

Molecular Identification of Snapper (Perciformes: Lutjanidae) Landed at Pondokdadap Fishing Port of Sendang Biru, Malang, Indonesia

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Abstract

Snapper is a type of demersal marine fish from the Lutjanidae family. The Lutjanidae family spread throughout the world and currently has 123 species in 21 genera, one of which is the *Lutjanus* genus (Miller and Thomas, 2007). To this day, the records of capture fisheries production data for snapper in Malang are still very limited to certain types. Morphological identification that has been carried out so far is still challenging to obtain accurate results because of the many similarities between the observed species or the loss of characteristics. Therefore, molecular identification is necessary to determine the types of snappers in this area and their conservation status. This study aims to determine the types of snappers using a molecular approach by Cytochrome Oxidase subunit I (COI) gene marker. Phylogenetic tree reconstruction and genetic distance calculations were performed using Mega X software through the neighbour-joining (NJ) algorithm. The results of the identification snapper based on a molecular approach with DNA barcoding revealed that the four snapper samples were *L. gibbus*, *L. rufolineatus*, *L. bengalensis*, and *L. erythropterus*. Based on the results of the compilation of the phylogenetic tree, it can be seen that the *L. bengalensis* sample is closely related to *L. rufolineatus* while *L. gibbus*, and *L. erythropterus* each form a separate clade from the two previous *Lutjanus* species. Based on their conservation status at the IUCN, the four species of snapper found are in the Least Concern category.

Keywords: diversity, gene, identification, phylogenetic, snapper

Introduction

Sendang Biru is one of the coastal areas that prioritise managing marine fisheries resources in Malang Regency, East Java (Andriyono *et al.*, 2019). The development makes Sendang Biru a centre for the capture industry (Aliviyanti *et al.*, 2021). Snapper is one of the fish catches obtained on this region (Luthfi *et al.*, 2016). Snapper is a type of demersal fish of the family Lutjanidae. The family Lutjanidae, spread throughout the world, currently has 123 species in 21 genera, one of which is the genus *Lutjanus* (Miller and Cribb, 2007). Based on morphology and habitat characteristics, 30 species of snapper from the genus *Lutjanus* found in Indonesian waters (Allen *et al.*, 2013, Halim *et al.*, 2020).

Snapper in nature plays the role as one of the large-sized apex predatory fishes that inhabit tropical coastal ecosystems around the world. Ecologically, the existence of this fish is important because it acts as a peak predator with extensive food habits. This fish can eat other small fish, *cephalopods*, crabs, shrimps, and other benthic crustaceans to control the stability of the aquatic ecosystem in which it lives (Simonsen *et al.*, 2015). Snapper is also one of the captured fishery commodities that is usually used as consumption fish sold in the form of fresh fish, fillets, and processed products (Oktaviyani, 2018). The production of this fish has increased every year. This follows data from the Central Statistics Agency (BPS) of Malang Regency (2020), where the total production of this fish reached 57.05 tons in 2018 and 2019 to 108.24 tons. Based on data from BPS

Malang Regency (2020), recording data on the production of capture fisheries for snapper in Malang is still very limited to certain types. This is due to the difficulty of identifying species in the field and at the time of simultaneous landing with other types of fish at the fish auction site. Identification of a species can be made morphologically as well as molecularly. Morphological identification that has been carried out so far is still challenging to obtain accurate results because of the many similarities between the observed specifications. In addition, the loss of distinctive features in observed species due to adaptation to the environment is also an obstacle in identifying a species morphologically (Prehadi *et al.*, 2015).

