

# Genetic diversity analysis and phylogenetic reconstruction of groupers *Epinephelus* spp. from Madura Island, Indonesia based on partial sequence of CO1 gene

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**Abstract.** Basith A, Abinawanto, Kusrini E, Yasman. 2021. Genetic diversity analysis and phylogenetic reconstruction of groupers *Epinephelus* spp. from Madura Island, Indonesia based on partial sequence of CO1 gene. *Biodiversitas* 22: 4282-4290. Groupers populations in Indonesia, particularly from Madura Island, East Java are indicated to be over-fished, thereby requiring data collection of more accurate genetic resources as an important step for grouper conservation. A total of 14 samples of the *Epinephelus* groupers were obtained from the fish landing port on Madura Island. The 617 bp CO1 gene sequence was utilized for genetic diversity analysis and phylogenetic tree reconstruction. Genetic diversity is based on the value of haplotype diversity (Hd) and nucleotide diversity ( $\pi$ ). Reconstruction of the phylogenetic tree includes neighbor-joining (NJ) implementing K2P substitution model, while maximum likelihood (ML) is conducted by implementing HKY+G+I substitution model, both of which were evaluated by employing a bootstrap of 1000 replications. Analysis of genetic distance between species indicated that the farthest distance between *E. heniochus* and *E. fasciatus* was 0.189, while the closest distance between *E. erythrurus* and *E. ongus* was 0.099. Intrapopulation genetic diversity indicated a high value with details of Hd=0.978 and  $\pi$ =0.12107. Furthermore, NJ and ML phylogenetic tree demonstrated similar topology in the observed *Epinephelus* spp. obtained from Madura Island grouped into 7 clades, that is *Epinephelus coioides*, *E. bleekeri*, *E. areolatus*, *E. erythrurus*, *E. heniochus*, *E. fasciatus*, and *E. ongus*.

**Keywords:** Cytochrome c oxidase 1, DNA barcoding, genetic diversity, groupers species, phylogenetic

## INTRODUCTION

Indonesia is acknowledged as one of the largest producers of reef fish trade worldwide, especially for grouper contributing 26.5% of the global capture production (Muldoon et al. 2016; Amorim et al. 2020). The high intensity of fishing has been due to the increasing demand for grouper, serving as an important economic commodity (Khasanah et al. 2019). Particularly, Madura Island is one of the potential grouper producers in East Java, Indonesia, generating 159.8 tons within 1179,166 fishing trips every fishing season (Sukandar et al. 2016). Status of sustainable management of fishery resources in the Madura Strait based on 5 dimensions of observation concludes that the sustainability scores for the ecological, economic dimensions and technology are <50 with status less sustainable (Hidayah et al. 2020).

Grouper in Indonesian waters is grouped into various genera, one of which is *Epinephelus*. The *Epinephelus* Bloch genus was placed under the subfamily of Epinephelinae in 1973, as one of three subfamilies in the family Serranidae, commonly acknowledged as groupers, rockcods, and seabasses (Heemstra and Randall 1993). Grouper identification based on morphological characters has been ambiguous leading to misidentification due to similarity in color and morphology, requiring accurate

identification for conservation management through molecular approach (Ma et al. 2016; Kline et al. 2011; Kamal et al. 2019).

The literature development of DNA barcoding indicates that a short fragment of CO1 could be utilized as an accurate marker of genetic variation to identify a wide range of animals at the species level. Becker et al. (2011) provide an overview of the progress of DNA barcoding applications according to taxonomy and geographic area. Approximately, 31.000 species of fish are currently known, 25% of which has been successfully processed, with at least one species from 89% of all families barcoded. Furthermore, Hanner et al. (2011) although currently less than 30.000 species are recognized and there is still species that need to be re-evaluated, new species are routinely discovered. Accurate assessment of species diversity has always been a major challenge for ichthyology biosystematics, especially the fact that morphological shifts that are almost always found throughout the developmental stage of the species, and sometimes sex, and perhaps more subtle shifts across geographic ranges. Imtiaz et al. (2017) explained that DNA barcoding has a high potential to identify species, resolve ambiguities in species identification, and assist in accurate species identification.

Molecular approaches using DNA barcodes have been widely preferred to identify groupers, especially in

selecting cytochrome c oxidase subunit 1 (CO1) gene sequence. Reports on the identification of grouper species, particularly *Epinephelus* spp., based on partial sequence of CO1 gene in various water areas continue to grow, some of which were in the Philippines (Alcantara and Yambot 2016), Vietnam (Durand et al. 2020), Malaysia (Aziz et al. 2016; Du et al. 2019), Thailand (Noikotr et al. 2013), China (Qu et al. 2018), India (Basheer et al. 2017; Deepti et al. 2018), Saudi Arabia (Trivedi et al. 2014) and the Great Barrier Reef of Australia (Johnson and Wilmer 2019). In more circumstances, the complete mitochondrial genome of some groupers has been successfully sequenced, such as *Epinephelus quoyanus* (Peng et al. 2014) and *Epinephelus awoara* (Qu et al. 2012).

