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Identification of Tropical Marine Diatom *Chaetoceros dayaensis* CBO from Bokor Island through Morphology and Genetic Marker Analysis

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Abstract— Microalgae are well-recognized by many researchers working on bioenergy resources due to its capability to produce high amounts of lipids as biodiesel precursors. Diatoms as a group of microalgae that specifically categorized by their silica valve could be more attractive to the researchers because in addition to high-lipid content, their silica features can be also utilized for wider applications, or used for diatoms bioproduct purification. Identification of some diatom species, such as the diatom genus *Chaetoceros*, as an initial step in diatom studies were difficult to accurately perform because of the unique morphology of silica frustules. Meanwhile, some candidate universal genetic markers of microalgae could also be used for the identification of diatoms with brittle frustules. In this research, we aimed to identify isolate CBO of tropical marine *Chaetoceros* CBO strain using morphological and genetic approaches. The isolate CBO of *Chaetoceros* sp. CBO was successfully obtained from sea region of Bokor Island, Kepulauan Seribu, Jakarta, and cultivated in our laboratory as culture stock for further use. Better resolution of the *Chaetoceros* CBO identification until species level was shown by genetic analysis toward two proposed gene markers for microalgae (*rbc*L-3P and V4 region of 18S rDNA) rather than by morphological. Specific DNA region for this specimen was found in V4 region of 18S rDNA genetic marker that could be recognized by MspA1I restriction enzyme. Hence, the RFLP (restriction fragment length polymorphism) method could be also used as an initial diagnostic tool for identification of this tropical marine *Chaetoceros dayaensis* CBO and for stock culture labelling purposes.

Keywords— Tropical marine diatom, SEM imaging, Genetic marker, DNA barcode.

1. INTRODUCTION

Marine diatoms are microalgae groups found in high-salinity water regions and recognized by their brown-colored appearance with various shapes of silica frustules [1]. In the seawater ecosystem, diatoms play an important role in the biogeochemical cycle of silica [2], so a large portion of the natural silica abundance may belong to fossilized marine diatoms' shells. The remains of the diverse fossilized exoskeletons of planktonic diatom have been sedimented in the form of natural soft siliceous sedimentary rock called diatomaceous earth [3]. Similar to *Chlorella* sp. as a popular green microalga in bioenergy research, such as study regarding genetic profiling related to triacylglycerol biosynthesis [4], the diatoms are also rich in lipid content so that many studies have been conducted on diatoms bioprocesses. For example, biodiesel produced by microalgae species categorized as marine diatoms, i.e., *Navicula* sp. and *Thalassiosira* sp., displays a cleaner flame of combustion than other biofuel sources [5,6]. The diatom group itself is called a tropical marine diatom because it originates and is subsequently isolated from the tropical sea region of Indonesia. A study on planktonic organisms inhabiting Jakarta Bay, a part of the Indonesian sea region rich in inorganic nutrients, shows that a huge number of marine diatoms live in the seawater bodies, with *Chaetoceros spp.* dominating the planktonic population [7]. Meanwhile, biochemical study of tropical marine diatom from Jakarta Bay, shows that Navicula

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salinicola NBO as a promising candidate for biodiesel, nutrition, and pharmaceutical [8].

Some difficulties may occur during the morphological identification of some diatoms because some specific parts of the silica frustules appear thin and brittle. The damaged parts of the silica frustule can provide a biased identification result. On the other hand, DNA barcoding is considered able to solve this problem because this method is used according to a genetic approach that can be applied to incomplete specimens [9]. Regarding the microalga groups, two candidate genetic markers have been proposed for microalga species identification, i.e., *rbc*L-3P (3' end region of large subunit of RuBisCo encoding gene) [10], and V4 region of 18S rRNA gene (V4 area of 18S ribosomal RNA encoding gene) [11]. These gene marker candidates have been proposed as universal markers for microalgae by considering the suitable length of the nucleotide sequence for a genetic marker [10] and a high resolution of phylogenetic analysis [12]. If applicable, the genetic marker candidates can be routinely employed to identify and label stock culture of existing tropical marine diatoms.

