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DNA barcoding insights into echinoderms from Situbondo Coral Reefs, East Java, Indonesia

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Abstract. *Aulia ED, Muzaki FK, Lee S-K, Khim JS, Park J. 2025. DNA barcoding insights into echinoderms from Situbondo Coral Reefs, East Java, Indonesia. Biodiversitas 26: 3434-3445.* Echinoderms are a key component of marine ecosystems, contributing to species richness and serving ecological functions. Given their importance, observing their diversity and ecology is essential. Despite the favorable coral reef conditions in Situbondo on Java Island, a comprehensive record of echinoderms has never been done. This research addresses this knowledge gap by collecting and identifying the species richness with morphology and molecular identification using the mitochondrial gene Cytochrome Oxidase I (COI) and 16S ribosomal RNA. Timed sampling for 40 minutes was carried out at intertidal reefs from depths between 0-5 m and in shallow reefs from 5-20 m, with a total of five sites. A total of 45 echinoderm species were identified, encompassing 6 Asteroidea, 5 Crinoidea, 8 Echinoidea, 15 Holothuroidea, and 11 Ophiuroidea. Of the 71 echinoderm samples collected, COI sequences were successfully amplified in 66% of the samples, while 16S rRNA sequences had a success rate of 53%. Notably, the COI sequences of five species and the 16S sequences of eight species were reported to GenBank for the first time, contributing to the expansion of publicly available reference data for echinoderm molecular identification. Furthermore, the present study highlights phylogenetic uncertainties based on maximum likelihood analysis of the COI sequences, particularly within two Asteroidea species and one Ophiuroidea species from the *Ophiolepis* clade. In addition, the findings suggest a potential range extension for *Diadema clarki* (Ikeda, 1939), indicating previously undocumented distribution patterns. These findings provide essential baseline data and monitoring for future marine biodiversity research, especially considering the area's significant coastal water utilization for tourism and fishing activities.

Keywords: Biodiversity, Echinodermata, marine invertebrate, molecular identification, phylogenetic analysis

INTRODUCTION

The phylum Echinodermata is exclusively marine invertebrates characterized by their typical “spiny-skinned” calcite skeleton, a distinctive water vascular system, and pentamerous radial symmetry in their adult forms (Clark and Rowe 1971; Pawson 2007). This widespread group, found in almost all marine habitats and depths, includes five living classes: Asteroidea (sea stars or starfish), Crinoidea (feather stars and sea lilies), Echinoidea (sea urchins and sand dollars), Holothuroidea (sea cucumbers), and Ophiuroidea (brittle and basket stars) (Pawson 2007; WoRMS 2024). Over the past two centuries, extensive research has uncovered the vast diversity within this phylum, with new species regularly being described (O’Loughlin and Bribiesca-Contreras 2015; Pineda-Enríquez et al. 2018; Lane and Vimono 2020; Zheng et al. 2022; Améziane et al. 2024). Global reviews on asteroids, ophiuroids, and other major echinoderm taxa highlight the remarkable contributions of the Indo-West Pacific region (Mah and Blake 2012; Stöhr et al. 2012; Summers et al. 2014). Studies of Indonesian echinoderm diversity have a longstanding history predating the 21st century (Aziz 1981; Hutomo and Moosa 2005). Recent regional research has also advanced our knowledge of echinoderm diversity across both shallow (Supono et al. 2014; Ali et al. 2017; Setyastuti et al. 2018; Aulia et al. 2021; Siburian et al. 2023) and deep-sea (Setyastuti and Wirawati 2018; Vimono and Lane 2021) environments. This remarkable diversity underscores the significant ecological roles that echinoderms play in marine ecosystems, including nutrient cycling (Purcell et al. 2016; Ennas et al. 2023), habitat provisioning (González and Borrero-Pérez 2020), as well as functioning as carnivores and herbivores (Lewis et al. 2018; Rojas-Montiel et al. 2020).

In species identification, morphological characteristics have long been the primary basis in taxonomic studies. However, the development of molecular techniques, particularly DNA barcoding, has improved the accuracy and efficiency of species identification by addressing key challenges in large-scale echinoderm research, including skeletal and color variability (Laakmann et al. 2016; Guzzi et al. 2023), morphological differences across developmental stages (Hodin et al. 2019; Goh et al. 2023), and the presence of cryptic and sibling species (Ward et al. 2008; Stöhr et al. 2020; Sonet et al. 2022). Furthermore, the

morphological and genetic variabilities of echinoderms have been studied to understand evolutionary processes, revealing the occurrence of speciation (Bribiesca-Contreras et al. 2019). In Indonesia, DNA barcoding has been applied to the identification of various echinoderm taxa, including overall echinoderms (Patantis et al. 2019; Nugroho et al. 2023), sea cucumbers (Amin et al. 2020; Sulardiono et al. 2022), and sea urchins (Dailami et al. 2023). Together, these applications of DNA barcoding highlight its growing significance in providing valuable baseline data for the study and conservation of Indonesian marine biodiversity.

However, despite the progress made in inventorying Indonesian echinoderm diversity, several locations in the western part of Indonesia particularly have yet to be explored (Hoeksema 2017; Madduppa et al. 2021; Aulia et al. 2024). Coastal areas of Situbondo, located in East Java, have been reported to have moderate to good coral reef cover (Hidayah and Nuzula 2019; Khusnah et al. 2019). However, records on marine benthic organisms in the areas remain limited, with echinoderm diversity being particularly understudied (Somma et al. 2017; Muzaki et al. 2023). Moreover, these regions are actively utilized for various anthropogenic activities such as fishing, diving, and snorkelling (Muthahharah and Adiwibowo 2015; Khusnah et al. 2019). For example, one site includes an ongoing coral restoration initiative at Batu Lawang, while others, such as Takat Palapa and Tanjung Pecaron, are well-known dive locations. The intertidal zones along the western and eastern parts of Pasir Putih are also frequently accessed on foot during low tide. Given the important roles of echinoderms, understanding their diversity is essential for assessing environmental conditions and establishing baseline data for potential changes (Lin et al. 2021).

Therefore, the present study aims to discover the species richness of echinoderms in the Situbondo coral reefs using both morphological and molecular identification methods. We used the mitochondrial genes Cytochrome Oxidase I (COI) and 16S ribosomal RNA due to their balanced conservation across related species and variation within species (Hebert et al. 2003b). In addition, these genes are widely used, resulting in a large and continuously growing repository of sequence data, making them a good proxy for identifying species (Ward et al. 2008; Hoareau and Boissin 2010). Furthermore, we discuss the insights gained from the molecular identification results, particularly in relation to the morphological characteristics.

