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DNA ISOLATION FOR PCR AMPLIFICATION AND MOLECULAR IDENTIFICATION OF MARINE SPONGES COLLECTED IN CON DAO ISLANDS, VIETNAM

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ABSTRACT

Molecular approaches based on DNA sequences have become increasingly indispensable in modern taxonomy, particularly for taxa with few or ambiguous diagnostic morphological characters, such as sponges. However, genomic DNA isolation from sponges is often hindered by abundant secondary metabolites. Here, we optimized a CTAB-based DNA extraction protocol by adjusting the concentrations of CTAB, SDS, and proteinase K. The optimized extraction buffer (4% CTAB, 1% SDS, and 5 µg/mL proteinase K) substantially improved DNA yield and purity. Applied to six sponge specimens from the Con Dao Islands, the protocol yielded DNA concentrations exceeding 200 ng/µL from 100–300 mg of tissue, with purity ratios indicative of high-quality DNA. DNA integrity was confirmed by successful PCR amplification of the mitochondrial cytochrome c oxidase subunit I (COI) gene fragment. Sequence analyses corroborated morphological identifications and enabled species-level assignment of four specimens. Together, these results demonstrate that the optimized extraction protocol provides a reliable and effective approach for obtaining high-quality genomic DNA from sponges, thereby facilitating molecular identification and supporting taxonomic and phylogenetic studies of marine sponges.

Keywords: Marine sponges, Con Dao Islands, DNA isolation, molecular identification, cytochrome c oxidase subunit I.

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INTRODUCTION

Marine sponges are among the earliest known metazoans, with fossil records dating back to approximately 630 million years ago (Maloof et al., 2010). They are distributed worldwide across diverse marine habitats. According to the World Porifera Database (de Voogd et al., 2025), more than 9,432 sponge species have been recognized, classified into 680 genera and four classes: Calcarea, Demospongiae, Hexactinellida, and Homoscleromorpha. In the Western Pacific region, including the East Sea of Vietnam, sponge diversity is considered high, with numerous species reported from tropical and subtropical waters (Hooper et al., 2000). Sponges play important ecological roles in marine ecosystems, such as nutrient cycling and habitat formation (Hill et al., 2013), and they are also a rich source of bioactive compounds with substantial pharmaceutical and biomedical potential (Thakur & Müller, 2004).

Early research on marine sponges in Vietnam was limited in scope. Lindgren (1898) recorded only 20 species from Nha Trang Bay. Later, Dawydoff (1952) documented 119 sponge taxa from the Indochina Sea, of which 102 were identified to species level (Thai Minh Quang, 2017). A more extensive survey conducted in 1977–1978 by Nguyen Van Chung et al. (1978) reported 160 sponge taxa along the coast of Vietnam, comprising 142 species across 18 genera. Hooper et al. (2000) later reported 176 sponge taxa belonging to the class Demospongiae in Vietnam, with 126 taxa identified to species level. More recently, Lim et al. (2016) and Quang (2017) compiled consolidated checklists, recording a total of 141 marine sponge species from Vietnamese waters. Building on these earlier studies, Thung (2024) synthesized data from multiple surveys and reported 205 sponge species in Vietnam, representing 85 genera, 56 families, and 22 orders across three classes, documented from 14 major islands. Despite a steady increase in documented species, Vietnam's marine biodiversity, spanning a 3,260 km coastline and more than 4,000 islands and islets, remains substantially underexplored with respect to sponge diversity.

Traditionally, morphology-based taxonomy has been the primary approach to studying sponge diversity. However, taxonomic classification and phylogenetic inference in sponges remain particularly challenging due to their simple body plans, the limited number of informative morphological characters, and the frequent occurrence of cryptic morphology (Hooper et al., 2000). To overcome these limitations, molecular approaches based on DNA sequence data have shown considerable promise (Wörheide et al., 2007). Among these, the use of specific DNA fragments, particularly the mitochondrial cytochrome c oxidase subunit I (COI; also cox1) gene, has proved especially effective for species identification across animal taxa, including sponges (Evans & Paulay, 2012). This molecular approach enhances species discrimination and complements traditional morphology-based taxonomy.

Deoxyribonucleic acid (DNA) extraction is a critical first step for a wide range of molecular applications, including DNA barcoding, molecular taxonomy, genomics, and other genetic studies (Gupta, 2019). Although numerous DNA isolation methods are available, the distinctive structural and biochemical characteristics of sponge tissues often complicate extraction, making the process more challenging than for many other metazoans. As a result, specialized reagents and tailored protocols are frequently required to obtain high-quality DNA from sponges. In our preliminary experiments, commercially available extraction kits failed to produce DNA of sufficient quality or quantity, often yielding low concentrations and highly fragmented DNA. Among manual DNA isolation approaches, protocols based on cetyltrimethylammonium bromide (CTAB) are widely used because they are cost-effective, scalable for high-throughput applications, and requires relatively small amounts of tissue (Krishnan et al., 2024).

Although originally developed for plant DNA extraction (Krishnan et al., 2024), the CTAB method has also been successfully applied to a wide range of marine taxa (Arseneau et al., 2017; Schiebelhut, 2017; Mishra et al., 2019). The strength of the

CTAB approach lies in its versatility; however, protocol optimization is often necessary for specific tissues or taxa to maximize DNA yield and purity. In this study, we developed an optimized DNA extraction protocol for marine sponges based on the conventional CTAB method by adjusting the concentrations of CTAB, SDS, and proteinase K. The optimized protocol was evaluated on six sponge specimens collected from the Con Dao Islands, Vietnam. The extracted DNA consistently met the quality and quantity requirements for downstream molecular analyses. Successful PCR amplification and sequencing of the mitochondrial cytochrome c oxidase subunit I (COI) barcode fragment further demonstrated the suitability of the extracted DNA for molecular identification. Together, these results highlight the utility of molecular approaches for future investigations of sponge genetic diversity in Vietnam.

MATERIALS AND METHODS

Materials

Sponges were collected from five sites around the Con Dao Islands using SCUBA at depths ranging from 3 to 10 m. Samples were kept on ice and transported to the laboratory of the Institute of Marine Biochemistry (now the Institute of Chemistry). Each voucher specimen was assigned a unique identification code, and reference photographs were taken prior to further processing.

Figure 1. Unidentified sponge specimens exhibiting distinct growth forms and textures. **A**, branching; **B**, massive; **C**, encrusting

For molecular analyses, tissue samples were stored at -20 °C until DNA extraction. To develop an efficient protocol for total genomic DNA extraction, three morphologically distinct but taxonomically unidentified sponge specimens were initially used (Fig. 1). The optimized protocol derived from these preliminary trials was subsequently evaluated on six additional sponge specimens (Table 1, Fig. 2). These latter specimens were taxonomically identified by Hoang Dinh Chieu & Luu Xuan Hoa at the Research Institute for Marine Fisheries, Vietnam.

