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Molecular identification of *Gracilaria* species (Gracilariales, Rhodophyta) obtained from the South Coast of Java Island, Indonesia

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Abstract. *Meinita MDN, Akromah N, Andriyani N, Setijanto, Harwanto D, Liu T. 2021. Molecular identification of* Gracilaria *species (Gracilariales, Rhodophyta) obtained from the South Coast of Java Island, Indonesia. Biodiversitas 22: 3046-3056.* The study of seaweeds diversity and species identification is an important component of marine resource management. However, seaweed identification based on morphological characteristics has several limitations. Besides, DNA barcodes or the partial sequences of cytochrome c oxidase I (COX1) have been proved to identify seaweeds at the species level. To date, *Gracilaria* species molecular diversity has not been studied in the South Coast of Java Island. Hence, this study aimed to identify the *Gracilaria* spp. obtained from 6 different beaches along the South Coast of Java, based on the COX1 gene. COX1 gene utilization for identifying and observing the genetic diversity, both intraspecific (genetic variation within species) and interspecific (variations between species) of Gracilaria species in this study has produced good results. A total of 13 seaweed samples collected from beaches in this study were identified as *Gracilaria salicornia*, *G. edulis*, *G. firma,* and *G. textorii*. The results of genetic diversity analysis conducted using the COX1 gene showed the intraspecific diversity of *G. edulis* obtained from the beaches of Kondang Merak, Kukup, Nusakambangan, and Karapyak was included in the moderate diversity category. Also, the intraspecific diversity of *G. salicornia* obtained from the beaches of Kondang Merak, Kukup, and Nusakambangan was included in the moderate diversity category, while the intraspecific diversity of *G. textorii* from Menganti and Karapyak Beach had no diversity.

Keywords: Barcode, COX1, *Gracilaria*, molecular, seaweeds

INTRODUCTION

Tropical sea exhibits high biodiversity providing a favorable habitat for many marine creatures, including seaweeds. As the largest archipelago country, Indonesia is a representative to describe the tropical biodiversity and is the second seaweed producer globally (FAO 2020). About 555 seaweed species are recorded in Indonesia, from which 55 have high economic value, belonging to the genera *Eucheuma* (J. Agardh), *Gracilaria* (Greville), and *Gelidium* (J. V. Lamouroux 1813) (Kadi 2004). Species of the genus *Gracilaria* are distributed from intertidal to subtidal habitats, occurring in most oceans. Their distribution spread from temperate to tropical waters, including Southeast Asian regions (Kim et al. 2010; Heo et al. 2011; Kongkittayapun and Chirapart 2011; Yang and Kim 2015). Members of the red algal family Gracilariaceae are high-quality raw material sources in the food-grade agar industry. Agar is widely used as a hydrocolloid in various industrial fields, especially in food, pharmaceuticals, cosmetics, and bioenergy (de Almeida et al. 2011; Meinita et al. 2017; Torres et al. 2019). Globally, agar production exceeds 14,500 tons annually (Porse and Rudolph 2017). Moreover, the increasing demand for seaweeds in food, cosmetics, and pharmaceutical industries has begun since 1980 (Bixler and Porse 2011; Porse and Rudolph 2017). As technology develops, the demand for agar raw materials also increases. *Gracilaria* spp. continuous utilization causes population decline and limited availability in nature, hence managing their biodiversity is necessary. Besides, biodiversity is an important aspect of resource management efforts related to long-term sustainable use (Nesbitt et al. 2010).

Indonesia is one of the largest producers of farmed *Gracilaria*, specially *Gracilariopsis longissimi* and *G. gigas* (Pambudi et al. 2010; Meinita et al. 2018). Unfortunately, there has not been a comprehensive study on Indonesian seaweed after Siboga and Snellius expedition. Most seaweed diversity studies in Indonesia were done partially in a certain area. Indonesian seaweed diversity is hard to be precisely reported as a part of other works or publications made several decades after the expedition. Its first published records were due to the expeditions in 1899 undertaken by Weber-van Bosse which discovered 782 seaweeds, consisting of 179 green algae, 134 brown algae, and 452 red algae (Hutomo and Moosa 2005). During the Snellius-II Expedition in 1984, herbarium species of marine alga was also collected from the Eastern part of the Indonesian Archipelago.

The study sites selected were the beaches of Kondang Merak, Kukup, Menganti, Nusakambangan, Karapyak, and Sayang Heulang which lie along the South Coast of Java Island and directly adjacent to the Indian Ocean. Moreover, the South Coast of Java Island has extreme conditions with steep topographic characteristics and strong waves, while the location also affects the *Gracilaria* spp diversity.

