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Short Communication

Esterase-D and chromosome patterns in Central Amazon piranha (*Serrasalmus rhombeus* Linnaeus, 1766) from Lake Catalão

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Abstract

This study presents additional genetic data on piranha (*Serrasalmus rhombeus* Linnaeus, 1766) complex previously diagnosed due to the presence of distinct cytotypes 2n = 58 and 2n = 60. Three esterase-D enzyme loci (*Est-D1*, *Est-D2* and *Est-D3*) were examined and complemented with chromosomal data from 66 piranha specimens collected from Lake Catalão. For all specimens the *Est-D1* and *Est-D2* loci were monomorphic. In contrast, the *Est-D3* locus was polymorphic with genotypes and alleles being differentially distributed in the previously described cytotypes and served as the basis for detecting a new cytotype (2n = 60 *B*). In cytotype 2n = 58 the *Est-D3* locus was also polymorphic and presented Mendelian allelic segregation with four genotypes (*Est-D3*^{11}, *Est-D3*^{12}, *Est-D3*^{22} and *Est-D3*^{33}) out of six theoretically possible genotypes, presumably encoded by alleles *Est-D3*^{1} (frequency = 0.237), *EsT-D3*^{2} (0.710) and *Est-D3*^{3} (0.053). A Chi-squared (χ^2) test for Hardy-Weinberg equilibrium was applied to the *Est-D3* locus and revealed a genetic unbalance in cytotype 2n = 58, indicating the probable existence in the surveyed area of different stocks for that karyotypic structure. A silent null allele *(Est-D3*^{0}) with a high frequency (0.959) occurred exclusively in the 2n = 60 cytotype. On the other hand, the new cytotype 2n = 60 *B* described here for the first time was monomorphic for the presumably fixed *Est-D3*^{3} allele. The data as a whole should contribute to the better understanding the *rhombeus* complex taxonomic status definition in the Central Amazon.

Key words: Brazilian Amazon basin, esterase enzymes, *Serrasalmus rhombeus* species complex, karyotype.

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Electrophoretic investigations of genetic markers such as proteins and enzymes, especially allozymes and isoenzymes, have been decisive in determining the taxonomic and population status of many organisms (Ferguson, 1980), with allozymes having been particularly useful for identifying fish species and their hybrids in natural and artificial populations (Ferguson *et al.,* 1995) and have been especially useful for identifying cryptic species (Allendorf and Utter, 1979; Lavery and Shaklee, 1991; Musyl and Keenan, 1996). Many enzymes, such as esterases, show pronounced differentiation in isoenzymatic patterns in many organisms, including plants (Anti, 2000), phytonematoids (Alonso and Alfenas, 1998), mollusks (Richardson *et al.*, 1982) and fish (Payne *et al.,* 1972; Reinitz, 1977; Solomon and Child, 1978; Ferguson, 1980).

Although S*errasalmus* is one of the most widespread and specious South American piranha genera (Machado Allison and Fink, 1995) only a few allozyme genetic studies have been carried out on this piscine group since the study of S*errasalmus spilopleura* lactate dehydrogenase (*LDH*), malate dehydrogenase (*MDH*) and glucose phosphate isomerase (*GPI*) isoenzyme patterns by Cestari (1996) on fish from the Paraná and Paraguai river basins.

Recent studies on Amazonian *Serrasalmus* species have revealed karyotypic divergence between and within populations of *S. spilopleura* and *Serrasalmus rhombeus* (Nakayama *et al.,* 2000, 2001, 2002; Centofante *et al.*, 2002). Nakayama *et al*. (2001) has suggested that *S. rhombeus* cryptic species may exist based on the two cytotypes (2n = 58 and 2n = 60) found at Lake Catalão located near the confluence of the Negro and Solimões rivers in the Brazilian state of Amazonas. In addition to being identified by their karyotypes (Nakayama *et al*., *op. cit*.) fish belonging to the *S. rhombeus* complex are also moderately distinguishable by parasite analysis (Van Every and Kritsky, 1992) but not by their 16S mitochondrial DNA (Ortí *et al*., 1996) but as yet there have been no isoenzyme studies on this complex.

