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Short Communication

Bioethanol Levels of Dragon Fruit (*Hylocereus polyrhizus*) Peel with the Addition of Blend Crude Cellulase Enzyme from *Trichoderma reesei* and *Aspergillus niger*

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ABSTRACT

The petroleum fuel crisis shows that Indonesia's fossil energy reserves are limited. It is necessary to develop an environmentally, friendly and sustainable alternative energy, one of which is bioethanol. This study aims to determine the bioethanol levels of dragon fruit (*Hylocereus polyrhizus*) peel with the treatment of cellulase enzymes from *Trichoderma reesei* and *Aspergillus niger*. This research was an experimental study that uses steps such as making dragon fruit peel substrate and filtrate, cellulose degradation with enzymes from *Trichoderma reesei* and *Aspergillus niger* and inoculating with yeast (*Saccharomyces cerevisiae*) with a fermentation time of 96 hours and then measured reducing sugar levels with the method of DNS, distillation, and the measurement of bioethanol levels using alcohol meters. The results have shown that using enzymes from *Trichoderma reesei* and *Aspergillus niger* can increase the reduction of 49.68 % sugar levels in the treatment of *T.reesei: A.niger* (3: 1) and produce the highest bioethanol level, which is 2.46 % in the treatment of *T.reesei: A.niger* (2: 1)

Keywords: *Aspergillus niger*, dragon fruit peel, cellulase, *Trichoderma reesei*

The increasing demand for ethanol for various industrial purposes such as alternative sources of energy, industrial solvents, cleansing agents and preservatives has necessitated increased production of this alcohol (Ali et al., 2011). In the current time, the importance of alternative energy sources has become even more necessary not only due to the continuous depletion of limited fossil fuel stock but also for a safe and better environment. With an inevitable depletion of the world's energy supply, there has been an increasing worldwide interest in alternative sources of energy (Lynd et al., 2017; Chandel et al. 2007; Wyman, 1999; Herrera 2004; Herrera, 2006; Lin, 2006; Vertes & Inui, 2006; Schubert, 2006; Dien, 2003).

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Currently, biomass-derived ethanol is produced at an industrial scale from sucrose and starch; however, this poses concerns about the potential competition with food and feed supplies (Hahn-Hägerdal et.al, 2006; Field, Campbell, J. E., & Lobell, 2008). Hence, other alternatives such as the production on fallow fields of crops and grasses to produce biofuels have recently attracted attention. In particular, the lignocellulosic materials such as agricultural wastes are considered to be the main potential sources of biomass for "second generation" bioethanol production (Hu.,et al., 2008; Hahn-Hägerdal et al., 2006; Sakai et al, 2007; Merino & Cherry, 2007; Goh et al, 2010).

Bioethanol is one of the renewable alternative fuels that have the potential to be developed in Indonesia. Bioethanol is produced from biomass fermentation processes aided by microorganisms.

The requirement to make bioethanol is a biological material that has sugar content (glucose, starch, and fibre) (Hambali et al, 2007) among other is dragon fruit peel. Dragon fruit including cactus or Cactaceae family, red dragon fruit peel contains sugar component around 8.4% and also other complex carbohydrates like cellulose around 68.3% (Jamilah et al., 2011).

Table 1. Sugar levels of Dragon fruit peel after treatment of *T.reesei* and *A.niger*

Cellulase ratio between *T. reesei* and *A. niger*	Sugar level (mg/mL)
Control	49.41 ± 3.94[a]
1:0	94.31 ± 3.56[de]
0:1	91.25 ± 2.29[d]
1:1	73.60 ± 1.72[b]
2:1	96.95 ± 4.98[de]
1:2	84.44 ± 4.69[c]
3:1	98.20 ± 2.83[e]
1:3	76.66 ± 3.56[b]

The same letter within each row do not differ significantly (p > 0.05) according to the Duncan test.

The cellulose degradation process can be done chemically or biologically using cellulolytic organisms originate from bacteria or fungi, degradation of cellulose into simpler sugars in the form of both cellobiose and glucose with the help of a catalyst. Hydrolysis can be carried out chemically (acid) or enzymatically, enzymatic hydrolysis using cellulase enzymes. Cellulase enzymes can be produced from cellulolytic microbes both mold and bacteria, while molds commonly used from *Trichoderma* and *Aspergillus*. *Trichoderma reesei* has been widely used for the production of commercial cellulase (Vandana and Anahit, 2014). Cellulase is a multi-component enzyme comprising of endoglucanase, which attacks cellulose in the amorphous zone and releases oligomers such as cellobiohydrolase, that liberate cellobiose from reducing and non-reducing ends also β-glucosidase, which hydrolyze cellobiose to glucose and play a key role in avoiding cellobiose inhibition and thus enhancing the hydrolysis rates of cellulose into glucose (David, 2008; Mehdi et al,, 2010; Sunkyu et al., 2010; Baljit, 2014; Veeresh and Wu, 2014). *Aspergillus niger* produces an enzyme that plays a role in accelerating the conversion of cellobiose to glucose, the enzyme β-glucosidase (Juhasz et al., 2003). The combination of cellulase enzymes from *T. reesai* and *A. niger* can be expected to increase the change of cellulose into glucose which is a material for making bioethanol.

This study aims to determine the levels of bioethanol dragon fruit peel (*Hylocereus polyrhizus*) with the treatment of cellulase enzymes from *Trichoderma reesei* and *Aspergillus niger*.

Dragon fruit peels were obtained from a local market in Indonesia. For analytical purposes, the Dragon fruit peels were made powder. *Trichoderma reesei* and *Aspergillus niger* culture were obtained from The Food & Nutrition Culture Collection (FNCC), Food and Nutrition Centre, Universitas Gadjah Mada. The strains were maintained on PDA and incubated at room temperature for seven days. (Safaria et al., 2013).

A total of 5 g of Dragon fruit peel powder were put into Erlenmeyer 250 mL and added 25 mL nutrition solutions which every 1000 mL contains 1.0 g yeast extract, 1.5 g peptone, 1.4 g $(NH_4)_2SO_4$; 2.0 g KH_2PO_4, 0.005 g $FeSO_4•7H_2O$, 5 mL solution CMC 1%. The flasks were sterilized for 15 minutes at 121°C. Two milliliters of spores (107-108 spores/ mL) were inoculated and incubates at room

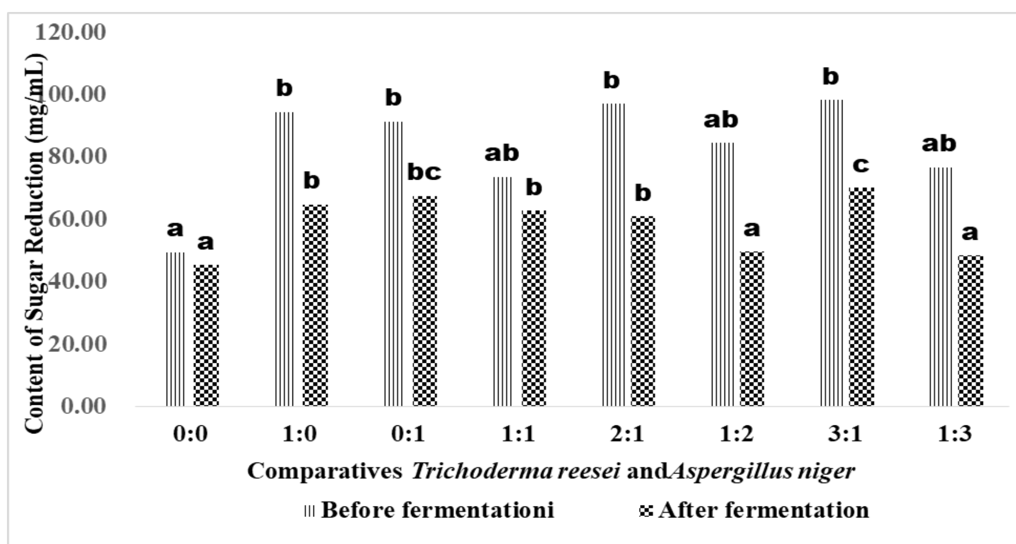

Figure 1. Reduced sugar levels before and after the treatment with *Saccharomyces cerevisiae*

temperature and static condition, for 6 days for *Trichoderma reesei* and 8 days for *Aspergillus niger* (Sri Winarsih et al., 2014).

Tween 80 0.1% solution was taken 100 mL and poured into the dragon fruit peel sample then stirred at 150 rpm for 120 minutes at room temperature. The solution was then centrifuged at 3000 rpm for 10 minutes. The supernatant obtained was used as a crude enzyme extract (Szendefy et al., 2006).

Dragon fruit peel porridge put into Erlenmeyer 100 mL then added the crude enzymes *T.reesei* and *A.niger* were added as much as 10% each according to the treatment with variations of 1: 0, 0: 1, 1: 1, 2: 1, 1: 2, 3: 1, 1: 3 using a measuring pipette aseptically and made three replications (21 Erlenmeyer), then stirred using a sterile glass stirrer. Erlenmeyer was covered with sterile cotton and coated with aluminum foil then incubated for 24 hours using an incubator at 37 °C. Hydrolysis results were measured for reducing sugar levels.

The culture of *Saccharomyces cereviceae* JCM 3012 was obtained from The Food & Nutrition Culture Collection (FNCC), Food and Nutrition Centre, Universitas Gadjah Mada. The strain was maintained on YM to keep medium (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, glucose 10 g/L) at 4 ºC. To carry-out the tests *S. cerevisiae* was grown overnight at 30 ºC on a rotary shaker (INNOVA 44, Incubator Shaker Series, New Brunswick Scientific) at 200 rpm, in tubes containing 20 ml YM medium.

Dragon fruit peel which produced on hydrolysis (21 Erlenmeyer treatment with crude enzyme mixture *A.niger* and *T.reesei*), each were added *Saccharomyces cerevisiae* at a dose of 10%, then each fermented for 96 hours for bioethanol production. The results of the fermentation treatment were measured by reducing sugar levels with the DNS method to compare sugar levels before and after treatment with *S. cerevisiae* (Jackson and Jayanthy, 2014).

Table 1 shown that reducing sugar from the dragon fruit peel produced after treatment *T. reesei* and *A.niger* were seen an increase because *T. reesei* and *A.niger* could produce cellulase enzymes, which can hydrolyze cellulose and hemicellulose into glucose. Enzymatic hydrolysis was regarded today as the most promising approach to liberating fermentable sugars in an energy-efficient way from the carbohydrates found in lignocellulosics in order to produce ethanol (Galbe and Zacchi, 2007).

Fig. 1 shown that reduced sugar decreased during the fermentation process with *S. cerevisiae.* The sugar content in the medium was continuously utilized by *S. cerevisiae* cells for the growth and formation of ethanol. The more reducing sugars used by *Saccharomyces cerevisiae* cells, the higher ethanol concentration produced and vice versa the less reducing sugars used, the lower the ethanol concentration. The increase in sugar concentration up to a certain level caused the fermentation rate to increase. However, the use of excessive sugar concentration will cause a steady fermentation rate, because the concentration of sugar used beyond the uptake capacity of the microbial cells. Generally, the maximum rate of ethanol production was achieved when using sugars at a concentration of 150 g/L. The initial sugar concentration also has been considered an important factor in ethanol production. (Zabed et al., 2016)

Fig 2. shown that the levels of bioethanol produced by fermentation are obtained by varying levels of bioethanol. The presence of fermented bioethanol was based on the opinions of Campbell, Reece, J.B., and Nitchel (2003), the results of *S.*

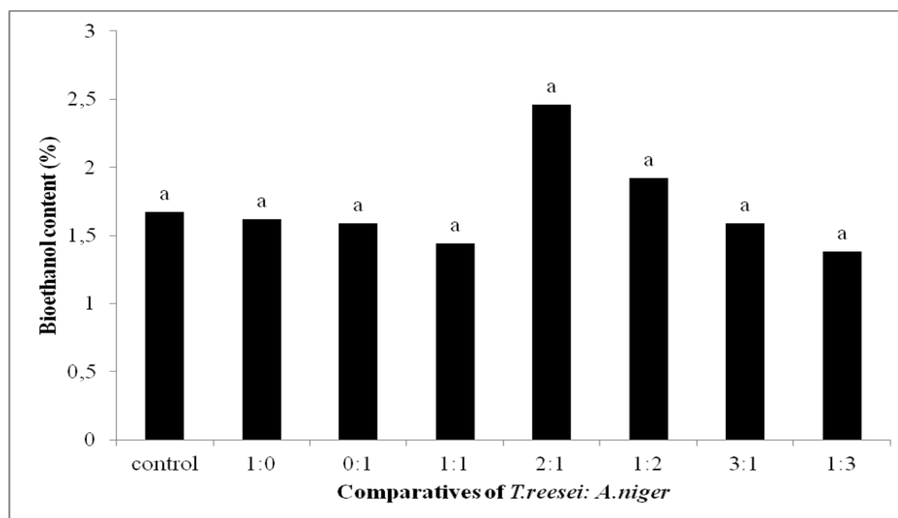

Figure. 2. Bioethanol levels of dragon fruit peel after fermentation using *Saccharomyces cerevisiae.* The DMRT test (p> 0.05) bioethanol levels were not differ significantly

cerevisiae metabolism in carbohydrate-based food sources such as sugar, starch, and cellulose are bioethanol. The presence of bioethanol indicates that the *S. cerevisiae* fermentation process is going well. According to the opinion (Wirahadikusumah, 2002) that the decomposition of carbohydrate or cellulose into pyruvate with the help of pyruvate decarboxylase enzyme which is reduced to bioethanol is through the event of glycolysis.

Enzymes from *Trichoderma reesei* and *Aspergillus niger* can increase reducing sugar levels 49.68 % in the treatment of *T.reesai: A.niger* (3: 1) and produce the highest bioethanol level, which is 2.46 % in the treatment of *T.reesai: A.niger* (2: 1)

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Short Communication

Diversity and Distribution of Herpetofauna in Banyu Nibo Waterfall, Nglanggeran, Gunung Kidul, Yogyakarta

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ABSTRACT

Banyu Nibo Waterfall is located near Nglanggeran, Gunung Kidul that well known for its eco-tourism development. The geographical and ecosystem condition could be providing a unique habitat for herpetofauna. Herpetofauna has important role in the ecosystem as food chain components and even as an environment bioindicator. The aim of this research is to assess the herpetofauna diversity in Banyu Nibo Waterfall as preliminary biodiversity data. The research was conducted using VES (*Visual Encounter Survey*) assisted by 500 meters transect line. As a result, 15 species were found, mainly distributed on the area with tree coverage.

Keywords: distribution, diversity, Gunung Kidul, herpetofauna, visual encounter survey

Herpetofauna (Reptiles and Amphibian) is one of the components which form the ecosystem, it has important roles in term of ecological and economical (Kusrini et al., 2003). The ecological role of herpetofauna is to maintain the balance of the ecosystem because the role of larger herpetofauna is as predators on the food chain. Some species also play a role as prey for the level of trophic in it. Some species of herpetofauna that are only found in certain specific habitat types (Iskandar, 1996).

Banyu Nibo Waterfalls is in Dusun Batur, Putat Village, Patuk District, Gunungkidul, Special Region of Yogyakarta. The height of this waterfall is about 50 meters and fast-medium water flow. This waterfall is located 150 away from the main road. The research was aimed to inventorying the diversity of herpetofauna in Banyu Nibo waterfall and to asses basic distribution of herpetofauna.

The research activity was carried out on 6-8 April 2018 with the location as an area open for ecotourism. The waterfall is full of rocks dominated by karstic and volcanic rocks with strong currents. In some parts, there are some puddles due to dammed water for irrigating rice fields. The sampling area is at an altitude of 241-306 masl. At the time of sampling, the weather was clear during the day with a little cloud cover and the temperature was around 28°C. During the night it is cloudy and drizzles with temperature around 26°C.

Materials used were 70% ethanol for preservative solution and specimen's euthanasia, GPS Garmin etrex as a coordinate pointer, syringes for injecting solutions, 2 kg plastic and sacks as a temporary specimen container, permanent markers as stationery for marking the obtained samples, and jam bottle as a container for preserved specimens.

Night and Day sampling were conducted three times to obtain both diurnal, nocturnal also cathemeral species. Visual encounter survey assisted with 500 meters transect line was conducted for 2 hours in the morning started at 08.00 a.m. and in the evening started at 07.00 p.m. Specimen was identified based on Das (2010) for reptile species while Kusrini (2013) and Iskandar (1998) for amphibian. The coordinates were recorded using Avenza Maps. ArcGIS 10.1 was used for mapping. The observation resulted in 79 individual composed

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Table 1. Herpetofauna species observed in Banyu Nibo waterfall

Order/Suborder	Species	Number of Individual	Frequently Founded Habitat
Order Squamata; Suborder Lacertilia	*Bronchocela jubata*	11	Woody Tree: *Pterocarpus indicus; Tectona* sp.; *Ficus* spp.
	Cyrtodactylus marmoratus	3	Rocks
	Draco volans	1	Palm
	Eutropis multifasciata	14	Rocks and grass
	Gehyra mutilata	1	Rocks
	Gekko gecko	6	Rocks
	Hemidactylus frenatus	2	Rocks and Banana leaf (*Musa sp.*)
	Varanus salvator	1	River stream
Order Squamata; Suborder Serpentes	*Dendrelaphis pictus*	6	Woody Tree: *Ficus* spp.
	Xenochropis vittatus	1	Pond
Order Anura	*Chalcorana chalconota*	14	Rocks and floor vegetation:*Urtica* sp., *Dendrocnide* sp., *Asplenium* sp.
	Duttaphrynus melanostictus	1	Rocks
	Fejervarya limnocharis	2	Grass
	Occidozyga lima	12	Water puddle
	Occidozyga sumatrana	4	Water puddle and rice field

of 15 species. (Table 1).

Based on Figure 1, we divided the area into two area i.e. area with tree coverage (agroforest and small rice paddy inside woodland) and open agricultural field. A large proportion of community was distributed in area within tree coverage mainly in agroforest, except for *Eutropis multifasciata, Hemidactylus frenatus,* and *Occidozyga sumatrana*.

A large proportion of reptiles (Figure 2) mainly observed in area with tree coverage because they were arboreal species i.e *Dendrelaphis pictus, Bronchocela jubata, Draco volans*. Large proportions of *Cyrtodactylus marmoratus, Gehyra mutilata, Hemidactylus frenatus* and *Gekko gecko* (familia Gekkonidae) also with their clutches, were commonly observed in rocks along the stream. The occurrence of rocks and tree become important for the Gekkonidae to put their clutches (Das, 2010). During daytime, we observed a large proportion of *Eutropis multifasciata* tend to bask in rocks and open areas to optimize the heat absorption from solar radiation.

We observed *Chalcorana chalconota* perched on rocks and vegetation along the river stream. The species prefers the perching site in 0-1 meters from river stream (9 individuals). This observation matched with observation by Kurniati & Sumadijaya, 2011 on Mount Salak. *Chlacorana chalconota* on Mount Salak often found in microhabitats where there are many herbaceous plants on the riverbank. The individual tends to choose site around 0-1 meters from river but without specific preference on vegetation if the leaf and petiole were strong enough. Meanwhile, *Occidozyga lima* and *Occidozyga sumatrana* tend to inhabit more calm water including small water puddle and ricefield until 15-20 meters away from river stream.

In conclusion, we observed 15 species of herpetofauna, which consists of five species of lizards (suborder Lacertilia), two species of snake (suborder Serpentes) and five species of frogs and toads (order Anura). Species communities were not evenly distributed, a large proportion of the community were distributed area with tree coverage i.e agroforest and forest area. *Eutropis multifasciata* and *Bronchocela jubata* were the most abundant reptiles in study sites. *Chalcorana chalconota* and *Occidozyga lima* were the most abundant amphibian in the study site.

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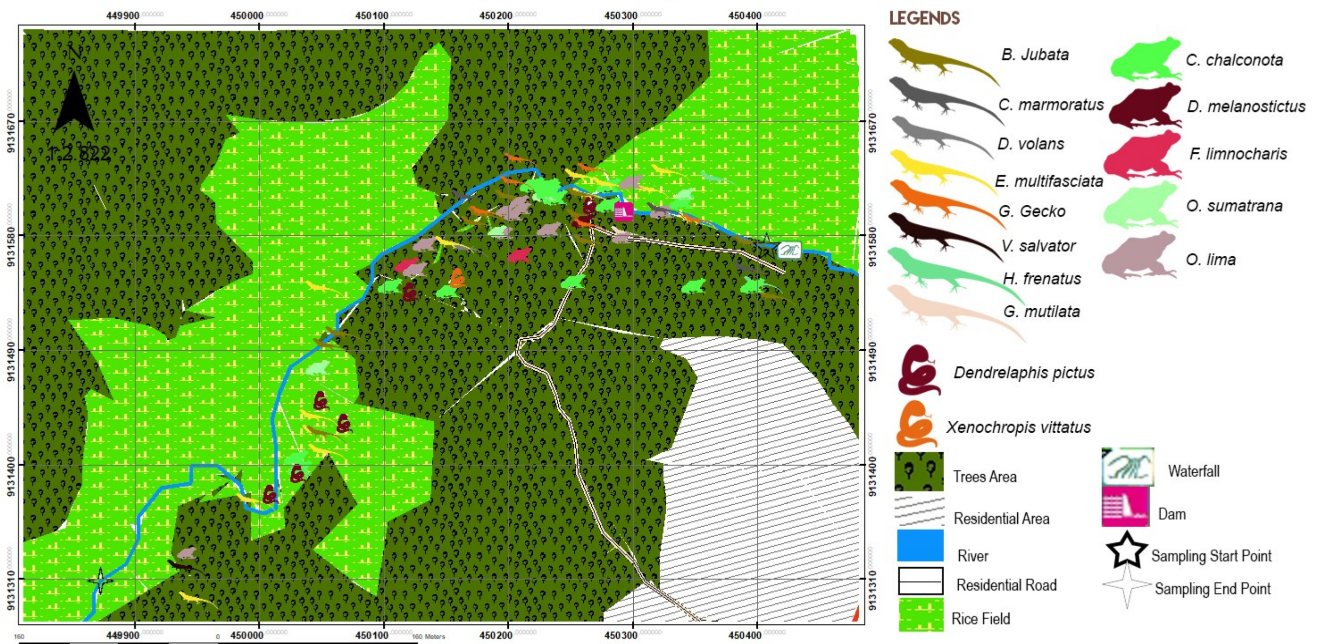
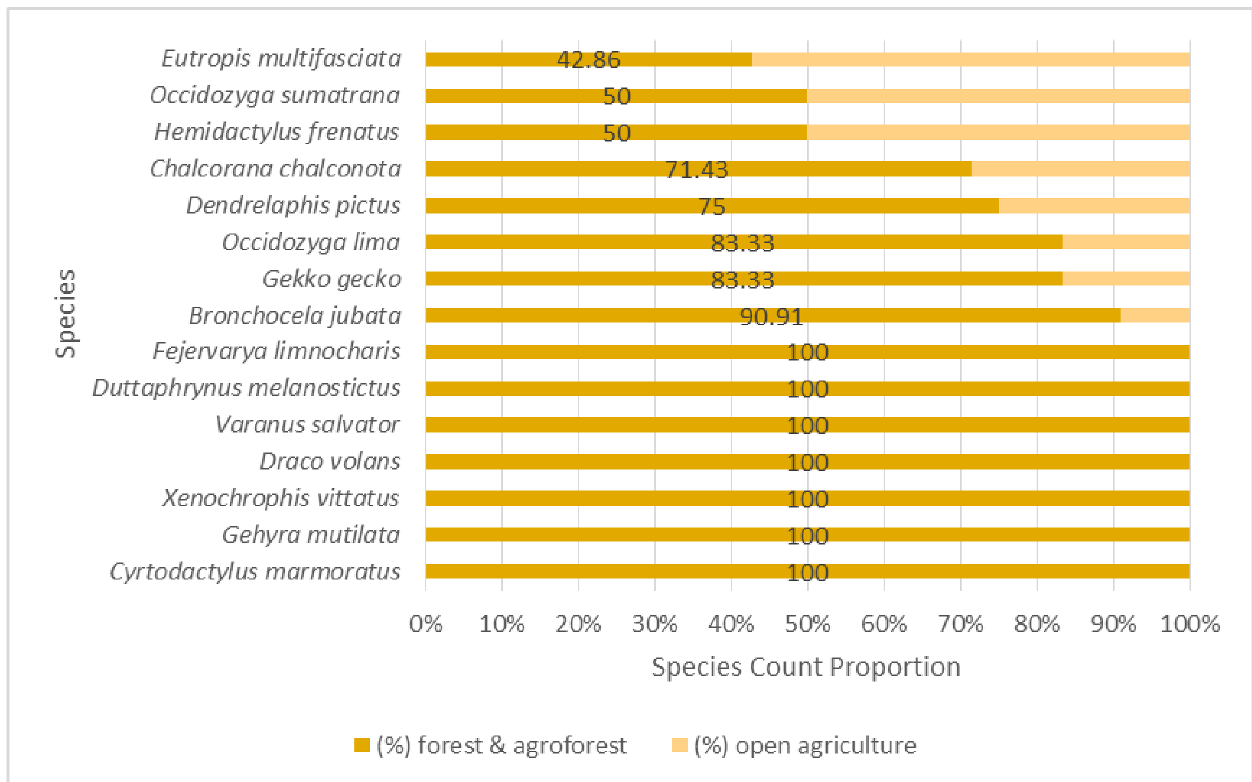


(a)



(b)

Figure 1. Herpetofauna Distribution Map in Banyu Nibo Waterfall (a), with individual count proportion each species between open agriculture and area with tree coverage (b)

(a)

(b)

(c)

(d)

Figure 2. Species commonly found in Banyu Nibo a. *Bronchocela jubata* b. *Eutropis multifasciata* c. *Chalcorana chalconota* (d) *Occidozyga lima*

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Research Article

Vegetation Composition of Savanna Ecosystem as a Habitat For The Komodo Dragon (*Varanus komodoensis*) on Padar and Komodo Islands, Flores East Nusa Tenggara Indonesia

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ABSTRACT

Tropical savanna and dry forest in Indonesia are an important type of ecosystems that supports various endemic wildlife of Indonesia including savannas at Padar and Komodo Islands which is home to the Komodo (*Varanus komodoensis*). The Komodo dragon is considered as "Vulnerable" by the International Union for Conservation of Nature. Studies with regards to the Komodo dragons' habitats are scarce, considering that these types of habitats are significant to support Komodo's existence, but yet are also very prone to conversion and disturbances. This paper elaborates the results of ecological study on the tropical savanna forest in Komodo National Park as habitat for the Komodo dragon.Vegetation sampling was conducted using nested plots 20 x 20 m, 10 x 10 m, 5 x 5 m and 2 x 2 m spread across the sampling sites. Data was analysed using PRIMER software which includes cluster analysis, analysis of similarity (ANOSIM) and similarity percentage (SIMPER). As many as 17 plant species which belongs to 11 families were identified in the sampling sites. These consist of six trees habitus, six shrubs, four grasses and one palm. Asteraceae, Fabaceae and Poaceae were the plant families which has high number of species. The result of cluster analysis shows that the similarity level of the two groups (Komodo and Padar) based on the results of cluster analysis is 60%. This result infers that there are similarities in terms of species composition in savanna on Komodo and Padar Island, however, each savanna still has its own species characteristics. This is confirmed by the ANOSIM test. The ANOSIM test results show the Global R value of 0.6.With the looming challenges from invasive alien plant species (IAPS), the Komodo Island's savanna has double threats to overcome. Hence conservation of the remaining savanna ecosystem is important.

Keywords: savanna, komodo, Padar, Flores, invasive alien plant species

INTRODUCTION

A range of ecosystem types and habitats that Indonesian archipelago has, created amazing species diversity and endemism. Nevertheless, swift and extensive habitat losses, together with the threatening challenge of climate change create a significant risk to the nation's biological diversity (Purwandana et al., 2014). Tropical savanna and dry forest in Indonesia are an important type of ecosystems that supports various endemic wildlife of Indonesia, some of which are under serious threat of extinction and have high conservation status according to IUCN categories, such as the wild Java Cattle (*Bos javanicus*) in the savanna of Baluran National Park in East Java, the endemic Bali Starling bird (*Leucopsar rotschildi*) in West Bali National Park savanna on Bali Island, and the Komodo Dragon (*Varanus komodoensis*) endemic only to Komodo Islands of East Nusa Tenggara. The Komodo dragon (*Varanus komodoensis*is) the world's largest lizard, of prominent conservation value as an umbrella species for protection of south-east Indonesian ecosystems (A. Ariefiandy et al., 2015).

Species with limited distribution or rapidly decreasing range margins are particularly sensitive to processes of global change (A. Ariefiandy et al., 2015; Davis et al., 2016). Given the high extinction risk facing such species, conservation program is likely to require multidisciplinary approaches that

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Figure 1. Location of the study in Komodo National Park, East Nusa Tenggara Province. Green circle point out Komodo and Padar Islands.

address both the species itself as well as their habitats (Estoque, Estoque, & Murayama, 2012). The endemic Komodo dragon (*Varanus komodoensisis)* the world's largest lizard, with a highly restricted range distribution. The Komodo dragon is considered as "Vulnerable" by the International Union for Conservation of Nature (IUCN, 2014). Clearing of coastal dry monsoon forest, savanna Landuse change, anthropogenic fire regimes and competition with humans for prey species, such as Timor deer (*Cervus timorensis florensis*), are thought to be threatening processes influencing the viability of historically small dragon populations. Populations that are particularly at threat are those restricted to habitats on western Flores Island (Jessop et al., 2004).

There have been numerous studies on the Komodo dragon. Many of these studies have been focused on the Komodo dragon itself ranging from its behavior, genetic, ontogeny, nesting, and demography, among others (Auffenberg, 1981; CIOFI et al., 2011; Jessop et al., 2004; Purwandana et al., 2014; Purwandana et al., 2016). However, studies with regards to the Komodo dragons' habitats are scarce, considering that these types of habitats are significant to support Komodo's existence, but yet are also very prone to conversion and disturbances. This paper elaborates the results of ecological study on the tropical savanna forest in Komodo National Park as habitat for the Komodo dragon. The aim is to characterize savanna ecosystems in terms of their structure, diversity and composition. By studying its habitat, it is hoped that it could also aim to raise awareness on the importance of Komodo dragon habitat conservation alongside protection of the Komodo dragon itself.

METHODS

Nusa Tenggara is a group of small and medium-sized islands stretching from Lombok Island in the west, to Tanimbar Island in the east. East Nusa Tenggara consists of several islands, with the main ones being Flores, Komodo and Sumba. Sampling will concentrate on Padar and Komodo Islands. Komodo Island is where the dominant population of the dragon lies (Figure 1).

Field vegetation sampling was conducted in April 2018. Nested plots 20 x 20 m, 10 x 10 m, 5 x 5 m and 2 x 2 m spread across the sampling sites to sample the vegetation (tree, poles, sapling and groundcovers) in savannas. In total there were ten nested plots. Plants were identifying the species and estimate the cover. Identification and plants material collection record conducted in the *Herbarium Baliensis* within the Eka Karya Botanical Garden – LIPI, as this botanical garden has an abundance of record collections from eastern parts of Indonesia, which is its specialty. Local environmental data for every plot also record, at a similar time of day (morning): soil pH and moisture; local microclimate (light intensity, air temperature, relative humidity, and wind velocity and heat stress index) and topography (altitude and slope).

Table 1. Tabulation of plant species, families, and habitus on Padar and Komodo Islands.

Plant species	Famili	Habitus	Padar Island	Komodo Island
Ageratina riparia	Asteraceae	Shrub	x	-
Alstonia sp	Apocynaceae	Tree	-	x
Anona muricata	Annonaceae	Tree	-	x
Chromolaena odorata	Asteraceae	Shrub	-	x
Coypha utan	Arecaceae	Palm	-	x
Crotalaria sp	Fabaceae	Shrub	x	-
Cymbopogon sp	Poaceae	Grass	x	-
Cyperus sp	Cyperaceae	Grass	x	-
Dysoxylum sp	Meliaceae	Tree	-	x
Glirisedia sepium	Fabaceae	Shrub	x	-
Hibiscus sp	Malvaceae	Shrub	x	-
Imperata cylindrica	Poaceae	Grass	-	x
Spondias dulcis	Anacardiaceae	Tree	-	x
Tamarindus indicus	Fabaceae	Tree	-	x
Themeda arguens	Poaceae	Grass	x	x
Tridax procumbens	Asteraceae	Shrub	x	-
Zizyphus jujube	Rhamnaceae	Tree	x	x

X sign means it was presence.

