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Table of Contents

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Short Communication

Biomass Estimation of Eaglewood (*Aquilaria filaria* (Oken) Merr.) in the Karst Ecosystem of West Papua

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ABSTRACT
Eaglewood is Indonesia's important trade commodity in the form of resins from several infected species of *Thymelaeaceae*. The basis to determine its international trade quota through CITES is derived from the estimated eaglewood-producing species grown in their habitat. This paper aims to estimate the biomass of eaglewood, *Aquilaria filaria*, in the karst ecosystem of West Papua. We conducted a plot-based method and calculated the biomass of *A. filaria* using a diameter-based allometric equation and simulated using a bootstrap procedure. The results showed that 15,500 tons of naturally infected eaglewood are estimated in the karst ecosystem of West Papua.

Keywords: Agarwood, allometry, bootstrap, CITES, gaharu

INTRODUCTION
One of Indonesia's forest-based important trade commodities exported to the Middle East is eaglewood. Eaglewood or agarwood or *gaharu* is a trading name of a solid resin produced mainly from the genera of *Aquilaria*, *Gyrinops*, and *Gonystilus* which belong to the *Thymelaeacea* (Hou 1960). The resins are naturally produced in the forest due to the infection of the wood by the fungus (Budi et al. 2010; Agustini et al. 2006) and produce highly valuable fragrant used for incense, perfume industry, as well as traditional medicines (Mohamed 2016). However, due to the increasing demand for eaglewood and the shrinking of its population in the forest, the international trade of eaglewood is regulated through the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2017). Therefore, the quota of naturally infected eaglewood producing trees in Indonesia is decided yearly by the management authority (the Ministry of Environment and Forestry) based on the consideration of the scientific authority (the Indonesian Institute of Sciences, LIPI).

LIPI, through the Secretariat of Scientific Authority for Biodiversity, provides the scientific-based evidence to decide the quota of eaglewood yearly based on the latest population study of the species. A recently published population study of the eaglewood producing tree by Destri et al. (2020) showed that the density of the tree and seedling of *Aquilaria filaria* was around 2.5 and 2.9 ha^{-1}, respectively. This density estimation was significantly lower compared to what the previous study suggests more than two decades

ago that reached 4.33 trees ha^{-1} in Papua (Soehartono 1997 in Donovan et al. 2004). Here, we provide biomass estimation of *A. filaria* as well as the biomass estimation of naturally infected *A. filaria* that grows in the karst ecosystem of West Papua (Destri et al. 2019; Destri et al. 2020; Soehartono et al. 2000). The biomass estimation, rather than its tree density (as estimated by Destri et al. 2020), is relatively applicable to decide the quota of eaglewood produced from *A. filaria*.

To estimate the eaglewood biomass in the karst ecosystem, we used data from the population study of *A. filaria* done in the karst forest of Natural Tourism Park of Beriat, South Sorong (West Papua) on 8-26 April 2019 (Destri et al. 2020). Our data was from 28 sampling units of 10 m x 10 m using purposive random sampling. Biomass estimation (AGB_{est}, in kg) was calculated using a generic allometry equation (Chave et al. 2014) based on the diameter of breast height (D, in cm) and tree height (H_{est}, in m) (Equation 1). As we did not measure tree height directly in the field, tree height was estimated using the D-based equation for the S.E. Asia region (Feldpausch et al. 2012) (Equation 2). We used the value of 0.347 (the average value of *Aquilaria*) as the specific wood density value (ρ) of *A. filaria* (Zanne et al. 2009; Chave et al. 2009), which is commonly used if the specific wood density of a species is unknown (Slik 2006). The two formulas used to estimate biomass are as follow:

$$AGB_{est} = 0.0673 \; x \; (\rho D^2 H)^{0.976} \quad \text{(Equation 1)}$$

$$H_{est} = 57.122 \; x \; (1 - \exp(1 - 0.0332 \; x \; D^{0.8468})) \quad \text{(Equation 2)}$$

To estimate total biomass and infected biomass per area, a bootstrap procedure with 1000 replications was done with a 95% confidence interval (Canty et al. 2019; DiCiccio et al. 1996). Extrapolation of biomass from the bootstrap method was then carried out to the whole estimated karst forest area in three regencies in West Papua (Sorong, South Sorong, and Teluk Bintuni). The area of karst was calculated based on physiographic maps of Papua bird's head region (Bartstra 1998) using ImageJ software (Schneider et al. 2012; Abràmoff et al. 2004). Further, the estimation of naturally infected *A. filaria* was calculated using the assumption of Gibson (1977) that stated only 10 % of *Aquilaria* in the forest is potentially naturally infected by the fungus and produces eaglewood.

Our simulation showed that the total biomass of *A. filaria* in the karst ecosystem of West Papua is estimated stable at *ca.* 221 kg ha^{-1} (Figure 1A). Further, the naturally infected *A. filaria* is estimated *ca.* 22 kg ha^{-1} (Figure 1B). Extrapolated to the karst area, the naturally infected biomass of *A. filaria* in West Papua is predicted at *ca.* 15,511 tons (Table 1). The highest potency within the Province of West Papua is predicted found in South Sorong Regency (9,193 tons), followed by Teluk Bintuni Regency (4,932 tons) and Sorong Regency (1,386 tons).

The biomass estimation shown in Figure 1 and Table 1 has some uncertainties. The uncertainties lie in the calculation and estimation of the karst area and the inaccuracy or uncertainties of protected areas within the karst areas. The uncertainties of the karst area in West Papua is relatively high. Up to date, the distribution of the karst ecosystem in West Papua is still lacking. We use the extrapolation method to estimate the area of karst in West Papua based on the physiographic maps of Bartstra (1998), therefore, causing the over/under-estimation of the karst area. Further, within the karst ecosystem, there are some protected areas where the extraction of eaglewood is prohibited. Ideally, the biomass of infected *A. filaria* calculation (as shown in Table 1) should exclude the area of the protected forests. The combination of those uncertainties results in the uncertainties of extractable biomass

estimation of *A. filaria* in West Papua.

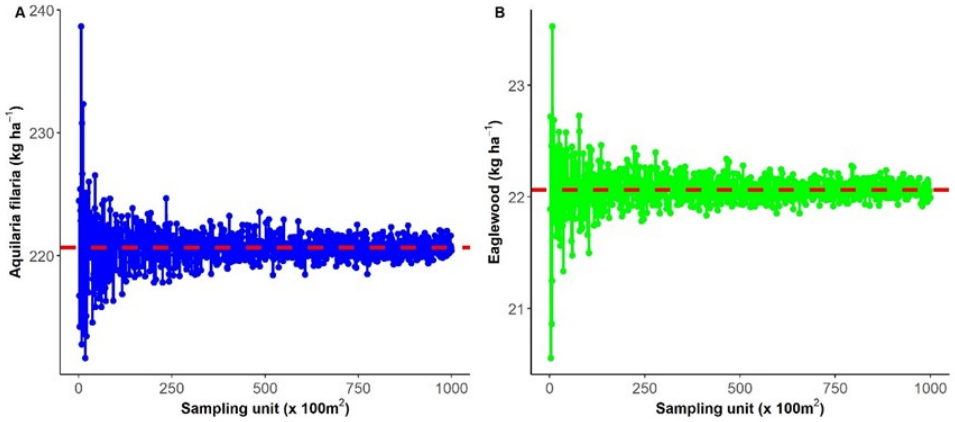

Figure 1. Biomass estimation simulation of *Aquilaria filaria* (panel A) and naturally infected *A. filaria* (panel B) in the karst ecosystem of West Papua along with the number of the sampling unit from the bootstrap procedure with a 95% confidence interval. The red dashed lines show the average biomass estimation of *A. filaria* and naturally infected of *A. filaria* (eaglewood).

Table 1. Estimation of karst area (ha), the biomass of *Aquilaria filaria* (ton), and biomass of naturally infected eaglewood from *A. filaria* in West Papua.

Regency	Area of karst forest (ha)	Biomass of *Aquilaria filaria* (ton)	Biomass of eaglewood (ton)
Sorong	62,718	13,861	1,386
South Sorong	415,991	91,933	9,193
Teluk Bintuni	223,147	49,316	4,932
Total	701,856	155,110	15,111

In conclusion, the maximum potency of naturally infected *A. filaria* in the karst ecosystem of West Papua is estimated at *ca.* 15,511 tons. This potency is still very high compared to the international trade quota of 2020 from CITES which only 490 tons. This present quota is equivalent to only 3.2% of the maximum potency in West Papua. However, even though the potency of eaglewood is still abundant, the sustainability concept must still be considered (Soehartono et al. 2002; Zhang et al. 2008; Soehartono et al. 2000). Therefore, the quota concept from CITES as well as the harvesting of eaglewood from its natural habitat must be tightened to conserve *A. filaria* in the future.

AUTHOR CONTRIBUTIONS

AHR, ZM, and DE have equal contribution to this work as the main contributor. AHR, ZM, and DE designed the project. AHR and ZM collected the data. AHR performed the analyses. AHR, ZM, and DE wrote, revised, and approved the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Short Communication

Antibacterial Activity of *Kecombrang* Flower (*Etlingera elatior* (Jack) R.M. Sm) Extract against *Staphylococcus epidermidis* and *Propionibacterium acnes*

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ABSTRACT
This study aimed to determine the antibacterial activity from the ethanol extract of *Kecombrang* flower (*Etlingera elatior* (Jack) R.M. Smith) against *Staphylococcus epidermidis* and *Propionibacterium acnes*. The extract was made by the maceration method with 70% ethanol as a solvent. Antibacterial activity test was carried out by the disk diffusion method with a concentration of 10%, 20%, 40%, and 80%. Meanwhile, the Minimum Inhibitory Concentration (MIC) was done at concentrations of 10%, 8%, 6%, 4%, and 2%. The results showed that the *Kecombrang* flower (*Etlingera elatior* (Jack) R.M.Smith) extract had antibacterial activity against *S. epidermidis* and *P. acnes*. The MIC for *S. epidermidis* is at a concentration of 4%, while in *P.acnes* cannot determine yet.

Keywords: antibacterial, ethanol extract, *Kecombrang* flower, *Propionibacterium acnes, Staphylococcus epidermidis*

Staphylococcus epidermidis and *Propionibacterium acnes* are known as commensals bacteria in human skin which can change into opportunistic (Nakase et al. 2014; Chessa et al. 2015). *Staphylococcus epidermidis* colonizes various areas of the skin, while *P. acnes* resides mainly in the pilosebaceous skin follicles. This microbial interplay, for instance, mediated through molecules involved in intercellular competition or communication, may have an impact on the fine balance of the skin ecosystem. A disturbed balance (dysbiosis) can impact skin health and might initiate or support the events that lead to skin disorders. One of such disorders is acne vulgaris, multifactorial disease of pilosebaceous units of the skin that affects adolescents (Christensen et al. 2016).

Propionibacterium acnes can be related to the initial stage of acne because it causes an increase in the lipogenesis originated in sebaceous glands. It induces inflammation and pustules on the skin (Neves et al. 2015; Blaskovich et al. 2019). Meanwhile, *S. epidermidis* also can be opportunistic when it enters the bloodstream (Nakase et al. 2014; Tabri 2019).

Acne treatment in skin clinics usually uses antibiotics that can overcome inflammation and kill bacteria such as tetracycline, erythromycin, doxycycline, and clindamycin (Nakatsuji et al. 2009; Doğan et al. 2017). However, these drugs have side effects such as irritation and allergy, while

long-term use of antibiotics can cause resistance, organ damage, and immune -hypersensitivity (Adawiyah et al. 2010; Tan et al. 2018; Dikicier 2019). These problems have led many researchers to discover and develop new sources for antimicrobial agents from natural products, e.g. medicinal plants (Abdallah 2011). Sadeek & Abdallah (2019) stated that some phytochemical compounds extracted from medicinal plants showed effective antibacterial potential against multi-drug-resistant pathogens and these compounds could be exploited as antibacterial drugs.

Indonesia is known as one of the countries that have many medicinal plants. One of them is *Kecombrang* (*Etlingera elatior* (Jack) R.M.Smith). *Kecombrang* is a spice plant that belongs to the Zingiberaceae Family and has been used in making medicine as well as flavour enhancers. This plant contains secondary metabolites such as phenols, flavonoids, glycosides, saponins, tannins, steroids, and terpenoids (Silalahi 2017; Juwita et al. 2018; Effendi et al. 2019). Those compounds are known as potential sources for antibacterial agents (Abdallah 2011; Sadeek & Abdallah 2019). Based on Farida & Maruzy (2016) report, *Kecombrang* flower has more antibacterial compounds compare to its rhizome, leaves, or fruit. *Kecombrang* fruit contains flavonoids only. *Kecombrang* leaves contain saponin and flavonoids. The rhizome of *Kecombrang* contains saponin, tannin, sterol, and terpenoid. Meanwhile, *Kecombrang* flower contains flavonoid, saponin, tannin, and terpenoid.

Some studies reported that *Kecombrang* flower extract has an antibacterial activity to some bacteria. Mackeen et al. (1997) reported that ethanol extract of *Kecombrang* flower can inhibit the growth of *Pseudomonas aeruginosa, Escherichia coli, Bacillus megaterium*, and *Cryptococcus neoformans*. Wijekoon et al. (2013) reported that *Kecombrang* flower extract with various solvents (water, 50% ethanol, and 96% ethanol) can inhibit the growth of *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Listeria monocytogenes*, and *Klebsiella pneumoniae*. Another research by Ghasemzadeh et al. (2015), showed that the ethanol and water extract of *Kecombrang* flower can inhibit *S. aureus, B. subtilis, E. coli, Salmonella* sp., *Micrococcus* sp., and *Proteus mirabilis*. Naufalin & Rukmini (2018) reported that the ethanol extract of *Kecombrang* flower has better antibacterial activity thBased on that background, this study was done to determine the antibacterial activity from the ethanol extract of *Kecombrang* flower against *S. epidermidis* and *P. acnes*. Since *Kecombrang* flower is high in containing polyphenol compounds, so it is best to use ethanolic solvents (Farida & Maruzy 2016). Ethanol can attract more polyphenol compounds than others (Tiwari et al. 2011). The outcome of the study is expected to show that ethanol extract of *Kecombrang* flower can be used as an alternative for acne treatment. This study was done by several steps, namely, plant source and preparation, extraction, phytochemical screening, disk diffusion test, and dilution method to determine Minimum Inhibitory Concentration (MIC).

The *Kecombrang* flower used in this research was obtained from *Kecombrang* plantation in Lubuk Begalung, Padang City, West Sumatra. About eight kg of the flowers was washed with clean water and cut into small pieces, then placed in a container and spread evenly for the drying process. The flowers were dried in the oven with a temperature of 40-50 ºC for 3 x 24 hours. The purpose of drying is to get the simplicia of flower that is not easily damaged and not overgrown with fungus in long-term storage (Sa'adah & Nurhasnawati 2015). The oven was chosen because it can keep at a controlled temperature and gave faster drying (Singh & Laishram 2010). The drying flower then mashed up into a homogenous powder to expand the contact between the solvent and the simplicial. This texture can speed up the extraction process because it was enlarging the contact between the powder

and the solvent (Depkes RI 1989).

The extraction of phytochemicals from *Kecombrang* was done using maceration method. Maceration was chosen because it is a very simple method and could be used for the extraction of thermolabile compounds (Zhang et al. 2018). *Kecombrang* flower powder as much as 500 g were macerated using 70% ethanol as a solvent with a concentration of 1:10. Ethanol was used because it can attract more polyphenol compounds than others (Tiwari et al. 2011). *Kecombrang* flower is high in containing polyphenol compounds, so it is best to use ethanolic solvents (Farida & Maruzy 2016). 70% ethanol is known more polar than pure ethanol because adding water to pure ethanol up to 30% could increase the polarity of ethanol. According to Velavan (2015), the higher concentrations of bioactive flavonoid compounds were detected in 70% ethanol due to their higher polarity than pure ethanol. Moreover, the higher the solvent polarity, the yield obtained will also increase (Noviyanty et al. 2019). The maceration was done 1 x 24 hours with occasional stirring. Remaceration was done for 2 x 24 hours using the same solvent. The filtrate from maceration then evaporated using rotary evaporator into a thick extract (Depkes RI 1995).
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hours with occasional stirring. Remaceration was done for 2 x 24 hours using the same solvent. The filtrate from maceration then evaporated using rotary evaporator into a thick extract (Depkes RI 1995).

The yield extract in this research was about 17.6%. The yield of an extract shows the number of active compounds extracted from the sample. The higher the yield extract, the active compounds contained in the extract also bigger (Harborne 1984; Hasnaeni et al. 2019). The calculation of the yield extract showed in Table 1.

Table 1. Calculation Yield of *Kecombrang* Flower Powder and Extract.

Sample	Flower powder (g)	Extract (g)	Yield (%)
Kecombrang flower	500	88	17.60

The extracted phytochemical was then screened for phytochemical contents based on Materia Medika Indonesia (Depkes RI 1989) and Pandey & Tripathi (2014). Screening includes testing for alkaloids, flavonoids, tannins, saponins, steroids, and triterpenoids. The results of the phytochemical screening showed that *Kecombrang* flower extract contains flavonoid, saponin, and tannin. While for alkaloid and steroid/triterpenoid, it shows negative results. These results following with Silalahi (2017) and Juwita et al. (2018), which stated that *Kecombrang* contains many secondary metabolites from terpenoids and phenolic groups, whereas alkaloid groups have not been reported. The phenolic compounds found in *Kecombrang* are flavonoid, saponin, tannin, and polyphenol. The result of the phytochemical screening showed in Table 2.

Table 2. Phytochemical Screening of *Kecombrang* Flower Extract.

Metabolites	Phytochemical screening	
	Powder	extract
Alkaloid	-	-
Flavonoid	+	+
Saponin	+	+
Tannin	+	+
Steroid/ Triterpenoid	-	-

(+) : contain tested metabolite; (-) : do not contain tested metabolites

The presence of flavonoids, saponin, and tannin in this research indicates that *Kecombrang* flower extract has the potential as an antimicrobial. As stated by Juwita et al. (2018) that the high potential antimicrobial activity possessed by *Kecombrang* is due to the presence of flavonoid, phenolic, and terpenoid contents.

The antibacterial activity test was carried out by the disk diffusion method on Mueller Hinton Agar (MHA). The MHA was poured into a petri dish and allowed to stand until the media hardens. After the media has hardened, the suspension of bacteria was pipetted about 1 ml onto a petri dish and spread evenly. The Bacterial suspension (9 x 10^7 CFU/ml) was suspended in NaCl 0.9%. After the bacterial suspension dried, sterile disk paper was inserted into a petri dish and 20 µl of the extract was dropped. The concentration of the extract was using 10%, 20%, 40%, and 80% based on

MacKeen et al. (1997) and Kusumawati et al. (2015) with modification. They tested the extract of *Kecombrang* flower and *Kecombrang* leaf (respectively) in concentrations of 20%, 40%, and 80% which showed positive results to the tested bacteria. The tested disks were then incubated for 24 hours at 37°C. The diameter of the inhibition zone was measured using a calliper. The clear zone that appears around the disk was measured as the inhibition zone (IZ) (Hudzicki 2016). The inhibition zones were analyzed statistically by Two-Way Anova and Least Significance Different (LSD) using MS Excel 2010 to see the significant effect of the differences concentrations.

The positive control used in this research is two antibiotics, named ciprofloxacin for *S. epidermidis* and clindamycin for *P. acnes*. Antibiotics are known as a substance that can inhibit the growth of bacteria (Pratiwi 2008). Ciprofloxacin is known as a broad-spectrum antibiotic from quinolone group. Quinolones are known can inhibit or disrupt the nucleic acid synthesis of the bacterial cell (Hogg 2005; Vidyavathi & Srividya 2018). Meanwhile, clindamycin is known as an antibiotic that can inhibit the protein synthesis of the bacteria. Clindamycin usually prescribes for acne treatment (Smieja 1998; Walsh et al. 2016). The use of different antibiotics in this research was due to in preliminary study ciprofloxacin gave a very wide inhibition zone in the dish containing *P. acnes*, so it can create ambiguous results. The negative control used in this test is DMSO 10% (v/v). DMSO is a surfactant that can dissolve polar and nonpolar materials. It also showed no antibacterial activity (de Brito et al. 2017). The measurements of the inhibition zone showed in Figure 1 and Table 3.

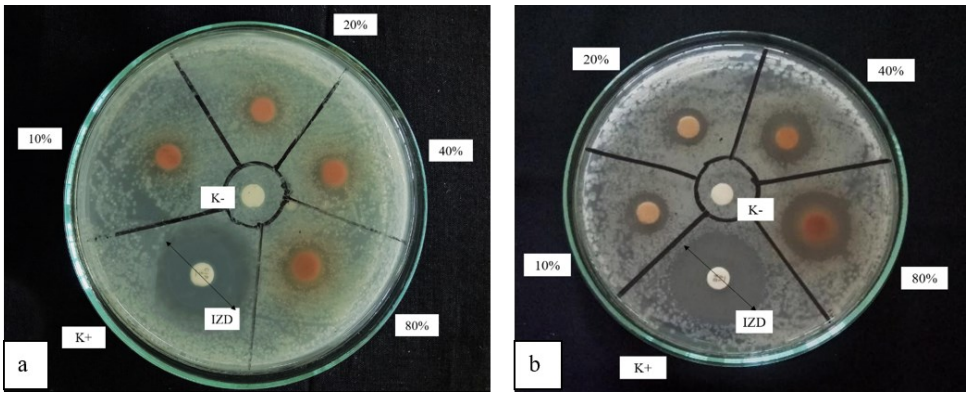

Figure 1. Inhibition zones of ethanol extract of *Kecombrang* flower (*Etlingera elatior* (Jack) R.M.Smith against (a) *S. epidermidis* and (b) *P. acnes*.; IZD: Diameter of Inhibition Zone; K+: positive control; K-: negative control; 10%, 20%, 40%, and 80% indicate the percentage test concentration of the ethanol extract of *Kecombrang* flower.

Table 3. Inhibition Zone (IZ) of *Kecombrang* Flower Extract against *Staphylococcus epidermidis* and *Propionibacterium acnes*.

Bacteria	Inhibition Zone (IZ) (mm)					
	10%	20%	40%	80%	Positive control	Negative control
Staphylococcus epidermidis	10.62±0.06^{b}	11.41±0.06 ^{b}	12.44±0.12 ^{b}	14.41± 0.02^{c}	28.67*± 0.17^{d}	-^{a}
Propionibacterium acnes	11.25± 0.05 ^{b}	11.46± 0.02 ^{b}	14.51±0.06 ^{b}	19.37± 0.11 ^{c}	26.31$^{\#}$±0.0 6 ^{d}	- ^{a}

10%, 20%, 40%, and 80%: concentration of *Kecombrang* flower extract; *: Ciprofloxacin 5 µg; #: Clindamycin 10 µg; Negative control: DMSO 10%; -: no inhibition zone; inhibition values are expressed as mean ± standard deviation. Different superscript letters show the significant differences ($p < 0,05$), and the same letters show no significant differences in the row.

The data in Table 3 showed that the ethanol extract of *Kecombrang* flower has the activity to inhibit the growth of *S. epidermidis* and *P. acnes*. The results showed that the inhibition zone became larger in line with the greater concentration of the extract. The Inhibition zone at a concentration of 80% showed significant inhibition against those two bacteria compared to other concentrations. The difference in diameter of inhibition zones at each concentration possibly was due to differences in the magnitude of active substances contained in the concentration. The active compounds in higher concentrations are more than the opposite (Lingga et al. 2016). Besides that, the size of the inhibition zone was also influenced by the level of sensitivity of the organism, the culture medium, the incubation conditions, and the diffusion rate of the antibacterial compound (Fitriah et al. 2017). Bacteria have different sensitivity against antibacterial agents. Usually, Gram-positive bacteria are more sensitive to antibacterial agents compared to Gram-negative bacteria. The Gram-negative bacteria have an outer membrane in their cell wall that is not easily penetrated or damaged by certain antibacterial compounds (Breijyeh et al. 2020). The culture medium can influence the inhibition zone because different nutrient contents in media are affecting the bacterial growth. The Composition of culture media exercises a key effect on the susceptibilities of microorganisms, such as free from inhibitor content or the magnesium levels (Daoudi et al. 2020). Besides the nutrient contents in media, agar thickness and inoculum volume can influence the diffusion rate of the antibacterial agent. The higher the thickness of agar layer, the smaller the zone diameter. Similarly, high inoculum concentration shows hazy growth on the media, whereas a low concentration of inoculum shows light and immeasurable zone (Dafale et al. 2016). The particle volume also can influence the diffusion rate. Small particles will diffuse faster than large ones. Other factor that influences the inhibition zone is the incubation conditions, such as pH and temperature. Higher temperatures will increase the diffusion process. The pH of a solvent must be adjusted to neutrality (6.0 – 8.0) or dissolved in buffer solutions because the bacterial growth might be inhibited in too acid or too alkaline media (Valgas et al. 2007). Antibacterial agents have four mechanisms to inhibit the growth of bacteria. First, by inhibiting the growth of cell walls. Secondly, changing the cell membrane permeability; then inhibits the protein synthesis; and the nucleic acids (Hogg 2005). According to Fitriah et al. (2017), each group of compounds can have different effects in inhibiting bacterial growth. The difference in the activity that occurs is caused by secondary metabolites contained having synergistic energy that is different depending on the nature and morphology of bacteria.

The *Kecombrang* flower extract in this research contains flavonoid, tannin, and saponin, which has different mechanisms to inhibit the growth of bacteria. According to Juwita et al. (2018), flavonoid compounds in *Kecombrang* (*Etlingera elatior* (Jack) R.M.Smith) have antibacterial activity by targeting of membrane cell wall due to its capability to composite with extracellular and soluble proteins. This mechanism is similar to saponin, which also attacks the bacterial cell membrane. Saponin can dissolve lipids in bacterial cell membranes (lipoprotein), which causes the bacterial cell to become lysis and death (Syafriana et al. 2019). Meanwhile, tannins act by disturbed the DNA gyrase, which is an enzyme that plays a role in DNA replication (Khameneh et al. 2019). However, we cannot determine yet which compounds have a significant effect in inhibiting bacterial growth because this research has only qualitative phytochemical data, not a quantitative one. So, we cannot find out whether these compounds are equal in quantity or one is higher than another.

The data in Table 3 also showed us that a minimum concentration (10%) can inhibit the two bacteria. Because of that, the antibacterial test

continued to the Minimum Inhibitory Concentration (MIC) value test. MIC value is the lowest concentration value that can inhibit bacterial growth (Dafale et al. 2016). The test was carried out using the solid dilution method which is observing the growth of the bacteria at the lowest concentration of IZ results onto agar media. The concentration used were 10%, 8%, 6%, 4%, and 2%. The liquid medium was mixed with 1 ml of bacterial suspension and 1 ml of predetermined extract concentration. The mixture of cell suspension, media, and extract was then homogenized and incubated for 24 hours at 37^0C. The results of the incubation were then observed for the presence or absence of bacterial colony growth on the media. If there is bacterial growth, the extract cannot inhibit bacterial growth. However, if the media remains clear, it shows that the extract could inhibit bacterial growth (Pratiwi 2008; Hudzicki 2016). The data of MIC was showed in Table 4.

Table 4. Minimum Inhibitory Concentration (MIC) Test of *Kecombrang* Flower Extract Against *Staphylococcus epidermidis* and *Propionibacterium acnes*.

Extract Concentrations	Bacteria	
	Staphylococcus epidermidis	*Propionibacterium acnes*
10 %	-	-
8 %	-	-
6%	-	-
4 %	-	-
2 %	+	-

-: no growth; +: growth

Data in Table 4 showed that MIC in *S. epidermidis* is at a concentration of 4% because, in a concentration of 2%, the bacteria showed growth. Meanwhile, the MIC value of *P. acnes* cannot be determined yet. It's due to at the lowest concentration of the test (2%), the *P. acnes* still showed no growth. The data in Tables 3 and 4 showed that *P. acnes* was more sensitive than *S. epidermidis* against the extract. This data aligned with Nishijima et al. (2000), which reported that *S. epidermidis* indeed more resistant than *P. acnes* when testing against several antibiotics. Based on that data, the tested concentration to *P. acnes* should be below 2% until it shows a MIC value.

In conclusion, the ethanol extract of *Kecombrang* flower (*Etlingera elatior* (Jack) R.M.Smith) can inhibit the growth of *S. epidermidis* and *P. acnes*. This data showed a potency of *Kecombrang* flower extract as an antibacterial agent against *S. epidermidis* and *P. acnes* that causes acne vulgaris. This research is a preliminary study, so further research is needed to ensure a more valid antibacterial activity, such as determining levels of flavonoid, tannin, and saponin. Besides that, it is also necessary to study the mechanism of inhibition or cell damage caused by the secondary metabolites to prove that the mechanisms mentioned above are suitable.

AUTHORS CONTRIBUTION

VS and RNP designed the study and carried out the laboratory work. VS, YSD, and RNP analysed the data and write the manuscript.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Short Communication

Seed and Germination Study of a New Guinea Endemic Plant Species *Grevillea papuana* Diels.

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ABSTRACT
Grevillea papuana is a culturally important endemic plant species of New Guinea highland. Although *G. papuana* conservation and propagation attempts were already conducted, this species seed and germination characters information were still very limited. This study aimed to provide information regarding *G. papuana* seed and germination biology. Seed characteristic and germination trials were conducted in this study. Data analysis was conducted descriptively, while germination parameters were also calculated. *G. papuana* has a light, elliptic, and winged seed. The species germination was low and ununiform with *phanerocotylar epigeal foliaceous* (PEF) seedling functional type.

Keywords: epigeal, phanerocotylar, germination, proteaceae

Grevillea papuana is a New Guinea endemic plant species that belong to the Proteaceae family (Makinson 2000; POWO 2019). This species is culturally essential for traditional construction, firewood, traditional medicine, and birth controller (Wiriadinata 1995; Priyono et al. 2002; Sukamto 2003; Arobaya & Pattiselanno 2007; Williams 2011; Jorim et al. 2012; Kuswantoro & Solihah 2017). *G. papuana* is also crucial for soil fertility as soil samples from the plant species dominated area produce a high *Rhizobium* population value (Purwaningsih 2005).

Ongoing harvesting for firewood and medical properties, low natural germination rate, and the absence of local community propagation attempt treat *G. papuana* wild population (Priyono et al. 2002; Sukamto 2003). Therefore, the plant species ex-situ conservation attempt is conducted in Bali and Cibodas Botanic Garden (Kuswantoro & Solihah, 2016). These two botanic gardens were chosen as they were situated in highland regions similar to *G. papuana* native distribution range in New Guinea highland. For example, Bali Botanic Garden is geographically situated in the Bedugul, a highland basin at an altitude of about 1200-1500 meters above sea level. However, this conservation attempt is still needed to be supported by propagation research (Priyono et al. 2002). *G. papuana* in-vitro and shoot cutting vegetative propagation research was already conducted by Sukamto (2003) and Kuswantoro and Solihah (2016), respectively. Generative

propagation research of the species was also reported by Priyono et al. (2002) and Kuswantoro and Solihah (2016).

Despite all these researches, research on *G. papuana* seed and germination characteristics is still needed to be done as to the best of our knowledge, little report on *G. papuana* seed characteristic is present, while no record regarding this species seeding functional type was reported. This condition is unfortunate as seed and germination characteristics can provide information regarding plant species adaptation strategies that is valuable for the plant conservation effort (Kuswantoro & Oktavia 2019; Handayani 2019). Due to its importance, germination biology and seedling functional type of several plant species such as *Euchresta horsfieldii*, *Pinanga arinasae*, *Areca vestiaria, Artabotrys hexapetalus,* and some Annonaceae species were already studied in Bali and Bogor Botanic Garden (Lestari & Asih 2015; Handayani 2017; Kuswantoro & Oktavia 2019; Handayani 2019). Thus, this study aimed to describe seed morphology, germination type, and seedling functional type of *G. papuana*. We hope that this study will contribute to the plant species conservation effort by enhancing our understanding of its seed and germination biology.

The study was carried out in November 2019-March 2020 at Bali Botanic Garden Seed Bank Laboratory. Seeds were harvested from the only *G. papuana* plant cultivated in the garden. Of all thirty-six seeds collected during the harvesting period, twenty-five seeds were randomly selected for morphometric and weight measurement. Seed measurement parameters used in this study are the seed length (SL) and seed wide (SW), which defined as the measurement of the seed length and wide, as well as total seed length (TL) and total seed wide (TW), which defined as the measurement of the total seed area, including the seed wing. Measurement of *G. papuana* seed conducted in this study is illustrated in Figure 1.

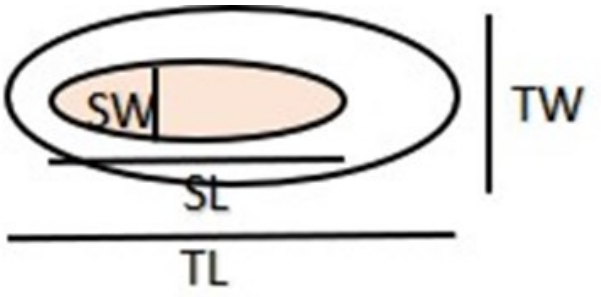

Figure 1. *Grevillea papuana* Seed Measurement

All thirty-six *G. papuana* seeds were then sown in a clear, closed plastic container with straw paper as the germination media. Before the sowing process, *t*he seeds, container, and straw papers were sterilized by dipping them into boiling water for about one and a half minutes. The seeds were then incubated at room temperature within the seed bank laboratory. The germination observation was carried out every day and terminated after 120 days after sowing.

Descriptive data analysis was conducted to describe *G. papuana* seed morphology, germination process, and seedling functional type. Moreover, calculation of the seed final germination percentage (FGP), the first day of germination (FDG), last day of germination (LDG), and time spread germination (TSG) values are also conducted. The calculation of germination parameters value is following Kader (2005) as follows:

$$FGP = \frac{\text{Total Number of Germinated Seed}}{\text{Total number of Sowed Seed}} \times 100\%$$

FDG = First day when germination occurred

LDG = Last day when germination occurred

TSG = time between first and last day of germination

Meanwhile, the seed germination type is defined following Tjitrosoepomo (2009), while the seedling functional type is defined according to Ibarra-Manríquez et al. (2001) and Pérez-Harguindeguy et al. (2016).

Grevillea papuana seeds are light, elliptic, and winged. *G. papuana* seed elliptic shape is based on its morphometric measurement result, which indicated that the seed averages total length value is about 1.87 times of the seed average total wide value (Table 1.). Meanwhile, the average seed weight value of *G. papuana* measured in this study which lighter than the seed weight of six *Grevillea* species studied by Auld and Denham (1999) highlight the seed lightness. *G. papuana* light seed is probably due to its small embryo and endosperm, reported by Priyono et al. (2002).

Table 1. *Grevillea papuana* Seed Characteristic Parameters Value

Seed Character Parameters	Average Value
Total Length (TL)	14.08 mm
Total Wide (TW)	7.52 mm
Seed Length (SL)	5.29 mm
Seed Wide (SW)	3.39 mm
Seed Weight	11.15 mg

Grevillea papuana seed is surrounded by a thin wing (Figure 2). This discovery corresponds with Priyono et al. (2002), who also reports wing in *G. papuana* seed. As *G. papuana* native range is in New Guinea highland, the species light and winged seed are in correspondence with Makinson (2000), who stated that a light, flat and winged seed is a strong trend in tropical and South Western Australia *Grevillea* species. The presence of wing in *G. papuana* seeds suggests that the seed is wind-dispersed. Our suggestion that *G. papuana* seeds are wind-dispersed is also based on our observation during this study seed collection period, which found that *G. papuana* mature seed pods were open to allow wind mediated dispersal (Figure 2).

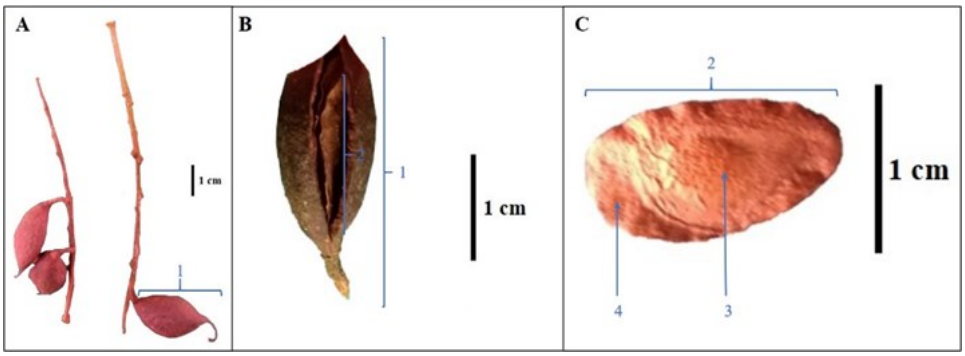

Figure 2. (A) *Grevillea papuana* Pods; (B) *Grevillea papuana* Open Pod Showing Seeds Ready to Disperse; (C) *Grevillea papuana* Seed. Remarks 1=Pod; 2= Whole Seed; 3=Seed; 4=Wing.

The result showed that *G. papuana* FGP value is 25%. Meanwhile, its FDG, LDG, and TSG values were 20, 117, and 97 days respectively (Table 2). FGP value indicates the seed germination rate, FDG, and LDG value indicates germination initiation and termination speed, while TSG value

indicates the time difference between fast and slow germination seed within the seed population (Kader 2005).

Table 2. *Grevillea papuana* Germination Parameter Value

Parameter	Value
Final germination percentage (FGP)	25%
First day of germination (FDG)	20 days after sowing
Last day of germination (LDG)	117 days after sowing
Time spread germination (TSG)	97 days

Several factors might cause *G. papuana* low FGP value acquired from this study. The presence of hard seed coat induced dormancy can be considered as one of the factors as Briggs et al. (2005) recorded that three types of seed coat induced dormancy potentially occurred in three *Grevillea* species. As seed anatomical study was not conducted in this study, we can not suggest that a hard seed coat is present in *G. papuana.* However, Priyono et al. (2002) reported that a hard seed coat does present in *G. papuana* seed. The presence of hard seed coats probably caused the low germination of *G. papuana* seed as research by Morris (2000), Morris et al. (2000), Baker et al. (2005), Guo et al. (2012), and Ma et al. (2015) stated that seed coat removal was able to increase *Grevillea* spp. germination rate.

Although a study by von Richter et al. (2001) suggested that fire-related cues were not required to produce a high germination rate in *G. kennedyana.* Studies by Morris (2000), Kenny (2000), Briggs and Morris (2008), and Guo et al. (2012), reported that fire-related cues such as smoke, temperature, and heat could improve the germination rate of some *Grevillea* species. Heat and smoke may also alleviate seed coat-induced dormancy in *G. juniperina* (Briggs et al. 2016). Thus, as there seems to be a various factor contributing to *Grevillea* low germination rate, further research needs to be conducted to understand the factor that contributes to the low FGP value of *G. papuana.*

Grevillea papuana FDG value acquired from this study is lower than *G. papuana* FDG value previously reported by Priyono et al. (2002) and Kuswantoro and Solihah (2016). *G. papuana* FDG value acquired from this study is comparable with the FDG value of another New Guinea highland plant species, *Pittosporum spicessens,* which was 19-21 days (Satyanti et al. 2015). Following the Annonaceae germination standard mentioned in Handayani (2019), based on its FDG value acquired from this study, *G. papuana* was grouped into fast germinated plant species. However, this study also reported a high TSG value in *G. papuana* germination, indicating low germination uniformity in its seed population. The low germination uniformity indicated low seed population quality, as Egli et al. (2010) suggested that germination uniformity is related to the seed population vigor.

Observation of *G. papuana* germination in this study revealed that the species seed germination is epigeal. At the same time, the species seedling functional type is *Phanerocotylar Epigeal Foliaceus* (PEF). According to Tjitrosoepomo (2009), epigeal germination is a type of germination in which, during the germination period, the cotyledon is elevated from the germination media. *Phanerocotylar* is defined by Ibarra-Manriquez et al. (2001) and Rifai (2004) as a condition in which the cotyledon is exposed during the germination period. Meanwhile, Ibarra-Manriquez et al. (2001) and Pérez-Harguindeguy et al. (2016) defined foliaceus as a condition in which the cotyledon shape and primary function are reassembling the shape and function of a leaf as a photosynthesis organ. The illustration of the *G. papuana* germination process observed during this study is presented in

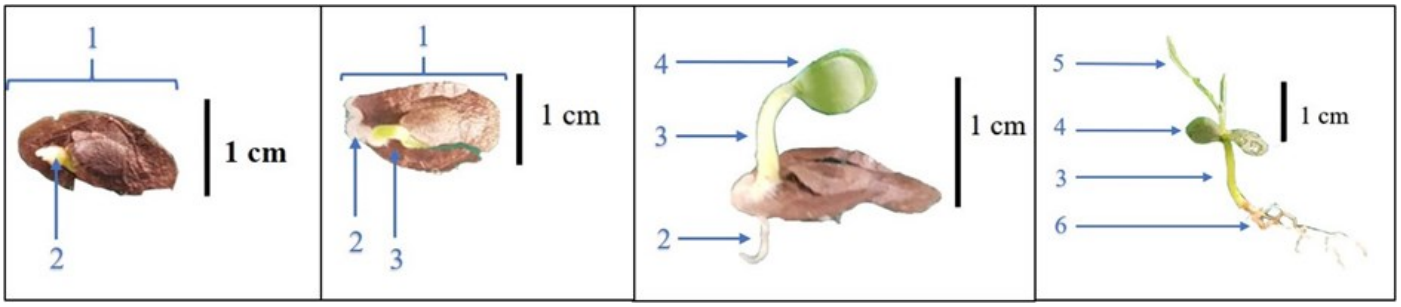

Figure 3. *Grevillea papuana* Germination Process. Remarks 1= Whole seed; 2= Radicle; 3= Hypocotyl; 4= Cotyledon; 5= Eophyll; 6= Roots.

Figure 3.

During this study, *G. papuana* cotyledon is observed as a pair of thin, leaf-like organs with green color. The thin and green cotyledon suggests that its primary function is as a photosynthetic organ. Satyanti et al. (2009) also supported this statement, suggested that *phanerocotylar* cotyledon is a photosynthetic cotyledon. These conditions supported our suggestion that *G. papuana* exhibit PEF seedling functional type.

Ibarra-Manriquez et al. (2001) report that PEF is the dominant seedling functional type in non-animal mediated seed dispersal plant species. The statement corresponded with this study result, which indicated that *G. papuana* has a wind-dispersed seed. *G. leucopteris* is another *Grevillea* species with a wind-dispersed seed (Lamont 1982). Ibarra-Manriquez et al. (2001) and Ressel et al. (2004) also stated that PEF is the dominant seedling functional type in pioneer species. PEF seedling functional type observed in this study suggests that *G. papuana* might also be a pioneer species. A study by Purwanto (2003) supports this suggestion, as they report *G. papuana* as one of the species that dominated secondary forest in Baliem Valley, Jayawijaya. This study finding further highlighted the seed biology study potency to provide information regarding plant species adaptation strategies, which important for the plant species conservation effort. In conclusion, this study record *G. papuana* light, elliptic and winged seed exhibited low and ununiform germination with epigeal germination type and *Phanerocotylar Epigeal Foliaceus* (PEF) seedling functional type.

AUTHORS CONTRIBUTION

F.K. contributes to seed collection, conducting trials, data collection and analysis, and manuscript writing.

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CONFLICT OF INTEREST

The author state that there is no conflict of interest regarding the research and the research funding.

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Short Communication

Identification of *Dendrobium* (Orchidaceae) in Liwa Botanical Garden Based on Leaf Morphological Characters

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ABSTRACT
Orchid is one of the most popular ornamental plants in the world. One of the orchid genera that is collected in a large number and known to have high morphological variations in the Liwa Botanical Garden is *Dendrobium*. However, to date, many *Dendrobium* collections have not been identified. Given the urgency of identification and the limitations of specimens in the field, especially flower organs, this study is important. This study aims to determine variations in morphological characters, phenetic relationships, and to identify *Dendrobium* collections based on leaf morphological characters in the Liwa Botanical Garden. Five accessions of *Dendrobium* were collected, namely CAT140, CAT 144, CAT 271, CAT 274, and IR015. Observation of 11 morphological characters leaves showed that leaf had high variations. The phenetic relationship based on the Gower similarity value and the UPGMA method shows that the *Dendrobium* in the Liwa Botanical Garden can be classified into 2 main groups formed with a similarity index value of 0.813. Based on Principle Component analysis values, it is known that the characters that have a large influence on grouping are the ratio of leaf length and width, leaf cross section, and leaf arrangement. The phenetic dendrogram topology is supported by the morphological character classification. The results of this study are expected to be basic information in the identification of natural orchids and conservation efforts in the Liwa Botanical Garden.

