DNA Barcoding and Phylogenetic Analysis of Commercially Important Groupers (Serranidae) in Raja Ampat using gene marker Cytochrome c Oxidase I (COI)

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Abstract

Groupers are one of the most important and commercial fishes; many of the species are classified as Life Reef Fish Trade (LRFT). The number of grouper species in these locations may be more diverse than at other sites. Identifying the species number of a grouper is essential for mapping reef fitness. However, morphological identification of groupers is relatively difficult. Molecular methods such as DNA barcoding could be an option and even serve as reliable validation. Therefore, this research was conducted to identify groupers belonging to the Serranidae family. The grouper samples were obtained from the fish market in Waisai, Raja Ampat, Indonesia. The DNA barcoding employed the COI genes to accurately identify groupers. The Qiagen DNeasy 96 tissue extraction kit was used to extract DNA, and Primer Fish F1/R1 was applied. The sequences were analysed using ClustalW in MEGA 7.0 and phylogenetic tree was generated using the NJ (Neighbour Joining) method. Eleven species of groupers belonging to five taxa were discovered in Waisai, Raja Ampat. Anyperodon, Cephalopholis, Epinephelus, Plectropomus, and Variola are the grouper genera. Epinephelus was the predominant genus within the serranidae family, with 51.4% of the total. The International Union for Conservation of Nature's (IUCN) Red List category puts the majority of grouper species in the 'Least Concerned' category. This study confirmed the effectiveness of DNA barcoding for species identification and the value of the COI gene for Grouper's phylogenetic research. The outcome offers information about fisheries resources, which should be helpful in managing fisheries in reef ecosystems.

Keywords: *Waisai, Reef Fish, Fish Trade, Mitochondrial DNA, DNA authentication*

Introduction

Raja Ampat is located in the Coral Triangle area (CT) and is part of the MPA, or Marine Protected Area (Allen, 2008). As a rich biodiversity area, it has special features such as unique and uncommon habitat, a delicate and sensitive environment, ecologically important habitats, and a high level of diversity (Asaad *et al*., 2017). Due to its richness, Raja Ampat has become a fishing ground for high-value reef fish, especially grouper.

Grouper, commonly known as Serranidae, is a luxury export reef fish. There are at least 77 reported species of Indonesian grouper (IUCN, 2024). The FAO (Food and Agricultural Organization of the United Nations) reported that grouper supplied more than 462,000 tons of fish production in 2017 and increased from 1960 to 610,000 tons per decade (Koenig *et al*., 2020). As a commercial target fish species, grouper may have significant pressure.

Based on the IUCN Red List, 163 species of grouper indicate that 20 species (12%) are 'Risk Extinction', 22 species (13%) are 'Near Threatened', and overall, all species (30%) are 'Data Deficient'. Approximately 70 percent of the overall wild catch is documented with limited detail, using more general classifications as "Groupers nei" (Amorim and Westmeyer, 2016). Identifying grouper species morphologically is a perplexing one. Combinations of color patterns and ontogenetic changes were attributes used to identify species (Heemstra and Randall, 1993).

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Advanced molecular techniques, such as DNA barcoding, help resolve ambiguities at the species level of identification (Pavan-Kumar *et al*., 2020). Bolaji *et al*. (2023) state that DNA barcoding has recorded great success in the identification of fish species. The cytochrome c oxidase subunit I (COI) gene sequences can be used to sort and identify fish and other invertebrates (Prehadi *et al*., 2015; Deepti *et al*., 2017; Ariyanti *et al*., 2023; Fadli *et al*., 2024). Other research used DNA barcoding methods to identify the genetic and phylogenic of structure of *Ephinepelus* spp. (Jefri *et al*., 2015), stingrays (Madduppa *et al*., 2016), Halmaheran walking shark (Madduppa *et al*., 2020), Eastern Atlantic seabream (Nuryanto *et al*., 2023), blue swimming crab (Joesidawati *et al*., 2023), and reef fish in Weh Island (Fadli *et al*., 2021). The application of DNA barcodes and genetic approach has helped improve our understanding of fisheries management (Hakim *et al*., 2023; Shetty and Shingadia, 2023) . Therefore, the objective of this research was to use DNA barcoding to accurately determine the species of grouper fish found in the specific region of interest. The grouper species from Raja Ampat was expected to support earlier findings and provide valuable information for implementing necessary management measures for priority grouper species.

