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Isolation of high lipid content microalgae from Wonorejo river, Surabaya, Indonesia and its identification using *rbcL* marker gene

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Abstract. *Saputro TB, Purwani KI, Ermavitalini D, Saifullah AF. 2019. Isolation of high lipids content microalgae from Wonorejo river, Surabaya, Indonesia and its identification using* rbcL *marker gene. Biodiversitas 20: 1380-1388.* Microalgae are unicellular organism which possesses various biologically active metabolites that used in food, cosmetic, pharmaceutical, nutraceutical, and bioenergy industries. One of the most important active metabolites found in microalgae is lipid which can be converted into biodiesel. The higher amount of lipid produced will increases the amount of biodiesel that can be obtained. Although, the production of active metabolites in microalgae has rather fluctuated as it is difficult to obtain the pure cultures of microalgae since it has a cryptic phenomenon. This problem can be solved through identification technique using molecular markers or DNA Barcoding. This research used *rbcL* gene as the molecular marker in species identification. *rbcL* gene encode *ribulose-1,5-bisphosphate carboxylase* (RubisCo) in chloroplast genome. Furthermore, microalgae were isolated from 3 different stations of Wonorejo river estuary, Surabaya, East Java, Indonesia. The isolated microalgae were then identified based on Freshwater algae: identification and use as bioindicators book. Eight isolates were successfully identified as *Mycrocystis* sp., *Nostoc* sp., *Chlorella* sp., *Tabellaria, Synedra, Nitzschia, Navicula,* and *Closteriopsis.* Out of eight, only 3 species that has a good potential to be developed as biodiesel sources *i.e Chlorella* sp., *Synedra* sp., and *Navicula* sp. that produce 21,20%, 20,30%, 27,20% of total lipids over their biomass. The genomic DNA from those three candidates were then isolated and used as a template to amplify *rbcL* gene. The obtained fragment of *rbcL* gene was sequenced and then submitted to the Basic Local Alignment Search Tool (BLAST). The result of BLAST used for alignment and phylogenetic tree construction. The result from BLAST shows the same results as the genus observed by microscopy observation. Overall, this research provides information about the genetic variety of microalgae in Wonorejo which has high potential to be further developed as biodiesel.

Keywords: Barcoding, microalgae, *rbcL* gene, Wonorejo river

INTRODUCTION

Microalgae are unicellular autotrophs microorganism which use CO_2 as carbon source and sun light for photosynthesis process (Spolaore et al. 2006). Microalgae grow in water environment (Hasler et al. 2012). Sanchez et al. (2007) and Ye et al. (2008) stated that microalgae contain several potential chemical compounds such as protein, carbohydrate, pigment (chlorophyll and carotenoids), amino acids, lipid, and hydrocarbon. Microalgae is one organism that has the potential to be used as raw material for biodiesel production since it can produce lipid (Teresa et al. 2010; Gouveia and Oliveira 2009).

Microalgae give a major benefit in biodiesel production as they have excellent ability in synthesizing the lipid. The pure content of lipid found in the dry biomass of certain microalgae species can reach up to 50%-80% with high rate of growth (Hu and Gao 2006; Hossain et al. 2008). According to Teresa et al. (2010), biodiesel is formed by ester alkyl obtained from transesterification process of hydrocarbon which can be obtained from animals, plants, and triacylglycerol (TAG) in algae. Furthermore, microalgae also contain active compounds which are often used in food industry, cosmetic, pharmaceutical and nutraceutical, such as phenol, terpenoids, sterol, flavonoids, and polysaccharides. In addition, microalgae also contain pigments (chlorophyll, phycobilisome, carotene), tocopherol, EPA and DHA (El-Baky et al. 2008). Besides, active compounds are potential antitumor and antimicrobials (Taskin et al. 2010). However, the production of those active compounds fluctuates as pure isolates are difficult to obtain due to the characteristics of cryptic species.

Cryptic species refer to one or more different species which are classified as one species because of share morphological similarities (Lahaye et al. 2008). The relatively high percentage of cryptic species makes the species morphological identification process challenging. To overcome this phenomenon, the usage of molecular markers as an approach for species identification is needed. Burja et al. (2001) stated that morphological identification is a common step in identifying organism. However, the usage of morphological characteristics in the identification has low validity. Schubart et al. (2001) reported that molecular technique is more accurate and efficient to be applied for identification.

Species identification method has rapidly developed from the usage of morphological identification up to molecular identification using the pieces of short DNA

known as "DNA barcode" (Hebert et al. 2003). The advantage of DNA barcoding technique relies on its reliability on identification and characterization of various species which cannot be identified using morphological method (Tudge 2000). The Consortium for the Barcode of Life (CBOL) recommends the use of two plastid genes which are *rbcL* and *matK* as the standard barcode (Hollingsworth et al. 2011). *rbcL* marker can be good marker for green microalgae identification (Wongsawad and Peerapornpisal, 2014; Hadi et al. 2016) use *rbcL* gene. Patel et al (2018) utilyze *rbcL* gene for Molecular Identification of Arthrospira and Dunaliella Isolates.

