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Malate dehydrogenase polymorphism in Amazon Curimatids (Teleostei: Curimatidae): Evidence of an ancient mutational event

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ABSTRACT

Electrophoretic mobilities and tissue expression of MDH (malate dehydrogenase) were found to be the same for 12 Curimatidae fish species from the Amazon basin. Similarities in the migration of isozymes produced by sMDH-A* and sMDH-B* allowed no differentiation among these species. Out of 12, six species showed two alleles for sMDH-B*. These alleles, named sMDH-B^*_{100} and sMDH-B^*_{85}, were present at different frequencies among the six species. B_{85} and/or $B_{100/85}$ phenotypes were generally more thermostable than B_{100} phenotypes. Based on the occurrence of a single allele (B^*_{85}) in six different species, their different frequencies, and their heat inactivation rates, the following hypotheses are suggested: a) the alleles are the result of a single mutational event occurring before the speciation process in this family, or b) they are cryptic alleles, a result of different mutational events.

INTRODUCTION

The enzyme malate dehydrogenase (L-malate: NAD$^+$ oxidoreductase, E.C.1.1.1.37) occurs in two different molecular forms in vertebrate and invertebrate cells: the mitochondrial form (m-MDH) and the soluble or cytoplasmic form (s-MDH). Two gene loci, MDH-A* and MDH-B*, have been found to encode s-MDH in almost all fish species studied (Wheat *et al.*, 1971; Whitt *et al.*, 1973; Place and Powers, 1978; Schwantes and Schwantes, 1982a; De Luca *et al.*, 1983; Fenerich-Verani *et al.*, 1990; Basaglia, 1991). Allo and/or isozymes have been described for both s-MDH and m-MDH of several fish species, demonstrating genetic variation (Bailey *et al.*, 1969; Wheat *et al.*, 1971; Aspinwall, 1974; Coppes *et al.*, 1987).

Three metabolic pathways include s-MDH functions: gluconeogenesis, lipogenesis and aerobic glycolysis (Bailey *et al.*, 1970). During aerobic metabolism s-MDH plays an important role transporting reductive power into mitochondria through the malate-aspartate shuttle (Zink and Shaw, 1968; Wheat *et al.*, 1971). The two different forms of s-MDH are distributed in the organism according to metabolic preferences of the tissue or organ in which they occur (reviewed by Basaglia, 1991). In addition, s-MDH isozyme activities are suggested to be temperature-regulated in fish.

The present paper describes the occurrence of a single allele (B^*_{85}), its frequency in six Curimatidae species, and its heat inactivation rate. Two hypotheses

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explaining the presence of these alleles are suggested and discussed.

MATERIAL AND METHODS

Animals

Four hundred and thirty five (435) specimens belonging to 12 species and four genera were analyzed electrophoretically for the MDH isozymes. The animals were captured in five different places in the Amazon basin: Solimões river (Ilha da Marchantaria, Lago do Rei and Catalão), Uatumã river, and Trombetas river (Figure 1). Species, number of analyzed animals and collecting places are presented in Table I. Five different tissues were analyzed: skeletal muscle, heart muscle, liver, retina, and brain. Samples were stored at -20°C until they were electrophoresed (up to two months).

Electrophoretic analysis

The samples were homogenized in 50 mM phosphate buffer, pH 7.0, and centrifuged at 18,000 *g* in a Sorvall RC-5B centrifuge, for 30 min at 4°C. Electrophoresis was performed according to Smithies (1955, 1959), using corn starch gel as described by Val *et al.* (1981). The buffer system was Tris-citrate (0.75 M Tris; 0.25 M citric acid), pH 6.9, as described by Whitt (1970). The samples were electrophoresed during 14 h (4 V/cm of gel length). The gel was sliced into two layers and stained for the MDH system and for ADH (alcohol dehydrogenase, E.C. 1.1.1.1). The isozymes were histochemically detected as described by Shaw and Prassad (1970). Cytoplasmic and mitochondrial MDH forms were identified by differential centrifugation, as described by Meizel and Markert (1967), using Tris/Cl-sucrose solution, pH 7.0.

Allelic frequencies

The allelic frequencies were obtained by direct observations of phenotypes, assuming codominant alleles and Mendelian inheritance. The deviation of Hardy-Weinberg equilibrium was verified by χ^2 tests, using one degree of freedom (Hedrick, 1983). Polymorphic species captured in different places were submitted to statistical comparisons through the Komogoroff and Smirnoff test (D-Statistics) as described in Sechs (1982). Nomenclature adopted for s-MDH subunits, structural genes, and alleles is according to Shaklee *et al.* (1989).

