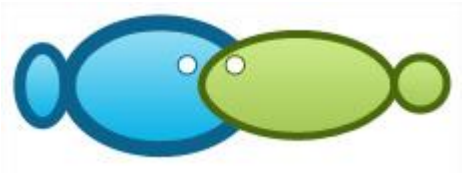

A modified method for high-purity genomic DNA isolation from sea cucumbers (*Holothuria fuscocinerea*) with ossicle removal

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Abstract. Sea cucumbers play important ecological and economic roles, making accurate species identification and genetic studies essential for their conservation and management. However, genomic DNA isolation in sea cucumbers is often challenged by the presence of ossicles and complex dermal structures. This study aimed to develop a modified method for high purity genomic DNA isolation by evaluating different tissue types and preparation techniques. A single specimen of *Holothuria fuscocinerea* from Karimunjawa was analyzed using anatomical and molecular approaches. Six extraction treatments were applied, including anterior tissue (P0), smooth muscle tissue (P1), dermal tissue treated with bleach (P2), homogenized muscle tissue (P3), gonad (P4), and body secretion (P5). DNA quality and concentration were assessed using Nanodrop and Quantus, followed by PCR amplification targeting the mitochondrial COI gene. Anatomical observations revealed the presence of buttons, rod, and tables ossicles, with button type being dominant. The results showed that dermal tissue treated with bleach as chemical methods for ossicle removal produced the highest DNA yield (4.76 ng μL^{-1} Nanodrop and 3.53 ng μL^{-1} Quantus) and relatively better purity compared to other tratments. PCR amplification of anterior tissue using the COIceF/ceR primer successfully generated clear DNA bands in all samples (100%), with a high identification success rate for *H. fuscocinerea*, indicating the suitability of the extracted DNA for molecular analysis. This study demonstrated that ossicle removal and appropriate tissue selection are critical factors in improving DNA extraction efficiency in sea cucumbers.
Keywords: COI gene, DNA extraction, molecular identification, ossicle, sea cucumber.

Introduction. Sea cucumbers are marine organisms that play an important role as detrivitores and bioturbators in marine ecosystems (Safitri et al., 2021). Their distribution in coastal waters can vary among locations, reflecting differences in habitat and environmental conditions (Pramithasari et al., 2024). Therefore, species identification and genetic studies of sea cucumbers are crucial to support sustainable resource management and conservation efforts (Yang et al., 2020). Molecular approaches using the cytochrome c oxidase subunit I (COI) gene are widely applied for sea cucumber species identification through DNA barcoding (Simbolon et al., 2021), due to its ease of amplification and high interspecific variation (Manampiring et al., 2024).

DNA purity is commonly assessed using the A260/280 absorbance ratio, which indicates potential contamination by proteins, RNA, or other compounds (Yulianti et al., 2024). However, genomic DNA isolation in sea cucumbers often faces several technical challenges (Daniels et al., 2023). One of the main factors is the presence of ossicles, calcium carbonate based (alkaline) components found in the dermal tissue (Tersol et al., 2023). Residual alkaline compounds can affect the purity of extracted genomic DNA (Yustina et al., 2025). Futhermore, variations in treatment methods significanly influence genomic DNA quality, highlighting the need for an optimized isolation protocol capable of overcoming these obstacles (Puch-Hau et al., 2019). In addition, primer performance may vary depending on sample condition and DNA quality (Veal et al., 2012). Poor DNA quality can lead to amplification failure or inconsistent sequencing results, which may compromise species identification accuracy (Johann et al., 2023).

Based on these challenges, this study aims to develop a modified method for genomic DNA isolation in *Holothuria fuscocinerea*. It is expected that this approach will prove a more effective strategy for molecular studies of organisms with similar tissue characteristics to sea cucumbers.

Material and Method

Description of the study sample. The sample used in this study consisted of a single sea cucumber specimen of *H. fuscocinerea* obtained from the laboratory collection of the Center of Marine Ecology and Biomonitoring for Sustainable Aquaculture (CE-MEBSA). The specimen was collected on 22 July 2025 from Karimunjawa, with sampling coordinates at 5.88238°S; 110.42931°E (Figure 1). The laboratory analysis was conducted starting in November 2025 until May 2026. The selection criteria for the specimen included fresh condition, non-decomposed tissue, living state at the time of collection, intact morphology, and non-faded coloration (Putri et al., 2024). The selected specimen was preserved in 70% ethanol to maintain body integrity, as this preservative is effective in inhibiting the growth of bacteria, fungi, and other microorganisms responsible for tissue degradation (Handayani et al., 2017). This study used two primer sets that is COI LCO1490/HCO2198 and COIceF/ceR, and six DNA extraction treatments based on different body tissues. These variations were designed to evaluate the effectiveness of each method in producing high quality DNA suitable for subsequent molecular analysis.

