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Molecular phylogeny inferred from mitochondrial DNA of the grouper *Epinephelus* spp. in Indonesia collected from local fish market

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Abstract. *Jefri E, Zamani EP, Subhan B, Madduppa HH. 2015. Molecular phylogeny inferred from mitochondrial DNA of the grouper* Epinephelus *spp. in Indonesia collected from local fish market. Biodiversitas 16: 254-263.* Groupers are widely distributed in the tropical and subtropical coastal waters, and are globally one of the most commercially important groups of marine fish, commanding high market price and are being heavily targeted in fisheries. Over fishing in Indonesia becomes a pivotal factor, which is seriously threatening the grouper biodiversity, as separate catch statistics are not reported for most species, and landings are often summarized as 'serranids' or 'groupers'. This lack of species-specific catch data is due to the difficulty of identifying many of the species. The focus of this study was the tracking of molecular phylogeny of *Epinephelus* spp. of the family Serranidae. DNA amplification using mitochondrial cytochrome oxidase I resulted in 526-base pairs long sequences all samples. A total of seven species were characterized that are (*Epinephelus areolatus, E. merra, E. fasciatus, E. longispinis, E. coioides, E. ongus* and *E. coeruleopunctatus*). All of which were found to belong to 7 different clades in the constructed phylogenetic tree. *E. ongus* is genetically closest to *E. coeruleopunctatus* with genetic distance 0.091 (9%), whereas the farthest genetic distance was successfully identified between *E. ongus* and *E. merra* with genetic distance 0.178 (18%). Migration activity on spawning and movement of larvae that are affected by Indonesian Through flow suspected as the cause of the closeness between species grouper *Epinephelus* spp. in the phylogeny tree from several Indonesian seas, although information about the location and time of *Epinephelus* spp. spawning activity sometimes difficult to obtain certainty. Fish identification using molecular phylogenetic approach has been successfully applied in this study. It seems need further application on this method to avoid misidentification and due to high variety of species landing at local fish market. Nevertheless, this study would be an important data in the genetic management for the sustainable conservation and trade of grouper (*Epinephelus* spp.) in Indonesia.

Key words: Coral triangle, DNA barcoding, phylogeny, taxonomy, seafood

INTRODUCTION

Grouper are generally found on coral and rocky reefs, but some species (e.g., *Epinephelus aeneus*) are commonly found on sandy, silty or muddy bottoms. The subfamily Epinephelinae includes 159 species in 15 genera (Allen and Adrim 2003). They can grow up to 2.5 m in length and 400 kg in weight (Heemstra and Randall 1993). Their desirable taste and high market value make them the most important mariculture fish species in Asia and around the world (Chiu et al. 2008). Groupers are also among the most important resources targeted by coastal fisheries in tropical and sub-tropical areas and they exhibit behavioral characteristics that make them vulnerable (Heemstra and Randall 1993). Since 1980, Indonesia is known as the third largest supplier of groupers with export destination countries such as Singapore, Hong Kong and China. The fishermen caught the groupers in almost all coral reef seas in Indonesia. This is because the trade of live grouper is highly profitable (Nuraini and Hartati 2006). However, One-third of the Epinephelinae, particularly the genera *Epinephelus* and *Mycteroperca*, have been listed as a threatened species, thereby emphasizing the threat faced by groupers worldwide (Morris et al. 2000).

The phylogenetic relationships among the fishes in the perciform tribe Epinephelinae (*Epinephelus*, Serranidae) are poorly understood because of the very numerous taxa that must be considered and the large, circumtropical distribution of the group. Knowledge of relationships within the Serranidae has been equally tenuous (Craig and Hastings 2007). Recently few questions were raised on the *Epinephelus* species on their morphological similarities and the species had extensive phonetic similarities, suggesting that some species in *Epinephelus* spp. might belong to a same species and group (Zhu and Yue 2008).

Over the last decade, the development of fish identification using molecular phylogenetic approach has been widely conducted. One of the molecular phylogenetic approaches that can be used is the mitochondrial DNA barcoding intended to distinguish species and identify specimens that are difficult to identify, such as larval stage, organ pieces or morphologically incomplete materials, using short gene sequences (Hebert et al. 2003). Mitochondrial DNA is a crucial marker allowing researchers to recognize and identify this Serranid species for the many advantages that it offers, Mitochondrial DNA has a high mutation rate than nuclear genome, inherited solely from the mother, present in large numbers in every cell. In that it allows researchers to elucidate the evolutionary relationship among species of groupers, without looking at the entire life cycle of grouper (Waugh 2007). For evaluating genetic diversity and phylogeny,

modern molecular biology has enabled comparisons between nucleotide and amino acid sequences of different populations. Many studies were carried out in this filed, such as this of (Ilves and Taylor 2008) on Osmeridae, (Sembiring et al. 2015) on sharks, (Akbar et al. 2014) on *Thunnus albacares*, (Ku et al. 2009) on *E. quoyanus*, and (Merritt et al. 1998) on *Epinephelus* and *Mycteroperca* species. Even some countries such as Egypt and South Africa also have been doing mithocondrial DNA to fish in some supermarkets. This is done to keep out of concern because of the high incidence of substitution and regulation of the circulation of species of fish, including grouper at the International level (Galal-Khallaf et al. 2014) and (Cawthorn et al. 2012). However, the *Epinephelus* spp. are often incorrectly identified in the field because of their closely related to the morphological features.

