Menara Perkebunan 2021, 89(1), 17-25
p-ISSN: 0125-9318/ e-ISSN: 1858-3768
DOI: http://dx.doi.org/10.22302/iribb.jur.mp.v89i1.408
Accreditation Number: 21/E/KPT/2018

Molecular identification and phylogenetic analysis of *Chlorella* isolates from Indonesia using *rbcL* gene

Identifikasi molekuler dan analisis kekerabatan isolat-isolat Chlorella *dari Indonesia menggunakan gen* rbcL

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Received 10 December 2020 / Accepted 27 April 2021

Abstrak

Identifikasi spesies memegang peranan penting dalam penyusunan database yang mumpuni baik untuk mendukung aplikasi dalam bidang bioteknologi maupun untuk kepentingan komersial. Identifikasi secara morfologi tidak mampu memberikan deskripsi yang cukup terutama untuk mikroalga uniseluler yang berukuran kecil. Gen rbcL *yang menyandikan* large unit of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) *telah banyak digunakan dalam* barcoding *tanaman dan sedang dikembangkan untuk identifikasi mikroalga secara molekuler. Dalam penelitian ini dilakukan identifikasi terhadap strain mikroalga hijau lokal asal Indonesia menggunakan marka* barcoding rbcL. *Mikroalga hijau yang diperoleh dari Yogyakarta, Serayu, Gondol, Ancol, Cilegon, dan Teluk Jakarta ditumbuhkan pada media f/2 kemudian dipanen untuk ekstraksi DNA. DNA yang diperoleh kemudian diamplifikasi dengan primer 1AB_rbcL dengan ukuran pita target 615 bp, dan dilakukan sequencing.* Barcoding *isolat DNA mikroalga hijau lokal telah berhasil dilakukan menggunakan pasangan primer 1AB_rbcL dengan kesamaan genetik mencapai 99% terhadap spesies dalam database Genbank. Dari enam isolat, TJ, G, S, C, dan A teridentifikasi sebagai* C. pyrenoidosa. *Hanya isolat CP dari Yogyakarta yang teridentifikasi sebagai* C. sorokiniana. *Sebagai* outgroup *digunakan sequence* rbcL *dari* Nannochloropsis gaditana. *Analisis filogenetik menunjukkan bahwa lima isolat* C. vulgaris *tergabung menjadi satu clade dan terpisah dengan spesies* C. sorokiniana *dari Yogyakarta.*

[Kata kunci: mikroalga hijau, identifikasi molekuler, pohon kekerabatan, rbcL]

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Abstract

Identifying the newly isolated species is crucial to establishing a reliable algal database with successful commercial applications for different biotechnological applications. Morphological identification does not give sufficient description, especially for tiny unicellular microalgae. The *rbcL* gene encodes the large unit of ribulose-1, 5-bisphosphate carboxylase /oxygenase (Rubisco) has been widely known for barcoding in plants and developed for microalgae molecular identification. In this study, we examined the local strains of green microalgae from Indonesia using the *rbcL* partial gene sequence to identify the strains. Green microalgae isolates originated from Yogyakarta, Serayu, Gondol, Ancol, Cilegon, and Teluk Jakarta were cultured in f/2 media and harvested for DNA extraction. The DNA extracted was proceeded to PCR using 1AB_rbcL primer pair to amplify the sequences of *rbcL* gene with target band located at 582 bp, followed by the sequencing of the PCR product was conducted. Molecular identification of local green microalgae isolates was successfully carried out using primers 1AB_rbcL with a genetic similarity of 99% toward identified species in the NCBI database. Among six isolates, TJ, G, S, C, and A isolates were identified as *C. pyrenoidosa*. Only CP isolate from Yogyakarta identified as *C. sorokiniana*. *Nannochloropsis gaditana rbcL* sequence was selected as an outgroup. The phylogenetic analysis indicated that the five isolates of *Chlorella* belong to one clade and clearly distinguished from *C. sorokiniana* isolate from Yogyakarta.

[Keywords: green microalgae, molecular identification, phylogenetic tree, *rbcL*]

Introduction

Green microalgae (Chlorophytes), including Chlorophyceae are important producers of oxygen in aquatic seawater and freshwater, which are often

used as bioindicators in waste water treatment, water monitoring, and ecological studies (Pasha *et al*., 2016). For decades, strains of *Chlorella* (Chlorophyceae, Chlorococcales) have served as model organisms in biochemical and plant physiology research. In addition, green microalgae have recently gained interest worldwide for biotechnological applications such as fuel, food, animal feed, and valuable chemical production (Khan *et al*., 2018). Recently, a member of Chlorophyceae, *Chlorella variabilis* gained more attention for the capacity to convert long-chain fatty acids to alkanes or alkenes in a light-dependent process (Huijbers *et al*., 2018).