Seaweeds morphology changes due to unstable environmental conditions (Kongkittayapun and Chirapart 2011). However, Indonesian *Gracilaria* spp. Identification is commonly based on morphological features. This process has some limitations that lead to the species misidentification due to their high phenotypic plasticity (Hassan et al. 2019). Correct identification is crucial for biodiversity studies, while misidentification influences the faults in determining biodiversity. Molecular identification based on genetic information using DNA sequences has more accuracy than morphological identification. Therefore, it is necessary because of being capable to provide more accurate information on a species genetic diversity. Some molecular studies of Gracilariaceae have been conducted (Liu et al. 2017; Sedanza et al. 2020; Wang et al. 2020), but not in Indonesia. A molecular study of this country's *Gracilaria* species was conducted using RAPD marker (Windarsih et al. 2019) to characterize their genetic profile with random single oligonucleotides, however, the RAPD also has limitations due to its low reproducibility.

COX1 (cytochrome c oxidase subunit 1) is one of the genes in mitochondrial DNA used to determine *Gracilaria* spp. genetic diversity. According to Sherwood et al. 2011, the COX1 gene is used to determine red seaweed species genetic diversity and the population's biogeographic structure. Several studies on *Gracilaria* spp. genetic diversity based on COX1 genes that have been carried out includes *G. firma, G. vermiculophylla, G. salicornia*, and *G. changii* (Yang et al. 2008; Yow et al. 2011; Gulbransen et al. 2012; Ng et al. 2017; Hifney et al. 2018; Song et al. 2019). The southern part of Java Island is known as a tropical area with a high seaweed diversity (Romdoni et al. 2018). Hence, this study investigated the natural seaweed potential along the aforementioned area.

MATERIALS AND METHODS

Study area

According to Figure 1, seaweed species were collected from Kondang Merak Beach (8°23'52.44"S 112°31'25.62"T), Kukup Beach (8°06'48.68"S 110°34'44.41"T), Menganti Beach, Nusakambangan Beach (7°45'15.29"S 109°01'04.68"T), Karapyak Beach (7°42'9.86"S 108°45'38.28"T) and Sayang Heulang Beach (7°38'16.34"S 107°41'50.30"T). These locations were selected to represent the coastal area in the Southern part of Java Island. Besides, samples were cleaned off dirt, sand, and periphyton, then washed with sterile seawater, stored in clean plastic, and coded, followed by storage in a freezer at-20ºC for further analysis.

Morphological identification

Morphological identification that involved DNA isolation and sequencing method was performed using the key of determination, based on the type and tip of thallus, and branching type.

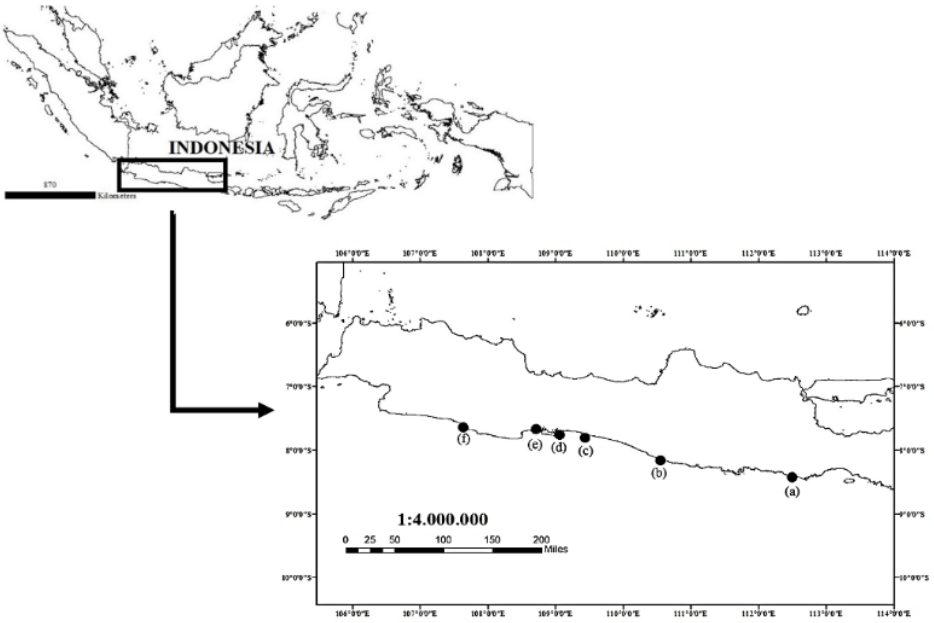

Figure 1. Location of the six beaches along the South Coast of Java Island, Indonesia: A. Kondang Merak, B. Kukup, C. Menganti, D. Nusakambangan, E. Karapyak, and F. Sayang Heulang

DNA isolation and sequencing

DNA was extracted from approximately 4 mg of algal powder ground in liquid nitrogen using the CTAB (Cetyl Trimethyl Ammonium Bromide) method. This isolate was then tested for quantity using spectrophotometers at 260 nm and 280 nm, to determine DNA purity and concentration. The purity was observed through A260:A280 ratio by agarose gel electrophoresis. However, quality analysis was carried out by agarose gel electrophoresis method, to determine DNA presence, integrity, and purity level from RNA contaminants. The DNA isolate was then amplified through the polymerase chain reaction (PCR) process, using COX1 primers, namely COX143F and COX11549R (Geraldino et al. 2006). The mitochondrial COX1 sequences were aligned and used to devise specific primers to amplify this gene region for *Gracilaria* (upstream COXI43F-5' TCA ACA AAT CAT AAA GAT ATT GGW ACT 3' and downstream COXI1549R-5' AGG CAT TTC TTC AAA NGT ATG ATA 3').

