Vol.49, n. 4 : pp. 589-598, July 2006
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Genetic Variability in Four Fish Species (*Pimelodus maculatus, Prochilodus lineatus, Salminus brasiliensis* and *Steindachneridion scripta*) from Uruguay River Basin

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ABSTRACT

The genetic variability of four fish species (Pimelodus maculatus, Prochilodus lineatus, Salminus brasiliensis and Steindachneridion scripta) collected in the upper Uruguay River basin was analyzed using the RAPD technique. A total of 118 amplified fragments was obtained, 11 for P. maculatus, 29 for P. lineatus, 45 for S. brasiliensis and 33 for S. scripta. Amplified fragments with monomorphic profile were not found in the studied species, except for S. brasiliensis, which presented seven monomorphic bands for Saltinho population. All species showed high levels of genetic variability among individuals.

Key words: RAPD, *Pimelodus maculatus, Prochilodus lineatus, Salminus brasiliensis, Steindachneridion scripta*

INTRODUCTION

The aquatic ecosystems are among those where life diversity expresses itself in an intense manner. The upper Uruguay River basin has a total area of approximately 365,000 Km^2, where 176,000 Km^2 are in Brazilian territory, being 46,000 Km^2 situated in Santa Catarina state and 130,000 km^2 in Rio Grande do Sul state. Recent studies in the basin described several fish species based on morphologic criteria. Actually, more than 100 fish species have been registered for only the upper Uruguay basin (Zaniboni-Filho and Schulz, 2003).

The aquatic ecosystems have suffered aggressions like predatory fishing, introduction of exotic species, deforestation, pollution and hydroelectric dam implementations. They have caused deep modifications in environment dynamics, jeopardizing the rich fish variety (Solé-Cava, 2001). The comprehension of genetic differences of native fish populations is fundamental for long term fisheries management. Informations derived from molecular genetic techniques shall contribute significantly for the preservation of aquatic genetic resources and sustainable development (Carvalho, 1993; Chiari, 1999; Martins et al, 2003; Almeida et al, 2003).

The RAPD procedure (Randomly Amplified Polymorphic DNA) has been instrumental to understand the genetic variability of fish populations (Bielawski and Pumo, 1997; Caccone et al., 1997; Callejas and Ochando, 1998; Dergam et al., 1998; Nadig et al., 1998; Liu et al., 1999; Prioli et al., 2002; Shikano and Taniguchi, 2002;

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Almeida et al., 2003; Bártfai et al., 2003; Hatanaka and Galetti Jr, 2003). This technique consists of amplification by PCR (Polymerase Chain Reaction) of random segments of genomic DNA using a single short primer of arbitrary sequence. RAPD can detect high levels of polymorphism and produce genetic markers (Welsh and McClelland, 1990; Williams et al., 1990).

To evaluate the genetic variability of *Pimelodus maculatus* (Lacépède,1803), *Prochilodus lineatus* (Valenciennes,1836), *Salminus brasiliensis* (Cuvier, 1817) and *Steindachneridion scripta* (Miranda Ribeiro, 1917) in the Uruguay River basin, RAPD analysis were performed. There are not previous reports in the literature about the genetic variability of these species for the Uruguay River basin. The results described here could be used for stocks maintenance of the studied species in hatchery programs.

MATERIAL AND METHODS

Samples

Fish samples from different locations from the upper Uruguay River basin were collected in 2001 and 2002 (Table 1 and Fig. 1). Tissue samples (muscle or fins) were individually preserved in ethanol 80%. The following specimens were analyzed: 13 of *P. maculatus*, 11 of *P. lineatus*, 22 of *S. brasiliensis* and 13 of *S. scripta*.

Genomic DNA extraction

Total genomic DNA was obtained from fish muscle or fin samples using CTAB buffer according to Doyle and Doyle (1987) and Dergam (1996) or using TNES-urea buffer (10 mM Tris-HCl pH 8.0; 125 mM NaCl; 10 mM EDTA; 0.5% SDS; 4 M urea) according to Asahida et al. (1996). Purified DNA was maintained at 4°C for immediate use or stored at −20° C. DNA concentration was estimated using a spectrophotometer (Hitachi model U2010).