To provide the tropical marine diatom strain with valuable characteristics, our study is initiated by capturing tropical diatom cells from a seawater region located in the northern Jakarta area, Indonesia. Afterwards, it must provide basic information about the strain for further stock culture labeling purpose. The main objective of this research is not only to identify the tropical marine diatom strains through morphological and genetic approaches but also to provide a simple and rapid method for stock culture identification using some proposed techniques related to algae and genetics research.

2. EXPERIMENTAL SECTION

2.1. Materials

A tropical marine diatom strain (labelled as CBO strain) isolated from Bokor Island (Jakarta, Indonesia), sterile seawater enriched with f/2 medium and inorganic silica content, ddH_2O, H_2O_2 30%, glacial acetic acid, ethanol p.a., cyano-cobalamin, thiamine hydrochloride, GeneRuler™ 1 kb DNA Ladder (Thermo Scientific), loading dye (Thermo Scientific), Tris-base, agarose, EtBr, GoTaq Green Master Mix (Promega), primers of *rbcL*-3P and V4 region of 18S rRNA gene.

2.2. Instrumentation

Thermal cycler for PCR (Bio-Rad), horizontal electrophoresis system (Bio-Rad), mini centrifuge U-320 R (BOEC Germany), autoclave MAC-601 (EYELA), salinity hydrometer (Warm Tone®), analytical balance (Ohaus Pioneer), luxmeter (Lutron LX-11-2), UV Illuminator (First Light), pH meter (Mettler Toledo), light microscope (Nikon YS 100), Scanning Electron Microscope JSM-6510LA (JEOL), and inverted microscope (Nikon DIAPHOT).

2.3. Procedure

2.3.1 Isolation and cultivation of the diatom cell

Tropical marine diatom stock culture was obtained from seawater near the coastal area of Bokor Island, Jakarta, Indonesia with the parameters displayed in **Table 1**.

Table 1. Sampling coordinate and seawater condition

Coordinate	Salinity (ppt)	Temperature (°C)	pH
S 05° 56. 927 E 106° 37. 723	35.0	29.1	7.84

The primary diatom cells were manually isolated under an inverted microscope and arbitrarily acquired from diverse planktonic microorganisms in seawater sample. Afterwards, single diatom cells were cultivated in sterilized f/2 medium [13] at room temperature and gradually scaled up to 25 mL medium and 100 mL medium, subsequently, as a stock culture.

The stock culture was routinely re-cultivated before the culture reached stationary phase with light exposure of 2000 lux, 12 h of light-dark cycles, without additional aeration. Moreover, the cell appearances were also observed under microscope to identify diatom cells morphology and to ensure that the stock culture was not contaminated by other types of diatoms or other microbes.

2.3.2 Morphological identification of silica frustules

Morphological information of tropical marine diatom specimens was obtained from two methods, i.e., observation of fresh diatom cells under a light microscope and analysis of dry diatom silica frustules through SEM imaging.

Regarding the identification through SEM approach, silica frustule from the diatom specimens was obtained by pre-treatment with acidic and peroxide using glacial acetic acid and 30% H_2O_2, subsequently, toward the harvested diatom biomass for 24 h [14]. Then, the wet silica frustules were dried at room temperature for 24-48 h. Afterwards, the dried frustules appearance was captured using a Scanning Electron Microscope JSM-6510LA (JEOL) at the SEM Laboratory of FMIPA ITB, Bandung, Indonesia.

2.3.3 DNA amplification and sequencing

The amplification of genetic markers was performed directly from fresh exponential diatom cells by disrupting the cells to release their DNA [15,16] by extending the duration of the initial denaturation phase of PCR [10].

Fresh diatom cells were mixed into GoTaq Green Master Mix (Promega) and related primers, i.e., *rbc*L-3P amplification using CfD [10] and DPrbcL7 [17] and V4 region of 18S rDNA amplification using M13F-D512for 18S andM13R-D978rev 18S [11]. Note that *rbc*L has 1400 bp of sequence length, because genetic marker should not have such a long sequence, so that only particular

region of the gene, i.e., *rbc*L-3P, was amplified by using these primers [10].