One alternative to identify that can be done in addition to morphological is molecular identification by DNA barcoding. DNA *barcoding* is a globally agreed method for identifying plant and animal species based on DNA sequence variations (Coissac *et al.*, 2016) from nitrogenous base pair regions in the *Cytochrome Oxidase* subunit I (COI) gene (Powers *et al.*, 2018). Since its introduction in 2003, the DNA barcoding technique has become the golden standard or the main standard for molecular taxonomy (Fadli *et al.*, 2020). DNA-based identification barcoding has been well received globally for its various advantages, such as being very simple and using a universal tool applicable to all organisms, both in fresh samples and processed products (Kress *et al.*, 2015). Some examples of research that utilizes the DNA barcoding technique include the use of DNA barcoding to identify fish larvae at different stages of development (Wibowo *et al.*, 2018), identification of the discovery of new and cryptic fish species (Farhana *et al.*, 2018), identification of fish species that have similar morphological characters (Bingpeng *et al.*, 2018).

DNA barcoding is effective for identifying a species with fast and accurate results based on the *Cytochrome Oxidase* subunit I (COI), even if specimen are larvae (Li *et al.*, 2016). The COI gene is one of the protein-encoding genes found in mitochondria that has a distinctive character in each species so it becomes a standard gene as a marker gene when identifying an animal species (Pentinsaari *et al.*, 2016). Therefore, the COI genes in DNA barcoding has two advantages, not only for species identification and for metabarcoding (Andújar *et al.*, 2018), (Tan *et al.*, 2019) as well. Therefore, research on the identification of snappers based on DNA markings of the COI gene needs to be carried out to provide genetic information. It is expected to be supporting data in the management of conservation areas and fishing zones in the waters of South Malang, especially from Sendang Biru.

Materials and Methods

Sampling of fish

A total of 4 samples were collected from traditional fish market of Pondokdadap fishing port at Sendang Biru, Malang in the middle of March 2020. All samples collected from the local traditional fisherman were dead upon purchase. The digital camera was used to take the individual photograph before further treatments. Morphologically, identification and species confirmation were carried out with molecular identification in this study. No specific permit was required for this study,

DNA extraction and PCR amplification

Each specimen has been collected based on the morphological characters and directly preserved in 90% ethanol for further experimental purposes. Genomic DNA extracted using an Accuprep® Genomic DNA Extraction Kit (Bioneer) according to the product guidelines. The pereiopod fin, around 1 cm tissues, was dissected and mixed with 6X lysis buffer, which was further homogenized by the TissueLyser II (Qiagen). Quantification of purified genomic DNA performed by nanoDrop (ThermoFisher Scientific D1000), aliquoted and stored at the -70°C for further analysis.

One set of universal fish primer targeting cytochrome c oxidase I (COI) region, BCL-BCH were used to obtain the partial sequences of each gene (Madduppa *et al.*, 2016). 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 purified with the AccuPrep®Gel purification kit (Bioneer, Korea).

Data analysis

Forward and reverse sequence were edited and aligned using MEGAX (Kumar *et al.*, 2018). All sequences were then aligned to the reference on GenBank database by BLASTN (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). 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 Mega X and genetic distance used a nucleotide substitution model by comparing a DNA sequence of one nucleotide with another nucleotide (Kumar *et al.*, 2018).

Result and Discussion

Morphological identification

The samples obtained from the Sendang Biru traditional fish market were 4 snappers with different species consisting of *Lutjanus bengalensis*, *L. rufolineatus*, *L. gibbus*, and *L. erythropterus*. The most striking difference between each species is each snapper sample's color and body pattern (Figure 1.). In addition to body color and pattern, morphological identification also observed morphometric and meristic characters in snapper samples (Table 1.).

Molecular identification

Molecular identification of snapper samples was carried out using the DNA barcoding method. The sequence data obtained were then analyzed and matched with the sequences found in GenBank at NCBI (National Center for Biotechnology Information) using BLASTN (Basic Local Alignment Search Tool Nucleotide) based on the degree of similarity (Table 2). Based on the results of the BLASTN analysis, sample MLKK1 was identified as having 96.72% similarity to the species *L. gibbus* (Humpback red snapper) access number MF409615, sample MLKK2 was identified to have 99.19% similarity to the species *L. rufolineatus* (Yellow lined snapper) access number MN870411, sample MLKK3 was identified as having 99.54% similarity to *L. bengalensis* (Bengal snapper) access number EU600137, while MLKK4 sample was identified to have 100% similarity to *L. erythropterus* (Crimson snapper) species access number GU673841.

