Challenges in Molecular and Morphological Identification of Sponge Species in Raja Ampat

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Abstract

Sponges (Phylum Porifera) are a group of highly diverse, sessile, and filter-feeding basal metazoans, except spongillids. The majority of sponges are marine species that play an important role in benthic habitat by maintaining the stability of marine ecosystems through nutrient cycling, habitat provision, and bioerosion. However, marine sponges are not easily identifiable because of their lack of species-level distinctive morphological features, which limits efforts to monitor actual species biodiversity. Indonesia is home to approximately 850 identified species of marine sponges, and Raja Ampat archipelago of West Papua province is known for its exceptional marine biodiversity. Despite the species abundance, the exact number of sponges in the region is not well-documented due to the lack of specific studies providing comprehensive data on their diversity. Therefore, this study aimed to conduct a DNA barcoding analysis using the mitochondrial cytochrome oxidase I (COI) gene as a marker, combined with morphological analyses of 22 individuals collected in the waters of Sapokreng, Besir, and Mansuar Islands in Raja Ampat. The results showed that 3 samples were identified at the species level (Halichondria sp. and Stylissa carteri) with good query cover and percent identity. This showed the possible presence of undescribed or cryptic species, suggesting a severe lack of reference data for both morphology and molecular analyses of marine sponges in the region. Consequently, the analysis showed the presence of a significant gap in the understanding of sponge biodiversity in Raja Ampat's waters.

Keywords: *DNA barcoding, genetic, spicules, sponge*

Introduction

Sponges (Phylum Porifera) are a highly diverse group (Van Soest *et al.*, 2012) of evolutionary basal invertebrate metazoans (Feuda *et al.*, 2017; Simion *et al.*, 2017) that are predominantly marine, except for the freshwater species in the monophyletic family Spongillidae (Masuda, 2006). These animals are distributed globally (Van Soest *et al.*, 2012) and can be found in tropical as well as subtropical waters (Sayori *et al.*, 2022). There are 4 classes of sponges (Ereskovsky and Lavrov, 2021), with the largest being Demospongiae comprising 81%. Sponges are sessile and filter-feeding organisms (Haris *et al.*, 2014; Larasati *et al.*, 2021), which has led to their use as water quality bioindicators (Girard *et al.*, 2021). Although there is a lack of fossil data, molecular clock and biomarker studies suggest that sponges were present before the Cambrian Explosion (Sperling *et al.*, 2010). These organisms have a long evolutionary history and play crucial roles in benthic ecosystems through nutrient cycling, habitat provision, and bioerosion, which contribute to the stability and health of marine environments (Pawlik *et al.*, 2013; Wulff, 2012).

Generally, sponges contain numerous bioactive compounds and synthesize toxic secondary metabolites as a form of self-defense. These metabolites, including alkaloids, terpenoids, and glycosphingolipids, show a variety of biological activities, such as antimicrobial, antiviral, and anticancer properties (Zhang *et al.*, 2017; Cheng-Sánchez and Sarabia, 2018; Varijakzhan *et al.*,

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2021). For example, sponges are among the most common marine organisms considered for cancer drug screening (Bashari *et al.*, 2019; 2021; Prabowo *et al.*, 2023). The bioactive potential of these compounds serves as a significant source for new drug discovery, showing potential importance (Ancheeva *et al.*, 2017; Putra and Hadi, 2017).

Despite the ecological importance and potential usefulness to humans, the biodiversity of sponges has not been widely explored compared to other marine biota (Sayori *et al.*, 2022). The lack of study is due to difficulty of identification and classification because of the absence of distinct morphological features (Cárdenas *et al.*, 2012; Yang *et al.*, 2019). This morphological ambiguity has significantly hindered accurate species-level biodiversity assessments, complicating conservation efforts and ecological studies (Van Soest *et al.*, 2012). Consequently, there is a need for advanced taxonomic methods, such as DNA barcoding, to improve the resolution of sponge biodiversity and enhance the understanding of their ecological roles (Vargas *et al.*, 2012; Boury-Esnault *et al.*, 2013; Morrow and Cárdenas, 2015).

