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Genetic variability in three species of *Gymnotus* Linnaeus, 1758 (Gymnotiformes: Gymnotidae) from Caracu stream of the upper Paraná River basin, Brazil

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The genetic variability of three *Gymnotus* species from the Caracu stream, a small tributary of the left margin of Paraná River (Brazilian upper Paraná River floodplain), was estimated with data of 17 putative allozyme *loci*, which were obtained by using corn starch gel electrophoresis of 10 enzymatic systems: Aspartate aminotransferase (E. C. 2.6.1.1), Alcohol dehydrogenase (E. C. 1.1.1.1), Esterase (E. C. 3.1.1.1), Glucose dehydrogenase (E. C. 1.1.1.118), Glycerol-3-phosphate dehydrogenase (E. C. 1.1.1.8), Isocitrate dehydrogenase (E. C. 1.1.1.42), L-Lactate dehydrogenase (E. C. 1.1.1.27), Malate dehydrogenase (E. C. 1.1.1.37), Superoxide dismutase (E. C. 1.15.1.1) and Sorbitol dehydrogenase (E. C. 1.1.1.14). The genetic diversity was estimated as *He* = 0.3458 for *G. pantanal*, *He* = 0.2481 for *G. inaequilabiatus*, and *He* = 0.3152 for *G. sylvius*. The most divergent species were *G. sylvius* and *G. pantanal* (D = 0.117), and the most similar were *G. inaequilabiatus* and *G. pantanal* (D = 0.051). The data indicates that the observed genetic variability was very low and the expected variability estimated for these three species is very high, and the genetic differences among them are small. The data suggest that the process of speciation which produced these three species is recent.

A variabilidade genética de três espécies de *Gymnotus* do riacho Caracu, um pequeno afluente da margem esquerda do rio Paraná (planície de inundação do alto rio Paraná) foi estimada com base em 17 *loci* aloenzimáticos, os quais foram obtidos utilizando eletroforese em gel de amido de milho em 10 sistemas enzimáticos: Aspartato aminotransferase (E. C. 2.6.1.1), Álcool desidrogenase (E. C. 1.1.1.1), Esterase (E. C. 3.1.1.1), Glicose desidrogenase (E. C. 1.1.1.118), Glicerol-3-fosfato desidrogenase (E. C. 1.1.1.8), Isocitrato desidrogenase (E. C. 1.1.1.42), L-Lactato desidrogenase (E. C. 1.1.1.27), Malato desidrogenase (E. C. 1.1.1.37), Superóxido dismutase (E. C. 1.15.1.1) e Sorbitol desidrogenase (E. C. 1.1.1.14). A diversidade genética foi estimada em *He* = 0.3458 para *G. pantanal*, *He* = 0,2481 para *G. inaequilabiatus*, e *He* = 0,3152 para *G. sylvius*. As espécies mais divergentes foram *G. sylvius* e *G. pantanal* (D = 0,117), e as mais semelhantes foram *G. inaequilabiatus* e *G. pantanal* (D = 0,051). Os dados mostram que a variabilidade genética observada é muito baixa, mas a esperada é muito alta e que as diferenças genéticas entre elas são pequenas. Os dados sugerem que o processo de especiação que originou as três espécies é recente.

Key words: Polymorphism, Genetic distance, Allozyme, Heterozygosity.

Introduction

The order Gymnotiformes comprises six families: Sternopygidae, Apteronotidae, Rhamphichthyidae, Hypopomidae, Gymnotidae and Electrophoridae (Mago-Leccia, 1994). The genus *Gymnotus* Linnaeus, 1758 includes 32 species, and possesses a larger geographical distribution, when compared to all the other Gymnotiformes. This distribution extends from Salado River in the Pampas of Argentina (36°S) to San Nicolas River of the southeast of Chiapas in Mexico (18°N). It is present in continental waters of all South American and Central American countries, except from Chile and Belize (Albert, 2001; Albert *et al*., 2005).