MATERIALS AND METHODS

Study area and sample collection

Echinodermata sampling was conducted during the day in the coral reefs on the northern coast of Situbondo at five different sites from July 15-16, 2023 (Table 1). This area is located within the Madura Strait, a semi-enclosed part of the Java Sea in the Indonesian archipelago (Figure 1). A timed roving diver sampling method was used, with each collection taking approximately 40 minutes (Andrimida 2022; Bravo et al. 2023). Samples were collected by SCUBA diving at 5-15 m depths at shallow reef sites, and by walking and hand-picking at 0-1 m depths at intertidal reef sites (Table 1). Several representative individuals of echinoderm species were photographed on-site and then preserved in absolute ethanol 70% (Ceríaco and Marques 2025). In cases of relatively low species abundance based on expert in-situ observations of rarity and ecological sensitivity, for DNA extraction targeted specific anatomical structures: part of the outer arms (Ophiuroidea and Crinoidea), tube feet (Asteroidea and Echinoidea), and tentacle (Holothuroidea) (Laakmann et al. 2016; Madeira et al. 2018).

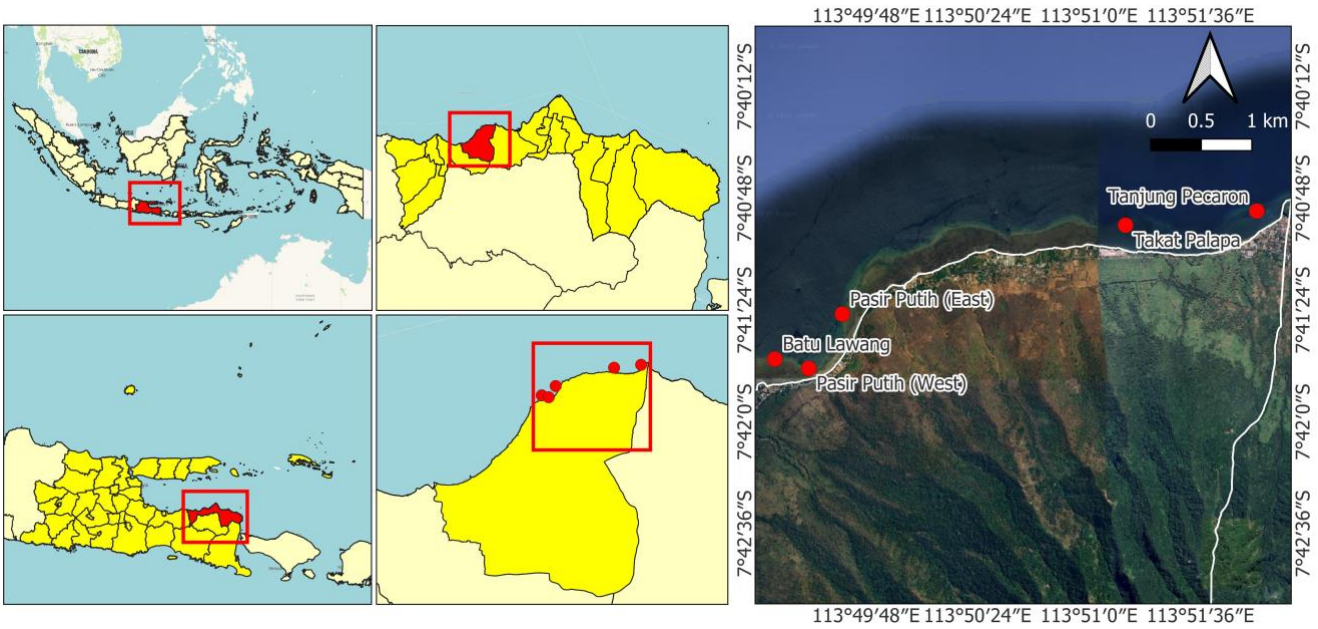

Figure 1. Echinodermata sampling sites at the Situbondo coral reefs, East Java, Indonesia

Table 1. Sampling sites, reef geomorphology, dates, coordinates, and depths for the echinoderms survey at Situbondo, Indonesia

Site	Site	Reef geomorphology	Date	Latitude	Longitude	Depth (m)
1	Batu Lawang	Shallow reef flats	16-07-2023	7°41'39.72"S	113°49'18.53"E	5-15
2	Pasir Putih (west)	Intertidal reef flats	15-07-2023	7°41'42.73"S	113°49'29.46"E	0-1
3	Pasir Putih (east)	Intertidal reef flats	16-07-2023	7°41'25.21"S	113°49'40.31"E	0-1
4	Takat Palapa	Shallow reef front	15-07-2023	7°40'56.59"S	113°51'11.79"E	10-20
5	Tanjung Pecaron	Shallow reef flats	15-07-2023	7°40'52.03"S	113°51'54.19"E	5-18

Morphological identification

Preserved echinoderm samples were photographed using a Leica Stereo Microscope at the Marine Ecology Laboratory, Korea Maritime and Ocean University, to observe detailed external features. Samples were identified based on characteristics specific to each class: namely, disk, arm plates, arm spines, and oral shields in asteroids, ophiuroids, and crinoids; spines and tentacles in holothuroids; spines and test form in echinoids; and coloration. These identifications were conducted based on original descriptions, identification guidebooks, and recent relevant references (Clark and Rowe 1971; Hoggett 1991; Colin and Arneson 1995; VandenSpiegel et al. 1998; Stöhr 2012; Fujita 2016; Wirawati et al. 2019; Virgili et al. 2020; Goharimanesh et al. 2021; Arbi et al. 2022). The scientific names were confirmed as listed in the World Register of Marine Species (WoRMS 2024). Afterwards, samples were stored in 95-99% ethanol and maintained at -80°C until DNA extraction procedures were initiated (Layton et al. 2016).