DNA barcoding is a method used to identify species by analyzing a short, standardized region of genetic material, typically from the mitochondrial COI gene in animals, which allows for rapid and accurate species identification by comparing the barcode sequence to a reference database (Hebert and Gregory, 2005). The method has also been applied to other marine biota, such as soft corals (Subhan *et al.*, 2022), sea cucumbers (Madduppa *et al.*, 2017; Yamana *et al.*, 2022), and gastropods (Setiamarga *et al.*, 2019). It has been successfully used to identify different sponges (Ngwakum *et al.*, 2021) and analyze the phylogenetic relationships of sponges (Vargas *et al.*, 2015).

As one of the largest archipelagos in the world and home to 2 biodiversity hotspots, Indonesia is estimated to have more than 700 species of sponges distributed in the western and central waters (Haris *et al.*, 2014; Sayori *et al.*, 2022; Putra *et al.*, 2023). Previous studies have been conducted on the distribution of sponges in the country using the DNA barcoding method. For example, Beking *et al.* (2013) identified *Suberites diversicolor* in Kalimantan and Papua. Schuster *et al.* (2017) used DNA barcoding to identify the sponge *Cinachyrella* spp. in East Kalimantan and West Papua. However, studies on the biodiversity of marine sponges in Indonesia remain severely lacking (Hutomo and Moosa, 2005; Chasanah, 2008).

The Raja Ampat archipelago, located in Indonesia's West Papua province, consists of over 610 small islands, atolls, and takas surrounding the main islands of Misool, Salawati, Batanta, and Waigeo (Sagai *et al.*, 2017). This region is a part of the Coral Triangle, recognized as the global epicenter of marine biodiversity (Subhan *et al.*, 2020). Raja Ampat's marine ecosystems include extensive coral reefs, mangroves, and seagrass beds, which support a wide variety of marine life. For example, there are 1,320 species of fish (Sagai *et al.*, 2017), 537 hard corals (Sagai *et al.*, 2017), 699 mollusks, 530 gastropods (Anggraeni, 2021), 6 seagrass, and 7 mangroves (Supriyadi *et al.*, 2018). Despite recent interest in the region, comprehensive biodiversity studies are still critically sparse, leading to incomplete, inadequate, and biased species lists that focus on certain animal groups. Sponges are recognized as part of the biodiversity, studies providing comprehensive data on their diversity are scarce, and the exact number of species remains unknown.

The potential ecological importance of sponges and their usefulness for human life, particularly in the exploration of secondary metabolite sources, show the need to explore the biodiversity of sponges. Due to the difficulties in identification, there is a need to continue developing appropriate methods to record and collect biodiversity data as a whole. Therefore, this study aimed to identify sponge species using both the DNA barcoding method and morphological observations to enhance knowledge and data related to sponges in Indonesia, particularly in the Raja Ampat region.

Materials and Methods

A total of 22 samples were collected from Raja Ampat (Figure 1), including Sapokreng (A1), Besir (A2), Mansuar (A3), with 4, 12, and 6 samples collected from each location respectively. Sampling was performed in October 2022 and analyzed in November 2022. Each sample was divided into 2 different preserves. Morphological samples were placed in a closed container and preserved using 5% buffered formaldehyde for 24 h, transferred to a container containing 80% ethanol solution (Nunez *et al.,* 2017). Meanwhile, the genetic samples were placed in a collection tube containing 96% ethanol for preservation (Ngwakum *et al.,* 2021).

