



Allozyme differentiation of two populations of the genus *Neoplecostomus* Eigenmann & Eigenmann, 1888 (Teleostei, Loricariidae) from the upper Paraná River basin, Brazil

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Abstract

Allozyme electrophoresis was used to examine 12 enzymatic systems in two populations of the genus *Neoplecostomus* from the Paraná River basin. Samples of *Neoplecostomus* sp. 1 were collected in Paraitinguinha stream of the Tietê River basin, in the municipality of Salesópolis, São Paulo State, and those of *Neoplecostomus* sp. 2 from São Domingos stream of the Rio Grande River basin, in the municipality of Muzambinho, Minas Gerais State. The genetic variability of the two populations was estimated by Nei's expected heterozygosity and was considered lower than average for populations of freshwater fish. The proportion of polymorphic loci was low (only 5.26% for the locus *Idh*). The low frequency of heterozygosity for both populations revealed a high fixation of alleles for each locus. Homozygote excess was observed in both populations. The values of Nei's genetic identity and the presence of loci with different allele frequencies in both populations may imply that the two populations belong to different species. The genetic variability between populations was compared to other data for loricariids.

Key words: allozymes, genetic variability, Neoplecostominae, Neotropical fishes.

Received: October 27, 2010; Accepted: March 30, 2011.

Introduction

The order Siluriformes is the most diverse and well-distributed within the Ostariophysi, and includes 3093 species, 478 genera and 36 families (Ferraris Jr, 2007). In the Neotropical region, there are 1648 nominal species grouped in 15 families (Reis *et al.*, 2003). The distribution of Neotropical Siluriformes appears to be limited by temperature since most of the species live in tropical areas, with few reaching the southern portion of South America or the northern edge of North America (Nelson, 2006). Many species of this order occur in small headwater streams with clear water, strong currents and a high oxygen content, while others have adapted to stagnant and often polluted waters in which oxygen levels are extremely low (Burgess, 1989; M.R. Britto, 2002, Doctoral thesis, Universidade de São Paulo, São Paulo, Brazil). Among the headwater fishes of the southeastern region of South America, representa-

tives of the subfamily Neoplecostominae are the most prominent.

There is controversy regarding which genera belong to the Neoplecostominae, although important progress has been made through the phylogenetic contributions of Montoya-Burgos *et al.* (1998), Cramer *et al.* (2008) and Chiacchio *et al.* (2009). Within the Neoplecostominae, representatives of the genus *Neoplecostomus* occur in the headwater streams of southern and southeastern Brazil. Langeani (1990), who reviewed the genus *Neoplecostomus*, recognized *N. microps* and *N. granosus*, and described *N. paranensis*, *N. espiritosantensis*, *N. ribeirensis* and *N. franciscoensis*. Bizerril (1995) subsequently described *N. variipictus* from the Paraíba do Sul River basin, and Zawadzki *et al.* (2008a) recently described three new species of *Neoplecostomus* (*N. corumba*, *N. selenae* and *N. yapo*) from the upper Paraná River basin.

Neoplecostomus species are morphologically very similar (Langeani, 1990), although some can be very different genetically, as shown by Zawadzki *et al.* (2004a), who compared *Neoplecostomus corumba* (*Neoplecostomus* sp. in that work) and *N. paranensis* using allozyme electropho-

resis. In view of the difficulty in identifying species of this genus, in the present study, two populations of *Neoplecostomus*, one from São Domingos stream of the Grande River in the municipality of Muzambinho, in Minas Gerais State, and another from Paraitinguinha stream of the Tietê River basin in the municipality of Salesópolis, São Paulo State (both in the upper Paraná River basin) were compared using allozyme gel electrophoresis in order to improve our understanding of the biodiversity within this genus.

