

Morphological variations and phylogenetic analysis of *Oryzias nigrimas* Kottelat, 1990 (Rice fish) from Lake Poso, Central Sulawesi, Indonesia

NOVALINA SERDIATI¹✉, DIANA ARFIATI², MAHENO SRI WIDODO², TRI JOKO LELONO²,
SAMLIOK NDOBE¹, RUDY SARANGA³

¹Faculty of Animal Husbandry and Fisheries, Universitas Tadulako. Jl. Soekarno-Hatta Km. 9, Tondo, Mantikulore, Palu 94148, Central Sulawesi, Indonesia. Tel.: +62-451-422611, Fax.: +62-451-422-844, ✉email: novalinaserdiati@untad.ac.id

²Faculty of Fisheries and Marine Science, Universitas Brawijaya. Jl. Veteran No.16, Malang 65145, East Java, Indonesia

³Department of Fishing Technique, Politeknik Kelautan dan Perikanan Bitung. Jl. Tandurusa, Bitung 95526, North Sulawesi, Indonesia

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Abstract. Serdiati N, Arfiati D, Widodo MS, Lelono TJ, Ndobe S, Saranga R. 2020. Morphological variations and phylogenetic analysis of *Oryzias nigrimas* Kottelat, 1990 (Rice fish) from Lake Poso, Central Sulawesi, Indonesia. *Biodiversitas* 21: 882-888. Rice fish (*Oryzias nigrimas* Kottelat, 1990) is a well-studied species endemic to Lake Poso, Sulawesi and identified as scarce. This study aims to identify the Rice fish of Lake Poso, Central Sulawesi based on morphological characters (15 morphometric characters and 5 meristic counts) and phylogenetic analysis using maximum-likelihood (ML) method based on mtDNA, COI gene. Based on the morphological analysis, it was showed that the meristic counts show significantly different only on the lateral line scale. However, the morphometric measurements show significantly different characteristics on postorbital distance, body depth, and length of jaw. Our phylogenetic analysis also showed that our samples are indeed a species of *O. nigrimas*. We suggest that the endemism of this Rice fish on Lake Poso is greatly influenced by the well-maintained genetic structure of the population.

Keywords: Indonesia, Lake Poso, morphological variations, *Oryzias nigrimas*, phylogenetic analysis

INTRODUCTION

Rice fishes are an important freshwater fish group, are significant species that were used as a model organism and well-studied species (Parenti 2008; Lin et al. 2016; Shima and Matani 2004; Wawrowski et al. 2011). Rice fishes belong to the Adrianichthyidae family are distributed and considered native to Southeast Asia, Japan, India and as far as Timor (Magtoon and Termvidchakorn, 2009). There are about 36 valid species, and some are considered rare and few of those were predicted to be extinct in the future (Mandagi et al. 2018). Recently, two genera of Rice fishes were distinguished by the genera of *Oryzias* and *Adrianichthys* (Mandagi et al. 2018). Some species of Rice fish especially the genus *Oryzias* are known to be endemic to Indonesia (Inoue and Takei 2003). A recent study showed that *Oryzias* fishes are divided into three species groups which are the latipes, javanicus, and the celebensis group (Takehana et al. 2005) which contain one of the endemic species in Celebensis (Sulawesi) i.e *Oryzias nigrimas* Kottelat, 1990 found on Lake Poso, Central Sulawesi of Indonesia.

Information on how the *O. nigrimas* end up in Lake Poso, and whether the speciation process takes place by the distinction of a population group are yet to be confirmed and despite the widely used of this species, their phylogenetic relationship of these genera is also poorly understood (Takehana et al. 2005). There are two techniques was officially used to explain the speciation process and to reconstruct the phylogenetic tree namely

morphological approach and molecular approach. The morphological characteristics of fish such as body shape, color pattern and number of scales can be used as preliminary methods to distinguish the species (Ikpeme et al. 2017). However, these methods are difficult and considered subjective to sever the species among the same genus. Therefore, the molecular approach is recently used to strengthen morphological approach by using DNA barcoding.

