

Genetic divergence between *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum* (Siluriformes: Pimelodidae) in the Paraná River Basin

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Abstract

Pseudoplatystoma corruscans (Spix and Agassiz, 1829) and *Pseudoplatystoma reticulatum* (Eigenmann and Eigenmann, 1889) are large migratory catfishes of high biological importance and great commercial value in South America. Because fertile crossbreeds can be artificially produced in hatcheries, a high genetic proximity between these two Pimelodidae species is conceivable. Possible escape of crossbred specimens from pisciculture stations is a serious environmental concern. Despite their importance, knowledge of *P. corruscans* and *P. reticulatum* biology, ecology, population diversity and genetics is limited. In the present work, the genetic divergence between *P. corruscans* and *P. reticulatum* populations from the Paraná River Basin was analyzed on the basis of polymorphisms in ISSR fragments and in the hypervariable sequence of the mitochondrial DNA (mtDNA) control region. Estimates of intraspecific haplotype ($h > 0.5$) and nucleotide diversities ($\pi < 0.01$) indicate that *P. corruscans* and *P. reticulatum* have survived a historical population decline, followed by a demographic expansion. The interspecific polymorphisms within the mtDNA control region and ISSR fragments were suitable as diagnostic molecular markers and could be used to discriminate the two species. A unique *Pseudoplatystoma* specimen, captured in the Upper Paraná River Floodplain, was identified by these DNA diagnostic markers as a hybrid *P. reticulatum* × *P. corruscans*, which possibly escaped from pisciculture. The integrity of the natural population of *P. corruscans* in the Upper Paraná River is at risk of genetic introgression or homogenization due to the presence of hybrids and the transposition of *P. reticulatum* upstream through the Canal da Piracema at Itaipu Dam. Data presented herein improve the understanding of the genetic relatedness between *P. corruscans* and *P. reticulatum* and represent potential tools for future programs of conservation and surveillance of genetic introgression events and the genetic integrity of these populations.

Keywords: *Pseudoplatystoma*, interspecific hybrid, D-loop, ISSR, Upper Paraná River.

Divergência genética entre *Pseudoplatystoma corruscans* e *Pseudoplatystoma reticulatum* (Pisces; Siluriformes) da Bacia do Rio Paraná

Resumo

Pseudoplatystoma corruscans Spix e Agassiz, 1829 e *Pseudoplatystoma reticulatum* Eigenmann e Eigenmann, 1889 são peixes migratórios de grande porte, com alta importância biológica e elevado valor comercial na América do Sul. Híbridos férteis são obtidos em cativeiro e, portanto, é esperada alta proximidade genética entre essas duas espécies de Pimelodidae. Escapes de espécimes híbridos a partir de estações de piscicultura representam um sério problema ambiental. Apesar da sua importância, conhecimentos sobre a biologia, ecologia, diversidade de populações e genética de *P. corruscans* e *P. reticulatum* são escassos. No presente trabalho, foi avaliada a divergência genética entre *P. corruscans* e *P. reticulatum* da Bacia do Rio Paraná, com base em fragmentos ISSR e na sequência *D-loop* do

DNA mitocondrial (mtDNA). As estimativas das diversidades intra-específicas haplotípica ($h > 0,5$) e nucleotídica ($\pi < 0,01$) evidenciaram que *P. corruscans* e *P. reticulatum* sobreviveram a um declínio populacional histórico, seguido de expansão demográfica. Os polimorfismos interespecíficos no mtDNA e nos fragmentos ISSR foram eficientes para diagnósticos e discriminaram as duas espécies. Um espécime de *Pseudoplatystoma* capturado na planície de inundação do Alto Rio Paraná foi identificado com esses marcadores moleculares como híbrido *P. reticulatum* × *P. corruscans*, que possivelmente escapou de psicicultura. A integridade da população de *P. corruscans* no Alto Rio Paraná está ameaçada, por introgressão ou homogeneização genética, pela presença de híbridos e pela transposição para montante de *P. reticulatum* através do Canal da Piracema em Itaipu. Os dados apresentados constituem um avanço na compreensão do parentesco entre *P. corruscans* e *P. reticulatum* e representam ferramentas em potencial para programas de conservação biológica, incluindo o monitoramento de introgressão e de integridade genética das populações.

Palavras-chave: *Pseudoplatystoma*, híbrido interespecífico, *D-loop*, ISSR, Alto Rio Paraná.

1. Introduction

The Pimelodidae catfishes *Pseudoplatystoma corruscans* (Spix and Agassiz, 1829), popularly known as 'pintado' or 'spotted sorubim', and *Pseudoplatystoma reticulatum* (Eigenmann and Eigenmann, 1889), known as 'cachara' or 'barred sorubim', are among the largest fishes in the main South American hydrographic basins. These abundant migratory fishes are important predators in these basins. Buitrago-Suárez and Burr (2007) recognized that the *Pseudoplatystoma* genus contains eight species. *P. reticulatum*, formerly *P. fasciatum*, is distributed throughout the Amazon and Paraná River Basins, while *P. fasciatum* is restricted to the Guyana region.