This study was aimed to identify the genetic and phylogenic structures of *Epinephelus* spp. collected from local fish market in Indonesia as inferred from mitochondrial DNA. By the results of this study we intended to support Indonesian government in their efforts in conservation of fish resources, particularly the genetic diversity, in accordance to the Indonesian Government Regulation No. 60/2007 (The Government of the Republic of Indonesia 2007), before groupers complete wiping-out from the Indonesian seas.

MATERIALS AND METHODS

Tissue sampling

A total of 39 groupers (*Epinephelus* spp.) muscle tissues or fin clip were collected from local fishermen and fish landing sites, or purchased from seven local fish market in Indonesia since January-April 2014. Lombok (n=12 samples) in January, Karimunjawa (n=11) in May, Lampung (n=4) in February, Kendari (n=3) in January, Madura (n=3) in April, Tanakeke (n=3) in February, and Numfor (n=3) in May (Fig.1). Fish were photographed and identified at species level and fin clip was sampled and preserved in 95% ethanol at -20^{o}C for further analysis. Grouper (*Epinephelus* spp.) samples were identified according to Heemstra and Randall (1993) based on morphometric characters (i.e. shapes, colors and fins).

DNA extraction and PCR reaction

DNA extraction was done based on commercial kit (DNeasy Blood & Tissue Kit, Qiagen Cat. No. 69504) with some modification according to the tissues (blood, fin or liver), or using 10% Chelex solution (Walsh et al. 1991) at 95^{o}C. Some tissue samples are not in good shape, and sometimes hard in the extraction process so it must be combine the best of both these techniques. The segment of mtDNA COI was amplified with the primer 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' (Sachithanandam et al. 2012). Polymerase Chain Reaction (PCR) was used to amplify approximately 526bp fragment of the mtDNA CO1 gene. The procedure was performed in 24 µL reaction mixture containing 2 µL 25 mM MgCl2, 2 µL 8µM dNTPs, 1.25 µL each primer pair 10 mM, 0,125 µL Taq DNA polymerase, 2.5 µL 10xPCR Buffer, 3 µL DNA template, 12.875 µL deionize water (ddH2O), The thermo cycler conditions were: pre-denaturation at 94^{o}C for 5 min, denaturation at 94^{o}C for 30 sec, annealing at 56^{o}C for 60 sec, extension at 72^{o}C for 60 sec and final extension at 72^{o}C for 7 min with 40x cycles (Sachithanandam et al. 2012). PCR products were separated on a 1% agarose gel, which had been stained with ethidium bromide and viewed under UV Transilluminator and documented. Sequence reactions were performed in both directions using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems), 8-10 µL purified PCR product, and 4-5 µL of either primer (3 µM)

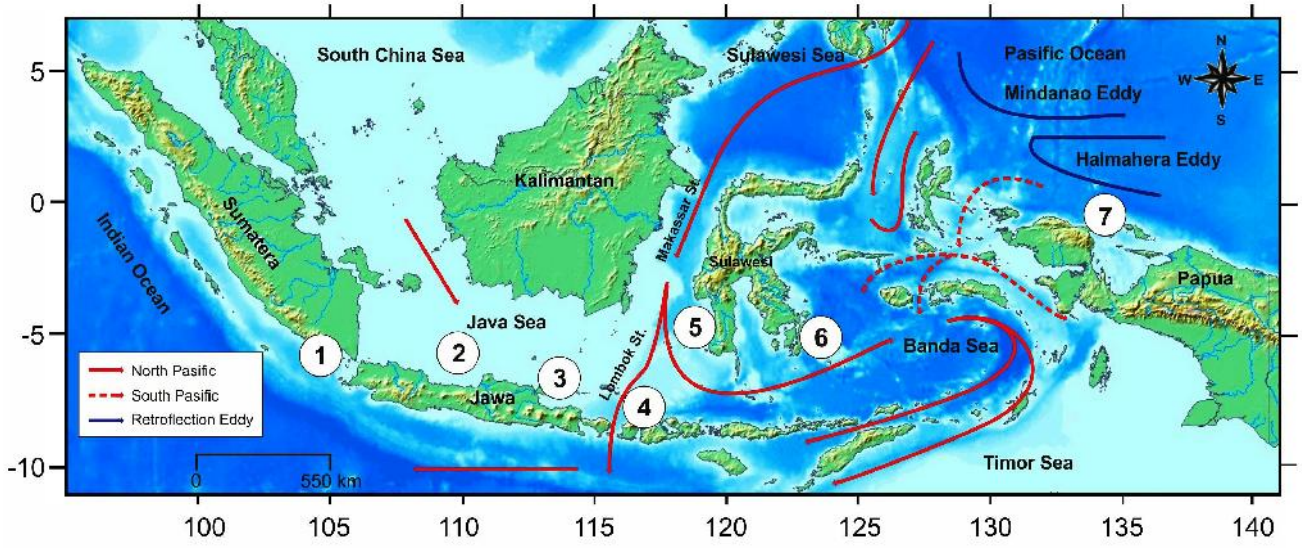

Figure 1. The sampling sites in Indonesia; 1. Lampung, 2. Karimunjawa, 3. Madura, 4. Lombok, 5. Tanakeke 6. Kendari 7. Numfor

per reaction. Sequence-reaction products were loaded into an ABI 3130xl automated sequencer (Applied Biosystems) at the Berkeley Sequencing Facility located in the United States (Sanger et al. 1977).

