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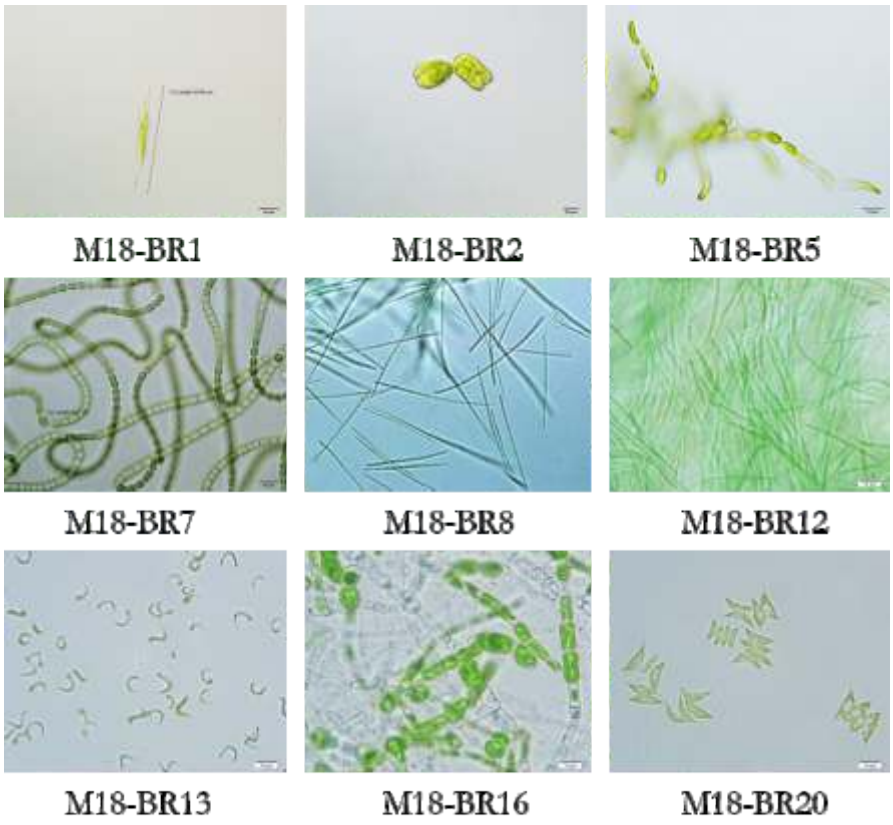

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Short Communication

Antihyperglycemic and Antioxidant Activity of Nanoemulsion Extracts of *M. affine* D. Don Leaves in Alloxan-Induced Rat

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ABSTRACT
This study determined the antihyperglycemic and antioxidant activity of nanoemulsion extracts of *M. affine* leaves in alloxan-induced rats. This research used 24 male Wistar rats around three months old which grouped as normal (untreated), negative control (treated with carboxymethyl cellulose sodium/Na-CMC,), positive control (treated with glibenclamide), and various concentration (30, 60, and 90%) of nanoemulsion extract of *M affine* leaves groups. The extract of *M. affine* leaves had an antioxidant activity with IC_{50} 5.30 ppm, categorized as a very strong antioxidant. Furthermore, the administration of this extract decreased glucose levels in antihyperglycemic rats. We concluded that *M. affine* leaves extract potential as antioxidants and be developed as an ingredient for diabetic drugs.

Keywords: diabetes, glucose levels, glibenclamide, *M. affine, Rattus norvegicus* strain wistar

Diabetes mellitus is a metabolic disorder that causes various complications. According to WHO, diabetic's incidences in Indonesia in 2000 were around 8.4 million and will be increased to 21.3 million in 2030 (Suharmiati, 2003). Currently, diabetes treatment is done by consuming synthetic drugs and maintaining a diet. Clinical management using synthetic drugs may cause irritation, resistance, and infection. Glibenclamide is an anti-hyperglycemic drug that is commonly used for the treatment of type 2 diabetes mellitus and has promising new medical indications. However, this drug is associated with high rates of serious hypoglycemic episodes as a result of its pharmacological activity (Hernández-Abad *et al.,* 2019). Glibenclamide spurs potassium channel activation (Assis *et al.,* 2018).

One of the natural approaches to overcome hyperglycemia is by consuming natural ingredients available in the environment. One of the plants which has antihyperglycemic potential namely "senggani" leaf from Aceh, Indonesia. Traditionally, Aceh people consume these leaves to reduce diabetes. *Melastoma malabathricum* leaf contains triterpene, alpha-amyrin, quercitrin, quercetin, and kaempferol-3-O- (2 ", 6 " - di-Op-trans-coumaroyl) - β glucoside (Hasnah *et al.,* 2010). Flavonoids play a role in preventing diabetes and its complications (Jack, 2012). Some research has proven that plants containing flavonoids are able to fight diabetes mellitus (Brahmachari, 2011). The administration of ethanolic extract of *Melastoma malabathricum* leaf by 400 mg/kg body weight can reduce glucose levels in diabetic mice (Sahara *et al.,* 2019), ethanolic extract of *M. malabathricum* leaves has significant antidiabetic and antihyperlipidemic activity in diabetic rats (Karappasamy *et al.*, 2014). However, this research uses *M. affine,* which belongs to the same genus as *M. malabathrium*, leaves extraction method carried out by nanoemulsion technique, one of the advantages of nanoemulsion technique is that it is easily absorbed by the small intestine wall thereby increasing the bioavailability of a compound. The rate of absorption of nano herbal in the human body can almost reach 100% (Poulain & Nakache, 1998). At present, there has never been reported the effect of

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nanoemulsion-based of *M. affine* leaves extract in diabetic conditions. The aim of this study was to determine the antihyperglycemic and antioxidant activity of nanoemulsion extracts of *M. affine* leaves in alloxan-induced rats.

The research was carried out at the Laboratory of Animal, Universitas Syiah Kuala. The preparation of *M. affine* leaves nanoemulsion extract was carried out at the Laboratory of Biology and Chemistry Faculty of Teacher Training and Education, Universitas Syiah Kuala, and Laboratory of Pharmacology, Faculty of Veterinary Medicine, Universitas Syiah Kuala. Antioxidant activity test was carried out at the Laboratory of Chemistry, Faculty of Teacher Training Education, Universitas Syiah Kuala.

This research used 24 male Wistar rats three months old with an average of body weight 180-200 gram. The rats were divided into 6 groups: N (normal group, untreated), NC (negative control, treated with carboxymethyl cellulose sodium/Na-CMC), PC (positive control, treated with glibenclamide), and nanoemulsion extract of *M affine* leaves groups: P1 (30 % of nanoemulsion extract of *M. affine* leaves), P2 (60 % of nanoemulsion extract of *M. affine* leaves), and P3 (90 % of nanoemulsion extract of *M. affine* leaves). The extract treatment was given orally once a day for 21 days.

Three kilograms of *M. affine* leaves were weighed, washed thoroughly, and air-dried for ± 3 days. After dry, these leaves were blended until smooth. Maceration was done with leaves powder and a 96 % ethanol ratio of 1:10 for 2 days to withdraw all the compounds contained in *M. affine* leaves. The preparation nanoemulsion of *M. affine* leaves extract includes the oil phase and water phase. The oil phase is a mixture of 180 mL of *M. affine* leaves extract and the water phase consists of 180 gram of maltodextrin, 18 mL tween 80, and 102 mL of phosphate buffer solution. Homogenization of the oil phase in the water phase was done using centrifugation at 15,000 rpm for 15 min (Safrida *et al*., 2020)

The Antioxidant activity test was done using the DPPH (2,2 –difenil -1-pikrilhidrazil) method. The test was carried with 0.5 mL sample solution at various concentrations (2, 4, 6, 8, and 10 ppm) to create a standard curve. 3.5 mL of DPPH was added to each solution. Homogenization was processed with vortex and incubated at 37 °C in a dark room. The absorbance was measured at the wavelength of 517 nm using a spectrophotometer (X Series UV VIS 100 DA-X) (Musri *et al.,* 2017).

The use of experimental animals in this study is in accordance with the code of ethics with ethical clearance (Ref: 5/KEPH/I/2020) from the Faculty of Veterinary Medicine, Universitas Syiah Kuala. Rats were kept in animal cages. Cages were cleaned before used by spraying formalin 10 % as a disinfectant. Cages were placed at room temperature and get indirect light. Animal cages were wire cages. Food and drink were administrated to rat in containers, cages were cleaned every day. The rats were acclimatized for 1 week and food was provided ad libitum. The initial blood glucose level was measured after being fasted for 8-12 h. Furthermore, rats were injected by intraperitoneal with alloxan 120 mg kg^{-1} body weight. Blood glucose was measured after alloxan induction. One week after alloxan injection, fasting blood glucose was measured to verify the incidence of hyperglycemic. Rats are declared hyperglycemic if glucose concentration above 200 mg dL^{-1}, the rats were treated with the extract of *M. affine* orally.

Rat's blood glucose examination was done using Gluco Dr strips. Blood was drawn from the tip of the rat's tail with a little massage and then pierced with a sterile needle ± as deep as ≤ 0.5 cm. Blood was taken from the tail of the rats and then dropped on a blood glucose strip. The blood glucose level was indicated on the numbers read on the blood sugar strips. Examination of blood sugar levels was done on day 0, 7, 14, and 21 after alloxan injection.

A compound has a very strong antioxidant if the IC50 is less than 50 ppm, strong (50-100 ppm), moderate (100-150 ppm), and weak (151-200 ppm). The smaller the IC50 value the higher the antioxidant activity (Musri *et al.,* 2017). The results showed that nanoemulsion extract of *M. affine* leaves has strong antioxidant activity with IC_{50} 5.30 ppm while the IC_{50} value of Vitamin C was 6.35 ppm (Table 1). This means that the antioxidant activity of *M. affine* extract is categorized as a very strong antioxidant. It was reported that methanolic extract of *Melastoma malabathricum* leaves has high antioxidants properties (Suhaimy *et al.,* 2017). Methanolic extract of *Melastoma malabathricum* leaves was considered as a compound which has a high antioxidant value (Mamat *et al*., 2013). *M. affine* is related to *M. malabathrium*, which belongs to the family Melastomataceae and genus Melastoma. Ethanolic extract of green grass jelly leaves was reported to have high antioxidant activity with IC_{50} 6.3 ppm (Mahadi *et al.,* 2018).

Nanoemulsion extract of *M. affine* leaves have very strong antioxidant activity. This extract has the potential to reduce glucose concentration in diabetic rats. The results showed that glucose levels decreased after the administration of nanoemulsion extract of *M. affine* leaves. The average blood glucose level of each treatment including N (normal group, untreated), NC (negative control, given

Table 1. Antioxidant Activity of Nanoemulsion Extract of *M. affine* Leaves.

No.	Control (EtOH, Absorbance)	Concentration (ppm)	Absorbance		% Inhibition		IC_{50}	
			Vitamin C	*M. affine* leaves	Vitamin C	*M. affine* leaves	Vitamin C	*M.affine* leaves
1	0.124	2	0.071	0.078	42.74	37.10	6.35ppm	5.30 ppm
2		4	0.064	0.074	48.39	40.32		
3		6	0.063	0.051	49.19	58.87		
4		8	0.058	0.047	53.23	62.10		
5		10	0.057	0.043	54.03	65.32		

carboxymethyl cellulose sodium/Na-CMC), PC (positive control, given glibenclamide), P1 (given 30 % nanoemulsion extracts of *M. affine* leaf), P2 (60 % nanoemulsion extracts of *M. affine* leaf), and P3 (90 % nanoemulsion extracts of *M. affine* leaf) can be seen in Table 2.

The results showed that the initial blood glucose level of rats was in the range of 75-98 mg/ dL. According to Wolfensohn and LIoyd (2013), the range of normal blood glucose levels in rats is 50-135 mg/dL. The results showed that the administration of alloxan caused increases in blood glucose in rats (Table 2). This is in line with a previous study by Safrida and Sabri (2019) that alloxan can induce diabetes in rats.

At the first week of observations, the amount of blood sugar in PC (positive control, treated with glibenclamide), and treated nanoemulsion extract of *M affine* leaves groups; P1 (30 % of nanoemulsion extract of *M. affine* leaves) and P2 (60 % of nanoemulsion extract of *M. affine* leaves) were decreased compared to diabetic rats, but had not reached normal blood sugar levels. The rat blood sugar levels in the P3 (90 % nanoemulsion extracts of *M. affine* leaves) treatment was almost close to normal rat blood sugar levels.

The administration nanoemulsion extract of *M. affine* leaves for 21 days was able to reduce the blood glucose level of rats to normal. This is presumably because *M. affine* leaves contain several bioactive compounds that have potency as antidiabetic. Mohd *et al.,* (2012) reported that *M. malabathricum* extract contains bioactive compounds namely ursolic acid, 2-hydroxyursolic acid, asiatic acid, glycerol-1,2-dilinolenyl-3-O-β-D galactopyranoside, glycerol 1,2-dilinolenyl-3-O- (4,6-di-O-isopropylidene) -β-D-galactopyranoside, methyl-2,5,6-trihydroxynaphtalene carbonate, flavonol glycoside derivatives, 2,5,6-trihydroxynaphtoic carbonic acid, kaempferol-3-O-(2 ', 6'-di-Op-trans-coumaroyl) -β-glucoside, and quercitrin.

Quercetin is a type of flavonoid, a subclass of flavonols. It has a hypoglycemic ability to inhibit the enzyme alpha-amylase in the hydrolysis of carbohydrates. Quercetin is able to inhibit glucose

Table 2. Average of Blood Glucose Concentration of Rats in Various Treatments.

Treatment	Initial blood glucose (mg/ dL)	Blood glucose after alloxan injection (mg/ dL)	Blood glucose levels (mg/dL)		
			Day 7	Day 14	Day 21
N	75,00 ±5,887^{a}	75,00 ± 5,887^{a}	96,25 ± 20,56^{a}	86,50 ± 1,91^{a}	76,00 ± 10^{a}
PC	85,25 ±5,315^{a}	263,25 ± 39,508^{b}	173,75 ± 14,31^{b}	82,50 ± 3,87^{a}	87,75 ± 4,11^{a}
NC	88,25 ±7,135^{a}	239,75 ± 41, 732^{b}	216,25 ± 46,77^{c}	247,50 ± 77,73^{b}	194,5 ± 5,32^{b}
P1	74,00 ±2,708^{a}	316,50 ± 35,519^{b}	157,00 ± 17,26^{b}	104,75 ± 6,50^{ab}	87,00 ± 4,24^{a}
P2	98,25 ± 8,995^{a}	307,00 ± 73,787^{b}	158,50 ± 11,95^{b}	98,25 ± 8,57^{a}	88,50 ± 8,18^{a}
P3	98,25 ± 6,130^{a}	259,50 ± 61,733^{b}	137,50 ± 39,49^{ab}	91,25 ± 8,53^{a}	82,5 ± 10,34^{a}

transport by intestinal GLUT2 and GLUT5 which have a function in the absorption of glucose in the small intestine, so that quercetin is able to reduce blood glucose. Some evidence showed that flavonoids from vegetables and medicinal plants have a beneficial effect on diabetes by increasing glycemic control, lipid profile, and antioxidant status. Mechanisms of antihyperglycemic effects include reduction of carbohydrate absorption from the small intestine, inhibition of tissue gluconeogenesis, elevation absorption of tissue glucose, stimulation of insulin secretion from beta cells, and protection of the islets of Langerhans against degeneration (Ghorbani, 2017). Antioxidants can repair cells by capturing free radicals thus protecting the islets of Langerhans against damage (Musri *et al.,* 2017)

The amount of rat blood glucose reached normal blood glucose levels after 14 and 21 days of treatment in all concentrations of nanoemulsion extract of *M. affine* leaves. Previous research by Karmilah (2018) showed that the ethanolic extract of *M. affine* leaves with the dose of 360 mg/g body weight could reduce the amount of blood sugar to normal with a duration of 28 days. According to Justina (2014), nanoemulsion is a technology that can change drug particles into a nano size. One of the advantages of nanoemulsion is that it is easily absorbed by the intestinal wall.

Nanoemulsion extract of *M. affine* leaves have high antioxidant activity and potentially reduce glucose levels in diabetic rats. Nanoemulsion extract of *M. affine* leaves has potency as a source of antioxidants and antihyperglycemic agents.

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Short Communication

Distribution record of *Musa borneensis* var. *sarawakensis* Becc. and *Musa campestris* var. *sarawakensis* Becc. in West Kalimantan, Indonesia

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ABSTRACT

Borneo Island has a large number of wild banana species. As a part of Borneo Island, West Kalimantan has limited information about the diversity of wild bananas. This research aims to update the record distribution of wild bananas from Bonti District of Sanggau Regency and to determine their morphological characteristics. Exploration method and resident information were used in this study. Two species of wild bananas have been identified and considered as new distribution records in West Kalimantan Province, namely-*Musa borneensis* var. *sarawakensis* with morphological character pseudostem red-purple colour, sparse black-purple blotches at petiole base, leaf base shape rounded on both sides, male bud red-purple colour and *Musa campestris* var. *sarawakensis* with morphological character pseudostem yellow-green colour, inflorescence erect, leaf base one side rounded and one-pointed, the dorsally pink-purple and ventrally pink-purple colour of bract.

Keywords: Bonti District, *Musa borneensis* var. *sarawakensis*, *Musa campestris* var. *sarawakensis,* Pisang Kera, wild banana

Musaceae is composed of three genera: *Ensente*, *Musa*, and *Musella*. The largest genus in *Musaceae* is *Musa*. Based on DNA analyses such as atpB-rbcl, rps16, trnL-F DNA sequences (Li *et al*., 2010), nuclear ribosomal (ITS) and chloroplast (trnL-F) (Liu *et al*., 2010), and ITS1-5.8S-ITS2 sequence (Hřibová *et al*., 2011), Häkkinen (2013) have restructured Musa species into two sections, sect. *Musa* and sect. *Callimusa*. Previously, based on chromosome number and morphological character the genus *Musa* has five sections, *Musa* sect. *Australimusa* 2n = 2x = 20; sect. *Callimusa* 2n = 2x = 20; sect. *Musa* 2n = 2x = 22; sect. *Rhodochlamys* 2n = 2x = 22 (Chessman, 1947) and sect. *Ingentimusa* 2n = 2x = 14 (Argent, 1976).

Musaceae is found in wet tropical lowland but some species were also found in higher latitude. Indonesia has many varieties of wild banana species, which are widely distributed in Sumatra, Java, Nusa Tenggara, Kalimantan, Sulawesi, and Papua islands (Nasution & Yamada, 2001), including three species of wild bananas in Sumatra Island, i.e. *Musa salaccensis*, *M. sumatrana*, and *M. halabanensis* (Meijer, 1961), eight species in Java island, i.e. *M. acuminata* with seven infraspecific taxa, *M. balbisiana*, *M. coccinea*, *M. ornata*, *M. salaccensis*, *M. sanguinea*, *M. textilis*, and *M. velutina* (Sulistyaningsih, 2016), eight species in Sulawesi Island, i.e. *M. balbisiana* and *M. itinerans* as new records (Sulistyaningsih *et al*., 2014), *M. acuminata* (with four infraspecific taxa as new record), *M. textilis*, *M. borneensis* (Hastuti *et al*., 2019), and *M. borneensis* var. *donggalensis* as a new species and rejected the endemic status of *M. borneensis* in Kalimantan (Sulistyaningsih, 2017) and one new species of *M. arfakiana* from Papua (Argent, 2010).

Borneo Island is the third largest island in the world. Borneo Island is located on the equator, has high mountains which provide many different habitats as part of the centre of the primary banana diversity centre, which has a large number of wild banana species (Häkkinen, 2004a). The exploration to find out the diversity of wild bananas in Borneo

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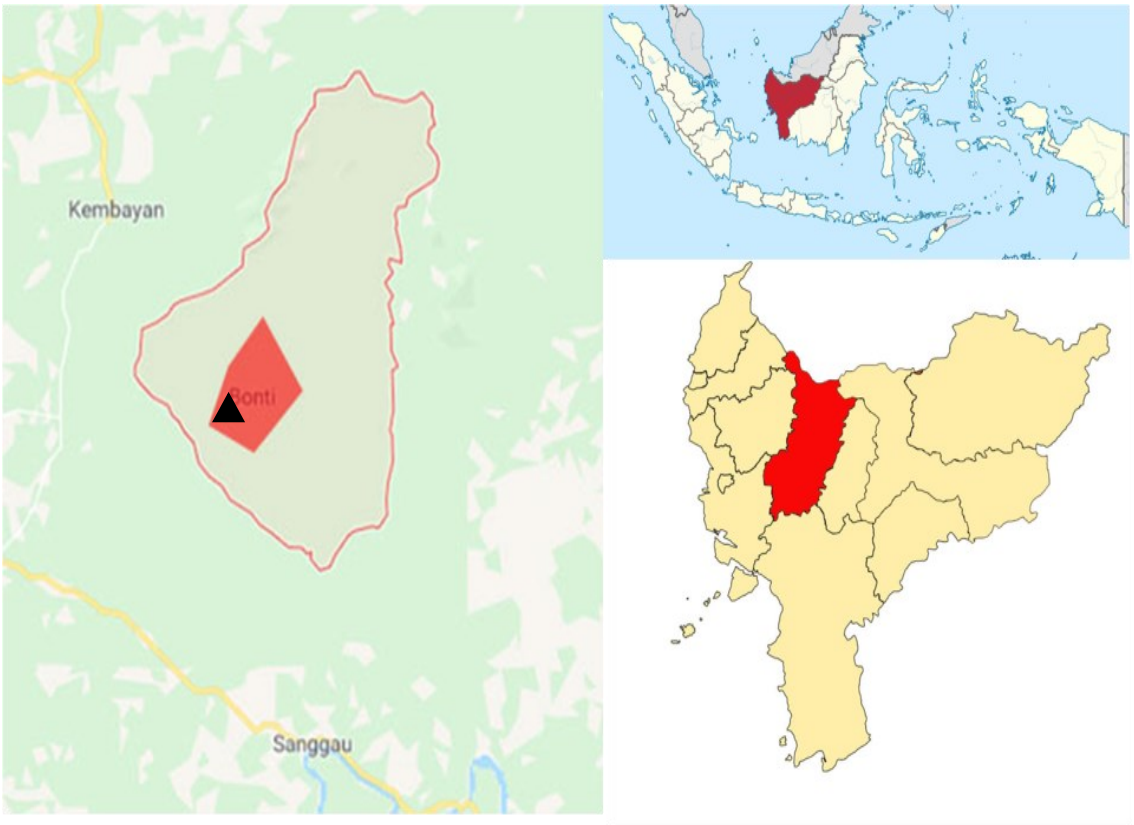

Figure 1. Study area in Bonti Village, Bonti District, Sanggau Regency,West Kalimantan, Indonesia.

Island has been carried out intensively in Sabah, Sarawak, and Brunei Darussalam. Häkkinen (2004a) also reported 20 species of endemic wild bananas were found in Borneo, but only 15 species have been previously described. Borneo Island is divided into three-state territories, namely Indonesia, Malaysia, and Brunei Darussalam. In Indonesia, the island of Borneo is known by the name Kalimantan which is divided into five provinces. However, the forest fire and resultant haze have potential impacts on Kalimantan's biodiversity such as habitat loss, forest fragmentation, and low sunlight on forest trees (Horrison *et al*., 2016). The exploration, inventory, and conservation of wild bananas in West Kalimantan need to be done before the wild banana species are lost due to forest fire.

Studies on wild bananas in West Kalimantan are still limited. Sulistyaningsih and Irawanto (2011) reported *M. campestris* var. *sarawakensis* Häkkinen or Pisang Kera in Nek Rokon Hill of Raya Pasi Natural Resources area, Singkawang-West Kalimantan. Previously, the distribution of *Musa campestris* was only considered in Sabah, Sarawak, and Brunei Darussalam (Häkkinen, 2004b). Moreover, Sunandar (2017) reported *Musa balbisiana* Colla or Pisang Klotok in Teluk Nibung Village, Kubu Raya District -West Kalimantan. Previously, *Musa balbisiana* Colla was known to be distributed in Java (Cheesman, 1948) and Sulawesi Island (Sulistyaningsih *et al*., 2014).

The information on the diversity and distribution of wild bananas in West Kalimantan are needed to improve the quality of cultivated banana using genetic manipulation in the future and for conservation management of wild bananas in West Kalimantan. This study aimed to update the record distribution of wild bananas from Bonti District of Sanggau Regency and to determine their morphological characteristics.

The study on wild bananas species were conducted in Bonti Village, Bonti District, Sanggau Regency, West Kalimantan, Indonesia (Figure 1). The study area was surrounded by Noyan and Kembayan Districts in the northern part, Parindu and Kapuas Districts in the southern part, and Tayan Hulu District in the western part. The average rainfall is 235 mm (BPS Sanggau, 2017). The topographic area in Bonti Sub-district is plains.

The exploration was carried out in March 2017. Morphological characters were documented with a digital camera. Morphological characterization was done under Descriptors for Banana (*Musa* spp.) from the International Plant Genetic Resources Institute (IPGRI, 1996). Morphological character records included the plant's general habit as well as characteristics of pseudostem, petiole, leaf, peduncle, male bud, male flower, fruit, and seed (shape and colour). Morphological characteristics obtained from the field were then crosschecked with references (Nasution & Yamada, 2001; Häkkinen, 2004b;

Häkkinen & Meekiong, 2005; Sulistyaningsih, 2017).

Based on the differences in 12 morphological characters, two species of wild banana were identified in Bonti District, West Kalimantan, i.e. *Musa borneensis* var. *sarawakensis* and *Musa campestris* var. *sarawakensis* (Table 1, Figure 2-3). Some morphological features of both species can be seen in Figures 2 and 3. In daily life, the villagers of Bonti District only utilized *M. borneensis* var. *sarawakensis* as food.

Musa borneensis var. *sarawakensis* has vernacular name Pisang Boha'(Bonti, Indonesia). However, local people in Sarawak called it Pisang hutan (Malay) or Baliek guun (Melanau) (Häkkinen & Meekiong, 2005). Geographically, *M. borneensis* var. *sarawakensis* located between 110^{0}32'56.781" E and 0^{0}24'47.893" N.

Characteristics: Mature pseudostem up to 4 m high, sheaths red-purple colour. Sucker Closed to parent and vertical growth. Petiole up to 42-93 cm long, petiole canal wide with erect margins, petiole bases corrugated auricles with sparse black-purple blotching. Leaf habit erects up to 400-470 cm long, 78-82 cm wide, colour of upper surface is green, lower surface is green-yellow, and leaf bases asymmetric and rounded on both sides, midrib dorsally yellow, midrib ventrally light green. Inflorescence first horizontal then pendulous, peduncle 37- 48 cm long, 8 cm in diameter, hairless, and red-green. Male bud rounded, normal male bud, apex obtuse and split with green tips, dorsally red-purple, ventrally yellow of bract, revolute bract behaviour, lifting two bracts at a time, and rachis position horizontal. Male flower compound tepal cream with cream lobes, free tepal translucent white, oval, with triangular apex, style straight, ovary straight. Fruit 6 fruits per hand, individual fruit 14 cm long, straight in shape, without any floral relicts, and apical part bottle-necked shaped. Seed obpyriform and brown (Figure 2). Variations morphology were found between *M. borneensis* var. *sarawakensis* in Bonti, West Kalimantan, and *M. borneensis* var. *sarawakensis* in Serian-Sri Aman, Sarawak. *Musa borneensis* var. *sarawakensis* in Bonti, West Kalimantan had red-purple pseudostem. Sucker closed to parent. Dorsally red-purple and ventrally yellow of bract (Table 1). However, *M. borneensis* var. *sarawakensis* in Serian-Sri Aman, Sarawak had purple-brown pseudostem. Sucker far from the parent plant. Dorsally pink-purple and ventrally yellow of bract (Table 1) (Häkkinen & Meekiong, 2005).

M. borneensis var. *sarawakensis* can be found on forest border in Bonti village, Sanggau District, West Kalimantan and considered as a new distribution record (Figure 2). Previously, *M. borneensis* was reported in Sarawak, Malaysia (Häkkinen & Meekiong, 2005) and Donggala-Central Sulawesi (Sulistyaningsih, 2017). In Serian-Sri Aman, Sarawak, *M. borneensis* var. *sarawakensis* was found on the roadside (Häkkinen & Meekiong, 2005).

Local people in Bonti village consume the young pseudostem of *M. borneensis* var. *sarawakensis*. The young pseudostem of *M. borneensis* var. *sarawakensis* boiled in water then cooked with coconut milk. Punan tribe consume the young pseudostem of *M. borneensis* var. *flavida* and as a land certificate (Sulistyaningsih & Wawo, 2011).

The key character of *M. borneensis* var. *sarawakensis* in Bonti, West Kalimantan is pseudostem red-purple colour, sparse black-purple blotches at petiole base, leaf base shape rounded on both sides, male bud red-purple colour**.**

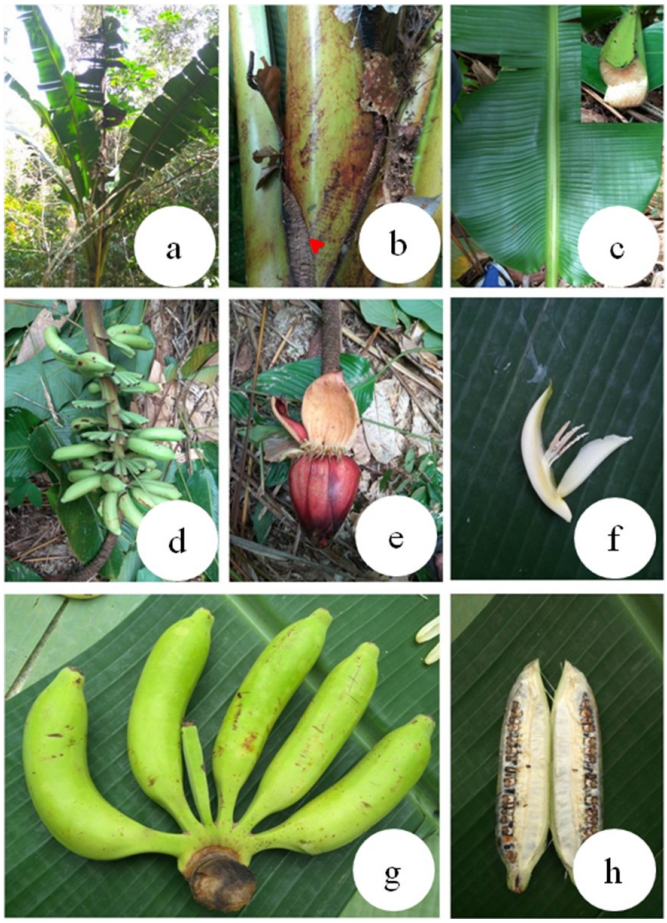

Figure 2. *Musa bornensis* var. *sarawakensis* in West Kalimantan. a. Habitus; b. Auricle; c. Leaf and petiole canal leaf; d. Bunch; e. Male bud; f. Male flower; g. A hand of fruits; h. Longitudinally section of fruit.

Another species of wild bananas have been identified namely-*Musa campestris* var. *sarawakensis*. *Musa campestris* var. *sarawakensis* has a vernacular name: Pisang Kera in Bonti District, Sanggau Regency. Local people in Nek Rokon Hill, Raya Pasi Natural Resource area, Singkawang, West Kalimantan also called it Pisang Kera

Table 1. Morphological characters of *Musa borneensis* var. *sarawakensis* and *Musa campestris* var. *sarawakensis*.

