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Molecular phylogeny of grouper of *Epinephelus* genus in Jayapura, Papua, Indonesia inferred from Cytochrome Oxidase I (COI) gene

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Abstract. *Dwifajri S, Tapilatu RF, Pranata B, Kusuma AB. 2022. Molecular phylogeny of grouper of* Epinephelus *genus in Jayapura, Papua, Indonesia inferred from Cytochrome Oxidase I (COI) gene. Biodiversitas 23: 1449-1456.* Grouper (Serranidae: Epinephelinae: *Epinephelus*) fish have high economic value, and are relatively overfished, yet have not received serious attention to determine conservation status by the International Union for Conservation of Nature (IUCN). DNA barcoding is an important molecular approach to identify Papua's grouper species. The objective of the present study was to analyze species diversity and molecular phylogeny of *Epinephelus* grouper based on DNA sequences of mitochondrial control region COI gene. Samples of grouper fish were collected from Hamadi, Sentani, and Youtefa local fish markets in Jayapura, Papua during August 2020. Grouper was morphologically identified, photographed and its fin was clipped and preserved for molecular analysis. Present study used primers, i.e., Fish R1 5'TAGACTTCTGGCCAAGAATCA3' and Fish F1 5'TCAACCAACCACAAAGACATTGGCA3'. Based on gene bank comparison at the sequence length 689 base pairs, the present study obtained seven species of grouper (Serranidae: Epinephelinae), namely *Epinephelus areolatus, Epinephelus coioides, Epinephelus episictus, Epinephelus kupangensis, Epinephelus macrospilos, Epinephelus melanostigma* and *Epinephelus merra*. The phylogenetic tree was composed of seven clades, where each grouper species represented each clade. The genetic distance between *Epinephelus kupangensis* and *Epinephelus melanostigma* was determined as the closest genetic distance (0.123) in the present study, while the farthest one was found between *Epinephelus episictus* and *Epinephelus merra* (0.161).

Keywords: DNA, fish, genetic relationship, morphology, Serranidae

INTRODUCTION

Coral reef is an important ecosystem in Indonesia that provides benefits to local communities in the tropics region. One of the most important ecological functions of coral reef ecosystems is fish habitat, especially for feeding ground, nursery ground, spawning ground, and shelter from predators (Yuliana et al. 2020; Mujiyanto et al. 2021). Therefore, there is a high level of biodiversity in coral reef ecosystems, especially in terms of fish species (Allen and Erdman 2012). Reef fish are a type of fish that are highly associated with coral reefs as their natural habitat. The abundance and biodiversity of reef fish are closely related to the actual condition of coral reefs (Paulangan et al. 2019). Damaged coral reefs cause a reduction in the abundance and diversity of fish (Tony et al. 2020; Ditzel et al. 2022). One of the reef fish groups with a high economic value is grouper fish (Khasanah et al. 2019).

Grouper is a group of reef fish belonging to the family of Serranidae and the subfamily of Epinephelinae. Grouper is naturally inhabits shallow-water habitats in coral reefs, estuary, mangrove, and seagrass, both in tropical and subtropical areas (Kamal et al. 2019). Earlier studies reported 110 species of grouper recorded in Indo-Pacific waters (Heemstra and Randall 1993). In more detail, Jefri (2015) reported 7 species of *Epinephelus* found in several waters in Indonesia. Most of the grouper found in Indonesia belong to the genus of *Epinephelus* (Tapilatu et al. 2021). This genus has an elongate, subcylindrical, or oblong body shape. The morphological features of this genus share high similarities with the genus of *Cephalopholis*. Therefore, several misidentifications potentially occur during morphological identification in between both genera. The use of morphological characteristics in the characterization of species must be supported by a molecular approach, because morphological characteristics alone are very limited. After all, traits are influenced by age and various environmental factors (Becker et al. 2015; Hulley et al. 2018).

