Identification of *Gracilaria* spp. in Gunungkidul Regency, Yogyakarta Indonesia Based on DNA *Barcoding* Target *Cytochrome Oxidase Subunit* 1

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Abstract

Seaweed is one of the most abundant biological resources in Indonesia. The number of species of Gracilaria spp. in Indonesia encourages the need for studies on diversity. This study aims to identify species diversity and phylogeny analysis of Gracilaria spp. found in Gunungkidul Regency, Yogyakarta. The sample collection was taken in February 2020 at Sepanjang and Krakal Beaches and in September 2020 at Siung and Watukodok Beaches, Gunungkidul, Yogyakarta. This research uses the methods of sample preservation, DNA isolation/extraction, DNA amplification, and COI mtDNA gene sequencing. Molecular identification of Gracilaria spp. successfully performed by DNA barcoding method using a commercial kit (Genaeid Genomic DNA Mini Kit (Plant)). Gracilaria spp. successfully amplified with primers GazF1 and GazR1 encoding the mtDNA COI gene at annealing temperature of 44 °C; 44.4 °C and 44.6 °C. Species Gracilaria spp. that found were G. salicornia (from Sepanjang and Krakal Beach), G. edulis (from Siung Beach), G. arcuata (from Watu Kodok Beach) and Gracilaria sp. (from Krakal Beach). The species of the Krakal Beach specimen of Gracilaria sp. is unknown, however it belongs to the genus Gracilaria, so further identification of the sample is needed. G. arcuata (from Watu Kodok Beach) is recognized as a species of G. arcuata, however it is 12.8 percent genetically distant from the source. Genetic diversity on the Sepanjang, Krakal, and Watu Kodok beaches is included in the high diversity category, while Siung Beach does not have diversity. This research found four species of Gracilaria spp. based on molecular identification in Gunungkidul, Yogyakarta.

Keywords: *COI, DNA barcoding, seaweed, mtDNA*

Introduction

Macroalgae or seaweed is one of the abundant biological resources in Indonesia. *Gracilaria* spp. is a species of red algae (Rhodophyceae) that has a potential role as agar material and has high economic value in Indonesia (Lyra *et al*., 2015). *Gracilaria* spp. widely used as an emulsifier, stabilizer, thickener, and various other functions in the food sector (Dita *et al*., 2020). It also has important economic value for producing ethanol, as abalone feed, immunostimulant for shrimp, waste management, biofilter, as well as pharmaceuticals and drugs (Oves *et al*., 2012; Handhani *et al*., 2017; Hengkengbala *et al*., 2018; Supriyantini *et al*., 2018). There are many important benefits of *Gracilaria* spp. and there are still many misunderstandings about the name. Therefore, it is necessary to identify species names clearly so that they can be used as guidelines in the breeding and utilization of *Gracilaria* spp.

Identification of seaweed with morphological characters is difficult to ascertain because of the level of its morphological plasticity (Anggraeni *et al*., 2008; Djakatara *et al*., 2018). According to Burdames and Ngangi (2014), morphological characteristics can be influenced by several environmental factors including waves, sunlight, air and water temperature, salinity, and pH. In addition, genetic factors can also affect the differences in the quality and characteristics of algae. The use of DNA sequences is expected to confirm morphological confusion. The use of DNA barcodes on mitochondrial COI genes has been successfully used to identify red algae (Clarkston and Saunders, 2010; Gall and Saunders, 2010; Sherwood *et al*., 2011; Annisaqois *et al*. 2018). The mitochondrial COI gene was used because it is more sensitive to reveal the population structure and diversity of red algae (Freshwater *et al*., 2010) and is a universal barcode marker (Kucera and Saunders, 2012; Yang *et al*., 2013). DNA barcoding also plays for identification of cryptic species, especially when morphological characters are difficult to analyze (Sherwood, 2008; Kim *et al*., 2010; Barcaccia *et al*. 2015).