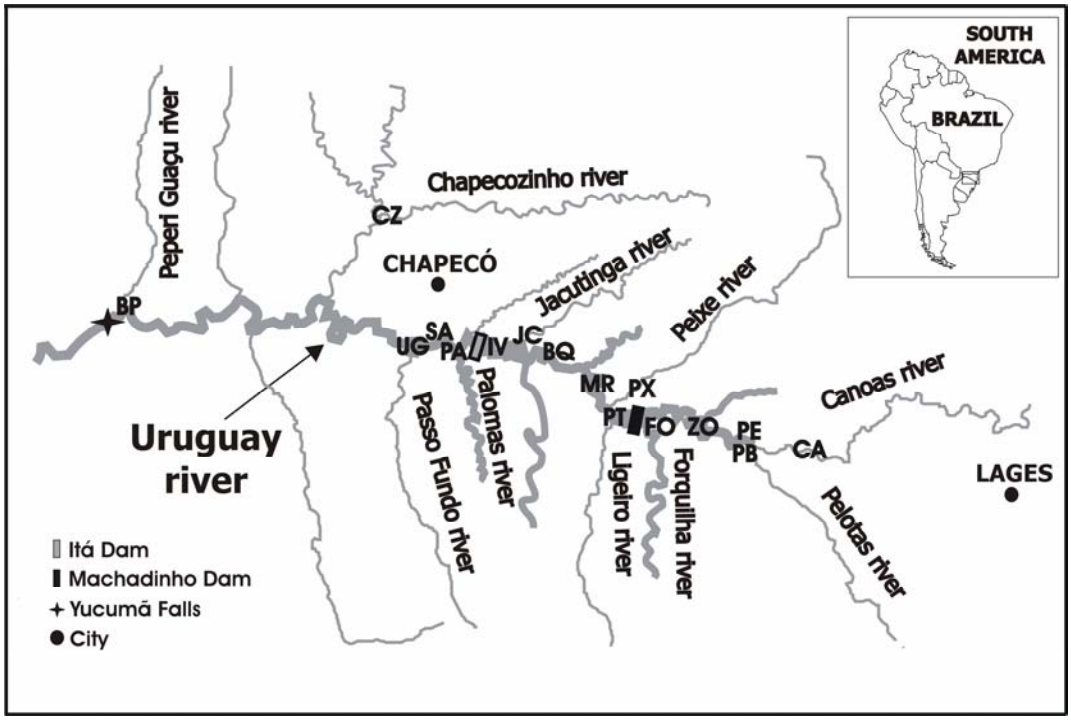

Figure 1 - Map of the sampling sites in the upper Uruguay River basin. BP = Barra do Peperi-Guaçu; CZ = Chapecozinho; UG = Uruguay Goio-En; SA = Saltinho; PA = Palomas; IV = Itá Velha; JC = Jacutinga; BQ = Barra dos Queimados; MR = Marcelino Ramos; PX = Peixe; PT = Pelotas-Túneis; FO = Forquilha; ZO = Zortéa; PE = Pelotas; PB = Pelotas-Barracão; CA = Canoas

RAPD analysis

DNA amplifications were performed in a final volume of 25 µl containing approximately 100 ng of genomic DNA, 1 µM of primer (Invitrogen), 0.2 mM of each dNTP, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$ and 1U of *Taq* DNA polymerase (Invitrogen). Eleven 10-mer primers were used to generate RAPD fragments (Table 2). PCR reactions were carried out in a thermal cycler (MiniCycler™ MJ Research) programmed for 94°C for 2 min., 44 cycles of 94°C for 1 min., 36°C for 30 sec., 72°C for 2 min. and finally 72°C for 7 min. One negative control (absence of template DNA) was performed for each set of amplifications. DNA amplification fragments were separated by electrophoresis at 80 V on 1.5% agarose gel with Tris-borate-EDTA buffer (Sambrook et al, 1989).

After 90 min, DNA fragments were stained with ethidium bromide and photographed under ultraviolet light with a digital photosystem (UVP). DNA fragments length was estimated by comparison with 1 kb ladder (λ DNA/Hind III, Invitrogen and DRIgestTM III, Amershan).

Table 1 - Sampling sites, codes, localities, habitat and sample size of fish species from the upper Uruguay River basin.

