

# Study of eight species of the Anostomidae family (Pisces, Characiformes) by RAPD analysis

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**ABSTRACT.** Random Amplified Polymorphic DNA (RAPD) techniques were applied to eight Anostomidae species to analyze the genetic variability and genetic similarity within and between species. The results obtained were compared to those from isoenzyme analysis executed for many of the species studied. The proportion of polymorphic loci obtained with 10 primers was over 40%, with the exception of *Leporinus amblyhynchus*. This is especially important since genetic variability is a population feature both for short term fitness of the individual and for the long term survival of populations. Through subsequent analysis of dendrograms it was possible to discriminate the two *Schizodon* species from those of the genus *Leporinus*, even though it was impossible to differentiate *L. obtusidens* and *L. elongatus*. RAPD analysis indicates that the two species are genetically very similar, supported too by isoenzyme data.

**Key words:** DNA, polymorphism, genetic variability, RAPD, Anostomidae, fish.

**RESUMO.** Estudo de oito espécies da família Anostomidae (Pisces, Characiformes) através da análise de RAPD. A técnica de Polimorfismo de DNA Amplificado ao Acaso (RAPD) foi utilizada na análise de oito espécies de peixe da família Anostomidae, com a finalidade de quantificar a variabilidade genética e estimar a similaridade genética dentro e entre essas espécies. Os resultados foram comparados com dados obtidos anteriormente com isoenzimas, para a maioria das espécies analisadas. A proporção de locos polimórficos obtida, com dez *primers*, está acima de 40 %, exceto para *Leporinus amblyhynchus*. Esse é um fato importante desde que a existência de variabilidade genética é uma condição importante para a sobrevivência das espécies no meio ambiente. Com dendrograma obtido, através de análise comparativa, foi possível discriminar as duas espécies de *Schizodon* daquelas do gênero *Leporinus*, no entanto não foi possível separar *L. obtusidens* e *L. elongatus*. A análise de RAPD indica que essas duas espécies são geneticamente muito similares, conclusão também sustentada por dados de estudos de isoenzimas.

**Palavras-chave:** DNA, polimorfismo, variabilidade genética, RAPD, Anostomidae, peixe.

The Anostomidae family comprises about twelve genera distributed exclusively in Central and South America (Greenwood *et al.*, 1966). In an ichthyofauna study of five localities along the Tibagi river, state of Paraná, Brazil, Bennemann *et al.* (1995) found nine species of the family Anostomidae: *Leporellus vittatus*, *Leporinus amblyhynchus*, *Leporinus elongatus*, *Leporinus friderici*, *Leporinus obtusidens*, *Leporinus octofasciatus*, *Leporinus striatus*, *Schizodon intermedius* and *Schizodon nasutus*. This is the largest family in the number of species described in this basin.

The Tibagi river basin in the state of Paraná (Brazil) is a rich hydrographical net with 65 main branches and hundreds of small tributaries. The river basin area has approximately 26.000 km<sup>2</sup> and

cover 13% of the surface of the state (cf. Bennemann *et al.*, 1995).

The karyotypes of several species of different genera of this family have been described in the relevant literature (Galetti Jr. *et al.*, 1981, 1984, 1995; Koehler *et al.*, 1997). The family Anostomidae has been studied by isoenzyme analysis (Panepucci *et al.*, 1984, 1987; Renno *et al.*, 1989, 1990; Monteiro *et al.*, 1991). Chiari and Sodré (1999) studied the genetic variability of five species of great importance to commercial fishing: *Leporinus elongatus*, *L. friderici*, *L. obtusidens*, *Schizodon intermedius* and *S. nasutus* from one locality of the Tibagi river basin, employing seven isoenzyme systems. These authors registered high identity rates between *S. intermedius* and *S. nasutus* (0.962) and between *L. elongatus* and *L.*

*obusidens* (0.949). They also stated that the estimated identity value between *L. friderici* and *S. intermedius* and *S. nasutus* was greater than the values observed between *L. friderici* and the other species of *Leporinus* under analysis. More recently Martins and Galetti Jr. (1998) used specific primers to amplify the 5S rDNA region to study the species *Leporinus elongatus*, *L. friderici* and *L. obtusidens*.