Many species of *Gracilaria* spp. are found in Indonesia, but few have been reported and still

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limited according to morphological identification. Research on the diversity of seaweed, especially *Gracilaria* spp., is useful as a guide in the management of marine biological resources and conservation. Previously, the molecular analysis of *Gracilaria* spp. from South Coast of Java Island, especially from Drini beach has been carried out (Meinita *et al*., 2021). However, the samples analyses from other area have not been carried out, therefore this research was aimed to identify the diversity of Gracilaria spp. in Panjang, Krakal, Siung and Watu Kodok beach, Gunungkidul Regency, Yogyakarta Indonesia based on DNA barcoding analysis using COI mtDNA gene (Cytochrome Oxidase I mitochondrial DNA).

Materials and Methods

Sampling was carried out in February-September 2020 at Sepanjang (1), Watu Kodok (2), Krakal (3), and Siung (4) Beach, Gunungkidul Regency, Yogyakarta (Figure 1). Morphological observations and molecular analysis were carried out at the laboratory of Aquatic Resources Management, Department of Fisheries, Universitas Gadjah Mada.

Procedure

Sampling and morphological identification

Samples of red algae *Gracilaria* spp. taken by tracing the coastline in the intertidal area with a distance of 10-15 m from the shoreline by looking at the morphology of the algae suspected of being *Gracilaria* spp. The samples found were then put into a plastic ziplock and labeled then stored in a cooling box containing ice cubes to be brought to the laboratory for further morphological and molecular analysis.

Morphological identification was carried out by observing and comparing the characteristics of the samples according to Silva *et al*. (1996); Lyer *et al*. (2004); Jha *et al*. (2009); Hassan *et al*. (2019) and using the algabase website of https://www.algaebase.org/ (Guiry and Guiry (2020). Several morphological characteristics were carefully observed, namely thallus shape, segment shape, seaweed shape, clumps, branching type, thallus length, and holdfast.

Sample preservation

Preservation is done by washing the sample with running water to remove the dirt attached on the algae. Furthermore, it was washed using aquabidest and then air-dried. Each sample of algae is put into a plastic clip and 96% ethanol is added until it is submerged.

DNA Isolation

DNA extraction of *Gracilaria* spp. was performed using a DNA extraction kit by following the Genomic DNA Mini Kit (Plant) Protocol from Genaeid. The DNA that has been obtained in the DNA isolationprocess is then separated by 1% agarose gel electrophoresis with 1X buffered TrisBorate-EDTA (TBE)

