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

Victor A. Tagliacollo [a,*], Rosana Souza-Lima [b], Ricardo C. Benine [c], Claudio Oliveira [a]

[a] Universidade Estadual Paulista, UNESP, Instituto de Biociências, Departamento de Morfologia, Distrito de Rubião Jr., s/no., CEP 18618–970, Botucatu, SP, Brazil
[b] Universidade do Estado do Rio de Janeiro, UERJ, Faculdade de Formação de Professores, Departamento de Ciências, Rua Dr. Francisco Portela, No. 1470, Patronato, CEP 24435-005, São Gonçalo, RJ, Brazil
[c] Universidade do Sagrado Coração, USC, Centro de Ciências da Saúde, Setor de Zoologia, Laboratório de Ictiologia, Rua Irmã Arminda, No. 10-50, CEP 17011-160, Bauru, SP, Brazil

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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

* Corresponding author. Present address: University of Louisiana at Lafayette, Department of Biology, Lafayette, LA 70504-2451, USA.
E-mail addresses: victor_tagliacollo@yahoo.com.br (V.A. Tagliacollo), rosanasl@yahoo.com.br (R. Souza-Lima), ricardo.benine@usc.br (R.C. Benine), claudio@ibb.unesp.br (C. Oliveira).

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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

MrBayes 3.1.2 only implements 1, 2 and 6 substitution rate models, it was not possible to implement some of the models chosen by jModelTest (Posada, 2008). Thus, correspondent models were selected as indicated by MrModelTest 3.2 (Nylander, 2004). Two runs of four independent MCMC chains were run with 50 million replicates each, sampling one tree every 1000 generation. The distribution of log likelihood scores was examined using Tracer 1.4 (Rambaut and Drummond, 2004) in order to determine stationarity for each search and to decide if extra runs were required to achieve convergence. The first 5 million generation (10%) were discarded as burn-in. The remaining trees were used to calculate a 50% majority-rule consensus topology. Branches with Bayesian posterior probabilities $\geqslant$95% were considered well supported.

1.3.4. Total evidence analysis based on parsimony (TE)

TE analysis was composed of molecular and morphological characters. Molecular data consisted of 4423 characters while morphological data consisted of 373 characters, of which 365 were published by Mirande (2010) and eight additional are proposed herein (supplementary material B). These additional characters were mainly based on osteological traits that were examined on cleared-and-stained (c&s) specimens prepared using the method outlined by Taylor and Van Dyke (1985). The resulting concatenated matrix was composed of 125 specimens (115 species) and 51 species coded also for morphological characters. Further information can be seen in Supplementary material B.

TE was conducted using TNT 1.1 (Goloboff et al., 2008). No differentially weighting or ordering of character states was adopted. Gaps and question marks were treated as a missing data. Phylogenies were obtained through searches using 'sectorial searches', 'ratchet', 'drift', and 'tree fusing' with their default values and employing a driven search with initial level set at 100 and checking level every three hits. Each of these searches was performed from 100 random addition sequences and TBR branch swapping. Consistency and retention indexes were calculated with the script 'stats'. Bootstrap was calculated from 1000 pseudoreplicates. Branches with bootstrap values higher than 70% were considerate well supported (see Hillis and Bull (1993) for justification).

2. Results

2.1. Dataset

Most of partial sequences of two mitochondrial (16S rRNA, CytB) and three nuclear (Myh6, RAG1, RAG2) genes used herein were published by Oliveira et al. (2011) while few others were published by Javonillo et al. (2010). From the species *Aphyocharax alburnus*, *A. anisitsi A. dentatus*, *A. nattereri*, *A.* sp, *A. pusillus*, *A. rathbuni*, *Prionobrama filigera*, *P. paraguayensis*, and *Leptagoniates pi*, sequences were generated in the present study. All sequences were deposited in GenBank under the accession number shown in Supplementary material A. Data matrix is deposited in the Dryad Repository at http://dx.doi.org/10.5061/dryad.v24r8753.

The resulting molecular dataset was comprised of 4423 characters. This dataset was partitioned into five blocks, each one representing a different gene. The overall mean of genetic distance ranged from 0.0695 ± 0.005 (Myh6) to 0.2295 ± 0.008 (CytB) suggesting that the analyzed sequences have enough genetic variation for phylogenetic studies. Genes and also their respective codon position were tested to investigate the occurrence of substitution saturation. The Iss index indicated that there are not saturations in the genes, however significant saturation was found for the 3rd codon position of the gene CytB in both asymmetrical (Iss.cA) and symmetrical (Iss.cS) topologies (data not shown). Although Iss is greater than Iss.cS, no statistical difference was found, which means that this codon position can be used in phylogenetic analyses (Xia et al., 2003; Xia and Lemey, 2009). Appropriate evolutionary models for the genes were investigated under the Akaike information criterion (AIC) and Bayesian information criteria (BIC). For 16S rRNA and CytB genes, both AIC and BIC selected the GTR + I + G as the best fit-model while to the other genes each criteria selected a different model. Therefore, the choice was to adopt models selected according to AIC (Posada and Buckley, 2004 for justification). For each gene partition, information content and characteristics such as: number of base pairs (bp) after alignment, base pair composition, overall mean genetic distance, substitution saturation (Iss index), nucleotide substitution models, α (shape) parameter of Γ distribution and proportion of invariant sites are shown in Table 1.

2.2. Model-based phylogenetic reconstructions

ML and BI show that excluding *Rachoviscus*, *Inpaichthys*, and *Leptagoniates pi*, Aphyocharacinae as suggested by Mirande (2010) constitute a statistically well supported group (Fig. 1). Additionally, molecular model-based hypotheses presume that Aphyocharacinae has two major clades being one comprised of *Paragoniates*, *Phenagoniates*, *Leptagoniates*, and *Xenagoniates* and, another comprised of *Aphyocharax* and *Prionobrama*. Evolutionary relationships among them are also statistically well supported and most genera are monophyletic, except *Leptagoniates* given that *L. pi* is the sister group of representatives of the subfamily Cheirodontinae (Fig. 1). At species level, interrelationships among some species of *Aphyocharax* are statistically poor supported under ML (mainly the relationships among *A. anisitsi*, *A. rathbuni*, *A. dentatus*, and *A.* sp.), however well support under BI analysis. Trees based on BI and ML analyses, bootstrap and posterior probabilities values are shown in Fig. 1.