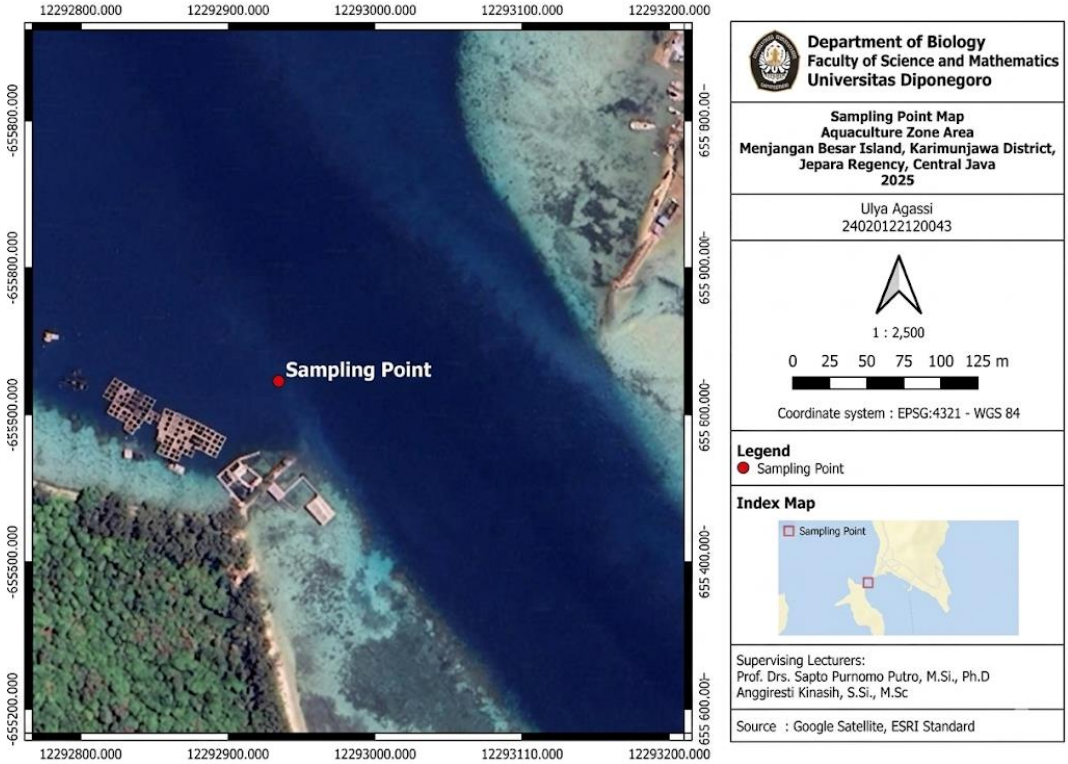

Figure 1. The location of sample collection (red circle) at Central Java, Indonesia.

Anatomical characterization and identification. The sea cucumber specimen was dissected along the dorsal and ventral sides under sterile conditions to expose the internal anatomical structures, including ossicles, for anatomical observation and tissue sampling (Figure 2). The dissected specimen was then prepared for subsequent anatomical observation and tissue sampling (Figure 3). Microscopic identification was carried out by examining the shape of ossicles isolated from the integument tissue. The sample was cut into approximately 2x2 cm sections and immersed in a bleach solution (NaClO) for 10-20 minutes (Hartati et al., 2016). Due to its strong oxidative properties, the bleach solution degraded the integument tissue, allowing the ossicles to separate

from the surroundingg matrix (Nurhidayatulloh et al., 2025). Following immersion, the dissolved integument formed a sediment (pellet) that separated from the bleach solution (supernatant) (Hartati et al., 2016). The supernatant was discarded and the pellet containing ossicles was rinsed with distilled water 3-5 times before being observed under a microscope (Hartati et al., 2016).

Figure 2. Preparation of the sea cucumber specimen prior to anatomical and molecular analysis. The sample was handled under sterile conditions and dissected to obtain tissue for further examination.

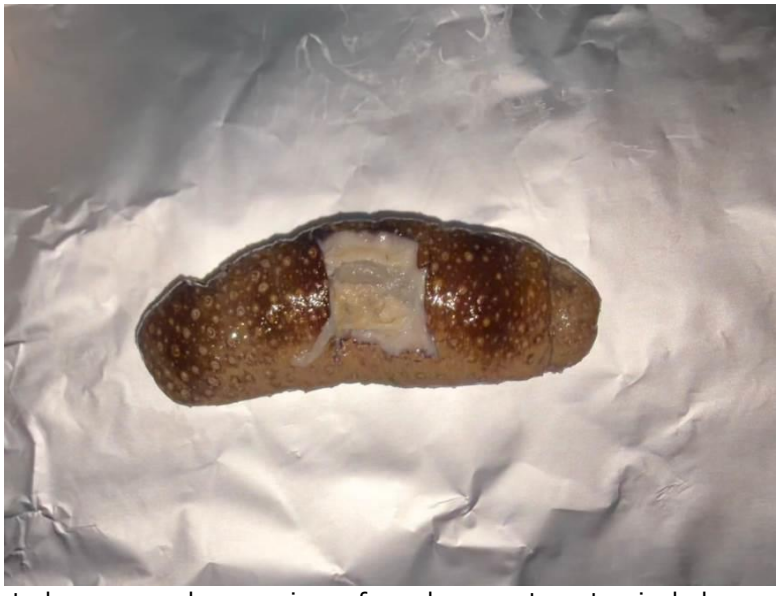

Figure 3. Dissected sea cucumber specimen for subsequent anatomical observation and tissue sampling.