Molecular identification

DNA data collection

Genomic DNA was extracted from Ca. 2 mg of 71 echinoderm individuals following the HiGene Genomic DNA prep kit (Biofact, Daejeon, Korea). The COI gene was amplified using the primer pair COIceF (5'-ACTGCCCACGCCCTAGTAATGATATTTTTTATGGTNATGCC-3') and COIceR (5'-TCGTGTGTCTACGTCCATTCCTACTGTRAACATRTG-3'), while the 16S gene was amplified using the primer pair 16sr (5'-ACGTAGATAGAAACTGACCTG-3') and 16sb (5'-GACGAGAAGACCCTGTGGAGC-3'). The Polymerase Chain Reaction (PCR) mixtures were composed of 25 µL volume containing 2 µL of genomic DNA, 12.5 µL BioFACT™ H Star Taq Master mix 2 buffer, 2 µL Primer_F/R Mixture (10 pmol/µL), and 8.5 µL distilled water. The PCR thermocycling to amplify the COI gene started with pre-denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 45 sec, 40 cycles of annealing at 51°C for 1 min 10 sec, and 40 cycles of extension at 72°C for 1 min 20 sec. The amplification ended with a final extension at 72°C for 5 min. The PCR condition for the 16S gene was pre-denaturation at 95°C for 15 min, 35 cycles of denaturation at 94°C for 45 sec, 35 cycles of annealing at 48°C for 40 sec, 35 cycles of extension at 72°C for 1 min, concluding with 1 cycle of extension at 72°C for 5 min. Afterwards, amplified PCR products were detected using agarose gel electrophoresis under UV light by staining with EcoDye™ DNA Staining Solution (Biofact). The sequencing of all PCR products was performed using a 3730xl DNA Analyzer at the Biofact sequencing facility.

DNA sequence and phylogenetic analyses

The sequences of the minimum 650 base pairs (COI) and 325 base pairs (16S) obtained in the study were submitted to the Basic Local Alignment Search Tool (BLAST) to find the best-matched species in the GenBank database. The COI sequences from this study (n = 47) and those best matched from the GenBank dataset (n = 45) were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm. Phylogenetic trees based on COI genes were constructed for each class, using the Maximum-Likelihood (ML) algorithm. Models for the analyses were selected based on the best fit according to the lowest Bayesian Information Criterion (BIC) value. Phylogenetic trees for Asteroidea, Crinoidea, and Ophiuroidea were constructed using the General Time Reversible (GTR) model with gamma distribution and invariant sites (G+I). For Echinoidea, the GTR model with invariant sites (I) was applied, while the Holothuroidea phylogeny was inferred using the Tamura-Nei model with gamma distribution and invariant sites (G+I). Bootstrap analyses were performed with 1000 replicates. Additionally, Kimura-2-Parameter (K2P) distances were calculated (Kimura 1980). All of the sequences and phylogenetic analyses were conducted using the Molecular Evolutionary Genetics Analysis (MEGA) version 11 (Tamura et al. 2021). Furthermore, a total of 37 COI (accession numbers: PP735393-PP735440) and 38 16S (accession numbers: PP784917-PP784947, PP812285-PP812292) sequences from this study were deposited into GenBank (Table 2).

RESULTS AND DISCUSSION

A total of 45 echinoderms were discovered in the Situbondo coral reefs, comprising six Asteroidea, five Crinoidea, eight Echinoidea, 15 Holothuroidea, and 11 Ophiuroidea (Table 2). Most of the samples were identified at the species level, while five of these were identified at the genus level. Among sites, species richness in the intertidal reef sites (sites 2 and 3) was relatively higher than in the shallow reef sites (sites 1, 4, and 5; Figure 2). Furthermore, the present study observed notable differences in the composition of echinoderm classes across different sites. Asteroids (starfish) and ophiuroids (brittle stars) demonstrated relatively high species diversity in intertidal reefs (sites 2 and 3). Relatively common species among these were the starfish *Culcita novaeguineae* and the brittle stars *Ophiolepis superba* and *Ophiocoma schoenleinii* (Table 2). In contrast, crinoids (feather stars) were exclusively found in shallow reefs (sites 1, 4, and 5) (Table 2).

Table 2. Echinodermata of Situbondo coral reefs

Species	Occurrence					GenBank accession number	
	1	2	3	4	5	COI	16S
Asteroidea							
Valvatida, Asterinidae							
Aquilonastra cepheus (Muller & Troschel, 1842)			+			PP735409*	PP784917*
Valvatida, Asteropseidae							
Asteropsis carinifera (Lamarck, 1816)		+	+			PP735410-11	PP784918-19
Valvatida, Oreasteridae							
Culcita novaeguineae (Müller & Troschel, 1842)	+	+		+	+	PP735412-13	PP784920-21
Valvatida, Ophidiasteridae							
Linckia laevigata (Linnaeus, 1758)	+	+				PP735414	-
Leiaster leachi (Gray, 1840)		+				-	-
Ophidiaster granifer (Lütken, 1871)		+				PP735415	-
Crinoidea							
Comatulida, Comatulidae							
Capillaster multiradiatus (Linnaeus, 1758)				+	+	PP735417	PP784923
Comanthus sp.					+	-	-
Comatulida, Colobometridae							
Cenometra bella (Hartlaub, 1890)				+	+	PP735416	PP784922
Colobometra perspinosa (Carpenter, 1881)	+			+	+	PP735418-20	PP784924-26
Comatulida, Mariametridae							
Dichrometra brachypecha (H.L. Clark, 1915)					+	PP735421	PP784927
Echinoidea							
Clypeasteroida, Clypeasteridae							
Arachnoides placenta (Linnaeus, 1758)		+	+			PP735422	PP784928
Diadematoida, Diadematidae							
Diadema setosum (Leske, 1778)	+	+	+	+	+	PP735423-25	PP784929-31
Diadema clarki (Ikeda, 1939)				+		PP735426	PP784932
Echinothrix calamaris (Pallas, 1774)				+	+	-	-
Camarodonta, Parasaleniidae							
Parasalenia gratiosa (A. Agassiz, 1863)		+				PP735427*	PP784933*
Camarodonta, Echinometridae							
Echinometra sp. C**		+		+		PP735428	PP784934*
Camarodonta, Temnopleuridae							
Mespilia globulus (Linnaeus, 1758)				+		PP735429	PP784935
Cidaroida, Cidaridae							
Plococidaris verticillata (Lamarck, 1816)	+					PP735430	PP784936
Holothuroidea							
Apodida, Synaptidae							
Euapta sp.				+		PP735431	-
Opheodesoma grisea (Semper, 1867)			+			PP735437	-
Synapta sp.	+					PP735440	-
Synaptula lamperti (Heding, 1928)	+			+	+	-	-
Apodida, Chiridotidae							
Polycheira sp.						-	-
Holothuriida, Holothuriidae							
Holothuria (Selenkothuria) moebii (Ludwig, 1883)			+			-	-
Holothuria (Mertensiothuria) hilla (Lesson, 1830)		+			+	PP735432	PP784938
Holothuria (Stauropora) fuscocinerea (Jaeger, 1833)	+		+			-	PP784937
Holothuria (Thymiosycia) impatiens (Forsskål, 1775)		+	+			PP735433	-
Holothuria leucospilota (Brandt, 1835)		+				PP735434	PP784939
Holothuria (Lessonothuria) pardalis (Selenka, 1867)			+			-	PP784940
Holothuria sp.					+	PP735435	PP784941
Labidodemas semperianum (Selenka, 1867)	+					PP735436*	PP784942
Synallactida, Stichopodidae							
Stichopus monotuberculatus (Quoy & Gaimard, 1834)		+	+		+	PP735439	PP784943,45,46
Stichopus ocellatus (Massin, Zulfigar, Hwai & Boss, 2002)				+		-	PP784947
Ophiuroidea							
Amphilepidida, Ophiotrichidae							
Macrophiothrix lorioli (A.M. Clark, 1968)	+	+				PP735393	-
Macrophiothrix koehleri (A.M. Clark, 1968)			+			PP735394	-
Ophiacanthida, Ophiocomidae							
Ophiomastix annulosa (Lamarck, 1816)		+	+			PP735395	PP812285*
Ophiomastix elegans (Peters, 1851)			+			PP735396	-