Amplicons were visualized by 1% agarose gel electrophoresis then sequenced using the same PCR primers for each genetic marker [10], excluding for V4 region of 18S rRNA gene using the M13F cloning vector [11]. The amplicons were sequenced at First Base, Malaysia.

The nucleotide sequences were proceeded and analyzed using DNA Baser Assembler v4 and BioEdit [18]. Poor chromatogram peaks of sequencing results were excluded for further analysis to avoid biased interpretation. Then, to provide similarity information, two nucleotide sequence databases were utilized in this research, i.e., BOLDSYSTEM (CBOL) and BLAST (NCBI). Currently, only the *rbc*L-3P gene marker is available in BOLDSYSTEM as it has been established as a standard genetic marker for plants by consensus.

3. RESULT AND DISCUSSION

3.1. Growth Profile of The Diatom Specimen

Gradual scaling-up of diatom cell was successfully conducted that was indicated by the appearance of a color medium changed from colorless to brown. **Fig. 1** shows the color change of the stock culture with 100 mL volume medium during 10 days of cultivation. Two medium types were separately used and compared during 100 mL scaling-up, i.e., f/2 medium and Walne medium.

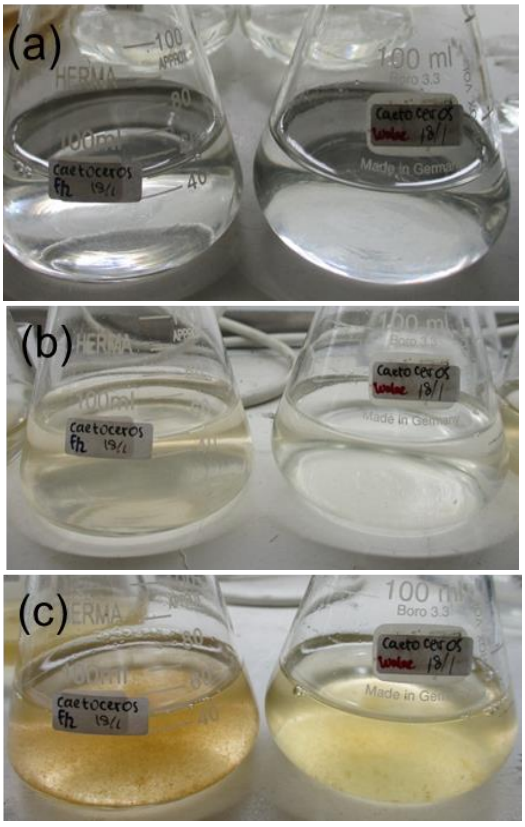

Fig. 1. The diatom from Bokor Island in the culture volume 25 mL within f/2 medium with silica (left) and walne medium with silica (right) [(a) day 0; (b) day 5, and (c) day 10]

Fig. 2. The diatom specimen in culture volume 1000 mL (Left to right: week 0, week 2, and week 3, respectively).

It can be seen from **Fig. 1**. that tropical marine diatom specimens looked healthier and grew well when cultivated within f/2 medium. Note that f/2 medium was also used from the initial step during obtaining this stock culture, i.e., isolation and cultivation of primary diatom cells. Hence, f/2 medium was regularly used for subsequent scaling-up and medium refreshment activities.

Scaling-up diatom culture from 100 mL to 1000 mL volume scale was also conducted in our laboratory as displayed in **Fig. 2**. However, the scaling-up of 1000 mL result showed that a significant colour change of the medium was reached after 3 weeks of cultivation. This slower growth rate indicates that scaling-up can optimally work at volumes below 1000 mL.

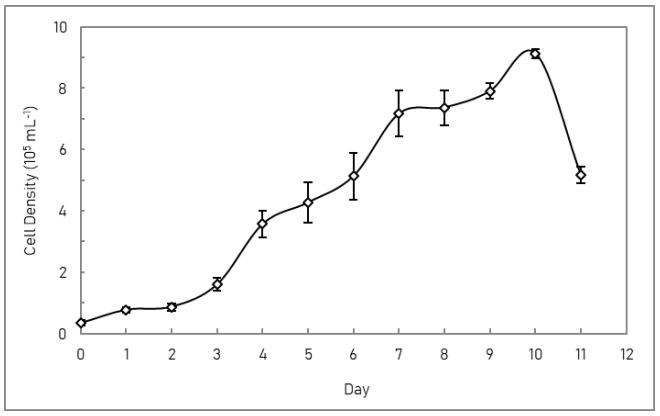

Fig. 3. Growth curve of tropical marine diatom specimen.

