

Molecular characterization and phylogenetic relationships among species of the genus *Brycon* (Characiformes: Characidae) from four hydrographic basins in Brazil

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ABSTRACT. *Brycon* is one of the main genera of Neotropical freshwater fish. In Brazil, *Brycon* species have been found in many hydrographic basins, such as the Amazon, Paraná, Paraguay, and Araguaia-Tocantins basins. We examined the phylogenetic relationships among the species *Brycon orbignyanus*, *B. hildebrandi*, *B. cf. pesu*, *B. cephalus*, *B. falcatus*, and *B. gouldingi*, using mitochondrial and nuclear molecular markers. Specimens of *B. orbignyanus* were collected in the Paraná River. Specimens of *B. hildebrandi* were collected in the Manso River. Specimens of *B. cephalus* were obtained from a fish farm, and

specimens of *B. cf. pesu*, *B. falcatus* and *B. gouldingi* were sampled in the Araguaia-Tocantins basin. DNA extraction was carried out using the phenol/chloroform method. Molecular polymorphism studies of *Brycon* species were carried out with the inter-simple sequence repeat (ISSR) technique, using the total DNA of six specimens of each species. In DNA amplification of *B. cf. pesu*, eight specimens were used. The partial sequence of mitochondrial cytochrome b was amplified by PCR. The PCR products were used directly in sequencing reactions. Each ISSR primer produced from 7 to 14 scorable and reproducible bands. The (GGAC)₃A and (GGAC)₃C primers produced the greatest number of species-specific bands. A 264-bp fragment, corresponding to the partial region of mitochondrial DNA cytochrome b, was sequenced and used for analysis. According to the phylogenetic tree obtained from the data, these *Brycon* species can be divided into two clades: one comprised only *B. cf. pesu*, and the second comprised the remaining *Brycon* species. We conclude that ISSR primers can be used for the identification of species-specific bands in fish, such as *Brycon* spp.

Key words: *Brycon*; Phylogenetic relationships; mtDNA cytochrome b; Inter-simple sequence repeat

INTRODUCTION

The genus *Brycon* belongs to the Bryconinae subfamily and is considered to be one of the main Neotropical fishes of freshwater systems. It comprises more than 40 species (Lima, 2003), widely distributed in Central and South America. Many of them represent important fishery resources. In Brazil, species of *Brycon* have been found in the majority of hydrographic basins, such as the Amazon, Paraná, Paraguay, and Araguaia-Tocantins basins. The present study included six species distributed in the basins mentioned: *Brycon orbignyanus*, *B. hilaarii*, *B. cf. pesu*, *B. cephalus*, *B. falcatus*, and *B. gouldingi*.

Brycon orbignyanus (Valenciennes, 1850), also called “piracanjuba”, is native to the Paraná River Basin. It is a big and migratory fish, which is of great economic importance. Populations of this species are abundant in the upper Paraná River basin and in its main tributaries, such as Grande, Tietê and Paranapanema Rivers. However, currently, *B. orbignyanus* is on the critically threatened species list (Machado, 2005). The reduction of their populations has been related to the construction of hydroelectric dams, destruction of riparian areas and predatory fishing.

Brycon hilaarii (Valenciennes, 1850), known as “piraputanga”, is a rheophylic fish, of great value for commercial fishing. It occurs in the Middle Paraná and Paraguay River basins. It has omnivorous habits, living in regions of both rapidly and slowly flowing waters, under fruitful trees and near aquatic plants.

Brycon cf. pesu Müller & Troschel, 1845 is distributed in the coastal basins of north-east South America, Orinoco River basin, and Amazon and Araguaia-Tocantins basins (Lima, 2003). It is a small fish, found in sandy and rocky areas in rapidly flowing waters and habitu-

ally feeds at river banks, mainly on terrestrial insects.

Brycon cephalus (Günther, 1869) is native to the Amazon basin and is also known as “matrinxã”. It is widely exploited in commercial activities (Izel et al., 2004).

Brycon falcatus Müller & Troschel, 1844 is an omnivorous species distributed in Guyana, Suriname and French Guiana Rivers, and the Amazon, Orinoco and Araguaia-Tocantins River basins.

Brycon gouldingi (Lima, 2004) is endemic to the Araguaia-Tocantins basin, where it feeds on fruits and insects.

