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A/C: ZOOLOGIA

VICTOR ALBERTO TAGLIACOLLO

SISTEMÁTICA FILOGENÉTICA DA SUBFAMÍLIA APHYOCHARACINAE (CHARACIFORMES, CHARACIDAE)

Botucatu
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Dissertação apresentada na forma de artigo junto ao curso de pós–graduação em Ciências Biológicas (Zoologia) da Universidade Estadual Paulista – UNESP, como requisito para obtenção do grau de Mestre em Ciências Biológicas (Zoologia)

Orientador: Prof. Dr. Ricardo Cardoso Benine
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COMISSÃO EXAMINADORA

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Prof. Dr. Ricardo Cardoso Benine

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Prof. Dr. Flavio César Thadeo de Lima

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Prof. Dr. Cristiano Rangel Moreira

Botucatu
Fevereiro, 2011

SUMMARY

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“Aprender é a única coisa de que a mente nunca se cansa, nunca tem medo e nunca se arrepende”

Leonardo da Vinci
1452–1519

DEDICATÓRIA

Ao meu amigo e ex–orientador
Gilson Luiz Volpato

AVISO

Esta dissertação é parte dos requerimentos necessários à obtenção do título de Mestre, área de Zoologia, e como tal, não deve ser vista como uma publicação no senso do Código Internacional de Nomenclatura Zoológica (apesar de disponível publicamente sem restrições). Desta forma, quaisquer informações inéditas, opiniões e hipóteses, bem como nomes novos, não estão disponíveis na literatura zoológica. Pessoas interessadas devem estar cientes de que referências públicas ao conteúdo deste estudo, na sua presente forma, somente devem ser feitas com a aprovação prévias do autor.

NOTICE

This dissertation is a partial requirement for the Master degree in Zoology and, as such, should not be considered as a publication in the sense of the International Code of Zoological Nomenclature (although it is available without restrictions). Therefore, any new information, opinions, and hypotheses, as well as new names, are not available in the zoological literature. Interested people are advised that any public reference to this study, in its current form, should only be done after previous acceptance of the author.

PHYLOGENETIC SYSTEMATIC OF THE SUBFAMILY APHYOCHARACINAE (CHARACIFORMES, CHARACIDAE)

Victor Alberto Tagliacollo

UNESP – São Paulo State University, Institute of Biosciences. Distrito de Rubião Jr., s/n, CxP 510, Cep 18618–000, Botucatu, São Paulo, Brazil.

e–mail: victor_tagliacollo@yahoo.com.br

ABSTRACT

The subfamily Aphyocharacinae comprises the genera A*phyocharax*, *Prionobrama*, *Paragoniates*, *Phenagoniates*, *Leptagoniates*, *Xenagoniates*, *Rachoviscus*, and *Inpaichthys*. This current arrangement based on a morphological analysis is congruent with most proposed morphological phylogenetic analyses, except for the inclusion of *Rachoviscus* and *Inpaichthys* which is tenuous and not always supported by data. In this current work the goal was to investigate the monophyly of the subfamily Aphyocharacinae and to recover relationships within it using molecular and morphological analyses. Using separate parsimony and Bayesian analyses of morphological, nuclear and mitochondrial genes, I recover new relationships within Aphyocharacinae. Independent analyses recovered similar topologies for molecular and morphologic datasets. Molecular based topologies showed common generic relationships: Aphyocharacinae, excluding the genus *Rachoviscus* and *Inpaichthys*, constitutes a monophyletic group. Moreover, Aphyocharacinae has two well–marked major branches. One composed by (*Paragoniates* (*Phenagoniates* (*Leptagoniates*+*Xenagoniates*))), while the other includes *Aphyocharax* and *Prionobrama*. Also, relationships among the genera were completely resolved and supported by strong statistical indexes. Based on molecular hypotheses, I had a specific monophyletic framework to conduct morphological analysis. This approach suggests that *Aphyocharacidium* sp. and *Microschemobrycon* cf. *casiquiare* are most probably the sister group of Aphyocharacinae. The morphological analysis conducted with 25 unweighted and unordered characters recovers one tree, resolved at generic–species level. A similar hypothesis found using molecular data is recovered with three well–marked morphological patterns based on: pectoral–fin girdle, neotenic characters and anal fin. Biogeography approach suggests the ancestral of the Aphyocharacinae arose in the Late Oligocene–Early Miocene. Thus, uplifts of northeastern Andes and respective changes in rivers courses explain aspects of probable geographic distribution such as: *Paragoniates* in Orinoco and Amazon; *Phenagoniates* only in *trans*–Andean rivers; and *Xenagoniates* and *Leptagoniates* in Orinoco and Amazon rivers respectively. To the other clade, catches of headwaters in Rio Mamoré were possibly responsible for the current presence of some species of *Aphyocharax* in Amazon and Orinoco and *Prionobrama filigera* in Amazon system basins.

Key words: Fishes, South America, Molecular systematic, Biogeography

INTRODUCTION

Characiformes comprises around 1400 species allocate into 237 genera with occurrence in Africa, south of North America, Central and South America (Reis *et al.*, 2003; Nelson, 2006; Eschmeyer, 2011). It is speculated that the origin of most its modern lineage arose more than 100 million year ago when Africa and South America still comprised a single landmass labeled Gondwana (Lundberg, 1998; Briggs, 2005; Lundberg *et al.*, 2010). The earliest taxonomic studies in Characiformes are dated from the 17th century (Marcgrave, 1648) and despite of the long history of the order, just a few is known about their phylogenetic relationships since most recent works take into account only specific group or not involve representatives of all groups of Characiformes (*e.g.* Ortí & Meyer, 1997; Calcagnotto *et al.*, 2005; Mirande, 2009; 2010). In relation to the number of families, Characiformes ranges from 14 (Reis *et al.*, 2003), 16 (Greenwood *et al.*, 1966) or 18 families (Nelson, 2006; Mirande, 2009). This discrepancy is due to how each author defines the family Characidae (Weitzman & Fink, 1983; Mirande 2009; 2010).

The family Characidae is the largest and most diverse in Characiformes with around 165 genera and more than 1,000 species (Reis *et al*., 2003; Nelson, 2006; Eschmeyer, 2011). Its representatives are geographically distributed from southern USA to northern Patagonia (Argentina) and many of them are popularly known as Tetra fishes. According to the most recent phylogenetic hypothesis (Mirande, 2010), Characidae comprises 23 possibly monophyletic subunits such as: Agoniatinae, Salmininae, Acestrorhynchinae, Cynodontinae, Bryconinae, Iguanodectinae, Heterocharacinae, Tetragonopterinae, Rhoadsiinae, Characinae, Gymnocharacinae, Aphyocharacinae, Aphyoditeinae, Cheirodontinae, Stevardiinae, *Pseudochalceus* clade, *Bryconops* clade, *Hyphessobrycon luetkenii* clade, *Astyanax paris* clade, *Bryconamericus scleroparius* clade, *Hemigrammus* clade, *Astyanax* clade, *Bramocharax* clade. Mirande's hypothesis indicates Bryconinae as the sister group of Salmininae that is congruent, in part, with most of molecular hypotheses (see Ortí & Meyer, 1997; Calcagnotto *et al*., 2005; Javonillo *et al*., 2010). Notwithstanding one year early, Mirande (2009) had already indicated a similar hypothesis to Characidae where he had also proposed the superfamily Characoidea comprised by Gasteropelecidae (including the genus *Clupeacharax* anteriorly allocate in Characidae *sensu* Lima & Zanata (2003)) and Serrasalmidae. Besides, in the same paper, Mirande (2009) included Acestrorhynchidae and Cynodontidae in Characidae; point out the subfamily Heterocharacinae; incorporates species of Glandulocaudinae within Stevardiinae; incorporates Stethaprioninae *sensu* Reis (2003) in the subfamily Tetragonopterinae; excludes Clupeacharacinae from Characidae; and redefines many groups, among them, the subfamily Aphyocharacinae.

The subfamily Aphyocharacinae was originally proposed by Eigenmann (1909) to include the genera *Coelurichthys* (= *Mimagoniates* Regan, 1907), *Odontostilbe* Cope, 1870, *Holoshesthes* (= *Odontostilbe* Cope, 1870), *Cheirodon* Girard, 1855 and *Aphyocharax* Günther, 1868, which comprises fishes with single series of well developed teeth on the premaxilla, mandible and maxilla. Since that time, Aphyocharacinae has been successively considered as valid or still included in the subfamilies Cheirodontinae and Characinae (Eigenmann, 1915; Weitzman, 1962; Géry, 1977; Souza–Lima, 2003; Mirande, 2009; 2010). The early history of Aphyocharacinae is a puzzle where many taxonomic equivocal occurred such as incorrect or not clear species descriptions and incongruence of name's priority between subfamilies (*e.g.* Cope, 1871; Steindachner, 1882; Ulrey, 1894; Eigenmann, 1915). Six year after Eigenmann (1909) proposed Aphyocharacinae, Eigenmann (1915) suggested the subfamily Cheirodontinae in order to comprise small characids with only one teeth row including those species anteriorly assigned to Aphyocharacinae. Eigenmann (1915) did not give reasons for using Cheirodontinae as a synonym to Aphyocharacinae. Thus, over years, Aphyocharacinae was being ignored and Cheirodontinae become widely adopted (*e.g.* Fowler, 1932; 1941; Gregory & Conrad, 1938; Géry, 1960). Weitzman (1962) grouped many genera in the subfamily Characinae including species of Aphyocharacinae (*sensu* Eigenmann, 1909), Cheirodontinae and Tetragnopterinae. Géry (1972) disagree with Weitzman's hypothesis and recognize, independently, Aphyocharacinae, Cheirodontinae and Tetragonopterinae. Later, Géry (1977) indicated that Aphyocharacinae is possibly related to the subfamily Paragoniatinae which was composed by *Prionobrama filigera* Fowler, 1913, *P. paraguayensis* (Eigenmann, 1914), *Rachoviscus crassiceps* Myers, 1926, *Paragoniates alburnus* Steindachner, 1876, *Phenagoniates macrolepis* Eigenmann & Wilson, 1914, *Leptagoniates steindachneri* Boulenger, 1887 and *Xenagoniates bondi* Myers, 1942. Still, Géry & Junk (1977) described *Inpaichthys kerri* suggesting that this new species has affinity with *Rachoviscus crassiceps* and "*Paragoniates* et al" (possibly "et al" refers to the species of the subfamily Paragoniatinae). Souza–Lima (2003; 2003b) adopted Aphyocharacinae as a monotype subfamily comprises only by the genus *Aphyocharax*. Besides, Souza–Lima (2003b) proposed nine valid species of *Aphyocharax*, eight undescribed species and three new generic combinations: *Prionobrama naterreri* (Steindachner, 1882); *Holoprion agassizii*

(Steindachner, 1882b) and *Holoprion melanotus* (Eigenmann, 1912). On the other hand, phylogenetic analysis by Quevedo (2006) pointed out the genus *Aphyocharax* is a paraphyletic group; in addition Quevedo's results also indicated that Paragoniatinae is a no–monophyletic subfamily since *Leptagoniates* is a polyphyletic genus and species of *Rachoviscus* are sister group of the genus *Hollandichthys* Eigenmann, 1910. Moreover, this author comments that *Prionobrama filigera*, *P. paraguayensis*, *Paragoniates alburnus*, *Phenagoniates macrolepis*, *Leptagoniates steindachneri* and *Xenagoniates bondi* should be moved into the subfamily Aphyocharacinae.

Currently, Aphyocharacinae is apparently a monophyletic group comprise by *Aphyocharax*, *Prionobrama*, *Paragoniates*, *Phenagoniates*, *Leptagoniates*, *Xenagoniates* and *Rachoviscus* and *Inpaichthys* (Mirande, 2009; 2010). Three ambiguous synapomorphies support it such as: (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; (3) six or less branched pelvic–fin rays (see Mirande, 2010). This current arrangement is congruent with most morphological phylogenetic analyses already proposed (Uj, 1990; Moreira, 2002; Quevedo, 2006), except for the inclusion of *Rachoviscus* and *Inpaichthys* which is tenuous and not always supported by data (Calcagnotto *et al*., 2005; Javonillo *et al*., 2010; see also Mirande, 2009; 2010). Thus, my goal was to investigate the monophyly of the subfamily Aphyocharacinae (*sensu* Mirande, 2009; 2010) and to recover relationships within it using rigorous molecular, morphological and biogeography analyses**.**

MATERIAL AND METHODS

Study Strategy

DNA genomic of characid species was extracted in order to amplify five partial genes (two mitochondrial and three nuclear). Subsequently, Parsimony and Bayesian molecular phylogeny analyses were conducted to investigate the monophyly of the subfamily Aphyocharacinae and to recover its internal relationships. Based on these molecular analyses, a specific monophyletic framework was used to conduct morphological analysis. As a final point, divergence data estimation was performed using fossils and palaeogeographic events.

