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ORIGINAL ARTICLE

Biology

Genetic variability of pure *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum* individuals in the Paraná and Paraguay River basins

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Abstract The presence of interspecific hybrid surubim (*Pseudoplatystoma corruscans* × *Pseudoplatystoma reticulatum*) in natural environments can put at risk the existence of the pure parental lineages. The aim of this study was to characterize the genetic variability of pure *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum* individuals from rivers in the Paraná and Paraguay basins in Brazil. Seventy-six pure individuals of *P. corruscans* and 16 of *P. reticulatum* were evaluated with six microsatellite loci for both species, along with one species-specific locus each for *P. corruscans* and *P. reticulatum*. Loss of heterozygosity was confirmed, and preservation measures for *P. corruscans* and *P. reticulatum* are needed in order not to lose further genetic variation. In addition, we confirmed that these markers are useful for the management of pure stocks in natural environments, for fish breeding, and in genetic conservation and improvement programs for these species.

Keywords Conservation · Crossbreeding · Fish · Microsatellite · Natural environment

Introduction

Freshwater fishes are responsible for 20–25 % of vertebrate biodiversity, and there is evidence that in South America more than 8000 species occur [1]. Catfishes are one of the most diverse freshwater groups, and the Pimelodidae family of catfishes, comprising more than 90 species and 30 genera [2], includes the genus *Pseudoplatystoma*, a group of migratory freshwater fishes of great commercial value [3]. *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum* are of particular economic importance in the regions where they occur, both in fishing and in captive production, owing to their palatability and the absence of intramuscular spines in their flesh [4].

The management of these species as fingerlings and through fattening is still a challenge, due to lack of knowledge of their environmental and physiological needs during growth, knowledge that is essential to improve their productive development. With respect to nutrition, for example, little is known about their nutritional demands and the digestibility of the food used in their diet, facts that could minimize the use of nutrients and reduce the costs of production, which are high [4].

Fish farmers have invested in the cultivation of interspecific hybrid surubim resulting from the cross of *P. corruscans* and *P. reticulatum*, aiming to take advantage of favorable characteristics of the parental species and improve development and the cultivation system [5–7]. These

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interspecific hybrid surubim can escape from breeding tanks or fishing ponds and invade the natural environment. The consequences of these escapes depend on the viability, performance, and reproductive capacity of the hybrids in nature. The hybrids can cause ecological instabilities and decrease the genetic integrity of the pure parental stock in the natural environment [8, 9] and, consequently, their genetic variability [10].

There are techniques for molecular identification that allow us to determine the genetic variability of a population of any species [11, 12]. Among these different techniques, microsatellite molecular markers have been considered proper molecular tools in the search for genetic variability [13, 14]. Microsatellite markers in fish have been used to study heterozygosity, the structure of populations with a focus on conservation and management, migration patterns, the effects of environmental fragmentation, reproductive systems, and phylogenetic relationships, among others factors [15, 16]. Before characterizing the genetic variability of pure populations; however, it important identify if the fishes are pure species or hybrids [17–19]. There have been studies of the genetic variability of *P. corruscans* and *P. reticulatum* using microsatellite molecular markers [12, 20, 21], but none have genetically identified the individuals in order to determine whether they were genetically pure before performing the study of genetic variability. This lack of genetic identification has occurred not only with *P. corruscans* and *P. reticulatum*, but also in studies of the "tambaqui" *Colossoma macropomum* [22, 23] and the "pacu" *Piaractus mesopotamicus* [24–27], which also have commercially produced hybrids. Against this background, our aim was to characterize the genetic variability of pure *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum* individuals in the Paraná and Paraguay River basins in Brazil. We believe these data are essential for the development of management policies and conservation practices for these species.