PCRs were carried out using the Thermal Cycler (USA), and the condition protocol used for these reactions consisted of an initial denaturation at 94°C for 10 min, followed by 35 amplification cycles, denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 2 min, and a final extension at 72°C for 10 min. Electrophoresis was used to determine the DNA doubling results in the amplification process. The agarose gel composition was 1.5% of 80 mL of 1x TBE buffer solution or 1.2 grams of agarose, 8 mL of 10x TBE, and 72 mL of distilled water, then all were mixed in a 250 mL Erlenmeyer, homogenized, heated, and boiled. About 5 μL of Florosafe was added to agarose before it was poured and solidified inside the electrophoresis chamber. TBE buffer 1x was added until the gel was submerged. Afterward, 10 μL of the amplified DNA sample was inserted into the well. Electrophoresis was run for 45 minutes at a voltage of 250 volts. Then agarose gel was taken and placed on a UV transilluminator. The products' sequences were determined and then used in making phylogenetic trees and analyzing genetic diversity.

BLAST analysis

Basic Local Alignment Search Tool (BLAST) was used to observe similarities in GenBank. This analysis was carried out using a web-based program, namely NCBI or National Center for Biotechnology (blast.ncbi.nih.nlm.gov) by entering the complete sample sequences which are the combined primary sequences of F and R, into the BLAST.

Phylogenetic trees

Phylogenetic trees were prepared by using neighbor-joining methods and bootstrap analysis (1,000 replicates) in the MEGA7 program.

Genetic diversity

Genetic diversity analysis was conducted by using the diversity of haplotypes. Hence, the alignment discovered included 28 sequences, namely 13 *Gracilaria* species from this study and 15 species of Gracilariaceae from GenBank. After multi-alignment, the Gracilaria species sequences were processed using the DNAsp 5.0 program to analyze intraspecific genetic diversity.

RESULTS AND DISCUSSION

Morphological identification

The red algal family Gracilariaceae covers approximately 230 species including Gracilaria, which are divided into 7 genera and also spread from temperate to tropical regions (Lyra et al. 2015; Guiry and Guiry 2020). The highest diversity of Gracilaria species is in the tropical area, specifically in Indo-Pacific and Western Antlantic region (Guiry and Guiry 2020). However, there is a lack of information and studies on the total number of Gracilaria species in Indonesia as a tropical country. In this study, a total of 13 species were collected from some sampling sites based on the preliminary morphological identification. These included 2 (*Gracilaria coronopifolia* and *Gracilaria Salicornia)* from Kondang Merak Beach, 2 (*Gacilaria corticata* and *Gracilaria debilis)* from Kukup Beach, 1 (*Gracilaria debilis)* from Menganti Beach, 3 (*Gracilaria debilis, Gracilaria gigas,* and *Gracilariopsis longissimi)* from Nusakambangan Beach, 3 (*Gacilaria corticata, Gracilaria corticate,* and *Gracilariopsis longissimi)* from Karapyak Beach, and 2 (*Gracilaria gracilis* and *Gracilaria blodgettii)* from Sayang Heulang Beach (Figure 2). Identification of the sample's morphological features such as thallus type, tip, and branching, etc. was carried out. It was observed that all samples showed similar branching type, either dichotomous or irregular (Figure 3). The *Gracilaria* species were observed based on morphological features including color, thallus habit, tips, segments, and texture, as well as holdfast, branching pattern, cell size gradation, cystocarp shape and size, nutritive filaments, pericarp, gonimoblast, spermatangial conceptacles, diameter, cell wall thickness (μm), cortex and medulla (Table 1).

Molecular identification

After the previous process, molecular identification was performed based on sequencing analysis, and it has more advantages than morphological identification. The molecular identification of algal species has been successfully applied to various red seaweeds, including *Gracilaria* species (Yang et al. 2008; Yow et al. 2011; Gulbransen et al. 2012; Ng et al. 2017; Hifney et al. 2018; Song et al. 2019). The *Gracilaria* samples DNA concentration values ranged between 22.5-1137 ng / μL, and DNA purity values ranged from 1.8-2.0. The DNA concentrations obtained were very diverse, but more than enough to be used in molecular analysis. The purity measurement aims to determine contaminants presence or absence, therefore when the value is below 1.8 and above 2.0, then the DNA is contaminated with RNA and protein. The purity values obtained were still in a good range, hence the DNA was used for molecular analysis.

