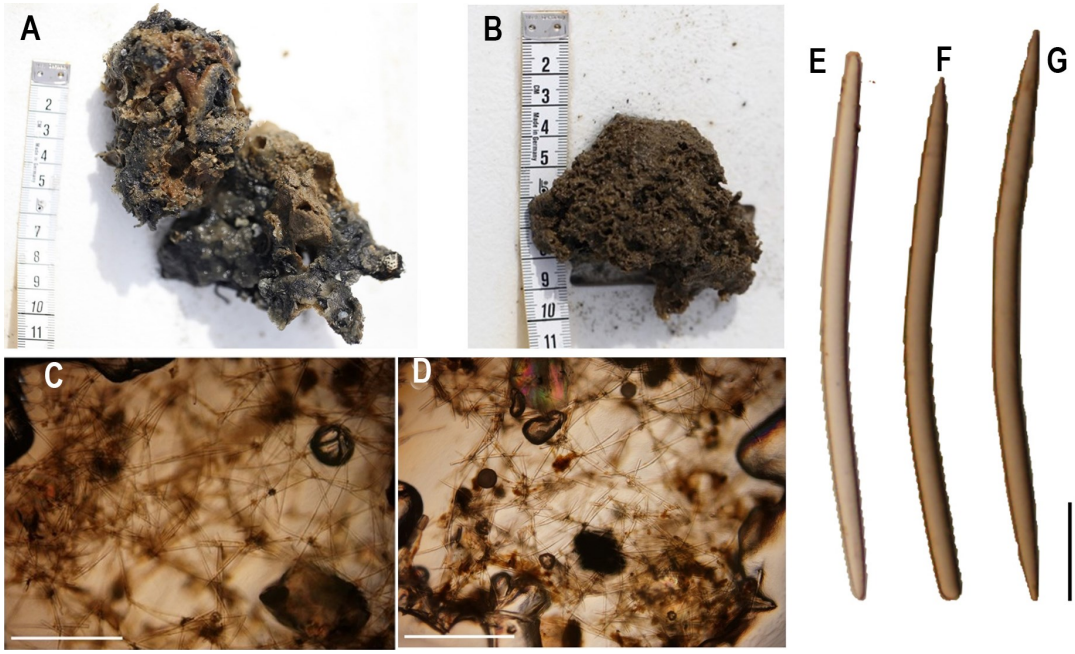

Figure 4. A. Specimen *Chalinula* sp. 1; B *Chalinula* sp. 2; C. Isotropic reticulation in *Chalinula* sp. 1; D. *Chalinula* sp. 2. E. Strongyles; F. Styles; G. Oxeas spicules. Scale Bar: C - D, 1=500 µm; E-G, 1=50 µm.

Table 3. Spicule type and size of *Chalinula* spp. specimens based on minimum–**mean**–maximum for 25 spicules.

Specimen	Strongyles	Styles	Oxeas
Chalinula sp. 1	–	328.9–**342.3**–365.7 µm × 12.9–**14.4**–16.5 µm	330.2–**358.5**–385.3 µm × 9.6–**13.4**–5.7 µm
Chalinula sp. 2	252.5–**269.3**–298.5 µm × 12.1–**12.9**–13.7 µm	225.3–268**.5**–292.7 µm × 8.8 –**12.8**–15.4 µm	215.5–**271.7**–300.1 µm × 4.5–**10.4**–14 µm
Chalinula renieroides (Schmidt 1868) type species of genus *Chalinula* (van Soest et al. 2018)	–	–	81–93.9–105 µm × 3.6–5.1–6.6 µm

Table 4. Spicule type and size of *Niphates* sp. specimens based on minimum–**mean**–maximum for 25 spicules.

Specimen	Styles	Oxeas
Niphates sp.	131.5–**176.7**–261.8 µm × 4.6–**6.1**–7.5 µm	247.1–**426.8**–617.9 µm × 6.6–**12.9**–18.9 µm
Niphates erecta (Duchassaing & Michelotti 1864) type species of genus *Niphates* (van Soest et al. 2018)	–	99–**139**–154 µm × 2–**5**–6 µm

Figure 5. A. Specimen of *Niphates* sp.; B. Plumoreticulate reticulation of skeleton; C Styles; D. Oxeas spicules. Scale Bar: B, 1=500 μm; C - D, 1=50 μm.

Figure 6. A. Specimen *Axinella* sp. 1; B *Axinella* sp. 2. C. Plumose reticulation of skeleton *Axinella* sp. 1; D *Axinella* sp. 1. E. Long styles; F. Long oxeas and common oxeas spicules. Scale Bar: C - D, 1=500 μm; E - G, 1=50 μm.

Axinella (two OTUs). *Axinella* sp.1 possesses a palmate body form, with several flattened digitate branches, 4 cm high by 2 cm wide, fused together at a common base with a short peduncle. Attaches directly to the substratum with its enlarged basal holdfast (Figure 6A). Its brown color in live specimens turns to olive green in alcohol. Oscules are small, less than 2 mm in diameter, with a slightly raised membranous lip, flushed, and positioned at the base of digitate fusion. The texture is hispid, brittle, easily crumbled and furry/brush. Skeleton, plumose in form (Figure 6C) and consisting of long styles; long oxeas and common oxeas spicules (Figure 6E, F, G & Table 5). *Axinella* sp.2 possesses a bushy microbenthic shape with

Table 5. Spicule type and size of *Axinella* spp. specimens based on minimum–**mean**–maximum for 25 spicules.

Specimen	Long styles	Long oxeas	Common oxeas
Axinella sp. 1	752.6–**992.8**–1113.4 µm × 12.2–**14.4**–23.2 µm	742.9–**949.9**–1008.7 µm × 12.9–**16.4**–19.5 µm	–
Axinella sp. 2	835.1–**900.4**–1001.4 µm × 16.1–**18.2**–20.3 µm	700.3–**823.9**–908.9 µm × 15.9 –**17.3**–19.1 µm	115.2–1**71.2**–200.5 µm × 12.1–**13.2**–14.6 µm
Axinella polypoides (Schmidt 1862) type species of genus *Axinella* (van Soest et. al 2018)	210–500 µm × 8–12 µm	–	270–420 µm × 5–12 µm

Table 6. Spicule type and size of *Spheciospongia* spp. specimens based on minimum–**mean**–maximum for 25 spicules.

Specimen	Long tylotes	Short tylotes
Spheciospongia sp. 1	546.1–**695.9**–764.9 µm × 37.8–**38.7**–39.3 µm	204.9–**259.9**–270.7 µm × 11.9–**12.4**–13.5 µm
Spheciospongia sp. 2	665.1–**788.4**–800.4 µm × 36.1–**38.2**–38.3 µm	200.3–**226.9**–238.9 µm × 10.9–**11.3**–12.1 µm
Spheciospongia vesparium (Lamarck 1815) type species of genus *Spheciospongia* (van Soest et. al 2018)	–	280–410 µm × 6–9 µm

Table 7. Spicule type and size of *Tedania* sp. specimens based on minimum–**mean**–maximum for 25 spicules.

Specimen	Styles	Tornotes	Onychaete
Tedania sp. 1	212.1–**255.9**–244.9 µm × 14.8 –**18.7**–19.9 µm	204.9–**229.9**–240.7 µm × 11.1 –**12.1**–13.3 µm	404.9–**459.9**–470.7 µm × 3.9–**4.4**–5.5 µm
Tedania anhelans (Vio in Olivi 1792) type species of genus Tedania subgenus Tedania (van Soest et. al 2018)	173–280.9 µm × 5–12 µm	140–302 µm × 2–7 µm	40–220 µm × 0.5–2 µm and 240–270 µm × 7–12 µm

arborescent to flattened digitate branching. Branching shows complex reticulation to thickly branching in more than one place. The body attaches directly to the substratum. It has a brown color when alive and pale brown when preserved. Oscules are not visible. Has a fibrous and hispid consistency, showing spicule projections on the surface. Skeleton also possesses a plumose form (Figure 6D) that consists of long styles; long oxeas and common oxeas spicules (Figure 6E, F, G & Table 5).

Spheciospongia (two OTUs). *Spheciospongia* sp. 1 (Figure 7A) possesses branching forms, which basally form a massive or bulb that is usually buried under the substratum. Oscules are conspicuous. Brown to black live specimens turn pale brown in ethanol. *Spheciospongia* sp. 2 possesses a massive form without oscules. Brown to black live specimens turn pale brown in ethanol (Figure 7B). Both *Spheciospongia* specimens possess a brittle and crumbly consistency. Likewise, their skeleton is irregular in form (Figure 7C, D) with spicules consisting of two classes of tylotes (long and short; Figure 7E, F & Table 6).

Tedania (one OTU) sponges possess branching forms with massive ramose, having irregular branches with flattened fistula-like projections. Live

Figure 7. A. Specimen *Spechiospongia* sp. 1; B *Spechiospongia* sp. 2. C. Irregular reticulation of skeleton *Spechiospongia* sp. 1; D *Spechiospongia* sp. 2. E. Long; F. Short tylotes spicules. Scale Bar: C - D, 1=500 μm; E-F, 1=50 μm.

specimens are orange turning beige in alcohol. Consistency is soft, easily torn and compressible. Oscula inconspicuous despite ostia observed in furrows of the corrugated surface of branched elevations (Figure 8A). Skeleton is predominantly plumose (Figure 8B), composed of tracts of style spicules (Figure 8C). Other spicule forms include tornotes (Figure 8D) and onychaetes (Figure 8E; Table 7).

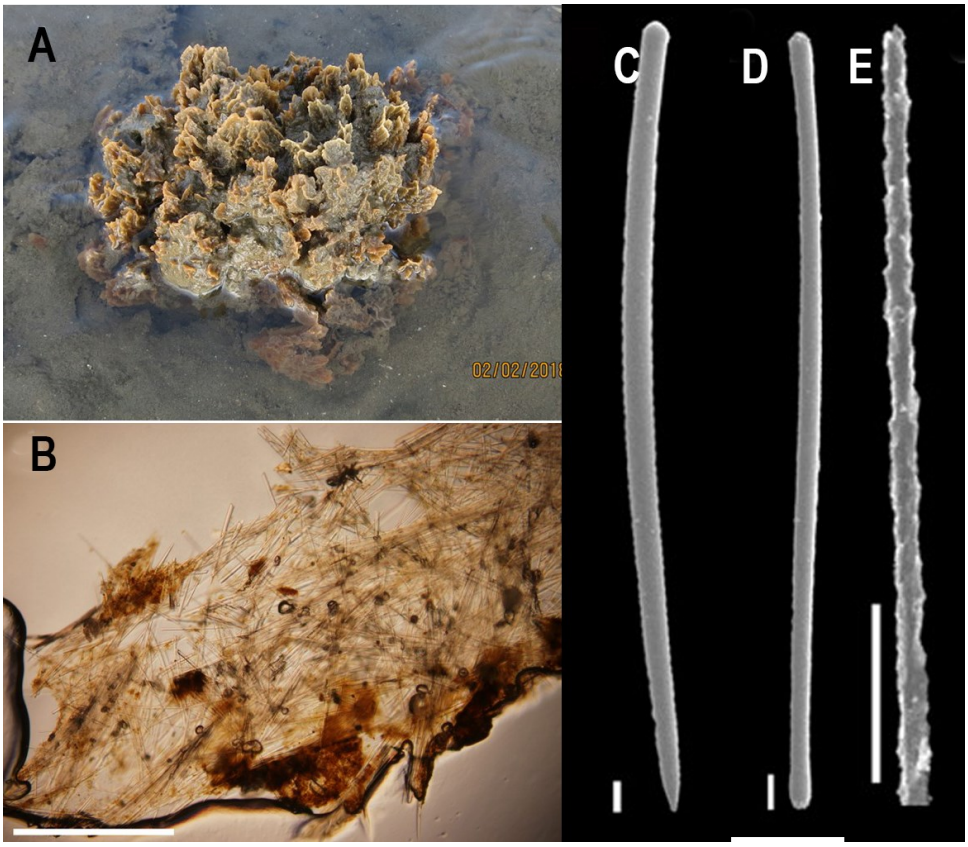

Figure 8. A. Specimen of *Tedania* sp.; B. Plumose reticulation of skeleton, C. Style; D. Tornote; E Onychaete spicules. Scale Bar: B1, 1=500 μm; C - E, 1=20 μm.

Discussion

Studies of sponge taxonomy for northwest Borneo are scarce to non-existent. This first known record for Brunei shows that the shallow-water estuarine conditions near Pulau Bedukang (including Oyster rocks) support at least 14 species. Studies from several other Southeast Asian regions, including coral ecosystems, show distinctly higher diversities, e.g., 33 species from Cebu, Philippines (Longakit et al. 2005), 118 species from Jakarta Bay, Indonesia (de Voogd & Cleary 2008), 126 species from the Eastern Gulf of Thailand (Kritsanapuntu et al. 2001), 197 from Singapore (Lim et al. 2012) and 299 species from Vietnam (Quang 2013). The low number of species reported at PB can be attributed to the highly turbid conditions in the estuary and the very small sampling area (100s of square meters), but possibly also the short sampling duration and the exclusion of cryptic and sciophilous specimens. Nonetheless, the dominance of Haplosclerida at PB is comparable to that of Singapore with 30% (Lim et al. 2012) and the Philippines with 28% (Longakit et al. 2005). This suggests that the species number for Brunei should increase significantly with more sampling effort that including samples from local coral reefs. When considering overlapping species, the PB sponges had a close affinity with Singapore, where inshore marine environments are turbid. Compared to Singapore, there is a similarly high number of *Haliclona* species, with *Haliclona* sp. 2 reported from an area known as the *Haliclona* "chimney" (Lim et al. 2012). *Haliclona* is considered to be a robust, generalist genus that shows unselective larval settlement and a preponderance for harsh environmental conditions, e.g., *Haliclona tenuiramosa* occurs in heavy metal polluted waters and *Haliclona tubifera* in thermally-stressful environments (Rao et al. 2009; Guzman & Conaco 2016). Our findings suggest that *Hippospongia* and *Axinella* also contain species suited to extreme habitats (variable turbidity and salinity).

Shallow-water estuarine ecosystems are typically variable and unpredictable (Barnes 1999; Steindler 2002; Lim et al. 2016; Marshall et al. 2016). The intertidal zone imposes conditions of periodic air exposure, variable temperatures, and greater irradiance (Steindler et al. 2002; Marshall et al. 2010). Despite these circumstances, the sponges recorded at PB were mostly intertidal and air exposed during low tides. Their abundance and ubiquitous nature in this heterogeneous habitat likely relate to plasticity in structural organization and morphology. Morphological remodeling could allow them to overcome the variable environmental conditions and ensure their survival. Species such as *Haliclona* sp. 3 apparently survive direct sun exposure by remaining buried in the mud. While sponges that occur encrusted on the surfaces of boulders usually become established in shaded 'refugia', reducing the threats of high temperature and desiccation (Barnes 1999). During air exposure when filter feeding is curtailed, alternative food sources are often needed. Some sponges adapted for periodic air exposure produce energy through photosynthesis by establishing associations with photosymbionts, including *Haliclona* sp. (Steindler et al. 2002).

Dredging activity poses an additional environmental risk to sponges, as sediments from the dredge or disposal site may smother the sponge surface, potentially affecting water filtration and light penetration (Pineda et al. 2017). With the dredging in the Brunei bay for the construction of a four-lane bridge in Pulau Muara Besar, together with the iconic bridge that transverses from Sungai Besar to Temburong, benthic organisms at PB were likely exposed during the sampling time to periodically high sediment loads (Marshall et al. 2016). Although sponge habitat-generalists may vary in body form to survive a variety of different environmental conditions, this capability is genetically predetermined (Swierts et al. 2013; Setiawan et al. 2016; Lopez-Legentil & Pawlik 2009). There are limited numbers of body

forms within this genetic framework, and only certain morphologies will survive the ensuring environmental conditions. Hence, the shift from a relatively mature and stable to a more unstable, less diverse community dominated by encrusting species may indicate chronic environmental stress (Easson et al. 2015). This observation is consistent with studies showing that morphological variation in sponge communities decreases as perturbation increases. High sediment conditions can clog the aquifer systems of sponges, and adversely affect pumping and reduce feeding efficiency. Consequently, sponges under high sedimentation conditions should be energetically stressed and should attempt to expel unwanted material, which presents a drain on their energy reserves and should impact growth. Pineda et al. (2017) suggested that high sediment loading particularly affects the massive form of encrusting and wide cup morphologies. The prevalence of the massive *Haliclona* sp. 1 at Oyster rocks (Figure 1) is, however, not consistent with this observation. Compensatory mechanisms for life in turbid conditions include elevated mucus production, psammobiotic growth forms, and the protrusion of spicules. Spicule protrusion functions to capture fine sediments and is seen in *Paratetilla bacca*, *Cinachyrella* spp. and *Raspailia* spp. (Becking et al. 2013). We also observed surfaces with protruding spicules in *Axinella* sp. Although the literature suggests that sponges are influenced by suspended sediment in multiple ways, there is a need for further exploration of these different responses.

Difficulties in this study in determining the species level of the sponges relate to the use of only a classical morphological approach. Traditional sponge identification has been based almost completely on their skeletal structure and organization (Hooper & van Soest 2002; Wörheide & Erpenbeck 2007). This proves to be a major problem because sponges exhibit a great diversity of skeletal types which often poorly distinguish species (McCormack et al. 2002; Pöppe et al. 2010; Xavier et al. 2010; Setiawan et al. 2016; Swierts et al. 2013). Taxonomists have also relied on morphological features such as color, shape, and skeletal elements to identify sponges. This is acknowledged to be difficult due to the lack of fixed diagnostic morphological characters, and limited knowledge of phenotypic plasticity among species (Hill & Hill 2002; Guardiola et al. 2016; Bell & Barnes 2000). Such limitations have become apparent during this study when attempting species identifications. For instance, the taxonomy of the Order Haplosclerida (Porifera: Demospongiae), which is a dominant group in this study, is made particularly difficult by their reduced spicule diversity and simplified skeletal structures (Redmond et al. 2007; Redmond et al. 2011; McCormack et al. 2002). This order is typically characterized as having a skeleton that reticulates in an isodictyal arrangement (triangular mesh) with unornamented diactinal megascleres (oxeas). In the genus *Haliclona* however, although the subordinates were diagnosed as *Haliclona* sp. for possessing isodictyal reticulation, spicules in species 1 and 3 did not conform to the features of having only analogous oxea. Palumbi (1986) suggested that the increase in the size and number of spicules are ecophenotypic responses to changing environmental stress such as waves. For this reason, reliability and "a sound classification" of the order are far from being established (Borchiellini et al. 2004; Redmond et al. 2011).

CONCLUSION

This preliminary study of shallow water and intertidal sponges of Brunei Darussalam recorded at least 14 species (OTUs). These mainly constituted the Haplosclerida, an order of sponges known to tolerate adverse and high sedimentation conditions, as are found in the Brunei Estuarine System (BES). This first inventory of these underexplored benthic animals is

considered useful to improve understanding of ecological and community responses to variable environmental conditions and general conservation issues. Despite the absence of definitive species determinations, our study suggests a significant diversity of sediment-tolerant sponges in the region. We advocate further investigation based on molecular taxonomy, as well as into the phenotypic plasticity of these sponges.

AUTHOR CONTRIBUTION

E.S. and D.J.M. designed and supervised the overall research project. D.R. collected, curated and captured the data for the sponges. All authors wrote and revised the manuscript. E.S. and D.J.M. read and approved the final paper.

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CONFLICT OF INTEREST

The authors declared that there is not any conflict of interest regarding this research work.

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Research Article

Astaxanthin-Producing Microalgae Identification Using 18S rRNA : Isolates from Bangkalan Mangrove Waters and Sowan Tuban Northern Waters, East Java, Indonesia

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ABSTRACT

Microalgae are a group of micro-sized photosynthetic organisms that range from prokaryotic cyanobacteria to eukaryotic algae. Microalgae are widely used as a source of natural food, cosmetic ingredients, food ingredients, and a source of pigments. This study aims to identify species of four microalgae isolates named B1, B2, B3, and S2 from Bangkalan Mangrove Waters and Sowan Tuban Northern Waters, and to determine their astaxanthin pigment concentration under 1 M NaCl. Species identification was carried out through a molecular approach by utilization of an 18S rRNA gene marker. A quantitative test of astaxanthin concentration was carried out by spectrophotometric analysis. Molecular identification results show that isolates B1 and B3 are closely related to *Chlorella* sp., while isolates B2 and S2 are closely related to *Picochlorum maculatum*. Moreover, under salinity stress condition of 1 M NaCl shown a significant decrement of astaxanthin production compared to the control treatment. At 1 M NaCl, the astaxanthin content of isolate B1 was $4x10^{-5}$ mgL^{-1}, isolate B2 was $2x10^{-5}$ mgL^{-1}, isolate B3 was $1x10^{-5}$ mgL^{-1}, and isolate S2 was $6x10^{-6}$ mgL^{-1}. All in all, isolate S2 has the highest astaxanthin among the other isolates at normal conditions, while under salt stress regime, isolate B1 shown to be the best source for astaxanthin.

Keywords: Astaxanthin, Bangkalan Mangrove Waters, *Chlorella* sp., *Picochlorum maculatum* Sowan Tuban Northern Waters

INTRODUCTION

Microalgae are plant-like protists commonly referred to as phytoplankton with a diameter of between 3-30 µm, single-celled or in simple colonies. The microalgae group is a large group that varies from prokaryotic cyanobacteria to eukaryotic algae scattered in many phyla. Microalgae are single-celled

organisms with simple structures that have the ability to convert carbon dioxide and water using solar energy into biomass. Microalgae can be found widely in various types of waters and are capable of multiplying their biomass rapidly during exponential times (Yanuhar 2016). Meanwhile, according to Guiry (2012), there is still uncertainty about what characteristics cause an organism to be classified as an algae and its taxonomic hierarchy. However, until June 2012 there were 44,000 algal species names that have been published with a conservative identification approach. This is the cause of the importance of having the ability to science algae systematics. Referring to Suparman (2012) identification using morphological data is an identification that is often used but this method is less effective because of a long time and often the appearance of the morphology of organisms is influenced by external factors so that the results of the identification obtained are not consistent. The shortcomings of this method have led to the identification and analysis of the relationship between organisms. Methods in identifying organisms have developed from the use of morphological identification up to the use of molecular identification based on some pieces of short DNA called "DNA barcode" (Hebert et al. 2003; Zulfahmi 2013). DNA Barcode refers to the use of standard short DNA regions to identify organisms even if the DNA is incomplete, damaged, or degraded (Hajibabaei et al. 2007). 18S rRNA gene is a common molecular marker for biodiversity studies because it is highly conserved within a single species (close to 100% similarity) and helps in species-level analyses.

Microalgae produce pigments that are focused on three types, namely chlorophyll, phycobillin, and carotenoids (Elfiza et al. 2019). Carotenoids are natural pigments produced in fungi, bacteria, algae, and plants but are not produced by animals. Carotenoids are used as ingredients in vitamin supplements, cosmetics, health food products, and addictive substances. Carotenoids that have been studied include β-carotene, lutein, lycopene, zeaxanthin, and astaxanthin (Coates et al. 2013). Astaxanthin with the structural formula 3,3'-dihydroxy-β, β-carotene-4,4'-dione is a ketocarotenoid that is synthesized in limited quantities in microalgae, plants, bacteria, and fungi. Unlike primary carotenoids (e.g., β-carotene, zeaxanthin, and lutein) which are components of the photosynthetic apparatus, which is in charge of collecting light energy and transferring energy to chlorophyll in photosynthesis (Demmig-Adams et al. 1996) astaxanthin is a secondary carotenoid that accumulates in cells when the cells are subjected to environmental stress or adverse culture conditions, such as high light, high salinity and nutritional deficiencies (Han et al. 2013). Salinity stress in the form of high salt concentrations in the medium will cause disruption of the osmotic balance and Na+ poisoning resulting in a decrease in the microalgal cell growth rate. Under conditions of salinity stress, microalgal cells will make physical and biochemical adjustments such as inhibition of photosynthesis, synthesis of macromolecular compounds, and adjustment of homeostasis. (Wang et al. 2018). Environmental stress causes Reactive Oxygen Species (ROS) in cells that cause damage to DNA, proteins, and cell membranes. ROS at the same time stimulate the emergence of secondary carotenoids such as astaxanthin which consume molecular oxygen during ROS synthesis so that it will directly reduce the formation of ROS (Collins et al. 2011). Environmental stress conditions will cause the accumulation of secondary carotenoids such as astaxanthin and ketolutein and result in the degradation of chlorophyll pigments and primary carotenoids (Mulders et al. 2015). The ratio value of the carotenoid pigments concentration to chlorophyll pigments concentration becomes an indicator of carotenogenesis in microalgae cells which will increase when microalgae cells are exposed to environmental

stress conditions due to the accumulation of secondary carotenoids (Chen et al. 2017).

Astaxanthin has been reported to have the most potent antioxidant activity compared to other carotenoids (Han et al. 2013). Astaxanthin antioxidant activity is the ability to capture reactive oxygen species (ROS) and neutralize free radicals in the cells because astaxanthin has thirteen conjugated double bonds arranged as single double bonds. Astaxanthin is widely used in nutraceuticals and pharmaceuticals for diseases associated with increased free radicals such as cancer, cardiovascular disease, degenerative diseases of the eye. Astaxanthin is also a common coloring agent in aquaculture to provide red pigmentation (Lorenz & Cysewski 2000; Guerin et al. 2003).

Some of the microalgae that produce astaxanthin in their cells, among others *Botryococcus braunii* which is equal to 0.01% astaxanthin by dry weight, *Haematococcus pluvialis* which is equal to 4% astaxanthin by dry weight, *Eremosphera viridis,* and *Trachelomonas volvocina* (Han et al. 2013), *Chlamydocapsa sp.* which is equal to 0.04% astaxanthin by dry weight (Leya et al. 2009), *Chloromonas nivalis* which is equal to 0.004% astaxanthin by dry weight (Remias et al. 2010), *Chlorella zofingiensis* which is equal to 0.7% astaxanthin by dry weight *Neochloris wimmeri* which is equal to 1.9% astaxanthin by dry weight, *Protosiphon botryoides* which is equal to 1.4% astaxanthin by dry weight, *Scotiellopsis oocystiformis* which is equal to 1.1% astaxanthin by dry weight (Orosa et al. 2000), *Chlorococcum* sp. which is equal to 0.7% astaxanthin by dry weight (Ma & Chen 2001), *Scenedesmus obliquus* which is equal to 0.3% astaxanthin by dry weight (Qin et al. 2008).

Indonesia is an archipelago with 17,508 islands, and a coastline of 81,290 km, which is united by a sea of 5.8 million km^2. However, the development of the marine and fisheries sector is still far from expectations, even though Indonesia's coastal and marine areas have enormous potential natural resources and have not been optimally utilized (Lasabuda 2013). Mangrove ecosystems are known as brackish forest ecosystems because they are found in water areas with salinity levels between 0.5 °/oo and 30 °/oo. This ecosystem is also known as a tidal forest ecosystem because it is found in areas that are affected by tides (Noor et al. 1999). Microalgae that live in brackish and marine ecosystems have the ability to adapt to living in saline environments. This research aims to explore and identify the species of microalgae from both mangrove waters located in Sepulu sub-district, Bangkalan Madura and the northern waters of Sowan, Tuban, East Java and then tested the concentration of astaxanthin produced by microalgae isolates after experiencing salinity stress. Based on a review of the literature, exploration of microlagae of Indonesia's local resources in Bangkalan mangrove waters and the northern waters of Sowan, Tuban East Java as a producer of astaxanthin has not been widely carried out until this article was compiled. In this study, microalgae that were isolated from both locations were induced to produce astaxanthin in vitro in high saline medium of 1 M or equal to 83 ‰ as a salinity stress.

MATERIALS AND METHODS

Microalgae sampling

The sampling of microalgae was carried out in December 2019 in mangrove waters, Sepulu sub district, Bangkalan Madura and in the northern waters of Sowan, Tuban, East Java. A sampling of microalgae was performed by the horizontally towing method, namely plankton net size 0.08 mm drawn horizontally by boat at a constant speed (10 km / hour) for a certain distance at a water depth of 0-50 cm. A sampling of microalgae in the northern waters

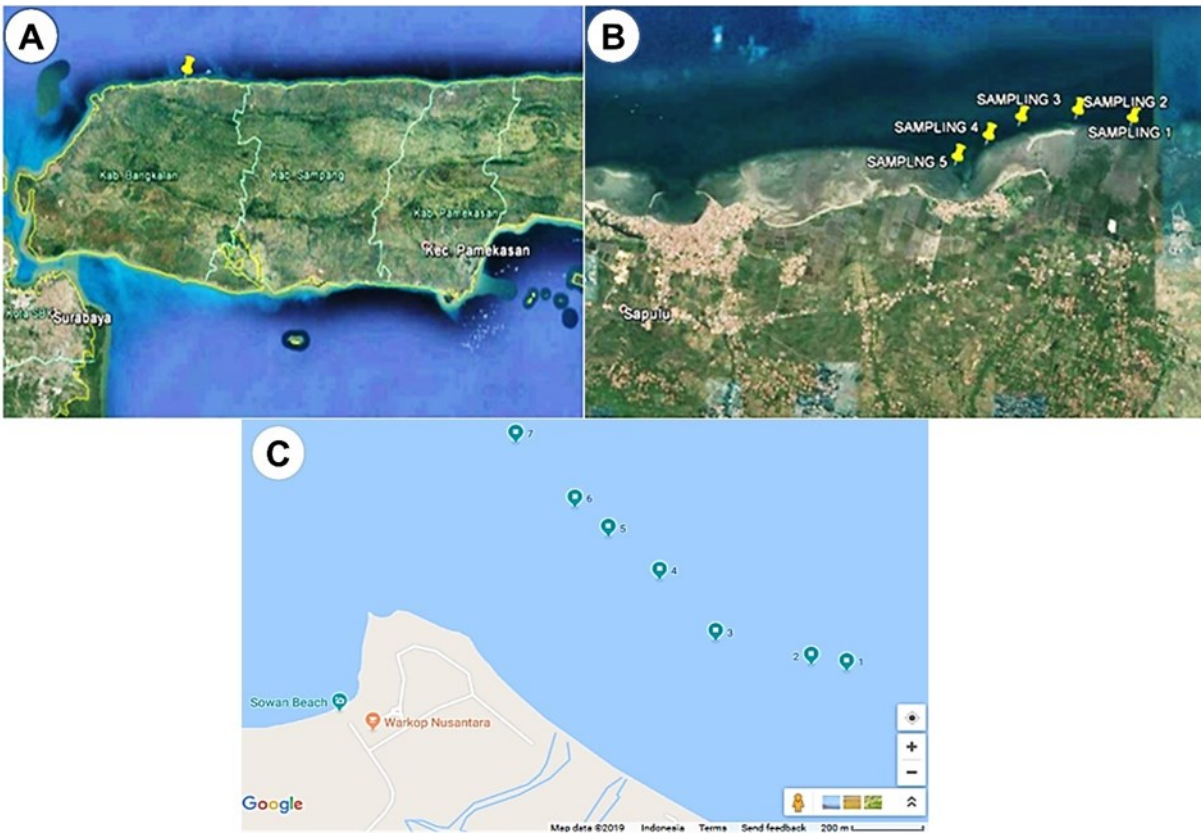

Figure 1. A) Sampling location seen from the north of Madura Island (6°52'48"S; 113°0'7"T) and B) sampling locations in the Bangkalan mangrove waters Madura, East Java with 5 sampling points. C) Sampling locations in the northern waters of Sowan Tuban, East Java with 7 sampling points.

of Sowan, Tuban was carried out at a distance of ± 500 meters from the coastline through seagrass and coral vegetation. Sampling was done by using a motorboat with a speed of 10 km / hour with a distance between stations along the 200 meters. Samples of microalgae from all stations were collected in one sampling bottle and then stored in a cooler box (Sulardiono et al. 2015). The sampling points in Bangkalan mangrove waters and northern waters of Sowan Tuban show in Figure 1.

