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Original Article

Molecular characterization and genetic relationships of seven piranha species of the genera *Serrasalmus* and *Pygocentrus* (Characiformes: Serrasalmidae) from Paraná-Paraguay, São Francisco and Tocantins River basins in Brazil

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Abstract

Genetic and phylogenetic relationships among seven piranha species of the genera *Serrasalmus* and *Pygocentrus* from the Paraná-Paraguay, São Francisco and Tocantins River basins were evaluated in the present study by partial sequences of two mitochondrial genes, Cytochrome b and Cytochrome c Oxidase I. Phylogenetic analysis of Maximum-Likelihood and Bayesian inference were performed. Results indicated, in general, greater genetic similarity between the two species of *Pygocentrus* (*P. nattereri* and *P. piraya*), between *Serrasalmus rhombeus* and *S. marginatus* and between *S. maculatus*, *S. brandtii* and *S. eigenmanni*. *Pygocentrus nattereri*, *S. rhombeus* and *S. maculatus* showed high intraspecific genetic variability. These species have each one, at least two different mitochondrial lineages that, currently, occur in sympatry (*S. rhombeus*) or in allopatry (*P. nattereri* and *S. maculatus)*. Species delimitation analysis and the high values of genetic distances observed between populations of *S. rhombeus* and of *S. maculatus* indicated that each species may corresponds to a complex of cryptic species. The non-monophyletic condition of *S. rhombeus* and *S. maculatus* reinforces the hypothesis. The geographic distribution and the genetic differentiation pattern observed for the piranha species analyzed herein are discussed regarding the geological and hydrological events that occurred in the hydrographic basins.

Keywords: mitochondrial DNA, Cytochrome b, Cytochrome c Oxidase subunit I, phylogeny, species complex.

Caracterização molecular e relações genéticas de sete espécies de piranhas dos gêneros *Serrasalmus* e *Pygocentrus* (Characiformes: Serrasalmidae) das bacias hidrográficas Paraná-Paraguai, São Francisco e Tocantins, no Brasil

Resumo

Relações genéticas e filogenéticas de sete espécies de piranhas dos gêneros *Serrasalmus* e *Pygocentrus* das bacias hidrográficas Paraná-Paraguai, São Francisco e Tocantins foram avaliadas com base em sequências parciais dos genes mitocondriais Citocromo b e Citocromo c Oxidase I. Foram realizadas análises filogenéticas de Máxima Verossimilhança e de inferência Bayesiana. Os resultados indicaram, em geral, maior similaridade genética entre as duas espécies de *Pygocentrus* (*P. nattereri* e *P. piraya*), entre *Serrasalmus rhombeus* e *S. marginatus* e entre *S. maculatus*, *S. brandtii* e *S. eigenmanni*. *Pygocentrus nattereri*, *S. rhombeus* e *S. maculatus* revelaram ter alta variabilidade genética intraespecífica. Essas espécies têm, cada uma, pelo menos duas linhagens mitocondriais que, atualmente, ocorrem em simpatria (*S. rhombeus*) ou alopatria (*P. nattereri* e *S. maculatus)*. Análises de delimitação de espécies e os altos valores de distância genética observados entre as populações de *S. rhombeus* e de *S. maculatus* indicam que cada espécie

pode, na verdade, corresponder a um complexo de espécies crípticas. A condição não-monofilética de *S. rhombeus* e *S. maculatus* reforça essa hipótese. A distribuição geográfica e o padrão de diferenciação genética observados para as espécies de piranhas analisadas são discutidos com relação aos eventos geológicos e hidrológicos que ocorreram nas bacias hidrográficas.

Palavras-chave: DNA mitocondrial, Citocromo b, Citocromo c Oxidase subunidade I, filogenia, complexo de espécies.

1. Introduction

Piranhas and closely related genera of pacus and tambaquis comprise the family Serrasalmidae (Ostariophysi: Characiformes), currently with 98 valid species in 16 genera (Fricke et al., 2019). The largest group of Serrasalmidae is comprised by the piranhas. Jégu (2003) estimated the number of piranhas at 38 recognized species. According to Freeman et al. (2007), "piranhas" or the "true piranhas" are a group of fishes that traditionally includes the genera *Serrasalmus* (24, perhaps 28 species), *Pristobrycon* (5), *Pygocentrus* (3 or 4) and *Pygopristis* (1). However, some authors also include the scale-eating genus *Catoprion* and the plant-eating genus *Metynnis* in the same group (e.g., Ortí et al., 1996; Nakayama et al., 2002).

Piranhas are freshwater Neotropical fish endemic from South America, occurring in the rivers of the Amazon, Orinoco, Guiana, Araguaia-Tocantins, Paraná-Paraguay and São Francisco basins (Jégu, 2003). Despite their ecological and economic importance, the taxonomy and systematic classification of piranhas and other serrasalmids are confusing. As a result, species identification and phylogenetic placement of many taxa are problematic (Freeman et al., 2007; Thompson et al., 2014).

