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Geographical genetics of *Pseudoplatystoma punctifer* (Castelnau, 1855) (Siluriformes, Pimelodidae) in the Amazon Basin

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ABSTRACT. Geographical genetics allows the evaluation of evolutionary processes underlying genetic variation within and among local populations and forms the basis for establishing more effective strategies for biodiversity conservation at the population level. In this study, we used explicit spatial analyses to investigate molecular genetic variation (estimated using 7 microsatellite markers) of *Pseudoplatystoma punctifer*, by using samples obtained from 15 localities along the Madeira River and Solimões, Amazon Basin. A high genetic diversity was observed associated with a relatively low F_{ST} (0.057; $P < 0.001$), but pairwise F_{ST} values ranged from zero up to 0.21 when some pairs of populations were compared. These F_{ST} values

have a relatively low correlation with geographic distances (r = 0.343; P = 0.074 by Mantel test), but a Mantel correlogram revealed that close populations (up to 80 km) tended to be more similar than expected by chance (r = 0.360; P = 0.015). The correlogram also showed a exponential-like decrease of genetic similarity with distance, with a patch-size of around 200 km, compatible with isolation-by-distance and analogous processes related to local constraints of dispersal and spatially structured levels of gene flow. The pattern revealed herein has important implications for establishing strategies to maintain genetic diversity in the species, especially considering the threats due to human impacts caused by building large dams in this river system.

Key words: Freshwater fish; Genetic diversity; Mantel test; Microsatellite; Population genetic structure; Spatial autocorrelation

INTRODUCTION

Geographical genetics aim to analyze patterns of genetic variation within or among populations by using explicit spatial analysis techniques that might be helpful to understand the ecological and evolutionary processes underlying such patterns (see Epperson, 2003; Diniz-Filho and Bini 2011). Starting from early applications of spatial autocorrelation analysis and Mantel tests (Sokal and Oden, 1978a,b; Sokal, 1979), the field later started applying more sophisticated statistical analyses, tested these procedures with simulation experiments, and developed more precise theoretical basis (see Epperson, 2003; Guillot et al., 2009 for reviews). More recently, the combination of genetic data and landscape features formed the field of landscape genetics (Manel et al., 2003; Holderegger and Wagner, 2008) that allows a better understanding of processes such as dispersal and gene flow patterns, with important conservation implications (Manel et al., 2010). All these fields, including geographical, landscape, and conservation genetics, are linked by both theoretical reasoning and use of similar statistical tools for spatial analysis (see Diniz-Filho et al., 2008; 2009).

Freshwater fish provide interesting opportunities for geographical genetic studies because, despite apparent habitat continuity and high dispersal, population structure can appear due to complex life cycles and migration (as in natal homing behavior, see Telles et al., 2011) or to hierarchical structure of aquatic systems in basins (Landeiro et al., 2011). Given the wide interference of human activities with aquatic environments affecting their spatial connection, such as dam building, as well as the importance of both water and fish as natural resources, the importance of population analysis of fish species becomes apparent.

Pseudoplatystoma punctifer (Castelnau, 1855; Siluriformes, Pimelodidae) is a large freshwater catfish, widely distributed in the Amazon Basin around the Amazon/Solimões River and is one of the most commercially important species in Amazon Basin (see also Crepaldi et al., 2006). It is a carnivore and migratory species, and some studies with a related species (*Pseudoplatystoma corruscans*) suggest a possible homing behavior associated with the migration patterns (see Barthem and Goulding, 1997; Pereira et al., 2009). Some previous studies investigated population genetic patterns in species of the genus *Pseudoplatystoma,* including Revaldaves et al. (2005), who developed microsatellite

primers for *P. corruscans* and tested its transferability to other species, including *Pseudoplatystoma fasciatum* (which is closely related, or even completely overlapped, with *P. punctifer* (see Buitrago-Suarez and Burr, 2007; Torrico et al., 2009; Carvalho-Costa et al., 2011). Saulo-Machado et al. (2011) recently developed specific microsatellite markers for *P. punctifer* and described some genetic parameters within a local population. Pereira et al. (2009) recently showed that a significant population differentiation occurred among local populations of *P. corruscans*, with an F_{ST} around 8% and a significant spatial pattern in genetic differentiation according to Mantel test, by using 6 local populations (and interpreted such patterns as a consequence of homing behavior). More recently, Carvalho et al. (2012) reported significant evolutionary units for *P. corruscans* from Paraná-Paraguay Basin compared with a population from São Francisco Basin.

