

Species Identification of the Helmet Urchin, *Colobocentrotus atratus* (Linnaeus, 1758) from the South Coast of Java, Indonesia Using DNA Barcoding

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Abstract

The diversity of sea urchins in Indonesia significantly differs throughout various aquatic environments. Sea urchins serve as a keystone species and as an indicator of coastal ecosystem health. *Colobocentrotus atratus*, commonly referred to as the helmet urchin, is a distinctive sea urchin endemic to the south coast of Java, originating from Hawaii. This research aims to identify the species, determine the genetic distance, and analyze the phylogenetic relationship of the *C. atratus* sea urchin that lives on the south coast of Java. Sampling occurred at Ngrumput Beach, Yogyakarta, Indonesia. Nucleotide sequence analysis was conducted on the COI mitochondrial DNA amplified using PCR. Nucleotide sequences ranging from 625 base pairs were obtained from three specimens of *C. atratus*. Molecular analysis indicates that these samples were identified as *Colobocentrotus atratus*. Phylogenetic analysis was performed by comparing the haplotype gene sequences of *C. atratus* from the south coast of Java with gene sequences of identical base pair length, utilizing *Diadema setosum* as the outgroup. The phylogenetic study results indicate that *C. atratus* from the south coast of Java is closely related to *C. atratus* from Sabang and France. This research provides essential data that can support future initiatives to preserve coastal ecosystems.

Introduction

Sea urchins are marine invertebrates that are capable of inhabiting deep-sea waters, kelp forests, coral reefs, and seagrass meadows (Lawrence, 2020). Its presence may serve as an indicator for determining the effects of global warming and ocean acidification, owing to its significant flexibility to environmental fluctuations (Uthicke et al., 2020; Asnicar et al., 2021). Sea urchins are herbivorous; hence their presence may regulate the population of macroalgae in the intertidal zone (Steneck, 2020). Sea urchins along the southern coast of Java comprise multiple species that's abundance and distribution are influenced by both abiotic and biotic factors within the marine ecosystem. *Colobocentrotus atratus* Linnaeus, 1758, is a distinctive species of sea

urchin dwelling in the rocky intertidal zone and is native to Hawaii. *C. atratus* performs as a grazer, hence shaping the composition of rocky intertidal ecosystems in Hawaii. It exhibits an indirect commensal relationship with the limpet species *Cellana sandwichensis*, which creates space for it (Bird, 2006). This sea urchin is referred to as the single urchin or helmet urchin (Pandey & Ganesh, 2016). *C. atratus*, commonly referred to as hā'uke'uke, serves as an enormous traditional food source for the native people of Hawaii (Wilbur & Moran, 2018).

Colobocentrotus atratus is extensively found in Hawaii and the Indo-West Pacific region, thriving in shallow waters and rocky surfaces. This sea urchin is essential to marine ecosystems, regulating algal growth and providing food for several predators. The Pacific Sea

urchin may inhabit and adapt to regions with strong waves and exposures (Wilbur & Moran, 2018; Santos & Flammang, 2008). Exposure to varied conditions in the environment enables *C. atratus* exhibit outstanding resilience and adaptation. The species' presence in many habitats demonstrates its significance in preserving ecological stability and encouraging biodiversity within marine environments. The morphology of this sea urchin is distinctive when compared to other species, as *C. atratus* possesses blunt spines and considerably smaller aboral spines that resemble tiles, along with a body shape like that of a limpet (Denny & Gaylord, 1996; Pandey & Ganesh, 2016; Wilbur & Moran, 2018; Santos & Flammang, 2008). Narvaez et al. (2022) suggest that the unique characteristics of sea urchins indicate significant plasticity as a response to environmental pressures. This plasticity enables them to survive in diverse maritime environments, demonstrating a remarkable capacity to alter their appearance in response to parameters such as temperature, salinity, and food sources. The investigation of *C. atratus* not only enhances our comprehension of echinoderm biology but also offers insights into the adaptability of marine organisms within dynamic ecosystems. *C. atratus* also thrives in rocky intertidal zones with severe wave conditions throughout the south coast of Java. Further investigation is required to figure out whether *C. atratus* on the south coast of Java is correctly identified as *C. atratus* and to elucidate its genetic relationship with the *C. atratus* in other population in the world.