DNA barcoding is a system designed to identify species accurately, quickly, and automatically by using short, standardized gene regions as internal species tags (Imtiaz et al. 2017). This molecular approach has been become very popular and rapidly developed in the last decade (De-Franco et al. 2012; Ulrich et al. 2013; Veneza et al. 2014). This approach may facilitate better identification results than morphological-based identification only. DNA barcoding is also a powerful tool to monitor biodiversity

and also construct molecular-based phylogeny of certain species (Pei et al. 2017). A molecular phylogenetic study aims to predict the existence of evolutionary relationships among tested species depicted in tree-like diagrams (Ramos et al. 2021). In addition, genetic and morphological information is vital to support conservation efforts and sustainable grouper trade in Indonesia (Jefri et al. 2015). The molecular phylogenetic tree also shows the estimation of genetic differences between the ancestor and the offspring (Makarenkov et al. 2006).

DNA barcoding using Cytochrome Oxidase subunit 1 (COI) markers has been previously reported to be used in grouper subfamily: Epinephelinae (Aziz et al. 2016), especially grouper of *Epinephelus* genus (Deepti et al. 2018; Qu et al. 2018; Ariyanti et al. 2019; Durand et al. 2020; Basith et al. 2021). Additionally, previous reports also perform the DNA barcoding and phylogenetic study on *Epinephelus* spp. from the water the Madura Island (Basith et al. 2021) and several water regions of Indonesia (Jefri et al. 2015). However, a similar study, specifically in Jayapura, Papua has never been carried out. Therefore, this study aimed to analyze species diversity and molecular phylogeny of *Epinephelus* grouper based on DNA sequences of mitochondrial control region COI gene. This study results may assist the local government to formulate specific policies for maintaining local grouper biodiversity and reducing overfishing of certain grouper species.

MATERIALS AND METHODS

Study sites

Samples in the form of grouper fish were collected from Hamidi, Sentani, and Youtefa local fish markets in Jayapura, Papua during August 2020 (Figure 1). Meanwhile, molecular analysis was carried out at the Genetics Laboratory of the University of Papua based in Manokwari, West Papua Province, Indonesia.

Sampling method

There were 13 samples of grouper fish collected from the local fish markets and landing stations. The number of grouper fish samples was collected randomly during September 2020 in several traditional markets and landing stations in Jayapura. The grouper samples collected were groupers fish with important economic value in the community. Then, the collected samples were selected for species representation based on morphological characters for molecular analysis. Sampling was initiated by conducting interviews with local fishers to ensure the quality of sample use. Groupers were then morphologically identified, photographed and their fin (either dorsal or caudal, for about 1-2 cm) was clipped, cleaned using sterile distilled water, and then preserved in 96% ethanol for molecular analysis.