Keywords: orchid, morphological leaf, identification, Dendrobium, UPGMA, Liwa Botanical Garden

Orchidaceae (orchids) is one of the biggest plant families that consist of approximately 25,000 species belongs to over 900 genera across the world. Orchids have high variations in the morphology of the flowers, leaves, and stems (pseudobulbs). *Dendrobium* is one of the orchid genera containing a large number of species (approximately 1500 species) widely spread across the world, from Japan, China, India, the Malacca Peninsula, Indonesia, the island of Papua, to Australia. This orchid has a charming flower (Dressler 1993; Kartikaningrum et al. 2004; Kumalawati et al. 2011; Hartati & Darsana 2015).

Dendrobium comes from the words "dendro" (tree) and "bios" (life). *Dendrobium* means orchids that grow on a living tree. *Dendrobium* has various shapes, sizes, and colors of flowers. Flowers that have bloomed can last in

one day to more than 30 days and each stem can have one to more than 20 flowers. Many *Dendrobium* grows at locations with an altitude less than 400 meters above sea level (Pang et al. 2012; De et al. 2015; Darmawati et al. 2018; Indraloka et al. 2019; Zahara & Win 2019; Yuan et al. 2020).

The Liwa Botanical Garden located in West Lampung Regency (Lampung, Sumatra island) is an institution that performs ex situ conservation of plants including orchids. Orchids have been given high priority in conservation because many orchids are threatened in the wild because of exploitation and overcollection for economic reasons that lead to the population decline and disappearance of many orchid species. Another threatening factor is deforestation causing the loss and damage of orchids, a natural habitat that will lead to the extinction of orchids. Liwa Botanical Garden has an important role in the preservation and conservation of plants including orchids. Many orchid species have been collected from the natural habitats for conservation purposes in the Liwa Botanical Gardens. However, many orchid species have not yet been identified in these gardens (Solihah 2015; Adi et al. 2019; Mahfut et al. 2019).

Identification of orchid species is important in orchid conservation. The present study focussed on the identification of some specimens of *Dendrobium* (one of the largest orchid genera) that will be based on the morphological characters as one of the important tools in the plant's taxonomic and systemic basis. The morphological characters that will be used in the present study are leaf morphology. Given the urgency of identification and the limitations of specimens in the field, especially flower organs, this study is important. This study aims to determine variations in morphological characters and phenetic relationships for the identification of *Dendrobium* in the Liwa Botanical Garden. The results of this study are expected to serve as basic information in the identification of natural orchids to support conservation in the Liwa Botanical Garden.

Sample collection

Sample collections that were identified based on leaf morphological characters included 5 accessions with sample codes CAT140, CAT 144, CAT 271, CAT 274, and IR015 (Table 1). *Dendrobium* samples were chosen based on orchid data that had not yet been identified. Overall, the sample accessions are native orchids to Lampung.

Table 1. List of accessions of *Dendrobium* samples in the Liwa Botanical Garden.

No. Acc.	Species	Origin Location
CAT140	*Dendrobium* sp.	Bukit Barisan Selatan National Park
CAT144	*Dendrobium* sp.	Seminung Forest
CAT271	*Dendrobium* sp.	Bukit Barisan Selatan National Park
CAT274	*Dendrobium* sp.	Bukit Barisan Selatan National Park
IR015	*Dendrobium* sp.	Bukit Barisan Selatan National Park

The morphological identification

The morphological identification was performed by direct observation to the leaf morphology characters including leaf shape, length (L) and width (W) tip shape, cross section, arrangement, edge shape, surface texture, symmetry, and arrangement (Dressler 1993; Kartikaningrum et al. 2004; Hartati & Darsana 2015).

Based on observations of morphological characters, orchid plants have a high variation. These variations were found in habitus, pseudobulb, leaves, and flowers (Dressler 1993; Kartikaningrum et al. 2004; Hartati & Darsana

2015). In this research characterization of the flower was not performed because limited specimens were obtained in the field and had not flowered yet.

Based on observations, *Dendrobium* leaves are known as the most varied organs (Table 2). Variations in leaf characters included leaf shape (S), length (L), width (W), tip shape, cross section, arrangement, edge shape, surface texture, and symmetry. Overall, the accession of *Dendrobium* samples at the Liwa Botanical Garden showed different morphological characters of the leaves, namely the cross-section. The leaf cross section of samples with accession numbers CAT 274, CAT 140, and IR 015 are semi terete, whereas that of a sample with accession number CAT 144 is terate and CAT 271 is flat. In addition, leaf arrangement was found in all samples are alternate.

Table 2. Leaf Type of Accession of *Dendrobium* samples in the Liwa Botanical Garden. Bar = 1 cm.

No. Acc	Leaf Shape
CAT140	
CAT144	
CAT271	
CAT274	
IR015	

The ratio difference between leaf length and width between the five accessions show that the shapes of the leaves of the five accessions are different. The complete identification of the morphology of the leaves of the *Dendrobium* accession sample at the Liwa Botanical Garden is presented in Table 3.

Table 3. Variation of morphological characters of the accession of *Dendrobium* samples in the Liwa Botanical Garden.

Morphology Character	CAT 274	CAT 144	CAT 140	IR 015	CAT 271
Leaf Shape (S)	Ovate	Triangular	Oblong	Lanceolate	Llinear
Length (L) and width (W) of leaf	L: 4,5 cm W: 1 cm	L: ±1,7 cm W: 0,5 cm	L: 9,5 cm W: 1,5 cm	L: 8 cm W: 2 cm	L: 8,5 cm W: 1,5 cm
Leaf Tip	Obtuse	Acuminate	Obtuse	Acute	Acuminate
Leaf Cross Section	Semi terete	Terete	Semi terete	Semi terete	Flat
Arrangement of Leaves	Alternate	Alternate	Alternate	Alternate	Alternate
Leaf Edge	Frayed (flat)	Frayed (flat)	Frayed (flat)	Frayed (flat)	Frayed (flat)
Leaf Surface Texture	Hairless (smooth)	Hairless (smooth)	Hairless (smooth)	Hairless (smooth)	Hairless (smooth)
Leaf Symmetry	Symmetry	Symmetry	Symmetry	Symmetry	Symmetry

Based on Table 3, it is known that most of the accessions of *Dendrobium* samples in the Liwa Botanical Garden showed different morphological characters in the leaves.

Phenetic Analysis

The Phenetic analysis is performed through cluster analysis methods and Principal Component Analysis (PCA). Cluster analysis begins with the morphological character scoring, then the Gower (Gower's General Similarity) similarity value is calculated which results in a matrix of similarity between accessions. Data matrix similarity is done by agglomerative hierarchical clustering using the UPGMA method and displayed in the form of a dendrogram.

Phenetic analysis on *Dendrobium* is performed through 2 methods, namely cluster analysis, and PCA. Cluster analysis begins with the morphological character scoring, then the Gower (Gower's General Similarity) similarity value is calculated which results in a matrix of similarities between accessions. Then the similarity matrix data is done by agglomerative hierarchical clustering using the UPGMA method. The results of cluster analysis of 5 *Dendrobium* accessions based on the characters produced by the dendrogram are presented in Figure 1.

Grouping the sample based on the level of similarity between accessions calculated using the gower coefficient formula and UPGMA was chosen for the clustering technique to produce a dendrogram showing 2 main groups formed with a similarity index value of 0.813 marked as group A and group B. Group A consists of CAT 144 which has a distinguishing character that distinguishes from group B, namely the cross section of the double leaf character (Figure CAT 144). Group B consists of CAT 140, CAT 271, IR 015, and CAT 274 which have symmetrical cross-section characters (Figure CAT 140, CAT 271, IR 015, and CAT 274). Group B is divided into 2 sub-groups with a similarity index value of 0.861 marked with B1 and B2

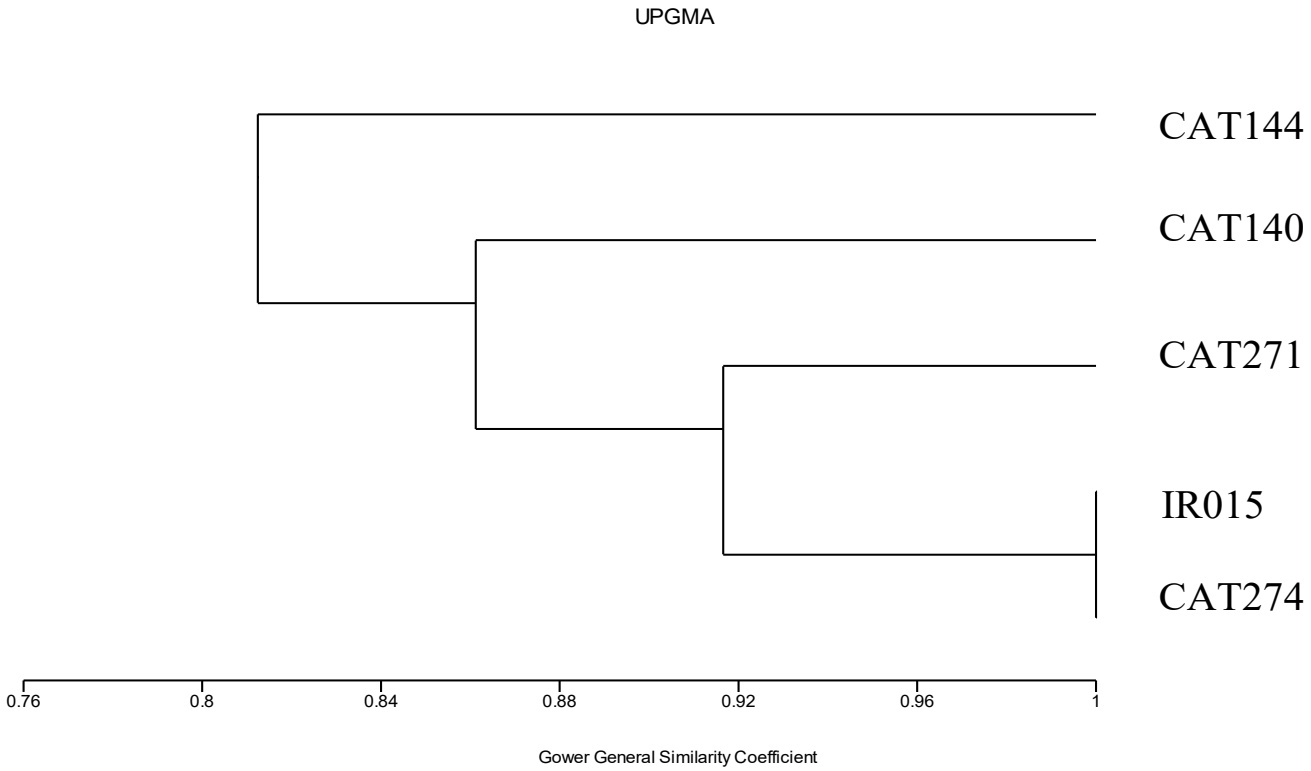

Figure 1. Dendrogram of 5 accessions *Dendrobium* samples from the Liwa Botanical Garden using UPGMA.

on the dendrogram. Characters that show the difference on ratio of the length and width of the leaf and leaf arrangement are same in all samples. Subgroup B1 consists of CAT 140, while subgroup B2 consists of CAT 271, IR 015, and CAT 274. B2 subgroups are divided into 2 namely B2a and B2b based on differences in leaf length and width ratios. The grouping of IR 015 and CAT 274 in one B2b group with a similarity level of 100% indicates that they are the same type. Based on the PCA values, it can be seen that the characters that have a large influence on grouping are the ratio of leaf length and width (PLD), leaf cross section (PMD), and leaf arrangement (DKD).

AUTHOR CONTRIBUTION

M is the main researcher who conceptualized and collected data. He did data analysis and interpretation and drafted and finalized this manuscript. S is the supervisor at Liwa Botanical Garden, he is with TTH and SW are provided the guidance from conceptualization of the research objectives, methodology, data collection, analysis, and interpretation of the results. All were key in the development, drafting later on and finalizing of this manuscript.

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CONFLICT OF INTEREST

The authors report no conflicts of interest regarding the research or the research funding.

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Research Article

Screening and Identification of Mushrooms Growth Promoting Bacteria on Straw Mushrooms (*Volvariella volvacea)*

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ABSTRACT
This research aimed to identify the indigenous Mushroom Growth Promoting Bacteria (MGPB) bacteria that can increase the growth of *Volvariella volvacea*. The research began by isolating indigenous MGPB from planting media of straw mushrooms in Karawang, Indonesia. The screening was performed to select bacterial isolates that can promote the highest growth of mushrooms by dual culture method on PDA media. There were 10 of the 58 highest bacterial isolates that have a positive effect on the vegetative growth of mushrooms. The 23K bacterial isolate was the most significant increase in mycelium growth compared to other isolates and bacteria-induced controls. A bacterial isolate 25K by gene analysis was identified by 16S rRNA (518F primer (5'- CCA-GCA-GCC-GCG-GTA-ATA-CG -3') and 800R primer (5'- TAC-CAG-GGT-ATC-TAA-TCC -3'). The result from gene analysis shows that there are ~1550 base pairs products. BLAST analysis and phylogenetic tree adjustment results show that the closest diversity of this bacterial isolate 25K is *Bacillus thuringiensis* serovar *konkukian str.* 92-27 (equality value = 99%).

Keywords: *Bacillus thuringiensis*, BLAST analysis, Indigenous bacteria, MGPB, *Volvariella volvacea*

INTRODUCTION
The factor of mushroom growth besides nutrient content on planting medium is an environmental factor. Environmental factors that are important for the growth and formation of fruit mushrooms body are temperature, moisture, light, and oxygen (Sukendro et al. 2001). All these environmental factors also support the growth of bacteria on the media of mushroom planting which helpful as a promoter of mycelium mushroom growth called MGPB (Mushrooms Growth Promoting Bacteria). Bacteria as MGPB can be found in the cover layer of planting medium which has been composted (Zeranejad et al. 2012).

The previous study reported that MGPB bacteria can induce the growth and productivity of fungi. Young et al. (2013) reported that bacteria in the growing medium and mycelium of *Agaricus blazei* can induce the

growth and productivity of the fungus. It was reported that bacteria that can induce *A. blazei* growth is *Actinobacteria* that present in the planting medium. However, there is a gap to look for MGPB that can increase the growth of *Volvariella volvacea* mycelium. *V. volvacea*, locally called straw mushroom is one of the high nutritional food mushrooms especially in the protein content. Straw mushrooms also have some good mineral content such as potassium and high phosphorus, coupled with the high enough riboflavin and thiamine, make the mushrooms more desirable and needed by consumers today (Haq et al. 2011).

Microbes become an important part of the growth of fungi. Research on the effect of bacteria on mushroom productivity has been widely practiced. Zeranejad et al. (2012) isolated and identified bacteria that can induce *Agaricus bisporus* mushrooms production. In this previous study, they found two strains of bacteria that can induce mushroom production. One of the identified strains of molecularly is *Pseudomonas putida.* A Previous study about the increasing production of straw mushrooms with the help of bacteria has been done by Payapanon et al. (2011), in this previous study found that the bacteria which contribute to increase the nutrition in the composting phase are *Paenibacillus* dan *Bacillus* sp. Another previous study by Familoni et al. (2018) shows that the bacteria were taken from the planting medium and oyster mushrooms fruit body (*Pleurotus ostreatus*) obtains some isolates that can give effect to the growth of thick fungal colonies. They found that there are *Pseudomonas putida, Streptomyces spp., Trichoderma spp., Penicillium italicum*; and others using random amplified polymorphic DNA analysis (RAPD) with 10 primers. However, there is a gap to identify the indigenous MGPB that can induce the growth of *Volvariella volvacea* using DNA sequence 16S rRNA gene analysis.

Based on the research gap in the background, this study aims to identify the indigenous MGPB that can induce the growth of *Volvariella volvacea* using DNA sequence 16S rRNA gene analysis.

MATERIALS AND METHODS

Screening of selected bacteria

The process of obtaining indigenous bacteria that can induce the mushroom growth began with a sampling of mushrooms substrate from four mushroom producing regions in Indonesia including Karawang, Cikampek, Subang, and Sukabumi. The substrate collection as a sample of harvesting and post-harvest was put into a sterile container so that no contamination from the outer bacteria. Bacterial isolation was performed by serial dilution method. After 24 hours, the grown and separated bacterial colonies were duplicated to be isolated by the four ways method. Then the pure isolate was separated into the NA medium inclined in the test tube (Cappuccino & Sherman 2005). The pure isolates were tested for their ability by the dual culture method. This method has been done to select bacteria that can increase the mycelia growth. This method used PDA in the Petri dish, and then bacteria cultured in the four sides of the medium which was about 4 cm with pieces of mycelium in the middle then incubated in an incubator with a temperature of 35^{o}C. According to Chang & Miles (2004), the method of tissue culture of straw mushrooms can be incubated at the temperature of 30-35^{o}C. The selected bacteria were the fastest bacteria to reach the edge of the Petri dish, so it can proceed to the next step.

Identification of selected bacteria

The identification was used the DNA sequence 16S rRNA method. DNA isolation in this research was performed by obtaining one selected bacterial

ose aged 24 hours into Eppendorf and resuspended with 100 µl Deion. The sample was heated at 96°C for 1 minute then incubated at -22°C for 3 minutes. The steps were repeated three times. Then the sample was centrifuged at 14,000 rpm for 5 minutes and the supernatant from the centrifugation was used as a template in the PCR (Baker et al. 2003; Araujo et al, 2001; Yuwono 2006). The amplification step of encoding gene 16S rRNA was used PCR kits with the composition including dH_2O 16,9 µl, 10 mM dNTP (dNTP mix) 0.50 µl, 5x KAPA2G buffer DNA polymerase 5 µl, *forward primers* 518F (5'- CCAGCAGCCGCGGTAATACG -3') and *reverse primers* 800R (5'- TACCAGGGTATCTAATCC -3') forward, and then added the KAPA2G robust (5U/ µl) 0,10 µl. The PCR condition used is pre-denaturation at 95°C, 5 minutes; the denaturation step is 95°C, 15 seconds; the annealing step is at 54°C, 90 seconds; and the elongation step is at 72°C, 60 seconds with the PCR process that consists of 25 cycles. The next step is the post PCR step at 72°C in 7 seconds and the stop PCR step at 4°C. DNA template of PCR result has performed the electrophoresis on 1% agarose gel and the formed band was seen by UV transilluminator after immersion in ethidium bromide solution (Yuwono 2006).

Sequence alignments and construct the phylogenetic tree

Researchers were used the bioinformatics software BIOEDIT v.7.0.8.0 to proceed with the sequence of nucleotide bases. The researchers analyzed it with the BLASTN (*Basic Local Alignment Search Tool Nucleotide*) program on the NCBI website (http://www.ncbi.nlm.nih.gov). The determination of the phylogenetic tree and bootstrap value were used a MEGA 6.06 software. The bootstrap value was used to determine the degree of confidence in the construction of a phylogenetic tree. If the bootstrap value at a low level (less than 75), so the sequence has a low confidence level, while if the bootstrap value is high level, so the sequence confidence level is also high (more than 75) (Dharmayanti 2011).

RESULTS AND DISCUSSION

The screening of the selected bacteria for straw mushroom growth

The isolation results were found 58 bacterial colonies that can be seen in Table 1 and ready to be screened using the dual culture method (see Figure 1).

Table 1. The isolation result from screening using the dual culture method.

Bacteria code	Average growth rate (mm/day)	Mycelium thickness
Control	6.50 ± 0.90	Thin
1S	4.75 ± 3.61	Thin
2S	6.33 ± 3.40	Thick
3S	6.75 ± 4.13	Thick
4S	4.58 ± 2.65	Thin
5S	6.75 ± 0.66	Thin
6S	6.83 ± 1.15	Thin
7S	5.92 ± 0.58	Thin
8S	5.75 ± 2.38	Thin
9S	5.42 ± 1.53	Thin

Table 1. Contd.

Bacteria code	Average growth rate (mm/day)	Mycelium thickness
10S	5.25 ≤ 1.39	Thin
11S	7.50 ≤ 1.32	Thick
12S	4.92 ≤ 0.80	Thin
13S	5.25 ≤ 0.90	Thin
14S	5.25 ± 1.64	Thin
15S	5.5 ± 1.80	Thin
16S	5.08 ± 1.38	Thin
17S	5.08 ± 1.23	Thin
18K	5.58 ± 0.63	Thin
19K	7.33 ± 0.29	Thin
20K	5.50 ± 0.66	Thin
21K	6.33 ± 1.59	Thin
22K	7.17 ± 1.28	Thick
23K	8.42 ± 1.18	Thick
24K	5.50 ± 1.56	Thin
25K	6.25 ± 1.73	Thin
26K	6.00 ± 1.52	Thin
27K	6.00 ± 1.00	Thin
28SB	7.00 ± 1.09	Thin
29SB	6.67 ± 1.44	Thin
30SB	7.67 ± 0.14	Thin
31SB	6.00 ± 0.87	Thin
32SB	6.67 ± 1.44	Thin
33SB	7.00 ± 2.54	Thick
34SB	6.08 ± 1.66	Thin
35SB	6.83 ± 2.02	Thin
36SB	5.25 ± 0.25	Thin
37SB	7.83 ± 0.88	Thin
38SB	6.17 ± 1.15	Thin
39SB	6.67 ± 0.14	Thin
40S	8.33 ± 0.72	Thick
41S	7.83 ± 1.53	Thick
42S	6.42 ± 1.23	Thin
43S	6.00 ± 1.98	Thin
44S	6.42 ± 1.01	Thin
45S	8.33 ± 1.89	Thin
46S	5.92 ± 0.76	Thin

Table 1. Contd.

Bacteria code	Average growth rate (mm/day)	Mycelium thickness
47K	8.00 ± 1.32	Thin
48K	6.00 ± 1.75	Thin
49S	3.17 ± 0.63	Thin
50K	6.75 ± 1.89	Thin
51C	6.92 ± 0.52	Thin
52C	**7.92 ± 0.38**	**Thick**
53C	5.50 ± 0.50	Thin
54C	**6.83 ± 1.51**	**Thick**
55C	5.25 ± 2.41	Thin
56C	4.42 ± 1.61	Thin
57S	5.17 ± 0.58	Thin
58K	4.33 ± 1.28	Thin

Figure 1 shows the difference in the length of mycelium between the four sides of bacteria. On the bacteria side of the 1S bacteria isolate, the mycelium does not move to the 1S bacteria isolate, the growth of the mycelium length is only about 20 mm and the growth rate of mycelium is about 4.75 mm/day. In the 2S and 3S bacteria isolates, the mycelium length moves to both sides of the bacterium, mycelium continues to grow until it passes through the bacteria-streaked side with a growth rate of about 6.75 mm/day. The 4S bacteria isolate shows the existence of a clear zone produced by bacteria so that the mycelium growth stopped until the clear zone. The growth rate of 4S bacteria isolate is slower than the other side, which is about 4.6 mm / day. Whereas in control, i.e., mycelium without bacterial cultures on the sides showed a widespread and thin mycelium growth with a growth rate of 6.5 mm / day.

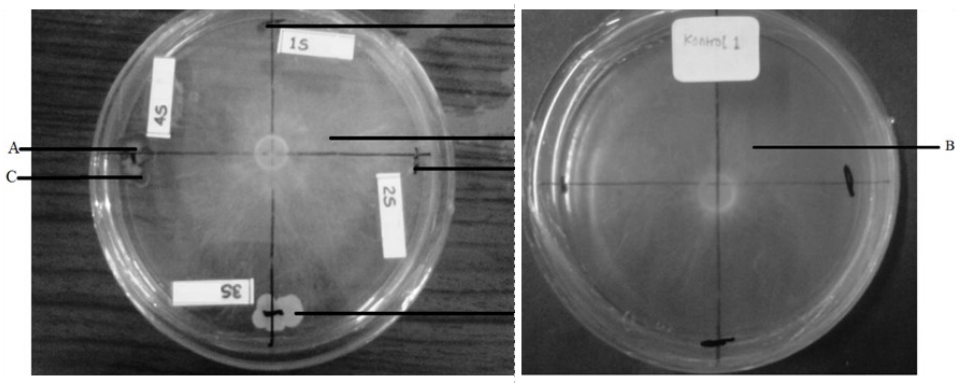

Figure 1. The screening of selected bacteria with *Dual Culture* method (left) and control (right) (A: Bacteria, B: Mycelium, C: Clear zone).

The results of the screening show two growth effects. The mycelium length and mycelium growth are faster and longer than control. Both of these effects may be possible due to the presence of bacteria on the PDA side of the medium. According to Pion et al. (2013), the growth of fungi may be affected by the presence of inhibiting or antagonistic bacteria or can induce

the growth of mycelium according to the mechanism and potential of the bacteria.

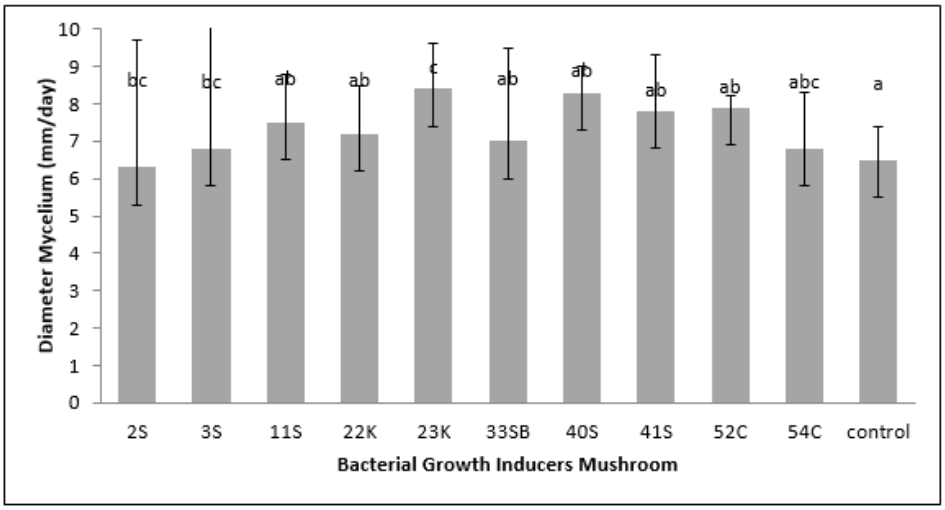

Figure 2. A Chart that diameter mushroom mycelium by indigenous bacteria (bar with the same letter are not significantly different at 5% Duncan test).

The dual culture method has been done to provide significant data on the difference in mushrooms mycelium growth by giving the bacteria treatment. From the 58 bacterial colonies, we selected the 10 highest growth rate average of mycelium and mycelium thickness (see Figure 2). The 10 bacteria isolate that can accelerate the growth of mycelium mushrooms, 23K bacteria has a faster rate of mycelium growth than controls and other bacteria isolates, which is about 8.4 mm / day. This finding is relevant to Payapanon et al. (2011) which found the presence of bacteria can affect the growth of fungi that exist around it by producing hormones and have the ability to dissolve phosphate for the availability of bacterial nutrients. The bacteria can also affect the growth of fungi because it acts as mushrooms growth-promoting bacteria (MGPB) to stimulate the growth of fungi (Zeranejad et al. 2012; Familoni et al. 2018).

Identification of Bacteria 23K

Bacteria 23K has the white colony color with the edge is rolled up. After four days, the edge of the Bacteria 23K colony will be formed the flagels (see Figure 3). These findings are relevant to Pakpahan et al. (2013) that found the characteristic of *Bacillus* is motile with the colonies' growth are spreading throughout the medium. The result of gram staining for bacteria 23K is characterized by a gram-positive purple color of bacterial cells under microscope observation and cell-shaped stem (bacillus).

Figure 4 shows that DNA of 23K bacteria isolate has an identical base length of ~ 1550 bp which be composed of both variable and conserved regions. The gene is large enough with sufficient interspecific polymorphisms of the 16S rRNA gene. Clarridge (2004) states that 500 and 1,500 bp are common lengths to be sequenced and compared. The sequences in databases have various lengths to provide valid measurements statistically.

Figure 5 shows that the construction of a 23K bacterial phylogenetic tree has the closest relationship with some *Bacillus* such as *Bacillus thuringiensis serovar strucia str.* 97-27, *Bacillus cereus*, and *Bacillus anthracis* with a bootstrap value of 76. *Bacillus thuringiensis* is one group with *Bacillus cereus* and *Bacillus anthracis* that have the ability to produce intracellular protoxin crystalline proteins (Roh et al. 2007). It can also be seen that *Bacillus cereus* has a close relationship to *Bacillus weihenstephanensis*, however the bootstrap value around

51. It means that the sequence has a low confidence level. Dharmayanti (2011) states that in the phylogenetic tree, the bootstrap value for each sequence is less than 75 so it can be categorized as having a low trust value.

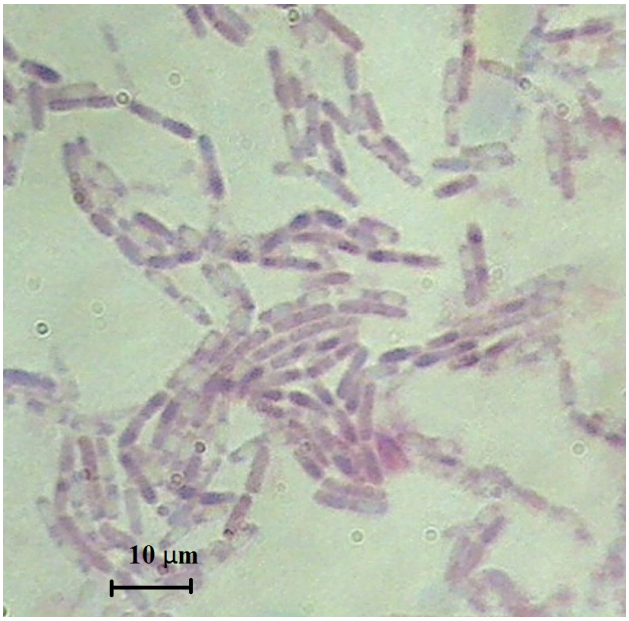

Figure 3. Morphology of bacterial 23K with magnification 100x.

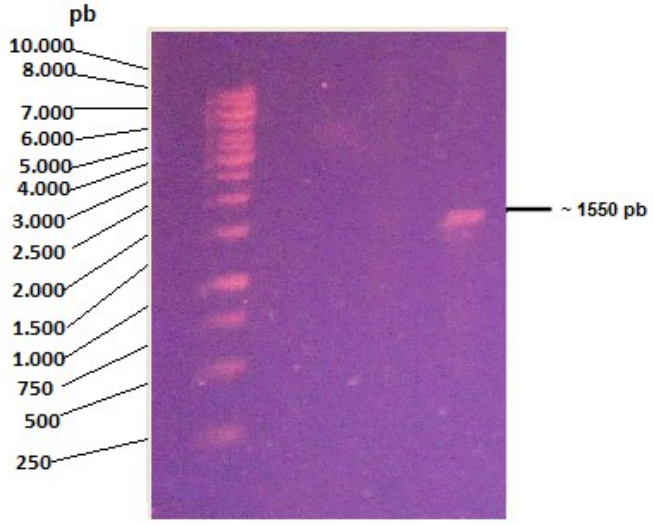

Figure 4. The result of amplification DNA 16sRNA for bacteria 23K.

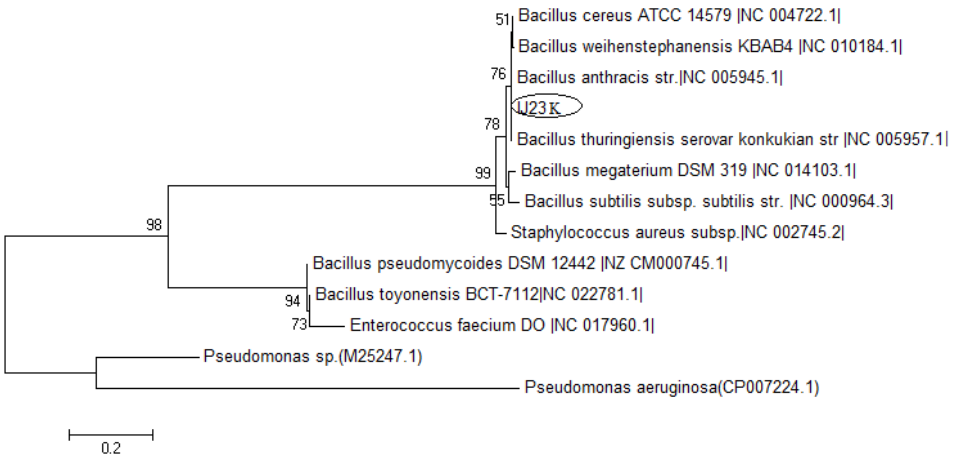

Figure 5. Phylogenetic tree bacteria 23K based on the 16S rRNA gene analysis using Maximum Likelihood method.

The phylogenetic tree shows that *Bacillus thuringiensis serovar konkukian str. 97-27* is one of the bacteria that closest to the Bacteria 23K. The classification of *Bacillus thuringiensis* in the NCBI as follows:

Kingdom : Bacteria
Division : Firmicutes
Class : Bacilli
Ordo : Bacillales
Family : Bacillaceae
Genus : *Bacillus*
Species : *Bacillus thuringiensis serovar konkukian str. 97-27*

Bacillus thuringiensis is one of the millions of soil bacteria with pathogen characteristics for insects (Hatmanti 2000). Toxic compounds for insects from *Bacillus thuringiensis* are specific to insect pests so they are harmless to other organisms and safe for humans (El-kersh et al. 2012). The growth temperature for these bacteria is between 15^{o}C - 40^{o}C with an optimum pH of 6.5 - 7.5 (Bernhard & Utz 1993). *Bacillus thuringiensis* undergoes is optimum growth at 3-30 hours from the onset of inoculation and at the 30th hour undergoes a static phase and decreases (Darwis et al. 2004).

CONCLUSION

Based on the results and discussion, it can be concluded that Bacteria 23K is the most significant bacteria that can increase the growth of mushrooms than other isolates in the mycelium phase in vitro. Based on the results of phylogenetic tree analysis, the 23K bacterial isolates have the closest to *Bacillus thuringiensis serovar konkukian str.* 97-27. It is necessary to test the generative phase of mushrooms with bacteria 23K in the controlled clusters of temperature and humidity.

AUTHORS CONTRIBUTION

I.J.S and I.N.P.A designed the research and supervised all the process, I.J.S and I.N.P.A collected and analyzed the data and wrote the manuscript.

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CONFLICT OF INTEREST

The authors don't have a conflict of interest.

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Research Article

Antifungal Activity of Bacterial Isolates from Straw Mushroom Cultivation Medium against Phytopathogenic Fungi

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ABSTRACT

Several bacteria were isolated from straw mushroom (*Volvariella volvacea*) cultivation medium. There were three potential isolates previously characterized and had a growth inhibition effect against *V. volvacea*. This screening result leads to further study of the inhibition activity against phytopathogenic fungi. This research aimed to investigate the antifungal activity of three bacterial isolates against three phytopathogenic fungi and identification of the bacteria. The methods used in this study were antifungal assay using co-culture method and disk diffusion assay using the filtrate of each bacteria. The profile of the antifungal compound was identified using ethyl acetate extract followed by evaporation and gas chromatography (GC-MS) analysis. Identification of each isolate was performed using 16S rDNA amplification and sequencing. Three phytopathogenic fungi *i.e Cercospora lactucae* (InaCC F168), *Colletotrichum gloeosporioides* (InaCC F304), and *Fusarium oxysporum* f.sp. *cubense* (F817) were co-cultured with bacterial isolates C2.2, C3.8, and D3.3. The C3.8 isolate has the highest average inhibition activity either using isolate and filtrate. The result is relatively consistent against three phytopathogenic fungi. The metabolite profile of the C3.8 isolate showed the Bis(2-ethylhexyl) phthalate as the main compound with 97% similarity. Bis(2-ethylhexyl) phthalate had a potential effect as an antibacterial and antifungal compound. According to EzBioCloud and GeneBank databases, the C2.2 isolate was identified as *Bacillus tequilensis*, C3.8 as *Bacillus siamensis*, and D3.3 as *Bacillus subtilis subsp. subtilis*. This study also showed the potential of *Bacillus siamensis* C3.8 as biocontrol against phytopathogenic fungi.

Keywords: antifungal, biocontrol, plant pathogen, bioactive compound, identification

INTRODUCTION

Mushroom cultivation is a process to grow fungus in the artificial cultivation medium to produce fruiting bodies. The main process is based on the solid fermentation of several substrates under controlled conditions. The bacteria and fungi have the major roles in converting raw materials into ready-to-use substrates, minimizing the contaminants, and inducing the development of fruiting bodies (Kertesz & Thai 2018; McGee 2018; Vieira & Pecchia 2018). The microbes present in the cultivation medium strongly influence the

fungal growth and the development of fruiting bodies (Carrasco & Preston 2020). The varieties of bacteria-fungal interaction in mushroom cultivation have been described as either positive or negative for the fungal growth, depend on the bacterial characteristics and the growth stage of the fungus (Frey-Klett et al. 2011)

The beneficial microbes in the mushroom cultivation medium can promote mycelial growth even increasing the yield of fruiting bodies. *Bacillus cereus* W34 previously reported has a growth-promoting ability and increases the yield of fruiting bodies in straw mushroom cultivation (Jemsi & Aryantha 2017). Several other bacteria from genera *Alcaligenes, Lysinibacillus, Paenibacillus, Pandorea, Pseudomonas*, *and Streptomyces* also were reported as potential mushroom growth-promoting bacteria (Xiang et al. 2017). However, several bacteria also have a detrimental effect on cultivated mushrooms. Some *Pseudomonas* species are the causal agents of blotch diseases in *Agaricus bisporus* fruiting body, which decrease the mushroom productivity. Those detrimental effects are depending on the fungal developmental stages (Frey-Klett et al. 2011).

Several bacteria and actinobacteria have the inhibition activity against fungi. *Streptomyces* is one of the common actinobacteria which produce antifungal compounds against plant pathogenic fungi, whether it is isolated from agricultural soil, desert soil, or marine sediment (Audinah & Ilmi 2019; Smaoui et al. 2012; Usha Nandhini & Masilamani Selvam 2013). *Bacillus subtilis* also was reported to have antifungal activity against several plant pathogenic fungi such as *Alternaria*, *Fusarium,* and *Colletotrichum* species by producing hydrolytic enzyme and antimicrobial peptides (AMPs) *i.e* iturin, bacillomycin, fengycin, surfactin, and mycosubtilin (Desmyttere et al. 2019; Mardanova et al. 2017).

According to previous research, 25 bacterial isolates from straw mushroom cultivation medium were screened for antifungal activity. The C3.8 has the highest inhibition activity in-vitro against *Volvariella volvacea* followed by C2.2 and D3.3 respectively (Masrukhin & Saskiawan 2020). As the prospect for future application, these three selected bacterial isolates will be tested against phytopathogenic fungi that causing major disease in Indonesia's important horticultural crops. Several major fungal diseases such as anthracnose in chili, leaf spots in cabbages and lettuce, and Panama disease (*Fusarium* wilt) in banana. *Cercospora lactucae* is the causal agent of cercospora leave spot disease in the lettuce which has wide geographic distribution (Nguanhom et al. 2015). *Colletotrichum gloeosporioides* is the major fungal pathogen in pepper which causing anthracnose disease in several important crops such as chili (*Capsium* spp.), black pepper (*Piper nigrum*), and grapefruit (*Citrus paradisi*) (Kurian et al. 2008; Than et al. 2008; Cruz-Lagunas et al. 2020). The third pathogen is *Fusarium oxysporum* f.sp. *cubense* that causing Panama disease, the most detrimental disease in banana (Dita et al. 2018). Therefore, this research aimed to characterize the antifungal activity of these three bacterial isolates from straw mushroom cultivation medium against plant pathogenic fungi *Cercospora lactucae* (InaCC F168), *Colletotrichum gloeosporioides* (InaCC F304), and *Fusarium oxysporum* f.sp. *cubense* (F817). In addition, we also conducted a profiling of its bioactive compound and molecular identification of the bacteria using 16S rDNA.

MATERIALS AND METHODS

Materials

The bacterial isolates used in this study were previously screened from 26 bacterial isolates isolated from *Volvariella volvacea* cultivation medium. There are three potential isolates that have growth inhibition activity against

Volvariella volvacea i.e C2.2, C.38, and D3.3. The three phytopathogenic fungi used in this study were collected from Indonesia Culture Collection (InaCC) fungal collection *i.e Cercospora lactucae* (InaCC F168), *Colletotrichum gloeosporioides* (InaCC F304), and *Fusarium oxysporum f.sp. cubense* (InaCC F817).

Methods

Antagonism Assay against Phytopathogenic Fungi

Antagonism assay was performed according to Oh and Lim (2018) with few modifications. The bacterial isolates were co-cultured with phytopathogenic fungi in Potato Dextrose Agar (PDA) medium. The phytopathogenic fungi were grown in PDA medium prior to antagonism assay and incubated at 30° C for 5 days. The phytopathogenic fungi were taken using cork borer 5 and placed in an 80 mm Petri dish containing PDA medium. Bacterial isolates were inoculated onto PDA-containing phytopathogenic fungi by streaking with a sterile 1 µL inoculating loop along a 30 mm line with a 20 mm distance. The radial growth of mycelium was measured using ImageJ (Schneider et al. 2012) and compared with the control treatment (without bacterial isolates). The mycelial growth inhibition was measured using the formula as follows:

$$Fungal\ growth\ inhibition = \frac{Rc - Ri}{Rc} X\ 100\%$$

Rc = Mycelial growth of control (phytopathogenic fungi without bacterial inoculation)
Ri = Mycelial growth of phytopathogenic fungi co-cultured with bacteria (Narayanasamy 2013).

Antagonism Assay Using Filtrate of Potential Isolates

Bacterial isolates were grown in Nutrient Broth (NB) medium and incubated for 2x24 hours to obtain the optimal growth for bacteria. The bacterial suspension was then centrifuged at 14000 rpm at 4°C temperature for 10 minutes and filtered using cellulose acetate membrane 0.2 µm. Antagonism assay was performed with a similar method and substitute the bacterial isolates with 6 mm sterile paper disk- containing 25 µL filtrate. The mycelial growth was measured using ImageJ software and growth inhibition was calculated similarly as above.

Data Analysis

Data was collected from the antagonism assay and calculated using Ms. Excel. Statistical analysis of the percentage of inhibition was calculated using ANOVA single factor and continued using LSD (least significant differences) with a 5% level of significance (α= 0.05).

Profiling of Antifungal Compound

The bacterial isolates were grown in Luria Bertani Broth and incubated for 48 hours. The bacterial suspension then was centrifuged at 14000 rpm for 10 minutes to precipitate bacterial cells. The supernatant was taken and syringe-filtered through a 0.2 µm membrane filter to make sure there were no bacterial cells were involved. Extraction of the potential antifungal compound was performed three times using ethyl acetate 1:1 (V/V) and shook vigorously at 120 rpm for two hours. Ethyl acetate was then evaporated using a rotary evaporator at 40°C. About 15 mg of evaporated samples were dissolved in 1 mL ethyl acetate. The concentrated samples were then analyzed for their metabolite profile using gas chromatography-mass spectrophotometry GCMS-QP 2010 Ultra (Shimadzu- Japan) with Rtx-5MS

column. The mass spectra of the compound then were compared with the National Institute of Standards and Technology database version 11 (NIST 11).

Molecular Identification of Potential Isolates

Identification of bacterial isolates was conducted by amplification of 16S rRNA using universal primer 27F/1492R (Jiang et al. 2006). Total Genomic DNA was extracted using a boiling method at 80°C for 10 minutes and was precipitated using DNA spin for 5 minutes. As much as 2-3 µL DNA genomic DNA was used as a DNA template for Polymerase Chain Reaction (PCR) amplification. DNA sequencing was conducted using Sanger sequencing through an outsourced sequencing service laboratory. The sequences obtained were analyzed using ChromasPro (Technylesium- AU) for quality checking and trimming process. The processed DNA sequences used for identification through BLAST-N in Genebank with restriction is set on sequences from type material (Altschul et al. 1997) and 16S-based ID in EzBioCloud (Yoon et al. 2017).

RESULTS AND DISCUSSION

Identification of the potential isolates

The identification was performed using two online databases *i.e* Genebank and EzBiocloud. The usage of the GeneBank database because GeneBank contains a huge number of 16s rDNA sequences, however, the status of the strain sequences in GeneBank is often not known (Christensen & Olsen 2018). Therefore, the EzBiocloud database was used as complementary and confirmation for all sequences previously identified using GeneBank. As mentioned by Yoon et al. (2017) the EzBiocloud contains quality controlled 16s rDNA sequences and genomes of type strain bacteria and archaea.

The identification result (table 1) shows that two online databases generate similar for 16S-based identification with close similarity. Isolate C2.2 was identified as *Bacillus tequilensis*, C3.8 as *Bacillus siamensis*, and D3.3 as *Bacillus subtilis subsp. subtilis*. The usage of two or more online databases including GeneBank is recommended for 16s RDNA identification because the interpretation of 16S rDNA sequences depends on the program used by the database provider (Park et al. 2012).

