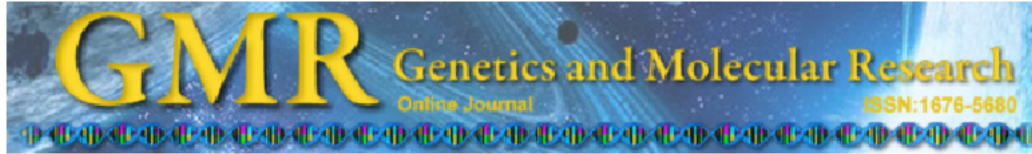

Molecular differentiation of species of the genus *Zungaro* (Siluriformes, Pimelodidae) from the Amazon and Paraná-Paraguay River basins in Brazil

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ABSTRACT. Fish species of the *Zungaro* genus (Siluriformes, Pimelodidae) are amongst the largest migratory fish in Latin America and have considerable economic importance for commercial fishing in Brazil. However, natural populations of this large catfish are experiencing a severe decline. There are significant taxonomical inconsistencies for this fish. Two geographically separated species of the fish were initially described, one endemic in the Amazon and another in the Paraná-Paraguay River basins. A taxonomic review had recently proposed that there is only one *Zungaro* species in Brazil, based on morphological data. We made a molecular study of *Zungaro* populations in an attempt

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to solve taxonomical inconsistencies and to analyze genetic diversity in natural populations of this genus. We analyzed two regions of the mitochondrial DNA (the control region and the ATPase 6 gene region) of individuals sampled from the Paraná-Paraguay River and Amazon River basins. Analyses based on *p*-distances and maximum likelihood phylogenetic models showed a genetic difference between populations corresponding to different species. Genetic differentiation between *Zungaro* populations was at the same level as that observed between other Siluriformes species, using the same DNA sequences. We conclude that *Zungaro* species of the Paraná-Paraguay River basin do not belong to the same species found in the Amazon basin. This finding has a significant implication for conservation of this fish, given that populations are disappearing at a high rate in the Paraná-Paraguay River basin, mainly due to impoundments.

Key words: Mitochondrial DNA; *Zungaro*; D-loop; ATPase 6

INTRODUCTION

Fish species of the *Zungaro* genus (Siluriformes, Pimelodidae) are piscivorous, with external fertilization and usually inhabit deep holes of lotic environments. These catfishes (popularly known as “Jaú” in Brazil) are among the largest species with migratory behavior in Neotropical rivers, reaching up to 150 kg (330 pounds) in weight and 144 cm (ca. 4.5 feet) in length (Agostinho et al., 2003). Not surprisingly, they have a great importance on commercial fishing and are highly appreciated for both regional culinary and recreational fishing. However, knowledge of the biology of this fish is still incomplete, particularly as related to its migratory behavior (Agostinho et al., 2003). Meanwhile, natural populations of *Zungaro* species have recently experienced a severe decline on population size mainly due to the construction of hydropower dams, since they prevent migration (Pelicice and Agostinho, 2008).

Additionally, there are taxonomic inconsistencies in the *Zungaro* genus in Brazil that can affect conservation of natural populations of this fish. A previous study has described two morphologically distinct species in major Brazilian rivers (Lundberg and Littmann, 2003): the species *Zungaro zungaro* (Humbolt, 1821), found in the Amazon basin, and the species *Zungaro jahu* (Ihering, 1898), restricted to the Paraná-Paraguay River basin. Moreover, this is the information available on FishBase (http://www.fishbase.org). Nevertheless, a review suggested that all populations of *Zungaro* genus in Brazil belonged to the species *Z. zungaro* (Graça and Pavanelli, 2007). To our knowledge, no previous study was able to morphologically differentiate two species (see Lundberg and Littmann, 2003; López et al., 2005; Britski et al., 2007; Ferraris, 2007; Graça and Pavanelli, 2007). Accordingly, information regarding the two species illustrated in FishBase is not sufficient to differentiate them, considering both morphology and aspects of their behavior (see http://www.fishbase.org).

In this context molecular biology can be useful to clarify the taxonomic status and answer ecological questions related to gene flow in natural populations. Several researchers have used molecular markers to differentiate species (e.g., Torres and Ribeiro, 2009). Mitochondrial DNA (mtDNA) is an effective marker for studies on animal evolution, phylogeography and

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population genetics (Agnese et al., 1997; Rokas et al., 2003; Azevedo et al., 2008; Ortí et al., 2008). Additionally, mtDNA has also been used to differentiate related species (Johns and Avise, 1998). In particular, the control region (D-loop) and the ATPase 6 gene region are among the most frequently used markers for this purpose (Meyer, 1994; Avise, 2004; Prioli et al., 2011).

