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DNA BARCODING OF FRESHWATER FISHES FROM LAKE LAUT TAWAR, ACEH PROVINCE, INDONESIA

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Background. DNA barcoding has been recognised for its usefulness in species identification. The method is used for fast and accurate species identifications by focusing analysis on a short standardized segment of the genome. The main objective of the barcode of life project is to provide a database of genetic sequences which can be used as a tool for universal species identification. Indonesia has at least 1300 freshwater fish species, however unfortunately no species has been barcoded as yet. In the presently reported study, we subjected to barcoding a total of 14 species of freshwater fishes from Lake Laut Tawar, Indonesia.

Materials and methods. On average, 10 random samples from each species were processed for DNA analysis. Approximately 655-bp were amplified from the 5′ region of the mitochondrial cytochrome *C* oxidase subunit I (COX1) gene. All obtained sequences were edited and aligned using MEGA 4.0 program. Nucleotide divergence among sequences was estimated based on Kimura 2-parameter distances. Unique haplotypes were determined using DnaSP Version 5.10.02 software, and the genetic relations among haplotypes were assessed by constructing a phenogram using the neighbour-joining method.

Results. A total of 31 haplotypes from 14 freshwater fish species were produced in this study. The read lengths were 626-bp, where 259 sites were polymorphic, 254 sites parsimony informative, and five singletons. No stop codons, deletions, or insertions were observed in any of the sequences. The nucleotide distance between species ranged from 7.1%—between *Puntius brevis* (Bleeker, 1850) and *Poropuntius tawarensis* (Weber et de Beaufort, 1916)—to 30.4%—between *Channa gachua* (Hamilton, 1822) and *Homaloptera* sp.—indicating that *P. brevis* and *P. tawarensis* are very closely related.

Conclusion. This study confirms the utility of COX1 gene in accurate identification of 14 species of freshwater fishes from Lake Laut Tawar, however, three species could not be identified to species level namely *Rasbora* sp. (local name: relo), *Homaloptera* sp. (ilie) and *Clarias* sp. (mud). It is suggested that future studies should incorporate morphometric methods to resolve the taxonomic status of these undetermined species.

Keywords: genetic distance, COX1 gene, depik, Cyprinidae

INTRODUCTION

Modern taxonomic work regularly employs internal anatomy, physiology, behaviour, genes, isozymes, geography, and morphological characters, which remain the cornerstone of taxonomic treatments (Ward et al. 2009). However, there are difficulties in relying primarily on morphology when attempting to identify fishes during various stages of their development for example larvae or when examining fragmentary-, partial-, or processed remains. Even when intact adult specimens are available, the morphological characters used to discern species can be so subtle that identification is difficult, even for trained taxonomists (Ward et al. 2009).

It is well recognised that DNA-based identification system or commonly known as DNA barcoding (Floyd et al. 2002, Hebert et al. 2003a), can aid the resolution of the vast diversity of life with its millions of species (Hebert et al. 2003a, 2003b, Tautz et al. 2003). It has much to offer

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to fisheries managers, especially in the provision of tools enabling unequivocal specimen identification and assessment of stock structure (Ward 2000). Presently, the barcode analysis is a cost-effective option for species identification in some situations and this will increasingly be the case as reference libraries are assembled and analytical protocols are simplified (Hajibabaei et al. 2005). The method promises fast and accurate species identifications by focusing analysis on a short standardized segment of the genome (Hebert et al. 2003a).

DNA barcoding has already been applied in many animals such as birds (Hebert et al. 2004b), springtails invertebrates (Hogg and Hebert 2004), skipper butterflies (Hebert et al. 2004a), blowflies (Whitworth et al. 2007), leaf beetles (Jurado-Rivera et al. 2009), nematodes (De Ley et al. 2005), amphibians (Vences et al. 2005a), ants (Smith et al. 2005), crustaceans (Lefébure et al. 2006), and scuttle flies (Boehme et al. 2010). In addition, DNA barcodes have been obtained for more than 8000 species of fish and the COI sequences deposited in the Barcode of Life Data Systems (BOLD) online workbench and repository (Ragnasingham and Hebert 2007).