Meanwhile in Indonesia, the application of partial sequence of CO1 gene in several grouper species has generally been reported by Jefri et al. (2015) at distant sampling points, in Lampung, Karimunjawa, Madura, Lombok, Tanakeke, Kendari, and Numfor. In addition, grouper sampling at more specific locations was also reported, some of which were in Aceh (Kamal et al. 2019), Pangandaran West Java (Ariyanti and Farajallah 2019), and West Papua (Tapilatu et al. 2021). Then to complement previous research, this study focuses on observing the genetic diversity and reconstructing the phylogenetic tree of various grouper types (*Epinephelus* spp.) found on Madura Island, East Java, Indonesia. The results of this study are expected to complement the identification results of grouper on Madura Island by utilizing a morphological approach (Basith et al. 2021), comprehensively providing important information, required for the development of management and conservation policies regarding the

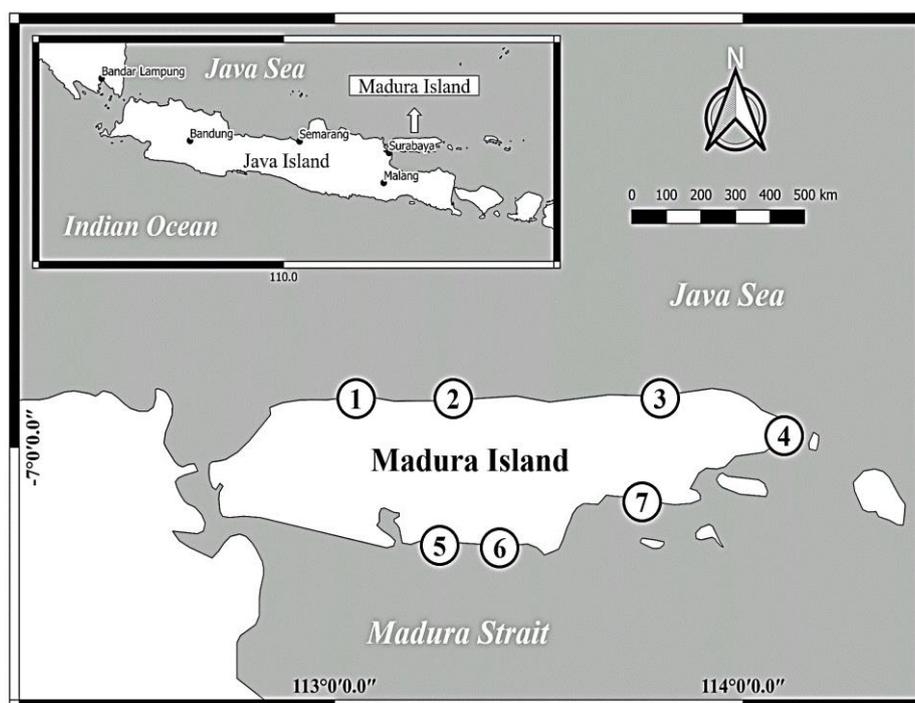
declining numbers of various grouper species on Madura Island.

## MATERIALS AND METHODS

### Sample collection

The sampling locations of groupers *Epinephelus* spp. from Madura Island, East Java Province, Indonesia are presented in Figure 1, containing the coordinate details of fish landing ports, which are: Banyu Sangkah ( $6^{\circ}52'59.42''$  S- $113^{\circ}1'45.58''$  E), Ketapang ( $6^{\circ}53'31.27''$  S- $113^{\circ}16'47.95''$  E), Ambunten ( $6^{\circ}53'16.82''$  S- $113^{\circ}44'17.68$  E), Dungkek ( $6^{\circ}58'45.36''$  S- $114^{\circ}5'56.12''$  E), Camplong ( $7^{\circ}13'4.61''$  S- $113^{\circ}20'28.83''$  E), Branta Pasisir ( $7^{\circ}13'26.38''$  S- $113^{\circ}26'55.41''$  E), and Prenduan ( $7^{\circ}6'34.52''$  S- $113^{\circ}40'35.55''$  E). Samples were collected from March to October 2019 from fishermen's catches at Fish Landing Ports on Madura Island, by ensuring that the grouper was obtained from the waters around the observed area.

