

Molecular Phylogeny of the *Opsariichthys* Group (Teleostei: Cypriniformes) Based On Complete Mitochondrial Genomes

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Shih-Pin Huang, Feng-Yu Wang, and Tzi-Yuan Wang (2017) The complete mitochondrial genomes of 76 species from 43 genera under Cyprinidae *sensu lato* were collected to reassess the molecular phylogeny of Opsariichthyinae *sensu* Liao et al. 2011. The mitogenomes of three species, *Candidia barbata*, *Opsariichthys evolans*, and *Opsariichthys pachycephalus*, were newly sequenced. Phylogenetic trees were reconstructed based on 13 concatenated multiple protein-coding genes with two ribosomal RNA genes. The concatenated dataset provided a new perspective on systematics and relationships. Tree topologies show that a monophyletic group containing *Parazacco*, *Candidia*, *Nipponocypris*, *Zacco*, and *Opsariichthys* should belong to the *Opsariichthys* group. In addition, the present results also strongly support that *Candidia* and *Nipponocypris* should be regarded as distinct genera within the *Opsariichthys* group. *Aphyocypris*, *Yaoshanicus*, *Nicholsicypris*, and *Parasbora* form a monophyletic group within Xenocyprididae, distinct from the *Opsariichthys* group. Furthermore, *Hemigrammocypripis* is nested with four species of *Metzia*, a genus of ex-Cultrinae in Xenocyprididae. In addition, two major types of distinct stripes - longitudinal and vertical - were observed among species of the *Opsariichthys* group and were highly correlated with molecular phylogenetic relationships. Such types of vertical and longitudinal stripes presented in the *Opsariichthys* group might have originated in an ancestor species, after which distinct vertical stripes might have been lost among these cyprinids but retained in the *Opsariichthys* group.

Key words: Molecular phylogeny, Mitochondrial genome, Freshwater fish, Cyprinidae, Opsariichthyinae.

BACKGROUND

Cyprinidae *sensu lato* (originally called family Cyprinidae) is the largest family of teleosts in the world, containing 3090 valid species (Eschmeyer et al. 2017). Several recent studies have been carried out to assess the phylogeny and systematics of this group and/or the rest of Cypriniformes based on molecular evidence (Tang et al. 2010 2013; Stout et al. 2016).

The taxonomic placement of several major subfamilies belonging in Cyprinidae *sensu lato* has undergone a large change. Several subfamilies, especially Danioninae and Cultrinae, were reported to be paraphyletic or polyphyletic (Tang

et al. 2010 2013; Stout et al. 2016). Some were subsequently renamed in an attempt to reflect their new taxonomic placements (Liao et al. 2011c). Among these were Opsariichthyinae, a group of minnows in Cyprinidae *sensu lato* occurring widely in East Asia that contains the genera *Aphyocypris*, *Candidia*, *Hemigrammocypripis*, *Nipponocypris*, *Opsariichthys*, *Parachela*, *Parazacco*, *Yaoshanicus* and *Zacco* (Liao et al. 2011c) (Fig. 1). Most of these genera were previously assigned to Danioninae. However, their taxonomic assignments have been continuously changed over recent years (Mayden et al. 2009; Tang et al. 2010 2013; Liao et al. 2011a; Stout et al. 2016). Among these common minnows, *Yaoshanicus*, *Nicholsicypris*,

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and *Pararasbora* were considered junior synonyms of *Aphyocypris* (Liao et al. 2011b), although Huynh and Chen (2013) still considered *Nicholsicypris* to be a valid genus. Fang et al. (2009) defined ex-Rasborinae, which included *Candidia*, *Nipponocypris*, *Opsariichthys*, *Parazacco*, and *Zacco*. Subsequently, Liao et al. (2011c) renamed ex-Rasborinae as Opsariichthyinae to include *Aphyocypris*, *Candidia*, *Hemigrammocypris*, *Nipponocypris*, *Opsariichthys*, *Parachela*, *Parazacco*, *Yaoshanicus* and *Zacco* (Fig. 1). Recently, these genera along with several other subfamilies (Cultrinae, Hypophthalmichthyinae, Squaliobarbinae, Xenocyprinae, parts of Alburninae, and Danioninae) were reclassified into Oxygastrinae (Tang et al. 2013). Subsequently, Kottelat (2013) proposed that Oxygastrinae was not available, and instead Hypophthalmichthyinae and Xenocyprinae were the earliest available names. Therefore, the genera and subfamilies were assigned to Xenocyprididae (Stout et al. 2016). These taxonomic placements and assignments will be used and discussed in this study.

The subfamilies Cultrinae, Xenocyprinae, Squaliobarbinae, Alburninae, and Opsariichthyinae were formerly in Cyprinidae *sensu lato* but considered a monophyletic group by Stout et al. (2016) and therefore reassigned to the family Xenocyprididae. However, the taxonomic placements and relatedness of subfamilies under Xenocyprididae remained ambiguous because there was an insufficient number of taxa and none of the taxonomic assignments were included. For example, two species from former subfamily Cultrinae (*Chanodichthys erythropterus* and *Parabramis pekinensis*) were nested with *Hypophthalmichthys molitrix*, *Ctenopharyngodon idella*, *Elopichthys bambusa*, and *Squaliobarbus curriculus*, which were part of the former subfamilies Leuciscinae and Squaliobarbinae (Stout et al. 2016). This classification was inconsistent with another study (Tang et al. 2013). In addition, the taxonomic status of *Hemigrammocypris* remained controversial. Liao et al. (2011c) proposed that it should be assigned to Opsariichthyinae. However, Tang et al. (2013) and Stout et al. (2016) proposed that it was

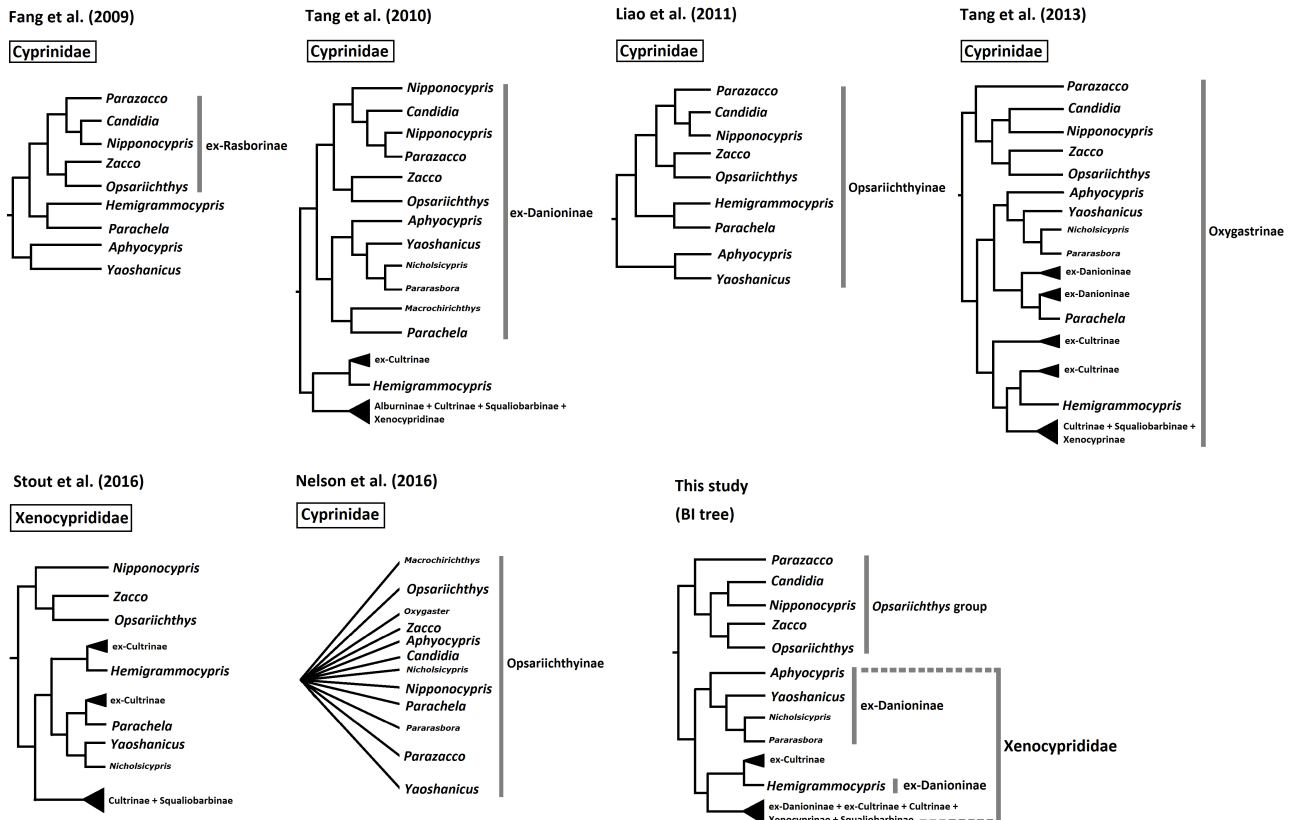


Fig. 1. Systematic positions of the *Opsariichthys* group and related genera from this and other studies.

closest to *Metzia*, a genus of ex-Cultrinae (Fig. 1). The systematic positions of these genera are summarized in figure 1.