Hardianto & Satriyo (2023) suggest that DNA barcoding offers significant advantages for species identification that morphological approaches and phylogenetic analyses cannot provide. Research has shown that it is effective in identifying species throughout larval, juvenile, and adult stages. This approach not only improves our knowledge of biodiversity but also supports conservation initiatives by delivering reliable information on species distribution and abundance. DNA barcoding has been employed in numerous studies to identify fish (Nursalim et al., 2022; Falah et al., 2023), mollusks (Hardianto & Satriyo, 2023), Echinodermata (Lee & Shin, 2012; Ward et al., 2008; Sonet et al., 2022; Probosunu et al., 2025), and seaweed (Saraswati et al., 2024; Susanti et al., 2022; Handy et al., 2020; Alshehri et al., 2019). Accurate and efficient species identification enables researchers to develop more effective management plans for the protection of marine ecosystems. This study will utilize COI to identify and validate the species of *C. atratus* found along the south coast of Java, as well as to ascertain its genetic variations and relationships with other species. The research findings are projected to provide genetic data that can serve as the basis for effective future conservation of sea urchins in coastal areas. Moreover, the results may enhance comprehensive ecological research concerning the adaptation of marine organisms to environmental variations.

Materials and Methods

Sampling and DNA Extraction

We directly collected sea urchin samples from their natural habitat, the intertidal zone of Ngrumput Beach on the south coast of Yogyakarta, Indonesia (Figure 1). We observed morphology in a total of twenty individuals and extracted three *C. atratus* individuals for molecular analysis. We carried out sampling activities for *C. atratus* during December 2023, believed to be the peak of its highest abundance. Samples were preserved using 95% ethanol and then taken to the laboratory for morphological observation and DNA extraction. Using tweezer forceps, we extracted DNA from approximately 50 milligrams of the tuberous section. We stored the tissue samples from the tube feet in 1.5 ml plastic test tubes. Next, we used the Favorgen Tissue Genomic DNA Extraction Mini Kit (Biotech Corp.) and followed the instructions that came with it to get the DNA.

Amplification and Sequencing

Colobocentrotus atratus samples were amplified using Polymerase Chain Reaction (PCR), specifically targeting mitochondrial DNA (mtDNA) cytochrome oxidase c subunit I (COI). PCR was performed with a total volume of 25 µL. Each PCR microtube contained: 3 µL of template DNA, 0.5 µL each of forward and reverse primers, 12.5 µL of Bioline Taq DNA polymerase master mix, and 8.5 µL of distilled H₂O. The primers used in *C. atratus* species were a combination of Echino F1 (5'-TTTCACTAATCATAAGGACATTGG-3') (Ward et al., 2008) as the forward primer and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3) (Folmer et al., 1994) as the reverse primer. The primer combination targets mtDNA COI which approximately amplifies a fragment of 657 bp (Ward et al., 2008). The PCR process begins with a pre-denaturation process of 94°C for 300 seconds, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 48°C for 30 seconds, and extension at 72°C for 60 seconds, then post-extension at 72°C for 300 seconds. The PCR results were then analyzed using DNA electrophoresis using 1% agarose gel to see the quality of the DNA. The sequencing process was carried out by sending PCR sample results to PT. Genetika Science Indonesia which was then sequenced by the first base Singapore. The DNA sequence results are stored in the GenBank National Center for Biotechnology Information (NCBI) with the accession number PV124837, PV124838, PV124839.

Data Analyses

The sequencing results were analyzed using the muscle sequencing method that provides high accuracy and speed, which is found in the MEGA-X software (Kumar et al., 2018). The analyst is expected to be able to perform a comprehensive analysis of the DNA

sequence results to find variations in the sequencing results (Edgar, 2004; Kumar et al., 2016). The nucleotide sequences were then analyzed using the BLAST (Basic Local Alignment Search Tool) program accessed through the NCBI website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to carry out the DNA barcoding approach, where species identification can be done by comparing the sequencing results in this study with data that has been stored in GenBank by considering percent identity and query cover. Furthermore, genetic distance analysis was carried out using pairwise distance in the MEGA-X software. Kinship analysis was carried out using a phylogenetic tree with the Neighbor Joining Tree method which is available on MEGA-X with the Bootstrap test phylogeny (1000x) and the Kimura 2-parameter method. Haplotype network analysis between species using the minimum spanning network (MSN) analysis method using PopArt software (Leigh & Bryant, 2015).