Materials and Methods

The study was conducted in Waisai, Kepulauan Raja Ampat, Papua Barat Daya Province, Indonesia (Figure 1.). These provinces are known as suppliers of dead groupers in Indonesia (KKP, 2018). 37 from 11 species of groupers were collected from traditional fish markets. Fish were selected based on the morphological diversity in the locations visited to refer to the Serranidae family group following Allen and Erdmann (2012). Individual fin clips were then collected by cutting the fins from each fish and preserved in 96% ethanol in 2 mL cryotubes.

DNA extraction and PCR assay

The DNA was extracted from fin clips using a commercial extraction kit, the Qiagen DNeasy 96 tissue, following the instructions provided by the manufacturer. The mitochondrial cytochrome oxidase I (COI) gene was PCR amplified using the forward primer Fish F1 R1 (TCA ACC AAC CAC AAA GAC ATT GGG AC) and the reverse primer (TAG ACT TCT GGG TGG CCA AAG AAT CA) (Ward *et al*., 2005). The primer was applied to 37 Waisai samples. PCR reaction mixtures of 25 µl were made. 3 µl of genomic DNA (extracted DNA), forward and reverse primers in 1 µl of each, 12.3 µl of Bioline's MyTaq DNA polymerase mix, and 9 µl of very pure water were mixed together. The 658-bp standard fragments of Cytochrome oxidase I (COI) was amplified using a thermocycler. The amplification process involves heating at 94°C for five minutes, then 40 cycles of denaturation at 94°C for 0.5 mins, annealing at 56°C for five minutes, and extending at 72°C for one minute. After the cycles, the mixture was held at 72°C for seven minutes as final extension and then cooled to 10°C (Sachithanandam *et al*., 2015). The amplicons, or PCR products, were put on a 1.5% agarose gel to check their quality and confirm the size of the amplicon. They were then sequenced using the Sanger method by sending the amplicons to a third party Genetika Science Malaysia for DNA sequencing.

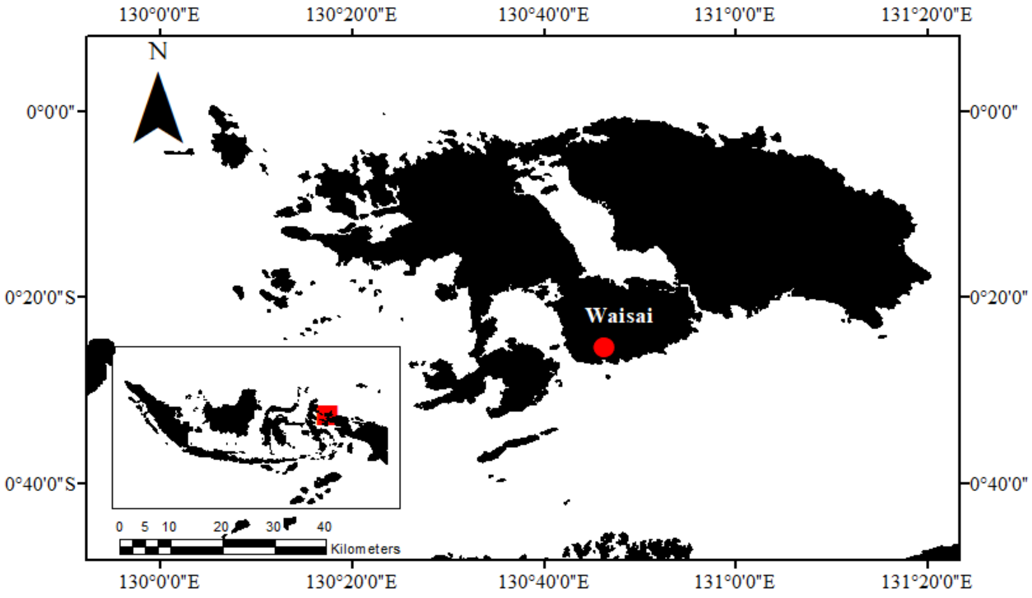

Figure 1. Location of the grouper samples from Waisai, Raja Ampat, Indonesia

Data analysis

All sequences were aligned using ClustalW in MEGA 7.0 software and then matched with the database of DNA sequences in GenBank (11 sequences) using BLAST. The distances within and between species were calculated using the P-distance and a phylogenetic tree was generated using the NJ (Neighbour Joining) method in 1000 bootstrapping. Several sequences of grouper species from GenBank were used to root the tree for comparative purposes. iTOL (Interactive Tree of Life) was used to develop the phylogenetic tree visualization.

Result and Discussion

Thirty-seven COI sequences (713 bp) of grouper from Waisai were shown in the study, covering 11 species across four genera in the Serranidae family. Similarity with 99–100% belonged to all sequences, except one sequence representing *Plecropomus leopardus* as 92% similar to BLAST NCBI. The lower E-value and high percentage of query coverage (Table 1) show successfully identified species-level designations for the grouper specimens.