In nature, *P. corruscans* and *P. reticulatum* coexist only in part of the Paraná hydrographic basin, at the Middle Paraná River Basin, including the Paraguay River sub-basin (Buitrago-Suárez and Burr, 2007). The genetic proximity between *P. corruscans* and *P. reticulatum* has been inferred primarily based on their known ability to generate fertile hybrids upon the induction of interspecific crosses in hatcheries. Because of their large size and remarkably appreciated meat quality, both species are of high commercial value and are greatly appreciated for sport fishery. Despite their biological and fishery importance, knowledge of the biology, ecology and genetics of *P. corruscans* and *P. reticulatum* is still quite limited.

The *P. corruscans* and *P. reticulatum* catfishes have been increasingly threatened by rapid and drastic environmental changes. Consequently, there has been a severe decline in the reproduction and capture of both species (Petrere-Jr. et al., 2002). Hence, there is now a greater interest in their reproduction in hatcheries for commercial purposes (Sato et al., 1997). Moreover, interspecific hybrids between *P. corruscans* and *P. reticulatum* can be generated in hatcheries, and eventual escapes into a natural environment are likely to occur. Particularly in the case of fertile hybrids, such escapes can represent a serious threat to the genetic integrity of local populations due to the risk of backcrossing and gene introgression (Avisé, 2004). In the Upper Paraná River Basin, where the *P. reticulatum* species does not naturally exist, escapes of *P. reticulatum* × *P. corruscans* fertile hybrids could be particularly menacing. Furthermore, the building of dams in the Upper Paraná River Basin has rapidly

isolated fish populations, and this is especially threatening to the dispersion of species that undergo long-distance reproduction migrations, such as *P. corruscans*. Therefore, monitoring of populations is becoming essential for preserving both *Pseudoplatystoma* species.

Molecular markers are fundamental for monitoring population genetic variability and for the surveillance of populations that are potentially at risk of losing their genetic integrity. In addition, they represent an important tool for studies of the genetic diversity and evolution of fishes. The availability of nuclear and mitochondrial DNA markers that are easily accessible and suitable for characterizing and discriminating *P. corruscans* and *P. reticulatum* is a fundamental requirement in the study and supervision of these populations. Moreover, these molecular markers could enable a better understanding of the genetic relatedness of these two species.

ISSR (Inter Simple Sequence Repeats) fingerprinting is a feasible molecular tool that does not require previous sequencing knowledge (Reddy et al., 2002). This technique uses a single primer containing the repetitive sequence of a microsatellite. The amplified DNA segment includes the nucleotide sequence situated between two microsatellites blocks, yielding a multilocus marker system useful for genetic diversity analysis (Maltagliati et al., 2006). The variable lengths of these amplified DNA sequences allow for the identification of differences between closely related species, although they reveal little information about the genetic variability within a single species (Reddy et al., 2002). In addition, the hypervariable domain of the control region (D-loop) in the mitochondrial genome has been a sequence of choice for taxonomic and genetic studies of populations and closely related vertebrates species, including fishes (Aboim et al., 2005; Oliveira et al., 2006). These nuclear and mitochondrial markers would be useful in evaluating genetic divergence between *P. corruscans* and *P. reticulatum* and could provide diagnostic loci for identifying first or advanced generations of interspecific hybrids.

The objective of the present work was to analyze the genetic divergence between *P. corruscans* and *P. reticulatum* populations from the Paraná River Basin and to identify diagnostic DNA markers suitable for

the discrimination of these two species. This proposed characterization could improve the understanding of the genetic relatedness between these *Pseudoplatystoma* species and may provide additional tools for future evaluations of hybridization and genetic introgression as well as for monitoring the genetic integrity of populations.

2. Materials and Methods

2.1. Fish sampling and DNA extraction

As shown in Figure 1, specimens of *Pseudoplatystoma corruscans* were sampled in the northern Paraguay River sub-basin (location 1) and in the floodplain of the Upper Paraná River Basin (location 2). *Pseudoplatystoma reticulatum* was sampled only in the northern Paraguay River sub-basin, as it does not exist in the Upper Paraná. In this study, a unique specimen of *Pseudoplatystoma* (2-PHybr1), which was characterized by concomitant patterns of spots and striped skin color, was also included. The 2-PHybr1 specimen was captured in nature, at the floodplain of the Upper Paraná River. Because of its morphological traits, which were intermediate between both species, the 2-PHybr1 specimen was hypothesized to be an interspecific hybrid of *P. reticulatum* and *P. corruscans*. Fish specimens were captured with gill-nets. Tissues were obtained from the adipose fins of the *P. reticulatum* and *P. corruscans* samples and from the muscles of the 2-PHybr1 specimen. Tissues were fixed

in ethanol and stored at -20°C . Samples ($\sim 100\text{ mg}$) were then macerated in liquid nitrogen and total DNA was extracted according to Whitmore et al. (1992), with few modifications (Prioli et al., 2002).