Fig. 3. displays the growth curve of a diatom specimen from a 100 mL volume of diatom culture with an exponential phase ending on day 10 of cultivation. According to this growth curve, the biomass for silica frustule collection was harvested before day 10 to reduce possibilities of damaged diatom frustule from dead cells appeared after SEM imaging.

3.2. Morphological Identification

Observation of fresh diatom cells under light microscope showed they appeared in a cylindrical shape attributed with extended antennae (**Fig. 4**), called as seta as a specific feature that can solely distinguish similar species within the subgenus *Chaetoceros* [19]. Therefore, the CBO specimen was identified until the genus level as *Chaetoceros* sp.

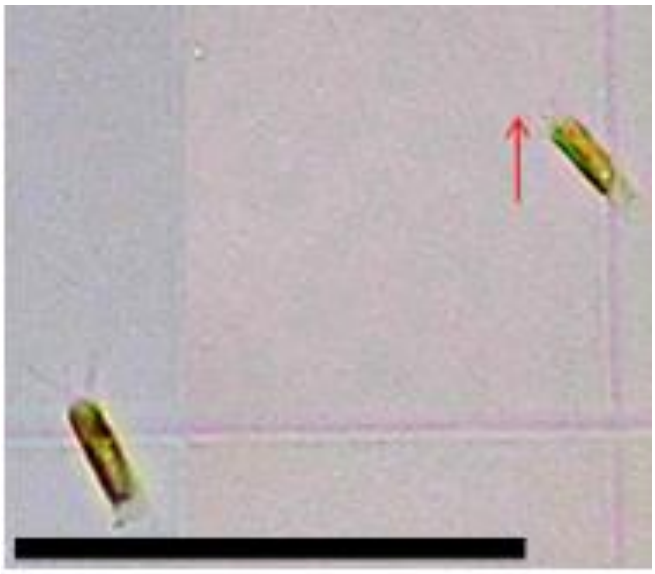

Fig. 4. Appearance of fresh *Chaetoceros* sp. CBO Cells (bar scale: 50 µm; red arrows indicate seta parts)

Fig. 5. shows the SEM imaging results with damaged silica frustules. The parts of cylindrical frustules were shown to be almost separated from each other with several parts indicated as broken seta frustules. The mechanical forces of mixing and centrifugation during acidic and peroxide pre-treatment contribute to the damaged frustule parts. Due to the disrupted frustule, further morphological identification based on SEM imaging results could not be performed.

3.3. Genetic Analysis

The DNA target of the two genetic markers has been successfully amplified as displayed in **Fig. 6.** Based on the sequencing results, two genetic markers of *rbc*L-3P and V4 region of 18S rDNA of *Chaetoceros sp.* CBO specimen had 854 bp and 599 bp, respectively. Currently, the nucleotide sequences of this specimen have also been recorded in the NCBI GenBank, i.e., *rbc*L-3P coded as MH125165.1 and V4 region of 18S rRNA gene coded as MH125164.1. **Fig. 7** shows a detailed phylogenetic tree of each genetic marker.

The specimen of our tropical marine *Chaetoceros* sp. CBO has the closest similarity with *Chaetoceros dayaensis* (KM407460.1) and *Chaetoceros cf. wighamii* (KT860958.1) according to *rbc*L-3P and V4 region of 18S rDNA, respectively. It is clear that *rbc*L-3P and V4 region of 18S rRNA gene results which identified the specimen as *Chaetoceros* cluster were consistent with the previous morphological identification results.

