NADP-DEPENDENT ISOCITRATE DEHYDROGENASE OF *Astyanax scabripinnis* (PISCES, CHARACIDAE) FROM THREE ALTITUDES AT GRANDE STREAM, CAMPOS DO JORDÃO, SP

MUNIN, F. S., SCHWANTES, M. L., SCHWANTES, A. R. and MOREIRA-FILHO, O.

Universidade Federal de São Carlos, Departamento de Genética e Evolução,
C.P. 676, CEP 3565-905, São Carlos, SP, Brazil

Correspondence to: Flavia Simone Munin, Universidade Federal de São Carlos, Departamento de Genética e
Evolução, C.P. 676, CEP 3565-905, São Carlos, SP, Brazil, e-mail: flaviamunin@hotmail.com

ABSTRACT

Electrokinetic, thermic, and kinetic properties of products of NADP-dependent isocitrate dehydrogenase (IDHP; EC 1.1.1.42) loci of *Astyanax scabripinnis* (Pisces, Characidae) collected at three different altitudes (700 m, 1,800 m, and 1,920 m) of Grande Stream at Campos do Jordão, State of São Paulo, Brazil, were analyzed. Two IDHP bidirectionally divergent loci, a single skeletal muscle, the *IDHP-A**, and a single liver *IDHP-B**, both polymorphic, were detected in the three different altitude populations. The variant allele **128* at the *IDHP-A** locus, had its highest frequency detected in the 1,920 m population (0.494). Among the nine variant alleles detected at the *IDHP-B** locus (**37*, **57*, **69*, **79*, **85*, **114*, **119*, **124*, and **140*), the **37* and 79 were detected only in 1,800 m population. Chi-square values showed that only the 700 m population is not in Hardy-Weinberg equilibrium for the *IDHP-A** locus, while for the *IDHP-B** locus, no population is. Homogeneity Chi^2 test indicated that the populations are significantly different in their A and B phenotype frequencies. Wright's FST mean value (0.036 and 0.32, *IDHP-A** and *IDHP-B**, respectively) was 0.178 for the three altitude populations which means that 82% of total genetic diversity was found among individuals of each one of the populations. Stability at environmental temperatures (16° to 21°C), and apparent K_m and V_{max} values of each A-phenotype skeletal muscle crude extract suggest different roles of A-isoforms during the increased lipogenesis that occurs in fish at low temperatures.

Key words: *Astyanax scabripinnis*, NADP-dependent isocitrate dehydrogenase, allozymes, thermostability, altitudes.

RESUMO

**Isocitrato desidrogenase NADP-dependente de *Astyanax scabripinnis*
(Pisces, Characidae) de diferentes altitudes do Ribeirão Grande, Campos do Jordão, SP**

Foram analisadas propriedades eletrocinéticas, térmicas e cinéticas dos produtos dos locos que codificam o isocitrato desidrogenase NADP-dependente (IDHP; E.C. 1.1.1.42) como *Astyanax scabripinnis* (Pisces, Characidae) em três diferentes alturas do Ribeirão Grande, Estado de São Paulo, Brasil. Nas populações das três altitudes foram detectados dois locos da IDHP, bidirecionalmente divergentes, um único em músculo esquelético, o *IDHP-A**, e um único em fígado, o *IDHP-B**, ambos polimórficos. O único alelo variante no loco *IDHP-A**, *A*128*, mostrou maior freqüência na população de 1.920 m (0,494). Dentre os nove alelos variantes detectados no loco *IDHP-B** (**37*, **57*, **69*, **79*, **85*, **114*, **119*, **124* e **140*), os alelos **37* e **79* apareceram somente na população de 1.800 m. Valores de Qui-quadrado revelaram que para o loco *IDHP-A** somente a população de 700 m não está em equilíbrio de Hardy-Weinberg, enquanto para o loco *IDHP-B** nenhuma população se encontra em

equilíbrio. Qui² da homogeneidade revelou que as populações são significativamente diferentes em suas freqüências fenotípicas para ambos os locos. Valor médio de FST de Wright (0,036 e 0,320, *IDHP-A** e *IDHP-B**, respectivamente) foi de 0,178 para populações das três altitudes, o que significa que 82% da diversidade gênica total foi detectada entre indivíduos da mesma população. Estabilidades em temperatura ambiente (16° a 21°C) e valores de K_m e V_{max} aparentes de extratos de músculo esquelético de cada fenótipo-A sugerem diferentes papéis das isoformas-A na lipogênese aumentada que ocorre nos peixes em baixas temperaturas.