The complete mitochondrial genome could be regarded as an alternative molecular marker for processing at a higher level of phylogenetic analysis (Saitoh et al. 2006; Mayden et al. 2009; Huang et al. 2016). In order to verify the systematic positions of Opsariichthyinae, a reassessment of molecular phylogenetic analysis was performed. We expected that mitogenomes would be useful for resolving these ambiguous relationships in Opsariichthyinae and Xenocyprididae. Fortunately, complete mitochondrial genomes of many species under Opsariichthyinae and Xenocyprididae have been sequenced (Jang-Liaw et al. 2013a b; Chang et al. 2016; Chen et al. 2016a b). However, none of these studies analyzed the complete mitochondrial genome. Recently, mitochondrial DNA has been frequently used for resolving the taxonomic and phylogenetic problems in East Asian cyprinids (Tsao et al. 2016; Huang et al. 2017). In order to verify taxonomic placement and assignment and attempt to provide a new molecular perspective on different genetic marks, this study analyzed more species and genera from Opsariichthyinae and family Xenocyprididae based on complete mitochondrial genomes.

Among East Asian common minnows, there are three major stripe patterns that can be roughly grouped. One is an indistinct stripe or band on the side of the body and the remaining two are a distinct vertical or longitudinal stripe or band on the side of the body. These minnows occur in five genera in East Asia: *Candidia*, *Nipponocypris*, *Opsariichthys*, *Parazacco*, and *Zacco*. Among these, *Candidia* is endemic to Taiwan, *Nipponocypris* is restricted to Japan and Korea, *Opsariichthys* is widely distributed in East Asia, *Parazacco* is restricted to southern China, and *Zacco* is distributed in northern China, Korea, and Japan. *Candidia*, *Nipponocypris*, and *Parazacco* have visible longitudinal stripes whereas *Opsariichthys* and *Zacco* have several vertically aligned stripes or bars (Wu 1977; Chen and Fang 1999; Nakabo 2013). Besides, all these color patterns are also found within *Danio* but not within single group of Cypriniformes (McCluskey and Postlethwait 2015). In this study, we attempted to understand whether the different stripe patterns were correlated with taxonomic relationships in these common minnows.

Candidia and *Nipponocypris* have been considered well-separated genera based on

molecular evidence (Liao et al. 2011c; Tang et al. 2013; Huynh and Chen 2013), but several contrary findings have been reported in recent years. For example, a phylogenetic tree of twelve Opsariichthyines species was reconstructed based on mitochondrial Cyt *b* and COI and nuclear RAG1 and Rh1 (Tang et al. 2010). Although they all belonged to a monophyletic group, *Candidia*, *Nipponocypris*, and *Parazacco* were nested together and therefore formed an indeterminate lineage. Yin et al. (2015) revealed the relationship of *Candidia*, *Nipponocypris*, *Opsariichthys*, *Parazacco*, and *Zacco* based on complete mitochondrial genomes. Remarkably, *Candidia* was only nested within *Nipponocypris*, which is consistent with Tang et al. (2010). In contrast, two Japanese species of *Nipponocypris* were assigned to *Candidia* (Nakabo 2013). Thus, this study also used further molecular research to reassess these inconsistencies and the validity of *Nipponocypris*.

MATERIALS AND METHODS

Whole mitogenome collection

Seventy-six species from 43 genera of Opsariichthyinae, Xenocyprididae, and related families were used to reassess their molecular phylogenetic analysis (Table 1). Among the East Asian common minnows, three species, *Candidia barbata*, *Opsariichthys evolans*, and *Opsariichthys pachycephalus*, were sequenced for the first time. Mitogenomes for the remaining nine species (*Aphyocypris chinensis*, *Hemigrammocypripis rasborella*, *Nicholsicypris normalis*, *Nipponocypris temminckii*, *Opsariichthys uncirostris*, *Pararasbora moltrechti*, *Parazacco spilurus*, *Yaoshanicus arcus*, and *Zacco platypus*), each of which is the type species of its genus, were retrieved from GenBank (Table 1). For the whole phylogenetic analysis, mitogenomes for *Cyprinus carpio* and five other cyprinids (Danioridae, Acheilognathidae, Gobionidae, Leucisidae, and Tincidae) were also obtained from GenBank (Table 1) and used as outgroups.

Mitogenomes by illumina shotgun sequencing

Specimens of *Candidia barbata*, *Opsariichthys evolans*, and *Opsariichthys pachycephalus* were collected from an upstream section of the Keelung River located in the Ruifang District of New Taipei City, Taiwan. Genomic DNA

was extracted from 100 mg of muscle tissue using a Roche DNA Isolation Kit (Indianapolis, IN, USA) following manufacturer instructions. Whole-genome shotgun sequencing was employed, and a 400-bp insert library was constructed using the Illumina standard protocol (San Diego, CA, USA). Paired-end sequencing was performed using the Illumina NextSeq system to obtain 1-2 Gb of raw reads from the libraries of *C. barbata*, *O. evolans*, and *O. pachycephalus*. The *de novo* assembly function of CLC Genomics Workbench vers. 7.0 (CLC Bio, Cambridge, MA, USA) was used to construct contigs. For each species, the mitogenome candidate contig was identified by using BLAST on all contigs to the nucleotide database downloaded from NCBI. All reads were mapped onto the

candidate contig and the mitogenome consensus sequence was extracted. MitoFish software was used to annotate protein-coding and RNA genes of the mitogenome consensus sequence (Iwasaki et al. 2013).

Phylogenetic analysis

Nucleotide sequence alignment was visually verified using BIOEDIT vers. 5.9 (Hall 2001). Sequence analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) vers. 7.0 (Kumar et al. 2016). MEGA 7.0 was also used for aligning sequences of different lengths and then manual modifications were performed before the phylogenetic analysis. All transfer (t)

