Short Communication

A phylogenetic analysis of *Brycon* and *Henochilus* (Characiformes, Characidae, Bryconinae) based on the mitochondrial gene 16S rRNA

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Abstract

The genus *Brycon*, the largest subunit of the Bryconinae, has 42 valid species distributed from southern Mexico to the La Plata River in Argentina. *Henochilus* is a monotypic genus, comprising a single species (*H. wheatlandii*) found in the upper Rio Doce basin. In the present study, partial sequences of the mitochondrial gene 16S were obtained for fifteen species of *Brycon* and for *Henochilus wheatlandii*. The results showed that the genus *Brycon* is paraphyletic, since *Henochilus* is the sister-group of *B. ferox* and *B. insignis*. The most basal species analyzed were the trans-Andean species *B. henni*, *B. petrosus*, and *B. chagrensis*.

Key words: phylogeny, molecular systematics, mitochondrial DNA, fish evolution, systematic.

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The subfamily Bryconinae includes 74 nominal species out of which 44 are currently considered as valid (Lima, 2003; 2004). The genus *Brycon* is the largest subunit of the Bryconinae, consisting of 42 valid species (Lima, 2003; 2004). Géry (1977) and Géry and de Rham (1981) delimited the subfamily as comprising the genera *Brycon, Catabasis, Chalceus, Chilobrycon, Salminus,* and *Triportheus.* In a more recent account, Lima (2003), partly based on Zanata (2000), restricted the subfamily Bryconinae to the genera *Brycon*, *Chilobrycon*, and *Henochilus*. A morphological and molecular study of *Henochilus wheatlandii* reinforced the hypothesis that this genus belongs to the subfamily Bryconinae (Castro *et al.*, 2004).

Species of *Brycon* are widely distributed from southern Mexico to the La Plata River in Argentina. They are particularly diverse in Panama, and in the trans-Andean rivers of Colombia and Ecuador. They also occur in the cis-Andean South America river basins as well as in the Atlantic and Caribbean coastal river drainages (Lima, 2003). Fishes of the genus *Brycon* are important for human consumption and sport fishing. Different species are commer-

cially caught (Carosfeld *et al.*, 2003) or raised in fish breeding programs (Wasko *et al.*, 2004). Species of *Brycon* are very sensitive to anthropogenic disturbances and several species are now considered as endangered in Brazil (Rosa and Lima, 2005). The genus *Henochilus* is monotypic and its single species, *H. whetlandii*, is currently known only from the upper Rio Doce Basin, a coastal drainage in the state of Minas Gerais, Brazil (Castro *et al.*, 2004).

The mitochondrial large subunit (16S) rRNA gene (16S rRNA) has been extensively used for the study of phylogenetic relationships, even among closely related taxa (Shimabukuro-Dias *et al.*, 2004). In the present study, a first attempt to infer a molecular-based phylogeny of the genera *Brycon* and *Henochilus* based on partial 16S rRNA mitochondrial sequences was conducted with the objectives to test the monophyly of *Brycon* and Bryconinae (sensu Lima, 2003), the relationship between *Brycon* and *Henochilus* and the relationship among trans- and cis-Andean species of *Brycon*.

DNA sequences of thirteen species of *Brycon* representing trans- and cis-Andean basins were examined (Table 1). Ten additional sequences determined by Castro *et al.* (2004) and Calcagnoto *et al.* (2005) were obtained from GenBank (Table 1). Based on the study of Calcagnoto *et al.* (2005) two species of the genus *Chalceus* were selected as outgroup.

Table 1 - Specimens sequenced in the present study and data obtained from GenBank. IAvHP = Colección de Peces dulceacuícolas del Instituto Alexander von Humboldt, Boyaca, Colombia; LBP = Laboratório de Biologia e Genética de Peixes, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo, Brazil; MZUSP = Museu de Zoologia, Universidade de São Paulo, São Paulo, Brazil; AMNH = American Museum of Natural History, USA.