Result

Morphology, Morphometric and Blast Analyses

Colobocentrotus atratus possesses a distinctive feature: its blunt aboral spines create a dense mosaic pattern that resembles a limpet throughout its entire aboral surface. This sea urchin features aboral spines,

referred to as ambitus spines, surrounding its oral side, which creates a resemblance to a helmet or hat. Consequently, this species is referred to as the helmet urchin. This sea urchin exhibits a uniform dark purple colour throughout its body. This analysis finds a diameter of around 5 cm. The spines of this sea urchin are flat and short. The oral surface features dark purple actinal spines and robust tube feet that adhere effectively to rocky ground (Figure 2). This species possesses multiple tube feet with significant adhesive strength to rocks. This study identified *C. atratus* in rocky intertidal zones characterized by severe wave exposure. This species also inhabits the small crevices of cliff rocks at the lowest tide line.

Based on the results of sequence editing with MEGA-X software, the results of DNA band sequencing were 625 bp in the three *C. atratus* samples. This was also confirmed by the electrophoresis results which showed DNA amplification results that were good enough for sequencing (Figure 3). The DNA sequencing results were then examined by comparing the samples in GenBank. Comparison of the sequencing results of the samples with the sequencing in Genbank was then analyzed using BLAST through the NCBI website, the results of the blast can be seen in Table 1.

The results of the BLAST analysis (Table 1) show the range of Query Cover in the range of 100%, while the percentage of identity is in the range of 99.52 - 99.68%.