Identification of microalgae isolates

Isolation, purification, and cultivation of microalgae

Isolation, purification, and cultivation were carried out at the Laboratory of Plant Bioscience and Technology, Department of Biology, Faculty of Science and Data Analysis, Sepuluh Nopember Institute of Technology. The medium used was f / 2 Guillard liquid medium for dilution and f / 2 Guillard solid medium for the streak plate method. Making liquid media was done by filtering seawater, respectively from the mangrove waters of Bangkalan Madura and northern waters of Sowan Tuban. The water from the sampling site was used as a solvent for Guillard f / 2 media. In making f/2 solid media, 10g / L (1%) agarose was added to the liquid medium (Thao et al. 2017). The microalgae samples were diluted using stratified dilution method in sterile f / 2 Guillard liquid medium. The stratified dilutions were carried out aseptically and obtained 10^{-2}, 10^{-3}, and 10^{-4} dilutions. A total of 5 drops of sample from each level of dilution were placed onto the surface of the sterile solid medium and leveled using drygalski (cell spreader). Isolates were incubated at room temperature (23-25°C) with 24: 0 light: dark photoperiod for 7-14 days (Azzahidah & Ermavitalini 2016). The isolate colonies that grew on the medium were transferred to a new sterile solid medium using the streak plate method. The selection of isolates was based on colony

morphology, colony color, and differences in cell morphology. The process was carried out continuously until pure isolates were obtained. Monoculture isolates were transferred gradually to liquid media. The ratio of isolates and media is 1:10. Cultivation is carried out by giving aeration everyday at room temperature (23-25˚C), with a light: dark photoperiod 24:0 for 14 days (Azzahidah & Ermavitalini 2016).

Genome DNA Extraction and determination of the concentration

The extraction of DNA genome using Genomic DNA Mini Kit (Plant) GeneaidTM following the protocol from the manufacture. Determination of DNA concentration using a nanodrop spectrophotometer using a ratio of 260 nm/280 nm. The purity value was obtained from the absorbance ratio at a wavelength of 260 nm which is absorbed by DNA with absorbance at a wavelength of 280 nm which is absorbed by the contaminants. Genome DNA was then treated with RNase to eliminate RNA and subjected to electrophoresis by using 0.8% of agarose gel in 0.5x TBE (Tris-Borat-EDTA) buffer.

Primers Design, PCR amplification, electroporation, and Gene Sequencing

In this research, the primers pair were designed at the new position and not using the universal primers. The DNA sequence of several 18S rRNA genes for green microalgae named *Chlorella sp.* (AB176665.1), *Haematococcus lacustris* (LT578169.1), *Chlamydomonas orbicularis* (AB511839.1), *Golenkinia sp.* (AF499924.1), *Mychonastes sp.* (AB727554.1) were retrieved from the NCBI database (www.ncbi.nlm.nih.gov). The sequence was then subjected to the MultAlin online software (http://multalin.toulouse.inra.fr/multalin/) for alignment purposes. The primers pair were chosen at the conserved region manually to customize the expected products. Neb calculator was used to calculating the melting temperature for each oligonucleotide sequence. The primer homodimer, heterodimer, and secondary structure were analyzed using the online software OligoAnalyzer (https://sg.idtdna.com/calc/analyzer). From the process, we obtain forward primer: 5'-CGATGGTAGG ATAGAGGCC-3', while the reverse primer is: 5'-CTAGGCATTCCTCGTT GAAG-3'.

The genome obtained during the extraction process was used as a template for the DNA amplification process. DNA amplification begins with the mixing of the primary reagents forward, reverse, ddH_2O, DNA template, and kit. DNA Amplification uses a PCR BIOER GeneTouch Thermal cycler and was carried out by setting the temperature and time according to the GoTaq Green Master Mix protocol. The steps of DNA polymerization with a PCR machine are carried out as follows: predenaturation step is set on 94˚C for 30 seconds and done in 1 cycle, denaturation step is set on 94˚C for 30 sec and done in 40 cycles, annealing step is set on 50˚C for 30 sec and done in 40 cycles, elongation step is set on 72˚C for 30 seconds and done in 40 cycles. PCR products were observed using electrophoresis. Moreover, 2% of agarose in 0.5x TBE buffer was used to visualize the DNA genome and PCR products. Electroporation processes were conducted at 100 volts for 35 min. DNA fragments were observed with a UV transilluminator. The correct size of the PCR product was then purified and subsequently sequenced using Sanger Method.

The Content of chlorophyll, carotenoids, and astaxanthin test

Preparation of microalgae culture and cultivation medium

The microalgae that were isolated from both sampling locations as a single colony and pure culture on solid medium were obtained from method 2.2.1, then cultured in liquid medium. microalgae isolated from the northern waters

of Sowan Tuban, East Java. The medium used for microalgae culture is seawater obtained from commercial providers. Seawater is filtered with a filter membrane, then the salinity is measured at 30-32 ppt (Endrawati et al. 2013) then added Walne fertilizer.

Determination of starter time and harvest time

Determination of harvesting time was carried out by making a culture of 500 ml with 10% by volume of pure microalgae culture. Microalgae cultures B1, B2, B3, and S2 were measured every 24 hours until they reached the death phase using a UV-Vis spectrophotometer at a wavelength of 680 nm. The starter period is a half exponential period of culture. Determination of harvest time is done by making growth curves of B1, B2, B3, and S2 microalgae cultures that were given stress treatment. The salinity treatment was carried out by giving NaCl with a concentration of 1 M. Culture growth was measured every 24 hours until it reached the death phase using a UV-Vis spectrophotometer at a wavelength of 680 nm. The statistical phase is defined as the harvest time for the culture.

Quantitative analysis of chlorophyll and carotenoids

A total of 4 ml of sample was taken and centrifuged at 3000 rpm for 10 min or 4000 rpm for 5 min. The supernatant was removed and the cells were put in 4 ml of distilled water to remove the salts in the biomass, then centrifuged. Washing in distilled water was carried out twice. The sample was suspended in 4 ml of ethanol solvent, then centrifuged for 10 min at a speed of 3000 rpm (Henriquez et al. 2007). The supernatant was taken to measure its absorbance using a spectrophotometer. The absorbance was measured at a wavelength (λ) 645 nm for chlorophyll b analysis, (λ) 662 nm for chlorophyll an analysis, (λ) 470 nm for carotenoid analysis. The blank used was 96% ethanol as a comparison (Pratama & Laily 2015). The amount of pigment is calculated based on the Lichtenthaler (1987) formula shown in Table 1. (Zawislak & Wierdak 2014).

Table 1. The formula for calculating the concentration of chlorophyll a, chlorophyll b, and carotenoids.

C_a	$11{,}75\ A_{662}$ -$2{,}350\ A_{645}$
C_b	$18{,}61\ A_{645} - 3{,}960\ A_{662}$
C_{x+c}	$1000\ A_{470} - 2{,}270\ C_a - 81{,}4\ C_b / 227$
C_a = chlorophyll a , C_b = chlorophyll b, C_{x+c} = total carotenoids	

Quantitative analysis of astaxanthin

A total of 10 ml of sample was taken and centrifuged at 7000 rpm for 5 min. The pellets were taken and then added 5 ml of 5% KOH in 30% methanol at a 75°C water bath for 10 min to remove chlorophyll. The remaining pellets were added 25 µl of acetic acid at 75°C for 10 min. The pellets were extracted using DMSO. At this stage, it is repeated several times until the colour sample fades. The pigment is measured using a spectrophotometer with a wavelength (λ) = 492 nm ($E^{1\%}_{1cm}$)=2220 and can be calculated by the formula (Li et al. 2019):

CA (mg.L-1) = (A492 x 1000/ $E^{1\%}_{1cm}$ x 100) x Va (mL) /10 (Ml) x f

CA : Concentration of astaxanthin;
Va : Volume of extract;
f : dilution rate

Data Analysis

The DNA sequences resulting from the sequencing were combined between reverse and forward primers using BioEdit software. Furthermore, the sequences that have been obtained were analyzed using the alignment contained in the Nucleotide Basic Local Alignment Search Tool (BLASTn) on the NCBI portal. Furthermore, phylogenetic trees were created using MEGA 7.0 software (Saputro et al. 2019). Phylogenetic trees were constructed using Neighbour-joining with bootstrap 1000 times. Meanwhile, quantitative data on chlorophyll, carotenoid, and astaxanthin concentrations were presented as the average of three repeated measurements. Data analysis was carried out by quantitative descriptive by comparing stress treatment with control without stress treatment.

RESULTS AND DISCUSSION

Results

The results of microalgae isolation from mangrove waters located in Sepulu sub-district Bangkalan and northern waters of Sowan Tuban, East Java, obtained 4 isolates that can be cultivated as pure culture in liquid media, namely isolates B1, B2, B3, and S2. Figure 2 below is the result of microscopic observations of the four isolates of microalgae under a microscope (Optilab Viewer) and microalgae isolate B1, B2, B3 and S2 in liquid culture.

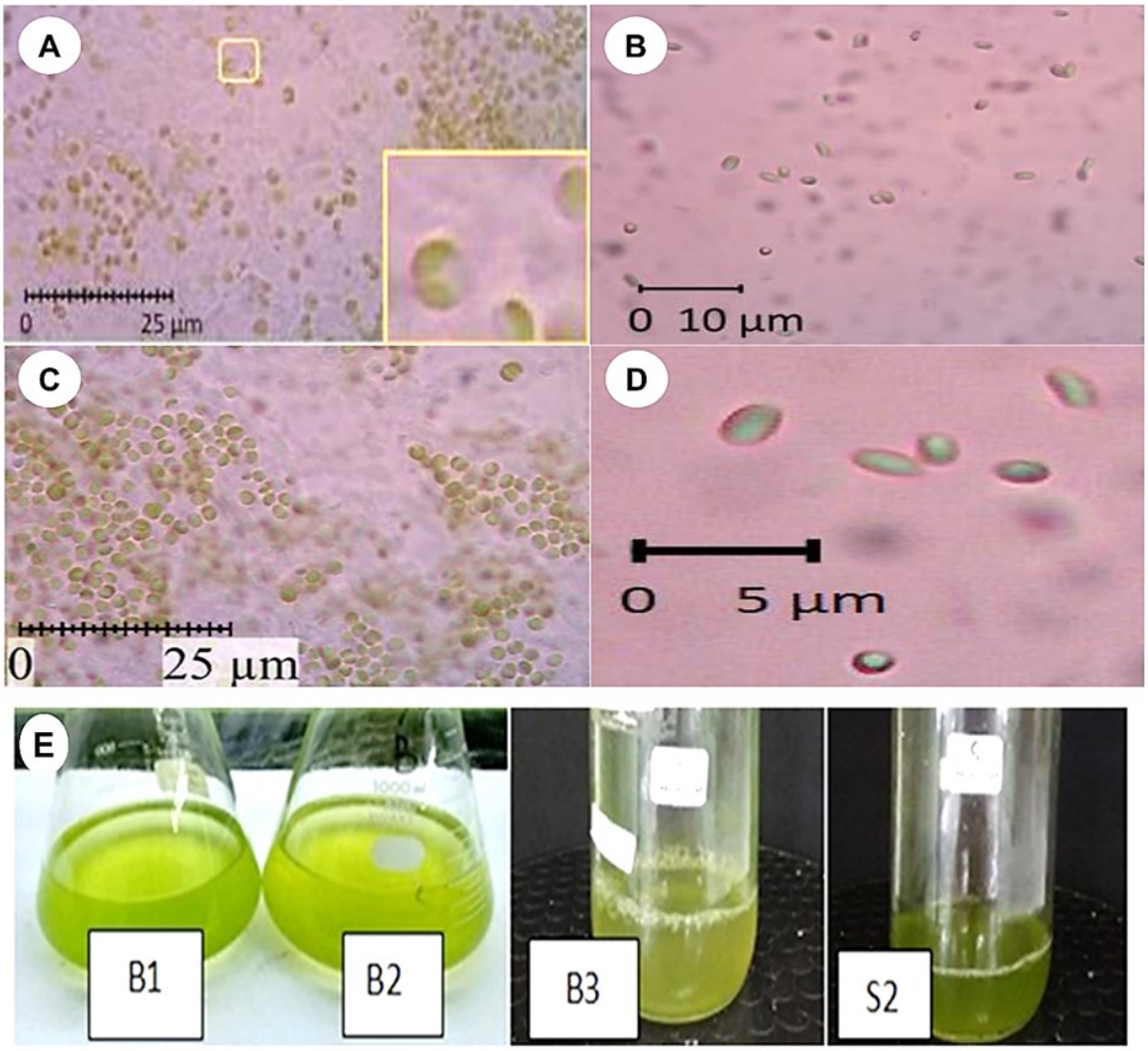

Figure 2. A). Microalgae cells isolate B1, B). Microalgae cells isolate B2, C). Microalgae cells isolate B3. D). Microalgae cells isolate S2, E). Microalgae isolate B1, B2, B3, and S2 in liquid culture.

The molecular identification stage of microalgae isolates using the 18S rRNA marker gene began by measuring the purity of the isolated DNA using a nanodrop spectrophotometer ND-1000 with a wavelength of 260 nm and 280 nm. The measurement results show that the purity value is in the range 1.97-2.13 and the concentration value is in the range of 14-24.3 ng/µL. These results indicate that the purity of the DNA sample and the DNA concentration have met the requirements as a template in the amplification process on the PCR machine (Sambrook et al. 1989). Purity with a value of 1.8-2.0 is the feasible value of the microalgae genome sample to go to the DNA amplification process and electrophoresis stages (Mustafa et al. 2016). The value of DNA purity affects the success of amplification because, with a good purity value, the DNA bands formed will be clearly visible on visualization under UV light. The following is the result of visualization of DNA bands on electrophoretic gel under UV light and Table of Differentiation rich among the samples and retrieved 18S rRNA (Figure 3).

The four isolates that have been identified then tested for the concentration of chlorophyll, carotenoid and astaxanthin pigments at salinity conditions with a concentration of 1 M NaCl. Testing the concentration of the pigment chlorophyll, carotenoids and astaxanthin can describe carotenoids in microalgae cells under stress, where microalgae cells under stress will accumulate secondary carotenoids including astaxanthin and reduce the concentration of chlorophyll pigments and primary carotenoids. (Chen et al. 2017).

Based on Figure 5A it can be seen that isolates B1, B2, B3, and S2 entered an adaptation phase (lag phase) on 1st day, then entered the logarithmic phase starting on 1st day to 12th day. The four isolates entered different stationary phases wherein isolate B1 and isolate B3 the stationary phase started from 12th day to 14th day, while isolates B2 and S2 started from 12th day to 13th day. The death phase of isolates B1 and B3 started on 14th day, while for isolates B2 and S2 the death phase started on 13th day. This is in accordance with the results of molecular identification which show that

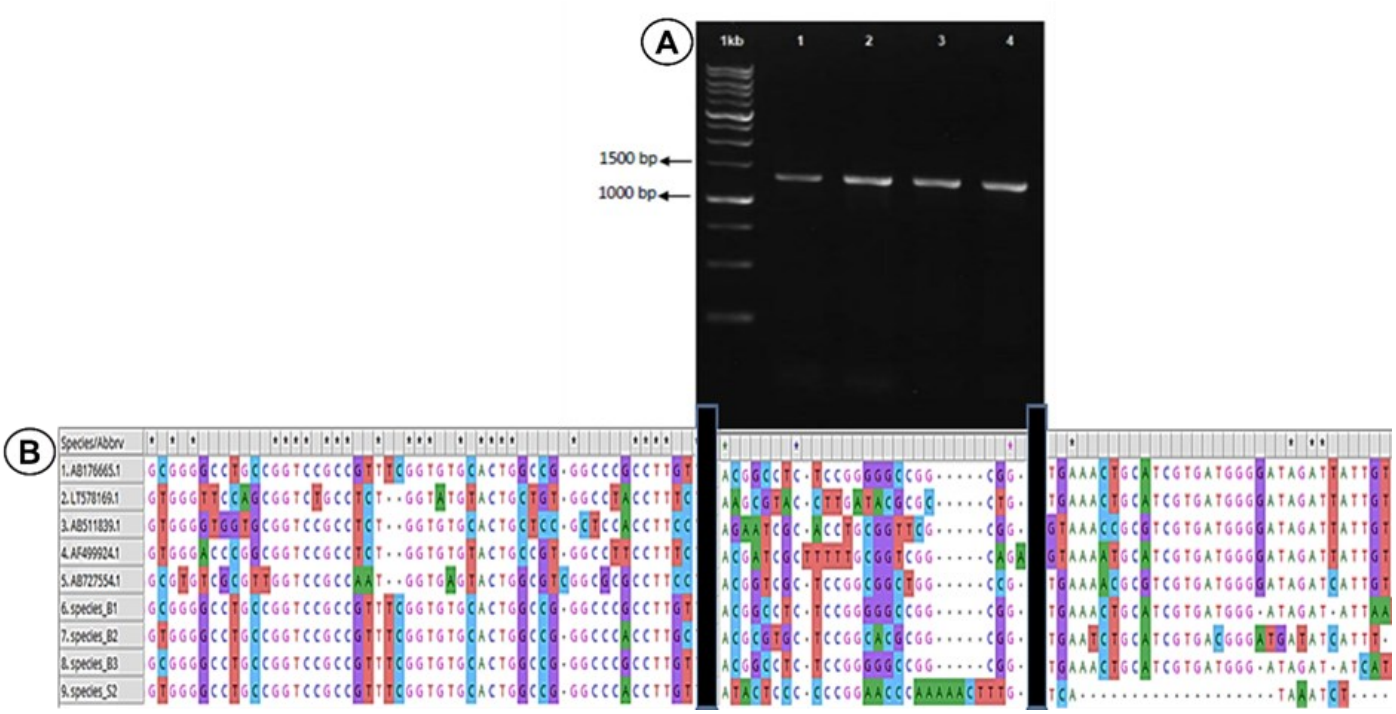

Figure 3. A) Visualization of electrophoretic DNA bands under UV light (1 is DNA band of microalgae isolate B1, 2 is DNA band of microalgae isolate B2, 3 is DNA band of microalgae isolate B3 and 4 is DNA band of microalgae isolate S2); B) The area has shown differentiation rich among the samples and retrieved 18S rRNA.

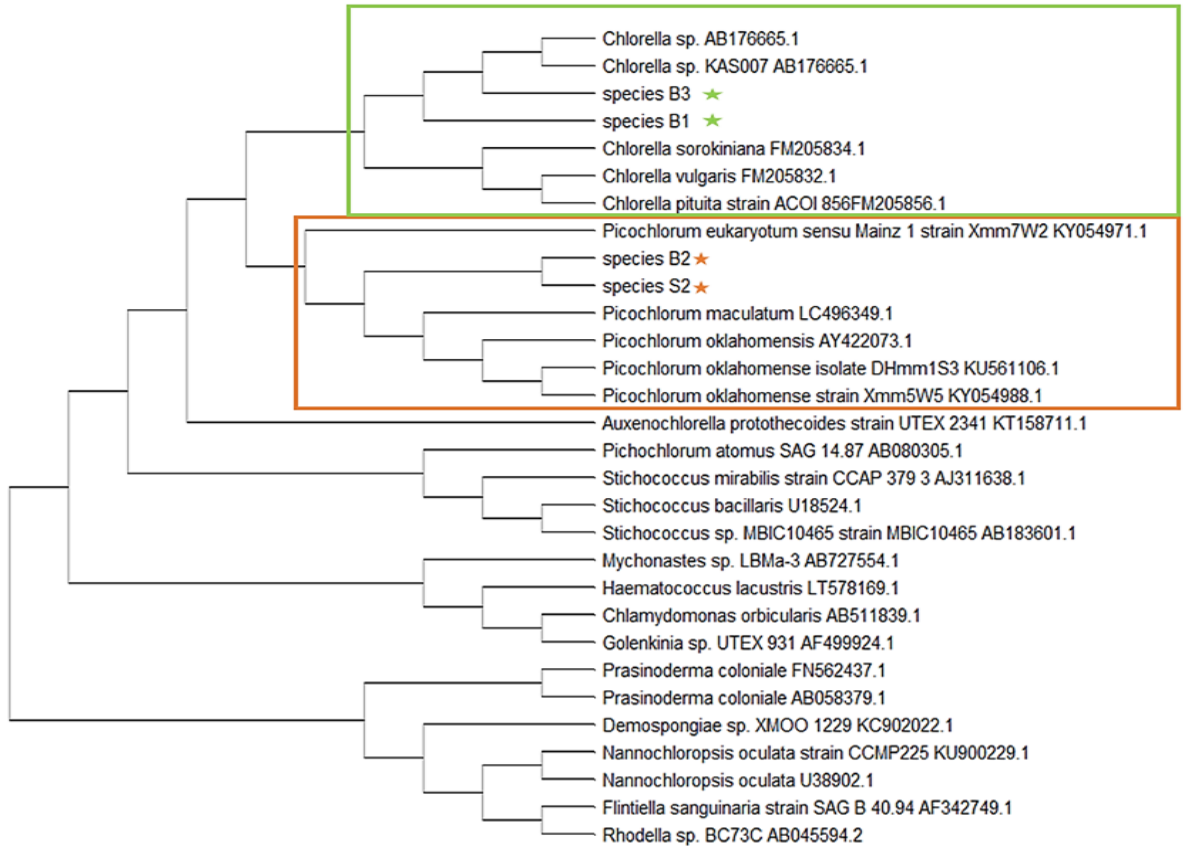

Figure 4. Phylogenetic tree construction based on the 18S-rRNA gene using the Neighbour Joining Tree bootstrap 1000x method.

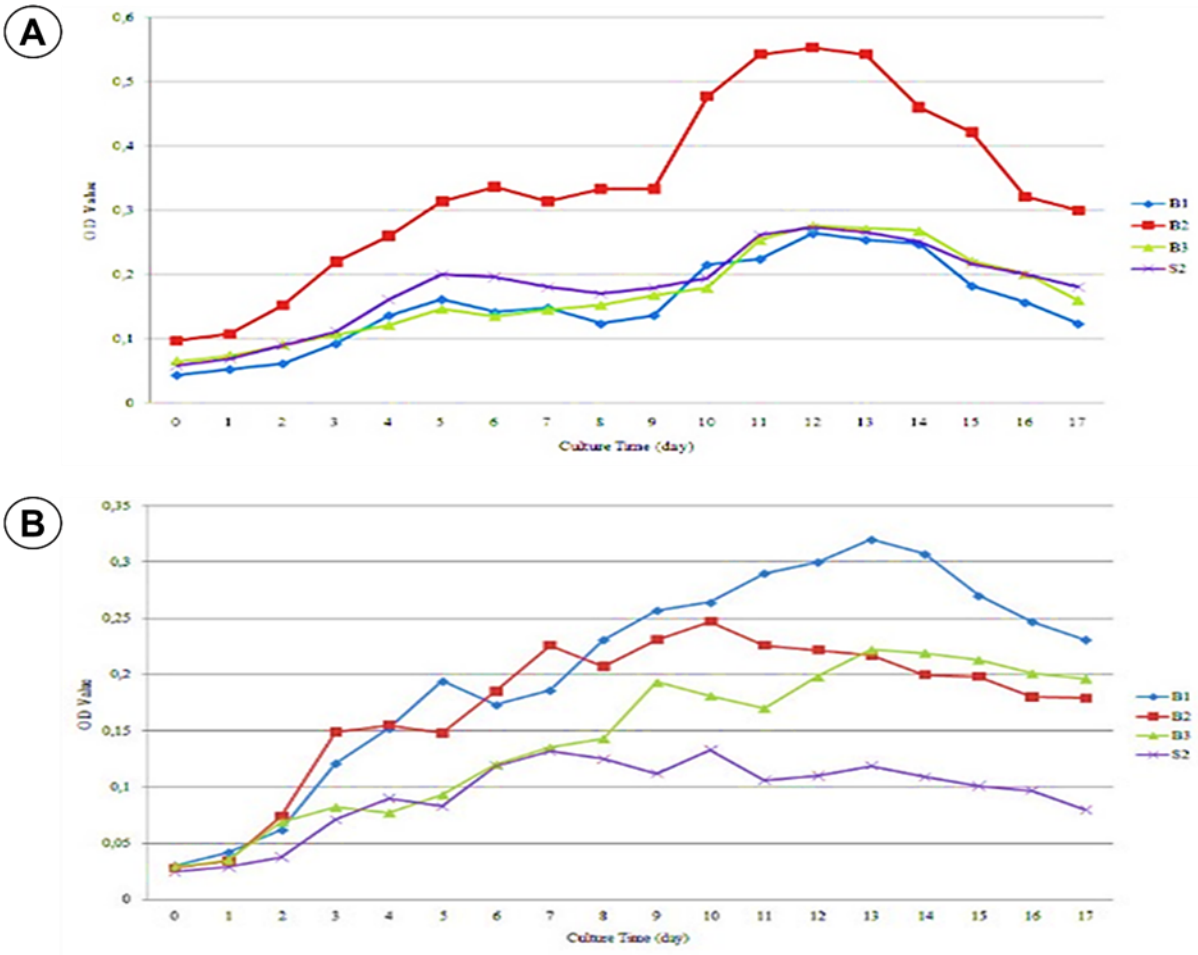

Figure 5. A). Growth curves of isolates B1, B2, B3, and S2 under normal conditions; B). Growth curves of isolates B1, B2, B3, and S2 under a salinity stress condition of 1 M NaCl

Table 2. Chlorophyll, carotenoid, and astaxanthin concentrations in isolates B1, B2, B3, and S2 under 1 M salinity stress conditions.

Isolates Name	Treatment	Chlorophyll Concentration (mgL^{-1})	Carotenoid Concentration (mgL^{-1})	Astaxanthin Concentration (mgL^{-1})
Isolate B1 (*Chlorella* sp.)	1 M NaCl	$18x10^{-2}\pm 0.5x10^{-2}$	$30.62x10^{-3}\pm2.1x10^{-3}$	$4x10^{-5}\pm0.9x10^{-5}$
	Control	$92x10^{-2}\pm 1.8\ x10^{-2}$	$138.41x10^{-3}\pm4.3x10^{-3}$	$40x10^{-5}\pm 2.3x10^{-5}$
Isolate B2 (*Picochlorum maculatum*)	1 M NaCl	$12x10^{-2}\pm1.96x10^{-2}$	$33.57x10^{-3}\pm2.03x10^{-3}$	$2x10^{-5}\pm0.2x10^{-5}$
	Control	$132x10^{-2}\pm2.9x10^{-2}$	$366.3x10^{-3}\pm4.2x10^{-3}$	$70x10^{-5}\pm4.3x10^{-5}$
Isolate B3 (*Chlorella* sp.)	1 M NaCl	$28x10^{-2}\pm1.2x10^{-2}$	$27.5x10^{-3}\pm1.6x10^{-3}$	$1x10^{-5}\pm0.1x10^{-5}$
	Control	$85x10^{-2}\pm1.65x10^{-2}$	$103.48x10^{-3}\pm5.2x10^{-3}$	$20x10^{-5}\pm1.6x10^{-5}$
Isolate S2 (*Picochlorum maculatum*)	1 M NaCl	$14x10^{-2}\pm0.4x10^{-2}$	$14.76x10^{-3}\pm1.9x10^{-3}$	$0.6x10^{-5}\pm0.04x10^{-5}$
	Control	$279x10^{-2}\pm3.4x10^{-2}$	$549.5x10^{-3}\pm6.9x10^{-3}$	$140x10^{-5}\pm5.5x10^{-5}$

isolates B1 and B3 are close relationships with a *Chlorella* sp., while isolates B2 and S2 are close relationships with *Picochlorum maculatum* (Figure 4).

Based on the growth curve, the time to take the starter culture is determined on the 6th day, which is half the exponential period of culture. Starters are used as seeds for making cultures for the mass production of microalgae (Perumal et al. 2012). While in Figure 4B. shows the OD value of the isolates with 1 M of NaCl salinity stress is lower than the OD value of the isolates without salinity stress in Figure 5A. Figure 5B shows that the harvest time and measurement of chlorophyll, carotenoid, and astaxanthin concentrations were carried out in the final exponential phase, namely on 13th day for isolates B1 and B3 and 10th day for isolates B2 and S2.

Table 2 above shows the results of measuring the concentration of chlorophyll, carotenoids, and astaxanthin in the four microalgae isolates under 1 M NaCl stress.

Discussion

The electrophoresis results in Figure 3A show that the microalgae DNA samples of isolates B1, B2, B3, and S2 can be amplified with the primer pairs that have been designed. The size of the DNA amplification results of each isolate was in the fragment size range between 1000 bp to 1500 bp. This is in accordance with the research of Rismiarti et al. (2016), who received electrophoretic fragments at ± 1250 bp in the analysis of the microalgae *Chlorella vulgaris* using the 18S rRNA marker gene. Good amplification results are indicated by a thick DNA band and there is little or no smear on the gel when visualized in ultraviolet light (Safitri et al. 2018) Phylogenetic tree construction was carried out to determine the groups and species relationships. The phylogenetic tree was designed using microalgae 18S-rRNA sequences from GenBank as BLASTn results, which showed that isolates B1, B2, B3, and S2 belonged to this group of sequences (in group). The phylogenetic tree shows that both isolates B1 and B3 have a very close relationship with *Chlorella* sp. while isolates B2 and S2 have a close relationship with *Picochlorum maculatum*. The genus Chlorella and the genus Picochlorum belong to the division Chlorophyta. The genus Chlorella has approximately 13 members of the species with a single green cell, spherical in shape with a diameter of 2 to 10 m, not flagellated. The chloroplasts contain the photosynthetic pigments chlorophyll a and b. In an optimal environment for growth, Chlorella will divide rapidly by requiring only carbon dioxide,

sunlight, water, and minerals (Scheffler 2007). According to Henley et al. (2004) members of the genus, Picochlorum is a species of small unicellular microalgae measuring 1-3 m, oval in shape, having chlorophyll pigment which is surrounded by a cell wall. Members of this genus are isolated from marine and estuary ecosystems with different salinity levels.

Based on the growth curve in Figure 5A, the time to take the starter culture is determined on the 6th day, which is half the exponential period of culture, because during the exponential period the cells are still actively dividing and can carry out metabolism maximally before entering the stationary phase or cell death (Agustini 2012; Widayat 2015). Starters are used as seeds for making cultures for the mass production of microalgae (Perumal et al. 2012). Figure 5B shows the OD value of the isolates with 1 M of NaCl salinity stress is lower than the OD value of the isolates without salinity stress in Figure5A. Giving 1 M of NaCl causes the final exponential phase shift to enter the stationary phase faster, which means that microalgae cultures enter the death phase faster. According to Erlina (2007), changing salt levels in water can cause growth barriers for culture. High salinity will result in Na^+ poisoning. Excessive Na^+ ions can inhibit K^+ uptake from the environment, where K^+ ions play a role in maintaining cell turgor and enzyme activity. Salinity stress also inhibits the absorption of water and elements through the osmosis process. The amount of water that enters the cell is reduced because the pressure outside the cell is higher than the pressure inside the cell, this situation makes the cell respond by attracting ions. But at the same time, water withdrawal from the vacuole continues which results in a reduced amount of water supply in the cell and results in cell shrinkage (Arisandi et al. 2011).

In this study, harvest time and measurement of chlorophyll, carotenoid, and astaxanthin concentrations were carried out in the final exponential phase, namely on 13th day for isolates B1 and B3 and 10th day for isolates B2 and S2. At the end of the exponential phase, microalgae experience the highest growth peak and cell density so that the biomass content in the cells will be higher (Isnansetyo & Kurniastuti 1995; Duong et al. 2012).

From Table 2, it can be seen that in all isolate cultures the concentration of all pigments decreased in the 1 M NaCl salinity treatment when compared to the control treatment. This study used salinity stress of 1 M NaCl or equal to 83 ‰ NaCl and the treatment without salinity stress (control) was equal to 35 ‰ NaCl. The optimum salinity value for producing chlorophyll in microalgae is 20 ‰ (Shang et al. 2018) with the ability of photosynthesis which can still take place at a salinity of 30 ‰ to produce a fairly high cell density (Sedjati et al. 2019). So based on this, it can be said that the 1 M NaCl treatment given is too high for microalgae to perform photosynthesis well. Too much NaCl treatment can be toxic and result in very disturbed cell metabolism.