Table 1. Species and their GenBank accession numbers used in this study

Species	Accession number	Source
<i>Opsariichthys</i> group		
<i>Parazacco spilurus</i>	KF971863	Chang et al. 2016
<i>Candidia barbata</i>	MG650169	This study
<i>Candidia pingtungensis</i>	KT725246	Yin et al. 2015
<i>Nipponocypris koreanus</i>	KJ427719	Chen et al. 2016a
<i>Nipponocypris sieboldii</i>	AB218898	Saitoh et al. 2006
<i>Nipponocypris temminckii</i>	KM213515	Chen et al. 2016b
<i>Zacco acanthogenys</i>	KT290890	Yin et al. 2015
<i>Zacco platypus</i>	AP012115	Miya et al. 2015
<i>Opsariichthys acutipinnis</i>	KT725245	Yin et al. 2015
<i>Opsariichthys bidens</i>	DQ367044	Wang et al. 2007
<i>Opsariichthys chengtui</i>	KT725244	Yin et al. 2015
<i>Opsariichthys evolans</i>	MG650170	This study
<i>Opsariichthys pachycephalus</i>	MG650171	This study
<i>Opsariichthys uncirostris</i>	AB218897	Saitoh et al. 2006
Xenocypridae		
<i>Hemigrammocypripis rasborella</i>	AP011422	Tang et al. 2010
<i>Metzia longinasus</i>	KF955011	Ma and Luo 2016
<i>Metzia mesembrinum</i>	NC_023797	Yuan et al. 2016
<i>Metzia formosae</i>	NC_022458	Lin et al. 2015
<i>Metzia lineata</i>	NC_031541	GenBank
<i>Aphyocypris chinensis</i>	AB218688	Saitoh et al. 2006
<i>Aphyocypris kikuchii</i>	JX184925	Jang-Liaw et al. 2013b
<i>Yaoshanicus arcus</i>	AP011398	Tang et al. 2010
<i>Nicholsicypris normalis</i>	AP011396	Tang et al. 2010
<i>Pararasbora moltrechti</i>	JX311312	Jang-Liaw et al. 2013a
<i>Macrochirichthys macrochirus</i>	NC_015551	GenBank
<i>Paralaubuca typus</i>	AP011211	Saitoh et al. 2011
<i>Ctenopharyngodon idella</i>	EU391390	Wang et al. 2008
<i>Elopichthys bambusa</i>	AP011213	Miya et al. 2015
<i>Hypophthalmichthys molitrix</i>	KJ729094	Farrington et al. 2015
<i>Hypophthalmichthys nobilis</i>	KJ746959	Farrington et al. 2015
<i>Squaliobarbus curriculus</i>	KC351187	Liu et al. 2013
<i>Xenocypris argentea</i>	AP011283	Mayden et al. 2009

Table 1. (continued)

Species	Accession number	Source
<i>Xenocypris davidi</i>	KF039718	Liu 2014
<i>Ischikauia steenackeri</i>	NC_008667	Saitoh et al. 2006
<i>Chanodichthys mongolicus</i>	KF826087	Wei et al. 2016
<i>Chanodichthys ilishaeformis</i>	NC_029722	Li et al. 2016
<i>Chanodichthys dabryi</i>	NC_021418	Zhang et al. 2014
<i>Culter erythropterus</i>	NC_024749	Chen et al. 2016
<i>Culter recurviceps</i>	NC_024277	GenBank
<i>Culter mongolicus</i>	AP009060	Saitoh et al. 2006
<i>Parabramis pekinensis</i>	KF857485	Duan et al. 2016
<i>Megalobrama amblycephala</i>	NC_010341	GenBank
<i>Megalobrama pellegrini</i>	NC_026458	Liu et al. 2016
<i>Hemiculter bleekeri</i>	NC_029831	GenBank
<i>Hemiculter leucisculus</i>	NC_022929	GenBank
<i>Hemiculter eigenmanni</i>	NC_029388	GenBank
Cyprinidae		
<i>Cyprinus carpio</i>	AP017363	Mabuchi 2016
Danionidae		
<i>Rasbora vaterifloris</i>	NC_015531	Tang et al. 2010
<i>Rasbora lateristriata</i>	NC_032723	Kusuma and Kumazawa 2016
<i>Rasbora trilineata</i>	NC_025336	Ho et al. 2016
<i>Rasbora steineri</i>	NC_020005	Chang et al. 2013
<i>Danio dangila</i>	NC_015525	Tang et al. 2010
<i>Danio erythromicron</i>	AP011419	Tang et al. 2010
<i>Danio rerio</i>	NC_002333	Broughton et al. 2001
Acheilognathidae		
<i>Acheilognathus macropterus</i>	NC_013711	Hwang et al. 2014
<i>Acheilognathus typus</i>	NC_008668	Saitoh et al. 2006
<i>Rhodeus ocellatus</i>	NC_011211	He et al. 2008
<i>Rhodeus lighti</i>	NC_024885	Wang et al. 2016
<i>Rhodeus sinensis</i>	NC_022721	Yang et al. 2015
<i>Rhodeus shitaiensis</i>	NC_022690	Li et al. 2015
<i>Tanakia limbata</i>	NC_025515	Luo et al. 2016
<i>Tanakia lanceolata</i>	NC_024566	Xu et al. 2016
Gobionidae		
<i>Hemibarbus barbus</i>	NC_008644	Saitoh et al. 2006
<i>Squalidus gracilis</i>	NC_024561	Liu et al. 2016
<i>Abbottina rivularis</i>	NC_023781	He et al. 2013
<i>Gobio gobio</i>	NC_008662	Saitoh et al. 2006
<i>Rhinogobio typus</i>	NC_024423	Yan et al. 2016
<i>Gnathopogon elongatus</i>	NC_008649	Saitoh et al. 2006
<i>Sarcocheilichthys variegatus microoculus</i>	NC_004694	Saitoh et al. 2003
Leucisidae		
<i>Leuciscus burdigalensis</i>	NC_029426	Hinsinger et al. 2015
<i>Acrocheilus alutaceus</i>	AP012086	GenBank
<i>Cyprinella lutrensis</i>	NC_008643	Saitoh et al. 2006
<i>Macrhybopsis storeriana</i>	NC_030485	Gaughan et al. 2016
Tincidae		
<i>Tinca tinca</i>	AB218686	Saitoh et al. 2006
<i>Tanichthys micagemmae</i>	NC_031631	GenBank
<i>Tanichthys albonubes</i>	NC_015539	GenBank

RNA genes were scanned with tRNAscan-SE 1.21 (Lowe and Eddy 1997).

Bayesian inference (BI) and maximum likelihood (ML) methods were employed for phylogenetic analyses in this study. ML analyses were carried out using MEGA 7.0 (Kumar et al. 2016). Branch support for ML trees were established via bootstrap analyses (with 1000 replications). The best-fit model for sequence evolution was selected using jModelTest v.2.1.3 (Darriba et al. 2012) in the BI analyses. The best-fit model of the ML analyses was selected using MEGA 7.0.

All aligned sequences were analyzed and phylogenetic trees were constructed with BI and ML methods. BI analyses were performed using MrBayes 3.0 (Ronquist and Huelsenbeck 2003) over a total of 10^6 replications. The posterior probabilities of each node were computed from the remaining 75% of all sampled trees.

RESULTS

New mitogenome annotation

The complete mitochondrial genomes of *Candidia barbata*, *Opsariichthys evolans*, and *Opsariichthys pachycephalus* were amplified and sequenced, obtaining respective lengths of 16,608, 16,656, and 16,612 bp. The complete mitochondrial genomes of these three species consisted of 37 genes, including 13 typical vertebrate protein-coding genes, 22 tRNA genes, two ribosomal (r) RNA genes, and one control region. All genes were encoded on the heavy strand except for the ND6 and eight tRNA genes (tRNA^{Gln}, tRNA^{Ala}, tRNA^{Asn}, tRNA^{Cys}, tRNA^{Tyr}, tRNA^{Ser1}, tRNA^{Glu}, and tRNA^{Pro}). An illustration of the complete mitochondrial genome of *Opsariichthys evolans* is shown in figure 2.

