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GENETIC VARIABILITY IN FIVE SPECIES OF ANOSTOMIDAE (OSTARIOPHYSI - CHARACIFORMES)

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ABSTRACT

Genetic variability was studied in five fish species (Anostomidae): *Schizodon intermedius* and *S. nasutus* and *Leporinus friderici*, *L. elongatus* and *L. obtusidens*, collected at one location on the Tibagi River (Paraná, Brazil). The protein data from seven systems coded collectively for 19 loci in the liver, muscle and heart. Nine of these loci were polymorphic. The estimated proportion of polymorphism loci ($\bar{P}$) varied from 16.7% in *S. intermedius* to 36.9% in *L. friderici*; the mean heterozygosity observed ($\bar{H}o$) was 0.027 ± 0.015 and 0.109 ± 0.042, respectively. The estimated value of the genetic identity among *L. friderici* and *S. intermedius* (0.749) and *S. nasutus* (0.787) suggested that these are "congeneric" species. Morphological characteristics indicate that these species belong to distinct genera, while isoenzymatic data show that they are very similar at the genetic/biochemical level.

INTRODUCTION

Anostomidae is one of the most abundant fish families in the Neotropical region (Vari, 1983). A large number of species from this family were found in a fish fauna survey carried out at five sampling points along the Tibagi River (Paraná, Brazil) by Bennemann *et al*. (1995). Five species, *Leporinus elongatus, L. friderici, L. obtusidens, Schizodon intermedius* and *S. nasutus,* were detected at one of these locations (Sertanópolis).

According to Garavello (1979), the Anostomidae are characterized by a large reduction in the number of teeth. The main character of the genus *Leporinus* is the presence of wide teeth arranged in steps and a prominent "symphysial" pair. Species of the genus *Schizodon* do not differ externally from the majority of species of the genus *Leporinus.* However, the pluricuspidate teeth form a continuous crenulate cutting border, and are quite characteristic of *Schizodon* (Géry, 1977).

Isozyme electrophoresis studies have been widely employed to analyze ontogenetic development, to quantify the genetic variability in natural fish populations for comparative analysis among populations, and also to trace phylogenetic relationships, e.g. Panepucci *et al.* (l984 and l987), Basaglia (1989), Rao *et al.* (1989), Renno *et al*. (1989), Degani and Veith (1990), Farias and Almeida-Val (l992), Verneau *et al.* (1994), Zawadzki (1996), Revaldaves *et al.* (l997) and Almeida and Sodré (l998), among many others.

The present study is a part of an integrated project for the restoration of the Tibagi River Basin - "Aspects of the Fauna and Flora of the Tibagi River Basin". The objective was to determine the electrophoretic profile and the distribution of expression in various tissues of the loci of seven protein systems and to investigate and quantify the genetic variability to estimate the genetic distance data and genetic identity for the five species of Anostomidae found by Bennemann *et al.* (1995) in Sertanópolis, PR.

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MATERIAL AND METHODS

L. elongatus, L. friderici, L. obtusidens, S. intermedius and *S. nasutus* specimens were collected monthly from the Tibagi River at Sertanópolis region, 2 km extension, PR, Brazil, between June 1994 and May 1996. Fish were collected by gillnets. Samples of liver, muscle, heart, and eye (analyzed for lactate dehydrogenase; LDH) were taken from the captured specimens and kept at -20°C. Individual samples from each tissue were homogenized in Tris-EDTA buffer, pH 7.0 (Degani and Veith, 1990), and centrifuged at 3.000 rpm for 15 min at 4°C.

Seven protein systems, carboxylesterases (EST- E.C. 3.1.1.1.), phosphoglucomutase (PGM- E.C. 5.4.2.2.), glycerol-3-phosphate dehydrogenase (G-3-PDH- E.C. 1.1.1.8), isocitrate dehydrogenase (IDHP- E.C. 1.1.1.42), LDH (E.C. 1.1.1.27), malate dehydrogenase (MDH- E.C. 1.1.1.37) and non-specific proteins (PT), were analyzed using the horizontal electrophoresis technique (Smithies, 1955) in starch gels (Val *et al*., 1981). Methodologies based on Shaw and Prasad (1970), Harris and Hopkinson (1976) and Lima and Contel (1990) were used for the preparation of the buffer systems and the staining (Table I).