No	Character	*M. borneensis* var. *sarawakensis* in this study	*M. borneensis* var. *sarawakensis* (Häkkinen & Meekiong 2005)	*M. campestris* var. *sarawakensis* in this study	*M. campestris* var. *sarawakensis* (Häkkinen 2004)
1	Mature pseudostem color	Red-purple	Purple brown	Yellow-green	Yellow red-purple
2	Petiole canal leaf	Wide with erect margin	Wide with erect margin	Straight with erect margins	Straight with erect margins
3	Leaf habit	Erect, Lamina up to 400-470 x 78-82 cm	Erect, Lamina up to 350 cm x 80 cm	Erect, Lamina up to 210-285 x 30-42 cm	Erect, Lamina up to 240 cm x 50 cm
4	Colour of upper surface leaf	Green	Green and shiny	Dark green	Green
5	Colour of lower surface leaf	Green-yellow	Medium green	Green	Yellowish-green
6	Leaf bases	Asymmetric; rounded on both sides	Asymmetric; both side rounded	Asymmetric; one side rounded and one-pointed of leaf bases	Asymmetric; both side rounded
7	Midrib	Dorsally yellow; ventrally light green	Dorsally light green to yellow; ventrally yellow	Dorsally yellow; ventrally green	Dorsally light-green; ventrally medium green
8	Inflorescence	First horizontal then pendulous	First horizontal then pendulous	Erect	Erect
9	Peduncle	Hairless, red-green	Hairless, light green yellow	Very hairy, red-purple	Very hairy, reddish-purple
10	Male bud	Rounded; Dorsally red-purple, ventrally yellow; revolute before falling	Rounded or cordate; dorsally pink-purple, ventrally yellow; revolute before falling	Ovoid; dorsally pink-purple, ventrally pink-purple; not revolute	Ovoid; dorsally purple, ventrally pale-purple; deflexed but not rolled back
11	Male flower	Compound tepal cream; free tepal translucent white, oval	Compound tepal cream to yellow; free tepal cream, oval	Compound tepal cream**;** free tepal translucent white**,** rectangular	Compound tepal watery green; free tepal translucent white, oblong
12	Fruits	Straight	Straight	Straight	Straight

(Sulistyaningsih & Irawanto, 2011). However, local people in Kuching, Sarawak, Malaysia called it Pisang Lengki (Häkkinen, 2004b). Geographically, *M. campestris* var. *sarawakensis* located between 110^{0}31'59.593" E and 0^{0}24'31.938" N.

Characteristics: Pseudostem sheaths yellow-green colour. Sucker closed to parent and vertical growth. Petiole up to 42-93 cm long, petiole canal straight with erect margins. Leaf habit erect up to 210-285 cm long, 30-42 cm wide, colour of upper surface dark green, lower surface green, and leaf bases one side rounded and one-pointed, midrib dorsally yellow, midrib ventrally green. Inflorescence erect. Peduncle red-purple in colour. Male bud ovoid, normal male bud, apex slightly pointed, dorsally pink-purple, ventrally pink-purple colour of bract, not revolute bract behaviour, lifting one at a time, and rachis position erect. Male flower compound tepal cream with yellow lobes, free tepal translucent white, rectangular, with obtuse apex, style straight, ovary straight. Fruit 5 fruits per hand, individual fruit18 cm long, straight in shape, without any floral relicts, and apical part blunt-tipped. The Seed is obpyriform and brown color (Figure 3). Variations morphology were found between *M. campestris* var. *sarawakensis* in Bonti, West Kalimantan and *M. campestris* var. *sarawakensis* in Kg. Jambu, Sarawak. *M. campestris* var. *sarawakensis* in Bonti, West Kalimantan had yellow-green pseudostem colour. Leaf bases one side rounded and one-pointed. The Dorsally pink-purple and ventrally pink-purple colour of bract (Table 1). However, *M. campestris* var. *sarawakensis* in Kg. Jambu, Sarawak had yellow-red purple pseudostem colour. Leaf bases both sides rounded. The dorsally purple and ventrally pale-purple colour of bract (Table 1) (Häkkinen, 2004).

Figure 3. *Musa campestris* var. *sarawakensis* in West Kalimantan. a. Habitus; b. Leaf and petiole canal leaf; d. Peduncle; e. Male flower; f. Longitudinally section of fruit; g. A hand of fruit.

M. campestris var. *sarawakensis* can be found on forest border in Bonti village, Sanggau District, West Kalimantan and also considered as a new distribution record (Figure 3). Previously, *M. campestris* var. *sarawakensis* was reported in Sarawak, Malaysia (Häkkinen, 2004b) and on foothill in Nek Rokon hill, Raya Pasi Natural Resource area, Singkawang, West Kalimantan (Sulistyaningsih & Irawanto, 2011).

Local people in Bonti village have not utilized *M. campestris* var. *sarawakensis*. However, local people in Keritan Ulu, Mongool, Senagang Ulu villages, Sabah, consume the heart of inner shoot and flower inflorescence as a salad with chilli sauce or sambal biris (Noweg *et al.*, 2003).

The Key character of *M. campestris* var. *sarawakensis* from Bonti West Kalimantan is pseudostem yellow-green colour, inflorescence erect, leaf base one side rounded, and one-pointed, the dorsally pink-purple and ventrally pink-purple colour of bract.

The two wild bananas species were successfully identified from Bonti district, Sanggau Regency, West Kalimantan i.e. *M. borneensis* var. *sarawakensis* and *M. campestris* var. *sarawakensis* and it is considered as new distribution records. The Conservation of *M. borneensis* var. *sarawakensis*, *M. campestris* var. *sarawakensis*, and other wild bananas in Indonesia is important to be prioritized both *in-situ* and/or *ex-situ* before it goes to extinction caused of deforestation and forests fragmentations. Conservation of wild bananas will provide long term and sustainable conservation of genetic diversity, that's important resources to improve the quality of cultivated banana using genetic manipulation in the future.

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Short Communication

Innovation of Natural Orchid Cultivation Technology for Tourism Development in Banyunganti Hamlet, Jatimulyo Village, Girimulyo Sub-District, Kulon Progo District, Yogyakarta

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ABSTRACT

Orchid is the best tourism icon which focused on nature-based tourism development in Indonesia. Banyunganti Hamlet is one of the tourism villages in Kulon Progo which has a high diversity of orchid species. Regarding this situation, guiding and assistance for villagers which is focused on the introduction of natural orchid species and its character, conventional propagation, and modern propagation by using household-scale tissue culture techniques (sowing seeds and planting) is important to give. The long-term goal for this activity was for empowering women in Dusun Banyunganti in line with the opening of the New Yogyakarta International Airport by the government.

Keywords: Orchid, Banyunganti Hamlet, Tour village, Kulon Progo district, Women's Group Orchid Farmer

Indonesia is a tropical country with mega biodiversity. One of them is the natural orchid which is scattered in almost all tropical rainforests, and one-sixth of the world's orchids population can be found in Indonesia. Some natural orchids are designated as superior areas, for example, *Vanda tricolor* var suavis is the superior orchid species of Sleman and Mount Merapi, Tiger orchids or *Grammatophyllum scriptum* endemic to Papua, *Phalaenopsis amboinensis* endemic orchid of Maluku, and others. Furthermore, orchid is a beautiful flowering ornamental plant and has a high economic value. It has the opportunity to be cultivated by the community to increase community income. It also can be expected as a local floriculture potency. Development in accordance with agroecological in this product will give better commercial value. As a result, Indonesian floriculture products able to compete at national and international levels.

The development of horticulture as a biological resource needs to consider the EfSD concept. The existence of these plants must always be maintained, some are reproduced to be cultivated in order to maintain their existence in nature and others can be traded, even exported as a source of foreign exchange. In the EfSD concept, youngster and local communities must be equipped with soft skills and capability to multiply superior plants, even innovate with science and technology, invent new technologies with science and technology, so that continuity and quality of products can be guaranteed (Sudibyo, 2009). For example, the addition of peptone into a cultured medium could increase the efficiency and speed of seed germination of Phalaenopsis and Dendrobium orchids in vitro (Semiarti *et al.*, 2014; Setiari *et al.*, 2016; Mose *et al.*, 2017; and Semiarti *et al.* 2017).

The Education for sustainable development program considers 3 pillars, namely economic sustainability, social justice, and environmental preservation including biodiversity and cultural diversity and culture (Sudibyo, 2009). The main road map of Community Service activities UGM

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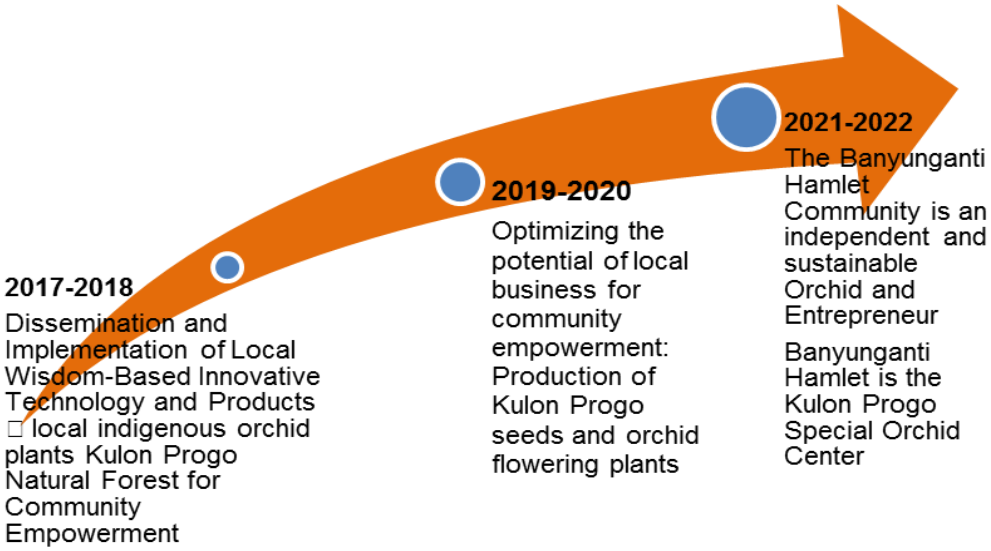

Figure 1. Road map of Community Service activities UGM Orchid Research Team.

Research Team for Multidisciplinary Orchids for the Establishment of Banyunganti Hamlet as Kulon Progo Orchid Center is as follow (Figure 1).

Banyunganti Hamlet is located in Jatimulyo Village, Girimulyo Sub-District, Kulon Progo District, Province of D.I. Yogyakarta. According to the Kulon Progo District Central Bureau of Statistics (2016), Jatimulyo Village has a land area of 16,2906 km^2 with 12 hamlets. Geographically, Jatimulyo Village, Kulon Progo District has boundaries, namely, the northern boundary is Magelang Regency, Central Java Province; the southern boundary is the Indian Ocean; the western border is Purworejo Regency, Central Java Province; and the eastern boundary is Sleman and Bantul Regencies, D.I Province. Yogyakarta. The population distribution in Jatimulyo Village is 6,648 people with 3,276 male and 3,372 women (BPS, 2016).

Banyunganti Hamlet is one of the potential tourism areas in Yogyakarta. As a result, this place has several tourist attractions including Mudal River Park, Kembangsoka Waterfall, and Kedung Pedut Waterfall. The Banyunganti Hamlet community, which mostly has a livelihood as farmers, formed a tourism awareness group ("Kelompok Sadar Wisata"/ POKDARWIS) which made the Banyunganti Hamlet community increasingly understand tourism villages and realized the importance of developing their area as a tourist destination. As a tourist village, Banyunganti Hamlet requires its local characteristics. Banyunganti Hamlet has other tourism potentials that have not been developed to its full potential which can be used as a local characteristic of natural orchids that are native to the natural forest of Banyunganti Hamlet.

Based on the exploration research conducted by the Biology Orchid Study Club (BiOSC), Banyunganti Hamlet has a high diversity of orchids. There are 23 species of natural orchids found in Banyunganti Hamlet of which the most common are *Spathoglotis plicata, Dendrobium crumenatum, Arundina graminifolia, Cymbidium bicolor*, and *Acriopsis liliifolia* (Figure 2). Unfortunately, the Banyunganti Hamlet community has not utilized the orchids as a regional characteristic due to a lack of public understanding of the important values and utilization of orchid's potentials. The introduction of orchids and their potential has been initiated through the Community Creativity Program for Community Service entitled "ANGGUN AYU Orchid of Banyunganti Hamlet: Development of the Forest Orchid Park" as a Means of Edutourism by students of the Faculty of Biology, Faculty of Cultural Sciences and the Faculty of Forestry. In addition, the community of Banyunganti Jatimulyo Village has also introduced a simple method of orchid cultivation through a grant from the Directorate of Community Service UGM with an Education for Sustainable Development (EfSD)-based community service program scheme funded by the UGM BPPTNBH in 2017, and community groups have formed orchid lover.

Even though orchid farmer groups have been formed, only a few were interested in orchid entrepreneurship as a source of family income. Therefore, UGM orchid research team initiates the application of research results on *in vitro* and *ex vitro* orchid cultivation (Semiarti *et al.*, 2014; Setiari *et al.*, 2016; Mose *et al.*, 2017; and Semiarti *et al.*, 2017) to educate local people in Banyunganti Hamlet and innovate it with appropriate technology to help the community grow seeds, grow plants, and accelerate flowering induction in Kulon Progo native orchid plants. As the Orchid Education Center, the Faculty of Biology has played an active role in the Tri Dharma Perguruan Tinggi activities in the field of engineering. The research result of lecturers in the

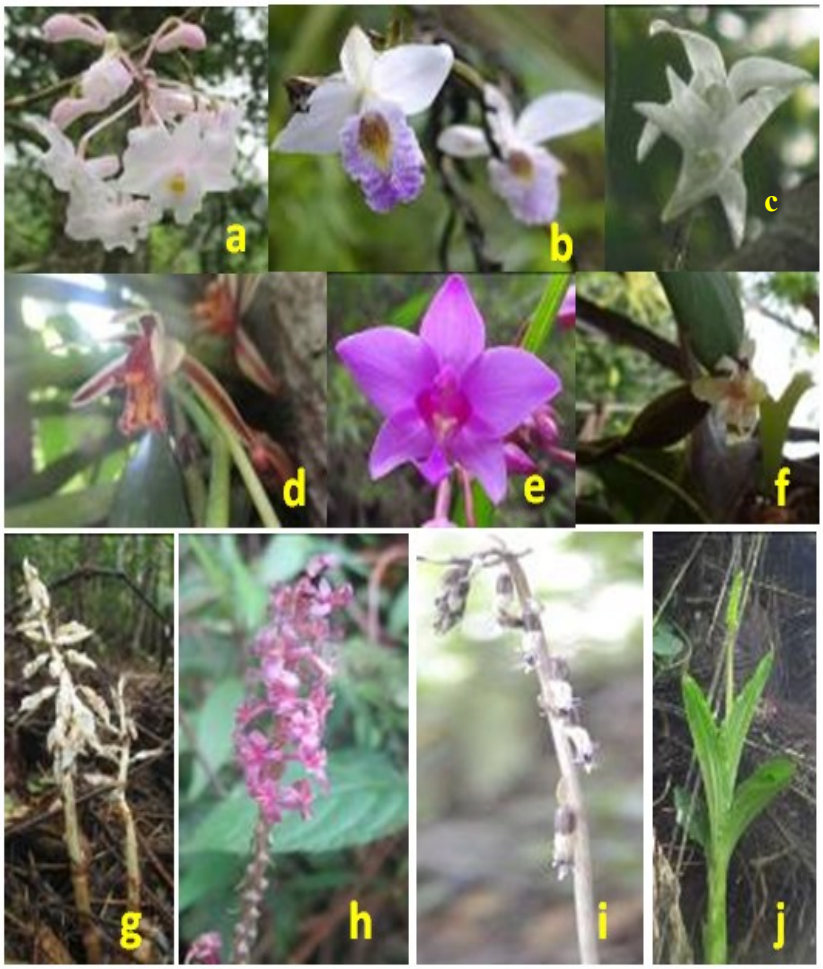

Figure 2. Orchid diversity in Banyunganti Hamlet. *Epiphytic* orchid (a-f). A.) *Dendrobium mutabile*, B.) *Arundina graminifolia*, C.) *Dendrobium crumenatum*. D.) *Cymbidium bicolor*, E.) *Spathoglottis plicata*, F.) *Flickingeria fimbriata*. Terrestrial orchids (g-h). G.) *Epipogium roseum*, H.) *Malaxis kobi*, I.) *Stereosandra javanica*, J.) *Dienia ophrydis* (After Wardhana, 2015).

orchid works have been published in popular media (newspapers and Facebook) and in national and international journals. Furthermore, some effective and efficient technologies for orchid propagation, prevention of pests and diseases, and induction of orchid flowering, which has been partially applied to the community, especially in collaboration with the Indonesian and National Provinces Orchid Society of Indonesia have been conducted as the Center of Excellent (CoE) of Orchids in Indonesia.

There were four main objectives for the community service, such as (1) increasing the knowledge and skills of Banyunganti Hamlet community about orchid cultivation techniques, especially the typical orchids of Kulon Progo District with the implementation of Appropriate Technology programs; (2) Facilitating lecturers and students of the Faculty of Biology and the Faculty of Agriculture UGM in mentoring and developing entrepreneurial programs and conservation of orchid biodiversity in Jatimulyo Village based on Appropriate Technology; (3) Realizing the collaboration between the people of the Banyunganti Hamlet, the Jatimulyo Village Government, UGM, the Indonesian Orchid Association of DIY Province, and the Study Group of the BiOSC students of the Faculty of Biology for the development of sustainable orchids in Banyunganti Hamlet; and (4) to increase the income of Banyunganti Hamlet community with sustainable orchid entrepreneurship.

The Empowering activity was held for 7 months, from May 2018 to October 2018. Training and education on orchid were carried out in the Banyunganti Hamlet, Jatimulyo Village, Girimulyo, Kulon Progo, D.I. Yogyakarta. The activities were divided into pre-implemented and implemented programs. Pre-implemented activity was held by intensive discussion and observation to determine the location, time, and programs. The discussion was an initial activity carried out with the aim of finalizing the idea that was initiated, namely conducting training and empowerment of the Banyunganti hamlet community with the theme of applying appropriate technology the results of research for the community to cultivate orchids. This is done to find the potential that exists in the hamlet in the form of potential cultural results, as

well as natural resources, in this case, the natural potential of forest orchids and other carrying capacity as a destination that supports the development of tourism in the region such as rivers, waterfalls, and forest area. In addition, this observation activity also aims to obtain information about community interests and the problems they face in terms of developing potential and carrying capacity in the area. This activity is expected to provide a general description of what will be done in the next stage in developing the potential that exists in the area in the form of training and community empowerment regarding orchid cultivation in Banyunganti Hamlet.

Moreover, the implementation program began with the management of permits and raising cooperation with related parties. Furthermore, the making of leaflets, posters, and banners will be installed in strategic locations so that they are easily accessible by the community. In addition, the making of discussion materials will be carried out through PowerPoint slides, guidebooks, and CDs, as well as preparation of facilities and infrastructure for orchid cultivation and entrepreneurship. Theories and practices that will be given to the community include how to cross orchid flowers to produce fruit and seeds, planting orchid seeds *in vitro*, transferring bottle orchid seeds to pots, and maintaining orchid plants in demonstration plots and the natural forest of Banyunganti Hamlet.

There are eight orchid's training programs that were implemented to provide supplies to local people to become successful orchid farmers, especially for the development of Banyunganti orchids and tips on successful orchid entrepreneurship (Table 1).

From all training programs were given to the Women Orchid Farmers of Banyunganti Hamlet, it was seen that the participants were very enthusiastic in participating in these programs, and had followed up by trying it out on their own in groups many times at the head of the Banyunganti hamlet's house. This is very positive because technology will be mastered well if it has been carried out many times and coupled with various innovations tailored to the conditions in the field.

In addition, men have worked together to create a simple greenhouse for their orchid house. Orchid seedlings were transferred from the bottle into community pots (compot) and adult plants as

Table 1. The Orchid Training Program is based on Appropriate Technology that was given to the Group of Women Orchid Farmers of Banyunganti Hamlet.

No.	Training Programs	Objectives	Outputs
1	Introduction of Orchid Parental and the Cross Technique.	The community can practice the care of conventional orchids independently to develop the potential of orchids in the Banyuganti Hamlet.	Some orchid breeders, Orchid siliques for seed production.
2.	Making sterile orchid *in vitro* culture medium.	People can prepare sterile medium for *in vitro* orchid cultivation on a household scale.	Medium *in vitro* ready to use.
3	*In vitro* seed plantation.	People can germinate orchid seeds in vitro on a household scale.	Some orchid seedlings production.
4	*In vitro* subculture/ overplanting.	People can subculture orchid plantlets *in vitro* on a household scale.	Orchid seedlings in bottles.
5	Hydroponic and Aeroponic Orchid Cultivation Training.	Local people can practice directly the installation of aeroponic and hydroponic orchid planting plants which are guided directly by agricultural experts.	Hydroponic and aeroponic orchids set by local people of Banyunganti Hamlet.
6	Orchid Entrepreneurship.	Local people know about the tips and tricks in building a business by looking at the potential of the Banyunganti Hamlet.	Tips for successful orchid entrepreneurs.
7	Nuance Orchids Souvenir Making.	Women Farmers Group Banyunganti Orchids can make nuanced souvenir trinkets orchid: hijab painting, orchid brooches, key holders.	Hijab painting, orchid brooches, key holders.
8	Introduction of an iT-based promotion system.	Women Farmers Group Banyunganti Orchids can make an iT-based promotion system.	An iT-based promotion system via WhatsApp, online shop, instagram, youtoube.

parental for breeding, as well as juvenile plants. So that it is expected that with continuous assistance from the Orchid Team of the Faculty of Biology UGM, the effort to establish Banyunganti Hamlet as the center of orchids in Kulon Progo can be realized.

In the long term, the program to produce and maintain the original orchid plant of Jatimulyo Village Natural Forest in a pilot plot or simple orchid house in Banyunganti Hamlet and ecotourism in "Taman Sungai Mudal" Park, can be equipped with orchid tourism facilities, orchid-nuanced souvenir shops, and online promotion systems will increase local orchid products typical of the Banyunganti Hamlet Forest, Jatimulyo Village and "Taman Sungai Mudal" Ecotourism by optimizing Orchid Masterplan demonstration and household-scale Laboratory, Kulon Progo forest orchid collection in its natural habitat for Orchid Tourism for the community.

Appropriate technology-based community empowerment activities from the results of orchid research implemented in Banyunganti Hamlet, Jatimulyo Village, Kulon Progo District have produced 8 programs that are continuously being developed by the Banyunganti Hamlet Women's Farmers Group. Intensive assistance needs to be carried out from the Orchid Team of the Faculty of Biology UGM, Indonesian Orchid Society Province of Yogyakarta, and the Regional Government of Kulon Progo Regency to support the sustainability of this activity so that the community can develop orchid tourism in their area independently and sustainably. To foster the interest of local people in developing orchid agribusiness in their area seriously, it is necessary to provide assistance from the government to complete the infrastructure and make a simple plant tissue culture laboratory to continuously grow plants and directly support "Bela_Beli Kulon Progo" and Yogyakarta International Air Port (YIA).

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Research Article

Species Diversity and Potential Utilization of Moraceae in Nglanggeran Ancient Volcano, Gunungkidul Regency, Yogyakarta

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ABSTRACT
Nglanggeran Ancient Volcano is one of the ecotourism areas in Gunungkidul Regency, Yogyakarta. This ancient volcano is one of the geological sites in Pegunungan Sewu. Pegunungan Sewu is a National Geopark in Indonesia and has been designated as a UNESCO Global Geopark since 2015. The determination of an area into a National Geopark and Global Geopark makes the biodiversity in the area must be protected and preserved, including the plant diversity, one of which is Moraceae. The ecological and economic importance of Moraceae in Nglanggeran Ancient Volcano has not been documented properly. This fact encourages the need to do this research. The aim of this research was to record species diversity of Moraceae and its potential uses. The research was conducted in August-November 2019 at Nglanggeran Ancient Volcano and followed by data analysis in Plant Systematic Laboratory, Faculty of Biology, Universitas Gadjah Mada. The samples were collected at two different tracks, covering the track to the peak and the pathway down. There were six species found, namely *Artocarpus altilis* (Parkinson) Fosberg, *Artocarpus heterophyllus* Lam., *Ficus benjamina* L., *Ficus septica* Burm. f., *Maclura cochinchinensis* (Lour.) Corner, and *Streblus taxoides* (Roth) Kurz. The potential uses of Moraceae by local people were identified from the interview and enriched by data from literature studies indicated that Moraceae plants were used as a food, medicine, for construction, soil protection plants, and houseplant.

Keywords: Moraceae, Nglanggeran Ancient Volcano, Plant potential uses, Species diversity

INTRODUCTION
Nglanggeran is an ancient volcano and a popular historic geological site in Pegunungan Sewu renowned as one of the ecotourism areas in Gunungkidul Regency, Yogyakarta. Pegunungan Sewu is a National Geopark in Indonesia and has been designated as a UNESCO Global Geopark since 2015 (Ministry of Energy and Mineral Resources, 2018). The concept of Geopark which was initiated by UNESCO applies a sustainable regional development concept that integrates three aspects of diversity, namely geodiversity, biodiversity, and cultural diversity. This concept aims at developing the economy of local communities based on the protection of the three diversities (UNESCO, 2014; Ministry of Energy and Mineral Resources, 2018). In this study, biological diversity is the main focus as presented as the inventory of species diversity. The cultural diversity aspect was observed by documenting the utilization of Moraceae species by local people. Meanwhile, the geological diversity aspect is not examined in this study because it is not under the scope and competence of the authors.

The determination of an area into a National Geopark and Global Geopark makes the biodiversity in the area must be protected and preserved, including the plant diversity, one of which is Moraceae. Moraceae is a family of Angiosperm consisted of 37 genera and approximately 1.100 species, with *Ficus* being the largest genus within the family with 600 species (Singh, 2010). Moraceae are distributed in tropics, subtropics, and temperate regions (Clement & Weiblen, 2009; Singh, 2010). *Ficus* has a critical ecological function in tropical rainforest ecosystem, also as an important food

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source for a wide range of frugivores (Datwyler & Weiblen, 2004; Harrison, 2005). The family is important for its fruits such as mulberry (*Morus alba* L., *Morus nigra* L.), fig (*Ficus carica* L.), and breadfruit (*Artocarpus altilis* (Parkinson) Fosberg). Leaves of *Morus* are widely known for rearing silkworms (Singh, 2010). The ecological and economic importance aspects of Moraceae in Nglanggeran Ancient Volcano have not been documented properly. This fact encourages the need to do this research. The aim of this research was to record species diversity of Moraceae and their potential uses, which covers the biological and cultural diversity aspects of geopark concept, whereas geological diversity was not included in this study due to the reasonable consideration of being out of the author's competence.

MATERIALS AND METHODS

Materials

The materials used in this study were plant samples collected from two different tracks of Nglanggeran, alcohol 70%, specimen label, plastic bags, herbarium envelope, herbarium sheet, and newspaper. The tools used in this study were garden scissors, cutter, oven, sprayer, digital camera NIKON J5, smartphone, GPS (Global Positioning System), altimeter, rope, board, cardboard, and identification books. Data on the potential use of Moraceae species was gathered using questionnaires collected from local people by the assistance of POKDARWIS (working group for tourism).

Methods

Plants specimens were taken from its natural population along two different tracks, covering the track to the peak and the pathway down (Figure 1). The specimens were documented by taking the photograph of the whole plant and its habitat before being preserved into dried herbarium specimens. Identifications were carried out based on the morphological character using an online database (Digital Flora of Eastern Ghats, Kew Herbarium Catalogue, Useful Tropical Plants Database) and identification books (Flora of Java, Flora Malesiana). Data on potential use and current utilization of Moraceae plants was obtained by gathering the information from the local community using a simple questionnaire and enriched with information from relevant literature. There were four questions in the questionnaire asked the local people, namely: (1) whether local people are allowed take plants in the tourist area or not; (2) what species commonly used by local people; (3) what plant parts that are usually used by local people; and (4) what is the common utilization of the plants by local people. The results obtained were presented as species descriptions and information on their potential uses.

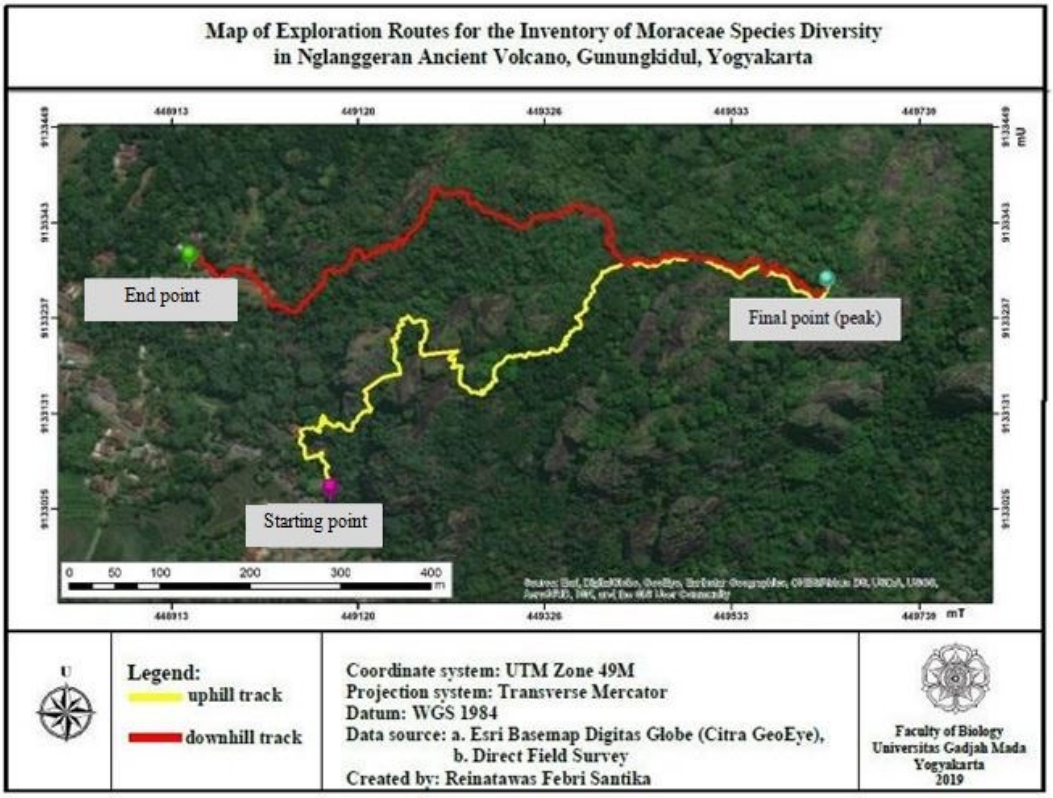

Figure 1. Tracks of specimen collection in Nglanggeran Ancient Volcano

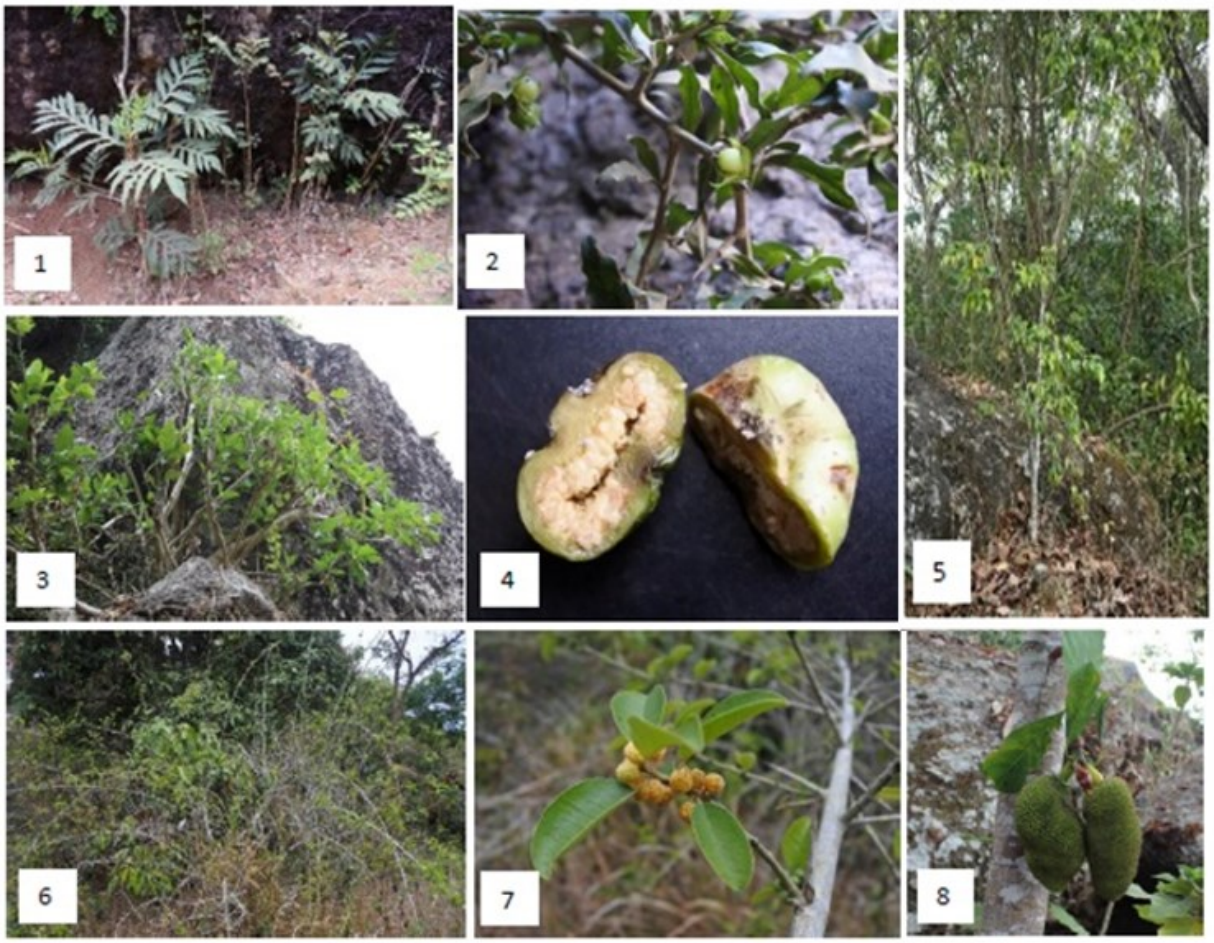

Figure 2. Species of Moraceae found in Nglanggeran were *A. altilis (1), S. taxoides (2), F. septica* habit *(3), F. septica* inflorescences *(4), F. benjamina (5), M. cochinchinensis* habit *(6), M. cochinchinensis* inflorescences *(7), A. heterophyllus (8).*

RESULTS AND DISCUSSION

Six species consisted of four genera of Moraceae were found during field exploration in Nglanggeran Ancient Volcano area (Figure 2). These species were *Artocarpus altilis* (Parkinson) Fosberg, *Artocarpus heterophyllus* Lam., *Ficus benjamina* L., *Ficus septica* Burm. f., *Maclura cochinchinensis* (Lour.) Corner, and *Streblus taxoides* (Roth) Kurz.

