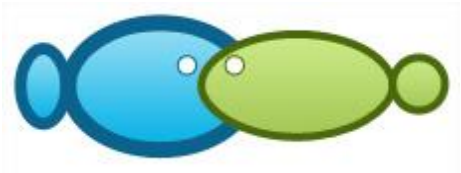

Phylogeny and molecular identification of brown macroalgae *Sargassum aquifolium* and *Sargassum henslowianum* in the coastal waters of Kora-Kora, North Sulawesi, Indonesia

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Abstract. *Sargassum* is a well-known genus of brown algae in Indonesia that has long been investigated due to its economic importance. To support its biological research, it is important to correctly identify and classify the species studied. Whilst morphological identification has been greatly contributed to taxonomy, it cannot distinguish *Sargassum* species accurately due to its plasticity. In this research, a molecular identification of *Sargassum aquifolium* and *Sargassum henslowlanum* in the coastal waters of Kora-Kora, Minahasa Regency, North Sulawesi Province, Indonesia is provided based on the genetic profile using plant/fungal DNA extraction kit. DNA was preserved at -20ºC for further analysis. The target DNA regions, namely ribulose-bisphosphate carboxylase gene, were amplified with universal DNA barcoding primers. The endemic brown macroalgae, *S. aquifolium* and *S. henslowlanum* were not confirmed as 100% similar to the reference *Sargassum* species, indicating genetic variations. The identification of this species is important, as it could be added into the vast taxonomic status of *Sargassum* sp. Moreover, it serves as a basis for gene stock identification for coastal resource management of Indonesian macroalgae.
Key Words: seaweed, genetic, monophyletic, Minahasa, DNA barcoding primer.

Introduction. *Sargassum* beds support high levels of biodiversity and productivity (Coston-Clements et al 1991). Knowledge of their genetic diversity is needed to effectively manage and conserve them. Several studies have explored the genetic diversity of *Sargassum polycystum* within its native range (Chan et al 2013; Kantachumpoo et al 2015), albeit with only a few samples from the coastal waters of Kora-Kora, Minahasa Regency, North Sulawesi Province. Each study requires identifying and classifying the species. Classical taxonomy has made significant contributions to the classification of species based on morphological features; nevertheless, traditional taxonomy is unable to correctly distinguish all species, notably closely related ones (Wang et al 2020). In particular, a wide range of morphological variations in the *Sargassum* causes confusion in classification issues (Widyartini et al 2017). Molecular identification of *Sargassum* is therefore urgent to clarify the morphological plasticity caused by the environmental factors (Kantachumpoo et al 2015).

In recent publications there is no information about *Sargassum henslowianum* in Mantehage Island (Kepel et al 2019a), Minahasa Peninsula (Kepel et al 2019b; Kepel et al 2020) and in the coastal waters of Likupang Marine Station, Tongkaina and Kora-Kora (Kepel et al 2024). This study aimed to prove the presence of *Sargassum aquifolium* and *Sargassum henslowianum* by using phylogenetic analysis and molecular identification in this site. The

genetic diversity of *Sargassum* sp. from the coastal waters of Kora-Kora was assessed based on the nuclear ribosomal DNA (rDNA) region involving the internal transcribed spacers (ITS1 and partial ITS2) and the 5.8S gene.

Material and Method

Sample collection. This study was conducted in June 2024. Samples of *S. aquifolium* (Figure 1) (FW1) and *S. henslowianum* (Figure 2) (FW2) were collected directly by hand. The research site was situated in the coastal waters of Kora-Kora, Minahasa Regency (Figure 3). Identified macroalgae were stored in proportional containers and labeled with classification information, location of discovery and collector. The samples characterization was based on the molecular identification.

Figure 1. Sample of *Sargassum aquifolium* from the coastal waters of Kora-Kora (FW1).

Figure 2. Sample of *Sargassum henslowianum* from the coastal waters of Kora-Kora (FW2).

