

**REVISTA TRÓPICA: Ciências Agrárias e Biológicas****Variabilidade genética em populações naturais de *Leporinus piau* (Anostomidae, Characiformes) da bacia do Rio Itapecuru**

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Resumo: A espécie *Leporinus piau* encontra-se amplamente distribuída na América do Sul ocorrendo com frequência na bacia do rio Itapecuru, Estado do Maranhão. Esta espécie constitui importante recurso pesqueiro de grande valor econômico. Na tentativa de conhecer os índices de variabilidade genética de *L. piau*, sequências do gene rRNA 16S foram obtidas de espécimes oriundos do alto, médio e baixo curso da bacia do rio Itapecuru. A análise de um fragmento de 481 pb revelou a presença de 10 haplótipos e uma elevada diversidade haplotípica $h = 0,7247$. A matriz de divergência genética para esses haplótipos mostrou altos índices variando de 0,2 a 1,9%, sendo que os mais elevados (0,9 a 1,9%) foram observados para o haplótipo três (H3) de ocorrência no alto, médio e baixo curso da bacia do rio Itapecuru. Altos níveis de variabilidade genética intra e interpopulacional e a ocorrência de haplótipos com elevados valores de divergência genética sugere uma diferenciação genética para esta espécie. No entanto, a análise molecular de variância não mostrou estruturação entre as populações de *L. piau* do Rio Itapecuru representando assim um único estoque.

Palavras-chave: genética de populações, rRNA 16S, mtDNA.

Genetic variability in wild populations of *Leporinus piau* (Anostomidae, Characiformes) from the Itapecuru River Basin

Abstract: The fish *Leporinus piau* is widely-distributed in South America, and is common in the Itapecuru River in the Brazilian state of Maranhão. The species is an important fishery resource of considerable economic value. In order to evaluate the genetic variability of *L. piau*, sequences of a 481-bp fragment of the rRNA 16S gene were obtained from specimens collected in the upper, middle, and lower Itapecuru basin. Analyses revealed the presence of ten haplotypes, and a

relatively high haplotype diversity ($h = 0.7247$). The matrix of genetic divergence among these haplotypes returned relatively high values, with indexes varying from 0.2% to 1.9%. The highest values (0.9-1.9%) all involved haplotype H3, which was found throughout the Itapecuru basin. High levels of genetic variability are observed in *L. piau*, both among and within populations, as well as the presence of highly divergent haplotypes, which suggest a marked pattern of genetic differentiation in this species. However, the analysis of molecular variance did not indicate structuring among populations, but rather that the *L. piau* populations sampled from the Itapecuru River represent a single stock.

Keywords: population genetics, 16S rRNA, mtDNA.

Introduction

The family Anostomidae encompasses 12 genera and approximately 140 species of Neotropical fishes (Géry, 1977; Sidlauskas & Vari, 2008), which are found in all of Brazil's major river basins. This group represents a fishery resource of considerable economic value. However, natural populations have declined drastically in recent years, emphasizing the need for the understanding of their genetic makeup as a tool for their conservation, and the development of future management programs. Knowledge of the genetic variability of natural populations is important for planning effective conservation programs capable of assuring their long term maintenance (Martins et al., 2003).

Leporinus Spix, 1829 is the most diverse anostomid genus, with some 87 (88 according to FishBase) species (Garavello & Britski, 2003; Froese & Pauly, 2011) distributed in South and Central America. Individuals of this genus are characterized by longitudinal stripes, bands or spots on the body, unblemished fins, and a caudal fin with scales at the base only. The teeth are uncusped and arranged asymmetrically in threes or fours in the premaxillary and dentary rows. The type locality of the South American species *Leporinus piau* Fowler 1941 is the Rio Salgado (or Icó) in the Brazilian state of Ceará (Garavello & Britski, 2003).

The morphology of the juveniles of *Leporinus* species is relatively homogeneous (Renno et al., 1989), while the adults are distinguished by subtle variations in coloration and certain morphometric traits. This obviously hampers the correct identification of the different species. Geographic variation in morphological patterns and coloration was found between populations of *L. friderici* from the Amazon and the Rio Paraná basin in Paraguay by Géry (1977). Garavello et al. (1992) reconfirmed this variation in a multivariate analysis of morphological traits.

