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Molecular phylogeny of Aphyocharacinae (Characiformes, Characidae) with morphological diagnoses for the subfamily and recognized genera

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ABSTRACT

The subfamily Aphyocharacinae was recently redefined to comprise eight genera: *Aphyocharax*, *Prionobrama*, *Paragoniates*, *Phenagoniates*, *Leptagoniates*, *Xenagoniates*, *Rachoviscus* and *Inpaichthys*. This new composition, however, is partially incongruent with published results of molecular studies especially concerning the positions of *Rachoviscus* and *Inpaichthys*. Our goal was to investigate the monophyly of Aphyocharacinae and its interrelationships using three distinct phylogenetic methodologies: Maximum-likelihood and Bayesian analyses of molecular data, and also Parsimony analysis of a concatenated molecular and morphological dataset. All tree topologies recovered herein suggest that *Rachoviscus*, *Inpaichthys* and *Leptagoniates pi* do not belong to the Aphyocharacinae. The remaining aphyocharacin taxa analyzed do form a monophyletic group, which is itself composed of two subgroups being one comprised of *Paragoniates*, *Phenagoniates*, *Leptagoniates* and *Xenagoniates*, and the other comprised of *Aphyocharax* and *Prionobrama*. Internal relationships among these genera are statistically well supported and morphological synapomorphies are presented at the generic level. All tree topologies also indicate that *Aphyocharacidium* is closely related to Aphyocharacinae suggesting that it should be included in this subfamily. As recognized in the present study, the Aphyocharacinae is diagnosed by a single morphological synapomorphy: two dorsal-fin rays articulating with the first dorsal pterygiophore.

1. Introduction

Characidae is the largest and most diverse family of Characiformes comprising around 200 genera and more than 1200 valid species geographically widespread from southern USA to northern Argentina (Reis et al., 2003; Eschmeyer, 2010). Phylogenetic relationships among characids, popularly known as "tetras", have been the subject of intense phylogenetic studies in recent years (Mirande, 2009, 2010; Javonillo et al., 2010; Oliveira et al., 2011). While these studies have generated many novels and well supported hypotheses of relationships, some of the results from the morphological and molecular analyses are incongruent, especially in relation to the composition of the subfamilies (e.g., Mirande, 2009; Oliveira et al., 2011).

Günther (1864) was the first author to propose a division of Family Characidae in 10 infra-families. In 1868, Günther described *Aphyocharax* as a new genus in infra-family Tetragonopterina. In a series of papers published around a century ago, Eigenmann (1909, 1910, 1912) included the blood-fin tetra *Aphiocharax* [sic] and some other genera of small-bodied characids (*Cheirodon*, *Coeluricthys*, *Holoprion*, *Holoshestes*, *Odontostilbe*, *Probolodus*, and *Aphyodite*) in the subfamily Aphiocharacinae [sic], based on similarities in the shape of the gill membranes, nares, fontanels, adipose fin, and maxillary teeth. Eigenmann (1915) later subsumed most of these species within a newly-recognized subfamily, the Cheirodontinae, a taxonomic arrangement that served as the basis of classification for many decades (e.g. Gregory and Conrad, 1938; Géry, 1960; Géry and Boutiére, 1964).

In his revision of the family Characidae, Géry (1977) recognized *Aphyocharax* as a member of a distinct subfamily, based on a laterally compressed body, anal fin of intermediate length, midbody position of dorsal fin, incomplete lateral line, and arrangement and shape of teeth in the oral jaws. Although he placed *Aphyocharax* in the monotypical subfamily Aphyocharacinae, Géry (1977) did note similarities between *Aphyocharax* and a newly created subfamily

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Paragoniatinae, in which he placed *Rachoviscus*, *Paragoniates*, *Phenagoniates*, *Leptagoniates*, *Xenagoniates* and *Prionobrama*. Géry and Junk (1977) described a new genus and species, *Inpaichthys kerri*, indicating its general resemblance with *Rachoviscus crassiceps* and "*Paragoniates* et al." ("et al." possibly referring to the other species of Paragoniatinae).

In the most complete osteological survey of Characidae to date, Mirande (2009, 2010) recognized eight genera in the Aphyocharacinae: *Paragoniates*, *Phenagoniates*, *Xenagoniates*, *Inpaichthys, Leptagoniates*, *Rachoviscus*, *Aphyocharax* and *Prionobrama*. The genera *Rachoviscus* and *Leptagoniates* were provisionally placed in the subfamily following previous reports published in the literature (e.g. Géry, 1977; Géry and Junk, 1977). Three synapomorphies supported the monophyly of Aphyocharacinae: (1) presence of synchondral articulation between lateral ethmoid and anterodorsal border of orbitosphenoid; (2) fourth infraorbital absent or much reduced and bordered posteriorly by third and fifth infraorbitals; and (3) six or less branched pelvic-fin rays (Mirande, 2010).

Mirande's composition of Aphyocharacinae is partly incongruent with two recently published molecular phylogenies of Characidae (Javonillo et al., 2010; Thomaz et al., 2010). Although not including many characid taxa, these studies indicated that *Inpaichthys* and *Rachoviscus* do not belong to the Aphyocharacinae. Javonillo et al. (2010) recovered *Aphyocharax* as the sister group of a clade comprised of *Exodon*, *Phenacogaster, Roeboides, Galeocharax, Cynopotamus* and *Tetragonopterus*. The authors additionally indicated that *Inpaichthys* and *Ctenobrycon* are sister taxa, while *Rachoviscus* is closer to a group including *Hollandichthys*. Thomaz et al. (2010) also recovered *Rachoviscus* as the sister group of *Hollandichthys*, however these genera were not close related to *Aphyocharax*. Oliveira et al. (2011) in the broadest molecular analysis of Characidae included all Aphyocharacinae genera proposed by Mirande (2010) in their study and again *Rachoviscus* and *Inpaichthys* do not appear as close related to the remaining Aphyochracinae.

