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Short Communication

Phylogenetic position of the enigmatic genus *Psilorhynchus* (Ostariophysii: Cypriniformes): Evidence from the mitochondrial genome

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1. Introduction

The Order Cypriniformes contains the planet's largest monophyletic group of freshwater fishes, with over 400 genera and 3000 species native to Asia, Europe, Africa, and North America (Nelson, 2006). Many of the species are of tremendous cultural, economic and scientific importance and because of this the order has received much recent attention in the area of molecular systematics (Šlechtová et al., 2005; Saitoh et al., 2006; Mayden et al., 2007; Rüber et al., 2007).

The order is currently divided into two superfamilies (following Siebert, 1987), the Cobitoidea, inclusive of the Balitoridae, Catostomidae, Cobitidae (sometimes recognized as two separate families: the Cobitidae and Botiidae; following Šlechtová et al., 2005) and Gyrinocheilidae, and the Superfamily Cyprinoidea, with the Cyprinidae and Psilorhynchidae (Nelson, 2006). Of all the families of cypriniforms the Psilorhynchidae, with one genus *Psilorhynchus* McClelland, has received least attention and has been the most problematic in its phylogenetic placement.

Psilorhynchus was created by McClelland (1839) and currently contains eight described species, *P. sucatio* (Hamilton, 1822), *P. balitora* (Hamilton 1822), *P. homaloptera* Hora and Mukerji, 1935, *P. pseudocheneis* Menon and Datta, 1964, *P. gracilis* Rainboth, 1983, *P. microphthalmus*

Vishwanath and Manojkumar, 1995, *P. arunachalensis* (Nebeshwar et al., 2007) and *P. amplicephalus* Arunachalam, Muralidharan and Sivakumar, 2007. The genus is restricted to the fast flowing streams of the Ganga–Brahmaputra Drainage of India, Nepal and Bangladesh and the Ayeyarwady River of Northern Myanmar and adjacent China (Rainboth, 1983). All of the species possess arched backs and flattened ventral surfaces, presumably adaptations for a benthic rheophilic lifestyle. On occasion *P. homaloptera*, *P. pseudocheneis* and *P. arunachalensis* are placed in a separate genus, *Psilorhynchoides* Yazdani, Singh and Rao (Yazdani et al., 1990; Nebeshwar et al., 2007) but this is not generally accepted and for the purpose of this investigation all species will be retained within *Psilorhynchus*.

The placement of the genus *Psilorhynchus* within existing cypriniform classifications has always been problematic (Conway and Mayden, 2007). Over the last century the genus has been placed in four different families (Balitoridae, Cobitidae, Cyprinidae, and Psilorhynchidae) and two subfamilies of the Cyprinidae (the Cyprininae and the Psilorhynchinae). Prior to Conway and Mayden (2007), no phylogenetic study, inclusive of *Psilorhynchus*, had been conducted outside of a doctoral dissertation (Siebert, 1987).

McClelland (1839) associated the genus with *Homaloptera*, a genus composed of morphologically similar species that occupy similar ecological niches. Day (1871) assigned *Psilorhynchus* to the subfamily Cyprininae, a group containing a highly diverse assemblage of species. Jordan

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(1919) placed *Psilorhynchus* in the synonymy of *Homaloptera*, but later (Jordan, 1923), rather confusingly, placed it in the Cobitidae. Hora (1920) carried out the first revision of *Psilorhynchus*. Later Hora (1925) removed *Psilorhynchus* from Cyprinidae, but refrained from placing the genus into the Homalopteridae (=Balitoridae after Kottelat, 1988) or the Cobitidae, and instead created a new family, the Psilorhynchidae, in light of their unique morphology. The erection of Psilorhynchidae has been widely accepted (Ramaswami, 1952; Yazdani et al., 1990), though Chen (1981) choose to place *Psilorhynchus* in a subfamily of the Cyprinidae, Psilorhynchinae, based on examination of *P. homaloptera*, a decision followed by Wu et al. (1981). Conway and Mayden (2007) reevaluated the osteological characters of the gill arches used in the unpublished work of Siebert (1987), a study that included species of *Psilorhynchus*. In this most recent and revised analysis of osteological characters of the gill arches, *Psilorhynchus* was identified as the sister group to a clade including cobitid and balitorid loaches, but bootstrap support for this relationship was weak (BS = 55). Other important works on the order, some of which examined *Psilorhynchus*, refrained from comment on the evolutionary relationships of the genus (Chen et al., 1984; Cavender and Coburn, 1992; Howes, 1991). Nelson (2006) chose to place the genus back under Psilorhynchidae, contrary to the previous (1994) edition which placed the genus within the cyprinid subfamily Psilorhynchidae, without discussion or supporting phylogenetic evidence.