Table 1. Sponge specimens and sampling sites around the Con Dao Islands.

No.	Sponge specimen ID	Sampling site (local name in Vietnamese)	Coordinates
1	HMCD02Da	Mũi Đá Trắng	8°39’33.0”N-106°36’26.4”E
2	HMCD02C	Mũi Chân Chim	8°41’28.6”N-106°38’59.2”E
3	HMCD02HT	Hòn Tre	8°44’20.2”N-106°35’10.0”E
4	HMCD04HT	Hòn Tre	8°44’20.2”N-106°35’10.0”E
5	HMCD04Ta	Hòn Tài, Vịnh Côn Sơn	8°38’19.0”N-106°37’40.7”E
6	HMCD04DT	Vịnh Đầm Tre	8°44’53.7”N-106°39’35.2”E

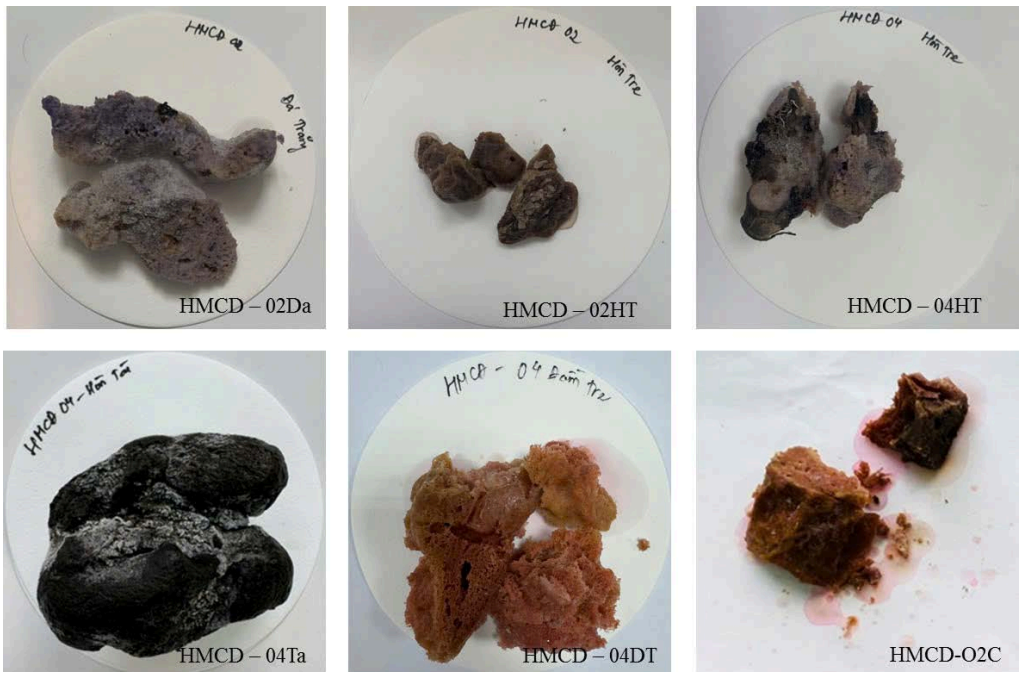

Figure 2. Six sponge voucher specimens from the Con Dao Islands used in this study. Specimens were photographed on 110 mm diameter Whatman filter paper for size reference

Methods

Optimization of the CTAB-based DNA extraction method for sponges

Frozen sponge tissues were carefully separated from substrates using a sterile scalpel under aseptic conditions. Samples were thoroughly rinsed with sterile distilled water to remove sand, debris, epibionts, and associated larvae. Excess moisture was removed by blotting with four layers of Whatman filter paper, followed by air-drying at room temperature (25 ^{o}C) for 2 h. Dried tissues (100–300 mg) were ground to a fine powder in liquid nitrogen using a sterile mortar and pestle. The homogenized tissue was suspended in 1.0 mL of sorbitol buffer (100 mM Tris-HCl, 300 mM sorbitol, 5 mM EDTA, and 0.5% NaH_2PO_4, w/v) and transferred to a new 2-mL microcentrifuge tube containing CTAB extraction buffer (Doyle & Doyle, 1987) with the following modifications: (i) CTAB concentrations of 1, 2, 4, 6 and 8% (w/v) were tested; (ii) SDS at concentrations of 0.5, 1.0, and 2.0% (w/v) was evaluated in combination with the optimized CTAB concentration; and (iii) proteinase K was added at final concentrations of 5 or 10 µg/mL. The mixture was incubated at 65 ^{o}C for 1 h, followed by centrifugation at 12,000 rpm for 10 min at 15 ^{o}C. The supernatant was transferred to a new 2 mL microcentrifuge tube, and 1 µL of RNase A (20 mg/mL) was added, followed by incubation at 37 °C for 30 min. Subsequently, 800 µL of chloroform:isoamyl alcohol (24:1, v/v) was added, the tube was gently inverted to mix, and the sample was centrifuged at 13,000 rpm for 10 min.

The upper aqueous phase was transferred to a fresh 2 mL tube, and DNA was precipitated by adding 1.6 mL of absolute ethanol (≥ 99.9%; Merck) together with 0.1 volume of 3 M sodium acetate. After overnight incubation at −20 °C, the DNA pellet was collected by centrifugation at 13,000 rpm for 15 min at 4 °C, washed with 500 µL of 70% ethanol, and centrifuged again at 13,000 rpm for 10 min at 4 °C. The pellet was air-dried, resuspended in 50 µL of sterile, nuclease-free water, and stored at −20 °C until further analysis.

Quantification of DNA

The concentration and purity of the extracted DNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). A 3 µL aliquot of each DNA sample was measured at 260 nm to determine DNA concentration, and purity was evaluated based on the A260/A280 ratio. DNA integrity was further examined by electrophoresis on 0.8% agarose gels.

Validation of the optimized CTAB DNA extraction method

PCR amplification of the COI gene fragment

Genomic DNA was extracted from six sponge specimens collected from the Con Dao Islands using the optimized CTAB protocol. After measuring concentration and purity, DNA samples were used as templates for PCR amplification of the COI gene fragment. Amplifications were performed using the gene specific primer pair LCO (5′-GATAGGAAC AGCATTTAGTATGC-3′) and RCO (5′-CCAA AGAACCAGAAAAGGTGTTG-3′). Each 20 µL PCR reaction contained 2.0 µL of 10× DreamTaq buffer (Thermo Fisher Scientific), 0.4 µL of forward primer (10 pmol), 0.4 µL of reverse primer (10 pmol), 0.2 µL of DreamTaq DNA polymerase (Thermo Fisher Scientific), 1.6 µL of dNTP mix (10 mM), 1.6 µL of $MgCl_2$ (25 mM), 1.0 µL of genomic DNA, and 12.4 µL of nuclease-free water. PCR cycling conditions consisted of an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 40 s, with a final extension at 72 °C for 10 min. A no-template negative control (DNA omitted) was included in each run to monitor potential contamination. PCR amplicons were resolved by electrophoresis on 1% agarose gels and visualized under UV illumination.