Genetic variability is an important feature of populations, both for short term fitness of individuals and for long term survival of the population, by allowing adaptation to changing environmental conditions. Genetic variation is similarly important in farmed populations since it concentrates on selective breeding and prevents loss of fitness due to inbreeding depression.

The development of Random Amplified Polymorphic DNA (RAPD) markers allows the examination of genome variation without *a priori* knowledge of DNA sequences (Williams *et al.*, 1990, Welsh and McClelland, 1990). This method is based on the hypothesis that single DNA primers of arbitrary nucleotide sequence can amplify genome DNA sequences in PCR (Mullis and Faloona, 1987) whenever locating regions of sufficient similarity are present at a favorable distance and in converging orientations on the two DNA strands. This method has been successfully used to detect genetic diversity within and among strains, species and subspecies and to assess genome variability (Bardakci and Skibinski, 1994, Naish *et al.*, 1995, Borowsky *et al.*, 1995, Bielawski and Pumo, 1997, Elo *et al.*, 1997, Almeida *et al.*, 2001).

However, its use in a phylogenetic context is not widely accepted, mainly because of the not-completely understood nature of RAPD polymorphism (Landry and Lapointe, 1998). Other limitations have been related to the standardization of the amplification conditions (Ferreira and Grattapaglia, 1996).

RAPD methodology has been applied in studies aiming at species identification whose morphological discrimination remains difficult (Takagi and Taniguchi, 1995; Dinesh *et al.*, 1995; Almeida *et al.*, 2001).

The present study is part of the integrated project "Aspects of Fauna and Flora in the Tibagi River Basin" undertaken by State University of Londrina. It aims at broadening knowledge about the various segments of animal and plant communities, their interrelationships and importance of the environmental factors, to provide a base for environmental education, to restore the river bank vegetation and recompose the food chain,

to restore the river basin. Although ichthyofauna surveys have been performed in this basin, estimates of genetic variability within and among populations of the representative species are practically nonexistent (Almeida and Sodr , 1998; Chiari and Sodr , 1999; Almeida *et al.*, 2001).

The objective of the present study is to quantify the genetic variability and to estimate genetic similarity by RAPD in the Anostomidae species of the Tibagi river basin, described by Bennemann *et al.* (1995), with the exception of *Leporellus vittatus*. Current data will be compared to previous isoenzyme data obtained by Chiari and Sodr  (1999).

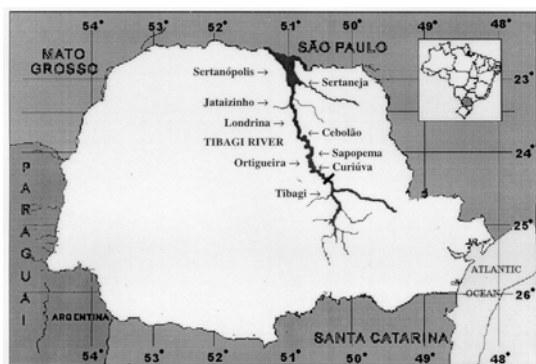
## Material and methods

**Species and localities.** Specimens of *Leporinus amblyrhynchus*, *L. elongatus*, *L. friderici*, *L. obtusidens*, *L. octofasciatus*, *L. striatus*, *Schizodon intermedius* and *S. nasutus* were collected between 1997 and 1998 from nine localities along the Tibagi river basin (Paran , Brazil): Tibagi, belonging to the upper Tibagi (UT); Sapopema, Curi va, Cebol o and Ortigueira, the middle Tibagi (MT); and Londrina, Jataizinho, Sert opolis and Sertaneja, the lower Tibagi (LT). Sert opolis and Sertaneja are locations with still waters due to the convergence of the Paranapanema river. The river is dammed by the Get lio Vargas hydroelectric plant downstream the Tibagi site (Figure 1). Samples of blood and muscle were removed from specimens immediately after capture and stored at -20 C until use. The number of specimens analyzed from each region is given in Table 1.

