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Allozyme analysis of the four species of *Hypostomus* (Teleostei: Loricariidae) from the Ivaí river, upper Paraná river basin, Brazil

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ABSTRACT. Allozyme electrophoresis analysis were performed in four species of *Hypostomus* (Loricariidae), *H. albopunctatus*, *H. hermanni*, *H. regani*, and *Hypostomus* sp. 1/NUP 5612 from the Ivaí river, a tributary of the upper Paraná river. The study of 14 *loci* revealed diagnostic characters and exclusive alleles in a low frequency. The heterozygosity ranged from 0.000 in *H. albopunctatus* to 0.199 in *H. hermanni*, which was higher than the heterozygosity in other samples of *Hypostomus* in literature, as well as in other fish groups. *Hypostomus albopunctatus* and *H. regani* revealed higher similarity (I = 0.804), while *H. hermanni* and *Hypostomus* sp. 1/NUP 5612 showed the least genetic identity (I = 0.569). All samples were genetically distinguished, despite there were several shared alleles. The F_{ST} value was 0.671, showing a high genetic differentiation among the samples. *Hypostomus* sp. 1/NUP 5612 was genetically distinguished from the three congeners by the *loci Adh-A* and *G3pdh-B* and by present rare exclusive alleles in other six enzymatic systems.

Keywords: catfish, heterozygosity, Neotropical fishes, genetic variability, Siluriformes.

Análise aloenzimática de quatro espécies de *Hypostomus* (Teleostei: Loricariidae) do rio Ivaí, bacia do alto rio Paraná, Brasil

RESUMO. Análises aloenzimáticas foram realizadas em quatro espécies de *Hypostomus* (Loricariidae), *H. albopunctatus*, *H. hermanni*, *H. regani* e *Hypostomus* sp. 1/NUP 5612 coletadas no rio Ivaí, um tributário da bacia do alto rio Paraná, através da técnica de eletroforese. O estudo de 14 *loci* gênicos revelou alelos diagnósticos e alelos exclusivos com uma baixa frequência. A heterozigosidade variou de 0,000 em *H. albopunctatus* a 0,199 em *H. hermanni*, a qual foi maior que a média para outras espécies de *Hypostomus*, como também para outros grupos de peixes já estudadas. *Hypostomus albopunctatus* e *H. regani* revelaram maior similaridade (I = 0,804), enquanto que *H. hermanni* e *Hypostomus* sp. 1/NUP 5612 mostraram a menor identidade genética (I = 0,569). Todas as populações foram geneticamente distintas apesar de apresentarem muitos alelos em comum. O teste de F_{ST} resultou em um valor de 0,671, indicando uma diferenciação significativa entre as populações. *Hypostomus* sp. 1/NUP 5612 foi geneticamente diferenciada das três congêneres pelos *loci Adh-A* e *G3pdh-B* e por apresentar alelos raros exclusivos em outros seis sistemas enzimáticos.

Palavras-chave: cascudos, heterozigosidade, peixes neotropicais, variabilidade genética, Siluriformes.

Introduction

The loricariid representatives of the genus *Hypostomus* are armored catfishes with a moderate to small and stout body, with non-depressed caudal peduncle, which usually supports an adipose fin (ARMBRUSTER, 2004). *Hypostomus*, with 128 valid species (ZAWADZKI et al., 2012), is distributed throughout Central and South America (FERRARIS JR, 2007). Several species of *Hypostomus* are morphologically very similar in shape and most of them present a high intraspecific variability in morphology and color pattern (OYAKAWA et al., 2005; ZAWADZKI et al., 2008a). Therefore, there are still many species with ill-defined specific *status*. Additionally, according to Weber (2003), based on molecular data, one-third of the current species of *Hypostomus* are still to be described.