A previous study by Widodo and Luthfi (2017) found 10 species of Moraceae in Nglanggeran Ancient Volcano, namely *Fatoua sp., Morus sp., Malaisia scandens* (Lour.) Planch., *Streblus asper*, *S. taxoides, M.cochinchinensis, F. benjamina, F.septica, F. montana* Burm. f., and *Artocarpus integra* (Thunb.) Merr. Results of this exploration showed that there were only six species found (Figure 2), namely *A. altilis, A. heterophyllus, F. benjamina, F. septica, M. cochinchinensis*, and *S. taxoides*.

The difference in the number of species found can be due to differences in exploration scope areas or exploration tracks, the presence of plants in areas that were too dangerous to explore (such as steep slope), and the difference of season when explorations were carried out. The six species found during the course of study was ranging from the lowest with only two individuals for *S. asper*, three for *A. altilis*, four for *A. heterophyllus*, five for *F. septica*, and eleven individualsfor both *F. benjamina* and *M. cochinchinensis*.

The results of this study showed that species diversity of Moraceae in Nglanggeran ancient volcano was low, as indicated by only six species was found. This number is notably lower compared to similar studies by Widodo and Lutfi (2017) who obtained 10 species of Moraceae in Nglanggeran. When compared to other locations, the number of *Ficus* species obtained in this study was only two, much lower than similar studies conducted in Prof. Soemitro Djojohadikusomo Conservation Forest West Sumatra which found 20 *Ficus* species (Nur'aini *et al.*, 2013). A study by Rahadiantoro and Siahaan (2016) on the diversity of Moraceae tree species in Telogo Dowo, Sempu Island recorded 14 species. The number of *Ficus* species found in this study was also very small compared to that obtained by Almulqu *et al.* (2018) in Mutis Timau Protected Forest, East Nusa Tenggara. Based on these comparisons, the diversity of Moraceae species in

Nglanggeran ancient volcano revealed in this study was obviously low.

The six species found in this study were collected from their habitat at the elevation 200-700 mdpl. *Artocarpus altilis* and *Artocarpus heterophyllus* were found at 200 mdpl, and these two species were also known planted by local people in their yard. *Ficus benjamina* was mostly found near the rocky karst areas at 200-670 mdpl. *Ficus septica* also found in similar habitat at 200-460 mdpl. *Maclura cochinchinensis* was found at 200-638 mdpl, while *Streblus taxoides* was found at 460 mdpl. The Morphological description of these six species was presented below.

Artocarpus altilis

An evergreen tree that is monoecious with heights up to 15-20 m, smooth bark, may reach a height of 4 m before branching, two large stipules enclosing the terminal bud, up to 30 cm, yellowing and falling when leaves fold or inflorescences emerge. Leaves obovate to broadly ovate, spirally arranged, thick leaves, leathery, margin entire, acuminate apex, top dark green, often glossy, pale green and dull underside with an elevated midrib and main veins. Mature leaves pinnatipartite with 3-8 segments, while juvenile leaves on young trees and new shoots of mature trees usually larger, more dissected, and more hirsute. Leaves sometimes smooth but often with few reddish hairs on midrib and veins. Male inflorescences have yellow, cylindrical, 7-30 cm lengths, composed of hundreds of flowers attached to the spongy core. Male flowers have a tubular calyx, apically 2-lobed, and elliptic anthers. Female flowers have a tubular calyx, ovary ovoid, long styled, and apically 2-branched. The fruit has a highly specialized structure, a syncarp, composed of 1500-2000 flowers attached to the fruit axis or core. Fruit globose to oblong, light green rind, yellowish-green or yellow when mature, flesh creamy white or pale yellow, seedless, but some forms seeded, seeds have a thin dark-brown outer skin (thick ± 0,5 mm) (Zhekun & Gilbert, 2003; Orwa *et al*., 2009a).

Artocarpus heterophyllus

An evergreen tree with heights up to 8-25 m, straight stemmed, branching near the base, canopy dense, and dome-shaped. Bark is greyish-brown, rough, and sometimes scaly, all living parts of the tree exude white latex when injured. Leaves are elliptic to obovate, glossy, dark green top, pale green or light green underside, entire margin, blunt apex, cuneate or pointed base, alternately arranged on horizontal branches, spirally arranged on ascending branches. An individual flower is attached on an elongated axis and forming a raceme inflorescence. Male inflorescences have oblong, cylindrical, or clavate with 2-7 cm length, peduncle 1-5 cm, yellowish, sterile flower has solid perianthium, fertile flower is tubular, and bi-lobed. Female inflorescence have oblong or cylindrical, rough, light to dark green skin, 5-15 cm length. Fruiting syncarp, pale yellow when young, yellowish-brown when mature, ellipsoid, globose, or irregularly shaped, covered by a rubbery rind and hard pyramidal, pointed or blunt spines. Syncarp composed of many fertilized ovaries developed into a yellow fruitlet. Unfertilized female flower developed into a hard strap or string-like structure, has white color, filling the space between fruitlets, called perigones. Seeds are oval-oblong or oblong-ellipsoid (Zhekun & Gilbert, 2003; Haq, 2006; Orwa *et al*., 2009b).

Ficus benjamina

A monoecious, tree or strangler, hemi-epiphytic, with height up to 35 m high, producing aerial roots, and have brown to greyish bark. Leaves spirally arranged with lamina elliptic, oblong, to ovate, acuminate apex, rounded to obtuse base, entire margin. Inflorescence hypanthodium, there are male, female, and gall flowers within the same inflorescence. Male flower has short pedicellate and long filament. Female flower is sessile with 3 calyx lobes, short style, and enlarged stigma. Gall flower has 3-5 calyx lobes, ovoid ovary, and short style. Infructescences is syconium, yellow to orange or dark red when mature (Zhekun & Gilbert, 2003; Berg *et al.,* 2006).

Ficus septica

A dioecious Tree or shrub with yellowish latex and pale brown to yellowish-brown bark. Branchlets are thick and cylindric. Red stipules, ovate to lanceolate, with 2-3 cm length, and membranous. Leaves are alternate, lamina oblong, ovate-elliptic, or obovate, acuminate, mucronate, or sometimes caudate apex with base cuneate, and entire margin. Inflorescence hypanthodium, there are male, female, and gall flowers within the same inflorescence. Male flower near apical pore has 2 or 3 calyx lobes, 1 stamen, short filament, and ellipsoid anther. Female flower has long pedicellate, 2 or 3 calyx lobes, long style, and clavate stigma. Gall flower has short and transparent calyx lobes, ovoid to globose ovary, and short style. Infructescences syconium, green to light brown, has white small spots in rind, apical pore open when mature (Zhekun & Gilbert, 2003; Berg *et al.,* 2006).

Maclura cochinchinensis

An evergreen shrub that has long branches, dioecious, erect, or scandent. Branches are brown greyish, with 4 cm long straight or curved thorns.

Lamina is elliptic-lanceolate to oblong, apex shortly acuminate to subacute, base obtuse, rounded, or cuneate, and entire margin, spirally arranged. Female and male inflorescences are capitulum with a diameter of 0,4-1 cm. Male flower has 4 calyx lobes, and short anthers. Female flower did not have calyx lobes, basally connate. Fruiting syncarp with a diameter of 2-5 cm, orange-reddish when mature (Zhekun & Gilbert, 2003; Berg *et al*., 2006)

Streblus taxoides

An evergreen shrub with heights up to 5 m, dioecious, much-branched, with thorns up to 1,5 cm long. Lamina is elliptic to oblong-lanceolate, acuminate to blunt apex, base is acuminate to obtuse, and entire margin. Male inflorescences spike or raceme, sessile. Male flower has short pedicellate, 4 calyx lobes, and globose anthers. Female flower is solitary, sometimes clustered, 4 calyx lobes, and apically style branched. Drupes globose, at first enclosed by enlarged calyx lobes (Zhekun & Gilbert, 2003; Berg *et al*., 2006).

The fruit of *Artocarpus altilis* (breadfruit) is used as food and source of carbohydrates. Young and ripe fruit of *Artocarpus heterophyllus* (jackfruit) is also used as food and its seeds are sometimes boiled or roasted for food. Leaves of breadfruit and jackfruit are used as animal feed. Their trunk is used for construction material (Berg *et al*., 2006).

Extracts and metabolites of breadfruit leaves, stems and fruit contain various useful active compounds such as antibacterial, anti-inflammatory, anti-diabetic, antifungal, and antioxidant activity (Sikarwar *et al*., 2014). Jackfruit root extract is used to treat skin diseases, asthma, and diarrhea. Its leaf extract contains flavonoids, anthocyanin, tannins, and proanthocyanidin which can increase glucose tolerance in diabetic patients (Haq, 2006). *Ficus benjamina* has potential uses as a medicinal plant, including its fruit extracts and latex which were used to treat skin diseases, inflammation, malaria, as antimicrobial, antinociceptive, antipyretic, hypotensive, and anti-dysentery (Imran *et al*., 2014). *Ficus septica* is used as traditional medicine for fever, headache, and stomach-ache (Ueda *et al*., 2009). *Ficus septica* also has been reported as having cytotoxic activity against HONE-1 nasopharyngeal cancer cells and gastric cancer cells NUGC (Damu *et al.,* 2005). *Maclura cochinchinensis* wood can be used to treat for fever (Atika & Salma, 2017). *Streblus taxoides* root is used to treat glandular swellings, and its trunk is used to treat elephantiasis (Kadavul & Divit, 2008).

Jackfruit has a function as a shade tree and can reduce the impact of rainfall on the soil. They are sometimes also planted on slopes and hills to help control soil erosion. The widespread root system can help the absorption of groundwater, and therefore reduce flooding (Haq, 2006). Intensive growth of *Ficus benjamina* roots can damage roads and sidewalks so it is less suitable as a shade plant on the roadside (Gilman & Watson, 2011). *Ficus benjamina* is widely cultivated as an ornamental plant. In Indonesia, *Streblus taxoides* are used for bonsai, but their use is already rare (Sulistyo, 2008). *Maclura cochinchinensis* woods are used as batik dye along with bark of *Ceriops tagal* and *Peltophorum pterocarpum* to make soga colors (Atika & Salma, 2017).

CONCLUSION

Species diversity of Moraceae in Gunung Api Purba Nglanggeran is low based on comparison to other studies, either those from the same area or other regions in Indonesia. Six species were found in this study namely *Artocapus altilis, Artocarpus heterophyllus, Ficus benjamina, Ficus septica, Maclura cochinchinensis,* and *Streblus taxoides*. The potential uses of Moraceae are food, medicine, for construction, soil protection plants, and houseplant.

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Research Article

Plant Conservation Based on *Tri Mandala* Concept on Homegarden at Pakraman Penge Village, Baru Village, Marga District, Tabanan Regency, Bali

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ABSTRACT
Penge Pakraman village is a traditional village that has the potency to become ecotourism. The emergence of new tourist attractions affects land changes to support tourism activities. This causes the number of plant species in nature to decrease. Plant conservation by utilizing local wisdom is one effort to reduce the decrease of plant species number. The application of *Tri Hita Karana* and *Tri Mandala* in Balinese daily life able to support plant conservation activity. The objective of this research was to determine the role of *Tri Mandala* concept in plant conservation at Pakraman Penge home garden. Data collection methods are carried out through observation plant location with inventory number and name of plants in house sample. Plant use continues with study literature. The results of an inventory of plant diversity in home gardens of Pakraman Penge village recorded 70 species of plants from 16 houses sample. The plant habitus varied from herbs, shrubs until trees. Plant species in the home gardens have functions for ceremonies (51%), medicinal (24%), ornamental (17%), food (6%), and spices (2%). However, based on the location, most plant species were found in *madya mandala* and the lowest were found in *utama mandala*. We also found plants with conservation status consist of least concerned (15 species), vulnerable (*Dracaena draco*), near threatened (*Cycas rumphii*), endangered (*Coffea arabica*). Therefore, the application of *Tri Mandala* concept on Balinese home gardens supports plant conservation and gives economic benefit in individual level.

Keywords: home garden, Pakraman village, plant conservation, *Tri mandala* concept

INTRODUCTION
Penge Pakraman village is a traditional village that still relies on Balinese custom conceptions especially in the design of gardens and houses. This village has the potency to become ecotourism (Prantawan & Sunarta, 2015). However, the emergence of new tourist attractions affects land changes to support tourism activities (Evita *et al.,* 2012). This causes the number of plant species to decrease. Plant conservation by utilizing local wisdom is one effort to reduce the decrease of plant species number (Leksono *et al.,* 2015). However, Balinese people need many kinds of plants to support their ceremonial activity. The applications of *Tri Hita Karana* and *Tri Mandala* in Balinese daily life are expected to support plant conservation activity and give economic benefits in Penge Pakraman village.

Tri Hita Karana's philosophy, which means three sources of goodness, is a reference for the Balinese people in their daily lives. This philosophy is also reflected in the division of space in traditional Balinese architecture known as the *Tri Mandala* concept. The purpose of this concept is to create a harmonious connection between God, Humans, and the environment. *Tri Mandala* divides space into three, namely: *utama mandala* (sacred) where the sacred area is for worshiping the God Almighty, *madya mandala* (middle) is an area where humans interact with society and *nista mandala* (profane) is a place of interaction with the environment (Aryani & Tanuwidjaja, 2013; Wastika, 2005).

Some research on the application of *Tri Mandala* and the use of home gardens in Bali has been carried out Widyastuti *et al.* (2020) studied

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about the suitability of plant placement based on *Tri Mandala* concept in Pura, Paramita *et al.* (2017) studied about medicinal plant use in home gardens, Pranditha *et al.* (2018) studied about plant placement based on *Tri Mandala* concept in Bangli home garden, and Sujarwo and Caneva (2015) studied about ethno botanical plants in traditional Balinese home gardens. However, research that focuses on the relation between *Tri Mandala* concept and plant conservation on Pakraman village is still rare. Therefore, the objective of this research was to determine the role of *Tri Mandala* concept in plant conservation at Pakraman Penge home garden.

MATERIALS AND METHODS

The study was conducted in Pakraman Penge Village, Baru Village, Marga district, Tabanan Regency, Bali from January-March 2020. This village was the only tourism village in Tabanan regency (Figure 1). A total of 16 houses were used as research samples. The selection of sample locations was done by purposive sampling of houses that apply the *Tri Mandala* concept. The Floor plan was drawn manually. Inventory of plant diversity was carried out referring to the division of *Tri Mandala* space, namely *utama mandala*, *madya mandala,* and *nista mandala*, including green space called *telajakan*.

The parameters measured in the field include the name of the species, the number of individuals of each species, and the location of growth. Plant specimens were identified in the field by the first author. Furthermore, unknown plant species were then made in vouchers and identified in herbarium Bali Botanic Garden. The scientific nomenclature used in this study was derived from the existing database (The Plantlist, 2020). A Literature study was conducted to find the utilization of each species of plant and divided into the following six use categories: ceremony, medicine, food, ornamental, protector (*tolak bala*), and spices. The same plant could fall into more than one category. However, the conservation status from each species checked with the International Union for Conservation Nature (IUCN) red list of threatened species website (IUCN, 2020).

Data Analysis

Standard statistical methods were used to calculate data using MS Office Excel. Furthermore, data from each location were analyzed to find the abundance of plant species in the village of Pakraman Penge by calculating the relative frequency (FR) values (Darma

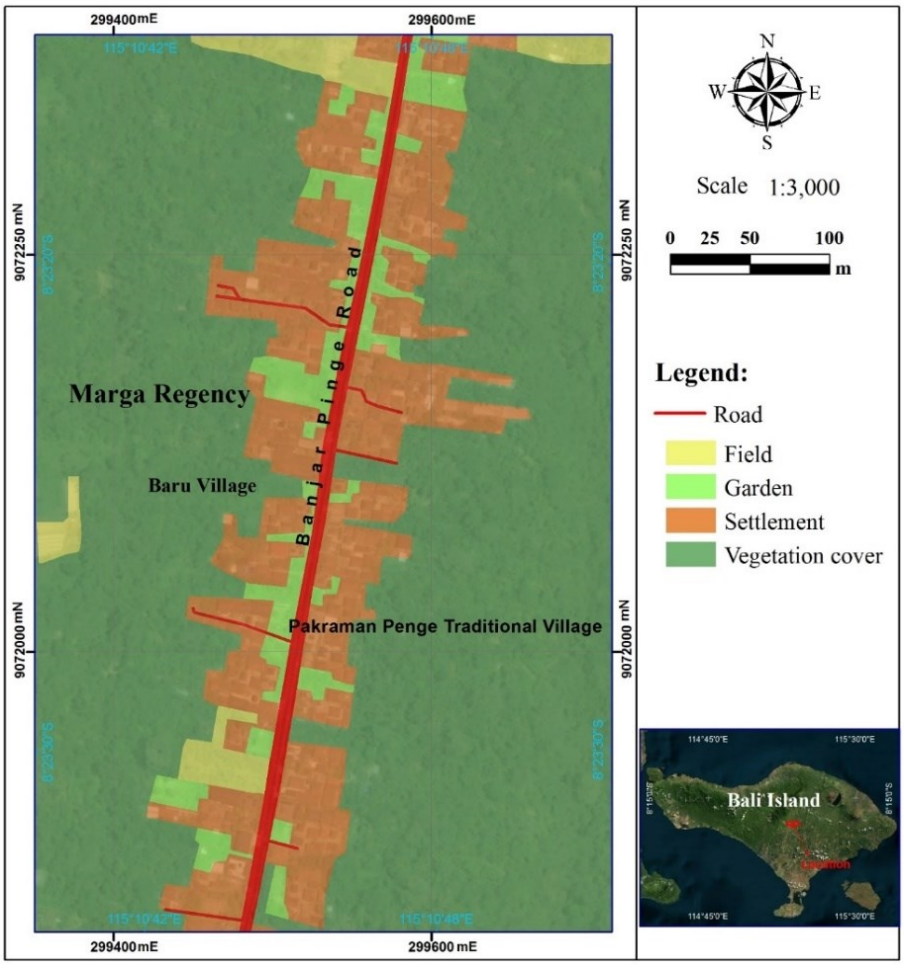

Figure 1. Map showing the location of Pakraman Penge village, Baru village, Marga district, Tabanan regency, Bali (Documentation by Rajif Iryadi, 2020).

et al., 2018). The Relative frequency used to determine the plant species distribution in home gardens.

$$\text{Relative frequency (FR)} = \frac{\text{Frequency one species}}{\text{Frequency all species}} \times 100\%$$

RESULTS AND DISCUSSION

Plant composition and cultivated plant uses in the Pakraman Penge Village home garden

The Pakraman village's concept is related to the garden space planted with various types of plants. One part of *Tri Hita Karana* concept is the harmonious relationship between humans and the environment which makes the concept of garden design to have various types of plants that have a special value for the surrounding community (Wisnumurti, 2017). Balinese garden has a high touch in terms of their culture and this plant has a function as a complement to *upakara* (ceremony plant), *usada* (medicinal plant) philosophy of placement, and enhancement of the aesthetics of the park. Therefore, the dominance of plant type determination is influenced by culture (Hazrinah *et al.,* 2016).

Plant inventory record 70 species belong to 43 families (Table 1). The most common family are Arecaceae (6 species) followed with Asparagaceae, Myrtaceae, Rubiaceae (4 species each), Fabaceae, Poaceae, Rutaceae (3 species each) and Annonaceae, Apocynaceae, Lythraceae, Moraceae, Pandanaceae, Phyllanthaceae, Zingiberaceae (2 species each). Our results showed a higher number of recorded plants in Pakraman Penge village home garden compared with the other 13 traditional home gardens village in Bali which only recorded 36 species. (Sujarwo & Caneva, 2015). This difference because the 13 village lead a traditional lifestyle and located near to the forest or natural areas so the number of plant species in their home garden is lower.

The most frequently used part are leaves, fruit, and flower (Table 1). This result in line with Ambarani *et al.* (2017) who mention that plant parts in Payangan home garden mostly used are leaves, flowers, and fruits because those parts are used in Hindu's ceremony.

Application of *Tri Mandala* concept on Traditional Architecture house at Pakraman Penge village

Based on observations of 16 houses that were sampled, it is known that the division of traditional architectural houses in the village of Pakraman Penge follows the *Tri Mandala* concept which consists of *utama mandala* /sacred, *madya mandala*/ middle, and *nista mandala*/profane. *Utama mandala* in the form of performance is to worship the greatness of God. *Madya mandala* is called *pekubonan* which is useful for the activity of its owner. Inside it was built a house consisting of *bale dangin*, *bale daja*, *bale dauh*, *bale tengah*, *paon* (kitchen), and *jineng* (granary). *Nista mandala* in the form of *tebe* is an area that serves as a place for raising livestock and growing plants that have large tree habit. In addition, there is also a green space called *telajakan* which is a barrier between the main road and the front of the house home garden (Figure 2). Yudantini (2012) revealed *telajakan* is an integral part of traditional housing patterns in an indigenous village in Bali, but often forgotten about in contemporary housing development in Bali.

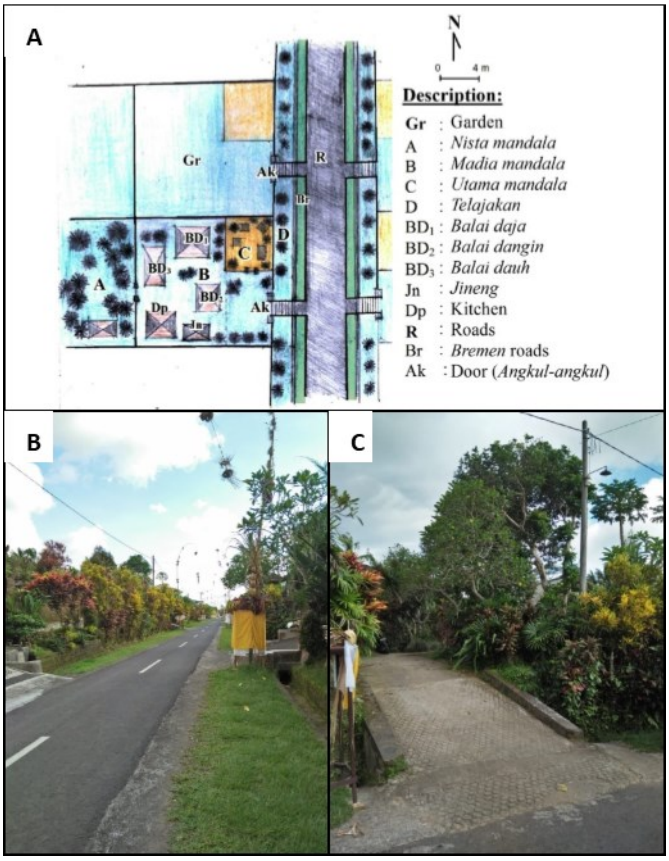

Figure 2. Home garden sketch at Pekraman Penge village, Baru village, Marga district, Tabanan regency, Bali (A). T*elajakan* planted with plant (B), 2C Home garden plant composition (Sketch and Photo by I Dewa putu Darma, 2020).

Plant preference and their location in Pakraman Penge village home garden

The home garden in Pakraman Penge village is dominated by ceremony and medicine plants (Figure 3). The garden provides quick and easy access to ceremony plants for their daily ritual religious, such as *Cordyline frutticosa, Cordyline terminalis, Cassia surratensis,* and *Michelia champaca*. The home garden also provides medicine function which has an advantage as the first curative before going to the

Table 1. Plant diversity in every sampled home garden in the Pakraman Penge village.

No	Species name (Local name) Family	Use	Areal				Total	FR (%)	Part of plant which usually used						Conservation status
			T	UM	MM	NM			Flower	Fruit	Leaf	Tuber	Stem	All part	
1	*Cordyline frutticosa* (L) A.Chev (Andong) Asparagaceae	Ceremony	1	1	1		3	2,29		1					LC
2	*Cassia surattensis* Burm.F. (Kembang kuning) Fabaceae	Ceremony	1	1	1		3	2,29	1						LC
3	*Averrhoa carambola* L. (Belimbing besi) Oxalidaceae	Medicine, Ceremony, Food			1	1	2	1,53		1 1 1	1 1				
4	*Pluchea indica* (L.) Less (Beluntas) Asteraceae	Medicine			1		1	0,76			1				
5	*Areca cathecu* L. (Buah) Arecaceae	Medicine, Ceremony				1	1	0,76		1 1					
6	*Nephelium lappa*ceum L. (Rambutan) Sapindaceae	Ceremony Food	1			1	1	0,76		1					LC
7	*Michelia campaca* L. (Campaka putih) Magnoliaceae	Ceremony	1		1	1	3	2,29	1						LC
8	*Acorus calamus* L. (Jangu) Acoraceae	Medicine, Ceremony			1		1	0,76				1 1			LC
9	*Moringa oleifera* Lam. (Kelor) Moringaceae	CeremonyMedicine, Food	1		1	1	3	2,29			1				
10	*Syzygium aromaticum* (L.) Merr.&L.M.Perry (Cengkeh) Myrtaceae	Medicine	1		1		2	1,53	1						
11	*Sauropus androgynus* (L.) Merr. (Kayu manis) Phyllanthaceae	Ceremony, Medicine			1		1	0,76			1				

Table 1. Contd.

No	Species name (Local name) Family	Use	Areal				Total	FR (%)	Part of plant which usually used						Conservation status
			T	UM	MM	NM			Flower	Fruit	Leaf	Tuber	Stem	All part	
12	*Piper betle* L. (Base) Piperaceae	Ceremony, Medicine			1		1	0,76			1 1				
13	*Alpinia galanga* (L.) Willd. (Isen) Zingiberaceae	Medicine			1		1	0,76				1			
14	*Allamanda cathartica* L.(Bunga ceblong) Apocynaceae	Ceremony	1	1	1		3	2,29	1						
15	*Gardenia jasminoides* J.Ellis (Jempiring) Rubiaceae	Ceremony	1	1	1		3	2,29	1						
16	*Plumeria acuminata* W. T. Aiton (Jepun) Apocynaceae	Ceremony, Medicine	1	1	1		3	2,29	1 1						
17	*Bougainvillea spectabilis* Willd. (Kembang kertas) Nyctagynaceae	Ceremony	1	1	1		3	2,29	1						
18	*Caesalpinia pulcherrima* L. (Sw) (Kemerakan) Fabaceae	Ceremony, Ornamental	1	1	1		3	2,29	1 1						
19	*Hibiscus rosa-sinensis* L. (Pucuk bang) Malvaceae	Ceremony, ornamental	1	1	1		3	2,29	1						
20	*Rhododendron mucronatum* (Bl.) G. Don (Rododendron) Ericaceae	Ceremony, ornamental	1		1		2	1,53	1						
21	*Cananga odorata* (Lam) Hook. f &Thomson (Sandat) Annonaceae	Ceremony, ornamental			1	1	2	1,53	1						LC
22	*Ixora coccinea* L. (Soka) Rubiaceae	Ceremony, ornamental	1	1	1		3	2,29	1 1						
23	*Medinilla speciosa* (Reinw. ex Bl.) Bl. (Trijata) Melastomataceae	Ceremony, ornamental		1	1		2	1,53		1	1				

Table 1. Contd.

No	Species name (Local name) Family	Use	Areal				Total	FR (%)	Part of plant which usually used						Conservation status
			T	UM	MM	NM			Flower	Fruit	Leaf	Tuber	Stem	All part	
24	*Musa paradisiaca* L(Pisang) Musaceae	Ceremony, Food				1	1	0,76			1		1		
25	*Zingiber officinale* Roscoe (Jahe) Zingiberaceae	Medicine			1		1	0,76				1			
26	*Psidium guajava* L. (Sotong) Myrtaceae	Medicine	1		1	1	3	2,29		1					LC
27	*Persea americana* Mill. (Alpukat) Lauraceae	Food, Medicine			1		1	0,76		1					LC
28	*Citrus aurantifolia* (Christm.) Swingle (Jeruk lengis) Rutaceae	Ceremony, medicine			1	1	2	1,53		1 1	1 1				
20	*Foeniculum vulgare* Mill. (Adas) Apiaceae	Food			1		1	0,76			1				LC
30	*Annona muricata* L.(Sirsak) Annonaceae	Medicine	1		1	1	2	1,53			1				LC
31	*Brugmansia* sp. (Kecubung) Solanaceae	Ornamental	1		1		2	1,53	1						
32	*Citrus maxima* (Burm.) Merr.(Jeruk Bali) Rutaceae	Ceremony, Medicine			1	1	2	1,53		1 1	1 1				LC
33	*Carica papaya* L.(Gedang) Caricaceae	Food			1	1	2	1,53		1					
34	*Saccharum officinarum* L. (Tebucemeng) Poaceae	Ceremony	1		1		2	1,53					1		
35	*Arenga pinnata* (Wurmb) Merr.(Aren) Arecaceae	Ceremony				1	1	0,76		1					

Table 1. Contd.