The decrease in photosynthesis due to high salinity treatment will result in a decrease in cell density. Photosynthesis is an important process that supports plant growth and its efficiency is a factor that determines the level of plant resistance to abiotic stress. The efficiency of photosynthesis is determined, among others, by photosystems, photosynthetic pigments, electron transport systems, gas exchange processes, and enzymes that participate in carbon metabolism (Tsai et al. 2019). Research on rice species that are sensitive to salinity showed that the salinity treatment resulted in the chlorophyll fluorescence parameter decreased significantly when compared to salinity tolerant species. When a large number of NaCl molecules enter plant cells, the chloroplast and thylakoid membrane systems are damaged and affect the photosynthesis process if the salt concentration cannot be

regulated to an optimal value (Tsai et al. 2019). In Caryophyllaceae plants that experienced a NaCl stress of 0.6% and 0.9%, there was a disturbance in chloroplast development (Ma et al. 2017). The salinity in this study was very high, so it is likely to cause damage to photosynthetic pigments and cause the concentration value to decrease significantly when compared to the treatment without salinity stress (control).

Pelah et al. (2004) stated that giving salinity stress can increase carotenoid production which will also increase the number of secondary carotenoid levels, especially astaxanthin. Astaxanthin is an antioxidant that is produced as a defense mechanism to survive from the presence of Reactive Oxygen Species (ROS). Salinity stress increases the production of ROS which can lead to metabolic disorders and cell damage (Moller et al. 2007; Jaleel et al. 2009; Miller et al. 2010; Habib et al. 2016; Hossain & Karl 2016). Excessive accumulation of ROS will react with suitable targets such as nucleic acids, proteins, lipids, and chlorophyll. ROS is a highly reactive form of molecular oxygen, including hydroxyl radicals (HO), superoxide (O_2^-) hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (Dowling & Simmons 2009; Shapiguzov et al. 2012; Saha et al. 2015). During stress, the production of ROS will increase due to differences in membrane fluidity which can hinder the transfer of electrons to photosystem II (PS II) (Chaves et al. 2009; Biswal et al. 2011; Silva et al. 2011; Jajoo 2013; Saha et al. 2015). Excess production of ROS when stress occurs in plants must be overcome by plants by producing antioxidants. Research by Ma et al. (2017) shows that salicylic acid as one of the growth regulators in plants has the effect of relieving poor growth and physiological conditions when plants experience salinity stress, namely by improving growth, increasing the activity of antioxidant synthesis enzymes, improving the development of stomata and chloroplasts. In Table 2, the carotenoid and astaxanthin concentrations did not increase in the 1 M NaCl salinity treatment. This may occur because the concentration of NaCl given is very high which results in disruption of the synthesis machinery of growth regulators such as salicylic acid so that it is unable to increase the activity of the enzyme synthesis of antioxidant pigments including carotenoids and astaxanthin. Thus, data collection on the concentration of chlorophyll, carotenoids, and astaxanthin in this study could not be used to describe the mechanism of carotenogenesis in microalgae cells subjected to NaCl concentration of 1 M. However, the results of this study showed that all isolates were able to synthesize astaxanthin even in small amounts and could be increased by salinity treatment at concentrations lower than 1 M or by other stress treatments. This research has supported the exploration of Indonesia's local natural resources that have the potential to produce astaxanthin.

CONCLUSION

Exploration of the diversity of microalgae obtained from mangrove waters in the area of Sepulu Bangkalan Madura and northern coastal waters of Sowan Tuban succeeded in isolating four microalgae isolates and identified molecularly with the 18 S rRNA gene marker. Isolates B1 and B3 were close to *Chlorella vulgaris*, isolate B2 had a close relationship with *Prasinoderma coloniale*, and isolate S2 had a close relationship with *Picochlorum oklahomense*. Giving salinity of 1 M NaCl had a bad effect, namely decreased growth and decreased levels of chlorophyll, carotenoids, and astaxanthin pigments in the culture of all microalgae isolates.

AUTHORS CONTRIBUTION

D.E. as a main and correspondence author designed the research, analyzed the data, and wrote the manuscript, S.Y.R, T.M., L.P.D.C.B collected the

data, T.B.S. analyzed the phylogenetic tree, H.P. and N are the promoter and co-promoter respectively in this research and both conduct supervised all the processes

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CONFLICT OF INTEREST

There is no conflict of interest in the research that has been carried out and the publication of this article.

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Research Article

Effect of Iron Toxicity on the Growth of *Calliandra calothyrsus* and *Leucaena leucocephala* Seedlings

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ABSTRACT
Iron (Fe) is a micro essential needed by plants in small amounts and can be toxic when available in large quantities. This study aimed to evaluate how Fe exposure affects the growth of *C. callothyrsu*s and *L. leucocephala* seedlings. This study used a completely randomized design with factorial, where the first factor consisted of two levels of seedlings (*C. calothyrsus* and *L. leucocephala*), and the second factor consisted of Fe concentration which consisted of 8 levels (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 1.75 mM). The results showed that treatment of seedlings species and concentration of Fe was able to significantly affect the growth parameters (height, root length, root dry weight, shoots, and plant dry weight) of seedlings. The control treatment (without Fe) showed the highest growth response compared to those treated with Fe exposure and an increase in Fe concentration was able to reduce all growth parameters in both seedlings. The 0.5 mM Fe concentration reduced all growth parameters of *C. calothyrsus* drastically, while in *L. leucocephala*, the Fe 0.75 concentration was able to decrease all growth parameters drastically. The tolerance index of both seedlings decreased with increasing Fe concentration. The rate of photosynthesis did not show a significant difference between treatments, meanwhile, it had a significant effect on chlorophyll affect chlorophyll (a, b, and total chlorophyll) and carotenoid content. The highest Fe content in *C. calothyrsus* seedlings was at a concentration of 1.5 mM (4.40%), while in *L. leucocephala* seedlings, the highest Fe content was at 1.7 mM (2.87%).

Keywords: *Calliandra calothyrsus,* Fe exposure, growth, *Leucaena leucocephala*, seedlings

INTRODUCTION
Iron (Fe) is a micro-essential element that plants require (Wintz et al. 2002; Rout & Sahoo 2015; Zhang et al. 2019). Fe is one of the important metals grouped into "trace elements" (Kabata-Pendias 2010). Metals which include "trace elements," including Fe, are needed by plants in low concentrations and play a part in a variety of critical plant processes, such as physiology and biochemistry, as well as enzyme cofactors and maintain the metabolic function of plant cells (Campbell et al. 2007; Cabral et al. 2015; Wu et al. 2017).

Fe is the fourth abundant element and approximately 5% of the earth's crust (Bernát 1983; Kerkeb & Connolly 2006). Generally, in the soil, Fe is found in the form of Fe3+, which has a low solubility (Conte & Walker 2011; Nogiya et al. 2016; Zhang et al. 2019). Meanwhile, High Fe^{2+} concentrations in the soil are linked to Fe toxicity (Khabaz-Saberi et al. 2010; Wu et al. 2014). Poor drainage, low nutrient content, acidic soil pH, and low cation exchange capacity (CEC) in the soil can contribute to Fe toxicity (Fageria et al. 2008). Fe content in soil is strongly influenced by soil pH; low soil pH can increase Fe content in the soil; on the other hand, high soil pH can reduce Fe content (Takahashi et al. 2001; Kusmana et al. 2013; Nogiya et al. 2016). Besides, aerobic conditions also make Fe unavailable to plants and vice versa; in anaerobic or inundated conditions, the availability of Fe increases and can be toxic (Gross et al. 2003; Silveira et al. 2009). Plants can be poisoned by high levels of Fe in the soil, which reduces plant productivity (Gross et al. 2003; Wu et al. 2017). However, Agricultural production has been hampered by iron deficit or deficiency (Hansen et al. 2006).

C. calothyrsus and *L. leucocephala* are quite important species from the Fabaceae family (legume) and both species are classified as multipurpose species (Shafiq et al. 2008; Awe et al. 2013; Zayed & Samling 2016). Legume species can grow and adapt well to dry and acid soils (Koutika et al. 2005; Stürm et al. 2007). Legume species such as *C. calothirsus* and *L. leucocephala* have fast growth (Sebuliba et al. 2012) and are widely used in agroforestry systems in tropical areas (Giller 2001; Bala et al. 2003) and revegetation activities (Lins et al. 2006). Legume species can fix nitrogen and become the types that are widely used for soil fertility restoration and are capable of producing relatively high biomass (Luna-Orea et al. 1996). Besides, *C. calothirsus* and *L. leucocephala* can be alternative feeds for livestock (Aganga & Tshwenyane 2003; Franzel et al. 2003; Herdiawan & Sutedi 2015), because their leaves have high nutritional value (protein 31.35%) (Lascano & Stewart 2003; Kabi & Bareeba 2008; Radrizzani et al. 2010; Novia et al. 2015). Information regarding the resistance of *C. calothirsus* and *L. leucocephala* to Fe exposure is not well known. Therefore, this study aims to evaluate how Fe exposure affects the growth of *C. callothyrsu*s and *L. leucocephala* seedlings.

MATERIALS AND METHODS

Materials

Materials used in this study include: lamtoro and calliandra seeds, zeolite, $Ca(NO_3)_2.4H_2O$, NH_4NO_3, KCl, $MgSO_4.7H_20$, KH_2PO_4, $MnSO_4.H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, H_3BO_3, $(NH_4)_6\ MO_7O_{24}.4H_2O$, Fe-EDTA, $FeSO_4.7H_2O$, KOH, HCL, acetone, distilled water, water, and hot water. Meanwhile, the tools consist of: pH meter, scale, sprout tub, styrofoam, aerator, container, hose, micropipette, measuring cup, spatula, mortar, portable photosynthetic analysis system (LI-COR model LI-6400 XT), spectrometer, centrifuge, scissors, ruler, camera and stationery.

Methods

Seed germination. Before the seeds of *C. calothirsus* and *L. leucocephala* were germinated, the seeds were first steeped in hot water (80 °C) for 15 minutes before being immersed in water (25-30 °C) for 24 hours. The seeds were sown in sprouts filled with zeolite. The seeds were watered twice a day depending on the humidity of the media. The tub of sprouts was placed in a place that is unexposed to direct sunlight until shoots appear. The seeds were weaned when they are ± two weeks old until 2-3 leaves appear.

Media preparation. The media used in this study was distilled water dissolved with several macro and micronutrients according to the nutrient solution developed by Sopandie (1999) consisting of: 1.0 mM NH_4NO_3, 1.5 mM $Ca(NO_3)_2.4H_2O$, 1.0 mM KH_2PO_4, 11.0 mM KCl, 0.4 mM $MgSO_4.7H_20$, 0.02 ppm $CuSO_4.5H_2O$, 0.05 ppm $ZnSO_4.7H_2O$, 0.50 ppm $MnSO_4.H_2O$, 0.01 ppm $(NH_4)_6$ $Mo7O_{24}.4H_2O$, and 0.50 ppm H_3BO_3.

Adaptation process for seedlings and Fe stress treatment experiments. The seedlings ready to wean were transferred to a container filled with media and maintained for ±14 days. Each seedling was placed on a perforated serophome, and the stems of the seedlings were wrapped around cotton so that the seedlings can stand upright. After 14 days of the adaptation test, the seedlings were transferred to a container that had been filled with media with a predetermined concentration of Fe. For Fe exposure used $FeSO_4.7H_2O$ solution. During the adaptation process and treatment experiment, the media was maintained at pH 4. The pH adjustment was carried out by adding 1 N HCl and 1 N KOH. Media addition was done when the media volume starts to decrease. The growing media was replaced after 14 days to keep seedling growth optimal.

Parameters. Parameters measured include plant height, root length, dry weight, photosynthesis, and chlorophyll content. Plant height was measured every week for four weeks, while root length was measured last week (end of observation). Photosynthesis was measured using a Licor (Li-6400 XT) portable photosynthesis system (morning around 09.00-11.00). After 30 days, the seedlings were plucked, the roots and shoots were separated, and the roots and shoots were roasted for two days at 80°C weighed to get the dry weight. The iron content in plant tissue was analyzed using the AAS (atomic absorption spectrophotometer) method.

Tolerance index. The tolerance index was determined based on the total dry weight of the plants treated with Al compared with the total dry weight of the control plants that were not treated with Al. Tolerance index calculation using the equation of Liu & Ding (2008) as follows:

$$\text{Tolerance index} = \frac{\text{Total dry weight of seedlings exposed to Fe}}{\text{Total dry weight of seedlings that were not exposed to Fe (control)}} \times 100\%$$

Analysis of chlorophyll and carotenoid content. Chlorophyll and carotenoids were analyzed using the method of Sims and Gamon (2002) with modification. Leaf samples were weighed 0.03-0.05 g and crushed using a mortar and added 2 ml of acetone (85:15%, Trs HCl 1%, pH 8) and centrifuged at 10.000 rpm for 5 minutes. Take 1 ml of supernatant and add 3 ml of tris acetone, then shake until homogeneous. The absorbance was measured at wavelengths (λ) 470, 537, 647, and 663 nm and was measured using a UV-VIS spectrophotometer. Chlorophyll and carotenoid values are expressed in mg/g. Chlorophyll and carotenoid content are determined based on equations (Sims & Gamon 2002).

$\text{Anthocyanin} = 0.08173*A_{537} - 0.00697*A_{647} - 0.002228*A_{663}$
$\text{Chl}_a = 0.01373*A_{663} - 0.000897*A537 - 0.003046*A_{647}$
$\text{Chl}_b = 0.02405*A647 - 0.004305*A537 - 0.005507*A663$

$$\text{Carotenoids} = \frac{(A470 - (17.1*(Chla + Chlb) - 9.479*Anthocianin))}{119.26}$$

Where, Ax is the absorbance at the measured wavelength.

Research design and data analysis. This study used a completely randomized design with factorial, where the first factor consisted of two levels of seedlings species, namely *C. callothyrsus* (A1) and *L. leucocephala* (A2). The second factor consisted of Fe concentration which consisted of 8 levels, namely: 0 mM (D0), 0.25 mM (D1), 0.5 mM (D2), 0.75 mM (D3), 1 mM (D4), 1.25 mM (D5), 1.5 mM (D6), and 1.75 mM (D7). Each treatment was repeated in 3 replications, and each replication consisted of 3 plant units. Data analysis using the Anova test followed by the Duncan Multiple's Range Test (DMRT) at a 95% confidence level ($\alpha = 5$).

RESULTS AND DISCUSSION

Plant Growth

One of the most important micronutrients for plant development and growth is iron (Curie & Briat 2003; Sahrawat 2005; Kobayashi & Nishizawa 2012). Fe functions as an enzyme cofactor in various cellular functions and is needed in many places in organs or cells (Roschzttardtz et al. 2013; Mahender et al. 2019). Fe can stimulate plant growth when it is available in low concentrations. Treatment of seedlings species and concentration of Fe had a significant effect on the growth response of seedlings (height, root length, root dry weight, shoots, and plant dry weight) (Table 1). The increase in Fe concentration was able to reduce all plant growth parameters in both seedlings.

All growth parameters of *C. callothyrsus* began to decrease drastically when the Fe concentration was 0.5 mM, while for *L. leucocephala* seedlings. The height parameter started to decrease when the Fe concentration was 0.75 mM, and the other parameters decreased drastically when the Fe concentration was 1 mM. *L. leucocephala* seedlings showed a higher growth response in all parameters compared to *C. callothyrsus* seedlings. High levels of Fe can be hazardous to plants, reducing their development and production and, in rare circumstances, causing plant death (Connolly & Guerinot 2002; Gross et al. 2003; Frei et al. 2016; Wu et al. 2017). The stage of plant growth and development, particularly the initial vegetative stage, which is associated with a decrease in plant height and accumulation of plant dry weight, has a major influence on Fe toxicity (Asch et al. 2005; Majerus et al. 2007; Quinet et al. 2012).

Fe toxicity affects the initiation of lateral roots by reducing the elongation and division of root cells (Li et al. 2016). Roots are the first organs to experience Fe toxicity, and root tips are the most sensitive sites for Fe toxicity (Li et al. 2015; Zhang et al. 2018; Onyango et al. 2019). Roots are the first organs to experience Fe toxicity, and root tips are the most sensitive sites for Fe toxicity (Li et al. 2015; Zhang et al. 2018; Onyango et al. 2019). High Fe concentration reduced the length of the plant roots (Table 1). This shows that the plant has experienced the impact of Fe toxicity. A considerable rise in Fe concentration reduced the growth of primary *Arabidops thaliana* roots (Zhang et al. 2018). High iron can induce ROS in the root tip zone area, which causes inhibition of plant root development (Onaga et al. 2016; Zhang et al. 2018). According to the findings of Effendy et al. (2015), Fe concentration had an effect on the root length of pineapple plants 1-5 weeks after treatment, and root length inhibition occurred as Fe concentration increased. The research by Shiwachi et al. (2006) also reported that the level of Fe 60 mg L^{-1} was able to reduce the number of roots up to 58% compared to controls. Excess iron can damage the root system in rice (Onyango et al. 2019). However, Majeed et al. (2020) reported that the Fe application could result in better root development, the application of Fe can result in better root proliferation and helps plants to produce more roots.

However, high concentrations of Fe can cause an imbalance of mineral nutrients that can affect plant growth and development (Shimizu et al. 2004; Audebert 2006; Mehraban et al. 2008). Fe poisoning can also inhibit nutrient uptake by harming the surface of the root epidermis (Jorgenson et al. 2013). The intake of nutrients such as P, K, Ca, and Mg can be reduced by increasing the Fe concentration (Sahrawat 2005; de Dorlodot et al. 2005; Fageria 2007; Fageria et al. 2008).

The dry weight of the plant is strongly influenced by the growth of shoots and plant roots. The results showed that increasing the concentration of Fe was able to reduce the dry weight of roots, shoots, and total seedlings (Table 1). The results of several studies reported that increasing the concentration of Fe was able to reduce the dry weight of roots and shoots of rice (Noor et al. 2016) and pineapple (Effendy et al. 2015). The poisoning of Fe has the potential to reduce plant biomass (Engel et al. 2012). Plant dry weight drop is related to increased Fe accumulation in the plant, which slows plant growth (Mehraban et al. 2008). Nenova (2006) also reported a reduction in the total dry weight of peas at a concentration of Fe^{2+} 40 mg L^{-1} at 44 days of age. However, the results of Bierschenk et al. (2020) reported that Fe exposure did not have a negative effect on straw biomass.

Tolerance Index

Each plant can respond differently to Fe exposure (Frei et al. 2016). The tolerance level of plants to Fe exposure is influenced by plant development, exposure intensity, exposure time, and climatic conditions (Engel et al. 2012). The tolerance index for both seedlings decreased with increasing Fe concentration (Figure 1). The tolerance index for *C. callothyrsus* decreased drastically when the Fe concentration was 0.5 mM, while the tolerance index

Table 1. Growth response of *C. calothyrsus* and *L. leucocephala* seedlings to Fe exposure after 4 weeks of treatment.

Treatment	Parameter				
	Heihgt (cm)	Root length (cm)	Dry root weight (g)	Dry shoot weight (g)	Dry total weight (g)
A1D0	7.56 ± 1.76 d	50.24 ± 7.06 c	0.50 ±0.26 ab	1.48 ± 0.48 b	1.99 ± 0.73 b
A1D1	7.33 ± 1.43 d	33.32 ± 6.93 d	0.34 ± 0.08 c	1.41 ± 0.42 b	1.75 ± 0.48 b
A1D2	1.44 ± 0.38 f	13.94 ± 3.14 efg	0.12 ± 0.03 de	0.29 ± 0.15 cd	0.39 ± 0.17 cde
A1D3	1.86 ± 0.45 f	10.56 ± 4.14 efgh	0.08 ± 0.03 ed	0.29 ± 0.09 cd	0.38 ± 0.12 cde
A1D4	0.83 ± 0.55 f	10.48 ± 1.80 efgh	0.05 ± 0.03 e	0.16 ± 0.03 d	0.22 ± 0.06 de
A1D5	0.94 ± 0.34 f	6.12 ± 0.74 gh	0.03 ± 0.02 e	0.08 ± 0.04 d	0.10 ± 0.04 e
A1D6	0.33 ± 0.10 f	8.96 ± 2.19 fgh	0..02 ± 0.01 e	0.05 ± 0.03 d	0.10 ± 0.04 e
A1D7	0.27 ± 0.23 f	4.76 ± 1.06 h	0.01 ± 0.01 e	0.05 ± 0.02 d	0.09 ± 0.06 e
A2D0	22.04 ± 2.41 a	87.78 ± 10.90 a	0.61 ± 0.14 a	1.89 ± 0.17 a	2.50 ± 0.31 a
A2D1	19.54 ± 1.81 b	58.34 ± 10.67 b	0.47 ± 0.05 b	1.43 ± 0.17 b	1.90 ± 0.21 b
A2D2	16.64 ± 2.14 c	36.78 ± 9.70 c	0.57 ± 0.15 ab	1.53 ± 0.32 b	2.02 ± 0.41 b
A2D3	6.96 ± 2.54 d	33.96 ± 12.07 d	0.20 ± 0.03 d	0.49 ± 0.10 c	0.70 ± 0.13 c
A2D4	4.00 ± 1.73 e	16.16 ± 3.43 ef	0.21 ± 0.11 de	0.32 ± 0.13 cd	0.53 ± 0.15 cd
A2D5	0.50 ± 0.37 f	18.74 ± 4.64 e	0.09 ± 0.05 ed	0.15 ± 0.05 d	0.24 ± 0.07 de
A2D6	1.10 ± 0.57 f	14.50 ± 1.45 efg	0.09 ± 0.01 ed	0.22 ± 0.03 cd	0.33 ± 0.04 cde
A2D7	0.33 ± 0.10 f	14.26 ± 3.48 efg	0.04 ± 0.01 e	0.11 ± 0.03 d	0.16 ± 0.03 de
P-value	**	**	**	**	**

Note: mean ± standard deviation, the different letters show a significant difference in the DMRT test results at the 5% level. ** significant effect at the 1% level. A1: *C. callothyrsus*, A2: *L. leucocephala*, D0: 0 mM, D1: 0.25 mM, D2: 0.5 mM, D3: 0.75 mM, D4: 1 mM, D5: 1.25 mM, D6: 1.5 mM, dan D7: 1.75 mM.

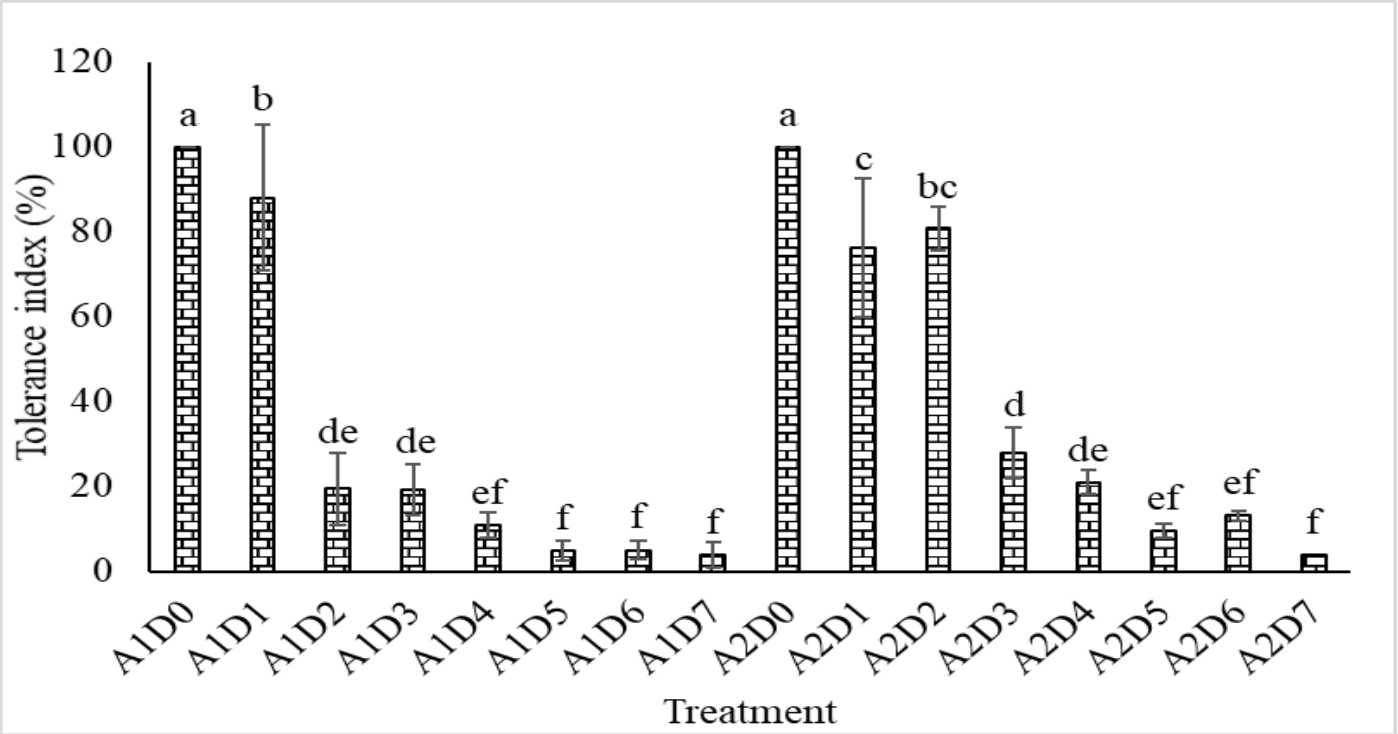

Figure 1. Tolerance index of *C. calothyrsus* and *L. leucocephala* seedlings to Fe exposure. The different letters above the bar chart show significant differences based on the DMRT test (α=5%). A1: *C. callothyrsus*, A2: *L. leucocephala*, D0: 0 mM, D1: 0.25 mM, D2: 0.5 mM, D3: 0.75 mM, D4: 1 mM, D5: 1.25 mM, D6: 1.5 mM, dan D7: 1.75 mM.

for *L. leucocephala* decreased drastically when the Fe concentration was 0.75 mM compared to the control treatment. The tolerance index for *L. leucocephala* was higher than that of *C. callothyrsus*. This shows that each seedling has a different tolerance to Fe exposure. Plants develop exclusion and inclusion strategies against Fe exposure by involving various complex physiological processes (Turhadi et al. 2018). Furthermore, excess Fe causes enhanced antioxidant and antioxidant enzymes such as ascorbate peroxidase, glutathione reductase, and peroxidation in rice (Fang et al. 2001; Stein et al. 2009).

Photosynthesis Rate

Fe exposure in seedlings did not show a significant difference in the rate of photosynthesis (Figure 2). The photosynthetic rate in all treatments varied and quite fluctuating. In species of *C. callothyrsus*, Fe concentrations of 0.25 and 1.5 mM were able to increase the rate of photosynthesis, while in species of *L. leucochepala* the presence of Fe exposure could increase the rate of photosynthesis. These results showed that Fe exposure to 2 mM was still able to stimulate the rate of photosynthesis in *C. callotyrsus* and *L. leucocephala* seedlings. Some concentrations of Fe can increase the rate of plant photosynthesis. Fe functions in plant physiological processes, namely photosynthesis and respiration as electron donors or acceptors (Kerkeb & Connolly 2006; Kobayashi & Nishizawa 2012; Zhai et al. 2014; Rout & Sahoo 2015; Wu et al. 2017). However, increasing the concentration of Fe can reduce the rate of plant photosynthesis. This is due to exposure to heavy metals such as Fe can affect photosystem II (PS), especially at PS II donor and acceptor sites, and inhibits the activity of oxygen evolution and trans-electron reactions (Pourrut et al. 2011; Deng et al. 2014). Kampfenkel et al. (1995) reported that Fe^{2+} stress was able to reduce the photosynthesis rate of *Nicotiana plumbaginofolia* plants by 40%.

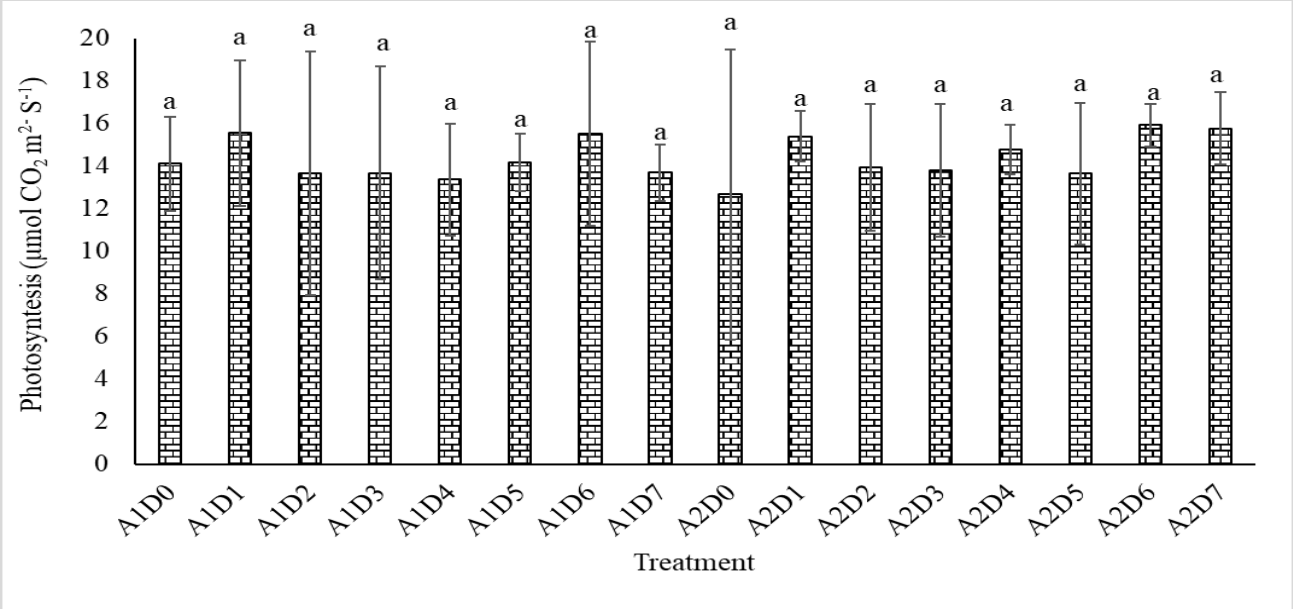

Figure 2. Photosynthesis rate of *C. calothyrsus* and *L. leucocephala* seedlings against Fe exposure. The letters above the bar chart show significant differences based on the DMRT test (α=5%). A1: *C. callothyrsus*, A2: *L. leucocephala*, D0: 0 mM, D1: 0.25 mM, D2: 0.5 mM, D3: 0.75 mM, D4: 1 mM, D5: 1.25 mM, D6: 1.5 mM, dan D7: 1.75 mM.

Chlorophyll and Carotenoid Content

Chlorophyll levels in plant leaves can be affected by Fe, which also plays a role in the creation of chloroplast ultrastructures (Bozorgi 2012), chlorophyll synthesis (Müller et al. 2015), maintenance of chloroplast structure and function (Jin et al. 2008; Jeong & Connolly 2009). Treatment of seedlings species and concentration of Fe had a significant effect on the content of chlorophyll a, b, total chlorophyll, and carotenoids (Table 2). However, both seedlings in several concentrations of Fe did not show a significant difference. In *L. leucocephala* seedlings, exposure to Fe was able to increase the content of chlorophyll and carotenoids. Meanwhile, the chlorophyll content of *C. callothyrsus* seedlings varied at various Fe concentrations. This shows that each plant has a different response to Fe exposure, especially to the chlorophyll content.

High Fe concentrations can reduce the chlorophyll content of plants and it is one symptom of Fe toxicity (Fageria et al. 2008; Dufey et al. 2009). This can be seen from the response of *C. calothyrsus* seedlings which showed a decrease when the Fe concentration was 1.75 mM. Decreases in chlorophyll content have also been reported in rice plants caused by Fe toxicity (Stein et al. 2009; Quinet et al. 2012; Turhadi et al. 2019). The decrease in plant chlorophyll content is caused by oxidative stress due to the high Fe content (Gajewska & Skłodowska 2007). Furthermore, it is assumed that the decrease in chlorophyll pigment is linked to the closure of the stomata, which results in a reduction in the photosynthetic process (Sairam & Saxena 2000; Quinet et al. 2012; Pereira et al. 2013).