The identification of green microalgae can be a pretty challenging task and often requires tedious microscopic examination of live cultured cells by a taxonomist (Hadi *et al*., 2016). Therefore, molecular identification provides a reliable solution to identify microalgae more accurately and faster to the species level (Yanuhar *et al*., 2019). DNA barcoding is an established technique for species identification, which identifies specimens based on DNA sequence similarity against a sequence database of previously identified species (Fei *et al*., 2020). This powerful technique has brought significant improvements to applications such as taxonomy, biosecurity, ecology, and food product regulation (Hadi *et al*., 2016). DNA-based identification is beneficial for microalgae species identification, which is crucial to establish a reliable algal database with successful commercial applications for different biotechnological applications (Ashour *et al*., 2019).

Potential Chlorophyta DNA barcodes have included chloroplast genes (*rbcL*, *Cp23S*, and *tufA*), nuclear genes (*18S rDNA*, *nuITS1*, and *nuITS2*), and mitochondrial DNA (*COI*) (Pawlowski *et al*., 2012). However, none of these markers were considered ideal for use across all lineages. The most commonly used DNA marker for Chlorophyta barcode is the *18S rDNA* sequence. As a result, there are (as of 7 December 2020) 1495 *18S rDNA* sequences listed for *Chlorella* alone in Genbank (NCBI). Many researchers are using the *18S rDNA* sequences as a DNA barcode for species identification. However *18S rDNA* is a highly conserved gene that is in general not considered appropriate for identification at the species level (Leliaert *et al*., 2012). Several species and varieties of green microalgae were initially characterized by the much more diverse *rbcL* gene (Suda *et al*., 2002; Fawley & Fawley, 2007; Fawley *et al*., 2015). Recently, researchers have shown that strains with identical *18S rDNA* sequences can have considerable variation in the *rbcL* gene (Wei *et al*., 2013; Carpinelli *et al*., 2014). Regardless of the unavailability of a universal PCR marker for DNA barcode, the *rbcL* marker is considered a good marker for green algae (Hadi *et al*., 2016).

Over 60% of Indonesia's territory is covered by water (Rahman *et al*., 2019). To be one of the enormous diversities in the world, not all of those areas had been explored yet. This present study was undertaken to (1) identify green microalgae isolates that originated from several places in Indonesia by using *rbcL* molecular markers as DNA barcodes, (2) construct a phylogenetic tree between species found in several places in Indonesia which previously analyzed by DNA barcoding.

Materials and Methods

Microalgae strains

Green microalgae originated from Yogyakarta (CP), Serayu (S), Gondol (G), Ancol (A), Cilegon (C), and Teluk Jakarta (TJ) were provided by the Laboratory of Bioindustry, Indonesian Research Institute for Biotechnology and Bioindustry (IRIBB). The microalgae were cultured with the inoculum amount (v/v) of 10% in f/2 liquid culture medium (Guillard & Ryther, 1962) in sterile conditions at 21°C under the illumination of 800–1000 lux. The microalgae were cultivated in artificial seawater (Monsterlaut ASW Salt Mix) with the following f/2 nutrients (from stock solution): 0.25 mL $NaNO_3$ (75 g L^{-1}), 0.25 mL NaH_2PO_4 (5 g L^{-1}), 0.25 mL Na_2CO_3 (30 g L^{-1}), 0.25 mL trace metal solution and 0.125 mL vitamins solution. Trace metal solution was prepared in pure water containing (per liter): 3.15 g $FeCl_3 \cdot 6H_2O$; 4.36 g $Na_2EDTA \cdot 2H_2O$; 0.1 mL $CuSO_4 \cdot 5H_2O$ (9.8 g L^{-1}); 0.1 mL $Na_2MoO_4 \cdot 2H_2O$ (6.3 g L^{-1}); 0.1 mL $ZnSO_4 \cdot 7H_2O$ (22 g L^{-1}); 0.1 mL $CoCl_2 \cdot 6H_2O$ (10 g L^{-1}); 0.1 mL $MnCl_2 \cdot 4H_2O$ (180 g L^{-1}). The vitamin solution was prepared in pure water containing (per liter): 40 mL thiamine HCl (0.05 g L^{-1}), 1 mL biotin (vitamin H) (0.1 g L^{-1}), and 0.1 mL cyanocobalamin (0.1 g L^{-1}). Cells of microalgae isolates were collected at the exponential growth at five days culture with centrifugation 10.000 rpm for 5 min and then transferred to 2 mL microtubes.

DNA extraction

DNA extraction and molecular analysis were conducted at Biochemistry and Biomolecular Laboratory, IRIBB. The DNA was extracted with the CTAB method described previously by Orozco-Castillo *et al*. (1994) and modified by Budiani *et al*. (2016) with supplementation of 1% β-mercaptoethanol into the lysis buffer. The CTAB lysis buffer was added (500 µl) into the sample and then agitated using vortex (without grinding) and incubated for 30 min at 65 °C. DNA were extracted with 500 µl Chloroform-Isoamyl Alcohol and precipitated in Isopropanol. The amount and purity of extracted DNA was determined using Nanodrop 2000 (Thermo Scientific) spectrophotometer. The purity of extracted DNA was assessed by the absorbance (A) ratio of 260 nm and 280 nm.

