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Genetic Diversity of *Gracilaria* spp. in the Intertidal Zone on the South Coast of Yogyakarta, Indonesia Based on DNA Barcoding with *rbc*L Marker

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ABSTRACT

***Gracilaria* spp. is a commercial seaweed utilized in various food, pharmaceutical, and health industries. Due to the high plasticity of seaweed, morphological identification must be validated by molecular identification using DNA barcoding. This study aims to identify the genetic diversity of *Gracilaria* spp. based on DNA barcoding with an *rbc*L marker. Samples were collected from six beaches, i.e., Trenggole, Drini, Siung, Wediombo, Nguyahan, and Ngedan, from September-November 2021. The methods in this study were sampling, sample preservation, morphological and tissue observations, DNA extraction, PCR and electrophoresis, and data analysis. The results showed four species of seaweed based on the *rbc*L marker: *Gracilaria* sp., *G. salicornia*, *G. edulis*, and *G. vieillardii*. Based on 18 samples confirmed by phylogenetics, five different species were found, namely *G. salicornia*, *G. edulis*, *G. vieillardii*, *Gracilaria* sp. 1, and *Gracilaria* sp. 2 The last two species, i.e., *Gracilaria* sp. 1 and *Gracilaria* sp. 2, from the intertidal zone of the south coast of Yogyakarta may have never been reported to GenBank.**

1. Introduction

Gracilariaceae belongs to Rhodophyta, which has seven genera and 236 species (Guiry and Guiry 2022). *Gracilaria* spp. contains agar with high economic potential because it is widely used for various industrial needs (Santelices 2014). However, identifying these seaweed species is often misunderstood due to the large species diversity, high morphological plasticity, and lack of diagnostic characteristics (Gurgel and Fredericq 2004), so it needs to be validated through molecular analysis through DNA barcoding. Molecular identification of *Gracilaria* spp. has been accomplished by Arbit *et al.* (2019), who found *G. verrucosa* and *G. gigas* in South Sulawesi, and Meinita *et al.* (2021) found *G. edulis*, *G. salicornia*, *G. textorii*, and *G. firma* in East Java, Yogyakarta, Central Java, and West Java. Furthermore, Wirawan *et al.* (2021) found *G. gracilis*, *G. vermiculophyla*, *G. salicornia*, *G. babae*, *G. isabellana*, *G. damaecornis*, *G. yoneshigueana*, and *G. edulis* in Serangan Island, Bali.

The ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) gene is a component of the DNA sequence found in chloroplast DNA (cpDNA) and potentially utilized as a DNA barcode (Newmaster *et al.* 2006). The *rbc*L marker has been used to identify *Gracilaria* spp. (Lyra *et al.* 2015; Kundu *et al.* 2017; Tampanguma *et al.* 2020; Freshwater *et al.* 2022).

Gracilaria sp. is distributed from the intertidal zone to the subtidal zone. *Gracilaria* spp. in Indonesia have been reported molecularly and morphologically, including *G. edulis*, *G. salicornia*, *G. verrucosa*, *G. gigas*, *G. textorii*, *G. firma*, *G. gracilis*, *G. vermiculophyla*, *G. babae*, *G. isabellana*, *G. damaecornis*, and *G. yoneshigueana* in Drini Beach in Yogyakarta, Kondang Merak in East Java, South Sulawesi, Kukup Beach in Yogyakarta, Nusakambangan Beach in Central Java, Karapyak Beach in West Java, Menganti Beach in Central Java, and Serangan Island in Bali, respectively (Romdoni *et al.* 2018; Arbit *et al.* 2019; Meinita *et al.* 2021; Wirawan *et al.* 2021). *Gracilaria* spp. is mostly found in the intertidal zone of Yogyakarta's south coast, but the molecular genetic diversity inventory has not been widely conducted. Therefore, this study aims to identify the genetic diversity of *Gracilaria* spp.

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molecularly by DNA barcoding using *rbc*L markers, supported by their morphological and histological characters.

2. Materials and Methods

2.1. Study Area

Samples were collected at the intertidal zone of the south coast of Yogyakarta, in Gunungkidul Regency from September to November 2021. Figure 1 illustrates the sampling location.

2.2. Sample Preservation

A portion of the thallus tip of *Gracilaria* spp. is taken for wet and dry preservation. Wet preservation was performed by immersing the sample in 70% ethanol for histological identification, whereas dry preservation was performed by immersing the sample in silica gel for DNA extraction (Tan *et al.* 2018). The remaining samples were subsequently frozen at -20°C for morphological analysis.

2.3. Morphological Identification

Morphological observations were made by comparing the sample's exterior appearance with identification sources from the algaebase (https://www.algaebase.org/) and other related papers. Several morphological characters of the thallus were observed, including thallus' color and length, type of tip, texture, and branching.

2.4. Photographs and Observations of Tissue Sample

Tissue observations were made with a modification of the research method by Welten *et al.* (2002) and Resendiz *et al.* (2019) by cutting the thallus of *Gracilaria* spp. and making the slide with dH_2O and 50% Karo (corn syrup). Tissue was observed using a binocular microscope (Nikon Eclipse E100LED MV R 742479) and documented and calibrated using an objective lens (1 DIV = 0.01 mm). Then, samples were observed under a camera microscope (Optilab Advance) with a magnification of 40X.

