

High levels of genetic variability and panmixia of the tambaqui *Colossoma macropomum* (Cuvier, 1816) in the main channel of the Amazon River

M. C. F. SANTOS*, M. L. RUFFINO† AND I. P. FARIAS*‡

*Universidade Federal do Amazonas (UFAM), Departamento de Ciências Biológicas, Laboratório de Evolução e Genética Animal (LEGAL), Mini Campus, ICB, Av. Gen. Rodrigo Octávio Jordão Ramos, 3000 – Coroado, 69077-000, Manaus, AM, Brasil and
†ProVárzea/Ibama, Rua Ministro João Gonçalves de Souza, s/n° Distrito Industrial 69075-830, Manaus, AM, Brasil

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In this study, the complete mitochondrial control region of 48 tambaqui *Colossoma macropomum* specimens from five localities along a 2200 km transect of the Amazon basin was analysed. High genetic variability was observed in all localities sampled. Analyses of molecular variance indicated that nearly all of the molecular variance was contained within localities, and estimates of gene flow among localities were high. These results suggest that the tambaqui forms a panmictic population along the Solimões-Amazon River channel and are in agreement with species-typical behaviour of semi-migratory movements driven by dispersal for feeding and reproduction during its life cycle. In spite of the observed high levels of genetic variability and mutation-drift equilibrium of the mtDNA control region, the tambaqui has experienced a 90% demographic decrease and reduction of size at maturity in the past two decades. Population estimates based on molecular markers therefore do not reflect its current demographic status but rather its status in ecological time.

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Key words: *Colossoma macropomum*; control region; genetic structure; genetic variability; mtDNA.

INTRODUCTION

The Amazonian ichthyofauna is very diverse and is represented by more than 3000 species known to science (Lundberg *et al.*, 2000). Fishery studies carried out by Falabella (1994) in the Amazon State (Brazil) showed that only 36 species are exploited for consumption, of which the tambaqui *Colossoma macropomum* (Cuvier, 1816) is economically one of the most important species in the region. Numbers of attributes including large size and good flavour make it highly popular. The tambaqui is the largest characid of the Amazon basin, reaching total lengths (L_T) >1 m and weighing >30 kg (Isaac & Ruffino,

‡Author to whom correspondence should be addressed. Tel.: +55926473244; fax: +55926474233; email: izeni_farias@ufam.edu.br

1996). In addition to the Amazon basin, the tambaqui is also distributed in the main rivers of the Orinoco basin (Venezuela). The tambaqui is considered a semi-migratory fish (Araújo-Lima & Ruffino, 2004), undertaking seasonal migrations to floodplains and floodplain lakes for reproduction and feeding (Araújo-Lima & Goulding, 1998). It is a highly fecund seasonally reproducing species, whose diet is based mainly on fruits and seeds from the flooded forests. Even though the production of this species in captivity has increased in the past few years, there are strong indications, such as the reduction in the landing of the fish in Amazonian markets and the continued reduction in the size of the fish captured, that the natural populations of tambaqui are suffering from overexploitation as reported by Isaac & Ruffino (1996) almost 10 years ago and re-enforced by Araújo-Lima & Ruffino (2004).

The genetic characterization of the wild population of this economically important species is fundamental for any policy that would regulate fishing activity. Therefore, the non-coding region of the mtDNA, the hypervariable control region, was used to determine the amount of genetic variability and the intra- and interpopulation genetic structure of tambaqui along 2200 km of the Solimões-Amazon River in the Amazon basin. The control region has a high substitution rate, providing some of the best data for studying the population structure and the levels of gene flow between populations of fishes (Fajen & Breden, 1992; Sivasundar *et al.*, 2001; Grunwald *et al.*, 2002; Sato *et al.*, 2003). In addition to addressing questions of population structuring, this study also gave an opportunity to test a recent hypothesis proposed by Araújo-Lima & Goulding (1998). This hypothesis is based on life history and ecological data of the tambaqui and states that in the main Amazon River only one stock and species exists. The present study also aims to provide genetic information that can contribute to management and conservation programmes.