In this study, we used explicit spatial methods to evaluate the population genetic structure of *P. punctifer* along the Madeira and Solimões Rivers in the Amazon Basin. The analyses were performed using microsatellite markers developed by Revaldaves et al. (2005) and allowed a better understanding of the evolutionary processes underlying genetic variation in the species, providing the basis for establishing optimal conservation strategies to maintain genetic variability of the species.

MATERIAL AND METHODS

Data

We analyzed samples of *P. punctifer* collected from 15 localities (populations in a loose sense, hereafter), mainly along the Madeira River and a few in the low Solimões River, close to Manaus, all in the Amazon Basin (Table 1; Figure 1). In these localities, a total of 180 individuals were collected, with sample sizes ranging from 3 to 76. We followed the taxonomic revision by Buitrago-Suarez and Burr (2007), who define this as a valid species for the Amazon/Solimões Basin and differentiated it from *P. fasciatum*, both geographically and morphologically. However, other more recent reviews and molecular phylogenetic analyses suggest that only *P. fasciatum* is a valid taxa (Torrico et al., 2009) or use *P. fasciatum* in a broad sense, including *P. punctifer* as a unit inside the later (Carvalho-Costa et al., 2011).

Genomic DNA was extracted from ethanol-fixed tissues following the protocol of Taggart et al. (1992). Seven microsatellite primers designed for *P. corruscans* Revaldaves et al. (2005; Table 2) were used. The genotyping experiments were performed in 15 µL reaction volume containing 15 ng template DNA, 260 µM each primer, 1 U *Taq* DNA polymerase (Phoneutria, BR), 210 µM each dNTP, 2.16 mg bovine serum albumin (BSA), and 1X reaction buffer (10 mm Tris-HCl, pH 8.3, 50 mm KCl, 1.5 mm $MgCl_2$). All PCR amplifications were performed in a 9700 thermal cycler (Applied Biosystems, CA, USA) by using the following conditions: 95°C for 5 min (one cycle), 94°C for 1 min, 52°C to 64°C (see Table 1) for 1 min, 72°C for 1 min (30 cycles); and 72°C for 7 min (one cycle). Forward primers were labeled with fluorescent dye (NED, 6-Fam, and Hex; Applied Biosystems). Amplified fragments were submitted to capillary electrophoresis at 9 V, 60°C for 22 min in the automated DNA sequencer ABI 3100 Genetic Analyzer from Applied Biosystem™, and fragments were automatically sized using the GeneMaper® v4.1 software (Applied Biosystems)

Table 1. Sampling localities and genetic characterization of *Pseudoplatystoma punctifer* populations.

No.	Population	N	*A*	*AR*	H_E	*f*
1	Madeira River (Porto Velho/RO)	11	7	3.8	0.776	0.164
2	Madeira River (Porto Velho/RO)	76	10	3.4	0.680	0.254*
3	Mamoré River (Guajará-Mirim/RO)	9	5	3.1	0.658	0.324*
4	Purus River (Lábrea/AM)	30	9.7	3.7	0.727	0.247*
5	Karipunas River (Porto Velho/RO)	11	5.7	3.2	0.666	0.260*
6	Igarapé São Simão (Porto Velho/RO)	8	5.8	3.6	0.743	0.208
7	São Lourenço River (Porto Velho/RO)	5	5	3.8	0.811	0.366
8	Abunã River (Fortaleza do Abunã/RO)	3	2.8	2.8	0.571	-0.083
9	Mamoré Foz do rio Sotério River (Costa Marques/RO)	4	2.7	2.5	0.553	0.032
10	Madre de Dios River (Santo Pablo/BOL)	3	2.8	2.8	0.714	0.600*
11	Madre de Dios River (Independência/BOL)	6	4.7	3.4	0.757	0.277
12	Alto Rio Solimões (Codajás/AM)	3	3.1	3.1	0.714	0.400*
13	Madeira River (Porto Velho/RO)	4	4.7	4	0.839	0.106
14	Amazonas River (Manaus/AM)	4	4.7	4	0.833	0.400
15	Guaporé River (Costa Marues/RO)	3	2.8	2.8	0.583	0.347

N = number of individuals; *A* = mean observed number of alleles; *AR* = allelic richness by rarefaction; H_E = expected heterozigosity and *f* = fixation index. *Significant at the 0.05 level.