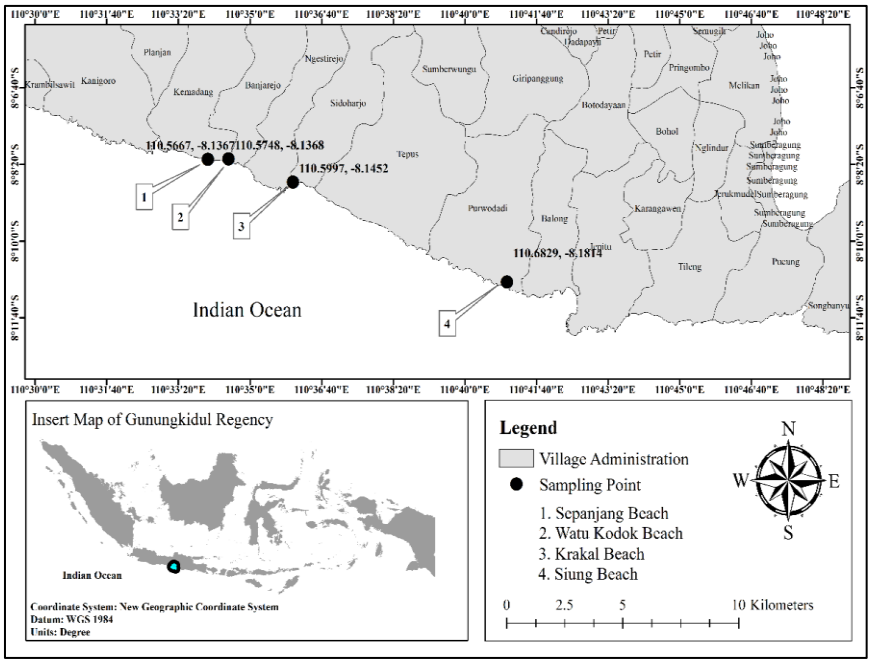

Figure 1. Sampling location in Gunungkidul Regency, Yogyakarta

solution (Nasihin *et al*., 2015). For genome electrophoresis, 5 L of the sample was taken and mixed with 1 L of loading dye and placed on the mold after the 100 bp DNA ladder, then the electrophoresis process was carried out for 20 minutes. The agarose gel was removed and placed on a UV-transilluminator to observe the presence of DNA bands. If there is DNA, it can be continued with the amplification process by PCR.

Amplification DNA

Amplification was carried out using a Thermal Cycler T100 Biorad combi block PCR machine (Whatman Biometra Germany). The steps in PCR consist of amplification of DNA products and checking of amplicon by electrophoresis. The primers used for the PCR process were primer pairs (GazF1 5'-TCAACAAATCATAAAGATATTGG-3' & GazR1 5'-ACTTCTGGATGTCCAAAAAAYCA-3') (Saunders and Lehmkuhl, 2005). Furthermore, the mixture is homogenized by tapping or by a spindown process using a mini centrifuge. After all homogenized, the mixture was put into a thermocycler for DNA propagation.

Pre-denaturation was carried out at 94°C for 5 mins. DNA denaturation was carried out at 94°C for 30 secs. Annealing was carried out at 44°C for samples A, B, C, D, E, and H, while samples F and G were at 44.6°C for 30 secs. Meanwhile, the extension process was carried out at a temperature range of 72°C for 1.25 mins. The three stages were repeated for 34 cycles. The last stage is post-extension at 72°C for 5 mins. After the PCR process is complete, the electrophoresis process is carried out again.

Gen COI Sequencing

The sequencing process is carried out by sending PCR DNA to the 1st base sequencing services through PT. Indonesian Genetics Science.

Genetic diversity

DNAsp 6.10 software was used to determine the diversity of haplotypes (Hd) (Nei, 1987) in each population.

Data analysis

Data analysis was carried out on the sequencing results, by editing the results through the MEGA-X software (Kimura, 1980; Kumar *et al*., 2018). The selected DNA segment is the best reading quality. After obtaining good quality DNA segments, the nucleotide sequences were analyzed using the BLAST (Basic Local Alignment Search Tool) program which is accessed directly from the MEGA-X software or can also be accessed through the NCBI website (http://blast.ncbi.nlm.nih. gov/Blast.cgi). The results of the BLAST analysis showed several species names and the degree of similarity of the nucleotide sequences of the corresponding DNA in the NCBI GenBank database.

The kinship analysis was carried out between samples of Gracilaria spp. obtained from the results of data analysis with *Gracilaria* spp. contained in GenBank which was analyzed using MEGA-X software. This kinship analysis uses the Pairwise Distance method by comparing the sample sequences with the Gracilaria spp sequences. on GenBank. Furthermore, based on the genetic distance, a phylogenetic analysis will be carried out using a phylogenetic tree that has previously been sequenced with ClustalW. This phylogenetic tree was made based on the sample nucleotide sequences and several *Gracilaria* spp. sequences. from GenBank. Making this phylogenetic tree using MEGA-X software with the Test Neighbor Joining Tree method.

Result and Discussion

Morphological identification

According to the morphological characters (Silva *et al*., 1996; Lyer *et al*., 2004; Jha *et al*., 2009; Hassan *et al*., 2019) of eight samples of *Gracilaria* spp., they were species of *G. salicornia*, *G. arcuata*, *G. canaliculata*, *G. foliifera* and *G. debilis*. (Figure 2).

