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Research Article

DNA Barcoding and Morphological Characters of Juvenile *Plectropomus* (Perciformes: Epinephelidae) Caught in Makassar Strait

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Abstract

The high economic value of groupers has made them a popular choice in both local and international markets. However, identifying grouper species is also challenging due to complex morphological variations especially in the juvenile phase. An integrative approach combining DNA barcoding and morphometric analysis was applied to improve species identification accuracy and provide additional information on grouper stocks. This research aims to gain a deeper understanding of the morphological and genetic diversity of groupers caught in the juvenile phase from the Makassar Strait. Samples of the genus *Plectropomus* (n=6) collected from a fish landing site in Pangkajene Kepulauan Regency were identified based on morphology and using molecular methods (DNA barcoding). Phylogenetic and haplotype network analyses were performed. For all specimens the morphometric-meristic and molecular analyses were consistent (98-100% similarity) to known *P. leopardus* and *P. oligacanthus* accessions from GenBank. However, phylogenetic analysis: *P. leopardus* clustered into two distinct lower-level clades, and notably, two *P. areolatus* (Taiwan) resolved within the *P. leopardus* clade, while two *P. laevis* (Philippines) the resolved within the *P. oligacanthus* clade. Haplotype network showed high intraspecific genetic diversity, with *P. leopardus* forming four distinct haplotype groups and *P. oligacanthus* forming two groups. These findings collectively indicate that misidentification may be common and highlight the urgent need for further investigation into geographic barriers to gene flow and the potential existence of cryptic species or subspecies within *Plectropomus*. This study is expected to provide critical support for sustainable fisheries management and the conservation of marine biodiversity in the Makassar Strait.

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1. Introduction

Groupers, family Epinephelidae (Moazzam and Osmany, 2023), are apex predators inhabiting shallow waters, including coral reefs, estuaries, mangroves, and seagrass beds (Farmer and Ault, 2011; Nadiarti *et al*., 2015; Rodemann *et al*., 2023). As apex predators, groupers play a crucial role in maintaining marine ecosystem balance (Sadovy de Mitcheson *et al*., 2013). Due to their high economic value, groupers are widely traded in both local and international seafood markets. High market demand has led to increased fishing efforts targeting groupers (Sadovy de Mitcheson *et al*., 2020; Ugarković and Dragičević, 2023). Indonesia, as the world's largest producer of groupers and snappers, contributes 96% of wild-caught grouper landings.

Coral groupers (genus *Plectropomus*) are iconic, high-value fish species that support valuable fisheries throughout the Indo-West Pacific and the Red Sea (Frisch *et al*., 2016; Frisch and Hobbs, 2007; Sadovy de Mitcheson *et al*., 2020; Shellem *et al*., 2021). The genus *Plectropomus* Oken, 1817 comprises eight currently accepted species: *Plectropomus areolatus* Rüppell 1830; *P. laevis* Lacepède 1801; *P leopardus* Lacepède 1802; *P. maculatus* Bloch 1790; *P. marisrubri* Randall and Hoese 1986; *P. oligacanthus* Bleeker 1855; *P. pessuliferus* Fowler 1904; and *P. punctatus* Quoy and Gaimard 1824 (WoRMS, 2025). All *Plectropomus* species are exploited both for local consumption and for export in the lucrative Live Reef Food Fish Trade (LRFFT) (Frisch *et al*., 2016). Two *Plectropomus* species (*P. leopardus* and *P. areolatus*) are among the ten most sought-after species in the live reef food fish (LRFF) trade in Southeast Asia (Fadli *et al*., 2021; Khasanah *et al*., 2019). The high demand for *P. leopardus* is primarily driven by its attractive red coloration and white flesh, making it a popular choice for Chinese New Year celebrations, where the colour red symbolizes good fortune (Frisch *et al*., 2016; Khasanah *et al*., 2019). Due to the demand for plate-sized fish, this trade often includes individuals in the juvenile and sub-adult phases (Frisch *et al*., 2016; Khasanah *et al*., 2019; Shimose and Kanaiwa, 2022). In addition, juvenile groupers (including *Plectropomus*) that do not meet export criteria are often sold on local markets (Achmad *et al*., 2023; Kadir *et al*., 2023; Nadiarti *et al*., 2021). According to the Union for Conservation of Nature (IUCN) Red List, *P. areolatus* is categorized as Vulnerable (Rhodes, 2018), while *P. leopardus* is listed as Least Concern (Choat and Samoilys, 2018) but with notes that some populations may be overexploited and at risk. Furthermore, spawning aggregations of these species are often easily located by and well-known to fishers, making them highly vulnerable to overfishing (Sadovy de Mitcheson *et al*., 2013). Therefore, effective management measures are needed to safeguard *Plectropomus* populations (Fadli *et al*., 2021; Kadir *et al*., 2023; Khasanah *et al*., 2019; Sadovy de Mitcheson *et al*., 2020).

In Indonesia, the high demand for groupers has led to overfishing (Herdiana *et al*., 2024; Khasanah *et al*., 2019), including juvenile exploitation (Achmad *et al*., 2023; Kadir *et al*., 2023). The Makassar Strait runs between the major land masses of Borneo (Indonesian Kalimantan and Malaysian Sabah) and Sulawesi and is traversed by the main branch of the Indonesian Throughflow, which connects the Pacific Ocean and Indian Ocean. Three Sulawesi provinces have western coasts facing the Makassar Strait from north to south: Central Sulawesi, West Sulawesi, and South Sulawesi. Forming part of Indonesian Fisheries Management Area FMA 713, the Makassar Strait is a major Indonesian fishing ground for reef-associated fish, in particular for groupers (Achmad *et al*., 2023; Kadir *et al*., 2023; Khasanah *et al*., 2019). Market surveys have shown that many groupers landed in South Sulawesi are caught during the juvenile stage (Kadir *et al*., 2023). Identifying grouper species can be challenging due to intra-species similarities and complex intra-species morphological variations, especially in the juvenile phase (Ariyanti and Farajallah, 2019; Frisch and Hobbs, 2007). Combining DNA barcoding and morphological characteristics is especially relevant when identifying cryptic (Cai *et al*., 2013) or easily confused groupers such as *Plectropomus leopardus* and *P. maculatus* (Harrison *et al*., 2017; He *et al*., 2018). Furthermore, such combined approaches can help identify biogeographical patterns and delimit stocks (Cuéllar-Pinzón *et al*., 2016; Fadli *et al*., 2023).