Morphological procedure

The body part of each sponge was cut into pieces measuring 0.5 cm and placed in the reaction tube. The samples obtained were treated with *natrium hypochlorite* to dissolve the sponges material, followed by rinsing with distilled water and ethanol. The suspension sediment was placed on a

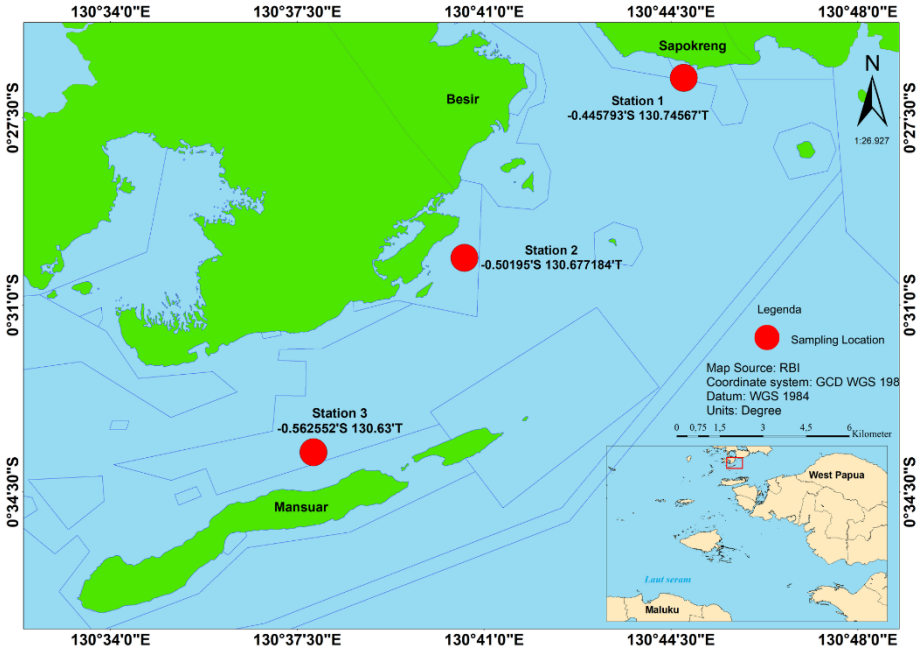

Figure 1. Sampling location at the Raja Ampat, West Papua

glass slide for observation under a microscope to study the spicules (Sayori *et al.,* 2022).

Genetic procedure

DNA was extracted using the Geneaid gSYNC extraction kit. The mitochondrial cytochrome oxidase I (COI) gene was (Polymerase Chain Reaction) PCR-amplified using the forward primer LCO 1490 (TAA ACT TCA GGG TGA CCA AAA AAT CA) and the reverse primer HCO 2198 (GGT CAA CAA ATC ATA AAG ATA TTG G) (Erpenbeck *et al.,* 2012). PCR amplifications were performed in 25 µL reaction mixture containing 2 µL DNA template, 8 µL ddH_2O, 1 µL each primer, and 13 µL MyTaq HS Red Mix (Bioline). The reactions were exposed to the following PCR profile, 35 cycles of denaturation (94^{0}C for 1 min), primer annealing (44^{0}C for 1 min), and extension (72^{0}C for 1 min) then 5 min at 72^{0}C as the final extension (Ngwakum *et al.,* 2021). The amplicon product was processed using the 1.5% electrophoresis agarose gel and was running at 100-volt voltage for 25 min (Kusuma *et al.,* 2016). The amplicon or PCR products were translated to nucleotide base following the Sanger method (Sanger *et al.*, 1977).

Morphological analysis

The identification of sponge types follows the guidelines provided in the book "Systema Porifera: A Guide to the Classification of Sponges" by John Hooper and Robert Van Soest (2002). This identification process includes examining the composition of spicules obtained from microscopic observations and the shape of the skeleton.