The largest *Gymnotus* diversity is found in the Amazonian basin, and most of them inhabit flooded areas (Albert & Crampton, 2001). However, little information on the diversity of species, distribution and populational structure of this genus in other Neotropical basins is available (Fernandes-

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Matioli *et al*., 2000).

The *Gymnotus* species are aggressive nocturnal predators of fishes and other small aquatic animals, and many of them are territorial. The males of at least two *Gymnotus* species build nests of foam and/or aquatic vegetation and guard them (Albert & Crampton, 2003). Males of *G. carapo*, for instance, either dig depressions in the substratum or make nests at the roots of aquatic macrophytes (Crampton & Hopkins, 2005).

The *Gymnotus* species have been object of ecological studies (Barbieri & Barbieri, 1984; Cognato & Fialho, 2006; Ferreira & Casatti, 2006), cytogenetic analysis (Foresti *et al*., 1984; Murofushi & Yosida, 1984; Fernandes-Matioli *et al*., 1997, 1998a, 1998b; Margarido *et al*., 2007), phylogenetic approaches (Albert, 2001; Fernandes-Matioli & Almeida-Toledo, 2001; Albert *et al*., 2005) and systematic studies (Albert & Miller, 1995; Campos-da-Paz, 1996, 2000; Albert *et al*., 1999; Fernandes-Matioli *et al*., 2000; Crampton & Albert, 2004; Fernandes *et al*., 2005). However, genetic variability estimates of the *Gymnotus* species have never been made. Estimates of genetic variability in natural populations are important in order to evaluate their evolutionary potentialities as well as to establish species conservation policies.

The upper Paraná River floodplain has 230 km of length, up to 20 km of width and is formed by several ponds, streams, Baía River and lower stretches of Paraná, Ivaí, Ivinheíma, Amambaí, and Iguatemi rivers (Agostinho & Zalewski, 1996). According to Agostinho & Júlio Jr. (1999), the genus *Gymnotus* is present in ponds and in some channels, rivers and streams that flow to Paraná River, being rarely found in the main valley of this river. In these rivers and streams it is possible to find three species of *Gymnotus*: *G. inaequilabiatus* (Valenciennes, 1839), *G. sylvius* Albert *et al*., 1999 and *G. pantanal* Fernandes *et al*., 2005.

The populations of these three species are found in syntopy; they inhabit rooted grasses and floating macrophytes in small creeks and the banks of larger black water rivers patches of floating macrophytes along channel and lake margins areas (Fernandes *et al*., 2005). In the Caracu stream, which runs into Paraná River near Porto Rico County (PR), these three species are in syntopy.

The aim of this work was to estimate the genetic variability of three *Gymnotus* species occurring in the Caracu stream by using the isozyme electrophoresis technique. We also aimed at evaluating their interspecific genetic differences.

Material and Methods

The specimens analyzed were collected from December 2005 to March 2006 along the Caracu stream (Fig. 1). The Caracu stream is a small tributary of Paraná River eastern side, located in Porto Rico County, Paraná State, Brazil. It is classified as a second-order river, according to the classification of Jeffries & Mills (1990). This small tributary has 5.2 km of length and its margins are degraded, except from a few forest stretches (Pavanelli & Caramaschi, 2003).

Fifty five specimens of *G. inaequilabiatus*, 40 of *G. pantanal* and 16 of *G. sylvius* were collected. The 111 specimens captured were deposited in the ichthyological collection of Nupelia (Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura) of Universidade Estadual de Maringá. Vouchers are NUP 3213 for *G. inaequilatiatus*, NUP 4184 for *G. pantanal*, and NUP 5962 for *G. sylvius*. This study was approved by the animal ethics committee of our institution and met all requirements under Brazilian environmental laws. Sampling was carried out under permission of the Brazilian environmental agency (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis - IBAMA), protocol number 137/2006.