This research aims to gain a deeper understanding of the morphological and genetic diversity of groupers of the genus *Plectropomus* caught in the juvenile phase from the Makassar Strait. Through this approach, it is hoped that a deeper understanding of the genetic and morphological diversity of these economically important groupers will be gained, ultimately supporting conservation efforts, sustainable fisheries management, and better resource utilization in the area.

2. Materials and Methods

2.1 Materials

2.1.1 The equipment

The equipment used in this study included callipers (precision1 mm), coolbox, gloves, scissors,

camera, labels, ziplock bags (16x25 cm), parafilm, marker pen (15 cm), Eppendorf tubes (1.5 ml), heating block, vortex, centrifuge, thermocycler (AB Applied Biosystems™ 2720 Thermal Cycler), chamber electrophoresis and power supply (Thermo scientific, USA), and UV transilluminator (Biologix, China).

2.1.2 The materials

Specimens of the groupers *Plectropomus leopardus* (n=4) and *P. oligacanthus* (n=2) were obtained from fishermen's catches. All specimens were caught in the Makassar Strait and landed at Maccini Baji Fish Auction Site (PPI) in Pangkajene Kepulauan (Pangkep) Regency in June and July 2024. This PPI serves a strategic function as a landing site for fish caught around several nearby islands, including Samatellu, Pala, Saugi, Sabangko, and Salemo. Consequently, samples collected from this location are considered representative of the broader grouper populations within the Spermonde Archipelago, in the southern reaches of Makassar Strait. The limited number specimens for *P. leopardus* (n=4) and *P. oligacanthus* (n=2) in this study reflects the availability of these species in fishermen's catches landed at the Maccini Baji PPI during the research period. The materials used in the molecular analysis included 10% Chelex (Bio-Rad, USA), 96% ethanol, template DNA extracted from the specimens, 1% agarose gel, FISH F1 forward and R1 reverse primers (IDT DNA), ddH2O, and dNTPs, MgCl2, buffer solution, and Taq polymerase enzyme (Ready Mix Bioline).

2.1.3 Ethical approval

This study does not require ethical approval because it does not use experimental animals.

2.2 Methods

2.2.1 Initial observation and sample preservation

The specimens collected were observed while fresh with clear colour patterns. Each specimen was photographed together with an object of known length and then weighed (precision 0.01 g). About 2 g of tissue was taken from the right pectoral fin of each specimen and preserved in 96% absolute ethanol for molecular analysis. These fin samples were sent to the Bionesia Laboratory in Denpasar, Bali for DNA barcoding analysis.

2.2.2 Identification and morphomeristic characters

The specimens were identified to species level based on morphological characters with references to Heemstra and Randall (1993). Morphometric and meristic data were collected at the Fisheries Biology Laboratory of Hasanuddin University in Makassar, Indonesia, with reference to Darwin *et al.* (2020). Morphometric characters measured using callipers (precision 1 mm) were: total length (TL), standard length (SL), head length (HL), snout length (SNL), dorsal fin height (DFD), dorsal fin base length (DFBL), eye diameter (ED), body depth (BD), pectoral fin length (PFL), pelvic fin length (VFL), pre-dorsal length (PDL), pre-pectoral length (PPL), pre-pelvic length (PVL), pre-anal length (PAL), anal fin base length (AFBL), anal fin length (AFL), (Figure 1) and weight (W, in g). Meristic characters counted included the number of dorsal fin rays, anal fin rays, pectoral fin rays, caudal fin rays, pelvic fin rays, lateral line scales (Figure 2), and vertebrae. The vertebrae were counted using digital X-ray radiographic images taken at the Laboratory of Makassar Pet Clinic (MPC), Makassar, Indonesia.

2.2.3 DNA barcoding

The DNA extraction and target gene fragment amplification were performed at the Bionesia laboratory in Denpasar, Bali, Indonesia. Genomic DNA was extracted from each grouper sample using the Chelex Method (10% Chelex). The target fragment of the cytochrome oxidase I (COI) mitochondrial gene was amplified through polymerase chain reaction (PCR) using the primer pair 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 26 µL, consisting of: 2 µL extracted DNA template, 1.25 µL of each primer at 10 mM concentration, 9 µL ddH2O, and 12.5 µL Ready Mix. The PCR profile comprised an initial denaturation at 95°C for 5 minutes, followed by 38 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, strand extension at 72°C for 45 seconds, and then a final extension at 72ºC for 5 minutes (Andriyono and Suciyono, 2020). The presence and quality of the amplified PCR products were verified using electrophoresis (1% agarose gel) stained with Nucleic Acid Gel Stain (GelRed®). PCR products showing clear DNA bands were sequenced using the Sanger method at PT. Genetika Science in Jakarta, Indonesia. The resulting chromatogram files were imported into MEGA 11 (Tamura *et al*., 2021) for analysis. For each specimen, the forward and reverse sequences were checked for quality cleaned, trimmed, aligned, and merged to produce a DNA barcode.

2.3 Analysis Data

The morphometric and meristic data were tabulated and analysed in Microsoft Excel 2021. The

