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DNA barcoding in molecular identification and phylogenetic relationship of beneficial wild Balinese red algae, *Bulung sangu* (*Gracilaria* sp.)

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ABSTRACT

Background: *Bulung sangu* (*Gracilaria* sp). is wildly widespread Rhodophyta in Bali and usually consumed as vegetable. *Bulung sangu* is reported for its bioactive compound scientifically proven as antioxidant, anti-inflammatory, and anti-hypercholesterolemia. Likewise, *Bulung sangu* as Rhodophyta is a potential source for food, fertilizer, cosmetic and pharmaceutical industry. *Bulung sangu* production is highly fluctuating and unable to meet its demands. Proper and correct cultivation methods based on molecular information are expected to increase the availability of *Bulung sangu* in Bali. Complete taxonomy is required to design the proper cultivation method. Meanwhile, *Bulung sangu* taxonomy is limited to the genus level. More information on *Bulung sangu* strain is needed, either does its relatedness.
Methods: Fresh *Bulung sangu* was collected from Serangan coastal area, Bali. DNA extraction was applied followed by PCR amplification using six combinations of COI primer sequence. DNA sequences obtained was evaluated to determine pairwise distance, percent of similarity and phylogenetic relationship compared to Gracilaria species registered in GenBank.
Results: PCR amplification produced 730 base pairs amplicons. Genetic distance and percentage of similarity obtained exhibit relatedness to *Gracilaria gracilis* with 0.487 of pairwise distance and 49.04% of similarity. The phylogenetic tree produced seven clades in which *Bulung sangu* and *Gracilaria gracilis* were in the same clade.
Conclusion: *Bulung sangu* showed closest relatedness to *Gracilaria gracilis.*

Keywords: COI gene, Gracilariaceae, Macroalgae, Molecular Identification, Rhodophyta.
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INTRODUCTION

Seaweed or macroalgae has long been a product widely consumed by people around the world. Along with science and technology development, seaweed has become very diverse both for food and non-food products. The Gracilariaceae has emerged as one of the families with economic potential. Indonesia has several varieties of *Gracilaria* sp. with different morphological and anatomical characteristics. *Gracilaria* sp. is claimed under various names such as *sango-sango, rambu kasang, janggut dayung, dongi-dongi, bulung embulung, agar-agar karang, agar-agar jahe*, and *Bulung sangu*. *Bulung sangu* is a *Gracilaria* sp. that grows wildly in Bali and is commonly consumed as a vegetable. *Gracilaria* sp. is also potentially developed in the cosmetics and pharmaceutical industries.[1] The previous study reported several beneficial phytochemicals in *Bulung sangu* ethanolic extract, which exhibited strong antioxidant and anti-inflammatory activity against UV-radiation-induced skin damage.[2] Several species of Gracilaria also reported various pharmacological activity, including immunostimulator, allelopathic activity, antibacterial, antifungal, and antiviral, effects for cardiovascular and gastrointestinal.[3–5]

The excessive growth of *Bulung sangu* has been reported in harvest season in many parts of coastal areas in Bali and will be decreasing in another season. Nowadays, seaweed production in Bali is unable to meet Indonesia's demand for seaweed. Local production is merely sufficient to supply the local markets and Java trading. *Bulung sangu* has not been cultivated yet in Bali, even though some coastal areas are felicitous for seaweed cultivation. Seaweed availability in Bali is highly fluctuating. Proper cultivation is expected to maintain the availability of seaweed so that it fulfills the global demands.

Genetic information is useful in cultivation management and increases cultivation and conservation success.[6] It provides a sufficient amount, especially to be developed in pharmaceutical and cosmetics needs. Genetic study is also needed for species that challenge extinction and limited distribution. Genetic helps in areas mapping for

species with high genetic variability as a conservation and species identification.[7] Understanding natural populations' ecology and genetic structure is essential to determine strategies for conservation, nurseries, and sustainable management.[8]

