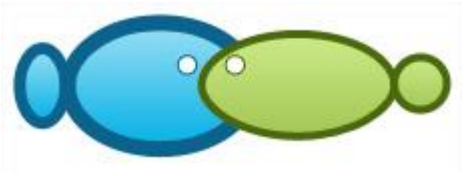

Species identification of echinoderms from Gili Ketapang Island by combining morphology and molecular data

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Abstract. Echinoderms are marine water invertebrates with diverse morphologies and grouped into five classes, but there are currently limited reports on the species from Gili Ketapang Island, East Java. Therefore, this study aims to identify these species, based on their morphologic and molecular characteristics and to determine the phylogenetics of echinoderms from Gili Ketapang Island. DNA barcodes, based on fragments of the 5' end of the cytochrome c oxidase subunit I gene (COI), have been used to support the morphological examination for the echinoderms' evolutionary lineages determination. A total of 16 echinoderm samples were collected and identified based on morphological characteristics. Subsequently, molecular characterization and identification were conducted using 503 bp of COI, by considering the gene similarity, sequence variation, genetic distance, phylogenetic topology, based on the Barcode of Life Data (BOLD) System. According to the results, the samples were identified as *Diadema setosum*, *Macrophiothrix longipeda*, *Archaster typicus*, *Echinometra mathaei*, *Holothuria atra*, *Linckia laevigata*, *Bohadschia argus*, and *Ophiactis savignyi*. Each species was associated with a particular DNA barcode cluster and the relationship among all was revealed. Automatic Barcode Gap Discovery (ABGD) analysis showed that the analyzed echinoderms were divided into eight species, and these new records established a sequences reference library for the Island.
Key Words: species identification, echinoderms, morphology, COI, phylogenetic.

Introduction. Echinoderms are marine water invertebrates with over 7000 extant species, 22 of which are grouped into five classes and have a wide range of morphologies (Laakmann et al 2017; Ward et al 2008). They can be found in habitats ranging from the deep sea to shallow intertidal areas, and in all climate zones. The phylum comprises five recent classes, namely Asteroidea (starfish or sea stars), Crinoidea (sea lilies & feather stars), Echinoidea (sea urchins), Holothuroidea (sea cucumbers), and Ophiuroidea (brittle & basket stars) with around 1,745;580; 900; 1,430 and 2,300 species, respectively (Fell 1962; Pawson 1966; Pawson 2007). Echinoderms play an important physical and biological role in the structure of marine benthic ecosystems in many regions (Layton et al 2016).

Echinoderms are also present in Indonesian coastal waters, and despite being abundant in Gili Ketapang Island, Probolinggo, there is no current database record of the species in this area. The first step in this study was to identify the species morphologically, with molecular reinforcement using DNA barcoding (Sonet et al 2022; Hubert et al 2008; Hubert et al 2019; Steinke et al 2009; Buhay 2009; Anzani et al 2019). Taxonomic uncertainties in morphological identification must be solved. However, the result needs to be confirmed through molecular identification, due to insufficient data on this taxon.

To support the morphology characterization and circumvent the taxonomic impediment, a standard molecular framework is proposed for species identification using the mitochondrial COI gene. DNA barcoding opened new perspectives in the inventory of echinoderms (Bribiesca-Contreras et al 2013; Laakmann et al 2017; Layton et al 2016; Sonet et al 2022; Sulardiono et al 2022; Ward et al 2008), and it can be used as a fast and rapid global bioscanner. Accurate identification of organisms is carried out based on partial COI gene sequence. However, the success of species identification using DNA barcoding is dependent on the accuracy of data in DNA barcode libraries, specifically GenBank and the BOLD System (Nur et al 2022), and of the ABGD analysis (Seth & Barik 2021). DNA barcoding clearly distinguishes the echinoderms species from barcodes in Australia, Canada, India, Thailand, Germany, and Indonesia (Ward et al 2008; Hubert et al 2008).

This is the first scientific study to confirm the molecular identification of echinoderms found in Gili Ketapang Island, based on COI DNA Barcode. The genetic information of echinoderms at the molecular level provides information for future genetic studies of populations and supports the conservation efforts, to maintain the local species' sustainability. Meanwhile, the information on genetic diversity is useful for increasing the biodiversity of Indonesian populations and promoting the genetic diversity. This study identifies the echinoderms species in East Java, a region which is known for having an exceptional marine diversity. Therefore, this region is of a remarkable importance for the completion of species' DNA barcode in the online databases available in the country.

Material and Method

Description of the study sites. According to Figure 1, echinoderms were obtained from Gili Ketapang Island, Sumberasih District, Probolinggo Regency, East Java, using a purposive sampling with a quadratic transect method. The samples were then brought to the laboratory and some of the dominant species were stored in 96% ethanol. The sampling at each station was carried out twice using the "square transect method".