Co-culture of potential isolates with phytopathogenic fungi

Co-culture of potential isolates was conducted as the first antagonism assay against phytopathogenic fungi. All isolates had an inhibition activity against *Cercospora lactucae* (InaCC F168). However, when it was conducted against

Table 1. Identification of three potential isolates based on Genebank and 16S-based ID- EzBioCloud.

Isolate code	GeneBank (NCBI)			EzBiolab (ChunLab)		
	Identification	Similarity	Accession	Identification	Similarity	Accession
C2.2	*Bacillus tequilensis* strain KCTC 13622	99.57	MN543830.1	*Bacillus tequilensis*	99.64	AY-TO01000043
C3.8	*Bacillus siamensis* KCTC 13613	99.71	KT781674.1	*Bacillus siamensis*	99.64	AJVF01000043
D3.3	*Bacillus subtilis subsp. subtilis* Str 168	99.42	CP053102.1	*Bacillus subtilis subsp. subtilis*	99.64	ABQL01000001

Colletotrichum gloeosporioides (InaCC F304) and *Fusarium oxysporum f.sp. cubense* (InaCC F817) only *Bacillus siamensis* C3.8 which significantly inhibited InaCC F304 (Figure 1).

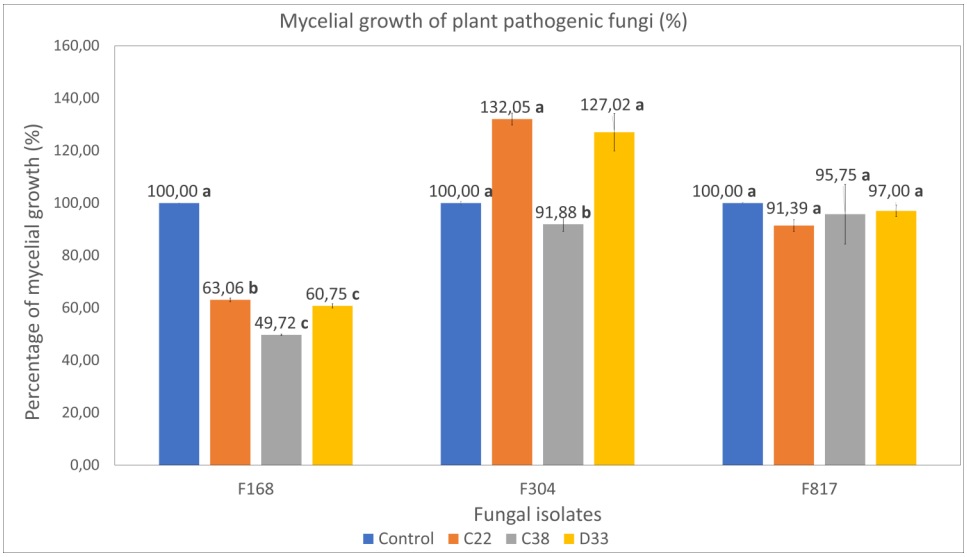

Figure 1. Mycelial growth of plant pathogenic fungi co-cultured with bacterial isolates. Bacteria: C2.2 (*Bacillus tequilensis*), C3.8 (*Bacillus siamensis*), D3.3 (*Bacillus subtilis subsp. subtilis*). Fungi: F168 (*Cercospora lactucae*), F304 (*Colletotrichum gloeosporioides*), and F817 (*Fusarium oxysporum* f.sp. *cubense*).

Many biological control agents have been developed through the screening of potential microbial isolates either from prokaryotes such as bacteria and actinobacteria or eukaryotes such as yeast and fungi. The screening of antifungal compounds can be performed through the co-culture method. This method is applied under the presumption that microbes interact with each other in a natural environment and compete for space and resources (Li et al. 2020; Oh & Lim 2018). The co-culture method can be complemented with the disk diffusion method to determine the active antifungal compound. The disk diffusion assay shows *Bacillus siamensis* C3.8 has the highest average inhibition activity among three isolates, either applied as whole isolates or filtrate followed by C2.2 and D3.3 respectively. However, in the antagonism assay using filtrate, the average is not significantly different among the three isolates tested (Figure 2).

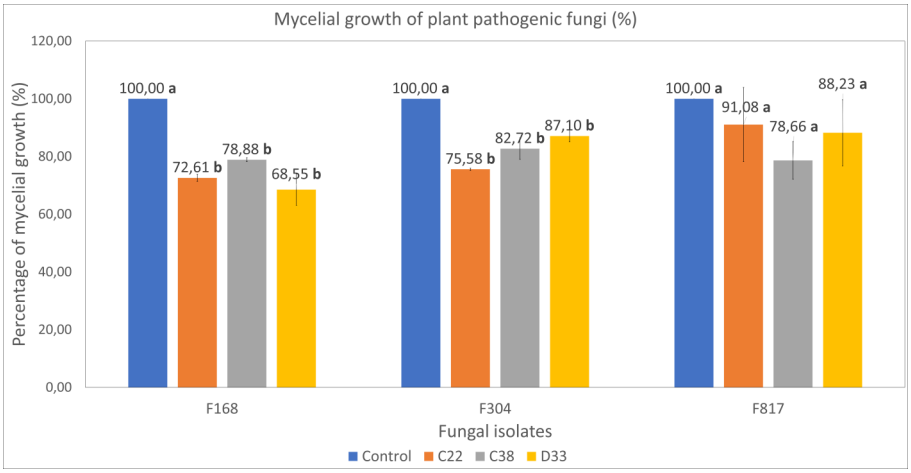

Figure 2. Mycelial growth of plant pathogenic fungi co-cultured with bacterial filtrate. Bacteria: C2.2 (*Bacillus tequilensis*), C3.8 (*Bacillus siamensis*), D3.3 (*Bacillus subtilis subsp. subtilis*). Fungi: F168 (*Cercospora lactucae*), F304 (*Colletotrichum gloeosporioides*), and F817 (*Fusarium oxysporum* f.sp. *cubense*).

Bacillus species are known as producers of a wide array of antagonistic compounds against other bacteria, fungi even viruses. Generally, the most important bioactive molecules are from non-ribosomal peptides, lipopeptide, polyketide compounds, bacteriocins, and siderophores (Fira et al. 2018). In this research, *B. siamensis* C3.8 has the highest and stable antifungal activity against three phytopathogenic fungi among three selected isolates. It is also supported by previous research that C.38 has the highest inhibition activity against *Volvariella volvacea* mycelial growth (Masrukhin & Saskiawan 2020). Previously Zhang et al. (2020) reported that *Bacillus siamensis* was able to inhibit *Botrytis cinerea* and *Rhizopus stolonifer* by producing volatile organic compounds (VOC) 2, 6-di-*tert*-butyl-4-methylphenol (BHT), and 2,4-di-*tert*-butylphenol (2,4-DTBP). Other *Bacillus* species, such as *B. amyloliquefaciens*, *B. tequilensis,* and *B. subtilis* were reported also have antagonism activity against *Candida albicans Magnaporthe oryzae* and *Penicillium roqueforti* by producing cyclic lipopeptide 6-2, iturin-like compound (Chitarra et al. 2003; Li et al. 2018; Song et al. 2013).

Profiling of active compound

Profiling of bioactive compounds showed that there were 16 active compounds detected in *B. siamensis* C3.8 with 91- 97 % similarity (data was not shown). However, there were four major bioactive compounds with the highest percentage of peak area. Bis(2-ethylhexyl) phthalate was the main major compound detected followed by 1-Heptacosanol, 1-Nonadecene, and E-15-Heptadecenal respectively (Table 2). Those bioactive compounds were previously described as antimicrobe and antifungal compounds. However, it needs further purification and assays to confirm that those bioactive compounds were responsible for *B. siamensis* C3.8 antifungal activity.

Bis(2-ethylhexyl) phthalate is an ester of phthalic acid which was widely used as a plasticizer in many materials. This compound is mostly considered as a pollutant due to the persistent characteristic and often found in the environment as the effect of extensive usage (Ortiz & Sansinenea 2018). Instead of environmental pollution, several researches have shown that Bis(2-ethylhexyl) phthalate is produced by microorganisms such as *Bacillus subtilis*, *Aspergillus awamori,* and crown flower (*Calotropis gigantea*). The bis(2-ethylhexyl phthalate has antimicrobe and antifungal characteristic against bacteria such as *Bacillus subtilis, Escherichia coli, Sarcina lutea, Shigella dysenteriae, Shigella sonnei, Staphylococcus aureus,* and *Aspergillus flavus* fungus (Habib & Karim 2009; M. M. Lotfy et al. 2018; W. A. Lotfy et al. 2018). According to this research, the isolate *B. siamensis* C.38 could produce Bis(2-ethylhexyl) phthalate which previously known has antifungal activity. This isolate is the potential to be applied as a biocontrol agent against phytopathogenic fungi, however, the other characteristics and the mode of action should be further studied.

Table 2. GC-MS profile of major active compound identified in *B. siamensis* C.38.

No	Retention time	% Area	Identified compound	Similarity (%)	Formula	Function
1	17.154	6.24	E-15-Heptadecenal	96	C17H32O	Antimicrobe (Abdel-Wahab et al. 2017)
2	18.634	9.02	1-Nonadecene	96	C19H38	Antimicrobe and Antifungal (Smaoui et al. 2012)
3	21.209	5.33	1-Heptacosanol	94	C27H56O	Antimicrobe (Chowdhary & Kaushik 2019)
4	22.337	22.39	Bis(2-ethylhexyl) phthalate	97	C24H38O4	Antimicrobe (M. M. Lotfy et al. 2018; W. A. Lotfy et al. 2018). Antifungal and antibacterial (Ortiz & Sansinenea 2018)

CONCLUSION

All bacterial isolates were identified through two online databases *i.e* Genebank and EzBioCloud. The identification result showed that C2.2 was identified as *B. tequilensis*, C3.8 as *B. siamensis,* and D3.3 as *B. subtilis subsp. subtilis*. All isolates had antifungal activity against *C. lactucae* (InaCC F168), *C. gloeosporides* (InaCC F304), and *F. oxysporum* f.sp. *cubense* (InaCC F817). The *B. siamensis* C3.8 had the highest and stable antifungal activity among three bacterial isolates. The Bioactive compound profile showed that Bis(2-ethylhexyl) phthalate was the main major compound detected in *B. siamensis* C3.8 and needs further purification and assays to confirm that this compound is responsible for the antifungal activity of C3.8.

AUTHORS CONTRIBUTION

All authors have reviewed the final version of the manuscript and approved it for publication. M and ALP were designed the study; M, ALP, IP, and MYN performed research and collected the data; M, ALP, TRS, MI, IP, IS and MYN analysed the data and wrote the paper. M and ALP are the main contributor of this manuscript.

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CONFLICT OF INTEREST

The authors state no conflict of interest from this manuscript.

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Research Article

Update on New Species and Record of Fishes in the Coral Triangle Region for the Last 10 Years (2008-2019)

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ABSTRACT
Updated data is an essential requirement for carrying out research, planning, and policy briefs. The Coral reef triangle region is one of the areas with the highest diversity of marine biota and the discovery of new species in this area are increasing every year, much of this information is already available. However, most of the data is not available per region and is still scattered. This study aims to create a checklist and assessment of new species and a new record of fishes from this region over the last ten years based on several aspects, including species composition, pattern of distribution, endemicity, and depth using every source of the report and secondary literature data. The current new species and a new record of fish in the last decades combined consists of 360 species (268 new species and 92 new records). The most speciose group of family dominated by Gobiidae (93), followed by Labridae, Pomacentridae and Serranidae (18), Apogonidae (17), Dasyatidae (15), and the rest were ranged from 1-9 species per family. More than half of new species and new records are found in Indonesia, followed by the Philippines, Papua New Guinea, and Solomon Island. The result shows that cryptobenthic Families especially Gobiidae from genus Trimma and eviota are dominated the trend of new species and new record discovery and it is expected to rise over time while there will also be an emergence of some possibly new endemic species from major and rare families from the eastern part of Indonesia (West Papua and Papua New Guinea). Thus, the eastern part of Indonesia (Papua, Maluku, Aru Sea, and Papua New Guinea) and the northern part of Indonesia (North Sulawesi and Philippine) are suitable for exploration for marine biodiversity discovery research in the future.

Keywords: composition, coral triangle area, distribution, fish, new record species

INTRODUCTION
The availability of updated data is an essential requirement in conducted research and planning policy briefs. The Coral Triangle area is one of the locations with the highest diversity of marine biota including fishes in the world. Currently, there are 2600 species of reef fishes and it is estimated that there will be more (Tornabene et al. 2015). The coral triangle region covers 37% of the total species of reef fishes in the world and the highest concentration is located in the eastern part of Indonesia to the Philippines (Allen 2007). This finding is in line with Halas & Winterbottom (2009) who explained that the position between West Sumatera to East Papua New Guinea and North Philippines is the area with the highest marine biodiversity

of fishes. Every year the discovery and record of new species of fishes in the coral triangle region especially Indonesia continue to increase in the form of journal publications. Despite categorized as new information, the knowledge about these species is still considered limited and as data collection and updates are rarely displayed in broad outline, making it difficult to find relevant information with all new species and records based out of the region to see the big picture of the current status and other information regarding this issue.

Some aspect of the outline of reef fish biodiversity has been discussed by (Allen 2007; Allen & Werner 2002; Eschmeyer et al. 2010) however this reported about 10 years ago and did not discuss details about new species, meanwhile (Drew & Amatangelo 2017) has addressed the diversity of reef fishes but still limited to the Melanesian region including Solomon Island, Vanuatu, Fiji, and Hawaii, this emerges the need of comprehensive data that provide essential information of new species and record of fishes data that can be beneficial for researchers, students, and stakeholders to support further research and policy. The purpose of this research is to create a checklist and assessment based on comprehensive data and outline related to the discovery progress of new species in the coral triangle area as a marine hotspot for biodiversity of marine biota in addition to information on new records of fishes for the last ten years.

MATERIALS AND METHODS

The scope of data is restricted to the year 2008 to the early half of 2019. Data collection was conducted using secondary literature study methods from FishBase (Froese & Pauly 2019), Eschmeyers Catalog of Fishes (Eschmeyers & Vander Laan 2019), and other related scientific publications and books. Data then compiled in the form of a checklist which includes ordo, families, genera, species name, country, discovery area, depth, year found, year of publication, status, collector, gear, and referral journal. The status in the checklist is a group in two terms which are new species and new record category, the new species is categorized as a species that newly discovered and described or re-described by taxonomist as a new species in the world or novel species based on the location of the holotype (main specimen for description) for the last 10 years. Meanwhile, the term new record refers to species that already being described by taxonomist, distributed and reported in some area outside of the coral triangle area before but recently found and reported in a new region for the first time in the last ten years (in this case coral triangle region) and/ or use as a paratype (supporting specimen). Thus, new species can also be categorized as a new record based on new species reported in some areas. However, because some of the species included in new records are not novel species in the last 10 years and already described before, and most publications conclude the term new species and new records as separate terms, then we group it in two different categories. Based on the data compiled, the point location maps and distribution were asses using QGIS software.

RESULTS AND DISCUSSION

Biodiversity and composition of fishes

According to data on new species and records of fishes for the last decade compiled in this study, the addition of species and new records is quite high for an area. This finding indicates that there is still a potential for high biodiversity that still needs to be explored. The proportion of combination of new species and new records fish found in each country which part of Coral Triangle Area (Indonesia, Philippines, Papua New Guinea, and Solomon

a. b.

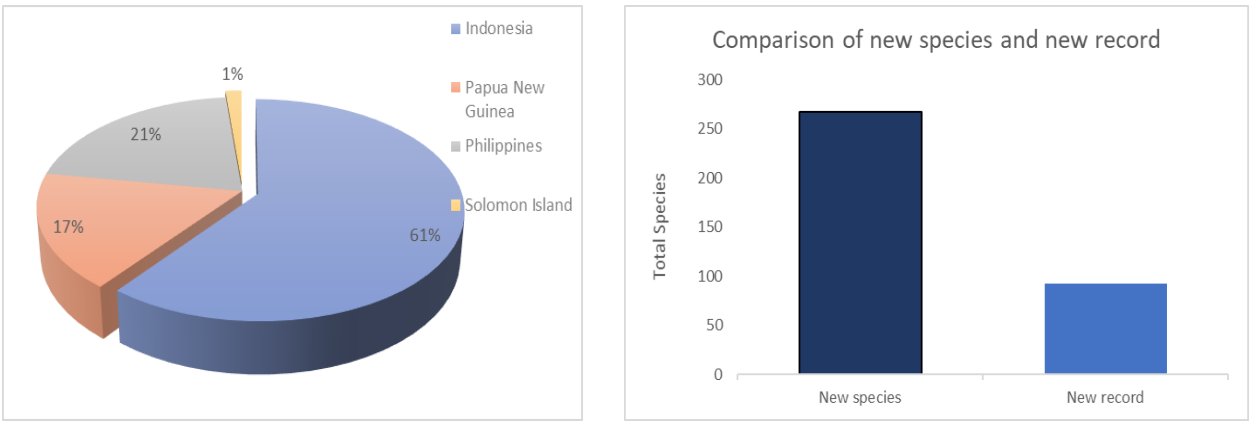

Figure 1. a. The proportion of new species and new records found in each country b. Comparison of new species and new records in the Coral Triangle Area.

Islands) are shown in Figure 1a. Figure 1b highlights the comparison of new species and a new record of fishes in the coral triangle area.

Based on the result of data for the last 10 years (mid-2008 to early 2019), a total number of 360 species which are the combination of new species and new record fishes were obtained consist of 31 orders, 80 families, and 170 genera from 4 countries which include Indonesia with 206 species, Papua New Guinea (58), Philippines (70) and Solomon Island (5) (Figure 1). The rest 21 species are the combined results of several countries in the coral triangle area with similar specimens of species in the form of paratypes based on observations during literature studies. For example, a new species *Parascombrops glossodon* is described based on holotype (main specimen for description) in Philippines. However, this species is also distributed in Indonesia and collected as a paratype (supporting specimen for reference). *Trimma maiandros* is a new record reported from the coral triangle region (Indonesia, Phillipines, Papua New Guinea, and Solomon Island) from previously reported in Japan, Australia, and Samoa (Hoese et al. 2011). Two hundred sixty-eight species are included in the new species category and 92 species are in the new record category (Figure 1b). The term of new record defines a species found in a new region that indicates a wider distribution record from previous reports. This could be caused by several factors including some species are described as new species only based on one or several specimens and as it is considered new there is limited knowledge about this particular species distribution (Peristiwady 2011) and limited records of monitoring and migration of fishes due to climate change (Gamito et al. 2016). In addition, the collection of new records data also have an important role in establishing a database, even though update of fishes record in monitoring results such as checklists and scientific journal publications are not always included in databases such as Fishbase, Catalog of Fishes and other.

From the total species and new records of fishes sorted by countries in coral triangle region, Indonesia contributed a total number of 57% represented by new species, new record in order (151, 55) followed by Philippines 20% (59,11), Papua New Guinea 16% (48,10) whereas Solomon Island contributed only 1% (5) and the rest 6% is the combination of some of these countries (1, 20). The trend of the total species addition is positively correlated with the total area of coral reefs and the total number of fishes that have been previously reported. Indonesia with an area of coral reefs reaching approximately 51.020 km2 has the highest total number of fish

species in the coral reef triangle region with a recorded number of about 2200 species, whereas Philippines reported consist of 25,060 km² coral reefs area and 1790 reef fishes species, Papua New Guinea consist of 13,840 km² with 1635 reef fishes species and Solomon Island with an area of coral reef about 5750 km² and recorded 1371 reef fishes species (Allen 2007). Areas with centers of diversity of coral reef fishes are located from Eastern Indonesia to Philippines, we highlighted Papua island in Indonesia as the region that contributes the most diversity of new species fishes, on a small part of the island alone such as the Birds Head Peninsula record approximately 1511 species and the data continues to increase (Allen & Erdmann 2009).

Figure 2 shows fish family composition based on the publication year (2008-2019) fluctuates and does not indicate an up or down trend. However, consistently the Gobiidae family is the most dominant with a total number of 93 species, followed by Labridae, Pomacentridae and Serranidae (18), Apogonidae (17), Dasyatidae (15), and the rest are ranges from 1-9 species per family. Gobiidae is a family with the most abundant number of species and during the last 10 years, Trimma and Eviota are the most common genera described as new species. Individuals from this genus belong to the cryptic type with a size of less than 5 cm and are associated with benthos (Goatley & Brandl 2017). Exploration of these genera can be categorized as new in recent decades by the growing development of scuba-diving equipment for ichthyologists. For example, the Trimma genus consists of only about ten species in 1970 increased to at least up to100 valid species in 2019 (Allen 2019). The number of species of this genus is increasing every year and in recent reports, species from this genera are included in the highly-endemic cryptic species which indicates the trend of species from these genera in the coral reef region will continue to be discovered. (Tornabene et al. 2015), Although this family genus is still largely ignored in the visual census (Brandl et al. 2018) due to its small-sized body, which is difficult to observe in the habitat.

Most Serranidae families are included in the new record status and the majority are recorded from North Sulawesi. This could be due to the condition of the region directly facing the Pacific Ocean and also consistent monitoring is carried out at several fish landing sites (Peristiwady 2011; Peristiwady et al. 2014; Uiblein et al. 2017; Peristiwady et al. 2018) which indicates consistent monitoring has the potential to add many new records of fish in Indonesia and other regions in the coral triangle area. Other families such as Labridae, Pomacentridae, and Apogonidae are families with the most abundance of species diversity in the world consist of 557, 422, and 381 valid species respectively (Eschmeyer & Vander Laan 2019). Most of the new species discovered in the world over the past ten years have been found in the coral triangle region, for example, 18 species out of 45 (world) for Labridae, 18 out of 33 (world) for Pomacentridae, and 18 out of 31 (world) for Apogonidae while most species and new records of Dasyatidae family are recorded from the coral triangle area which is 15 species out of 20 species (world).

The publication year (Figure 2) shows the average number of species added each year ranges from 20-30 species. The least number of species in 2018-2019 due to the data records in some databases are not consistently updated for the latest years. Data collection from secondary sources from journal publications conducted by the author only consists of half the year 2019 which we expected the total of species in 2019 would be increasing in Figure 2. The high number of new species published in 2012 is due to the publication of the book by Allen and Erdmann (2012) that adds up the description of 25 species and several new fish records that are mostly

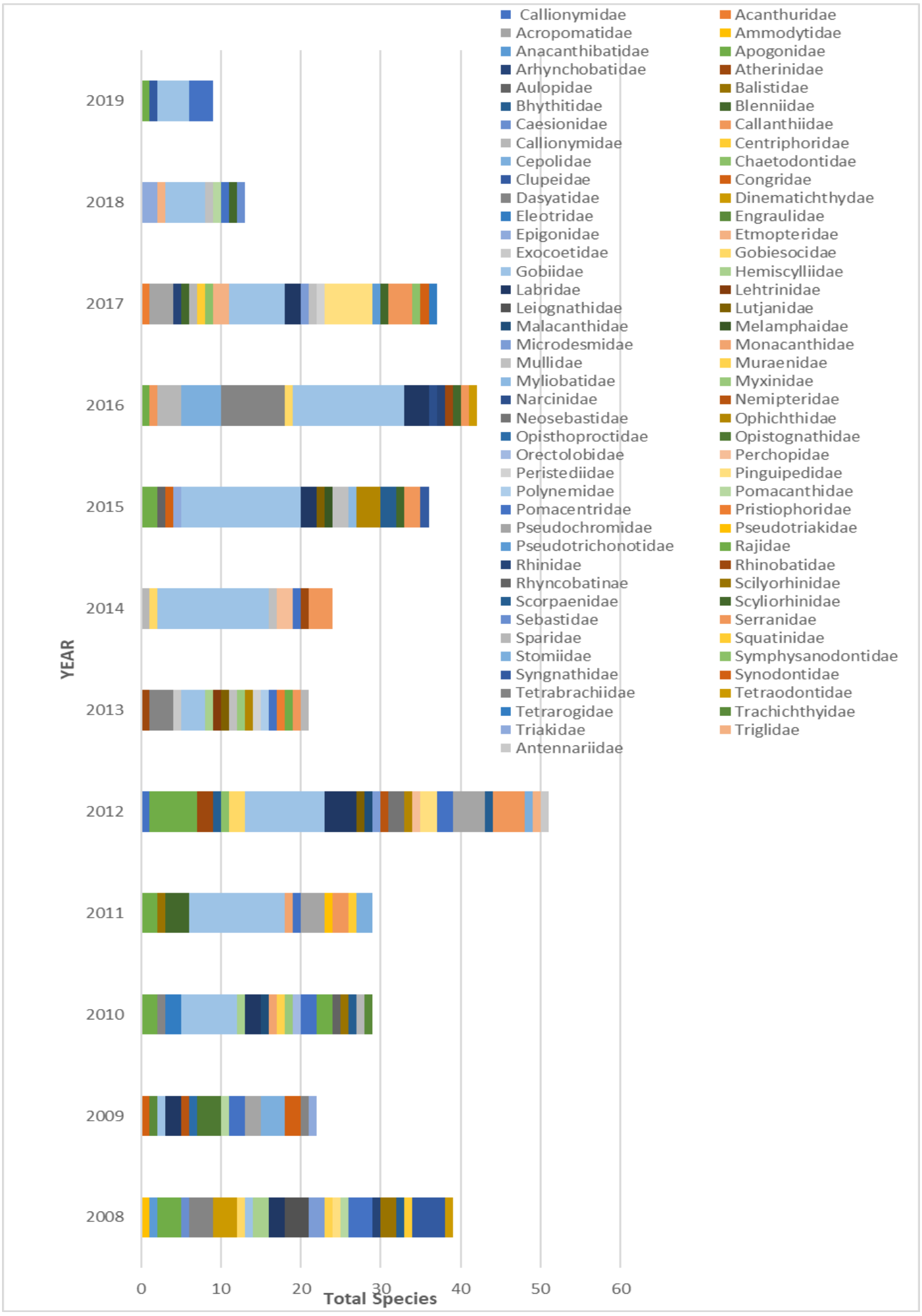

Figure 2. Family composition based on publication year.

originating from countries in the coral reef triangle region.

Distribution of new species and record of new fishes

New species and new records of fishes found in the Coral Triangle also are distributed in several countries and regions other than coral reef triangle areas such as the Southwest Pacific (New Zealand), Indian Ocean (Africa), Eastern Indian Ocean (Australia), and Countries in the Pacific (Japan, Taiwan, China) and South Pacific Ocean (Solomon Island, Vanuatu, Hawaii) (Table 1). This indicated the distribution patterns of some recent fish records referring to fairly wide distribution especially for species with high larval dispersal rate, swimming ability, and no habitat fragmentation (barrier) (Franco et al. 2012). For example some specious fishes from family Gobiidae, Pomacentridae, Serranidae, Labridae, and others which are distributed in almost all region of ocean in the world (Hixon & Randall 2019) in line to the statement by Kulbicki et al. (2013) that this phenomenon caused by extensive dispersal patterns and other evidence of evolution. The pattern of new records of fishes found fits the location and sea area, for example, new records found in the region of Sulawesi, Indonesia distributed fairly in the Pacific Ocean region like Japan (21), Taiwan (10), China (3), and Palau (7) (Burhanuddin et al. 2017; Peristiwady et al. 2014) (Table 1). On the other hand, new records found in the West Papua region and parts of Java and Nusa Tenggara have similarities with species recorded from Australia (12), New Caledonia (8), and other Micronesia region (Vanuatu, Fiji, Samoa, etc) (Last et al. 2006, 2008; Last & White 2008).

Table 1. New species and records of fishes and number of species in coral triangle area which are also distributed in other countries.

Country	Distribution			
	New Species	Number of species	New record	Number of species
Indonesia	Australia	5	Caribbean	1
	India	4	Africa	2
	Japan	2	Australia	12
	Malaysia	3	Japan	21
	Brunei	1	Taiwan	10
	Maldives	1	New Caledonia	8
	Srilanka	1	China	3
	Japan	4	New Zealand	1
	Myanmar	1	Fiji	1
	Vanuatu	2	Maldives	2
	Singapore	3	Samoa	3
	Thailand	2	Palau	7
	Palau	1	Polinesia	1
	China	1	Singapore	1
	Taiwan	1	Somalia	1
	Tanzania	1	India	1
	Africa	1	Oman	1
	Papua New Guinea	18	Pakistan	1
	Philippines	14	Srilanka	1
	Solomon Island	8	Thailand	1
			Brunei	2
			Papua New Guinea	5
			Philippines	4
			Solomon Island	3

Table 1. Contd.

Country	Distribution			
	New Species	Number of species	New record	Number of species
Papua New Guinea	Malaysia	1	Australia	6
	Japan	1	China	1
	Vanuatu	1	France	1
	Indonesia	6	India	1
	Phillipines	2	Japan	2
	Solomon Island	2	Kenya	1
			Korea	1
			Africa	2
			Fiji	2
			Vietnam	1
			Thailand	1
			Fiji	1
			Samoa	1
Philippines	Australia	1	Australia	4
	Brunei	1	Japan	5
	Hawaii	1	China	1
	Malaysia	3	New Caledonia	1
	Japan	3	Taiwan	1
	Nicaragua	1	Vanuatu	3
	Fiji	1	Maldives	1
	French Polynesia	1		
	Vanuatu	1		
	New Caledonia	1		
	Indonesia	6		
	Papua New Guinea	2		
	Solomon Island	1		
Solomon Island	New Caledonia	1		
	Papua New Guinea	1		

The present study indicates that several new species are currently considered endemics. Allen et al. (2018) reported that some species recorded in the last 10 years in the West Papua region, especially Fakfak Peninsula such as *Scorpaenodes bathycolus* (Scorpaenidae), *Chrysiptera giti*, *Pomacentrus fakfakensis* (Pseudochromidae), *Paracheilinus nursalim* (Labridae), and *Pomacentrus bellipictus* (Pomacentridae) are indicated belong to the local microendemic fishes. Some unique species from Hemischylidae or walking sharks consist of only 17 species globally, and 4 of them are described in the last ten years originating from the eastern part of Indonesia (Ternate and Papua). The rest that has been described previously are scattered mostly in West Papua and Papua New Guinea. This is due to their limited swimming and dispersal ability resulting in most species of this genus has a restricted distribution (Allen et al. 2013) and potentially be put in the endemic species category. Family of Pomacentridae such as *Chromis Athena*, *Chromis unipa,* and *Pomacentrus Fakfakensis* are endemic species found only in West Papua, as well as *Pseudochromis ammeri* (Pomacentridae), while some species that are classified as endemic from the Ambon and Merauke regions including *Hemytrigon longicauda* (Dasyatidae) and *Sueviota bryozophila* (Gobiidae), from Philippine

Branchiostegus saitoi (Malacanthidae) 2012 and *Pseudochromis eichleri* (Pseudochromidae) 2012 are some examples of endemic species from that region. It is estimated that more endemic species will be expected, as several species are still being studied and some data are needed before they are included as endemic species.

Figure 3 shows that mostly major families (most abundance species) are spread in almost all countries in the coral reef triangle area, most families

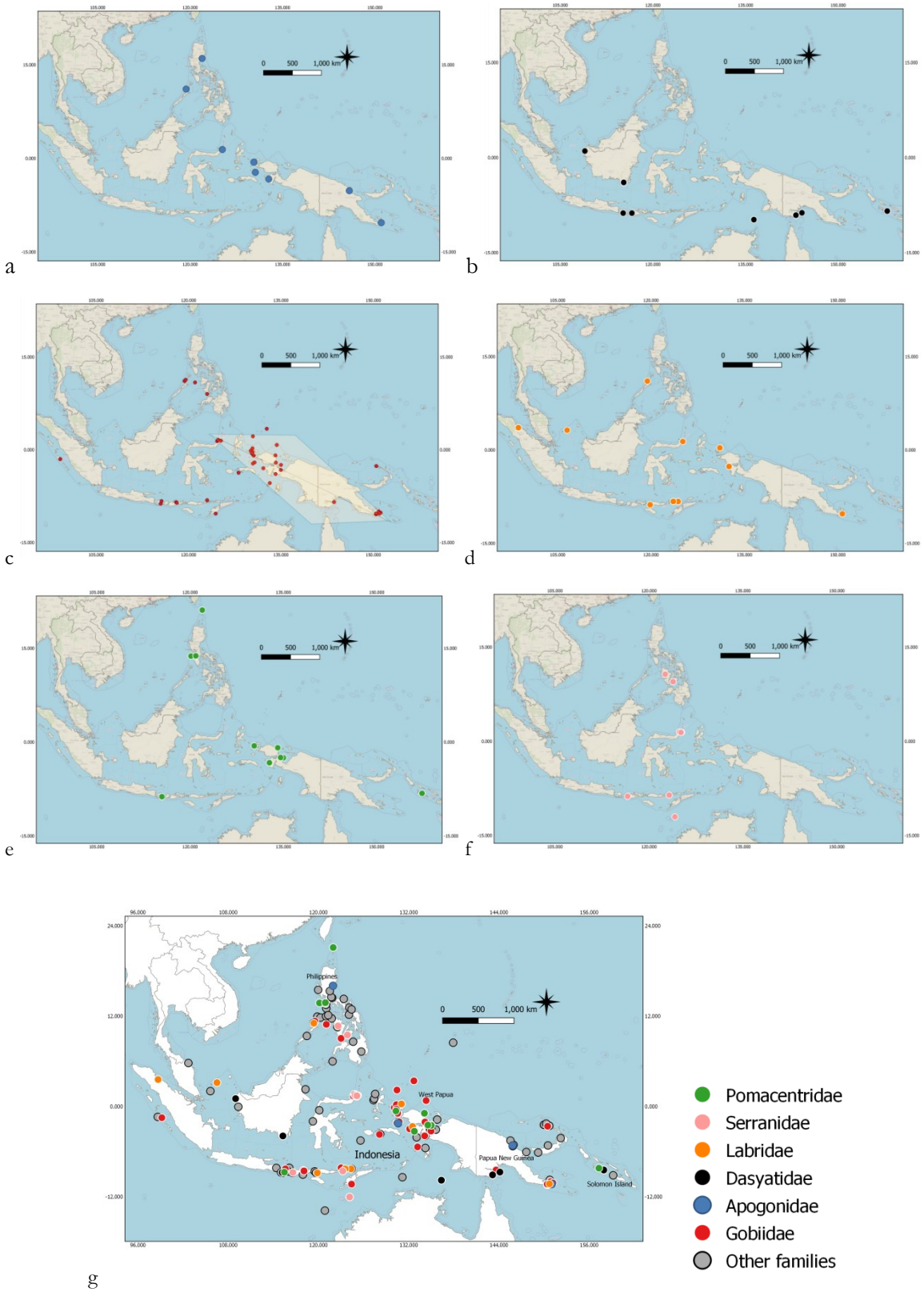

Figure 3. Location points for new species and records of fishes in several major families a. Apogonidae, b. Dasyatidae, c. Gobiidae, d. Labridae, e. Pomacentridae, f, Serranidae, g. Combination of major and the other family.

such as Gobiidae are found in many regions of West Papua, Papua New Guinea towards Sulawesi and Philippines that can be correlated with their habitats which are generally located in coral reef areas, sand, rubble, and seagrass beds due characteristic of the species living patterns that tend to hide from predators (Herler et al. 2011) the existence of species from these families in coral reefs contributes significantly to the transfer of energy to the ecosystems due to their fast life cycle and as the source of energy or food for marine biota, due to some of the properties of these species are specialized in certain types of coral (Herler et al. 2011) it created the possibility of endemic species records from the Gobiidae families is expected to rise over time, while it is also correlated directly to the damage of coral reefs that could threaten the existence of some species in this family that specialize in specific types or species of coral reefs. A total of 15 species from the Dasyatidae or stingray family have a somewhat different location pattern compared to some other families, because these new species are found in Kalimantan, Java and the lower part of the island of Papua (Papua New Guinea and Solomon Island, nonetheless there is no striking difference for families such as Labridae, Serranidae, and Apogonidae which are found in almost all regions in Indonesia, Philippines, Papua New Guinea, and Solomon Island, but in terms of the abundance, the majority of all new species found are located in West Papua province, Indonesia especially locations such as Raja Ampat and Birdhead Peninsula (FakFak), Misool, parts of Maluku and the Aru Sea, North Sulawesi (Lembeh strait and Bunaken), East Nusa Tenggara, and West Nusa Tenggara followed by parts of Papua New Guinea (Milne Bay, Alotau) and Philippine (Palawan, Iloilo), this finding is in line with (Allen 2007) who report a large degree of diversity and also endemicity which lead to conclusion that these area are very potential as a hostspots for studying marine biodiversity, especially reef fishes, and a potential destination to explore existing marine biodiversity for researcher due to the fact that new species and record discovery at this region is consistent every year and continues raise up.

Classification of fishes based on depth

All kind of different species and new records of fishes are found in a different range of depth which was shallow water (0-50m), intermediate (50-200m), and deep water (>200m) but not all are shown in Table 2 due to limited data in the publication journal. In general, the majority of the species are had associated habitats in shallow water and the rest are found in-depth more than 50 m. One of the factors is coastal fish are the most abundant in the world (Eschmeyer et al. 2010). Most species that live in shallow water associated with the reef fish family including Gobiidae, Apogonidae, Blennidae, etc. Species found in-depth range around 50-200 m are classified as a demersal benthopelagic (Mauchline & Gordon 1986) that live in the deep sea and forage in the coral reef ecosystems such as species from Serranidae families, Mullidae, and Dasyatidae. Despite the fact that some other species classified as reef fish and associated with shallow water can be found in these depths such as Pomacentridae and several species of Gobiidae (Prokofiev 2017; Pyle & Earle 2008). Other species that are located at a depth of>200 m are a type that is classified as deep-sea fish including Cepolidae families (William & Johnson 2016), Stomiidae (Flynn & Klepadlo 2012), Trigilidae, and some new records from the Serranidae family including Plectranthias (Peristiwady et al. 2018) from North Sulawesi. Plectranthias is probably a rare collection because most of the valid species of this genus are described only from one or two specimens in the world (Eschmeyer et al. 2010).

Table 2. New species and record of fishes based on depth.

Country	Range of depth		
	0-50 m	50-200 m	>200 m
Indonesia	109	42	14
Papua New Guinea	43	10	12
Philippines	18	24	23
Solomon Island	5	0	0

Collection of specimens from fishes at different depths requires different methods and approaches, from various fishing gear compiled in the checklist, it can be concluded that trawling and hook and line are the majority of fishing gear used to collect deep-sea fish at depths> 200 m, while hook and line, clove oil and collections from the market (fishermen) become a collection method for fish in the depths of 50-200 m, moreover fishes in shallow water are usually collected by hand, hand net, spear anesthetized with clove oil, and rotenone during SCUBA. However, the use of rotenone in recent years has been controversial (Robertson & Smith-Vaniz 2008); hence the use of Clove oil is becoming a more environmentally friendly choice for collecting and anesthetized small fish in the coral reef ecosystem (Robertson & Smith-Vaniz 2010).

CONCLUSION

This study supports the application of the marine biodiversity informatics database to give a comprehensive outline related to the discovery progress of new species in the coral triangle area as a marine hotspot for biodiversity of marine biota in addition to information on new records of fishes for the last ten years. Here author demonstrated analysis from a different type of information that was formed from the data compilation including the composition and distribution of new species and records of fishes. This study found that the trend in the total of species addition is positively correlated with the total area of coral reefs in a particular region or country and the total number of coral fishes previously reported and distributed in that region. The Majority of new species found are located in the eastern part of Indonesia (Papua, Maluku, Aru Sea, and Papua New Guinea) and the northern part of Indonesia (North Sulawesi and Philippine), in addition, the endemic species from these locations (West Papua) is estimated to increase. It is a suitable area for exploration for marine biodiversity research in the future and important to be prioritized as a protected reserve in the coral reef region.

AUTHORS CONTRIBUTION

F.Y., T.P. and P.S.I. collected the data; F.Y. and T.P. analyzed the data; F.Y. wrote the paper; all authors have reviewed the final version of the manuscript and approved it for publication.

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CONFLICT OF INTEREST

The authors state there is no conflict of interest.

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Research Article

Avifaunal Diversity and Community Structure in Universitas Brawijaya Forest, East Java, Indonesia

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ABSTRACT
Avifauna inhabiting the mountainous forest ecosystem is severely threatened by anthropogenic disturbances, especially in the Java island of --Indonesia. Yet, efforts to monitor the avifauna diversity are lacking, including in one of the mountainous forest areas, Universitas Brawijaya Forest (UBF). In this study, information about diversity, community structure, feeding specialization, and conservation status of avifauna is presented. Observations were conducted from December 2019 to February 2020 on two designated tracks with different degree of disturbances. Data were analyzed based on their conservation status, local distribution, feeding specialization (Jaccard similarity index), species richness, total abundance, species diversity (Shannon-Wiener diversity index), and importance value index (IVI). A total of 51 species from 27 families were identified. Two species at risk (one Near Threatened and one Endangered) and 9 protected avifauna were noted. This study can be used as the baseline data for future conservation management in the UBF.

Keywords: biodiversity, diet types, Malang, tropical bird

INTRODUCTION
Avifauna (birds) is one of the animal groups that has a diverse morphology and has been adapted to various landscapes (Coates et al. 2000). Even more, the avifauna can be used as a bioindicator to assess environmental changes (Kurniawan & Arifianto 2017). In this case, the decline of avifauna populations will reflect detrimental changes in the ecosystems (Labe et al. 2018). Several characteristics of avifauna, e.g., feeding specialization, ecological niche, abundance, density, and diversity in populations are considered as the major indicators of environmental change. Thus, avifauna promises good assessment tools to reflect the quality of the environment (Scott 2010).

Currently, there are 494 species of avifauna recorded in Java, 28% of them are confined in mountainous areas (MacKinnon et al. 2010). In this mountainous area, avifauna provides ecosystem services by spreading plant seeds, controlling insect populations, pollinating flowering plants, etc.; while several species act as apex predators (Fjeldsa et al. 2012). However, the habitat of avifauna in mountainous areas has been severely impacted by land

conversion, deforestation, and illegal hunt (Kurniawan & Arifianto 2017; Kementerian Lingkungan Hidup dan Kehutanan 2019; Iskandar et al. 2019). Due to the anthropogenic effects, avifauna conservation is urgently needed.

Universitas Brawijaya Forest (UBF) serves as an education forest under the management of Universitas Brawijaya. These areas span 554 ha with elevations ranging between 800–1200 m asl (Putri et al. 2019), comprising both a tropical and sub-montane zone (Gőltenboth et al. 2006). Due to its location at the intersection of the agroforestry ecosystem of the Arjuno mountain slope, and the conservation area of Raden Soerjo Forest Park, the UBF serves as an important bird and biodiversity area (IBA) (BirdLife International 2020). Prominent threats to the biodiversity in UBF have been reported, e.g., volcanic activity and forest fire (Febriandhika et al. 2019). Yet, the information on avifauna and their community in UBF are unavailable. Therefore, we monitored the avifauna in the UBF to provide the baseline data for avifauna conservation management, based on diversity, conservation status, feeding specialization, and community structure.

MATERIALS AND METHODS

Study area

The research was conducted on the west side of the UBF which administratively belongs to Tawang Argo in Karangploso of East Java. Field observation was carried out on two designated tracks. The starting point of these tracks was around the residential area (7°49'30.47" S; 112°34'43.45" E). Track 1 leads to the highest point of UBF near Raden Soerjo Forest Park area (7°49'14.58" S; 112°34'43.41" E), with an approximate track length of 0.5 km. Whereas Track 2 leads to Mountain Mujur site (7°49'27.62" S; 112° 34'56.31" E) with approximately similar length to Track 1 (Figure 1). Track 2 is adjacent to the main road and had a higher disturbance than Track 1, the latter being far from human activities. The habitats of UBF are dominated by human settlement, coffee plantation, pine forest, and sub-montane forest (Figure 2).