**Table 1.** Number of analyzed individuals of eight Anostomidae species from different regions of Tibagi river basin

Species	Abbreviation	Lower Tibagi (LT)	Middle Tibagi (MT)	Upper Tibagi (UT)	Total
<i>Leporinus amblyrhynchus</i>	Lam	-	4	-	4
<i>Leporinus elongatus</i>	Lel	9	8	-	17
<i>Leporinus friderici</i>	Lfr	4	-	-	4
<i>Leporinus obtusidens</i>	Lob	9	8	-	17
<i>Leporinus octofasciatus</i>	Loc	-	9	6	15
<i>Leporinus striatus</i>	Lst	-	-	6	6
<i>Schizodon intermedius</i>	Sin	10	-	-	10
<i>Schizodon nasutus</i>	Sna	9	3	4	16

**DNA extraction.** DNA was extracted from blood samples following the method described by Fairbanks *et al.* (1993); muscle samples were processed according to method described by Shiozawa *et al.* (1992), both with modifications described by Almeida *et al.* (2001).



**Figure 1.** Map of the Tibagi river basin with designated collection regions

### Amplification conditions and electrophoresis.

A set of ten decamer primers (OPAK 19 and 20; OPAM 04, 10 and 13; OPW 02, 06, 08, 13 and 16, from Operon Technologies, Alameda, CA) were selected for the study of DNA variability, on the basis of the number of bands obtained and also on their ability to produce consistent fragment patterns.

The amplification conditions were based on Williams *et al.* (1990) with some modifications. Amplification reactions were carried out in a total volume of 15  $\mu$ L containing approximately 12–15 ng of template DNA; 0.25  $\mu$ M primer, 3 mM  $MgCl_2$ , 0.25 mM dNTP (Promega Biotec), and 1 unit of *Taq* DNA polymerase (GIBCO BRL) in the reaction buffer supplied (100 mM Tris-HCl pH 8.3; 500 mM KCl). Control reactions were run containing all components except genome DNA.

DNA amplification was performed using an MJ (model PTC-100) thermal cycler. For the first cycle, denaturation, annealing and extension were 92°C for 4 min, 40°C for 1.5 min and 72°C for 2 min, respectively. Denaturation time was decreased to 40 sec. for the following 40 cycles.

RAPD products were resolved by electrophoresis in 1.4% agarose gels run with TBE buffer (0.89 M Tris, 0.89 M boric acid and 0.08 M EDTA, pH 8.3). Electrophoresis was conducted at 3 V  $cm^{-1}$ . Gels were stained with ethidium bromide and photographed under UV light using black and white Kodak film.

RAPD patterns of specimens were compared within and among populations by analyzing one selected primer in each gel with all the individuals of each species collected in the different regions. Comparative analysis among species (interspecific) was performed by using one selected primer in each gel, with four randomly selected individuals of each species, except *Schizodon nasutus* with six individuals analyzed, two individuals from each region.

**Data analysis.** Genetic variability was estimated by the proportion of polymorphic loci calculation ( $\bar{P}$ ), according to Ayala and Valentine (1979) for species collected in a single region only, and by the mean proportion of polymorphic loci ( $\bar{P}_m$ ) for species collected in more than one region.

Each individual was scored for the presence or absence of amplification products. Data were entered into a binary matrix and a pairwise similarity matrix was constructed using the Jaccard's similarity (J) index (Sneath and Sokal, 1973). J values were calculated for the number of shared bands between two individuals divided by the sum of all the bands. An UPGMA cluster based on J values was generated using the NTSYS - PC (Numerical Taxonomy System, Applied Biostatistics, Setauket, New York) computer application software (Rohlf, 1988).

### Results

All the primers examined produced different RAPD fragment patterns. The number of fragments (RAPD loci) generated per primer varied between seven and twenty-seven.