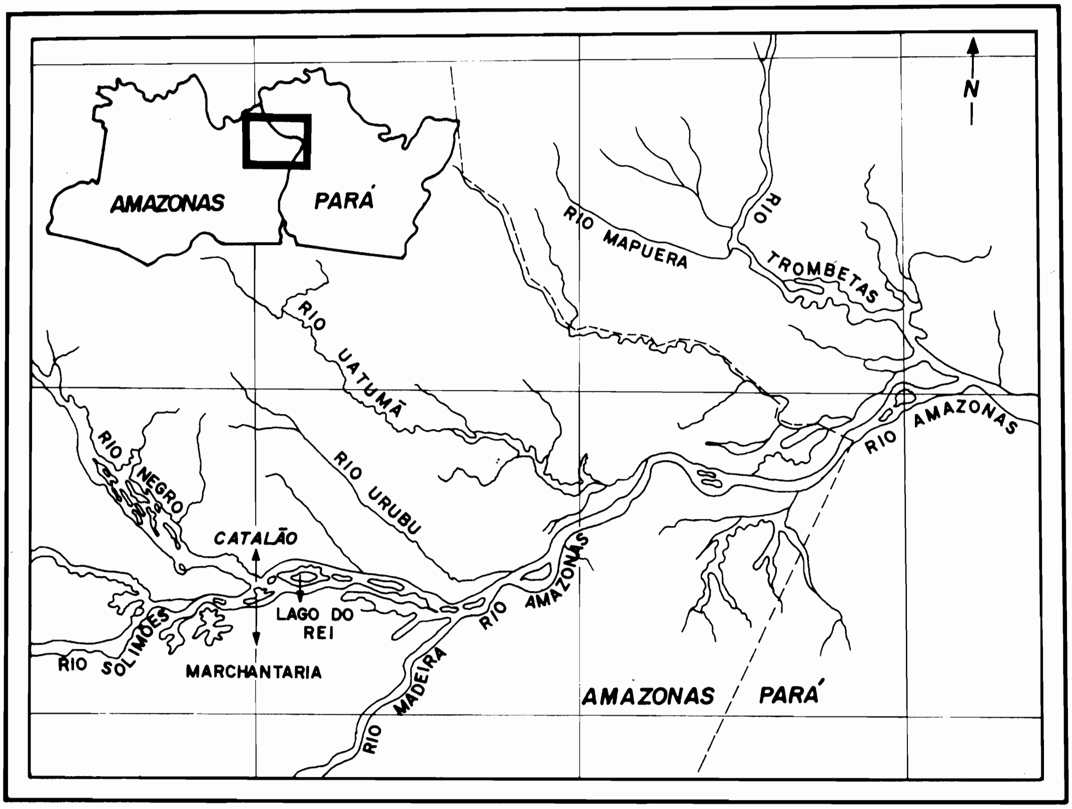

Figure 1 - Map of Amazon basin showing the places where species were captured.

Table I - Fish genera and species analyzed, showing collecting places and numbers of captured individuals.

	Solimões/Amazonas				
	Marchantaria	Catalão	Lago do Rei	Uatumã	Trombetas
Order Characiformes					
Family Curimatidae					
Genus *Potamorhina*					
P. pristigaster	-	-	37	-	-
P. altamazonica	26	45	16	-	-
P. latior	23	67	31	-	-
Genus *Curimata*					
C. ocellata	-	-	-	8	-
C. vittata	-	7	-	-	7
C. knerii	-	-	7	-	-
C. cyprinoides	-	-	-	-	21
C. innornata	-	-	-	23	-
Genus *Psectrogaster*					
P. rutiloides	-	92	4	-	-
P. amazonica	-	7	1	-	-
Genus *Curimatella*					
C. alburna	-	1	11	-	-
C. meyeri	-	3	5	-	-

Heat inactivation

Skeletal muscle extracts from the different phenotypes of these polymorphic species were incubated at 50°C (water bath) for different periods of time (10, 20, 30, 40, 50 or 60 min) and assayed spectrophotometrically. Time zero was included as the control. Enzyme assays were performed in 100 mM potassium phosphate buffer, pH 7.0, using a Zeiss PM6 spectrophotometer attached to a Shimadzu TB-85 thermobath. Enzyme activities were recorded by measuring the decrease of NADH concentration (starting with 1.3×10^{-4} M) at 340 nm, as the MDH reduced 0.33 mM oxaloacetate at 30°C. Triplicate values were obtained and presented as means of residual activity (%), considering the activity obtained at time zero to be 100%.