Data analysis

Sequences data were analyzed using MEGA 6.0.5 program edited and aligned using Clustal W to see the diversity of their nucleotide bases (Tamura et al. 2013). Sequence analysis was done along with reference sequences of various species belonging to the family Serranidae retrieved from NCBI (National Center for Biotechnology Information) GenBank. Aligned sequences were also subjected to nucleotide BLAST (Basic Local Alignment Search Tool) search to know the identity. Phylogeny tree was constructed using phylogenetic analysis of Neighbor Joining (NJ) and Maximum Likelihood (ML) methods with Kimura 2-parameter evolution model and 1000x bootstrap replications (Tamura et al. 2004). *Cephalopholis cyanostigma* was used as an out-group when constructing the phylogenetic tree.

RESULTS AND DISCUSSION

Molecular characteristics

The processes of extracting and sequencing were conducted on 39 *Epinephelus* spp. Sequences that have been aligned were then followed by BLAST analysis on the National Center for Biotechnology Information (NCBI). The results obtained seven species, namely *Epinephelus areolatus, Epinephelus merra, Epinephelus ongus, Epinephelus fasciatus, Epinephelus coioides, Epinephelus coeruleopunctatus* and *Epinephelus longispinis,* each species showed 99%-100% similarity value (Table 1).

Table 1. Summary of identified species at specific locations after BLAST in *National Center for Biotechnology Information* (NCBI), and their IUCN status. Number of samples per location is shown in bracket. LN is Least Concern.

Species	Locations (number)	Code	BLAST (%)	IUCN Status
Epinephelus areolatus	Karimunjawa (5)	EJ-KRM-01-*Epinephelus areolatus*	100	LN
		EJ-KRM-02-*Epinephelus areolatus*	100	LN
		EJ-KRM-03-*Epinephelus areolatus*	100	LN
		EJ-KRM-04-*Epinephelus areolatus*	99	LN
		EJ-KRM-05-*Epinephelus areolatus*	99	LN
	Lombok (5)	EJ-LBK-12-*Epinephelus areolatus*	100	LN
		EJ-LBK-13-*Epinephelus areolatus*	99	LN
		EJ-LBK-14-*Epinephelus areolatus*	100	LN
		EJ-LBK-15-*Epinephelus areolatus*	99	LN
		EJ-LBK-16-*Epinephelus areolatus*	99	LN
	Madura (3)	EJ-MDR-01-*Epinephelus areolatus*	99	LN
		EJ-MDR-02-*Epinephelus areolatus*	99	LN
		EJ-MDR-05-*Epinephelus areolatus*	99	LN
	Kendari (1)	EJ-KDR-03-*Epinephelus areolatus*	99	LN
	Lampung (1)	EJ-LPG-04-*Epinephelus areolatus*	100	LN
E. merra	Karimunjawa (3)	EJ-KRM-27-*Epinephelus merra*	99	LN
		EJ-KRM-28-*Epinephelus merra*	99	LN
		EJ-KRM-29-*Epinephelus merra*	100	LN
	Tanakeke (2)	EJ-TNK-01-*Epinephelus merra*	100	LN
		EJ-TNK-03-*Epinephelus merra*	100	LN
	Kendari (2)	EJ-KDR-13-*Epinephelus merra*	100	LN
		EJ-KDR-14-*Epinephelus merra*	99	LN
	Lombok (4)	EJ-LBK-01-*Epinephelus merra*	99	LN
		EJ-LBK-02-*Epinephelus merra*	99	LN
		EJ-LBK-03-*Epinephelus merra*	99	LN
		EJ-LBK-11-*Epinephelus merra*	99	LN
	Numfor (2)	EJ-NMP-03-*Epinephelus merra*	99	LN
		EJ-NMP-05-*Epinephelus merra*	99	LN
E. ongus	Tanakeke (1)	EJ-TNK-02-*Epinephelus ongus*	100	LN
	Karimunjawa (1)	EJ-KRM-58-*Epinephelus ongus*	100	LN
	Lombok (1)	EJ-LBK-10-*Epinephelus ongus*	100	LN
E. fasciatus	Lombok (2)	EJ-LBK-08-*Epinephelus fasciatus*	99	LN
		EJ-LBK-09-*Epinephelus fasciatus*	99	LN
	Lampung (2)	EJ-LPG-03-*Epinephelus fasciatus*	99	LN
		EJ-LPG-05-*Epinephelus fasciatus*	99	LN
E. coioides	Karimunjawa (2)	EJ-KRM-45-*Epinephelus coioides*	100	LN
		EJ-KRM-46-*Epinephelus coioides*	99	LN
E. coeruleopunctatus	Numfor (1)	EJ-NMP-02-*Epinephelus coeruleopunctatus*	99	LN
E. longispinis	Lampung (1)	EJ-LPG-02-*Epinephelus longispinis*	100	LN