The values of estimated genetic variability (proportion polymorphic loci) to each species is shown in Table 2, *Leporinus amblyrhynchus* exhibited the smallest value  $\bar{P} = 29.3\%$ , while *L. elongatus* displayed the highest ( $\bar{P}_m = 58.7\%$ ). Species collected in more than one region showed higher values of  $\bar{P}$ .

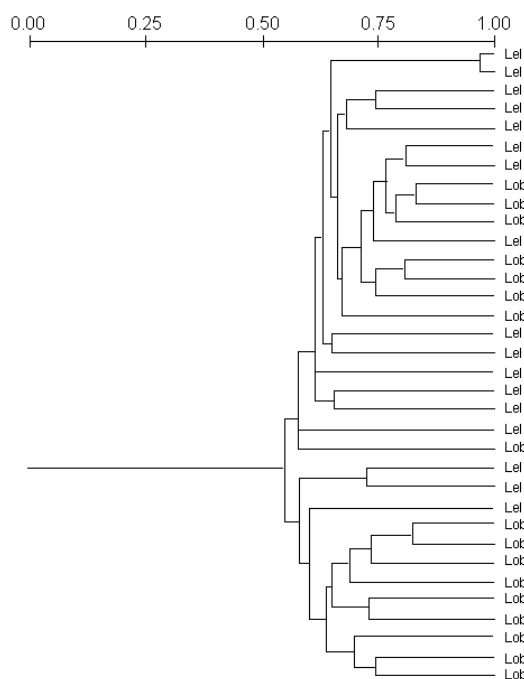
Clusters among individuals of each species collected in just one region from the basin ranged from 0.697 to 0.956 in genetic similarity (data not shown).

The individual dendrograms of species collected in more than one region, except *L. obtusidens*, showed that specimens of different regions were clustered amongst themselves, proving that no genetic differences segregate the populations (data not shown). In this case, clustering among individuals of each species ranged from 0.591 to 0.979.

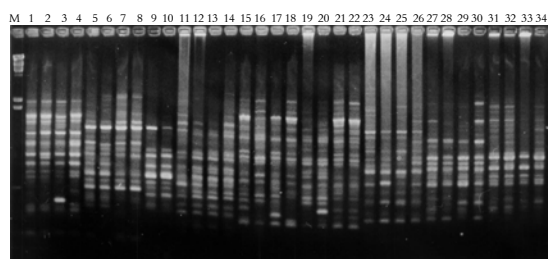
Due to the similarity observed in the fragment patterns produced by primer OPW 13, first used in analysis of *Leporinus elongatus* and *L. obtusidens*, the same species were re-analyzed in the same gel using all primers for a better comparison. The comparative dendrogram constructed for these two species from the RAPD patterns (Figure 2) shows that the individuals of each species do not cluster together.

In the comparative analysis (Figure 3) it can be noted that each species, except *L. elongatus* and *L. obtusidens*, presented a distinct RAPD pattern. The dendrogram constructed (Figure 4) clearly shows the separation of the two genera (*Schizodon* and

*Leporinus*), and also the separation of the two species of genus *Schizodon* and four of the six species of *Leporinus*. As previously commented, *L. elongatus* and *L. obtusidens* were not separable.



**Figure 2.** Dendrogram for *Leporinus elongatus* and *Leporinus obtusidens* obtained by the Jaccard coefficient method UPGMA

