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Phylogenetic Position of the Enigmatic Genus *Psilorhynchus* (Ostariophysi: Cypriniformes): Evidence from the Mitochondrial Genome

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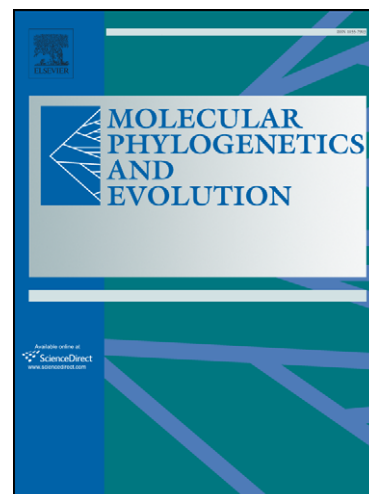
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1 **Phylogenetic Position of the Enigmatic Genus *Psilorhynchus* (Ostariophysi:**
2 **Cypriniformes): Evidence from the Mitochondrial Genome**

3

4 **Short Communication**

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43 The Order Cypriniformes contains the planet's largest monophyletic group of
44 freshwater fishes, with over 400 genera and 3,000 species native to Asia, Europe,
45 Africa, and North America (Nelson, 2006). Many of the species are of tremendous
46 cultural, economic and scientific importance and because of this the order has received
47 much recent attention in the area of molecular systematics (Šlechtová et al., 2005;
48 Saitoh et al., 2006; Mayden et al., 2007; Rüber et al., 2007).

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

56 *Psilorhynchus* was created by McClelland (1832) and currently contains eight
57 described species, *P. sucatio* (Hamilton 1822), *P. balitora* (Hamilton 1822), *P.*
58 *homaloptera* Hora and Mukerji 1935, *P. pseudecheneis* Menon and Datta 1964, *P.*
59 *gracilis* Rainboth 1983, *P. microphthalmus* Vishwanath and Manojkumar 1995, *P.*
60 *arunachalensis* (Nebeshwar, Bagra and Das 2007) and *P. amplicephalus* Arunachalam,
61 Muralidharan and Sivakumar 2007. The genus is restricted to the fast flowing streams
62 of the Ganga-Brahmaputra Drainage of India, Nepal and Bangladesh and the
63 Ayeyarwady River of Northern Myanmar and adjacent China (Rainboth, 1983). All of
64 the species possess arched backs and flattened ventral surfaces, presumably adaptations

65 for a benthic rheophilic lifestyle. On occasion *P. homaloptera*, *P. pseudecheneis* and *P.*
66 *arunachalensis* are placed in a separate genus, *Psilorhynchoides* Yazdani, Singh and
67 Rao (Yazdani et al., 1990; Nebeshwar et al., 2007) but this is not generally accepted and
68 for the purpose of this investigation all species will be retained within *Psilorhynchus*.

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

76 McClelland (1839) associated the genus with *Homaloptera*, a genus composed of
77 morphologically similar species that occupy similar ecological niches. Day (1871)
78 assigned *Psilorhynchus* to the subfamily Cyprininae, a group containing a highly
79 diverse assemblage of species. Jordan (1919) placed *Psilorhynchus* in the synonymy of
80 *Homaloptera*, but later (Jordan, 1923), rather confusingly, placed it in the Cobitidae.
81 Hora (1920) carried out the first revision of *Psilorhynchus*. Later Hora (1925) removed
82 *Psilorhynchus* from Cyprinidae, but refrained from placing the genus into the
83 Homalopteridae (=Balitoridae after Kottelat, 1988) or the Cobitidae, and instead
84 created a new family, the Psilorhynchidae, in light of their unique morphology. The
85 erection of Psilorhynchidae has been widely accepted (Ramaswami, 1952; Yazdani et
86 al. 1990), though Chen (1981) choose to place *Psilorhynchus* in a subfamily of the

87 Cyprinidae, Psilorhynchinae, based on examination of *P. homaloptera*, a decision
88 followed by Wu et al. (1981). Conway and Mayden (2007) reevaluated the osteological
89 characters of the gill arches used in the unpublished work of Siebert (1987), a study that
90 included species of *Psilorhynchus*. In this most recent and revised analysis of
91 osteological characters of the gill arches, *Psilorhynchus* was identified as the sister
92 group to a clade including cobitid and balitorid loaches, but bootstrap support for this
93 relationship was weak (BS = 55). Other important works on the order, some of which
94 examined *Psilorhynchus*, refrained from comment on the evolutionary relationships of
95 the genus (Chen et al., 1984; Cavender and Coburn, 1991; Howes, 1991). Nelson (2006)
96 chose to place the genus back under Psilorhynchidae, contrary to the previous (1994)
97 edition which placed the genus within the cyprinid subfamily Psilorhynchidae, without
98 discussion or supporting phylogenetic evidence.