There is a lack of information and study regarding the molecular identification and diversity of Indonesian seaweed including Gracilaria species. Most of such studies only focus on ecological aspect of seaweed, while the species identification was performed based on their morphological features. Some Indonesian seaweed studies conducted using RAPD method, determined the genetic diversity and productivity of G. coronopifolia collected from Anyer Beach, Banten, Indonesia (Windarsih et al. 2019). However, RAPD utilization has some weaknesses, such as unreliable, because it gives a different product when repeated. In this study, to overcome the weaknesses, DNA barcoding method was employed using COX1 gene. It has been previously shown that the COX1 gene is applicable in identifying red seaweed species (Yang et al. 2013; Yoon et al. 2014). This gene is a relatively short DNA portion and is easily amplified and sequenced with one pair of primers. The COX1 gene is one gene with a 1422 bp length out total of 53 genes with a 26,898 bp length contained in mitochondrial DNA (Lee et al. 2015). The amplification using the COX1 gene with COX143F and COX11549R primers from the thirteen *Gracilaria* spp. samples were successful, while DNA bands with 1422 bp size were produced. This condition showed the amplification was progressing well and had to be continued for the sequencing step.

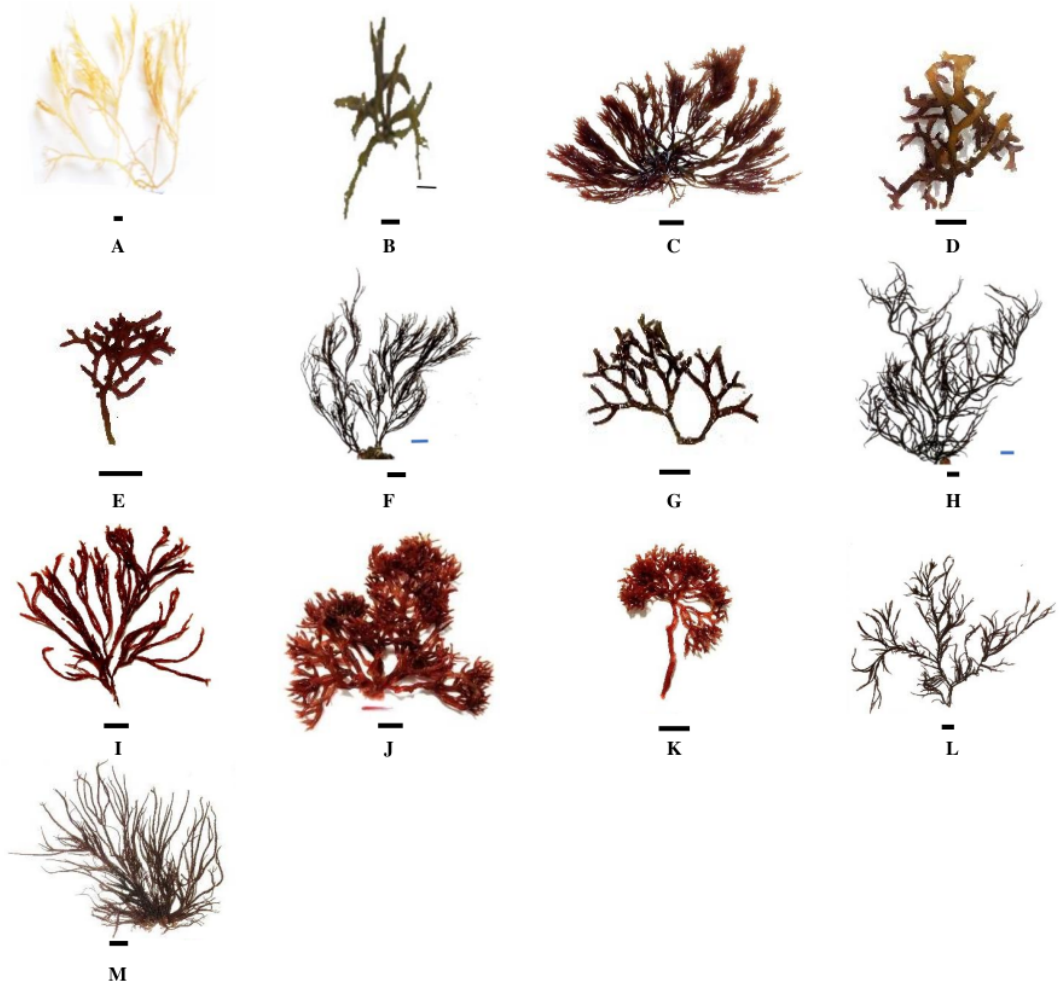

Figure 2. Voucher species of *Gracilaria* spp. collected along the South Coast of Java Island, Indonesia. A. KM1: *Gracilaria coronopifolia* Kondang Merak; B. KM3: *Gracilaria salicornia* Kondang Merak; C. K1: *Gracilaria corticata* Kukup; D. K2: *Gracilaria debilis* Kukup; E. M1: *Gracilaria debilis* Menganti; F. NK1: *Gracilaria gigas* Nusakambangan; G. NK2: *Gracilaria debilis* Nusakambangan; H. NK3: *Gracilaria coronopifolia* Nusakambangan; I. F1: *Gracilaria coronopifolia* Karapyak; J. F4: *Gracilaria corticata* Karapyak; K. F6: *Gracilaria corticata* Karapyak; L. SH4: *Gracilaria gracilis* Sayang Heulang; M. SH6: *Gracilaria blodgettii* Sayang Heulang. Scale bars = 1 cm

