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Research Article

Genetic variability of *Hypostomus* (Teleostei, Loricariidae) from the Ribeirão Maringá, a stream of the upper Rio Paraná basin, Brazil

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Abstract

Hypostomus strigaticeps and two morphotypes of *Hypostomus* were collected from Ribeirão Maringá, a small tributary of the Rio Pirapó, an effluent of the upper Rio Paraná. The three populations were analyzed by allozyme electrophoresis that allowed the scoring of 25 loci from 14 enzyme systems. Heterozygosity values (H_e) were 0.028 in *H. strigaticeps*, 0.027 in *Hypostomus* sp. 1 and zero in *Hypostomus* sp. 2. Several diagnostic loci and fixed differences were observed for each population at loci Acp-A, Gcdh-A and Mdhp-A. Thus, all populations were genetically distinct, although there were many common alleles. The unbiased genetic identities of Nei (I) were estimated as 0.780 for *Hypostomus* sp. 1 and *H. strigaticeps*, 0.357 for *H. strigaticeps* and *Hypostomus* sp. 2 and 0.322 for *Hypostomus* sp. 1 and *Hypostomus* sp. 2. The data indicate that the two morphotypes are distinct species from *Hypostomus strigaticeps.*

Key words: allozymes, heterozygosity, *Hypostomus*, Loricariidae, systematic.

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Introduction

The loricariid genus *Hypostomus* is considered one of the most complex of the Neotropical ichthyofauna (Gosline, 1947; Reis *et al.,* 1990; Mazzoni *et al.,* 1994). In the upper Rio Paraná basin, which is the stretch of the Paraná hydrographic basin above Itaipu Dam, there are several small and medium-sized tributaries whose ichthyofauna is usually isolated by biotic and abiotic factors. Furthermore, geographically close tributaries harbor different loricariid species, popularly known as cascudos. The usual absence of some headwater loricariid species in the main channel of great rivers could lead to the assumption that waterfalls at the mouth of the tributaries and the different ecological parameters of big rivers would act as barriers to many small and medium-length cascudos. At the same time, that geographical isolation of populations plays an important role in speciation, it can also lead to extinction of small populations. Therefore, characterization of the distribution and the genetic constitution of the many morphotypes of *Hypostomus* found in many headwaters of the upper Rio Paraná basin is required for future conservation guidelines. Nowadays, although several species of the genus *Hypostomus*, such as *H. regani* (Ihering, 1905), *H. ancistroides* (Ihering, 1911) and *H. margaritifer* (Regan, 1907), are widespread and well characterized, many morphotypes are endemic to one or a few small tributaries of the upper Rio Paraná basin. Thus, while a taxonomical review is necessary, genetic works could improve the knowledge about the genetic variability and reproductive isolation among the many forms observed.

In the Ribeirão Maringá (Figure 1) there are three syntopic morphotypes belonging to the genus *Hypostomus*: *H. strigaticeps* (Regan, 1907) and two other populations, probably two non-described species. Reproductive isolation among populations has been investigated using the allozyme electrophoresis technique. This methodology has been used to discriminate known species (Zawadzki *et al.,* 2001) and to reveal new species (Fisch-Muller *et al.,* 2001) of Loricariidae and its strength is a focus on analysis of sympatric species. Thus, this work aims to verify the existence of reproductive isolation among these three populations found in Ribeirão Maringá as well as to discuss their genetic variability and the interpretation of allozyme data in the systematics of *Hypostomus*.