Sampling sites	Codes	Localities	Habitat	Sample size
Pimelodus maculatus				**13**
Barra dos Queimados	BQ	Itá	Reservoir	01
Itá Velha	IV	Itá	Reservoir	01
Forquilha	FO	Machadinho	Reservoir	02
Pelotas-Barracão	PB	Machadinho	Reservoir	01
Zortéa	ZO	Machadinho	Reservoir	01
Peixe	PX	Itá	Reservoir	04
Pelotas-Túneis	PT	Uruguay River	River	01
Canoas	CA	Canoas River	River	02
Prochilodus lineatus				**11**
Saltinho	SA	Uruguay River	River	04
Palomas	PA	Palomas River	River	07
Salminus brasiliensis				**22**
Foz do Chapecozinho	CZ	Chapecó River	River	01
Jacutinga	JC	Jacutinga River	River	08
Palomas	PA	Palomas River	River	01
Barra do Peperi-Guaçu	BP	Peperi-Guaçu River	River	01
Uruguay Goio-En	UG	Uruguay River	River	01
Marcelino Ramos	MR	Uruguay River	River	01
Saltinho	SA	Uruguay River	River	09
Steindachneridion scripta				**13**
Pelotas-Túneis	PT	Uruguay River	River	02
Saltinho	SA	Uruguay River	River	03
Pelotas	PE	Uruguay River	River	07
Uruguay Goio-En	UG	Uruguay River	River	01

Data analysis

RAPD profiles were compared among individuals of the same species, and reproducible fragments were selected for analysis. DNA bands were scored as present (1) or absent (0) in order to estimate Jaccard Similarity Coefficient among the individuals. Data analysis was performed with the NTSYS program version 2.02 (Rohlf, 1992). Genetic similarity dendrograms among the individuals were constructed from the Jaccard Similarity Coefficient data by the UPGMA clustering method.

RESULTS

Nine out of eleven primers employed in *P. maculatus, P. lineatus, S. brasiliensis* and *S. scripta* allowed estimation of genetic similarity (Table 2).

Pimelodus maculatus

For *P. maculatus*, six fragments were observed after amplification with primers A02 and and five for primer A16 (Table 2). The dendrogram obtained through RAPD data analysis (Fig. 2) showed that the samples of *P. maculatus* were grouped in 0.34 to 1.00 genetic similarity interval. From the thirteen samples, twelve presented a similarity coefficient higher than 0.62. Nine samples presented similarity equal to 1.00, forming a group for the PX-PT-BQ-IV-PB locations, a group for the FO-ZO, and other for the CA-PX.The collected individuals in FO and ZO (sites located in Machadinho reservoir) formed a very distinct group. Individuals from sites IV, BQ, PT and PX, that were located between Itá and Machadinho dams along the old Uruguay River watercourse, formed a highly similar cluster. Specimens collected in PX were similar to most clusters suggesting that this region harbored a diverse population.

Prochilodus lineatus

Genetic analysis of *P. lineatus* was carried over with six primers that generated two to eight fragments per primer (Table 2), and all of them were polymorphic. *P. lineatus* samples were grouped in an interval from 0.03 to 1.00 of genetic similarity (Fig. 3). From the eleven analyzed specimens, four presented similarity coefficient higher than 0.50. Two Saltinho site specimens were grouped with Jaccard Similarity Coefficient equal to 1.00.

Salminus brasiliensis

Forty five RAPD fragments were obtained after the amplification with nine primers (Table 2), and all of them were polymorphic for *Salminus brasiliensis*. DNA fragment number varied from three to eight per primer. The 22 *S. brasiliensis* samples were grouped in a large interval from 0.06 to 0.96 (Fig. 4).

Table 2 - Primer sequences and number of DNA fragments obtained for the analyzed species from the upper Uruguay River basin.

Primers	Sequence (5'-3')	Number of DNA fragments			
		Pimelodus maculatus	***Prochilodus Lineatus***	***Salminus brasiliensis***	***Steindacheridion scripta***
A01	CAGGCCCTTC	-	04	04	-
A02	TGCCGAGCTG	06	08	05	04
A03	AGTCAGCCAC	-	05	06	07
A04	AATCGGGCTG	-	-	06	02
A10	GTGATCGCAG	-	-	-	-
A11	CAATCGCCGT	-	03	03	06
A16	AGCCAGCGAA	05	-	04	06
F03	CCTGATCACC	-	02	05	05
F04	GGTGATCAGG	-	-	08	03
F05	CCGAATTCCC	-	07	04	-
F07	CCGATATCCC	-	-	-	-
Total		**11**	**29**	**45**	**33**

- : absence of amplified fragments.