The *rbcL* genes in plants are the code of the subunit of *ribulose*-1,5-*bisphosphate carboxylase* (RubisCo) enzymes in chloroplast genomes (Judd et al. 2002), and they are universal genes that can be found in almost all of lower plants and upper plants. This recommendation strengthens the reason for using *rcb*L gene in phylogenetic and in relationship analysis of plants within one genus. Research on microalgae identification has been frequently conducted. Furthermore, research on the identification of microalgae obtained from Wonorejo river, Surabaya, Indonesia based on the variety of *rbcL* has never been conducted. The aims of this research was to study the genetic variability of microalgae in Wonorejo river using *rbcL* gene and investigating the potentials of microalgae species in Wonorejo river as biodiesel material by lipid analysis. The result of this research is expected to give comprehensive information about microalgae obtained from Wonorejo river in 2018 and information about the potentials usage of microalgae as biodiesel materials.

MATERIALS AND METHODS

Microalgae collection

This research was conducted from April to September 2018. Samples of microalgae were obtained from 3 stations of Wonorejo River, Surabaya, East Java, Indonesia. The coordinate of station 1 was 7°18'30.8 S and 112°49'56.1 E; station 2 was 7°18'37.3 S and 112°50'19.0 E; while station 3 was 7°18'21.4 S and 112°50'34.2 E (Figure 1). The first location was the estuary located 2000 m away from river downstream. The second location was at the point of contact between fresh water and salt water under the influence of daily tidal, 700 m from the downstream of the river. The third location was the middle estuary in which salt water and fresh water were mixed. Plankton nets 45 µm and diameter of 31 cm and 100 cm nest length were used to collect microalgae samples. Microalgae were collected by pulling the nest horizontally from the surface. The collection was repeated twice for each station. The collected samples were then put into 30 mL sterilized bottles and were stored in a cool box to be isolated and identified in the laboratory. Microalgae samples were put into different bottles and were preserved using 4% formalin.

Sterilization of room and medium

Any kinds of tool which were used had been previously sprayed with 70 alcohol and put into the Laminar Air Flow Cabinet (LAFC). Then, room sterilization was done by turning on the UV lamp in LAFC for 2 hours. LAFC blower was turned on for 30-60 minutes, and the tables were also sprayed with 70% alcohol before they were cleaned using sterilized tissue. Aseptic procedures were administered in LAFC. Culture bottles which were filled with medium were sterilized with lids closed. The sterilization process was conducted using autoclave at 121°C of temperature and 1.5 atm pressure of 20 minutes before they were stored in the inoculation room.

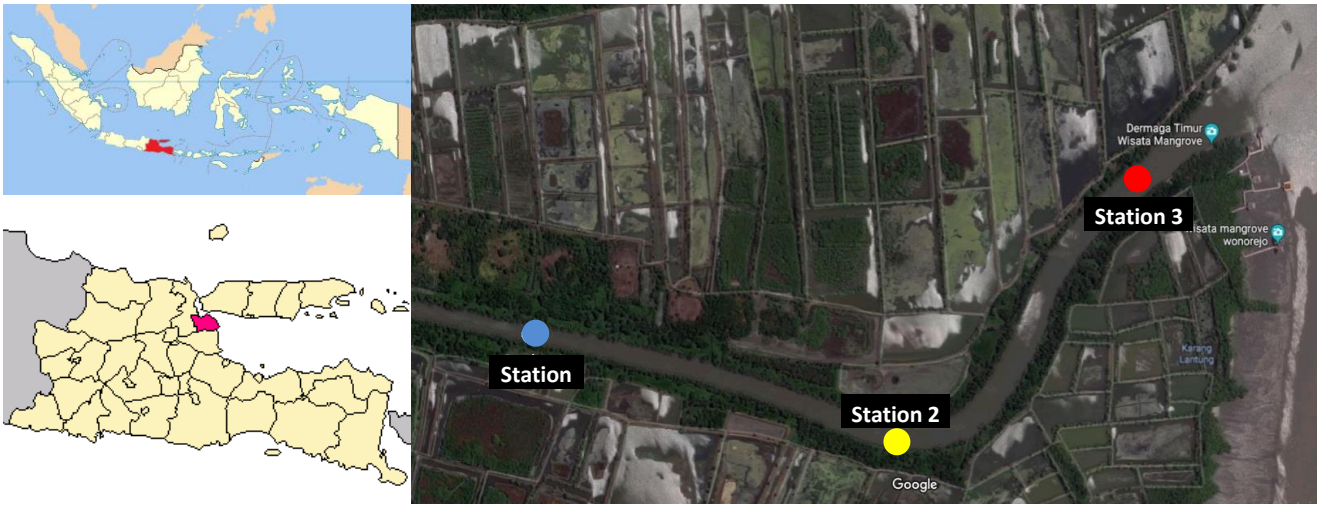

Figure 1. The location of microalgae sampling in Wonorejo River, Surabaya, East Java, Indonesia