Table 1
Information content and characteristics of each gene partition.

	GENES				
	16S rRNA	CytB	Myh6	RAG1	RAG2
Bp after alignment	540	918	700	1275	1051
Π_A	0.3873	0.3377	0.2817	0.2866	0.2737
Π_C	0.1939	0.3368	0.2261	0.2230	0.2185
Π_G	0.1863	0.0612	0.2250	0.2244	0.2242
Π_T	0.2324	0.2643	0.2673	0.2660	0.2837
Overall mean genetic distance (*p*-distance)	0.1183 ± 0.009	0.2295 ± 0.008	0.0695 ± 0.005	0.0904 ± 0.004	0.0802 ± 0.005
Substitution saturation	Iss < Iss.c	Iss < Iss.c	Iss < Iss.c	Iss < Iss.c	Iss < Iss.c
Nucleotide substitution model	GTR	GTR	TrN	TPM2uf	TPM2uf
α (shape) parameter of Γ distribution	0.4130	0.3650	0.2760	0.6810	0.6480
Proportion of invariant (I) sites	0.5330	0.4890	–	0.2510	0.2550

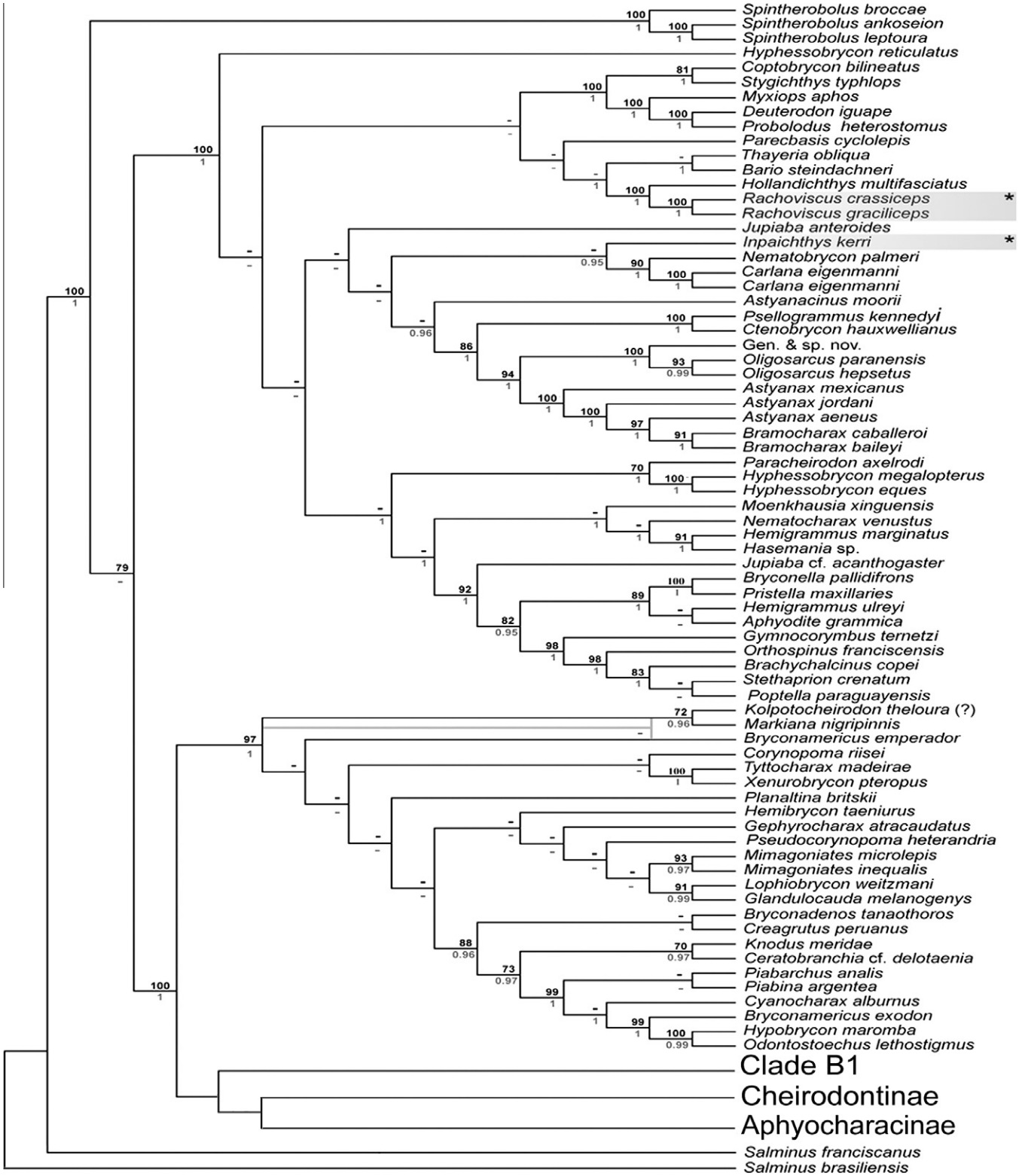

Fig. 1. ML (black topology) and BI (grey topology) analyses showing similar relationships among characid species. In this study results indicate that Aphyocharacinae (*sensu* Mirande, 2010) is not monophyletic since *Inpaichthys* is more closely related to *Nematobrycon* and *Carlana*. Molecular data also suggest that *Rachoviscus* and *Hollandichthys* are sister groups while *Leptagoniates pi* is the sister group of the Cheirodontinae. Dashed square limits Aphyocharacinae as proposed herein. Black numbers (above) correspond to bootstrap values and gray numbers (below) to posterior probability values. "∗" indicates species that do not belong to the Aphyocharacinae as recognized herein.

