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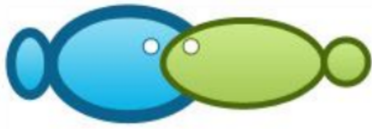

DNA barcoding of tilapia fish from Merauke, Papua and Malang, East Java-Indonesia

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Abstract. Fish identification investigation is an important component of fisheries management and aquaculture. The purpose of this study is to identify tilapia fish existing in Indonesia on the basis of nucleotide composition, polymorphic sites, haplotype grouping, nucleotide BLAST, and phylogenetic tree analyses for cytochrome c oxidase I gene. This required a DNA barcoding process, which involves the production of PCR amplicons from COI gene to generate a sequence data, which is subsequently used to ascertain and distinct the organism from other species. The tilapia fish samples were collected from Merauke, Papua and Malang, East Java, and DNA amplification results showed COI gene fragment sequences, characterized by a length of 654 base pairs. A total of three haplotypes were identified from the twelve samples, with mutases occurring at 56 points (polymorphism). The nucleotide composition, polymorphic sites, haplotype grouping, BLAST analysis, and phylogenetic tree constructed by all 12 individuals were used to categorize the samples into three species, including *Oreochromis mossambicus*, *Oreochromis niloticus* and *Oreochromis urolepis*. In addition, the genetic distance between *O. mossambicus* and both *O. niloticus* and *O. urolepis* were 0.0523 and 0.0401, respectively, while between *O. niloticus* and *O. urolepis* was 0.0592. The results indicated DNA barcodes as an effective identification approach for tilapia fish, and the results have a potential for application in aquaculture and during the management of fisheries resource in Indonesia.
Key Words: tilapia fish, *Oreochromis*, COI gene, DNA barcoding, phylogenetic.

Introduction. Tilapia is a very important aquaculture species used as a substitute for all forms of wild-caught fish, and as an aquatic “chicken”, widely accepted worldwide (Fitzsimmons et al 2011). In addition, reports have acknowledged this as the most highly domesticated farmed fish (Prabu et al 2019), with superior environmental adaptation abilities, as evidenced by the wide range of biological responses to different conditions, both in culture and in nature (Schofield et al 2011; Grammer et al 2012). Moreover, tilapia is a known group of freshwater fish widely consumed and developed in Indonesian aquaculture. This has been used in several forms at aquaculture farmlands, including the Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) and Mujair (*Oreochromis mossambicus* Peters, 1852).

Tilapia, Tilapiine fishes have a huge species diversity, and are grouped into three main genera: *Oreochromis* (arena-spawning maternal mouthbrooders), *Sarotherodon* (paternal or biparental mouthbrooders), and *Tilapia* (substrate spawners) (Trewavas 1983; Canonico et al 2005). Previous reports show extensive investigation on Tilapiine fish diversity, using both morphological methods (Trewavas 1983; Ndiwa et al 2016) and molecular markers (Arifin et al 2007; Tibihika et al 2020). These discrepancies in procedure have led to contradictory patterns in species description, and identification through morphometric as well as meristic characters have been implicated in misidentification, taxon ambiguities and fluctuation in total species number. Furthermore, the main culprits for this phenomenon include phenotypic and genotypic plasticity, cryptic

diversity or possible hidden species and variation in color pattern at different stages of life within the same species (Barman et al 2018). According to Syaifudin et al (2015), the numerous varied species and sub-species, alongside the extensive use of interspecies hybrids, prompts the need for proper identification based on DNA barcoding.

This is a potential method to identify species exhibiting a sufficient level of variation and ensures proper discrimination by a short universal DNA sequence from mitochondrial cytochrome c oxidase subunit I (COI) gene (Hebert et al 2003). According to Hebert & Gregory (2005), DNA barcoding is a system designed to provide accurate, fast and automatable species identification. Furthermore, the technology is used as a solution to speed up the pace of discovery and opens new perspectives in conservation (Tautz et al 2003). This method presents several advantages compared to the morphological character approach (Hubert et al 2015), and is extensively applied in current plant, microbes, and animal studies.

