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The diversity of *Sargassum* spp. from the south coast of Yogyakarta, Indonesia, based on morphological characters and DNA Barcoding ITS2 nrDNA

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Abstract. *Saraswati M, Adharini RI, Hardianto E. 2024. The diversity of* Sargassum *spp. from the south coast of Yogyakarta, Indonesia, based on morphological characters and DNA Barcoding ITS2 nrDNA. Biodiversitas 25: 2733-2739.* The genus *Sargassum* grows abundantly on the south coast of Yogyakarta, but diversity of species in the genus has not been reported. This research aimed to determine the diversity of the genus *Sargassum* based on morphological characters and molecular analysis. Several samples were collected from two beaches on the south coast of Yogyakarta, namely Trenggole and Wediombo Beach. After observing the morphological characters, five individuals that have morphological differences were found and continued for molecular identification. The morphological analysis showed a characteristic of greenish-brown color, clear phylloids with serrated edges, and rounded vesicles. The results of DNA barcoding identification of *Sargassum* in Trenggole Beach were highly similar at 99.67% to *Sargassum aquifolium* (Turner) C.Agardh (MG731834.1), 99.68% to *Sargassum yinggehaiense* Tseng and Lu (KP101256.1), and 98.99% to *Sargassum polycystum* C.Agardh (MG731836.1). Whereas at Wediombo Beach were highly similar at 99.68% to *S. yinggehaiense* (KP101256.1) and 99.67% to *S. aquifolium* (MG731834.1). Based on phylogenetic analysis, the species were separated impacting the biodiversity of the genus *Sargassum* in the south coast of Yogyakarta, Indonesia. Environmental factors might influence the morphotype, biodiversity, genetic diversity, and distribution of *Sargassum* spp. The results described herein provide a foundation for developing better conservation strategies for the target species in the future.

Keywords: Diversity, molecular, morphology, phylogenetic, *Sargassum*, south coast of Yogyakarta

INTRODUCTION

The genus *Sargassum* also known as brown algae is widely distributed area and has a high diversity level (Van Tu 2023). The genus is reported to be found in the Indian, Pacific, and Atlantic Oceans, as well as in tropical, subtropical, and temperate climates (Yip et al. 2020). *Sargassum* was stated to be a key species for the coral reef ecosystem (Yip et al. 2018) and has dominant habitats in intertidal and subtidal areas that are still influenced by tides and have currents and waves with depths of up to 10 m (Groisillier et al. 2014; Teagle et al. 2017). Several previous research reported the identification of brown algae in wide locations, e.g., *Sargassum muticum* (Yendo) Fensholt found in Europe (Tanniou et al. 2014), *Sargassum aquifolium* (Turner) C.Agardh, *Sargassum granuliferum* C.Agardh*;*, *Sargassum polycystum* C.Agardh*;*, and *Sargassum siliquosum* J.Agardh in Singapore (Low and Chou 2013), *Sargassum xochitlae* in Mexican coastal waters (Gonzáles-Nieto et al. 2020), and some various species from East Asia (Cho et al. 2012; Van Tu 2020). Meanwhile, in Indonesia especially in the south coastal waters of Yogyakarta, morphological identification of several species from the genus *Sargassum* was reported e.g., *Sargassum duplicatum* J.Agardh, *Sargassum echinocarpum* J.Agardh, *Sargassum binderi* Sonder, and *Sargassum cinereum* J.Agardh (Sodiq and Arisandi 2020). However, there have been no reports regarding the identification of brown algae diversity using molecular analysis at this location.

Morphological variations in the genus *Sargassum* are wide, so that the boundaries of species and varieties are still confusing and cause difficulties in their taxonomy and classification (Widyartini et al. 2017). Habitat, season, and differences at the population level influence the phenotypic variation (Gonzáles-Nieto et al. 2020). Furthermore, morphological plasticity also caused difficulties in identifying *Sargassum* spp. (Yap-Dejeto et al. 2022). Thus, determining the genetic characteristics of algae can be done through DNA molecular analysis. DNA-based molecular identification shows sufficient information to provide taxonomic identifications and algae diversity (Huang et al. 2017).

DNA barcoding and phylogenetic analysis is one of the genetic tools that are very useful in identifying into species level. These methods provide an efficient identify existing species and discover unknown species with the help of analysis of sequence variation in a conserved domain region of DNA (Castaneda et al. 2023; Hardianto and Satriyo 2023). Previous research on DNA barcodes combined with phylogenetic analysis has been conducted on several aquatic species in crustacean (Irwani et al. 2020; Hanamura et al. 2024), shellfish (Castaneda et al. 2023; Hardianto and Satriyo 2023), fish (Nursalim et al. 2022) and also algae (Camacho et al. 2015; Sulistiyani et al.