Bioinformatic analysis

Molecular data analysis includes using Mega 11 software to input sequence results obtained from the sequencing stage (Tamura *et al.,* 2021). The sequence data is edited using the Clustal W method. Subsequently, the sequence data is compared with the information available in GenBank at National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tools (BLAST) (Dailami *et al.*, 2021). The BLAST results are used to determine the species type. Moreover, BLAST results with higher identity values indicate greater species similarity (Simbolon *et al.,* 2021). Data accuracy can be assessed based on 3 criteria, namely query coverage approaching 100%, e-value or error rate reaching 0, and percent identification approaching 100% (Gaffar and Sumarlin, 2020). The phylogenetic tree was constructed using the Maximum Likelihood method with 1000 bootstraps on the Mega11 software (Vargas *et al.,* 2015).

Result and Discussion

Spicule characteristic

A total of 22 samples were identified as spicule types and genetic analysis was sequenced targeting

the COI gene. Based on the analysis, 5 samples (A2.G.024, A2.G.027, A2.G.028, A3.G.033, A3.G.040) were identified as sponge biota. One way to identify sponge morphology is by examining the shape of the spicules in the collected samples. The spicule shapes were observed using a microscope to analyze the spicules in each sample. Sponge spicules can be categorized based on size into megasclera and microsclera (Trianto *et al.,* 2016). Megasclera are spicules that contribute to forming the main skeleton of sponges and structural support for the cortex or as a defense against predators (Schuster *et al.,* 2015).

In sample code A2.G.024, megasclere spicules with the *monoaxon* form *Hastate oxea* were identified (Figure 2A). This shape is characterized by its elongated structure with a slightly pointed tip at each end (Trianto *et al.,* 2016). The spicule type found in the sponge *Halichondria bowerbanki* in the British Isles by Ackers *et al.* (2007) is similar. After microscopic observation, sample A2.G.027 was found to possess megasclera spicules with the *Styloid* spicule type, featuring one pointed and blunt end, the spicules were long, and the edges were smooth (Figure 2B). This was similar to the report by Pereira *et al.* (2023) which found a spicule in *Stylissa carteri* with long, smooth, and curved style. Sample A2.G.028 (Figure 2C) also contains spicules of the *Hastate oxea* shape, like those in sample A2.G.024, characterized by elongated form with pointed ends. Meanwhile, sample A3.G.033 showed Fusiform style spicules (Figure 2D), which are long with one end pointed and the other end curved. Additionally, *Prodiaene tetraxon* megasclera spicules were found in sample A3.G.033, characterized by 4 axes with 1 branched end (Figure 2E). A3.G.040 contained spicules with a *Strongyle* shape (Figure 2F), with both blunt and curved ends. Based on the observations of 5 samples, the identified spicule shapes were classified as *monoaxon* and t*etraxon* spicules, typically found in sponges from the Demospongiae class (Lukowiak, 2020). Genetic analysis also showed that the type of sponge found belonged to the Demospongiae class, correlating with the observations of spicule type.