RESULTS

A three-banded pattern was observed in all 12 species. It suggests a dimeric molecule encoded at two distinct loci: sMDH-A* and sMDH-B*. The products of sMDH-A* loci were present at higher intensities in liver, suggesting tissue specificity. In addition, they were present in all other tissues as the principal component. The products of sMDH-B* did not predominate over sMDH-A* products in any tissue, suggesting a unidirectionally divergent pattern for these duplicated genes. Though the isozyme AB occurred in all tissues, there were less sMDH-B* products in skeletal muscle and retina. The s-MDH isozymes presented very similar electrophoretic mobilities for all species.

Different mobilities of mitochondrial MDH were observed for *Potamorhina latior* and *Curimata vittata*. These differences suggest divergence among regulatory genes compared with the conservative configuration of the structural genes.

Out of 12 species, six presented polymorphic loci: *Potamorhina pristigaster, Potamorhina altamazonica, Curimata ocellata, Curimata cyprinoides, Curimata innornata,* and *Psectrogaster rutiloides*. Similar allelic forms (allozymes with identical mobilities) were found for sMDH-B* orthologous loci in all polymorphic species (Figure 2). The allelic forms were termed B^*_{100} and B^*_{85}. Although the alleles occurred at different frequencies, the common allele was always the same, i.e., the most anodic one (B^*_{100}) (Table II and Figure 2). Statistical comparisons of polymorphic species captured in different places showed no difference among frequencies. Deviation from Hardy-Weinberg equilibrium was not observed in any polymorphic species.

Thermal inactivation analysis suggested that, except in *C. cyprinoides* and *P. rutiloides*, B^*_{85} products ($B_{100/85}$ and $B_{85/85}$) were more resistant to temperature exposure than B^*_{100} products (Figure 3). The $B_{85/85}$ or $B_{85/100}$ products sustained their activity longer than $B_{100/100}$ products (Table III). However, no correlation between phenotypic half-life (time necessary to decrease activity down to 50% of the initial) and allelic frequency or distribution was observed. The highest frequency of B^*_{85} allele occurred in *C. cyprinoides* in which B_{100} did not lose 50% of its activity within 60 min and B_{85} lost most of its activity with less than 15 minutes of heat exposure (Table II). Species with low allelic frequencies for B^*_{85} would express B_{85} allozymes which do not lose their activity during the 60 min of heat exposure, as occurred in *C. innornata* (Tables II and III, Figure 3).

Table II - sMDH-B^* allelic frequencies (MDH-B_{100} and MDH-B_{85}) in species of Curimatidae.

Species	p(B_{100})	q(B_{85})	n
Potamorhina pristigaster	0.7432	0.2568	37
*Potamorhina altamazonica**	0.9713	0.0287	87
P. altamazonica[c]	0.9556	0.0440	45
P. altamazonica[lr]	0.9688	0.0312	16
P. altamazonica[m]	1.0000	0.0000	26
Potamorhina latior	1.0000	0.0000	121
Curimata ocellata	0.6250	0.3750	8
Curimata vittata	1.0000	0.0000	7
Curimata knerii	1.0000	0.0000	7
Curimata cyprinoides	0.5714	0.4286	21
Curimata innornata	0.9348	0.0652	23
Psectrogaster amazonica	1.0000	0.0000	8
*Psectrogaster rutiloides**	0.9844	0.0156	96
P. rutiloides[c]	0.9837	0.0163	92
P. rutiloides[lr]	1.0000	0.0000	4
Curimatela alburna	1.0000	0.0000	12
Curimatela meyeri	1.0000	0.0000	

*The species *Potamorhina altamazonica* and *Psectrogaster rutiloides* had their allelic frequencies combined in order to allow comparative analysis among other species since there were no significant differences among collecting places. [c]Catalão; [lr]Lago do Rei; and [m]Marchantaria.

DISCUSSION

Similar electrophoretic mobilities among orthologous isozymes reflect a conservative system.