2.3. Total evidence phylogenetic reconstruction

As with the ML and BI phylogenies, TE parsimony-based analysis suggests that *Rachoviscus, Inpaichthys*, and *Leptagoniates pi* do not belong to Aphyocharacinae (Fig. 2). Furthermore, it presumes that the subfamily has two statistically well supported clades being one comprised of *Paragoniates*, *Phenagoniates*, *Leptagoniates*, and *Xenagoniates* and, another comprised of *Aphyocharax* and

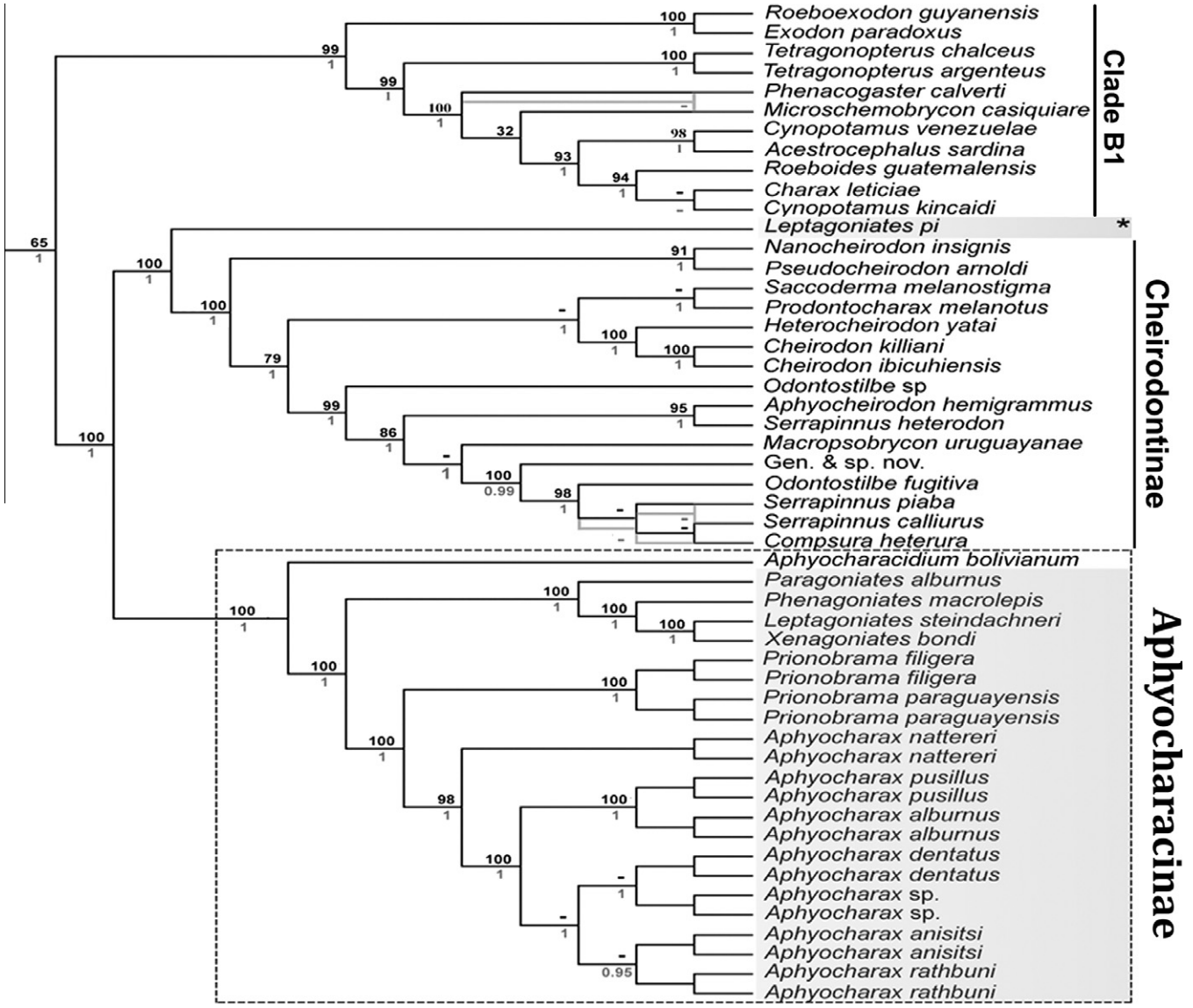

Fig. 1 (*continued*)

Prionobrama (Fig. 2). At species level, only a single discrepancy is observed between model-based and parsimony-based phylogenies (Figs. 1 and 2). Although statistically poor supported under ML analysis, model-based hypotheses suggest that *A. anisisti* and *A. rathbuni* are together the sister group of *A. dentatus* and *A.* sp. (Fig. 1). Alternatively, TE analysis indicates that *A. dentatus* and *A.* sp are both the sister group to *A. rathbuni*, and all three species together are the sister group of *A. anisitsi* (Fig. 2). Strict consensus among 16 most parsimonious trees, length, number of informative characters, consistency and retention indexes, and bootstrap values are presented in Fig 2. Further details can be seen in the Fig. 3 and in Supplementary material B.

3. Discussion

3.1. Monophyly of Aphyocharacinae (sensu Mirande, 2010)

Recent morphological hypotheses on the taxonomic composition of Aphyocharacinae propose that the subfamily is comprised of eight genera: *Aphyocharax*, *Inpaichthys*, *Leptagoniates*, *Paragoniates*, *Phenagoniates*, *Prionobrama*, *Rachoviscus*, and *Xenagoniates* (Mirande, 2009, 2010). This arrangement, however, is incongruent with some recently published molecular phylogenies of the Characidae (Javonillo et al., 2010; Thomaz et al., 2010; Oliveira et al., 2011). The main disagreement refers to the position of the monotypic *Inpaichthys*, and the two known species of *Rachoviscus*. As shown in previously published phylogenies (Javonillo et al., 2010; Thomaz et al., 2010; Oliveira et al., 2011), molecular model-based hypotheses recovered herein suggest that *Rachoviscus* constitutes the immediate sister group of *Hollandichthys* (Fig. 1) and that *Inpaichthys* belongs to a clade comprised of *Nematobrycon* plus *Carlana* (Fig. 1). Despite some uncertainty as to which species is the sister taxon to *Inpaichthys*, the molecular phylogenies concur that this species does not belong to Aphyocharacinae (Javonillo et al., 2010; Oliveira et al., 2011).