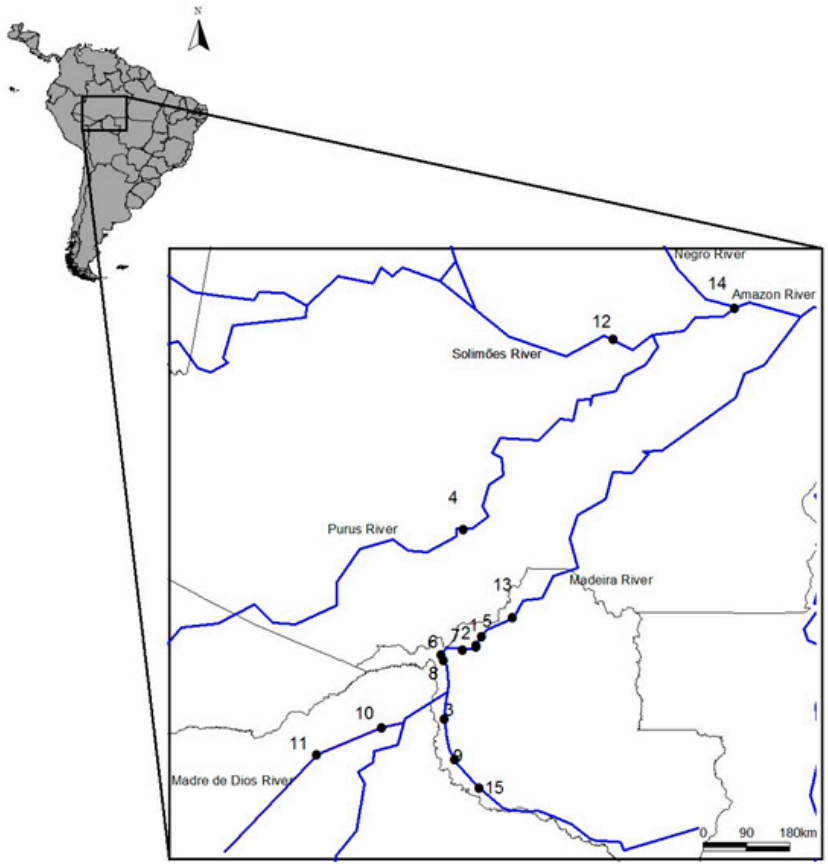

Figure 1. Localities from which samples of *Pseudoplatystoma punctifer* were obtained for the molecular analyses.

Table 2. Microsatellite panels (1 and 2), markers (locus), forward primer sequence, fluorescent dye and annealing temperature prime (TA) used in the study.

No.	Marker	Primer sequence	Fluorescente dye	TA(°C)	Multiplex
1	Pcor 1 - F	AAACCCGAGGATAACCAGTC	6-FAM	64	1
2	Pcor 2 - F	GATATGCAAATAAGAAGGTC	6-FAM	58	
3	Pcor 5 - F	GACTAAGATTACACAGAGATTC	NED	60	
4	Pcor 8 - F	ACACCATACGCACACACTCG	HEX	62	
5	Pcor 7 - F	ATGCTGGGATACGCTCAGAC	NED	64	2
6	Pcor 10 - F	TTTAAGACAGCACAGCCTGTGGGG	6-FAM	62	
7	Pcor 21 - F	TCACCGAGAGGTCTGACCATGA	HEX	64	

Data Analysis

Standard descriptive statistics of genetic parameters, including number of alleles (A), allelic richness by rarefaction (RA), observed and expected heterozigosities (H_O and H_E), and fixation indices (*f*) were obtained for each local population by using the FSTAT 2.9.3.2 software (see Goudet, 2002). Following Telles et al. (2011), we tested the correlation between these metrics and geographic distance from a fixed point in the basin, to determine a reduction in genetic diversity (taking into account the variation in sample sizes).