Genetic distance

Genetic distance data obtained from seven species ranged from 0.091 (9%) to 0.178 (18%) (Table 2). According to Nei (1972), the closer the genetic distance of a species with other species means that the COI gene similarity is closer and the value of genetic distance is still at the middle limits. The results of data analysis showed that the closest genetic distance was *E. ongus* with *E. coeruleopunctatus* at 0.091 (9%) and the farthest genetic distance was *E. merra* with *E. ongus* at 0.178 (18%).

Phylogeny tree

Phylogeny tree was constructed from 39 sequences obtained from the Indonesian seas and added with *GeneBank* sequences of 31 individuals presented in Table 1 and 3. The addition of 31 sequences from other countries was aimed to strengthen the position of the sequences from Indonesia in the phylogeny tree. Phylogenetic is a description of relationship based on DNA sequence composition or protein which resembles to that of a tree to estimate the past evolution process (Baldauf 2003). The reconstruction of *Epinephelus* spp. phylogeny tree was conducted using MEGA 6.0.5 software with the bootstrap NJ and ML methods. Both tree construction methods showed similar topologies with only minor differences at deeper nodes. The results showed seven clades; *Epinephelus areolatus, E. merra, E. fasciatus, E. longispinis, E. coioides, E. ongus* and *E. coeruleopunctatus.*

Table 2. Genetic distance between the 7 species identified in the study

No.	Species	1	2	3	4	5	6	7
1	*Epinephelus areolatus*	-	*	*	*	*	*	*
2	*E. merra*	0.152	-	*	*	*	*	*
3	*E. coioides*	0.163	0.176	-	*	*	*	*
4	*E. ongus*	0.166	**0.178**	0.117	-	*	*	*
5	*E. fasciatus*	0.145	0.148	0.144	0.168	-	*	*
6	*E. coeruleopunctatus*	0.160	0.150	0.123	**0.091**	0.163	-	*
7	*E. longispinis*	0.151	0.160	0.165	0.178	0.157	0.135	-

Table 3. *GeneBank* data information of the *Epinephelus* spp. included in this analysis, location and accession number from *National Center for Biotechnology Information* (NCBI)

Species	Locations	Access number	References
Epinephelus areolatus	Luzon, Philippines	KC970469	Alcantara and Yambot 2014
E. areolatus	South China Sea, China	FJ237757	Zhang and Hanner 2012
E. areolatus	South China Sea, China	FJ237756	Zhang and Hanner 2012
E. merra	Luzon, Philippines	KC970471	Alcantara and Yambot 2014
E. merra	Queensland, Australia	DQ107898	Ward et al. 2005
E. merra	French Polynesia	JQ431721	Hubert et al. 2012
E. coioides	Pangasinan, Philippines	KF714940	Alcantara and Yambot 2014
E. coioides	Andaman, India	JX674987	Sachithanandam et al. 2012
E. coioides	Andaman, India	JX674982	Sachithanandam et al. 2012
E. coioides	Andaman, India	JX674983	Sachithanandam et al. 2012
E. coioides	Queensland, Australia	DQ107891	Ward et al. 2005
E. ongus	Queensland, Australia	DQ107858	Ward et al. 2005
E. ongus	Queensland, Australia	DQ107859	Ward et al. 2005
E. ongus	Queensland, Australia	DQ107872	Ward et al. 2005
E. ongus	Cuba	FJ583398	Steinke et al. 2009
E. ongus	Okinawa, Japan	JF952725	Zhang and Hanner 2012
E. fasciatus	Luzon, Philippines	KC970470	Alcantara and Yambot 2014
E. fasciatus	Queensland, Australia	DQ107874	Ward et al. 2005
E. fasciatus	Arabian Sea	FJ459562	Lakra et al. 2011
E. fasciatus	Arabian Sea	FJ459561	Lakra et al. 2011
E. fasciatus	India	EU392208	Lakra et al. 2011
E. coeruleopunctatus	Pomene, Mozambique	JF493438	Steinke et al. 2009
E. coeruleopunctatus	Madagascar	JQ349962	Hubert et al. 2012
E. coeruleopunctatus	Madagascar	JQ349961	Hubert et al. 2012
E. coeruleopunctatus	Viti Levu Island, Fiji	KF929848	Bentley and Wiley 2013
E. coeruleopunctatus	Andaman, India	JX674991	Sachithanandam et al. 2012
E. longispinis	India	KJ607970	Mandal et al. 2014
E. longispinis	Kerala, India	EF609521	Lakra et al. 2011
E. longispinis	Kerala, India	EF609522	Lakra et al. 2011
E. longispinis	South Africa	HM909800	Steinke et al. 2009
E. longispinis	Pomene, Mozambique	HQ945868	Steinke et al. 2009