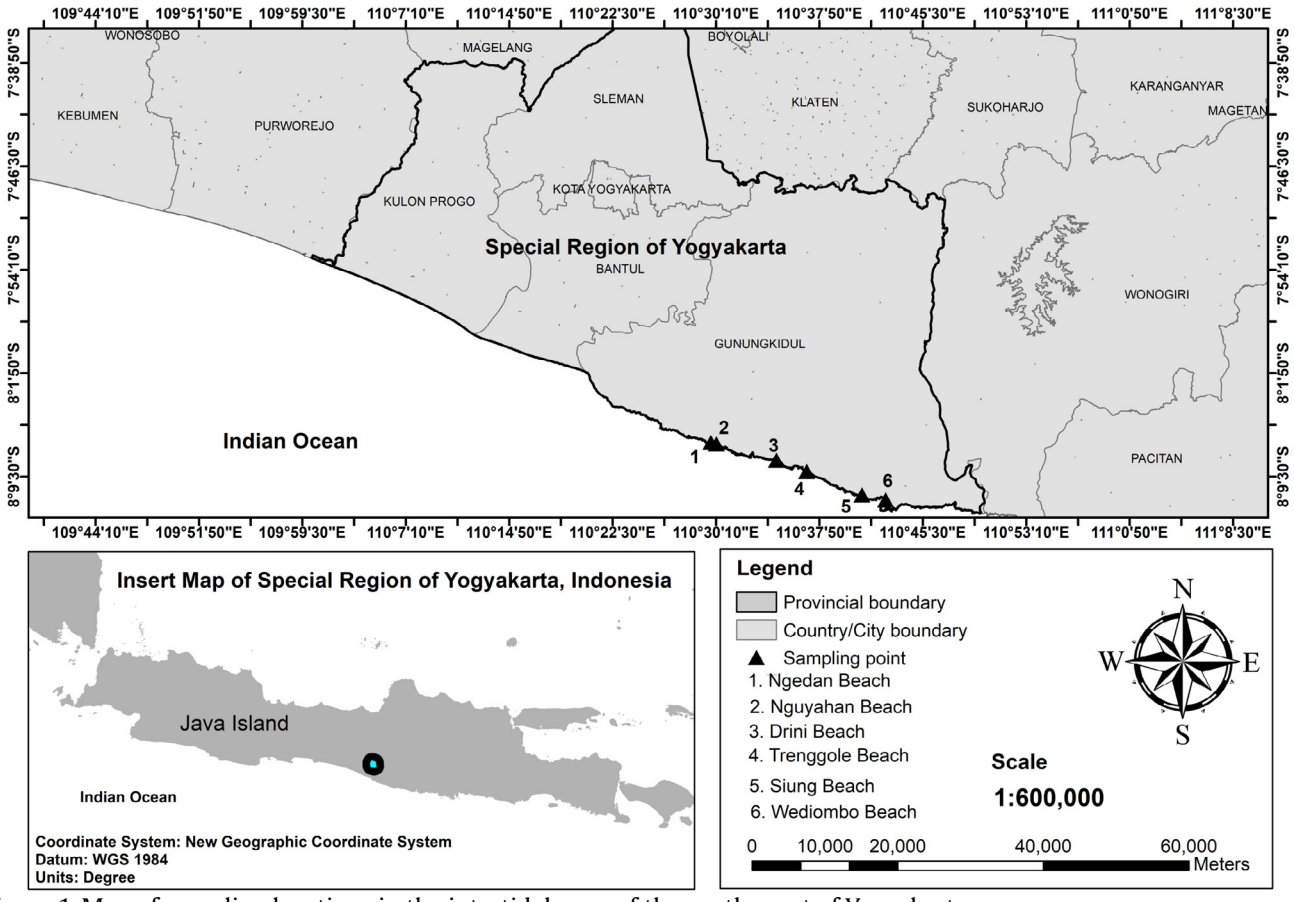

Figure 1. Map of sampling locations in the intertidal zone of the south coast of Yogyakarta

2.5. Molecular Identification

The tip of the 10-25 mg dried seaweed thallus was used for DNA extraction using a DNA extraction kit (Genomic DNA Mini Kit (Plant) Protocol from Geneaid) following ISO 9001:2008 QMS Certificate No. QAIC/TW/50077. The amplicon DNA obtained from the DNA extraction is then separated by 1% agarose gel electrophoresis. DNA amplified using a PCR machine (Thermal Cycler T100 Biorad combi block, Whatman-Biometra Germany). The markers used for the PCR process are Oligonucleotide marker (*rbc*L F7 (5'-AACTCTGTAGTAGAACGNACAAG-3') and *rbc*L R753 (5'-GTATATGAAGGTCTAAAAGGTGG-3') (Gavio and Fredericq 2002). The PCR process was performed by pre-denaturation (94°C, 5 minutes), denaturation (94°C, 30 seconds), annealing (44°C, 30 seconds), and extension (72°C, 1 minute) for 34 cycles. The last stage is post-extension at 72°C for 5 minutes. Furthermore, the DNA electrophoresis process was performed again to see the DNA bands, then continued with the sequencing process.