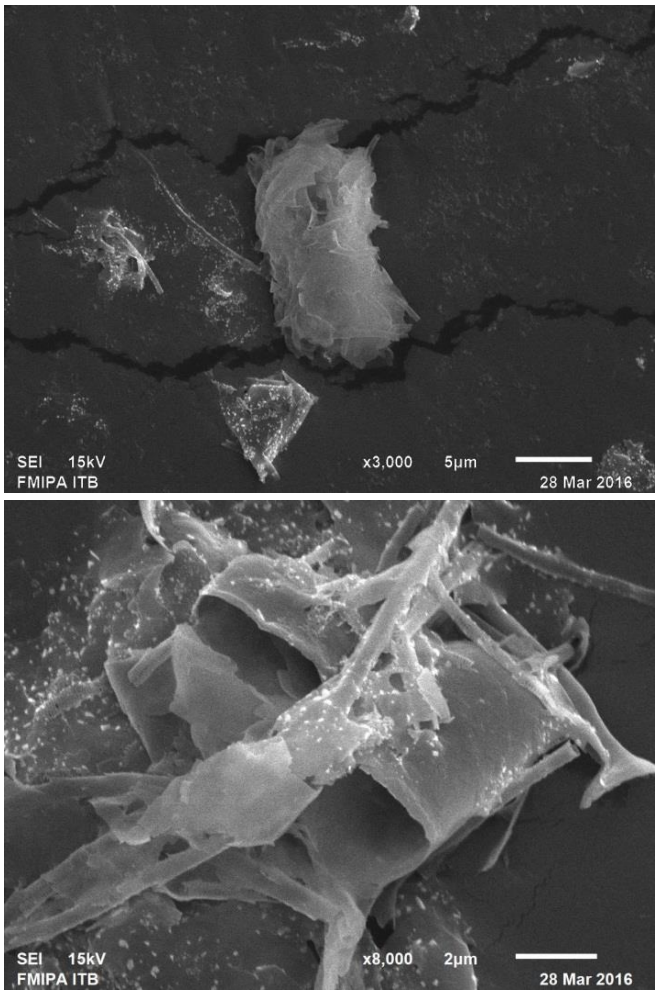

Fig. 5. SEM imaging result of silica frustule from tropical marine *Chaetoceros* sp. CBO

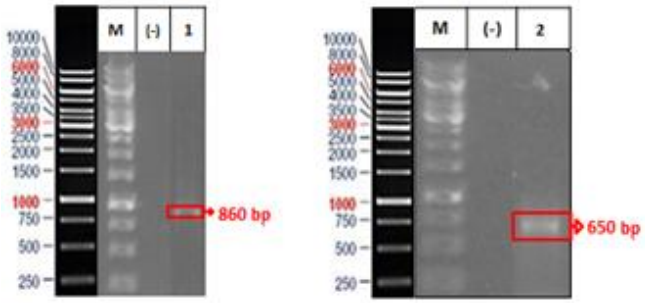

Fig. 6. Electropherogram of genetic marker amplicons, M = GeneRuler™ 1 kb DNA Ladder; (-) = negative control; 1 = rbcL-3P; 2 = V4 region of 18S rDNA.

The specimen of our tropical marine *Chaetoceros* sp. CBO has the closest similarity with *Chaetoceros dayaensis* (KM407460.1) and *Chaetoceros cf. wighamii* (KT860958.1) according to *rbc*L-3P and V4 region of 18S rDNA, respectively. It is clear that *rbc*L-3P and V4 region of 18S rRNA gene results which identified the specimen as *Chaetoceros* cluster were consistent with the previous morphological identification results.

Meanwhile, genetic identification results based on the BOLDSYSTEM database show that our

Chaetoceros CBO strain has the closest similarity with *Chaetoceros lorenzianus*. **Table 2** summarizes the identification results based on genetic marker analysis.