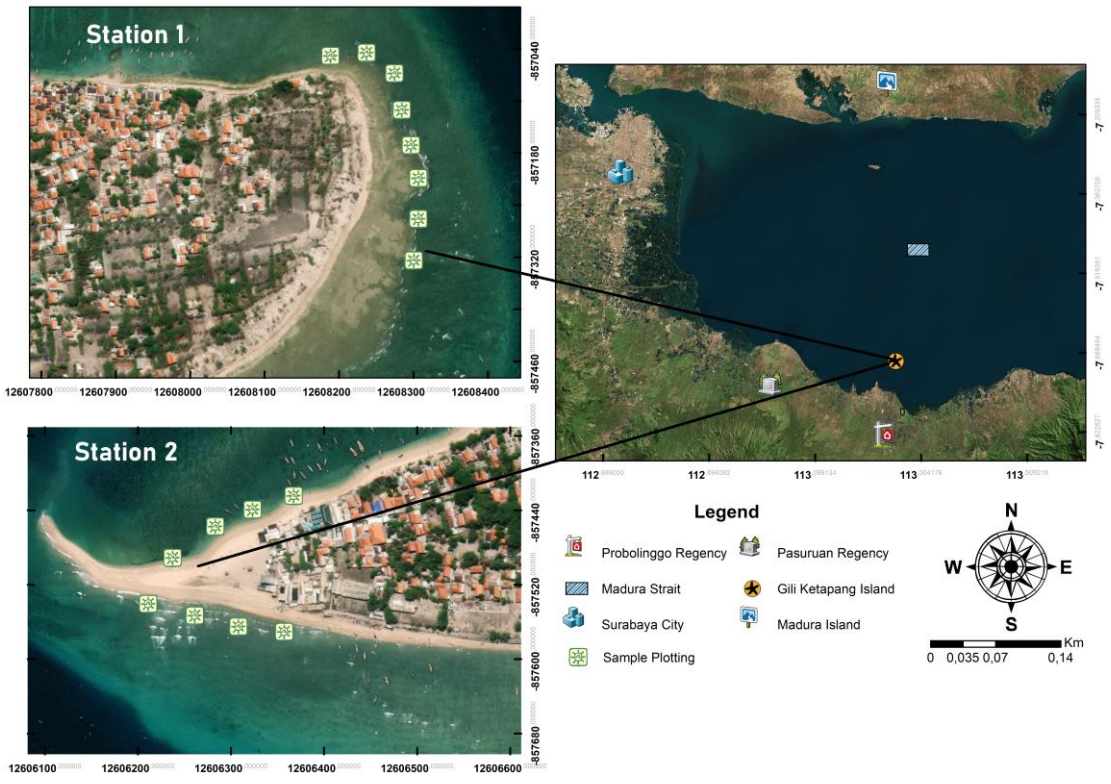

Figure 1. The location of echinoderms from Gili Ketapang Islands, Indonesia.

The transect was drawn perpendicular to the coastline for 100 m, and a 1 x 1 m paragon frame was used for the sampling plot. Observation plot points were marked 10 m along the transect line. The distance between the transects was of 30 m, and the distance between stations was of 50 m. The observations were made at low tide and the number of species and individuals of the echinoderms population were recorded in the frame of each observation plot. Additionally, the type of substrate was also recorded in order to describe the habitats determining the fauna's local distribution. Each specimen was placed in ice, frozen on site, and transported to the Zoology Laboratory, ITSNU University, Indonesia. Voucher specimens were deposited at the ITSNU specimens repository, with voucher numbers Gili 1-16".

Morphological measurements. The identification process was carried out by observing the samples' morphology directly using a microscope. Characteristics such as tube-shaped feet and the number of arms for Asteroidea, arm shape and tentacle scale for Ophiuroidea class, and the presence of anus and ambulacral shape for Echinoidea can be observed by referring to the identification key book Monograph of Shallow Water Indo-West Pacific echinoderms (Clark & Rowe 1971) and Sypnotic Keys to the Genera of Ophiuroidea (Fell 1962).

DNA extraction and sequencing. The total DNA (whole genome) from tissue samples was extracted using the DNA Isolation Kit (Roche), with several modifications. A fragment of approximately 503 bp (base pairs) from the mtDNA COI region was amplified by a gradient PCR using universal EchinoF1–5'TTTCAACTAATCATAAGGACATTGG-3 and EchinoR1–5'-CTTCAGGGTGTCCAAAAAATCA-3'. EchinoF1 and EchinoR1 are newly designed modifications of the primers (Folmer et al 1994; Ward et al 2008). The HotStart PCR method usually employs a Kapa master mix and two Taq master mixes. This was carried out in 35 cycles, each consisting of a double thread attachment process (pre-denaturation), denaturation, annealing, extension, and final elongation at 95, 94, 45, 72 and 72°C for 3 mins, 45 secs, 45 secs, 2 mins, and 10 mins, respectively. The products were checked on 1% agarose gel (0.5 g agarose and 50 mL TAE Buffer) mixed with 4 µL Ethidium Bromide (EtBr), as a dye. About 3 µL of the PCR samples were mixed with 1 µL of loading dye, then poured into an agarose well. The electrophoresis was performed using an electrophoresis machine with a voltage of 220 V and a current of 400 mA, for 25 minutes. The PCR products were also purified with a Qiagen purification kit according to the manufacturer's instructions and subsequently sequenced at First Base, Malaysia.

Data analysis. After obtaining echinoderms from Gili Island, DNA sequencing was continued by conducting the main analysis, namely chromatography, using the FinchTV chromatogram viewer, and translating proteins online through the ExPASy web server (Duvaud et al 2021). The data of partial COI gene sequences were checked through the Basic Local Alignment Search Tool (BLAST) (Boratyn et al 2012). They were analyzed using a bioinformatics software, involving the alignment stages of the *Nemacheilus* spp. The partial COI gene sequences were aligned using the Clustal X program (Larkin et al 2007) and then checked again manually with the BioEdit program (Hall 1999). The results were then identified online through the BOLD System (Ratnasingham & Hebert 2007). The phylogenetic tree was reconstructed with MEGA X version 10.2.6 (Kumar et al 2018) following the Neighbor-Joining (NJ) and Maximum-Likelihood (ML) methods. NJ reconstruction was calculated by using the Kimura 2-Parameter (K2P) substitution model (Saitou & Nei 1987), and the rate of variation among sites was modeled with a Gamma distribution. Meanwhile, the evolutionary history was inferred by using the ML method and K2P model (Kimura 1980). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985) was selected to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was shown next to the branches. Codon positions included were 1st+2nd+3rd+Noncoding, and there was a total of 503 positions in the final dataset. Similarity values were reviewed through GenBank (Sayers et al 2022) to be compared with close relative echinoderms species. The purpose of making

phylogenetic trees was to visualize the grouping of different and related species. The similarity values were calculated as follows: similarity percentage = (1-Genetic Distance) x 100.

Results

Morphological identification. A total of 16 echinoderm sample specimens were collected and identified based on morphological characteristics. Characteristics such as tube-shaped feet and the number of arms for Asteroidea, arm shape and tentacle scale for Ophiuroidea class, and the presence of anus and ambulacral shape for Echinoidea can be observed by referring to the identification key book Monograph of Shallow Water Indo-West Pacific echinoderms (Clark & Rowe 1971) and Sypnotic Keys to the Genera of Ophiuroidea (Fell 1962).

***Echinometra mathaei* (Blainville, 1825)**
Material examined. Gili 1, Gili 2. 22 July 2022. 07°40'33.25''S 113°15'42.19''E station 2. Collector: Endik Deni Nugroho & Reza Ardiansyah (Figure 2a).
Diagnosis. The upper part of the dome-shaped body of *Echinometra mathaei* is slightly flat, the lower part is light but contains a mouth for burrowing into hard calcareous substrates, and reaches a diameter of 38-55 mm. It has a slightly short spiny shell, thick and increasingly pointed, with a sharp tip. The shell is hard and oval, with a white ring at the base of the spine, and perforated tubercles.
Coloration. The color of the spines is white, green, light brown, greenish-brown, and dark brown. The smooth surface and pointed tip appear light brown, while the shell is dark brown.
Habitat. Sandy soil and coral reefs.