Species	Locality	Collection number	Genbank
Brycon amazonicus	Tomo River, Colombia	IAvHP 4490	DQ530607
Brycon aff. *atrocaudatus*	Santa River, Macate, Ancash, Peru	LBP 1356	DQ530602
Brycon chagrensis	Río Llano Sucio, Santa Rita Arriba, Colón, Panama	LBP 2749	DQ530614
Brycon ferox	Mucuri River, Espírito Santo, Brazil	Not preserved	DQ530609
Brycon henni	Santacruz River, Santander, Colombia	IAvHP 4355	DQ530612
Brycon insignis	Paraíba do Sul River, Caçapava, São Paulo, Brazil	Not preserved	DQ530610
Brycon moorei	Rancheria River, Guajira, Colombia	IAvHP 40	DQ530608
Brycon nattereri	Itaúna River, Baenpedi, Minas Gerais, Brazil	Not preserved	DQ530611
Brycon opalinus	Paraibuna River, São Luiz do Paraitinga, São Paulo, Brazil	Not preserved	DQ530603
Brycon orbignyanus	Paraná River, São Paulo, Brazil	Not preserved	DQ530606
Brycon orthotaenia	São Francisco River, Bahia, Brazil	Not preserved	DQ530605
Brycon pesu	Aripuanã River, Mato Grosso, Brazil	MZUSP uncat.	DQ530604
Brycon petrosus	Río Llano Sucio, Santa Rita Arriba, Colón, Panama	LBP 2750	DQ530613
Acestrorhynchus sp.	Unavailable	Not cited	AY787956
Astyanax bimaculatus	Unavailable	Not cited	AY787955
Brycon hilarii	Unavailable	Not cited	AY787976
Bryconops sp.	Unavailable	Not cited	AY787985
Chalceus erythrurus	Unavailable	AMNH233413	AY787990
Chalceus macrolepidotus	Unavailable	AMNH233404	AY787999
Exodon paradoxus	Unavailable	AMNH233426	AY788013
Hemibrycon beni	Unavailable	AMNH233328	AY788020
Henochilus wheatlandii	Rio Santo Antônio, São Sebastião do Rio Preto, Minas Gerais, Brazil	MZUSP73105	AY170135
Triportheus angulatus	Unavailable	AMNH233403	AY788082

Tissues were obtained from adipose fin biopsy and stored in 95% ethanol until their use. One to three individuals of each species were used for DNA extraction, amplification and sequencing. Total DNA from ethanol-preserved tissues was extracted in accordance with the protocol described by Taggart *et al.* (1992) modified by using smaller amounts of EDTA in the extraction buffer (0.1 M NaCl, 0,05 M Tris-HCl e 0.01 M EDTA, pH 8.0). PCR amplifications of partial 16S rRNA were performed with primers 16Sa-L and 16Sb-H (Palumbi *et al.*, 1991). Symmetric amplifications were carried out in 50 μL reactions containing 2.0 mM MgCl$_2$, 0.25 mM each dNTP, 0.7 μM of each primer, Taq polymerase (2 units) and 200 ng of DNA template. Amplification reactions were performed with a regime of 94 °C for 3 min and then 35 cycles of 60 s at 94 °C denaturation, 60 s at 51 °C annealing, and 90 s at 72 °C extension, followed by a final 5 min at 72 °C. PCR products were checked in 1% agarose gels and purified using the E.Z.N.A.® System (Omega Bio-Tek, Inc). The purified PCR products were sequenced with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the protocols provided by the manufacturer. Electrophoresis of the purified samples was performed in an ABI Prism 3100 DNA Sequencer (Applied Biosystems).

The sequences were aligned with the software Clustal W (Thompson *et al.*, 1994), as implemented in the program DAMBE (Xia and Xie, 2001), with the following multiple alignment options: DNA transitions weight equal to 0.5, IUB DNA weight matrix, option "delay divergent sequences" equal to 30, three gap open penalties (5, 10, and 15), and three gap extension penalties (0.1, 1.0, and 2.0). Nucleotide saturation was analyzed by plotting the number of observed transitions (Ti) relative to that of transversions (Tv) against genetic distance values (Kimura, 1980). Genetic distance analyses were based on a hierarchical hypothesis test of alternative models implemented with Modeltest 3.7 (Posada and Crandall, 1998).