The gene loci nomenclature adopted in this study was proposed by Shaklee *et al.* (1990). Each locus is designated by the abbreviation of the enzyme name in italics, followed by an Arabic number and an asterisk. The loci and alleles which coded the most anodic isoenzyme were designated by the number *1* or letter *A*.

BIOSYS-1 application software (Swoford and Selander, 1981) was used for statistical analyses. The ge-

Table I - Electrophoretic conditions and buffers used for the different protein systems.

Protein systems	Buffer systems		Migration time (hours)	(Volts/ cm)
	Bridge	Gel		
MDH LDH	Tris-citrate pH 7.0 0.155-0.043 M	66.7 ml of bridge buffer/ liter	17	3.5
PGM G-3-PDH	TEMM pH 7.4 0.22 M	1:10 bridge buffer	6	4.0
IDHP	Phosphate- citrate pH 5.9 0.25-0.15 M	1:40 bridge buffer	17	3.5
EST PT	Borate pH 8.0 0.3 M	Tris-citrate pH 8.0 0.017-0.0023 M	6	4.0

netic variability was estimated by calculating the proportion of polymorphic loci ($\bar{P}$) (99% criterion). The observed intralocus and mean heterozygosities ($\bar{H}o$) were obtained by direct counting. Expected intralocus and mean heterozygosities ($\bar{H}e$) were calculated according to Nei (1972). The Nei genetic distance and identity were also calculated by BIOSYS-1, and the genetic distance values were used to construct a dendrogram by the unweighted means method (UPGMA).

RESULTS

Of the seven protein systems, 19 loci were detected for *L. friderici* and 18 were detected for the other species. All of them exhibited anode migration (Figure 1).

Analysis of each isoenzymatic system of different tissues from one individual sample showed differential expression of each locus (Table II) regarding both number and color intensity of bands.

Of the 19 loci sampled, nine were polymorphic: *PT-1*, PGM-1** and *PGM-2** for the five species, *PGM-3** for the three *Leporinus* species, *PT-2** for *S. nasutus*, *IDHP-1** for *L. elongatus* and *EST-3*, LDH-A** and *LDH-B** for *L. friderici* (Table III).

The loci *PT-1** and *PGM-1** in *S. intermedius*; *PT-2** in *S. nasutus; PGM-2** in *S. intermedius*, *L. elongatus* and *L. obtusidens* and *PGM-3** in the three *Leporinus* species were not at Hardy-Weinberg genetic equilibrium, as indicated by the significantly different genotypic frequencies ($P < 0.05$).

Leporinus friderici showed the largest proportion of polymorphic loci ($\bar{P}$ = 36.8%) and *L. elongatus* showed the largest expected mean heterozygosity ($\bar{H}e = 0.142 \pm 0.054$) (Table IV). The greatst genetic identity value (0.962) was observed between *S. intermedius* and *S. nasutus* (TableV). From all three species of *Leporinus* analyzed, *L. friderici* was the one which had the smallest genetic distance from *S. intermedius* and *S. nasutus* (0.251 and 0.213, respectively) (TableV), which is shown in a dendrogram (Figure 2) constructed with D values by the UPGMA method.

DISCUSSION

Simultaneous analysis of three different tissues (liver, muscle and heart) of the same individual allowed the detection of a greater number of gene loci compared with other studies, even though the number of protein systems was small. Three loci were detected for PGM. The majority of studies with fish describe only one locus, as found by Renno *et al*. (1989) for four species of *Leporinus.* Four loci were found for G-3-PDH in the two *Schizodon* species and in *L. friderici*, and three loci in *L. obtusidens* and *L. elongatus*; Renno *et al*. (1989, 1990) observed a single locus in *L. friderici* muscle.