Because of the extreme morphological divergence characteristic of *Psilorhynchus*, the sister group relationships of this genus is of extreme interest to systematic ichthyologists, fish biologists, and evolutionary biologists alike. The traditional phenetic or pure divergence philosophy of classifying this genus as to its rank has not been successful because of the extreme morphological divergence of its included species. Curole and Kocher (1999) argued that mitochondrial genome sequences are most informative at deep nodes in phylogenetic studies, and hence the resolution of supraspecific taxa. Herein, for the first time, we incorporate nearly complete mitochondrial genome sequences of a species of *Psilorhynchus* in a phylogenetic analysis including representatives of all families of the Order Cypriniformes. In addition we provide a new mitochondrial genome for *Misgurnus anguillicaudatus*.

2. Methods

2.1. Sequence analysis

Total genomic DNA was isolated from specimens of *Psilorhynchus homaloptera*, *Gyrinocheilus aymonieri*, and *Misgurnus anguillicaudatus*; other gene sequences from whole mt-genomes were obtained from GenBank. Ingroup taxa and sources of sequences used in this study are provided in Table 1. Gene content and arrangement corresponds to the mitochondrial genome of common carp

(*Cyprinus carpio*) (X61010). Protein-coding genes were identified by similarity of predicted amino-acid sequence with known mitochondrial protein sequences. Five species from other families of Ostariophysi were used as outgroups, including *Pseudobagrus tokiensis* (AB054127), *Phenacogrammus interruptus* (AB054129), *Eigenmannia* sp. (AB054131), *Chanos chanos* (AB054133), and *Gonorynchus greyi* (AB054134). Total genomic DNA was extracted from muscle tissue using QIAamp tissue kit (Qiagen, Germany) following manufacture's protocol. The mitochondrial genome DNA was amplified in its entirety using long PCR (Miya and Nishida, 1999) with primers of Miya and Nishida (2000) and Inoue et al. (2001) for two reactions. Long PCR was performed in a PTC-100 programmable thermal controller (MJ Research, USA); reactions were carried out in 25 μ l reaction volume containing 2.5 μ l of 10 \times LA PCR buffer II (Takara), 0.8 mM of dNTPs, 2.5 mM of MgCl₂, 0.5 mM of each primer, 0.625 U LA *Taq* polymerase (Takara) and approximately 20 ng template DNA. The thermal cycle profile was: pre-denaturation at 94 $^{\circ}$ C for 2 min and 30 cycles of denaturation at 98 $^{\circ}$ C for 10 s, annealing and extension combined at the same temperature (68 $^{\circ}$ C) for 16 min, and electrophoresis on a 0.8% agarose gel (Promega, USA). The long PCR products were diluted in sterilized distilled water for subsequent use as PCR templates. We used 24 different primer pairs that amplify contiguous, overlapping segments to amplify the entire mitochondrial genome. Eleven primer pairs were versatile, based on the complete mitochondrial genome of six ray-finned fish species, following Miya and Nishida (2000). PCR reactions were carried out in 25 μ l reaction volume containing 2.5 μ l of 10 \times PCR buffer (Takara), 0.4 mM of dNTPs, 1.8 mM of MgCl₂, 0.2 mM of each primer, 1 U of *Taq* polymerase (Takara) and 1.0 μ l of long PCR products as template. The thermal cycle profile was: pre-denaturation at 94 $^{\circ}$ C for 2 min and 30 cycles of denaturation at 94 $^{\circ}$ C for 15 s, annealing at 52 $^{\circ}$ C for 15 s, extension at 72 $^{\circ}$ C for 30 s, and final extension at 72 $^{\circ}$ C for 5 min. PCR products were electrophoresed on 1.0% agarose gel (Promega). Double strand PCR purified products were subsequently used for direct cycle sequencing with dye-labeled terminators (ABI). PCR primers were used for sequencing. All sequencing reactions were performed according to the manufacturer's instructions. Labeled fragments were analyzed on a model MegaBACE 1000 DNA sequencer (GE Healthcare Biosciences, USA).