As reported by Mirande (2010), *Rachoviscus* was provisionally included within Aphyocharacinae based on general similarity between *R. crassiceps* and *Inpaichthys kerri* in overall body shape, coloration and, as previously noted by Géry and Junk (1977), the presence of non-aligned premaxillary teeth. This latter trait, in particular, is notable as it is also present in *Hollandichthys multifasciatus*, and may therefore represent evidence for close

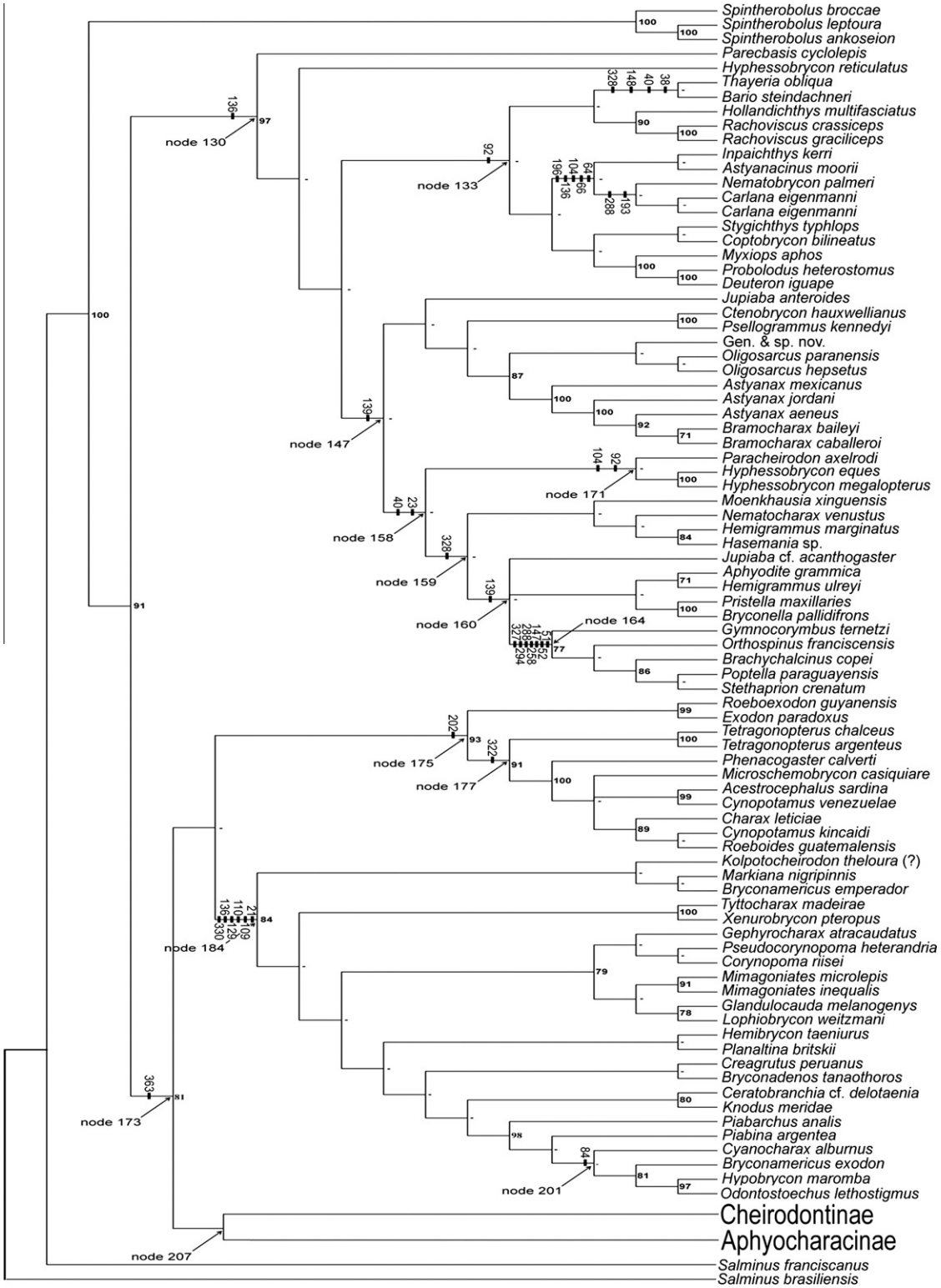

Fig. 2. Strict consensus of 16 most parsimony trees (18338 steps; 2006 parsimony-informative, CI: 0.2296; RI: 0.5832) based on TE analysis of 4423 molecular and 373 morphological characters concatenated. As with the model-based phylogenies, this cladogram suggests that *Inpaichthys*, *Rachoviscus* and *Leptagoniates pi* do not belong to Aphyocharacinae. Dashed square limits Aphyocharacinae as proposed herein. This arrangement is supported by a single morphological synapomorphy (ch. 266: 1 > 0; RI: 0.62 RC: 0.43). Numbers in front of the branches correspond to bootstrap values. Numbers on the black bars indicate morphological synapomorphies. Further information can be seen in Supplementary material B.

relationship between *Rachoviscus* and *Hollandichthys* as already proposed by other authors (Javonillo et al., 2010; Thomaz et al., 2010; Oliveira et al., 2011).

Mirande (2010) also suggested that *Inpaichthys* be included within Aphyocharacinae based on three morphological synapomorphies. Nevertheless, the TE parsimony-based analysis

