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**Submission date:** 20-Sep-2022 10:56AM (UTC+0700)

**Submission ID:** 1904241696

**File name:** JIPK.pdf (1.91M)

**Word count:** 6775

**Character count:** 36843



Short Communication

## Low Genetic Diversity Study on Leopard Coral Grouper *Plectropomus leopardus* (Perciformes: Serranidae) from Sulawesi, Indonesia

Andi Aliah Hidayani<sup>1</sup>, Asmi Citra Malina A. R. Tasakka<sup>1</sup>, Widyastuti Umar<sup>2</sup>, Md. Jobaidul Alam<sup>3</sup>, Amit Kumer Neogi<sup>4</sup>, and Sapto Andriyono<sup>5\*</sup>

<sup>1</sup>Fisheries Department, Faculty of Marine Science and Fisheries, Universitas Hasanuddin, Makassar, South Sulawesi, 90245. Indonesia

<sup>2</sup>Marine Science Department, Faculty of Marine Science and Fisheries, Universitas Hasanuddin, Makassar, South Sulawesi, 90245. Indonesia

<sup>3</sup>Department of Fisheries, Ministry of Fisheries and Livestock. Bangladesh

<sup>4</sup>Communicable Disease Programme, Bangladesh Rural Advancement Committee (BRAC), BRAC Centre, Dhaka, 1212. Bangladesh

<sup>5</sup>Department of Marine, Fisheries and Marine Faculty, Universitas Airlangga, Surabaya, East Java, 60115. Indonesia



### ARTICLE INFO

Received: January 12, 2022

Accepted: March 19, 2022

Published: March 25, 2022

Available online: August 30, 2022

\*) Corresponding author:

E-mail:  
[sapto.andriyono@fpk.unair.ac.id](mailto:sapto.andriyono@fpk.unair.ac.id)

### Keywords:

Biodiversity  
Coral Reef Fish  
Grouper  
Wallacea  
Sulawesi

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### Abstract

Bar-cheek coral trout (*P. leopardus*) is the flagship of the grouper in the live fish market in Asia. Unfortunately, the potential of the grouper is still partly produced from natural catches. Even though hybridisation activities have also started to be carried out, there still have not been many studies on the genetic diversity of these fish. The application of molecular identification has been widely applied in marine aquatic animal species, which are very likely to occur due to errors in terms of shape and colour in the morphological character. DNA information has been beneficial efforts to the breeding program and develop grouper aquaculture activities. DNA barcoding was used for the molecular identification and haplotype analysis of *P. leopardus* from two locations in Gorontalo, Sulawesi, Indonesia. A total of 14 fish samples were collected from two traditional fish markets around Kwandang and Sumalata Gulf in the northern part of Gorontalo Province, Sulawesi. This study identified and found three haplotypes from both regions. Molecular identification using Cytochrome c Oxidase subunit I (COI) gene region on mitochondrial DNA. Besides Mega7 for phylogenetic reconstruction, the data analysis using DnaSP6, Arlequin Ver.3.5.2.2, and Network 5.0.1.1. The first Haplotype is a mixed population between the Kwandang Gulf and the Sumalata Gulf, then the Kwandang Gulf haplotype and the Sumalata Gulf haplotype. The genetic distance between Kwandang Gulf haplotype and Sumalata Gulf haplotype is 0.003984, classified as a shallow genetic distance and needs more samples from another region to figure out leopard coral grouper around Indonesia.