Phylogentic tree reconstruction

Based on the results of the phylogenetic tree reconstruction (Figure 2.), samples of snapper landed at the Pondokdadap Sendang Biru Fishing Port, obtained 4 clades formed in the family Lutjanidae with the genus *Lutjanus*. The clade *L. bengalensis* is phylogenetically close to the clade *L. rufolineatus*, while the clade *L. gibbus* is closely related to the clade *L. erythropterus*. Both *L. bengalensis* (MLKK3) and *L. rufolineatus* (MLKK2) has low genetic distance with close species from other region (Table 3.), but *L. erythropterus* from Malang has significant genetic distance with sam species from Australia (0.18) and Malaysia (0.19). As a fish associated with coral reef ecosystems, *Lutjanus* fish species make coral reefs a habitat for rearing ground and feeding ground (Halim *et al.*, 2020, Tony *et al.*, 2020). The coral reef habitat will experience different speciation in each region. The Indian Ocean area has different characteristics from the Malaysian waters (South China Sea) and the Australian area which is influenced by the Pacific Ocean which is the main barrier in the distribution of shallow marine fish species. This pattern is known as allopatric speciation (Rocha and Bowen, 2008).

The diversity of potential marine fish in Indonesia needs serious attention (Suman *et al.*, 2017). Not only in sustainable management (Atmaja and Nugroho, 2017), accurate species determination is also a must in providing a valid database at the species level. Many morphological identifications have been carried out. However, in marine fish species there are morphological similarities in both shape and color which causes confusion and inconsistency in naming fish species. In this study,

Table 1. Morphometric and Meristic Measurements of Snapper Samples

Parameters	Sample ID			
	MLKK1	MLKK2	MLKK3	MLKK4
Total length	26,3 cm	26,8 cm	27,5 cm	26,4 cm
Standard length	21,5 cm	21,5 cm	22,5 cm	20,5 cm
Head length	8 cm	8,3 cm	8,6 cm	8,2 cm
Height	8,5 cm	8,6 cm	10,8 cm	11,2 cm
Head height	6,5 cm	6,8 cm	9,2 cm	8,6 cm
Tail base height	2,5 cm	2,8 cm	3,0 cm	2,8 cm
Dorsal fin	D.X, 14	D.X, 14	D.XI, 14	D.XI, 14
Pectoral fin	P.17	P.16	P.16	P.17
ventral fin	V.I,6	V.I,6	V.I,6	V.I,5
anal fin	A.III,8	A.III, 8	A.III, 8	A.III, 9
caudal fin	C.18	C.18	C.20	C.18

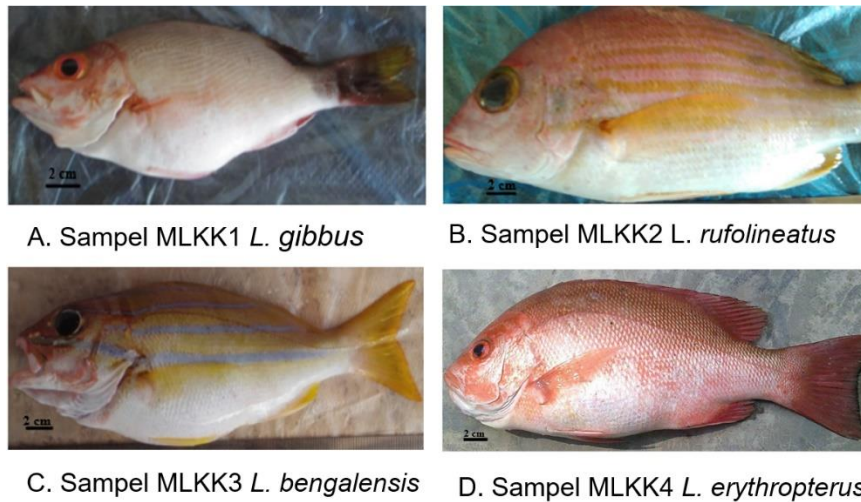


Figure 1. Four species of Snappers Landed at Pondokdadap Fishing Port in traditional fish market of Sendang Biru, South Malang.