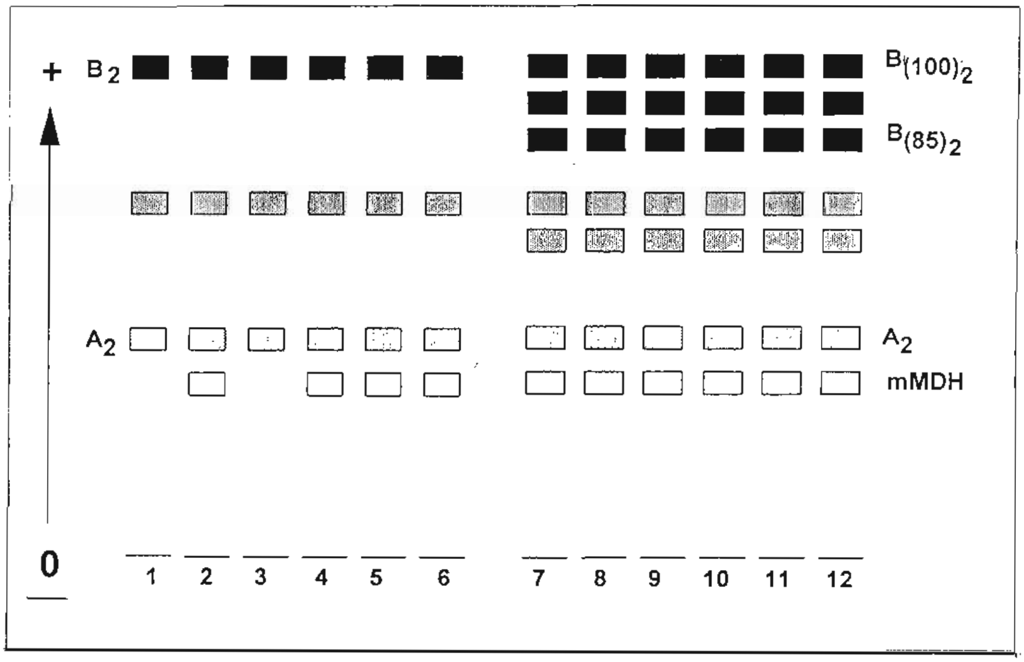

Figure 2 - Diagram of electrophoretic migration of MDH iso/allozymes for all studied species, showing monomorphic species (left) and heterozygote phenotypes $A_{100}/B_{100/85}$ for polymorphic species (right). O = Origin.