DNA sequencing and analysis

Successful PCR products were purified using the GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Fisher Scientific) according to the manufacturer's instructions and subjected to bidirectional Sanger sequencing (1st BASE, Singapore). Raw chromatogram files were visually inspected and edited to remove ambiguous base calls using BioEdit, and forward and reverse reads were assembled into consensus sequences in MEGA version 11.

Phylogenetic relationships among the six sponge specimens (Table 1) were inferred together with reference sequences retrieved from GenBank. Multiple sequence alignment was performed using the ClustalW algorithm implemented in MEGA 11 (Tamura et al., 2021). Clustering trees were reconstructed using the neighbor-joining (NJ) method with 1,000 bootstrap replicates to assess node support. Species-level assignments were inferred based on clustering with conspecific reference sequences and pairwise sequence identity between study sequences and reference sequences within the same NJ clades. Pairwise sequence identities were calculated using the Multiple Sequence Alignment module implemented in DNAMAN X (Lynnon BioSoft).

RESULTS

Optimization of the CTAB extraction method for DNA isolation from sponges

Effect of CTAB concentrations

Isolation of high-quality DNA from marine sponges is inherently challenging and often unsuccessful across many species, due to the abundance of phenolic compounds and other secondary metabolites (Varijakzhan et al., 2021), as well as complex tissue architecture and the presence of robust mineral spicules that hinder mechanical disruption (Langenbruch, 1983). CTAB, a cationic surfactant, facilitates DNA extraction by disrupting membrane lipids thereby promoting cell lysis and nuclear release. At low ionic strength, CTAB precipitates nucleic acids together with secondary metabolites (Sambrook & Russell, 2001), whereas at higher salt concentrations it preferentially forms complexes with polysaccharides, reducing their co-extraction with DNA (Kishor et al., 2020).

Previous studies have recommended the use of 2% CTAB in extraction buffers to

achieve effective cell lysis (Doyle & Doyle, 1987). In the present study, CTAB concentrations of 1, 2, 3, 4, and 6% were evaluated for their effects on DNA yield and purity. Among these treatments, 4% CTAB produced the highest DNA yield with acceptable purity, yielding concentrations ranging from 109.30 ± 7.66 to 112.56 ± 6.9 ng/µL and A260/A280 ratios between 1.56 ± 0.12 and 1.71 ± 0.05. In contrast, extraction using 1% CTAB resulted in substantially lower DNA yields (28.20 ± 3.90 to 32.10 ± 8.68 ng/µL) and reduced purity, with A260/A280 ratios below 1.38 ± 0.06). The 2% CTAB buffer produced intermediate yields (47.00–55.00 ng/µL) with A260/A280 ratios ranging from 1.40–1.50. At 6% CTAB, DNA yields were also relatively high (77.74 ± 9.86 to 96.10 ± 4.29 ng/µL), with A260/A280 ratios of 1.70 ± 0.07 to 1.75 ± 0.11 (Table 2).

Table 2. Effect of different CTAB concentrations on DNA yield and purity obtained from marine sponge specimens

CTAB concentration	Specimen	DNA concentration (ng/µL)	A260/A280 ratio
1%	A	28.2 ± 3.9	1.36±0.04
1%	B	32.80 ±5.35	1.33 ± 0.05
1%	C	29.10 ± 8.68	1.38 ± 0.06
2%	A	47.00 ± 6.09	1.44 ± 0.11
2%	B	55.46 ± 6.88	1.38 ± 0.16
2%	C	50.30 ± 3.00	1.51 ± 0.06
4%	A	109.30 ± 7.66	1.56 ± 0.12
4%	B	91.46 ± 3.75	1.71 ± 0.05
4%	C	112.56 ± 6.9	1.62 ± 0.09
6%	A	77.73 ± 9.86	1.75 ± 0.11
6%	B	85.1 ± 5.9	1.72 ± 0.04
6%	C	96.1 ± 4.29	1.70 ± 0.07

Note: Data represent the mean of three independent DNA extractions ± standard deviation.

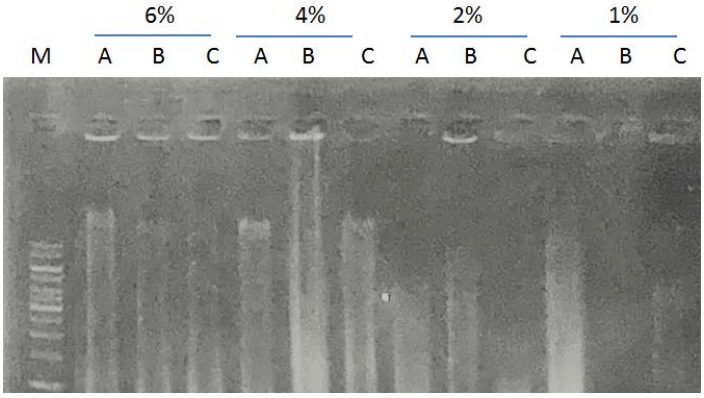

Figure 3. Agarose gel electrophoresis of DNA extracted from sponge specimens using different CTAB concentrations (1%, 2%, 4%, and 6%). Lanes **A**-**C** correspond to three specimens shown in Figure 1; **M**, 1kb DNA ladder

Collectively, these results indicate that increasing CTAB concentration enhances DNA yield, with 4% CTAB providing the optimal balance between DNA yield and purity for sponge tissue extractions.

NanoDrop measurements confirmed successful DNA extraction from all sponge specimens across the tested CTAB concentrations. However, at 1% and 2% CTAB, DNA yields were extremely low and not detectable by agarose gel electrophoresis (Fig. 3). Electrophoretic profiles further showed that DNA integrity was compromised at 6% CTAB,, as indicated by smeared bands (Fig. 3). Although DNA quality generally improved with increasing CTAB concentration, A260/A280 ratios remained below the optimal range of 1.8–2.0. Based on these observations, 4% CTAB was selected as the optimal concentration, providing a suitable compromise between DNA yield and quality for subsequent optimization steps.

Effect of SDS addition to the 4% CTAB buffer

An important consideration in DNA extraction is that not all protocols efficiently release DNA from protein–nucleic acid complexes. Sodium dodecyl sulfate (SDS), an anionic surfactant, disrupts lipid and protein components of cellular membranes, thereby enhancing cell lysis and nucleic acid release. In addition, SDS denatures proteins, reducing their interaction with DNA and facilitating subsequent isolation and purification steps. Through these combined effects, the incorporation of SDS into the CTAB extraction buffer has been shown to increase the total DNA yield recovered from sponge tissues (Gross-Bellard et al., 1973).