The DNA barcoding process from COI gene is widely adopted in animal species identification, including for tilapia (Syaifudin et al 2015; Sogbesan et al 2017; Iyiola et al 2018; Silva et al 2020; Ude et al 2020). This technique has been used and is proposed as useful during freshwater fish documentation in Indonesia (Hubert et al 2015). However, little attention has been paid to the potential application with tilapias from Indonesia, especially at Merauke-Papua and Malang-East Java. The purpose of this study, therefore, was to identify tilapia fish existing at Merauke and Malang-Indonesia, based on the nucleotide composition, polymorphic sites, haplotype grouping, nucleotide BLAST, and phylogenetic tree analyses of the COI gene through DNA barcoding.

Material and Method

Sample collection. Figure 1 shows the tilapia fish samples collected, while Figure 2 shows the sampling location for *Oreochromis* species from the two regions (Merauke-Papua and Malang-East Java, Indonesia). In Malang, three specimens were obtained, while nine were acquired from three different sites in Merauke. These sites were Barki village (four specimens), Bian river (three specimens), and Blorep (two specimens). Subsequently, tissue samples and pectoral fins were collected and preserved in 96% ethanol, prior to storage.

Figure 1. Sample of tilapia fish collected from Merauke, Papua (9 samples) and Malang, East java (3 samples).

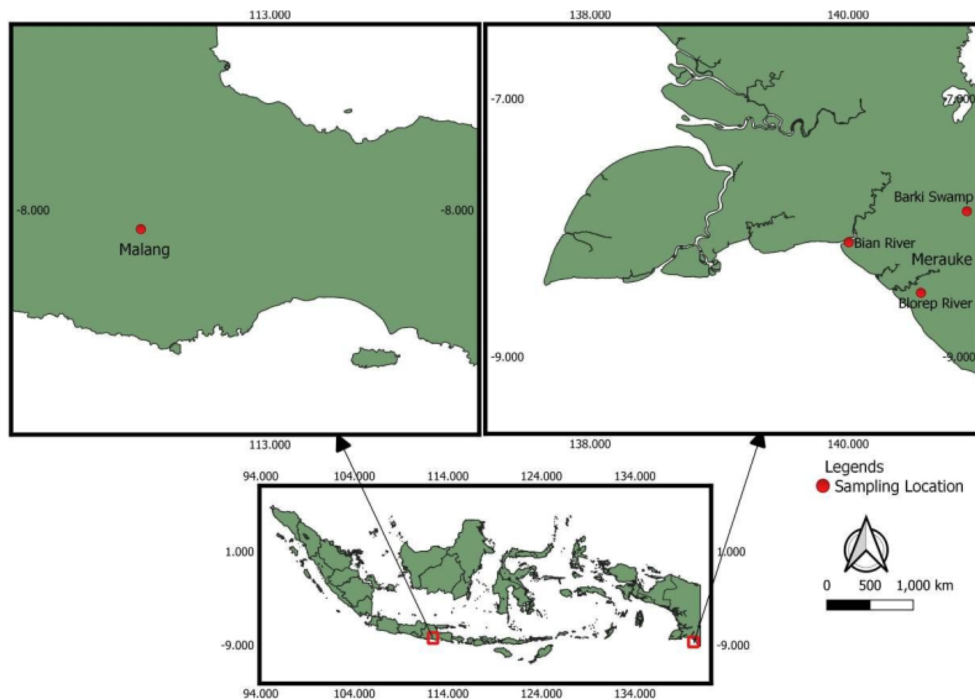

Figure 2. The sampling location of *Oreochromis* fish in Merauke, Papua and Malang, East Java Indonesia.

