RESEARCH ARTICLE

Molecular Characterization of *Caulerpa racemosa* (Caulerpales, Chlorophyta) from Indonesia Based on the Plastid *tuf*A Gene

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Abstract

The green seaweed *Caulerpa racemosa* is a seaweed of high prospect that is being given emphasis by the Indonesian government. However, *C. racemosa* in Indonesia may include multiple species level-entity exhibiting morphological overlap and require molecular analysis for species identification. Molecular documentation of species richness of indigenous populations of *C. racemosa* is essential to underpin cultivar development and conservation of the species to avoid overharvesting. The present study aimed to determine the genetic diversity of *C. racemosa* and document the haplotype network of the specimens from four different locations (Bintan Island, Jepara, Takalar and Osi Island) using the chloroplast *tuf*A gene. Twenty individuals from four areas were collected and amplified with the chloroplast-encoded gene *tuf*A for species identification against publicly available data. The identification of *C. racemosa* based on the *tuf*A gene showed that the species found in four locations were *C. cylindracea* (previously *C. racemosa* var. *cylindracea*), *C. macra* (previously *C. racemosa* var. *macra*), *C. racemosa*, and *C. oligophylla* (previously *C. racemosa* var. *lamourouxii*). This study records the existence of *C. cylindracea* in Takalar and Jepara, Indonesia for the first time. The most diverse *C. racemosa* species was in Osi Island, where the exploitation of this seaweed is very low. In contrast, the lowest number of *C. racemosa* varieties were found in Takalar, where exploitation is very high. There were only minor light variations of *Caulerpa* species in the *tuf*A gene in four different sites with only four haplotypes found, and each haplotype corresponded to another species.

Keywords: genetic diversity, haplotype, *Caulerpa macra*, *Caulerpa oligophylla*, *Caulerpa cylindracea*

Introduction

Caulerpa is one of the most widely distributed marine green algae in the tropical and warm-temperate seas (Draisma et al., 2014; Rushdi et al., 2020; Darmawan, Fajarningsih, Sihono, & Irianto, 2020). The genus of *Caulerpa* currently consists of 174 recognized species (Guiry & Guiry, 2021), many of which are associated with an exceptional capacity to adapt to a wide range of environmental factors such as temperature, depth, irradiance, and substrates. As such, *Caulerpa* can be found from the intertidal to subtidal zones (Baleta & Nalleb, 2016).

Caulerpa is a multinucleate siphonous green alga that differs from other coenocytic green algae, in a way that it has a trabeculae, or cell wall ingrowths that creates an anastomosing network for structural support (Zubia, Draisma, Morrissey, Varela-Álvarez, & De Clerck, 2020). The plants in the genus *Caulerpa* have the same basic morphological structure, which consists of rhizophores, stolon, and fronds (assimilators) (Manas et al., 2015; Zubia et al., 2020). The rhizophores of *Caulerpa* attach to rigid substrate or anchor to the unconsolidated substrate. The shape of stolon can be different from one species to another, such as smooth (glabrous) or covered in scale-like appendages (squamiferous). The fronds (assimilators) stand upright from the stolon. It has a branchlet (ramuli) with various arrangements and shapes (Zubia et al., 2020). The differences in gross morphology of fronds, stolons, and rhizoids and the form and size of ramuli were used to identify most species (Manas et al., 2015).

Some species of the genus *Caulerpa* are economically important because they can be sold in the local and international markets. Compared to *Gracilaria*, *Eucheuma*, and *Sargassum*, the production

of *Caulerpa* seaweed for the international trade is very small (less than 0.5%) from the total seaweed trade globally (Zubia et al., 2020). Caulerpa's two different species from the genus *Caulerpa* with a significant economic value are *C. racemosa* and *C. lentilifera* (Estrada, Bautista, & Dionisio-Sese, 2020). *C. racemosa* has a bigger vesiculate branchlet and is often called sea grapes or grape algae. In comparison, *C. lentilifera* has a smaller vesiculate branchlet and is usually called green caviar (Zubia et al., 2020). *C. lentillifera* has ramuli with globose tips constricted at the base and arranged imbricated in four rows. In contrast, *C. racemosa* has highly variable ramuli, can be stipitate or substipitate, and arranged irregularly - distichous, multiseriate, or imbricate (Estrada et al., 2020).