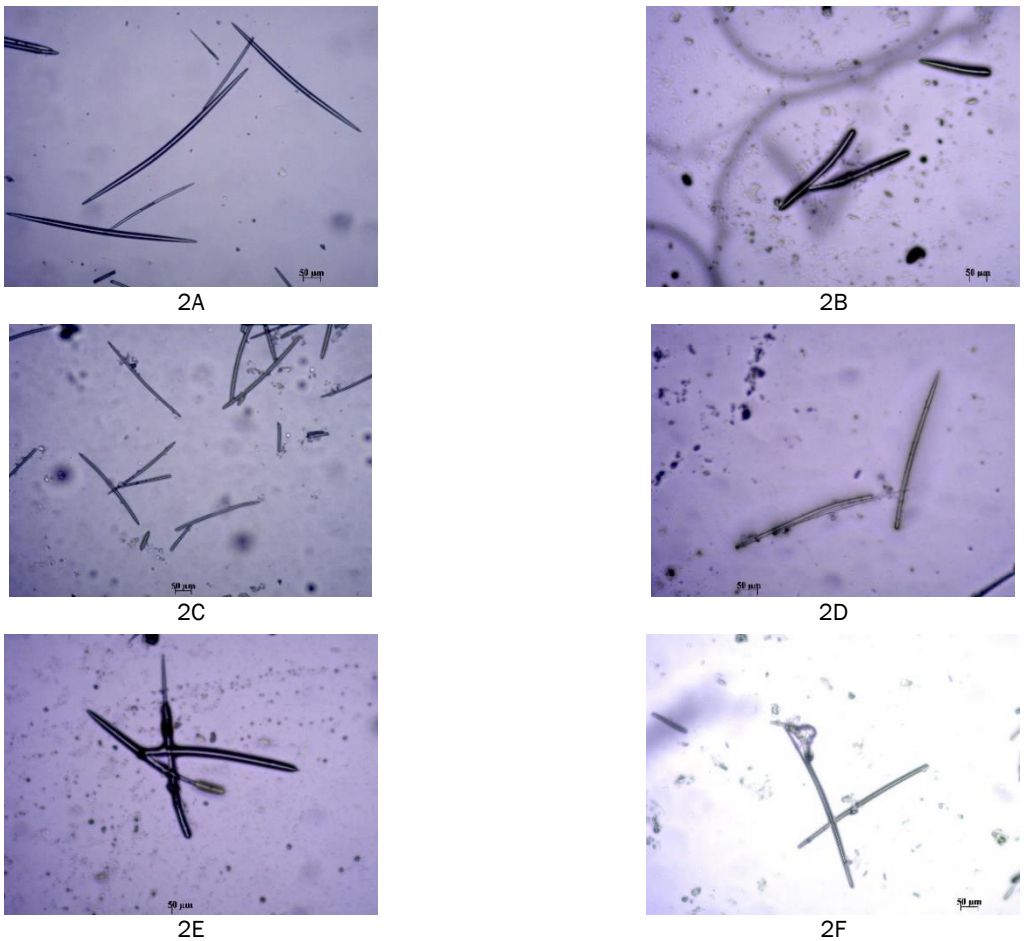

Figure 2. Spicules identified from sample A2.G.024 (2A), A2.G.027 (2B), A2.G.028 (2C), A3.G.033 (2D,2E), and A3.G.040 (2F) at 10x magnification

Table 1. Identification of species of sponges using BLAST

Code sample	Query cover (%)	Percent identity (%)	Species
A2.G.024	100	99.85	*Halichondria* sp.
A2.G.027	100	100	*Stylissa carteri*
A3.G.033	100	97.10	*Stylissa carteri*

Molecular characteristic

The nucleotide base sequences were obtained through the extraction process, Polymerase Chain Reaction (PCR), and sample sequencing. Subsequently, the resulting sequences were analyzed using the BLAST in Genbank at NCBI to determine the species of sample based on similarity level in the Genbank database. The analysis results of the sponge samples from Raja Ampat waters are presented in Table 1.

Molecular study successfully identified 3 out of the 22 samples as sponge species. The samples showed high suitability and accuracy, as indicated by 3 key parameters, namely a query coverage value approaching 100%, an e-value (error rate) close to 0, and a percent identity near 100% (Gaffar and Sumarlin, 2020). Query coverage shows the extent of similarity between the length of the sample nucleotide bases and those in the Genbank database (Wehantouw *et al.*, 2017). Percent identity denotes the correlation between the sequence from the sample and the sequence in the database (Gaffar and Sumarlin, 2020). Wehantouw *et al.* (2017) stated that the expected value provided information on the error rate or estimated homology value based on statistical measures of the shared sequence with the database. A higher e-value indicates a lower level of sequence homology and vice versa.