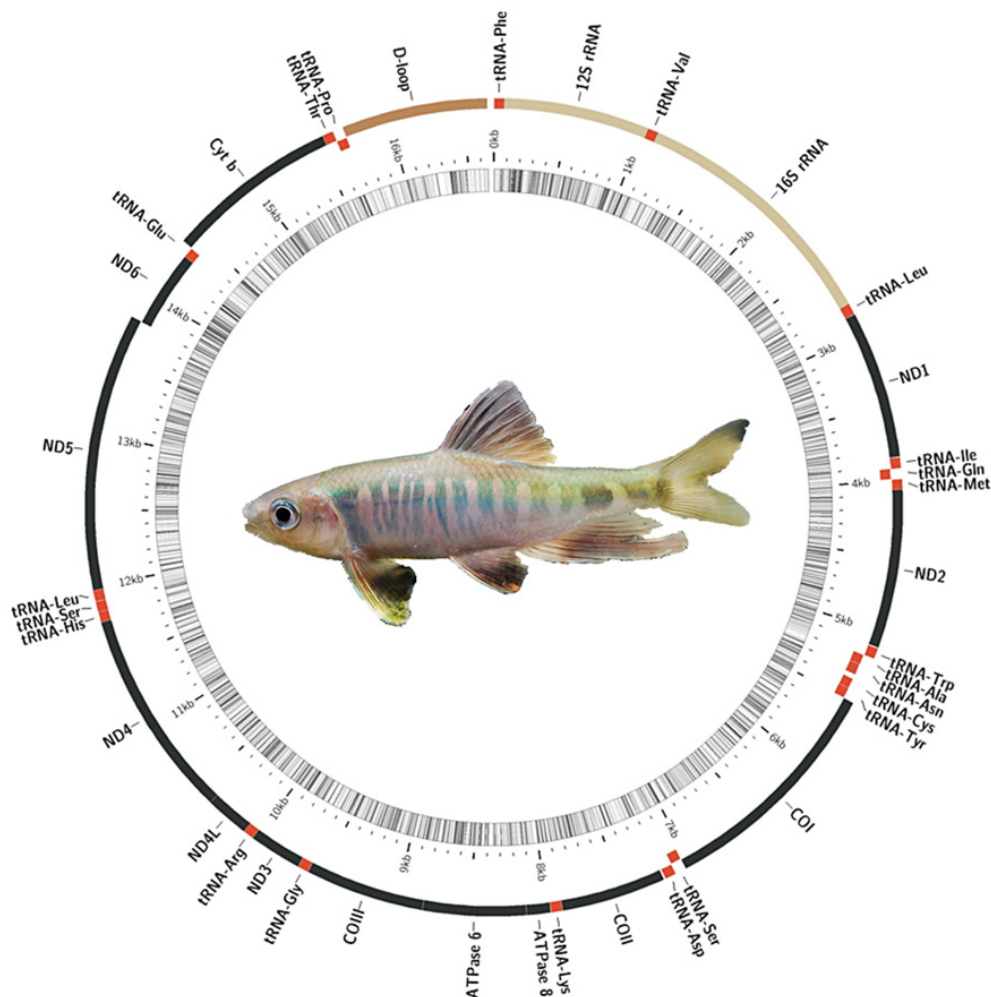


Fig. 2. Mitogenome map of *Opsariichthys evolans* as a representative species of the *Opsariichthys* group.

Molecular phylogeny of the *Opsariichthys* group

In order to assess the molecular phylogeny of Opsariichthyinae and Xenocyprididae, aligned sequences combined with 13 protein-coding genes and two rRNA genes were used. The lengths of the combined 13 protein-coding genes and two rRNA genes from 76 species were 13,903-14,087 bp in total. This alignment contained 13,287 total mutations and 7,507 polymorphic (segregating) sites, calculated by DNA sequence polymorphisms with DnaSP vers. 5 (Librado and Rozas 2009). The ML tree was reconstructed using concatenated protein-coding genes and rRNA gene sequences based on GTR+G+I models. The HKY+G models were selected as the best-fit models for the BI tree reconstructions based on the concatenated protein-coding genes and rRNA gene sequences.

The phylogenetic trees reconstructed using the BI or ML methods based on combined protein-coding and rRNA genes produced slightly different tree topologies (Figs. 3 and 4). BI tree topology revealed that Danionidae is the ancestral group. The remaining OTUs separated into two major lineages. The first one contained the sister pair Xenocyprididae and the *Opsariichthys* group, which contained five genera of Opsariichthyinae *sensu* Liao et al. 2011 (Figs. 1 and 3). The second lineage contained the four families Acheilognathidae, Leuciscidae, Tincidae, and Gobionidae. The Xenocyprididae in the first lineage can be divided into seven clades, three of which followed the traditionally accepted classification (Cultrinae, Xenocyprinae, Squaliobarbinae) while the other four of which were inconsistent (one clade for ex-Cultrinae and ex-Danioninae + ex-

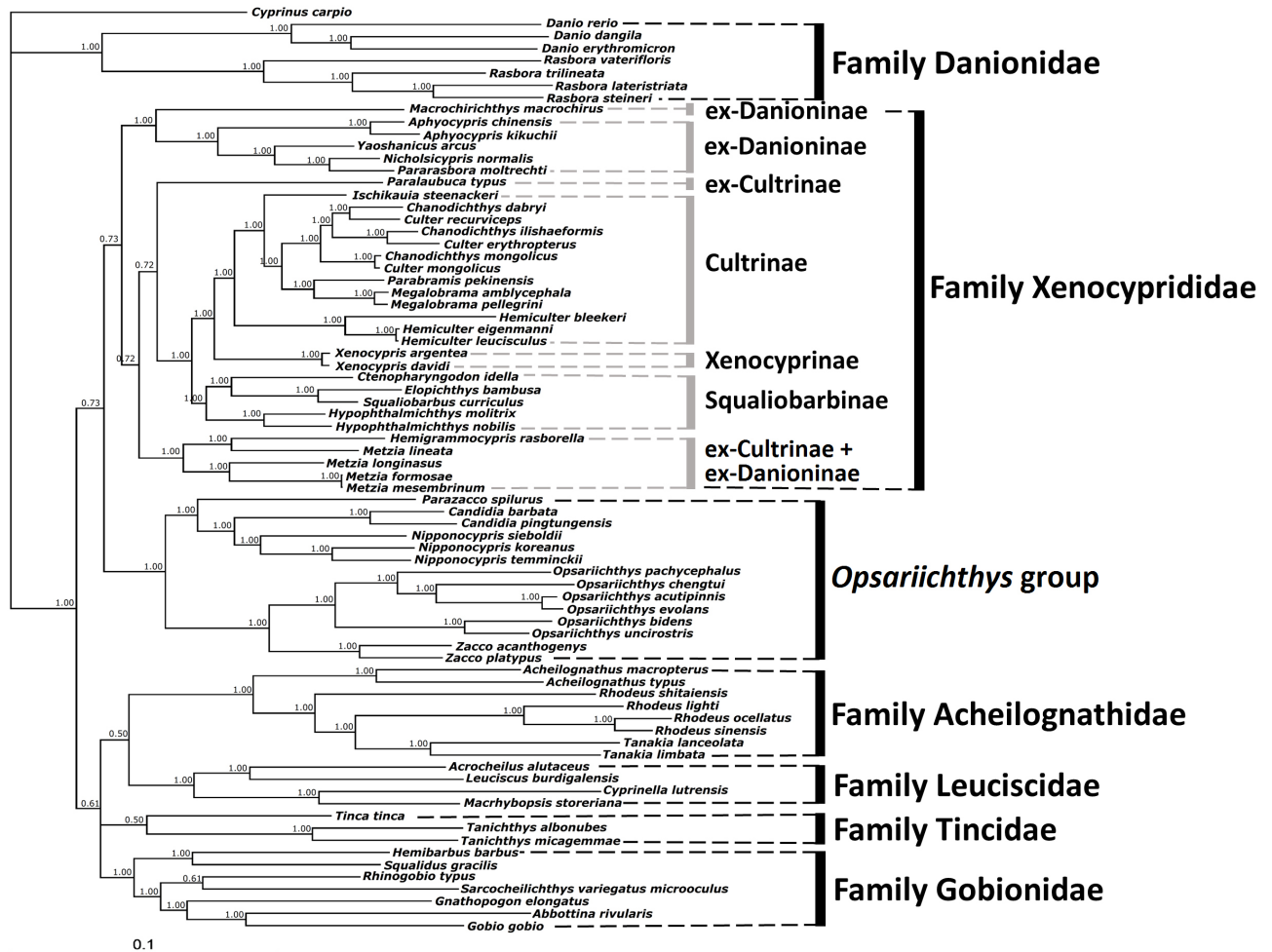


Fig. 3. Molecular phylogenetic tree of the *Opsariichthys* group and related families based on 13 concatenated protein-coding genes and two rRNA gene sequences reconstructed with Bayesian inference (values above the branch are posterior probabilities). Subfamily and family names follow those in Liao et al. (2011c), Nelson et al. (2016), Tang et al. (2013) and Stout et al. (2016).

Cultrinae, two clades for ex-Danioninae) (Fig. 3). The *Opsariichthys* group contained two major clades, with *Parazacco* being sister to *Candidia* + *Nipponocypris* in the first clade and *Zacco* and *Opsariichthys* a sister pair in the second clade (Fig. 3).