Table 3. Effects of SDS addition to 4% CTAB extraction buffer on DNA yield and purity in marine sponge specimens

Specimen	A	B	C	A	B	C	A	B	C
Proteinase K	0.5%	0.5%	0.5%	1.0%	1.0%	1.0%	2.0%	2.0%	2.0%
DNA concentration (ng/µL)	105.40 ± 5.76	118.32 ± 7.82	122.10 ± 8.73	186.26 ± 8.88	192.9 ± 10.26	204.52 ± 12.00	157.70 ± 9.42	167.46 ± 9.54	162.45 ± 1.8
A260/A280	1.82 ± 0.12	1.79 ± 0.09	1.68 ± 0.13	1.95 ± 0.21	1.85 ± 0.18	1.81 ± 0.09	1.87 ± 0.15	1.95 ± 0.12	2.04 ± 0.14

Note: Data are mean ± SD of three independent DNA extractions (n = 3). SD: standard deviation.

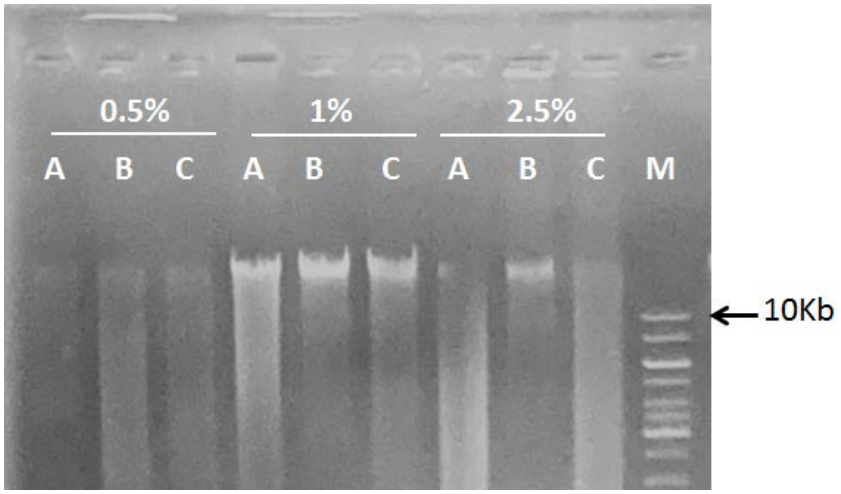

Figure 4. Agarose gel electrophoresis of DNA extracted from sponge specimens using a 4% CTAB extraction buffer supplemented with SDS at the concentrations of 0.5, 1.0, or 2.0%. Lanes **A–C** correspond to the three specimens shown in Figure 1; **M**, 1kb DNA ladder

Incorporating SDS into the 4% CTAB extraction buffer markedly improved DNA yield and quality across all tested concentrations (0.5%, 1.0%, and 2.0%). The highest overall DNA quality was observed at 1% SDS, with concentrations of approximately 200 ng/µL and A260/A280 ratios exceeding 1.8. At 0.5% SDS, DNA yields ranged from 105.40 ± 5.76 to 122.10 ± 8.73 ng/µL, with A260/A280 ratios of 1.68 ± 0.13 to 1.82 ± 0.12. At 2% SDS, DNA yields increased further (157.46 ± 9.54 to 167.46 ± 9.54 ng/µL), accompanied by improved A260/A280 ratios ranging from 1.87 ± 0.15 to 2.04 ± 0.14 (Table 3). Overall, these results indicate that increasing SDS concentration enhances DNA yield and purity, with 1% SDS providing the most consistent balance between high concentration and optimal purity across specimens. Agarose gel electrophoresis (Fig. 4) confirmed that SDS supplementation enhanced the performance of the 4% CTAB buffer; with the most intense DNA bands consistently occurred consistently observed at 1% SDS across all three specimens.

Effect of proteinase K addition to CTAB buffer supplemented with 1% SDS

Proteinase K is a broad-spectrum proteolytic enzyme that facilitates protein digestion, thereby promoting cell lysis and the release of DNA into solution. This function is particularly important when extracting DNA from tissues with rigid structural matrices, such as marine sponges. In the present study, incorporation of proteinase K into the 4% CTAB buffer supplemented with 1% SDS further improved both the yield and purity of DNA isolated from sponge specimens. On average, DNA concentrations of approximately 220 ng/µL were obtained across all tested proteinase K concentrations. Notably, A260/A280 ratios ranged from 1.8 to 2.0, consistent with accepted standards for high-quality DNA (Table 4).

Table 4. Effects of proteinase K concentration on DNA yield and purity from marine sponge specimens using a 4% CTAB + 1% SDS extraction buffer

Specimen	A	B	C	A	B	C
Proteinase K	5 µg/mL	5 µg/mL	5 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL
DNA concentration (ng/µL)	190.35 ± 12.5	232.80 ± 10.55	242.10 ± 8.68	182.00 ± 6.09	225.46 ± 6.88	249.30 ± 3.00
A260/A280	1.86 ± 0.12	1.92 ± 0.15	9.58 ± 0.11	8.52 ± 0.14	9.33 ± 0.09	2.01 ± 0.15

Note: Data are mean ± SD of three independent DNA extractions (n = 3).

The DNA egel electrophoresis profiles for the three specimens (Fig. 5) indicated no substantial difference in DNA yield between the two protein K concentrations tested. However, DNA isolated with 5 µg/mL proteinase K showed higher integrity, with less fragmentation, compared to DNA obtained with 10 µg/mL. Accordingly, 5 µg/mL proteinase K was selected as a component of the optimized CTAB-based DNA extraction protocol for marine sponges.

DNA extraction is a fundamental step in molecular biology, particularly for taxonomic and phylogenetic analyses, where DNA yield and purity are critical determinants of downstream success, including in studies of marine sponges (Gupta, 2019). A variety of approaches have been applied for sponge DNA isolation, ranging from conventional protocols to commercial kits. In our experiments, however, the Gene JET Total DNA Isolation Kit did not yield DNA of sufficient quality from sponge tissues, likely due to abundant secondary metabolites and polysaccharides that interfere with kit reagents and compromise purity and yield (data not shown). Similarly, the conventional CTAB protocol employing 2% CTAB, although widely used, was ineffective for sponge DNA isolation in this study (Table 2).

In contrast, the optimized protocol developed here, which incorporated a higher

CTAB concentration (4%), markedly improved DNA yield. As a cationic surfactant, CTAB facilitates cell lysis by interacting interacting with membrane lipids to release cellular amd nuclear contents; at low ionic strength it can also precipitate nucleic acids, whereas at higher salt concentrations it forms complexes with polysaccharides (Sambrook et al., 2001). When combined with SDS, CTAB further reduced protein and secondary metabolite contamination, resulting in DNA of improved purity. Collectively, these modifications enhanced extraction efficiency and yielded DNA suitable for downstream molecular applications.