Palavras-chave: *Astyanax scabripinnis*, isocitrato desidrogenase NADP-dependente, alozimas, termo-estabilidade, altitudes.

INTRODUCTION

The dimeric enzyme isocitrate dehydrogenase found in all vertebrate cells in the Krebs cycle exists in two forms: one NAD (EC 1.1.1.41), and the other NADP-dependent (EC 1.1.1.42) (Lowenstein & Smith, 1962). In the presence of a divalent cation (Mn^{2+} or Mg^{2+}) both forms catalyze the oxidative decarboxilation of isocitric acid to α-ketoglutarate. The cytosolic NADP-dependent isocitrate dehydrogenase (IDHP) function is to provide NADPH for lipid biosynthesis, and α-ketoglutarate for glutamate and glutamine formation (Saxrud *et al.*, 1996). Multiple forms of IDHP encoded by unlinked gene loci have been described in fish (Allendorf *et al.*, 1977; Van-Beneden *et al.*, 1981; Perdices *et al.*, 1996; Doadrio & Carmona, 1998; Chiari & Sodré, 1999; Kohlmann & Kersten, 1999; Renesto *et al.*, 2000).

The small characid fish *Astyanax scabripinnis* is excellent for evolutionary studies due to its remarkable karyotypic diversity, wide geographical distribution, presence of supernumerary B chromosome, and incidence of natural triploidy (Moreira-Filho & Bertollo, 1991; Maistro *et al.*, 1992; Fauaz *et al.*, 1994). Based on the divergence of karyotypical numbers, variation in chromosome morphology, C-banding patterns, and the location of NORs observed in seven populations of *A. scabripinnis* from Parana-panema, São Francisco, and Tiete rivers, Moreira-Filho & Bertollo (1991) proposed the *scabripinnis* complex. The occurrence of genetic variation has also been shown in previous studies focused on six protein-coding loci within these populations of *A. scabripinnis* (Schwantes *et al.*, 1994).

The present study is part of an integrated project entitled "Genetic Bases of Diversity in the *scabripinnis* complex". Its objective was to compare electrophoretic patterns, stability at environmental temperature (16°

to 21°C), and some kinetic parameters of IDHP of *A.scabripinnis* populations from three different altitudes at Grande Stream, Campos do Jordão, SP, Brazil, in an attempt to distinguish these populations and determine if those parameters might confer adaptability of individuals to a particular habitat.

MATERIAL AND METHODS

Five hundred eighty-nine *A. scabripinnis* individuals caught at ponds located at three altitudes of Grande Stream were analyzed: 204 individuals from 700 m; 210 from 1,800 m; and 175 from 1,920 m. The water temperature and dissolved O_2 measured at each altitude varied from 16°C and 10.4 mg/ml at 1,920 m, to 18°C and 9.8 mg/ml at 1,800 m, and 21°C and 9.5 mg/ml at 700 m (Landini *et al.*, 2002). Liver and white muscle from each individual were dissected immediately after capture and kept at –20°C. A small piece of each tissue was homogenized (w/v) in 50 mM phosphate potassium buffer (pH 7.0), using a Potter-Elvejhem tissue grinder, and then centrifuged at 27,000 g for 30 min at 4°C in a Sorvall RC5B centrifuge. The resulting crude extracts were used for electrophoretic and spectrophotometric analyses. Electrophoreses were carried out employing horizontal gels containing 14% (w/v) corn starch prepared according to Val *et al.* (1981), using the pH 6.9 Whitt (1970) buffer system. A voltage gradient of 5 V/cm was applied for 12-14 h at 4°C. After electrophoreses, the gels were sliced lengthwise and the lower halves incubated in an IDHP staining solution, described by Shaklee *et al.* (1989) and modified by us, consisting of 25.8 mM isocitric acid, 0.218 mM NADP, 0.456 mM PMS, 0.4 mM MTT, 0.1 mM $MgCl_2$, 1.5% agar-agar, and a 100 m Tris-HCl pH 7.4 buffer. Nomenclature of IDHP gene loci and iso/allozymes was taken from Shaklee *et al.* (1990). Alleles were designated