2.2. Amplification and analysis of ISSR loci

DNA fragments were amplified by PCR of total DNA samples using the primers $(\text{GGAC})_3\text{T}$, $(\text{GGAC})_3\text{C}$ and $(\text{GGAC})_4$ as previously described (Gupta et al., 1994), with few modifications. The ISSR analysis included 19 specimens of *P. corruscans*, 18 specimens of *P. reticulatum* (both from the northern Paraguay River sub-basin) and the 2-PHybr1 specimen. The PCR reaction mix consisted of Tris-KCl (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, 1.0 U *Taq* DNA polymerase (Invitrogen), 15 ng DNA and sterilized water to a total volume of 13 μL . PCR amplification was performed in 5 cycles of 94°C for 45 seconds, 51°C for 1 minute and 72°C for 1 minute, immediately followed by 30 cycles of 94°C for 45 seconds, 50°C for 1 minute, 72°C for 1 minute. PCR amplification reproducibility was tested in at least two independent reactions. In addition, one to two samples of PCR products, previously amplified and analyzed with the same primer, were included in each agarose gel. A sample consisting of the PCR reaction mix without DNA was included as a negative control in each experiment. PCR amplified DNA fragments were separated by electrophoresis in an agarose gel (1.4%), which

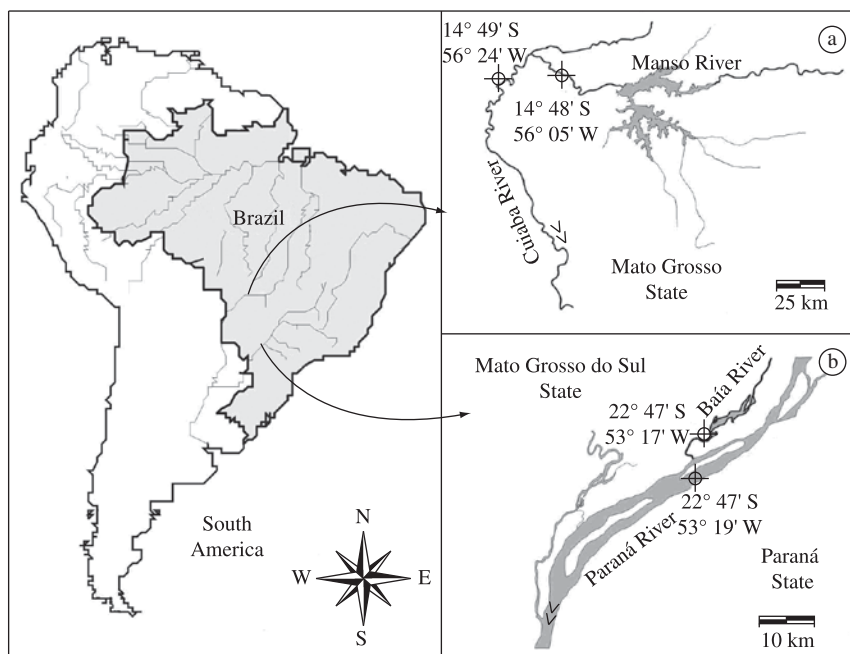


Figure 1. Map displaying the sampling locations from which *Pseudoplatystoma* specimens were obtained. At Location a, situated in the northern Paraguay River Basin, *P. corruscans* and *P. reticulatum* were sampled in the Manso and Cuiabá Rivers. At Location b, situated in the floodplain of the Paraná River Basin, *P. corruscans* was sampled in the Baía River and the 2-PHybr1 specimen was captured in the Paraná River.

was stained with ethidium bromide (20 µg/100 mL), at 5 V.cm⁻¹ for 4-5 hours. Electrophoresis profiles were visualized with UV irradiation and photographed with a Kodak EDAS-290. Analysis of ISSR fragment polymorphisms was based on the presence or absence of accurate steady DNA bands on the agarose gel. Specimens were compared within and among populations. The Nei and Li genetic similarity was estimated using NTSYS 1.7 and was utilized in both neighbor-joining clustering and principal coordinates analysis. Indexes of molecular diversity F_{ST} were estimated based on the average genetic diversity over all haplotype loci using Arlequin v. 3.11.

2.3. Sequencing and analysis of mitochondrial DNA

A fragment of mtDNA, approximately 460 base pairs (bp) in length, was PCR amplified from DNA samples of seven *P. corruscans* specimens from the Paraguay River sub-basin and 15 specimens from the Upper Paraná River Basin, of seven *P. reticulatum* specimens, and of the PHybr-1 specimen. For PCR, the D-loop L 5'-AGAGCGTCCGGTCTTGTAAC-3' and H16498 5'-CCTGAAGTAGGAACAGATG-3' primers were used. The PCR reactions were performed according to Prioli et al. (2002) and DNA sequencing reactions were performed according to Oliveira et al. (2006). For each specimen, the fragment was amplified in two independent reactions and then bi-directionally sequenced. Quality check, assembly and alignment of DNA sequences were performed using Vector NTI Suite 6.0 (Informax Inc/Invitrogen Life Technologies™). DNA sequences were aligned using CLUSTALW. The HKY+I evolutionary model on MODELTEST 3.7 was used to assess the phylogenetic relationships among the mitochondrial haplotypes. Analyses were based on the maximum likelihood and neighbor-joining methods using the programs PHYML and PAUP* 4.0b10, respectively. The haplotype diversity (h) and the nucleotide diversity (π) of haplotypes were calculated on DNAsp 4.5. Using Arlequin 3.11, the genetic divergence between the *P. corruscans* populations in the Upper Paraná River and the Paraguay River basins were estimated by molecular F_{ST} and genetic variability partitioning was tested by analysis of molecular variance.

