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MORPHOLOGICAL AND MOLECULAR COMPARISON OF AREOLATE GROUPER (*Epinephelus areolatus*) FROM SAUDI ARABIA AND INDONESIA

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ABSTRACT

Grouper (subfamily Epinephelinae) is one of the largest groups of fish in the oceans. Identification of groupers, especially the *Epinephelus*, is conducted based on morphological characteristics (color, pattern, body shape, and size. However, the identification process is difficult to differentiate morphologically because of their similar characteristics. One method that can be applied is DNA barcoding. This study aimed to compare groupers from Saudi Arabia and Indonesia. Morphological and molecular identification results show that the grouper from this study (from Yanbu, Saudi Arabia, and Lamongan, Indonesia) was *Epinephelus areolatus* (areolate grouper). Morphologically, grouper samples from Yanbu (Saudi Arabia) were as follows: dorsal fin X-XI/12-15; anal fins II-III/8-9; pectoral fins 13-15; pelvic fin I-5; lateral line scales 48-53; vertebrae 24. Meanwhile, the meristic results of groupers from Lamongan (Indonesia) were as follows: dorsal fins X-XI/15-17; anal fins II-III/8; pectoral fins 16-19; pelvic fin I-5; lateral line scales 48-53; vertebrae 24. The morphological differences between *E. areolatus* from Saudi Arabia and Indonesia were its spots and caudal fin. Molecular results on *E. areolatus* showed different clades. Samples from Saudi Arabia belonged to the Western Indian Ocean clade while Indonesia belonged to the Western Pacific. This showed that there were morphological and molecular differences between *E. areolatus* from Yanbu (Saudi Arabia) and Lamongan (Indonesia). The COI gene sequences of areolate grouper were submitted to NCBI (accession number PP388919.1 for Lamongan and PP388920.1 for Saudi Arabia). This research data can be used as a reference for conservation.
Keywords: biodiversity, COI gene, evaluation of species, morphology, grouper

Perbandingan secara Morfologi dan Molecular pada Kerapu Ekor Putih (Epinephelus Areolatus) dari Arab Saudi dan Indonesia

ABSTRAK

Ikan kerapu (subfamily Epinephelinae) merupakan salah satu kelompok ikan terbesar di dunia. Identifikasi kerapu, khususnya genus Epinephelus, dilakukan berdasarkan karakteristik morfologi seperti warna, bentuk tubuh, dan ukurannya. Namun, proses identifikasinya kadang sulit untuk dibedakan secara morfologi karena memiliki karakteristik yang sangat mirip. Salah satu metode yang dapat diaplikasikan adalah penggunaan DNA barcoding. Penelitian ini bertujuan untuk membandingkan ikan kerapu dari Arab Saudi dan Indonesia. Hasil identifikasi morfologi dan molekuler menunjukkan bahwa kerapu dari penelitian ini (dari Yanbu, Arab Saudi dan Lamongan, Indonesia) adalah Epinephelus areolatus (kerapu sirip putih). Secara morfologi, sampel kerapu ekor putih dari

Yanbu Arab (Saudi) adalah sebagai berikut: sirip dorsal X-XI/12-15; sirpi anal II-III/8-9; sirip pectoral 13-15; sirip pelvic I-5; sisik linea lateralis 48-53; vertebrae 24. Sementara hasil meristik dari kerapu dari Lamongan (Indonesia) adalah sebagai berikut: sirip dorsal X-XI/15-17; sirip anal II-III/8; sirip pectoral 16-19; sirip pelvic I-5; sisik linea lateralis 48-53; vertebrae 24. Perbedaan E. areolatus dari Arab Saudi dan Indonesia secara morfologi adalah dari bintik dan ekor caudal-nya. Hasil molekuler pada E. areolatus menunjukkan perbedaan clade. Sampel dari Arab Saudi termasuk clade Western Indian Ocean sementara Indonesia termasuk Western Pacific. Hal ini menunjukkan bahwa terdapat perbedaan morfologi dan molekuler antara E. areolatus dari Yanbu (Saudi Arabia) dan Lamongan (Indonesia). Sekuens gen COI dari DNA ikan kerapu ekor putih pada penelitian ini telah didaftarkan ke NCBI (PP388919.1 untuk Lamongan dan PP388920.1 untuk Arab Saudi). Data penelitian ini dapat dijadikan sebagai referensi untuk konservasi.

Kata kunci: *biodiversitas, COI gen, evaluasi spesies, kerapu, morfologi*

INTRODUCTION

Grouper (subfamily Epinephelinae) is one of the largest groups of fish in the oceans (Tavakoli-Kolour *et al.*, 2022). Grouper consists of 15 genera and more than 159 species have been reported (Tapilatu *et al.*, 2021). Groupers can be found in coral reefs and shallow water (mangrove, estuary, seagrass) in sub-tropical or tropical regions (Dwifajri *et al.*, 2022; Osuka *et al.*, 2022). The existence of groupers is important for the formation of coral reef ecosystems and acts as a predator in the waters (Hackradt *et al.*, 2020). Not only for the ecosystem, the existence of groupers is also important for the world economy. This is because this fish has a high price, tastes good, and is popularly consumed by the public (Khasanah *et al.*, 2020). Therefore, various countries such as Iran, Saudi Arabia, and Indonesia produce a lot of grouper fish both through fishing and aquaculture (Squalli, 2020; Glamuzina and Rimmer, 2022).