The FAO species catalog Vol. 16 Groupers of the world (Serranidae family, Subfamily Epinephelinae) (Heemstra and Randall, 1993) have been applied as a reference to analyze the morphological characteristics of groupers in this study. Based on the morphological characteristics, grouper fish samples were grouped into *Epinephelus coioides*, *E. bleekeri*, *E. areolatus*, *E. heniochus*, *E. erythrus*, *E. fasciatus* and *E. ongus*. The grouper samples were undertaken with a pectoral fin of 15-20 grams for preservation in absolute ethanol (96%), further prepared for molecular analysis. Molecular analysis was conducted at the Research and Genetics Laboratory, in State Islamic University of Malang, Indonesia.



**Figure 1.** Map of Madura Island, East Java, Indonesia and the position of survey location for fishing ports, comprising: 1. Banyu Sangkah (BNS), 2. Ketapang (KTP), 3. Ambunten (AMB), 4. Dungkek (DGK), 5. Camplong (CMP), 6. Branta Pasisir (BRP), and 7. Prenduan (PRD)

### DNA extraction and PCR reaction

Grouper fin samples that preserved in absolute ethanol were first washed using aquadest and TE buffer, then crushed manually by adding liquid nitrogen. Extraction of total DNA in this research sample was performed through the Wizard® Genomic DNA Purification Kit (Promega Corporation) by following the protocol. The universal primer for partial sequence of CO1 gene for PCR was developed by Ward et al. (2005) with forward primer sequence of Fish F1 5'-TCAACCAACCACAAAGACA TTGGCAC-3' and reverse sequence of Fish R1 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'. PCR amplification was performed with MyTaq HS Red Mix (Bioline) with composition details of 9.5  $\mu$ L ddH<sub>2</sub>O, 12.5  $\mu$ L MyTaq HS Red Mix, 1  $\mu$ L 10 M forward primer (Fish F1), 1  $\mu$ L 10 M reverse primer (Fish R1), and 1  $\mu$ L sample DNA template.

The PCR cycles were sequentially initiated with predenaturation at 95°C (1 minute), denaturation at 96°C (15 seconds), annealing of CO1 primer at 55°C (30 seconds), elongation of target genes at 72°C (45 seconds), and finalized by maintaining 4°C until the PCR product was utilized. The main amplification parts (denaturation, annealing, and elongation) were repeated for 35 cycles. The CO1 gene sequence amplicon was confirmed by using agarose gel electrophoresis with 1% agarose gel at a voltage of 100 V for 25 minutes, along with a comparison marker of 100 bp DNA ladder (Intron). Gene purification was performed through a gel extraction method with the standard protocol of GeneJET Gel Extraction and DNA cleanup Micro Kit (the thermo scientific). Meanwhile, nucleotide sequencing was carried out by the service of 1st BASE (Singapore).

### Data analysis

#### *Sequences editing, validation, and alignment*

Partial sequence of CO1 gene along 600-700 bp were obtained from 14 groupers of *Epinephelus* spp. from Madura Island. Each sequence was initially translated into an amino acid sequence to check and remove the stop codon in the middle of the sequence (Song et al. 2008). Sequence examination and editing were conducted by applying the BioEdit software (Hall 1999), in addition to manual careful examination. Each sequence sample was validated by using online facility the Basic Local Alignment Search Tool (BLAST) of GenBank/NCBI and the Barcode of Life Data System (BOLD System). A total of 16 accessions from GenBank (NCBI) were selected as in group and out group for the phylogenetic tree reconstruction. Multiple sequences alignment was performed by using the ClustalW algorithm (Thompson et al. 1994) in MEGA X software version 10.2.6. (Kumar et al. 2018).

#### *Sequence composition, genetic diversity and genetic distance*

Analysis of intrapopulation genetic diversity based on partial sequence of CO1 gene in 14 groupers of *Epinephelus* spp. from Madura Island was performed with DnaSP software version 6.12.03 (Rozas et al. 2017). Information on genetic diversity was analyzed through items, such as: the value of nucleotide diversity ( $\pi$ ), the

number of polymorphic sites (S), the haplotype analysis (haplotype diversity (Hd), the number of haplotypes (nHap), and the variance of haplotype diversity (Nei 1987). In addition, MEGA X software version 10.2.6 was employed to calculate the nucleotide frequencies, transition/transversion rate ratio (k), transition/transversion rate ratio bias (R), and probability and pattern of nucleotide substitution by applying the maximum composite likelihood method. The estimated value of the Gamma Distribution was calculated by using the HKY+G substitution model (Hasegawa et al. 1985) with complete deletion options for gaps and missing data. Genetic distance analysis between species was conducted on the 7 validated species of *Epinephelus* spp. obtained from Madura Island. Genetic distance calculation was performed by using the maximum composite likelihood method with a bootstrap of 1000 replications (Felsenstein 1985).