Ingroup and outgroup criteria selection

The ingroup was chosen based on the most recent phylogenetic hypotheses proposed by Mirande (2009; 2010). Following these hypotheses, Aphyocharacinae comprises eight genera: A*phyocharax*, *Prionobrama*, *Paragoniates*, *Phenagoniates*, *Leptagoniates*, *Xenagoniates*, *Rachoviscus*, and *Inpaichthys*.

The outgroup was chosen based on Mirande's hypotheses although other phylogenies were also taken into account (*e.g.* Calcagnotto *et al*., 2005; Javonillo *et al.*, 2010). Following Mirande's hypotheses, Aphyoditeinae plus Cheirodontinae are both sister group of Aphyocharacinae, consequently *Prodontocharax melanotus* Pearson, 1924; *Cheirodon interruptus* (Jenyns, 1842); *Serrapinnus kriegi* (Schindler, 1937); *Odontostilbe fugitive* Cope, 1870; *Aphyocharacidium* sp. Géry, 1960; and *Microschemobrycon* cf. *casiquiare* Böhlke, 1953 were selected. Moreover, *Brycon falcatus* Müller & Troschel, 1844; *Salminus brasiliensis* (Cuvier, 1816) and *Lignobrycon myersi* (Miranda Ribeiro, 1956) were choose due to their basal position among characid fishes (see Ortí & Meyer, 1997; Javonillo *et al.*, 2010; Mirande, 2009; 2010). The species such as *Nematobrycon palmeri* Eigenmann, 1911, *Astyanax mexicanus* (De Filippi, 1853), *Hyphessobrycon eques* (Steindachner, 1882), *Moenkhausia xinguensis* (Steindachner, 1882), *Hemigrammus marginatus* Ellis, 1911 and *Phenacogaster* sp. Eigenmann, 1907 were added in order to verify relationship of *Rachoviscus* and *Leptagoniates* since they were not included by Mirande (2009; 2010). Besides, there are phylogenies that report species of *Rachoviscus* and *Leptagoniates* more related to Characinae, Gymnocharacinae or even Tetragnopterinae (*e.g.* Quevedo, 2006; Javonillo *et al*., 2010).

Tissue samples

Tissue samples of species of Aphyocharacinae and subfamilies Aphyoditeinae, Bryconinae, Characinae, Cheirodontinae, Gymnocharacinae, *Astyanax* clade, and Tetragonopterinae are listed in Appendix 1. *Brycon falcatus* was used as root following its basal position among characid fishes (Ortí & Meyer, 1997; Calcagnotto *et al*., 2005; Mirande 2009; Javonillo *et al*., 2010; Mirande, 2010).

DNA sequence data

DNA was extracted from muscle tissue preserved in ethanol with Wizard Genomic DNA Purification Kit (Promega). The partial sequences of the genes 16S rRNA (16S, 700 pb) and cytochrome b (CytB, 900 pb) were amplified using one round of 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–distillated water. Cycles of amplification were programmed with the following profile: (1) 3 min at 94°C (initia denaturation), (2) 30 s at 94°C, (3) 45s at 48–54°C, (4) 80s at 72°C, and 5 min at 72°C (final elongation). Steps 2–4 were repeated 35 times. On the other hand, 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 PCRs. The first was conducted using external primers while the second was conducted using internal primers (see Table 1). PCR amplifications were performed in 50 μl reactions consisting of 5 μl 10x 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) 45s at 50–54°C (4) 80s 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® (USB Corporation).

Sequencing reactions were performed with the Big Dye Terminator Cycle Sequencing Ready Reaction 3.1 Kit (Applied Biosystems) following instructions of the manufacturer and they were loaded on an automatic sequencer 3130–Genetic Analyzer (Applied Biosystems). Sequences will be deposited in GenBank. Individual sequences were analyzed with BioEdit 5.0.9 (Hall, 1999).

Table 1. Primers used to amplify the five gene regions sequenced in this study

Gene region	name	Primers sequence	Literature
16S rRNA	16Sar	5’-ACG CCT GTT TAT CAA AAA CAT-3’	Kocher *et al.* (1989)
	16Sbr	5’-CCG GTC TGA ACT CAG ATC ACG T-3’	
CytB	L14841	5’-CCA TCC AAC ATC TCA GCA TGA TGA AA 3’	Present study
	H15915b	5’-AAC CTC CGA TCT TCG GAT TAC AAG AC 3’	
Myh6	Myh6F329	5’- CCG CMT GGA TGA TCT ACA C - 3’	Li *et al.* (2008)
	Myh6A3R1	5’- ATT CTC ACC ACC ATC CAG TTG AA- 3’	
	Myh6bA3F2	5’- GGA GAA TCA RTC KGT GCT CAT CA - 3’	
	Myh6bA3R2	5’- CTC ACC ACC ATC CAG TTG AAC AT - 3’	
RAG 1	RAG12510F	5’- TGG CCA TCC GGG TMA ACA C – 3’	Li *et al.* (2007)
	RAG14090R	5’- CTG AGT CCT TGT GAG CTT CCA TRA AYT T – 3’	
	RAG1b2535F	5’- AGC CAG TAC CAT AAG ATG TA – 3’	
	RAG1b4078R	5’- TGA GCC TCC ATG AAC TTC TGA AGR TAY TT- 3’	
RAG 2	RAG2164F	5’ – AGC TCA AGC TGC GYG CCA T – 3’	Li *et al.* (2007)
	RAG2R2R6	5’- TGR TCC ARG CAG AAG TAC TTG – 3’	
	RAG2b176F	5’ – GYG CCA TCT CAT TCT CCA ACA – 3’	
	RAG2b1387R2	5’ - GGT CCA YGC YCA RTG CAT GG – 3’	

Phylogenetic molecular analyses

The sequences were aligned separately for each gene with on–line Multiple Sequence Comparison by Log–Expectation – MUSCLE (Edgar, 2004). Two phylogenetic reconstruction methods were used: Parsimony and Bayesian analyses.

Parsimony analysis was conducted in TNT 1.1 (Goloboff *et al.*, 2008). None weighting or ordering of character states were adopted and gaps were treated as a missing data. Phylogenies were constructed under the 'new technology search' methodology (Goloboff, 1996; 1999) using the options 'sectorial search', 'ratched', 'drift', and 'tree fusing' with their default values and employing a driven search with initial levels setting at level 100 and checking level every three hits. Bremer support was calculated with the script 'bremer' (Bremer, 1988) and consistency and retention indexes with the script 'stats'. Bootstrap (Felsenstein, 1985) was calculated with 1000 replications. Branches with bootstrap values higher than 70% were considerate well–supported (Hillis & Bull, 1993).

Bayesian phylogenetic inference was conducted in MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Four chains each were run simultaneously for 10 x 10^6 generations. Every 1000th generation was sampled and the asymptote of likelihood score was detected with the 'SUMP' command. After a graphical analysis of the evolution of the likelihood scores, and checking for the stationary of all model parameters, the first 2 x 10^6 generations (20%) were discarded as burn–in. The remaining trees were used to calculate 50% majority–rule consensus. Bayesian posterior probabilities ≥ 95% were considered well–supported. Appropriate substitution models for each gene were estimated with the Akaike Information Criterion method (Posada & Buckley, 2004) as implemented in Modeltest 3.6 (Posada & Crandall, 1998). Genes and their respective distance model can be seen in Table 2.

Table 2. Genes and their respective distance model.

Genes	Distance models	Partition of sequences (pb)
16S rRNA	GTR+I+G	1 - 552
CytB	TVM+I+G	553 - 1358
Myh6	TrN+I+G	1359 - 2099
RAG 1	TVMef+I+G	2100 - 3347
RAG 2	TrNef+I+G	3348 - 4309

Morphological procedures

Phylogenetic morphological analysis was based on osteological and morphological external characters of 16 taxa that included all genera (except *Rachoviscus* and *Inpaichthys*) plus *Microschemobrycon* cf. *casiquiare* and *Aphyocharacidium* sp. which represent the most probable sister group of Aphyocharacinae (see Fig. 1).

Examined specimens belong to the following institutions: ANSP, Academy of Natural Sciences of Philadelphia, Philadelphia; BMNH, Natural History Museum, British Museum of Natural History, London; CAS, California Academy of Sciences, San Francisco; FMNH, Field

Museum of Natural History, Chicago; LBP, Laboratório de Biologia e Genética de Peixes, Botucatu; MZUSP, Museu de Zoologia, Universidade de São Paulo, São Paulo (further information see Appendix 2).

Many characters were reinterpreted from data available in the literature (Malabarba, 1998; Zanata & Vari, 2005; Toledo–Piza, 2007; Mirande, 2009; Mirande, 2010) and others are firstly proposed herein. Osteological analyses were conducted on cleared–and–stained species prepared using method outlined by Taylor & Van Dyke (1985). Species were dissected under a stereomicroscope and structures were removed following suggestion of Weitzman (1974). Drawings were made on pictures captured with aid of a photographer camera connected to a stereomicroscope. Osteological nomenclature follows Weitzman (1962) and Weitzman & Fink (1983).

Morphological phylogenetic relationships were inferred using cladistic methodology (Hennig, 1966; Nelson & Platnick, 1981; Wiley *et al*., 1991; Swofford *et al*., 1996). The matrix (see Appendix 3) was constructed in Mesquite 2.7 (Maddison & Maddison, 2005). Parsimony analysis was conducted using TNT 1.1 (Goloboff *et al*., 2008) without weighting or ordering of character states and under default parameters. Phylogenetic trees were constructed using the 'implicit enumeration' (Goloboff, 1996; 1999) with the script 'function collapse tree after the search'. Consistency and retention indexes were calculated with the script 'stats'.

Divergence data estimation based on fossil and geological records

The estimation of evolutionary rate based on molecular divergence of sequences was done in two step: (1) using only information of †*Lignobrycon ligniticus* (23 Ma) which is the sister group of *Lignobrycon myersi* (Malabarba, 1998b); (2) using palaeogeographic event, in case, the uplift of Merida Andes (10–8 Ma) within the clade composed by *Phenagoniates*, *Leptagoniates* and *Xenagoniates* (see Fig. 1 – 2).

Divergence data estimation was performed in BEAST 1.6.1 (Drummond & Rambaut, 2007) using the uncorrelated lognormal model of molecular estimation (Drummond *et al.*, 2006). This method uses a global sampling strategy (MCMC) to maximize both phylogenetic inference and molecular rate estimation on branches simultaneously (Drummond *et al.*, 2006) eliminating, therefore, problems associated with selecting a tree topology and branch lengths *a priori*.

To conduct the analysis, substitution model parameter values were selected according to the results of Modeltest 3.6 (Posada & Crandall, 1998). Genes and their respective distance model can be seen in Table 2. Mutation rate was not fixed and an uncorrelated lognormal relaxed molecular–clock model was selected. The analysis started with an UPGMA tree and considered the Yule process tree prior. Input files were generated with BEAUti 1.6.1 (Rambaut & Drummond, 2007a). Two runs of 6 x 10^6 generations each were executed, sampling every 1000 generations and a burn–in of 10% of samples. Results of the two runs were displayed and combined in Tracer 1.3 (Rambaut & Drummond, 2005) to check for stationary.

RESULTS

Parsimony and Bayesian phylogenetic reconstruction

Trees based on Parsimony and Bayesian analyses, number of base pairs and phylogenetic informative bases pairs, shortest length tree, consistency and retention indexes, values of bootstrap and Bremer support and values of posterior probabilities are provided in Fig. 1.

Parsimony and Bayesian phylogenetic analyses based on molecular data showed that excluding *Rachoviscus crassiceps* and *Inpaichthys kerri*, Aphyocharacinae constitutes a monophyletic group well supported statistically (Fig. 1). Besides, Aphyocharacinae has two major lineages being one composed by (*Paragoniates* (*Phenagoniates* (*Leptagoniates*+*Xenagoniates*))) and the other composed by *Aphyocharax* and *Prionobrama*. Evolutionary relationships among them were also completely elucidated (Fig. 1). In addition, all genera had their monophyletic status corroborated (Fig. 1), except *Leptagoniates* because I was not able to evaluate the species *Leptagoniates pi*. Only five valid species of Aphyocharacinae (*sensu* Mirande 2009; 2010) were not included in molecular analyses: *Leptagoniates pi*, *Rachoviscus graciliceps*, *Aphyocharax yekwanae*, *A. erythrurus* and *A. gracilis*. This last species is probably extinct according to Souza–Lima (2003b) while *Leptagoniates pi* has been pointed out as the sister group of *Phenacogaster franciscoensis* (Quevedo, 2006).