Table 1. Morphological characteristics of *Gracilaria* species obtained in this study

Morphological characteristics	*G. coronopifolia*	*G. salicornia*	*G. corticata*	*G. debilis*	*G. gigas*	*G. verrucosa*	*G. gracilis*	*G. blodgettii*
Color	Yellowish to brownish red	Dark green to greenish-brown	Purple-red	Dark green to blackish-red	Dark green to blackish-red	Yellowish to brownish-red	Yellowish to brownish-green	Yellowish to brownish-green
Thallus habit	Not observed	Erect, 6.5 cm in length	Erect, 14 cm in length	10 cm in length	10-15 cm in length	Erect, 30 cm in length	Erect, 20 cm (<1 m depth) – 100 cm (>1m in depth) in length	200 mm in length
Thallus tips	Pointed	Not observed	Acute, sometimes with proliferations	Not observed		Attenuated and ultimate small	Taper to an acute point	Attenuate
Thallus segments	Absent	Present, 6 articulated segments. the segment base attenuated, the apex obtuse, often dilated or club-shaped	Narrow segments, segments usually 2 – 4 mm wide	Not observed	Not observed	Not observed	Absent	Absent
Texture of thallus	Not observed	Soft to slightly cartilaginous, smooth	Almost cartilaginous	Not observed	Soft to slightly cartilaginous, smooth	Not observed	Cartilaginous	Not observed
Holdfast	Discoid	Irregularly discoid	Discoid	Not observed		Not observed	No holdfast	Discoid
Branching pattern	Irregularly	Irregularly subdichotomous to trichotomous to alternate	Mostly dichotomous, up to many orders	Di-or sub-dichotomously	Di-or sub-dichotomously	Irregularly	Repeatedly and irregularly branched, up to four orders	Irregular, slightly constricted at the base, enlarged at the middle and become attenuate at the tip
Cell size gradation	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
Cystocarp shape and size	Not observed	Dome-shaped, with ostiole at top	Not observed	Dome-shaped, scattered over the mature parts of the thallus	Not observed	Sub-spherical, elevated and scattered over the thallus	Cystocarps scattered irregularly over the surface of all branches; mature cystocarps ostiolate, up to 1mm high and 1.2mm wide	Not observed
Nutritive filaments	Not observed	Absent	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
Pericarp	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	Pericarp tissue up to 220µm and 13 cell layers thick	Not observed

Gonimoblast	Not observed	Composed of loosely arranged ovoid vacuolate cells bearing clusters of carposporangia at periphery in short branched chains	Not observed	Not observed	Not observed	Not observed	Gonimoblast parenchyma dense, central cells up to 20μm wide	Not observed
Spermatangial conceptacles	Not observed	Confined to unevenly distributed soral patches, forming deep pits embedded in the cortex, each with a narrow opening to the outside and with slender compressed cortical cells attached to the outer conceptacle wall	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
Diameter	1-2 mm	3 mm	Not observed	Not observed	Not observed	Not observed	0.8 mm	1-2 mm
Wall thickness (μm)	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
Cortex	1–2 layer of small rounded cortical cells	1-2 layers	1-2 layers	1–2 layers	Not observed		Cortex of large branches 1-3 cells thick, the cells darkly staining, up to 14μm long and 10μm wide. Subcortex 1–2 cells thick, cells lightly staining, globose, up to 80μm long and 40μm wide	2–3 layers of small rounded cortical cells
Medulla	3–4 layers of parenchymatous cells	Large central cells	Large central cells	Large central cells	Not observed	Not observed	Medulla in this region up to 7 cells wide, the cells lightly staining, polygonal to spherical, increasing in size towards the center, up to 320μm long and 240μm wide	3–4 layers of parenchymatous cells
Habitat	Attach to net of cage culture	Lower mid littoral zone, rock polls	Lower mid littoral zone and infra littoral fringe	Upper mid littoral zone, tide pools	Lower mid littoral zone, tide pools	Lower mid littoral zone, tide pools	Not observed	Attached to root of mangrove Trees
Distribution in this study	Kondang Merak Beach	Kondang Merak Beach	Kukup Beach, Karapyak Beach	Kukup Beach, Menganti Beach, Nusakambangan Beach	Nusakambangan Beach	Nusakambangan Beach, Karapyak Beach	Sayang Heulang Beach	Sayang Heulang Beach