2.6. Data Analysis

The sequencing results were then analyzed by editing through the MEGA-X software (Kimura 1980; Kumar *et al.* 2018). After that, the nucleotide sequences were analyzed using the BLAST (Basic Local Alignment Search Tool) program, accessed directly from the MEGA-X software or the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Next, kinship analysis was conducted using the Pairwise Distance method by comparing the research data with GenBank data. The phylogenetic tree was made based on the nucleotide sequences of the sample and several sequences of *Gracilaria* sp. from GenBank. These sequences were aligned using ClustalW to produce an equivalent sequence size. Finally, this phylogenetic tree was made using MEGA-X software with the Maximum Likelihood (ML) statistical method with Bootstrap test phylogeny (1,000 replicates) and Kimura 2-parameter nucleotide substitution type model.

3. Results

3.1. BLAST Molecular Analysis

Using the *rbc*L marker, the molecular identification of 18 *Gracilaria* spp. specimens revealed multiple distinct species (Table 1). Generally, three species of *Gracilaria spp., G. salicornia, G. edulis*, and *G. vieillardii*—were identified based on the *rbc*L marker with the highest percent identity. After editing and cutting the *rbc*L marker, the sequence size was 685-719 bp. The *rbc*L cover marker query results for the entire sample show a value of 100%. Furthermore, the percent identity for the *rbc*L marker is 95.54-100%.

3.2. Phylogenetic Tree Analysis

A phylogenetic tree was created from 18 sequences of *Gracilaria* spp. From this study, 17 sequences of the *rbc*L gene from GenBank (Figure 2). The interspecific genetic range of the *rbc*L marker is (0-13.8%). Types of *Gracilaria* spp. identified based on the results of

Table 1. *Gracilaria* spp. based on *rbc*L marker-BLAST in the intertidal zone of the south coast of Yogyakarta, Indonesia

Sampling location (Beach)	Sample code	Identified species	Sequence size (bp)	Query cover (%)	Percent identity (%)	Accession number reference
Trenggole	FT1	*Gracilaria* sp.	702	100	95.59	AY049316
	FT2	*Gracilaria* sp.	702	100	95.59	AY049316
Drini	FD1	*Gracilaria* sp.	686	100	95.63	MT939893
	FD2	*Gracilaria* sp.	685	100	95.77	AY049316
	FD3	*Gracilaria salicornia*	690	100	99.86	KF831118
	FD4	*Gracilaria* sp.	690	100	95.80	AY049316
	FD5	*Gracilaria salicornia*	690	100	99.86	KF831118
	FD6	*Gracilaria edulis*	690	100	100.00	NC_046041
	FD7	*Gracilaria vieillardii*	709	100	97.74	AY737437
Siung	SF2	*Gracilaria* sp.	707	100	95.76	AY049316
	SF4	*Gracilaria edulis*	699	100	99.86	NC_046041
	SB1	*Gracilaria vieillardii*	708	100	97.60	AY737437
Wediombo	WF1	*Gracilaria* sp.	719	100	95.55	MT939893
	WF3	*Gracilaria* sp.	719	100	95.55	MT939893
Nguyahan	NF3	*Gracilaria edulis*	690	100	100.00	NC_046041
Ngedan	NgeF2	*Gracilaria* sp.	718	100	95.54	AY049316
	NgeF3	*Gracilaria* sp.	718	100	95.54	AY049316
	NgeF4	*Gracilaria edulis*	690	100	100.00	NC_046041