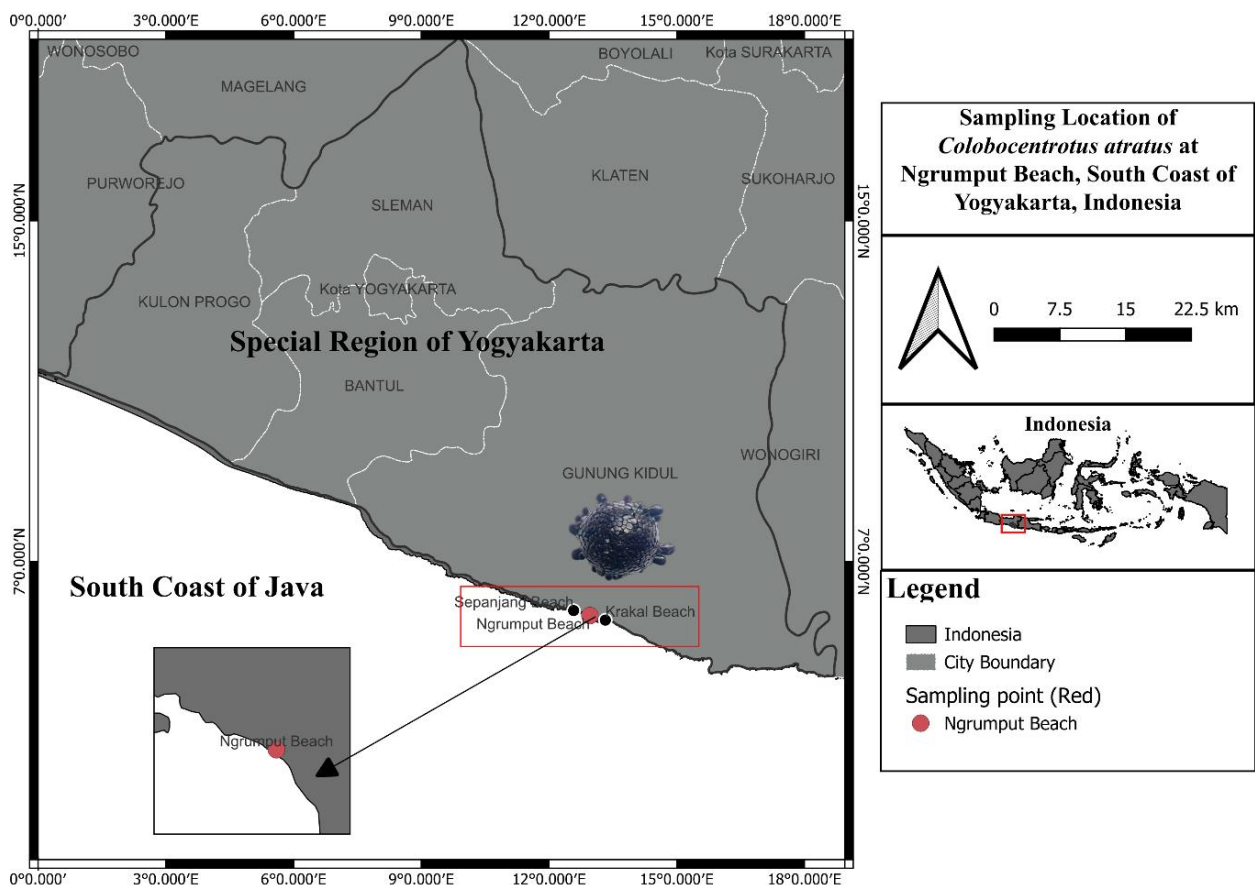


Figure 1. Sampling location of *C. atratus* at Ngrumput Beach, south coast of Yogyakarta, Indonesia.

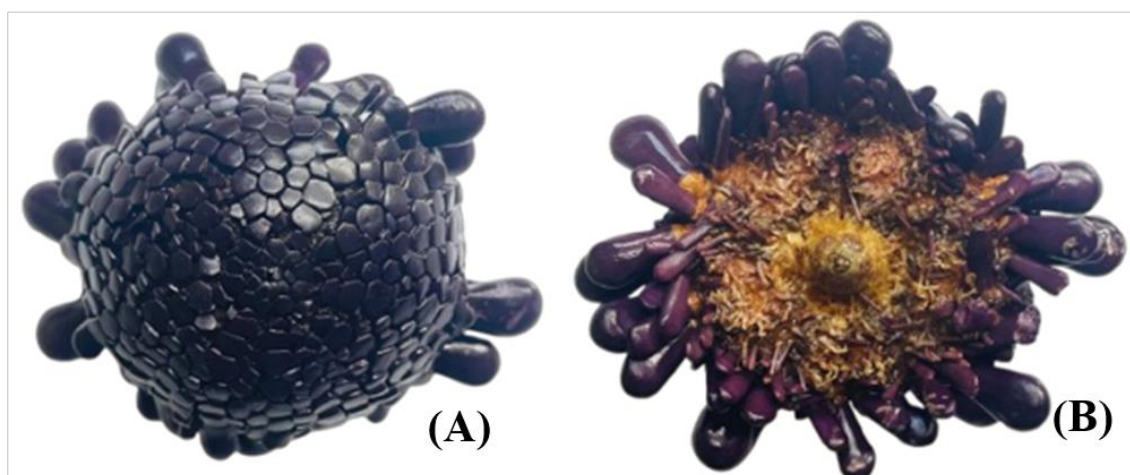


Figure 2. Morphological picture of *C. atratus* that collected from the natural habitat. (A) body view and (B) oral view of *C. atratus*..

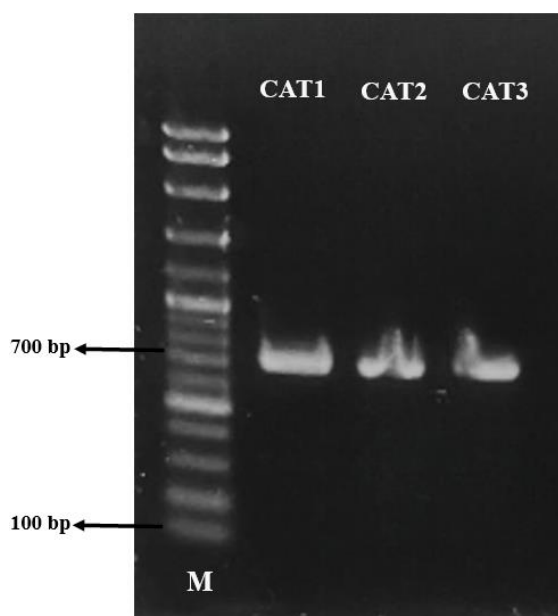


Figure 3: Visualization of COI gene electrophoresis results of *C. atratus*. (M=DNA ladder; CAT1= sample 1 *C. atratus*; CAT2 = sample 2 *C. atratus*; CAT3 = sample 3 *C. atratus*).

The results of the base ratio obtained from the sequencing results excluding out of group are the Adenine ratio: 26.0%, Thymine ratio: 27.9%, Guanine ratio: 19.1%, and Cytosine ratio: 27.0% with a total base of 625.0 (Table 2).