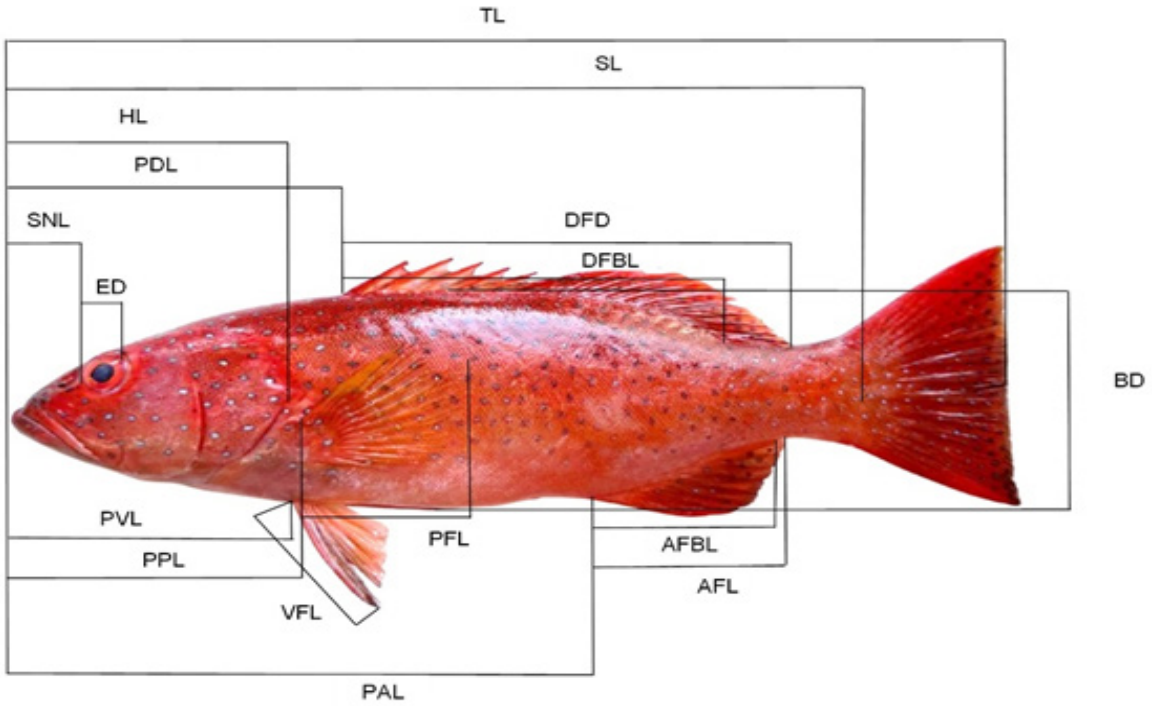


Figure 1. Morphometric characteristics measured for groupers from the Makassar Strait.

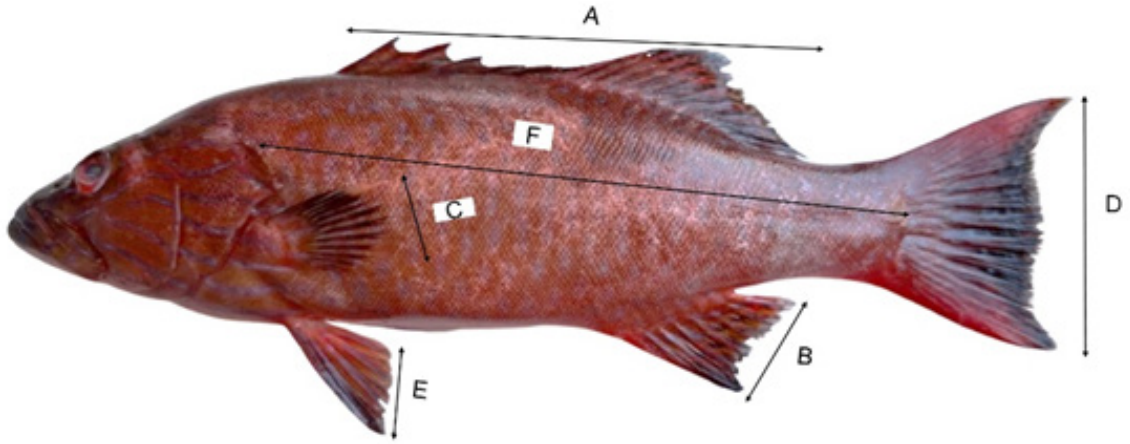


Figure 2. Meristic counts used in this study. Spines and rays: (A) Dorsal fin, (B) Anal fin, (C) Pectoral fin, (D) Caudal fin, (E) Pelvic fin; Scale counts: (F) Lateral line.

ratios of morphometric characters were calculated relative to standard length (SL) and head length (HL)and analysed descriptively. Homologous sequences for the grouper DNA barcodes were obtained from the NCBI GenBank nucleotide repository using the BLAST-n routine and downloaded as FASTA files. The DNA barcode sequences from the grouper specimens and downloaded GenBank accession sequences (250 sequences) represented the entire dataset of homologous sequences for the genus *Plectropomus* in the GenBank database. These sequences were aligned (ClustalW routine) with the DNA barcode sequences obtained from this study and a *Neighbor-Joining* phylogenetic tree was generated in MEGA 11 (Tamura *et al*., 2021) using the Kimura 2-parameter routine with 1000 bootstraps test replicates (Kimura, 1980). Haplotype networks were constructed using DNASP v6 (Librado and Rozas, 2009) and Network v5.0.1.1. (Fluxus Technology) following Madduppa *et al*. (2020). DNASP v6 was used to identify the haplotypes in DNA sequence data set. Network was used to visualise these relationships in the form of a haplotype network diagram.

3. Results and Discussion

3.1 Results

3.1.1 Morphological characters

Morphologically, both *P. leopardus* and *P. oligacanthus* (Figure 3) specimens exhibited elongated and slender body shapes. However, we observed distinct colour patterns in our specimens: *P. leopardus* (Figure 3A) had a bright red body with small dark blue spots, while *P. oligacanthus* (Figure 3B) had a brownish-red body with horizontal blue lines on the back of the head and vertical blue lines along the body.

The morphometric and meristic characteristics of *P. leopardus and P. oligacanthus* collected from the Makassar Strait are presented in Table 1. *Plectropomus leopardus* (n=4) standard length ranging from 175 to 232 mm, and meristic formula of D, VII-VIII+11-12; A, III+8; P, 13–16; V, I+5; C, 14-16. The standard length of *P. oligacanthus* (n=2) ranged from 220 to 225 mm, with a meristic formula of D, VII-VIII+11-12; A, III+8; P, 12–13; V, I+5-6; C, 16-17. Both *P. leopardus* (Figure 4A) and *P. oligacanthus* (Figure 4B) had a total of 24 vertebrae, consistent with previous reports on grouper vertebrae counts.

3.1.2 Molecular identification

The DNA barcodes obtained from Makassar Strait *Plectropomus* specimens had a nucleotide base pair length of 684 base pairs. The closest GenBank accessions obtained from the BLAST routine belonged to two species: *P. leopardus* and *P. oligacanthus* (Table 2), with high sequence similarity 98-100%.