***Diadema setosum* (Leske, 1778)**
Material examined. Gili 3, Gili 4. 22 July 2022. 07°40'33.39''S 113°15'40.63''E station 1. Collector: Endik Deni Nugroho & Reza Ardiansyah (Figure 2b).
Diagnosis. The body is round pentagonal and flat, with a black appearance. The spines are longer than the body, the surface is sharp, and the tip is pointed and brittle, while the secondary spines are short. The top contains five white dots which are present in each segment, and the tubercle's shape is crenulate. The mouth is below and the anus is on top, facing the rounded shell's apex, and has 12 to 14 pairs of equally irregular pores. The spines are relatively large, usually hollow, long, cylindrical, thin, and fragile, with a rough surface and an average length between 1-16 cm.
Coloration. Black color, with black spines extending upwards, but the underside is shorter. The top has five white dots, with one white dot between each segment.
Habitat. *Diadema* lives in flat sand zones, algae growth areas, and usually in large groups amidst coral reefs.

***Holothuria atra* (Jaeger, 1833)**
Material examined. Gili 5, Gili6. 22 July 2022. 07°40'34.14''S 113°15'43.40''E station 2. Collector: Endik Deni Nugroho & Reza Ardiansyah (Figure 2c).
Diagnosis. This type of sea cucumber has a round body cross-section, the ventral part tends to be flat, and the anus is round. The skin is soft and thick, containing spicules with a reduced disc and a moderate spire bearing a 'Maltese cross', and rosettes but no buttons. The dominant type of spicules has the form of table plates and they can be found in almost all parts of the integument. Other forms are rods, cylindrical with rounded ends, and they have terminal plates, rosettes, a smooth tegument, and a pliable body wall, while the ventral body surface is lined with long, compact tube feet.
Coloration. Black sea cucumber with black tentacles, frequently covered with sand.
Habitat. *Holothuria* is found in areas containing coarse sandy soil and the body has a length of ±20 cm, and a width of ±4 cm. It usually lives covered by fine sand, in rocky and sandy waters, overgrown with seagrass.
***Macrophiothrix longipeda* (Lamarck, 1816)**

Material examined. Gili 7, Gili8. 22 July 2022. 07°40'37.24''S 113°15'44.12''E Station 1. Collector: Endik Deni Nugroho & Reza Ardiansyah (Figure 2d).
Diagnosis. Macrophiothrix has five simple unbranched arms. Oral papillae are absent and each jaw is covered by a more or less dense group of apical papillae (Ophiotrichidae). The ventral and dorsal arm plates are horizontally flexible, well-developed, and usually complete. The shapes of dorsal arm plate can be either rhomboidal, hexagonal, oval or polygonal. The width is doubled between the juvenile (<5 mm) and adult (10-20 mm) stages, with most species measuring ±35 mm (Macrophiothrix). There have very short, sharp spines along the vertical side of each arm. Every vertical row is equipped with a pair of spines on each side. The radial shield (disc) is flat or slightly convex and covered with granules. The aboral arm plates are more than twice as wide as they are long, with a straight median region. They have laterodistal angles, and they are widest distally. The oral arm plates are octagonal, and the distal edge is straight. Each arm contains ten thorny spines, and the lowest looks like a comb.
Coloration. The disc appears bluish with dark blue spots, radial shields are spotted, the ventral surface is light blue with dark spots, and the arms are banded in grey and pale blue color.
Habitat. Under coral boulders or in burrows or crevices.

***Bohadschia argus* (Jaeger, 1833)**
Material examined. Gili 15, Gili16. 07°40'49.53''S 113°14'38.29''E Station 2. 22 July 2022. Collector: Endik Deni Nugroho & Reza Ardiansyah (Figure 2e).
Diagnosis. Bohadschia argus has spicules that generate the effect of branched rods. The body is sausage-shaped or cylindrical with smooth, tough, leathery skin, can grow up to 2 feet (0.61 m) long, and there are rows of tube feet on the underside. Surrounding the mouth, at the anterior end, there is a ring of paddle-shaped, black tentacles fringed with white color. The body is decorated with round patterns at the base of each papilla, and the anus tends to be positioned on the dorsal side, without teeth. It has a 16 cm length, black round papilla scattered in the bivium, and podia scattered on the trivium, with a ventral mouth. The spicules are rose, of rod or plate-shaped types, with perforations of a 5 m size.
Coloration. The lower surface is light yellow-brown, while the upper surface appears grey or grey-brown with a striking pattern of spots ringed with white color.
Habitat. Sand habitats, hand collections, and intertidal areas.

***Archaster typicus* (Müller & Troschel, 1840)**
Material examined. Gili 11, Gili12. 07°40'49.53''S 113°14'38.29''E Station 1. 22 July 2022. Collector: Endik Deni Nugroho & Reza Ardiansyah (Figure 2e).
Diagnosis. *Archaster typicus* has five pointed arms with a flat body and a rough back structure. Also possesses an 8.7 cm sleeve length and 3 cm width. The body is covered by spines on the inferolateral part and colored grayish-white with black spots along the dorsum. The back is covered by papillae. On the ventral side, there are two rows of tubular feet equipped with suckers, containing an inframarginal plate covered with flat spines similar to fish scales. It has smooth spines that are blunt and flat.
Coloration. The body is brownish white, while the dorsal part and the fine spines are white.
Habitat. Lives under rocks at a depth of 2-6 m and on 33 m deep coral reefs.

***Linckia laevigata* (Linnaeus, 1758)**
Material examined. Gili 13, Gili14. 07°40'40.41''S 113°15'44.46''E Station 1. 22 July 2022. Collector: Endik Deni Nugroho & Reza Ardiansyah (Figure 2f).
Diagnosis. *Linckia laevigata* has five cylindrical arms with a bright or light blue body color, and yellow tube feet, which can also be white. The dorsal side contains one madreporite, smooth spicules, and an anus. The circumference of the central canal is star-shaped with a very striking color and contrast to the environment. Each arm is

elongated and slender, measuring about 15 cm or more, and the spicules are shaped similarly to fine spines.
Coloration. Blue and non-massive body circumference.
Habitat. Sand and coral reef substrate.