The work described in the present paper used karyotype and esterase-D isoenzyme patterns to provide addi-

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tional genetic information on the *S. rhombeus* complex in order to complement studies on the taxonomic status of the Central Amazon *rhombeus* complex.

Between the 2nd of February 2000 and 16th of April 2001 we collected 66 *Serrasalmus rhombeus* (Linnaeus, 1766) specimens from Lake Catalão in the Brazilian state of Amazonas (03°09'47" S; 58°54'29" W, Figure 1), the specimens belonged to the two karyotypic groups reported by Nakayama *et al*. (2001) and comprised 37 specimens of karyotype 2n = 58 and 29 specimens with a 2n = 60 karyotype.

Kidney cell mitotic chromosomes were prepared and analyzed using the air-drying technique (Bertollo *et al*., 1978) and skeletal muscle protein extracts and starch-gel electrophoresis were used to detect the esterase-D *Est-D1*, *Est-D2* and *Est-D3* loci using standard gel and electrode electrophoretic buffers (Ridgway *et al*., 1970) and the staining procedure described by Hopkinson *et al*. (1973).

Hardy-Weinberg expectations were calculated using the Chi-square (χ^2) test to verify the population gene balance for the 2n = 58 karyotype based on allelic segregation on the *Est-D3* polymorphic locus. This locus was also used to estimate the 2n = 60 karyotype null (recessive) allele frequency using the square root of the null genotype frequency as calculated using the Tools for Population Genetic Analyses" (TFPGA) program developed by Miller (1997).

Morphologically distinct species lacking chromosomal differences and morphologically cryptic species with chromosomal differences have been reported in piranha populations from the Central Amazon (Nakayama *et al*., 2000, 2001, 2002), with Nakayama *et al*. (2001) suggesting the existence of two cryptic species (2n = 60 and 2n = 58) for piranhas taxonomically identified as *S. rhombeus*.

We found three electrophoretic activity *S. rhombeus* esterase-D zones, presumably coded for by the three loci *Est-D1*, *Est-D2* and *Est-D3* (Table 1). The *Est-D1* and *Est-D2* loci were monomorphic in all specimens and presented genotypes $Est\text{-}D1^{11}$ and $Est\text{-}D2^{11}$, presumably encoded by the fixed alleles $Est\text{-}D1^{1}$ and $Est\text{-}D2^{1}$, while the *Est-D3* locus had polymorphic genotype and allele distributions which were differentiated and highly congruent with the identified distinct cytotypes (Table 1, Figure 2).

We found that for the 2n = 58 (46M-SM+2ST+10A) cytotype (Nakayama *et al*., 2001) *S. rhombeus* specimens the *Est-D3* locus presented Mendelian polymorphism and allelic segregation and showed four genotypes ($Est\text{-}D3^{11}$, $Est\text{-}D3^{12}$, $Est\text{-}D3^{22}$ and $Est\text{-}D3^{33}$) out of the six theoretically expected genotypes. The four genotypes were presumably encoded by the $Est\text{-}D3^{1}$, $Est\text{-}D3^{2}$ and $Est\text{-}D3^{3}$ alleles (Table 1), of which $Est\text{-}D3^{2}$ was the most commonly observed (f = 0.710). On the other hand we found that in *S. rhombeus* specimens with the 2n = 60 (44M-SM+6ST+10A) cytotype (Nakayama *et al*. 2001) the *Est-D3* locus presented a silent null allele ($Est\text{-}D3^{0}$) at the very high frequency of 0.959 (with no electrophoretic bands being detected exclusively for this cytotype) in addition to the low frequency