#### *Phylogenetic tree reconstruction*

Reconstruction of the phylogenetic tree based on partial sequence of CO1 gene was conducted on a total of 30 sequences (included in group and out group accessions obtained from GenBank). Reconstruction of phylogenetic tree was performed by using MEGA X version 10.2.6. Meanwhile, the applied methods for phylogenetic tree reconstruction were Neighbor-Joining (NJ) and Maximum-Likelihood (ML). NJ reconstruction was calculated by using K2P substitution model (Kimura 2-Parameter) (Kimura, 1980), Gamma Distribution (G), and pairwise deletion gaps/missing data treatment. Meanwhile, ML reconstruction was performed through Hasegawa-Kishino-Yano+Gamma Distributed with Invariant (HKY+G+I) substitution model, based on the best-fit substitution model using the BIC (Bayesian Information Criterion) and AICc (Akaike Information Criterion corrected) values (Hasegawa et al. 1985; Nei and Kumar 2000). Both of phylogenetic trees were evaluated by employing a bootstrap of 1000 replications (Felsenstein 1985).

## RESULTS AND DISCUSSION

### PCR product visualization and sequence validation

The PCR product visualized using 1% agarose gel electrophoresis indicated partial sequence of CO1 gene in 14 species of *Epinephelus* spp. grouper on Madura Island, was successfully amplified at 650-700 bp long (Figure 2), according to Ward et al. (2005), affirming that the CO1 universal primer produced an amplicon of 655 bp. Upon the completion process of multiple sequences alignment and minor editing, the sequence length that was employed in this study with an amplicon of 612 bp; in contrast to other previous studies by Jefri et al. (2015) using a 526 bp sequence, Basheer et al. (2017) using a 655 bp, and Tapilatu et al. (2021) using a 623 bp in their respective analyses. Sequence validation results conducted through the online facility of BLAST (NCBI) and the BOLD system indicated that the sequence samples matched the available accessions in the database with query cover in the range of 97-100% (Table 1).

The validation results depicted that the 14 samples were grouped into 7 species, including: *E. coioides*, *E. bleekeri*, *E. areolatus*, *E. heniochus*, *E. ongus*, *E. erythrurus* and *E. fasciatus*. In accordance with the results of the sequence validation, the similarity of the sequences reported by Jefri et al. (2015) was in the range of 99-100% and Tapilatu et al (2021) was in the range of 98-100%. All species of *Epinephelus* spp. in this study are species that are commonly found in the waters of the Western Indo-Pacific, especially in Indonesian waters, according to the geographical distribution illustrated by Heemstra and Randall (1993). Specifically, the findings of *E. areolatus* in this study were in accordance with the findings in two samples of *E. areolatus* from Madura Island reported by Jefri et al. (2015).

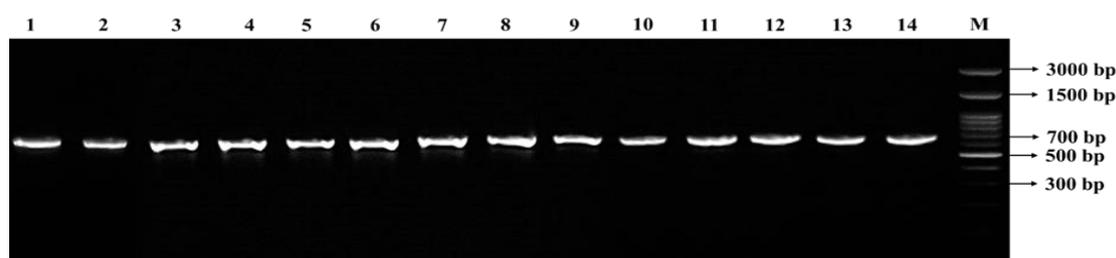
### Sequence composition and genetic diversity

The nucleotide composition of partial sequence of CO1 gene of *Epinephelus* spp. indicated that percentage figures were in accordance with the Teleosts reported by Ward et al. (2005). Percentage of bases (G, C, A, and T) in *Epinephelus* spp., respectively were 17.69%, 29.37%, 23.88%, and 29.06%, while the percentage of bases in Teleost was 18.31%, 28.75%, 23.58%, and 29.38%. Furthermore, the percentage of G+C content in partial sequence of CO1 gene of *Epinephelus* spp. was 47.06%,

while on Teleost was 47.1%, thereby indicating that the universal primer for partial sequence of CO1 gene developed by Ward et al. (2005) was conducted through accurate calculations, successfully applied in *Epinephelus* spp. groupers. The details of the genetic diversity analysis are summarized in Table 2.

