

Distribution and phylogeny of rice fish and their relatives belonging to the suborder Adrianichthyoidei in Sulawesi, Indonesia

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Introduction

The suborder Adrianichthyoidei consists of three families, Oryziidae, Adrianichthyidae and Horaichthyidae. Family Oryziidae is widely distributed from India to the Far East. Horaichthyidae is found on the west coast of India. Adrianichthyidae is found only in lakes Poso and Lindu in Central Sulawesi, Indonesia. Rice fish and their relatives have been reported in Sulawesi by Weber and de Beaufort (1922) and Aurich (1935). They reported three species of the genus *Oryzias* (*O. matanensis*, *O. marmoratus* and *O. celebensis*) and three species of adrianichthyids belonging to the genera *Adrianichthys* and *Xenopoecilus* (*A. kuryti*, *X. saracinorum* and *X. poptae*). Recently, Kottelat (1990a,b) found three new species of the rice fish (*O. nigrimas*, *O. orthognathus* and *O. profundicola*), and one species of adrianichthyid, *X. oophorus* in Sulawesi. Altogether 10 out of the 19 species of the suborder Adrianichthyoidei are found in Sulawesi. These fish are very important for the study of phylogenetic relationships and

species differentiation in the suborder Adrianichthyoidei that is endemic to Asia.

The genus *Oryzias* is divided into three groups (the monoarmed, the biarmed and the fused chromosome groups) mainly on the basis of the cytogenetic data (Uwa, 1986). The rice fish in Sulawesi are members of the fused chromosome group. Field surveys investigating the existence of adrianichthyids in Sulawesi were carried out at several lakes and rivers along the Trans Sulawesi Highway from Manado to Ujung Pandang in 1991 and 1992. We were able to collect live and fixed specimens and the DNA of six species of the genus *Oryzias* and one species of the genus *Xenopoecilus*. In this paper, we describe the distribution of adrianichthyoids in Sulawesi and the phylogenetic relationships among them.

Materials and Methods

Collection of fish

Table 1 shows a list of samples, scientific names, nearest towns for sampling, habitat and sampling

Table 1. List of species, nearest towns, habitats and methods for sampling in the field surveys.

Species	Nearest Town	Habitat	Sampling Method
<i>O. nigrimas</i>	Tentena (Poso)	lake, shallow area	small scoop net
<i>X. oophorus</i>	Tentena (Poso)	lake, surface region	light fishing
<i>O. marmoratus</i>	Timampu (Towuti)	lake, shallow area	small scoop net
<i>O. profundicola</i>	Timampu (Towuti)	lake, pebble bottom area	scoop net
<i>O. matanensis</i>	Soroako (Matano)	lake, shallow area	small scoop net
<i>O. celebensis</i>	Malino (Malino)		
	Enrekang (Buru)	river and lake	small scoop net
<i>O. javanicus</i> (?)	Jenepono (Tammanroya)	river and salt field	small scoop net
<i>O. javanicus</i>	Tangerang, Jave	irrigation canal	small scoop net

Names of rivers and lakes from which we collected the samples are shown in parenthesis.

methods in the field surveys. These fish with the exception of *X. oophorus* are maintained at Shinshu University, Niigata University, University of Tokyo and Aichi University of Education.

DNA isolation

The total DNA of each fish was isolated using the method described by Schartl (1990). Briefly, the tissues of liver, gonad and muscle were homogenized with about 0.5 ml of lysis buffer [0.2M NaCl, 0.5 M EDTA pH 8.0 + 1 drop/ml of proteinase K solution (10 mg/ml)] using a plastic pestle. DNA samples can be kept for more than one month in this lysis solution. In the laboratory, the samples were diluted with two volumes of distilled water in a 1.5 ml sampling tube. DNA was extracted with phenol-chloroform and precipitated with ethanol. Each DNA sample was dissolved in 10 mM Tris, 1 mM EDTA pH 8.0.