Cover data was used to test the differences in plant community composition between savannas. The data square-root transformed prior to constructing a resemblance matrix based on Bray-Curtis similarity (Valessini 2009). A cluster ordination diagram then generated based on the resemblance matrix. The result of the cluster ordination was tested for significance using one-way ANOSIM (analysis of similarity). SIMPER (Similarity Percentage) analysis then used to explore the relative contribution of individual species to dissimilarity among savannas. This multivariate analysis makes use of the PRIMER V.6 package (Clarke & Gorley, 2005). Correlations between floristic and local environmental gradients were explored using BEST (Bio-Env) module in PRIMER V.6.

RESULTS AND DISCUSSION

According to Purwandana *et al.*(2014), there are four main vegetation communities in Komodo National Park. Tropical monsoon forest dominates areas above 500–700 m. At lower elevations deciduous dry monsoon forest occurs in valley floors. Savanna woodland and savanna grassland occupy drier areas of the islands, although. perhaps what Purwandana *et al.*(2014) meant is that savanna woodlands are true savanna, whereas what they meant by savanna grasslands is a true grassland. Komodo dragon utilizes both dry monsoon forests and savannas in the Komodo National Park KNP). Komodo dragons preferentially use deciduous monsoon forest and savanna, as a consequence of their thermoregulatory requirements and the location of their prey (Achmad Ariefiandy et al., 2014).

In this study, the tropical savanna plant communities of the Padar and Komodo Islands in Flores East Nusa Tenggara have been characterized. As many as 17 plant species which belongs to 11 families were identified in the sampling sites (savannas in Padar and Komodo Islands). These consist of six trees habitus, six shrubs, four grasses and one palm (Table 1). Asteraceae, Fabaceae and Poaceae were the plant families which has a high number of species compared to other families (Figure 2). These numbers are quite high when we compare to the number of plant species and families found in the western and wetter parts of Indonesia such as savanna in Baluran and Alas Purwo (in East Java), Bali Barat (Bali) and Rinjani (Lombok). Sutomo (2017) found as many as 43 plant species within 26 families across the four savannas including one fern, seven grass or grass-like plants and two forbs.

However, there seems to be a separation between the two groups (Komodo and Padar). This result infers that there are similarities in terms of species composition in savanna on Komodo and Padar Island, however, each savanna still has its own species characteristics. This is confirmed by the ANOSIM test. The ANOSIM test results show the Global R value of 0.6. In Padar Island, the tree layer

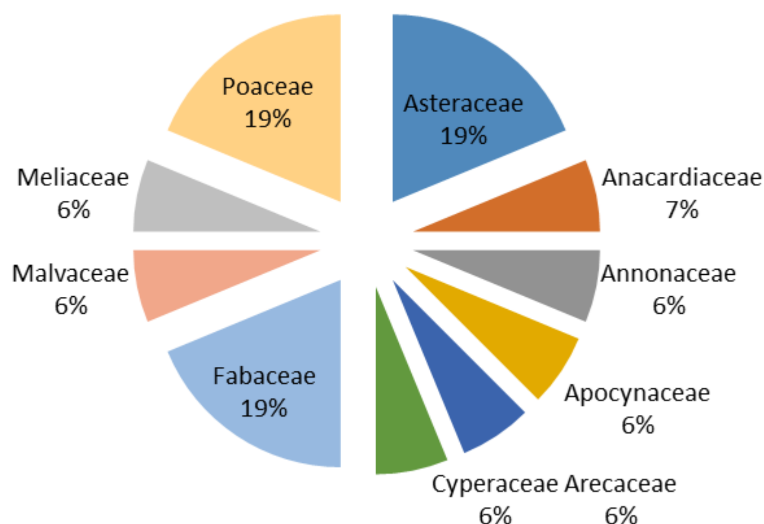

Figure 2. The Proportion of number of species each family has on Savannas in Padar and Komodo Islands.

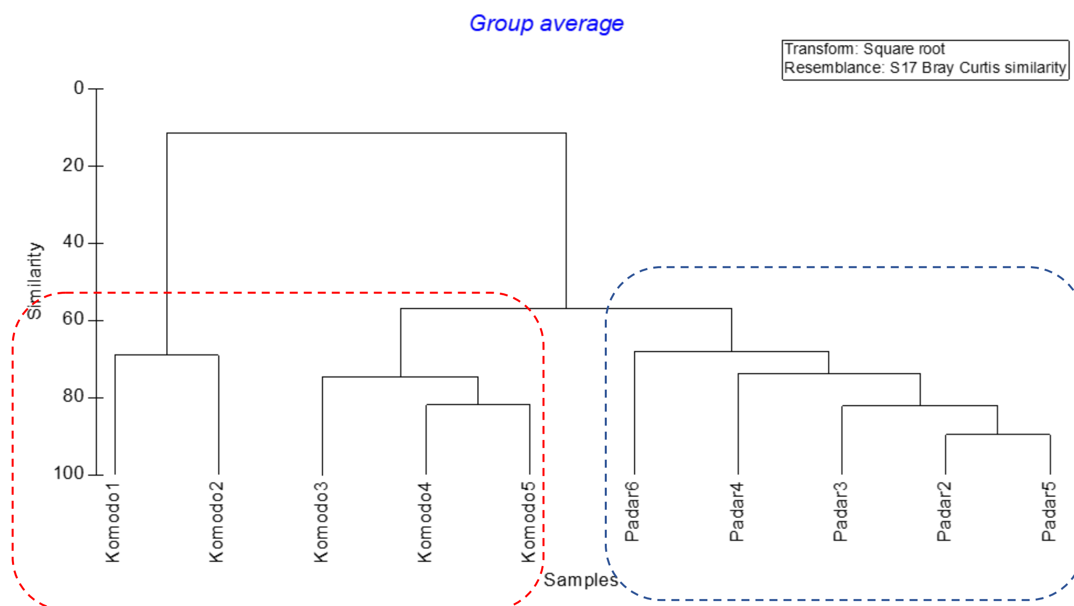

Figure 3. Result of Cluster analysis based on species composition data on Padar and Komodo Islands. R_{ANOSIM} = 0.6

is occupied by *Zizyphus jujuba*, the groundcover was compose of grasses such as *Cymbopogon, Cyperus* and *Themedea arguens*, whereas the shrub layer were occupied by *Crotalaria, Glirisedia, Hibiscus, Tridax procumbens* and also the invasive alien species *Ageratina riparia* (*Eupatorium riparium*) (Table 1). *Ziziphus* is also the dominant tree species in Savanna in Baluran East Java (Sutomo & van Etten, 2016). At Komodo Island, the tree layer is of *Anona muricata, Dysoxylum sp, Spondias dulcis, Tamarindus indicus* and also *Zizyphus jujuba* (Table 1). The grass layer on Komodo was from *Themedea arguens* and *Imperata cylindrica* species, whereas the shrub layer is occupied by invasive alien species *Chromolaena odorata* (*Eupatorium odoratum*) or known as "Kriyuh". The savanna on Komodo also has palm species (*Coryphautan*) (Table 1).

Asteraceae, Fabaceae and Poaceae were the plant families which has a high number of species compared to other families (Figure 2). This numbers are quite high when we compare to the number of plant species and families found in the western and wetter parts of Indonesia such as savanna in Baluran and Alas Purwo (in East Java), Bali Barat (Bali) and Rinjani (Lombok). Sutomo (2017) found as many as 43 plant species within 26 families across the four savannas including one fern, seven grass or grass-like plants and two forbs.

The results of cluster analysis using savanna vegetation data on both islands in the Komodo National Park show that the similarity level of the two groups (Komodo and Padar) based on the results of cluster analysis is 60% (Figure 3).

In Padar Island, *Themeda arguens* grass and

Table 2. Important species in each savanna at Padar and Komodo Islands as analyzed by SIMPER. Av.Abund = Average abundance; Av.Sim = Average similarity; Sim/SD refers to consistency; Contrib% = Percentage of contribution; Cum% = Cumulative percentage

Pulau Padar					
Species	**Av.Abund**	**Av.Sim**	**Sim/SD**	**Contrib%**	**Cum.%**
Themeda arguens	14.36	54.09	11.89	72.39	72.39
Crotalaria sp	5.57	18.51	2.39	24.78	97.16
Pulau Komodo					
Species	**Av.Abund**	**Av.Sim**	**Sim/SD**	**Contrib%**	**Cum.%**
Chromolaena odorata	6.62	23.35	3.05	49.76	49.76
Themeda arguens	10.15	16.84	0.62	35.90	85.66
Imperata cylindrica	2.54	2.53	0.32	5.39	91.05

Crotalaria shrub plays an important contribution in the species composition configuration with 72.39% and 24.78% contribution respectively (Table 2).

In Komodo Island, *Themeda arguens* grass position was replaced by the IAS *Chromolaena odorata* with 49.76% contribution, whereas *Themeda* 35.90% and *Imperata cylindrica* grass 5.39%. *Imperata cylindrica* is categorized as native invasive species. SIMPER analysis showed that eight species were mostly responsible for the dissimilarity between the two sites, Padar and Komodo (Table3). *Themeda arguens* appear in both islands but with different abundance. *Themeda* is abundant at Padar compare than Komodo. This perhaps due to the appearance of other grass species such as the native invasive *Imperata cylindrica* and also perhaps due to the competition with the alien invasive shrubs *Chromolaena odorata* (Table 3). The tree layer in Padar is characterized by the Bekul or *Zizyphus* whereas on Komodo it is the tamarind (*Tamarindus indicus*) (Table 3).

Figure 4. Bekul (*Zizyphus jujuba*). *Zizyphus* is one of a well-known tree species that characterized savanna vegetation.

As a comparison, in Baluran National Park in East Java, the Bekol Savanna, the dominant grass layer is the groundcover layer, which characterized by two grasses *Polytrias indica* and *Dichanthium caricosum* (Sutomo, 2017), whereas the Pangandaran savanna in West Java, the groundcover layer is of the characteristic of the secondary succession toward forest habitus, which *Eleusine indica* grass and the low creeping grasses *Ischaemum rugosum* dominates (Rosleine & Suzuki, 2013).

The presence of invasive alien species is notable in most of the savannas. Similar problems also occur in other savannas such as savanna in West Bali with the *Chromolaena odorata* and Baluran with the *Acacia nilotica* (Caesariantika, Kondo, & Nakagoshi, 2011; Sutomo, 2017). Caesariantika noted that the invasion of *Acacia nilotica* in Baluran NP has decreased its native/local species diversity. Shannon-Wiener species diversity index in Padar's (0.57) savanna is lower than Komodo's (0.699) savanna (Figure 5).

Table 3. Average of *Pulau Padar* and *Pulau Komodo* Groups

Species	**Group Pulau Padar Av. Abund.**	**Group Pulau Komodo Av. Abund.**
Themeda arguens	14.36	10.15
Chromolaena odorata	0.00	6.62
Crotalaria sp	5.57	0.00
Imperata cylindrica	0.00	2.54
Glirisedia sepium	1.20	0.00
Zizyphus jujuba	1.13	0.93
Alstonia sp	0.00	1.00
Tamarindus indicus	0.00	1.09

dissimilarity = 65.24

This phenomenon is perhaps due to several factors as described by Hill (2011). The Komodo Island has more types of ecosystems than Padar. Thus the recruitment of seasonally dry tropical forest (SDTF) species on Komodo's islands to the savannas is possible hence the species add to the Komodo's savannas species pool. This is the possible explanation, in the results section we can see that other tree species which are uncommon for

savanna tree layer exist on the Komodo Island savanna such as *Alstonia scholaris, Annona muricata, Dysoxylum* sp and *Spondias dulcis*. In contrast, the Padar Island only has savanna as its ecosystem type, and the water body that divides the two islands create a barrier for recruitments from the SDTF on Komodo Island.

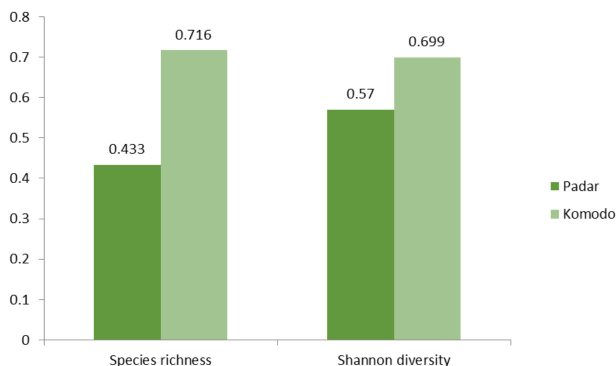

Figure 5. Shanon-Wiener species diversity index for Padar and Komodo Islands's savanna.

The consequence of the phenomenon on Komodo Island's various ecosystems types is that there is a possibility that the savanna might convert and transition to a STDF. This phenomenon has been observed in Baluran Savanna as reported by Sutomo & van Etten (2016). This might not be beneficial for the Komodo dragon as they use both ecosystems type for their habitat. With the looming challenges from IAS the Komodo Island's savanna has double threats to overcome. Hence conservation of the remaining savanna ecosystem is important.

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Research Article

Conservation, Phytoremediation Potential and Invasiveness Status of Bali Botanic Garden Aquatic Plant Collection

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ABSTRACT
Bali Botanic Garden (BBG) aspires to conduct conservation and research of eastern Indonesian plant species, including the aquatic plant species. These were important as aquatic plant species could be ecologically threatened, beneficial or even dangerous. As scientific data of BBG aquatic plant species collection was limited, we proposed this study to provide researchers and garden managers with data to conduct research, collection and maintenance of the garden aquatic plant collection. The study was carried out by sourcing list of BBG collected plant species data for its aquatic plant species. Literatures study was then carried out to gain information regarding the plant species' heavy metal phytoremediation, conservation and invasiveness status while data analysis was conducted descriptively. The study result showed that 38 collection numbers of aquatic plant species collected in BGG were placed in five sites within the garden with 94% of all the aquatic plants collection came from Lesser Sunda Islands. Eleven aquatic plants species were listed as Least Concern by IUCN Red List. Fourteen species of collected aquatic plants were proved to possessed phytoremediation potential toward numerous heavy metal pollutants, while six species were listed as an invasive alien plant species in Indonesia. All of the provided data should be enabled the botanic garden stakeholders to come up with ideas in the research and maintenance effort of BBG aquatic plant collection.

Keywords: aquatic plant, botanic garden, conservation, heavy metal, invasive

INTRODUCTION

Aquatic plants species were an important constituent of the wetland ecosystem (Lacoul and Freedman, 2006). Evidence of the statement could be found in a study by Ismail *et al.* (2018) and Jha (2013) that stated the importance of the aquatic plant to influence the fish population, as well as food sources for numerous bird species. The importance of aquatic plant species for the ecosystem was not limited as shelter and food provider, but also to eliminate environmental pollutant known as phytoremediation (Peuke and Rennenberg, 2005). Alkorta and Garbisu (2001) study shows that good organic remediation result from plant species has drawn people attention to phytoremediation. Furthermore, Sumiahadi and Acar (2018) have listed several crop plants species including aquatic plant species as *Pistia stratiotes* and *Eichhornia crassipes* were already assessed for its phytoremediation potential toward heavy metal pollutant

Despite all of its environmental importance, some aquatic plant species may also treat the ecosystem as they were considered as invasive alien plant species. Hulme (2011) described alien species as an organism that present beyond its past or present origin area and dispersal potential and invasiveness as the establishment of those alien species which caused a detrimental effect on its new region. The presence of invasive aquatic plant species could not be overlooked as it could decrease the wetland yield and services as well as altering its cycle and chemistry (Keller *et al.*, 2018). Invasive aquatic plant species induced environmental change was evidenced in the presence of *Myriophyllum aquaticum* that decreased the Dissolve Oxygen level and was in correlation with the diversity of epiphytic invertebrates and alien fish species (Kuehne *et al.*, 2016). *Hydrilla verticillata, E. crassipes, P. stratiotes,*

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Mimosa pigra and *Salvinia molesta* were listed as Indonesian important aquatic invasive alien plant species (Tjitrosoedirdjo, 2005).

Although of all the above-mentioned facts, numerous aquatic plant species were threatened to extinction. In Europe for example, one aquatic plant species namely *Trapa annosa* was ranked as Extinct while five other aquatic plant species were ranked as Critically Endangered, eight species listed as Endangered and 13 species considered as Vulnerable by IUCN Red List (Bilz *et al.*, 2011). Bali Botanic Garden (BBG) as an ex-situ conservation site has collected 22.432 plant specimens with 9.037 collection-number which some of them were aquatic plant species. Besides the living specimens, the garden collection was boosted even more by numerous amounts of herbarium, seed and nursery collection. However, scientific study regarding BBG aquatic plant species collection was scarce. Thus we propose this study to fill the gap. This study aims to describe aquatic plant species conservation in BBG as well as its phytoremediation potential to heavy metals pollutants and invasive status. We believe that the study result will offer baseline data for collection -based research of BBG aquatic plant species as well as enabled the garden manager to conduct research-based collection and management of the aquatic plant species.

MATERIALS AND METHODS

Materials

The latest list of plant species collected in BBG (July 2019) were acquired from BBG Registration Unit. The online database of The Plant List (2013) and IUCN (2019) were used to determine the scientific name of the plant species and conservation status. Setyawati *et al.* (2015) and Tjitrosoedirdjo *et al.* (2016) were used to determine the invasiveness status of the aquatic plant species in Indonesia. Various scientific publications were also consulted to gain information regarding the plant species phytoremediation potential. The comparison of BBG aquatic plant collection and Purwodadi Botanic Garden (PBG) aquatic plant collection acquired from Puspitasari and Irawanto (2016) was also conducted.

Methods

The acquired list of plant species collection in BBG was sourced for the garden aquatic plant species collection. Obtained aquatic plant species data was then matched with The Plant List (2013) website online source to verify each plant species binomial names while IUCN (2019) website online source was consulted to acquire the plant species conservation status. Numerous scientific literatures available on the internet was then sourced for the aquatic plant species phytoremediation potential. Setyawati *et al.* (2015) and Tjitrosoedirdjo *et al.* (2016) studies were also sourced to provide the invasiveness status of BBG aquatic plant species in Indonesia. All of the acquired data were then analyzed and presented descriptively as tables, charts and figures. Jaccard Similarity Index (JSI) was used to compare aquatic plant collection identified until its species-level collected in BBG and PBG, and calculated following Mueller-Dombois and Ellenberg (2016) as follows:

$$JSI = \frac{A}{A + B + C} \times 100\% \quad (1)$$

A = Common species
B = Unique species in BBG
C = Unique species in PBG

RESULTS AND DISCUSSION

Aquatic Plant Conservation in BBG

Currently, BBG conserves 38 collection number of aquatic plants, consisting of 12 families. Complete aquatic plant taxa collected by BBG presented in Table 1. BBG aquatic plant collection was placed in five locations within the garden. Separated

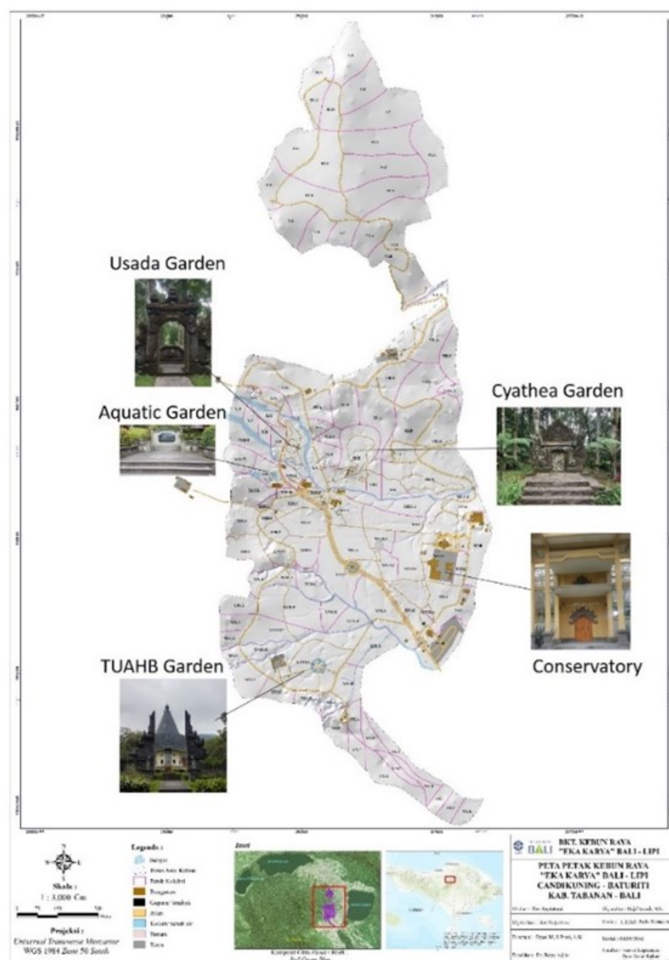

Figure 1. Aquatic Plant Species Location in BBG (©Bali botanic Garden)

Table 1. Aquatic Plant Taxa Collected by BBG

Species Name	Location	Origin
Acanthaceae		
Acanthus ebracteatus Vahl	Usada Garden	Bali
Acanthus ilicifolius L.	Usada Garden	Bali
Acoraceae		
Acorus calamus L.	Aquatic Garden, TUAHB Garden	Bali, Timor
Alismataceae		
Sagittaria lancifolia L.	Aquatic Garden	Bali
Araceae		
Cyrtosperma beccarianum A. Hay	Conservatory	Papua
Cyrtosperma sp.	Conservatory	Bali
Pistia stratiotes L.	Aquatic Garden	Bali
Cyperaceae		
Cyperus involucratus Rottb.	Aquatic Garden	Bali
*Cyperus papyru*s L.	Aquatic Garden	Bali
Cyperus haspan L.	Aquatic Garden	Bali
Cyperus sp.	Aquatic Garden	Sumba
Cyperus sp.	Aquatic Garden	Papua (Waigeo Is.)
Cyperus sp.	Aquatic Garden	Bali
Fimbristylis umbellaris (Lam.) Vahl	Aquatic Garden	Bali
Schoenoplectiella mucronata (L.) J.Jung & H.K.Choi	Aquatic Garden	Sumba
Haloragaceae		
Myriophyllum aquaticum (Vell.) Verdc.	Aquatic Garden	Bali
Marsileaceae		
Marsilea polycarpa Hook. & Grev.	Cyathea Garden	Bali
Menyanthaceae		
Nymphoides indica (L.) Kuntze	Aquatic Garden	Bali
Nymphaeaceae		
Nymphaea elleniae S.W.L. Jacobs	Aquatic Garden	Bali
Nymphaea "Mrs. C.W. Word"	Aquatic Garden	Bali
Nymphaea pubescens Willd.	Aquatic Garden	Bali
Pontederiaceae		
Eichornia crassipes (Mart.) Solms	Aquatic Garden	Bali
Pontederia cordata L.	Aquatic Garden	Bali
Salviniaceae		
Azolla pinnata R.Br.	Cyathea Garden	Bali
Salvinia adnata Desv.	Cyathea Garden	Bali
Thypaceae		
Typha angustifolia L.	Aquatic Garden	Bali

placement of aquatic plant specimens in BBG was possible since Indonesian Botanic Garden arranged its collection placement based on the plant taxonomy, utilization, origin and another category or its combination (Hadimuljono *et al.*, 2014). In case of BBG aquatic plant species, most of the specimen was placed based on its habitus similarity at the Aquatic Garden while some of the others were placed based on its taxonomy and utilization. Species that were placed based on its taxonomical status were the *A. pinnata*, *M. polycarpa* and *S. adnata* which placed in Cyathea Garden which contain fern collection of BBG (Figure 1). Aquatic plant species that placed based on its utilization as medical plant were the *A. ebracteatus* and *A. ilicifolius,* those were placed in Usada Garden which contain Balinese traditional medicinal plant species (Figure 1). Another plant species that placed based on its utilization was the *A. calamus* which also placed in Taman Upacara Adat Hindu Bali (TUAHB) Garden which contains plant species utilized for the Balinese Hindu ceremony due to its ceremonial use (Figure 1). Another aquatic plant species placed outside the Aquatic Garden was *Cyrtosperma beccarianum* which placed in the Conservatory as representative of the aquatic plant species, Conservatory purpose was to describe the plant species evolution process.

BBG Aquatic Garden was the garden primary site to conserve its aquatic plant specimen with 71% of all the aquatic plant collection number placed in the site (Figure 2). This was not surprising as the Aquatic Garden was purposively built to conserve

BBG aquatic plant specimen. However, as mentioned before, some of the aquatic plant species were also placed in other sites within the BBG such as Taman Upacara Adat Hindu Bali (TUAHB) Garden, Usada Garden and Cyathea Garden which contain 8% of the aquatic plant collection-number respectively (Figure 2). Another site that contains aquatic plant species was the Conservatory which contain 5% of the aquatic plant collection number (Figure 2).

Member of Acoraceae and Cyperaceae families were composed the most collection number with nine collection-number respectively (Figure 3). Most aquatic plant specimens were collected from Bali island (84%) followed by plant specimen collected from Papua (6%), Sumba and Timor with 5% respectively (Figure 4). The aquatic plant collection site data shows us that BBG aquatic plant was collected only from Lesser Sunda Islands (Bali, Sumba and Timor) and Papua. As BBG objective is to conserve plant species from the eastern part of Indonesia, the botanic garden needs to collect aquatic plant species from another part of the region such as the Sulawesi and Moluccas.

Thirty-three aquatic plant collection-number in BBG were already identified until its species-level while four and one collection-number were identified until its genus level and as a hybrid plant specimen namely *Nymphaea* "Mrs. C.W. Word" respectively. Twenty-one species was consisting of the 33 collection-number identified until species level. The number of aquatic plants identified until its species level in BBG was almost the same as the number of aquatic species identified until its species level collected by the PBG with 17 species (Puspitasari and Irawanto, 2016). Of all plant species collected in BBG *A. calamus* was the most collected specimen with nine collection number, followed by *A. ilicifolius*, *Cyperus involucratus*, *Nymphoides indica* and *Pontederia cordata* with two collection number respectively (Figure 5).

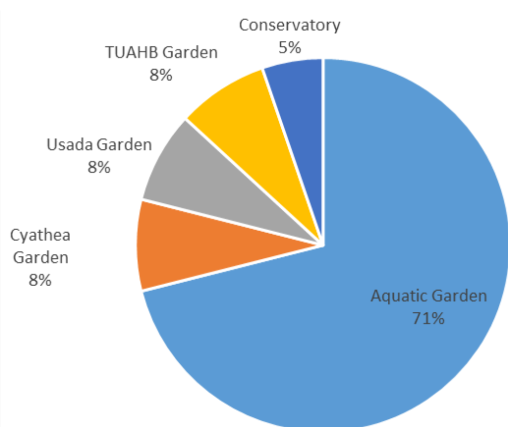

Figure 2. Aquatic Plant Species placement in Bali Botanic Garden

Jaccard similarity index calculation result shows that BBG and PBG aquatic plant species similarity is 11,76%. Four common species were present in these two gardens, namely *A. ilicifolius, A. calamus, Sagittaria lancifolia* L. and *Typha angustifolia*. Low similarity value between BBG and PBG means that aquatic plant collection in these two botanic gardens was very different. The result was unsurprising as both botanic gardens were situated in two very different altitudes. The presence of common plant species may suggest those species' high tolerance toward temperature gradient.

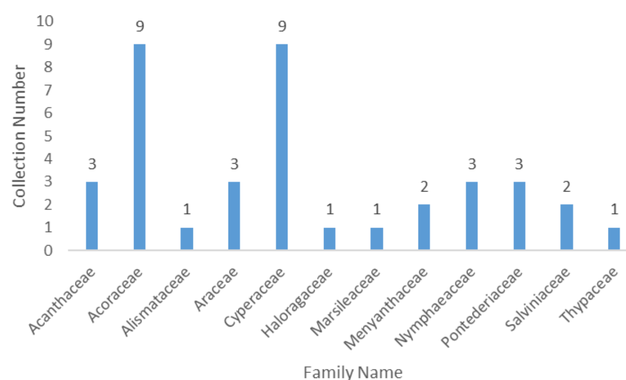

Figure 3. Aquatic Plant Family Collected in Bali Botanic Garden

Eleven aquatic plant species collected in BBG were listed in IUCN Red List as Least Concern (Table 2). Purnomo *et al.* (2015) stated that Indonesian Botanic Gardens were able to collect 24% and cultivated 25% of Indonesian threatened plant species which defined with its Vulnerable and above IUCN Red List status with BBG was mentioned to collect more than 20 of the listed species. The absence of aquatic plant species with IUCN Red List status of Vulnerable and above in BBG collection means that aquatic plant conservation in the garden was still not able yet to conserve aquatic plant species considered as high priority species for conservation. However,

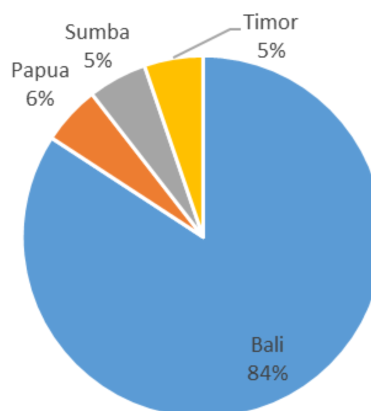

Figure 4. BBG Aquatic Plant Specimen Collection Sites

conservation of aquatic plant species in BBG was still able to conserve plant species with decreasing population in the wild namely *A. ebracteatus* (Ellison *et al.*, 2010). BBG was also able to conserve *A. calamus* which was important to traditionally cure several illnesses in Bali (Oktavia *et al.*, 2017). The conservation effort of these species was important to safeguard the plant species from extinction.

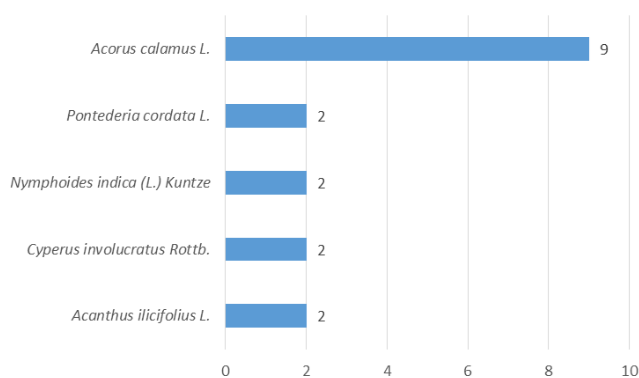

Figure 5. Five Most Collected Aquatic Plant Species in Bali Botanic Garden

Phytoremediation Potential of BBG Aquatic Plant Collection

Literatures studies suggested that 14 aquatic plant species collected in BBG was already assessed for its phytoremediation potential. The literatures also suggest that the plant species were able to accumulate 12 heavy metal pollutants, namely Arsenic (As), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Cesium (Cs), Copper (Cu), Iron (Fe), Mercury (Hg), Manganese (Mn), Nickel (Ni), Lead (Pb) and Zinc (Zn). A complete list of aquatic plant species collected in BBG with metal pollutant that they able to accumulate based on our literatures study were presented in Table 3.

To the best of our knowledge, no research regarding aquatic plant species remediation potential has been done in BBG. This was unlike what happened in PBG which already researched the aquatic plant phytoremediation such as Irawanto *et al.* (2015) and Irawanto and Mangkoedihardjo (2015) which study the potential of *A. ilicifolius* and *Coix lacryma-jobi* to accumulate Pb and Cd. Another example of an aquatic plant phytoremediation research conducted in PBG was the phytoremediation study of *Lemna minor* and *Ceratophyllum demersum* to Pb polluted water (Munandar *et al.*, 2018) and the study of *S. molesta* and *P. stratiotes* phytoremediation toward Cu (Baroroh *et al.*, 2018).

The current absence of aquatic plant species phytoremediation research in BBG open possibilities for future research in that field as phytoremediation potential of BBG aquatic plant species which not listed in Table 3, were probably not yet assessed. Even if the plant species phytoremediation potential were already assessed, further research in phytoremediator aquatic plant species will still important to conduct, as Gratão *et al.* (2005) and Vasavi *et al.* (2010) stated that research on aquatic plant mutant impact on the environment must be conducted as genetically modified phytoremediator plant species were possible to produce.

Invasive Alien Plant Species Status of BBG Aquatic Plant Collection

According to Setyawati *et al.* (2015) and Tjitrosoedirdjo *et al.* (2016), six aquatic plant species collected in BBG were listed as Invasive Alien Plant Species in Indonesia. Salviniaceae has contributed two species while Pontederiaceae, Cyperaceae, Haloragaceae and Araceae contributed one species respectively. Complete list of BBG aquatic plant species collection listed as invasive alien species was presented in Table 4..