Cite this as: Hidayani, A. A., Tasakka, A. C. M. A. R., Umar, W., Alam, M. J., Neogi, A. K., & Andriyono, S. (2022). Low Genetic Diversity Study on Leopard Coral Grouper *Plectropomus leopardus* (Perciformes: Serranidae) from Sulawesi, Indonesia. *Jurnal Ilmiah Perikanan dan Kelautan*, 14(2):349-359. <http://doi.org/10.20473/jipk.v14i2.32815>

## 1. Introduction

Coral trout and groupers are closely associated with coral reef ecosystems (Lieske and Myers, 2002). The Leopard coral grouper *Plectropomus leopardus* (Lacepède, 1802) is a grouper that is currently receiving a high level of attention in aquaculture activities in Indonesia (Andamari et al., 2007; Halim, 2001). This fish commodity is the flagship of the grouper types known in the live fish market in Asia and some countries globally (Chiu et al., 2008). Exciting market potential of grouper species with a significant development shown at 8-16% in aquaculture activities in the 1900s (Pomeroy, 2002). Some reasons that make grouper an aquaculture commodity are that this fish can weigh up to 400 kg and reach up to 2.5 meters in body length (Heemstra, 1993). At the juvenile stage, the superiority of groupers, other than as consumption fish, is also an attractive ornamental fish with a promising export market potential (Halim, 2001). As both consumption fish and ornamental fish, the trade-in fresh live grouper has become an essential orientation with very lucrative market prices to export these fish to several countries in Southeast Asia such as Singapore, Hong Kong, and China (Nuraini and Hartati, 2006), United States, and Europe (Halim, 2001). Taiwan's species of bar-cheek coral trout is the leading export destination with around 82%, while the rest goes to other Asian countries (Andamari et al., 2007). Interestingly, this market potential is still partly generated from the catch of nature, which ultimately led to the grouper exploitation, which is relatively high. Several species of the genus *Epinephelus* are now classified as endangered species (Morris et al., 2000), including giant groupers (*E. lanceolatus*) and Napoleon wrasse (*C. undulatus*), which are currently vulnerable since 1996 by IUCN (Halim, 2001).

Aquaculture activities on leopard coral grouper were conducted to reduce the high level of fishing activity. With the increased demand and market prices of grouper, studies on aquaculture development on various aspects have been carried out, such as reproductive aspects (Andamari et al., 2007), larvae (Melianawati et al., 2015), and growth of grouper in floating net culture (Kongkeo et al., 2010; Rimmer et al., 2013). Coral reefs are ideal for many fish, including this coral grouper, with a high diversity among grouper fish groups. Research on a variety of groupers in the waters of Bali shows considerable potential with 54 species (5.5%) of the composition of reef fish (Allen and Erdmann, 2013), while the Wallacea region in the Togeian and Banggai islands complex shows a similarity of 46 species (5.6%) of reef fish composition in the area (Allen, 2001). Nonetheless, global research on groupers shows very high diversity in 102 species (Serranidae), including 30 species of *Plectropomus* (Allen and Adrim, 2003).

The study of genetic diversity by phylogenetic relationships between fish in Family Epinephelinae (Serranidae) is still needed and essential because of the complexity of this family members (Craig and Hastings, 2007). The distribution of grouper habitat spread across the Indo-pacific regions (Heemstra and Randall, 1993; Unsworth et al., 2007; Van Herwerden et al., 2002) offers opportunities for greater diversity between species due to differences in geographic distribution, characteristic of sea waters, as well as the unique conditions in each water especially the waters in the islands. The reef fish speciation process will also be very diverse, supporting diversity created in coral reef ecosystems (Rocha and Bowen, 2008). So the study of genetic variation is essential enough to support leopard coral grouper aquaculture activities.

Molecular-based genetic studies have recognised the differences in the order of the nucleotide bases in their DNA. This molecular identification is called DNA barcoding. The ability of DNA barcoding applies not only to intact and fresh specimens; identification can still be made even on fragmented tissue pieces (Hebert et al., 2003). This identification process is the advantage of DNA barcoding in overcoming bias when morphological identification is carried out on reef fishes with morphological similarities. DNA barcoding is increasingly widespread and can be applied to processed products with challenging morphology identification (Giusti et al., 2017; Pepe et al., 2007). With almost 100% accuracy, DNA barcoding applications have become very famous and can be used in various environmental conditions (Meyer and Paulay, 2005).