2.2. Phylogenetic analysis

Multiple alignments were prepared for all sequences using Clustal X (Thompson et al., 1994) at default settings. We used Gblocks (Castresana, 2000) to extract regions of defined sequence conservation from the gene specific alignments and generated a single file of concatenated conserved regions. Phylogenetic analyses were performed using maximum parsimony (MP) as implemented in PAUP*—version 4.0b10 (Swofford, 2002) and partitioned Bayesian

Table 1
The mitochondrial genome sequences for analysis in this study

Species	Source (voucher Specimen)	Accession No.
Order Cypriniformes		
Family Cyprinidae		
<i>Opsariichthys biden</i>	Wang et al. (NRMT20042)	DQ367044
<i>Mylopharyngodon piceus</i>	Peng et al. (NRMT2071)	DQ026435
<i>Megalobrama amblycephalus</i>	Peng et al. (IHB305004)	DQ026433
<i>Sarcocheilichthys variegates microoculus</i>	Saitoh et al. (2003)	AB054124
<i>Distoechodon tumirostris</i>	Peng et al. (IHB303084)	DQ026431
<i>Rhodeus</i> sp.	Peng et al. (IHB305003)	DQ026430
<i>Danio rerio</i>	Broughton et al. (2001)	AC024175
<i>Cyprinus carpio</i>	Chang et al. (1994)	X61010
<i>Carassius auratus langsdorffi</i>	Murakami et al. (1997)	AB006953
Family Psilorhynchidae		
<i>Psilorhynchus homaloptera</i>	Aquarium trade	DQ026436*
Family Catostomidae		
<i>Myxocyprinus asiaticus</i>	Peng et al. (IHB305001)	AY986503
<i>Carpiodes carpio</i>	Broughton and Reneau (2006)	NC005257
Family Balitoridae		
<i>Crossostoma lacustre</i>	Tzeng et al. (1992)	M91245
Family Cobitidae		
<i>Cobitis striata</i>	Saitoh et al. (2003)	AB054125
<i>Lefua echigonia</i>	Saitoh et al. (2003)	AB054126
<i>Misgurnus anguillicaudatus</i>	This study (N/A)	DQ026434*
Family Gyrinocheilidae		
<i>Gyrinocheilus aymonieri</i>	This study (IHB305002)	DQ026432*
Out-group		
<i>Pseudobagrus tokiensis</i>	Saitoh et al. (2003)	AB054127
<i>Phenacogrammus interruptus</i>	Saitoh et al. (2003)	AB054129
<i>Eigenmannia</i> sp.	Saitoh et al. (2003)	AB054131
<i>Chanos chanos</i>	Saitoh et al. (2003)	AB054133
<i>Gonorynchus greyi</i>	Saitoh et al. (2003)	AB054134

* New sequence in this study.

inference (BI) as implemented in MrBayes parallel version v3.1.1 (Huelsenbeck and Ronquist, 2001). For each MP search, optimal trees were obtained by heuristic searches with random stepwise addition sequences followed by TBR swapping for 100 replications (Swofford, 2002). Node support was assessed using the bootstrap procedure (Felsenstein, 1985) under MP criterion, based on 100 pseudo-replicates of heuristic searches as described above. Likelihood ratio tests (Goldman, 1993), as implemented in MrModeltest 2.2 (Nylander, 2004), were used to choose models for each gene partition in Partitioned BI. Partitions were defined based on putative gene functional constraints and selective pressures. We assigned separate properties to each protein-coding gene, to each ribosomal RNA gene (12S and 16S), and to all tRNA genes together. Three data

matrices (1, using structure DNA sequences only; 2, using protein-coding gene sequences only; and 3, using all aligned sequences, excluding D-Loop region) were used in phylogenetic analyses (Fig. 1). In addition, as phylogenetic analyses of protein-coding genes can be biased from homoplasy at third codon positions due to multiple substitutions in transitions (Saitoh et al., 2006) and/or due to base composition bias across taxa (Lockhart et al., 1994; Chen et al., 2003), we also used a “down-weighting scheme” for the third codon position. At this position this scheme employed converting nucleotides A and G into purine (R) and T and C into pyrimidine (Y) (or RY-coding) for both MP and BI (Fig. 1).