Evolutionary relationships among species of piranhas have been the subject of several publications with morphological (Machado-Allison, 1983), cytological (Nakayama et al., 2002, 2012), parasitological (Van Every and Kritsky, 1992) and molecular markers (Ortí et al., 1996, 2008; Freeman et al., 2007; Hubert et al., 2007; Thompson et al., 2014; Machado et al., 2018; Mateussi et al., 2019). Morphological and molecular approaches agree with the hypothesis that the genera of piranhas *Pygopristis*, *Pygocentrus*, *Pristobrycon* and *Serrasalmus* together with *Catoprion*, represent a monophyletic unit of the family Serrasalmidae. However, relationships within the group are not yet well established and at least *Serrasalmus* and *Pristobrycon* may be non-monophyletic (Ortí et al., 1996, 2008; Freeman et al., 2007; Thompson et al., 2014).

Genetic characterization of piranha species is therefore essential to aid in the elucidation of genetic relationships and identification at species level. Hence, the goal of our research was to use partial sequences of the mitochondrial genes Cytochrome b (*cytb*) and Cytochrome c Oxidase subunit I (*coI*) to characterize and to elucidate the genetic and phylogenetic relationships of seven piranha species of the genera *Serrasalmus* (*S. maculatus* Kner, 1858; *S. marginatus* Valenciennes, 1837; *S. eigenmanni* Norman, 1929; *S. rhombeus* Linnaeus, 1766 and *S. brandtii* Lütken, 1875) and *Pygocentrus* (*P. nattereri* Kner, 1858 and *P. piraya* Cuvier, 1819) from the Upper Paraná, Upper Paraguay, São Francisco and Tocantins River basins, in Brazil.

2. Material and Methods

2.1. Sample collection

Sixty-four piranha specimens of the genera *Serrasalmus* and *Pygocentrus* were collected at four hydrographic basins: Upper Paraná, Upper Paraguay, Tocantins and São Francisco Rivers (Figure 1; Table 1). Specimens were anaesthetized and subsequently sacrificed by overdosing of clove oil. Muscle samples were preserved in 96% ethanol and maintained at -20 °C before DNA extraction. *Piaractus brachypomus* was used as outgroup in phylogenetic analysis (GenBank accession numbers: AY791429 for *cytb* and FJ978042 for *coI* sequences).

2.2. DNA isolation, PCR amplification and sequencing

Genomic DNA was isolated from muscle tissues using the phenol-chloroform protocol (Green and Sambrook, 2012). After DNA quantification, mitochondrial DNA fragments were amplified by Polymerase Chain Reaction

Figure 1. Sample collection sites of the piranha genera *Serrasalmus* and *Pygocentrus* in Brazil. Numbers correspond to the local sample of the respective river basin: (1) Upper Paraná River; (2) Upper Paraguay River; (3) Tocantins River; and (4) São Francisco River.

Table 1. Piranha species of the genera *Serrasalmus* and *Pygocentrus* analyzed in the present study.

Species	Sampling sites	Code	N	Voucher	Collection date	GenBank accession no.	
						cytb	*coI*
S. maculatus	Upper Paraguay basin (Manso River)	PY	7	NUP 884	Jul., 2006	KP256436-442	KP256372-378
S. maculatus	Upper Paraná basin (Garças Lagoon)	PR	3	NUP 4208	Feb., 2006	KP256443-445	KP256379-381
S. maculatus	Upper Paraná basin (Baía River)	PR	4	NUP 4208	Feb. & Nov., 2006	KP256446-449	KP256382-385
S. maculatus	Upper Paraná basin (Floodplain)	PR	5	NUP 4208	Feb. & Nov., 2006	KP256450-454	KP256386-390
S. maculatus	Tocantins River	TO	7	UNT 8175	May, 2007	KP256455-461	KP256391-397
S. marginatus	Upper Paraguay basin (Manso River)	PY	4	NUP 885	Jul., 2006	KP256462-465	KP256398-401
S. marginatus	Upper Paraná basin (Garças Lagoon)	PR	2	NUP 6374	Feb., 2006	KP256466-467	KP256402-403
S. marginatus	Upper Paraná basin (Floodplain)	PR	5	NUP 6374	Feb. & Nov., 2006	KP256468-472	KP256404-408
S. rhombeus	Tocantins River	TO	6	—	Oct. & Nov., 2005	KP256473-478	KP256409-414
S. eigenmanni	Tocantins River	TO	5	—	Dec., 2005	KP256479-483	KP256415-419
S. brandtii	São Francisco River	SF	4	—	Oct., 2009	KP256484-487	KP256420-423
P. nattereri	Upper Paraguay basin (Manso River)	PY	4	NUP 886	Jul., 2006	KP256488-491	KP256424-427
P. nattereri	Tocantins River	TO	4	UNT 8148	Mar., 2006	KP256492-495	KP256428-431
P. piraya	São Francisco River	SF	4	—	Oct., 2009	KP256496-499	KP256432-435

Code = sample site abbreviation; N = number of analyzed specimens; Voucher: NUP = number of catalogue at Ichthyological Collection of the Nupelia (Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura)/Universidade Estadual de Maringá; UNT = number of catalogue at Laboratory of Ichthyology and Systematic of Universidade Federal do Tocantins.