Determination of extraction samples. A various tissue types of the sea cucumber were selected for genomic DNA extraction, including the anterior tissue (P0), smooth muscle tissue (P1), dermal tissue treated with bleach (P2), homogenized muscle tissue (P3), gonad (P4), and body secretion (P5) (Figure 4).

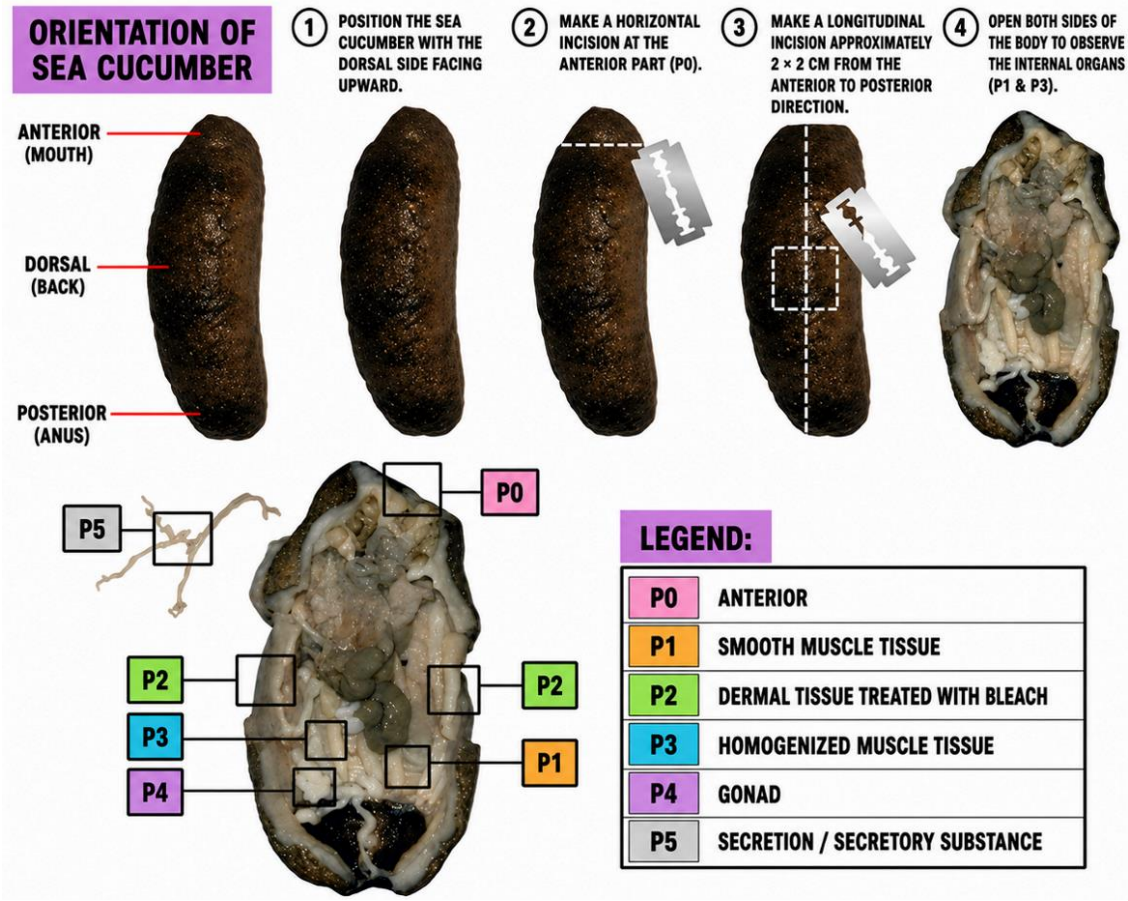

Figure 4. Determination of samples from sea cucumber body parts.