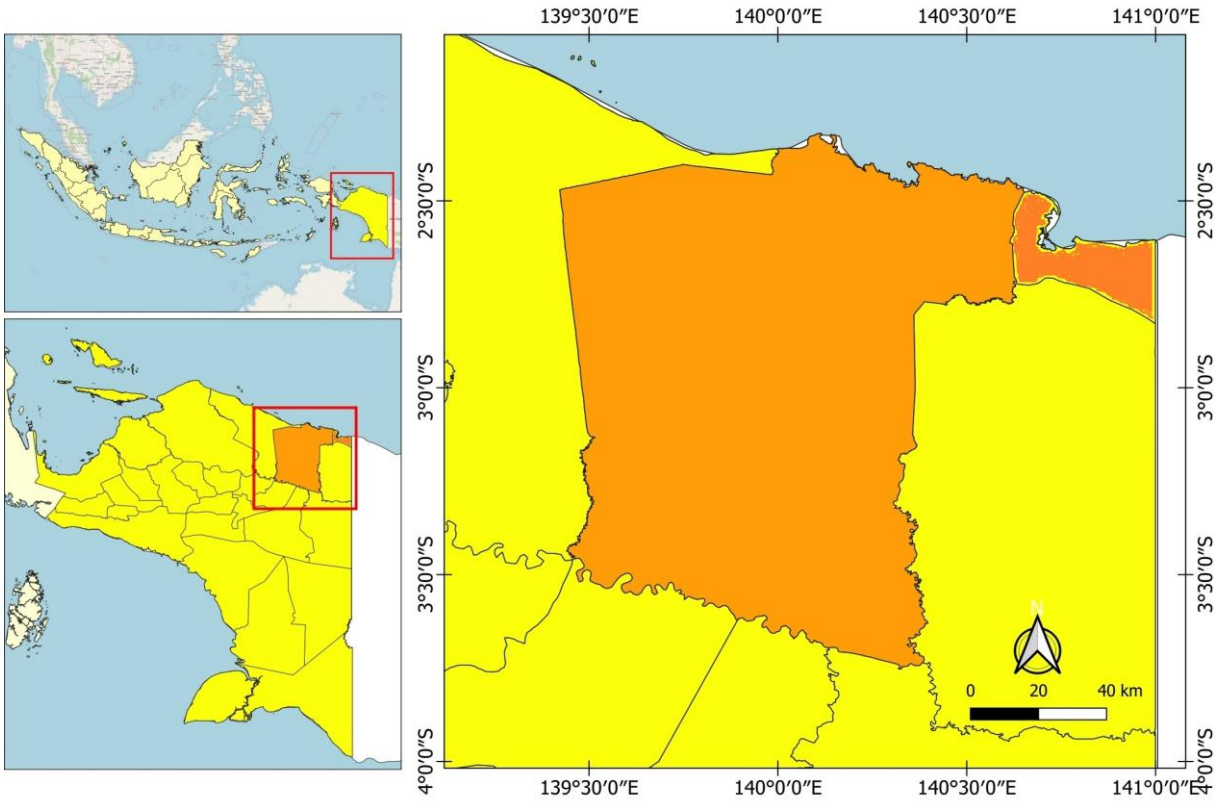

Figure 1. Sampling site of grouper of *Epinephelus* genus in Jayapura, Papua, Indonesia

Morphological identification

Grouper samples were then identified based on their morphometric and meristic characteristics. Earlier studies by Kusuma et al. (2021) reported that morphometric characteristics play a significant role in distinguishing groups of fish and differentiate one group from another. Both morphometric and meristic characteristics were powerful tools for identifying *Rutilus frisii* kutum fish (Kashefi et al. 2012) and flying fish (Rathipriya et al. 2016). All fish were photographed and then morphologically measured, especially in the variable of total body length (TBL), standard body length (SBL), head length (HL), body height (BH), tail height (TH), snout length (SL), eye diameter (ED), the distance between two eyes (DTE), body length before dorsal fin (BLDF), body length before ventral fin (BLVF), body length before anal fin (BLAF), tail length (TL), dorsal fin base length (DFBL), anal fin base length (AFBL), ventral fin base length (VFBL), pectoral fin base length (PFBL), upper tail fin length (UTFL), middle tail fin length (MTFL), lower tail fin length (LTFL) and the distance from the eye to the gill cover (DEGC).

Molecular analysis

The clipped fins were reduced to 2 mm, and further, the tissue of the clipped fines was extracted using the Geneaid gSYNC DNA extraction kit. The extraction results were then amplified in a polymerase chain reaction (PCR) using primers, i.e., Fish F1 5′TCAACCAACCACAAAGACATT GGCAC3' and Fish R1 5'TAGACTTCTGGGTGGC CAAAGAATCA3' (Ward et al. 2005). The target gene obtained was Mitochondrial DNA Cytochrome Oxidase subunit I (mtDNA COI) gene. One µL DNA template was reacted to 12 µL My Taq HS Red Mix 2x, 10.5 µL ddH2O and 0.5 µL each primer pair. The present study showed thermocycler conditions like denaturation at 98ºC, annealing at 57ºC, and extension at 72ºC with 35x cycles. The products of PCR were then electrophoresed in 1% agarose at 100 Volt for 30 minutes. The separated DNA molecules were then visualized by using UV light and documented.

