Genetic diversity and phylogenetic reconstruction of grouper (Serranidae) from Sunda Land, Indonesia

Sapto Andriyono¹, Adrian Damora², Andi A. Hidayani³

¹Department of Marine, Fisheries and Marine Faculty, Universitas Airlangga C Campus Jl. Mulyorejo Surabaya East Java, 60115, Indonesia
²Department of Aquaculture, Faculty of Marine and Fisheries, Universitas Syiah Kuala, Jl. Putroe Phang, Darussalam, Banda Aceh 23111, Aceh, Indonesia
³Fisheries Department, Faculty of Marine Science and Fisheries, Hasanuddin University. Jl. Perintis Kemerdekaan KM. 10, Makassar, South Sulawesi, Indonesia.

*Corresponding Author: sapto.andriyono@fpk.unair.ac.id

INTRODUCTION

A grouper is a group of fish that inhabit in the coral reef ecosystems and rocky shallow waters as their primary habitat. Several species found in shallow waters that have sandy mud substrate, which makes the grouper habitat variation quite diverse. The diversity of grouper habitat also makes groupers have a variety of species. As reported in previous studies, the waters of Bali have Serranidae diversity of 54 species or about 5.5 % of the composition of reef fish (Allen and Erdmann, 2013). Other studies reported
that the Epinephelinae subfamily has 159 species, including 15 genera of *Cephalopolis* and 31 genera of *Epinephelus* (Allen and Adrim, 2003).

Groupers are an intimate group in the aquaculture industry because it can reach a significant size in weight of up to 400 kg and the total length of 2.5 meters (Heemstra, 1993), this fish became an essential commodity of aquaculture in Asia and some countries in the world (Chiu et al., 2008), both with floating net systems and in ponds that show an increase of 8-16 % starting in the 1900s (Pomeroy, 2002). The high demand for grouper, both for consumption and for ornamental fisheries, makes grouper aquaculture more attractive with high market prices and export-oriented (Halim, 2001). The Indo-Malayan Archipelago region plays a vital role in the grouper supply chain worldwide. It has been estimated that about 80 % of the world’s production of groupers was reported from Asia, mainly from Indonesia, the Philippines and Malaysia with a steadily increasing number of products every year (Craig et al., 2011; Sadovy de Mitcheson et al., 2013; Yulianto et al., 2015; Alcantara and Yambot, 2016; and Kadir et al., 2018). Increased export values have been felt since 1980, with export values to several Asian countries (Singapore, Hong Kong, and China) (Nuraini and Hartati, 2006), United States, and Europe (Halim, 2001). The high level of human exploitation of groupers causes at least one-third of the genus of the subfamily Epinephelinae, especially the *Epinephelus* and *Mycteroperca* genera listed as endangered species (Morris et al., 2000) and requires very strict licensing and approval. For example, the giant groupers (*E. lanceolatus*) and the Napoleon wrasse (*C. undulatus*), have become vulnerable fish since they were established in 1996 by IUCN (Halim, 2001). In addition, out of 163 grouper species across the globe, 12 % (20 species) are at risk of extinction, and 13 % (22 species) are considered to be nearly threatened based on the IUCN Red List criteria (Craig et al., 2011).

The limitations of the study of phylogenetic relationships between fish in subfamily Epinephelinae (*Epinephelus*, Serranidae) are still vital, given the complexity of the members of this family (Craig and Hastings, 2007). *Epinephelus* distribution is quite extensive, around the Indo-west Pacific and Indo-Pacific (Heemstra and Randall, 1993; Van Herwerden et al., 2002; and Unsworth et al. 2007). In general, people only mention groupers, which are indicated by the spots on their body parts. The fish have a brownish to attractive red colour associated with their diverse habitats from shallow water areas and very colourful coral reef ecosystems (Unsworth et al., 2007). DNA barcoding believed to be one of the methods used globally to identify molecular approaches for animals and plants. This identification, the DNA in mitochondrial, becomes a remarkable sequence that is considered capable of being a marker and has been accepted as a global bio-identification system for animals (Hebert et al., 2003 and Ward et al., 2005). This identification is beneficial in specimens that are difficult to identify morphologically, such as larval stage, and organ fragments or morphologically incomplete specimens (Hebert et al., 2003). Various advantages of DNA coding are straightforward and useful universal tools that include all the animals both in the form of fresh and processed product samples (Pepe et al., 2007 and Giusti et al., 2017). The accuracy of DNA-based identification is nearly 100 %, which indicates that this method can prove the identification of specimens under different environmental conditions (Meyer and Paulay, 2005). The barcoding system uses sequences that have a diversity in the single region of mitochondrial DNA, cytochrome c oxidase subunit I gene
Genetic diversity and phylogenetic reconstruction of grouper (Serranidae)