Table 2. BLASTN Results of Snapper Samples with NCBI GenBank Database

No.	Sample Code	Species Name/ Common Name	No. Access GenBank	Identity (%)
1.	MLKK1	<i>Lutjanus gibbus</i> / Humpback red snapper	MF409615	96,72%
2.	MLKK2	<i>Lutjanus rufolineatus</i> / Yellow lined snapper	MN870411	99,19%
3.	MLKK3	<i>Lutjanus bengalensis</i> / Bengal snapper	EU600137	99,54%
4.	MLKK4	<i>Lutjanus erythropterus</i> / Crimson snapper	GU673841	100%

apart from observing specimens based on morphological characteristics, a molecular approach was used to improve data accuracy in identification at the species level. Of the 4 specimens collected, the morphological characteristics showed that the specimens were able to be identified based on their morphometric characteristics, so that all samples were identified as *L. bengalensis*, *L. rufolineatus*, *L. gibbus*, and *L. erythropterus*. To increase accuracy in identification, we also carried out molecular identification of the COI gene section (Andriyono and Suciyo, 2020) which has been agreed as a universal area for identification at the species level.

Morphological observations on the four samples showed that the MLKK1 sample had similarities with *Lutjanus gibbus*/ humpback red snapper, also known as jinaha snapper. The distinctive features or key morphological identification of this fish are having a compressed body shape with a grayish red body color, the caudal fin is clearly branched with dark red rounded lobes, and on the dorsal fin there are 10 hard spines and 13-14 soft spines (Thi et al., 2015). The MLKK2 sample has similarities with *Lutjanus rufolineatus*/ yellow lined snapper, also known as badur snapper. The key to morphological identification of the *L. rufolineatus* species is that there are 6 yellow stripes on each side of its body, it has a pale red compressed body shape, the tail is brownish yellow, and on the

dorsal fin there are 10 hard spines and 12-13 soft spines (Allen et al., 2013).

The MLKK3 sample is similar to the *Lutjanus bengalensis*/bengal snapper species, also known as yellow snapper with a key identification. The body of this fish is compressed, the body color is bright yellow with 4 grayish white stripes on each side of the body, there is a deep groove on the front operculum, the caudal fin is broad with a straight tip, has 11 spines and 12- 14 soft rays on the dorsal fin (Iwatsuki et al., 2016). The MLKK4 sample has similarities to the *L. erythropterus*/ crimson snapper or often referred to as the red snapper. The identification key in common in the form of having a pink to dark red compressed body shape from the tip of the head to the tail. Another characteristic are the tip of the snout is slightly pointed and relatively small. Besides, the preoperculum notch is not very pronounced and on the dorsal fin there are 11 hard spines and 16-17 soft spines (Sarkar et al., 2021).

Reconstruction of the phylogenetic tree of snapper samples landed at the Pondokdadap Fishing Port, Sendang Biru, obtained 4 clades formed in the family Lutjanidae with the genus *Lutjanus*. The clade *L. bengalensis* is phylogenetically close to the clade *L. rufolineatus*, while the clade *L. gibbus* is closely related to the clade *L. erythropterus*. In the phylogenetic tree reconstruction, there is also a clade

of the *Nemipterus virgatus* species as a comparison or outgroup. Reconstruction of phylogenetic trees is supported by the results of genetic distance analysis in a species (Akbar and Labenua 2018). The results of the genetic distance analysis showed that the MLKK3 sample was close to the *L. bengalensis* EU600137 (China) and LC075762 (Indian Ocean) samples, with a genetic distance of 0.00 (zero). The MLKK2 specimen was closely related to the *L. rufolineatus* specimen MN870411 (Indonesia) and had a genetic distance of 0.01. The MLKK1 specimen was closely related to *L. gibbus* MN870581 (Ambon, Indonesia), MK566973 (France) and MF409615 (Reunion) with a genetic distance of 0.01 each. The MLKK4 specimen was closely related to *L. erythropterus* specimens GU673841 (Indonesia) and GU673202 (Australia) with a genetic distance of 0.00 and 0.01, respectively. Research on the Lutjanidae species in peninsular Malaysia (Malacca Strait and South China Sea) also shows that there is a variation in genetic distance (Halim et al., 2022).