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Material and Methods

From August, 2001 to April, 2002, 17 specimens of *H. strigaticeps*, 17 of *Hypostomus* sp. 1, and 13 of

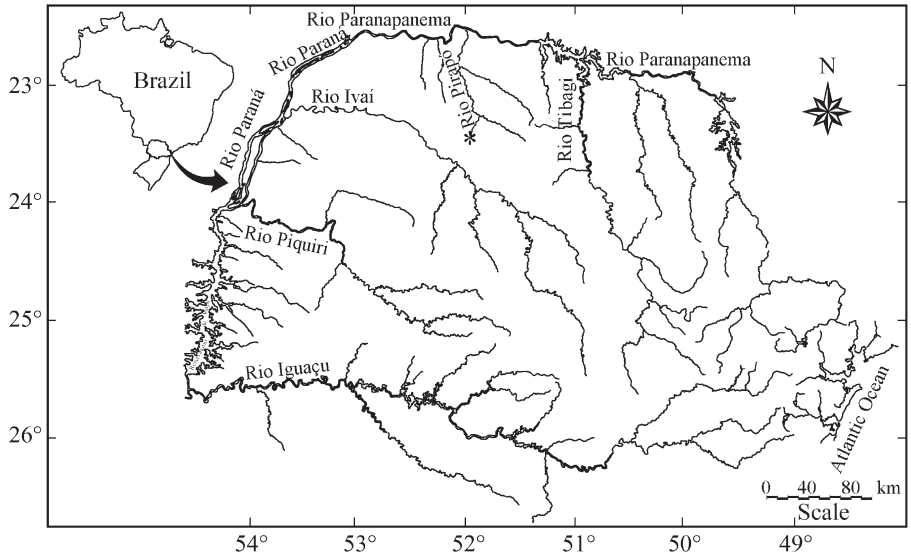

Figure 1 - Collection site of the three populations of *Hypostomus* from the Ribeirão Maringá (asterisk), Rio Pirapó, Rio Paranapanema, upper Rio Paraná basin.

Hypostomus sp. 2 were sampled in the Ribeirão Maringá, which is a small effluent of the Rio Pirapó, Rio Paranapanema, upper Rio Paraná basin. Samples of white skeletal muscle, liver and heart tissues were removed from the fishes immediately after capture. Tissues were stored in liquid nitrogen until analyses. Electrophoretic procedures were detailed in Zawadzki *et al.* (1999). The nomenclature used was proposed by the International Union of Biochemistry and Molecular Biology (1992). Data were analyzed using POPGENE 1.31 software (Yeh and Boyle, 1997). The genetic interpretation of the enzymatic patterns was based on the quaternary structure of the enzymes described by Ward *et al.* (1992).

Voucher specimens were deposited in the collection of Nupelia (Research Center in Ichthyology, Limnology and Aquaculture) of the State University of Maringá. Specimen list was arranged in the text as follows: museum acronym and catalogue number, number of specimens as a parenthetical remark, standard length range in millimeters, locality, collection date and collector.

Voucher specimens

Hypostomus strigaticeps: NUP 2831, (20), 75.45-147.96, Ribeirão Maringá (23°25' S, 51°55' W), 20.xii.2001, Cláudio H. Zawadzki and Weferson J. Graça; *Hypostomus* sp. 1: NUP 2830 (30), 53.32-88.98, Ribeirão Maringá (23°25' S/51°55' W), 20.xii.2001, Cláudio H. Zawadzki and Weferson J. Graça; *Hypostomus* sp. 2: NUP 2832, (8), 113,36-139,15, Ribeirão Maringá (23°25' S, 51°55' W), 20.xii.2001, Cláudio H. Zawadzki and Weferson J. Graça.

Results

A total of 14 enzyme systems encoded by 25 loci were assayed for the three *Hypostomus* populations, allowing the detection of 52 alleles. Table 1 shows the enzymatic systems analyzed and Table 2 shows the allele frequencies of the three populations analyzed.

