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Lactate dehydrogenase isozyme pattern of the Mediterranean killifish *Aphanius fasciatus* (Teleostei, Cyprinodontidae)

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ABSTRACT

Electrophoretic analyses of the tetrameric lactate dehydrogenase isozyme patterns of the endangered cyprinodontid fish *Aphanius fasciatus* were carried out. Organ and tissue samples of ten individuals of each sex were analysed by cellulose acetate electrophoresis and isoelectric focusing. Isozyme electrophoretic patterns indicated that three LDH loci (*Ldh*-A, *Ldh*-B and *Ldh*-C) were active and showed differential tissue expressions. No differences between sexes were observed. The *Ldh*-A and *Ldh*-B loci were expressed in all tissues analysed, and the A subunits possessed a net negative charge greater than B subunits. Heterotetramers including subunit A were not observed, but a complex heterotetramer banding pattern of subunits B and C was detected. On the basis of the staining intensity of the electromorphs, *Ldh*-A predominated in all tissues analysed, with the exception of the liver, where *Ldh*-B predominated. Instead, *Ldh*-C was characterised by high relative anodal mobility and was expressed only in the eye. Furthermore, at this locus two alleles were observed in specimens analysed. The LDH banding pattern of *A. fasciatus* was consistent with that of advanced teleosts and it showed an inversion of the net negative charge of products of the *Ldh*-A and *Ldh*-B loci.

KEY WORDS: *Aphanius fasciatus* - Cyprinodontidae - LDH - Isoelectric focusing - Cellulose acetate electrophoresis - Quaternary structure - Isozymes.

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INTRODUCTION

Aphanius fasciatus Nardo, 1827 is a small cyprinodontid fish (7-8 cm maximum total length) typical of brackish-water habitats along Mediterranean coasts. It also has been recorded in inland fresh-waters (Parenti & Tigano, 1993) and in Tunisian oases (Kraiem, 1983). Its geographical distribution includes the Mediterranean basin with the exception of the easternmost and westernmost parts (Villwock, 1982; Bianco, 1995). In recent years the species assumed relevance for conservation purposes, due to the progressive deterioration of coastal habitats as a result of human impact and the introduction of the exotic competitor *Gambusia affinis*, occurred in many European countries for malaria mosquito control in the first decades of the century (Bianco, 1995). For these reasons, *A. fasciatus* has been listed as endangered by both the 'Berne Convention' and the 'Fauna-Flora-Habitat 92/43/CEE Directive', relative to the conservation of wildlife and natural environment in Europe.

The aim of the present investigation was to gain information on the isozyme pattern of lactate dehydrogenase (LDH; E.C. 1.1.1.27) in *A. fasciatus*. The LDH multilocus isozyme system represents an example of gene duplication which led to homologous proteins in fishes (Markert *et al.*, 1975). In fact, *Ldh*-A and *Ldh*-B loci of fishes appear to have originated from a gene duplication event occurred early in the evolution of the vertebrate line (roughly 500 million years ago); these loci have been shown to be homologous to those of higher vertebrates (Markert *et al.*, 1975; Whitt *et al.*, 1975; Fisher *et al.*, 1980; Coppes *et al.*, 1990). Some groups of teleosts, mammals and birds also possess a third locus (*Ldh*-C), restricted to specific tissues (Markert *et al.*, 1975; Coppes *et al.*, 1990). Three loci for LDH were observed in previous works dealing with the allozyme genetic polymorphism of *A. fasciatus* (Comparini *et al.*, 1983), of which one (*Ldh*-3 ≡ *Ldh*-C) was biallelic (Maltagliati, 1998a, b).

MATERIALS AND METHODS

Twenty adult individuals of *Aphanius fasciatus* (10 males and 10 females) were collected from a brackish-water habitat at Elba Island, Italy (42°48'N; 10°19'E) in May 1997 using small fish-traps baited with anchovy fillets. Alive specimens were transported to the laboratory where they were killed in distilled water and ice, and stored at −80° C before use. Samples of epaxial muscle, liver, eye, heart, intestine, gills, testis, and ovary were excised from single thawed individuals to be analysed by cellulose acetate electrophoresis and isoelectric focusing (IEF). Portions of tissue were homogenised with a glass rod in three volumes of extracting buffer (see Maltagliati, 1998b). Then homogenates were centrifuged for 10 min at 4000 *g*. Supernatant fractions were applied to cellulose acetate membranes for electrophoresis and polyacrylamide gel with ampholites (Servalyt® Precotes®, 300 µm thick, pH range 3-10) for IEF. Care was taken to keep sample temperature below 5° C at all stages of preparation to preserve enzyme activity. Electrophoretic running conditions are reported in Table I. Enzyme activity was visualised by using slight modifications of