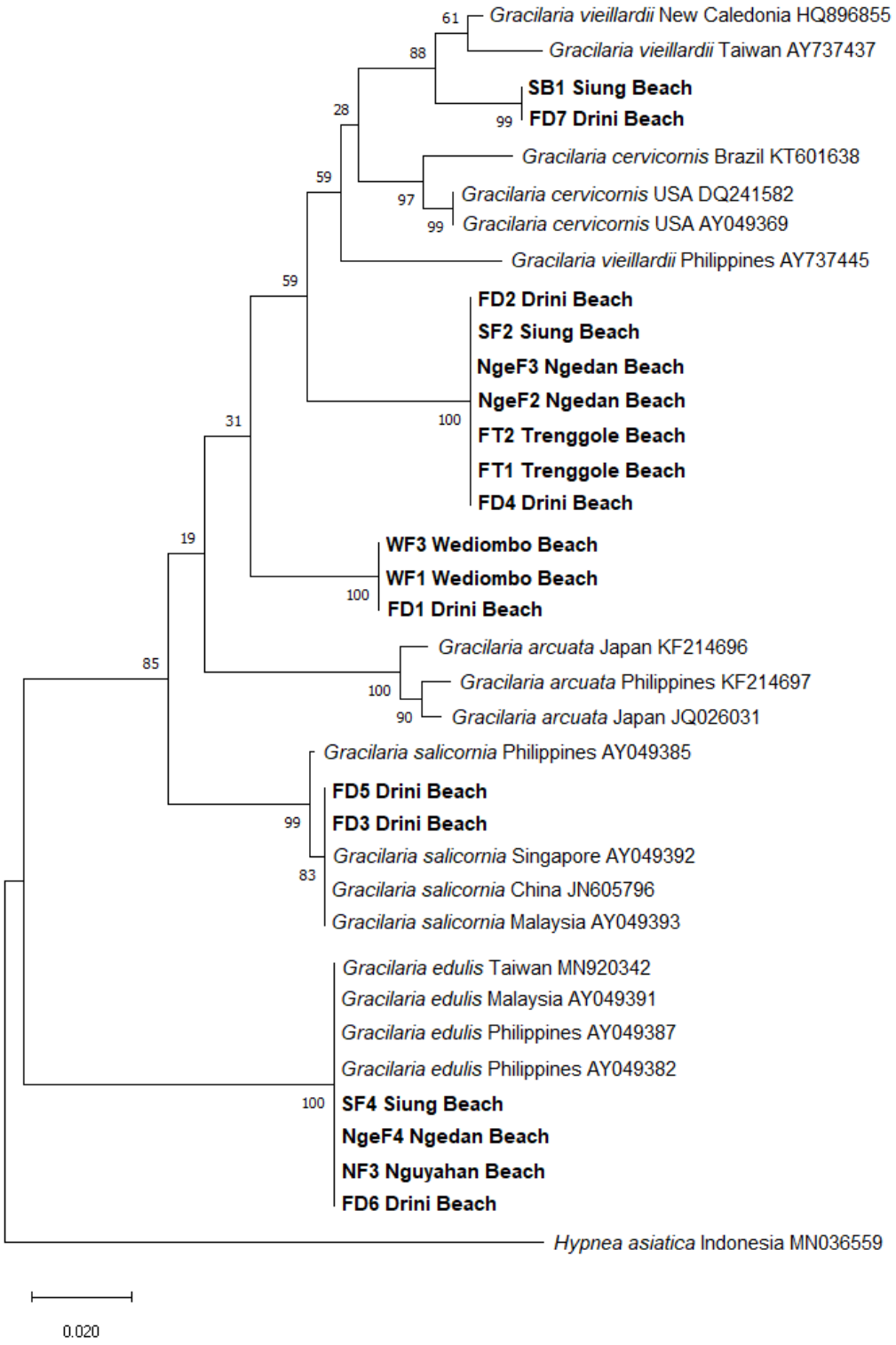

Figure 2. Phylogenetic tree of 18 sequences of *Gracilaria* spp. in this study and 17 sequences of *Gracilaria* spp. of the *rbc*L marker from GenBank

the phylogenetic marker of *rbc*L (Figure 2) were *G. salicornia* and *G. edulis*. Intraspecific genetic distances in *G. salicornia* and *G. edulis* were 0% (*rbc*L marker). Specimens SB1 and FD7 were identified as *G. vieillardii* with the *rbc*L marker, as evidenced by the lower intraspecific distance (1.6-3.0%). Specimens FD4, FD2, FT1, NgeF2, NgeF3, SF2, and FT2 have a genetic distance of 0% (*rbc*L marker) identified as *Gracilaria* sp. 1. While FD1, WF3, and WF3 specimens were monophyletic with 0% intraspecific genetic distance (*rbc*L marker) identified as *Gracilaria* sp. 2.

3.3. Morphological and Histological Observations

The phylogenetic trees (based on *rbc*L marker) were used to describe morphological and histological observations, which revealed five species of *Gracilaria* spp. from the intertidal zone of the south coast of Yogyakarta, Indonesia: *Gracilaria salicornia, Gracilaria edulis, Gracilaria vieillardii, Gracilaria* sp. 1, and *Gracilaria* sp. 2 Figures 3 to 7 show each of these species. Meanwhile, the comparison of morphological characters can be seen in Table 2.

4. Discussion

4.1. Molecular Analyses Using the *rbc*L Marker

The *rbc*L gene is part of the DNA sequence located in cpDNA (chloroplast DNA) and can potentially be used as a DNA barcode because of its ease of amplification and analysis (Newmaster *et al.* 2006). The *rbc*L marker in this study had a larger sequence size, query cover, and percent identity (Table 1). These results indicate that the *rbc*L gene has many characters for phylogenetic analysis. Furthermore, the *rbc*L marker has a low mutation rate compared to other barcodes, an advantage of the *rbc*L gene. In addition, the *rbc*L marker also has a high degree of similarity between species (Sundari and Papuangan 2019).