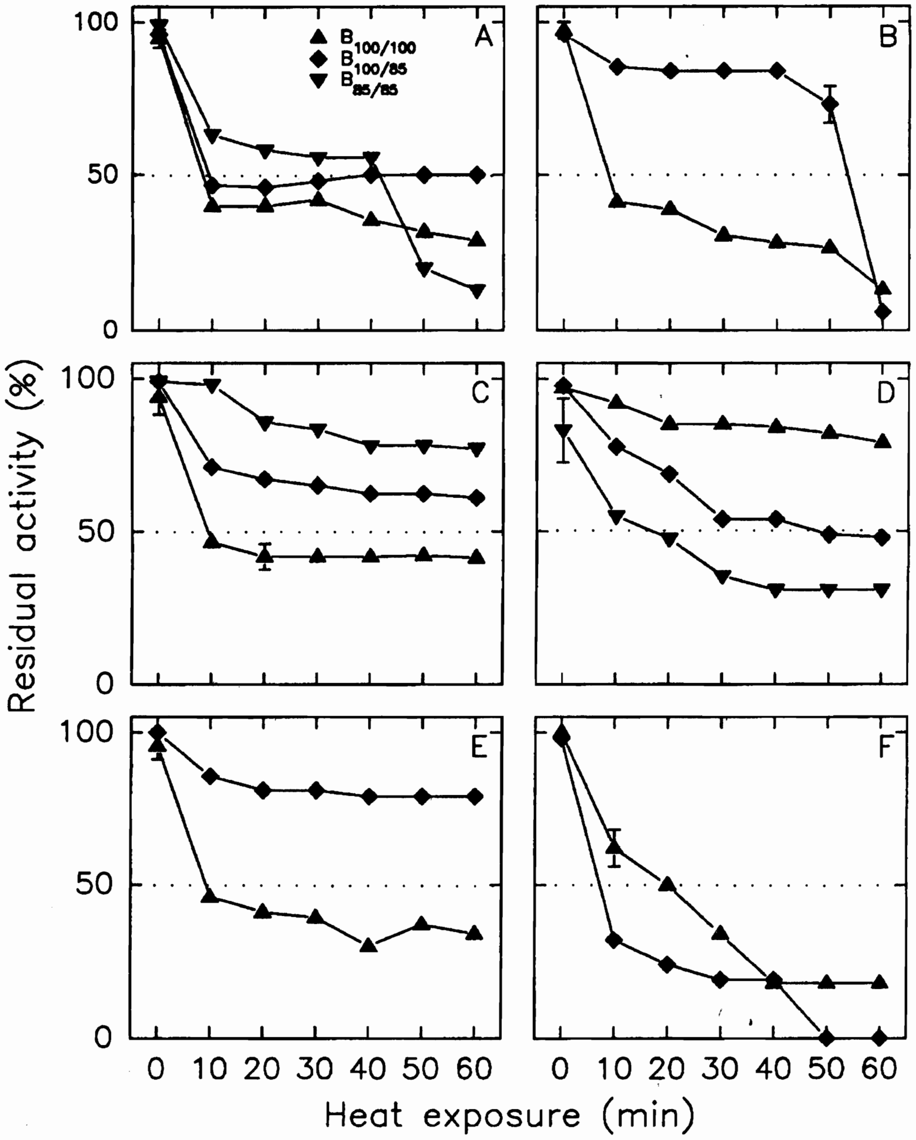

Figure 3 - Residual activity (%) of MDH enzymes from different phenotypes of *Potamorhina pristigaster* (A); *Potamorhina altamazonica* (B); *Curimata ocellata* (C); *Curimata cyprinoides* (D); *Curimata innornata* (E); and *Psectrogaster rutiloides* (F) after exposure to 60°C for six different periods of time (10, 20, 30, 50 or 60 min). Symbols indicate mean ± SEM. Dotted line indicates half-life.

Table III - Half-life* values for all analyzed phenotypes in polymorphic fish species. *Minutes of heat exposure to reduce enzyme activity by 50% (see Figure 3 and text for explanation).

Species	$B_{(100)2}$	$B_{(100/85)}$	$B_{(85)2}$
Potamorhina pristigaster	9.0	10.0	42.0
Potamorhina altamazonica	8.5	54.0	-
Curimata cyprinoides	> 60.0	48.0	17.5
Curimata innornata	9.0	> 60.0	-
Curimata ocellata	9.5	> 60.0	> 60.0
Psectrogaster rutiloides	7.5	20.0	-

The main differences among the species studied were in the specific mobilities of m-MDH. *P. latior* and *C. vittata* presented similar mobilities for m-MDH and sMDH-A2, constrasting with other species where s-MDH isozymes can be easily distinguished from m-MDH isozymes by electrophoresis (Bailey *et al.*, 1969; 1970; Rainboth and Whitt, 1974; Fisher *et al.*, 1980; Schwantes and Schwantes, 1982a; Basaglia, 1991).

Different metabolic roles for the products of duplicated genes (isozymes) have been claimed for many enzymes. The gene duplication event as described by Ohno (1970) took place early in the evolution of vertebrates, allowing the differentiation of structural and regulatory genes. As a consequence new fates in biochemistry and physiology of the organisms, including the specialization of their isozymes have occurred. Tissue specificities are similar for both paralogous isozymes sMDH-A2 and sMDH-B2 in all 12 Curimatidae species. This characteristic has been described for other teleosts (Fisher *et al.*, 1980; Coppes *et al.*, 1987; Basaglia, 1991), reflecting a unidirectionally divergent pattern for duplicated genes. Bidirectionally divergent patterns for s-MDH duplicated genes, where sMDH-A2 predominates in liver and sMDH-B2 predominates in skeletal muscle, have also been described in fish (Bailey *et al.*, 1969; 1970; Aspinwall, 1974; Fisher *et al.*, 1980; De Luca *et al.*, 1983; Coppes *et al.*, 1987). This differentiation in gene expression among species or groups of species is explained by different evolutionary pressures (Whitt, 1987). The physiological meaning of these different types of tissue distribution has not been ascertained yet. Wheat *et al.* (1971) suggested that s-MDH activity is regulated by the control of sMDH-B2 synthesis. However, this idea remains to be tested.

Organic thermal compensation may be achieved by immediate changes in metabolic rates and/or by changes in enzyme concentrations during acclimation. Compensatory adjustments may also be built up during evolutionary time (Hochachka and Somero, 1984). Thermostability of A2 and the apparent activation of B2 isozymes at low temperatures have been described for different species of teleosts (Schwantes and Schwantes, 1982ab; Hines *et al.*, 1983; Whitt, 1983). Such a compensatory mechanism has been reported as a common feature during thermal acclimation (Hochachka, 1967; Somero and Hochachka, 1969; Tsukuda, 1975; Yamawaki and Tsukuda, 1979ab).