There are many challenges in identifying grouper species. This is due to cryptic speciation in groupers (Félix-Hackradt *et al.*, 2022). Cryptic species are species that are difficult to differentiate morphologically because they have very similar characteristics (Shin and Allmon, 2023). For example, residents in the Persian Gulf or Oman Sea regions sometimes find it difficult to differentiate orange-spotted grouper (*Epinephelus coioides*) from similar species such as areolate (*E. areolatus*), dusky-tail grouper (*E. bleekeri*), and brown-spotted grouper (*E. chlorostigma*) because they both have spots with almost similar colors on his body (Tavakoli-Kolour *et al.*, 2022). This confuses identifying grouper species. Misidentification of grouper species often occurs not only by residents but also by scientists (Félix-Hackradt *et al.*, 2022). Usually, grouper identification is done by observing their color, body pattern, body shape, morphology variations, fin elements, and size (Tapilatu *et al.*, 2021). However, this identification method has a weakness. It is very dependent on expert taxonomists to see visuals and understand taxonomic information from publications and books (Teletchea, 2010). This is the reason that morphological identification is considered a traditional method in the current era (Hallam *et al.*, 2021).

DNA barcoding is one of the latest methods that can be used to identify fish more accurately than morphological methods. This method has the advantages of having high accuracy, and cost-efficiency, and can be used on any stage of fish, whether eggs, larvae, or adults (Ward *et al.*, 2009; Hallerman, 2021). COI (Cytochrome Oxidase subunit I) is a gene target used to differentiate fish between species. COI gene is used because of its low variation within species compared to between species so it is easy to differentiate (Valen *et al.*, 2021). The COI gene also has a low mutation rate compared to the cytochrome b gene (Riyadini *et al.*, 2020). The use of COI of this gene is often used to identify groupers

(Fadli *et al*., 2021; Liang *et al*., 2021; Tavakoli-Kolour *et al*., 2022). This research aims to identify grouper fish with an orange-spotted pattern (areolate grouper) from Saudi Arabia and Indonesia morphologically and molecularly. Apart from distinguishing grouper species between Saudi Arabia and Indonesia, this research also aims as a conservation reference for grouper.

Conservation is a crucial aspect to preserve species diversity from human threats (Miqueleiz *et al*., 2020). Biodiversity preservation can be done by understanding the characteristic traits of fish through morphological and molecular identification (Ali *et al*., 2020). Biodiversity preservation in areolate grouper is very important because every year this fish experiences a decline in abundance due to human activities (Simbolon *et al*., 2020). This fish is one of the groupers with high demand and the price reaches 11 to 25 USD kg^{-1} (Yusuf *et al*., 2023). Until now, areolate grouper has not been successfully seeded so its sustainability continues to be threatened (Vicente, 2020).

This research sample was taken from Saudi Arabia and Indonesia. The distribution of areolate grouper is in Indo-Pacific waters, including Saudi Arabia and Indonesia (Andriyono *et al*., 2020). The two countries have different climates, where Saudi Arabia has 2 climates, tropical and sub-tropical while Indonesia is tropical (Adyasari *et al*., 2021; Faraj *et al*., 2023). Differences in living environments can cause fish of the same species to have different body shapes (Amoutchi *et al*., 2023). Therefore, this study also compared the morphological and molecular information of areolate grouper from Saudi Arabia and Indonesia. Appropriate species identification will contribute to formulating efficient conservation strategies through understanding marine species diversity, especially areolate grouper.

RESEARCH METHODS

Time and Research Location

This research was carried out from November 2023 to January 2024. The samples taken were 20 areolate grouper fish from Yanbu Waters, Saudi Arabia (23°58'18.985"N, 37°54'28.618"E) and the Java Sea or north of Lamongan Regency, Indonesia (6°47'18.171"S, 112°21'4.2978"E). The research locations are shown in Figure 1. The fish were taken and put in a cool box to preserve them according to previous research procedures and taken to the laboratory for morphological identification (Astuti *et al*., 2022). Morphological identification for samples from Saudi Arabia was carried out at the Faculty of Marine Sciences, King.

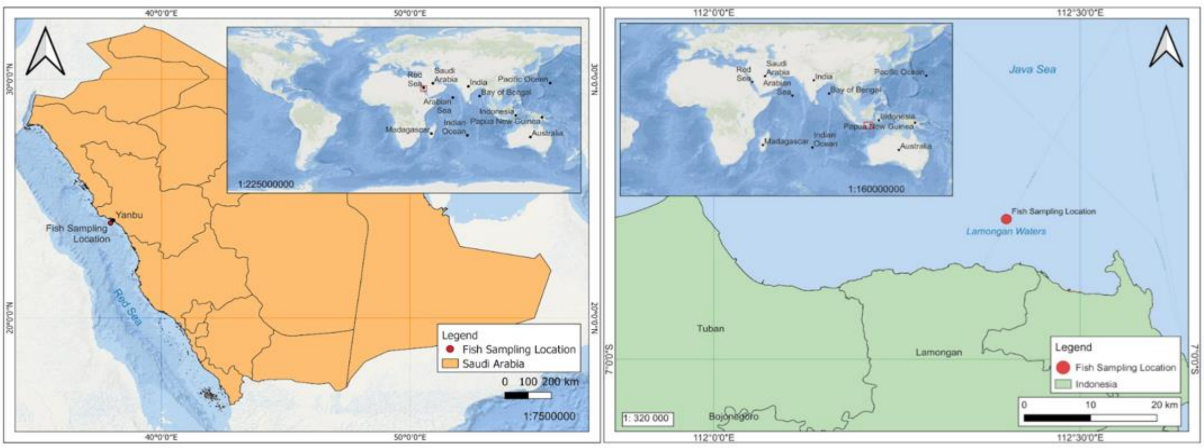

Figure 1. Sampling site of this study. (Left) from Yanbu, Saudi Arabia; (Right) from Lamongan, Indonesia.

