

Konservasi genetik dua spesies kerapu (*Epinephelus*) menggunakan DNA barkoding di Teluk Palu, Indonesia

[Genetic Conservation of two grouper species (*Epinephelus*) through DNA barcoding in Palu Bay, Indonesia]

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ABSTRACT | Grouper resources in various Indonesian waters face several issues, primarily due to decreased total catch and overexploitation of stocks. The risk of overfishing can impact genetic diversity. Therefore, understanding the population structure of grouper is crucial as an initial step in identifying potential genetic declines. Information on population structure is obtained through molecular approaches using the Cytochrome Oxidase subunit I as a DNA marker. The research aims to reconstruct the phylogenetics of grouper and analyze their genetic structure in Palu Bay, Central Sulawesi, for genetic conservation purposes. The collected samples consisted of two species from the genus *Epinephelus*. Fin samples were taken approximately 10 gr and processed for DNA extraction. The extraction method followed the DNeasy Tissue Kits protocol. The extraction results were then analyzed by Polymerase Chain Reaction followed by DNA sequencing using the Sanger dideoxy method. Phylogenetic reconstruction used MEGA X software, while haplotype diversity (Hd), nucleotide diversity (Pi), and fixation index (F_{st}) analysis used ARLEQUIN 3.1 software. Phylogenetic reconstruction found two different grouper species clades, *Epinephelus coioides* and *E. bontoides*. Genetic diversity in *E. coioides* was found to be moderate, while it was low in *E. bontoides*. Genetic conservation approaches through hybridization can be applied to both grouper species in Palu Bay as an effort to recover declining genetic resources.

Key words | Conservation, DNA barcoding, *Epinephelinae*, grouper

ABSTRAK | Sumber daya ikan kerapu di berbagai perairan Indonesia menghadapi berbagai permasalahan, terutama karena penangkapan berlebihan. Risiko penangkapan berlebihan, dapat berdampak pada penurunan keragaman genetik. Oleh karena itu, penting untuk memahami struktur populasi ikan kerapu sebagai langkah awal dalam mengidentifikasi potensi penurunan genetik. Informasi terkait struktur populasi diperoleh melalui pendekatan molekuler dengan menggunakan Cytochrome Oxidase subunit I sebagai DNA marker. Tujuan penelitian untuk merekonstruksi filogenetik ikan kerapu dan menganalisis struktur genetiknya di Teluk Palu, Sulawesi Tengah untuk keperluan konservasi genetik. Sampel yang dikoleksi terdiri dari dua spesies yang berasal dari genus *Epinephelus*. Sampel sirip kemudian diambil jaringannya (berukuran kurang lebih 10 gr) dan dilakukan proses ekstraksi untuk mengisolasi DNA. Metode ekstraksi yang digunakan adalah mengikuti protokol DNeasy Tissue Kits (Qiagen). Hasil ekstraksi kemudian dianalisis PCR (Polymerase Chain Reaction) menggunakan protokol laboratorium BIONESIA. Proses pembacaan DNA (sequencing) menggunakan metode Sanger dideoksi. Rekonstruksi filogenetik menggunakan software MEGA X sementara analisis keanekaragaman haplotipe (Hd), keanekaragaman nukleotida (Pi), dan nilai indeks fiksasi (F_{st}) menggunakan software ARLEQUIN 3.1. Rekonstruksi filogenetik menemukan dua clade spesies ikan kerapu yang berbeda yaitu *E. coioides* dan *E. bontoides*. Keragaman genetik pada *E. coioides* kategori sedang sedangkan *E. bontoides* ditemukan rendah. Pendekatan konservasi genetik dapat diterapkan pada kedua spesies kerapu di Teluk Palu sebagai upaya pemulihan sumber daya genetik yang mengalami penurunan.

Kata kunci | Konservasi, DNA barkoding, *Epinephelinae*, ikan kerapu

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INTRODUCTION

Grouper are true bone fish and belong to the sub-family Epinephelinae (Iswarya Deepti *et al.*, 2017). Groupers are targeted fish commodities and also serve as bioindicators of coral reef ecosystem

health (Dimarchopoulou *et al.*, 2021). Grouper fishing is generally small-scale, involving multiple species and gears (Achmad *et al.*, 2023; Chong-Montenegro & Kindsvater, 2022). However, grouper fisheries face serious challenges, especially due to the decline in total catch and overexploitation of stocks (Amorim *et al.*, 2020; Bravo-

Calderon *et al.*, 2021). Because Top-end market demand for groupers has driven increasingly widespread and intensive exploitation (O'Neill *et al.*, 2019; Rowan, 2023). This situation is further complicated by the lack of adequate regulation (unregulated) and often unreported fishing (Halim *et al.*, 2019), worsening the situation and potentially harming the sustainability of grouper populations and disrupting the balance of coral ecosystems (Ferse *et al.*, 2014).