Amplification of the rbcL *gene and sequencing*

The oligonucleotide primers 1AB_rbcL were amplified using modified forward primer 5'-TCIGCIAARAACTAYGGTCG-3' and reverse primer 5'-GGCATRTGCCAIACRTGRAT-3 as described previously (Ghosh & Love, 2011). This set of primers amplified DNA from Chlorophyta, Eglenozoa, and certain bacteria, including Cyanobacteria with a product size ~615 bp. The PCR protocol included an initial denaturation of 3 min at 95 °C followed by 40 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min 30 s at 72 °C, and a 15 min 72 °C of final extension step. The PCR products were visualized on 0.8% agarose gel electrophoresis stained by GreenSafe DNA Gel Stain (Canvax Biotech). The purified DNA was commercially sequenced (First Base Lab, PT. Genetika Science) using Applied Biosystems™ BigDye™ Terminator v3.1 Cycle Sequencing Kit with the same forward and reverse primer used in the PCR process.

Molecular data analysis

Computer-assisted sequence analysis and assembly were performed in Geneious Prime Software. Each consensus sequence measured in this study was Basic Local Alignment Search Tool (BLAST) against the sequence in the Genbank database in NCBI. The percentage of identical sites was being evaluated. The phylogenetic analysis was carried out by constructing the neighbor-joining tree with Tamura-Nei distances. *Nannochloropsis gaditana* was selected as an outgroup (Tamura & Nei, 1993). A phylogenetic test using the Bootstrap method (1,000 replicates) was used.

Results and Discussion

DNA extraction and molecular identification

Genomic DNA of six isolates originated from Teluk Jakarta (TJ), Gondol, Cilegon, Serayu, Ancol, and Yogyakarta (Indonesia) were extracted and quantified with spectrophotometry analysis. Table 1 showed the concentration of DNA extracted and the purity of each sample. The result indicated that DNA concentrations for all isolates are considered enough. PCR requires 1-1000 ng (10^4-10^7 molecules) of DNA template, generally for a 25 µl PCR reaction, 50-125 ng of genomic DNA will be required (Lorenz, 2012). All extracted samples by the ratio of A260/A280 indicate good purity. However, the ratio of A260/A230 was too low, indicating that absorb at 230 nm, such as EDTA from the culture media or carbohydrates from the cell extract (NanoDrop Inc., 2007).

The extracted DNA of CP seemed unreasonably too high with high viscosity, making it difficult to pipette. For quality check, CP genomic DNA was then run in agarose gel electrophoresis, and the result was shown in Figure 1. The genomic DNA of CP isolates present in a similar size to lambda DNA Marker around 48.5 kbp. However,the visualization was a faint band that may indicate low DNA amount, with a bright smear at the bottom of agarose gel due to high RNA contamination. DNA quality and purity will have a substantial effect on a successful PCR experiment. DNA extraction contaminants are common inhibitors in PCR and should be carefully avoided. Common DNA extraction inhibitors of PCR, including RNA, salts, and detergents, in all probability, will be problematic for PCR (Lorenz, 2012).

The PCR reaction and visualization were carried out to verify that the microalgae strains belong to the Chlorophyta using general primer for green microalgae. The PCR results of the six isolates using the *rbcL* gene showed an amplified band with a length of ~615 bp (Figure 1). DNA sequences from forward and reverse direction were trimmed by an error probability limit of 5% in Geneious Prime software. Each sequence pair was *de novo* assembled to create a consensus sequence and then analyzed using nucleotide BLASTn to see the query sample homology with microalgae species in the nucleotide collection (nr/nt) database. The sequences were also submitted to BOLD for comparison of species identification against Genbank (Table 2).

Table 1. Concentration and purity of extracted DNA
Tabel 1. Konsentrasi dan kemurnian DNA yang diekstrak

Sample ID *ID sampel*	DNA Concentration (ng µl^{-1}) *Konsentrasi DNA* (ng µl^{-1})	A260/A280 ratio *Rasio A260/A280*	A260/A230 ratio *Rasio A260/A230*
CP	453.8	1.87	1.77
S	171.3	1.88	1.35
G	153.6	1.74	1.04
A	49.9	1.84	1.02
C	54.1	1.92	1.76
TJ	50.0	1.82	1.08

Note: CP= Yogyakarta isolate, S=Serayu isolate, G=Gondol isolate, A=Ancol isolate, C=Cilegon isolate, TJ=Teluk Jakarta isolate
Keterangan: CP= isolat Yogyakarta, S= isolat Serayu, G= isolat Gondol, A= isolat Ancol, C= isolat Cilegon, TJ= isolat Jakarta