Population structure was analyzed using Weir's (1996) analysis of variance of allele frequencies, allowing obtaining analogous of Wright's *F*-statistics (F_{ST}, F_{IS}, and F_{IT}). Parwise F_{ST} between localities was also obtained and was the basis for further spatial analyses by using Mantel tests (see below).

Spatial patterns were analyzed using Mantel test (Sokal, 1979; Manly, 1985, 1997), which is actually a matrix correlation between genetic (F_{ST}) and geographic distances tested by matrix randomization. The statistical significance of Mantel's coefficient (Pearson's correlation among matrices) was tested using 5000 random permutations. However, recent analyses (e.g., Perez et al., 2010; Legendre and Fortin, 2010) showed low statistical power of Mantel test. An unbiased and more informative analysis for evaluating spatial patterns in F_{ST} is building a Mantel correlogram, in which F_{ST} is compared with model matrices, in which 1 indicates if the pairs of local populations are connected at a given distance, and zero elsewhere. Here, 7 model matrices were built, linking populations situated at increasing geographical distances. Mantel tests and correlograms were performed in SAM 4. 0 (Rangel et al., 2010; see www.ecoevol.ufg.br/sam).

RESULTS

There was a high level of within population polymorphism in *P. punctifer* (Table 1), with mean number of alleles varying from 2.7 to 10, although allelic richness was noted in localities (i.e., taking into account the variation in sample sizes), with values per loci ranging from 2.5 to 4 (although, as expected due to large variations in sample size, the rarefacted number varied less). For each of the 7 markers (loci), there was a considerable variation in Hardy-Weinberg (HW) equilibrium levels, and some of the local populations showed very high fixation indices *f*, indicating deviations from equilibrium. Expected heterozigosity was very high in local populations, ranging from 0.553 to 0.839. A high global value of *f* (0.254; $P < 0.05$) was noted, with values within local populations ranging from 0 to 0.6, indicating non-random mating and low effective population sizes (similar to those found by de Abreu et al., 2009 and Pereira et al., 2009 for related species).

The correlation between within-locality genetic parameters and distance from a fixed point were not significant, although correlation with RA was more negative and only marginally significant (r = -0.428; P = 0.144). This indicates a lack of strong decrease in genetic diversity toward waterheads (even after taking into account variation in sample size, by using the rarefied richness).

There was a significant differentiation among local populations, with an overall F_{ST} of 0.057 (P < 0.01). However, pairwise F_{ST} between local populations varied around this overall value, ranging from values close to zero (actually -0.048, due to sampling issues) up to 0.214 (between local population of Abunã River and upper Solimões). In general, pairwise F_{ST} values were only marginally correlated with geographic distances at logarithmic scale by Mantel test (r = 0.342; P = 0.064 with 1000 permutations; Figure 2A). However, Mantel correlogram showed a significant correlation (r = 0.360; P = 0.012 with 1000 permutations) between F_{ST} and connections at the smallest distance class (0 < D < 70 km); the correlations stabilized around non-significant values after this initial distance class up to 200 km (Figure 2B). However, despite non-significant, there was an increase in autocorrelation at the fourth distance class, suggesting the presence of 2 "bumps" of genetic variability in space (see Legendre and Legendre, 2012).