Seaweeds are known for their phenotypic plasticity, which means the same species of seaweeds can exhibit morphological variations under different environmental conditions (Estrada et al., 2020). Like other seaweed species, *C. racemosa* has a high level of phenotypic plasticity, making it one of the most taxonomically problematic species (Fama, Procaccini, Olsen, & Stam, 2000; Belton et al., 2014; Estrada et al., 2020; Zubia et al., 2020). Taxonomic issues associated with phenotypic plasticity has been reported in many algal groups (Sauvage et al., 2013; Dumilag et al., 2018; Belton et al., 2019; Ningrum & Chasani, 2021). The phenotypic plasticity can lead to misidentifications, nomenclatural quagmires, incorrect biodiversity estimates, and confusing classification schemes (Belton et al., 2014). Many of these issues have since been elucidated or resolved with the advance of molecular identification and characterization techniques.

The correct identification and characterization of seaweed species are essential to overcoming the taxonomic ambiguity of seaweed species, especially for those with economic interests. The growth factor and biochemical compounds can be differentiated and measured between areas and across the environmental condition. In Indonesia, the molecular identification of local seaweed has only been sporadically employed (Zuccarello & Paul, 2019). The advent of molecular tools has provided researchers with an independent and convenient method to delimit and identify species in taxonomically challenging groups (Belton et al., 2014). In recent years, the use of molecular identification techniques has improved our understanding of the biodiversity of *Caulerpa* (Fama et al., 2000; Žuljeviæ et al., 2003; Dumilag et al., 2019; Wynne, Verbruggen, & Angel, 2009; Sauvage, Wynne, Paul, & Fredericq, 2014). Today, species discrimination in *Caulerpa* is generally based on several genomic loci, with the chloroplast gene *tuf*A being used almost universally (de Senerpont Dormis et al., 2003; Kazi, Reddy, & Jha, 2013; Sauvage et al., 2013; Belton, Prud'homme Van Reine, Huisman, Draisma, & Gurgel, 2014; Belton, Draisma, Prud'homme van Reine, Huisman, & Gurgel, 2019; Dumilag et al., 2019).

The majority of the islands of Indonesia are located within the Coral Triangle, which is a hotspot for marine flora and fauna. This region, western tropical Atlantic, and Australia's southern coast (Sauvage et al., 2013) have been identified as areas with a high diversity of *Caulerpa*. Despite this, the molecular characterization of *C. racemosa* in Indonesia remains poor. Although morphological identification has identified 32 species of *Caulerpa* from the western to eastern parts of Indonesia, including 11 different morphotypes of *C. racemosa* (Atmadja & Prud'homme van Reine, 2014), these were not supplemented with genetic data. Owing to the commercial importance of *Caulerpa* in the international market, the Indonesian government has developed strategies to strengthen the country's position in the seaweed market (Ministry of Marine Affairs and Fisheries, 2017). The complete information about species richness and genetic biodiversity in Indonesia is essential to anticipate the overexploitation of *C. racemosa* in the future and obtain new strains to improve varieties with more desirable characteristics through tissue culture. Despite the biodiversity assessment, genetic diversity research is essential for exploiting and conserving *C. racemosa*. This study aimed to identify specimens of *C. racemosa* from Bintan Island, Jepara, Takalar, and Osi Island, Indonesia, using the *tuf*A DNA barcode and determining the haplotype network of the specimens in these locations.

Materials and Methods

Sample Collection

C. racemosa were collected from October 2018 – September 2019 at four different locations from the western to the eastern part of Indonesian waters. The sampling locations were Bintan Island (Riau Islands), Jepara (Central Java), Takalar (South Sulawesi), and Osi Island (Maluku) (Figure 1). Five specimens were collected from each site. The samples of *C. racemosa*, attached to the substrates, were collected from the subtidal zone. Individuals collected were at least 10 m apart to avoid the collection of ramets. Individuals were cleaned using seawater and desiccated using silica gel with a sample:silica gel ratio of 1:10. Samples were transferred to the laboratory for molecular analysis.