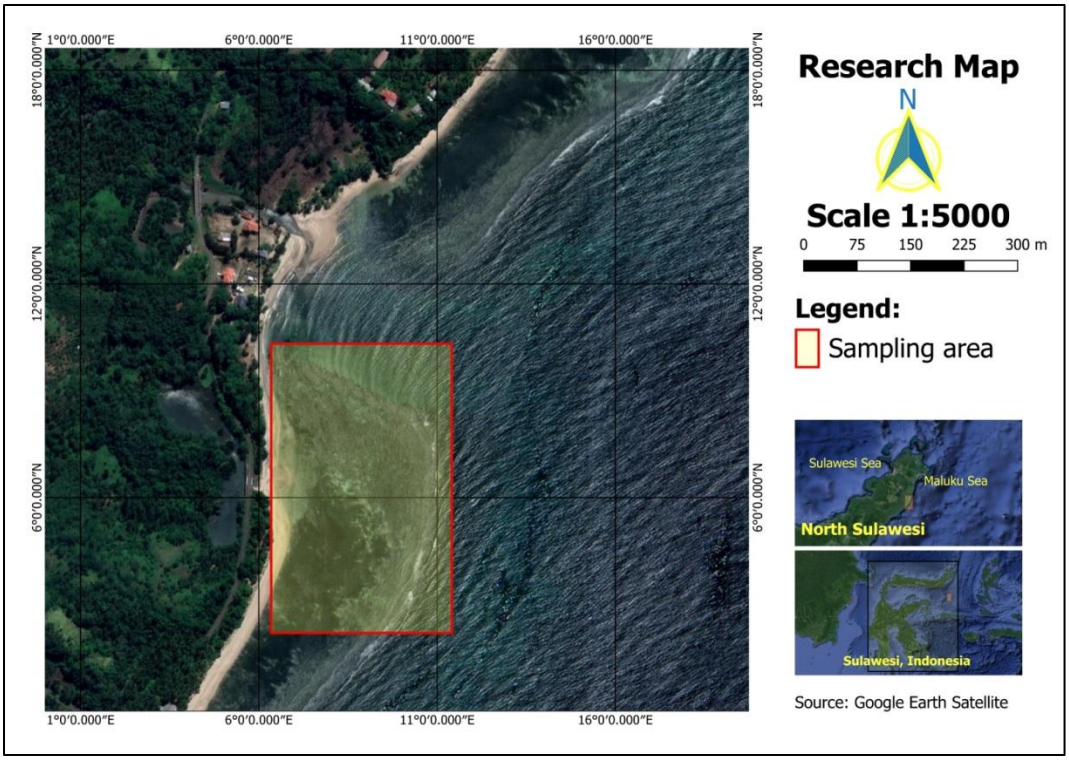

Figure 3. Sampling sites of *Sargassum henslowianum* from the coastal waters of Kora-Kora.

DNA extraction, isolation, and amplification. Total genomic DNA of plant samples was extracted using Plant/Fungal DNA extraction mini kit (Geneaid, catalogue GP 1000).

Data analysis. The bidirectional sequencing process was performed using PCR primers from 1st Base(Malaysia). Chromatograms were edited using Geneious Prime v2024.0.5 and alignment was performed using the MUSCLE software (Edgar 2004).

Primary design. New primers were designed to amplify the COI gene in *Sargassum* (Table 1).

Table 1

Primers design for COI gene amplification in *Sargassum*

Primer name	*Primer sequence*	*Melting temperature*	*Direction*	*Amplicon length*
FW-COI-SargF	5'- TTT CTA CAA AYC AYA ARG ATA TTG G-3'	57.2°C - 62.2°C	Forward	715 bp
FW-COI-SargR	5'- TAC ACC TCA GGR TGT CCR AAA AAC CA-3'	66.8°C - 69.5°C	Reverse	

PCR. The PCR kit used MyTaq™ HS Red Mix (Bioline). Each 40 µL PCR reaction contained 1x MyTaq™ HS Red Mix (Bioline), 1.5 nmol of each primer, and 2 µL of sample DNA. Heating was carried out using a TGradient thermocycler (Biometra) with an initial denaturation temperature of 95°C (3 minutes), followed by 35 cycles ofdenaturation at 95°C (20 seconds),

primer attachment at 50°C (20 seconds) and DNA elongation at 72°C (30 seconds). PCR results were observed using 0.8% agarose gel electrophoresis. PCR success is indicated by a single band of 710 bp.

Results and Discussion

Genetic characteristics. A genetic identification was carried out to confirm genetics of brown macroalgae from the coastal waters of Kora-Kora. Cytochrome Oxidase gene Subunit 1 (COI) is a barcode DNA commonly used as a reference in genetic identification. The COI gene is also used in tree phylogenetic analysis, genetic diversity, history evolution, and population genetics (Hebert et al 2003). Electrophoresis of mitochondrial DNA Cytochrome Oxidase 1 (CO1) genes of brown macroalgae with amplicon lengths is approximately 600-700 bp. Genetic sequencing results identified the brown macroalgae samples from the coastal waters of Kora-Kora as *S. aquifolium* and *S. henslowianum*. The alignment of the nucleotide sequences of mitochondrial DNA CO1 gene from samples was compared with the other data available in the NCBI database. The profiles of DNA bands were determined by using the FW-COI-SargF/FW-COI-SargR primers (Figure 4).