Therefore, our goal was to molecularly differentiate individuals of *Zungaro* from the Paraná-Paraguay River and Amazon basins in Brazil. This is essential to solve taxonomic uncertainties and prevent frequent changes on the scientific classification of the species. As a consequence, the almost inexistent information about the genetic diversity of natural populations of *Zungaro* species was amplified, allowing for proper evaluation of their conservation status in Brazil.

MATERIAL AND METHODS

Individuals of the *Zungaro* genus were sampled from the Manso River, located at the Paraná-Paraguay River basin, and the Tocantins River in the Amazon basin (Figure 1). Four and three individuals (from the Paraná-Paraguay River and Amazon basins, respectively) were analyzed for the D-loop region (the control region), whereas 11 and 5 (equally from the Paraná-Paraguay River and Amazon basins, respectively) were analyzed for ATPase 6 gene region. This number of individuals was sufficient for our analyses of molecular differentiation given the results (see Results section).

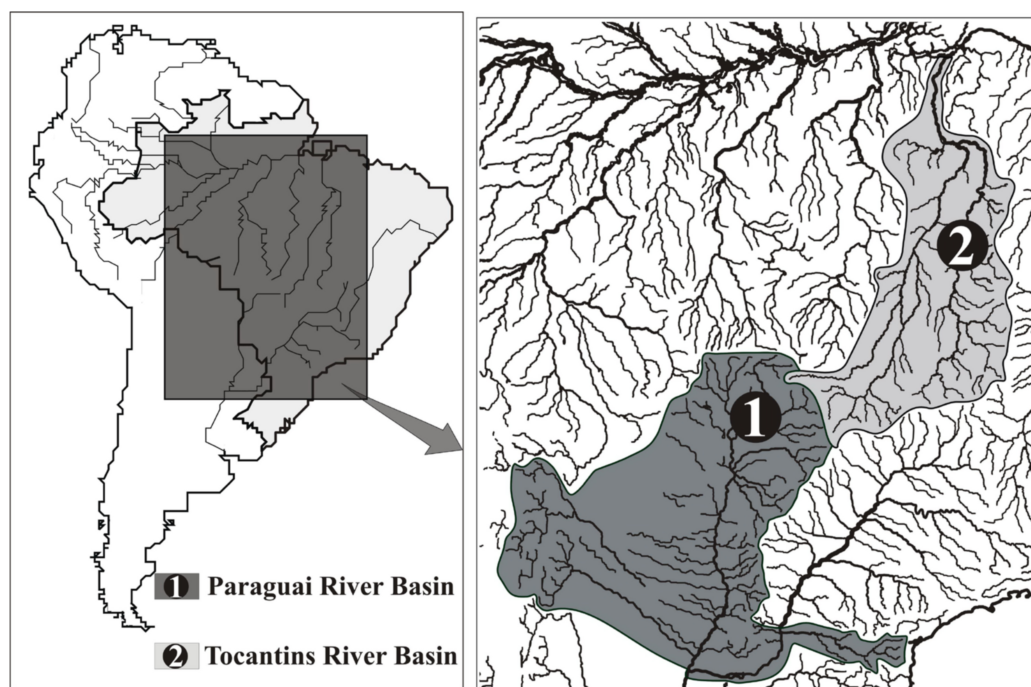

Figure 1. South America map indicating the sampling areas of *Zungaro* individuals. Numbers indicate sampling basins: 1 = Paraguay River basin, a sub-basin of Paraná-Paraguay River basin, where Manso River is located; 2 = Tocantins River basin, a sub-basin of Amazon basin where Tocantins River is located.