Maximum-parsimony (MP) based phylogenetic analyses were performed using the software PAUP* beta version 4.0b10 (Swofford, 2002) with heuristic searches using random addition of sequences and the tree bisection and reconnection (TBR) algorithm. In all analyses, the character-state optimization method employed was the acceler-

ated transformation (ACCTRAN). Parsimony trees were generated using 1:1 and 1:2 Ti/Tv ratios, considering gaps either as missing data or as a fifth base. Bootstrap resampling (Felsenstein, 1985) was applied to assess support for individual nodes using 1000 replicates with 100000 random additions and TBR branch swapping. Decay indexes (Bremer, 1988) were calculated with SEPAL (Salisbury, 2001).

Maximum-likelihood (ML) based phylogenetic relationships were estimated using the software PAUP* beta version 4.0b10 (Swofford, 2002). The GTR model (Rodríguez *et al.*, 1990), incorporating rate variation (G) and PINVAR with four G-distributed rate classes (Swofford *et al.*, 1996), was utilized for all likelihood analyses based on a hierarchical hypothesis test of alternative models implemented with Modeltest 3.7 (Posada and Crandall, 1998). The Ti/Tv ratio, gamma shape parameter, and proportion of non-variant sites were estimated by maximum likelihood from a maximum parsimony tree. Gaps were considered as missing data. Bootstrap resampling was applied to assess support for individual nodes using 100 replicates with 10000 random additions and TBR branch swapping.

Bayesian analyses using the Markov chain Monte Carlo technique were performed by MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). We used the GTR + G + I model, and initiated an analysis from a random starting tree run for one million generations. The log-likelihood scores were found to stabilize after 10,000 generations. Therefore, we discarded the initial 10,000 generation trees and sampled one out of every 100 generations from the remaining 0.99 million generations (9900 trees) to calculate posterior probabilities for each branch of the Bayesian tree.

The combined sequence data of the 23 taxa resulted in 490 characters, out of which 302 were constant and 108 were parsimony-informative. The transitions/transversions (Ti/Tv) ratio observed was 1.8. The results from the analysis of plotting transitions and transversions against genetic distance do not suggest the occurrence of nucleotide saturation (Figure 1).
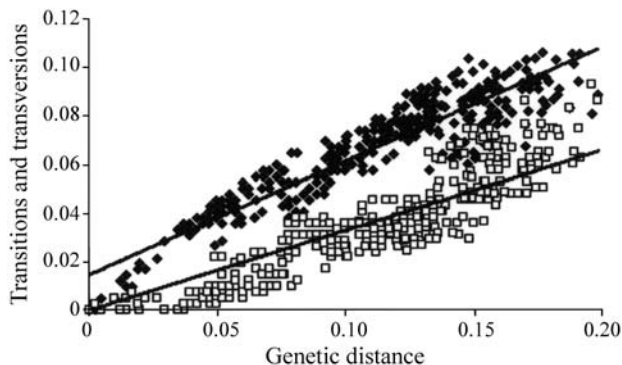

Figure 1 - Frequency of observed transitions and transversions versus genetic distance (Kimura, 1980) of the 16S rRNA gene. Transitions are black squares, transversions are open squares.

Eight MP heuristic searches were conducted employing all data or excluding 23 characters belonging to one segment (8 base pairs) without a direct alignment solution, including or excluding gaps, and considering the Ti/Tv ratios of 1:1 or 1:1.8. The resultant phylogenies were largely congruent. The best resolved consensus MP tree obtained from the analysis of 1000 bootstrap replicates including all data and considering gaps as a fifth base and the Ti/Tv ratio of 1:1 is presented in Figure 2a. For this tree the results obtained were: tree length (TL) = 478, consistency index (CI) = 0.5774, and retention index (RI) = 0.5992. Bayesian analysis of all the trees sampled at stationarity was -2735.129. The consensus Bayesian tree is shown in Figure 2b. ML analyses resulted in a consensus phylogeny very similar with those obtained in the Bayesian analyses (-Ln likelihood = 1592.036). The values higher than or equal to 50% found in the 100 bootstrap replicates of ML analysis are shown in Figure 2b.