Molecularly, the majority of the groupers belong to the genera *Epinephelus* (51.4%) (Figure 2.). *Epinephelus* comprises five species in this study, including *E. areolatus*, *E. bleekeri*, *E. coioides*, *E. malabaricus*, and *E. ongus*. *E. areolatus* contributed to a significant number of specimens. Other seranids were barcoded as *Plectropomus*, which includes *P. maculatus* and *P. leopardus*; V. *albimarginata*, one of two species in the genus *variola*; *Cephalopolis cyanostigma* and C. *miniata*, both in the genus *Cephalopolis*. *Anyperodon leucogrammicus* was also identified as a lone representative of its respective genera.

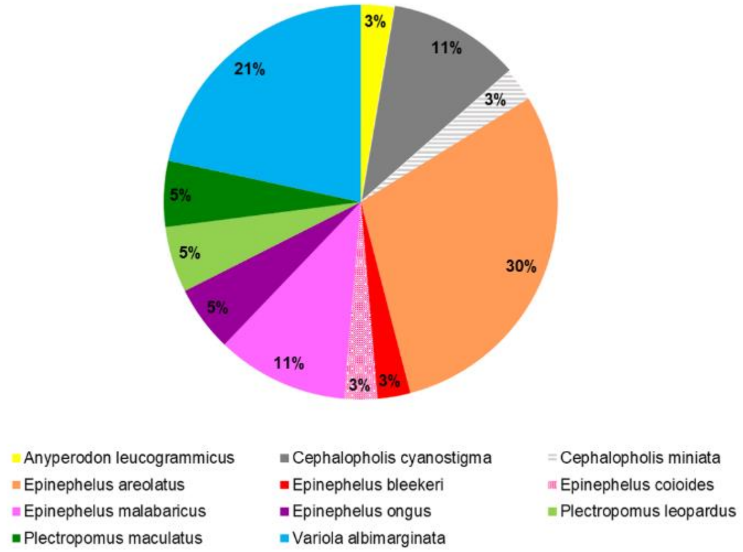

Figure 2. The composition of grouper species on Waisai Raja Ampat fish landings using DNA barcoding (COI)

Table 1. List of the BLAST results and IUCN status of Waisai, Raja Ampat grouper species

Species	Total number of individual	Query cover (%)	E. Value	BLAST (%)	Status IUCN
Anyperodon leucogrammicus	1	95	0	100	LC
Cephalopholis cyanostigma	4	92-97	0	99-100	LC
Cephalopholis miniata	1	99	0	100	LC
Epinephelus areolatus	11	96-100	0	99-100	LC
Epinephelus bleekeri	1	94	0	100	LC
Epinephelus coioides	1	99	0	100	LC
Epinephelus malabaricus	4	95-98	0	99-100	LC
Epinephelus ongus	2	95-99	0	100	LC
Plectropomus leopardus	2	96-98	0	92-100	LC
Plectropomus maculatus	2	97-98	0	100	LC
Variola albimarginata	8	96-99	0	99-100	LC

*LC: Least Concerned

All species of serranids are clustered with their respective species in the phylogenetic tree. *Paranthias pascalus* was the outgroup of the grouper from the Waisai site. Based on sequence data, the *Epinephelus* was distinct into two clades by neighbour-joining analysis. The first clade includes an internal node uniting *Epinephelus areolatus* and *Epinephelus bleekeri*. The second clade comprises *Anyperodon leucogrammicus, Epinephelus coioides, Epinephelus malabaricus,* and *Epinephelus* ongus. *Anyperodon leucogrammicus* was monophyletic with *Epinephelus*, which was supported by strong bootstrap analysis (0.88 or equivalent 88%). *Cephalopolis is* also distinct into two clades: *Cephalopolis cyanostigma* and *Cephalopolis miniata. Plectropomus* was separated into two clades, which are *Plectropomus leopardus* and *Plectropomus maculatus*. A strong bootstrap existed for the splitting of these two clades (1 or equivalently 100%). The eight individuals of species V. *albimarginata* under the genus *Variola* were in one clade in the phylogeny tree.

Epinephelus coioides and *E. malabaricus* sometimes were difficult to distinguish based on color pattern. The monophyletic relationship between both of them was shown in the phylogeny tree and also shows close genetic distance (Table 2.). Govindraju, GS Jayasankar, 2004 state that *E. coioides* has a very close genetic relationship with *E. malabaricus*. The close genetic relationship also shows in E. *bleekeri* and E. *areolatus*. E. *bleeker*i is sometimes confused with E. *areolatus* (CSIRO, 2014). The genetic distance of eleven species ranged from 0.038 to 0.209, and within Epinephelus genera, there is a close genetic distance compared to other genera (Table 2).