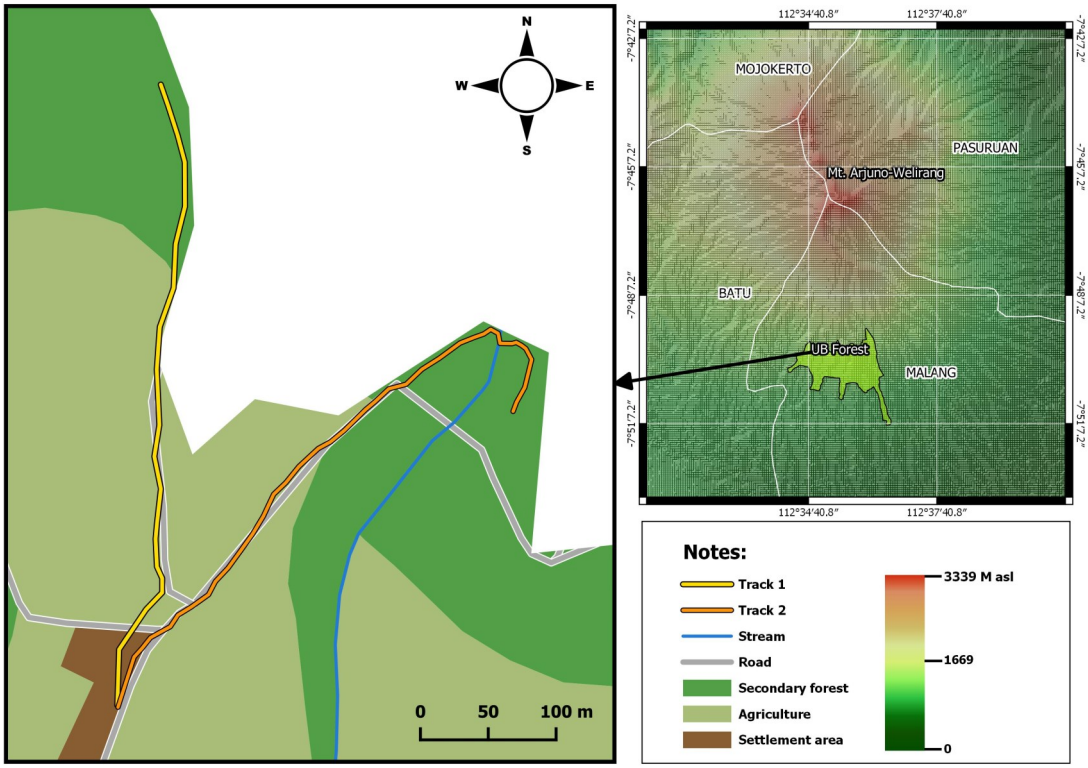

Figure 1. Study sites of avifauna monitoring in the UBF showing the observation track, land use pattern, and elevation.

Figure 2. Representative habitats in the UBF: A) human settlement, B) coffee plantation, C) pine forest, and D) sub-montane forest.

Field observation and species identification

Avifauna observation was conducted in December 2019–February 2020 for two consecutive days every week. The observation was started in the morning (06.00–08.00 am) due to the peak activities of birds (MacKinnon et al. 2010). The line transect method (Buckland et al. 2008) was chosen, then observed by 3–5 observers. The observation was aided by standard equipment, i.e., Binocular 10x50D, camera (NIKON D5200), and telephoto lens (Tamron 150-600 mm). The sounds of the birds were recorded using Sony ICD-PX240. The identification was based on the bird morphology (size, proportion, shape, beak, leg, and color pattern) following several references (MacKinnon et al. 2010; Kurnianto et al. 2013; Prasetya et al. 2018), Burungnesia v.3.0. application (Andriutomo et al. 2020), and sound confirmed by Xenocanto database (https://xeno-canto.org//). The individual encounters, their habitats, and feeding specialization on both tracks were noted, then subjected for further analysis.

Data analysis

The avifauna species were grouped by taxa. The conservation status was based on International Union for Conservation of Nature (IUCN 2020) and national laws under the Permen LHK No.106 (Balai KSDAE 2018). The local distribution categorization (e.g., migrant and resident) of birds was based on Burungnesia v.3.0 (Andriutomo et al. 2020). The feeding specialization of birds was categorized as follows: carnivores, insectivores, frugivores, granivores, and nectarivores (MacKinnon et al. 2010), were clustered using the presence or absence matrix by the Jaccard similarity index through Unweighted Pair Group Method with Arithmetic mean (UPGMA) analyzed on PAST3 software. The data were further analyzed to estimate the species richness, total abundance, Shannon-Wiener diversity index following their respective categorization (Heip et al. 1998), along with the importance value index (IVI) analysis (Misra 1980) to investigate the community structure on both tracks.

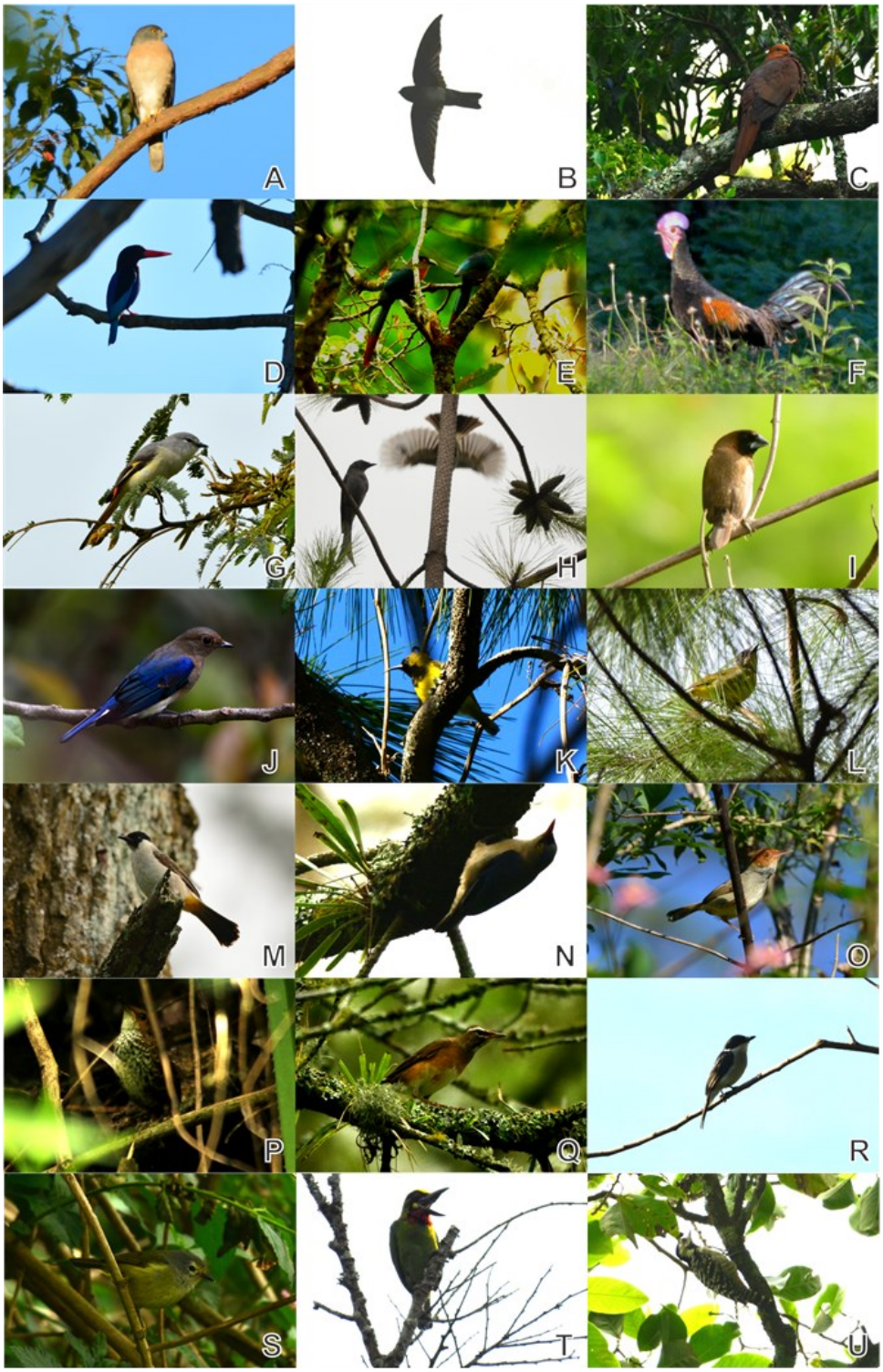

Figure 3. Documentation of representative avifauna species in UBF: A) *A. soloensis* [Accipitridae], B) *C. linchi* [Apodidae], C) *M. emiliana* [Columbidae], D) *H. cyanoventris* [Alcedinidae], E) *P. curvirostris* [Cuculidae], F) *G. varius* [Gallidae] G) *P. cinnamomeus* [Camphephagidae], H) *D. leucophaeus* [Dicruridae], I) *L. leucogastroides* [Estrildidae], J) *C. cyanomelana* [Muscicapidae], K) *C. jugularis* [Nectariniidae], L) *P. fulvocincta* [Pachycephalidae], M) *P. aurigaster* [Pycnonotide], N) *S. frontalis* [Sittidae] O) *O. sepium* [Sylviidae] P) *P. pusilla* [Timaliidae], Q) *T. obscurus* [Turdidae], R) *H. hirundinanceus* [Vangidae], S) *H. javanica* [Zosteropidae], T) *P. javensis* [Capitonidae], U) *D. analis* [Picidae].

RESULTS AND DISCUSSION

Avian Community in UBF

Field observation from both tracks counted a total of 1525 individuals of birds under 51 species from 27 families in UBF (Table 1). This number

represented 27% of 186 species in the mountainous habitat range of East Java (MacKinnon et al. 2010; Prasetya et al. 2018). The representative avifauna documentation is shown in Figure 3.

The IUCN conservation status shows that 49 species were categorized as least concern, one species (i.e., *P. javensis*) categorized as near-threatened and one species (i.e., *N. bartelsii*) categorized as endangered (Table 1). Those two species, along with the other seven species (i.e., *A. soloensis, A. gularis, S. cheela, P. ptilorynchus*, *P. guajana, H. javanica,* and *M. armillaris),* were classified as protected birds according to the national law of Indonesia (Table 1). Based on the local distribution, eight species (i.e., *A. soloensis, A. gularis, P. ptilorynchus, C. saturatus, C. cyanomelana, M. dauurica, F. mugimaki*, and *T. obscurus*) were migratory while the remainders were resident species (Table 1). This migratory avifauna originated from North Asian Peninsula which comes in large flocks temporarily to avoid winter from their native place during November–February cycle (Elphick 2011). The UBF provides an ideal resting place for this migratory avifauna.

Table 1. Checklist of the avifauna in the UBF based on conservation status, local distribution, and feeding specialization. Abbreviation as follows: Conservation status: IUCN Status = least concern (LC), near threatened (NT), endangered (EN); National law status = protected (P), non-protected (NP). Local distribution= migrant (M), resident (R). Feeding specialization= carnivores (C), insectivores (I), granivores (G), frugivores (F), nectarivores (N).

Taxa	Common name	IUCN Status	National law status	Local distribution	Feeding specialization				
					C	I	F	G	N
Accipitridae									
Accipiter soloensis	Chinese sparrowhawk	LC	P	M	√				
A. gularis	Japanese sparrowhawk	LC	P	M	√				
Spilornis cheela	Crested-serpent eagle	LC	P	R	√				
Nisaetus bartelsii	Javan-hawk eagle	EN	P	R	√				
Pernis ptilorynchus	Oriental-honey buzzard	LC	P	M	√				
Apodidae									
Collocalia linchi	Cave swiftlet	LC	NP	R		√			
Hemiprocnidae									
Hemiprocne longipennis	Grey-rumped treeswift	LC	NP	R		√			
Columbidae									
Streptopelia chinensis	Spotted dove	LC	NP	R			√	√	
Macropygia emiliana	Ruddy cuckoo-dove	LC	NP	R			√	√	
M. ruficeps	Little cuckoo-dove	LC	NP	R			√	√	

Table 1. Contd.

Taxa	Common name	IUCN Status	National law status	Local distribution	Feeding specialization				
					C	I	F	G	N
Alcedinidae									
Halcyon cyanoventris	Javan kingfisher	LC	NP	R	√	√			
Todiramphus chloris	Collared kingfisher	LC	NP	R	√	√			
Cuculidae									
Pahenicophaeus curvirostris	Chestnut-breasted malkoha	LC	NP	R		√			
Cuculus saturatus	Himalayan cuckoo	LC	NP	M		√			
Cacomantis merulinus	Plaintive cuckoo	LC	NP	R		√			
C. sepulcralis	Rusty-breasted cuckoo	LC	NP	R		√			
Gallidae									
Gallus varius	Green junglefowl	LC	NP	R	√	√	√	√	
Aegithinidae									
Aegithina tiphia	Common Iora	LC	NP	R		√			
Campephagidae									
Coracina larvata	Sunda cuckooshrike	LC	NP	R		√	√		
Pericrocotus cinnamomeus	Small minivet	LC	NP	R		√			
Cettidae									
Horornis vulcanius	Sunda bush-warbler	LC	NP	R		√			
Dicaeidae									
Dicaeum sanguinolentum	Blood-breasted flowerpacker	LC	NP	R		√			√
Dicruridae									
Dicrurus leucophaeus	Ashy drongo	LC	NP	R		√			
Estrildidae									
Lonchura leucogastroides	Javan munia	LC	NP	R			√	√	
L. punctulate	Scaly-breasted munia	LC	NP	R			√	√	
Locustellidae									
Locustella montis	Sunda grasshopper-warbler	LC	NP	R		√			
Muscicapidae									
Brachypteryx leucophrys	Lesser shortwing	LC	NP	R		√			
Myophonus caruleus	Blue whistling-thrush	LC	NP	R		√			
Enicurus velatus	Sunda forktail	LC	NP	R		√			
Ficedula westermanni	Little-pied flycatcher	LC	NP	R		√			
Cyanoptila cyanomelana	Blue-and-white flycatcher	LC	NP	M		√			
Muscicapa dauurica	Asian-brown flycatcher	LC	NP	M		√			
Ficedula mugimaki	Mugimaki flycatcher	LC	NP	M		√			

Table 1. Contd.

Taxa	Common name	IUCN Status	National law status	Local distribution	Feeding specialization				
					C	I	F	G	N
Nectariniidae									
Cinnyris jugularis	Olive-backed sunbird	LC	NP	R		√			√
Pachycephalidae									
Pachycephalia fulvotincta	Rusty-breasted whistler	LC	NP	R		√			
Pittidae									
Pitta guajana	Javan-banded pitta	LC	P	R		√			
Pcynonotidae									
Ixos virescens	Sunda bulbul	LC	NP	R		√	√		√
Pycnonotus aurigaster	Sooty-headed bulbul	LC	NP	R		√	√		√
P. goiavier	Yellow-vented bulbul	LC	NP	R		√	√		√
Sittidae									
Sitta frontalis	Velvet-fronted nuthatch	LC	NP	R		√			
Sylviidae									
Orthotomus sepium	Olive-backed tailorbird	LC	NP	R		√			
O. sutorius	Common tailorbird	LC	NP	R		√			
Timaliidae									
Pnoepyga pusilla	Pygmy-wren babbler	LC	NP	R		√			
Malacocincla sepiarium	Horsfield's babbler	LC	NP	R		√			
Cyanoderma melanothorax	Crescent-chested babbler	LC	NP	R		√			
Turdidae									
Turdus obscurus	Eye-browed thrust	LC	NP	M		√			
Vangidae									
Hemipus hirundinanceus	Black-winged flycatcher shrike	LC	NP	R		√			
Zosteropidae									
Heleia javanica	Javan Grey-throated White-eye	LC	P	R		√	√	√	√
Capitonidae									
Megalaima armillaris	Flame-fronted barbet	LC	P	R			√	√	
Psilopogon javensis	Black-banded barbet	NT	P	R			√	√	
Picidae									
Dendrocopos analis	Freckle-breasted woodpecker	LC	NP	R		√			

The clustering on feeding specialization reveals as follows: carnivores covered 3 families (1 family is specific), insectivores covered 23 families (16 families are specifics), frugivores covered 7 families, granivores covered 5 families, and nectarivores covered 4 families (Figure 4). The insectivore dominated the niche (along with 7 families that are non-specifics) due to the presence of agroforestry plantation (e.g., coffee plantation and vegetable agroforestry) in UBF which attract insects, providing a nutrition source for avifauna. As for the secondary forest, it provides canopies for avifauna to perch to feed on prey (Poo et al. 2012). Moreover, UBF provides a suitable condition (i.e., temperature, humidity) for the insect's abundance (Rosenthal 2004; Jaworski & Hilszczański 2013).

Track 1 had higher species richness than Track 2 (Figure 5). A total of 31 bird species were found on both tracks, but twelve species can only be found on Track 1, i.e., *S. cheela, P. ptilornychus, H. longipennis, G. varius, A. tiphia, D. sanguinolentum, F. westermanni, P. fulvotincta, I. virescens, H. javanica,* and *M. armillaris*. While 8 species can only be found on Track 2, i.e., *A. gularis, N. bartelsii, C. larvata, L. leucogastroides, L. punctulate, M. dauurica, M. caruleus,* and *E. velatus*. The total abundance shows the higher individual of birds founds on Track 1 than on Track 2 (Figure 5). The Diversity index showed a high value on Track 1 (H' > 3) than on Track 2 (H' < 3) (Heip et a1. 1998) (Figure 5).

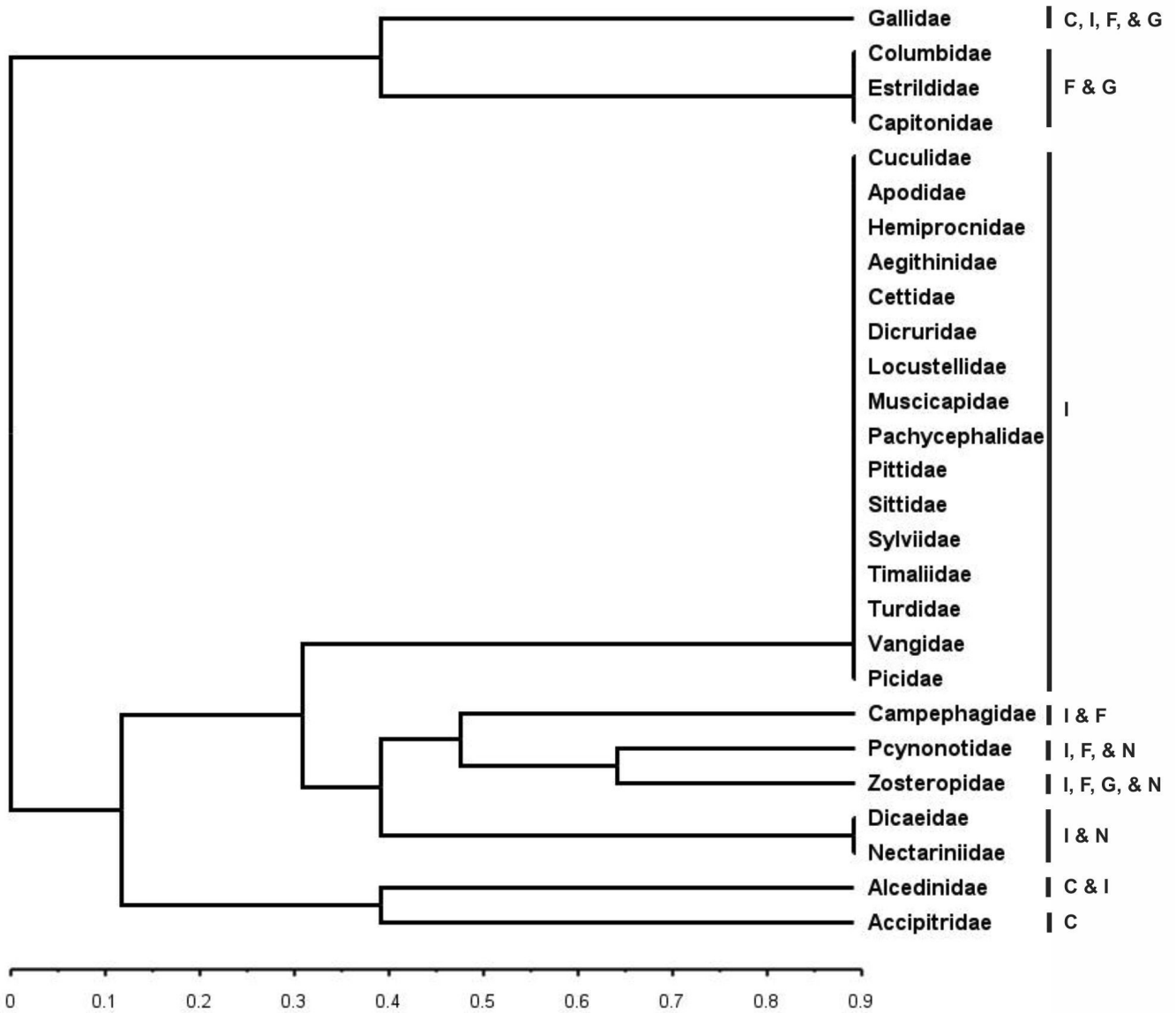

Figure 4. Clustering of feeding specialization based on the family of avifauna in the UBF. Abbreviation as follows: carnivores (C), insectivores (I), frugivores (F), granivores (G), and nectarivores (N).

The enormous amount of species diversity highlights the importance of UBF as a refuge spot for avifauna of mountainous forests in Java.

The range on the important value index reflects the actual conditions on each observation track. It shows that out of 27 families, Pycnonotidae had the highest important value on both tracks followed by Campephagidae and Timaliidae, respectively (Figure 6). Pycnonotidae and Campephagidae have similar common strategies which are living in colonies (except for *C. larvata* that are living in solitary). However, Pycnonotidae is more adaptive due to the wider range of feeding specialization (i.e., insectivore, frugivore, and nectarivore), while Campephagidae is limited to insectivore and frugivore (MacKinnon et al. 2010).

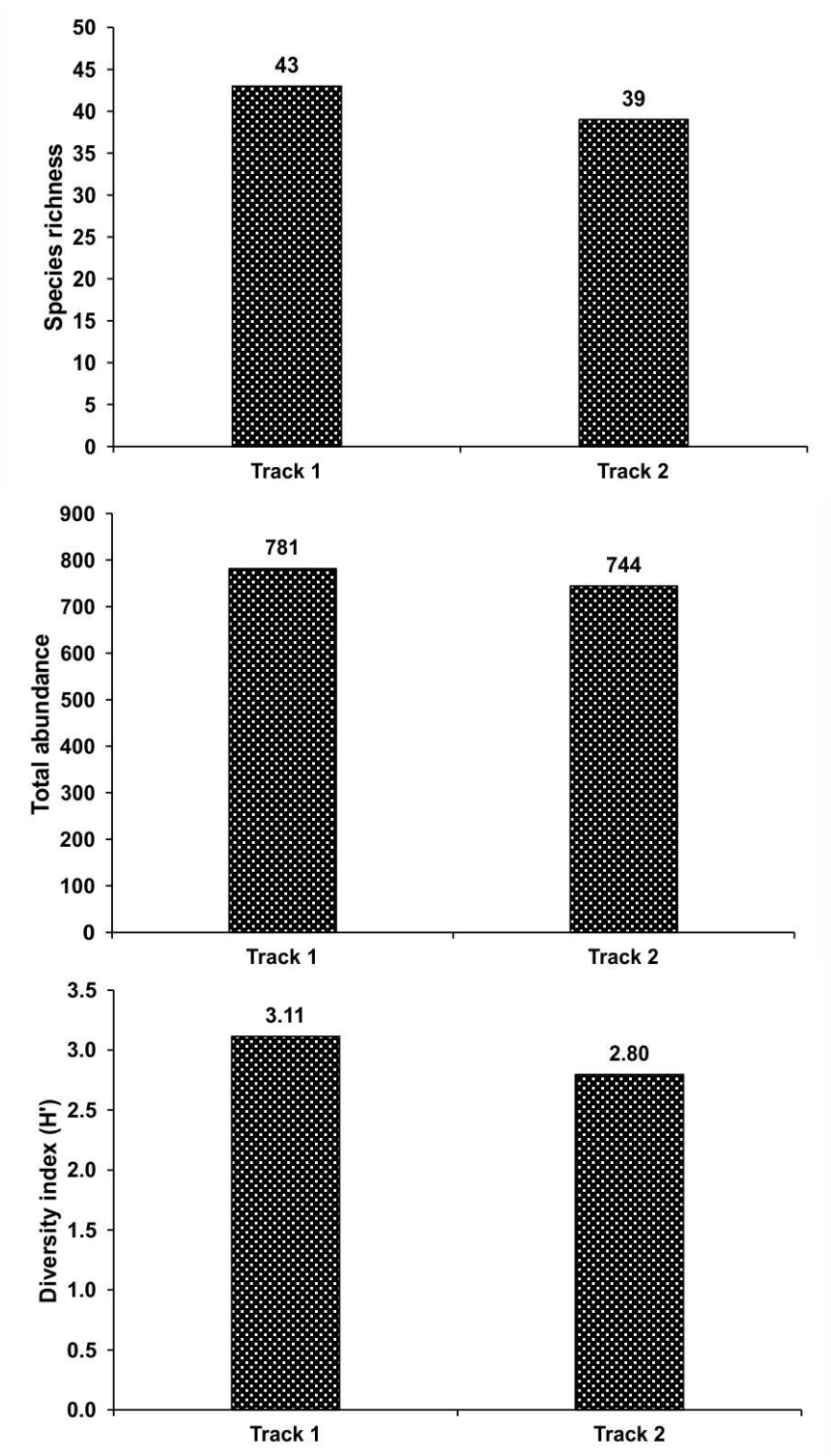

Figure 5. Comparison of species richness, total abundance, and diversity index of avifauna on both two tracks in the UBF.

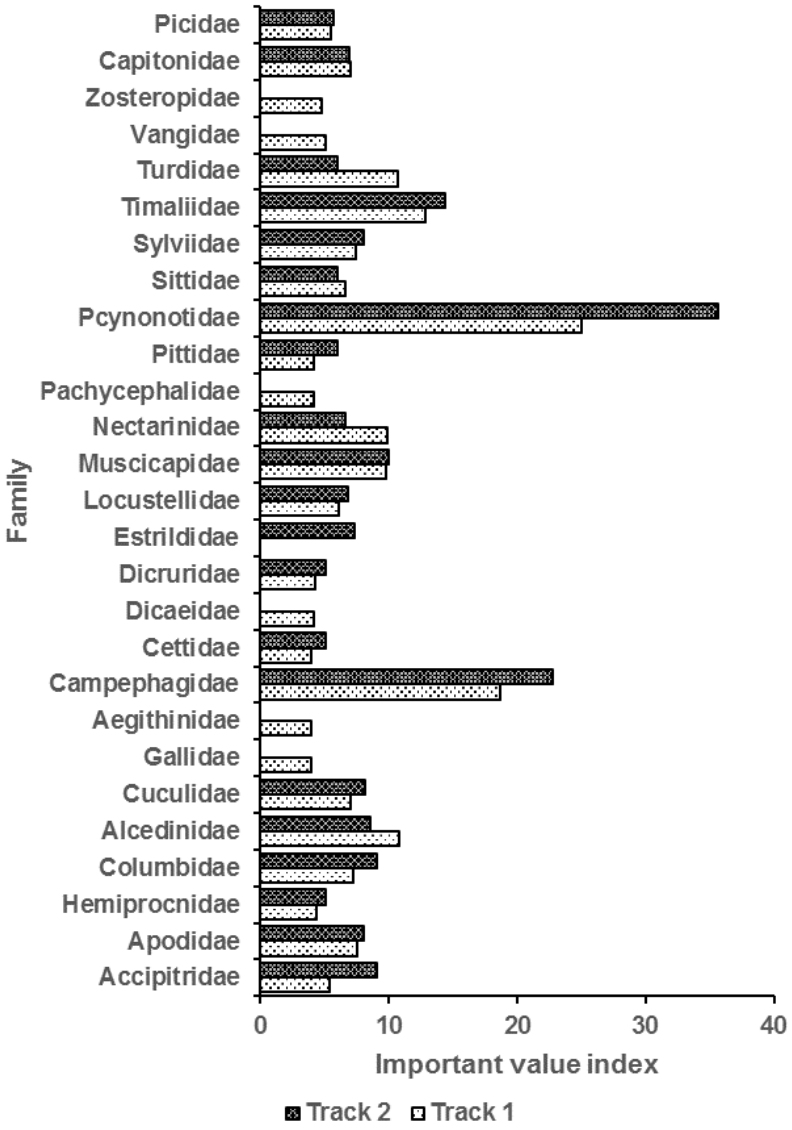

Figure 6. Comparison of the Important value index (IVI) of avifauna on two study tracks in the UBF.

Factor affecting the avifauna community and future conservation

The differences of avifauna composition—based on many indicators observed on both tracks, are associated with three main factors, i.e., habitat and vegetation composition, interactions between species, and presence of stress (Scott 2010). The UBF vegetation is comprised of two different ecosystems. The transition between the lower montane forest (upper side area) and the agricultural area (lower side area) affects the vegetation structures required for avifauna daily activities. The activities of each avifauna species are also related to the adaptation response of other bird species and the interactions among species (e.g., association, predation, and competition), thus shaping the community structure (Sutherland et al. 2004). Despite that, both tracks had different degrees of disturbances. Track 1 had less human disturbance due to its difficult terrain and dense canopy (Figure 2D), while Track 2 had higher human disturbance due to its location near the main road (Figures 2A, 2C). The disturbed areas have been reported to reduce the abundance and the amount of avifauna (Nuñeza et al. 2019). Moreover, Track 2 habitat is isolated by the main roads crossing the natural valley. The absence of a green corridor may limit avifauna mobility in exploring the diverse habitats. Also, birds prefer to fly higher or crossing over quiet roads to avoid the risk of being killed or struck (Reijnen & Foppen 1997).

The conservation effort of avifauna in UBF must be developed immediately through the collaborative and coordinative approach (Kristanti et al. 2017) to minimize the negative effect of anthropogenic disturbances (Estrada et al. 1997). Several things that could improve the conservation of avifauna in UBF, e.g., synergistic collaborations between the local community, institutions, and researchers. It can be succeded through regular monitoring, education, good governance, also by improving research effort in the UBF area.

CONCLUSION

A total of 51 species from 27 families were identified. Two species at risk (one Near Threatened and one Endangered) and 9 protected avifauna were noted. This study can be used as the baseline data for future conservation management in the UBF. The study on microclimatic factors, spatial and temporal distribution, and species interaction of avifauna is recommended in the future.

AUTHORS CONTRIBUTION

A.N. and M.F. designed the research, collect and analyzed the data. A.N. wrote the initial manuscript. L.S. and A.S.K. reviewed, revised, and proofread the final manuscript. N.K. supervised all the process.

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CONFLICT OF INTEREST

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome.

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Research Article

Callus induction and secondary metabolite profile from *Elephantopus scaber* L.

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ABSTRACT
Elephantopus scaber L. is a plant that has potential as traditional medicine. Callus induction and production of secondary metabolite content can be increased by culture callus using plant growth regulators. This study was purposed to investigate the effect of IBA and kinetin concentration on the induction and secondary metabolite profile of callus from *E. scaber* L. leaves. Leaves explant of *E. scaber* L. were cultured on MS medium with various combination concentrations of IBA and kinetin for 6 weeks and then callus was extracted using methanol. Secondary metabolite content from the resulting extract was analyzed using the phytochemical screening method. The result showed that the treatment of IBA 2.0 mg/L and kinetin 1.0 mg/L and treatment of IBA 2.0 mg/L and kinetin 2.5 mg/L are the fastest combination concentration to induce callus at 5.33 ± 0.577 days. Treatment of IBA 2.0 mg/L and kinetin 2.5 mg/L produced callus with the highest fresh weight and dry weight at 0.7016 ± 0.0588 grams and 0.0766 ± 0.0062 grams, respectively. The morphology of calluses grown during this study was compact with various colors appearance, such as light green, brownish green, and brown. Secondary metabolite content of methanol extract of callus *E. scaber* L. are flavonoids, alkaloids, terpenoids, and saponins.

Keywords: Callus, *Elephantopus scaber* L., IBA, kinetin, secondary metabolites

INTRODUCTION
Elephantopus scaber L. is a type of plant that can be easily found in Indonesia (Nurtamin et al. 2018). This plant is also potentially used as a traditional medicine to treat influenza, headaches, cough, fever, diarrhea, jaundice, hepatitis, laryngitis, anemia, and fluor albus (Arisandi & Andriani 2006; Wang et al. 2014). In previous studies, showed that *E. scaber* L. can reduce LDL (Low Density Lipoprotein) level and also promising hepatoprotection activity in mice (Daisy et al. 2007; Ho et al. 2012; Nasri 2012; Puspita 2004; Sankar et al. 2001; Sulastri 2008). The leaves of *E. scaber* L. containing terpenoids, flavonoids, saponins, and tannins which function as antibacterial and antiinflammatory (Bigham et al. 2003; Grayson 2000; Lim et al. 2006; Nurtamin et al. 2018).

There are an increasing use and unrestricted exploitation of natural population for medicinal purposes due to the high medicinal value of this plant, there has been causing an increase in the use (Abraham & Thomas

2015). Poor seed viability and early death of young seedlings in these natural environmental conditions can be inhibiting factors for the propagation of this species (Parashurama et al. 2013). Therefore, alternative propagation methods are necessary for the rapid multiplication and conservation of this plant. One of them is the technique of plant tissue culture. This method has been recognized as an alternative for plant propagation, production, characterization, elite clones, and secondary metabolites in a limited period (Abraham et al. 2010; Cheruvathur & Thomas 2014; Kumar & Thomas 2012; Murthy et al. 2014).

Plant propagation and an efficient callus induction have been standardized for an ethnomedicinal plant (Abraham & Thomas 2015). In previous studies was reported that callus of *E. scaber* L. can be induced by adding 1.5 mg L-1 2,4-Dichlorophenoxy acetic acid and 1.5 mg L-1 kinetin, or also can be induced by adding 5.0 µM 2,4- Dichlorophenoxy acetic acid and 0.5 µM kinetin (Abrahan & Thomas 2015; Rout & Sahoo 2013). In this study, callus induction of *E. scaber* L. is using the addition of indole butyric acid/IBA (auxin) and kinetin (cytokinins) as plant growth regulators. The purpose of this study was to investigate the effect of IBA and kinetin concentration on the induction and secondary metabolite profile of callus from *E. scaber* L. leaves.

MATERIALS AND METHODS

Materials

In this study, the plant materials used were the young leaves of *Elephantopus scaber* L. obtained from the Bratang flower market, Surabaya, Indonesia. Which had been identified in the plant physiology laboratory, Universitas Airlangga. The chemical materials for Murashige and Skoog (MS) medium, chlorox, alcohol 70%, IBA (PhytoTechnology Laboratories®, US), kinetin (PhytoTechnology Laboratories®, US), methanol, filter paper, distilled water, pH indicator strips, liquid detergent, aluminium foil, KOH 1 N, and HCl 1 N.

Callus induction

Preparation of MS medium with addition of IBA and kinetin

MS medium was prepared by adding macronutrient stock, 1 mL micronutrient stock, 5 mL iron stock, 4 mL vitamin in 500 mL distilled water was homogenized. Then, added 100 mg myoinositol and 30 grams of sucrose, after all the materials have dissolved then added distilled water to 1 L and the concentration of IBA and kinetin according to the specified treatment was added (Table 1). Furthermore, the acidity of the medium solution was adjusted to the range 5.6-5.8 using pH indicator strips, then 8 grams of agar powder was added to condense the solution, then it was covered with aluminium foil and labelled. The medium was sterilized using an autoclave at a pressure of 1.2 atm and a temperature of 121°C for 15 minutes (Manuhara 2014).

Table 1. Combination concentration of IBA and kinetin applied to each treatment group.

Treatment (mg/L)	Code
IBA 0.0 + Kinetin 0.0	$I_{0.0}K_{0.0}$
IBA 0.5 + Kinetin 0.5	$I_{0.5}K_{0.5}$
IBA 0.5 + Kinetin 1.0	$I_{0.5}K_{1.0}$
IBA 0.5 + Kinetin 1.5	$I_{0.5}K_{1.5}$
IBA 1.5 + Kinetin 0.5	$I_{1.5}K_{0.5}$

Table 1. Contd.

Treatment (mg/L)	Code
IBA 1.5 + Kinetin 1.0	$I_{1.5}K_{1.0}$
IBA 1.5 + Kinetin 1.5	$I_{1.5}K_{1.5}$
IBA 2.0 + Kinetin 0.5	$I_{2.0}K_{0.5}$
IBA 2.0 + Kinetin 1.0	$I_{2.0}K_{1.0}$
IBA 2.0 + Kinetin 2.5	$I_{2.0}K_{2.5}$

Planting leaves explant of *E. scaber* L.

E. scaber L. leaves were washed with liquid detergent for five minutes then rinsed three times using tap water, then it was soaked in 70% alcohol for 5 minutes and rinsed using sterile distilled water three times, then soaked in 20% chlorox for 10 minutes and rinsed using sterile distilled water again three times. Then the leaves explant was cut (± 1 cm^2) and planted on MS medium for 6 weeks of culture periods, at a regulated temperature of 25 ± 2°C and lighting 3000-3500 lux for 24 hours (Manuhara 2014).

Analysis of secondary metabolites content

Callus extraction

The dried callus was weighed and recorded, then mashed into powder. Then 5 mL of methanol was added to the vial containing the callus powder to be macerated for 24 hours. Then the extract was filtered using filter paper. The extract was concentrated until the remaining volume of 2 mL. Extraction was also carried out on *E. scaber* L. leaves that had dried with the same extraction method. This is necessary to compare the compounds contained in the mother plant and callus of *E. scaber* L.

Identification of secondary metabolites content using phytochemical screening

Flavonoids compounds test

The methanol extract from callus *E. Scaber* L. has put in as much as 1 mL into a test tube, then 0.5 mL concentrated HCl and 4 pieces of Mg bands were added. The appearance of red, orange, or green indicates the presence of flavonoids in the sample extract (Kristanti et al. 2008).

Alkaloids compounds test

1 mL of the methanol extract from callus *E. scaber* L., 3 drops of Mayer's reagent, and 3 drops of chloroform were added to 3 test tubes and then homogenized until blended. The positive alkaloid content was indicated by the presence of white sediment in a test tube (Darwis 2000).

Terpenoids and steroids compounds test

3 drops of sample methanol extract of *E. scaber* L. callus were put on a spot test board. Then added 3 drops of anhydrous acetate (Ac2O) and a drop of concentrated sulphuric acid (H_2SO_4) into it. After well homogenized the terpenoids content was marked by red or brown colors, while steroids were indicated by blue color (Kristanti et al. 2008).

Saponins compounds test

The Forth method was used to analyze the saponins content by putting 1 mL methanol extract into the test tube then added 5 ml hot water and the test tube was shaken until 60 seconds. The extracted sample was positive for saponins if it showed foam that had formed in the test tube for 30 seconds (Darwis 2000).

RESULTS AND DISCUSSION

Based on the result in this study, shown that callus can be induced in all treatments except control (Table 2). The table shows that the fastest callus induction time period was 5.33 ± 0.577 days in combination with IBA 2.0 mg/L + kinetin 1.0 mg/L and IBA 2.0 mg/L + kinetin 2.5 mg/L. Whereas the slowest callus induction period was 8.0 ± 0 days in combination with IBA 1.5 mg/L + kinetin 1.5 mg/L. Explants planted on MS medium without the addition of growth regulators (as a control) did not form a callus until the end of the observation time at the sixth weeks after planting. The percentage of explant forming callus in each treatment was 100%.

Table 2. Mean callus induction time and percentage of explant forming callus of *E. scaber* L. leaves from various combination concentration of IBA and kinetin treatments.

Treatment (mg/L)	Mean callus induction time (days)	Percentage of explant forming callus (%)
$I_{0.0}K_{0.0}$	0 ± 0^{a}	0
$I_{0.5}K_{0.5}$	6.66 ± 0.577^{bc}	100
$I_{0.5}K_{1.0}$	7.33 ± 0.577^{cd}	100
$I_{0.5}K_{1.5}$	7.66 ± 0.577^{cd}	100
$I_{1.5}K_{0.5}$	6.66 ± 0.577^{bc}	100
$I_{1.5}K_{1.0}$	5.66 ± 0.577^{b}	100
$I_{1.5}K_{1.5}$	8.0 ± 0^{cd}	100
$I_{2.0}K_{0.5}$	6.66 ± 0.577^{bc}	100
$I_{2.0}K_{1.0}$	5.33 ± 0.577^{b}	100
$I_{2.0}K_{2.5}$	5.33 ± 0.577^{b}	100

*) Number followed by different letters indicating significant difference based on Mann-Whitney test at 5% significant level.

Callus culture is used to obtain callus from explants grown in a controlled environment. The formation of callus is to induce certain plant parts by providing growth regulators. Callus induction is caused by cuts or explant slices in response to hormones both exogenously and endogenously. The selection of growth regulators is one of the important factors in determining the formation of plant callus. IBA has chemical properties and more stable mobility in plants, besides the longer working power. This is causes that IBA more successful to be used. In addition, the cytokinins play a role in stimulating cell division and callus proliferation. Kinetin added to culture medium increases the rate of protein synthesis so that it encourages cell enlargement and division (mitosis). Interaction between the role of auxin and cytokinin which are both added to the medium with the right combination will cause the explants to experience callus induction (Rosyidah et al. 2014).

Table 3. Mean fresh weight and dry weight of *E. scaber* L. callus.

Treatment (mg/L)	Mean	
	Fresh weight (grams)	Dry weight (grams)
$I_{0.0}K_{0.0}$	0 ± 0^{a}	0 ± 0^{a}
$I_{0.5}K_{0.5}$	0.1850 ± 0.0103^{b}	0.0288 ± 0.0046^{b}
$I_{0.5}K_{1.0}$	0.2627 ± 0.0117^{b}	0.0525 ± 0.0027^{d}
$I_{0.5}K_{1.5}$	0.2473 ± 0.0069^{b}	0.0400 ± 0.0088^{bcd}
$I_{1.5}K_{0.5}$	0.2652 ± 0.0481^{b}	0.0416 ± 0.0141^{bcd}
$I_{1.5}K_{1.0}$	0.2696 ± 0.0222^{b}	0.0431 ± 0.0055^{cd}
$I_{1.5}K_{1.5}$	0.2332 ± 0.0217^{b}	0.0362 ± 0.0028^{bc}
$I_{2.0}K_{0.5}$	0.2215 ± 0.0219^{b}	0.0352 ± 0.0045^{bc}

Table 3. Contd.

Treatment (mg/L)	Mean	
	Fresh weight (grams)	Dry weight (grams)
$I_{2.0}K_{1.0}$	0.3215 ± 0.0108^{b}	0.0440 ± 0.0092^{cd}
$I_{2.0}K_{2.5}$	0.7016 ± 0.0588^{bc}	0.0776 ± 0.0062^{e}

*) Number followed by different letters indicating significant difference based on Mann-Whitney test at 5% significant level.

The addition of a combination concentration of IBA and kinetin growth regulators on fresh weight and dry weight of callus in each treatment was different. The highest mean fresh weight of callus was found in the treatment of IBA 2.0 mg/L + kinetin 2.5 mg/L with 0.7016 ± 0.0588 grams, this treatment also had the highest average dry weight with a weight of 0.0776 ± 0.0062 grams. While the treatment of IBA 0.5 mg/L + kinetin 0.5 mg/L had the lowest fresh weight and dry weight of callus with 0.1850 ± 0.0103 grams and 0.0288 ± 0.0046 grams, respectively. Table 3 shows that there is a tendency to increase the average fresh weight and dry weight of callus along with an increase in the concentration of growth regulators IBA and kinetin.

In this study, the addition of a combination concentration of IBA 2.0 mg/L + kinetin 2.5 mg/L growth regulators produced the highest mean fresh weight and dry weight, 0.7016 ± 0.0588 grams and 0.0776 ± 0.0062 grams, respectively. This is presumably because at these concentrations the endogenous auxin and cytokinin conditions become balanced so that it can produce a good amount of callus mass formation (George & Sherrington 2007). The results of fresh weight and dry weight of callus were obtained from a combination of the lower concentration of IBA compared to kinetin. This is in line with previous studies that produce fresh weight and dry weight at a ratio of IBA combination of 1 mg/L + kinetin 1.5 mg/L with fresh weight 0.4688 grams and IBA concentration 1 mg/L + kinetin 2 mg/L with dry weight 0.0895 grams (Rohmatin 2014).

Table 4. Callus morphology of *E. scaber* L. at 6th weeks of the culture period (bar = 1 cm).

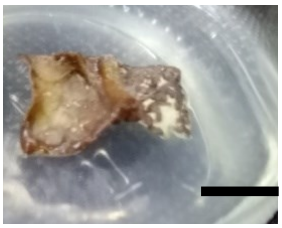
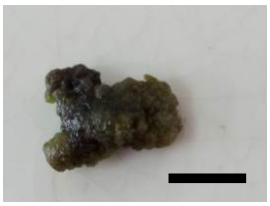
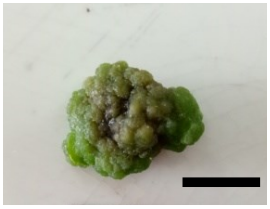
No.	Treatment (mg/L)	Figure	Description of callus morphology
1.	$I_{0.0}K_{0.0}$		The leaves explant planted on MS medium was colored brown and failed to induce callus
2.	$I_{0.5}K_{0.5}$		The brown callus has compact texture
3.	$I_{0.5}K_{1.0}$		The brown callus has compact texture

Table 4. Contd.