Due to the extraordinary diversity and complexity of the Characidae, with large amounts of morphological homoplasy and character state reversals (Malabarba, 1998; Zanata and Vari, 2005; Toledo-Piza, 2007; Mirande, 2010), previous phylogenetic studies of the group have been forced to sample a relatively small proportion of all known taxa (Malabarba and Weitzman, 2003; Mirande, 2009, 2010; Javonillo et al., 2010; Thomaz et al., 2010; Oliveira et al., 2011). This strategy is called the "basal exemplar approach", which selects representative species from among what are perceived to be the major distinct clades (Albert et al., 2009). Here we build on the results of these previous phylogenetic studies, which allowed us to concentrate efforts on a far less-inclusive efforts set of species. Thus this is the first analysis of morphological and molecular data for all aphyocharacin genera, with data for most species. Our aims were to investigate the monophyly and interrelationships of Aphyocharacinae (sensu Mirande, 2010) using model-based phylogenetic analyses of molecular data, and also do total evidence analysis by parsimony.

1.1. Ingroup and outgroup criteria selection

Ingroup taxa were selected based on phylogenies proposed by Mirande (2009, 2010). Following these hypotheses, Aphyocharacinae comprises eight genera: *Aphyocharax*, *Inpaichthys, Leptagoniates, Paragoniates, Phenagoniates, Prionobrama*, *Rachoviscus* and *Xenagoniates*.

Outgroup taxa were selected based on phylogenies proposed by Oliveira et al. (2011). Following their hypotheses, Characidae (node 37) is a well supported clade comprised of four monophyletic units. All species of these four clades were selected as a distinct outgroups and, in addition, two species of *Salminus* were included as extra outgroups.

1.2. DNA extraction and sequencing

Total DNA was extracted from muscle tissue preserved in ethanol with DNeasy Tissue Kit following manufacturer's instructions. Partial sequences of the genes 16S rRNA (16S, 700 pb) and cytochrome b (CytB, 900 pb) were amplified using one round of polymerase chain reaction (PCR). PCR amplifications were performed in 50 μl reactions consisting of 5 μl 10 x reaction buffers, 1 μl dNTP mix at 10 mM each, 1 μl of each primer at 10 μM, 0.2 μl Taq DNA Polymerase 1 U of Polymerase per reaction, 1 μl DNA, and 40.8 μl of double-distilled water. Cycles of amplification were programmed with the following profile: (1) 3 min at 94 °C (initial denaturation), (2) 30 s at 94 °C, (3) 45 s at 48–54 °C, (4) 80 s at 72 °C, and 5 min at 72 °C (final elongation). Steps 2–4 were repeated 35 times. Additionally, sequences of myosin heavy chain 6 gene (Myh6, 750 pb), recombination activating gene 1 (RAG 1, 1250 pb) and recombination activating gene 2 (RAG 2, 950 pb) were amplified through two rounds of PCR. The first was conducted using external primers while the second was conducted using internal primers (Supplementary material A). PCR amplifications were performed in 50 μl reactions consisting of 5 μl 10× reaction buffers, 1 μl dNTP mix at 10 mM each, 1 μl of each primer at 10 μM, 0.2 μl Taq DNA Polymerase 1 U of Polymerase per reaction, 1 μl DNA, and 40.8 μl of double-distillated water. Cycles of amplification were programmed with the following profile: (1) 3 min at 94 °C (initial denaturation), (2) 30 s at 94 °C, (3) 45 s at 50–54 °C (4) 80 s at 72 °C, and 5 min at 72 °C (final elongation). Steps 2–4 were repeated 37–40 times. Products of all amplification were identified on a 1% agarose gel. PCR products were purified with the ExoSap-IT®. Sequencing reactions were performed with the Big Dye Terminator Cycle Sequencing Ready Reaction 3.1 Kit following instructions of the manufacturer, and were loaded on an automatic sequencer 3130-Genetic Analyzer in the Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo. Consensus sequences were assembled and edited in BioEdit 7.0.9.0 (Hall, 1999). Where uncertainty of nucleotide identity was detected, IUPAC ambiguity codes were applied.

1.3. Sequencing alignment and phylogenetic analyses

1.3.1. Sequence data

Consensus sequences of each gene were independently aligned using MAFFT v. 5.3 (Katoh et al., 2002, 2005) and, then, alignments were inspected by eye for any obvious misreading. To evaluate the occurrence of substitution saturation, the index of substitution saturation (Iss) was estimated in DAMBE (Xia and Xie, 2001) as outline by Xia et al. (2003) and Xia and Lemey (2009). Overall genetic distances (Tamura 3-parameter) among sequences were calculated in Mega 5.04 (Tamura et al., 2011) and appropriate evolutionary models were estimated by jModelTest under default parameters (Posada, 2008).

1.3.2. Maximum-likelihood (ML)

ML was conducted in RAxML (Stamatakis, 2006) using the web servers RaxML BlackBox (Stamatakis et al., 2008). Random starting trees were applied for ML tree search and all other parameters were set on default values. ML analyses were conducted under GTR + G given that RAxML only applies such a model (Stamatakis et al., 2008). Topological robustness was investigated using 1000 non-parametric bootstrap replicates. Branches with bootstrap values higher than 70% were considered well supported (see Hillis and Bull (1993) for justification).

1.3.3. Bayesian inference (BI)

BI was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Because