Data analysis

Obtained sequencing results were edited by using the MEGA 7.0.26 software. Data were then compared with the DNA in the gene bank of National Center for Biotechnology Information (NCBI) to confirm the obtained grouper species name. The kinship between populations could be determined based on the genetic distance (Nei 1972), species identification, and phylogenetic tree reconstruction by using the Neighbor-joining method (Tamura et al. 2011). The Kimura 2-parameter was used to analyze genetic distance with 1,000 bootstrap replications. The outgroup in the present study was *Variolla albimarginata*. The phylogeny tree was constructed by using MEGA 7.0.26 application (Kumar et al. 2016). The results of molecular identification were then compared with morphological identification results.

RESULTS AND DISCUSSION

Morphological characteristics

Morphological characteristics of the grouper of *Epinephelus* genus are described in Table 1. There were six species observed with a variation in terms of head length between 6 and 12 cm. All observed groupers from the genus of *Epinephelus* had a rounded tail fin, except *Epinephelus areolatus* with the emarginate tail fin. An earlier study by Kusuma et al. (2021), Craig (2011) reported that groupers of the Epinephelus genus had several spot colors on their body, such as brown, yellow, red, and white. The upper and lower jaws were equipped with sharp and strong teeth. The mouth was broad oblique upwards, with the lower lip slightly exceeding the upper lip. The body height on the first dorsal fin was usually higher than the body height on the anal fin.

Molecular characteristics

The molecular approach by using NCBI database resulted in 13 samples of grouper fish from the genus of *Epinephelus* (Table 2). The result showed narrow variation in terms of similarity, i.e., 92.94-99.69%. The results could be similar to NCBI database sequences if there were 97%-100% similarity, while 92-96% similarity was categorized as sufficiently similar and lower than 91% was known as insignificant similar (Bhattacharjee et al. 2012). Thus, 13 samples obtained in the current study could be identified as seven species of grouper fish; *Epinephelus episticus*, *Epinephelus kupangensis*, *Epinephelus macrospilos*, *Epinephelus merra*, *Epinephelus coiodes*, *Epinephelus areolatus*, and *Epinephelus melanostigma*. Interestingly, five samples were significantly identified as *Epinephelus areolatus.*

Genetic distances

The genetic distance by using Kimura 2-parameter analysis resulted in variation of genetic distance among seven collected groupers. The closest genetic distance was observed between *Epinephelus kupangensis* and *Epinephelus melanostigma* for about 0.123. In contrast, the farthest genetic distance was found between *Epinephelus episictus* and *Epinephelus merra* for about 0.161 (Table 3). An earlier study by Nei (1972) stated that the smaller the genetic distance, the higher the similarity between observed species and *vice versa*.

Phylogenetic tree

Phylogenetic trees describe genetic relationships and reconstruct the past evolutionary history of extant species or taxa, based on current data, such as morphology or molecular information (data sequences) (Jarvis et al. 2017). The phylogeny tree was constructed from 12 individual sequences obtained in the present finding and added 32 individual sequences from GenBank (Table 4). The downloaded sequence data showed a strong relationship with the existing sequence data, evidenced by the 99-100% similarity. The addition of 32 individual sequences from other countries was used to strengthen the position of obtained sequences in the present finding collected from several marine areas in Jayapura.

Table 1. Morphological characteristics of grouper of *Epinephelus* genus

Species	Head length (cm)	Abdominal fin spines	Tail fin shape
Epinephelus merra	6	11 hard and 13 soft fins	Rounded
Epinephelus macrospilos	11	12 hard and 14 soft fins	Rounded
Epinephelus areolatus	9	9 hard and 15 soft fins	Emarginated
Epinephelus melanostigma	12	11 hard and 15 soft fins	Rounded
Epinephelus kupangensis	11	11 hard and 15 soft fins	Rounded
Epinephelus coioides	8	9 hard and 15 soft fins	Rounded
Epinephelus epistictus	11.5	11 hard and 15 soft fins	Rounded

Table 2. The results after specimen data comparison to gene bank of National Center for Biotechnology Information (NCBI)