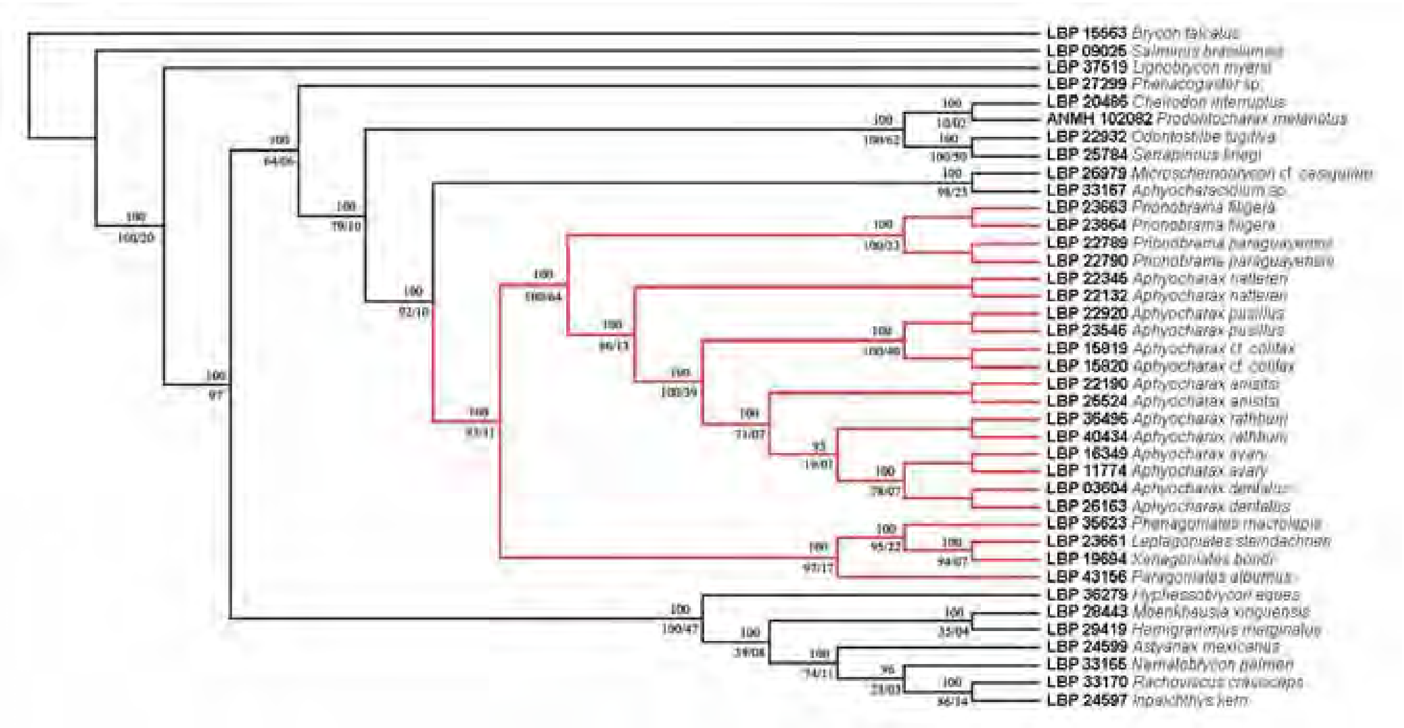


Figure 1. Consensus between Parsimony (one single tree; pb = 4329; pb informative = 1167; shortest length = 5366 steps; CI = 0.42; RI = 0.64) and Bayesian molecular analyses based on two mitochondrial and three nuclear genes. The numbers below each branch correspond to bootstrap values and Bremer support, respectively. The numbers above correspond to posterior probabilities values (8 x 10^6 generations). Branches in red color comprise the genera of Aphyocharacinae new def. as proposed here.

Morphological phylogenetic approach

Most parsimony tree, shortest length tree, consistency and retention indexes are provided in Fig. 2.

Parsimony analysis based on morphological analysis resulted in a similar topology to those produced by molecular data. The only discrepancy occurred in the internal nodes among the species of *Aphyocharax* that were not well elucidated (Fig. 2) in contrast with the molecular phylogeny topologies (Fig. 1). The species of *Aphyocharax* aggregated within a polytomy and *A. nattereri* showed yet three autopomorphies (ch. 8; ch. 9; ch. 18). These autopomorphies, in addition to the phylogenetic position of *Aphyocharax nattereri* proposed by molecular trees (Fig. 1), could indicate a new taxonomic status to the species; however more characters are still required in order to clarify internal relationships on the morphological point of view. Besides, all genera also had their monophyletic status corroborated, except *Leptagoniates* because I was not able to investigate *Leptagoniates pi*. Species of the genus *Rachoviscus* and *Inpaichthys kerri* were not included due to their most probably phylogenetic relationship with *Hemigrammus marginatus*, *Hyphessobrycon eques*, *Moenkhausia xinguensis*, *Nematobrycon palmeri* and *Astyanax mexicanus* (Fig. 1). Consequently, it was assumed that Aphyocharacinae (*sensu* Mirande, 2010) is no–monophyletic group and species of *Rachoviscus* and *Inpaichthys kerri* were withdrawn of morphological approach. Only three valid species of Aphyocharacinae was not included herein: *Leptagoniates pi*, *Aphyocharax yekwana* and *A. gracilis*. The inclusion of *Leptagoniates pi* and *Aphyocharax yekwana* will be made when exemplars are available to clean and stained. In relation to *Aphyocharax gracilis*, only the holotype and one paratype specimen are known what become impracticable clean and stained. The list of synapomorphies that supports the monophyly of Aphyocharacinae and its internal relationships is detailed below. Complete list of characters can be seen in Appendix C.

Monophyly of subfamily Aphyocharacinae

Character 03 (0>1): No contact between the second and third pos–cleithrum.

Character 04 (0>1): Third pos–cleithrum slender, without associated lamella.

Character 06 (0>1): Postero–ventral margin of cleithrum convex, covering the first pectoral–fin rays.

Monophyly of clade formed by the genera *Aphyocharax* and *Prionobrama*

Character 07 (0>1): Fourth infraorbital absent or reduced and bordered posteriorly by third and fifth infraorbitals.

Character 25 (0>1): Lateral line interrupted, with only the last scale of the series also perforated.

Monophyly of the genus *Aphyocharax*

Character 10 (0>1): *Trigemino–facialis* foramen narrow, as a cleft almost completely limited by prootic and pterosphenoid.

Character 11 (0>1): Ischiatic process curved laterally.

Autapomorphies of *Aphyocharax nattereri*

Character 08 (0>1): Rhinosphenoid absent or not ossified.

Character 09 (0>1): Contact between frontals anteriorly to frontal fontanel absent or only contact in the most anterior border.

Character 18 (0>1): Laterosensory canal on dentary reaching only half of dentary length.

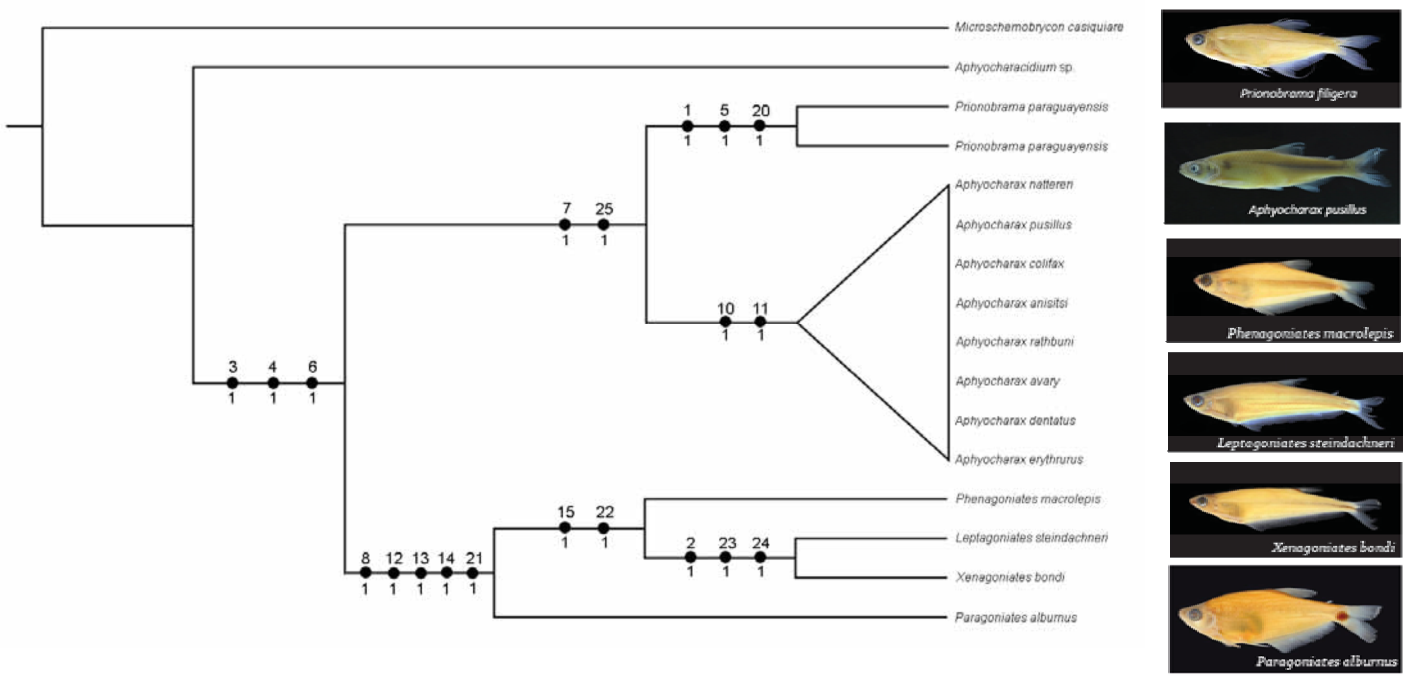


Figure 2. Single cladograma set up by parsimony analysis and based on 25 morphological characters (length 26 steps; CI = 0.923; RI = 0.963). Numbers below correspond to state of character and above to caracter as proposed in Appendix C. Pictures taken from Quevedo (2006), except *Aphyocharax pusillus*.

Monophyly of the genus *Prionobrama*

Character 01 (0>1): Coracoid length as wide as its height

Character 05 (0>1): Mesocaracoid inclined in relation to the position of cleithrum

Character 20 (0>1): Anterior border of metapterygoid bifurcated.

Monophyly of clade formed by the genera *Paragoniates*, *Phenagoniates*, *Leptagoniates* and *Xenagoniates*

Character 08 (0>1): Rhinosphenoid absent or not ossified.

Character 12 (0>1): 38 to 75 branched anal–fin rays.

Character 13 (0>1): Anal–fin origin anterior to the vertical trough first dorsal–fin ray.

Character 14 (0>1): First and second hypural fused.

Character 21 (0>1): 9–11 supraneurals.

Autapomorphy of *Paragoniates alburnus*

Character 17 (0>1): Symphyseal dentary teeth absent.

Monophyly of clade formed by the genera *Phenagoniates*, *Leptagoniates* and *Xenagoniates*

Character 15 (0>1): dentary teeth pedunculated.

Character 22 (0>1): 8–11 pre–caudal vertebrae.

Autapomorphy of *Phenagoniates macrolepis*

Character 16 (0>1): Medial border of maxillary bone with a medial longitudinal lamella.

Monophyly of clade formed by the genera *Leptagoniates* and *Xenagoniates*

Character 02 (0>1): Third pos–cleithrum absent.

Character 23 (0>1): 30–40 caudal vertebrae.

Character 24 (0>1): Lateral line complete.

Autapomorphy of *Xenagoniates bondi*

Character 19 (0>1): Ectopterygoid teeth present

Biogeography: estimated dates

According to the biogeography analysis, that one which maximize both phylogenetic inference and molecular rate estimation simultaneously (Drummond *et al.*, 2006), Aphyocharacinae appeared as a monophyletic group which has two major lineages being one composed by (*Paragoniates* (*Phenagoniates* (*Leptagoniates*+*Xenagoniates*))) and the other composed by *Aphyocharax* and *Prionobrama* (Fig. 3 – 4). Besides, generic relationships among them were also similar to those produce by molecular (Fig. 1) and morphological approaches (Fig. 2). The only discrepancy occurred in the internal nodes among the species ((*Aphyocharax avary* + *A. dentatus*)(*A. rathbuni* + *A. anisitsi*). Molecular hypotheses suggested *Aphyocharax anisitsi* is the sister group of the clade formed by *A. rathbuni* (*A. avary* + *A. dentatus*).

Nonetheless, the two independent calibrations resulted into similar estimated dates for the origin of the subfamily Aphyocharacinae and its respective branches (Fig. 3 – 4). Calibration using the uplift of Merida Andes (Fig. 3) suggested that Aphyocharacinae arose approximately 21.0 Ma ago; the two major branches probably arose around 19.7 Ma ago; *Paragoniates alburnus* become a distinguish fishes in 14.9 Ma ago while *Phenagoniates macrolepis* in 11.6 Ma ago; *Leptagoniates steindachneri* and *Xenagoniates bondi* develop into independent species around 8.3 Ma ago; and lineages of *Aphyocharax* and *Prionobrama* split around in 13.8 Ma ago. Otherwise, calibration using the fossil species *Lignobrycon ligniticus* (Fig. 4) suggested that Aphyocharacinae arose approximately 23.2 Ma ago; the two major lineages probably appeared around 21.0 Ma ago;

Paragoniates alburnus become a distinguish lineage 15.5 Ma ago while *Phenagoniates macrolepis* lineage originated 11.4 Ma ago; *Leptagoniates steindachneri* and *Xenagoniates bondi* develop into independent species around 8.1 Ma ago; and lineages of *Aphyocharax* and *Prionobrama* split around in 14.7 Ma ago.