Table 2. Conservation Status of Aquatic Species Collection in BBG Based on IUCN Red List

Species Name	Conservation Status	Literatures
Acanthus ebracteatus Vahl	Least Concern	Ellison *et al.* (2010)
Acanthus ilicifolius L.	Least Concern	Juffe Bignoli (2011)
Acorus calamus L.	Least Concern	Lansdown (2014)
Azolla pinnata R. Br.	Least Concern	Gupta and Beentje (2018)
Cyperus haspan L.	Least Concern	Gupta and Lansdown (2018)
Cyperus papyrus L.	Least Concern	Beentje and Lansdown (2018)
Nymphaea pubescens Willd.	Least Concern	Gupta (2011)
Nymphoides indica (L.) Kuntze	Least Concern	Karuppasamy *et al.* (2019)
Pistia stratiotes L.	Least Concern	Lansdown (2019)
Schoenoplectiella mucronata (L.) J.Jung & H.K.Choi	Least Concern	Lansdown (2013)
Typha angustifolia L.	Least Concern	Zhuang (2011)

Table 3. Phytoremediation Potential of BBG Aquatic Species Collection

Plant Species	Pollutant	Literatures
Acanthus ebracteatus Vahl	Cu	Wahwakhi *et al.* (2017)
Acanthus ilicifolius L.	Pb, Cd	Irawanto *et al.* (2015)
Acorus calamus L.	Cd	Jeelani *et al.* (2017)
	Cu	Lu *et al.* (2018); Sun *et al.* (2013)
	Cr, Zn, Fe	Sun *et al.* (2013)
	Pb	Ma *et al.* (2019)
Azolla pinnata R. Br.	Pb	Mandakini *et al.* (2016)
	Cd	Mandakini *et al.* (2016); Rai (2008); Arora *et al.* (2004); Sood *et al.* (2012); Talebi *et al.* (2019)
	Cr	Mandakini *et al.* (2016); Rai (2008); Arora *et al.* (2006); Sood *et al.* (2012)
	Ni	Mandakini *et al.* (2016); Arora *et al.* (2004); Sood *et al.*; (2012); Talebi *et al.* (2019)
	Hg	Rai (2008); Mishra *et al.* (2009); Rai and Tripathi (2009); Sood *et al.* (2012)
	Zn, Cu	Talebi *et al.* (2019)
Cyperus involucratus Rottb.	Cu, Zn, Ni, Mn, Cd, Pb	Kaewtubtim *et al.* (2016)
	Cr	Meeinkuirt *et al.* (2017); Kaewtubtim *et al.* (2016)
Cyperus papyrus L.	As	Jomjun *et al.* (2011)
Eichhornia crassipes (Mart.) Solms	Cu	Lu *et al.* (2018); Hu *et al.* (2007); Sood *et al.* (2012)
	Pb	Ma *et al.* (2019)
	Hg	Sood *et al.* (2012); Molisani *et al.* (2006); Skinner *et al.* (2007)
	Cd	Mishra *et al.* (2007); Sood *et al.* (2012); Verma *et al.* (2008)
	Cr	Verma *et al.* (2008); Paiva *et al.* (2009); Sood *et al.* (2012); Mishra and Tripathi (2009); Sumiahadi and Acar (2018)
	Ni	Verma *et al.* (2008); Sood *et al.* (2012)
	As	Alvarado *et al.* (2008); Sumiahadi and Acar (2018)
	Zn	Mishra and Tripathi (2009); Sumiahadi and Acar (2018)
	Cs, Co	Saleh (2012); Sumiahadi and Acar (2018)
Myriophyllum aquaticum (Vell.) Verdc.	Ni, Pb, Zn	Harguinteguy *et al.* (2015); Harguinteguy *et al.* (2013)
	Co, Cu, Fe, Mn	Harguinteguy *et al.* (2013)
Nymphaea pubescens Willd.	Pb, Zn, Co, Cd	Kabeer *et al.* (2014)
Pistia stratiotes L.	Cu	Lu *et al.* (2018); Baroroh *et al.* (2018)
	Hg	Mishra *et al.* (2009); Molisani *et al.* (2006); Skinner *et al.* (2007); Sood *et al.* (2012)
	Cr	Verma *et al.* (2008); Mufarrege *et al.* (2010); Sood *et al.* (2012); Serang *et al.* (2018); Akter *et al.* (2014); Sumiahadi and Acar (2018)
	Cd	Verma *et al.* (2008); Sood *et al.* (2012); Das *et al.* (2014); Sumiahadi and Acar (2018)
	Ni	Verma *et al.* (2008); Mufarrege *et al.* (2010); Sood *et al.* (2012)
	Zn	Mufarrege *et al.* (2010); Sood *et al.* (2012)
	As	Sumiahadi and Acar (2018); Farnese *et al.* (2014)
Pontederia cordata L.	Cr, Fe, Cu, Zn	Sun *et al.* (2013)
Sagittaria lancifolia L.	Cr	Serang *et al.* (2018)
Salvinia adnata Desv.	Pb	George and Gabriel (2017); Kumari *et al.* (2017); Ranjitha *et al.* (2016)
	Hg	Kumari *et al.* (2017)
	Cr, Cd	Ranjitha *et al.* (2016)
	Cu	Ranjitha *et al.* (2016); Baroroh *et al.* (2018)
Typha angustifolia L.	Mn	Kaewtubtim *et al.* (2016)
	Cr, Zn, Cu	Bareen and Khilji (2008); Sood *et al.* (2012)
	As	Jomjun *et al.* (2011)

Table 4. Aquatic Species Collection of BBG Listed as Invasive Alien Plant Species in Indonesia

Species Name	Family	Origin	Literatures
Azolla pinnata R. Br.	Salviniaceae	Tropical Asia	Setyawati *et al.* (2015)
Eichhornia crassipes (Mart.) Solms	Pontederiaceae	Tropical South America	Setyawati *et al.* (2015); Tjitrosoedirdjo *et al.* (2016)
Fimbristylis umbellaris (Lam.) Vahl.	Cyperaceae	South East Asia	Setyawati *et al.* (2015)
Myriophyllum aquaticum (Vell.) Verdc.	Haloragaceae	South America	Setyawati *et al.* (2015)
Pistia stratiotes L.	Araceae	Africa or South America	Setyawati *et al.* (2015); Tjitrosoedirdjo *et al.* (2016)
Salvinia adnata Desv.	Salviniaceae	South America	Setyawati *et al.* (2015); Tjitrosoedirdjo *et al.* (2016)

Eichhornia crassipes, P. stratiotes and *S. adnata* were considered as three of the 75 important invasive alien plant species in Indonesia by Tjitrosoedirdjo *et al.* (2016). All the three species were also listed as important aquatic invasive alien plant species by Tjitrosoedirdjo (2005). *E. crassipes* was even listed by Lowe *et al.* (2000) as one of the worst invasive alien plant species. Originated from the Tropical South America *E. crassipes* was first introduced in 1894 to Bogor Botanic Garden and now present throughout Indonesia (Tjitrosoedirdjo *et al.*, 2016; Hulme 2011; Setyawati *et al.*, 2015). As with the *E. crassipes*, *P. stratiotes* was also placed in the BBG Aquatic Garden and also widely distributed in Indonesia with Africa or South America were considered as probable origin region for the species (Setyawati *et al.*, 2015; Tjitrosoedirdjo *et al.*, 2016). Both *E. crassipes* and *P. stratiotes* were firstly introduced to BBG in 2004 while Darma *et al.* (2017) reported the present of both plant species in *Tri-Danau* which consist of three lakes near BBG namely the Beratan, Buyan and Tamblingan.

Unlike the two previously mentioned important aquatic invasive alien plant species collected in BBG, *S. adnata* was placed in Cyathea Garden. Popularly known also by its synonym name of *S. molesta,* the plant species was originated from South America and distributed throughout Indonesia (Setyawati *et al.*, 2015; Tjitrosoedirdjo *et al.*, 2016). *S. adnata* was firstly introduced to BBG in 2012. The introduced specimens were collected from Buyan Lake. This was following Darma *et al.* (2017) which reported that a large portion of Buyan Lake was covered by *S. adnata*. However, the lake revitalization program carried out by the government was able to free most of the lake water bodies previously occupied by aquatic weeds including *S. adnata*.

As data of the three species, first present in the Tri-Danau region was absent, we were unable to conclude whether the *P. stratiotes and E. crassipes* specimen in the lakes were actually came from the BBG. However, careful management of all invasive aquatic plant species which presented in BBG collection was needed to prevent the invasive plant species escape. Management of aquatic invasive alien plant species in BBG could be done as suggested by Heywood and Sharrock (2013) in *the European Code of Conduct for Botanic Gardens on Invasive Alien Species*.

CONCLUSION

Aquatic plant species conservation in BBG is able to conserve plant species listed by IUCN Red List, as well as aquatic plant with phytoremediation and invasive potential. Eleven species of aquatic plant species in BBG are listed as Least Concern by IUCN Red List, fourteen species have phytoremediation potential and six species are listed as invasive alien plant species. The majority of the aquatic plant species are collected from Bali Island. Resulted data from this study can be used as a baseline data to conduct collection-based research in the future. On the other hand, this data also provides the garden manager with invasiveness status of aquatic plant collection to be monitored accordingly. The list also suggests that BBG should explore more aquatic plant species from another island in the eastern Indonesia region to enrich its collections.

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Research Article

Tree Species Composition and Natural Regeneration Status in South Eastern Bangladesh

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ABSTRACT

The study aimed to quantify and discuss the current condition of the tree species composition and natural regeneration of southeast parts of Bangladesh (Cox's Bazar North Forest Division). A total of 121 stems having dbh ≥10cm and 3481 stems of regenerating tree species (dbh <10cm) per hectare were recorded. A large trees comprised of 17 species belonging to 10 families and 14 genera and 30 regenerating tree species belonging to 19 families and 27 genera have been found. The forests were highly non-uniform, with three or four species represented most of the stands. The values of diversity indices indicated limited plant diversity, which is dominated by two or three tree species. Stems of 10-30 cm dbh contributed almost 90% of the total stem density, whereas more than 80% of the total basal area still belonged to trees with dbh 100 cm or above. *Dipterocarpus turbinatus* was the most dominant species which have the highest Importance Value Index (IVI) with 135.82 and embodied 37.71% of the total stand density and 72.19% of total basal area. The study will provide scientific basis for the future implementation of forest conservation strategies in tropical forests of Southeast Asia, particularly in Bangladesh. This study may also pave the way to further research on regeneration potentials of the native species for conservation and enhancement of forests in future.

Keywords: tree species composition; biodiversity index; South-East Asia; tropical forest; natural regeneration status.

INTRODUCTION

Tropical forests are often considered as the world's most species-rich plant communities and are both ecologically and economically important for the livelihood of local communities. However, vegetation covers are rapidly decreasing in the tropical areas. Due to increasing anthropogenic pressure, tropical forests are depleting at an alarming rate by 1–4% of their current land area (Laurance et al. 1998). In South and Southeast Asia, from 2010 to 2015 the net forest loss was about 25% higher compared to 1990 (Keenan et al. 2015).

Bangladesh lies within the tropical forest region of southeast Asia, covering 2.5 million ha of forest lands managed by the forest department, land ministry and other individuals (Sobuj & Rahman 2011). The forests of this country have been severely damaged over the past several decades by both biotic and abiotic disturbances affecting the regeneration and dynamics population. The major causes of forest degradation in Bangladesh are agricultural expansion, over-extraction of wood and non-wood resources, deforestation, urbanization, and inappropriate management practices (Hasan & Alam 2006). The forest degradation in Bangladesh has brought about an alarming rate of biodiversity reduction.

Quantitative information on tree species composition and distribution is important to understand the structure of a forest community and also for formulating conservation strategy for the community (Malik et al. 2014). Species diversity and regeneration status of tree species largely portray the nature of the forest community, as they provide resources and habitat for almost all other forest species (Cannon et al. 1998). The very existence of species in a community depends on the regeneration status. In forest management, regeneration study describes the current condition as well as possible future changes in forest composition (Malik & Bhatt 2016; Sharma et al. 2014).

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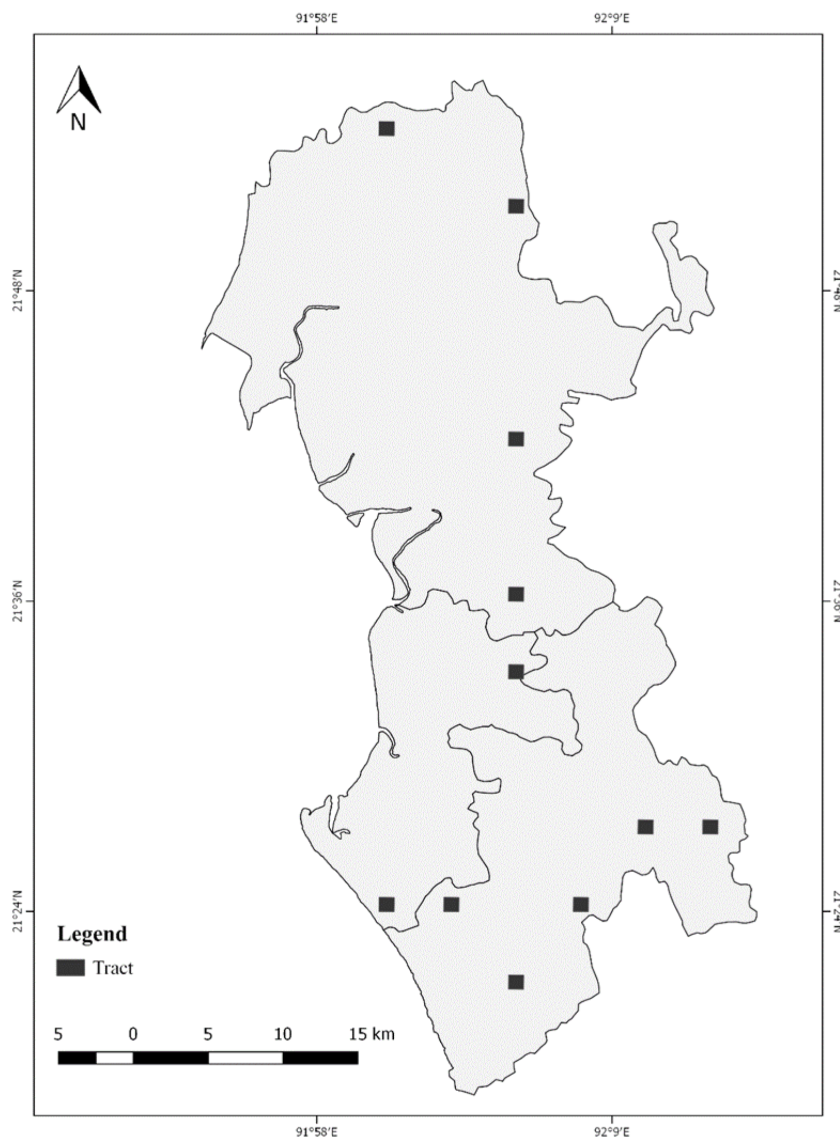

Figure 1. Location of tracts in Cox's Bazar North Forest Division

Well-timed and precise information on forest resources is essential for sustainable forest policy. The southeast parts of Bangladesh have natural forest patches, which are degrading at an alarming rate, and thus require a due attention. A rationalized strategy based on scientific information and complete knowledge of species distribution is required for these forests management. Therefore, the study was designed to provide quantitative information of the species composition, diversity, stem and basal area distribution of tree species in different diameter classes, and natural regeneration of this region.

MATERIALS AND METHODS

Study site

The study was conducted in the forests of Cox's Bazar North Forest Division, lying along the north-eastern coast of the Bay of Bengal. Its geographical location is between 20°30´ and 22° N latitude and between 91°45´ and 92°15´ E longitude. Cox's Bazar was initially established as a separate forest division on April, 1st 1920 (Choudhury 1969). The forests in the region are mostly tropical semi-evergreen types. It covers 74780.96 ha of forest area, which 62352.01 ha is managed as a reserve and 12428.95 ha as a protected forest, including 17 ranges, 66 beats, 74 blocks, 9 beat-cum-check stations and 2 check stations.

Sampling design

The sampling units were selected using random sampling design followed by Food and Agriculture Organization (FAO). The sampling units are termed as Tracts. The whole area was divided into units or tracts, 500 × 500m in size, distributed throughout Cox's Bazar North forest division at an interval of 3-minute latitude and 2.5-minute longitude. From them, 11 tracts were selected randomly for the study (Figure 1).

Methods

The Tract represents a square of 500 × 500m (25 ha) within which the field data was collected. Each Tract comprised of 4 Plots with the dimension of 20 × 100m (0.2 ha), one at each corner of the tract. The coordinates of the tracts and plots were precisely located using GPS for future relocation. A total of 8.8 ha area has been surveyed for this study. Information on species name, measurements of diameter and height of individual trees, and regeneration data were collected from the field. The measurements of trees having a diameter at breast height (dbh) larger than 30cm were collected along the central line of the plot. Measurements involved both left and right sides from the central line on a 10m wide extension. Additionally, two circular subplots were also established at the centre of each plot. Trees having 10-30cm dbh were measured inside sub-plot 1 (10m radius) and measurement of regeneration was taken inside sub-plot 2 (3.99 m radius) (Figure 2).

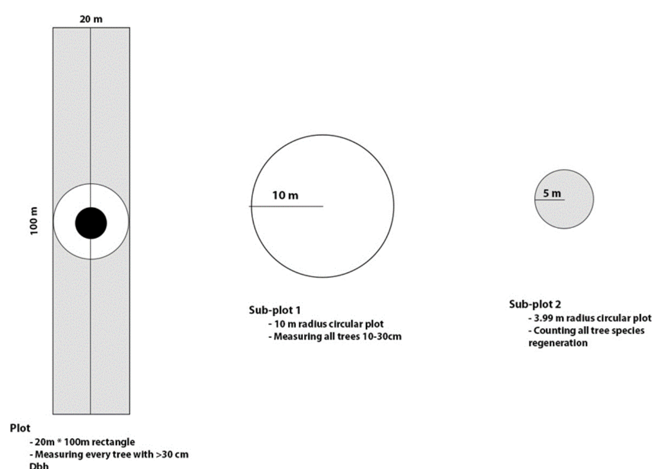

Figure 2. Plot design for data collection

The collected information was used to measure the basal area, Relative Frequency (RF), Relative Density (RD), Relative Dominance (RDo) and Relative Abundance (RA), which were then used to calculate IVI of each species. For this purpose, the following formulas were used (Dogra et al. 2009; Nebel et al. 2001):

$$\text{Basal area} = \frac{\pi \times dbh^2}{4} \quad (1)$$

$$\text{Frequency} = \frac{\text{Total no. of quadrats of sampling units in which the species occurs}}{\text{Total no. of quadrats}} \quad (2)$$

$$\text{Relative Frequency (RF)} = \frac{\text{Frequency of occurence of each species}}{\text{Sum of frequencies of occurence of all species}} \times 100 \quad (3)$$

$$\text{Relative Density (RD)} = \frac{\text{Total no. of individual of each species}}{\text{Total no. of individuals of all species}} \times 100 \quad (4)$$

$$\text{Relative Dominance (RDo)} = \frac{\text{Basal area of all individuals of species}}{\text{Total basal area of all individuals of all species}} \times 100 \quad (5)$$

$$\text{Abundance} = \frac{\text{Total no. of individuals of each species in all quadrats}}{\text{Total no. of quadrats in which the species occured}} \quad (6)$$

$$\text{Relative Abundance (RA)} = \frac{\text{Abundance of each species}}{\text{Total abundance of all quadrats}} \times 100 \quad (7)$$

For calculating IVI of tree species having dbh 10cm or above, the following equation has been used. For the IVI calculation of regeneration (dbh <10cm) instead of RDo, RA has been used.

IVI = RF + RD + RDo

Diversity indices are useful tools to understand the vegetative structure of a natural forest by providing information on the composition and status of vegetation in the designated area. The species richness was accessed using The Margalef's diversity index (Clifford & Stephenson 1975) and Menhinick's index (Whittaker 1977). The Shannon's index (H) (Shannon 1948) and Simpson's index of domination (D) (Simpson et al. 1949) were used to determine species diversity. The species evenness index (Pielou 1975) was also measured in the study.

$$\text{Shannon's Index (H)} = -\sum p_i \ln(p_i) \quad (8)$$

$$\text{Simpson's Index (D)} = \sum p_i^2 \quad (9)$$

$$\text{Species evenness index, E} = \frac{H}{Ln(S)} \quad (10)$$

$$\text{Margalef's index, } D_{Mg} = \frac{(S-1)}{ln(N)} \quad (11)$$

$$\text{Menhinick's index, } D_{Mn} = \frac{S}{\sqrt{N}} \quad (12)$$

Where:

p_i = the ratio of number of individuals of one species to the total number of individuals of all species

N = the total number of individuals,

S = the total number of species in the study area.

RESULTS AND DISCUSSION

A total of 131 individual stems with dbh ≥ 10 cm has been enumerated in 4.4 ha vegetation areas of

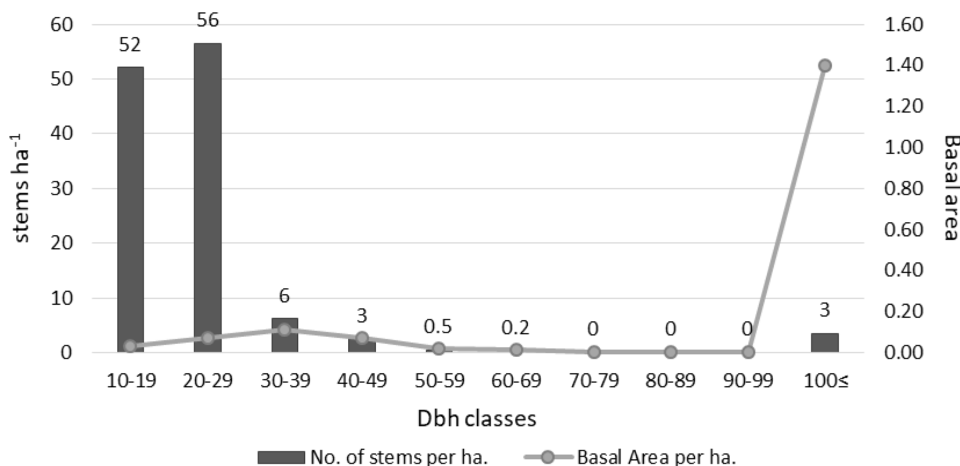

Figure 3. Distribution of tree density and basal area (m^2) per ha. in different diameter classes

Cox's Bazar North forest division, which were represented by 17 tree species belonging to 14 genera and 10 families. The figure 3 shows that stems with dbh between 10--30 cm contributed almost 90% of the total stem density. However, more than 80% of the total basal area still belonged to trees with dbh 100 cm or above. Stems of all dbh classes in the forests are not evenly distributed. There is no stem belonging to 70-99 cm dbh classes and rapid decline in stem density from ≥30 cm diameter class can be observed. The reason might be that trees were likely to be cut down, legally or illegally, after reaching diameter of 30 cm because of high market demand and easy transportation of logs.

Table 1. Density, basal area and tree diversity indices of Cox's Bazar North forest division

Index	Value
Density (Stems ha^{-1})	121
Basal Area (m^2ha^{-1})	11.533
Menhinick's index	0.329
Margalef's index	2.028
Shannon's diversity index	1.735
Shannon's maximum diversity index	2.833
Simpson's index	0.238
Shannon's equitability index or species evenness index	0.613

Species Diversity Analysis

The stem density and basal area of the vegetation covers in Cox's Bazar North forest division were 121 trees ha^{-1} and 11.533 m^2ha^{-1} respectively. The value of species evenness index was 0.613. The Shannon's diversity index was 1.735. The Menhinick's and Margalef's indices were 0.329 and 2.028 respectively. The Simpson's index value was 0.238. The values of Shannon's, Menhinick's and Margalef's indices indicate limited plant diversity. On the other hand, Simpson's index value shows the species were not uniformly distributed; but dominated by 2 or 3 tree species (Table 1).

Structural Composition of Tree Species

Among the 17 tree species, four species embody 88% of the stand density (*Dipterocarpus turbinatus* 37.71%, *Acacia auriculiformis* 24.05%, *Syzygium grande* 15.51%, *Syzygium cumini* 10.93%). Similarly, these four species represent 87% of the total basal area, where *Dipterocarpus turbinatus* alone comprises 72.19%. *Dipterocarpus turbinatus* has the highest stem density (46 stems ha^{-1}) followed by *Acacia auriculiformis* (29), *Syzygium grande* (19), and *Syzygium cumini* (13). The maximum relative density was in *Dipterocarpus turbinatus* (37.71%) followed by *Acacia auriculiformis* (24.05%), *Syzygium grande* (15.51%), and *Syzygium cumini* (10.93 %). The highest relative frequency was found in *Dipterocarpus turbinatus* (25.93%) followed by *Syzygium cumini* (11.11%), *Acacia auriculiformis* (7.41%), and *Syzygium grande* (7.41%). The study area was mainly dominated by *Dipterocarpus turbinatus* with relative dominance 72.19%, followed by *Syzygium grande* (5.62%), *Acacia auriculiformis* (5.43%), and *Anthocephalus chinensis* (3.96%). The importance value index (IVI) has been observed highest in *Dipterocarpus turbinatus* (135.82), followed by *Acacia auriculiformis* (36.89), *Syzygium grande* (28.54), and *Syzygium cumini* (25.97). The other species with low IVI values indicates their infrequency in the forests. The figure 4 and figure 5 illustrate the relative frequency, relative density, relative dominance and IVI, and the stem density and basal area per ha., respectively, for the top 7 tree species in the study area.

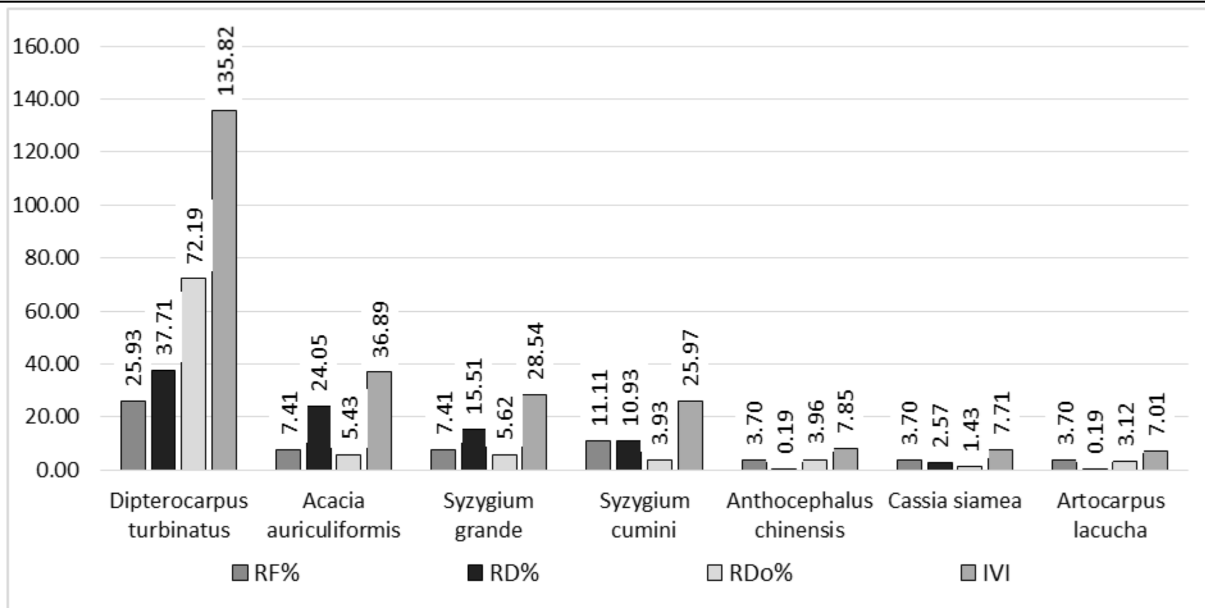

Figure 4. Relative frequency, Relative density, Relative dominance, and IVI of the top 7 tree species in the study area

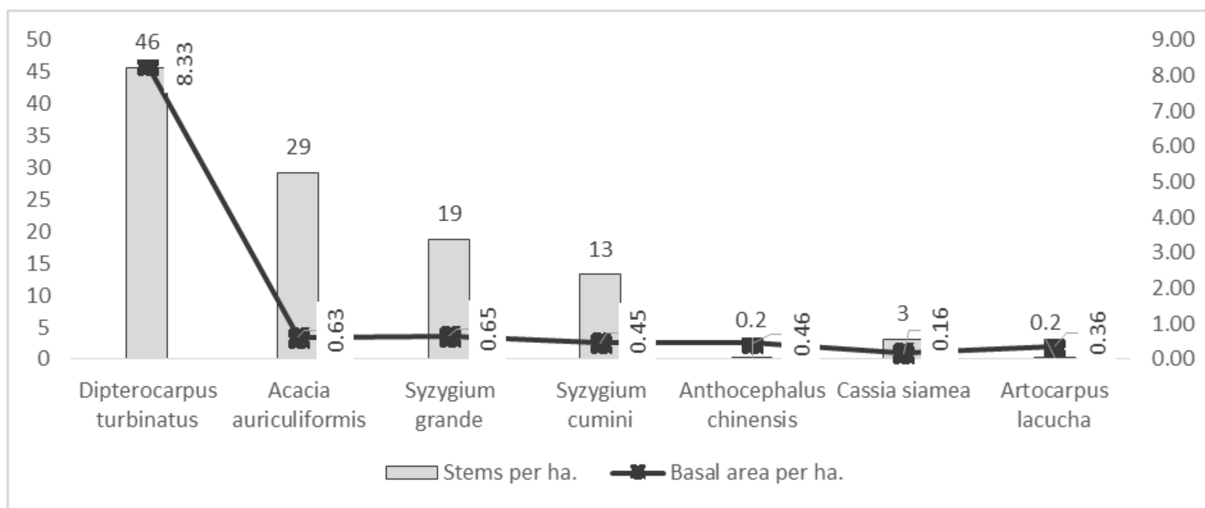

Figure 5. Stem density and Basal area per ha. of the top 7 tree species in the study area

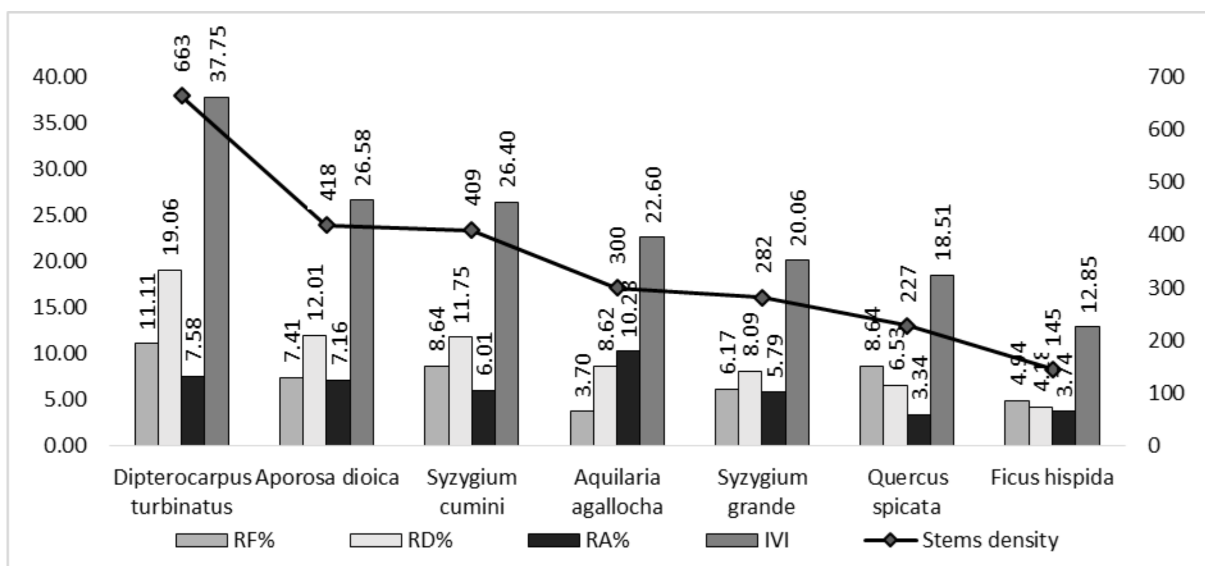

Figure 6. Stem density, Relative frequency, Relative density, Relative abundance, and IVI of the top 7 regeneration species in the study area

Quantitative Structure of Naturally Regenerated Seedling

Thirty tree species have shown regeneration in the study area. *Dipterocarpus turbinatus* has the highest regeneration status with 663 stems ha^{-1}, followed by *Aporosa dioica* (418), *Syzygium cumini* (409), *Aquilaria agallocha* (300), and *Syzygium grande* (282). These five species represent about 60% of the total regeneration. The study showed that *Dipterocarpus turbinatus* has the highest relative frequency (11.11%), followed by *Quercus spicata* (8.64%), *Syzygium cumini* (8.64%), and *Aporosa dioica* (7.41%). The highest relative density was found in *Dipterocarpus turbinatus* (19.06%) followed by *Aporosa dioica* (12.01%), *Syzygium cumini* (11.75%), and *Aquilaria agallocha* (8.62%). Relative abundance was highest in *Aquilaria agallocha* (10.28%), followed by *Dipterocarpus turbinatus* (7.58%), *Aporosa dioica* (7.16%), and *Syzygium cumini* (6.01%). The IVI was also highest in *Dipterocarpus turbinatus* (37.75), followed by *Aporosa dioica* (26.58), *Syzygium cumini* (26.40), and *Aquilaria agallocha* (22.60). Regeneration of the following five species was not found in the study: *Anthocephalus chinensis, Bauhinia acuminate, Dipterocarpus alatus, Ficus benghalensis, and Litsea glutinosa.* The stem density, relative frequency, relative density, relative dominance and IVI of the top 7 regeneration species have been shown in Figure 6.