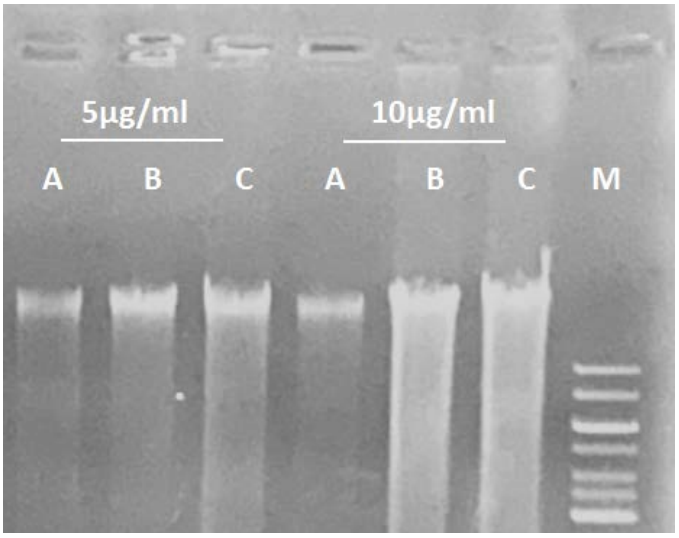

Figure 5. Agarose gel electrophoresis of DNA extracted from sponge specimens using a 4% CTAB extraction buffer supplemented with 1.0% SDS and proteinase K at concentraions of 5.0 or 10.0 µg/mL. Lanes **A–C** correspond to the three specimens shown in Figure 1; **M**: 1 kb DNA ladder

Further improvements were observed when SDS and proteinase K were incorporated into the extraction buffer. SDS increased DNA yields by disrupting cellular membranes and denaturing proteins, thereby reducing nuclease activity (e.g., Rnases, Dnases) that can degrade DNA (Akinwale & Babarinde, 2019). Proteinase K, routinely employed for protein-rich or structurally resilient tissues, further enhanced DNA integrity and recovery by facilitating efficient protein digestion (Saenger, 2013). In summary, the optimized CTAB-based protocol produced high-quality genomic DNA from marine sponge specimens and performed reliably across multiple species, This protocol is therefore suitable for downstream applications including PCR amplification, DNA sequencing, integrative taxonomic assignment, and phylogenetic analyses.

Validation of the optimized CTAB DNA extraction method

DNA concentration and quality

To validate the efficiency of the optimized protocol, genomic DNA was extracted from six sponge specimens from the Con Dao Islands (Table 1). DNA concentration (ng/µL) and purity (A260/A280, A260/A230) were measured with a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). Although yields varied among specimens, all extracts obtained with the optimized CTAB protocol exceeded 200 ng/µL, ranging from 210 to 438 ng/µL (Table 5). The A260/A280 ratio is a primary indicator of nucleic acid purity, with values near 1.8 indicating pure DNA and lower values suggesting protein contamination. The

A260/A230 ratio is a secondary metric; values of ~2.0–2.2 indicate minimal contamination by organic compounds or residual reagents (e.g., phenol, carbohydrates). In this study, all DNA extracts exhibited consistently high purity, with A260/A280 ratios ranging from 1.79 to 1.88 and A260/A230 ratios ranging from 1.98 to 2.11. Collectively, these results demonstrate that the optimized CTAB protocol effectively yields high-quality genomic DNA from marine sponge specimens, providing reliable templates for downstream applications, such as PCR and sequencing.

Table 5. Concentration and purity of genomic DNA from six sponge specimens extracted with the optimized CTAB protocol

No.	Sample ID	DNA concentration (ng/µL)	Purity	
			A260/A280	A260/A230
1	HMCD02Da	421 ± 10.8	1.81 ± 0.01	2.09 ± 0.04
2	HMCD02C	229 ± 3.9	1.80 ± 0.05	2.00 ± 0.03
3	HMCD02HT	298 ± 8.4	1.82 ± 0.02	1.98 ± 0.1
4	HMCD04HT	438 ± 49.4	1.88 ± 0.04	2.11 ± 0.09
5	HMCD04Ta	343 ± 9.1	1.86 ± 0.02	2.02 ± 0.07
6	HMCD04DT	210 ± 3.9	1.79 ± 0.05	2.04 ± 0.03

Note: Data are mean ± SD (n = 3); measured on NanoDrop 2000c (Thermo Fisher Scientific).

DNA integrity

DNA integrity was evaluated by agarose gel electrophoresis (Fig. 6). All samples yielded high-molecular-weight DNA, characterized by distinct bands exceeding 10 kb and minimal evidence of degradation or shearing. These results indicate that the optimized CTAB-based protocol effectively preserves DNA integrity during extraction and provides material suitable for downstream applications such as PCR and Sanger sequencing. DNA degradation can result from multiple chemical and physical factors, with nucleases posing a particular risk by rapidly cleaving genomic DNA into smaller fragments. Such degradation typically manifests as smeared bands on agarose gels, reflecting extensive fragmentation. To mitigate these effects, strict sample preservation under cold or dry conditions is essential (Rossman et al., 2011). In addition, the inclusion of chelating agents such as EDTA in extraction buffers helps inhibit nuclease activity by sequestering divalent metal ions required for nuclease function and by reducing metal-catalyzed radical formation.

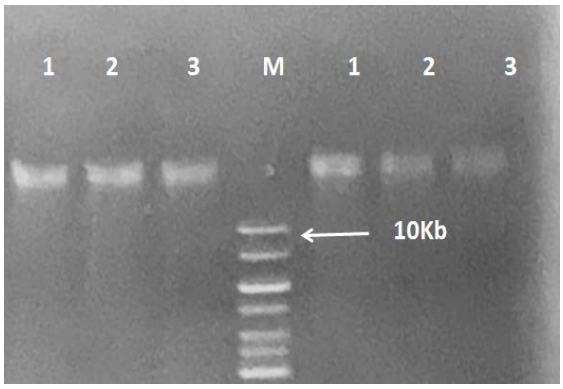

Figure 6. Agarose gel electrophoresis (0.8%) of total DNA extracted from six marine sponge specimens using the optimized CTAB protocol. Lanes **1–6**: DNA extracts from sponge samples corresponding to the order listed in Table 1; **M**: 1kb DNA ladder

Physical stresses during extraction can also compromise DNA integrity. Mechanical shearing may occur through vigorous pipetting, vortexing, or other harsh handling steps, underscoring the importance of gentle manipulation (Malentacchi et al., 2016). Repeated freeze–thaw cycles and prolonged storage further contribute to fragmentation. In this study, we observed that DNA shearing in sponge samples was minimized by using the optimized extraction buffer composed of 4% CTAB, 1% SDS, and 5 µg/mL proteinase K. This combination improved DNA yield and purity while preserving genomic DNA integrity, ensuring suitability for downstream molecular applications.