These fishes are important for the aquarium trade where they are known as "plecos". They occur in a wide range of habitats, from still waters to fast running mountain streams. In greater water bodies they are usually found along lateral bank rivers with slower water current speed (BURGESS, 1989), but several species are

also found in rapids. According to Suzuki et al. (2000) these fishes exhibit several reproductive strategies, mainly parental care as egg and larvae brooders or nest guarders, and its sucker-like mouth allow them to feed on algae and detritus.

Studies with allozymes have been carried out to assess the reproductive isolation and the genetic variability among populations of fish (LIMEIRA et al., 2009; PAIVA et al., 2005; PHILIPPSEN et al., 2009; RENESTO et al., 2007; ZAWADZKI et al., 2004a), as well as to reveal new species in high species-rich fish genus as it is the case of the Neotropical genera *Hypostomus* (ZAWADZKI et al., 2004b, 2008b) and *Neoplecostomus* (LUCENA et al., 2012; REUSING et al., 2011).

In samplings in the Ivaí river, a tributary on the left margin of upper Paraná river, southern Brazil, four samples of the genus *Hypostomus*, *H. albopunctatus* (Regan), *H. hermanni* (Ihering), *H. regani* (Ihering) and also specimens of a putative not described species assigned to as *Hypostomus* sp. 1/NUP 5612 were caught. In the rivers and streams of the Neotropical region it is common to find two or more species of *Hypostomus*, which due to the aforementioned morphological variation are often difficult to be identified by ichthyologist. The main objective of this work is to verify if the allozyme data can be useful to genetically differentiate the four morphotype/species, as well as to estimate the level of genetic variability of the analyzed samples.

Material and methods

Specimens of *Hypostomus* sp. 1/NUP 5612, *H. albopunctatus*, *H. hermanni* and *H. regani* (Figure 1) were collected in the Ivaí river (23º40'28"S, 52º07'09"W) (Figure 2), a tributary of the left margin of the upper Paraná river, State of Paraná, Brazil. Voucher specimens were deposited in the collection of fish of the Nupélia (Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura) from the Universidade Estadual de Maringá: NUP 5614 for *H. albopunctatus*; NUP 4842 for *H. hermanni*; NUP 4360 for *H. regani*; and NUP 5612 for *Hypostomus* sp. 1/NUP 5612.

Samples of white skeletal muscle and liver were withdrawn from the fish, homogenized, centrifuged and then applied in horizontal starch gel (15%) according to detailed procedures in Zawadzki et al. (1999) and in polyacrylamide gel vertically (11%) (LAPENTA et al., 1995) in order to perform the electrophoresis. Thereafter, the gels were stained for nine enzymatic systems (Table 1), according to Zawadzki et al. (1999). The data were analyzed using POPGENE 1.31 software (YEH et al., 1999). The genetic interpretation of the enzymatic patterns was based on the quaternary structure of the enzymes described by Ward et al. (1992).