TABLE I - Aphanius fasciatus. *Experimental conditions for electrophoresis and isoelectric focusing of lactate dehydrogenase isozymes*

	Electrophoresis	Isoelectric focusing
Substrate	Cellulose acetate membrane	Polyacrylamide gel
pH	Constant (7.8)	Variable (3 to 10)
Voltage	Constant (450 V)	Variable (200 to 1500 V)
Power	Variable (8 to 6 W)	Constant (4 W)
Current	Variable (4 to 6 mA)	Variable (7 to 4 mA)
Running time	25 min	175 min

the histochemical technique of Richardson *et al.* (1986). To determine if there were differences in LDH activity between sexes, calibration of isozymes was accomplished by running male and female individuals on the same electrophoretic substrate. The absence of nothing dehydrogenase was verified by incubating the cellulose acetate membrane in all the staining components with the exception of lactate. The nomenclature used to describe the LDH isozymes of *A. fasciatus* is that outlined in Shaklee *et al.* (1973).

RESULTS AND DISCUSSION

The tissue specific LDH isozyme patterns for *Aphanius fasciatus* are presented in Figure 1. Cellulose acetate electrophoresis migrations were anodal and no substantial differences in isozyme patterns between sexes were found. The IEF provided no additional information, thus confirming the resolution power of cellulose acetate electrophoresis. The results of the present study highlighted the occurrence of three LDH loci in *A. fasciatus*. Within the Cyprinodontidae three LDH loci have been recorded in *A. iberus* (Doadrio *et al.*, 1996;

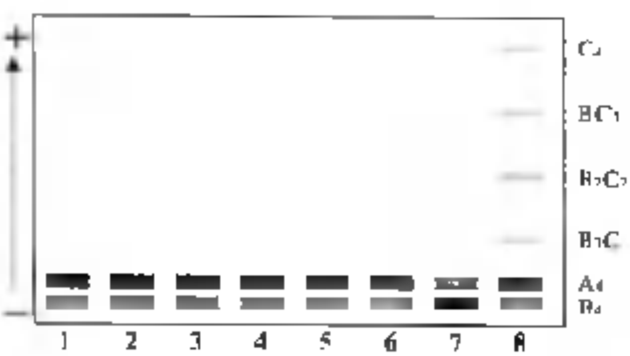

Fig. 1 – Schematic diagram of lactate dehydrogenase isozyme pattern in various tissues of *Aphanius fasciatus* after cellulose acetate electrophoresis. 1, epaxial muscle; 2, heart; 3, intestine; 4, gills; 5, testis; 6, ovary; 7, liver; 8, eye. The arrow indicates the direction of the migration. Products of loci *Ldh-B* and *Ldh-C* form heteropolymeric isozymes. Grey intensity represents band staining intensity.

Maltagliati, 1998b), *A. dispar* (Kornfield & Nevo, 1976) and several species of the genus *Cyprinodon* (Echelle *et al.*, 1987; Echelle & Echelle, 1993a, b; Ashbaugh *et al.*, 1994). In addition, the presence of three LDH loci has been demonstrated in many other teleost families (Shaklee *et al.*, 1973; Markert *et al.*, 1975).