Abdulaziz University, Jeddah while samples from Indonesia were conducted at the Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya

Morphological Identification

Determining the sampling sites was done using purposive sampling, the place where areolate grouper was most commonly found (Fadhilah *et al.*, 2020). The locations of this study were in a coral area since Epinephelinae such as the areolate grouper was often found there (Andriyono *et al.*, 2020). Groupers were found at depths of 1-100 m in coral areas (Ergüden *et al.*, 2021). The groupers that had been taken were placed in a cool box with ice, tagged, and taken to the laboratory for morphological identification as described in a previous study (Astuti *et al*., 2022).

Morphological identification was carried out by previous studies (Darwin *et al*., 2020). The identification method consisted of morphometric and meristic tests. Morphometric tests included total length, standard length, head length, eye diameter, body depth, snout length, pectoral fin length, pelvic fin length, preanal length, pre-pectoral length, dorsal base, anal base, dorsal spine height, soft dorsal spine height, and anal spine height. (Elamin *et al*., 2016) while the meristic test includes dorsal fin (spines, rays), anal fin (spines, rays), pectoral fin rays, pelvic fin rays, lateral line scales, and vertebrae (Darwin *et al*., 2020). The results of the morphometric and meristic tests were compared with other reported areolate grouper data (Heemstra and Randall, 1993; Elamin *et al*., 2016; Darwin *et al*., 2020). After the morphology test was completed, the areolate grouper samples obtained were subjected to molecular identification.

Molecular Identification

The pectoralis fins of the groupers were cut and stored in 96% ethanol for further analysis as described in a previous study (Fadli *et al*., 2021). The fin samples were sent to Oseanogen Baruga Indonesia (Bogor, Indonesia) for molecular identification. These samples were extracted to obtain the pure DNA needed and do not experience degradation (Rasmussen and Morrissey, 2008). The protocol for performing DNA extraction was to use the Gene Aid gSYNC extraction kit according to the manufacturer's procedures. The primers used in this study were Fish F1 (5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3') and Fish R1 (5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3') (Ward *et al*., 2005). This primer was a COI (Cytochrome C Oxidase Subunit I) gene with 650 bp (base pairs) as target DNA. After the extraction process was complete, the PCR (Polymerase Chain Reaction) process (BiosystemsTM VeritiTM 96-Well Thermal Cycler, Thermo Fisher Scientific) was conducted. This PCR aimed to amplify and duplicate the required DNA target so that the amount was sufficient to be used in this case for molecular identification (Kotsanopoulos *et al*., 2021). The steps of PCR in this study were predenaturation (94°C for 2 minutes), denaturation (94°C for 45 seconds), annealing (45°C for 45 seconds), extension (72°C for 1.5 minutes), and final extension (72°C for 10 minutes) for 40 cycles of amplification. The results of this PCR would be visualized using 2% agarose gel electrophoresis. This process used 25 µg/mL EtBr (Ethidium Bromide) and 1-kb-marker which was applied with a voltage of 1000 v for 20 minutes. The electrophoresis result was placed under UV light to see the bands. If there were bands then sequencing was carried out in the same place (Oseanogen Baruga Indonesia, Bogor, Indonesia).

The sequencing results obtained were analyzed with MEGA X and compared with NCBI (National Center for Biotechnology Information) using the BLAST (Basic Local Alignment Search Tool) method. The results of BLAST will show similarity and query cover with species listed on NCBI. Phylogenetic tree analysis was carried out with MEGA X with the ClustalW program.

The method used was Kimura 2-Parameter (K2P) with Maximum Likelihood settings and a total bootstrap of 1000 (Fadli *et al.,* 2021).

RESULTS AND DISCUSSION

Morphological Identification of Grouper Samples

Morphological analysis on samples from Yanbu (Saudi Arabia) and Lamongan (Indonesia) was done. The research results showed that the groupers taken in this study were as follows:

Family	Serranidae (Swainson, 1839)
Genus	*Epinephelus* (Bloch, 1793)
Species	*Epinephelus areolatus* (Forsskål, 1775) Areolate grouper