Almost every bony fish has the *LDH-C** locus, which is believed to have originated through a duplication of the *LDH-B** locus (Basaglia, 1989; Rao *et. al*., 1989). The *LDH-C** locus in bony fish shows different tissue regulation patterns in different taxons. Primitive orders of bony fish have a generalized tissue expression, while representatives of more advanced orders show a specialized tissue pattern (Whitt, 1975; Kettler and Whitt, 1986; Basaglia, 1989; Rao *et al*. 1989). *LDH-C** locus activity was not detected in the tissues (liver, muscle, heart and eye) analyzed for the lactate dehydrogenase system. The absence of the *LDH-C** locus was also observed by Panepucci *et al*. (1984) in a study on lactate dehydrogenase in several species of the Anostomidae, including those of the present study (except for *S. intermedius*), and by Renno *et al*. (1989) in four *Leporinus* species (including *L. friderici*).

Divergences from Hardy-Weinberg equilibrium in the expected genotypic frequencies may occur due to mutation, natural selection, preferential crossing, loss or gain of migrants, genetic drift, and/or methodological errors. Methodological errors could explain the deviations shown mainly by the loci *PT-2**, *PGM-1** and *PGM-3**, because of the difficulties in electrophoretic profile interpretation. The deficiency in heterozygotes for these loci may be due to errors in typing. According to Crouau-Rou (1988) (Apud Lima, 1989), many studies have shown significant levels of heterozygote deficiency in natural populations, but these deficiencies are usually observed in some allozymic loci and/or in only some samples of a species, while for other loci the genotypic proportions are in equilibrium.

In the analysis of the genetic variability data, the proportion of polymorphic loci ($\bar{P}$) in the five fish species from the family Anostomidae studied varied from 16.7% in *S. intermedius* to 36.8% in *L. friderici*. These data are in agreement with the literature. Renno *et al*. (1989) ob-