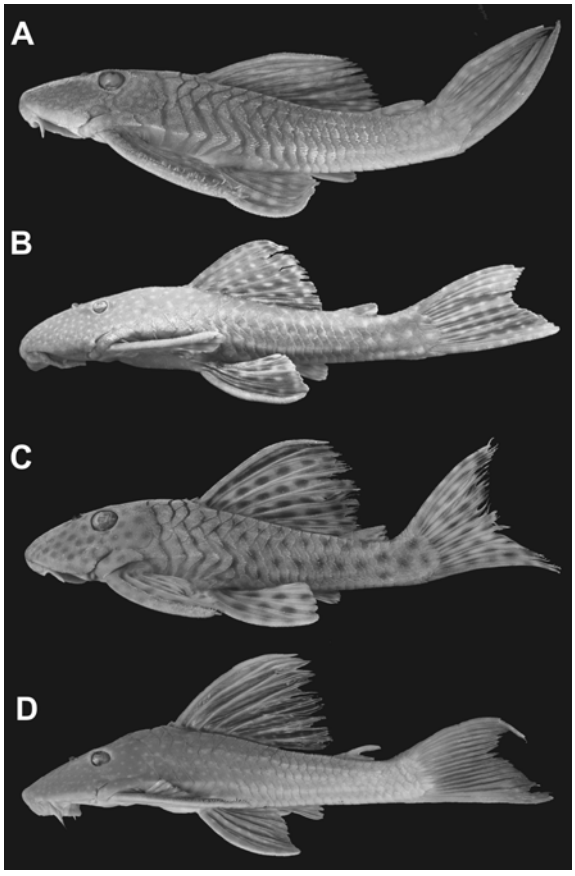

Figure 1. Lateral view of the four *Hypostomus* species from the Ivaí river, upper Paraná river basin, Brazil. A) *Hypostomus* sp. 1/NUP 5612, NUP 5612, SL = 131.1 mm; B) *H. albopunctatus*, NUP 5614, SL = 148.1 mm; C) *H. hermanni*, NUP 6409, SL = 152.6 mm; and D) *H. regani*, NUP 4979, SL = 227.9 mm. SL - means standard length.

Results

The study of the four samples of *Hypostomus* from the Ivaí river, through nine enzymatic systems allowed the analysis of 14 *loci*, which presented a total of 40 alleles. Some diagnostic *loci* were detected among the species: *Adh-A* and *G3pdh-B* in *Hypostomus* sp. 1/NUP 5612, *sAat-B* and *Gcdh-A* for *H. hermanni* and *Est-2* for *H. albopunctatus*. An allele of *locus sAat-A* which expressed a band with a slower cathodic migration for *H. albopunctatus* also differentiated this species from *H. regani* (Table 2).

Variations in allelic frequencies also occurred among the samples of *Hypostomus* analyzed. The

allele *Est-2-d* was diagnostic for *H. albopunctatus,* while the more frequent allele of *Hypostomus* sp. 1/NUP 5612 (*Est-2-c*) and that of *H. hermanni* (*Est-2-e*) allowed the differentiation in most of their individuals from *H. regani* (allele *Est-2-f*). The same case was also verified for *locus sMdh-B* of *Hypostomus* sp. 1/NUP 5612 in relation to the other species, as the most common allele (*sMdh-B-c*) was exclusive for this species. Furthermore, in *Hypostomus* sp. 1/NUP 5612 some exclusive alleles were detected in low frequency (Table 2) (*sAat-B-a*, *Acp-A-a*, *Adh-A-b*, *Est-1-b*, *sMdh-A-b*, *sMdh-B-a*), usually in heterozygosis.

All the *loci* in this study presented more than one allele in at least one species, except *locus G3pdh-A*, which presented the same fixed allele in all samples. The Esterase presented the highest number of alleles per *locus*. *Locus Est-2* presented five different alleles in *H. hermanni* and three alleles in *Hypostomus* sp. 1/NUP 5612.