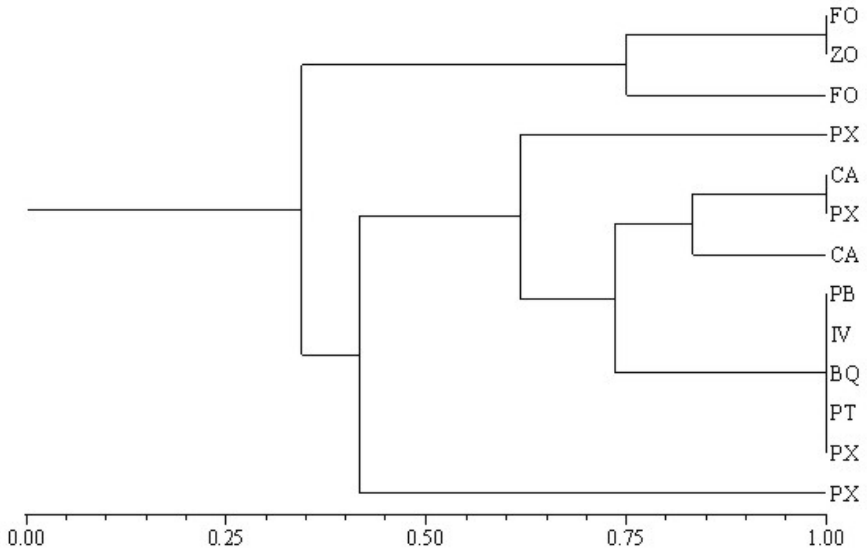

Figure 2 - Dendrogram obtained with Jaccard Similarity Coefficient and UPGMA method for *Pimelodus maculatus* individuals collected in upper Uruguay River basin. Codes of sampling sites are listed in Table 1.

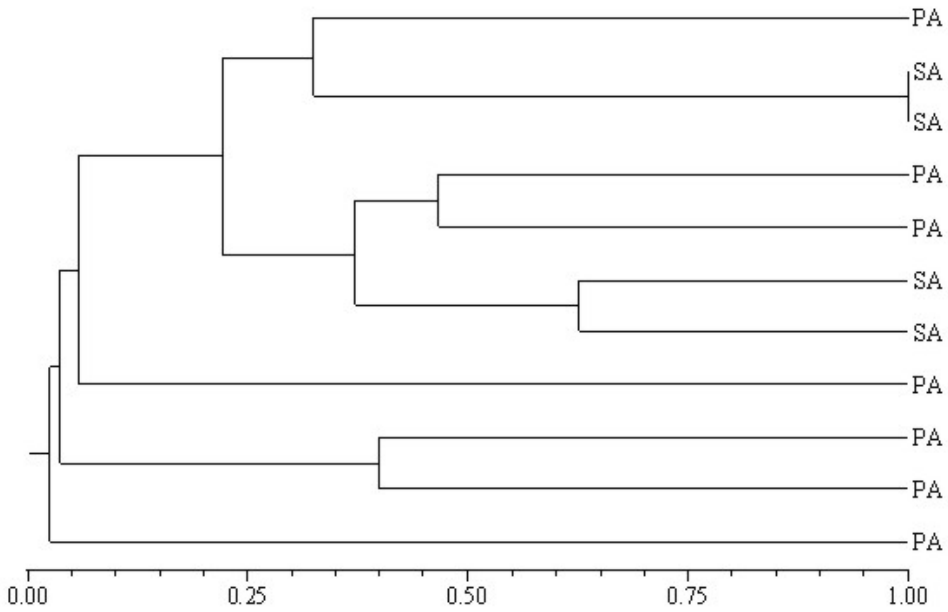

Figure 3 - Dendrogram obtained with Jaccard Similarity Coefficient and UPGMA method for *Prochilodus lineatus* individuals collected in upper Uruguay River basin. Codes of sampling sites are listed in Table 1.