No	Species name (Local name) Family	Use	Areal				Total	FR (%)	Part of plant which usually used						Conservation status
			T	UM	MM	NM			Flower	Fruit	Leaf	Tuber	Stem	All part	
36	*Phyllanthus buxifolius* (Blume) Mull.Arg.(Kayu sisih) Phyllanthaceae	Ceremony, protector	1		1		2	1,53		1				1	
37	*Schefflera elliptica* (Blume) Harms (Kayutulak) Araliaceae	Ceremony, protector	1		1		2	1,53						1	LC
38	*Cordyline terminalis* (L.) Kunth (Andong gadang) Asparagaceae	ceremony	1	1	1		3	2,29			1				
39	*Pandanus* sp. (Pandan meduwi) Pandanaceae	Ceremony, protector			1		1	0,76			1			1	
40	*Erythrina hypaphorus* Boerl.ex Koord (Dadap) Fabaceae	Ceremony, medicine				1	1	0,76			1		1		
41	*Cocos nucifera* L. (Kelapa) Arecaceae	Ceremony, medicine	1			1	2	1,53			1				
42	*Dendrocalamus asper* (Schult.) Backer (Tiing Betung) Poaceae	Ceremony				1	1	0,76					1		
43	*Artocarpus integer* (Thunb)Merr. (Nangka) Moraceae	Ceremony food	1			1	2	1,53		1	1				
44	*Caryota mitis* Lour.(Uduh) Arecaceae	Ceremony	1		1		2	1,53			1				LC
45	*Manilkara zapota* (L.) P. Royen (Sabo) Sapotaceae	Ceremony	1			1	2	1,53		1					
46	*Syzygium* sp. (Jambu) Myrtaceae	Ceremony			1	1	2	1,53		1					
47	*Durio zibethinus* L. (Duren) Myrtaceae	Ceremony				1	1	0,76		1					
48	*Garcinia* x *mangostana* L. (Mangis) Clusiaceae	Ceremony, Medicine	1			1	2	1,53		1					

Table 1. Contd.

No	Species name (Local name) Family	Use	Areal				Total	FR (%)	Part of plant which usually used						Conservation status
			T	UM	MM	NM			Flower	Fruit	Leaf	Tuber	Stem	All part	
49	*Codiaeum variegatum* (L.) Rumph.ex A.Juss.(Puring) Euphorbiaceae	Ceremony	1	1	1		3	2,29			1				LC
50	*Nymphaea* sp. *(*Tunjung) Nymphaeaceae.	Ceremony			1		1	0,76	1						
51	*Graptophyllum pictum* (L.) Griff. (Temen) Acanthaceae	Ceremony	1	1	1		3	2,29			1				
52	*Ficus rumphii* Blume. (Ancak) Moraceae	Ceremony	1				1	0,76			1				
53	*Dracaena angustifolia* Roxb. (Kayusugih) Asparagaceae	Ceremony	1	1			2	1,53			1				
54	*Punica granatum* L. (Delima) Lythraceae	Ceremony			1		1	0,76		1					LC
55	*Citrus limon* (L.) Osbeck (Lemo) Rutaceae	Spices			1		1	0,76		1	1				
56	*Pandanus amaryllifolius* Roxb. (Pandan arum) Pandanaceae	Ceremony			1	1	2	1,53		1					
57	*Schizostachyum brachycladum* (Kurz) Kurz (Bambu tali) Poaceae	Ceremony				1	1	0,76					1		
58	*Syzygium polyanthum* (Wight) Walp. (Jangarulam) Myrtaceae	Spices				1	1	0,76			1				
59	*Dracaena draco* L. (Prakso) Asparagaceae	Ornamental	1		1		2	1,53						1	Vu
60	*Cyrtostachys lakka* Becc. (Palem merah) Arecaceae	Ornamental	1		1		2	1,53						1	

Table 1. Contd.

No	Species name (Local name) Family	Use	Areal				Total	FR (%)	Part of plant which usually used						Conservation status
			T	UM	MM	NM			Flower	Fruit	Leaf	Tuber	Stem	All part	
61	*Cyathea contaminans* (Wall. ex Hook.) Copel (Paku lemputu) Cyatheaceae	Orna-mental	1				1	0,76						1	
62	*Cycas rumphii* Miq. (Pakis Aji) Cycadaceae	Ceremony	1	1			2	1,53			1				NT
63	*Morinda citrifolia* L (Tibah) Rubiaceae	Medicine	1			1	2	1,53		1					
64	*Rhapis excelsa (Thunb.) Henry (Beregu)* Arecaceae	Orna-mental	1		1		2	1,53						1	
65	*Clerodendrum paniculatum* L. Lamiaceae	Orna-mental	1				1	0,76						1	
66	Sansiviera sp. Ruscaceae	Orna-mental			1	1	2	1,53						1	
67	*Cuphea lyssopifolia* Kunth (White) Lythraceae	Orna-mental				1	1	0,76						1	
68	*Ophiopogon jaburan* (Siebold) Lodd Aspragaceae	Orna-mental	1		1		2	1,53						1	
69	*Rosa* sp (Mawar) Rosaceae	Ceremony, Orna-mental			1		1	0,76	1						
70	*Coffea arabica* L. (Kopi) Moraceae	Food				1	1	0,76		1					En
	Total		39	16	49	27	131	100	15	22	24	3	5	11	

Note: T: Telanjakan, UM: Utama Mandala, MM: Madya Mandala, NM: Nista Mandala, FR: relative frequency. LC: Least Concern, NT: Near Threatened, En: Endanger

health center. This result in line with Sujarwo and Caneva, (2015) who found medicine function as number two plant function in a traditional village in Bali after vegetables.

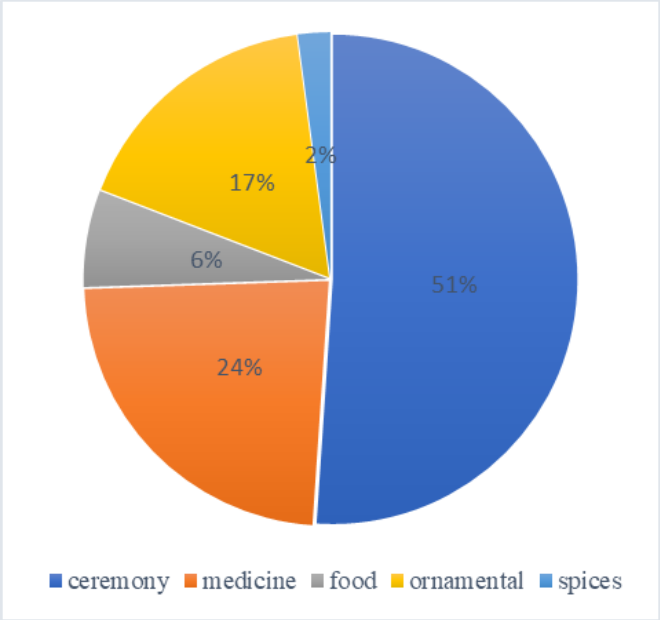

Figure 3. Plant species percentage base on function.

However, the abundance of species classified in three categories consists of high abundance with index is 2.29, middle abundance with index is 1.76 and low abundance with index is 1.53 (Table 1). High abundance means this plant species found in three areas, middle abundance means this plant species found in two areas and low abundance means this plant species only found in one area. The following fifteen species are with high abundance consist of *Cordyline fruticosa* (Local name (LN): Adong), *Cassia surattensis* (LN: Kembang kuning), *Allamanda cathartica* .(LN: Bunga ceblong), *Gardenia jasminoides* (LN: Jempiring), *Plumeria acuminata* (LN: Jepun), *Bougainvillea spectabilis* (LN: Kembang kertas), *Caesalpinia pulcherrima* (LN: Kemerakan), *Hibiscus rosa-sinensis* (LN: Pucuk bang), *Ixora coccinea* (LN: Soka), *Cordyline terminalis* (LN: Andong gadang), *Codiaeum variegatum* (LN: Puring), and *Graptophyllum pictum* (LN: Temen). These 15 species are abundant because they are found in more than one location in one house. This index related to the abundance of this plant which means this plant mostly planted in home gardens and support conservation concept because this plant can survive and used regularly for human life. Five of these abundant plants are included in the status of least concern at conservation status IUCN (*Cordyline fruticosa*, *Cassia surattensis Psidium guajava*, *Michelia champaca,* and *Codiaeum variegatum* (IUCN, 2020)), there are distributed mostly from Indonesia (Lim, 2015; GBIF, 2020). This result in accordance with Ambarani *et al.* (2017) who also found *Cordyline fruticosa* as the most abundant plant in Payangan home gardens. The result also shows that *madya mandala* is a space with the highest number of plant species, consists of 49 species (37%), followed by 39 species of *telajakan* (30%), 27 species of *nista mandala* (21%) and 16 species of *utama mandala* (12%) (Figure 4). *Madya mandala* and *telajakan* show the highest number of plant species because the area can be planted with plants from every category while *utama mandala* is only planted with ceremonial plants. This result in accordance with Yudantini (2012) who said that *telajakan* in indigenous villages in Bali is planted with spiritual and economic function. However, Kato *et al.* (2019) found *telajakan* plant function in northern Denpasar as aesthetic, economic, and ritual (ceremony).

This result also in accordance with Pranditha *et al.* (2018) which states that based on the *Tri Mandala* philosophy it is better to place plants whose flower parts are used in the ceremony are preferably planted in the *utama mandala* area because there is a family temple for praying located. This result also in line with Ambarani *et al.* (2017) who mention *madya mandala* in Payangan home garden has the highest plant number.

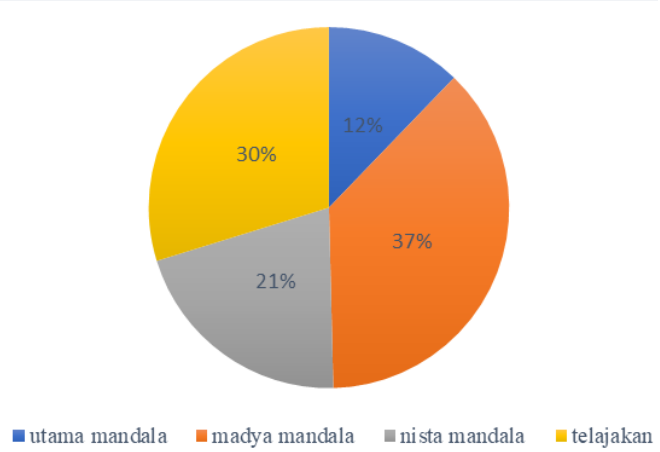

Figure 4. Plant species percentage on traditional architecture house based on *Tri Mandala* conception.

Tri Mandala concept relation with plant conservation and economic benefit

The structure and composition of vegetation in the home garden of Pekraman Penge village is a representation of the art of local community architecture in the processing of their home garden. The *Tri Mandala* concept is important to maintain because it has the meaning of socially, environmentally, and economically sustainable use (Aryani & Tanuwidjaja, 2013). The people in Pakraman Penge Village use more of the remaining land in their homes by planting plants for the needs

of Hindu religious ceremonies because all respondents are Hindu. This is in accordance with the definition of home gardens according to (Hakim, 2014) which states that the home garden is the land around the settlement which is managed by the family of the house owner intensively-semi-intensively to support the fulfillment of the diversity of needs of the homeowner that can be facilitated by the function of the home garden.

Ceremony plants can be found in *utama mandala*, *madya mandala*, *nista mandala,* and *telajakan*. Fruit plants such as *Musa paradisiaca*, *Carica papaya*, *Averrhoa carambola* found in *madya mandala* while screen plant such as *Artorcapus integra*, *Arenga pinnata*, *Dendrocalamus asper* found in *nista mandala*. Moreover, ceremony, medicine, and ornamental plant function are also found in *telajakan* (Table 1). This result not in accordance with Sardiana in Ambarani *et al.* (2017) who said ceremony plants should be planted at *utama mandala* because this location is a sacred place. However, the plant placement in *madya mandala* and *nista mandala* in accordance with Sardiana in Ambarani *et al.* (2017) who said *madya mandala* should be planted for fruit or flower tree and *nista mandala* should be planted with screen plant function. Furthermore, plant placement in *telajakan* accordance with Yudantini (2012) who said *telajakan* should be planting with aesthetic plants, rituals, and medicines.

In addition, several plants in Pakraman Penge village has conservation status such as *Dracaena draco* which has an ornamental function, has vulnerable conservation status found in *telajakan* and *madya mandala*, *Coffee arabica* which has a function as beverages, has endangered conservation status found in *nista mandala* and the last were *Cycas rumphii* which has a function for ceremonies has near threatened status found in *telajakan* and *utama mandala*. Mogea *et al.* (2001) said vulnerable status means this plant suffers a high risk of extinction in nature, Endangered status means this plant runs a very high risk of extinction. This founding shows that *Tri Mandala* concept in Pakraman Penge village has a role to conserve plant especially plant with conservation status.

CONCLUSION

This study documented the relationship between *Tri Mandala* concepts with plant conservation in the home garden of Pakraman Penge village. In all, 70 plant species were documented, 18 of them have conservation status. Most of the plant functions as ceremonial plants used in Balinese daily life. *Tri Mandala* concept able to support plant conservation in home gardens and give economic benefit

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Research Article

Ectoparasite Infestation among Stray Cats around Surabaya Traditional Market, Indonesia

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ABSTRACT

This study was conducted to determine the prevalence of ectoparasite infestation among stray cats around Surabaya traditional markets. A total of 305 stray cats were collected around 17 traditional markets in Surabaya City and were examined for the presence of fleas with a fine-toothed flea comb. Surveys were conducted during May-June 2019. 228 of 305 stray cats (74.75%) were infested with one species of ectoparasite. The average number of *C. felis* in every cat was 2.54, while the number of *F. subrostratus* in every cat was 0.33. Additional data about the gender, pregnancy/ maternity, and bodyweight of every cat were recorded. The result of chi-square test shows that there is a significant difference between gender, pregnancy status, and bodyweight by the occurrence of ectoparasites (p=0.008; p=0.00; p=0.00). A total of 878 ectoparasites consisting of flea and lices, namely *Ctenocephalides felis* (88.27%) as the dominant ectoparasite, followed by *Felicola subrostratus* (11.73%). The highest infection rate (prevalence) of ectoparasite was found in Pucang Market (16.81%), while the lowest prevalence was found in Mulyorejo Market (0.8%). Coinfection was observed in only a few cats (1.63%). Multiple Regression showed that pregnancy is the most influential factor in the occurrence of fleas (p=0.000). These results should be taken into account among health workers to prevent a possible outbreak of zoonotic diseases caused by fleas.

Keywords: *Ctenocephalides felis*, ectoparasite, *Felicola subrostratus*, market

INTRODUCTION

Zoonotic infectious diseases caused by bacteria, viruses, and parasites that are transmitted from animals to humans are still some of the major public health problems. Tick fever, mange, leishmaniasis, and ascariasis are the diseases that often infect domestic animals, such as cats and dogs, and have the potential to spread to humans (Colombo *et al.,* 2011). Ectoparasites, as a group of animals in the Arthropoda phylum, cause the manifestation of skin diseases in dogs and cats (Akucewich *et al.,* 2002).

The common cause of skin disorders and anemia is blood-sucking, and the main consultations in small animal practice are ectoparasite infestations, especially flea infestations (Dyrden & Rust, 1994). *Ctenocephalides felis* is a flea that can transmit a tapeworm *Dyplidium caninum* (Pugh, 1987). Epidemiological surveys were already reported worldwide, but Indonesia is still limited. Only one study that reported ectoparasite distribution in the dogs from Indonesia, showing that *Rhipicephalus sanguineus* was the most manifested tick (Hadi & Soviana, 2015). Study in the USA also reported *Rhipicephalus sanguineus* as a predominant tick in dogs with the prevalence of 94.3%, and *Amblyomma americanum* as a predominant tick in cats with the prevalence of 74% (Burroughs *et al.,* 2016). In addition, studies in the USA showed a high prevalence of ectoparasite in cats caused by fleas (*Ctenocephalides felis*, *Pulex* spp., *Cediopsylla simplex*, and

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Nosopyllus fasciatus) and ticks (*Amblyomma americanum*, *Ixodes scapularis*, *Dermacentor variabilis*, *Rhipicephalus sanguineus*, and half of them were still in immature stages (Thomas *et al.,* 2016).

Some studies reported that *C. felis* is the most common external parasites found on dogs and cats, such as a report from New Zealand that showed mostly cat and dog infected by *C.felis* (Chandra *et al.,* 2017). This is also supported by studies in Nigeria (Omonijo & Sowemimo, 2017) and Ethiopia (Kumsa *et al.,* 2019). *Ctenocephalides felis* is known as a blood-feeder and an important vector of various pathogens, most of which are zoonotic such as *Yersinia pestis*, *Rickettsia typhi, R. felis, R. conori, Bartonella clarridgeiae*, and *B. henselae* (Beugnet & Marié, 2009; Boudebouch *et al.,* 2011; Chandra *et al.,* 2017; Lappin & Hawley, 2009; Shaw *et al.,* 2004). Frequent flea species reported from some countries such as in Germany were *C. felis*, *C. canis*, and *Archaeopsylla erinacei* (Visser *et al.,* 2001). A study in Mexico showed both *C. felis* and *C. canis* were manifested on dogs and cats (Cruz-Vazquez *et al.,* 2001). Four subspecies were already identified such as *C. felis damarensis*, *C. felis strongylus* that were mostly found in East Africa, *C. felis orientis* that was found in Australia and India, *C. felis felis* that spread in all continents except Antarctica (Shakya *et al.,* 2019).

Recent studies have certainly shown zoonoses in companion animal (Dantas-Torres & Otranto, 2014; ElSeify *et al.,* 2016; Kumsa *et al.,* 2019; Thomas *et al.,* 2016). However, it needs to elevate knowledge about the prevention and management of companion animals. Companion animals or pets could be a new potential health threat due to the frequent interaction with humans (Diakou *et al.,* 2017). Stray cats are almost found in many locations, including Surabaya as an urban area that is located in the East Java Province, Java Island. Surabaya has many traditional markets to support the daily needs of citizens. Markets were chosen as study areas regarding the possibilities of stray cats living in direct contact with human food. Markets and food courts are often visited by stray cats to support their survival. This different geographical area could lead to a different distribution of flea species. Thus, this study aimed to investigate the infestation of flea among stray cats around Surabaya traditional markets.

MATERIALS AND METHODS

Study Area

The survey was conducted from May to June 2019 in 17 traditional markets in Surabaya. Detail of coordinate locations can be seen in Table 1.

Table 1. Detail of sampling location.

No	Name of Market	Coordinate
1	Dinoyo	-7.937004, 112.608421
2	Gubeng	-7.264635, 112.752541
3	Pacar Keling	-7.259755, 112.759060
4	Karang Menjangan	-7.269295, 112.760920
5	Manyar	-7.280465, 112.762291
6	Pandegiling	-7.276136, 112.734760
7	Ngagel	-7.291142, 112.746650
8	Pucang	-7.283782, 112.753590
9	Banyu Urip	-7.274693, 112.720839
10	Simo	-7.267122, 112.713544
11	Jojoran	-7.272445, 112.766158
12	Menur	-7.280580. 112.762244
13	Keputih	-7.289643, 112.799469
14	Mulyorejo	-7.263779, 112.775044
15	Blauran	-7.256133, 112.733423
16	Asemrowo	-7.252092, 112.715279
17	Indrakila	-7.260296, 112.755938

Ectoparasite Collection

Random sampling was conducted in each market, in which samples were chosen by surrounding all market areas. Each cat was examined for the presence of ectoparasites by combing their fur using a fine-toothed flea comb for 5 min for each cat (Zakson *et al.,* 1995). Ear swabs were also conducted with an additional time of 5 min. Once the combing was completed, flea combs were placed in a white tray and ectoparasites fell into the tray covered by white paper. Each ectoparasite was then separately placed into a vial bottle filled with 70% ethanol for species identification. Afterward, the vial bottle was labelled with the number of cats, details of location, the name of the collector, and the time of collection. The collectors also recorded the gender, maternity, and bodyweight of each cat. When the cats had been checked, they were marked with a red rope around their neck to avoid double sampling.

Laboratory Examination

Samples were kept in 70% ethanol for identification. Samples were brought to the Laboratory of Animal Histology, Biology Department, Faculty of Science and Technology, Universitas Airlangga. Each sample was immersed in a slightly warm 5% potassium hydroxide (KOH) solution for 10-15 min. Then, samples were placed in 35% alcohol solution for 5 min to adjust pH, then moved to the series of 50, 70, 90, 95, and 100% ethyl alcohol solutions for dehydration for 5 min, respectively. After that, samples were cleared in xylene twice for 5 min to obtain transparency. The processed samples were mounted in Entellan® new 107961 Merck Millipore on microscope slides then they were identified to the species level under a stereomicroscope. Identification was made using the keys of the CDC flea identification key (2019) and the keys in the following references (Bowman *et al.,* 2002; Lewis, 1966; Soulsby, 1982; Wall *et al.,* 1997).

Statistical Analysis

Statistical analysis was done using SPSS IBM version 21. Chi-square test was used to analyze the difference between gender, bodyweight, and pregnancy status by the occurrence of ectoparasite. Significance levels were noted if p-value shows equal to or less than 0.05. Multiple Regression was applied to find out which factors were most influential on the occurrence of ectoparasite. The most influential factor is the factor that has the smallest p-value and the largest odds ratio among the other. Distribution of fleas was also figured out using ArcGIS 10.3 version.

RESULTS AND DISCUSSION

Distribution of ectoparasite that infected stray cats in Surabaya traditional markets

The infection rate of stray cats with ectoparasites from the study area was 74.75% of the 305 cats. A total of 878 ectoparasites were found, consisting of 775 *Ctenocephalides felis* (88.27%), 103 of *Felicola subrostratus* (11.73%) shown in Table 2. Almost all (99%) cats have a single infection and co-infection was seen in only five cats (1.63%). Coinfection was found in four study areas namely Pandegiling, Ngagel, Banyu Urip, and Jojoran (Table 3). The value of bodyweight was categorized by cut-off points. Cut-off points of bodyweight were determined by the roc curve. The optimal cut off is 2.87. If bodyweight > 2.87, bodyweight is classified as high. There was a significant relationship between bodyweight and the presence of ectoparasites (p = 0.00). There was a significant relationship between the gender of cats and the presence of ectoparasites (p = 0.008). Pregnancy also had a strong relationship with the presence of ectoparasites with a significance level (p = 0.00). All of them were proven by the Chi-square test (Table 4).

Multivariate tests showed that female cats were more highly infected than male cats (P=0.004; OR=2.896). Low Bodyweight was more highly infected than a high bodyweight cat (P=0.005; OR:2.988). A Pregnant cat was more highly infected than an unpregnant cat (P=0.000; OR:6.789). Among three variables, pregnancy factor was the most influential factor in the occurrence of ectoparasite because it had the smallest p-value and larger odds ratio than the other variables. All of

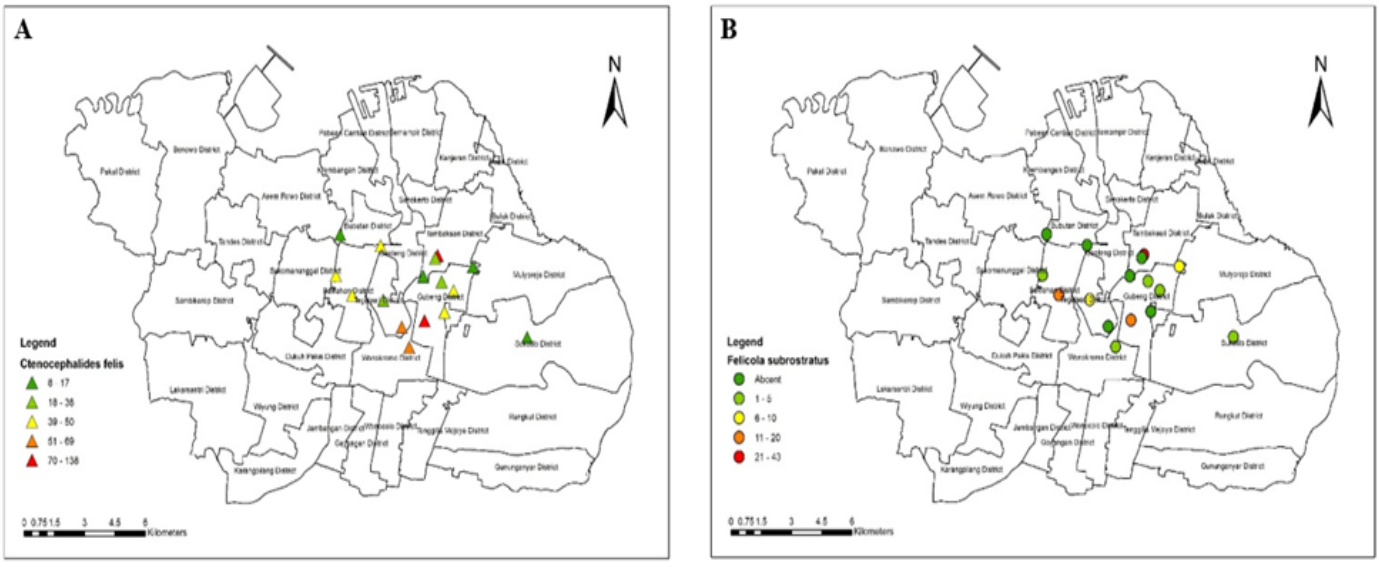

Figure 1. Mapping of *Felicola subrostratus* (A) and *Ctenocephalides felis* (B) distribution using ArcGIS 10.3 version: dark green dot indicates that *Felicola subrostratus* was not found; green dot indicates flea was found from one to five; yellow dot indicates flea was found from six to ten; orange dot indicates flea was found from eleven to twenty; red dot indicates flea was found from twenty-one to forty-three (Source: ArcGIS 10.3 version).

Table 2. Number of cats and ectoparasites collected in each study area.

Number	Name of Market	Number of Cats examined	Number of positive cats	Percentage of positive cats (%)	Number of Ectoparasites	Number of *C. felis*	Percentage of *C.felis*(%)	Number of *F. subrostratus*	Percentage of *F.subrostratus*
1.	Dinoyo	16	16	100	61	61	100	0	0
2.	Gubeng	12	4	33.3	15	15	100	0	0
3.	Pacar Keling	41	30	73.17	159	116	72.59	43	37.06
4.	Karang Menjangan	12	11	91.67	30	29	96.67	1	3.33
5.	Manyar	12	8	66.67	14	13	92.87	1	7.1
6.	Pandegiling	14	13	100	47	38	80.85	9	19.1
7.	Ngagel	31	18	58.06	70	69	98.57	1	1.43
8.	Pucang	48	39	81.25	155	138	89.02	17	10.96
9.	Banyu Urip	26	21	80.76	52	41	78.84	11	21,16
10.	Simo	12	8	66.67	51	46	90.19	5	9.8
11.	Jojoran	16	14	81.25	48	47	97.91	1	2.08
12.	Menur	10	9	100	46	46	100	0	0
13.	Keputih	10	6	70	13	8	61.5	5	38.46
14.	Mulyorejo	10	2	20	17	8	47.05	9	52.95
15.	Blauran	16	14	100	50	50	100	0	0
16.	Asemrowo	8	5	62.5	17	17	100	0	0
17.	Indrakila	10	10	100	33	33	100	0	0
Total	17 markets	305	228	-	878	775		103	-

them were proven by the Multiple binary regression test (Table 5). The distribution of *Felicola subrostratus* in the sampling site was shown in Figure 1A, whereas the distribution of *Ctenocephalides felis* was shown in Figure 1B.

This study was the first report from Surabaya, Indonesia, and it strongly indicated a high infection rate of flea *Ctenocephalides felis* (85.6%) and a low infection rate of *Felicola subrostratus* (14.4%) on the stray cats around traditional markets. Morphology of flea found among stray cat populations show in Figure 2. The average percentage of infection caused by findings are in line with recent worldwide studies that show *C. felis* as predominant species infected stray cats (Chandra *et al.,* 2017; Kumsa *et al.,* 2019; Omonijo & Sowemimo, 2017). Fleas can act as important vectors of diseases and can produce troublesome bites. *Ctenocephalides felis* is the most common nuisance fleas in cats distributed worldwide. This species can lay their eggs up to 25

Figure 2. Flea species found among stray cat populations A. *Ctenocephalides felis* and B. *Felicola subrostratus* observed with a stereo microscope with magnification 10x.

Table 3. Prevalence of coinfection in cats collected from every study site.

Number	Name of Market	Number of Cats examined	Number of positive cats	Infection rate within all study site (%)	Number of cats with single infection	Percentage of single infection (%)	Number of cats with double infection / coinfectio	Percentage pf coinfection (%)
1.	Dinoyo	16	16	6.89	16	100	0	0
2.	Gubeng	12	4	1.7	4	100	0	0
3.	Pacar Keling	41	30	12.9	30	100	0	0
4.	Karang Menjangan	12	11	4.74	11	100	0	0
5.	Manyar	12	8	3.44	8	100	0	0
6.	Pandegiling	14	13	6.03	12	92.85	1	7.15
7.	Ngagel	31	18	7.75	17	94.44	1	5.56
8.	Pucang	48	39	**16.81**	39	100	0	0
9.	Banyu Urip	26	21	9.05	20	95.23	1	4.77
10.	Simo	12	8	3.44	8	100	0	0
11.	Jojoran	16	14	5.6	12	84.61	2	**15.39**
12.	Menur	10	9	4.3	9	100	0	0
13.	Keputih	10	6	3.01	6	100	0	0
14.	Mulyorejo	10	2	**0.8**	2	100	0	0
15.	Blauran	16	14	6.89	14	100	0	0
16.	Asemrowo	8	5	2.15	5	100	0	0
17.	Indrakila	10	10	4.3	10	100	0	0
Total		305	228		223	-	5	-

Table 4. Chi-square Test between independent variable and ectoparasite manifestation.

Variable			df	Total	Ectoparasites				p-value
					Negative (n=77)		Positive (n=228)		
					N	%	n	%	
Gender	Male		1	91	54	59.3	37	40.7	
	Female	Pregnant	1	134	5	3.7	129	96.3	=0.000
		Unpregnant	1	80	18	22.5	62	77.5	
Bodyweight	Low (< 2.85)		1	171	72	42.1	99	57.9	=0.000
	High (>= 2.85)			134	5	3.7	129	96.3	

Table 5. Multiple binary regression model of factors associated with ectoparasite manifestation.

Variable	Odds ratio	df	95% confidence interval		Standard error	P-value
			Lower	Upper		
Female vs Male	2.896	1	1.407	5.960	0.368	0.004
Low vs high weight body	2.988	1	1.403	6.354	0.386	0.005
Pregnant vs unpregnant	6.789	1	2.383	19.339	0.534	=0.000

eggs a day during a month so that the prevalence of ectoparasite still exist (Service, 2008).

The high infection rate of *Ctenocephalides felis* (85.6%) in this study is similar to other studies in Iran (ElSeify *et al.,* 2016); Israel (Salant *et al.,* 2014), and Nigeria (Omonijo & Sowemimo, 2017). The infection rate of this species was reported worldwide and varied, 25,6% in United Kingdom (Abdullah *et al.,* 2019), and 20.68% in Iraq borderline area (Bahrami *et al.,* 2012). In this study, we didn't check DNA samples of flea, while another survey in UK showed that most *C. felis* contained pathogens such as *Bartonella henselae*, *Bartonella clamidgeiae*, *Dipylidium caninum*, *Mycoplasma haemofelis*, and *Mycoplasma haemocanis* (Abdullah *et al.,* 2019). Urban area in Cuernavaca, Mexico, shows infection rate about 92.3% (Cruz-Vazquez *et al.,* 2001), United Kingdom during 2005 was 98.93% (Bond *et al.,* 2007), and Greece was 97.4% (Koutinas *et al.,* 1995). This survey was conducted during dry season, and the findings are in line with other studies showing *C. felis* as a predominant flea species during all seasons (Akucewich *et al.,* 2002; Chesney, 1995; Clark, 1999). This is also supported by the study result in urban areas in Germany (Liebich *et al.,* 1985; Visser *et al.,* 2001) and Denmark (Kristensen *et al.,* 1978).