Genetic differentiation has been observed in *Leporinus* specimens based on sequences of the control region (Martins et al., 2003), as well as in analyses of isoenzymatic markers (Renno et

al.,1989; Renno et al., 1990; Chiari & Sodr , 1999). Chiari & Sodr  (2001) analyzed the intra- and inter-specific genetic variability of eight Anostomidae species based on RAPD markers, while Renno et al. (1991) studied the polymorphism of mitochondrial genes among the *L. friderici* populations of the rivers of French Guiana using RFLP markers. The latter study revealed the existence of two distinct groups of populations, reconfirming their previous findings using isoenzymes.

Population-level analyses of *Leporinus* species have been conducted primarily in the Paran  River basin (Chiari & Sodr , 1999; Martins et al., 2003), and little is known of the genetic constitution of the stocks of *L. piau* Fowler 1941, which is endemic to the Brazilian Northeast.

This study has analyzed the genetic variability of the *L. piau* populations from the upper, middle and lower Itapecuru River based on the sequencing of a fragment of the rRNA 16S gene of the mitochondrial genome. This is the first analysis of sequences of the mitochondrial genome for this species. The results might be useful not only for the characterization of *L. piau*, but also as support for recovery efforts, including the maintenance of the genetic diversity of this fish species.

Material and Methods

Sampling and molecular procedures

Thirty-one specimens of *L. piau* were collected from a number of different locations in the Itapecuru River basin in the Brazilian state of Maranh o, representing the full length of the river (Table 1 and Figure 1). Specimens were identified to species level according to the relevant literature (Fowler, 1941; Piorski et al., 1998) and the voucher specimens were deposited in the fish collection of the Zoology Museum of S o Paulo University (voucher specimen: MZUSP 104576). A small sample of muscle tissue was extracted from each specimen and preserved in 95% ethanol prior to the extraction of DNA. Total DNA was isolated from these samples, using the phenol-chloroform protocol modified from that originally developed by Sambrook & Russel (2001). A 481-bp fragment of the rRNA 16S mitochondrial gene was amplified by PCR and sequenced using the ABI PrismTM dye terminator cycle sequencing reading reaction in an ABI 377 automatic sequencer (Applied Biosystems, Foster City, CA). A more detailed description of the PCR and sequencing procedures is given in Fraga et al. (2007).

Table 1. Specimens of *L. piau* investigated in this study. N = number of specimens.

Population	Coordinates	N	Specimens
Upper Itapecuru	6°22'S/44°22'W	9	Lep18 (6); Lep 28; Lep 30; Lep 46
Middle Itapecuru	5°26'S/43°52'W	13	Lep1 (4); Lep8 (2); Lep48; <i>Lep49</i> ; Lep 51; Lep 52; Lep53 (3)
Lower Itapecuru	4°44'S/43°27'W	9	Lep2 (6); Lep86; Lep87; <i>Lep89</i>

Specimens shared by all three populations in bold; Specimens shared by two populations in italics. Numbers of specimens sharing identical sequences are in parenthesis.

