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DNA Barcoding of Six Commercially Important Groupers (Epinephelidae) from Langsa, Aceh, Indonesia

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ABSTRACT

Groupers are among the fish groups that are difficult to recognize due to their high morphological similarities. Therefore, molecular techniques, particularly DNA barcoding, are extensively utilized to differentiate this fish group. This study aimed to analyze and validate six grouper species belonging to the Epinephelidae family that were harvested from Langsa district waters in Aceh province, Indonesia, based on DNA barcode data. It was conducted from June to December 2021, with the fish specimens collected from fishers at fish landing sites and the fish market in Langsa City, Aceh province. A total of 22 grouper sequences belonging to six species were generated, namely *Epinephelus coioides*, *E. bleekeri*, *E. malabaricus*, *E. erythrurus*, *E. sexfasciatus* and *Mycteroperca poecilonotus* (formerly *Epinephelus poecilonotus*). Genetic distance within these species ranged from 0.10 to 0.73% (average: 0.40%). Notably, *E. malabaricus* and *E. coioides* exhibited the closest genetic kinship (4.07%), while *E. sexfasciatus* and *M. poecilonotus* displayed the greatest genetic distance (19.33%). This study provides the first DNA reference for grouper in Langsa district, Indonesia, with significant implications for future sustainable grouper management.

1. Introduction

Groupers are demersal fishes (Heemstra and Randall 1993) that generally predominantly inhabit shallow waters, such as coral reefs, estuaries, mangroves, and seagrasses (Heemstra and Randall 1993; Craig *et al.* 2011; FRCI 2021; Froese and Pauly 2023). Geographically, grouper can be found in tropical and sub-tropical waters across the Indo-Pacific, Atlantic, Mediterranean, and Red Sea (Craig *et al.* 2011; Froese and Pauly 2023). A total of 163 grouper species have been recorded worldwide (Craig *et al.* 2011), with Indonesian waters hosting at least 77 (FRCI 2021).

Epinephelus is a highly abundant genus with significant global commercial importance (Behera *et al.* 2015). These fish species are considered promising for intensive aquaculture due to their rapid growth, high consumer demand, nutritional value, and efficient feed conversion ratio (Ganeshalingam *et al.* 2023). As a fish group, grouper possesses substantial economic value in Indonesia and worldwide, with its production steadily increasing yearly (Cawthorn and Mariani 2017; Rimmer and Glamuzina 2017). For example, the total volume of grouper exports in Indonesia witnessed a 19.77% increase, equivalent to 1,216.56 kg, during the second quarter of 2021 compared to the same period in 2020 (Suhana 2022). This upward trend is also evident in grouper fisheries in Aceh province, with production escalating from 1,631.3 tons in 2017 to 3,771.8 tons in 2021 (DKP-Aceh 2022).

Langsa City is in the Eastern Aceh region, directly adjacent to the Malacca Strait. This region is renowned for its mangrove ecosystem. The mangroves in Langsa cover an area of 6,172.42 hectares (Iswahyudi *et al.* 2019), with 119 hectares designated as the Mangrove Forest Park (BPS-Langsa 2023). Capture fisheries play

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a vital role in Langsa City, with a production volume of 1,227.39 tons in 2022 (https://statistik.kkp.go.id/). Groupers are one of the most frequently caught fish species and land in Langsa City fishing port (Arkham *et al.* 2021).

Groupers pose a challenge in identification due to their overlapping morphological characteristics. Therefore, molecular approaches, particularly DNA barcoding, are widely employed to distinguish different grouper species. Over the past two decades, several studies have utilized DNA barcoding to investigate grouper worldwide. For example, Alcantara and Yambot (2016) conducted DNA-barcoding study on 27 grouper species in the Philippines. Aziz *et al.* (2016) focused on ten grouper species in peninsular Malaysia for their DNA barcoding study. Basheer *et al.* (2017) DNA barcoded 36 grouper species in Indian waters. In another study, Iswarya Deepti *et al.* (2018) employed DNA barcoding to identify five grouper species in Visakhapatnam, located on the central-eastern coast of India. Similar studies have also been conducted in Indonesian waters. For instance, Jefri *et al.* (2015) investigated seven grouper species in Central and Eastern Indonesia. Basith *et al.* (2021) conducted DNA barcoding study on seven grouper species in Madura, Tapilatu *et al.* (2021) on 16 species in Northern Papua, and Dwifajri *et al.* (2022) on 7 species in Jayapura, Papua.