Material and Methods

Twenty-nine specimens of *Neoplecostomus* sp. 1 (Figure 1A) were collected in Paraitinguinha stream (Tietê River basin) at 23°30'39,84" S/45°51'32,22" W and an altitude of 786 meters in the municipality of Salesópolis, São Paulo State (Figure 2). Twenty specimens of *Neoplecostomus* sp. 2 (Figure 1B) were collected in São Domingos stream (Grande River basin), 21°20'47,22" S/46°28'00,79" W and an altitude of 1021 meters in the municipality of Muzambinho, Minas Gerais State (Figure 2). Specimens of the two populations reported here differed morphologically from the four species of *Neoplecostomus* described for the upper Paraná River basin by the following characters: (1) a well-developed adipose fin distinguished them from *N. corumba* and *N. paranensis* that have a reduced/absent adipose fin or no adipose fin, respectively, and (2) homogeneously dispersed hypertrophied odontodes in the dorsal region of the head and not bordered by swollen skin *vs.* more hypertrophied odontodes in front of the eyes

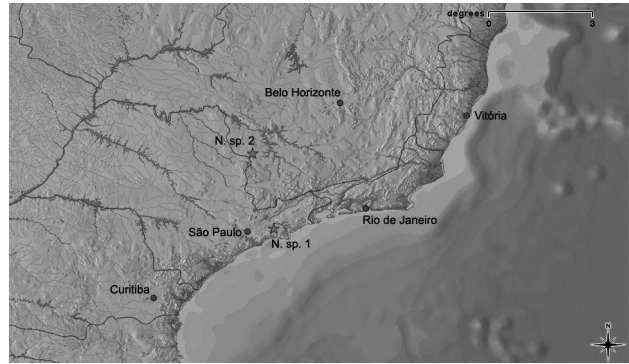


Figure 2 - Geographic location of sampling sites for *Neoplecostomus* sp. 1 (N. sp. 1) and *Neoplecostomus* sp. 2 (N. sp. 2).

and the lateral margin of the snout surrounded by swollen skin in *N. selenae*, and more hypertrophied odontodes bordered by hypertrophied skin only on the lateral margin of the snout in *N. yapo*.

The fish were frozen in liquid nitrogen and transported to the Universidade Estadual de Maringá. Voucher specimens were deposited in the ichthyological collection of the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupélia) of the Universidade Estadual de Maringá (*Neoplecostomus* sp. 1 under accession number NUP 6102 and *Neoplecostomus* sp. 2 under accession number NUP 6103).

Samples of liver and white muscle were homogenized with a plastic pestle in polypropylene tubes (1.5 mL) containing 100 µL of 0.02 M Tris-HCl, pH 7.5. To the liver samples 100 µL of carbon tetrachloride (CCl₄) was added



Figure 1 - A) *Neoplecostomus* sp. 1, CP = 102.2 mm, collected in the Paraitinguinha stream, Salesópolis, SP, NUP6103; B) *Neoplecostomus* sp. 2, CP = 75.2 mm, collected in São Domingos stream, Muzambinho, MG, NUP 6102.

to facilitate homogenization of this fatty tissue (Pasteur *et al.*, 1988). Aliquots of the protein extracts were applied to 15% corn starch (Penetrose 50) gels (Val *et al.*, 1981) by using small (4 mm x 8 mm) Whatman® 3 MM filter paper strips soaked in the samples followed by horizontal electrophoresis under refrigeration. Two buffer solutions were used: 0.135 M Tris/0.043 M citric acid, pH 7.0 (TC), diluted 15 times during preparation of the gel, and 0.18 M Tris/0.1 M boric acid/0.004 M EDTA, pH 8.6 (TBE), diluted four times during preparation of the gel.

The gels were run for 17 h (current of ~50 V at the ends of the gel). After electrophoresis, the gels were cut horizontally into two slices that were then incubated with specific histochemical solutions to detect the bands of enzyme activity in each system, according to standard protocols (Murphy *et al.*, 1996).

The genetic interpretation of the electrophoretic profiles was based on the structure of each enzyme, according to Ward *et al.* (1992). Genetic variability was estimated by calculating the expected (He) and observed (Ho) heterozygosities, according to Nei (1978), as well as genetic identity (I) and distance (D), which were calculated from the allele frequencies. All analyses were done using the software GENEPOP 1.31 (Yeh *et al.*, 1997).