No.	Treatment (mg/L)	Figure	Description of callus morphology
4.	$I_{0.5}K_{1.5}$		The brown callus has compact texture
5.	$I_{1.5}K_{0.5}$		The brownish green callus has compact texture
6.	$I_{1.5}K_{1.0}$		The brownish green callus has compact texture
7.	$I_{1.5}K_{1.5}$		The light green callus has compact texture
8.	$I_{2.0}K_{0.5}$		The brownish green callus has compact texture
9.	$I_{2.0}K_{1.0}$		The brownish green callus has compact texture
10.	$I_{2.0}K_{2.5}$		The light green callus has compact texture

All treatments produced callus with a compact callus texture while the control in this study did not experience growth so that no callus was formed. The callus color of the *E. scaber* L. explants observed in each treatment was brownish, brownish green, and light green. Observation of callus morphology at the sixth weeks after culture was presented in Table 4. For

efficient callus induction and plant regeneration, callus would be developed in light conditions rather than in the dark. In previous studies, callus induction on *Nicotina tabacum* L., *Stelechocarpus burahol,* and *Brassica napus* L. which showed the best callus induced in the light conditions, while explants grown in the dark did not show optimum results and some of the treatment was not even callus induced (Siddique & Islam 2015; Habibah et al. 2018; Afshari et al. 2011). The abilities of photosynthesis increased plant morphological features gradually in *Ginkgo biloba* L. (Yang & Chen 2014). According to their reports and claimed that the callus developed in light condition might carry photosynthetic pigments that influenced the growth and development of callus.

The compact callus texture is an effect of cytokinins which play a role in nutrient transport. The cytokinin transport system from the basal to the apex will carry water and nutrients through the transport vessels and influence the osmotic potential in the cells. The addition of sucrose will flow through the phloem vessels and causes turgor pressure. This pressure arises due to differences in solution concentration so that water and nutrients such as sucrose from the medium will enter the cell through osmosis. This will make the cell walls more rigid so that the callus cells will become compact. Callus *E. scaber* L. was induced at different lengths of time, but each callus in each treatment had the same characteristic, the initial color of the light green callus was shown in each early induction. As the culture period increases until the sixth weeks the callus appears to have a variety of colors. These color changes indicate changes in the growth phase of cells and cell regeneration power that is influenced by low nutrient intake during culture and the presence of explant tissue injury that triggers the callus to experience stress so that the callus is brown, while the green color of the callus indicates the formation of chlorophyll in the tissue (Payghamzadeh & Kazemitabar 2011).

Based on Table 5 it can be seen that the *E. scaber* L. leaves extract which was used as a positive control, produces secondary metabolites of flavonoids, alkaloids, terpenoids, and saponins. In the flavonoid test, all treatments are positive contained flavonoids except for the treatment of IBA 0.5 mg/L + kinetin 0.5 mg/L. This is due to the provision of kinetin with low concentrations that cause flavonoid compounds cannot be identified by using phytochemical screening methods. In the alkaloids, saponins,

Table 5. Phytochemical screening of *E. scaber* L. callus extract supplemented with combination concentration of IBA and kinetin plant growth regulator.

Treatment (mg/L)	The presence of secondary metabolites from the callus extract				
	Flavonoids	**Alkaloids**	**Steroids**	**Terpenoids**	**Saponins**
Leaves extract of *E. scaber* L.	+	+	-	+	+
$I_{0.0}K_{0.0}$	-	-	-	-	-
$I_{0.5}K_{0.5}$	-	+	-	+	+
$I_{0.5}K_{1.0}$	+	+	-	+	+
$I_{0.5}K_{1.5}$	+	+	-	+	+
$I_{1.5}K_{0.5}$	+	+	-	+	+
$I_{1.5}K_{1.0}$	+	+	-	+	+
$I_{1.5}K_{1.5}$	+	+	-	+	+
$I_{2.0}K_{0.5}$	+	+	-	+	+
$I_{2.0}K_{1.0}$	+	+	-	+	+
$I_{2.0}K_{2.5}$	+	+	-	+	+

terpenoids, and steroids tests all treatments showed positive results containing these compounds. Kinetin is one of the growth regulator types from cytokinin. In previous studies, authors stated that cytokinin and low auxin concentration have a possible synergistic interaction on accumulation and distribution of recorded secondary metabolites, but at low kinetin concentration not shown an optimum accumulation and distribution of recorded secondary metabolites (Radić et al. 2016). The concentration of IBA and kinetin in this study also affected to secondary metabolites profile in the methanol extract of *E. scaber* L. callus. The accumulation of secondary metabolites contained in *E. scaber* L. callus was influenced by the increase in the biosynthetic pathway process in each type of secondary metabolites therein. IBA and kinetin added from outside the cell (exogenous) will have a more effective impact if they can be balanced with the levels of auxin and cytokinin hormones in the explant cells (endogenous). In previous studies also stated that the production of secondary metabolites in vitro will significantly increase if added exogenous of various types and concentrations of auxin and cytokinins (Radić et al. 2016; Jamwal et al. 2018; Jain et al. 2012).

According to Harborne (2006), terpenes are generally fat-soluble compounds. Then based on the level of solubility, in testing compounds, terpenes are withdrawn with ether. But in this study, the withdrawal of terpenes was carried out using methanol as a solvent. This is because methanol is a universal solvent so that it can dissolve polar and nonpolar compounds. Terpenoids have antibacterial activity, inhibit cancer cells, inhibition of cholesterol synthesis, anti-inflammatory, menstrual disorders, skin disorders, liver damage, and malaria (Rumondang 2013). In previous studies, *E. scaber* L. leaves contained saponins, terpenoids, polyphenols, flavonoids luteolin-7-glucoside, epipriedelinol, lupeol, stigmaserin, lupeol acetate, deoxyelephantopin, isodeoxyelephantopin compounds (Asmaliyah et al. 2010; Yuniarti 2008). These leaves also contain secondary metabolites of terpenoids and flavonoids which act as antibacterial. In previous study, showed that terpenoid compounds have antibacterial activity, there are monoterpenoid linalool, phytol, hardwicklic acid diterpenoid, triterpenoid glycoside, and triterpenoid saponin (Grayson 2000; Lim et al. 2006; Bigham et al. 2003).

CONCLUSION

The combination of IBA and kinetin plant growth regulators has a significant effect on the induction time, the percentage of explants forming callus, fresh weight, dry weight, morphology, texture, and secondary metabolite profile of *E. scaber* L. leaves callus. The optimum combination treatment is the concentration IBA 2.0 mg/L and kinetin 2.5 mg/L with a mean of fresh weight callus at 0.7016 ± 0.0588 grams and dry weight callus at 0.0776 ± 0.0062 grams. *E. scaber* L. callus extract contains secondary metabolites such as flavonoids, alkaloids, terpenoids, and saponins.

AUTHORS CONTRIBUTION

J.J. designed the research and supervised all the process, D.A.W. collected and analyzed the data, E.S.W.U. designed the research and supervised the data, and N.I.Z. analyzed the data and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research Article

The Use of DNA Barcoding and Phylogenetic Analysis to Improve Identification of *Usnea* spp. Based on ITS rDNA

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ABSTRACT
Lichen of the genus *Usnea* is quite common being used as a traditional herbal remedy. This genus is characterized by thallus, which is very similar among the species, leads to some difficulties in distinguishing them. In Indonesia, such research report on the availability of this genus based on their morphological characteristic is minimal. This might be due to too high morphological similarities among them. The molecular character, which is based on the DNA Barcode of Internal Transcribed Spacer (ITS) rDNA sequences, with its conserved region (5.8S) and varied region (ITS1 and ITS2), are becoming essential characters on identifying as well as analyzing the phylogenetic. The current study then proposed to identify and draw the species dendrogram of species within the *Usnea* genus obtained from Mount Lawu Forest of Central Java and Turgo Forest of Yogyakarta based on their phylogenetic and phenetic analysis. The dendrogram was constructed with UPGMA using the simple matching coefficient, whereas the phylogenetic tree was constructed with Maximum Likelihood (ML) using Kimura-2 parameter with 1000 bootstrap. The data were unable to draw phenetic relationships among the subgenus *Usnea* and *Eumitria* members. The phylogenetic tree shows the primary two clades, distinguishing the subgenus *Usnea* and *Eumitria*. The ITS rDNA sequence was able to identify most of the *Usnea* species.

Keywords: *Usnea*, DNA Barcode, ITS rDNA, Phylogenetic

INTRODUCTION
Lichen is an outstanding successful group of symbiotic organisms comprised of algae (phycobiont) and fungal (mycobiont) (Zachariah & Varghese 2018). Lichen of the genus *Usnea* is used as traditional herbal remedies in Solo and Yogyakarta, with of local name of "Kayu Angin". The local people collect the thallus from the forests of Turgo Hill and Mount Lawu. They use Usnea as one component of all herbal medicines, such as cholesterol, diabetes, gout, maternity, high blood pressure, skin, clods, and heart disease (Jannah 2019a).

Usnea (Parmeliaceae) has been distinguished with approximately 600 species worldwide (Hawksworth et al. 1995). Articus (2004) grouped the

Usnea members as inadequate taxonomy since they have many similarities among the species. The thallus of this genus is very similar among species. At this level, there is exceptionally high plasticity on their morphological characters to respond to environmental factors, which leads to a very complicated effort in drawing a clear boundary among the species (Clrec 1998). The situation leads to species identification limitation based on their morphological characters but not on the genus' family.

Divakar et al. (2006) stated there were sister species cases due to high similarities between two species of *U. florida* and *U. subfloridana*. These two species could only be distinguished on the presence or absence of their reproductive organs where *U. florida* has more sexual organs than another. The reproductive organ, such as apothecia, is an essential character for species-level identification, but the prolonged growth during the life phase becomes an obstacle to identification (Clerc 1998; Swinscow & Krog 1978). Based on the above facts, molecular analysis with DNA Barcodes is needed to strengthen and support species identification quickly, precisely, and accurately.

Research on the identification of *Usnea* in Indonesia using DNA barcodes from ITS rDNA has not been reported yet. However, the ITS rDNA region has been used extensively in the study of lichenized fungi overseas, including in assessing the species boundaries as well as testing the correlation between genetic and morphological diversity in species complexity (White et al. 1990; Korabecna et al. 2007; Del-Prado et al. 2010; Kelly 2011; Jannah 2019b). The molecular characters based on Internal Transcribed Spacer (ITS) rDNA sequences that have conserved regions (5.8S) and varied regions (ITS1 and ITS2) are needed to strengthen and support the identification and phylogenetic analysis (Articus et al. 2002; Ohmura 2002). The objective of this research was to carry out a phylogenetic analysis of *Usnea* species.

MATERIALS AND METHODS

Materials

The samples used in this research were fresh thallus obtained through the explorative method. A total of 16 examined specimens in the present study were collected at Mount Lawu Forest (LW) (East Java) and Turgo Hill Forest (T) (Yogyakarta) (Table 1).

Table 1. List of *Usnea* spp. examined and location of origin.

No.	Species	Accession Number	Origin
1	*U. himalayana*	LW1	Mount Lawu Forest
2	*U. himalayana*	LW2	Mount Lawu Forest
3	*U. pectinata*	LW3	Mount Lawu Forest
4	*U. rubrotincta*	LW4	Mount Lawu Forest
5	*U. himalayana*	LW5	Mount Lawu Forest
6	*U. fragilescens*	LW6	Mount Lawu Forest
7	*U. baileyi*	LW7	Mount Lawu Forest

Table 1. Contd.

No.	Species	Accession Number	Origin
8	*U. nidifica*	LW8	Mount Lawu Forest
9	*U. fragilescens*	LW9	Mount Lawu Forest
10	*U. nidifica*	LW10	Mount Lawu Forest
11	*U. himalayana*	LW11	Mount Lawu Forest
12	*U. baileyi*	T1	Turgo Hill Forest
13	*U. bismolliuscula*	T2	Turgo Hill Forest
14	*U. baileyi*	T3	Turgo Hill Forest
15	*U. bismolliuscula*	T4	Turgo Hill Forest
16	*U. bismolliuscula*	T5	Turgo Hill Forest

Methods

The *Usnea* thallus' total DNA was extracted using a modified CTAB method that we developed by adding High Salt-TE. It is challenging to get pure DNA from the *Usnea* thallus since there has never been a manuscript on DNA isolation from lichens in Indonesia. The following is the DNA isolation method that we developed. Total DNA of lichen was extracted from the thallus using a modified CTAB method. As many as 0.05 gr of Usnea thallus was powderized with additional liquid nitrogen. The powders are transferred into a 1.5 ml tube, added 400 µl of pre-heated 2X CTAB buffer, and incubated for 65 ºC for 10 minutes. Then, added 400 µl of chloroform/isoamyl alcohol solution 24:1, homogenized gently for 5 minutes at room temperature, then centrifuged at 12,000 rpm for 1 minute. The supernatant, in turn, was added with 250 µl isopropanol and centrifuged speed of 12,000 rpm for 1 minute. The supernatant was removed, added 100 µl of High Salt-TE, incubated at 65 ºC until dissolved, then added 800 µl 100% ethanol, incubated at -20ºC for 15 minutes, centrifuged 15,000 rpm 15 minutes at 4ºC and the supernatant removed and the pellet dried. After the pellet was added with 300-500 µl of 70% ethanol added, centrifuged at 15,000 rpm for 3-5 minutes 4ºC, the supernatant was removed, and the pellet was drained. A volume of 300-500 µl 70% ethanol was added into the pellet, centrifuged 15,000 rpm for 3-5 minutes at 4ºC, and the pellet is dried again. Finally, the pellet was dissolved by adding 50 µl of buffer pH TE 8. The purified DNA is stored at -20 °C.

Fungal nuclear ITS rDNA was amplified using the primer ITS 1 and ITS 4 (White et al. 1990). The PCR amplifications using KAPPA 2G™ Fast ReadyMix (2x) were performed with a program of initial denaturation for 2 min at 95°C, followed by 25 cycles of 0.5 min at 95°C, 0.5 min at 56.3°C, 1 min at 72°C, and a final elongation for 1 min at 72°C. The PCR product was electrophoresed in 1% agarose gel stained with 1 µl good view at 50 Volt for 40 minutes and visualized through UV-trans illuminator. The sequencing process was carried out at First Base, Singapore, through the PT Genetika Science Indonesia service.

The ITS rDNA sequence was analyzed and edited using Bioedit and DNA Baser. The homology level was determined through BLAST online. The sequence alignment was analyzed using Clustal-X and nucleotide similarity was executed through Phydit. The phylogenetic tree reconstruction was carried out using Maximum Likelihood (ML) with Kimura-2 parameter and 1000 bootstrap replications on MEGA-5.05. The Automatic Barcode Gap Discovery (ABGD) was carried out through http://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html using Jukes-Cantor (JC69) distance.

RESULTS AND DISCUSSION

Seven species were found based on morphological characters, namely *U. pectinata* (LW3), *U. rubrotincta* (LW4), *U. himalayana* (LW1, LW2, LW5, LW11), *U. fragilescens* (LW6, LW9), *U. nidifica* (LW8,LW10), *U. baileyi* (LW7,T1,T3), and *U. bismolliuscula* (T2, T4, T5). These species of *Usnea* come from subgenus *Usnea* (Figure 1) dan *Eumitria* (Figure 2).

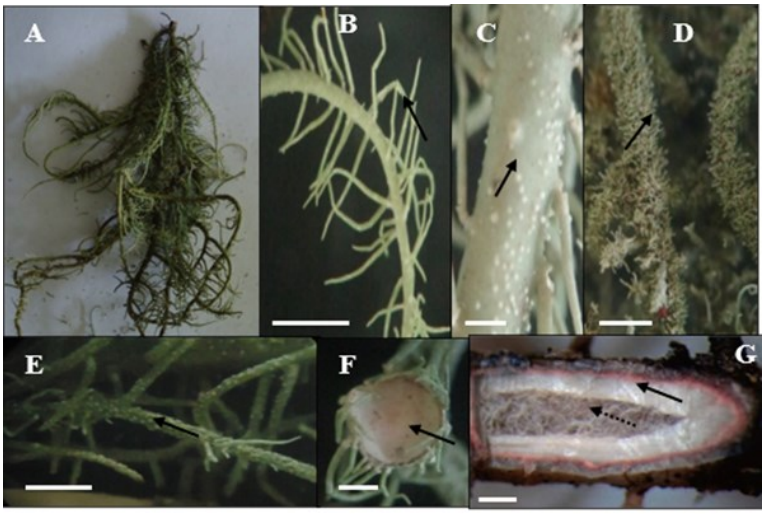

Figure 1. Morphological structure of *U. baileyi* (subgenus *Eumitria*). A. thallus, B. fibril, C papillae, D. isidia, E. soralia, F. apothecia, G. compact medulla & fistulose central axis. Bar=0.5 mm.

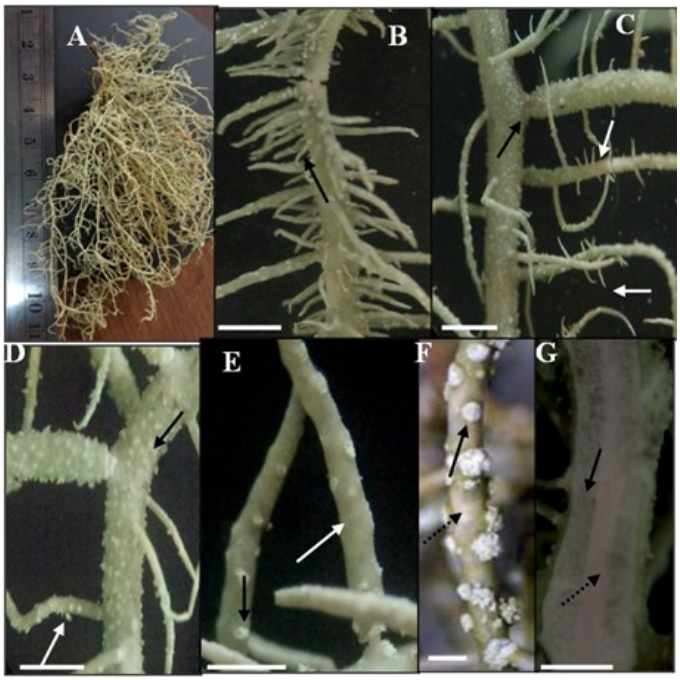

Figure 2. Morphological structure of *U. fragilescens (*subgenus *Usnea)*. A. thallus, B. fibril, C. branch, D. white isidia, and black papillae, E-F. soralia, F. apothecia, G. loose medulla & solid central axis. Bar=0.5 mm.

The dendrogram generated from morphological characters showed that all specimens are divided into two large clades (clade A and B), separating *U. baileyi* (clade A) and the other *Usnea* species (clade B) (Figure 3). Morphologically, *U. pectinata* is closely related to *U. bismolliuscula*, *U. rubrotincta* is closely related to *U. fragilescens*, *U. himalayana* is closely related to *U. nidifica*, and *U. baileyi* is distantly related to those clustered in clade B.

The morphological character distinguishing both clades is the central axis type. Clade A consists of *U. baileyi* with a fistulose central axis, while the clade B member has a solid central axis. The morphological differences between the subgenus *Usnea* and *Eumitria* are not always apparent in some species. *Usnea pectinata* and *U. baileyi* belong to the subgenus *Eumitria* but both are separated into distinct clades because *U. pectinata* having a solid central axis. The clustering result based on morphological characters in this study could not reflect the overall relationship among *Usnea* species. It can not separate the member of subgenus *Usnea* and *Eumitria* accordingly.

Sokal et al. (1963), stated that the phenetic approach in determining the relationship between individuals is based on the existing similarity of characteristics without comparing the characters that are homology (characters inherited from ancestors to their descendants) and homoplation (characters obtained as a result of the adaptation to the same environment). The existence of convergent evolution is obtained because of the adaptation process to the same habitat conditions and divergent evolution that makes the gains different forms from the same ancestor due to different environmental habitats resulting in the phenetic method almost unable to describe the true relationship among species (Campbell et al. 2008).

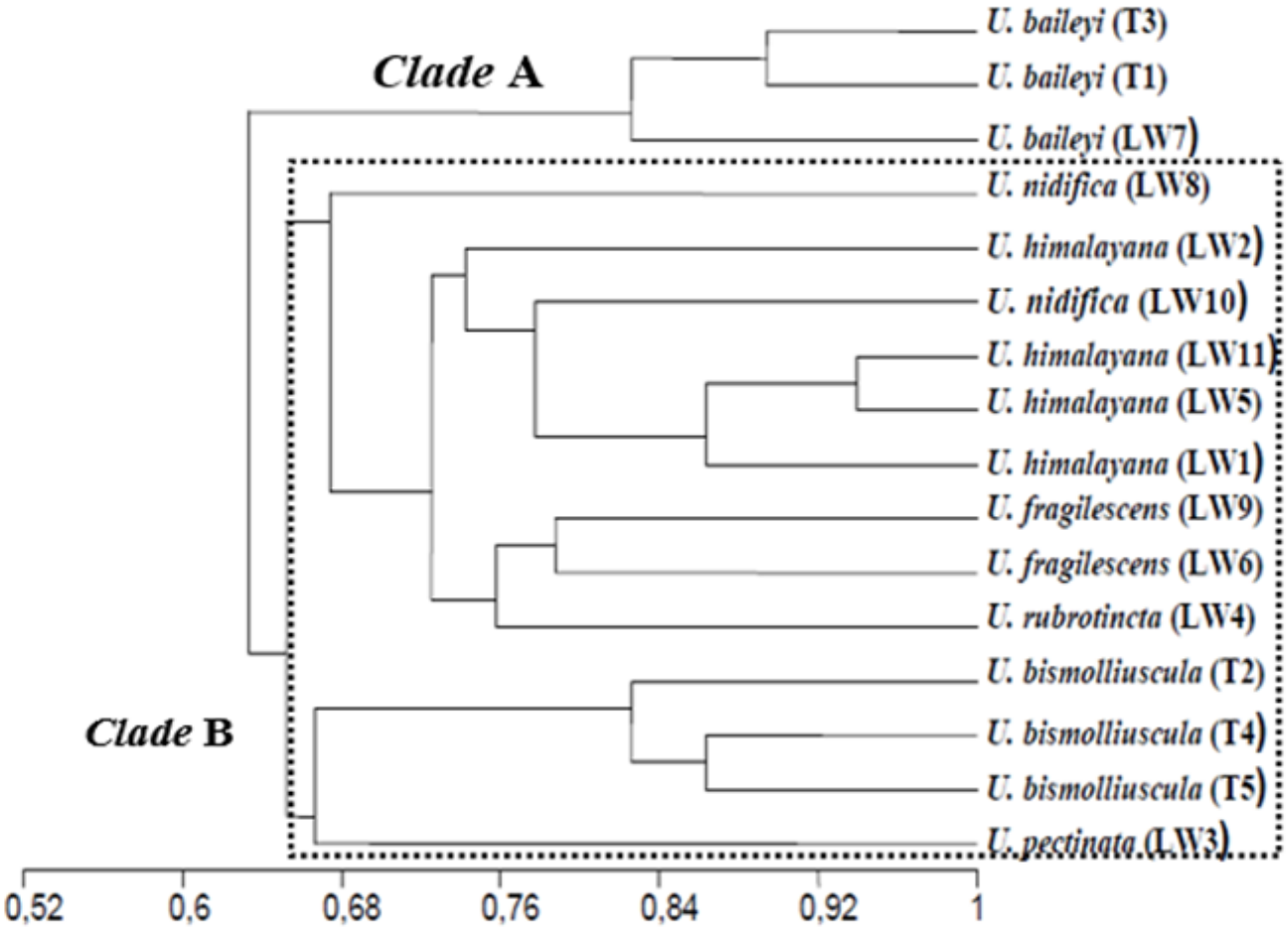

Figure 3. The dendrogram of *Usnea* is based on the UPGMA method using Simple Matching Coefficient.

The identification of *Usnea* used by the people around Yogyakarta and Solo, which comes from Turgo Hill forest and Mount Lawu forest, was carried out based on molecular data inferred from the Internal Transcribed Spacer (ITS) rDNA sequence. ITS rDNA sequences are widely used for species-level identification, capable of differentiating inter and intra-species, and determine the relationship of species through the differences of the conserved region and the similarity of variable region (White et al. 1990; Korabecna et al. 2007); Del-Prado et al. 2010; Kelly 2011).

The amplification of ITS rDNA from the *Usnea* genome was successful. The result of PCR visualized using agarose shows a clear strong band, which means that the ITS rDNA has been successfully amplified. ITS sequence length is ± 559 bp. The results of the PCR products that have been obtained and are under the target sequence length (ITS rDNA) are then sequenced to read the ITS rDNA nucleotide sequence from each sample (Figure 4). The sequencing results were then analyzed with BLAST software on the NCBI Gene Bank to determine the homology level of the ITS rDNA sequences obtained from the Gene Bank database, besides being able to show that the sequence obtained was true ITS rDNA. This can be proven if the homology level of the ITS rDNA sequence obtained with the ITS rDNA sequence in Gene Bank shows high compatibility between 94-99%. This high compatibility means that the target sequence obtained is the ITS rDNA sequence lichen genus *Usnea*.

The phylogenetic tree was constructed using Maximum Likelihood (ML) and 1000x bootstrap methods. This method is used to identify differences in genetic distance and analyze the similarity between samples. The ITS rDNA sequence was able to identify most of the *Usnea*, but do not support the separation of some species in *Usnea*. The topology of the phylogenetic tree that was formed showed that ITS rDNA was able to separate species in each sub-genus into groups to form one clade. In the results of the phylogenetic tree reconstruction, it is clear that the species included in the Usnea subgenus are grouped into one clade (monophyletic), and separate from the *Eumitra* subgenus (Figure 4). The results of this study indicate that the genetic distance between *Usnea* species is meager. The lowest genetic distance of 0 is found in *U. pectinata* and *U. baileyi* (T1 and T3) (Table 2).

Figure 4. Results of 496 bp ITS rDNA sequences (1-188 bp for ITS1, 189-346 5.8S, & 347-496 for ITS2) of *U. baileyi*.

Table 2. Genetic distances of *Usnea* spp. based ITS rDNA sequences.

No	Specimen	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*U. himalayana* (LW1)															
2	*U. himalayana* (LW2)	0,002														
3	*U. pectinata* (LW3)	0,123	0,121													
4	*U. himalayana* (LW11)	0,006	0,008	0,131												
5	*U. bismolliuscula* (T5)	0,079	0,077	0,138	0,086											
6	*U. himalayana* (LW5)	0,000	0,002	0,123	0,006	0,079										
7	*U. baileyi* (LW7)	0,113	0,111	0,032	0,116	0,128	0,113									
8	*U. fragilescens* (LW9)	0,066	0,063	0,125	0,063	0,059	0,066	0,110								
9	*U. baileyi* (T1)	0,123	0,121	0,000	0,131	0,138	0,123	0,032	0,125							
10	*U. bismolliuscula* (T2)	0,079	0,077	0,138	0,086	0,000	0,079	0,128	0,059	0,138						
11	*U. baileyi* (T3)	0,123	0,121	0,000	0,131	0,138	0,123	0,032	0,125	0,000	0,138					
12	*U. bismolliuscula* (T4)	0,079	0,077	0,138	0,086	0,000	0,079	0,128	0,059	0,138	0,000	0,138				
13	*U. nidifica* (LW8)	0,004	0,002	0,123	0,010	0,079	0,004	0,113	0,065	0,123	0,079	0,123	0,079			
14	*U. nidifica* (LW10)	0,019	0,017	0,123	0,025	0,084	0,019	0,113	0,063	0,123	0,084	0,123	0,084	0,019		
15	*U. fragilescens* (LW6)	0,066	0,063	0,125	0,063	0,059	0,066	0,110	0,000	0,125	0,059	0,125	0,059	0,065	0,063	
16	*U. rubrotincta* (LW4)	0,070	0,068	0,125	0,072	0,067	0,070	0,103	0,041	0,125	0,067	0,125	0,067	0,070	0,068	0,041

The ABGD species delimitation method retrieved four "initial partitions" and five "recursive partitions" with all samples of *U. bismolliuscula* are monospecific. Only when values of intraspecific divergence (P) were lower than 0.0046 was a higher number of groups suggested (5) and only by the recursive analysis resulting in the split of *U. nidifica* in two different lineages; both represented the specimens from Lawu (Table 3, Figure 5).

The topology of the phylogeny based on ITS rDNA sequences showed that within the clade of subgenus *Usnea*, *U. himalayana* is closely related to *U. nidifica* and *U. bismolliuscula* is more closely related to *U. rubrotincta* than *U. fragilescens*. Within the clade of subgenus *Eumitria*, *U. pectinata* is closely related to *U. baileyi* (Figure 6).

In the *Usnea* subgenus clade, it shows *U. himalayana* and *U. nidifica* to form one clade, which means that among these species, they have the same ancestor (monophyletic) supported by a very high bootstrap value of 99%. The bootstrap shows that *U. himalayana* is very closely related to *U. nidifica.* The phylogenetic topology shows *U. himalayana* (LW 2) separated from its fellow species and forming a clade with *U. nidifica* (LW8), which is also separated from fellow species *U. nidifica* (LW 10). This indicates that *U. himalayana* (LW2) and *U. nidifica* (LW 10) began to navigate from their fellow

Table 3. The specific composition of the four and five groups hypotheses derived from ABGD analysis.

Group	Four group hypothesis species composition	Group	Five group hypothesis species composition
1	*U. himalayana* (LW1), *U. himalayana* (LW2), *U. himalayana* (LW11), *U. himalayana* (LW5), *U. nidifica* (LW8), *U. nidifica* (LW10)	1	*U. himalayana* (LW1), *U. himalayana* (LW2), *U. himalayana* (LW11), *U. himalayana* (LW5), *U. nidifica* (LW8)
2	*U. pectinata* (LW3), *U. baileyi* (LW7), *U. baileyi* (T1), *U. baileyi* (T3)	2	*U. pectinata* (LW3), *U. baileyi* (LW7), *U. baileyi* (T1), *U. baileyi* (T3)
3	*U. bismolliuscula* (T5), *U. bismolliuscula* (T2), *U. bismolliuscula* (T4)	3	*U. bismolliuscula* (T5), *U. bismolliuscula* (T2), *U. bismolliuscula* (T4)
4	*U. fragilescens* (LW9), *U. fragilescens* (LW6), *U. rubrotincta* (LW4)	4	*U. fragilescens* (LW9), *U. fragilescens* (LW6), *U. rubrotincta* (LW4)
		5	*U. nidifica* (LW10)

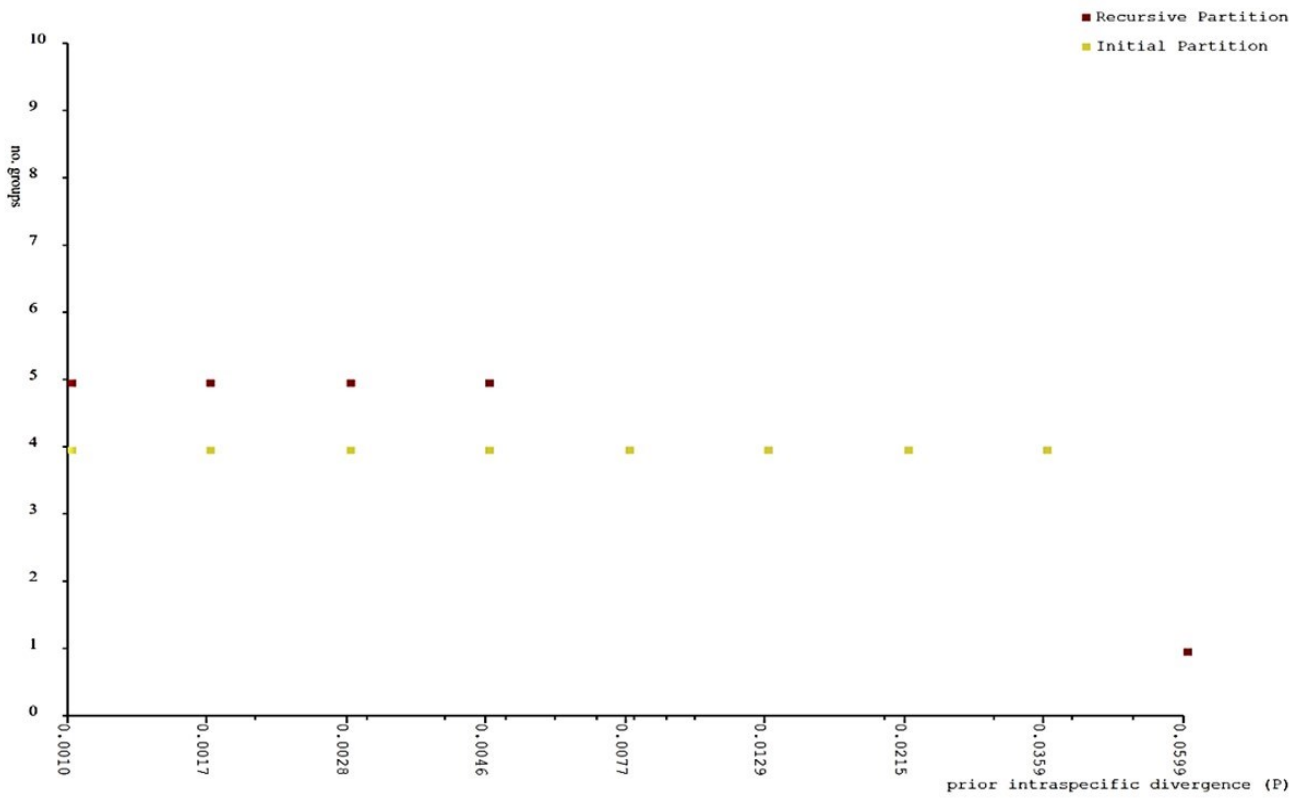

Figure 5. Results of the Automatic Barcode Gap Discovery (ABGD) analysis showing the four partitions (initial) also 5 and 1 (recursive) recovered. Nearly all partitions suggested the different number of species (≠ No of groups) with all representatives of *U. bismolliuscula* rendered monospecific. Only recursive partitions rendered a higher number of species (5) when P values were ≤ 0.0046.

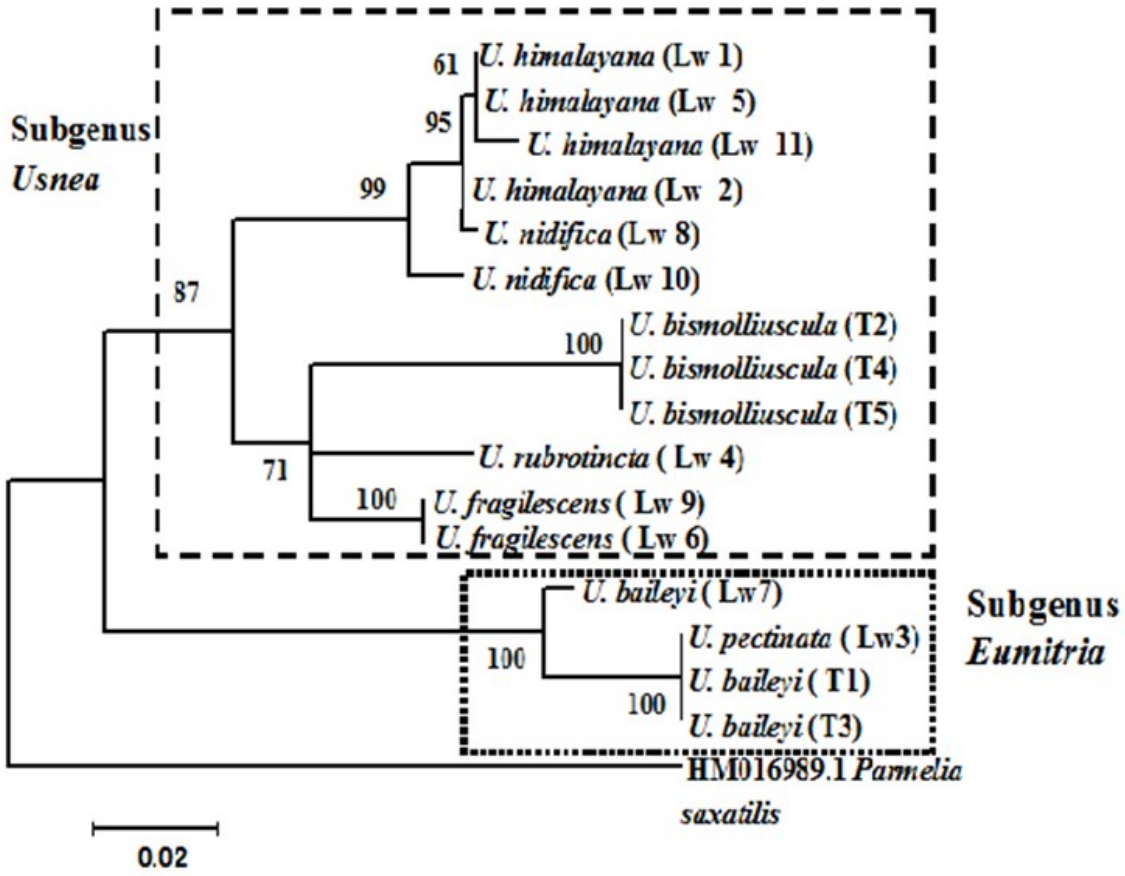

Figure 6. Phylogram obtained from a Maximum Likelihood analysis, evolution model Kimura-2 parameter with 1000 bootstrap replications, showing the phylogenetic relationship among *Usnea* based on internal transcribed spacer (ITS) rDNA.

species. This is in line with the opinion of Ohmura (2012) which stated that *U. himalayana* and *U. nidifica* have very close genetic relationships both morphologically and molecularly. The morphology between *U. himalayana* and *U. nidifica* is only distinguished by the absence of soralia in *U. himalayana*.

The alignment results of 515 bp ITS rDNA of *U. himalayana* with *U. nidifica* showed that there were substitutions, namely ten transitions and three transversions (72, 486, and 490 base positions) (Table 4). The alignment results showed the insertion of 12 nucleotide *U. himalayana* (LW 1) (TTCTACGTCGGT) in the 79th to 90th base positions (Table 5).

The subgenus *Eumitria* clade consists of 4 specimens (LW3, LW7, T1, and T3), which are divided into two species, namely *U. pectinata* and *U. baileyi*. The phylogenetic tree topology formed shows that *U. baileyi* (T1 and T3) formed one clade with *U. pectinata* (LW3) supported by a very high bootstrap value of 100%. Instead, *U. baileyi* separated from its fellow species and was supported by a value high bootstrap of 100%. It can be said that, based on ITS rDNA, *U. baileyi* (T1 and T3) is more closely related to *U. pectinata*, than to its fellow species (LW7). Ohmura (2002) stated that phylogenetic result based on ITS rDNA sequences strongly suggests that the close relationship between *U. pectinata* and *U. baileyi*.

Table 4. Nucleotide base substitution in ITS rDNA of *U. himalayana* and *U. nidifica*.

Sample	Variation of Nucleotide Base												
	57	59	68	71	72	103	107	202	237	351	359	486	490
LW 1 (*U. himalayana*)	C	G	T	A	C	G	T	G	G	C	T	G	G
LW 2 (*U. himalayana*)	.	.	.	.	.	.	C	.	.	.	.	.	.
LW 11 (*U. himalayana*)	T	.	.	.	.	.	.	.	.	.	C	.	T
LW 5 (*U. himalayana*)	.	.	.	.	.	.	.	.	.	.	.	.	.
LW 8 (*U. nidifica*)	.	.	.	.	.	.	C	.	.	.	.	T	.
LW 10 (*U. nidifica*)	.	A	C	G	G	A	C	A	A	T	.	.	.

Note: green shows transition and red shows transversion.

Table 5. Insertion in ITS rDNA nucleotide bases of *U. himalayana* and *U. nidifica*.

Sample	Variation of Nucleotide Base											
	79	80	81	82	83	84	85	86	87	88	89	90
LW 1	T	T	C	T	A	C	G	T	C	G	G	T
LW 2	-	-	-	-	-	-	-	-	-	-	-	-
LW 11	-	-	-	-	-	-	-	-	-	-	-	-
LW 5	-	-	-	-	-	-	-	-	-	-	-	-
LW 8	-	-	-	-	-	-	-	-	-	-	-	-
LW 10	-	-	-	-	-	-	-	-	-	-	-	-

Table 6. Nucleotide similarity in ITS rDNA of *U. baileyi* and *U. pectinata.*

	***U. pectinata*(LW3)**	***U. baileyi* (LW7)**	***U. baileyi* (T1)**	***U. baileyi* (T3)**
***U.pectinata*(LW3)**	---	15/499	0/499	0/499
***U. baileyi* (LW7)**	96.99	---	15/499	15/499
***U. baileyi* (T1)**	100.00	96.99	---	0/499
***U. baileyi* (T3)**	100.00	96.99	100.00	---

Table 7. Nucleotide Base Substitution in ITS rDNA of *U. baileyi* and *U. pectinata.*

Sample	Variation of Nucleotide Base													
	35	44	51	92	137	334	346	361	378	436	439	443	446	448
***U.pectinata*(LW3)**	G	C	T	A	A	C	T	G	A	T	T	T	T	T
***U. baileyi* (LW7)**	T	T	C	G	G	T	C	A	G	A	C	C	A	C
***U. baileyi* (T1)**	.	.	.	.	.	.	.	.	.	.	.	.	.	.
***U. baileyi* (T3)**	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Note: green shows transition and red shows transversion.

This statement is reinforced by the results of the similarity analysis of nucleotide bases, from the compared 494 nucleotide bases ITS rDNA, which shows that between *U. pectinata* and *U. baileyi* (T1 and T3) there is not a single difference in nucleotide bases. At the same time, *U. baileyi* (LW7) and its fellow species only had a 96.99% similarity of nucleotide bases (15 of 499 differences in compared nucleotide) (Table 6).

Usnea baileyi (LW7), which separates from its fellow species, is indicated by a rather long branch in the phylogenetic tree topology, indicating that this species is starting to experience divergence. The alignment result supports the divergence, showing the existence of substitution in the form of 11 transitions and three transversions (G-T, T-A) at the position of the 35th, 436th, and 446th nucleotide bases (Table 7).

The results of this study are in line with what Ohmura (2002) did, that *U. pectinata* and *U. baileyi* shared a common ancestor to form one clade, which was supported by a boost value of 99%. A similar case was reported by Articus et al. (2002), the unclear separation between *U. florida* and *U. subfloridana* also in *U. rigida* and *U. barbata*. Based on a phylogenetic approach using β-tubulin and ITS-LSU, *U. floridana* and *U. subfloridana* clustered in one clade supported with high bootstrap values. This also happened to *U. barbata* and *U. rigida*, so the concept of species pair was proposed among them.

Based on the results of this study, it shows that in some *Usnea* species such as *U. himalayana* with *U. nidifica*, also in *U. pectinata* and *U. baileyi* indicates the starting of species divergence. It is necessary to carry out further comprehensive research so that the position of the taxon can be clearly seen. In the genus *Usnea*, only a few morphological characters are distinguished from one species to another, and most of them are only distinguished by one character. Some of them are only distinguished by their reproductive organs, whereas the environment very much influences the appearance of reproductive organs. The determination of the species name in the genus *Usnea* follows the concept of species presented by Motyka et al. (1936-1938)

in Clerc (1998), that was "A species is a strong character that has a little variation between them (1 character = 1 species)". The result of this study needs further and thorough observation related to the nomenclature in the genus *Usnea*.

CONCLUSION

The ITS rDNA sequence can be utilized to strengthen the identification and investigation of relationships within the *Usnea* genus. The ITS rDNA sequence was able to identify most of the *Usnea* spp. However, it cannot distinguish between *U. baileyi* and *U. pectinata*. So that further research is needed by using more distinguishable sequences with a faster evolutionary rate so that differences in species of *Usnea* can be solved.

AUTHORS CONTRIBUTION

Contribution of the author: **M.J.** designed the research, collected and analyzed the data, and wrote the manuscript, **M.R.H.** analyzed the data and wrote the manuscript, **R.S.K.** and **N.S.N.H.** supervised all the processes.

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CONFLICT OF INTEREST

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

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Research Article

The Abundance of Fish Species in Branched (*branching*) and Table (*tabulate*) Coral Habitat on Tanjung Beach, Muna Island, Southeast Sulawesi

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ABSTRACT
This research aimed to study the types and abundance of fish which was found in branched and table coral habitat. The quadrate transect method was used at the location of the habitat of branched and table corals. The observation was carried out for 3 days. It was conducted 3 times/day, in the morning, the afternoon, and the evening with ± 10 minutes of observation time for each observation. The method used was transect quadrat 5 m x 5 m with direct observation techniques by means of snorkeling. The types and numbers of individual fish were recorded using the visual census method. Snorkeling was done straightforwardly and looking around by following the contour of the reef. In branching coral habitat, 11 species of fish were found with a total of 66 individuals. On the other hand, on the tabulate reef habitat, there were 9 species of fish with a total of 50 individuals. Both habitats have different types of fish. In the branched coral habitats there were more individual than in the table reef habitats. Fish species found in branched coral habitat and table type fish habitat were different. In branched coral habitats were found more reef fish species than in table coral habitats. These results illustrated that the condition of the coral reefs at Tanjung Beach is still in good condition.

