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MOLECULAR AND PHYLOGENETIC IDENTIFICATION OF MARINE MICROALGAE INFERRED BY 18S RDNA GENE

NAZIA ABDUL KADAR[1], SITTI RAEHANAH MUHAMAD SHALEH[1*] and EMMA SUALI[2]

[1]Borneo Marine Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia
[2]Faculty of Engineering, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia
[*]E-mail: sittirae@ums.edu.my

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ABSTRACT

Microalgae are microscopic organisms that are usually identified based on the morphological features. However traditional classification are complicated by high levels of morphological plasticity and convergence that will lead to uncertain identification especially at the species level. Therefore, molecular marker is advantageous to distinguish and correctly identify microalgae at the lowest taxonomic level based on the genetic information and phylogenetic analyses. In the present study, we have characterized five strains of microalgae that were deposited in Borneo Marine Research Institute based on 18S rDNA gene sequences. The BLAST results of microalgae sequences showed high percentage similarities that revealed the species as *Chaetoceros gracilis* (99.77%), *Thalassiosira weissflogii* (99.70%), *Nannochloropsis oceanica* (100%) and *Isochrysis* sp. (99.97%). Combination of both classical and genetic approach is vital to clearly identify microalgae at generic and species levels.

Key words: 18S rDNA, microalgae, *Chaetoceros*, *Thalassiosira*, *Nannochloropsis*, *Isochrysis*

INTRODUCTION

Microalgae is a heterogeneous group of single-celled photosynthetic organism that is important in food, feed, healthcare, industry and also energy. They have been used as live feeds in aquaculture industry, especially for molluscs, crustacean larvae, juvenile finfish, zooplankton in mariculture food chains and many more (Gouveia *et al*., 2015; Han *et al*., 2015; Sarker *et al*., 2016). Most common methods for identification and characterization of microalgae species are by using light microscopy and transmission electron microscopy (Bongiovani *et al.,* 2014). However, morphological resemblances and plasticity have frequently led to inaccurate species identification. For instance, taxonomic identification of *Nannochloropsis* species is challenging due to the small cell size and simple structure, the difficulties in fixing the cells for transmission electron microscopy (Hibberd, 1981) and the lack of sexual reproduction.

The application of molecular markers as an important tool in the detection and identification of microorganisms has been internationally recognized. Molecular techniques permit an assessment of the validity of the morphological species concept for many types of microorganisms; and most commonly employed gene is the small-subunit ribosomal RNA gene (16S rRNA in prokaryotes and 18S rRNA in eukaryotes). There have been a number of studies done on the molecular identification of microalgae (Manoylov, 2014; Lee *et al.,* 2015; Tragin *et al.,* 2016). Nuclear-encoded small subunit ribosomal RNA gene (18S rDNA) sequences have been used widely to infer the phylogenetic relationships among taxa at different hierarchical rank (Haddad *et al*., 2014). There are several species of microalgae in the algae research laboratory in Borneo Marine Research Institute, Universiti Malaysia Sabah. *Chaetoceros* sp., *Nannochloropsis* sp., *Nitzschia* and *Thalassiosira* were collected by the algae research laboratory and some were donated by the Fisheries Department. In order to reconfirm the species of the microalgae

* To whom correspondence should be addressed.

collection, we performed DNA sequencing and phylogenetic analyses.

MATERIALS AND METHODS

Microalgae purification and propagation

Microalgae culture procedures were carried out under sterile conditions to avoid cross-contamination. The microalgae samples in the laboratory collection were purified by using the streaking method, whereby each of the algae strain were streaked onto the sterile plate containing Walne media and silicate (only for diatom) and 1.5% agar aseptically. After that, the plate was incubated at 25°C until algae colonies appeared. The selected single colony was transferred into test tubes containing 10 mL fresh media. The microalgae cultures were kept as pure stock culture. In the microalgae propagation process, it was first carried out in a 100 mL flask and followed by 500 mL and finally in 1000 mL. Walne media were used with silicate addition for the diatom strains. In a temperature controlled room (25°C), all cultures were maintained under continuous illumination at low light intensity ~80 µmol m^{-2} s^{-1}. To accelerate the growth, cultures of 1 L volume were vigorously aerated while for the lower volume, an orbital shaker was used.