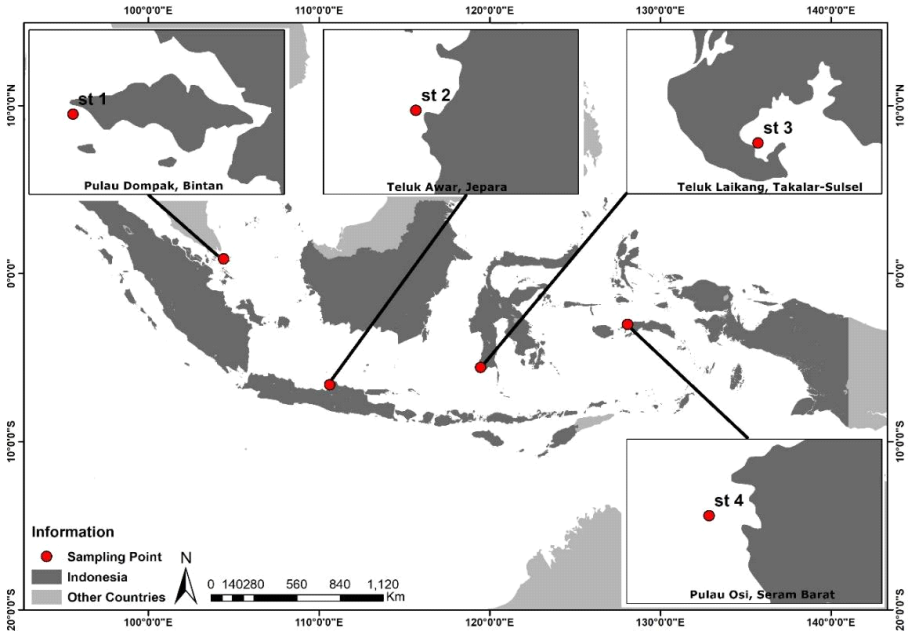

Figure 1. Map showing sampling locations.

DNA Extraction, Amplification, and DNA Sequencing

All dried algal samples were extracted using the cetyltrimethylammonium bromide (CTAB) method (Zuccarello & Lockhorst, 2005; Zuccarello & Paul, 2019). A microcentrifuge tube containing 500 µL of CTAB extraction buffer (2% CTAB, 0.1 M Tris–HCl with pH 8, 1.4 M NaCl, 20 mM EDTA, 1% PEG 8000) plus 50 µg RNAse A and 80 µg Proteinase K (Promega, Madison, USA) was used to place the sample. The samples were ground and homogenized with a microcentrifuge pestle. Samples were then heated to 55-60 °C for 30 min and mixed occasionally. An equal volume of the solution consists of chloroform:isoamyl alcohol (24:1) was added to the samples. Samples then spun at 12,000 rpm for 5-10 min. The aqueous phase was then extracted with an equal volume of chloroform: isoamyl alcohol (24:1), spun for 5 min at 12,000 rpm and removed to a new tube. An equal volume of 100% isopropanol was added to the samples. The tube was inverted occasionally and placed at room temperature for 30 min. The samples were spun for 30 min at 12,000 rpm and decanted. The DNA pellet was washed in 70% ethanol, air-dried, and 50 µL of 0.1×TE buffer was added. The sample was then frozen (-20 °C) until use.