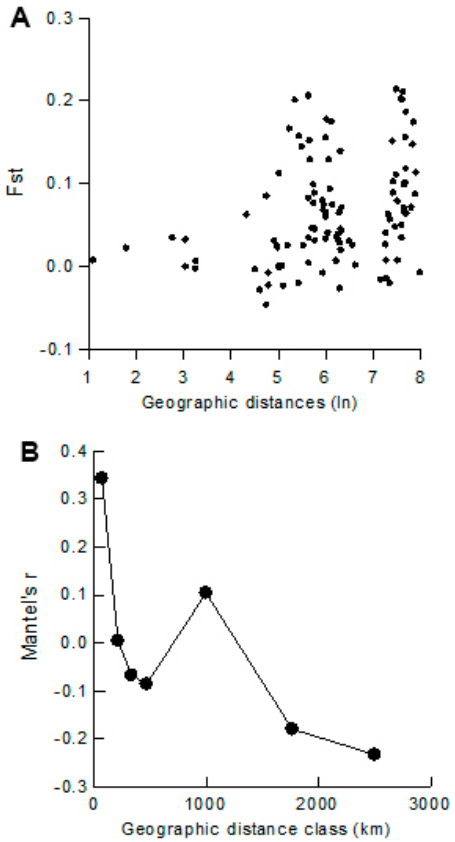

Figure 2. Relationship between pairwise F_{ST} and geographic distance among samples (Mantel correlation equal to 0.342; P = 0.064 with 1000 permutations) (**A**) and Mantel correlogram with 7 distance classes, revealing high positive autocorrelation in the small geographic distances (**B**).

DISCUSSION

Population differentiation in *P. punctifer*

Our analyses of *P. punctifer* revealed a significant genetic diversity both within and among populations throughout the Madeira, and for a few populations in the Solimões River in Manaus. Even after considering the large variation in sample sizes (with some local populations with only 3 individuals), it was possible to detect some interesting spatial patterns. Actually, there was no correlation (r = 0.046; P = 0.88) between sample sizes and distance from Manaus; therefore, spatial patterns in genetic variation among local populations cannot be artifacts of sample sizes.

Despite the low level of genetic differentiation in the populations, some pairs of populations showed levels of differentiation as high as 0.21. This value was within the range usually observed in other related species, such as *P. corruscans* in the Amazon basin and *P. reticulatum* in the Paraguay River (Pereira et al., 2009; Saulo-Machado et al., 2011; see also Carvalho et al., 2012 for a comparison of *P. corruscans* between Parana and São Francisco basins). However, in this study, F_{ST} values tended to be slightly correlated with geographical distances according to Mantel test. Nonetheless, this relationship was actually not linear (and this explained the lack of statistical significance of Mantel test), and correlograms revealed that closer populations in geographical space, at less than 200 km, tend to be more genetically similar than expected by chance. The correlogram obtained for *P. punctifer* suggested an exponential decrease of genetic similarity with increasing geographic distance, in which close populations are genetically similar, but more distant populations are not necessarily the most different (the difference is random at broad geographical scales). This spatial pattern has been in turn associated with isolation - by-distance or stepping-stone models - in distinct spatial evolutionary and spatial scales (Sokal and Wartenberg, 1983; Hardy and Vekemans, 1999; Epperson, 2003). In general, the combination of statistically significant spatial heterogeneity (measured using F_{ST} and analogous statistics) and significant spatial patterns (evaluated here by Mantel test and correlograms) is indeed a strong indication of evolutionary processes driving geographic variation if based on molecular-genetic studies (rather than phenotypic data, which can be due to phenotypic plasticity only). If the analysis is coupled with a more specific evaluation of spatial patterns by correlograms, this can allow a more effective inference of evolutionary processes underlying genetic variation (Sokal and Oden, 1978b; Sokal et al., 1997).

An exponential decrease of genetic similarity with geographical distances, as observed here for F_{ST} along *P. punctifer* populations, reveals a local (rather than long-distance) structure, usually associated with processes that cause restricted dispersal, such as isolation-by-distance. Inferring the details and origins of the patterns might be difficult on the basis of this isolation-by-distance like pattern, for example, to distinguish if this is a current pattern caused by restricted gene flow or if it better reflects deep time evolutionary relationships among closer populations in geographical space, related to historical barriers. However, ruling out some other processes, such as long distance migration or adaptation to environmental variation might be possible (although this last process is unlikely for microsatellite markers). Moreover, the 2 bumps in the correlograms suggest 2 or more groups of slightly distinct mean genetic values.

Long distance migration or processes of range expansion (e.g., Excoffier and Ray, 2008) tend to generate more clinal patterns in which there is positive Mantel correlations at small geographical distances coupled with negative significant Mantel correlations at broad distance (So-

kal and Oden, 1978b). Similarly, migration processes (and particularly homing behavior) tend to generate a clear and strong loss of genetic diversity within populations, which was not observed in this study (see Batista and Alves-Gomes, 2006; Batista et al., 2010; Telles et al., 2011).