These two samples had an elongated and robust body shape following Epinephelinae in general (Wu *et al*., 2020). The genus *E. areolatus* has a body depth at the dorsal fin that is longer than the depth at the anus, in contrast to *Plectropomus* or *Mycteroperca* which have the same or shorter body depth at the dorsal fin compared to a depth at the anus (Heemstra and Randall, 1993). The heads of groupers in samples from these two countries also did not have concave like *Cromileptes* (Cao *et al*., 2023). The caudal fin in these two samples was truncated, to slightly marginate or concave so it belongs to *Epinephelus* (Randall and Ben-Tuvia, 1983). The caudal fin in other genera of groupers, *Cephalopolis,* is rounded (Nakamura *et al*., 2020). Areolate grouper has another name, yellow-spotted rockrod, because it has a white or gray color with yellow or brownish-yellow spots and a white caudal fin (Darwin *et al.,* 2020; Boddington *et al*., 2021). This characteristic is by the sample in this study which showed a grayish color with brownish yellow spots. Sometimes these characteristics made people misidentify areolate grouper with similar *Epinephelus* such as brown-spotted grouper (*E. chlorostigma*), dusky tail grouper (*E. blekeeri*), and orange-spotted grouper (*E. coioides*) because of their spots with similar colors (Tavakoli-Kolour *et al*., 2022). *E. bleekeri* has a white to brownish-gray body color with brown spots, while *E. chlorostigma* has a darker body color and darker (brown) spots (Darwin *et al*., 2020). *E. coioides* is white-brown with orange or reddish-brown spots (Wang *et al*., 2007). The spots on *E. areolatus* are also further apart and not as many compared to *E. geoffroyi* or *E. chlorostigma* which have more spots and closer distances (Randall *et al*., 2013). When compared with the others, areolate grouper has a white color on the caudal fin so it is easy to distinguish (Darwin *et al*., 2020). Areolate grouper has a pectoral fin longer than the pelvic fin, a pelvic fin that approaches and even reaches the anus, and a smaller front head than those on the operculum (Heemstra and Randall, 1993). The sample images and morphological comparisons are shown in Figure 2.

Epinephelus areolatus or areolate grouper can be found in the Red Sea, Persian Gulf, Indo-Pacific Archipelago, Indian Ocean, and South China Sea (Rothman *et al.,* 2016). The habitats of *E. areolatus* are coral reefs, rocky reefs, seagrass, and sediment bottoms (Lin *et al*., 2022; Yusuf *et al*., 2023). Not only in the ocean, *E. areolatus* can also be found in coastal water (Boddington *et al*., 2021). *E. epinephelus* lives at depths of less than 200 m (Yusuf *et al*., 2023). This fish is an important commercial fish in the world and one of the most sought-after fish in seafood trade activities (Vicente, 2022). Unfortunately, this fish cannot yet be cultivated, although until now researchers are still trying to make breeding of this fish successful (Vicente, 2020).

The standard length of grouper results in Yanbu (Saudi Arabia) showed values of 26.7-31.1 cm while Lamongan (Indonesia) showed 16.8-21.7 cm. This value is around 3 times of the head length (8.3-10.3 cm for Yanbu and 6.7-8.1 cm for Lamongan). A previous study