3. Results

The amplified ISSR nuclear DNA fragments and the mtDNA sequences were analyzed in the *P. corruscans* and *P. reticulatum* populations and in the 2-PHybr1 specimen. The ISSR analysis included the 28 most intense, well-defined and repeatable DNA fragments, which varied from 400 bp to 2 kb. Of those 28 loci, six (21.4%) were monomorphic and exclusive to *P. corruscans*, and seven (25%) were monomorphic and exclusive to *P. reticulatum* (Table 1).

Therefore, 46.4% of the analyzed ISSR fragments could be used to discriminate *P. corruscans* and *P. reticulatum*, clearly excluding the other species. This

suggests that these ISSR fragments may be used as diagnostic molecular markers (Table 1). In addition, both species shared nine (32.1%) monomorphic and two (7.1%) polymorphic loci. There were two polymorphic loci exclusive to *P. reticulatum*, one locus was polymorphic in *P. corruscans* and monomorphic in *P. reticulatum*, and one locus was identified as monomorphic in *P. corruscans* and polymorphic in *P. reticulatum*. In the 2-PHybr1 specimen, the ISSR primers amplified 22 DNA fragments, of which three fragments were exclusive to *P. corruscans* and five were exclusive to *P. reticulatum* (Table 1). An electrophoresis profile of fragments generated with the (GGAC)₃T primer is illustrated in Figure 2.

The genetic differentiation pattern based on the Nei and Li genetic distance is illustrated by the dendrogram in Figure 3, and is complemented by the principal coordinates graph (Figure 4). The *P. corruscans* and *P. reticulatum* species were grouped in two distinct major clusters, which were clearly separated from each other. The 2-PHybr1 specimen displayed an intermediate position between the two species, with a small deviation toward the *P. reticulatum* side. The relative position of 2-PHybr1 to the *P. reticulatum* species indicates that this specimen contains a higher number of *P. reticulatum* ISSR fragments.

The intra-population haplotype molecular diversity index, based on the average gene diversity over all haplotype loci, was estimated to be 0.0413 (± 0.0312) for *P. corruscans* and 0.0726 (± 0.0478) for *P. reticulatum*. Therefore, based on the ISSR markers, the *P. corruscans* population is genetically more homogenous than the *P. reticulatum* population. Several bootstrap values were higher than 0.5 in the *P. reticulatum* species, indicating a rate of polymorphism higher than that of *P. corruscans* (Figure 3). This was also evident in the principal coordinate analysis (Figure 4). The estimate of the haplotype molecular diversity index increased to 0.3269 (± 0.1710) in the *P. corruscans* and *P. reticulatum* specimens when analyzed together as a group. The molecular fixation index F_{ST} , based on ISSR data, was estimated to be 0.9031 by comparing pairs of *P. corruscans* and *P. reticulatum* population samples. The Nei and Li genetic distance estimates, as expressed by the similarity complement, varied from zero to 0.51 at the individual level. Based on ISSR fragments, the Nei and Li genetic distance between *P. corruscans* and *P. reticulatum* populations was 0.3659, a value that would be expected for distinct, yet closely related, species. The Nei and Li genetic distance was estimated to be 0.2195 between *P. corruscans* and the 2-PHybr1 specimen, and 0.1364 between *P. reticulatum* and 2-PHybr1. Therefore, numbers of the ISSR fragments derived from each of the *Pseudoplatystoma* species were unequal in the 2-PHybr1 specimen.

The amplified mtDNA fragment (~470 bp) consisted of a partial sequence of the *tRNA^{Thr}* gene, the complete sequence of the *tRNA^{Phe}* gene, and 360 bp comprising the hypervariable domain of the control region (GenBank accession numbers FJ024050 to FJ024079). The number

Table 1. Approximate length in base pairs (bp) of ISSR fragments of *P. corruscans*, *P. reticulatum* and the hybrid specimen 2-PHybr1. The presence or absence of a DNA fragment in the agarose gel is indicated by + or –, respectively. The DNA fragment in a species can be monomorphic (m) or polymorphic (p).

		Primer (GGAC) ₄										
Fragments (~bp)	2000	1800	1500	1100	800	780	700	650	600	550	450	
<i>P. corruscans</i>	+p	–	+p	+m	+m	–	–	+m	–	+m	+m	
<i>P. reticulatum</i>	+m	+p	+p	+m	–	+m	+m	–	+m	+m	+m	
Hybrid	+	+	+	+	+	–	+	–	+	+	+	
		Primer (GGAC) ₃ C										
Fragments (~bp)	1800	1500	1100	1000	900	800	600	500	450	–	–	
<i>P. corruscans</i>	+m	+p	+m	+m	+m	+m	–	+m	+m	–	–	
<i>P. reticulatum</i>	+p	+p	+m	–	+m	+m	+m	–	+m	–	–	
Hybrid	+	+	+	+	+	+	+	–	+	–	–	
		Primer (GGAC) ₃ T										
Fragments (~bp)	2000	1700	1300	800	650	550	450	400	–	–	–	
<i>P. corruscans</i>	–	–	+m	+m	–	+m	+m	–	–	–	–	
<i>P. reticulatum</i>	+m	+m	–	+m	+m	+m	–	+m	–	–	–	
Hybrid	+	–	–	+	+	+	+	–	–	–	–	