Discussion

The study reveals the poor forest condition of Cox's Bazar North forest division. Usually, tropical forests are species rich with low frequent occurrence (Pitman et al. 1999). However, the study has found more than 50% of species were comprised of only one individual and more than 60% of total population were represented by two species.

The species diversity of the study area (H= 1.735) is lower than some tropical forests. According to other studies, the Shannon–Wiener diversity index of tropical rainforests of Xishuangbanna, China and tropical moist forests of Mizoram, northeast India were 3.45, 4.08 and 4.37 respectively (Devi et al. 2018; Lü et al. 2010). Same conditions can be observed in other forest areas of Bangladesh, where the index values were as follows: Fashiakhali WS 2.06, Sitapahar RF 2.98, Chunati WS 3.27-3.58, and Dudhpukuria-Dophachari WS 4.45 (Das et al. 2018; Hossain & Hossain 2014; Hossain et al. 2013; Nath et al. 2000). These values suggest the poor condition of species diversity in the forest division.

The stem density is severely affected by natural and anthropogenic disturbances. In 1988, Haque and Alam recorded 215 stems ha^{-1} in Cox's Bazar forest division (Haque & Alam 1988), which was much higher than the present stem density 121 stems ha^{-1}. It is lower than the density range 245– 859 stems per hectare for tropical forests as suggested by Campbell (Campbell et al. 1992). The basal area of the study area (11.53 m^{2}h^{-1}) is also lower than other tropical forests, i.e. in tropical rain forest of western India 30.87 m^{2}h^{-1}, in tropical forest Malaysia 32-51 m^{2}h^{-1} (Sha 1990; Swaine et al. 1987).

Due to human intervention, the establishment of new seedlings and their transition to mature stands are very poor. If this continues, the local species diversity will decline followed by the introduction of exotic species. The native people are dependent on the forest for fuel woods and other non-timber products and are often ignorant of the adverse effect this trend of logging on the forest. Regulations on logging operation and illegal felling can ensure recovery of forest structure, though it may take a long time and supports from local people. A well-developed management system and enrichment programs can further ensure the achievement of the desired goal.

CONCLUSION

Tree composition and their regeneration status show the overall well-being of the local forest community. The overall aim of the study was to provide quantitative structure of tree composition and regeneration status of southeastern Bangladesh. A total of 17 tree species (having dbh 10 cm or above) and 30 regenerating tree species were found in the study area. The study has revealed that the species diversity (H= 1.735) and stem density (121 stems ha^{-1}) of Cox's Bazar North forest division were lower than some of the tropical forests of Southeast Asia. The forests were largely populated by young trees with 10-30cm dbh (~90% of total density), though trees with dbh 100 cm or above hold more than 80% of the total basal area. A quick decline in stem density after reaching dbh 30 cm has been witnessed, due to illegal felling. *Dipterocarpus turbinatus* was the most valued species for both mature stand and regeneration composition. Effective and timely measures should be taken to conserve the forests using the latest technologies and adaptable management system. Planning forest policies and decision-making requires up-to-date information on forests and land uses, which can be obtained through continuous assessment and monitoring system. The study may be helpful for the future implementation of forest inventory and silvicultural techniques. This study may pave the way for further research on regeneration potentials of the native species for conservation and enhancement of forests in the future.

DECLARATION 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

Characteristics of Shell Bone as an Identification Tool for Turtle Species (Reptiles: Testudines) in Java, Borneo, and Sumatra

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ABSTRACT
There are 42 species of turtles; including sea turtle, freshwater turtle, tortoise, and softshell turtle (Reptilia: Testudines) living in Indonesia today. Turtles have economic values for quite a long time and it has led to illegal trade such as smuggling of carapace and plastron bones. Identification is needed to find out more details about the turtle species. Turtles have shells as unique features with different characteristics on each species. Shell's identification usually relies on the shape of carapace and plastron. The purpose of this research is to understand turtle shell morphological characters and determine the diagnostic characters of each species. We conducted visual observation on specimen collections from the Laboratory of Animal Systematics, Faculty of Biology, Universitas Gadjah Mada and Museum of Biology, Faculty of Biology, Universitas Gadjah Mada. The result shows that each turtle species have their own unique shell bone's characters, therefore it can be used to determine each turtle species of Indonesia.

Keywords: turtle, bones, carapace, plastron

INTRODUCTION

There are 42 species of turtles (including sea turtle, freshwater turtles, tortoise, and softshell turtle) living in Indonesia today. However, only five families can be found in the western part of Indonesia (Java, Borneo, and Sumatra) there are: Cheloniidae (sea turtles), Dermochelyidae (leatherback sea turtles), Geoemydidae (freshwater turtles), Testudinidae (tortoises), and Trionychidae (softshell turtles) (Iskandar, 2000; Iverson, 1992; Rhodin *et al.*, 2017). Turtles are a group of taxa that has unique characters and easily distinguished from other reptile groups. They are characterized by external features such as the absence of teeth and the most well-known is the shell as 'home'. Turtle's shell consists of two parts: the upper part (dorsal) that covers the back is called carapace and the lower part (ventral) is called plastron. The carapace and plastron have a function to protect the head, limbs, tail, and internal organs of turtle. Turtle's shell originates from a combination of vertebral columns with ribs, sternum and collarbone, and pelvis. The turtle's shell is determined in three general forms based on the nature and level of ossification: hard shells, softshells, and thick skin. As a result, each species of turtles has different shape and characteristics (Das, 2010).

Turtles have been a commodity for humans since ancient times. Most turtles are traded as pets, but some are processed as meat consumption. The high market demand (either export and local consumption) for turtles has triggered illegal hunting and trade (Iskandar, 2000). The turtle meat trade usually leaves shell bones and it can be used for species identification. The identification of turtle species using shells is generally based on the typical characteristics found in turtle shell bones (Sobolik & Steele, 1996). The paleontologists have used the shell bone to identify fossil turtle. Consequently, there are

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some fossils of turtles in Indonesia that have been identified, among them are *Megalochelys* sp. and *Hardella isoclina* Dubois, 1908 (Das, 1997; Joordens *et al.*, 2009; Setiyabudi, 2009).

The purpose of this study is to understand the morphological characteristics of shell bone from several turtle species to determine the diagnostic characters. These characters can be an alternative to identify turtle species. The obtaining results are expected to help people identify shell bones of turtle species, especially for wildlife scientific and management authority (identify illegal trafficking evidence).

MATERIALS AND METHODS

Materials

We examined 11 shell bones specimens from eight turtle's species. Specimens' collection of the Laboratory of Animal Systematics, Faculty of Biology, Universitas Gadjah Mada includes: 1) two species of sea turtles Family Cheloniidae (*Eretmochelys imbricata* and *Chelonia mydas*); 2) one species of fresh water turtle Family Geoemydidae (*Cuora amboinensis*); 3) two species of land tortoises Family Testudinidae (*Manouria emys* and *Indotestudo forstenii*); and 4) two species of softshell turtle Family Trionychidae (*Amyda cartilaginea* and *Dogania subplana*), all with a complete shell bone. Specimens' collection of the Museum of Biology, Faculty of Biology, Universitas Gadjah Mada , includes: 1) one species of sea turtle Family Cheloniidae (*Lepidochelys olivacea*); 2) one species of land tortoises Family Testudinidae (*Manouria emys*); and 3) two species of softshell turtle Family Trionychidae (*Amyda cartilaginea* and *Dogania subplana*), all with a complete shell bones. For comparison, we used the description of other species: 1) sea turtle Family Cheloniidae (*Caretta carreta*) and Family Dermochelyidae (*Dermochelys coriacea*); 2) freshwater turtle Family Geoemydidae (*Batagur baska*, *Orlitia borneensis*, *Heosemys spinosa*, and *Leucocephalon yuwonoi*); 3) softshell turtle Family Trionychidae (*Chitra chitra* and *Pelochelys cantorii*) taken from Lydekker (1889), Pritchard *et al.* (2009), and Eckert *et al.* (2012). Complete shell bones are generally composed of carapace and plastron (Figure 1.). The carapace consists of nuchal, peripherical, neural, pleural, suprapygal, pygal and supracaudal. The plastron consists of epiplastron, entoplastron, hyoplastron, hypoplastron, xiphiplastron, and bridge. Each fragment is connected by a suture (Brinkman *et al.,* 2017; Pritchard *et al.,* 2009; Sobolik & Steele, 1996).

Methods

We conducted visual observation on the turtle's shell bones and literature study to determine the diagnostic character of each species. These characters can be found from each part of carapace and plastron fragments. We also compared the scute sulci and grid on the shell bone's surface. On scaly turtles, scute sulci (Figure 1) are found as a place where the scales attach. In softshell turtles, a pattern will form a grid on the surface of the shell as the skin layer attaches (Iskandar, 2000; Sobolik & Steele, 1996).

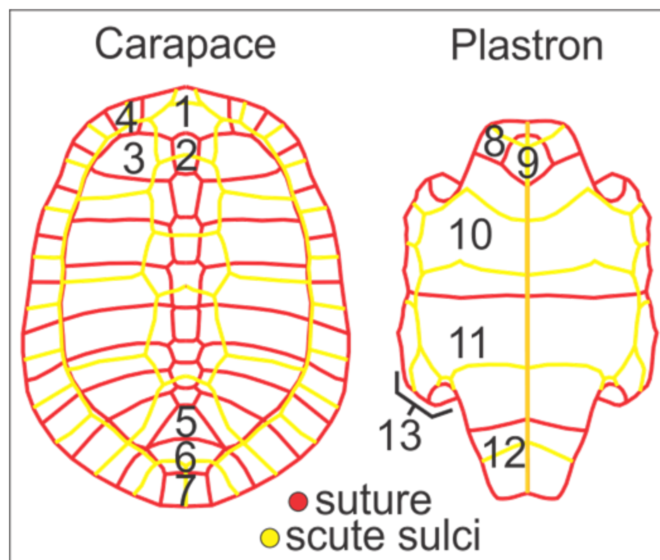

Figure 1. Complete shell bone specimen. Left: dorsal (carapace) view and right: ventral (plastron) view, with description: 1. Nuchal, 2. Neural, 3. Pleural, 4. Peripherical, 5. Suprapygal, 6. Pygal, 7. Supracaudal, 8. Epiplastron, 9. Entoplastron, 10. Hyoplastron, 11. Hypoplastron, 12. Xiphiplastron, 13. Bridge (modified from Lydekker, 1889)

RESULTS AND DISCUSSION

Sea Turtle Specimen members of the Family Cheloniidae (examined specimens and literature study)

The sea turtle group (family Cheloniidae) consists of four species spread throughout the Indonesian sea. This group is supported by several characters, including the number of plates (scutes) of the carapace and the different parts of plastron. Plastron is divided into three parts. The first part consists of epiplastron and entoplastron which are fused. Other parts are hyoplastron, hypoplastron, and xiphiplastron which are fused, each of them is divided into right and left. The plastron has a fontanel in the middle which split hyo-hypo-xiphiplastron right and left, however, the plastron in sea turtle has no bridge (Figure 1).

Lepidochelys olivacea has a solid carapace, where other groups of sea turtles have carapace with fontanel as adults (Figure 2). Plastron of *Lepidochelys olivacea* has a wide size, without long sutures

resembling thorns on hyoplastron or hypoplastron. Entoplastron has a wide triangle shape. *Caretta caretta* has a carapace character with small fontanels. In adults, sometimes there is no fontanel, similar to *Lepidochelys olivacea* (Figure 2). Plastron of *Caretta caretta* has a slim morph, with long thorn-like sutures on hyoplastron or hypoplastron.

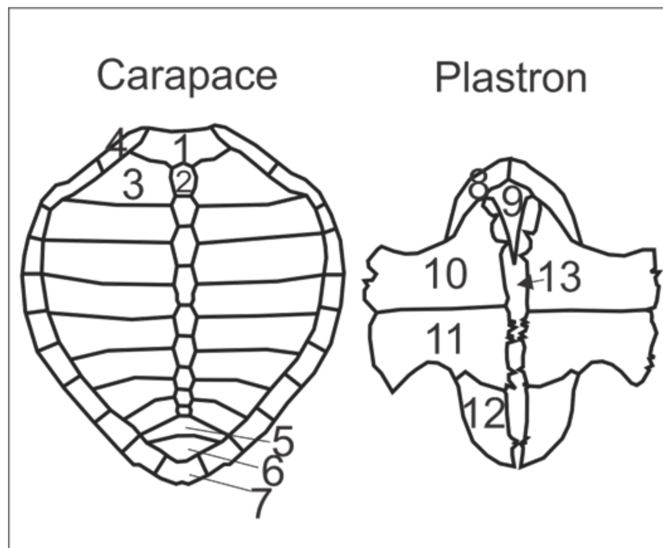

Figure 2. Specimens of *Lepidochelys olivacea* from Museum of Biology UGM. Left: dorsal (carapace) view and right: ventral (plastron) view, with description: 1. Nuchal, 2. Neural, 3. Pleural, 4. Peripherical, 5. Suprapygal, 6. Pygal, 7. Supracaudal, 8. Epiplastron, 9. Entoplastron, 10. Hyoplastron, 11. Hypoplastron, 12. Xiphiplastron, 13. Fontanel which split between hyo-hypoplastron (Personal documentation, drawn from real skeletal specimen collection of the Museum of Biology, Universitas Gadjah Mada).

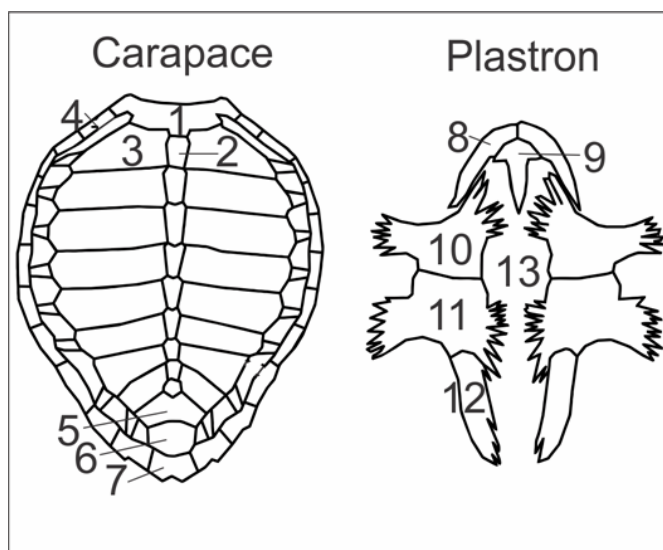

Figure 3. Specimens of *Caretta caretta.* Left: dorsal (carapace) view and right: ventral (plastron) view, with description: 1. Nuchal, 2. Neural, 3. Pleural, 4. Peripherical, 5. Suprapygal, 6. Pygal, 7. Supracaudal, 8. Epiplastron, 9. Entoplastron, 10. Hyoplastron, 11. Hypoplastron, 12. Xiphiplastron, 13. Fontanel which split between hyo-hypoplastron (modified from Valente, 2007).

Sutures of *Caretta caretta* tend to cluster on each side. Entoplastron has a tack-like shape, with a fairly wide center. *Eretmochelys imbricata* has many fontanels in the carapace. Fontanels are used for forming the flexibility of carapace bones so they can resist the underwater pressure when diving. This character also found in *Chelonia mydas*. The pleural part has a dagger-like shape, with the rib cage covered by a carapace shield plate (Figure 4).

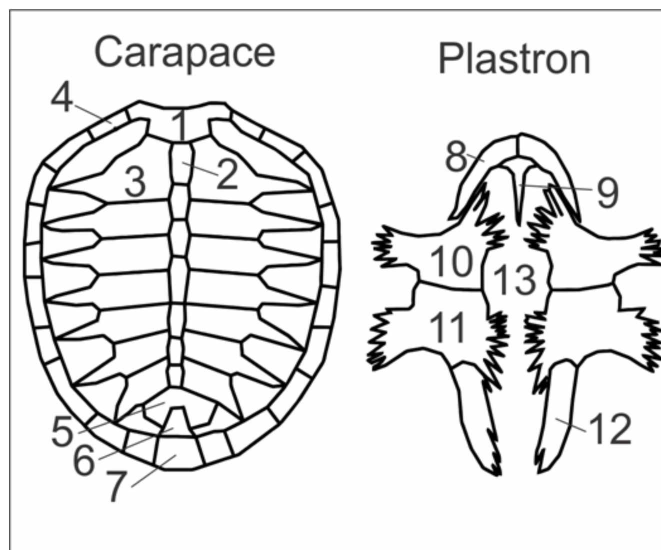

Figure 4. Specimens of *Eretmochelys imbricata* from the Laboratory of Animal Systematics, Faculty of Biology, UGM. Left: dorsal (carapace) view and right: ventral (plastron) view, with description: 1. Nuchal, 2. Neural, 3. Pleural, 4. Peripherical, 5. Suprapygal, 6. Pygal, 7. Supracaudal, 8. Epiplastron, 9. Entoplastron, 10. Hyoplastron, 11. Hypoplastron, 12. Xiphiplastron, 13. Fontanel which split between hyo-hypoplastron (modified from Wyneken, 2001).

The plastron of *Eretmochelys imbricata* has a morphological form that resembles *Caretta caretta* (Figure 3). However, the distinguishing character is in the entoplastron section. The entoplastron of *Eretmochelys imbricata* has a narrower shape in the middle. The last species of sea turtle group is *Chelonia mydas*. The carapace of *Chelonia mydas* is not completely ossified, so there are many fontanels. In the pleural part, the rib cage is partially exposed, because it is not covered with a carapace shield plate (Figure 5).

Plastron of *Chelonia mydas* has the least morphology with ossification compared to other species of sea turtles. Hyoplastron has not many spines but extends individually on each spine. The spine of a suture in the hypoplastron looks more clustered and slightly dense. *Chelonia mydas* entoplastron has the slimmest form than the other sea turtles, resembling a plus sign (Figure 5).

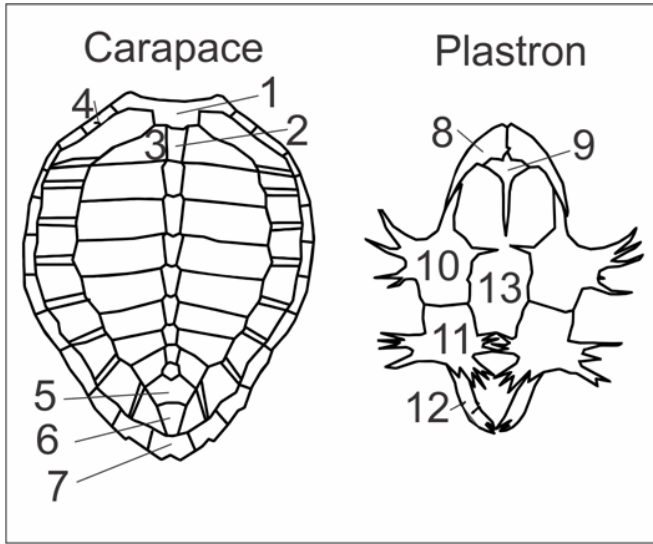

Figure 5. Specimens of *Chelonia mydas* from Museum of Biology UGM. Left: dorsal (carapace) view and right: ventral (plastron) view, with description: 1. Nuchal, 2. Neural, 3. Pleural, 4. Peripherical, 5. Suprapygal, 6. Pygal, 7. Supracaudal, 8. Epiplastron, 9. Entoplastron, 10. Hyoplastron, 11. Hypoplastron, 12. Xiphiplastron, 13. Fontanel which split between hyo-hypoplastron (modified from Grace, 2012).

Sea Turtle Specimens members of the Family Dermochelyidae (literature study)

The leatherback sea turtle group (Dermochelyidae) only has one species that still alive today, it is *Dermochelys coriacea*. This group shows the level of neotony skeletons that are more advanced than other species members of the order Testudines. Only the nuchal bone has no sutural contact with other bones and relatively intact. Pleural bone, which in other members of the Testudines generally forms together with the ribs in a composite bone structure, is not found in this species. The ribs are thin, so they have morphology as found in hard-shelled turtle embryos. Peripheral, suprapygal, pygal, and supracaudal bone were not found in this group (Figure 6).

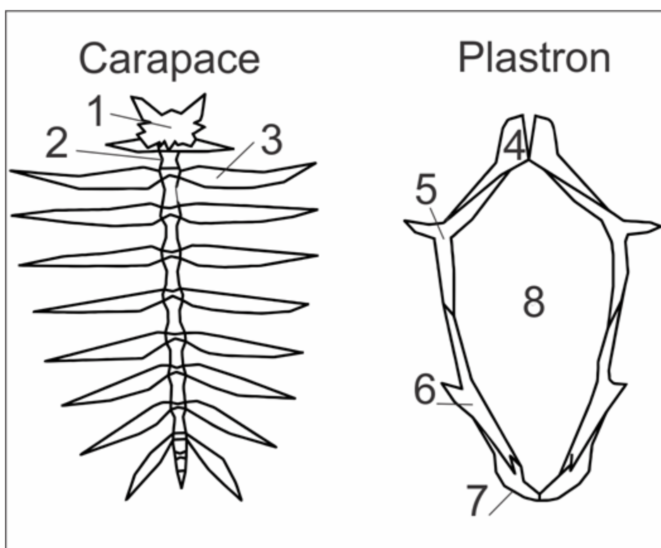

Figure 6. Specimens of *Dermochelys coriacea*. Left: dorsal (carapace) view and right: ventral (plastron) view, with description: 1. Nuchal, 2. Neural, 3. Pleural, 4. Epiplastron, 5. Hyoplastron, 6. Hypoplastron, 7. Xiphiplastron, 8. Very large fontanel (modified from Eckert *et al.,* 2012).

Dermochelys coriacea has plastron without entoplastron. But the remaining elements (epiplastron, hyoplastron, hypoplastron, and xiphiplastron) are reduced to a narrow structure, fused to form a ring around a large and open middle fontanel (Figure 6). Leatherback turtle shells thus obtain very high flexibility from their reinforced core structures. It appears that the shell, especially the plastron, has a very large reduced ossification, can resist substantially to accommodate the extreme pressure at a deep sea.

Freshwater turtle specimens' members of the Family Geoemydidae (literature study)

The group of freshwater turtles (family Geoemydidae) is the group that has the most members in western Indonesia. This turtle has the most complete diagnostic character among the other members of the Testudines, for example, taken in the *Batagur baska* (Figure 7).

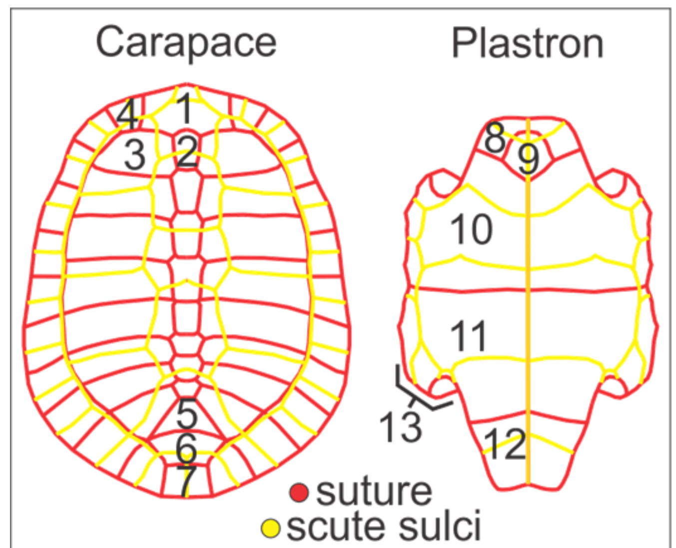

Figure 7. Specimens of *Batagur baska*. Left: dorsal (carapace) view and right: ventral (plastron) view, with description: 1. Nuchal, 2. Neural, 3. Pleural, 4. Peripherical, 5. Suprapygal, 6. Pygal, 7. Supracaudal, 8. Epiplastron, 9. Entoplastron, 10. Hyoplastron, 11. Hypoplastron, 12. Xiphiplastron (modified from Lydekker, 1889).

In the members of this group, the whole shell is covered with keratin scales, so that in the shell bone you will see a line of sticky scales called scute sulci. In this group, there is no fontanel when adults. In addition, pleural is arranged uniformly because

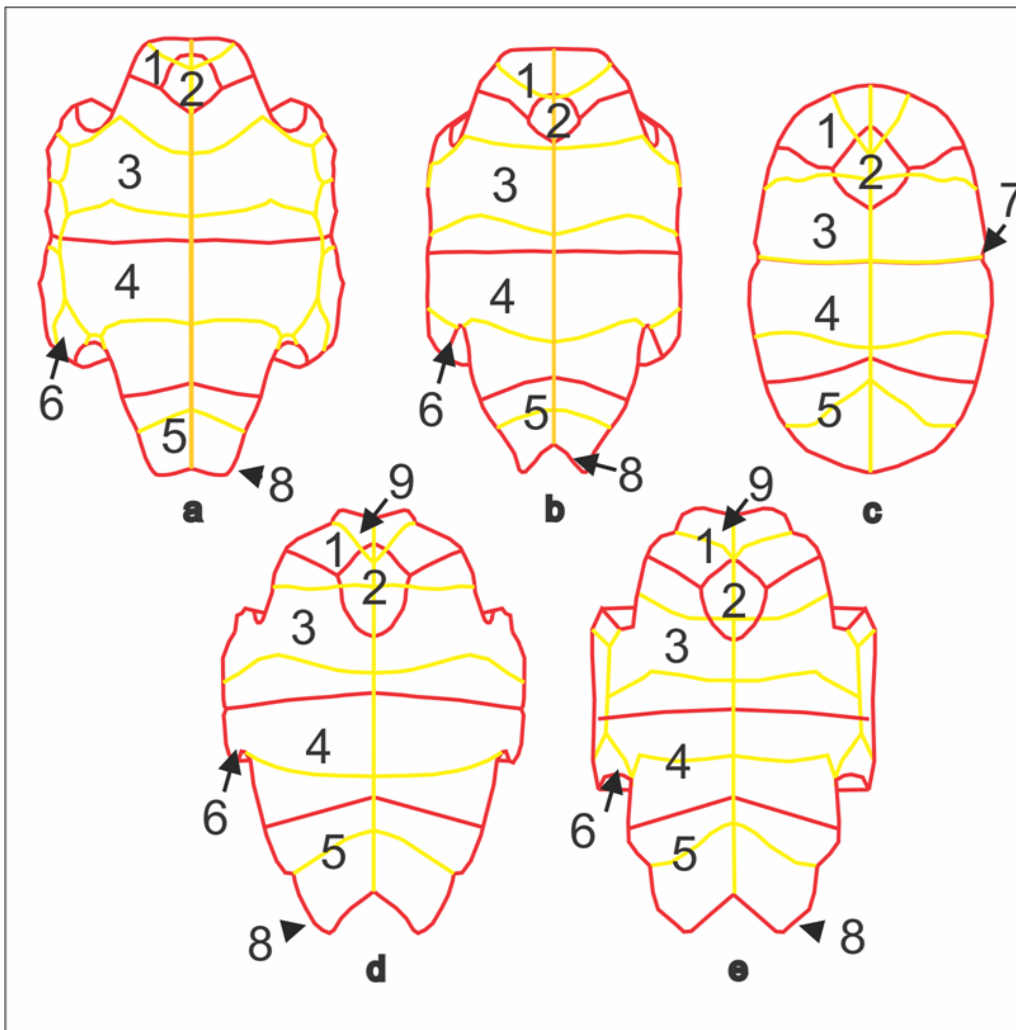

Figure 8. Plastron of various species members of the Geoemydidae. a. *Batagur baska*, b. *Orlitia borneensis*, c. *Cuora amboiensis*, d. *Heosemys spinosa*, e. *Leucocephalon yuwonoi*, with descriptions: 1. Epiplastron, 2. Entoplastron, 3. Hyoplastron, 4. Hypoplastron, 5. Xiphiplastron, 6. Bridge, 7. Hinge, 8. Anal notch, 9. Gular notch (modified from Lydekker, 1889; Pritchard *et al.*, 2009; & personal documentation).

the morphology is relatively the same. The carapace section is very difficult to use as a distinguishing character between species. Then the character diagnostic focuses on the plastron morphological characters (Figure 8).

At the *Batagur baska*, the diagnostic characters found in epiplastron and xiphiplastron forms that are elongated and narrow, likely to be slender. The shell suture is only owned by young individuals. On adult specimens, the shell bone fragments fused and the suture developed into ankylosis form. In the hyoplastron fragment, there are scute sulci which do not cross entoplastron. The bridge part is elongated and clear, so the overall shape of the plastron resembles a plus sign. The anal notch is not too concave (Figure 8a). In *Orlitia borneensis*, the diagnostic character that can be seen clearly is the hyoplastron part which has scute sulci, and do not cross entoplastron. In addition, the anal notch looks very concave (Figure 8b).

Cuora amboinensis has a diagnostic character in the hyoplastron section that only has one scute sulci and cut entoplastron. Dual scute sulci which generally found in hyoplastron of turtles, only found a single in *Cuora amboinensis*, as a result, one of the scute sulci which has fused with suture in part called hinge (Figure 8c). *Heosemys spinosa* has a diagnostic character in the form of gular notch and anal notch which form to be concave. Two scute sulci found in the hyoplastron, one of which is cutting the entoplastron (Figure 8d). In *Leucocephalon yuwonoi*, the diagnostic character is seen from the form of Y scute sulci (three branches) in hyoplastron and hypoplastron (Figure 8e)

Tortoise specimens' members of Family Testudinidae (examined specimens and literature study)

The group of tortoises (family Testudinidae) has a diagnostic character that has many similarities to members of the freshwater turtle (family Geoemydidae). The two groups can be distinguished based on the form of pleural arrangement. In members of the Testudinidae have a pleural shape

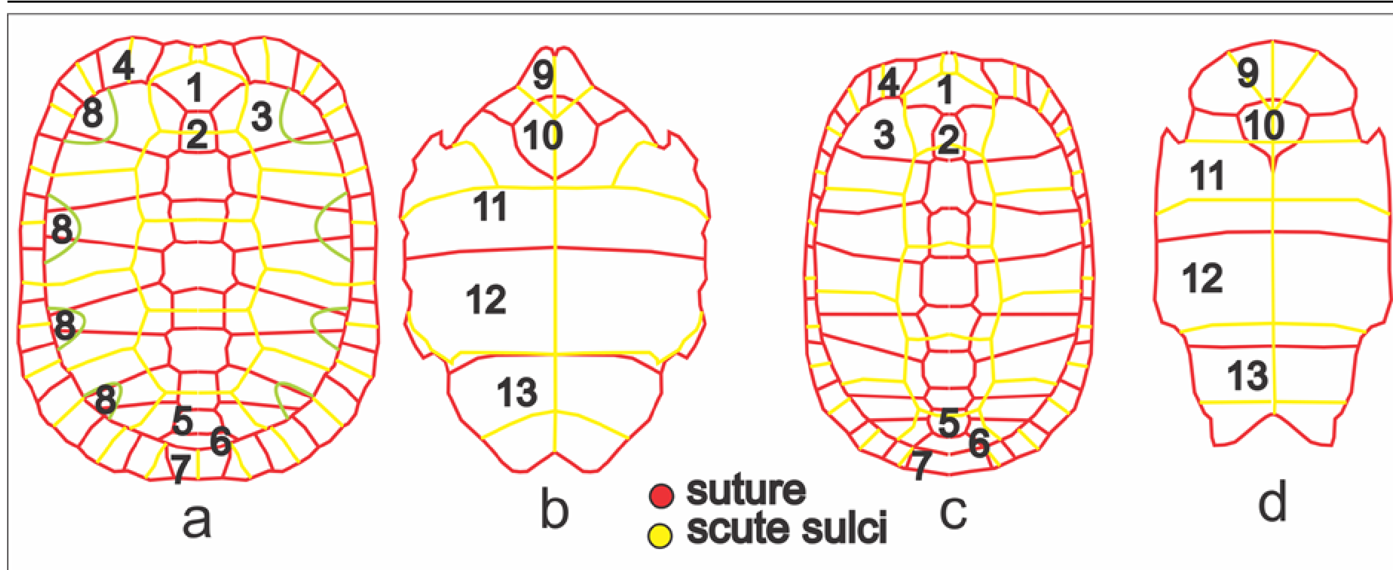

Figure 9. Shell bone specimens' members of Testudinidae. a. *Manouria emys* carapace, b. *Indotestudo forstenii* carapace, c. *Manouria emys* plastron, d. *Indotestudo forstenii* plastron, with descriptions: 1. Nuchal, 2. Neural, 3. Pleural, 4. Peripherical, 5. Suprapygal, 6. Pygal, 7. Supracaudal, 8. Parts that geodesics, 9. Epiplastron, 10. Entoplastron, 11. Hyoplastron, 12. Hypoplastron, 13. Xiphiplastron (modified from Brinkman, 2017; & Personal documentation).

that is not the same between the base of the pleural which is connected with the neural, and the pleural end that is connected with peripherical. So that the pleural shape that belongs to this family tends to be trapezoidal. The pleural arrangement is found in the carapace forms a zigzag pattern (Figure 9). There are two species of tortoises which was found in Indonesia, there are *Manouria emys* and *Indotestudo forstenii*. Although *Indotestudo forstenii* is not found in western Indonesia, the same genus *Indotestudo elongata,* is found in the Southeast Asia region near to western Indonesia, so we include it in this study.

