DNA barcode and phylogenetics of green humphead parrotfish (*Bolbometopon muricatum*) in North Maluku waters

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Abstract. The green humphead parrotfish (*Bolbometopon muricatum*) is one of the large species inhabiting coral reefs in North Maluku waters, Indonesia. The declining fish populations due to excessive fishing has caused the green humphead parrotfish to be listed in the Red List of IUCN in the vulnerable category since 2012. The species could be highly endangered, bordering extinction in the future. Studies on the genetic identification of green humphead parrotfish could be considered critical in the policy of sustainable conservation and fish culture. This research is designed for the identification and analysis of the genetic relationship of green humphead parrotfish based on the COI (cytochrome-c-oxidase subunit I) gene. DNA samples were collected from 4 locations in North Maluku, Ternate Island, Morotai Island, Bacan Island and Sanan Island. The DNA from samples was extracted and the COI gene was amplified using PCR (Polymerase Chain Reaction). Furthermore, the amplicon was sequenced to observe the similarities with the NCBI GenBank database. The results of this study showed that the green humphead parrotfish from this study had high similarities (98-100%) with the green humphead parrotfish with the reference access no. KY235362.1. Based on the phylogenetic tree, the green humphead parrotfish originating from North Maluku has a genetic relationship with the green humphead parrotfish from the database, but with different molecular characters.
Key Words: COI genes, genetic identification, parrotfish.

Introduction. The green humphead parrotfish (*Bolbometopon muricatum*) is a species that plays an important role in the sustainability of coral reef ecosystems (Hamilton et al 2008). This species is part of the Scaridae family. It is a demersal and herbivorous species, eating algae off the coral reefs and, by this, indirectly removing sand sediments from corals (Bellwood et al 2003). It has a mouth resembling the one of a parrot and no strong jaws (Roff et al 2017). Thus, when eating algae, it does not damage the coral reefs with its bite. It brings benefits for coral reefs by removing the algae, cleaning them and allowing a better growth.

The green humphead parrotfish is categorized as the largest species from the parrot fish group, reaching 1.5 m and 75 kg (Munoz et al 2014). Morphologically, it is simply identified by the form of the mouth, head and color of its body (Munoz et al 2014). This fish has the form of the mouth resembling the beak of a parrot. It has strong plate gears on the upper and lower jaws. It is called "plate gear" because the teeth structure of the fish is concatenated with a space in the middle part (Adrim 2008). It has a typical form of the head, with a prominent bump on the forehead, similarly with the Napoleon fish (*Cheilinus undulatus*) (Munoz et al 2014). However, looking more closely, the forms of the bump between the 2 species are different, the head of the green humphead parrotfish being vertically more compressed, while the one of Napoleon fish is more rounded (Munoz et al 2012). In addition, the green humphead parrotfish has a body contrasting color, bluish green (Hamilton 2003). These two characteristics in both form and color are commonly found in adult green humphead parrotfish (Figure 1). In the juvenile phase, the fish tends to be grey or brown.

The classification of the green humphead parrotfish is described by Valenciennes (1840): kingdom Animalia, phylum Chordata, class Actinopterygii, order Perciformes,

family Labridae, genus *Bolbometopon*, species *Bolbometopon muricatum*. *Bolbometopon muricatum* is a synonym with *Bolbometopon muricatus*, *Callyodon muricatus*, and *Scarus muricatus*.

Figure 1. Green humphead parrotfish (*Bolbometopon muricatum*); source: https://www.iucnredlist.org/species/63571/12692575.

In some waters, the population of green humphead parrotfish is declining because of excessive fishing. This fish is not only for sale as an ornamental fish, but also for consumption among local people. Donaldson & Dulvy (2004) argued that, though the fish can have a large size and long life, the level of its sustainability is very low and it is vulnerable to fishing activities. Considering that the existence of the fish is very influential for coral reef ecosystems, since 2012 it has been included on the IUCN Red List, in the vulnerable category (https://www.iucnredlist.org/species/63571/17894276).