Genomic DNA isolation. Total genomic DNA was isolated using DNA Easy Blood and Tissue isolation kit (Qiagen), in accordance with the standard protocol from the factory. Meanwhile, approximately 20 mg of tissue samples were cut and mixed with 180 μL of ATL buffer, and 20 μL of proteinase K was added. This was then heated at 56°C until the samples dissolved, followed by the addition of 200 μL of AL buffer and 200 μL of ethanol 96%. The resulting mixture was then transferred into a mini spin column in a 1.5 mL tube and centrifuged at 8000 rpm for 1 minute. Furthermore, 500 μL of AW1 buffer was added and centrifugation was carried out for 1 minute at 8000 rpm, to wash the column. The flow-through liquid was then discarded and 500 μL of AW2 buffer was added, before centrifugation for 3 minutes at 14000 rpm. Subsequently, the spine was removed and placed in a new microtube, 200 μL of AE buffer was added. This was followed by incubation at room temperature for 1 minute and centrifugation at 8000 rpm for 1 minute. The liquid was then collected and stored in the freezer until use.

COI gene amplification. The amplification of cytochrome oxidase subunit 1 gene fragments was performed using the PCR technique. The primers used in the PCR process were Fish BCL (forward) (5'-TCA ACYAATCAYAAAGATATYGGCAC-3') and Fish BCH (reverse) (5'-ACTTCYGGGTGRCCR AARAATCA-3') as described by Baldwin et al (2009). The master mix (50 μL) used was the "Go Taq Green PCR-Mix" product from Promega, comprising ddH_2O (18 μL), forward (2.5 μL) and reverse (2.5 μL) primers, DMSO (1 μL), Go Taq Green PCR Mix 2x (25 μL) and Template DNA (2 μL). Meanwhile, the temperature profile for the technique had three main stages. These were, the first or initial stage, (80°C for 10 seconds followed by 94°C for 3 minutes), the second, carried out in up to 40 cycles with three temperatures, denaturation (94°C for 30 seconds), annealing (50°C for 30 seconds), as well as extension (72°C for 45 seconds), and the last stage, final extension (72°C for 5 minutes, followed by cooling at 37°C for 1 minute) (Dailami et al 2018; Pranata et al 2018).

DNA electrophoresis. Electrophoresis of the PCR product was carried out in 1% agarose gel in Sodium-Boric (SB) Buffer. This was created by dissolving 0.5 grams of in 50 mL of pH 8 SB-Buffer and heating with a microwave for 1 minute. The resulting solution was then poured into a comb as a gel well mold. Subsequently, 4 µL of PCR product was mixed with loading dye and added to the mold immersed in SB buffer, while 4 µL of 1 Kb Gene Ruller from Thermo scientific was used as a marker in the first line well. The Electrophoresis process was carried at 100 volts for 30 minutes and DNA staining was achieved by immersing the Gel in EtBr solution for 15-20 minutes, and rinsing with distilled water, while visualization of DNA bands was performed with a UV Transilluminator and documented using a digital camera.

Data analysis. The electropherograms and the nucleotide sequences were proofread by MEGA X software (Kumar et al 2018), and the forward and reverse sequences were aligned into one contig. Meanwhile, comparison with NCBI database was carried out using basic local search alignment (BLAST) (Morgulis et al 2008). The phylogenetic tree was reconstructed using the Neighbor-Joining method, and the genetic distances were calculated with the Kimura 2-parameter, while the phylogenetic tree was tested using bootstrap with 1000 replication, and all evolutionary analyses were conducted in MEGA X software (Kumar et al 2018).

Results and Discussion. Figure 3 shows the 12 amplicons of COI gene fragments from tilapia fish samples successfully amplified with a length of about 600-700 base pairs. These amplicons were found to have the same size as the other studies performed with the same primer (Fish-BCH and BCL) used to amplify the COI gene from *Cirrhilabrus marinda* (Allen et al 2015), *Cirrhilabrus cf ryukyuensis* (Dailami et al 2018), *Oryzias nebulosus* and *Oryzias nigrimas* (Serdiati et al 2019), and *Rhincodon typus* (Toha et al 2020).