In the carapace of *Manouria emys* has a diagnostic character of geodesics, where thickening of the ossification occurs in the area around scute sulci. Whereas other parts (including the part of the suture) that are not close to scute sulci will be thinning out (Figure 9a). Epiplastron forms a spear. Hyoplastron has a scute sulci pattern forming Y (Figure 9c). Not many characteristic in the carapace *Indotestudo forstenii*, except the overall shape is more elongated compared to *Manouria emys* (Figure 9b). The diagnostic character of this species is the presence of a thick epiplastron forming a bumper (Figure 9d).

Softshell Turtle Specimens members of Family Trionychidae (examined specimens and literature study)

Members of the softshell turtle (family Trionychidae) have a distinctive shell character compared to other members of the order Testudines. There is a much reduction in shell configuration, loss of scales and replaced by soft and rough skin. Shells have no peripherical bones so the ribs are exposed. Supracaudal was not found, but the bone was replaced by a pair of pygal bones. The plastron consists of seven parts, epiplastron, entoplastron, hyoplastron, xiphiplastron hypoplastron. Overall, the plastron is not connected, except in the hyoplastron and hypoplastron which are joined by sutures. Entoplastron has a shape like a boomerang. Epiplastron elongated and narrow (Figure 10).

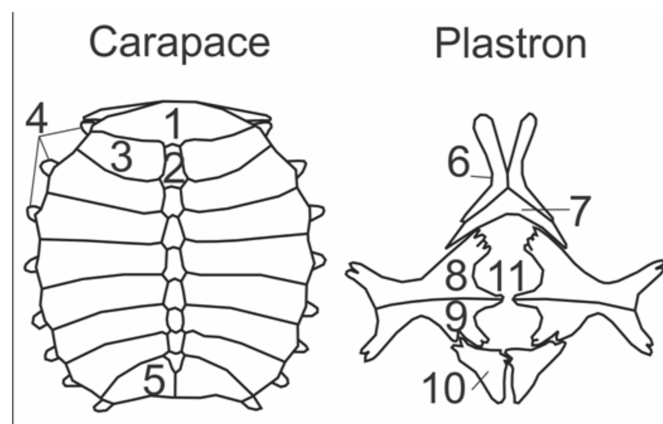

Figure 10. Specimens of *Amyda cartilaginea*, left: dorsal (carapace) view and right: ventral (plastron) view, with description: 1. Nuchal, 2. Neural, 3. Pleural, 4. Ribs, 5. Pygal, 6. Epiplastron, 7. Entoplastron, 8. Hyoplastron, 9. Hypoplastron, 10. Xiphiplastron, 11. Fontanel (redrawn from Iskandar, 2000)

In softshell turtles, the basic character for species identification is the latticed patterns on shell bone's surface. This pattern can be found in all parts of the carapace and plastron of this group. In western Indonesia, there are four species of softshell turtles: *Amyda cartilaginea*, *Dogania subplana*, *Chitra chitra*, and

Table 1. Shell bone diagnostic character.

No	Species	Characters	Remarks
Family Cheloniidae			
1	*Lepidochelys olivacea*	Carapace	Solid with no fontanel
		Plastron	Without long suture spines
		Entoplastron	Wide triangle shape
2	*Caretta caretta*	Carapace	Partly fontanelled
		Plastron	Grouped long suture
		Entoplastron	Tack-like shape (wide at center)
3	*Eretmochelys imbricata*	Carapace	Fontanelle 50% pleural
		Pleural	Dagger-like shape,Covered ribs
		Plastron	Grouped long suture
		Entoplastron	Thin tack-like shape
4	*Chelonia mydas*	Carapace	Fontanelle 50% pleural
		Pleural	Uncovered ribs
		Plastron	Long suture and not grouped
		Entoplastron	Cross-like shape
Family Dermochelyidae			
5	*Dermochelys coriacea*	Carapace	Only consists of nuchal, neural and pleural
		Pleural	Dagger-like shape
		Plastron	Ringed fontanel without entoplastron
Family Geomydidae			
6	*Batagur baska*	Shell	Ankylosis
		Plastron	Epiplastron and xiphiplastron are elongated and slender
7	*Orlitia borneensis*	Hyoplastron	Scute sulci without cuts entoplastron
8	*Cuora amboinensis*	Hyoplastron	Single scute sulci with hinge
		Plastron	Without bridge
9	*Heosemys spinosa*	Hyoplastron	Scute sulci cuts entoplastron
10	*Leucocephalon yuwonoi*	Hyo-hypoplastron	Triangular scute sulci, Y shape
Family Testudinidae			
11	*Indotestudo forstenii*	Plastron	Epiplastron bumper shape
12	*Manouria emys*	Carapace	Geodesics
Family Trionychidae			
13	*Amyda cartilaginea*	Shell pattern	Mosaic with hill
14	*Dogania subplana*	Shell pattern	Near the neural is mosaic, near the marginal is vermiform with protuberance
15	*Chitra chitra*	Shell pattern	Near the neural is an overlapping circle, near the marginal is vermiform
16	*Pelochelys cantorii*	Shell pattern	Near the neural is a circle with bold a border, near the marginal, is vermiform

Pelochelys cantorii. In *Amyda cartilaginea*, it has the same pattern between neural and marginal parts. The pattern looks random and irregular to form a mosaic. In addition, there is a hill (in the illustration shown by a thicker line) which is scattered and longitudinally parallel to the neural (Figure 11a). In *Dogania subplana*, the approach to neural patterns looks similar to the mosaic-shaped pattern (cf. *Amyda cartilaginea*). But the obvious difference is based on a deep and firm crater on *Dogania subplana*. The approaching marginal shell pattern looks slightly sloping and vermiform, with the presence of nodules (Figure 11b). *Chitra chitra* has a circular pattern that overlaps at the neural part but towards the marginal direction, the pattern changes into a vermiform form (Figure 11c). *Pelochelys cantorii* has a pattern that is not different from *Chitra chitra*. In the neural part, the pattern is dominated by a circle that has clear

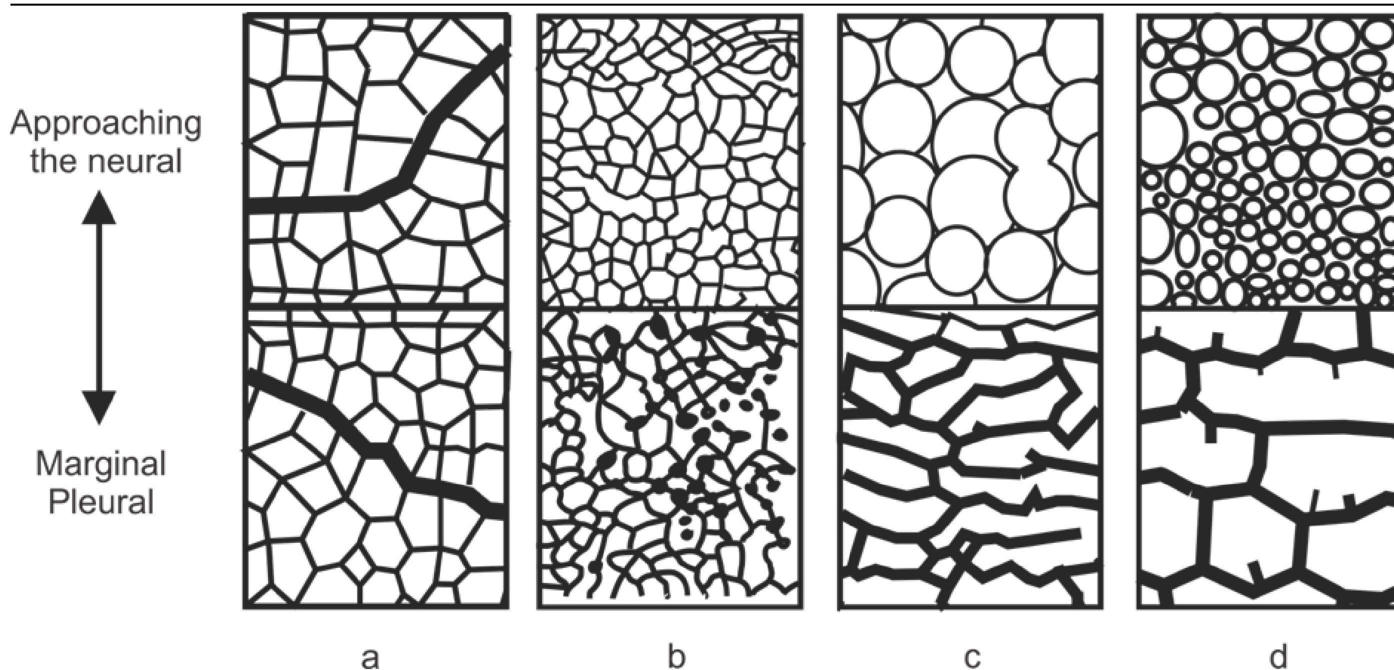

Figure 11. Shell pattern of the Trionychidae members. Above: approaching neural, bottom: marginal pleural, a. *Amyda cartilaginea*, b. *Dogania subplana*, c. *Chitra chitra*, d. *Pelochelys cantorii* (modified from Pritchard *et al*., 2009)

boundaries and does not overlap. Towards marginal, the pattern is replaced by a sloping vermiform form (Figure 11d).

Diagnostic Character of each Turtle Shell

Based on the results of the analysis, we determined the diagnostic characteristics of each species (Table 1).

CONCLUSION

This research is important in contributing to the turtle's species identification based on their shell bones composition. The sea turtles species can be identified by the carapace's fontanelle form and location, carapace's bone solidity, and the different forms and groups of plastron's suture spines. Softshell turtles species are identified from their shell pattern with or without a mosaic hill, vermiform shape, and their protuberance. Freshwater and semi-aquatic turtles species are identified from the shape of scute sulci. These characters are useful to determine the species of turtle which are sold by illegal trading or fragments fossil found in Java. Further studies with more specimens from other turtle species are needed to make a complete identification key.

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Research Article

Genetic Diversity Analysis of *Rhacophorus margaritifer* (Schlegel, 1837) in Baturraden, Purwokerto, Central Java, Indonesia Revealed by Based on RAPD Marker

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ABSTRACT
Rhacophorus margaritifer is an endemic species of arboreal frog in Java. Previous studies found this frog in several locations of Baturraden, namely in primary forest of Ketenger area, along Pancuran Pitu tourism track, and Baturraden Botanical Garden. There were still limited studies of molecular diversity of *R. margaritifer* and no prior data from population in the southern slope of Mount Slamet. This study aimed to look at the genetic polymorphism and determine the locus diversity of *R.margaritifer* population in Baturraden by using the PCR-RAPD technique. Frog tissue samples were taken from three populations in Kalipagu hiking trail (HPK 01), Baturraden hiking trail (HPK 02), and Baturraden Botanical Garden (KRB). DNA was extracted using the Chelex method. Molecular characterization was performed based on RAPD markers. The RAPD marker band pattern was changed to binary data 0-1 and analysed using Arlequin software ver.3.5. A total of 19 frog individuals were obtained during sampling at three locations. High genetic diversity had been observed in all populations with gene diversity range from 0.9643 in HPK 01 population to 1.0000 in both KRB and HPK 02 populations. A high locus variation was also observed for all populations with values of 0.159524 in KRB; 0.165816 in HPK 01; 0.192857 in HPK 02, respectively. AMOVA indicated no genetic difference among populations of *R. margaritifer* (p=0.50244).

Keywords: Baturraden, genetic diversity, RAPD, *Rhacophorus margaritifer*

INTRODUCTION
Rhacophorus margaritifer is a frog of Rhacophoridae family. It is an endemic species in Java and distributed throughout West Java to East Java (Iskandar, 1998). Previous studies found this species in several locations in the southern slope of Mount Slamet, namely in primary forest of Ketenger, Baturraden (Riyanto, 2010), Pancuran Pitu tourism track (Puspitasari *et al.*, 2017), and thematic area of Baturraden Botanical Garden (Avani, 2018).

There were still limited studies of molecular diversity of *R. margaritifer*. There is no prior data from *R. margaritifer* populations in the southern slope of Gunung Slamet, including Baturraden area. The information on species molecular diversity plays an important role in species conservation and can be used to determine specific conservation method (Funk *et al.*, 2012; Angulo & Icochea, 2010; Loeschcke *et al.*, 1994; Schonewald-Cox *et al.*, 1983). Molecular characterization of *R. margaritifer* enables to identify any diversity from individuals with the similar morphology. Molecular diversity can be discovered by detecting DNA polymorphism using Random Amplified Polymorphic DNA technique. RAPD detects DNA polymorphism based on PCR (Polymerase Chain Reaction). The RAPD primer randomly amplifies a compatible sequence of DNA genome to produce various fragment lengths (Telles *et al.*, 2006). RAPD is widely used since it is relatively fast, simple, and affordable (Anggereini, 2008; Williams *et al.*, 1990). RAPD also helps to identify genetic variation in an animal. Snell & Evans (2006) used RAPD to differentiate the larva of *Rana arvialis* and *Rana temporaria* that were morphologically similar. Padhye *et al.* (2012) reported variations on the population of *Hylarana malabarica* in northern

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Figure 1. *Rhacophorus margaritifer* (Schlegel, 1837)

West Ghat, India based on RAPD markers. Therefore, this study aimed to look at the genetic polymorphism and determine the locus diversity of *R. margaritifer* population in Baturraden using the PCR-RAPD technique. No genetic data are available on Amphibian in Baturraden, Purwokerto, Central Java, Indonesia. Therefore, it is the first report about genetic diversity of Amphibian from that area with special emphasis on *R. margaritifer*. The informations obtained in this study are vital to sustaining *R. margaritifer* since that species plays an important role in Baturraden ecosystem.

MATERIALS AND METHODS

Materials

Materials used in this study were *Rhacophorus margaritifer* (Figure 1), aquadest, 70% ethanol, absolute ethanol, GPS, headlamp, flashlight, nets, specimen bag, caliper, stationary and camera for the field sampling. Chelex 5%, Dithiothreitol (DTT), and proteinase-K for DNA isolation; Nuclease Free Water/ddH_2O, Buffer PCR, $MgCl_2$ solution, dNTPs mix, Taq polymerase, DNA Template, and RAPD primers for amplification RAPD marker. TAE 1X solution, agarose, aquadest, Ethidium Bromide (EtBr), loading dye, and DNA ladder for DNA visualization. Freezer, micropipette and tips, thermomixer, centrifuge, electrophoresis apparatus, analytical scale, UV transilluminator, beaker glass, Erlenmeyer, microwave, 0.2 mL & 1.5 mL microtube, thermal cycler (PCR), parafilm paper, gloves, vortex, scissors, tray and comb, sprayer, microtube rack, label, and phone camera for genetic diversity analysis.

Methods

This study was done from February to July 2019 in the Baturraden subdistrict, Banyumas. Sampling was carried out at the Baturraden Botanical Garden (KRB), Kalipagu Hiking Trail (HPK 01) and Baturraden Hiking Trail (HPK 02) (Figure 2). Frog samples of *Rhacophorus margaritifer* (6 samples from KRB, 8 samples from HPK 01, 5 samples from HPK 02) were obtained using a Purposive Sampling Technique. Digiti web and flap tissues were cut and stored in absolute alcohol.

DNA extraction used the Chelex method (Walsh *et al.*, 1991). The materials used for extraction were 100 µL Chelex 5%, 5 µL DTT 0.1 M, 4 µL Proteinase K, and *R. margaritifer* tissue. The mixture of solution and tissue was incubated at 56°C 1,000 rpm for 4 hours followed by centrifugation of 13,000 rpm 2 min to separate DNA from other components. At the end, it was re-incubated at 95°C for 10 min to inactivate the Proteinase enzyme.

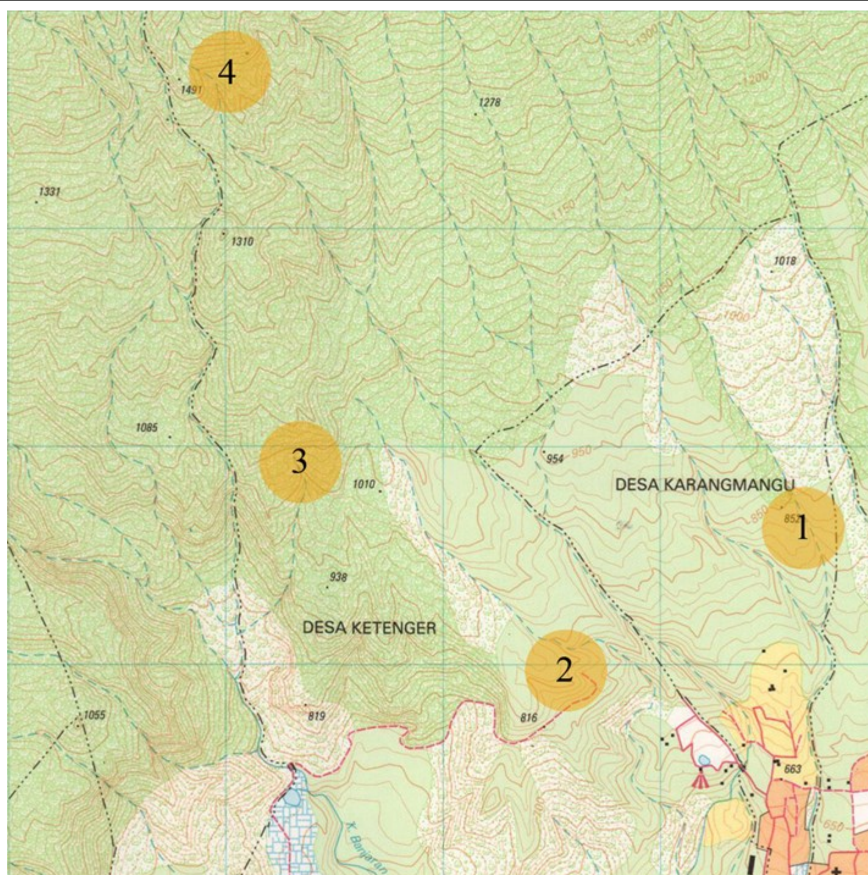

Figure 2. Sampling locations; 1. Baturraden Botanical Garden (KRB); 2. Pancuran Pitu; 3. Kalipagu Hiking Trail (HPK 01); 4. Baturrraden Hiking Trail (HPK02).

Diversity analysis was evaluated by RAPD markers amplification (Williams *et al.*, 1990). The PCR composition was 17,875 µL ddH2O, 2.5 µL reaction buffer 10X, 1.5 µL MgCl2 50 mM, 1 µL dNTPs 10 M, 1 µL RAPD Primer, 0.125 µL Taq Polymerase, and 1 µL DNA template. The PCR-RAPD program ran at Pre-Denaturation 95°C for 2 min, (Denaturation 95°C for 35 s, Annealing 39/41° C for 35/40 s, Extension 72°C for 1 min repeated 35 cycles), Final Extension at 72°C for 5 min, and storage at 8°C for 5 min. The primers used were selected from 26 primers to choose the 5 best primers, namely OPB 19, OPAH 04, GEN 23, OPA 08, and OPAH 02.

The PCR-RAPD results were evaluated with 2% agarose gel electrophoresis in TAE 1X solvent. The RAPD markers pattern were changed to binary data 01 based on the presence or absence of amplified loci. Intra-population analysis is based on gene and locus diversity while inter-population analysis is based on AMOVA (Analysis Molecular of Variance) in the Arlequin ver. software. 3.5 (Excoffier & Lischer, 2010).

RESULTS AND DISCUSSION

A total of 19 individuals of *R. margaritifer* individuals were obtained from 3 locations; 8 individuals from Kalipagu Hiking Trail (HPK 01), 5 individuals from Baturraden hiking trails (HPK 02), and 6 individuals from Baturraden Botanical Garden (KRB). The distance range among the three locations was 1.725 – 3.125 km. Snout-Vent Length (SVL) of the frog samples were ranged between 35 – 60 mm. The frog populations in three locations had noticeable morphological differences. Some individuals had white dots on the dorsal and vague patterns on the dorsal and hind limbs. Hoffman & Blouin (2000) stated that many anurans showed polymorphism of color and pattern on the dorsal part of the body.

Visualization of the DNA extractions showed that the DNA genomes of *R. margaritifer* were successfully extracted from the web tissues using the Chelex method. DNA genome of samples J6, J7, J8, J9, 10, and J11 were rather vague; it was assumed that the DNA was absorbed inside the gel prior the electrophoresis. Another possibility was the low quality of the DNA during four months of storage. DNA genome of the frog was shown as smears, which was DNA with similar length and indistinct. Nuryanto *et al.* (2012) stated that smears resulted from DNA fragmentation during tissue preservation and physical treatment during DNA extractions. However, DNA smears resulted from extraction with Chelex method can be used for RAPD marker amplification, as stated in the previous studies in *Penaeus monodon* (Prastowo *et al.*, 2009), *Polymesoda*

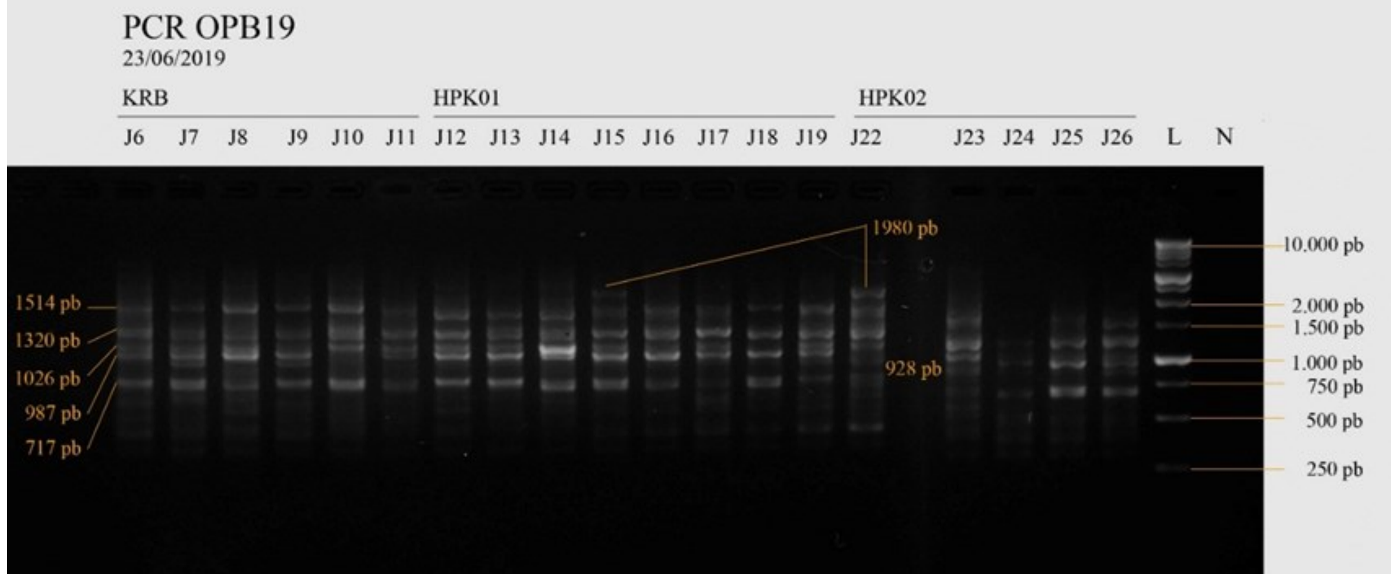

Figure 3. Visualization Results of PCR-RAPD using OPB 19 Primer on Three *Rhacophorus margaritifer* Populations. L. DNA Ladder; N. Negative Control; KRB. Baturraden Botanical Garden; HPK 01. Kalipagu Hiking Trail; HPK02. Baturraden Hiking Trail.

erosa (Nuryanto & Susanto, 2010), catfish (Nuryanto *et al.*, 2012), and *Osphronemus goramy* (Khairunisa, 2015).

Selection was done in total 26 RAPD primers, OPA 01, OPA 02, OPA 03, OPA 04, OPA 05, OPA 06, OPA 07, OPA 08, OPA 09, OPA 10, OPA 11, OPA 20, OPB 01, OPB 02, OPB 19, OPAC 14, OPAH 01, OPAH 02, OPAH 04, OPAH 08, OPAH 09, GEN 11, GEN 12, GEN 13, GEN 14, and GEN 23. Several extracted genomes, J5, J7, J8, and J9, were used as templates. J5 was a sample collection of previous studies in the Laboratory of Animal Taxonomy, Jenderal Soedirman University. Every primer used for PCR was equipped with a negative control. RAPD primers were selected based on the ability to amplifying the best RAPD bands.

PCR result of primer selections showed that despite different expressions of the bands, almost all primers could amplify the DNA sample of *R. margaritifer*, except GEN 13. Primers that gave the best results were OPAH 02, GEN 23, OPA 01, OPA 05, OPAH 04, OPA 08, OPB 19, GEN 14, and OPAH 08. This result indicated several complementary sites within *R. margaritifer* DNA genome with the RAPD sequences. RAPD primers attached to the different complementary sequences of DNA template and generated various bands. Different results of bands pattern on each primer referred to specific amplification of the DNA genome. RAPD primers were specifically amplified segments on DNA templates that had the complementary sequence with the primer. Every amplified segment was considered as locus. Locus was the result of primer amplification attached on opposite 3' end template. The number and size of the bands depended on the number of primer attachment sites along with the DNA template (Williams *et al.*, 1990; Kumar & Gurusubramanian, 2011).

Primer selection meant to select the primers that were capable to produce polymorphic, uncontaminated band patterns since the primers had been used eight to ten years. Evidence of contamination showed by the visualization of amplification product stained with ethidium bromide. If the negative control had shown amplified bands, then it had been contaminated. Contamination occurred on primers OPB 01, OPAC 14, OPAH 02, OPA 09, OPA 02, OPA 04, OPA 10, OPA 11, OPA 20, OPAH 09, GEN 11 and GEN 13. Contamination might be originated from the environment, reagent, or PCR operator (Hu, 2016). New reagents might be required if the reagent were contaminated. Best 5 amplified primers were randomly selected, namely OPB 19, OPAH 04, OPA 08, GEN 23 and OPAH 02. Primer OPAH 02 was selected despite the contamination since it was assumed as the result of environment and operator contamination.

There were 7 loci in the electrophoresis result of PCR-RAPD using OPB 19 primer (Figure 3). The lengths of the loci were 1980 bp, 1514 bp, 1320 bp, 1026 bp, 982 bp, 928 bp, and 717 bp. Locus allele 1980 bpb was present in sample J5 and J22, while locus allele 928 bp was present in sample J7, J9, J19, J22, J23, and J24. Locus 1320 bp and 717 bp had conserve alleles that were amplified in all samples. Meanwhile, locus allele 1026 bp was absent in sample J18, J25 and locus allele 982 bp was absent in sample J10, J22, J23.

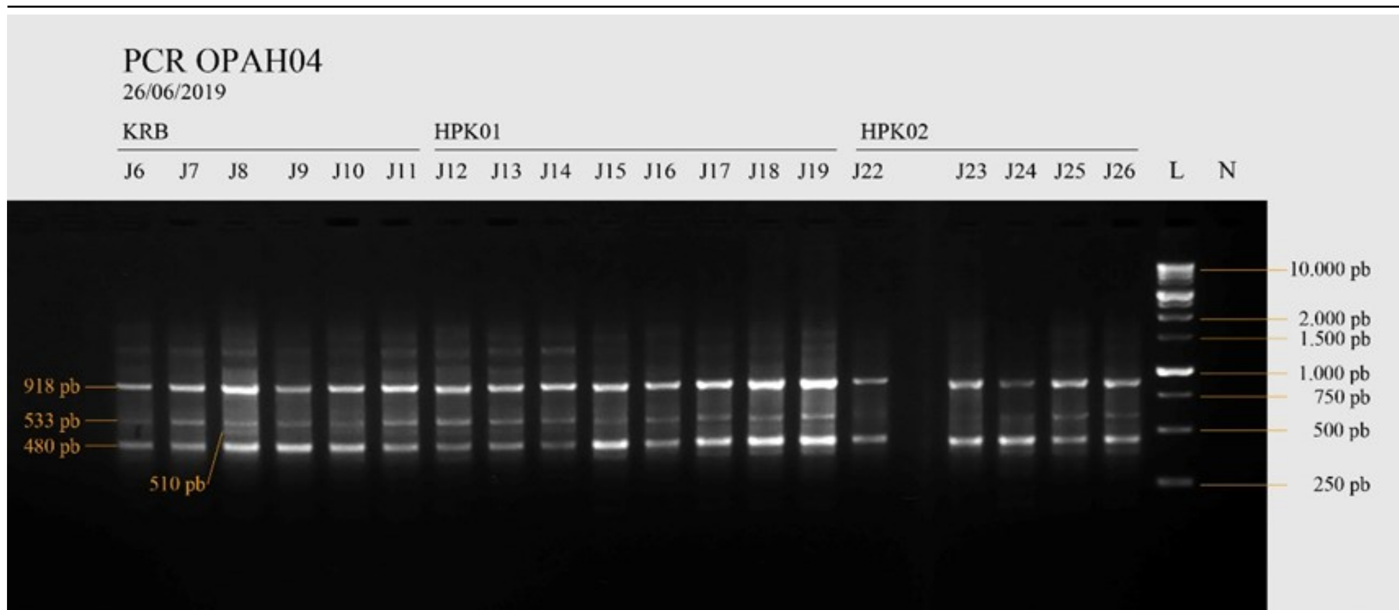

Figure 4. Visualization Results of PCR-RAPD using OPAH 04 Primer on Three *Rhacophorus margaritifer* Populations. L. DNA Ladder; N. Negative Control; KRB. Baturraden Botanical Garden; HPK01. Kalipagu Hiking Trail; HPK02. Baturraden Hiking Trail.

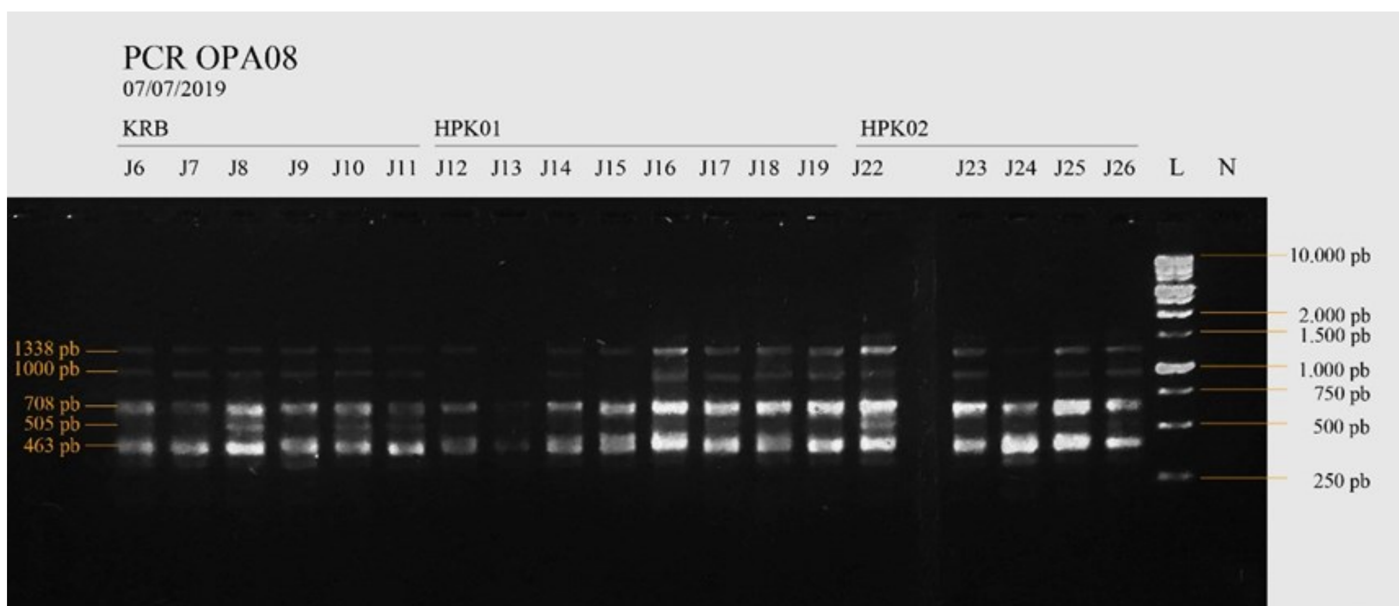

Figure 5. Visualization Results of PCR-RAPD using OPA 08 Primer on Three *Rhacophorus margaritifer* Populations. L. DNA Ladder; N. Negative Control; KRB. Baturraden Botanical Garden; HPK01. Kalipagu Hiking Trail; HPK02. Baturraden Hiking Trail.