Fish genetic research in the waters of North Maluku was previously conducted. Akbar et al (2014), Akbar et al (2018a) and Akbar & Aris (2018) studied the genetic diversity of yellowfin tuna (*Thunnus albacares*) in Maluku Sea and North Maluku waters. Aris et al (2017) observed the genetic diversity of yellowfin tuna in North Maluku waters. Akbar et al (2018b) studied the genetic population and the phytogeography of bigeye tuna (*Thunnus obesus*) in Maluku Sea, Indonesia. Akbar & Labenua (2018) studied the genetic diversity of skipjack tuna (*Katsuwonus pelamis*) in North Maluku waters. Studies on parrotfish were conducted by Saad et al (2013) in the Red Sea. However, studies on the genetic information of the green humphead parrotfish were not conducted in the waters of Indonesia, to our knowledge. Madduppa et al (2014) stated that genetic markers can be used to determine the origin and the relationship in measuring the recruitment and connectivity. Thus, they can provide important information about the

dynamics of the population. This research is expected to bring information about the identification of green humphead parrotfish in North Maluku waters, Indonesia.

DNA barcoding refers to a technique that has been widely used for the identification of living creatures to species level. The technique uses DNA fragments with certain sizes standardized as the species marker. DNA barcoding is quick and accurate, although the accuracy is highly influenced by the completeness of the DNA barcode reference itself (Hebert et al 2013). DNA barcoding has a wide spectrum of potential in its development, such as the study of the life phase of species, conservation status, taxonomy, biogeography and biodiversity inventory (Delrieu-Trottin et al 2019). DNA barcoding is efficient in differentiating the cryptic speciation, a number of species that have identical characteristics or very similar morphologically, but differ genetically. Cryptic speciation frequently causes problems related to scientific synonyms of the species (Bickford et al 2007). The aims of this research were to identify the green humphead parrotfish in North Maluku waters using DNA barcoding, construct the phylogenetic tree and to analyze the genetic relationship between the green humphead parrotfish from North Maluku waters and from other waters. This research could help the conservation of the species. Collecting scientific data such as the genetic identity of a species is important in establishing policies regarding the conservation and management of sustainable fisheries.

Material and Method

Sample collection. This research was conducted from June 2018 to December 2019. The sampling of fish was conducted in 4 locations in North Maluku waters: Ternate Island, Morotai Island, Bacan Island, and Sanana Island (Figure 2). The DNA analyses were conducted in the Laboratory of BIONESIA (Biodiversitas Indonesia) Bali with the amplification of the COI gene.

Figure 2. Sampling locations in North Maluku waters; 1 - Morotai Island; 2 - Ternate Island; 3 - Bacan Island; 4 - Sanana Island.

Samples were obtained from fishermen (bycatch). The samples were photographed and the morphological characteristics (total length, standard length, head length, head height and body width) were determined. For preservation, 3 cm of the pectoral fin were collected and stored in a tube containing 96% ethanol (Akbar et al 2014). The equipment used for the extraction included a 1.5 mL tube, a set of micropipettes along with their tips, vortex, centrifuge, freezer and incubator. The equipment used for the amplification and electrophoresis included a PCR thermo cycler machine, vortex, micro centrifuge, PCR tubes, refrigerators, a set of gel moulding trays, digital scales, microwaves, stirrers, 100-volt power supplies, and UV transilluminators. The materials used in this research included TNES solution (Tris-base 10 mM, NaCl 125 mM, EDTA 10 mM pH 8, and SDS 0.5%), PCIAA (Phenol Chloroform Isoamil Alcohol), TE (Tris EDTA), Mix PCR, primer forward and reverse COI genes, agarose gel, TBE buffer, and fluorosave.

In this research, the identification and the analysis of the genetic diversity of green humphead parrotfish used COI gene (cytochrome-c-oxidase subunit I) - a part of the mitochondrial genome proposed as a universal marker gene for species identification. The sequence of the COI gene is stored in the Barcode of Life Database system (BOLD, http://www.boldsystems.org/) and many journals and scientific articles have published information about the use of COI gene to identify and discover new species (Pentinsaari 2016).

DNA extraction. The extraction was conducted using DNeasy Blood & Tissue Kit A centrifuge at 8000 rpm for 1 min was used (Barber et al 2006). The fluid in the collection tube was removed and the spin column was placed into a new 2 mL collection tube, where 500 µL of Buffer AW1 was added. Furthermore, the mix was centrifuged at 8000 rpm for 1 min and the fluid from the collection tube was removed. The spin column was placed in a new 2 mL collection tube and 500 µL of Buffer AW2 was added. The mix was again centrifuged at 14000 rpm for 3 min. The fluid in the collection tube was removed. The spin column in the new 1.5 mL tube was removed. The DNA elusion was conducted by adding 100 µL of ddH2O in the central part of the spin column membrane. The incubation was at room temperature for 2 hours. Again, the mix was centrifuged at 8000 rpm for 1 min. The final step was repeated by adding 100 µL of ddH2O, to obtain a final volume of 200 µL. Thus, the extraction solution was ready for amplification.