Molecular approach in fish was developed based on its rapid and accurate to identify the species using universal primers. Many fishes were identified and characterized by using this molecular approach including genus of *Labiobarbus* (Panprommin et al. 2019) and Taiwan Fish (Bingpeng et al. 2018). Furthermore, molecular approach also has been used to determine biodiversity in several natural waters. Mitochondrial DNA that generally used as DNA barcoding is cytochrome subunit I (COI). COI gene is a highly conserved region that has been used across all levels of an organism to identify species, differing by only a few sequences (variable sites) (Folmer et al. 1994). COI sequences can be used as primary barcodes to classify and identify both freshwater fish and marine fish (Viswambharan et al. 2015; Bingpeng et al. 2018).

The used of both morphological characteristics and phylogenetic analysis are a synergistic combination to determine the identity of these freshwater fish by the comparison between phylogeny trees and morphometric and meristic characters (Kim et al. 2010; Marzouk et al. 2016). Hence, in this study, we assess the morphometric

and meristic characters of *O. nigrimas* from several sites in Lake Poso, Central Sulawesi, Indonesia, as well as molecular character of cytochrome subunit I (COI) for phylogenetic reconstruction. We also discuss the reliability of our morphological methods that are used for analyzing the *O. nigrimas* and compare our genetic data to the available DNA sequence.

MATERIALS AND METHODS

Field location and samples collecting

Sampling was carried out in the 3 sites, namely Watudilana, Tolambo, Taipa of Lake Poso, Central Sulawesi, Indonesia. As much as 15 samples of *O. nigrimas* (Rice Fish) were collected from each sampling site and preserved for morphological analysis and DNA extraction purposes. The preferred sites were marked its coordinates using GPS and projected as shown in Figure 1.

Morphometrics and meristic analysis

As much as 15 samples in each population sites were measured using morphological analysis. The morphological analysis consists of 15 morphometric measurements and 5 meristic counts following to Holden and Raitt (1974). Morphometric characters include characteristics as follows: Standard length (SL), total length (TL), head length (HL), Preorbital Distance (PRD), Eye diameter (ED), Post orbital Distance (POD), Predorsal distance (PD), pre-pelvic distance (PPED), pre-pectoral distance (PPD), pectoral fin length (PFL), Body depth (BD), dorsal fin length (DFL), Anal fin length (AFL), length of jaw (PRg), and Tail stem height (Figure 2). Meristic characters include the number of scales along the lateral line, the number of dorsal, ventral, pectoral and anal fin fingers. Data were analyzed using Oneway ANOVA and further tested using Duncan Test on SPSS 13 for Windows.