PCR amplification of genomic DNA extracted using the optimized CTAB method

The suitability of the isolated genomic DNA for downstream applications was evaluated through PCR amplification of the mitochondrial cytochrome c oxidase subunit I (COI) gene. Clear, intense amplicons of the expected size (~600 bp) were consistently obtained from all extracts (Fig. 7), confirming that the DNA isolated with the optimized CTAB protocol was of sufficient quality and purity for reliable molecular analyses.

DNA quantity and quality are critical for validating extraction protocols, as high-quality DNA is essential for reliable downstream applications such as PCR. In animal tissues, contaminants such as polysaccharides and polyphenols can co-purify with DNA and inhibit key enzymes, including DNA polymerases and restriction endonucleases (Furukawa & Bhavanandan, 1983). Polyphenolic compounds, which are abundant in sponge tissues (Pereira & Cotas, 2023), are particularly problematic because they can strongly interfere with PCR if not effectively removed. In this study, successful amplification of the COI fragment from all samples indicates that the optimized CTAB-based protocol effectively removes inhibitory substances and yields DNA of sufficient purity for molecular analyses.

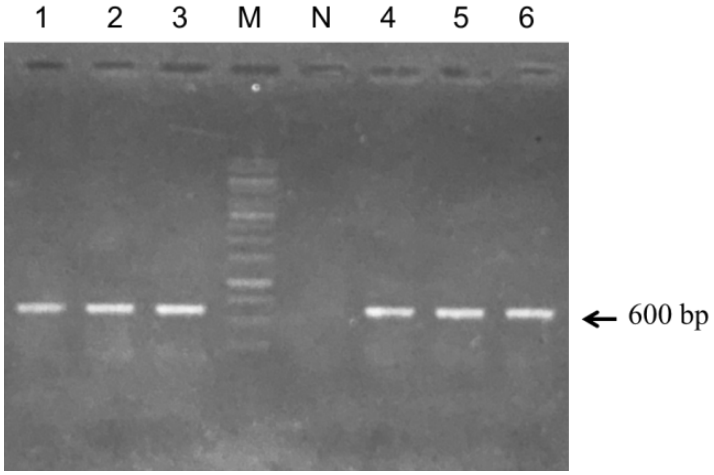

Figure 7. PCR amplification of the mitochondrial COI gene fragment (~600 bp) using genomic DNA extracted from sponge specimens. Lanes 1–6: HMCD02Da, HMCD02C, HMCD02HT, HMCD04HT, HMCD04Ta, and HMCD04DT; M: 1kb DNA ladder; N: negative control

Sequence homology and species identification

To evaluate the suitability of the extracted DNA for molecular identification, the amplified COI fragments were sequenced. All six specimens yielded reads of 638 bp; after trimming primer regions, 598 bp of high-quality sequence were retained for analysis. These sequences were compared with reference sequences available in GenBank to assess sequence similarity and infer

taxonomic placement (Table 6). Relationships among the study specimens and reference taxa based on the aligned COI sequences are presented in Figure 8.

Table 6. Morphological and COI-based identifications for the 6 sponge specimens

Sample ID	Morphological identification	COI-based identification	
		% similarity (best match)	Species (GenBank accession number)
HMCD02Da	*Haliclona* sp.1	96.80%	*Haliclona amboinensis* (KX894494)
HMCD02C	*Xestospongia testudinaria*	100%	*Xestospongia testudinaria* (HQ452959)
HMCD02HT	*Haliclona* sp.2	97.49%	*Haliclona amphioxa* (AJ843892)
HMCD04HT	*Haliclona* sp.1	97.96%	*Haliclona amboinensis* (KX894494)
HMCD04Ta	*Aaptos* sp.	100%	*Aaptos suberitoides* (MH784603)
HMCD04DT	*Xestospongia testudinaria*	100%	*Xestospongia testudinaria* (HQ452959)

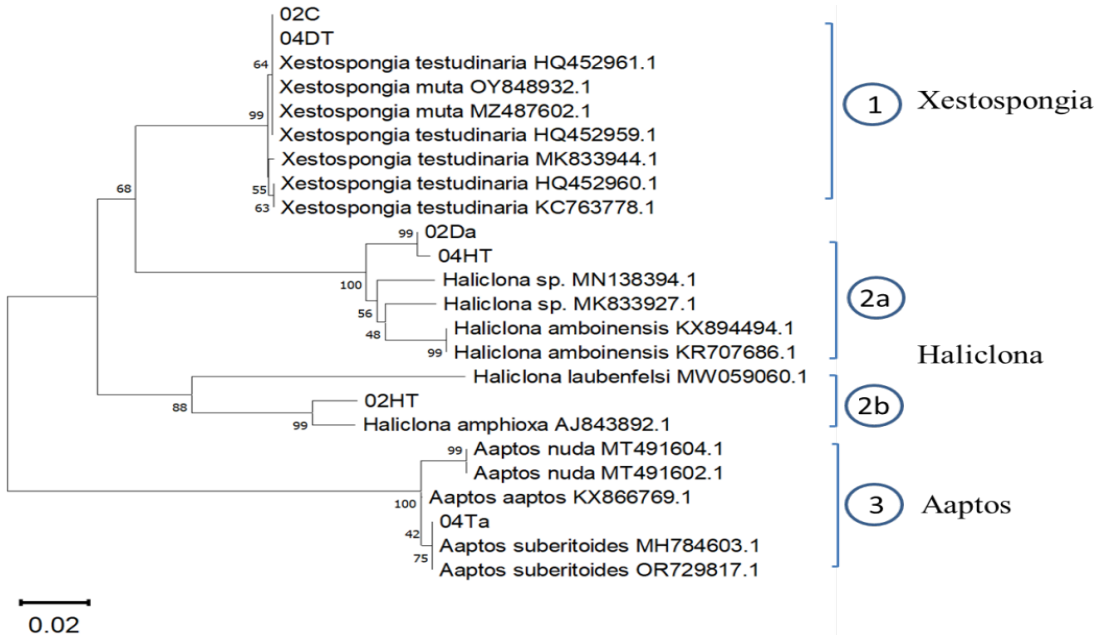

Figure 8. Neighbor-joining (NJ) tree showing relationships among sponge specimens and reference taxa based on partial COI sequences, reconstructed in MEGA version 11. Reference taxa are annotated with their GenBank accession numbers. Clade numbers (open circles) indicate to genus-level groupings shown on the right. Bootstrap support values was estimated from 1,000 replicates; and only values ≥ 50% are shown at the corresponding nodes. Sequence labels 02C, 04DT, 02Da, 04HT, 02HT, and 04Ta correspond to specimens HMCD02C, HMCD04DT, HMCD02Da, HMCD04HT, HMCD02HT, and HMCD04Ta, respectively

Alignments between the COI sequences of the six sponge specimens and reference taxa within each clade (Fig. 8) revealed high sequence similarity, ranging from 96.8% to 100%. COI sequences of specimens HMCD04DT and HMCD02C (04DT and 02C) were 100% identical to the reference sequence of *Xestospongia testudinaria* (GenBank accession number HQ452959), confirming their assignment to this species. The COI sequences of specimens HMCD02Da and HMCD04HT (02Da and 04HT) showed ~ 97%

and ~ 98% identity, respectively, to *Haliclona amboinensis* (GenBank accession number KX894494), supporting their identification as *H. amboinensis* and consistent with their morphological classification as *Haliclona* sp. 1. The COI sequence of specimen HMCD02HT (02HT) exhibited 97.49% identity to the reference sequence of *Haliclona amphioxa* (GenBank accession number AJ843892), indicating placement within genus *Haliclona*. Finally, the COI sequence of specimen HMCD04Ta (02Ta) was 100% identical to *Aaptos suberitoides* (GenBank accession number MH784603), confirming its species-level identification (Table 6).