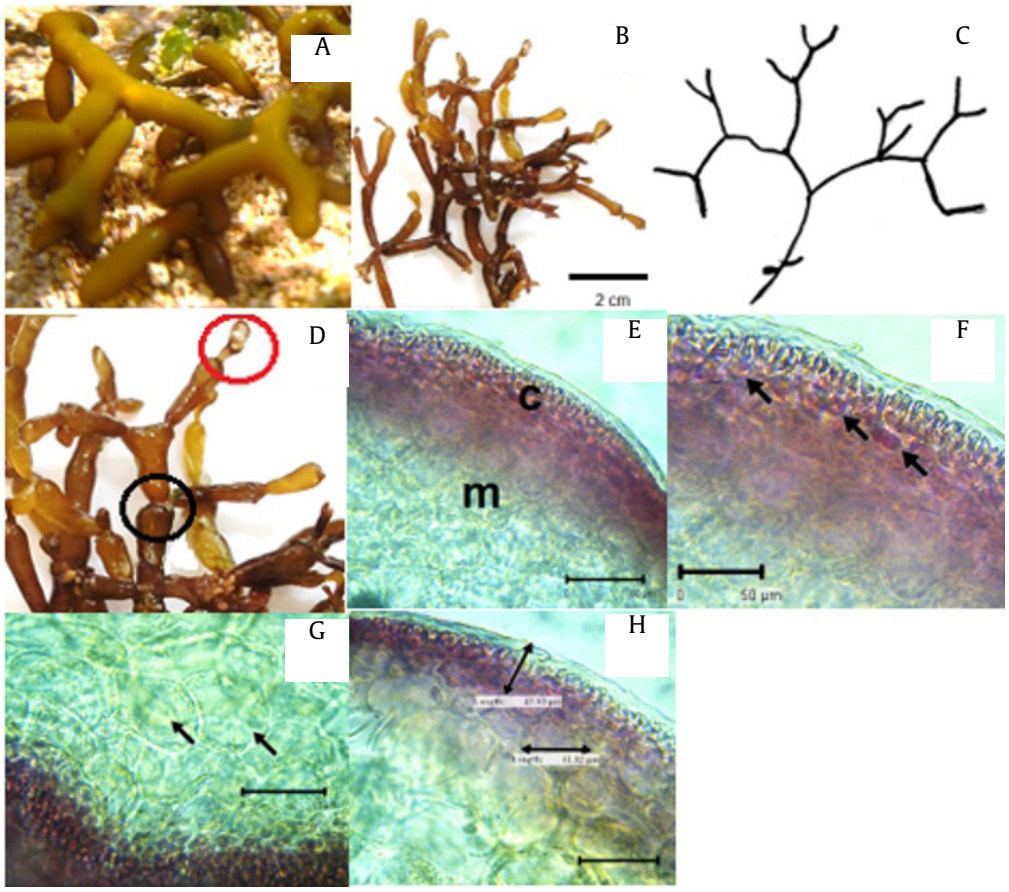

Figure 3. *Gracilaria salicornia* specimen in this study: (A) habitat, (B) morphology of the sampling location, (C) branching, (D) Thallus tip with proliferation (red circle) and segments (black circle), (E) Cortex (c) and medulla (m), scale bar = 50 µm, (F) cortex layers, scale bar = 50 µm, (G) medullary cells (arrow), scale bar = 50 µm, and (H) cortex length and medulla diameter, scale bar = 50 µm

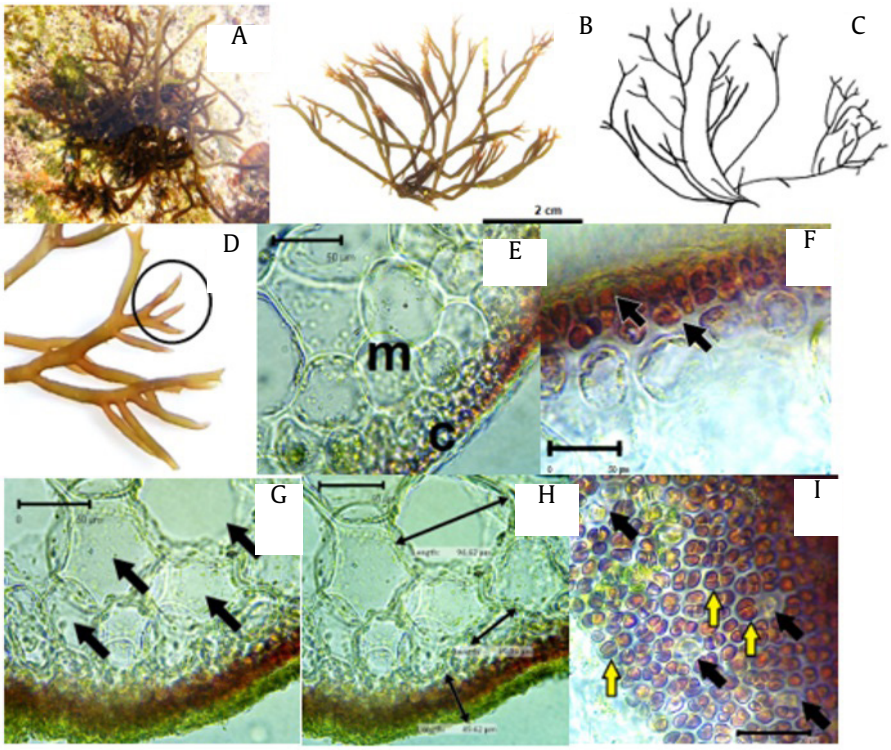

Figure 4. *Gracilaria edulis* specimen in this study: (A) habitat, (B) morphology of the sampling location, (C) branching, (D) the tip of the thallus was tapered with proliferation, (E) cortex (c) and medulla (m), scale bar = 50 µm, (F) cortex layers, scale bar = 50 µm, (G) medullary cells (arrow), scale bar = 50 µm, (H) cortex length and medulla diameter, scale bar = 50 µm, (I) spermatangial (black arrow) and bi-sporangial (yellow arrow), scale bar = 50 µm