DNA isolation, extraction, and quantification

Protocol. DNA was extracted using the Zymo Research Quick-DNA™ Miniprep Plus Kit with minor modification:

1. Pretreatment was carried out using different tissue types. P0 samples were obtained from anterior dermal-to-muscle tissue and homogenized using a mortar and pestle;
2. P1 samples were obtained from muscle tissue and homogenized using a mortar and pestle;
3. P2 samples were obtained from dermis tissue, immersed in bleach solution for 10 minutes, rinsed with distilled (aquades) water 3 times, and subsequently homogenized using a mortar and pestle;
4. P3 samples were obtained from muscle tissue and finely minced using scissors sterilized with an infrared sterilizer;
5. P4 samples were obtained from gonad tissue and homogenized using a mortar and pestle;
6. P5 samples were obtained from secretory tissue and homogenized using a mortar and pestle;
7. Approximately ±25 mg of each tissue samples was transferred into a microcentrifuge tube containing 95 µL nuclease-free water, 95 µL Solid Tissue Buffer, and 10 µL Proteinase K;
8. The samples were mixed thoroughly by vortexing for 15 seconds and incubated at 55°C for 3 hours until the tissues were completely solubilized. The samples were mixed thoroughly before proceeding to the next step;
9. To remove insoluble debris, the samples were centrifuged at 12,000 for 1 minute, and the aqueous supernatant was transferred into a clean microcentrifuge tube;
10. Subsequently, 2x of Genomic Binding Buffer (400 µL for 200 µL mixture) were added to the supernatant and mixed thoroughly by vortexing for 15 seconds;

11. The mixture was transferred into a Zymo-Spin™ IIC-XLR Column placed in a collection tube and centrifuged at 12,000 for 1 minute. The flow-through was discarded;
12. Subsequently, 400 µL DNA Pre-Wash Buffer was added into the spin column placed in a new collection tube, followed by centrifugation at 12,000 for 1 minute. The flow-through was discarded;
13. The samples was washed with 700 µL g-DNA Wash Buffer and centrifuged at 12,000 for 1 minute. The flow-through was discarded;
14. An additional 200 µL g-DNA Wash Buffer was added into the spin column and centrifuged again at 12,000 for 1 minute. The collection tube with the flow through was discarded;
15. The spin column was transferred into a clean microcentrifuge tube, and 50 µL DNA Elution Buffer was added directly onto the matrix. The column was incubated at room temperature for 5 minutes and centrifuged at maximum speed for 1 minute to elute the DNA;
16. The eluted DNA was either used immediately for downstream molecular analysis or stored at −20°C for future use.

Treatment with RNAse

1. For P2 and P3 samples, after incubation at 55°C, 5 µL RNAse was added into each sample tube;
2. The sample were subsequently incubated at 37°C for 15 minutes;
3. A volume of 430 µL Genomic Binding Buffer (2x the sample volume) was added into the samples and mixed thoroughly by vortexing for 15 seconds before proceeding to the next extraction step.

DNA quantification was performed using a Nanodrop spectrophotometer at a wavelength ratio of A260/280 nm and using a Quantus fluorometer, with 1 µL of DNA elution buffer used as a blank for calibration (Yustina et al., 2025). DNA quality was assessed based on optical density (OD) values, where a ratio of 1.8-2.0 indicated pure DNA (Yulianti el al., 2024). Value below 1.8 suggested protein contamination, while value above 2.0 indicated RNA contamination (Yulianti el al., 2024). DNA concentration was recorded in ng μL^{-1} and purity values were used as indicators of extract quality for subsequent amplification (Yulianti el al., 2024).

Genomic DNA amplification and visualization

Conventional PCR assays. In order to validate the DNA quality, we amplified a fragment corresponding to Cytochrome c Oxidase subunit I (COI) gene:

1. PCR reaction was prepared in a total volume of 50 µL consisting of DNA template, primers, PCR master mix, nuclease free water, and other reaction components as listed in Table 1 and Table 2;
2. The primers used in this study were COIceF (5'-ACT GCC CAC GCC CTA GTA ATG ATA TTT TTT ATG GTN ATG CC-3') and COIceR (5'-TCG TGT GTC TAC GTC CAT TCC TAC TGT RAA CAT RTG-3') (Kautsari et al., 2024), as well as LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TTA ACT TCA GGG TGA CCA AAA AAT CA-3') (Chakraborthy et al. 2024);
3. PCR amplification was carried out using a thermal cycler with a pre denaturation step at 95°C for 3 minutes;
4. The amplification process was continued for 35 cycles consisting of denaturation at 95°C for 20 s, annealing at 52°C for COIceF/ceR primers and 54°C for LCO1490/HCO2198 primers for 30 seconds, and extension at 72°C for 20 seconds;
5. A final extension step was performed at 72°C for 1 minute;
6. The amplified PCR products were subsequently visualized using 1% agarose gel electrophoresis to confirm the successful amplification of the target DNA fragments according to Dailami et al. (2025).

Table 1

PCR reaction with COIceF/ceR primer

Samples	*COIceF/ceR*				
	DNA template	*Primer forward*	*Primer reverse*	*PCR mix*	*ddH_2O*
P0	2	1.25	1.25	25	20.5
P1	ND	ND	ND	ND	ND
P2	18	1.25	1.25	25	12.5
P3	7.5	1.25	1.25	25	15
P4	ND	ND	ND	ND	ND
P5	9	1.25	1.25	25	13.5

Note: the volume used is microliters (µL); ND - not detected due to insufficient volume.