The seven clades formed grouping and showed solid phylogeny tree, each clade indicated the bootstrap value of 100% both on the NJ method and ML method (except; *E. coeruleopunctatus* at 99%) (Figures 2 and 3). Each group; *E. merra* clade formed from Numfor, Karimunjawa, Tanakeke, Kendari and Lombok, with additional samples from Philippines (KC970471), Australia (DQ107898) and French Polynesia (JQ431721). *E. fasciatus* clade formed from Lombok and Lampung as well as additional samples from Philippines (KC970470), Australia (DQ107874), India (EU392208) and the Arabian Sea (FJ459561 and FJ459562). *E. areolatus* clade formed from Lombok, Lampung, Karimunjawa, and Madura and additional samples from Philippines (KC970469) and China (FJ237756 and FJ237757), there is also a sample from Lombok and Lampung formed a separate sub-clade (EJ-LBK-13 and EJ-LPG-04). *E. longispinis* clade formed from Lampung with additional samples from India (KJ607970, EF609522 and EF609521), South Africa (HM909800) and Mozambique (HQ945868) with bootstrap value of 100%. A similar case also occurred in *E. coioides* clade, the sample comes from Karimunjawa with additional samples from Philippines (KF714940), Australia (DQ107891) and India (JX674982, JX674983 and JX674987). Then *E. ongus* clade formed from Karimunjawa, Tanakeke and Lombok with additional samples from Australia (DQ107858, DQ107859 and DQ107872), Japan (JF952725) and Cuba (FJ583398). And the latter with bootstrap value 99% in both methods NJ and ML. The last *E. coeruleopunctatus* clade formed from Numfor with additional samples from Mozambique (JF493438), Madagascar (JQ349961 and JQ349962), Fiji (KF929848) and India (JX674991).

Discussion

The fragment length from PCR amplification using COI with Fish R1 and Fish F1 primers from 39 samples was 526bp (*basepairs*). Previous research has also conducted studies and obtained a fragment length of 582bp on *Epinephelus septemfasciatus* (Guan et al. 2014), *Epinephelus longispinis* at 516bp *Epinephelus ongus* at 522bp and *Epinephelus areolatus* at 318bp (Sachithanandam et al. 2012). The different sequence length is determined by the difference of quality DNA in each sample collected, but it does not affect the results of the sequence analysis in each sample. In fact, several DNA barcoding studies using fish samples obtained from some supermarkets also show good sequence results (300-600bp) as long as the collection and storage processes are well conducted (Filonzi et al. 2010). Shark tissues were collected from three fisheries landing site in Java Islands, Indonesia also showed 600-700bp a total of seven species from 59 individuals was identified (Prehadi et al. 2014) and other part in Indonesia (Sembiring et al. 2015). Even, the tissue from the museum also showed the base pairs length although shorter than fresh tissue (Zein et al. 2013).

Fish identification is traditionally based on morphological features. However, in many cases, fish and their diverse developmental stages are difficult to identify using morphological characteristics alone. Molecular DNA identification techniques have been developed and proven to be analytically powerful. As a standardized and universal method, DNA barcoding will correct an error in grouper identification based on morphological analysis (Zhang and Hanner 2012). In addition to *E. merra*, this species has a relatively small body (grow up to 28 cm in length) and live up to 25 m in depth, while *E. ongus* can grow to nearly 1 m in 100 m depth (Heemstra and Randall 1993) (Figure 4). The current classification of the *Epinephelus* genera is primarily based on different morphological traits: the number of anal fin rays (7-10), the shape of caudal fins (rounded and truncate) and the head length (2.1-2.5 in standard length) (Table 4). The use of morphological characteristics to identify grouper species and then reconstruct phylogenetic relationships is very complex and not always satisfactory (Maggio et al. 2004).

Morphological analysis of seven species in this study showed a difference; although there are some species nearly as visually and size, but with the analysis of mitochondrial DNA is very helpful correcting genetic distance between species, especially of each species. Heemstra and Randall (1993) stated that *E. merra* can be distinguished from the other reticulated groupers by its pectoral-fin pattern of conspicuous black dots that are largely confined to the rays of the fin. *E. areolatus* has often been confused with *E. chlorostigma*, which is also covered with brown spots and has a truncate or emarginate caudal fin with a white posterior margin. *E. ongus* also sympatric with *E. coeruleopunctatus* has a similar color pattern, but the caudal and anal fins have only a few white spots (confined mainly to proximal part of these fins). Genetic distance and phylogenetic tree also showed a strong proximity between both.

Groupers (*Epinephelus* spp.) are distributed in the tropical and subtropical regions of African to Indo-Pacific oceans. Madduppa et al. (2012) stated that the diversity of grouper in the reef slope was higher than in the lagoon. This shows that the characteristics of the habitat were instrumental in shaping the fish community. Their distribution territory is limited, they live in solitary, sedentary and territories in the coral reef ecosystem that cause the genetic distance of *Epinephelus* spp. is not too far.

Although sometimes they are found to migrate several kilometers for the spawning process to a more conducive seas for 1 to 2 weeks aggregation, *Epinephelus* spp. migrate to form massive spawning aggregations at specific locations and during specific periods (Erisman et al. 2014). It is not surprising that *Epinephelus* spp. exhibits considerable intraspecific variation based on scale counts and color pattern.