At least 76 grouper species are distributed across nearly all Indonesian waters, with 3 species reported as vulnerable (Razi *et al.*, 2021). Some species have been reported to experience population declines, including *Epinephelus coioides* in Saleh Bay (Efendi *et al.*, 2020) and Sulawesi Sea (Achmad *et al.*, 2022), *E. fuscoguttatus* in Takabonerate National Park (Fatma *et al.*, 2021), *Plectropomus leopardus* in Cendrawasih Bay National Park (Bawole *et al.*, 2018), and *P. oligacanthus* in Wakatobi National Park (Patanda *et al.*, 2017). The decline in grouper populations not only endangers their sustainability but also has serious economic impacts, such as reduced income for fishermen and threats to the grouper business continuity (Condini *et al.*, 2018; Frisch *et al.*, 2016). Therefore, grouper conservation efforts are necessary, with genetic conservation being one of the most important approaches.

Genetic conservation plays a crucial role in preventing species extinction, especially in populations with high genetic diversity, which provides resilience over time (Benjamin *et al.*, 2016). In the context of grouper resource conservation, the initial critical stage is identification, where taxonomic clarity and identification serve as the primary foundation (Madduppa *et al.*, 2016; Saleky & Dailami, 2021). Identification processes in fish can be conducted through two approaches: morphology and molecular techniques. However, the morphological approach in grouper fish sometimes faces challenges in differentiating cryptic species with high morphological similarity

(Jefri *et al.*, 2015). Therefore, the molecular approach using DNA barcoding becomes an effective solution for revealing cryptic species and enhancing species identification accuracy (Abbas *et al.*, 2016; Sarhan *et al.*, 2019).

The molecular approach based on DNA barcoding not only provides more accurate species identification but also offers in-depth genetic information, such as phylogenetic analysis and genetic structure (Dwifajri *et al.*, 2022). Phylogenetic reconstruction enables understanding of genetic relationships between species within a population and among different populations, while genetic structure provides information related to genetic exchange among different populations, thus determining population status (Akbar & Labenua, 2020). Combining both pieces of information can be the initial stage in grouper genetic conservation (Alcantara & Yambot, 2016; Almodóvar *et al.*, 2023). With a deeper understanding of genetic diversity and inter-population relationships, conservation efforts can be more targeted and effective in maintaining grouper population sustainability.

Several studies related to grouper DNA barcoding in Indonesia have been conducted in various waters on the islands of Sumatra, Java, Sulawesi, and Papua (Basith *et al.*, 2022; Fadli *et al.*, 2021; Jefri *et al.*, 2015; Tapilatu *et al.*, 2021), successfully uncovering phylogeographic origins (Fadli *et al.*, 2023), genetic diversity (Basith *et al.*, 2021; Hidayani *et al.*, 2022), and kinship relationships of groupers (Andriyono *et al.*, 2020; Kamal *et al.*, 2019). Therefore, similar research was conducted with the aim of reconstructing the phylogenetics of groupers and analyzing their population structure in Palu Bay, Central Sulawesi, for genetic conservation purposes. Additionally, the findings from this study also serve as baseline data related to the types of groupers in Indonesia.