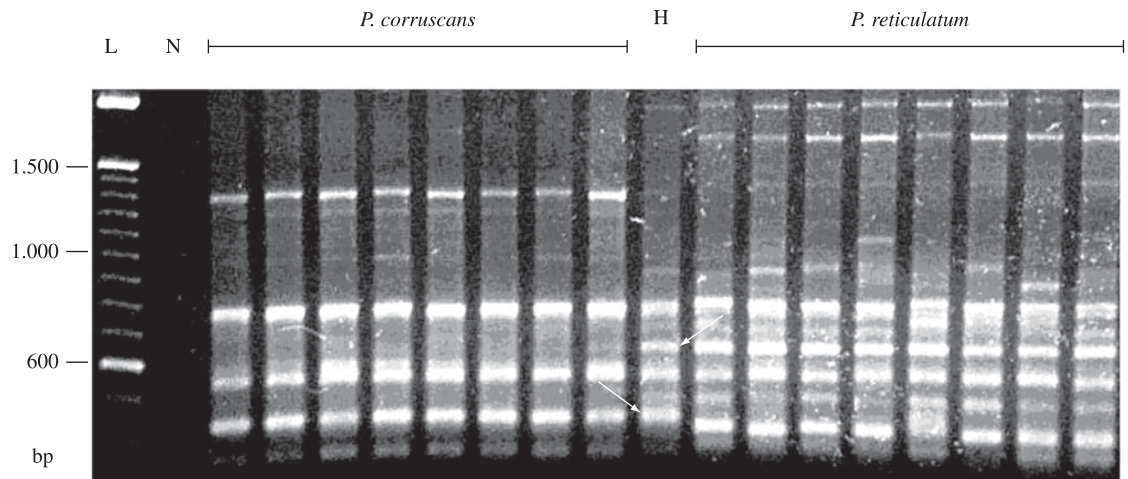


Figure 2. Electrophoresis profiles, obtained with the ISSR primer (GGAC)₃T, of *P. corruscans* and *P. reticulatum* from the northern Paraguay sub-basin. Lane H, *P. reticulatum* × *P. corruscans* hybrid (2-PHybr1); Lane N, negative control without DNA; Lane L, molecular mass markers (Ladder 100 bp). Arrows indicate ISSR fragments exclusive to either *P. corruscans* (left) or *P. reticulatum* (right), which were inherited by the hybrid.

of nucleotide polymorphisms was low among the *tRNA* sequences of *P. corruscans* and *P. reticulatum*. These sequences were therefore not informative and were not included in the analysis. The analyzed mitochondrial control region was rich in A-T nucleotides, which is typical of D-loop mtDNA sequences. The region consisted of: T = 33.8, C = 17.7, A = 36.5 and G = 11.9. As shown in Table 2, the CLUSTALW alignment of the mtDNA hypervariable sequence of the control region revealed 41 polymorphic nucleotide sites, which were distributed in 16 haplotypes. The haplotypes were separated into two

haplogroups. As is characteristic of the mtDNA control region, most changes were single nucleotide substitutions, with transitions being the most frequent type ($R = si/sv = 3.3$). Of the 16 haplotypes identified, nine were from *P. corruscans* ($h = 0.814$; $\pi = 0.007$) and seven were from *P. reticulatum* ($h = 0.952$; $\pi = 0.007$). The two *Pseudoplatystoma* species were discriminated from each other by a total of 19 nucleotide sites. The mtDNA control region of the 2-PHybr-1 specimen was similar to that of the *P. reticulatum* species (Table 2).

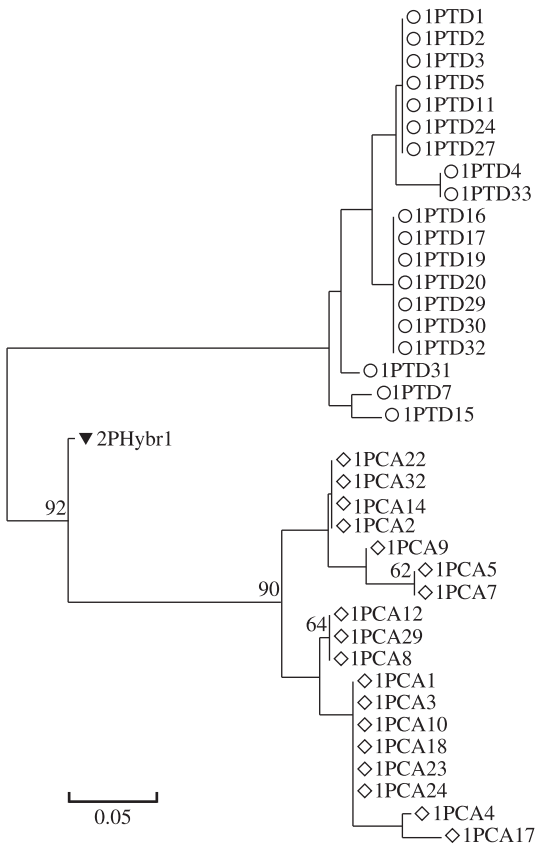


Figure 3. Neighbor-joining dendrogram based on Nei and Li similarity coefficients obtained from ISSR markers of *Pseudoplatystoma*: (○) *P. corruscans* and (◇) *P. reticulatum* from the northern Paraguay Basin; (▼) *P. reticulatum* × *P. corruscans* hybrid captured in the Upper Paraná Basin. Numbers above branches are bootstrap values based on 1,000 replicates.