Several loci showed diagnostic alleles. The loci Acp-A, Gcdh-A and sMdhp-A were diagnostic for the three populations and can be elected as good genetic markers for a rapid discrimination among them. Furthermore, the alleles sAta-B-*a*, G3pdh-A-*b*, G3pdh-B-*b*, G6pdh-A-*b*, G6pdh-B-*b*, Gpi-A-*c*, Gpi-B-*b*, mIcdh-A-*c*, Ldh-A-*a*, Ldh-B-*a*, sMdh-B-*a* and mMdhp-A-*b* are exclusive to *Hypostomus* sp. 2. The allozyme encoded by the allele *a* of locus Ldh-A in *Hypostomus* sp. 2 migrates to the same position of allele *b* of locus Ldh-B in *Hypostomus* sp. 1 and *H. strigaticeps* (Figure 2). The allele Adh-A-*a* distinguished *Hypostomus* sp. 1 from the other populations analyzed. In addition to loci Acp-A, Gcdh-A, sMdhp-A and Adh-A, *Hypostomus* sp. 1 and *H. strigaticeps* were discriminated by clear allele frequency differences at loci Gpi-A and mIcdh-A. No locus was consistently polymorphic for all populations. The loci mMdh-A, sMdh-A, Per-1, Per-2, Per-3 and Sod-A were monomorphic and similar for the three populations.

The genetic variability for the three populations is shown in Table 2. Six loci were polymorphic ($P_{0.99}$ criterion) in one or more populations (Table 2). The frequency of polymorphic loci ranged from zero in *Hypostomus* sp. 2 to 0.20 in *Hypostomus* sp. 1 and *H. strigaticeps*. The expected mean heterozygosity ranged from zero in *Hypostomus* sp. 2 to 0.028 in *H. strigaticeps*. All the polymorphic loci analyzed were in Hardy-Weinberg equilibrium. The unbiased genetic identity of Nei (1978) for the three populations is shown in Table 3. The most similar populations were *H. strigaticeps* and *Hypostomus* sp. 1

Table 1 - Names, number of enzyme commission (E.C.n.), tissues, buffers, quaternary structure (Q.S) and number of loci of each enzyme of the three *Hypostomus* populations from the Ribeirão Maringá, upper Rio Paraná basin. L = liver; M = muscle; H = heart; TBE = Tris/borate/EDTA (pH 8.7) (Boyer *et al.* 1963); TC = Tris/citrate (pH 7.0) and TEM = Tris/EDTA/maleic (pH 7.4) (Shaw and Prasad 1970).

Enzyme (Abbreviation)	E.C. n°	Tissue	Buffer	Q. S.	Loci
Acid phosphatase (ACP)	3.1.3.2	L	TC	Dimeric	1
Alcohol dehydrogenase (ADH)	1.1.1.1	L	TBE	Dimeric	1
Aspartate transaminase (ATA)	2.6.1.1	L, H, M	TEM	Dimeric	2
Glucose 1-dehydrogenase - NAD+(GCDH)	1.1.1.118	L	TEM	Dimeric	1
Glycerol-3-phosphate dehydrogenase (G3PDH)	1.1.1.8	L, H, M	TC	Dimeric	2
Glucose-6-phosphate dehydrogenase (G6PDH)	1.1.1.49	L	TBE	Tetrameric	2
Glucose-6-phosphate isomerase (GPI)	5.3.1.9	L, H, M	TC	Dimeric	2
Isocitrate dehydrogenase - NADP+(ICDH)	1.1.1.42	L, H, M	TC	Dimeric	2
L-Lactate dehydrogenase (LDH)	1.1.1.27	H, M	TC	Tetrameric	2
Malate dehydrogenase (MDH)	1.1.1.37	L, H, M	TC	Dimeric	3
Malate dehydrogenase - NADP+(MDHP)	1.1.1.40	L, H, M	TC	Tetrameric	2
Peroxidase (PER)	1.11.1.6	L, H	TC	Tetrameric	3
Phosphoglucomutase (PGM)	5.4.2.2	L, H, M	TC	Monomeric	1
Superoxide dismutase (SOD)	1.15.1.1	L, H, M	TBE	Dimeric	1

(I = 0.780) and the most divergent were *Hypostomus* sp. 1 and *Hypostomus* sp. 2 (I = 0.322).