***Ophiactis savignyi* (Müller & Troschel, 1842)**
Material examined. Gili 9, Gili10. 07°40'33.25''S 113°15'42.19''E Station 2. 22 July 2022. Collector: Endik Deni Nugroho & Reza Ardiansyah (Figure 2g).
Diagnosis. *Ophiactis savignyi* has broad, square teeth, but lacks an anus. In the aboral region, there are only granular skin that wraps around the disc and the six arms. The arm length is 2 cm, the disc diameter is 5 mm, the aboral disc distinct spineless, and two to seven arms are present. Radial shields are large and cover a broad area at the distal end. The oral shields are larger than those on the aboral side, but they do not meet in the middle. The aboral arm plates are elliptical with rounded lateral margins, and the distal edge is convex with a prominent median lobe. The six spines on the short arm are rugose and spiculated. The arms are sharply demarcated from the central disc, jointed, flexible, and usually variegated with intermittent dark and light markings. The disc of the animal is 3.8-5 mm and darkly pigmented with rough-tipped spines on the disc top. The arms range from 16.3 to 20 mm and have small, rough spines running along them. The oral surface of the central disc contains one to three oral papillae that are flat and scaly.
Coloration. The disc has regular dark and light green markings, arms are similarly banded and ventrally white. The oral is lighter than the aboral surface, while the disc is variegated with light and dark colors, and the arms are banded yellow to blue-green. There is a dark spot on either side of the median lobe.
Habitat. Dominates sponge habitats and competes with other brittle stars for space.

Molecular identification
PCR Product visualization and sequence validation (BOLD System and GeneBank). The PCR product visualized with 1% agarose gel electrophoresis indicated that the partial COI gene sequence in the 16 echinoderms was successfully amplified. These samples confirmed that the COI universal primer produced an amplicon of 655 bp. After multiple sequence alignments and minor editing, the sequence length used was an amplicon of 501 bp. The results of the sequence validation conducted through the online facility of BLAST (NCBI) and the BOLD system showed that the sequence samples matched available accessions in the database with a query cover in the range of 97-100%, as presented in Table 2. The validation results revealed that the 16 samples were grouped into eight species, including *Diadema setosum, Macrophiothrix longipeda, Archaster typicus, Echinometra mathaei, Ophiactis savignyi, Ophiactis savignyi, Linckia laevigata*, and *Bohadschia argus*. The observations results of the echinoderms in this study were in accordance with the *Archaster typicus* sample from Gili Ketapang Island reported by Buyami et al (2020). Moreover, seven species have not been recorded before.

Sequence composition and genetic diversity. One of the characteristics of the COI DNA barcode in species identification is the concern for amplification of "COI-like sequence" or pseudogenes derived from mitochondria (nuclear mitochondrial DNA segment-NUMTs) (Buhay 2009). To confirm whether this sequence really comes from the mitochondrial DNA COI gene, a consensus sequence analysis was carried out with the protein translation because COI is a coding region. No insertions and deletions (INDELs) were found in the echinoderms alignment results. Additionally, to confirm the presence of NUMTs, the heterozygosity of the chromatogram peaks was carefully examined (Bensasson et al 2001). The COI gene barcode sequence showed the nucleotide base composition of GC was less than AT (Table 3). The composition of GC nucleotide bases was between 60.33-60.88%, as presented in Table 3. This is related to the results of Nugroho et al (2017), stating that Nomei fish found in North Kalimantan waters had more AT nucleotide bases than GC. Sari et al (2021) stated that the average value of *Donax faba*'s nucleotide base composition of GC was 48.61%, while the average value of the

nucleotide base composition of AT was 51.38%. The variations can be related to the metabolic physiology of these organisms.

Table 1
Gene Bank accession numbers for COI sequences of echinoderms with references

Species	*Voucher code*	*Locality*	*Gene Bank ACC.*
Echinometra mathaei	Gili1	Gili River, Probolinggo	OP458572 (This study)
Echinometra mathaei	Gili2	Gili River, Probolinggo	OP458597 (This study)
Diadema setosum	Gili3	Gili River, Probolinggo	OP458756 (This study)
Diadema setosum	Gili4	Gili River, Probolinggo	OP458786(This study)
Holothuria atra	Gili5	Gili River, Probolinggo	OP458824 (This study)
Holothuria atra	Gili6	Gili River, Probolinggo	OP458809 (This study)
Macrophiothrix longipeda	Gili 7	Gili River, Probolinggo	OP474056 (This study)
Macrophiothrix longipeda	Gili 8	Gili River, Probolinggo	OP474055 (This study)
Ophiactis savignyi	Gili 9	Gili River, Probolinggo	OP474055 (This study)
Ophiactis savignyi	Gili 10	Gili River, Probolinggo	OP474056 (This study)
Archaster typicus	Gili11	Gili River, Probolinggo	OP477066 (This study)
Archaster typicus	Gili12	Gili River, Probolinggo	OP477067 (This study)
Linckia laevigata	Gili13	Gili River, Probolinggo	OP646485 (This study)
Linckia laevigata	Gili14	Gili River, Probolinggo	OP646486 (This study)
Bohadschia argus	Gili15		OP646487 (This study)
Bohadschia argus	Gili16		OP646488 (This study)
Echinometra mathaei	Khark St.6	Persian Gulf	LC194864
Diadema setosum	M19SET_JGLCO_A	Sulawesi Selatan	MK296422
Macrophiothrix longipeda	REU0182		GU480574.1
Ophiactis savignyi	1789Hai		AF331616
Holothuria atra	PFKK6		MF787761
Archaster typicus	MZH_HP.1088		MN690254.1
Linckia laevigata	haplotype F		EU816374.1
Bohadschia argus	FAO 032		EU848263.1

Table 2

Validation of the partial sequence of the CO1 gene of echinoderms obtained from Gili Ketapang Island, East Java Province, Indonesia through the BLAST (GenBank/NCBI) and the BOLD System online facilities