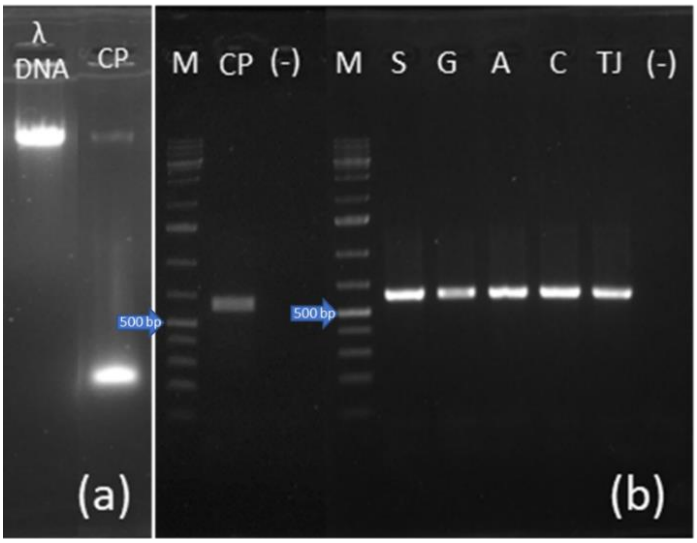

Figure 1. Visualization of (a) quality check of CP genomic DNA against lambda DNA marker and (b) PCR amplification of six isolates using the *rbcL* gene at 615 bp. M= 1kb+ DNA marker, (-) = negative control (NFW), CP= Yogyakarta isolate, S=Serayu isolate, G=Gondol isolate, A=Ancol isolate, C=Cilegon isolate, TJ=Teluk Jakarta isolate

Gambar 1. Visualisasi (a) pengecekkan kualitas *DNA genom CP terhadap lambda DNA dan (b) hasil amplifikasi dengan PCR dari enam isolat menggunakan gen* rbcL *pada 615 bp. M= 1kb+* DNA marker, *(-) = Kontrol negatif (NFW), CP= isolat Yogyakarta, S= isolat Serayu, G= isolat Gondol, A= isolat Ancol, C= isolat Cilegon, TJ= isolat Teluk Jakarta*

Table 2. Species identification based on BLASTn and BOLD system v4 analysis
Tabel 2. Identifikasi spesies berdasarkan analisis di BLASTn dan BOLD system v4

Isolates *Isolat*	BLAST-NCBI *BLAST-NCBI*			BOLD system v4 *BOLD system v4*		
	Species *Spesies*	Similarity *Kemiripan*	E-value *Skor-E*	Species *Spesies*	Similarity *Kemiripan*	E-value *Skor-E*
CP	*Chlorella sorokiniana*	94.70	0	*Chlorella sorokiniana*	95.55	0
S	*Chlorella pyrenoidosa*	99.36	0	*Dicloster acuatus*	92.65	0
G	*Chlorella pyrenoidosa*	99.36	0	*Dicloster acuatus*	92.64	0
A	*Chlorella pyrenoidosa*	99.51	0	*Dicloster acuatus*	92.88	0
C	*Chlorella pyrenoidosa*	99.04	0	*Dicloster acuatus*	92.33	0
TJ	*Chlorella* sp.	99.04	0	*Dicloster acuatus*	92.33	0

Note: CP= Yogyakarta isolate, S=Serayu isolate, G=Gondol isolate, A=Ancol isolate, C=Cilegon isolate, TJ=Teluk Jakarta isolate
Keterangan: CP= isolat Yogyakarta, S= isolat Serayu, G= isolat Gondol, A= isolat Ancol, C= isolat Cilegon, TJ= isolat Teluk Jakarta

The base sequence homology analysis of five isolates (TJ, G, C, S, and A) showed a high level of up to 99%. The query cover varies between 96-100% and an E-value of 0.0 (Table 2). Based on the BLASTn analysis and phylogenetic tree construction of prior species in the database, TJ isolate was identified as *Chlorella* sp. The C, AS, G, A, and C isolates were identified as *C. pyrenoidosa*. Only CP isolate from Yogyakarta showed a moderate homology level of 94.57%, with a query cover of 95% with *C. sorokiniana* MK842150.1. In line with the previous study, a homology level of 99-100% and the E-value is smaller than e-0.4 from BLASTn defined as identical and identified as the species (Yanuhar *et al*., 2019). Yanuhar *et al*. (2019) identified local green microalgae isolate as *C. vulgaris* using *rbcL* gene sequence. The sequence homology analysisshows homology of 99% of 589 bases and an E-value of 0.0.

Chromatogram of CP sequence also shown to have a low quality compared to another sequence (data not shown). The *rbcL* sequence of CP isolate has a pretty low base call quality, by Phred quality score below 20 throughout the reverse direction sequence and on the upstream and downstream of forward direction sequence. A Phred quality score is a measure of the quality of identifying the nucleobases generated by automated DNA sequencing (Shi *et al*., 2016). A Phred Score of 20 is acceptable, which means that whatever it qualifies is 99% accurate with a 1% chance of error

and is just as valuable as one sequence with a quality score above 40 (error probability of 1 in 10,000) (Richterich, 1998). Besides, there are many multi-peaks (data not shown), which also contribute to the low base call quality. The primary cause of multi-peaks in the sequence data is a contamination of the culture or specimen with other living organisms or genetic material. Low base call quality may contribute to the low homology level compared to other isolates.

