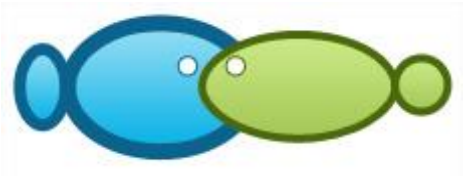

DNA barcoding, phylogenetic tree and genetic distances of manggabai (*Glossogobius aureus*) from Limboto Lake, Indonesia

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Abstract. Manggabai fish (*Glossogobius aureus*) is a local fish from Lake Limboto with economic value. In line with the damage to the lake's ecosystem, the pressure on local fish is increasing. The diminishing population of manggabai shows this over time. In general, the local community views manggabai as an endemic fish. Manggabai conservation efforts need to be analyzed from the DNA aspect. This study aimed to explore the identity of manggabai species, analyze genetic distances and construct phylogenetic trees. Fish samples were collected from Lake Limboto and transported to a laboratory of the Faculty of Fisheries and Marine Sciences of Gorontalo State University, Gorontalo, for morphometric and meristic analyses. Tissue was sampled for DNA barcode analysis, using PCR. DNA sequencing was based on PCR. The analyses show that all sampled manggabai specimens from Limboto Lake are a single species, namely *G. aureus*. The DNA barcoding results are supported by a similarity test with reference sequences of 99-100%, phylogenetic tree sequences of individual manggabai samples, and individual reference fish sequences showing kinship.
Key Words: DNA barcode, fish species identity, Limboto lake, similarity index.

Introduction. Lake Limboto is an ecosystem resource located in Gorontalo Province, Indonesia, covering the Gorontalo Regency and Gorontalo City areas. Physiographically, Lake Limboto is in a lowland area surrounded by limestone hills with 23 tributaries as inlets and one as an outlet. Limboto Lake is essential for the region because it has multiple ecological, social, and economic functions. Therefore, the government has made Limboto Lake a national strategic area.

According to Hasim & Mopangga (2018), Lake Limboto has a high economic value that comes from the potential of ecotourism and fishery biological resources. Based on field information, there are three types of local fish in the waters of Lake Limboto, namely payangka, manggabai, and huluu. The three types of fish are consumed and have economic value. Fazrin et al (2020) reported that manggabai are under ecological pressure. The same was conveyed by Hasim et al (2021), with payangka and manggabai populations having decreased due to the declining quality of the Limboto Lake ecosystem. Limnologically, the water quality of the Lake Limboto has decreased in quality (Hasim et al 2017; Lihawa & Mahmud 2017).

Much research has been carried out on Lake Limboto, mainly covering aspects of cultivation (Hasim 2013; Hasim et al 2017a), lake management (Hasim 2012), water quality parameters (Hasim et al 2015; Hasim et al 2017b; Krismono et al 2018), lake geology (Yunginger et al 2018). On the other hand, local fish species, especially manggabai, are still a mystery. Unfortunately, the types of endemic fish in these waters have yet been confirmed with a genetic approach.

DNA can help quickly and accurately identify a species. According to Stoeckle (2003), every living organism has standard gene markers from the cytochrome gene c oxidase subunit 1 (COI) (Hebert et al 2003). DNA barcoding using sequence variations within the 648 bp region of the COI gene is a tool for species identification (Hebert et al 2003). COI is shown to have low intraspecific variation, but high interspecific divergence

between closely allied taxa (Ward 2012). Most of the fauna species studied can be distinguished using DNA barcodes (Hikam et al 2021).

DNA barcoding has advantages over morphological approaches in identifying species (Layton et al 2016). Obstacles in the morphological approach are possible damages to the sample (Schander & Willasen 2005) and the sample's age (Webb et al 2006). These obstacles can be overcome through a genetic approach with DNA barcoding. DNA barcoding has been shown to be an effective tool for species identification in various vertebrate groups (Vieira de Carvalho 2014), including fish. DNA barcoding have also been used to identify pinhead fish (*Aplocheilus panchax*) and parrot fish (*Anabas testudineus*) (Mustikasarai & Agustiani 2021). This study aimed to explore the identity of manggabai, to analyze the genetic distance and the phylogenetic tree using DNA barcoding.