Based on the key parameters, 3 samples met the specified characteristic requirements (Table 1). Samples with codes A2.G.024, A2.G.027, and A3.G.033 had a query coverage of 100%, a similarity level of 97.10% to 100%, and an e-value of 0. One of these samples was identified as *Halichondria* sp., while 2 were identified as *Stylissa carteri*. Meanwhile, A2.G.028 and A3.G.040 were identified as sponge species. Sample A2.G.028 was identified as *Xestospongia deweerdtae* with a percent identity value of 95.57%, but the query coverage value was very low at 15%. A3.G.040 was identified as *Theonella* sp. with a percent identity of 98.80%. However, the resulting query coverage was relatively low at 69%. The low query coverage was consistent with the sequence quality of the sequencing analysis carried out. Sample A2.G.028 showed a length of 1,777 bp and produced the longest DNA sequence compared to others. The sequence quality was low, as indicated by the chromatogram, which showed good quality only for approximately 699 bp. The rest of the sequence had poor quality, with the chromatogram being unstable and low. This issue also occurred in sample A3.G.040, where the total sequence length was 241 bp, and the chromatogram with good quality was only approximately 150 bp. This sample had the shortest sequence length compared to other sponges, which ranged from 699 bp to 700 bp. Although the sequence similarity values for these samples were high (95.57% and 98.80%), the results had low validity due to inadequate query coverage.

Based on the morphological identification of the spicule type, both samples were indicative of sponge biota. This was because spicules with the monoaxon *Hastate oxea* (Figure 2C) shape were observed in A2.G.028, and *Strongyle* (Figure 2F) in sample A3.G.040. The spicules detected in A2.G.028 and A3.G.040 are commonly found in sponges of the Demospongiae class. Generally, the size of spicules is often used to distinguish between species within the same genus, as spicules can vary in size across different taxa, and within the same genus (Lukowiak, 2020). Swierts *et al.* (2013) stated that sponge species with the same morphological lineage showed evolutionary differences. In these cases, molecular studies have shown the existence of genetically differentiated lineages in morphologically identical samples. Several reports on sponge plasticity through DNA sequencing must still be supported by morphological analysis (Balansa and Rieuwpassa, 2022). Therefore, both data types are equally important when reporting on sponge plasticity to ensure more accurate results.

As presented in Table 1, 17 samples analyzed by BLAST did not yield results for sponge species, but were identified as mollusks, arthropods, and rotifers. This occurs because sponges are filter-feeding, which causes many non-target macro and microorganisms, to be associated with sponges. Therefore, non-target organisms can be extracted during the laboratory analysis process, making the DNA sequence difficult to interpret or belonging to non-target organisms (Vargas *et al.*, 2012). Based on the sequence data analyzed by BLAST, the phyla (Mollusca, Arthropoda, and Rotifera) detected are associated with sponges, as organisms from these phyla are commonly found in sponge habitats. Sponges typically live and grow on

the substrate of water bottoms, attaching to hard surfaces such as corals and rocks (Marzuki, 2018). This condition allows metabolic waste from non-target phyla to be filtered by sponges. As filter feeders, sponges obtain food in the process, allowing non-target DNA to be extracted during laboratory analysis.

Based on the results, 3 samples were found to be sponge biota with good accuracy. Additionally, spicules can help complement genetic data. Samples identified as sponge species also contained sponge spicules, namely monoaxon and tetraxon. These spicules are typically found in sponges of the class Demospongiae. However, this data cannot sufficiently explain the specific morphological characteristics. Therefore, external morphological data such as texture, shape, color, and life form are recommended for future studies to provide a higher level of accuracy.

Phylogenetics tree

Genetic information is used as a key to environmental conservation. Changes in environmental conditions can cause variations in species composition in ecosystems, thereby influencing genetic information (Kusuma *et al.,* 2016). The phylogenetic reconstruction was made using the Kimura 2-Parameter model with the Maximum Likelihood with 1000 bootstrap on all of sequences detected as sponge biota.