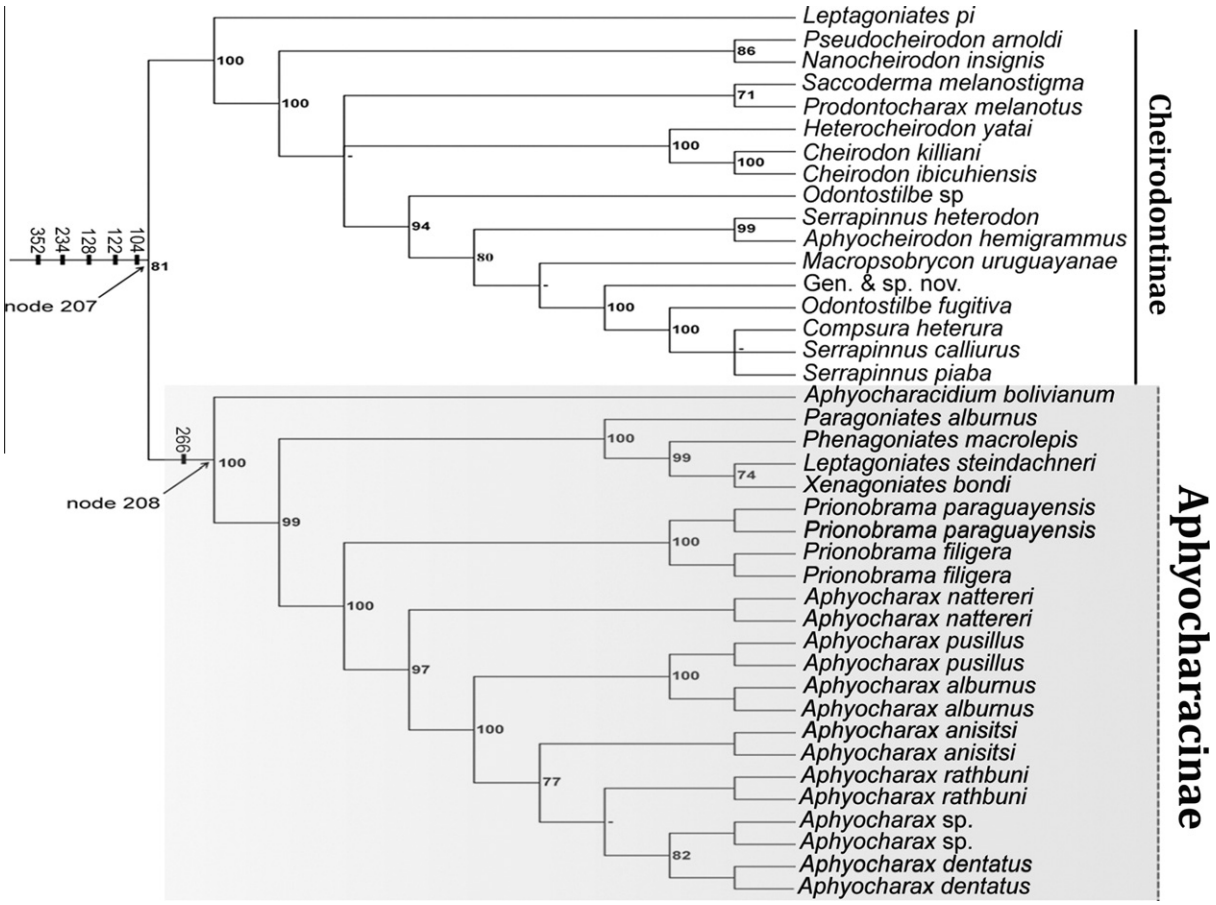

Fig. 2 (*continued*)

recovered in the present study shows that *Inpaichthys* belongs to a clade comprised of *Nematobrycon*, *Carlana*, and *Astyanacinus*, supported by five morphological synapomorphies (Fig. 2). One of these synapomorphies is the absence or reduction of the fourth infraorbital canal bone (ch. 66: 0 > 1) that is derived independently in a clade comprised of *Aphyocharax* and *Prionobrama* (Fig. 3). The reduction of the infraorbital series in different characiform groups, especially the fourth infraorbital bone, is widely thought to be homoplastic (Vari, 1995; Zanata and Vari, 2005; Toledo-Piza, 2007; Mirande, 2010). Other synapomorphies for the clade comprised of *Inpaichthys* and its close relatives include aspects of the third infraorbital canal bone (ch. 64: 1 > 0), premaxilla (ch. 104: 0 > 1), maxillary teeth (ch. 136: 0 > 1), and gill rakers (ch. 196: 0 > 1). Although these characters are homoplastic, most are not optimized as basal within Aphyocharacinae.

3.2. Monophyly of Aphyocharax, Prionobrama, Paragoniates, Phenagoniates, Leptagoniates, and Xenagoniates

The clade comprised of *Aphyocharax*, *Prionobrama*, *Paragoniates*, *Phenagoniates*, *Leptagoniates steindachneri,* and *Xenagoniates*, but excluding *Inpaichthys*, *Rachoviscus*, and *Leptagoniates pi* is a statistically well supported group in both the model-based and parsimony-based analyses (Figs. 1 and 2); additionally, this clade is supported by eleven morphological synapomorphies (Fig. 3), including seven identical to those proposed by Mirande (2010). In relation to internal relationships, phylogenies recovered in this study are congruent with molecular hypotheses of Oliveira et al. (2011), however slightly different from morphological hypotheses of Mirande (2009, 2010) in relation to the sister group of Aphyocharax.

3.3. Aphyocharax as the sister to Prionobrama

The morphological phylogenies of Mirande (2009, 2010) proposed that the three species of *Aphyocharax* studied form a natural group that is the sister group of a clade comprised of *Prionobrama*, *Paragoniates*, *Phenagoniates*, and *Xenagoniates*. Including also *Leptagoniates steindachneri*, which shares a general resemblance to *Xenagoniates*, this group is similar to that proposed by Géry (1977). Herein, phylogenetic analyses recovered identical relationships to that published by Oliveira et al. (2011), and a very similar topology to those proposed by Mirande (2009, 2010), except for the relationship between *Aphyocharax* and *Prionobrama* (Figs. 1 and 2). While morphological phylogenies indicated that *Aphyocharax* is the sister group of all remaining aphyocharacins (Mirande, 2009, 2010), molecular phylogenies have placed *Aphyocharax* as the sister to *Prionobrama* (Oliveira et al., 2011; Figs. 1 and 2).