Ctenocephalides felis was also reported as an ectoparasite that infected many mammals other than cats and dogs, such as red foxes (*Vulpes vulpes*), black rats (*Rattus rattus*), European rabbits (*Oryctolagus cuniculus*), and brown rats (*Rattus novergicus*). Meanwhile, in native species, *C. felis* was known infecting American opossums (*Virginia opossum*, *Didelphis virginianam*, *Didelphis marsupialis*); North American gray foxes (*Urocyon cinereoargenteus*), and Australian brushtail possums (*Trichsuorus vulpecula*) (Clark *et al.,* 2018).

Coinfection in five cats that were examined shows the distribution of *Felicola subrostratus*. This is in line with the survey reported in Greece and UK (Bond *et al.,* 2007; Koutinas *et al.,* 1995). Common coinfection was reported in the studies in Mexico and Germany (Beck *et al.,* 2006; Bond *et al.,* 2007; Cruz-Vazquez *et al.,* 2001). The species infecting found from the investigations on England include *Pulex irritans* coinfected with *C. felis* (Bond *et al.,* 2007). The low prevalence of *Felicola subrostratus* was in line with the previous investigations in Brazil (De Castro & Rafael, 2006; Morales-Malacara & Guerrero, 2007). The prevalence of *Felicola subrostratus* was higher than in United States which was only 1% (Thomas *et al.,* 2016); Florida roughly 1% (Akucewich *et al.,* 2002), and Thailand (4.2%). On the contrary, this prevalence was less than the investigations conducted by Salant *et al.* (2014) in Israel (14.4%). Increased prevalence may be because of different habitats between two populations.

This study highlighted the average number of *C. felis* in every cat was 2.54, while the average number of *F. subrostratus* was 0.33. The prevalence of *F. subrostratus* is not common, supported by low prevalence that has been reported from all continents, from Asia (Amin-Babjee, 1978; Eduardo *et al.,* 1977; Mustaffa-Babjee, 1969; Shanta, 1982), Europe (Trotti *et al.,* 1990), and Australia (Coman *et al.,* 1981). Highly infection rate of ectoparasites was more common in female stray cats (62.62%) than male stray cats (37.3%); this finding is supported by the study results from Sahimin (2012) in Kuala Lumpur. Sahimin (2012) also found *Ctenocephalides felis, Felicola subrostratus*, *Heterodoxus spiniger*, *Haemophysalis bispinosa*, and *Lynxacarus radovskyi* in their survey; and found that female stray cats were more likely to be infested with ectoparasites than in male stray cats (OR 2.8; $p<0.004$) (Aldemir, 2007). The prevalence of ectoparasite infestation was higher in female than male stray cats (89.3 % and 40%, respectively). This finding is in line with the study in Ismailia city which reported a greater ectoparasitic infestation in female stray dogs (AbuZeid *et al.,* 2015). Although there was no significant association between ectoparasitic infestation with sex, females domestic dogs from Erzurum, Turkey, tended to be more frequently infected by ectoparasites, especially by *C. canis* (Aldemir, 2007). It is believed some female behavioral factors would be responsible for this tendency, such as confining of female pets during the reproductive period that could favor re-infections by fleas in domestic areas (Aldemir, 2007).

Season and environmental factors affected the various prevalence manifestation of ectoparasite (Dyrden & Rust, 1994). The high prevalence of ectoparasite in this study may be affected by the dry season. Studies from Sahimin (2012) shows high prevalence of ectoparasite during dry season than in rainy season. Insemination and fertilization of flea can be affected by the host's body temperature and the occurrence of food around the host (Dean & Meola, 2002). The optimum temperature for the fertilization of fleas was 38°C, meaning the common temperature of cat and dog (Yue *et al.,* 2002). *C. felis* has a specific ability that supports it to move from one infected-host to each other with an average jumping speed of 3.6 m/s, jumping height of 13.2 cm, and jumping length of 19.9 cm (Cadiergues *et al.,* 2000). Association between bodyweight and the occurrence of ectoparasite in this study with p-value =0.000 shows the possibility of fleas jumping from one cat to another cat. This specific ability can also lead to the movement of fleas to humans regarding direct contact in traditional markets. The movement

speed of each cat is different from each other and can be affected by some factors, such as pregnancy status. In this study, we found that pregnancy status shows a positive association with the occurrence of flea (p-value=0.000).

Market as a place that provides possible direct contact between flea-infected stray cats and humans must be considered regarding the occurrence of flea-borne diseases. Since *C. felis* found with high infection rate has been shown to transmit murine typhus and also has been implicated as a vector of plague, *Bartonella henselae*, which is the etiologic agent of cat scratch disease (Dyrden and Rust, 1994; Jameson *et al.,* 1995; Schrierfer *et al.,* 1994; Sorvillo *et al.,* 1993). This finding should be a baseline for flea management control. Flea allergic dermatitis is the most common nuisance caused by fleas in cats and dogs (Lee *et al.,* 1997). Those fleas can also bite humans and cause heavy inflammation (Youssefi and Rahimi, 2014). Six students from Malaysia were reported to be affected by flea allergic dermatitis (Chin *et al.,* 2010).

The high infection rate of stray cats with ectoparasites was affected by the high temperature and humidity of Surabaya which is 68%-84%, with a temperature of 27.8°C and 30.5°C. Reproductivity of flea will increase in humidity range of 80% and temperature of 27°C (Silverman *et al.,* 1981). The possibility of fleas to infect humans must be a consideration (O'Neal *et al.,* 2014). Serologic examination on stray cats in Yunani showed infection with some pathogens, such as *Bartonella henselae* (58,8%), *Rickettsia* spp. (43,2%), *Leishmania infatum* (6,1%), *Ditofilaria immitis* (4,7%), and *Ehrlichia canis* (2%) (Diakou *et al.,* 2017). However, the prevalence of flea-borne disease in stray cats still get limited consideration among health workers due to insufficient information about zoonotic diseases. The distribution of *C. felis* was mostly not affected by global warming (Roy *et al.,* 2009). This study was also supported by Maina *et al.* (2016) who have found 37.2% of squirrel and cats were infected with *C. felis*. Billeter and Metzger (2017) argued the possibility of fleas as a vector of *R. typhi*, but still not completed with the data distribution in humans so that additional study is important to reveal any association between murine typhus and flea. The lesion caused by cat's paws results in cat scratch disease (CSD) that is brought by *C. felis* (McElroy *et al.,* 2010), but the prevalence among cats is still unclear. Laboratory studies showed *C. felis* as the secondary vector of *Yersinia pestis*, though the efficiency was not as high as *Xenopsylla cheopis* (Eisen *et al.,* 2008). During the plague investigation in Uganda, Eisen *et al.* (2008) found that *C. felis* was the main fleas in rodents. In addition, *C. felis* is also known as a vector of a flea tapeworm, *Dipyridium caninum*. Humans can be infected if they ingest cysticercoids of *D. caninum*. High prevalence was associated with the occurrence of infected dogs or cats as their pet (Pan American Health Organization, 2003).

The importance of identifying flea in companion animals due to the role of the flea to transmit pathogens to humans with the historical note resulting in human plagues and black death (Bubonic Plague) (Gubler, 2009) and many impacts of the occurrence of flea in the environment such as nuisance, anemia, allergic reactions, and discomfort (Iannino *et al.,* 2017).

CONCLUSION

It can be concluded that the high prevalence of ectoparasites on the stray cats in Surabaya traditional markets must be a consideration among health workers as early mitigation and prevention of vector-borne diseases. Serologic and molecular test for the pathogens in stray cats should be conducted for the early detection of vector-borne zoonotic diseases.

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Research Article

Leaf Vein Density of Tree Saplings Composing Lower Canopy in Tropical Forest Reflects Their Ecophysiological Characteristics

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ABSTRACT
One factor affecting the survival of a species in a tropical ecosystem is its ability to respond to environmental conditions, which depend on their ecophysiological performances. Plants ability to transport water as a major environmental factor would determine their survival. The anatomy of xylem inside leaves and stem as water conductive tissue will dictate the rate of water transport through the plant stem and leaves. Leaf vein, which contains xylem vessels, dictates water transport through leaves and plant's ability to control water loss through stomata. This research found that tree saplings composing a lower canopy of tropical forests have different ecophysiological attributes. Pioneer species, such as *Cinnamomum* sp., *Diospyros macrophylla*, *Castanopsis costata*, *Elateriospermum tapos,* and *Ziziphus* sp., have higher leaf vein density than primary species, such as a member of genus *Garcinia*, *Shorea*, *Dipterocarpus,* and *Syzigium*. It implies that pioneer species might have higher rates of water transport and consequently, higher rates of photosynthesis. If forest vegetation was more opened, then pioneer species may dominate the area as they are more tolerant of light. The Composition of forest vegetation with different ecophysiological characteristics may affect the forest dynamics and hydrological cycle.

Keywords: Ecophysiology, leaf vein density, tropical forest ecosystem, water transport

INTRODUCTION
The efficiency of water to be transported in plants from the soil to the stem and then leaves partly affects plants ability to survive in their environments. This efficiency is driven by several aspects, i.e. the capacity of roots to absorb water from the ground, the rate of water movement in the xylem to the canopy, and the effectiveness of plants to control transpirational water loss from the stomata (Atwell *et al.,* 1999). The structural design of xylem, which is the water conductive tissue, will dictate how water is transported through the stem (Tyree & Zimmerman, 2002). Therefore it will drive water transport efficiency (Tyree & Ewers, 1996). The design of xylem includes dimensions of the vessels, hydraulic conductivity, and vulnerability to the formation of embolism. Hydraulic conductivity is the rate at which water can be transported through xylem at a given pressure (Tyree & Ewers, 1996). As the structural design of xylem may affect the flow of water from the root to the stem and finally to the leaf, it may consequently dictate stomatal conductance, leaf gas exchange, and water potential.

Variation in hydraulic architecture of plants may partially affect the height that can be attained by the plant and their distributions along environmental gradients and (Tyree & Ewers, 1996). Plants with different growth forms, such as epiphytes, vines, and trees have different hydraulic architecture characteristics that result in different ecological and physiological adaptations. Hydraulic architecture of woody plants, such as lianas, primary hemi-epiphytes, shrubs, and trees have been extensively studied (Drake & Franks, 2003; Ewers *et al.,* 1991; Patiño *et al.,* 1995; Tng *et al.,* 2018; Tyree & Ewers, 1996; Tyree & Zimmerman, 2002). Studies on hydraulic architecture of non-woody plants,

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especially climbing plants, have also been conducted (Ganthaler *et al.,* 2019). These studies include observation of vessel size and density and hydraulic capacity of species of climbing rattans (Fisher *et al.,* 2002; Tomlinson *et al.,* 2001), climbing aroids *Monstera acuminata* (Lopez-Portillo *et al.,* 2000), and invasive climbing species *Merremia peltata (Yansen et al., 2015).* Embolism in palm xylem *Rhapis excelsa* (Sperry, 1986) and the vine *Rhipidocladum racemiflorum* (Cochard *et al.,* 1994) due to drought condition has also been observed.

However, published researches so far on plant hydraulic architecture were more focused on the individuals and species/group of species levels or growth forms. The contribution of plant hydraulic variations on spatial vegetation dynamics has not been widely discussed. As the hydraulic efficiency could affect plants ability to survive and to regulate water loss from the leaf, information on spatial distribution of hydraulic characteristics of plants composing tropical forests can be used to predict future vegetation dynamics and perhaps its effect on the hydrological cycle.

Leaf vein architecture as part of plant hydraulic has been studied for the last two decades. It has received more attention as it is linked to the physiology, ecology, and evolution of terrestrial plants (Price *et al.,* 2014; Sack & Holbrook, 2006). Leaf vein architecture, including vein size and density, and hydraulic conductivity, might play as the main constraint in water transport for photosynthesis and transpiration. If this architecture could restrict water transport, then the evolutionary strategy to form more adaptive leaf vein architecture for certain environmental conditions may dictate the fitness of certain species (Boyce *et al.,* 2009; Tabassum *et al.,* 2016). Leaf hydraulic capacity is very much related to the ability of species to utilize water and to exchange carbon in different habitat and vegetational zones (Sack *et al.,* 2005; Bodribb *et al.,* 2007; Pagano & Storchi, 2015). Therefore, leaf hydraulic architecture is crucial information in observing forest vegetation dynamics, as well as predicting the response of vegetation to environmental changes. This research aimed to observe leaf vein characteristics of tree saplings composing a lower canopy of tropical forests. Those characteristics were then related to their ecophysiological characteristics, such as light-demanding pioneer species and shade-tolerant primary species (Goodale *et al.,* 2012; Whitmore, 1998).

MATERIALS AND METHODS

This research was conducted in the protected forest of Boven Lais Kemumu, North Bengkulu, Bengkulu Province, Indonesia, which is located between 102° 11'50" - 102°25'40" E (east longitude) and 3°15'24" - 3°33'15" S (south latitude). This forest has a high biodiversity of plants with different characteristics. Twenty plots of 10 x 10 m were placed systematically from the forest edge into the intact area. These 20 plots were put on four lines; hence one line consisted of five plots with 40 m distance between plots.

All tree saplings in every plot were recorded and tagged. Tree saplings are categorized as to have < 10 cm dbh (diameter of breast height), and > 3 m tall. Sapling diameter and height were measured. Tree saplings were chosen as the object of this research since it is assumed that saplings are on their optimum growth and they will dominate the ecosystem in the future. Ten fully expanded leaves of each sapling were taken as samples to be analysed their leaf vein characteristics.

Leaf samples were stored in a container containing alcohol. Those leaves were then cleaned with NaOH and water. Fractions of leaves were placed under a microscope (Olympus) and photos were taken. Leaf vein characteristics were observed using ImageJ software (National Health Institute, USA). Observed leaf vein characteristics include leaf vein level, and leaf vein density per area (mm/mm^2). Environmental conditions were also monitored, including humidity, temperature, and light intensity under the canopy.

RESULTS AND DISCUSSION

The location of the research had relatively dense canopy cover with varied vegetational strata from seedlings to trees. The measurement of environmental conditions shows that observed tree saplings grow under a canopy with high humidity, mild temperature, and low light intensity. No differences in humidity, light intensity, and temperature between forest edge and intact vegetation (Figure 1).

Twenty fives species (13 families) of tree saplings were found on the location (Table 1). *Garcinia* and *Cinnamomum* were two commonly genus found on the location. Tree saplings compose the lower stratum of the forest. The range of the diameter of observed saplings was 4 cm to 9 cm and the range of height was 4 m to 8 m.

Tree saplings were distributed from the forest edge into a more intact canopy. Species such as *Cinnamomum* sp., *Garcinia* sp., *Dipetrocarpus gracilis,* and *Exoecaria bantamensis* were found from forest edge into intact canopy area (Table 2). As explained before, no differences in humidity, light intensity, and temperature between forest edge and intact vegetation (Figure 1). Many factors may affect the

Table 1. Species and number of saplings per species found at study plots in the protected forest of Boven Lais Kemumu.

No	Species	Family	Number of individuals
1	*Artocarpus heterophyllus*	Moraceae	1
2	*Azadirachta* sp.	Meliaceae	6
3	*Castanopsis costata*	Fagaceae	4
4	*Cinnamomum obtusifolium*	Lauraceae	4
5	*Cinnamomum* sp. 1	Lauraceae	13
6	*Cinnamomum* sp. 2	Lauraceae	2
7	*Diospyros macrophylla*	Ebenaceae	4
8	*Dipterocarpus gracilis*	Dipterocarpaceae	5
9	*Elateriospermum tapos*	Euphorbiaceae	2
10	*Elmerillia tsiampacca*	Magnoliaceae	2
11	*Excoecaria bantamensis*	Euphorbiaceae	8
12	*Fragraea racemosa*	Loganaceae	3
13	*Garcinia* sp. 1	Clusiaceae	10
14	*Garcinia* sp. 2	Clusiaceae	2
15	*Lannea coromandelica*	Anarcadiaceae	1
16	*Litsea* sp.	Lauraceae	2
17	*Macaranga gigantea*	Euphorbiaceae	2
18	*Shorea leprosula*	Dipterocarpaceae	1
19	*Shorea multiflora*	Dipterocarpaceae	1
20	*Shorea siamensis*	Dipterocarpaceae	4
21	*Syzigium oides*	Myrtaceae	3
22	*Syzigium* sp.	Myrtaceae	5
25	*Ziziphus* sp.	Rhamnaceae	1

development of vegetation in a tropical forest, e.g. intra and inter-specific competition, predation, *niche* differentiation, disturbances, and stochastic recruitment (Brokaw & Busing, 2000; Goodale *et al.,* 2012; Nathan *et al.,* 2008; Silvestrini & dos Santos, 2015). With relatively similar environmental conditions in most of the studied forest ecosystem, every species would have similar opportunities to grow both on the edge or more to the middle part of the forest.

The level of leaf vein of observed tree saplings ranges between 3 to 5 levels (Figure 2). Saplings with more leaf vein levels usually have more complex vein arrangement (Figure 3a-c), although they do not necessarily have more dense veins. On the other hand, some other species have a simple leaf vein arrangement (Figure 3d). The range of leaf vein density of observed saplings was 0.02 to > 0.3 mm/ mm^2 (Figure 2). Leaf vein density of 0.3 mm/mm^2 means that 30% of the leaf area consists of veins. In this research, species found to have high leaf vein density include *Cinnamomum* sp., *Diospyros macrophylla*, *Castanopsis costata*, *Elateriospermum tapos,* and *Ziziphus* sp. On the other hand, *Garcinia*, *Shorea*, *Dipterocarpus,* and *Syzigium* tend to have low vein density.

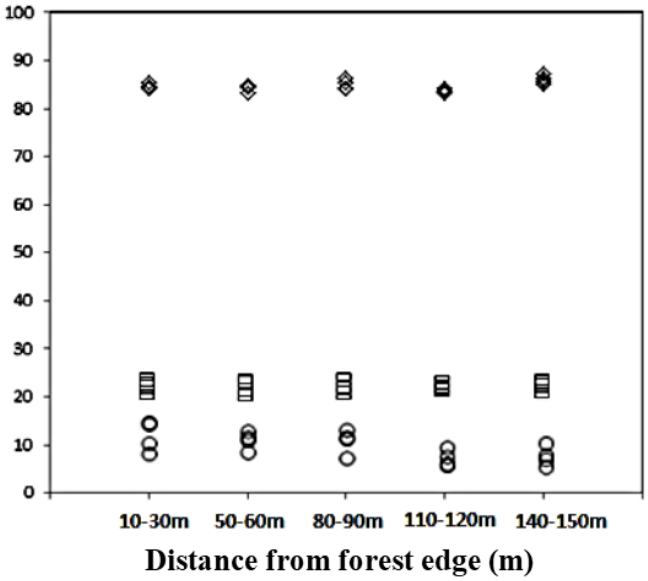

Figure 1. Humidity (◊) (%), temperature (□) (˚C), and light intensity (○) (Watt/m^2) of research site with different distances from the forest edge.

Based on their ecophysiological characters, tree sapling species occurring on the studied area can be categorized as pioneer and primary species, following characterization by Whitmore (1998).

Table 2. The distribution of species based on their distance from the forest edge.

Distance from forest edge (m)				
10 - 30 m	50 - 60 m	80 - 90 m	110 - 120 m	140 - 150 m
Castanopsis costata	*Castanopsis costata*	*Artocarpus heterophyllus*	*Azadirachta* sp.	*Azadirachta* sp.
Castanopsis costata	*Cinnamomum obtusifolium*	*Azadirachta* sp.	*Azadirachta* sp.	*Azadirachta* sp.
Castanopsis costata	*Cinnamomum obtusifolium*	*Cinnamomum obtusifolium*	*Cinnamomum* sp. 1	*Azadirachta* sp.
Cinnamomum sp. 1	*Cinnamomum obtusifolium*	*Cinnamomum* sp. 1	*Cinnamomum* sp. 1	*Dipterocarpus gracilis*
Dipterocarpus gracilis	*Cinnamomum* sp. 1	*Cinnamomum* sp. 1	*Cinnamomum* sp. 1	*Dipterocarpus gracilis*
Dipterocarpus gracilis	*Cinnamomum* sp. 1	*Cinnamomum* sp. 1	*Cinnamomum* sp. 1	*Elateriospermum tapos*
Elateriospermum tapos	*Cinnamomum* sp. 1	*Cinnamomum* sp. 2	*Cinnamomum* sp. 2	*Excoecaria bantamensis*
Elateriospermum tapos	*Cinnamomum* sp. 1	*Cinnamomum* sp. 2	*Cinnamomum* sp. 2	*Garcinia* sp. 1
Fragraea racemosa	*Cinnamomum* sp. 1	*Dipterocarpus gracilis*	*Diospyros macrophylla*	*Garcinia* sp. 1
Litsea sp.	*Cinnamomum* sp. 1	*Dipterocarpus gracilis*	*Diospyros macrophylla*	*Garcinia* sp. 1
Macaranga gigantea	*Elmerillia tsiampacca*	*Dipterocarpus gracilis*	*Diospyros macrophylla*	*Garcinia* sp. 1
Syzigium sp.	*Excoecaria bantamensis*	*Elmerillia tsiampacca*	*Diospyros macrophylla*	*Garcinia* sp. 1
Syzigium sp.	*Excoecaria bantamensis*	*Excoecaria bantamensis*	*Garcinia* sp. 1	*Shorea leprosula*
Syzigium sp.	*Excoecaria bantamensis*	*Excoecaria bantamensis*	*Garcinia* sp. 1	
Unknown species 1	*Excoecaria bantamensis*	*Litsea* sp.	*Garcinia* sp. 1	
Unknown species 2	*Excoecaria bantamensis*	*Macaranga gigantea*	*Garcinia* sp. 1	
Unknown species 2	*Fragraea racemosa*	*Shorea multiflora*	*Garcinia* sp. 1	
	Fragraea racemosa	*Shorea siamensis*	*Garcinia* sp. 1	
	Shorea siamensis	*Shorea siamensis*	*Garcinia* sp. 1	
	Shorea siamensis	*Syzigium oides*	*Garcinia* sp. 2	
	Syzigium oides	*Syzigium oides*	*Lannea coromandelica*	
	Syzigium sp.	Unknown species 2	Unknown species 1	
	Syzigium sp.	Unknown species 2	Unknown species 2	
	Unknown species 2	*Ziziphus* sp.		

Pioneers are light-demanding and fast-growing species. Their seeds germinate when the environmental conditions favour and the seedlings then quickly grow (Goodale *et al.,* 2012; Silvestrini & dos Santos, 2015). On the other hand, primary species are shade tolerant (Franklin, 2003). In this research, some species can be categorized as pioneer species, including *Cinnamomum* sp., *Diospyros macrophylla*, *Castanopsis costata*, *Elateriospermum tapos,* and *Ziziphus* sp. Primary species found in the research site include genus *Garcinia*, *Shorea*, *Dipterocarpus,* and *Syzigium*.

The responses of plants to genotype, age, ontogeny, and environmental heterogeneity result in differences in the ecophysiological performance by the plants (Goodale *et al.,* 2012; Zotz, 2000; Zotz *et al.,* 2001). Light intensity, CO_2 uptake, variation in temperature, and humidity, soil fertility and nutrient cycling are prominent environmental entities that affect the dynamics of individuals, as well as the population of plants. The interactions between these environmental factors result in a certain micro-climate for the plants to live in. As the environment may vary seasonally and spatially, plants must respond continually and consequently adapt to the change in environmental conditions (Dickison, 2000). Genetic properties and environmental factors will dictate the nature of responses by the plants.

Pioneer species were found to have higher leaf vein density than primary species. High leaf vein density may provide these pioneer species with a higher capacity to transport water (Boyce *et al.,* 2009; Price *et al.,* 2014; Sack *et al.,* 2005; Sack & Holbrook, 2006). Consequently, pioneer species may have higher rates of photosynthesis and growth. Vegetation is an important part of the hydrological cycle. Forest plants take water up from the ground to the forest canopy. Most of the water then transpires into the air. The amount of water regulated by the vegetation transportation and

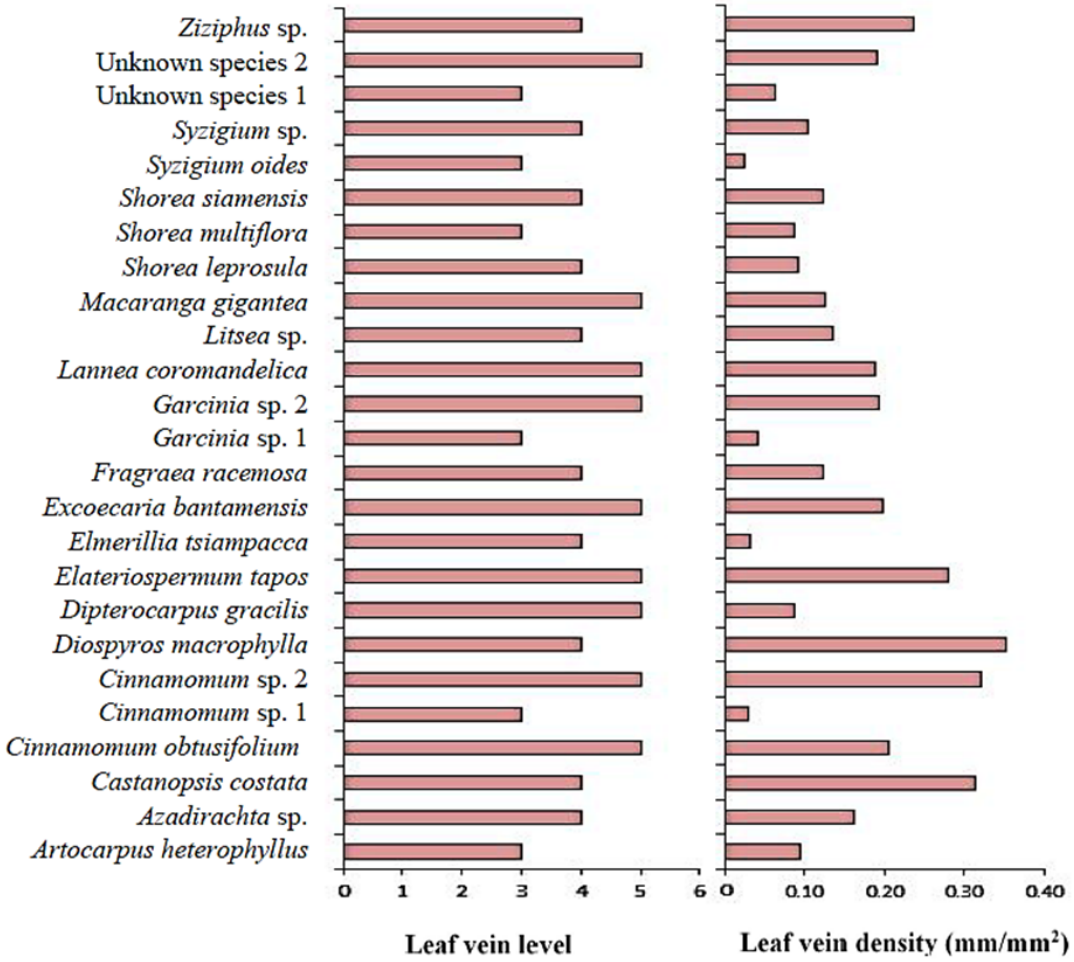

Figure 2. Leaf vein level and density of tree saplings composing lower canopy at the protected forest of Boven Lais Kemumu.

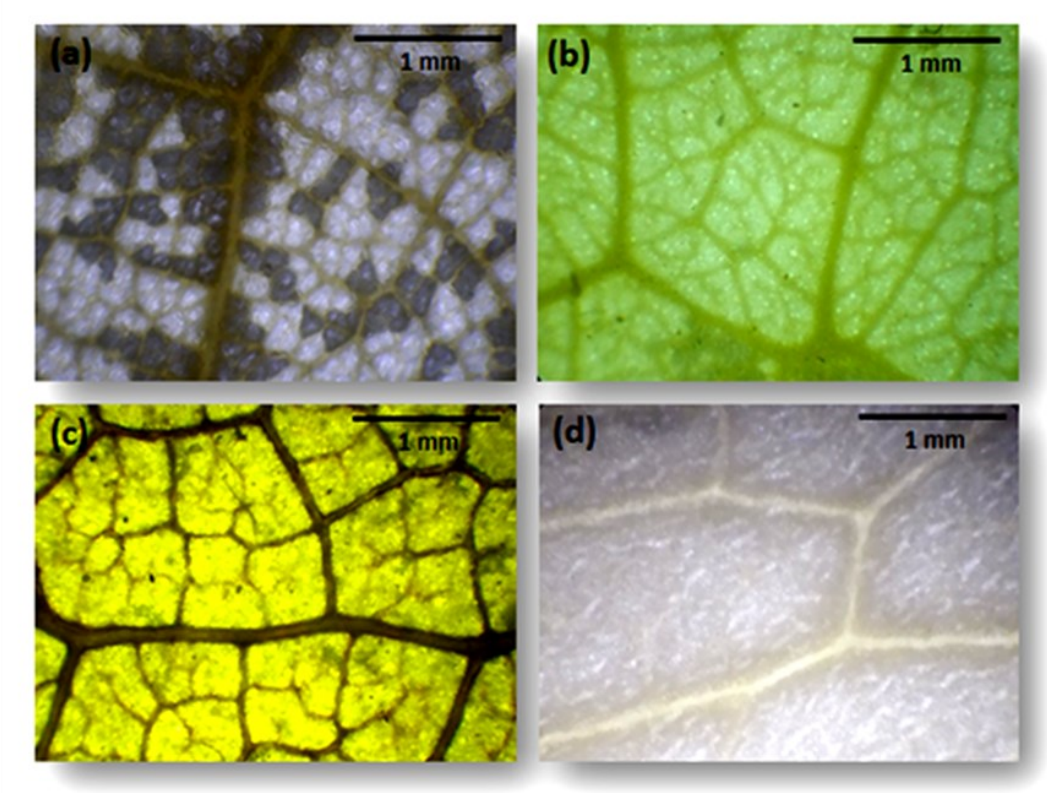

Figure 3. Examples of leaf veins of several tree saplings species found on the protected forest of Boven Lais Kemumu. The species are pioneer species (a) *Cinnamomum obtusifolium* and (b) *Exoecaria bantamensis,* and primary species (c) *Lannea coromandelica* and (d) *Shorea multiflora* .

transpiration process in the soil-plant-atmosphere continuum will affect the hydrological cycle in general. Therefore, different compositions of vegetation with different ability to transport water will contribute differently to the hydrological cycle. If the ecosystem becomes more opened, pioneer species may dominate as they are more light-demanding. As they are physiologically more water demanding to support high rates of photosynthesis, the domination of pioneer species (if happening) will hypothetically affect the hydrological cycle.

CONCLUSION

Tree saplings composing a lower canopy of tropical forest have different ecophysiological attributes and leaf vein characteristics. Pioneer species, such as *Cinnamomum* sp., *Diospyros macrophylla*, *Castanopsis costata*, *Elateriospermum tapos,* and *Ziziphus* sp., have higher leaf vein density than primary species, such as *Garcinia*, *Shorea*, *Dipterocarpus,* and *Syzigium*. As leaf vein density may affect the capacity of plants to transport water, pioneer species might have higher rates of water transport and higher rates of photosynthesis. Consequently, the composition of forest vegetation with different ecophysiological characteristics will affect the forest dynamics and in the long-term hydrological cycle. Future research is directed to measure water conductivity and transpiration rates. Then, total water transport and transpiration will be spatially analysed and the contribution of the vegetation to the hydrological cycle can be simulated.