(PCR), using aliquots of total DNA. *Cytb* region was amplified with the primers H16498 (5'-CCT GAA GTA GGA ACC AGA TG-3'; Meyer et al., 1990) and L14841 (5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3'; Kocher et al., 1989), whereas *coI* gene was PCR amplified using the primers H7152 (5'-CAC CTC AGG GTG TCC GAA RAA YCA RAA-3'; Ivanova et al., 2007) and L6448-F1 (5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3'; Ward et al., 2005). Reactions were performed in a total volume of 25 µL containing Tris-KCl buffer (20 mM Tris-HCl pH 8.4 and 50 mM KCl), 1.5 mM $MgCl_2$, 2.5 µM of each primer, 0.1 mM of each dNTP, 2.5 units of DNA *Taq* polymerase, 30 ng of genomic DNA and deionized and autoclaved water. PCR profile in thermal cycler included an initial pre-melt of 4 min at 94 °C, followed by 40 cycles of 15 s denaturation at 94 °C, 30 s of annealing at 59-61 °C (for *cytb*) or 52-55°C (for *coI*) and 2 min extension at 72 °C, ending with a 10 min extension at 72 °C.

The amplification efficiency was confirmed on 1% agarose gel. Samples were then purified with polyethylene glycol. Resulting fragments were once more amplified with primers L14841 or L6448-F1 for *cytb* or *coI* regions, respectively. Approximately 50 ng of the final products were directly used in the nucleotide sequencing reactions using the DYEnamic ET Dye Terminator Kit (Amersham Biosciences) in the automatic sequencer MegaBACE 1000 (Amersham Biosciences), following manufacturer instructions.

2.3. Sequence and phylogenetic analysis

Partial nucleotide sequences of *cytb* and *coI* were edited and aligned separately by Clustal Omega (Sievers et al., 2011) and BioEdit 7.0.1 (Hall, 2011) and, when necessary, adjusted manually. Genetic diversity indices such as polymorphic sites, number of transitions and transversions, *p*-distances and nucleotide compositions were obtained with MEGA7 (Kumar et al., 2016).

After checking for congruency among tree topologies derived from the single-gene phylogenies, analyses of Maximum-Likelihood (ML) and Bayesian (BA) inferences were based on the concatenated sequences of *cytb* and *coI*. The best-fit model of nucleotide evolution was estimated by PartitionFinder 2.1 software (Lanfear et al., 2012). Best-scoring ML trees were estimated with the raxmlGUI software (Silvestro and Michalak, 2012), using rapid bootstrap algorithm, autoMRE function for resamplings, and the partition set defined by the PartitionFinder 2.1. BA trees were calculated using the uncorrelated lognormal relaxed-clock model implemented in BEAST 1.8.2 with an input file generated in BEAUti 1.8.0 (Drummond et al., 2012). The Yule process of speciation, which assumes a constant speciation rate among lineages, was applied as a tree prior. A Monte-Carlo Markov chain (MCMC) of 50,000,000 generations was performed and sampled every 1,000 generations. Results were checked using Tracer 1.6

(Rambaut et al., 2014; ESS >200). The final trees were calculated after 20% of burn-in. Tree was edited with FigTree (Rambaut, 2010). Support for nodes was determined using posterior probabilities (PP; calculated by BEAST).

2.4. Species delimitation analyses

Three species delimitation methods were applied to the *coI* sequences only using either sequence-based estimations (Automatic Barcode Gap Discovery – ABGD) or topology-based analyses based on the ML or BA inferences (Poisson Tree Process – PTP, and General Mixed Yule Coalescent Model – GMYC). The ABGD method was conducted on the online server (Puillandre et al., 2012) using the default parameters and the Kimura model (K80; 2.0) of nucleotide substitution. The PTP model was implemented on the online server (Zhang et al., 2013) using the best-scoring ML tree constructed on raxmlGUI, as mentioned before. The GMYC method was implemented using the ultrametric tree based on the Bayesian inference constructed in BEAST, as mentioned above. Tracer 1.6 software was used to check for chain convergence and the effective sampling size (ESS>200). The identification of significant clusters was implemented in Rstudio software (RStudio Team, 2016) by using the splits package (Ezard et al., 2009).