Amplification of the mitochondria cytochrome b gene and sequencing

A region of about 500 bp from the mitochondria cytochrome b gene was amplified by the polymerase chain reaction (PCR) (Innis *et al.*, 1990) in program temperature control system PC-700 (Astec). The amplification of the mitochondria cytochrome b gene was performed in 100 µl of 50 mM KCl, 10 mM Tris HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM MgCl₂, 0.1 mM each of dNTPs, 10 pmol each of primers, DNA template (0.1–1 µg) and 1 unit Taq DNA polymerase (Promega). The Cy-bF1 primer (5'-aggcctgtggcttgaaaaacca-3') and the Cy-bR2 primer [5'-caatgaattgagggggatttcagt-3'] were used for amplification. The temperature regimen for 30 or 40 cycles was 1 minute at 95°C for denaturation, 1 minute at 55°C or 45°C for annealing and 1 minute at 70°C for elongation.

Sequencing of the amplified products was performed using SequiTherm Cycle sequencing Kit (Epicentre Technologies). The Cy-bR2 primer, the Cy-bIF primer [5'-gtggcccatatctgccgaga-3'] and the Cy-bIR primer [5'-tataagtatgagccgtagta-3'] were also used for sequencing.

Comparison of the sequences and construction of a phylogenetic tree

Multiple alignment of sequences was done using ODN, a program package for molecular evolutionary analysis and database search of DNA and amino acid sequence installed in a nixp UNIX system [DNA data bank of Japan (DDBJ), National Institute of Genetics] (Ina, 1991). MEGA

(Molecular Evolutionary Genetic Analysis Version 1) (Kumar *et al.*, 1993) was used for the construction of a phylogenetic tree. The cytochrome b sequence of *Micropoiterus salmoides* (Whitmore *et al.*, 1993) was used as an outer group to construct the phylogenetic tree.

Results and Discussion

Distribution of adrianichthyids in Sulawesi

Fig. 1 shows the collection sites of adrianichthyids in Sulawesi. None of adrianichthyids were found in North Sulawesi but they were found in Central and South Sulawesi. Fish of the genus *Oryzias* are common in ditches, ponds and paddy fields in Asia. By contrast, fish of *Oryzias* in Sulawesi live in lakes and each species is endemic to one lake with the exception of *O. celebensis*. *Adrianichthys* and *Xenopoecilus* are found only in lakes in Central Sulawesi. These findings suggest that the speciation of fish of the genus *Oryzias* in Sulawesi seems to occur mainly in lakes.

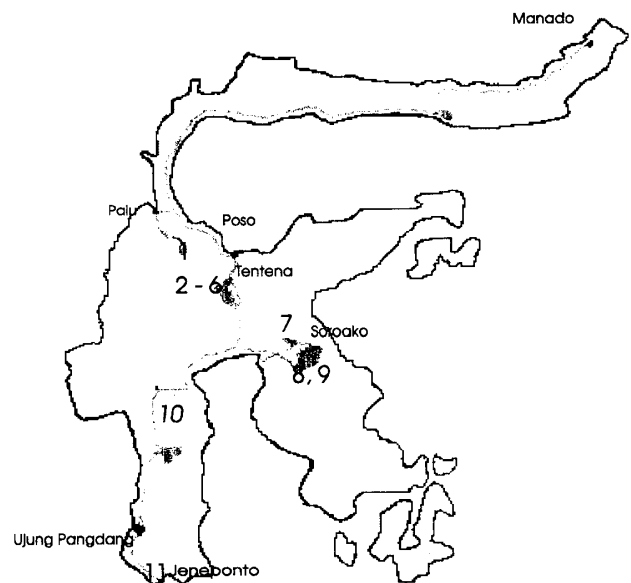


Fig. 1. Collection sites of the suborder Adrianichthyoidei in Sulawesi. 1: *X. saracinorum*, 2: *X. oophorus*, 3: *X. poptae*, 4: *A. kruti*, 5: *O. nigrimas*, 6: *O. orthognathus*, 7: *O. matanensis*, 8: *O. marmoratus*, 9: *O. profundicola*, 10: *O. celebensis*, 11: *O. javanicus*(?).