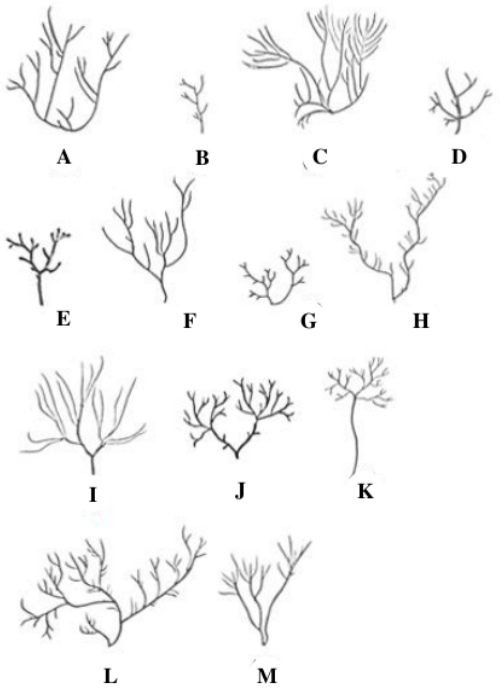

Figure 3. Branching type of *Gracilaria* spp. collected along the South Coast of Java Island, Indonesia. A. KM1: *Gracilaria coronopifolia* Kondang Merak; B. KM3: *Gracilaria salicornia* Kondang Merak; C. K1: *Gracilaria corticata* Kukup; D. K2: *Gracilaria debilis* Kukup; E. M1: *Gracilaria debilis* Menganti; F. NK1: *Gracilaria gigas* Nusakambangan; G. NK2: *Gracilaria debilis* Nusakambangan; H. NK3: *Gracilaria coronopifolia* Nusakambangan; I. F1: *Gracilaria coronopifolia* Karapyak; J. F4: *Gracilaria corticata* Karapyak; K. F6: *Gracilaria corticata* Karapyak; L. SH4: *Gracilaria gracilis* Sayang Heulang; M. SH6= *Gracilaria blodgettii* Sayang Heulang)

The blast analysis search results can be seen in Table 2, which showed the 13 samples consisted of 5 species, namely *Gracilaria edulis*, *Gracilaria salicornia*, *Gracilaria textorii*, *Gracilaria firma* and *Hypnea* sp. Romdoni et al., 2018 studied seaweed diversity based on their morphological features in two beaches along the Southern Part of Java Island, i.e. Drini and Kondang Merak. They found that Rhodophyta was the most abundant seaweed in both beaches, including *G. salicornia* and *G. edulis* also. This current study confirmed *G. salicornia* and *G. edulis* presence in Kondang Merak Beach through molecular identification using Cox 1 gene marker.

There are some differences between morphological and molecular identification, as shown in Table 3. Some studies showed Gracilaria species identification based on the morphological approach often led to misidentification. The misidentification of three Gracilaria species in Malaysia i.e. *G. blodgettii*, *G. arcuata*, and *G. changii*, was observed by Hassan et al. 2019. Molecular identification based on 672 bp CO1-5P gene sequence analysis indicated those three Gracilaria species need to be identified as one, namely *Gracilaria blodgettii* (Harvey 1853). Ng et al. (2017) validated two Gracilaria species, i.e. *G. changii* and *G. firma*.

Some study teams investigate the mitogenome sequence and complete plastid genome to obtain detailed molecular and phylogenetic information on Gracilaria species. Mitogenomes sequence is one of the fundamental approaches used to analyze the taxonomy and phylogeny of red algae (Boo et al. 2016). Sedanza et al. (2020) determined that the complete *G. edulis* mitogenome length was 25,708 bp and this species clustered together with *G. changii* and *G. salicornia*. Wang et al. (2020) found *G. chilensis* plastid genome sequence was 185,640 bp and it had a closer relationship with *Gracilaria tenuistipitata* var. liui in Gracilaria. Also, Liu et al. (2017) found the complete mitochondrial genome of *G. chilensis* was 26,897 bp and that *G. chilensis*, *G. salicornia*, and *G. changii* shared a closer relationship than *G. vermiculophylla* in the genus Gracilaria. Song et al. (2016) observe *G. salicornia* and found the mitogenome length was 25,915 bp containing 50 genes. Gracilaria species misidentification occurred due to high plasticity. since they have simple morphological features, lack of distinct morphological feature differentiation and complex heteromorphic reproduction cycle. Hence, identifying them solely based on morphological features is difficult (Zhao et al. 2013).

Phylogenetic analysis

Molecular identification using the COX1 gene can be further seen from the phylogenetic tree (Figure 4). To compare other data, 15 COX1 sequences were obtained from GenBank. The phylogenetic tree results showed each of the 13 sample and comparative species sequences assessed using the COX1 gene had different genetic distances, both intraspecific and interspecific. Overall, the intraspecific genetic distance ranged from 0-1.0%, while the interspecific genetic distance of *Gracilaria* spp. ranged from 10.4-14.7%. Also, the interspecific genetic distance of the genus *Gracilaria* and *Hypnea* ranged between 16.3-20.5%. This value is comparable to the study of *G. vermiculophylla* presence in the western Atlantic region where the intraspecific genetic distance of *Gracilaria* species ranged between 0.0% and 1.73% (Gulbransen et al. 2012).

