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Morphology, molecular, and nutritional value of *Amphora* sp. from coastal water of the grouper cultivation center (Situbondo, Indonesia)

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ABSTRACT

A thorough and specific identification of diatoms genera can provide valid information for further development and use. The purpose of this study is to identify morphology, molecular, and nutritional value. This study, is based on light microscopy (LM) images, DNA-barcoding using primer (ITS-L and ITS4) and nutritional value of diatom ST02. After being morphologically and molecularly analyzed, the diatom was then named as *Amphora* sp. The nutritional value of *Amphora* sp. ST02 is fairly good with protein of 13.66%, and 14.2% carbohydrate. These results provide information about the *Amphora* sp. genera isolated from the coastal water of the Grouper Cultivation Center, Situbondo, Indonesia. Moreover, the nutritional value can be used as fundamental information for utilization and development for the food and health field.

Key words *: Diatom, DNA-barcoding, ITS, Aquaculture.*

Introduction

Diatom are single eukaryotic cell that can be easily found in all aquatic habitats. It has various body length (~1-500 mm) and has a role as a producer in the food chain (Scala and Bowler, 2001). In general, the role of diatoms ecology are as a primary producer (Goldman, 1993), nutrient cycle, stabilization of aquatic sediments (Haubois *et al.* 2005), marine silica cycle (Nelson *et al*. 1995), and biomonitoring of watershed (Herlory *et al.*, 2013). In recent years, the potential of diatom as antioxidants (Xia *et al.* 2014; Hemalatha *et al.*, 2013; Smerilli *et al.*, 2019) immunostimulants, and natural antiviral agents has also been investigated (Manzo *et al.*, 2019; Bergél *et al.*, 1999).

According to Mann and Drop (Mann and Droop, 1996), diatom has around 200,000 species with various characters, which are spread in the sea, brackish and fresh water. Specific and accurate species iden-

tification of diatom is crucial to study its character, function, and use. Morphologically, diatoms have frustules containing silica, and are divided into two groups, namely centric and pennate, and are divided into three classes namely Coscinodiscophyceae (centric diatoms), Fragillariophyceae (araphid diatoms) and Bacillariophyceae (raphid diatoms) (Mishra *et al.* 2017).

Diatom have a tiny size and thus, made the morphological identification of species challenging. According to Guo *et al.*, (2015) the small diatom size can provide ambiguous information at the genus or species. Evans *et al.,* (2007) also revealed that morphological identification cannot always be used as a reliable indicator of species and phylogenetic relationship. DNA barcoding is a technique or method used to detect species based on the DNA sequence of a predetermined species sequence (Hebert *et al.* 2003). DNA-based identification is beneficial for uncovering vague diversity at the taxonomic level, as well as identifying species whose structural characteristics are challenging to observe (Bock *et al*., 2011).

The *internal transcribed spacer* (ITS) is one of the DNA potential target sequences in the DNA barcoding method. According to Godhe *et al.* (2006) and Vanormelingen *et al.* (2007) the ITS sequence is considered useful in defining intraspecific or population-level differences due to rapid evolution. Also, according to Guo *et al.* (2015) ITS is a potential diatom that can produce the highest percentage of parsimony-informative sites (%PI), that is 85.84%, compared to other primers CO1 (82.14%), rbcL (41.01%) and UPA (14.97%). This study aims to identify morphological and molecularl diatom ST02 specimens located in Brackishwater Aquaculture Development Centre (Situbondo, Indonesia) using ITS primers. Also, to provide information on the nutritional character of *Amphora* sp. ST02.

Materials and Methods

Culturing of diatom

Diatom samples with code ST02 was obtained from the Natural Feed Laboratory, Brackishwater Aquaculture Development Centre which was isolated from the beach waters of the Grouper Aquaculture Center (7°40'59.7"S 113°52'13.9"E), Situbondo, Indonesia in 2015 and cultured intensively. Diatom ST02 was cultivated in a semi-mass manner with an initial stocking density of inoculant ± 100 cell/ml x 10^4 in a 350-liter sterile seawater medium and administering nutrients (fertilizers, micro metals, and vitamins) using a dose of each nutrient, i.e. 1 ml/l culture media.

Diatom cultivation is carried out for five days. Optimal conditioned water quality according to diatom growth: Temperature of 27 - 31 ºC, pH 5.5 - 7.4, salinity 26 - 31 ppt, light 2500 - 5000 lux, and nutrients (nitrogen, phosphorus, silica, iron, and vitamins). During the culture period, diatom growth can be observed by calculating the density (cell/mL) by taking samples on the wall or base of the media using a pipette of 5-10 points. 5 m. of the sample was then transferred into a container and observed under an Olympus BX51 microscope (4x magnification) and counted using a hand counter (Sujarwani *et al.,* 2016). The growth rate is calculated using Andersen's formula (2005) as follow:

$$r = \frac{In(\frac{Nt}{No})}{\Delta t} \quad .. (1)$$

where*r* is the growth rate, N_0 is the size of the population at the beginning of the time interval, N_t is the size of the population at the end of the time interval, and Δ_t is the length of the time interval (t_t-t_0).