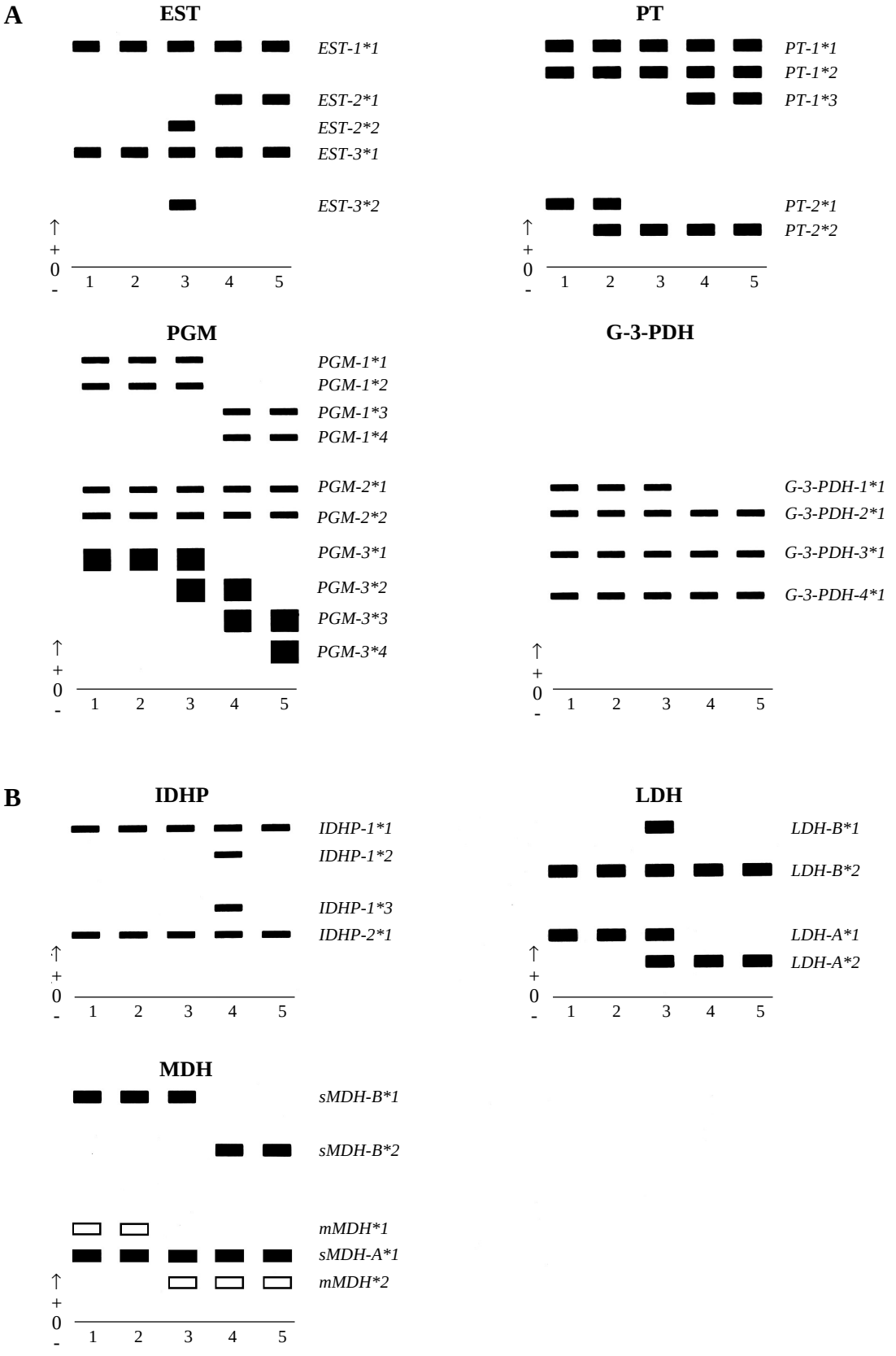

Figure 1 - Representation of all the alleles from different loci of the seven protein systems analyzed in five species of Anostomidae. **A:** Carboxylesterases **(EST)**; nonspecific proteins **(PT)**; phosphoglucomutase **(PGM)**; glycerol-3-phosphate dehydrogenase **(G-3-PDH)**. **B:** Isocitrate dehydrogenase **(IDHP)**; lactate dehydrogenase **(LDH)**; malate dehydrogenase **(MDH). Samples: 1-** *Schizodon intermedius*, **2-** *S. nasutus*, **3-** *Leporinus friderici*, **4-** *L. elongatus* and **5-** *L. obtusidens.*

Table II - Tissue distribution of the expression of different loci identified in *Schizodon intermedius, S. nasutus, Leporinus elongatus, L. friderici* and *L. obtusidens.*

Loci	*S. intermedius*			*S. nasutus*			*L. elongatus*			*L. friderici*			*L. obtusidens*		
	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
*EST-1**	+	+++	+	+	+++	+	+	+++	+	+	+++	+	+	+++	+
*EST-2**	0	0	0	0	0	0	++	+	+	++	+	+	++	+	+
*EST-3**	+	+++	+	+	+++	+	+	+++	+	+	+++	+	+	+++	+
*PT-1**	++	0	0	++	0	0	++	0	0	++	0	0	++	0	0
*PT-2**	++	0	0	++	0	0	++	0	0	++	0	0	++	0	0
*PGM-1**	0	++	0	0	++	0	0	++	0	0	++	0	0	++	0
*PGM-2**	++	0	0	++	0	0	++	0	0	++	0	0	++	0	0
*PGM-3**	++	+++	++	++	+++	++	++	+++	++	++	+++	++	++	+++	++
*G-3-PDH-1**	++	0	++	++	0	++	0	0	0	++	0	++	0	0	0
*G-3-PDH-2**	+/-	+/-	++	+/-	+/-	++	+/-	+/-	++	+/-	+/-	++	+/-	+/-	++
*G-3-PDH-3**	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
*G-3-PDH-4**	++	++	0	++	++	0	++	++	0	++	++	0	++	++	0
*IDHP-1**	0	+++	0	0	+++	0	0	+++	0	0	+++	0	0	+++	0
*IDHP-2**	+++	0	+	+++	0	+	+++	0	+	+++	0	+	+++	0	+
*LDH-A**	+++	+/-	++	+++	+/-	++	+++	+/-	++	+++	+/-	++	+++	+/-	++
*LDH-B**	+/-	++	++	+/-	++	++	+/-	++	++	+/-	++	+	+/-	++	++
*sMDH-A**	+	+++	++	+	+++	++	+	+++	++	+	+++	+	+	+++	++
*sMDH-B**	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++
*mMDH**	+/-	+/-	++	+/-	+/-	++	+	+	++	+	+	++	+	+	++

M = Muscle; L = liver; H = heart; +++ = the locus is strongly expressed; ++ = intermediate expression; + = low expression; +/- = expression is not always observed; 0 = the locus is not expressed.