However, studies on DNA barcoding of grouper are scarce in the Aceh region. Only two previous studies have been conducted on this topic, namely those by Kamal *et al.* (2019) and Fadli *et al.* (2021), which validated five and 26 grouper species, respectively. These previous studies did not cover Langsa region. Hence, this study aimed to analyze and validate grouper species harvested from the waters of Langsa City using the COI gene (DNA barcodes).

2. Materials and Methods

2.1. Sample Collection

The sampling process was conducted in Langsa, Aceh, from June to December 2021. Grouper specimens were collected from local fishers, fish landing sites, and the fish market to ensure they originated from the local seas, as shown in Figure 1. Morphological identification of the fish samples was carried out to determine the species level (Heemstra and Randall 1993; Craig *et al.* 2011; Froese and Pauly 2023). Fin clips were taken from representative samples and preserved in 96% ethanol in a 2 ml microcentrifuge tube. The sample sizes ranged from three to five individuals. Tissue and whole body vouchering and

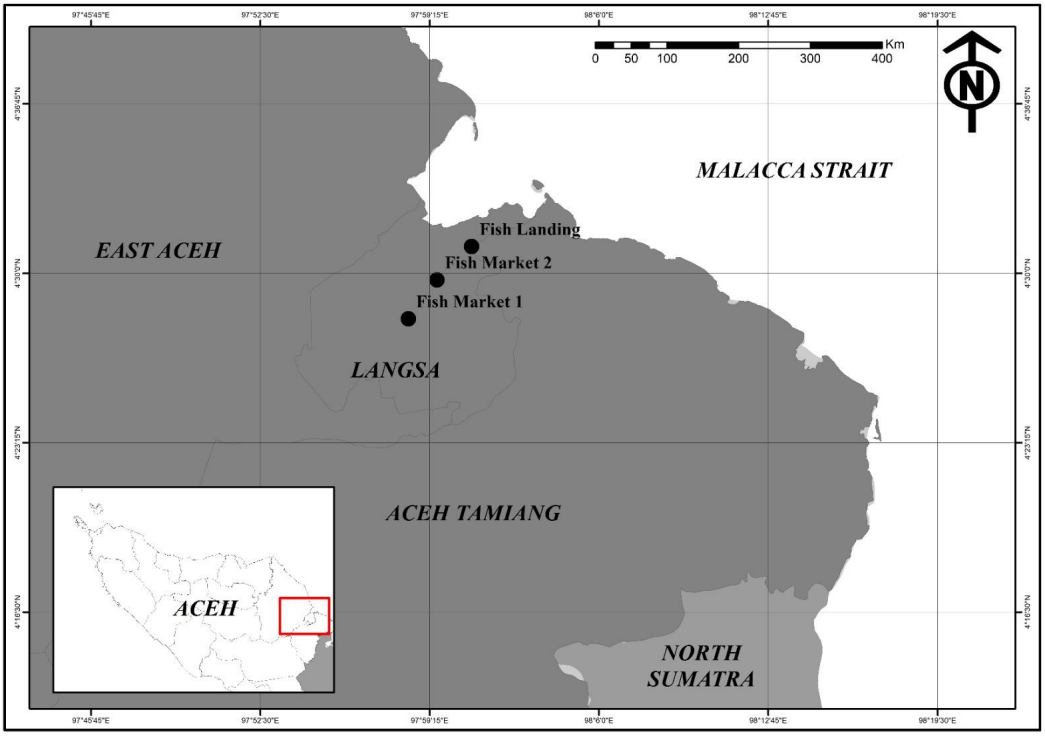


Figure 1. Sampling site of grouper in Langsa, Aceh (black point: sampling sites)

documentation was performed following the Fish-BOL protocol (Steinke and Hanner 2011) and was stored at the Faculty of Marine and Fisheries, Syiah Kuala University at Banda Aceh, Indonesia.