The addition of sequences from GenBank was carried out to strengthen the results of phylogenetic tree construction. Specifically, sequence data from GenBank, including the species *Leuconia nivea*, were incorporated into the phylogenetic tree as an outgroup. The selection of sequences for the outgroup should have a close correlation with the sequences used for analysis in phylogenetic tree construction, showing significant differences from others. Moreover, using outgroup sequences that are excessively divergent from the sequences being analyzed can lead to errors in phylogenetic tree predictions (Dharmayanti, 2011). Phylogenetic tree reconstruction using the Maximum Likelihood method (Figure 3) showed the presence of 4 major clades, namely *Stylissa carteri*, *Halichondria* sp., *Xestospongia deweerdtae*, and *Theonella* sp. Specifically, the phylogenetic tree indicated that the sponge *Stylissa carteri* in sample code 33 shared a clade with *Stylissa carteri* A2.G.027. This suggested that both sequences might belong to the same species but with different haplotypes. The presence of these haplotype differences is beneficial for the survival of an organism, allowing adaptation to environmental changes to reduce the risk of extinction (Saleky *et al.*, 2020). These haplotype differences are reflected in the genetic distance observed in the analysis.

Genetic distance analysis was conducted to assess the genetic distance between species. The results showed that the lowest genetic distance was between *Halichondria* sp. and *Stylissa carteri*, at 0.263 (Table 2). Furthermore, genetic distance for species identified with outgroups was very high. This is because the outgroups do not belong to the same class as the species under analysis. The phylogenetic tree includes numbers at the end of branches, which represent the bootstrap values. Subari (2021) stated that the bootstrap value was used to test the reliability of model data set. A lower bootstrap value indicates that the sequence used to construct the phylogenetic tree is not reliable. According to Saleky *et al.* (2021), a higher bootstrap value in the phylogenetic tree shows better reconstruction. When the bootstrap value is above 70%, the branching is permanent or significant. Meanwhile, values above 95% indicate that the branching is accurate and consistent (Yonatika *et al.*, 2020). In the phylogenetic tree reconstruction, the bootstrap values for each clade range from 89% to 100%. Therefore, the phylogenetic trees for identification are reliable and accurate for *Stylissa carteri* (97%), *Halichondria sp.* (99%), and *Xestospongia deweerdtae* (100%).

Distribution of sponges in Indonesia

Sponges are marine organisms that are not frequently explored compared to coral reefs or others (Sayori *et al.*, 2022). According to Bell *et al.* (2015), this group of organisms has been considered neglected in marine environments. Several publications related to sponges in Indonesia have been conducted

Table 2. Genetic Distance of Sponge Detection

	Halichondria sp.	*Stylissa carteri*	*Xestospongia deweerdtaae*	*Theonella* sp.	*Leuconia nivea*
Halichondria sp.					
Stylissa carteri	0.263				
Xestospongia deweerdtaae	0.378	0.371			
Theonella sp.	0.416	0.462	0.398		
Leuconia nivea	10.763	15.602	16.433	17.964	