The six species of *Brycon* cited are important components of the Brazilian ichthyofauna and are of importance in commercial fishing, aquatic biodiversity and for maintaining the equilibrium of food chains in Brazilian freshwater ecosystems. However, some species, such as those found in the Araguaia-Tocantins basin, have been scarcely studied, and little is known about their biology and genetics. Besides, there are no reported phylogenetic studies involving the six species mentioned.

The possibility to quantify genetic variability by the direct analysis of polymorphism of DNA fragments has stimulated the advance of population genetics and the genetics of evolutionary processes. DNA polymorphism, associated with morphological and ecological studies, has been useful in demonstrating ecological genetics diversity and the genetics of the evolution process in species and subspecies. DNA amplification by PCR (polymerase chain reaction) and the sequencing of DNA fragments are the main methodologies. ISSR (inter-simple sequence repeat) method is based on amplification of DNA fragments by means of a single primer composed of a short microsatellite sequence with one to four degenerate nucleotides anchored at the 5' or 3' end, as described by Gupta et al. (1994). ISSR molecular markers (Schulz et al., 2004; Maltagliati et al., 2006; Pazza et al., 2007) and mitochondrial DNA (mtDNA) sequences have been revealed as important tools in studies regarding the genetic structure of populations, phylogeographic relations and phylogenetic reconstruction in fish (Reeves and Bermingham, 2006; Haponski and Stepien, 2008; Almada et al., 2009; Kawamura et al., 2009).

The aim of the present article was to study the phylogenetic relationships among the species *B. orbignyanus*, *B. hiliarii*, *B. cf. pesu*, *B. cephalus*, *B. falcatus*, and *B. gouldingi*, using mitochondrial and nuclear molecular markers.

MATERIAL AND METHODS

Biological material

Specimens of *B. orbignyanus* were collected in the Paraná River, in the region of Guaira. Specimens of *B. hiliarii* were collected in the Manso River. Specimens of *B. cephalus* were obtained from a fish farm, and specimens of *B. cf. pesu*, *B. falcatus* and *B. gouldingi* were sampled in the Araguaia-Tocantins basin. Specimens of *B. cf. pesu* were collected in the fish ladder of the Lajeado Hydroelectric Plant (9°46'S 48°21'W), built on the Tocantins River, and in the Palmas River, its tributary. Specimens of *B. falcatus* and *B. gouldingi* were collected in the fish ladder of the Lajeado Hydroelectric Plant (Figure 1).