2.2. DNA Extraction, Primer, and PCR Assay

DNA extraction was conducted using the modified CTAB protocol (Grewe *et al.* 1993). The final concentrations of the extracted DNA samples were measured using an NP80 Implen Nanophotometer (https://www.implen.de/). For PCR amplification of the COI gene, primers F1, R1, F2, and R2 from Ward *et al.* (2005) were used, as shown in Table 1. The PCR reactions were set up in a 25 µL master mix containing 8.5 µL of ddH2O water, 12.5 µL of MyTaq Red Mix, 2.0 µL of DNA template, and 1 µL of each primer. Amplification was performed using a Sensoquest gradient thermal cycler (https://www.sensoquest.de/). The thermal cycling conditions consisted of an initial denaturation step at 95°C (2 minutes) followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 49.7-56°C (45 seconds), elongation at 72°C (1 minute), and a final extension step at 72°C (10 minutes) before termination of the reaction at 4 oC. The successfully amplified PCR product was sent to PT. Genetika Sciences in Jakarta for sequencing.

2.3. Data Analysis

All COI sequences obtained were subjected to trimming and alignment using MEGA 6.06 software (Tamura *et al.* 2013). The aligned sequences were then translated into protein to ensure accurate alignment and to identify the presence if any of stop codons. Additionally, this software was used to analyze the base composition and determine the number of variable sites. Pairwise genetic distance, including conspecific, congeneric, and confamilial distance, were calculated using the Kimura-2-parameter (K2P) model, as implemented in MEGA 6.06 (Kimura 1980; Tamura *et al.* 2013). The haplotype distributions were summarized using DnaSP 5.10 software (Rozas *et al.* 2003; Librado and Rozas 2009).

Table 1. COI universal primer sets of Ward *et al.* (2005) were used to identify grouper samples collected in Langsa, Aceh, Indonesia

Name	Primer sequence 5'-3'
Fish F1	TCA-ACC-AAC-CAC-AAA-GAC-ATT-GGG-AC
Fish R1	TAG-ACT-TCT-GGG-TGG-CCA-AAG-AAT-CA
Fish F2	TCG-ACT-AAT-CAT-AAA-GAT-ATC-GGC-AC
Fish R2	ACT-TCA-GGG-TGA-CCG-AAG-AAT-CAG-AA

Initial species identifications were conducted by comparing the COI sequences using BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST) and the BOLD Identification System (IDS) (https://www.boldsystems.org/). The Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.* 2012) was used for species delimitation by determining the number of operational taxonomic units (OTUs) based on pairwise sequence distance among individuals within the dataset (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html). Furthermore, the Neighbor-Joining method (NJ) was used to establish phylogenetic relationships among grouper samples using MEGA 6.06 software (Tamura *et al.* 2013). *Cephalopholis aurantia* (Sample ID: 3 27 1) and *Variola albimarginata* (Sample ID: KC2) sequences were used as outgrups to root the trees (Fadli *et al.* 2021).

3. Results

3.1. Species Composition

In total, 22 grouper sequences belonging to six species were generated in this study, namely *Epinephelus bleekeri, E. coioides, E. malabaricus, E. erythrurus, E. sexfasciatus* and *Mycteroperca poecilonotus* (formerly *Epinephelus poecilonotus*). There were no differences in identification between morphological and DNA barcode (Table 2 and Figure 2).