by numbers with *100 representing the most frequent allele. Subsequent numbers refer to their relative mobility. Electrophoretic data were used in Chi^2 homogeneity tests among the three different altitude populations and analyzed for Hardy-Weinberg equilibrium. A locus was considered polymorphic if the frequency of the most common allele was equal to or less than 0.99 (P_{99}). The observed intralocus heterozygosity (*Ho*) was obtained by direct counting. Expected intralocus heterozygosity (*He*) was calculated according to Nei (1978). Deviations from random-mating expectations within populations were estimated for both loci using F_{is} (Wright, 1978), and the statistical significance of each F_{is} was tested using the relationship $\chi^2 = NF_{is}^2$; d.f. $= (a - 1)(s - 1)$, where N is the total number of individuals sampled at the locus, a is the number of alleles at the locus, and s is the number of subpopulations (Nei, 1977). Genetic differentiation among populations was estimated as Wright's standardized variance in allele frequencies (F_{st}) (Wright, 1978), and the significance of F_{st} estimates was tested using the relationship with $\chi^2 = 2NF_{st} + (a - 1)$ with $(a - 1)(s - 1)$ degrees of freedom (Workman & Niswander, 1970).

Temperature dependency of both A-phenotypes (A100 and A128) of skeletal muscle (the IDHP-A is the single isoform in skeletal muscle) crude extracts was tested by subjecting each sample to several different temperatures, for 3 min, in two ways. In the first test, glass tubes containing 10 µL of extract of each phenotype were incubated at 25°, 30°, 35°, 40°, 45°, 50°, and 55°C in the presence of the coenzyme (0.18 mM NADP, 1 mM MgCl$_2$, and 20 mM Tris-HCl buffer pH 8.5) for 3 min. Immediately afterward, their residual activities were examined at 25°C through the change in absorbance at 340 ηm in an HP-8452A diode array spectrophotometer using a temperature-controlled cuvette holder. In the second test, extracts of each phenotype were incubated in the spectro-photometer thermocell at 10°, 15°, 20°, 25°, 30°, 40°, and 50°C, according to Williamson (1998). In both cases, the reactions were triggered by addition of the substrate (23 µM isocitric acid).

The kinetic parameters of IDHP were determined at 25°C by measuring NADP reduction at 340 ηm for 3 min, according to Williamson (1998). The assays were carried out in a 20 mM Tris-HCl buffer pH 8.5 containing 0.18 mM NADP, 1 mM MgCl$_2$, and varying concentrations of isocitrate (288 nm to 23 µm). All assays were performed in triplicate and initiated by adding 10 µL of enzyme (crude extract) to 1.0 ml of assay medium. Apparent Michaelis-Menten constants (K_m) and maximum catalytic rates (V_{max}) were calculated by the Lineweaver Burk method using double-reciprocal plots of velocity vs. substrate concentrations.

Statistical differences among thermal stability, and K_m, and V_{max} values detected in skeletal muscle crude extracts of *A. scabripinnis* A-phenotypes were determined by the non-parametric Kruskal-Wallis (Sokal & Rohlf, 1981) and Mann-Whitney tests (Zar, 1974) with a probability level of 0.05 chosen as the limit of statistical significance.