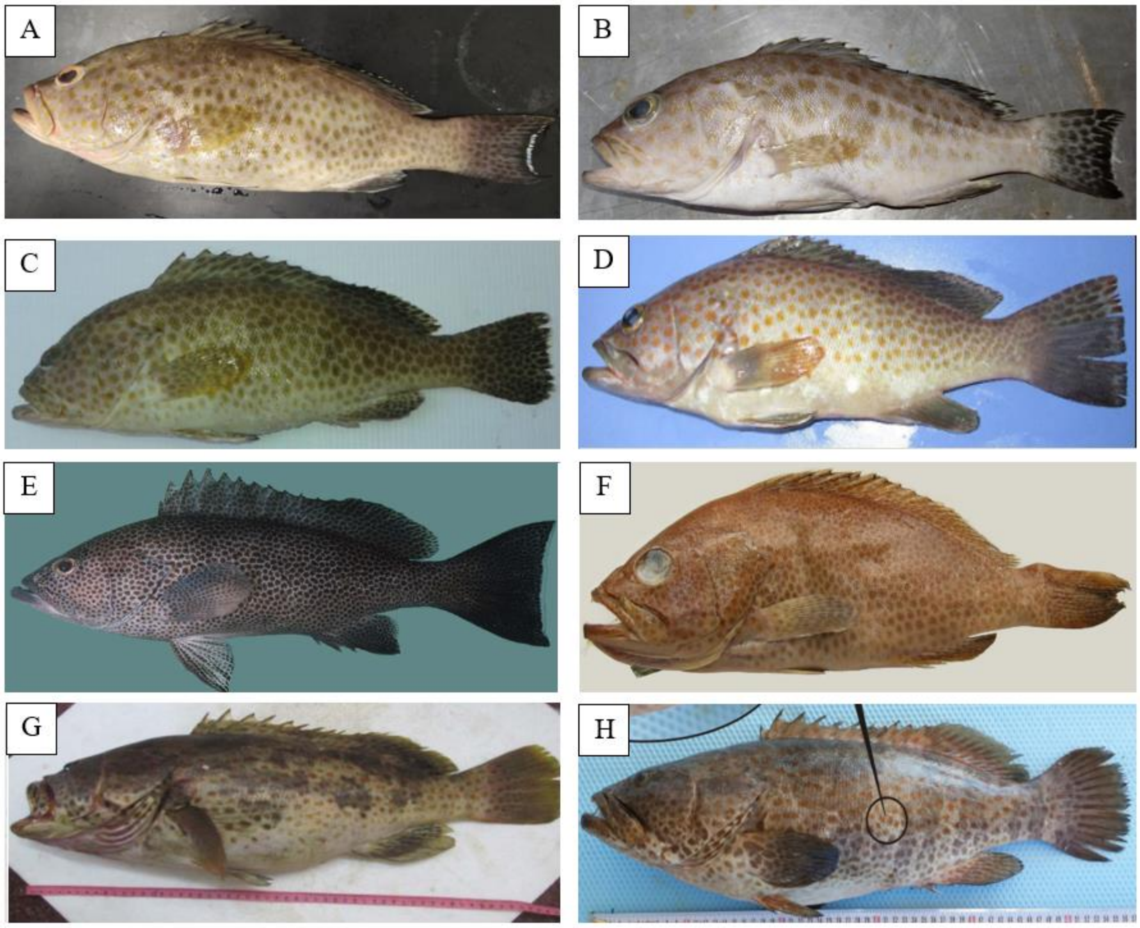

Figure 2. *Epinephelus* grouper morphology comparison. (A) This study from Yanbu (Saudi Arabia); (B) This study from Lamongan (Indonesia); (C) *E. areolatus* (Osman *et al*., 2018); (D) *E. bleekeri* (Saha *et al*., 2022); (E) *E. chlorostigma* (Randall *et al*., 2013); (F) *E. geoffroyi* (Randall *et al*., 2013); (G) *E. fuscoguttatus* (Bunawan *et al*., 2015); (H) *E. coioides* (Gökoğlu and Özvarol, 2015).

stated that *E. areolatus* has a head length of 2.7-3.3 times the standard length (Allen and Erdmann, 2012). The body depth of groupers in Yanbu (3.3-4.1 cm) and Lamongan (4.5-5.8 cm) was also smaller than the head length. This is due to the previous study's statement that this species' body depth is less than its head length (Heemstra and Randall, 1993). The value is not much different from other *Epinephelus* such as *E. bleekeri*, *E. chlorostigma*, and *E. coioides* from previous studies (Ghosh *et al*., 2017; Saleh *et al.*, 2017). The *Epinephelus* genus can be differentiated based on skin color and morphometrics, but this often confuses identification, so a meristic test or DNA barcoding is needed (Ma and Craig, 2022). The morphometric results of groupers in this study and comparison with previous studies can be seen in Table 1.

The meristic results of grouper samples from Yanbu (Saudi Arabia) were as follows: dorsal fin X-XI/12-15; anal fins II-III/8-9; pectoral fins 13-15; pelvic fin I-5; lateral line scales 48-53; vertebrae 24. Meanwhile, the meristic results of grouper samples from Lamongan (Indonesia)were as follows: dorsal fin X-XI/15-17; anal fins II-III/8; pectoral fins 16-19; pelvic fin I-5; lateral line scales 48-53; vertebrae 24. The meristic results were not much different from those reported by previous studies (Heemstra and Randall, 1993; Darwin et al., 2020). Compared with

Table 1. Morphometric parameters of areolate grouper collected from Indonesia and Saudi Arabia in this study compared with previous studies

Morphometric Parameter	Yanbu, Saudi Arabia (this study)	Lamongan, Indonesia (this study)	*E. areolatus* (Hassan and Ahmad, 2023)	*E. bleekeri* (Saha *et al*., 2023)	*E. chlorostigma* (Saleh *et al*., 2017)	*E. coioides* (Ghosh *et al*., 2017)
Total length (cm)	34.8-36.3	18.1-24.4	20.5	21.9-26.5	17-62.9	14.19-83.08
Standard length (cm)	26.7-31.1	16.8-21.7	17	18-22	14-53.3	11.6-69.75
Head length (cm/%SL)	8.3-10.3 /21,57-22.5	6.7-8.1 /37.3-40	- /37	- /42.73–42.91	5.71-18.42 /-	4.61-32.03 /-
Body Depth (cm/%SL)	3.3-4.1 /12.34-13.2	4.5-5.8 /26.7-28.6	- /35	- /31.56–31.68	4.8-19.5 /-	-
Pectoral fin length (cm/%SL)	7.0-7.8 /22.54-25.42	2.9-3.7 /17.1-18.3	- /22	- /-	3.2-9.84 /-	2.48-13.92 /-
Pelvic fin length (cm/%SL)	3.4-4.1 /	2.0-2.3 /	- /17	- /-	2.69-8.95 /-	2.06-10.64 /-
Preanal length (cm/%SL)	17.5-17.7 /56.99-65.42	9.7-12.4 /57.14-60	- /72	- /69.44–69.47	- /-	- /-
Prepectoral length (cm/%SL)	8.8-9.1 /29.3-32.9	6.2-6.9 /31.8-37.1	- /35	- /36.67–36.68	- /-	- /-
Dorsal base (cm/%SL)	17.4-17.9 /57.63-65.05	9.5-10.5 /48.4-56.5	- /-	- /58.32–58.33	- /-	- /-
Anal base (cm/%SL)	10.6-11.4 /36.7-39.63	6.4-7.0 /32.3-38.1	- /-	- /15.50–15.56	- /-	- /-
Dorsal spine height (cm/%SL)	4.2-4.5 /14.49-15.7	1.6-2.4 /9.5-11.4	- /12	- /12.22–12.25	- /-	- /-
Soft dorsal height (cm/%SL)	4-4.3 /14.96-15.36	1.4-1.8 /8.29-8.33	- /12	- /-	- /-	- /-
Anal spine height (cm/%SL)	3.4-3.6 /12.14-13.46	1.3-1.8 /7.7-8.3	- /6	- /11.67–11.76	- /-	- /-
Eye diameter (cm/%HL)	1.9-2.0 /29,85-32,20	1.2-1.6 /17.91-20.00	- /15	- /19.39–19.52	1.1-2.85	0.83-3.02 /-
Snout length (cm/%HL)	3.3-3.9 /55.93-58.73	1.3-1.9 /19.40-23.46	- /-	- /15.57–15.64	1.06-6 /-	0.88-9.98 /-
Total weight (gram)	501.28-532.42	99.32-134.65	185	140-169.1	-	-