Material and Method

Description of the study sites. This research was conducted in August-September 2022. It took place in two locations, namely water quality sampling at Lake Limboto with 7 stations and meat sampling for genetic analysis as well as morphometric and meristic observations at the Laboratory of the Faculty of Fisheries and Marine Sciences, State University of Gorontalo, Indonesia. The research locations are presented in Figure 1. There were 5 study stations: station 1 (Pentadio), station 2 (Bulato), station 3 (Bungalow), and station 4 (Dembe) and station 5 (Tabumela Jaya).

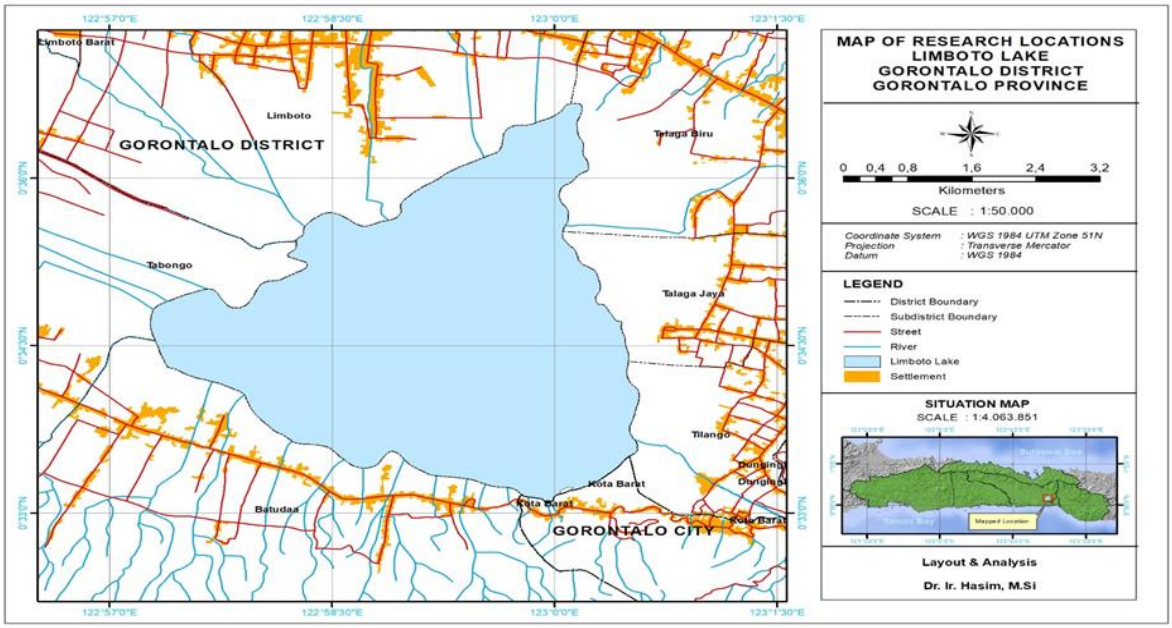

Figure 1. The Limboto Lake research location

Tools and materials. The tools used in this study were scissors, tweezers, digital scales, tubes, rulers, stationery, tissues, and cameras. The material used in this study was manggabai fish (*Glossogobius giuris*) 96% alcohol (Dailami et al 2021a).

Procedure sampling. Live fish samples were obtained from fishermen using traditional tools made from tires and bamboo and transported using plastic packing to the laboratory, to collect flesh and heart samples. 8 individuals were sampled for DNA analysis. DNA samples were stored in a tube with 96% alcohol to maintain their durability (Toha et al 2021). In addition, morphometric and meristic observations were carried out at the Faculty of Fisheries and Marine Sciences Laboratory, State University of Gorontalo.

Measurement of water chemical and physical parameters. Measuring the physical and chemical parameters of the water was carried out at each station. Water quality measurements were carried out directly. The method of measuring water quality parameters can be seen in the table below.