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

109 addition we provide a new mitochondrial genome for *Misgurnus anguillicaudatus*.

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111

112 **Methods**

113 **Sequence analysis**

114 Total genomic DNA was isolated from specimens of *Psilorhynchus homaloptera*,

115 *Gyrinocheilus aymonieri*, and *Misgurnus anguillicaudatus*; other gene sequences from

116 whole mt-genomes were obtained from GenBank. Ingroup taxa and sources of

117 sequences used in this study are provided in Table 1. Gene content and arrangement

118 corresponds to the mitochondrial genome of common carp (*Cyprinus carpio*)

119 (X61010). Protein-coding genes were identified by similarity of predicted amino-acid

120 sequence with known mitochondrial protein sequences. Five species from other

121 families of Ostariophysi were used as outgroups, including *Pseudobagrus tokiensis*

122 (AB054127), *Phenacogrammus interruptus* (AB054129), *Eigenmannia* sp.

123 (AB054131), *Chanos chanos* (AB054133), and *Gonorynchus greyi* (AB054134). Total

124 genomic DNA was extracted from muscle tissue using QIAamp tissue kit (Qiagen,

125 Germany) following manufacture's protocol. The mitochondrial genome DNA was

126 amplified in its entirety using long PCR (Miya and Nishida, 1999) with primers of Miya

127 and Nishida (2000) and Inoue et al. (2001) for two reactions. Long PCR was performed

128 in a PTC-100 programmable thermal controller (MJ Research, USA); reactions were

129 carried out in 25 µl reaction volume containing 2.5 µl of 10 X LA PCR buffer II

130 (Takara), 0.8 mM of dNTPs, 2.5 mM of MgCl₂, 0.5 mM of each primer, 0.625 units LA

131 Taq polymerase (Takara) and approximately 20 ng template DNA. The thermal cycle
132 profile was: pre-denaturation at 94°C for 2 minutes and 30 cycles of denaturation at
133 98°C for 10 seconds, annealing and extension combined at the same temperature (68°C)
134 for 16 minutes, and electrophoresis on a 0.8% agarose gel (Promega, USA). The long
135 PCR products were diluted in sterilized distilled water for subsequent use as PCR
136 templates. We used 24 different primer pairs that amplify contiguous, overlapping
137 segments to amplify the entire mitochondrial genome. Eleven primer pairs were
138 versatile, based on the complete mitochondrial genome of six ray-finned fish species,
139 following Miya and Nishida (2000). PCR reactions were carried out in 25 µl reaction
140 volume containing 2.5 µl of 10 × PCR buffer (Takara), 0.4 mM of dNTPs, 1.8 mM of
141 MgCl₂, 0.2 mM of each primer, 1 unit of Taq polymerase (Takara) and 1.0 µl of long
142 PCR products as template. The thermal cycle profile was: pre-denaturation at 94°C for
143 2 minutes and 30 cycles of denaturation at 94°C for 15 seconds, annealing at 52°C for
144 15 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 5 minutes.
145 PCR products were electrophoresed on 1.0% agarose gel (Promega). Double strand
146 PCR purified products were subsequently used for direct cycle sequencing with
147 dye-labeled terminators (ABI). PCR primers were used for sequencing. All sequencing
148 reactions were performed according to the manufacturer's instructions. Labeled
149 fragments were analyzed on a model MegaBACE 1000 DNA sequencer (GE
150 Healthcare Biosciences, USA).