**Table 2.** Summary analysis of intrapopulation genetic diversity based on partial sequence of CO1 gene of *Epinephelus* spp. obtained from Madura Island, East Java Province, Indonesia

Parameter	Analysis result
Nucleotide frequencies	
Adenine (A)	23.88%
Thymine (T)	29.06%
Cytosine (C)	29.37%
Guanine (G)	17.69%
G+C content	47.06%
Transition/transversion rate ratios (k)	4.73 (purines) 6256 (pyrimidines)
transition/transversion bias (R)	3.021
Nucleotide diversity ( $\pi$ )	0.12107
Number of polymorphic sites (S)	141
Haplotype diversity (Hd)	0.978
Number of haplotypes (nHap)	12
Variance of haplotype diversity	0.00119
Gamma distribution	0.12018



**Figure 2.** Amplicon visualization of partial sequence of CO1 gene of *Epinephelus* spp. obtained from Madura Island, East Java Province, Indonesia on 1% gel electrophoresis. Note: M: DNA ladder 100 bp, 1: MDR-CMP103, 2: MDR-CMP105, 3: MDR-CMP207, 4: MDR-CMP208, 5: MDR-CMP209, 6: MDR-KTP011, 7: MDR-AMB013, 8: MDR-AMB014, 9: MDR-BRP016, 10: MDR-BNS022, 11: MDR-BNS023, 12: MDR-PRD224, 13: MDR-DGK026, 14: MDR-DGK027

**Table 1.** Validation of partial sequence of CO1 gene of *Epinephelus* spp. obtained from Madura Island, East Java Province, Indonesia through the BLAST (GenBank/NCBI) and the BOLD System online facilities

Local accession number	Species	GenBank (NCBI)			BOLD system
		Query cover (%)	E-value	% identity	Similarity (%)
MDR-CMP207	<i>Epinephelus coioides</i>	97	0	99.5	100
MDR-KTP011	<i>E. coioides</i>	97	0	96.9	100
MDR-AMB013	<i>E. coioides</i>	100	0	98	100
MDR-BRP016	<i>E. coioides</i>	100	0	96	99
MDR-PRD224	<i>E. coioides</i>	98	0	97.8	99
MDR-CMP208	<i>E. bleekeri</i>	97	0	99.8	99
MDR-AMB014	<i>E. bleekeri</i>	98	0	98	100
MDR-DGK026	<i>E. areolatus</i>	99	0	98.5	100
MDR-DGK027	<i>E. areolatus</i>	98	0	97.4	99
MDR-BNS022	<i>E. erythrurus</i>	98	0	96.5	100
MDR-BNS023	<i>E. erythrurus</i>	98	0	97.2	100
MDR-CMP209	<i>E. heniochus</i>	100	0	96.9	100
MDR-CMP103	<i>E. fasciatus</i>	99	0	100	100
MDR-CMP105	<i>E. ongus</i>	99	0	96.9	98

Based on the value of haplotype diversity (Hd) 0.978 with nucleotide diversity ( $\pi$ ) 0.12107, it is concluded that the genetic diversity of *Epinephelus* spp. grouper was relatively high. The diversity value was not much different from Basheer et al. (2017) with Hd value of 0.9830 and  $\pi$  value of 0.14465. Genetic diversity refers to the interpretation result of isolation ecologically, behaviorally and physically, comprising the limited number of individuals and selecting certain traits (Mignon-Grasteau et al. 2005). Populations with high genetic diversity have a better chance of survival, in which high value of genetic diversity illustrates that the population of *Epinephelus* spp. on Madura Island was in good condition, further indicating high chance of survival and adaptation to environmental quality disturbances including over-fishing pressure. However, this study consisted of multiple species, thereby encouraging future research specifically observing the genetic diversity of each *Epinephelus* spp. species.

Genetic diversity is influenced by distribution and habitat. Further, Crandall et al. (2008) explained that the widely marine biota, could have high genetic variation. In addition, habitat characteristics determine the genetic diversity of groupers, as revealed by Madduppa et al. (2012) indicating that the genetic diversity of the groupers that live on the slopes of coral reefs are higher than those living on the lagoons. Complementing the expression, Manel et al. (2020) systematically revealed that habitat complexity drives genetic diversity of marine fish, especially temperatures found at sea level, thereby explaining the high genetic diversity in demersal fish (less than 200 meters deep).

### Genetic distance and phylogenetic tree

Genetic distance refers to the ratio of genetic differences between species or populations (Dogan and Dogan 2016). Based on the genetic distance matrix of 7 species of *Epinephelus* spp. groupers obtained in Madura Island, the closest distance is found between *E. ongus* and *E. erythrurus* with a value of 0.099. In contrast, the farthest distance is navigated between *E. fasciatus* and *E. heniochus* with a value of 0.189 (Table 3). Thus, a smaller genetic distance value generates a more similar appearance partial sequence of CO1 gene compared between the two species.