Table 1

The parameters of water quality at Limboto Lake

No	*Parameter*	*Unit*	*Observation method*	*Tool*
1	Depth	M	Direct observation	Meter
2	Brightness	M	Direct observation	Secchi disk
3	Temperature	^oC	Direct observation	Digital thermometer
4	DO	mg L^{-1}	Direct observation	DO meter
5	pH	-	Direct observation	pH meter digital

DNA barcoding. DNA barcoding begins with DNA isolation using the Qiagen method. After isolation, electrophoresis is continued to determine the isolated results' quality and quantity. Furthermore, cytochrome oxidase sub unit I (COI) gene amplification was carried out using polymerase chain reaction (PCR). A pair of primers is used to amplify the target organism's DNA. The amplification process was carried out in a thermocycler using the Hot-start and Gold programs (Saiki et al 1988) following a modified protocol (Barber & Erdmann 2000). PCR conditions were denaturation at 95^{o}C for 1 min, annealing at 48-55^{o}C for 1 min, and chain elongation at 72^{o}C for 1.5 min. The next stage is electrophoresis to determine the success of the PCR results. Electrophoresis was carried out for 30 min at 80 volts with 1 x TAE buffer. The results of the electrophoresis were determined using UV light at a wavelength of 254 nm and photographed with a Polaroid camera. The PCR products were purified using Shrimp Alkaline Phosphatase (Amersham Biosciences Corporation, Arlington Heights, Illinois, USA) and Exonuclease (Amersham) (SAP/EXO) for 30 min at 37^{o}C followed by 80^{o}C for 15 min. Determination of nucleotide sequences was carried out using Big Dye 3.0 terminator chemistry (Applied Biosystems). The sequencing product was purified by the isopropanol precipitation method and visualized on the ABI377 automated sequencer (Applied Biosystems).

Data analysis. The COI mtDNA sequencing results were analyzed on an ABI 377 automated sequencer (Applied Biosystems). Forward and reverse sequences were corrected and compiled in Sequencher 4.0 (GeneCodes Corporation, Ann Arbor, Michigan, USA). Sequencing was performed using ClustalW (Thompson et al 1994). The results of the COI gene analysis obtained were analyzed for similarity with reference data using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast) (Fahmi et al 2017). Furthermore, for the analysis of molecular characteristics (nucleotide composition, polymorphic sites, etc.) and genetic distance analysis, the next step was to construct a phylogenetic tree using the neighbor-joining method with the Kimura 2-parameter model, bootstrap 1000x. All of these analyses were performed with Mega software's help (Dailami et al 2021b).

Results and Discussion. Manggabai from Limboto Lake has a superiorly positioned mouth, has pointed caudal fin with black spots, light-colored pointed soft pectoral fins, soft anal fin, dark at the end. The shape is elongated with the upper part darker, while it is relatively light in color towards the belly. In general, manggabai has a dark yellow color. It has a cylindrical body shape with a small head and slightly protruding eyes, a high snout, and cycloid-shaped scales. It presents a caudal fin, two dorsal fins, an anal fin, two pectoral fins, and two pelvic fins. The caudal fin has a round shape with a blackish-white pattern (Figure 2).

Figure 2. Limboto Lake manggabai fish.

The condition of the aquatic environment at the Manggabai fishing location is presented in Table 2.

Table 2

The water quality of parameters at the study site

Station	*Water quality parameters*				
	Depth (m)	*Brightness (m)*	*Temperature (°C)*	*DO (mg L^{-1})*	*pH*
Pentadio	0.98	0.23	28	5.1	7
Bulota	2.28	0.26	28.3	5.6	7
Bungalow	1.66	0.31	27.6	5.9	7
Dembe	3.1	0.32	28.1	5.3	7
Tabumela Jaya	2.83	0.37	28	6.1	7

Note: DO - dissolved oxygen.

Table 2 shows that DO, pH and temperature are in a range that supports fish survival (Hasim et al 2017). However, the brightness in several places is less than 30 cm. Low brightness indicates high dissolved and suspended solids in Lake Limboto. Certain conditions will negatively affect the survival of fish.

According to Zhang & Hanner (2012), ideal DNA barcoding must be strong, i.e., DNA fragment sequences must be almost identical between individuals of the same species, and different between species. Therefore, the length of the band with the same relatives is chosen in the sequencing analysis. Based on sequence analysis of 10 samples of manggabai, 8 had good quality DNA bands of ±650 bp in length. At the same time, two samples were ignored because they had a DNA band length of less than ±100 bp. Therefore, the 2 were not included in this analysis. The similarity index of individual sequences of manggabai fish samples is presented in Table 3.