Two intriguing features regarding the polymorphic species are denoted in this study. First, although the alleles exhibited different frequencies among the species studied, they always had the same rare or common status. Secondly, a higher thermostability was observed for the rare allele in four polymorphic species. A third feature is that there was no correlation between the allelic frequencies and the degree of thermostability.

Cryptic alleles have been described for many species (Singh *et al.*, 1976; Coyne *et al.*, 1978; Ayala, 1982; Kreitzman, 1983; Lewontin, 1985). However, different mutational events in orthologous loci producing allozymes with similar electrophoretic mobilities in six closely related species is a very remote hypothesis. A more feasible idea is that a single mutational event occurred before the speciation in the Curimatidae. Minor differences in thermal properties may be the result of few aminoacid replacements not affecting electrophoretic mobility.

According to Vari (1988) the speciation events in this family precede the final uplift of the Andean mountains; therefore, preceding the Amazon basin formation. Thus, at least for the Curimatidae, the appearance and formation of a complex hydrographic basin such as the Amazon were not the main cause of its high diversity.

Functional diploidization and/or gene silencing have been described in tetraploid groups as powerful tools in systematic studies. Such events allow the identification of the primitive character as the expression of the duplicated gene and the derived one as the expression of a single locus (Buth, 1983; 1984; Ferris, 1984). The presence of a new allele may not be compared with a duplicated locus because they are the result of different genetic and evolutionary processes. However, the similarities among sMDH-B* loci products and the appearance of alleles with similar mobilities in this family indicate that a single mutational event in the ancestral lineage of the group has occurred. Considering that the main speciation event in this group occurred in the Miocene (23 MYR ago, Vari, 1988) the first alleles had time to become differentiated. Thus, by analogy, all species may have first displayed a polymorphic state, and the currently monomorphic species have lost their polymorphic status.

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RESUMO

O presente trabalho descreve os padrões eletroforéticos da MDH (malato desidrogenase) de 12 espécies da família Curimatidae (Teleostei) que ocorrem na bacia amazônica. Os padrões se assemelham quanto à migração das enzimas ortólogas não permitindo diferenciação dentre as diversas espécies estudadas. Das 12 espécies analisadas, seis apresentaram locos polimórficos. Os alelos encontrados foram denominados sMDH-B^{*}_{100} e sMDH-B^{*}_{85} de acordo com suas mobilidades relativas e suas freqüências dentre as espécies. Todos os fenótipos foram submetidos a ensaios de inativação térmica. Em geral, aqueles que possuem o alelo

B^{*}_{85} são mais estáveis que os que possuem o alelo B_{100} nas espécies polimórficas. Baseados na ocorrência de um único alelo para as seis espécies polimórficas bem como em suas freqüências e diferentes termoestabilidades, sugerimos que esse alelo seja o resultado de um único evento mutacional ocorrido na espécie ancestral da família. A outra hipótese, a nosso ver, menos provável, seria a de mutações independentes nas diversas espécies em tempos diferentes.

REFERENCES

Aspinwall, N. (1974). Genetic analysis of duplicate malate dehydrogenase loci in the pink salmon, *Oncorhynchus gorbuscha*. *Genetics 76*: 65-72.

Ayala, F.J. (1982). Molecular polymorphism: How much is there and why is there so much? *Devel. Genet. 4*: 379-391.

Bailey, G.S., Cocks, G.T. and **Wilson, A.C.** (1969). Gene duplication in fishes: malate dehydrogenase of salmon and trout. *Biochem. Biophys. Res. Comm. 34*: 605-612.

Bailey, G.S., Wilson, A.C., Halver, J.E. and **Johnson, C.L.** (1970). Multiple forms of supernatant malate dehydrogenase in salmonid fishes: biochemical, immunological and genetic studies. *J. Biol. Chem. 245*: 5927-5940.

Basaglia, F. (1991). Malate dehydrogenase isozymes in fifteen sparidae species (Perciformes, Teleostei). *Comp. Biochem. Physiol. 98B*: 9-19.

Buth, D.G. (1983). Duplicate isozyme loci in fishes: Origins, distribution, phyletic consequences and locus nomenclature. In: *Isozymes: Current Topics in Biological and Medical Research*. (Rattazzi, M.C., Scandalios, J.G. and Whitt, G.S., eds.). Vol. 10. Alan R. Liss, New York, pp. 381-400.

Buth, D.G. (1984). The application of electrophoretic data in systematic studies. *Ann. Rev. Ecol. Syst. 15*: 501-522.