We found one species closely related to but not definitely identifiable as *O. javanicus* from the Tammanroya river system, close to Jenebonto (see Fig. 1). *O. javanicus* is widely distributed over the Malaysian Peninsula, Borneo, Java and Lombok (Weber and de Beaufort, 1922; Iwamatsu *et al.*, 1982). If this species is truly *O. javanicus*, this will be the first recorded finding of *O. javanicus* in Sulawesi. Whitten *et al.* (1987) reported that no

official lists have been compiled by the Fisheries Office for the last 30 years in Sulawesi, and suggested that *A. kuryti* and *X. poptae* might be extinct or critically endangered in Lake Poso. Kottelat (1990a) also reported that he could not find *A. kuryti* and *X. poptae* in Lake Poso. Thus, the survey of these fish was essential not only for the basic study of them, but also for the conservation of these endangered species. Gill nets of three different mesh sizes, cast nets, bottom traps, angling, and light fishing were all used for the collection of fish. In spite of every effort, these fish, especially *A. kuryti* and *X. poptae*, could not be found. We could observe only one specimen of *X. poptae* which was collected by Mr. Sigilipu in 1990. These results indicate that *A. kuryti* and *X. poptae* may be endangered. The limnological conditions of Lake Poso were as good as the oligotrophic lakes in tropical areas and there is no anthropogenic pollution (Okino *et al.*, 1992). Therefore, the most probable threat to these fish is competition between endemic and introduced fish.

Phylogenetic relationship of the genus Oryzias and relative

Fig. 2 shows the DNA sequence of the cytochrome b gene in fish of *Oryzias* and *Xenopoeilus*. Genetic distance was calculated by the Tamura and Nei's model (Tamura and Nei, 1993). As shown in Table 2, the d values within *Oryzias* in Sulawesi (0.025–0.143) are smaller than those between *Oryzias* in Sulawesi and *X. oophorus* (0.248–0.258) and those between *O. latipes* and *Oryzias* in Sulawesi (0.246–0.278). Genetic distances are almost the same between the genus *Oryzias* in Sulawesi and *O. latipes* and between the genus *Oryzias* in Sulawesi and *X. oophorus*. The d value between *Micropterus salmoides* (outer group) and the other species examined are 0.256–0.281. This value is similar to the d value within adrianichthyids. To clarify the phylogenetic relationship within adrianichthyids, it is necessary to obtain the sequence data of the more conservative molecules like the ribosomal RNA gene. The result also indicates that there are very large genetic differences within the genus *Oryzias*.

Fig. 3 shows the phylogenetic tree deduced from cytochrome b sequence using the neighbor-joining method (Saitou and Nei, 1987). Tree topology using the UPGMA method (data not shown) is the same as that obtained from the neighbor-joining method. Bootstrapping values of each branch within the genus *Oryzias* in Sulawesi