Table 3

BLAST results and DNA barcoding of 8 individual nucleotide sequences of the COI gene of manggabai

Code	*BLAST*		*Source*
	Species	*Identity percentage (%)*	
MB30_3_2	*Glossogobius aureus,*	100	Zhang (2011), Zhang & Hanner (2012)
MB30_2_2	*Glossogobius aureus*	99.85	Ndobe et al (2022)
MB30_1_2	*Glossogobius aureus*	98.99	Fu'Adil et al (2020)
MB2451	*Glossogobius aureus*	99.27	Fu'Adil et al (2020)
MB24_4_1	*Glossogobius aureus*	99.41	Fu'Adil et al (2020)
MB24_3_1	*Glossogobius aureus*	99.41	Fu'Adil et al (2020)
MB24_2_1	*Glossogobius aureus*	99.55	Fu'Adil et al (2020)
MB23_1_1	*Glossogobius aureus*	99.85	Ndobe et al (2022)

According to Abdulmalik-Labe & Quilang (2019), it is often incorrect to morphologically distinguish *G. aureus* and *G. giuris* because of their almost identical shape and color. However, using the DNA barcoding approach, the accuracy is more reliable in identification. Based on Table 3, the individual sequences of manggabai are identified as *G. aureus* with a similarity index of 99-100%. The information clarifies two aspects. Firstly, manggabai is not an endemic fish to Lake Limboto as understood by local people because it has a high similarity with similar fish in other places. Secondly, the scientific name of manggabai in Limboto Lake commonly used by local and Indonesian people is *G. giuris*. However, a search through Genbank showed that the scientific name of the species is *G. aureus*. The existence of differences in the 8 individual samples compared to the GenBank references indicate that the COI gene sequence is sufficient to be used as a DNA barcode for manggabai fish. The genetic distance analysis was carried out for eight samples of manggabai from Lake Limboto and 8 similar fish from the GenBank, presented in Figure 3.

No		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	MG407394.1																
2	MG407393.1	0.000															
3	MW498627.1	0.000	0.000														
4	EF607394.1	0.002	0.002	0.002													
5	MG407403.1	0.002	0.002	0.002	0.003												
6	MG407395.1	0.002	0.002	0.002	0.003	0.000											
7	MW498632.1	0.002	0.002	0.002	0.003	0.003	0.003										
8	EF609360.1	0.002	0.002	0.002	0.003	0.003	0.003	0.000									
9	MB30_3_2	0.002	0.002	0.002	0.000	0.003	0.003	0.003	0.003								
10	MB30_2_2	0.002	0.002	0.002	0.000	0.003	0.003	0.003	0.003	0.000							
11	MB30_1_2	0.002	0.002	0.002	0.000	0.003	0.003	0.003	0.003	0.000	0.000						
12	MB2451	0.002	0.002	0.002	0.000	0.003	0.003	0.003	0.003	0.000	0.000	0.000					
13	MB24_4_1	0.002	0.002	0.002	0.000	0.003	0.003	0.003	0.003	0.000	0.000	0.000	0.000				
14	MB24_3_1	0.002	0.002	0.002	0.000	0.003	0.003	0.003	0.003	0.000	0.000	0.000	0.000	0.000			
15	MB24_2_1	0.002	0.002	0.002	0.000	0.003	0.003	0.003	0.003	0.000	0.000	0.000	0.000	0.000	0.000		
16	MB23_1_1	0.002	0.002	0.002	0.000	0.003	0.003	0.003	0.003	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

Figure 3. The genetic distance of Manggabai from Limboto Lake and GenBank.

According to Doğan & Doğan (2016), genetic distance is based on the analysis of gene differentiation between populations. According to Nei (1972), genetic distance is divided in the distance outside the population and the distance between populations. Figure 3 shows that the 8 manggabai fish samples present no genetic distance, meaning that they are of the same species. On the other hand, variations in the genetic distance outside the population are also present. Overall, the genetic distance outside the population has an interval between 0-0.003. According to Lante et al (2012), a smaller genetic distance in a population shows a more uniform population. Furthermore, a considerable genetic distance indicates high genetic diversity and that the stocks available in nature are in high number. This follows the condition of the manggabai fish, which has a low genetic distance in Lake Limboto, and the population continues to decline. According to Hasim et al (2021), manggabai are experiencing severe pressure due to extractive utilization. The phylogenetic tree also supports that all manggabai specimens come from one species (Figure 4).