The genetic divergence between the two haplogroups was examined by phylogenetic analysis. Using the model HKY + I and the parameters Nst = 2, TRatio = 5.3882, Rates = equal and Pinvar = 0.7345, maximum likelihood and neighbor-joining trees were created (Figure 5). The two haplotype groups represented by *P. corruscans* and *P. reticulatum* were supported by a 100% bootstrap rate. The 2-PHYbr1 haplotype was included in the *P. reticulatum* group. The intraspecific HKY + I genetic distance was estimated to be 0.007 between the *P. corruscans* and *P. reticulatum* species. The interspecific distance was 0.085, nearly eight times larger than the intraspecific distance.

4. Discussion

The genetic divergence between *P. corruscans* and *P. reticulatum* was estimated using ISSR and mtDNA polymorphisms. Monomorphic ISSR fragments exclusive to each species, consisting of 46.4% of the analyzed

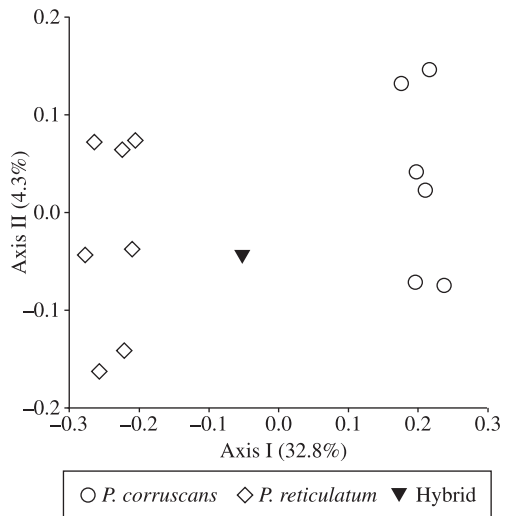


Figure 4. Dispersion in principal coordinates analysis (PCO) based on complements of Nei and Li similarity from ISSR markers of *Pseudoplatystoma*: (○) *P. corruscans* and (◇) *P. reticulatum* from the northern Paraguay Basin; (▼) *P. reticulatum* × *P. corruscans* hybrid captured in the Upper Paraná Basin.

fragments, and 19 polymorphic nucleotide sites of the mtDNA control region clearly discriminated the two closely related *P. corruscans* and *P. reticulatum* species. These two species both have 2n = 54 chromosomes, but differences were detected in the nucleolar organizer regions (Porto-Foresti et al., 2007). The genetic parameters obtained with D-loop and ISSR indicate that the two *Pseudoplatystoma* species are genetically differentiated to an extent that seems consistent with distinct, yet closely related species. As the efficiency of the ISSR technique in the characterization of closely related fish species has been demonstrated, these results are accurate and reliable (Maltagliati et al., 2006).

The monomorphic and exclusive ISSR fragments could be used as molecular diagnostic markers capable of discriminating these two species. The ISSR data are in agreement with a previous study that identified many steady, monomorphic RAPD fragments exclusive to either *P. corruscans* or to *P. reticulatum* (Prioli et al., unpublished data). The genetic differentiation pattern demonstrated that *P. corruscans* and *P. reticulatum* have accumulated DNA polymorphisms in their nuclear and mitochondrial genomes, which were suitable for characterizing each species and useful as diagnostic DNA markers.

In contrast, because *P. corruscans* and *P. reticulatum* generate potentially fertile crossbreds in hatcheries, it is reasonable to assume that they must share a high degree of chromosomal (Porto-Foresti et al., 2007) and genomic similarities. Such genetic proximity between *P. corruscans* and *P. reticulatum* is evident as 32.1% of the monomorphic ISSR fragments were present in both species, 88.6% identity was found in the analyzed sequences of the differ-

