Molecular Identification of the Brown Algae *Sargassum* sp. from the Lombok Coastal Waters

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Abstract

Sargassum is a well-known genus of brown algae in Indonesia that has long been investigated due to its economic importance. To support its biological research, it is important to correctly identify and classify the species studied. Whilst morphological identification has been greatly contributed to taxonomy, it cannot distinguish Sargassum species accurately due to its plasticity. Therefore this current study aims to apply molecular-based identification to Sargassum collected from Lombok coastal waters using an ITS2 DNA barcode. Fresh algae were gathered from Ekas Bay (EB) and Aan Cape (AC) in Lombok. DNA was first purified, and then its gene product was amplified using ITS2 primers. DNA sequences were examined and traced using the Basic Local Alignment Search Tool (BLAST). DNA sequences were processed in MEGA-X to reconstruct the phylogenetic tree and to estimate the genetic distance. Three species were successfully identified to be *Sargassum cf granuliferum*, *Sargassum polycystum,* and *Sargassum oligocystum*. The base length obtained ranged from 521 to 637 bp, with a similarity percentage of 99.25 to 100%. The phylogenetic tree exhibited each recognized Sargassum species was clustered with the same species from the GenBank. Interspecies genetic distance was 0,000-0,0039, while amongst Sargassum species it ranged from 0,0136-0,2395. The genetic distance between Sargassum and *Ulva adherens* (outgroup) was >1. Sargassum species found in Lombok were closely related to Sargassum cf. granuliferum isolate ZT050, *Sargassum polycystum* isolate ZT058 and *Sargassum oligocystum* isolate BTM036 from the southern shores of mainland Singapore, the Southern Island of Singapore and Hainan, China, respectively.

Keywords: Sargassum, ITS2, DNA Barcoding, Identification molecular, Brown algae

INTRODUCTION

Marine macroalgae are benthic plants that inhabit coastal waters albeit not the main source of primary productivity they are ecologically and economically important. Brown algae, particularly the genus Sargassum, are ubiquitous in coastal waters, particularly in Indonesia (Widyaswari *et al*., 2021). Atmadja and Prudhomme van Reine (2014) discovered 150 species of brown algae in Indonesian seas, of which Sargassum is the most prominent genus with 57 species.

Sargassum thrives as floating beds in the ocean or along the coast with coral reef bottoms and strong tides in tropical and subtropical oceans (Rosado-Espinosa *et al*., 2020). . The distribution is in the intertidal zone, upper littoral zone, and sublittoral zone (Achmadi & Arisandi, 2021). Some live attached to rocks or other hard objects, while others are attached to the seafloor by a root-like structure known as a holdfast (Kumalasari *et al*., 2018). For the best growth when cultured, water depth should be between 0.5 to 10 meters (Muslimin & Sari, 2018). The appropriate water temperature is 25-35 °C with a salinity of 32 - 33.5 ‰ (Fajri, 2020). According to Setyawidati *et al*., (2018) Sargassum biomass reaches its peak in May and June during the dry season. Nutrients, substrate, quantity, quality of light, pH, salinity, temperature, and hydrodynamics are the most essential factors influencing the growth of these algae (Puspita *et al*., 2020).

Several studies have been conducted on the economic significance of Sargassum such as for medical (Kurnialahi *et al*., 2020), functional food (Husni *et al*., 2019), agriculture (Ghazali *et al*., 2019), and aquaculture purposes (Hulpa *et al*., 2021).

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Each study requires identifying and classifying the species. Classical taxonomy has made significant contributions to the classification of species based on morphological features; nevertheless, traditional taxonomy is unable to correctly distinguish all species, notably closely related ones (Wang *et al*., 2020). In particular, a wide range of morphological variations in the Sargassum causes confusion in classification issues (Widyartini *et al*., 2017). Molecular identification of Sargassum is therefore urgent to resolve morphological plasticity cause the environmental factors (Kantachumpoo *et al*., 2015).