Phylogeny tree

A strong clade indicated by the bootstrap value of 100% both on the NJ method and on the ML method (except for *E. coeruleopunctatus* at 99%) (Figures 2 and 3). In *E. areolatus* clade, there were sub-clades with the bootstrap value of 99% (EJ-LBK-13 and EJ-LPG-04), in which geographically the species belonged to Lombok and Lampung seas but has a close phylogeny with a bootstrap

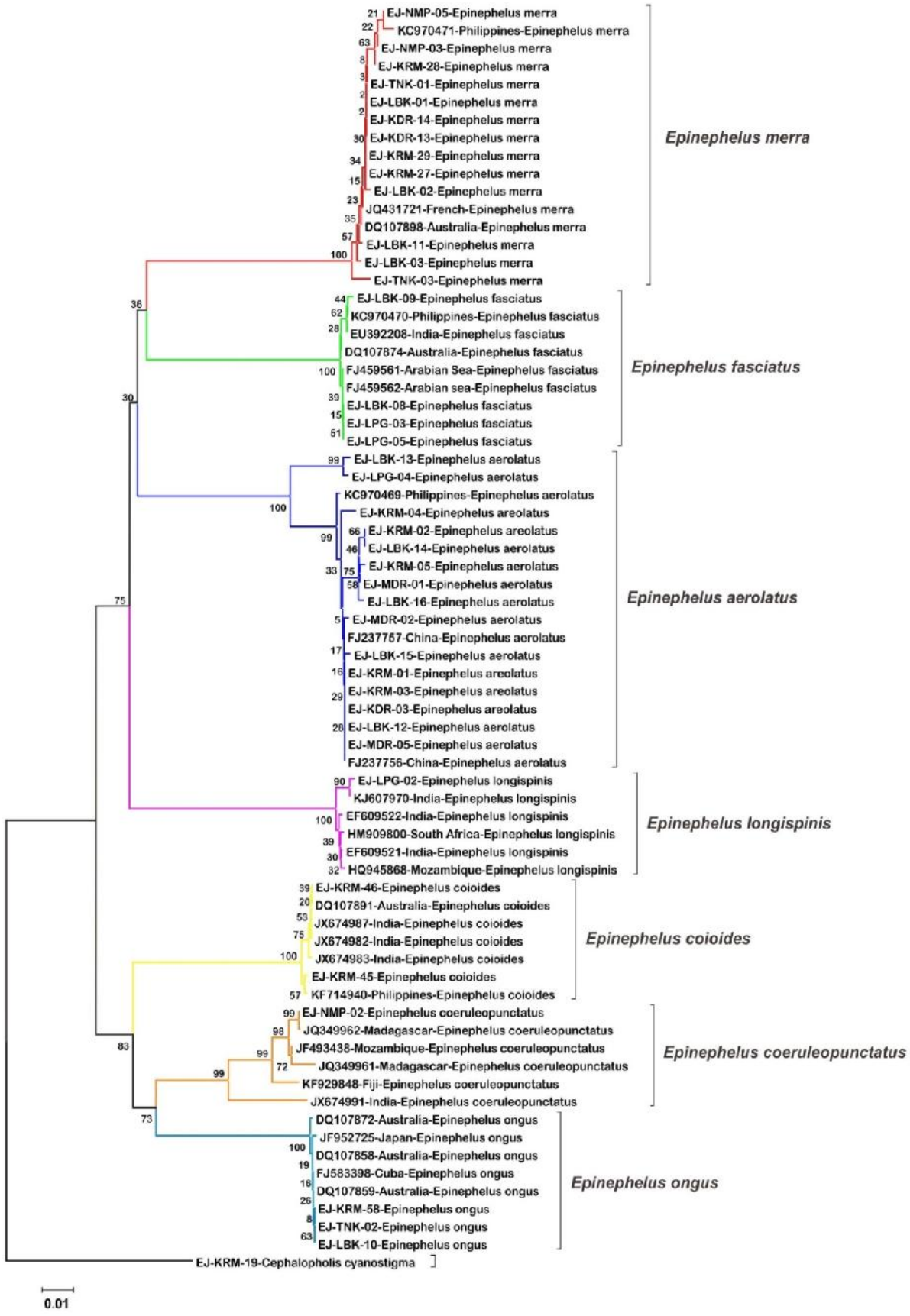

Figure 2. Neighbor-Joining tree using 39 *Epinephelus* spp. grouper sequences from Indonesia based on the mtDNA CO1 and added 31 sequences from *GeneBank* with *Cephalopholis cyanostigma* as out-group. Note: KDR = Kendari, LPG = Lampung, LBK = Lombok, TNK = Tanakeke, KRM = Karimunjawa, MDR = Madura, NMP = Numfor.