Zymograms of samples of heart, intestine, gills, testis and ovary of *A. fasciatus* were substantially identical, with an intense band corresponding to the homotetramer A_4 and a lighter band, with a slightly lower anodal mobility, corresponding to B_4 (Fig. 1). However, some differences in band staining intensity among the tissues analysed were observed. Band staining intensity was assumed to be related to the enzyme concentration, hence the differences observed among tissues could be attributed to differential concentrations of LDH and related to tissue specificity. Alternatively, this could be explained by reductions of enzyme activity occurring during the homogenisation procedure. Zymograms of liver tissue showed inversion of band staining intensity, with electromorphs corresponding to isozyme B_4 being more intense than A_4 ones (Fig. 1). Like its counterparts in many other teleosts, the eye banding pattern of *A. fasciatus* exhibited a highly anodal electrophoretic mobility which is characteristic of neural tissue LDH. In fact, zymograms of eye homogenate showed an additional banding pattern determined by the products of locus *Ldh-C*, characterised by high anodal electrophoretic mobility. The complexity of eye banding pattern is determined by heteropolymerisation among products of the *Ldh-B* and *Ldh-C* loci. Six isozymes were characteristic of eye: the slowest and the fastest corresponding to the homotetramers B_4 and C_4, respectively, and intermediate electromorphs corresponding to the homotetramer A_4 and heterotetramers B_3C, B_2C_2 and BC_3, proceeding from the less to the more anodal (Fig. 1). Although many teleosts show an irrestrictive association of subunits A and B with a pentaisozymic system which characterises mammals and birds, in the majority of them, as well as in *A. fasciatus*, a tetrameric restriction or instability happens (Markert & Faulhaber, 1965; Markert *et al.*, 1975; Coppes de Achaval, 1984; Coppes *et al.*, 1990). Subunits C produced by locus *Ldh-C*, which is assumed to be originated from a duplication of gene *Ldh-B*, do not form heteropolymers with subunits A (Markert *et al.*, 1975).

In primitive teleosts, *Ldh-C* is expressed in various organs and tissues, but is localised in specific tissues in advanced teleosts (Shaklee *et al.*, 1973; Markert *et al.*, 1975; Coppes de Achaval, 1984). In fact, although some teleosts belonging to the Cypriniformes and Gadiformes express a cathodal *Ldh-C* isozyme which is characteristic of liver tissue, in most teleosts the highly anodal homopolymer C_4 is expressed only in eye and neural tissue (Markert & Faulhaber, 1965; Coppes de Achaval, 1984; Coppes *et al.*, 1990; Mork & Giaever, 1995). *A. fasciatus* is no exception to this trend, with *Ldh-A* being predominant in muscle, heart, intestine, gills and go-

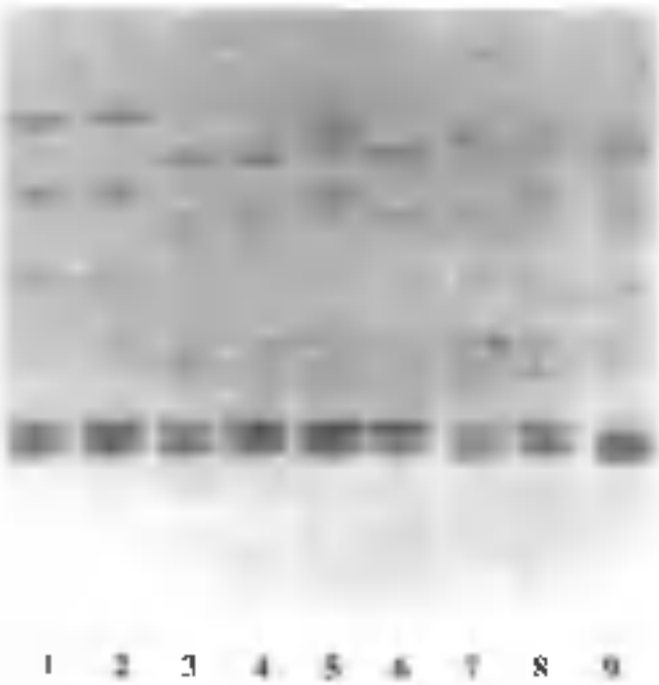

Fig. 3. Lactate dehydrogenase isozyme pattern in *Aphanius fasciatus* after histochemical staining following cellulose acetate electrophoresis of nine specimens. Individuals 3, 7, 8, 9 are heterozygotes for locus *Ldh-C*; 1, 2 are homozygotes for the fast allele, and 4, 5, 6 are homozygotes for the slow allele. See Figure 4 for explanation of the isozyme patterns.