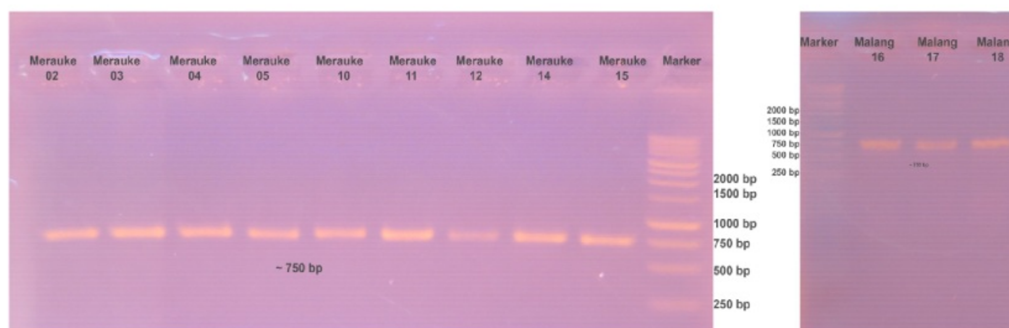

Figure 3. Tilapia fish were collected from Merauke and Malang.

In addition, all the DNA sequences of tilapia's COI gene fragments from Merauke (9 samples) and Malang (3 samples) were successfully obtained. According to the nucleotide composition presented in Table 1, the sequence's total length is 654 base pairs (bp), while samples with haplotype 1 have a different nucleotide percentage compared to the haplotype 2 and 3 equivalents. For instance, the percentage of nucleotide C differs between haplotypes 1 (29.4%) and 2(29.7%). This variation is influenced by DNA polymorphism, a phenomenon occurring in cases where each individual nucleotide is different.

Table 2 shows the 46 polymorphism points (7 transversion and 39 transition mutations) obtained from the twelve sample sequences, while Table 3 shows the consequent grouping of the sequences into three different haplotypes. Transversion mutation tends to have a higher probability of occurrence and a greater impact on amino acid changes, compared to the transition counterpart. However, in this study, transition was found to occur more frequently.

Table 4 shows the results of comparison between the DNA sequences of the obtained samples and the GenBank (NCBI) counterpart. The NCBI BLAST on this sequence resulted in high identity (mostly 100%) with the same score and query cover for several species. However, Meiklejohn et al (2019) and Pentinsaari et al (2020) both reported a possibility of incorrect identification data on the NCBI sequences caused by the *O. niloticus* and *O. mossambicus* sequence submitter.

Table 1

Nucleotide composition of 654 bp sequence of COI gene from tilapia samples

Sample	*Accession number*	*Percentage (%)*				*Total*	*Haplotypes*
		T	*C*	*A*	*G*		
Merauke02	MW206002	28.9	29.4	24.5	17.3	654	1
Merauke03	MW206003	28.9	29.4	24.5	17.3	654	1
Merauke04	MW205900	28.9	29.7	24.5	17.0	654	2
Merauke05	MW205901	28.9	29.7	24.5	17.0	654	2
Merauke10	MW206004	28.9	29.4	24.5	17.3	654	1
Merauke11	MW205902	28.9	29.7	24.5	17.0	654	2
Merauke12	MW205903	28.9	29.7	24.5	17.0	654	2
Merauke14	MW205904	28.9	29.7	24.5	17.0	654	2
Merauke15	MW206005	28.9	29.4	24.5	17.3	654	1
Malang16	MW206006	28.9	29.4	24.5	17.3	654	1
Malang17	MW206001	28.9	29.7	24.5	17.0	654	2
Malang18	MW205999	28.6	29.8	24.2	17.4	654	3
Avg.		28.9	29.5	24.4	17.1	654	-