Microalgae isolation, purification and identification

The samples were then isolated and identified in the Bioscience and Plant Technology Laboratory, Department of Biology, Faculty of Science, Institut Teknologi Sepuluh Nopember (ITS), Surabaya, Indonesia. Sample isolation was conducted using dilution method, pour plate and streak plate to liquid medium and solid medium of the sterilized sea water. The production of the sterilized sea water was done by filtering the samples of Wonorejo river water using 0,45 µm media pore filter paper prior to the sterilization (Isnansetyo and Kurniastuty 1995). The sterilization was conducted for 45 minutes using the autoclave at 1.5 atm pressure and temperature of 120ºC.

As much as 10 mL of microalgae samples which had been adapted were gradually diluted into 10^{-1} to 10^{-4} test tube containing the sterilized salt water. Purification of microalgae samples was conducted using the streak plate method. In this method, the sterilized salt water was added agar with final concentration as much 1.5%. 2 mL of microalgae sample obtained from 10^{-4} dilution was poured using the pour plate method into the sterilized solid salt water. The isolates were then incubated for 7-14 days. The colonies of microalgae isolate grown in the media were then purified and sterilized using 16 streak plate method to obtain only one species of microalgae indicated by the appearance of colonies that grew in separated areas. Those isolates were incubated within the temperature 25-27ºC under 50 lux neon light at 12:12 light and dark cycle of photoperiods for 7 - 14 days (Nurhatika et al. 2018). Pure culture of microalgae from agar plates was then grown in the liquid medium that is suitable for the growth of the expected microalgae in the test tube.

The colonies of microalgae which grew separately in solid media were taken using inoculating loop to be put in drops of culture media in object glass and were then observed under binocular microscope (Olympus CX-21) at 400x zoom to identify the morphological characteristics. The morphological identification was administered based on the book Bellinger and Siege (2010).

Biomass and total lipid determination

Measurement of fresh and dry weight was carried out on day-16. Measurements were taken by taking 100 mL of microalgae culture, then centrifuging at 3000 rpm for 10 minutes and taking the pellets and measured as fresh weight. Then, pellet in the oven at 80° C for 24 hours. Dry biomass is then weighed as dry weight. Measurement of lipid content was carried out on the 16th day with the extraction process. Microalgae lipids were measured by following the method carried out by Bligh and Dyer (1959) which had been modified in terms of the centrifuge speed being changed to 3000 rpm for 10 minutes.. This method uses methanol and chloroform solvents. Testing of microalgae lipid content was carried out by means of dried microalgae biomass added with 2 mL of pure water, 5 mL of methanol and 2.5 mL of chloroform, then shaken with a shaker for 1 night. After completion, add back with 2.5 mL of pure water and 2.5 mL of chloroform. The next step was centrifuged again at a speed of 3000 rpm for 10 minutes and lipid deposits (pellets) were taken and placed in a test tube, then heated at a temperature of 200°C to remove the added chemical mixture previously. The lipid content of microalgae grown in the appropriate medium was analyzed by the formula used by Weldy and Huesemann (2007) to obtain lipid weight. Calculation of total microalgae lipids is:

$$\text{Lipid content} = \frac{\text{lipid weight}}{\text{dry weight microalgae}} \text{ x100\%}$$

While lipid productivity was measured by using this following formula :

$$Productivity\ \left(gr/\frac{lt}{day}\right) = \frac{(Lwx10)}{16}$$

Note : gr = grams; lt = litres; LW = lipid weight

DNA extraction and amplification of *rbcL* gene

The DNA analysis was conducted in the Bioscience and Plant Technology Laboratory, Biology Department of ITS and in the Molecular Laboratory of Universitas Airlangga Hospital, Surabaya. DNA extraction process was using the CTAB 3% (Cetyl trimethyl ammonium bromide) methods adapted from Saputro et al. (2016). Genomic DNA obtained in the DNA extraction process was used as a template in the PCR process. Forward *rbcL* gene = 5'-TAA AAC ATT CCA AGG TCC TGC-3', Reverse *rbcL* gene = 5'-TTG TTC TAC GTA AAG AAG CCC-3'. The primer pairs F*rbcL* and R*rbcL* were obtained from primer design process using nucleotide sequences of *rbcL* genes from the genebank database (NCBI). The Nucleotide Data of *rbcL* Gene in several microalgae were used to design the primer pairs *Oscillatoria tenuis* (FN813332); *Oscillatoria sancta* (FN813331) *Calothrix desertica* (AB075906) *Melosira varians* (KM999081) *Coscinodiscus concinnus* (HQ912545) *Fragilaria* sp. *HGC-2007-2* (EU090046) *Synedra ulna* (HQ912454) *Thalassionema frauenfeldii* (JX401251) *Nannochloropsis oceanica* (HQ710610) *Chlorella vulgaris* (EU038286). Those nucleotide sequences of *rbcL* genes from different microalgae were then analyzed using the Multalin online software. The software showed the similarity level of the nucleotides and recommended the most suitable consensus to be used. Areas with high similarity that flank the low similarity areas were then used as the primer pairs. PCR was performed using Rotor-GeneQ PCR. The component of PCR, i.e. 12,5 µL Bioline ready mix; 1,5 µL of 10 nM forward primer *rbcL* gene; 1,5 µL of 10 nM reverse primer *rbcL* gene; 7,5 µL of sterile ddH_2O and 2 µL of DNA template. PCR reaction was programmed for 30 cycles. PCR products were observed using Mupid-exu DNA electrophoresis submarine electrophoresis system. Electrophoresis using 2% agarose gel that contains 5 µL Cybr Save and runs at 50 volts. Separated DNA fragments were observed with the Biostep UV Light Transilluminator.