Table 2

PCR reaction with COI LCO1490/HCO2198 primer

Samples	*COI LCO1490/HCO2198*				
	DNA template	*Primer forward*	*Primer reverse*	*PCR mix*	*ddH_2O*
P0	2	1.25	1.25	25	20.5
P1	ND	ND	ND	ND	ND
P2	18	1.25	1.25	25	12.5
P3	7.5	1.25	1.25	25	15
P4	ND	ND	ND	ND	ND
P5	9	1.25	1.25	25	13.5

Note: the volume used is microliters (µL); ND - not detected due to insufficient volume.

Results. Anatomical observations of sea cucumber specimens revealed the ossicle within the integumentary tissue with distinct morphological characteristics (Figure 5). Isolated ossicle exhibited a variety of shapes, including buttons, rod, and tables. The most frequently observed were button type ossicles, characterized by smooth rims, irregular contours, and threepairs of holes (Borrero-Pérez & Vanegas-González, 2019).

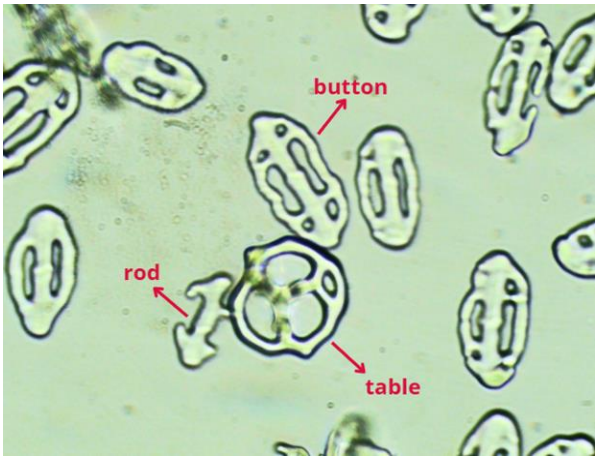

Figure 5. Ossicle shape in sea cucumber sample collected from Karimunjawa.

DNA purity and concentration across different tissue types. The spectrophotometrics analysis showed variation in DNA purity and concentration across different extraction treatment (Table 3). Based on the A260/280 ratio, all treatments showed poor purity values as seen from the fairly high standard deviation values. In terms of DNA concentration measured using the Nanodrop spectrophotometer, P2 produced the highest yield (4.76 ng μL^{-1}), followed by P1 and P3, while P0 and P5 showed relatively low concentrations and P4 resulted in a negative value. Quantus measurements showed P2 again yielding the highest concentration (3.53 ng μL^{-1}), while P1 and P4 were not detected (ND), likely due to insufficient DNA quantity. The superior DNA yield observed in P2 suggested that the bleach pretreatment effectively reduced ossicle contamination prior to extraction. By facilitating the separation of ossicles from the dermal tissue, the treatment reduced the amount of mineral material present during

extraction. Therefore, the chemical pretreatment applied in P2 likely contributed to the higher DNA concentration obtained in this case.

Table 3
Nanodrop and quantus spectrophotometer results of sea cucumber sample to measure DNA purity and concentration (ng μL^{-1})

Extraction treatment	*Spectrophotometer Nanodrop (average)*		*Quantus*
	A260/280	*Concentration (ng μL^{-1})*	*Concentration (ng μL^{-1})*
P0	4.1±2.304	1.7	0.133
P1	2.81±1.020	2.81	ND
P2	2.30±0.560	4.76	3.53
P3	2.43±0.665	2.78	0.860
P4	1.59±0.026	-6.93	ND
P5	4.67±0.100	1.1	0.0597

Note: P0 (anterior dermis tissue); P1 (muscle tissue ground with mortar and pestle); P2 (dermal tissue soaked in 10% bleach solution); P3 (muscle tissue ground with infrared sterilizer); P4 (gonad); P5 (secretions); ND (not detected because the DNA samples volume is insufficient).

DNA visualization of PCR products using electrophoresis. Based on the visualization results (Figure 6), DNA bands were clearly observed alongside the DNA marker. The marker functioned as a reference for fragment size. The PCR amplification results were evaluated by comparing the position of the DNA bands to the marker. The presence of distinct and well defined bands indicates that the PCR amplification was successful.