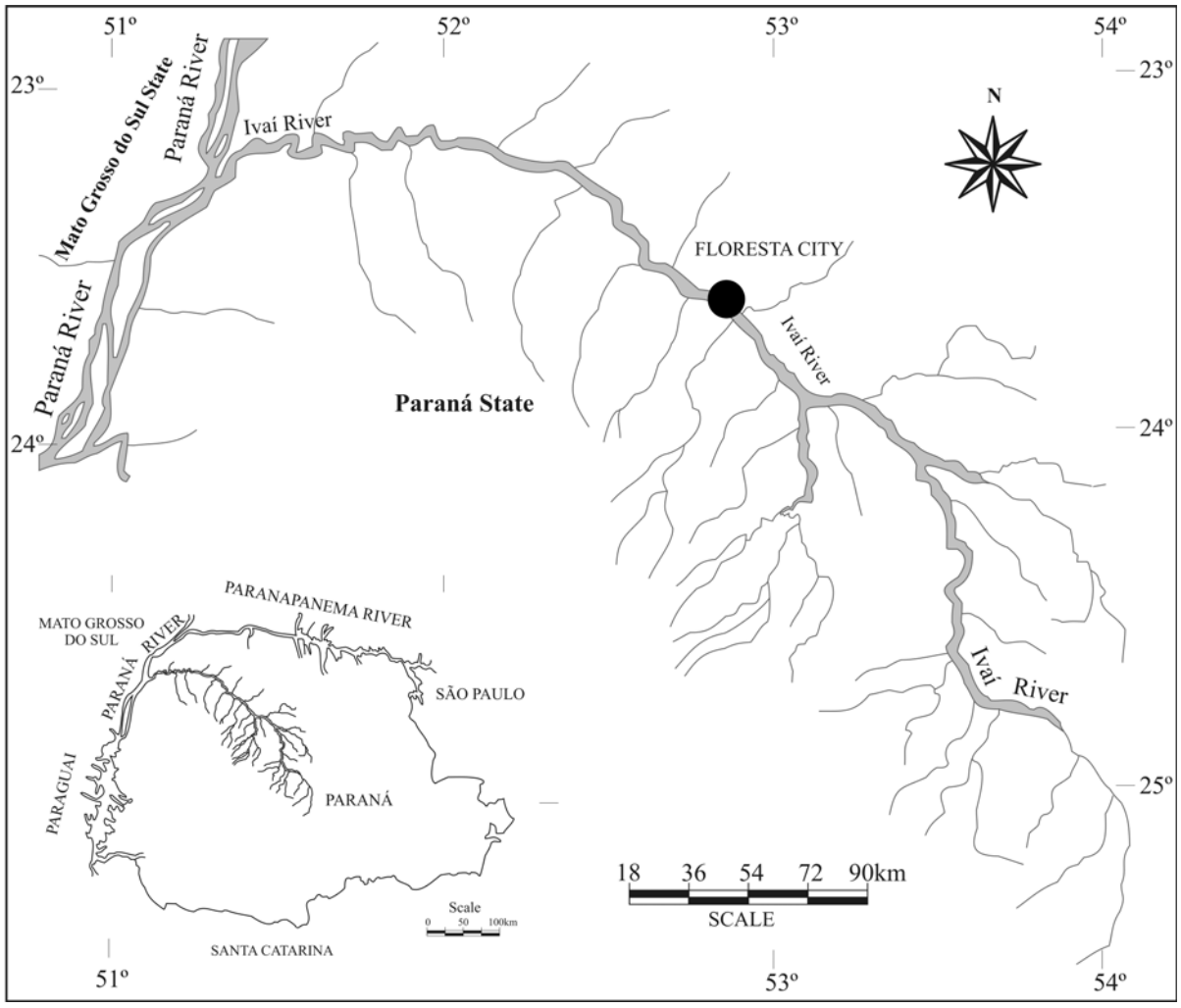

Figure 2. Partial map of Paraná State showing the collecting point (black dot) in the Ivaí river.

Table 1. Name, Enzyme commission number (no. E.C.), tissue, buffer and quaternary structure (Q.S.) of enzymes analyzed in starch and polyacrylamide* gel; (L = liver; M = muscle; 1 = Tris-borate-EDTA; 2 = Tris-citrate; 3 = Tris-EDTA-maleate; 4 = Tris-HCl; 5 = Tris-Glicine).

Enzyme (abbreviation)	nº E.C.	Tissue	Buffer	Q.S.
Alcohol dehydrogenase (ADH)	1.1.1.1	L	1	Dimeric
Aspartate aminotransferase (AAT)	2.6.1.1	L	3	Dimeric
Esterase (EST)*	3.1.1.1	L	4; 5	Monomeric
Acid Phosphatase (ACP)	3.1.3.2	L	2	Monomeric
Glycerol-3-phosphate dehydrogenase (G3PDH)	1.1.1.8	L	2	Dimeric
Glucose-1-dehydrogenase - NAD^+ (GCDH)	1.1.1.118	L	3	Dimeric
L-Lactate dehyidrogenase (LDH)	1.1.1.27	M	2	Tetrameric
Malate dehydrogenase (MDH)	1.1.1.37	L; M	2	Dimeric
Superoxide dismutase (SOD)	1.15.1.1	L	1	Dimeric

Table 2. Allele frequencies, number of analyzed specimens (N), number of polymorphic *loci* (P), proportion of polymorphic *loci* (P%), mean number of allele per *locus* (K), average observed heterozygosity (*Ho*) average expected heterozygosity (*He*) of four populations of *Hypostomus* from Ivaí river. SD = Standard deviation.