The collected tissues were homogenized with plastic sticks in 1.5 mL micro centrifuge tubes containing 100 µL of Tris/0.02MHCl buffer, pH 7.5. Carbon tetrachloride (CCl4) was added to the homogenized liver samples due to the large amounts of fat present in the tissues (Pasteur *et al*., 1988). The homogenized samples were centrifuged at 45.114 x g for 30 min at temperatures between 1°C and 5°C and the supernatants submitted to horizontal electrophoresis in 15% corn starch gel (Val *et al*., 1981).

Ten enzymatic systems were evaluated (Table 1). Enzyme nomenclature followed the proposals of Murphy *et al*. (1996). Electrophoreses conditions were according to the following authors: Boyer *et al*. (1963) for AAT, ADH, EST, GDH, SOD; Shaw & Prasad (1970) for G3PDH, IDH, LDH, MDH, and SORB.

Standard histochemical staining procedures were used to visualize specific enzymes (Aebersold *et al*., 1987). The genetic interpretation of the gels was based on the quaternary structure of the enzymes (Ward *et al*., 1992). The data was analyzed with POPGENE software, version 1.31 (Yeh *et al*., 1999). The genetic variability was estimated by using Nei's unbiased heterozygosity (*He*) or gene diversity (Nei, 1978).

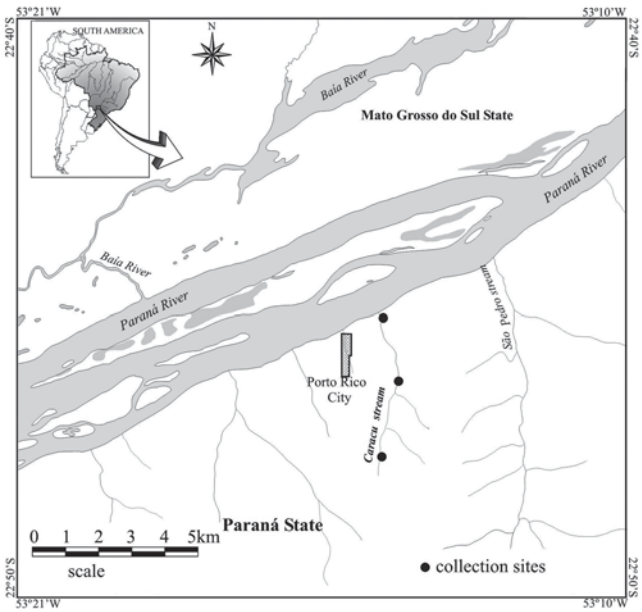

Fig. 1. Localization of Caracu stream where the *Gymnotus* specimens were collected.

Table 1. Electrophoretic conditions for 10 enzymatic systems assayed for *Gymnotus inaequilabiatus*, *G. sylvius* and *G. pantanal* from Caracu stream, Paraná State, Brazil.

Enzyme	Tissue	Buffer	Time	References
AAT, EST, ADH, GDH, SOD	Liver, Kidney	Tris/borate/EDTA 0.045/0.025/0.001M, pH 8.6	7 hours	Boyer *et al.* (1963)
G3PDH, LDH, SORB	Liver, Kidney	Tris/citrate 0.009/0.003M, pH 7.0	14 hours	Shaw & Prasad (1970)
IDH, MDH	Liver, Muscle, Gill	Tris/citrate 0.009/0.003M, pH 7.0	14 hours	Shaw & Prasad (1970)

The observed (*Ho*) and expected (*He*) heterozygosities for each putative *locus* and the overall *loci* means were also calculated. Genotypic frequencies were tested for Hardy-Weinberg equilibrium, by using the chi squared (χ^2) test. The Genetic Identity and the genetic distance were calculated according to Nei (1972). The comparison between the heterozygosities of these three species was made through a test for paired data (Nei, 1987).