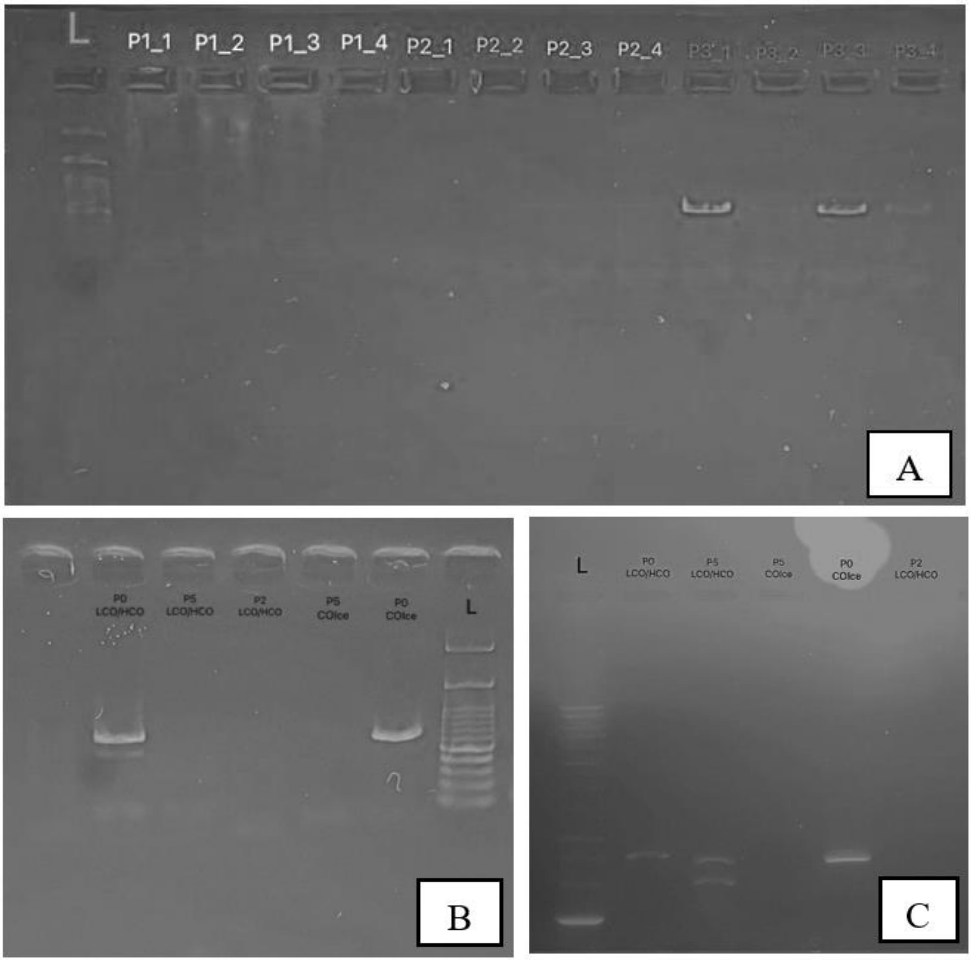

Figure 6. Visualization by electrophoresis, namely A) P1, P2, P3 with primers COIceF/ceR; B) P0_1, P2_1, and P5_1 with primers COIceF/ceR and LCO1490/HCO2198; C) P0_2, P2_2, and P5_2 with primers COIceF/ceR and LCO1490/HCO2198.

As shown in Table 4, amplification success varied among samples and primer pairs. The COI ceF/ceR primers produced amplification in P0 and P3, with success rates of 100% and 50%, respectively, whereas the COI LCO1490/HCO2198 primers produced amplification in P0 and P5, with success rates of 100% and 33.3%, respectively. In addition, the BLAST results for P0 (Table 5) showed high sequence similarity to *H. fuscocinerea* (96.00%) for the COIceF/ceR primers and to *Terebralia sulcata* (99.70% and 99.24%) for the COI LCO1490/HCO2198 primers.

Table 4

Primer effectiveness in amplification of sea cucumber sample from Karimunjawa

Sample	*COI ceF/ceR*			*COI LCO1490/HCO2198*		
	n	n_1	n_1/n (%)	*n*	n_1	n_1/n (%)
P0	2	2	100	3	3	100
P1	4	0	0	ND	ND	ND
P2	4	0	0	2	0	0
P3	4	2	50	ND	ND	ND
P5	2	0	0	3	1	33.3

Note: n = replicates; n1 = amplification with primers (1 = if it appears; 0 = if it does not appear); n_1/n(%) = percentage of success; ND = not detected because the DNA sample volume is insufficient.