Gracilaria spp. found in some beaches of Gunungkidul Regency, Yogyakarta have different morphological characteristic. The samples of A, D, and H were identified as *G. salicornia* because their cylindrical thallus were yellowish red to brownish in color and the length of upright cylindrical thallus were up to 5 cm. The tip of the thallus is blunt with little growth and a narrow segment is found at the base. The texture of the thallus is smooth with subdichotomous branches, and it is not filamentous. The holdfast type is disc-shaped with habitat in the intertidal zone with rocky sand and coral substrates (Silva *et al*., 1996; Lyer *et al*., 2004; Yang *et al*., 2013; Guiry and Guiry, 2020; Tega *et al*., 2020).

Samples of B and F identified as *G. arcuata* because the cylindrical thallus is reddish brown when it is fresh, with a small upright cylindrical thallus with length up to 2-3 cm. The tip of the thallus is pointed with two or more branches on each thallus. It has a smooth thallus texture, with forms dense and irregular growth, and the thallus is not filamentous. The holdfast type is disc-shaped with a habitat in the intertidal zone with rocky sand and coral substrates (Liao, 2018; Hassan *et al*., 2019; Guiry and Guiry, 2020).

The sample of C was identified as *G. canaliculata* (synonym with *G. crassa*) because the thallus is brownish red, with the upright thallus and the length up to 3 cm. The thallus is cylindrical or oval with a cartilaginous substance. The tip of the thallus is pointed with two or more branches on each thallus. Thallus texture is smooth branching irregular or dichotomous to trichotomies and has the main branch with a blunt apex, holdfast firmly attached to rocks or corals, and the thallus is not filamentous (Lyer *et al*., 2004; Silva *et al*., 1996; Guiry and Guiry 2020).

The sample of E was identified as *G. foliifera* because the cylindrical thallus is yellowish red, with a small cylindrical upright thallus, and the length up to 5 cm. The thallus is small and forms a dense clump. The tip of the thallus is pointed with dichotomous branches, while the thallus has irregular branches. The texture of the thallus is slightly rough, and it is not filamentous. The holdfast type is disc-shaped with habitat in the intertidal zone with sand and coral substrates (Jha *et al*., 2009; Kundu *et al*., 2017). The sample of G was identified as *G. debilis* by its reddish cylindrical thallus, with a long, upright, and small cylindrical thallus type with a length up to 5-7 cm. the tip of the thallus is pointed with two or more branches of each thallus, and the texture of the thallus is slightly rough. It has forms dense and irregular growth. The holdfast type is disc-shaped found in the intertidal zone with rocky sand and coral substrates (Jha *et al*., 2009).

Based on the results of morphological observations in Figure 2, it showed that there were variations in the characteristics of the morphology of each sample of *Gracilaria* spp. found in Gunungkidul Regency, Yogyakarta. The difference between samples is mostly the shape of the thallus and branching. According to Triastinurmiatiningsih *et al*., (2011), it is due to their genetic diversity. *Gracilaria* spp. is one of the red algae species with simple morphological features, lacks differentiation of morphological features, and has a complex heteromorphic reproductive cycle. Therefore, it is difficult to identify these species based on morphological features alone (Zhao *et al*., 2013), therefore requires reconfirmation using molecular characters Bast *et al*. (2014).

Figure 2. The species identification result using morphologycal character for Gracilaria spp. Samples from Gunungkidul Regency, Yogyakarta, Indonesia. (A. *G. salicornia*; B. *G. arcuata*; C. *G. canaliculata*; D. *G. salicornia*; E. *G. foliifera*; F. *G. arcuata*; G. *G. debilis*; H. *G. salicornia*) Scale bar: 2 cm.