The content of Fe in the plant tissue

Several metal chelators and transporters are required for Fe uptake by plant roots and translocation to various plant tissues (Quinet et al. 2012). Fe content in both seedling tissues was quite fluctuating at various Fe concentrations (Figure 3). In *C. calothyrsus* seedlings, the highest Fe content was when the concentration was 1.5 mM (4.40%), while in *L. leucocephala* seedlings, the highest Fe content was at 1.7 mM (2.87%). The Fe content in the two seedlings is different for each Fe concentration, and this shows that each plant has the other ability to absorb Fe. Fe can be toxic when it

Table 2. Chlorophyll and carotenoid content of *C. calothyrsus* and *L. leucocephala* seedlings to Fe exposure.

Treatment	Parameters			
	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll (mg/g)	Caretoneoids (mg/g)
A1D0	3.42 ± 0.43 abcde	1.89 ± 0.26 abc	5.30 ± 0.68 abcd	1.16 ± 0.13 abc
A1D1	4.48 ± 0.43 a	2.46 ± 0.22 a	6.82 ± 0.70 a	1.35 ± 0.11 a
A1D2	4.00 ± 0.35 abcd	2.19 ± 0.17 ab	6.18 ± 0.51 abc	1.32 ± 0.06 a
A1D3	4.32 ± 0.46 ab	2.39 ± 0.27 a	6.69 ± 0.73 ab	1.37 ± 0.14 a
A1D4	3.12 ± 1.28 cde	1.77 ± 0.74 abc	4.89 ± 2.02 bcd	1.02 ± 0.23 abc
A1D5	2.99 ± 0.51 de	1.61 ± 0.33 bc	4.61 ± 0.84 dc	0.94 ± 0.18 bc
A1D6	4.21 ± 1.48 abc	2.38 ± 0.87 a	6.59 ± 2.35 ab	1.38 ± 0.49 a
A1D7	2.42 ± 0.33 e	1.36 ± 0.24 c	3.78 ± 0.57 d	0.84 ± 0.15 c
A2D0	3.23 ± 0.07 bcde	1.89 ± 0.08 abc	5.16 ± 0.15 abcd	1.07 ± 0.03 abc
A2D1	3.65 ± 0.10 abcd	2.09 ± 0.08 ab	5.78 ± 0.18 abc	1.10 ± 0.04 abc
A2D2	3.58 ± 0.22 abcd	2.07 ± 0.20 ab	5.64 ± 0.43 abc	1.08 ± 0.12 abc
A2D3	3.53 ± 0.33 abcde	2.05 ± 0.19 ab	5.58 ± 0.52 abcd	1.08 ± 0.10 abc
A2D4	3.63 ± 0.11 abcd	2.11 ± 0.08 ab	5.73 ± 0.19 abc	1.11 ± 0.06 abc
A2D5	3.93 ± 0.25 abcd	2.23 ± 0.11 ab	6.16 ± 0.36 abc	1.21 ± 0.05 abc
A2D6	3.62 ± 0.32 abcd	2.10 ± 0.26 ab	5.71 ± 0.57 abc	1.50 ± 0.16 abc
A2D7	3.68 ± 0.77 abcd	2.19 ± 0.47 ab	5.87 ± 1.24 abc	1.30 ± 0.19 ab
P-value	*	*	*	*

Note: mean ± standard deviation, the different letters show a significant difference in the DMRT test results at the 5% level. *: significant effect at the 5% level. A1: *C. callothyrsus*, A2: *L. leucocephala*, D0: 0 mM, D1: 0.25 mM, D2: 0.5 mM, D3: 0.75 mM, D4: 1 mM, D5: 1.25 mM, D6: 1.5 mM, dan D7: 1.75 mM.

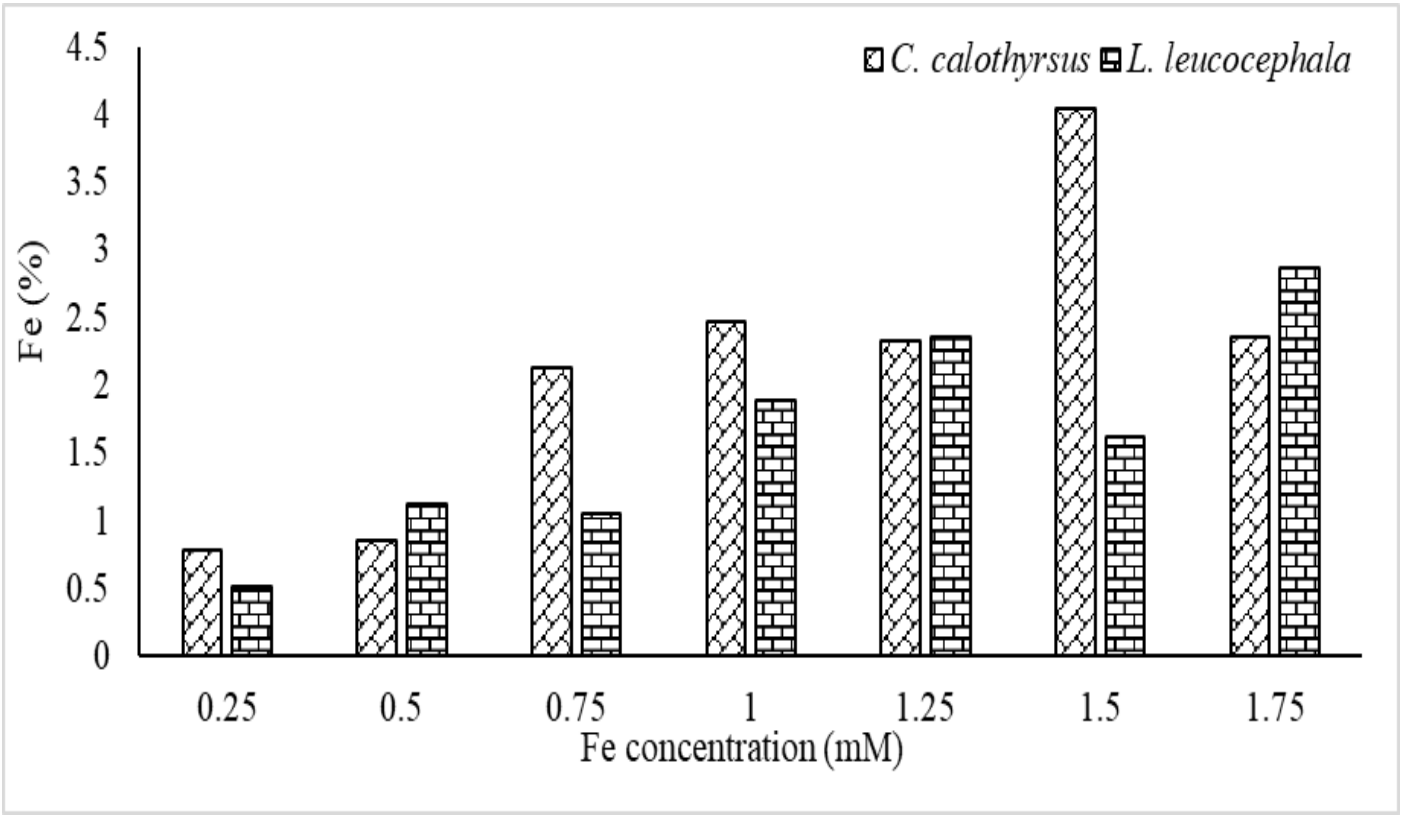

Figure 3. Fe content in the seedling tissue of *C. calothyrsus* and *L. leucocephala*

accumulates high enough in plant tissue (Connolly & Guerinot 2002). When plants absorb too much Fe, it can induce a shift in the redox cell balance, which can lead to alterations in the plant's morphological, biochemical, and physiological properties, as well as death (Hell & Stephan 2003; Onyango et al. 2019). Fe poisoning can cause Fe uptake in shoots and roots to be reduced (Dufey et al. 2009).

CONCLUSION

Treatment of seedlings species and concentration of Fe was able to significantly affect the growth parameters (height, root length, root dry weight, shoots, and plant dry weight) of seedlings. The increase in Fe concentration was able to reduce all growth parameters in both seedlings. The 0.5 mM Fe concentration reduced all growth parameters of *C. calothyrsus* drastically, while in *L. leucocephala*, the Fe 0.75 concentration was able to decrease all growth parameters drastically. The tolerance index of both seedlings decreased with increasing Fe concentration. The rate of photosynthesis did not show a significant difference between treatments, meanwhile, it had a significant effect on chlorophyll affect chlorophyll (a, b, and total chlorophyll) and carotenoid content. The highest Fe content in *C. calothyrsus* seedlings was at a concentration of 1.5 mM, while in *L. leucocephala* seedlings, the highest Fe content was at 1.7 mM.

AUTHORS CONTRIBUTION

S.W. conceptualization, methodology, review and supervised all the process, M.A.S. collected and analyzed the data and wrote the manuscript, L.S. conceptualization, and review, I.W. Conceptualization, and review.

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CONFLICT OF INTEREST

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

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Research Article

Molecular Identification of Mudskipper Fish (*Periophthalmus* spp.) from Baros Beach, Bantul, Yogyakarta

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ABSTRACT
Mudskipper fish is amphibious fish belonging to the family Gobiidae. Coastal communities widely consume mudskipper to meet their animal protein needs. Mudskipper is primarily cryptic species that are morphologically difficult to identify and distinguish from other mudskipper fish species. Consequently, it can be confused with the naming of mudskipper fish species and can affect the conservation efforts of the fish in their habitat. One of the molecular approaches that can be used to identify the fish species quickly and accurately is DNA barcoding using the *COI* mitochondrial gene. However, the research on the identification of mudskipper fish in Indonesia is still very limited. Therefore, this study aimed to identify 26 mudskipper fish from Baros Beach, Bantul, Yogyakarta, using *COI* mitochondrial gene as a molecular marker for DNA barcoding. The method used in this study was a PCR method with universal primers, FishF2 and FishR2. The data obtained were then analyzed using GeneStudio, DNASTAR, BLAST, Identification Engine, Mesquite, MEGAX, and BEAST. The analysis was conducted to obtain similarity, genetic distance and reconstruct a phylogenetic tree. The result revealed that all 26 samples of mudskippers collected from Baros Beach were identified in one genus, namely *Periophthalmus,* and consisted of 3 species, namely *P. kalolo* (16 samples), *P. argentilineatus* (9 samples), and *P. novemradiatus* (1 sample). Furthermore, this study also discovered a suspected cryptic species in *P. argentilineatus* with a genetic distance of 5.46-5.96% between clade E, F compared with clade G. Further morphological studies are needed to confirm the species status of these three clades before solidly proclaim that they are cryptic species.

Keywords: *COI*, cryptic species, DNA barcoding, mudskipper

INTRODUCTION
Mudskippers fish are classified into order Gobiiformes, family Gobiidae, and subfamily Oxudercinae (Murdy 1989; Van der Laan et al. 2014). The Gobiidae family comprises 258 genera and 1,850 fish species distributed in tropical and sub-tropical areas. One of the genera is *Periophthlamus* which consists of 19 valid species (Froese & Pauly 2019). According to Pormansyah et al. (2019), 11 valid species of *Periophthalmus* have been recorded and spread over 7 major islands in Indonesia, namely Sumatera, Kalimantan, Java, Sulawesi, Moluccas, Lesser Sunda, and Papua.

The mudskippers have few characteristics similar to those amphibious animals so that they are commonly recognized as amphibian fish. However, mudskipper's amphibious lifestyle is assisted by morphological and physiological adaptations such as aerial vision and olfaction, increased ammonia tolerance, terrestrial locomotion via pectoral fins, and enhanced immunological protection against pathogens (You et al. 2014; You et al. 2018). Coastal communities widely consume mudskippers because the fish contain high-quality sources of protein, minerals, and some vitamins (Andem & Ekpo 2014; Kanejiya et al. 2017; Mahadevan et al. 2021).

Cryptic species are commonly found in the family Gobiidae (Thacker 2003). Previous studies showed that cryptic species has occurred in genus *Elacatinus*, *Tigrigobius*, *Trimma*, *Eviota* using *COI* mitochondrial gene (Victor 2010; Victor 2014; Winterbottom et al. 2014; Tornabene et al. 2015), genus *Ponticola* using *Cyt-b* mitochondrial gene (Vasil'eva et al. 2016) and genus *Mugilogobius* using three mitochondrial markers (*ND5*, *Cytb*, and *D-loop*) (Huang et al. 2016). Moreover, cryptic species are also observed in mudskippers *Boleophthalmus pectinirostris* from the western Pacific coast of East Asia and the Strait of Malacca in Malaysia using Mitochondrial *ND*5 gene and nuclear *RAG-1* gene (Chen et al. 2014), *Periophthalmus argentilineatus* from East-African and Indo-Malaya using two mtDNA markers (*D-loop* and *16S* rDNA) and one nDNA region (*RAG-1*) (Polgar et al. 2014), and *Periophthalmus argentilineatus* from Bogowonto Lagoon, Yogyakarta, Indonesia using *COI* mitochondrial gene as a DNA barcoding marker (Arisuryanti et al. 2018). Since cryptic species are morphologically similar, distinguishing them solely on morphological characteristics is nearly impossible. Furthermore, the inaccurate identification of cryptic species can lead to taxonomic and conservation problems. In the last few years, molecular biology approaches have been applied for identifying fish species due to the limits of morphological identification methods. The most widely used method is DNA barcoding using the *COI* gene as a DNA barcoding marker (Hogg & Hebert 2004). DNA barcoding is a method for quickly identifying species by examining a short specific target gene. This method can identify hidden species that previously cannot be identified by the conventional identification method (Hebert et al. 2003). Sequences from the mitochondrial *COI* gene are now considered highly desirable for the precise identification of freshwater fish species (Dahruddin et al. 2017; Hutama et al. 2017; Arisuryanti et al. 2020). This is because accurate species database information can be used to develop conservation programs for cryptic species.

Baros Beach has natural barriers such as land and different newly planted mangrove area that separate sub-populations of mudskipper fish. Moreover, there is no genetic information on mudskipper fish in this area. Therefore, this study aimed to identify mudskippers from Baros Beach Yogyakarta, Indonesia, using the *COI* mitochondrial gene as a DNA barcoding marker.

MATERIALS AND METHODS

Sample collection

A total of 26 mudskippers (code MBR) were collected from 4 different stations (A, B, C, and D) in Baros Beach (Figure 1) during August 2019 and December 2020. These stations were chosen by purposive sampling to represent the natural barriers in Baros Beach (Table 1). The sampling of mudskipper fish was performed using a hand net. Then, the sample was cleaned and documented (Figure 2). Each mudskipper fish sample was then placed in a plastic clip, placed in a cooler (coolbox), and transported to the Laboratory of Genetics and Breeding (Faculty of Biology, Universitas Gadjah

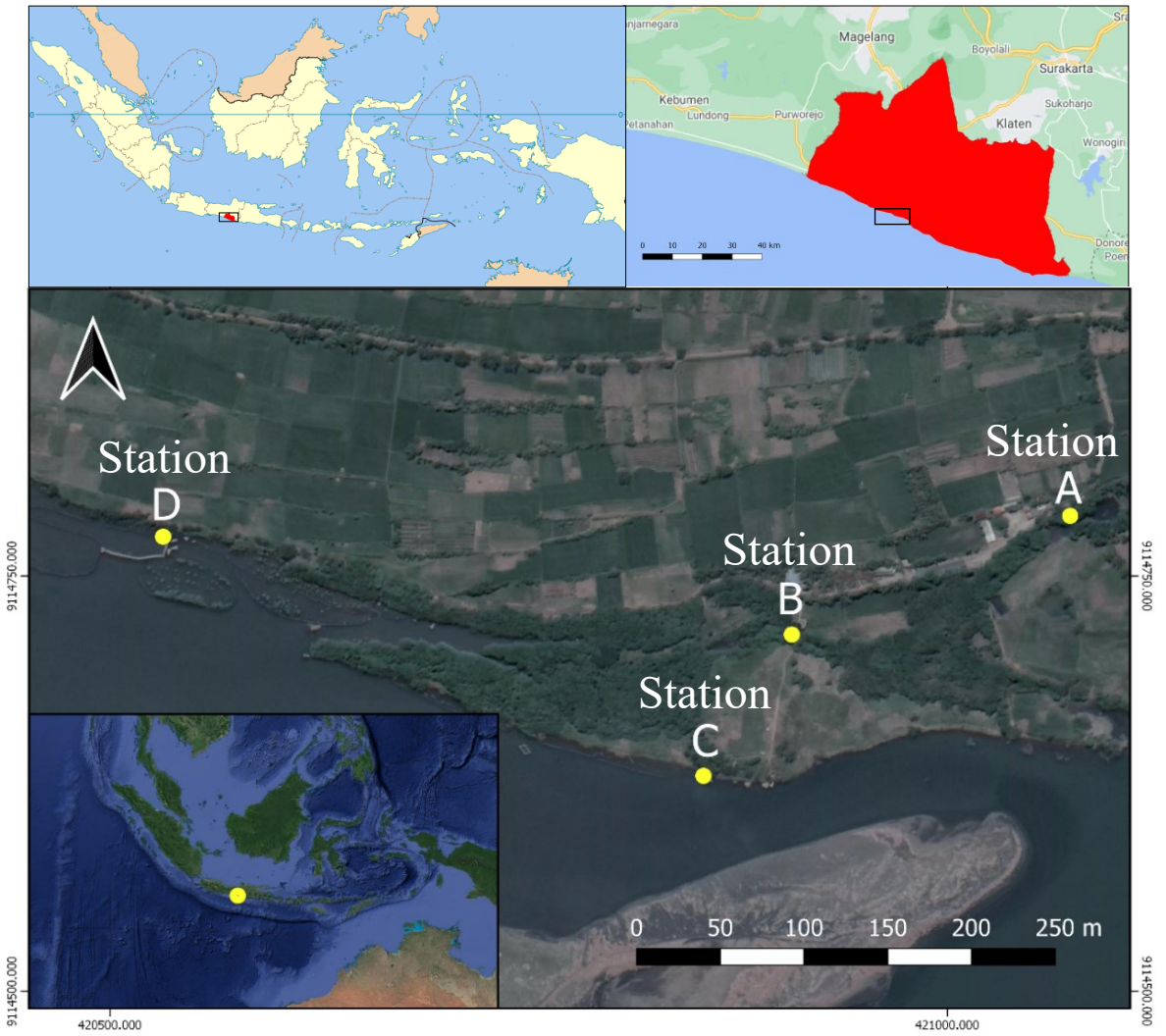

Figure 1. Map of sampling location for collecting mudskippers at Baros Beach, Bantul Yogyakarta, Indonesia.

Table 1. Coordinates and description of the sampling station.

Station name	Coordinates	Description
Station A	8°00'27.4"S 110°17'02.2"E	Brackish muddy pond surrounded by mangrove trees
Station B	8°00'29.9"S 110°16'56.2"E	Brackish muddy pond surrounded by mangrove trees
Station C	8°00'32.7"S 110°16'54.6"E	Baros Beach Lagoon with a newly planted mangrove area
Station D	8°00'27.9"S 110°16'44.2"E	A newly planted mangrove area

Mada) to be preserved with 99% ethanol and stored at -20°C until used in the following process.

DNA extraction

The genomic DNA of each specimen was extracted from tissue muscle on the back of the gills above the pectoral fins using DNeasy blood and tissue kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's protocols.

DNA Amplification

The partial *COI* mitochondrial gene was amplified using primers FishF2 (5'-TCGACTAATCATAAAGATATCGGCAC-3') and FishR2 (5'-ACTTCAG GGTGACC GAAGAATCAGAA-3') (Ward et al. 2005) with T100 Thermal-

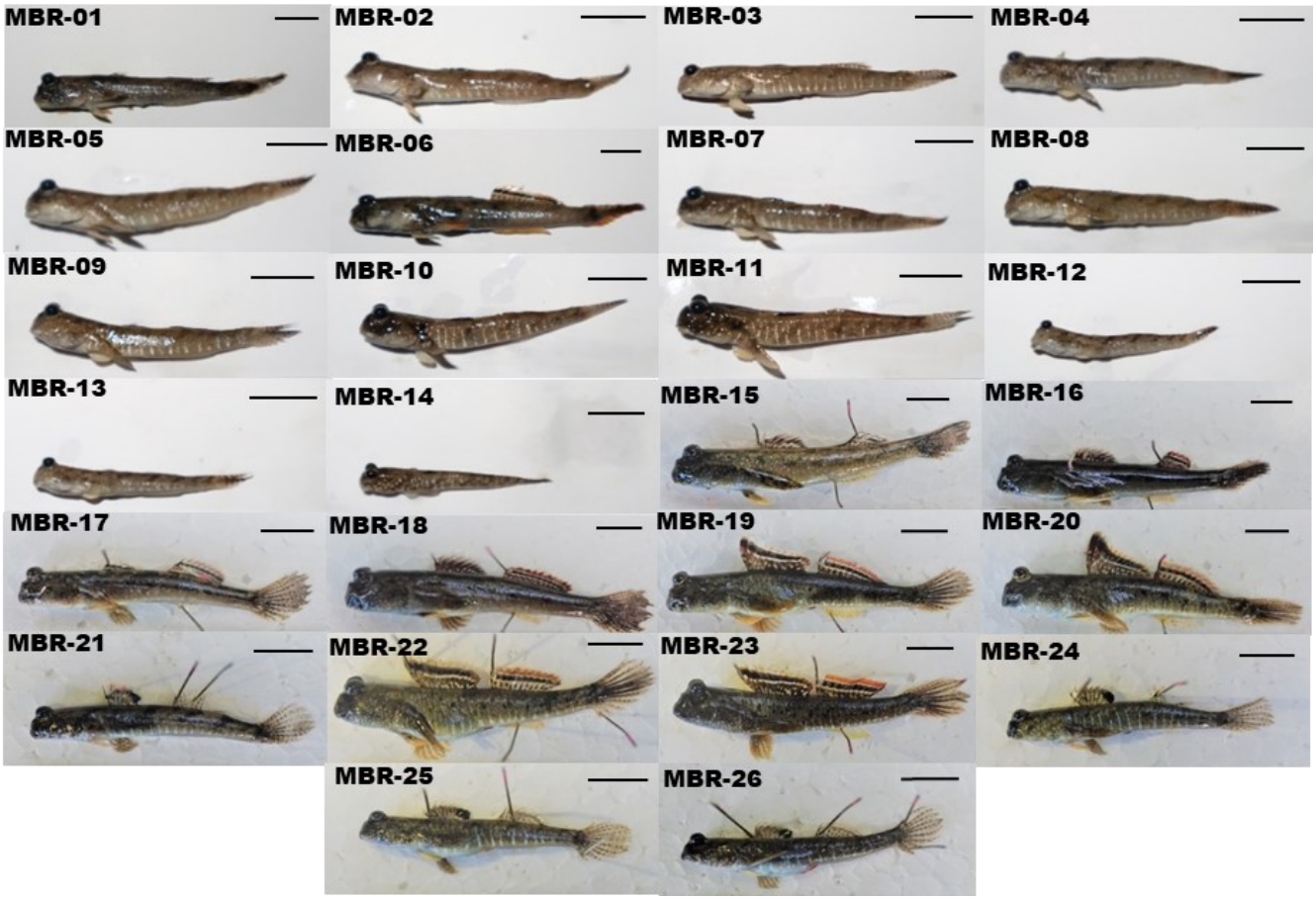

Figure 2. Mudskipper fish collected from Baros Beach. Bar = 1 cm.

Cycler (Biorad). The MyTaq HS Red Mix PCR kit (Bioline) was used for the polymerase chain reaction (PCR), and PCR amplifications were performed in 50 µL reaction volumes containing 10-100 ng of genomic DNA, 25 µL MyTaq HS Red Mix PCR, 1 mM $MgCl_2$, 0.6 µM of each primer and 11 µL double distilled water (ddH2O). Negative control was set up by omitting template DNA from the reaction mixture to assess the efficiency of the DNA amplification. PCR amplification conditions were 2 min predenaturation at 95°C, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 50°C for 30 sec, and extension at 72°C for 30 sec. A final extension of 5 min at 72°C was performed (Arisuryanti et al. 2020).

Electrophoresis and Sequencing

The electrophoresis of PCR products was run on a 1% agarose gel stained with Florosafe (Bioline) and buffered with Tris-acetate EDTA (TAE) at 50 volts for 20 minutes. Visualization was conducted under U.V. light. All amplification samples were 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).

Sequence Editing

Data obtained from DNA sequencing results were edited in the GeneStudio program and validated with SeqMan and EditSeq on the DNASTAR program (DNASTAR Inc. Madison, USA). Sequencing reactions were carried out on each individual using both forward and reverse primers. Chromatograms were inspected manually to check ambiguous bases and stop codons.

Sequence Alignment

The mudskipper *COI* sequences were then aligned using Opal on Mesquite v.3.51 program (Maddison & Maddison 2018) and ClustalW on the MEGAX program (Kumar et al. 2018).

Nucleotide Composition & Genetic Distance

The composition of the *COI* nucleotides was calculated using the MEGAX program. Genetic distance was analyzed using the MEGAX program with the Kimura-2 Parameter (K2P) model and summarized in a Neighbor-Joining (NJ) tree, which is the standard methodology used in barcoding studies with bootstrap 1,000 replicates (Hebert et al. 2003).

Phylogenetic Relationship

The reconstruction of the phylogeny tree was analyzed using the Neighbor Joining and Maximum Likelihood methods with 1,000 bootstrap using the MEGAX program (Kumar et al. 2018) and Bayesian Inference using the BEAST program (Suchard et al. 2018). The Bayesian Information Criterion (BIC) implemented in jModelTest 2.1.10 (Darriba et al. 2012) was used to determine the best fit evolutionary model. This study's most optimal sequence substitution model is HKY with invariant sites (HKY + I) on the Bayesian Information Criterion (BIC). The Markov Chain Monte Carlo (MCMC) was run for 10^6 generations to estimate the posterior probabilities distribution with a sampling frequency set to every 1,000 generations. The consensus trees were visualized in FigTree 1.4.4 (Rambaut 2019).

RESULTS AND DISCUSSION

PCR amplification & sequence identification of mudskipper from Baros Beach

The result showed that the amplification of the *COI* mitochondrial gene of 26 mudskipper fish from Baros Beach produced fragment length around 700 bp (Figure 3). The consensus sequence results from the chromatogram editing process were between 666-708 bp, which can be translated into 222-236 amino acids. According to the results (Table 2), all 26 mudskippers collected from Baros Beach were identified in one genus, namely *Periophthalmus*, and consisted of three species: *P. kalolo* (16 samples), *P. argentilineatus* (9 samples), and *P. novemradiatus* (1 sample). The similarity of the samples compared to the data in GenBank was 99.39-100% for *P. kalolo*, 97.70-100% for *P. argentilineatus*, and 100% for *P. novemradiatus*. All of the mudskipper samples collected and investigated from Baros Beach have been registered in GenBank with accession number MZ606679-MZ606687 for *P. argentilineatus*, accession number MZ614840-MZ614855 for *P. kalolo*, and accession number MZ614856 for *P. novemradiatus*.

Sequence Alignment

The alignment of sixteen *P. kalolo* sequence samples from Baros Beach yielded a clean sequence (a sequence that resulted after the alignment and cutting process) of 684 bp, meanwhile in nine *P. argentilineatus* sequence samples from Baros Beach resulted in a clean sequence of 648 bp. These clean *COI* gene sequences of each species were then used for intrapopulation analysis.

The *COI* sequences alignment of mudskippers from Baros Beach and other Indonesian regions recorded in GenBank and BOLD resulted in a fragment length of 579 bp for *P. kalolo* and *P. argentilineatus*, 651 bp for *P. novemradiatus*, respectively. This result was then subjected to intraspecies analysis (nucleotide composition and genetic distance). For phylogenetic tree

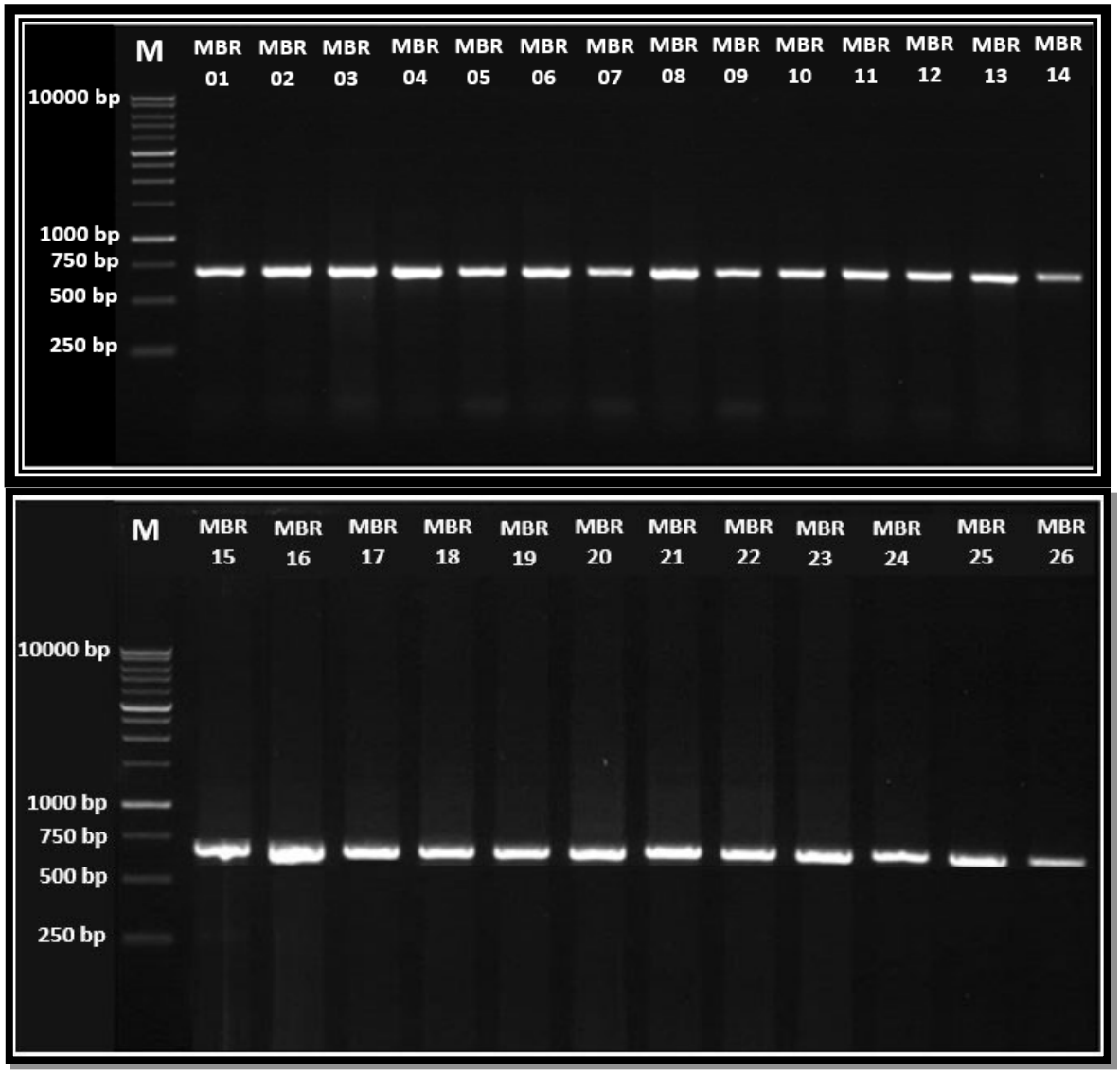

Figure 3. PCR amplification result of *COI* mitochondrial gene of mudskipper fish from Baros Beach migrated in 1% agarose electrophoresis. MBR 01 to 26 is the sample code. M is marker visualized from DNA ladder 1kb (GENEAID).

analysis, the clean *COI* sequences (570 bp) of 44 samples representing 3 species, namely, *P. kalolo, P. argentilineatus,* and *P. novemradiatus* from Baros Beach and other Indonesian regions recorded on GenBank and BOLD were used. One *COI* sequence of *Boleophthalmus boddarti* (accession number KU692377) was used as an outgroup.