Loci	Allele	*Hypostomus* sp. 1/NUP 5612	*H. albopunctatus*	*H. hermanni*	*H. regani*
sAat-A	*a*	0.285	1.000	0.026	—
	b	0.714	—	0.736	1.000
	c	—	—	0.210	—
	d	—	—	0.026	—
sAat-B	*a*	0.023	—	—	—
	b	0.976	1.000	—	1.000
	c	—	—	1.000	—
Acp-A	*a*	0.047	—	—	—
	b	0.952	1.000	1.000	1.000
Adh-A	*a*	—	—	0.789	0.444
	b	0.071	—	—	—
	c	0.928	—	—	—
	d	—	1.000	0.210	0.555
Est-1	*a*	0.071	—	0.368	—
	b	0.023	—	—	—
	c	0.904	1.000	0.631	1.000
Est-2	*a*	0.119	—	0.052	—
	b	0.071	—	0.105	—
	c	0.809	—	—	—
	d	—	1.000	—	—
	e	—	—	0.631	—
	f	—	—	0.052	1.000
	g	—	—	0.157	—
Gcdh-A	*a*	1.000	1.000	—	1.000
	b	—	—	1.000	—
G3pdh-A	*a*	1.000	1.000	1.000	1.000
G3pdh-B	*a*	—	—	0.947	0.666
	b	1.000	—	—	—
	c	—	1.000	0.052	0.333
Ldh-A	*a*	—	—	0.026	0.111
	b	1.000	1.000	0.973	0.888
sMdh-A	*a*	0.928	1.000	1.000	1.000
	b	0.071	—	—	—
mMdh-A	*a*	—	—	0.368	—
	b	1.000	1.000	0.631	1.000
sMdh-B	*a*	0.023	—	—	—
	b	0.261	1.000	1.000	1.000
	c	0.714	—	—	—
Sod-A	*a*	—	—	0.210	—
	b	1.000	1.000	0.789	1.000
N		21	13	19	9
P		8	0	8	3
P %		57.14	0.00	57.14	21.43
K (SD)		1.785 (0.801)	1.000 (0.000)	1.928 (1.206)	1.214 (0.425)
Ho (SD)		0.051 (0.073)	0.000 (0.000)	0.172 (0.262)	0.031 (0.080)
He (SD)		0.126 (0.158)	0.000 (0.000)	0.199 (0.224)	0.085 (0.183)

The genetic variability of the species analyzed presented values from 0.000 for *H. albopunctatus* to 0.199 for *H. hermanni* (Table 2). From the allele frequencies, the values of genetic identity (I) and genetic distance (D) were calculated (Table 3) and it was observed that *Hypostomus* sp. 1/NUP 5612 and *H. hermanni* (D = 0.563) were the most genetically divergent. A dendrogram based on the genetic distance between the four samples is represented in Figure 3. *Hypostomus albopunctatus* and *H. regani* were the most similar samples analyzed (D = 0.218). The F_{ST} value among the samples were 0.671.

Table 3. Genetic identity (I) (above diagonal), genetic distance (D) (below diagonal) of Nei (1978) between four species of *Hypostomus* from the Ivaí river.