Ophiomastix pictum (Müller & Troschel, 1842)		+	+			PP735402*	PP812290
Ophiomastix flaccida (Lyman, 1874)			+			-	-
Ophiocoma schoenleinii (Müller & Troschel, 1842)	+	+			+	PP735407-08	PP812291-92*
Ophiacanthida, Ophiodermatidae							
Ophiarachnella gorgonia (Müller & Troschel, 1842)	+					PP735397	PP812286*
Ophiacanthida, Ophiacanthida incertae sedis*							
Ophiodyscrita instratus (Murakami, 1944)	+					PP735401*	PP812289*
Amphilepidida, Hemieuryalidae							
Ophioplocus imbricatus (Müller & Troschel, 1842)			+			PP735398-400	PP812287-88*
Amphilepidida, Ophiolepididae							
Ophiolepis superba (H.L. Clark, 1915)	+	+	+	+		PP735403-06	-
Total species per site	14	18	17	13	13		

Note: *First reported sequences of the species to GenBank, **Temporary name: An as-yet-unnamed cryptic species of Echinometra

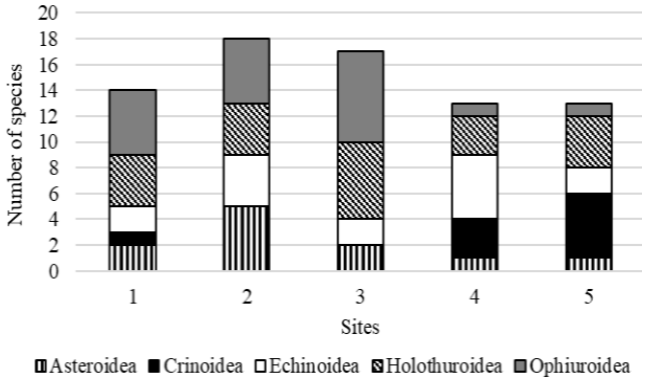

Figure 2. Number of Echinodermata species across different sites within the coral reefs of Situbondo, East Java, Indonesia

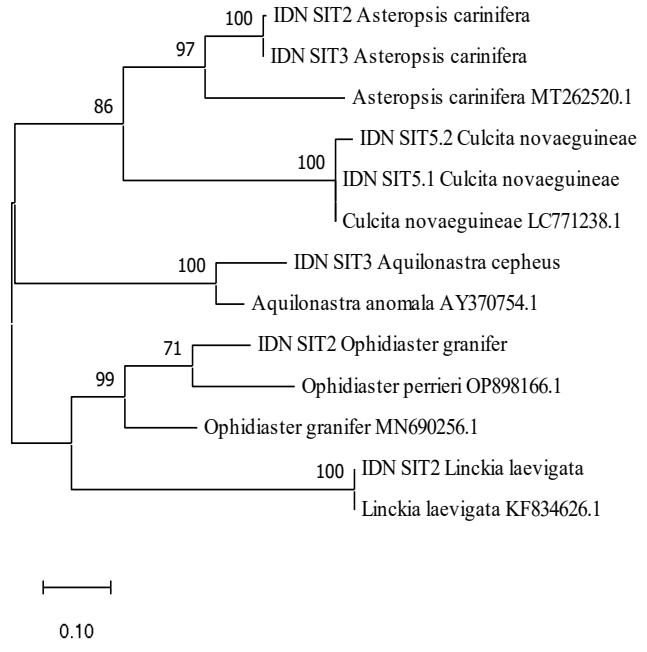

Figure 3. Phylogenetic tree based on maximum likelihood analysis of the COI sequences of Asteroidea from this study (n = 7) and the best-fit sequences available from GenBank (n = 6). The tree is drawn to scale, with branch lengths indicating the number of substitutions per site and bootstrap values exceeding 70% displayed at the corresponding branches (1000 replicates). Evolutionary analyses were conducted in MEGA11

Species identification using DNA barcoding

Of the total samples processed, 47 COI sequences were successfully amplified, corresponding to a success rate of 66%, while 38 sequences of the 16S rRNA gene were obtained, yielding a success rate of 53%. The remaining samples showed either weak amplification or complete amplification failure. Notably, the COI sequences of five species from this study (*Aquilonastra cepheus*, *Parasalenia gratiosa*, *Labidodemas semperianum*, *Ophiomastix pictum*, *Ophiodyscrita instratus*) were reported in GenBank for the first time. For the 16S genes, eight sequences were newly reported, including *A. cepheus*, *P. gratiosa*, *Echinometra* sp. C, *O. annulosa*, *O. schoenleinii*, *O. gorgonia*, *O. instratus*, and *O. imbricatus*. The overall phylogenetic analysis across all classes showed consistent results between morphological and molecular identification, with 64% of successfully amplified 16S sequences and 79% of COI sequences matching reference sequences in GenBank. However, ambiguous placements or unresolved topologies were observed in Asteroidea and Ophiuroidea. Therefore, only the phylogenetic trees for these two classes are presented and discussed in detail.