At present, the cytochrome c subunit I gene (COI) in the region of mitochondrial DNA is a universal gene marker for species identification. We successfully analysed the genetic diversity on the COI Sequence of leopard coral grouper of Gorontalo (*P. leopardus*) to avoid breeding activities of this grouper from the close region in Sulawesi. This condition is possible because many researchers have deposited genetic information in the GenBank database. GenBank has become central to depositing diversity from all parts of the world. Scientists have demonstrated their effectiveness in conducting DNA barcoding on freshwater fish (Hubert et al., 2008; Kadarusman et al., 2012), marine fish (Alcantara et al., 2016; Yambot, 2016; Andriyono et al., 2020a; Bingpeng et al., 2018; Lakra et al., 2011; Wang et al., 2012; Ward et al., 2005), and also deep-sea fish (Morita, 1999). Due to the mitochondrial DNA having a higher mutation rate and inheriting the maternal gene, researchers can study the evolution between species and within the same species (Waugh, 2007). This report identified the leopard coral grouper *P. leopardus* from two areas in Gorontalo through a molecular approach. In addition, the analysis

of the resulting COI sequence is to determine the genetic distances and the number of haplotypes between the two populations. We hope this information on DNA barcoding of the leopard coral grouper *P. leopardus* can add genetic data to potential marine fish from Wallacea (Sulawesi) to be developed in aquaculture activities.

## 2. Materials and Methods

### 2.1 Sampling Site

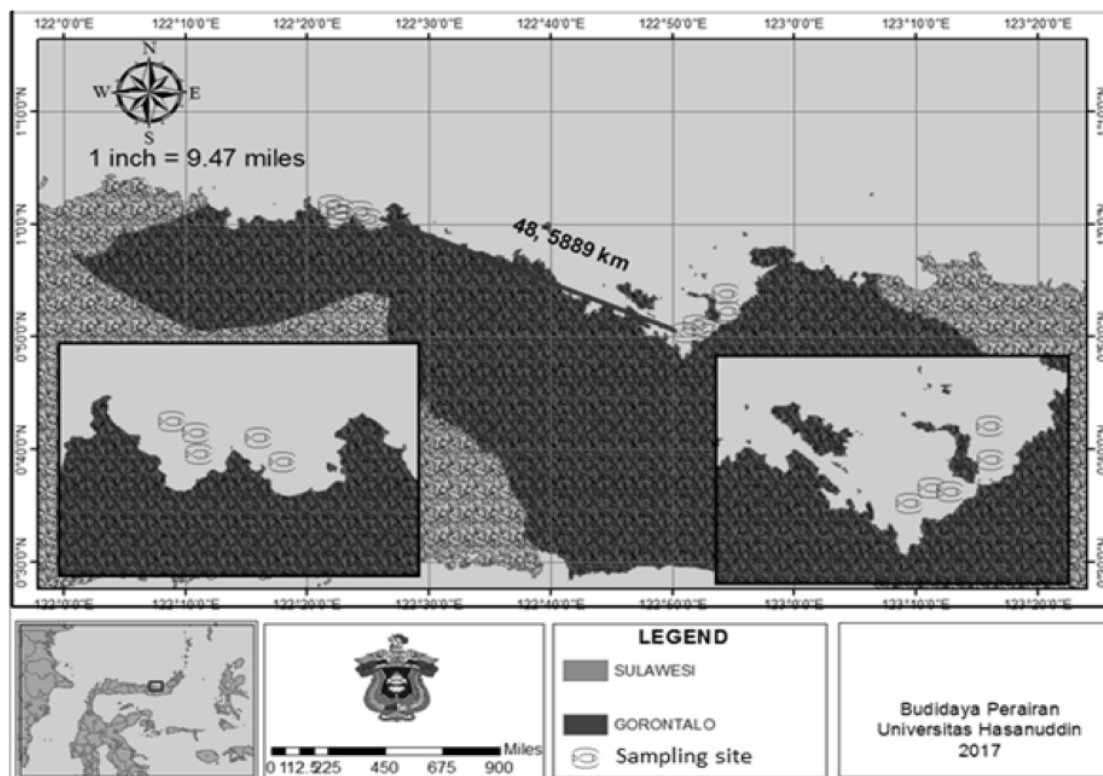
A total of 14 fish samples were collected from the two traditional fish markets around the Kwandang Gulf (TK; 0°53'39" N 122°54'40" E) and the Sumalata Gulf (TS; 1°00'53" N 122°25'10" E) in the northern part of Gorontalo Province, Sulawesi, during July 2019 (Figure 1). Morphological identification was conducted according to the guideline from FAO (Heemstra, 1993), and species confirmation has been carried out with molecular identification through the COI gene region. This study did not apply for a specific permit. All sample collected from local traditional fish market which was dead upon purchasing. All samples have been photographed with a digital camera.