Results

In this work 10 enzymatic systems were analyzed in 111 specimens of *Gymnotus*, 55 *Gymnotus inaequilabiatus*, 40 *Gymnotus pantanal* and 16 *Gymnotus sylvius*. The allele frequencies are summarized in Table 2 and the estimates of genetic variability of these species in Table 3. Table 4 shows the values of the χ^2 test for homogeneity of the allele frequencies among the three species, and Table 5 presents the values of genetic identity and genetic distance of Nei (1972).

In most of the enzymatic systems it was possible to detect more than one *locus* except for GDH, G3PDH and SORB, which presented just one. The Malate dehydrogenase (MDH) enzymatic system presented three *loci*; one of them (*Mdh-B*) is monomorphic.

All the *loci* presented more than one allele in at least one population, and the *locus Sorb-1* presented four alleles for the population of *G. inaequilabiatus*. No diagnostic *locus* was found to differentiate these three species in the enzymatic systems analyzed. However, some alleles with low frequency and exclusive for each population were detected: *Ldh-1(c)* e *Sorb-1(d)* for *G. inaequilabiatus*, *Aat-1(b)*, *Aat-2(b)* e *Est-1(c)* for *G. pantanal* and *G3pd-1(a)* for *G. sylvius*. None of the polymorphic *loci* is in Hardy-Weinberg equilibrium.

The observed heterozygosity (*Ho)* measures revealed that there were heterozygotes in only three of the seventeen *loci* in *G. pantanal* and *G. inaequilabiatus*, while no heterozygote was detected in *G. sylvius*, probably due to the low number of analyzed individuals. On the other hand, the number of polymorphic *loci* was 13 (76.47%), 15 (88.24%) and 14 (82.35%) for *G. inaequilabiatus*, *G. pantanal* and *G. sylvius*, respectively.

The estimates of genetic variability showed that *G. sylvius* presented the smallest heterozygosity observed and the smallest average number of alleles per *locus* (Na = 1.88). *Gymnotus pantanal* presented the largest expected heterozygosity (*He*), the largest proportion of polymorphic *loci* (88.24%), and also the largest average number of alleles per *locus* (Na = 2.18), while *G. inaequilabiatus* presented intermediate values of genetic variability. The expected heterozygosity for *G. pantanal* is significantly greater than the expected for *G. inaequilabiatus* ($t = 2.88$; 16 d.f.; $p < 0.05$). On the other hand, the expected heterozygosity for *G. pantanal* is not significantly greater than the expected for *G. sylvius* ($t = 0.505$; 16 d.f.; $p > 0.05$), and the expected for *G. sylvius* is not significantly greater than the expected for *G. inaequilabiatus* ($t = 1.35$; 16 d.f.; $p > 0.05$). The expected heterozygosity measures are much higher than the

Table 2. Allele frequencies for three *Gymnotus* species from Caracu stream, Paraná State, Brazil. Bold type indicates exclusive alleles for each species.

Locus	Allele	*G. inaequilabiatus*	*G. pantanal*	*G. sylvius*
Aat-1	*a*	1.0000	0.9615	1.0000
	b		**0.0385**	
Aat-2	*a*	1.0000	0.9474	1.0000
	b		**0.0526**	
Est-1	*a*	0.7182	0.5526	0.6667
	b	0.2818	0.1184	0.3333
	c		**0.3289**	
Est-2	*a*	0.7308	0.8250	0.8333
	b	0.2692	0.1750	0.1667
Adh-1	*a*	0.6389	0.3750	0.3333
	b	0.3333	0.1667	0.6667
	c	0.0278	0.4583	
Gdh-1	*a*	0.0182	0.0250	0.16667
	b	0.9636	0.9500	0.8333
	c	0.0182	0.0250	
G3pd-1	*a*			**0.5000**
	b	1.0000	1.0000	0.5000
Idh-1	*a*	0.0952	0.2381	0.3333
	b	0.9048	0.7619	0.6667
Idh-2	*a*	0.0217	0.4103	
	b	0.9783	0.5897	1.0000
Ldh-A	*a*	0.0400	0.3243	0.2000
	b	0.9400	0.6757	0.8000
	c	**0.0200**		
Ldh-B	*a*	0.0816	0.0385	0.2500
	b	0.9184	0.9615	0.7500
Mdh-A	*a*	0.5455	0.4615	0.1667
	b	0.4545	0.3590	0.6667
	c		0.1795	0.1667
Mdh-B	*a*	1.0000	1.0000	1.0000
Mdh-C	*a*	0.3491	0.4359	0.6000
	b	0.6509	0.5641	0.4000
Sorb-1	*a*	0.5435	0.3333	0.4000
	b	0.2826	0.1905	0.6000
	c	0.1304	0.4762	
	d	**0.0435**		
Sod-1	*a*	0.7812	0.6786	
	b	0.2188	0.3214	1.0000
Sod-2	*a*	0.6735	0.5714	0.2000
	b	0.3265	0.4286	0.8000