Discussion

The mean genetic variability estimated for the three studied populations (0.018) was considered quite below the mean found by Ward *et al.* (1992) for freshwater fishes (H_e = 0.051). In another allozyme survey carried out in the Ribeirão Keller, a tributary of the Rio Ivaí basin, Zawadzki *et al.* (2004a) found higher values of *He* for four morphotypes of the genus *Hypostomus*: 0.068 in *H. hermanni* (Ihering, 1905), 0.143 in *Hypostomus* sp. 1, 0.059 in *Hypostomus* sp. 2, and 0.091 in *Hypostomus* sp. 3. In addition to the higher mean expected heterozygosity found by those authors, (0.088) it should be noted that the three non-described morphotypes were not the same as those studied in this work. Although the heterozygosity values of most species of *Hypostomus* fall into the Ward's average, some of them, such as *H. margaritifer* (H_e = 0.106), *H. regani* (H_e = 0.098), and *Hypostomus* sp. 1 (H_e = 0.107) from the Itaipu Reservoir show higher values (Zawadzki, 2001).

Contrary to these findings, the loricariid *Neoplecostomus paranensis* (Langeani, 1990) wich were restricted to small headwater streams presented zero H_e values (Zawadzki *et al*., 2004b). Population isolation promoted by some efficient barrier was used to explain the unusual lack of genetic variability in *N. paranensis*. Here, the low heterozygosity values for *H. strigaticeps* and *Hypostomus* sp. 1 and the zero heterozygosity value found for *Hypostomus* sp. 2 also indicate that similar events can be acting to isolate these three morphotypes from other populations of *Hypostomus*. Consequently, isolated populations, especially small ones, may have their heterozygosity eroded by inbreeding process or by chance (Solé-Cava, 2001).

The recording of 17 loci as diagnostic genetic markers, 68% from the 25 studied loci, is strong evidence of the usefulness of allozyme data in the systematics of the loricariid genus *Hypostomus* as was pointed out by Zawadzki *et al.* (2000). According to Weber (2003), the genus *Hypostomus* comprises 107 valid species and shows a particularly high intraspecific variability in morphology and color pattern that prompted most of these species to be misunderstood in terms of variability. This phenomenon makes it impossible for most Neotropical ichthyologists to correctly identify *Hypostomus* morphotypes. Here, we highlight the potential use of allozyme data for the acquisition of genetic markers for some morphologically variable and syntopic *Hypostomus* populations.

Although *Hypostomus strigaticeps* and *Hypostomus* sp. 1 have light dots over a darker background and both of them fit the *Hypostomus regani* group of species *sensu* Muller and Weber (1992), for both, the dots can range from clear round to a striped pattern, and in *Hypostomus* sp. 1 these light markers may even be absent. Morphometrically, they diverge in dorsal-fin spine lengths that ranges from 1.65 to 1.82 in the pre-dorsal length in *Hypostomus* sp. 1 *vs.* 1.26 to 1.28 in *H. strigaticeps*.

Hypostomus sp. 1 and *H. strigaticeps* are distinguished, in addition to Acp-A, Gcdh-A and sMdhp-A, by different fixed alleles at locus Adh-A. Furthermore, differential expression was observed in LDH isozymes of *Hypostomus* sp. 1 that showed higher expression of locus

Table 2 - Allele frequencies, sample number (n), number of alleles per locus (K), frequency of polymorphic loci ($P_{0.99}$), mean expected heterozygosity (H_e), mean observed heterozygosity (H_o) of *Hypostomus strigaticeps* and two morphotypes from Ribeirão Maringá.