Polymerase Chain Reaction (PCR) amplification was performed in a master mix of 25 µL containing 1.5 µL of *tuf*A forward primer (DNA sequence of forwarding PCR primer), and 1.5 µL of *tuf*A reverse primer (5'TGA AAC AGA AMA WCG TCA TTA TGC CCT TCN CGA ATM GCR AAW CGC 3') (Kazi et al., 2013), 12.5 µL KOD FX Neo buffer (Toyobo, Japan), 5 µL 2 mM dNTP, 0.5 µL KOD FX Neo (Toyobo), and 4.5 µL PCR water. PCR amplification conditions involve an initial denaturing step at 94 °C for 3-5 min; followed by 10 cycles of 94 °C for 30 s, 55 °C for 1 min, which is reduced by 1 °C per cycle, and 72 °C for 30-60 s; followed by 25 cycles of 94 °C/30 s, 45 °C/30 s, 72 °C /30 s; and a final extension of 72 °C for 5 min (Zuccarello & Paul, 2019). Amplification was verified based on a 1% agarose gel electrophoresis. Samples were purified, and Sanger sequenced at 1st Base Laboratories (Malaysia).

Phylogenetic and Haplotype Network

The phylogenetic reconstruction was built using the 20 samples, which were sequenced in both directions. All samples were edited, trimmed to equal length, and extracted to an alignment in MEGA 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). *Tuf*A sequences from various *C. racemosa* with 41 sequences were downloaded from National Center of Bioinformatics Institute (NCBI), edited, and added to the sample's alignments. A sequence of *Caulerpella ambigua* (GenBank Accession Number AJ417963) was added to the alignment and used as an outgroup. The multiple-sequence alignment (MSA) was performed to have a final alignment that is more suitable for the phylogenetic analysis (Castresana, 2000). The MSA was created using the Clustal IW (Thompson, Higgins, & Gibson, 1994) and then optimized visually. Sequence similarity searches were conducted for all samples against representative *Caulerpa* species from the

database in National Centre for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) (*http://www.ncbi.nlm.nih.gov*). The species identification followed Belton et al. (2014) for a naming system based on reinstated epithets. A phylogenetic tree (maximum-likelihood) was constructed based on the General Time Reversible model with 1000 bootstrap replicates using MEGA 6 (Nei & Kumar, 2000).

A haplotype network using 20 *C. racemosa* from four sites in Indonesia was calculated using the Median Joining method implemented in Network v5.0 (Bandelt, Forster & Rohl, 1999). The connectivity of *C. racemosa* was analyzed to determine the connection of the genetic compositions in each site. The names of species in each location were provided in a proper map to show a clear, transparent precise distribution among populations.

Results and Discussion

Phylogenetic

Twenty sequences of 741 bp fragments of chloroplast *tuf*A gene of *C. racemosa* were obtained from four sampling sites across the Indonesian archipelago. The result of *Caulerpa* species identification is shown in Table 1. *Tuf*A sequence of *Caulerpa* species from Bintan Island shared 100% similarity with *C. oligophylla* (FM956043), which was previously known as *C. racemosa* var. *lamourouxii* (Belton et al., 2014) and 99% similarity with *C. racemosa* (FM956051) in the GenBank database, which is previously sequenced from Thousand Islands, Java Sea, Indonesia. In Jepara, *Caulerpa* species presented 100% similarity with *C. oligophylla* (FM956043) from Thousand Islands, Java Sea, Indonesia, and 100% similarity with *C. cylindracea* (JN817677) from Montgomery reef, Western Australia, Australia, which was previously known as *C. racemosa* var. *cylindracea* (Belton et al., 2014). Three different species of *Caulerpa* were found in Osi Island. *Tuf*A sequence of *Caulerpa* from Osi Island showed 100% similarity with *C. oligophylla* (FM956043) from Thousand Islands, Java Sea, Indonesia; 100% similarity with *C. macra* (KF256089), which was previously known as *C. racemosa* var. *macra* (Belton et al., 2014) and 99% similarity with *C. racemosa* (FM956052), both sequenced from Berau Delta, East Kalimantan, Indonesia. All samples sequenced from Takalar had 100% similarity with *C. cylindracea* (JN817677) from Montgomery reef, Western Australia, Australia (Sauvage et al., 2013; Belton et al., 2014).