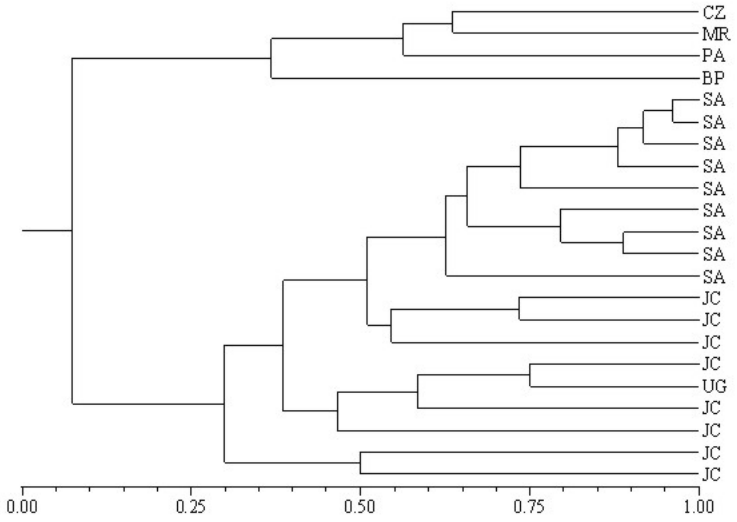

Figure 4 - Dendrogram obtained with Jaccard Similarity Coefficient and UPGMA method for *Salminus brasiliensis* individuals collected in upper Uruguay River basin. Codes of sampling sites are listed in Table 1.

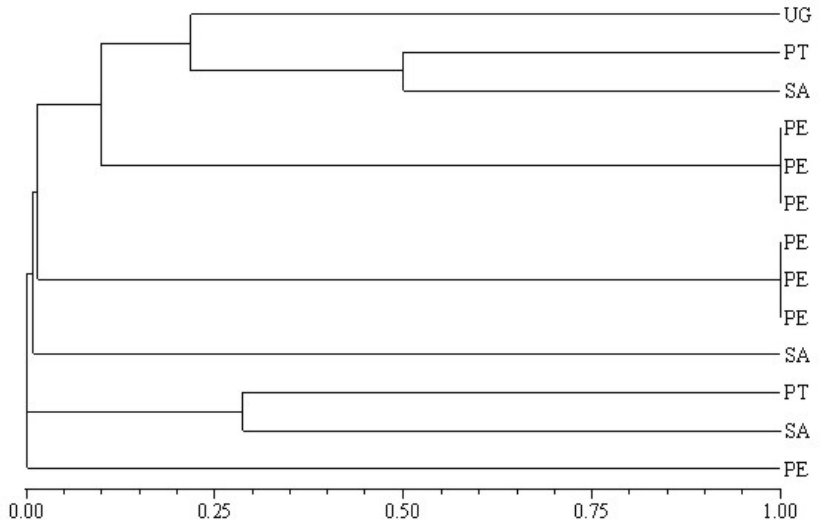

Figure 5 - Dendrogram obtained with Jaccard Similarity Coefficient and UPGMA method for *Steindachneridion scripta* individuals collected in upper Uruguay River basin. Codes of sampling sites are listed in Table 1.

When the nine samples from Saltinho were analyzed in separate, 38 bands were observed, from which seven were monomorphic. One of these monomorphic bands occurred only for Saltinho population. From the 22 analyzed specimens, 20 (91%) presented similarity coefficient higher than 0.50.

Steindachneridion scripta

For this species, seven primers generated a total of 33 RAPD fragments (Table 2) and all of them were polymorphic. The higher number of DNA fragments (07) was obtained with the A03 primer and the lowest (02) with the A04 primer (Table 2). Specimens of *S.scripta* clustered in an interval

from 0.00 to 1.00 genetic similarity coefficients. From the 13 analyzed specimens, eight presented similarity coefficient higher than 0.50.
The samples from the Pelotas River presented highest genetic similarity coefficient, six samples presented a similarity coefficient equal to 1.00, although these samples were grouped in two different groups (Fig. 5).