Coppes, Z.L., Schwantes, M.L.B. and **Schwantes, A.R.** (1987). Adaptative features of enzymes from the family Scianidae (Perciformes) - I. Studies of soluble malate dehydrogenase (s-MDH) and creatine kinase (CK) of fishes from the south coast of Uruguai. *Comp. Biochem. Physiol. 88B*: 203-209.

Coyne, J.A., Felton, A.A. and **Lewontin, R.C.** (1978). Extent of genetic variation at a highly polymorphic esterase locus in *Drosophila pseudobscura*. *Proc. Natl. Acad. Sci.* USA *75*: 5090-5093.

De Luca, P.H., Schwantes, M.L.B. and **Schwantes, A.R.** (1983). Adaptative features of ectothermic enzymes. IV. Studies on malate dehydrogenase of *Astyanax fasciatus* (Characidae) from Lobo reservoir (São Carlos, São Paulo, Brasil). *Comp. Biochem. Physiol. 74B*: 315-324.

Fenerich-Verani, N., Schwantes, M.L.B. and **Schwantes, A.R.** (1990). Patterns of gene expression during *Prochilodus scrofa* (Characiformes, Prochilodontidae) embryogenesis. I. Lactate dehydrogenase. *Comp. Biochem. Physiol. 97B*: 235-246.

Ferris, S.D. (1984). Tetraploidy and the evolution of the catostomid fishes. In: *Evolutionary Genetics of Fish* (Turner, B.J., ed.). Plenum Publishing Corp., New York, pp. 55-93.

Fisher, S.E., Shaklee, J.B., Ferris, S.D. and **Whitt, G.S.** (1980). Evolution of five multilocus isozyme systems in the chordates. In: *Animal Genetics and Evolution* (Vorontsov, N.N. and von Brink, J.M., eds.). vol. 52: 73-85.

Hedrick, P.W. (1983). *Genetics of Population* (Lipsett, S., ed.). Science Books International, Boston, pp. 629.

Hines, S.A., Philipp, D.P., Childers, W.F. and **Whitt, G.S.** (1983). Thermal kinetic differences between allelic isozymes of malate dehydrogenase (MDH-B) of largemouth bass, *Micropterus salmonoides. Biochem. Genet. 21*: 1143-1151.

Hochachka, P.W. (1967). Organization of metabolism during temperature compensation. In: *Molecular Aspects of Temperature Adaptation*. (Prosser, C.L., ed.). American Association for the Advancement of Science Symposium Series, 84: 177-203, Washington D.C.

Hochachka, P.W. and **Somero, G.N.** (1984). *Biochemical Adaptation*. Princeton Univ. Press, Princeton, pp. 537.

Kreitzman, H. (1983). Nucleotide polymorphism at the alcohol dehydrogenase locus of *Drosophila melanogaster*. *Nature 304*: 412-417.

Lewontin, R.C. (1985). Population genetics. In: *Evolution*. (Greenwood, P.J., Harvey, P.H. and Slatkin, M., eds.). Cambridge University Press, Cambridge, pp. 3-18.

Meizel, S. and **Markert, C.L.** (1967). Malate dehydrogenase isozymes of the marine snail, *Ieyanasse obsoluta*. *Arch. Biochem. Biophy. 127*: 753-765.

Ohno, S. (1970). The enormous diversity in genome sizes of fish as a reflection of nature's extensive experiments with gene duplication. *Trans. Amer. Fish. Soc. 99*: 120-130.

Place, A.R. and **Powers, D.A.** (1978). Genetic bases for protein polymorphism in *Fundulus heteroclitus* (L.). I. Lactate dehydrogenase (Ldh-B), malate dehydrogenase (Mdh-B), glucosephosphate isomerase (Gpi-B), and phosphoglucomutase (Pgm-A). *Biochem. Genet. 16*: 577-591.

Rainboth, W.J. and **Whitt, G.S.** (1974). Analysis of evolutionary relationships among shiners of the subgenus *Luxilus* (Teleostei, Cypriniformes, Notropis) with the lactate dehydrogenase and malate dehydrogenase isozyme systems. *Comp. Biochem. Physiol. 49B*: 241-252.

Schwantes, M.L.B. and **Schwantes, A.R.** (1982a). Adaptative features of ectothermic enzymes - I. Temperature effects on the malate dehydrogenase from a temperate fish *Leiostomus xanthurus*. *Comp. Biochem. Physiol. 72B*: 49-58.