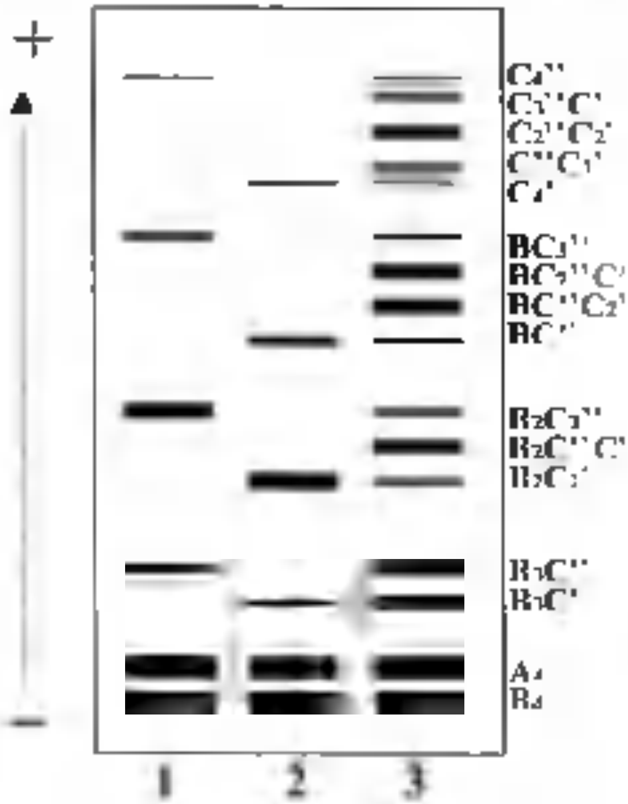

Fig. 4. Schematic diagram of eye lactate dehydrogenase isozyme pattern in *Aphanius fasciatus* after cellulose acetate electrophoresis. 1: homozygote pattern for fast allele at *Ldh-C* locus (genotype C'C'); 2: homozygote pattern for slower allele (genotype C''C''); 3: heterozygote (genotype C'C''). Differences of band staining intensity are not reported.

nads, *Ldh-B* in liver, and *Ldh-C* exclusive to the eye and neural tissue. Therefore, this species represents a typical 'two-isozyme' fish (*sensu* Whitt, 1970), having only the two homopolymeric isozymes in all tissues, with the exception of eye and neural tissue, and the heteropolymers A_3B, A_2B_2, and AB_3 being absent. The LDH banding pattern of *A. fasciatus* is consistent with that of advanced teleosts in the evolutionary scheme proposed by Markert *et al.* (1975) and reviewed by Coppes de Achaval (1981). Furthermore, with respect to most vertebrates, *A. fasciatus* showed isozymes A_4 and B_4 reversed, i.e., A_4 isozyme possessed a net negative charge greater than B_4. However, the inversion of net negative charge of products of the *Ldh-A* and *Ldh-B* loci is also a characteristic of many other teleosts and probably does not play, as suggested by Markert & Holmes (1969), an important physiological role.

Both the *Ldh-A* and *Ldh-B* loci were monomorphic in *A. fasciatus*, while two alleles were detected in the *Ldh-C* locus. The complex banding pattern of heterozygotes is shown in Figures 3 and 4. Campanini *et al.* (1988) observed these loci which were monomorphic in three populations of *A. fasciatus* from Sardinian and upper Adriatic coasts. Conversely, Maltagliati (1998a, b) observed polymorphism in the *Ldh-C* locus (there denominated *Ldh-3*) in five populations from central and upper Tyrrhenian coastal brackish water habitats. The inconsistency of these studies may be due to the absence of polyallelism at *Ldh-C* locus in the populations analysed by Campanini *et al.* (1988). Alternatively these authors might have been unable to detect polymorphism, given that they homogenised whole individuals or cephalic halves. This procedure could have caused some electromorphs relative to eye to go undetected.

At *Ldh-C* locus, eight out the 20 individuals assayed in the present investigation were homozygotes for the fast allele, three for the slow allele, and nine were heterozygotes. Despite the small number of individuals analysed, these genotypic proportions were in accordance with the Hardy-Weinberg equilibrium and consistent with results of a previous allozyme study, where the two *Ldh-C* alleles were detected in a sample of 30 individuals from the same population as that analysed here (Maltagliati, 1998a). Therefore, *Ldh-C* can be considered a reliable genetic marker for estimating the levels of genetic variability and investigating the genetic structure of the species. The present study may represent a basis useful for planning further investigations on *A. fasciatus*, in particular for addressing the questions of the correspondence between the dependence of allele frequencies on environmental factors such as temperature or oxygen concentration and the genotypic distribution of these alleles

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