The plastid-encoded *rbcL* gene has been widely used for DNA barcoding in green microalgae. This gene is a part of the DNA sequence located in cpDNA. It is considered standard DNA barcoding markers in the plant due to their universality, relatively high overall sequence quality, low cost, and high discrimination (Carneiro *et al*., 2019). The *rbcL* gene also provides many characters to study phylogenetic because it has a total length of 1400 bp (Basith, 2015). This sequence has a low level of mutation compared with other barcodes in cpDNA, with high similarity between species (Sundari & Papuangan, 2019).

Saputro *et al*. (2019) isolated microalgae from 3 different stations of Wonorejo river estuary, Surabaya, East Java, Indonesia, and identified eight isolates as *Mycrocystis* sp., *Nostoc* sp., *Chlorella* sp., *Tabellaria*, *Synedra*, *Nitzschia*, *Navicula*, and *Closteriopsis* using *rbcL* gene as the molecular marker (Saputro *et al*., 2019). However, genuinely universal primers are not available for *rbcL*, although some primers can be used on a wide range of taxa (Hall *et al*., 2010). A combination of morphological and molecular approaches may provide better understanding of species identification in microalgae.

Phylogenetic tree analysis

Construction of phylogenetic tree was performed on *rbcL* gene sequences of *Chlorella* from Indonesia, including six *Chlorella* isolates from this study and collected sequence from literature and Genbank (Table 3). A total of 11 *Chlorella* isolates from Indonesia were aligned with *Nannochloropsis gaditana*'s *rbcL* gene sequence using Geneious Alignment Program with cost matrix at 65% similarity. Alignment of 12 sequences was then used to construct phylogenetic tree with Tamura-Nei genetic distance mode and the NeighborJoining Tree method.

According to the phylogenetic tree, these strains were grouped into five clusters. Based on the phylogenetic tree in Figure 3, it was found that the *Chlorella* strains (G, S, C, TJ, and A) were clustered in the same clade with high genetic similarity between strains of up to 99%, except between *Chlorella* sp. strain Teluk Jakarta (TJ) and *C. pyrenoidosa* strain Cilegon (C) which has similarity level at 98.98% (Table 4). These high genetic similarities showed that a DNA "barcoding gap" did not exist in the *rbcL* gene between G, S, C, TJ, and A strains, indicating that *Chlorella* sp. strain TJ fall into the same species of *C. pyrenoidosa*. Therefore, all of these five isolates originated from quite distant region, especially *C. pyrenoidosa* strain G, which originated from different Bali island. In contrast, others were distributed in Western and Central Java. The bootstrap value between branches is also moderately high (72.1%), which supports that these strains come from a different population. This result proved how wide the distribution of this species, from West Java to Bali.

Chlorella sorokiniana strain Yogyakarta (CP) fell into a distinct cladeconsistent with the taxonomic assignments. In comparison, the construction of phylogenetic of four priors *Chlorella* sp. datasets from Genbank formed into three different clades, which indicated that they come from a different population. Whereas, *C. vulgaris* strain Situbondo clustered in the same clade with *Chlorella* sp. MH048633 indicated these have closer lineage but come from different population as seen by the bootstrap value of 72.4%.

Table 3. Sequences of *rbcL* gene in microalgae used for phylogenetic tree construction
Tabel 3. Sekuens gen rbcL *dari microalgae yang digunakan dalam konstruksi pohon kekerabatan*

Species *Spesies*	Accession Number *Nomor aksesi*	Origin *Asal*	Reference *Sumber pustaka*
Nannochloropsis gaditana	AB052735.1	Capetown, South Africa	Genbank
Chlorella sp.	MH048635	Indonesia ocean	Genbank
Chlorella sp.	MH048634	Indonesia ocean	Genbank
Chlorella sp.	MH048633	Indonesia ocean	Genbank
Chlorella sp.	MH048632	Indonesia ocean	Genbank
Chlorella vulgaris	-	Situbondo, Indonesia	(Yanuhar *et al*., 2019)
Chlorella sorokiniana	-	Yogyakarta, Indonesia	This study
Chlorella pyrenoidosa	-	Gondol, Indonesia	This study
Chlorella pyrenoidosa	-	Serayu, Indonesia	This study
Chlorella pyrenoidosa	-	Cilegon, Indonesia	This study
Chlorella sp.	-	Teluk Jakarta, Indonesia	This study
Chlorella pyrenoidosa	-	Ancol, Indonesia	This study