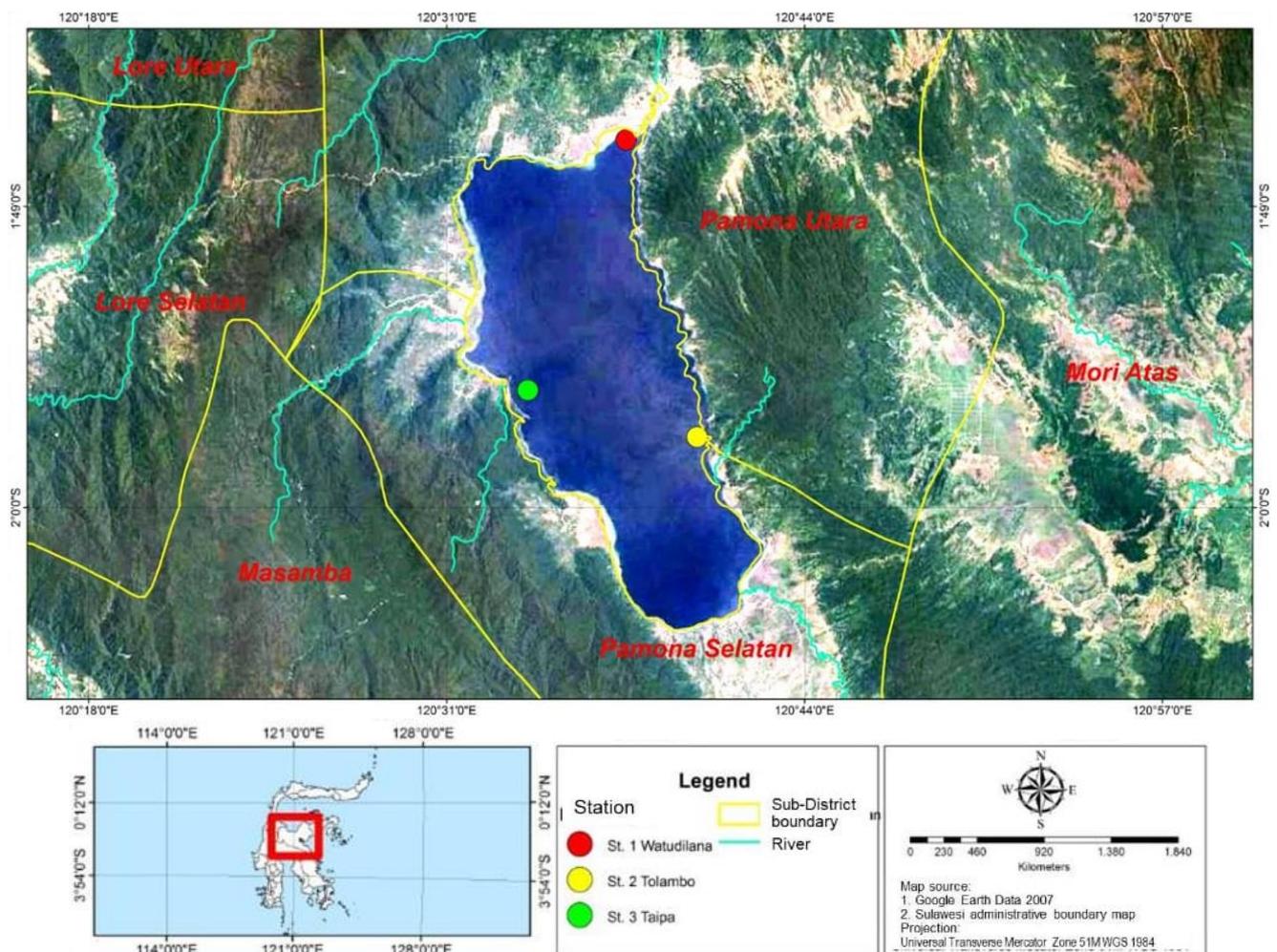


Figure 1. Sample collection of Rice fish (*Oryzias nigrimas*) location on Lake Poso, Central Sulawesi, Indonesia, the colored dots indicated the specific sampling sites (Notes: red: Watudilana, yellow: Tolambo, green: Taipa, yellow line: district boundaries, blue line: river flows)

DNA extraction, amplification, and sequencing

The total genomic DNA was extracted from part of the caudal fin from each of fish. DNA isolation was carried out using the GENE AID kit (Tissomic DNA Mini.). The DNA Isolation steps were following its manufacturer protocol for animal tissue. DNA Amplification was performed with PCR Thermocycler (Bio-Rad) using two COI primers. We amplified and sequenced the mitochondrial DNA specifically the COI gene using following primers: BCL: 5'-TCA ACY AAT CAY AAA GAT ATY GGC AC-3' and Fish BCH: 5'-ACT TCY GGG TGR CCR AAR AAT CA-3'. PCR reaction was carried out in total volume 50 μ l consisting of 2 μ l DNA (100 ng/ μ l), 2.5 μ l; Forward Primer (10 μ M); 2.5 μ l Reverse Primer (10 μ M), 18 μ l ddH₂O and 25 μ l Go Taq Green (Promega). the amplifications were carried out under the following condition: 1 cycle of pre-denaturation at 94 °C for 5 minutes, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds, extension at 72 °C for 45 seconds, subsequently followed by a final extension at 72 °C for 7

minutes. The final samples were sequenced using the BigDye® Terminator v3.1 (Applied Biosystem) First base Malaysia.