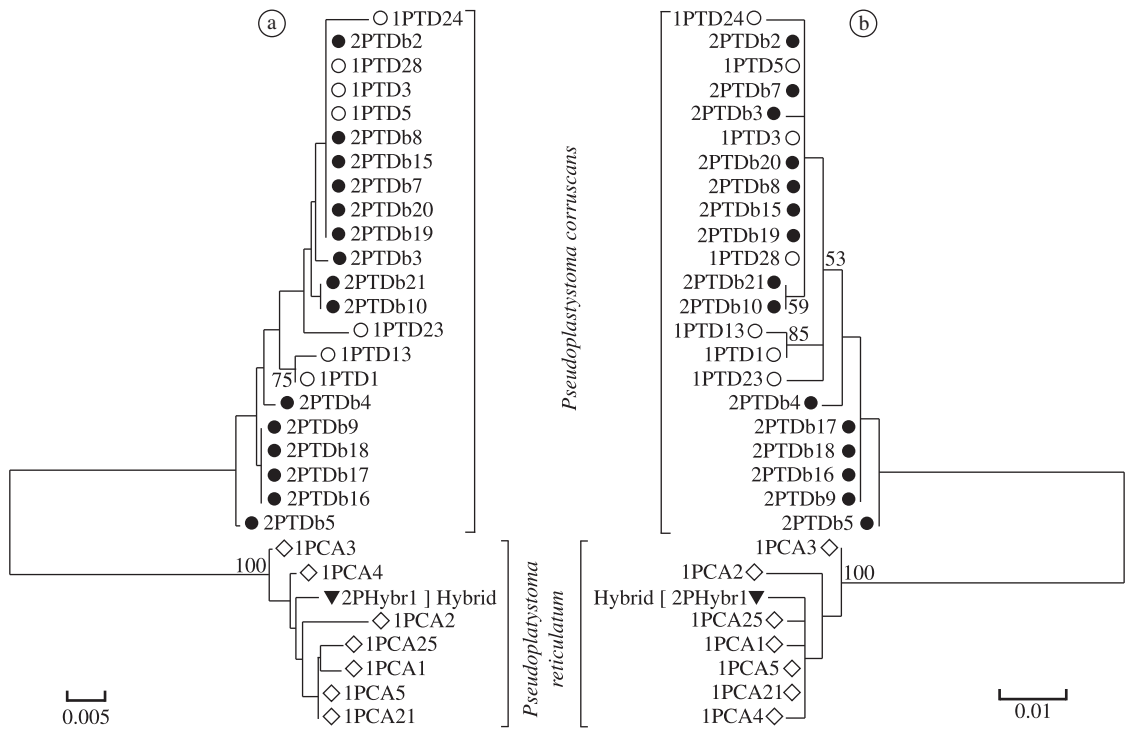


Figure 5. a) Neighbor-joining; and b) maximum likelihood dendrograms, obtained with the HKY + I model, from the hypervariable domain of the mtDNA control region (D-loop) haplotypes: (○) *P. corruscans* and (◇) *P. reticulatum* from the northern Paraguay River sub-basin; (●) *P. corruscans* from the Upper Paraná Basin; (▼) *P. reticulatum* × *P. corruscans* hybrid. Numbers above branches are bootstrap values based on 1,000 replicates.

ent mtDNA control regions and low nucleotide variation was observed in the tRNA sequences. In addition, NTS 5S rDNA markers are homogenous between these two species (Prioli et al., unpublished data).

The presence of both monomorphic ISSR fragments exclusive to *P. corruscans* or to *P. reticulatum* in the 2-PHybr1 specimen demonstrated that this individual is likely a hybrid between these two species. The data reject the possibility that the 2-PHybr1 specimen could be a hybrid between *P. corruscans* and another Amazonian *Pseudoplatystoma* species. Analysis of the 2-PHybr1 specimen shows that ISSR markers are also suitable for recognizing *P. reticulatum* × *P. corruscans* hybrids.

The mtDNA data was unequivocal in demonstrating that the *P. reticulatum* species was the mother in the interspecific cross that generated the 2-PHybr1 specimen. Cytogenetic analysis was also shown to be useful for identifying *P. reticulatum* × *P. corruscans* hybrids whose mothers were *P. reticulatum* (Porto-Foresti et al., 2007). In the present study, the unequal genetic distances between the 2-PHybr1 specimen and the parental species indicate that this hybrid is likely not a first generation descendent (Figures 2 and 3). This inference could be verified with further analysis of more ISSR markers.

The *Pseudoplatystoma corruscans* and *P. reticulatum* populations showed high values of haplotype diversity (*h*) and low values of nucleotide diversity (π). According

to Grant and Bowen (1998), a combination of $h > 0.5$ with $\pi < 0.01$ indicates a recent reduction of the population followed by a demographic expansion. For reasons unknown, the *P. corruscans* and *P. reticulatum* species were apparently reduced to one or a few related haplotypes. Most likely, these two species independently survived a severe populational decline. Coronel et al. (2004) also found evidence of a reduction in a *P. reticulatum* (formerly *P. fasciatum*) population of the Bolivian Amazon region. Presently, the genetic integrity of the *P. corruscans* and *P. reticulatum* natural populations of the Paraná/Paraguay hydrographic basin could be endangered by reciprocal genetic introgression.

The 2-PHybr1 specimen was captured in the floodplain of the Upper Paraná River Basin (Luz et al., 2004). In the Cuiabá River Basin, where the two species occur in sympatry, a *P. reticulatum* × *P. corruscans* hybrid was registered by Veríssimo et al. (2005). The occurrence of these hybrids in the Paraná and Manso rivers likely results from fishes escaping from farming stations. Accidental introduction of *Pseudoplatystoma* interspecific hybrids into a natural environment could be a threat to the genetic integrity of local populations due to the potential risk of backcrossing and subsequent gene introgression. This threat would be even more drastic in the Upper Paraná River Basin, where, due to geographic isolation, the *P. reticulatum* species does not exist naturally.

Table 2. Nucleotide polymorphisms (41 sites) in the hypervariable sequence (~360 bp) of the mtDNA control region (D-loop) in *Pseudoplatystoma* populations. Sampling locations (1 or 2) are indicated by the first number in each specimen identification: 1. Upper Paraná River Basin, 2. Paraguay River sub-basin. Haplotypes: Hapl-PTD = *P. corruscans*; Hapl-PCA = *P. reticulatum*. Entire sequences can be found at GenBank at accession numbers FJ024050 to FJ024079.