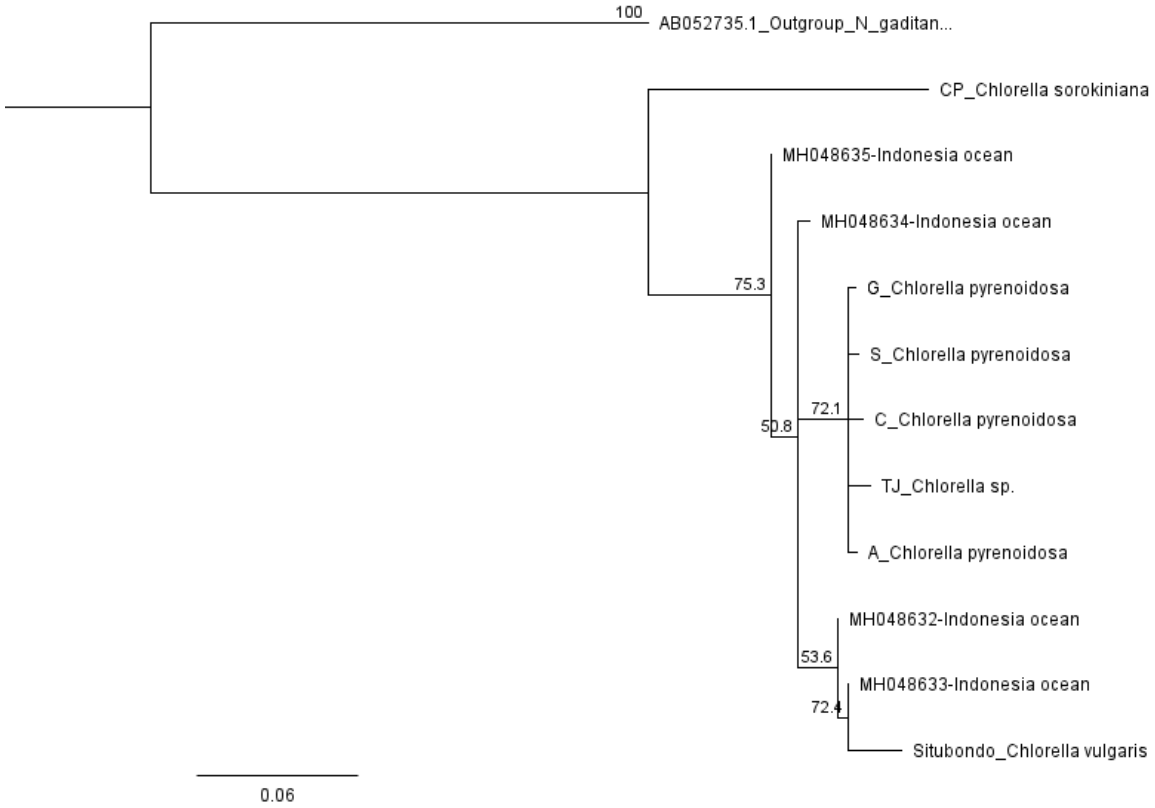

Figure 3. Neighbour-Joining (NJ) phylogenetic tree based on *rbcL* gene of local *Chlorella* strains from Indonesia. The tree was constructed by the neighbor-joining (NJ) method in the Geneious Prime software based on the multiple sequence alignment by Geneious alignment program. Bootstrap support values of 1000 replicates (%) are shown at the nodes

Gambar 3. Pohon kekerabatan Neighbour-Joining (NJ) berdasarkan gen rbcL *strain lokal* Chlorella *dari Indonesia. Pohon disusun dengan metode* neighbor-joining *(NJ) dalam software Geneious Prime berdasarkan* multiple sequence alignment *oleh* Geneious alignment program. Bootstrap support values *sebanyak 1000 ulangan (%) ditampilkan pada percabangan*

Table 4. Genetic similarity matrix among local *Chlorella* strains
Tabel 4. Matriks kekerabatan genetik antar strain Chlorella *local*

Strain	TJ	Cv Sit.	S	MH-35	MH-34	MH-33	MH-32	G	CP	C	A	Nga
TJ		92.86	99.44	98.06	98.06	98.06	98.06	99.12	86.19	98.98	99.12	63.44
Cv Sit.			94.74	99.04	99.36	99.42	99.42	92.98	77.68	91.38	91.38	66.05
S				99.04	99.04	99.04	99.04	99.52	86.19	99.60	99.44	63.85
MH-35					99.72	99.72	99.72	98.08	84.47	97.14	97.14	71.39
MH-34						100.00	100.00	98.08	84.47	97.14	97.14	71.39
MH-33							100.00	98.08	84.47	97.14	97.14	70.26
MH-32								98.08	84.47	97.14	97.14	70.26
G									86.23	99.24	99.36	63.67
CP										86.35	85.95	63.97
C											99.36	62.52
A												63.93
Nga												

Note: CP= Yogyakarta isolate, TJ=Teluk Jakarta isolate, G=Gondol isolate, C=Cilegon isolate, S=Serayu isolate, A=Ancol isolate, Cv Sit=*Chlorella vulgaris* strain Situbondo, MH-35=*Chlorella* sp. MH048635, MH-34=*Chlorella* sp. MH048634, MH-33=*Chlorella* sp. MH048633, MH-32=*Chlorella* sp. MH048632, Nga=*Nannochloropsis gaditana*

Keterangan: TJ=isolat Teluk Jakarta, G=isolat Gondol, C=isolat Cilegon, S=isolat Serayu, A= isolat Ancol, CP= isolat Yogyakarta, Cv Sit=Chlorella vulgaris *strain Situbondo, MH-35*=Chlorella *sp. MH048635, MH-34*=Chlorella *sp. MH048634, MH-33*=Chlorella *sp. MH048633, MH-32*=Chlorella *sp. MH048632, Nga*=Nannochloropsis gaditana

The phylogeny helps combine knowledge of biological diversity for structural classifications and generate insight into the diversity and distribution (Leaché, 2013). Based on the constructed phylogenetic tree, genetic relations of species within the population and between populations can be recognized. Phylogenetic reconstruction performed by Prehadi *et al*. (2015) showed that the analysis was helped in the distribution analysis of shark landed in Muncar. They found that *Carcharhinus brevipinna* landed in Muncar was divided into two clades with the high bootstrap value indicated that these individuals might come from different populations.