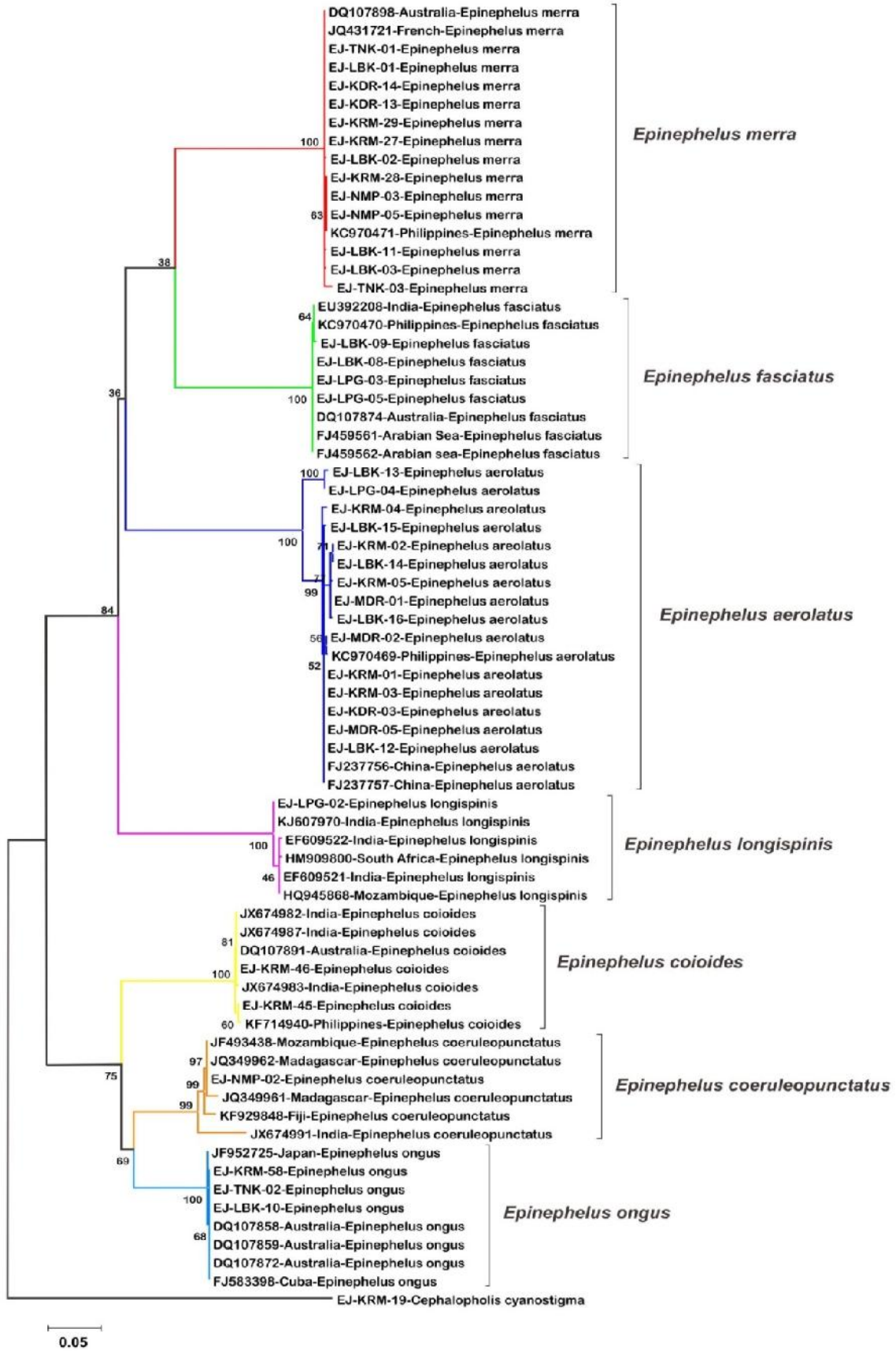

Figure 3. Maximum Likelihood tree using 39 *Epinephelus* spp. grouper sequences from Indonesia based on the mtDNA CO1 and added 31 sequences from *GeneBank* with *Cephalopholis cyanostigma* as out-group. Note: KDR = Kendari, LPG = Lampung, LBK = Lombok, TNK = Tanakeke, KRM = Karimunjawa, MDR = Madura, NMP = Numfor.