Schwantes, M.L.B. and **Schwantes, A.R.** (1982b). Adaptative features of the ectothermic enzymes - II. The effects of acclimation temperature on the malate dehydrogenase of the spot, *Leiostomus xanthurus*. *Comp. Biochem. Physiol. 72B*: 59-64.

Sechs, L. (1982). *Applied Statistics: a Handbook of Techniques*. Springer Verlag, New York, pp. 299-306.

Shaklee, J.B., Allendorf, F.W., Morizot, D.C. and **Whitt, G.S.** (1989). Genetic nomenclature for proteins-coding loci in fish: Proposed guidelines. *Trans. Amer. Fish. Soc. 118*: 218-277.

Shaw, R.R. and **Prassad, R.** (1970). Starch gel electrophoresis of enzymes. A compilation of recipes. *Biochem. Genet. 4*: 297-320.

Singh, R.S., Lewontin, R.C. and **Felton, A.A.** (1976). Genetic heterogeneity within electrophoretic ``alleles'' of xanthine dehydrogenase in *Drosophila pseudobscura. Genetics 84*: 609-629.

Smithies, O. (1955). Zone electrophoresis in starch gel: group variation in the serum protein of normal human adults. *J. Biochem. 61*: 629-641.

Smithies, O. (1959). An improved procedure for starch gel electrophoresis: further variation in the serum proteins of normal individuals. *J. Biochem. 71*: 585-587.

Somero, G.N. and **Hochachka, P.W.** (1969). Isoenzymes and short-term temperature compensation in poikilotherms: activation of lactate dehydrogenase isoenzyme by temperature decreases. *Nature 223*: 194-195.

Tsukuda, H. (1975). Temperature of the relative activities of liver lactate dehydrogenase isozymes in goldfish acclimated to different temperatures. *Comp. Biochem. Physiol. 52B*: 343-345.

Val, A.L., Schwantes, A.R., Schwantes, M.L.B. and **De Luca, P.H.** (1981). Amido hidrolisado do milho como suporte eletroforético. *Ciênc. Cult. 33*: 992-996.

Vari, R.P. (1988). The Curimatidae, a lowland neotropical family (Pisces: Characiformes): distribution, endemism and phylogenetic biogeography. In: *Proceedings of a Workshop on Neotropical Distribution Patterns. Academia Brasileira de Ciências*, Rio de Janeiro, pp. 343-377.

Wheat, T.E., Childers, W.F., Miller, E.T. and **Whitt, G.S.** (1971). Genetic and *in vitro* molecular hybridization of malate dehydrogenase isozyme in an interspecific bass (*Micropterus*) hybrid. *Anim. Blood. Grp. Biochem. Genet. 2*: 3-14.

Whitt, G.S. (1970). Developmental genetics of the lactate dehydrogenase isozymes of fish. *J. Exp. Zool. 175*: 1-3.

Whitt, G.S. (1983). Isozymes as probes and participants in developmental and evolutionary genetics. In: *Isozymes: Genetics and Evolution*. (Rattazzi, M.C., Scandalios, J.G. and Whitt, G.S., eds.). Alan R. Liss Inc., New York, pp. 1-40.

Whitt, G.S. (1987). Species differences in isozyme tissue patterns: their utility for systematic and evolutionary analysis. In: *Isozymes: Current Topics in Biological and Medical Research*. (Rattazzi, G.C.; Scandalios, J.G. and Whitt, G.S., eds.). Alan Liss Inc., New York, pp. 1-26.

Whitt, G.S., Miller, E.T. and **Shaklee, J.B.** (1973). Developmental and biochemical genetics of the lactate dehydrogenase isozymes in fishes. In: *Genetics and Mutagenesis of Fish*. (Schroder, J.H., ed.). Springer-Verlag, Berlin, pp. 243-276.

Yamawaki, H. and **Tsukuda, H.** (1979a). Significance of the variation in isozymes of liver lactate dehydrogenase with thermal acclimation in goldfish. I. Thermostability temperature dependence. *Comp. Biochem. Physiol. 62B*: 89-93.

Yamawaki, H. and **Tsukuda, H.** (1979b). Significance of the variation in isozymes of liver lactate dehydrogenase with thermal acclimation in goldfish. II. Effect of pH. *Comp. Biochem. Physiol. 62B*: 94-95.

Zink, M.W. and **Shaw, D.A.** (1968). Regulation of malic enzyme and malic dehydrogenase in *Neurospora crassa. Canad. J. Microbiol. 14*: 907-912.

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