In the class Asteroidea, among six assigned morphospecies, the COI gene was amplified in five species, and the 16S gene in three species (Table 2). However, no amplification occurred for either COI or 16S genes in *Leiaster leachi*. The K2P distance of 13 COI sequences (494-645 base pairs) was analyzed, comprising seven sequences from this study and six from GenBank. The interspecific divergence values ranged from 0.088 to 0.295, with most values exceeding the commonly reported species delimitation threshold (e.g., 2-3%). The pairwise genetic distances based on the Kimura 2-parameter model (Table 3), revealed low intraspecific divergence values, particularly in *C. novaeguineae* (0.003 and 0.025) and *L. laevigata* (0.000). These low divergence values indicate high sequence similarity among conspecific individuals of our samples and reference sequences. In contrast, *A. carinifera* and *O. granifer* showed relatively high genetic divergence from known sequences (Figure 3), with K2P distances of 0.166-0.167 and 0.164, respectively (Table 3). Notably, the divergence between *A. cepheus* and *A. anomala* was 0.088, suggesting a close taxonomic relationship within the genus.

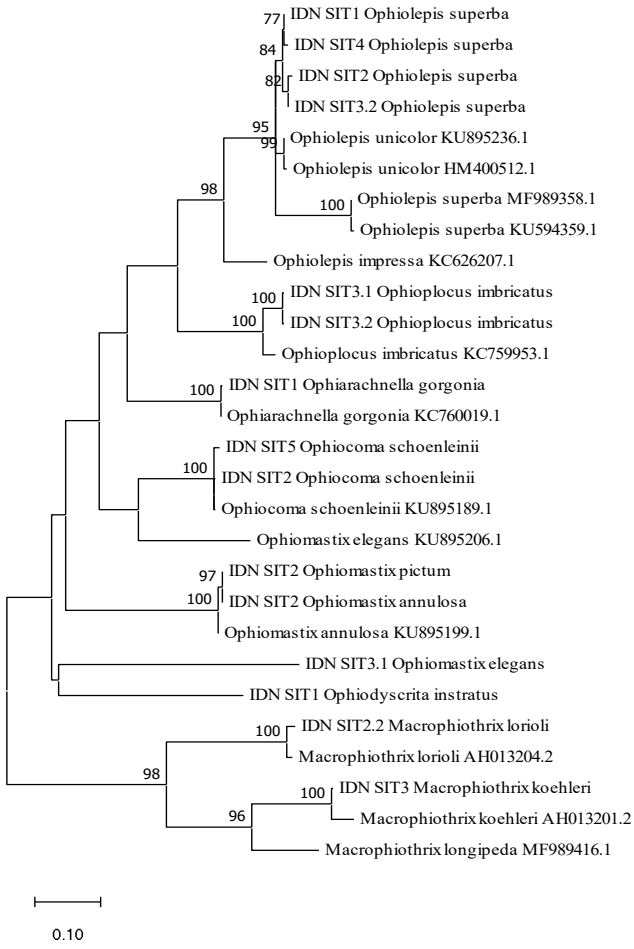

Figure 4. Phylogenetic tree based on maximum likelihood analysis of the COI sequences of Ophiuroidea from this study (n = 15) and the best-fit sequences available from GenBank (n = 13). Branch lengths in the scaled phylogenetic tree represent the number of substitutions per site, with bootstrap values above 70% shown at the respective nodes (based on 1000 replicates). Evolutionary analysis was performed using MEGA version 11

For Ophiuroidea, COI sequences were successfully amplified in ten out of 11 identified species, while 16S sequences were obtained for six species, five of which are reported to GenBank for the first time (Table 2). Maximum likelihood analysis of the COI sequences showed that six morphospecies aligned with their respective reference species (Figure 4). These species include *O. imbricatus*, *O. schoenleinii*, *O. annulosa*, *M. lorioli*, and *M. koehleri*, with intraspecific divergence ranging from 0.005-0.051 (Table 4). Although the average of interspecific divergence based on K2P distance was 0.235, incongruencies were observed in *O. superba*, which grouped more closely with *O. unicolor* (K2P distances: 0.018-0.034 vs. 0.084-0.093; Table 4). Additionally, two species *O. pictum* and *O. instratus* were submitted to GenBank for the first time, with *O. instratus* showing no close match in the available sequences.

In contrast to the phylogenetic uncertainties observed in Asteroidea and Ophiuroidea, the remaining echinoderm classes, Crinoidea and Echinoidea, exhibited clear genetic clustering and strong concordance between molecular and morphological identifications. In Crinoidea, the COI and 16S genes were successfully amplified in four out of five morphospecies. Analysis of COI sequences (633-669 bp) revealed low pairwise genetic divergence (K2P: 0-0.026), supporting reliable species-level identification. However, one morphospecies (*Comanthus* sp.) failed to yield amplifiable DNA. In Echinoidea, both mitochondrial markers were successfully amplified for all seven morphospecies. COI-based analysis delineated distinct clades corresponding to each species, with minimal intraspecific divergence (K2P: 0-0.015). Notably, molecular data confirmed the identities of two previously genus-level designations: *Diadema* sp. as *D. clarki* and *Echinometra* sp. as *Echinometra* sp. *C*, a recognized lineage within the *E. mathaei* species complex. In the case of the sea urchin *P. gratiosa*, both COI and 16S sequences were reported to GenBank for the first time.

In Holothuroidea, COI and 16S genes were successfully amplified in nine and eight out of 15 morphologically assigned species, respectively (Table 2). Five species matched their respective GenBank references, while three (*Holothuria* sp., *Euapta* sp., and *Synapta* sp.) could only be resolved to the genus level. The COI sequence of *L. semperianum* was also newly submitted to GenBank. Overall, intraspecific genetic divergence was low (K2P: 0.001-0.015), except within the Synaptidae clade, which exhibited notably higher variability (K2P: 0.031-0.183), suggesting greater genetic differentiation within this group.