Based on the standards from Nei (1972), the genetic distance of *Epinephelus* spp. grouper obtained in Madura Island is categorized into low (0.01-0.09) and medium (0.1-0.99), generally similar to *Epinephelus* spp. genetic distance calculations reported by Jefri et al. (2015). The value of genetic distance proved that the partial sequence of CO1 gene provides a fast and effective DNA barcode technique for species identification, especially in addressing morphological confusion or ambiguity, in line with the report of Noikotr et al. (2013). Furthermore, Madduppa et al. (2012) assumed that the lifestyle of *Epinephelus* spp. was classified as a limited, solitary, sedentary and territorial distribution in coral reef ecosystems indicating less different genetic distance.

A total of 16 accessions were downloaded from GenBank (NCBI) as in in-group and out-group access for reconstruction of phylogenetic tree of *Epinephelus* spp. (Table 4). Reconstruction of NJ and ML phylogenetic trees indicates similar topology in all samples of *Epinephelus* spp., restrictedly grouped into 7 species, in line with the results of validation applying the BLAST (NCBI) and BOLD system (Table 1) and in line with the calculation of genetic distance between species (Table 3). The phylogenetic tree of *Epinephelus* spp. in both NJ and ML, forms a clear and unambiguous branching pattern (Figures 3 and 4). This robust reconstruction of the phylogenetic tree is in line with previous study (Jefri et al. 2015).

The two phylogenetic trees indicate that *E. ongus* and *E. erythrurus* species form their own monophyletic branches; however, since the two species are connected at the same node, the formed branches formed indicated closeness. The branching position is in line with the calculation of the genetic distance of 0.099, indicating the closeness between *E. ongus* and *E. erythrurus*. Moreover, Jefri et al. (2015) reported that the closest genetic distance was also found between *E. ongus* and *E. coeruleopunctatus* with a value of 0.091. In addition, Heemstra and Randall (1993) explained that *E. ongus* and *E. erythrurus* each had a maximum length of 40 cm and 45 cm, indicating that the total length of the two was not much different. Furthermore, *E. ongus* has the opportunity to be misidentified with *E. coeruleopunctatus*, *E. summana* and *E. corrallicola*.

**Table 3.** Pairwise genetic distance matrix of the 7 species of *Epinephelus* spp. obtained in Madura Island, East Java Province, Indonesia

No.	Species	1	2	3	4	5	6	7
1.	<i>Epinephelus coioides</i>	-	*	*	*	*	*	*
2.	<i>E. bleekeri</i>	0.149	-	*	*	*	*	*
3.	<i>E. areolatus</i>	0.169	0.116	-	*	*	*	*
4.	<i>E. erythrurus</i>	0.121	0.130	0.137	-	*	*	*
5.	<i>E. heniochus</i>	0.135	0.164	0.17	0.146	-	*	*
6.	<i>E. fasciatus</i>	0.155	0.145	0.153	0.151	0.189	-	*
7.	<i>E. ongus</i>	0.133	0.160	0.170	0.099	0.150	0.182	-