RESULTS

Electrophoretic patterns of *A. scabripinnis* IDHP collected at 700 m; 1,800 m; and 1,920 m of Grande Stream at Campos do Jordão, showed two bidirectionally divergent loci, the skeletal muscle-single *IDHP-A**, and the liver-single *IDHP-B**, both polymorphic (Fig. 1; Table 1).

Fig. 1A shows the three *IDHP-A** phenotypes, detected in skeletal muscle of the populations from the three altitudes due to the presence of a *128 variant allele. The highest frequency of this variant allele was detected in the 1,920 m population (0.494) (Table 1). Chi-square (χ^2) values obtained indicate that only the 700 m population were not in Hardy-Weinberg genetic equilibrium (HWE) for the IDHP-*A** locus, as indicated by the significantly different genotypic frequencies (Table 2). Figure 1B shows the twenty-one *IDHP-B** phenotypes detected in these individuals due to the presence of nine variant alleles: *37, *57, *69, *79, *85, *114, *119, *124, and *140. The 700 m population was fixed for six of these eight variant alleles: *57, *69, *85, *119, *124, and *140 (Table 1). The specimens collected from 1,800 m were fixed for six among the eight variant alleles: *37, *57, *69, *79, *85, and *114 (Table 1). The specimens collected from 1,920 m were fixed for seven of the variant alleles: *57, *69, *85, *114, *119, *124, and *140 (Table 1). The χ^2 values obtained (Table 2) indicate that the three altitude populations are not in HWE for this locus. Among these variant alleles two altitude-exclusive alleles were detected: the *37 and *79, in 1,800 m population.

Homogeneity Chi^2 test indicated that the populations are significantly different in their A and B phenotype frequencies (Table 3).
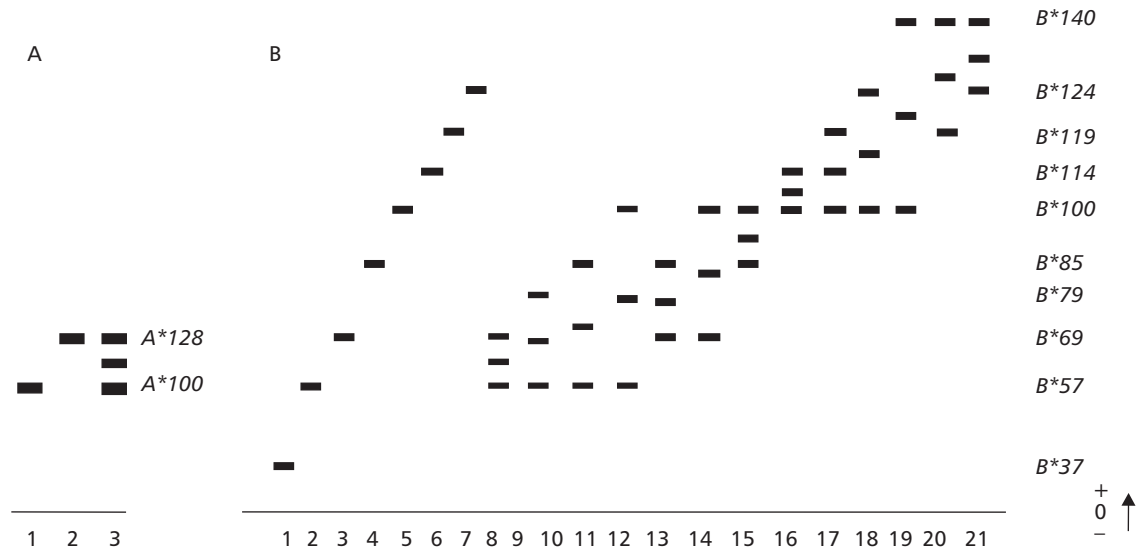
Fig. 1 — Schematic representation of IDHP phenotypes detected in populations of *A. scabripinnis* and their genetic interpretation. (A) skeletal muscle, and (B) liver extracts. A: 1. A100; 2. A128; 3. A100/128. B: 1. B37; 2. B57; 3. B69; 4. B85; 5. B100; 6. B124; 7. B140; 8. B57/69; 9. B57/79; 10. B57/85; 11. B57/100; 12. B69/85; 13. B69/100; 14. B85/100; 15. B85/140; 16. B100/114; 17. B100/124; 18. B100/140; 19. B124/140.