3. Results

The total length of the concatenated sequences of partial *coI* (549 bp) and *cytb* (592 bp) genes was of 1,141 bp long, without indels. A total of 167 variable sites were identified within the 64 analyzed specimens (excluding outgroup); 157 of them were parsimony-informative. Polymorphic sites and diagnostic nucleotides are presented in Supplementary Material 1. Sequence analysis revealed a ratio of transitions and transversions of R = si/sv = 8.26 and a proportion of bases of T = 26.7%, C = 32.5%, A = 24.1% and G = 16.7%.

Concatenated *cytb* and *coI* sequences were efficient in discriminating the piranha species, revealing high bootstrap or posterior probabilities values supporting clades in both dendrograms (Figure 2) as well as consistent *p*-distance

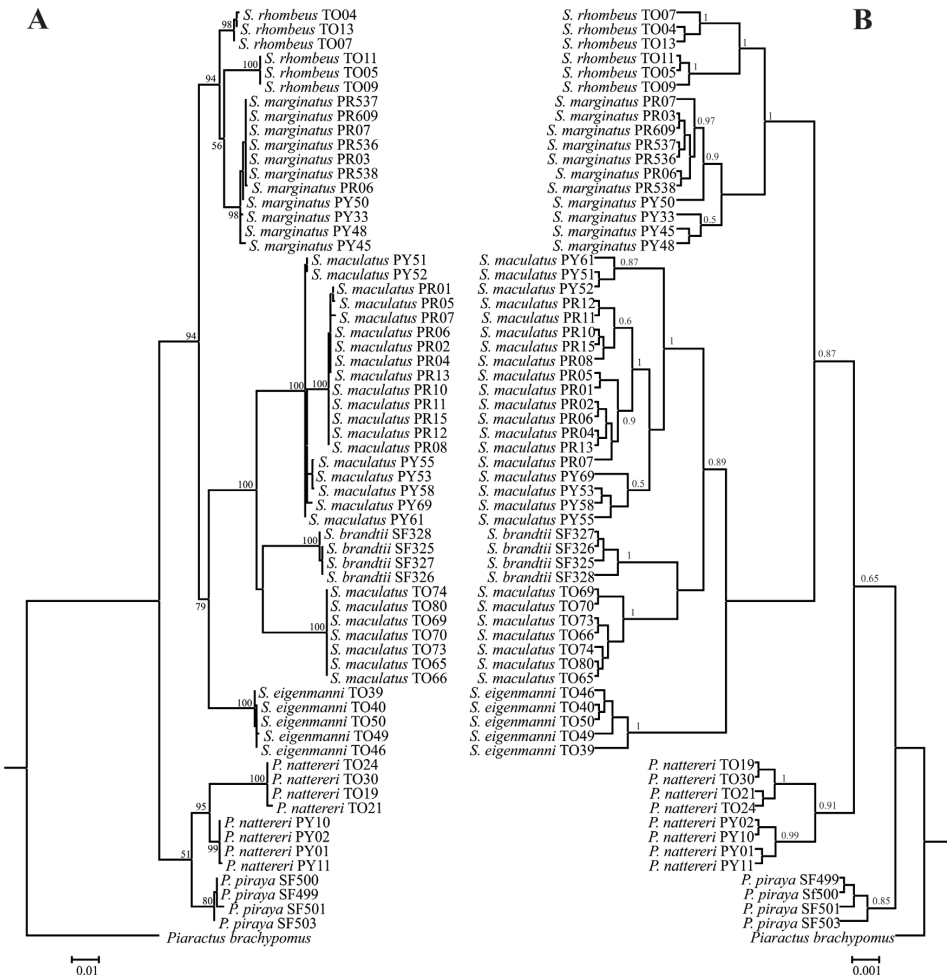

Figure 2. Maximum-likelihood (ML; A) and Bayesian (BA; B) phylogenetic trees inferred from the concatenated sequences of Cytochrome b and Cytochrome c oxidase I mitochondrial genes of *Serrasalmus* and *Pygocentrus* species. Values near branches indicate bootstrap and posterior probabilities support values for nodes in the ML and BA analysis, respectively.

values (Table 2). Single-gene topologies obtained with *cytb* or *coI* sequences were similar with each other (data not shown) and with the topology based on the concatenated sequences. Only two taxa were problematic regarding their positions in the trees: *S. eigenmanni* and *S. brandtii*. In the ML dendrogram obtained with *coI* sequences only, *S. eigenmanni* was the sister group of *S. rhombeus* and *S. marginatus* (see Figure 3), instead of *S. maculatus* and *S. brandtii* (as in Figure 2). *Serrasalmus brandtii*, on the other hand, was closer to *S. maculatus*