2022; Sakti et al. 2024). DNA barcoding combined with phylogenetic analysis are promising benefits for species authentication lies in the ability to identify early stages that cannot be done by using morphological descriptions and interspecies connections.

In this research, we conducted Internal Transcribed Spacer 2 (ITS2) sequence data to identify genetic distances determine, species authentication, and analyze phylogenetic relationships of the target species. Identifying *Sargassum* is challenging because of its diversity due to plasticity morphology, so it is hard to distinguish (Chalini et al. 2021). Molecular research based on DNA barcoding is essential to avoid misunderstanding the morphological identification of species diversity and obtain more precise and accurate results. Therefore, this research aimed to determine the diversity of *Sargassum* spp. based on morphological characters and DNA barcoding in the south coast of Yogyakarta as a basis for its sustainable management and utilization.

MATERIALS AND METHODS

Sampling site and material collections

Sampling was conducted from September to October 2021 in the south coast of Yogyakarta, Indonesia (Figure 1). Sampling was carried out by walking along the coastline and *Sargassum* spp. found were put in a plastic ziplock and labeled using a permanent marker. The labeled sample was put into a cooling box before being tested at the laboratory of Aquatic Resources Management, Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta.

Samples of *Sargassum* spp. were collected from two locations, Wediombo beach which represents the western coastal area, and Trenggole beach which represents the eastern coastal area. Data related to the number of samples studied and accession numbers from the DNA Data Bank of Japan are presented in Table 1. We took several samples of brown algae from the target location and after selection, we choose five samples of *Sargassum* spp. for morphological and molecular analysis.

Procedures

Morphological characters observation

Sargassum spp. samples collected from Trenggole and Wediombo Beaches were grouped based on their morphological appearances. Furthermore, each group was observed for its morphological characteristics, including stipe, phylloid, and air bladders. The specimens then were made dry herbaria, then deposited at the laboratory.

Table 1. Details of two sampling sites, number of samples collected for DNA analysis, and accession number for ITS2 nrDNA sequence

Location site	Number of samples	Collecting date	Sample code	DDBJ Accession Number
Trenggole, Gunung Kidul District, Yogyakarta	3	September 2021	T1	LC836050
			T2	LC836051
			T3	LC836052
Wediombo Gunung Kidul District, Yogyakarta	2	October 2021	W1	LC836053
			W2	LC836054