Information about *Bulung sangu* taxonomy is limited to the genus level. High morphological variation in the wild and the lack of morphological diagnostics have misidentified seaweed strains, impacting the wrong cultivation method and declining productivity and quality of cultivated seaweed.[1] Strain identification relying on morphological analysis is relatively difficult to establish due to simple morphology and anatomy, convergence, rampant phenotypic plasticity, and alternation of heteromorphic generations.[9] Identification based on complete taxonomy helps to correctly and effectively identify the seaweed strains.[1] DNA Barcoding provides fast and accurate system for species identification. Instead of using whole-genome, DNA Barcoding uses short DNA sequence generated from the standard region of the genome known as marker. The specific locus used in DNA Barcodes must be determined on most taxa that are sought and sequenced without using specific Polymerase Chain Reaction (PCR) primers.[10] Several markers have been developed previously to identify algae, such as Internal Transcribed Spacer (ITS) in the ribosomal cistron, rubisco operon, and variable parts of large ribosomal cistron subunits; nevertheless, those markers serve unsatisfactory shortcoming. The lack of universal markers resulted in multiple, independent, and not easily comparable system. Nonetheless, molecular detection using markers designed from the gene cytochrome oxidase subunit I (COI) was reported generating powerful ally in the identification of red algal species.[9] This research was aimed to map the relatedness of *Bulung sangu* using COI gene as the barcoding marker.