Sequence alignment and phylogenetic analysis

The sequenced of the mtDNA gene COI (ab1 format) were analyzed using Sequence Scanner (Applied Biosystem) and converted into fasta format using CLUSTAL X. The sequences were aligned in MEGA X version (Kumar et al. 2018) and Bioedit (Hall, 1999) software, with additional sequences from NCBI (Table 1). Genetic distance was analyzed by pair-distance using MEGA X (Kumar et al. 2018). The phylogenetic relationships were analyzed by the maximum-likelihood (ML) method using MEGA X (Kumar et al. 2018). The analyses were performed based on the Kimura-2 parameter distance (Kimura, 1980). To evaluate the robustness of internal branches of the trees, 1000 bootstrap replication was executed, using the heuristic algorithm.

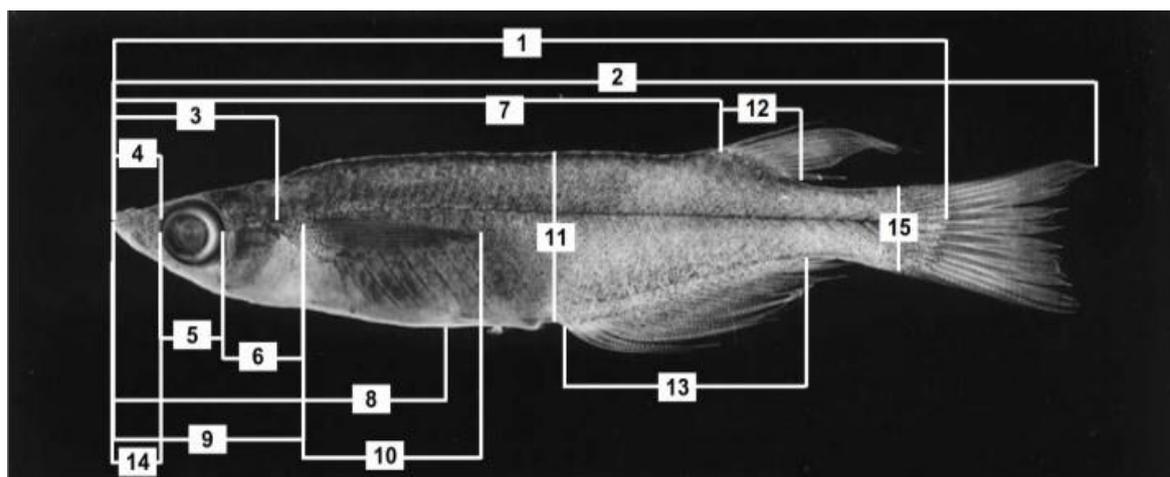


Figure 2. A total of 15 morphometrical characters of *Oryzias nigrimas* that are measured, notation and abbreviations as follows: 1. Standard length (SL), 2. total length (TL), 3. head length (HL), 4. Preorbital Distance (PRD), 5. Eye diameter (ED) 6. Post orbital Distance (POD), 7. Predorsal distance (PD), 8. pre-pelvic distance (PPED), 9. pre-pectoral distance (PPD), 10. pectoral fin length (PFL), 11. Body depth (BD), 12. dorsal fin length (DFL), 13. Anal fin length (AFL), 14. length of jaw (PRg), and 15. Tail stem height.

Table 1. List of Rice Fish *Oryzias* spp. used in this study.

Samples	Locality	Accession	Sources
<i>O. nigrimas</i> 1	Tolambo, Lake Poso, Sulawesi	-	This study
<i>O. nigrimas</i> 2	Watudilana, Lake Poso, Sulawesi	-	This study
<i>O. nigrimas</i> 3	Taipa, Lake Poso, Sulawesi	-	This study
<i>O. nigrimas</i>	Aichi, Higashiyama Zoo, Japan	LC153106.1	Direct submission
<i>O. nebulosus</i>	Aichi, Higashiyama Zoo, Japan	LC153098	Direct submission
<i>Oryzias</i> sp.	Aichi, Higashiyama Zoo, Japan	LC153107.1	Direct submission
<i>O. marmoratus</i>	Aichi, Higashiyama Zoo, Japan	LC154797.1	Direct submission
<i>O. sarasinorum</i>	Aichi, Higashiyama Zoo, Japan	LC154798.1	Direct submission
<i>O. matanensis</i>	Aichi, Higashiyama Zoo, Japan	LC153099.1	Direct submission
<i>O. celebensis</i>	Sulawesi and Timor, Japan	JX311942.1	Parenti et al. 2013
<i>O. woworae</i>	Muna Island, Sulawesi, Japan	JX311940.1	Parenti et al. 2013
<i>O. asinua</i>	Asinua river, Sulawesi, Japan	JX311929.1	Parenti et al. 2013