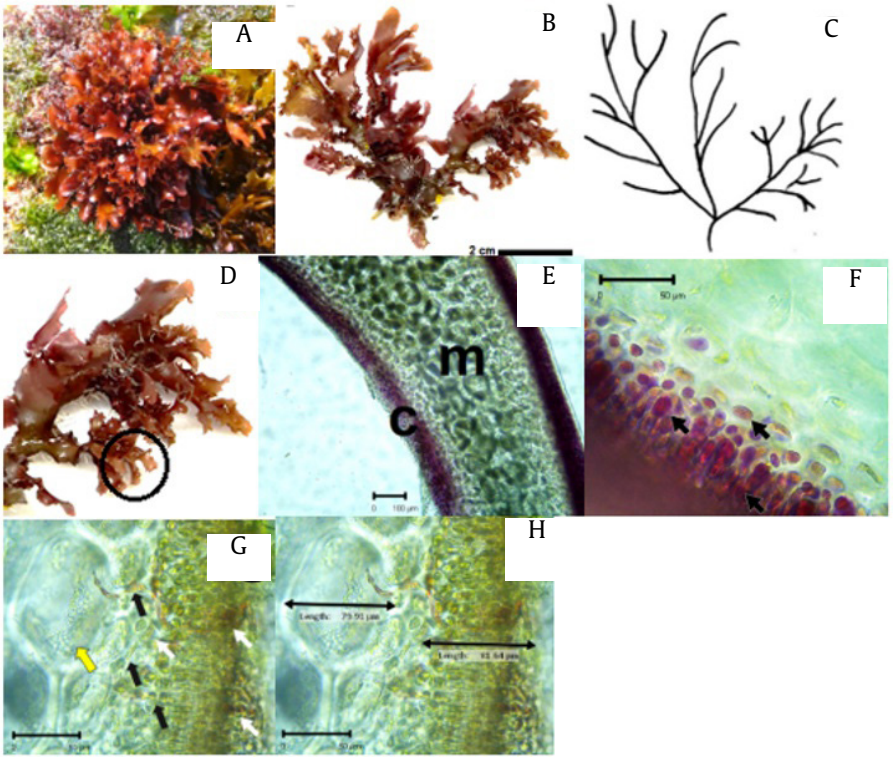

Figure 5. *Gracilaria vieillardii* specimen in this study: (A) habitat, (B) morphology of the sampling location, (C) branching, (D) the tip of the thallus is blunt, slightly weak and withered and jagged all around (black circle), (E) Cortex (c) and medulla (m), scale bar = 100 µm, (F) cortex layers, scale bar = 50 µm, (G) medullary cells (yellow arrow) and conjuncture cells fusiform (black arrow), scale bar = 50 µm, and (H) cortex length and medulla diameter, scale bar = 50 µm

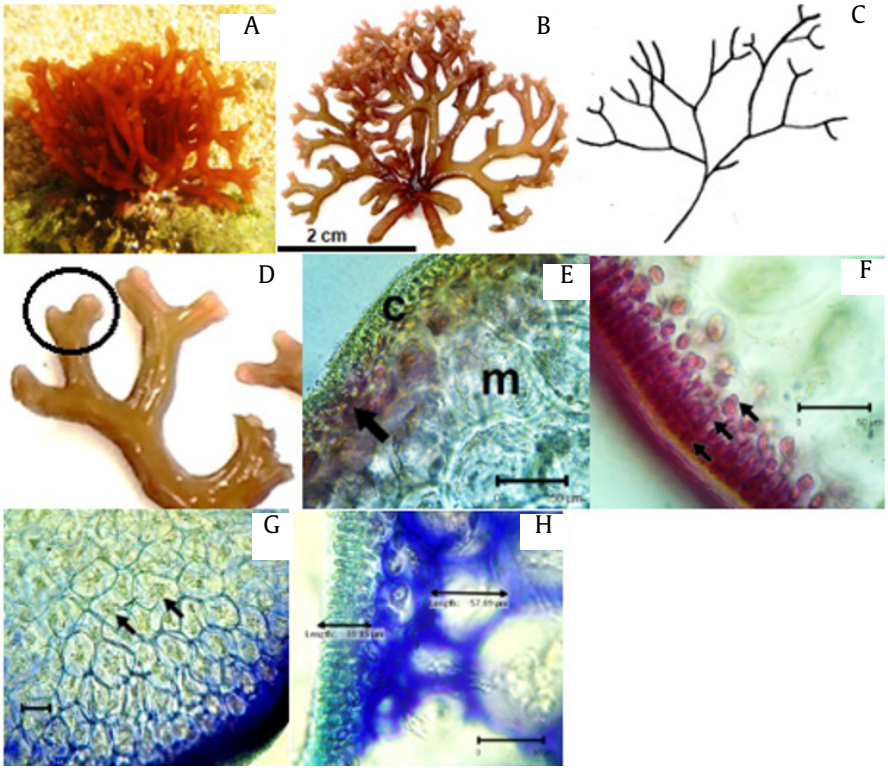

Figure 6. *Gracilaria* sp. 1 specimen in this study: (A) habitat, (B) morphology of the sampling location, (C) branching, (D) the tip of the thallus is blunt, (E) cortex (c) and medulla (m) and spermatangia (arrow), scale bar = 50 µm, (F) cortex layers, scale bar = 50 µm, (G) medullary cells (arrow), scale bar = 100 µm, and (H) cortex length and medulla diameter, scale bar = 50 µm