Based on the conservation status that refers to the IUCN (International Union for the Conservation of Nature and Natural Resources), *L. gibbus*, *L. rufolineatus*, *L. bengalensis*, and *L. erythropterus* species are included in the Least Concern or low risk category (IUCN Red List, 2021). Least Concern is a species that has been evaluated but its status is still under the status of almost endangered or it can be said that it does not fall into any category. The IUCN conservation status categories include the category of extinction (EX), category of extinction in the wild (EW), category of critically (CR), category of threatened or critical (EN), category of vulnerable (VU), category of near threatened (NT), the category of low risk (LC) and the category of lack of information (DD) (<https://www.iucnredlist.org/>). Then based on their trading status according to CITES, these four snapper species are included in the Not Evaluated category, so that they are still classified as safe for international trade.

Table 3. Genetic Distance of Snapper COI gene Sequences from Sendang Biru with Snapper COI gene Sequences on NCBI GenBank

No.	Name of Species	1	2	3	4	5	6	7	8	9	10	11	12	13
1.	MLKK1 <i>Lutjanus gibbus</i>													
2.	MN870581 <i>L. gibbus</i> (Ambon)	0,01												
3.	MK566973 <i>L. gibbus</i> (Perancis)	0,01	0,00											
4.	MF409615 <i>L. gibbus</i> (Reunion)	0,01	0,00	0,00										
5.	MLKK2 <i>Lutjanus rufolineatus</i>	0,17	0,16	0,16	0,16									
6.	MN870411 <i>L. rufolineatus</i> (Ambon)	0,16	0,15	0,15	0,15	0,01								
7.	MLKK3 <i>Lutjanus bengalensis</i>	0,17	0,16	0,16	0,16	0,07	0,06							
8.	EU600137 <i>L. bengalensis</i> (China)	0,17	0,16	0,16	0,16	0,06	0,05	0,00						
9.	LC075762 <i>L. bengalensis</i> (Indian Ocean)	0,17	0,16	0,16	0,16	0,07	0,06	0,00	0,00					
10.	MLKK4 <i>Lutjanus erythropterus</i>	0,18	0,17	0,17	0,17	0,17	0,16	0,18	0,17	0,18				
11.	GU673841 <i>L. erythropterus</i> (Australia)	0,18	0,17	0,17	0,17	0,17	0,16	0,18	0,17	0,18	0,18	0,00		
12.	GU67202 <i>L. erythropterus</i> (Malaysia)	0,19	0,18	0,18	0,18	0,18	0,17	0,19	0,18	0,19	0,19	0,01	0,01	
13.	KP112336 <i>Nemipterus virgatus</i> (China)	0,24	0,23	0,23	0,23	0,21	0,20	0,21	0,21	0,21	0,21	0,21	0,21	0,20

Conclusion

Based on morphological and molecular identification, the types of snappers that landed from Sendang Biru, South Malang waters were *Lutjanus bengalensis*, *L. rufolineatus*, *L. gibbus*, and *L. erythropterus*. Based on the results of the compilation of the phylogenetic tree, it can be seen that the *L. bengalensis* sample is closely related to *L. rufolineatus* while *L. gibbus*, and *L. erythropterus* each form a separate clade from the two previous *Lutjanus* species. Based on their conservation status at the IUCN, the four species of snapper found are in the Least Concern category, while based on their trading status on CITES, these four species are in the Not Evaluated category.

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