RESULTS AND DISCUSSION

Meristic count and morphometric measurement analysis of *Oryzias nigrimas* (Rice fish) from Lake Poso, Central Sulawesi

The results of the meristics count (Table 2) and morphometrics measurement (Table 3) are shown below. Our analysis showed that meristics counts on the number of dorsal fin ray, ventral fins ray, pectoral fin ray, and anal fin ray are not significantly different to make submission that the species were morphologically different. However, the lateral line scale is significantly different, between the *O. nigrimas* (Rice fish) from Tolambo and Watudilana population. Subsequently, our analyses on morphometric measurement did not show any significant differences in characters except postorbital distance, body depth, and length of jaw. The difference on lateral line scale counts suggests that this character may be influenced by environmental conditions and also both physical and composition of these water (Quilang et al. 2007; Wark et al. 2010).

Measurement of morphometric and meristic characteristics can be used to determine the taxonomic and systematics of fish (Simon et al. 2010). Meristic counts are based on more primitive characters than morphometric measurements and would, therefore, provide stronger

evidence for speciation. In addition, species status would only be accepted if there was no overlap in the range of at least one of the investigated meristic characters or morphometric ratios (Fakunmoju et al. 2014). Environmental changes greatly affect the freshwater fish adaptation, that manifested on the changes of morphological features (Braich and Akhter 2015). No significantly different in morphological characteristics of *O. Nigrimas* other than caused by the similarity habitat or environmental factors may also be associated with pronounced differences in resource acquisition and predator escape (Seebacher et al. 2016).

Genetic distance and Phylogenetic analysis of *O. nigrimas* (Rice fish) from Lake Poso, Central Sulawesi

Based on our genetic distance analyses of 651 bp of CO1 mtDNA genes of *O. nigrimas* (Rice fish) from 3 different sampling sites in Lake Poso, Central Sulawesi, it was shown that our samples have low genetic distance (at a value of 0.005) to the *O. nigrimas* (Table 4). It was indicated that the *O. nigrimas* from 3 different sampling sites (Tolambo, Watudilana, Taipa) from Lake Poso, are identically similar to *O. nigrimas*. It was considered that there was no genetic isolation occur and genetic structure are well maintained to form the single species (Braich and Akhter, 2015) in Lake Poso.

Table 2. Meristic count of Rice fish *Oryzias nigrimas* from several sites in Lake Poso, Central Sulawesi (Mean±SD).

Meristic count	Watudilana	Tolambo	Taipa
Lateral line scale	19.92 ^b ± 2.11 (17-23)	18.17 ^a ± 1.27 (16-21)	18.83 ^{ab} ± 1.27 (17-21)
Dorsal Fin ray	8.50 ^a ± 1.24 (7-11)	8.42 ^a ± 1.31 (7-11)	8.92 ^a ± 1.24 (7-11)
Ventral fin ray	5.08 ^a ± 0.90 (4-6)	4.83 ^a ± 1.03 (4-6)	4.92 ^a ± 1.00 (4-6)
Pectoral fin ray	14.33 ^a ± 1.78 (12-17)	14.33 ^a ± 1.67 (12-17)	14.17 ^a ± 1.64 (12-16)
Anal fin ray	23.17 ^a ± 1.40 (22-25)	22.75 ^a ± 0.87 (22-24)	22.75 ^a ± 0.62 (22-23)

Note: Each variable using a 5% significance with the same superscript letter is not significantly different.