DNA extraction

Genomic DNA was extracted using Nucleospin® Plant 11 kit (Macherey-Nagel, Germany). The microalgae cultures were harvested using repeated centrifugation at approximately 11000 × *g* for 1 min. The supernatants were discarded and the pellet was subjected to DNA extraction following the manufacturer's instructions. The pellet was suspended in 400 µL buffer PL. The mixture was vortexed thoroughly until the pellet dissolved into the buffer. Ten µL of RNase A solution was added to the mixtures and incubated at 65°C for 10 min. After incubation, a NucleoSpin® filter column (violet ring) was placed into 2 mL collection tube and the mixtures were loaded into the column and centrifuged for 2 min at 11000 × *g*. The upper layer aqueous which contains the DNA was transferred into a new 1.5 mL microcentrifuge tube. For DNA binding, 450 µL of buffer PC was added into the microcentrifuge tube and mixed thoroughly by pipetting. This buffer PC was able to create the ideal binding conditions to the silica membrane in the binding plate. A NucleoSpin® filter column (green ring) was then placed into a new 2 mL collection tube and all the mixture was loaded into the column and centrifuged for 1 min at 11000 × *g*, and the flow-through was discarded. Following that, DNA washing was carried out, with first washing done by adding 400 µL of PW1 into the column (green ring), followed by centrifugation for 1 min at 11000 × *g* and the flow-through was discarded. Next, 700 µL of PW2 was added into the column (green ring), centrifuged for 1 min at 11000 × *g* and the flow-through was discarded. After that, another 200 µL of buffer PW2 was added into the column (green ring), centrifuged for 1 minute at 11000 × *g* and the flow-through was discarded. Finally, the NucleoSpin® filter column (green ring) was placed into a new 1.5 mL microcentrifuge tube. A total of 50 µL of buffer PE was pipetted onto the membrane and incubated for 5 minutes at 65°C and centrifuged at 11000 × *g* for 1 min. This step was repeated with another 50 µL buffer PE and eluted into the same microcentrifuge tube. The DNA sample was kept in -20°C until further use.

PCR amplification and sequencing

The 18S rDNA region was amplified using universal primer 18SCOMF1 (forward), 5'-GCTTGT CTCAAAGATTAAGCCATGC-3' and 18SCOMR1 (reverse), 5'-CACCTACGGAAACCTTGGTTA CGAC-3' (Zhang *et al.,* 2005). PCR amplification was conducted using GoTaq® DNA Polymerase (Promega) in 50 µl reaction volume, containing 1× PCR buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 200 µl DNTPs (Promega), 1 pmol of each primer, 1.25 U of GoTaq® DNA Polymerase (Promega) and 5 µL of template DNA (Table 1). The PCR thermocycling conditions were as follows; initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing at 66°C for 40 seconds and extension at 72°C for 30 seconds and final extension at 72°C for 10 min. The PCR products were electrophoresed on 1.5% (w/v) agarose gel using 1 kb DNA ladder (Promega) as standard size before being stored at -20°C.

Successful amplification products were purified using DNA-spin™ Plasmid DNA Purification Kit (iNtRON). Before sending the plasmids for sequencing, the purity and concentration were determined using spectrophotometer. Those PCR products which fulfilled the minimum requirement (purity ~1.9-2.0, concentration > 100 ng/mL) were sent to the AITBiotech Pte. Ltd., Singapore for DNA sequencing using Applied Biosystems 3730XL. Each plasmid was subjected to bi-directional sequencing method. The sequencing of all the plasmids used the M13 forward (-20) and M13 reverse (-24) primers.

Table 1. Reagents used per reaction for 18S rDNA PCR amplification of the microalgae

PCR reagents	Initial concentration	Final concentration	Volume (µL)
PCR buffer (Promega)	5×	1×	10
$MgCl_2$	25 mM	1.5 mM	3
dNTPs	10 mM	200 µM	1
Primer 18SCOMF1	10 µM	1 µM	5
Primer 18SCOMR1	10 µM	1 µM	5
Go*Taq* DNA polymerase	2.5 unit/µL	1.25 U	0.25
DNA template	10-30 µg/mL	≤1 µg	5
Sterile distilled water	–	–	20.75
		Total	50

Table 2. Results of DNA sequencing identification and BLAST similarity percentage

No	Laboratory identification	DNA Sequencing Results	Percentage similarities (%)
1	*Chaetoceros calcitrans (P)*	*Chaeotoceros gracilis*	99.77
2	*Thalassiosira (M)*	*Thalassiosira weissflogii*	99.67
3	*Nannochloropsis (A)*	*Nannochloropsis oceanica*	100
4	*Nannochloropsis (J)*	*Nannochloropsis oceania*	100
5	*Nitzschia (F)*	*Isochrysis* sp.	99.90