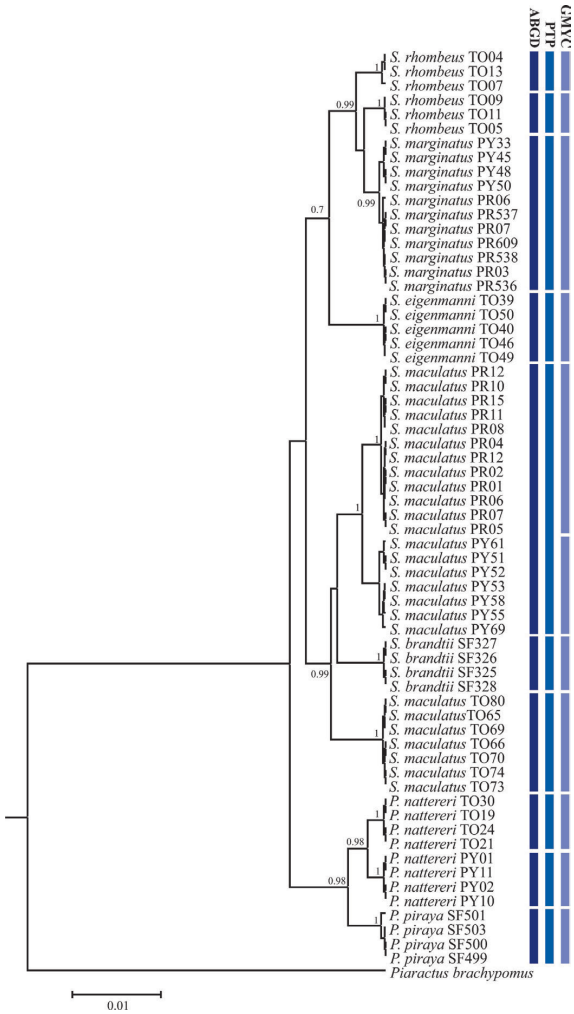

Figure 3. Species delimitation analyses based on the Cytochrome c Oxidase I sequences of *Serrasalmus* and *Pygocentrus* species from Upper Paraná (PR), Upper Paraguay (PY), São Francisco (SF) and Tocantins (TO) River basins, using Automatic Barcode Gap Discovery (ABGD), Poisson Tree Processes (PTP), and General Mixed Yule Coalescent (GMYC) methods. Bayesian (posterior probability) support values for each node are presented in the Bayesian tree.

Table 2. Mean *p*-distance values (%) between species of the piranha genera *Serrasalmus* and *Pygocentrus*, based on the concatenated sequences of the mitochondrial DNA genes Cytochrome b and Cytochrome c Oxidase I.

Species	1	2	3	4	5	6	7
1. *S. maculatus* PY and PR							
2. *S. maculatus* TO	4.01						
3. *S. marginatus* PY and PR	4.73	4.43					
4. *S. rhombeus* TO	4.69	4.41	1.68				
5. *S. eigenmanni* TO	4.64	4.41	3.02	3.55			
6. *S. brandtii* SF	3.63	3.74	4.67	4.75	4.22		
7. *P. nattereri* PY and TO	5.74	5.97	4.60	4.59	4.60	5.72	
8. *P. piraya* SF	5.71	5.78	4.16	4.25	4.33	5.85	2.48

PY = Upper Paraguay River basin; PR = Upper Paraná River basin; TO = Tocantins River; SF = São Francisco River.

from Paraná-Paraguay basin (data not shown), rather than *S. maculatus* from Tocantins, in the ML tree based on *cytb* sequences. All the analyzed species of *Serrasalmus* were grouped in a monophyletic clade in the ML and BA trees. The same was observed for *Pygocentrus* in the ML analysis (Figures 2 and 3).

The two species of the genus *Pygocentrus* formed a basal clade in both dendrograms, and the relationships among specimens were mostly corroborated in the ML and BA analyses. *Pygocentrus nattereri* and *P. piraya* constituted each one a monophyletic group and they presented a genetic distance from each other of 2.48% (Table 2). In addition, *P. nattereri* was separated in two clades, each one corresponding to the populations of Tocantins and Upper Paraguay River basins. This fact was corroborated by the values of genetic distance: although variability within each group was low (0.04% to 0.08%), the genetic distance between the two populations of *P. nattereri* was of 2.25% (Table 3).