The new classification of the Epinephelidae family was sub revised the *Anyperodon leucogrammicus* as *Epinephelus leucogrammicus*. Anyperodon monophyletic with E. *coioides*, *E. ongus*, and *E. malabaricus*. The result also shows the position of *Anyperodon* and *Epinephelus* in the phylogenetic tree, similar to Ma and Craig (2018); Wang *et al*. (2022). The research showed genera *Anyperodon* and *Epinephelus* clustered with a strong monophyletic branch. The inclusion of the monotypic *Anyperodon* within *Epinephelus* is consistent with all previous molecular studies (Ma and Craig, 2018). *Anyperodon leucogrammicus* has the homotypic synonym *Epinehelus leucogrammicus* (NCBI, 2023). Based on genetic distance, *Anyperodon* has a close genetic relationship with *Epinephelus coioides* compared to other *Epinephelus* genera (Table 2.). The monophyletic relationship also shown in *Plectropomus maculatus* and *leopardus*, as is the case with *Cephalopolis cyanostigma* and *miniate, E bleekeri* DD (IUCN, 2024).

The eleven species sampled in Waisai, Raja Ampat, are categorized as 'Least Concern' (LC) according to the Red List of IUCN (International Union for the Conservation of Nature's) for Threatened Species. Nevertheless, CITES did not assess all grouper species ((http://www.cites.org/, accessed June 10, 2023). All the serranidae species described are part of the Live Reef Food Fish Trade (LRFFT), together with other valuable fish species (Bellwood *et al*., 2004; Sadovy de Mitcheson, 2019). Epinephelinae under family Serranidae, was the target of a high price, which means the IUCN state of all species that mention in this research could potentially change to the dangerous level state. Since the LC category encompasses 80% of teleostei and takes into account the fisheries in this region, it is also "unsafe" to disregard the management (IUCN, 2019). Most grouper fisheries have been ongoing for many generations. Sadovy de Mitcheson *et al*. (2013) propose that the IUCN criteria should take into account the risk of extinction due to unregulated fishing pressure.

Table 2. Genetic distance of groupers species in Waisai, Raja Ampat

Species	1	2	3	4	5	6	7	8	9	10	11
Cepalopholis cyanostigma	-	*	*	*	*	*	*	*	*	*	*
Epinephelus areolatus	0.172	-	*	*							
Epinephelus bleekeri	0.163	0.112	-	*	*	*	*	*	*	*	*
Epinephelus coioides	0.154	0.155	0.138	-	*	*					
Epinephelus malabaricus	0.161	0.160	0.140	0.038	-	*	*	*	*	*	*
Epinephelus ongus	0.165	0.155	0.150	0.109	0.114	-	*	*	*	*	*
Variola albimarginata	0.174	0.183	0.185	0.171	0.177	0.196	-	*	*	*	*
Cepalopholis miniata	0.170	0.168	0.155	0.183	0.175	0.176	0.187	-	*	*	*
Plectropomus maculatus	0.182	0.169	0.192	0.195	0.193	0.188	0.187	0.195	-	*	*
Plectropomus leopardus	0.189	0.179	0.186	0.186	0.193	0.189	0.190	0.209	0.050	-	*
Anyperodon leucogrammicus	0.175	0.147	0.141	0.112	0.133	0.129	0.179	0.172	0.176	0.178	-