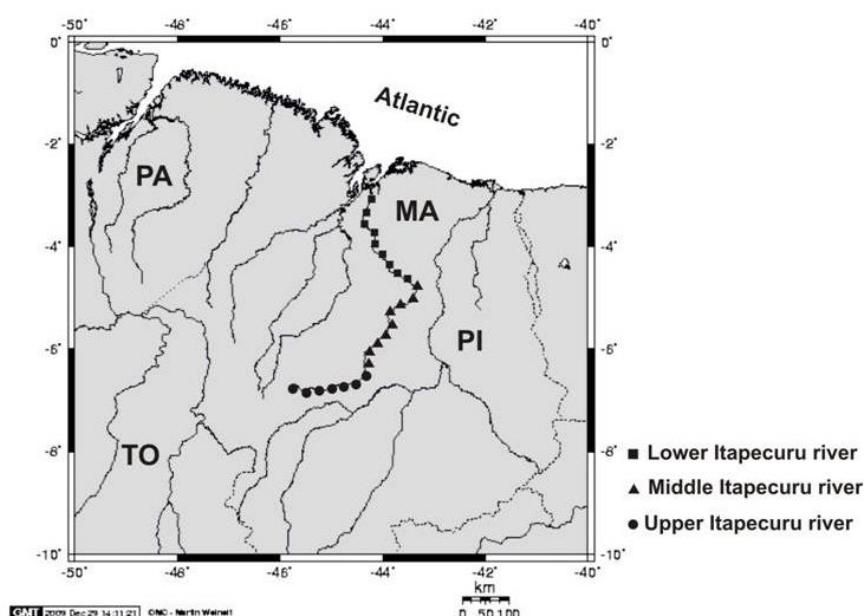


Figure 1. Map of the Brazilian state of Maranhão (MA), showing the Itapecuru River and sample sites.

Data analyses

Sequences of the fragment of the rRNA 16S gene were edited and aligned in the BIOEDIT program version 7.0.5.3 (Hall, 1999). Saturation of the data was estimated using DAMBE program (Xia & Xie, 2001), which plots transition and transversion rates against genetic divergence, based on the JC69 model. The indexes of genetic variability were estimated using DnaSP 4.10 (Rozas et al., 2003). The parameters estimated were: number of haplotypes (h), haplotype diversity (Hd), the number of segregating sites (S), and pairwise nucleotide diversity (π).

Phylogenetic analyses were run in MEGA v.4.0 (Tamura et al., 2007) and PAUP 4.0b10 (Swofford, 2003), using both distance (Neighbor-Joining) and character (Maximum Parsimony and

Maximum Likelihood) approaches. MODELTEST 3.7 program (Posada & Crandal, 1998) was used to select the evolutionary model that best fit the data, in this case, GTR modeled by the gamma distribution with the following parameters: Base=(0.32060.2433 0.2226), N st = 6, R mat = (0.9428 3.2387 2.8927 0.2582 9.1226) Rates=gamma, Shape = 0.1795 and the proportion of invariable sites equal to zero. The MODELTEST parameters were used in the Maximum Likelihood (ML) and Neighbor-Joining (NJ) analyses for phylogenetic reconstruction, in addition to the generation of the genetic divergence matrix. The significance of the clades was estimated through bootstrap analysis (Felsenstein,1985).The haplotype network was generated in NETWORK 4.5.1.0 (Fluxus Technology Ltd. at www.fluxus-engineering.com) using the Median Joining method (Bandelt et al., 1999).Sequences of *Schizodondis similis* (voucher specimen: MZUSP 104577) from the Itapecuru River and *Prochilodus nigricans* (GenBank: AY788075) were used as outgroups.

Analyses of molecular variance, or AMOVA were conducted to examine the genetic structure of the *L. piau* populations using ARLEQUIN 3.01(Excoffier et al.,2006). The significance of χ^2 - statistics was determined via nonparametric permutation (1000 permutations).

Results

A fragment of 481 base pairs was obtained from the rRNA 16S mitochondrial gene of the 31 *L. piau* specimens collected in the Itapecuru basin. The nucleotide composition of the fragment was 21.4% thymine, 24.0% cytosine, 32.2% adenine and 22.4% guanine. The plot of transition and transversion rates against genetic distance indicated that the data were not saturated (data not shown).

The polymorphism analysis revealed the presence of ten haplotypes and 11 variable sites in the sequences of the 31 *L. piau* specimens analyzed. One haplotype (H1) was by far the most frequent, being recorded 16 times in samples from the upper, middle and lower stretches of the Itapecuru River. Two other haplotypes (H3 and H5) were each recorded three times, but while H5 was recorded only in the middle Itapecuru, H3 was found throughout the basin. A number of unique haplotypes were observed in all three stretches of the river (Table2 and Figure 2).

Table 2. Polymorphic sites of the ten haplotypes identified in three *L. piau* populations from the Itapecuru River.