Keywords: abundance, branched coral, reef fish, table coral, transect square

INTRODUCTION
The coral reef ecosystem is one of the marine ecosystems in habited by various type of marine life. The coral reef ecosystems are part of the marine ecosystem which is the place of life for various types of biota (Burke et al. 2012). Biota that lives on coral reefs is a community that consists of various tropical levels, where each component in this community is interdependent with each other and forming a complex ecosystem. One type of biota that lives on coral reefs is reef fish, with a high level of species diversity (Odum 1996). Coral reefs have ecological functions as a spawning ground, nursery

ground, and feeding for reef fish. Therefore the damage of coral reefs will affect the diversity and abundance of reef fish.

Corals have a variety of different growth forms. Various types of coral growth are affected by the intensity of sunlight, current waves, and sediment. The form of stony coral growth is divided into Acropora and non-Acropora corals. This type of branching coral is a type of *Acropora* coral (English et al. 1994). Branched coral is a type of coral with longer branches like tree branches. One type of branching coral is *Acropora abrolhosensis*. Table coral is a branching coral with a horizontal and flat direction like a table. This coral is supported by a rod that is centered or resting on one side which forms a flat angle. One type of table coral is *Acropora latistella* (Veron 2000).

The current condition of coral reef ecosystems in various territorial waters in Indonesia is in quite a rapid degradation. Southeast Sulawesi until 2016 having serious damage to coral reefs, around 80% of coral reefs are in a severely damaged condition (Department of Marine Affairs & Fisheries of Southeast Sulawesi Province 2016). The condition of coral reefs in Indonesia was around 6.39% in the very good condition level, 23.40% good, 35.06% damaged, and 35.15% in very critical conditions (Giyanto et al. 2017).

Tanjung Beach is one of the coastal areas in the Tongkuno district of Muna Regency, Southeast Sulawesi which is bordered by Buton Strait and has quite high fisheries resources, where around 80% of the people in this area work as fishermen. We identified there were four types of corals in Tanjung Beach. There was growth type with ramified (branching), solid growth type (massive), leaf type (foliose), and table form (tabulate). Coral growth type on the coast of Tanjung is dominated by branching form and the shape of table corals that are still in good condition (Department of Marine Affairs & Fisheries of Southeast Sulawesi Province 2016).

This is related to the interest of various species of fish in certain coral habitats that provide a variety of needs such as, places to find food, shelter, breeding places, and others. The presence of fish species against certain coral habitats may also be related to fluctuations in environmental conditions. Several studies reveal that the factors that influence the presence of fish (community structure and abundance of fish) in a reef community include a high and low percentage of coral cover (Bell & Galzin 1984). Besides the physical condition of the waters such as currents, brightness, and temperature were greatly affect the existence of reef fish (Nybakken & Bertness 2005).

The purpose of this study was to determine the types and abundance of fish found in branched coral habitat and table coral habitat.

MATERIALS AND METHODS

Materials

Animals

Fish species are found in branched coral habitats and reef fish species in tabulated reef habitats on Tanjung Beach, Muna Island Southeast Sulawesi.

Instruments

In this study, instruments used were boats or canoes as transportation during observation, GPS (Global Position System) to determine the coordinates of the research location, underwater writing instruments (water proof) to record observational data, underwater cameras to take documentation pictures, hand refractometer to measure sea water salinity, thermometer to measure sea water temperature, roller meter, and ping pong ball to measure current velocity, raffia rope to make square plots in coral reef communities, and coral fish identification book for identification.

Methods

This research was conducted from December 2017 to January 2018. The coordinate points of coral fish observation in branching coral habitats are 05° 36'42.5 LS and 122° 42'59 BT, observation of coral fish in table coral habitats, namely 05° 36'42.5 LS and 122° 44'51 BT (Figure 1).

Data collection for reef fish was carried out using the quadrat transect method 5 m x 5 m. Observation of reef fish using a visual census method by snorkeling on the surface of the water straight ahead and following the contour of the reef (poking left and right) (English et al. 1994). The visual census was carried out for 3 days, in which 3 observations/day, namely morning, afternoon, and evening, with a length of observation of ± 10 minutes for each observation. Fish found during the visual census were documented, their species observed and the number of each individual was counted.

The collected data analyzed quantitatively, which was measured against the parameters of the reef fish community, namely the abundance and Similarity Index (IS) (Muller-Dumbois & Ellenberg 1974).

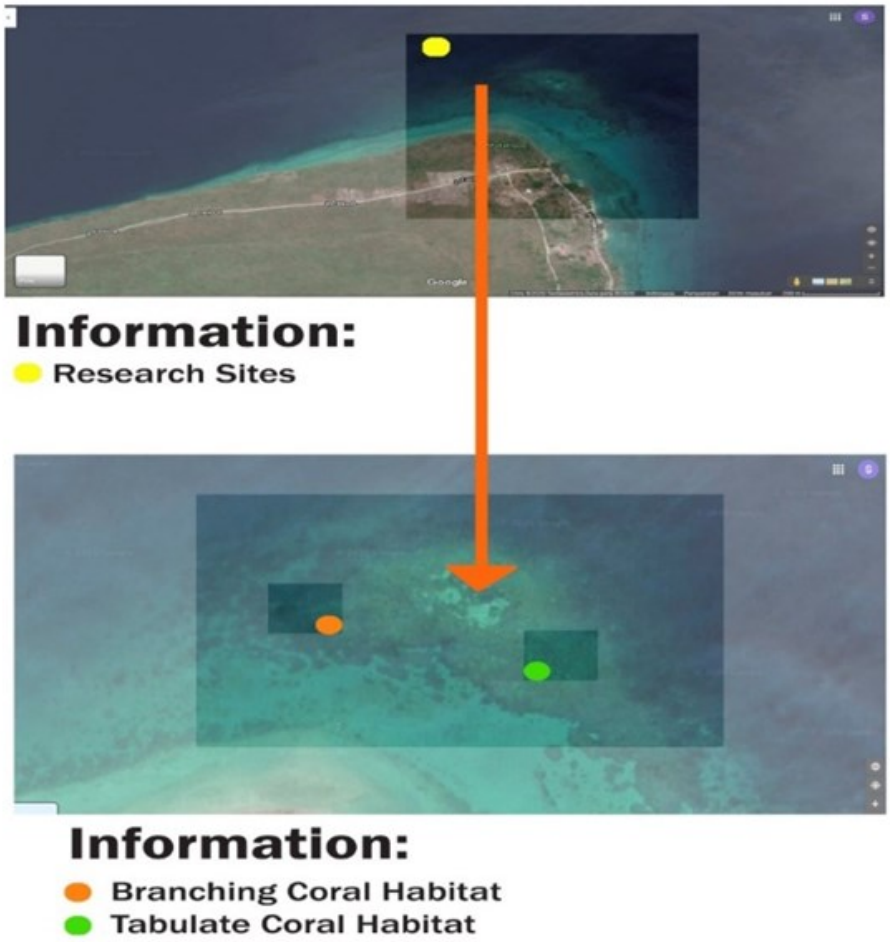

Figure 1. Research location for reef fish in Tanjung Beach, Muna Island, Southeast Sulawesi.

RESULTS AND DISCUSSION

The physical condition of the environment at Tanjung Beach

Physical environmental condition is one of the indicators that greatly affect the life and survival of various biota. Based on the results of the survey, the condition of the coral at Tanjung beach was dominated by 2 types of coral, namely the type of branching coral (branching) and the type of table coral

(tubulate). The existence of several types of reef fish in the community of table and branching corals is certainly influenced by water conditions or environmental parameters. Physical environment parameters for branching and tubulate coral types include water temperature, current velocity, and salinity. Environmental parameters measured in branching and tabulate coral habitats are presented in Table 1.

Table 1. The Measurement result of environmental parameters in the research location.

No.	Location	Parameters		
		Water Temperature (°C)	Salinity (‰)	Current speed (m s^{-1})
1.	Branching Coral	31	31.88	0.20
2.	Tabulate Coral	30	31.00	0.21

The physical condition of the environment in branching coral habitat (branching) and table reef habitat (tabulate) relatively has similar measurements. The physical condition of these waters is still in the optimal range of marine life to survival, especially reef fish. The optimal temperature for the reef fish to grow is between 25-32 °C, this temperature range is generally found in tropical climates such as Indonesia (Whitten et al. 1984). The classified current speeds into several categories, very fast (>1 m/sec), fast (0.5-1 m/sec), moderate (0.25-0.5 m/sec), slow (0.1-0.2 m/sec) and very slow (<0.1 m/sec) (Laevastu & Hayes 1982). The currents can spread fish larvae as well as the food for reef fish such as plankton and currents scatter eggs and also larvae of various aquatic animals to reduce food competition with their mothers (Koesoebiono 1981).

Fish species found in branching and tabulate coral habitat

The highest presence and abundance were found in branching coral habitats compared to table coral habitats. 11 species of branching corals and 9 species of table corals were found. Fish species found in branching and tabulate coral habitat were presented in Table 2.

Table 2. Fish species found in branching and table reef habitat.

Family	Name of Species	Branching Coral	Tabulate Coral
Pomacentridae	*Amblyglyphidodon curacao*	12	8
	Amblyglyphidodon aureus	-	5
	Dischistodus melanotus	-	5
Siganidae	*Siganus vulpinus*	4	5
Pomacanthidae	*Chaetodontoplus mesoleucus*	5	-
Chaetodontidae	*Heniochus varius*	4	-
	Chaetodon triangulum	-	3
Labridae	*Cheilinus fasciatus*	5	-
	Halichoeres chloropterus	-	11
	Thalassoma lunare	-	8
Scaridae	*Scarus bleekeri*	4	-
	Scarus schlegeli	-	3
	Scarus Oviceps	-	2

Table 2. Contd.

Family	Name of Species	Branching Coral	Tabulate Coral
Lutjanidae	*Lutjanus biguttatus*	5	-
	Lutjanus monostigma	3	-
Apogonidae	*Apogon chrysopomus*	12	-
	Cheilodipterus artus	8	-
Nemipteridae	*Scolopsis margaritifera*	4	-
	Total of individual numbers	**66**	**50**

Based on Table 2, 18 species of reef fish were found and divided into 9 families. 11 species of branching corals and 9 species of table corals were found. There were 2 types of reef fish with the highest individual chopping on branching coral types, namely *Amblyglyphidodon curacao* 12 individual and *Apogon chrysopomus* 12 individual. On the other hand, there were 3 types of reef fish with the highest individual chopping on table coral, namely 11 individuals of *Halichoeres chloropterus*, 8 individuals of *Amblyglyphidodon curacao*, and 8 individuals of *Thalassoma lunare*.

The discovery of several species of fish with various individual numbers in the branched corals habitat can illustrate that there are a lot of micro habitats in branching corals. This allows these reef fish to use them together based on their needs such as spawning, foraging, and protection. Besides, the complex form of corals, branched, and the cavernous structure makes this type of coral attractive for fish to form their colony (Allen 1997; Thresher 1984).

Based on Table 2, there are 2 types of fish found in these 2 types of coral. These two species of fish are *Amblyglyphidodon curacao* and *Siganus vulpinus*. From this result observation *Amblyglyphidodon curacao* and *Siganus vulpinus* conjectured attracted with branching coral habitat and tabulate coral habitat. The species of reef fish would show a preference or suitability to the two habitats, it means the suitable habitat determines the abundance of these fish (Rondonuwu 2014).

The fish preference for certain habitats causes species diversity in coral reefs (Triana 2004). The complexity of habitat structure and environment of coral reefs plays an important role in the structure of the reef, therefore, fish communities enabling to share a habitat with many species and the existence and resilience of a fish species in one or more habitats depending on the habitat carrying capacity (Chabanet et al. 1997; Lee & Shin 2013).

The fish similarity index in branching and tabulate coral habitat

The similarity index analysis of reef fish species in branching and table coral habitats showed very different results. The results of the index calculation of fish species similarity between branched coral habitat (branching) and table reef (tabulate) are presented in Table 3.

Table 3. Similarity and dissimilarity index of branching and tabulate coral habitat.

IS/ID	Branching Coral	Tabulate Coral
Branching Coral	-	79.31
Tabulate Coral	20.69	-

Based on Table 3, the Similarity Index (IS) value between types of fish in branched coral habitat and table coral habitat was obtained 20.69. The results of analysis about the similarity of coral fish communities by using the

similarity coefficient (Similarity) of Sorensen show that the similarity of the community is relatively different in branching coral habitat and tabulate coral habitat. The similarity value <50% or 0.49 <0.50 there are differences in community structure from the two coral habitats (Odum 1996). This means, a low similarity index showing an indication that the types of fish occupied in branched coral habitat and table coral habitat are not the same. This means that the calculation of the similarity index aims to compare the composition and variations in the quantitative value of species at a location or habitat. This proves that the structure of the habitat complexity of branching and table corals in the research location is still in good condition (Ford et al. 2017).

CONCLUSION

In branching coral habitat founded 11 species of fish. *Amblyglyphidodon curacao* and *Apogon chrysopomus* are types of fish that have the highest individual numbers that were 12 species, while *Lutjanus monostigma* is a type of fish that has the lowest number of individual counts, namely 3. In the tabulate coral habitat, 9 species of fish were found. There are 3 types of fish with the highest individual number namely *Halichoeres chloropterus* 11 species, *Amblyglyphidodon curacao* 8 species, and *Thalassoma lunare* with 8 species. Conversely, the lowest individual numbers were *Chaetodon triangulum* and *Scarus schlegeli,* each of them with 3 species, and *Scarus oviceps* with 2 species. The Similarity index of fish in branching coral habitat and tabulate coral habitat is 20.69%. These results illustrate that the condition of the coral reefs at Tanjung Beach is still in good condition.

AUTHORS CONTRIBUTION

MT conduct research in the field, collected and analyzed the data and wrote the manuscript. MS guides and designs studies and carries out fieldwork.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Research Article

Potential Screening of Bacteriocinogenic-Lactic Acid Bacteria from Mangrove Sediment of Logending Beach for Fisheries Product Preservation

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ABSTRACT
The meat and fisheries products have high nutritional content which is highly ideal for bacterial growth. Lactic Acid Bacteria (LAB) have several potential advantages as a bio-preservative agent in the food industry because they produce antimicrobial substances against pathogenic bacteria e.g. bacteriocin. Our previous study has succeeded in isolating and characterizing LAB from the mangrove sediments of Logending Beach, Kebumen. This present study aimed to determine the activity of bacteriocinogenic-LAB against food-borne pathogens and their potential for fisheries product preservation. The study consisted of five serial stages, as follows: screening of LAB isolates, cell-free supernatant production and its inhibition activity, extraction of partially purified bacteriocin, bacteriocin confirmation against proteolytic enzymes, and in-vitro test of partially-purified bacteriocin against *Listeria monocytogenes, Shigella flexneri,* and *Salmonella typhi.* A total of 25 out of 99 isolates were able to grow on MRSA+1% $CaCO_3$ medium. Initial screening showed that the cell-free supernatant of 14 LAB isolates was able to inhibit the growth of *S. thypi*, *S. flexneri*, and *L. monocytogenes*. There was an increased inhibitory activity of partially purified bacteriocin when compared with the cell-free supernatant which was statistically different ($p<0.01$). It indicated that the purification was successfully performed. Bacteriocin expressed a lower inhibition against *S. typhi* than *L. monocytogenes* and *S. flexneri*. The ANOVA test showed that each indicator pathogenic-bacterium expresses a very significant sensitivity to the partially purified bacteriocin.

Keywords: Bacteriocin, fisheries product, LAB, mangrove sediments, food-borne pathogens

INTRODUCTION
The human population in the world is increasing rapidly which influences the high demand for food availability. In this modern era, the safety, quality, and health profile of food products are being a big concern. The meat and fisheries products have high nutritional content. On the other hand, they are highly ideal for bacterial growth e.g. *Listeria monocytogenes, Salmonella* spp., and *Shigella* spp. which cause food-borne diseases (Jennison & Verma 2004; Novotny et al. 2010).

Bacterial contamination of seafood and its products affects the nutritional properties or undesirable organoleptic changes. The contamination may take place during harvesting, handling, preparation, processing, transportation, and storage. Many food treatments have been applied to control bacterial contamination, such as salt administration, smoking, canning, freezing, and vacuum application. However, these treatments are not sufficient to kill pathogenic microorganisms since some bacteria have been reported to be resistant to high salt concentration, drying, freezing, and heat processes (Dupard et al. 2006).

Bio-preservation is a promising approach to control bacterial contamination and extend the shelf life of foods, including the application of living microorganisms and their metabolites (Moradi et al. 2020). Bacteriocin, a secondary metabolite of Lactic Acid Bacteria (LAB), has several potential advantages as a bio-preservative agent in the food industry including (a) generally recognized as safe substances, (ii) inactive and nontoxic on eukaryotic cells, (iii) inactivated by digestive proteases, (iv) usually pH and heat-tolerant, and (v) have a relatively broad antimicrobial spectrum against many food-borne pathogenic and spoilage bacteria (Siedler et al. 2019).

LAB are reported to be able to produce antimicrobial substances, such as carbon dioxide, diacetyl, acetaldehyde, ethanol, hydrogen peroxide, and bacteriocin (de-Vuyst & Leroy 2007; Liao & Nyachoti 2017). Bacteriocin has bactericidal or bacteriostatic effects (Cheong et al. 2014; Gerez et al. 2013; Yang et al. 2012) against various foodborne pathogens, such as *Staphylococcus aureus, Bacillus cereus, L. monocytogenes, Clostridium botulinum,* and *E. coli* (Aunpad et al. 2007; Dobson et al. 2012).

LAB are commonly isolated from fermented food products, milk, and the intestinal tract of humans or animals (Dobson et al. 2012; Kusharyati et al. 2020). Meanwhile, only a few studies isolated LAB from soils, water, and plants. Mangroves are unique ecosystems that harbor unique and diverse microorganism groups, such as fungi, actinomycetes, and bacteria. Our previous study has succeeded in isolating and characterizing LAB from the mangrove sediments of Logending Beach, Kebumen. This present study aimed to determine the activity of bacteriocinogenic-LAB against food-borne pathogens and their potential for fisheries product preservation.

MATERIALS AND METHODS

The study consisted of five serial stages, as follows: screening of LAB isolates, cell-free supernatant production and its inhibition activity, production of partially-purified bacteriocin, bacteriocin confirmation against proteolytic enzymes, and in-vitro test of partially purified bacteriocin against *L. monocytogenes, Shigella flexneri,* and *Salmonella typhi.*

Screening of bacteriocinogenic-LAB

One loop of isolates originating from mangrove sediments of Logending Beach Kebumen was grown on the de-Man Rogose Sharpe Agar (MRSA) medium (Oxoid) which was supplemented with 1% $CaCO_3$. The spot inoculation was performed triplicate in each dish. Bacterial incubation was carried out for 48 hours at 37^{o}C. Isolates that expressed a clear zone around the growing colony were assumed as Lactic Acid Bacteria. The bacterial characterization was performed following Hendrati et al. (2017). The selected isolates were stocked on MRSA slant medium. The pathogenic bacteria, *S. typhi*, and *S. flexneri* were re-cultured using Salmonella Shigella Agar (SSA) medium (Merck), meanwhile, *L. monocytogenes* using Nutrient Agar (NA) medium (Merck).

Pre-screening of LAB's inhibition activity

One loop of LAB isolate was inoculated in MRS Broth (MRSB) medium and incubated for 18 hours at 37°C. The bacterial culture was centrifuged for 10 minutes at 10,000 rpm to obtain a bacterial cell-free supernatant (CFS). Its inhibition activity was evaluated using Kirby's Bauer method by following Hendrati et al. (2017). The pathogenic bacteria were inoculated on Nutrient Broth (NB) medium (1% v/v), then was incubated for 8 hours at 37°C. The pathogenic bacteria were spread on NA medium. A 6 mm diameter paper disc was dropped by 20 µL of cell-free supernatant, then was placed into the pathogenic bacterial lawn-medium. The assay was performed triplicate in each dish. Incubation was carried out for 24 hours at 37°C. The formed inhibition zone was observed and calculated.

Production of partially-purified bacteriocin

One loop of LAB isolates was inoculated into 10 mL MRSB medium, then was incubated for 18 hours at 37°C. This culture was used as an inoculum. One milliliter (~ 10^8 CFU/mL) of inoculum was inoculated into 100 mL MRSB medium, then was incubated at 37°C for 24 hours. The 24-hour LAB culture was cold-centrifuged (Thermo Scientific) at 10,000 rpm, 4°C for 10 minutes. The supernatant was salted out by adding ammonium sulfate. The mixture was homogenized using a magnetic stirrer. The 50% ammonium sulfate was slowly added to gradually precipitate the bacteriocin until the end of saturation. The precipitated bacteriocin was separated from the mixture by performing a cold-centrifugation at 10,000 rpm for 15 minutes. Then, the partially-purified bacteriocin was dissolved into 2 mL of 0.1 M phosphate buffer saline (PBS) with pH 5.3.

Bacteriocin confirmation against proteolytic enzymes

A total of 200 µL partially-purified bacteriocin was mixed with 20 µL of proteolytic enzyme solution (one gram of proteolytic 'papain' enzymes was dissolved into 1 mL of 0.1 M PBS with pH 5.3), then was incubated at 37°C for 2 hours. Its inhibition activity was evaluated using Kirby's Bauer method by following Hendrati et al. (2017). The degraded bacteriocin did not show a clear zone around the paper disc (Nithya et al. 2012).

In-vitro test of partially-purified bacteriocin against pathogenic bacteria

The inhibition activity of crude bacteriocin was evaluated using Kirby's Bauer method following by following Hendrati et al. (2017). The diameter of the inhibition zone expressed by CFS and partially-purified bacteriocin were then compared to evaluate the success of bacteriocin extraction.

Data analysis

The data of inhibitory activity of partially-purified bacteriocin against pathogenic bacteria was analyzed by using an analysis of variance (ANOVA) at 95% and 99% levels of confidence.

RESULTS AND DISCUSSION

The consumption of seafood in various countries is increasing since the global population is increasing rapidly (Elbashir et al. 2018). Food spoilage results in undesirable odor, texture, and appearance which make it unfit for consumption (Odeyemi et al. 2020). LAB is effective to enhance the shelf life of food products and inhabit undesirable microorganism growth in food (Xi et al. 2018). Therefore, LAB has been broadly applied in various food industries. Food industries commonly use individual and/or consortium of different LAB, including genera *Lactococcus* sp., *Streptococcus* sp., *Pediococcus* sp.,

Enterococcus sp., *Lactobacillus sp., Leuconostoc* sp., and *Weissela* sp. (Hamad et al. 2020; Mokhtar et al. 2016; Nithya et al. 2012).

In this present study, we screened and determined the inhibitory activity of bacteriocinogenic-LAB against food-borne pathogens. A total of 99 bacterial isolates originating from mangrove sediment of Logending Beach (unpublished data) were grown on MRSA+1% $CaCO_3$ as a selective medium for Lactic Acid Bacteria groups. A total of 25 isolates were able to grow on MRSA+1% $CaCO_3$ medium and expressed a clear zone around the colonies. It indicated the ability of bacteria to grow and dissolve the $CaCO_3$ in the medium (Mahulette et al. 2016). Gram-positive, non-spore-forming, and Catalase negative isolates were selected for further analysis (data not shown). Pringsulaka et al. (2012) described a LAB as Gram-positive, non-spore-forming, cocci, cocci-bacilli, or rods-shape cells, have anaerobic respiration, and Catalase-negative.

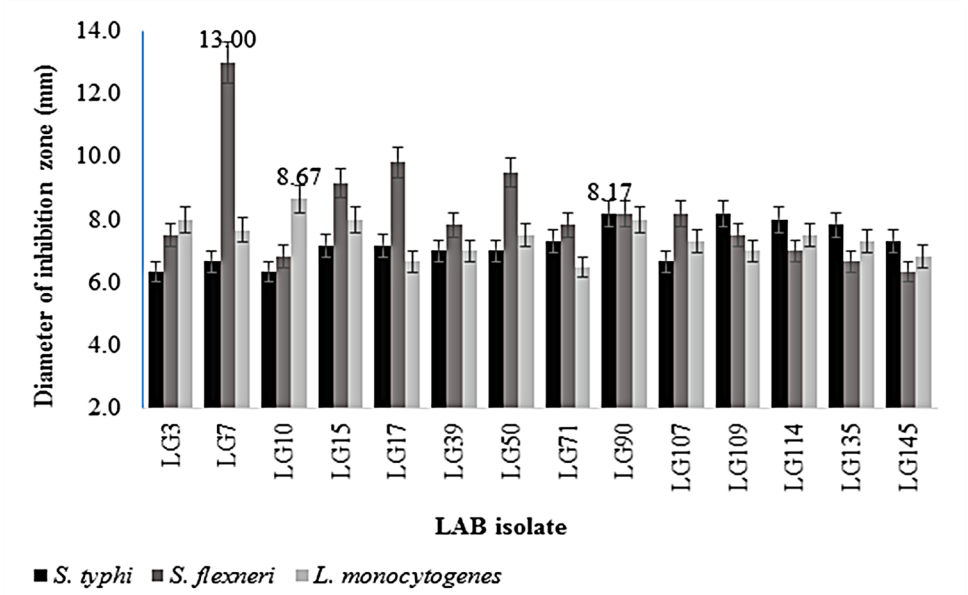

Figure 1. The diameter of the inhibition zone of cell-free supernatant against pathogenic bacteria.

Garrido et al. (2012) reported three major foodborne pathogens affecting people worldwide are *Salmonella* spp., *Shigella* spp., and *Listeria monocytogenes*. Salmonella genus is the leading cause of food-borne outbreaks and remains a major public health concern. *Salmonella* spp. are Gram-negative, rod-shaped, facultatively anaerobic, usually motile, Catalase-positive, and Oxidase-negative. Salmonella produces enterotoxins and causes inflammatory reactions and diarrhea. Symptoms often start 12-72 hours after the ingestion of contaminated food. Salmonella infections from the consumption of seafood products are most commonly associated with raw, undercooked, and poorly cooked seafood (Iwamoto et al. 2010).

Shigella spp. are reported as the third most frequently isolated bacteria from foodstuffs (WHO 2005). *Shigella flexneri* is reported as responsible for shigellosis outbreaks in developing countries (Shahin et al. 2018). *Shigella* spp. are Gram-negative, rod-shaped, non-motile, Oxidase-negative, and non-lactose fermenting bacteria. Shigella produces enterotoxin 1 and 2 which causes watery loose stool, fever, abdominal pain, and bloody diarrhea (Scallan et al. 2011).

Listeria monocytogenes is a Gram-positive, rod-shaped, non-spore-forming, Catalase-positive, and glucose fermenter. The bacterium can adapt to various environmental conditions such as a broad range of temperatures, pH, and high salt content (Aspri et al. 2017). Food including seafood

contamination by *L. monocytogenes* is recognized as a public health and food safety concern since early 1981. The transmission to humans is mainly through the consumption of ready-to-eat foods (Miya et al. 2010).

The LAB cell-free supernatant was tested against both Gram-negative and positive pathogenic bacteria i.e. *S. typhi, S. flexneri,* and *L. monocytogenes.* A total of 14 LAB isolates could inhibit all tested pathogenic bacteria. This is indicated by the presence of a clear zone or zone of inhibition around the paper disc (previously dropped by cell-free supernatant) on the bacterial lawn. The largest inhibition against *S. typhi* was shown by LAB isolate LG109 with an average diameter of 8.17 mm. The largest inhibition against *S. flexneri* was shown by LAB isolate LG7 with an average diameter of 13.00 mm. While the largest inhibition against *L. monocytogenes* was shown by LAB isolate LG10 with an average diameter of 8.67 mm. All LAB isolates express a broad and strong inhibition (Figure 1). Pan et al. (2009) categorized the more than 6 mm clear zone expresses a strong inhibition activity, a 3-6 mm clear zone as moderate, while a 0-3 mm clear zone as weak inhibitory. Some research reported the antimicrobial activity of bacteriocin against both Gram-negative and positive bacteria (Cotter et al. 2005; Duranti et al. 2017; Martinez et al. 2013).

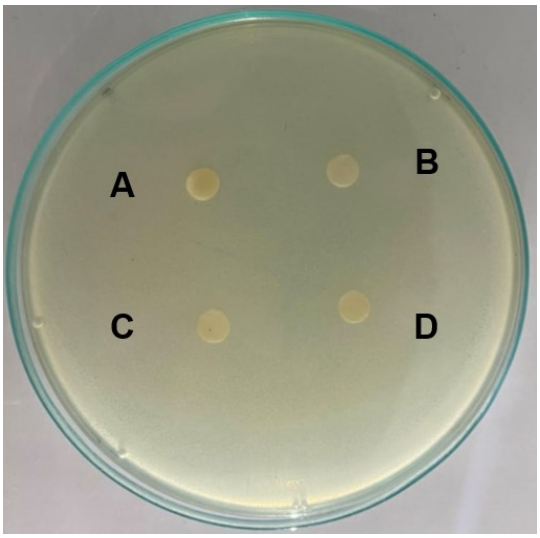

Figure 2. Bacteriocin confirmation against proteolytic enzymes, no clear zone was shown around the paper disc (A: isolate LG50, B: isolate LG135, C: isolate LG73, D: isolate LG17).

The presence of bacteriocin was confirmed by mixing the cell-free supernatant with proteolytic enzymes. Confirmatory tests of CFS extracted from all LAB isolates showed no clear zone presence around the paper disc which indicated the loss of their inhibition activity against pathogenic bacteria (Figure 2). Bacteriocin is proteinaceous in nature which could be inactivated by a proteolytic enzyme (Nithya et al. 2012; Yang et al. 2012). Therefore, it was agreed that bacteriocin is present in the cell-free supernatant.

Cell-free supernatant may contain bacteriocins, organic acids, enzymes, alcohols, and low-molecular-mass substances which are the main metabolites responsible for the antimicrobial action of LAB (Chen et al. 2003). The salted -out method was performed to partially purify the bacteriocin and removing other substances. To evaluate the success of bacteriocin partial purification, we monitor their inhibition activity against pathogenic bacteria. The partially-purified bacteriocin showed a bigger clear zone than the cell-free supernatant (Figure 3). However, the total inhibition zone diameter was varied among isolates. The largest inhibition zone was shown by LAB isolate LG7 against

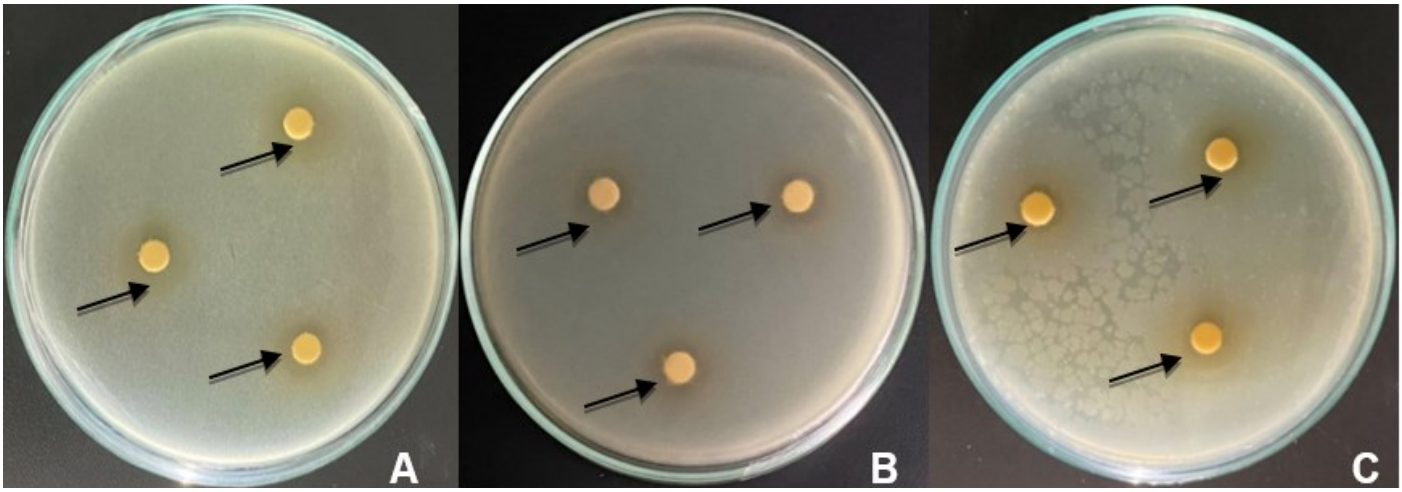

Figure 3. The inhibition zone of partially-purified bacteriocin against *S. typhi* (A), *S. flexneri* (B), *L. monocytogenes* (C), (black arrow pointed the formed clear zone).

S. flexneri with an average diameter of 14.67 mm, followed by LAB isolate LG71 against *L. monocytogenes* with 15.17 mm in diameter.

Bacteriocin can inhibit the growth of pathogenic bacteria due to the electrostatic interactions between positively charged bacteriocin and negatively charged cytoplasmic membrane lipids. The hydrophobic part of the bacteriocin will enter the cytoplasmic membrane by forming pores. This pore formation will cause the failure of the proton motive force (PMF). PMF is a proton that forms energy to be used in various cell activities including bacterial cell metabolism (Chen et al. 2003; Perez et al. 2014). On the other hand, the bacteriocin-producing bacteria has its immunity to the produced bacteriocin with a specific immune system (Martinez et al. 2013).

There was an increased inhibitory activity of all the partially purified bacteriocin when compared with the cell-free supernatant (Figure 4). It indicated that the purification was successfully performed. Also, these results were statistically significant ($p<0.01$). The lower activity of cell-free supernatant might be due to the presence of various metabolites. Moradi et al. (2020) suggested that the biological activity of bacteriocin is attributed to the purification process. Moreover, different purification methods of bacteriocin might result in different levels of purification and yields.

The inhibitory action of bacteriocin can vary between identical species within- and inter-genera (Castellano et al. 2004). The data showed that bacteriocin expressed a lower inhibition against *S. typhi* than *L. monocytogenes* and *S. flexneri*. The ANOVA test showed that each pathogenic bacterium expresses a very significant sensitivity to the partially-purified bacteriocin (Table 1). The high sensitivity was shown by *S. flexneri, L. monocytogenes,* and *S. typhi*, respectively. Interestingly, the inhibitory activity of bacteriocin against *S. flexneri* (Gram-negative) was higher than *L. monocytogenes* (Gram-positive bacteria). Gram-negative bacteria are generally resistant to the bacteriocin produced by Gram-positive bacteria due to their outer membrane acts as an effective and protective barrier (Cao-Hoang et al. 2010). However, Caridi (2002) studied *Lactobacillus paracasei* and its strong activity of bacteriocin against *Escherichia coli.*

Table 1. DMRT test of bacterial sensitivity to partially-purified bacteriocin.

Pathogens	Average of total inhibition zone (mm)
S. typhi	8.83 ± 1.09^{a}
L. monocytogenes	9.30 ± 1.57^{b}
S. flexneri	10.38 ± 1.58^{c}

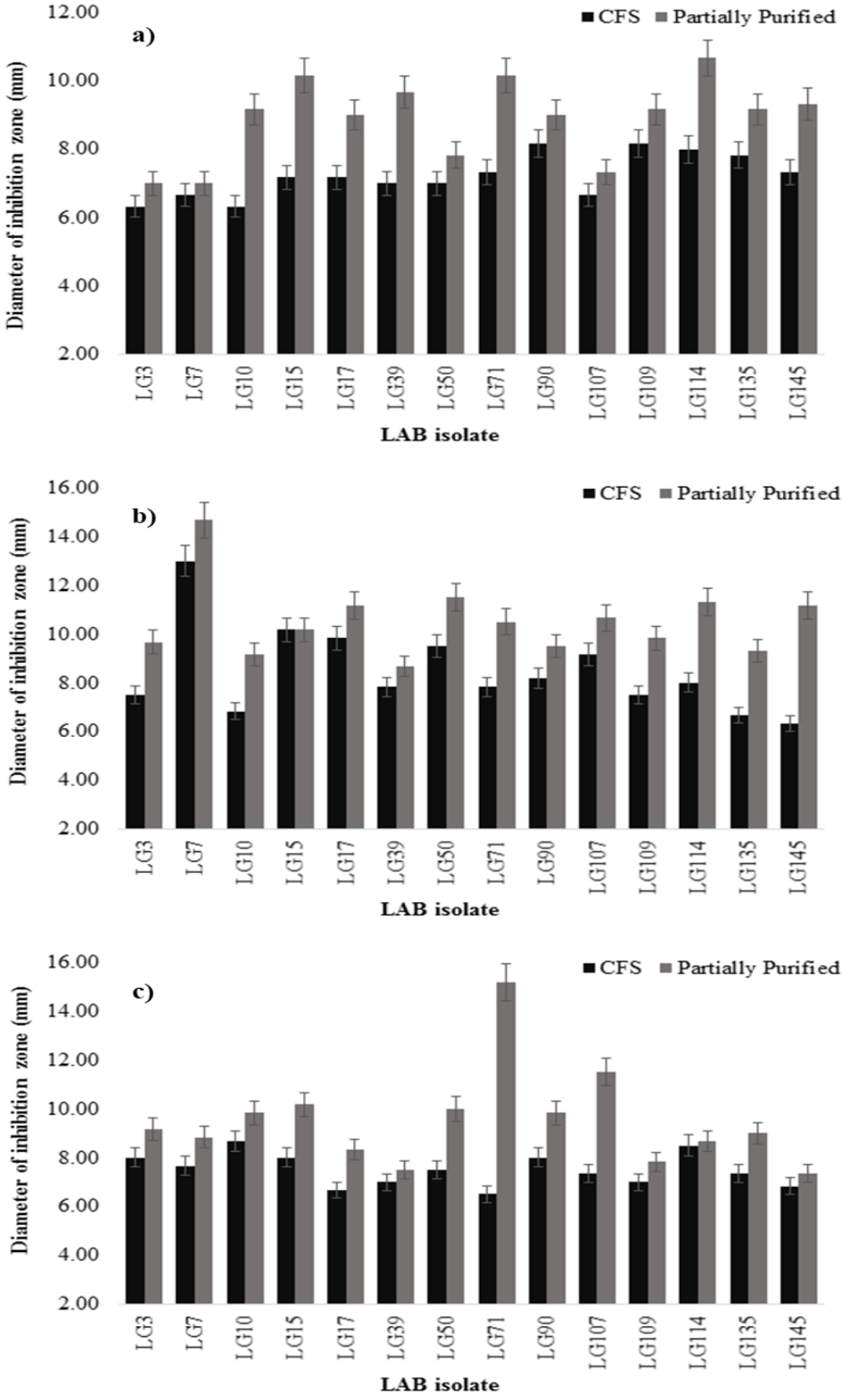

Figure 4. The comparison of inhibition zone of cell-free supernatant (CFS) and partially purified bacteriocin against *S. typhi* (a), *S. flexneri* (b), *L. monocytogenes* (c).

In general, Gram-positive bacteria are more sensitive to bacteriocin. The cell wall structure of Gram-positive bacteria has lipopolysaccharide, lipoprotein, and phospholipid composition which is lower than Gram-negative bacteria. The simpler cell wall structure of Gram-positive bacteria is facilitating the easier activity of bacteriocin (Cao-Hoang et al. 2010; Usmiati & Marwati 2007). Although, some Gram-positive bacteria have been reported resistant to LAB bacteriocins (e.g. nisin, lactocin, enterocin), such as *L. monocytogenes, L. innocua, Staphylococcus aureus, Clostridium botulinum,* and *Bacillus cereus* (Garsa et al. 2014; Vignolo et al. 2000).

This present study showed a wide range of antimicrobial activity against food-spoilage and/or food-borne pathogens, both Gram-positive and Gram-negative bacteria. It indicating that bacteriocin produced by LAB from mangrove sediments are suitable as bio-preservatives for food including fisheries products. Previous independent studies have reported the application of bacteriocinogenic-LAB form nature origin as bio-preservative, such as *Lactobacillus curvatus* BCS35 marine origin in fish bio-preservation (Gómez-Sala et al. 2016), *Leuconostoc* sp. application in ground meat to reduce *E. coli* contamination (Koo et al. 2015). Our present paper did not study the species-specific effects which possibly influence the bacteriocin's efficacy. Therefore, in-vivo studies are urged to perform. As well as, the study to confirm the type and classes of produced bacteriocins, since LAB produces a variety of bacteriocins (Elayaraja et al. 2014).

CONCLUSION

Our study found 14 bacteriocinogenic-LAB isolates originating from mangrove sediments, Logending Beach Kebumen had a wide range of antimicrobial activity against pathogenic bacteria *S. typhi, S. flexneri,* and *L. monocytogenes*. The ANOVA test showed a statistical difference of inhibitory activity between cell-free supernatant and partially-purified bacteriocin and their relationship to the type of indicator pathogenic-bacteria.

AUTHORS CONTRIBUTION

Conceptualization: DFK; Methodology: TDS, AM, and AR; Investigation: TDS and AM; Writing—original draft preparation: DFK, TDS, and AR; Writing—review and editing: AR.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest in this study.

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Research Article

Ethnobotanical and Phytochemical Study of Bayur (*Pterospermum javanicum* Jungh.) on Sasak Tribe around Mount Rinjani National Park, West Lombok as a Conservation Effort

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ABSTRACT
Pterospermum javanicum Jungh. (Bayur) is a species belongs to the Pterospermum genera (Malvaceae). Several species of Pterospermum had been reported for their ethnobotanical usage, but the studies about ethnobotany information of Bayur and its secondary metabolite compounds were still limited which have been published. This study aimed to observe the ethnobotanical usage of Bayur and to examine the phytochemical contents of the acetone extract of Bayur flower from West Lombok. The ethnobotany information of Bayur was obtained through interviews with local people and more information on the ethnobotanical records of *P. javanicum* (Bayur) was conducted by reviewing the scientific literature. The chemical compounds of Bayur flower were analysed by the GC-MS method. The results revealed that Bayur was used by the Sasak community around Gunung Rinjani National Park West Lombok for various purposes, such as traditional medicines, beverages, rigging, and building material. The phytochemical analysis showed that the acetone extract of Bayur flower from West Lombok contained 38 identified chemical components, representing 93.78% of the total compounds. The major contents of them were Lupeyl acetate (10.68%), p-n-Amylphenol (8.16%), Lauric acid (7.31%), N-(Methyl-d2)- Aniline (5,82%), and Pentanal (5.07%). This report was the first publication about the phytochemical contents of Bayur flower. It is expected that this study gives further information on the potentials of Bayur, especially about its secondary metabolite to support and prove the truth of the cultural concept of society in utilizing Bayur as a medicinal plant.

Keywords: Bayur Flower, Ethnobotany, GC-MS Analysis, *Pterospermum javanicum*

INTRODUCTION
Malvaceae is a family of the flowering plant which is comprised of around 85 genera and 1500 species, distributed widely in the tropical and temperate region (Rahman & Gondha 2014) It has been a long time known that several species of Malvaceae have been reported as traditional medicines in many countries to treat various human diseases and disorder (Al Muqarrabun & Ahmat 2015). In India, *Pterospermum acerifolium* L. Willd with the local name 'Muchukunda' traditionally was used to treat for blood

troubles, inflammation, ulcer, tumors, leprosy, for smallpox eruptions (Panda & Dutta 2011), analgesic, for the treatment of diabetes, gastrointestinal disorders, bronchitis, cough, cephalic pain, migraine, as a haemostatic and antimicrobial agent (Datta et al. 2011). In Malaysia, *Scaphium macropodum* known as Kembang Semangkuk Jantung has been reported to possess medicinal properties to treat intestinal infections, diarrhea, throat aches, asthma, dysentery, fever, coughs, inflammation, and urinary illness (Al Muqarrabun et al. 2013). In Eritrean, the root *Dombeya torrid* with the local name Songua was used for the treatment of asthma, cough, and skin wound (Yemane et al. 2017).

Pterospermum is one of the genera belonging to the Malvaceae family, consists of approximately 30 species. It is distributed from the eastern Himalayas, South China, and Southeast Asia (Wilkie 2013). Several species of this genus have been reported for their ethnobotanical usage, especially for traditional medicine. For example, *P. diversifolium* traditionally was used to treat skin diseases (Salempa 2012) and dysentery (Hidayathulla et al. 2011). *P. heyneanum* Wall was used as traditional medicine for leucorrhoea disease (Al Muqarrabun & Ahmat 2015), and *P. subpeltatum* was used as a cure for dysentery, toothache, and itchy drug (Salempa et al. 2009). In India, the leaves of *P. acerifolium* are widely used for diabetes treatment and as a haemostatic (Chatterjee et al. 2012). While the flowers of *P. suberifolium* are used for treating hemicrania, migraine, cephalalgia, pharyngitis, inflammations, cough, bronchitis, and skin diseases and Its leaves were used for headache treatment (Khare 2007).