Materials and methods

Fish samples

Pseudoplatystoma corruscans and *Pseudoplatystoma reticulatum* were collected in 2007, 2008, 2011, and 2012 in Mato Grosso do Sul: the Dourados and Ivinhema Rivers (part of the Paraná River basin), and Negro, Aquidauana, Miranda, and Paraguay Rivers (part of the Paraguay River basin) (Fig. 1). They were identified by fishermen of Colônia de Pescadores Artesanais Profissionais "Z-10" of Fátima do Sul/MS and researchers of University Estadual of Mato Grosso do Sul (UEMS) according to the difference of phenotypic features. The caudal fin was collected for DNA analyses.

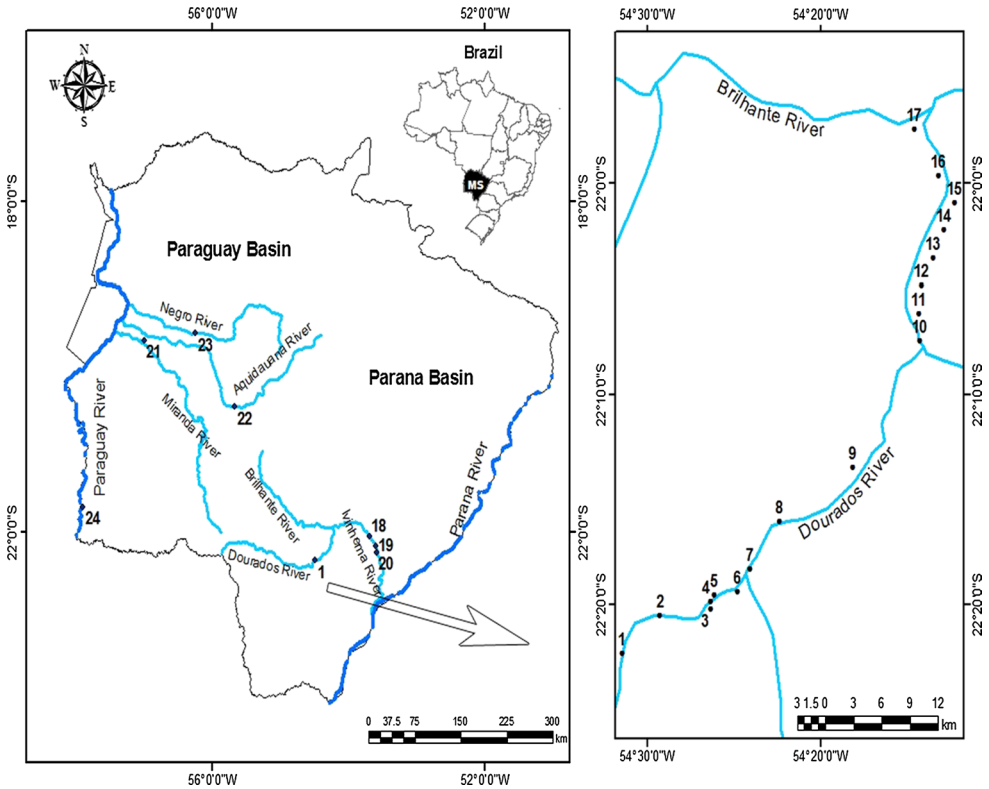

Fig. 1 Map of the biological material collection sites. Basin of Paraná River: Dourados River (*1–16*), Ivinhema River (*18–20*). Basin of Paraguay River: Miranda River (*21*), Aquidauana River (*22*), Negro River (*23*), Paraguay River (*24*), Vaini et al. [19]

Genetic identification

The DNA extraction process, multiplex-PCR, and PCR–RFLP using RAG2, GLOBINA, EF1α, and 18S nuclear genes, as well as the 16S rRNA mitochondrial gene, were performed in Vaini et al. [19]. For this study, only *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum* identified as genetically pure were used to evaluate the genetic diversity with microsatellite molecular markers.

Genetic variability

Genetic variability was evaluated with 76 pure individuals of *P. corruscans* and 16 pure individuals of *P. reticulatum*, using six microsatellite molecular markers common to both species (Pcor 01, Pcor 05, Pcor 10, Pcor 21, Pcor 23, and Pcor 28) as well as two species-specific markers (Pcor 02 specific for *P. corruscans* and Pcor08 specific for *P. reticulatum*) (Table 1), as previously described [12, 21].