Table III - Allelic frequencies of the 19 loci observed in *Schizodon intermedius, S. nasutus, Leporinus elongatus, L. friderici* and *L. obtusidens.*

Loci	Alleles	*S. intermedius*	*S. nasutus*	*L; friderici*	*L. elongatus*	*L. obtusidens*
EST-1*	EST-1*1	1.000	1.000	1.000	1.000	1.000
*EST-2**	*EST-2*1*	——-	——-	——-	1.000	1.000
	*EST-2*2*	——-	——-	1.000	——-	——-
*EST-3**	*EST-3*1*	1.000	1.000	0.692	1.000	1.000
	*EST-3*2*	——-	——-	0.308	——-	——-
	*PT-1*1*	0.604	0.375	0.375	0.400	0.333
*PT-1**	*PT-1*2*	0.396	0.625	0.625	0.440	0.528
	*PT-1*3*	——-	——-	——-	0.160	0.139
*PT-2**	*PT-2*1*	1.000	0.800	——-	——-	——-
	*PT-2*2*	——-	0.200	1.000	1.000	1.000
	*PGM-1*1*	0.526	0.750	0.800	——-	——-
*PGM-1**	*PGM-1*2*	0.474	0.250	0.200	——-	——-
	*PGM-1*3*	——-	——-	——-	0.667	0.818
	*PGM-1*4*	——-	——-	——-	0.333	0.182
*PGM-2**	*PGM-2*1*	0.820	0.750	0.700	0.700	0.615
	*PGM-2*2*	0.180	0.250	0.300	0.300	0.385
	*PGM-3*1*	1.000	1.000	0.313	——-	——-
*PGM-3**	*PGM-3*2*	——-	——-	0.688	0.405	——-
	*PGM-3*3*	——-	——-	——-	0.595	0.882
	*PGM-3*4*	——-	——-	——-	——-	0.118
*G-3-PDH-1**	*G-3-PDH-1*1*	1.000	1.000	1.000	——-	——-
*G-3-PDH-2**	*G-3-PDH-2*1*	1.000	1.000	1.000	1.000	1.000
*G-3-PDH-3**	*G-3-PDH-3*1*	1.000	1.000	1.000	1.000	1.000
*G-3-PDH-4**	*G-3-PDH-4*1*	1.000	1.000	1.000	1.000	1.000
	*IDHP-1*1*	1.000	1.000	1.000	0.667	1.000
*IDHP-1**	*IDHP-1*2*	——-	——-	——-	0.250	——-
	*IDHP-1*3*	——-	——-	——-	0.083	——-
*IDHP-2**	*IDHP-2*1*	1.000	1.000	1.000	1.000	1.000
*LDH-A**	*LDH-A*1*	1.000	1.000	0.957	——-	——-
	*LDH-A*2*	——-	——-	0.043	1.000	1.000
*LDH-B**	*LDH-B*1*	——-	——-	0.114	——-	——-
	*LDH-B*2*	1.000	1.000	0.886	1.000	1.000
*sMDH-A**	*sMDH-A*1*	1.000	1.000	1.000	1.000	1.000
*sMDH-B**	*sMDH-B*1*	1.000	1.000	1.000	——-	——-
	*sMDH-B*2*	——-	——-	——-	1.000	1.000
*mMDH**	*mMDH*1*	1.000	1.000	——-	——-	——-
	*mMDH*2*	——-	——-	1.000	1.000	1.000

Table IV - Statistical analysis of the genetic variability of *Schizodon intermedius, S. nasutus, Leporinus elongatus, L. friderici* and *L. obtusidens.*

Variable	*S. intermedius*	*S. nasutus*	*L. friderici*	*L. elongatus*	*L. obtusidens*
L	18	18	19	18	18
N	148	22	27	57	34
NA	1.2 (0.1)	1.2 (0.1)	1.4 (0.1)	1.4 (0.2)	1.3 (0.1)
$\bar{P}$ (0.99)	16.7	22.2	36.8	27.8	22.2
$\bar{H}o$	0.027 (0.015)	0.055 (0.031)	0.109 (0.042)	0.075 (0.036)	0.056 (0.036)
$\bar{H}e$	0.072 (0.038)	0.092 (0.040)	0.132 (0.046)	0.142 (0.054)	0.090 (0.042)