The phylogenetic tree, including homologous GenBank accessions (Figure 5) shows the grouper specimens from the Makassar Strait nested within the expected clades. The four *P. leopardus* sequences from the Makassar Strait (yellow highlight in Figure 5) all nested within the same high-level *P. leopardus* clade, but were placed in two lower-level clades. One specimen clustered with accession MK777629 from Vietnam and JN021314 (unknown origin), while three clustered with accessions from Bali (JN313060) and an unknown location (JQ420074). Similarly, the two *P. oligacanthus* sequences from the Makassar Strait (green highlight in Figure 5) were both placed within the high-level *P. oligacanthus* clade. One of the two

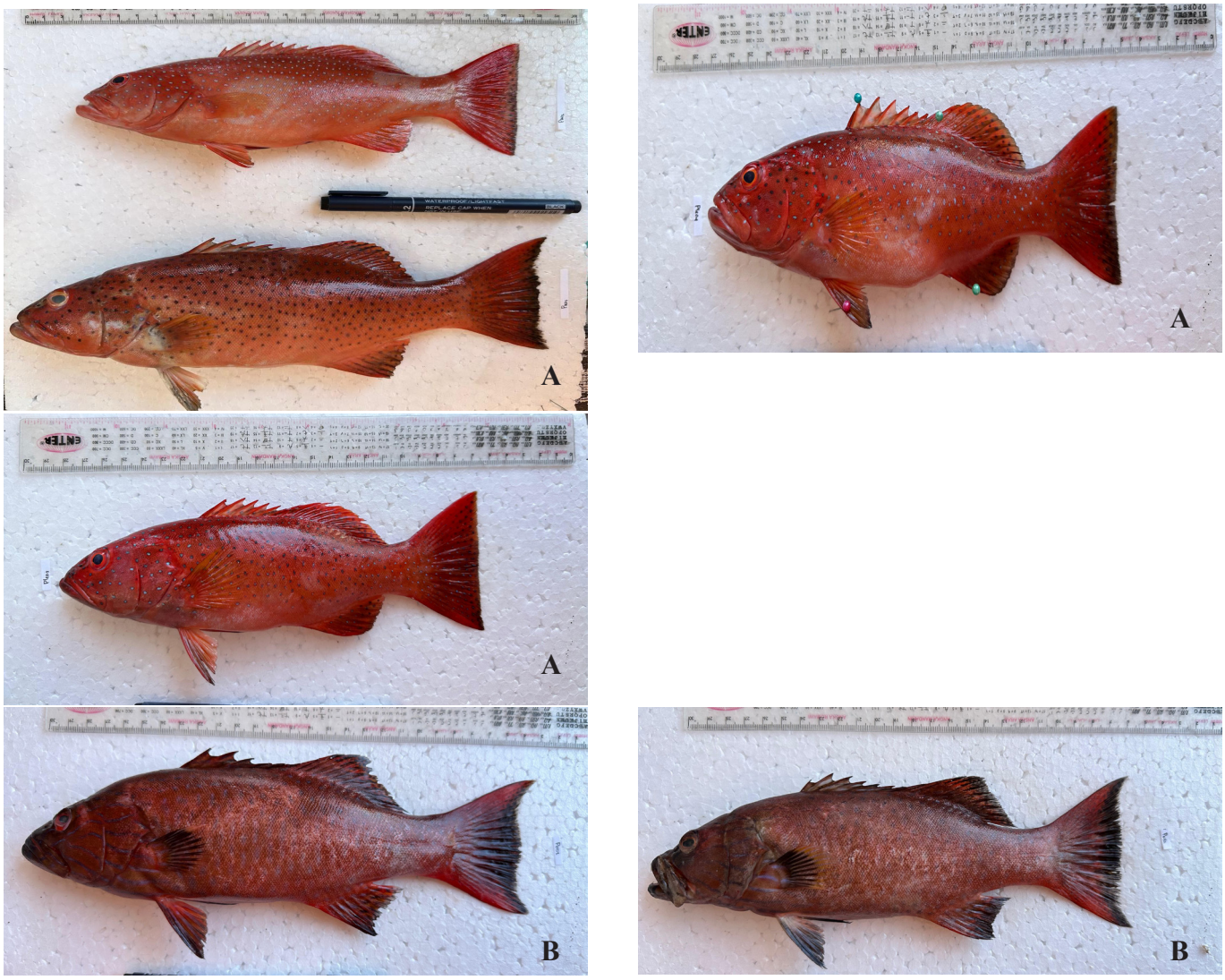


Figure 3. Six juvenile grouper specimens from Makassar Strait. (A) *Plectropomus leopardus*, (B) *Plectropomus oligacanthus.*

Table 1. Morphometric and meristic characters of *Plectropomus* from Makassar Strait (lengths in mm)

No.	Characters	*Plectropomus leopardus*			*Plectropomus oligacanthus*	
		This Study	Heemstra and Randall (1993)	Sujatha and Shrikanya (2012)	This Study	Heemstra and Randall (1993)
Morphometrics						
1	TL	218-292	-	476	252-257	-
2	SL	175-232	120-500	387	220-225	160-510
3	HL /SL (%)	33.7-35.3	32.3-37.0	33.8	32.5-33.0	32.3-37.0
4	SNL/HL (%)	28.1-34.6	27.8-35.7	19.8	31.4-31.7	27.8-35.7
5	DFD /SL (%)	7.3-7.9	-	4.1	5.8-6.0	-
6	DFBL /SL (%)	43.3-45.3	37.0-45.5	45.9	31.5-33.5	24.0-31.5
7	ED /HL (%)	15.2-18.0	-	12.2	15.1-16.4	-
8	BD /SL (%)	26.9-31.9	25.6-34.5	29.9	25.3-27.3	25.6-33.4
9	PFL /SL (%)	15.4-17.4	14.7-19.5	17.5	13.9-14.8	13.4-17.6
10	AFL /SL (%)	19.6-23.1	-	-	20.4-20.7	-
11	VFL/SL (%)	14.1-15.6	-	15.5	15.5-15.7	15.3-24.7
12	PDL /SL (%)	38.6-40.8	-	39.5	36.4-36.5	-
13	PPL /SL (%)	33.7-34.8	-	32.8	32.4-33.7	-
14	PVL /SL (%)	33.0-35.4	-	33.3	31.4-31.9	-
15	PAL /SL (%)	65.8-72.8	-	65.1	66.3-67.9	-
16	AFBL /SL (%)	15.0-15.4	-	16	13.0-13.6	-
17	Weight (g)	133.46-369.90	-		198.1-262.5	-
Meristics						
18	Dorsal fin spines (D)	VII-VIII	VII-VIII	VIII	VII-VIII	VII-VIII
19	Dorsal fin rays (D)	11-12	10-12	11	11-12	10-12
20	Anal fin spines (A)	III	III	III	III	III
21	Anal fin rays (A)	8	8	8	8	8
22	Pectoral fin rays (P)	13-16	-	16	12-13	14-16
23	Caudal fin rays (C)	14-16	15-17	16	16-17	-
24	Pelvic fin spines (V)	1	-	I	I	-
25	Pelvic fin rays (V)	5	-	5	5-6	-
26	Linea lateralis scales	110-117	88-99	118	105-115	86-96
27	Vertebrae	24	-	-	24	-