Mirande's (2010) hypothesis of relationship between *Prionobrama* and all remaining aphyocharacins is supported by seven non-exclusive synapomorphies. The TE parsimony-based phylogeny, alternatively, indicates that *Aphyocharax* and *Prionobrama* form a monophyletic group based on three morphological synapomorphies (ch. 66: 0 > 1; ch. 138: 1 > 0; ch. 366: 0 > 1). One of these synapomorphies is remarkable and unique; the lateral line is interrupted with a single perforated scale on the posterior region of caudal peduncle (ch. 366: 0 > 1; Fig. 4). This atypical state has not been reported to any other characid species (Malabarba, 1998; Zanata and Vari, 2005; Mirande, 2009, 2010), and therefore represents strong

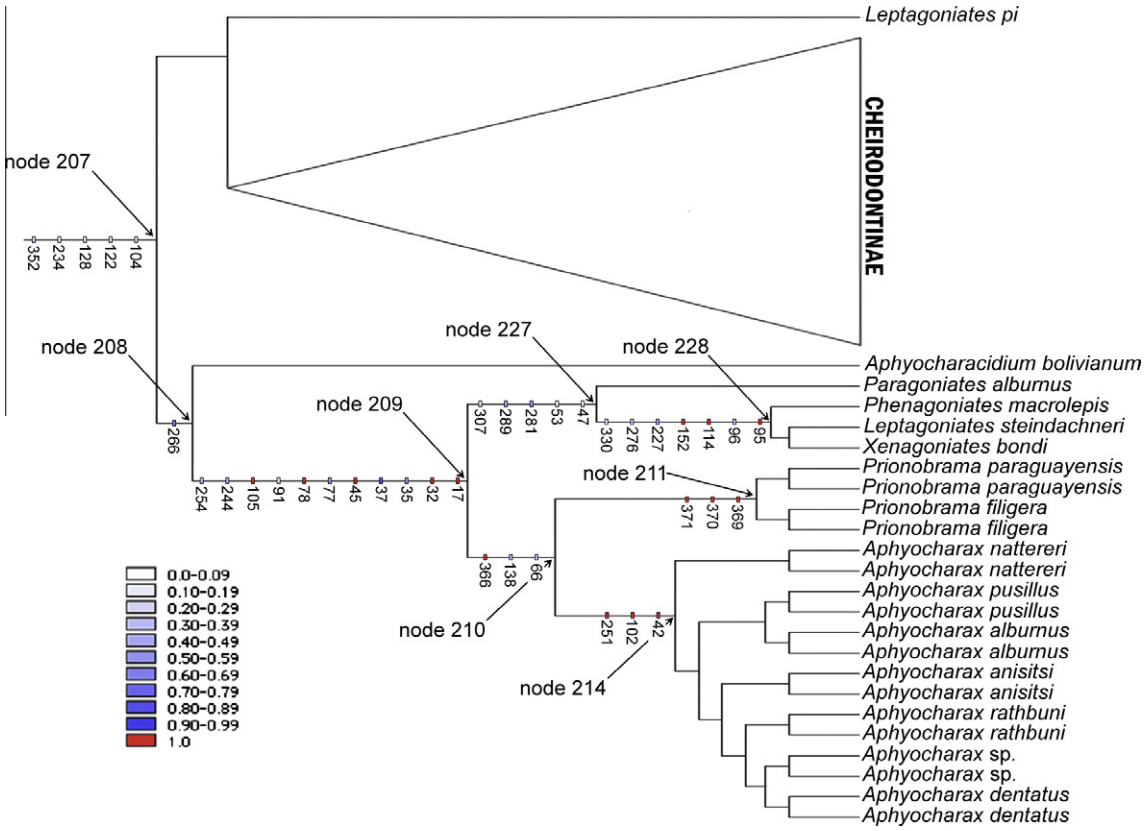

Fig. 3. Cladogram of the subfamily Aphyocharacinae (as defined in the present study) showing morphological synapomorphies along its branches. Numbers correspond to characters reported by Mirande (2010), except 366 (0 > 1), 369 (0 > 1), 370 (0 > 1), and 371 (0 > 1) which are proposed here. See legend for symbols and RI values on branches.

evidence for a close relationship between *Aphyocharax* and *Prionobrama*. Another synapomorphy is the absence or reduction of the fourth infraorbital canal bone (ch. 66: 0 > 1; Fig. 5). This character state is one of the synapomorphies proposed by Mirande (2010) for the clade he called Aphyocharacinae; however it is only shared by *Inpaichthys*, *Aphyocharax*, and *Prionobrama*, with a reversion to the condition of having well developed fourth infraorbital in other aphyocharacin species (Mirande, 2010). If *Inpaichthys* is excluded from consideration as argued above, the absence or reduction of this infraorbital bone may be viewed as shared only by *Aphyocharax* and *Prionobrama*. A third synapomorphy recovered is the presence of a single large cusp on anterior maxillary teeth (ch. 138: 1 > 0). Although a single cusp is also present in *Aphyodite grammica*, *Exodon paradoxus*, and *Acestrocephalus sardine*, the tooth morphology of *Paragoniates*, *Phenagoniates*, *Leptagoniates* and *Xenagoniates* is distinct due to the presence of three or more small cuspids.

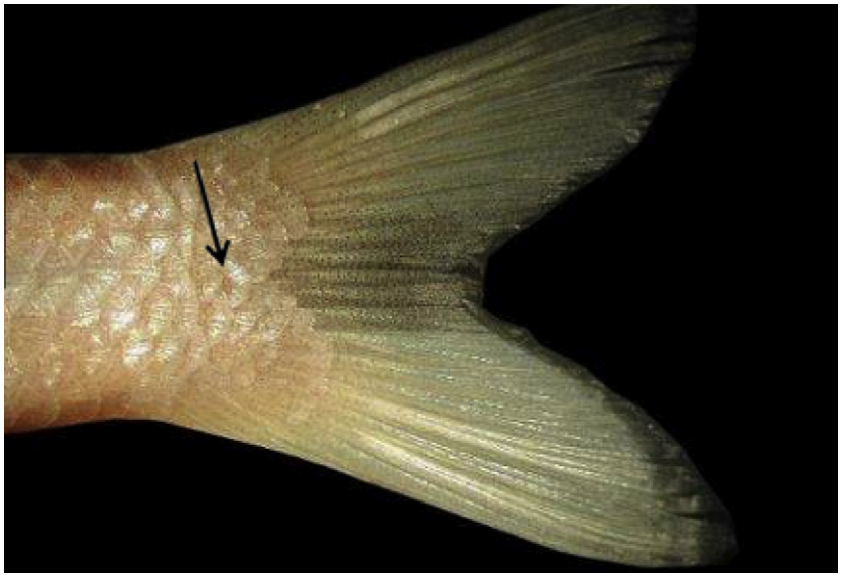

Fig. 4. Character 366 (0 > 1; CI: 1.0/RC: 1.0): lateral line interrupted with only the last scale of the series perforated (USNM 361472, *Aphyocharax pusillus*).