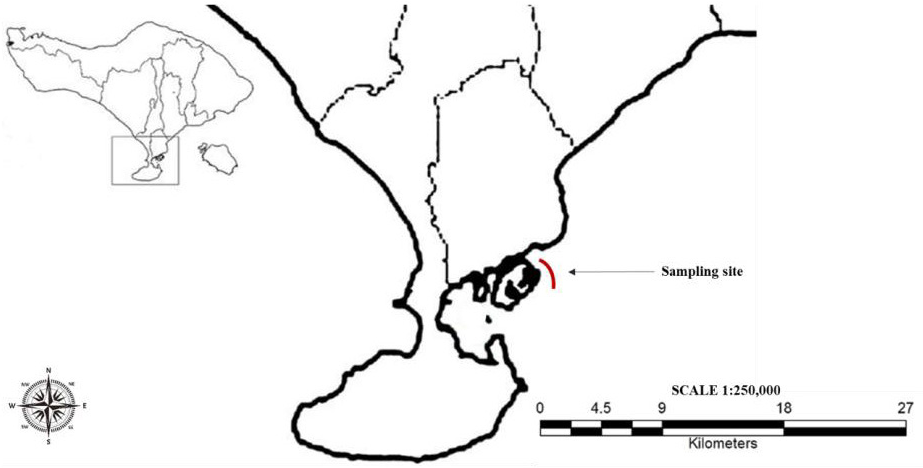

Figure 1. The sampling sites in Serangan (shows in arrow), -8.719715,115.242034

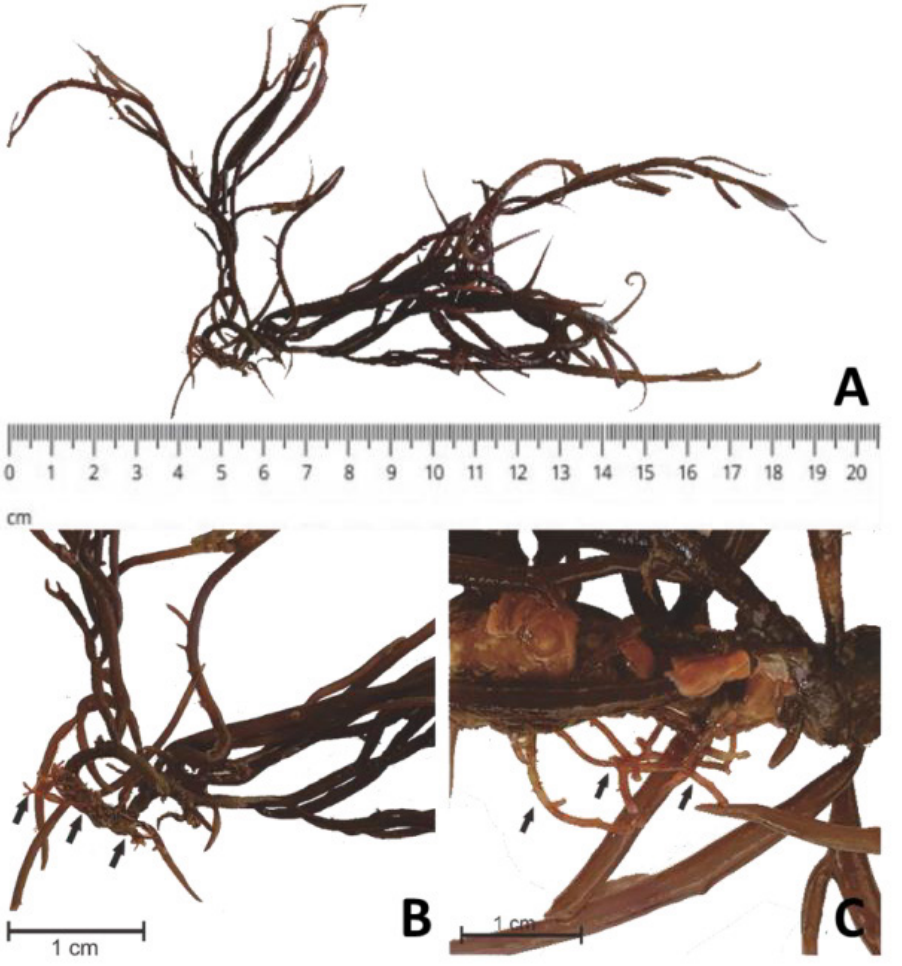

Figure 2. Thallus of *Bulung sangu* (A). *Bulung sangu* lives by attaching itself to plants or other seaweed using holdfast (arrow), which has a root-like structure in a true plant (B), thus wraps the thallus on other seaweed (C)

MATERIAL AND METHODS

Study area

The study was Test-tube Lab Research, carried out from July 2019 until April 2020 on Serangan Island (Bali), Central Laboratory of Genetic Resources Universitas Udayana and Laboratory of Genetica Science Indonesia.

Algae material

Samples of fresh *Bulung sangu* were collected from Serangan coastal area, Bali

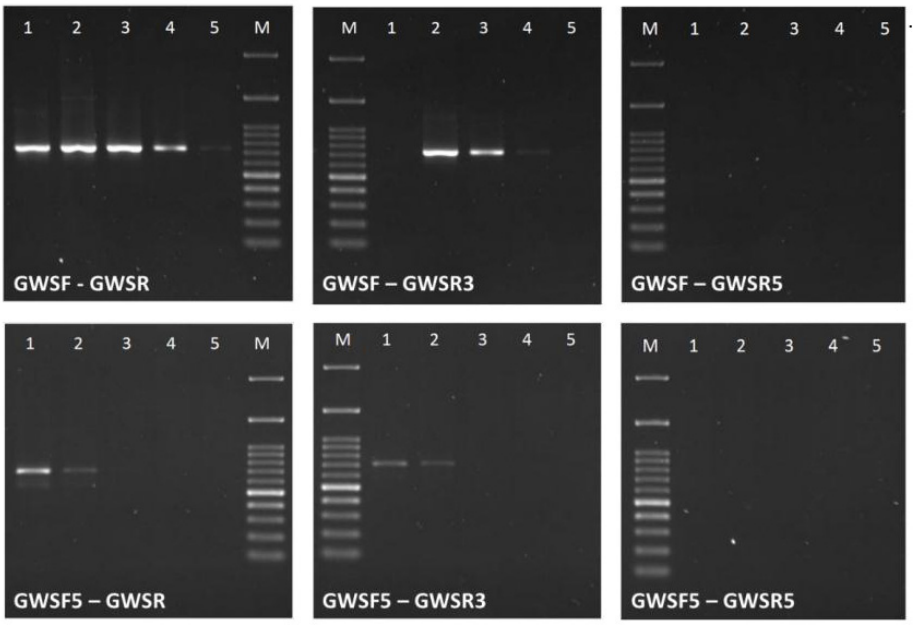

Figure 3. Electrophoresis of PCR products. Primer optimization generated six primer combinations. Combination of GWSF-GWSR that was annealed in temperature of 50°C produced clear band. Primer combination of GWSF-GWSR5 and GWSF5-GWSR5 did not produced amplification.

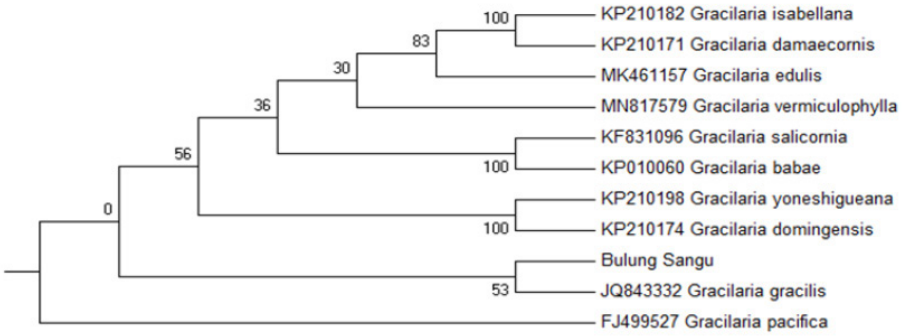

Figure 4. Phylogram of *Bulung sangu* constructed using maximum parsimony

(Figure 1). *Bulung sangu* samples were taken from the sea, about 500 – 1,000 m from the seashore, in September 2019. Collected *Bulung sangu* were cleaned to remove dirt and salt. Samples were placed in the icebox and kept in -20°C refrigerators in the laboratory until further analyses.