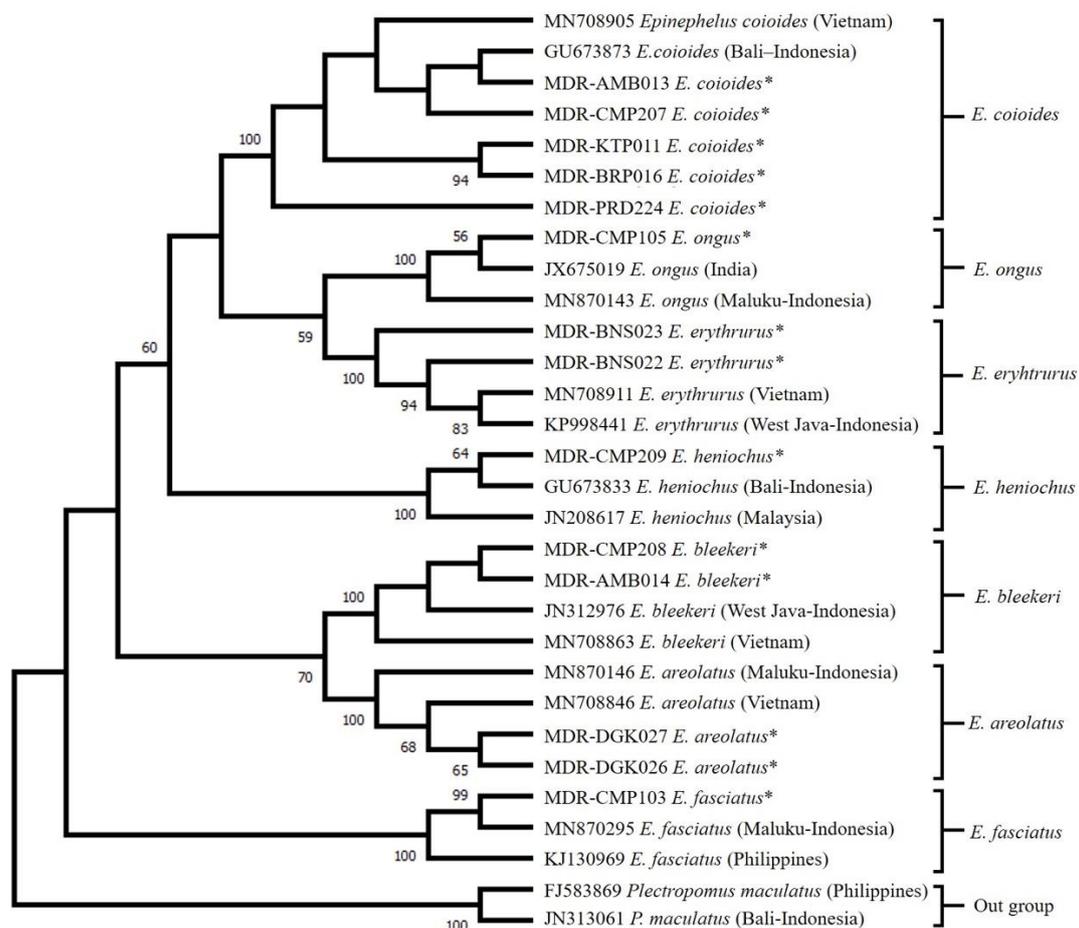
**Table 4.** List of species accessions of in group and out group obtained from GenBank (NCBI)

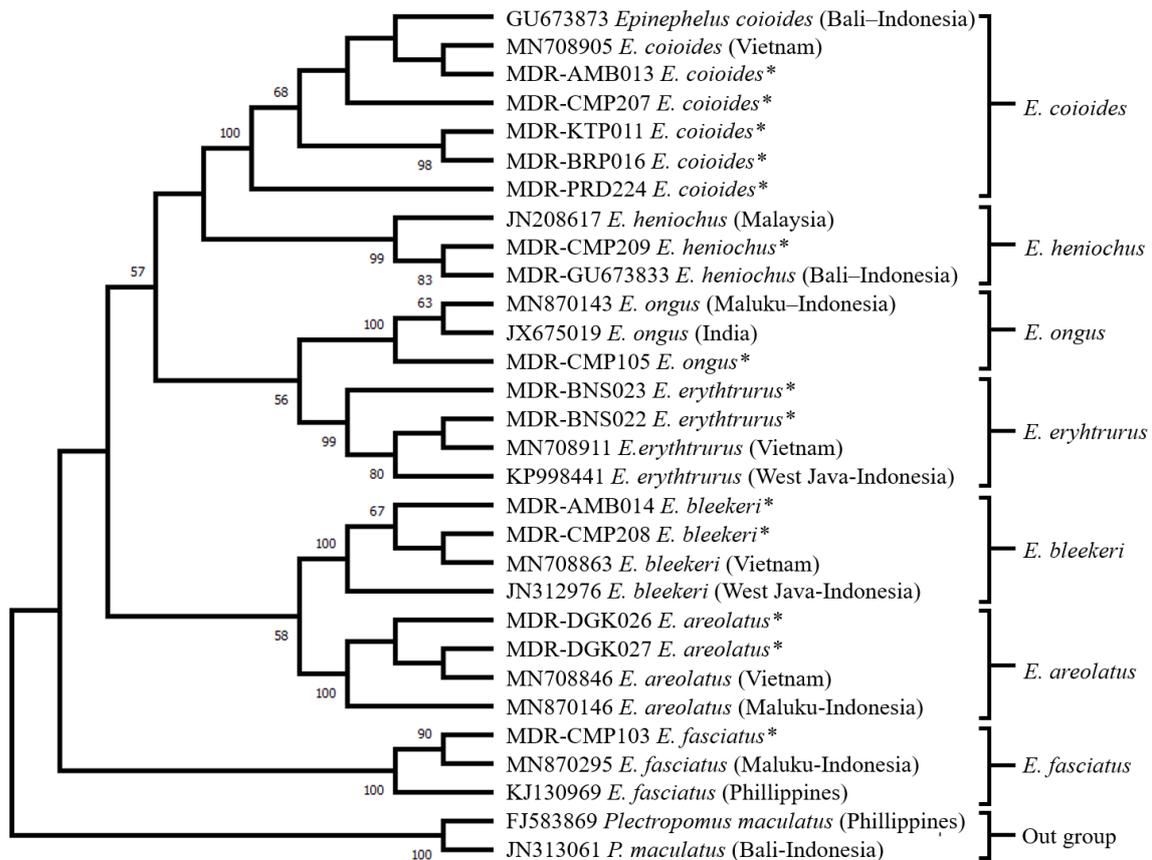
Species	Locality	Accession
<b>In group accessions</b>		
<i>Epinephelus coioides</i>	Bali, Indonesia	GU673873
	Vietnam	MN708905
<i>E. bleekeri</i>	West Java, Indonesia	JN312976
	Vietnamese	MN708863
<i>E. areolatus</i>	Maluku, Indonesia	MN870146
	Vietnam	MN708846
<i>E. erythrurus</i>	West Java, Indonesia	KP998441
	Vietnam	MN708911
<i>E. heniochus</i>	Bali, Indonesia	GU673833
	Malaysia	JN208617
<i>E. fasciatus</i>	Maluku, Indonesia	MN870296
	Philippines	KJ130969
<i>E. ongus</i>	Maluku, Indonesia	MN870143
	India	JX675019
<b>Out group accessions</b>		
<i>Plectropomus maculatus</i>	Bali, Indonesia	JN313061
<i>P. maculatus</i>	Philippines	FJ583869