<i>O. nigrinus</i>	ATG GCA AAC ATT CGA AAA ACC CAT CCG CTT CTT AAA ATC GCA AAC GAT
<i>O. profundicola</i>T.....C.....A.....A.....
<i>O. celebensis</i>G.....C.....T.....C.....
<i>O. matanensis</i>A.....A.....
<i>O. marmoratus</i>T.....A.....C.....
<i>O. latipes</i>	...C...C... ..C...G.T.A...T... ..
<i>X. oophorus</i>	...C..T... ..C...A...T.A...T...T..C
<i>M. salmoides</i>G.C.C.....C...T.A.A.....C
<i>O. nigrinus</i>	CGG TTA GTA GAC CTT CCG GCA CCG TCG AAC ATC TCT GTG TGA TGA AAC
<i>O. profundicola</i>	..T... ..T...C..A... ..T... ..A... ..G...
<i>O. celebensis</i>	..A... ..C... ..A... ..A... ..A... ..G...
<i>O. matanensis</i>	..T... ..A... ..A... ..A... ..G...
<i>O. marmoratus</i>	..T... ..T..C..A... ..T... ..T... ..A... ..G...
<i>O. latipes</i>	..T.C.G..C... ..A..C..T..A... ..T..A..T... ..
<i>X. oophorus</i>	..T... ..C...C..C..A..A... ..T..A..A... ..G...
<i>M. salmoides</i>	..A.C.C..C... ..C..C..T..A..A..T... ..G..C... ..
<i>O. nigrinus</i>	TTC GGC TCT CTT CTA GGC CTT TGC TTA GCG GCT CAA ATC GTT ACA GGG
<i>O. profundicola</i>	...G... ..C... ..A... ..A... ..A... ..A...
<i>O. celebensis</i>	...A..C..C... ..C... ..A... ..A... ..A... ..A...
<i>O. matanensis</i>	...T... ..C... ..A... ..C... ..A... ..A...
<i>O. marmoratus</i>	...G... ..C... ..C... ..A... ..G... ..A...
<i>O. latipes</i>	..T..T... ..C..A... ..T... ..T..C..G... ..A..C..G..C...
<i>X. oophorus</i>	..T... ..C.T.G... ..A..C... ..G... ..T.C..G... ..T..C..C
<i>M. salmoides</i>C..G..G... ..C... ..C... ..A.A.C... ..C.C... ..C
<i>O. nigrinus</i>	CTA TTT TTA GGC ATG CAC TAC ACC TCT GAC ATT GCC ACA GCA TTT TCC
<i>O. profundicola</i>	T..... ..A..... ..T..... ..T..... ..
<i>O. celebensis</i>A..... ..T..... ..T..... ..
<i>O. matanensis</i>T..... ..A..... ..T..... ..
<i>O. marmoratus</i>	T..... ..T..A... ..T..... ..T..... ..
<i>O. latipes</i>	..T... ..C.T... ..A..T..T..A..C... ..C... ..C... ..C..G
<i>X. oophorus</i>	..C..C..G..A..A..T... ..G... ..C..T... ..C..A
<i>M. salmoides</i>	T... ..C.C.C..T..A... ..T... ..T... ..T..C..A..C... ..C..A
<i>O. nigrinus</i>	TCA GTG GCC CAT ATT TGC CGA GAC GTT AAT TAC GGA TGA CTA ATC GGT
<i>O. profundicola</i>C... ..A... ..C... ..T... ..T... ..T...
<i>O. celebensis</i>	...A..T..C..C..T... ..T... ..T... ..T... ..T..C
<i>O. matanensis</i>	...A... ..C... ..T... ..T... ..T... ..T...
<i>O. marmoratus</i>C... ..C... ..T... ..T... ..T...
<i>O. latipes</i>T..A..C..C... ..G..T... ..C... ..C... ..G
<i>X. oophorus</i>	..C..T... ..T... ..T..A..C... ..C... ..C... ..C... ..G
<i>M. salmoides</i>	..C..T... ..C... ..T..C..T..A..C... ..C... ..T... ..T..A
<i>O. nigrinus</i>	AAC ATA CAT GCT AAC GGG GCT TCT TTC TTT TTT ATT TGT ATT TAC CTT
<i>O. profundicola</i>A..... ..A..... ..A..... ..A..... ..A.....