Total genomic DNA extraction

Total genomic DNA of *Bulung sangu* was extracted and purified using the Zymo Plant & seed DNA extraction kit with the procedure following the kit's protocol. The sample used was a dry sample of 150 mg. Analysis of DNA quality and quantity was performed by 1% gel electrophoresis and optical density using a 260 and 280 nm spectrophotometer, respectively.

COI gene amplification

Amplification was optimized using five primers, consisting of two forward primers (GWSF and GWSF5) and three reverse primers (GWSR, GWSR3, and GWSR5)[11] in six primer combinations. Primer sequences were shown in Table 1. The annealing temperature for each primer combination was optimized to determine the optimum temperature for further analysis. Primer combination and annealing temperature used in optimization are shown in Table 2. Genetica Science, Indonesia synthesized primers. PCR amplification was held using Toyobo KOD FX Neo Kit. Amplification reaction followed the kit procedure with one µL of DNA template was used. PCR conditions were used as follows: 1 cycle of 95°C for 2 min, 35 cycles of 95°C for 10s, annealing temperature (Table 2) for 30 sec and 72°C for 1 min. The PCR products (2 µl) were visualized on 1% agarose gel. Primer combination resulting in amplification was then cloned in eight colonies of pTA2 vector plasmid.

Data analysis

The COI sequence was determined by bidirectional sequencing. The obtained sequence was then analyzed using the BLAST (Basic Local Alignment Search Tool) program in the NCBI (National Center for Biotechnology Information) database (https://www.ncbi.nlm.nih.gov/). Ten accession numbers of Gracilaria species registered in the NCBI database with the highest percent identity to *Bulung sangu* sequence were used to determine pairwise distance, percent of similarity and construct the phylogenetic tree. Sequences were aligned using Muscle, under default parameter using MEGA program (version 6.0). Genetic distance and percent similarity were then obtained following the phylogenetic tree construction by maximum parsimony. The reliability of the branches was evaluated with non-parametric bootstrapping (1000 replication).

RESULTS

Bulung sangu was obtained in the intertidal zone, attached to rocks, plants or other seaweed, and is commonly found buried in the sand. *Bulung sangu* is pink to dark brownish red (mahogany color). Exposure to sunlight can fade the dark color of *Bulung sangu*, making it transparent green or white. The thallus is a clump with an irregular branching type and cylindrical with sharpened talus edges, with the length ranges from 7-18 cm, diameter ranges from 1-1.5 mm and 0.1-0,5 mm for the tapered ends of the thallus. *Bulung sangu* lives by attaching itself to plants or other seaweed using holdfast that resembles a root, thus wrapping the thallus to the attached object (Figure 2).

Table 1. Five COI markers used to generate DNA fragment by PCR reactions

Primer	Sequence 5′ ✇ 3′	Length
GWSF	TCC CAG TCA CGA CGT CGT TCA ACA AAY CAY AAA GAT ATY GG	41
GWSF5	ACA AAY CAY AAI GAT ATY GG	20
GWSR	GGA AAC AGC TAT GAC CAT GGG RTG TCC RAA RAA YCA RAA	39
GWSR3	GGA AAC AGC TAT GAC CAT GGG RTG TCC AAA IAA YCA RAA	39
GWSR5	TCA GGR TGN CCI AAR AAY CA	20

Table 2. Primer combinations and annealing temperature used in optimization

Primer Combination	Annealing Temperature (°C)				
	1	2	3	4	5
GWSF – GWSR	50	53	56	59	62
GWSF – GWSR3	50	53	56	59	62
GWSF – GWSR5	50	53	56	59	62
GWSF5 – GWSR	48	51	54	57	60
GWSF5 – GWSR3	48	51	54	57	60
GWSF5 – GWSR5	48	51	54	57	60

Table 3. Pairwise distance and percent of similarity of *Bulung sangu* and other species registered in GenBank