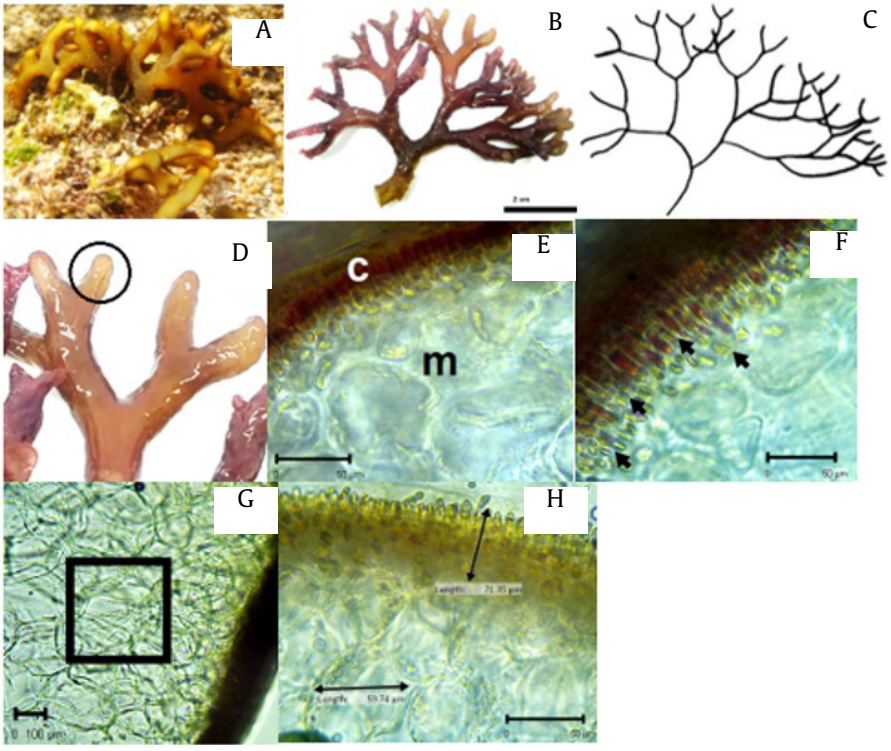

Figure 7. *Gracilaria* sp. 2 specimen in this study: (A) habitat, (B) morphology of the sampling location, (C) branching, (D) blunt tip of the thallus, (E) cortex (c) and medulla (m), scale bar = 50 µm, (F) cortex layers, scale bar = 50 µm, (G) medullary cells (box), scale bar = 100 µm, and (H) cortex length and medulla diameter, scale bar = 50 µm

Table 2. Comparison of morphology and histology of Gracilaria spp. from the intertidal zone of the south coast of Yogyakarta.

Species are Identified	Thallus color and length (n = 18)	Thallus type	Thallus tip	Thallus texture and branching	Number of cortex layers	Cortex cell shape	Medullary cell size	Medullary cell count	Medulla shape	Cortex length (n = 18) (µm)	Medulla diameter (n = 18) (µm)	Carposporangia and cystocarps
G. salicornia (FD3, FD5)	Yellow to brown (2.5-5.5 cm)	Upright cylindrical with 2-3 segments	Blunt with little growth	Smooth and sub-dichotomous	2-4 layers	Small ovoid and outer cortical is more elongated and pigmented	Uniform	4-5 to the middle	Oval to round and not pigmented	40.29-47.93	41.92-44.93	Not found
G. edulis (NF3, FD6, SF4, NgeF4)	Dark green, yellow, or-ange to brown (3.3-9 cm)	Upright cylindrical and not segmented	Taper	Smooth like cartilage and irregular	1-5 layers	Small ovoid and outer cortical is more elongated and pigmented	Uniform and bigger middle cell	3-4 to the middle	Oval to round and not pigmented	43.82-79.58	32.82-94.77	No cystocarps Visible spermatangial and bisporangial
G. vieillardii (SB1, FD7)	Maroon to dark brown and greenish at the edges of the thallus (3 cm)	Leafy sheets and not seg-mented	Blunt, slightly wilted, and jagged at the edges	A little rough and opposite to whorled with quite thick side branches	3-4 layers	Small ovoid and outer cortical is more elongated and pigmented	Irregular cells with fusiform conjuncture cells	1-2 after cortex and 3 middle cells	Oval to round and not pigmented	81.64-171.89	79.91-278.60	Not found
Gracilaria sp1. (FD4, FD2, FT1, NgeF2, NgeF3, SF2)	Yellowish orange to brownish maroon (2.4-4.7 cm)	Upright cylindrical and not segmented	Blunt	Smooth stripped-like cartilage and dichotomous to sub-dichotomous	2-5 layers	Small ovoid and outer cortical is more elongated and pigmented	Uniform and bigger middle cell	6-9 to the middle	Oval to round and not pigmented	34.78-265.92	57.09-146.55	No cystocarps but visible spermatangial
Gracilaria sp2. (FD1, WF3, WF1)	Yellowish green to brownish red (2-4.5 cm)	Upright cylindrical and not segmented but contains little water in the thallus	Blunt	Smooth as cartilage and dichotomous	2-4 layers	Small ovoid and outer cortical is more elongated and pigmented	Uniform and bigger middle cell	3-9 to the middle	Oval to round and not pigmented	41.77-71.35	36.41-106.94	Not found