Loci	Allele	*H. Strigaticeps* (n = 17)	*Hypostomus* sp. 1 (n = 17)	*Hypostomus* sp. 2 (n = 13)
sAta-A	*a*	0.117	0.058	****
	b	0.882	0.941	1.000
sAta-B	*a*	****	****	1.000
	b	0.970	1.000	****
	c	0.029	****	****
Acp-A	*a*	****	****	1.000
	b	****	1.000	****
	c	1.000	****	****
Adh-A	*a*	****	1.000	****
	b	1.000	****	1.000
Gcdh-A	*a*	****	1.000	****
	b	1.000	****	****
	c	****	****	1.000
G3pdh-A	*a*	1.000	1.000	****
	b	****	****	1.000
G3pdh-B	*a*	1.000	1.000	****
	b	****	****	1.000
G6pdh-A	*a*	1.000	1.000	****
	b	****	****	1.000
G6pdh-B	*a*	1.000	1.000	****
	b	****	****	1.000
Gpi-A	*a*	1.000	0.218	****
	b	****	0.781	****
	c	****	****	1.000
Gpi-B	*a*	1.000	1.000	****
	b	****	****	1.000
mIcdh-A	*a*	0.088	0.941	****
	b	0.911	0.058	****
	c	****	****	1.000
sIcdh-A	*a*	0.058	****	****
	b	0.941	0.970	****
	c	****	0.029	1.000
Ldh-A	*a*	****	****	1.000
	b	1.000	1.000	****
Ldh-B	*a*	****	****	1.000
	b	1.000	1.000	****
mMdh-A	*a*	1.000	1.000	1.000
sMdh-A	*a*	1.000	1.000	1.000
sMdh-B	*a*	****	****	1.000
	b	1.000	1.000	****
sMdhp-A	*a*	****	****	1.000
	b	1.000	****	****
	c	****	1.000	****
mMdhp-A	*a*	1.000	1.000	****
	b	****	****	1.000
Per-1	*a*	1.000	1.000	1.000
Per-2	*a*	1.000	1.000	1.000
Per-3	*a*	1.000	1.000	1.000
Pgm-A	*a*	0.058	0.029	****
	b	0.911	0.970	1.000
	c	0.029	****	****
Sod-A	*a*	1.000	1.000	1.000
K	_	1.24 (0.52)*	1.20 (0.41)*	1.00 (0.00)*
$P_{0.99}$	_	20.0	20.0	00.0
H_o	_	0.021 (0.053)*	0.022 (0.088)*	0.000 (0.000)*
H_e	_	0.028 (0.062)*	0.027 (0.074)*	0.000 (0.000)*

*Number in parentheses is the respective standard error.

Ldh-A than the other populations of *Hypostomus* analyzed which showed no or very weak expression at this locus in the TC-7.0 buffer (Figure 2). Zawadzki *et al.* (1999) found, in three *Hypostomus* species from the Rio Iguaçu, a divergent expression pattern: in *Hypostomus* cf. *commersoni* (Valenciennes, 1840) and H. *myersi* (Gosline, 1947) the least anodal LDH-B was less stained and the most anodal LDH-A was the most stained; in *Hypostomus derbyi* (Haseman, 1911) the least anodal LDH-B was most stained and the most anodal LDH-A was weakly or not stained. Here, the LDH expression in *Hypostomus* sp. 2 is similar to that found in *H. derbyi*. However, in *Hypostomus* sp. 1 the least stained was not the least anodal but, instead, it was the most anodal. We have two possible explanations for this phenomenon, both isozymes encoded by these loci increased equally their anodal mobility and inverted their staining intensity, or LDH-A was kept in the same position and only the LDH-B increased its mobility, since it is the most anodal and both maintained their expression intensity pattern. Murphy *et al.* (1996) showed that the LDH expression in different buffers. In Tris/Citrate pH 7.0 buffer only the Ldh-B locus isozyme was stained for a variety of species. When these same organisms were run in a Tris/Citrate/Borate pH 8.2 buffer, both the most anodal Ldh-A and the least anodal Ldh-B were equally expressed.