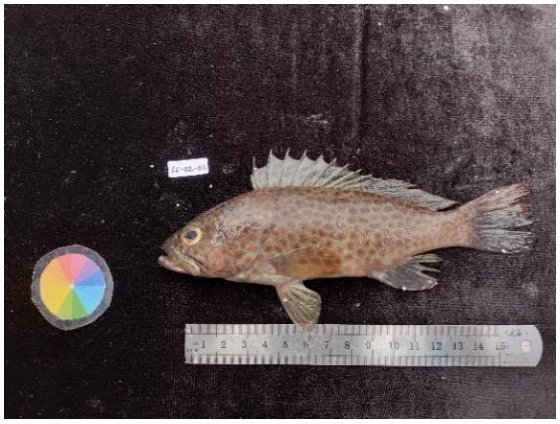
3.2. Cytochrome Oxidase Subunit I Diversity Assessment

The COI sequences obtained had a read length of 639 base pairs (bp) with an average nucleotide composition of A = 24.95%, T = 29.47%, C = 27.79%, and G = 17.79%, as shown in Table 3. Among the aligned sequences, 478 sites were conserved, while 161 sites exhibited variation. Out of these variable sites, 148 were parsimony informative, and 13 were singletons. No insertions, deletions or stop codons were detected in the 22 sequences. The mean GC content was 45,58%, while AT content was higher at 54.42%. The GC content decreased in the order of first position codon, second, to third position codon, as shown in Table 3.

Genetic distance within species based on the COI gene ranged from 0.10% to 0.73%, with a mean distance of 0.40%. Furthermore, Table 4 presents the pairwise comparisons of the COI gene using the Kimura-2-parameter (K2P) distance, both within species and between different grouper species.

Table 2. Comparison of fish identification in the Epinephelidae family based on morphological and DNA barcoding data

n	Species		Similarity (%)	
	Morphology	DNA barcode	BLAST	BOLD
3	*E. bleekeri*	*E. bleekeri*	99.84-100.00	100.00
5	*E. coioides*	*E. coioides*	99.84-100.00	99.84-100.00
4	*E. erythrurus*	*E. erythrurus*	99.84-100.00	100.00
3	*E. malabaricus*	*E. malabaricus*	99.53-100.00	99.65-100.00
4	*E. sexfasciatus*	*E. sexfasciatus*	99.69-100.00	99.84-100.00
3	*M. poecilonotus*	*M. poecilonotus*	98.90-99.84	99.63-100.00
Total 22				



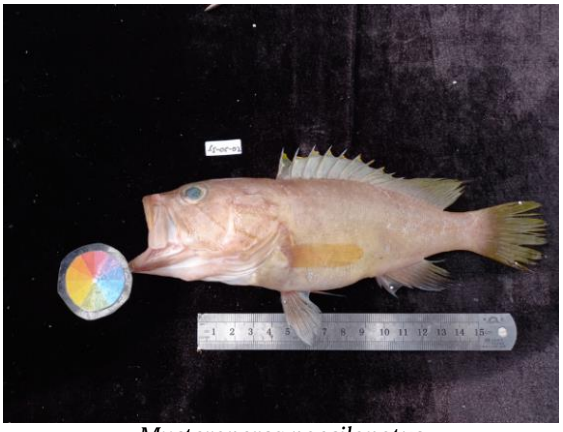
Epinephelus bleekeri

Epinephelus coioides

Epinephelus erythrurus

Epinephelus malabaricus

Epinephelus sexfasciatus



Mycteroperca poecilonotus

Figure 2. Grouper species found in Langsa

3.3. Species Delimitation

Species validation using BLAST and the BOLD database demonstrated a high similarity ranging from 98.90 to 100.00%, indicating accurate species-level identification based on the COI gene. Furthermore, Barcoding Gap Analysis revealed that all observed species had a maximum intra-species distance of less than 2%, supporting the distinction among different species. The mean kinship distance was calculated to be 10.65%, 27 times higher than the average intra-species distance of 0.40%. The final analysis also illustrated that all specimens showed high distance values to their closest kin, indicating a "Barcoding Gap" among six grouper species observed (Table 5 and Figure 3A).

The ABGD analysis resulted in the identification of six OTUs using an initial partition on prior (P) intra-specific divergence ranging from P = 0.001 to P = 0.0359, as performed in ABGD, as shown in Figure 3B.