There were 4 loci in the profile RAPD marker using OPAH 04 primer (Figure 4). The lengths of the loci were 918 bp, 533 bp, 510 bp, and 480 bp, respectively. The loci present in the result of PCR-RAPD using OPAH 04 had three conserve alleles in all samples.

There were 5 loci in the electrophoresis result of PCR-RAPD using OPA 08 primer (Figure 5). The lengths of the loci were 1338 bp, 1000 bp, 708 bp, 505 bp, and 463 bp. Loci 707 bp and 463 bp had conserved alleles that were present in all samples. Locus allele of 505 bp was present in samples J8, J10, J11, and J22. Locus allele of 1338 bp was absent in sample J13, while locus allele of 1013 bp was also absent in samples J12, J13, J15, and J24.

Six loci were present in the electrophoresis result of PCR-RAPD using GEN 23 primer (Figure 6). The lengths of the loci were 1077 bp, 1000 bp, 699 bp, 515 bp, 485 bp, 272 bp. Locus 1077 bp was absent in sample J24, while 515 bp was absent in samples J6, J16, J17, J18, and J23. Locus 699 bp was conserved locus present in all samples. Locus 1000 bp was present only in sample J23. Meanwhile, locus 485 bp and 272 bp were present in sample J12, J24, and J25.

Six loci were present in the electrophoresis result of PCR-RAPD using OPAH 02 primer (Figure 7). The lengths of the loci were 1468 bp,

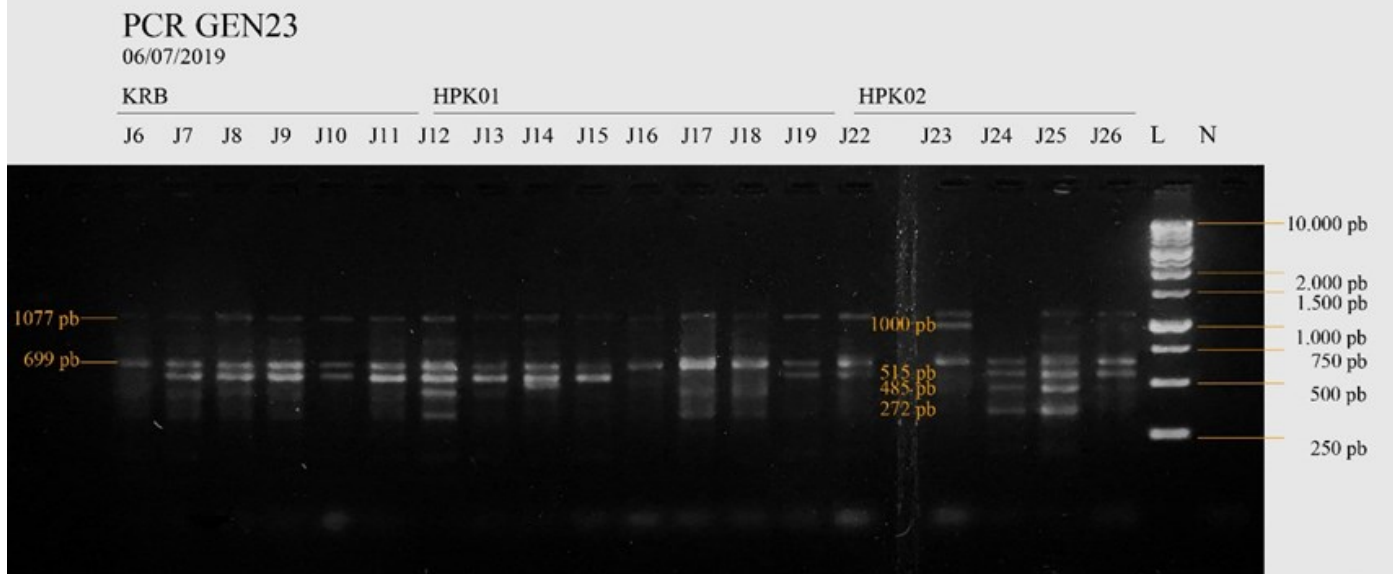

Figure 6. Visualization Results of PCR-RAPD using GEN 23 Primer on Three *Rhacophorus margaritifer* Populations. L. DNA Ladder; N. Negative Control; KRB. Baturraden Botanical Garden; HPK01. Kalipagu Hiking Trail; HPK02. Baturraden Hiking Trail.

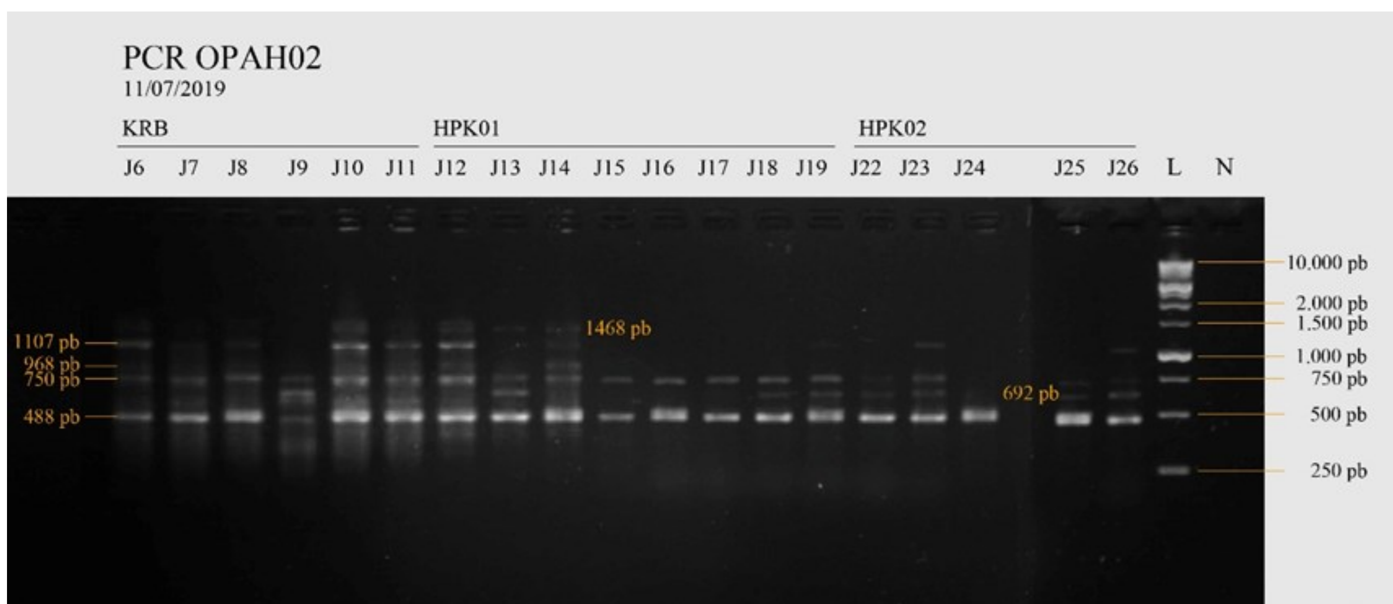

Figure 7. Visualization Results of PCR-RAPD using OPAH 02 Primer on Three *Rhacophorus margaritifer* Populations. L. DNA Ladder; N. Negative Control; KRB. Baturraden Botanical Garden; HPK01. Kalipagu Hiking Trail; HPK02. Baturraden Hiking Trail.

1107 bp, 750 bp, 692 bp, and 488 bp. Locus 1468 bp was present in samples J6, J8, J10, J12, J13, and J14. Locus 1107 bp was present in samples J6, J7, J8, J10, J12, J13, J14, J19, J23, and J26. Locus 968 bp was present in samples J6, J8, J12, and J14. Locus 692 bp was present in samples J9, J13, J18, J19, J22, J23, J25, and J26. Locus 750 bp and 499 bp were present in all samples. There was no band in the negative control of PCR-RAPD using all primers. This meant all bands of *R. margaritifer* DNA produced were specific and usable for analysis.

Amplification results of RAPD markers in part of the samples were smeared although clear bands still present. Smear on the PCR-RAPD result might be caused by Taq polymerase concentration and less optimal DNA template (Williams *et al.*, 1990). Visualised bands also had different contrast aside from smear. The differences of contrast in each sample indicated that extracted DNA genome had different concentrations.

Most of the amplified locus were polymorphic, with the most common allele frequency was more or less than 95%. However, monomorphic locus was also present in amplification result of every primer. This result indicated that those alleles were still [onserved within the DNA genome of *R. margaritifer.*

Table 1. Polymorphism in each primer

Primer	Band length (bp)	Total amplicon produced by individual	Total number of individual	Common allele frequency (%)	Average polymorphism in each primer
OPB 19	1980	2	19	89.47%	
	1514	18	19	94.74%	
	1320	19	19	100.00%	
	1026	17	19	89.47%	
	982	16	19	84.21%	
	928	6	19	68.42%	
	717	19	19	100.00%	91.58%
OPAH 04	918	19	19	100.00%	
	533	19	19	100.00%	
	510	1	19	94.74%	
	480	19	19	100.00%	98.68%
OPA 08	1352	18	19	94.74%	
	1013	15	19	78.95%	
	708	19	19	100.00%	
	505	4	19	78.95%	
	463	19	19	100.00%	90.53%
GEN 23	1077	18	19	94.74%	
	1000	1	19	94.74%	
	699	19	19	100.00%	
	515	14	19	73.68%	
	485	3	19	84.21%	
	272	3	19	84.21%	88.60%
OPAH 02	1468	6	19	68.42%	
	1107	9	19	52.63%	
	968	4	19	78.95%	
	750	18	19	94.74%	
	692	8	19	57.89%	
	488	19	19	100.00%	75.44%

The polymorphic allele was an indication of nitrogen base variation within the DNA genome of *R. margaritifer*. Polymorphic evaluation using OPB 19 and GEN 23 primers managed to point more polymorphic sites within HPK 01 and HPK 02 populations, while OPA 08, OPAH 02, and OPAH 04 primers were able to point polymorphic sites within KRB population. Polymorphic sites of each primer were present in different samples. This could be interpreted that the whole sample had various DNA sequences. Allele might be present and absent because of the differences in complementary sites of RAPD primers. The differences of complementary sites in each sample might be the result of mutation and recombination (Kumar & Gurusubramanian, 2011). Insertion, deletion, or substitution of nitrogen base might cause the primer fails to attach if the original complementary site changed. Mutation also caused new attachment site. Williams *et al.* (1990) stated that the change of one nitrogen base, a single change in primer or DNA template, might affect the primer attachment in DNA genome.

Intra populational analysis using Arlequin *ver.* 3.5 showed high genetic diversity in all populations. Gene diversity in KRB population was 1.0000 ±0.0962 with the average value of locus variation was 0.159524 ±0.105553. Genetic diversity in HPK 01 population was 0.9643 ±0.0772 with the average value of locus variation was 0.165816 ±0.103543. Meanwhile, genetic diversity in HPK 02 population was 1.0000 ±0.1265 with the average value of locus variation was 0.192857 ±0.130697. Although the genetic diversity of KRB population was higher than HPK 01, the average value of locus variation was higher in HPK 01 population than KRB. Sample of HPK 01 population had higher polymorphic sites than sample of KRB population that lead to this result. Meanwhile, the number of polymorphic locus of HPK 02 and HPK 01 were the same, despite higher locus variation in HPK 02. This was caused by the number of gene copy of HPK 01 was higher than haplotype. If genetic diversity value was almost 1 it meant that the population has higher genetic diversity (Nei, 1987). In this case, all three

Table 2. Inter and intra population genetic diversity of *R. margaritifer* in Baturraden

Population	Samples	Haplotype	Loci	Polymorphic Site	Genetic diversity	Locus diversity
KRB	6	6	28	10	1.0000	0.159524
HPK 01	8	7	28	12	0.9643	0.165816
HPK 02	5	5	28	12	1.0000	0.192857

P-value = 0.50244±0.01368

populations of *R. margaritifer* had high genetic diversity that enabled the species to be conserved. A species need to have genetic diversity to adapt with the environmental changes (Nuryanto & Susanto, 2010).

Analysis Molecular of Variance (AMOVA) indicated that there was no genetic difference among populations of *R. margaritifer* in Baturraden (P-value = 0.50244 ±0.01368). This result indicated that the *R. margaritifer* populations in Baturraden were not fragmented. It is could be due to that high gene flow was occurred among *R. margaritifer* populations in Baturraden since lack of prominent barriers among the populations. Short distance (less than 5 km) enabled the migration of individuals among populations and lead high gene flow to occur among populations. Previous studies had shown that geographical distance was significantly correlated with population structure (Silva *et al.*, 2007; Telles *et al.*, 2006).

CONCLUSION

High genetic diversity had been observed in all populations of *R. margaritifer* in Baturraden, namely 100% in KRB and HPK 02 populations and 96.43% in HPK 01 population. A high locus variation was also observed for all populations with a value of 15.9524% in KRB; 16.5816% in HPK 01; and 19.2857% in HPK 02. There was no genetic difference among populations of *R. margaritifer*.

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Research Article

Optimation of *Spirulina* sp. Growth in Walne Media with Variation of Urea and $NaHCO_3$ Supplements

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ABSTRACT
One alternative biofuel to substitute fossil fuels is bioethanol. Microalgae *Spirulina* sp. contains high carbohydrates, which has 17-25% potential to produce bioethanol. Urea and $NaHCO_3$ can be used as additional nutrients sources of nitrogen and carbon to *Spirulina* sp. cultivation. Deficiency of nitrogen causing the cell's enzymes change that shown through decreased lipid and chlorophyll synthesis. While deficiency of carbon can affect the growth rate. In this research, the growth rate of *Spirulina* sp. is analyzed using Optical Density (OD) method. The growth rate calculation is used to measure the growth of microalgae cells shown in the growth curve. This was a laboratory-scale method using CRD with 4 treatments and 5 replications namely treatment A addition of 0.36 g/500 ml urea without addition of $NaHCO_3$, treatment B addition of 0.043 g/500 ml $NaHCO_3$ without addition of urea, treatment C addition of 0.36 g/500 ml urea and 0.043 g/500 ml $NaHCO_3$, and control without addition of urea or $NaHCO_3$. The results indicated that addition of urea and $NaHCO_3$ didn't affect to OD and *Spirulina* sp. growth rate. The highest growth rate was treatment A with 0.00906/day of growth rate followed by treatment C which has 0.00865/day of growth rate. Treatment B and control treatment (K) showed a low growth rate. The maximum OD value obtained in treatment C was 0.674 cells/ml on the 10th day. This research can be used as the reference to larger scale of *Spirulina* sp. cultivation in the field of bioethanol production.

Keywords: Cultivation, *Spirulina* sp., growth rate, optical density (OD).

INTRODUCTION
As the time flies, biofuel is highly needed for transportation and industry. The fuel that is often used to fulfill human needs is fossil fuel that can't be renewed such as coal, natural gas, and petroleum. In fact, its availability in nature is decreasing. Meanwhile, using biofuel as alternative energy can replace the use of fossil fuel.

The alternative energy is produced from other resources that will not run out and sustainable if it is managed properly. One of the biofuels called bioethanol, made from fermented sugar liquid from carbohydrate source by using microorganisms. Bioethanol developed from wastes that still contains carbohydrate (Seftian *et al.* 2012). According to Dewi (2016), bioethanol can be produced from variety of plants such as corn, cassava, potatoes, sugar cane, sorghum, and algae.

Microalgae is a potential energy source in the future because it contains carbohydrates that can be processed into several types of compounds such as biodiesel, bioethanol, and methane (Melanie and Diini, 2015; Hadiyanto and Maulana, 2012). Microalgae is an unicellular organism. It has chlorophyll and utilizes the process of photosynthesis to produce biomass. Microalgae is widely used to produce food supplements because of its high protein. The advantages of microalgae compared to other organisms are able to produce biomass and energy supplies in a short time, only need a small area, can be grown on non-productive land, and high growth rate so it is easily to cultivate (Hadiyanto and Maulana, 2012).

Spirulina sp. is a type of microalgae with carbohydrates and high protein. According to Christwardana *et al.* (2012), *Spirulina* sp. contains about 17-25% of carbohydrate and 56-62% of

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protein. The high content of carbohydrate from this microalga cause *Spirulina* sp. potentially produce bioethanol as a renewable energy product. The advantages possess by *Spirulina* sp. from other types of microalgae are relatively fast to reproduce and easier to harvest because it has a large biomass size (Syaichurozzi and Jayanudin, 2016). Cultivation of *Spirulina* sp. can use sea water, fresh water, or brackish water. Cultivation requires appropriate culture media and contains nutrients for *Spirulina* sp. *Spirulina* sp. also requires additional nutrients both macro and micro nutrients for their survival. Those macro nutrients are N, P, C, H, O, Ca, Mg, Na, and K, while the micro nutrients are Fe, Mn, Cu, Zn, B, and cyanocobalamin (Sari *et al.*, 2012).

According to Widianingsih (2008), carbon (C) and nitrogen (N) are the most important elements for *Spirulina* sp. to growth. The carbon is used in respiration, an energy source, and raw material for the formation of additional cells. The lack of carbon in the growth media affects the growth rate. Nitrogen play a role in the formation of proteins and nucleic acid. Ambarwati *et al.* (2018), also say that nitrogen play a role to stimulate vegetative growth and increase cell number. The deficiency of nitrogen can cause the enzymes in cells change that shown through decreased lipid synthesis and chlorophyll synthesis (Juneja *et al.*, 2013). Urea and $NaHCO_3$ can be used as additional nutrients to fulfill the survival of *Spirulina* sp. Urea is a source of nitrogen (N) while $NaHCO_3$ is a source of carbon (C). Urea and $NaHCO_3$ are more economical and easier to obtain than other N and C sources.

The maximum density of *Spirulina* sp. can be seen through the measurement of OD (Optical Density) on growth media. OD value is proportional to the population density of microalgae. Density measurement used to analyze the growth rate of microalgae using OD method. According to Prayitno (2006), the calculation of growth rate used as a measure growth speed of microalgae cells. The results of OD measurements during this cultivation period can be shown through a microalgae curve. The growth curve used as a determinant when microalgae enter the highest density peak. The application of urea and $NaHCO_3$ are mixed in a *Spirulina* sp. medium at certain doses. One of the growth media of *Spirulina* sp. is Walne's media. According to Widianingsih (2008), Walne's media is a good culture media for *Spirulina* sp. growth. This research is to determine the effect of urea and $NaHCO_3$ supplies on OD and the growth rate of *Spirulina* sp., to find out the value of *Spirulina* sp.growth rate with urea and $NaHCO_3$ supplements, and to find out the maximum OD value of *Spirulina* sp.

MATERIALS AND METHODS

This was a laboratory scale method using Completely Randomized Design (CRD) with 4 treatments and 5 replications namely; treatment A addition of 0.36 g/500 ml urea without addition of $NaHCO_3$, treatment B addition of 0.043 g/500 ml $NaHCO_3$ without addition of urea, treatment C addition of 0.36 g/500 ml urea and 0.043 g/500 ml $NaHCO_3$, and control without addition of urea or $NaHCO_3$. Microalgae used in this study was *Spirulina* sp. from Balai Pengembangan Teknologi Perikanan Budidaya (BPTPB) Cangkringan, Yogyakarta. This study used Walne media as basic media. Cultivation was carried out for 10 days with batch culture method. The standardization of *Spirulina* sp. cells at the beginning of cultivation cannot be uniform, so the standardization in this study is the volume of inoculants and the volume of media. Optical density (OD) of *Spirulina* sp. measured every 24 hours for 10 days cultivation using Spectrophotometer UV-1800 Shimadzu wavelength 680 nm according to the research of Syaichurozzi and Jayanuddin (2016). The volume of sea water as a solvent of Walne media is 300 ml, volume inoculant 200 ml, Walne media, and vitamin B_{12} addition respectively 0.3 ml, pH 8.5, salinity 27‰, aeration, and lighting every day for 24 hours.

Spirulina sp. cultivation tools used 500 ml culture bottles, aerator, two 40-watt tube light lamps on each rack, and aeration hose arranged as in Figure 1. The cultivation tools were designed in a such way to adjust BPTPB Cangkringan laboratory conditions, which was the place to do the research.

Researchers used the formula presented by Hirata *et al.* as referred by Kawaroe *et al.* (2009); Kawaroe *et al.* (2015); and Syaichurozzi & Jayanudin (2016) which uses the same formula to calculate the growth rate as follows:

$$k = \frac{\log \frac{N_1}{N_0}}{T_1 - T_0} \times 3.22$$

Where:

k = growth rate(/day)
N_1 = microalgae density at time t
N_2 = microalgae density at time 0
3.22 = constant
T_1 = observation time at time t
T_0 = observation time at time 0

Data analysis was performed by quantitative descriptive methods and simple statistics by calculating the average value of Optical Density (OD) to get the growth rate and curve making. Data analysis technique used to obtain the most optimal nutrient addition for *Spirulina* sp. conducted

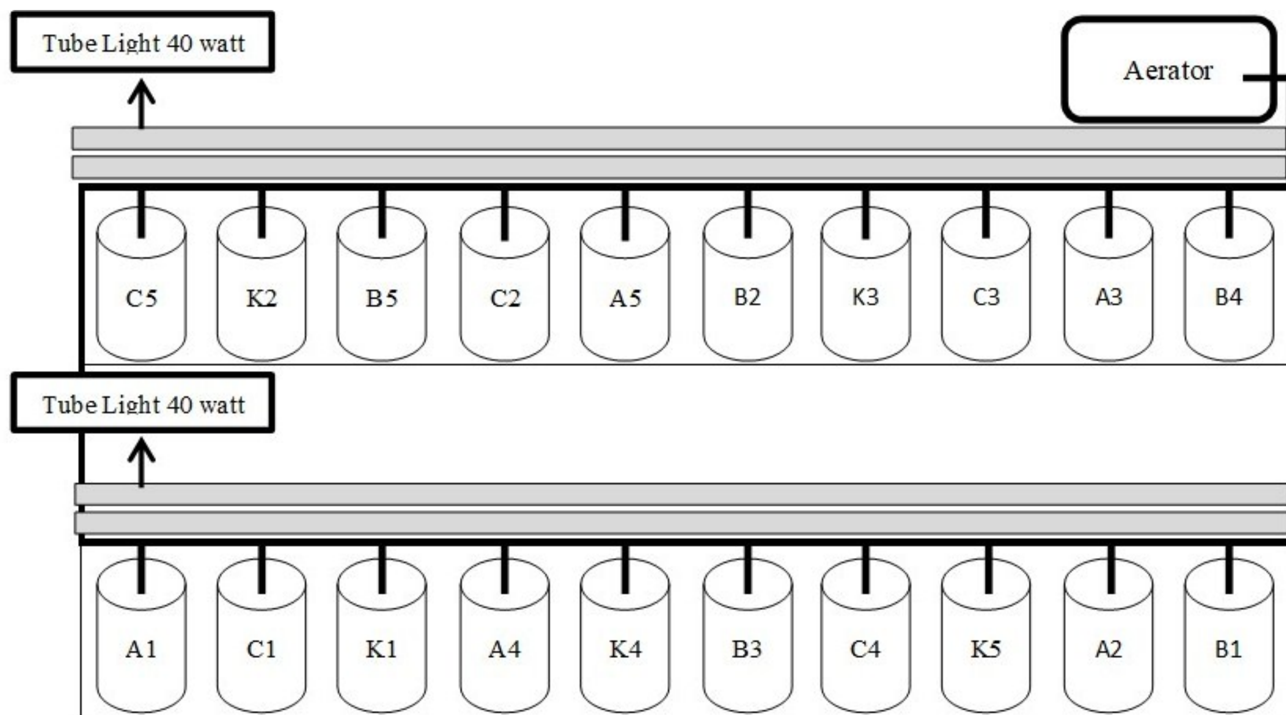

Figure 1. Design of cultivation tools.

quantitatively using the ANOVA test on IBM SPSS Statistics version 20. In this study, the X variable is the addition of urea and $NaHCO_3$, while the Y variable represents the Optical Density (OD) value. Before the ANOVA test, normality tests and homogeneity tests were carried out to ensure that the data are normal and homogeneous.

RESULTS AND DISCUSSION

The Density and Growth Pattern of *Spirulina* sp.

Density data were calculated starting from the 0th day of cultivation to the 10th day. Based on Figure 2, it's shown that the highest density value on treatment K (control) was on the 6th day which was 0.492 cells/ml, treatment A on the 10th day which was 0.672 cells/ml, treatment B on the 7th day which was 0.494 cells/ml, and treatment C on the 10th day which was 0.674 cells/ml. The density of *Spirulina* sp. at the beginning cannot be formed so that it has a different value in each treatment. However, differences in density are not very different from one another.

Increased density on day 0 to 1st day indicates that *Spirulina* sp. has adapted to new growth media. Suyono and Winarto (2006), mentioned that in the process of microalgae cell adaptation, they have utilized the nutrients in the media, although not yet optimal. This phase is called the lag phase (induction phase). Muyassaroh (2018) also stated that the growth of *Spirulina* sp. marked with a bluish-green color in the growth media, while a yellowish color in the growth media shows that *Spirulina* sp. has experienced a phase of death. The thicker bluish greencolor on the media shows that the growth of *Spirulina* sp. increased both in terms of size and number of cells.

Based on the growth curve from Figure 2, it can be seen that the growth of *Spirulina* sp. each treatment has a different pattern. The density of *Spirulina* sp. the treatment K (control) continued to increase since the first day of cultivation and experienced a peak on the 6th day, but decreased from the 7th day to the 10th day. The density of *Spirulina* sp. treatment A continued to increase from the 1st day of cultivation to the 5th day, then down to the 9th day, and increased again on the 10th day. The density of *Spirulina* sp. treatment B continued to increase since the 1st day of cultivation and experienced a peak on the 7th day, then decreased from the 8th to the 10th day, while the density of *Spirulina* sp. treatment C has increased since the 1st day of cultivation and continues to increase until the 10th day. In treatments A and C, the highest peak was on the 10th day but the possibility could still increase until an unknown day. *Spirulina* sp. culture will stay alive as long as the nutrients in the growth media are still available. Decreased density of *Spirulina* sp. in treatment A on the 6th day until the 9th day was caused by aeration on the A3 culture bottle died, thereby reducing the average value of density.

Table 1. Growth Rate of *Spirulina* sp. in Control and 3 Treatment.

Sample	Day										ẋ
	1	2	3	4	5	6	7	8	9	10	
K	0.02302	0.01650	0.01157	0.00943	0.00618	0.00356	-0.00043	-0.00214	-0.01058	-0.00941	0.00477
A	0.02501	0.02804	0.01045	0.01015	0.00566	-0.00042	-0.00049	-0.00017	0.00060	0.01183	0.00906
B	0.02904	0.00416	0.01852	0.01342	0.00382	0.00133	0.00192	-0.00510	-0.01156	-0.00736	0.00482
C	0.02518	0.02629	0.01328	0.00052	0.00727	0.00218	0.00343	0.00227	0.00422	0.00188	0.00865

Note: A = medium with 0.36 g/500 ml urea; B = medium with 0.043 g/500 ml $NaHCO_3$; C = medium with 0.36 g/500 ml urea and 0.043 g/500 ml $NaHCO_3$; K = control medium without addition of urea and $NaHCO_3$.

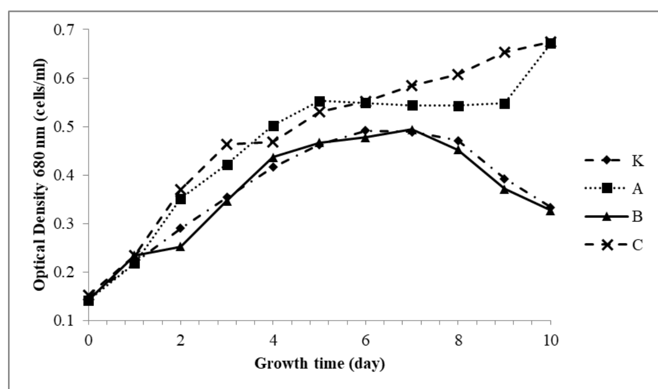

Figure 2. Growth pattern of *Spirulina* sp. based on average density of control and treated medium. A = medium with 0.36 g/500 ml urea ; B = medium with 0.043 g/500 ml $NaHCO_3$; C = medium with 0.36 g/500 ml urea and 0.043 g/500 ml $NaHCO_3$; K = control medium without addition of urea and $NaHCO_3$.

The data density of *Spirulina* sp. above showed that each treatment has a different standard deviation. The standard deviation on treatment A, B, C and control treatment (K) in a row were 0.161, 0.115, 0.168, and 0.114. The observations showed that treatments A and C were treated with the highest OD values compared to treatments K (control) and B. Treatment A and C contained the addition of urea nutrients as a source of N (nitrogen). The nitrogen content in urea added in treatments A and C has been shown to increase the growth of *Spirulina* sp. when compared with the control treatment (K). According to Ambarwati *et al.* (2018), the addition of N elements in the cultivation of microalgae with the right amount can optimally increase the population of microalgae. Rauf *et al.* (in Ambarwati *et al.*, 2018) state that in the growth of microalgae, N element plays a role in stimulating vegetative growth and increasing the number of microalgae cells. The increase in growth seen in the growth curve in treatments A and C proved an increase in the growth of *Spirulina* sp. until the last day of cultivation.

Based on the results of normality and homogeneity tests, data on *Spirulina* sp. are normal and homogeneous ($p > 0.05$). However, the results of the analysis using the ANOVA test showed a significant value is 0.132 ($p > 0.05$), so that further tests could not be carried out. These results indicate that the addition of nutrients in the form of urea and $NaHCO_3$ does not affect the density of *Spirulina* sp.

Growth Rate of *Spirulina* sp.

In this study, calculation of the growth rate (growth rate/day) is used to determine the rate of growth of *Spirulina* sp. per day. The results of the calculation of the growth rate are presented in Table 1.

Based on the *Spirulina* sp. growth rate data above showed that each treatment has a different standard deviation as same as the density. The standard deviation on treatment A, B, C, and control treatment (K) in a row that are 0.01041, 0.01241, 0.00971, and 0.01082. The average growth rates of *Spirulina* sp. in each treatment are presented in Figure 3.

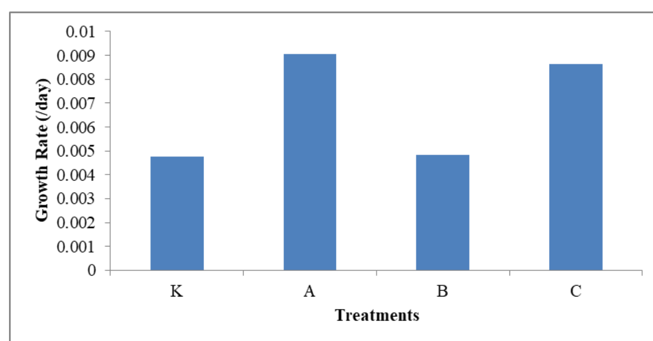

Figure 3. Comparison of the average growth rate of *Spirulina* sp. in control and treatments medium. A = medium with 0.36 g/500 ml urea; B = medium with 0.043 g/500 ml $NaHCO_3$; C = medium with 0.36 g/500 ml urea and 0.043 g/500 ml $NaHCO_3$; K = control medium without addition of urea and $NaHCO_3$.

Observation of growth rate (k) *Spirulina* sp. in each treatment has different results. The highest average growth rate of *Spirulina* sp. was in treatment A which was 0.00906/day followed by treatment C which was 0.00865/day, while the lowest average

growth rate of *Spirulina* sp. was in the control treatment (K) which was 0.00477/day followed by treatment B which was 0.00482/day. Based on the results of normality and homogeneity, growth rate data of *Spirulina* sp. normal and homogeneous (p> 0.05). However, the results of the ANOVA test showed a significant value is 0.707 (p> 0.05), so that further tests could not be carried out. Based on the ANOVA test results, the addition of nutrients such as urea and $NaHCO_3$ did not affect the growth rate of *Spirulina* sp.