DNA sequencing

The good quality of PCR product was then sequenced. DNA sequencing was performed to identify the sequence of *rbcL* gene. The sequencing procedure was conducted in Genetica Science following the procedure applied by manufacturer. The principal concept of Sanger method is by terminating the synthesized DNA using dideoxynucleotides which put into different tubes that terminated the synthesis process of the modified bases. Each tube has one type of ddNTP, ddNTP did not contain -OH group at tip 3' which stopped the synthesis of the primer in sequences that did not have any -OH groups. This termination produced chain terminating dideoxynucleotide that formed some fragments through electrophoresis by identifying the types of the dideoxynucleotide to be used in the termination process.

Data analysis

This research was conducted in the form of descriptive-explorative research. Data obtained from observation process were then analyzed and presented in the forms of descriptions about microalgae species. The species of microalgae were identified up to taxa species using Belilinger and Siege (2010). Meanwhile, data on the sequences of microalgae obtained from the blast analysis were used to determine the match rate between DNA sequence and the data from the genebank. The sequence resulted from Blast were then entry into MEGA7 software and aligned by using muscle to shows the differentiation among sequences. Moreover, aligned sequence were used as materials for tree arrangement by using Neighbor-Joining method. Figure 3 is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated.

RESULTS AND DISCUSSIONS

Physical condition of research sites

Wonorejo river is one of river estuary in the eastern part of Surabaya that is directly adjacent to the Madura Strait. The estuary of Wonorejo river which belongs to the coastal area of East Surabaya has narrow water and broad area that receives enough sun light throughout the year. Mangrove dominates the ecosystem in the river which gives positive benefits for the environment and the society (Balai Lingkungan Hidup Surabaya 2011). The estuary of Wonorejo river is mostly muddy substrate as the result of natural sedimentation from the sludge that flows along the river (Balai Lingkungan Hidup Surabaya 2011). The muddy substrate in the river influences the level of water turbidity, triggering varieties in the productivity of phytoplankton. Sampling was carried out on 2 February 2018. Microalgae sampling was carried out in three locations of Wonorejo river. Sampling location was determined by salinity content of each area (Ermavitalini et al. 2017). Salinity value increased from station 1 to station 3. Station 1 has the lowest salinity value which is 0‰, while has the highest salinity content with 20‰ (Nurhatika et al. 2018). Salinity gradients change dynamically following changes in river water flows, tides and coastal waters (Nybakken and Bertness 2005). The volume and flow of fresh water from the upstream of the river is considered to affect the low salinity value at station 1. On the contrary, the tides and reduced exposure to fresh water from the river causing high salinity at stations 2 and stations 3. The presence of salinity in the estuary produces salinity gradients, ranging from seawater dominance to the freshwater dominance in downstream. Salinity values in the Wonorejo river estuary waters show a minimum range according to the definition by Isnansetyo and Kurniastuty (1995) which states that the optimal salinity for plankton is between 20-35%. The sampling location was carried out at several points to obtain samples that represented both the area and the sample group so that the overall research location was obtained.

In the sampling process, several things need to be considered, particularly time, place, light intensity and physical parameters. Microalgae growth will be optimum at 09.00 - 11.00 WIB, the growth of microalgae will be faster along with the increasing amount of sunlight entering the water column. The availability of light in greater amounts causes phytoplankton to be more active in photosynthesis (Nagasoe et al. 2006). At that time microalgae will accumulate on the surface of water to carry out photosynthesis. Wonorejo has air humidity between 70-80%. Furthermore, Wonorejo air temperature ranges from 28-36ºC.. The pH of Wonorejo waters ranges from 6-8 (Ermavitalini et al. 2017). The pH value shows the normal value for the surface of Indonesian waters which generally ranges from 6.0 to 8.5 (Aksornkoae 1993). This condition is the optimal temperature and pH range for microalgae life so that it has no effect in distinguishing the high and low abundance of microalgae.