Molecular identification of algae is accomplished by recognizing the nucleotide base sequence of algae using a marker gene (primer). Brown alga Sargassum was identified using an ITS2 primer for molecular identification. This primer is commonly used to determine brown algae, according to Yoshida *et al*., (2000) and Kantachumpoo *et al*., (2015). ITS2 is appropriate for cryptogamae plants (Schultz *et al*., 2006), simple to amplify, and has sufficient variability to distinguish closely related species (Tan *et al*., 2020; Yao *et al*., 2010). The identification process was initiated by extracting DNA to obtain a DNA template. Ecological circumstances determine sargassum's morphology variations, possibly causing polymorphisms within and between species. Polymorph species are influenced by genetic and environmental variables (Widyartini *et al*., 2017), hence it needs a more accurate technique.

DNA taxonomy was initiated by Deák, (1999). Thereafter, Hebert *et al*. (2003) proposed the concept of DNA barcoding. DNA barcoding on Sargassum is required to avoid misidentification because of its polymorphism and cryptic species. This work attempts to employ DNA barcoding to identify phylogenetic relationships of Sargassum from Lombok coasts, where morphological identification is sometimes less accurate. DNA analysis was established by building a phylogenetic tree using aligned nucleotides and GenBank data and then estimating the genetic distance of each sample. The phylogenetic tree in this research was built using the Neighbor-Joining (NJ) class category technique with 1000 Bootstraps. The Bootstrap number reflects MEGA-tree-reconstruction repetitions. Neighbour-Joining Tree can investigate genetic distances between species based on the nitrogen base shifts (Nuha *et al*., 2020). In reconstructing the phylogenetic tree, it was required to outgroup the species.

MATERIALS AND METHODS

Samples of algae were collected either through snorkelling or manually collecting them directly during low tide. Sargassum species identification used molecular techniques following the Kantachumpoo method (Kantachumpoo *et al*., 2015).

DNA Extraction and Purification

Sargassum DNA was extracted, purified and amplified using the Phire Plant Direct PCR Master Mix Kit. The Implementation procedures followed the kit instructions. The 2 mm^2 of fresh algae filoid were placed in a PCR tube containing 20 µl of dilution buffer. Algae filoid was crushed with the micropipette tip until the solution changed in color. The solution was centrifuged for 5 minutes at 9500 rpm. The supernatant (0,5 µl) was transferred to 20 µl of PCR reaction as a DNA template. The employed PCR reaction formula can be found in Table 1.

Table 1. PCR Reaction Formula

Components	Volume (µl)
2X Phire Plant Direct PCR Master Mix	10
Primer Forward	0.5
Primer Reverse	0.5
Supernatant from the dilution buffer	0.5
H_2O	8.5

Table 2. The PCR cycle for Sargassum sp.

Phase	Temperature (°C)	Time	Cycle
Initial denaturation	98	5 minutes	1
Denaturation	98	5 seconds	40
Annealing	61.9	5 seconds	
Extension	72	20 seconds	
Final	72	1 minute	1
Extension	4		1

The primer used in this process is Internal Transcripted Spacer 2 (ITS2), which is 5.8S BF (5'CGATGAAGAACGCAGCGAAATGCGAT-3') and 25BR2 (5'-TCCTCCGCTTAG TATA TGCTTAA) (Kantachumpoo *et al.,* 2015), produced by PT Genetika Science, Jakarta, Indonesia. The DNA was amplified by means of a thermal cycler applying the cycle protocols provided in Table 2 with annealing temperature justification.

Thereafter, electrophoresis was carried out to ensure that the amplification had been successful. Agarose gel (1%) was prepared by dissolving 0.3 g of agarose powder in 30 ml of 10x Tris Borate EDTA (TBE) buffer, then heated. The agarose was allowed to harden. Electrophoresis was performed by pipetting 5 µl of DNA (PCR product) and 100bp ladder DNA marker as a molecular weight marker into each well. After the 1x TBE buffer solution was electrified (80 volts, 30 minutes), the gel was carefully soaked in Ethidium bromide (EtBr) for 20 minutes and then rinsed with 10x TBE buffer. The gel was observed under Ultra Violet (UV) light (gel imaging).

DNA *sequencing*

DNA sequencing's goal was to determine the sequence of nucleotide bases from the DNA amplification product. This process was conducted by PT Genetika Science, Jakarta, Indonesia. The pure DNA was traced and analyzed automatically (ABI 3130XL, Applied Biosystem). The DNA sequences were compared with The National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) database tracking tool.

RESULT AND DISCUSSION

Sargassum sampling was performed in Ekas Bay and Aan Cape of Lombok coastal waters. Four species of Sargassum have been collected from those locations (Figures 1). The whole, fresh Sargassum was frozen at -20°C for further analysis.