ACC number sample	*Species*	*Gene bank NCBI*			*Bold system*
		Query cover (%)	*E-Value*	*Identity (%)*	*Similarity*
OP458572	*Echinometra mathaei*	97	0	99.5	100
OP458597	*Echinometra mathaei*	98	0	97.9	100
OP458756	*Diadema setosum*	97	0	99.36	99.84
OP458786	*Diadema setosum*	99	0	98.36	99.76
OP458824	*Holothuria atra*	97	0	97.4	100
OP458809	*Holothuria atra*	96	0	98.6	100
OP474056	*Macrophiothrix longipeda*	97	0	98.4	99
OP474055	*Macrophiothrix longipeda*	98	0	98.5	100
OP474055	*Ophiactis savignyi*	98	0	100	100
OP474056	*Ophiactis savignyi*	98	0	100	100
OP477066	*Archaster typicus*	95	0	98	99
OP477067	*Archaster typicus*	95	0	98.8	99
OP646485	*Linckia laevigata*	99	0	98.9	99
OP646486	*Linckia laevigata*	99	0	98.2	99
OP646487	*Bohadschia argus*	97	0	99	100
OP646488	*Bohadschia argus*	97	0	99	100

A universal primer for the partial COI gene sequence was developed from Folmer et al (1994). Modifications were carried out through accurate calculations and the model was successfully applied to echinoderms from Gili Ketapang Island. The details of the genetic diversity analysis are summarized in Table 4. The percentage of bases (G, C, A, and T) in all the samples was higher for AT than in GC composition, as demonstrated in Table 3. Furthermore, the percentage of G+C content in the partial COI gene sequence was 45.0%. The details of the genetic diversity analysis are summarized in Table 3. The base composition analysis of the partial COI gene sequence revealed that AT content (54.44%) is higher than GC (48.26%). Related data were observed in Australian (Ward et al 2005), Canadian (Steinke et al 2009), Cuban (Lara et al 2010), and Bangladeshian fish species (Ahmed et al 2020). In this study, Clusters 1 and 2 were resolved as sister taxa with a bootstrap confidence interval of 99%, and the genetic diversity was very low or less than 2%. A genetic distance value <2% indicates the presence of species that are different from other group members, while a value <3% means the group or cluster comes from the same species (Wong et al 2009).

Overall, species belonging to the same genera are grouped together with the related species. Phylogenetic relationships were shown in the NJ and ML trees in Figures 3 and 4. Each species was associated with a specific DNA barcode cluster and the relationship among all was revealed. Closer species in terms of genetic divergence were clustered at the same nodes and the distance between the terminal branches of both trees, which consisted of two divergent clusters. Bootstrap values higher than 99% indicated the high genetic variation within species. Based on the values of haplotype diversity (Hd) 0.978 with nucleotide diversity (π) 0.10532, the echinoderms' genetic diversity was concluded to be relatively high between species. A summary of the genetic distance of echinoderms in Gili Ketapang waters is presented in Table 4. The mean intraspecific genetic distance ranged from 0.003 (*Diadema setosum*) to 0.024 (*Bohadschia argus*), as presented in Table 4. Based on K2P, the genetic distance found between the eight types of echinoderms showed higher values (0.299) than the threshold separating the species.