Pterospermum javanicum Jungh. is a species of the genus Pterospermum known by the local name of Bayur. This species grows and spreads mainly in the lowland areas of Indonesia. Thus far, the wood of *P. javanicum* (Bayur) has high commercial prospects because it is classified as a wood-producing plant with durability class IV and a specific gravity of 0.35-0.70. The wood of *P. javanicum* (Bayur) is commonly used as a material for making plywood, furniture, shipping, bridges, pulp, and paper (Salempa 2012). This causes the presence of *P. javanicum* (Bayur) in natural habitats increasingly threatened due to illegal logging (Hidayat 2014). Therefore, further research on the other potentials of *P. javanicum* (Bayur) will be very useful as supplementary information to increase public and government awareness in efforts to conserve the *P. javanicum* (Bayur) population.

Ethnobotany information showed that *P. javanicum* (Bayur) has several traditional functions in Indonesia and Malaya, but the scientific data has not been recorded properly. In Lombok, especially in areas inhabited by the Sasak tribe, the use of roots and stems of Bayur for traditional drinks and ethnomedicine have led to over-exploitation of Bayur in its natural habitat, so that the population of Bayur in the forest has decreased significantly. Therefore, one of the ways to overcome the overexploitation of Bayur is to encourage and increase community awareness to conserve Bayur plants.

Flowers are reproductive plant organs that are produced continuously and can be used without having to cut down the plants. Therefore, by studying the potency of Bayur flower as a substitute for roots and stems as a medicinal ingredient, it is hoped that it can reduce Bayur plants pruning for medicinal purposes to increase Bayur replantation in natural habitat. Thus far, the ethnobotany study of P. *javanicum* (Bayur) has been observed limitedly. There is only one scientific journal that reports on the phytochemical content of *P. javanicum* bark (Praptiwi & Fathoni 2017), whereas the phytochemical contents of other parts of *P. javanicum* (Bayur) have never been conducted, including from its flower. Accordingly, the purposes of this research were to explore the ethnobotany information of *P. javanicum* (Bayur) and the phytochemistry potential of its flower. Therefore, the results of this study are

expected to explore the potentials of *P. javanicum* (Bayur) flower, so that the plants can continue to be preserved.

MATERIALS AND METHODS

Ethnobotany Study of *Pterospermum javanicum* Jungh.

Pterospermum javanicum Jungh (Bayur) material was collected in the natural forest of Kembang Kuning, Jeruk Manis Village, Sikur Regency, around Gunung Rinjani National Park, Lombok Island, West Nusa Tenggara Province Indonesia (S 08°31'19.18" E116°25'28.50"; altitude 794 masl) on April 2014.

The plant was identified by a taxonomist of Bali Botanic Garden, Mr. Ida Bagus Ketut Arinasa, M.Si., and the herbarium voucher was deposited in the herbarium of Tabanan Hortus Botanicus Balinese (THBB). The plant scientific name was verified using an online database (e.g. The Plant List, 2018 and The International Plant Names Index, 2018) and the description was described based on morphological observations and compare the characteristics with the available literature. We collected the potential uses of *Pterospermum javanicum* Jungh. through interviews with local people and information in related documents. More information on the ethnobotanical records of *P. javanicum* (Bayur) was conducted by reviewing the scientific literature (such as Google Scholar and Google Book) (Sujarwo 2018).

Plant Material dan Sample Preparation

Fresh materials of flowers were chopped in small size and dried without direct sun irradiance for several days until the materials were completely dry. One hundred grams of dried materials of *P. javanicum* (Bayur) flower was extracted with acetone by maceration method and the extract suspension was filtered by filter paper (Azwanida 2015). This extract was analyzed with a GC-MS method to identify the phytochemical components. GC-MS method to identify the phytochemical components.

GC-MS Analytical Conditions and Identification of Chemical Compounds

The acetone extract of *P. javanicum* (Bayur) flower was analyzed using the GC-MS method with the condition according to Andila et al. (2018). The GC-MS equipment was model Shimadzu GC- MS-QP2010 with an Rtx 5ms capillary column (60.0 m x 25 mm with 0.25 µm thickness) and Carrier gas UHP Helium. The conditions of GC setting was column oven at temperature of 50 ^{o}C (±5 minutes to 280 ^{o}C, injection temperature at 280 ^{o}C, injection mode: split, total program time: 50 minute, flow control mode : Linear velocity, pressure : 101.0 KPa, Total Flow : 46.5 mL/Min, Column Flow: 0.85 mL/min, linear velocity: 23.7 cm/sec, purge flow :3.0 ml/min, split ratio : 1: 50,total sample injection : 1 µL. The MS conditions were ion source temperature: 200 ^{o}C, interface temperature: 280 ^{o}C, solvent cut time: 1.5 min, and detector temperature 280 ^{o}C. Mass spectra fragmentation patterns were used to identify the chemical compounds.

Data Analysis

Information on the ethnobotany studies of *P. javanicum* (Bayur) was obtained through interviewing with local informants and reviewing scientific literature database. The data obtained were tabulated and discussed descriptively. While to determine the names of chemical compounds of GC-MS results were approved by comparing each retention time indices, and mass spectra fragmentation patterns data with those from computer library WILEY7.LIB and open published literature (Andila et al. 2018).

RESULTS AND DISCUSSION

Description of *Pterospermum javanicum* Jungh. (Bayur)

The name of *Pterospermum javanicum* Jungh. (Bayur) was described as a medium-size to a large tree, Emergent up to 59 m tall and 54 cm diameter at breast height (DBH). Stipules 5 mm long. Leaves alternate, simple, triple-veined, undersurface whitish-brownish, hairy, leaf base asymmetrical. Flowers 100 mm diameter, yellowish, with very long and narrow petals, flowers placed in racemes. Fruits 104 mm long, green-brown, hairy, dehiscent capsules filled with many winged seeds (Whitmore 1972; Wilkie 2013). This species occurs scattered in undisturbed to disturbed (open sites) mixed dipterocarp forests, up to 1,400 m of altitude. Usually on ridges or river banks. It was also found on limestone. Distributed from India, Myanmar, Thailand, Malaya, Sumatra, Java, Borneo, and New Guinea (Whitmore 1972).

Ethnobotany Study of *Pterospermum javanicum* Jungh. (Bayur)

Based on interviews, the potential use of Bayur (*P. javanicum*) on Sasak Tribe around Gunung Rinjani National Park, West Lombok was described in Table 1. Their usage specifically as traditional medicine and traditional beverage (Salempa et al. 2014). Based on the collected data of Bayur application, informants also utilize the wood for furniture, house, boat, and bridge material. They are also use the wood for weathercock (weather control tools), *berugak*, and *lumbung*. Weathercock is usually placed in the garden near the house and it makes a nice and unique sound. A *berugak*, or gazebo, stands out front, while a *lumbung*, or rice barn, sits in the back. A *berugak* usually is square or rectangular with a thatch (*alang-alang*) roof, timber floor, and either four poles (*sekepat*) or six poles (*sekenem*), often of jackfruit or coconut wood. The berugak is an open-air structure which is separated from the house and it is part of the philosophy of life of the island's indigenous Sasak people.

Comparative analysis with previous research on the ethnobotany study of Bayur both in the Lombok region and in other areas was also described in this paper. Johnson (1998) in his book "Ethnobotany Desk Reference" revealed that In Java and Malaya, *P. javanicum* (Bayur) has been used as a dentifrice and to treat several human diseases such as dysentery, gingivitis, inflammation, sore, sprains, and abdomen disorder. In Lombok, especially for the local community around the Mount Rinjani area, the root of *P. javanicum* (Bayur) was used as raw material for traditional beverage, with a local name of "Tuak Bayur". The local people believe that this traditional beverage can be used for diabetes treatment (Hidayat 2014). Bayur root added to palm sap (*Arenga pinnata*) serves as a tuak preservative. Another study also found that the Sesaot tribe community in West Lombok (West Nusa Tenggara) uses the mixture of roots of *P. javanicum* (Bayur) and water to treat haemorrhoids (Hidayat & Pendit 2012). While Sukenti et al. (2016)

Table 1. Potencial Use of *P. javanicum* Jungh. on Sasak Tribe in around Gunung Rinjani National Park, West Lombok..

NO	Part of Plant	Potencial Use			
		Traditional Medicine	Food and Beverage	Rigging material	House construction/ furniture
1	Bark	+	+	+	-
2	Leaf	-	-	-	-
3	Root	+	-	-	-
4	Stem	+	-	-	-
5	Wood	-	-	-	+

found that the local cuisine of the Sasak tribe in Lombok Island used stem bark of *P. javanicum* (Bayur) as a traditional beverage. In West Nusa Tenggara, Bayur was also used as a building material and food flavoring (Dharma et al. 2017). Specifically, the medicinal use of *P. javanicum* base on the literature study was performed in Table 2.

Table 2. Medicinal Use of *P. javanicum* Jungh.

No.	Part of Plant	Medicinal use	Processes/ preparation	References
1.	Root	Diabetes drug	Roots are sliced into small pieces, then mixed in Bayur Tuak and drunk	(Hidayat 2017)
		Haemorrhoid	(base on scientific research)	(Hidayat & Pendit 2012)
		Tonic	Mixed in bayur tuak (tradisional drink)	Interview; (Dharma et al. 2017); (Hidayat & Pendit 2012)
2.	Bark	Dysentery, Toothache, Ulcers and sprains	(as an ingredient of thnomedicine)	(Heyne 1987)
		Antibacterial	(base on scientific research)	(Praptiwi & Fathoni 2017)
		Antioxidant	(base on scientific research)	(Saefudin et al. 2013)

The Chemical Constituents of Acetone Extract of The Flower of *P. javanicum* (Bayur) from Lombok

This study was carried out to determine the possible chemical compounds of acetone extract of *P. javanicum* (Bayur) flower from Lombok by GC-MS method. The complete result of the GC-MS analysis was shown in Table 3 and its chromatogram in Figure 1. This study revealed that the acetone extract of *P. javanicum* (Bayur) flower contained 38 identified chemical components, consisting of 93,78% of the total determined contents. The dominant compounds were Lupeyl acetate (10.68%), p-n-Amylphenol (8.16%), Lauric acid (7.31%), N-(Methyl-d2)-Aniline (5,82%), and Pentanal (5.07%). As far as the author's investigation, this report was the first publication about the phytochemical contents of *P. javanicum* flower.

Moreover, Higher plants produce both primary and secondary metabolites. Adopting a phytochemical analysis approach for preliminary screening of plant's secondary metabolites seemed to be reasonable to obtain comparative information about the main group of secondary metabolites among those plants (Edriss et al. 2012). Plant secondary metabolites exhibit a wide composition of biological and pharmacological properties. Because of this, some medicinal products were derived from them and used to treat various infections and diseases (Wink 2015).

In this research, the study about screening phytochemical properties of *P. javanicum (Bayur)* flower was conducted by the GC-MS method. This method was chosen according to a previous study about phytochemical properties of bark extract of *P. javanicum* by Praptiwi and Fathoni (2017). The results showed that the flower of *P. javanicum* (Bayur) contained 38 identified chemical components with main properties consisted of Lupeyl acetate (10.68%), p-n-Amylphenol (8.16%), Lauric acid (7.31%), N-(Methyl-d2)-

Aniline (5,82%), and Pentanal (5.07%). These components were quite different from the chemical compounds contained in Bark of *P. javanicum*. Praptiwi and Fathoni (2017) reported that the phytochemical compounds in the bark of *P. javanicum* (Bayur) consisted of 31 identified chemical compounds with dominant properties: stigmast-4-en-3-one (19.68%), γ-sitosterol (19.81%), and 4,22-Cholestadien-3-one 9 (9.09%). Thus far, there are only two of these publications which have reported on the phytochemical contents of *P. javanicum*.

Some of the compounds identified in Bayur flower extract have pharmacological potential. Lupeyl acetate has potential as an anticancer, Leishmanicidal, and anti-inflammatory. (Suwito et al. 2016) reported that Lupeyl acetate, a major constituent of the bark of *Artocarpus integra* has anticancer activity. Juárez-vázquez et al. (2020) also found that Lupeol Acetate isolated from *Cnidoscolus tehuacanensis* showed potential as a Leishmanicidal and anti-inflammatory. Several compounds such as p-n-Amylphenol (Shapiro & Guggenheim 1998), lauric acid (Anzaku et al. 2017), acetic acid (Iroha et al. 2011), ethyl methacrylate (Rawlinson et al. 2010) and p-Vinylguaiacol are known to have antibacterial activity. p-Vinylguaiacol (Ravikumar et al. 2012), and 2,6-Dimethoxyphenol (Yang et al. 2016) showed high antioxidant activity, while Lauric acid and Linoleic acid (Choi 2014) have potential as medicine for cancer treatment (Lappano et al. 2017). Several compounds have also been identified to have anti-inflammatory activity, among others: p-Ethylguaiacol (Zhao et al. 2019), p-Vinylguaiacol (Ravikumar et al. 2012), 4-Allyl-2,6-dimethoxypheno (Xie et al. 2015), Eicosane and pentadecane (Okechukwu 2020). Eicosane and pentadecane were able to show very strong analgesic, and antipyretic effects (Okechukwu 2020). Indole was used for several diseases, such as muscle relaxants, anti-leukemic, neurotransmitter in the CNS, and also in the cardiovascular and gastrointestinal systems (Kaushik et al. 2013). These data support the fact that *P. javanicum* has been used by the Sasak people around Gunung Rinjani National Park as a traditional medicine to treat diseases.

As comparative studies, several secondary metabolite profiling of other species in Pterospermum genera have been recorded in scientific journals. Al Muqarrabun and Ahmat, (2015) found that *P. heyneanum* (stem) contained terpenoids (Cyclopterospermol, 30 norcyclopterospermol, 30-norcyclopterospermone), and Miscellaneous compounds (n-octacosanol, 3-hydroxy-5-methoxy-2-methylbenzoquinone. While Salempa (2012) reported that the chloroform fraction of *P. subpeltatum* extract contained steroid, a non -aromatic compound. Other studies revealed that flowers of *P. acerifolium* contained various chemical compounds, among other Phenyl propanoid (p-coumaric acid), flavonoids (Apigenin, 3'-methoxy-apigenin, Apigenin-7-b-O-neohesperidoside, 56 Luteolin, Luteolin 7-O-glucoside, Luteolin-7-b-O-neohesperidoside, Vitexin, Pterospermin B, Pterospermin A, Trans-tiliroside), Terpenoids ((6R,9S)-3-oxo-a-ionol-b-D-glucopyranoside, Linalool-3-rutinoside, Friedelan-3a-ol, Friedelan-3b-ol, b-amyrin), and miscellaneous compounds (1-undecene, pterospermin C, and (3R,4R,5S)-3,4-dihydroxy-5-methyl-dihydrofuran-2-one) (Dixit et al. 2011). While the bark of *P. acerifolium* was rich in bioactive constituents like phenol (2.36%), alkaloid (2.10%), flavonoid (1.84%), and tannin (2.16%). *P. acerifolium* also contained high total antioxidant content in its bark (Rath et al. 2014) and leaves (Chatterjee et al. 2012; Sannigrahi et al. 2010).

Based on the results of this study, it can be seen that the chemical content contained in Bayur flowers can be associated with different pharmacological activities including antibacterial, antioxidant, anti-inflammatory, and analgesic properties, which can justify and confirm the indication of traditional therapeutic preparations based on plants. So it is

hoped that bayur flowers can be used as an alternative material for traditional medicinal materials other than roots and bark which in practice tends to damage and kill the plants.

Table 3. The result of GCMS analyses obtained from acetone extract *of Pterospermum javanicum* Jungh. flower.

Peak	Chemical Name	R.Time	Conc. Relative (%)	SI
1	2-Propynoic Acid	1.733	1.07	98
2	Acetic acid	2.170	3.81	97
3	Cyclohexanone	8.641	2.87	87
4	IZAL	9.816	4.09	96
5	Corylon	10.303	1.58	95
6	N-(Methyl-d2)-Aniline	11.078	5.82	82
7	Pentanal	11.243	5.07	87
8	3-Ethyl-2-hydroxy-2-cyclopenten-1-one	11.488	0.93	92
9	m-Ethylphenol	12.126	1.34	79
10	Creosol	12.295	1.51	94
11	Ethyl methacrylate	12.588	1.15	81
12	1-hydroxy-4,4-dimethyl-cyclohexanecarbonitrile	12.745	0.76	68
13	p-Ethylguaiacol	13.173	0.88	86
14	Indole	13.485	2.28	95
15	p-Vinylguaiacol	13.549	2.86	87
16	4,4-Dimethylbut-2-enolide	13.694	0.69	77
17	2,6-Dimethoxyphenol	13.891	3.53	90
18	n-Tetradecane	14.086	2.02	79
19	3-Methylindole	14.341	0.93	92
20	1,2,4-Trimethoxybenzene	14.725	0.65	82
21	Phenol, 2-methoxy-3-(2-propenyl)-	14.775	1.09	93
22	benzenemethanol, .alpha.-(2-aminocyclopentyl)-	15.158	0.55	69
23	Lauric acid	15.590	7.31	97
24	1,2-Epoxy-3-propyl acetate	15.658	1.06	78
25	4-methyl-2,5-dimethoxybenzaldehyde	15.725	1.29	70
26	4-Allyl-2,6-dimethoxyphenol	16.762	0.55	92
27	n-phenyl-n'-furaldehyde hydrazone	17.052	0.73	71
28	Coniferyl alcohol	17.158	3.74	85
29	1-Hexadecyne	18.007	0.82	84
30	Palmitic acid	18.404	4.51	96
31	decane, 2,3,5,8-tetramethyl-	18.542	1.72	87
32	Nonadecane	19.163	0.49	92
33	Linoleic acid	19.520	4.44	94
34	Stearic acid	19.633	1.66	95
35	Octacosane	19.758	0.71	90
36	Myristamide	19.823	0.43	85
37	p-n-Amylphenol	24.005	8.16	86
38	Lupeyl acetate	42.128	10.68	87
	Total		93.78	
	Phenol		12.7	
	Terpenoid (Triterpene)		10.68	

Note: R.Time: Retention Time; Conc. Relative: Concentration Relative; SI: Similarity Index.

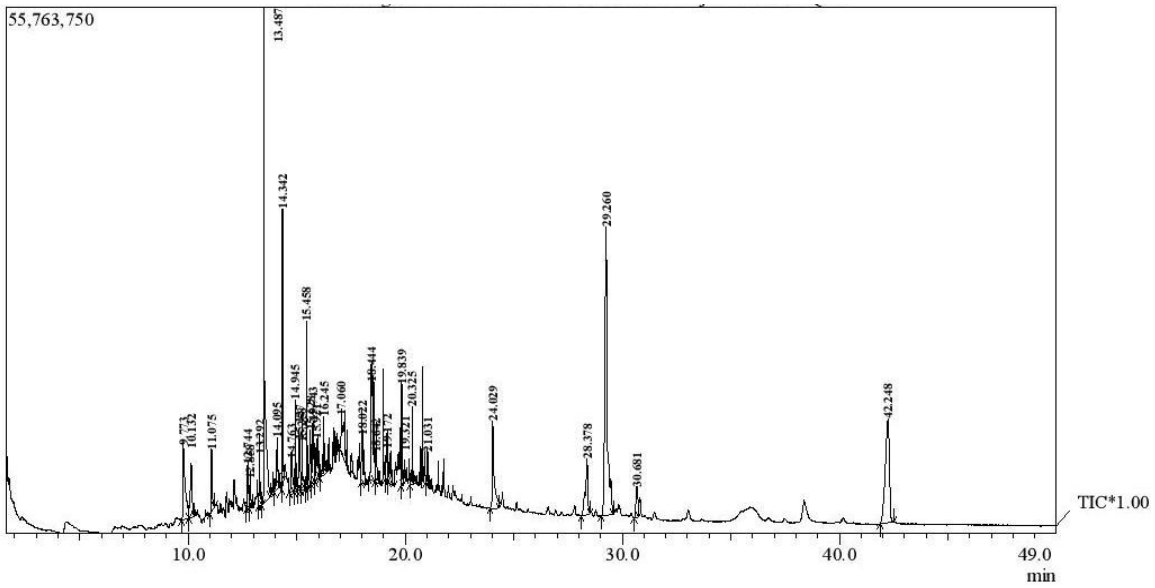

Figure 1. GCMS chromatogram of the acetone extract of *Pterospermum javanicum* Jungh flower.

Pterospermum javanicum Jungh. Conservation Issues

Since there are no major threats envisaged for this taxon, there are no recommendations on conservation action at this point. This species was evaluated as Least Concern (Ganesan 2020). But the utilization of *P. javanicum* Jungh. from the forest by the Sasak people also needs to be evaluated in terms of the long term conservation prospects for this species.

Nowadays, conservation requires a multidisciplinary approach, including the integration of local indigenous knowledge. It is a very important thing for conservation and sustainable management of natural resources, especially for the medicinal used of forest products instead of pure timber harvesting (Nahdi et al. 2016; Pieroni et al. 2014; Shrestha & Kimberly 2017). So that the utilization of *P. javanicum* as traditional medicine and beverage by the local community in Lombok can be a sustainable basis in order to conserve the *P. javanicum* population in those areas. In addition, by knowing the chemical content of Bayur flowers, it is hoped that the community can use it as an alternative material for making traditional medicines because the use of flowers is much safer for plant survival when compared to the use of their stem bark or roots.

CONCLUSION

The investigation about ethnobotany studies showed that *P. javanicum* (Bayur) have been used by the local community in Lombok for various ethnobotanical purposes: traditional medicine and beverages, rigging, and house (building) material.

The phytochemical analysis revealed that the acetone extract of *P. javanicum (Bayur)* flower from Sumbawa contained 38 identified chemical components, representing 93,78% of the total compounds. The major contents of them were Lupeyl acetate (10.68%), p-n- Amylphenol (8.16%), Lauric acid (7.31%), N-(Methyl-d2)-Aniline (5,82%), and Pentanal (5.07%). This report was the first publication about the phytochemical contents of *P. javanicum* (Bayur) flower. This present study also indicated that *P. javanicum* (Bayur) flower contained phenolic compounds and triterpenoid, lupeyl acetate which had great potential for human medicine. By knowing the chemical content of Bayur flowers, it is hoped that the community can use it as an alternative material for making traditional medicines and support the conservation of Bayur.

AUTHORS CONTRIBUTION

P. S. A. designed the research, wrote the manuscript and supervised all the process. T. W. designed the research, wrote the manuscript, and reviewed the manuscript, I G. T. designed and collected the ethnobotani data. I P. A. H. W. designed and collected the ethnobotani data.

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CONFLICT OF INTEREST

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

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Research Article

Predicting the Distribution of Sunda Pangolin (*Manis javanica* Desmarest, 1822) in Way Canguk Research Station, Bukit Barisan Selatan National Park, Lampung

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ABSTRACT
The distribution of a species can help guide the protection activities in their natural habitat. Conversely, the lack of information on this distribution makes the protection strategy of this species difficult. The research was conducted in Way Canguk Research Station, Bukit Barisan Selatan National Park from January until March 2018. The purposes of this research were to create a distribution prediction map of Sunda pangolin (*Manis javanica*) and estimating the environment variables that most influenced the probability of the distribution. Fourteen points of camera trap coordinates were used for presence data with nine types of environment variables such as elevation, slope, understorey, canopy cover, distance from roads, distance from rivers, distance from villages, food source, and distance from the threat. The result of maxent showed an Area Under the Curve (AUC) value of 0.909 categorized as very good. The highest probability of Sunda pangolin distributions was in the Pemerihan Resort and Way Haru Resort area, while the dominant environmental variables included the distance from the village, the canopy cover, and the distance from threat with the value 47.7; 25.85; and 15.8%, respectively. Prediction maps and environment variables can help to identify the population of Sunda pangolin in the wild and can provide input for the national parks to prioritize protection areas for Sunda pangolin from the increased poaching.

Keywords: *Manis javanica*, maxent, species distribution, Way Canguk research station

INTRODUCTION
A pangolin is a type of mammals of the Order of Pholidota having scales (Payne et al. 1985). There are four types of pangolins distributed in Asia (*Manis javanica, Manis craussicaudata, Manis pentadactyla*, and *Manis culionensis*) and four in Africa (*Manis temminckii, Manis tricuspis, Manis gigantea*, and *Manis tetradactyla*). *Manis javanica* or Sunda pangolin are distributed on the islands of Sumatra, Java, and Kalimantan (Challender 2014). Geographically, Sunda pangolins can be found at different altitudes: 10-100 masl (TEAM camera traps photo findings), 350-900 masl (Wirdateti et al. 2013), and 1170 masl (Manshur 2015).

Generally, a Sunda pangolin is a shy animal, not aggressive, and solitary, although on some occasions they found more than one individual. Sunda pangolins have an important value as a natural controller of the insect population, such as ants and termites. They can eat up to 70 thousand ants and termites per year, and they play a role in improving soil quality (Rodrigues 2011). Sunda pangolins also have high economic values, their scales are used for traditional medicine and the meat is cooked for exotic food (Sawitri & Takandjandji 2016). In Malaysia, the scales of Sunda pangolin used for asthma medication and protection from witchcraft, whereas, in Indonesia believed to protect from harmful magic (Chong 2020). Poachers used snares, traps, or dogs to catch on the Sunda pangolin (Newton et al. 2008). They are one of the protected and are included in the Appendix I Convention on International Trade in Endangered Species (CITES) due to illegal hunting since 2000 (Sawitri et al. 2012). The IUCN Red-List established Sunda pangolin as critically endangered in 2013.

One of the habitats of Sunda pangolins is Bukit Barisan Selatan National Park (BBSNP). This research is the first ecological study about Sunda pangolins in this area since 1997. Wirdateti et al. (2013) was researched the distribution and population of Sunda pangolin in Tanggamus and West Lampung, but that was not in the BBSNP area. Studies on Sunda pangolins are very rarely done in Indonesia, especially in Sumatra. So far, most of the studies on pangolins were about genetics (Nie et al. 2009; Wirdateti et al. 2013; Wirdateti & Semiadi 2017), physiology (Cahyono 2008), behavior in the captivity (Febriyanti 2016), and trades (Takandjanji & Sawitri 2016).

However, the information about the ecological nature of Sunda pangolins was found to be lacking, including the information about the distribution of Sunda pangolin in a certain region. The purposes of this research are to predict the distribution pattern of Sunda pangolins and to study the environmental variables that influenced the presence of Sunda pangolin in the BBSNP. By evaluating the distribution of Sunda pangolins, BBSNP can determine the priority areas for the protection and conservation of Sunda pangolin.

METHODS

Research location

The research was conducted at Way Canguk Research Station, Bukit Barisan Selatan National Park (BBSNP) (Figure 1) from January - March 2018. The range of altitude of this research station was 0-100 masl (Endarwin 2006).

Data collection

Presence data of Sunda pangolin (direct observation)

The presence was used for maxent analysis, and they were recorded not only by direct encounter with the species (the primary sign of existence) but also by coordinates point of presence of species from camera traps (the secondary sign of existence). Several Sunda pangolin photos were caught by camera trap installed by WCS-IP through the TEAM (Tropical Ecology Assessment and Monitoring program) from 2010-2017. Fourteen presence data of Sunda pangolin were recorded from TEAM camera trap coordinate points from 2010-2017. The camera trap installation area included three resorts: Pemerihan Resort, Way Haru Resort, and Way Nipah Resort with a total of 60. All camera trap locations were evaluated to obtain environment variables, and camera traps points were recorded using Microsoft Office Excel with three columns: species, longitude, and latitude in CSV format. Additionally, UTM (*Universal Transverse Mercator*) for a geographic coordinate system was

used.

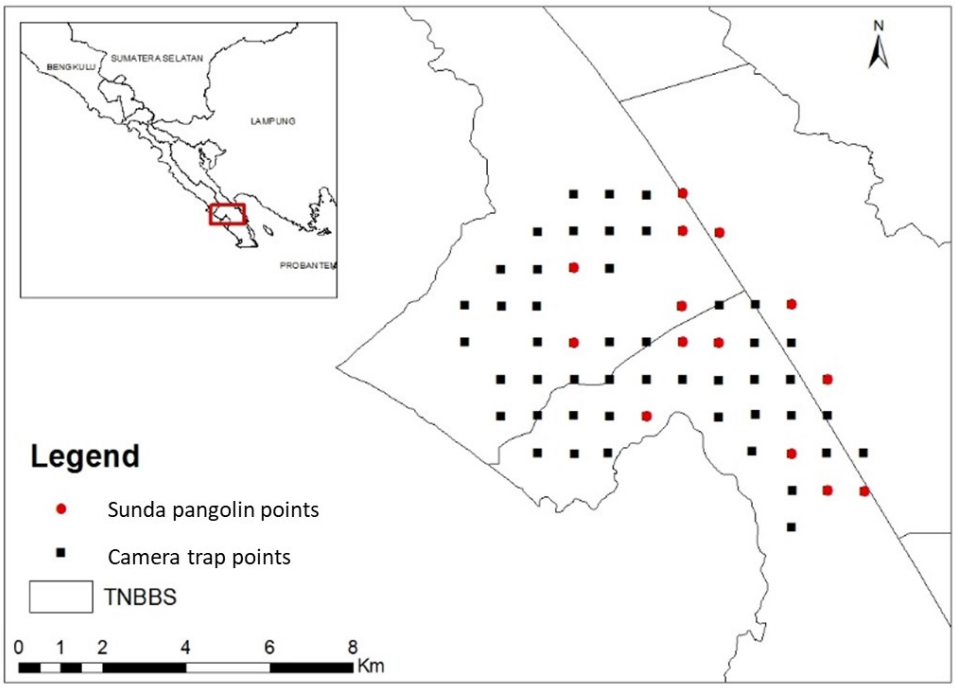

Figure 1. Research location in Bukit Barisan Selatan National Park.

Environment variables

The data of environment variables were obtained directly and indirectly. Some parameters, such as tree vegetation survey, food sources, understorey, canopy cover, and distance from threat were recorded directly. Tree vegetation survey used a 20 m x 20 m grid, and points of camera trap were used as a center point. Measurement of DBH (Diameter at the Breast Height) was carried out 1.3 m above the ground and only the trees having the DBH > 20 cm were surveyed. Food sources data were obtained from ants and termite nests in every camera trap point. Understorey data were obtained using a gridded sheet sized 1 m x 1 m which was divided into 16 squares, and camera trap points were used as the center point.

The distance from the camera trap point to obtain the data was 10 meters each in four directions following the compass direction. Canopy cover data were obtained using spherical densiometer model C in four directions based on compass direction, and camera traps points were used as the center point. Distances from threat data were obtained from traps coordinate point directly in the field and human photos of presence coordinate point at camera trap were also used.

Four data from the environment variables were recorded directly to Microsoft Office Excel with CSV format, and then ArcGIS was used for the deterministic interpolation (Arctoolbox –interpolation – IDW). The processed data were in raster format, and then they were converted into ASCII format (Arctoolbox – Conversion Tools – From Raster – Raster to ASCII). Distances from villages, road, and river data were obtained from Rupa Bumi Indonesia (RBI) map with a scale of 1:200.000 in point and line shapes. Slope and elevation data were obtained from USGS Explorer from DEM (Digital Elevation Model) 30 arc second via https://earthexplorer.usgs.gov/ website.

Data analysis

Maxent Ver. 3.4.1k at https://biodiversityinformatics.amnh.org/open_source/maxent/ (free version) was used. The maximum entropy is a

species distribution model that uses two data sets: presence data and environment variables (Elith et al. 2006; Phillips et al. 2006). For presence data in this study, direct presence data and indirect presence data were used. Direct presence data was finding the species directly in the field, while the indirect presence data included camera trap coordinates, scratches, footprints, and feces data. The environment variable data used direct data or indirect data. Direct data of environment variable were obtained in the field, for example, food sources (ants and termite nest) of Sunda pangolins, and indirect data used the GIS layer of RBI maps. Direct data of the environment variables used the interpolation in the ArcGIS menu for the estimated values. Interpolation is generally divided into two, deterministic interpolation and geostatistical methods. Deterministic interpolation is a deterministic calculation that is used to measure values based on the data obtained from the field. Deterministic interpolation has various choices of menus at ArcGIS desktops such as IDW (Inverse Distance Weighting), Natural neighbour, Trend, and Splind. Geostatistical methods are based on an autocorrelated statistic model (statistical relationship based on measured points) (ArcGIS Dekstop 2020). Environment variables were obtained using the GIS layer and using Euclidean Distance. Euclidean Distance describes each cell-to-source relationship or set of sources based on straight line distance (ArcGIS Dekstop 2020).

Maxent estimates environment variables that had important roles in the prediction model, like an environment variable based on the Jackknife curve (Phillips et al. 2006). In addition, there was a response curve that had an important role in showing the presence probability of a species to the environment variables (Tarjuelo et al. 2014). The prediction was improved based on AUC (*Area Under the Curve*) value. AUC is a curve that shows the probability of a species to maps (Baldwin 2009). A prediction is acceptable if the AUC value is above 0.75. AUC values according to Baldwin (2009) can be seen in Table 1.

Table 1. Area Under the Curve (AUC) value classification.

AUC value	Model performance
0,9 – 1,0	Very good
0,8 – 0,9	Good
0,7 – 0,8	Medium
0,6 – 0,7	Not good

Presence data of the 14 coordinate points containing Sunda pangolin photos from the TEAM camera trap were saved in CSV format. The environment variable data were compiled using ASCII (asc) format with the same extent and cell size (depend on the data) in order running in maxent software. Cell size and extent are environment settings contained in tools at ArcGIS software. Cell size refers to raster size and extent refers to a range of feature data or specified raster (ArcGIS Dekstop 2020). All geographic coordinate systems used the UTM projection format. Presence data of Sunda pangolin were recorded in CSV format and environment variable in asc format. The data result was the prediction distribution of Sunda pangolins and the prediction of environment variables that most influenced the Sunda pangolins presence at the research site.

RESULTS AND DISCUSSION

Prediction distribution map of Sunda pangolin

One of the results of the maxent model was AUC values that shaped the graph, and AUC could easily compare the performance of one model with

another. The black line on the AUC value was a random prediction, while the red line meant the value of training data. The AUC value of this study showed that the prediction of Sunda pangolin distribution was 0.909 (Figure 2). Based on the classification of AUC values from Araujo and Gausan (2006), the AUC value was categorized as very good.

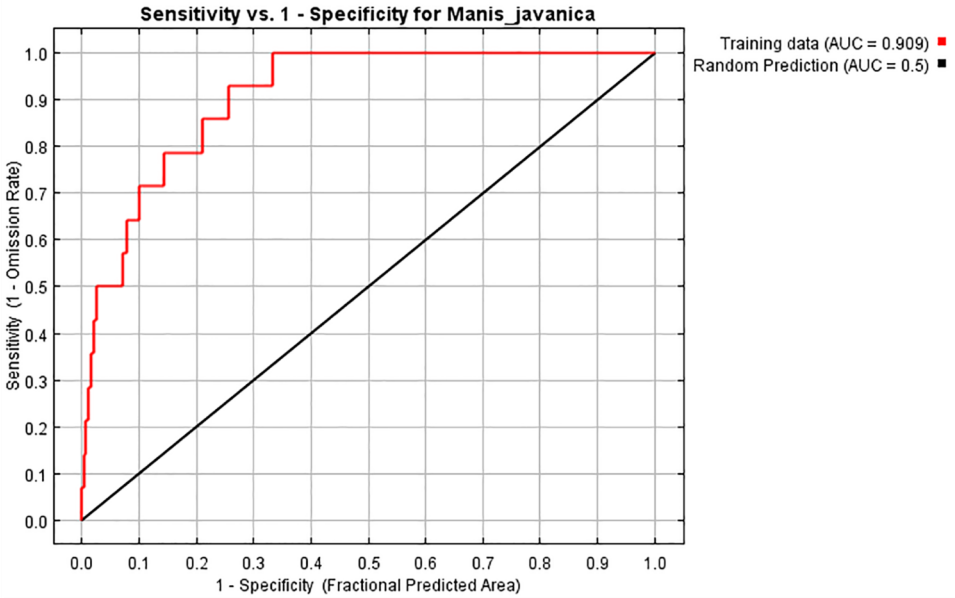

Figure 2. The sensitivity and 1-sensitivity graph of Sunda pangolin.

Figure 3 shows the prediction of the distribution of Sunda pangolin using the Maxent's analysis. The color gradations generated in the Maxent analysis provided separate information for the prediction of the presence of Sunda pangolins. The green color predicted a low distribution of Sunda pangolins presence, the orange color showed a moderate prediction

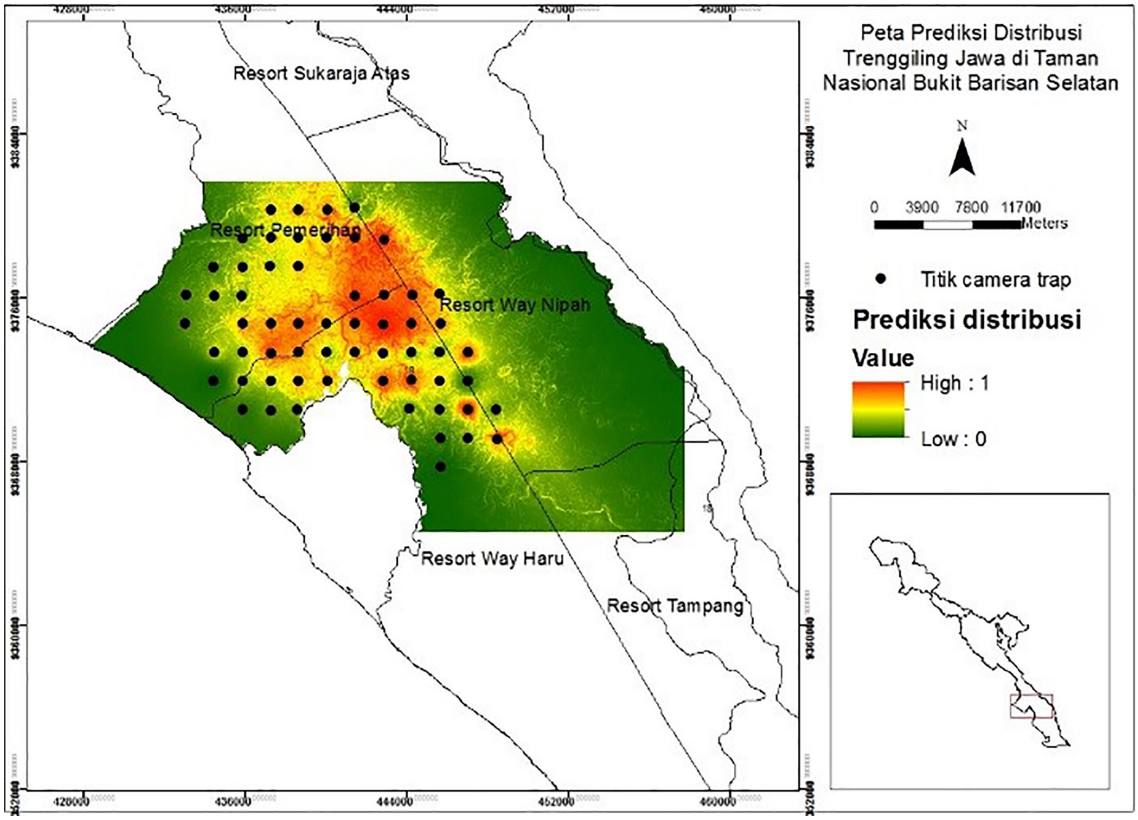

Figure 3. Prediction distribution of Sunda pangolin in BBSNP.

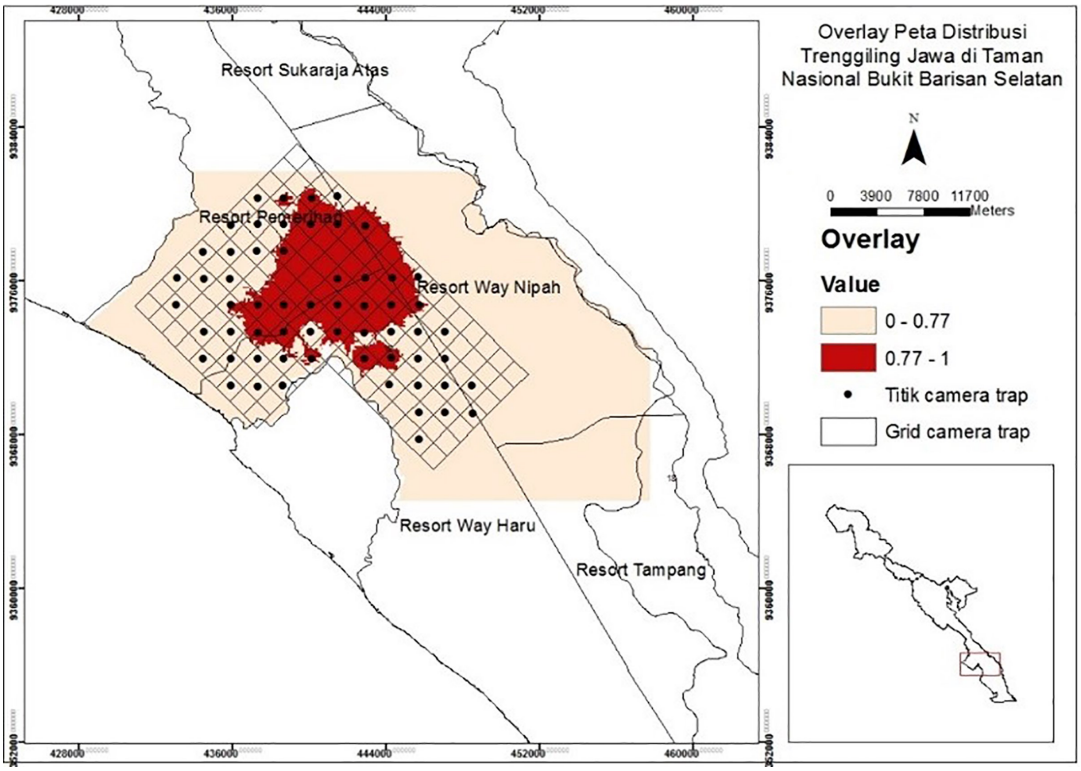

Figure 4. The overlay of distribution of Sunda pangolin in BBSNP.

distribution of Sunda pangolins, and the red color indicated as a high distribution of Sunda pangolin. The highest prediction of the Sunda pangolin was found in the Pemerihan Resort and Way Haru Resort area as indicated with the red color.

The results of the prediction distribution map, especially the line of the prediction area, were more visible using an overlay. An overlay is an overlapping thematic map process with different geographical layers to decide a spatial conclusion. The red color showed the prediction distribution area of Sunda pangolin with the probability of >0.77 (Figure 4). The highest prediction for the presence areas of Sunda pangolin was in the Pemerihan resort and Way Haru resort area. Both resorts were known to be the habitat for several endangered and protected species such as wild cats, sumatran tigers, and Sunda pangolins (Putri 2017).

Sunda pangolin tends to be found on difficult (or steep) lanes and slopes, and in this study, there were 14 points of Sunda pangolin presence found using TEAM camera traps which were located in this difficult condition. Manshur (2015) stated that slopes had an important role as an environmental variable affecting the presence of Sunda pangolin for as much as 72%. The slope was used by Sunda pangolins as an anti-predator strategy which was to roll away to protection. The slope used by Sunda pangolins ranged from 0-70 degrees (as seen from the result of the Maxent analysis), and the speed at which a Sunda pangolin roll itself could reach 15 km/ minute (Manshur 2015).

A direct encounter with Sunda pangolins did not occur in this study. However, scratches on trees and one scale of Sunda pangolin near a branching river. The scratches of Sunda pangolin that were found in the field could not use as presence data, because they were difficult to distinguish from those of the sun bear (Figure 5). The Pangolin Specialist Group (PSG) team in 2017, which conducted observations for two years in Thailand, was also still unsure whether the scratch came from Sunda pangolin. They assumed that the Sunda pangolin's scratches had 2 to 3 lines that stuck to

trees or soil. Sunda pangolin scratches seen in Ragunan Zoo also had 2 to 3 lines.

Figure 5. The scratches form of Sunda pangolin in Ragunan zoo.

Contribution of the environmental variables

Maxent provides the metric to determine the importance of environment variables in contribution percentages. The results showed that the distance from the village had the highest percentage of 47.8%, followed by canopy cover and distance from the threat having a percentage of 25.8% and 15.8% respectively (Table 2).

Table 2. Contribution percentage of environment variables of Sunda pangolin.

Variable	Contribution percentage
Village	47,4
Canopy cover	25,8
Threat	15,8
River	5,9
Slope	4,3
Food source	0,7
Road	0,2
Understorey	0
Elevation	0
Total	**100%**

Maxent model estimates that environmental variables have an important role in the resulting prediction model, i.e, environmental variables based on the contribution percentages of the jackknife test results. Based on the percentage of contribution, three environmental variables were most influential in the presence of Sunda pangolins: distance from the village, canopy cover, and distance from the threat.

Based on the distance response curve from the village (Figure 6), the probability of the presence of Sunda pangolin increased the further the distance from the village was. The probability of the presence of Sunda pangolin continued to increase until the distance from the road reached 9500

m (red line). Sunda pangolins tend to avoid the center of the crowd caused by other animals and humans in order to protect themselves from predators, such as big cats (Wang 2016). Additionally, Sunda pangolins are a solitary animal which does not like to appear in groups. Sunda pangolins use the distance of village as a strategy to avoid humans (Manshur 2015).