Discussion

This study presents the first molecular-based assessment of echinoderm diversity in the coral reefs off the northern coast of Situbondo, East Java. Furthermore, in contrast to earlier studies that focused on commercial sea cucumbers (Setyastuti et al. 2014) or listed only a few sea urchin species (Somma et al. 2017), our findings reveal a broader taxonomic spectrum, underscoring the ecological importance of this region. Among the echinoderm groups observed, holothuroids exhibited particularly high species richness, and their dominance of holothuroids in our survey is particularly noteworthy given that Situbondo's coastal waters serve as one of the source locations for the sea cucumber trade in Indonesia (Setyastuti and Purwati 2015). Interestingly, none of the commercially important species previously recorded by Setyastuti et al. (2014) —*Actinopyga lecanora* (Jaeger, 1833), *Holothuria excellens* (Ludwig, 1875), *H. scabra* (Jaeger, 1833), *H. turriscelsa* (Cherbonnier, 1980), *Stichopus vastus* (Sluiter, 1887), *S. quadrifasciatus* (Massin, 1999), and *S. noctivagus* (Cherbonnier, 1980))—were encountered in our study, where all holothuroid species differ from those previously reported. This discrepancy suggests an increase in species richness but also raises questions about the absence of these species from our survey. Possible explanations for this absence include variations in sampling effort, variations in depth and timing of sample collection, and changes in local environmental conditions, such as a decline in abundance or potential local extinction (Petović and Krpo-Ćetković 2016; Prescott et al. 2017; Wolfe and Byrne 2022; Kalidi et al. 2023; Sobczyk et al. 2023; Pérez-Lloréns and Mouritsen 2024; Prata and Christoffersen 2024).

Table 3. Pairwise distances between 13 sequences of Asteroidea from this study and GenBank based on the Kimura 2-parameter model with 1000 bootstrap replicates

Sample	1	2	3	4	5	6	7	8	9	10	11	12
1. IDN_SIT3_Aquilonastra_cepheus												
2. IDN_SIT2_Asteropsis_carinifera	0.216											
3. IDN_SIT3_Asteropsis_carinifera	0.220	0.003										
4. IDN_SIT5.1_Culcita_novaeguineae	0.229	0.212	0.212									
5. IDN_SIT5.2_Culcita_novaeguineae	0.236	0.212	0.212	0.025								
6. IDN_SIT2_Linckia_laevigata	0.252	0.244	0.246	0.238	0.249							
7. IDN_SIT2_Ophidiaster_granifer	0.252	0.253	0.253	0.227	0.227	0.256						
8. Aquilonastra_anomala_AY370754.1	0.088	0.222	0.224	0.218	0.227	0.255	0.251					
9. Asteropsis_carinifera_MT262520.1	0.235	0.168	0.166	0.208	0.212	0.257	0.283	0.229				
10. Culcita_novaeguineae_LC771238.1	0.229	0.210	0.210	0.003	0.025	0.236	0.225	0.218	0.208			
11. Linckia_laevigata_KF834626.1	0.252	0.252	0.255	0.244	0.256	0.000	0.261	0.256	0.261	0.242		
12. Ophidiaster_perrieri_OP898166.1	0.255	0.233	0.231	0.242	0.242	0.248	0.152	0.239	0.261	0.242	0.256	
13. Ophidiaster_granifer_MN690256.1	0.242	0.240	0.237	0.265	0.271	0.235	0.164	0.228	0.295	0.265	0.244	0.188

Table 4. Pairwise distances between 28 sequences of Ophiuroidea from this study and GenBank based on the Kimura 2-parameter model with 1000 bootstrap replicates

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1. IDN_SIT2.2_Macrophiothrix_lorioli																											
2. IDN_SIT3_Macrophiothrix_koehleri	0.218																										
3. IDN_SIT2_Ophiomastix_annulosa	0.287	0.289																									
4. IDN_SIT3.1_Ophiomastix_elegans	0.292	0.296	0.267																								
5. IDN_SIT1_Ophiarachnella_gorgonia	0.271	0.275	0.205	0.312																							
6. IDN_SIT3.1_Ophioplocus_imbricatus	0.267	0.282	0.206	0.332	0.172																						
7. IDN_SIT3.2_Ophioplocus_imbricatus	0.264	0.278	0.204	0.327	0.168	0.003																					
8. IDN_SIT1_Ophiodyscrita_instratus	0.285	0.274	0.277	0.255	0.226	0.237	0.233																				
9. IDN_SIT2_Ophiomastix_pictum	0.287	0.289	0.000	0.267	0.205	0.206	0.204	0.277																			
10. IDN_SIT1_Ophiolepis_superba	0.300	0.322	0.233	0.326	0.203	0.199	0.199	0.251	0.233																		
11. IDN_SIT2_Ophiolepis_superba	0.300	0.305	0.233	0.311	0.199	0.206	0.206	0.251	0.233	0.020																	
12. IDN_SIT3.2_Ophiolepis_superba	0.303	0.310	0.233	0.311	0.199	0.204	0.204	0.253	0.233	0.016	0.005																
13. IDN_SIT4_Ophiolepis_superba	0.298	0.312	0.226	0.321	0.207	0.204	0.204	0.251	0.226	0.012	0.014	0.016															
14. IDN_SIT2_Ophiocoma_schoenleinii	0.261	0.299	0.214	0.278	0.197	0.210	0.210	0.259	0.214	0.202	0.209	0.206	0.206														
15. IDN_SIT5_Ophiocoma_schoenleinii	0.266	0.300	0.213	0.275	0.196	0.215	0.210	0.249	0.213	0.203	0.209	0.207	0.207	0.012													
16. Macrophiothrix_koehleri_AH013201.2	0.265	0.034	0.297	0.318	0.287	0.304	0.297	0.281	0.297	0.300	0.293	0.293	0.290	0.297	0.300												
17. Macrophiothrix_longipeda_MF989416.1	0.224	0.159	0.293	0.286	0.277	0.278	0.273	0.297	0.293	0.287	0.284	0.280	0.287	0.280	0.273	0.177											
18. Macrophiothrix_lorioli_AH013204.2	0.021	0.242	0.307	0.318	0.256	0.266	0.263	0.293	0.307	0.307	0.300	0.303	0.310	0.263	0.270	0.265	0.231										
19. Ophiomastix_elegans_KU895206.1	0.275	0.310	0.193	0.280	0.210	0.202	0.200	0.256	0.193	0.208	0.223	0.223	0.208	0.190	0.194	0.334	0.294	0.296									
20. Ophiarachnella_gorgonia_KC760019.1	0.280	0.282	0.212	0.317	0.008	0.182	0.178	0.235	0.212	0.210	0.205	0.205	0.214	0.197	0.196	0.284	0.289	0.260	0.216								
21. Ophioplocus_imbricatus_KC759953.1	0.279	0.284	0.203	0.319	0.188	0.051	0.051	0.239	0.203	0.192	0.192	0.188	0.196	0.211	0.218	0.300	0.285	0.287	0.199	0.190							
22. Ophiomastix_annulosa_KU895199.1	0.286	0.289	0.010	0.268	0.216	0.201	0.199	0.276	0.010	0.223	0.230	0.230	0.223	0.212	0.211	0.294	0.291	0.303	0.200	0.223	0.203						
23. Ophiolepis_unicolor_KU895236.1	0.298	0.318	0.231	0.316	0.202	0.204	0.204	0.247	0.231	0.025	0.020	0.022	0.019	0.209	0.210	0.304	0.292	0.310	0.214	0.208	0.190	0.228					
24. Ophiolepis_unicolor_HM400512.1	0.301	0.314	0.257	0.311	0.211	0.206	0.206	0.243	0.257	0.034	0.028	0.030	0.025	0.209	0.210	0.308	0.291	0.318	0.223	0.214	0.190	0.259	0.004				
25. Ophiolepis_superba_MF989358.1	0.296	0.313	0.235	0.305	0.220	0.204	0.204	0.247	0.235	0.084	0.092	0.092	0.086	0.219	0.222	0.294	0.285	0.310	0.235	0.227	0.209	0.232	0.090	0.097			
26. Ophiolepis_superba_KU594359.1	0.298	0.316	0.239	0.305	0.227	0.208	0.208	0.252	0.239	0.090	0.093	0.093	0.088	0.217	0.219	0.294	0.287	0.310	0.238	0.233	0.214	0.238	0.088	0.097	0.005		
27. Ophiolepis_impressa_KC626207.1	0.283	0.293	0.247	0.318	0.179	0.186	0.186	0.266	0.247	0.119	0.131	0.129	0.125	0.224	0.229	0.289	0.304	0.286	0.212	0.186	0.179	0.247	0.123	0.123	0.176	0.183	
28. Ophiocoma_schoenleinii_KU895189.1	0.261	0.303	0.210	0.278	0.193	0.204	0.204	0.256	0.210	0.196	0.202	0.200	0.200	0.005	0.011	0.304	0.275	0.270	0.190	0.193	0.207	0.208	0.203	0.201	0.213	0.211	0.224