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Total DNA of samples (from muscle tissues) was obtained based on the phenol/chlorophorm methodology (Monesi et al., 1998) and resuspended in 50 μL TE diluted (1:10) buffer (1 mM Tris-HCl, pH 8, 0.1 mM EDTA) with 20 μg/mL RNase. Fragments of mtDNA were amplified by PCR, using aliquots of the total DNA (Prioli et al., 2011). The first fragment of approximately 2000 bp was amplified by the primers H1091 (5'-ATAGTGGGGTATCTAATCCAGTT-3') (Kocher et al., 1989) and L15774 (5'-CAACATGAATTGGAGGTATACCAGT-3') (Shields and Kocher, 1991). This fragment encompasses the $tRNA^{thr}$ and $tRNA^{Pro}$ genes (responsible for codifying tRNA of threonine and proline amino acids, respectively) and a sequence of a hypervariable region 5' of the heavy ribbon of the mtDNA control region (D-loop). The second fragment of approximately 1000 bp comprises gene codings for ATPase 6 and ATPase 8 subunits. This fragment was amplified by the primers H8331 (5'-AAAGCRTYRGCCTTTTAAGC-3') and L9236 (5'-GTTAGTGGTCAKGGGCTTGGRTC-3') (Hughes and Hillyer, 2006).

Both fragments were amplified in two independent reactions for further sequence determination and analysis, always using replicates. The reaction solution was composed of Tris-KCl buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 2.5 μM of each primer, 0.1 mM of each dNTP, 2.5 U *Taq* DNA polymerase per reaction, 30 ng genomic DNA, and deionized and autoclave water was used to make a final volume of 25 μL. Amplifications of fragments were executed in a thermocycler through 40 cycles, with the following temperature profiles: an initial cycle of 4 min at 94°C followed by 40 cycles of 15 s at 94°C , 30 s at 59°C and 2 min at 72°C, finishing with a 10-min cycle at 72°C.

Resulting fragments were once more amplified with the primers H8331 and L15774 (for ATPase 6 gene and D-loop regions, respectively). Approximately 50 ng DNA after each PCR was directly used in nucleotide sequencing reactions in an automatic sequencer MegaBace (Amersham), following manual instructions. Nucleotide sequences, shaped as a chromatogram, were aligned using CLUSTAL W (Thompson et al., 1994) and manually edited with the BIOEDIT software (Hall, 1999). Using similarity, D-loop sequences were confirmed with available accesses in the GenBank, whereas tRNAs were identified using the tRNAScan-SE software (Lowe and Eddy, 1997). The number of polymorphic nucleotides and *p*-distances (percentages of nucleotide differences) were calculated with the MEGA 4.0 software (Tamura et al., 2007). Matrices of *p*-distances, for both regions of the mitochondrial genome, were used to group analyses using the neighbor-joining algorithm. Bootstrap analyses were based on 1000 resamplings.

Phylogenetic analyses were executed by comparing individual pairs. Since the evolutionary meaning of DNA sequence deletions is poorly known (Nei and Kumar, 2000), sites with deletions were not considered in the analysis of each comparison.

PAUP 4.0 (Swofford, 2002) and Modeltest 3.7 (Posada and Crandall, 1998) softwares were used to select evolutionary models through Akaike information criterium (AICc) and Bayesian information criterium (BIC). Phylogenetic relationships among haplotypes were obtained using the PHYML software (Guindon and Gascuel, 2003) and expressed as dendrograms. The maximum likelihood procedure, based on 500 bootstraps, was combined with the model mentioned above. Dendrograms and *p*-distances were calculated only for D-loop and ATPase 6 subunit regions, given that the sequence encompassing tRNAs is a highly conserved region. For that region, we only searched for punctual nucleotide modifications.

Sequences from other fish species obtained in GenBank were selected to serve as comparison basis for *Zungaro* sequences in this study. Sequences were aligned and only the

same base pairs used in *Zungaro* individuals for each region (D-loop or ATPase 6 gene regions) were compared among individuals selected in GenBank to ensure that analyses were reliable. In addition, only sequences available from articles published in Thompson-ISI indexed journals (http://www.webbofscience.com) were selected for comparison. GenBank access numbers of sequences used are listed in Table 1.

Table 1. D-loop control region of mtDNA sequence from two Siluriformes genera, *Pseudobagrus* and *Hipostomus*, and ATPase 6 gene region of mtDNA sequence from two Siluriformes genera of the Ariidae family, *Potamarius* and *Cathorops*, with their respective GenBank accession numbers.