Comparison of morphological and molecular identification

Morphological examination identified the six sponge specimens as belonging to four distinct species, *Haliclona* sp. 1, *Haliclona* sp. 2, *Aaptos* sp., and *Xestospongia testudinaria*, representing three genera (*Xestospongia*, *Haliclona*, and *Aaptos*). Among these, two specimens, HMCD02C and HMCD04DT were confidently assigned to species level based on diagnostic morphological characters (Table 6). Overall, there was a high degree of concordance between morphological and molecular results, with COI sequence analysis providing effective support for species-level identification and reinforcing the value of integrating morphological and molecular approaches in sponge taxonomy. The provisional labels *Haliclona* sp. 1 and *Haliclona* sp. 2 were assigned to distinguish two morphologically and genetically distinct operational taxonomic units within the genus *Haliclona*. These specimens could not be confidently assigned to described species based solely on morphological characters, and were therefore treated as separate morphospecies pending further taxonomic resolution

The mitochondrial cytochrome c oxidase subunit I (COI) marker has been widely applied for species identification across a broad range of sponge taxa (Ngwakum et al., 2021) and other marine invertebrates, including soft corals (Subhan et al., 2022), sea cucumbers (Yamana et al., 2022), and gastropods (Setiamarga et al., 2019). In Vietnam, COI-based molecular analyses have corroborated morphological identifications of sponge specimens collected from Quang Tri, Thua Thien Hue, and Nha Trang, notably confirming the identity of *Xestospongia testudinaria* (Dat et al., 2018). Together with the present study, these findings underscore the utility of COI sequences for resolving taxonomic uncertainty and improving the accuracy of species-level assignments in marine sponges.

The Con Dao Islands, an archipelago of sixteen islands in Ba Ria-Vung Tau province (now administratively part of Ho Chi Minh city), southern Vietnam, comprise approximately 14,000 ha of surrounding waters and are noted for diverse, well-preserved marine ecosystems. To date, 1,725 marine species have been documented from the region, including 360 corals, 130 polychaetes, 116 crustaceans, 187 mollusks, and 115 echinoderms (Nguyet & Bau, 2023). However, marine sponges from the Con Dao Islands remain poorly studied, with few taxonomic records currently available. The present study represents the first application of molecular identification to sponge specimens from this archipelago.

Among the identified taxa, *X. testudinaria* has been recognized as one of the eight most widespread marine sponge species in Vietnam, based on an analysis of 1,500 sponge specimens collected from 14 island systems between 2003 and 2021. These include Ha Long, Cat Ba, Bai Tu Long (northeastern coast); Hon Me, Con Co (north-central coast); and Hai Van-Son Cha, Cu Lao Cham, Ly Son, Van Phong Bay, Ca Na Bay, and Phu Quy Island (south-central coast) (Thung, 2024). Although the genus *Haliclona* was reported as common in that survey, neither *H. amboinensis* nor *H. amphioxa* appeared in the species list for Vietnamese island waters. Further investigations are therefore required to confirm the occurrence and distribution of these species in the Con Dao Islands.

In addition, *A. suberitoides*, previously recorded from Hai Van-Son Cha (Thua Thien-Hue) and Phu Quy (Binh Thuan), has been recognized as one of sixteen common sponge

species distributed across the South China Sea (Lim et al., 2016). Our record from the Con Dao Islands further supports the view that regional sponge biodiversity is both high and underexplored. Collectively, these findings underscore the need for comprehensive biodiversity assessments and integrative, molecular-based taxonomic studies to fully document the sponge fauna of the Con Dao Islands and to evaluate their potential for biotechnology and natural product research.

CONCLUSION

An optimized method for DNA isolation from marine sponges was developed by modifying the conventional CTAB extraction buffer. The optimized buffer, supplemented with 4% CTAB, 1% SDS, and 5 µg/mL proteinase K, substantially improved both DNA yield and purity. The protocol consistently produced high-quality genomic DNA that served as a reliable template for PCR amplification and sequencing of the COI barcode fragment, enabling genus- and species-level identification of the examined specimens. These results demonstrate the effectiveness and practical applicability of the optimized protocol for molecular identification and sequence-based analyses of marine sponges, and provide a foundation for broader downstream applications in sponge taxonomy and biodiversity research. Further validation across a wider range of taxa and preservation conditions would be valuable.

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REFERENCES

Akinwole M., Babarinde I., 2019. Assessing Tissue Lysis with Sodium Dodecyl Sulphate for DNA Extraction from Frozen Animal Tissue. *Journal of Forensic Research*, 10: 3.

Dat T. T. H., Cuc N. T. K., Cuong P. V., 2018. Molecular taxonomy of some sponges (Demospongiae) using ribosomal (18s rRNA) and partial mitochondrial (COI) genes. *Academia Journal of Biology*, 40(4): 67−75. https://doi.org/10.15625/2615-9023/v40n4.12705

de Voogd N. J. A., Boury-Esnault B., Cárdenas N., Díaz P. M.-C., Dohrmann M., Downey R., Goodwin C., Hajdu E., Hooper J. N. A., Kelly M., Klautau M., Lim S. C., Manconi R., Morrow C., Pinheiro U., Pisera A. B., Ríos P., Rützler K., Schönberg C., Turner T., Vacelet J., van Soest R. W. M., Xavier J., 2025. World Porifera Database. http://www.marinespecies.org/porifera on 20/04/2025

Doyle J. J., Doyle, J. L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue, *Phytochemical Bulletin*, 19 (1): 11−15.

Evans N., Paulay G., 2012. DNA Barcoding Methods for Invertebrates, DNA barcodes: methods and protocols, pp. 47−77.