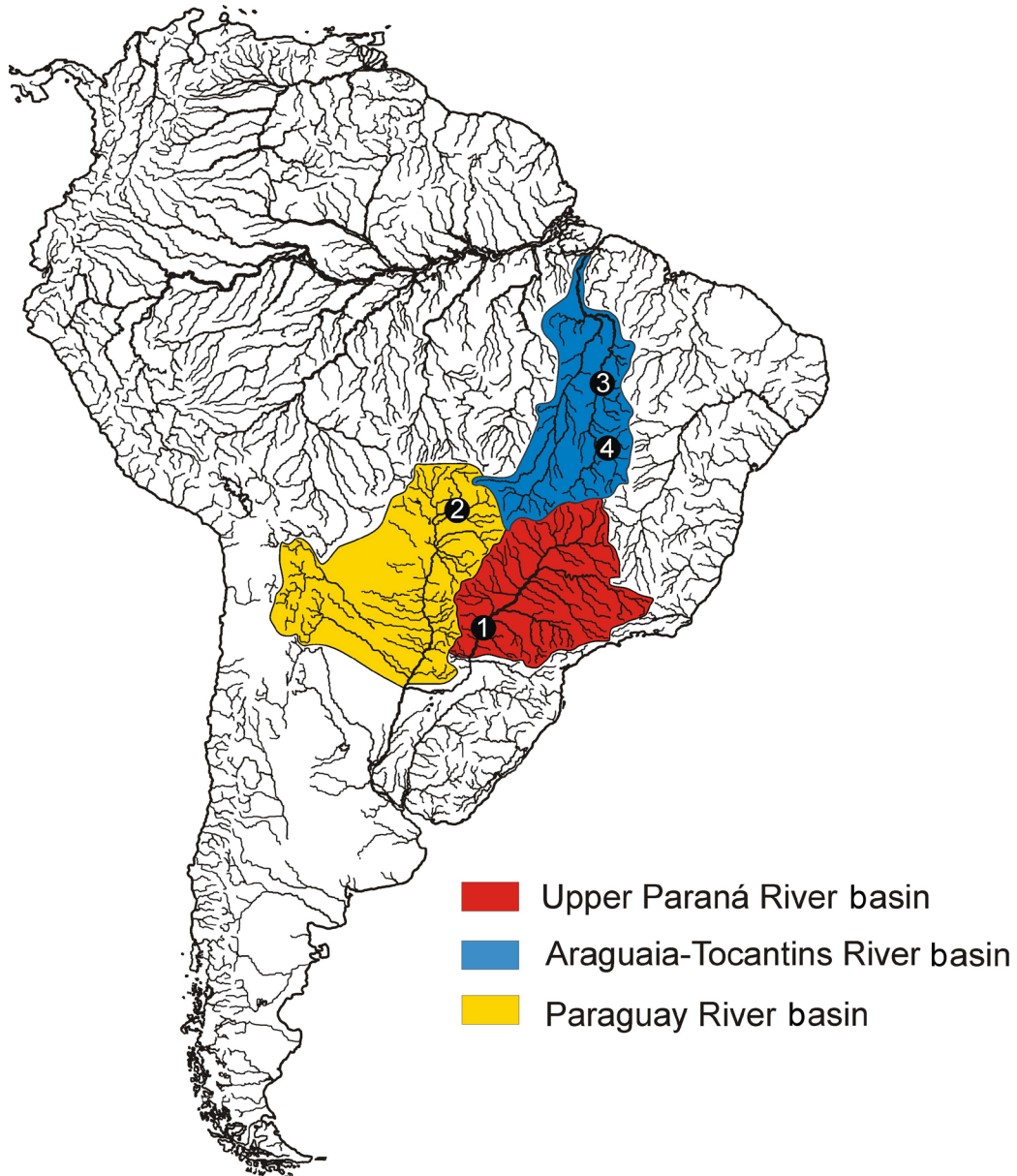


Figure 1. Araguaia-Tocantins basin. 1 = Guaira; 2 = Manso River; 3 = Lajeado Hydroelectric Plant fish ladder; 4 = Palmas River.

DNA extraction

DNA extraction was carried out using the phenol/chloroform method (Monesi et al., 1998). Samples of muscle tissue were macerated in liquid nitrogen. Samples were homogenized in 500 μ L TH buffer (10 mM Tris-HCl, 60 mM NaCl, 10 mM EDTA, 5% sucrose, 0.15 mM spermine, and 0.15 mM spermidine) plus 500 μ L PS buffer (Tris-0.2 M HCl, 30 mM EDTA, 2% SDS, and 5% sucrose) and 5 μ L proteinase K (20 μ g/ μ L), and then were incubated in a shaking water bath at 37°C for approximately 1 h. Next, DNA was purified through washes with phenol/chloroform (1:1) and chloroform. DNA was then precipitated using saline (0.2 M NaCl) and chilled ethanol. The precipitate was submitted to several centrifugations with 70 and 100% ethanol, and finally resuspended in TE (1 mM Tris, 0.1 mM EDTA) containing RNase. DNA of all samples was later quantified on a 0.8% agarose gel stained with ethidium bromide (20 μ g/100 mL). DNA samples were quantified by comparison with known quantities of phage λ DNA (Invitrogen Life Technologies™, Carlsbad, CA, USA), on agarose gel (0.8%) stained with ethidium bromide (20 μ g/100 mL).

Inter-simple sequence repeat and data analysis

ISSR primers composed of the tetranucleotide repeats (AACC)₄, (GGAC)₃C, (GGAC)₃A, (GGAC)₃T, and (GGAC)₄ were selected for analysis. The PCR technique was used for the amplification of the total DNA of six specimens of each species. In DNA amplification of *B. cf. pesu*, eight specimens were used. The amplification method was in accordance with the protocol described by Fernandes-Matioli et al. (2000). In each experiment, a sample without template DNA was included as a negative control. Aliquots (10 μ L) of PCR amplification products were applied on 1.4% agarose gels stained with ethidium bromide (20 μ g/100 mL), and separated by electrophoresis in TBE buffer (Tris-borate) at 5 V/cm for 3-4 h. Amplified fragments were visualized under UV irradiation and photographed for later analysis. Sizes of fragments were estimated by comparison with a standard 100-bp ladder (Invitrogen).