For each partitioned BI, four independent MCMC chains were performed with 3×10^6 replicates, sampling one tree per 100 replicates for each run. The results from two runs were compared to evaluate the degree of convergence in tree topologies and in log likelihoods. This procedure was repeated until stationarity was observed. Initial trees with non-stationary log likelihood values were discarded as part of burn-in; the remaining trees from two independent runs were used to construct a 50% majority rule consensus tree. Resulting *a posteriori* probabilities were used as a measure of node support in addition to the measures through MP bootstrap.

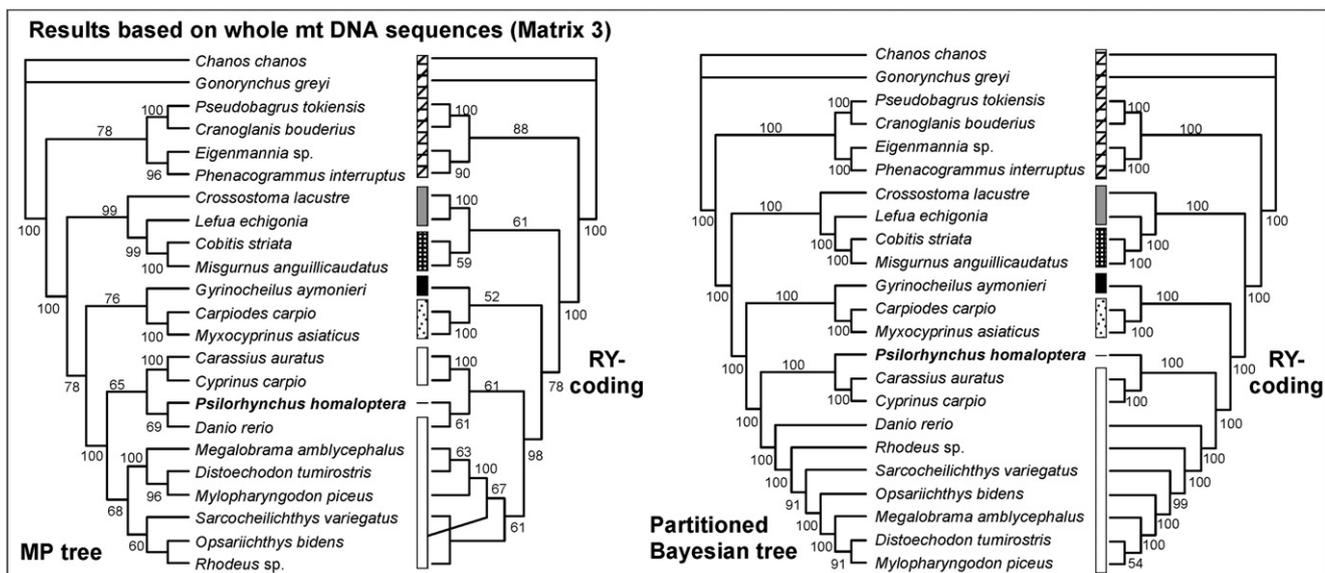
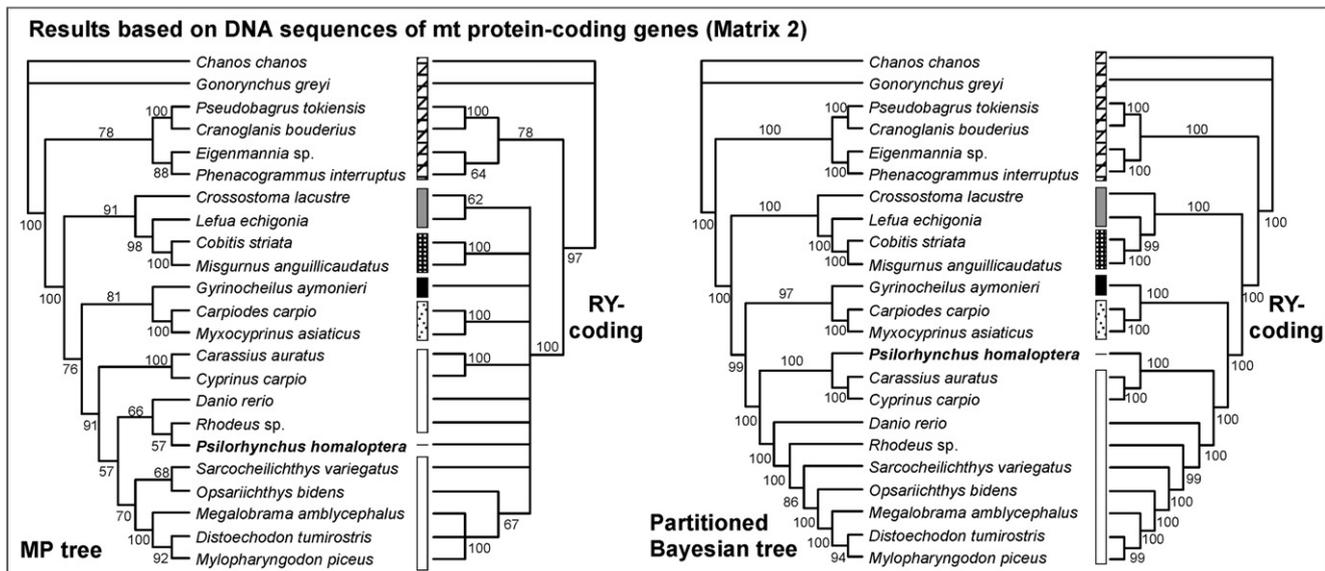
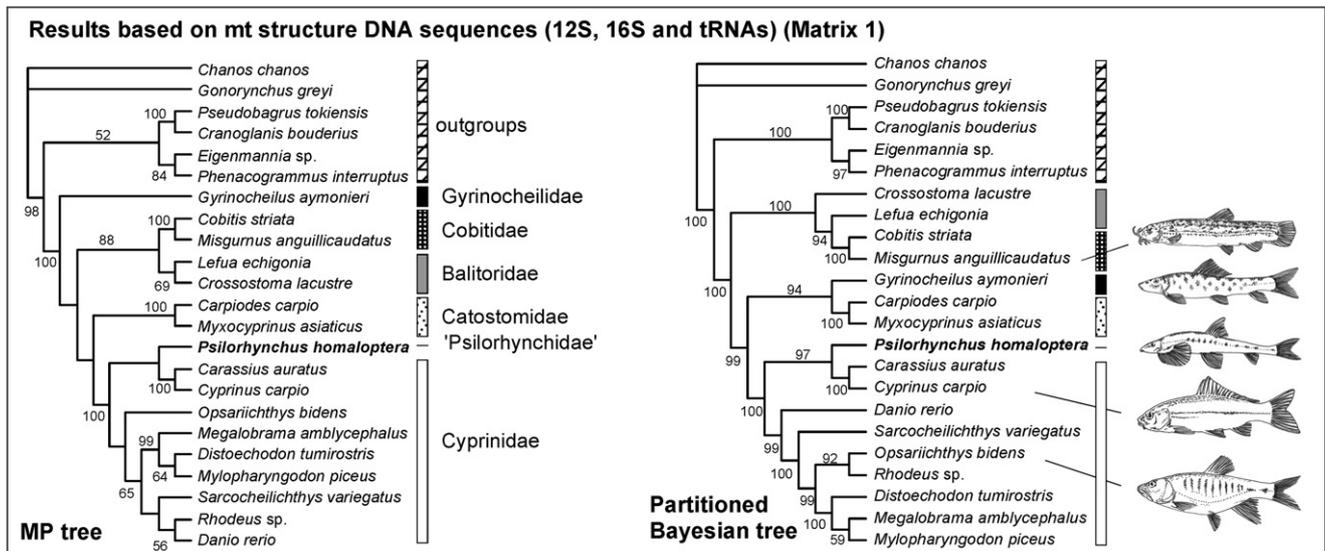
3. Results

A total of 14,768 bp were aligned in the mitogenome sequences for all taxa. Of these, 7174 sites were constant and 6428 were phylogenetically informative. MP analysis yielded a single tree (Tree length, 36473; CI, 0.36; RI, 0.38; Fig. 1).