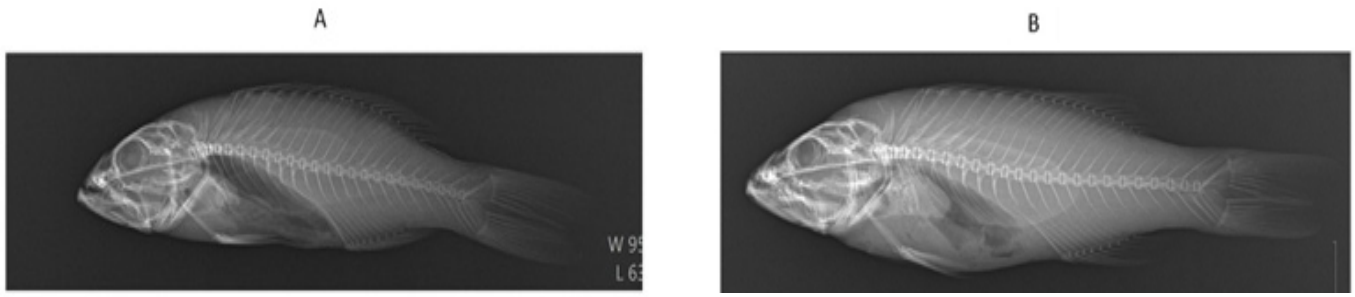


Figure 4. X-ray showing *Plectropomus* skeletons with 24 vertebrate. (A) *Plectropomus leopardus,* (B) *Plectropomus oligacanthus.*

Table 2. DNA barcodes of six groupers of the genus *Plectropomus* from the Makassar Strait and closest GenBank accessions (BLAST)

Makassar Strait Sequence			Closest GenBank BLAST Result		
Lab ID	**Species**	**Bp**	**Accession Number**	**Query cover (%)**	**Identity (%)**
UNH24-PAN001	*Plectropomus oligacanthus*	684	OR524581	98%	100%
UNH24-PAN002	*Plectropomus oligacanthus*	684	MN708953	99%	100%
UNH24-PAN003	*Plectropomus leopardus*	684	KJ101555	100%	100%
UNH24-PAN004	*Plectropomus leopardus*	684	JQ420074	100%	100%
UNH24-PAN005	*Plectropomus leopardus*	684	KJ101556	100%	100%
UNH24-PAN006	*Plectropomus leopardus*	684	MN708946	99%	100%