The matrix of Nei and Li similarity means within and among *Brycon* species was obtained from ISSR data, using the Mantel-Struct 1.0 program (Miller, 1999). The principal coordinate plot was constructed from Nei and Li distances with the FAMD program (Schlüter and Harris, 2006).

Mitochondrial DNA and data analysis

PCR amplifications of a partial cytochrome b sequence were performed with the primers H15149 5'-CCCCTCAGAATGATATTTGTCCTCA-3' and L14841 5'-CCATCCAA CATCTCAGCATGATGAAA-3' (Kocher et al., 1989), as described by Prioli et al. (2002). Five samples of *B. hylarii*, *B. orbignyanus* and *B. cephalus* were analyzed. Six specimens of *B. cf. pesu* and four specimens of *B. falcatus* were sequenced. The sequencing of *B. gouldingi* specimens was not successful, and they were thus excluded from analysis. Amplifications were carried out in 25- μ L reaction mixtures containing Tris-KCl buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 1.5 mM MgCl₂, 5 μ M of each primer, 0.1 mM of each dNTP, 2.5 U *Taq*-DNA polymerase (Invitrogen), 30 ng genomic DNA and deionized autoclaved water to make the volume up to 25 μ L. Amplification reactions were performed with a regime of 94°C for 4 min, 50°C for 30 s and 72°C for 1 min and 30 s, and then 40 cycles of 30 s at 94°C, 30 s at 56°C, 1 min and 30 s at 72°C,

followed by a final cycle of 1 min at 94°C, 1 min at 56°C and 7 min at 72°C. PCR products were checked on 1% agarose gels. The DNA samples (of approximately 50 ng) amplified in each PCR were used directly in sequencing reactions with BigDye Terminator Cycle Sequencing, in an ABI-3100 automatic sequencer (Perkin Elmer), according to manufacturer instructions.

The sequences were aligned with the MEGA 4.0 program (Tamura et al., 2007), and edited with the BIOEDIT program (Hall, 1999). A partial cytochrome b sequence of *B. hilarii* (AY 791370) was included in the analysis. Based on the study of Margarido and Galetti-Junior (1999), *Salminus brasiliensis* (AY 791438) was selected as the outgroup.

The evolutionary model was selected using PAUP 4.0 (Swofford, 2002) and MODELTEST 3.7 programs (Posada and Crandall, 1998). Akaike Information Criterion with correction and Bayesian Information Criterion procedures were performed.

The evolutionary model selected was also used to estimate the genetic distances and to construct a dendrogram with the neighbor-joining algorithm, based on 10,000 replicates. The phylogenetic tree was obtained with the MEGA 4.0 program (Tamura et al., 2007).

RESULTS

Inter-simple sequence repeat

Of the total of five ISSR primers, four yielded species-specific ISSR profiles in at least one of the species studied (Figure 2). The (GGAC)₃T primer did not produce clear amplifications and was excluded from analyses. The amplified fragments varied in length from 300 to 1900 bp (Table 1). Each primer used produced from 7 to 14 scorable and reproducible bands for 43 polymorphic loci. Around 37% of the bands detected were species-specific. The (GGAC)₃A and (GGAC)₃C primers produced the greatest number of species-specific bands. (GGAC)₃C primer yielded 5 species-specific bands in 6 species of *Brycon* analyzed.