MATERIALS AND METHODS

SAMPLING AND DATA COLLECTION

All tambaqui samples were collected from fish landed at market in Tabatinga (Upper Solimões), Coari and Parintins (Middle Amazonas), Oriximiná and Santarém (Lower Amazonas) during surveys conducted by the ProVárzea/Ibama Project (Fig. 1). Samples were obtained only from small local operators, sampling a total of 48 tambaqui individuals. Tissue sample was preserved in 95% ethanol in the field. From each sample, the total DNA was extracted following a standard proteinase K, phenol-chloroform extraction protocol of Sambrook *et al.* (1989). The control region was amplified *via* the polymerase chain reaction (PCR) using the primers F-TTF (5'-GCCTAAGAG-CATCGGTCTTGTA-3') and F-12R (5'-GTCAGGACCATGCCTTTGTG-3') designed by Sivasundar *et al.* (2001). The PCR reactions were carried out in 25 µl total volume and consisted of 1 µl of total genomic DNA (*c.* 100 ng DNA), 2 µl of each primer (2 µM), 2.5 µl of 10× Buffer (Tris-KCL 200 mM pH 8.5), 3 µl of MgCl₂ (25 mM), 2.5 µl of deoxynucleotide triphosphate (10 mM), 0.2 µl *Taq* DNA polymerase (5 U µl⁻¹) and 11.8 µl of H₂O. The PCR condition consisted of 35 cycles of denaturation at 93° C for 1 min, annealing at 55° C for 1 min and extension at 72° C for 2 mins. The additional internal primer CMF2 (5'-CATCTGGTTCCTATTTTCAGG-3') was designed for the present study and was used for sequencing. The PCR products were purified using GFX kit (GE Healthcare, São Paulo, Brazil), cycle-sequenced using

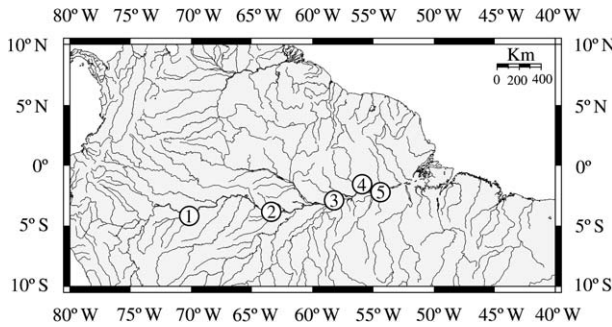


FIG. 1. Sample areas (1, Tabatinga; 2, Coari; 3, Parintins; 4, Oriximiná; 5, Santarém) of the tambaqui collected in the main channel of the Amazon River and analysed in the present study.

DYEnamic™ ET dye terminator kit (GE Healthcare, São Paulo, Brazil) and electrophoresed on a MegaBACE 1000 DNA sequencer. Sequences were edited and aligned in the programme BioEdit Version 5.0.6 (Hall, 1999).

DATA ANALYSIS

Indices of genetic variability for all populations were estimated based on number of haplotypes, nucleotide diversity (Nei, 1987), haplotype diversity (Nei & Tajima, 1981) and theta from segregating sites (Θ_s) calculated using the programme DnaSP (Rozas *et al.*, 2003). The level of geographic structuring of genetic variation was tested using an analysis of molecular variance (AMOVA) with 10 000 permutations (Excoffier *et al.*, 1992) using haplotype frequency and sequence divergence. AMOVA produces estimates of covariance components and calculates Φ_{ST} , which are analogues of Weir & Cockerham's F_{ST} estimator (Weir & Cockerham, 1984). For AMOVA, *a priori* hierarchical structure was defined and implemented in the programme Arlequin 3.0 (Excoffier *et al.*, 2005). Significance was evaluated after adjusting probabilities with Bonferroni correction for multiple comparisons (Rice, 1989). Migration rates between populations were calculated using Wright's (1951) standard approximations: $N_f m \approx 0.5[(mtF_{ST})^{-1} - 1]$ for a mitochondrial (mt) marker, where m is the per individual rate of migration and N_f is the effective size of the female portion of the population. Calculations were made using Arlequin 3.0 (Excoffier *et al.*, 2005). The Mantel (1967) test was used to assess the significance of association between genetic and geographic distances by correlating pairwise Φ_{ST} values against geographic distance. Statistical significance was tested using 10 000 random permutations as implemented in Arlequin 3.0 (Excoffier *et al.*, 2005).

The Tajima's D (Tajima, 1989) and Fu's F_S (Fu, 1997) tests were also applied to evaluate whether mutations are neutral or under influence of selection and whether the sequence data deviate significantly from the expectations of a population expansion model. The Tajima's D test is based on the relation between the number of segregating sites and the average pair-wise sequence divergence, while Fu's F_S test is based on the probability of observing a given number of alleles, given θ_π . In general, the F_S statistic is more sensitive in detecting demographic events than Tajima's D statistic. Both tests were calculated assuming 10 000 simulations implemented in the programme Arlequin 3.0 (Excoffier *et al.*, 2005).