151

152 **Phylogenetic analysis**

153 Multiple alignments were prepared for all sequences using Clustal X (Thompson et al.,
154 1997) at default settings. We used Gblocks (Castresana, 2000) to extract regions of
155 defined sequence conservation from the gene specific alignments and generated a
156 single file of concatenated conserved regions. Phylogenetic analyses were performed
157 using maximum parsimony (MP) as implemented in PAUP* - version 4.0b10
158 (Swofford, 2002) and partitioned Bayesian analysis (BI) as implemented in MrBayes
159 parallel version v3.1.1 (Huelsenbeck and Ronquist, 2001). For each MP search, optimal
160 trees were obtained by heuristic searches with random stepwise addition sequences
161 followed by TBR swapping for 100 replications (Swofford, 2002). Node support was
162 assessed using the bootstrap procedure (Felsenstein, 1985) under MP criterion, based
163 on 100 pseudo-replicates of heuristic searches as described above. Likelihood ratio
164 tests (Goldman, 1993), as implemented in MrModeltest 2.2 (Nylander, 2004), were
165 used to choose models for each gene partition in Partitioned BI. Partitions were defined
166 based on putative gene functional constrains and selective pressures. We assigned
167 separate properties to each protein-coding gene, to each ribosomal RNA gene (12S and
168 16S), and to all tRNA genes together. Three data matrices (1, using structure DNA
169 sequences only; 2, using protein-coding gene sequences only; and 3, using all aligned
170 sequences, excluding D-Loop region) were used in phylogenetic analyses (Fig. 1). In
171 addition, as phylogenetic analyses of protein-coding genes can be biased from
172 homoplasy at third codon positions due to multiple substitutions in transitions (Saitoh
173 et al., 2005) and/or due to base composition bias across taxa (Lockhart et al., 1994;
174 Chen, et al., 2003), we also used a "down-weighting scheme" for the third codon

175 position. At this position this scheme employed converting nucleotides A and G into
176 purine (R) and T and C into pyrimidine (Y) (or RY-coding) for both MP and BI (Fig. 1).

177 For each partitioned BI, four independent MCMC chains were performed with 3 x
178 10^6 replicates, sampling one tree per 100 replicates for each run. The results from two
179 runs were compared to evaluate the degree of convergence in tree topologies and in log
180 likelihoods. This procedure was repeated until stationarity was observed. Initial trees
181 with non-stationary log likelihood values were discarded as part of burn-in; the
182 remaining trees from two independent runs were used to construct a 50% majority rule
183 consensus tree. Resulting *a posteriori* probabilities were used as a measure of node
184 support in addition to the measures through MP bootstrap.

185

186 **Results**

187 A total of 14,768 bp were aligned in the mitogenome sequences for all taxa. Of
188 these, 7,174 sites were constant and 6,428 were phylogenetically informative. MP
189 analysis yielded a single tree (Tree length = 36473, C.I. = 0.36, R.I. = 0.38; Fig. 1).

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

195 In the resulting phylogenies of all analyses the Cypriniformes was supported as a
196 monophyletic group (Fig. 1). Within the order none of the analyses supported the

197 traditionally recognized Superfamily Cobitoidea (= Gyrinocheilidae + Catostomidae +
198 Cobitidae + Balitoridae). Gyrinocheilidae was either the sister group to Catostomidae
199 (most analyses) or the basal sister group within Cypriniformes (MP of ribosomal genes
200 only), although the latter resolution had no support. In the former trees Gyrinocheilidae
201 plus Catostomidae was sister to the monophyletic Cyprinoidea, family Cyprinidae plus
202 *Psilorhynchus*. The Balitoridae was always resolved as a paraphyletic group relative to
203 the monophyletic Cobitidae.

204 In all BI analyses, including the RY-coding scheme, and in MP analysis of
205 ribosomal genes *Psilorhynchus* was strongly supported as occurring within the
206 Cyprinoidea (Cyprinidae) sister to *Cyprinus carpio* (common carp) plus *Carassius*
207 *auratus* (goldfish), two representatives of the Tribe Barbini of the Subfamily
208 Cyprininae (Chen et al., 1984). This clade was well supported as the basal-most lineage
209 within the family. In MP analyses of protein-coding genes and with all genes combined
210 *Psilorhynchus* was either sister to *Rhodeus* or *Danio*, respectively, suggesting likely
211 long-branch attraction of *Psilorhynchus* in the non-model-based MP analyses. In
212 neither of these analyses was there strong branch support for the placement of
213 *Psilorhynchus*.