Specimen code	Species	Query cover	Similarity	Accession
SM-KRP-PSR 01-JYP-20	*Epinephelus episictus*	100%	98.75%	KU722931.1
SM-KRP-PSR 01-JYP-19	*Epinephelus kupangensis*	99%	99.69%	MH328251.1
SM-KRP-PSR 01-JYP-18	*Epinephelus macropilos*	100%	97.25%	KM226279.1
SM-KRP-PSR 01-JYP-15	*Epinephelus merra*	100%	98.78%	MW034059.1
SM-KRP-PSR 01-JYP-16	*Epinephelus merra*	98%	99.22%	MF185547.1
SM-KRP-PSR 01-JYP-12	*Epinephelus coioides*	99%	99.54%	KY315402.1
SM-KRP-PSR 01-JYP-06	*Epinephelus coioides*	99%	92.94%	MF185482.1
SM-KRP-HAMADI-JYP-05	*Epinephelus areolatus*	99%	99.40%	KC466080.1
SM-KRP-PSR 01-JYP-05	*Epinephelus areolatus*	99%	99.38%	MN708831.1
SM-KRP-PSR 01-JYP-05 B	*Epinephelus areolatus*	98%	99.69%	MN708840.1
SM-KRP-PSR 02-JYP-05	*Epinephelus areolatus*	98%	99.69%	MN708839.1
SM-KRP-PSR 01-JYP 09	*Epinephelus areolatus*	99%	98.77%	MN870146.1
SM-KRP-HAMADI-JYP-03	*Epinephelus melanostigma*	99%	99.69%	MH707772.1

Table 3. Genetic distance among collected grouper of *Epinephelus* genus

Spesies	1	2	3	4	5	6
Epinephelus episictus						
Epinephelus kupangensis	0.131					
Epinephelus macrospilos	0.157	0.138				
Epinephelus merra	**0.161**	0.147	0.147			
Epinephelus coioides	0.128	0.131	0.155	0.152		
Epinephelus areolatus	0.149	0.128	0.152	0.147	0.152	
Epinephelus melanostigma	0.138	**0.123**	0.147	0.133	0.128	0.125

Discussion

Morphological characteristics

The grouper of *Epinephelus* genus in the present finding possessed rounded tail fins, except the *E. areolatus*, similar to a previous study by Craig et al. (2011). All observed fish also shared similarities, such as superior mouth position type. Some species such as *E. merra, E. macrospilos* and *E. episictus* had a fusiform body shape while *E. areolatus*, *E. melanostigma*, *E. coioides* and *E. kupangensis* had elongated body shapes. However, there was a more complex variation in terms of body color and spots. Based on the previous report by Craig et al. (2011), the grouper of *Epinephelus merra* has a pale brown color covered with dark brown or reddish-brown spots. Some spots on the body merged to form a horizontal line with a darker color. Spots on the fins were small. The dorsal, pectoral, and tail fins were yellowish brown. The grouper *Epinephelus episictus* had a brown to dark gray body with small brownish-black spots on the back. The front of the gill cover firmly resembled a vertical line. The dorsal, anal, and caudal fins were black, while the pectoral fin was light. The grouper of *Epinephelus macrospilos* showed grayish-brown body color with dark brown spots. Spots on the dorsal fin and tail base were black. The dorsal fin tip was soft. Anal and caudal fins were yellowish brown. The pectoral fins have black spots. The *Epinephelus areolatus* grouper had dark brown to yellowish color on the underside of the fish head and body. Yellowish-brown spots arranged tightly on the top and bottom, and the tail fin shape was emarginated. The grouper of *Epinephelus melanostigma* had a yellowish-brown body-color with blackish-brown spots scattered throughout the body, head, and fins. There were spots with lighter color on the abdomen and under the operculum. There was a large black spot under the base of the dorsal fin. *Epinephelus coioides* possessed pale brown body color with many orange to bright yellow spots along the body, head, and fins. There were five vertical lines on the body with a darker color faintly forming the letter "H" in each row. Lastly, the grouper of *Epinephelus kupangensis* had pale grayish-brown with five dark brown vertical lines that extended from the base of the dorsal fin to the abdomen. There were blackish-brown spots observed on the upper body of the head to the back (Tucker et al. 2016).