Genetic Distance and Network Analyses

The genetic distance obtained varies between 0.01 to 0.02. The largest genetic distance was found between *C. atratus* from the south coast of Java with the outgroup species *Diadema setosum* (0.26) from Jordan (KU496327.1) (Table 3). Haplotype network analysis (Figure 4) showed that there were 4 connected haplotypes, from the results of the haplotype network it can be seen that *C. atratus* on the south coast of Java

has the same haplotype as those in Sabang Indonesia and La Reunion France (Figure 4).

Phylogenetic Tree

The results of the phylogenetic tree of sea urchin on the south coast of Java contain three clusters (Figure 5). Each species forms a separate cluster and is different with a bootstrap value of 100%. Samples CAT1-CAT3 are closely related to *C. atratus* Sabang Indonesia, La Reunion France, and Hawaii USA. The outgroup sample also has no kinship with sea urchins on south coast Java, as they are different species. The construction of the phylogenetic tree obtained a scale bar result of 0.02.

Table 1. Percent identity of nucleotide bases in *Colobocentrotus atratus* and other sea urchins found on coast Java from BLAST (NCBI)

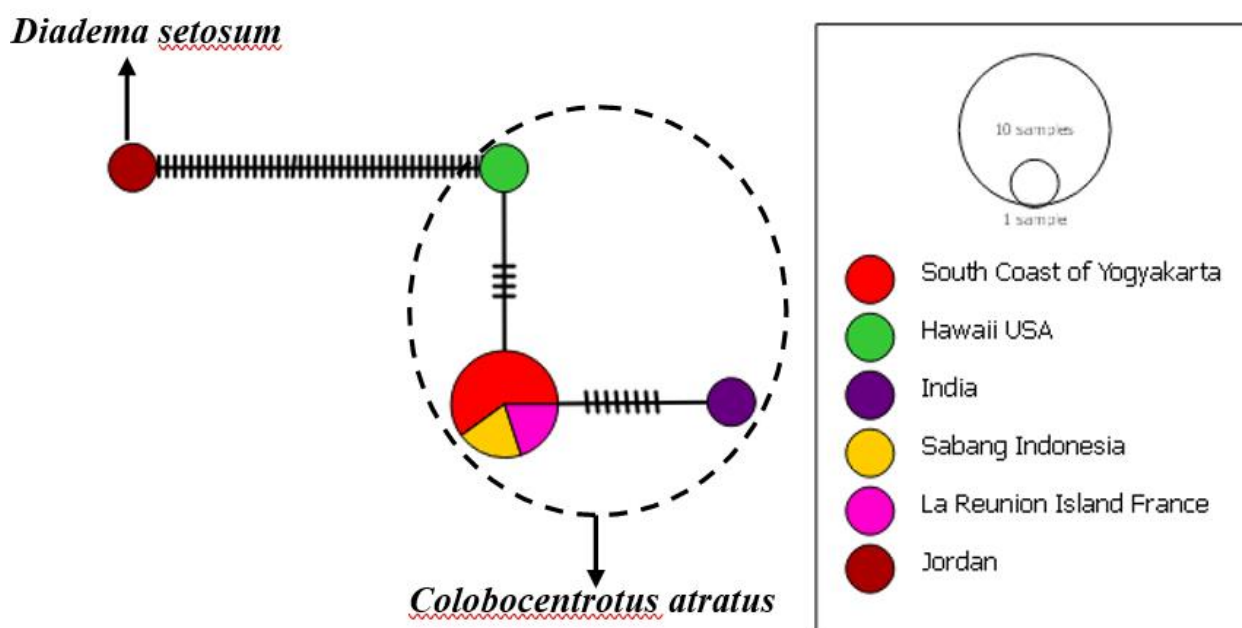
Sample code	Sampling Location	Acession Number	Query cover (%)	Percent identity (%)	References Accession Number	Species Identification
CAT1	Ngrumput Beach, Southern	PV124837	100	99.68	OR098295.1	<i>C. atratus</i>
CAT2	Coast of Yogyakarta,	PV124838	100	99.52	OR098295.1	<i>C. atratus</i>
CAT3	Indonesia	PV124839	100	99.52	OR098295.1	<i>C. atratus</i>

Table 2. Nucleotide composition (%) of COI gene amplified of *C. atratus* from southern coast of Yogyakarta