### 2.2 DNA Extraction

Each specimen has been collected and directly preserved in 90% ethanol for other experimental purposes. The anal fin, around 1 cm of tissue, was dissected and mixed with 6X lysis buffer, further homogenised by the TissueLyser II (Qiagen). According to the manufacturer's guidelines, genomic DNA was extracted using Accuprep® Genomic DNA Extraction Kit (Bioneer). Quantification of purified genomic DNA performed by nanoDrop (ThermoFisher Scientific D1000), aliquoted and stored at the -70°C for further analysis.

### 2.3 PCR Condition and Data Analysis

One set of universal fish primer have targeting cytochrome c oxidase I (COI) region. Those Primers are LCO (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') with a target sequence of 720 base pairs (Folmer et al., 1994). The PCR mixture (20µL) included 11.2 µL ultra-pure water, 1 µL primer forward and reversed (0.5 µM), 0.2 µL Ex Taq DNA polymerase (TaKaRa, Japan), 2 µL 10X ExTag Buffer, 2 µL dNTPs



**Figure 1.** Distribution of sampling site at the Kwandang Gulf and the Sumalata Gulf in northern part of Sulawesi, Indonesia

1 (1  $\mu$ M, TaKaRa, Japan), and 2  $\mu$ L genomic DNA as a template. The PCR condition was 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), and Sanger sequencing was performed at 1<sup>st</sup>BASE (Singapore).

#### 2.4 Phylogenetic and Haplotype Analysis

Reverse and forward sequences were cut at each end using Chromas (<http://technelysium.com.au/wp/chromas/>). In addition to cutting the ends, this software can also ensure good sequence results. After cutting, reverse the reverse sequence can be combined with the forward line via online software via the website [https://www.bioinformatics.org/sms/rev\\_comp.html](https://www.bioinformatics.org/sms/rev_comp.html). The forward and reverse sequence numbering is also done online through the website <https://www.ebi.ac.uk/Tools/msa/elustalo/>. All sequences were aligned, including reference sequences from the GenBank database by BASTN (Table 1). The pairwise evolutionary distance among the family is determined by the Kimura 2-Parameter algorithm in Mega7. The Neighbor-joining (NJ) tree was constructed, and 1000 bootstrap analysis was carried out by Mega7 (Kumar et al., 2016). This software can also generate the genetic distance and even nucleotide composition (AT-GC percentage). Besides, haplotype analysis using DnaSP6 (Librado and Rozas, 2009) to measure the number of Haplotypes, than the genetic and nucleotide diversity using Arlequin Ver.3.5.2.2 (Excoffier et al., 2005). The network between each Haplotype was analysed using Network 5.0.1.1