PCR Amplification of ITS2 rDNA

The DNA template with forward and reverse primers of ITS2 were amplified using PCR. The range for amplified DNA fragments of Sargassum studied was between 500 and 1000 base pairs (bp). The quality of PCR output was then checked under UV light (Figure 2). ITS2 is called a mini barcode due to its small length target base pair (about 700 bp) (Arulandhu *et al*., 2019). ITS2 DNA barcoding identifies short region genes with efficient DNA barcodes, 700 base pairs in one partial gene region. (Ballesteros *et al*., 2021).

DNA Sequencing

According to the results of the forward and reverse primers' base alignment, the base length obtained was between 521 and 637 bp (Table 3).

Sequencing data were processed using MEGA-X 10.1.8. This program analyzes molecular sequences by cutting, aligning and computing genetic distances. Sequence alignment and phylogenetic tree results are depicted (Kumar *et al*., 2018). The sequence alignment results were

submitted to BLAST to compare the sequence outputs studied with BLAST database sequences. BLAST is a breakthrough tool for internet-based sequence analysis (Ren *et al*., 2019). The determining factor of species identification is similarity percentage and the acceptable similarity percentage is more than 99% (Normand *et al*., 2018). Based on the BLAST data, three species of Sargassum were identified as *Sargassum cf. granuliferum*, *Sargassum polycystum,* and *Sargassum oligocystum*, with a similarity percentage of 99.25-100% to *S. polycystum* are closely related to Sargassum cf. granuliferum isolate ZT050, *Sargassum polycystum* isolate ZT058 and *Sargassum oligocystum* isolate BTM036 from the southern shores of mainland Singapore, the Southern Island of Singapore (Yip *et al* 2018) and Hainan, China.

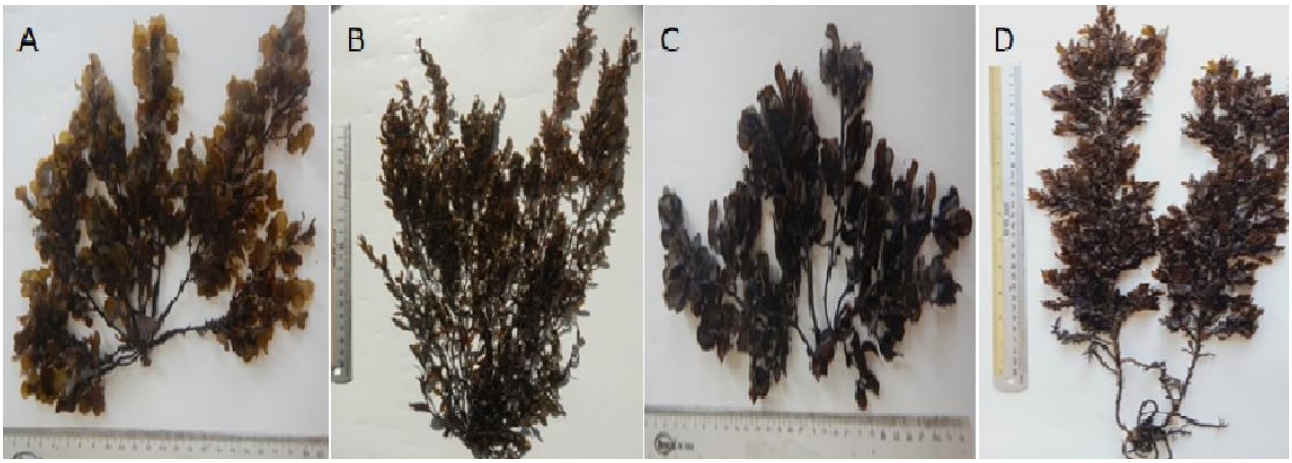

Figure 1. Sargassum found in Lombok coastal waters (Sulistiyani *et al*., 2021)
(Note: Species A and B sampled from Akas Bay; Species C and D sampled from Aan Cape of Lombok coastal waters)