Taxon	1	2	3	4
1 - *Hypostomus* sp. 1/NUP 5612	—	0.711	0.569	0.768
2 – *Hypostomus albopunctatus*	0.341	—	0.583	0.804
3 - *Hypostomus hermanni*	0.563	0.539	—	0.734
4 - *Hypostomus regani*	0.264	0.218	0.308	—

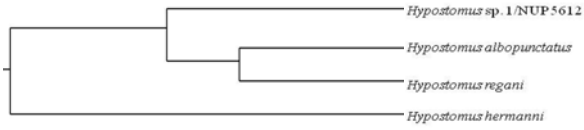

Figure 3. UPGMA dendrogram from the similarity indices of Nei (1978) for the four *Hypostomus* population from the Ivaí river.

Discussion

Hypostomus sp. 1/NUP 5612 morphotype is a small to medium-lengthened species bearing pale spots over body and fins, large mandibles and large eyes. It is distributed throughout Ivaí river and several tributaries of this river and, apparently, is restricted to this system. The similar pale-spotted species inhabiting Ivaí river basin are *H. albopunctatus*, *H. regani* and *H. strigaticeps*. *Hypostomus regani* (Figure 1D) is the most likely to be morphologically confounded to *Hypostomus* sp. 1/NUP 5612 (Figure 1A), mainly the young specimens. *Hypostomus* is traditionally known as having a complex taxonomy, which is mainly due to the incomplete descriptions and broad morphological variation of many of its species (OYAKAWA et al., 2005; WEBER, 2003).

The study of enzymatic *loci* has been used to discover or to confirm the existence of sibling species or species with dubious taxonomic *status* in sympatric populations of several organisms (THORPE; SOLÉ-CAVA, 1994) and in syntopic morphotypes of *Hypostomus* (ITO et al., 2009; ZAWADZKI et al., 2008b). In another study with allozyme data of the genus *Hypostomus*, Paiva et al. (2005) found the *loci* Acp-A, Gcdh-A and sMdhp-A as diagnostic for three species, *H. strigaticeps*, *Hypostomus* sp. 1 and *Hypostomus* sp. 2 from Maringá stream, a tributary to Pirapó river, Paranapanema river basin. Furthermore, 12 alleles also served as diagnostic for *Hypostomus* sp. 2 in that work. The allele Adh-A-*a* was diagnostic for *Hypostomus* sp. 1 as it clearly distinguished the two morphotypes from each other and from *H. strigaticeps* (PAIVA et al., 2005).

In the present work, besides the diagnostic *loci* *Adh-A* and *G3pdh-B* in *Hypostomus* sp. 1/NUP 5612, changes in allelic frequencies also revealed a possible

differentiation between the species analyzed. The alleles *Est-2-c* were the most frequent at *Est-2 locus* and exclusive for *Hypostomus* sp. 1/NUP 5612 and *Est-2-f* was the most frequent and exclusive for *H. hermanni*. *Est-2-d* was fixed and exclusive for *H. albopunctatus*. The *sMdh-B-c* allele also allowed the differentiation of most specimens of *Hypostomus* sp. 1/NUP 5612 from the other three species. Besides, we verified exclusive alleles in low frequencies in *H. hermanni*: *sAat-A-c*, *Est-2-g*, *mMdh-A-a* and *Sod-A-a* (Table 2).

In *Hypostomus* sp. 1/NUP5612, the exclusive alleles *sAat-B-a, Acp-A-a*, *Adh-A-b*, *sMdh-A-b* and *sMdh-B-a* were recorded with low frequencies (Table 2). The low frequency of some exclusive alleles, together with the fact that part of these *loci* are not in Hardy-Weinberg equilibrium in these species, could characterize a population which is in the process of stabilization after some undetermined disturbing event (eg. drastic drought, pollution or local overfishing).