The maximum-likelihood tree was used for the molecular evolution models produced a phylogeny with a log-likelihood score of –1879.91. There are six clades of *Caulerpa* found based on the phylogenetic tree using the maximum-likelihood method (Figure 2). The phylogram clustered all *tuf*A sequence of *C. cylindracea* in clade one, including Takalar and Jepara samples. Two samples from Osi Island clustered in clade two with other sequences of *C. macra* from Genbank. Clade three (*C. oligophylla*) consisted of samples from Bintan Island, Jepara, and Osi Island. The population of *C. racemosa* from Osi Island and Bintan Island was clustered in clade five. Meanwhile, clade four clustered *tuf*A sequence of *C. macrodisca* and clade six of *C. chemnitzia* consisted only sequences from the Genbank (Figure 2).

Two different species of *Caulerpa* (*C. chemnitzia* and *C. macrodisca*) which were previously reported in three other sites in Indonesia (Thousand Island, Java Sea; Berau Delta, East Kalimantan, and West Papua) (Sauvage al., 2013; Belton et al., 2014) were not found in this study. This study thus reports of the existence of *C. cylindracea* in Takalar and Jepara, Indonesia for the first time. Even though the location is distant geographically, *C. cylindracea* from Takalar and Jepara had high similarity with *C. cylindracea* from Australia. Domingues, Hilsdorf, Shivji, Hazin, and Gadig (2017) found that there was no relationship between genetic distance and geographic distance. The ability of seaweed to drift along with ocean currents is one of the essential characteristics that allows the seaweed to spread over long distances resulting in complex biogeographic patterns (De Bruyn, Martin, & Lefeuvre, 2014).

Information about the existence of *C. cylindracea* in Indonesia is essential. *C. cylindracea* has been recently identified as an independent species (Belton et al., 2014). It was formerly recognized as *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman & Boudouresque (Verlaque et al., 2003). *Caulerpa cylindracea* was classified as invasive species in several sites in the world, such as in Canary Island (Sangil & Juan, 2020), Algeria (Boumediene & Lotfi, 2019), and especially in the Mediterranean (Bernardeau-Esteller et al., 2020; Rizzo, Pusceddu, Bianchelli, & Fraschetti, 2020; Sinopoli et al., 2020). *C. cylindracea* and *C. taxifolia* were among the fastest spread rates of marine bio-invasions (Ruitton et al., 2005), and notable for *C. cylindracea*, and it exhibits an impressive and constant expansion from the beginning of its first appearance in the Mediterranean (Montefalcone, Morri, Parravicini, & Bianchi, 2015). This seaweed can have a negative impact on the marine environment because it can (*C.*

Tabel 1. Identification of *Caulerpa racemosa* samples using *tuf*A primer

No	Location	Sample Name	GPS Coordinates		Date of Collection	Sample Identification	Genbank Accession number
1	Bintan Island	Bintan 01	0°52'28.58"	N	September 2019	*Caulerpa oligophylla*	FM956043
			104°25'3.37"	E			
2	Bintan Island	Bintan 02	0°52'26.62"	N	September 2019	*Caulerpa racemosa*	FM956051
			104°25'3.08"	E			
3	Bintan Island	Bintan 03	0°52'24.59"	N	September 2019	*Caulerpa oligophylla*	FM956043
			104°25'6.26"	E			
4	Bintan Island	Bintan 04	0°52'25.52"	N	September 2019	*Caulerpa oligophylla*	FM956043
			104°25'7.68"	E			
5	Bintan Island	Bintan 05	0°52'26.80"	N	September 2019	*Caulerpa racemosa*	FM956051
			104°25'8.11"	E			
6	Jepara	Jepara 01	06º36'49.36"	S	October 2018	*Caulerpa oligophylla*	FM956043
			110º38'10.03"	E			
7	Jepara	Jepara 02	06º36'51.99"	S	October 2018	*Caulerpa oligophylla*	FM956043
			110º38'04.10"	E			
8	Jepara	Jepara 03	06º36'51.32"	S	October 2018	*Caulerpa cylindracea*	JN817677
			110º38'04.22"	E			
9	Jepara	Jepara 04	06º36'49.73"	S	October 2018	*Caulerpa oligophylla*	FM956043
			110º38'05.15"	E			
10	Jepara	Jepara 05	06º36'50.91"	S	October 2018	*Caulerpa oligophylla*	FM956043
			110º38'04.63"	E			
11	Osi Island	Osi 01	03º01'30.60"	S	December 2018	*Caulerpa racemosa*	FM956052
			128º04'39.90"	E			
12	Osi Island	Osi 02	03º01'48.55"	S	December 2018	*Caulerpa macra*	KF256089
			128º05'31.45"	E			
13	Osi Island	Osi 03	03º01'48.07"	S	December 2018	*Caulerpa oligophylla*	FM956043
			128º05'31.49"	E			
14	Osi Island	Osi 04	03º01'47.62"	S	December 2018	*Caulerpa macra*	KF256089
			128º05'31.44"	E			
15	Osi Island	Osi 05	03º01'31.55"	S	December 2018	*Caulerpa oligophylla*	FM956043
			128º04'39.76"	E			
16	Takalar	Takalar 01	05º35'1.37"	S	November 2018	*Caulerpa cylindracea*	JN817677
			119º27'54.33"	E			
17	Takalar	Takalar 02	05º35'1.07"	S	November 2018	*Caulerpa cylindracea*	JN817677
			119º27'55.23"	E			
18	Takalar	Takalar 03	05º35'3.47"	S	November 2018	*Caulerpa cylindracea*	JN817677
			119º27'53.48"	E			
19	Takalar	Takalar 04	05º35'3.58"	S	November 2018	*Caulerpa cylindracea*	JN817677
			119º27'54.00"	E			
20	Takalar	Takalar 05	05º35'0.81"	S	November 2018	*Caulerpa cylindracea*	JN817677
			104°25'8.11"	E			