The amplification reactions were done in a final volume of 25.0 µl, and the mix of each microsatellite locus consisted of 7.5 µl of ultra-pure water (Fermentas, Waltham, MA, USA), 0.15 µM of forward and reverse primer, 12.5 µl of PCR Master MIX (50 U/ml of Taq polymerase DNA, 400 µM of dNTP, and 3 mM of $MgCl_2$) (Fermentas) and 20 ng of DNA.

PCR reactions were performed on a Bio-Rad MyCycler thermal cycler. The protocol used for the amplification reactions was adapted from Revaldaves et al. [12] and Pereira et al. [21], and consisted of the following steps for the Pcor01, Pcor21, Pcor23, and Pcor28 markers: one cycle at 95 °C for 5 min; one cycle at 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 10 s, totaling 30 repetitions; and a final cycle at 72 °C for 10 min. For the Pcor10 marker it was: one cycle at 95 °C for 5 min; one cycle at 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 30 s, totaling 30 repetitions; and a final cycle at 72 °C for 10 min. The amplicons were visualized in a 7 % polyacrylamide gel dyed with 0.2 % silver nitrate. We used a 10-bp DNA ladder (Invitrogen, Carlsbad, CA, USA) as a reference to identify the size of the fragments.

Statistical analysis

The allele frequencies were estimated by using CERVUS 3.0 software [28]. The parameters of locus diversity were estimated for all microsatellites: expected (He) and observed (Ho) heterozygosity, polymorphic information content (PIC), and Hardy–Weinberg equilibrium (HWE). FSTAT 2.9 software [29] was used to calculate allelic richness (AR). The estimated coefficient of endogamy (F_{IS} and F_{ST}) and the population structures were evaluated by the analysis of molecular variance (AMOVA) implemented on ARLEQUIN 3.1 [30]. Based on the genotype results from the six microsatellite loci, individuals were grouped into a determined number of populations and assigned probabilistically to infer groups by the Bayesian methodology implemented in STRUCTURE 2.3 [31].

The tests were performed based on an admixture model where the allelic frequencies were correlated. To choose the appropriate number of inferred populations, several analyses were done with K (number of inferred populations) varying from 2 to 6 and 300,000 interactions (burn-in period of 3000), with three independent repetitions for each analysis. The values of *K* were inferred from the magnitude of the ΔK function of *K* with the assistance of STRUCTURE HARVESTER web 0.6 software [32], following the model proposed by Evanno et al. [33].

Results

The data analysis from the 92 analyzed individuals of both species for each microsatellite locus (Pcor01, Pcor02, Pcor05, Pcor08, Pcor10, Pcor21, Pcor23, and Pcor28) are shown in Table 2. The description of the alleles found in *P. corruscans* and *P. reticulatum* using six microsatellite molecular markers common to both species are shown in the supplementary material (S1). All loci were polymorphic, with a total of 57 alleles for *P. corruscans* and 27 alleles for *P. reticulatum*. The average number of alleles per locus was 8.14 for *P. corruscans* (varying from 6 to 11 for markers Pcor01 and Pcor02, and Pcor10, respectively) and 3.86 for *P. reticulatum* (varying from 2 to 5 for markers Pcor10 and Pcor05, and Pcor08 and Pcor23, respectively).

For *P. corruscans,* the observed heterozygosity (Ho) varied from 0.27 (Pcor02) to 0.70 (Pcor23) and the expected heterozygosity (He) varied from 0.66 (Pcor28) to 0.87 (Pcor10). For *P. reticulatum*, Ho varied from 0.06 (Pcor10) to 0.94 (Pcor23) and He varied from 0.18 (Pcor28) to 0.74 (Pcor23). PIC of the studied loci for *P. corruscans* was highly informative, with an average value of 0.73 (varying from 0.62 in Pcor28, to 0.84 in Pcor10). However, for *P. reticulatum*, the PIC was only moderately informative, with an average of 0.49 (varying from 0.16 in Pcor28, to 0.67 in Pcor23).