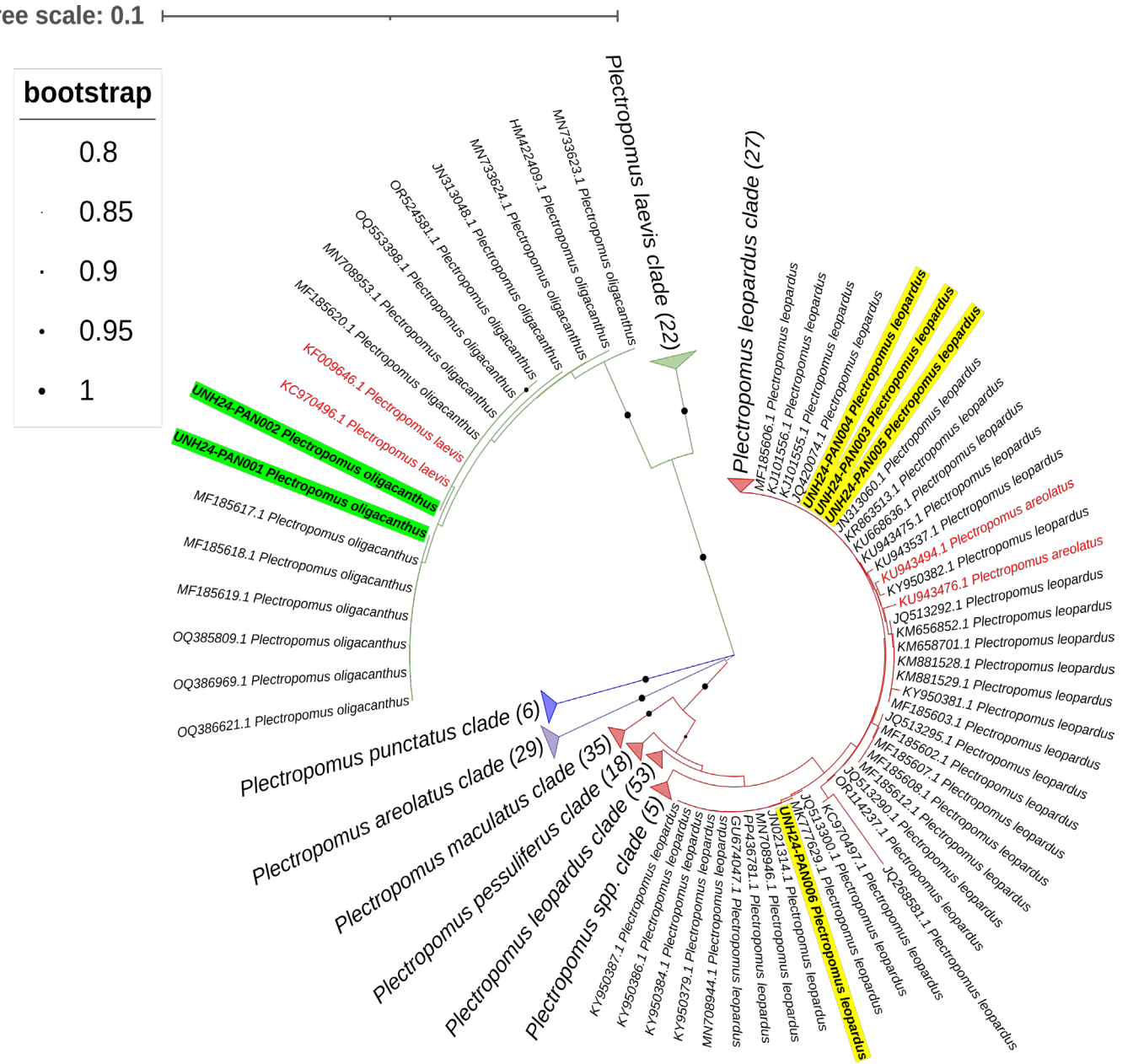
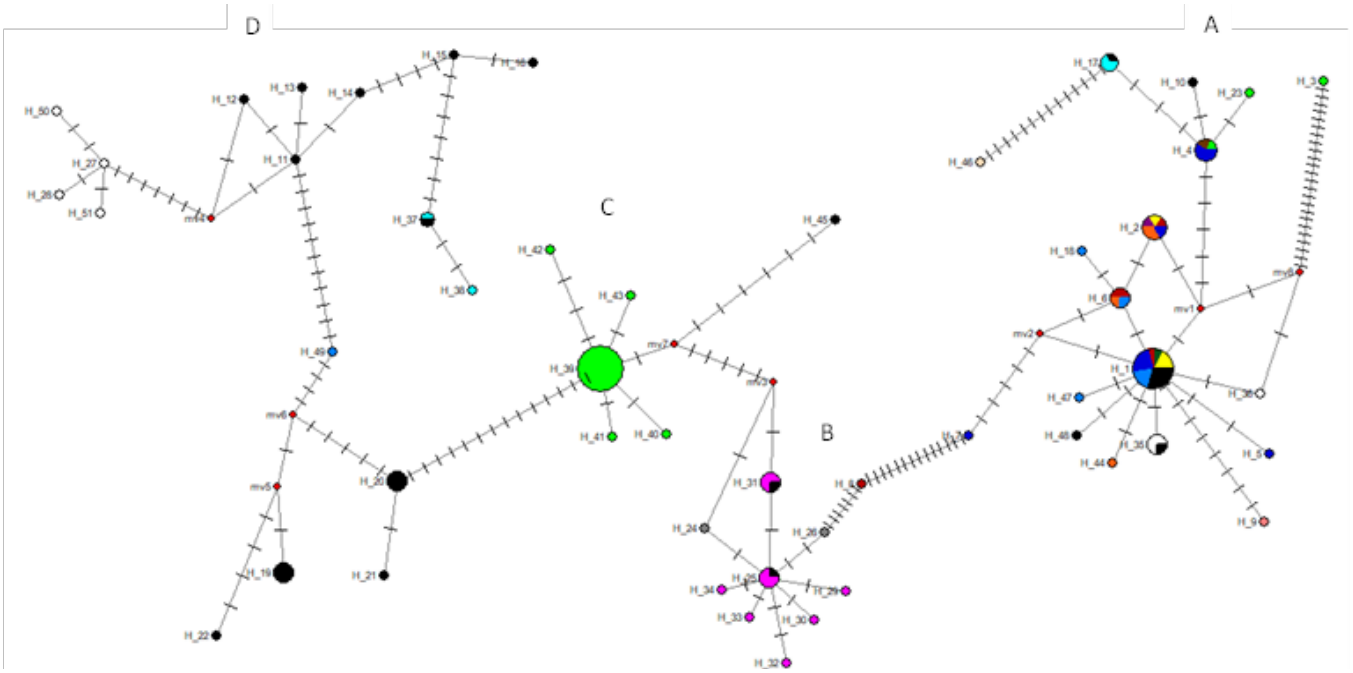


Figure 5. Phylogenetic reconstruction of the genus *Plectropomus* with sequences from Makassar Strait (n=6) and selected homologous GenBank accessions (n=250). Highlights indicate Makassar Straits sequences (yellow = *Plectropomus leopardus*; green = *P. oligacanthus*); red font = GenBank accessions assigned to clades of a different taxon.

sequences clustered with sequences from an unknown location (MF185617, MF185618, and MF185619).

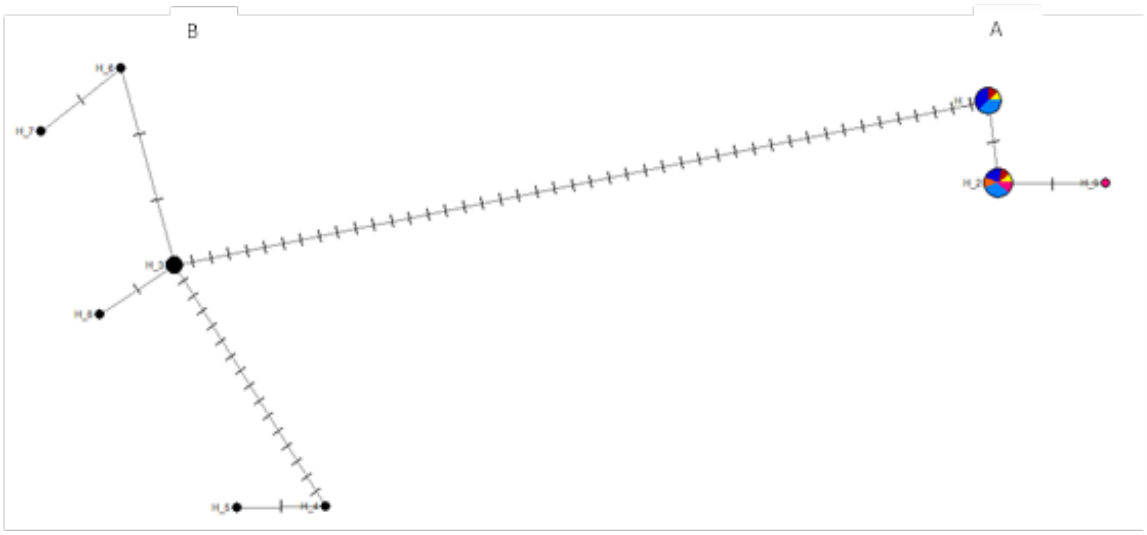
3.1.3 Haplotype network

The *Plectropomus leopardus* haplotype network data set comprised 117 sequences, of which four sequences came from the Makassar Strait and 113 were GenBank accessions from various regions. The *P. oligacanthus* haplotype network data set was much smaller, comprising 26 sequences, two sequences from the Makassar Strait, and 24 GenBank accessions from various regions. This disparity in number reflects the relative paucity of data on *P. oligacanthus* com pared to *P. leopardus*, including in terms of sequences submitted to NCBI GenBank. The haplotype analysis reveals a high genetic diversity within the populations of *P. leopardus* (A-D in Figure 6) and *P. oligacanthus* (A-B in Figure 7), as indicated by the formation of several haplotype groups.