The females inbreeding effective population size (N_{ef}) based on coalescence theory using Watterson's estimate of the population genetic parameter Θ from s and assuming a mutation rate of 2.0×10^{-8} mutations per site per year for the control region was estimated; this estimate is an average derived from other teleost species (Donaldson & Wilson, 1999; Sato *et al.*, 2003). Sato *et al.* (2003) estimated $2.2\text{--}4.3 \times 10^{-8}$ mutations per site per year, while Donaldson & Wilson (1999) estimated 1.8×10^{-8} mutations per

site per year. The mutation rate of 2.0×10^{-8} mutations per site per year is therefore a conservative estimate and results in a conservative upper bound estimate of inbreeding effective population size.

Nucleotide sequence data were imported into MEGA version 3.1 (Kumar *et al.*, 2004) to construct a neighbour-joining (NJ) tree of the haplotypes using a selected model of nucleotide evolution as determined by the MODELTEST programme (Posada & Crandall, 1998).

RESULTS

A total of 1077 base pairs (bp) of the control region were obtained from 48 samples of tambaqui (GenBank accession numbers DQ480027–DQ480074). The control regions of three individuals of the tambaqui (Par14, Ori38 and Tab46) had insertions of 99 bp, 34 bp and 68 bp, respectively, and therefore, these insertions were not included in the main dataset, which was used to carry out population analyses. Of the 1077 bp analysed, 71 variable sites were observed with 59 transition- and 12 transversion-type mutations. The mean frequency base composition was 22% C, 31% T, 32% A and 14% G, confirming an under representation of guanine as is normally observed in all mitochondrial genome (Zhang & Hewitt, 1996). A total of 47 haplotypes were found and nearly all sequenced individuals had a unique haplotype, with only one shared haplotype between one Tabatinga and one Parintins individual. Uncorrected pair-wise sequence divergence ranged from 0.1 to 1.5%.

No effect of phylogenetic saturation was observed in the control region data. The Mantel test (Mantel, 1967) showed no association of genetic and geographic divergence ($r = -0.446$, $P = 70.05$), which would be expected if haplotypes or clades of haplotypes were randomly distributed over a geographic landscape. NJ topology showed what appears to be a random distribution of haplotypes from different geographic localities distributed on the unrooted NJ network (Fig. 2).

The genetic variability of the tambaqui was estimated based on genetic parameters and on polymorphism analysis of DNA as shown in Table I. The results show a high rate of genetic variability for all populations sampled, with all populations showing high values of haplotype diversity and low values of nucleotide diversity. AMOVA identified that the greatest genetic variation was within sampled populations, and no genetic differentiation was observed between populations ($\Phi_{ST} = -0.0099$; $P > 0.05$). Table II shows the pair-wise comparisons obtained for Φ_{ST} and the $N_f m$ values between pairs of populations. Φ_{ST} values are inversely proportional to the $N_f m$ values, in other words, the greater the number of migrants, the smaller Φ_{ST} values are. All Φ_{ST} fixation indexes are low and show no significant pair-wise differences; the tambaqui populations are not differentiated, supporting the hypothesis of no genetic structuring and of panmixia along the 2200 km transect. Pair-wise $N_f m$ values approach infinity and indicate very high levels of flow between the populations.

The results of the Tajima's D and Fu statistical tests were not significant ($P > 0.05$), suggesting that the tambaqui populations analysed are in mutation-drift genetic equilibrium with regard to the mtDNA haplotypes. The inbreeding effective population estimated from the population genetic parameter Θ_S suggests *c.* 371 400 effective female individuals of tambaqui.