Species	Fragment	Genbank accession No.	Reference
Pseudobagrus taeniatus	D-loop	AB097696	Watanabe and Nishida (2003)
Pseudobagrus aurantiacus	D-loop	AB097694	Watanabe and Nishida (2003)
Pseudobagrus nudiceps	D-loop	AB097693	Watanabe and Nishida (2003)
Pseudobagrus ichikawai	D-loop	AB097692	Watanabe and Nishida (2003)
Pseudobagrus tokiensis	D-loop	AB097691	Watanabe and Nishida (2003)
Hypostomus affinis	D-loop	AJ318358	Montoya-Burgos (2003)
Hypostomus punctatus	D-loop	AJ318357	Montoya-Burgos (2003)
Hypostomus commersoni	D-loop	AJ318356	Montoya-Burgos (2003)
Hypostomus nigromaculatus	D-loop	AJ318355	Montoya-Burgos (2003)
Hypostomus watwata	D-loop	AJ318352	Montoya-Burgos (2003)
Hypostomus plecostomus	D-loop	AJ318351	Montoya-Burgos (2003)
Hypostomus fonchii	D-loop	AJ318350	Montoya-Burgos (2003)
Hypostomus boulengeri	D-loop	AJ318344	Montoya-Burgos (2003)
Potamarius grandoculis	ATPase 6	DQ990657	Betancur et al. (2007)
Potamarius nelson	ATPase 6	DQ990656	Betancur et al. (2007)
Potamarius izabalensis	ATPase 6	DQ990654	Betancur et al. (2007)
Cathorops tuyra	ATPase 6	DQ990652	Betancur et al. (2007)
Cathorops hypophthalmus	ATPase 6	DQ990651	Betancur et al. (2007)
Cathorops multiradiatus	ATPase 6	DQ990650	Betancur et al. (2007)
Cathorops aguadulce	ATPase 6	DQ990648	Betancur et al. (2007)
Cathorops arenatus	ATPase 6	DQ990647	Betancur et al. (2007)
Cathorops spixii	ATPase 6	DQ990646	Betancur et al. (2007)
Cathorops steindachneri	ATPase 6	DQ990644	Betancur et al. (2007)
Cathorops fuerthii	ATPase 6	DQ990641	Betancur et al. (2007)
Cathorops dasycephalus	ATPase 6	DQ990639	Betancur et al. (2007)

Analyses were made on the D-loop region of two Siluriformes genera, *Pseudobagrus* (with five species) and *Hypostomus* (with eight species). Considering the ATPase 6 gene region, two other Siluriformes genera from the Ariidae family were used: *Potamarius* (with three species) and *Cathorops* (with nine species). All individual names were checked in the FishBase database (Froese and Pauly, 2007) to avoid comparisons between synonym species. The review by Marceniuk and Menezes (2007) was used for individuals from the Ariidae family. According to these authors, *Potamarius usumacintae* is synonym of *P. grandoculis*, and the last name should be used. Therefore, we used the *P. usumacintae* sequence (indicated at GenBank) but with the correct species name (*P. grandoculis*).

RESULTS

The *Zungaro* sequences used here were added to GenBank database (see access numbers in Table 2). By amplifying D-loop fragments, two sequences were selected for analyses, the first comprising the control region of D-loop (~355 bp) and the second corresponding to $tRNA^{Thr}$ and $tRNA^{Pro}$ genes (140 bp) (Table 2). The main reason for choosing these sequences was their location at the best quality segment in the whole sequence (after manual edition).

Despite being short sequences compared to the total fragments amplified (ca. 2000 bp for D-loop region), this strategy was used for high rigor analysis and to avoid misinterpretations, thus enhancing reliability of the results. In addition, the selected sequence was sufficient to discriminate populations (see below). After aligning, two nucleotide substitution points were identified in tRNAs sequences, discriminating individuals of different sampling sites.

Table 2. *Zungaro* individuals, their sampling sites and GenBank accession numbers (AN) for both D-loop control region and ATPase 6 gene region.

Zungaro individuals	Sampling site	D-loop AN	ATPase 6 AN
ZgrMS01	Manso River (Paraná-Paraguay River basin)	-	FJ794946
ZgrMS02	Manso River (Paraná-Paraguay River basin)	-	FJ794947
ZgrMS03	Manso River (Paraná-Paraguay River basin)	-	FJ794948
ZgrMS04	Manso River (Paraná-Paraguay River basin)	FJ797691	FJ794955
ZgrMS05	Manso River (Paraná-Paraguay River basin)	FJ797692	FJ794949
ZgrMS06	Manso River (Paraná-Paraguay River basin)	FJ797693	FJ794956
ZgrMS07	Manso River (Paraná-Paraguay River basin)	-	FJ794950
ZgrMS08	Manso River (Paraná-Paraguay River basin)	-	FJ794951
ZgrMS09	Manso River (Paraná-Paraguay River basin)	FJ797694	FJ794952
ZgrMS10	Manso River (Paraná-Paraguay River basin)	-	FJ794953
ZgrMS11	Manso River (Paraná-Paraguay River basin)	-	FJ794954
ZgrTo02	Tocantins River (Amazon basin)	FJ797695	FJ794957
ZgrTo03	Tocantins River (Amazon basin)	FJ797696	FJ794958
ZgrTo04	Tocantins River (Amazon basin)	-	FJ794959
ZgrTo05	Tocantins River (Amazon basin)	FJ797697	FJ794960
ZgrTo06	Tocantins River (Amazon basin)	-	FJ794961