Table 3. Genetic variability estimates for each *locus* of the *Gymnotus* species from Caracu stream, Paraná State, Brazil. H_o = observed heterozygosity; H_e = unbiased expected heterozygosity; SD = standard deviation; N = number of specimens; Na = mean number of alleles per *locus*; Ne = effective number of alleles per *locus*; P = proportion of polymorphic *loci*.

	G. inaequilabiatus		*G. pantanal*		*G. sylvius*	
Locus	H_o	H_e	H_o	H_e	H_o	H_e
Aat-1	0.0000	0.0000	0.0000	0.0754	0.0000	0.0000
Aat-2	0.0000	0.0000	0.0000	0.1011	0.0000	0.0000
Est-1	0.0182	0.4085	0.0263	0.5800	0.0000	0.4848
Est-2	0.0000	0.3973	0.0500	0.2924	0.0000	0.3030
Adh-1	0.0000	0.4867	0.0000	0.6348	0.0000	0.4848
Gdh-1	0.0000	0.0714	0.0000	0.0975	0.0000	0.3030
G3pd-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.5455
Idh-1	0.0000	0.1744	0.0000	0.3717	0.0000	0.4848
Idh-2	0.0000	0.0430	0.0000	0.4902	0.0000	0.0000
Ldh-A	0.0000	0.1156	0.0000	0.4443	0.0000	0.3556
Ldh-B	0.0000	0.1515	0.0000	0.0754	0.0000	0.4286
Mdh-A	0.0000	0.5004	0.0000	0.6340	0.0000	0.5455
Mdh-B	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Mdh-C	0.0943	0.4588	0.2051	0.4982	0.0000	0.5333
Sorb-1	0.0435	0.6193	0.0000	0.6411	0.0000	0.5333
Sod-1	0.0000	0.3472	0.0000	0.4442	0.0000	0.0000
Sod-2	0.0000	0.4444	0.0000	0.4987	0.0000	0.3556
mean	0.0092	0.2481	0.0166	0.3458	0.0000	0.3152
SD	0.0246	0.2166	0.0504	0.2379	0.0000	0.2237
N	55		40		16	
Na±SD	2.05 ± 0.80		2.18 ± 0.64		1.88 ± 0.48	
Ne±SD	1.44 ± 0.46		1.71 ± 0.61		1.60 ± 0.37	
P	76.47%		88.24%		82.35%	

Table 4. Chi-square test for homogeneity of allele frequencies for *G. inaequilabiatus*, *G. pantanal* and *G. sylvius* from Caracu stream, Paraná State, Brazil. Bold type indicates the *loci* which were statistically different. χ^2 = chi-square value; DF = degree of freedom.