Beyond holothuroids, this study reveals previously undocumented diversity of ophiuroids, echinoids, and asteroids in the Situbondo reef ecosystem. Notably, several of these groups showed greater species richness than has previously been reported in adjacent areas, such as in Taman Nasional Baluran, the eastern coast of Situbondo (Huda et al. 2017; Setiawan et al. 2019). When compared to other regions of similar spatial scope, such as Kri in Raja Ampat (Siburian et al. 2023) and the southern part of Lombok (Bahri et al. 2021), overall species richness in Situbondo was higher. While asteroid richness was relatively comparable, fewer holothuroid and echinoid species were recorded in Kri (Siburian et al. 2023). A review of previous studies also shows that only 29 echinoderm species have been documented in Karimunjawa and the northern part of Central Java (Aulia et al. 2024). In contrast, surveys conducted over broader areas reported higher species richness, with 74 species in Kepulauan Seribu (Aulia et al. 2024), and 76 species in North Sulawesi (Supono et al. 2014). Furthermore, although crinoids exhibited the lowest richness among all classes in this study (five species), their presence is noteworthy, as there has been no prior record of this group from Situbondo or the northern coast of East Java (Aulia et al. 2024). For comparison, eight crinoid species have been reported from Kri, Raja Ampat (Siburian et al. 2023), while more focused studies on crinoids from Bangka Island documented as many as 39 species (Virgili et al. 2020) and 31 species from Ambon and Lombok (Kogo et al. 2019). Given the scarcity of studies addressing echinoderm diversity in East Java, despite it having the largest coral reef area in Java (Arbi et al. 2022; Aulia et al. 2024), our findings fill a critical knowledge gap and emphasize the need for continued biodiversity monitoring and habitat-specific assessments in Indonesian reef ecosystems.

Molecular approaches are essential in addressing the challenges associated with morphological identification, which often involves distinguishing between species with varied and overlapping characteristics (Ward et al. 2008; Thuy and Stöhr 2016; Laakmann et al. 2016; Foo et al. 2021; Chacón-Monge et al. 2024). Advances have been made in this area, especially for commercially important sea cucumbers (Patantis et al. 2019). Sulardiono et al. (2022) further revealed that three locally named sea cucumbers from Karimunjawa, Central Java, were genetically identified as a single species, *S. monotuberculatus*. In the Philippines, Alcudia-Catalma et al. (2020) also showed the utility of DNA barcoding in resolving taxonomic ambiguities among key sea cucumber species. Similarly, our study clarified that brittle stars initially identified as a single species of *Macrophiothrix* in fact represent two distinct species, *M. lorioli* and *M. koehleri*. In contrast, for other echinoderm groups in Indonesia, molecular efforts remain limited. A study on environmental DNA (eDNA) of Indonesian marine biota by Madduppa et al. (2021) highlighted the high proportions of unidentified echinoderm taxa, underscoring the need for more comprehensive molecular analyses. In other regions, molecular approaches have been applied to broadly document echinoderm diversity, though suggested as complementary to morphological identification (Laakmann et al. 2016; Nethupul et al. 2022; Sonet et al. 2022; Chacón-Monge et al. 2024). Collectively, these findings highlight the importance of expanding sequence databases and advancing integrative taxonomic frameworks that aligns traditional taxonomy with the growing relevance of DNA-based research. Contributing to this effort, our study provides new COI and 16S rRNA gene sequences for several species, some of which are reported in GenBank for the first time.

Beyond species-level identification, molecular data also provide valuable insights into population genetics and biogeographic distributions (McGaughran 2015; Casillas and Barbadilla 2017; Zheng et al. 2022; Rodríguez-Barreras 2024). Previous studies have investigated the genetic structure of echinoderm populations across the Indo-Malay Archipelago (Coppard et al. 2021; Vimono et al. 2023), highlighting the importance of molecular approaches in understanding genetic connectivity and evolutionary relationships among echinoderm populations. Notably, studies of sea urchin diversity in the Coral Triangle have revealed the presence of *Diadema clarki* in Sulawesi, the eastern part of Indonesia (Moore et al. 2019), which was previously restricted to Japan (Chow et al. 2016). Our findings similarly confirmed the presence of *D. clarki* (Figure 5), with genetic evidence (K2P distances: 0-0.011) supporting its presence and suggesting a broader distribution range than previously supposed. These findings underscore the critical role of molecular techniques in advancing our understanding of echinoderm diversity and biogeography in Indonesia. In line with this, as marine biodiversity research advances, many species now considered endemic may be found elsewhere, potentially lowering endemicity rates (Costello et al. 2017).