Kottelat and Whitten (1996) reported that among the east-, south-, and southeast Asian nations, Indonesia has the largest number of freshwater fishes (1300), of which 114 species occur in Aceh waters (Muchlisin and Siti-Azizah 2009). Unfortunately none of those species have been barcoded. Hence, the presently reported study examined the patterns of DNA sequences of freshwater fishes from Lake Laut Tawar based on the barcoding cytochrome oxidase subunit I (COX1) gene. The fish fauna in Lake Laut Tawar is unique due to its geographical isolation and environmental conditions and therefore, the greater likelihood of species endemism, for example *Rasbora tawarensis* Weber et de Beaufort, 1916 and *Poropuntius tawarensis* (Weber et de Beaufort, 1916) (see Muchlisin and Siti-Azizah 2009, Muchlisin et al. 2012). Currently, the indigenous fish populations in Lake Laut Tawar, Indonesia are threatened by both biological- and physical factors, i.e., presence of introduced species, pollution, overharvesting, and habitat loss. Unfortunately, very limited information on fish populations in Lake Laut Tawar is available. In many situations, genetic data are the best way to determine whether a species is worth a special protection under the Endangered Species Act (ESA) or other form of conservation status (Matoso et al. 2004), as it allows calculated decisions on the best course of action to be taken for protection and conservation (Leuzzi et al. 2004) as well as managing different stocks (Salini et al. 2006).

MATERIALS AND METHODS

Study area. Lake Laut Tawar (04°36′43″N, 096°55′25″E) is situated in Central Aceh, Aceh Province. It is located approximately 1200 m above sea level. The lake is an old volcanic caldera of 16 km in length, 4 km in width with an average depth of 35 m and a maximum predicted depth of 80 to 115 m. It is surrounded by mountains reaching over 2000 m. Several short tributaries discharge into Lake Tawar, the main outflow being the Peusangan River (Muchlisin et al. 2010). The watershed is covered by forests, which are increasingly affected by deforestation and agricultural land conversion.

Sample collection and preparation. Explorative survey was conducted based on initial report by local fishermen during November 2009, February 2011, and August 2012. Gillnet (mesh size of 19, 37, and 75 mm), hooks, acting nets (mesh size of 25, 50, and 75 mm) and traditional traps (bubu) were used to catch the fish samples in the lake. The fishes caught were identified based on their morphological differences (Kottelat et al. 1993). On average, 10 random samples from each species were processed for DNA analysis. Approximately 1 cm^2 of caudal fin tissue was taken from each specimen using sterile scissors to avoid contamination among specimens. The tissues were placed into 2.0 mL tubes containing TNES-urea buffer, labelled and transported to the laboratory. There the tissues were further minced into small pieces in order to enhance lysis activity. The samples were preserved at least two weeks prior to DNA extraction. Voucher specimens were preserved in 96% ethanol and deposited at Laboratory of Ichthyology, Syiah Kuala University, Banda Aceh, Indonesia.

Specimens for the study were collected under licences from the Marine and Fisheries Affair of Aceh Province, Indonesia. Experimental protocols involving the use of live fish were in accordance with the Syiah Kuala University and Universiti Sains Malaysia's guidelines.

DNA extraction. Genomic DNA was isolated using Aqua Genomic DNA solution following the manufacturer's protocol. Approximately 50 µL of tissue in TNES-urea buffer was transferred into a sterile 1.5 mL tube. A 100 µL of Aqua Genomic DNA solution was added and the tissue was homogenized at room temperature followed by addition of 15 µL of 100% isopropanol. The mixture was then vortexed and incubated at 60ºC for 10 min. After incubation, the mixture was revortexed for 30 s and then centrifuged at 12 000 rpm for 5 min. Then, the clear supernatant was transferred into a new 1.5 mL tube. One volume of 100% isopropanol was added and revortexed for 30 s. The mixture was recentrifuged at 12 000 rpm for 2.5 min. The supernatant was discarded and the tubes were rinsed twice with 70% ethanol. The tubes were flipped and tapped several times on a piece of paper towel to remove the remaining ethanol. After that, 60 µL of deionized water was added and pipetted up and down to suspend the DNA. The DNA was kept at –20°C prior to use.