(COI), and then deposited to the GenBank database. The GenBank has become central to
deposit diverse taxa from all parts of the world. With the increase of molecular
databases, scientists have demonstrated their effectiveness in conducting DNA barcoding
from freshwater fish to deep-sea fish (Ward et al., 2005 and Lakra et al., 2011).
Previous research has shown that mitochondrial DNA has a higher mutation rate
compared to nuclear DNA by inheriting the maternal gene. Thus, researchers can obtain
handy data for studying evolution between species, even within the same species
(Waugh, 2007). In this research, we performed the molecular identification of several
grouper from seven sampling sites (Java, Bali Island, and Aceh) with the COI gene
region to understand the diversity and measure the genetic distance of each species,
especially in the genus Epinephelus, Cephalopholis, and Variola.

MATERIALS AND METHODS

Sampling site
A total of 8 fish samples were collected from the five traditional fish markets
around Jawa Island during July 2019. In the northern part of Java, samples were obtained
from the Banten (6°0'50.00"S-106°10'21.00"E), and Gresik (6°52'56.65"S-
112°12‘15.87"E), while Southern Java was represented samples from Malang
(8°26’06.65”S-112°40’55.31"), the Banyuwangi (8°12’07.52”S-114°23’07.18”E), and
Bali (8°45’23.00”S-115°10’05.68”E). Here, we also collected a specimen from the
Kutaradja fish traditional market in Aceh, the westernmost province of Sumatera
(5°35’07.00”N-95°19’07.00”E). Morphologically identification conducted according to
the guideline from FAO (Heemstra, 1993), and species confirmation has been carried out
with molecular identification carried out in this study using the COI gene region. No
specific permit was required for this study due to collect from the local traditional fish
market were dead upon purchasing. Before dissected, all specimen has been
photographed by the digital camera.

DNA extraction and PCR
Each specimen has been collected and directly preserved in 90 % ethanol for
further experimental purposes. Around one cm tissues was taken from the anal fin of each
specimen, dissected and mix with 6X lysis buffer, which was further homogenized by the
TissueLyser II (Qiagen). Genomic DNA extracted using an Accuprep® Genomic DNA
Extraction Kit (Bioneer) according to the manufacturer’s guidelines. Quantification of
purified genomic DNA performed by Nanodrop (Thermofisher Scientific D1000),
aliquoted and stored at -70°C for further analysis.

PCR condition and Data Analysis
One set of universal fish primer targeting cytochrome c oxidase I (COI) region,
FISH-BCL (5’-TCA ACY AAT CAY AAA GAT ATY GGC AC-3’) and FISH-BCH (5’-
TAA ACT TCA GGG TGA CCA AAA AAT CA-3’) (Baldwin et al., 2009 and Handy
et al., 2011), used to obtain the partial sequences of COI gene. The PCR mixture (20µL)
included 11.2 µL ultra-pure water, 1 µL primer forward and reverse (0.5 µM), 0.2 µL Ex
Taq DNA polymerase (TaKaRa, Japan), 2 µL 10X ExTag Buffer, 2 µL dNTPs (1 µM,
TaKaRa, Japan), and 2 µL genomic DNA as template. The PCR condition carried out
under the following setting: 95°C for 5 min in initial denaturation, followed by denaturation at 95°C for 30 s in 40 cycles, 50°C for 30 s in annealing, and 72°C for 45 s in extension step, and a final extension at 72°C for 5 min. The PCR products were purified with the AccuPrep®Gel purification kit (Bioneer, Korea).

**Phylogenetic analysis**

All sequences were aligned, including reference sequences from the GenBank database (Table 1). The pairwise evolutionary distance among the family determined by the Kimura 2-Parameter method. The Neighbor-joining (NJ) tree constructed, and 1000 bootstrap analysis was carried by Mega7 program (Kumar et al., 2016).