COX1 genes utilization for analysis of genetic diversity, both intraspecific (genetic variation within species) and interspecific (variations between species) has produced good results. This can be seen from the distance or genetic variation both intraspecifically and interspecifically. The intraspecific diversity of *G. salicornia* was 0-1%, *G. edulis* was 0 to 0.6%, and *G. firma* was 0-2%, while that of *G. textorii* was 0-10.6%. According to Yang and Kim 2015, intraspecific diversity has a genetic distance ranging from 0-2.6%. A very high intraspecific genetic distance in the COX1 gene causes errors in the identification process (Robba et al. 2006).

Table 2. The BLAST results obtained from the Cox1 gene partial sequences for each sample from six different beaches in Indonesia were compared with the results of morphological identification results

Sampling location	GPS coordinate	Code	Genbank strain	No. accession	Similarity
Kondang Merak	8°23'52.44"S	KM1	*Gracilaria edulis*	**KY995645**	99,68 %
	112°31'25.62"T	KM3	*Gracilaria salicornia*	**KF831096**	100 %
Kukup	8°06'48.68"S	K1	*Gracilaria edulis*	KY995645	99,67%
	110°34'44.41"T	K2	*Gracilaria salicornia*	**KF831096**	100%
Menganti	7°45'32.18"S	M1	*Gracilaria textorii*	**NC_037892**	90,2%
	109°25'19.41"T				
Nusakambangan	7°45'15.29"S	NK1	*Gracilaria edulis*	**KY995645**	99,68 %
	109°01'04.68"T	NK2	*Gracilaria salicornia*	**KF831096**	100 %
		NK3	*Gracilaria firma*	**KY315283**	100 %
Karapyak	7°42'9.86"S	F1	*Gracilaria edulis*	**NC_037889**	99,42%
	108°45'38.28"T	F4	*Gracilaria edulis*	**KY995645**	99,42%
		F6	*Gracilaria edulis*	**KY995645**	99,67%
Sayang Heulang	7°38'16.34"S	SH4	*Hypnea sp.*	**EU240816**	90,27 %
	107°41'50.30"T	SH6	*Hypnea sp.*	**EU240816.1**	90,27 %

Table 3. Comparison of *Gracilaria* species morphological and molecular identification

Sampling location	GPS coordinate	Code	Morphological identification	Molecular identification
Kondang Merak	8°23'52.44"S	KM1	*Gracilaria coronopifolia*	***Gracilaria edulis***
	112°31'25.62"T	KM3	*Gracilaria salicornia*	***Gracilaria salicornia***
Kukup	8°06'48.68"S	K1	*Gracilaria corticata*	*Gracilaria edulis*
	110°34'44.41"T	K2	*Gracilaria debilis*	*Gracilaria salicornia*
Menganti	7°45'32.18"S	M1	*Gracilaria debilis*	*Gracilaria textorii*
	109°25'19.41"T			
Nusakambangan	7°45'15.29"S	NK1	*Gracilaria gigas*	***Gracilaria edulis***
	109°01'04.68"T	NK2	*Gracilaria debilis*	***Gracilaria salicornia***
		NK3	*Gracilaria verrucose*	***Gracilaria firma***
Karapyak	7°42'9.86"S	F1	*Gracilaria verrucosa*	*Gracilaria edulis*
	108°45'38.28"T	F4	*Gracilaria corticata*	*Gracilaria edulis*
		F6	*Gracilaria corticata*	*Gracilaria edulis*
Sayang Heulang	7°38'16.34"S	SH4	*Gracilaria gracilis*	***Hypnea* sp.**
	107°41'50.30"T	SH6	*Gracilaria blodgettii*	***Hypnea* sp.**

However, interspecific diversity between *G. salicornia* and *G. firma* ranged from 13.9-14.7%, *G. salicornia* and *G. edulis* was 12.4-12.9%, *G. firma* and *G. edulis* was 13.5-14.1%, *Gracilaria textorii* and *G. salicornia* was 11.8-13.9%, *G. textorii* and *G. edulis* was 12.9-13.9%, while *G. textorii* and *G. firma* was 10.6 to 12.9%. Yang and Kim, 2015 conducted a molecular analysis using mitochondrial COX1 and plastid rbcL genes for identifying the Gracilaria species obtained from the Asia–Pacific region. The range of intra-and inter-specific divergence for COX1 gene was 0–2.6 % and 2.8–15.7 %, respectively. RbcL data showed that the interspecific divergences ranged from 1.1 to 14.8 % and divided into two large clades, such as Gracilaria sensu lato and Gracilariopsis (Yang and Kim 2015).

Low nucleotide variations in molecular markers within and between species are prerequisites for coding (Saunders 2005; Robba et al. 2006).