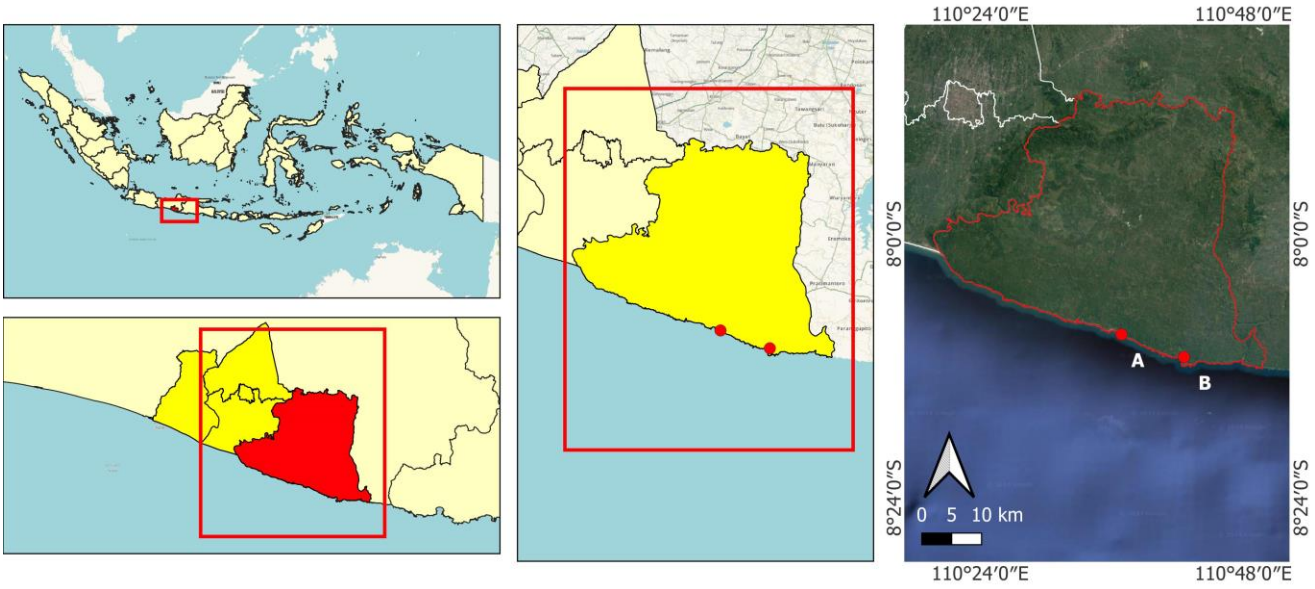

Figure 1. Sampling location of *Sargassum* spp. from Trenggole and Wediombo Beaches, south coast of Gunung Kidul, Yogyakarta, Indonesia. A. Trenggole; B. Wediombo

DNA extraction

Approximately 10 mg of *Sargassum* was collected and ground with a mortar until smooth. The DNA extraction process followed the procedure in the genomic DNA Mini Kit (Plant) Protocol from Genaeid. DNA extraction quality checking was used electrophoresis using 1% agarose gel for 20 minutes, and the results were photographed on a Benchtop UV Transilluminator to determine the presence or absence of isolated DNA.

DNA amplification and sequencing

The amplification of ITS2 was performed using specific primers 5.8S-BF (forward) (5'-CGATGAAGAACGCAG CGAAATGCGAT-3') and 25BR-2 (reverse) (5'-TCCTCCCGCTTAGTATATGCTTAA-3') (Mattio et al. 2008). Polymerase chain reaction (PCR) were performed in a total volume of 50 μL containing 25 μL PCR mix (Geneaid), 2 μL each primer forward and reverse which are the concentration of each primer are 10 pmol, 2 μL of DNA, and 19 μL aquabides (Prasetyo and Kusumaningrum 2014). The reactions were placed in a PCR Thermocycler T100™ Bio-Rad (Germany) and performed in the following PCR setting: initial denaturation process at 95°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 40 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 45 seconds, and final extension at 72°C for 7 minutes. The sequencing process was then carried out by sending PCR results to the PT. Genetika Science Indonesia.

Data analysis

Morphological analysis was carried out by comparing the characteristics of the sample obtained with https://www.algaebase.org. the morphological characteristics and suspected species are written and documented in a table and descriptively analyzed. Sequence data were aligned using muscle alignment software for the molecular data analysis using MEGA X (Kumar et al. 2018). Default alignment parameters and the sequences were adjusted manually to avoid mismatches. The sequences were analyzed for their identity using BLAST (Basic Local Alignment Search Tool) in NCBI (National Center for Biotechnology Information). We also aligned sequence data to calculate Kimura's two-parameter (K2P) distance with 1,000 bootstrap replications, to analyze the pairwise genetic difference, nucleotide composition and to construct phylogenetic tree by the Neighbor Joining (NJ) methods. Haplotype network analysis among species was constructed by the analysis of minimum spanning network (MSN) using PopART (Leight and Bryant 2015).

RESULTS AND DISCUSSION

Morphological characters

Three types of *Sargassum* from Trenggole Beach with different morphological characteristics and two types of *Sargassum* from Trenggole Beach were identified (Table 2). However, there are slight similarities in morphological characteristics in samples T1 and W2, which have cylindrical stipe, alternate branch type, oval and wide phylloid, jagged edges phylloid, and air bladder. Sample T2 has a long and cylindrical stub, an alternate branch type, jagged edges, and pointed ends of the phylloid. In comparison, sample T3 has a phylloid-like trumpet as the main characteristic. The W2 sample has a cylindrical stipe, dichotomous branch type, oval and wide phylloid, jagged and wavy edges of the phylloid, and air bladder.

DNA barcoding and haplotype network

The results of electrophoresis from the five samples from Trenggole Beach and Wediombo Beach using a pair of ITS2 primers are shown in Figure 2. The electrophoresis results of samples W1, W2, T1, and T2 in UV transilluminator tools all showed a size of approximately more than 600 bp. However, only T3 showed a size of less than 600 bp.