L = Number of loci shown; N = number of individuals analyzed; NA = mean number of alleles per locus; $\bar{P}$ = proportion of polymorphic loci; $\bar{H}o$ and $\bar{H}e$ = mean heterozygosity observed and expected (Nei, 1972), respectively; standard deviation given in parentheses.

Table V - Nei's (1972) genetic distance (below diagonal) and genetic identity (above) among *Schizodon intermedius, S. nasutus, Leporinus friderici, L. elongatus* and *L. obtusidens.*

Species	*S. intermedius*	*S. nasutus*	*L. friderici*	*L. elongatus*	*L. obtusidens*
S. intermedius	——	0.962	0.749	0.568	0.570
S. nasutus	0.038	——	0.787	0.581	0.588
L. friderici	0.251	0.213	——	0.676	0.671
L. elongatus	0.432	0.419	0.324	——	0.949
L. obtusidens	0.430	0.412	0.329	0.051	——

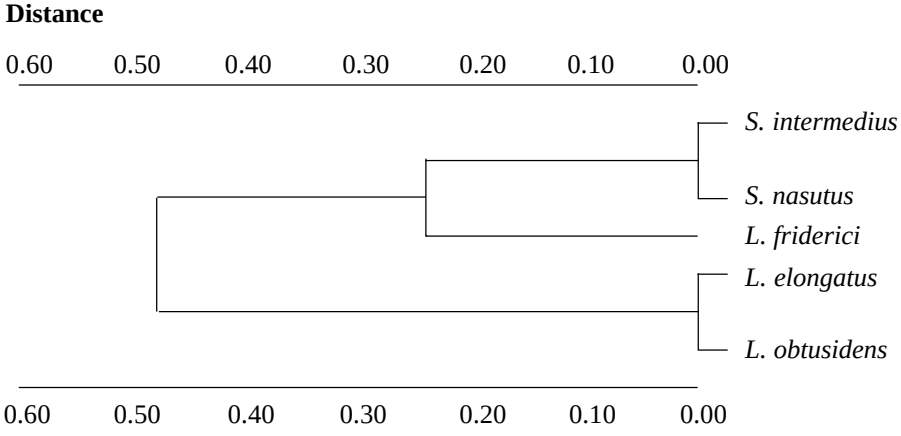

Figure 2 - Dendrogram obtained by the UPGMA method for genetic distance (Nei, 1972) for the five species of Anostomidae.

served a $\bar{P}$ value of 33% for *L. friderici* collected in French Guiana. Nevo (1978) estimated from data in the literature a mean $\bar{P}$ value of 15.2%, for 51 species of Teleostei. Studies carried out in fish species from Brazilian rivers show $\bar{P}$ values with a wide range of variation: Zawadzki (1996) found values from 11.45 to 19.23% in three species of the genus *Hypostomus* (Iguaçu River); Revaldaves *et al.* (1997) observed 33.3% for *Prochilodus lineatus* (Paraná River) and Almeida and Sodré (1998) observed values from 6.67 to 20% in three *Pimelodidae* species (Tibagi River).

Ward *et al.* (1992) estimated an $\bar{H}e$ value of 0.051 for about 150 marine and fresh water fish species. The mean heterozygosity observed in the present study varied from 0.027 ± 0.015 in S. *intermedius* to 0.109 ± 0.042 in *L. friderici*, and the corresponding expected values ranged from 0.072 ± 0.038 in *S. intermedius* to 0.142 ± 0.054 in *L. elongatus*. The $\bar{H}e$ values for Neotropic fish vary from 1.1% in *Hypostomus derbyi*, a sedentary species (Zawadzki, 1996), to 13.2% in *Prochilodus lineatus* (Revaldaves *et al*., 1997). Renno *et al.* (1989) estimated $\bar{H}e$ at 12% for *L. friderici*. Thus, the values observed in the five species of Anostomidae do not diverge significantly from those observed in other fish species. The variation in the values, according to Lewontin (1974) and Nei

(1978), could be due to the fact that $\bar{H}e$ is greatly affected by the choice and number of loci analyzed.