According to Mayr (1963), new genes are incorporated in populations in heterozygous condition, and the homozygotes usually only become frequent after a long time. Three *loci* with alleles in similar frequencies were found in *H. derbyi* from Iguaçu river, *GDH-1**-A (0.036), *GPI-2**-C (0.071) and *IDHP-1**-C (0.036) (ZAWADZKI et al., 1999) and the alleles Gpi-B-*a* (0.031), mMdh-A-*b* (0.063) and sMdh-B-*a* (0.031) in *Hypostomus* sp. 3 from Keller stream (ZAWADZKI et al., 2004b). According to Thorpe and Solé-Cava (1994), in sympatric populations, significant differences in any *locus* between two morphs represent a barrier to gene flow and, at least, partial reproductive isolation. In organisms with sexual reproduction by cross fertilization, this variation indicates that the two populations should be considered as different species.

In *H. albopunctatus*, all the analyzed *loci* were monomorphic (Table 2) and consequentially, presented a genetic variability with zero value (*He* = 0.000). Probably, inbreeding due to its sedentary habits, could explain the absence of heterozygotes for this sample. Zawadzki et al. (1999) found the value of *He* = 0.011 for *H. derbyi* and 0.017 for *H. myersi* of Iguaçu river and suggested that inbreeding could have contributed for the maintenance of low levels of heterozygosity. In *Hypostomus* sp. 2 from Maringá stream (PAIVA et al., 2005) and *Neoplecostomus paranensis* of Hortelã stream (ZAWADZKI et al., 2004a), both species belonging to the family Loricariidae, all *loci* were also monomorphic, that is, *He* equal to zero. According to the authors, inbreeding would be acting in these species, due to a probable isolation by some geographical barriers in these small tributaries.

However, genetic drift events as bottleneck and founder effect can not be rejected. Increasing the analyzed specimens number of each of these aforementioned homozygote populations could probably reveal some rare heterozygote alleles, although it would not be expected to considerably change the allelic frequency levels.

A comparison among the values of average heterozygosity for 84 species of tropical fish, in relation to reproductive strategies (LASSALA; RENESTO, 2007), showed that the group sedentary or short distance migrants with parental care (as is also the case of the species of *Hypostomus*) had the smallest average heterozygosity (0.046), followed by migratory species of long distance and without parental care (0.064). The highest mean *He* (0.081) was for the sedentary or short distance migrants species and without parental care group. These results indicate that the parental care could be associated with low values of heterozygosity. According to these authors, species exhibiting parental care tend to be less variable. In species without parental care, few offspring are likely to survive, and a greater genetic variability is important to face environmental challenges.

The genetic variability in *H. albopunctatus* from the Itaipu reservoir (ZAWADZKI et al., 2005), was slightly higher (He = 0.031) than those found in this work for *H. albopunctatus* (He = 0.000). Also, the total mean values obtained herein for *H. albopunctatus* are still low when compared to the value of average heterozygosity for 195 species of fish from several regions of the world (WARD et al., 1992) that was *He* = 0.051, or with the average value of heterozygosity (*He* = 0.046) for 49 species of freshwater fish by Ward et al. (1994).

The values of *He* for *Hypostomus* sp. 1/NUP 5612 (0.126), *H. hermanni* (0.199) and *H. regani* (0.085) in this work, different from *H. albopunctatus*, were considerably higher than the average found by Ward et al. (1992, 1994). The value of *He* in *H. regani* from the Corumbá reservoir (0.056) (ZAWADZKI et al., 2008c) were slightly above the average of Ward et al. (1992, 1994), while the population of *H. regani* from the Itaipu reservoir presented *He* value higher than the average, 0.078 (ZAWADZKI et al., 2008b). These *H. regani* populations were previously isolated by several waterfalls along the Paraná river and, currently by several reservoirs. Thus, they would have suffered different evolutionary rates which could have occasioned the differences in the heterozygosity values.