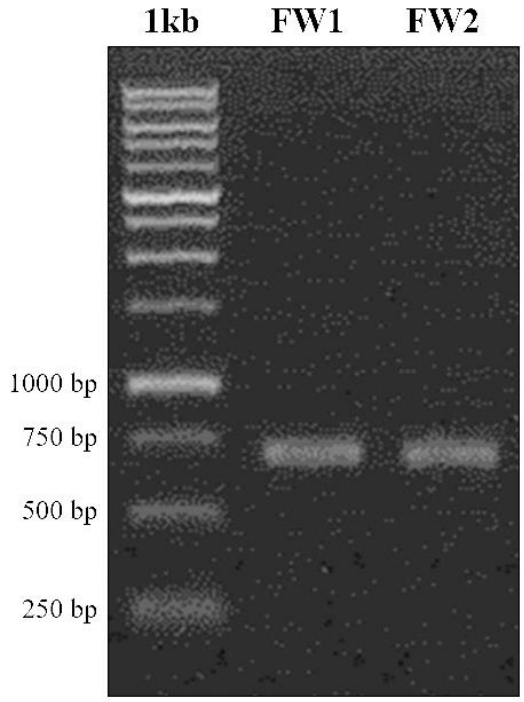

Figure 4. Profiles of DNA bands that use FW-COI-SargF/ FW-COI-SargR primers.

The results showed that the brown macroalgae analyzed morphologically matched the molecular analysis, thus corresponding to same species. The proofreading results from the forward and reverse sequences combined with the sequence of the sample is presented in Figure 5.