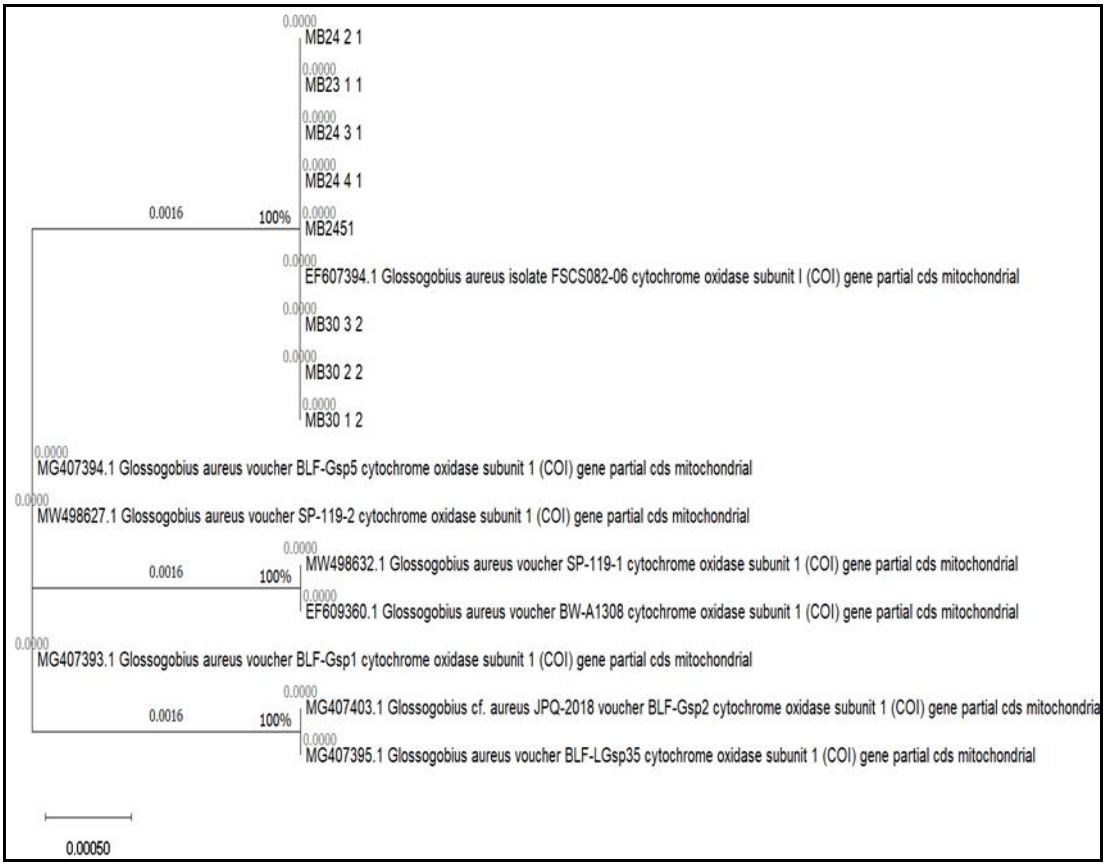

Figure 4. The phylogenetic tree of *Glossogobius aureus*.

This phylogenetic tree shows that Manggabai fish consists of three main groups. The first group consists of the eight samples of manggabai from Limboto Lake and EF607394.1 from GenBank with 100% bootstrap. Group two consisted of MW498632.1 and EF609360.1 with 100% bootstrap, and group three consisted of MG407403.1 and MG407395.1 with 100% bootstrap. The 100% bootstrap value shows that the model data set is used very well for constructing the phylogenetic tree (Dharmayanti 2011). MG407394.1, MW498627.1 and MG407393.1 are in the initial fork. This condition confirms that the genetic distance of the groups is 0.000. Abdulmalik-Labe & Quilang (2019) note that *G. giuris* and *G. aureus* are in different branches in the phylogenetic tree, meaning that they are not the same species. In this research, all belong samples belong to *G. aureus* species.

Conclusions. This study succeeded in identifying manggabai through DNA barcoding. All the manggabai specimens in this study belonged to *Glossogobius aureus* species. A similarity test with reference sequences, molecular characters, genetic distance, and phylogenetic tree sequences of individual manggabai samples and individual reference fish sequences supported the DNA barcoding results.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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