Table 5

BLAST results of P0

Primer	*Scientific name*	*Accession number*	*Query cover (%)*	*E value*	*Percentage identity (%)*
COIceF/ceR	*Holothuria fuscocinerea*	MK562386	90	0.0	96.00
	Holothuria fuscocinerea	PP652084	75	0.0	96.00
COI LCO1490/HCO2198	*Terebralia sulcata*	HE680673	97	0.0	99.70
	Terebralia sulcata	AM932807	97	0.0	99.24

Discussion. The A260/280 absorbance ratio was used as an indicator of genomic DNA purity. The lowest ratio was observed in P4 (gonad), indicating protein contamination and low DNA integrity. Therefore, the use of proteinase K is recommended for improving DNA purity in gonad samples. Samples derived from dermal tissue containing ossicle showed high A260/280 ratios (> 2), suggesting contamination, likely from RNA or other compounds. Treatment P0 (dermis) exhibited the highest ratio, reflecting greater contamination compared to muscle tissues (P1 and P3). In contrast, P1 and P3 showed lower ratios, indicating that muscle tissue particulary when processed using infrared treatment (P3) is more suitable for obtaining purer DNA, although RNAse treatment is still recommended. In terms of DNA concentration, P2 produced the highest yield in both Nanodrop (4.76 ng μL^{-1}) and Quantus (3.53 ng μL^{-1}) measurements, likely due to the use of 10% bleach, which effectively removed ossicle, combined with RNAse treatment.

According to Yustina el al. (2025), ossicle, which are components from calcium carbonate based (alkaline), can reduce the quantity of DNA, so they need to be eliminated. Compared with the findings of Ogiso-Tanaka et al. (2025), the present study provides a different approach to addressing ossicle-related problems during DNA extraction. Ogiso-Tanaka et al. (2025) reported that DESS containing EDTA caused the dissolution or degradation of calcareous ossicles and recommended avoiding ossicle-containing tissues for DNA extraction. Similarly, the present study showed that ossicle removal using 10% bleach resulted in the highest DNA concentration in P2 (4.76 ng μL^{-1} by Nanodrop and 3.53 ng μL^{-1} by Quantus). However, unlike the previous study, which relied on EDTA during preservation, the present study directly removed ossicles before DNA extraction.

Both COIceF/ceR and LCO1490/HCO2198 successfully amplified sea cucumber sample, although their amplification success varied among treatments. Using COIceF/ceR, amplification was achieved in P0 (100%) and P3 (50%), whereas LCO1490/HCO2198 successfully amplified P0 (100%) and P5 (33.3%). However, successful amplification did not always result in accurate species identification. BLAST

analysis showed that sequences generated using COIceF/ceR were consistent with the organism, whereas sequences obtained using LCO1490/HCO2198 matched different species. Therefore, despite producing PCR amplicons, LCO1490/HCO2198 exhibited lower specificity, while COIceF/ceR was more reliable for the identification of sea cucumber sample from Karimunjawa.

The sea cucumber sample analyzed in this study were moleculary identified as *H. fuscocinerea* based on COI sequence analysis. Therefore, the following high purity genomic DNA with ossicle removal method is recommended for genomic DNA extraction from *H. fuscocinerea* dermal tissue. The anterior region (dermal to muscle tissue) of *H. fuscocinerea* represents an appropriate tissue source for molecular identification because it can provide sufficient DNA quality for downstream analysis until BLASTn. Genomic DNA concentration and purity can be further improved by a bleaching treatment to remove calcareous ossicles from the anterior tissue. In addition, RNase treatment is useful for reducing RNA contamination, thereby improving the purity of the extracted DNA.

Recommended method for high purity genomic DNA with ossicle removal. Consequently, the combination of appropriate anterior tissue selection, bleaching treatment for ossicle removal, and RNase treatment can enhance the quality and suitability of DNA for subsequent PCR amplification and molecular identification. The recommended method for high-purity genomic DNA extraction with ossicle removal is illustrated in Figure 7:

1. Collect dermal tissue until the muscle tissue from anterior region of *H. fuscocinerea*;
2. Soak the tissue in sodium hypochlorite (NaOCl) solution for 5 minutes to remove the calcareous ossicles (Brown et al., 2025);
3. Wash the tissue with destilled water 5 times to remove residual bleach containing dissolved ossicles;
4. Finely mince the tissue using scissors sterilized with an infrared sterilizer prior to DNA extraction using the Zymo Miniprep Plus Kit with modified RNase treatment;
5. Transfer approximately ±25 mg of minced muscle tissue into a microcentrifuge tube containing 95 µL nuclease free water, 95 µL Solid Tissue Buffer, and 10 µL proteinase K;
6. Mix the samples thoroughly by vortexing for 15 seconds and incubate at 55°C for 3 hours until the tissues are completely solubilized. Mix the samples again before proceeding to the next step;
7. To remove insoluble debris, centrifuge the samples at 12,000 × g for 1 minute and transfer the aqueous supernatant into a clean microcentrifuge tube;
8. After incubation at 55°C, add 5 µL of RNase into each sample tube;
9. Incubate the samples at 37°C for 15 minutes;
10. Add 430 µL of Genomic Binding Buffer (2× volume) into the samples and mix thoroughly by vortexing for 15 seconds;
11. Transfer the mixture into a Zymo-Spin IIC-XLR Column placed in a collection tube and centrifuge at 12,000 × g for 1 minute. Discard the flow through;
12. Add 400 µL of DNA Pre Wash Buffer into the spin column placed in a new collection tube, followed by centrifugation at 12,000 × g for 1 minute. Discard the flow through;
13. Wash the samples with 700 µL of genomic DNA Wash Buffer and centrifuge at 12,000 × g for 1 minute. Discard the flow through;
14. Add an additional 200 µL of genomic DNA Wash Buffer into the spin column and centrifuge again at 12,000 × g for 1 minute. Discard the collection tube containing the flow through;
15. Transfer the spin column into a clean microcentrifuge tube and add 50 µL of DNA Elution Buffer directly onto the matrix. Incubate the column at room temperature for 5 minutes and centrifuge at maximum speed for 1 minute to elute the DNA;
16. Use the eluted DNA immediately for downstream molecular analysis or store it at 20°C for future use.