<i>O. celebensis</i>	..T... ..A... ..C..A... ..A..... ..C... ..C... ..
<i>O. matanensis</i>A..... ..A..... ..A..... ..A..... ..A.....
<i>O. marmoratus</i>A..... ..A..... ..A..... ..C... ..C... ..
<i>O. latipes</i>	..T... ..A... ..C... ..T... ..T..C..C..C..C... ..
<i>X. oophorus</i>	..T..G..C..A..T..T... ..A..T..C... ..C... ..C... ..C...
<i>M. salmoides</i>T... ..T..T..A..C... ..C... ..C... ..C... ..G
<i>O. nigrinus</i>	CAT ATC GGT CCG GGA CTT TAC TAC GGC TCA TAT CTA TAC AAA GAA ACC
<i>O. profundicola</i>C..T..G... ..T... ..T... ..T... ..T... ..
<i>O. celebensis</i>T..A..A... ..T... ..T... ..C..T... ..TT... ..
<i>O. matanensis</i>C..T..G... ..T... ..T... ..C.T.G... ..G... ..
<i>O. marmoratus</i>C..T..G... ..T... ..T... ..T... ..T... ..
<i>O. latipes</i>	..C..T..A..A..C.T.G... ..A..C... ..C.T.G... ..G... ..A
<i>X. oophorus</i>T..C... ..G... ..T... ..C... ..C... ..T... ..G... ..
<i>M. salmoides</i>C..G... ..T.A..T..T..T... ..C..T... ..G... ..A
<i>O. nigrinus</i>	TGA AAC ATC GGG GTG GTC CTC CTT CTT CTA GTA ATA ATG ACA GCA TTC
<i>O. profundicola</i>A..... ..A..... ..A..... ..A..... ..A.....
<i>O. celebensis</i>T... ..T... ..C.T.A... ..A..... ..T... ..T...
<i>O. matanensis</i>A..... ..A..... ..A..... ..G... ..A... ..
<i>O. marmoratus</i>A..... ..T... ..T... ..A..... ..A..... ..A.....
<i>O. latipes</i>T.G... ..T..A..T..T... ..A... ..A... ..A..G..T... ..
<i>X. oophorus</i>G... ..A... ..T.T.A... ..G.TC... ..A.T.T..C..T
<i>M. salmoides</i>A... ..A... ..C... ..C... ..C... ..C... ..T... ..C...
<i>O. nigrinus</i>	GTC GGG TAT GTA CTT CCA TGA GGC CAA ATA TCA TTC TGA GGG GCT ACA
<i>O. profundicola</i>	..T... ..C... ..G... ..A... ..T... ..T... ..T... ..T...
<i>O. celebensis</i>	..T... ..C... ..G... ..A... ..T... ..T... ..T... ..T...
<i>O. matanensis</i>	..T..A... ..T... ..G... ..G... ..T... ..T... ..T... ..T...
<i>O. marmoratus</i>	..T... ..C... ..G... ..A... ..T... ..T... ..T... ..T...
<i>O. latipes</i>	..A..T... ..C..T..A..C... ..G... ..T... ..T... ..C..T
<i>X. oophorus</i>	..T..A... ..T..G..C..A... ..A... ..C... ..G... ..C... ..C..C
<i>M. salmoides</i>	..A... ..C... ..C... ..C... ..A... ..C... ..T... ..C...
<i>O. nigrinus</i>	GTA ATT ACC AAC CTC TTA TCA GCT GTC CCT TAC GTT GGC AAC GTT
<i>O. profundicola</i>T... ..G... ..A... ..C... ..T..A... ..C... ..
<i>O. celebensis</i>	..G... ..T... ..G... ..C..A... ..C... ..T..A... ..T... ..
<i>O. matanensis</i>T... ..G... ..A... ..T... ..C... ..T... ..T... ..
<i>O. marmoratus</i>G... ..A... ..T... ..C... ..T... ..T... ..T... ..
<i>O. latipes</i>C... ..G... ..T..C... ..A... ..C... ..G... ..C... ..C
<i>X. oophorus</i>	..T... ..G... ..G..C..A... ..A... ..A... ..T... ..C...
<i>M. salmoides</i>	..T... ..T... ..T... ..C..G..C... ..A... ..C... ..T... ..ACC