S. intermedius was the species, among those analyzed, with the lowest $\bar{P}$ and $\bar{H}o$ values (Table IV). The fish fauna survey carried out by Bennemann *et al.* (1995) at five locations on the Tibagi River shows that *S. intermedius* specimens were present only in Sertanópolis. *L. friderici* and *L. elongatus*, with $\bar{H}o$ values 10.9 and 7.5%, respectively, could be found at Sapopema, Londrina and Sertanópolis (Bennemann *et al*., 1995). According to Zimmerman (1987), high heterozygosity levels and polymorphism may be expected for fish species which are dispersed among several places in one river. The suggestion of a large population would also decrease the probability of inbreeding.

Thorpe (1982), using data available in the literature, established a distribution index for the genetic identity frequencies of Nei (1972) for genetic divergence estimates among conspecific populations (0.95 to 1.0), among species of the same genus (0.35 to 0.85) and among genera of the same family (0.0 to 0.60). According to this index, the values of identity obtained for *L. friderici, L. elongatus* and *L. obtusidens* (Table V) show that they may be considered genetically distinct biological species of the same genus. The values obtained between *S. intermedius* and *S. nasutus* compared with *L. obtusidens* and *L. elongatus* (Table V) are in the identity interval for species of the genera of one family. Although the identity values between *S. intermedius* and *S. nasutus* (0.962) and between L. *elongatus* and *L. obtusidens* (0.949) are at the limit of the Thorpe's index for conspecific populations, the morphological characteristics make it clear that they are distinct species (Géry, 1977; Shibatta, O.A., personal communication). The estimated identity value between *L. friderici* and *S. intermedius* and *S. nasutus* (Table V) corresponds to the value for congeneric species; however, they are classified as species of distinct genera, and at the genetic-biochemical level, these species share a great number of loci. The dendrogram constructed (Figure 2) from the D values shows that *L. friderici* is the species within *Leporinus* that is genetically most similar to the genus *Schizodon*.

The present study, part of the integrated project "Aspects of Fauna and Flora on the Tibagi River Basin" carried out by Universidade Estadual de Londrina for the restoration of the basin, conflicts with government projects to construct five hydro-electric plants along the Tibagi River. These plants may be barriers to the dispersion of fresh water organisms, specially migratory species. Besides the impact caused by water flow control, they jeopardize survival, successful mating, and gene flow, which surely will alter the genic frequencies in the fish species. Thus, the maintenance of the environmental heterogeneity of the Tibagi River is important for the preservation of aquatic organisms. The construction of hydro-electric plants will lead to a reduction in the genetic variability of migratory species, such as *L. friderici*, perhaps reaching levels lower than those observed for *S. intermedius*, a species apparently restricted to a single locality on the Tibagi River.

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RESUMO

A variabilidade genética de 5 espécies da família Anostomidae pertencentes aos gêneros *Schizodon* (*S. intermedius* e *S. nasutus*) e *Leporinus* (*L. friderici*, *L. elongatus* e *L. obtusidens*) coletadas em uma localidade do rio Tibagi (Paraná, Brasil) foi analisada comparativamente utilizando dados protéicos de 7 sistemas que codificam l9 locos no fígado, músculo e coração. Dos locos identificados, 9 são polimórficos, com valores estimados de proporção de locos polimórficos ($\bar{P}$) que variaram de 16.7% em *S. intermedius* a 36.85% em *L. friderici*, e a heterozigosidade média observada ($\bar{H}o$) foi de 0.027 ± 0.015 e 0.109 ± 0.042, nessas mesmas espécies. O valor estimado de identidade genética (I) entre *L. friderici* e *S. intermedius* (0.749) e *S. nasutus* (0.787) sugere que estas são espécies congenéricas. As características morfológicas determinam que estas espécies pertencem a gêneros distintos, no entanto os dados de identidade e distância genética obtidos demonstram que essas três espécies, no nível genético-bioquímico, têm uma maior similaridade.

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