Electrophoresis was conducted on a 0.8% agarose gel at 100 V for 45 min to assess the success of DNA extraction. The gel was stained with ethidium bromide prior to visualization for the presence of the extracted DNA, indicated by the presence of a band in a gel documentation system (GENE FLASH, Syngene Bio Imaging). The quality and quantity of extracted DNA were assessed using a spectrophotometer (SQ-4802, Unico).

PCR amplification. Approximately 655-bp were amplified from the 5′ region of the mitochondrial cytochrome oxidase subunit I (COX1) gene using the primer pairs following Ward et al. (2005). FishF1 5′TCAACCAACCACAAAGACATTGGCAC3′ and FishR1 5′TAGACTTCTGGGTGGCCAAAGAATCA3′

The 25 µL PCR reaction mix contained 17.65 µL of deionized water, 2.25 µL of 10 X PCR buffer, 3.0 µL of $MgCl_2$ (25 mM), 0.25 µL of each primer (0.01 mM), 0.5 µL of mixed DNTP (0.05 mM), 0.1 µL of *Taq* polymerase, and 1.0 µL of DNA template. Amplifications were performed using a Mastercycler® Eppendorf gradient thermal cycler (Brinkmann Instruments, Inc). The thermal regime consisted of an initial step of 2 min at 95°C followed by 35 cycles of 0.5 min at 94°C, 0.5 min at 54°C, and 1 min at 72°C, followed in turn by 10 min at 72°C, and then cooled to 4°C (Ward et al. 2005).

After amplification, the PCR products were run on 1.2% agarose gel electrophoresis for 45 min and then visualized using GENEFLASH® Syngene Bio Imaging. The most clarified products were selected for purification.

The purification of satisfactory PCR products was conducted using purification kits (PCR Clean-up System, Promega) by following the manufacturer's protocol. The products were sent for sequencing to a service provider (Centre for Chemical Biology, Universiti Sains Malaysia, CCB-USM) (Model, ABI 3730XL).

Mitochondrial DNA data analysis. All obtained sequences were edited and aligned using MEGA 4.0 program (Tamura et al. 2007). Multiple sequence alignments were performed on the edited sequences by Cluster *W* which is integrated in the MEGA 4.0 program. The haplotypes were produced using DnaSP Version 5.10.02 software (Rozas et al. 2003) and the genetic relations among haplotypes were assessed by constructing phylogenetic trees.

RESULTS

A total of 112 products were successfully sequenced by FishF1 and FishR1 primers. Due to the limited number of species, phylogenetic relations among species were not conducted. Nevertheless, a phylogenetic tree was constructed to assess the monophyly of presumed species through NJ method and also to support the genetic distance data. The GenBank accession numbers were listed in Table 1. Of these 14 species, three could not be identified to species level namely *Rasbora* sp. (local name: relo), *Homaloptera* sp. (ilie), and *Clarias* sp. (mud). The *Clarias* sp. sequences have been confirmed to the GenBank data, and BLAST results showed approximately 89% identity (98% query coverage) with sequences of *Clarias batrachus* (**L.**) in GenBank (accession No. GQ466403). Therefore herein this species is referred to as *Clarias* sp. In addition there were two undetermined species namely *Rasbora* sp. and *Homaloptera* sp. (<84% identity and 99% query coverage with BLAST results with the most genetically similar species). Each individual clustered within its presumed species, family and order forming monophyletic clusters.