All nodes had high posterior probabilities of 0.72-1.00 in the lineage Xenocypridae + *Opsariichthys* group of the BI tree. Relatively low posterior probabilities of 0.50-0.61 occurred at the nodes among the families Acheilognathidae + Leuciscidae + Tincidae + Gobionidae. However, the posterior probability value was as high as 1.00 at the node that separated these two major lineages (Fig. 3).

Similar to the BI tree, the ML tree topology revealed that the family Danionidae was the

ancestral group but the remainders were divided into three major lineages (Fig. 4). *Macrochirichthys macrochirus* (ex-Danioninae) formed the first lineage. The second lineage contained the Xenocypridae lineage, which separated into six clades and three of six clades followed the traditionally accepted classification (Cultrinae, Xenocyprinae, Squaliobarbinae). The third lineage contained the *Opsariichthys* group, Acheilognathidae, Leuciscidae, Tincidae, and Gobionidae. The ML tree had three different groupings shown in all three lineages when compared to the BI tree in the ML tree, *Macrochirichthys macrochirus* (ex-Danioninae) formed the first lineage and was outside of the other two lineages. Moreover, the *Opsariichthys* group was sister to Acheilognathidae +

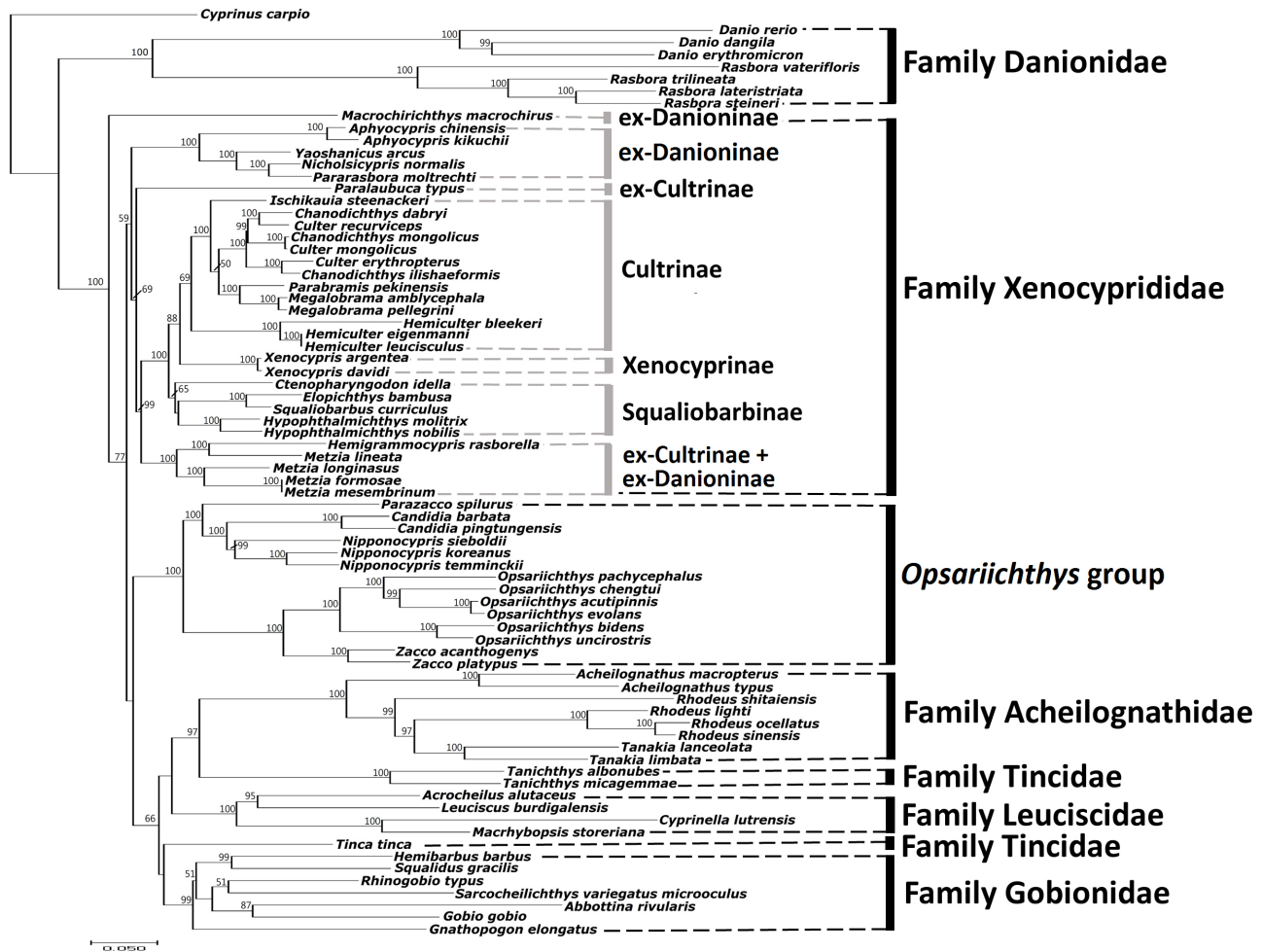


Fig. 4. Molecular phylogenetic tree of the *Opsariichthys* group and related families based on 13 concatenated protein-coding genes and two rRNA gene sequences reconstructed with the maximum likelihood method (values below the branch are bootstrap numbers, bootstrap values less than 50 not shown). Subfamily and family names follow those in Liao et al. (2011c), Nelson et al. (2016), Tang et al. (2013) and Stout et al. (2016).

Leuciscidae + Tincidae + Gobionidae. On the other hand, the ML tree revealed that Tincidae was not monophyletic; instead, it was divided into two clades, but with only low bootstrap support (Fig. 4).

In short, the BI tree revealed the *Opsariichthys* group is sister to Xenocyprididae (Fig. 3). However, the ML tree revealed the inconsistent lineage *Opsariichthys* group + Acheilognathidae + Leuciscidae + Tincidae + Gobionidae (Fig. 4). These results indicate that higher taxonomic levels might still be unsettled. Nevertheless, five genera in the *Opsariichthys* group (*Parazacco*, *Candidia*, *Nipponocypris*, *Zacco*, and *Opsariichthys*) indeed formed a monophyletic group distinct to Xenocyprididae (Stout et al. 2016) and other closely-related families in Cypriniformes (Fig. 4).

DISCUSSION

Molecular evidences (Figs. 3 and 4) reveal a monophyletic *Opsariichthys* group and further comparative morphological studies might be needed to clarify this taxonomic level in the future.

Opsariichthys group is a monophyletic group

In order to verify taxonomic placements and assignments in the *Opsariichthys* group, related species from Xenocyprididae (Stout et al. 2016; Tang et al. 2013) were included (Table 1; Fig. 1). Among the two phylogenetic trees reconstructed, the BI tree topology revealed a relatively stable and reliable grouping based on higher posterior probability values and reliable tree topology (Fig. 3). The mitogenomic phylogeny suggests that *Opsariichthys* is monophyletic and sister to Xenocyprididae. Acheilognathidae, Leuciscidae, Tincidae, and Gobionidae are closely related families as outgroups.

Our results show that *Parazacco*, *Candidia*, *Nipponocypris*, *Zacco*, and *Opsariichthys* comprise a stable monophyletic group distinct from Xenocyprididae in both BI and ML trees (Figs. 3, 4). Moreover, all these members were previously confirmed as a monophyletic group (Tang et al. 2013; Stout et al. 2016). Therefore, we propose the *Opsariichthys* group contains *Parazacco*, *Candidia*, *Nipponocypris*, *Zacco*, and *Opsariichthys*.

In addition, Liao et al. (2011b) assigned *Aphyocypris* and *Yaoshanicus* to subfamily Opsariichthyinae based on a single mitochondrial cytochrome *b* gene. Nelson et al. (2016) proposed that several additional genera, including *Candidia*,

Macrochirichthys, *Nicholsicypris*, *Nipponocypris*, *Oxygaster*, *Parachela*, *Pararosbora*, and *Parazacco*, would be assigned to Opsariichthyinae if monophyly of the entire clade is confirmed. Tree topology clearly shows that only *Parazacco*, *Candidia*, *Nipponocypris*, *Zacco*, and *Opsariichthys* should be assigned to a monophyletic group, the *Opsariichthys* group (Figs. 3 and 4). Tang et al. (2013) also showed similar results with different topology (Fig. 1).