The genetic structure of populations was analyzed using Bayesian statistics with the aid of the Structure program. The grouping of $k = 3$ (Fig. 2) corresponds to the real k, according to the methodology proposed by Evanno et al. [33], showed that the *P. reticulatum* populations collected in the Paraguay River basin (Negro, Aquidauana, Miranda and Paraguay Rivers) and in the Paraná River basin (Dourados and Ivinhema Rivers) represent the same population.

Table 1 Microsatellite marker panel by locus, allele size, GenBank accession number, primer sequences, annealing temperature, repetitions, and references

Locus	Allele size (bp)	GenBank accession number	Primer sequence (5′–3′)	Annealing temperature (°C)	Repetitions	References
Pcor01	100–135	AY737063	F:AAACCCAGGATAACCAGTC R:AGCGTGCTACTAACACAAAC	55	$(TC)_9GC(TC)_9$	Revaldaves et al. [12]
Pcor02[a]	175–200	AY737064	F:GATATGCAAATAAGAAGGTC R:TCTTCTGGCTTTTCCTCCTCT	55	$(AG)_{19}$	Revaldaves et al. [12]
Pcor05	135–170	AY737067	F:GACTAAGATTACACAGAGATTC R:CTTGGTGGGGAAACAGGC	55	$(TC)_8CC(TC)_{15}$	Revaldaves et al. [12]
Pcor08[b]	160–180	AY737070	F:ACACCATACGCACACACTCG R:TGAGGTCGGGTGATAAGGTC	56	$(AC)_{12}$	Revaldaves et al. [12]
Pcor10	100–195	AY737072	F:TTTAAGACAGCACAGCCTGTGGGG R:AAGACAGCGCCATAGAGTTCTGCC	49	$(GTCG)_{15}(GT)_9CC$	Revaldaves et al. [12]
Pcor21	100–150	AY737078	F:TCACCGAGAGGTCTGACCATGA R:CTGTGGTTAACCAGCTAGCAC	55	$(GT)_{13}$	Revaldaves et al. [12]
Pcor23	85–160	EF635878	F:TCCACTCACTAGGAAATGTTCTG R:CCAGCTCACAATATGCAACC	55	$(AC)_{13}$	Pereira et al. [20]
Pcor28	100–155	EF635879	F:TGATAGTACTGATCTCTCGCTGTC R:AAAGCTGCCTGCAGTCTCG	55	$(TC)_9$	Pereira et al. [20]

[a] Specific to *Pseudoplatystoma corruscans*

[b] Specific to *Pseudoplatystoma reticulatum*

Discussion

The applicability of molecular tools to identify hybrid and pure-bred individuals in natural environments [18, 34] makes it possible to evaluate genetic variability in fish. This can bring more reliability to the results because morphological identification alone may not be enough to discriminate a pure individual from a hybrid one, as demonstrated by Vaini et al. [19]. Molecular tools for the genetic identification of hybrid and pure surubins were, therefore, used in the current study, prior to the study with microsatellite molecular markers, to evaluate the genetic variability of these populations.

The genotype data about Ho and allelic richness demonstrated that there is genetic variability in the populations of *P. corruscans* and *P. reticulatum* in this study. Barker [35] suggests that, in studies of genetic diversity, a locus must have at least four different alleles in order to reduce the standard error of genetic distance estimates.

The results of the selected marker analyses indicated that seven loci of microsatellites were viable and informative for characterizing the genetic diversity of *P. corruscans*, because they presented six or more alleles per locus, but for *P. reticulatum* this situation did not obtain because the markers exhibited only two alleles, perhaps because of the small population sample (16 individuals). Similar results were found by Abreu et al. [20].