Notes: SL (Standard Length), HL (Head Length).

other *Epinephelus* species, meristic results showed no difference. According to previous studies, *Epinephelus* has 10-11 dorsal fin spines, 7-10 anal fin rays, and a lack of trisegmental pterygio-phores (Ma and Craig, 2018). Another study also provided that meristic data such as vertebrae in the genera Epinephelus showed values that were not significantly different (Lim *et al*., 2016). However, the pectoral fin results showed that there were fish with fewer rays in the grouper samples from Yanbu (13-15 rays). Meristic differences are possible due to differences in geography, life history, phyletic position, biome, and environmental factors such as salinity or temperature (Lim *et al*., 2016). Meristic results and comparisons with previous studies can be seen in Table 2.

Comparison of Grouper Samples from Saudi Arabia and Indonesia

The comparison of the morphology of grouper samples from Yanbu (Saudi Arabia) and Lamongan (Indonesia) is shown in Figure 3. The difference between the grouper from Yanbu (Saudi Arabia) and Lamongan (Indonesia) was the spot (Figure 3, red color). The spots of grouper from Yanbu were smaller but more numbers, and closer distance between each other. This was different from the grouper sample from Lamongan in that the spots were larger, fewer in number, and wider distance between spots. Apart from spots, the caudal fin also had differences. The top and bottom of the Yanbu grouper's caudal fin showed a longer size than the middle part while the Lamongan grouper's caudal fin was a straight, parallel shape. Previous studies stated that pattern variations in *Epinephelus areolatus* were caused by fish size and geographical distribution (Rothman *et al*., 2016). For example, *E. areolatus* found in South Korea has darker and larger spots (Kim and Song, 2010). Meanwhile, *E. areolatus* found in the Gulf of Suze, near the Red Sea, has smaller spots and is brighter in color (Osman *et al*., 2018).

Molecular Identification of Areolate Grouper Samples

The electrophoresis result of this study can be seen in Figure 4. This showed that the COI gene of grouper from Yanbu (Saudi Arabia) and Lamongan (Indonesia) could be amplified. By using the COI gene as a primer, this research obtained 652 bp of sequences

Table 2. Meristic parameter of orange-spotted grouper collected from Indonesia and Saudi Arabia in this study compared with the previous study

Meristic Parameter	Yanbu, Saudi Arabia (this study)	Lamongan, Indonesia (this study)	*E. areolatus* (Heemstra and Randall, 1993)	*E. areolatus* (Darwin *et al*., 2020)	*E. bleekeri* (Saha *et al*., 2023)	*E. chlorostigma* (Wu *et al*., 2020)	*E. coioides* (Ghosh *et al*., 2017)
Dorsal fin (spines)	X-XI	X-XI	XI	XI	XI	XI	-
Dorsal fin (rays)	12-15	15-17	15-17	14-16	17	16	-
Anal fin (spines)	II-III	II-III	III	III	III	III	-
Anal fin (rays)	8-9	8	8	8	8	8	-
Pectoral fin rays	13-15	16-19	17-19	17-19	19	18	18
Pelvic fin rays	I-5	I-5	-	-	I-5	I-5	I-5
Lateral line scales	48-53	48-53	49-53	49-53	-	52	-
Vertebrae	24	24	-	24	33	-	

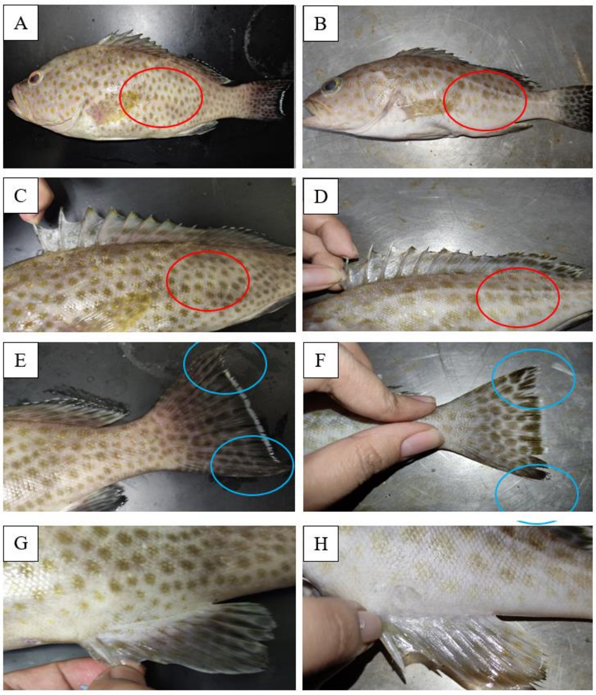

Figure 3. Comparison of *E. areolatus* morphology from Yanbu (Saudi Arabia) and Lamongan (Indonesia). (A) *E. areolatus* from Yanbu (Saudi Arabia); (B) *E. areolatus* from Lamongan (Indonesia); (C) Dorsal fin of *E. areolatus* from Yanbu (Saudi Arabia); (D) Dorsal fin of *E. areolatus* from Lamongan (Indonesia); (E) Caudal fin of *E. areolatus* from Yanbu (Saudi Arabia); (D) Caudal fin of *E. areolatus* from Lamongan (Indonesia); (E) Anal fin of *E. areolatus* from Yanbu (Saudi Arabia); (D) Anal fin of *E. areolatus* from Lamongan (Indonesia). Red color: spots different (Yanbu: smaller and more in number; Lamongan: bigger and less in number).