<table>
<thead>
<tr>
<th>Species name</th>
<th>Genetic distance within species</th>
<th>Genetic distance between species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clade I (Epinephelus)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. areolatus</td>
<td>0.000</td>
<td>0.152-0.181</td>
</tr>
<tr>
<td>E. merra</td>
<td>0.004</td>
<td>0.157-0.185</td>
</tr>
<tr>
<td>E. ongus</td>
<td>0.000</td>
<td>0.107-0.184</td>
</tr>
<tr>
<td>E. poecilonotus</td>
<td>0.000</td>
<td>0.107-0.119</td>
</tr>
<tr>
<td>E. ongidos</td>
<td>0.000</td>
<td>0.119-0.181</td>
</tr>
<tr>
<td><strong>Clade II (Cephalopholis and Variola)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. miniata</td>
<td>0.000</td>
<td>0.089-0.216</td>
</tr>
<tr>
<td>C. sonnerati</td>
<td>0.000</td>
<td>0.089-0.174</td>
</tr>
<tr>
<td>C. cyanostigma</td>
<td>0.002</td>
<td>0.150-0.202</td>
</tr>
<tr>
<td>V. albimarginata</td>
<td>0.000</td>
<td>0.173-0.206</td>
</tr>
</tbody>
</table>

**RESULTS**

A total of 11 successful identification samples consisted of three genera, Epinephelus (5), Cephalopholis (3), Variola (1). In this study, the genus Epinephelus was more dominated the general catches of fishers, including E. coioides, E. ongus, E. poecilonotus, E. areolatus, E. merra. Meanwhile, other types are only in small quantities.

**Tabel 1.** The genetic distance of Epinephelinae compare to the reference from GenBank database

Genetic distance analysis was carried out using Mega 7, which aligned all obtained queries (Table 1). The results of this analysis provide a description of the distance between species (interspecific) and in the same species (intraspecific). Epinephelus areolatus obtained from Aceh is not different from the reference sequences originating from Saudi Arabia (KU499597) by the genetic distance is zero. The same thing also happens to another genus Epinephelus, which also has genetic distance 0 with reference in the GenBank database except in E. merra. In E. merra there is a slight difference, although identification still refers to the same species. Genetic distance with specimens originating from Japan (AP005991) is only 0.004, indicating that E.merra species is an Indonesian haplotype. Whereas in Cephalopolis species,
only *C. cyanostigma* was found in Indonesian haplotypes with a genetic distance of 0.002 with reference specimens from the Philippines (KU668647).

**Phylogenetic reconstruction**

In the phylogenetic tree produced, two clades have been formed consisting of the genus *Epinephelus*, and the other clade consists of *Cephalopholis* and *Variola* (Figure 1). Morphologically, the *Epinephelus* group is dark grey to dark brown, while *Cephalopholis* and *Variola* are bright red body-colour (Figure 2). Although morphologically, the two clades show differences, sometimes it is challenging for researchers and the public to distinguish each species. Besides, the giving of various regional names also adds to the complexity of naming. So that the molecular identification carried out in this study is expected to help ensure the types of fish identified and traded in traditional markets in several areas. The certainty of the name in the specie here is also essential in various scientific writings because it deals with scientific information that will be read by the general public, especially in academic purposes.

![Figure 1. Phylogenetic tree of Epinephelinae including references from the GenBank database](image)

**Epinephelinae status in IUCN and CITES**

Almost all grouper species in this study have the status of Least Concern (LC), while the grouper in international trade (CITES) is not evaluated (Table 2). However, *E. merra* has been reported as a causative agent of Ciguatera fish poisoning in several countries (Lewis, 1986). This fish poisoning occurs in some countries such as Thailand (Toyoda et al., 1981), the Philippines (Montijo et al., 2020), Hong Kong (Sadovy, 1997), Australia (Gillespie et al., 1986), and other countries (Randall, 1958; Chan 2015; and Gaboriau et al., 2014). However, there have been no reports of ciguatera in Indonesia (Chan, 2015).
TABLE 2. IUCN and CITES status of all grouper specimens

<table>
<thead>
<tr>
<th>Species name</th>
<th>English name</th>
<th>Distribution</th>
<th>IUCN</th>
<th>CITES</th>
<th>Threat to humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. areolatus</td>
<td>Areolate grouper</td>
<td>Indo-Pacific</td>
<td>LC</td>
<td>NE</td>
<td>Harmless</td>
</tr>
<tr>
<td>E. merra</td>
<td>Honeycomb grouper</td>
<td>Indo-Pacific</td>
<td>LC</td>
<td>NE</td>
<td>Reports of ciguatera poisoning</td>
</tr>
<tr>
<td>E. ongus</td>
<td>White-streaked grouper</td>
<td>Indo-West Pacific</td>
<td>LC</td>
<td>NE</td>
<td>Harmless</td>
</tr>
<tr>
<td>E. poecilonotus</td>
<td>Dot-dash grouper</td>
<td>Indo-West Pacific</td>
<td>LC</td>
<td>NE</td>
<td>Harmless</td>
</tr>
<tr>
<td>E. oioides</td>
<td>Orange-spotted grouper</td>
<td>Indo-West Pacific</td>
<td>LC</td>
<td>NE</td>
<td>Harmless</td>
</tr>
<tr>
<td>C. miniata</td>
<td>Coral hind</td>
<td>Indo-Pacific</td>
<td>LC</td>
<td>NE</td>
<td>Harmless</td>
</tr>
<tr>
<td>C. sonnerati</td>
<td>Tomato hind</td>
<td>Indo-Pacific</td>
<td>LC</td>
<td>NE</td>
<td>Harmless</td>
</tr>
<tr>
<td>C. cyanostigma</td>
<td>Blue-spotted hind</td>
<td>Western Pacific</td>
<td>LC</td>
<td>NE</td>
<td>Harmless</td>
</tr>
<tr>
<td>V. albimarginata</td>
<td>White-edged lyre tail</td>
<td>Indo-Pacific</td>
<td>LC</td>
<td>NE</td>
<td>Harmless</td>
</tr>
</tbody>
</table>