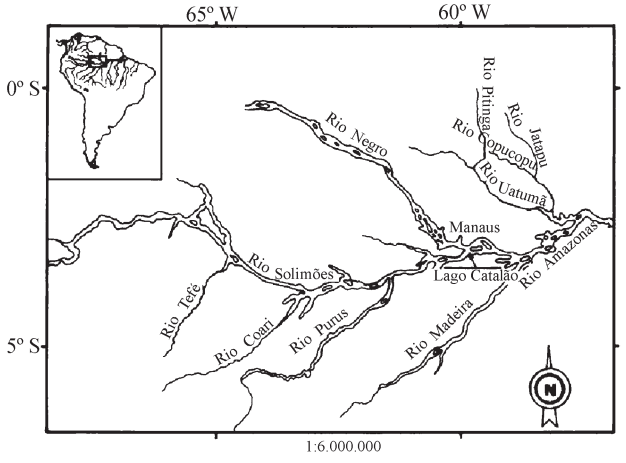

Figure 1 - Location of Lake Catalão in the Brazilian Central Amazon where specimens of the piranha *Serrasalmus rhombeus* were sampled.

Table 1 - Cytotype and allele frequency distributions for esterase-D loci *Est-D1*, *Est-D2* and *Est-D3* for the 2n = 58 (n = 37 specimens), 2n = 60 (n = 25) and 2n = 60 *B* (n = 4) *Serrasalmus rhombeus* karyotypes from Lake Catalão in the Brazilian Central Amazon. The expected number of some *Est-D3* polymorphic locus genotypes are shown in parentheses beside observed numbers.

	S. rhombeus cytotype		
Esterase-D loci	2n = 58	2n = 60	2n = 60 *B*
	Est-D1 genotype (n)		
$Est\text{-}D1^{11}$	37	25	4
	Est-D1 allele frequency		
$Est\text{-}D1^{1}$	1.000	1.000	1.000
	Est-D2 genotype		
$Est\text{-}D2^{11}$	37	25	4
	Est-D2 allele frequency		
$Est\text{-}D2^{1}$	1.000	1.000	1.000
	Est-D3 genotypes		
$Est\text{-}D3^{11}$	8 (4.223)	0	0
$Est\text{-}D3^{12}$	9 (15.878)	0	0
$Est\text{-}D3^{13}$	0 (0.676)	0	0
$Est\text{-}D3^{22}$	19 (14.926)	2 (0.042)	0
$Est\text{-}D3^{20}$	0	0 (1.966)	0
$Est\text{-}D3^{23}$	0 (1.270)	0	0
$Est\text{-}D3^{33}$	1 (0.027)	0	4
$Est\text{-}D3^{00}$	0	23 (22.990)	0
	Est-D3 allele frequencies		
$Est\text{-}D3^{1}$	0.237	0.000	0.000
$Est\text{-}D3^{2}$	0.710	0.041	0.000
$Est\text{-}D3^{3}$	0.053	0.000	1.000
$Est\text{-}D3^{0}$	0.000	0.959	0.000

Hardy-Weinberg $\chi^2_{(3)}$ value 44.443 for $p \leq 0.001$.