Sample	T	C	A	G	Total
CAT1	28.0	26.9	26.1	19.0	625
CAT2	27.8	27.2	25.8	19.2	625
CAT3	27.8	27.0	26.1	19.0	625
<i>Diadema setosum</i> (out of group)	31.3	24.7	24.1	19.9	623
Overall	28.7	26.5	25.5	19.3	624.5

Table 3. Pairwise genetic distances of *Colobocentrotus atratus* including out of groups (*D. setosum*) and DNA data bank data of mitochondrial DNA COI

No	Species and Sample Code	Genetic Distance						
		1	2	3	4	5	6	7
1	<i>Colobocentrotus atratus</i> -Ngrumput1							
2	<i>Colobocentrotus atratus</i> -Ngrumput2	0.00						
3	<i>Colobocentrotus atratus</i> -Ngrumput3	0.01	0.00					
4	<i>Colobocentrotus atratus</i> -Sabang_Indonesia-OR098295.1	0.00	0.00	0.00				
5	<i>Colobocentrotus atratus</i> -La_Reunion_France-GU480562.1	0.00	0.00	0.00	0.00			
6	<i>Colobocentrotus atratus</i> -Hawaii_USA-MW278676.1	0.02	0.02	0.02	0.02	0.02		
7	<i>Diadema setosum</i> -Jordan-KU496327.1	0.26	0.26	0.26	0.26	0.26	0.26	

**Figure 4.** Haplotype network of *C. atratus* compares with DNA data bank data. These analyses using a minimum spanning network analysis (Yogyakarta= South Coas of Java).

Discussion

Sea urchins are often found in the intertidal zone of waters around the world, including in Indonesia. The diversity of sea urchins in Indonesia varies widely in each water, as evidenced by several previous studies. Based on several previous studies on sea urchins, it was reported that Java Island is inhabited by the following species: *Diadema setosum*, *Diadema antillarum*, *Diadema savingii*, *Echinometra mathaei*, *Echinothrix calamaris*, *Echinothrix diadema*, *Phyllacanthus* sp., *Asthenosoma varium*, *Tripneustes gratilla*, *Echinometra oblonga*, *Heterocentrotus trigonarius*, and *Stomopneustes variolaris* (Sasongko et al., 2020; Supono & Arbi, 2012; Triacha et al., 2021; Wijaya et al., 2022; Suryanti et al., 2020; Aimatuzzahro et al., 2020). Meanwhile, previous research by Suryanti et al. (2020) conducted on the south coast of Yogyakarta reported that the species inhabiting these waters were *Echinometra mathaei*, *Echinometra oblonga*, *Heterocentrotus trigonarius*, *Echinothrix calamaris*, *Diadema setosum*, and *Stomopneustes variolaris* and *C. atratus*.

Colobocentrotus atratus exhibits a distinctive and a typical morphology relative to other sea urchins. The spines on the aboral surface are blunt and create a structure similar to a limpet, but the oral surface features blunt, flat spines that surround the body, giving this sea urchin a helmet-like appearance. Consequently, this species is referred known as the helmet urchin (Denny & Gaylord, 1996; Santos & Flammang, 2008; Pandey & Ganesh, 2016; Wilbur & Moran, 2018). This study determined the diameter of *C. atratus* to be 5 cm, while other research indicated a diameter range of 4.5 cm to 7.5 cm (Pandey & Ganesh, 2016; Nurcahyo et al., 2024). The distinctive form of *C. atratus* results from exposure to intense sea waves and currents, enabling

the tiny body of *C. atratus* to adapt and thrive in tightly coral reef crevices.

Hydrodynamic forces make the morphology of this sea urchin unusual, as it lowers wave impacts and minimizes damage to its spines. Santos & Flammang (2008) suggested that the morphology of *C. atratus* likely serves as a mechanism for avoiding various hydrodynamic forces, such as wave impact. The distinctive shape of *C. atratus* appears to help reduce the stresses generated by water motion, hence enabling this species to endure in regions of intense wave exposure (Denny & Gaylord, 1996). The tube feet that firmly attach to the rocks enable this sea urchin to endure intense waves and escape predators. Santos & Flammang (2008) assert that *C. atratus* possesses a more optimal dome-shaped shell and exhibits superior adhesive strength of its tube feet relative to other species. The genus *Colobocentrotus* comprises three species: *Colobocentrotus atratus*, *Colobocentrotus mertensii*, and *Colobocentrotus pedifer*.