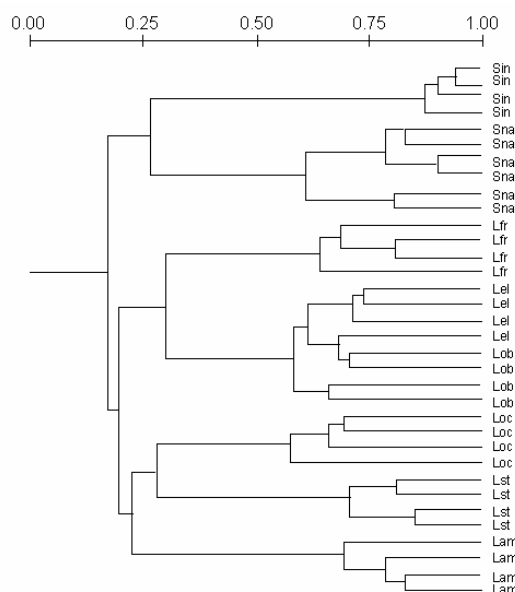


**Figure 3.** Comparative analysis of RAPD markers among individuals of eight Anostomidae species, amplified by primer OPAM 04. Columns: M = molecular weight markers DNA  $\lambda$  Hind III; 1-4= *S. intermedius*; 5-10= *S. nasutus*; 11-14= *L. friderici*; 15-18= *L. elongatus*; 19-22= *L. obtusidens*; 23-26= *L. octofasciatus*; 27-30= *L. striatus* and 31-34= *L. amblyrhynchus*

## Discussion

Among the eight species, *Leporinus amblyrhynchus* had the smallest value of estimated genetic variability. It is worth mentioning that this species was only collected in the middle Tibagi and with a small number of individuals. Nowadays this species

retains one pocket of abundance, (personal communication from ichthyologists of the State University of Londrina) and is considered the most vulnerable to changing environmental conditions (Agostinho *et al.*, 1997).



**Figure 4.** Dendrogram for eight Anostomidae species, *S. intermedius*, *S. nasutus*, *L. friderici*, *L. elongatus*, *L. obtusidens*, *L. octofasciatus*, *L. striatus* and *L. amblyrhynchus*, obtained by the Jaccard coefficient method UPGMA.

**Table 2** Number of polymorphic loci and estimated proportion of polymorphic loci for the eight Anostomidae species

Species	N° Polymorphic loci			Total of loci analyzed	$\bar{P}$ or $\bar{P}m^*$ (%)
	LT	MT	UT		
<i>L. amblyrhynchus</i>	-	36	-	123	29.3
<i>L. elongatus</i>	93	69	-	138	58.7*
<i>L. friderici</i>	69	-	-	149	46.3
<i>L. obtusidens</i>	80	67	-	138	53.1*
<i>L. octofasciatus</i>	-	66	65	119	55.1*
<i>L. striatus</i>	-	-	57	139	41.0
<i>S. intermedius</i>	56	-	-	123	45.5
<i>S. nasutus</i>	102	34	65	162	41.4*

$\bar{P}$  = Proportion of polymorphic loci;  $\bar{P}m$  = Mean proportion of polymorphic loci; LT = Lower Tibagi; MT = Middle Tibagi and UT = Upper Tibagi

Almeida (1998) studied, through RAPD, six species of fish from the Pimelodidae family, hailing from the Tibagi river basin. The author also reported low values of genetic variability in two species of restricted range: *Pimelodella aff. gracillis* ( $\bar{P}$  = 18.8%) and *Pinirampus pinirampu* ( $\bar{P}$  = 27.2%); the other four analyzed species from more than one locality presented values of estimated genetic variability from  $\bar{P}m$  = 43.2% to 53.7%. These values are compatible with those described in this work (Table 2). Small and isolated populations

suffer from genetic variability, greatly limited by genetic drift and inbreeding (Ferguson *et al.*, 1995).

Chiari and Sodr  (1999) used seven isoenzyme systems to quantify the genome variability in five species of the family Anostomidae from the lower Tibagi. *Schizodon intermedius* was the species with the lowest genetic variability ( $\bar{P} = 16.7\%$ ) and mean heterozygosity observed ( $\bar{H}_o = 2.7\%$ ); *Leporinus friderici* had the greatest variability ( $\bar{P} = 36.8\%$  and  $\bar{H}_o = 10.9\%$ ). In the present work a high degree of variability in *L. friderici* ( $\bar{P} = 46.3\%$ ) was also reported, with only four individuals analyzed, while *S. intermedius*, with ten individuals, had a value of ( $\bar{P} = 45.5\%$ ). These same authors obtained  $\bar{P} = 27.8\%$  and  $22.2\%$ ,  $\bar{H}_o = 7.5\%$  and  $5.6\%$  for *L. elongatus* and *L. obtusidens*, respectively. Data obtained by quantifying the genetic variability through RAPD (Table 2) are comparable with those obtained through isoenzymes (Chiari and Sodr , 1999) with regard to these species.