Molecular analysis

COI gene electrophoresis results from *Gracilaria* spp. on UV irradiation showed a size of about 720 bp (Figure 3). Based on the results of sequence editing with MEGA-X software, the most parallel sizes were obtained in samples A, B, C, and D, i.e. 670 bp, while samples E, F, G, and H were 675 bp, previously from the entire sample the size of the sample was 720 bp. The next analysis then the DNA sequence of the sample were checked by comparing the samples in GenBank. Sequence analysis was performed using BLAST analysis through the website of the National Center for Biotechnology Information (NCBI), National Institute for Health. The result was shown in Table 1.

Table 1 shows the range of query cover obtained in this study ranged from 83-100%, while percent identity was in the range between 90.15-99.69%. According to Holman (2004), the minimum value of identity similarity is at least 95%. Therefore, the species sample C was still unidentified, therefore its name remained *Gracilaria* sp. The samples of *Gracilaria* spp. with >95% identity were *G. salicornia* (A and D) and *G. edulis* (E). Stoeckle (2003) states that the DNA barcoding method is highly dependent on the availability of accurate reference sequences for comparison. So that some of these things can trigger the low percentage of identity in samples C, G, and H. For this reason, further identification with phylogenetic methods is needed to compare the identified species with sources in GenBank.

Phylogenetic analysis

Huang *et al*. (2016) stated that phylogenetic trees describe the evolutionary lineages of species, organisms or from a single common ancestor. Genetic distance is considered low if it has a value between 0.010-0.099; medium 0.100-0.99; and a high of 1.00-2.00 (Nei, 1972). Based on the phylogenetic tree, it showed the genetic relationship between species in one population and between populations. Eight sample sequences were found in this study and twenty-five sequences of *Gracilaria* spp. COI gene from GenBank created a phylogenetic tree as shown in Figure 4.

Species that have been identified based on the highest percent identity similarity (Table 1.) are *G. salicornia* (A and D) and *G. edulis* (E), while specimens B and F confirmed based on BLAST results are not *Gracilaria* spp., but *Hypnea nidulans*. These results were confirmed by phylogenetic analysis by comparing the results from this study with sources on GenBank. The *G. salicornia* identified from Sepanjang (A) and Krakal (D) beaches were confirmed to be *G. salicornia* because they were monophyletic or located on the same branch as *G. salicornia* from China and Thailand with low intraspecific genetic distance values 0-0.003 (0-0.3%). The E specimen from Siung Beach confirmed by phylogenetic analysis is indeed *G. edulis* because it is located in the same branch as *G. edulis* from the Philippines with a low intraspecific genetic distance value of 0-0.005 (0-0.5%). Meanwhile, specimens B (from Sepanjang Beach)