Five genera reassigned into Xenocyprididae

Liao et al. (2011c) classified five genera into Opsariichthyinae, which was placed into Xenocyprididae in two later studies (Tang et al. 2013; Stout et al. 2016). Our present results also revealed that *Aphyocypris*, *Yaoshanicus*, *Nicholsicypris*, and *Pararosbora* are monophyletic with high support at the nodes (1.00 in BI, and 100 in ML), which was confirmed in Tang et al. (2010, 2013). *Yaoshanicus*, *Nicholsicypris*, and *Pararosbora* should be considered to be junior synonyms of *Aphyocypris* (Liao et al. 2011b). In addition, our study revealed *Hemigrammocypripis* is nested with four species of *Metzia* and congruent with previous studies (Tang et al. 2013; Stout et al. 2016).

Candidia and *Nipponocypris* are distinct genera

All valid species of *Candidia* and *Nipponocypris* were used in this study to reassess their relatedness. The BI and ML trees both showed that *Candidia* and *Nipponocypris* were well separated with high support (1.00 in BI, and 65 in ML); our results were consistent with several previous studies (Huynh and Chen 2013; Liao et al. 2011c; Tang et al. 2013). Morphologically, these two genera can be easily distinguished: *Candidia* has maxillary barbels, which are absent in *Nipponocypris* (Chen and Fang 1999; Nakabo 2013). The present study thus strongly suggests that they should be regarded as distinct genera.

Evolutionary implications of the color pattern

Among all studied species, only those in the *Opsariichthys* group have distinct longitudinal or vertical stripes. Most of them are known to have color dimorphism, especially *Opsariichthys* and *Zacco* (Chen and Chang 2005). This study's tree topologies revealed that the type of stripe pattern on the sides of the body was highly correlated with

molecular phylogeny (Fig. 5). The BI tree showed that *Parazacco*, *Candidia*, and *Nipponocypris* share similar longitudinal stripe patterns. *Opsariichthys* and *Zacco* both share similar vertical stripe patterns, although independent bars always appeared in *Opsariichthys* and the otherwise typically smaller bars are usually fused into a single wide bar in *Zacco*, but both could be defined as the same type of color pattern. Otherwise, members of Xenocypridae have only an indistinct longitudinal stripe.

These representative stripes or bars can also be found in several groups under Cypriniformes, such as *Danio*, *Crossocheilus* and *Acrossocheilus*. The genus *Danio*, a primitive cyprinids, already shows these stripe and bar patterns of the *Opsariichthys* group (McCluskey and Postlethwait 2015). Among these, *D. erythromicron* and *D. choprae* have distinct vertical stripes and *D. nigrofasciatus* and *D. rerio* have distinct longitudinal ones. Furthermore, *Danio* is the earliest offshoot in tree topologies (Tang et al. 2013; Stout et al. 2016). Therefore, we hypothesize that the types of vertical and longitudinal stripes presented in the

Opsariichthys group might have originated from an primitive ancestor, then distinct vertical stripes might have been lost among these cyprinids but retained in the *Opsariichthys* group.

CONCLUSIONS

The phylogenetic trees in this study provide a new perspective on the systematics of the *Opsariichthys* group and its sister group Xenocypridae, which includes the related taxa from the following groups, all of which are under Cyprinidae *sensu lato*: Cultrinae, Xenocyprinae, Squaliobarbinae, ex-Danioninae and ex-Cultrinae. The *Opsariichthys* group formed a stable monophyletic group, which includes five genera: *Parazacco*, *Candidia*, *Nipponocypris*, *Zacco*, and *Opsariichthys*. Our results also strongly suggest that *Candidia* and *Nipponocypris* be regarded as distinct genera within this family. Lastly, *Aphyocypris*, *Yaoshanicus*, *Nicholsicypris*, *Hemigrammocypripis* and *Pararasbora* belong to Xenocypridae.

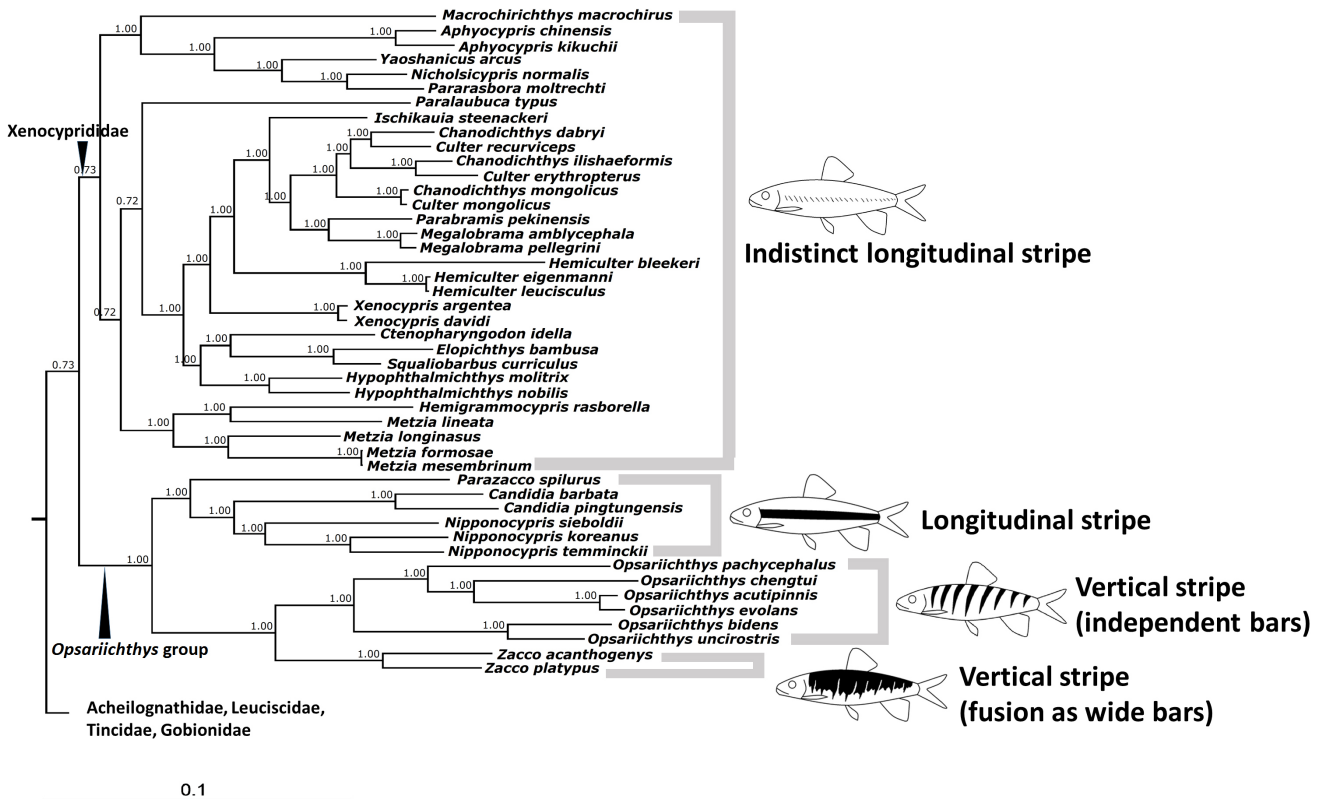


Fig. 5. Correlation between stripe patterns and molecular relationships in the *Opsariichthys* group (tree reconstructed based on Bayesian inference in this study).