In *P. corruscans*, He for each locus was significantly higher than Ho, indicating a loss of heterozygosity and genetic structure. In *P. reticulatum,* Ho for each locus was significantly higher than the He, indicating high heterozygosity. Microsatellite markers that show average PIC values greater than 0.7 are considered highly informative [36], so, except for Pcor01 and Pcor28, all the other studied loci were considered highly informative.

The allele fixation indices in the population (F_{IS}), i.e., the endogamy indices, had an average of 0.27 for *P. corruscans* (varying from 0.14 in Pcor23 and Pcor28, to 0.41 in Pcor01), and an average of 0.06 for *P. reticulatum* (varying from −0.39 in Pcor21, to 0.66 in Pcor28). Except for Pcor28, all the *P. corruscans* loci were in Hardy–Weinberg equilibrium (HWE), but no locus showed equilibrium in *P. reticulatum*.

The *P. corruscans* population exhibited a greater number of alleles per locus (8.50) and greater allelic richness (5.51) when compared to the *P. reticulatum* population, which presented smaller values: 3.67 and 3.63, respectively. The analysis of the population structure showed a difference of 19.10 % between populations (F_{ST}) of *P. corruscans* and *P. reticulatum*, 7.66 % between individuals in the populations (F_{IS}), and 73.23 % total individuals (F_{IT}), and the estimated genetic differentiation based on F_{ST} was significant ($p < 0.001$), indicating that the species under study are genetically independent, thus highlighting the importance of their preservation.

The analysis of genetic structure of species using STRUCTURE software showed that the *P. reticulatum*

Table 2 Number of individuals (*N*), number of alleles (A), allelic richness (AR), observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC), Wright statistics (F_{IS}) and Hardy–Weinberg equilibrium (HWE) of the seven microsatellite loci of *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum*

Locus	*N*	A	AR	Ho	He	F_{IS}	PIC	HWE
Pseudoplatystoma corruscans								
Pcor01	64	6	4.48	0.40	0.69	0.41	0.70	***
Pcor02	59	6	4.20	0.27	0.74	−0.20	0.69	***
Pcor05	70	8	7.00	0.44	0.81	0.45	0.78	***
Pcor10	65	11	8.74	0.60	0.87	0.31	0.84	***
Pcor21	76	9	6.70	0.68	0.80	0.15	0.77	***
Pcor23	74	10	7.05	0.70	0.81	0.14	0.78	***
Pcor28	76	7	5.14	0.56	0.66	0.14	0.62	NS
Average ± DP		8.14 ± 1.95	6.18 ± 1.64	0.52 ± 0.15	0.76 ± 0.07	0.27 ± 0.14	0.73 ± 0.08	
Pseudoplatystoma reticulatum								
Pcor01	16	4	4.00	0.69	0.67	−0.02	0.60	NS
Pcor05	16	5	4.97	0.50	0.68	0.28	0.61	NS
Pcor08	16	5	4.98	0.75	0.67	−0.15	0.61	NS
Pcor10	14	2	2.00	0.35	0.30	−0.18	0.25	NS
Pcor21	16	3	3.00	0.87	0.64	−0.39	0.54	NS
Pcor23	16	5	4.98	0.94	0.74	−0.28	0.67	NS
Pcor28	16	3	2.86	0.06	0.18	0.66	0.16	NS
Average ± DP		3.86 ± 1.21	3.82 ± 1.22	0.59 ± 0.31	0.55 ± 0.22	0.06 ± 0.39	0.49 ± 0.20	

Pcor02 specific to *Pseudoplatystoma corruscans*, *Pcor08* specific to *Pseudoplatystoma reticulatum*, *NS* not significant