### 3. Results and Discussion

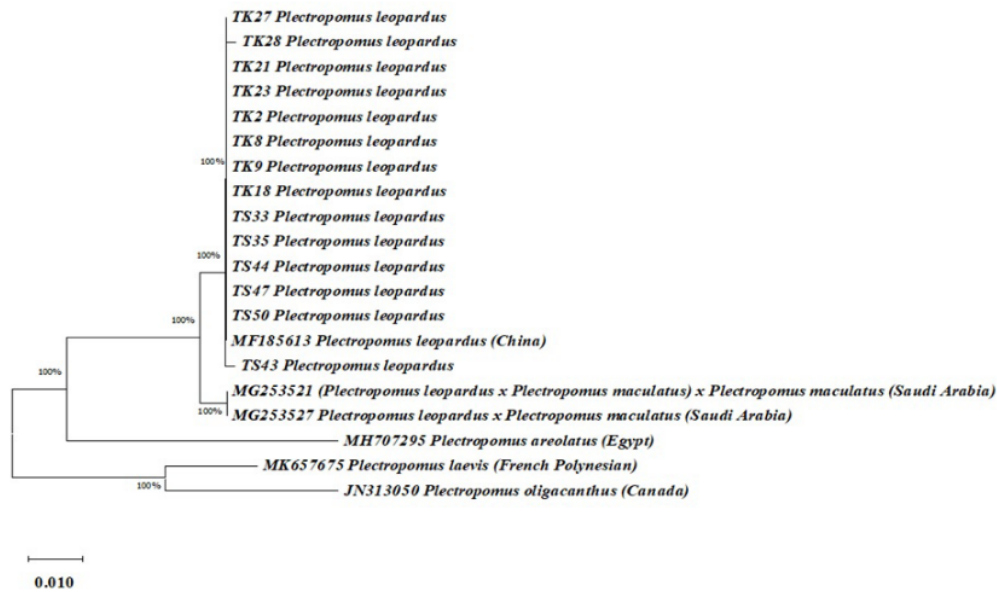
#### 3.1 Species Identification and Phylogenetic Reconstruction

In this study, 14 species of *P. leopardus* were identified as fisheries commodities and, at the same time, were potential candidates for cultured fish (Sun et al., 2015). Molecular identification is expected to reduce misidentification in the cryptic species of the genus *Plectropomus*. In previous studies, *P. maculatus* has morphological and colour characteristics almost the same as *P. pessuliferus* (Gharbawi et al., 2019). Research on molecular identification in the genus *P. leopardus* has been done before on mitochondrial DNA and nuclear DNA (Santos et al., 2013), in addition to the use of microsatellite has also been carried out to identify differences in *P. leopardus* and *P. maculatus* species

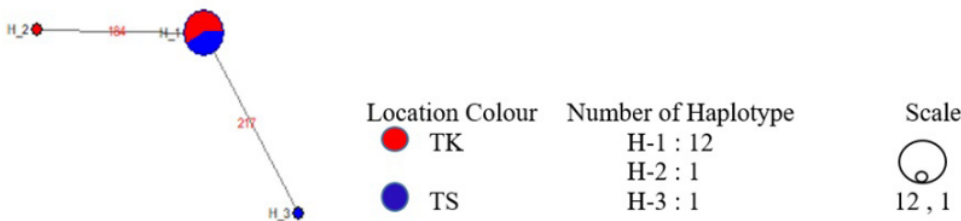
(Harrison et al., 2014).

Molecular identification has been identically made to identify reef fish (Andriyono et al., 2019; Lakra et al., 2011; Wang et al., 2012; Ward et al., 2005; Zhang and Hanner, 2012), which have similar morphological properties (Hubert et al., 2012). DNA barcoding using COI gene region is advantageous for identifying reef fish species at the larvae stage, which are very difficult to distinguish (Hubert et al., 2010). Among the Serranidae group, the Epinehelus group had already gained attention in molecular identification (Andriyono et al. 2020b), breeding (Sugama et al., 2004; Toledo, 2002), and aquaculture (Pierre et al., 2008). This identification method approach is advantageous in differentiating the level of species. In addition to obtaining accurate information up to the species level, the genetic data generated can also be used to analyse a population's biodiversity potential (Carvalho and Hauser, 1998). Marine waters, which are open ecosystems that allow genetic exchange between populations, also will enable a population to adapt and have different characteristics from their peers even though genetic mutations occur pretty long. The specialisation process on coral reef ecosystems is quite complex and exciting to continue to study biodiversity and various influential factors (Leray et al., 2010; Rocha and Bowen, 2008).