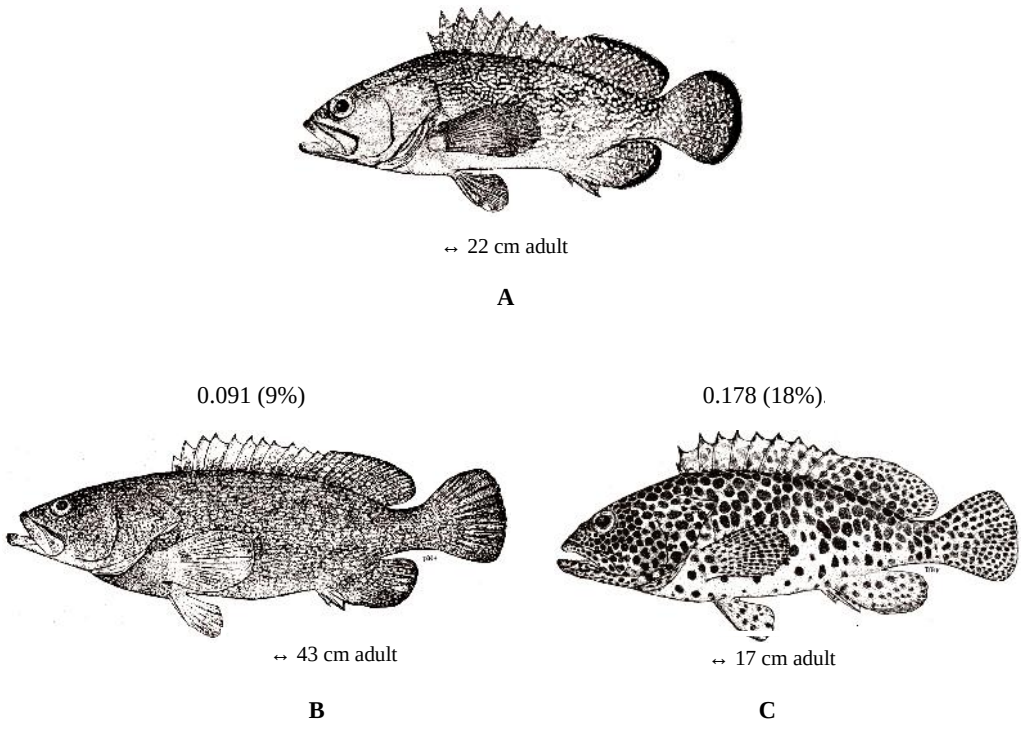

Figure 4. Three species of grouper *Epinephelus* spp. The closest genetic distance (0.091 or 9%) between *Epinephelus ongus* (A) and *Epinephelus coeruleopunctatus*, and furthest genetic distance (0.178 or 18%) between *Epinephelus ongus* (A) and *Epinephelus merra* (C) (after Heemstra and Randall 1993)

Table 4. The main morphological characters to identify grouper (*Epinephelus* spp.) based on Heemstra and Randall (1993)

Species	Head length (inch)	Anal fin rays	Shape of caudal fins
Epinephelus areolatus	2.4 to 2.8	III spines and 8 rays	Truncate or slightly
E. merra	2.3 to 2.6	III spines and 8 rays	Rounded
E. coioides	2.3 to 2.6	III spines and 8 rays	Rounded
E. ongus	2.3 to 2.5	III spines and 8 rays	Rounded.
E. fasciatus	2.3 to 2.6	III spines and 8 rays	Slightly to moderately rounded
E. coeruleopunctatus	2.3 to 2.5	III spines and 8 rays	Rounded
E.longispinis	2.4 to 2.6	III spines and 8 rays	Convex

value of 99%. Other samples from the Philippines (KC970469) and China (FJ237757 and FJ237756) were also joined in one large clade, indicating that several groupers of *E. areolatus* species from Indonesia, the Philippines and China were still have a close kinship. No significant differences from these seas were due to the limited distribution and territorial-nature of this grouper species in accordance with the results of (Heemstra and Randall 1993).

The other clades, *E. longispinis* showed the existence of two sub-clades. EJ-LPG-02 and KJ607970 from Lampung and India were formed their own sub-clade, whereas EF609522, EF609521, HM909800 and HQ945868 from India, South Africa and Mozambique formed other sub-clades. Sachithanandam et al. (2012) stated that *E. longispinis* of Andaman India also showed almost similar character of South African as well as Arabian sea species. The existence of a large sub-clade is suspected because of the considerable differences in geography, even though *E. longispinis* is a geographically distributed species in the continental areas and islands in the Indian Ocean region from Kenya to South Africa and the Banda Sea, including Madagascar, Comoros, Maldives, India to Sri Lanka (Heemstra and Randall 1993). Spawning migration activity for a long time and affected by Indonesian Through flow that suspected for causing the phylogeny tree has proximity of some of these seas. It is known that grouper species is a protogynous hermaphrodite (Craig et al. 2011), although the location and timing of grouper spawning activity is sometimes difficult to find the information (Golbuu and Friedlander 2011).

The results of phylogeny tree either using NJ or ML methods were also strengthened the data from the analysis of genetic distance, whereas the closest (*E. ongus* and *E. coeruleopunctatus*) were on the same branch (with the bootstrap values of 72% (ML) and 73% (NJ)) with a

different clade. Meanwhile, the farthest *E. ongus* and *E. merra* were in a different clade and the same large branch (with the bootstrap values of 73% (ML) and 82% (NJ)). *E. fasciatus* and *E. coioides* clades also showed no significant differences in the position of the phylogeny tree from the results obtained in the analysis of genetic distance, even though they had merged with several sequences from outside Indonesian seas. Craig and Hastings (2007) also corroborate that *Epinephelus* spp. are monophyly of the remaining tribes.

The present study suggested that event morphologically the species *Epinephelus* spp. are difficult to differentiate due to key features are quite similar, but they were confirmed by the molecular analysis results. Mitochondrial COI gene, as an ideal region for species barcode, DNA barcode may be used for the rapid analysis for the commercial purposes especially confirmation for the particular species. This study would be an important data in the genetic management for the sustainable conservation and trade of grouper (*Epinephelus* spp.) in Indonesia.

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