Specimen	Haplotypes	Identification	00111111578889999911111111111122223333333333 34234581315715678012233356674779012233455 002556752315084843423035
2-PTD3	Hapl-PTD-I	<i>P. corruscans</i>	GCTATGTATTACCCAAATTACCAA-CCAATAAGATCTACCA
2-PTD28	Hapl-PTD-I	<i>P. corruscans</i>
2-PTD5	Hapl-PTD-I	<i>P. corruscans</i>
1-PTDb2	Hapl-PTD-I	<i>P. corruscans</i>
1-PTDb19	Hapl-PTD-I	<i>P. corruscans</i>
1-PTDb20	Hapl-PTD-I	<i>P. corruscans</i>
1-PTDb7	Hapl-PTD-I	<i>P. corruscans</i>
1-PTDb8	Hapl-PTD-I	<i>P. corruscans</i>
1-PTDb15	Hapl-PTD-I	<i>P. corruscans</i>
2-PTD24	Hapl-PTD-II	<i>P. corruscans</i>C.....-...T.....
1-PTDb3	Hapl-PTD-III	<i>P. corruscans</i>T.....-.....
1-PTDb10	Hapl-PTD-IV	<i>P. corruscans</i>T.....-.....
1-PTDb21	Hapl-PTD-IV	<i>P. corruscans</i>T.....-.....
2-PTD23	Hapl-PTD-V	<i>P. corruscans</i>G.....G-...C.....
2-PTD1	Hapl-PTD-VI	<i>P. corruscans</i>	A.....G.T.....-.....
2-PTD13	Hapl-PTD-VII	<i>P. corruscans</i>	A.....G.T.....-T.....
1-PTDb4	Hapl-PTD-VIII	<i>P. corruscans</i>G.....-T...G.....
1-PTDb9	Hapl-PTD-IX	<i>P. corruscans</i>	.T.....G.....-T.....
1-PTDb16	Hapl-PTD-IX	<i>P. corruscans</i>	.T.....G.....-T.....
1-PTDb17	Hapl-PTD-IX	<i>P. corruscans</i>	.T.....G.....-T.....
1-PTDb18	Hapl-PTD-IX	<i>P. corruscans</i>	.T.....G.....-T.....
1-PTDb5	Hapl-PTD-IX	<i>P. corruscans</i>	.T.....G.....-T.....
2-PCA1	Hapl-PCA-I	<i>P. reticulatum</i>	ATCTATCG.CGTTTG.TC.GTTG.T.TT..GG..C.CTTGG
2-PCA25	Hapl-PCA-II	<i>P. reticulatum</i>	ATCTATCG.CGTTTGTC..TTG.T.TT..GG..C.CTTGG
2-PCA5	Hapl-PCA-III	<i>P. reticulatum</i>	ATCTATCG.CGTTTG.TC..TTG.T.TT..GG..C.CTTGG
2-PCA21	Hapl-PCA-III	<i>P. reticulatum</i>	ATCTATCG.CGTTTG.TC..TTG.T.TT..GG..C.CTTGG
2-PCA4	Hapl-PCA-IV	<i>P. reticulatum</i>	ATCTATCG.CGTTTG.TC..TTG.T.TT..GG...CTTGG
1-PHybr1	Hapl-PCA-V	Hybrid	ATCTATCG.CGTTTG.T.G.TTG.T.TT..GG..C.CTTGG
2-PCA2	Hapl-PCA-VI	<i>P. reticulatum</i>	ATCTATC..CGTTTG.TC..TTG.T.TT..GGAGCTCTTGG
2-PCA3	Hapl-PCA-VII	<i>P. reticulatum</i>	ATCTATC..CGTTT..TC..TTG.T.TT..GG..C.CTTGG

The Canal da Piracema, a fish pass system recently built in the Itaipu Dam, could be allowing the introduction of *P. reticulatum* into the Upper Paraná River (Graça and Pavanelli, 2007; Makrakis et al., 2007), increasing the possibility of gene transfer between the two *Pseudoplatystoma* species. The free marketing of young *P. reticulatum* × *P. corruscans* hybrids represents an additional genetic risk. If relatively large numbers of *P. reticulatum* and hybrid specimens reach the Upper Paraná River, the *P. corruscans* native population will be exposed to the risk of local extinction by genetic homogenization and replacement by a population of inter-specific hybrids.

P. corruscans and *P. reticulatum* have accumulated ISSR and mtDNA nucleotide polymorphisms. These polymorphisms are sufficient for clearly discriminating the two species, for identifying a hybrid specimen captured in nature and as molecular diagnostic markers. These polymorphisms are therefore useful for genetic analysis, discrimination of these species and studies of threatened populations. Data presented herein improve the understanding of the genetic relatedness between the *P. corruscans* and *P. reticulatum* species. Furthermore, our results comprise potential tools for periodical evaluation of hybridization and genetic introgression or genetic homogenization, as well as for monitoring the genetic

integrity of populations and programs of biological conservation.

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