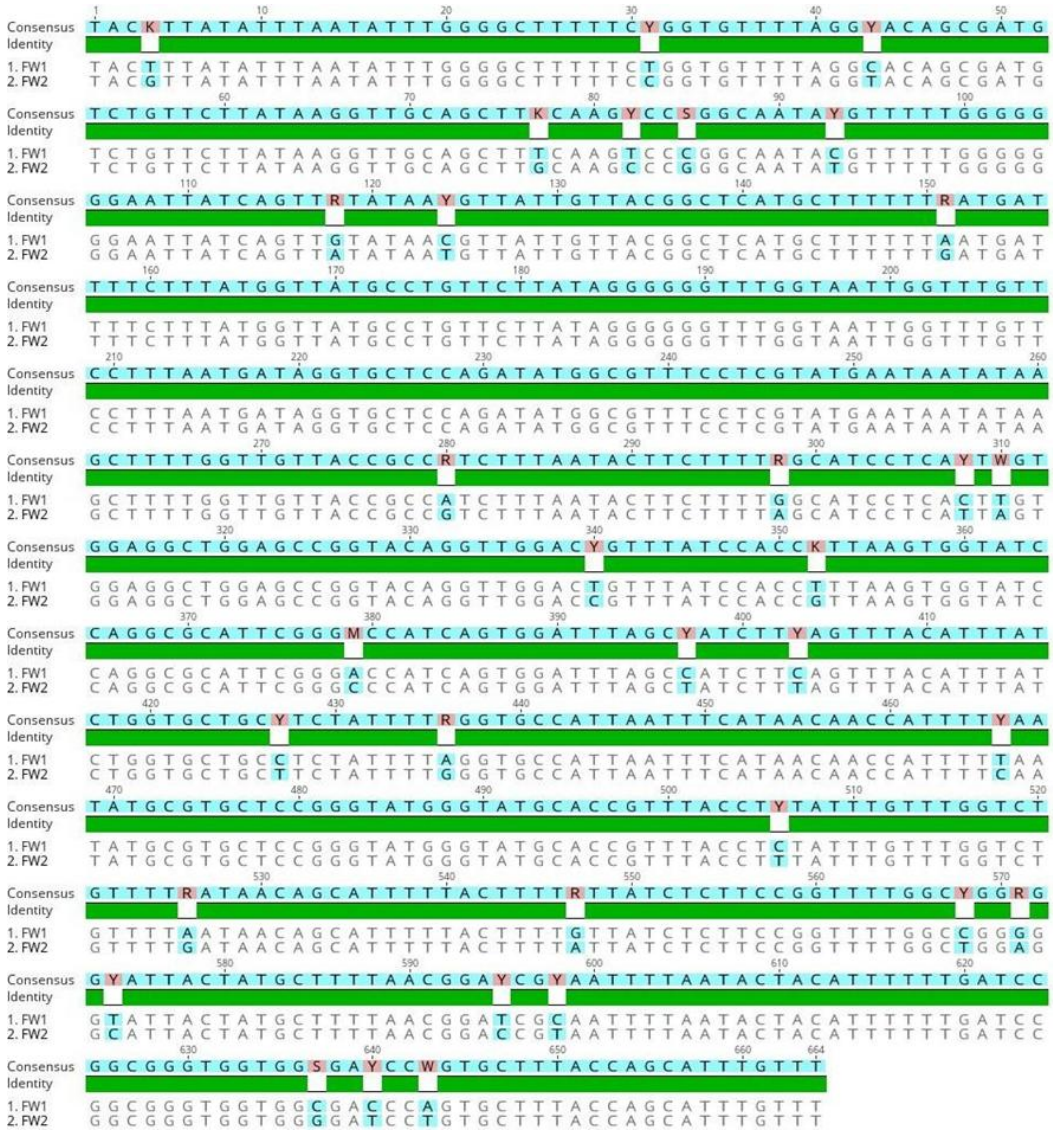

Figure 5. Proofreading results of forward and reverse sequence of samples.

The brown macroalgae sampled from coastal waters were identified in the GenBank, using the BLAST method. The samples were identified as *S. aquifolium* (FW1) and *S. henslowianum* (FW2) with a query cover value of 99.25%, E-value of 0.0 and identity value of 99% (Table 2). Based on the results of BLAST analysis, it can be concluded that these DNA sequences have a high degree of similarity to the DNA sequences available in the Genbank. According to Claverie & Notredame (2003), if the E-value is less than 0.4, then the DNA sequence has a high similarity or homology.

Table 2

Nucleotide sequence identified through BLAST analysis

Sample code	*Species outcome*	*BLAST*			
		Access code of NCBI	*Query cover (%)*	*E-value*	*Identity value (%)*
FW1	*S. aquifolium*	NC_033408.1	100	0	99.25
FW2	*S. henslowianum*	NC_063981.1	100	0	99.25

Phylogeny and relatedness. Genetic identification results show that brown algae sample from the coastal waters of Kora-Kora was identified as *S. aquifolium* and *S. henslowianum*. The alignment of the nucleotide sequences of mitochondrial DNA CO1 gene from samples with the other data available in the NCBI database is presented in Figure 3. Figure 3 showed that the brown macroalgae samples from the coastal waters of Kora-Kora were in different clades and they were identified as *S. aquifolium* and *S. henslowianum*. The phylogenetic tree confirmed that the identified specieswere *S. aquifolium* and *S. henslowianum*.