Table 3. Measurement of 15 morphometric characters of Rice fish *Oryzias nigrimas* from several sites in Lake Poso, Central Sulawesi (Mean±SD)

Morphometric character	Watudilana	Tolambo	Taipa
Total Length	51.36 ^a ± 3.45	51.03 ^a ± 3.00	52.51 ^a ± 2.14
Standard Length	44.30 ^a ± 4.88	40.96 ^a ± 3.70	43.93 ^a ± 4.04
Head length	11.80 ^a ± 1.72	12.17 ^a ± 0.98	12.31 ^a ± 0.97
Preorbital Distance	2.93 ^a ± 0.45	2.96 ^a ± 0.47	2.77 ^a ± 0.41
Eye diameter	3.88 ^a ± 0.71	3.83 ^a ± 0.67	4.15 ^a ± 0.58
Post orbital distance	3.03 ^{ab} ± 0.57	2.79 ^a ± 0.59	3.37 ^b ± 0.47
Predorsal distance	32.75 ^a ± 5.15	30.24 ^a ± 4.07	32.61 ^a ± 3.46
Pre-pelvic distance	18.35 ^a ± 4.55	17.24 ^a ± 3.38	19.27 ^a ± 2.38
Pre-pectoral distance	12.59 ^a ± 1.75	13.19 ^a ± 0.92	13.25 ^a ± 0.75
Pectoral fin length	3.25 ^a ± 0.53	3.22 ^a ± 0.80	3.35 ^a ± 0.67
Body depth	4.98 ^{ab} ± 0.97	4.36 ^a ± 1.00	5.25 ^b ± 1.04
Dorsal fin length	13.38 ^a ± 1.26	13.61 ^a ± 1.31	13.87 ^a ± 1.16
Anal fin length	5.63 ^a ± 1.15	5.46 ^a ± 1.18	6.17 ^a ± 1.56
Length of jaw	8.21 ^b ± 1.78	6.64 ^a ± 1.30	7.79 ^{ab} ± 1.15
Height of tail stem	3.37 ^a ± 0.80	3.45 ^a ± 0.54	3.24 ^a ± 0.51

Note: Each variable using a 5% significance with the same superscript letter is not significantly different.

This condition is will threaten the sustainability of *O. nigrimas* in the future since genetic diversity is the only way to predict whether *O. nigrimas* can adapt to future conditions. On the other hand, reduced genetic diversity may cause decreased population viability and increased extinction, especially for populations faced with stressful environmental conditions (Martinez et al. 2018). The lower genetic diversity of freshwater fish relative to marine fish also reported by DeWoody and Avise (2000) and Martinet et al (2018). Large genetic diversity of marine species suggests that marine fishes may inhabit more productive environments that allow for higher carrying capacities. In addition, larger population sizes also suggest that marine environments may be more stable and thus may be less sensitive to or experience smaller amounts of genetic drift (April et al. 2012).

Our phylogenetic analysis based on successfully amplified CO1 genes (651 base pairs) also showed *O. nigrimas* (Rice fish) from 3 different sampling sites in Lake

Poso, Central Sulawesi are nested within the same clade of another *O. nigrimas* (Figure 3) with a well-supported bootstrap on the nodes (maximum-likelihood bootstrap value: 94). The bootstrap value indicates the consistency of data, manifested in percentages. Low values mean that the alignment gives a different tree topology on each sampling. The considered well- supported bootstrap value for maximum likelihood analysis is at >75% (Yang, 1994). However, based on the observations of our alignment, it was shown that there were variations in several sites, yet inadequate to differentiate the samples. According to Kres and Erickson (2012), the CO1 gene has the capability to distinguish the taxa up to species level because of the highly conserved and variations of the region. These variations of nucleotides can be used as a character that distinguishes the species. The high variation of nucleotides among a sequence of samples is an effective tool to identify Rice fish species (Zhang and Hanner 2012).

Table 4. Genetic distances of *Oryzias nigrimas* from Lake Poso, Central Sulawesi and additional sample *Oryzias* spp. from the Gene bank database inferred by p-distance.