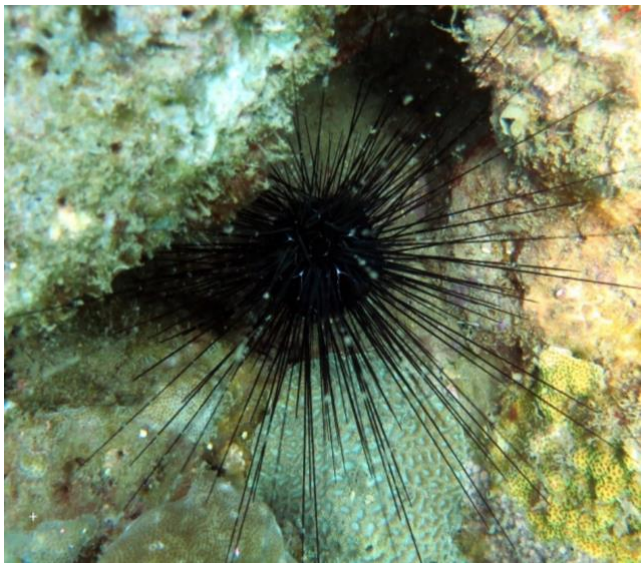

Figure 5. *Diadema clarki* in Situbondo coral reef

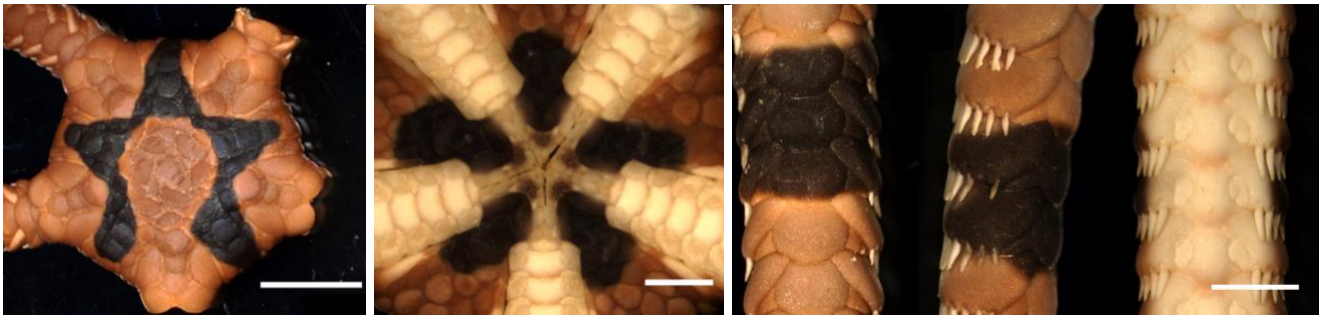

Figure 6. *Ophiolepis superba* from Situbondo coral reef: A. Dorsal disk surface, B. Ventral disk surface, C. Dorsal arm plates, D. Lateral arm plates, and E. Ventral arm plates. Scale bars a, 5 mm; b-e, 2 mm

Despite the molecular insights, challenges in species delimitation persist due to the historical reliance on phenotypic and morphological traits (Colin and Arneson 1995; Pawson 2007; Stöhr et al. 2012). Sequence divergences based on the COI gene vary widely across taxa (Hebert et al. 2003b), with an average divergence of 6.8% among invertebrates (Hebert et al. 2003a). However, studies focusing on complete COI sequences in Asteroidea have reported even higher divergences, sometimes exceeding 20% at the genus level (Yasuda et al. 2006; Lopes et al. 2016). In our study, COI-based comparisons with GenBank references revealed high K2P distances (up to ~16%) between two morphologically assigned sea star species (*A. carinifera* and *O. granifer*), indicating ambiguity in the divergence thresholds used for species delimitation. Similar patterns have been reported in other echinoderms; morphologically similar or variable species with high genetic divergence have been observed such as in starfishes (*Luidia* and *Freyastera*), sea urchins (*Echinometra*), and brittle stars (*Ophiolepis*), which may represent unrecognized or undescribed species, or reflect past taxonomic confusion (Hoareau et al. 2013; McClanahan and Muthiga 2020; Shilling et al. 2022; Zhang et al. 2025). Although our result suggest possible cryptic diversity or taxonomic inconsistencies, the limited number of samples necessitates further investigation that ideally incorporating additional molecular markers, broader geographic sampling, and detailed morphological re-evaluation to clarify species boundaries.

A similarly complex case was encountered in the brittle stars *O. superba* and *O. unicolor*. Initial morphological identification based on coloration and patterning (Figure 6) matched published descriptions of *O. superba*, distinguished by its deep buff color with a well-defined deep purple star-like pattern and banded arms (Pineda-Enríquez et al. 2018). However, closer examination showed that our samples had an arm spine range of 4-6, which partially overlaps with the ranges reported for *O. superba* (6-7) and *O. unicolor* (5-7) (Pineda-Enríquez et al. 2018). Given this overlap, the arm spine counts in our samples are somewhat intermediate but align more closely with *O. unicolor*, particularly considering other morphological and molecular evidence. This discrepancy suggests that previous morphological diagnoses of *O. superba* may not fully reflect its intraspecific variability or that these two species may form part of an unresolved complex. Given that *O. unicolor* has predominantly been recorded in northwestern Australia, further studies with larger sample sizes and broader geographical coverage are necessary to resolve this potential species complex issue.

In conclusion, this study provides a comprehensive assessment of echinoderm diversity in the coral reefs of Situbondo, East Java, through integrated morphological and molecular approaches, offering valuable baseline data for future biodiversity research and monitoring. The addition of new COI and 16S gene sequences to GenBank represents an important step in addressing the molecular data gap for Indonesian echinoderms. While the findings demonstrate the utility of molecular tools in resolving species boundaries and uncovering overlooked diversity, several cases also foreground ongoing challenges in species delimitation. These ambiguities, particularly in taxa such as asteroids and ophiuroids, emphasize the importance of integrative taxonomic frameworks. Finally, the study's limited sample size and geographic coverage highlight the need for further research to better understand echinoderm diversity, genetic connectivity, and evolutionary history in this understudied region.

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