The clade constituted by *Pygocentrus* was the sister group of all species of the genus *Serrasalmus* in dendrograms. As observed for *P. nattereri*, genetic isolation of populations from Tocantins River and Paraná-Paraguay hydrographic basin was also observed for *S. maculatus*. Specimens of *S. maculatus* from the Paraná-Paraguay River basin formed a clade distinct from the population of Tocantins River (Figure 2) and showed high genetic distance values (4.01%; Table 2). However, no significant differentiation was observed between *S. maculatus* of Paraná and Paraguay Rivers basins. These results were corroborated by the genetic distance values (Table 3). The population of *S. maculatus* from Tocantins River diverged from the populations of Paraná and Paraguay basins by mean values of 4.20% and 3.69%, respectively. On the other hand, the genetic differentiation between *S. maculatus* from Paraná and Paraguay Rivers was only 1.04%. In addition, *S. brandtii* was placed within the clade formed by *S. maculatus* (Figure 2), indicating the non-monophyletic condition of *S. maculatus*. *Serrasalmus eigenmanni* was defined as the sister group of the clade formed by *S. maculatus* and *S. brandtii*.

Serrasalmus rhombeus and *S. marginatus* were genetically close to each other and formed a major clade that was further divided into three sub-clades: one formed exclusively by *S. marginatus* and two formed by *S. rhombeus* (Figure 2). The two clades of *S. rhombeus* were distant from each other by a value of genetic *p*-distance of 1.78% (Table 3). Values of such magnitude were also identified between *S. rhombeus* and *S. marginatus* (*p*-distance = 1.68%; Table 2). Additionally, *S. rhombeus* was non-monophyletic in the ML (Figure 2A) and BA (Figure 3) trees.

The three species delimitation analyses were mostly congruent and returned ten (for ABGD and PTP methods) or eleven (GMYC) lineages for *Serrasalmus* and *Pygocentrus* species (Figure 3). All methods split both *P. nattereri* and *S. rhombeus* into two subgroups. However, *S. maculatus* was divided into two (ABGD and PTP) or three (GMYC) lineages, depending on the approach.

4. Discussion

4.1. Delimitation of piranha species

All of the piranha species analyzed herein were discriminated based on the *cytb* and *coI* mitochondrial DNA (mtDNA) sequences and based on the species delimitation analyses (ABGD, PTP and GMYC). Genetic *p*-distance mean values between species varied from 1.68% to 5.97% (Table 2). The genetic differentiation between *P. nattereri* and *P. piraya* was evident. Although the value of *p*-distance detected between the species were low (2.48%), this is an expected result, since the speciation event leading to *P. nattereri* and *P. piraya* occurred recently, during the last 2.6 ± 0.2 Ma (Mateussi et al., 2019).

Table 3. Mean *p*-distance values (%) within (diagonal) and between (below the diagonal) populations of the piranha genera *Serrasalmus* and *Pygocentrus*, based on the concatenated sequences of the mitochondrial genes Cytochrome b and Cytochrome c Oxidase I.

Species/ Population	1	2	3	4	5	6	7	8	9	10	11	12
1. *S. maculatus* PY	0.29											
2. *S. maculatus* PR	1.04	0.11										
3. *S. maculatus* TO	3.69	4.20	0.00									
4. *S. marginatus* PY	4.41	4.77	4.33	0.17								
5. *S. marginatus* PR	4.55	4.92	4.48	0.23	0.02							
6. *S. rhombeus* TO 1	3.95	4.29	4.09	1.40	1.50	0.01						
7. *S. rhombeus* TO 2	4.98	5.34	4.73	1.84	1.94	1.78	0.00					
8. *S. eigenmanni* TO	4.43	4.76	4.41	2.93	3.08	3.15	3.94	0.07				
9. *S. brandtii* SF	3.44	3.74	3.74	4.58	4.72	4.44	5.06	4.22	0.04			
10. *P. nattereri* PY	5.19	5.32	5.67	4.14	4.27	3.87	4.40	4.22	5.47	0.04		
11. *P. nattereri* TO	6.14	6.26	6.26	4.90	5.04	4.77	5.30	4.98	5.98	2.25	0.08	
12. *P. piraya* SF	5.65	5.74	5.78	4.07	4.20	3.94	4.55	4.33	5.85	1.77	3.20	0.17

PY = Upper Paraguay River basin; PR = Upper Paraná River basin; TO = Tocantins River; SF = São Francisco River.

We also detected high genetic variability within *P. nattereri*, as previously reported by Mateussi et al. (2019) and Luz et al. (2015). Specimens of *P. nattereri* were separate in the dendrograms according to the sample locality. Values of *p*-distance observed between populations of *P. nattereri* (Paraguay and Tocantins River basins; 2.25%; Table 3) were lower than the values identified between species of *Serrasalmus*, suggesting that the existing polymorphism in *P. nattereri* is related to intra-specific variability, which is commonly observed in geographically isolated populations. Distinction between populations of *P. nattereri* of different river basins has already been discussed (Fink, 1993; Fink and Zelditch, 1997), appointing to shape differences consistent with the geographical variation of a widely distributed species (Fink and Zelditch, 1997). However, all species delimitation analysis recovered two mtDNA lineages among *P. nattereri* specimens (Figure 3). Similarly, Mateussi et al. (2019) detected at least five lineages of *P. nattereri* in five hydrographic river basins (Amazonas, Guaporé, Itapecuru, Paraná-Paraguay and Tocantins-Araguaia). The authors also concluded that these lineages can be potentially sibling species, a special case of cryptic species, representing the *P. nattereri* species complex (Mateussi et al., 2019). Finally, the monophyletic condition of *P. nattereri* confirmed in the studies of Fink (1993) and Ortí et al. (1996) was also observed in our analysis (Figure 2).