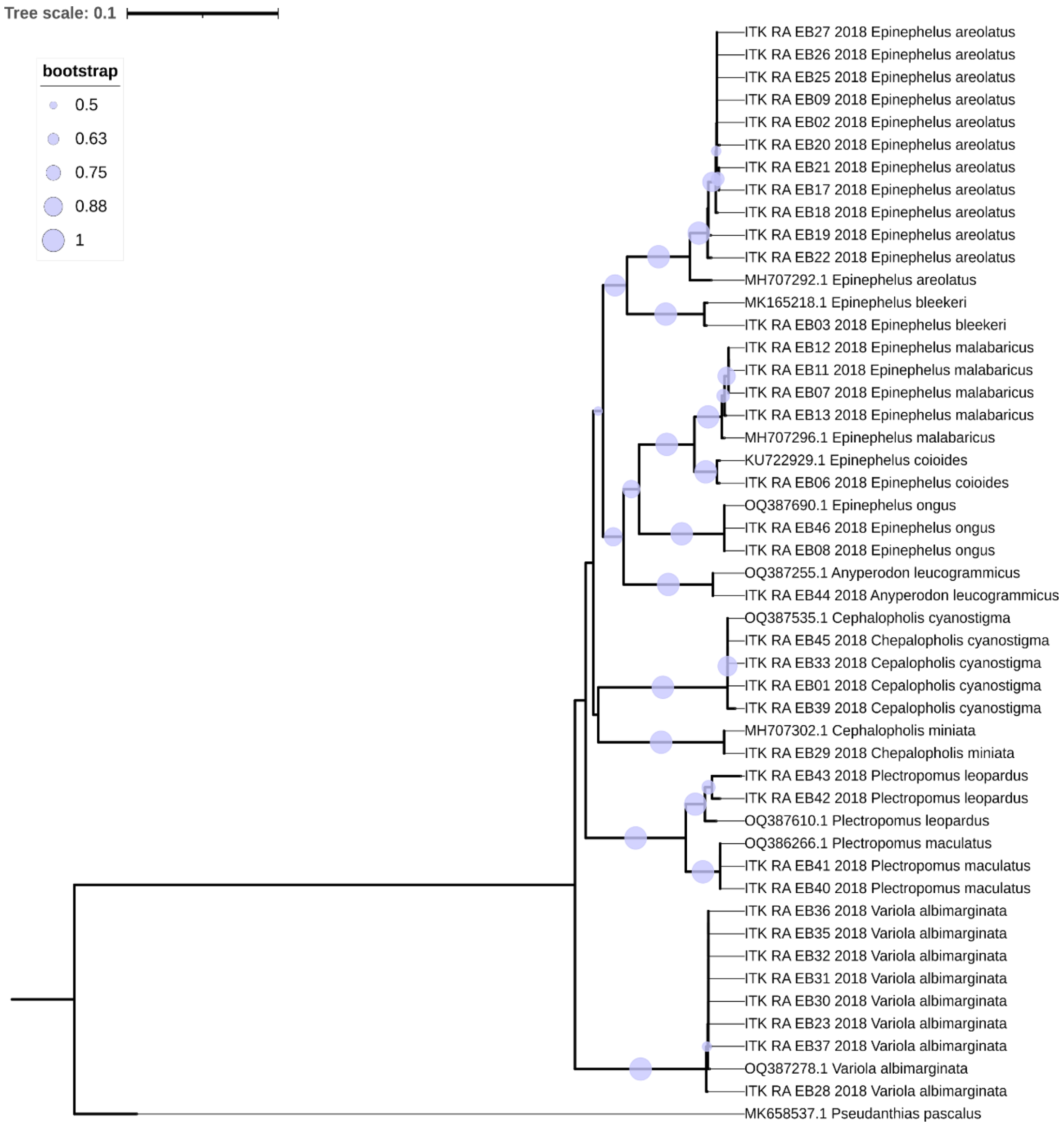

Figure 3. Neighbour Joining (NJ) tree of eleven species grouper from Waisai Raja Ampat (1000 bootsrap)

Validation of groupers using DNA barcoding was able to accommodate the ambiguity of species traded in Raja Ampat as luxury fish. The outcome was helpful in expanding knowledge about DNA barcoding-identified grouper species in Raja Ampat. Previously, research was done on Salawati Island on eight fish that identified eight species under 3 genera; other research was done in the northern part of the Bird's Head Seascape (BHS) of Papua, including Raja Ampat and in six neighboring areas, 16 species were identified under 5 genera (Tapilatu *et al.*, 2021). DNA barcoding was also extremely useful in the quality monitoring of ecologically important fish (Schoelinck *et al.*, 2014). In addition, the result also provides information for the government or other stakeholder in fishery resources, which should be valuable in managing sustainable fisheries.

Conclusion

COI genes successfully identified groupers from the fish market in Waisai Raja Ampat and revealed eleven species under five genera. E*pinephelus* comprises five species, including *E. areolatus, E. bleekeri, E. coioides, E. malabaricus*, and *E. ongus*. *E. areolatus*. Plectropomus is comprised of *P. maculatus* and *P. leopardus*. *V. albimarginata* under the genus *Variola. Cephalopolis cyanostigma* and *C. miniata* under *the Cephalopolis* genera. *Anyperodon leucogrammicus* was also identified, which is monotypic with Epinephelus. The genetic distance of eleven species ranged from 0.038 to 0.209; the closest distance was between *Anyperodon leucogrammicus* and *Epinephelus coioides*. The species are listed as Least Concerned based on IUCN status.

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