DISCUSSION

The present study is the first that used RAPD to estimate genetic variability in fish species from the upper Uruguay River basin. RAPD markers are efficient in species discrimination, particularly in cases where morphologic characteristics have low resolving power (Callejas and Ochando, 1998). Besides the classical application on estimates of genetic similarity among populations, RAPD markers have been used successfully to determine species-specific traits in fishes (Dinesh et al., 1993; Takagi and Taniguchi, 1995; Callejas and Ochando, 1998) and for reconstructing phylogenetic relations among species and sub-species (Bardakci and Skibinski, 1994; Callejas and Ochando, 1998).
Since heterozigotes are not separated from homozigotes due to the dominant character from the RAPD markers, the absence of amplification of a band in two genotypes does not necessarily represent genetic similarity between them. Jaccard Similarity Coefficient, which has been widely used for the RAPD markers statistical analysis, takes in consideration only the discordance and positive concordance, regarding the presence or absence of bands, without considering the negative concordance (Duarte et al., 1999), and it is thus suitable for genetic similarity analysis of populations.
All species showed high levels of genetic variability among individuals. Natural populations with high genetic variability are usually considered as suitable to compose fish culture stocks. According to Toledo-Filho et al. (1992) hatchery stocks may be derived from wild populations from the same basin when they show low levels of inbreeding and high biological potential to adapt to the reservoir conditions. The advantages of stocks with high genetic variation reside in better changes to adapt to environmental challenges (Meffe and Carroll, 1997).

Pimelodus maculatus
(Lacépède,1803), order Siluriformes, family Pimelodidae. Almeida et al. (2003) performed studies in the *P. maculatus* population structure for the Tietê and Paranapanema Rivers and obtained genetic identity values around 0.96 for the Tietê and 0.91 for the Paranapanema River. These values were similar to those reported by Thorpe and Solé-Cava (1994), who found that 98% of the populations of the same species showed a genetic identity higher than 0.85.
This species is abundant and widespread in the Uruguay River basin (Zaniboni Filho and Schulz, 2003). According to Dergam et al. (2002), widely distributed species might present genetic variation patterns which are not expected as a result of their evolutionary histories. Genus *Pimelodus* is considered to be complex, where taxonomy of species and populations similar to the type-species *P. maculatus* is not well established at the species level (Lundberg et al., 1991, Almeida et al., 2003). According to Azpelicueta (1995) *P. maculatus* has been confused with the *P. absconditus* due to similar color patterns. Despite the morphological similarity, the collected individuals could be discriminated very well from both species found in Uruguay River.

Prochilodus lineatus
(Valenciennes,1836), order Characiformes, family Prochilodontidae. High levels of variability found among the individuals could be associated with the migratory species behavior and with the population size. According to Sivasundar et al. (2001), *Prochilodus* are known for their high swimming capacity and their ability to migrate upstream and even overcoming rapids and falls.
The knowledge regarding the *Prochilodus* population genetics is still scanty (Hatanaka and Galetti Jr, 2003). Genetic variability of *P. lineatus* captured in three regions of the Paraná River basin was evaluated by isoenzyme patterns (Revaldaves et al., 1997). Genetic differences among samples from the three regions were not observed, suggesting unique genetic profile spread along the studied area. High genetic variability among individuals were observed in Uruguay River even when they were collected in the same location, suggesting the existence of different populations in the same region.

Salminus brasiliensis

(Cuvier, 1817), order Characiformes, family Characidae. Data about genetic variability for this species have not been found in the literature. *S. brasiliensis* samples were clustered in a large interval of genetic similarity and the majority (91%) with similarity coefficient higher than 0.50. Although the high variability was observed, samples collected in Saltinho location showed monomorphic fragments and one fragment was specific for this population, indicating a possible Saltinho specific population marker. DNA fragments obtained from RAPD may be used as species-specific or population-specific markers if they are present in all samples of a species (Hadrys et al., 1992).

Genetic variability estimation included 100% polymorphic sampled fragments. According to Zaniboni Filho and Schulz (2003), *S. brasiliensis* occured along the whole Uruguay River and before the construction of Itá dam, only the Yucumã Falls was a barrier for the species migration during the low-water period. According to Nei (1977), high polymorphisms values are expected for species that present populations of large sizes and occupies heterogeneous environments, once the inbreeding possibilities are smaller.