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Research Article

UV Mutagenesis as a Strategy to Enhance Growth and Lipid Productivity of *Chlorella* sp. 042

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ABSTRACT
Microalgae appeared to be an alternative feedstock for renewable biodiesel production due to their capability to accumulate considerable amounts of lipids. In this study, mutagenesis using UVC light with different periods was applied to *Chlorella* sp. 042 to produce a microalgae strain with high lipid productivity of 45, 60, and 75 min. The Nile red fluorescence method was conducted to select a *Chlorella* sp. mutant with high neutral lipid and generated one mutant from every UV mutation period, M45-06, M60-02, and M75-21. All of the mutants have higher growth rates than the wild type. *Chlorella* sp. 042 M60-02 achieved the highest lipid productivity, with 34 mg L^{-1} day^{-1}. Furthermore, as other major biochemical components, carbohydrate and protein contents were determined. Our results showed that all the mutants enhance their carbohydrate and protein contents compared to the wild type. However, mutations for more than 60 min do not intensely change the protein content of mutant microalgae. Gas chromatography-mass spectrophotometry analysis revealed that M60-02 mutant has similar FAME profiles with the wild type, which contain palmitic acid (C16:0), stearic acid (C 18:0), oleic acid (C18:1), and linoleic acid (C18:2). These results demonstrate that the UV mutation of *Chlorella* sp. 042 for 60 min is suitable as a source of biodiesel production.

Keywords: Biodiesel, *Chlorella* sp., Fatty acids, Lipid productivity, UV mutagenesis

INTRODUCTION
The limited availability of fossil energy sources and the negative impact of fossil fuels on the environment forced researchers to explore alternative and renewable energy sources capable of producing low carbon dioxide emissions (Tan *et al.*, 2017). Biodiesel is a mono-alkyl ester with a long-chain fatty acid, which is a derivative of animal fats or wastes cooking oil or vegetable oils (Yusuf & Yaakub, 2010; Balat, 2011; Abbaszaadeh *et al.*, 2012; Faried *et al.*, 2017). The eminences of biodiesel compared to fossil fuels are its renewability, toxic-free, sulfur-free, and have better lubricity (Aransiola *et al.*, 2014; Goh *et al.*, 2019). However, these materials, especially those originating from vegetable oil, cause new problems due to their implications on food security and commodity prices (Goh *et al.*, 2019). Besides, biodiesel production using crops as raw material requires extensive agricultural land and one of the causes of deforestation (Rawat *et al.*, 2013).

Microalgae oil produced can be used as an alternative feedstock for biodiesel in terms of social and economic aspects. Microalgae are capable of accumulating oil with a shorter harvesting time and less volume of water demand and can be done in open land (Chen *et al.*, 2018; Rawat *et al.*, 2013). Oil productivity from algae is twenty times higher than oilseed plants based on a one-hectare area. Therefore, a more viable biodiesel feedstock (Chisti, 2007; Ahmad *et al.*, 2011; Antoni *et al.*, 2007; Feng *et al.*, 2011; Rawat *et al.*, 2013). Some microalgae with high lipid productivity have been identified as candidates for biodiesel production such as *Chlamydomonas* sp., *Scenedesmus* sp., *Nannochloropsis oculata*, *Dunaliella salina*, *Botryococcus braunii*, and *Chlorella* sp. (Chisti, 2007; Hosseini Tafreshi & Shariati, 2009; Van Vooren *et al.*, 2012; Yoo *et al.*, 2010; Sarayloo *et al.*, 2017). Nevertheless, biodiesel

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production from large-scale microalgae is still not economically feasible (Sarayloo *et al.*, 2017). Therefore, it is important to find candidates for microalgae that have high lipid content with high biomass production (Zhang *et al.*, 2010).

To obtain microalgae strain with high lipid content and biomass production, various strategies can be applied. Some of them include performing screening and species characterization of lipid producing microalgae (Vigeolas *et al.*, 2012; Nascimento *et al.*, 2013) followed by optimization of culture condition and cultivation techniques, and via metabolic engineering (Trentacoste *et al.*, 2013) followed by the screening of lipid producing microorganism using lipophilic dye (Vigeolas *et al.*, 2012). Screening and species characterization to derive robust strain require intensive effort, and many times, they fail to provide strain with high biomass and high lipid production. Meanwhile, metabolic engineering requires the elucidation of lipid metabolism processes and other related metabolism pathways as well as the knowledge about carbon flux in cellular processes (Trentacoste *et al.*, 2013). Targeted genetic engineering is limited due to the lack of the genomic sequence and the detailed study about lipid-producing-microalgae that can be manipulated (Vigeolas *et al.*, 2012).

In addition to those approaches, the effort to increase lipid production is also done via mutagenesis, either physically or chemically. This approach is simple and does not require genomic information as required in genetic engineering. Mutagenesis performed physically using UV irradiation is preferred to chemical mutagenesis. The former is considered more benign either for the operator or the environment, faster, and more effective (Fang *et al.*, 2013; Sivaramakrishnan & Incharoensakdi, 2017). UV mutagenesis has been applied to microalgae and other oleaginous microorganisms such as yeast to provide strain with improved lipid production (Tapia *et al.*, 2012; Sharma *et al.*, 2014; Liu *et al.*, 2015; Sivaramakrishnan & Incharoensakdi, 2017).

In this study, *Chlorella vulgaris*, either the mutagenized or wild type, is investigated for its lipid production as well as lipid productivity. This strain is acknowledged for its ability to grow in various conditions and its resistance to invaders (Pauline *et al.*, 2006; Liang *et al.*, 2009; Sarayloo *et al.*, 2018). It also contains lipids mainly as triacylglycerol. Major fatty acids produced by *C. vulgaris* are saturated and monounsaturated fatty acids like palmitic acid, palmitoleic acid, stearic acid, and oleic acid, which are compatible to be employed as biodiesel feedstock (Yeh & Chang, 2011; Sarkar & Shimizu, 2015; Sarayloo *et al.*, 2018). Besides, this species produces valuable metabolites such as protein, β-carotene, astaxanthin, as well as several types of polyunsaturated fatty acids (PUFAs) (Chacon-Lee & Gonzalez-Marino, 2010; Singh & Cu, 2010; Sarayloo *et al.*, 2018).

Previous studies showed that applying mutagenesis to *Chlorella* sp. for 30 min increased its growth rate and lipid productivity to be 0.257 day^{-1} and 11 mg L^{-1} day^{-1} concerning wild type (growth rate was 0.196 day^{-1} and lipid productivity was 9 mg L^{-1} day^{-1}) (Rahman *et al.*, 2020). In this study, we conducted UV mutagenesis to *Chlorella* sp. isolated from East Kalimantan River for 45, 60, and 75 min. We observed and determined the optimum period for UV mutagenesis concerning growth and lipid productivity. We also characterized fatty acid profiles of the mutants.

MATERIALS AND METHODS

Microalgae strain and growth condition

Wild type *Chlorella* sp. 042 was isolated from the Wain River, East Kalimantan, Indonesia. The cells were inoculated into 500 mL photobioreactors containing 400 mL AF6 medium (140 mg $NaNO_3$, 22 mg NH_4NO_3, 30 mg $MgSO_4.7H_2O$, 10 mg KH_2PO_4, 5 mg K_2HPO_5, 10 mg $CaCl_2.2H_2O$, 2 mg Fe-Citrate, 2 mg Citric acid, and 1,000 mL distilled water). The culture condition was maintained at 25 °C under continuous light (800 Lux) with continuous aeration. The growth curves of the microalgae were made by daily measurement of optical density at 750 nm using a UV-Vis spectrophotometer (Shimadzu PharmaSpec UV-1700).

UV mutagenesis

The mutagenized cell was conducted by 5 mL of *Chlorella* sp. 042 in the exponential growth phase. It was placed in the open petri dishes and exposed to the UV irradiation (Germicidal lamp, UVC 30 W, Philips) at a distance of 25 cm for 45, 60, and 75 min. The mutagenized mutants were kept in the darkroom for 24 h, to avoid light induction of cell recovery. The mutagenized cells were grown in AF6 agar medium and incubated under continuous light for 2-3 weeks in advance of single colonies appeared.

Screening of mutants

The single developed colonies on the agar plate were selected and transferred into a sterile 96-wells plate containing 200 µL of AF6 medium. Plates were incubated under constant light with agitation at 150 rpm for 7 days. Cell densities of each culture were measured using Varioscan™ LUX multimode

microplate reader (Thermo Fisher Scientific). The amount of neutral lipid was determined using modified Chen *et al.* (2009) methods by following Nile Red fluorescence in cell suspensions diluted to the concentration of approximately 0.1 at 750 nm.

Determination of cell dry weight and lipid content

The cell dry weight of *Chlorella* sp. 042 was determined gravimetrically. Aliquot of 10 mL culture was transferred to a 15 mL falcon tube of a known mass and centrifuged at 8000 rpm for 10 min. The supernatant was discharged, and the remaining cell pellets were dried at 60 ℃ for 24 h. The dry weight of cell pellets was determined gravimetrically (Rahman *et al.*, 2020).

Total lipid content was determined by performing lipid extraction prior to gravimetric analysis. Lipid was extracted according to Ryckebosch *et al.* (2012) with modification. A solution of chloroform and methanol (1:1, v/v) was added to the lyophilized cell pellet and mixed. Water was added to the homogenized mixture until the final concentration of chloroform-methanol-water of 2:2:1 (v/v) was reached. The upper layer (lipid) was separated by centrifugation (8000 rpm, 10 min) and evaporated at room temperature. The dried lipid layer was then determined gravimetrically (Ryckebosch *et al.*, 2012).

FAME analysis

Transesterification of microalgae lipids was performed before analysis using GC-MS. Derivatization of lipid into its methyl ester was done according to Lewis *et al.* (2000), with modification. Reagent, consisting of methanol: chloroform: chloric acid (10:1:1), was added to a lyophilized microalga cell, into duplicate, screw-capped reaction tubes. The tubes were mixed using vortex and heated at 90 ℃ for 2 h in a water bath. After the reaction was completed, the reaction mixtures were set to cool until it reached 25 ± 2 ℃ and to which the water was added and mixed. To each reaction mixture, hexane was added, mixed, and was left alone until it reached phase separation. The hexane addition step was repeated twice, and the upper layer (organic phase) was being collected and subjected to GC-MS analysis. This step was done for both mutant and wild type lipids.

FAME analysis using GC-MS (Shimadzu QP 2010 Ultra, DB-23 column) was performed qualitatively. The oven temperature was 50 ℃ for 1 min and increased at 25 ℃ min^{-1} up to 180 ℃ for 4 min. An increment of 5 ℃ min^{-1} followed the step until 235 ℃ for 5 min. The temperature of the ion source was set to 230 ℃, with an interface temperature of 250 ℃ and the solvent cut time of 2 min. The peaks derived from GC-MS analysis were assigned according to the reference of FAME mix C14-C22 components.

Determination of Carbohydrate and Protein

Carbohydrate determination was held using the phenol-sulfuric acid method, according to Dubois *et al.* (1956), with modification. Wet biomass was incubated with 2.25 % sulfuric acid at 90 ℃ for 70 min. The sugar solution (0.5 mL) was pipetted to the reaction tube. 5 % phenol and 96 % sulfuric acid were mixed with the sugar solution. The absorbance of the mixed solution was measured using a UV-Vis spectrophotometer at 490 nm.

The determination of protein content was done using the dye-binding assay (Bradford, 1976). Phosphate buffer (pH 7) was added to microalgae wet biomass. The cell was then disrupted using sonication for 15 s and repeated until 10 cycles. Bradford reagent was added to 0.3 mL of supernatant. The solution was determined using the UV-Vis spectrophotometer at 595 nm.

Statistical Analysis

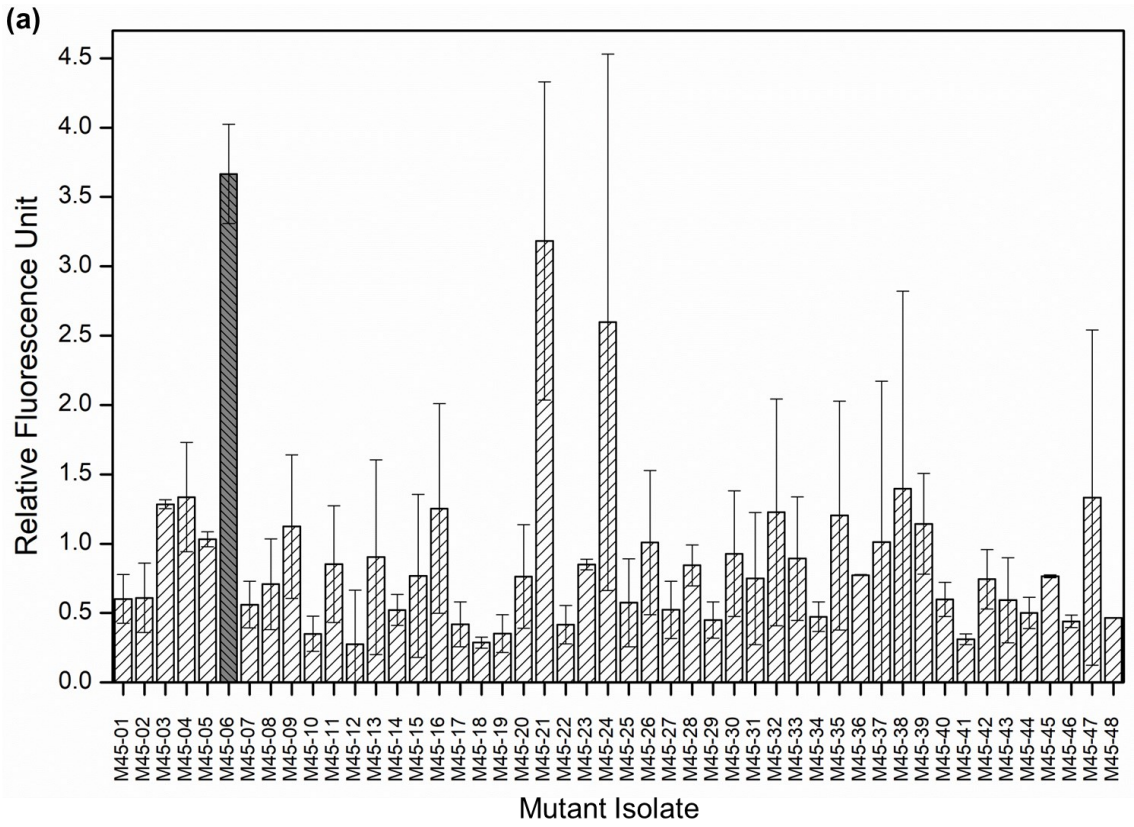
To determine the significant difference among groups ($P< 0,05$), all average values of mutants were analyzed against the control employing one way ANOVA and t-test analysis by using Microsoft Excel Software.

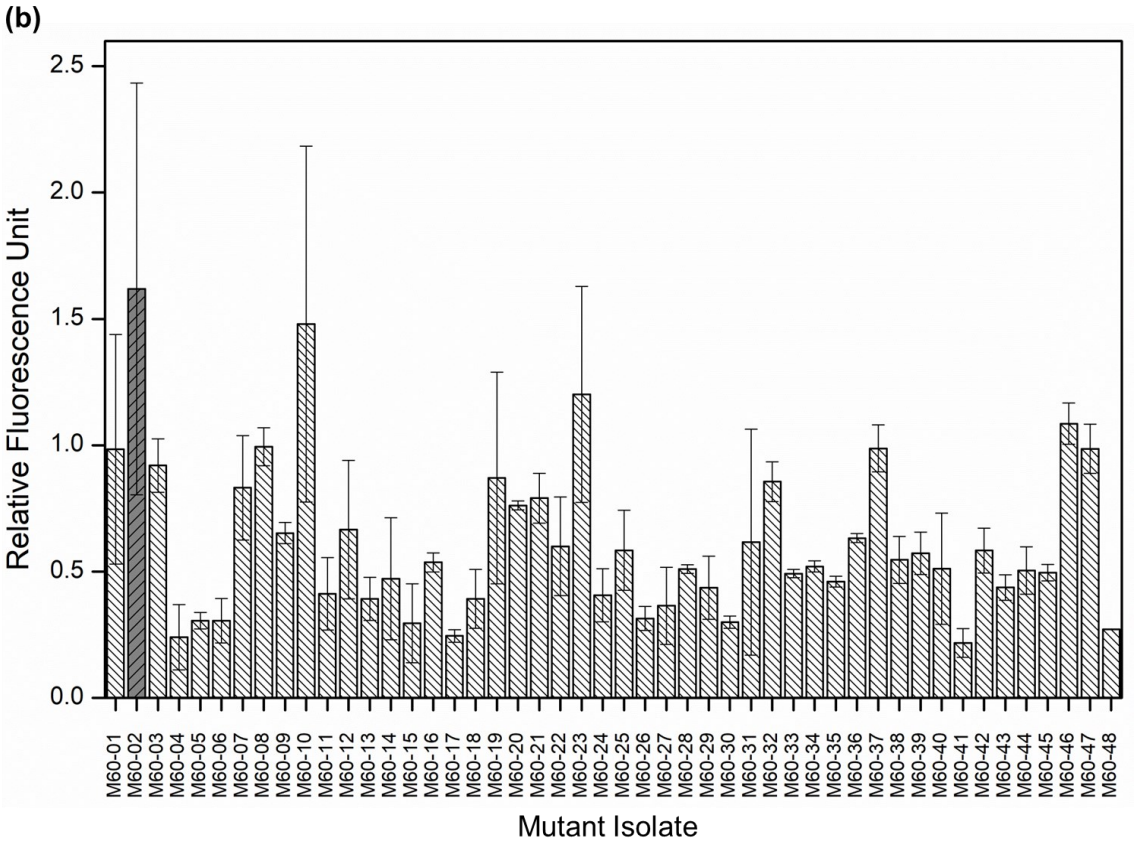
RESULTS AND DISCUSSION

Screening of mutants

Mutagenized green microalgae, *Chlorella vulgaris*, were screened for its lipid content using the Nile red method. Nile red dye has been applied to detect neutral lipid in various oleaginous microorganisms including yeasts (Sitepu *et al.*, 2014; Rostron *et al.*, 2015), bacteria (Alves *et al.*, 2017), and microalgae (Chen *et al.*, 2009; Huang *et al.*, 2009; Satpati & Pal, 2014). This hydrophobic benzophenoxazone dye emits fluorescence as it contacts lipid bodies and organic solvents (Halim & Webley, 2015). Such a characteristic makes it useful to be applied in lipid staining to detect lipid content. In this study, DMSO was used as a carrier for the Nile red dye. The use of DMSO as well as other cell pre-treatments such as cell grounding in liquid nitrogen and the use of methanol, acetone, and ethanol as a solvent were proved to increase fluorescent intensity compared to those without pre-treatment (Chen *et al.*, 2009). The pre-treatments applied to make it possible for the Nile red method to be applied to stain and detect lipid content in green algae are known to have thick and rigid cell walls (Chen *et al.*, 2009). The use of this

Nile Red method also enables high throughput screening, which is difficult to achieve when applied by the conventional gravimetric method. According to fluorescence intensity measured for 48 colonies, generated from 45, 60, and 75 min UV radiation (Figure 1), three colonies showed prominent results, indicating high lipid content. The three colonies are M45-06, M60-02, and M75-21, which originated from 45, 60, and 75 min radiation, respectively. The three colonies were considered as potential candidates for lipid production and subjected to further analysis.





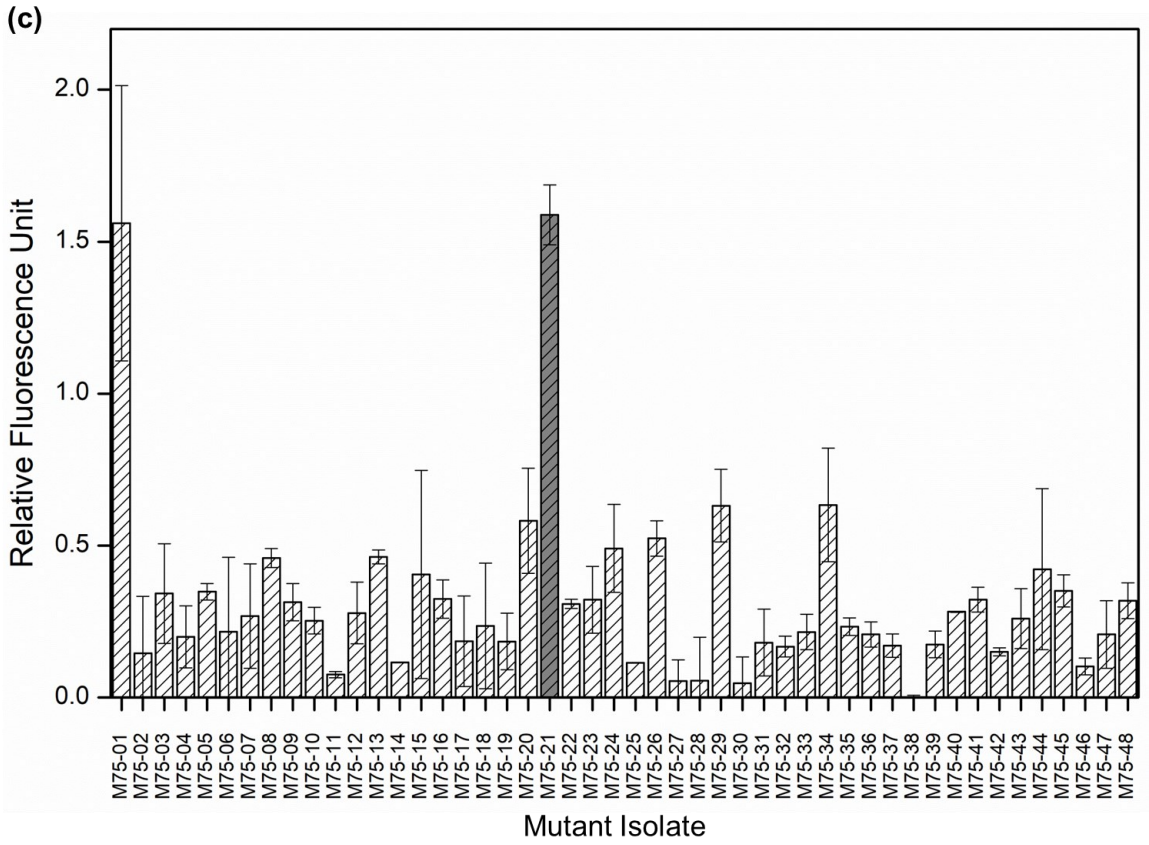


Figure 1. Relative amounts of lipids in *Chlorella* sp. 042 after UV mutagenesis: (a). M45-06 mutant; (b) M60-02 mutant; and (c) M75-21 mutant.

Cell dry weight and lipid contents

The growth rate of *Chlorella* sp. 042 was measured daily using a UV-Vis spectrophotometer. Both wild type and mutants showed an increase in growth rate until the last day of cultivation (Figure 2). The wild type achieved a growth rate of 0.503 day^{-1}, while all mutants exhibited a higher growth rate with 0.641, 0.783, and 0.7369 day^{-1} for M45-06, M60-02, and M75-22, respectively. The mutant M60-02 showed the highest growth rate of all. Previous studies showed that UV mutagenesis to the *Chlorella* sp. increased the biomass productivity and the lipid content, and thus, this strategy suitable for biodiesel production (Liu *et al.*, 2015; Sivaramakrishnan *et al.*, 2017). Our study found a similar result in which all of the mutants have higher biomass productivity (P<0.05) than the wild type (29.95 mg L^{-1} day $^{-1}$). The M60-02 mutant achieved the highest biomass productivity (P<0.05) with a value of 61.8 mg L^{-1} day $^{-1}$. The biomass productivity achieved was also in line with the growth rate in which the mutant M60-02 with the highest biomass productivity also showed the highest growth rate, which increased by two-fold concerning the wild type. The biomass productivity, lipid content, and lipid productivity of *Chlorella* sp. 042 wild type and mutants are provided in Table 1.

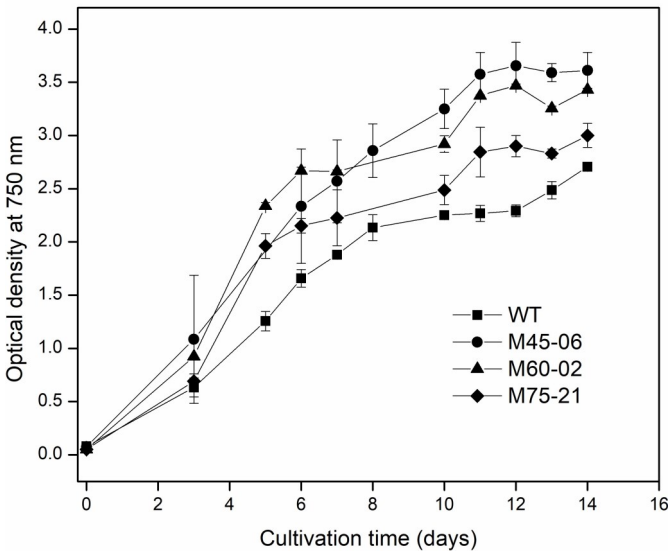


Figure 2. Growth curve of *Chlorella* sp. 042 wild type and mutants.

The lipid content of M45-06, M60-02, and M75-21 showed higher value (P<0.05) than wild type (30.8 %), 60.5 %, 55 %, and 40.6 %, respectively. The lipid content of M60-02 and M75-21 were lower than those of M45-06 (Table 1). However, the biomass productivity of the latter was lower than other mutants. Occasionally, microalgae with a high growth rate have low lipid content, whilst microalgae with a low growth rate have high lipid content (Liu *et al.*, 2015). In microalgae, there

Table 1. Lipid content, biomass productivity, and lipid productivity of *Chlorella* sp. 042 wild type and mutants.

Strain	Lipid Content* (%)	Biomass Productivity* (mg L^{-1} day^{-1})	Lipid Productivity* (mg L^{-1} day^{-1})
WT	30.8 ± 1.44[a]	29.95 ± 1.77[a]	9.34 ± 0.97[a]
M45-06	60.5 ± 3.8[b]	50.7 ± 9.92[b]	27.2 ± 0.6[b]
M60-02	55.1 ± 7.2[c]	61.8 ± 3.5[c]	34.0 ± 1.9[c]
M75-21	40.6 ± 4.4[d]	51.1 ± 0.5[d]	20.7 ± 0.2[d]

are three steps responsible for lipid biosynthesis, including fatty acid synthesis, acyl chain prolongation, and lipid formation. Acetyl-CoA carboxylase (ACC) is one of the essential enzymes in fatty acid synthesis. This enzyme catalyzes the biotin -dependent carboxylation of acetyl-CoA to form malonyl-CoA, which is considered a step-in fatty acid synthesis (Sendl *et al.*, 1992; Li & Cronan, 1993; Kim, 1997; Davis *et al.*, 2000; Courchesne *et al.*, 2009). Exposure of UV irradiation to the microalgae may affect the ACC and nutrient fixation. Thus, enhancing the lipids content. High lipid content in UV-mutagenized-*Chlorella* sp. was observed to have higher ACC concentrations than those of the untreated *Chlorella* sp. (Liu *et al.*, 2015).

As shown in Table 1, the lipid productivity for UV mutation microalgae was higher than the value for the wild type (9 mg L^{-1} day^{-1}) (P<0.05). Mutant M60-02 has the highest value of lipid productivity of 34 mg L^{-1} day^{-1} (P<0.05). The total lipid production is proportional to the biomass productivity and the lipid content, which is a significant parameter for large-scale lipid production of microalgae (Fan *et al.*, 2014). Lipid productivity is also a determining factor for the cost-effective production of biodiesel (Rahman *et al.*, 2020). The results suggest that the UV irradiation strategy for 60 min can result in the most favorable lipid productivity (Table 1).

Carbohydrate and protein contents

The major biochemical component of microalgae consisted of proteins, carbohydrates, and lipids, and their composition depends on the strain and culture condition (Behrens & Kyle, 1996; Vigeolas *et al.*, 2012). Since we perform UV mutagenesis, which can alter the metabolism of microalgae, the carbohydrates and protein assay are required to know the effect of the mutation on those compounds. Our study found that the M60-02 and M75-21 (P<0.05) carbohydrates and protein content were higher than those of the wild type after 14 days of cultivation. The results are shown in Figure 3, the carbohydrates content increased along with period times of mutation M60-02, and M75-21 which contained 38 % and 40 %, respectively. The carbohydrate content of M45-06 (34.5 ± 20 %) was not significantly different compared to the wild type (24 ± 0.39 %). The M60-02 and M75-21 mutants showed higher protein content compared to the wild type (4.55 ± 0.2 %). However, the M60-02 and M75-21 mutants exhibited similar results (P>0.05), 6.76 % ± 0.04 % and 6.93 ± 0.16 %, respectively. Further study at the genome level needs to be performed to identify the underlying biochemical process affected

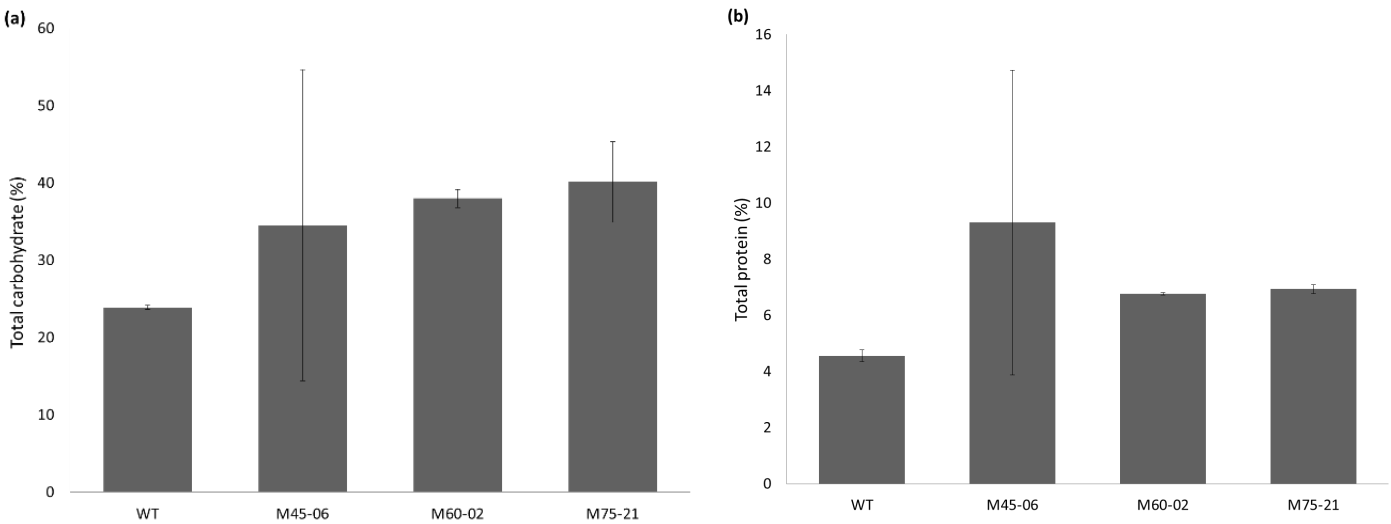


Figure 3. Primary metabolites content of *Chlorella* sp. 042 wild type and mutants: (a) carbohydrate and (b) protein.

by UV mutagenesis.

Fatty acid profiles of mutagenized microalgae

The fatty acid contents of lipids from both mutants and wild type can be inferred by the FAMEs detected by GC-MS analysis. The fatty acids contained in lipids determine the properties of biodiesel (Knothe, 2009). In this study, the analysis performed qualitatively and gave insight into the lipid's fatty acid profile and whether the mutagenesis affects the fatty acid profile of the strain. The result obtained in this study showed that the fatty acid contained in the lipids of both mutant and wild type are similar, with minor differences in which we did not observe oleic acid (C 18:1) in M45-06 and linoleic acid (C18:2) in M75-21. The same result was also shown by the study performed by Vigeolas *et al.* (2012) on *Chlorella sorokiniana* and *Scenedesmus obliquus*. In that study, both mutants and wild type strains produced the same type of fatty acids. However, the composition of certain fatty acids varies in mutant and wild type.