Nucleotide Composition

Intrapopulation

Both species (*P. kalolo* and *P. argentilineatus*) had different nucleotide compositions of T, C, A, and G. In *P. kalolo*, the average nucleotide compositions of T, C, A, and G were 30.82%, 26.75%, 24.60%, and 17.83%, meanwhile in *P. argentilineatus* were 31.19%, 26.23%, 25.46%, and 17.11% respectively. Both species had A+T content higher than G+C, but the G+C content was higher in *P. kalolo* (44.58%) than in *P. argentilineatus* (43.35%). The difference in T, C, A, and G nucleotides between samples of *P. kalolo* were 0-1.02 %, 0-1.17%, 0-0.43 %, and 0.15-0.59 % respectively, meanwhile in *P. argentilineatus*, were 0-1.08%, 0.15-1.08%; 0.16-0.47%, and 0-0.30% respectively. Therefore, the nucleotide analysis indicated that there were variations in the intrapopulation of *P. kalolo* and *P. argentilineatus* from Baros Beach based on the *COI* mitochondrial gene.

Table 2. Species identification based on GenBank database using BLAST and BOLD identification.

No	Sample Code	Identified Species from GenBank/BOLD	Similarity (%)	Query Cover (%)	Accession Number	References
1	MBR-01	*Periophthalmus argentilineatus*	97.97	95	KU692745 KU692746	Dahruddin et al. (2017)
2	MBR-02	*Periophthalmus kalolo*	99.85	92	KU692750	Dahruddin et al. (2017)
3	MBR-03	*Periophthalmus kalolo*	99.54	92	KU692750	Dahruddin et al. (2017)
4	MBR-04	*Periophthalmus kalolo*	99.85	92	KU692747 KU692751	Dahruddin et al. (2017)
5	MBR-05	*Periophthalmus kalolo*	100	94	KU692753	Dahruddin et al. (2017)
6	MBR-06	*Periophthalmus novemradiatus*	100	92	KU692756 KU692759 KU692760 KU692763 KU692764	Dahruddin et al. (2017)
7	MBR-07	*Periophthalmus kalolo*	99.85	92	KU692747 KU692751	Dahruddin et al. (2017)
8	MBR-08	*Periophthalmus kalolo*	100	92	KU692752	Dahruddin et al. (2017)
9	MBR-09	*Periophthalmus kalolo*	100	92	KU692750	Dahruddin et al. (2017)
10	MBR-10	*Periophthalmus kalolo*	99.69	92	KU692753 MT439601 MT439602	Dahruddin et al. (2017); Arisuryanti et al. (2018)
11	MBR-11	*Periophthalmus kalolo*	99.85	92	KU692747 KU692751	Dahruddin et al. (2017)
12	MBR-12	*Periophthalmus kalolo*	99.54	93	KU692747 KU692751 KU692752	Dahruddin et al. (2017)
13	MBR-13	*Periophthalmus kalolo*	99.69	94	KU692747 KU692751	Dahruddin et al. (2017)
14	MBR-14	*Periophthalmus kalolo*	99.85	92	KU692752	Dahruddin et al. (2017)
15	MBR-15	*Periophthalmus argentilineatus*	97.70	94	KU692745 KU692746	Dahruddin et al. (2017)
16	MBR-16	*Periophthalmus argentilineatus*	97.85	92	KU692745 KU692746	Dahruddin et al. (2017)
17	MBR-17	*Periophthalmus argentilineatus*	97.70	92	KU692745 KU692746	Dahruddin et al. (2017)
18	MBR-18	*Periophthalmus argentilineatus*	97.78	95	KU692745 KU692746	Dahruddin et al. (2017)
19	MBR-19	*Periophthalmus argentilineatus*	100	92	KU692745 KU692746	Dahruddin et al. (2017)
20	MBR-20	*Periophthalmus argentilineatus*	99.69	92	KU692745 KU692746	Dahruddin et al. (2017)
21	MBR-21	*Periophthalmus kalolo*	99.69	92 92 92	KU692753 MT439601 MT439602	Dahruddin et al. (2017); Arisuryanti et al. (2018)
22	MBR-22	*Periophthalmus argentilineatus*	100	94	KU692745 KU692746	Dahruddin et al. (2017)

Table 2. Contd.

No	Sample Code	Identified Species from GenBank/BOLD	Similarity (%)	Query Cover (%)	Accession Number	References
23	MBR-23	*Periophthalmus argentilineatus*	99.86	99	MW514024	Rha'ifa et al. (2021)
24	MBR-24	*Periophthalmus kalolo*	99.39	92	KU692752 KU692751 KU692747	Dahruddin et al. (2017)
25	MBR-25	*Periophthalmus kalolo*	99.85	92	KU692751 KU692747	Dahruddin et al. (2017)
26	MBR-26	*Periophthalmus kalolo*	99.85	92	KU692750	Dahruddin et al. (2017)

Intraspecies

The analysis showed that all *P. kalolo* samples had different T, C, A, and G nucleotide compositions. The differences of T, C, A, and G nucleotides between samples were 0-0.87%, 0-1.04%, 0-0.35%, and 0-0.34%, respectively. The composition of A+T was greater than G+C. The GC content from all *P. kalolo* samples ranged from 43.18% to 44.21%. The maximum GC content was observed in *P. kalolo* from Tulungagung (KU692748) and from Cilacap (KU692749). In addition, *P. kalolo* from Tulungagung (KU692747, KU692751, KU692755) had nucleotide compositions similar to one another. Furthermore, *P. kalolo* from Cilacap (KU692753) and Bogowonto (MT439602, MT439601) had the same nucleotide composition. This variation in nucleotide composition indicated variations between *P. kalolo* from the Baros Beach and *P. kalolo* from other Indonesian regions recorded in GenBank and BOLD based on the mitochondrial *COI* gene.

All *P. argentilineatus* samples had different T, C, A, and G nucleotide compositions. The differences of T, C, A, and G nucleotide between samples were 0.17-0.69%, 0-0.52%, 0-0.19%, and 0.02-0.3%, respectively. The composition of A+T was greater than that of G+C. The GC content from all *P. argentilineatus* samples ranged from 42.14% to 42.66%. The maximum GC content was observed in *P. argentilineatus* from Pandeglang (KU692744). Furthermore, *P. argentilineatus* from Pandeglang (KU692745-46) and *P. argentilineatus* from Bogowonto (MT439599) had the same nucleotide composition. This variation of nucleotide composition indicated that there were intraspecies variations of *P. argentilineatus* from Baros Beach and *P. argentilineatus* from other Indonesian regions recorded in GenBank and BOLD based on the mitochondrial *COI* gene.

P. novemradiatus Baros Beach and *P. novemradiatus* from other Indonesian regions recorded in GenBank and BOLD had relatively similar nucleotide compositions of T, C, A, and G. The differences in T, C, A, and G nucleotides between samples were 0-0.30%, 0.15-0.46%, 0-0.15%, and 0%, respectively. The composition of A+T being greater than the composition of G+C. The GC content from all *P. novemradiatus* samples ranged from 46.24% to 46.70%. This variation in nucleotide composition indicated variations of *P. novemradiatus* from Baros Beach and *P. novemradiatus* from other Indonesian regions recorded in GenBank and BOLD based on the mitochondrial *COI* gene.

Phylogenetic analysis

The tree reconstruction results yielded identical tree topologies (Figure 4). The results of the jModelTest2 analysis revealed that the most optimal

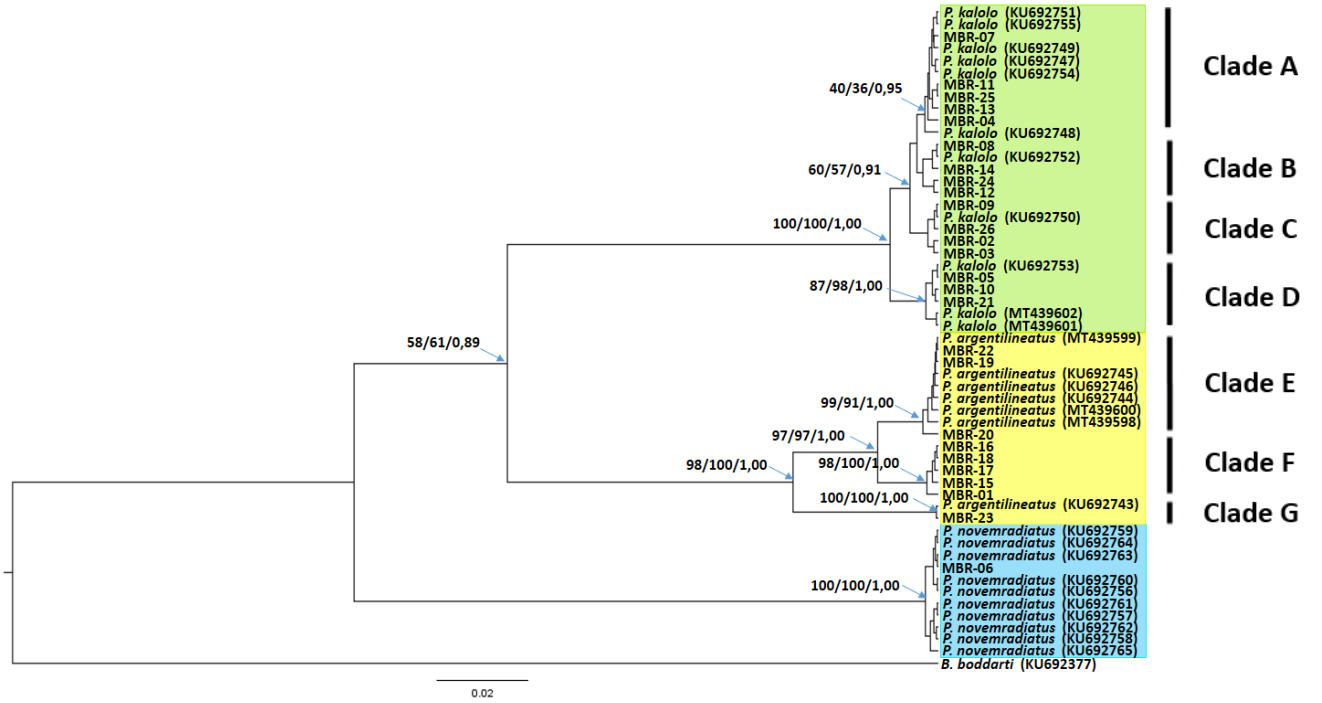

Figure 4. Phylogenetic tree reconstruction based on Neighbor-Joining (NJ), Maximum-Likelihood (ML), Bayesian Inference (BI) topology *Periophthalmus* spp. and outgroup based on *COI* gene sequences (570 bp). The node represented the number bootstrap (NJ and ML) and Bayesian Posterior Probability (Bayesian Inference).

sequence substitution model is HKY with invariant sites (HKY + I) on the Bayesian Information Criterion (BIC).

The phylogenetic tree reconstruction of *P. kalolo* from Baros Beach and *P. kalolo* from other regions in Indonesia recorded on GenBank and BOLD formed 4 different clades, namely Clade A, B, C, and D. The formation of these 4 clades were supported by a bootstrap value of 100% in the Neighbor-Joining and Maximum Likelihood methods. In addition, the posterior probability value is 1.00 in Bayesian Inference. Bootstrap and posterior probability results showed that the formation of these 4 clades was powerful and rigid. The results of the grouping above were consistent with previous studies conducted by Arisuryanti et al. (2018), which showed that *P. kalolo* from Bogowonto formed a group with *P. kalolo* from Cilacap (KU692753) and separated from *P. kalolo* from Tulungagung (KU692747-48, KU692750-52, KU692755) and other *P. kalolo* from Cilacap (KU692753 and KU692754).

The phylogenetic tree reconstruction of *P. argentilineatus* from Baros Beach and *P. argentilineatus* from regions in Indonesia recorded on GenBank and BOLD formed 3 different clades, consisting of clade E, clade F, and clade G. The formation of these 3 clades was supported by a bootstrap value of 98% in the Neighbor-Joining method, 100% in Maximum Likelihood, and posterior probability value of 1.00 on Bayesian Inference. Bootstrap and posterior probability results showed that the formation of these 3 clades was powerful and rigid. The separation of clade E and clade F was supported by morphological data in which *P. argentilineatus* members of clade E had a pointed first dorsal fin shape, while members of clade F had a rounded first dorsal fin shape.

The reconstruction of the phylogenetic tree of *P. novemradiatus* from Baros Beach with *P. novemradiatus* from other Indonesian regions recorded on GenBank and BOLD formed one clade of *P. novemradiatus*, which was supported by a bootstrap value of 100% in the Neighbor-Joining and Maximum Likelihood methods, and a posterior probability of 1.00 on the Bayesian Inference.

Genetic Distance

The lowest genetic distance was between *P. kalolo* clade B and C with an average of 0.66% (0.35-1.05%), while the highest genetic distance was between clade A and D with an average of 1.90% (1.40-2.29%) (Table 3). According to Zemlak et al. (2009), the threshold for intraspecies genetic distance in fish species is 3.5%. If it exceeds 3.5%, it is considered a different species. Based on the criterion from Zemlak above, *P. kalolo* from Baros Beach and *P. kalolo* from the Indonesian regions recorded in GenBank were still classified as the same species (conspecific).

The average genetic distance of *P. argentilineatus* between clade E and clade F was 2.51% (1.94-2.84%), between clade E and clade G was 5.96% (5.62-6.19%), and then between clade F and clade G was 5.46% (5.24-5.62%) (Table 4). Therefore, according to Zemlak's criterion, clade E and clade F were still classified as the same species (conspecific) because their genetic distance was less than 3.5%, while clade E and clade F compared with clade G were suspected to be cryptic species because their genetic distance was more significant than 3.5%. This finding was consistent with the study conducted by Arisuryanti et al. (2018) and Rha'ifa et al. (2021), who discovered that *P. argentilineatus* in Indonesia was divided into two clades separated by a genetic distance > 3,5%. The results of the study further showed that *P. argentilineatus* in Indonesia are suspected to be cryptic species. Further studies are needed to examine whether *P. argentilineatus* in Indonesia should be categorized into different species or categorized into one species with several sub-species.

The average genetic distance of *P. novemradiatus* was 0.13% (0-0.30%). Based on the Zemlak criterion, *P. novemradiatus* from Baros Beach and *P. novemradiatus* from other Indonesian regions recorded in GenBank were still classified as the same species (conspecific).

Table 3. Percentage of an intra-species genetic distance of *P. kalolo* from Baros Beach and *P. kalolo* from other regions in Indonesia recorded in GenBank and BOLD based on the *COI* mitochondrial gene.

	Clade A	*Clade* B	*Clade* C	*Clade* D
Clade A				
Clade B	0.72 (0.35-1.05)			
Clade C	0.98 (0.52-1.40)	0.66 (0.35-1.05)		
Clade D	1.90 (1.40-2.29)	1.62 (1.22-1.93)	1.39 (1.05-1.75)	

Table 4. Percentage of an intraspecies genetic distance of *P. argentilineatus* from Baros Beach and *P. argentilineatus* from other regions in Indonesia recorded in GenBank and BOLD based on the *COI* mitochondrial gene.

	Clade E	Clade F	Clade G
Clade E			
Clade F	2.51 (1.94-2.84)		
Clade G	5.96 (5.62-6.19)	5.46 (5.24-5.62)	

CONCLUSION

The identification of 26 mudskippers fish from Baros Beach revealed that they were identified in 1 genus, namely *Periophthalmus,* and consists of 3 species, namely *P. kalolo* (16 samples), *P. argentilineatus* (9 samples), and *P. novemradiatus* (1 sample). The similarity of the samples compared to the data in GenBank was 99.39-100% for *P. kalolo*, 97.70-100% for *P. argentilineatus*, and 100% for *P. novemradiatus*. The genetic relationship results showed that *P. kalolo* and *P. novemradiatus* from Baros Beach were still classified into their respective species. The phylogenetic tree showed that *P. argentilineatus* from Baros Beach consisted of 3 clades, consisting of clade E, clade F, and clade G. The average genetic distance between clade E and clade F was 2.51%, between clade E and clade G was 5.96%, and between clade F and clade G was 5.46%. That result revealed cryptic species on *P. argentilineatus* examined in this study. Further morphological studies are required to confirm the species status of these 3 clades before solidly proclaim that they are cryptic species.

AUTHORS CONTRIBUTION

T.A. planned, designed the research work, and supervised all the processes. KWA performed laboratory works (collecting samples, DNA extraction, PCR amplification, agarose gel electrophoresis, data analysis, and writing manuscript).

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CONFLICT OF INTEREST

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

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Research Article

Morphological, Histological, and Protein Profiling of Tea Embryo Axis at Early Stage of Culture

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ABSTRACT
Tissue culture is an alternative choice of plant propagation either through somatic embryogenesis or *in vitro* organogenesis techniques. TRI2025 tea clone has been cultured successfully, however, the scientific information related to morphology, histology, and protein profile at an early event of culturing time has not been reported yet. This study aimed to determine the differences between those pathways, in the context of morphology, histology, and protein profile. The explants were the embryo axis of TRI2025 tea clone cultured on two different induction mediums; somatic embryogenesis and *in vitro* organogenesis induction medium. The results showed that most of the explants cultured on A medium developed to be a globular-like structure at 11-day after culture (DAC), while all explants cultured on B medium showed the initiation stage of *in vitro* organogenesis. Histological analysis showed meristem reconstruction at shoot apical meristem (SAM) and root apical meristem (RAM) at 11-DAC at explants cultured on B medium, while explants cultured on A medium showed callusing at 21-DAC. Protein profile analysis using SDS-PAGE showed protein bands of 54 and 81 KDa that only appeared at explants cultured on A medium start from 14-DAC, and those two protein bands thought to be a differentiator at the early stages of the two tissue culture techniques. Thus, these parameters can be used as early detection for plant tissue culture, especially in tea.

Keywords: *auxin*, *Camellia sinensis*, cytokinin, micropropagation, tissue culture

INTRODUCTION
Tea is known as one of the major healthy crops cultivated in the world and its propagation can be through generative or vegetative techniques. One alternative choice of its vegetative propagation is through tissue culture, both somatic embryogenesis or *in vitro* organogenesis. There have been many reports that provided information about the success of tea propagation, with different culture conditions, such as differences in plant growth regulators (PGRs), incubation time, and other tissue culture conditions (Seran et al. 2006; Kaviani 2013; Gonbad et al. 2014; Eskundari et al. 2018).

TRI2025 tea clone belongs to the Cambod type (Kumar et al. 2014) and resistant to drought (Munivenkatappa et al. 2018). This clone is also suitable to be planted in various elevations but has high catechin content at higher altitudes of tea plantations (Mitrowihardjo et al. 2012). Those reasons make it to be one of the commonly cultivated tea clones in many plantations in the world, including Indonesia (Indonesian Tea and Quinine Research Center 2006; Haq & Mastur 2018).

Somatic embryogenesis is known as tissue culture technique that requires somatic cells as explants that then be induced its further development as the embryogenesis development pathway. On the other hand, *in vitro* organogenesis is likely the previous one, but its further development is different. One crucial point is the root-apical axis development that only occurs at explants induced through somatic embryogenesis (Bassuner et al. 2006). In tea, there were many reports about somatic embryogenesis and *in vitro* organogenesis using different clones (Gunasekare & Evans 2000; Sandal et al. 2005; Seran et al. 2006; Kaviani 2013; Gonbad et al. 2014), and TRI2025 tea clone has been reported for source clone of various explants; through both techniques (Akula & Dodd 1998; Seran et al. 2006; Eskundari et al. 2018). Besides specific clone requirements, successful of somatic embryogenesis and *in vitro* organogenesis also require specific PGRs to promote the explant's growth and development. Several studies *in vitro* related to somatic embryogenesis and *in vitro* organogenesis of tea plants were reported using PGR(s), such as auxin and cytokinin (Mondal et al. 1998; Gunasekare & Evans 2000; Sandal et al. 2005; Seran et al. 2006; Kaviani 2013; Gonbad et al. 2014).

Auxin is known as PGR that acts as an inducer of embryogenesis, exactly on apical-basal axes formation through dynamic gradient concentration (Bowman & Floyd 2008). Many transcription factors and hormonally related genes to auxin, such as *Agamous-15* (*AGL15*), *Leafy Cotyledone1* and *2* (*LEC1*; *LEC2*), and *Yucca* (*YUC*) gene are related to the somatic embryogenesis signal transduction through somatic embryogenesis pathway (Horstman et al. 2016). The LEC2 protein was reported to have a role in activating the gene encoding the MADS-box transcription factor called AGL15 (Braybrook et al. 2006) and vice versa (Zheng et al. 2009). In addition, the LEC2 protein is associated with *YUC* gene activation (Wojcikowska et al. 2013). One kind of auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), is known well as a herbicide but it can be useful for many plant tissue cultures for its low concentration addition on a culture medium (Mahalakshmi et al. 2003; Aslam et al. 2011; Kaviani 2013). Its role related to somatic embryogenesis (also to zygotic embryogenesis) is well correlated with its capability to induce cell expansion, cell cycle, and morphogenesis in plant tissue culture (Raghavan 2004). Especially in tea's tissue culture, 2,4-D is usually added to the culture medium to induce somatic embryogenesis, either by callusing pathway or directly (Kaviani 2013; Eskundari et al. 2018).

Cytokinin is known to have a role in initiating adventitious shoots (Raven & Johnson 2009). At shoot initiation, it plays an important role in proliferation and differentiation of cells around the shoot meristem through the encoding shoot meristem-related gene; *Shoot Meristemless* (*STM*) gene (Dello Ioio et al. 2008) via KNOX protein pathway (Scofield et al. 2013). Cytokinin is in relation to root initiation, particularly in meristem cell differentiation (Kyozuka 2007) in terms of cell growth initiation from differentiation zone (differentiation zone; DZ) towards the elongation and differentiation area (elongation and differentiation zone; EDZ) (Dello Ioio et al. 2008). Benzylaminopurine (BAP) is one kind of cytokinin, belongs to the purine class, and has an adenine-like configuration (Raven & Johnson 2009). BAP deficiency was reported to be associated with a decline in the ability to

initiate shooting (Werner et al. 2008). In tea's tissue culture, the use of BAP has been widely reported, such as using shoot tips and axillary buds of TRI2025 from the field as explants (Widhianata & Taryono 2019); with leaf explants of TRI2024 and TRI2025 (Farida & Muslihatin 2017); also cotyledon explants of TRI2025, Malabar2, Cinyuruan 143, and Kiara8 clone (Putra et al. 2015).

Morphology analysis is often used for knowing spatio-temporal information of explant development, while histological one is usually used to determine the process of cell development (Ogita et al. 2004; Qosim et al. 2013). These have also been used in tea's research using embryo axes of TRI2025 tea clones without removal of their growth points as explants and lead to both somatic embryogenesis and *in vitro* organogenesis (Seran et al. 2006). The surface ultrastructure of many plants derived tissue cultured has been reported widely, such as in *Passiflora* spp. (Carvalho et al. 2014), *Zea mays* (Mendez et al. 2009), and *Canna indica* L. (Wafa et al. 2016). In tea, but not derived from tissue culture, there were many reports about surface ultrastructure analysis such as white tea leaves (Ekayanti et al. 2017), trichomes of green tea (Liang et al. 1993), and polysaccharides of green tea (Yi et al. 2011).

Protein profiling has a relation with spatio-temporal aspects. In tissue culture, there were many reports about protein profiling, such as in *Medicago truncatula* (Imin et al. 2005), *Coffe spp.* (Lopez et al. 2012), and hybrid larch (Han et al. 2014). There were several reports related to tea's proteomics from the field, such as proteomics of albino tea leaves (Li et al. 2011), proteomics of tea leaf related to abscisic acid application and drought stress (Zhou et al. 2014), proteomics of tea shoot and leaves from the field Li et al. (2015), and proteomics of purple shoots tea leaves (Zhou et al. 2016). Eskundari et al. (2019) reported protein profiling of tea's tissue culture at callus, somatic embryo, globular-like structure (GLS), and initiation of leaves stages.

Tissue culture of TRI2025 tea clone using embryo axis as explant has been successfully done but the scientific information about its early event of somatic embryogenesis and *in vitro* organogenesis has been not reported yet. This research aims to analyze morphology, histology, and protein profile at an early stage of somatic embryogenesis and *in vitro* organogenesis of embryo axis of TRI2025 tea clone.

MATERIALS AND METHODS

Materials

Tea seeds of TRI2025 clone with an age average of eight months after seed-set forming were collected from the polyclonal garden of PT Pagilaran (Batang, Central Java-Indonesia) and used for initial explants. These were later sterilized using an antimicrobe agent (Agrept 20WP; Streptomycin sulphate 20%) and antifungal agent (Dithane M-45; Mankozeb 80%) then soaked into 96% alcohol for about 10 minutes. These sterilized seeds later were burned just a little moment and their seed coat was crushed using a walnut breaker.

Methods

Culture condition

The embryo axis was taken from the cotyledonary region of uncoated seeds then its two growth points at SAM and RAM were cut using a scalpel. This removed growth points of axis embryo later to be cultured on two different induction media; somatic embryogenesis and *in vitro* organogenesis induction medium (or called by A and B medium, respectively). The A medium was MS

medium added with 2 mg L^{-1} of 2,4-D (Eskundari et al. 2018) and the other was ½ MS media added with 2 mg L^{-1} of BAP (according to our best result of preliminary study). Both of these media were adjusted to 5.6 of pH prior to autoclaving. The culture condition was at light condition (with light intensity at 3250 lm, 90 lm/W (Philips TLD 36/865 cool daylight)) and with the temperature at 23ºC. This experiment has been repeated three times, with details that the experiment consisting of 20 explants to be cultured on each induction medium. The cultured explants were taken for morphological and histological analysis at 0, 11, and 21-DAC, as well as for protein profiling at 0, 3, 7, and 14-DAC.

Morphological analysis

Morphological analysis was conducted using a light microscope and scanning electron microscope (SEM). For morphological analysis using a light microscope was conducted by observing the samples under a light microscope and documented by a digital camera (Canon PowerShot A2500). For morphological analysis using SEM microscope, the prepared samples for SEM analysis were carried out following Fernando et al. (2007) with some modifications. The water content of the first explants was reduced by a vacuum dryer (PELCO 813-600), later coated the vacuumed explants with platinum (JEOL JEC-3000FC), and analyzed the explants with SEM (SEM JLM-6510LA). Time of morphological observation was at 0, 11, 21- DAC both by light and SEM microscope. Forty times (500 µm) and 100x (100 µm) at magnification were used at 11-DAC, while 22x (1 mm) at magnification was used at 21-DAC of this SEM microscope analysis.

Histological analysis

Histological analysis was carried out following Jensen (1962) at 0, 11, and 21-DAC. Explants fixed in a formaldehyde solution: glacial acetic acid: 70% ethanol at 5: 5: 9 for 24 h and dealcoholisation were done by soaking explants in alcohol-xylol mixture successively (3: 1; 1: 1; 1: 3) as well put in xylol (change twice, 30 minutes for each change). The xylol was then removed and replaced with a xylol: paraffin mixture 1: 9 at 57°C for 24 h. The explants were added to paraffin pure at a temperature of 57°C for 24 hours and cloaked by replacing it with new paraffin, and finally, after an hour a block was made. In the last stage, explants were sliced with rotary microtome with a thickness of 10-20 µm and stained with Safranin 1% and Fast Green 0,1% (Sass 1958). The slices obtained were observed under a light microscope and documented using a digital camera (Canon PowerShot A2500).

Protein extraction and profiling analysis

Protein extraction was carried out by grinding explants added with 300 µL of phosphate saline buffer (PBS). The scour was centrifuged at 9,000 rpm for 5 min at 4ºC. The supernatant was taken to obtain crude protein extract, the concentration was measured using the Biorad method (Bradford 1976). The next step was protein separation which done using the SDS-PAGE method. SDS-PAGE electrophoresis was performed according to Maniatis et al. (1982) with modification; that began with the making of resolving gel followed by stacking gel formation. The well was made by placing a comb at the corner of one side of the stacking gel. A protein mixture with a buffer with a total volume of about 25 µL was injected into the well, which was previously treated by heating followed by a short cooling. For the standard marker, 5 µL marker (Fermentas) was used. The process of separating proteins using this method was done for about 2 hours with a voltage of about 100 Volts. Staining of the electrophoresis results was done by

immersion in 0.1% brilliant blue Coomasie solution overnight and continued with de-coloration through instant heating in the microwave. The protein bands formed were then analyzed using ImageJ software (www.imageJ.com).

RESULTS AND DISCUSSION

Morphological and Histological Analysis

Both growth points were removed partially but the corpus region of the SAM was still a little left (Figure 1A). It was done in order to determine the pattern of explant development. It can be shown that the procambium and ground meristem layers were still intact as seen at Figure 1B and histological analysis showed that the cutting of the lower growth point still left the root meristem part of the quiescent center (QC) area (Figure 1B). Maybe it was the cause of reconstruction meristem took place.

The unique thing that only happened to almost explants cultured on A medium was the visible globular-like structure; a slightly transparent white near the shoot meristem (Figure 1C, white cycle). This transparent globular-like structure was the main differentiator of explants cultured on A medium and this phenomenon had been reported (Eskundari et al. 2018). It was likely that this globular-like structure was the result of the presence of 54 kDa and 81 kDa protein. On the other hand, it did not occur in explants cultured on B medium but all explants cultured on B medium then showed the development of *in vitro* organogenesis (Figure 1D; the area that delimited by black dotted lines) started at 7-DAC.

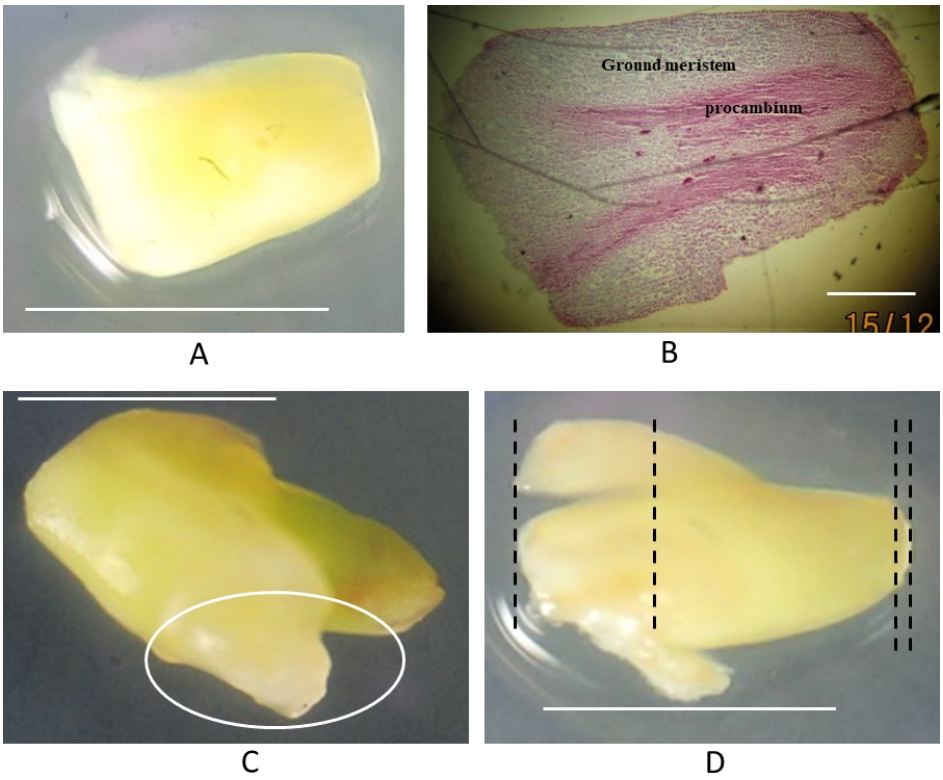

Figure 1. Morphological analysis using light microscope. Explant at 0-DAC (A); histological analysis at 0-DAC (B); at 7-HST on A media (C); at 7-HST on B media (D). Bars: A,C, D = 2.5mm; B = 0.5mm.