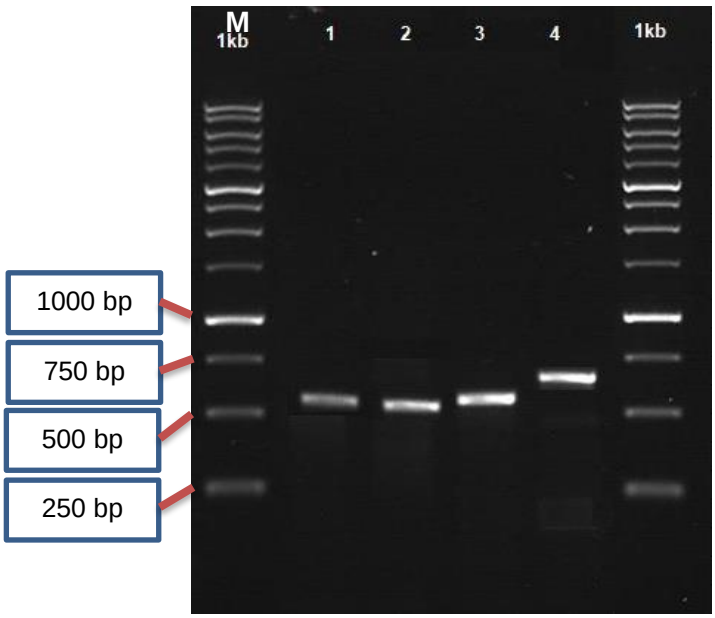

Figure 2. Gel Imaging of Electrophoresis
(Note: M: DNA ladder, 1: Species A, 2: Species B , 3: Species C, 2: Species D)

Tabel 3. BLAST result of Sargassum

Species	Location	Species identified	Accession Number	Sequence Length (bp)	Ident (%)	Query Cover (%)
1	Ekas Bay	*Sargassum cf. granuliferum*	OL339345	546	99.63	99
2		*Sargassum polycystum*	OL339346	521	99.62	100
3	Aan Cape	*Sargassum polycystum:*	OL339347	536	99.25	99
4		*Sargassum oligocystum*	OL339348	637	99.37	99

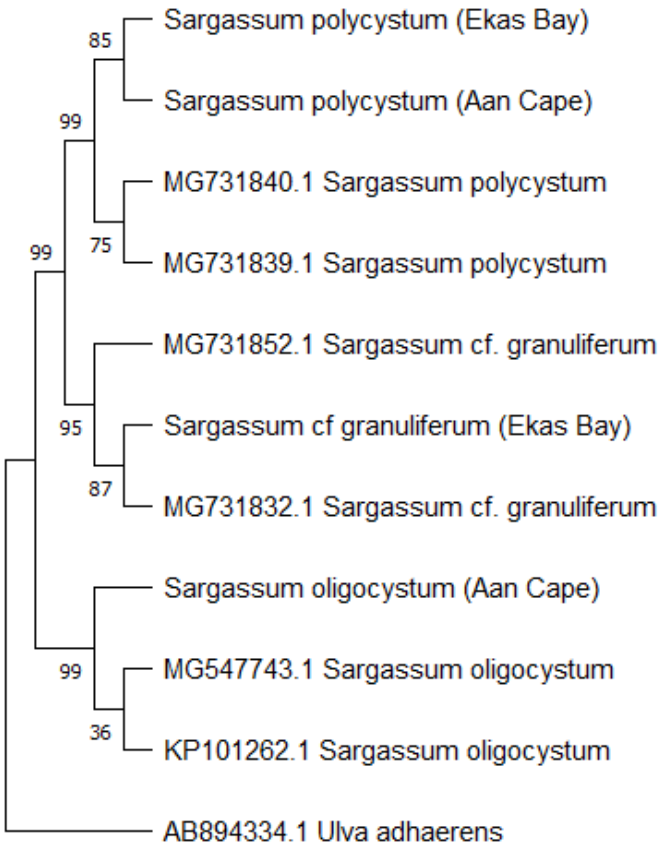

Figure 3. Phylogenetic Tree of identified *Sargassum* sp.

Phylogenetic Tree

The phylogenetic tree demonstrated that each identified Sargassum species was clustered on one division with the same species of the GenBank (Figure 3). The reconstruction of the Sargassum phylogenetic tree was designed to identify the taxonomy of algae-based on DNA sequencing. This reconstruction was accomplished by adjusting sequencing data using MEGA X . Alignment utilized ClustalW to study nucleotide diversity (Kumar *et al*., 2018). Species from the class Ulvophyceae (*Ulva adhaerens*) were chosen as an outgroup for comparison.