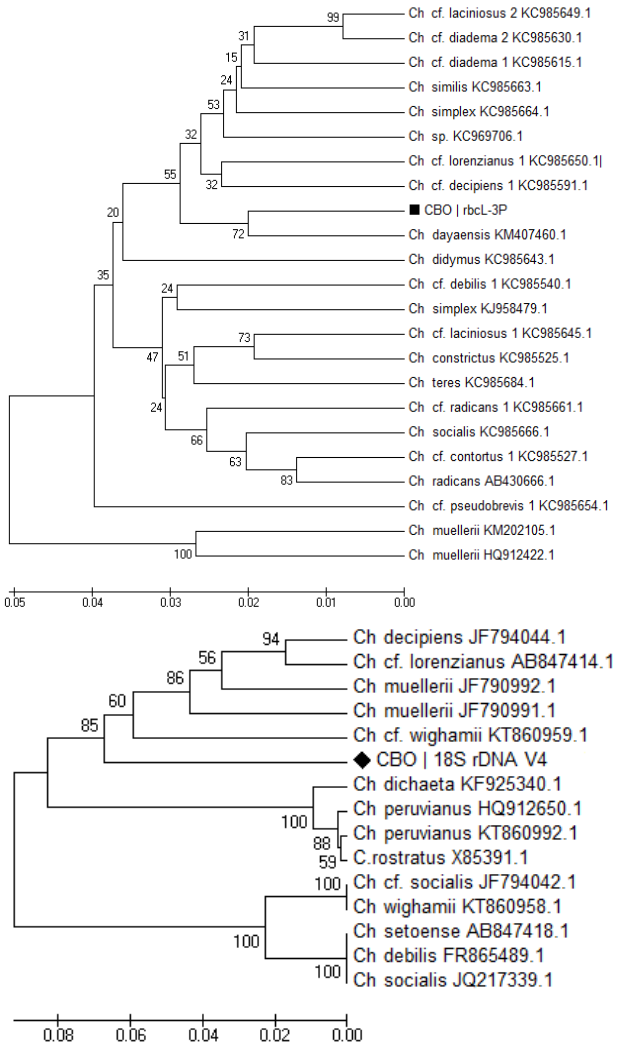

Fig 7. Phylogenetic tree of *Chaetoceros* sp. CBO based on *rbc*L-3P (top) and V4 region of 18S rRNA gene (right-bottom). Note that *Chaetoceros* is abbreviated as *Ch.*

In this study, *rbc*L-3P had better identification resolution, or broader nucleotide sequence records in the databases of GenBank and BOLDSYSTEM, than other genetic markers. Considering that GenBank currently has more *rbc*L database than *rbc*L recorded in the BOLDSYSTEM, the tropical marine diatom from Bokor Island is confirmed as *Chaetoceros dayaensis* CBO.

Meanwhile, although the V4 region of 18S rRNA gene showed correct identification until the genus level, this gene marker displayed lower resolution at the species level than *rbc*L-3P. Possibly, the plastids nucleotide sequences are still widely used and submitted to GenBank for microalgal identification because *rbc*L is mainly proposed as a universal DNA barcode for plants according to BOLDSYSTEM. However, this study still could not precisely decide that *rbc*L-3P is more accurate than V4 region of 18S rRNA gene in diatom identification because the quantity between both types of genetic sequences recorded in the GenBank database is still considered to be unequal.

Table 2. The Closest Similarity of *Chaetoceros* sp. CBO Specimen based on Two Nucleotides Database

	BLAST (%similarity)	BOLDSYSTEM (%similarity)
*rbc*L-3P	V4 region of 18S rDNA	*rbc*L-3P
Ch. dayaensis (96%)	*Ch. cf. wighamii* (85%)	*Ch. lorenzianus* (95,77%)

Remarks: *Chaetoceros* is abbreviated as *Ch.*

Fig. 8 displays two relevant genetic markers, i.e., *rbc*L-3P and V4 region of 18S rRNA gene sequences of the tropical marine *Chaetoceros dayaensis* CBO from Bokor Island that had been visually converted into illustrative DNA barcodes. The illustrative barcodes consist of combination of different colors representing each base of nucleotide.