Mitogenome sequences provided well resolved relationships within the order, except for MP analyses of protein-coding genes under RY-coding; BI analyses were not influenced by the RY-coding (Fig. 1). Furthermore, it is apparent that much of the phylogenetic structure within the order in MP analyses is derived from information at third-codon positions and ribosomal genes.

In the resulting phylogenies of all analyses the Cypriniformes was supported as a monophyletic group (Fig. 1). Within the order none of the analyses supported the traditionally recognized Superfamily Cobitoidea (= Gyrinocheilidae + Catostomidae + Cobitidae + Balitoridae). Gyrinocheilidae was either the sister group to Catostomidae (most analyses) or the basal sister group within Cypriniformes (MP of ribosomal genes only), although the latter resolution had no support. In the former trees Gyrinocheilidae plus Catostomidae was sister to the monophyletic Cyprinoidea, family Cyprinidae plus *Psilorhynchus*. The Balitoridae was always resolved as a paraphyletic group relative to the monophyletic Cobitidae.

In all BI analyses, including the RY-coding scheme, and in MP analysis of ribosomal genes *Psilorhynchus* was strongly supported as occurring within the Cyprinoidea (Cyprinidae) sister to *Cyprinus carpio* (common carp) plus



Carassius auratus (goldfish), two representatives of the Tribe Barbini of the Subfamily Cyprininae (Chen et al., 1984). This clade was well supported as the basal-most lineage within the family. In MP analyses of protein-coding genes and with all genes combined *Psilorhynchus* was either sister to *Rhodeus* or *Danio*, respectively, suggesting likely long-branch attraction of *Psilorhynchus* in the non-model-based MP analyses. In neither of these analyses was there strong branch support for the placement of *Psilorhynchus*.

In addition to *Psilorhynchus*, *Cyprinus*, and *Carassius*, Cyprinidae is represented by an additional seven species, together representing the purported subfamilies (sensu Howes, 1991) Acheilognathinae (*Rhodeus*), Cultrinae (*Megalobrama*), Cyprininae (*Mylopharyngodon*), Gobioninae (*Sarcocheilichthys*), Leucisinae (*Distoechodon*), and Rasborinae (*Opsariichthys*, *Danio*). In addition to the Barbini, all phylogenetic resolutions also supported the historically proposed major Cyprinidae clade Leuciscini (Chen et al., 1984), that, in this study, included species endemic to eastern Asian (*Opsariichthys biden*, *Mylopharyngodon piceus*, *Megalobrama amblycephalus*, *Distoechodon tumirostris*), from northern cold water habitats (*Sarcocheilichthys variegates microoculus*, *Rhodeus* sp.), and the model organism species *Danio rerio* (zebrafish). Relationships amongst the different subfamilies varied depending upon the analysis, however strongest support for relationships was observed in BI of protein-coding and total sequences. For both of these analyses, including RY-coding, neither Cyprininae nor Rasborinae was monophyletic. *Danio* (“Rasborinae”) formed the basal sister-group to the other taxa and ladderized sister group relations of Acheilognathinae (*Rhodeus*), Gobioninae (*Sarcocheilichthys*), “Rasborinae” (*Opsariichthys*), Cultrinae (*Megalobrama*), and Leucisinae (*Distoechodon*) plus “Cyprininae” (*Mylopharyngodon*) (Fig. 1). BI of ribosomal genes revealed the clade Acheilognathinae (*Rhodeus*) plus “Rasborinae” (*Opsariichthys*) sister to the clade Leucisinae (*Distoechodon*) sister to Cultrinae (*Megalobrama*) plus “Cyprininae” (*Mylopharyngodon*). The only relationships in MP analysis with substantive nodal support included the clade Cultrinae (*Megalobrama*), sister to Leucisinae (*Distoechodon*) plus “Cyprininae” (*Mylopharyngodon*) in analyses of protein-coding genes and the total matrix (Fig. 1).