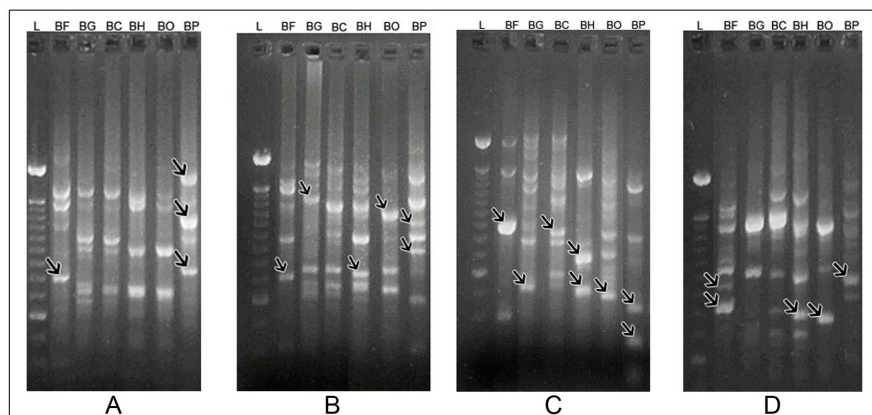


Figure 2. Electrophoretic profiles of the inter-simple sequence repeat of six species of *Brycon*. **A.** Primer (AACC)₄. **B.** Primer (GGAC)₃A. **C.** Primer (GGAC)₃C. **D.** Primer (GGAC)₄. L = Molecular weight marker fragments (100-bp ladder, Invitrogen Life Technologies, Carlsbad, CA, USA); BF = *Brycon falcatus*; BG = *B. gouldingi*; BC = *B. cephalus*; BH = *B. hilarii*; BO = *B. orbignyanus*; BP = *B. cf. pesu*. Arrows indicate species-specific bands.

Table 1. Approximate length in bp of inter-simple sequence repeat fragments of *Brycon falcatus*, *B. gouldingi*, *B. cephalus*, *B. hilarii*, *B. orbignyanus*, and *B. cf. pesu* specimens.

Primer (GGAC) ₄														
Fragments (~bp)	1650	1400	1350	1150	1050	920	800							
<i>B. falcatus</i>	-	-	+m	-	+m	+p	+m							
<i>B. gouldingi</i>	-	-	+m	-	+m	-	-							
<i>B. cephalus</i>	+m	+m	-	-	+p	-	+m							
<i>B. hilarii</i>	+m	+m	-	-	+p	-	+m							
<i>B. orbignyanus</i>	-	+m	-	-	-	-	+m							
<i>B. cf. pesu</i>	-	-	-	+p	+m	+p	-							
Primer (AACC) ₄														
Fragments (~bp)	2000	1750	1700	1400	1250	1050	900	850	800	720				
<i>B. falcatus</i>	-	-	+m	+p	+p	-	-	-	+m	-				
<i>B. gouldingi</i>	-	+p	-	+p	-	+p	-	-	-	+m				
<i>B. cephalus</i>	-	+m	-	-	-	+p	-	-	-	+m				
<i>B. hilarii</i>	-	+p	+m	+m	-	-	-	-	-	+m				
<i>B. orbignyanus</i>	-	+m	+m	+m	-	-	-	-	-	+m				
<i>B. cf. pesu</i>	+m	-	-	-	+m	-	+p	+m	-	-				
Primer (GGAC) ₃ C														
Fragments (~bp)	2300	1200	850	800	750	650	600	570	550	520	470	350		
<i>B. falcatus</i>	-	-	+m	-	-	-	-	-	-	-	-	-		
<i>B. gouldingi</i>	+m	-	-	+p	+m	-	-	+p	-	-	-	-		
<i>B. cephalus</i>	+m	+m	-	+m	+m	-	+p	-	-	-	-	-		
<i>B. hilarii</i>	-	-	-	-	+m	+m	-	-	+m	-	-	-		
<i>B. orbignyanus</i>	-	+p	-	+p	-	-	+p	-	-	+m	-	-		
<i>B. cf. pesu</i>	-	-	-	+p	-	+p	-	-	-	-	+p	+m		
Primer (GGAC) ₃ A														
Fragments (~bp)	1900	1800	1600	1500	1350	1220	1150	1100	950	900	750	720	700	650
<i>B. falcatus</i>	+m	-	+m	+m	-	-	-	+p	+m	-	-	-	+m	-
<i>B. gouldingi</i>	-	+m	-	-	+m	-	-	-	-	-	-	+m	-	+m
<i>B. cephalus</i>	-	-	-	-	-	+m	-	+m	-	-	-	+m	-	+m
<i>B. hilarii</i>	-	-	-	+p	-	+p	-	+m	-	-	-	+m	-	+m
<i>B. orbignyanus</i>	-	-	-	-	-	+m	-	+p	-	-	+m	-	-	+m
<i>B. cf. pesu</i>	-	+m	-	-	+m	-	+p	-	+p	+p	+m	-	-	-

Presence or absence of a DNA fragment on the agarose gel is indicated by + and -, respectively. The DNA fragment present in a species can be monomorphic (m) or polymorphic (p).