Growth Phase of *Spirulina* sp.

Based on the growth pattern curve of *Spirulina* sp. in Figure 2, it can be analyzed the growth phases of *Spirulina* sp. which include:

Lag Phase / Induction Phase

According to Suyono and Winarto (2006), the lag phase is also referred to as the resting phase. On the growth pattern curve *Spirulina* sp. in Figure 3.2 the lag phase of each treatment occurred on day 0 and the 1st day. The density of *Spirulina* sp. in the control treatment (K) on day 0 was 0.147 cells/ml, treatment A was 0.142 cells/ml, treatment B was 0.143 cells/ ml, and treatment C was 0.153 cells/ml. The density of each treatment whichincreased on the 1st day of cultivation where the control treatment (K) reached 0.219 cells/ml, treatment A reached 0.218 cells/ml, treatment B reached 0.235 cells/ml and treatment C reached 0.235 cells/ml. Differences density in each treatments are control treatment (K) of 0.072 cells / ml, treatment A of 0.076 cells/ml, treatment B of 0.092 cells/ml, and treatment C of 0.082 cells/ml. The increase in density on day 0 to the 1st day shows that the culture of *Spirulina* sp. has adapted to the media and its cultural environment. As according to Suyono and Winarto (2006), the lag time depends on cell viability, which is the possibility of cells to be able to live adjusting their environmental conditions. At a young inoculant (exponential phase), *Spirulina* sp. the possibility is still viable and adapts more quickly to the environment, whereas at the age of the older inoculants (stationary phase), the lag phase will last longer. *Spirulina* sp. culture which is used as an inoculant in this study is an inoculant with the age of 10 days which is likely to be in an exponential phase so that the lag phase only lasts for 1 day.

Log Phase

In this phase, the number of *Spirulina* sp. has increased rapidly. According to Suyono and Winarto (2006), this phase proves that microalgae cells have successfully adapted to the new growth media and utilize the nutrients contained in the media. Based on the growth pattern of *Spirulina* sp. in figure 3.2, the exponential phase of control treatment (K) lasts from the 1st day to the 5th day, treatment A takes place from the 1st day to the 5th day, treatment B takes place from the 1st to the 6th day, and treatment C lasts from the 1st day to the unknown day if cultivation time is continued.

Stationary Phase

In this phase, the growth of *Spirulina* sp. stationary or permanent. This shows that the growth rate of *Spirulina* sp. the same as the rate of death. Based on the growth pattern of *Spirulina* sp. in figure 3.2, the stationary phase of the control treatment (K) lasts from the 6th day to the 7th day, treatment A takes place from the 5th to the 9th day, treatment B takes place on the 7th day, and C treatment is thought to have not yet reached the stationary phase.

Death Phase

In this phase, the decreased growth of *Spirulina* sp. higher than the stationary phase, so the growth pattern tends to decrease. Based on the growth pattern of *Spirulina* sp. in figure 3.2, the death phase in the control treatment (K) lasted from the 8th day to the 10th day, in treatment A it was thought that it had not yet experienced a death phase because the cell density increased again on the 10th day, in treatment B the death phase lasted since the 8th day to the 10th day, and treatment C has not yet experienced a phase of death because cell density still continues to increase until the last day of cultivation.

Maximum OD of *Spirulina* sp.

In this study, the density of *Spirulina* sp. measured every 24 hours for 10 days of cultivation. This is done to get the maximum density (maximum OD) of each treatment. In the control treatment (K), the density of *Spirulina* sp. was on the 6th day with a value of 0.492 cells/ml. In treatment A, the density of *Spirulina* sp. the highest was on the 10th day with a value of 0.672 cells/ml. In treatment B, the highest density of *Spirulina* sp. was on the 7th day with a value of 0.494 cells/ml, while in treatment C the highest density of *Spirulina* sp. was on the 10th day with a value of 0.674 cells/ml. The difference in density is due to each treatment. The growth media is different which causes the nutrient content in the growth media is also different. Thus, this is in accordance with the statement of Widianingsih (2008), that differences in cell density are caused by differences in nutrient content in growth media.

CONCLUSION

The results indicated that addition of urea and NaHCO3 didn't affect to OD and *Spirulina* sp.

growth rate. OD illustrates population density meanwhile, the increasing OD indicates the growth of Spirulina sp. The growth of Spirulina sp. per unit of time is the growth rate. The highest growth rate was treatment A with addition 0.36 g/500 ml of urea without addition of $NaHCO_3$ supplements which had 0.00906/day, followed by treatment C with addition 0.36 g/500 ml of urea and 0.043 g/500 ml of $NaHCO_3$ supplements which had 0.00865/day. Treatment B with addition of 0.043 g/500 ml of $NaHCO_3$ without addition of urea and the control treatment showed a low growth rate, which were 0.00482/day and 0.00477/day. The maximum OD value obtained in treatment C with addition of 0.36 g/500 ml of urea and 0.043 g/500 ml of $NaHCO_3$ supplements was 0.674 cells/ml on the 10th day.

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Research Article

Effect of *Arthrospira maxima* and *Chlorella vulgaris* to Lipid Profile and Visceral Fat Index Alteration in Streptozotocin-Induced Hyperglycemia Rats

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ABSTRACT
Arthrospira maxima and *Chlorella vulgaris* contain protein, carbohydrates, antioxidants, omega-3 fatty acids, and many micronutrients. Those compounds have potency of antidiabetic and hypolipidemic activity. This study aimed to evaluate the effect of A. *maxima* and C. *vulgaris* powder administration on alteration of body weight, lipid profile, glucose levels, and visceral fat index of hyperglycemia rats. Twenty male rats were divided into 5 groups i.e. negative control (NC), hyperglycemia control (HC), metformin (M), A. *maxima* (AR), and C. *vulgaris* (CH). Body weight and visceral fat index were measured and calculated by semianalytic and analytical scales. Serum glucose levels were measured by Easy Touch GCU (Glucose, Cholesterol, Uric acid). Lipid profile levels were measured using the photometric enzymatic method. The results showed no differences in body weight between groups, except in AR group was found significantly decreased in body weight on the 20th day. Glucose serum, total cholesterol, HDL and triglyceride levels in microalgae treatment groups were not significantly different be compare to control group. LDL levels of D30 significantly different from D0, but neither between groups. The visceral fat index of a control group was higher compared to that of a microalgae group and significantly different. In conclusion, the administration of microalgae A. *maxima* and C. *vulgaris* for 30 days are effective to reduce visceral fat index but not effective to maintain body weight, glucose level, as well as not effective to improve lipid profile.

Keywords: *Arthrospira maxima, Chlorella vulgaris*, lipid profile, glucose serum level, visceral fat index

INTRODUCTION
In recent years, the prevalence of diabetes mellitus (DM) continues to increase and become a major health problem in all countries (Aizzat et al., 2010). Type 2 diabetes mellitus (T2DM) is the most common type of diabetes, around 90% total incidence of diabetes mellitus (Ekoe, 2019). T2DM develops through a combination of genes, and environmental factors arise as a manifestation of the hyperglycemia phenotype (Hupfeld & Olefsky, 2016). Hyperglycemia is one of the main symptoms of DM. Pharmaceutical factory products which most widely used to overcome the state of hyperglycemia are metformin (Hossain & Pervin, 2018).

Nowadays, the demand of microalgae is increasing in the pharmaceutical field (Udayan et al., 2017). Microalgae that are often used are *Arthrospira* and *Chlorella,* which contain important nutrients including carbohydrate, lipid, protein, nucleic acid, minerals (especially iron), γ-linolenic acid, and antioxidants (Udayan et al., 2017; Yousefi et al., 2019).

Several studies have assessed the potential cytotoxic effects of *Arthrospira* in vitro. There were no adverse effects in rats that were fed A. *maxima* up to 30% in food for three months (Bigagli et al., 2017). A meta-analysis conducted by Serban et al. (2016), showed a significant effect of supplementation with *Arthrospira* in reducing plasma concentrations of total cholesterol, LDL, triglycerides, and increasing HDL levels.

Chlorella's role is to prevent dyslipidemia in high-fat feed rats. The treatment for 8 weeks showed a decrease in total cholesterol, LDL, and triglyceride

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levels (Jong-Yuh & Mei-Fen, 2005). *Chlorella* consumption is useful in preventing the development of type 2 diabetes mellitus. The research conducted by Aizzat et al. (2010), showed that the administration of *Chlorella vulgaris* during 4 weeks in diabetic rats has no hypoglycemic effect but has a protective function against STZ-induced rats by reducing oxidative DNA damage and lipid peroxidation.

Measurement of serum cholesterol levels has a major function in the diagnosis and treatment of several diseases such as cardiovascular disease, hypothyroidism, and diabetes (Narwal et al. 2019). In some studies, there is a relationship between lipid profile levels and type 2 diabetes (Pantoja-Torres et al*.,* 2019). Therefore, this study aimed to evaluate the effect of A. *maxima* and C. *vulgaris* administration for 30 days to serum lipid profile, visceral fat index, glucose serum level, and body weight of hyperglycemia rats induced by single low-dose of streptozotocin.

MATERIALS AND METHODS

Microalgae *A. maxima* and *C. vulgaris* powder were obtained from Blue-Green Algae Biotechnology. Male Wistar rats at 15 weeks old, 200-300 gram body weight from Integrated Laboratory Research and Testing (Laboratorium Penelitian dan Pengujian Terpadu, LPPT) UGM. This research was approved by UGM Animal Ethics Commission with certificate number 00167/04/LPPT/1/2018.

Experimental Design

The animal models were maintained in middle photoperiode (12L-12D cycles) and room temperature. The rats routine feed and drinking with Reverse Osmosis (RO) water ad libitum. The twenty male rats were randomly divided into five groups with 4 replicants. Every group of animals were taken care of in a communal cage and acclimated for a week. A group of rats for normal control (NC). Four other groups were induced hyperglycemia using single low-dose of STZ was 30 mg/kg b.w. in 0.1 M citrate buffer pH 4.5 intraperitoneally. After that, without any other treatments for hyperglycemia control (HC) group. Metformin (M) group treated with metformin 10 mg/kg b.w. as medical treatment. AR group treated with *A. maxima* 2,500 mg/kg b.w. CH group treated with *Chlorella vulgaris* 2,500 mg/kg b.w. Blood glucose levels were measured three days and seven days after induction. This level of glucose later was marked as day 0 (0th). Metformin and microalgae were given orally once a day at 3.00 p.m. - 4.00 p.m. The treatment lasts for 30 days.

Body weight and Visceral Fat Index

Body weight was measured every 10 days. At the end of the experiment, the rats were anesthetized briefly before sacrificed. The adipose tissues were collected and measured its weight to obtained visceral fat index. Visceral fat index was calculated using the formula below:

$$\frac{weight\ of\ visceral\ fat}{body\ weight - weight\ of\ visceral\ fat} \times 100\% \quad (1)$$

Biochemical Analysis

Serum glucose level and serum lipid profile were measured at the start, 15th, and 30th days. Blood samples were collected from the supra-orbital sinus of rats after 8 hours fast. Serum glucose level measured using Easy Touch GCU (Glucose, Cholesterol, Uric acid). Lipid profile consists of total cholesterol, HDL-C, LDL-C, and triglycerides. Total cholesterol was measured using enzymatic photometric CHOD-PAP method. While HDL-C, LDL-C, and TG were measured using enzymatic photometric GPO method.

Statistical Analysis

Body weight, blood glucose level, and lipid profile data were analyzed using One way ANOVA, followed by Duncan test at a significance level of 5%

RESULTS AND DISCUSSION

The results of this research are body weight, serum glucose level, lipid profile, and visceral index shown at the figures and tables below. The age of rats used was 15 weeks with high carbohydrate diet in the previous research period (Hartantyo et al*.*, 2018). Hartantyo et al. (2018) showed that high carbohydrate diet caused metabolic disorders such as obesity and hyperglycemia. In obese animals, insulin resistance can increase with age (King & Austin, 2017). Microalgae used in the study were *A. maxima* and *C. vulgaris*. Some nutrients in A. *maxima* and C. *vulgaris* are omega 3, omega 6, linoleic acid, EPA, DHA, tannins, saponins, Zn, and Fe (Widiyanto et al., 2018). There were no adverse effects or negative effects during the treatment. All of the test animals were kept alive during the treatment.

Body Weight

Body weight of the test animal measurements were used as a parameter for growth. The body weight range of rats was 200-300 grams to minimize mortality (Mu'allimah, 2017). The body weight rats of all groups continued to increase until the 30th day. Body weight gain was linear with age (Stevani, 2017). Fluctuations showed in body weight gain or decrease during microalgae and metformin intervention

Table 1. Serum glucose level of hyperglycemia Wistar male rats (*Rattus norvegicus* Berkenhout,1769) treated with A. *maxima* and C. *vulgaris* on days (-7), 0, 15, and 30 of treatments

Group	Serum Glucose Level (mg/dL)				Δ (D30-D0) (%)
	D(-7)	D0	D15	D30	
NC	81.25 ± 12.69^{a,x}	**87.00 ± 4.08^{a,x}**	105.50 ± 10.60^{a,y}	95.75 ± 8.77^{a,xy}	10.06 ± 10.88
HC	**249.50 ± 99.95^{c,x}**	295.75 ± 55.09^{b,x}	183.75 ± 104.98^{a,x}	185.75 ± 75.26^{a,x}	-37.19 ± 17.03
M	237.25 ± 105.75^{bc,x}	292.00 ± 134.32^{b,x}	192.75± 82.10^{a,x}	223.25 ± 127.06^{a,x}	-23.54 ± 39.92
AR	189.50 ± 14.39^{bc,x}	210.25 ± 79.19^{ab,x}	175.25 ± 62.95^{a,x}	187.25 ± 110.62^{a,x}	-10.94 ± 107.28
CH	139.25 ± 33.46^{ab,x}	277.25 ± 185.48^{b,x}	142.25 ± 62.89^{a,x}	152.75 ± 68.16^{a,x}	-44.91 ± 33.88

Note : ^{a,b,c} = differences between days, ^{x,y,z} = differences between groups, (-) = decreasing of levels, bold : significant differences between groups or days, NC : negative control, HC : hyperglycemia control, M : metformin, AR : A. *maxima*, and CH : C. *vulgaris*

(Figure 1.) However, there were no significant differences between the treatment groups at Δ1 and Δ3. Whereas at Δ2, the *Arthrospira maxima* group had a significant decrease in weight. Δ : the difference between two data; Δ1: body weight difference between D0 and D10; Δ2: body weight difference between D10 and H20; Δ3: body weight difference between D20 and D30.

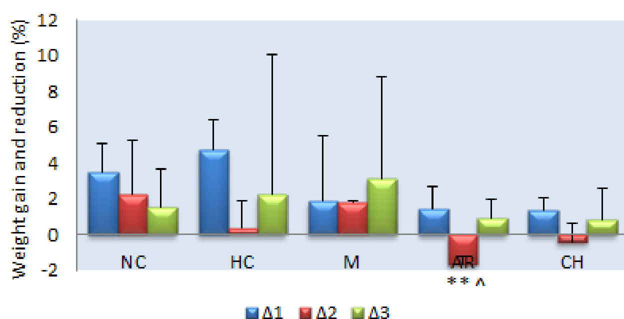

Figure 1. Body weight of hyperglycemia Wistar male rats (*Rattus norvegicus* Berkenhout, 1769) treated with A. *maxima* and C. *vulgaris* for 30 days; Δ1: BW difference between D0 and D10; Δ2: BW difference between D10 and H20; Δ3: BW difference between D20 and D30; ** = significantly different between groups, ^ = significantly different between deltas. NC: negative control, HC: hyperglycemia control, M: metformin, AR: A. *maxima*, and CH: C. *vulgaris*

The smallest or lowest weight gain was at Δ2. This is related to high serum glucose levels in D10 to D20 (day 10 and day 20 of treatment) and rats undergoing early diabetes. So that the increase of body weight was only slightly due to the limitations of cells utilizing glucose to be converted into energy. At the end of the treatment, blood glucose levels had improved so that the body weight began to increase again even though the increase in body weight was lower than at the beginning of the treatment. Body weight reductions of 1.70 ± 0.48% and 0.45 ± 1.11% in the AR and CH groups, respectively, can be caused by the saponin in both microalgae. Although it can not be certain that saponin is the single cause. According to Stevani (2017), saponins can suppress and maintain body weight in the normal range by inhibiting the activity of the pancreatic lipase enzyme thereby preventing the accumulation of fat in the body.

Serum Glucose Levels

Streptozotocin (STZ) has been widely used to induce hyperglycemia in experimental animals by inhibit insulin secretion because it is analogous to glucose (Radenkovic et al., 2015). Pancreatic beta cells have a high concentration of glucose transporter 2 (GLUT2). STZ has the same structure with glucose, so it will absorbted by pancreatic beta cells (Papich, 2016).

In this study, induction of hyperglycemia was used single low-dose 30 mg/kg b.w. of STZ. Some previous studies used higher STZ dose variations to induce type 2 diabetes mellitus (Ghadge et al., 2016; Rouhi et al., 2017). Meanwhile, according to Radenkovic et al (2015), the most widely dose used to induce diabetes is 35-80 mg/kg b.w. intraperitoneally and 35 mg/kg b.w. intravenously. The recommended dose is 60 mg/kg b.w. intraperitoneally. Based on these recommendations, it can be assumed that the effect of a single low-dose STZ induction will only temporarily increase glucose levels.

The decreased of glucose levels in the HC group was happen because this is still the initial stage

of induction to diabetes, so that insulin resistance and impaired glucose tolerance can still return to normal glucose tolerance (Hupfeld & Olefsky, 2016). Whereas in the M and AR groups respectively decreased by 23.54 ± 39.92% and 10.94 ± 107.28% (Table 1.). The cause of decreased serum glucose levels in the microalgae group is explained as follows. According to Jong-Yuh & Mei-Fen (2005), *Chlorella* can reduce glucose levels by reducing the level of DNA damage and lipid peroxidation. Whereas according to Vo et al. (2015), the water-soluble *Arthrospira* fraction is effective in reducing serum glucose levels in a fasting state.

Lipid Profile

Lipid profiles are the main data for this research. Acording to Table 2. showed the normal levels for each of the Wistar rat lipid profile parameters (*Rattus norvegicus* Berkenhout,1769) for males (Stevani, 2017) and females (Karima & Mulyati, 2019). Normal levels showed as two normal line in the result's figures, that is upper and lower normal limits.

Table 2. Normal ranges of lipid profile parameters in male and female Wistar rats (*Rattus norvegicus* Berkenhout,1769)

Lipid Profile Parameters	Normal range of lipid profile levels (mg/dL)	
	Karima & Mulyati, 2019	Stevani, 2017
Total cholesterol levels	75.86 – 82.00	53.70 – 73.60
HDL-C levels	59.08 – 64.60	14.90 – 30.90
LDL-C levels	53.72 – 57.92	18.70 – 36.90
Triglycerides levels	46.10 – 65.52	48.00 – 167.00

Total Cholesterol Levels

Cholesterol is part of lipids, with the main structure of steroids. Cholesterol is the basic structure for the synthesis of steroid hormones such as estradiol, progesterone, testosterone, and cortisol. Cholesterol levels in the blood are closely related to the dynamics of steroid hormone levels. It is also related to the individual reproduction phase and stress conditions. Cholesterol levels in the blood of test rats with the treatment of *A. maxima* and *C.vulgaris* are presented in the Table 3.

Based on Table 3., total cholesterol level of the microalgae group was higher than the HC and M groups, and almost the same as the NC group at the end of the treatment (D30). Total cholesterol levels of the HC group increased in D0. This is linear to statement of Rouhi et al. (2017) that the state of diabetes can increase total cholesterol levels. An increase of total cholesterol levels in hyperglycemia individuals due to the state of insulin resistance can reduce the level of cholesterol absorption (Andrade et al., 2019). Even the CH groups in D15 and D30 were slightly higher than normal levels of total cholesterol (Stevani, 2017). NC group levels also exceed normal levels at D0 and D30. However, when viewed from D15, the total cholesterol level in the CH group was lower at D30. The increase and decrease are not significantly difference.

Cholesterol levels of negative control and microalgae group higher than the hyperglycemia control group caused by high cholesterol synthesis and absorption (Andrade et al., 2019), as well as the accumulation of cholesterol metabolism in the body (Stevani, 2017). An increase in cholesterol levels also caused by an increase in LDL levels. This is related to the function of LDL as a cholesterol transporter from the liver to the peripheral cells. Therefore, factors that influence total LDL concentrations will affect total cholesterol. So, an increase in LDL levels is proportional to an increase in total cholesterol levels (Arifah, 2006).

HDL-C levels

At D30, all groups except AR group showed an increase in HDL levels. NC and CH groups had HDL levels exceeding the normal range. The group with the highest HDL levels was *Chlorella* group, followed by the negative control, *Arthrospira*, hyperglycemia control, and metformin group (Table 4.).

According to Tabel 4. when compared between the two microalgae groups, the *Chlorella vulgaris* group (CH group) has higher HDL-C levels than AR group. The omega 3 content may be higher in *Chlorella* than *Arthrospira*. The results are consistent with the statement of Harris & Jacobson (2009), that omega 3 has a minor effect on HDL however, omega 3 can increase HDL levels, although not significantly. Mechanisms that possible to increase HDL levels are HDL receives cholesterol from peripheral cells and takes it to the liver for bile production (Wickramasinghe & Weaver, 2018). HDL has two mechanisms in transporting cholesterol esters to the liver i.e. direct and indirect reverse transport. Reverse transportation is indirectly mediated by Cholesterol Esters Transfer Protein (CETP) (Jim, 2013). According to Purnomo (2014), omega-3 can reduce CETP activity so that the transfer of ester cholesterol from HDL to VLDL, IDL, and LDL also decreases. In addition, according to Riggs and Rohatgi (2019), Apo A1 is the main protein constituent of HDL particles, mediating reverse cholesterol transport. Then decrease the concentration of VLDL and apo B due to the fall of

CETP which is influenced by omega-3. This result has implications for the secretion of apo B and VLDL which slows down into circulation or faster VLDL catabolism in the liver (Ryu et al., 2014). So that HDL levels increase.

LDL-C Levels

LDL lipoprotein is commonly used as an indicator of health-related to the heart and blood vessels. LDL is a part of lipid in blood circulation that carries a lot of cholesterol and triglyceride components. The two components have a large proportion, consequently forming molecules that are also large. In blood circulation, this is often seen as a barrier to the rate of circulation. The measurements of LDL-C levels of this research at D0, D15, and D30 were showed in Table 5.

Based on Table 5., all groups have a significant difference increase in LDL-C levels when compared to D0. According to previous research, A. *maxima* and C. *vulgaris* have the effect of lowering LDL cholesterol levels (Jong-Yuh & Mei-Fen, 2005; Torres-Duran *et al.,* 2007; Karima & Mulyati, 2019). The results in this study are not in accordance with the study. However, based on a meta-analysis in patients with type 2 diabetes who were given an omega-3 diet, they have a significant difference increase of LDL cholesterol levels (Chaves et al., 2019).

LDL cholesterol increase caused by two things: an increase in the number of LDL particles or an increase in LDL particle size, with each particle carrying more cholesterol. Larger LDL particles are less atherogenic than smaller particles, the denser characteristics found in the hypertriglyceridemia state (Harris & Jacobson, 2009). This is in accordance with Purnomo (2014), which states that in the process of catabolism of VLDL to LDL, there are two types of LDL produced, large VLDL will produce small, dense, and atherogenic LDL. Whereas a small VLDL will produce large and non-atherogenic LDL. In Metformin groups, the difference in increasing LDL-C is the highest compared with the other groups. Metformin has an effect on reducing blood glucose levels. Through certain mechanisms, then blood glucose is converted to triglycerides so that levels gradually increase with time. In group Microalgae, there was a slight increase compared to control.

Triglyceride Levels

Triglycerides are part of a lipid with a structured form of glycerol and fatty acids. In general, high triglyceride levels also indicate an unhealthy circulation system. Associated with LDL-C levels, the possibility of triglycerides is moving dynamically on free TG with TG on LDL-cholesterol (LDL-C). The results of measurements of triglyceride levels at D0, D15, and D30 are shown in Table 6.

Triglyceride levels in the microalgae group were lower than the hyperglycemia group. Whereas in the metformin group the triglyceride levels were higher but also decreased in D30. This is caused by the ability of metformin to improve glucose levels. Increasing the utilization of peripheral glucose has the potential to reduce the production of fatty acids and triglycerides (Srinivasan et al., 2018). C. *vulgaris* can lower triglyceride levels better than A. *maxima* because the ratio of omega-6 / omega-3 to C. *vulgaris* is lower than A. *maxima* (Gonzalez-Periz et al., 2009; Zanwar et al., 2018)

Previous studies have revealed that *Arthrospira* and *Chlorella* can reduce triglyceride levels (Jong-Yuh & Mei-Fen, 2005; Ou et al., 2012; Karima & Mulyati, 2019). The decrease of triglyceride levels may be influenced by omega-3 content in both microalgae (Ghadge et al., 2016). *Chlorella* is a good source of dietary fiber that affects lymphatic cholesterol and triglyceride absorption by increasing intestinal viscosity, changing the composition of bile acid pools or producing fermented products in the intestine (Ryu et al., 2014).

A potential mechanism by which omega-3 fatty acids (FA) in both microalgae were affecting hepatic triglyceride (TG) metabolism. Feeding omega-3 FA in mice has been shown can inhibit lipogenesis and diacylglycerol acyltransferase (DGAT) activity, phosphatidic acid phosphohydrolase (PA), and hormone-sensitive lipase; and to stimulate oxidation, phospholipid synthesis, and degradation of apolipoprotein (apo) B. The result is a very low level of TG lipoprotein (VLDL) secretion. Nonesterified fatty acid serum (NEFA) also provides FA for TG synthesis (Harris & Jacobson, 2009).

Visceral Fat Index

The visceral fat index shows the amount of fat found in the abdominal cavity. The fat appears to envelop or cover the internal organs. These fats can indicate the presence of excess glucose in the blood that is in a chronic state, the glucose is then converted to glycogen and fat. The visceral fat index is often associated with metabolic syndrome, impaired lipid and glucose metabolism, and cardiovascular disease (Hameed & AbdulQahar, 2019). It is a good predictor of visceral adiposity associated with T2DM. Serves as an indicator of fat distribution and an indicator of adipose tissue function that can change due to insulin resistance (Babiker et al., 2018).

The visceral fat index of hyperglycemia group

Table 3. Total cholesterol level of hyperglycemia male Wistar rats (Rattus norvegicus Berkenhout, 1769) with A. maxima and C. vulgaris treatment on days 0, 15, and 30 of treatments.

Groups	Total Cholesterol Levels (mg/dL)			Δ (D30-D0) (%)
	D0	D15	D30	
NC	77.50 ± 12.34^{a,x}	68.68 ± 6.05^{a,x}	74.20 ± 9.50^{a,x}	-4.26 ± 5.62
HC	59.68 ± 10.17^{a,x}	69.43 ± 8.24^{a,x}	67.60 ± 11.09^{a,x}	13.27±12.97
M	58.98 ± 11.08^{a,x}	61.90 ± 14.05^{a,x}	67.03 ±16.40^{a,x}	13.65 ± 0.09
AR	67.90 ± 4.24^{a,x}	68.78 ±11.66^{a,x}	73.48 ± 21.08^{a,x}	8.22 ± 31.52
CH	70.35 ± 16.89^{a,x}	75.65 ± 16.72^{a,x}	74.78 ± 12.70^{a,x}	6.30 ± 21.49

Note : ^{a,b,c} = differences between days, ^{x,y,z} = differences between groups, (-) = decreasing of levels, bold : significant differences between groups or days, NC : negative control, HC : hyperglycemia control, M : metformin, AR : A. *maxima*, and CH : C. *vulgaris*

Table 4. HDL cholesterol levels of hyperglycemia male Wistar rats (Rattus norvegicus Berkenhout, 1769) with A. maxima and C. vulgaris treatment during 30 days.

Groups	HDL-C Levels (mg/dL)			Δ (D30-D0) (%)
	D0	D15	D30	
NC	34.18 ± 6.76^{ab,x}	36.83 ± 4.21^{a,x}	34.20 ± 4.54^{a,x}	0.06 ± 8.34
HC	29.30 ± 6.73^{a,x}	33.18 ± 3.22^{a,x}	30.18 ± 3.54^{a,x}	3.00 ± 22.28
M	26.60 ± 3.98^{a,x}	29.88 ± 4.38^{a,x}	30.05 ± 4.89^{a,x}	12.97 ± 18.43
AR	39.35 ± 5.56^{c,x}	32.93 ± 4.66^{a,x}	33.83 ± 6.54^{a,x}	-14.03 ± 28.31
CH	35.78 ± 7.20^{ab,x}	35.48 ±5.54^{a,x}	40.50 ± 13.15^{a,x}	13.19 ± 42.22

Note : ^{a,b,c} = differences between days, ^{x,y,z} = differences between groups, (-) = decreasing of levels, bold : significant differences between groups or days, NC : negative control, HC : hyperglycemia control, M : metformin, AR : A. *maxima*, and CH : C. *vulgaris*

Table 5. LDL-cholesterol levels of hyperglycemia male Wistar rats (*Rattus norvegicus* Berkenhout,1769) treated with A. *maxima* and C. *vulgaris* during 30 days

Groups	LDL-C Levels (mg/dL)			Δ (D30-D0) (%)
	D0	D15	D30	
NC	**14.65 ± 2.45^{a,x}**	22.80 ± 2.32^{a,y}	23.78 ± 3.47^{a,y}	62.32 ± 19.82
HC	**13.13 ± 3.83^{a,x}**	23.68 ± 4.91^{a,y}	21.55 ± 2.59^{a,y}	64.13 ± 58.42
M	**10.95 ± 3.00^{a,x}**	20.10 ± 0.16^{a,y}	23.58 ± 2.93^{a,y}	**115.34 ± 63.80**
AR	12.63 ± 2.21^{a,x}	21.05 ± 5.14^{a,x}	20.70 ± 9.87^{a,x}	63.90 ± 96.39
CH	**13.43 ± 5.14^{a,x}**	22.60 ± 5.30^{a,y}	22.65 ± 4.15^{a,y}	68.65 ± 78.60

Note : ^{a,b,c} = differences between days, ^{x,y,z} = differences between groups, bold : significant differences between groups or days, NC : negative control, HC : hyperglycemia control, M : metformin, AR : A. *maxima*, and CH : C. *vulgaris*

showed the lowest compared to other groups especially normal control or nomale group. Visceral fat index of all groups was lower than normal controls. All hyperglycemia treatment groups became less fatty. This may be due to the body cells experiencing glucose deficiency so that there is no conversion to glycogen or fat. The results of treatment with *A maxima* and *C. vulgaris* are better than hyperglycemia conditions. Furthermore, between the two kinds of microalgae, *A maxima* is better than *C vulgaris* (Figure 2.).

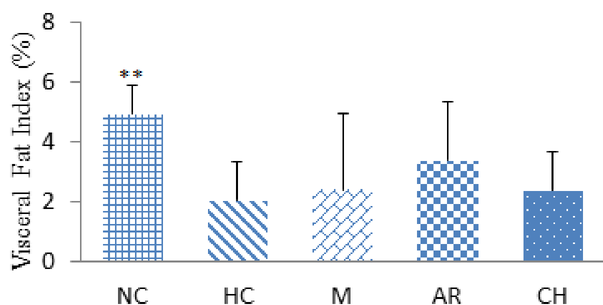

Figure 2. Visceral fat index of hyperglycemia male Wistar rats (*Rattus norvegicus* Berkenhout, 1769) treated with *A. maxima* and *C. vulgaris* after 30 days of treatment; sign (**) = significantly different between treatment groups. NC : negative control, HC : hyperglycemia control, M : metformin, AR : A. *maxima*, and CH : C. *vulgaris*

According to Eckel (2018), the main sites for the regulation of insulin and glucagon are liver, fat tissue, and muscle. The liver functions in the process of glycogenesis, glycogenolysis, gluconeogenesis, and ketogenesis. While fat cells function for lipolysis (Eckel, 2018). Because there is a disruption in energy production, alternative energy sources are used, namely the adipose tissue. So the adipose tissue mass in the HC group is low.

Increasing the utilization of peripheral glucose has the potential to reduce the production of fatty acids and triglycerides (Srinivasan et al., 2018). The results of this study indicate that the omega-3 content reduces triglyceride levels. When triglyceride levels go down, triglyceride stores in adipose tissue will also decrease. Therefore, the visceral fat index in the microalgae group was lower than the NC group but was already higher than the HC visceral fat index due to induction hyperglycemia using metformin and microalgae.