The phylogenetic tree also better understands the taxonomy and describes the evolutionary lineage of species, organisms, or one distinct ancestor. Rahman *et al*. (2019) conducted a phylogenetic analysis of microalgae isolated from Wakatobi Marine National Park, Indonesia. The BLAST result of *18S DNA* sequences showed that the isolated microalgae strain was closest to *Chlorella* sp. KMCC 185 with a similarity of 99% and the query cover of 99%. Therefore, the phylogenetic analysis showed the correlation of isolated strain with other species from *Chlorella* genus, which has no flagella, no mucilage, and eyespot. Based on the morphological, physiological, and phylogenetic analysis of the isolated microalgae,the isolate belongs to *Chlorella volutis*.

Conclusion

Six local microalgae isolates from different places in Indonesia were identified based on the *rbcL* gene. Microalgae strain TJ, G, S, C, and A isolates were identified as *C. pyrenoidosa*. Only CP isolate from Yogyakarta identified as *C. sorokiniana*. The phylogenetic analysis indicated that the five isolates of *Chlorella* belong to the same clade and are separated to *C. sorokiniana* isolate from Yogyakarta. It can be concluded that the *rbcL* gene can be used as a DNA marker for molecular identification of species in the genus *Chlorella*.

Acknowledgments

This research was funded by Palm Oil Fund Management Agency as part of the research grant contract number PRJ-18/DPKS/2020.

References

Ashour M, ME Elshobary, R El-Shenody, AW Kamil & AEF Abomohra (2019). Evaluation of a native oleaginous marine microalga Nannochloropsis oceanica for dual use in biodiesel production and aquaculture feed. *Biomass Bioenergy 120*, 439–447.

Basith A (2015). Peluang gen *rbcL* sebagai DNA barcode berbasis DNA kloroplas untuk mengungkap keanekaragaman genetik padi beras hitam (*Oryza sativa* L.) lokal Indonesia. *Seminar Nasional XII Pendidikan Biologi FKIP UNS*, 938–941.

Budiani A, RA Putranto, H Minarsih, I Riyadi, Sumaryono & B Abbas (2016). Kloning dan karakterisasi daerah promoter gen penyandi ADP glucose pyrophosphorylase dari Metroxylon sagu rendemen patitinggi dan rendah. *Menara Perkebunan 84*(1), 1–12.

Carneiro C, DM Moura, F Brambach, K Jair, H Bado, KV Krutovsky, H Kreft, SS Tjitrosoedirdjo, IZ Siregar & O Gailing (2019). Integrating DNA barcoding and traditional taxonomy for the identification of Dipterocarps in remnant lowland forests of Sumatra. *Plants 8*(461), 1–14.

Carpinelli EC, A Telatin, N Vitulo, C Forcato, M D'Angelo, R Schiavon, A Vezzi, GM Giacometti, T Morosinotto & G Valle (2014). Chromosome scale genome assembly and transcriptome profiling of *Nannochloropsis gaditana* in nitrogen depletion. *Mol Plant 7*(2), 323–335.

Fawley KP & MW Fawley (2007). Observations on the diversity and ecology of freshwater *Nannochloropsis* (Eustigmatophyceae), with descriptions of new taxa. *Protist 158*(3), 325–336.

Fawley MW, I Jameson & KP Fawley (2015). The phylogeny of the genus *Nannochloropsis* (Monodopsidaceae, Eustigmatophyceae), with descriptions of *N. australis* sp. Nov. and *Microchloropsis* gen. Nov. *Phycologia 54*(5), 545–552.

Fei C, S Zou, T Wang, C Wang, ND Kemuma, M He, SA Amin, & C Wang (2020). A quick method for obtaining high-quality DNA barcodes without DNA extraction in microalgae. *J Appl Phycol 32*(2), 1165–1175.

Ghosh S & NG Love (2011). Application of *rbcL* based molecular diversity analysis to algae in wastewater treatment plants. *Bioresour Technol 102*(3), 3619–3622.

Guillard RR & JH Ryther (1962). Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (cleve) Gran. *Can J Microbiol 8*(1140), 229–239.

Hadi SIIA, H Santana, PPM Brunale, TG Gomes, MD Oliveira, A Matthiensen, MEC Oliveira, FCP Silva & BSAF Brasil (2016). DNA barcoding green microalgae isolated from neotropical inland waters. *PLoS ONE 11*(2),

1–18.