change the structure of marine communities (Sangil & Juan, 2020). Despite this negative impact on the environment, the *C. cylindracea* found in Takalar became one of the seaweed commodities developed by the community. Local people utilized this type of seaweed not only for local consumption but also for economic values. The community cultured this seaweed in a pond and marketed it locally and overseas (Perryman, Lapong, Mustafa, Sabang, & Rimmer, 2017). The molecular identification of *C. cylindracea* can improve the utilization of this kind of seaweed by searching the best characteristic of *C. cylindracea* for economic value through tissue culture.

Haplotype Network

Caulerpa species in four different sites showed a slight variation in the *tuf*A gene with only four haplotypes recorded, and each haplotype corresponded to various other species. Haplotype one, which corresponded to *C. oligophylla*, was dominant and

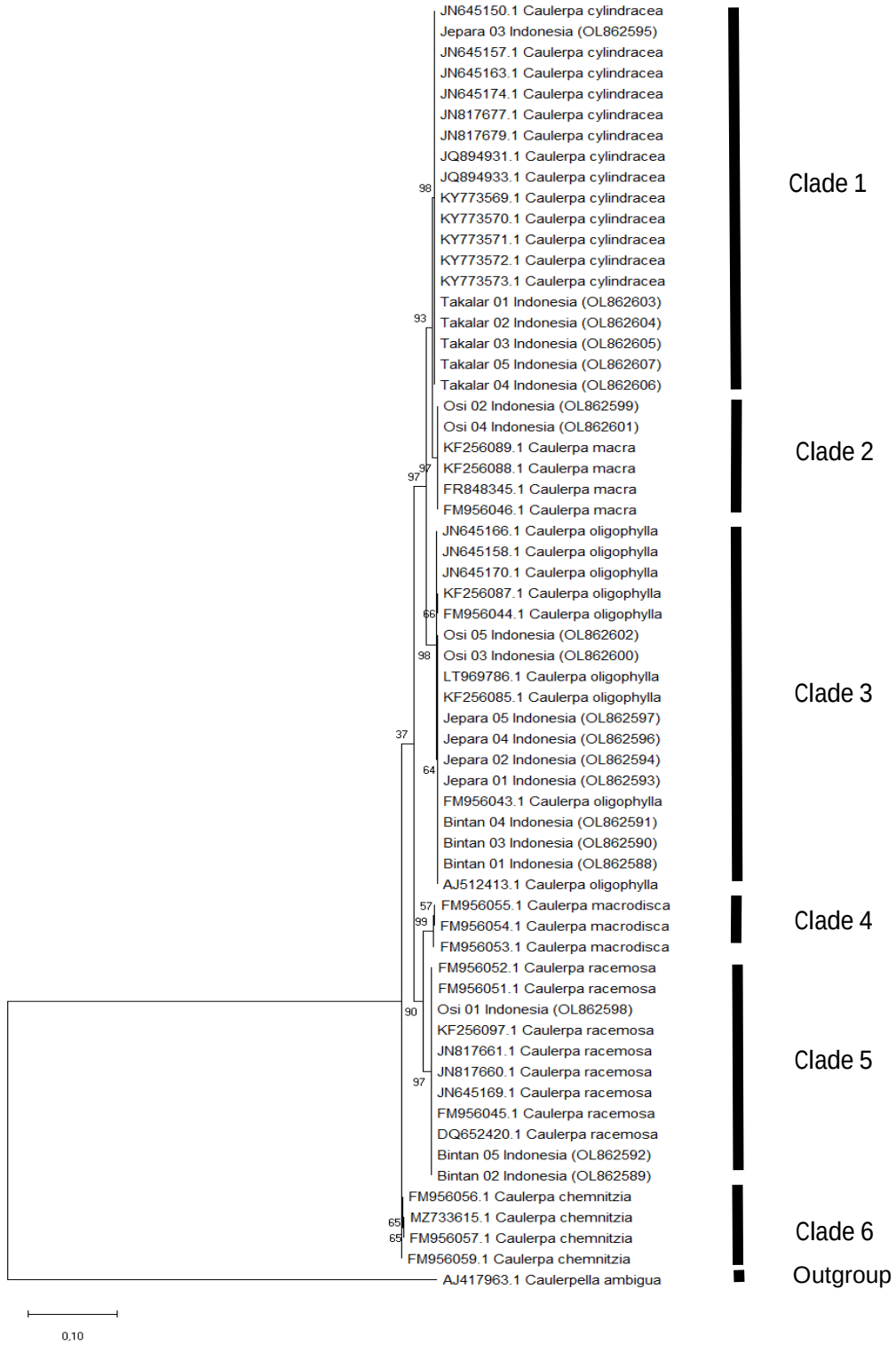

Figure 2. Maximum-livelihood phylogeny of partial DNA sequences of *C. racemosa* samples from four sites plus identified sequences from Genbank. Accession numbers are given.

cylindracea) were found in two different sites. Two samples of *Caulerpa* from Osi Island formed a distinct group characterized by haplotype four corresponding with *C. macra*. Osi Island had the most diverse species of *Caulerpa* (three species), while Takalar had a low genetic variation with only one species of *Caulerpa*. The haplotype network in four locations and the species distribution map can be seen in Figures 3 and 4.

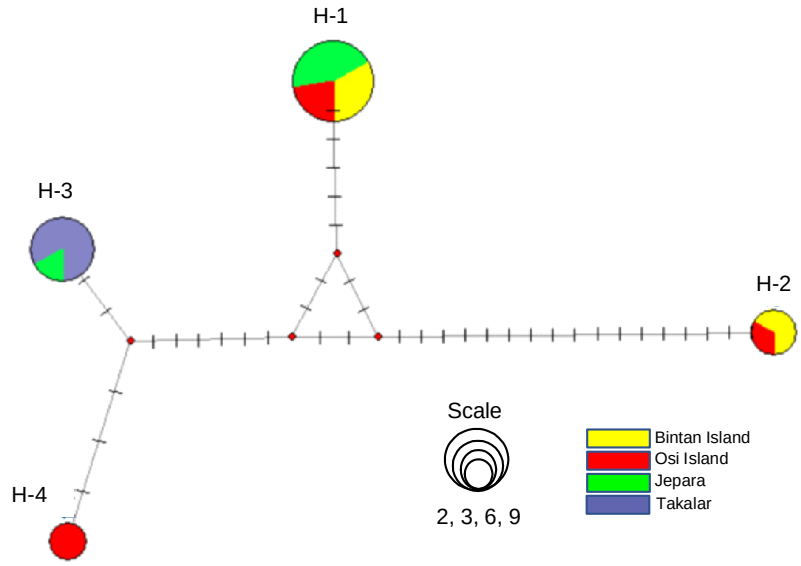

Figure 3. Haplotype network of *C. racemosa* showing the distribution of the haplotypes in four geographical regions in this study (Note: The scale of the diameter of the circle correspond with the number of species found).