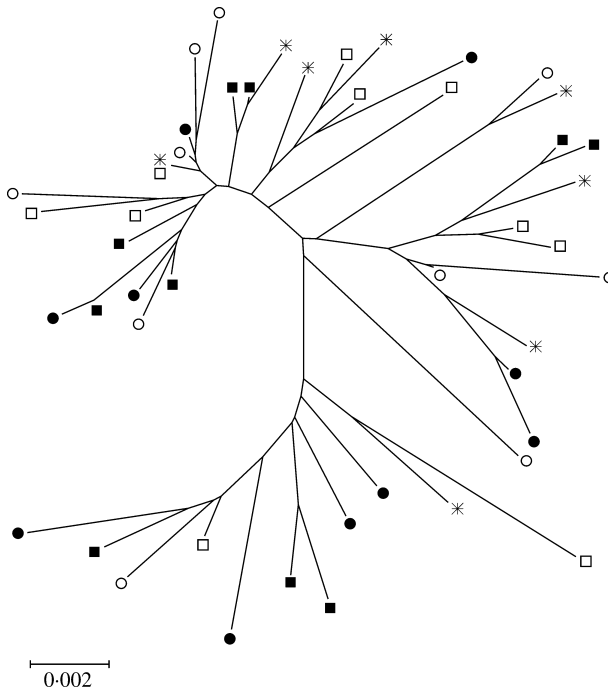


FIG. 2. Unrooted neighbour-joining trees showing the relationship of 47 tambaqui haplotypes estimated from Tamura-Nei distance matrix. The geographic origin of haplotypes is identified: *, Tabatinga; ■, Coari; □, Parintins; ○, Oriximiná; ●, Santarém (see Fig. 1).

DISCUSSION

The present results suggest that all the tambaqui sampled along the main channel of the Amazonian River present one large panmictic, genetically diverse species. In a panmictic population, the level of genetic diversity is expected to be proportional to the effective population size and the mutation rate, assuming they are constant through time (Avice *et al.*, 1988). Therefore, the high level of mtDNA polymorphisms and large number of segregating sites compared with haplotype number observed in tambaqui may be related to the high female effective population size over evolutionary time for this species (*c.* 371 400 effective female individuals). The same pattern was observed by Sivasundar *et al.* (2001) who analysed populations of another characiform species (*Prochilodus* spp.) from the Paraná, Amazonas, Orinoco and Magdalena basins using the mtDNA control region. They found that the mtDNA control region was highly variable and that all sequenced individuals had unique haplotypes, with a sequence divergence varying from 0.3 to 3.6%. The same result was also observed in *Leporinus elongatus* Valenciennes, 1850 from the Paraná River in Brazil (Martins *et al.*, 2003), where the authors found high level of haplotype diversity with large number of unique haplotypes. This pattern is also found in short-distance migratory marine fishes (Turner *et al.*, 2002; Ely *et al.*, 2005), which are estimated to have very large population sizes. Thus, high levels of genetic diversity seem to be commonly observed in migratory fishes with

TABLE I. Main genetic patterns of *Colossoma macropomum* populations

Population localities	Number of <i>n</i>	Number of haplotypes	Mean \pm s.d. gene diversity (<i>h</i>)	Mean \pm s.d.		Tajima's D test	Fu's F_S test
				nucleotide diversity (π)	Θ_s		
Tabatinga	8	8	1.000 \pm 0.063	0.011 \pm 0.001	0.0128	-0.435	-1.690
Coari	10	10	1.000 \pm 0.045	0.012 \pm 0.001	0.0121	0.061	-2.727
Parintins	10	10	1.000 \pm 0.045	0.011 \pm 0.001	0.0128	-0.339	-2.809
Oriximiná	10	10	1.000 \pm 0.045	0.012 \pm 0.001	0.0137	-0.626	-2.767
Santarém	10	10	1.000 \pm 0.044	0.012 \pm 0.001	0.0118	0.451	-2.607
All	48	47	0.999 \pm 0.004	0.012 \pm 0.0005	0.0148	-0.651	-40.970*

n, number of individuals; Θ_s , theta derived from number of segregating sites; *, significant at the 5% level.

large panmictic populations. This is because large effective population size and high migration rates minimize the effect of genetic drift as a force that lowers intra-lineage genetic diversity.

The high values of $N_f m$ and low values of Φ_{ST} encountered in this study indicate that there is an intense genetic exchange occurring among all the tambaqui populations, and this exchange is sufficient to prevent genetic differentiation. Consequently, the absence of a population structure can be suggested for the tambaqui, supporting the hypothesis of a single and large panmictic population occupying the main channel of the Solimões and Amazon rivers as suggested by Araújo-Lima & Goulding (1998). The high gene flow observed in the tambaqui probably results from its adaptation to the life in the floodplain (locally known as várzea). The tambaqui life cycle directly depends on seasonal flooding of the Amazonian basin. During the flood period, the tambaqui disperses into the nutrient-rich floodplain to seek food (Goulding & Carvalho, 1982). The tambaqui is an *r*-strategist; the adults are highly fecund and produce thousands of offspring. According to the hypothesis of Araújo-Lima & Goulding (1998), adults, large juveniles and larvae of tambaqui stay in flooded habitats during the high-water (flood) season to feed and to grow. During the low-water season, the juveniles stay in the floodplain (nursery areas), while the adults migrate to adjacent rivers or main river channels. The adults stay in the river channel looking for a suitable site to spawn. When the flood season starts