The DNA barcoding analysis results shown in Table 1 show that the nucleotide sequences of three *C. atratus* sequencing samples observed at Ngrumput Beach, Yogyakarta, Indonesia, are identical, showing a match of 99.52% to 99.68% with *C. atratus* from Sabang, Indonesia (OR098295.1), La Reunion Island, France (GU480562.1), and Hawaii (MW278676.1). In DNA barcoding analysis, the requirement for authentication at the species level is sequence similarity or percent identity exceeding 97%, while similarities below this threshold are used for recognition at the genus level (Wong & Hanner, 2008; Syaifudin et al., 2021; Probosunu et al., 2025). Based on this study, it shows that species classification using DNA barcoding combined with morphological characters is very precise and accurate. The use of the COI gene is also considered very appropriate and effective because of its low

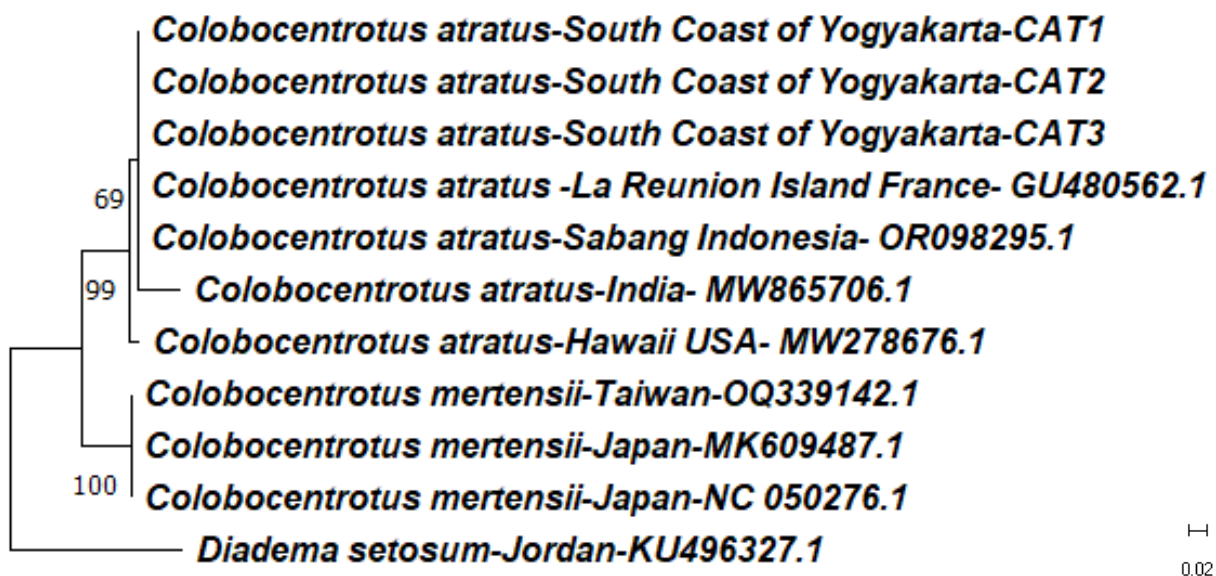


Figure 5. Phylogenetic tree of *C. atratus* south coast of Java with other sea urchins using Neighbor joining (NJ) method using Bootstrap 1000 X.

intraspecific variation and high interspecific variation values, especially in marine animal taxa (Ward et al., 2005). Previous studies have also shown that DNA coding functions to authenticate species carried out on various taxa (Hardianto & Satriyo, 2023; Hanamura et al., 2024; Saraswati et al., 2024; Probosunu et al., 2025). This approach is beneficial for species identification and sustainable fisheries resource management.

Genetic distance was also used in this study to determine the genetic correlation between *C. atratus* specimens collected from the study site and other samples from the DNA database. Genetic distance values ranged from 0.01 to 0.02. A genetic distance difference of 4% (0.04) is considered as a threshold for molecularly similar species (Hardianto & Satriyo 2023; Hanamura et al., 2024). Greater population uniformity was observed when the genetic distance between individuals was smaller. Conversely, higher genetic distances between individuals within a group would result in greater levels of population diversity. The largest genetic difference (0.26) was observed between *C. atratus* inhabiting Indonesia and the outgroup species *Deadema setosum* from Jordan (KU496327.1).