*** Significant

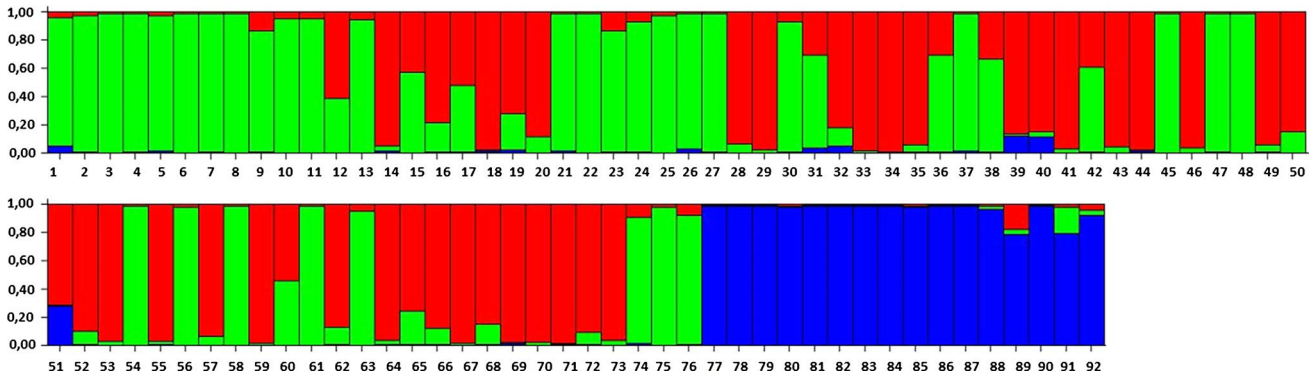

Fig. 2 Overview of individuals from *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum*. Each individual was represented by a *vertical bar* divided into segments that correspond to the relative proportion of genome referred to each species. *P. corruscans* basin of Paraguay River (Negro: *1* and *2*; Aquidauana: *3* and *4*; Miranda: *5–9*; Paraguay *10*); basin of Paraná River (Ivinhema: *11–28*; Dourados: *29–76*). *P. reticulatum* basin of Paraguay River (Negro: *77–83*; Aquidauana: *84–87*; Miranda: *88–89*; Paraguay: *90*); basin of Paraná River (Ivinhema: *91*; Dourados: *92*)

populations collected in the Paraguay River basin (Negro, Aquidauana, Miranda and Paraguay Rivers) and in the Paraná River basin (Dourados and Ivinhema Rivers) represent the same population. However, the *P. corruscans* populations collected in the same rivers as *P. reticulatum* proved to be a single population (Fig. 2), with the ones from the Paraná basin forming a subpopulation.

Abreu et al. [20] and Bignotto et al. [37] observed genetic differences in populations of *P. corruscans* and *P. reticulatum* using molecular markers, thus, there was a need to consider each taxon as a unit for conservation and management with its own characteristics, and a need to conserve the different habitats in order to preserve the genetic variability of these species. Although these species

are subjected to high fishing pressure, they can not be considered at risk because they are in relatively intact environments in the Paraguay River basin (Pantanal ecosystem) [38]. Studies like this highlight the importance of the preservation of these species, as well as the value of genetic techniques in conservation and breeding programs and in stock evaluation in natural environments, in order to minimize the loss of variation in these genetic resources. They also represent a tool for making changes in Brazilian environmental legislation related to the production of hybrid species from native parental types.

Fish farmers have been mistakenly using interspecific catfish hybrids as broodstocks [18]. Superior performance or desirable characteristics associated with hybrid vigor might be lost in post-F1 individuals because of introgressive hybridization that reduces heterosis obtained in F1 hybrids and because post-F1 hybrids show reduced offspring viability due to high mortality rates [7]. Therefore, there is a need to preserve pure individuals of *P. corruscans* and *P. reticulatum* in the basins of the Paraná and Paraguay Rivers with broodstocks in order to assure their genetic variability, since a loss of heterozygosity in these species was confirmed with the use of molecular marker microsatellites. Vaini et al. [19] identified interspecific hybrid surubim in the rivers analyzed in this work, and we have identified low heterozygosity in pure individuals of *P. corruscans* and *P. reticulatum*; thus, it is necessary to have conservation measures for species in the natural environment. These results about genetic diversity in pure species are relevant to introduction of management practices and conservation of these species.

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