TABLE II. Pair-wise comparison of $N_f m$ (above) and Φ_{ST} (below) values between the population localities of the tambaqui

Population localities	Tabatinga	Coari	Parintins	Oriximiná	Santarém
Tabatinga	—	∞	∞	∞	13.9
Coari	-0.015	—	∞	∞	∞
Parintins	-0.052	-0.021	—	∞	50.1
Oriximiná	-0.046	-0.009	-0.026	—	21.1
Santarém	0.034	-0.011	0.009	0.023	—

∞ , infinity.

again, eggs and larvae are passively transported into the floodplain areas. Recent data from Lima & Araújo-Lima (2004) found that adults of several characiform species, including the tambaqui, also migrate from nutrient-poor rivers to spawn in nutrient-rich rivers; larvae and juvenile individuals were found in nutrient-rich rivers only, indicating that spawning activity was restricted to that river type. This dynamic cycle seems to happen in all the tributaries of the Amazon River where the tambaqui occurs (Loubens & Panfili, 1997) and such migrations and the passive transport of eggs and larvae are probably contribute to the high levels of genetic exchange and lack of population structuring observed among the localities analysed.

Junk *et al.* (1989) were the first group to propose that the interconnection of the river channels and floodplains driven by the flood 'pulse' is the main force triggering the interactions among the major biota in Central Amazon várzea system (floodplain areas). It is likely that this 'pulse' system of the várzea contributes to the observed genetic pattern in tambaqui and in other species of fishes. The lack of population structuring seems to be a general pattern for many species of fishes from the Amazonian várzea, especially in the main channel of the Amazon River, which is the main corridor of the Amazon basin system. The exact role of the floodplain in promoting high gene flow will have to be carried out through a rigorous comparative analysis of different várzea species.

Results of other studies carried out to date on várzea fishes suggest lack of population structure and high gene flow. Batista *et al.* (2005) used the mtDNA control region to characterize the interspecific and intraspecific genetic variability of two catfish species along the main Solimões/Amazonas channel. They showed that the dourada *Brachyplatystoma rousseauxii* (Castelnaud, 1855) and the piramutaba *Brachyplatystoma vaillantii* (Valenciennes, 1840) have very high levels of genetic polymorphism in all localities sampled along the main channel of the Amazon River. Although there are differences in the total amount of genetic diversity observed in these two species, both species, as in the tambaqui, show high levels of gene flow between localities and appear to form large panmictic populations. Hrbek *et al.* (2005) using ND1 and ATP 6 and 8 mtDNA genes to study the population genetic structure of the pirarucu *Arapaima gigas* (Schinz, 1822) detected low levels of genetic variability as a signal of the overexploitation, however, they also observed lack of population structuring and concluded that the pirarucu probably forms a large panmictic population.

The abundant genetic exchange among the five localities sampled for tambaqui along the 2200 km of the Solimões-Amazon River in the Amazon basin is the consequence of widespread gene flow between localities. A more definitive conclusion, however, may be reached with larger sample sizes that include also the main tributaries of the Amazon River and with faster evolving molecular marker such as microsatellite loci.

As the non-coding mtDNA control region is unlikely to be under selection, the non-significant values ($P > 0.05$) obtained from the Tajima D and Fu F_S statistical tests indicate that the sampled populations of tambaqui are in a genetic equilibrium, *i.e.* apparently there is no pressure of selection on the wild population with regard to the DNA mitochondrial haplotypes nor have they

experienced significant demographic expansion or contraction. In addition, high genetic variability was observed in all the wild population of tambaqui.