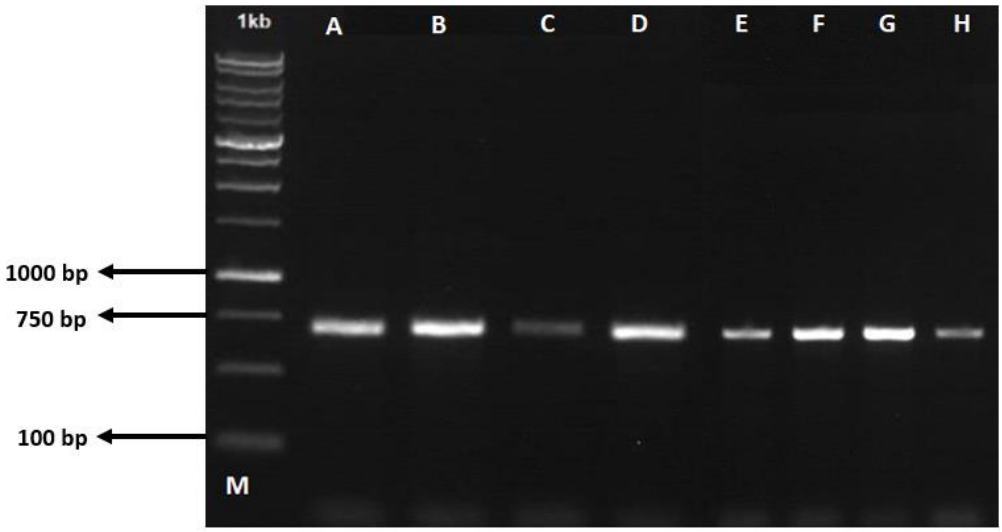

Figure 3. Visualization of the COI gene electrophoresis results of *Gracilaria* spp.
Note: M= DNA Ladder with size 1kb (bp); A= Sample 1 *Gracilaria* spp. from Sepanjang beach; B= Sample 2 *Gracilaria* spp. from Sepanjang beach; C= Sample 1 *Gracilaria* spp. from Krakal beach; D= Sampel 2 *Gracilaria* spp. from Krakal beach; E= Sampel 1 *Gracilaria* spp. from Siung beach; F= Sampel 1 *Gracilaria* spp. from Watu Kodok beach; G= Sampel 2 *Gracilaria* spp. from Watu Kodok beach; H= Sampel 3 *Gracilaria* spp. from Watu Kodok beach

and F (from Watu Kodok Beach) were confirmed not *Hypnea nidulans* from the Philippines with a low intraspecific genetic distance value of 0-0.013 (0-1.3%).

Figure 4 shows that the sampel C from Krakal Beach was identified as *Gracilaria* sp. because of one clade with other species of *Gracilaria* sp. and is located in a different branch of another genus (*Hypnea* sp.). However, the species in this specimen is still unknown because the C specimen is located on a different branch from the other *Gracilaria* sp. species (*G. salicornia, G. arcuata, G. debilis, G. foliifera and G. edulis*) so that based on phylogenetic analysis it is possible that the C specimen is not this species and further identification is needed. Specimen G from Wediombo Beach was confirmed not to be a *Gracilaria* spp., but indeed a *H. bullata* because it is monophyletic with *H. bullata* from India with a low intraspecific genetic distance but quite far apart (7.7%). This can happen because *Gracilaria* sp. has high plasticity (capable of adapting its morphological shape according to its environment) (Veeragurunathan *et al*., 2015) and shares a morphological similarity with the genus Hypnea (especially in the cylindrical thallus shape). Meanwhile, the specimen H previously identified as *G. arcuata* was confirmed to be *G. arcuata* because it is located on the same branch as *G. arcuata* from Japan and the Philippines with intraspecific distance values that are quite far apart, namely 0-0.128 (0-12, 8).

There is different result in morphological (Figure 2.) and molecular identification of *Gracilaria* spp. The diifferent identification of *Gracilaria* spp. occurs due to high plasticity. Therefore, it is difficult to identify these species based on morphological features alone (Zhao *et al*., 2013). Morphological differences among seaweed species, and their ability to change morphology vegetatively (called plasticity) in different environments make morphological identification quite difficult (Veeragurunathan *et al*., 2015). So in this case it is necessary to confirm the species using molecular analysis, especially the DNA barcoding method. This molecular analysis has been widely applied to reveal the biodiversity of seaweeds in the world (Zuccarello *et al*., 2019). Molecular characters can be used to detect variations in genotypes or even genes so that between cultivars can be clearly distinguished (Edison *et al*., 2004). In addition, the use of molecular COI also has drawbacks, namely low differences between species and high mutations when compared to other markers (e.g. rbcL) (Sundari and Papuangan, 2019) and sometimes missing datasets during phylogenetic analysis (Freshwater *et al*., 2010).