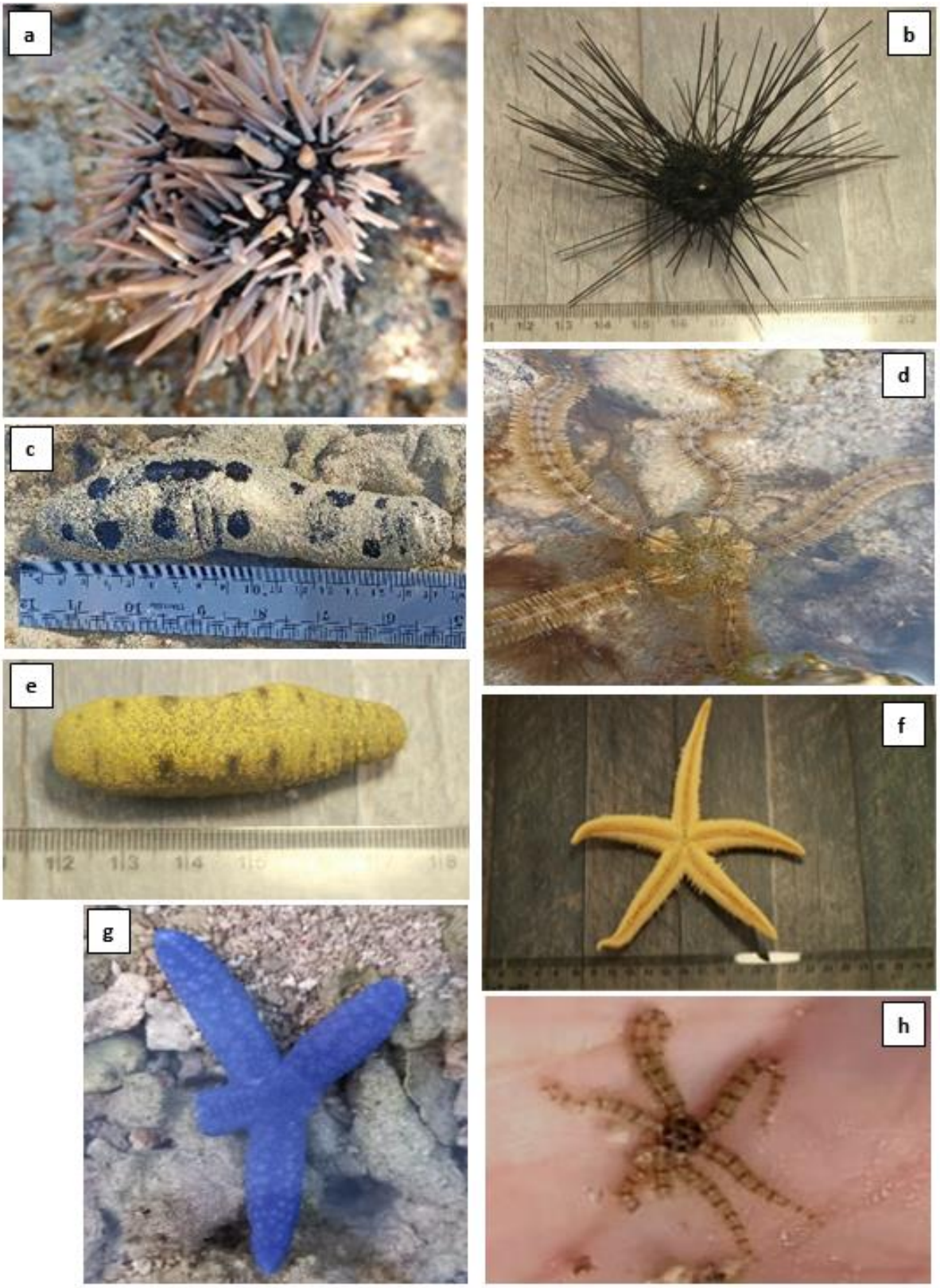

Figure 2. Echinoderms species in Gili Ketapang Island. a. *Echinometra mathaei* voucher Gili 1, b. *Diadema setosum* voucher Gili 3, c. *Holothuria atra* voucher Gili 5, d. *Macrophiothrix longipeda* voucher Gili 7, e. *Bohadschia argus* voucher Gili 15, f. *Archaster typicus* voucher Gili 11, g. *Linckia laevigata* voucher Gili 13, h. *Ophiactis savignyi* voucher Gili 9.

Table 3
The nucleotide base composition of the echinoderm COI gene from Gili Ketapang Island

Species	Conserved sites (bp)	Nucleotide composition		Total of amino acid
		AT	GC	
Echinometra mathaei	501	58.04	41.96	167
Echinometra mathaei	501	58.00	42.00	167
Diadema setosum	501	54.56	45.44	167
Diadema setosum	501	54.26	45.74	167
Holothuria atra	501	55.13	44.86	167
Holothuria atra	501	56.14	43.86	167
Macrophiothrix longipeda	501	56.46	43.54	167
Macrophiothrix longipeda	501	56.42	43.58	167
Ophiactis savignyi	501	55.80	44.20	167
Ophiactis savignyi	501	56.00	44.00	167
Archaster typicus	501	54.12	45.88	167
Archaster typicus	501	55.45	44.54	167
Linckia laevigata	501	55.46	44.54	167
Linckia laevigata	501	55.42	44.58	218
Bohadschia argus	501	54.80	46.20	218
Bohadschia argus	501	56.00	44.00	218

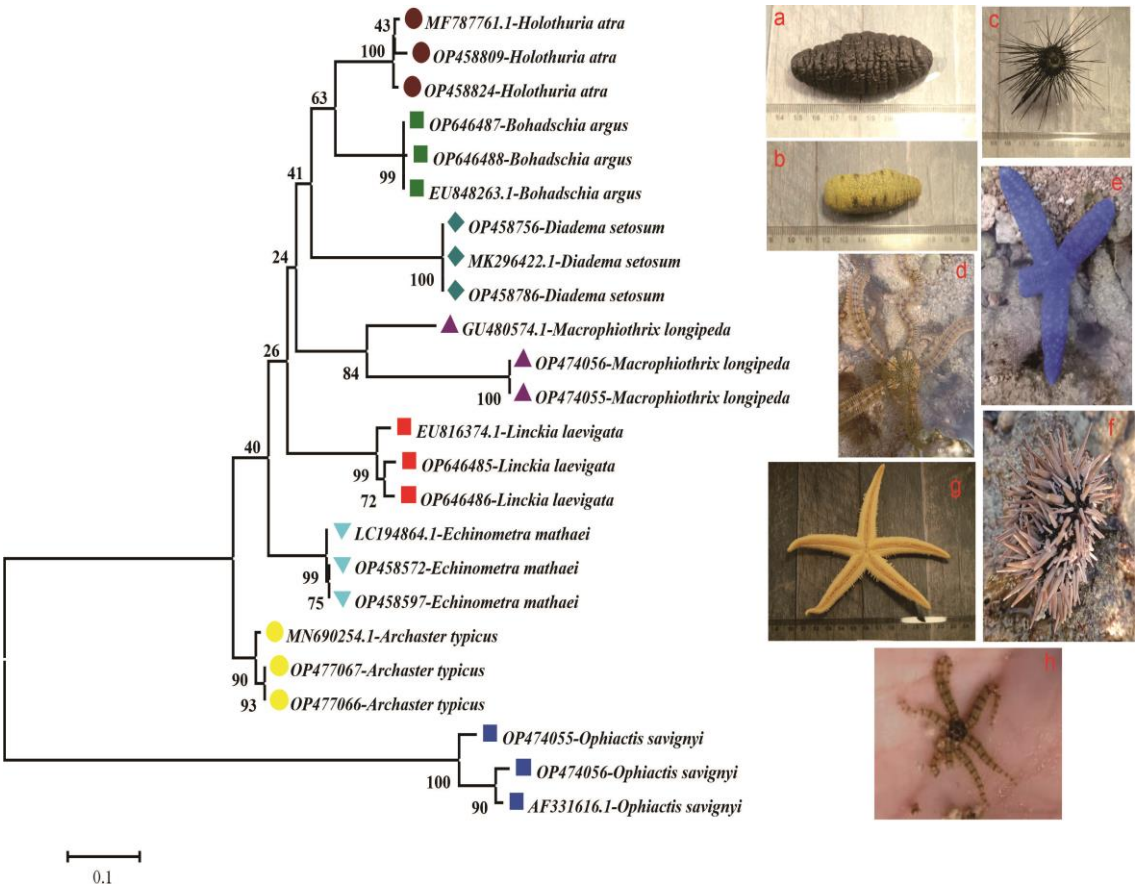

Figure 3. Phylogenetic topology using the Neighbour Joining Method with the Kimura 2 Parameter Model calculation model using 1000x bootstrap repetitions: a. *Holothuria atra* voucher Gili 5, b. *Bohadschia argus* voucher Gili 15, c. *Diadema setosum* voucher Gili 3, d. *Macrophiothrix longipeda* voucher Gili 7; e. *Linckia laevigata* voucher Gili 13, f. *Echinometra mathaei* voucher Gili1, g. *Archaster typicus* voucher Gili 11, h. *Ophiactis savignyi* voucher Gili 9.