Table 2

Polymorphic sites of COI gene of *Oreochromis* sample from Merauke and Malang

ID				*1*	*1*	*1*	*1*	*1*	*1*	***2***	***2***	*2*	*2*	*2*	*2*	*2*	*2*	*2*	*2*	*2*	*3*	***3***	*3*
	4	*4*	*9*	*0*	*0*	*6*	*7*	*8*	*9*	***0***	***0***	*4*	*4*	*5*	*6*	*6*	*7*	*8*	*8*	*9*	*0*	***1***	*1*
	2	*6*	*0*	*5*	*8*	*2*	*1*	*0*	*8*	***1***	***4***	*0*	*3*	*2*	*1*	*4*	*9*	*2*	*5*	*7*	*0*	***2***	*8*
Merauke02	T	T	C	G	T	G	A	T	G	**A**	**A**	A	T	T	C	C	C	T	C	C	G	**G**	T
Merauke03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Merauke04	.	C	T	A	C	A	G	C	A	**C**	**C**	.	.	C	T	.	T	.	T	T	A	**T**	C
Merauke05	.	C	T	A	C	A	G	C	A	**C**	**C**	.	.	C	T	.	T	.	T	T	A	**T**	C
Merauke10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Merauke11	.	C	T	A	C	A	G	C	A	**C**	**C**	.	.	C	T	.	T	.	T	T	A	**T**	C
Merauke12	.	C	T	A	C	A	G	C	A	**C**	**C**	.	.	C	T	.	T	.	T	T	A	**T**	C
Merauke14	.	C	T	A	C	A	G	C	A	**C**	**C**	.	.	C	T	.	T	.	T	T	A	**T**	C
Merauke15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Malang16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Malang17	.	C	T	A	C	A	G	C	A	**C**	**C**	.	.	C	T	.	T	.	T	T	A	**T**	C
Malang18	C	C	.	.	.	A	.	.	A	.	**T**	G	C	.	.	T	.	C	.	.	A	.	.
ID	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*4*	*4*	*4*	*4*	*4*	***4***	*5*	*5*	*5*	*5*	***5***	*5*	***5***	*5*	***6***
	2	*3*	*4*	*5*	*6*	*6*	*7*	*9*	*1*	*2*	*4*	*6*	*8*	***9***	*0*	*1*	*2*	*3*	***3***	*4*	***8***	*8*	***3***
	7	*6*	*2*	*7*	*3*	*9*	*5*	*3*	*1*	*0*	*4*	*2*	*0*	***8***	*4*	*3*	*8*	*1*	***7***	*6*	***5***	*8*	***9***
Merauke02	G	T	A	C	T	G	C	C	G	T	T	A	C	**C**	A	T	A	A	**A**	A	**C**	A	**T**
Merauke03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Merauke04	.	.	G	T	C	A	T	.	A	.	C	G	.	**A**	G	C	.	.	**T**	G	.	.	**A**
Merauke05	.	.	G	T	C	A	T	.	A	.	C	G	.	**A**	G	C	.	.	**T**	G	.	.	**A**
Merauke10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Merauke11	.	.	G	T	C	A	T	.	A	.	C	G	.	**A**	G	C	.	.	**T**	G	.	.	**A**
Merauke12	.	.	G	T	C	A	T	.	A	.	C	G	.	**A**	G	C	.	.	**T**	G	.	.	**A**
Merauke14	.	.	G	T	C	A	T	.	A	.	C	G	.	**A**	G	C	.	.	**T**	G	.	.	**A**
Merauke15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Malang16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Malang17	.	.	G	T	C	A	T	.	A	.	C	G	.	**A**	G	C	.	.	**T**	G	.	.	**A**
Malang18	A	C	.	.	C	.	T	T	.	C	C	.	T	**A**	G	.	G	G	.	G	**T**	C	.

The column number is the nucleotide position. A **bold** letter indicates the transversion.