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REFERENCES

- Broughton RE, Milam JE, Roe BA. 2001. The complete sequence of the zebrafish (*Danio rerio*) mitochondrial genome and evolutionary patterns in vertebrate mitochondrial genome. *Genome Res* **11**:1958-1967.
- Chang HY, Yuan LY, Hu TL, Chien CI, Lin YC, Tseng SF, Chang TW, Wang WK. 2016. Complete mitochondrial DNA genome of *Zarazacco spilurus* (Cypriniformes: Cyprinidae). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:165-166.
- Chang CH, Tsai CL, Jang-Liaw NH. 2013. Complete mitochondrial genome of the Chinese rasbora *Rasbora steineri* (Teleostei, Cyprinidae). *Mitochondrial DNA* **24**:183-185.
- Chen IS, Chang YC. 2005. The photographic guide of inland water fishes of Taiwan. Vol. I. Cypriniformes. Sheichuan Press, Keelung. (in Chinese)
- Chen IS, Fang LS. 1999. The freshwater and estuarine fishes of taiwan. National Museum of Marine Biology and Aquarium, Pingtung. (in Chinese)
- Chen IS, Liu YW, Huang SP, Shen CN. 2016a. The complete mitochondrial genome of the Korean minnow *Nipponocypris koreanus* (Cypriniformes, Cyprinidae). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:708-710.
- Chen IS, Tsai WL, Huang SP, Chen HM. 2016b. The complete mitochondrial genome of Temminck's minnow *Nipponocypris temminckii* (Cypriniformes, Cyprinidae). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:1542-1544.
- Chen L, Li B, Zhou L, Zhao G. 2016. The complete mitochondrial genome sequence of Predatory carp *Chanodichthys erythropterus* (Cypriniformes: Cyprinidae). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:1119-1120.
- Darriba D, Taboada GL, Doallo R, Posada D. 2012. Jmodeltest 2: More models, new heuristics and parallel computing. *Nat Methods* **9**:772.
- Duan X, Zhang X, Song W, Wang Y, Gao Z, Wang W. 2014. The complete mitochondrial genome sequence of *Parabramis pekinensis strenosoma* (Cypriniformes: Cyprinidae). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:86-87.
- Eschmeyer WN, Fricke R, Laan R van der (eds). 2017. CATALOG OF FISHES: GENERA, SPECIES, REFERENCES. <http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp>. Electronic version Accessed 22 Mar 2017.
- Fang F, Noren M, Liao TY, Kallersjo M, Kullander SO. 2009. Molecular phylogenetic interrelationships of the south asian cyprinid genera *Danio*, *Devario* and *Microrasbora* (Teleostei, Cyprinidae, Danioninae). *Zool Scr* **38**:237-256.
- Farrington HL, Edwards CE, Guan X, Carr MR, Baerwaldt K, Lance RF. 2015. Mitochondrial genome sequencing and development of genetic markers for the detection of DNA of invasive bighead and silver carp (*Hypophthalmichthys nobilis* and *H. molitrix*) in environmental water samples from the United States. *PLoS One* **10**(2):e0117803. doi:10.1371/journal.pone.0117803.
- Gaughan S, Johnson R, Wang J, Wachholtz M, Steffensen K, King T, Lu G. 2016. The complete mitochondrial genome of the silver chub, *Macrhybopsis storeriana*. *Mitochondrial DNA Part B Resour* **1**:789-790.
- Hall T. 2001. Bioedit: A user-friendly biological sequence alignment editor and analysis, version 5.09. Department of Microbiology, North Carolina State University, North Carolina.
- He HY, Wang J, You P. 2013. The complete nucleotide sequence of mitochondrial genome of *Abbottina rivularis* (Cypriniformes: Cyprinidae). *Acta Zootaxonomica Sinica* **38**:695-704.
- He S, Gu X, Mayden RL, Chen WJ, Conway KW, Chen Y. 2008. Phylogenetic position of the enigmatic genus *Psilorhynchus* (Ostariophysi: Cypriniformes): evidence from the mitochondrial genome. *Mol Phylogenet Evol* **47**:419-425.
- Hinsinger DD, Debruyne R, Thomas M, Denys GPJ, Menesson M, Utge J, Dettai A. 2015. Fishing for barcodes in the Torrent: from COI to complete mitogenomes on NGS platforms. *DNA Barcodes* **3**:170-186.
- Ho CW, Liu MY, Chen MH. 2016. Complete mitochondrial genome of *Rasbora trilineata* (Cypriniformes, Cyprinidae). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:1755-1757.
- Huang SP, Chen IS, Jang-Liaw NH, Shao KT, Yung MMN. 2016. Complete mitochondrial reveals a new phylogenetic perspective on the brackish water goby *Mugilogobius* group (Teleostei: Gobiidae: Gobiionellinae). *Zool Sci* **33**:566-574.
- Huang SP, Zhao YH, Chen IS, Shao KT. 2017. A new species of *Microphysogobio* (Cypriniformes: Cyprinidae) from Guangxi Province, southern China. *Zool Stud* **56**:8. doi:10.6620/ZS.2017.56-08.
- Huynh TQ, Chen IS. 2013. A new species of cyprinid fish of genus *Opsariichthys* from Ky Cung - Bang Giang River basin, northern Vietnam with notes on the taxonomic status of the genus from northern Vietnam and southern China. *J Mar Sci Tech Suppl* **21**:135-145.