DNA amplification. DNA amplification was conducted using PCR (polymerase chain reaction). The extracted sample was amplified at the COI locus (cytochrome oxidase I) by the Gold (Bioline) method. The steps are: pre-denaturation at 94^{o}C for 3 min, denaturation at 94^{o}C for 3 seconds, annealing at 50^{o}C for 30 seconds, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 2 min. The PCR process was repeated for 38 cycles (Barber et al 2006). The following two primers were used: forward primer jgLCO1490 with the nucleotide sequence of TITCIACIAAYCAYAARGAYATTGG and reverse primer jgHCO2198 with the nucleotide sequence of TAIACYTCIGGRTGICCRAARAAYCA (Geller et al 2013).

Sequencing. The samples amplified using the PCR method were sequenced in the facilities of sequencing service to obtain their nucleotide sequence using the "sanger sequencing" method (Sanger et al 1977).

Data analysis. The identification of green humphead parrotfish was also conducted using the phylogenetic tree approach to determine the genetic relationship and the history of evolution of the fish in North Maluku waters with a number of other species. All 9 sample sequences were aligned with Multiple Sequence Alignment using the Crustal O program. The genetic distance among the samples was calculated to describe the differences of nucleotide alkali between the population and the diversity among species. The genetic distance among the samples of green humphead parrotfish in North Maluku waters was analyzed using Kimura's 2 parameter model Pairwise Distance (Kimura 1980).

The analysis of the phylogenetic tree was constructed using the Neighbour-Joining method based on the nucleotide of the COI gene of green humphead parrotfish samples from North Maluku Sea and the reference database having the high value of haplotype. The Neighbor-Joining method has been widely used to obtain accurate phylogenetic relationships from the phylogenic signals coming from the DNA sequence. Dodson et al (2015) stated that the historical divergence and genetic connectivity create a different race in which, later on, genetic mixture from the gene pool previously isolated can occur. DNA barcoding can help learning the species diversity and genetic distances based on the reference database (Delrieu-Trottin et al 2019).

Results and Discussion

Morphological characteristics and genetic identification using the COI gene. Morphological characteristics showed variations between locations, although the differences in characteristics appear low (Table 1). Species sizes at each site were relatively similar. Species sizes were included in the small category. The COI genes of 9 samples of green humphead parrotfish DNA were successfully amplified and resulted in an amplicon sized 637-639 bp. Table 2 presents the identification based on the similarities of genes with the ones in the database with the BLASTn program that can be accessed through NCBI (https://www.ncbi.nlm.nih.gov/). The results showed the conformity of samples with the species *Bolbometopon muricatum* reference with a similarity level of 98-100% (Table 2).

Table 1

Morphological characteristics of green humphead parrotfish (*Bolbometopon muricatum*) in North Maluku

No	*Species*	*Location*	*Number of samples*	*Morphological characteristic (cm)*				
				Total length	*Standard length*	*Head length*	*Head height*	*Body width*
1	Green humphead parrotfish (*Bolbometopon muricatum*)	Morotai	1	29	24	10.7	5.3	3.5
			2	29.6	26	10.6	5.4	3.7
2		Ternate	1	28.5	23	8.6	3.4	3.1
			2	3.2	28	12.5	7.3	5.6
			3	30.3	26	12.5	7.4	5.4
3		Bacan	1	30.6	28	12.5	7.3	5.9
			2	28.8	28	8.7	3.4	2.7
4		Sanana	1	27.3	24	8.6	4.4	3.2
			2	31.6	27	11.3	7.4	4.7

Table 2

Sample identification of green humphead parrotfish (*Bolbometopon muricatum*) in North Maluku

Sample	*Similarity (%)*	*Species reference*	*GenBank access number*
Tt1	100	*Bolbometopon muricatum*	KY235362.1
Tt2	100	*Bolbometopon muricatum*	KY235362.1
Tt3	98	*Bolbometopon muricatum*	KY235362.1
Mrt1	99	*Bolbometopon muricatum*	KY235362.1
Mrt2	98	*Bolbometopon muricatum*	KY235362.1
Bcn1	98	*Bolbometopon muricatum*	KY235362.1
Bcn2	98	*Bolbometopon muricatum*	KY235362.1
Snn1	100	*Bolbometopon muricatum*	KY235362.1
Snn2	100	*Bolbometopon muricatum*	KY235362.1

Note: Tt - Ternate Island; Mrt - Morotai Island; Bcn - Bacan Island; Snn - Sanana Island.