TABLE 1

Allelic frequencies observed at *IDHP-A** and *IDHP-B** loci from *A. scabripinnis* collected from three altitudes.

	Altitude populations		
	700 m	**1,800 m**	**1,920 m**
IDHP-A*			
100	0.725	0.617	0.506
128	0.275	0.383	0.494
IDHP-B*			
37	0	0.008	0
57	0.049	0.212	0.100
69	0.303	0.242	0.150
79	0	0.031	0
85	0.227	0.212	0.250
100	0.340	0.292	0.417
114	0	0.003	0.044
119	0.032	0	0.027
124	0.039	0	0.006
140	0.010	0	0.006

Table 2 shows a deficit of heterozygotes for both *IDHP-A** ($Ho = 0.235$, $He = 0.398$ for 700 m; $Ho = 0.471$, $He = 0.473$ for 1,800 m; $Ho = 0.440$, $He = 0.500$ for 1,920 m) and *IDHP-B** ($Ho = 0.305$, $He = 0.736$ for 700 m; $Ho = 0.477$, $He = 0.765$ for 1,800 m; $Ho = 0.500$, $He = 0.729$ for 1,920 m) loci

of the three populations. F_{is} values (Table 4) showed for both loci, a 16.4% and 16.9% heterozygote deficiency. F_{it} mean value (0.314) showed a 31.4% heterozygote deficiency in total population. Wright's F_{st} mean value was 0.178 (0.036 and 0.32 for *IDHP-A** and *IDHP-B** loci, respectively) among the 3 sampled populations, i.e., 17.8% of the total gene diversity ($H_t = 0.615$) was attributed to differences between populations. The remaining gene diversity $(1 - 0.178 = 0.822)$ was due to within-total population variability.

Fig. 2 shows temperature dependency of both homozygous phenotypes detected for the *IDHP-A** of *A. scabripinnis*. When both isoforms were exposed to 25°, 30°, 35°, 40°, 45°, 50°, and 55°C, its response was significantly different (p = 0.0265). While the *A*128*-isoform showed maximum activity when incubated at 35°C, the *A*100* did so at 45°C (Fig. 2A). When extracts of each phenotype were incubated in the spectrophotometer thermocell at 10°, 15°, 20°, 25°, 30°, 40°, and 50°C, both isoforms increased their activities (Fig. 2B), but in the physiological temperature interval of the three altitude populations (16° to 2°C)(Landini *et al.*, 2002), significant differences between them (p = 0.0286) were detected. While *A*100*-isoform chan-

ged its residual activity from 6% to 15%, the *A*128*-isoform changed from 16% to 35%.

In order to compare the effect of substrate (288 ηm to 23 μm) concentrations on IDHP activity, we examined the responses of the apparent Km and V_{max} of isocitrate on both A-phenotype skeletal muscle extracts. The K_m and V_{max} values obtained for the A128 phenotype extracts ($K_m = 0.109 \times 10^{-5}$ M; $V_{max} = 62 \times 10^{-9}$ moles x liter^{-1} x min^{-1}) were higher than those obtained for the A100 ($K_m = 0.037 \times 10^{-5}$ M; $V_{max} = 25 \times 10^{-9}$ moles x liter^{-1} x min^{-1}). However, these differences are not significantly different (p = 0.1297).

DISCUSSION

The IDHP bidirectionally divergent loci detected in *A. scabripinnis* have also been described in other fish (Perdices *et al.*, 1996; Chiari & Sodré, 1999; Kohlmann & Kersten, 1999; Renesto *et al.*, 2000). In the fish species *Fundulus heteroclitus* (Van-Beneden *et al.*, 1981) and in the genus *Leuciscos* (Doadrio & Carmona, 1998), 3 IDHP loci, 2 liver-specific and 1 skeletal muscle-specific, were described. In rainbow trout, muscle and liver forms are codified by 4 loci (Allendorf *et al.*, 1977).