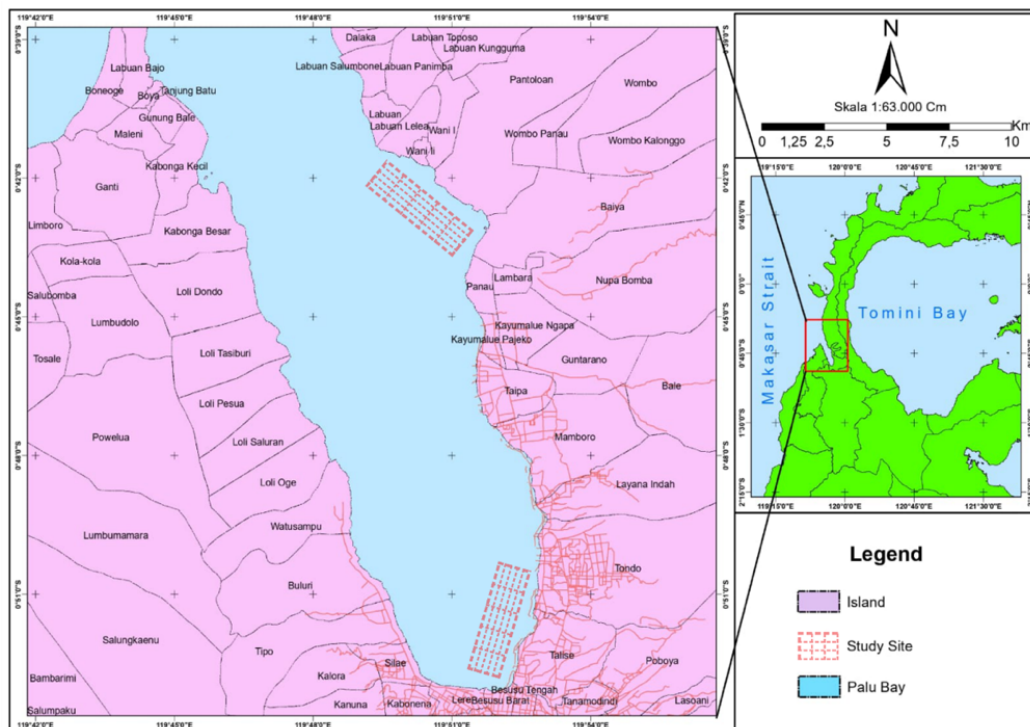


Figure 1. *Epinephelus* grouper fishing grounds in Palu Bay

MATERIAL AND METHODS

Sample collection

Sample collection of grouper fish was conducted in August 2023 in Palu Bay (Figure 1). The collected samples consisted of two species

from the genus *Epinephelus* (Figure 2). The samples were photographed, measured for total length using a Cadwell fish ruler, and fin sections were then stored in tubes filled with 96% ethanol solution for preservation. The extraction process was carried out at the Genetics Laboratory, Indonesian Biodiversity, Bali.

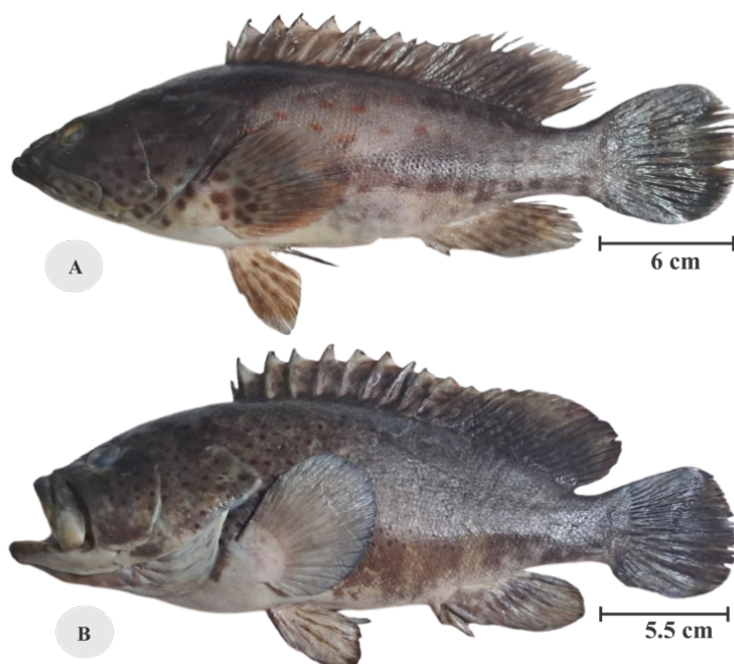


Figure 2. The specimen of *Epinephelus* groupers from Palu Bay, (A) *E. coioides* and (B) *E. bontoides*

DNA extraction and amplification

Fin samples were then taken for tissue (approximately 10 gr) and processed for DNA extraction. The extraction method followed the DNeasy Tissue Kits (Qiagen) protocol. The extracted DNA was then stored in a refrigerator at -20°C. The extraction results were further analyzed through the next step, PCR (Polymerase Chain Reaction). The PCR process used the BIONESIA laboratory protocol. Primers used for fish sample amplification 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). The total PCR reaction volume was 25 µL, consisting of a mix: 2 µL of template DNA from the extraction, 1.25 µL of each primer at a concentration of 10 mM, 9 µL ddH₂O, 12.5 µL Ready Mix. This reaction mix was then amplified using an Applied Biosystems™ 2720 Thermal Cycler machine. The temperature and time profile for the PCR protocol were as follows: Initial Denaturation: 94 °C for 3 minutes, followed by Denaturation: 94 °C for 30 seconds, Annealing: 50°C for 30 seconds, and Extension: 72 °C for 60 seconds. The Denaturation to Extension stages were repeated for 38 cycles, with the final stage being Final Extension: 72 °C for 2 minutes.