The result of molecular identification using DNA barcoding methods of *Sargassum* spp. showed that samples T1 and W2 were identified as *S. aquifolium* with a query cover of 99% with percentage identity of 99.67%. Samples of T2 and W1 were identified as *S. yinggehaiense* with a query cover of 100% with percentage identity of 99.68%. Meanwhile, sample of T3 identified as *S. polycystum* with a query cover of 99% with percentage identity of 99.68% (Table 3).

We also carried out haplotype network analysis using a minimum spanning network (MST) (Figure 3) and genetic distance analysis using the Kimura 2-parameter and 1000 bootstraps (Table 4). The results show that each sample that we molecularly identified showed quite significant differences in haplotype network and genetic distance. This indicates that our species identification shows results that are quite good in accordance with and strengthen the results of other analyses.

Phylogenetic analysis

The genetic relationship among *Sargassum* species was analyzed using MEGA-X software with the Neighbour-joining Tree method. Phylogenetic analysis of the collected samples was compared with *Sargassum* spp from other countries based on the Genbank database (Figure 4).

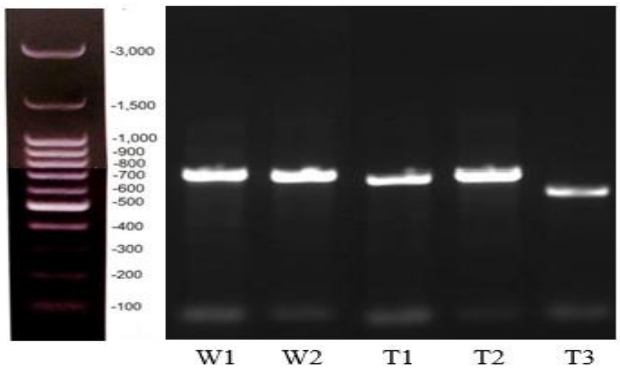

Figure 2. The results of the electrophoresis visualization of the amplification (PCR) of *Sargassum* using ITS2 primers at Trenggole and Wediombo Beaches, Yogyakarta, Indonesia

Table 2. Morphological characters of *Sargassum* spp. from Trenggole and Wediombo Beaches, Yogyakarta, Indonesia

Thallus	Phylloid	Sample code	Sample Origin (beach)	Morphological Characters
		T1	Trenggole	Cylindrical stipe, alternate branch type, oval and wide phylloid, jagged edges phylloid, phylloid start from stipe base, and has an air bladder.
		T2	Trenggole	Long and cylindrical stipe, alternate branch type, jagged edges, and pointed ends of phyloid.
		T3	Trenggole	Cylindrical stipe, dichotomous branch type, and phylloid like a trumpet.
		W1	Wediombo	Cylindrical stipe, dichotomous branch type, oval and wide phylloid, jagged and wavy edges of phyloids, has air bladder.
		W2	Wediombo	Cylindrical stipe, alternate branch type, oval and wide phylloid, jagged edges of phylloid, and has air bladder.

Table 3. Molecular Identification using DNA barcoding of *Sargassum* spp. from south coast of Yogyakarta

Sample code	Identified species	Query cover (%)	Percent identity (%)	Accession number	Origin
T1	*Sargassum aquifolium* (Turner) C.Agardh	99	99.67	MG731834.1	Singapore
T2	*Sargassum yinggehaiense* Tseng and Lu	100	99.68	KP101256.1	China
T3	*Sargassum polycystum* C.Agardh	99	98.99	MG731836.1	Singapore
W1	*Sargassum yinggehaiense* Tseng and Lu	100	99.68	KP101256.1	China
W2	*Sargassum aquifolium* (Turner) C.Agardh	99	99.67	MG731834.1	Singapore

Sargassum aquifolium from Wediombo and Trenggole beaches is closely related to *S. aquifolium* from New Caledonia, Singapore, Vanuatu, and The Solomon, but closely related to *S. aquifolium* from New Zealand. *S. polycystum* from Trenggole is closely related to *S. polycystum* from China, Singapore, France, Vanuatu, and Fiji. *S. yinggehaiense* from Wediombo and Trenggole beaches are closely related to each other and closely related to *S. cristaefolium* from Taiwan and Iran and *S. duplicatum* from Japan.