Figure 6. Environment variable response curve of the village distance of Sunda pangolin.

Besides the distance of the village, the contribution percentage of the environment variables showed that canopy cover was the second most important environment variable affecting the probability of Sunda pangolin's existence. Based on the canopy cover response curve, the probability of the presence of Sunda pangolin increased the higher the canopy cover was (Figure 7), shown by the consistent increase of the red line. The presence of Sunda pangolins increased when canopy cover values ranged from 70-90%. The high level of canopy cover density is a special habitat type used by Sunda pangolins to obtain food resources and to use as security strategies from both competitions and predators (Manshur 2015).

At the time of the study, the family of Dipterocarpaceae dominated the area where Sunda pangolins were present at the TEAM camera trap coordinates. This family grows at 0-800 masl with a wet climate and high humidity (Fajri 2008). One type of burrow made by Sunda pangolin was located under a tree trunk that had a hole near the ground. Pangolins make burrows in trees and use wood from this family because this type of wood is resistant to cold temperatures; thus, the temperature in the wood used as the burrow is still warm.

The third environment variable affecting the probability of Sunda pangolin was the distance from threats. Based on the distance from the threat response curve, the probability of the Sunda pangolin decreased the farther the distance from the threat was (Figure 8), shown by the downward red line. The Sunda pangolin is an animal that does not have many activities during the day and spends the afternoon resting and remaining silent. Sunda pangolin are difficult to find if there is no threat from natural or human predators. In addition, if the intensity of threat is too high the pangolin will go out of its burrow (based on the maxent analysis). In Indonesia, the population of Sunda pangolin in nature is still unknown. Researchers were difficult to meet Sunda pangolin, thus they used poachers to help them to research Sunda pangolin. Poachers used their steps or threats to find Sunda pangolin. The threats such as poacher using dog, traps, or used fire smoke.

Based on camera traps data, there were human illegal activities on nine camera traps coordinate.

The area of the Sunda pangolins prediction distribution map had a high level of hunting. This was evidenced by the discovery of traps made of rope, nylon straps, and iron strings. In addition, traces of poachers' tents, weapons, traps for birds, rifles or firecrackers, and animals were found. The research from Pangolin Specialist Group (PSG) team in 2017 showed that every 5 minutes, there was one pangolin take from nature. Sunda pangolin is one of the animals that most traded because it has higher economic value. Sunda pangolin tends to roll up the body when they feel threatened, and human was easy to catch. The knowing of the situation can encourage the national park and SMART patrol team to give more attention to the patrolling of the resort, thus the danger of Sunda pangolin hunting can be minimized.

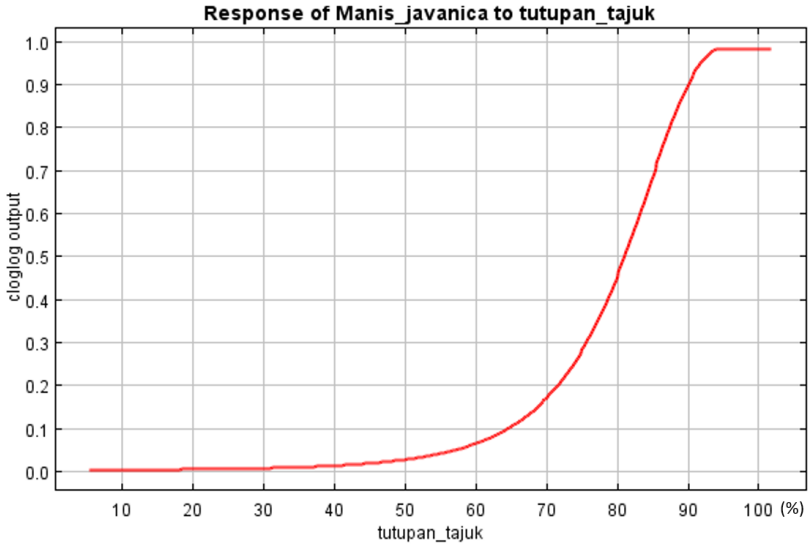

Figure 7. Environment variable response curve of canopy cover of Sunda pangolin.

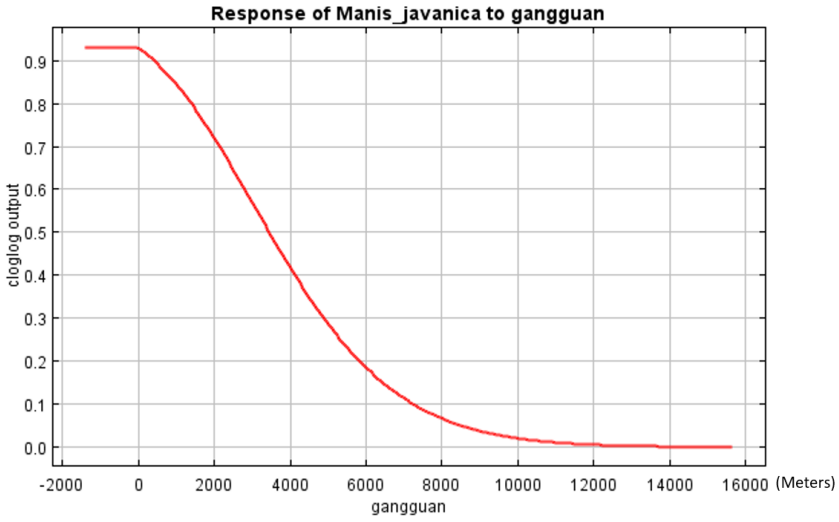

Figure 8. Environment variable response curve of the threat distance of Sunda pangolin.

CONCLUSION

Sunda pangolins tended to like with tight canopy cover of trees, found in this study. As solitary animals, Sunda pangolins tended to avoid the crowd. They would leave the burrow to look for food, and threats also made Sunda pangolins go out of their burrow and this made it was for them to be caught.

The tree environmental variables that most influence the distribution of Sunda pangolin are the distance of village (47.8%), canopy cover (25.8%), and distance from threat (15.8%). Poachers used smokes or dogs to achieve this condition. Pemerihan and Way Haru resorts were the highest prediction distribution of Sunda pangolins but they also had too much hunting and poaching. Both resorts were still the site of frequent illegal logging and trees falling naturally.

AUTHOR CONTRIBUTION

S.D.A designed the research and analyzed the data. All authors wrote the manuscript.

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CONFLICT OF INTEREST

The authors don't have conflict of interest.

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Review Article

Nutrigenomic and Biomolecular Aspect of *Moringa oleifera* Leaf Powder as Supplementation for Stunting Children

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ABSTRACT

Stunting is a global health problem. Based on WHO data, there are 161 million children who experience stunting. Breastmilk supplementation in the form of *Moringa oleifera* leaves powder is known to be beneficial in suppressing the stunting incidence. *Moringa oleifera* leaves powder contains protein, micronutrients, and minerals such as calcium, iron, sodium, vitamins C and E, beta carotene, and antioxidants (flavonoid acids, phenolic acids, glucosinolates, isothiocyanates, and saponins). The use of *Moringa oleifera* leaves powder in stunting cases has been carried out, but further studies in the aspects of nutrigenomics and molecular biology have not been conducted. This study is a literature review of relevant articles from www.pubmed.com, www.sciencedirect.com, and scholar.google.com. The author used keywords "stunting", "nutrigenomics", "biomolecular", and "*Moringa oleifera*". Pathogenic microbe such as *Shigella* and pathogenic *E. coli* ingestion can cause changes in DNA sequences in the stunting pathogenesis. *Moringa oleifera* leave powder can pass through nutrigenomic and biomolecular mechanisms. Some macro and micromolecules of *Moringa oleifera* leaves powder such as folate plays a role in DNA methylation; vegetable protein and fatty acids act as promoters in DNA sequences; vitamins act as cofactors for enzymes, antioxidant, and antiinflammation. Seeing various potential mechanisms in the aspects of nutrigenomic and molecular biology, *Moringa oleifera* leaves powder can be used in overcoming stunting. Further research is needed to give more review about cytokines and molecules included in this literature review.

Keywords: biomolecular, *Moringa oleifera,* nutrigenomic, stunting

INTRODUCTION

Stunting is a chronic nutritional deficiency problem that starts at early development which causes growth retardation thus the children will be shorter than it should be based on their age development (De Onis & Branca 2016). In addition to physical suffering, stunting also causes cognitive growth disorders (Zhang & Chandola 2017).

Stunting remains a global health problem to date. Based on the WHO data, there are 161 million stunted children worldwide (World Health Organization 2015). Indonesia is the fifth country with the most stunting

cases as many as 26% in 2015 which affected children under five years old (Kemenkes 2013; Kemenkes 2018).

When stunting is not addressed properly, it will become a serious problem for the government because it relates to the nation's future human resources. The Indonesian government has launched nutritional interventions that improve public education and specific supplementary nutrition. Specific nutritional intervention is done by providing complementary foods of breastmilk (Mitra 2015).

Giving the right complementary foods is known to be effective in suppressing the incidence of stunting. Food that can be given as complementary foods is *Moringa oleifera* leaves powder (Kuswanto & Widanti 2018). *Moringa oleifera* or Moringa plants are abundant in Indonesia (Dhakar et al. 2011). *Moringa oleifera* is a commodity that is widely grown in Indonesia which is potentially used as economically cheap complementary foods. The use of *Moringa oleifera* leaves has been carried out in a number of communities. For example, a study conducted in Yogyakarta reported that adding *Moringa oleifera* powder to baby food made a positive increase of body mass index to an average value around of 13-14 children from 30 respondents (Rahayu & Nurindahsari 2018). The study conducted at the Piyungan Community Health Centre showed a similar result. Mothers with higher educational status tend to choose complementary food that rich in *Moringa oleifera* powder for their babies which can increase toddler height by 0.476 cm to 0.594 cm (Muliawati & Sulistyawati 2019). *Moringa oleifera* contains minerals (such as calcium, iron, and sodium), vitamins C and E, beta carotene, as well as antioxidants (flavonoid acids, phenolic acids, glucosinolates, isothiocyanates, and saponins) (Leone et al. 2015). Various compounds in *Moringa oleifera* leaves powder can affect genomic processes (transcriptomic, proteomic, and metabolomic) as well as inflammatory processes that occur specifically in stunting (Susanto et al. 2017). Fulfilment of nutrients for infants needs to be done so that it does not cause inflammation and disruption of genomic processes that occur in stunting.

The utilization of *Moringa oleifera* leaves powder for stunting is currently being carried out, but there is no further study summarize the nutrigenomics and molecular biology aspects. Therefore in this literature review, we will discuss the nutrigenomic and molecular biology aspects of *Moringa oleifera* leaves powder as supplementation for stunted children. The review is written because no literature thoroughly discusses the ingredients contained in *Moringa oleifera* leaves against the pathophysiology of stunting.

MATERIALS AND METHODS

The writing method used was a literature review with relevant articles from search engines such as pubmed.com, sciencedirect.com, and scholar.google.com. We searched for the keywords "*stunting*", "*nutrigenomics*", "*biomolecular*", and "*Moringa oleifera*". Based on the search results, we filtered the literature and only use articles in proceedings or journals both in English and Bahasa Indonesia. Then sorting was done by understanding the abstract and article contents. Inclusion criteria were all research articles concerning the relationship of nutrition to *Moringa oleifera* with stunting. Articles exceeding the last 10 years were eliminated unless there was no new research that contradicts the contents of the article. Of the 71 articles reviewed, 60 articles were found to be suitable as references.

RESULTS AND DISCUSSION

Stunting

Stunting is a state of malnutrition so that individuals will be short below the

average growth chart. This condition is one of the contributors that cause an increase in early childhood death due to malnutrition which continues into the terminal phase (Susanto et al. 2017).

The level of parental education influences preferences in offering food for children's nutrition. Most parents only provide exclusive breastfeeding for up to six months, which makes breast milk not enough to support the nutrition of children. On the other hand, lack of socio-economic conditions makes parents unable to provide decent food for their children, so that children tend to be malnourished. Complementary foods are very important to be given to babies under one year old because complementary foods will support the maturation of myelination of brain development, thereby reducing cognitive deficits in child development (Soetjiningsih 1995).

Complementary foods should be given to infants, especially those 6-24 months old, to meet the nutritional needs of the child (Mufida et al. 2015). Most parents do not understand how to arrange nutritional needs according to the child's age so that children's nutrition is prone to developing failure (Maki A 2014). This is a reason to provide children's nutrition during the growth period because it will be an investment in the future condition of children (Shekar et al. 2017). In the process of child development, sufficient micronutrient content in complementary foods is needed so that fortification is necessary (Kim et al. 2009). Micronutrients and proteins play important role in the body, so if a child is suffering micronutrient deficiency, it triggers decreasing amino acid regulation in the body (Reddy et al. 2018). In addition, protein functions as a stabilizer and buffer in the body, so it has a role as a guard in the immune system such as antibodies (Laurus et al. 2016).

If the nutrient requirement containing protein and micronutrients is not fulfilled, the child will be susceptible to stunting. Stunting tends to increase susceptibility to infection due to the release of proinflammatory cytokines in the form of IL-1, IL-6, and IL-8 which cause children to have the potential to develop metabolic syndrome in the future (Abd El-Maksoud et al. 2017). This incident requires the ability of parenting care to provide proper nutrition to children so that children do not become malnourished (Glover-Amengor et al. 2017).

Moringa oleifera Leaves

Moringa oleifera is a cultural-magical-medical plant that has many bioactive substances and can live in a variety of soil conditions (Daba 2016). According to research in Uganda, the *Moringa oleifera* plant can be used to cure 24 chronic metabolic diseases and one of them is stunting (Kasolo et al. 2010). The nutrient of *Moringa oleifera* is fairly complete, namely protein, micronutrients, natural minerals, and antioxidants (Leone et al. 2015). This is supported by research in Tanzania, the nutritional content of *Moringa oleifera* is higher than oranges, eggplants, spinach, cabbage, and peanuts (Shija et al. 2019). The nutritional value per 100 g of *Moringa oleifera* leaves extract consists of carbohydrate 9.1 g, dietary fiber 2.1 g, fat 1.7 g, and protein 8.1 g. The vitamin content of *Moringa oleifera* are vitamin A 80 µg, thiamine 0,103 mg, riboflavin 0,112 mg, niacin 1.5 pantothenic acids 0.48 mg, vitamin B6 0.129 mg, folate 41 µg, and vitamin C 8,6 mg (Abbas et al. 2018). *Moringa oleifera* is relatively easy to be made into powder and efficient because it only dried with the sun and then blended with the machine, the nutritional content is similar when compared to conventional methods (method with additional 35-55^{0}C heating in a machine) (Susanto et al. 2017). Thus, this plant is practically used in households, contains enough polyphenols for infant nutrition, and can meet iron needs for anemia (Teshome et al. 2009; Zongo et al. 2013; Rahayu & Nurindahsari 2018). Five grams of *Moringa oleifera* powder as complementary food at a two-phase meal every day already

fulfil 75% of the nutritional needs of babies after breastfeeding (Amagloh et al. 2012). The use of *Moringa oleifera* leaves powder for overcoming stunting for 4 months is considered feasible (Rosha et al. 2016). Side effects such as diarrhoea rarely complained because *Moringa oleifera* leaves powder also act as antidiarrheal (Joung et al. 2017).

Moringa oleifera leaves have the potential benefit to modify epigenetics. They also have benefits as active substances in the nutrigenomic approach, besides its anti-inflammatory, antioxidant, and antianemic effect.

Stunting can be triggered by the presence of contaminants in food because there are pathogenic microbes. The contaminated food will enter the digestive tract which triggers an imbalance in the composition of the intestinal microbiota to change structure, function, and ability to gut epithelial regeneration that changes metagenomic and metatranscriptomic processes. There are two pathways from these metagenomic and metatranscriptomic changes. The first pathway explains the disruption of tight junctions and enterocytes that increase intestinal permeability and then increase microbial translocation. The increased translocation of microbe increase lipopolysaccharide (LPS), Endocab, sCD14 receptor for LPS, sCD163 receptor for LPS, and trigger gut mucosa inflammation. The inflammation of mucosa can increase neopterin, ATT (specific protein), myeloperoxidase (MPO), and can trigger systemic cascade and subsequent increased level of C-reactive proteins (CRP) and alfa 1-acid glycoprotein (AGP). This process increases the child's nutritional needs and creates growth hormone resistance, increases hepcidin, and decreases EPO (erythropoiesis). The second pathway explains the occurrence of metagenomic and metatranscriptomic changes and causes intestinal villi

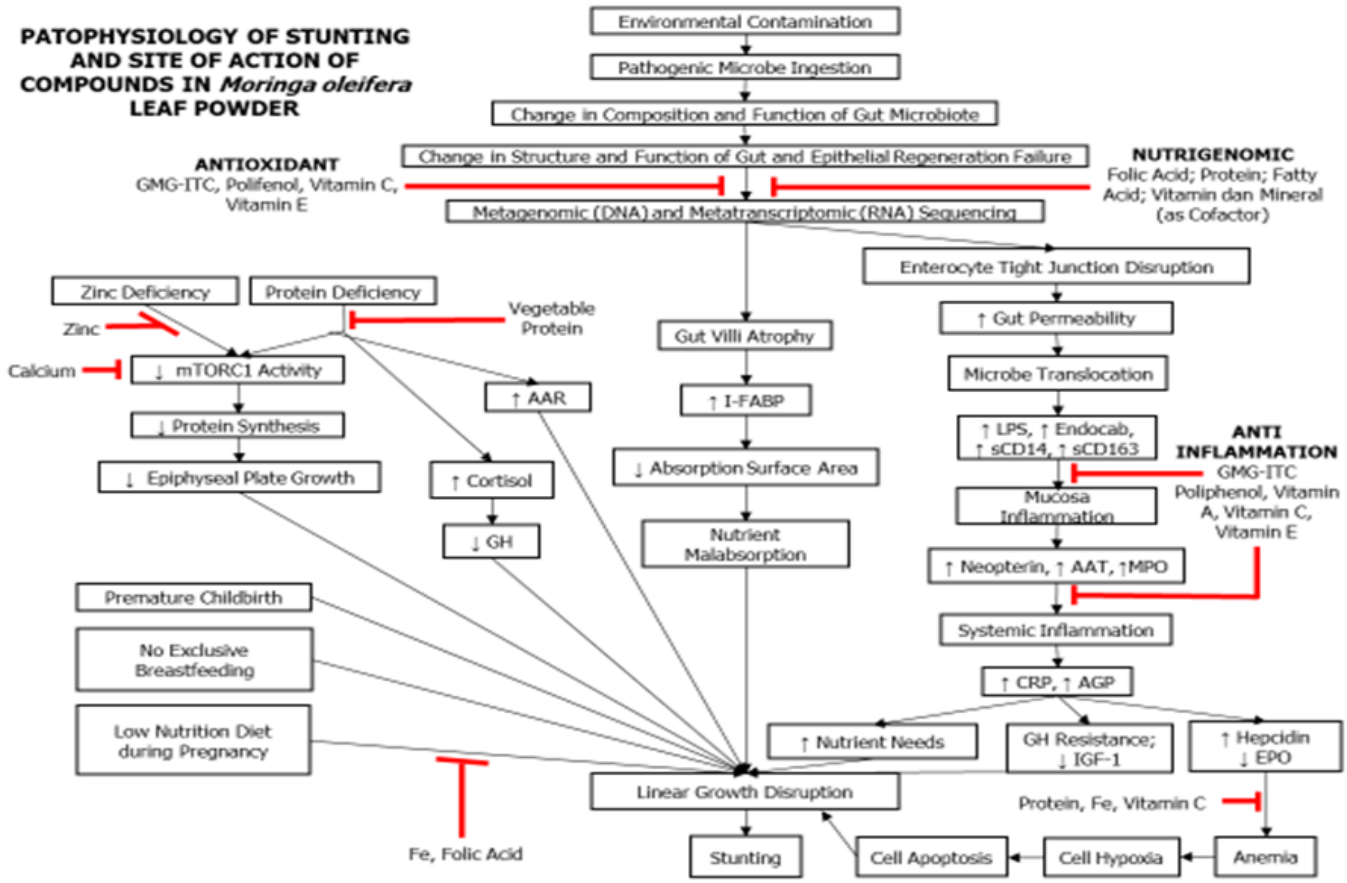

Figure 1. Mechanism Action Review of *Moringa oleifera* leaves powder in Stunting Pathophysiology at Molecular, Cellular, and Physiological Level via Nutrigenomic, Antioxidant, Antiinflammation, and Antianemia Mechanism (Sedgh et al. 2000; Mohan 2013; Ma et al. 2020; Prendergast et al. 2015; Gonzalez et al. 2015; Saini et al. 2016; Denardo et al. 2015).

atrophy can increase intestinal fatty-acid binding protein (iFABP) and decreases the absorption surface area which causes malabsorption of nutrients consumed by children. Increased hepcidin and decreased EPO will cause anemia which triggers cell hypoxia and cell apoptosis causes malabsorption of the nutrients consumed. As a result, there is an increase in nutrition due to cell apoptosis, which if not fulfilled the nutritional adequacy disrupts the linear growth of the child so that it becomes stunting. In addition, stunting can be caused by zinc deficiency and protein deficiency as well as premature births that do not receive exclusive breastfeeding and lack nutrition during pregnancy. *Moringa oleifera* has antioxidant and nutrigenomic mechanisms that inhibit the metagenomic and metatranscriptomic sequences. In addition, the anti-inflammatory effect of *Moringa oleifera* inhibits mucosal and systemic inflammation. Another content in the form of calcium inhibits the decrease in mTOR complex 1 (mTORC1) activity which increases protein synthesis and increases the growth of the epiphyseal plate which results in increased linear growth of children. This explanation is described in Figure 1. (Sedgh et al. 2000; Mohan 2013; Ma et al. 2020; Prendergast et al. 2015; Gonzalez et al. 2015; Saini et al. 2016; Denardo et al. 2015; Stefano et al. 2019).

Mechanism in Nutrigenomic Aspect

The pathogenesis of stunting involves the changes in deoxyribonucleic acid (DNA). The genome can be modified through nutrigenomics where the nutrients affect the genome stability. Nutrigenomics is closely related to epigenetics. Nutrigenomics is the study of the effects of substances contained in food on gene expression. Whereas epigenetics refers to variations derived from DNA that regulate chromosome architecture and modify gene expression without changing the underlying DNA sequence. Epigenetics can be distinguished from genetic mutations. Epigenetics does not produce a change in the nucleotide sequence because they consist of modifications such as DNA methylation, histone deacetylation, gene silencing by microRNA (miRNA), and maintaining chromosome stability (Gonzalez et al. 2015; Saini et al. 2016). DNA wrapped around a protein called a histone. Modification of histones refers to how closely the DNA strands are wrapped around the histones. Modified histones are known to affect protein transcription, DNA repair processes, DNA replication, and chromatin condensation. Dietary factors directly influence these epigenetic mechanisms (Saini et al. 2016). Nutrition can change gene expression in several ways, such as: acting as a ligand for transcription factor receptors; being metabolized in the primary or secondary metabolic pathways thereby changing the concentration of substrates or intermediates; and changing the signal transduction pathway. There are three important influences that can change the way genes are expressed, namely what genes are turned on, how the messages in genes are transcribed, and what post-translocation effects in cells by the expression of these genes (Gonzalez et al. 2015).

In nutrigenomics aspects, the folate contained in *Moringa oleifera* leaves powder plays a role in epigenetic modification such as DNA methylation, through the pathway as described in Figure 2 and Figure 3. Figure 2 described approximately 50% of S-adenosylmethionine (SAM) is metabolized in the liver. SAM is metabolized to S-adenosylhomocysteine (SAH) which is then metabolized to homocysteine. Homocysteine can either be metabolized to cystathionine and then cysteine or methionine. *Moringa oleifera* leaves powder also contains vegetable protein which acts as a ligand in the DNA sequence promoter which then starts DNA transcription for protein production again (Huang et al. 2014). This reproduced protein plays a role in overcoming stunting (Ma et al. 2020). In addition to protein, the fatty acids in

Moringa oleifera leaves powder also have a similar ability to affect DNA transcription (Mohan 2013). The fatty acids contained in *Moringa oleifera* leaves powder is ω-3 Poly Unsaturated Fatty Acid (PUFA) which is of α-linolenic as shown in Figure 4. Long chain ω-3 fatty acids such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) are synthesized de novo in organisms from α-linolenic fatty acids (Rodríguez-Cruz & Serna 2017). The vitamins and minerals contained in *Moringa oleifera* leaves powder also play a role in nutrigenomics because one of its functions is to increase enzyme efficiency. Vitamins are cofactors for enzymes, enzymes classified as protein, and proteins can be changed due to genetic changes and these genetic changes affect protein function. The formation of an active cofactor requires an active enzyme, moreover for the enzyme to function at its maximum capacity, an adequate active cofactor must necessarily exist (Gonzalez et al. 2015).

Folic Acid has a role in the nutrigenomic process. This folic acid enters the nucleus which triggers dihydrofolic acid (DHF) to be converted into tetrahidrofuran (THF). Later, THF with the help of vitamin B6 and vitamin B2 will be converted to 5-10 methylene THF and combine with betaine and homocysteine to become B12. Vitamin B12 and B6 will be converted into methionine which then is converted into S-adenosylmethionine (SAM) and by the DNA methyltransferase (DNAMTase) and SAM enzymes enter the cell nucleus and undergo epigenetic modification which plays a role in the nutrigenomic process (Huang et al. 2014). This explanation is described in Figure 2.

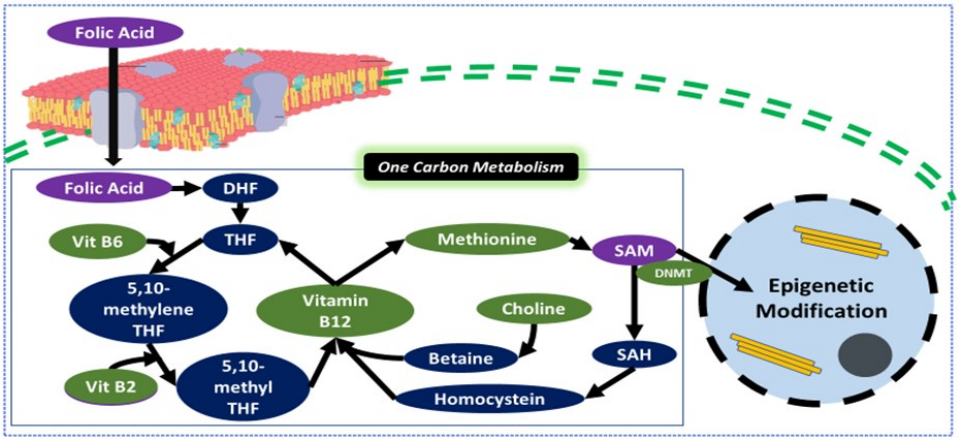

Figure 2. S-adenosylmethionine (SAM) Produced in One Carbon Metabolism of Folic Acid, Act in Cell Nucleus to Induce Epigenetic Modification (Modified From: Huang et al. 2014).

S-adenosylmethionine (SAM) that enters the nucleus causes genomic methylation and metabolic methylation. The genomic methylation cause chromatin remodeling and histone modifications. Histone modification trigger activated gene silencing stunting. An increase in mTOR complex 1 (mTORC1) in the presence of glucose, growth factor, and amino acids also increase mTORC1 activity which activates Unc -51 Like Autophagy Activating Kinase 1 (ULK1) and causes a decrease in autophagy so that cells do not eat themselves and then trigger 4E-Binding Protein (4EBP) to synthesize protein and contribute to child development (Huang et al. 2014). This explanation is described in Figure 3.

The fatty acids contained in *Moringa oleifera* leaf powder is ω-3 Polyunsaturated Fatty Acid (PUFA) which is α-linolenic as shown in Figure 4. Long chain ω-3 fatty acids such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) are synthesized de novo in organisms from α-

linolenic fatty acids (Rodríguez-Cruz and Serna, 2017). EPA and DHA trigger peroxisome proliferator-activated receptor gamma (PPAR-γ) activity which then enter the nucleus to induce adiponectin production. EPA and DHA also increase beta-oxidation and reduce lipid accumulation through adenosine monophosphate protein kinase (AMPK) activity. Inhibition of prostaglandins E2 (PGE2) formation also occurs through arachidonic acid (AA) decrease. EPA and DHA also inhibit lipopolysaccharide (LPS) and increase the activity of G-proteins couples receptors 120 (GPR120) so that it inhibits nuclear factor kappa beta (NFKB) and do not form interleukin-6 (IL -6) and monocyte chemoattractant protein 1 (MCP1). The vitamins and minerals contained in *Moringa oleifera* leaf powder also play a role in nutrigenomics because one of its functions is to increase enzyme efficiency. Vitamins are cofactors for enzymes, enzymes classified as protein, and proteins can be changed due to genetic changes and affect protein function. The formation of an active cofactor requires an active enzyme, moreover for the enzyme to function at its maximum capacity, an adequate active cofactor must necessarily exist (Gonzalez et al. 2015).

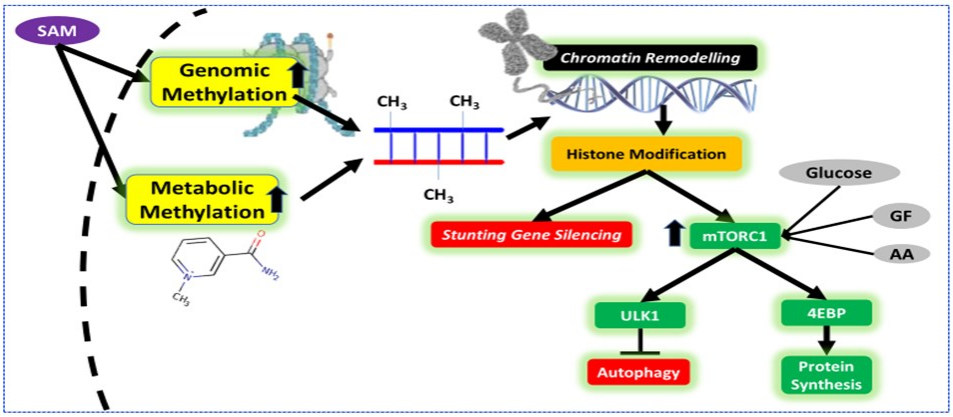

Figure 3. Mechanism of Action of SAM in Nutrigenomics (Modified From: Huang et al. 2014).

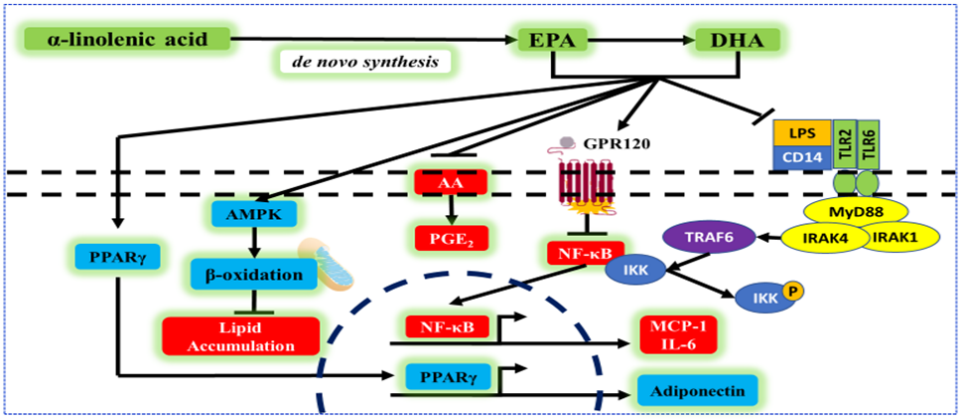

Figure 4. Mechanism of Action of α-linolenic acid in Nutrigenomics (Modified From: Rodríguez-Cruz & Serna 2017).

Mechanism in Biochemical Aspect

Antioxidants are compounds that inhibit oxidation and thus prevent oxidative stress which causes DNA damage (Wang et al. 2017). The antioxidant agents contained in Moringa oleifera leaves powder are polyphenols, vitamin C, vitamin E, and glucomoringin-isothiocyanate (GMG -ITC). Polyphenols induce antioxidant effects through the mechanism of hydrogen atom transfer (HAT), single electron transfer (SET), and transition metal chelation (TMC) (Leopoldini et al. 2011). Polyphenols are useful in

reducing lipid peroxide and increasing the concentration of glutathione (GSH), along with decreasing the activity of SOD and catalase enzymes. In addition, polyphenols can also reduce the concentration of serum malondialdehyde (MDA) (Ma et al. 2020). *Moringa oleifera* leaf powder rich in polyphenol (such as flavonoids quercetin, isorhamnetin, and kaempferol glycosides) and phenolic acids (Sedgh et al. 2000; Ma et al. 2020). Vitamin C and E work together as antioxidants. Vitamin E converts lipid free radicals such as peroxyl (LOO•) and alkoxyl (LO•) into lipid hydroperoxide (LOOH) and lipid hydroxide (LOH) through the transfer of hydrogen atoms to its structure. Vitamin E which has lost its hydrogen atom will be renewed again by vitamin C so that vitamin E contains the hydrogen atom again and is ready to convert free radicals again (Valko et al. 2004).

GMG-ITC is one of the dominant types of glucosinolate found in *Moringa oleifera* leaves powder. GMG that is converted to ITC has active antioxidant effects on the body (Figure 5). ITC causes an increase in expression of erythroid 2-related factor 2 (Nrf2) which then translocates to the nucleus and releases its bond with KEAP1 in the cytoplasm. Inside the nucleus, Nrf2 binds to small Maf proteins (sMaF) and undergo phosphorylation which triggers the transcription of several compounds that are essential in the process of lowering oxidative stress. Compounds that will be enhanced are superoxide dismutase-1 (SOD-1), GSH/GSR, NAD(P)H:quinone oxidoreductase 1 (NQO1), glutaredoxin (GLRX), heme oxygenase-1 (HO-1), thioredoxin reductase/thioredoxin (TXNRD/TXN), and nicotinamide adenine dinucleotide phosphate (NADPH) (Saini et al. 2016).

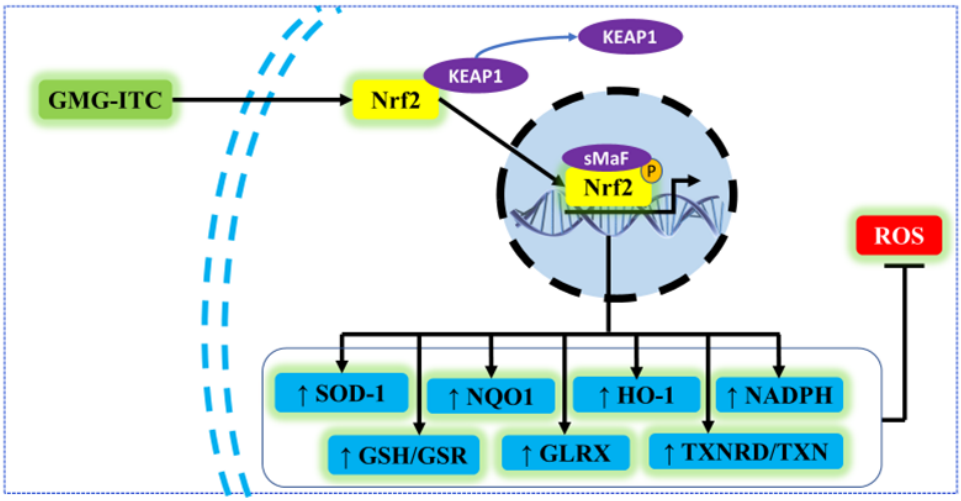

Figure 5. Mechanism of Action of GMG-ITC as an Antioxidant (Modified From: Saini et al. 2016).

Inflammation also plays an important role in the pathophysiology of stunting, by inducing Growth Hormone (GH) resistance, an increase in the body's nutritional need, and also anemia, and all of these results in cell apoptosis as shown in Figure 1. Inflammation begins with the binding of lipopolysaccharides (LPS) to TLR4. The anti-inflammatory agents in *Moringa oleifera* leaf powder which plays a role in inhibiting stunting are vitamin E, vitamin C, GMG-ITC, and All-E lutein as described in Figure 6 (Prendergast et al. 2015). Vitamin E and C play a role in inhibiting NF-κB, where vitamin C acts directly inside the nucleus and vitamin E through PKC-θ inhibition. Meanwhile, GMG-ITC and All-E lutein reduce TLR4 expression which will cause a decrease in NF-κB activation through MAPK (ERK/JNK) and IκBα. The suppression of NF-κB will inhibits the expression of messenger RNA (mRNA) and results in decreased inducible nitric oxide synthase (iNOS),

cyclooxygenase-2 (COX-2), and thereby decrease the release of TNFα, IL-1β, IL-6, and IL-8 (Mohan 2013). GMG-ITC will also enhance IKK activation which will reduce the phosphorylation of IκB and will repeat the process of NF-κB inhibition again. This explanation is described in Figure 6. (Mohan 2013).

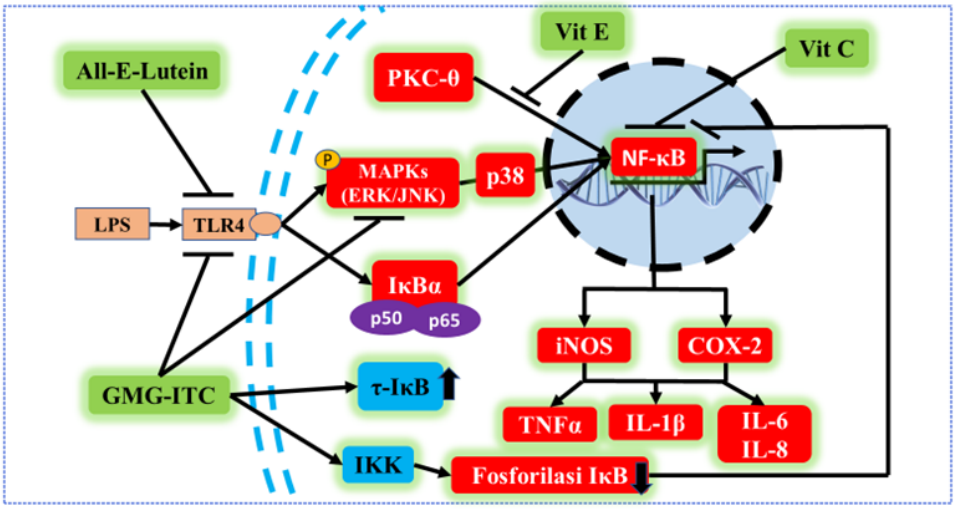

Figure 6. Mechanism of Action of GMG-ITC, All-E-Lutein, Vitamin C, and Vitamin E as An Antiinflammation

Moringa oleifera may acts as an anti-anemia through direct and indirect mechanisms. The indirect mechanism occurs through the depletion of IL-6 synthesis which is induced by GMG-ITC, thereby inhibits hepcidin expression in hepatocytes (Ganz & Nemeth 2011). The direct anti-anemia mechanism involves several active compounds in *Moringa oleifera* leaves powder, such as vegetable protein, iron, and vitamin C (Idohou-Dossou et al. 2011; Saini et al. 2014). Vegetable protein will be converted to amino acid (AA) in the digestive tract which then contributes to erythropoiesis through the synthesis of transferrin, protoporphyrin, and globin (Idohou-Dossou et al. 2011). Iron, in addition to its role in erythropoiesis (the formation process of erythrocytes with iron that binds oxygen), may also suppress the synthesis of hepcidin mRNA in hepatocytes. Reduced hepcidin level raises the ferroprotein (FPN) activity. It facilitates iron to enter enterocytes into the blood plasma and trigger erythropoiesis in the bone marrow. This effect incites the potential of the iron contained in *Moringa oleifera* leaves powder to be better than the iron contained in ferric citrate (a potent drug for anemia at the moment) (Saini et al. 2014). Vitamin C acts as a nonheme iron enhancer. Iron in *Moringa oleifera* leaves powder is classified as nonheme iron (Fe^{3+}) because it comes from a plant. In order to enter the enterocytes, this type of iron needs to be converted into the form of Fe^{2+}. The conversion of Fe^{3+} to Fe^{2+} occurs with the help of duodenal cytochrome b (DCYTB), a ferric reductase enzyme. This enzyme requires vitamin C as a cofactor by donating its electrons and reduce Fe^{3+} to Fe^{2+}, therefore Fe^{2+} may be transported by divalent metal transporter-1 (DMT-1). The vitamin C compound is already contained in *Moringa oleifera* leaves powder to facilitate the conversion of the iron and help its absorption inside the gut (Idohou-Dossou et al. 2011).

The Advantages of *Moringa oleifera* Leaves Powder

The development of innovative use of *Moringa oleifera* leaves powder as an additional ingredient for complementary food has the potential to fulfil the baby's nutritional needs and prevent stunting. The use of *Moringa oleifera* leaves powder as an alternative to supplement food for stunting children may

ease the country's burden in the massive import of green beans and milk which is also difficult to reach by the middle to lower economic class (Nasmiati et al. 2014).

According to the survey results of food consumption in Central Java Province, the majority of people consume foods containing vegetable protein compared to animal protein meat that relatively more expensive (Rosha et al. 2016). The vegetable protein that is often consumed is in the form of processed soybeans. According to a previous study by Swarinastiti et al. (2018), tempeh consumption alone causes a 4.49-times risk of stunting in children, because tempeh is low in amino acids (methionine and cysteine) and if the protein synthesis is not optimal, the susceptibility of growth faltering will be higher (Swarinastiti et al. 2018). Based on the latest research, the dietary formulation to improve stunting condition is still by a combination of staple foods (corn, rice porridges, and/or yams) with vegetable protein sources (tofu and tempeh), and add *Moringa oleifera* leaves powder to complete all of the nutritional needs with the ratio of 60:30:10 to meet the minimum calories requirement by WHO that is 200-300 kcal (Stefano et al. 2019; Netshiheni et al. 2019).

The recommended food processing for children is steaming because it is easily pulverized and no protein denaturation compared to the deep frying method which may lower the micronutrient contents by 5-40% (Mohan 2013). Vegetable protein can be given starting from 6 months old, meanwhile animal protein can be safely given at the age of eight months old because it contains gluten that is difficult to digest. In addition, meat that is available in the market may contain preservatives such as monosodium glutamate (MSG) (Ganasen et al. 2018). MSG may trigger a slowdown in linear growth that causes suboptimal growth in children (Chakraborty 2019). Preserved meat may also induce metabolic syndrome in the future, which is also a complication of stunting (Chibisov et al. 2019). *Moringa oleifera* leaves powder contains zinc and iron, thereby is the potential to be the main source of complementary food (Mawouma et al. 2017).

The Limitations of *Moringa oleifera* Leaves Powder

In the process of giving *Moringa oleifera* leaves powder as a complementary food, the limitation that arises is the bitter taste. On the other hand, the bitter taste is a form of taste recognition in children. This process also supports the development of children's taste sensation. To overcome this limitation, the consumption of *Moringa oleifera* leaves powder can be done by adding the powder in several food segments and also estimates the children's eating habits and times (Probowati et al. 2016).

Complementary food consumption can be adjusted by arranging a daily schedule, such as a combination of fruit juice with honey and additional *Moringa oleifera* leaves powder in the morning, and a combination of porridge with milk or coconut milk and additional *Moringa oleifera* leaves powder in the afternoon or evening. This consumption should be done before breastfeeding in order to avoid the feeling of fullness from the baby and eventually refuse to eat (Oyeyinka & Oyeyinka 2018). The principle of giving complementary food fortification of *Moringa oleifera* leaves powder is to provide a good experience for the children and minimize the bad experience (Loya & Nuryanto 2017). The effects of this *Moringa oleifera* leaves powder will be optimal if the consumption takes place constantly every day for four months (Joung et al. 2017).

This literature review has discussed some pathways that are considered to treat stunting, such as nutrigenomics, anti-inflammatory, antioxidants, and antianemia. However, cytokines and molecules included in this literature

review have not been widely discussed, so it is necessary to conduct further literature studies.

CONCLUSION

Moringa oleifera leaves powder is very beneficial in preventing stunting. The consumption of *Moringa oleifera* leaves powder as a fortification in complementary food has the potential through nutrigenomic and biology molecular aspects with its mechanisms as an antiinflammation, antioxidant, and antianemia agent. Therefore, further research related to these aspects must be developed in the future.

AUTHORS CONTRIBUTION

Data gathering and idea owner of this study was conducted by Agus Indra Yudhistira Diva Putra, Nyoman Budhi Wirananda Setiawan, Made Indira Dianti Sanjiwani, Agung Wiwiek Indrayani. Writing and submitting manuscript was carried out by Agus Indra Yudhistira Diva Putra, Nyoman Budhi Wirananda Setiawan, Made Indira Dianti Sanjiwani, Agung Wiwiek Indrayani. In addition, editing and final draft approval was conducted by Agung Wiwiek Indrayani and Ida Ayu Ika Wahyuniari.

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CONFLICT OF INTEREST

There is no competing interest regarding manuscript.

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