Results

We analyzed 12 enzyme systems (Table 1) in two populations of *Neoplecostomus* and obtained 19 loci (Table 2) with a total of 29 alleles. Of the 49 individuals analyzed, 29 belonged to the morphotype *Neoplecostomus* sp. 1, collected in Paraitinguinha stream, and 20 to *Neoplecostomus* sp. 2, collected in São Domingos stream. The elec-

Table 1 - Name, Enzyme Commission number (E.C. No.), tissue, buffer, and number of loci for each enzyme in *Neoplecostomus* sp. 1 from Paraitinguinha stream (Salesópolis, SP) and *Neoplecostomus* sp. 2 from São Domingos stream (Muzambinho, MG).

Enzyme (abbreviation)	E.C. No.	Tissue	Buffer	Loci
Aspartate aminotransferase (AAT)	2.6.1.1	L	TBE	1
Acid phosphatase (ACP)	3.1.3.2	L	TBE	1
Alcohol dehydrogenase (ADH)	1.1.1.1	L	TBE	1
Esterase (EST)	3.1.1.1	L	TBE	2
Glycerol-3-phosphate dehydrogenase (G3PDH)	1.1.1.8	M	TC	2
Glucose dehydrogenase (GDH)	1.1.1.47	L	TBE	1
Isocitrate dehydrogenase (IDH)	1.1.1.42	M	TC	1
L-lactate dehydrogenase (LDH)	1.1.1.27	M	TC	2
Malate dehydrogenase (MDH)	1.1.1.37	M	TC	3
Peroxidase (PER)	1.11.1.6	L	TBE	2
Phosphoglucomutase (PGM)	5.4.2.2	M	TC	1
Sorbitol dehydrogenase (SORB)	1.1.1.14	L	TC	2

L – liver, M – muscle, TBE – Tris/borate/EDTA buffer, pH 8.7 (Boyer *et al.*, 1963) and TC – Tris/citrate buffer, pH 7.0 (Shaw and Prasad, 1970).

trophoretic patterns of the 12 enzyme systems obtained in this study were similar to those reported by Zawadzki *et al.* (2004a).

The two populations differed at nine (*Aat*, *Acp*, *Adh*, *Gdh*, *Idh*, *Mdh-C*, *Pgm* and *Sorb-1-2*) of the 19 loci. These loci were diagnostic, *i.e.*, they possessed alleles for each morphotype with a frequency of 100%. As shown in Table 2, *Neoplecostomus* sp. 2 (from Muzambinho) was monomorphic at all 19 loci, whereas *Neoplecostomus* sp. 1 (from Salesópolis) was monomorphic at all but one locus (5.26% polymorphism; only the *Idh* loci showed allelic variation). The expected heterozygosity (He) for *Neoplecostomus* sp. 1 was 0.0069, while the observed heterozygosity (Ho) was 0.0073.

Table 2 - Allele frequencies at 19 loci in *Neoplecostomus* sp. 1 from Paraitinguinha stream (Salesópolis, SP) and *Neoplecostomus* sp. 2 from São Domingos stream (Muzambinho, MG).

Loci	Alleles	Salesópolis (N = 29)	Muzambinho (N = 20)
<i>Aat</i> *	<i>a</i>	-	1.0000
	<i>b</i>	1.0000	-
<i>Acp</i> *	<i>a</i>	-	1.0000
	<i>b</i>	1.0000	-
<i>Adh</i> *	<i>a</i>	-	1.0000
	<i>b</i>	1.0000	-
<i>Est-1</i>	<i>a</i>	1.0000	1.0000
<i>Est-2</i>	<i>a</i>	1.0000	1.0000
<i>Gdh</i> *	<i>a</i>	-	1.0000
	<i>b</i>	1.0000	-
<i>G3pdh-1</i>	<i>a</i>	1.0000	1.0000
<i>G3pdh-2</i>	<i>a</i>	1.0000	1.0000
<i>Idh</i> *	<i>a</i>	-	1.0000
	<i>b</i>	0.9310	-
	<i>c</i>	0.0690	-
<i>Ldh-A</i>	<i>a</i>	1.0000	1.0000
<i>Ldh-B</i>	<i>a</i>	1.0000	1.0000
<i>Mdh-A</i>	<i>a</i>	1.0000	1.0000
<i>Mdh-B</i>	<i>a</i>	1.0000	1.0000
<i>Mdh-C</i> *	<i>a</i>	-	1.0000
	<i>b</i>	1.0000	-
<i>Per-1</i>	<i>a</i>	1.0000	1.0000
<i>Per-2</i>	<i>a</i>	1.0000	1.0000
<i>Pgm</i> *	<i>a</i>	1.0000	-
	<i>b</i>	-	1.0000
<i>Sorb-1</i> *	<i>a</i>	-	1.0000
	<i>b</i>	1.0000	-
<i>Sorb-2</i> *	<i>a</i>	-	1.0000
	<i>b</i>	1.0000	-