Table 3. Summary statistics of nucleotide frequencies of COI sequences of samples collected from Langsa

(%)	Min	Mean	Max	SD
G	16.90	17.79	18.62	0.44
C	27.07	27.79	29.42	0.53
A	24.26	24.95	25.51	0.34
T	27.70	29.47	30.52	0.64
GC	43.97	45.58	48.04	0.88

Table 4. Pairwise comparisons of COI genes based on mean K2P distance (%) within species (bold) and among grouper species

Species	1	2	3	4	5	6
E. bleekeri	**0.00**					
E. coioides	15.30	**0.00**				
E. erythrurus	13.93	12.63	**0.01**			
E. malabaricus	14.89	4.07	12.19	**0.00**		
E. sexfasciatus	15.27	16.53	16.29	16.51	**0.00**	
M. poecilonotus	16.26	14.36	15.66	15.16	19.33	**0.01**

Additionally, the NJ tree analysis revealed that all presumed species formed monophyletic clusters, and the relationships between genera were well resolved. Each group of similar individuals displayed a high bootstrap support value of >99%, as shown in Figure 4.

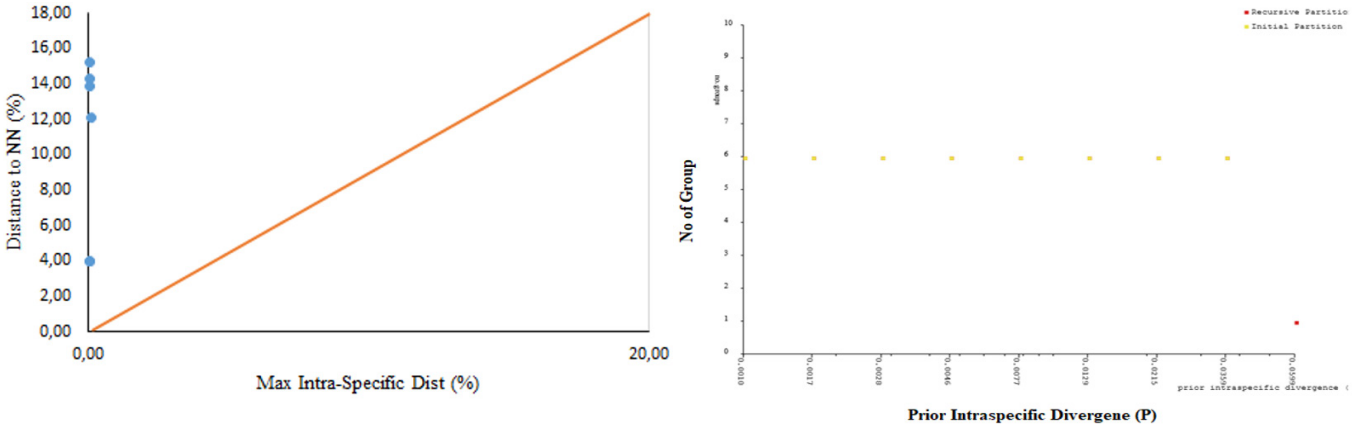
4. Discussion

This study successfully validated the COI gene as a reliable tool for identifying six commercially important species of grouper harvested from the waters of Langsa City, Indonesia. This is the first report on *E. malabaricus, E. erythrurus* and *M. poecilonotus* (formerly *E. poecilonotus*). It is also a complementary molecular data of groupers found in Aceh water, Indonesia.

The BLAST and BOLD analysis results, with sequences displaying 98.90-100.00% similarity to those in the database, confirm the validity of the identified species among six grouper species. The morphological and molecular taxonomy identifications were found to be consistent in this study. This might be explained by the obvious physical differences between the observed species (Craig *et al.* 2011; Froese and Pauly 2023). In addition, the number of species reported in this study is comparable with studies by Deepti *et al.* (2018), who barcoded five grouper species from Visakhapatnam (India), and Basith *et al.* (2021), who DNA barcoded seven grouper species from Madura (Indonesia). However, the number of reported species is lower compared to studies by Alcantara and Yambot (2016) and Tapilatu *et al.* (2021), who barcoded 26 and 16 species of commercial grouper from the Philippines and Papua (Indonesia), respectively. The lower number of species found in Langsa is attributed to the limited coral coverage and the presence of mangroves surrounding the coastal area of Langsa