Considering the D-loop region, sequences revealed 19 substitution points, 15 transversions and four transitions. This rate (R = transversions/transitions = 3.75) corroborates with expectations for the fragment corresponding to the D-loop region (Nei and Kumar, 2000). Individuals from Tocantins River presented an insertion of a cytosine and 16 substitutions. The polymorphisms of the D-loop region among Manso individuals, using pairwise comparisons, were always up to one polymorphic site (*p*-distance = 0.3%). When Manso individuals were compared to Tocantins ones, 16-17 polymorphic sites were found (*p*-distances = 4.5-4.8%).

The values of D-loop region polymorphic sites for some species from different Siluriformes genus, *Pseudobagrus* and *Hypostomus*, were at the same level with those obtained for the same D-loop region from *Zungaro* of Manso and Tocantins Rivers. Similar values were also observed in half the relationships among *Pseudobagrus* species (*p*-distances up to 5.0%). The differentiation of *Zungaro* haplogroups from Manso and Tocantins Rivers was sometimes clearer than the differentiation of species from *Hypostomus* and *Pseudobagres* genus, considering the dendrogram constructed with *p*-distance values of the D-loop region (Figure 2). This dendrogram grouped *Zungaro* individuals according to sampling rivers, with a bootstrap of 100%. The dendrogram constructed using evolutionary models displayed a similar pattern and therefore was omitted.

Considering that the ATPase 6 gene region is more preserved, few differences were observed between related groups. A good-quality sequence of 204 bp (edited from an amplified fragment of ca. 1000 bp) was selected for analysis (see reasons for editing sequences above). Five nucleotide substitutions between individuals from the two rivers (Manso and Tocantins Rivers) were found. Individuals of the same river always had the

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same nucleotide sequence. This partial ATPase 6 gene sequence corresponds to 68 amino acids. Despite these nucleotidic differences, no amino acid substitution was evidenced considering the previewed polypeptide.

Figure 2. Neighbour joining tree based on *p*-distances of partial D-loop nucleotidic sequences of mitochondrial DNA from eight *Hypostomus* species, five *Pseudobagrus* species and *Zungaro* (Zgr) individuals sampled in Manso and Tocantins Rivers, with a bootstrap of 100%. Sequences of *Hypostomus* and *Pseudobagrus* species were obtained in GenBank.

The differentiation between haplotypes from Paraná-Paraguay River and Amazon basins represents a *p*-distance of 2.5%. This value is higher than those found to differentiate three Siluriformes species of the *Potamarius* genus (*P. grandoculis*, *P. nelson*, and *P. izabalensis*), and similar to some *p*-distances between Siluriformes species of the *Cathorops* genus. Values of *p*-distances between species of *Cathorops* genus were highly variable, varying from 1.5% (between *C. spixii* and *C. arenatus*) to 17.6% (between *C. dasycephalus* and *C. hypophthalmus*). Differences observed when comparing *Zungaro* individuals from both rivers were higher than those observed for *Potamarius* species considering the dendrogram constructed with *p*-distance values of the ATPase 6 region (Figure 3). In addition, these differences were at the same level of those observed for some *Cathorops* species (Figure 3). Once more, the dendrogram constructed using evolutionary models was similar and, therefore, omitted.

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Figure 3. Dendrogram constructed using *p*-distances of partial ATPase 6 nucleotidic sequences from nine *Cathorops* species, three *Potamarius* species and *Zungaro* (Zgr) individuals sampled in Manso and Tocantins Rivers, with a bootstrap of 100%. Sequences of individuals from *Cathorops* and *Potamarius* genus were obtained in GenBank.