N = number of specimens analyzed. Asterisks indicate loci with allele frequencies that differed significantly between populations.

Based on the gene frequencies, the genetic identity (I) and distance (D) were 0.5281 and 0.6384, respectively. The Nei (1978) genetic distance represents the average number of nucleotide substitutions per locus (detectable by electrophoresis) that have accumulated in populations since they diverged from a common ancestor, *i.e.*, the substitution is proportional to evolutionary time (Dobzhansky *et al.*, 1977; Thorpe, 1982; Thorpe and Solé-Cava, 1994).

The negative value of F_{IS} (-0.0741) indicated an excess of heterozygotes for the *Idh* locus in the *Neoplecostomus* sp. 1 population. On the other hand, the mean F_{IT} value (0.9844) indicated an excess of homozygotes for both species. According to Wright (1978), the F_{ST} statistic reflects genetic differentiation between two populations. The average F_{ST} score for the loci analyzed was 0.9855, indicating marked genetic differentiation between the two samples; for nine loci (*Aat*, *Acp*, *Adh*, *Gdh*, *Mdhc*, *Pgm*, *Sorb-1* and *Sorb-2*) the F_{ST} value was 1.00.

Discussion

According to Thorpe and Solé-Cava (1994), populations belonging to the same species have genetic identity values (I) > 0.85, whereas those belonging to different genera have I < 0.35 and species belonging to the same genus have I values of 0.35-0.85. The I value for *Neoplecostomus* sp. 1 and *Neoplecostomus* sp. 2 was 0.5281 (with D = 0.6384), indicating that these populations belong to two species of the same genus. *Neoplecostomus* species are morphologically very similar (Langeani, 1990), but very different genetically, as shown by Zawadzki *et al.* (2004a). The detection of fixed divergent alleles in syntopic populations of diploid organisms generally reflects a restricted gene flow and, consequently, the existence of different biological species (Richardson *et al.*, 1986; Murphy *et al.*, 1996). As shown here, nine of the 19 loci surveyed were diagnostic (Table 2), leading us to conclude that the two populations studied represented different species.

In contrast to the marked genetic divergence seen here between the two populations, other studies based on allozyme characters in allopatric populations of loricariid fishes have found no diagnostic markers. For example, Zawadzki *et al.* (2008b) found no fixed diagnostic markers for three populations of *Hypostomus regani* from the Corumbá River, Itaipu Reservoir (both in the upper Paraná River basin) and Manso River (in the Paraguay River basin). Similarly, Limeira *et al.* (2009) found no fixed markers in two populations of *Rineloricaria pentamaculata* above and below an 80 m high waterfall on the Ivaí River. Strieder *et al.* (2009) also found no fixed markers for four populations of *N. yapo* along tributaries of the Tibagi and Pirapó rivers. The lack of genetic divergence in these loricariid populations highlights the relevance of the marked differentiation seen between *Neoplecostomus* sp. 1 and *Neoplecostomus* sp. 2. The finding that almost half of the surveyed loci were fixed to different alleles in each pop-

ulation suggests that there are strong geographic barriers to *Neoplecostomus* fish that try to move from the headwaters of the Tietê River basin to the headwaters of the Grande River basin, or vice versa. Since specimens of *Neoplecostomus* occur only in medium to small headwater streams, we believe that the main channel of large rivers such as the Tietê, Paraná and Grande acts as a barrier to free dispersion.