Electrophoresis and DNA reading

The PCR results were then visualized on a 1% Agarose gel stained with Nucleic Acid Gel Stain (GelRed®). Samples that were positive (showing DNA bands) underwent DNA reading (sequencing) using the Sanger dideoxy method at a Sequencing facility.

Table 1. The DNA sequences used in the phylogenetic reconstruction of grouper fish are based on the National Center for Biotechnology Information (NCBI).

No	Species	Location	Accession Number	Reference
1	<i>Epinephelus bontoides</i>	Philippines	OQ386667.1	Bemis <i>et al.</i> (2023)
2	<i>Epinephelus bontoides</i>	Taiwan	KU943511.1	Chang <i>et al.</i> (2017)
3	<i>Epinephelus bontoides</i>	Taiwan	KM656842.1	Ma <i>et al.</i> (2016)
4	<i>Epinephelus coioides</i>	Malaysia	KR863505.1	Aziz <i>et al.</i> (2016)
5	<i>Epinephelus coioides</i>	Malaysia	KR863506.1	Aziz <i>et al.</i> (2016)
6	<i>Epinephelus coioides</i>	Malaysia	KR863507.1	Aziz <i>et al.</i> (2016)
7	<i>Epinephelus coioides</i>	Philippines	KJ013039.1	Alcantara & Yambot (2016)

Data analysis

The sequencing process yielded sequence data (Ab.1 file). The obtained sequences were edited and aligned (alignment) using the ClustalW method in MEGA X software (Kumar *et al.*, 2018). Each base arrangement was checked manually to ensure all data used were of good quality. The data were then matched with the existing database at GenBank NCBI through the Basic Local Alignment Search Tool (BLAST) available on the NCBI website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Table 1).

In addition to the BLAST method, the data were further analyzed using a phylogenetic tree to observe the kinship relationships among

samples and to confirm the BLAST results for species-level identification. The phylogenetic tree was created using the Neighbor-joining (NJ) method with 1000 bootstrap replications in MEGA X software (Kumar et al., 2018). Genetic distance values were then analyzed using the p-distance method to compare one sample with another.

The analysis of haplotype diversity (H_d), nucleotide diversity (P_i), and fixation index (F_{st}) was conducted using ARLEQUIN 3.1 software (Excoffier et al., 2005). Genetic structure was analyzed using Analysis of Molecular Variance (AMOVA) employing 1,000 permutations and a significance level of 0.05 (Madduppa et al., 2021).

RESULTS

Phylogenetic reconstruction

The nucleotide matching results and the phylogenetic tree of the samples against the GenBank database showed that BIOSUB237.001 was identified as the species *E. coioides* in the GenBank database (NCBI), while BIOSUB237.002 was identified as the species *E. bontoides*, with nucleotide sequence lengths obtained from PCR amplification of 667 bp and 631 bp, respectively (Table 2). The

identity values for both *E. coioides* and *E. bontoides* species were very high at 99%.

Table 2. Results of COI gene identification in GenBank/NCBI

Species	GenBank (NCBI)		
	Base Pairs (bp)	Query Cover (%)	Identity (%)
<i>E. coioides</i>	667	95	99
<i>E. bontoides</i>	631	94	99

Phylogenetic reconstruction of both species using the Neighbor-Joining (NJ) method with the Kimura's Two-Parameter model generally found that all species clusters showed very high relatedness between the test animal sequences and the NCBI database, as evidenced by bootstrap values reaching 100 (Figure 3). The reconstructed phylogenetic tree consists of two clades, where the *E. coioides* clade from Palu Bay shows similarity with *E. coioides* found in Malaysia and the Philippines. Meanwhile, the *E. bontoides* clade shows similarity with *E. bontoides* found in the Philippines and Taiwan. These findings indicate that although each population group is geographically separated in the waters, *E. coioides* shows close kinship relationships with *E. coioides* populations in Southeast Asia and shares a common ancestor, as does *E. bontoides*.