B. gouldingi and *B. cephalus* exhibited the most similar ISSR electrophoretic profile, followed by *B. hilarii* and *B. orbignyanus*. The most genetically diverse species, according to the ISSR data, were *B. falcatus* and *B. cf. pesu*.

The table of similarity means (Table 2) shows that the divergence among individuals of the same species is lower than that for individuals of different species. The principal coordinate plot and the dendrogram obtained from ISSR data (Figures 3 and 4) separated the six species studied. *Brycon hilarii* and *B. orbignyanus* are the most closely related, whereas *B. cf. pesu* is the most divergent.

Table 2. Nei and Li similarity means based on inter-simple sequence repeat molecular markers, within (diagonal) and among (lower left) species of *Brycon*.

Species	<i>B. falcatus</i> (1)	<i>B. gouldingi</i> (1)	<i>B. cephalus</i> (2)	<i>B. hilarii</i> (3)	<i>B. orbignyanus</i> (4)	<i>B. cf. pesu</i> (1)
<i>B. falcatus</i> (1)	0.0660					
<i>B. gouldingi</i> (1)	0.8027	0.0786				
<i>B. cephalus</i> (2)	0.7808	0.4626	0.0422			
<i>B. hilarii</i> (3)	0.6824	0.5463	0.3690	0.0735		
<i>B. orbignyanus</i> (4)	0.7434	0.6511	0.3970	0.4125	0.0814	
<i>B. cf. pesu</i> (1)	0.7434	0.7334	0.9257	0.9001	0.9113	0.1693

1 = Araguaia-Tocantins River basin; 2 = Amazon River basin; 3 = Paraguay River basin; 4 = Paraná River basin.

According to the phylogenetic tree obtained from cytochrome b sequences, the *Brycon* species studied were separated into two clades. One comprised only *B. cf. pesu* and the second comprised the other species: *B. hilarii*, *B. orbignyanus*, *B. cephalus*, and *B. falcatus*.

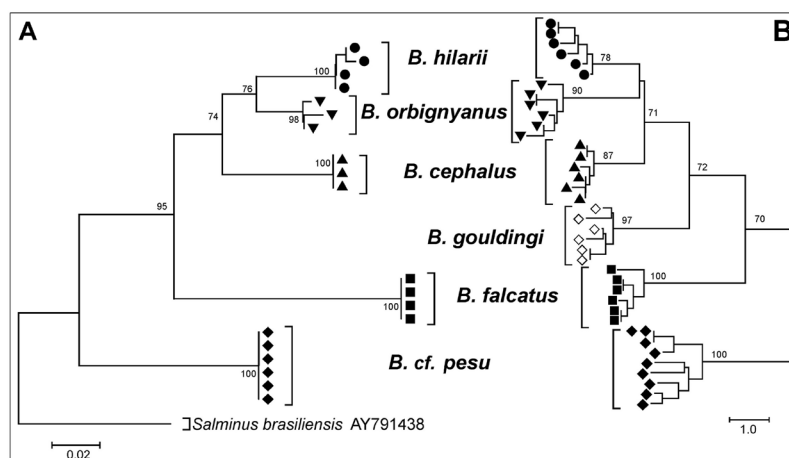


Figure 4. Neighbor-joining dendrograms constructed from the Tamura and Nei + I distance matrix among the mitochondrial cytochrome b sequences (A) and from inter-simple sequence repeat markers based on Nei and Li distances (B) of the *Brycon* species. Numbers above branches are bootstrap values, as based on 10,000 replicates.

DISCUSSION

The data obtained by the analysis of cytochrome b sequences corroborated the results determined by the ISSR data. The phylogenetic trees constructed from ISSR and cytochrome b data produced the same clades. *Brycon gouldingi*, studied only with ISSR markers, occupies an intermediary position in evolutionary terms, when compared with the other *Brycon* species.