LC: Least Concern
NE: Not Evaluated

DISCUSSION

Grouper has become a leading commodity in aquaculture in several Asian countries and several other continents (Halim, 2001). The success of aquaculture is demonstrated by many studies on the reproductive biology that are sufficiently detailed (Andrade et al., 2003 and Andamari et al., 2007, ), hatchery (Rimmer, 2000, and Sugama et al. 2012), larval rearing to a variety of grow-up system (Fukuhara, 1989 and Pomeroy, 2002). Several studies have shown good results in hatching several species of groupers such as E. coioides, E. fuscoguttatus, Plectropomus leopardus, and Cromileptes altivelis (Rimmer, 2000), E. fuscoguttatus (Sugama et al., 2017). Research on the grow-up system also showed excellent results both in the floating net system (Baliao et al., 2000) and in the aquaculture system in the pond (Baliao et al., 1998).

Trade-in grouper in the live fish becomes a superior commodity because the price is quite high when compared to fresh dead fish. The demand for live fish forms continues to increase, so fishers prefer to sell live fish (Halim, 2001). However, many aquaculture activities are currently conducting such as breeding between species, which are expected to produce variants that have growth and other good traits such as disease resistance, good growth, resistance to extreme environments, and at the same time, making sterile fish (Hickling, 1968). For example, the crossing of tiger grouper and E. fuscoguttatus and E. polyphasekion grouper, which produce hybrid grouper with excellent growth performance (James et al., 1999). However, please note that the pure parent lines that will be breeding must be well known so that the study of this breeding effect can be done well. Other breeding was also developed on E. costae with E. marginatus (Glamuzina et al., 2001), Plectropomus leopardus with Plectropomus maculatus (Frisch and Hobbs, 2007), and E. coioides with E. lanceolatus (Kiriyakiet et al., 2011 and Sutthinon et al., 2015).
Some breeding activities between *Epinephelus* species have been carried out in Indonesia. One of them is a hybrid between *E. microdon* (male) and *E. fuscoguttatus* (female) by producing seeds that have the title grouper cantik (*Ismi, 2014*). Another type of hybrid is the grouper cantang, which is a hybrid of *E. fuscoguttatus* and *E. lanceolatus* (*Shapawi et al., 2019*). Another hybrid type is the kustang grouper, which is breeding of *Cromileptes altivelis* with *E. coioides* (*Yu et al., 2004*). Breeding between *E. microdon* and *E. lanceolatus* has been carried out, which produces a new type of hybrid grouper (*Sutarmat and Yudha, 2016*). Also, back-cross hybrids have also been carried out between hybrids (*E. coioides* x *E. lanceolatus*) breeding with *E. lanceolatus* (*Luan et al., 2016*). Back-cross breeding efforts are carried out to produce seeds that are more productive and have the advantage of better traits such as a high survival rate than the previous generation.

Due to the intensive exploitation of grouper species in the world, only a small number of studies on the genetic diversity of the species have been carried out. In Indonesia, although intensive aquaculture activities are carried out, only a few types of grouper have been reported about genetic studies such as *E. coioides* (*Antoro et al., 2006*), *E. siulius* (*Parenrengi and Tenriulo, 2008*), *Cromileptes altivelis* (*Susanto et al., 2011* and *Sugama et al., 2017*) and several other types of grouper *E. areolatus*, *E. merra*, *E. ongus*, *E. fasciatus*, *E. coioides*, *E. coeruleopunctatus*, and *E. longispinis* (*Jefri et al., 2015*). Nevertheless, barcoding studies of several types of grouper have been carried out from some regions in Java and Bali (*Andriyono et al., 2020*), Makassar (*Parenrengi and Tenriulo, 2008*), Lampung and Papua (*Jefri et al., 2015*). This report is the first report involving specimens from Aceh and at the same time comparing with references from the GenBank database.
The use of genetic information from the GenBank database provides a picture of the similarities between species groups, even though the species has a large habitat distribution in the Indo-Pacific region (Randall and Heemstra, 1991 and Unsworth et al., 2007). It is estimated, the grouper included in coral reef fish associated has experienced speciation that allows it to have variations in its genetic composition (Rocha and Bowen, 2008). Specialization of reef fishes has illustrated that open access sea also has its boundaries and niches so that many species will be different and adapt to each type of coral reef ecosystem. In this study, only two types (E. merra and C. cyanostigma) were found to be Indonesian haplotypes and differ from the same species from the alignment of the DNA reference sequence.