The expected heterozygosity of *Hypostomus* sp. 1/NUP 5612 (0.126) may be similar to those

obtained by Zawadzki et al. (2005) for *H. margaritifer* (0.106) and *Hypostomus* sp. 1 (0.107) from the Itaipu reservoir, or even, with *Hypostomus* sp. 1 (0.143) from the Keller river (ZAWADZKI et al., 2004b). However, none of these results are close to the genetic variability reported for *H. hermanni* in this work (0.199), which seems to be one of the greatest values of *He* ever found for a species of this genus. According to Ward et al. (1994), the estimated values of heterozygosity expected from an average of 107 species of fish, varies from zero to 0.05 for 54% of the species, from 0.05 to 0.10 for 30%, from 0.10 to 0.15 for 12% and exceeding 0.15 there was only 4% of the species analyzed by these authors. In the Corumbá reservoir, Zawadzki et al. (2008b) also verified a significant variation in heterozygosity among ten species of *Hypostomus* analyzed, ranging from 0.009 in *H. iheringii* to 0.099 in *Hypostomus* sp. 4. These authors reported that the different *He* values revealed a lack of uniformity in the patterns of genetic variability among species of this genus. Although *Hypostomus* sp. 1/NUP 5612 presented exclusive alleles and the same percentage of polymorphic *loci* of that *H. hermanni*, the highest genetic variability was found in this last species due to a greater number of alleles per *locus* (1.928) (Table 2) as, for example, in *loci* Est-2 and Sod-A.

From the allele frequencies the values of identity (I) and genetic distance (D) were calculated (Table 3) and it was observed that *Hypostomus* sp. 1/NUP 5612 and *H. hermanni* (D = 0.563) were the most genetically divergent. The genetic distance (D) of Nei (1978) estimates the average number of nucleotide substitutions per *locus*, detectable by electrophoresis and accumulated in populations since they diverged from a common ancestral, that is, the distance should be proportional to the evolutionary time (DOBZHANSKY et al., 1977; THORPE; SOLÉ-CAVA, 1994). Contrary to the genetic distance, the genetic identity (I) represents the proportion of the products of the genes that are not differentiated by electrophoretic procedure (DOBZHANSKY et al., 1977) and its value varies from 0 to 1. According to Thorpe and Solé-Cava (1994), 85% of the values of *I* between species of the same genus exceed 0.35 and 97% of the values are below 0.85. Among species of different genus 77% of the values are less than 0.35, while for 98% of populations of the same species exceeds 0.85. These values when compared to the similarity between *Hypostomus* sp. 1/NUP 5612 and *H. hermanni* (I = 0.569), between *Hypostomus* sp. 1/NUP 5612 and *H. albopunctatus* (I = 0.711) and between *Hypostomus* sp. 1/NUP 5612 and *H. regani* (I = 0.768) corroborate the distinction among this morphotype and the three nominal species. Additionally, the differentiation is supported by the high F_{ST} value (0.6707) among the analyzed samples. According to allozyme data, F_{ST} values higher than 0.15 are considered indicative of significant differentiation among populations (FRANKHAM et al., 2008).

The genetic distance among the species studied in the present work resulted in a dendrogram (Figure 3) which showed *H. albopunctatus* and *H. regani* as the most similar (D = 0.218). An analysis of the same both species, but from the Itaipu reservoir by Zawadzki et al. (2005), presented practically the same distance between them (D = 0.219), despite a greater number of enzymatic systems (14) and *loci* number (25) that have been analyzed in that study. Zawadzki et al. (2005) also found that *H. albopunctatus* and *H. regani* from Itaipu reservoir were genetically similar (I = 0.803), as well as *H. albopunctatus* and *H. regani* from the Ivaí river in the present work (I = 0.804). This data corroborates the statement of Thorpe and Solé-Cava (1994), that there is a clear relationship between taxonomic divergence and genetic distance.

Conclusion

These results highlight the usefulness of allozyme data to obtain genetic markers to Neotropical fish, as well as to inferences on the heterozygosity and evolutionary genetics among species of *Hypostomus*. The allozyme data, therefore, indicate herein that the four species of *Hypostomus* from the Ivaí river present biochemical diagnostic markers which allow to genetically differentiate them.

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