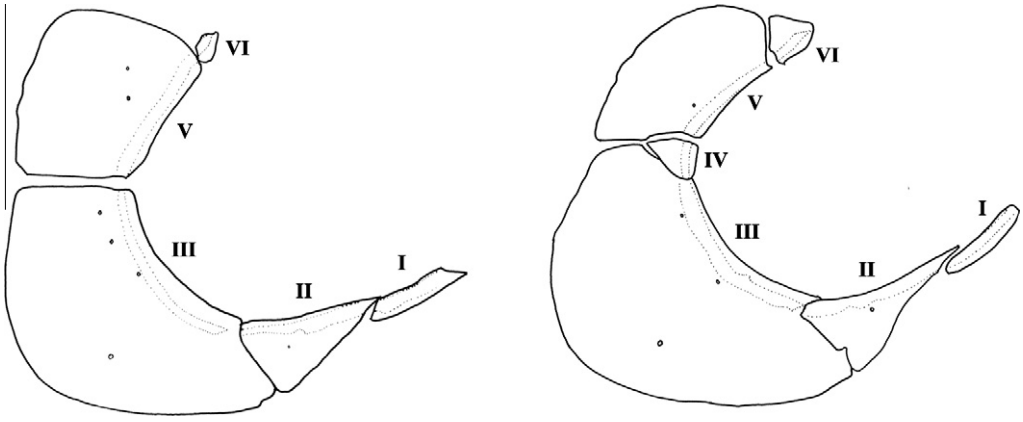

Fig. 5. Character 66 (0 > 1; CI: 0.40/RC: 0.31): Fourth infra-orbital absent (LBP 4046, *Aphyocharax pusillus*) or reduced (LBP not cataloged, *Prionobrama filigera*).

3.4. Monophyly of Paragoniates, Phenagoniates, Leptagoniates, and Xenagoniates

The clade comprised of *Paragoniates*, *Phenagoniates*, *Leptagoniates steindachneri*, and *Xenagoniates* is statistically well supported based on both mode-based and parsimony-based analyses (Figs. 1 and 2). Five morphological synapomorphies reinforce its monophyly (Fig. 3), of which four were reported by Mirande (2010). Furthermore, internal relationships recovered support the hypotheses of Mirande (2009, 2010) and Oliveira et al. (2011).

The monophyly of the clade comprised of *Phenagoniates*, *Leptagoniates steindachneri* and *Xenagoniates* is statistically well supported (Figs. 1 and 2); additionally seven synapomorphies reinforce its monophyly (Fig. 3). Among these synapomorphies, six are identical to those recovered by Mirande (2010). One additional synapomorphy is the higher number of dorsal-fin pterygiophores (ch. 276: 0 > 1), which evolved independently in *Prionobrama*, and also in a clade comprised of some species of Stevardiinae (Fig. 2).

The monophyly of the clade comprised of *Leptagoniates steindachneri* plus *Xenagoniates* is also statistically well supported (Figs. 1 and 2). Although morphological synapomorphies are not yet known for this clade, in part because *Leptagoniates steindacheneri* was not examined by Mirande (2010), both species share at least three unusual traits which could be evidence of their relationship. These traits include a high number (30–40 vs. 15–17) of caudal vertebrae, absence of the third postcheithrum, and a complete lateral line. The former two characteristics are currently known only from species that are distantly related to Aphyocharacinae (see Mirande, 2009, 2010). Also, a complete lateral line has been concluded to represent the plesiomorphic state in many characid taxa (Malabarba, 1998; Zanata and Vari, 2005; Toledo-Piza, 2007; Mirande, 2010). However, a complete lateral line may be a synapomorphy in the case of *Leptagoniates steindachneri* plus *Xenagoniates*, since other aphyocharacins possess an incomplete lateral line. Future studies may test this hypothesis, which if confirmed, would represent the first case where a complete lateral line was regained within the Characidae.

3.5. Monophyly of Aphyocharax and Prionobrama and polyphyly of Leptagoniates

As previously recognized by Mirande (2010) Aphyocharacinae was thought to include four monotypic genera and four genera with multiple species: *Prionobrama* (2 spp.), *Rachovischus* (2 spp.), *Leptagoniates* (2 spp.), and *Aphyocharax* (11 spp.). Excluding *Rachoviscus* due to its close relation to *Hollandichthys*, results of this study indicate that *Prionobrama* and, most probably, *Aphyocharax* are both monophyletic, while *Leptagoniates* is polyphyletic (Figs. 1 and 2).

The monophyly of *Prionobrama* is statistically well supported (Fig. 1 and 2); additionally three morphological synapomorphies reinforce this hypothesis (Fig. 3). According to the TE parsimony-based phylogeny *P. paraguayensis* and *P. filigera* share characteristics of the pelvic girdle and suspensorium including: coracoid as long as deep (ch. 369: 0 > 1), mesocoracoid oriented obliquely with respect to the posterior margin of the cleithrum (ch. 370: 0 > 1) and bifurcated anterior border of the metapterygoid (ch. 371: 0 > 1; Fig. 6).

The monophyly of *Aphyocharax* is also well supported statistically (Figs. 1 and 2); additionally three morphological synapomorphies reinforce this hypothesis (Fig. 3). Although four described species (*A. erythrurus*, *A. yekwanae*, *A. gracilis*, and *A. agassizii)* were not included in this study, these species share the features of red fin coloration, modest body elongation, single series of tricuspid teeth on the premaxilla and mandible, and maxilla with teeth on up to two thirds of its ventral margin that characterize the genus *Aphyocharax*. The synapomorphies recovered herein are identical to those proposed by Mirande (2010), which include: trigeminofacialis foramen narrow, as a cleft with sphenotic almost excluded from its margin (ch. 42: 0 > 1), dorsal projection of maxilla overlaps the second infraorbital (ch. 102: 0 > 1), and dorsal development of third postcleithrum not projecting dorsally to posterior region of scapula (ch. 251: 0 > 1).