Table 4. Grouper sequence data downloaded from the National Center for Biotechnology Information (NCBI)

Species	Location	Access No.
Epinephelus epistictus	Philippines	KU705388.1
Epinephelus kupangensis	USA	MH328250.1
Epinephelus kupangensis	USA	MH328251.1
Epinephelus macrospilos	South Africa: KwaZulu-Natal	JF493445.1
Epinephelus macrospilos	India	KM226277.1
Epinephelus macrospilos	Indonesia	JN312977.1
Epinephelus macrospilos	India	KM226278.1
Epinephelus macrospilos	India	KM226279.1
Epinephelus merra	China	MF185546.1
Epinephelus merra	China	MF185547.1
Epinephelus merra	Philippines	KC970471.1
Epinephelus merra	China	MW034053.1
Epinephelus merra	China	MW034059.1
Epinephelus coioides	Australia: Western Australia	DQ107879.1
Epinephelus coioides	Indonesia: Maluku, Ambon	MN870453.1
Epinephelus coioides	Philippines: Luzon	KJ013039.1
Epinephelus coioides	China	KY315402.1
Epinephelus coioides	China	MF185482
Epinephelus coioides	Australia: West Coast	MK092070.1
Epinephelus coioides	India	MF383176.1
Epinephelus coioides	India	MF383175.1
Epinephelus coioides	Myanmar	MH235639.2
Epinephelus areolatus	Vietnam	MN708831.1
Epinephelus areolatus	Vietnam	MN708839.1
Epinephels areolatus	Vietnam	MN708840.1
Epinephelus areolatus	Indonesia: Maluku, Ambon	MN870146.1
Epinephelus areolatus	Philippines: Luzon	KC970469.1
Epinephelus melanostigma	Indonesia	HQ564438.1
Epinephelus melanostigma	Madagascar: Antananarivo	JQ349966.1
Epinephelus melanostigma	India	KM226281.1
Epinephelus melanostigma	Okinawa	MH707769.1
Epinephelus melanostigma	Japan: Okinawa	MH707771.1

Morphology-molecular agreement in genetic distance

Present findings reported that both groupers, *Epinephelus melanostigma* and *Epinephelus kupangensis*, had the closest genetic distance with a value of 0.123. The genetic distance was categorized as the medium genetic distance, according to a previous study by Nei (1972). The closer the genetic distance, the higher the similarity could indicate that specific species might share a similar ancestral origin. Our molecular finding was also in agreement with morphological results. They shared similarities in the form of elongated mouth shape, rounded tail shape, body spots, and the same dorsal fin shape. However, the grouper *Epinephelus melanostigama* had a yellowish-brown body color. In contrast, the grouper of *Epinehelus kupangensis* had a grayish-brown body color.

In contrast, the farthest genetic distance was found in *Epinephelus merra* and *Epinephelus episictus* for about 0.161. The wider the genetic distance, the lower the similarity. This argument was proved by the molecular approach and the morphological approach. The fish body color of both species was very different, where the *Epinephelus merra* grouper had a pale brown color with brown spots on the body, tail, and fins. In contrast, the *Epinephelus episictus* grouper had a brown and dark gray body with a small black spot. Additionally, both species also differed in terms of fin shape, especially in dorsal, pelvic, pectoral, and anal fin (Figure 2).

Molecular characteristics based phylogenetic tree

The phylogenetic analysis of the present study resulted in the construction of phylogenetic tree from seven clades, i.e., *Epinephelus episictus* as the 1st clade, *Epinephelus kupangensis* as the 2nd clade, *Epinephelus macrospilos* as the 3rd clade, *Epinephelus merra* as the 4th clade, *Epinephelus coioides* as the 5th clade, *Epinephelus areolatus* as the 6th clade, and lastly, *Epinephelus melanostigma* as the 7th clade.