Phylogenetic analyses

The sequencing results provided by AITBiotech were in the form of nucleotide sequences. To analyze the sequences, both forward and reverse sequences were assembled and aligned using SeqMan of Lasergene software (DNASTAR, 7.1). The DNA sequences (consensus) which assembled during the contig analysis were further analyzed by using BLAST (Basic Local Alignment Search Tool) analysis (www.ncbi.nlm.nih.gov/BLAST). Then, phylogenetic tree was constructed using neighbor joining method, with Kimura two-parameter distance matrix and 1000 bootstrap replicates to determine the genus and the strain of the microalgae by comparing with the sequences retrieved from the GenBank.

RESULTS AND DISCUSSION

PCR amplifications and sequencing

Regions of the 18S rDNA were successfully amplified in all five microalgae strains, namely *C. calcitrans* (P), *Thalassiosira* (M), *Nannochloropsis* (A), *Nannochloropsis* (J) and *Nitzschia* (F). The amplified PCR products ranged from size 1292-1346 bp. All the sequences were deposited in Genbank with accession numbers KY399775-KY399779.

Identification of microalgae

Nucleic acid sequences of the 18S rDNA genes were analyzed using BLAST to determine the sequences percentage similarities with the sequences available in the GenBank. All of the determined sequences corresponded to known species with a high sequence similarity. Table 2 showed the results of DNA sequencing identification of microalgae samples and the BLAST similarity percentage. Each of the DNA sequences were analyzed to determine their respective genus of microalgae. The obtained DNA sequences were aligned with the corresponding sequences from the GenBank. Each of the microalgae sequences showed high percentage similarities with its respective genus sequences references except for sample *Nitzschia* (F); *Chaetoceros calcitrans* (99.77%), *Thalassiosira* (M) (99.70%), *Nannochloropsis* (A) (100%), *Nannochloropsis* (J) (100%) and *Nitzschia* (F) (99.97% with genus *Isochrysis)* (Figure 1). Apparently the *Isochrysis* sp. culture was mislabeled as *Nitzschia* due to the golden brown colour of the culture that was observed through the naked eye. The microalgae were characterized again under light microscopy with magnification of 400× to distinguih the morphological characteristics. *Chaetoceros gracilis* has an irregular square shape cell with four long setae*, Thalassiosira weissflogii* has barrel shape or short rectangular cells that forms

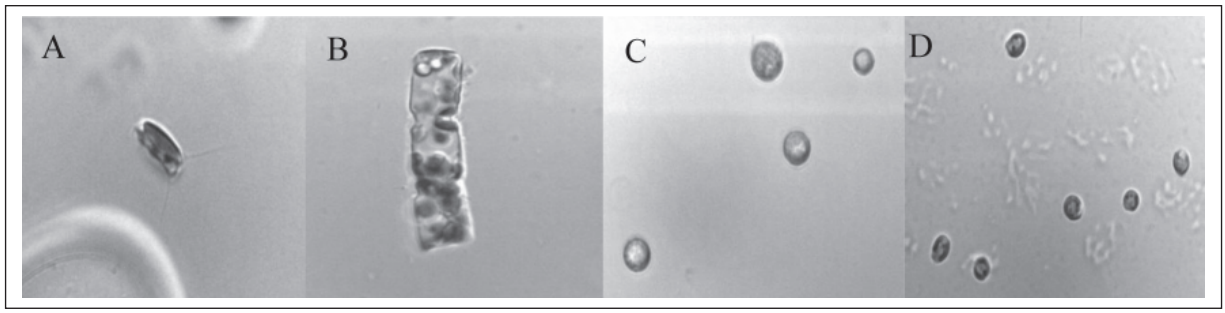

Fig. 1. Light microscope (400×) pictures of microalgae. A= *Chaetoceros gracilis, B= Thalassiosira weissflogii, C=Nannochloropsis oceanica* and D= *Isochrysis* sp. Scale bar = 20 µm.