Although 371 400 female inbreeding effective individuals may not seem small, this number is small compared with the number of tambaqui landed in Manaus, the capital of Amazonas state, and just one of the Amazonian cities where tambaqui are landed. Isaac & Ruffino (1996) report that the average catch size of the tambaqui is 40.89 cm, which corresponds to an average mass of 1.42 kg per individual landed. Petrere (1983) reported 22 000 to 30 000 t of tambaqui landed in Manaus during 1976–1978. This translated to 7.7 to 10.4 million individuals per year landed in Manaus. Merona & Bittencourt (1988) estimated 8.4 million individuals (12 000 t) landed in 1976, which decreased to 3.5 million individuals in 1986 (5000 t). Batista (2004) reports a 1994–1996 yearly average of 1.5 million individuals (2236 t) landed in Manaus. Over the 1976–1996 year period studied, the total tonnage of tambaqui landed in Manaus decreased by nearly 90%, while at the same time fishing effort increased and population of the city of Manaus quadrupled. Tambaqui is among the 10 most exploited fish species in the Amazon region (Ruffino & Isaac, 1994) and heavily overexploited. To have been able to support this level of fishing, even if unsustainable in the long-term, it is clear that historical census sizes in the Amazon basin must have been enormous.

Effective population sizes estimate the ideal number of individuals that would produce the observed patterns of genetic variation in a studied sample; they do not necessarily reflect current census numbers. In natural populations, inbreeding effective population sizes are often an order of magnitude smaller than census sizes (Crandall *et al.*, 1999; Frankham *et al.*, 2002), although they can be smaller (Turner *et al.*, 2002) or even larger than census sizes (Gerber & Templeton, 1996). Assuming an order of magnitude difference between effective and census sizes, the current 371 400 inbreeding female effective size would be equivalent to *c.* 7 million individuals. The total number of individuals harvested in the 1970s was larger than the current estimated total population, and the number of individuals caught in the 1990s was *c.* 20% of the current estimated population. The 90% decrease in census population size therefore most likely resulted from unsustainable harvest levels.

In spite of clear and drastic reduction in census size of the tambaqui, the molecular data do not detect a signature of a significant population reduction. There are at least three main explanations for such a pattern: (1) the current demographic population reduction may be masked by a previous demographic event that left a strong signature on the molecular data, such as a strong historical demographic expansion (*e.g.* pacific crabs studied by Lavery *et al.*, 1996); (2) the large-scale exploitation of the past 30 years is too short relative to their generation time to be detected by the studied mtDNA segment; (3) because of extremely large historical census sizes and their longevity, the tambaqui still have relatively large census sizes (even if it is only 10% of estimated census sizes from 30 years ago) and so reduction in genetic diversity due to genetic drift will be difficult to detect over ecological time scales.

The inbreeding effective population size inferred from mtDNA markers of the tambaqui does not reflect its current demographic status. The effective population size measured in this study is based on the coalescent theory and is also

referred as 'long-term or historical effective population size' (Garrigan *et al.*, 2002). Therefore, the effective population size of the tambaqui is relevant in historical but not ecological time. It reflects the historical status of the tambaqui due to lag in response of genetic diversity as it comes to equilibrium with the tambaqui's current census size. Similar genetic patterns have also been observed in the Atlantic cod *Gadus morhua* L., 1758, a species that has had a long history of intense commercial exploitation, and in the early 1990s, fishery stocks collapsed, yet inbreeding effective population sizes inferred from DNA data remain high and show no significant decrease over time (Ruzzante *et al.*, 2001; Poulsen *et al.*, 2006). Therefore, the lack of observed signal of population reduction in the mtDNA markers should not be interpreted as lack of overexploitation of the tambaqui and used by fisheries managers as a justification to continue fishing of tambaqui at its current levels. In contrast to neutral molecular markers, key life-history traits, such as body size and age at first reproduction, which are known to have decreased significantly in the tambaqui over the past 30 years, are likely to be accompanied by changes in key components of the tambaqui genetic architecture. Studies of these candidate loci are therefore likely to be more informative with respect to the understanding of genetic changes associated with fishing pressure. Unfortunately, knowledge of the genomic architecture of the tambaqui is non-existent, and thus, this research area cannot be currently pursued.

Although the data are not sufficiently powerful to detect the signature of recent overexploitation, they are powerful in inferring population structuring or lack thereof. Using similar sample sizes, the study of Neotropical fishes by different authors such as Bermingham & Martin (1998) and Perdices *et al.* (2002) were able to detect phylogeographic structuring, while other authors such as Sivasundar *et al.* (2001) and Hrbek *et al.* (2005) were unable to detect structuring with similar or larger number of individuals over larger geographic distances. In agreement with the study of vertebrates of the Amazonian floodplain (Batista *et al.*, 2005; Cantanhede *et al.*, 2005; Hrbek *et al.*, 2005; Pearse *et al.*, 2006; Vasconcelos *et al.*, 2006), the tambaqui also shows no discernable pattern of population structuring across the Amazonian landscape.

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