Discussion

The morphological characteristics of all samples revealed many similarities, leading to the identification of samples T1 and W2 as *S. aquifolium*. Several morphological similarities between samples T1 and W2 include having a 30 cm long thallus, dominant brown pigment, and regularly alternating branches. Apart from that, the leaves from samples T1 and W2 are broadly oval in shape, the edges of the leaves are serrated with slightly tapered tips, and the air sacs are round to oval in shape. Nguyen and Boo (2020) and Van Tu (2020) examined the morphological characteristics of brown algae and their distribution in Vietnam, have confirmed the morphological characteristics of the species *S. aquifolium*. Meanwhile, samples T2 and W1 showed significant differences in morphological characters compared to other samples. We identified samples T2 and W1 as *S. yinggehaiense* based on their morphological characteristics. The species has a yellowish-brown stem-shaped thallus, spiny leaf tips, and a disc-shaped handle (Widyartini et al. 2017). In contrast to the others, sample T3 was morphologically identified as *S. polycystum* with small, short, trumpet-like, and phylloid-like stems as its main characteristics (Mattio et al. 2015; Van Tu 2020).

Brown algae has a very wide distribution, because it has a high tolerance to various environmental conditions, such as temperature, salinity, and radiation (Zou et al. 2018). In addition, *Sargassum* has more diverse species than other algae species, and the morphology between species is very similar to each other, so it is difficult to differentiate species based on their morphology (Prabha et al. 2012). The high morphological similarity, even though molecularly it shows different species, shows that *Sargassum* has high plasticity. Therefore, environmental conditions influence its morphological form (Prabha et al. 2012). Therefore, we need to use both morphological and molecular character approaches to confirm the species differences.

Table 4. Pairwise genetic distances of *Sargassum* spp. in each location based on K2P defined in this study

Specimen Code-Species	Genetic Distances				
	T1	T2	T3	W1	W2
T1- *S. aquifolium*	0.00				
T2-*S. yinggehaiense*	0.66	0.00			
T3-*S. polycystum*	0.04	0.67	0.00		
W1-*S. yinggehaiense*	0.66	0.00	0.67	0.00	
W2-*S. aquifolium*	0.00	0.66	0.04	0.66	0.00

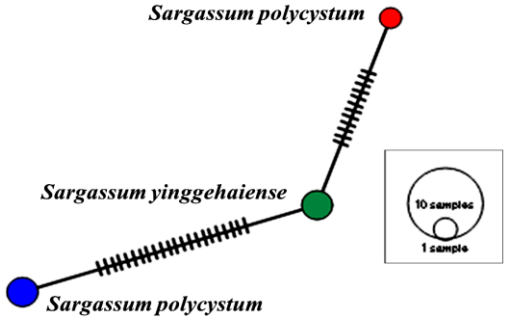

Figure 3. The results of haplotype network analysis using ITS2 primers at Trenggole and Wediombo Beaches, Yogyakarta, Indonesia

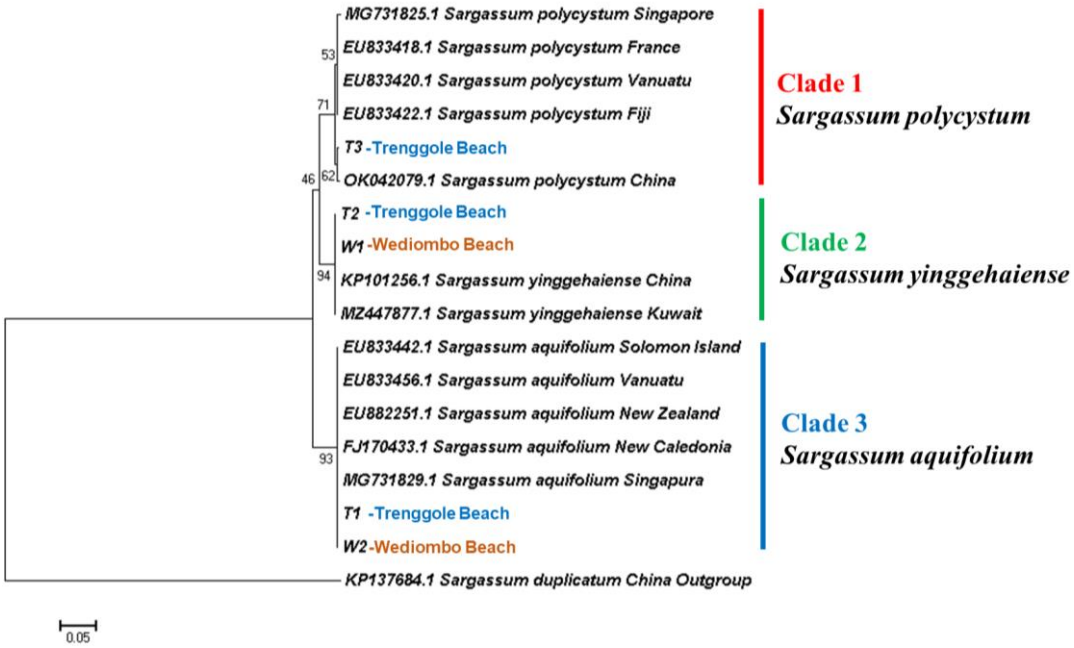

Figure 4. Phylogenetic tree of *Sargassum* spp. compared to the GenBank database using Neighbor-joining (NJ) with Bootstrap 1000×