Efforts to improve the phenotypic characteristics are carried out by breeding with the closest relatives in one genus that are expected to be beneficial for the supply of animal protein needed by humans. The artificial breeding activities for *P. leopardus* species has been carried out because they naturally experience reproductive cycles that are almost the same as those of their relatives of *P. maculatus*. This situation allow and provides a hybridization potential for these two species (Frisch and Van Herwerden, 2006). The practice of *P. leopardus* breeding activities in Indonesia currently has not been reported scientifically yet from a number of studies and produces new varieties, but several species of the genus Serranidae family are more prevalent in mariculture and breeding between the genus *Epinephelus*. For instance, *E. fuscoguttatus* and *E. polyphekadion* grouper have successfully carried-out breeding that produces fish-hybrid groups with excellent growth performance (James et al., 1999). Breeding of the genus *P. leopardus* with *P. maculatus* has been carried out in Great Barrier Reef Australia by in vitro to find out its potential development in aquaculture activities. In this breeding activity, the most critical activity is the treatment of larvae which are still very difficult and need to get serious attention so that they are not only successful in spawning but also in rearing larvae (Frisch and Hobbs, 2007).



**Figure 2.** Phylogenetic tree of *P. leopardus* from Sulawesi generated by Neighbor-joining (NJ) algorithm on Mega7



**Figure 3.** Connectivity between haplotype of *P. leopardus* from two population in Gorontalo water, Sulawesi Indonesia

**Table 1.** Gene and Nucleotide diversity generated from Arlequin V3.5

Parameter	TK ( $\pm$ SD)	TS ( $\pm$ SD)
Gene diversity	0.2500 $\pm$ 0.1802	0.3333 $\pm$ 0.2152
Nucleotide diversity (average over loci)	0.000495 $\pm$ 0.000702	0.000660 $\pm$ 0.000869

Phylogenetic tree analysis showed the results of all sample sequences produced grouped in *P. leopardus* species and fused into one clade of the same species, including Chinese Haplotype. In this analysis, it was found that two species is possible to become an Indonesian (Sulawesi) haplotype, namely in TS43 and TK28, which has a slight difference from its ancestors (Figure 2). In this phylogenetic, it is also known that the results of breeding between *P. leopardus* and another species within genus *Plectropomus* will produce a different haplotype than pure strains of *P. leopardus* (from wild).

In addition to molecular identification, the results of this study found three haplotypes from the Kwandang Gulf and Sumalata Gulf populations (Figure 3) through haplotype analysis with the help of DnaSP v5 (Librado and Rozas, 2009) and Arlequin 3.5.2.2 software (Excoffier et al., 2005). In phylogenetic tree, it was also seen that the TS43 population separated from the main clade in the *P. leopardus* group (Figure 1). This finding is a haplotype that has been found and becomes new information in the biodiversity of *P. leopardus* in Indonesia, especially from the Wallace region. In the

TK28 population, possible haplotypes in this population are also found, although in general, the populations in the two gulf water in northern Gorontalo region. This information is expected to be developed with in-depth research on these two populations to conduct a further study of the biological characteristics of *P. leopardus*.

### 3.2 Genetic Diversity and Haplotype Connectivity

Information on genetic diversity in Coral trout groupers fish groups in Kwandang Gulf (TK) is lower than genetic diversity in Sumalata Gulf (TS) populations. The same thing also results in nucleotide diversity which shows that the population of Sulamata Gulf has 0.000165 higher than the population in Kwandang Gulf (Table 1). In general, the value of nucleotide diversity found is still very low. Previous research stated that the diversity value was 0.8-1 (high), 0.5-0.7 (medium) and less than 0.4 (low) (Nei, 1987). Referring to the grouping, the value of nucleotide diversity both location is very low.