In this study, microscopic observations showed callusing at explants cultured on A medium especially started at 21-DAC, and this callus occurred at the surrounding root regions (Figure 2A; white dotted line) and was never found at surrounding apical regions. On the other hand, there was no callus formation that preceded shoot or root *in vitro* organogenesis. This was

probably caused by not adding auxin to B media so that did not make the accumulation of auxin-related proteins such as *PLT3*, *PLT5*, *PLT7*, and *CUP -SHAPED COTYLEDONE1* (*CUC1*) also *CUC2* (Gordon et al. 2007) and in the end, it would not eventually activate meristem-related genes such as *STM* genes and *PIN-FORMED1* (*PIN1*) (Ikeuchi et al. 2016). On the contrary, the direct *in vitro* organogenesis that occurred in this study was probably due to the accumulation of the expression of wound-encoding genes (*WOUND INDUCED DEDIFFERENTIATION*; *WIND*) that will activate cytokinin-related genes; *ARR1* and *ARR2*, and their downstream genes such as *ESR1*, then will eventually activate genes related to shoot meristems (Iwase et al. 2011). Another possible cause was an accumulation of endogenous cytokinins induced by injury (Crane & Ross 1986) so that initiated development of plants *in vitro* organogenesis. At 120-DAC, although not all explants can be induced their somatic embryos capability, some lead to the development of somatic embryogenesis (had been reported, please see Eskundari et al. 2018). The globular stage can be seen histologically with the presence of a globular structure that was initially formed through embryogenic callus (Figure 2B). The surface ultrastructural analysis also clearly demonstrated the globular embryo stage with the spherical structure (Figure 2C).

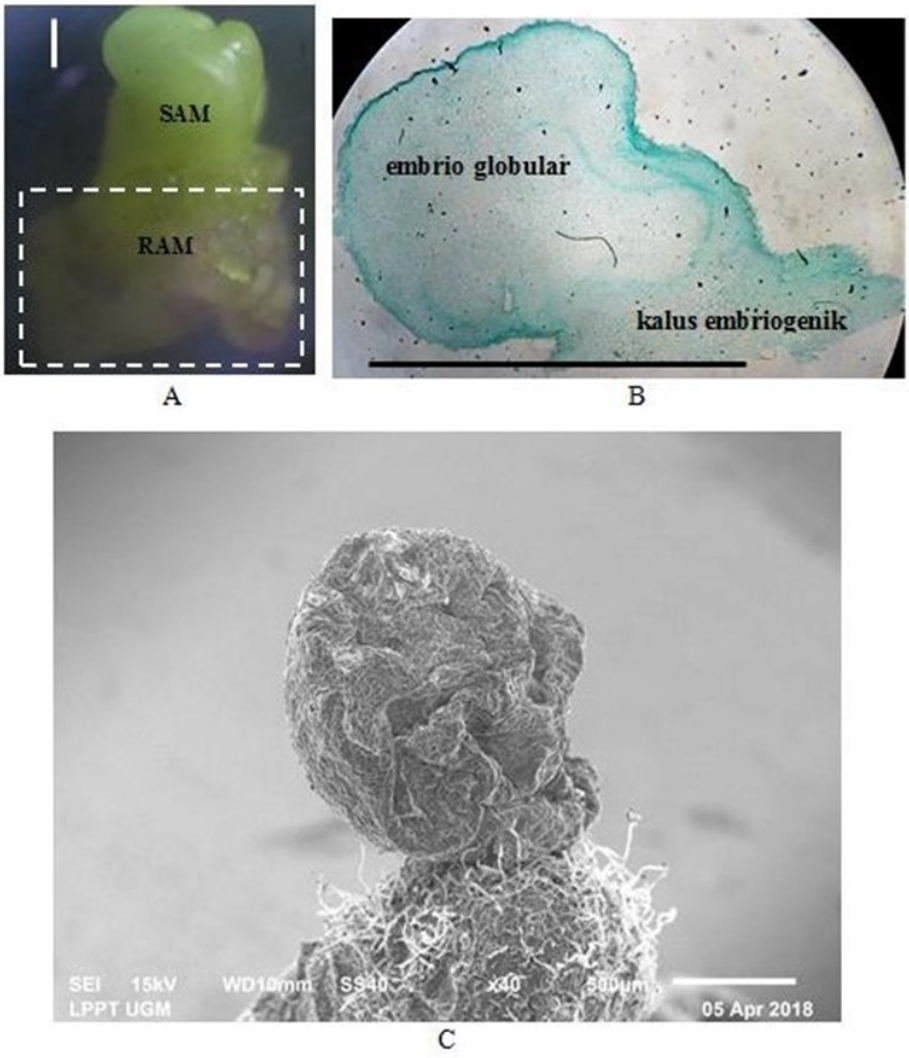

Figure 2. Morphological and histological analyses of tea's embryo axis explant cultured on A medium. Analysis using a light microscope; explant cultured on A medium at 21-DAC with callusing marked by white dotted lines (A); histological analysis of globular embryo at 120-DAC (B); surface ultrastructure of a globular embryo at 120-DAC (C). Bars: A: 0.5mm; B: 10 mm; C: 500 µm.

The development stage of *in vitro* organogenesis was seen at 11-DAC which was characterized by the development of shoots and roots on explants cultured on B medium, especially marked by arising both of leaf primordial and root elongation (Figure 3A). Furthermore, shoot and root *in vitro* organogenesis were seen at 11-DAC through meristem reconstruction; one mechanism of *in vitro* organogenesis was caused by removing some of the functional meristem areas (Reinhardt et al. 2003; Sena et al. 2009). Histological analysis showed the development of both SAM and RAM, and leaf primordial as one characteristic of plant development (Figure 3B). *In vitro* shoot, organogenesis was seen with the emergence of leaf primordial, preceded by reconstruction. In this research, SAM reconstruction was seen as the result of rearranging the missing part of the meristem. It later resulted in the initiation of leaf development by invading the adjacent empty area that was preceded by cell division. Furthermore, the reconstruction of the meristem also occurred in the RAM region. This can be proven by the development of RAM that comes from a division of surrounding cells then resulted in re-formed of the RAM region. This phenomenon was also reported by Sena et al. (2009) in *Arabidopsis* with the removal of the QC region (one of the parts included in RAM). Surface ultrastructure analysis at 11-DAC confirmed the occurrence of plant development especially at the initiation stage of *in vitro* organogenesis. At SAM region, visible developing regions at the former incision were marked by a rising plateau-like structure that later developed to be leaf primordial (Figure 3C). On the other hand, at RAM region, visible developing regions were also seen that marked by root lengthening at elongation zone (white arc; Figure 3D).

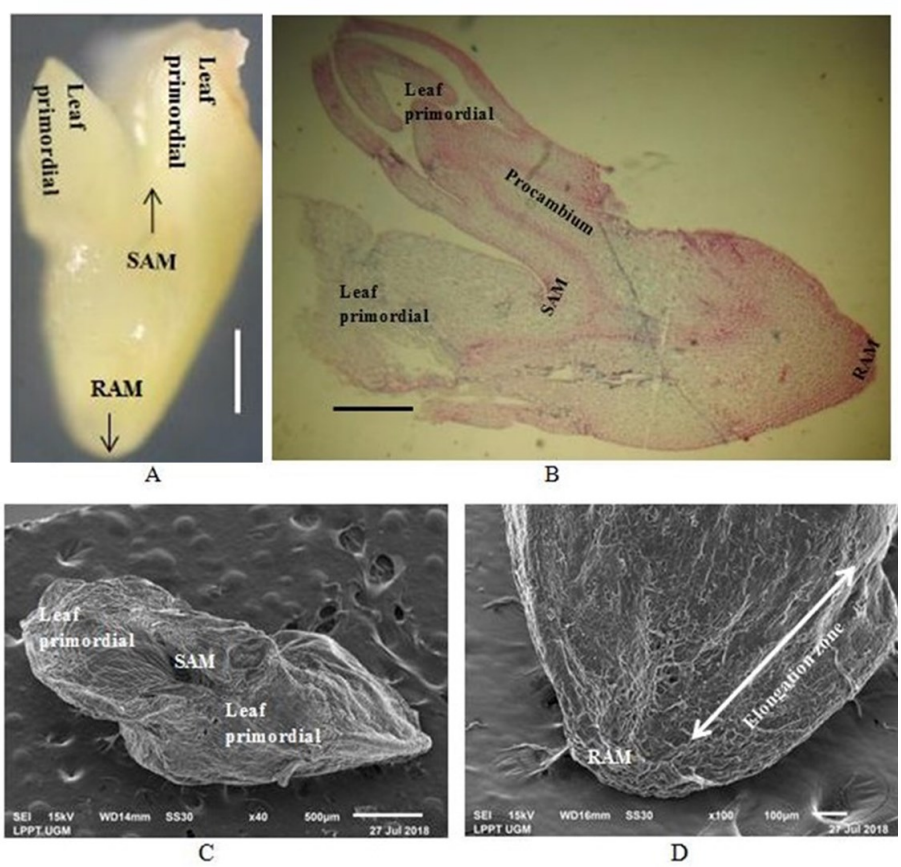

Figure 3. Morphological and histological analyses of tea's embryo axis explant cultured B medium at 11-DAC. Morphological analysis of explants cultured on B medium (A); histological analysis of explant cultured on B medium (B); SAM region morphology was analyzed using SEM (C); RAM region morphology was analyzed using SEM (D). Bars: A-C: 0.5mm; D: 500 μm; E: 100 μm.

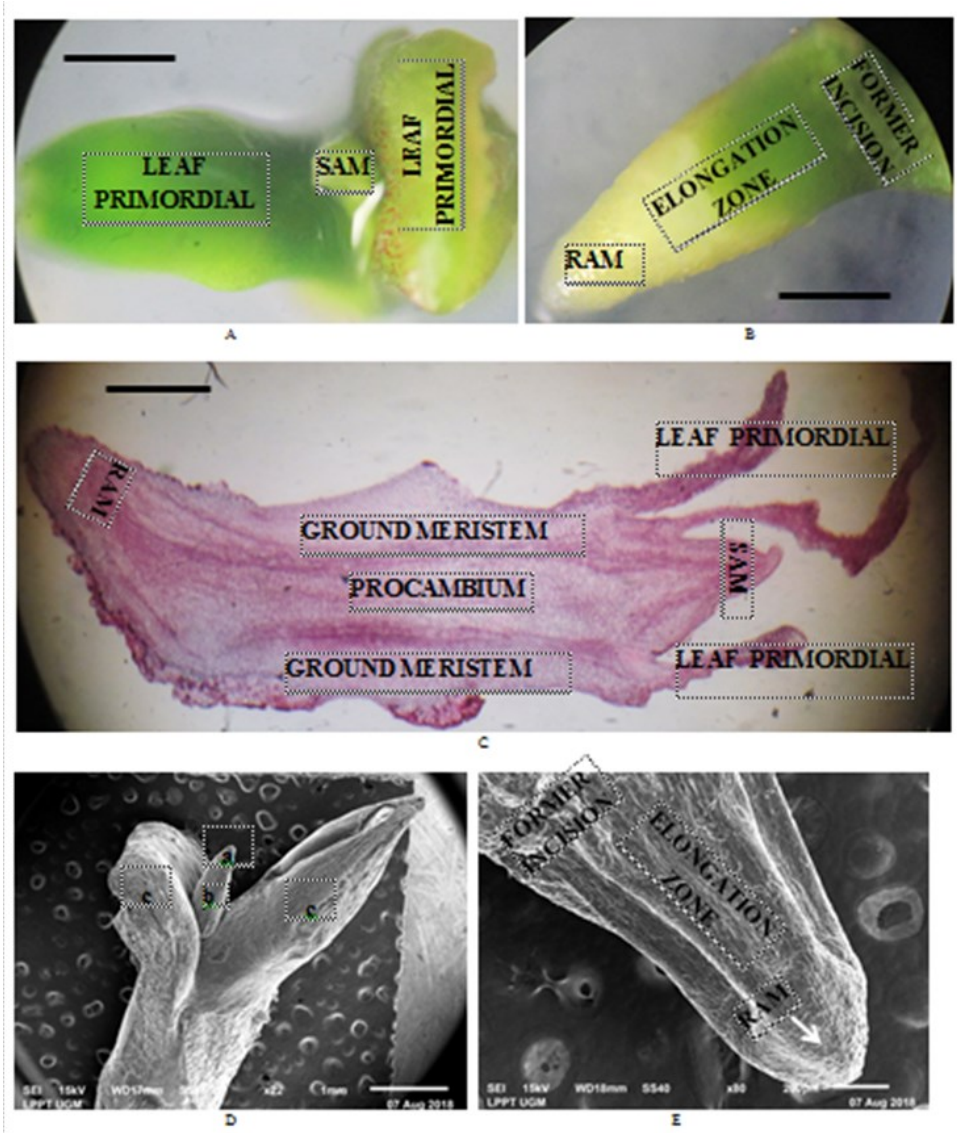

Figure 4. Morphological and histological analysis of planlet cultured on B medium at 21-DAC. The Upper region of planlet (A); lower one (B); histological analysis (C); upper region using SEM (D); lower one (E). a: former incision; b: SAM; c: leaf promordial. Bars: A-C: 0.5mm; D: 1mm; E: 200μm.

The plantlets that formed at 21-DAC on B medium were marked by the root and shoot formation, then followed by the initiation of leaf development (Figure 4A-B). The histological analysis showed that leaf development started with initiation of one leaf primordial with three rosette leaves (Figure 4C). Histological analysis also showed that the roots formed were the main roots, not the lateral roots. This was probably due to cutting in some regions of the root meristem so that the root meristem was still be maintained. Next, the explant development can be seen by increasing at volume and size of the explants, also the development of shoot and root that started from the former incision region (Figure 4D-E). It was also seen that there was root development at the elongation stage.

Morphological analysis using SEM was often used to examine the development of plant derived tissue cultures, such as in passion fruit (Biasi et al. 2000), *Lolium perrene* L. (Bradley et al. 2001), and *Passiflora* (Fernando et al. 2007). In this study, we proved the successful method without standard preparation, just directly to be vacuumed and coated with platinum then finally were observed using SEM. The results of SEM images were not so bad, so that, this would be an alternative method for SEM analysis, especially for limited laboratory equipment.

The time for initiation of somatic embryogenesis and *in vitro* organogenesis was vary in many plants derived from tissue culture. Kaviani (2013) reported that somatic embryo occurred directly at embryonic axes of tea after two months cultured on MS medium containing 1 µM 2,4-D. Tahardi et al. (2003) also reported that cotyledon explants of Yabukita tea's variety can be induced in its somatic embryo at four weeks after culturing on MS medium supplemented with BAP. Ali et al. (2016) reported that indirect *in vitro* organogenesis of ginger was at 15-20 -DAC for root initiation and at 30-40 -DAC for shoot initiation. Initiation of *in vitro* organogenesis of soybean occurred at four weeks after culture (Joyner et al. 2010) while petiole of *Gerbera jamesonii* Bolus ex. Hook f. needed 28-DAC to induce its shoot *in vitro* organogenesis (Hasbullah et al. 2008). In this study, some explants showed globular embryo at 120-DAC for explants cultured on A medium and initiation of *in vitro* organogenesis at 11-DAC followed by planlet formation at 21-DAC for explants cultured on B medium.

The ability of this PGR; 2,4-D, for induction somatic embryogenesis might be due to its role in plant development that irregular distribution of auxin must be established first in order to initiate embryo formation. This irregular (or asymmetrical) auxin distribution results from differential transport (Márquez-López et al. 2018). Next, at *in vitro* organogenesis, it has been proposed that at shoot initiation, cytokinin plays a important role in proliferating and differentiating cells within SAM through influencing the expression of key regulator gene at SAM; *SHOOT MERISTEMLESS* (*STM*) gene (Dello Ioio et al. 2008). Its deficiency revealed the reduction of shoot initiation that can be seen by the difference of nuclear DNA (Werner et al. 2008). Then, cytokinin also has an important role in initiating differentiation at RAM (Kyozuka 2007) especially to promote cells moving from differentiation zone (DZ) to elongation and differentiation zone (EDZ) (Dello Ioio et al. 2008).

Many functions of auxin were in line with the results of this study that can be proven by the presence of somatic embryos at some explants cultured on A medium. Later, two functions of cytokinin both at SAM and RAM were also in line with the results of this study. *In vitro* organogenesis, both shooting and rooting can be achieved by culturing the explants just into MS medium supplemented with BAP alone. These results suggested that the capability of cytokinin alone for promoting two kinds of *in vitro* organogenesis simultaneously. *In vitro* organogenesis is one way for propagating any part of a plant to be a partial or whole planlet, including for tea plant. This way was easy for rapid propagation of tea but with some limitations, such as hyperhydricity (Gonbad et al. 2014). In tea *in vitro* organogenesis, a shoot regeneration commonly to be induced first, and then root organogenesis was done subsequently (Ranaweera et al. 2013; Gonbad et al. 2014).

Protein Profiling

It was clear that there were at least two different protein bands in explants cultured on both mediums, namely protein bands with MW of 54 and 81 KDa (Figure 5). Both of these protein bands were visible vaguely at 7-DAC and were seen clearly at 14-DAC at explants cultured on A medium. This was likely to provide information that at beginning of the explants cultured on both mediums would induce the similar proteins up to 7-DAC and later started from 7-DAC there were different proteins involved in this different medium culture resulted in different developmental pathways further.

During 7 until 14-DAC, these two 54 and 81 KDa protein bands were only appeared at explants cultured on A medium, but these were not found at explants cultured on B medium. These two differentiating protein bands

were probably related to the key proteins that direct the explants to the pathway of somatic embryogenesis development. Probably, that somatic embryogenesis and *in vitro* organogenesis have different developmental pathways and those two protein bands were probably protein candidates for somatic embryo direction pathway. These results were correlated to PGRs that are believed to affect the development of explants in tissue culture by influencing the levels of auxin and cytokinin of the explants so that they can cause differences in gene expression that in turn produce different proteins (Mendez-Hernandez et al. 2019). The differences in the proteins that are formed are what will lead the explants to their development, whether through somatic embryogenesis or *in vitro* organogenesis.

Besides the differences, there were similarities in protein profile between explants cultured on A and B medium. One similarity between them was the protein bands of 69 KDa that appeared at 0, 3, 7, and 14 DAC. This similarity probably showed the similarity between the two pathways within two weeks of culturing. This protein band was predicted as HSP70 protein that has a function against abiotic stress (Usman et al. 2017) and this protein has also been reported in rice (Sarkar et al. 2013).

2,4-D is known as herbicide and strong synthetic auxin; has an important role in the induction of somatic embryogenesis. This 2,4-D is correlated with the expression genes related to somatic embryogenesis, such as *YUC* and *LEC2* genes (Wojcikowska et al. 2013). Several types of *YUC* genes were reported to be one of the genes expressed within seconds to 12 h after wounding to both light and dark conditions and induce auxin biogenesis in mesophyll regions and competent cells (Chen et al. 2016). Ledwon & Gaj (2009) reported that expression of the *LEC2* gene at *Arabidopsis thaliana* was increased between 1 until 15-DAC but reached the maximal level of its expression at 5-DAC. Related to this case, it probably related to protein bands with MW of 54 KDa that only found at explants cultured on A medium but need further analysis; that was corroborated by the report of Qin et al. (2020) that the presence of YUC protein in *Isatis indigotica* was with a MW of 38.64-49.77 KDa.

Protein bands around 81 KDa were also reported to appear in zygotic immature peanut embryos cultured on MS medium supplemented with 2,4-D at 0 to 15 DAC with fluctuating band thickness (Rani et al. 2005). This showed that there was a similarity in the protein bands obtained in this study with that, namely the protein band of 81 KDa, and this protein was thought to be a protein involved in the somatic embryogenesis process. Probably, this 81 KDa protein was the same protein as the protein that Sung & Okimoto (1981) reported in carrot somatic embryo culture as a somatic embryo marker protein with a MW of 77 KDa. In addition, Hakan-Mo et al. (1996) reported that in *Picea abies* there was a somatic embryogenesis marker protein with a MW of 85 KDa from embryogenic cells with high density and secreted as extracellular proteins.

Unlike 2,4-D as auxin that induces somatic embryo initiation, BAP is an example of exogenous cytokinins that is widely used in tissue culture, especially to induce *in vitro* organogenesis. BAP has the role as an activator that produces proteins for the development of *in vitro* organogenesis, such as *Wuschel* (*WUS*), *Enhancer of Shoot Regeneration* (*ESR1*), and *ESR2* genes, that in turn leads to shoot development (Ikeuchi et al. 2016). Gallois et al. (2004) reported collaboration between the key meristem gene, *SHOOTMERISTEMLESS* (*STM*), and *WUS* that would eventually lead to the induction of *in vitro* organogenesis. The *ESR1* and *ESR2* genes were reported to be responsible for shoot organogenesis but the expression patterns of the two are different. The *ESR1* gene was expressed when the hypocotyl was cultured on shoot inducing medium (SIM) media until the 4th

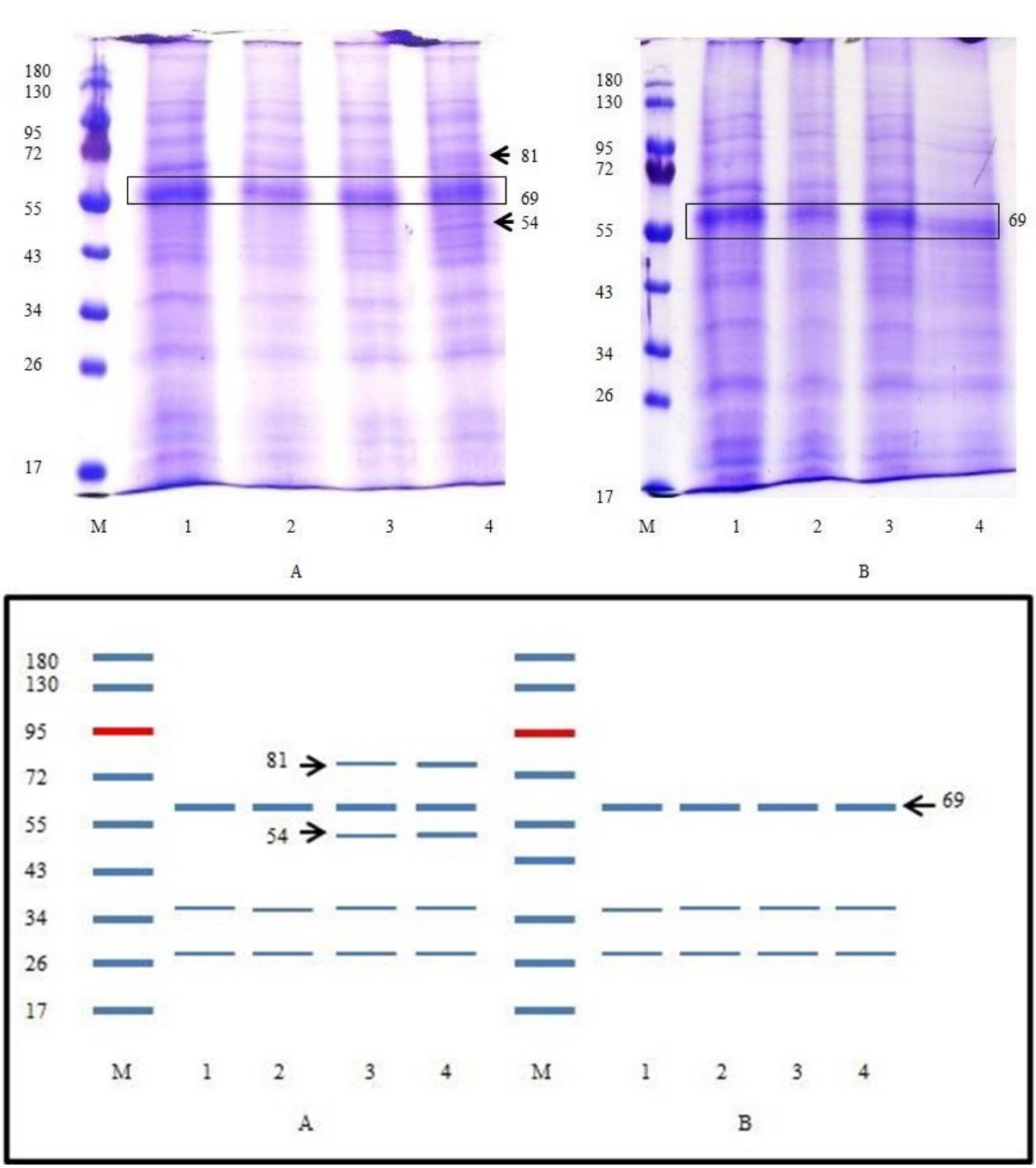

Figure 5. Protein profiling of explants on 0, 3, 7, and 14-DAC (up) and its representative (down). Cultured on somatic embryogenesis induction media (A); cultured on organogenesis induction media (B). M= marker; 1= 0-DAC; 2= 3-DAC; 3= 7-DAC; 4= 14-DAC. Left = explants cultured on MS media supplemented with 2,4-D; right = supplemented with BAP. MW of protein bands was in KDa.

day, while the *ESR2* gene was expressed after the 4th day of culturing on SIM medium (Matsuo et al. 2011).

Protein profiling can be used as an initial screening for the process of growth and development of plant tissue culture (Utami et al. 2007) as well as morphological and histological analysis that can be used for explaining any microscopic changes in detail (Koyuncu & Balta 2004). This was in line with the results of this study that there were different protein bands between those two pathways regeneration started from 7-DAC and this can be proven by morphological observations using light microscopy and SEM as well by histological analysis.

CONCLUSION

This research showed that the two tissue culture techniques have different aspects at morphological, histological, and protein profile. At the early event of culturing, explants cultured on A medium showed the presence of a globular-like structure, while the explants cultured on B medium showed the initiation stage of *in vitro* organogenesis. The histological analysis showed that some of the explants cultured on A medium lead to the development of globular stage, while at explants cultured on B medium showed the meristem reconstruction at SAM and RAM. Protein profile analysis using SDS-PAGE showed protein bands of 54 and 81 KDa only appeared at explants cultured on A medium started from 7-DAC, and those two protein bands thought to be a candidate of different key protein at the early stages of those two tissue culture processes.

AUTHORS CONTRIBUTION

R.D.E, T.T., D.I., and Y.A.P. designed the research. R.D.E. collected-analyzed the data and wrote the manuscript. T.T., D.I., and Y.A.P. supervised all the processes.

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CONFLICT OF INTEREST

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

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Research Article

Detection of Knockdown-resistance Mutations (V1016G and F1534C) in Dengue Vector from Urban Park, Surabaya, Indonesia

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ABSTRACT

An urban park is potentially a source of vector-borne disease transmission due to it being a natural and artificial mosquito breeding habitats combined with people's continuous presence. Thus, this study aims to screen the occurrence of knockdown-resistance (*kdr*) mutant alleles (V1016G and F1534C) in mosquito populations collected from urban parks in Surabaya, Indonesia. Cross sectional study was conducted in July 2019. A total of 28 ovitraps were installed in seven urban parks, having four ovitraps installed in each park. In total, 1,662 eggs were collected, and only 187 emerged into adult mosquitoes, consisting of 97 *Aedes (Stegomyia) aegypti* and 90 *Aedes (Stegomyia) albopictus*. All-female adult mosquitoes (n=55) were tested using allele-specific polymerase chain reaction assay (AS-PCR) to detect voltage gated sodium channel (VGSC) gene mutations. This study found no mutations in Valine to Glysine mutation in point 1016 (V1016G) and Phenylalanine to Cysteine in point 1534 (F1534C) alleles in both two species. All of mosquito samples have wild type genotype of *kdr* alleles (V1016V and F1534F). Data were analysed using R Studio 1.4 Version by *Genetics* package. Results showed that the frequency of resistant alleles (G1016 and C1534) was zero, and the frequency of susceptible allele was 1 (V1016 and F1534). Insecticide bioassay could not be established due to the limited number of adult mosquitoes, so insecticide resistance status could not be determined. However, this study can be used as preliminary monitoring for the vector control program.

Keywords: dengue, insecticide, *kdr* allele, mosquito, Surabaya, urban parks

INTRODUCTION

Mosquitoes can spread and carry diseases so that it can making them as one of the deadliest animals. Many mosquito-borne diseases still show an increasing number, including dengue, Zika, chikungunya, West-Nile Virus, malaria, and yellow fever. Half of the population worldwide have lived in an area where mosquitoes are present. Four genus are commonly found as the vector of mosquito-borne diseases, such as *Aedes, Culex, Mansonia*, and *Anopheles*. Genus of *Aedes* can transmit chikungunya virus, Zika virus, dengue virus, lymphatic filariasis, and yellow fever virus (World Health Organization 2020b). Two species of *Aedes*, including *Aedes (Stegomyia) aegypti* and *Aedes (Stegomyia) albopictus*, are the important vector of arboviral disease (Reinert et al. 2009). Dengue infection was a disease transmitted by both of them and became a burden for public health (Simmons et al. 2012).

Data from WHO in 2020 shows that approximately 390 million people have been estimated infected by dengue infection and distributed across 128 countries (World Health Organization 2020a)Indonesia was also endemic to dengue infection, increasing its annual incidence rate from 0.05/100,000 in 1968 to 78.8/100,000 in 2016. However, the case fatality rate was decreased from 41% in 1968 (Nathin et al. 1988) to 1.21% in 2004 (World Health Organization 2006).

Currently, there is no effective vaccine that can be a prophylaxis for all age groups. Dengvaxia (CYD-TDV) is a live recombinant tetravalent dengue vaccine by Sanofi-Pasteur that was first licensed and can be used for individuals 9 to45 years old of age individuals in endemic areas. The efficacy of this vaccine was varied, with the highest efficacy being against dengue serotypes 3 and 4 (71.6% and 76.9%) followed by dengue serotypes 1 and 2 (54.7% and 43%) (World Health Organization 2020c). Following that, the primary method to prevent dengue infection transmission is through vector control. A meta-analysis study showed that varieties of dengue control variation significantly reduces dengue risk, such as house screening, water-container cover, and community-based environmental management. Interestingly, indoor residual spraying (IRS) did not significantly reduce dengue risk, while the use of insecticide aerosol and mosquito coils was associated with increased dengue risk. In line with that, skin repellent, insecticide-treated bed nets were also had no effects (Bowman et al. 2016). Thus, the efficacy of vector control remained in question and maybe can vary in different geographical areas.

The primary method of dengue infection control in Indonesia is the combination of environmental management (eradicating larval mosquito, covering the water-container, draining the bathtub regularly) and thermal fogging. In Indonesia, Organophosphates have been used for a long decade to control adult mosquitoes. Malathion, as the derivative of Organophosphate, was used to control adult mosquitoes, while temephos was used as larvacides. Malathion was firstly introduced in Indonesia in 1969, while temephos was introduced in 1980 for dengue control (Hardjanti et al. 2015).The intensive and massive use of insecticide can lead to the sensitivity decreasing of mosquito population against commonly-used insecticide. This phenomenon can be called as insecticide-resistance mechanism (World Health Organization 2012).There are four types of insecticide-resistance: metabolic resistance, target-site resistance, cuticular resistance, and behavioural resistance (Corbel & Guessan 2013).

Metabolic resistance is a mechanism in insects enzyme system to naturally detoxify from insecticides exposure. The over-expression of enzymes that can detox and amino acid substitution within the enzyme can increase the enzyme's affinity, resulting in metabolic resistance (David et al. 2007; Hemingway et al. 2004). The decreasing effectivity of the site of action

in mosquito to bind insecticide called target-site resistance, such as the target site of carbamates and organophosphate insecticides is acetylcholinesterase (AChE) in the nerve cell (Fournier 2005). The insect can also develop cuticular resistance as the impact of the reduced uptake of insecticide and the increasing thickness of the cuticle, but the study of this resistance is still limited in *Anopheles* genus (Djouaka et al. 2008). Another mechanism of insecticide resistance contributing to vector control failure is behavioural resistance, that is the mosquito's ability to avoid insecticide exposure. The investigation of this mechanism was also limited and only found in *Anopheles* population (Russell et al. 2011).