Haplotype	Segregating sites	Number of haplotypes in each population		
		UpperItapecuru	Middle Itapecuru	Lower Itapecuru
	112222223			
	21824445696			
	15590786206			
H1	TGAAATTCATT	6	4	6
H2	G.	1	-	-
H3	.AG..CCT...	1	1	1
H4	...G...T..C	1	-	-
H5	C	-	3	-
H6	T...	-	2	-
H7	AC	-	1	-
H8	C...C.C	-	1	-
H9	C.....	-	1	1
H10	A.....	-	-	1

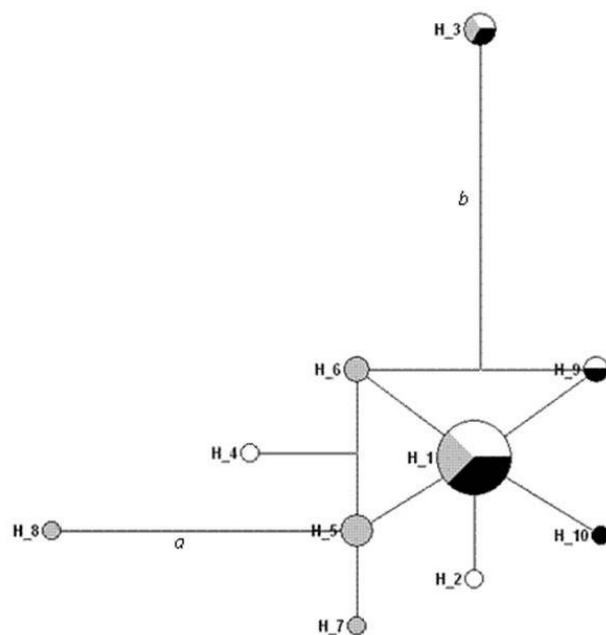


Figure 2. Median-joining haplotype network. The haplotypes are represented by circles. The diameter is proportional to their frequency. The white, gray and black shading corresponds to samples from the upper, middle, and lower Itapecuru, respectively. Each branch corresponds to a single mutation, except those marked *a* (two mutations) and *b* (three mutations).

Haplotype diversity was relatively high ($H_d = 0.8718$) in the *L. piau* population of the middle Itapecuru, with the same value of $H_d = 0.5833$ being recorded for both the upper and lower basin. The diversity of the whole sample was $H_d = 0.7247$. By contrast, nucleotide diversity (π) was

relatively low, varying from 0.00320 for the population from the lower Itapecuru, to 0.00448 for the middle portion of the river (Table 3).

Table 3. Parameters of genetic diversity for the *L. piau* populations sampled in this study. n = number of specimens; h = number of haplotypes; S = number of polymorphic sites.

Population	n	h	S	Hd	π
Upper Itapecuru	9	4	8	0.5833 (0.183)	0.00415 (0.00329)
Middle Itapecuru	13	7	9	0.8718 (0.067)	0.00448 (0.00300)
Lower Itapecuru	9	4	6	0.5833 (0.183)	0.00320 (0.00262)
Total	31	10	11	0.7247 (0.082)	0.00395 (0.00245)

Standard deviations are in parentheses.

The phylogenetic relationships among the 10 haplotypes resulted in trees with similar topology characterized by strong groupings (bootstrap values of 86-100%) among the *L. piau* haplotypes of the Itapecuru basin (Figure 3). Haplotype H3, which was observed in the upper, middle and lower stretches of river, was the most divergent of the haplotypes (Figure 2).