The phylogenetic relationships within Characidae are still poorly known, and even monophyly is uncertain (Weitzman and Malabarba, 1998). A molecular phylogenetic analysis conducted by Calcagnoto *et al.* (2005) recovered the family Characidae as a characiform clade, though excluding the subfamily Serrasalminae (sensu Jégu, 2003). In the cited molecular study, fishes of the genus *Chalceus* were found to be the sister-group of all remaining species of characids. This latter finding, however, is contradicted on morphological grounds, since Zanata and Vari (2005) concluded that *Chalceus* is the basal-most member of Alestidae, a family of African characiforms. The remaining characid clades found in the analysis conducted by Calcagnoto *et al.* (2005) are: 1 - *Acestrorhynchus* and *Bryconops*; 2- *Brycon* and *Salminus*; 3- *Triportheus*; 4- *Bryconamericus*, *Knodus*, *Creagrutus*, *Hemibrycon*, *Gephyrocharax*, and *Mimagoniates*; 5- *Aphyocheirodon*, *Cheirodon*, *Cheirodontops*, *Prodontocharax*, *Exodon*, and *Roeboides*; and 6- *Astyanax*, *Astyanacinus*, *Inpaichthys*, *Moenkhausia*, *Hemigrammus*, and *Hyphessobrycon*. Unfortunately, although we lack published detailed morphological phylogenies with which Calcagnoto *et al.* (2005) results could be compared, it is worthy to note the apparent congruence of such results with current views on characid phylogeny based on morphology. For instance, the basal-most clades within Characidae, *Chalceus* excepted, are *Acestrorhynchus* and *Bryconops*, *Brycon* and *Salminus*, and *Triportheus*, all taxa (with the exception of *Acestrorhynchus*) supposed to be basal on morphological grounds (see *e.g.*, Malabarba and Weitzman 2003). The clade formed by *Brycon* and *Salminus* suggested the non-monophyly of *Brycon*, since *Salminus* was recovered as the sister taxon of *B. hilarii*, with an unidentified species of *Brycon* as the sister taxon of the latter clade (Calcagnoto *et al.*, 2005). Species of the genus *Salminus* were not included in the present analysis since morphological studies suggest that this genus has several plesiomorphic charac-

a)