Based on phylogenetic and molecular analysis, it can be seen that *Gracilaria* spp. found in Gunungkidul Regency, Yogyakarta based on COI gene targets, namely *G. salicornia* (from Sepanjang and Krakal Beach), *G. edulis* (from Siung Beach), *G. arcuata* (from Watu Kodok Beach) and *Gracilaria sp*. (from Krakal Beach). Romdoni *et al*. (2018) conducted research on seaweed diversity based on morphological characteristics on two beaches along the southern part of Java Island, namely Drini Beach, Yogyakarta, and Kondang Merak Beach, East Java.

The study showed that Rhodophyta was the most abundant seaweed on coasts of Gunung Kidul, one of which was *G. salicornia* and *G. edulis*. This study confirmed that *G. salicornia* and *G. edulis* species were also found in Yogyakarta (Sepanjang, Krakal, and Siung Beach) by molecular identification using the COI gene marker. The same result was also confirmed in the study by Meinita *et al*. (2021) found that *G. salicornia* and *G. edulis* were found in Kukup Beach, Yogyakarta.

Gracilaria sp. from Krakal Beach phylogenetically confirmed it is indeed the genus Gracilaria, but the exact species is not yet known, so further identification of the sample is needed. *G. arcuata*

Table 1. Percent identity of nucleotide base on *Gracilaria* spp. from BLAST (NCBI)

Sample Code	Morphologically identified species	Moleculary identified species	Query Cover (%)	Percent Identity (%)	References Accession Number	Sampling location
A	*G. salicornia*	*Gracilaria salicornia*	99	99,68	KF831096.1	Sepanjang Beach
B	*G. arcuata*	*Hypnea nidulans*	83	98,47	FJ694913.1	Sepanjang Beach
C	*G. canaliculata* (synonim *G. crassa*)	*Gracilaria* sp.	95	90,15	KT357392.1	Krakal Beach
D	*G. salicornia*	*Gracilaria salicornia*	99	99,68	KF831096.1	Krakal Beach
E	*G. foliifera*	*Gracilaria edulis*	100	99,69	MZ336086.1	Siung Beach
F	*G. arcuata*	*Hypnea nidulans*	83	98,47	FJ694913.1	Watu Kodok Beach
G	*G. debilis*	*Hypnea bullata*	90	93,12	MT996223.1	Watu Kodok Beach
H	*G. salicornia*	*Gracilaria arcuata*	99	91,69	MT996223.1	Watu Kodok Beach