Table 5. The mean and maximum intraspecific values for each grouper species were compared with the closest kinship distance

Species	Mean intra-species (K2P%)	Mean intra-species (K2P%)	Nearest neighbour	Distance to nearest neighbour (K2P%)
Epinephelus bleekeri	0.00	0.00	*Epinephelus erythrurus*	13.93
Epinephelus coioides	0.00	0.00	*Epinephelus malabaricus*	4.07
Epinephelus erythrurus	0.01	0.01	*Epinephelus malabaricus*	12.19
Epinephelus malabaricus	0.00	0.00	*Epinephelus coioides*	4.07
Epinephelus sexfasciatus	0.00	0.00	*Epinephelus bleekeri*	15.27
Mycteroperca poecilonotus	0.01	0.01	*Epinephelus coioides*	14.36



A B

Figure 3. (A) Maximum intraspecific divergence (K2P%) in the COI barcode region plotted against nearest neighbour distance (%K2P) for six grouper morphospecies studied in this study. Dots above the diagonal line indicate species with barcode gaps, (B) the number of genetically different OTUs according to the intraspecific divergence values generated by ABGD based on K2P

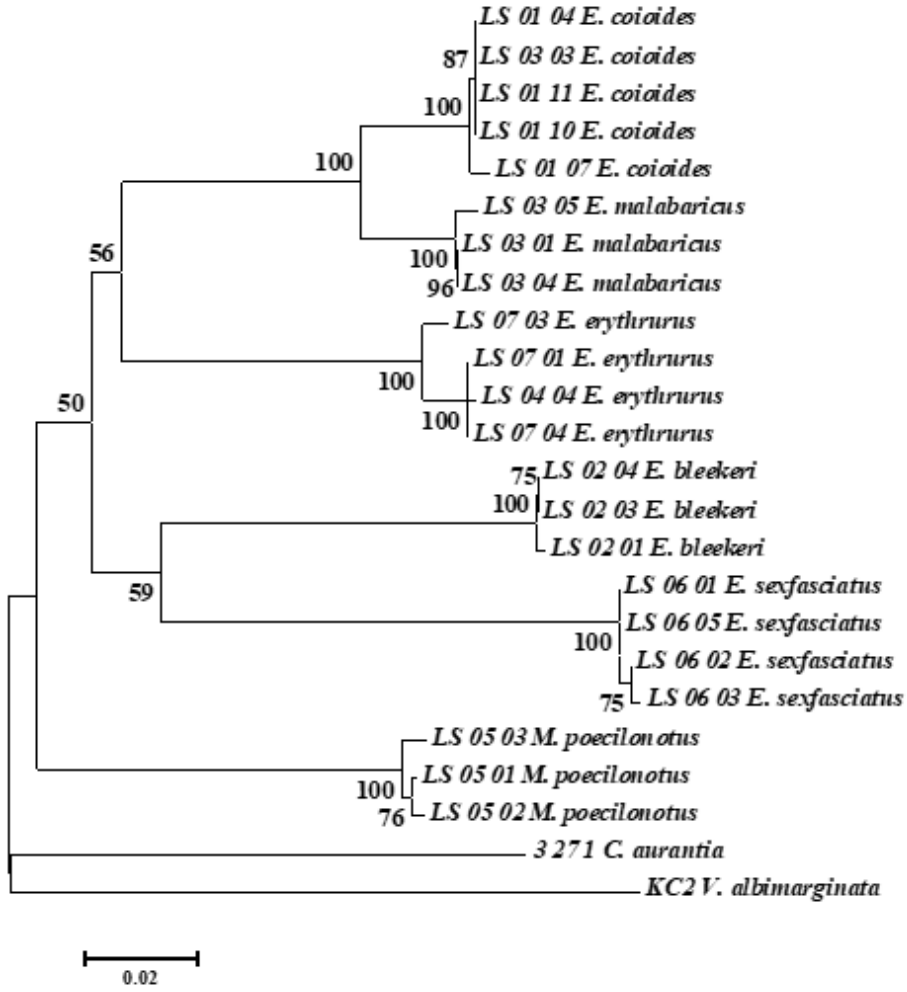


Figure 4. NJ COI barcode tree for all grouper samples. Bootstrap values <50% are not shown, and the scale bar illustrates the percentage divergence calculated based on the K2P model