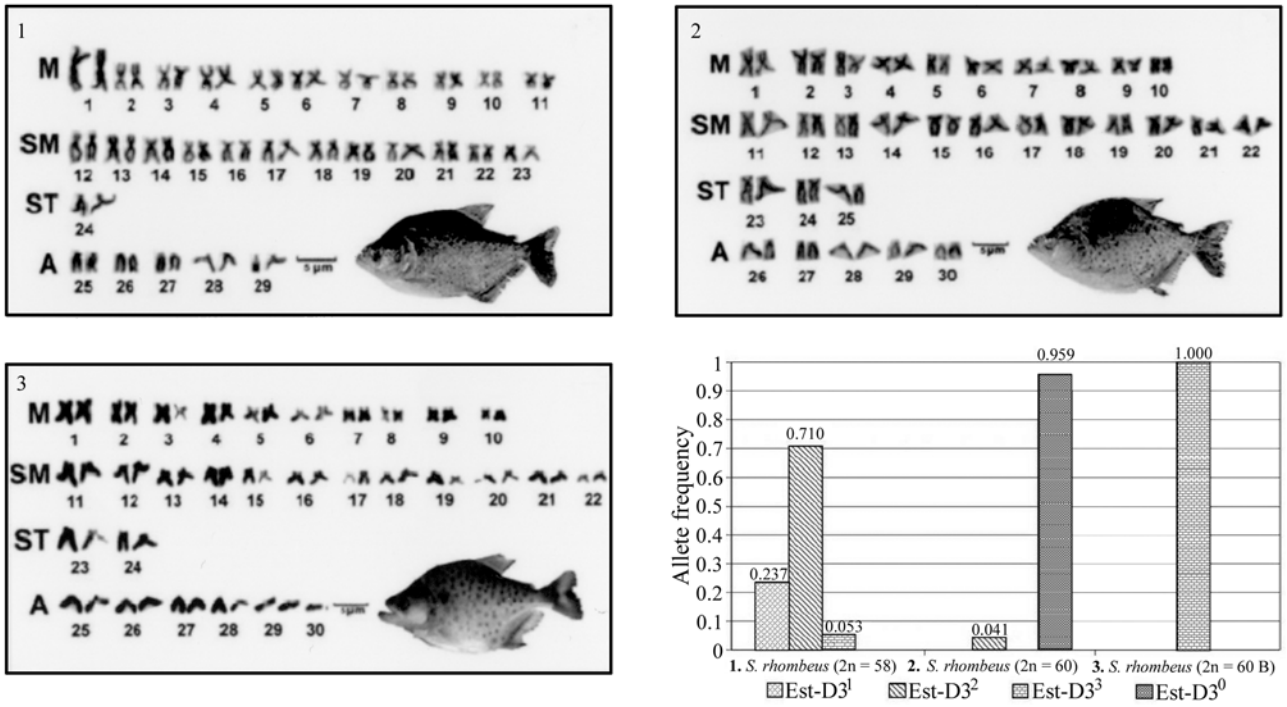

Figure 2 - Allele frequency distributions on *Est-D3* locus shown in three piranha *Serrasalmus rhombeus* cytotypes from Lake Catalão. 1. cytotype 2n = 58; 2. cytotype 2n = 60; 3. cytotype 2n = 60 *B*.

(f = 0.041) *Est-D3*^{2} allele. This atypically high *Est-D3*^{0} silent null allele frequency for the 2n = 60 *S. rhombeus* population is about 2.5 times higher than the 0.40 described by Aquino-Silva *et al.* (1998) for a null allele detected at a soluble malate dehydrogenase (*sMDH-B2**) locus in *Geophagus brasiliensis* (Cichlidae, Perciformes) and far higher as compared with the low frequencies (under 5%) of null alleles at allozyme loci in natural *Drosophila melanogaster* populations (Voelker *et al*., 1980; Langley *et al*., 1981). Despite the excess of *Est-D3*^{22} homozygotes and a corresponding deficiency of predictable *Est-D3*^{20} heterozygotes on the *Est-D3* locus, the observed number of the *Est-D3*^{00} null genotype for the 2n = 60 cytotype showed good agreement with the statistical expectation, discarding the possibility of the *Est-D3*^{0} silent null allele being interpreted as a technical artifact (Table 1). Although there are some examples in the literature associating the presence of null alleles with possible mildly deleterious effects to its carriers (see Aquino-Silva *et al*.,1998), this kind of association involving the silent *Est-D3*^{0} allele in the 2n = 60 cytotype could only be effectively tested for by crossing experiments with *Est-D3*^{22} and *Est-D3*^{00} homozygotes.

The new *S. rhombeus* cytotype reported here for the first time, 2n = 60 *B* (44M-SM+4ST+12A) revealed monomorphism for the presumably fixed allele *Est-D3*^{3}, and was detected in all four *S. rhombeus* specimens examined, although this allele can only be definitively reported as fixed following the screening of the *Est-D3* locus in a larger population of the *S. rhombeus* 2n = 60 cytotype.

The Chi-squared (χ^2) test for Hardy-Weinberg equilibrium used to check the genetic balance in the *S. rhombeus* 2n = 58 population revealed a highly significant statistical difference ($\chi^2_{(3)} = 44.443$ for $p \leq 0.001$) due to an excess of homozygotes and a corresponding deficiency of heterozygotes at the *Est-D3* locus (Table 1). Cestari (1996) also found highly significant departures from Hard-Weinberg equilibrium regarding the allele frequency distributions of two polymorphic glucose phosphate isomerase loci (*GPI-A** and *GPI-B**) examined in the *S. spilopleura* `a' cytotype, which appears to be an endemic cytotype of the Brazilian upper Paraná River basin, this genetic disequilibrium also being due to homozygote excess and heterozygote deficiency as was the case for the *S. rhombeus* *Est-D3* locus studied by us.