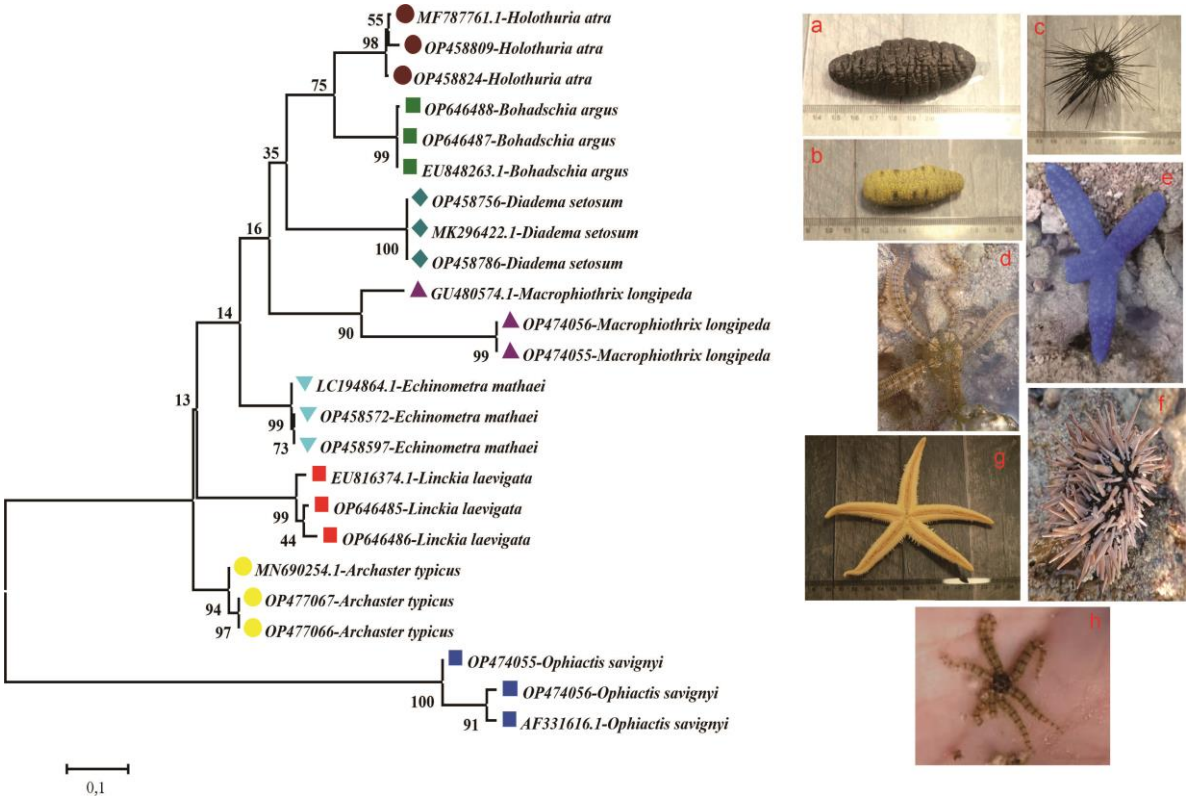

Figure 4. Phylogenetic topology using the Maximum Likelihood Method with the Kimura 2 Parameter Model calculation model using 1000x bootstrap repetitions: a. *Holothuria atra* voucher Gili5, b. *Bohadschia argus* voucher Gili15, c. *Diadema setosum* voucher Gili 3, d. *Macrophiothrix longipeda* voucher 7; e. *Linckia laevigata* voucher Gili 13, f. *Echinometra mathaei* voucher Gili1, g. *Archaster typicus* voucher Gili 11, h. *Ophiactis savignyi* voucher Gili 9.

Table 4

Pairwise genetic distance matrix of the 8 species of echinoderms obtained in Gili Ketapang Island, East Java Province, Indonesia

No	*Species*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	*Holothuria atra*	0.021							
2	*Bohadschia argus*	0.189	0.024						
3	*Macrophiothrix longipeda*	0.345	0.082	0.02					
4	*Linckia laevigata*	0.282	0.245	0.245	0.023				
5	*Echinometra mathaei*	0.280	0.279	0.220	0.236	0.002			
6	*Diadema setosum*	0.286	0.285	0.233	0.236	0.305	0.003		
7	*Linckia laevigata*	0.284	0.278	0.227	0.236	0.285	0.225	0.024	
8	*Archaster typicus*	0.248	0.289	0.222	0.236	0.256	0.260	0.240	0.002

Figures 5A & 5B show that the ABGD method identified three groups for Echinoderm specimens with the initial approach, while the barcode gap threshold was calculated by analyzing the dataset of the partial COI gene sequence. The value of the barcode gap distance was 0.025, in accordance with the results of the ABGD grouping which divided the species into clusters (Figure 5C). Each cluster was grouped according to its species, without overlapping, as demonstrated in Figure 5. Different haplotypes from the same location indicated that the selected individuals had a heteroplasmic type of mtDNA. Evolutionary

relationships using the MJN method were further analyzed between haplotype groups, and eight groups were formed (1, 2, 3, 4, 5, 6, 7, and 8).