DISCUSSION

Molecular approach: the monophyly of Aphyocharacinae

The subfamily Aphyocharacinae, up to this study, comprised the genus A*phyocharax*, *Prionobrama*, *Paragoniates*, *Phenagoniates*, *Leptagoniates*, *Xenagoniates*, *Rachoviscus* and *Inpaichthys* (Mirande, 2009; 2010). This current arrangement is congruent with most molecular phylogenetic analyses already proposed, except for the inclusion of *Rachoviscus* and *Inpaichthys* which is tenuous and not always supported by data (Calcagnotto *et al*., 2005; Javonillo *et al*., 2010; see also Mirande, 2009; 2010). Molecular topology shown here (Fig. 1) corroborates most of previous hypotheses; nevertheless it disagrees with *Rachoviscus* and *Inpaichthys* as representatives of the subfamily Aphyocharacinae. Both Parsimony and Bayesian molecular analyses point out that *Rachoviscus crassiceps* and *Inpaichthys kerri* are most probably related to *Hemigrammus marginatus*, *Hyphessobrycon eques*, *Moenkhausia xinguensis*, *Nematobrycon palmeri* and *Astyanax mexicanus* than to the other species of Aphyocharacinae. Consequently, *Rachoviscus* and *Inpaichthys* were withdrawn from Aphyocharacinae, which is comprised from now on by the genera *Paragoniates*, *Phenagoniates*, *Leptagoniates*, *Xenagoniates*, *Aphyocharax* and *Prionobrama*. *Inpaichthys kerri* and *Rachoviscus crassiceps* have two non–aligned premaxillary teeth rows which are very similar to those seen in *Rachoviscus graciliceps* and *Hollandichthys multifasciatus*. These species also share: lateral line interrupted; fourth and sixth infra–orbital absent; premaxilar with two

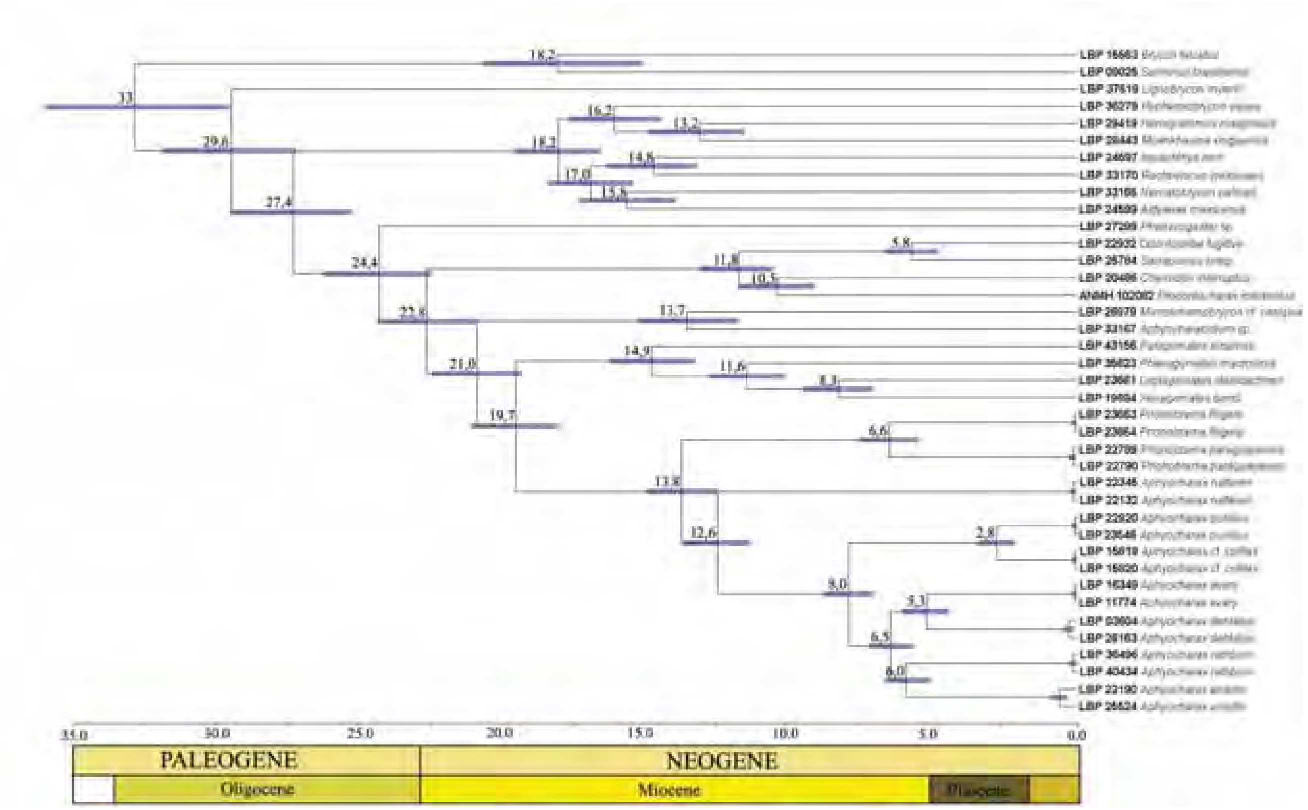


Figure 3. Biogeographic analysis of Aphyocharacinae based on two mitochondrial and three nuclear genes and using palaeogeographic event, in case, the uplift of Merida Andes (10 – 8 Ma) within the node composed by *Phenagoniates macrolepis*, *Leptagoniates steindachneri* and *Xenagoniates bondi.* Root age 33 Ma – oldest Characidae fossil known according to Malabarba & Malabarba (2010).

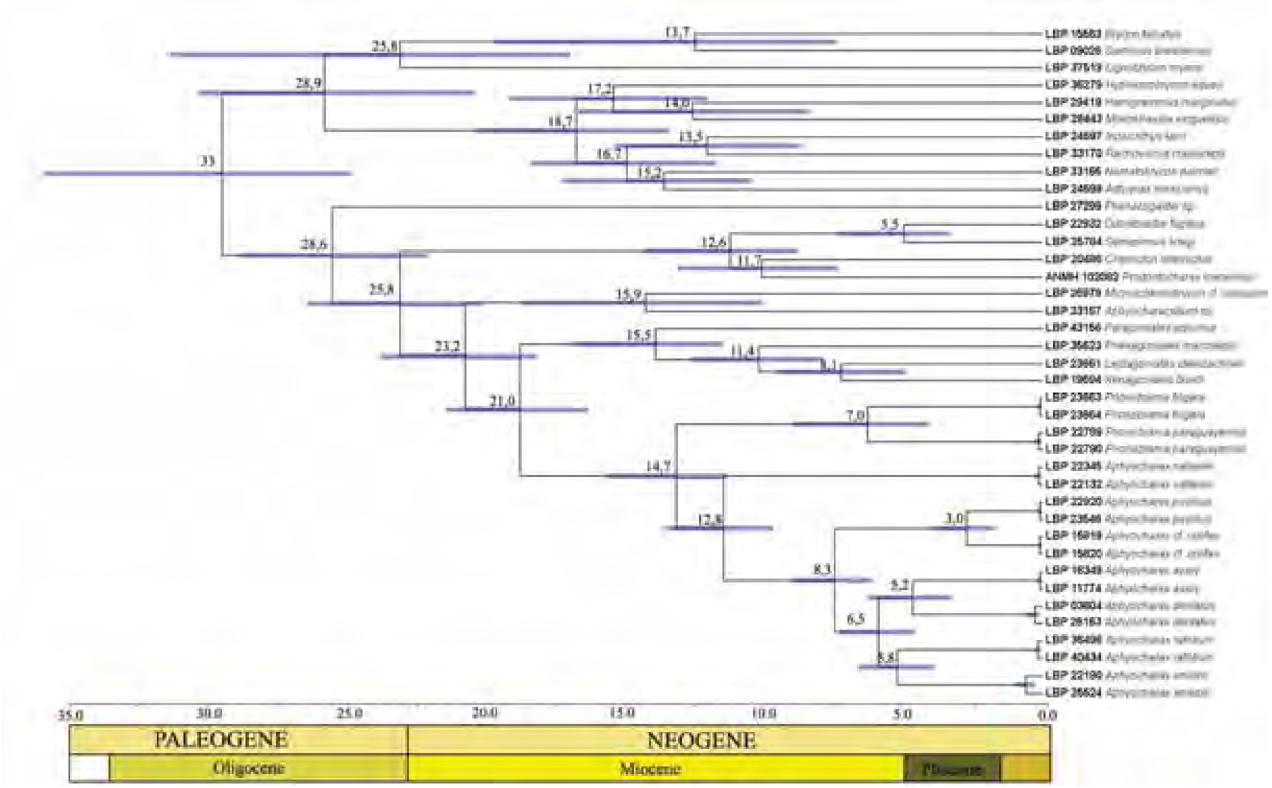


Figure 4. Biogeographic analysis of Aphyocharacinae based on two mitochondrial and three nuclear genes and using the fossil record, in case the species †*Lignobrycon ligniticus* within the node composed by *Lignobrycon myersi.* Root age 33 Ma – oldest Characidae fossil known according to Malabarba & Malabarba (2010).

teeth rows where outer row has normally two teeth; canal sensorial absent on first infra–orbital; and foramen scapular completely rounded by scapula. According to Bertaco (2003), most of these characters are found in species of *Pseudochalceus*, *Nematobrycon* and others of the genus *Hollandichthys*. Moreover, none of the features reported above were verified in representatives of the subfamily Aphyocharacinae, except for the fourth infra–orbital which is absent in *Prionobrama paraguayensis* and species of *Aphyocharax*. However, many other species also has fourth infra–orbital absent including those so far related to Aphyocharacinae such as *Hasemania* nana (Reinhardt, 1874), *Hemigrammus erythrozonus* Durbin, 1909, *Hoplocharax goethei* Géry, 1966 (see Mirande, 2009; 2010). Still, Vari (1995) mentioned that the absence of the fourth infra–orbital is a synapomorphy to Ctenoluciidae while Mirande (2010) reports it as a synapomorphy to Gasteropelecidae and to some species of Serrasalmidae; a synapomorphy to the subfamily Aphyocharacinae; an autopomorphy to *Hoplocharax goethei*, *Hyphessobrycon pulchripinnis*, *Hemigrammus erythrozonus*, *Aphyodite grammica* and *Nematobrycon palmeri*. Indeed, as already proposed by other authors (Zanata & Vari, 2005; Mirande, 2009; 2010) the reduction of infra–orbital series is highly homoplastic, with independent origins, and it appears to be the case between the clada composed by (*Rachoviscus* + *Inpaichthys*) and (*Aphyocharax* + *Prionobrama*).

From the phylogenetic point of view, there is consensus that *Inpaichthys kerri* and *Rachoviscus crassiceps* are most probably related to species of *Moenkhausia*, *Astyanax*, *Pseudochalceus*, *Hollandichthys* and *Nematobrycon* as well as, in part, proposed here (Bertaco, 2003; Calcagnotto *et al.*, 2005; Quevedo, 2006; Javonillo *et al*., 2009). Javonillo *et al.* (2009) included in their molecular analysis *Rachoviscus graciliceps* and *Rachoviscus crassiceps* which appeared as the sister group of *Hollandichthys* which, in turn, is the sister–group of *Moenkhausia* plus species of *Thayeria*. Quevedo (2006) included in his morphological analysis *Rachoviscus graciliceps* and *Rachoviscus crassiceps* which appeared as a sister group of species of *Hollandichthys*. Calcagnotto *et al.* (2005) included in their molecular analyses *Inpaichthys kerri* which appears as a sister group of *Moenkhausia* and those as a sister group of *Astyanax bimaculatus* plus *Astyanacinus*. Bertaco (2003) included in his morphological analysis *Rachoviscus graciliceps* and *Rachoviscus crassiceps* which appeared as the sister group of *Aphyocharax anisitsi* and this as the sister group of *Nematobrycon palmeri*. The synapomorphies that support the clade composed by *Rachoviscus* plus *Aphyocharax anisitsi* are: sixth infraorbital absence and rhinosphenoid absence. However, the sixth infraorbital is present in all species of the genus *Aphyocharax* while the absence of rhinosphenoid is an autapomorphy of only *Aphyocharax nattereri* (more information see morphological discussion section). Consequently, phylogenetic relationship between *Rachoviscus* and *Aphyocharax* proposed by Bertaco (2003) is incongruent.