Hypostomus sp. 2 was the most divergent population and among the 25 loci analyzed it was distinguished by 12 fixed different alleles. Morphologically, *Hypostomus* sp. 2 is also easily distinguished, bears small dark dots on the body and head over a light background and is morphologically similar to *Hypostomus ancistroides*. Together with *H. ancistroides*, *Hypostomus* sp. 2 fits the *Hypostomus*

Table 3 - Nei's unbiased genetic identity *I* are shown above the diagonal and genetic distance *D* are shown below the diagonal.

Populations	*H. strigaticeps*	*Hypostomus* sp. 1	*Hypostomus* sp. 2
H. strigaticeps	****	0.780	0.357
Hypostomus sp. 1	0.248	****	0.322
Hypostomus sp. 2	1.304	1.133	****

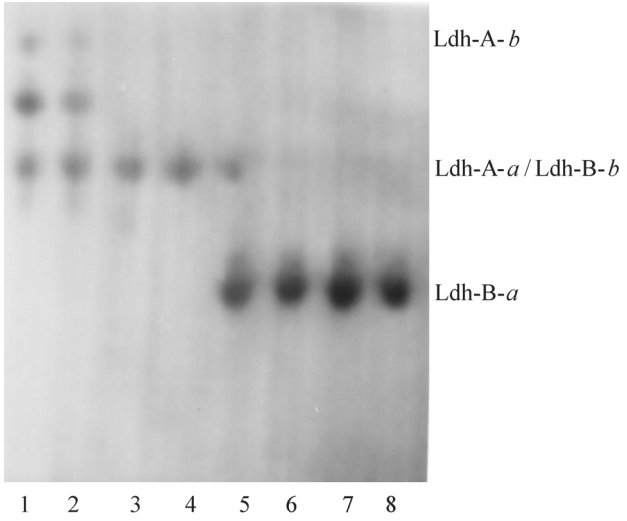

Figure 2 - Lactate dehydrogenase of muscle plus heart tissues of the three *Hypostomus* populations from Ribeirão Maringá: *Hypostomus* sp. 1 (1 and 2), *H. strigaticeps* (3 and 4) and *Hypostomus* sp. 2 (5 to 8).

plecostomus group *sensu* Muller and Weber (1992). However, *Hypostomus* sp. 2 can be allozimically diagnosed from *H. ancistroides* by fixed differences at loci G3pdh-A and G3pdh-B, because *Hypostomus* sp. 2 has the alleles G3pdh-A-*b* and G3pdh-B-*b* while, according to Zawadzki (2001), *H. ancistroides* from the Itaipu Reservoir has the alleles G3pdh-A-*a* and G3pdh-B-*a*.

Thus, the allozyme genetic markers found in this work permit us to raise the three populations from the Ribeirão Keller to biological distinct species because, in addition to the morphological differences, they present elevated values of allozyme differentiation and do not share alleles at many of the loci scored. According to Avise and Aquadro (1982) and Richardson *et al.* (1986), populations differing at about 10% of loci generally represent distinct species. In fact, *Hypostomus* sp. 1 and *Hypostomus* sp. 2 are probably new species of the genus *Hypostomus* whose taxonomic studies are in process (C.H. Zawadzki and C.S. Pavanelli, personal communication). The status of these three biological species inferred by the electrophoresis technique confirms the usefulness of allozyme data as a tool to discriminate species of the complex family Loricariidae as considered by Zawadzki *et al.* (1999; 2000; 2002).

As in other fluvial basins of the Neotropics, in the headwater streams of the upper Rio Paraná basin there is a great magnitude variation of morphological and color types of *Hypostomus*. In addition, many species are cited in the basin (Weber, 1986; 2003) and many usual diagnostic characters often overlap in the many morphotypes or in nominal species of *Hypostomus*. This phenomenon raises difficulties to the correct identification of the morphotypes and expresses the need for taxonomical revision. From this perspective, biochemical and molecular studies are valuable tools in generating species differentiation by the acquisition of genetic diagnostic markers, as well as insights into the genetic variability and species relationship of Neotropical freshwater fishes.

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