A classical explanation for homozygote excess in population samples is the Wahlund effect caused by the mixture of genetically distinct populations (Wahlund, 1928). Our *S. rhombeus* cytotype 2n = 58 data indicates the probable existence of different stocks of this karyotypic structure within the Lake Catalão. Teixeira *et al*., (2002) have shown highly statistically significant departures from genetic equilibrium due to homozygote excess at the transferrin locus (*Tf*) in seven out of eight Central Amazon population samples of the piscine *Plagioscion squamosissimus* (pescada in Portuguese), including three out of the four Lake Catalão *P. squamosissimus* population samples collected which showed three genetically discreet subpopulations of this species. Lake Catalão is an ecotone

formed by the mixture of acid and dark water from the Negro river and clear waters from the Solimões river but may also be viewed as an area of mixing of genetically distinct fish populations since this area is widely known as a stopping place and passage corridor in the migratory route of several Central Amazon fish species.

Although esterase isoenzyme patterns are usually species-specific, especially in fish (Payne *et al*., 1972; Reinitz, 1977; Solomon and Child, 1978; Ferguson, 1980), our investigation showed no cytotype-specific fixed allele in the three esterase-D loci for the three piranha karyotypic structures examined. Generally, species are typically fixed for different alleles on the same locus, while co-specific populations typically differ in regard to the same allele frequencies (Smith *et al*., 1981).

Gradual frequency differences in the *A*125* and *B*210* alleles at two *GPI* loci detected in *S. spilopleura* caught between the upper Paraná River (cytotype `a') and the lower Paraná River (cytotype `b' and cytotype `c') led Cestari (1996) to suggest that there may be interbreeding between fish from these two sites, supporting the hypothesis of a hybrid origin for the `c' cytotype. However, our esterase-D and chromosome data do not support the existence of different *S. rhombeus* piranha species in Lake Catalão and there was no indication of hybridization among the *S. rhombeus* cytotypes examined. Thus once cytotype-specific fixed alleles are detected in any *S. rhombeus* isoenzyme patterns different taxonomic units with species status will have to be formally recognized.

Several cases have been described in the literature where genetic polymorphism seems to be shared between a pair of species while closely related species might be expected to show higher levels of shared polymorphism (see Clark, 1997). Nakayama *et al*. (2001) considered *S. rhombeus* to be a cryptic species, with imperceptible morphological differences among the three cytotypes examined by us and it follows that the occurrence of a *Est-D3* locus polymorphism shared among these cytotypes would reasonably be expected to follow the same pattern as that seen for the 2n = 58 cytotype i.e. segregation of alleles following a Mendelian model which did not occur. Regarding our study, recent and ongoing differentiation of the distinct diploid number of piranhas might explain the very high frequency (95.90%) of the $Est\text{-}D3^{0}$ silent null allele only seen in cytotype 2n = 60, the high frequency (71%) of the $Est\text{-}D3^{2}$ allele only occurring in cytotype 2n = 58, the apparent fixation (despite the small number of specimens) of the $Est\text{-}D3^{3}$ allele only detected in the new 2n = 60 *B* cytotype and the absence of different cytotype-specific fixed alleles at the Esterase-D loci (Table 1, Figure 2). Additionally, our data may suggest that these Central Amazon piranhas karyotypic groups partially represent isolated populations, or populations which have been isolated for an insufficient period of time for the fixation of different cytotype-specific alleles.

A character applied for identifying taxonomic units with species status should occur in all members of the species and not in other species, *i.e.*, be a unique fixed allele or its product. Consequently, various distinct genetic and molecular techniques such as chromosome, DNA and protein studies should be complemented with meristic-morphometric studies in order that the taxonomic status of Central Amazon *rhombeus* complex can be elucidated.

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