TABLE 2

Chi² analysis for Hardy-Weinberg equilibrium, observed and expected heterozygosities for IDHP loci from *A. scabripinnis* from three altitudes. *N* – sample size, d.f. – degrees of freedom, *Ho* – observed intralocus heterozygosity, *He* – expected intralocus heterozygosity.

Altitude (N)	Number of alleles	χ^2	d.f.	*Ho*	*He*
IDHP-A*					
700 m (204)	2	34.170**	1	0.235	0.398
1800 m (210)	2	0.002	1	0.471	0.473
1920 m (175)	2	2.515	1	0.440	0.500
IDHP-B*					
700 m (204)	7	49.745**	1	0.305	0.736
1800 m (210)	7	45.401**	1	0.477	0.765
1920 m (175)	8	10.230*	1	0.500	0.729

* p < 0.05; ** p < 0.001.

TABLE 3

Homogeneity Chi² tests for comparing the IDHP phenotype frequencies detected in the three altitude populations.

|	700 m x 1,800 m	700 m x 1,920 m	1,800 m x 1,920 m	700 m x 1,800 m x 1,920 m
IDHP-A*				
χ^2	30.76**	49.09**	10.66*	62.7**
d.f.	2	2	2	4
IDHP-B*				
χ^2	16.29*	11.84*	12.49*	25.56*
d.f.	4	4	4	8

*p < 0.05; **p < 0.001.

TABLE 4

Wright's *F*-statistics analyses (Wright, 1978) for both IDHP loci for *A. scabripinnis* from three altitudes.

	F_{is}	$\chi^2 F_{is}$	d.f.	F_{it}	F_{st}	$\chi^2 F_{st}$	d.f.	H_i	H_s	H_t
IDHP-A*	0.164	15.862*	2	0.194	0.036	42.243*	2	0.382	0.457	0.474
IDHP-B*	0.169	12.006	18	0.435	0.320	2,436	18	0.427	0.514	0.756

Wright's F_{is} fixation index measures the deviation of genotypic frequencies from the HWE of individuals relative to its subpopulaton. Wright's F_{it} fixation index measures the deviation of genotypic frequencies from the Hardy-Weinberg equilibrium of individuals relative to the total populaton. Wright's F_{st} fixation index measures the degree of heterogeneity among populations. * p < 0.001.

The polymorphism at the IDHP loci here detected was also detected in other fish (Perdices *et al.*, 1996; Doadrio & Carmona, 1998; Chiari & Sodré, 1999; Kohlmann & Kersten, 1999; Renesto *et al.*, 2000). Only the *IDHP-A** genotypic frequencies of the 1,800 m and 1,920 m populations did not differ from Hardy-Weinberg expectations. The *IDHP-A** genotypic frequencies from the 700 m population, as well as the *IDHP-B** genotypic frequencies from the three altitude populations differed from Hardy-Weinberg expectations. In cases where the genotypic frequencies did not differ from HWE for more than one sample of a population, it could be suggested that each sample represented either an independent subpopulation within which mating was random or was a random sample from a larger panmictic unit (Carvalho, 1993). Except for the *IDHP-A** genotypic frequencies of 1,800 m and 1,920 m populations, this is not applicable to our sample. According to Chiari & Sodré (1999), divergences from Hardy-Weinberg equilibrium in the expected genotypic frequencies may occur due to mutation, natural selection, assortative mating, loss or gain of migrants, genetic drift, and/or methodological errors.