214 In addition to *Psilorhynchus*, *Cyprinus*, and *Carassius*, Cyprinidae is represented
215 by an additional seven species, together representing the purported subfamilies (sensu
216 Howes, 1991) Acheilognathinae (*Rhodeus*), Cultrinae (*Megalobrama*), Cyprininae
217 (*Mylopharyngodon*), Gobioninae (*Sarcocheilichthys*), Leucisinae (*Distoechodon*), and
218 Rasborinae (*Opsariichthys*, *Danio*). In addition to the Barbini, all phylogenetic

219 resolutions also supported the historically proposed major Cyprinidae clade Leuciscini
220 (Chen et al., 1984), that, in this study, included species endemic to eastern Asian
221 (*Opsariichthys biden*, *Mylopharyngodon piceus*, *Megalobrama amblycephalus*,
222 *Distoechodon tumirostris*), from northern cold water habitats (*Sarcocheilichthys*
223 *variegates microoculus*, *Rhodeus* sp.), and the model organism species *Danio rerio*
224 (zebrafish). Relationships amongst the different subfamilies varied depending upon the
225 analysis, however strongest support for relationships was observed in BI of
226 protein-coding and total sequences. For both of these analyses, including RY-coding,
227 neither Cyprininae nor Rasborinae was monophyletic. *Danio* (“Rasborinae”) formed
228 the basal sister-group to the other taxa and ladderized sister group relations of
229 Acheilognathinae (*Rhodeus*), Gobioninae (*Sarcocheilichthys*), “Rasborinae”
230 (*Opsariichthys*), Cultrinae (*Megalobrama*), and Leucisinae (*Distoechodon*) plus
231 “Cyprininae” (*Mylopharyngodon*) (Fig. 1). BI of ribosomal genes revealed the clade
232 Acheilognathinae (*Rhodeus*) plus “Rasborinae” (*Opsariichthys*) sister to the clade
233 Leucisinae (*Distoechodon*) sister to Cultrinae (*Megalobrama*) plus “Cyprininae”
234 (*Mylopharyngodon*). The only relationships in MP analysis with substantive nodal
235 support included the clade Cultrinae (*Megalobrama*), sister to Leucisinae
236 (*Distoechodon*) plus “Cyprininae” (*Mylopharyngodon*) in analyses of protein-coding
237 genes and the total matrix (Fig. 1).

238

239 Discussion

240 DNA sequences have been extremely important in elucidating relationships of

241 species and supraspecific groups of all forms of life, particularly those that have been
242 difficult to resolve using only morphological characters. Because species of
243 *Psilorhynchus* are extremely divergent morphologically from other members of the
244 order, the application of molecular data and phylogenetic methods are proposed to be of
245 high value in resolving their relationships at higher levels.

246 Liu et al (2004), using mitochondrial control region, reconstructed relationships
247 within Cypriniformes and placed Cyprinidae as sister to all other families with
248 Balitoridae sister to Cobitidae, but neither Psilorhynchidae nor Gyrinocheilidae were
249 represented. Saitoh et al. (2003), based on whole mitochondrial genome sequences,
250 reconstructed the phylogeny of ostariophysan fishes but among the Cypriniformes,
251 only Cyprinidae, Cobitidae and Balitoridae were included. Later, Saitoh et al. (2006)
252 examined additional diversity within the Cypriniformes for whole mitogenome
253 sequences but did not include *Psilorhynchus*. Thus, with the exception of the
254 morphological analysis of Conway and Mayden (2007), wherein the genus was
255 considered to be closely related to certain members of the Cobitoidea (based on
256 gill-arch osteology), the phylogenetic position of this enigmatic genus has yet to be
257 tested within a phylogenetic framework with potentially informative molecular
258 characters.

259 Molecular analyses using model-based and partitioned BI analyses of either
260 mitochondrial ribosomal genes, protein-coding genes, or combined data identify
261 *Psilorhynchus* as closely related to members of the subfamily Cyprininae.
262 *Psilorhynchus* exhibits a high degree of molecular divergence, analogous to its

263 observed morphological divergence, and is highly divergent genetically from members
264 of the Cyprinidae examined herein. The varied relationships of *Psilorhynchus* in MP
265 analyses, relative to BI analyses, is likely indicative of erroneous relationships due to
266 long-branch attraction accompanying its genetic divergence relative to *Danio* and
267 *Rhodeus*.