A total of 31 haplotypes were produced, of these 8 haplotypes were contributed by *Rasbora tawarensis* and 6 haplotypes by *Rasbora* sp. While *Channa gachua* (Hamilton, 1822); *Clarias gariepinus* (Burchell, 1822); *Homaloptera* sp.; *Oreochromis niloticus* (Linnaeus, 1758); and *Osteochilus kahajanensis* (Bleeker, 1856) were contributed two haplotypes of every species. In addition, *Anguilla marmorata* (Quoy et Gaimard, 1824); *Xiphophorus hellerii* (Heckel, 1848); *Channa striata* (Bloch, 1793); *Clarias* sp.; *Poropuntius tawarensis*; *Puntius brevis* (Bleeker, 1849); and *Cyprinus carpio* L. were contributed a single haplotype of each species (Table 1).

The read lengths were 626-bp where 259 sites were polymorphic, 254 sites parsimony informative, and five singletons, no stop codons, deletions, or insertions were observed in any of the sequences. The mean conspecific, congeneric, and confamilial nucleotide divergences were 0.15%, 2.53%, and 5.63% (Table 2). Thus level of divergence among congeneric species was about 17 times higher than among conspecific fish. Interestingly, while higher order comparisons of within genus, family and order approximated 20% in genetic distance, *Poropuntius tawarensis* and *Puntius brevis* were only 7.1 % genetically distant, being much lower than intra-genera comparisons within *Rasbora*, *Channa*, and *Clarias* (Table 3).

DISCUSSION

A total of 14 freshwater fishes from Lake Laut Tawar were successfully barcoded. Among the 14 species barcoded fishes, four were introduced fishes i.e., *Clarias gariepinus*, *Oreochromis niloticus*, *Xiphophorus hellerii*, and *Cyprinus carpio* (see Lowe et al. 2000, Anonymous 2011), and the rest indigenous species, two being endemic i.e., *Rasbora tawarensis* and *Poropuntius tawarensis*. Genetic study on *C. carpio* from Turkey and Uzbekistan were reported by some researchers (Murakaeva et al. 2003, Kohlmann et al. 2005, Memiş and Kohlmann 2006), and *X. hellerii* was reported by Mejía et al. (2012), however, the genetic characteristic of wild *C. carpio*, *X. hellerii*, *C. gariepinus*, and *O. niloticus* from Lake Laut Tawar, Indonesia have not previously been evaluated. This study was the first report on the genetic status of freshwater fishes in Aceh region Indonesia, except for Rasbora group in Lake Laut Tawar which was reported by Muchlisin et al. (2012).

The study revealed the monophyly of each presumed taxon at the species, genera and family levels (Fig. 1). Thus the barcode approach provided additional important data for the precise identification of fishes in Lake Laut Tawar. Ward et al. (2005) reported that phylogenetic COX1 sequences could effectively cluster most congeneric and confamilial species. This was observed in previous studies for example in Australian fishes (Ward et al. 2005), Canadian freshwater fishes (Hubert et al. 2008), Indian carangid fishes (Persis et al. 2009), freshwater fishes from Mexico and Guatemala (Valdez-Moreno et al. 2009), and Cuban freshwater fishes (Lara et al. 2009).

Theoretically, genetic divergences should increase with increasing taxonomic levels. As expected, this was not supported by the presently reported study; i.e., com-

Table 1

List of the studied fish species from Lake Laut Tawar, Indonesia and their GenBank accession numbers for COX1 sequences