City (BPS-Langsa 2023). Most grouper species are reef-associated fish (Craig *et al.* 2011; Froese and Pauly 2023). However, six grouper species recorded in this study prefer muddy estuaries and mangroves for habitat (Froese and Pauly 2023).

The preliminary observation in this study revealed interesting findings regarding the geographical structuring of *E. erythrurus* and *M. poecilonotus* from different regions. The analysis indicated that the *E. erythrurus* sequences showed a high similarity of 99.84% with those from China (MF185498) and India (MW810350), but they were relatively distant from those from Madura, Indonesia (97.88%-ON357980). Congruently, *M. poecilonotus* sequences had a high similarity to barcodes from the South China Sea, China (99.84%-FJ237769), and the Philippines (99.80%-KJ594985) but were far removed from the sequences from India (91.71%-KM226286 and KM226287). These findings suggest the existence of geographical variations or population differentiation between *E. erythrurus* and *M. poecilonotus* from different regions.

The mean GC content of 45.58% observed in this study for grouper species is comparable to the GC values reported for grouper specimens from the Philippines (45.16%) (Alcantara and Yambot 2016). However, grouper specimens from India exhibited a slightly higher GC value of 46.06% (Basheer *et al.* 2017). In addition, it is interesting to note that four of the observed species (*E. bleekeri, E. coioides, E. malabaricus* and *E. sexfasciatus*) showed no genetic variations. This lack of genetic diversity may be attributed to various factors, including overfishing, bottleneck events, or extreme environmental pressures (Pinsky and Palumbi 2014; Ketchum *et al.* 2016). The low genetic diversity value is a sign that grouper in Langsa is already experiencing overexploitation. Fadli *et al.* (2021) also showed some grouper species in different part of Aceh (*Cephalopolis sonnerati, E. coeruleopunctatus, E. melanostigma, E. tauvina, Plectropomus leopardus* and *Variola louti*), which displayed a lack of COI genetic variation attributable to the overfishing.

Genetic distance analysis revealed that *Epinephelus malabaricus* and *E. coioides* exhibited the closest kinship, with genetic distance of 4.07%. On the other hand, *E. sexfasciatus* and *M. poecilonotus* displayed the farthest genetic distance, with a value of 19.33%. The result is parallel with earlier studies (Ma and Craig 2018; Froese and Pauly 2023). *Epinephelus malabaricus* and *E. coioides* share similar characteristics and are often misidentified as each other. Both species have a light grey to yellowish-brown colouration with numerous small brown spots (Froese and Pauly 2023). Rimmer and Glamuzina (2017) reported that the cultured grouper identified as *E. malabaricus* in Taiwan and Thailand is actually *E. coioides*.

Overall, genetic data obtained from DNA barcoding in this study could play a crucial role in supporting grouper fisheries management in Aceh, particularly in the waters of Langsa City, Indonesia.

In conclusion, this study successfully recognized six grouper species belonging to the genus *Epinephelus* in Langsa district, Aceh province, Indonesia. Sequences of *E. malabaricus, E. erythrurus* and *M. poecilonotus* were generated for the first time, contributing to the molecular data of grouper species found in Aceh. The determination of the closest kinship between *E. malabaricus* and *E. coioides* (4.07%) and the furthest kinship between *E. sexfasciatus* and *M. poecilonotus* (19.33%) provides valuable insights into genetic relationships and diversity among the studied grouper species. By employing DNA barcoding, this study successfully validated the identification of six grouper species harvested from the waters of Langsa City in Aceh province.

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