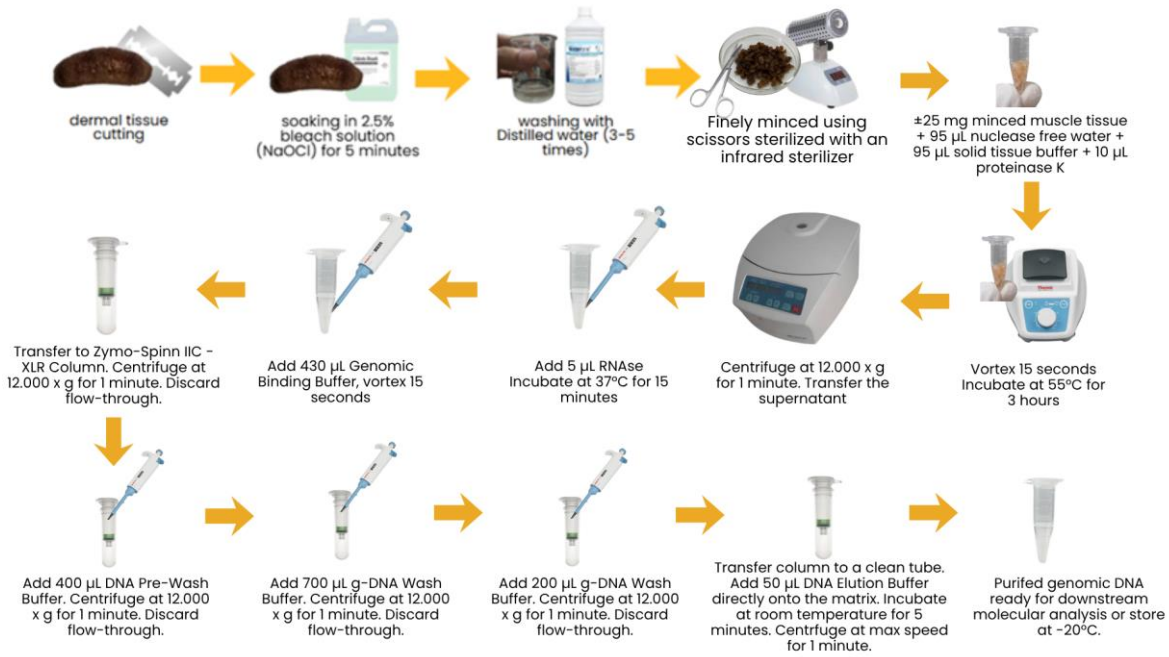

Figure 7. Recommended method for high purity genomic DNA extraction with ossicle removal.

Conclusions. This study provides a novel modified approach for genomic DNA isolation from *Holothuria fuscocinerea* by integrating anatomical observation, tissue selection, and chemical pretreatment. Anatomical observations revealed three types of calcareous ossicles in the dermis of *H. fuscocinerea* that may interfere with DNA extraction. Genomic DNA isolation from the anterior dermal-to-muscle tissue, combined with sodium hypochlorite treatment for ossicle removal and RNase treatment to reduce RNA contamination, produced DNA with sufficient yield and quality for PCR amplification. The extracted DNA successfully enabled *H. fuscocinerea* identification using the COIceF/ceR primer pair. This integrated approach highlights the importance of tissue-specific pretreatment as a novel strategy for improving molecular analysis of sea cucumbers with complex calcareous dermal structures.

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Authors Contributions. UA: Conceptualization, development and implementation of the molecular research, Molecular Methodology, Investigation, Formal Analysis, Data Curation, interpretation of molecular data, Writing Original Draft, and Editing; AK: Conceptualization, development and refinement of the research methodology, Molecular Methodology, Investigation, Data Curation, Supervision of the research process, interpretation and evaluation of the results, and Editing, Project Administration, and Finalization of the manuscript; SPP: Conceptualization, development of the initial research idea, Investigation, Sample Collection and provision of research materials, Supervision, Project Administration, contextual interpretation of the research findings, and Editing.

Conflicts of Interest. The authors declare that there is no conflict of interest.

Data Availability. The data supporting the findings of this study are available from the first author upon reasonable request.

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