Individuals were clustered in two well-defined groups (Fig. 4): one formed by SA-JC-UG individuals (group 1) and another by individuals collected in BP-CZ-PA-MR (group 2). Jacutinga individuals did not form a unique cluster with direct similarity, once the clustering were sequential, grouped in a range from 0.30 to 0.73. The cluster formed by Saltinho population was in a range from 0.63 to 0.96, showing high genetic similarity among individuals. Group 2 did not present high internal similarity, but it was well different from group 1. According to Carvalho (1993), if populations inhabit similar environments or stay in communication, through migration and gene flow, they may be genetically homogeneous. In face of this, the non-separation of individuals could suggest the existence of gene flow between the populations of Saltinho and Jacutinga.

The sites where group 2 individuals were collected presented distinct characteristics. Uruguay basin as a dynamic habitat may favor the existence of structured populations. Despite the variability and the fact that *S. brasiliensis* constitute a long distance migratory species, there may be some kind of structure organization among the individuals, indicating the occurrence of distinct populations. According to Zaniboni Filho and Schulz (2003), this species can be found in large shoals immediately downstream of natural obstacles, during the upstream migration related to the reproductive behavior.

Steindachneridion scripta

(Miranda Ribeiro, 1917), order Siluriformes, family Pimelodidae. The samples from the Pelotas River were the ones that presented highest genetic similarity coefficients, although these samples were grouped in two different groups. No study was found in the literature about the genetic variability of Steindachneridion scripta, although for S. brasiliensis. Thorpe (1982) determined Nei's genetic identity indices and found standard genetic diversity values for co-specific populations (0.95 to 1.0) for species of the same genus (0.35 a 0.85) and for genera of the same family (0.0 a 0.60). According to these indices, the individuals collected in the Pelotas River were unique, except one sample, which matched co-specific population levels.

Uruguay River basin fish populations

Knowledge related to the population structure, through genetic variability estimations, are important in the natural populations conservation efforts and recovery programs. Although future studies are necessary in order to comprehensively characterize and identify the Uruguay River basin fish populations, a hypothesis can be raised for the high genetic diversity found within the species populations under study. The data suggested that these natural populations were isolated by geographic accidents in the past, accumulating polymorphisms and genetic divergences proportional to the demographic factors and the period of isolation.

The Uruguay River basin has been over the sedimentary and volcanic rocks which originated the geological formation known as Serra Geral, represented by the presence of basaltic material that spread over a wide area from the end of the mesozoic age to the tertiary. The upper Uruguay River profile is escalated and, due to its geological formation presents some significant nip in its main stream bed, as in some of its affluents (ANEEL, 2003; Zaniboni Filho and Schulz, 2003). The Uruguay River is composed by a sequence of wells and waterfalls, the largest is Yucumã Falls (1,215 km). There were two others: Salto Grande

Falls that disappeared in 1979 with the construction of Salto Grande dam and Augusto César strait that disappeared with the Itá dam construction, whose reservoir filling begun in December 1999 (CARU, 1993; Zaniboni Filho and Schulz, 2003). Due to these environmental changes, the fish populations formerly separated by natural accidents during the low-water period, spread along the basin in new reservoirs. In order to complement the data described here, fish populations are under analysis by use of telemetry of selected individuals.

ACKNOLEDGEMENTS

This work received financial support from Tractebel Energia. MSR and MAK were supported by fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.

RESUMO

A diversidade da vida se expressa de modo extraordinário nos ecossistemas aquáticos. A bacia do alto rio Uruguai é um exemplo desta condição, onde há registro de mais de 100 espécies de peixes. A compreensão das diferenças genéticas entre as diversas populações nativas é fundamental para a manutenção de seus estoques. A variabilidade genética de quatro espécies de peixes (*Pimelodus maculatus*, *Prochilodus lineatus*, *Salminus brasiliensis* e *Steindachneridion scripta*) coletadas na bacia do alto rio Uruguai foi analisada utilizando-se a técnica de RAPD. Obteve-se um total de 118 fragmentos amplificados, sendo 11 para *P. maculatus*, 29 para *P. lineatus*, 45 para *S. brasiliensis* e 33 para *S. scripta*. Fragmentos de caráter monomórfico não foram encontrados para as espécies estudadas, com exceção de *S. brasiliensis* de Saltinho que apresentou sete bandas monomórficas para estes indivíduos. As análises estatísticas mostraram altos níveis de variabilidade genética entre os indivíduos das espécies estudadas.

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Received: December 17, 2004;
Revised: October 03, 2005;
Accepted: April 04, 2006.