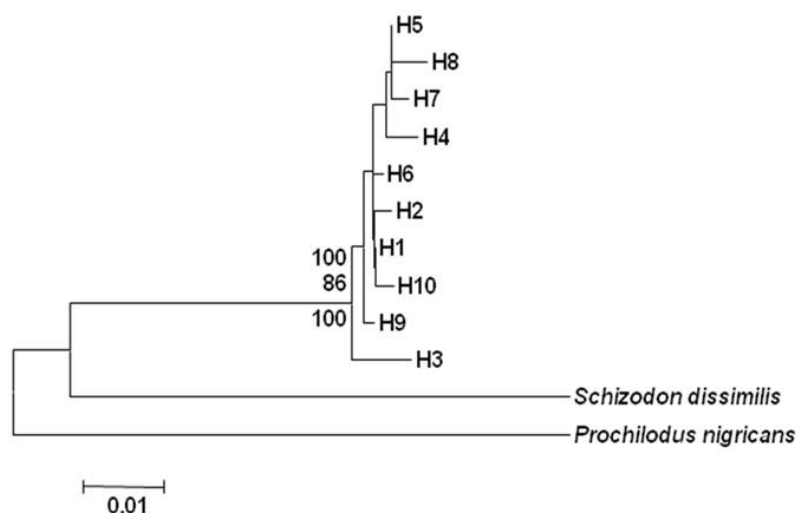


Figure 3. Consensus tree derived from the three different methods (MP, NJ and ML) employed in the present study, based on a 481 bp fragment of the rRNA 16S gene. The numbers above the branch represent bootstrap values for MP and ML, and the number below the branch corresponds to the bootstrap value for NJ, based on 1000 replicates.

The diversity matrix revealed elevated values of divergence among the ten different *L. piau* haplotypes, ranging from 0.2% to 1.9%. The highest values (0.9-1.9%) were recorded for comparisons involving the specimens represented by Lep30 (haplotype H3), which was distinct from all other haplotypes (Table 4).

However, the AMOVA did not indicate any structuring among populations ($\Phi_{ST} = -0.02703$, $P > 0.05$), given that most of the variation was found within populations. The lack of genetic structuring among the *L. piau* samples corroborated these results and indicate clearly the existence of a single population with high levels of gene flow within the Itapecuru basin.

Discussion

Our results demonstrated high levels of haplotype diversity, but low nucleotide diversity (Table 3) for the sample of *L. piau*, further reinforcing the findings of earlier studies, which identified the genus *Leporinus* as one of the most diverse of the Anostomidae (Martins et al., 2003; Morelli et al., 2007). Similar results were obtained by Martins et al. (2003), who studied *D-loop* sequences in six populations of *L. elongatus* from the Paraná River, and detected high levels of haplotype (0.958) and nucleotide (0.0351) diversity.

Table 4. Percentage nucleotide divergence generated by PAUP for 16S rRNA in two anostomid species and one prochilodontid using the GTR + G model with parameters Base=(0.3206 0.2433 0.2226), Nst=6, Rmat=(0.9428 3.2387 2.8927 0.2582 9.1226) Rates=gamma, Shape=0.1795, Pinvar=0.

Haplotype	% Nucleotide divergence											
	1	2	3	4	5	6	7	8	9	10	11	12
1 H1- <i>L. piau</i> Lep20	-											
2 H2- <i>L. piau</i> Lep28	0.2											
3 H3-<i>L. piau</i> Lep30	1.1	1.4										
4 H4- <i>L. piau</i> Lep46	0.7	0.9	1.4									
5 H5- <i>L. piau</i> Lep53	0.2	0.4	1.4	0.4								
6 H6- <i>L. piau</i> Lep8	0.2	0.4	0.9	0.4	0.4							
7 H7- <i>L. piau</i> Lep48	0.4	0.4	1.6	0.7	0.2	0.7						
8 H8- <i>L. piau</i> Lep52	0.7	0.9	1.9	0.9	0.4	0.9	0.7					
9 H9- <i>L. piau</i> Lep49	0.2	0.4	0.9	0.9	0.4	0.4	0.7	0.9				
10 H10- <i>L. piau</i> Lep87	0.2	0.4	1.4	0.9	0.4	0.4	0.7	0.9	0.4			
11 <i>S. dissimilis</i>	16.5	17.0	17.9	18.7	17.2	17.2	17.7	17.5	17.2	17.0		
12 <i>P. nigricans</i>	18.0	18.6	19.5	19.5	18.7	18.0	19.3	19.9	18.0	18.6	25.7	-

Despite the fact that the values of haplotype diversity recorded in this study were similar to those recorded by Martins et al. (2003), it is important to remember that the different regions of the mitochondrial genome analyzed have distinct evolutionary rates. The control region is hypervariable, and, as it does not play a role in codification, mutation rates tend to be two to five times higher than in other portions of the mitochondrial genome (Meyer, 1993). These relatively high rates of nucleotide substitution make this region a preferred tool for population studies in fish (Meyer, 1993; Sivasundar et al., 2001). By contrast, the more conservative rRNA 16S gene, which

has an evolutionary rate of approximately 1% per million years (Tringali et al., 1999), is expected to present relatively low levels of haplotype and nucleotide diversity. This was not the case in the present study, however, which recorded elevated values of genetic variability in the rRNA 16S gene in *L. piau*.