Closest match species	Pairwise distance	Similarity	Acession Number
Gracilaria gracilis	0.487	49.04%	JQ843332.1
Gracilaria vermiculophyla	0.497	48.59%	MN817579.1
Gracilaria salicornia	0.507	47.12%	KF831096.1
Gracilaria babae	0.509	46.72%	KP010060.1
Gracilaria isabellana	0.501	47.44%	KP210182.1
Gracilaria damaecornis	0.503	47.28%	KP210171.1
Gracilaria yoneshigueana	0.526	45.20%	KP210198.1
Gracilaria domingensis	0.526	45.20%	KP210174.1
Gracilaria pacifica	0.507	47.28%	FJ499527.1
Gracilaria edulis	0.499	47.92%	MK461157.1

The molecular-based method for *Bulung sangu* identification was held using five COI markers consisting of two forward and three reverse primers. DNA amplification resulting in six combinations of the forward and reverse primer. Optimization of six primer combinations showed amplification in four combinations. A combination of GWSF and GWSR produced a clear band in annealing temperatures of 50°C, 53°C and 56°C (Figure 3). The sequence was then generated on PCR products with GWSF and GWSR primer and amplified at a temperature of 50°C for producing the clearest band. Amplification produced 730 base pairs of amplicons. The DNA sequence was analyzed using the BLAST program. The species with the highest identity matches, listed in Table 3, were used to determine pairwise distance, percent of similarity and construct the phylogenetic tree. Pairwise distance and the similarity of *Bulung sangu* and ten Gracilaria species from GenBank are shown in Table 3. Phylogenetic was then constructed, as shown in Figure 4.

DNA Barcoding uses a short region of mitochondrial DNA as a barcode to amplify genes, following by sequencing and matching the unidentified sequences with known sequences in BOLD (The Barcode of Life Data System) or NCBI (National Center for Biotechnology Information) libraries. DNA barcoding is an efficient and advanced method for species identification and can identify species' complexity in a population.[11,12] Genetic is needed to identify species with high phenotypic.[13] DNA barcoding can identify organisms in various developmental stages in animals.[14–16] Also, DNA barcoding can be powerful in the identification of cryptic species.[17]

DISCUSSION

At present, the molecular-based method is used to identify *Bulung sangu* (*Gracilaria* sp.) specimens, which are wildly distributed in Bali coastal. The phenotypic traits may lead to misidentifications and will be more reliable to identify with the molecular identification approach. *Bulung sangu* belongs to the *Gracilariaceae* species in which is the potential commodity in Indonesia. There is no species concept of *Gracilaria* sp. in Indonesia, usually termed *Gracilaria* sp. as red seaweed. There are several types of *Gracilaria* sp. in Indonesia without any differentiation, even for each type's different morphological appearance. The general morphology of *Gracilaria* sp. is characterized by red or dark red color and irregular branching type.

Gracilaria sp. generally live in the eulittoral zone, or the lower zublittoral zone, in sandy and sedimentary areas that protect it from waves. *Gracilaria* sp. is sometimes found free at high tide and can adapt to various growing conditions. *Gracilaria* sp. are widely distributed in various tropical countries such as Indonesia, Thailand, Viet Nam, China, Namibia, southern Chile, and Canada's Atlantic coast. Indonesia, Argentina and Brazil also have several types of wild *Gracilaria* sp. In Indonesia, *Gracilaria* sp. is collected wild or cultivated.[18]

Genetic distance exhibits the closest relatedness between *Bulung sangu* and *Gracilaria gracilis* (JQ843332) with the smallest pairwise distance value than other species (Table 3). A similar result was generated in the similarity percentage, which shows the highest percentage of *Bulung sangu* and *G. gracilis*. The phylogram produced seven clades in which *Bulung sangu* and *G. gracilis*. were in the same clade, as well. The same node

produced in *Bulung sangu* clade showed the common ancestor with *G. gracilis*. In this present study, the molecular identification using the COI gene showed weak bootstrap support for *Bulung sangu* and *G. gracilis*.