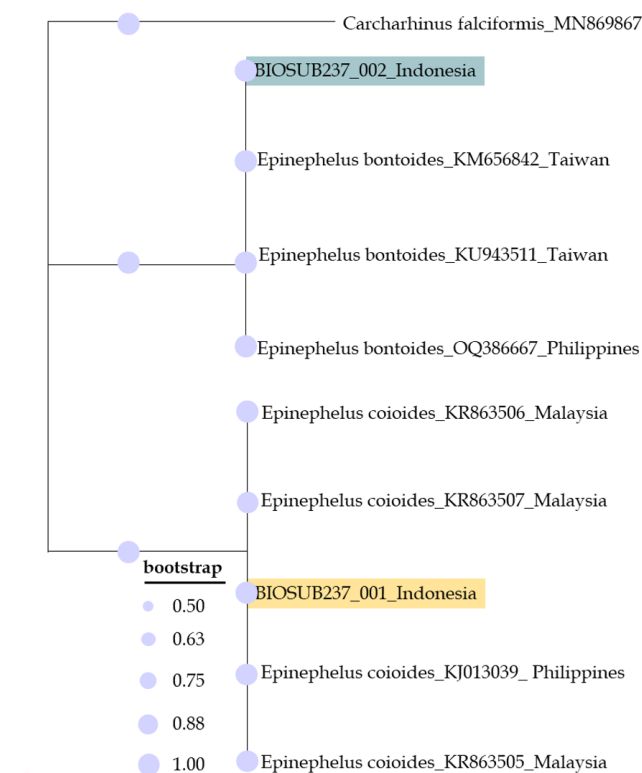


Figure 3. Neighbor-Joining phylogenetic tree of the *Epinephelus* sequences from Palu Bay with selected NCBI GenBank grouper accessions

Population structure

The results of the fixation index (F_{st}) analysis using Analysis of Molecular Variance (AMOVA) on *E. coioides* from Palu Bay (Indonesia) with the Philippines yielded 0.9909, as did Indonesia with Malaysia. Similarly, for *E. bontoides* from Indonesia with Taiwan, the F_{st} value was 0.9909, as well as for Indonesia with the Philippines (Table 3).

The fixation index values for both species, with $F_{st} > 0.05$, indicate a high level of genetic flow between populations. The haplotype diversity (H_d) of *E. coioides* is 0.6730 and the nucleotide diversity (P_i) is 0.0022, whereas the haplotype diversity of *E. bontoides* is 0.2317 and the nucleotide diversity is 0.0015 (Table 4). The haplotype and nucleotide diversity in *E. coioides* are greater than in *E. bontoides*.

Table 3. Pairwise Fixation index (F_{st}) of *Epinephelus*

<i>Epinephelus coioides</i>			
Site	Philippines	Indonesia	Malaysia
Philippines	-	-	-
Indonesia	0.9909	-	-
Malaysia	0.2317	0.9909	-
<i>Epinephelus bontoides</i>			
Site	Taiwan	Indonesia	Philippines
Taiwan	-	-	-
Indonesia	0.9909	-	-
Philippines	0.9909	0.9909	-

Table 4. Haplotype diversity and Nucleotide diversity Generated Arlequin 3.1

Parameter	<i>Epinephelus coioides</i>	<i>Epinephelus</i>
Gene diversity (Hd)	0.6730	0.2317
Nucleotide diversity	0.0022	0.0015

DISCUSSION

Phylogenetic and population structure

All specimens could be identified to the species level with a high degree of species similarity. A higher identity value indicates a higher species similarity. According to Hebert *et al.* (2003), species identification below 97% indicates different species. Thus, the BLAST results obtained in this study demonstrate a high level of species similarity validation. This confirms that the COI gene is effective for identifying grouper species. Molecular identification using the COI gene in groupers has also been conducted by Basith *et al.* (2021), who successfully identified 7 grouper species in Madura Island. Similarly, Fadli *et al.* (2021) successfully identified 26 grouper species in the waters of Aceh. Dwifajri *et al.* (2022) specifically identified groupers from the genus *Epinephelus* in the waters of Papua and found 7 species: *E. areolatus*, *E. merra*, *E. episictus*, *E. kupangensis*, *E. coioides*, *E. macrospilos*, and *E. melanostigma*. These findings make significant contributions to the understanding and documentation of groupers in Indonesian waters.