The distribution of E. merra is quite extensive with significant habitats found in the Indo-Pacific region including South Africa to French Polynesia and even in the central Pacific (Randall and Heemstra, 1991; Craig et al., 2011; and Muths and Bourjea, 2011) which generally inhabit waters bring (<20 m depth). Its natural habitat is a coral reef area and becomes essential in artisanal fisheries as a source of protein and food for coastal communities (Heemstra, 1993). Studies on E. merra diversity also indicate genetic variation in Madagascar, the Maldives, and small islands in the West Indian Ocean (Muths et al., 2015).

Meanwhile, C. cyanostigma was also identified in Maluku waters, which has a reasonably high diversity of the genus Cephalopolis with 11 species inhabiting the waters of this region (Limmon et al., 2017) and North Sulawesi (Tokeshi et al., 2013). Based on previous reports, the genus Cephalopholis consists of 22 species that have habitat distribution in the Pacific Ocean region (Heemstra, 1993), which tend to have a cryptic habit on coral reef ecosystems (Shpigel and Fishelson, 1989). Of the 22 known species of Cephalopholis, only nine species have been studied in terms of their biology. Many studies on several aspects of biology, including sexual maturity (Shapiro 1987), spawning (Donaldson, 1989), territoriality and their ecology (Shpigel and Fishelson, 1991), and sex change and population structure of Cephalopholis (Siau, 1994). Meanwhile, this report is the first report on genetic distance in C. syanostigma in Indonesia based on the COI sequences that show the existence of different haplotypes with the same species in the Philippines. The haplotype was formed due to geographical different, the Philippine species is an Indo West-pacific species, while the sample in this study is a species of the Indian ocean. This result needs to get attention for further research on genetic variation of Cephalopholis in Indonesian regions with a more significant number of samples.

The haplotypes found in E. merra and C. cyanostigma can be known by phylogenetic tree analysis, which shows a slight distance with sequence reference (Figure 1). This study is quite helpful in giving an idea of the haplotype formed. Also, through this phylogenetic tree, it is known that the genus Epinephelus forms a separate clade separating from Cephalopolis and Variola. In this study, Cephalopholis and Variola are in the same clade but separated in several branches. Morphologically, these two genera can be easily distinguished by observing the caudal fin (Figure 2). In Variola species have caudal fin lunate (Baldwin, 2003), than Cephalopholis have rounded caudal fin (Allen, 2015). Thus, the phylogenetic tree places the two genera in separate branches (Figure 1). Although the number of samples is still small, this report
reinforces that the Epinephelinae group has several clades, which are genetic variations in the grouper.

**CONCLUSION**

In this study, we found genetic variation in *E. merra* and *C. cyanostigma* that showed the existence of Indonesian haplotypes. This result was figure-out genetic distance of some species in this report. The genetic distance of *E. merra* and *C. cyanostigma* are 0.004 and 0.002, which is slightly different from sequences from Japan and the Philippines, respectively. It is necessary more in-depth studies of *C. cyanostigma*, due to the limited study of genetic variation, especially in Indonesia. The study of *C. cyanostigma* is very supportive in efforts to develop this species as an aquaculture commodity in the future, such as *Epinephelus*. An in-depth study of biological characteristics, including reproductive biology, ecology, and other specific characteristics, will be beneficial in both conservation and domestication.

**ACKNOWLEDGMENTS**

The authors would like to express our sincere gratitude for the initiation of collaborative research between Airlangga University (Surabaya), Syiah Kuala University (Aceh), and Hasanuddin University (Makassar) which have given great importance to the development of studies in Indonesia. Fisheries studies in Java, Sumatra, Sulawesi, Kalimantan, and Papua require the support of many collaborators to produce quality and beneficial research for Indonesia.

**REFERENCES**


Hickling, C.F. (1968). The farming of fish: Pergamon Press. 88pp