The same pattern of genetic differentiation identified in *P. nattereri* was also observed for the specimens of *S. maculatus*, i.e., population of *S. maculatus* from Tocantins River basin was segregated from the populations of Paraná-Paraguay system. In the case of *S. maculatus*, *p*-distance values were relatively higher than in *P. nattereri.* ABGD, PTP and GMYC approaches split *S. maculatus* in two or three distinct lineages, as previously described by Bignotto et al. (2019). The high values of genetic distance and the differentiation of *S. maculatus* haplogroups from Tocantins and Paraná-Paraguay River basins, associated with the non-monophyletic condition of *S. maculatus* observed in the dendrograms, may constitute evidence for more than one species within *S. maculatus* (Bignotto et al., 2019). Similarly, Machado et al. (2018) did not recover the monophyletic condition of the species.

In addition to the wide geographic distribution, *S. maculatus* has high karyotypic variability (Cestari and Galetti Junior, 1992; Nakayama et al., 2000; Centofante et al., 2002; Nakayama et al., 2002). The different cytotypes identified in *S. maculatus* are related to the different hydrographic basins, characterizing differences among populations. Consequently, these evidences support a complex of cryptic species for *S. maculatus* (Nakayama et al., 2000).

Results obtained in the present study also revealed the close relationship between *S. maculatus* and *S. brandtii* (*p*-distance ranging from 3.63% to 3.74%; Table 2), corroborating previous studies (Cestari and Galetti Junior, 1992; Hubert et al., 2007). Cestari and Galetti Jr. (1992b) reported that *S. brandtii* of the São Francisco River and *S. maculatus* from the Upper Paraná River have similar karyotypes. According to Hubert et al. (2007), the event that gave rise to *S. brandtii* and *S. maculatus* occurred around 8 Ma.

The low genetic *p*-distance values observed between *S. rhombeus* and *S. marginatus* (1.68%; Table 2) were inferior to those detected among other species of piranhas analyzed in the present study. Values of such magnitude could be explained by two hypotheses. First, specimens namely *S. rhombeus* and *S. marginatus* might represent only one species with wide intraspecific variability. Another more reasonable explanation is that *S. rhombeus* and *S. marginatus* have undergone a recent speciation process, which impeded the accumulation of mtDNA polymorphisms. Both *S. rhombeus* and *S. marginatus* have been previously described, are well-defined species, and are morphologically distinct from each other (Jégu, 2003), which gives support for the second hypothesis.

Analysis clearly divided specimens of *S. rhombeus* from Tocantins River basin into two clades. Furthermore, *p*-distance values detected between these two groups of *S. rhombeus* are in the same magnitude of interspecific genetic divergence observed between *S. rhombeus* and *S. marginatus*. Additionally, ML and BA trees revealed the non-monophyletic condition of *S. rhombeus*. Thompson et al. (2014) and Machado et al. (2018) also did not obtain the monophyly of *S. rhombeus*. Hence, in the present study, we found evidence for at least two sympatric mitochondrial lineages of *S. rhombeus* that occur in the Tocantins River basin which diverged recently from each other. We suggest additional studies combining molecular and morphological data to confirm if both groups of *S. rhombeus* should be considered different species.

Cytogenetic studies also reported the high variability within *S. rhombeus* (Nakayama et al., 2001, 2012). Different *S. rhombeus* cytotypes were found in both allopatry and sympatry, suggesting that each cytotype represents a different fish species (Nakayama et al., 2001). Nakayama et al. (2001) also noted that differences in parasite species supported the recognition of a cryptic species of piranha within *S. rhombeus*. Our results agree with previously described data: according to Géry (1976), *S. rhombeus* is a species complex (*rhombeus* group) comprising six to nine species (Nakayama et al., 2001). Consequently, caution should be used in any decision regarding the species complex of the *rhombeus* group.

Therefore, mtDNA sequences (*cytb* and *coI*) were sufficient to characterize and discriminate all of the piranha species analyzed herein from the Upper Paraná, Upper Paraguay, São Francisco and Tocantins River basins, with additional evidence for the possible occurrence of cryptic species in *S. maculatus*, *S. rhombeus* and *P. nattereri*.