Phylogenetic reconstruction demonstrates connectivity among waters in Malaysia, Taiwan, the Philippines, and Indonesia. Similar findings were also obtained by Andriyono *et al.* (2020) and Jefri *et al.* (2015), indicating population mixing of *E. coioides* caught in Sunda

Land and Karimunjawa with *E. coioides* in Malaysia and the Philippines. As for *E. bontoides*, this is the first publication from Indonesia, so the information is still very limited. Connectivity among waters allows gene flow between waters, especially for species that are widely distributed in the Indo-Malayan Archipelago (Fadli *et al.*, 2023). Gene flow among populations can ensure the stability of fish community structure and increase species diversity (Lu *et al.*, 2023). However, gene flow in groupers may be hindered by increasing water temperatures (Hassanien & Al-Rashada, 2021).

Referring to the genetic diversity values proposed by Nei (1987), the genetic diversity in *E. coioides* is found to be moderate, whereas in *E. bontoides*, it is low. Low genetic diversity is also found in the species *Plectropomus leopardus* caught in Kwandang Bay and Sumalata Bay, Gorontalo, which are 0.2500 and 0.3333, respectively (Hidayani *et al.*, 2022). Population structure in both studied grouper species is very high but inversely related to their low diversity values, indicating that both species essentially have small population sizes and very specific habitat preferences. Confirmation of this finding has been obtained from the study by Antoro *et al.* (2006) on *E. coioides*, which also collected samples in the areas of Sibolga, Lampung, Jepara, and Flores. Highly specific habitat preferences also provide opportunities for inbreeding within the population, which in turn can result in heterozygosity deficiencies (Yang *et al.*, 2022).

A decrease in genetic diversity in commercial fish is important when populations decline by $\geq 30\%$ in the wild, which can lead to reduced genetic variation and decreased adaptive potential in facing environmental pressures and natural condition changes (Petit-Marty *et al.*, 2022). The impact extends to fisheries productivity, which tends to significantly decline if genetic decline continues (Hauser *et al.*, 2002). Therefore, protecting and restoring genetic diversity is key to maintaining the sustainability of fisheries resources and ecosystem stability.

Genetic conservation

Genetic Conservation Genetic information on *E. coioides* and *E. bontoides* populations indicates a decrease in genetic diversity in both species. While referring to the IUCN Red List, the status of both species is Least Concern (Table 5). The difference lies in the fact that the population trend of *E. coioides* has already declined (Amorim *et al.*, 2018), whereas information about the population trend of *E. bontoides* is still unknown (Russell, 2018).

Table 5. The Conservation Status of *E. coioides* and *E. bontoides* Based on the IUCN Red List

Species	Common Name	IUCN Status	Population Trend	Threats	Source
<i>E. coioides</i>	Orange-spotted Grouper	LC	Decreasing	1. Residential & commercial development 2. Biological resource use 3. Pollution	Amorim <i>et al.</i> (2018)
<i>E. bontoides</i>	Palemargin Grouper	LC	Unknown	1. Biological resource use	Russell (2018)

Hybridization for conservation purposes is implemented as an effort to restore genetic resources that have declined (Carlson *et al.*, 2014). The success of hybridization for conservation purposes has been illustrated in a case study by Fitzpatrick *et al.* (2016) on the species *Poecilia reticulata*, where hybridization in natural habitats can enhance survival and recruitment. Referring to this study, a similar

approach can be applied to both grouper species in Palu Bay. It is important to note that hybridization should preferably be intra-species hybridization involving individuals from different waters to avoid inbreeding, which can reduce genetic quality by increasing the number of homozygotes and decreasing the number of heterozygotes (Suryani & Arya, 2019; Vega - Trejo *et al.*, 2015).

Basically, hybridization in economically important grouper species has long been the focus of development by several experts, such as in the species *E. bruneus* × *E. lanceolatus* (Murata et al., 2017), *E. coioides* × *E. lanceolatus* (Huang et al., 2016; Kiriyaakit et al., 2011), and *Cephalopholis fulva* × *Paranthias furcifer* (Bostrom et al., 2002). However, hybridization in these species is still limited to cultivation needs and has not reached the same level of importance in the context of genetic conservation. An important note before engaging in hybridization for conservation purposes is to prepare comprehensive scientific information regarding (1) the frequency of hybrid genotype compared to natural populations in different generations; (2) the richness of alleles and heterozygosity; (3) species diversity in relation to ecological functions and competition; and (4) tracking individuals acting as males and females (Chan et al., 2019).

CONCLUSION

The nucleotide matching and phylogenetic tree analysis of the samples against the GenBank database identified two species, namely *E. coioides* and *E. bontoides*. Genetic diversity in *E. coioides* was found to be moderate, while in *E. bontoides* it was found to be low. The genetic conservation approach through hybridization can be applied to both grouper species in Palu Bay as an effort to restore declining genetic resources.

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