The analysis of the genetic distance among the samples of the green humphead parrotfish in North Maluku waters. Genetic distance indicates that the entire sample is closely related (Table 3). Genetic proximity is possible due to the strong genetic flow between species. Low genetic differences indicate that the entire population comes from the same ancestry. The results of the analysis were supported by phylogenetic reconstruction, where there were no separate groups between species

Table 3

The estimation of genetic distance among the sequences of COI gene of green humphead parrotfish (*Bolbometopon muricatum*) samples

Sample	*Tt1*	*Tt2*	*Tt3*	*Mrt1*	*Mrt2*	*Bcn1*	*Bcn2*	*Snn1*	*Snn2*
Tt1	-								
Tt2	0.005								
Tt3	0.000	0.005							
Mrt1	0.003	0.005	0.009						
Mrt2	0.000	0.005	0.000	0.009					
Bcn1	0.000	0.005	0.000	0.009	0.000				
Bcn2	0.000	0.005	0.000	0.009	0.000	0.000			
Snn1	0.008	0.010	0.014	0.017	0.014	0.014	0.014		
Snn2	0.008	0.010	0.014	0.017	0.014	0.014	0.014	0.000	

Note: Tt - Ternate Island; Mrt - Morotai Island; Bcn - Bacan Island; Snn - Sanana Island.

Phylogenetic tree of green humphead parrotfish. The phylogenetic tree can be seen in Figure 3. The COI gene sequence of the sample of Green Humphead Parrotfish from North Maluku Islands showed a high similarity (98-100%) with the species *B. muricatum* that has been deposited in GenBank with the access number KY235362.1. There is only one whole genome of the species *B. muricatum* deposited in GenBank, from Taiwan. The species *B. muricatum* has a wide spreading area, particularly in Pacific Ocean regions. Initially, this species had a high abundance, yet, as time went by, the population of parrotfish has been declining in some areas, such as in the Pacific islands of the United States, Indian Ocean, and Eastern Africa due to continual fishing activity. Donaldson & Dulvy (2004) affirmed that the population decline has been accelerated by the low rate of replacement of parrotfish stock in nature. This, together with its role as the main bioeroder towards the coral reefs and for the sustainability of ecosystem resilience, become strong reasons to issue a policy for the protection of this species.

The identification of species using the DNA barcode of the COI gene has been widely used by researchers because it is an accurate and quick method. Da Silva Ferrette et al (2019) used DNA barcoding to identify the composition of the Batoidea fish (superorder of stingray) in the small-scale fisheries industry in the Southwest Atlantic region and Southeast Brazil. Based on the Kimura 2 parameter model, the lowest value of the genetic distance was 0% (0.000) and the highest was 1.7% (0.017), with the average of ±0.7% (0.007). The lowest genetic distance (0%) was present in the case of the green humphead parrotfish from the areas of Ternate and Bacan, and Ternate and Morotai. By contrast, the highest genetic distance of the fish was present between green humphead parrotfish from Sanana and Morotai. Tallei et al (2016) stated that a lower value of genetic distance between two organisms suggests a closer genetic relationship. Overall, the low genetic distance among the areas showed that green humphead parrotfish in North Maluku Islands have a high closeness according to their genetic relationship.