4.2. Relationships among hydrographic basins

The mitochondrial haplotypes of both *P. nattereri* and *S. maculatus* populations were related to the Amazon (Tocantins) or Paraná-Paraguay hydrographic basins, i.e., the genetic diversity is geographically structured and there are no shared haplotypes among rivers. The biogeographical pattern and the genetic differentiation found in the

populations of both species can be explained by vicariance and/or dispersion events.

According to Hubert and Renno (2006), the Paraná-Paraguay system split from the Amazon at 10 Ma, in the Late Miocene. Therefore, during the formation of these basins, the definitive separation of the two populations of both *S. maculatus* and *P. nattereri* was possible due to vicariance. Similarly, dispersal events between different drainages were hypothesized to promote post-dispersal allopatric speciation once the connections ceased (Hubert et al., 2007). During the last 10 Ma, headwater-capture events occurred between the Amazon and the Paraná systems (Lundberg et al., 1998). Several studies have reported the possible connection between the Amazon and the Paraná-Paraguay systems (e.g., Aquino and Schaefer, 2010). Therefore, vicariance and dispersion events could be related to the genetic differentiation of the populations of both *S. maculatus* and *P. nattereri* of the Amazon and the Paraná-Paraguay River basins.

The biogeography associated to the distribution of the haplotypes of both species (*S. maculatus* and *P. nattereri*) indicate that the populations are isolated geographically, reproductively and/or by distance for enough time so that mutations could be accumulated in the mtDNA molecule. Hubert et al. (2007) suggested that at least *P. nattereri* colonized the Paraná River at 2 Ma, which justifies the low genetic divergence identified in the populations of *P. nattereri*. However, the differentiation between the populations of *S. maculatus* must have occurred earlier, due to the higher genetic divergence detected for this species.

Fishes from the same river basin tend to be most closely related, whereas populations of fishes from adjacent localities within basins should be most similar (Lovejoy and Araújo, 2000). Such genetic pattern was identified for both *S. maculatus* and *S. marginatus*, species that occur in the Paraná-Paraguay hydrographic basin. Within the Upper Paraná River or the Upper Paraguay River, populations of both *S. maculatus* and *S. marginatus* revealed minor values of genetic distance. However, comparisons of populations between these localities showed only slightly higher values of genetic distance, characterizing polymorphisms within a species.

Probably, gene flow between populations of the Paraná-Paraguay hydrographic basin of *S. marginatus* and also of populations of *S. maculatus* has occurred until recently or may still occur. Occasional migrations from upstream to downstream and/or incorporation of the subpopulation from the Itaipu region to the population of the Upper Paraná River may have contributed to the attenuation of the genetic differentiation between these populations. The Guaíra Falls (Seven Falls) has characterized an efficient geographical barrier against the migration from downstream to upstream (Júlio Júnior et al., 2009). However, migration from upstream to downstream could frequently happen.

Genetic similarity was higher between the piranha species from the São Francisco River (*S. brandtii* and *P. piraya*) and the Paraná-Paraguay hydrographic basin (*S. maculatus* and *P. nattereri*) than with species occurring in the Tocantins River (*S. maculatus* and *P. nattereri*) (Table 3). This could be explained by the hydrological and geological history. Events of headwater capture were identified after the separations of the Upper Paraná and the São Francisco River basins (Hubert and Renno, 2006), which could enable ichthyofauna exchange between these two hydrographic basins. However, until now, headwater capture events have not been identified between São Francisco and Tocantins River basins.

Another two situations arose in the region and promoted fish dispersion between hydrographic basins. First, in the 1960s, the artificial transposition of the Piumhi River, originally from the Upper Paraná River basin, to the São Francisco River and, consequently, the entire fish fauna of this river was transposed to the São Francisco River (Moreira-Filho and Buckup, 2005). Second, Blanco et al. (2010) suggested that, in the same region prior to transposition of the Piumhi River, there may have been a natural connection causing mixture of the ichthyofauna. During floods, it is likely that connections between the Grande River (Paraná hydrographic basin) and the São Francisco River through the Cururu wetland occurred, representing a natural migratory route for fish (Blanco et al., 2010).

The results of the present study suggest that the biogeographic pattern identified for the piranha species analyzed of *Serrasalmus* and *Pygocentrus* agree with the geological and hydrological events previously documented in the literature. The separation of the hydrographic basins, as well as the headwaters events and dispersion routes among the different river systems that occurred in the past, may have strongly influenced the distribution and genetic differentiation of the piranha species.

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Supplementary Material

Supplementary material accompanies this paper.

Supplementary Material 1. Polymorphism of nucleotide sites in the concatenated sequences of the mitochondrial genes Cytochrome c oxidase I (1 to 550 bp) and Cytochrome b (551 to 1,141 bp) of piranha species of the genera Serrasalmus and Pygocentrus from the Upper Paraná (PR), Upper Paraguay (PY), São Francisco (SF) and Tocantins (TO) River basins. Letters in bold represent diagnostic nucleotides for a species and/or population.

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