Locus	χ^2	DF	Probability
Aat-1	2.659713	2	0.264515
Aat-2	6.447368	2	**0.039808**
Est-1	48.136494	4	**0.000000**
Est-2	2.551745	2	0.279187
Adh-1	44.904085	4	**0.000000**
Gdh-1	8.684470	4	0.069489
G3pd-1	49.820000	2	**0.000000**
Idh-1	7.230631	2	0.026908
Idh-2	43.211523	2	**0.000000**
Ldh-A	26.460840	4	**0.000026**
Ldh-B	4.539571	2	0.103334
Mdh-A	25.588794	4	**0.000038**
Mdh-B	0.000000	0	1.000000
Mdh-C	3.232424	2	0.198650
Sorb-1	22.288405	6	**0.001073**
Sod-1	20.238961	2	**0.000040**
Sod-2	9.079258	2	**0.010677**

Table 5. Genetic identity (above diagonal) and genetic distance (below diagonal) of Nei (1972), for three *Gymnotus* species from Caracu stream, Paraná State, Brazil.

Species	1	2	3
1 - *G. inaequilabiatus*	-	0.950	0.908
2 - *G. pantanal*	0.051	-	0.889
3 - *G. sylvius*	0.097	0.117	-

heterozygosity values obtained for the three species. This fact reveals homozygote excess. The homogeneity test (Table 4) showed that the allele frequencies in the three species differed statistically in ten out of the seventeen *loci* studied.

Discussion

The band pattern obtained for the 10 analyzed systems and the *loci* number detected in *Gymnotus* are very similar to those obtained for other species of neotropical fish (Zawadzki *et al*., 2001). The presence of exclusive alleles indicates that these three species are in process of genetic divergence.

The estimates of expected average heterozygosity for *G. inaequilabiatus* (0.248), *G. pantanal* (0.346) and *G. sylvius* (0.315) are high when compared to the values described by Ward *et al*. (1994) for 59 species of freshwater fish (0.046), and to the values detected by Machado & Renesto (2007) for nine species of fish from the floodplain of the upper Paraná River (0 to 0.147).

The fact that no *locus* is in the Hardy-Weinberg equilibrium and the presence of a homozygote excess may be due to inbreeding, once these species are of sedentary habits. Homozygote excess have also been found in the *Hypostomus* species from the upper Paraná and Paraguay River basins, which also have sedentary habits (Zawadzki *et al*., 2002, 2004, 2005; Paiva *et al*., 2005; Renesto *et al*., 2007)

The genetic distance and genetic identity values among the three species of *Gymnotus* showed that the populations with larger genetic divergence were *G. sylvius* and *G. inaequilabiatus* (D = 0.117), and the genetically closest populations were *G. inaequilabiatus* and *G. pantanal* (D = 0.051). Nei's genetic distance, D, is claimed to estimate the average number of electrophoretically detectable substitutions per *locus* and, given certain assumptions, to be linearly proportional to evolutionary time (Thorpe & Solé-Cava, 1994).

The genetic (I) identity, which varies from 0 to 1, represents the proportion of the gene products that are not electrophoretically detectable (Thorpe, 1982). The genetic identity of Nei (1972) varies from 0.80 and 1.00 between two populations of the same species and from 0.02 to 0.98 between two species of the same genus. Thorpe concluded that the critical level for I values distinguishing between species and genera appears to be around 0.35. If allopatric populations of uncertain status usually have genetic identities below 0.85 it is unlikely that they should be considered conspecific, while nominate species with I values above 0.85 should be considered doubtful if there is no other evidence of their specific status. The values of genetic identity (Table 5) among the species analyzed in this work are inside the limits of the same species populations. However, these species possess morphological and chromosomal differences and a combination of different microsatellite patterns (Fernandes, 2000) that allow us to affirm that they are distinct from each

other. Besides, the three species are syntopic and no intermediate types are found among them. It reveals the absence of crossing and reproductive isolation among them. Considering the distance values and the genetic identity obtained in this work we can estimate that the speciation event leading to the origin of these species is very recent.

We consider further studies using molecular markers to be needed for confirming or refuting the low genetic variability estimated for these species.

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