Culture and identification of microalgae

The initial stage that needs to be conducted before microalgae species identification is to ensure that the microalgae isolates used are one type of microalgae (monoculture). In the preliminary experiments microalgae culture was carried out using river water from the microalgae collection site. However, the observations show that the media is unable to grow microalgae. This is due to the lack of nutrients found in the river water, so that additional nutrients are needed in the growing media. In this study, the medium was supplemented with 220 mg of Murashige and Skoog Basal Medium with vitamin and Biotech agar 1.5% was used to solidify the medium. As previously known that MS medium has a mineral salt content in the form of macronutrients such as NH_4^+, NO_3^- and PO_4^- or micronutrients such as Cu^{2+}, I^- and Zn^{2+}. Furthermore, it also contains vitamins that can accelerate the growth of microalgae (Bell et al. 2009). After incubation for 14 days, microalgae grow in green, brown, blue, red and white (data not showed). After 14 days of incubation on solid media, several types of microalgae can grow well. In general, the microalgae has green, brown, blue, red and white colors. Then the growing microalgae are transferred to solid media which has the same

composition as the liquid medium used in the previous stage but replaces the biotech agar with Walne fertilizer. It aims to obtain microalgae monoculture. This transfer to the new plate is done up to four times. The results of purification with the media plate will be transferred to liquid media. Water from the sampling site was utilized as liquid medium with the addition of Walne fertilizer. Based on morphological identification results it is known that there are 8 microalgae genera, with one species per genera (Figure 2).

Based on the observation, *Microcystis* sp. can be described as small cells (only a few micrometers in diameter), which do not have individual sheaths. The protoplast of *Microcystis* has a bright blue-green color, looks dark or brown because of the optical effect of gas-filled vesicles used as a distinguishing characteristic when observed using a light microscope. *Nostoc* sp. has a filamentous form, did not grow straight and has no branch, scattered the color of the colonies of blue-green or gray with yellow heterosis cells or like olives. The shape of round vegetative cells, in some samples of *Nostoc* colony Petri dishes can be seen clearly. The colonies that appear on the petri dish are thick, gray or black, the color and shape of the colony is different from the surroundings, shiny, and mucilage looks like a gel or mucus. *Chlorella* cells were round or ovoid in groups, with diameter ranges from 2-8 µm, green in color, and the cell wall is hard consisting of cellulose and pectin, and has a protoplasm in cup shape. *Chlorella* can move but is very slow so that the observation seems to be motionless. *Chlorella* has chlorophyll, stores food reserves in the meal sac or pirenoid and has a strong cell wall composed of cellulose polysaccharides with a matrix of hemicellulose and pectin. *Tabellaria* has box-like shape, transparent in color, unicellular, it moves gliding on the substrate. *Tabellaria* has a colony or individual, basically bilateral simitris (pennales). The cell wall consists of two hemispheres, or valves that cover each other. The cell wall consists of a layer of inner pectin and a layer of silica (SiO_2) on the outside.