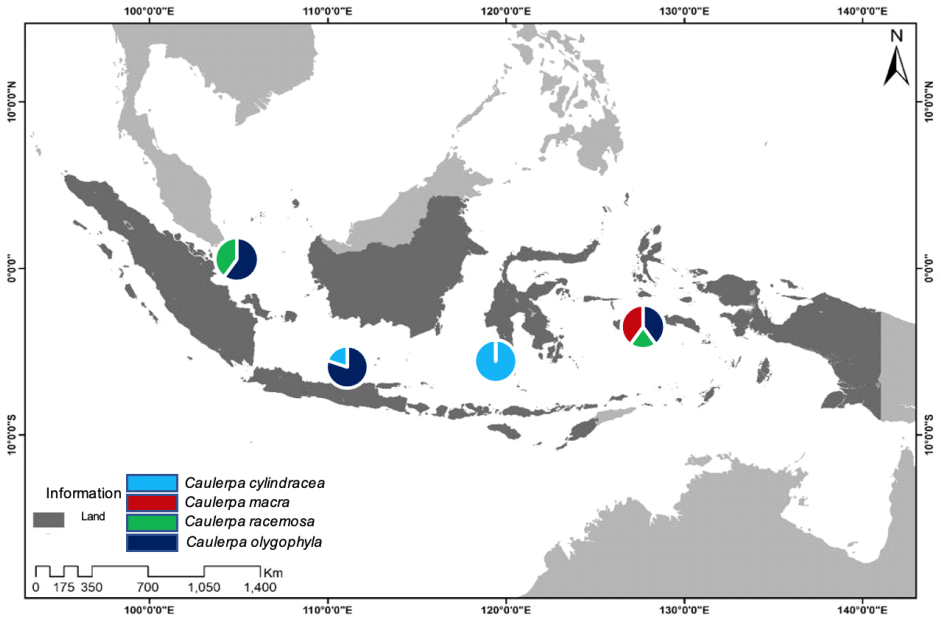

Figure 4. Species distribution of *C. racemosa* across the population in four geographical regions in this study.

Some of the population of *Caulerpa*, for example, *C. oligophylla* in Bintan, Jepara, and Osi Island, had a similar haplotype composition. This relationship was suspected due to their dispersal capacity supported by the movement of the current. One of the factors affecting algal phylogeography is long-distance dispersal, which can lead to widely distributed species and can be anthropogenic or natural (Sherwood & Zuccarello, 2016). Previous studies have shown that dispersal capacity by fragmentation of *C. taxifolia* (Checcerelli & Chinelli, 1999; Rushdi et al., 2020) and *C. racemosa* (Checcerelli & Piazzi, 2001) was likely to contribute to the spread of those algae in the Mediterranean Sea. The haplotype of *C. cylindracea* in Takalar was relatively homogenous, which was dominated by haplotype 3. This condition may be influenced by the Indonesian Through Flow (ITF). Indonesian Through Flow (ITF) plays an essential role in marine biogeography in the Wallacea line region, namely the Makassar Strait, and can be a significant barrier to species dispersal patterns (Hall, 2009; Chasani, 2017).

Conclusion

Identification of *C. racemosa* variant samples using the *tuf*A gene in this study found four different *Caulerpa species: C. racemosa, C. macra, C. oligophylla,* and *C. cylindracea*. The genetic diversity of *Caulerpa* species in four other locations (Bintan Island, Jepara, Osi Island, and Takalar) showed a little haplotype diversity. *Caulerpa* species in all areas consisted of four haplotypes, and each haplotype corresponded to a different species. This study has revealed the first record of *C. cylindracea* found in Indonesia.

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