Fig. 2. DNA sequences of the mitochondrial cytochrome b genes of *Oryzias* and *Xenopoeilus* in Sulawesi, and *Micropterus salmoides*. 477 nucleotides from the first ATG codon are shown.

Table 2. Genetic distances among species of *Oryzias* and *Xenopoecilus* in Sulawesi, *Oryzias lapites*, and *Micropterus salmoides*.

OTU	1	2	3	4	5	6	7	8
1 <i>O. nigrimas</i>		0.0951	0.1429	0.0810	0.1005	0.2585	0.2551	0.2718
2 <i>O. profundicola</i>	8.81		0.1270	0.0503	0.0257	0.2659	0.2587	0.2706
3 <i>O. celebensis</i>	12.58	11.31		0.1288	0.1302	0.2789	0.2557	0.2562
4 <i>O. matanensis</i>	7.55	4.82	11.53		0.0669	0.2467	0.2534	0.2673
5 <i>O. marmoratus</i>	9.22	2.52	11.53	6.29		0.2608	0.2486	0.2696
6 <i>O. lapites</i>	21.38	21.80	22.43	20.55	21.38		0.2452	0.2817
7 <i>X. oophorus</i>	21.17	21.17	21.17	20.96	20.55	20.55		0.2667
8 <i>M. salmoides</i>	22.01	22.01	21.17	21.80	22.01	22.64	21.80	

Distances (upper-right) were calculated according to the Tamura and Nei model (Tamura and Nei, 1993). The values for the percent divergence are shown in lower-left. OTU means the operational taxonomic unit.

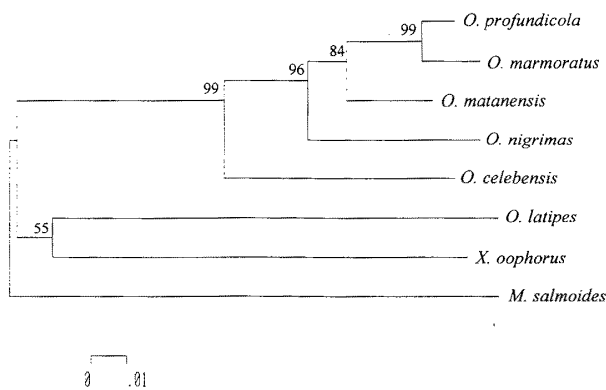


Fig. 3. Phylogenetic tree of the fish genera *Oryzias* and *Xenopoecilus* as deduced by the neighbor-joining method. Numbers at each branch indicate the percentage of times a node was supported in 1000 bootstrap pseudoreplications by neighbor-joining. *Micropterus salmoides* is used for an outer group. A scale bar indicates 0.01 unit of tree length.

are high. These results indicate that the genus *Oryzias* in Sulawesi is monophyletic. Although the tree topology obtained by isozyme analysis (Naruse *et al.*, 1992) is different from that obtained by cytochrome b sequencing, and is the same as the tree topology obtained by karyotyping (Uwa, 1992), all these studies support the monophyly of the *Oryzias* species in Sulawesi. Judging from the phylogenetic tree constructed by the neighbor-joining method, *O. marmoratus*, *O. profundicola* and *O. matanensis* are closely related to each other. This result coincides with their distributions. *O. marmoratus* and *O. profundicola* live in the same lake (Lake Towuti), and *O. matanensis* lives in Lake Matano. These two lakes are connected by a river. *O. marmoratus* and *O. profundicola* have very similar nucleotide sequences (d value is 0.02). *O. profundicola* has been described by

Kottelat in 1990. He also suggested the existence of hybrids between the two species in his paper (Kottelat, 1990b). It is possible that *O. profundicola* is not a real species, and detailed experiments including hybridization, electrophoretic analysis and comparison of genomic genes are required.

Due to the collaboration of many researchers distributed throughout Southeast Asia and the Far East over 10-year period, almost all fish belonging to the genus *Oryzias* have been collected and maintained in several laboratories in Japan. These fish provide not only good opportunity for evolutionary study but also for study on genetics, development biology and physiology.

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