Molecular approach: internal relationships among species of Aphyocharacinae

Mirande (2009; 2010) pointed out species of the genus *Aphyocharax* as the sister–group of *Prionobrama* (*Paragoniates* (*Phenagoniates* + *Xenagoniates*)). A similar hypothesis was proposed by Quevedo (2006) which indicated *Aphyocharax* as a paraphyletic group which is the sister group of (*Prionobrama* (*Paragoniates* (*Leptagoniates* (*Phenagoniates* + *Xenagoniates*))). The no–monophyly of *Aphyocharax* is discussed by Souza–Lima (2003b) who argues that *Aphyocharax nattereri* is a miniaturization of *Prionobrama*. Besides, the monophyly of the genus *Leptagoniates* has been put in check by Quevedo (2006), especially due to synapomorphies shared by *Lepatogoniates pi* and *Phenacogaster franciscoensis*. Molecular topologies proposed herein refute all these hypotheses since they indicate *Aphyocharax* as the sister group of *Prionobrama*; *Leptagoniates* as the sister–group of *Xenagoniates*; and the monophyly of all genera of the subfamily Aphyocharacinae, except *Leptagoniates* which I was not able to investigate the species *Leptagoniates pi* (Fig. 5).

The relationship between *Aphyocharax* and *Prionobrama* is strongly supported by statistical indexes (bootstrap and posterior probabilities) and by Bremer support (Fig. 5). Furthermore, Souza–

Lima (2003b) suggested, at least, six features shared among them being one an exclusive synapomorphy (see further information in morphological section). On the other hand, most of synapomorphies proposed by Mirande (2009; 2010) in favor of the clade composed by *Prionobrama* (*Paragoniates* (*Phenagoniates* (*Leptagoniates* + *Xenagoniates*))) are ambiguous. This clade comprises those species of Paragoniatinae (*sensu* Géry, 1977) supported by seven (six ambiguous) synapomorphies, including 25 or more branched anal–fin rays and anal–fin origin extended anteriorly ventral to dorsal–fin (Mirande, 2010). To this last character, species of *Prionobrama* have their anal–fin normally anterior or on the vertical line trough the first dorsal–fin ray, however never posterior as suggested by Mirande (2010). In relation to branched anal–fin rays, species of *Aphyocharax* have 12–22 branched anal–fin rays while *Paragoniates*, *Phenagoniates*, *Leptagoniates* and *Xenagoniates* share more than 38 branched rays.

The relationship between *Leptagoniates* and *Xenagoniates* is also strongly supported by bootstrap, posterior probabilities and Bremer values (Fig. 5). These species can be easily confused to each other because they share three peculiar characters: body elongates, elongates anal–fin (range iv/v–56/72 rays) and third post–cleithrum absent. Despite evidences, Quevedo (2006) suggested that *Leptagoniates steindachneri* is the sister–group of *Phenagoniates macrolepis* plus *Xenagoniates bondi* and just one non–ambiguous synapomorphy (ch. 88 [1] – anterior–ventral border of maxillary with bigger and tricuspid teeth and postero–ventral border with smaller and conical teeth) supports the relationship between the last two species.

Moreover, all genera of the subfamily Aphyocharacinae had their monophyletic status corroborated, except *Leptagoniates* which should be more studied. The monophyly of *Leptagoniates* has been put in check since *Lepatogoniates pi* shares, at least, eleven ambiguous synapomorphies with *Phenacogaster franciscoensis* (Quevedo, 2006). Other points that call my attention are the position of anal–fin origin in relation to dorsal–fin origin and the body shape. *Leptagoniates pi* has a high number of anal–fin rays that rarely reach few millimeters ahead of dorsal–fin origin and its

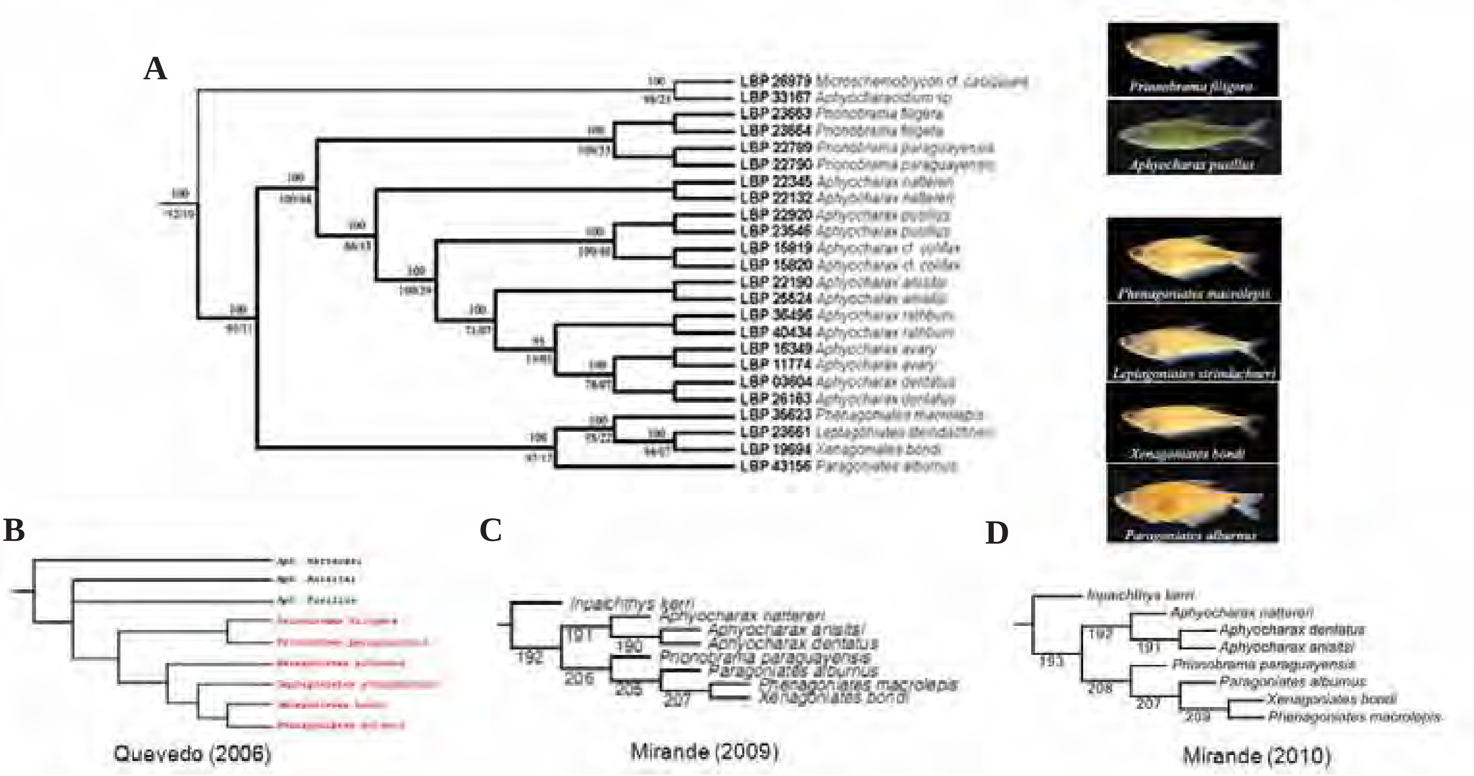


Figure 5. (**A**) Cladogram of interrelationships of Aphyocharacinae plus *Microschemobrycon* cf. *casiquiare* and *Aphyocharacidium* sp. (further information see Fig. 1). (**B**) Clado C by Quevedo (2006) showing phylogenetic hypothesis to the subfamily Paragoniatinae (*sensu* Géry, 1997). (**C**) and (**D**) Mirande's hypotheses to the subfamily Aphyocharacinae. Pictures taken from Quevedo (2006), except *Aphyocharax pusillus*.

body shape is triangular, not elongate as in *L. steindachneri*, being similar to those found in species of *Phenagoniates*. On the other hand, *Aphyocharax* is a monophyletic genus given that *Aphyocharax nattereri* do not belong to the genus *Prionobrama* (Fig. 5) as suggest Souza–Lima (2003b). This author proposed the new combination, named *Prionobrama nattereri*, based on four principal features (including humeral spot absent) which seem to be more plesiomorphies than synapomorphies shared with species of *Prionobrama*.

Morphological approach: morphological phylogeny supporting internal relationships among Aphyocharacinae

Morphological data also pointed out *Aphyocharax* as the sister–group of *Prionobrama*. Two synapormophies (ch. 7; ch. 25) support their relationship being that the character 25 is an exclusive synapomorphy. One perforated scale isolated on most posterior region of caudal peduncle (ch. 25) has not been reported to other species of characid up to this moment (Géry, 1977; Mirande, 2010). Still, Souza–Lima (2003b) suggests that this trait represents an indicative in favor of the monophyly of *Aphyocharax* and *Prionobrama* which was already proposed by Uj (1990) and Moreira (2002).

Besides, morphological data also indicated *Leptagoniates* as the sister–group of *Xenagoniates*. Three synapomorphies support their relationship such as: third postcleithrum absent (ch. 2), eighth to eleven precaudal vertebrae (ch. 22) and lateral line complete (ch. 24). This last character state has been reported as plesiomorphic (see Malabarba, 1998; Zanata & Vari, 2005; Toledo–Piza, 2007; Mirande, 2009; 2010). Still, according to Quevedo (2006), the lower number of precaudal vertebrae (ch. 22) is synapopmorphic for *Leptagoniates* and *Xenagoniates*. Finally, third post–cleithrum absent (ch. 2) is rarely spread among species of Characidae (Mirande, 2010) being it shared only by *Rhaphiodon vulpinus* Agassiz, 1829 (Cynodontinae), *Triportheus nematurus* (Kner, 1858), *T. pantanensis* Malabarba, 2004 (Bryconinae) and *Piabucus melanostomus* Holmberg, 1891 (Iguanodectinae).

Aphyocharax and *Prionobrama* are also monophyletic genera supported by two and three synapomorphies respectively. Species of *Aphyocharax* are distinguishes by a foramen *trigemino–facialis* limited by prootic and pterosphenoid (ch.10) and ischiatic process curved laterally (ch. 11). Mirande (2010) point out *Aphyocharax* as a monophyletic group reporting three synapomorphies, being one of them the character 10. Yet, the ischiatic process curved laterally (ch. 11) is firstly proposed herein and it also represents a synapomorphy to the genus *Aphyocharax* which had been already suggested as monophyletic, in part, by Souza–Lima (2003; 2003b). Species of *Prionobrama* are distinguished by a coracoid length as large as its depth (ch.1), mesocaracoid inclined (ch.5) and region anterior of metapterygoid bifurcated (ch. 20). These characters are firstly proposed here and they could be added to the eight synapomorphies indicated by Mirande (2010) to the genus *Prionobrama*.

Given these information, *Aphyocharax nattereri* should not be transferred to the genus *Prionobrama* as suggested by Souza–Lima (2003) because it shares synapomorphies of *Aphyocharax*. Yet, *Aphyocharax nattereri* has three autapomorphies (ch. 8; ch. 9; ch.18) in addition to other three proposed by Mirande (2010) which can indicate a new taxonomic status considering also molecular hypotheses showed herein (Fig. 1). At moment, it will be kept inside its genus until more morphological characters elucidate their internal relationships.

Morphological approach: comments about evolutionary patterns

Subfamily Aphyocharacinae

The six species comprised in Aphyocharacinae (Fig. 1 – 2) have a reduction of postero–ventral border of escapular girdle which can be seen through its three synapomorphies: no contact between the second and third pos–cleithrum (ch. 3); third pos–cleithrum slender, without associated lamella (ch. 4); Postero–ventral border of cleithrum convex and covering the first pectoral–fin rays (ch. 6). These synapomorphies together represent important evidences in favor of the monophyly of Aphyocharacinae.

Aphyocharax and Prionobrama

The body size of both *Aphyocharax* and *Prionobrama* are normally bigger than 32.0 mm SL in adults. On the other hand, these species have features of miniature fishes (*sensu* Weitzman, 1997) such as: lateral line incomplete; numbers of anal–fin rays reduced when compared to their closest larger relatives; teeth and row teeth simplified; bones head reduced to small (*e.g* fourth infra–orbital absent or reduced; rhinosphenoid absent). Thus, adopting Weitzman's criteria, *Aphyocharax nattereri* (11.9 – 31.0 mm SL see Souza–Lima, 2003b) and *Aphyocharax rathbuni* (17.0 – 26.9 mm SL see Souza–Lima, 2003b) are miniature species. Besides, the ancestral of *Aphyocharax* and *Prionobrama* was probably a miniature animal since the sister–group of Aphyocharacinae proposed here (*Aphyocharacidium* sp. and *Microschemobrycon* cf. *casiquiare*) is composed by species smaller than 32.0 mm SL (see Géry, 1973).