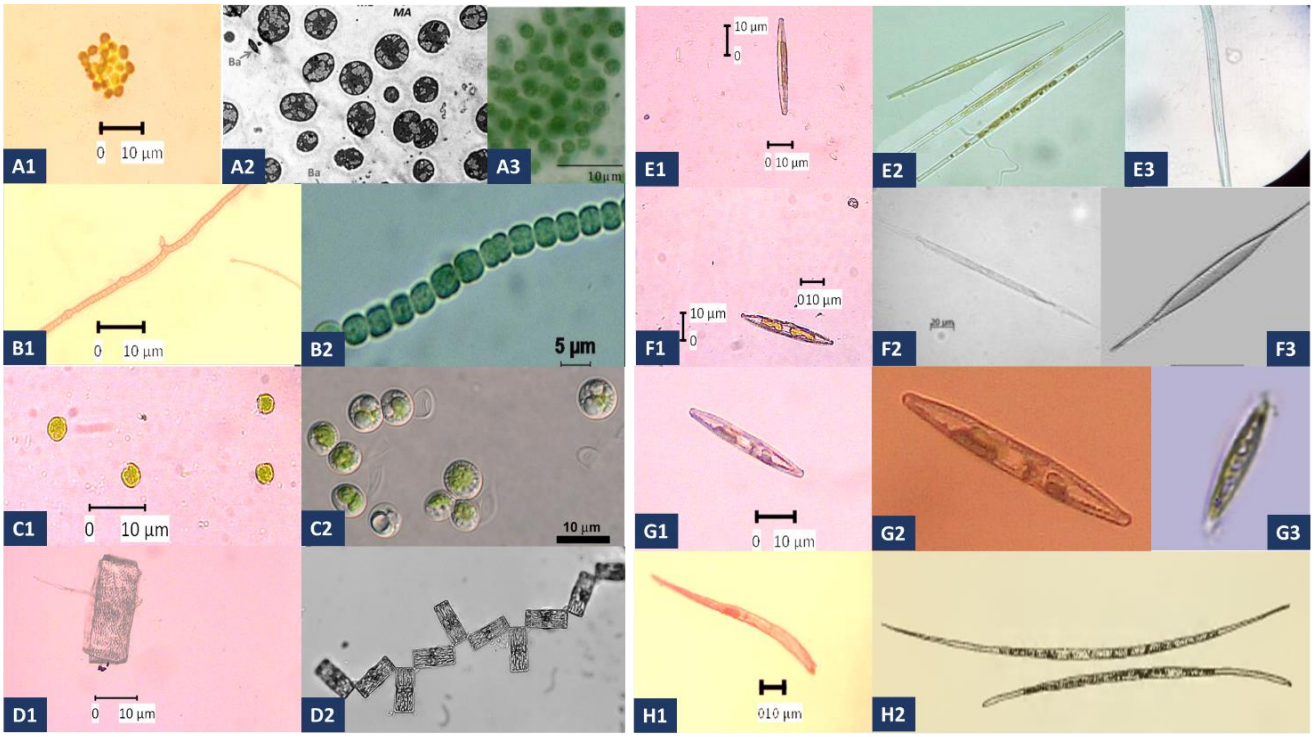

Figure 2. Observed microalgae is identified microalgae in this research compare to the literature (A1) observed *Microcystis* sp., M = 400x (A2) *Microcystis* (Parveeni 2013), (A3) *Microcystis* (Vijayan 2015); (B1) observed *Nostoc* sp., *M* = 100x, (B2) *Nostoc*, M= 400x (Hendrayanti et al. 2012); (C1) observed *Chlorella* sp., M = 100x, (C2) *Chlorella*. (Gomaa 2014); (D1) observed *Tabellaria* sp., M = 100x, (D2) *Tabellaria* (Hein, 1990); (E1) observed *Synedra* sp., M = 100x, (E2) *and* (E3) *Synedra* (Isti'anah 2015); (F1) observed *Nitzschia* sp., M = 100x, (F2) *Nitzschia* (Wulandari et al. 2014) *and* (F3) *Nitzschia* (Kociolek 2011); (G1) observed *Navicula* sp., M = 40x, (G2) observed *Navicula* sp., M = 400x, (G3) *Navicula* (Wood et al. 2016); (H1) observed *Closteriopsis* sp., M = 100x, (H2) *Closteriopsis* (Prescott 1954).

Based on the observation, *Synedra* was unicellular, elongated, like a needle and immobile. The cell is surrounded by the characteristics of a diatom silica shell consisting of two counters. In the lateral view, it appears narrow, rectangular, needle-shaped in the view of the valve or narrow schiffchenförmig. The cells are individual, but can be put together by jelly for stellate groups. Cells are not curved or bent. Inti is located in the center. There are two longitudinal plastids by golden brown fucoxanthin. *Nitzschia* has a shape like clear threads and a cell plasma containing chloroplasts for photosynthesis. *Navicula* characteristics at the time of observation are the jagged edges on the inside of the cell wall consisting of two hemispheres or valves that close together. The dominant carotene pigment is xanthophyll which gives a golden color. The observed *Closteriopsis* is shaped like a needle at the point of the two pointed edges. *Closteriopsis* as plankton, a solitary cell, is needle-shaped and slightly curved, long and gradually tapering to a regular endpoint.

Based on the data obtained, there are several genera found in various stations (1, 2 and 3), namely *Navicula*, *Nostoc*, and *Nitzschia*. This is because these three genera can live in various conditions, especially in waters that contain lots of minerals. *Navicula* is able to protect itself from toxic substances in the waters. Therefore, these genera can live in waters that experience pollution (John et al. 2002). *Nitzschia* has a high tolerance and adaptation to the aquatic environment and be able to live in a polluted environment. Determination of the type of microalgae based on the results of observations of light microscopy has a high level of difficulty, because microalgae have high plasticity which is influenced by environmental conditions or habitats. In unfavorable environmental conditions, the morphology of microalgae can be different from its morphology in normal conditions. This phenomenon will increase the bias in identification process (Umayah and Purwantara 2006). This conventional characterization has increased the possibility that microalgae that have the same phenotype are identified to be the same species, even though they are not genetically similar.

Lipid concentration

In general, microalgae have lipid content varying from the lowest to the highest, which is 1.9% to 40%. However, different culture and time conditions can alter lipid content. Under normal growth conditions microalgae lipid content of 10-30% dry weight (Schenk et al. 2008). According to Matsumoto et al. (2010) and Elumalai et al. (2011), the red color indicates the presence of polar lipids or chlorophyll and the yellow color shows the presence of neutral lipids containing hydrocarbons and triacylglycerols in microalgae isolates. Neutral lipids containing microalgae biomass are the basic ingredients of biodiesel (Matsumoto et al. 2010). In this study, the determination of lipid will be carried out quantitatively. The details result of lipid content on microalgae can be seen in Table 1.