As noted above, the monophyly of *Leptagoniates* was not confirmed since *L. steindachneri* belongs to Aphyocharacinae while *L.*

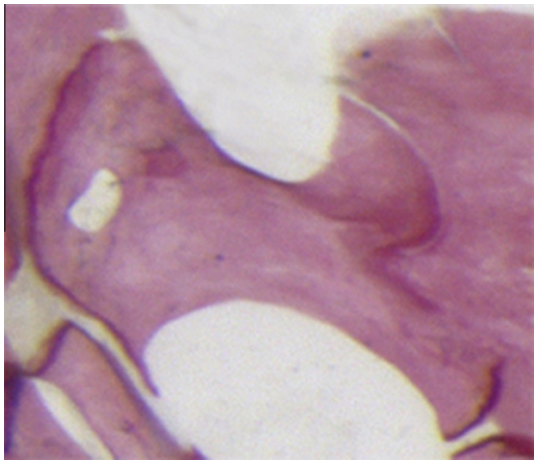

Fig. 6. Character 371 (0 > 1; CI: 1.0/RC: 1.0): Anterior border of metapterygoid bifurcated (LBP 3230, *Prionobrama paraguayensis*).

pi is more closely related to species of Cheirodontinae (Figs. 1 and 2). The subfamily Cheirodontinae is currently diagnosed by four synapomorphies including the lack of a humeral spot, presence of a pseudotympanum, pedunculate teeth, and a single row of regular teeth on the maxilla (Malabarba, 1998). Both *L. pi* and *L. steindachneri* possess all these characteristics, and, additionally, share a high number of anal-fin rays (usually more than 40). Nevertheless, *L. steindachneri* has an elongate and slender body, like that of *Xenagoniates*, whereas the body shape of *L. pi* is much deeper. Also, unlike *L. steindachneri*, the fin rays in *L. pi* rarely transcend a vertical line through the last dorsal-fin ray. Furthermore, these species can be distinguished by the number of vertebrae: 13 vs. 10 pre-caudal vertebrae in *L. pi*, and 27 vs. 38 caudal vertebrae in *L. steindachneri*.

3.6. Phylogenetic position of Aphyocharacidium

The phylogenetic results of this study indicate that *Aphyocharacidium bolivianum* is the sister group to Aphyocharacinae (*sensu* Mirande, 2010 – excluding *Rachoviscus* and *Inpaichthys*) (Figs. 1 and 2). This hypothesis is the same as that of Oliveira et al. (2011). According to Mirande (2010), *Aphyocharacidium* is a member of the Aphyoditeinae, which is itself the sister to Cheirodontinae, and these subfamilies together represent the sister group of Aphyocharacinae. However, the molecular results of Oliveira et al. (2011) and those presented herein suggest that Aphyoditeinae is not monophyletic.

Although Mirande (2010) recognized eight genera in Aphyoditeinae, the position of three of these genera (*Leptobrycon*, *Oxybrycon*, *Tyttobrycon*) was provisional due to a lack of material available for examination. The species sampling of Aphyoditeinae used in this study included four of the five genera and species examined by Mirande (2010): *Aphyodite grammica*, *Microschemobrycon casiquiare*, *Parecbasis cyclolepis*, and *Aphyocharacidium bolivianum*. The results are similar to those of Oliveira et al. (2011), since they suggest that *Aphyodite grammica* is closely related to *Hemigrammus ulrey*, *Microschemobrycon casiquiare* is closely related to Characinae (*sensu* Lucena and Menezes, 2003), and *Parecbasis cyclolepis* is the sister group of a clade comprised of *Thayeria*, *Bario*, *Hollandichthys*, and *Rachoviscus* (Figs. 1 and 2). The molecular phylogenies additionally suggest that *Aphyocharacidium bolivianum* is more closely related to Aphyocharacinae than Cheirodontinae (Figs. 1 and 2).

Under a TE parsimony analysis, the relationship between *Aphyocharacidium bolivianum* and all remaining aphyocharacins (as recognized herein) is supported by a single morphological synapomorphy: two dorsal-fin rays articulating with the first dorsal pterygiophore (ch. 266: 1 > 0). Although this state is polymorphic in some characid species (see also Mirande, 2010) it is invariable in *A. bolivianum* and other aphyocharacin species examined ($n = 45$ specimens – see Supplementary material B). Consequently, character 266 is taken as evidence for a close relationship between *A. bolivianum* and other aphyocharacins.

4. Conclusion

The relationships recovered in this study are similar to many of those proposed by Mirande (2009, 2010), with some notable exceptions. The results largely concur with the inclusion of *Aphyocharax*, *Prionobrama*, *Paragoniates*, *Phenagoniates*, *Leptagoniates*, and *Xenagoniates.* However, the subfamily Aphyocharacinae as proposed herein differs from that of Mirande (2010) in that it excludes: (1) *Inpaichthys*, which is closer to the distantly related characid taxa *Nematobrycon* and *Carlana*; (2) *Rachoviscus*, which is the sister group to the distantly related characid taxon *Hollandichthys*; and (3) *Leptagoniates pi*, which is more closely related to species of Cheirodontinae than to the type of the genus *L. steindachneri*. The results also differ from Mirande (2010) in recognizing a sister-group relationship between *Aphyocharax* and *Prionobrama*. In conclusion, this study is the first to propose the inclusion of *Aphyocharacidium* within the Aphyocharacinae. Given the phylogenetic results recovered herein, we propose to include *Aphyocharacidium* within the subfamily Aphyocharacinae, rather than place it in Aphyoditeinae. Under this proposal the Aphyocharacinae includes seven genera (*Aphyocharacidium*, *Aphyocharax*, *Prionobrama*, *Paragoniates*, *Phenagoniates*, *Leptagoniates*, and *Xenagoniates*) diagnosed by a single morphological synapomorphy: two dorsal-fin rays articulating with the first dorsal pterygiophore.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.04.007.

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