Table 3
Haplotype grouping from 12 samples of *Oreochromis* fish collected from Merauke and Malang

Haplotype	*Sample*
Haplotype 1	Merauke 2, Merauke 3, Merauke 10, Merauke 15, Malang 16
Haplotype 2	Merauke 4, Merauke 5, Merauke 11, Merauke 12, Merauke 14, Malang 17
Haplotype 3	Malang18

Table 4
Basic local alignment search tools of all samples in 3 haplotypes

ID Samples	*NCBI Blast*	*Max score*	*Query cover*	*Identity*	*Accession number*
Haplotype 1: Merauke2, Merauke3, Merauke10, Merauke15, Malang16	*O. mossambicus*	1208	100%	100%	MG438458.1
	O. mossambicus	1208	100%	100%	MF189954.1
	O. mossambicus	1208	100%	100%	KU565862.1
	O. mossambicus	1208	100%	100%	KU565856.1
	O. mossambicus	1208	100%	100%	KU565855.1
Haplotype 2: Merauke4, Merauke5, Merauke11, Merauke12, Merauke14, Malang17	*O. niloticus*	1208	100%	100%	MT079202.1
	O. niloticus	1208	100%	100%	MG407418.1
	O. niloticus	1208	100%	100%	MG407416.1
	O. niloticus	1208	100%	100%	MG407413.1
	O. niloticus	1208	100%	100%	MG407410.1
Haplotype 3: Malang18	*O. urolepis*	1208	100%	100%	MF509598.1
	O. urolepis	1208	100%	100%	KM438540.1
	O. urolepis	1208	100%	100%	KM438539.1
	Oreochromis sp.	1208	100%	100%	HM067614.1
	O. niloticus x O. mossambicus	1208	100%	100%	DQ426668.1

According to the results of BLAST analysis in GenBank data, haplotype 1 is 100% identical with sequence of *O. mossambicus* (MG438458.1) (Panprommin et al 2019), while haplotype 3 is 100% similar with the sequence of *O. niloticus* (MT079202.1) (Yadav et al 2020), and with *O. urolepis* (MF509598.1) (Mohd Zharif et al 2017). However, this is unclear for *O. niloticus* and *O. mossambicus* in Merauke and Malang, and most people assume the two have the same morphological appearance. Interestingly, some uncommon species (*O. urolepis*) used as brood stock by farmers were found in Malang, and the creation of hybrid species from unclear brood stock species in fry production tends to result in uncertainty with regard to quality of fish.

Figure 3 shows the creation of a phylogenetic tree by adding the sequence data from GenBank for *O. niloticus* and *O. mossambicus*, using the neighbor joining tree method and a Kimura 2 parameter, as well as the differentiation of the 3 haplotypes into 3 prominent clades, strongly supported by 100% of the bootstrap value.

Table 5 shows the results obtained from the calculation of genetic distance between the clades, using the same parameter as the tree. The genetic distance between *O. mossambicus* and *O. niloticus* is 0.0523, while the distances from the two species to *O. urolepis* are 0.0401 and 0.0592, respectively. Therefore, *O. mossambicus* has a closer relationship to *O. urolepis* (0.0401) compared to *O. niloticus* (0.0523), in accordance with the phylogenetic tree. Sequence of *O. aureus* was used as the outgrup of this phylogenetic tree and it shows the most farther distance from three other species (0.0690-0.734).