The phylogenetic tree obtained from cytochrome b sequences separated the *Brycon* species into two clades. One comprised only *B. cf. pesu*, the most basal group. In other words, the bifurcation, which originated this species, is older than the other group of *Brycon* species analyzed. The second clade is composed of the other species: *B. hilarii*, *B. orbignyanus*, *B. cephalus*, and *B. falcatus*. Besides, the results demonstrated that the species of the genus *Brycon* belong to a monophyletic group. Phylogenetic studies with *B. pesu*, *B. hilarii*, *B. orbignyanus*, and other *Brycon* species, based on the mitochondrial gene 16S rRNA, also supported the monophyletic status of the *Brycon* species, although considered the paraphyletic status of the genus (Hilsdorf et al., 2008).

The phylogenetic relationships among the *Brycon* species analyzed could be related to their geographic distributions, as well as to their morphologic similarity. The Paraná River and Paraguay River basins, along with the Uruguay River, belong to the Prata River basin. The Paraguay River is the main tributary of the Paraná River, where individuals of *B. orbignyanus* were obtained. The Manso River, where *B. hilarii* specimens were collected, joins in its river mouth with the Cuiabazinho River to form the Cuiabá River (Neto et al., 1993). This river, in turn, is one of the main tributaries of the Paraguay River.

Among the four basins where *Brycon* species were obtained, the most geographically close are the Paraná River and the Paraguay River basins. Therefore, *B. orbignyanus*

and *B. hilarii*, belonging to these basins, are more closely related than the other species. This is demonstrated by the dendrogram, because both species have their origins in the same bifurcation. The Manso River, as well as Paraná River, in some parts of their courses, have characteristics of a plain river (Neto et al., 1993). Such environments, with similar characteristics, allowed the origin of species with similar habits and morphological features, such as *B. hilarii* and *B. orbignyanus*. Hilsdorf et al. (2008) studied species of the genus *Brycon* with the mitochondrial gene 16S rRNA and also demonstrated that *B. hilarii* and *B. orbignyanus* are sister groups.

The Amazon and the Araguaia-Tocantins basins are interconnected. The Amazon basin is composed of the Amazon River and its tributaries, while the Araguaia-Tocantins basin is composed of the Tocantins and Araguaia Rivers, and their tributaries. The Tocantins River arises in Goiás State, passing by Tocantins, Maranhão and Pará States, until arriving in the mouth of the Amazon River, where it empties its water. *B. cf. pesu* and *B. falcatus* belong to the Araguaia-Tocantins basin and are also found in the Amazon basin.

The analyses of mtDNA cytochrome b data demonstrated that *B. cf. pesu* and *B. falcatus*, within the group analyzed, have origins in older clades. Both species are very morphologically diverse, since *B. cf. pesu* is a small fish while *B. falcatus* is a big fish. Besides, the phylogenetic tree shows that the distance between *B. falcatus* and the clade comprised of large-sized species, *B. orbignyanus*, *B. hilarii* and *B. cephalus*, is smaller than the distance between *B. falcatus* and *B. cf. pesu*. However, the phylogenetic analysis indicates that *B. falcatus* is more closely related to the large-sized species than to *B. cf. pesu*, a small-sized fish.

According to the results, *B. cephalus* and *B. hilarii* diverged from a common ancestor, that branched out into two lineages: the older originated *B. cephalus*, and the most recent was divided into two branches. One originated *B. orbignyanus* and the second originated *B. hilarii*. *B. cephalus* is restricted to the Amazon basin and is closely related to *B. orbignyanus* and *B. hilarii*, since the Amazon basin is also connected with the Paraná-Paraguay basin. Besides, the three species mentioned are large and have similar external morphology and habits.

Based on the results obtained in the present study, we can conclude that ISSR primers are efficient in detecting species-specific loci in fish, and thus, they may be used as tools to assist in the identification of species, as reported by other authors (Schulz et al., 2004; Maltagliati et al., 2006). The application of these markers is important in identifying species, as well as detecting genetic variability, in order to preserve threatened species and to reestablish stocks with low genetic diversity for conservation purposes.

In the *Brycon* species analyzed here, the (GGAC)₃A and (GGAC)₃C primers were the most effective in discriminating species-specific bands.

Among the species studied, *B. hilarii* and *B. orbignyanus* are the most closely related. *B. cf. pesu* is of the oldest origin and greatest genetic distance. Both inferences were corroborated by nuclear and mitochondrial markers. The phylogenetic relationships found among *Brycon* species can be associated with geographic distribution, as well as with the morphology of the species.

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