Information on genetic diversity in coral trout groupers fish groups in TK is lower than genetic diversity in TS populations. The same thing also results in nucleotide diversity which shows that the population of TS has 0.000165 higher than the population in TK (Table 1).

The haplotype analysis identified three haplotypes. The most common haplotype (12 samples) dominated both populations (TK and TS), while haplotype two was found in the TK population (1) and Haplotype three in the TS population (1). This result can be seen clearly in the connectivity analysis of the two grouper populations (Figure 3).

Genetic distances that are not too large are found on different haplotypes for TK and TS populations, and mixed haplotypes. Genetic distance ranges from 0.00-0.003984. The most considerable distance was found in species TK28 and TS43 of 0.003984 (Table 2). This study is the first study conducted on *P. leopardus* species, although there have been previous studies comparing the genetic distance between *P. leopardus*, *P. maculatus* and *P. oligacanthus* from Bali waters (Suryani and Arya, 2019). From these results, it is estimated, the initial population is a mixed population between TK and TS, but with the development of time and changes in the marine environment causing mutations and allowing the existence of haplotypes in each region. It is clearly seen that TK28 and TS43 have genetic distance from all other currently available *P. leopardus* homologous sequences. When compared with sequences in the GenBank database, TK28 has a genetic distance of 0.001988 with MF185613, while TK28 has a greater genetic distance 0.003984 with TS43 (Table 2).

**Table 2.** The genetic distance of each sample and several sequences from the GenBank database generated by Mega7

	TK21	TK23	TK27	TK28	TK02	TK08	TK09	TK18	TS33	TS35	TS43	TS44	TS47	TS50
<b>TK21</b>														
<b>TK23</b>	0													
<b>TK27</b>	0	0												
<b>TK28</b>	0,002	0,002	0,002											
<b>TK02</b>	0	0	0	0,002										
<b>TK08</b>	0	0	0	0,002	0									
<b>TK09</b>	0	0	0	0,002	0	0								
<b>TK18</b>	0	0	0	0,002	0	0	0							
<b>TS33</b>	0	0	0	0,002	0	0	0	0						
<b>TS35</b>	0	0	0	0,002	0	0	0	0	0					
<b>TS43</b>	0,002	0,002	0,002	0,004	0,002	0,002	0,002	0,002	0,002	0,002				
<b>TS44</b>	0	0	0	0,002	0	0	0	0	0	0	0,002			
<b>TS47</b>	0	0	0	0,002	0	0	0	0	0	0	0,002	0		
<b>TS50</b>	0	0	0	0,002	0	0	0	0	0	0	0,002	0	0	
<b>MF185613</b>	0	0	0	0,002	0	0	0	0	0	0	0,002	0	0	0

This study, genetic distance character difference between each haplotype is not too far (Table 2). From this information, it is estimated that there is a limited mutation that causes genetic differences in the *P. leopardus* species, which description of the diversity of leopard coral grouper in both regions in Gorontalo Sulawesi. The effects of environmental stressors in the two regions need to be examined to determine the factors that influence the emergence of the Haplotype. This difference in character may also be due to the low migration rate in North Gorontalo, resulting in limited space for individuals in the population due to certain obstacles. This situation causes limitations in interacting and if it lasts long enough to cause local adaptation to form separate sub-populations such as in Kwandang Bay and Sumalata Bay. Besides, close attention needs to be paid to breeding and aquaculture activities so that the breeding process able to allowed the negative impact on the loss of pure *P. leopardus* genetic strain in Indonesia. Previous study related to the cross-breeding activities between several *Plectropomus* are carried out, but only cross-breeding between *P. leopardus* and *P. maculatus* has been scientifically reported (Frisch and Van Herwerden, 2006; Frisch and Hobbs, 2007). This breeding activity is possible because of the genetic proximity between *P. leopardus* and *P. maculatus*, compared to *P. oligacanthus* (Suryani and Arya, 2019) and *P. areolatus* (Saad et al., 2012) which separates from the two previous species.