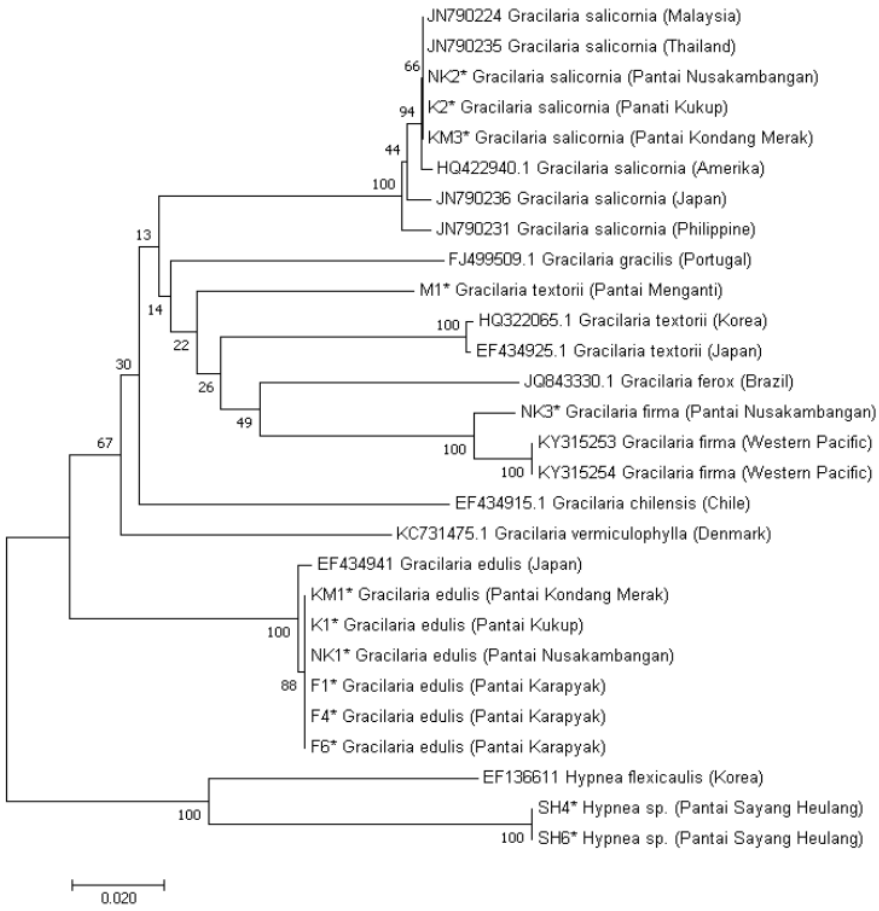

Figure 4. Phylogenetic tree of *Gracilaria* spp. collected along the South Coast of Java Island, Indonesia

Molecular identification of the seven samples obtained from the six beaches showed one *Hypnea* sp. and four *Gracilaria* spp. Furthermore, the *Hypnea* sp. consisted of two samples from Menganti and Pantai Sayang Heulang Beach. Also, the *Gracilaria* spp. consisted of 12 samples, namely *G. salicornia* (from Kondang Merak, Kukup, and Nusakambangan Beach), *G. edulis* (from Kondang Merak, Kukup, Karapyak, and Nusakambangan Beach), *G. firma* (from Nusakambangan Beach) and *G. textorii* (from Karapyak and Menganti Beach). Moreover, genetic diversity analysis was performed by observing haplotype diversity (Hd). Based on the molecular identification results, *Gracilaria* spp, intraspecifically, consisted of three species namely *G. edulis*, *G. salicornia* and *G. textorii*. But, interspecifically, it consists of four species namely *G. salicornia*, *G. edulis*, *G. firma* and *G. textorii*. Intraspecific genetic diversity analysis of *Gracilaria* spp was carried out using DNAsp 5.0 application. This showed *G. edulis* and *G. salicornia* obtained from the beaches of Kondang Merak, Kukup, Nusakambangan, and Karapyak had a diversity value of 0.53 and 0.67, respectively, and both belonged to the moderate diversity category, while the intraspecific diversity of *G. textorii* obtained from Menganti and Karapyak Beach had a value of 0 or no diversity. Valtueña et al. 2014 stated low genetic diversity is caused by inbreeding or an inbreeding within a population.

The results of genetic diversity analysis conducted using the COX1 gene showed the intraspecific diversity of *G. edulis* obtained from the beaches of Kondang Merak, Kukup, Nusakambangan, and Karapyak was included in the moderate diversity category. Also, the intraspecific diversity of *G. salicornia* obtained from the beaches of Kondang Mera, Kukup, and Nusakambangan was included in the moderate diversity category, while the intraspecific diversity of *G. textorii* from Menganti and Karapyak Beach had no diversity. Further study is recommended to be conducted on genetic variability of seaweed *Gracilaria*

spp. obtained from Kondang Merak, Nusakambangan, and Sayang Heulang Beach using genes other than the COX1. Among the 230 Gracilaria species found globally, only 150 have been described and identified (Lyra, 2015; Guiry and Guiry 2020). In Indonesia, few Gracilaria species have been studied and identified, hence there is a need for more exploration and identification efforts. Species molecular identification is an important step that we must conduct before exploring and utilizing the Gracilaria species. By identifying the Gracilaria species we can do more explorations and develop Gracilaria species to become high-value products.

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