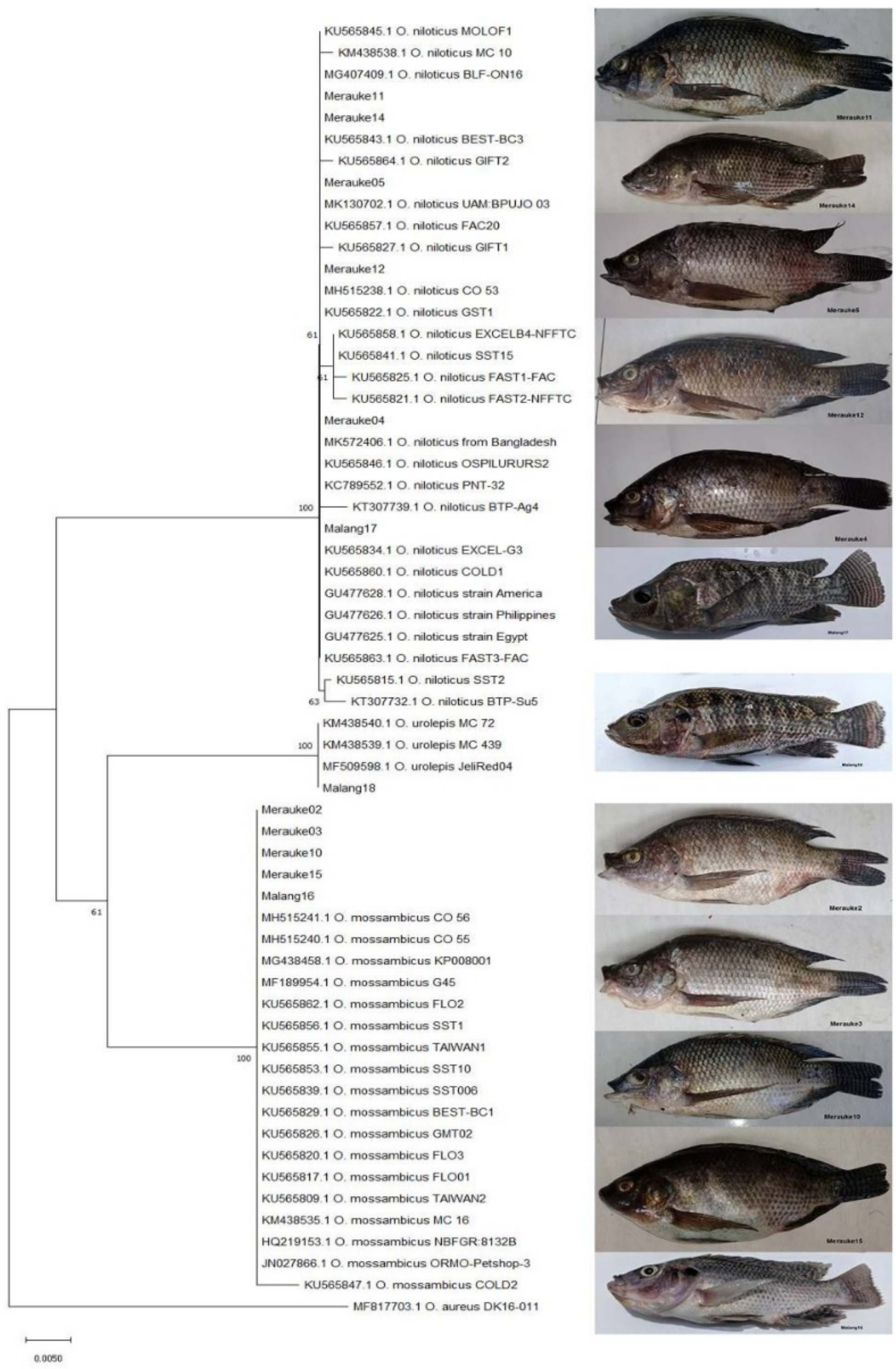

Figure 3. Phylogenetic tree of tilapia fish collected from Merauke and Malang with the data GenBank as a comparison.

Table 5

Genetic distance between clades

Clade	*1*	*2*	*3*	*Outgroup*
1 (*O. niloticus*)				
2 (*O. urolepis*)	0.0592			
3 (*O. mossambicus*)	0.0523	0.0401		
Outgrup (*O. aureus*)	0.0734	0.0691	00690	

Conclusions. This study successfully identified the samples from Merauke and Malang through the DNA barcoding approach. The three species found were *Oreochromis nilotius*, *Oreochromis mossambicus,* and *Oreochromis urolepis*. Meanwhile, Merauke residents had mistaken the samples for Mujaer or *O. mossambicus*, and the fish vendor in Malang had identified his wares as Nila or *O. niloticus*. Also, a total of three haplotypes were found in all the specimens, based on the 56 polymorphic sites caused by mutation, and the tilapia collected from Merauke exhibited low genetic variety.

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