Paragoniates alburnus, *Phenagoniates macrolepis*, *Leptagoniates steindachneri* and *Xenagoniates bondi*

These species have an elongate anal fin with more than 38 branched rays. *Paragoniates alburnus* has ii/v–38/50 rays; *Phenagoniates macrolepis* has iii/vi–46/56 rays while *Leptagoniates steindachneri* and *Xenagoniates bondi* have iv/v–56/72 and iv/v–58/68 rays respectively. Based on their relationships, the values suggest a progressive increase of anal–fin rays that can be also seen dividing pre–dorsal length by pre–anal length (data not shown). The increase produces independent consequences such as: position of anal–fin origin in relation to dorsal–fin origin, reduction of pre–caudal vertebrae and increase of caudal vertebrae. For that reasons, I put in check the monophyly of the genus *Leptagoniates* (see also Quevedo, 2006), especially due to *Leptagoniates pi* which has the anal–fin origin anterior to vertical line trough last dorsal–fin ray; 13 pre–caudal vertebrae (*vs*. 10 in *L. steindachneri*) and 27 caudal vertebrae (*vs*. 38 in *L. steindachneri*).

Biogeography approach: A brief introduction

Phylogenetic analysis of molecular data provides two distinct kinds of information such as branching order (tree topology) and branch lengths (molecular divergence). Translating branch lengths to absolute age (years) requires an estimate evolutionary rate which is calculated through molecular divergence among the sequences (molecular clock). Such rates can be assumed *a priori*, however this approach has its problems (Ho, 2007). A useful technique is to estimate a rate specifically for the genes and clade in question using at least one or, whether possible, more calibration points. Nowadays, there are sophisticated methods that relax the assumption of the molecular clock and allow rates of evolution to vary across the branch of a phylogenetic tree (*e.g.* Drummond *et al*., 2006; Yang & Rannala, 2006). Two types of calibration points have been used: stratigraphic information from fossils and radiometric dating of palaeogeographic events.

Fossil is widely used in evolutionary and biogeographical studies to estimate minimum clade ages (*e.g.* Arratia, 1999; Albert & Fink, 2007) and it can be used as a minimum calibration points for molecular rate estimates (Near *et al.*, 2005). Notwithstanding, paleontological record from characid species is relatively poor, especially considering their high diversity. The oldest characid known is an undescribed species treated as “chacarid new species” (Malabarba & Malabarba, 2010). Although it is still not formally described, “characid new species” is important because provides a minimum age for the origin of the family Characidae (age of the cladogram root). Besides, there are more three fossil of characid species described from Brazilian Tremembé formation which could provide addition useful information: *Brycon avus* Woodward, 1898; *Megacheirodon unicus* (Travassos & Silva Santos, 1955) and *Lignobrycon ligniticus* (Woodward, 1898)

Phylogeny relationships of †*Brycon avus* are unclear since monophyly of the genus *Brycon* is still discussed (Calcagnotto *et al.*, 2005; Mirande, 2009; Javonillo *et al.*, 2010). †*Megacheirodon unicus* is proposed as the sister–group of *Amazonspinther dalmata* Bührnheim *et al.*, 2008 and species of *Spintherobolus* (Malabarba, 1998; Malabarba, 1998b; Bührnheim *et al.*, 2008), however a recent unpublished molecular hypothesis (Mariguela, 2010) pointed out that *Spintherobolus* does not belong to the subfamily Cheirodontinae. Fortunately, phylogeny relationships of †*Lignobrycon ligniticus* is well–understood from the cladistic point of view. This fossil has been proposed as sister group of *Lignobrycon myersi* (the only extant species of the genus) based on nine synapomorphies (Malabarba, 1998b). Thus, currently only †*Lignobrycon ligniticus* is a characid fossil trust enough to be employed as a calibration point for molecular rate estimates.

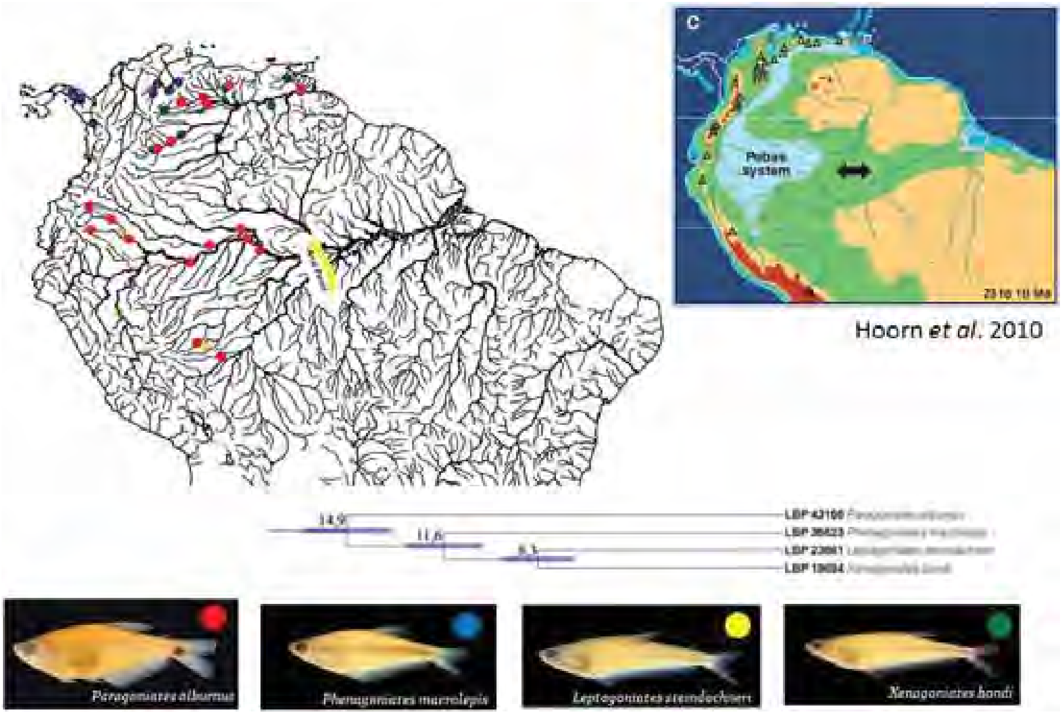
The other typical type of calibration is based on radiometric dating of palaeogeographic events. This method uses dates of palaeogeographic events provided by geological data as minimum age for the divergence of sister taxa. For freshwater fishes, geographical events that separate river basins provide important barriers to gene flow and dispersal. Consequently, the orogenies of the Eastern Cordillera of Colombia and Merida Andes represent the most reliable option for palaeogeographic calibration because they were relatively rapid, spatially extensive, long–lived, impermeably to fishes, and associated with well–accepted geological dates (Hoorn *et al.*, 1995; Lundberg, 1998; Hoorn *et al.*, 2010; Lovejoy *et al.*, 2010). Other reasonable points are the separation of Paraná and Amazon drainage basins by the rise of Chapare Buttress (Butler *et al.*, 1995; DeCelles & Horton, 2003) and the separation of the Orinoco and Amazon Rivers into their modern drainage basin through the rise of the Vaupes Arch (Hoorn, 1993; Hoorn *et al.*, 1995; Diaz de Gamero, 1996; Mora *et al.*, 2010). Notwithstanding, these points should be used with caution because exchanges of headwater tributaries and multiple stream capture events have probably been common throughout the intervening period (Lundberg *et al.*, 1998). Unfortunately, not all fishes clade of Characidae have living representatives in the Magdalena or Maracaibo drainages (see Albert *et al.*, 2006); in addition few phylogenetic hypotheses have been published involving genera or subfamilies of characids that have *cis*/*trans* Andean distribution (Bermingham & Martin, 1998; Lucena & Menezes, 1998; Malabarba, 1998; Vari & Harold, 2001).

*Biogeography approach: evolutionary history of Aphyocharacin***ae**

The subfamily Aphyocharacinae arose during Oligocene–Miocene boundary around 23 – 19 Ma (Fig. 3 – 4) possibly in the Oligocene river system. The Oligocene river system was a trunk river system flowing northward through the Andean foreland basins (Lundberg *et al*., 1998). This Oligocene Subandean River incorporates river systems draining the emergent Andes from the west and south as well as Cratonic rivers flowing in from the east (Wesselingh & Hoorn, *in press*). Initially, northwestern parts of the present western Paraná drainage were also incorporated into this Oligocene Subandean River. Stream capture shifted the drainage divide between the proto–Paraná and Subandean river systems northward toward the Michicola Arch in eastern Bolivia (Lundberg *et al*., 1998). River courses in eastern Amazonia were largely unchanged during the Oligocene, with the major watershed located at the Purus Arch (Wesselingh & Hoorn, *in press*). To the west of the Purus Arch, rivers drained toward the Andean foreland basin zone and were deflected northward toward the Caribbean (Hoorn *et al.*, 2010). Although the Andes were low and discontinuous at that time, there are as yet no indications that river systems actually crossed these mountains and emptied directly into the pacific (Hoorn *et al.*, 2010; Wesselingh & Hoorn *in press*). The ancestral of Aphyocharacinae possibly evolve in this Oligocene environment up to the separation of the Paraná and Amazon drainage basins by the rise of Chapare Buttress (Butler *et al*., 1995; DeCelles & Horton, 2003). Thus, the rise of Chapare Buttress elucidates the origin of two major branches.

The clade composed by *Paragoniates* (*Phenagoniates* (*Leptagoniates* + *Xenagoniates*)) arose in early Miocene around 22 – 18 Ma (Fig. 3 – 4) and their evolution occurred within the Pebas Lake system. During those times, the uplift of the Central Andes created the Pebas system, lowland habitats composed by lakes and swamps that covered an area of more than 1.5 million km^2 comprising much of the current western Amazonian lowlands (Wesselingh *et al*., 2002; Hoorn *et al.*, 2010). Fishes inhabited this environment were able to move freely over almost the entire western Amazonian region and many of the presently separated drainage (*e.g.* Magdalena and Maracaibo Lake) also comprised the environment (Hoorn *et al.*, 1995; Lundberg *et al.*, 1998; Hoorn *et al.*, 2010). The Pebas system was bordered by lowland rainforest and the Purus Arch (at circa 62°) was an effective barrier to dispersal. Species of this major branch have probably a limited dispersal movement; consequently their current geographic distribution is similar to the ancestral (Fig. 6). There are yet, at least, three other geological events related to the historic biogeography of this clade. The orogeny of the Eastern Cordillera of Colombia and isolation of the Magdalena dranaige basin at circa 12 – 11 Ma ago (Hoorn *et al*., 1995; Guerreiro, 1997; Hoorn *et al*., 2010); the orogeny of the Merida Andes and respective isolation of Maracaibo Lake around 10 – 8 Ma ago (Colletta *et al*., 1997; Lundberg *et al*., 1998; Hoorn *et al*., 2010) and changes in Amazon and Orinoco rivers courses caused by the rise of Vaupes Arch around 8 – 6 Ma ago (Hoorn *et al*., 1995; Mora *et al*., 2010). These events explain aspects of current geographic distribution such as: *Paragoniates* in Orinoco and Amazon; *Phenagoniates* only in *trans*–Andean rivers; and *Xenagoniates* and *Leptagoniates* in Orinoco and Amazon rivers respectively.

The clade composed by species of *Aphyocharax* and *Prionobrama* arose in early Miocene around 22 – 18 Ma (Fig. 3 – 4) and their evolution occurred within the current Paraguay drainages system. During those times, the pronounced bending of the Central Andes resulted from the contact of an underlying structure, Chaparé Buttress, between the Andean thrust front and the subsurface edge of the Brazilian Shield along the northern edge of a preexisting Paleozoic basin separating the Pebas Lake system from Paraguay system (Butler *et al*., 1995; DeCelles & Horton, 2003). The formation of the Paraguay basin was strongly influenced by the Andean orogeny along the western



Hoorn *et al.* 2010

Figure 6. Distribution map of the first major clade. See that the current distribution is comparable to the Pebas system which was delimited by the Purus Arch at circa 62°. Besides, uplifts of northeastern Andes (12 – 8 Ma) and respective changes in river courses (8 – 6 Ma) explain aspects of their current distribution such as *Paragoniates* in Orinoco and Amazon; *Phenagoniates* only in *trans*–Andean rivers; and *Xenagoniates* and *Leptagoniates* in Orinoco and Amazon rivers respectively.