The finding that *Neoplecostomus* sp. 2 was monomorphic for the 19 loci while *Neoplecostomus* sp. 1 was polymorphic at only a single locus (*Idh*) contrasted with other studies in which the percentage of polymorphic loci in loricariids was generally greater than that observed here. Zawadzki *et al.* (2004a) reported that several loci (*Gpi-B*, *Ldh-B* and *Pgm-A*) were polymorphic in a population of *Neoplecostomus* sp. (= *N. corumba*) whereas no polymorphism was observed in *N. paranensis*. Zawadzki *et al.* (1999) found that the percentage of polymorphic loci in three populations of *Hypostomus* from the Iguaçu River basin ranged from 20 to 40%, whereas Paiva *et al.* (2005) detected 20% polymorphic loci in *H. strigaticeps* and *Hypostomus* sp. 1 and no polymorphism in *Hypostomus* sp. 2 from Ribeirão Maringá.

The H_e values in the family Loricariidae vary considerably. Zawadzki *et al.* (1999) found low H_e values of 0.011 in *Hypostomus derbyi* and 0.017 in *H. myersi* from the Iguaçu River basin, but an extremely high value ($H_e = 0.107$) for another species of *Hypostomus* (*Hypostomus* sp.) from the Itaipu Reservoir in the Paraná River basin (Zawadzki *et al.*, 2005). The H_e of *Neoplecostomus* sp. 1 in the present study was 0.0069, a low value when compared to that observed by Zawadzki *et al.* (2004a) for *Neoplecostomus* sp. ($H_e = 0.030$) and also low in relation to other loricariids from the Paraná-Paraguay River basin (Zawadzki *et al.*, 2002, 2004b; Paiva *et al.*, 2005; Renesto *et al.*, 2007; Ito *et al.*, 2009; Strieder *et al.*, 2009). The H_e for the population of *Neoplecostomus* sp. 2 was zero, as also reported for *N. paranensis* from Hortelã stream (Zawadzki *et al.*, 2004a). The genetic variability in *Neoplecostomus* sp. 1 and *Neoplecostomus* sp. 2 is low when compared to the average H_e of 0.051 estimated for 195 species of different fish species around the world (Ward *et al.*, 1992).

According to Zawadzki *et al.* (2004a), the unusual absence of allozyme genetic variability in *N. paranensis* from Hortelã stream could be explained by the endogamic process and ecological restrictions imposed by geographical or environmental barriers to species of *Neoplecostomus*. Likewise, in the present study, the low levels of genetic variability for the two populations of *Neoplecostomus* may indicate that they are mainly sedentary and probably restricted to small areas.

Loricariids are generally non-migratory (Burgess, 1989; Montoya-Burgos, 2003). Moreover, the short length of most *Neoplecostomus* species and the currently re-

stricted distribution of their populations (limited to headwaters) point towards a low rate of migration (Chiachio *et al.*, 2008). The sedentary nature of these fish leads to mating within the same family group and results in low genetic variability. These characteristics suggest a possible restricted range and reduced gene flow among *Neoplecostomus* species compared to other fish species. A reduction in gene flow and genetic events, such as inbreeding, may favor rapid speciation and endemism (Strieder *et al.*, 2009). In addition, other evolutionary forces may also affect *Neoplecostomus* species. For example, stochastic events such as genetic drift could lead to the fixation of alternative alleles (Kerr and Wright, 1954) in these presumably small populations and may provide a reasonable explanation for the low intraspecific and high interspecific variation in *Neoplecostomus* species. Our findings suggest that other genetically-differentiated populations may be revealed as more headwater streams in southeastern Brazil are sampled.

Acknowledgments

The authors thank Guilherme Costa e Silva and Renato Devidé for helping to collect the specimens, Suzana Paiva for helping with laboratory experiments, Nupélia (Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura) for logistical support and Anderson L. Alves for reading and reviewing this manuscript. We also thank Corn Products Brazil for supplying Penetrose 50. This work was partially supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grant no. 484716/20996-9) and by an individual grant to Claudio Henrique Zawadzki (CNPq grant no. 306066/2009-2).

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Associate Editor: Fábio de Melo Sene

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