Harvesting diatom

Diatom ST02 which has been cultured for 5 days was traditionally harvested and dried in a room with temperature of 31-33 ºC for 3 days until it forms a dry solid, then it was blended to form powder and stored in a glass cabinet at temperature of 25-30 ºC.

Microscopy

The livinge diatom ST02 was isolated using a pipette and placed on a slide for a light microscope, then analyzed using an Olympus BX51 microscope (100x magnification) equipped with an Olympus DP73 camera with oil immersion (NA = 1.30) (Olympus Optical Co. Ltd., Japan) (Kesici *et al.* 2013)

DNA Isolation and polymerase chain reaction (PCR)

The total isolation of DNA diatom ST02 was carried out using a NEXpreptm Cell/Tissue DNA Mini kit (NexK-3000). The DNA isolation procedure followed the protocol provided by the kit provider and

Table 1. Nutrient manufacturing materials (fertilizers, micro metals and vitamins) for diatom ST02 culture.

Materials	Dose (g)	Manufacturing Method	
Fertilizer	KNO_3	750	All chemicals were mixed with 10 L of boiled water, then stirred and stored in a sterile container.
	NaH_2PO_4	50	
	Na_2EDTA	50	
	$FeCl_3$	31.5	
	Silik ate	300	
Heavy metal solution	21		All chemicals were mixed with 10 L of boiled water, then stirred and stored in a sterile container.
	Zn Cl_2		
	$CoC1_2$, $6H_2O$	20	
	$(NH_4)_6$, MO_7, O_{24}, $4H_2O$	9	
	$CuSO_4$, $5H_2O$	20	
Vitamin	Vitamin B1	1000	All chemicals were mixed with 10 L of boiled water, then stirred and stored in a sterile container.
	Vitamin B12	50	

was stored at -20 ºC until further analysis. The isolated DNA was then tested qualitatively and quantitatively. The qualitative test was carried out using electrophoresis with agarose gel 1%, while the quantitative test was carried out using a UV-Vis spectrophotometer nanodrop (Basyuni *et al.*, 2019).

The isolation results were amplified using a Biorad PCR machine in a 30 µL solution consisting of ddh_2O, PCR mix (NexProtm e PCR, NexG-2000), primers, and DNA templates. Amplification was carried out by controlling temperature, predenaturation carried at 94 ºC for 5 minutes, then continued with 30 cycles consisting of denaturation at 94 ºC for 30 seconds, annealing at 56 ºC for 30 seconds, extension at 72 ºC for 90 seconds and ended with post elongation at 72 ºC for 5 minutes (Al-Daoude *et al.* 2014).

Primers used in this test are ITS-L (5'-TCG TAA CAA GGT AGG CGT TTC TG-3 ‘) as the forward and ITS-4 (5’-TCC TCC GCT TAT TGA TAT GC-3') as the reverse. The PCR results where then sequenced using the first-base sequencing service.

Squence analysis

Sequence analysis of PCR amplicons was performed using sequence scanner software to determine the QV peak. The sequences obtained were compared with the data in GeneBank using the NCBI BLAST algorithm (Table 2). The phylogenetic tree reconstruction was carried out using the Distance-based method of *Neighbor-Joining* (NJ) (Tamura *et al.*, 2004) using Mega X v.10.0.5 software (Kumar *et al.*, 2018). Bootstrap consensus tree analysis concluded from 1000 replications. Phylogenetic studies include *Phaedactylum tricornutum*, as an *outgroup*.

Proximate diatom

The chemical composition of ST02 diatom powder was determined based on the AOAC method (1995). Protein determined by nitrogen dose, ash content was analyzed by calcination in a 105 ºC oven for 24 hours, and crude fat content was analyzed by the Kjeldahl method.

Results and Discussion

Diatom culture

Inoculant from cultured diatom ST02 has good growth. diatom ST02 growth reached 240 x 10^4 cell/cm^2 on the 5th day of culture, with 156 g of concentrate. Reasonable growth rates, indicating diatom ST02 in optimal conditions. In comparison, the study from Fahruddin *et al.* (2012) using 100-liter culture media, it produced growth of 210 x 10^4 cell/cm^2 after 6 days culture period, whereas diatom sp. ST02 has a growth value of 240 x 10^4 cell/cm^2 on the 5th day. These results also provide evidence that *Amphora* sp. can be cultured properly with simple culture techniques on a small or large scale.