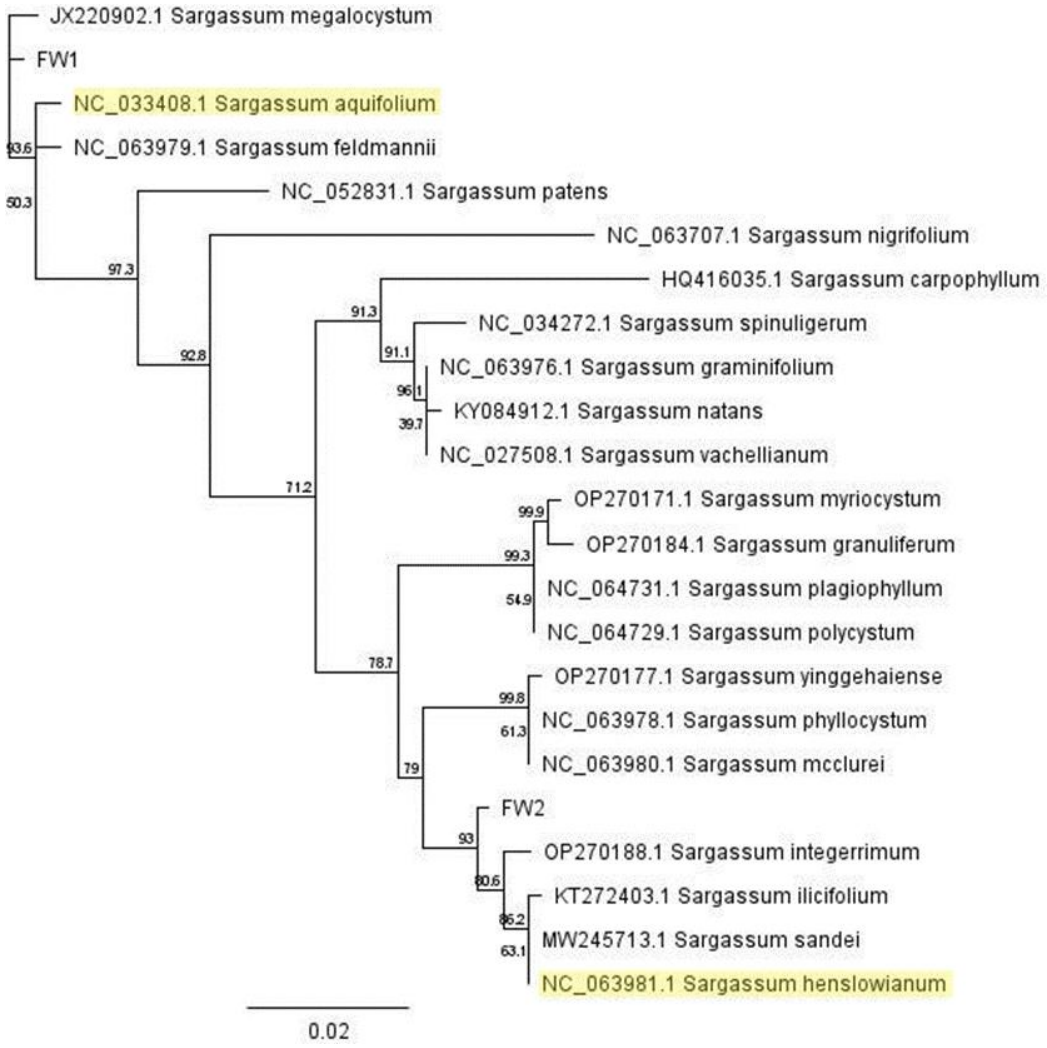

Figure 6. Phylogenetic tree of *Sargassum aquifolium* (FW1) and *Sargassum henslowianum* (FW2).

In recent years, a wealth of bioactive components such as polysaccharides, phlorotannins, and phytosterols have been identified in *S. henslowianum* (Cuong et al 2015). The polysaccharides in *S. henslowianum* mainly consist of fucoidan which possess a wide range of biological activity, such as anti-inflammatory or anticoagulant effect (Hou et al 2001; Hwang et al 2011). Bi et al (2019) found that *S. henslowianum* had an antioxidant activity.

Brown algae have a very wide distribution because it has a high tolerance to various environmental conditions, such as temperature, salinity, and radiation (Zou et al 2018). Species' morphology is very similar, it is difficult to differentiate species based on morphologic criteria (Prabha et al 2012). The genetic distance between monophyletic species was small, therefore it is assumed that their genetic relationship was significant. In both *S. aquifolium*

and *S. henslowianum*, the distance was 0.0079. Different species had different ranges of genetic distances. Moreover, genetic distances between ingroup and outgroup species were greater than 1, indicating genetic differences. The small ingroup genetic differencesuggests close relationship, hence the DNA profiles presumablycorrespond to the same species (Tallei et al 2017). Meanwhile, the genetic distance between the algae genus was greater than between species. Genetic distance is even larger when the samples are compared to outgroup species from other classes. Tallei et al (2017) stated that samples with large genetic distances are assumed to be of distinct species and even genera.

The source of genetic variation, is generally caused by migration, recombination, mutation, population size, and natural selection (Barton 2010). Genetic distance is used to form a phylogeny tree. The phylogeny tree is constructed based on the p-distance method. Results of phylogeny analysis determined a real separation between Indonesian species with outgroup species. Genetic resources are an important key for a species to survive, and the crisis of biodiversity starts from the decreasing level of genetic diversity of a species (Riyadini et al 2020).

Conclusions. The brown macroalgae species found in the coastal waters of Kora-Kora were identified as *S. aquifolium* and *S. henslowianum*, and after phylogenetic analysis, the results obtained showed 97-98% similarity with other *Sargassum* species found inGenBank.

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Conflict of interest. The authors declare no conflict of interest.

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