### 3.3 Implication on Fisheries Sector

The study of genetic diversity will be interesting in terms of efforts to protect and conserve germplasm in an area. Several economically important fish species have been hybridized to increase genetic diversity in order to obtain varieties that have good characteristics such as fast growth, resistance to disease and other traits that are adapted to the needs of crossbreeding carried out (Hickling, 1968). For example, grouper has been found many types of groupers from crosses that provide benefits for the development of grouper aquaculture in Indonesia. Cantang grouper, is a hybrid type of grouper *E. fuscoguttatus* - *E. lanceolatus* (Sutarmat and Yudha, 2016). Besides the beautiful grouper species, the grouper hybrid *E. fuscoguttatus* - *E. crodon* (Ismi, 2014; Yudha and Sutarmat, 2017) and the hybrid *E. fuscoguttatus* - *E. polyphkadion* (Muzaki et al., 2016). Other examples of successful hybridization are *Epinephelus costae* - *E. marginatus* (Glamuzina et al., 2001) and *Plectropomus leopardus* with *Plectropomus maculatus* (Frisch and Hobbs, 2007). In this study, the study was conducted on sunu grouper which allows

hybridization between populations. This low diversity provides information so that interbreeding activities are carried out with the same species from other populations to avoid inbreeding which can reduce the quality of subsequent filians (Valtuena et al., 2014).

The Bar-cheek coral trout, which is currently the object of research, is one of the species that begun to be developed as a cultivation commodity. Studies on the genetic diversity of leopard coral grouper are still very limited, however, this study must continue because genetic diversity is an important indicator to determine whether a population is able to adapt to environmental changes, climate change and the spread of various diseases. From previous research, it is stated that genetic diversity is an important factor for a population to grow, reproduce and also carry out a good regeneration process. High genetic diversity will be directly proportional to the ability to adapt to environmental changes (Perwati, 2009).

The existence of conservation areas, marine protected areas is very significant in maintaining genetic diversity in Indonesia. A number of marine National Parks have demonstrated better management efforts such as the Kepulauan Seribu National Park (Agustina, 2019), Waktobi National Park (Patanda and Rahmani, 2018), and Takabonerate National Park (Akhmad et al., 2016). In addition, several newly formed marine conservation areas have also received high attention from the government, academics and community social institutions (NGOs) such as the Sawu Sea National Park (Rahman et al., 2020), Cendrawasih Bay National Park (Syamsudin, 2018) and much more. The government has also consistently had a legal umbrella in the management of conservation areas with Law no. 5 of 1990 and Presidential Decree number 32 of 1990.

## 4. Conclusion

In this study, 14 fish samples were identified as *P. leopardus*, consisting of eight samples from TK and six TS samples. Analysis of *P. leopardus* diversity found three haplotypes in both study areas. The most common haplotype of leopard coral grouper was shared between both locations. This study needs to be improved with a large number of samples both in other areas in North and South Sulawesi which allows to see the influence of geographical position on the diversity of *P. leopardus* in Sulawesi.

## Acknowledgment

The authors would like to deliver our sincere

gratitude for the initiation of collaborative research between Faculty of Marine Science and Fisheries, Universitas Hasanuddin (Makassar) and Faculty of Fisheries and Marine, Airlangga University (Surabaya), which have given essential activities to the development of marine fish biodiversity studies in Indonesia.

### Authors' Contributions

The contribution of each author as follows, Andy, Asmi, and Widya; collected the data, drafted the manuscript, and designed the sampling. Jobaidul, Sapto, and Amit; data analysis, plotting and setting all the figure. Arji and Sapto; research conceptual, data analysis, and critical revision of the manuscript. All authors discussed the results and contributed to the final manuscript.

### Conflict of Interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of this manuscript. This paper does not contain any studies humanly related or animal performed by any of the authors.

### Funding Information

No research funding.

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