Based on the results obtained, it was found that microalgae isolates which had the highest lipid content were *Navicula* sp. that was 27,2% and *Chlorella* was 21,20%, while the highest lipid productivity was *Navicula* sp., which was 6.49 mgl^{-1} day^{-1} and *Chlorella* sp. that is 3.34 mgl^{-1} day^{-1}. It can be seen that microalgae which have the potential to be used as biodiesel are *Navicula* sp. and *Chlorella* sp. The percentage of the four components varies depending on the type of microalgae. Microalgae lipid content depends on the type of microalgae, average growth and microalgae culture conditions (Chisti 2007). *Navicula* sp. has high lipid content because *Navicula* sp. has a high growth rate. *Navicula* sp. can live in various conditions, especially in waters that contain lots of minerals. This is because *Navicula* sp. is able to protect itself from toxic substances in the waters. Therefore, the genus can live in waters that experience pollution (John et al. 2002). *Chlorella* sp. and *Synedra* sp. also have high lipid content above 20% because the average growth of those two microalgae was also high on the medium used. These genera can live in a variety of conditions, especially in waters that contain lots of minerals. *Nitzschia*, *Tabellaria* sp, and *Closteriopsis* sp. have moderate growth rates. While *Nostoc* sp. has a low growth average because it requires a specific place of growth with good nutrition value, this condition was strengthened by evidence that *Nostoc* sp. only found in station 3 (Table 1).

Table 1. The total lipid content of observed microalgae

Genus	General shape	Station			Dry biomass (mgs)	Lipid biomass (mg)	Lipid content (%)	Lipid productivity (mg l^{-1} day^{-1})
		1	2	3				
Mycrocystis sp.	Ovoid or spherical	-	+	+	20.30±1.79	3.35±0,37	16,5±1.52	2.09 ±0,065
Nostoc sp.	Filamentous, single cell is spherical or ovoid	-	-	+	3.85±0.32	0.07±0,02	1.80±0.17	0.04±0.004
Chlorella sp.	Round	-	+	+	25.20±1.38	5.34±0,31	21.20±1.03	334±0.014
Tabellaria sp.	Cuboid	-	-	+	16.90±0.80	1.84±0,02	10.90±1.71	1.15±0.029
Synedra sp.	Elongated and needle-like	-	+	+	21.30±1.53	4.32±0,17	20.30±2.01	2.70±0.012
Nitzschia sp.	Lanceolate	+	+	+	20.50±0.36	3.38±0,61	16.50±1.24	2.11±0.014
Navicula sp.	Boat	+	+	+	38.20±1.13	10.39±0,92	27.20±1.37	6.49±0.019
Closteriopsis sp.	Needle	-	-	+	13.10±0.57	1.06±0,12	8.10±0.42	0.66±0.014

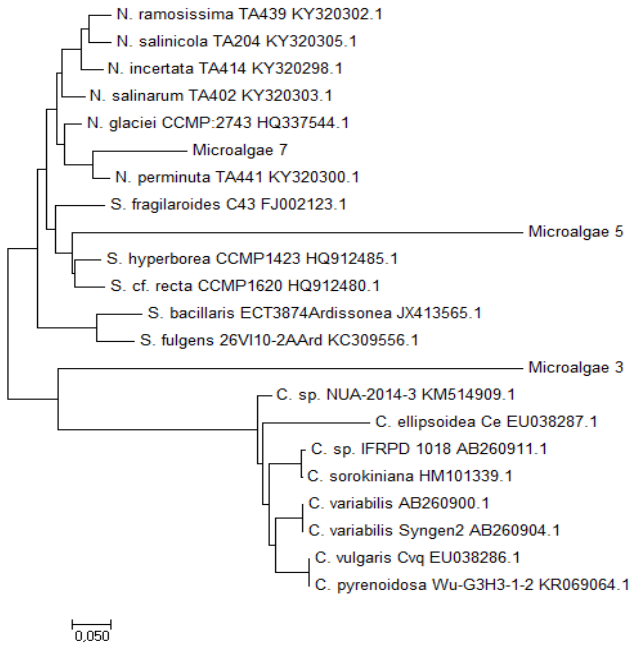

Figure 3. Neighbour-Joining (NJ) phylogenetic tree based on the chloroplast encoded *rbcL* gene. Microalgae 3 is *Chlorella* sp., Microalgae 5 is *Synedra* sp., Microalgae 7 is *Navicula* sp.

Previous research states that the lipid content of the microalgae due to the influence of differences in temperature, salinity, and light intensity has been widely carried out with the result that microalgae can grow well at salinity of 20-25 ppt, whereas to produce maximum total lipids it takes 10-15 ppt salinity while for temperature treatment of microalgae can grow well at a temperature of 20-30ºC, while to produce a maximum lipid a good temperature is 15ºC (Chisti 2007). Nurhatika et al. (2018) observed that *Nannochloropsis* sp. and *Nitzschia* sp. from Wonorejo have high intracellular lipid accumulation. However, in this case, there is still a possibility that the genus not found in this study actually exists, because the amount is too little to be counted and might not be filtered when the sample was taken.