b)
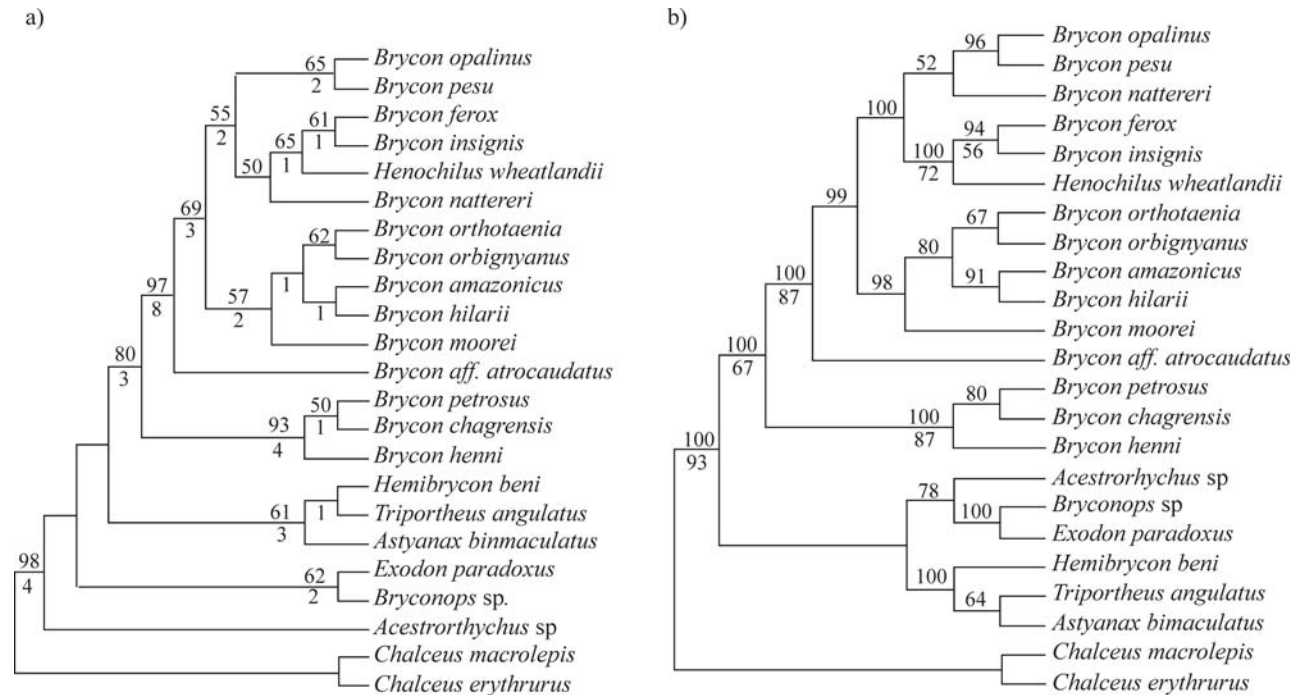
Figure 2 - Phylogenetic analyses of *Brycon* and *Henochilus* with Maximum Parsimony (MP) and Bayesian analysis (MB). (a) Consensus MP tree produced when all data were included, gaps were treated as a fifth base and the Ti/Tv ratio of 1:1 (TL = 478, CI = 0.5774, RI = 0.5992). Numbers above branches are bootstrap values based on 1000 replicates. Values below 50% are not shown. Numbers below branches represent Bremer support index values. (b) Consensus MB tree. Numbers above nodes are posterior probabilities recovered by the Bayesian analysis. Numbers below branches are bootstrap values based on 100 replicates in maximum likelihood analyses. Values below 50% are not shown.

ters conveying a basal position within Characidae (Lima, 2006).

The results of the four methods employed to reconstruct the phylogeny of Bryconinae were largely congruent, but in general with low bootstrap support (Figure 2). The better scores obtained in the Bayesian analysis were expected since unlike the parsimony and distance methods, maximum likelihood and Bayesian inference take full advantage of the information contained in an alignment of DNA sequences when estimating parameters of an evolutionary model (Huelsenbeck and Ronquist, 2005). The high support values obtained in the Bayesian analyses reinforce the findings of Simmons and Miya (2004) that demonstrated that the study of one gene in a large dataset with this kind of method results in a better resolved phylogeny. However, the general interpretation of posterior probabilities obtained with Bayesian analysis has been subject of criticism since higher values are sometimes obtained for low supported branches (Suzuki *et al.*, 2002; Simmons *et al.*, 2004), and thus results obtained by this method should be carefully analyzed.

Our results showed that the species of the genus *Brycon* belong to a monophyletic group (Figure 2) that is well supported, but the genus, as currently defined, is paraphyletic as discussed below. The only topological difference among phylogenetic analytical methods found in the ingroup was the position of *B. nattereri* that could not be resolved with any of the analyses conducted (Figure 2).