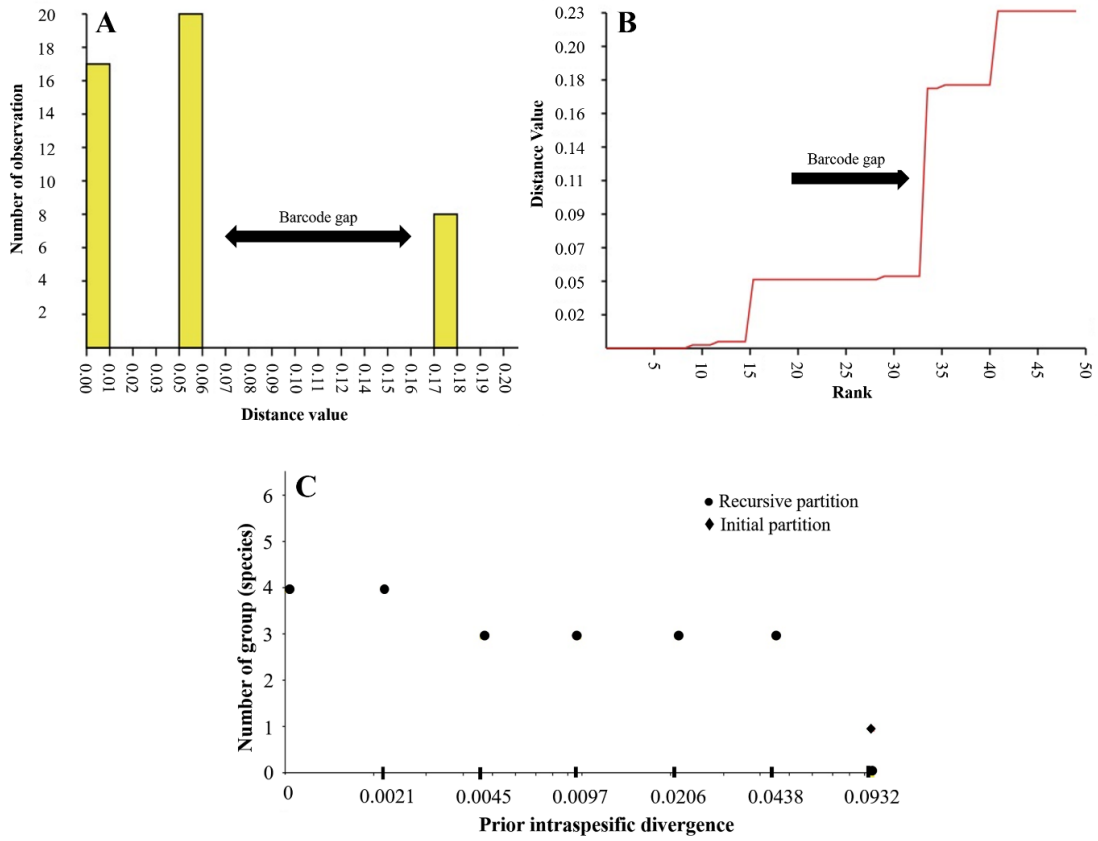

Figure 5. Barcode gap analysis between echinoderms generated by automatic barcode discovery gap discovery. Distribution of K2P distances and between each pair of specimens for the COI gene (a) distance histogram (b) rank distance and (c) number of PSHS obtained for each previous intraspecific divergence.

Discussion. A total of 16 samples from eight different echinoderm species belonging to eight genera were already identified by morphological characteristics. More than 1/3 of the species investigated showed that Asteroidea had the highest species number (116), followed by Holothuroidea (54), Echinoidea (11), and Echinoidea (2). The same order in species richness by classes was observed for the eastern English Channel, Bristol Channel, and Irish Sea (Ellis et al 2000). This current study demonstrated a concordance of the morphological identification with the genetic analysis results for about 94% of the investigated North Sea echinoderm species. The results underlined the reliability of the species delimitation, based on the morphological features diagnostic presently applied for most of the species. However, the COI divergences effectively revealed deep lineage splits within various nominal species of starfish.

The COI gene partial sequence had been successfully amplified and validated using online database facilities. This is consistent with Chow et al (2016) and Folmer et al (1994), who showed that the developed primer generated partial COI gene sequences of 655 bp in length. The validation results showed that the 16 samples were classified as *Diadema setosum*, *Macrophiothrix longipeda*, *Archaster typicus*, *Echinometra mathaei*, *Ophiactis savignyi*, *Linckia laevigata*, and *Bohadschia argus*. In line with these findings, the sequences determined by Catalma et al (2020) had a similarity of >98%, while Patantis et al (2019) reported 95-100%. All the echinoderms studied are new records in Gili Ketapang Island, and 100% of the eight species can be separated by their COI barcodes.

The degree of gene or genome difference between species or populations is referred to as genetic distance (Dogan & Dogan 2016). Based on the results, all samples have genetic variation between species. The closest values were also found between species, while the greatest values were observed between various classes. This distance demonstrates that the partial COI gene sequence has a high degree of accuracy in distinguishing between Indonesian echinoderms. The sequence also provides a fast and effective DNA barcode technique for species identification, particularly when addressing the morphological identification, which is in line with the report of Uthicke et al (2010). The variation between species within a genus was 0.024 for *Bohadschia*, and 0.024 for *Holothuria*, in the class of Holothuroidea. The value obtained for *Bohadschia* was comparable with the value of 0.111±0.028, obtained by Kim et al (2013) and with the value of 0.1205, obtained by Uthicke et al (2010). The current results are in line with Ward et al (2008) and Uthicke et al (2010): the DNA sequences of individuals belonging to the same species have a sequence difference level <0.02 (or 2%).

Finally, the phylogenetic analysis results showed that the samples were segregated from each other based on relatedness. The divergence between echinoderm groups had high probabilities, bootstrapping, ML (100), and NJ (99). Therefore, the segregation suggested that the two clusters should be classified into separate groups, and these results were similar to the genetic distance analysis values. No insertions/deletions or codon stops were observed after the nucleotide translation, suggesting that the view of all amplified sequences denotes functional mitochondrial COI sequences. The average length of the amplified sequences was larger than 503 bp, the limit typically observed for nuclear DNA sequences originating from mtDNA (NUMT) (Buhay 2009). All species could be distinguished based on individual DNA barcodes, indicating that this study confirmed the effectiveness of partial COI gene sequence for *Nemacheilus* spp. identification.

The ABGD analysis at a prior maximal distance of 0.035 also delineated the species into separate partitions, and these results supported the separation of the phylogenetic tree of echinoderms in groups. The combination of genetic distance, phylogenetics, and ABGD analysis supported the identification process. In conclusion, the targeted use of DNA barcoding and morphological evidence was confirmed as efficient and reliable tools for the identification of echinoderms species.

This study becomes the first to report on the morphology, genetic identification, and phylogenetic reconstruction of echinoderms from Gili Ketapang using partial COI gene sequence. Furthermore, there is a possibility for conservation management of echinoderms by grouping animal units according to species and genetic entity. A molecular approach using DNA barcoding supports the identification results based on a morphological approach in Echinoderm and an accession number has been obtained from GenBank (NCBI) database. The identified morphology and molecular characteristics of echinoderms were found on this island. Through this study, a reliable DNA barcode reference library was established, which could be used to assign Echinoderm species by screening sequences. This could help to achieve better monitoring, conservation, and management of echinoderms in Indonesia.

Conclusions. This study has successfully identified the baseline data for echinoderms found in Gili Ketapang Islands. A total of 16 echinoderm samples were collected and identified based on morphological characteristics: *Diadema setosum*, *Macrophiothrix longipeda*, *Archaster typicus*, *Echinometra mathaei*, *Holothuria atra*, *Linckia laevigata*, *Bohadschia argus*, *and Ophiactis savignyi*. Automatic Barcode Gap Discovery (ABGD) analysis showed that the echinoderms were divided into eight species, and these new records established a sequence reference library for the Island.

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Conflict of interest. The authors declare no conflict of interest.

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