4. Discussion

DNA sequences have been extremely important in elucidating relationships of species and supraspecific groups of all forms of life, particularly those that have been difficult to resolve using only morphological characters. Because species of *Psilorhynchus* are extremely divergent morphologi-

cally from other members of the order, the application of molecular data and phylogenetic methods are proposed to be of high value in resolving their relationships at higher levels.

Liu et al. (2002), using mitochondrial control region, reconstructed relationships within Cypriniformes and placed Cyprinidae as sister to all other families with Balitoridae sister to Cobitidae, but neither Psilorhynchidae nor Gyrinocheilidae were represented. Saitoh et al. (2003) based on whole mitochondrial genome sequences, reconstructed the phylogeny of ostariophysan fishes but among the Cypriniformes, only Cyprinidae, Cobitidae and Balitoridae were included. Later, Saitoh et al. (2006) examined additional diversity within the Cypriniformes for whole mitogenome sequences but did not include *Psilorhynchus*. Thus, with the exception of the morphological analysis of Conway and Mayden (2007), wherein the genus was considered to be closely related to certain members of the Cobitoidea (based on gill-arch osteology), the phylogenetic position of this enigmatic genus has yet to be tested within a phylogenetic framework with potentially informative molecular characters.

Molecular analyses using model-based and partitioned BI analyses of either mitochondrial ribosomal genes, protein-coding genes, or combined data identify *Psilorhynchus* as closely related to members of the subfamily Cyprininae. *Psilorhynchus* exhibits a high degree of molecular divergence, analogous to its observed morphological divergence, and is highly divergent genetically from members of the Cyprinidae examined herein. The varied relationships of *Psilorhynchus* in MP analyses, relative to BI analyses, is likely indicative of erroneous relationships due to long-branch attraction accompanying its genetic divergence relative to *Danio* and *Rhodeus*.

The results of these analyses differ somewhat from the results of previous molecular investigations (Saitoh et al., 2006). For example, the Superfamily Cobitoidea was not recovered as a monophyletic grouping as Gyrinocheilidae and Catostomidae were found to be more closely related to members of the Cyprinoidea (Cyprinidae and *Psilorhynchus*) than to other cobitoids (Balitoridae and Cobitidae). Members of the Cobitidae and Balitoridae form a monophyletic group, sister to all other cypriniforms examined, but the Balitoridae (*Crossostoma lacustre* and *Lefua echigonia*) was not recovered as monophyletic. Recent independent analyses using multiple nuclear and mitochondrial genes (Mayden et al., 2007) provide similar results.

The most recent morphological investigation of the phylogenetic position of *Psilorhynchus*, derived from characters of gill-arch osteology (Conway and Mayden, 2007), concluded that *Psilorhynchus* was more closely related to

Fig. 1. Most Parsimony (MP) tree and 50% majority rule consensus tree of all post burn-in trees from partitioned Bayesian analyses depicting phylogenetic relationships of species of Cypriniformes and outgroup taxa employed in this study. Phylogenetic analyses were conducted with different data matrices according to the proprieties of sequences and character weighting schemes (see Section 2 for details). The target taxon, *Psilorhynchus homaloptera*, in this study is marked in bold. Numbers on the branches of topology represent the resulting MP bootstrap values and Bayesian posterior probabilities, respectively. Values below 50% are not shown.

members of the Superfamily Cobitoidea (Cypriniformes minus Cyprinidae) than to members of the Cyprinoidea (Cyprinidae); the latter being its currently proposed position by Nelson (2006). Previous morphological investigations had suggested the contrary, that *Psilorhynchus* was more likely affiliated with the Cyprinidae amongst Cypriniformes, and placed accordingly within the cyprinid subfamily Psilorhynchinae (Chen, 1981; He and Chen, 1997). Phylogenetic analyses of extensive mitochondrial sequence data presented herein, however, supports the placement of *Psilorhynchus* within Cyprinidae, rendering this family paraphyletic if Psilorhynchidae is recognized as a separate family for this genus (Nelson, 2006). Based on these results, we recommend that the genus should be recognized in a separate subfamily of Cyprinidae, Psilorhynchinae, as suggested previously by Chen (1981). Only after additional taxa currently contained within the family Cyprinidae have been examined in a similar thorough manner can we confidently identify if the clade containing *Psilorhynchus* warrants separate family recognition.

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