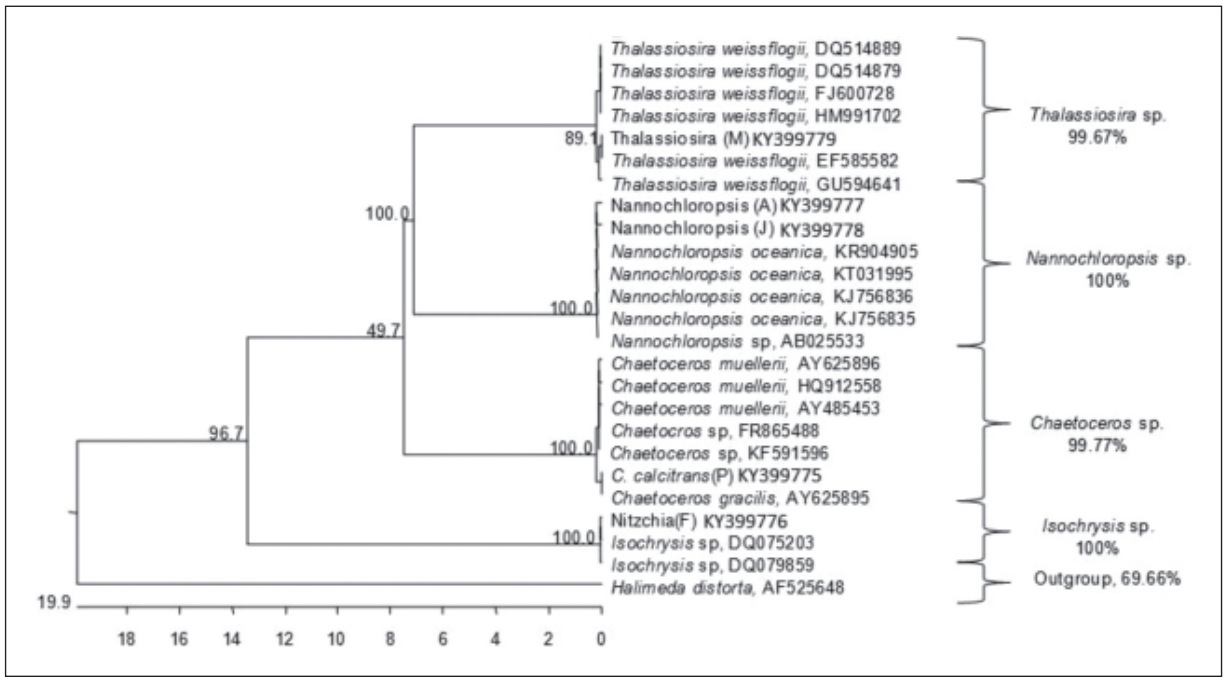

Fig. 2. Phylogenetic tree constructed based on 24 18S rDNA gene sequences. Numbers at the tree nodes indicates bootstrap values of 1000 replicates. The distance scale is represented by the horizontal branches. *Halimeda distorta* is used as outgroup.

chain, *Isochrysis* sp. has a spherical and motile cell and finally *Nannochloropsis oceanica* has a green and spherical cell.

Phylogenetic analyses

The neighbor joining phylogenetic tree for the genus determination of the microalgae in this study is shown in Figure 2. It is clear that all the sequences obtained have a high level of homology with other sequences belonging to the same species which were deposited in the GenBank. The phylogenetic tree analysis showed that *Chaetoceros calcitran* (P) from the algae research laboratory had high nucleotide percentage similarities with *Chaetoceros* sp., *Chaetoceros muellerii* and *Chaetoceros gracilis* with 99.77% similarities. Meanwhile *Thalassiosira (*M) from the algae research laboratory had high nucleotide percentage similarities with *Thalassiosira weissflogii* (99.67%). Both *Nannochloropsis* samples (A) and (J) have high nucleotide percentage similarities with *Nannochloropsis oceanica* and *Nannochloropsis* sp. with percentage similarities of 100%. In exception of sample *Nitzschia* (F), all the other samples were identified as the said species of the microalgae. From this analysis, the DNA of the *Nitzschia* (F) has high nucleotide similarities with the *Isochrysis* sp. with 99.90% of percentage similarities.

The results from the DNA sequencing showed that four of the microalgae were identified as the said species. Meanwhile one sample showed different species than the said species (Table 2). The present study successfully confirmed the microalgae strain available at the algae research laboratory in Borneo Marine Research Institute by employing molecular approach using sequences of the 18S

rDNA gene of microalgae. Correct identification of the microalgae strain is vital for successful cultivation at larger scale and also for accurate live feed strain used in the aquaculture industry.

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