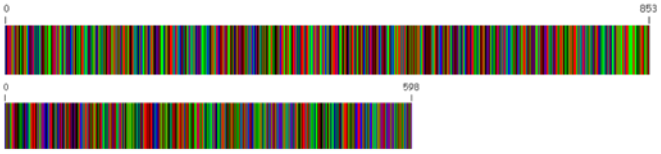

Fig. 8. DNA barcode of the tropical marine *Chaetoceros dayaensis* CBO from Bokor Island based on rbcL-3P (top) and V4 region of 18S rDNA (bottom)

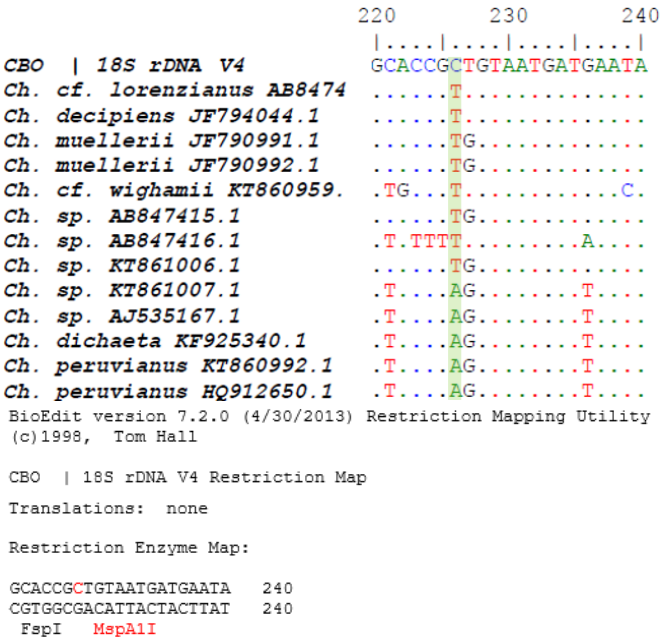

Fig. 9. Specific markers at the 226th base of V4 region of 18S rRNA gene (top, highlighted) and its restriction enzyme map recognition (bottom, font in red).

Furthermore, a specific DNA region for this specimen was in the V4 region of 18S rRNA gene that could be recognized by the MspA1I restriction enzyme, i.e., located at 226th base pair as displayed in **Fig. 9.** Sequence of CmG'CkG was recognized by the MspA1I enzyme. Thus, the enzyme could also recognize CCGCTG sequence in the 226th position of the V4 region of 18S rRNA gene.

Consequently, prior to performing the primary method by gene marker sequencing, another relatively simpler method of RFLP (restriction fragment length polymorphism) method could be also used as an initial diagnostic tool for this tropical marine *Chaetoceros dayaensis* identification.

4. CONCLUSION

Our tropical marine *Chaetoceros dayaensis* CBO stock culture from Bokor Island was successfully identified to the species level using a genetic approach of two genetic markers for microalgae, i.e., *rbc*L-3P and V4 region of 18S rRNA gene, with *rbc*L-3P showing a higher resolution of species identification. Our study revealed that our diatom DNA barcodes were completely acquired using the direct single-cell PCR method. This means that DNA barcoding of diatoms could be conducted easily without laborious work of the serial steps of DNA extraction and purification. Moreover, additional findings regarding specific base regions recognized by restriction enzymes could provide a simpler technique for stock culture identification, i.e., the RFLP method, which did not require relatively costly and time-consuming DNA sequencing.

Our study was still unable to optimally exploit the illustrative DNA barcodes. Advanced studies are required to develop a fast and robust scanning system based on DNA barcode patterns. In addition, the RFLP method for our specimen-specific base had not been conducted in this study. Further research can be conducted to support our assumptions regarding a simpler and easier technique for stock culture identification. Meanwhile, regarding accurate morphological analysis as the fundamental species identification, a proper method for diatom morphological identification through SEM imaging still needs to be established because there may still be a misinterpretation of genetic marker information. Further studies are necessary to provide an appropriate technique for silica frustule purification without damaging its native shapes.

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CONFLICT OF INTEREST

We have no conflicts of interest to disclose regarding this research.

AUTHOR CONTRIBUTIONS

ES conducted the experiment and wrote the manuscript. YR, ZN, SS, and S revised the manuscript. All authors agreed to the final version of this manuscript.

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† Dedicated to the memory of Sutomo, M.Si. (2019)