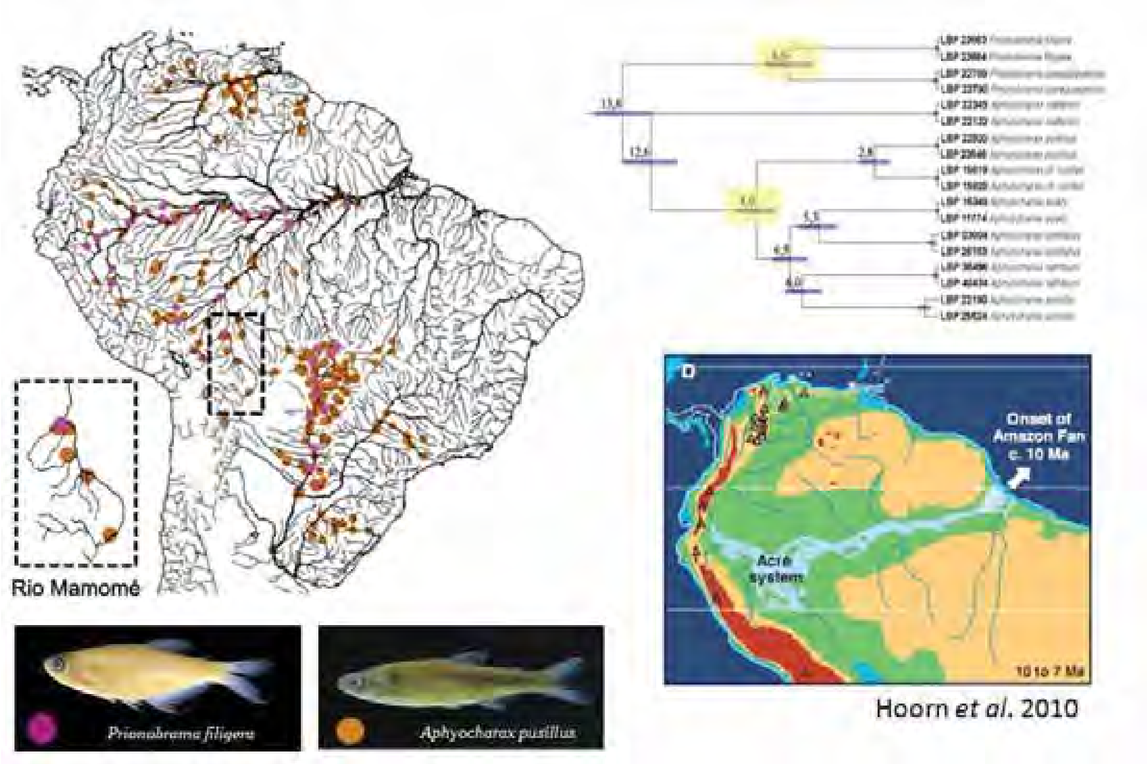


Figure 7. Distribution map of species of *Prionobrama* and *Aphyocharax*. Purple circle corresponds to the genus *Prionobrama* while orange circle corresponds to the genus *Aphyocharax*. See that the current distribution of *Prionobrama* in Amazon drainages is comparable to Acre system what suggests a more recent colonization, after the end of Pebas system period. Besides, *Prionobrama* and *Aphyocharax* are sympatric in the Rio Mamoré suggesting a possible catch event of headwaters by Amazon system around 6 – 8 Ma ago.

margin of South America (Uba *et al.*, 2006; Menezes *et al.*, 2008). Since the Miocene, the influences of the Andean uplift in this region have been quite pronounced, controlling the long–term evolution of the Chaco–Pantanal foreland basins (Uba *et al*., 2006). The genus *Aphyocharax* and *Prionobrama* possibly evolve in Paraguay rivers system environment (Fig. 7) and exchanges of headwater tributaries between the modern Paraguay basin and Mamoré–Madeira drainages was relatively common between 8 – 6 Ma (Lovejoy *et al*., 2010). There are evidences of faunal exchanges between the Mamoré and Paraguay basins such as: the loricariid *Otocinclus vittatus* (Schaefer, 1997), the aspredinid *Pterobunocephalus depressus* (Friel, 2003) and gymnotid *Gymnotus pantanal* Fernandes *et al*., 2005. Thus, I believe that species of *Aphyocharax* and *Prionobrama* colonized the Amazon landscape posteriorly.

CONCLUSION

The subfamily Aphyocharacinae *sensu* Mirande (2009; 2010) is a non–monophyletic group. For that reason, I propose the removal of the genera *Rachoviscus* and *Inpaichthys* which must go back to the status of *incertae sedis* in Characidae. With the removal, Aphyocharacinae comprises only the genera *Aphyocharax*, *Prionobrama*, *Paragoniates*, *Phenagoniates*, *Leptagoniates* and *Xenagoniates.* This new arrangement with six genera have three well–marked evolutionary pattern being one base on reduction of pectoral–fin girdle, another on extension of anal–fin and the last based on maintenance of neotenic characters. Because of the maintenance of some neotenic characters and also due to the relationship of *Aphyocharacidium* sp. and *Microschemobrycon* cf. *casiquiare* as most probably sister group of Aphyocharacinae, I assume the ancestral of the genus *Prionobrama* and *Aphyocharax* was a miniature species as well as *Aphyocharax nattereri* and *Aphyocharax rathbuni* are nowadays. By the same token, extension of anal–fin and its independent consequences suggests that *Leptagoniates* is not a monophyletic genus. Finally, Aphyocharacinae should be arisen in the early Miocene (23 – 19 Ma ago). Thus, the separation of the Paraná and

Amazon drainage basis by the rise of *Chapare Buttress* elucidate the origin of its two major branches. For the first major branch, uplifts of northeastern Andes and respective changes in rivers courses explain aspects of geographic distribution such as *Paragoniates* in Orinoco and Amazon basin; *Phenagoniates* only in *trans*–Andean rivers; and *Xenagoniates* and *Leptagoniates* in Orinoco and Amazon rivers respectively. For the second major branch which comprises only *Aphyocharax* and *Prionobrama*, catches events of headwaters near of Chapada do Parecis (Mamoré – Paraguay basin) and dispersal events may explain their modern geographic distribution across Paraguay, Paraná, Araguaia, Amazon and Orinoco Rivers.

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APPENDIX 1. List of species included in molecular analyses. LBP numbers correspond to the specimens used in the present study

Characidae (Mirande, 2010)	**Museum vouchers**	**species**	**Locality** (informations avaiable in the lots)	**Geographic Position**
Aphyocharacinae	LBP 25524	*Aphyocharax anisitsi*	Brasil / Barra do Ribeiro / RS / Rio Guaíba	30°17' 07'' S 58°18' 02'' W
	LBP 22190	*Aphyocharax anisitsi*	Brasil / Aquidauana / MS / Rio Paraguai	19°34' 33'' S 56°14' 49'' W
	LBP 11774	*Aphyocharax avary*	Brasil / Barra do Garça / MT / Rio Araguaia	15°54' 18'' S 52°19' 24'' W
	LBP 16349	*Aphyocharax avary*	Brasil / Aragarças / GO / Rio Amazonas	15°53' 35'' S 52°15' 00'' W
	LBP 3604	*Aphyocharax dentatus*	Brasil / Corumbá / MS / Rio Paraguai	19°34' 63'' S 57°01' 12'' W
	LBP 26163	*Aphyocharax dentatus*	Brasil / Cáceres / MT / Rio Paraguai	16°06' 66'' S 57°44' 33'' W
	LBP 22132	*Aphyocharax nattereri*	Brasil / Aquidauana / MS / Rio Paraguai	19°34' 54'' S 56°15' 16'' W
	LBP 22345	*Aphyocharax nattereri*	Brasil / Aquidauana / MS / Rio Paraguai	19°34' 17'' S 56°14' 44'' W
	LBP 22920	*Aphyocharax pusillus*	Brasil / Cruzeiro do Sul / AC / Rio Juruá	07°37' 20'' S 72°47' 42'' W
	LBP 23546	*Aphyocharax pusillus*	Brasil / Mâncio Lima / AC / Rio Japiim	07°34' 29'' S 72°55' 25'' W
	LBP 40434	*Aphyocharax ratbuni*	Brasil / Cárceres / MT / Rio Paraguai	16°03'14'' S 57°48'32'' W
	LBP 36496	*Aphyocharax ratbuni*	Brasil / Barão de Melgaço / MT / Rio Cuiába	16°11'39'' S 55°48'25'' W
	LBP 15819	*Aphyocharax* cf. *colifax*	Venezuela / Caicara del Orinoco / Bolivar / Rio Orinoco	07°39' 07'' N 66°10' 34'' W
	LBP 15820	*Aphyocharax* cf. *colifax*	Venezuela / Caicara del Orinoco / Bolivar / Rio Orinoco	07°39' 07'' N 66°10' 34'' W
	LBP 24597	*Inpaichthys kerri*	not available	not available
	LBP 23661	*Leptagoniates steindachneri*	Brasil / Mâncio Lima / AC / Rio Juruá	07°26' 35'' S 73°03' 33'' W
	LBP 43156	*Paragoniates alburnus*	Venezuela / Guarico / Rio Orinoco / Rio Manapire	07°52'04'' N 66°12'40'' W
	LBP 35623	*Phenagoniates macrolepis*	Venezuela / Machiques de Perijá / Zulia / Rio Apon	10°09' 42'' N 72°25' 58'' W
	LBP 23663	*Prionobrama filigera*	Brasil / Mâncio Lima / AC / Rio Juruá	07°26' 35'' S 73°03' 33'' W
	LBP 23664	*Prionobrama filigera*	Brasil / Mâncio Lima / AC / Rio Juruá	07°26' 35'' S 73°03' 33'' W
	LBP 22789	*Prionobrama paraguayensis*	Brasil / Sto Antônio do Leverger / MT / Rio Paraguai	15°54' 03'' S 56°01' 17'' W
	LBP 22790	*Prionobrama paraguayensis*	Brasil / Sto Antônio do Leverger / MT / Rio Paraguai	15°54' 03'' S 56°01' 17'' W
	LBP 33170	*Rachoviscus crassiceps*	Brasil / Guaratuba / PR / ------	25°55' 28'' S 48°36' 40'' W
	LBP 19694	*Xenagoniates bondi*	Venezuela / Caicara del Orinoco / Bolivar / Rio Orinoco	07°38' 11'' N 66°19' 04'' W
Aphyoditeinae	LBP 36167	*Aphyocharacidium* sp	Brasil / São Gabriel da Cachoeira / AM / Rio Negro	00°05' 10'' S 66°49' 03'' W
	LBP 26979	*Microschemobrycon* cf. *casiquiare*	Brasil / Almeirim / PA / Rio Jari	00°29' 09'' S 52°41' 29'' W
Astyanax clade	LBP 24599	*Astyanax mexicanus*	not available	not available
Bryconinae	LBP 15563	*Brycon falcatus*	Venezuela / Caicara del Orinoco / Bolivar / Rio Orinoco	07°30' 51'' N 66°09' 20'' W
	LBP 09025	*Salminus brasiliensis*	Brasil / Pirassununga / São Paulo / Rio Paraná	21°53' 37'' S 47°22' 01'' W
	LBP 37519	*Lignobrycon myersi*	not available	21°55' 38'' S 47°22' 04'' W
Characinae	LBP 27299	*Phenacogaster* sp	Brasil / Santa Filomena / PI / Rio Parnaíba	09°09' 51'' S 45°51' 15'' W
Cheirodontinae	LBP 20486	*Cheirodon interruptus*	Brasil / Rio Grande/ RS / -----	32°09' 07'' S 52°06' 24'' W
	AMNH 102082	*Prodontocharax melanotus*	Bolivia / Santa Cruz / -----	17°41' 29'' S 63°38' 96'' W
	LBP 22923	*Odontostilbe fugitiva*	Brasil / Cruzeiro do Sul / AC / Rio Juruá	07°37' 20'' S 72°47' 42'' W
	LBP 25764	*Serrapinnus kriegi*	Brasil / Aquidauana / MS / Rio Paraguai	19°34' 17'' S 56°14' 45'' W
Tetragonopterinae	LBP 29419	*Hemigrammus marginatus*	Brasil / Pontal / SP / Rio Grande	29°56' 50'' S 48°08' 52'' W
	LBP 12707	*Hyphessobrycon eques*	Brasil / Coxim / MS / Rio Paraguai	18°25' 42'' S 54°50' 03'' W
	LBP 28443	*Moenkhausia xinguensis*	Brasil / Paranatinga / MT / Rio Xingu	13°49' 00'' S 53°15' 00'' W
Gymnocharacinae	LBP 33165	*Nematobrycon palmeri*	not available	not available