Epinephelus merra *Epinephelus kupangensis*

Epinephelus episictus *Epinephelus melanostigma*

A **B**

Figure 2. Morphological appearance of four selected groupers. A. The farthest genetic distance was found between *Epinephelus merra* and *Epinephelus episictus*. B. The closest genetic distance was found between *Epinephelus kupangensis* and *Epinephelus melanostigma*

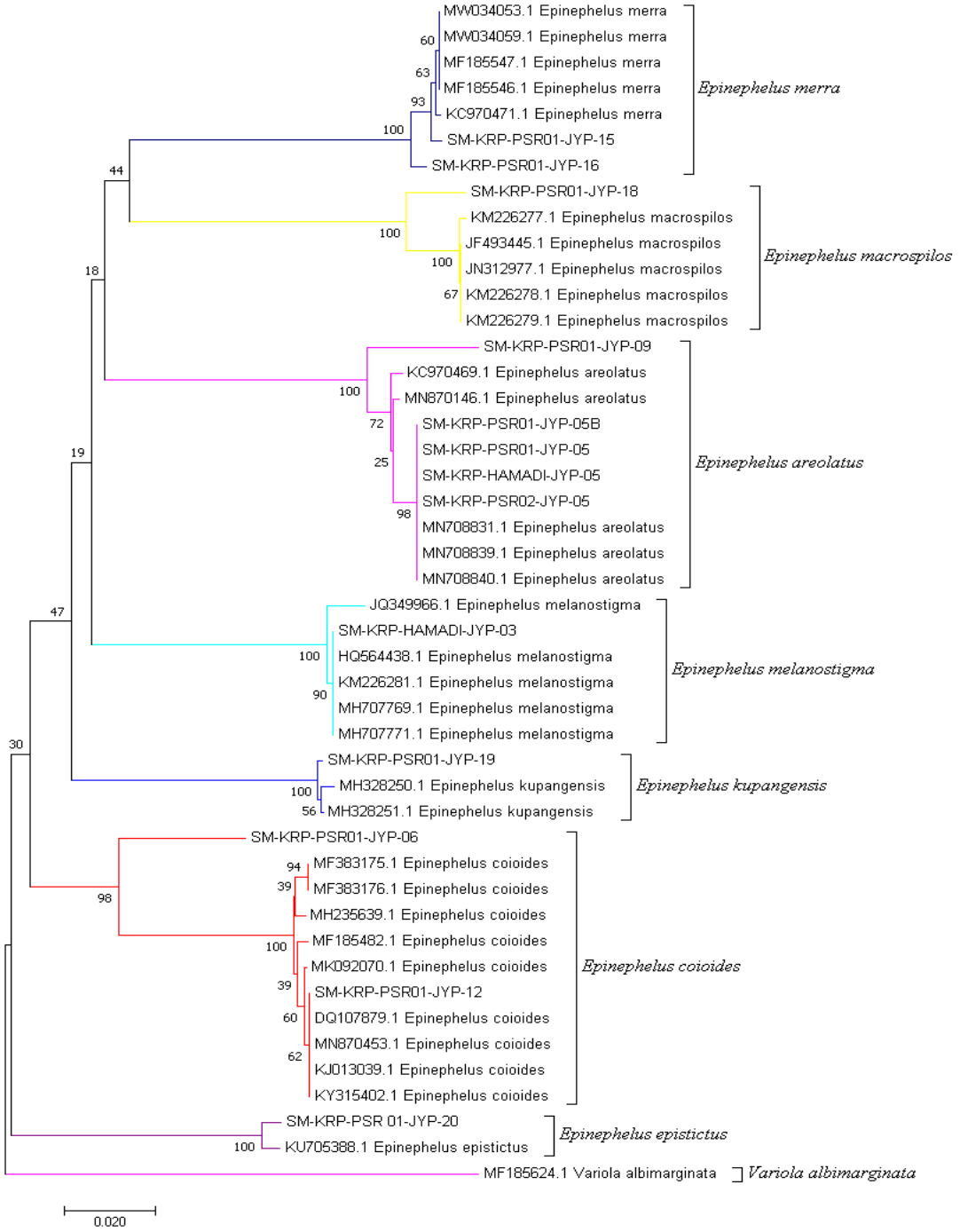

Figure 2. Reconstruction of the phylogenetic tree of grouper of *Epinephelus* genus by using the Neighbor-joining method, with 1000x bootstrap and *Variolla albimarginata* as an outgroup