CONCLUSION

In conclusion, the administration of microalgae A. *maxima* and C. *vulgaris* were effective to reduce the visceral fat index but not effective to maintain body weight, glucose serum levels, as well as not effective to improve lipid profile.

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Research Article

RAPD Analysis for Genetic Variability Detection of Mutant Soybean (*Glycine max* (L.) Merr)

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ABSTRACT

This study aimed to detect and evaluate the genetic mutation from mutagenized soybean by RAPD markers. Soybean seeds of "Grobogan" variety were treated with two different concentrations of EMS (0.5% and 1%) and three incubation times (4, 6 and 8 h). DNA whole-genome was isolated from young leaf seedling with the Qiagen DNeasy Plant Mini Kit. Twenty OPA primers (OPA-1 to OPA-20) were used for DNA amplification. The results showed that EMS treatments successfully generated genetic variation in soybean, which indicated by high values of PIC, EMR, and MI. RAPD primers that effective to detect the mutation were OPA-2, OPA-07, OPA-10, OPA-11, OPA-12, OPA-13, OPA-14, OPA-15, OPA-16, OPA-18 and OPA-20. The band expression of those primers was exhibited a stronger intensity along with increasing of EMS concentration and incubation time used in this study. Treatment of 0.5% EMS in 6 hours incubation was successfully generated soybean mutants with the lowest genetic similarity compared to the wild-type. Thus, this study provides a new approach to generate genetic variability in soybean and has the potential to improve for soybean breeding program.

Keywords: ethyl methane sulphonate, genetic mutation, RAPD, soybean.

INTRODUCTION

Soybean is one of the major foods crops commodities and can be considered as the cheapest source of protein in Indonesia (Setiawan, 2013). The productivity of soybean in Indonesia was still low and less profit if compared to rice and corn. This may be due to the lack of development and attention of soybean seed industry, land optimization, pricing policy for locals, exporters, and importers in the market (Kristanti *et al.* 2017). Furthermore, more than 60% of Indonesia's soybean consumption is still needed to be imported during 2012 – 2016 (Pusdatin, 2015; Wulandari, 2016). Therefore, The Indonesian government in 2015 has optimized the soybean farmland to enhance domestic soybean production (Suherman, 2014). However, about 40% of the soybean farmland is considered as suboptimal and drylands (Balitbangtan, 2005; Wulandari, 2017).

Optimization of dry land for soybean farming has many challenges, especially the limited availability of soybean genotypes which tolerate the drought stress. Increasing the variability and adaptability of soybean by mutation breeding, and followed by selection may become one of the methods to solve the problem. Mutation breeding has been used in recent years as an alternative technique in generating new variability and development of crop species and varieties (Khan and Tyagi, 2013). One of the soybean varieties that have high yielding is Grobagan. Furthermore, Grobogan varieties have early maturity (76 days) and larger pod (Krisdiana, 2013)

Genetic mutations can be induced by chemical mutagenic agents using some mutation reagents (Oladosu *et al.* 2016) and physic mutagens by gamma rays, x-rays, and ultraviolet (Soeranto *et al.* 2001;

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Aisyah *et al.* 2005; Sreeju *et al.* 2011). Genetic mutations in soybean crops have been shown to increase the germination rate, viability, survival, yield, quality contributing characters such as pod setting, branching, protein, and oil content, also resistant to drought and diseases (Khan and Tyagi, 2013).

Ethyl Methane Sulfonate (EMS) is an alkylating chemical mutagen that has been shown effectively causes a high frequency of gene mutation. At low concentration, EMS generates G/C to C/G or G/C to T/A transversion, or A/T to G/C transition by pairing errors of 7-ethylguanine and 3-ethyladenine, respectively. Based on codon usage, EMS may also induce nonsense and missense mutations (Behera et al. 2012). If compared to other mutagens, genetic mutations induced by EMS were proven to be more effective than gamma rays in rice, lentil, mungbean (Singh et al. 2001), pea (Shah et al. 2009) and urad bean (Thilagavathi and Mullainathan, 2009). In addition, mutations using EMS in pea (Girija et al. 2013) and soybean (Khan and Tyagi, 2010) also caused mutations higher than the combination of gamma rays and EMS.

Recently, the effective detection of induced genetic mutations is still difficult to be measured. Many laboratories could not afford it since it is a very lengthy task, and due to spatial and technical limitations. Mutation detection in plants can be performed through phenotypic and genetic approaches. However, mutation detection using the phenotypic approach has many limitations and generated non-fixed character (Garcia et al. 2016). Therefore, the utilization of the genetic approach becomes a more reliable method and suitable for early mutation detection in the plant. Some genetic approaches which can be used for mutation detection are Random Amplification Polymorphic DNA (RAPD) (Ashraf et al. 2007), Restriction Fragment Length Polymorphism (RFLP), Inter-Simple Sequence Repeat (ISSR) (Mishra et al. 2014), DNA sequences (Wahyudi et al. 2013; Nikmah et al. 2016), etc.

RAPD based detection of genetic polymorphism is a molecular marker that is based upon a variation of the primer annealing sites in polymerase chain reaction/PCR. Primers were modified by even a single nucleotide which produces different banding patterns to detect genetic polymorphisms (Behera et al. 2012). It is a genetic marker which commonly used for mutation detection (Ashraf et al. 2007) and genetic variation study (Sundari et al. 2017; Kawar et al. 2009). RAPD marker has proven to be more reproducible than another genetic marker such as ISSR and RFLP; and no need to know the background of the genome being analyzed (Kumari and Thakur, 2014). The previous study proved that RAPD had been successfully used for mutation detection in rice (Ashraf et al. 2007), melon (Daryono et al. 2011), and sugarcane (Kawar et al. 2009).

In the present study, the seeds of soybean "Grobogan" variety were subjected to EMS-induced mutagenesis with combination treatments of different concentrations and incubation time to create variability. This study was aimed to detect and evaluate genetic mutation occurred in generated mutants seedlings of soybean, compared to the wild-type using RAPD marker. The finding of this study was expected to be useful in supporting the soybean breeding researches in Indonesia.

MATERIALS AND METHODS

Plant Material

Plant material used in this study was soybean "Grobogan" variety. It was obtained from Grobogan District, Central Java and released in 2008 by Grobogan District Local Government, Seed Control and Certification Service of Central Java and Central Java Province Local Government as superior soybean variety (Decreed 238/Kpts/SR.120/3/2008). The superiorities of soybean "Grobogan" variety is early matured (76 days), large seed size (about 18 g/100 seeds), and tolerant in fed rain dry land; with a potential yield up to 4.2 tonnes/ha (Rahajeng and Adie, 2013; Balitkabi, 2016; Balitbangtan, 2016).

Soybean mutation induction by EMS

A total of 40 Grobogan varieties soybean seeds were used in this study. About 30 soybean seeds were previously soaked in 40 ml of phosphate buffer 100 mM at 4 ℃ for 24 h. Then, it was added with new 40 ml phosphate buffer 100mM and treated with a combination of 2 variations of EMS concentration (0.5% and 1%) and 3 variations of incubation time (4, 6 and 8 h). The combination of treatments are as follows: 1) 5 seeds soaked with 0.5% EMS and incubated for 4 h; 2) 5 seeds soaked with 1% EMS and incubated for 4 h; 3) 5 seeds soaked with 0.5% EMS and incubated for 6 h; 4) 5 seeds were soaked with 1% EMS and incubated for 6 h; 5) 5 seeds soaked with 0.5% EMS and incubated for 8 h and 6) 5 seeds soaked with 1% EMS and incubated for 8 h. The 10 seeds left was soaked with aquadest for 24 hours as a control group.

After treatment, the EMS-induced mutant soybean seeds and control were sown on the germination tray. On 30th days after sown, each seedling was transplanted in a polybag containing growing media, i.e. sands, husks, and composts. The

Table 1. DNA sequences, GC content and annealing temperature of OPA primers used in this study

Primer	Sequences ('5-'3)	GC Content (%)	T_A (°C)
OPA-01	CAGGCCCTTC	70	36.4
OPA-02	TGCCGAGCTG	70	40.7
OPA-03	AGTCAGCCAC	60	35.0
OPA-04	AATCGGGCTG	60	35.1
OPA-05	AGGGGTCTTG	60	32.6
OPA-06	GGTCCCTGAC	70	35.2
OPA-07	GAAACGGGTG	60	33.2
OPA-08	GTGACGTAGG	60	31.1
OPA-09	GGGTAACGCC	70	37.4
OPA-10	GTGATCGCAG	60	33.1
OPA-11	CAATCGCCGT	60	36.7
OPA-12	TCGGCGATAG	60	34.0
OPA-13	CAGCACCCAC	70	37.7
OPA-14	TCTGTGCTGG	60	34.3
OPA-15	TTCCGAACCC	60	34.2
OPA-16	AGCCAGCGAA	60	38.3
OPA-17	GACCGCTTGT	60	35.7
OPA-18	AGGTGACCGT	60	36.2
OPA-19	CAAACGTCGG	60	34.2
OPA-20	GTTGCGATCC	60	33.5

young EMS-induced mutants and wild-type soybean leaf on 60 days after transplant were taken for DNA extraction and genetic test in Molecular Biology Laboratory of State Islamic University of Maulana Malik Ibrahim, Malang.

DNA extraction

About 0.1 g of young soybean leaf was used for DNA extraction. DNA whole genome was extracted with the Qiagen DNeasy Plant Mini Kit (Promega™) following its manual instruction. DNA yields were confirmed qualitatively using electrophoresis separation on 1% agarose gels, stained with 2 μg/ml of Ethidium bromide (EtBr) in TBE buffer, then visualized under UV transilluminator. Geneon 100bp Plus Blue DNA ladder was used as a standard marker.

PCR RAPD

Twenty primers of OPA (Operon Technologies) were used for RAPD amplification. Total reaction volume for PCR RAPD was 10 ml containing 3 ml ddH2O, 5 ml PCR MasterMix Thermo scientific, 1ml of OPA primer (10 pmol) and 1 ml DNA template (5-25 ng). The PCR amplification was carried out in thermal cycler BioRad. One cycle of initial denaturation was conducted at 94 ℃ for 5 min, then followed by 45 cycles of denaturation for 30 sec at 94 ℃, annealing temperatures were varied each primer for 30 sec (Table 1), the extension for 90 seconds at 72 ℃ and terminated by final extension for 7 min at 72 ℃.

Data analysis

The effectiveness of EMS to induce mutation in soybean seeds in this study was detected by polymorphism, clustering and distance method. The data were gathered based on the presence and the absence of amplification product bands, as shown on the agarose gel. It was scored as "1" for the present band and "0" for the absent band of each primer. The binary data matrix was then subjected to clustering and distance method using an unweighted pair group method with arithmetic mean (UPGMA) algorithm and Jaccard's coefficient similarity (Hammer et al. 2001) with PAST (Paleontological Statistics) software. The percentage of polymorphism was calculated by dividing the polymorphic bands shown by each primer with a total of scored bands x 100. The presence of the mutation was also checked with a qualitative method by comparing the band thickness of each primer.

Polymorphism information content (PIC), effective multiplex ratio (EMR) and marker index (MI), were also performed to evaluate the effectiveness of each primer used. PIC Value for each primer was calculated with the formula: $PIC_i = 2f_i (1-f_i)$, where PIC_i is the polymorphism information content of the primer i, f_i is the frequency of primer fragment that was present and $1-f_i$ is the frequency of primer fragment that was absent. EMR was calculated by using formula

Table 2. Analysis results of polymorphism and effectivity of RAPD primers used in this study

Primer	nB	nPB	PB (%)	PIC	EMR	MI
OPA-01	8	7	87.50	0.50	56	28.00
OPA-02	4	4	100.00	0.41	16	6.56
OPA-03	7	5	71.40	0.47	35	16.45
OPA-04	6	5	83.30	0.49	30	14.7
OPA-05	3	2	66.70	0.50	6	3.00
OPA-06	3	2	66.70	0.30	6	1.80
OPA-07	5	5	100.00	0.43	25	10.75
OPA-08	7	6	85.70	0.43	42	18.06
OPA-09	7	5	71.40	0.29	35	10.15
OPA-10	7	7	100.00	0.47	49	23.03
OPA-11	6	6	100.00	0.46	36	16.56
OPA-12	5	5	100.00	0.45	25	11.25
OPA-13	7	7	100.00	0.50	49	24.5
OPA-14	4	4	100.00	0.50	16	8.00
OPA-15	4	4	100.00	0.44	16	7.04
OPA-16	4	4	100.00	0.44	16	7.04
OPA-17	4	2	50.00	0.41	8	3.28
OPA-18	6	6	100.00	0.23	36	8.28
OPA-19	3	1	33.30	0.20	3	0.60
OPA-20	5	5	100.00	0.50	25	12.50
Total	**105**	**92**	**1716.07**	**8.42**	**530**	**231.55**
Average	**5.25**	**4.60**	**85.80**	**0.42**	**26.5**	**11.57**

Note: nB=Number of Band, nPB=Number of Polymorphic Band, PB%=percentage of Polymorphic Band, PIC=Polimorphic Information Content, EMR=Effective multiplex ratio, and MI=Marker index.

EMR= η.β, where η is the total number of fragments per primer and β is the fraction of polymorphic fragments (Laurentin and Karlovsky, 2007). MI was calculated with formula MI= PIC x EMR (Varshney et al. 2007). Pearson correlation test was also performed using SPSS 16.0 subjected to the values of PIC, EMR, and MI.

RESULTS AND DISCUSSION

RAPD Profiles

RAPD is a technique based on the PCR method to identify genetic variation. RAPD has been used as an assessment intraspecific variation since 1990 (Kumari and Takur, 2014). Moreover, in recent years, RAPD was also used for genetic mapping, taxonomic study and even used to detect genetic mutation in the treated plant (Dhakshanamoorthy et al. 2014). Here, we used RAPD markers to assess genetic diversity among EMS-induced soybean mutants. This study will be useful as basic information for further soybean breeding programs since it will make it easy to select where the soybean which has high genetic variation can be used for crossbreeding.

About twenty OPA primers were selected for RAPD amplification to detect polymorphism and genetic diversity among EMS soybean mutants. In total, about 105 bands were detected, in which 92 bands were considered polymorphic. Each primer generated bands ranged from 3 to 8 bands, with an average of 5.25 bands (Table 2). The minimum of 3 bands was resulted by OPA-5, OPA-6, and OPA-19 primers, whereas the maximum of 8 bands was generated by OPA-01 (Figure 1). Polymorphism percentages were ranged from 33.30% to 100%, with an average of 85.80%. OPA-19 showed the lowest polymorphism percentage; it only generated 1 polymorphic band out of 3 total bands (Table 2). Whilst, about 11 primers showed 100% polymorphic band percentage i.e. OPA-2, OPA-07, OPA-10, OPA-11, OPA-12, OPA-13, OPA-14, OPA-15, OPA-16, OPA-18 and OPA-20.

The values of PIC, EMR and MI can be used to identify what primer is the most informative to detect polymorphism. PIC values were varied from 0.20 to 0.50, with an average of 0.42. The lowest PIC value was obtained by OPA-19, whereas the highest PIC value was generated by OPA-01, OPA-05, OPA -13, OPA-14 and OPA-20 (Table 2). EMR values

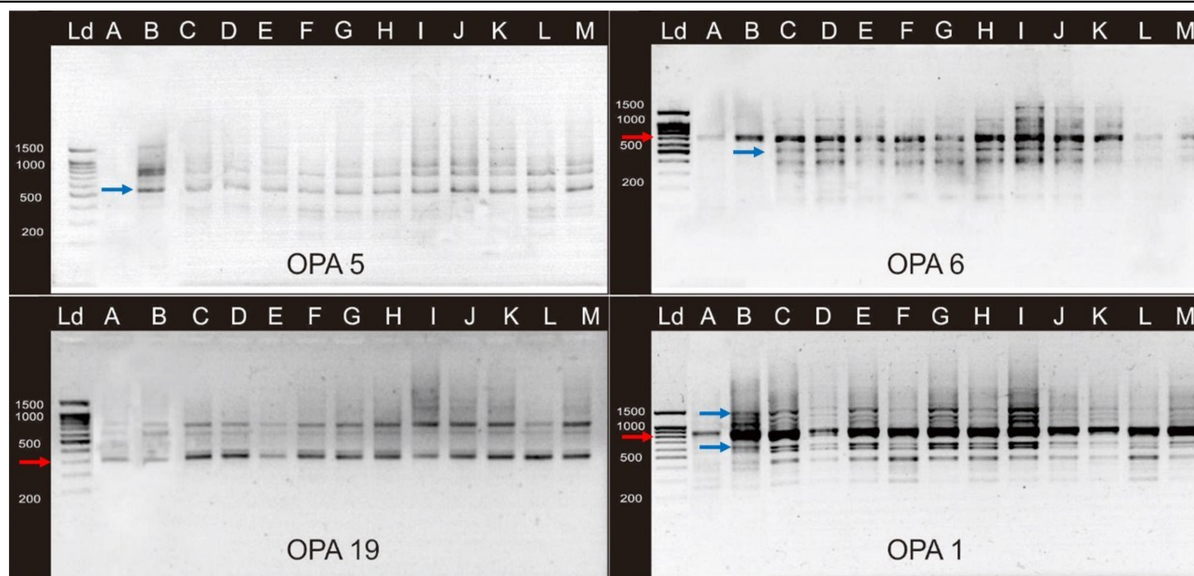

Figure 1. RAPD band profiles of the minimum bands (OPA 5, 6 and 19), a maximum band (OPA 1), the polymorphic band (blue arrow) and monomorphic band (red arrow). Ld: DNA Ladder, A & B: control/ wild-type, C & D: 0.5% EMS and 4 hours incubation, E & F: 1% EMS and 4 hours incubation, G & H: 0.5% EMS and 6 hours incubation, I & J: 1% EMS and 6 hours incubation, K & L: 0.5% EMS and 8 hours incubation, M: soaked with 1% EMS and incubated for 8 hours

were ranged from 3 to 56, with an average of 26.5. OPA-03 had the highest EMR value (56), whereas OPA-19 had the lowest EMR value (3). A similar tendency was also observed for the MI value. The MI values were ranged from 0.6 to 28, with an average of 11.57. OPA-01 was observed as the highest MI value, while OPA-19 was observed as the lowest MI value (Table 2).

Polymorphism information content (PIC) was measured to evaluate the discriminatory power of the RAPD marker. PIC is determined by the ability of the primer to generate polymorphism in the population depending on their distribution frequency (Nagy et al. 2012). Thus, PIC is equivalent to genetic diversity. The maximum number of PIC for RAPD marker is 0.5 because of two alleles per locus are assumed in RAPD analysis. In this study, we found that the average PIC value was 0.421 and about 80 % of the primers have PIC more than 0.4. This fact indicated that there was genetic diversity in soybean mutant in this study caused by EMS treatment. Furthermore, the number of EMR and MI also resulted in high values. It was also indicated that EMS treatments were successfully induced high genetic variation in soybean mutants. The result of this study is similar and comparable to some previous studies in terms of mutation detection using the RAPD marker in Jatropha curcas (Dhakshanamoorthy et al. 2014), Vitis vinifera (Maia et al. 2009) and Vigna unguiculata (Kolade et al. 2016).

Some RAPD primers used in this study has detected polymorphisms among soybean mutants and wild-type. The appearance of new bands (soybean mutants) or disappearance of bands in wild -type were observed (Figure 1). The thickening band, along with EMS concentration and incubation time were also observed (Figure 2). The appearance of new bands in soybean mutants may be related to the changes in oligonucleotide priming site due to mutation, deletion and homolog recombination (Dhakshanamoorthy et al. 2014). Furthermore, Atienzar et al. (2010) revealed that only 10 % mutation of one locus could emerge a new band. Therefore, in this study, EMS has successfully induced mutations on soybeans indicated by the appearance of new bands in soybean mutants assessed with RAPD markers.

The disappearance of regular bands was also detected by OPA 08 and OPA 10 primer (Figure 2). The disappearance of regular bands in soybean mutants may cause by DNA damage, modified bases, base oxidation, point mutation, and even chromosomal rearrangements induced by EMS (Dhakshanamoorthy et al. 2014). Taq polymerase can't bind DNA products due to some possible reasons including dissociation of the enzyme complex, polymerase blockage and disassociation of the enzyme complex, which may lead to the loss of the band (Atienzar and Jha, 2006). The appearance of new bands indicated the occurrence of mutations, whereas the disappearance of the band indicated the DNA damage (Dhakshanamoorthy et al. 2014). Both events were lead to genetic variations among soybean mutants compared to the wild-type.

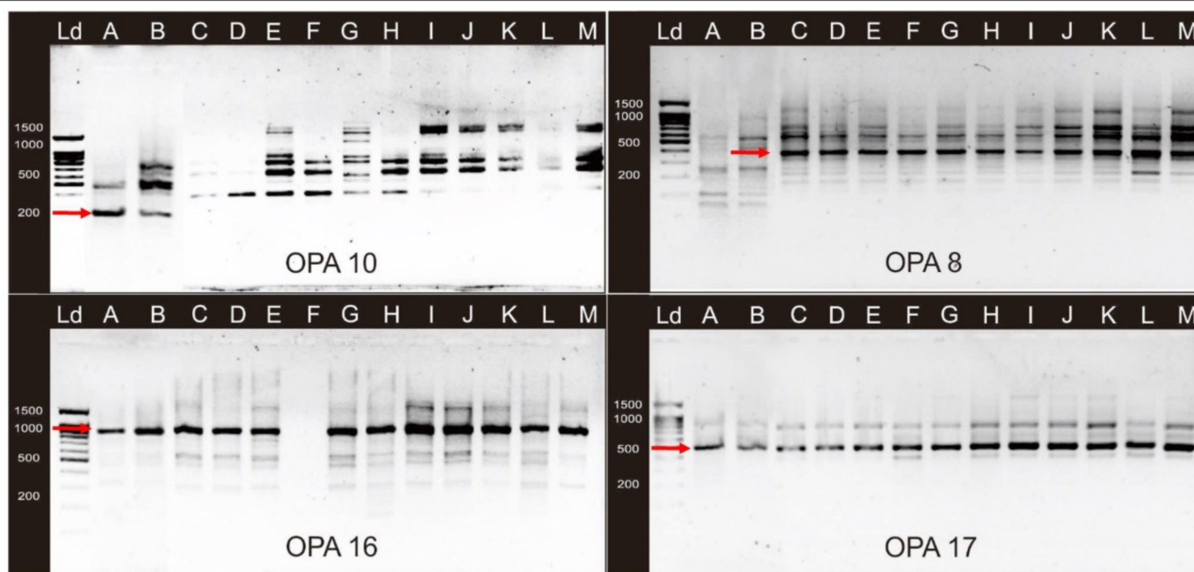

Figure 2. RAPD profiles of appearance of bands in soybean mutants (OPA 8), disappearance band in soybean mutant (OPA 10) and thickening bands along with the increasing of EMS concentration and incubation time (OPA 16 and 17). Ld: DNA Ladder, A & B: control/ wild-type, C & D: 0.5% EMS and 4 hours incubation, E & F: 1% EMS and 4 hours incubation, G & H: 0.5% EMS and 6 hours incubation, I & J: 1% EMS and 6 hours incubation, K & L: 0.5% EMS and 8 hours incubation, M: 1% EMS and 8 hours incubation.

Pearson correlation tests were performed to determine the relationships between pairs of continuous variables of PIC with EMR, PCI with MI, and EMR with MI. Result showed that there was a positive correlation between PIC with EMR (r = 0.308) but not significant (p= 0,187> 0,05). Whilst, PIC and MI had positive and significant correlation (r = 0,529; p= 0.016 <0.05). A positive and significant correlation (r= 0.948; p = 0,000 <0.05) were also found between EMR and MI.

In this study, we found a significant positive correlation between EMR and MI (r=0.948, p=0.05). It suggested that EMR, MI, and PIC are relevant to be used as markers for evaluation of genetic mapping and phylogenetic study (Dhakshanamoorthy et al. 2014). In addition, this study has also proved that RAPD marker is suitable for detection of genetic diversity in a breeding population (Biswas et al. 2010), as well as for mutation detection in plants (Dhakshanamoorthy et al. 2014; Kolade et al. 2016; Maia et al. 2009).

Qualitative tests based on band thickness showed that the band expression of some primers has resulted in stronger intensity with the increasing of EMS concentration and incubation time (Figure 2). The thickening bands were observed at 900 bp of OPA-16, and OPA-17 at 500 bp and 900 bp. Furthermore, the disappearance of the band in soybean mutants was observed by OPA 10 at 300bp and OPA 08 at 200bp (Figure 2).

Cluster analysis result

The genetic similarity among EMS-induced soybean based on RAPD marker was ranged from 0.23 to 0.84 (Table 3). All of the soybean mutants were observed to have low similarity with the wild-type. Meanwhile, it has high similarity amongst mutants. The lowest genetic similarity was observed at a pair of wild-type (A) and soybean mutants of 0.5% EMS and 6 hours incubation (H), whereas, the highest genetic similarity was observed at a pair of soybean mutants of 1 % EMS and 6 hours incubation (J) and soybean mutants of 0.5% EMS and 8 hours incubation (K).

Clustering analysis was conducted to 11 EMS-induced soybean mutants and control/ wild-type, performed using Jaccard's algorithm. One soybean mutant (1 % EMS and 8 hours incubation) was not included in the clustering analysis because the seeds cannot grow caused by the toxicity of EMS. This condition was in accordance with the result of Li et al. (2017) that a high concentration of EMS showed reduced emergence and physiological damage, including inhibition of the main stem even failed to grow. The dendrogram of genetic relationship resulted in 2 major groups (Figure 3). The wild-type soybean became the first group, which act like an outgroup. The second group was consist of all soybean mutants, which can be separated into 3 subgroups based on EMS concentration and incubation time. The subgroup 1 was comprised of soybean mutants generated by 0.5 % EMS and 1 % EMS and incubated for 4 hours and 6 hours incubation. The subgroup 2 was consists of soybean mutants generated by 0.5 % EMS and 1% EMS for 6 hours and 8 hours incubation. The subgroup 3 was

Table 3. Jaccard's coefficient similarity among EMS induced soybean mutants and wild-type

	A	B	C	D	E	F	G	H	I	J	K	L	M
A	-												
B	0.63	-											
C	0.25	0.39	-										
D	0.31	0.42	0.77	-									
E	0.29	0.42	0.75	0.76	-								
F	0.32	0.37	0.67	0.69	0.74	-							
G	0.27	0.42	0.65	0.65	0.74	0.67	-						
H	0.23	0.36	0.76	0.77	0.73	0.70	0.59	-					
I	0.24	0.40	0.75	0.66	0.76	0.62	0.68	0.61	-				
J	0.30	0.40	0.76	0.79	0.75	0.69	0.69	0.67	0.80	-			
K	0.28	0.39	0.72	0.74	0.72	0.62	0.63	0.59	0.78	0.84	-		
L	0.38	0.42	0.63	0.67	0,63	0.79	0.65	0.62	0.57	0.65	0.60	-	
M	0.30	0.42	0.69	0.69	0.70	0.66	0.64	0.59	0.79	0.83	0.78	0.64	-

Note: A & B: control/ wild-type, C & D: 0.5% EMS and 4 hours incubation, E & F: 1% EMS and 4 hours incubation, G & H: 0.5% EMS and 6 hours incubation, I & J: 1% EMS and 6 hours incubation, K & L: 0.5% EMS and 8 hours incubation, M: 1% EMS and 8 hours incubation.

consists of the mixture of soybean mutants generated by 0.5 % EMS and 1 % EMS for 4 hours, 6 hours and 8 hours incubation.

The similarity index and clustering method were performed to ascertain the degree of genetic relationship among soybean mutants and wild-type. The lowest similarity index was observed by a pair of wild-type and soybean mutants from treatments of 0.5% EMS and incubated for 6 hours (similarity index = 0.23). It was indicated that those treatments generated soybean mutants with the highest genetic distance compare to the wild-type. Whilst, the highest similarity index was observed by soybean mutants with 1 % EMS and incubated for 6 hours and soybean mutants with 0.5 % EMS and incubated for 8 hours (similarity index = 0.59). It was indicated that both treatments resulted in quite similar soybean mutants with low genetic distance. Moreover, this study was proven that EMS treatments successfully resulted in the potential variation among soybean mutants compared to the wild-type. It was supported by dendrogram which showed different clustering between wild-type and soybean mutants, and also amongst soybean mutants in different concentrations of EMS and incubation time.

EMS is an effective mutagen which caused transitions of DNA bases G/C to A/T (Greene et al. 2003). Therefore, it was led to a mispairing of complementary bases and caused a high frequency of gene mutation (Behera et al. 2012). EMS has been widely used as genetic variability induction in several plants like Asteranctha longifolia (Behera et al. 2012), Vigna unguiculata (Girija et al. 2013), Cucumis sativus (Wang et al. 2014) and Jatropha curcas (Dhakshanamoorthy et al. 2014). In this study, EMS was also proven to induce genetic variability in the soybean plant.

Another method that can be used to increase the genetic variability of soybean is somatic embryogenesis (somatoclone). This method utilizes the polyethylene glycol (PEG) as an osmotic solution. Somotoclone method has been proven to derived new varieties of soybean that tolerance against drought stress (Sunaryo et al. 2016). However, this technology requires a high cost and advance tool that makes it difficult to be implemented by the farmer.

RAPD marker effectively detected variability and have been adopted in a population study, plant systematic (Arif et al. 2010) and plant breeding (Fei et al. 2014). Moreover, it has some other advantages such as it is applicable for anonymous genome, low DNA quantities and resulted in a high number of DNA fragments (Kumari and Thakur, 2014). However, it has some disadvantages regarding their low sensitivity and reproducible, which caused unstable results; the different study will result in a different outcome. In spite of the contrary argument regarding their usage for genetic diversity study,

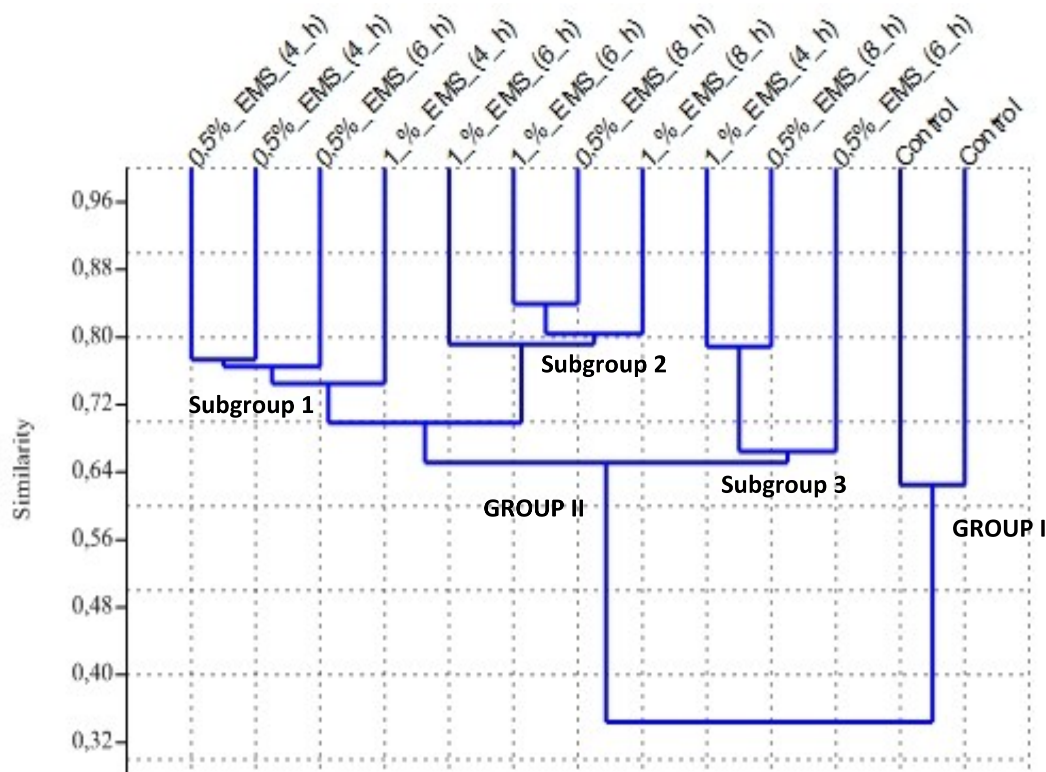

Figure 3. Dendrogram generated from Jaccard's coefficient similarity showing the genetic relationship of 11 EMS induced soybean mutants and control/ wild-type.

RAPD was still recommended and acceptable for detecting genetic variability than AFLP, ISSR and SSR (Sun and Wong, 2001).

CONCLUSION

EMS was an effective chemical mutagen to induce potential mutations in soybean. In this study, RAPD marker is still applicable and relevant to detect genetic mutations while also can be used as an alternative genetic approach. The occurrence of mutations in this study was shown by genetic variability, clustering and band appearance that all of them were obtained by RAPD marker.

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