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

277 The most recent morphological investigation of the phylogenetic position of
278 *Psilorhynchus*, derived from characters of gill-arch osteology (Conway and Mayden,
279 2007), concluded that *Psilorhynchus* was more closely related to members of the
280 Superfamily Cobitoidea (Cypriniformes minus Cyprinidae) than to members of the
281 Cyprinoidea (Cyprinidae); the latter being its currently proposed position by Nelson
282 (2006). Previous morphological investigations had suggested the contrary, that
283 *Psilorhynchus* was more likely affiliated with the Cyprinidae amongst Cypriniformes,
284 and placed accordingly within the cyprinid subfamily Psilorhynchinae (Chen, 1981; He

285 and Chen, 1997). Phylogenetic analyses of extensive mitochondrial sequence data
286 presented herein, however, supports the placement of *Psilorhynchus* within Cyprinidae,
287 rendering this family paraphyletic if Psilorhynchidae is recognized as a separate family
288 for this genus (Nelson, 2006). Based on these results, we recommend that the genus be
289 recognized in a separate subfamily of Cyprinidae, Psilorhynchinae, as suggested
290 previously by Chen (1981). Only after additional taxa currently contained within the
291 family Cyprinidae have been examined in a similar thorough manner can we
292 confidently identify if the clade containing *Psilorhynchus* warrants separate family
293 recognition.

294

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301 (www.cypriniformes.org).

302

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409

410 **Table 1. The mitochondrial genome sequences for analysis in this study**

411

412	Species	Source (Voucher Specimen)	Accession No
413	Order Cypriniformes		
414	Family Cyprinidae		
415	<i>Opsariichthys biden</i>	Wang et al. (NRMT20042)	DQ367044
416	<i>Mylopharyngodon piceus</i>	Peng et al. (NRMT2071)	DQ026435
417	<i>Megalobrama amblycephalus</i>	Peng et al. (IHB305004)	DQ026433
418	<i>Sarcocheilichthys variegates microoculus</i>	Saitoh et al. (2003)	AB054124
419	<i>Distoechodon tumirostris</i>	Peng et al. (IHB303084)	DQ026431
420	<i>Rhodeus</i> sp.	Peng et al. (IHB305003)	DQ026430
421	<i>Danio rerio</i>	Broughton et al. (2001)	AC024175
422	<i>Cyprinus carpio</i>	Chang et al. (1994)	X61010
423	<i>Carassius auratus langsdorfii</i>	Murakami et al. (1997)	AB006953
424	Family Psilorhynchidae		
425	<i>Psilorhynchus homaloptera</i>	Aquarium trade	DQ026436*
426	Family Catastomidae		
427	<i>Myxocyprinus asiaticus</i>	Peng et al. (IHB305001)	AY986503
428	<i>Carpiodes carpio</i>	Broughton et al. (2003)	NC005257
429	Family Balitoridae		
430	<i>Crossostoma lacustre</i>	Tzeng et al. (1992)	M91245
431	Family Cobitidae		

432	<i>Cobitis striata</i>	Saitoh et al. (2003)	AB054125
433	<i>Lefua echigonia</i>	Saitoh et al. (2003)	AB054126
434	<i>Misgurnus anguillicaudatus</i>	This study (N/A)	DQ026434*
435	Family Gyrinocheilidae		
436	<i>Gyrinocheilus aymonieri</i>	This study (IHB305002)	DQ026432*
437	Out-group		
438	<i>Pseudobagrus tokiensis</i>	Saitoh et al. (2003)	AB054127
439	<i>Phenacogrammus interruptus</i>	Saitoh et al (2003)	AB054129
440	<i>Eigenmannia</i> sp.	Saitoh et al (2003)	AB054131
441	<i>Chanos chanos</i>	Saitoh et al (2003)	AB054133
442	<i>Gonorynchus greyi</i>	Saitoh et al (2003)	AB054134

443 *New sequence in this study

444

445

446 **Figure Legends**

447

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