Location colour legend	Number of sequences per haplotype				Scale
This study	H-1	17	H-25	4	
Indonesia_Aceh	H-2	6	H-31	4	
Indonesia_Sulawesi	H-3	1	H-35	4	
Indonesia_Bali	H-4	5	H-36	1	21, 17, 6, 5,4,3, 2,1
Malaysia	H-5	1	H-37	2	
Thailand	H-6	4	H-38	1	
Philippines	H-17	3	H-39	21	
Vietnam	H-18	1			
India	H-7 to H-16		1 each		
Australia	H-19 to H-20		4 each		
New Caledonia	H-21 to H-24		1 each		
China	H-26 to H-30		1 each		
Japan	H-32 to H-34		1 each		
USA	H-40 to H-51		1 each		
Taiwan					
Unknown origin					

Figure 6. Haplotype network for *Plectropomus leopardus* from Makassar Strait and homologous GenBank accessions.



Location colour legend		Number of sequences per haplotype				Scale
This study		H-1	8	H-3	3	
Indonesia_Bali		H-2	9			
Philippines		H-4 to H-9		1 each		9, 8, 3, 1
Vietnam						
China						
Micronesia						
Unknown origin						

Figure 7. Haplotype network for *Plectropomus oligacanthus* from Makassar Strait and homologous GenBank accessions.

3.2 Discussion

3.2.1 Morphological characters

Morphological data can serve as basic information for identifying species traits and names. However, significant changes throughout the life cycle often lead to species misidentification (Ding *et al*., 2006; Heemstra and Randall, 1993). Morphological similarities, especially in the juvenile phase, suggest that individuals of several grouper species, including the genus *Plectropomus*, may fall under the same description (Zhu and Yue, 2008). All species of the genus *Plectropomus* exhibit certain similarities, such as the elongated and slender body shape and emarginate tail (Heemstra and Randall, 1993).

The colouration of individual fish can vary due to many factors, including the characteristics of their habitat, the time of day, and the activity in which they are engaged (Cai *et al*., 2013). In particular, organisms residing in shallow waters are believed to have darker body colours due to direct sunlight exposure and the accumulation of certain carotenoids compared to those living in deeper waters (Shimose and Kanaiwa, 2022), and in fish, colour brightness tends to increase with habitat depth (Frisch *et al*., 2016). Despite variability between individuals and changes in individual colouring, for example, during spawning (Samoilys and Squire, 1994), there are general species-specific differences in colouration and colour patterns (Cai *et al*., 2013; Heemstra and Randall, 1993). The differences in pattern we observed are consonant with the respective species descriptions (Heemstra and Randall, 1993), while the base body colour seems to be consonant with the depth-colour trend described by (Shimose and Kanaiwa, 2022), as the brighter-coloured *P. leopardus* typically inhabits depths of up to 100 m, while the darker *P. oligacanthus* is generally found at depths of 50 m or less (Rome and Newman, 2010).

Morphomeristic data on *P. leopardus* and *P. oligacanthus* are still limited, as reflected in the paucity of comparative data. In particular, there is a lack of morphometric data on juveniles. According to Kadir *et al*. (2023), the grouper fishery in South Sulawesi is dominated by juveniles and subadults, although there is a need for research on size at first maturity, as fishing pressure can influence growth and maturation (Kuparinzen and Merilä, 2007; Trippel, 1995). The

slight differences in morphometric ratios could be attributed to ontogenetic factors, as the specimens from the Makassar Strait were small individuals in the juvenile (immature female) phase of the protogynous life cycle. For *P. leopardus*, the traits described by Heemstra and Randall (1993) were obtained from mostly larger individuals, although the range stated includes juveniles, sub-adults, and mature specimens; the 476 mm SL specimen reported by Sujatha and Shrikanya (2012) was also likely in the mature female or male phase. There is no mention of the size range of specimens used in the description of *P. oligacanthus* in Heemstra and Randall (1993).

In Heemstra and Randall (1993), *P. leopardus* (SL 120 to 500 mm) dorsal fin ray counts covered a larger range (D, VII-VIII+10-12), while the dorsal fin rays and spines reported by Sujatha and Shrikanya (2012) for an individual (476 mm SL) from India was within the range in this study (D, VIII+11). *P. oligacanthus* collected from the Makassar Strait were within the range reported by Heemstra and Randall (1993).The pectoral fin ray count was lower (12-13) for *P. oligacanthus* in this study compared to Heemstra and Randall (1993) (14-16). However, this is not considered a diagnostic character, and is not given for *P. leopardus* in Heemstra and Randall (1993).

Vertebrate counts from this study are consistent with previous reports on grouper vertebrae counts. At the genus level, eleven genera in the tribe Epinephelidae are reported as having 24 vertebrates, including the genera *Plectropomus*, *Cephalopholis*, *Epinephelus*, and *Variola* (Leis, 1986). At the species level, reports of grouper species with 24 vertebrates include *Epinephelus areolatus* from India Darwin *et al.* (2020), *E. septemfasciatus* (Nagano *et al.*, 2007) and *E. bruneus* from Japan (Iwasaki *et al.*, 2018), *E. awoara* and *E. tukula* from China (Chen *et al.*, 2021).

3.2.2 Molecular identification

The present study successfully utilized DNA barcoding of the mitochondrial cytochrome oxidase subunit I (COI) gene to identify *Plectropomus* species from the Makassar Strait, confirming the presence of *P. leopardus* and *P. oligacanthus*. The high sequence similarity (98-100%) observed between the sequences obtained from our specimens and their nearest GenBank accessions directly confirmed our initial morphological identifications. This consistency between molecular and morphological results underscores the effectiveness of the COI gene as a reliable molecular marker for species identification in groupers like juvenile (Hebert *et al.*, 2003; Sachithanandam *et al.*, 2022; Ward *et al.*, 2005). Furthermore, this finding highlights the value of an integrated approach, combining molecular and morphological characteristics, for accurate species identification, particularly in taxonomically challenging groups (Ariyanti and Farajallah, 2019).