In line with the genetic distance analysis, haplotype network analysis revealed negligible differences in species analysis (Figure 4). The barcode variation within *C. atratus* is rather low compared to the sequence variation between species and outgroup species. Genetic distance is a measure of the proportion of genetic differences between species or populations (Dogan et al., 2016). Consequently, lower genetic distance values result in more similar partial sequences of the COI gene (Sonet et al., 2022). The assembled sea urchin phylogenetic tree is shown schematically in Figure 5. This study assessed the evolutionary dynamics and degree of relatedness of the species, categorizing them into three distinct clusters. The species *C. atratus* was isolated from *D. setosum* (KU496327.1) with bootstrap values greater than or equal to 100%. One cluster was formed within the species *C. atratus* from Indonesia, France, and Hawaii.

Although *C. atratus* has been documented in several countries in Asia, Europe and America, studies on its molecular and evolutionary history are rare in the literature, especially in Indonesia. A previous study conducted by Pandey and Ganesh (2016) documented the reappearance of *C. atratus* in the Andaman Islands, India using a morphological approach. In addition, Sonet et al. (2022) documented the DNA barcoding process for several echinoderm species, including *C. atratus*, which is specific to the South Coast of Africa. Nurcahyo et al. (2024) conducted a comprehensive analysis of the community structure of echinoderms, including *C. atratus* on the south coast of Yogyakarta, Indonesia. Recently, Probosunu et al. (2025) used a molecular approach in the identification of Echinodermata on the south coast of East Java in the species *Triplaneustes gratilla*. Determining the phylogenetic status of an organism is very important to provide information in

identification research and resource management. *Colobocentrotus atratus* is a species that offers valuable insights into the ecological and environmental conditions and contributes to the marine food chain (Pandey & Ganesh et al., 2016). Assume that the phylogeny of *C. atratus* has been fully elucidated. In such a situation, baseline data will be provided to facilitate further research aimed at maintaining the balance of the marine ecosystem. Molecular studies revealed a strong genetic relationship between all samples and *C. atratus*. In a previous study using sequence data from an identical molecular marker (COI gene), similar findings were reported (Probosunu et al., 2025). A bootstrap score above 70% indicates that the data has a high degree of stability (Lemey et al., 2009). The reconstructed phylogenetic tree produced a bar scale of 0.05. Based on the findings of Syaifudin et al. (2021), a phylogenetic tree with a scale of 0.01 indicates the genetic distance at which nucleotides change every 100 base pairs. The obtained phylogenetic structure reveals genetic distances characterized by nucleotide sequence variations occurring five times every 100 base pairs. Various aquatic species have been described using DNA barcoding, which shows greater variation within congeneric species than among conspecific individuals (Ward et al., 2005; Hardianto & Satriyo, 2023; Castaneda et al., 2023). Thus, this method can accurately distinguish a group of morphologically unique species in marine organisms (Hardianto & Satriyo, 2023; Castaneda et al., 2023; Probosunu et al., 2025).

Conclusion

In the light of species identification, molecular techniques such as DNA barcoding and phylogenetic analysis are more accurate and dependable than morphological methods. The current effort used DNA barcoding and phylogenetic analysis to prove that the specimens found on Ngrumput Beach in Yogyakarta were from a certain species. This finding would clarify the specimens' evolutionary position and provide essential information for further species research. DNA Barcoding analysis compared haplotypes from other DNA data banks at NCBI; the analyses verified that the samples identified as *Colobocentrotus atratus* (match range 99.52-99.68%). The DNA barcoding analysis conducted on specimens from Ngrumput Beach, Yogyakarta, has not only identified the species as *C. atratus* but also provided valuable insights into its evolutionary position within the broader ecological framework. By comparing haplotypes with data from established DNA banks at NCBI, this research underscores the significance of genetic diversity in local marine ecosystems and highlights potential avenues for future research programs in Indonesia. These findings emphasize the necessity of integrating molecular methods into conservation efforts, paving the way for a more comprehensive understanding of biodiversity and its preservation in this unique coastal region.

Ethical Statement

Not applicable.

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Author Contribution

First Author: Data curation, Formal analysis, investigation, writing original draft; Second Author: Conceptualization, Supervision, methodology, writing, review, editing; Third Author: Methodology; supervision, review; Fourth Author: supervision, writing, review, editing.

Conflict of Interest

Authors hereby declare that there is no conflict of interest.

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