The content of lipid from both strains (Table 2) consists of saturated fatty acid, palmitic acid (C16:0) accompanied by stearic acid (C18:0), and unsaturated fatty acid components, oleic and linoleic acid. The result resembled the fatty acids profile of immobilized *C. vulgaris* obtained in Abu Sepian *et al.* (2017). Lipid from *C. vulgaris* was mainly composed of saturated fatty acids (60%), consisting of palmitic and stearic acid, and the remaining portion (40%) was comprised of unsaturated fatty acid, consisted of oleic, linoleic, and linolenic acid (Abu Sepian *et al.*, 2017). The more significant proportion of saturated fatty acids will contribute to the higher cetane number of the fuel. More specifically, the chain length and degree of unsaturation will affect the cetane number (Knothe, 2010). The higher the cetane number, the shorter the ignition delay time, which is one of the desired properties of a fuel (Knothe, 2009). The presence of palmitic and stearic acid thus may contribute to biodiesel with the desired property. However, further analysis is required to determine the exact composition of each fatty acid. Besides, the FAMEs derived from transesterification are also required to fulfil several other criteria like viscosity, cold flow, oxidative stability, and lubricity as determined in a certain standard of biodiesel (e.g., ASTM D6751, EN 14214) to be eligible for use and commercialization (Knothe, 2009).

CONCLUSION

Based on Nile red fluorescence assay, we found three microalgae mutants from three periods of mutation time, 45 min, 60 min, and 75 min, and there are M45-06, M60-02, and M75-21, respectively. The growth rate for all mutants is higher than the wild type after 14 days of cultivation, so does the lipid productivity. The M60-02 mutant achieved the highest lipid productivity, with 34 mg L^{-1} day^{-1}. Surprisingly, carbohydrate and protein contents of the mutants (M60-02 and M75-21) are also higher than the wild type. We found no difference in FAMEs profile of M60-02 mutant and the wild type that included palmitic acid, stearic acid, oleic acid, and linoleic acid. Finally, our results indicate that random mutation by UV mutagenesis for 60 min is suggested as the best way for generating microalgae mutant with higher growth rate and lipid productivity for environmentally friendly and sustainable energy sources such as biodiesel.

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Table 2. Fatty Acid Methyl Ester (FAMEs) profile of wild type and mutant.

FAMEs	Strains			
	WT	M45-06	M60-02	M75-21
C16:0	**V**	**V**	**V**	**V**
C18:0	**V**	**V**	**V**	**V**
C18:1	**V**	**-**	**V**	**V**
C18:2	**V**	**V**	**V**	**-**

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Research Article

Ulva lactuca Linnaeus Potentially Promotes Reproductive Indices and Depressive-like Behavior of Hypertriglyceridemia Male Wistar Rats (*Rattus norvegicus* Berkenhout, 1769)

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ABSTRACT

Excessive consumption of fatty foods can lead to hyperlipidemia, which is often coupled with hypertriglyceridemia (HTG), a condition where blood plasma triglyceride (TG) levels elevated beyond normal levels. This condition may disturb physiological functions of the body, such as reproductive functions, and other physiological imbalances leading to chronic stress and depression. *Ulva lactuca* is a potential natural treatment for HTG, as it contains various nutrients to aid physiological functions. This seaweed also has high levels of Cd, which can increase depression. Therefore, research on the potential benefits of *U. lactuca* should be followed by an investigation of its health risks. This research aimed to examine the effects of HTG and treatment with *U. lactuca* on reproduction and depressive-like behavior of male Wistar rats (*Rattus norvegicus* Berkenhout, 1769). The data collected in this research include body weight, serum TG concentration, gonadosomatic index (GSI), serum testosterone concentration using competitive ELISA, and depressive-like behaviors assessed using the Forced Swim Test (FST) and Open Field Test (OFT). Data were analyzed using One-Way ANOVA followed by DMRT, independent- and paired-samples t-test, and Kruskal-Wallis H test with a significance value of α=0.05. Body weight, serum TG and testosterone concentration, GSI, and depressive-like behaviors were increased by the HTG condition. *Ulva lactuca* at the1500 mg/kg BW/day did not significantly affect body weight, testosterone concentration, and depressive-like behaviors of HTG rats. Meanwhile, this treatment significantly increased the GSI and depressive-like behaviors of healthy rats. These results suggest that *Ulva lactuca* treatment not only enhances gonad growth and development but also increases depressive-like behaviors.

Keywords: depression, hypertriglyceridemia, male reproduction, triglycerides, *Ulva lactuca*

INTRODUCTION

The definition of an unhealthy lifestyle often involves the habit of smoking, lack of physical exercise, excessive consumption of alcohol and artificial sweeteners, as well as consumption of foods with a high-fat content (Ashakiran & Deepth, 2012). In developing countries, cooking oil is often used repeatedly, therefore it goes through repeated heating, which may cause physical and chemical alterations of the oil. These physical changes include the darkening of color, turning the yellow-tinted oil to black, and increasing viscosity. Chemical changes include the breaking of double bonds on the carbon chain of fatty acids, transforming unsaturated fatty acids to saturated fatty acids, and the increase of free fatty acids (Suroso, 2013).

Consuming high saturated fatty acids and free fatty acids foods increase health risks such as hyperlipidemia, hypertriglyceridemia (HTG), atherosclerosis, obesity, coronary heart disease, and other cardiovascular diseases (Blesso & Fernandez, 2018). One of the most common conditions arising from excessive consumption of foods that were cooked in overused frying oil is hyperlipidemia. This condition is often paired with HTG, an elevation of blood plasma triglyceride (TG) levels beyond the

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normal level. The Endocrine Society 2010 defines this as more than 150 mg/dL (Berglund *et al.*, 2012). Hypertriglyceridemia may have a negative effect on the functions of certain organs, such as the liver, heart, kidneys, and reproductive organs (Lockman *et al.*, 2012; Minguez-Alarcon *et al.*, 2017). High concentrations of TG also increase its chances of crossing the brain-blood barrier, inducing central resistance of leptin (Banks *et al.*, 2018), thus leading to low leptin levels in the brain, a condition found in depressive patients (Ge *et al.*, 2018).

Natural substances are often preferred for the prevention and treatment of health conditions, are usually considered to be safer, causing minimum side effects. *Ulva lactuca*, commonly called the "sea lettuce", is a species of seaweed that has been used as food in Vietnam (White & Wilson, 2015) as well as a traditional medicine in China (Tseng & Chang, 1984). This seaweed contains high amounts of antioxidants, proteins at 10-21 g/100 g dry weight, as well as antibacterial, antifungal, and antitumor properties (Erniati *et al.*, 2010). The antihyperlipidemic and antioxidant properties of *U. lactuca* are found in its polysaccharides (Sathivel *et al.*, 2008; Hassan *et al.*, 2011) and ethanolic extract (Widyaningsih *et al.*, 2016). Mulyati *et al.* (2019) found that *U. lactuca* is quite abundant in nutrients, comprised of macronutrients, micronutrients, secondary metabolites, and antioxidants, although cadmium (Cd) levels were also identified to be higher than the recommended limit regulated by *Badan Pengawas Obat dan Makanan* (BPOM) (2018) for plant-based foods. Cadmium is a heavy metal that potentially increases depressive symptoms (Lamtai *et al.*, 2018; Scinicariello & Buser, 2015), amongst other health risks. This research aims to examine the effects of HTG and treatment with *U. lactuca* on reproduction and depressive-like behaviors of male Wistar rats (*Rattus norvegicus* Berkenhout, 1769).

MATERIALS AND METHODS

Materials

Twenty 12-weeks old male Wistar rats (*Rattus norvegicus* Berkenhout, 1769) with a body weight range of 200-300 grams obtained from LPPT UGM unit 4 were used as animal models. Materials administered into the animals included: saturated fat comprised of overused cooking oil and beef fat (1:1) for HTG induction; *U. lactuca* obtained from the south coast of Gunungkidul, D.I. Yogyakarta, which were air-dried and grounded at the Laboratory of Animal Physiology, Faculty of Biology, UGM, to create a powdery texture; and over-the-counter drug Gemfibrozil for treatment of HTG. AD II pellets and reverse osmosis water (RO) were both given *ad libitum*. Materials for data collection included: Testosterone ELISA Kit TE373S (Calbiotech), 20x20x40 cm^3 glass chambers, a 40x40x40 cm^3 opaque black box, YI Action Camera (XiaoYi), semi-analytical and analytical balances, centrifuge, dissecting set, ketamine-xylazine/ cocktail (1:1), distilled water, NaCl 0.9% solution, neutral buffered formalin (NBF) 10%.

Methods

This research was conducted under the Ethical Clearance certificate number 00046/04/LPPT/ VIII/2019, issued by the Institutional Committee of Animal Use and Care (ICAUC) of Universitas Gadjah Mada.

Animal Treatment

This experiment was conducted for 54 days. The first 14 days was the induction period, and the next 40 days was the treatment period. All treatments were given orally. Twenty rats were divided into 5 groups: H, HTG-induced only; H.O, HTG-induced and treated by Gemfibrozil (10 mL/kg BW/day); H.U, HTG-induced and treated by *U. lactuca* (1500 mg/kg BW/day); S.U, not HTG-induced and treated by *U. lactuca* (1500 mg/kg BW/day); K.S, healthy control, not HTG-induced and not treated. Saturated fat was used for HTG induction at 15 mL/kg BW/day during the induction period and 7.5 mL/kg BW/day during the treatment period. Rats in S.U and K.S groups were given distilled water at the same dose as the saturated fat.

Body Weight

Body weight was measured using a semi-analytical balance. This data was used to calculate the amount of saturated fat, Gemfibrozil, *U. lactuca* (Eq. 1), and ketamine-xylazine cocktail (Eq. 2-4) needed for administration. Body weight was also used to calculate the gonadosomatic index (GSI).

$$daily\ intake = daily\ dose \times body\ weight \quad (1)$$

Daily dose of saturated fat is 15 mL/kg BW or 7.5 mL/kg BW, Gemfibrozil is 10 mg/kg BW, and *U. lactuca* is 1500 mg/kg BW.

$$volume\ of\ ketamine\ (mL) = 2 \times volume\ of\ ketamine\ (mL) \quad (2)$$

$$volume\ of\ ketamine\ (mL) = weight\ of\ ketamine\ (mg) \div ketamine\ concentration \quad (3)$$

$$weight\ of\ ketamine\ (mg) = dose \times body\ weight \quad (4)$$

Ketamine dosage for anesthesia is 50 mg/kg BW. For euthanasia, the dosage is doubled. The concentration of ketamine solution used was 100 mg/mL.

Serum TG Concentration

Blood serum was obtained from centrifugation (10,000 rpm, 10 min) of blood samples collected from the retro-orbital plexus of rats on D-0, D-14, D-34, and D-54. Triglyceride concentrations in the blood serum were determined using Triglycerides FS (DiaSys) based on its protocol (DiaSys, 2015) at LPPT UGM unit 2.

Serum Testosterone Concentration

Testosterone concentrations of blood serum collected on D-0 and D-54 were determined by competitive ELISA using Testosterone ELISA Kit TE373S (Calbiotech).

Forced Swim Test

This procedure was done based on the protocol by Yankelevitch-Yahav *et al.* (2015) using glass chambers sized 20 x 20 x 40 cm^3. The chambers were filled with clean water set at room temperature up to 30 cm in depth. Video recording using YI Action Camera (XiaoYi) was done throughout the testing. The rat behavior recorded in this test was immobility, the rat floats, only performing movements to keep its nose above the water.

Open Field Test

The procedure for this test was modified from Sestakova *et al.* (2013). Each rat was placed in the center of a 40 x 40 x 40 cm^3 opaque black wooden chamber. The video camera was placed directly above the chamber to record the rat's behavior, 5 min for each rat. After 5 min, the rat was removed and the chamber was sterilized with 70% ethanol, before placement of the next rat. The number of crossings between the central area (20 x 20 cm^2) and outer area, frequency of rearing, and duration of freezing were recorded.

Gonadosomatic Index (of testes soaked in NBF for 24 hours)

Upon necropsy, the testes were collected, washed in NaCl 0.9% solution, then stored in NBF 10%. An incision was made on each testis' capsule, 2 hours after collection. After 24 hours of collection, the testes were weighed using an analytical balance. Gonadosomatic Index is calculated using Eq. (5) based on Nurhidayat *et al.* (2017).

$$GSI = \frac{testes\ weight}{(body\ weight - testes\ weight)}\ x\ 100\% \quad (5)$$

Data Analysis

Quantitative data were statistically analyzed using SPSS v.16 with the One-Way ANOVA followed by DMRT for body weight, TG D-0, D-20, and D-40 treatment, depressive-like behaviors in OFT, and GSI; paired-samples T-test for D-0 of HTG-induction and testosterone concentration, or Kruskal -Wallis H Test for depressive-like behavior in FST. A value of $p \leq 0.05$ was considered significant statistically. Video recordings from OFT were analyzed using idTracker (Cajal Institute, Spain).

RESULTS AND DISCUSSION

Body Weight

Body weight was measured on day-0 (D-0) to record the initial body weight, day-14 (D-14) to examine the effect of HTG induction, day-34 (D-34) to examine the effects of 20 days of HTG treatment, and day-54 (D-54) to determine the GSI. On D-0 and D-14, the mean body weight of H is the lowest and it is significantly different amongst the other groups, but on D-34, it is no longer significantly different from the other groups (Table 1). According to Jung and Yoo (2018), HTG is related to obesity and

Table 1. Body weight of HTG male Wistar Rats (*Rattus norvegicus* Berkenhout, 1769) on D-0, D-14, D-34, and D-54.

Group	Body weight (g)			
	D-0	D-14	D-34	D-54
H	262.25±7.69^{a,w}	276.25±8.33^{a,w}	322.25±12.05^{a,x}	338.00±16.21^{a,x}
H.O	301.25±10.36^{b,w}	308.25±13.22^{ab,w}	337.50±11.87^{a,wx}	358.75±14.38^{a,x}
H.U	321.75±13.06^{b,w}	330.00±15.93^{b,w}	358.75±16.13^{a,w}	372.75±20.42^{a,w}
S.U	305.25±3.20^{b,w}	323.50±9.18^{b,wx}	346.50±9.73^{a,xy}	370.25±9.92^{a,y}
K.S	294.25±14.30^{b,w}	311.50±15.12^{ab,wx}	342.75±12.74^{a,xy}	360.25±12.27^{a,y}

Data were presented as mean±SE. The different superscript letters note a significant difference at $p<0.05$: **ab** compares values between groups within the same day, **wxy** compares values between days within the same group. H: HTG-induced, H.O: HTG-induced with Gemfibrozil treatment, H.U: HTG-induced with *U. lactuca* treatment, S.U: not HTG-induced with *U. lactuca* treatment, K.S: healthy control, not HTG-induced nor given treatment. D-0 – D-14: HTG induction period; D15 D-15-D-54: treatment period with maintained HTG induction.

cardiovascular disorders. Obesity is defined as a condition of excessive fat build up in the body, with a high body mass index (Ofei, 2005). Consumption of foods with high Fe content such as *U. lactuca*, which has Fe content of 873.72 mg/kg (Mulyati *et al.*, 2019), has potency to increase body weight by approximately 7 grams/day (Aukett *et al.*, 1986). Yokus and Gedik (2016) reported that iron therapy can be used to increase Hb, body weight, and ferritin.

Serum TG Concentration

The three groups induced with HTG for 14 days showed 24.3% of serum TG concentration elevation (Table 2), but not statistically significant ($p>0.05$). Table 3 showed the increasing and decreasing serum TG concentration in all groups during the treatment periods. Until D-20 of the treatment period, all groups showed a reduction of serum TG concentration, as the daily dose of saturated fat was reduced by 50%. This was seen in the H group, whose TG concentrations decreased from 293.40 mg/dL to 170.38 mg/dL. This design implicates a person with HTG who limits their fat intake to reduce their blood TG concentration.

From D-0 to D-20, treatment with Gemfibrozil and *U. lactuca* also showed a significant decrease in TG concentrations ($p<0.05$). This is because Gemfibrozil is intended as a hypolipidemic or antihyperlipidemic drug, especially targeting TGs. Frick *et al.* (1987) stated that Gemfibrozil could reduce TG in the blood by 35%, while Vinik and Colwell (1993) found the decrease to be 26.4%. These findings confirm that Gemfibrozil may treat HTG.

Ulva lactuca's main polysaccharide, ulvan, has potency as a lipid antiperoxidative and antihyperlipidemic (Kidgell *et al.*, 2019). In addition, the content of secondary metabolites in *U. lactuca* such as terpenoids, sterols, carotenoids, and polyphenols can also act as antihyperlipidemic properties (Silva *et al.*, 2013). The content of omega-3 (0.1% relative), omega-6 (0.1% relative), omega-9 (0.1% relative), and several unsaturated fatty acids such as pentadecenoic acid (11.60% relative), heptadecanoic acid (7.58% relative), and linolelaidic acid (18.54% relative) (Mulyati *et al.*, 2019) could reduce TG concentration in blood plasma approximately 25-50% if consumed regularly (Shearer *et al.*, 2012). Therefore, the TG

Table 2. Serum TG concentration of male Wistar Rats (*Rattus norvegicus* Berkenhout, 1769) during the 14 day-HTG induction period.

Group	Mean TG concentration (mg/dL)		Δ Mean TG concentration		Sig. (2-tailed)
	D-0	D-14	mg/dL	%	
H	207.08±25.63	273.63±33.49	66.55	24.32	0.129
H.O					
H.U					

H: HTG-induced, H.O: HTG-induced with Gemfibrozil treatment, H.U: HTG-induced with *U. lactuca* treatment, S.U: not HTG-induced with *U. lactuca* treatment, K.S: healthy control, not HTG-induced nor given treatment. Sig. (2-tailed) < 0.05 shows significant difference.

Table 3. Serum TG concentration of HTG male Wistar Rats (*Rattus norvegicus* Berkenhout, 1769) during the 40 day treatment period.

Group	Serum TG concentration (mg/dL)		
	D-0*	D-20*	D-40*
H	**293.40±72.50$^{a.w}$**	**170.38±15.61$^{c.w}$**	206.72±29.30$^{ab.w}$
H.O	286.38±48.84$^{a.x}$	**122.50±5.44$^{ab.w}$**	**130.83±14.40$^{a.w}$**
H.U	199.41±23.25$^{a.x}$	103.80±8.19$^{a.w}$	146.70±15.17$^{ab.w}$
S.U	**196.05±44.28$^{a.w}$**	131.65±7.15$^{ab.w}$	182.20±16.74$^{ab.w}$
K.S	233.80±30.68$^{a.w}$	151.02±17.60$^{bc.w}$	**227.17±34.06$^{b.w}$**

Data are presented as mean±SE. Different superscript letters note significant difference at $p<0.05$: **abc** compares values between groups within the same day, **wx** compares values between days within the same group. H: HTG-induced, H.O: HTG-induced with Gemfibrozil treatment, H.U: HTG-induced with *U. lactuca* treatment, S.U: not HTG-induced with *U. lactuca* treatment, K.S: healthy control, not HTG-induced nor given treatment. *D-0, D-20, and D-40 of the treatment period.

concentration decrease in the H.U group was significant between D-0 and D-20 ($p < 0.05$).

However, at D-40 all groups have different TG concentrations. This might be caused by sensitization towards the administered fat. Sensitization is the opposite of tolerance, where there will be an increase in response when treated by a substance with the same dose continuously or repeated dose (Tomek & Olive, 2018). In addition, individual differences in TG concentrations can be influenced by differences in physiological sensitivity to the treatment or even the environment (André *et al.*, 2018; Nistiar *et al.*, 2012).

Gonadosomatic Index (of testes soaked in NBF for 24 hours)

This index shows the growth and development of the reproductive system, which correlates with sexual maturity (Barber and Black, 2006). In this research, an increase of testes weight was followed by an increase of GSI (Table 4). Based on Table 4, GSI of H and H.U groups were not significantly different ($p > 0.05$), although GSI of H group was greater than H.U group. This may be caused by the presence of the cadmium (Cd) contaminant in *U. lactuca*, which is 11-12 times higher than the recommended limit by BPOM 2018 (Mulyati *et al.*, 2019). Cadmium can inhibit the performance of bioactive nutrients in *U. lactuca* such as selenium (Se) and zinc (Zn) to enhance the male reproductive profile (Dewantari, 2013). According to Elhafeez *et al.* (2019), chronic exposure of Cd to Wistar rats caused a decrease of GSI because it can interfere with the HPG axis regulation by increasing the occurrence of lipid peroxidation, which further causes atrophy. In addition, according to El-Shahat *et al.* (2009), the presence of Cd in the body increases the risk of degenerative cell death (necrosis). Furthermore, it causes a decreasing number of Sertoli cells in the seminiferous tubules. A low GSI also can be caused by abnormal hormone levels, which might be influenced by the use of anti-lipid drugs such as Gemfibrozil. Lee *et al.* (2019) and Semet *et al.* (2017) found that Gemfibrozil may cause hormonal imbalance in the body, therefore, inhibiting the growth and development of organs in the reproductive system.

Gonadosomatic index of S.U group was the highest amongst the other groups, suggesting that *U. lactuca* increases the growth and development of the reproductive system. *Ulva lactuca* contains nutrients including Zn and Se. Zinc maintains sexual function and increases spermatogenesis. In fact, lack of Zn causes a reduction in testosterone production and shrinking of the testes, while Se serves as an antioxidant and increases fertility in males (Dewantari, 2013). Our results suggest that there is a dual effect by *U. lactuca*, where it increases GSI in healthy rats, but reduces GSI in HTG rats.

Serum Testosterone Concentration

The highest rise in testosterone concentration was shown in H group (Figure 1). An increase in testosterone concentration happens as a result of age maturity (Alvarado *et al.*, 2019; Stanworth & Jones, 2008). An increase in testosterone is followed by testes development. Cholesterol is a precursor of testosterone. Sources of cholesterol include de novo cholesterol synthesis, plasma membrane cholesterol, LDL, and HDL cholesterol, as well as lipid droplets (Hu *et al.*, 2010). Triglycerides are different from cholesterol, although Freeman and Ontko (1992) state that TG is stored in cells as lipid droplets as cholesteryl ester. Indirectly, these lipid droplets can also be used in steroidogenesis.

An increase of testosterone concentration was also found in H.U group, although only at 1.90% ($p > 0.05$). The presence of Cd in *U. lactuca* can prevent the bioactive nutrients found in *U. lactuca* such as Se and Zn, which play roles in increasing fertility, spermatogenesis, as antioxidants that can prevent the oxidation of sperm cells, and able to increase the production of testosterone hormone (Dewantari, 2013). According to Zeng *et al.* (2004), the presence of contaminants such as Cd in the human body can cause a decrease in the hormone testosterone, but not significant.

A significant decrease ($p < 0.05$) in testosterone

Table 4. Gonadosomatic Index (GSI) of male Wistar Rats (*Rattus norvegicus* Berkenhout, 1769).

Group	Total testes weight (gram)	GSI* (%)
H	2.16±0.36	0.655 ± 0.122^{ab}
H.O	**1.34±0.14**	**0.375 ± 0.033^{a}**
H.U	1.92±0.39	0.518 ± 0.094^{ab}
S.U	**2.82±0.24**	**0.773 ± 0.141^{b}**
K.S	1.62±0.31	0.455 ± 0.184^{a}

Different superscript letters note a significant difference at $p < 0.05$. H: HTG-induced control, H.O: HTG-induced with Gemfibrozil treatment, H.U: HTG-induced with *U. lactuca* treatment, S.U: not HTG-induced with *U. lactuca* treatment, K.S: healthy control, not HTG-induced nor given treatment. *GSI of testes soaked in NBF 10% for 24 hours.

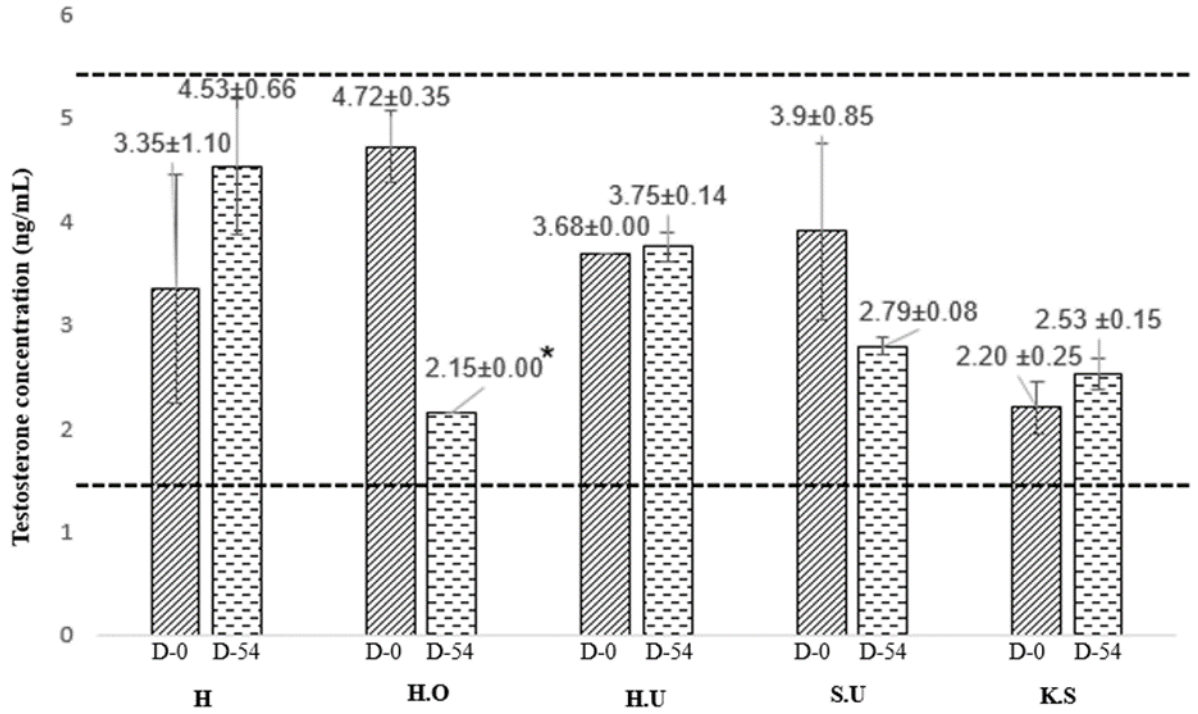

Figure 1. Serum testosterone concentration of male Wistar rats (*Rattus norvegicus* Berkenhout, 1769) on D-0 and D-54 of induction (D-40 of treatment).
Data are presented as mean±SE. H: HTG-induced control, H.O: HTG-induced with Gemfibrozil treatment, H.U: HTG-induced with *U. lactuca* treatment, S.U: not HTG-induced with *U. lactuca* treatment, K.S: healthy control, not HTG-induced nor given treatment. *significant difference with D-0.

concentration was shown in H.O group. This is caused by the effect of Gemfibrozil as an antilipid, that can decrease its precursor, cholesterol, about 11 % (Frick *et al.*, 1987). Gemfibrozil affects exocrine function in spermatogenesis by altering spermatogenic cells and/or Sertoli cells. The effect of this drug on the endocrine system of the testes is by transforming or damaging Leydig cells and the hormone regulation of the Hypothalamus-Pituitary-Gonad axis (Semet *et al.*, 2017). Lee *et al.* (2019) found that Gemfibrozil given to *Oryzias latipes* significantly reduces testosterone concentrations of this freshwater fish. De Keyser *et al.* (2015) also states that the consumption of other cholesterol-lowing drugs such as statin significantly drops cholesterol concentration in humans and rats. Carrier *et al.* (2018) link male reproduction to depression by anxiolytic and antidepressant-like properties of testosterone.

Anxiety-like and Depressive-like Behaviors

Planchez *et al.* (2019) state that behavioral despair is a symptom of major depressive disorder that can be assessed as a depressive-like behavior in animal models through FST. Duration of immobility in FST reflects behavioral despair, as the administration of antidepressants reduces the duration of immobility (Cryan & Holmes, 2005). The highest duration of immobility is shown in S.U group, suggesting that treatment with *U. lactuca* increases behavioral despair in this group, although this pattern is not shown in H.U group, which is not significantly different from, and in fact slightly lower than, H group (Figure 2a). *Ulva lactuca* might increase depressive-like behaviors because of its high Cd content (Mulyati *et al.*, 2019). Lamtai *et al.* (2018) has found that injection of Cd into rats at 0.25 – 1 mg/kg BW for 8 weeks increases depressive-like behaviors, while Scinicariello and Buser (2015) found that increased blood Cd levels increased possibilities of depressive symptoms in 20 – 39 years old men and women. Lamtai *et al.* (2018) discussed in their paper that chronic exposure to Cd could lead to Cd accumulation in the brain, where Cd inhibits enzymes of the serotonergic system, reducing serotonin levels. Low serotonin levels are more likely to be found in individuals with depression (Kamel *et al.*, 2011).

Thigmotaxis, the preference to be near the chamber's walls, is a sign of anxiety (Seibenhener & Wooten, 2015). Thigmotaxis can be visually observed in Figure 3 and supported by crossing frequency (Figure 2b). Fewer crossings to the central area mean that the rat tends to stay in the outer area, closer to the walls. Both H.U and H groups have the lowest values for crossing and rearing frequency (Figure 2b-c), and the highest values for the freezing duration (Figure 2d). These parameters illustrate

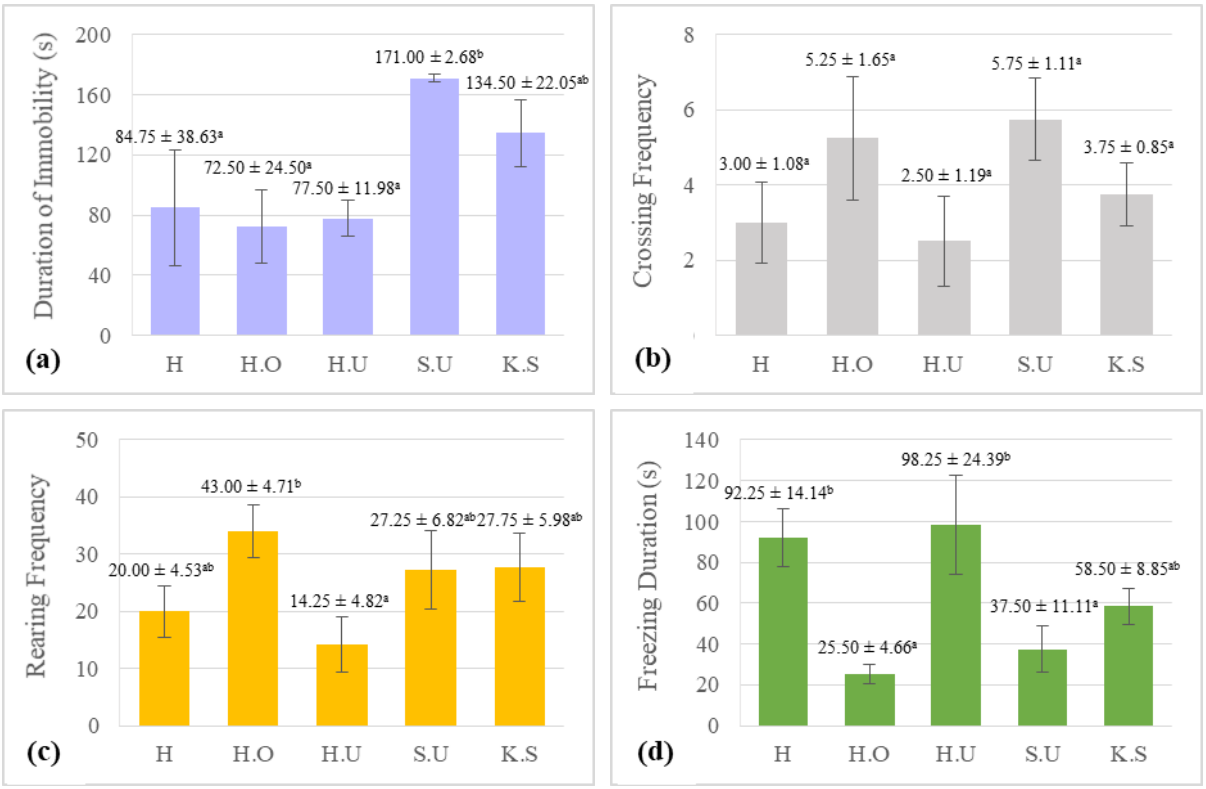

Figure 2. Behavioral data recorded from FST is the duration of immobility (**a**). Behavioral data recorded from OFT: number of movements crossing over between the central area and the outer area (**b**), frequency of rearing (**c**), and freezing duration (**d**). Data are presented as mean±SE. Different superscript letters note a significant difference at $p<0.05$. H: HTG-induced control, H.O: HTG-induced with Gemfibrozil treatment, H.U: HTG-induced with *U. lactuca* treatment, S.U: not HTG-induced with *U. lactuca* treatment, K.S: healthy control, not HTG-induced nor given treatment.