One of the reasons for the significant heterogeneity obtained when IDHP loci of the three *A. scabripinnis* altitude populations were compared by homogeneity chi-square tests could be the existence of variant alleles detected in only one of the populations. Since these analyzed populations are relatively small, genetic drift could be responsible for disappearance/maintenance of these alleles. Meanwhile, the existence of alleles found in specific populations and lack of homogeneity in their A and B phenotype frequencies suggest a reduced or nonexistent gene flow among these groups, characterizing populations in an isolating process. According to Avise *et al.* (1986), if localized fish populations inhabit similar environments or remain interconnected through migration and gene flow, they may display largely homogeneous arrays of phenotypic or genetic traits. If, however, they are exposed to contrasting environmental conditions and/or exchange only a few migrants (Stephen & McAndrew, 1990), appreciable population differentiation may arise. As most of the variant alleles were found to occur at low frequencies in the populations, genetic drift could easily result in low levels of variation.
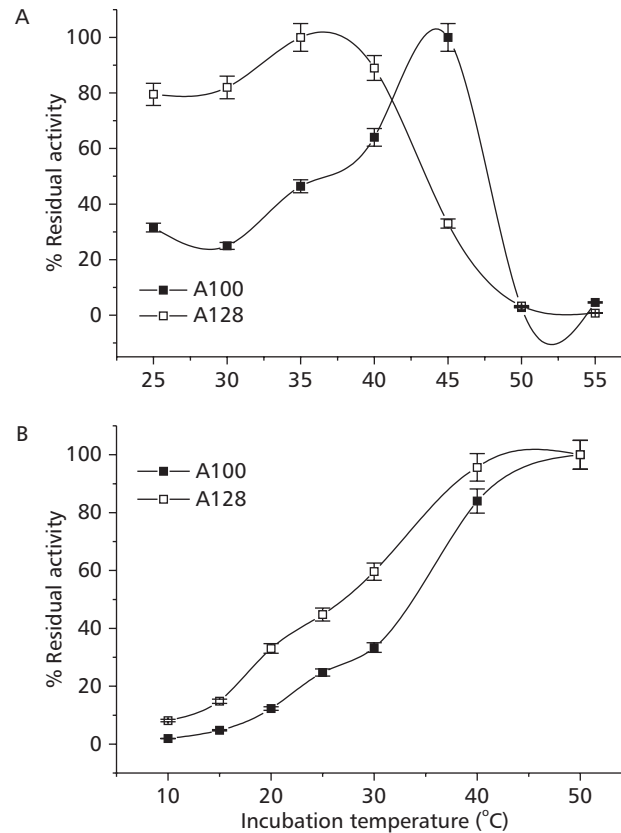
Fig. 2 — Temperature dependency of homozygous phenotypes detected for the *IDHP-A** of *A. scabripinnis.* A – extracts of each phenotype were incubated at 25°, 30°, 35°, 40°, 45°, 50°, and 55°C in the presence of coenzyme (0.18 mM NADP, 1 mM MgCl$_2$, and 20 mM Tris-HCl buffer pH 8.5) for 3 min and their residual activities were examined at 25°C. B – extracts of each phenotype were incubated in the spectrophotometer thermocell at 10°, 15°, 20°, 25°, 30°, 40°, and 50°C, for 3 min and their residual activities were examined at each one of their incubation temperatures. In both tests, reactions were started with addition of 23 µM isocitric acid.

According to Crouau-Roy (1988), many studies like ours have shown significant levels of heterozygote deficiency in natural populations, but these deficiencies are usually observed in some allozymic loci and/or in only some samples of a species, while for other loci the genotypic proportions are in equilibrium. These differences could signify independence in the evolutive process and, consequently, reproductive isolation. According to Engelbrecht *et al.* (1997), fit to Hardy-Weinberg proportions is an important but not an absolute criterion for inferring the genetic nature of populations because several factors may contribute to achieving deviations of genotype frequencies from expected Hardy-Weinberg proportions. These include natural selection, small effective population, sampling error, and anthropogenic influence. A small effective population (as here analyzed) may result in a decrease in the number of alleles through selection and inbreeding. The isolation of populations because of human activity or natural conditions may increase the probability of mating between relatives. This in turn increases the frequency of homozygous individuals and decreases the frequency of heterozygous individuals compared with those that may be expected from random mating. Also, inadequate sampling may result in the inclusion of more than one population in a particular river, a situation referred to as the Wahlund effect (Wahlund, 1928) which could result in Hardy-Weinberg discrepancies.