These molecular identification results confirm the validity of previous morphological identification results. The overall molecular analysis led to the identification of samples T1 and W2 as *S. aquifolium*, T2 and W1 as *S. yinggehaiense*, and T3 as *S. polycystum*. The results of BLAST analysis show that the nucleotide sequences of the five samples observed show identification that is identical to the gene bank sequence data (Table 3). Samples T1 and W2 showed a percent identity of 99.67% with a query cover of 99% matching *S. aquifolium* from Singapore (MG731834.1). Samples T2 and W1 showed a 99.68% percent identity, with a query cover 100% matching *S. yinggehaiense* from China (KP101256.1). Meanwhile, sample T3 showed a percent identity of 98.99% with a query cover of 99%, which matched *S. polycystum* from Singapore (MG731836.1). The species level uses sequence similarity higher than 97% as an authentication criterion, while the genus level uses similarity lower than that for identification. These results show that species differentiation using DNA barcoding is very accurate. Researchers have proven that DNA barcoding successfully identifies various individuals at the species level, including macroalgae (Camacho et al. 2015; Thu et al. 2019). Meanwhile, ITS markers have been proven effective for identifying various species of macroalgae (Mattio 2008; Camacho et al. 2015; Sakti et al. 2024).

The genetic distance data substantially supports the DNA barcoding data (Table 4). We assume that the genetic relationship is significant because the genetic distance between species is quite high. *S. aquifolium* (T1 and W2) has a genetic distance of 0.66 to *S. yinggehaiense* (T2 and W1) and 0.04 to *S. polycystum* (T3). Meanwhile, the genetic distance between *S. yinggehaiense* (T2 and W1) and *S. polycystum* (sample T3) was 0.67. Hubert et al. (2014) stated that differences in genetic distance of less or equal to 3% indicate molecularly identical species. The smaller genetic distance between individuals in a population, the more uniform it is. Conversely, the greater an individual's genetic distance in a population, the more diverse the population will be. Similar to the genetic distance analysis, the network haplotype analysis showed less within-species spacing (Figure 3). This result shows that the nucleotide sequence variation between species in the genus *Sargassum* is quite high. Genetic distance shows the ratio of genetic differences between species or populations (Dogan and Dogan 2016). Therefore, a smaller genetic distance value makes the appearance of some ITS genes more difficult to differentiate between the two species (Sulistiyani et al. 2021).

The phylogenetic analysis confirmed the formation of three main clades, with each clade confirming a different species. *S. aquifolium* (T1 and W2) were in clade 3 along with other *S. aquifolium* samples from the gene bank (Singapore, New Caledonia, Vanuatu, The Solomons, and New Zealand). Gene bank data from China, Singapore, France, Fiji, and Vanuatu establish a relationship between *S. polycystum* (T3) in clade 1. This proves that *Sargassum* is very easy to spread in various habitats, even though the distance is quite long (Schell et al. 2024). Meanwhile, *S. yinggehaiense* (T2 and W1) showed in clade 2. Oceanographic factors such as temperature, salinity, dissolved oxygen, depth, and currents can influence the distribution pattern of *Sargassum* spp. The majority of species in the genus *Sargassum* will colonize larger areas both naturally and anthropogenically, spreading almost throughout the world (Stieger-Pouvreau et al. 2023).

In conclusion, based on morphological and molecular analysis, we found three species from the genus *Sargassum* on the south coast of Yogyakarta, namely *S. aquifolium, S. yinggehaiense*, and *S. polycystum.* The results of molecular and morphological analyses in this study complement each other, which strengthens the argument that morphological and molecular analyses are very important for identifying species. Apart from that, this research also confirms and strengthens the argument that the use of ITS markers is very suitable for identifying macroalgae. These results led to the decision to include programs in Indonesian research that identify phylogenetically linked haplotypes.

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