Although Pereira et al. (2009) interpreted significant spatial structure of R_{ST} as evidence of homing behavior for *P. corruscans*, under homing behavior, although population differentiation is expected, a geographic spatial structure of genetic differentiation will occur only if there are other confounding historical factors and connections among the waterheads creating an exponential decrease (Telles et al., 2011). The decrease in genetic polymorphism (rarefied allelic richness) is expected under homing behavior, but it was not statistically significant in this study, despite the slight tendency or reduction or allelic richness toward waterheads. Due to the variation in sample sizes, our results actually do not rule the possibility that this decrease exists (because rarefied richness might not be able to deal with such strongly unbalanced sample size, as in our study). Nonetheless, the strong geographical pattern and exponential decrease seems to rule out migration and range expansion and consequently explanations should be focused on restricted gene flow among adjacent local populations, which also has important implications for conservation genetics of the species. Although Barthem and Goulding (1997) indicated that this species was migratory, our molecular analyses revealed that these migration and gene flow processes affect reproductive behavior and genetic variability at a relatively small geographical scale (i.e., not at Amazonian basin scale). Indeed, Isaac and Rufino (2000) suggested that migration of *Pseudoplatystomas* does not seem to occur throughout the entire basin, but mainly at a location in the main rivers toward smaller neighboring waterheads. This can explain the structure observed in this study, because this migration pattern would generate regional autocorrelation structure across the basin, and is also consistent with the 2 bumps in the correlograms.

Conservation Implications

The correlogram shape indicated thus that the local population of *P. punctifer*, despite inhabiting a continuous environment along the Madeira River, is locally structured and shows genetic differentiation at small geographical distances. As mentioned above, further analyses using mtDNA markers and a phylogeographical analyses based on a coalescent reasoning might be required for better understanding the origins of the observed patterns, as well as the relatively roles of restricted current and historical gene flows in this pattern. This is the basis of the phylogeographical definition of evolutionary significant units (ESUs) for improving the within-specific conservation strategies (see Moritz, 1994), but this has only been applied at broader geographical and taxonomic scales.

However, Diniz-Filho and Telles (2002; see also Diniz-Filho and Telles, 2006; Diniz-Filho and Bini, 2011) proposed a more general strategy for defining operational units for conservation for finer-scale, continuously distributed local populations, on the basis of autocorrelation. This approach clearly applies to *P. punctifer*, and the shape of the correlogram suggests that populations situated at distances smaller than 200 km tend to be genetically similar. This indicates that optimizing sampling strategies for *ex-situ* conservation (i.e., tissue collections or programs for monitoring fluctuations in genetic diversity as surrogates of demographic parameters) can be possible if this distance can be used as a minimum distance between samples. Alternatively, definition of operational units suggest that, for *P. punctifer*, would be necessary

to maintain at least one local population preserved within intervals of 200 km and associated with known reproductive area to ensure the preservation of genetic diversity of the species in the region, on the basis of the genetic system analyzed herein. Indeed, because population differentiation in general is low, all populations are similar and generating cost-effective complementary analysis methods in all these conservation strategies is necessary.

CONCLUSIONS

Our analyses revealed significant spatial heterogeneity of molecular genetic variation in *P. punctifer,* coupled with short-distance spatial patterns. This combination reveals that local restrictions to dispersal exist, due to current or historical spatially structured levels of gene flow. Independent of the evolutionary processes underlying this pattern, there are important conservation implications for such findings. By considering the human impacts in the region, notably the establishment of large dams in the Amazon Basin, the patterns observed here should be viewed and interpreted with care. On one hand, long-distance migration and historical patterns of genetic differentiation can be ruled out, at least in the geographical extent analyzed in this study, and creates a relatively uniform population throughout the basin. On the other hand, limited gene flow suggests that impacts at large distances can disturb populations locally and eliminate parts of the genetic diversity of the species. Considering cost-benefit analysis and using the results of this study to define optimum strategies, it might be necessary to maintain viable populations and maintain genetic diversity at current levels.

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