Furukawa K., Bhavanandan V. P., 1983. Influences of anionic polysaccharides on DNA synthesis in isolated nuclei and by DNA polymerase α: correlation of observed effects with properties of the polysaccharides. *Biochim. Biophys. Acta*., 740(4): 466−475. https://doi.org/10.1016/0167-4781(83)90096-9

Gross-Bellard M., Oudet P., Chambon P., 1973. Isolation of high-molecular-weight DNA from mammalian cells. *European Journal of Biochemistry*, 36(1): 32–38

Gupta N., 2019. DNA Extraction and Polymerase Chain Reaction. *J. Cytol*, 36(2): 116−117. https://doi.org/10.4103/joc.joc_110_18

Hill M. S., Hill A. L., Lopez J., Peterson K. J., Pomponi S., Diaz M. C., Thacker R. W., Adamska M., Boury-Esnault N., Cárdenas P., Chaves-Fonnegra A., Danka E., De Laine B. O., Formica D, Hajdu E, Lobo-Hajdu G, Klontz S, Morrow CC, Patel J, Picton B, Pisani D, Pohlmann D., Redmond N. E., Reed J., Richey S., Riesgo A., Rubin E., Russell Z., Rützler

K., Sperling E. A., di Stefano M., Tarver J. E., Collins A. G., 2013. Reconstruction of family-level phylogenetic relationships within Demospongiae (Porifera) using nuclear encoded housekeeping genes. *PLoS One*, 8(1): e50437. https://doi.org/10.1371/journal.pone.0050437

Hooper J. N. A., van Soest R. W. M., 2000. Systema Porifera. A guide to the classification of sponges, New York: Kluwer Academic/Plenum Publishers, pp. 1810.

Kishor R., Mazumder P. B., Heikrujam J., 2020. The Chemistry Behind Plant DNA Isolation Protocols. In: Boldura O-M, Balta C, Awwad N (eds) Biochemical Analysis Tools - Methods for Bio-Molecules Studies. IntechOpen, Rijeka. https://doi.org/10.5772/intechopen.92206

Langenbruch P.F., 1983. Body structure of marine sponges. *Marine* Biology, 75(2): 319−325. doi: 10.1007/BF00406018

Lim S. C., Putchakarn S., Thai Q., Wang D., Huang Y., 2016. Inventory of sponge fauna from the Singapore Strait to Taiwan Strait along the western coastline of the South China Sea. *Raffles Bulletin of Zoology Supplement*, 34: 104−129.

Malentacchi F., Pizzamiglio S., Ibrahim-Gawel H., Pazzagli M., Verderio P., Ciniselli C. M., Wyrich R., Gelmini S., 2016. Data and performances evaluation of the SPIDIA-DNA Pan-European External Quality Assessment: 2nd SPIDIA-DNA laboratory report. *Data Brief*, 6: 980–984. http://dx.doi.org/10.1016/j.dib.2016.01.062

Maloof A. C., Rose C. V., Beach R., Samuels B. M., Calmet C. C., Erwin D. H., Simons F. J., 2010. Possible animal-body fossils in pre-Marinoan limestones from South Australia. *Nature Geoscience*, 3(9): 653−659. http://dx.doi.org/10.1038/ngeo934

Nguyen Van Chung, Dao Tan Ho, Le Minh Trong, Ton That Thong, Tran Dinh Nam, Nguyen Van Luom, 1978. A review of the preliminary surveys on benthos in Vietnam. *Collection of Marine Research Works*: 57−72 (In Vietnamese).

Nguyet N., Bau N., 2023. Con Dao's Positional Resources: Potential and Orientation in Value Promotion. *IOP Conference Series: Earth and Environmental Science*, 1247(1): 012014. http://dx.doi.org/10.1088/1755-1315/1247/1/012014

Ngwakum B. B., Payne R. P., Teske P. R., Janson L., Kerwath S. E., Samaai T., 2021. Hundreds of new DNA barcodes for South African sponges. *Systematics and Biodiversity*, 19(7): 747−769. doi: 10.1080/14772000.2021.1915896

Pereira L., Cotas J., 2023. Therapeutic Potential of Polyphenols and Other Micronutrients of Marine Origin. *Mar. Drugs*, 21(6): 323. https://doi.org/10.3390/md21060323

Rossmanith P., Röder B., Frühwirth K., Vogl C., Wagner M., 2011. Mechanisms of degradation of DNA standards for calibration function during storage. *Appl. Microbiol. Biotechnol*. 89(2): 407–417. https://doi.org/10.1007/s00253-010-2943-2

Saenger W. 2013. Chapter 714 - Proteinase K. In: Rawlings ND, Salvesen G (eds) Handbook of Proteolytic Enzymes (Third Edition). Academic Press, pp. 3240−3242. https://doi.org/10.1016/B978-0-12-382219-2.00714-6

Sambrook J, Russell D. W., Russell D. W., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Setiamarga D. H., Nakaji N., Iwamoto S., Teruya S., Sasaki T., 2019. DNA barcoding study of shelled gastropods in the intertidal rocky coasts of central Wakayama Prefecture, Japan, using two gene markers. *GEOMATE Journal. J.*, 17(62): 9−16. https://doi.org/10.21660/2019.62.4521

Subhan B., Ferse S., Dzulfannazhir F., Izza L. M., Anggraini N. P., Santoso P., Madduppa H., 2022. DNA barcoding of the soft coral, Clavularia inflata, shows two major groups

across Indonesian coral reefs. *Ilmu Kelautan Indonesian Journal of Marine Sciences*, 27(1): 1−12. http://dx.doi.org/10.14710/ik.ijms.27.1.1-12

Tamura K., Stecher G., Kumar S., 2021. MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*, 38(7): 3022−3027. https://doi.org/10.1093/molbev/msab120

Thai Minh Quang, 2017. Overview of marine sponges research in Vietnam. *Vietnam Journal of Marine Science and Technology*, 17(4A): 98−107 (In Vietnamese with English summary). http://dx.doi.org/10.15625/1859-3097/17/4A/13265

Thakur N., Müller W., 2004. Biotechnological potential of marine sponges. *Current Science*, 86(11): 1506–1512.

Thung D. C., 2024. Species composition and distribution of Sponges in some islands in the Vietnam sea. *Vietnam Journal of Marine Science and Technology*, 24(4): 375−385. doi: 10.15625/1859-3097/21471

Varijakzhan D., Loh J. Y, Yap W. S., Yusoff K., Seboussi R., Lim S.E., Lai K. S., Chong C.M., 2021. Bioactive Compounds from Marine Sponges: Fundamentals and Applications. *Mar. Drugs*, 19 (5): 246. https://doi.org/10.3390/md19050246

Worheide G., Erpenbeck D., Menke C., 2007. The Sponge barcoding project: aiding in the identification and description of poriferan taxa. Porifera research: Biodiversity, innovation, and sustainability, 28: 123−128.

Yamana Y., Thandar A. S., Hayashibara T., Setiamarga D. H. E., 2022. A new species of dendrochirotid holothuroid from deep water of southern Japan, with the erection of a new genus, Satsumaocnus (Echinodermata: Holothuroidea: Dendrochirotida: Cucumariidae: Colochirinae). *Zootaxa*, 5209(2): 270−284. https://doi.org/10.11646/*zootaxa*.5209.2.7