Detection of insecticide resistance in mosquito populations can be conducted through various methods, such as biochemical assay and bioassays using WHO diagnosed doses, dose-response bioassays, and molecular assays. Molecular assays are the most sensitive way to predict the possibility of insecticide-resistance in the future. This method can determine the frequency of resistant allele in a population (Ranson et al. 2011). Point mutations of *kdr* have been revealed in some countries worldwide, such as point mutations G923V (Glisin to Valin) and I1011M (Isoleusin to Metionin) found in Brazil, Guyana, and Martinique (Brengues et al. 2003), L982W (Leusin to Tryptofan) found in Vietnam (Brengues et al. 2003), F1534C (Phenylalanin to Cystein) found in Indonesia and Thailand (Kawada et al. 2014; Wuliandari et al. 2020), and V1016G (Valin to Glisin) widely distributed in Indonesia and Thailand (Brengues et al. 2003; Rajatileka et al. 2008; Wuliandari et al. 2015). The mutations are associated with the exposure of permethrin and DDT. In several regions of Indonesia, mutant allele was reported in Kuningan (Jakarta), Padang, Samarinda, Pontianak, Mataram, Denpasar, Dompu, West Manggarai, and East Sumba with the frequency 0.73; 0.6; and 0.02 respectively for V1016G, S989P, and F1534C (Amelia-Yap et al. 2019). As the first place where dengue infection was found in 1968, Surabaya also used malathion as the insecticide for vector control, but the resistant alleles report in mosquito from these populations is still yet detected. This study aims to screen the occurrence of knockdown-resistance (*kdr*) mutant alleles in Surabaya, Indonesia. The public urban park is the chosen study site due to its importance value as the public facility.

MATERIALS AND METHODS

Materials

Mosquito eggs from the field were reared until they emerged to be adult mosquitoes, consisting of two species, namely *Aedes (Stegomyia) aegypti* and *Aedes (Stegomyia) albopictus*. Rearing processes were in accordance to the guidelines mentioned in procedure paragraph. Materials that were used in this research were QIAamp Viral RNA Extraction Kit (Qiagen, Hilden, Germany), NEXscript™ RT-PCR 2x Master Mix (NEX™ Diagnostic, USA), Nuclease Free Water (NFW), 70% ethanol, agarose gel, ethidium bromide, TBE buffer, sugar solution, cotton, and labelled paper. Instruments used were micropipette, tray, plastic pipette, board marker, microcentrifuge, freezer -80^{o}C, vortex mixer, biosafety cabinet level-2, thermal cycler Bio Rad, Erlenmeyer, gel documentation imaging system, and stereo microscope.

Methods

Study Area

Surabaya is in Java Island and is highlighted as the most populated city in East Java, resided by two million citizens of various ethnicities. This situation can lead to urbanization and affect the transmission of mosquito-borne diseases, including dengue infection. This study was conducted in seven

Table 1. Details of parks that have successfully had mosquito hatched eggs, collection date, and their coordinates.

Collection Site	District	Coordinates	Collection Date
Apsari	Genteng	7°15'52.34"S 112°44'34.22"E	15072019
Harmoni	Sukolilo	7°17'40.04"S 112°48'13.16"E	08072019
Lansia	Gubeng	7°16'18.85"S 112°45'17.74"E	22071019
Pelangi	Gayungan	7°19'39.23"S 112°43'52.34"	15072019
Persahabatan	Wonokromo	7°16'39.93"S 112°45'07.44"E	15072019
Prestasi	Genteng	7°15'41.30"S 112°44'34.25"E	22072019
Wira Agung	Wonokromo	7°17'56.29"S 112°44'16.73"E	15072019

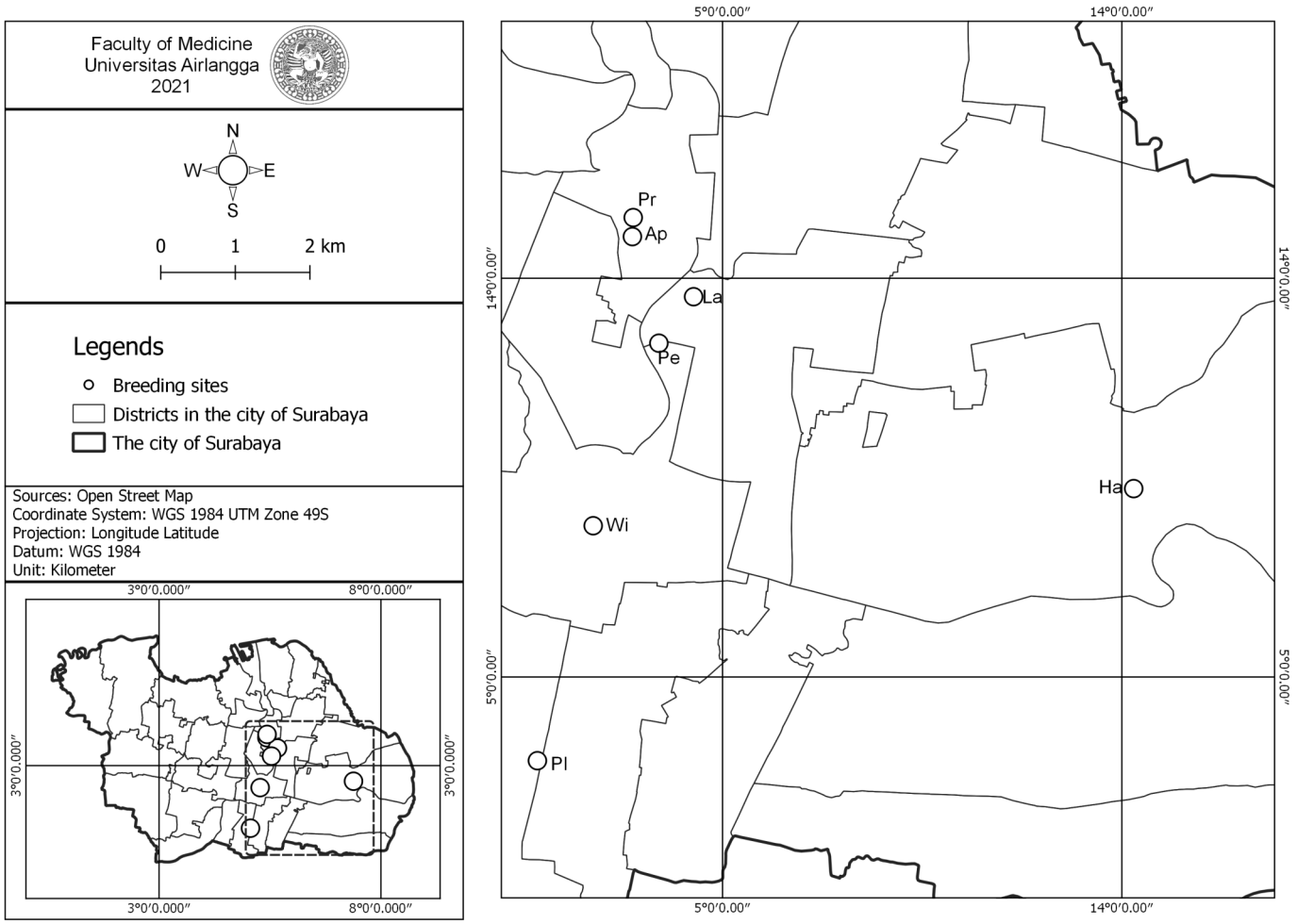

Figure 1. Geographical map of Surabaya City, seven urban parks were marked with circle (Ha: Harmoni Park, Pr: Prestasi Park, La: Lansia Park, Pe: Persahabatan Park, Wi: Wira Agung Park, Pl: Pelangi Park). Figure was created with QGIS 3.10.4 Version.

urban parks in Surabaya, Indonesia. Above are the coordinate of the sampling site (Table 1) and the geographical map was shown in Figure 1.

Procedures

Mosquito Collection and Rearing

Ovitrap surveillance method was adopted from the following guidelines (Imam et al. 2014). Ovitraps were installed in some of the public urban parks distributed in Surabaya (Figure 1) and designed with black colour to attract mosquitoes to lay their eggs, but only some of the eggs from several parks were successfully attached (Table 1). A total of 28 ovitraps were installed in which 4 ovitraps were installed in each park. Observation of attached eggs in filter paper was done every seven days. All filter papers are labelled based on the date of collection and the collection site. After that, they would be

brought to Entomology Laboratory, Institute of Tropical Disease, Universitas Airlangga. Mosquito rearing was set based on the mosquito rearing guidelines (Imam et al. 2014). Adult mosquitoes were identified using an identification key from the Indonesian Ministry of Health (Indonesian Ministry of Health 1989). All of study design in present study was approved by Ethical Committee Faculty of Medicine, Universitas Airlangga with the referee number of 24-934/UN3.14/PPd/2013.

RNA Extraction

Detection of the dengue virus on the mosquito that were caught was also conducted, but the result will be reported in another study. In regards to the double purpose, RNA extraction was conducted. After mosquito identification was made, mosquitos were extracted using QIAamp® Viral RNA by Qiagen, Germany, based on the manufacturer's instruction to extract RNA from mosquito specimens.

AS-PCR Assays for the Detection of kdr Mutant Alleles (V1016G and F1534C)

After cDNA from the procedure of 2.2.2.2 were obtained, genotyping process was conducted. Genotyping of *kdr* mutant alleles to detect mutant in codon 1016 and 1534 were performed using Allele-specific PCR assays following previous guidelines (Stenhouse et al. 2013; Yanola et al. 2011) AS-PCR was conducted by using forward and reverse primer below (Table 2).

Reactions were performed using PCR Thermocycler with following stage: 94^{o}C in 2 min, 35 cycles of 30 sec in 94^{o}C, 55^{o}C in 30 sec, 72^{o}C in 30 sec, and final elongation 72^{o}C in 2 min for the detection of V1016G. For the detection of F1534C, PCR was performed with the stage of 94^{o}C in 2 min, 35 cycles of 30 sec in 94^{o}C, 30 sec in 60^{o}C, 30 sec in 72^{o}C, and elongation step with 72^{o}C in two minutes. PCR products were then loaded into 1.5% agarose gel.

Data Analysis

Data were analysed using R Application of 4.0.4 Version, using packages *"HardyWeinberg"* to analyse the frequency of resistant allele.

RESULTS

A total of 55 fema le *Aedes (Stegomyia) aegypti* and *Aedes (Stegomyia) albopictus* were tested, consisting of 37 *Aedes (Stegomyia) aegypti* and 18 *Aedes (Stegomyia) albopictus*. The composition of mosquitoes in every park was shown in Figure 2. The electrophoresis results were shown in Figure 3. The comparison between our result and the reference was shown in Figure 4 and Figure 5.

Table 2. The sequences of oligonucleotides used to amplify fragments of the VGSC gene (Stenhouse et al. 2013; Yanola et al. 2011).

	Primer sequence	Product (bp)
Gly1016f	5'-ACCGACAAATTGTTTCCC-3'	
Gly1016r	5'GCGGGCAGGGCGGCGGGGGCGGGG CCAGCAAGGCTAAGAAAAGGTTAACTC-3'	60
Val1016r	5'-GCGGGCAGCAAGGCTAAGAAAAGGT TAATTA-3'	80
Cys1534f	5'GCGGGCAGGGCGGCGGGGGCGGGGCCTCTACTTT-GTGTTCTTCATCATGTG3'	113
Cys1534r	5'TCTGCTCGTTGAAGTTGTCGAT3'	
Phe1534f	5'GCGGGCTCTACTTTGTGTTCTTCATCATATT3'	93

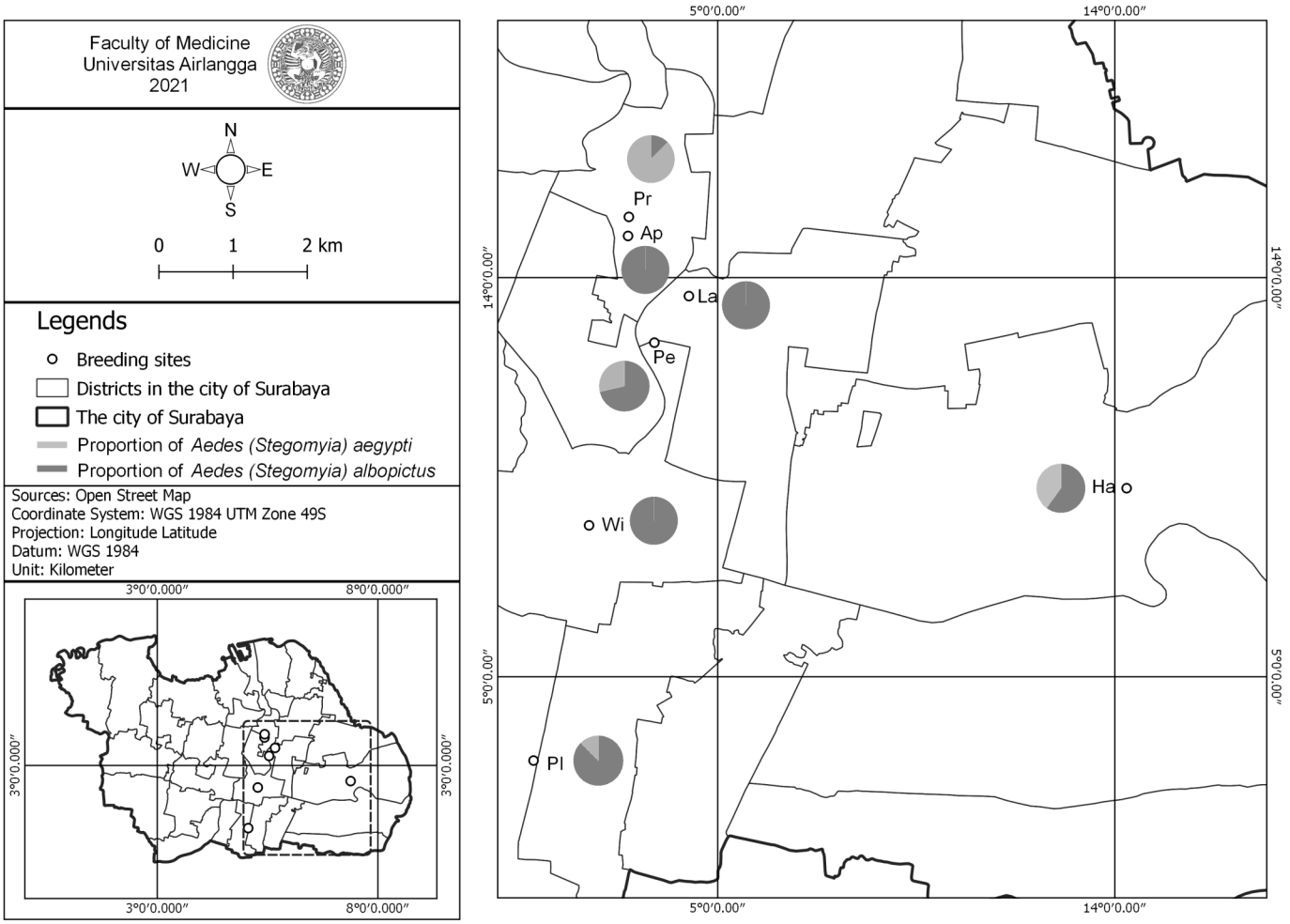

Figure 2. The distribution of mosquito species, dark grey indicated the occurrence of *Aedes (Stegomyia) albopictus*, meanwhile light grey indicated the occurrence of *Aedes (Stegomyia) aegypti*. Ha: Harmoni Park, Pr: Prestasi Park, La: Lansia Park, Pe: Persahabatan Park, Wi: Wira Agung Park, Pl: Pelangi Park). (Figure was created with QGIS).

It was genotyped to only female mosquitoes due to the important role of female mosquitoes as the vector of mosquito-borne diseases. We estimated that random mating was occurred in this population and VGSC is not sex-linked. The results showed that no point mutation was detected in

Table 3. The distribution of species in every park, AS-PCR result (SS: Susceptible allele/V1016V/F1534F/Homozygous wildtype, SR: Susceptible resistant/V1016G/F1534C/Heterozygous mutant, RR: Resistant resistant/G1016G/C1534C/ Homozygous mutant).

Sampling Site	*Aedes (Stegomyia) aegypti*	*Aedes (Stegomyia) albopictus*	n (pool)	Genotype			Allele Frequency	
				SS (%)	SR (%)	RR	S	R
Apsari Park	5	0	3	3 (100)	0 (0)	0 (0)	1	0
Harmoni Park	12	8	2	2 (100)	0 (0)	0 (0)	1	0
Lansia Park	3	0	1	1 (100)	0 (0)	0 (0)	1	0
Pelangi Park	7	1	1	1 (100)	0 (0)	0 (0)	1	0
Persahabatan Park	5	2	1	1 (100)	0 (0)	0 (0)	1	0
Prestasi Park	1	7	1	1 (100)	0 (0)	0 (0)	1	0
Wira Agung Park	4	0	2	2 (100)	0 (0)	0 (0)	1	0
Total	37	18	11	11	0	0		

all of the samples. In other words, all the mosquitoes collected have homozygous wildtype of V1016V and F1534F, as shown in Table 3 below. If the AS-PCR results shows the homozygous wildtype/V1016V/F1534F, it can be symbolized as genotype SS (means that this pool represents susceptible allele), heterozygous mutant/V1016G/F1534C symbolized as genotype SR (means that this pool represents susceptible and resistant allele), homozygous mutant/G1016G/C1534C symbolized as genotype RR (means that this pool represents resistant allele).

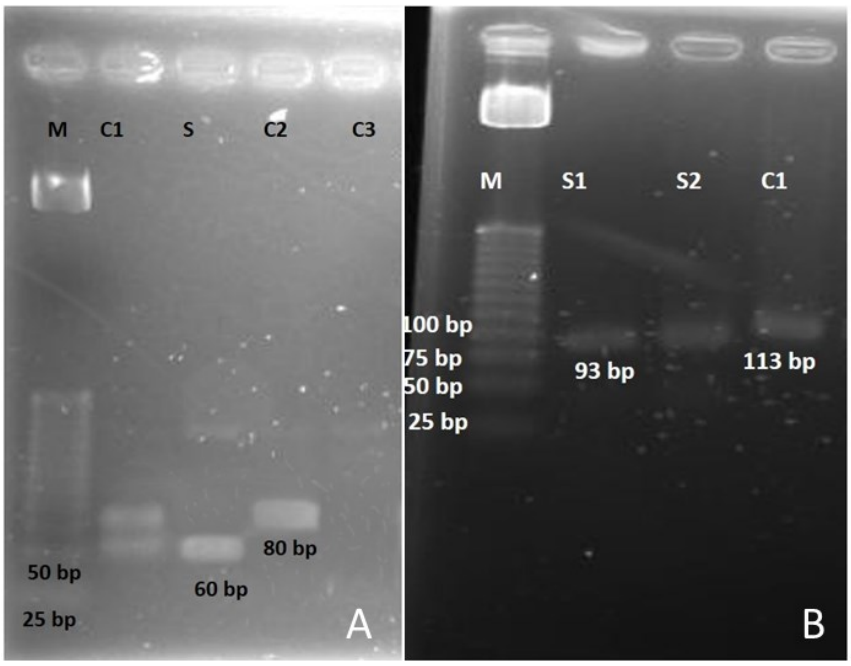

Figure 3. The electrophoresis results of the detection of kdr mutant allele (A: V1016G and B: F1534C). Amplified products could be differentiated by size (60 bp for V1016, 80 bp for G1016), 93 bp for F1534 and 113 bp for C1534.

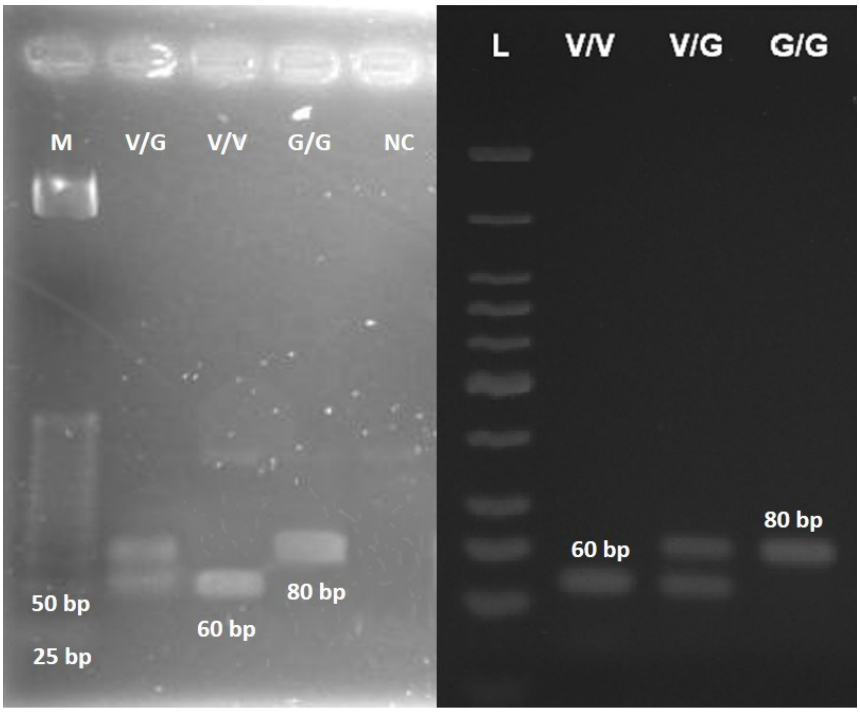

Figure 4. The representative of the electrophoresis of V1016G. Study result compared with the reference, left figure was the study result, right figure was the figure from reference (Stenhouse et al. 2013). M=marker of 25 bp; V1016V = Homozygous wildtype; V1016G =Heterozygous Mutant; G1016G = Homozygous Mutant.

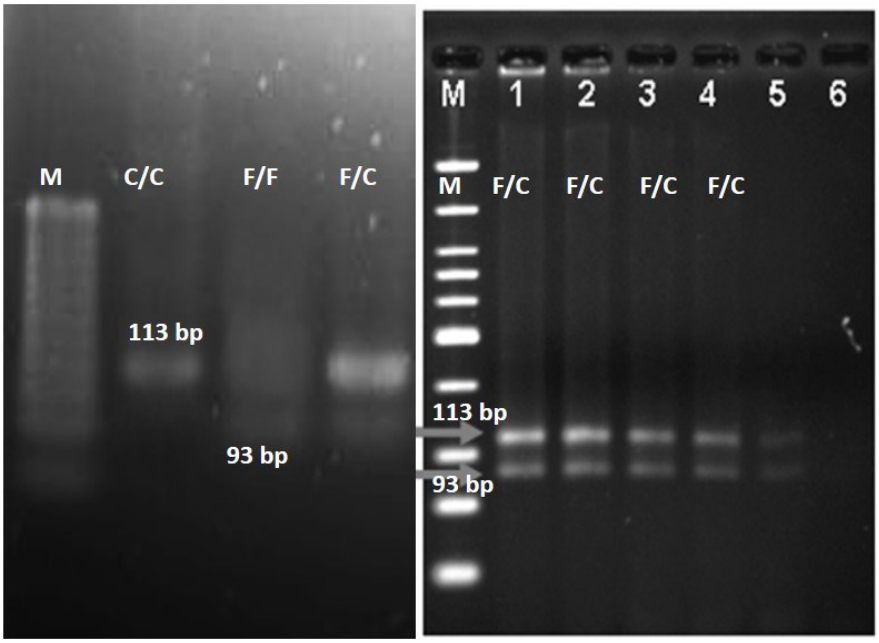

Figure 5. The representative of the electrophoresis of F1534C. Study result compared with the reference, left figure was the study result, right figure was the figure from reference (Yanola et al. 2011). M=marker of 25 bp; F1534F= Homozygous wildtype; F1534C=Heterozygous Mutant; C1534C= Homozygous Mutant.

DISCUSSIONS

This study highlighted that no *kdr* mutant alleles (V1016G and F1534C) have been found from the study site. This study was the first report of *kdr* mutation screening in Surabaya, East Java Province, Indonesia. Some of *kdr* mutant alleles in Indonesia have been reported from many regions and showed various mutations on V1016G, F1534C, and S989P. A study from Amelia-Yap shows the occurrence of mutation on V1016G and S989P in mosquitoes collected from Kuningan, Padang, Denpasar, Samarinda, Mataram, and Sumba Timur (Amelia-Yap et al. 2019). Pointing out and detecting mutation of Val1016Gly/V1016G and Phe1534Cys/F1534C is important, including in national and local areas. The Allele-Specific PCR Assay (AS-PCR) can be implemented to detect *kdr* mutant and provide a rapid result, accurate, and cost-effective genotyping *(*Lee et al. 2016). Rapid results and the precise target can be used to figure out the VGSC gene of mosquitoes in a population. However, this method cannot stand alone and must be combined with WHO tube test Bioassay to capture information about the susceptibility status of mosquito against insecticides (Corbel & Guessan 2013).World Health Organization (WHO) Tube Test Bioassay against insecticides require too much number of samples. Instead, AS-PCR was used for the screening of *kdr* mutant alleles in the mosquito, using the previous method (Stenhouse et al. 2013). *Kdr* screening using Allele-Specific PCR assay is a very sensitive method that can provide early warning for future resistance detection that is suitable to use given the limited number of mosquitoes (Ranson et al. 2011). In this study, not all the mosquito eggs that were rearing successfully emerge into adult mosquitoes. Thus, AS-PCR assay screening method was adopted.

Surabaya is a dengue-endemic area and the first place where dengue cases were found in 1968. Management of dengue infection in Indonesia, including in Surabaya, was focused on vector control, which can be broken down into three methods: physical control, chemical control, and biological control. Physical control can be done by washing bathtubs regularly every seven days before the mosquito eggs become adult mosquitoes, larval

elimination through program one house one larval observer, and closing water containers. Fogging activity (hot-fogging) was applied in some Indonesian regions as chemical control, except in Surabaya where they used Ultra Low Volume spray (cold-fogging). Some chemical insecticides in fogging and ULV were from the organophosphate group (Malathion and methylpirimiphos), Pyrethroid (Cypermethrine, Lamda-cyhalotrine, Cyflutrine, Permethrine, S-Bioalethrine, and its derivation). In comparison, chemical control in goal for killing mosquito larvae was temephos from the organophosphate group and piriproxifen (Indonesian Ministry of Health 2017).

Since insecticides have been used for many decades, long exposure to insecticides has been a major public health problem. Continuously using the same insecticide for a long period led to mosquitoes' development resistance to insecticide exposure. In this case, every country should be responsible for their vector control program. World Health Organization (WHO) defined resistance as an insect's ability to survive against the effect of insecticides through natural selection and mutations (World Health Organization 2012). Other countries in South-East Asia were also facing the same problem. In the South-East Asia region, the number of V1016G mutations have been reported from Cambodia, Laos, Myanmar, Malaysia, Singapore, Thailand, and Vietnam. While the report from Philippine and Timor Leste are still not available (Amelia-Yap et al. 2018). Other mutations were also reported, such as mutations in Thailand that show mutations in codon 1011, where isoleucine become valin (I1011V) (Rajatileka et al. 2008). Some resistant alleles frequencies, including F1534C and V1016G have been reported in various range. A cross sectional study in India in 2015, shows the frequency of F1534C(C) and V1016G(G) were 0.51 and 0.18 respectively (Kushwah et al. 2020). In other part of India, it also has been reported the frequency of F1534C(C) was around 0.41-0.79, combined with the new point mutation T1520I(I) with the frequency of 0.13 (Kushwah et al. 2015). Researches about point mutation in the voltage-gated sodium channel of dengue vector were rapidly increase, a report from Taiwan found a novel point mutation, D1794Y that occurs with the V1023G mutation (Chang et al. 2009). Meanwhile, a study in Mexico successfully revealed the co-occurence of point mutation V1016I and F1534C was associated with pyrethroid resistance during 16 years of observation (Saavedra-Rodriguez et al. 2018). The derivative of permethrin (λ-cyhalothrin) was also reported to be the causative of *kdr* point mutation (V419L) in Colombia, with the frequency ranging from 0.06 to 0.46 (Granada et al. 2018).

Study of knockdown-resistance (*kdr*) alleles in Indonesia may give another impact on vector control method. In Yogyakarta, a dengue-endemic city, the frequency of *kdr* mutant alleles V1016G and F1534C in the area where Wolbachia will be released as vector control method was firstly measured and shows high frequencies of V1016G mutation, but F1534C was low detected. The result of *kdr* screening can figure out population background, so that the vector control that may be released can be suitable (Wuliandari et al. 2020). Since having the first case of dengue infection in 1968 in Jakarta and Surabaya, Indonesia has applied some vector control, but it still needs to be improved so that the dengue outbreaks can be avoided. The number of dengue incidence rate (IR) in Indonesia was increased from 0.05 to 40 per 100,000 populations in 2013. The highest epidemic was reported in 2010, with the IR value of 85.7/100,000 population (Haryanto 2018). Some risk factors of dengue infection in Indonesia were various breeding sites especially during rainy season, the mobility of citizens inside or outside the country, and the low level of awareness toward health and hygiene lifestyle (Setiati et al. 2006). Indonesia is a suitable place for some

vector's growth and development as a tropical country, including mosquitoes. Although this study had not found *kdr* mutant allele in *Aedes* (*Stegomyia*) *aegypti* and *Aedes* (*Stegomyia*) *albopictus*, it does not take alleles in another district/region into account, so regular surveillance in other endemic areas are strongly suggested. *Kdr* mutant alleles in points 989, 1534, and 1016 was a leading factor that causes mosquitoes to develop resistance phenotype against insecticide (Harris et al. 2010; Srisawat et al. 2010). AS-PCR assay contributes to the early detection for future warning resistance in the mosquito that cannot be detected by bioassay methods. Thus, the presence of a single/double *kdr* mutant allele should be considered. In other cases, the negative result/the absence of *kdr* mutant allele did not lead to complacency because of the specific target that are being examined (Corbel & Guessan 2013). The absence of *kdr* mutant allele in one point does not reflect another absence of mutation in another point. Hence, this study might be used as complement data for the vector control program.

CONCLUSION

Result of present study confirmed that no mutations were found in Valine to Glysine in point 1016 (V1016G) and Phenylalanine to Cysteine in point 1534 (F1534C) alleles in both two species, *Aedes (Stegomyia) aegypti* and *Aedes (Stegomyia) albopictus* collected form urban parks, Surabaya, Indonesia. All of mosquito samples have homozygous wild type genotype of *kdr* alleles (V1016V anf F1534F).

AUTHORS CONTRIBUTION

SF was responsible for conceptualization, data collection, and manuscript preparation. SS was responsible for supervision, investigation of the data, and manuscript preparation. BU was responsible for data analysis and manuscript preparation. THS was responsible for data collection and manuscript preparation. HA was responsible for manuscript preparation and grammatical check. AA was responsible for data validation and manuscript preparation. PW was responsible for data validation and manuscript preparation. SS was responsible for investigation of the data and manuscript preparation.

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CONFLICT OF INTEREST

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

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Research Article

Rafflesia patma Blume in Pananjung Pangandaran Nature Reserve, West Java: Population Structure, Distribution Patterns, and Environmental Influences

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ABSTRACT
Rafflesia patma is an endemic plant of Pangandaran, West Java which is protected because of its rare status. The purpose of this research is to study the population structure, distribution patterns, and the effect of the physical environment of abundance *R. patma* in Pananjung Pangandaran Nature Reserve, West Java. The method used in this research was a survey method with a purposive sampling technique. Sampling was conducted using quadrat plots. The population pattern distribution was defined by a standardized Morisita index, and the analysis of abiotic environmental factors was determined by Principal Component Analysis (PCA) using PAST3. The results showed that there were 114 *R. patma* individuals scattered in several research areas in Pananjung Pangandaran Nature Reserve, they were Gua Parat (3 individuals), Cilegon (13 individuals), Pasir Putih (12 individuals), Badeto (48 individuals), and Curug Leutik (38 individuals). The distribution pattern of *R. patma* in Pananjung Pangandaran Nature Reserve was clustered with the Morisita index value (Id) > 1. Based on the PCA analysis, results that support the classification of the cluster analysis were obtained. Based on four abiotic environmental conditions analyzed, the most dominant character in influencing the distribution patterns and population structure of *R. patma* is light intensity.