The phylogenetic reconstruction, incorporating a comprehensive dataset of 250 homologous *Plectropomus* sequences from GenBank, revealed more nuanced genetic patterns than simple species confirmation. All specimens from the Makassar Strait nested within their respective high-level species clades (*P. leopardus* and *P. oligacanthus*), the *P. leopardus* sequences from the Makassar Strait (highlight yellow) were distributed across two distinct lower-level clades. One specimen grouping with sequences from Vietnam (MK777629.1) and from USA (JN021314.1), and three others clustering with accessions from Bali (JN313060.1) and another unknown location, reveals intra-species complexity and shows that the grouper specimens from the Makassar Strait nested within the expected clades. Overall, *P. leopardus* has several subclades and is most closely related to the *Plectropomus* spp. clade, containing accessions submitted under various names. The closest sister clade to *P. leopardus*, labelled as *Plectropomus* spp., comprises sequences submitted under a variety of names, raising doubts on the accuracy of source specimen identification. Furthermore, two *P. areolatus* specimens (KU943494 and KU943476) from Taiwan (red font) (Chang *et al.*, 2017) resolved within the *P. leopardus* clade. These examples indicate challenges in *Plectropomus* species identification, while the tree structure indicates the possibility of cryptic species within populations currently considered as *P. leopardus*, highlighting and the need to clarify the taxonomy of this genus, combining classical morphology-based and molecular taxonomy approaches.

Similarly, *P. oligacanthus* sequences from the Makassar Strait (green highlight) were both placed within the high-level *P. oligacanthus* clade. Two specimens clustering with accessions from unknown location (MF185617.1, MF185618.1, and MF185619.1). The sister clade closest to *P. oligacanthus* was *P. laevis*, and two *P. laevis* accessions from the Philippines (KC970496 and KF009646) (red font) resolved within the *P. oligacanthus* clade with 100% identity to several *P. oligacanthus* sequences. This reinforces the need for taxonomic clarification within the genus *Plectropomus*.

3.2.3 Haplotype network

Haplotype networks are employed in population genetics to visualize intraspecific relation-

ships, infer population biogeography, and elucidate evolutionary history (Leigh and Bryant, 2015). Furthermore, haplotype networks are frequently used to clarify relationships exposed through phylogenetic analyses and to visualize relationships between differences in DNA sequence data and other factors, such as place of origin (Posada and Crandall, 2001; Teacher and Griffiths, 2011).

The haplotype network analysis of *P. leopardus* revealed four distinct haplotype groups. Group A comprised 18 haplotypes from various geographic locations; each bar on the lines connecting haplotypes indicates one mutation. Therefore, fewer bars on lines connecting haplotype groups indicate a closer genetic relationship; for instance, haplotypes H-1 and H-6 are separated by one mutation. Conversely, longer lines indicate a more distant relationship, as seen between haplotypes H-1 and H-9 with 8 mutations. All sequences from this study were in group A; however, other Sulawesian specimens, including sequences from other studies with source specimens from the Spermonde Islands in the Makassar Strait, were placed in both group A and group B. The large number of mutations between groups may indicate cryptic species or ongoing speciation within this species. Irrespective of whether these groups represent one or more species-level taxa, the network structure also indicates the need for stock delineation incorporating molecular methods.

The haplotype analysis of *P. oligacanthus* shows two groups (A and B) separated by 44 mutations. The first group consists of three haplotypes originating from different countries. The second group comprises six haplotypes from several unknown locations. The sequences from this study were both placed in group A (H-1 and H-2). *P. oligacanthus* is described in Heemstra and Randall (1993) as a widespread but rare species, with a patchy reported distribution likely due to its scarcity. The low number of sequences available, combined with the haplotype network structure, indicates a need for further research on this species, including voucher DNA barcoding and genetic population structure for stock delineation. Furthermore, the high proportion of GenBank accessions with no data on the geographic origin of the source specimen highlights the importance of metadata when submitting nucleotide sequence data to GenBank.

Haplotypes evolve over time, but tend to diverge over time between populations separated by barriers that limit migration, restricting the movement of individuals within the population due to certain barriers, leading to the formation of separate sub-populations (Hidayani *et al*., 2022). The observed patterns indicate barriers in both species. Mutations leading to new haplotypes and their distribution across populations can be influenced by biogeographic and oceanographic conditions, especially factors that can disrupt gene flow. In the case *of P. leopardus*, such factors may have led to the formation of subspecies within *P. leopardus*, with at least one study (Cai *et al*., 2013) considering that two distinct subspecies or species within *P. leopardus* as currently recognized can be differentiated based on colour patterns and mtDNA analyses including barcoding. Overall, the haplotype networks indicate high genetic diversity within both *Plectropomus* species studied, and indicate this diversity may be linked, at least in part, to genetic barriers separating populations in different regions, as well as intra-population diversity (Hughes *et al*., 2008).

4. Conclusion

This study highlights the importance of combining DNA barcoding and morphometric-meristics approaches in identifying grouper species, particularly in the genus *Plectropomus*. This approach succeeded in identifying juvenile groupers caught in the Makassar Strait. The genetic analysis clearly separated the species while revealing high intraspecies genetic diversity within *P. leopardus* and *P. oligacanthus,* possibly indicating cryptic species or sub-species. These findings provide insights into population structure that can inform conservation and sustainable fisheries management, while highlighting the need for further research to understand *Plectropomus* taxonomy and biogeography and to delineate grouper stocks in this region, and underscore the importance of accurate and comprehensive metadata in nucleotide database submissions.

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Authors' Contributions

All authors have contributed to the final manuscript. The contribution of each author was as follows: MM, NNK, AAAH, AAH, and WU conceived and planned the research, collected the data, drafted the manuscript, and designed the figures. AMM contributed to the research concept, data analysis, figure design, and critical revision of the article. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

All the authors declared no conflict of interest with respect to the publication of this manuscript.

Declaration of Artificial Intelligence (AI)

The author(s) affirm that no artificial intelligence (AI) tools, services, or technologies were employed in the creation, editing, or refinement of this manuscript. All content presented is the result of the independent intellectual efforts of the author(s), ensuring originality and integrity.

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