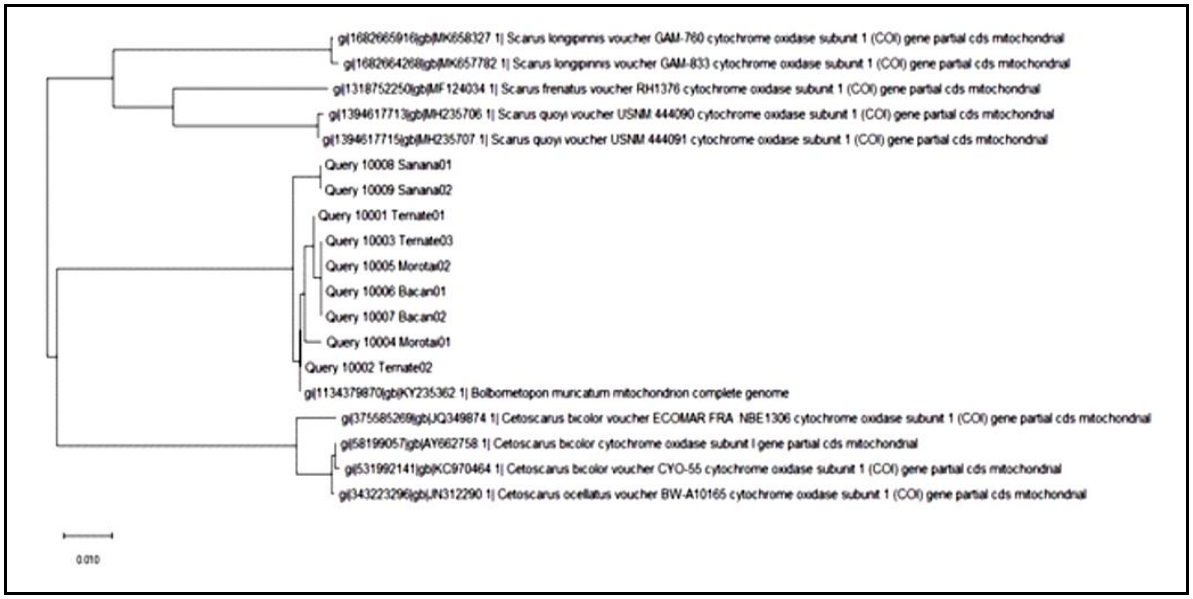

Figure 3. Phylogenic tree of green humphead parrotfish (*Bolbometopon muricatum*) in North Maluku Waters and the reference haplotype of the GenBank.

The phylogenetic tree showed 3 main clades: the family of Scaridae including *Scarus longipinis* and *Scarus frenatus*, *Bolbometopon muricatum*, and *Cetoscarus bicolor*. The samples of green humphead parrotfish originating from North Maluku waters are mainly the same with the species *Bolbometopon muricatum* (KY235362.1). However, of 9 samples, it was found that samples Sanana1 and Sanana2 were at the branch that was different from the other 7 samples. This might be caused by the evolutionarily different genetic distance. Westneat & Alfaro (2005) explained that, traditionally, parrotfish is part of the Scaridae family. Though the phylogenetic and evolution analyses are still in progress, today, parrotfish can be accepted as part of the class Cheilini (sub-family Scarinae, family Lambridae). Based on the World Register of Marine Species (marinespecies.org), sub-family Scarinae presents 5 genera: *Bolbometopon, Cetoscarus, Chlorurus, Hipposcarus,* and *Scarus*. On the other hand, the sub-family Sparisomatinae, the other sub-family of the family Scaridae, also has 5 genera: *Calotomus, Cryptotomus, Leptoscarus*, *Nicholsina* and *Sparisoma*. The naming of species in the Scaridae family has synonyms, as recorded in Fishbase (2019) (https://www.fishbase.in/). *Bolbometopon muricatum* is synonymous with *Bolbometopon muricatus, Callyodon muricatus* and *Scarus muricatus*. Meanwhile, *Cetoscarus bicolor* (Figure 3) is synonymous with *Bolbometopon muricatus, Callyodon muricatus* and *Scarus muricatus.* As the species is included into the Red List of IUCN, being an international concern, the barcode DNA with COI gene can be used as a tool to identify green humphead parrotfish that is a commodity in the capture fisheries of Indonesia and for better conservation strategies. Considering the phylogenic tree, *B. muricatum* originating from North Maluku waters had a molecular character different from the reference database. Hence, there is a need for more information about the genetic diversity of parrotfish originating from North Maluku, Indonesia.

Conclusions. From the analysis of the COI gene, 9 samples of parrotfish from the North Maluku Sea were identified as *B. muricatum*, with the level of similarity between 98%-100% with reference KY235362.1 from the GenBank. The genetic distance of *B. muricatum* from North Maluku waters is in the low category (average of 0.7%). Thus, it can be concluded that the genetic relationship is close. The phylogenic tree of parrotfish in North Maluku Sea has a close genetic relationship with a parrotfish from Taiwan (access number KY235362.1 from GenBank), but the fish have a different molecular character. There is a need for the publication of the sequence of COI gene of parrotfish from the North Maluku Sea for further studies.

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