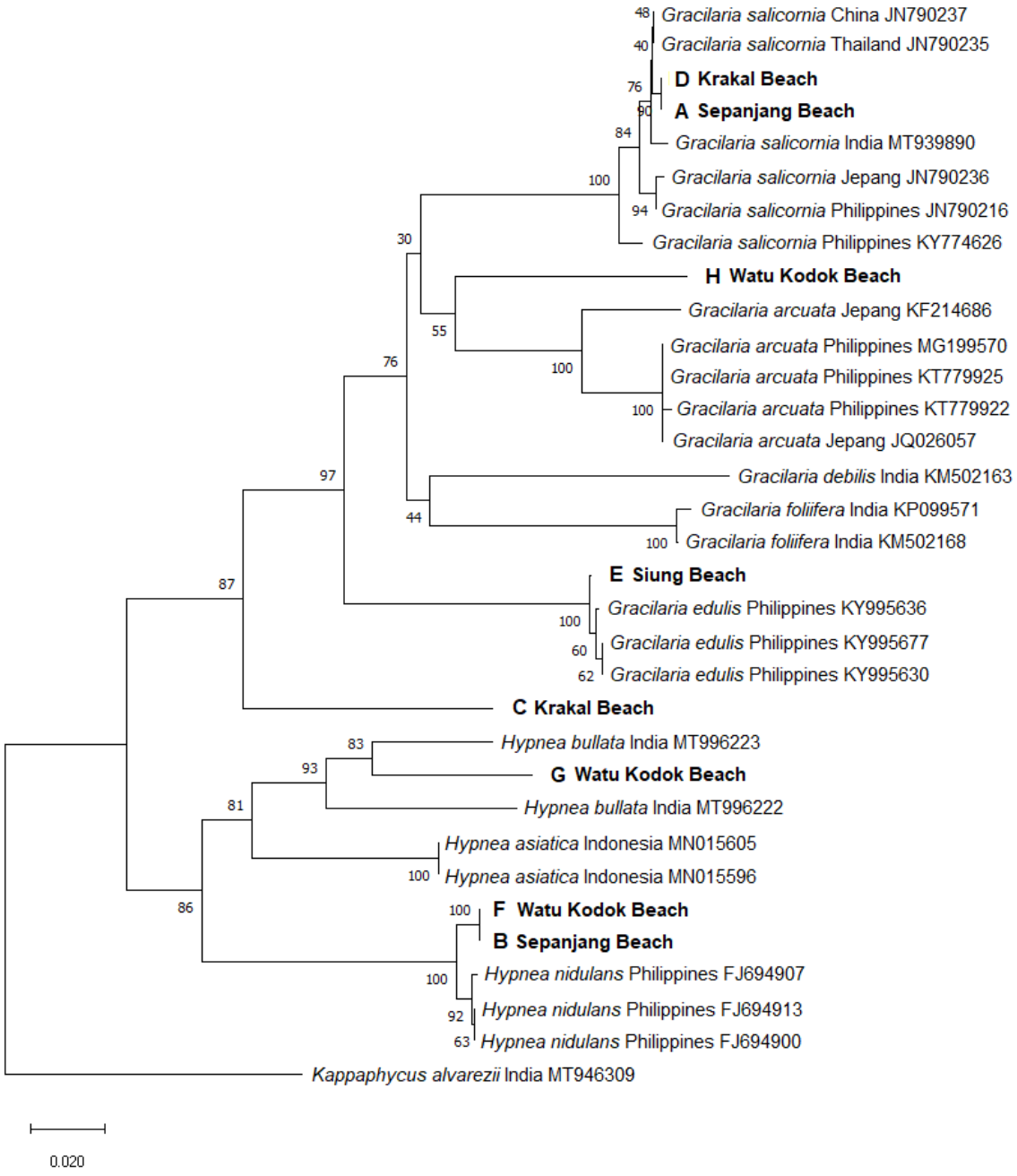

Figure 4. The phylogenetic tree of the *Gracilaria* spp. From Gunung Kidul Beach with other seaweed species using the Neighbor joining (NJ) method with Bootstrap 1000X.

(from Watu Kodok Beach) is confirmed to be *G. arcuata*, but it has a genetic distance that is far a drift from the source (12.8%) refer to Yang and Kim (2015) who stated that intraspecific diversity has a genetic distance ranging from 0-2.6%. Therefore that a more in-depth identification analysis is needed.

Genetic diversity for haplotype observations (Hd) was described by Nei (1987) and the value of genetic diversity ranging from 0.8 to 1 belong to high category, while values from 0.5 to 0.7 are classified in the medium category, and 0.1-0.4 is in a low category. The analysis of the diversity of *Gracilaria* spp. from the Sepanjang, Krakal, and Watu Kodok beaches in this study showed the value of haplotype diversity (Hd) 1 and was included in the high diversity category, while Siung Beach had a value of 0 or no diversity. The low level of haplotype diversity in Siung

Beach is possible because of inbreeding, which can give negative impact for the population because it can reduce the genetic diversity of a population (Valtuena *et al*., 2014).

Conclusion

Based on COI gene targets, *Gracilaria* spp. found in Gunungkidul Regency, Yogyakarta were identified as *G. salicornia*, *G. edulis*, *G. arcuata* and *Gracilaria* sp. The genetic diversity of COI genes of Gracilaria species in the Sepanjang, Krakal, and Watu Kodok beaches regions were in the high diversity category, while Siung Beach did not have the diversity. Molecular identification in Gracilaria confirm the morphological identification which is difficult to ascertain due to its plasticity.

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