Chiari & Sodr  (1999) also found evidence of elevated levels of genetic polymorphism in their analysis of isoenzymatic markers in species of the genera *Leporinus* (*L. elongatus*, *L. friderici* and *L. obtusidens*) and *Schizodon* (*S. intermedius* and *S. nasutus*). The largest proportion of polymorphic loci was found in *L. friderici* (36.8%) reinforcing earlier findings on its high levels of diversification. Chiari & Sodr  (2001) also recorded a high level of polymorphism in *L. elongatus* (58.7%) in their analysis of RAPD markers. They recorded a value of 46.3% for *L. friderici*, although only four samples were included in the analysis.

High levels of haplotype diversity and low levels of nucleotide diversity have been reported in a number of species of marine fish by Grant & Bowen (1998), based on the analysis of sequences of mitochondrial DNA. These authors conclude that the contrasting diversity values were the result of either population expansion following a long period of reduced effective population size or rapid population growth accompanied by an increase in the retention of new mutations. Grant & Bowen (1998) noted that many of the species exhibiting this contrasting pattern of diversity appear to have originated in the Pliocene or early Pleistocene.

Overall, then, it seems clear that there is a single population of *L. piau* in the Itapecuru River basin, in northeastern Brazil, with no evidence of genetic structuring. The data presented here revealed a strong grouping among the *L. piau* haplotypes in all the different methodological approaches (Figure 3). However, one haplotype (H3) was relatively divergent, and was separated from the most common haplotype (H1) by five mutational steps (Table 2 and Figure 2), but the data are not sufficient to determine whether this was due to an ancient genetic division of the haplotypes.

Similarly high indexes of genetic diversity (0.077 and 0.060, respectively) were recorded by Martins et al. (2003) in their analysis of sequences of the control region of *L. elongatus* from the Paranapanema and Paran -Ayolas rivers in Brazil and Paraguay. A relatively low value (0.016) was recorded, however, for the population from the municipality of Gua ra (Paran  River).

In French Guiana, Renno et al. (1989) recorded genetic divergence of 0.004 between two populations of *L. friderici*, using isoenzymatic markers. These authors recorded much higher values, however, when comparing populations of this species from distinct regions, such as the Mogi-Gua u River in the Paran  basin and Januaca Lake in the Amazon basin (0.239), and between Brazil and French Guiana, 0.222 (Renno et al., 1990). Within French Guiana, however, these authors recorded a distance of 0.029 between two distinct clades. In their subsequent study of mitochondrial DNA using RFLP markers, Renno et al. (1991) identified a discontinuity in the gene flow in the

central region of French Guiana, associated with the Guianan aquatic refuge in the region of the Kourou River.

The indexes of genetic divergence recorded in the present study are closely similar to those reported by Martins et al. (2003) for *L. elongatus* from the Paraná River basin. The differences in the values are probably related to the analysis of the much more variable control region (*D-loop*) and different populations distributed within the basin. Clearly, then, species of the genus *Leporinus* present considerable differentiation both within and between populations, based on the analysis of different markers (Renno et al., 1989, 1990, 1991), a pattern also confirmed in the present study, in which considerable divergence was detected among the different sequences, reflecting the within-population variability in *L. piau*.

Indexes of genetic variability are important tools for the evaluation of the vigor of natural populations, and provide fundamental parameters for the development of conservation and management strategies, given the importance of the genetic makeup of a population with regard to its ability to adapt to a given environment (Ryman, 1991). Given the importance of *L. piau* as a fishery and aquaculture resource, and the drastic decline in their natural populations in recent years, the results of the present study of the rRNA 16S gene in *L. piau* provides potentially important insights into the genetic differentiation of this species, which may even have taxonomic implications for the genus as a whole. Any such developments will also have important implications for the development of successful management and conservation programs.

Conclusion

The present analysis has revealed that the *L. piau* population sampled from the upper, middle and lower Itapecuru River basin represents a single stock that occupies a total area of 52,972.1 km², which may be particularly important for the reproductive cycle of the species, and demands special attention with regard to its conservation status.

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