GenBank accession number(s)	Species	Common name (local name)	Family	N_h	n
HM100243–HM100250	*Rasbora tawarensis* (Weber et de Beaufort, 1916)	Laut Tawar rasbora (depik or eas)	Cyprinidae	8	23
HM345923–HM345928	*Rasbora* sp.	(relo)	Cyprinidae	6	13
HM345943, HM345944	*Osteochilus kahajanensis* (Bleeker, 1856)	(peres)	Cyprinidae	2	8
HM345940	*Cyprinus carpio* (Linnaeus, 1758)	common carp (mas)	Cyprinidae	1	6
HM231326	*Poropuntius tawarensis* (Weber et de Beaufort, 1916)	(kawan)	Cyprinidae	1	5
HM345937	*Puntius brevis* (Bleeker, 1849)	(kepras)	Cyprinidae	1	5
HM345935, HM345936	*Homaloptera* sp.	(ilie)	Balitoridae	2	6
HM345929	*Anguilla marmorata* (Quoy et Gaimard, 1824)	giant mottled eel (denung. ileah)	Anguillidae	1	1
HM345932	*Clarias* sp.	(mud)	Clariidae	1	6
HM345933, HM345934	*Clarias gariepinus* (Burchell, 1822)	north African catfish (dumbo)	Clariidae	2	7
HM345941, HM345942	*Oreochromis niloticus* (Linnaeus, 1758)	Nile tilapia (mujair)	Cichlidae	2	6
HM345930	*Xiphophorus hellerii* (Heckel, 1848)	green sword tail (buntok)	Poecilidae	1	10
HM345931	*Channa striata* (Bloch, 1793)	striped snakehead (gabus)	Channidae	1	8
HM345938, HM345939	*Channa gachua* (Hamilton, 1822)	dwarf snakehead (lokot)	Channidae	2	8
Total	14		7	31	112

N_h = total number of haplotypes, n = total number of individuals.

parisons between species, genera, and families gave very similar values, probably due to very limited sample sizes at each taxonomic level. Furthermore, the limited number of species in this study did not permit further inference to be made on the phylogenetic relations. However, despite this obvious limitation, one interesting observation is the genetic relatedness between *Puntius brevis* and *Poropuntius tawarensis*. The nucleotide distance between species belonging to different genera ranged from 7.1% (between *Puntius brevis* and *Poropuntius tawarensis*) to 30.4% (between *Channa gachua* and *Homaloptera* sp.), indicating that *Puntius brevis* and *Poropuntius tawarensis* are very closely related. This is even lower than the comparison within the genus *Rasbora* (9.6%), while within *Clarias* (16.5%) species, the genetic distances were much higher.

Puntius and *Poropuntius* are morphologically very similar, and the scale pores in the *Poropuntius* is the main character to distinguish between these two genera (Kottelat et al. 1993). However, local researchers regard them both as belonging to *Puntius* in general (Personal communication from a local authority). Further studies need to be done to confirm whether these two species indeed belong to the same genus. However, based on the genetic distances observed in this study, *Puntius brevis* and *Poropuntius tawarensis* are most likely different species but possibly should be assigned to the same genus. In addition, the caudal, dorsal, and anal fins of *P. tawarensis* were more colourful than the deeper-forked caudal fin of *P. brevis*.

A single giant mottled eel, *Anguilla marmorata*, was caught by fishermen and barcoded in this study. In recent times, fishermen have reported rare catches of this species from the lake. This was probably a consequence of a dam construction in the Peusangan River, the main discharge of the lake. Giant mottled eel is known as a catadromous fish (McDowall 1988), with the adults migrating from freshwater habitats to offshore spawning areas and the translucent larvae (glass eel) are drifting into the coastal area and returning to its river of origin to mature (Chino and Arai 2010). However, the dam hinders movement of this migratory fish, resulting in failure to spawn and no recruitment, hence reducing their population drastically. In recent years, no juvenile giant mottled eel has been caught and the rare catches are mostly larger in size (Personal communication from a local authority), evidently no recruitment has occurred. In addition, according to local fishermen, the lake mahseer or locally known as pedih (probably the *Neolissochilus* sp.) which is known to be indigenous to the lake, is now very rarely caught. This species could be moving towards extinction in the lake.