Keywords: distribution pattern, habitat, nature reserve, population structure, *R. patma* Blume

INTRODUCTION
Rafflesia patma is a unique plant that lives in the tropical rainforest ecosystem of Malesiana. The plants do not have any stem, leaves, roots, or photosynthetic organs (Wicaksono et al. 2016). *R. patma* is a parasitic plant on the tree as the host in its habitat. The nutritional needs in its life cycle are taken from the host plant. The intake of nutrients from the host plant is carried out using suction roots to absorb photosynthate into the body organs of *R. patma*. The characteristic of *Rafflesia* is a rare plant species must receive a priority for conservation because the population in nature is small (rare) and is an endemic species. Scarcity of this species is caused by *Rafflesia* having different biological characteristics from other plants, namely having an annual life cycle, being a parasite in certain liana species, and breeding

difficult. This leads to the notion that *Rafflesia* chose certain environmental conditions to support its breeding and growth (Hidayati & Walck 2016; Priatna et al. 1989).

The population of *Rafflesia* in nature is very small. The small number coupled with the sporadic and inconsistent flowering seasons causes the scarcity of male and female flowers to bloom at the same time. In the case of *R. Patma*, in Pangandaran (West Java) the population is dominated by male flowers (Hidayati et al. 2000), which means that the chances of pollination between male and female flowers are reduced. This opportunity is further reduced by the short bloom period, around 4-5 days. Consequently, the pollen viability of male flowers will be very limited. Nais (2001) stated that the viability of *R. keithii* pollen was only 72 hours after being separated from the flower. This means that during this time the pollinating insects must find the female flowers in bloom and pollinate them. It is not yet known how long the viability of *R. patma* pollen is.

Rafflesia has specific life characteristics so that it requires suitable habitat to support its growth, distribution, and sustainability. Anthropogenic factors that contribute to declining populations of *Rafflesia* are deforestation and harvesting by locals due to their perceived medicinal properties (Nais 2001; Yahya et al. 2010). The current human influence tends to be negative and leads to their extinction. This is shown by *Rafflesia* and its habitat which are disposed to disturbance and extinction caused by humans. Protection to avoid the extinction of *Rafflesia* and its habitat must be done. Basic data is needed as a reference for the efforts to preserve *Rafflesia*, both in terms of numbers and in terms of preserving its intact habitat.

The purpose of this study is to analyze the population structure, distribution patterns, and the effect of the physical environment of abundance *R. Patma* in Pananjung Pangandaran Nature Reserve, West Java. It is hoped that the data obtained from this study can be an adequate source of information so that it can be used as a policy basis for the conservation of *R. Patma* and its habitat in Pananjung Pangandaran Nature Reserve, West Java.

MATERIALS AND METHODS

Study Area

This research was conducted from January to April 2020 in Pananjung Pangandaran Nature Reserve, Pangandaran Regency, West Java, which is divided into 5 locations, namely Gua Parat, Badeto, Cilegon, Curug Leutik, and Pasir Putih. Pananjung Pangandaran is a peninsula on the South Coast of West Java, bordering Central Java. The location of the Pananjung Pangandaran Nature Reserve is located between 108°50' to 109°55' E and 7° 40' to 7°45' S. This area is bordered by the Indian Ocean to the south, Ciamis Regency to the north, Teluk Pangandaran or Pananjung in the east, and Parigi Bay to the west (Figure 1).

Methods

The method used in this study was a survey method with purposive sampling technique and sampling using quadrat plots. The sampling area was determined based on different habitat types (coastal forest including Gua Parat and Pasir Putih; lowland forest including Curug Leutik, Badeto, and Cilegon). The quadrat used is 20 x 20 m in each location with five repetitions. The data collected were the number of living and dead buds, the number of flowers in live bloom, and dead flowers. Measurement of abiotic environmental factors includes temperature and humidity measured using a

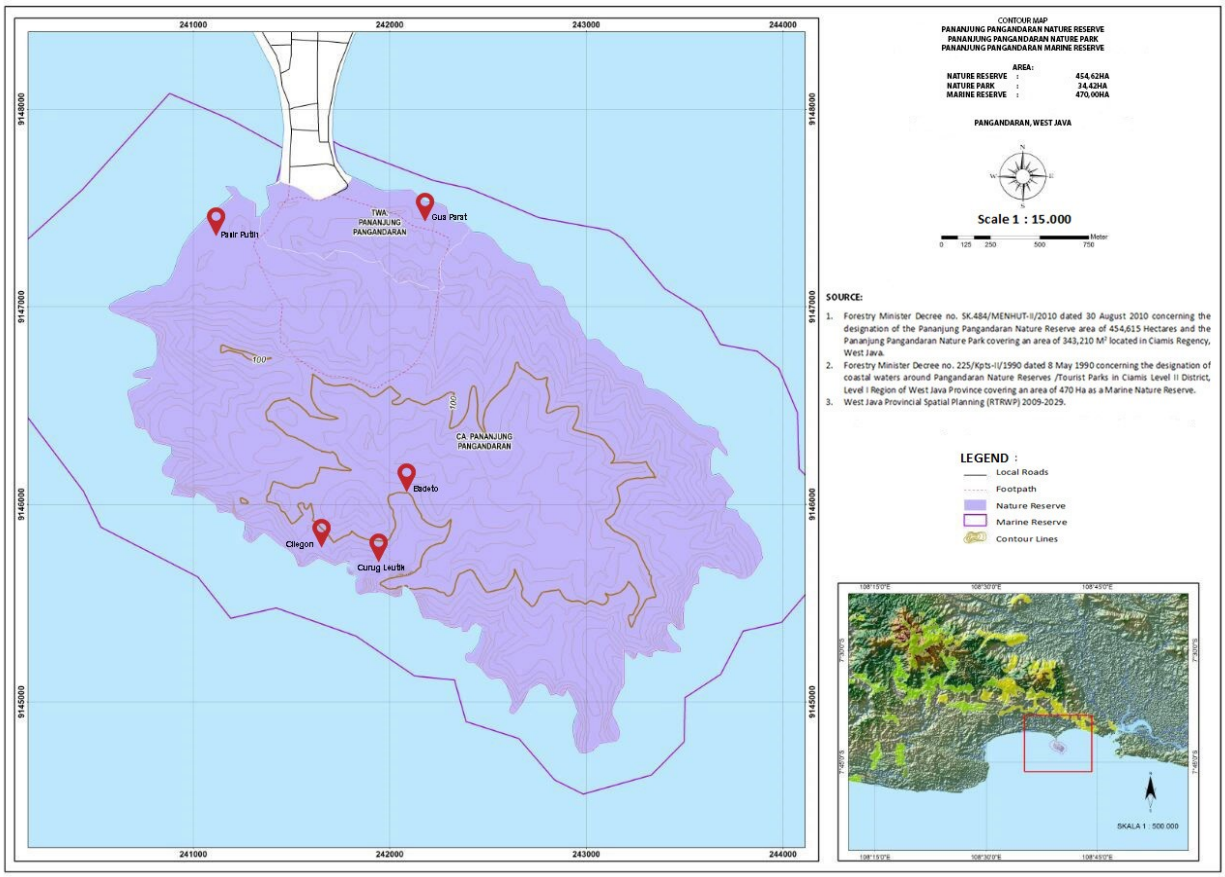

Figure 1. Map of Pananjung Pangandaran Nature Reserve, West Java, Indonesia.

thermo hygrometer, light intensity measured using a lux meter, and pH using a pH meter by measuring directly in the field on each sample plot.

Data analysis

Distribution pattern index

To find out the pattern of *R. patma* distribution, it can be calculated using the standardized Morisita Index (Krebs 1989). The standardized Morisita dispersion index (Ip) ranges from -1 to 1, with 95% confidence limits at 0.5 and -0.5.

Below was the formula used in this study:

$$Id = n\ ((\Sigma xi^2 - \Sigma xi) / (\Sigma xi)^2 - \Sigma xi))$$

Information:

Id : Morisita Index
n : Number of all plots
xi : Number of individual species on i-th plot

Standardized of Morista index calculated by equation:

Ip : 0.5 + 0.5 ((Id – Mc) / (n – Mc)) ; if Id ≥ Mc > 1
Ip : 0.5 ((Id – 1) – (Mc – 1)) ; if Mc > Id ≥ 1
Ip : 0.5 ((Id – 1) / (Mu – 1)) ; if 1 > Id > Mu
Ip : 0.5 + 0.5 ((Id – Mu) / (Mu)) ; if 1 > Mu > Id

Continuously distribution pattern has been shown by Mu and Mc:

$$Mu = (x^2_{0.975} - n + \Sigma xi) / (\Sigma xi) - 1$$
$$Mc = (x^2_{0.025} - n + \Sigma xi) / (\Sigma xi) - 1$$

Information:

Mu : Morista index on uniform pattern of distribution
Mc : Morista index on clustered pattern of distribution Index

$x^2_{0.975}$: The value of x^2 with n-1 degree of freedom and confidence interval of 97.5%
$x^2_{0.025}$: The value of x^2 with n-1 degree of freedom and confidence interval of $x^2_{0.025}$
xi : Number of individual species on i-th plot

Principal component analysis (PCA)

The correlation between abiotic factors and the *Rafflesia patma* population was obtained using Principal Component Analysis (PCA) with software PAST3 (Trianto & Purwanto 2020). PCA analysis was carried out to determine the physical factors that had the most influences based on the points of *R. patma*, including temperature, humidity, light intensity, and soil pH. The PCA results will show the factor that most influences the abundance of *R. patma*.

RESULTS AND DISCUSSION

Population structure of *Rafflesia patma*

The results of the study found that there were 114 individuals of *R. patma* in several research areas in Pananjung Pangandaran Nature Reserve, including Gua Parat (3 individuals), Badeto (48 individuals), Cilegon (13 individuals), Curug Leutik (38 individuals), and Pasir Putih (12 individuals) (Figure 2).

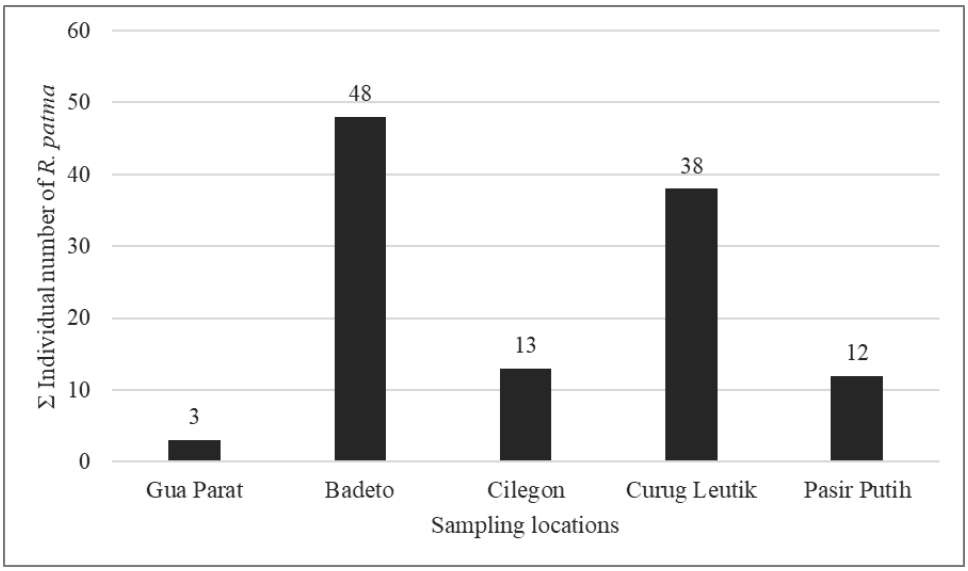

Figure 2. The number of *Rafflesia patma* individuals in the research area in Pananjang Pangandaran Nature Reserve.

The number of individuals found varied in each region and did not show a uniform trend in the number of individuals. This is due to the presence of different habitat conditions. The highest number of *R. patma* individuals was in the Badeto area and the lowest was in the Gua Parat area. Mursidawati & Irawati (2017) report that Gua Parat is one of the areas that are very close to human activities. This area is around the entrance gate of the nature reserve (<10 meters). The forest in this area is already fragmented by footpaths that are made to facilitate access to beaches or other tourist areas. Almost every day visitors pass through the tourist area. The population of *R. patma* in this area is found on the left and right of the road to the beach. Some of them grow slightly into the forest, but some of them grow right on the side of roads that are disposed of being walked on and trampled by visitors. Compared to the Badeto area (80-130 m asl) with the highest individual of *R. patma*, this area is one of the areas with a good condition, although it is often passed by visitors. The frequency of visitor arrivals in the

Badeto area is not as intensive as in Gua Parat and Pasir Putih, because the road to this area is quite far. This area is often visited by visitors, especially during the holiday season or weekends (Mursidawati & Irawati 2017). As additional information, Badeto Forest still has a dense population of vegetation (dense) with multi-layered strata. The buds or flowers of *R. patma* are scattered along the river to the waterfall directly into the sea.

Based on the results, the number of *R. patma* individuals observed was higher than the study of Lestari & Rianto (2017) which consisted of 47 *Rafflesia* individuals (67% alive and 33% dead) at the Rhino-Camp Sukaraja Atas Bukit Barisan Selatan National Park (TNBBS), Susatya (2011) which consisted of 59 individuals, and Suwartini et al. (2008) consist of 57 individuals. Compared to previous studies at the same research location in Pananjung Pangandaran Nature Reserve, the results obtained were less than Mukmin & Hikmat (2009) consisting of 312 individuals of *R. patma*. This population decrease is due to the existence of illegal logging and hunting of wild animals in the habitat of *R. patma* which is carried out regularly. This condition resulting in the disruption of the habitat of *R. patma* which leads to the destruction of its natural habitat.

The percentage mortality of *R. patma* found in this study consisted of 54 individuals or 47.37% of dead buds, 28 individuals or 24.56% of live buds, and 32 individuals or 28.07% of rotten blooms (Figure 3, 4). Compared with the research of *R. patma* in other areas that have been carried out, the mortality rate in this study was classified as moderate, especially in the percentage of mortality *R. patma* before the flowers bloom. The mortality rate of *R. patma* in the Bojonglarang Jayanti Nature Reserve is included in the low mortality category, which is only around 12.22% (Ali et al. 2015). Susatya (2011) revealed that the *Rafflesia* mortality rate is low if it is in the range of 20 -37% of deaths, and high if it is in the range of 80-100% of deaths.

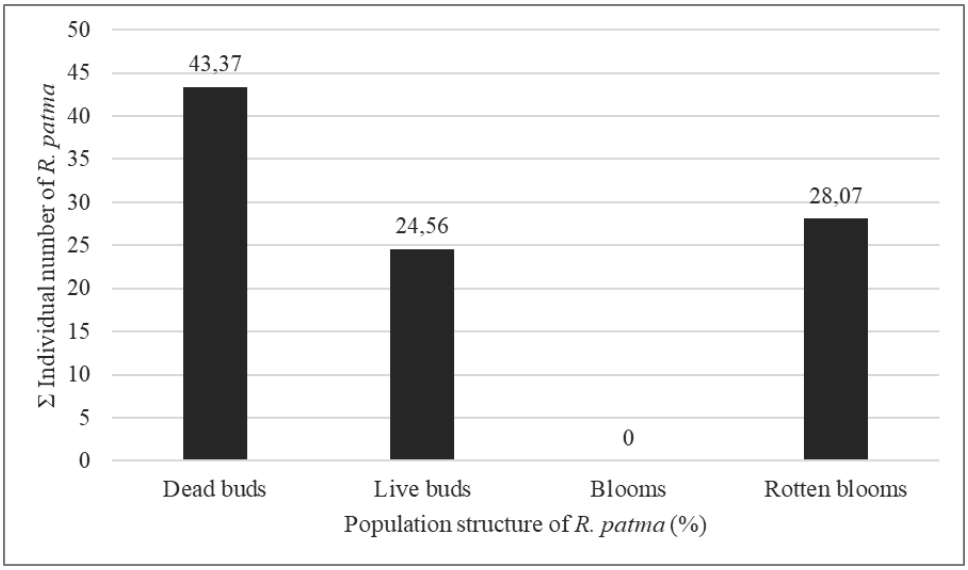

Figure 3. The population structure of *Rafflesia patma* in Pananjung Pangandaran Nature Reserve (Januari-April 2020).

In this study, the individual conditions of *R. patma* that died were different. The perigon condition of *R. patma* was black indicates that the knob has been blooming for more than one week and finally rot (Figure 4). Lestari & Rianto (2017) suggested that the mortality that occurred in the *Rafflesia* flower was caused by several factors, such as the disturbance of wild animals or humans and the inadequate distribution or distribution of nutrients from the host to the *Rafflesia* knob. The highest mortality rate was on knobs less than 3 cm in diameter, whereas most knobs with a size of > 16

Figure 4. Condition of *Rafflesia patma* population: A. Dead buds, B. Live buds, and C. Rotten blooms.

cm were able to survive (Nais 2001). The mortality of *Rafflesia* knobs generally varied from 60% to 90% (Sofiyanti et al. 2007). Furthermore, Ramadhani et al. (2017) show that 2-3 *Rafflesia* knobs die before bloom. Throughout the year, *R. patma* is always present and is found in various phases of development, from the bud (knob) phase to fruit (Mursidawati & Irawati 2017).

Distribution pattern of *Rafflesia patma*

In an ecosystem, there are three basic patterns of distribution of a species that has been recognized, namely: random, clustered, and uniform (Ludwig & Reynolds 1988). To identify the spatial distribution pattern of a species, various distribution indices can be used, including the ratio of variance and mean, clumping index, green coefficient, standardized Morisita index. One index that is often used is the Morisita index because the results of simulation research prove that this index is the best method for measuring the spatial distribution pattern of an individual independent of population density and sample size (Morisita 1962). The standardization of the Morisita index is an improvement of the Morisita index by placing an absolute scale between -1 to 1. The distribution pattern of organisms in a region varies widely, including organisms from members of the Rafflesiaceae family.

Members of the Rafflesiaceae family are entirely parasitic plants, including Sapria and Rhizanthes (Nikolov & Davis 2017). This clan is popular in tropical areas, especially Southeast Asia including the Philippines and Thailand. *Rafflesia* can be found both in primary forests and secondary forests. Its spread is highly dependent on the spread of its host tree, namely Tetra stigma. In general, *Rafflesia* can be found in hosts that live near water sources. Land altitude and slope where it grows varies greatly depending on the species ranging from 5 m asl (*R. patma* in West Java) to 1400 m asl (*R. pricei* in Sabah and *R. rochusenii* in West Java). The spread of *Rafflesia* in Indonesia covers the regions of Sumatra, Kalimantan, and Java (Mursidawati & Irawati 2017), one of which is in the Pananjung Pangandaran Nature Reserve.

The population of *R. patma* in this study was found in five observation areas in Pananjung Pangandaran Nature Reserve, namely Gua Parat (2.63%), Badeto (42.10%), Cilegon (11.40%), Curug Leutik (33.33%), and Pasir Putih (10.53%). Mursidawati & Irawati (2017) suggested that the population of *R. patma* in Pangandaran Nature Reserve spread over several very specific areas. *R. patma* was found growing on hosts along the river. Some of them grow some distance from the river. In the Pasir Putih location, generally, the flowers found, both medium and past flowering, are male. The same thing was also found in Upper Badeto. The dominance of male flowers in the Pangandaran area has also been reported by Hidayati et al. (2000), that the

Table 1. Distribution pattern of *Rafflesia patma* in Pananjung Pangandaran Nature Reserve.

Location	Σxi	Σxi²	Id	Mu	Mc	Ip	Distribution
Nature reserve	114	4070	1,535	0,97	1,06	0,56	Clustered

Captions:
Σxi = Total number of individual species on i-th plot
Σxi² = Total quadrate of number of individual species on i-th plot
Id = Morisita Index
Mu = Morisita index on uniform pattern of distribution
Mc = Morisita index on clustered pattern of distribution
Ip = Standardized of Morisita Index

area around waterfall and Rajamantri appears to have several mixed colonies between male and female.

According to the population level, the distribution pattern of *R. patma* in Pananjung Pangandaran Nature Reserve is clustered with the Morisita index value > 1 (Table 1). Brower et al. (1990) explained that the distribution patterns of species in a population can be divided into three categories, namely random, uniform, and clustered. It is said to be clustered if the Morisita index value (Id) > 1. This is in accordance with Bullock et al. (2008) statement that plant populations in nature are more often spread out in groups.

The distribution pattern of *R. patma* is related to the system and its physiological conditions in a region. Mursidawati & Irawati (2017) and Pelser et al. (2013) explained that *Rafflesia* reproduces with seeds whose distribution is assisted by wind, water, and animals, including insects, hedgehogs, squirrels to wild boar, which are still estimated and need further research. This is supported by the data that the *Rafflesia* fruit rind is hard and tough, it can only be cracked by these animals. Physiographic conditions are also thought to be able to significantly influence the distribution of *Rafflesia*. This is supported by the research of Mukmin & Hikmat (2009) that *R. patma* in the Pananjung Pangandaran Nature Reserve is found mostly near the Cikamal River and Tributary at the closest distance of 0.50 m from the water source. Other factors that also affect the distribution of *R. patma* are soil conditions or edaphic factors. Soil is a medium for the growth and development of plants. The soil conditions that directly affect plants are fertility. Indicators of soil fertility, among others, are seen from the content of humus or organic matter, nutrients, soil texture and structure, and the amount of water in the soil's pores (Triana et al. 2017).

Environmental conditions

The success rate of an individual to be able to live (grow and reproduce) is influenced by physical or abiotic environmental factors. The results showed that the average temperature at the location where *R. patma* was found in the Pananjung Pangandaran Nature Reserve was in the range of 28.42 °C, humidity 84.52%, soil pH equal to 5.72, and light intensity of 1.080,5 lux. (Table 2).

The abiotic conditions obtained in this study were included in the normal range to affect the growth of *R. patma*. Ramadhani et al. (2017) stated that the abiotic conditions of temperature (25-29 °C), 90% humidity and acidic pH (5.5) were able to guarantee the breeding and growth of *Rafflesia* in Rhino-Camp Sukaraja Atas Bukit Barisan Selatan National Park (TNBBS). Simamora et al. (2017) also reported that air humidity (76-84%) and air temperature (19.5-24.4 °C) were able to support the growth of *Rafflesia* in Batang Gadis National Park, North Sumatra. Meanwhile, the *Rafflesia* species

Table 2. Abiotic conditions of the Pananjung Pangandaran Nature Reserve.

Locations	Abiotic Factors			
	Temperature (°C)	Humidity (%)	Light intensity (lux)	pH
Gua Parat	28	84,8	1.013	5,54
Badeto	29	80,6	1.081,6	5,8
Cilegon	27,4	86,4	1.052,8	5,58
Curug Leutik	28,2	82,4	1.120,6	5,58
Pasir Putih	29,4	88,4	1.134,6	6,1
Total	142	422,6	5.402,6	28,6
Average	28,4	84,52	1.080,5	5,72

found by Lestari et al. (2014) in Meru Betiri National Park grew in temperature conditions of 25-29 °C with a humidity of 79-96%.

Data of abiotic environmental conditions with the number of *R. patma* individuals in five research areas in Pananjung Pangandaran Nature Reserve were further analyzed using the Principal Component Analysis (PCA) method with PAST3 software. The purpose of this analysis is to see the dominant character that affects the variation of the individual grouping pattern of *R. patma*. The correlation analysis between groups yielded the Eigenvalue and % Variance shown in Table 3.

Table 3. Result Eigenvalue and % Variance.

PC	Eigenvalue	% Variance
1	2451.77	99.595
2	9.57712	0.38904
3	0.398346	0.016181
4	0.0028106	0.00011417

The main component that can be used and represents the data in the analysis process Principal Component Analysis (PCA) is the value on PC1. A study by Bascos et al. (2019) revealed that the number of main components that can be used or is considered sufficiently representative, that is if the % variance produced is more than 70%.

The results of the analysis showed that the abiotic environmental conditions could affect the distribution patterns and abundance of the *R. patma* population in an area. Grouping also occurs because of the role of each character being analyzed. According to Jolliffe (2002) principal component analysis is an analytical technique that is often used in taxonomic research because this technique can identify the role of each character in each formed group.

The results of the principal component analysis are shown in the PCA diagram (Figure 6). Based on the results of the PCA analysis, results were obtained that supported grouping based on cluster analysis. Based on the four abiotic environmental conditions analyzed, the most dominant character in influencing the distribution patterns and population structure of *R. patma* in Pananjung Pangandaran Nature Reserve is light intensity. This can be seen from the length of the resulted line (Figure 5), and the picture of Loading Plot of Component 1 (Figure 6) produced.

Light intensity greatly affects the level of air temperature in an area. The data found in this study; many light intensities occur due to the

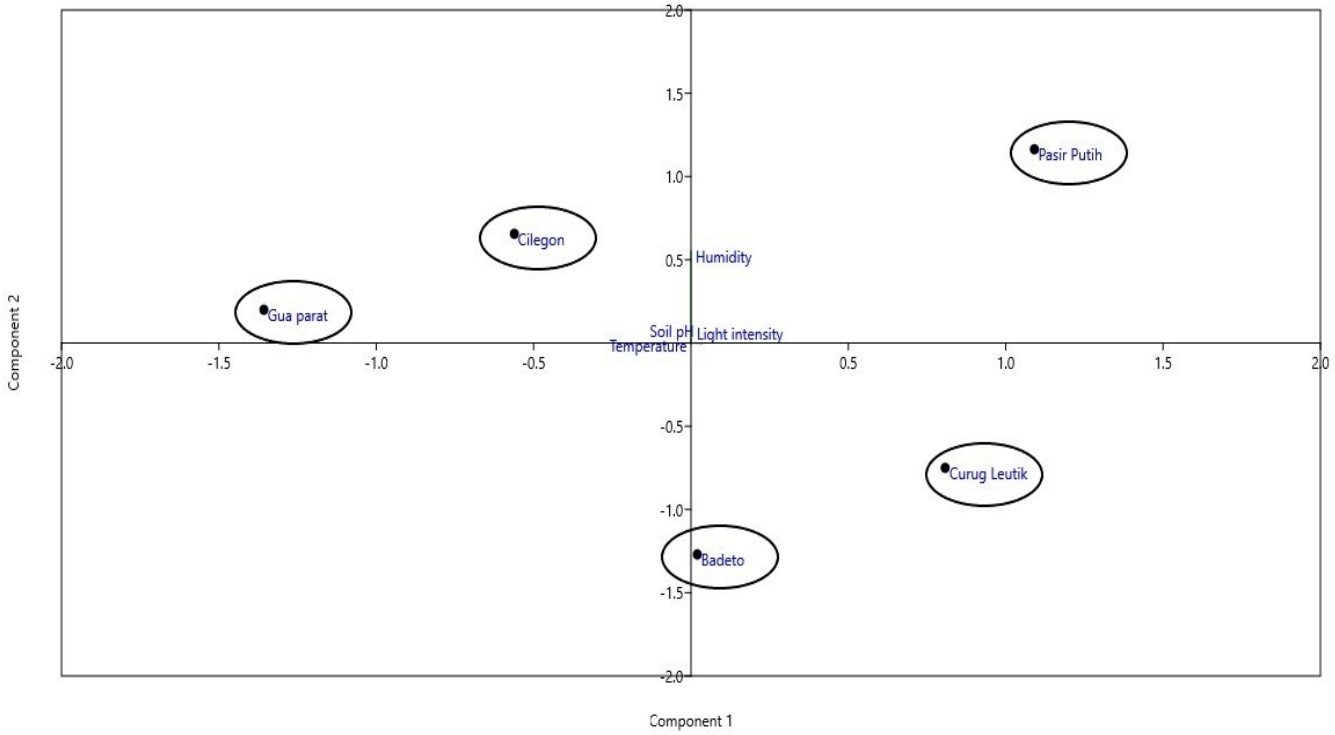

Figure 5. Biplot analysis on Principal Component Analysis (PCA) of abiotic factors that affect the abundance of *Rafflesia patma*.

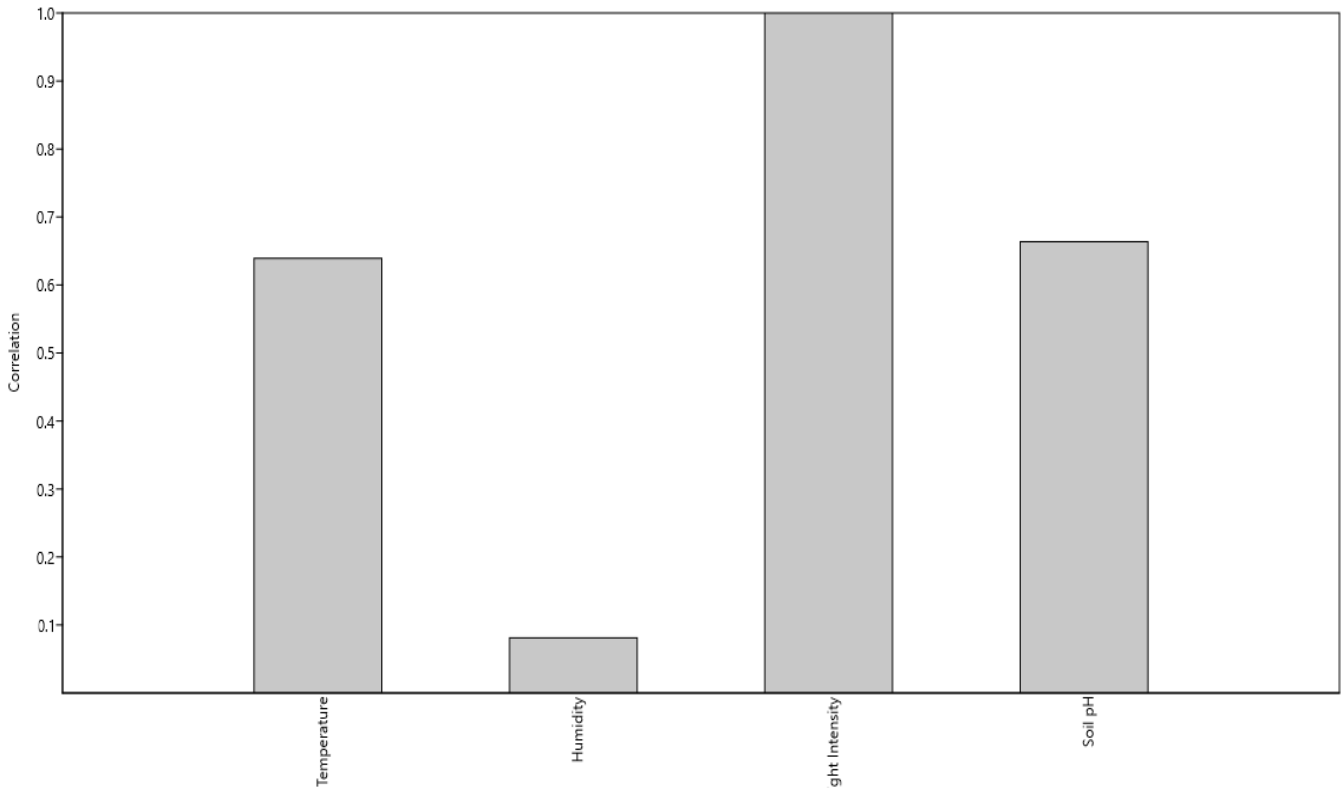

Figure 6. The Loading Plot of Component 1

openness of the forest canopy which can affect the level of sunlight reaching the forest floor. Sunlight that enters the forest floor can give a negative impact on *Rafflesia* survival. Exposure to the forest floor directly causes excessive evaporation and the soil surface becomes too humid so that the *Rafflesia* knob becomes dry and does not support *Rafflesia* growth (McNaughton & Wolf 1990; Banerjee & Linn 2018).

CONCLUSION

There were 114 individuals of *Rafflesia patma* found in Pananjung Pangandaran Nature Reserve, including 54 dead buds, 28 live buds, 32 rotten blooms, and no blooming flowers were found. The distribution pattern of *R. patma* in Pananjung Pangandaran Nature Reserve is clustered with the Morisita index value (Id)> 1. The result of PCA analysis also showed that light intensity became the most affecting abiotic environmental factor on the growth of *R. patma*.

AUTHORS CONTRIBUTION

B.A.A. designed the research, collected and analyzed the data. B.A.A., S.H., and P wrote, revised, and approved the manuscript. S.H. and P supervised all the processes.

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CONFLICT OF INTEREST

The authors state there is no conflict of interest.

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