Tuney and Sukatar (2010) confirmed that DNA extraction and amplification of the *rbc*L gene of *Gracilaria* spp. are often difficult to obtain in large quantities and lack ideal qualities for molecular analysis is one of the factors contributing to the low number of species found in the *rbc*L marker. The capability of the *rbc*L marker to discriminate between species (discrimination power) is proven limited (Lyra *et al.* 2015; Rodríguez-Prieto *et al.* 2016; Kundu *et al.* 2017; Tampanguma *et al.* 2020; Freshwater *et al.* 2022). Despite its limited discrimination power, the *rbc*L marker has a successful amplification rate for *Gracilaria* spp. and can be sequenced.

According to Hebert *et al.* (2003), a genetic distance of ≤ 3 percent suggests that two species are molecularly identical. Furthermore, Gurgel and Fredericq (2004) suggested that morphologically distinct species usually have an interspecific *rbc*L divergence of more than 2%. Using this definition, it can be demonstrated that *G. salicornia* and *G. edulis* have an intraspecific genetic distance of 0.0-0.7 percent (< 2%) compared to GenBank species. It implies that the species is found to be molecularly identical and confirmed as the same species. Moreover, specimens SB1 and FD7 were more genetically identical to *G. viellardii* (1.6-3.0%) than to *G. cervicornis* (0.0-10.8%); hence it is conceivable that the specimen was a member of the *G. viellardii* species. In the species *Gracilaria* sp. 1 and *Gracilaria* sp. 2, intraspecific genetic distance was 0% for *rbc*L markers, indicating that they are molecularly identical.

The *rbc*L marker can be used to identify red algae species, but the loci from this section lack the universality feature of the barcode marker. They may result in identification errors if used to distinguish closely related species. The benefit of the *rbc*L marker is that it enriches the core dataset already accessible to researchers and complements the barcode of phylogenetic analyses that may be absent. In this scenario, the usage *rbc*L marker may be superior for the phylogenetic study of evolutionary relationships.

4.2. Morphological and Histological Analyses

Gracilaria sp. 1, as determined from the analyses of *rbc*L markers, must be validated by matching morphological characteristics. *Gracilaria* sp. 1 is more closely related to *G. arcuata* than *G. manilaensis, G. cervicornis*, and *G. viellardii* (FAO 1996a, 1996b; Dreckmann and Sentíes 2009; Lin *et al.* 2012 Ratnasingham and Hebert 2014b; Yang and Kim 2015; Reine 2016; Hessami *et al.* 2020; Palomares and Pauly 2022a, 2022b). However, the phylogenetic analysis confirmed that it was not *G. arcuata*, so based on these results, the species of *Gracilaria* sp. 1 was still unknown, and further confirmation was required by conducting another DNA barcoding molecular analysis with other markers, as well as a more thorough characterization of the species before it could be reported.

The morphology of *Gracilaria* sp. 2 is similar to that of *G. arcuata* in branching (i.e., dichotomous). However, the texture of the thallus is distinct: *G. arcuata* has a denser thallus, whereas *Gracilaria* sp. 2 has slightly water-filled. Further compared with *G. viellardii*, the thallus morphology of the three are different, i.e., *G. viellardii* has a sheet thallus (Lin *et al.* 2012). In contrast, *Gracilaria* sp. 2 has an upright cylindrical thallus. Therefore, the identification of *Gracilaria* sp. 2 species must be compared to the identification source, as a phylogenetic tree is unsuitable. *Gracilaria* sp. 2 has morphological similarities with *G. canaliculata*. According to Lyer *et al.* (2004), *G. canaliculata* has a yellowish-red thallus, is cylindrical with short dichotomous branches, and has a bigger thallus size than *G. arcuata*. Thus, the exact species of *Gracilaria* sp. 2 remains uncertain, and further molecular identification is required using DNA barcoding with other primers.

G. edulis and *G. salicornia* were earlier discovered on the south coast of Yogyakarta, i.e., on Drini Beach and Kukup Beach, respectively (Romdoni *et al.* 2018; Meinita *et al.* 2021). Following previous research, the same species was discovered in the same location in the current investigation. However, prior research limited the identification method to morphological and molecular identification with a single marker (i.e., *rbc*L) without histological analysis. Therefore, this study is the first to identify *Gracilaria* spp. molecularly utilizing *rbc*L markers combined with histological identification over the southern coast of Yogyakarta, Indonesia.

Four species of *Gracilaria* sp., i.e., *G. salicornia, G. edulis*, and *G. vieillardii*, were identified by applying the *rbc*L marker. The *rbc*L marker can be utilized to distinguish between species with the benefit of high interspecies similarity and low mutation rates and as barcodes supplement for phylogenetic analyses that might be missing. In addition, the *rbc*L marker can be employed as a complement for species identification, allowing for species identification using a second marker if it is not detected on the first.

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