Morphological and Molecular Identification of Diatom

Microscope imaging results show that the diatom ST02 has the morphological characteristics of semi-elliptical and dorsiventral valves, the dorsal margins curved smoothly, horizontally near the center of the valve. Diatom length ranges from 14-17 µm, frustule width 7-11 µm, and valve 3-5 µm (Fig. 1). Morphological observation using light microscopy (LM) imaging (Fig. 1) shows that diatom ST02 has the same shape with *Halamphora yongxingensis* from

Yongxing Island in the South China Sea (Jiang *et al.,* 2015), with length of 9.18 - 18.2 µm. However, diatom ST02 has a larger frustule width of 7-11 µm, which is more likely to have similarities with *Halamphora subturgida* (Hustedt) Levkov which has a frustule width of 7-11 µm and valve 2.9-4.5 µm (Sala *et al.,* 2006). Furthermore, Sala *et al.* (2006) stated that *H. subturgida* is a tropical freshwater species different from *Amphora* sp, which is marine water species. Identification based on LM image has limitations and difficulties in identifying details and it is only able to identify frustules.

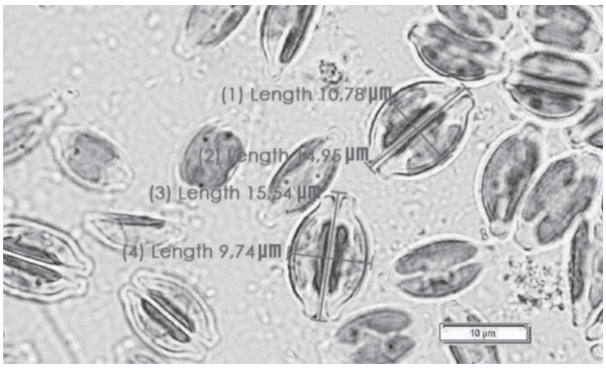

Fig. 1. Diatom ST02 images obtained from light microscope (Olympus BX53) with magnification 100x.

DNA Isolation and Sequence ITS Diatom

DNA-based molecular identification were carried out to support the findings from the morphological analysis. The results of the isolation of DNA diatom ST02 were analyzed qualitatively to determine the quality of DNA from isolation. The results shown in Fig. 6 (ISO) show, a band with a length >1500 bp indicating, the qualitative separation of DNA diatom ST02 is good. Also, DNA bands in the early part of the migration indicates that the band is total DNA. The results from quantitative DNA analysis using nanodrop UV-Vis spectrophotometer showed good results with a concentration value of 375.20 ng/µL and purity of 1.03. This result is considered quite good, and the DNA template can continue on DNA Amplification.

DNA amplification of diatoms ST02 with ITSL and ITS4 produced DNA bands around 900 bp (Fig. 2, ITS). Amplicon DNA results were then purified and used as the target DNA in sequencing. Purification and sequencing electrograms are then translated into nitrogen bases to facilitate the analysis of target sequences. The forward and reverse sequences are then combined into one to get the complete rDNA region sequence, and show the results of the 885 bp sequence. Sequences are deposited in GenBank with accession number (MN592659).

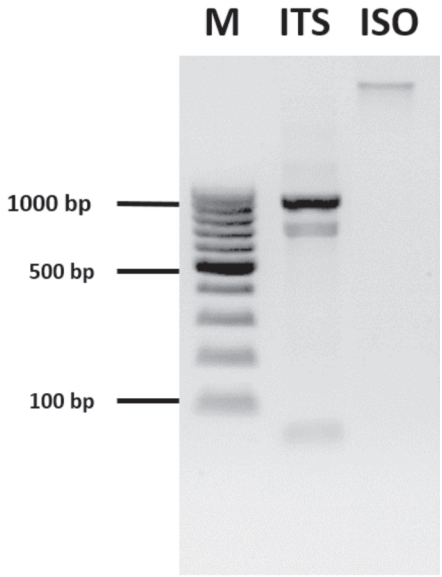

Fig. 2. Electrogram isolation of DNA diatom ST02 >1500 bp. (ISO), ± 900 bp.*internal transcribe spacer* (ITS), and 1000 bp (M) marker.