- Hwang DS, Lee WO, Lee JS. 2014. Complete mitochondrial genome of the freshwater fish, *Acanthorhodeus macropterus* (Cypriniformes, Cyprinidae). *Mitochondrial DNA* **25**:11-12.
- Iwasaki W, Fukunaga T, Isagozawa R, Yamada K, Maeda Y, Satoh TP, Sado T, Mabuchi K, Takeshima H, Miya M, Nishida M. 2013. Mitofish and mitoannotator: A mitochondrial genome database of fish with an accurate and automatic annotation pipeline. *Mol Biol Evol* **30**:2531-2540.
- Jang-Liaw NH, Tsai CL, Chang CH, Watanabe K. 2013a. Complete mitochondrial genome of the Moltrecht's minnow, *Aphyocypris moltrechti* (teleostei, cyprinidae), in comparison with *A. kikuchii*. *Mitochondrial DNA* **24**:117-119.
- Jang-Liaw NH, Tsai CL, Watanabe K. 2013b. Complete mitochondrial genome of the Kikuchi's minnow *Aphyocypris kikuchii* (Teleostei, Cyprinidae). *Mitochondrial DNA* **24**:11-13.
- Kottelat M. 2013. The fishes of the Inland Waters of Southeast Asia: A catalogue and core bibliography of the fishes known to occur in freshwaters, mangroves and estuaries. *Raffles B Zool* **27**:1-663.
- Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Bio Evol* **33**:1870-1874.
- Kusuma WE, Kumazawa Y. 2016. Complete mitochondrial genome sequences of two Indonesian rasboras (*Rasbora aprotaenia* and *Rasbora lateristriata*). *Mitochondrial DNA Part A* **27**:4222-4223.
- Li F, Shao KT, Lin YS, Chang CH. 2015. The complete mitochondrial genome of the *Rhodeus shitaiensis* (Teleostei, Cypriniformes, Acheilognathidae). *Mitochondrial DNA* **26**:301-302.
- Li S, Liu X, Bai Y, Huang T, Wang J, Qu H, Chen L, Jiang W. 2016. The complete mtDNA genome of *Erythroculter ilishaeformis*: genome characterization and phylogenetic analysis. *Mitochondrial DNA Part B* **1**:39-40.
- Liao TY, Kullander SO, Fang F. 2011a. Phylogenetic position of rasborin cyprinids and monophyly of major lineages among the danioninae, based on morphological characters (Cypriniformes: Cyprinidae). *J Zool Syst Evol Res* **49**:224-232.
- Liao TY, Kullander SO, Lin HD. 2011b. Synonymization of *Pararasbora*, *Yaoshanicus*, and *Nicholsicypris* with *Aphyocypris*, and description of a new species of *Aphyocypris* from Taiwan (Teleostei: Cyprinidae). *Zool Stud* **50**:657-664.
- Liao TY, Unlu E, Kullander SO. 2011c. Western boundary of the subfamily danioninae in Asia (Teleostei, Cyprinidae): Derived from the systematic position of *barilius mesopotamicus* based on molecular and morphological data. *Zootaxa* **2880**:31-40.
- Librado P, Rozas J. 2009. Dnasp v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**:1451-1452.
- Lin HD, Lin FJ, Chiang TY, Lee TW. 2015. The complete mitochondrial genome sequence of *Metzia formosae* (Cypriniformes, Cyprinidae). *Mitochondrial DNA* **26**:257-258.
- Liu GD, Chen IS, Zhu JQ, Shen CN. 2016. The complete mitochondrial genome of small sliver gudgeon *Squalidus gracilis* (Teleostei, Cyprinidae). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:603-604.
- Liu QL, Xu BH, Xiao TY, Su JM, Yao YB, Liu YJ. 2013. Complete mitochondrial genome of the Xiangjiang barbel chub *Squaliobarbus curriculus*: comparative analysis of the genetic variation associated with geographical population. *Mitochondrial DNA* **24**:654-656. doi:10.3109/19401736.2013.772167.
- Liu X, Xiao K, Zhao X, Liu J, Zhang X, Guo W, Chen L, Du H. 2016. The complete mitochondrial genome of the *Megalobrama pellegrini* (Teleostei: Cyprinidae). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:3069-3070.
- Liu Y. 2014. The complete mitochondrial genome sequence of *Xenocypris davidi* (Bleeker). *Mitochondrial DNA* **25**:374-376. doi:10.3109/19401736.2013.809429.
- Luo Y, Cao X, Zhu Y. 2016. The complete mitochondrial genome of *Tanakia limbata* (Cypriniformes: Cyprinidae). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:1713-1714.
- Lowe TM, Eddy SR. 1997. tRNAscan-SE: A program for improved detection of transfer RNA genes in genomic sequence. *Nucl Acid Res* **25**:955-964.
- Ma Q, Luo W. 2016. The complete mitochondrial genome of a cyprinid fish; *Metzia longinasus* (Teleostei, Cypriniformes). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:185-186.
- Mabuchi K. 2016. Complete mitochondrial genomes of five introduced strains of common carp (*Cyprinus carpio*) in Japan with 29 diagnostic SNPs distinguishable by restriction enzyme analysis. *Mitochondrial DNA Part B* **1**:261-263.
- Mayden RL, Chen WJ, Bart HL, Dosey MH, Simons AM, Tang KL, Wood RM, Agnew MK, Yang L, Hirt MV, Clements MD, Saitoh K, Sado T, Miya M, Nishida M. 2009. Reconstructing the phylogenetic relationships of the earth's most diverse clade of freshwater fishes - order Cypriniformes (Actinopterygii: Ostariophysii): A case study using multiple nuclear loci and the mitochondrial genome. *Mol Phylogenet Evol* **51**:500-514.
- McCluskey BM, Postlethwait JH. 2015. Phylogeny of zebrafish, a "model species," within *danio*, a "model genus". *Mol Biol Evol* **32**:635-652.
- Miya M, Sato Y, Fukunaga T, Sado T, Poulsen JY, Sato K, Minamoto T, Yamamoto S, Yamanaka H, Araki H, Kondoh M, Iwasaki W. 2015. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. *R Soc Open Sci* **2**(7):150088. doi:10.1098/rsos.150088.
- Nakabo T. 2013. Fishes of Japan with pictorial keys to the species. Third edition ed. Tokai University Press, Tokyo. (in Japanese)
- Nelson JS, Grande TC, Wilson MV. 2016. Fishes of the world. John Wiley & Sons.
- Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**:1572-1574.
- Saitoh K, Miya M, Inoue JG, Ishiguro NB, Nishida M. 2003. Mitochondrial genomics of ostariophysan fishes: perspectives on phylogeny and biogeography. *J Mol Evol* **56**:464-72.
- Saitoh K, Sado T, Dosey MH, Bart Jr HL, Inoue JG, Nishida M, Mayden RL, Miya M. 2011. Evidence from mitochondrial genomics supports the lower Mesozoic of South Asia as the time and place of basal divergence of cypriniform fishes (Actinopterygii: Ostariophysii). *Zool J Linn Soc* **161**:633-662.
- Saitoh K, Sado T, Mayden RL, Hanzawa N, Nakamura K,

- Nishida M, Miya M. 2006. Mitogenomic evolution and interrelationships of the Cypriniformes (Actinopterygii: Ostariophysi): The first evidence toward resolution of higher-level relationships of the world's largest freshwater fish clade based on 59 whole mitogenome sequences. *J Mol Evol* **63**:826-841.
- Stout CC, Tan M, Lemmon AR, Lemmon EM, Armbruster JW. 2016. Resolving Cypriniformes relationships using an anchored enrichment approach. *BMC Evol Biol* **16**:244.
- Tang KL, Agnew MK, Hirt MV, Lumbantobing DN, Sado T, Teoh VH, Yang L, Bart HL, Harris PM, He S, Miya M, Saitoh K, Simons AM, Wood RM, Mayden RL. 2013. Limits and phylogenetic relationships of East Asian fishes in the subfamily Oxygastrinae (Teleostei: Cypriniformes: Cyprinidae). *Zootaxa* **3681**:101-135.
- Tang KL, Agnew MK, Hirt MV, Sado T, Schneider LM, Freyhof J, Sulaiman Z, Swartz E, Vidthayanon C, Miya M, Saitoh K, Simons AM, Wood RM, Mayden RL. 2010. Systematics of the subfamily Danioninae (Teleostei: Cypriniformes: Cyprinidae). *Mol Phylogenet and Evol* **57**:189-214.
- Tsao YF, Lin WW, Chang CH, Ueda T, Jang-Liaw NH, Zhao YH, Kao HW. 2016. Phylogeography, historical demography, and genetic structure of the rose bitterling, *Rhodeus ocellatus* (Kner, 1866) (Cypriniformes: Acheilognathidae), in East Asia. *Zool Stud* **55**:49. doi:10.6620/ZS.2016.55-49.
- Wang B, Wang S, Hu M, Wang F. 2016. Complete mitochondrial genome of *Rhodeus lighti* (Cypriniformes: Cyprinidae). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:1497-1498.
- Wang C, Chen Q, Lu G, Xu J, Yang Q, Li S. 2008. Complete mitochondrial genome of the grass carp (*Ctenopharyngodon idella*, Teleostei): insight into its phylogenetic position within Cyprinidae. *Gene* **424**:96-101.
- Wang X, Wang J, He S, Mayden RL. 2007. The complete mitochondrial genome of the Chinese hook snout carp *Opsariichthys bidens* (Actinopterygii: Cypriniformes) and an alternative pattern of mitogenomic evolution in vertebrate. *Gene* **399**:11-19.
- Wei M, Yang Y, Yu P, Pan D, Wan Q. 2016. The complete mitochondrial genome of Mongolian redbfin, *Chanodichthys mongolicus* (Cypriniformes: Cyprinidae): genome description and related phylogenetic analyses. *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:20-21.
- Wu HW. 1977. The cyprinid fishes of China. People's Press, Shanghai. (in Chinese)
- Xu X, Cao X, Zhu Y. 2016. The complete mitochondrial genome of *Tanakia lanceolata* (Cypriniformes: Cyprinidae). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:867-868.
- Yan SX, Xiong MH, Zhu B, Tian H, Liao XL, Shao K. 2016. The complete mitochondrial genome of *Rhinogobio typus* (Teleostei, Cyprinidae, Gobioninae). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:698-699.
- Yang X, Ma Z, Xie L, Yang R, Shen J. 2015. Complete mitochondrial genome of the Chinese bitterling *Rhodeus sinensis* (Cypriniformes: Cyprinidae). *Mitochondrial DNA* **26**:647-648.
- Yin W, Cao K, He H, Fu C. 2015. Four complete mitochondrial genomes of the genera *Candidia*, *Opsariichthys*, and *Zacco* (cypriniformes: Cyprinidae). *Mitochondrial DNA Part A* **27**:4613-4614.
- Yuan LY, Wang WK, Lee CH, Chiu KH, Li YM, Kuo PC, Chang HY. 2016. Complete mitochondrial DNA genome of *Metzia mesembrinum* (Cypriniformes: Cyprinidae). *Mitochondrial DNA A DNA Mapp Seq Anal* **27**:214-215.
- Zhang X, Ma Z, Chen D, Xu W, Yang R. 2014. The complete mitochondrial genome of *Culter dabryi* (Cyprinidae: Cultrinae). *Mitochondrial DNA* **25**:98-99.