Navicula sp. and *Chlorella* sp. have the best potential to be developed into high lipid content of microalgae. Chisti (2007) stated that microalgae which have a lipid content 30% can produce oil as much as 58,700 L/ha, greater than corn 172 L/ha, and palm oil 5950 L/ha. All those three candidates of microalgae then extracted its DNA genome and sequenced its nitrogen bases to ensure the species of microalgae. The other five microalgae were not further sequenced, since they produce only small percentage of lipid. The sequence was analyzed using BLAST and then aligned with other genera members obtained from NCBI to build phylogenetic tree using MEGA 7 software. Details information about phylogenetic tree can be shown in Figure 3.

Thus molecular result indicates similar characteristic by morphological identification, it was confirmed that isolated microalgae were *Chlorella* sp. microalgae 3, *Synedra* sp. for microalgae 5 and *Navicula* sp. for microalgae 7 where those three microalgae subjected in the right groups. Schubart et al. (2001) state the advantages of using molecular technique including better accuracy and efficiency. DNA barcode is one of molecular technique that frequently used in species identification process (Hebert et al. 2003). DNA barcode has various applicative functions including for ecological survey (Dick and Kress 2009), cryptic taxon identification (Lahaye et al. 2008), confirmation of medicinal plant samples (Xue and Li 2011), and also can be used to identify and distinguish the level of an organism, starting from species level up to subspecies level. DNA barcoding technique can identify and characterize various species which cannot be done using morphological analysis (Tudge 2000). The combination of microscopy observation and DNA barcode is more directed identification of microalgae.

Discussion

Microorganism classification and identification are important steps to conduct. Traditionally, microbes are characterized based on their phenotypes (Madigan et al. 2000), yet this method is vulnerable to identification errors. The classification of organism used to be conducted based on morphological characteristics as the indicator. However, at this present time, there has been a strong tendency to apply new approaches such as chemotaxonomy and molecular approach which are considered able to complete the available classification system (Schubart et al. 2001). Methods in identifying organism have developed from the use of morphological identification up to the use of molecular identification based on some pieces of short DNA called “DNA barcode” (Hebert et al. 2003). DNA Barcoding refers to the use of the standard short DNA region in identifying an organism in the most efficient and accurate way. Furthermore, DNA barcoding is able to identify an organism even though its DNA is incomplete or damaged, even degraded or processed DNA can still be used in DNA barcoding (Hajibabaei et al. 2007). DNA barcode has some applicative functions including for ecological survey (Dick and Kress 2009), taxon identification in cryptic (Lahaye et al. 2008), confirmation of medicinal plants (Xue and Li 2011), and DNA barcoding technique is also able to identify and distinct an organism based on its species up to subspecies phase. DNA barcoding is advantageous as it can be used in the identification and characterization of various species which cannot be morphologically identified (Tudge 2000).

Relationship among genus can be efficiently arranged by applied the DNA barcodes. The gene which is used as a molecular marker should be the moderately conserved gene which has sufficient varieties in each species. Generally, COI, 16s-RNA, and Cytb are recommended for animal identification using molecular markers. All of the genes are the parts of mitochondria. COI is cytochrome c oxidase gene found in mitochondria which is also a part of the complex cytochrome oxidase subunit that also takes a part in electron transfer chain. Some research has shown that the sequences of COI effectively identifies Animalia

kingdom as 95% of the animals have been successfully divided into their species level using COI sequence (Ratnasingham and Hebert 2007).

In the Plantae group, a number of genes are commonly used in phylogenetic analysis and plant identification including the microsatellites, *rbcL* gene, *gap*C, *ndh*F, matK, and *psa*A. One of molecular markers which are frequently used in phylogenetic analysis of plant is *rbcL*. *rbcL* gene in plant is the code of *ribulose*-1,5-*bisphosphate carboxylase* (RubisCo) enzyme subunit in its chloroplast genome (Judd et al. 2002), besides it is also a universal gene found in almost any plant. Thus, the use of this gene effectively identifies a variety of plants. *rbcL* gene is a coding gene of a photosynthesize enzyme namely the *large* subunit *Ribulose-1,5-bisphosphate carboxylase* (RuBisCo) or RUBP carboxylase (Judd et al. 2002), which plays a significant role in the earlier steps of Calvin cycle in photosynthesizing process.

The effectiveness of *rcb*L gene used in phylogenetic analysis of flowering plants also indicates the evolutional relationship among organism groups (taxon). Besides, this gene is also an eternal gene found almost in any Angiospermae (Stefanovic 2005). Consortium Barcode Of Life (CBOL) also recommends the use of *rcb*L gene as barcode marker in plant identification by combining it with *mat*K gene (CBOL 2009) regarding to the fact that based on their existences, the effectiveness and the mutation sustainability rate, and also those genes should be found in almost any species of plants. In the field of systematics, DNA barcode can be employed to identify plant species and to be used in phylogenetic analysis and population analysis (Hajibabaei et al. 2007).

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