Table 2. Results of the diatom ST02 alignment with BLAST

Accession	Description	Max. Score	Total Score	Query Coverage (%)	E value	Max. Ident (%)
KJ845340.2	*Amphora* sp. MACC 9	1352	1352	97	0	94.93
JF834543.1	*Amphora* sp. PP-2011	1334	1334	98	0	94.3
KR905764.1	*Amphora* sp. HN09	1332	1332	97	0	94.58
KR905762.1	*Amphora* sp. BQW2	1271	1271	98	0	93.12
KR905763.1	*Amphora* sp. HN08	1192	1192	94	0	92.4
GQ330308.1	*Amphora salina*	298	298	20	2E-76	96.7
MH810165.1	*Halamphoracalidilacuna*	287	364	27	5E-73	95.6
GQ330309.1	*Amphora* sp. CCMP1405	265	265	19	2E-66	94.3
MF159100.1	*Phaeodactylumtricornutum*	257	257	18	4E-64	95.1

The sequence of the diatom ST02 sequence was then compared with the sequence in GeneBank using BLAST. The comparison was performed by aligning with six sequences *Amphora* sp., one sequence *Amphora salina*, one sequence *Halamphora caldicuna*, and one sequence as *Phaeodactylum tricornotum* in GeneBank. Of the nine sequences, the highest similarity value produced by *Amphora* sp. MACC 9 isolates is from the west coast of India with similiarity value of 94.93% (Table 2). Then a phylogenetic tree was formed using Mega X software with Neighbor-joining (NJ), Jukes-Cantor model substitution, and Bootstrap 1000 value of as shown in Fig. 3.

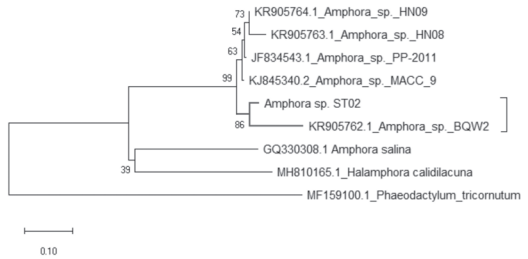

Fig. 3. *Neighbor-joining* (NJ) phylogenetic tree between *Amphora* sp. and the other related taxa in GenBank database with 1000 bootstrap values. *Phaedactylum tricornutum* is considered *outgroup.*

The phylogenetic tree reconstruction using ITS markings shows two large groups of phylogenetic branches. The first group with 99%, and the second group with 44% (Fig. 3). Diatom ST02 in this study has genetic proximity to *Amphora* sp. BQW2 with a bootstrap value of 85% (red line), which means it has very good data accuracy. Based on these results, diatom ST 02 was identified as *Amphora* sp. and named *Amphora* sp. ST02 (according to isolate code).

Characteristics of Nutritional Content

The nutritional content of *Amphora* sp. ST02 has a reasonably good value (Fig. 4). The highest value of nutritional content is ash (51.51%), then water content (17.94%), crude fiber (14.7%), protein (13.66%), carbohydrates (14.2%), and fat (0.81%). The protein value of *Amphora* sp. ST02 (13.66%), lower than some strains and other research results such as: CTM 20023 (54%) (Chtourou *et al.*, 2015), *Amphora* sp. (43.8%) (Khatoon *et al.*, 2009), *Amphora* sp. (27.62%) (Boukhris *et al.*, 2017). The protein content is almost the same as *Amphora coffeaformis* (15.6%) (Lee *et al.*, 2009), (15.74%) (El-Sayed *et al.*, 2018), and

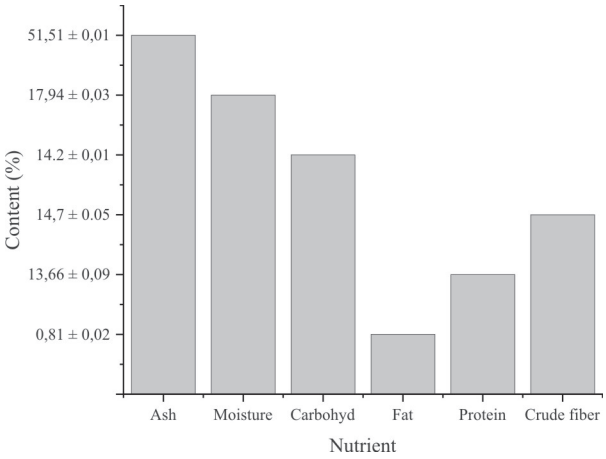

Fig. 4. Proximate composition of *Amphora* sp. ST02

is higher than *Amphora* sp. from Indonesian waters studied by Fahruddin *et al.* (2012) (8.18%). With a higher protein value, the utilization of *Amphora* sp. ST02 will have a better effect, in its use as a natural feed of Abalone (Sujarwani *et al.*, 2016; Khotimah *et al.*, 2018) and postlarva supplement *Penaeus monodon* (Khatoon *et al.* 2009).

Conclusion

Based on morphological and molecular identification results, the diatom ST02 was identified *Amphora* sp. This strain has the potential to be developed in the field of aquaculture and health with fairly good nutritional value.

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