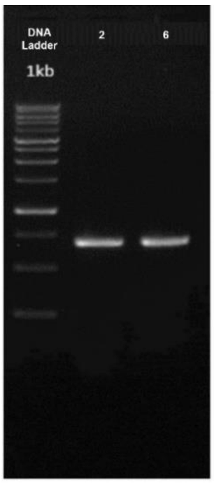

Figure 4. The electrophoresis of COI gene from grouper samples in this study. 1. Marker; 2. Grouper from Yanbu (Saudi Arabia); 4. Grouper from Lamongan (Indonesia).

from grouper samples of Yanbu, Saudi Arabia. Compared with other sequences from NCBI, the grouper obtained from Yanbu was *Epinephelus areolatus*. This was because this sample had 100% similarity to *E. areolatus* with accession number MH707739.1. DNA sequences of *E. areolatus*' COI gene in Saudi Arabia in NCBI had been reported in a previous study, 8 fish by Rabaoui *et al.* (2019) from Saudi waters in the Gulf and 1 by Coker *et al*. (2017) from Farasan Island on the Red Sea. This study was the first reported COI gene from Yanbu, Saudi Arabia, Red Sea. Previously, research related to DNA identification in *E. areolatus* from Yanbu itself had been carried out using 12S rRNA and Otx1B (Shaikh-Omar *et al*., 2020; Shaikh-Omar *et al*., 2022). The report of the COI gene in *E. areolatus* in Yanbu is very important for the conservation of this fish species (Petit-Marty *et al*., 2021). This COI gene is also reported to have better performance than other genes in differentiating organisms (Mohanty *et al*., 2015; Koroiva and Santana, 2022). This research complements the reported gene in *E. areolatus* from Yanbu, Saudi Arabia. The similarity of this specimen is shown in Table 3.

Using the COI gene as a primer, this research obtained 667 bp of areolate grouper sample sequences from Lamongan, Indonesia. Both forward and reverse clusters were used to obtained sense and antisense sequences. This process should be done to obtain valid COI gene sequences for comparison between Lamongan and Yanbu grouper. Compared with other sequences from NCBI, the grouper obtained from Lamongan, Indonesia is *Epinephelus areolatus*. This sample had 99.85% similarity to *E. areolatus* with accession number KF009591.1. There were 28 COI genes of *E. areolatus* from Indonesia reported to NCBI. The reports of *E. areolatus* in Indonesia were obtained from various places such as Maluku, Aceh, Madura, and even Papua (Limmon *et al*., 2020; Basith *et al*., 2021; Andriyono *et al*., 2022; Dwifajri *et al*., 2022). This sample was taken from Lamongan, East Java, Indonesia. This location was close to the Java Sea. The existence of this fish in the Java Sea and its surroundings has been reported by previous studies (Basith *et al*., 2021; Yusuf *et al*., 2023). However, research related to *E. areolatus*' DNA from Lamongan had never been reported and this study was the first. The similarity of this specimen is shown in Table 4.

The results of the phylogenetic tree can be seen in Figure 5. The phylogenetic tree functions to help classify organisms into taxonomic groups based on evolutionary relationships, not just morphological

Table 3. Identification result (Query Coverage and Percent Identity) of grouper sample from Yanbu, Saudi Arabia compared with the same genus and family using BLAST

Sample	Query Cover (%)	Similarity (%)	Accession Number
Epinephelus areolatus	100	99.85	MH707739.1
Epinephelus areolatus	99	97.08	KF009591.1
Epinephelus bleekeri	94	89.50	KU722926.1
Epinephelus geoffroyi	94	89.16	MH331752.1
Epinephelus fuscoguttatus	94	84.81	KU722932.1
Plectropomus leopardus	99	82.00	KJ130973.1
Plectropomus areolatus	100	81.90	MF185598.1

Table 4. Identification result (Query Coverage and Percent Identity) of grouper sample from Lamongan, Indonesia compared with the same genus and family using BLAST

Sample	Query Cover (%)	Similarity (%)	Accession Number
Epinephelus areolatus	97	99.85	KF009591.1
Epinephelus areolatus	97	99.54	MN708836.1
Epinephelus bleekeri	92	89.66	KU722926.1
Epinephelus geoffroyi	92	89.50	MH331752.1
Epinephelus fuscoguttatus	92	86.13	KU722932.1
Plectropomus leopardus	98	83.51	KJ130973.1
Plectropomus areolatus	96	82.89	MF185598.1