DISCUSSION

The taxonomy of indigenous *Zungaro* populations in Brazil still shows discrepancies to be solved. Previous studies have morphologically identified two different species of *Zungaro* from the Paraná-Paraguay River and Amazon basins (Lundberg and Littmann, 2003). However, a taxonomic review considered that *Z. zungaro* is the only species in Brazil (e.g., Graça and Pavanelli, 2007). Overall, our results support the suspicion that *Zungaro*

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individuals from the Paraná-Paraguay River and Amazon basins represent two distinct taxa. They possibly belong to closely related species, but not to the same species. Further taxonomic studies are necessary to conclude on the taxonomy of this genus.

The analysis of ATPase 6 fragment from *Zungaro* indicated a low magnitude of differences, when compared to D-loop sequences. This finding was expected *a priori* since D-loop region is highly variable. Therefore, both intra- and interspecific differences should be low (Avise, 2004). Even so, genetic distances between individuals from Paraná-Paraguay River and Amazon basins were of the same level as those of differentiate related species from other Siluriformes genera. In fact, other studies using mtDNA have reported similar levels of differentiation between related species (Oliveira et al., 2006; Samonte et al., 2007).

Even with a consistent genetic distance, there is a phylogenetic proximity between *Zungaro* individuals from the Paraná-Paraguay River and Amazon basins, due to equal nucleotidic sequences of $tRNA^{Pro}$ and $tRNA^{Thr}$ mitochondrial genes. This is an expected result for individuals of related species. In fact, identical sequences of $tRNA^{Pro}$ gene were found among A, C and D cytotypes of *Hoplias* aff. *malabaricus* (Perioto, 2004). It is clear that the cytotypes of *H.* aff. *malabaricus* are distinct, although undescribed, species (Perioto, 2004).

The genetic differentiation and high morphologic similarity of *Zungaro* individuals analyzed here can be explained by the origin of the populations. The separation of both the Paraná-Paraguay River and Amazon basins occurred in Late Miocene, in the last 10 Ma (Hubert and Renno, 2006). Therefore, during the formation of the two basins, the definitive separation of two *Zungaro* populations was possible through vicariance. After this event, genetic differentiation would initiate. However, there is some capture of *Zungaro* individuals in the source streams of the Paraná-Paraguay River and Amazon basins (Räsänen et al., 1995), where little and temporary communication exists even after the establishment of these two macro-basins. Therefore, populations could possibly migrate across basins during wet seasons. An original population of the Amazon basin could have colonized the Paraná-Paraguay River basin after dispersion via source streams. However, at some specific time, genetic flux between *Zungaro* populations must have been interrupted, and populations from different basins were isolated through vicariance. This hypothesis seems possible given that accumulated genetic differences are, apparently, in much lower levels than those expected for a period of 10 Ma, corresponding to the separation of the Paraná-Paraguay River and Amazon basins (Hubert and Renno, 2006).

Independently of the mechanism, the magnitude of differences found between individuals suggests that *Zungaro* populations from the Paraná-Paraguay River and Amazon basins have been geographically isolated for a sufficient amount of time for speciation to occur. The accentuated geographic pattern in distribution of the haplotypes was evidenced in all constructed trees relating individuals from the two basins. Therefore, the suspicion that the *Zungaro* species from the Paraná-Paraguay River basin does not identify with the species encountered in the Amazon basin deserves a detailed taxonomic investigation. Our findings are extremely relevant, since this fish is highly threatened in the Paraná-Paraguay River basin (Okada et al., 2005; Alves, 2006), and the introduction of *Zungaro* individuals from the Amazon basin may not be a valid re-population effort. If *Zungaro* from Amazon is introduced in the Paraná-Paraguay River basin, native populations can decay even more due to competition. Moreover, given that *Zungaro* from different River basins seems to be closely related species, hybrids can be generated, affecting the genetic structure of native populations of this fish. Therefore, conservation efforts should be urgently improved due to the possible existence of

an endemic and highly threatened species of *Zungaro* in the Paraná-Paraguay River basin. In fact, the recent impoundments in this basin are contributing to the rapid population decrease of migratory fish, such as *Zungaro* species (Okada et al., 2005; Pelicice and Agostinho, 2008). When hydropower dams are constructed, fish ladders can not be fully effective to conserve populations of migratory fish (Pelicice and Agostinho, 2008). The protection and restoration of critical habitats in those sub-basins of the Paraná-Paraguay River basin still free from damming seems to be the best conservation strategy (Pelicice and Agostinho, 2008). Furthermore, other studies on the genetic diversity of this species within locations of the Paraná-Paraguay River basin are fundamental to subsidize management efforts aiming at the recovery of natural populations of *Zungaro* species.

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