APPENDIX 2. List of species included in morphological analyses

Characidae (Mirande)	**Lots analyzed**	**species**	**n° specimes analyzed**	**Locality** (informations avaiable in the lots)	**Geographic Position**
Aphyocharacinae	**CAS** 59697	*Aphyocharax anisitsi*	1 (holotype)	Paraguai/ Central / Assunção / Laguna Pasito at Asunción	25º23' S 57º37' W
	LBP 3764	*Aphyocharax anisitsi*	10 (3 c&s)	Brasil / Aquidauana / MS / Rio Paraguai / rio Negro	19°34'33'' S 56°14'49'' W
	LBP 6568	*Aphyocharax anisitsi*	10	Brasil / Marilena / PR / Rio Paraná / Lagoa marginal	22°37'57'' S 53°03'09'' W
	MZUSP 27067	*Aphyocharax anisitsi*	1 (1c&s)	Argentina / Colonia Carlos Pelegrini / Laguna Ibera	not available
	MZUSP 42757	*Aphyocharax anisitsi*	1 (1 c&s)	Brasil / Barão de Melgaço / MT / rio Cuiabá	not available
	ANSP 39217	*Aphyocharax avary*	1 (holotype)	Brasil / Rio Madeira / ca. 200 mi. east of 62°20'W	not available
	LBP 2480	*Aphyocharax avary*	10	Brasil / Aragarças / GO / Rio Araguaia	15°53'35' S 52°15'00'' W
	LBP 1587	*Aphyocharax avary*	9	Brasil / Barra do Garça / MT / Rio Araguaia / rio das Garças	15°54'18'' S 52°19'24'' W
	MZUSP 29405	*Aphyocharax avary*	2 (2 c&s)	Brasil / Calama / RO / Rio Madeira / Paraná do Caraparu	not available
	MZUSP 27977	*Aphyocharax avary*	1 (1 c&s)	Venezuela / San Fernando de Apure / Apure / 5 km de San Fernando	not available
	ANSP 139596	*Aphyocharax colifax*	10	Venezuela / Bolívar, Sand bar along Rio Mato	07°02' N 65°13' W
	FMNH 97153	*Aphyocharax colifax*	4 (paratypes)	Venezuela / Bolívar / Middle Oris River / Turumban campsite	not available
	LBP 2293	*Aphyocharax* cf. *colifax*	2	Venezuela / Bolívar / Caicara Del Orinoco / Rio Orinoco	07°39'06'' N 66°10'34'' W
	MZUSP 27067	*Aphyocharax colifax*	1 (1 c&s)	Venezuela / Corrientes / Colonia Carlos Pelegrini / Laguna Ibera	not available
	CAS 59722	*Aphyocharax dentatus*	1 (holotype)	Paraguai / Central / Assunção / Rio Paraguay / Laguna Pasito at Asunción	not available
	LBP 5112	*Aphyocharax dentatus*	10 (2 c&s)	Brasil / Cáceres / MT / Rio Paraguai / Lagoa Bairro caiçara	16°06'66'' S 57°44'33'' W
	LBP 20	*Aphyocharax dentatus*	6	Brasil / Corumbá / MT / Rio Paraguai / Rio Miranda	19°34' S 57°01' W
	MZUSP 16865	*Aphyocharax dentatus*	2 (2 c&s)	Brasil / Pirassununga / SP / rio Mogi Guaçú / Cachoeira de Emas	not available
	ANSP 175687	*Aphyocharax erythrurus*	1	Guiana / Essequibo River / sandbars in vicinity of Maipuri campsite	04°34'17'' N 58°35'17'' W
	ANSP 175668	*Aphyocharax erythrurus*	1	Guiana / Essequibo River / sandbar 800 m downstream from Essequibo campsite	04°45'43'' N 58°45'52'' W
	FMNH 53579	*Aphyocharax erythrurus*	1 (holotype)	Guiana / Upper Demerara-Berbice Region / Rockstone / Rockstone sandbank	not available
	MZUSP 27067	*Aphyocharax erythrurus*	1 (1 c&s)	Venezuela / Corrientes / Colonia Carlos Pelegrini / Laguna Ibera	not available
	ANSP 68864	*Aphyocharax gracilis*	1 (holotype)	Bolivia / Tarija / Rio Pilcomayo / tributary of the Paraguay at Villa Montes	not available
	ANSP 68865	*Aphyocharax gracilis*	4 (paratypes)	Bolivia / Tarija / Rio Pilcomayo / tributary of the Paraguay at Villa Montes	not available
	BMNH 1867.6.13.46	*Aphyocharax pusillus*	1 (holotype)	Peru / Xeberos	not available
	LBP 4046	*Aphyocharax pusillus*	10 (3 c&s)	Brasil / Cruzeiro do Sul / AC / rio Juruá / rio Moa	7°37'20'' S 72°47'42'' W
	LBP 4097	*Aphyocharax pusillus*	3	Brasil / Mâncio Lima / AC / rio Juruá / Rio Japim	07°34'28'' S 72°55'24'' W
	MZUSP 26144	*Aphyocharax pusillus*	1 (1c&s)	Peru / Loreto / Coronel Portillo / Rio Ucayali / Bagazan	not available
	MZUSP 30540	*Aphyocharax pusillus*	1 (1 c&s)	Brasil / Calama / RO / Rio Madeira / Poço da Angélica	not available
	FMNH 57922	*Aphyocharax nattereri*	1 (holotype)	Brasil / Cáceres / MT	16°4' S 57°41' W
	LBP 3786	*Aphyocharax nattereri*	10 (3 c&s)	Brasil / Aquidauna / MS / Rio Paraguai / lagoa marginal do Rio Negro	19°34'17'' S 56°14'44'' W
	MZUSP 9167	*Aphyocharax nattereri*	1 (1c&s)	Brasil / Santarém / PA / Rio Maicá	not available
	CAS 76467	*Aphyocharax rathbuni*	1 (holotype)	Paraguai / Arroyo / Chagalalina	not available
	LBP 7608	*Aphyocharax rathbuni*	4 (1 c&s)	Brasil / Barão de melgaço / MT / La Plata basin / Lagoa marginal do rio Cuiabá	16°11'39'' S 55°48'25'' W
	MZUSP 25234	*Aphyocharax rathbuni*	1 (1c&s)	Brasil / Itiquira / MS / Lagoas internas do sistema Piquiri	not available
	LBP 4526	*Inpaichthys kerri*	4 (2 c&s)	Aquarium tank without any information	not available

APPENDIX 2. List of species included in morphological analyses (continue)

Aphyocharacinae	**LBP** 4137	*Leptagoniates steindachneri*	2 (1 c&s)	Brasil / Mâncio Lima / AC / Rio Juruá / Rio Moá	07°26'35'' S 73°03'33'' W
	MZUSP 26085	*Leptagoniates steindachneri*	1	Peru/ Ucayal / Prov. Coronel Portilho	not available
	ANSP 149493	*Paragoniates alburnus*	10	Venezuela / Bolivar / Rio Orinoco / W side of Isla Fajardo near Palua	not available
	LBP 9208	*Paragoniates alburnus*	6 (1 c&s)	Venezuela / Cabruta / Guarico / Rio Orinoco / Rio Manapire	07°52'04'' N 66°12'40'' W
	MZUSP 17649	*Paragoniates alburnus*	1 (1 c&s)	Brasil / Amazonas / AM / Manacabi / rio Japurá	02°45' S 64° 52' W
	MZUSP 26707	*Paragoniates alburnus*	1 (1 c&s)	Peru / Ucayali / Coronel Portillo / Ivita – Pucallpa / Pueblo de Neshuya	not available
	ANSP 134909	*Phenagoniates macrolepis*	2 (2 c&s)	Venezuela / Motatan / 30 Km N Trujillo	not available
	ANSP 150124	*Phenagoniates macrolepis*	5	Venezuela / Motatan / 30 Km N Trujillo	not available
	LBP 6105	*Phenagoniates macrolepis*	1	Venezuela / Zulia / Machiques de Perijá / Lago Maracaibo / Rio Apon Medio	10°09'42'' N 72°25'58'' W
	ANSP 8073	*Prionobrama filigera*	1 (holotype)	Peru / Pebas	not available
	ANSP 134927	*Prionobrama filigera*	2 (2 c&s)	Peru / Morona	not available
	LBP 4139	*Prionobrama filigera*	2	Brasil / Mâncio Lima / AC / Rio Juruá / Rio Moa	07°26'35'' S 73°03'33'' W
	LBP, not catologed yet	*Prionobrama filigera*	6 (2 c&s)		
	MZUSP 43465	*Prionobrama filigera*	1 (1 c&s)	Brasil / cachoeira de Santo Antônio / RO/ Rio Madeira	not available
	LBP 3230	*Prionobrama paraguayensis*	10 (3 c&s)	Brasil / Nobre / MT / Rio Cuiabazinho / Lagoa marginal	14°40'32'' S 56°13'14'' W
	LBP 3960	*Prionobrama paraguayensis*	4	Brasil / Santo Antônio do Leverger / MT / Rio Paraguai / Baía do Poço	15°54'03'' S 56°01'17'' W
	LBP 8127	*Rachovischus crassiceps*	7 (3 c&s)	Brasil / Itanhaém / SP / Rio Itanhaém / lagoa temporária	24°13'55'' S 46°55'14'' W
	ANSP 127504	*Xenagoniates bondi*	1 (1 c&s)	Colombia / Rio Meta / Quebrada la venturosa between la balsa and puerto Lopez	04°05' N 72°58' W
	ANSP 131652	*Xenagoniates bondi*	5	Colombia / Rio Meta / Quebrada la venturosa between la balsa and puerto Lopez	04°05' N 72°58' W
	LBP 3074	*Xenagoniates bondi*	3 (1 c&s)	Venezuela / Bolivar / Caicara del Orinoco / Rio Orinoco	07°38'11'' N 66°19' 04'' W
Aphyoditeinae	**ANSP** 159710	*Miscroschemobrycon casiquiare*	5	Venezuela / Bolivar / Backwater at Rio Caura near Puerto Las Manjadas	07°38'18'' N 64°50'25'' W
	MZUSP	*Miscroschemobrycon casiquiare*	15 (2 c&s)	Brasil / Amazonas / AM / Rio Urubaxi /Rio Negro	00°31'00'' S 64°50' 00'' W
	MZUSP 90002	*Aphyocharacidium* sp	15 (2 c&s)	Brasil / Tanguará da Serra / MT / Rio Sepotuba / Rio Paraguai	14°30'04'' N 57°34'38'' W

APPENDIX 3. Character matrix for species of the subfamily Aphyocharacinae *sensu* Mirande (2009) present study and other two species (outgroup) of the subfamily Aphyoditeinae

Taxa		characters																								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
	Microschemobrycon casiquiare *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Aphyocharacidium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
APHYOCHARACNAE (*sensu* present study)	*Aphyocharax anisitsi*	0	0	1	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	Aphyocharax avary	0	0	1	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	Aphyocharax colifax	0	0	1	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	Aphyocharax dentatus	0	0	1	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	Aphyocharax erythrurus	0	0	1	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	Aphyocharax nattereri	0	0	1	1	0	1	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1
	Aphyocharax pusillus	0	0	1	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	Aphyocharax rathbuni	0	0	1	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	Prionobrama filigera	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
	Prionobrama paraguayensis	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
	Paragoniates alburnus	0	0	1	1	0	1	0	1	0	0	0	1	1	1	0	0	1	0	0	0	1	0	0	0	0
	Phenagoniates macrolepis	0	0	1	1	0	1	0	1	0	0	0	1	1	1	1	1	0	0	0	0	1	0	1	0	0
	Leptagoniates steindachneri	0	1	-	-	0	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	1	1	0
	Xenagoniates bondi	0	1	-	-	0	1	0	1	0	0	0	1	1	1	1	0	0	0	1	0	1	1	1	1	0

*indicate the root

APPENDIX 4. List of morphological characters

1. Ratio between coracoid length and coracoid height

(0) length bigger than height (1) length as big as height

2. Third pos–cleithrum

(0) present (1) absent

3. Second and third pos–cleithrum

(0) there are contact between them (1) there are not contact between them (–) not applicable

4. Form of third pos–cleithrum

(0) with a posterior lamella (1) slender, without associated lamella (–) not applicable

5. Position of mesocaracoid when compared it within the cleithrum

(0) parallel (1) inclined

6. Postero–ventral board of cleithrum

(0) concave, not covering the first pectoral–fin rays (1) convex, covering the first pectoral–fin rays

7. Fourth infraorbital

(0) present, well developed (1) absent or reduced and boarded posteriorly by third and fifth infraorbitals.

8. Rhinosphenoid

(0) present, ossified (1) absent or not ossified

9. Contact between frontals anteriorly to frontal fontanel

(0) present (1) absent or only contact in the most anterior border

10. Trigemino–facialis foramen

(0) broad, dorsally limited by sphenotic (1) narrow, as a cleft almost completely limited by prootic and pterosphenoid

11. Ischiac process

(0) straight (1) curved laterally

12. Number of branched anal–fin rays

(0) 10 to 35 rays (1) 35 to 75 rays

13. Anal–fin position

(0) posterior to vertical line through last dorsal–fin ray (1) anterior to vertical line trough last dorsal–fin ray

14. Fusion between the first and second hypural

(0) present (1) absent

15. Shape of dentary teeth

(0) not pedunculated (1) pedunculated

16. Border medial of maxillary bone

(0) straight or almost straight, without a lamella (1) within a medial longitudinal lamella

17. Symphyseal dentary teeth

(0) present (1) absent

18. Extension of laterosensory canal on dentary

(0) piercing almost entire length of dentary (1) reaching only half of dentary length

19. Ectopterygoid teeth

(0) absent (1) presente

20. Border anterior of metapterygoid

(0) convex (1) bifurcated

21. Number of supraneural

(0) 4–8 supraneural (1) 9–11 supraneural

22. Number of pre–caudal vertebrae

(0) 12–15 vertebrae (1) 8–11 vertebrae

23. Number of caudal vertebrae

(0) 20–25 vertebrae (1) 30–40 vertebrae

24. Lateral line

(0) interrupted (1) complete

25. One perforated scale, isolated, on most posterior caudal peduncle

(0) absent (1) present