similarities (Neves *et al*., 2020). The phylogenetic tree result showed that the grouper samples from Yanbu (Saudi Arabia) and Lamongan (Indonesia) in this study were in the same clade as *E. areolatus* (MN870557.1 and MT076840.1). This indicated that the COI gene could differentiate between one grouper species and another. The COI gene has been used to detect grouper species. COI is a gene target used to differentiate fish between species. COI gene is used because they have low variation within species compared to between species, so they are easy to differentiate (Valen *et al*., 2021). The use of molecular techniques for identification is very important because this method is more accurate than morphological which can sometimes be subjective depending on the ability of the person observing (Behrens-Chapuis *et al*., 2021). For example, residents in the Persian Gulf or Oman Sea regions sometimes find it difficult to differentiate orange-spotted grouper (*E. coioides*) from similar *Epinephelus* such as *E. areolatus*, *E. blekeeri*, and *E. chlorostigma* because of their spots with almost similar colors on their body (Tavakoli-Kolour *et al*., 2022). Uniquely, the results of this study showed that the sample from Saudi Arabia had a different clade from Indonesia. The phylogenetic tree results indicated that samples from Yanbu were in the Western Indian Ocean clade like samples from Saudi Arabia (KU499782.1), India (KJ607969.1), and United Arab Emirates (MT076840.1). Meanwhile, samples from Lamongan were in the Western Pacific clade such as Indonesia (MN870557.1), Vietnam (MN708836.1), and China (FJ237755.1). This is due to the previous study's statement that there is significant geographic separation in this species (Rothman *et al*., 2016). The sequences of areolate grouper were submitted to NCBI (accession number PP388919.1 for Lamongan and PP388920.1 for Saudi Arabia).

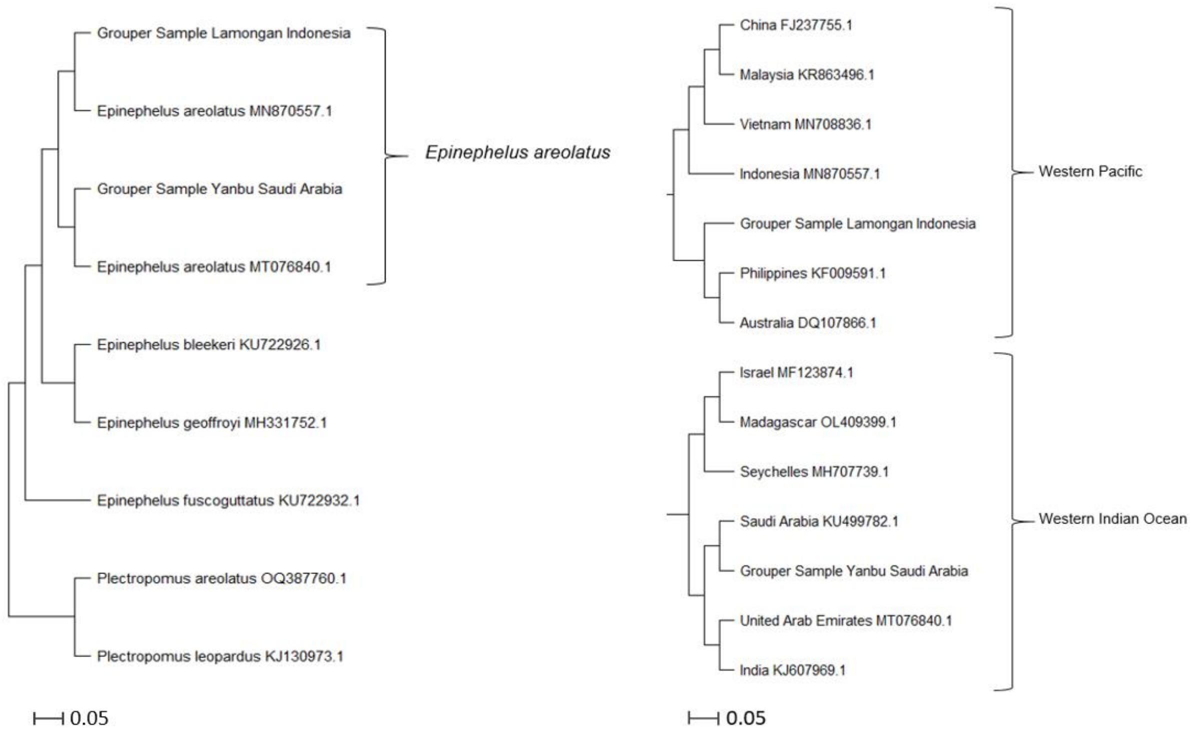

Figure 5. Phylogenetic tree of areolate grouper from this study. (Left) Relationship of *E. areolatus* from this study with another grouper; (Right) Relationships of *E. areolatus* from Western Pacific and Western Indian Ocean.

CONCLUSION

It could be concluded that the grouper from this study (from Yanbu, Saudi Arabia, and Lamongan, Indonesia) was *Epinephelus areolatus* (areolate grouper) morphologically and molecularly. Morphologically, grouper samples from Yanbu (Saudi Arabia) were as follows: dorsal fin X-XI/12-15; anal fins II-III/8-9; pectoral fins 13-15; pelvic fin I-5; lateral line scales 48-53; vertebrae 24. Meanwhile, the meristic results of groupers from Lamongan (Indonesia) were as follows: dorsal fins X-XI/15-17; anal fins II-III/8; pectoral fins 16-19; pelvic fin I-5; lateral line scales 48-53; vertebrae 24. The morphological differences between *E. areolatus* from Saudi Arabia and Indonesia were its spots and caudal fin. Molecular results on *E. areolatus* showed different clades. Samples from Saudi Arabia belonged to the Western Indian Ocean clade while Indonesia belonged to the Western Pacific. This showed that there were morphological and molecular differences between *E. areolatus* from Yanbu (Saudi Arabia) and Lamongan (Indonesia). The sequences of areolate grouper were submitted to NCBI (accession number PP388919.1 for Lamongan and PP388920.1 for Saudi Arabia). This research data can be used as a reference for conservation.

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