Gracilaria gracilis (JQ843332) was described in Pajuçara, AL, Brazil[19] *G.gracilis* (Stackhouse), Steentoft, L.M.Irvine & Farnham describes as cartilaginous, cylindrical, soft purple fronds, to 500 mm long, one or several arising from small, fleshy, perennial discoid holdfast. *G. gracilis* can be attached on rocks and stones, in intertidal and subtidal zones, especially on sandy shores. *G. gracilis* is branching very irregular, sparse or profuse, with up to 2 mm of diameter and apices pointed, with the length of thallus can reach 60 cm in nutrient-rich habitats.[20] Both *Bulung sangu* and *G.gracilis* share the irregularly branched thallus with curved edges. They are found buried in the sand to protect itself from sun exposure. Thallus in both species can fade to green or white in sun exposure. *G. gracilis* distributes in the north-eastern Atlantic, known from southern Norway southwards to northern Spain. *G. gracilis* also reported known outside the area including Italy, Brazil, Irish, Caspian Sea (Western Asia), and Singapore.[19,21–24] In Indonesia, *G. gracilis* is reported grows in Sulawesi, and cultivated in several parts in Java.[25,26]

G. gracilis is reported for various valuable products, including arachidonic acid, protein, carbohydrates and high level of glutamic acid along with all types of amino acid except tryptophan, isoleucine, and asparagine.[25,27] Several *G.gracilis* bioactivities are reported, including cholinesterase inhibitor, strong antioxidant activity, and antimicrobial activity against *Candida* sp., *Enterococcus faecalis, Staphylococcus aureus, Streptococcus epidermidis, Pseudomonas aeruginosa,* and *Eschericia coli*.[27–30]

According to our phylogenetic analysis, the partial sequences of COI of *Bulung sangu* is now known, which was previously unknown in Indonesia. The molecular methods using DNA sequencing technologies have been successfully developed to study their phylogenetic relationship and classify living organisms' unkonown species. Alignment of COI sequence of *Bulung sangu* revealed it closely related to other *Gracilaria* sequences and it to be 49.04% identical with *G. gracilis* data in the Genbank database. Identification results in a low percentage of similarity. Generally, the name with the most closely related species is suggested to the organism identified with at least 97% similarity.[31] These result does not show the homology between *Bulung sangu* and *G. gracilis*, thus *Bulung sangu* considered different to other *Gracilaria* species registered in the GenBank.

Hebert et al. firstly reported using the COI gene as a marker in DNA Barcoding in animals.[32] The use of the COI gene as a barcode for identification of macroalgae was reported by Saunders, where the use of the gene locus resulted in accurate and powerful identification.[9] Hebert et al. reported that using this marker in animals successfully identifies unknown species to a higher taxon level. COI can be used as a marker for the identification of species with high phenotypic plasticity. COI gene has two advantages to be used as a marker in animals: the primer's universality for amplifying the 5' end of various animal species and a broad phylogenetic level. The COI gene has a relatively fast divergence rate, makes it suitable to be designed as a universal primer on certain parts of the gene. COI also has a low prevalence of indels so that it can adequately facilitate alignment across phyla.[32]

Two molecular markers have been proposed as standard markers for species identification of macroalgae: COI and UPA, the universal plastid amplicon, domain V of the 23 rRNA gene.[33] Hebert et al. reported that the use of COI markers shows its ability to identify animal species. Likewise, COI markers are also suitable for identifying red algae, brown algae and green algae.[9,34–38] COI results in more sensitive markers for delimiting species than UPA.[39] Compare with other markers such as rbcL, the mitochondrial marker COI is also more effective, variable and exhibited a larger barcoding gap with closely related species.[35,40] In identifying intraspecific divergences, COI markers can identify 0-2 base pairs and more than 30 base pairs in interspecific divergences.[41]

A molecular approach can determine the phylogenetic identification of *Bulung sangu*. Further study is still needed to clarify the species of *Bulung sangu*. Several other COI universal primer as designed by Saunders and Moore[41] is suggested to obtain comparative and more reliable data for *Bulung sangu* molecular sequences before declaring *Bulung sangu* as new wild species of *Gracilaria* seaweed.

CONCLUSION

Molecular identification of *Bulung sangu* using COI primer produces genetic distance, similarity percentage and phylogenetic tree of *Bulung sangu* which shows the closest relationship to *Gracilaria gracilis*. However, the results also show that *Bulung sangu* differs genomically from other *Gracilaria* species registered in GenBank. As an amplifier and comparative study, molecular identification can be carried out with other universal COI primers, such as the primer series of COTF (forward) and CoTR (reverse).

DISCLOSURE

Conflict of interest

We declare that there are no competing interests.

Author contributions

IGPW and MMVS conceptualized the research and research design, IGPW defined the intellectual contest, MMVS and AAKK conducted literature search, MMVS and INW conducted experinmental studies, MMVS acquisitioned data, IGPW and INW analyzed data, MMVS prepared the manuscript, AAKK edited the manuscript, IGPW and INW reviewed the manuscript. All authors have read and agreed to the published version of the manuscript, and guarantee the research and publication.

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Ethical statement

None

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