	1	2	3	4	5	6	7	8	9	10	11	12
<i>O. nigrimas</i> _Tolambo	0.000											
<i>O. nigrimas</i> 2_Watudilana	0.000	0.000										
<i>O. nigrimas</i> 3_Taipa	0.000	0.000	0.000									
<i>O. nigrimas</i> _Japan	0.005	0.005	0.005	0.000								
<i>O. nebulosus</i> _Japan	0.027	0.027	0.027	0.025	0.000							
<i>Oryzias</i> sp._Japan	0.063	0.063	0.063	0.065	0.073	0.000						
<i>O. marmoratus</i> _Japan	0.103	0.103	0.103	0.105	0.107	0.107	0.000					
<i>O. sarasinorum</i> _Japan	0.113	0.113	0.113	0.107	0.105	0.122	0.112	0.000				
<i>O. matanensis</i> _Japan	0.125	0.125	0.125	0.123	0.121	0.112	0.072	0.107	0.000			
<i>O. celebensis</i> _Sulawesi	0.129	0.129	0.129	0.125	0.121	0.127	0.140	0.118	0.144	0.000		
<i>O. woworae</i> _Muna	0.127	0.127	0.127	0.125	0.133	0.131	0.129	0.122	0.132	0.137	0.000	
<i>O. asinua</i> _Sulawesi	0.127	0.127	0.127	0.125	0.133	0.128	0.134	0.125	0.140	0.137	0.009	0.000

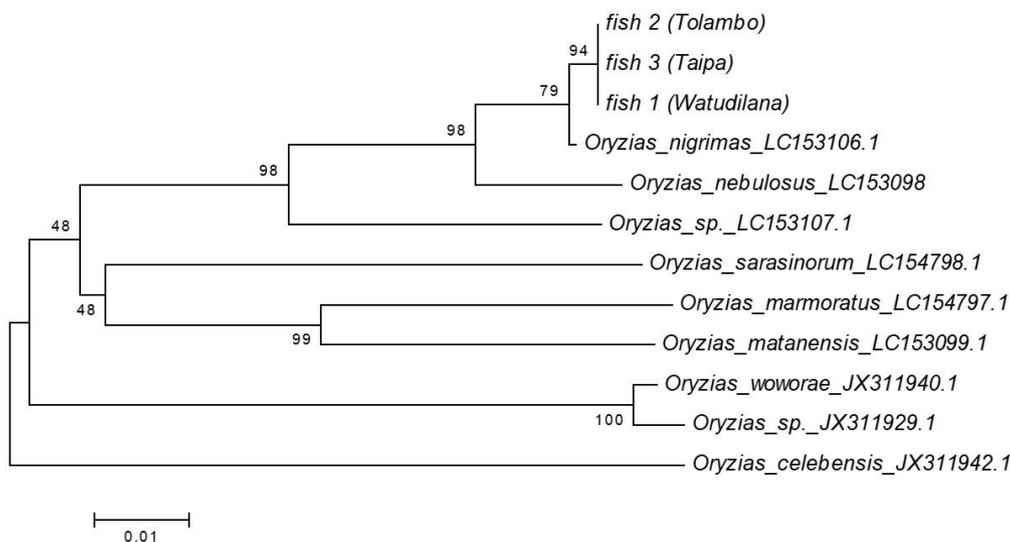


Figure 3. Phylogenetic trees of *Oryzias nigrimas* (Rice fish) from Lake Poso, Central Sulawesi based on maximum-likelihood (ML) methods

Further, a polymorphism that occurs in DNA sequences of the CO1 gene may also be used as tools in distinguishing and identify the species in a population (Liu et al. 2017; Wang et al. 2011). The presence of polymorphism in Rice fish serves as a marker for the other Rice fish of Lake Poso other populations. The endemicity of this Rice fish will greatly be influenced by well-maintained population, whether it was reduced or lost due to natural or external causes

To conclude, our morphological analysis showed that the meristic counts show significantly different only on the lateral line scale. However, the morphometric measurements show significantly different characteristics on postorbital distance, body depth, and length of jaw. Our phylogenetic analysis also resolved that the samples that were analyzed are indeed an endemic species of *O. nigrimas*. We suggest that the endemicity of this Rice fish on Lake Poso is greatly influenced by the well-maintained genetic structure of the population. Future studies examining the genetic diversity in *O. nigrimas* should examine using alternative markers. Application of next-generation sequencing may help to inquire into whether patterns of genome-wide genetic diversity in *O. nigrimas* show similar relationships with our study. Finally, we expect there are future studies that pay attention to well-resolved phylogenies and thereby remove additional noise from the analysis.

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