Heterozygote deficiency was showed in our sample through the F_{is} (16.7%), F_{it} (31.4%), and F_{st} (17.8%) values. Only 17.8% of the total gene

diversity was attributed to differences between populations, i.e., 82.2% of the remaining gene diversity was due to within-total population variability. According to Katoh & Foltz (1994), the high genetic differentiation (F_{st} = 0.321) among populations of the fish *Valencia hispanica*, excluding the mixed population, suggests reduced gene flow, allowing populations to adapt to local environments. The population subdivision (F_{st} = 0.178) detected among *A. scabripinnis* populations indicates a interruption of genetic exchange among them.

Spatial variation has a central role in evolutionary biology, particularly in studies of population differentiation and speciation (Baumgartner, 1986). Clinal variation, a spatial gradient in the frequency of a gene, genotype, or phenotype (Endler, 1977), as here detected for the *IDHP-A*128*, is common in natural populations and is characteristic of secondary contact zones between divergent conspecific populations. Because many geographic clines involve a significant latitudinal difference in allele frequencies, some authors have concluded that varying environments in assorted geographic regions differentially selected individuals with specific enzyme phenotypes (Philipp *et al.*, 1985). Because most of the species analyzed in these studies were ectothermic, the most obvious component of the environment which might exert differential selective pressure among geographic regions of different latitudes is temperature. Thermal stability of proteins of animals from various habitats is quite different (Klyachko & Ozernyuk, 1998). Such stability in proteins of different species correlates with environmental temperature (Alexandrov, 1977; De-Luca *et al.*, 1983; Swezey & Somero, 1982). In the present case, the *IDHP-A*128* allele, which showed a spatial gradient in its frequency in *A. scabripinnis* collected at different altitudes (0.275 at 700 m; 0.383 at 1,800 m; and 0.494 at 1,920), seemed thermolabile at high temperatures (maximum activity at 35°C), but in the over-all physiological temperature interval (16°-21°C) was more active than the *IDHP-A*100* allele, predominant in all altitudes. On the other hand, apparent K_m values obtained for the product of this most common allele showed a substrate affinity threefold higher (K_m = 0.037 x 10^{-5} M) than that of the *A*128* (K_m = 0.109 x 10^{-5} M).

In the light of Tetragonopterinae fossil records from Pleistocene prior to the Mantiqueira mountain lifting, the existence of the three-altitude common alleles here detected could be explained. Also, the results here obtained showing divergences from Hardy-Weinberg equilibrium for both loci, significant heterogeneity among the three altitude populations, altitude exclusive alleles, heterozygote deficiency, and F_{is} estimates could suggest that these fishes constitute solitary populations with low gene flow among them and a reproductively isolating process.

The *IDHP-A*100* was the most frequent allele in the three altitude populations, with its highest frequency in the 700 m population (0.725), where the water temperature was the highest (21 C) and the dissolved O_2 was the smallest (10.4 mg/L). Its product, the A100-isoform, showed maximum activity at 45°C, and in the range of the Grande Stream physiological temperature, 6% to 15% of its maximal activity. Since its K_m value was the lowest detected (highest affinity to substrate), the *IDHP-A*100* product may function to provide NADPH for biosynthetic reactions, like increased lipogenesis which occurs in fish at low temperatures (Knipprat & Mead, 1968), and α-ketoglutarate required for glutamate and glutamine formation.

The *IDHP-A*128* has its highest frequency in the 1,920 m population (0.494), where water temperature was the lowest (16°C) and dissolved O_2 was the highest (9.5 mg/L). Its product, the A128-isoform, showed maximum activity at 35°C, and in the range of the Grande Stream physiological temperature, 16% to 35% of its maximal activity. Since its K_m value was the highest detected (lowest affinity to substrate), the *IDHP-A*128* product may function as another and less efficient option for providing NADPH for biosynthetic reactions.

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