We were unable to find data in the literature about estimates of genetic variability in *Leporinus octofasciatus*, *L. striatus* and *L. amblyrhynchus*. This may be the first study of its kind in the species.

Studies with the purpose of estimating genetic variability, which may also supply knowledge about the structure of the populations, are of extreme importance in the conservation and recuperation of natural populations. High levels of genetic variability may be used to indicate which populations of fish are more appropriate in the formation of stocks used in aquiculture (Toledo-Filho *et al.*, 1992). The species studied presented estimated genetic variability ( $\bar{P}$ ) greater than 40%, with the exception of *L. amblyrhynchus*. This is an important fact since genetic variability is a population feature, both for short term fitness of individuals and for long term survival of the populations.

It is therefore necessary to maintain this genetic variability, which is threatened by the possible construction of five hydroelectric plants in the Tibagi river. The barriers would alter the intensity, duration and period of the high water, reducing the nutrients available from the area's seasonal flooding. They would establish highly unstable thermal and hydrodynamic conditions in the segments immediately downstream from the plants and further interrupt the migratory patterns of several species of fish (Agostinho *et al.*, 1992).

RAPD analysis has been used with success in the identification of species in several organisms including fish. Borowsky *et al.* (1995) tested the use of this technique to separate species of the genus *Xiphophorus* and concluded that RAPD is very

efficient for phylogenetic analysis at species and population levels. Almeida *et al.* (2001) utilized seven primers to distinguish two species of different genera of the family Pimelodidae, *Iheringichthys labrosus* and *Pimelodus* aff. *absconditus*, with high morphological resemblance.

With ten primers it was possible to separate sharply the two genera (*Schizodon* and *Leporinus*); in Chiari and Sodr  (1999) *L. friderici* was more similar to the genus *Schizodon* than to the genus *Leporinus*. It was also possible to separate the two *Schizodon* species (*S. intermedius* and *S. nasutus*), isoenzymatically impossible, and the *Leporinus* ones (*L. octofasciatus*, *L. striatus*, *L. amblyrhynchus* and *L. friderici*), with the exception of *L. elongatus* and *L. obtusidens* (Figure 4). The dendrogram (Figure 2) demonstrates that the genetic similarity of some individuals of *L. obtusidens* are closer to *L. elongatus* than other individuals of *L. obtusidens* and vice-versa. These species are morphologically very similar, although they have two distinguishing characteristics, or rather, height of body and length of snout (Garavello, 1979). Cytogenetic analysis also showed a high degree of homology between *L. elongatus* and *L. obtusidens* (Galetti Jr. *et al.*, 1981; 1995, Koehler *et al.*, 1997). Studies about the molecular characterization of the 5S rDNA in *Leporinus friderici*, *L. elongatus* and *L. obtusidens* from the Mogi-Gua u river (S o Paulo, Brazil), revealed great similarity between *L. elongatus* and *L. obtusidens* (Martins and Galetti Jr., 1998). Isoenzyme studies revealed the same electrophoretic patterns for the two species with regard to lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) (Pannepucci *et al.*, 1984 and Monteiro *et al.*, 1991, respectively).

Analyzing seven isoenzymatic systems, including LDH and MDH, Chiari and Sodr  (1999) obtained a total of eighteen loci in *Leporinus elongatus* and *L. obtusidens*. These species were only different in the alleles of two loci (*PGM-3\** and *IDHP-1\**) and the estimated values of identity and genetic distance of Nei (1972) were  $I = 0.949$  and  $D = 0.051$ . This fact suggested great genome homology between them. Results obtained in this research by the employment of RAPD markers reinforce the existence of such a high degree of genome similarity.

According to Weising *et al.* (1995), consistent results have been described in many cases, where two or more techniques were used for the investigation of the same material. The data of genetic variability in the present study agreed to those obtained by Chiari and Sodr  (1999), since the

species which presented large genetic variability for isoenzymes, gave similar results to RAPD markers.

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