The populations of two other introduced species i.e., *Oreochromis mossambicus* (Peters, 1852) and *Ctenopharyngodon idella* (Valenciennes, 1844) have also decreased in the lake. They were not caught during the 2009–2012 sampling, although present in the initial sampling in 2008. The *C. idella* (grass carp) is a native to larger coastal rivers of eastern Asia (20–50°N, 100–140°E) (Fischer and Lyakhnovich 1973), it was introduced to Europe for aquatic weed control and to improve fish production (Van Zon 1977). According to Pípalová (2006) that the likelihood of natural grass carp reproduction is limited to few water bodies, and in the wild they only spawn in the native range of its habitat. Therefore, no recruitment could occur in the tropical zone, as confirmed by our observations in Lake Laut Tawar; like the giant mottled eel, the rare catches of grass carp were also bigger in size. This supports the argument that there was no new recruitment for both fishes. In addition, the decreasing *O. mossambicus* population may be due to competition with *O. niloticus*; both fishes have the same feeding habit and habitat occupation. The presence of *O. niloticus* in Lake Laut Tawar may also have a negative effect to the fish community in general. Further studies are needed to evaluate the trophic and habitat overlap between *O. niloticus* with other fishes in Lake Laut Tawar.

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Table 2

Summary of genetic divergences (K2P percent) within and between various taxonomic levels based on the studied fish species from Lake Laut Tawar, Indonesia

Taxonomic level	Genetic distance (K2P percent)					
	Comparison within			Comparison between		
	Min	Max	Mean ± SE	Min	Max	Mean ± SE
Species	0	1.00	0.15 ± 0.06	7.10	30.4	22.9 ± 2.00
Genera	0	11.00	2.53 ± 0.29	7.10	29.30	22.60 ± 2.00
Families	0	13.20	5.63 ± 0.52	22.80	29.3	25.40 ± 1.90
Order	0	16.00	10.08 ± 0.73	23.30	28.40	26.10 ±1.80

Data are from 112 sequences from 14 species, 11 genera, 6 families, and 5 orders; SE = standard error of the mean.

Table 3

Percentage K2P sequence divergence between species in 613-bp consensus sequences of COX1 gene (below diagonal) based on the studied fish species from Lake Laut Tawar, Indonesia

No.	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	*Anguilla marmorata*	*n/a*													
2.	*Rasbora* sp.	23.5	*0.5*												
3.	*Rasbora tawarensis*	22.8	9.6	*0.2*											
4.	*Xiphophorus hellerii*	24.2	27.8	27.9	*0*										
5.	*Channa striata*	25.7	22.5	23.6	24.7	*0*									
6.	*Channa gachua*	28.0	27.9	27.2	29.7	20.6	*0*								
7.	*Clarias* sp.	26.0	24.1	24.8	29.4	25.4	27.3	*0*							
8.	*Clarias gariepinus*	27.3	24.6	25.1	27.6	26.1	30.2	16.9	*1.0*						
9.	*Homaloptera* sp.	25.3	23.4	23.3	26.2	28.1	30.4	26.1	26.4	*0.1*					
10.	*Poropuntius tawarensis*	21.8	19.8	18.9	26.5	22.1	22.6	23.7	21.8	25.3	*0*				
11.	*Puntius brevis*	21.3	19.5	18.7	27.7	23.7	22.1	22.1	22.4	24.0	**7.1**	*0*			
12.	*Cyprinus carpio*	22.2	19.6	19.6	23.5	23.9	26.2	24.2	25.1	21.5	13.3	15.4	*0*		
13.	*Oreochromis niloticus*	24.0	24.7	23.0	22.8	22.9	26.5	24.4	25.0	23.9	25.3	23.6	23.9	*0.1*	
14.	*Osteochilus kahajanensis*	25.8	20.3	21.6	25.1	26.7	27.3	26.4	23.2	24.5	16.9	19.0	16.5	24.4	*0*
	Average within species = 0.15		Average between species = 22.9												

Italic values on diagonal are nucleotide divergence among individuals within species.