All clade showed a bootstrap value of 100%, except for *Epinephelus coioides* with a sample code SM-KRP-PSR 01-JYP-06 for about 98% (Figure 3). In the first clade of *Epinephelus merra*, there was a 100% subclade with sample code SM-KRP-PSR-01-JYP-15 and SM-KRP-PSR-01-JYP-16. In the second clade of *Epinephelus macrospilos*, there was a 100% subclade of 100% with the sample code SM-KRP-PSR 01-JYP-18. In the third clade of *Epinephelus areolatus*, there was has a 100% subclade with five sample codes; SM-KRP-PSR 01-JYP-09, SM-KRP-PSR 01-JYP-05B, SM-KRP-PSR 01-JYP-05, SM-KRP-HAMADI-JYP-05, and SM-KRP-PSR 02-JYP-05. In the fourth clade of *Epinephelus melanostigma*, there was a 100% subclade with a sample code SM-KRP-HAMADI-JYP-03. In the fifth clade of *Epinephelus kupangensis* there was a 100% subclade with a sample code SM-KRP-PSR 01-JYP-19. In the sixth clade of *Epinephelus coioides*, there was a subclade of 100% with a sample code SM-

KRP-PSR 01-JYP-12. In the seventh clade of *Epinephelus episictus*, there is a subclade of 100% with a sample code SM-KRP-PSR 01-JYP-20. Interestingly, *Variola albimarginata* with code MF185624.1 was arranged as the desired design, i.e., to be an outgroup.

The phylogenetic tree results using the Neighbor Joining method's phylogenetic tree results could strengthen data from genetic distance analysis, where the closest genetic distance found in between *Epinephelus melanostigma* and *Epinephelus kupangensis* formed paraphyletic tree branches with a bootstrap value of 100%. While the farthest genetic distance found between *Epinephelus merra* and *Epinephelus episictus* was formed polyphyletic tree branches with a bootstrap value of 100%. The results also seemed to have no variation after several sequences from outside Indonesian waters had been combined.

Molecular implications for conservation of marine biological resources

The traded groupers in Jayapura traditional markets and landing stations are groupers with high economic value. This would increase grouper fishing by fishers in Jayapura and surrounding areas and cause over-fishing of groupers. Over-fishing consequently has a negative impact on the marine biodiversity, in particular fish biodiversity in Jayapura. Therefore, this research is expected to assist in identifying grouper species traded in the community to be used as a database on the existence of groupers species that have important economic value and are targeted for fishing in Jayapura.

The molecular approach was a useful tool to help the identification of species with more accurate results rather than only a morphological approach (Bingpeng et al. 2018). The combination of both morphology and molecular-based approaches could reduce the taxonomic uncertainty on observed species. Li et al. (2019) combined of molecular and morphological data, it can be concluded that two different species are considered as a single species with a high intraspecific morphological variation. This taxonomic uncertainty could lead to several problems due to the need for taxonomic certainty data to make a distribution map of endemic and invasive species, update identification of species with economic value, and legal protection policy for endangered species. The grouper biodiversity was also damaged due to a lack of conservation programs and rapid overfishing, habitat loss, global warming (Mavruk et al. 2020) dan Climate Change (Johnson 2018).

The genetic data of groupers from Jayapura water resulting from the present study was important information for policymakers to formulate a sustainable conservation management system for local marine biological resources. This step was per government regulation, such as Government Regulation of the Republic of Indonesia Number 60 Year 2007 deal with the conservation of fish resources. The fish conservation was carried out based on the principles of benefit, justice, partnership, equity, integration, openness, efficiency, and sustainable sustainability. Fish breeding also became an alternative strategy that was highly recommended for both protected and unprotected fish species in order to maintain the quantity of marine biological resources. The improvement should not only be based on quantity, but also the quality, thus present finding recommended several suggestions, i.e., the implementation of strict regulation deals with the size limit, only catch adult fish based on its morphology, fishing only in the peak season and determination of conservation zone for no fishing activity at all. The conservation zone should be made by all stakeholders, i.e., government, private and local society.

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