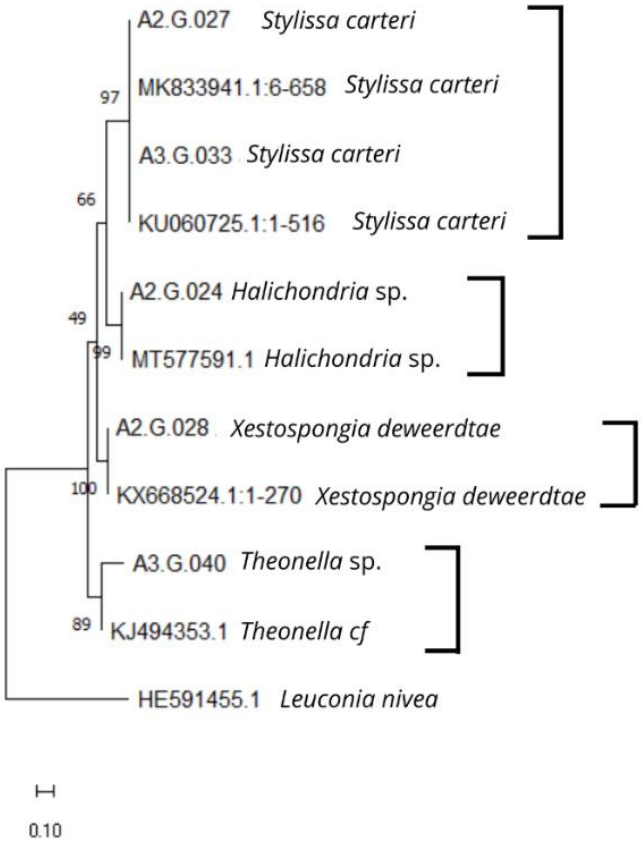

Figure 3. Phylogenetic tree using the Maximum Likelihood method with bootstrap 1000

in western region (the Thousand Islands, Riau Islands, Central Java, and East Java) as well as central Indonesia (Sulawesi and Kalimantan). Investigations on sponges in eastern Indonesia have also been carried out, with 11 species found in Manokwari (Sayori *et al.*, 2022) and 3 in Misool, Papua (Cleary *et al.*, 2017).

A literature review was conducted regarding sponge species activities in Indonesia. The results showed that the distribution of studies was mostly concentrated in Java and Sulawesi compared to Sumatra, Kalimantan, Nusa Tenggara, and eastern regions. This suggests that reports on sponges in Indonesian waters have not been comprehensive. Aulia *et al.* (2021) reported that the study conducted by de Voogh *et al.* (2006) predominantly focused on western and central Indonesia (Sayori *et al.*, 2022). According to literature from 2005-2021, the number of published sponge species in Indonesian waters is 340, with 104 sponge genera, the dominant being *Haliclona*, *Callyspongia*, *Petrosia*, *Clathria*, and *Xestospongia*. Data collection on the distribution of sponge species has used various methods, including Point Intersect Transect (PIT), belt transect, morphological observation, and spicule purification. However, data collection related to sponges using DNA barcoding remains very limited.

The use of DNA barcoding to identify sponge species in Indonesian waters is limited due to the challenges associated with identifying sponges using this method, as it may detect non-target DNA (Vargas *et al.*, 2012). The application of DNA barcoding in identifying new sponge species was demonstrated by Becking *et al.* (2013) and Schuster *et al.* (2017) in studies conducted in the waters of Papua and Kalimantan. Becking *et al.* (2013) used mitochondrial DNA to identify the sponge species *Suberites diversicolor* and investigated its phylogeography across various locations in Indonesian waters. Schuster *et al.* (2017) conducted a study on the distribution, diversity, and mobility of intron I in the sponge class *Tetractinellida*. The use of DNA barcoding methods in sponge studies is crucial for advancing knowledge about sponge genetic information. As stated in the 2010 Nagoya Protocol, the importance of genetic resources and their use was recognized (Darmadi and Mirza, 2016). Based on molecular studies with good DNA quality, 3 samples were identified as sponge species, particularly *Halichondria* sp. and *Stylissa carteri* (Table 1).

Conclusion

In conclusion, this study showed that the DNA barcoding method successfully identified 3 out of 22 samples processed from Raja Ampat Waters with good query coverage and percent identity. A total of 2 samples did not have good quality from BLAST results. Furthermore, observations of spicule types found *Hastate oxea, Styloid, Fusiform style, Strongyle,* and *Prodiane* spicules from 5 samples, indicating the presence of sponge biota. The use of spicule type observation methods could complement morphological and genetic data, strengthening the understanding of sponge species. However, there were still challenges in identifying sponge species molecularly and morphologically. The challenges included the possibility of detecting other biota because sponges had filter feeders. Therefore, ensuring high-quality DNA and selecting target sponge biota were recommended for successful identification.

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