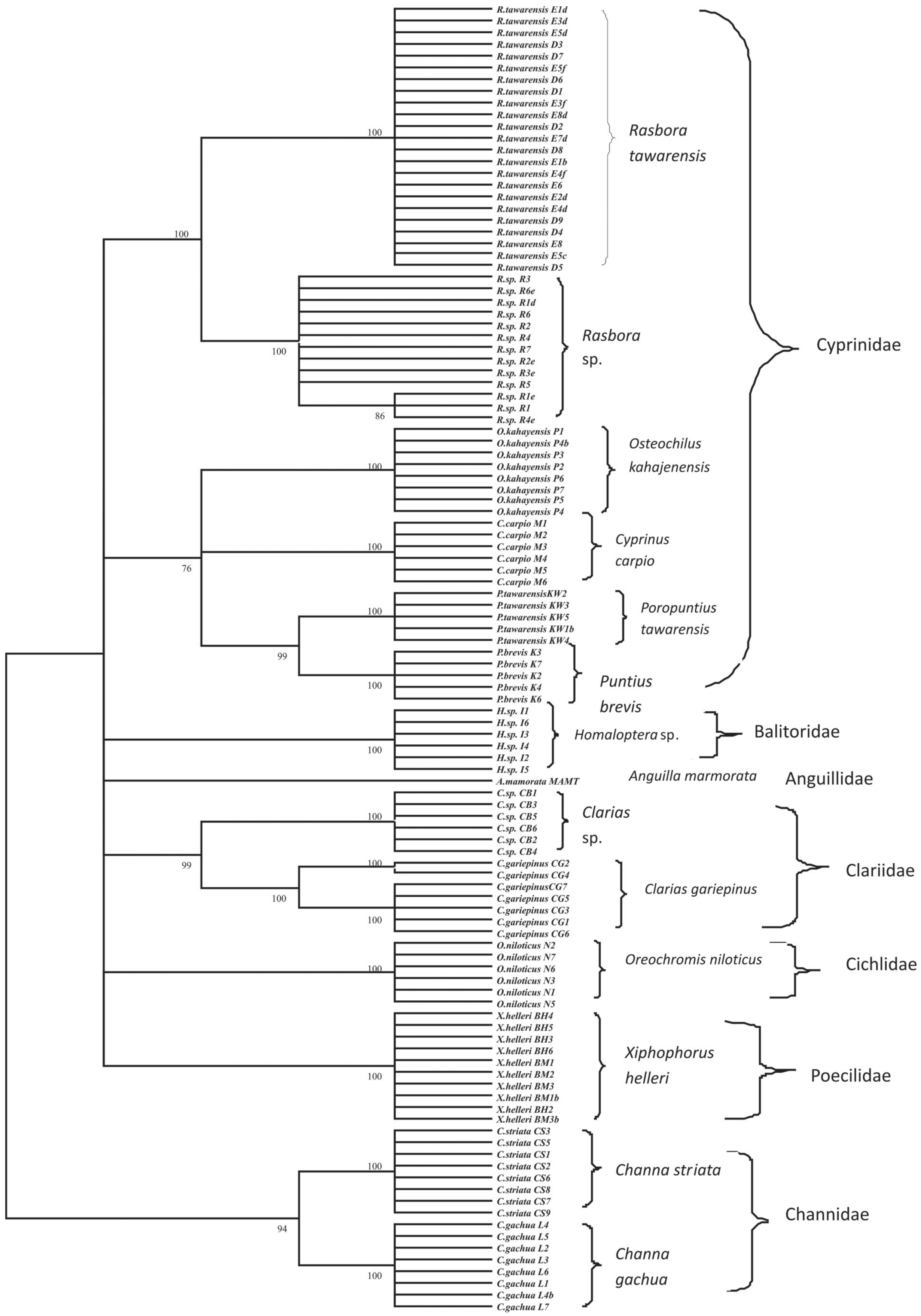

Fig. 1. The 75% majority-rule consensus phylogenetic tree haplotypes based on 626-bp COX1 sequence between individuals (based on the studied fish species from Lake Laut Tawar, Indonesia). Numbers above branches represent bootstrap confident level for NJ (1000 replicates)

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