Hall JD, K Fučíková, C Lo, LA Lewis & KG Karol (2010). An assessment of proposed DNA barcodes in freshwater green algae. *Cryptogamie, Algologie 31*(4), 529–555.

Huijbers MME, W Zhang, F Tonin & F Hollmann (2018). Light-Driven enzymatic decarboxylation of fatty acids. *Angew Chem Int 57*, 13648–13651.

Khan MI, JH Shin & JD Kim (2018). The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microb Cell Fact 17*(36), 1–21.

Leaché AD (2013). phylogenetic trees made easy: a how-to manual by Barry G. Hall. *Q Rev Biol 88*(1), 47–48.

Leliaert F, DR Smith, H Moreau, MD Herron, H Verbruggen, CF Delwiche & O De Clerck (2012). Phylogeny and molecular evolution of the green algae. *CRC Crit Rev Plant Sci 31*(1), 1–46.

Lorenz TC (2012). Polymerase chain reaction: Basic protocol plus troubleshooting and optimization strategies. *J Vis Exp 63*, 1–15.

NanoDrop Inc (2007). *260/280 and 260/230 Ratios NanoDrop® ND-1000 and ND-8000 8-Sample Spectrophotometers - Technical Support Bulletin T009*. 8–9. http://www.bio.davidson.edu/projects/gcat/protocols/NanoDrop_tip.pdf.

Orozco-Castillo C, KJ Chalmers, R Waugh & W Powell (1994). Detection of genetic diversity and selective gene introgression in coffee using RAPD markers. *Theor Appl Genet* 87: 934-940.

Pasha F, S Saggu & MF Albalawi (2016). Future perspectives of DNA barcoding in marine zooplanktons and invertebrates. *In*: S Trivedi, A A Ansari, SK Ghosh & H Rehman (eds.), *DNA Barcoding in Marine Perspectives: Assessment and Conservation of Biodiversity*. Switzerland, Springer International Publishing p. 169–179.

Pawlowski J, S Audic, S Adl, D Bass, L Belbahri, C Berney, SS Bowser, I Cepicka, J Decelle, M Dunthorn, AM Fiore-Donno, GH Gile, M Holzmann, R Jahn, M Jirků, PJ Keeling, M Kostka, A Kudryavtsev, E Lara, J Lukes, DG Mann, EAD Mitchel, F Nitsche, M Romeralo, GW Saunders, AGB Simpson, AV Smirnov, JL Spouge, RF Stern, T Stoeck, J Zimmermann, D Schindel, & C de Vargas (2012). CBOL protist working group: barcoding eukaryotic richness beyond the animal, plant, and fungal kingdoms. *PLoS Biol 10*(11), 1–5.

Prehadi, A Sembiring, EM Kurniasih, Rahmad, D Arafat, B Subhan & HH Madduppa (2015). DNA barcoding and phylogenetic reconstruction of shark species landed in muncar fisheries landing site in comparison with southern Java fishing port. *Biodiversitas 16*(1), 55–61.

Rahman DY, S Praharyawan, S Raharjo, F Fadiyah & D Susilaningsih (2019). Morphology and molecular characterization of newly isolated microalgae strain Chlorella volutis LIPI13-WKT066 from Wakatobi Islands and its potential use. *Ann Bogor 23*(1), 13–19.

Richterich P (1998). Estimation of errors in "raw" DNA sequences: a validation study. *Genome Res 8*(3), 251–259.

Saputro TB, KI Purwani, D Ermavitalini & AF Saifulloh (2019). Isolation of high lipid content microalgae from wonorejo river, Surabaya, Indonesia and its identification using *rbcL* marker gene. *Biodiversitas 20*(5), 1380–1388.

Shi H, W Li & X Xu (2016). Learning the comparing and converting method of sequence phred quality score. In: *Proceedings of the 6th International Conference on Management, Education, Information and Control (MEICI 2016)*. Shenyang, China. p, 260–263.

Suda S, M Atsumi & H Miyashita (2002). Taxonomic characterization of a marine *Nannochloropsis* species, *N. oceanica* sp. nov. (Eustigmatophyceae). *Phycologia 41*(3), 273–279.

Sundari N & N Papuangan (2019). Amplification and analysis of *Rbcl* gene (Ribulose-1,5-Bisphosphate Carboxylase) of clove in Ternate island. *IOP Conf. Series: Earth and Environmental Science*, *276*, 1–7.

Tamura K & M Nei (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol 10*(3), 512–526.

Wei L, Y Xin, D Wang, X Jing, Q Zhou, X Su, J Jia, K Ning, F Chen, Q Hu & J Xu (2013). *Nannochloropsis* plastid and mitochondrial phylogenomes reveal organelle diversification mechanism and intragenus phylotyping strategy in microalgae. *BMC Genomics 14*,1–17.

Yanuhar U, NR Caesar & M Musa (2019). Identification of local isolate of microalgae *Chlorella vulgaris* using Ribulose-1,5-

Bisphosphate Carboxylase/Oxygenase large subunit (*rbcL*) Gene. *IOP Conf. Series: Materials Science and Engineering 546*, 1–6.