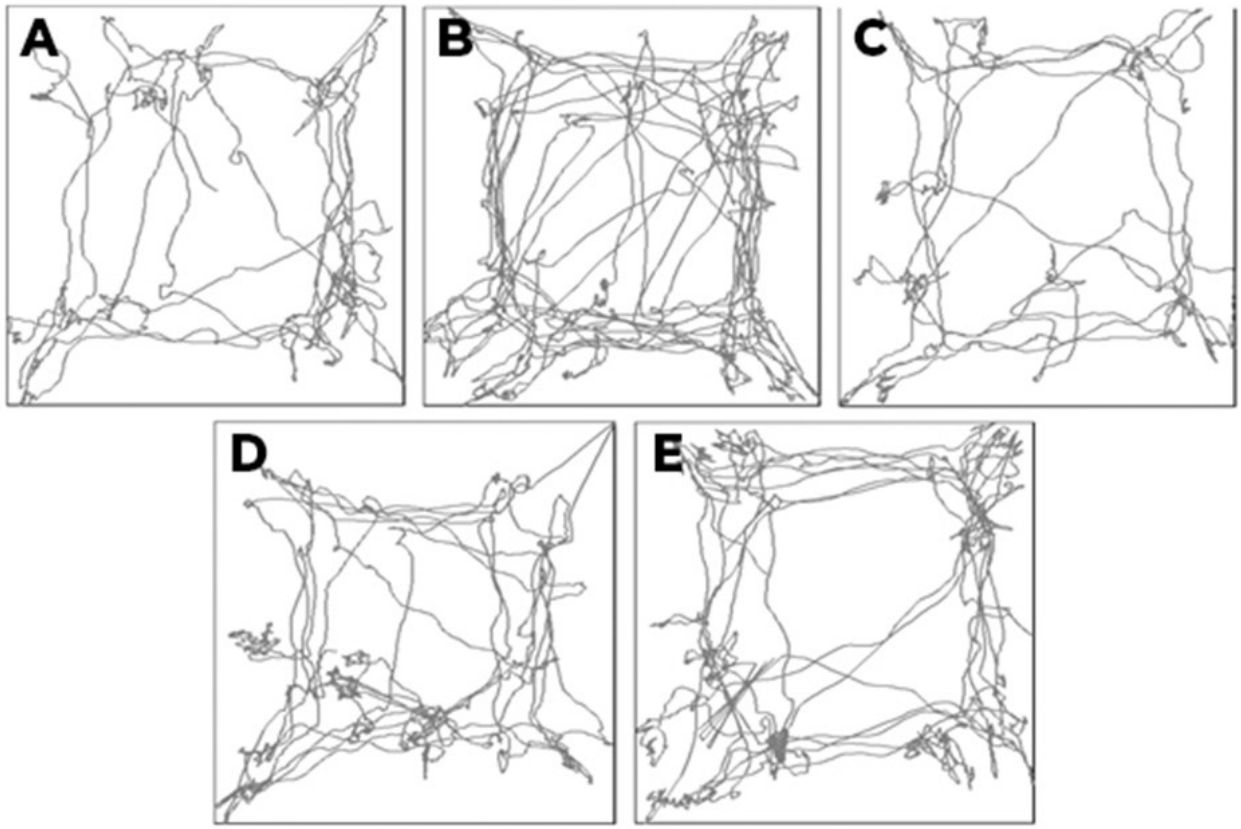

Figure 3. Trajectory pattern in OFT of rats from group H (a), H.O (b), H.U (c), S.U (d), and K.S (e) obtained from idTracker. Lines in each box represent the paths made by the animal on the chamber floor. H: HTG-induced control, H.O: HTG-induced with Gemfibrozil treatment, H.U: HTG-induced with *U. lactuca* treatment, S.U: not HTG-induced with *U. lactuca* treatment, K.S: healthy control, not HTG-induced nor given treatment.

exploratory activities in a novel environment (Choleris *et al.*, 2001; Ennaceur, 2014; Sestakova *et al.*, 2013). Furthermore, Magara *et al.* (2015) found that a rat model of depression showed less exploratory activities and more reactive coping (freezing, motionless behavior) when first introduced to a new cage, compared to healthy Sprague-Dawley rats, suggesting that exploratory behaviors in OFT can indicate depressive-like symptoms. High levels of TG in blood could cause TG to cross the blood-brain barrier (BBB), where it creates leptin resistance in the brain, through what might be an allosteric or post-receptor mechanism (Banks *et al.*, 2018). Leptin is a peptide hormone that has been found to have antidepressant properties (Lawson *et al.*, 2012; Lu, 2007). This might explain the increased depressive-like behaviors in the HTG-induced rats. These behaviors were lowered in H.O group, possibly because Gemfibrozil was able to lower and maintain serum TG.

In analyzing these behaviors, it must be noted that rearing has been interpreted as a result of both an anxiogenic and anxiolytic treatment in different studies (Ennaceur, 2014). A decrease in thigmotaxis of H.O suggests that Gemfibrozil has anxiolytic properties, further suggesting that in this research, rearing behavior is not an anxiety-like behavior, because H.O has the highest rearing frequency (Figure 2c). Freezing is considered a defensive behavior in avoiding danger (Choleris *et al.*, 2001), as well as a physiological sign of fear (Sestakova *et al.*, 2013). A decrease in the number of crossing and rearing, added with an increase of freezing in H.U group (Figure 2b-d), insignificant to H group, suggests that the *U. lactuca* treatment did not aid in reducing anxiety-like behaviors in HTG rats. Results from OFT also did not show that the *U. lactuca* treatment was able to reduce anxiety-like behaviors in healthy rats, except in crossing frequency, which is highest in S.U group, although insignificant to K.S group. The high level of Cd in *U. lactuca* (Mulyati *et al.*, 2019) might contribute to these results, canceling out the potential antihypertriglyceridemic effect in *U. lactuca*.

CONCLUSION

Hypertriglyceridemia increases body weight, serum TG and testosterone concentration, GSI, and depressive-like behaviors in FST and OFT. Treatment of HTG using *U. lactuca* at 1500 mg/kg BW/day did not show significantly different results compared to HTG controls. Meanwhile, an increase in GSI, testosterone concentration, and depressive-like behaviors can be observed compared to healthy controls. These results suggest that this treatment not only enhances gonad growth and development but also increases depressive-like behavior.

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Research Article

Epilithic Microalgae Isolated from Biofilm on Borobudur Temple Stone

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ABSTRACT
Borobudur Temple is a historical heritage building located in an open area and made of porous building materials (stone materials). This condition makes the Borobudur Temple susceptible to various problems related to degradation and weathering. Biodeterioration of Borobudur Temple may be caused by activities of living organisms present in the biofilm of stone. Continuous monitoring and evaluation need to be carried out by observing and isolating the growth of micro-organisms, including epilithic microalgae. Therefore, this study aims to isolate and identify epilithic microalgae from the biofilm on Borobudur Temple stones. Epilithic microalgae were isolated to obtain a uni-algae and maintained under culture conditions. The morphological of microalgae were observed using light microscopy, while the 18S rRNA gene sequence determined the molecular identification of microalgae for eukaryotic and 16S rRNA sequence for prokaryotic. A total of nine epilithic microalgae were successfully isolated from the biofilm of Borobudur Temple stones. The isolated were identified as *Ankistrodesmus falcatus*, *Tetraselmis cordiformis*, *Pseudendoclonium arthropyreniae*, *Anabaena cylindrica*, *Nostoc gelatinosum*, *Oscillatoria limnetica*, *Messastrum gracile*, *Stigeoclonium aestivale*, and *Scenedesmus acuminatus*. This is the first study for the identification of microalgae from Borobudur temple stones. The isolates will be collected and will be used as a source for further study.

Keywords: 16S rRNA gene, 18S rRNA gene, epilithic algal, molecular identification, phylogeny, subaerial

INTRODUCTION
Borobudur Temple is located in Borobudur Village, Borobudur District, Magelang Regency, Central Java Province. This historical heritage temple compound was built in an open area on a modified hill, with a height of 265 meters above sea level, with a length of 121.66 meters, width 121.38 meters, and a height of 34.50 meters. The structure of the Borobudur Temple consists of nine terraces and a main stupa at the top. There are six rectangular terraces and three circular terraces, covering the Kamadhatu, Rupadhatu, and Arupadhatu levels. Borobudur Temple built using the andesite stone material from rivers around the Borobudur Temple with a total of ± 2,000,000 pieces of stones (Banindro, 2015; Salazar, 2018).

The restoration of Borobudur Temple has been carried out twice, the Dutch East Indies government carried out the first restoration under the leadership of Van Erp, and the second restoration was carried out by the Indonesian government chaired by Soekmono (Voûte & Voute, 1973; Gunarto, 2007; Banindro, 2015). Rehabilitation of Borobudur Temple has also been done after Mount Merapi's eruption in 2010 to clean up volcanic ash, which is chemically acidic and can damage this historic temple stone. Demolition of stone blocks has been carried out to improve the clogged water and drainage system, which is clogged with volcanic dust mixture mixed with rainwater, followed by reforestation and planting of trees in the surrounding environment to stabilize the temperature (Yulianto *et al.*, 2013; Khoirunnisa, Warsono, & Suryaningsih, 2014). After the Borobudur Temple has been restored and rehabilitated, it does not mean that the temple maintenance has been completed. There is no

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guarantee that the Borobudur Temple is free from damage, degradation, and weathering processes. Therefore, continuous monitoring and evaluation need to be carried out by observing and isolating the growth of micro-organisms, including epilithic microalgae covered on Borobudur Temple stones.

Epilithic microalgae are part of a group of peripheral microalgae that live attached to various substrates such as rocks/stones, corals, gravel, and other hard objects. The development and ability of epilithic microalgae are very dependent on the presence and condition of the substrate. Microalgae attached to temple stone are more permanent than microalgae attached to a living substrate because the living substrate will experience development and death. In contrast, in the substrate, inanimate objects do not experience changes such as damage or death. The presence of epilithic microalgae on surfaces over time can cause substantial damage, including physicochemical damage and aesthetic discoloration of stone objects such as facades of buildings and monuments (Garside, 2010; Bertuzzi *et al.*, 2017; Matteucci *et al.*, 2019).

Several studies have been reported that eukaryotic green micro-algae are the dominant organisms on the biofilm of monumental stones of temperate regions, where microalgae like the Cyanophyta group predominantly occur on similar substrates in the tropics (Song, Kim & Lee, 2012; Keshari & Adhikary, 2013; Nakajima, Hokoi & Ogura, 2015; Villa *et al.*, 2016). Research on the isolation and identification of microorganisms associated with moss on the Surface of the Borobudur Temple Stone has been carried out on the Actinomycetes group, as one of the monitoring and exploration of microorganism biodiversity in Borobudur Temple (Putri, Purbani, & Habibi, 2020). However, studies of epilithic microalgae isolated from biofilms in Borobudur Temple stones have never been reported. Studies on microbial populations living, including epilithic microalgae on stones of Borobudur Temple, need to be done as a starting point for successful conservation management and control. One of the most important steps in studying the epilithic microalgae ecology of Borobudur Temple stones is to identify the microalgae involved in biological damage/ biodeterioration. Therefore, this study aims to isolate and identify epilithic microalgae from the biofilm on Borobudur Temple stone, as a database that can complement studies on micro-organism in particular that can weather and reduce the aesthetic value of temples in Indonesia.

MATERIALS AND METHODS

Materials

The materials were used for this study: a sterile swab, scalpel, screw cap and bottles, Pasteur pipette, object and cover glass, rubber bulb, microscope (Olympus CKX41 and Olympus BX5), Olympus DP26 cameras, corning well cell, fluorescent lamps, shaker incubator, shaking bath, centrifuge, vortex mixer, microtube 2.0 ml, beat bitter, micropipette and tips, thermal cycler (PCR), electrophoresis apparatus, UV transilluminator, Gel Doc, BG11 medium, Genomic DNA mini kit (Plant) Geneaid, Go Taq Green kit, and universal primer 18S rRNA and 16S rRNA gene.

Methods

Samples were collected from the exposed biofilm stone surfaces of the different tiers of Borobudur Temple by gently scrapping the surfaces of stone with a sterile swab and scalpel. Each sample was placed into the BG11 medium (Keshari & Adhikary, 2013). Epilithic microalgae were isolated to obtain a uni-algae culture using the capillary micro-pipetting method under the light microscope (Olympus CKX41). The culture was maintained for 10-14 days at 25^{o}C below 30 µmol m^{-2} s^{-1} fluorescent light with 12h light cycle:12h dark in a shaker incubator (Anderson, 2005; Barsanti & Gualtieri, 2014). Morphological characteristics of epilithic microalgae were observed regularly under a microscope using Olympus BX5 light microscopy linked to the Olympus DP26 camera and a personal computer with the CellSens Standart application. The epilithic microalgae characteristics were then analyzed descriptively using a microalgae identification book (Naselli-Flores & Barone, 2009; Barsanti & Gualtieri, 2014; Kaštovský *et al.*, 2019).

Microalgae were identified based on the 18S rRNA gene sequence (for eukaryotic) and 16S rRNA gene sequence (for prokaryotic). Firstly, as much as 10 ml of microalgae culture was centrifuged for 4 minutes at 3000 rpm, a temperature below 10^{o}C. Genomic DNA was extracted using the Genomic DNA mini kit (Plant) Geneaid, following Genetica Science manufacturing protocol. Amplification was performed using 18S rRNA primers, 18SF, and 18SR for eukaryotic microalgae (Tale *et al.*, 2014). A partial gene sequence of 16S rRNA was amplified using universal primers, Forward 27F Algae, and Reverse 1510R for prokaryotic microalgae (Marsh & Nakatsu, 2014). The PCR composition was 12.5 µl GoTag® Green Master Mix, 10 µl Nuclease Free Water (NFW), 0.5 µl primer, 0.5 µl DMSO, and 1 µl DNA template. The PCR condition includes pre denaturation at 94°C for five minutes, 35 cycles of

denaturation at 94°C for one minute, annealing at 63°C (for eukaryotic) and 55°C (for prokaryotic) for one minute, and extension at 72°C for one minute, then final extension at 72°C for 10 minutes, and storage at 4°C (Ma *et al.*, 2008; Tale *et al.*, 2014).
The PCR products were visualized on 1% agarose gel under UV-transilluminator using Mupid Electrophoresis. Then, the gene fragment was sequenced by Macrogen. inc. The DNA sequence similarities were analyzed using the BLASTN program on the NCBI database server (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree was constructed using Neighbor-Joining (NJ), 1000 bootstrap welding methods with the application of the Molecular Evolutionary Genetics Analysis (MEGA) program (Kumar *et al.*, 2018).

RESULTS AND DISCUSSION

We successfully isolated and purified nine isolates of epilithic microalgae from the biofilm on Borobudur Temple stone during this study. Based on isolates' morphological characteristics under a light microscope, the isolates identified to the division Chlorophyta and division Cyanophyta. Among the nine isolates, six isolates belong to Chlorophyta (or green algae) division, and three isolates belong to the Cyanophyta division. The isolates code belongs to the Chlorophyta division were M18-BR1, M18-BR2, M18-BR5, M18-BR13, M18-BR16, and M18-BR20, while the isolates code belongs to Cyanophyta were M18-BR7, M18-BR8, and M18-BR12 (Figure 1).

Molecular identification was carried out to support the identification of morphology and determine which types of isolates to the species level. Six isolates were identified based on the 18S rRNA gene sequence, and three isolates were identified based on the 16S rRNA gene sequence. The 18S rRNA gene sequence was determined, and BLAST analysis was performed, which confirmed that the isolate M18-BR1 has similarities with *Ankistrodesmus falcatus,* M18-BR2 has similarities with *Tetraselmis cordiformis*, M18-BR5 has similarities with *Pseudendoclonium arthropyreniae*, M18-BR13 has similarities with *Messastrum gracile,* M18-BR16 has similarities *Stigeoclonium aestivale*, and M18-BR20 has similarities with *Scenedesmus acuminatus*. The similarity values of these isolates were between 99.06-99.68 of the closest strain type (Table 1.).

Three isolates (M18-BR7, M18-BR8, and M18-BR12) were identified based on the 16S rRNA gene. These isolates have similarities with *Anabaena cylindrica* (M18-BR7), *Nostoc gelatinosum* (M18-BR8), and *Oscillatoria limnetica* (M18-BR12). The isolates have a homology percentage of 100, 99.31 %, and 99.04 % of the closest strain type (Table 2).

The Neighbor-Joining method's phylogeny tree construction was made with 1000 bootstrap replications in the Kimura 2 Parameter model, to

Figure 1. Microscopic photographs of epilithic microalgae in the biofilm of the Borobudur Temple stone.

Table 1. Identification result of epilithic microalgae isolates based on 18S rRNA gene.

Isolate code	Number of nucleotides	Sequence result	Percentage of homology
M18-BR1	609	*Ankistrodesmus falcatus* (MK159026.1)	99.06
M18-BR2	610	*Tetraselmis cordiformis* CCAC 0051 (MK460468.1)	99.93
M18-BR5	601	*Pseudendoclonium arthropyreniae* SAG467-2 (MF034609.1)	99.66
M18-BR13	609	*Messastrum gracile* CCMA UFSCar 622 (KT833593.1)	99.34
M18-BR16	624	*Stigeoclonium aestivale* EP SAG477-20 (KU948222.1)	99.64
M18-BR20	606	*Scenedesmus acuminatus* (AB037088.1)	99.68

Table 2. Identification result of epilithic microalgae isolates based on 16s rRNA gene.

Isolate code	Number of nucleotides	Sequence result	Percentage of homology
M18-BR7	1377	*Anabaena cylindrica* NIES19 (AF247592.1)	100
M18-BR8	1390	*Nostoc sp.* Leptogium gelatinosum cyanobiont (DQ185232.1)	99.31
M18-BR12	1381	*Oscillatoria limnetica* MR1 (AJ007908.1)	99.04

emphasize the identification process of the Basic Local Alignment Search Tool (BLAST). A phylogenetic tree showed that microalgae isolates were affiliation to nine species, the results obtained as shown in Figures 2 and 3.

Ankistrodesmus falcatus is a species of Chlorophyta in the family Selenastraceae. It is needle -like in shape, with gradually tapering ends. As seen in the morphology of the M18-BR1 isolate in Figure 1. Cells mostly of four arranged in cruciate flocky mucilaginous groups could be two to many cells in a colony. M18-BR2 isolate was identified as *Tetraselmis cordiformis* which belongs to the phylum Chlorophyta. These isolates are characterized by chloroplasts of intense green color, their marked cell bodies, the presence of pyrenoids in the chloroplasts, and skeletal walls produced by scales. Isolates M18-BR5 has a green appearance of cell morphology, unipolar or bipolar germination, the irregular shape of the filament, with branching cylinder attached to the surface, can be split into different directions and has a parietal chloroplast with a pyrenoid. Molecularly, this isolate was identified as *Pseudendoclonium arthropyreniae*.

M18-BR13 was identified as *Messastrum gracile* which belongs to phylum Chlorophyta in the family Selenastraceae. Morphologically, the cells are narrow, fusiform to semilunate in shape, the ends gradually tapered, curved. Colonies are solitary or multicellular with irregularly separated cells. There is one parietal chloroplast with a cell wall covered by a thin layer of diffuse mucus. M18-BR16 isolate has morphological characteristics of branched filamentous thalli, without rhizoid. The filamentous cells are cylindrical or spherical, containing chloroplasts of the parietal plate with pyrenoids. The branches are unilateral or alternating, not opposite, ending in sharp cells or hairs, with a thick gelatinous sheath. This isolate was identified as *Stigeoclonium aestivale*. InaCC M131 isolate is a green cell, with an elliptical and spindle shape, and has chloroplasts containing pyrenoid. This isolate was identified as *Scenedesmus acuminatus*.

In this study, we are showing for the first time the biodiversity of epilithic microalgae on the biofilm of Borobudur Temple Stone, identified by morphological and molecular traits. Chlorophyta was the dominant taxa from the biofilm on Borobudur Temple stone, with six genera (*Ankistrodesmus, Tetraselmis, Pseudendoclonium, Messastrum, Stigeoclonium,* and *Scenedesmus*). The genera are mostly soil algae. Biological colonization of the stone containing micro-organisms such as microalgae can originate from the surrounding soil, contaminating the stone after excavation. Also, stone inoculation can occur due to increased infiltration of groundwater and windblown dendrites. According to Soares *et al.* (2019), the Chlorophyta division is a cosmopolitan type that is easy to breed and adapt. They often occur in stone monuments and building stone walls, which are anthropogenic surfaces. Some of them are tolerant of harsh environmental conditions, including temperature fluctuations. This trait also causes Chlorophyta to be more diverse than other groups.

Two orders from the Cyanophyta group were also found from the biofilm in Borobudur temple stone, namely Nostocales and Oscillatoriales. *Anabaena* and *Nostoc* included in the Nostocales group were isolated from samples examined from the Borobudur temple stone's biofilm. Anabaena has

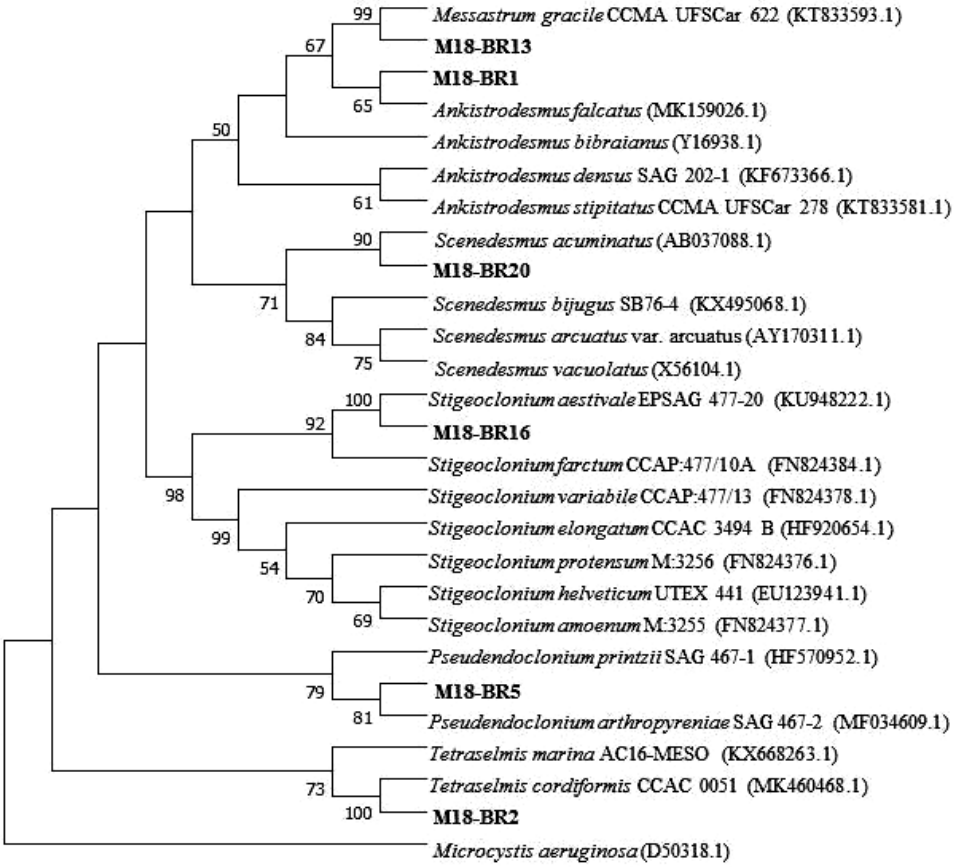

Figure 2. The phylogenetic tree of six selected epilithic microalgae isolates based on 18S rRNA gene sequences.

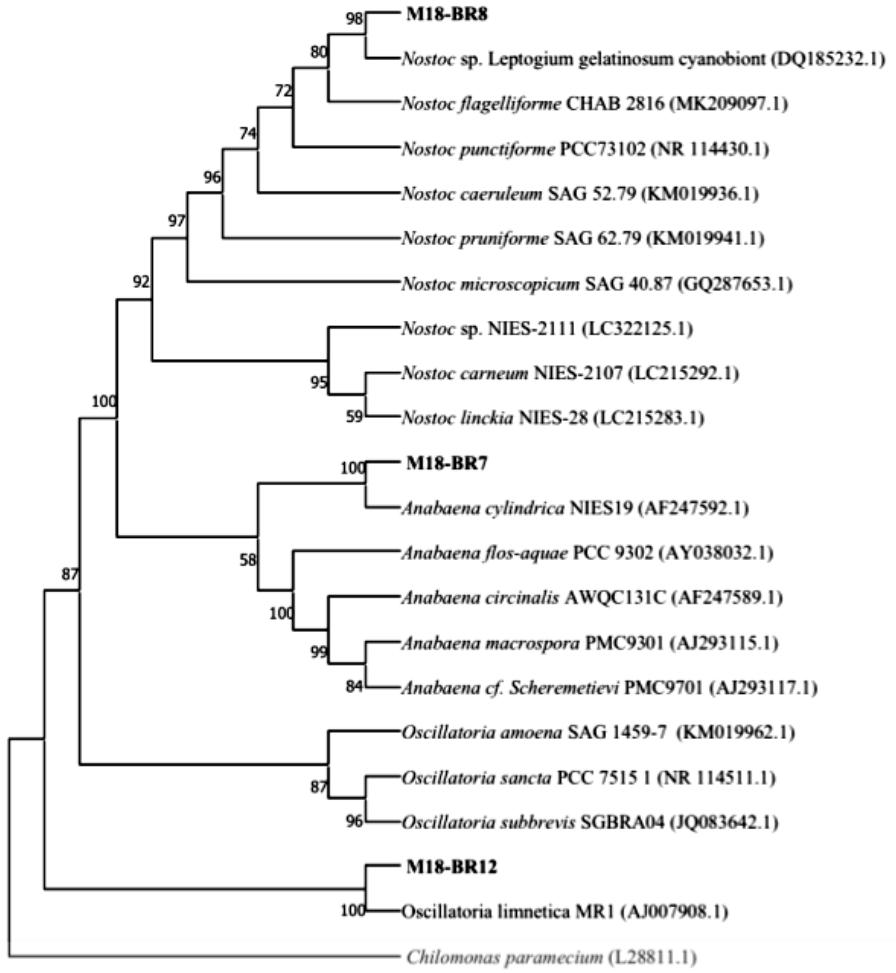

Figure 3. The phylogenetic tree of three selected epilithic microalgae isolates based on 16S rRNA gene sequences.

characteristic filaments with a long chain of vegetative cells ranging from square to round, or cylindrical. Heterocyst forms with a slightly higher length compared to vegetative cells. Nostoc also has cells arranged in beaded chains. Nostoc also has cells arranged in bead chains in the form of unbranched filaments. There are heterocysts with a filamentous structure that is twisted and folded by itself to form a spherical structure. Kaštovský *et al.* (2019) stated that epilithic Cyanophyta, such as Nostocales have thick outer envelopes and protective pigments to survive extreme environmental conditions such as cold, hot, and dry stone surfaces along with cryptoendollithic lichens, fungi, and bacteria. The *Oscillatoria limnetica* identified in this study completely lacks a sheath, and the cells may contain small vacuoles. The cells are typically considerably longer than broad, although the length to width ratio varies. Average filament length is quite steady across seasons. Occasionally the filaments are slightly curved, but never tightly coiled. The cells touch each other but are not closely joined as in most *Oscillatoria* species. Adhikary (2000) and Pandey (2011) reported that the Cyanophyta group belonging to the genera *Gloeocapsa*, *Lyngbya*, *Oscillatoria*, and *Tolypothrix* is the main component of the biofilm. These epilithic microalgae are enveloped by a colored sheath layer and occurred binding with finely textured soil particles on the temple stones. The microalgae communities that inhabit these stones are filamentous microalgae that can induce carbonate formation and cement deposition. In the present study, the Cyanophyta that colonized the temple stones were dominated by filamentous forms such as *Oscillatoria, Nostoc,* and *Anabaena*.

According to several studies, Chlorophyta and Cyanophyta can develop easily on stone surfaces because of their photoautotrophic nature. They are considered pioneering inhabitants of stone colonization, giving rise to colored patinas and incrustations (Tomaselli *et al.*, 2000; Vázquez-Nion *et al.*, 2016; Villa *et al.*, 2016; Popović *et al.*, 2018; Gallego-Cartagena *et al.*, 2020). The epilithic microalgae identified in this study are known to have a water reservoir in the form of a gelatinous sheath, bound by a strong molecular force, thus allowing the microalgae to colonize stone even in dry conditions. This sheath causes adhesion to the substrate. Sometimes the sheath can be pigmented and colored, which is an expression of various ecology and environmental adaptation stages, such as light intensity, temperature, nutrient availability, and cell age. Reduction in chlorophyll and phycocyanin, as well as increased carotenoids under low nitrogen conditions, can cause the epilithic microalgae to turn yellow-brown. Colored patinas and incrustations because of epilithic microalgae cause aesthetic damage to the temple building (Wynn-Williams *et al.*, 2002; Mayer, Dubinsky, & Iluz, 2016; Sonina *et al.*, 2018).

Many factors can cause the high number of taxa contained in the biofilm on the Borobudur Temple stone. One of them is the physicochemical properties of rocks that support the formation of photosynthetic communities such as epilithic microalgae that live in it, especially light, which affects the total community biomass. The availability of water also determines the successful colonization of green algae and Cyanophyta and allows subaerial biofilms to be formed by micro-organisms. According to Young *et al.* (2008) and Pinheiro *et al.* (2019), the population or community of immobilized micro-organisms on the surface of a stone, including green algae and Cyanophyta, can live in biofilms because biofilms can introduce large amounts of water into their structures so that the humidity and temperature balance is maintained. They play a role in binding cells to the substrate (adhesion) and cells to other particles together (cohesion).

Epilithic microalgae from biofilms in Candi rocks can be considered deteriogenic, because patinas that produce various colors can be aesthetically damaging (Javaherdashti *et al.*, 2009). Macedo *et al.* (2009) stated that the inhibition of epilithic microalgae colonization reduces the growth and heterotrophicity of fungi and bacteria and supports the accepted colonization sequence. These photosynthetic micro-organisms can provide nutrients for other communities' growth through the accumulation of the resulting biomass. They contribute to stone breaking directly and through synergistic interactions with heterotrophic micro-organisms such as fungi and bacteria. So the isolation and identification of epilithic microalgae are very important for further research on the control and prevention of biodeteriogenic processes.

CONCLUSION

Chlorophyta was the dominant taxa from the biofilm on Borobudur Temple stone, consisting of *Ankistrodesmus falcatus, Tetraselmis cordiformis*, *Pseudendoclonium arthropyreniae*, *Messastrum gracile, Stigeoclonium aestivale*, and *Scenedesmus acuminatus*.. Three species from the Cyanophyta group were also found, namely *Anabaena cylindrica*, *Nostoc gelatinosum*, and *Oscillatoria limnetica*. This study may help evolve a managerial plan for preventing the growth of epilithic microalgae in exposed temple stones and historic buildings to prevent damage. The isolated epilithic microalgae were then preserved and stored in the Indonesian Cultural Collection (InaCC) for further study on the ecology and physiology of

certain species of this micro-organism to understand their role in the process of stone colonization and biodeterioration.

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