The results of the phylogenetic analysis shown in Figure 3 revealed that the same species of Sargassum referred to the same clade, but if the species were distinct, the clade would be different. In this study, *S. cf. granuliferum* is in the same clade as *S. cf. granuliferum* with the access codes MG.731852.1 and MG.731832.1. Both *S. polycystum* investigated belonged to the same clade as *S. polycystum* MG731840.1 and MG73189.1. A similar pattern was also found in *S. oligocystum* species,

Table 4. The Genetic distance of the identified Sargassum

Algae Species	1	2	3	4	5	6	7	8	9	10	11
*S._cf_granuliferum_*(EB)											
*S. polycystum_*(EB)	0.0137										
*S. polycystum_*(AC)	0.0137	0.0020									
*S. oligocystum_*(AC)	0.2313	0.2138	0.2202								
MG731852.1_*S. cf._granuliferum*	0.0020	0.0159	0.0159	0.2031							
MG731832.1_*S. cf. granuliferum*	0.0000	0.0137	0.0137	0.2248	0.0020						
MG731840.1_*S. polycystum*	0.0136	0.0039	0.0039	0.2165	0.0119	0.0117					
MG731839.1_*S. polycystum*	0.0136	0.0039	0.0039	0.2165	0.0119	0.0117	0.0000				
MG547743.1_*S. oligocystum*	0.2395	0.2217	0.2283	0.0020	0.2107	0.2328	0.2244	0.2244			
KP101262.1_*S. oligocystum*	0.2313	0.2138	0.2202	0.0000	0.2031	0.2248	0.2165	0.2165	0.0020		
AB894334.1_*Ulva_adhaeren*	1.3844	1.3871	1.3977	1.3489	1.3399	1.3863	1.3799	1.3799	1.3653	1.3482	-

the species studied by reference (MG547743.1 and KP101262.1) were in the same clade. So, it is suggested that each species is monophyletic. When the same species are in the same branching, it can be assumed that they shared a common ancestor. According to Paterson *et al*. (2020), species from the same clade are monophyletic.

Outgroup species do not belong to the same clade as other Sargassum species, according to the phylogenetic tree. There is no correlation in the clade between the ingroup and outgroup. This suggests that Sargassum is a homoplasy with the outgroup species, which have quite distinct morphologies. Caetano-Anollés *et al*. (2018) explained this theory by implying that homoplasy is highly probable if the phylogenetic distance between ingroup and outgroup taxa is large.

The genetic distance data substantially supported the phylogenetic tree data (Table 4). Since the genetic distance between monophyletic species was small, it is assumed that their genetic relationship was significant. In *S. cf. granuliferum*, the distance was between 0.000 and 0.0020, while it was between 0.0020 and 0.0039 in *S. polycystum* and between 0.000 and 0.0020 in *S. oligocystum*. Different species had different ranges of genetic distance amongst themselves. In the correlation between *S. cf. granuliferum* and *S. polycystum*, the genetic distance between the two species was close (0.0136–0.2202), whereas the range with *S. oligocystum* was greater (0.02031–0.2395). Then, comparing the genetic distance between the ingroup and the outgroup, the species *U. adhaerens* with *S. cf. granuliferum* at 1.3844, with *S. polycystum* at 1.3871, and with *S. oligocystum* at 1.3489. Genetic distances between ingroup and outgroup were greater than 1, indicating genetic differences.

The small genetic difference between the same species suggests that they are closely related, hence they are presumably the same species (Tallei *et al*., 2017). Meanwhile, the genetic distance between the Algae genus was greater than between species. Genetic distance will be larger in the range when compared to outgroup species from other classes. This phenomenon was thought to be due to differences between species and even between classes. This trend was described by Tallei *et al*., (2017), stating that species with large genetic distances are assumed to be distinct species and even genera.

CONCLUSION

Molecular identification of Sargassum using DNA Barcode ITS2 demonstrated that the brown algal Sargassum of Lombok coastal waters is closely related to Sargassum cf. granuliferum isolate ZT050, *Sargassum polycystum* isolate ZT058, and *Sargassum oligocystum* isolate BTM036 from the

southern shores of mainland Singapore, the Southern Island of Singapore and Hainan, China, respectively.

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