The next closest genetic distance was found between *E. areolatus* and *E. bleekeri* with a value of 0.116. Although according to Nei (1972) standard, the genetic distance of

the two species is moderate, the branching of NJ and ML phylogenetic trees in both species indicated a close relationship at the same node. These two phylogenetic trees are in line with the previous study, conducted by Darwin et al. (2020) highlighting the proximity of the phylogenetic tree branching positions among *E. areolatus*, *E. bleekeri*, and *E. chlorostigma*. On the other hand, based on morphology, identification of *E. areolatus* presents a high chance of being misidentified as *E. bleekeri* and *E. chlorostigma* (Heemstra and Randall 1993; He et al. 2013).

The application of partial sequence of CO1 gene in this study indicates an accurate match between the genetic distance values and the phylogenetic tree, in which the combination of the two has been in accordance with the level of morphological similarity between *Epinephelus* spp. species, as found between *E. ongus* and *E. erythrurus* and between *E. areolatus* and *E. bleekeri* in this study. Thus, it is perceived that closer genetic distance equals to closer phylogenetic branching, indicating more similar morphology of *Epinephelus* spp., thereby generating misidentification. The conclusion regarding the suitability between the phylogenetic branching proximity and morphological similarities is in accordance with the study conducted by Tapilatu et al. (2021).

**Figure 3.** Neighbor-Joining (NJ) phylogenetic tree of *Epinephelus* spp. The asterisk (\*) denotes the sequence of *Epinephelus* spp. obtained from Madura Island, East Java Province, Indonesia



**Figure 4.** Maximum-Likelihood (ML) phylogenetic tree of *Epinephelus* spp. The asterisk (\*) denotes the sequence of *Epinephelus* spp. obtained from Madura Island, East Java Province, Indonesia

Kamal et al. (2019) explained that grouper from Peukan Bada waters had been misidentified due to its morphological ambiguity, thereby requiring the application of partial sequence of CO1 gene for authentication. Previously, the potential misidentification of groupers had been reported by Heemstra and Randall (1993). This indicates a phenomenon of phenotypic plasticity in groupers, referring to the ability of one gene to produce more than one phenotype due to exposure to different environmental conditions (Kamal et al. 2019). Similarly, the phenomenon of phenotypic plasticity was observed in *E. itajara* and *E. adscensionis*, and possibility to occur in all grouper species (Craig et al. 2009; Kline et al. 2011).

Hence, this study becomes the first to report on the genetic diversity and phylogenetic reconstruction of *Epinephelus* spp. from the coastal area of Madura Island based on partial sequence of CO1 gene. Although the genetic diversity of grouper on Madura Island is relatively high, the decrease in diversity due to habitat destruction and over-fishing has been inevitable. The results of this study indicate that a molecular approach using the partial sequence of CO1 gene supports the identification results based on a morphological approach in *Epinephelus* spp. found in Madura Island, and soon will be submitted to the GenBank (NCBI) database. Future research is encouraged

to emphasize the integration of molecular and morphological approaches to improve the resolution of grouper species identification. Comprehensively, all the information collected will support the management of grouper fishery genetic resources management, especially in Madura waters.

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