Brycon species from the trans-Andean Neotropical region presented some interesting patterns. A well supported clade found in all phylogenetic analysis was composed by *B. henni*, a species occurring in the Rio Magdalena Basin in Colombia, and *B. petrosus* and *B. chagrensis*, two species occurring in the Atlantic coastal river drainages of Panama. Additionally, the species herein identified as *B.* aff. *atrocaudatus* was recovered as the sister group of all remaining species analyzed in this study, although without strong statistical support. This result is congruent with the hypothesis of Zanata (2000), whereby most species of *Brycon* from trans-Andean South and Central America form a clade, but more than two lineages exist in the Neotropical region. *B. moorei*, the remaining trans-Andean species present in our analysis, was recovered as the sister group to species distributed in the Amazon, São Francisco, and Paraná River Basins, which are discussed below.

Henochilus wheatlandii was recovered among the species of *Brycon*, as the sister species of *B. insignis* and *B. ferox*, corroborating the monophyly of Bryconinae but rejecting the monophyly of *Brycon*. In a phylogenetic analysis of the relationships of *Brycon* and allied genera, Zanata (2000) identified seven morphological synapomorphies that delimited a clade formed by all examined species of *Brycon* (including *B. pesu*) along with *Chilobrycon*, a *Brycon*-like genus from the western slopes of the Andes (Rio Tumbes basin at the Peruvian-Ecuadorian border; Gé-

ry and de Rham, 1981). As noticed by Lima (2003) and Castro *et al.* (2004), *Henochilus* is morphologically very similar with *Brycon* and *Chilobrycon*, however distinct from the latter primarily by the possession of three series of teeth in the premaxillary bone (the generalized condition within *Brycon*), versus two series of premaxillary teeth in *Henochilus*. Therefore, the recovery of *Henochilus* within *Brycon* in the present analysis, although for the first time proposed, is not really surprising if we consider the fact that *Henochilus* is the putative sister-group of *Chilobrycon* (Lima, 2003). The reduction of the number of series of premaxillary teeth may represent an autapomorphy of *Henochilus*. Consequently, both *Chilobrycon* and *Henochilus* are possible synonyms of *Brycon*. However, we prefer not to synonymize both genera herein and prefer to settle the question in further investigations.

Zanata (2000) found the species *Brycon pesu* to be the sister species of the remaining *Brycon* species. In the present study this hypothesis was rejected since *B. pesu* was recovered as the sister species of *B. opalinus*, this clade being sister to other *Brycon* species found in coastal rivers of South America (*B. insignis*, *B. ferox*, and *Henochilus wheatlandii*), and in the upper Rio Paraná River basin (*B. nattereri*).

The remaining *Brycon* species were divided into two clades without strong statistical support. The first clade is composed of the species occurring in the relatively small river basins of eastern Brazil (*B. insignis*, *B. ferox*, and *B. opalinus*), plus *B. nattereri*, found in the upper Rio Parana Basin. This result partially corroborates the hypothesis of Zanata (2000) who suggested that *B. insignis* and *B. opalinus* form a monophyletic group, although Zanata (2000) recovered *B. nattereri* as the sister group of the clade discussed below.

The second *Brycon* clade is composed by the remaining species of *Brycon*. The species *B. moorei* occurring in the Rio Magdalena Basin in trans-Andean South America was recovered as the sister species of the other species. *Brycon orbignyanus* (La Plata Basin) was recovered as the sister species of *B. orthotaenia* (Rio São Francisco Basin). *Brycon hilarii* (Rio Paraguay Basin) was recovered as the sister species of *B. amazonicus*. The monophyly of this clade is also well supported morphologically (Zanata, 2000). The presence of *B. moorei* as the basalmost member of this clade is suggestive of the undoubtedly complex history of the genus *Brycon*, demonstrating the apparent non-monophyly of trans-Andean and cis-Andean components of the genus.

The present result points to the fact that *Brycon* in its present sense is not monophyletic. Further studies including additional species, mainly *B. falcatus* (the type species of the *Brycon*), as well as *Chilobrycon deuterodon*, and sequences of different genes coupled with a detailed morphological phylogenetic hypothesis will be necessary for a better understanding of the interrelationships within the

species of *Brycon,* and also to refine the definition of the species in the subfamily Bryconinae.

Acknowledgments

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