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Source: Zoological Science, 14(3) : 415-418

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.415

ZOOLOGICAL SCIENCE **14**: 415–418 (1997)

[Short Communication]

First Description of Two Genetic Loci in *Leuciscus cephalus* (Cyprinidae) from Italy

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ABSTRACT—The biochemical-genetic analysis of 19 enzyme systems coded by 33 structural gene loci in the diploid *Leuciscus cephalus* have revealed the existence of two genetic loci never described in other cyprinid fishes. The electromorphs of mitochondrial aspartate aminotransferase (mAAT) and fumarate hydratase (FH) in larvae and in different adult tissues of *Leuciscus cephalus* suggest that these enzymes are coded by two loci each, at variance of available literature data on cyprinids, in which a single structural locus is known to code the mAAT and FH enzymes. These observations are confirmed for the adults of 8 additional Italian cyprinids.

INTRODUCTION

Electrophoretic analysis of protein variability is a rutinely utilized method to study population genetics, systematics and to reconstruct the phylogeny of different taxa from an evolutionary perspective. Along these perspectives, iso- and allozymatic characterization of fish populations have rapidly increased in the last few decades (Utter, 1991). In particular, papers dealing with the biochemical genetics of cyprinid hybrids have contributed to reveal several instances of hybridization, introgression and polyploidy along with the evolution of the group. Features of teleosts are the differential gene expression of homologous loci expecially in Salmoniformes and Cypriniformes (Ohno, 1970; Schultz, 1980; Buth, 1983) and ancestral tetraploydization events followed by functional diploidization in Palearctic cyprinids (Ferris and Whitt, 1977; Woods and Buth, 1984). Although unusual, in diploid teleost fishes also species-specific gene duplications have been described (Kuhl *et al.*, 1976; Turner *et al.*, 1980; Dowling and Moore, 1985).

Electrophoretic studies of enzymes and structural proteins of North American cyprinids are quite numerous (Buth, 1984; Buth *et al.*, 1991), whereas extensive biochemical-genetic analysis of European taxa are scant. In the context of a wide project, aimed to acquire a better morphological, cytological and genetic characterization of the Italian freshwater ichthyofauna - particularly felt for cyprinids (Gandolfi *et al.*, 1991) - we started an electrophoretic analysis using electromorphs as genetic markers for fish species identification and comparison. Differential tissue and developmental expression of a multilocus isozyme is a common feature; in this case it is necessary to analyze tissue and ontogenetic patterns of expression to define the homology among protein and loci (Shaklee *et al.*, 1990). In order to relate literature data on other cyprinid species with ours, we preliminarily analyzed 19 enzyme systems during ontogenesis and in different adult tissues of *Leuciscus cephalus*. This taxon is one of the most abundant species among autochthonous cyprinids and its diploid constitution has been well documented (Fontana *et al.*, 1970; Cataudella *et al.*, 1977). All enzymes gave zymogram patterns whose description and genetic interpretation are in general agreement with literature data (Buth, 1984; Buth *et al.*, 1991), with the exception of mitochondrial aspartate aminotransferase and fumarate hydratase which were known to be coded by a single locus each in cyprinids. This paper reports particularly on these enzyme systems, since our findings and interpretation are at variance with published data.

MATERIALS AND METHODS

Twenty-seven adult specimens of *Leuciscus cephalus* were collected in the Reno river (Bologna; Fig. 1), and either directly analyzed or stored at -80°C until dissected for electrophoretic analysis. Twenty individuals captured in the same place were maintained in a fish tank untill reproduction; after spawning, about 100 eggs were reared in 10 l aerated aquaria and, at hatching, the larvae fed on natural plancton or commercial foodstuffs. Samples of developing embryos and larvae at the time of endogenous (9-11 mm total length, 7 days) and exogenous feeding (13-15 total length, 28 days) were stored in a deep-freezer until electrophoresis. Developmental stages were scored following Economou *et al.* (1991).

Samples of adult tissues (white epiassial muscle, liver, heart, eye) or the whole larva were mechanically homogenized with an equal volume of O.1 M Tris-HCl, pH 7.5 and centrifuged at 12000 rpm for

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Fig. 1. Map showing the collecting area (Reno river).

10 min at room temperature; 2-3 µl of the supernatant were loaded on cellulose acetate membranes (Cellogel, 17 × 17 cm) previously equilibrated with either TEC 0.075 or TBE run buffers (Meera Khan, 1971) for aspartate aminotransferase (AAT; C.E. 2.6.1.1) and fumarate hydratase (FH; C.E. 4.2.1.2), respectively. Electrophoresis was carried out at constant voltage (220 V) and lasted either 4 or 5 hr for AAT and FH, respectively. Staining procedures followed Meera Khan *et al.* (1982).

The genetic interpretation of the observed phenotypes took into account the known quaternary structure and the proposed genetic interpretations of the same enzymes in other fishes. Enzyme and locus nomenclature follows the recommendations of Shaklee *et al.* (1990) and in particular the system proposed and adopted for diploid and polyploid cyprinids (Buth, 1983, 1984; Buth *et al.*, 1991).

RESULTS AND DISCUSSION

Both fresh and frozen samples showed the same zymogram patterns, thus excluding the possibility of protein degradation due to storage procedures.

The dimeric aspartate aminotransferase is known to exist in two distinct forms in teleost fishes, supernatant and mitochondrial. The former, sAAT, migrates anodally, whereas mAAT moves cathodically under electrophoresis at pH 7.0 (Schmidtke and Engel, 1972).

In all analyzed specimens two distinct zones of activity were found; the anodal one was scored as the supernatant or cytoplasmic fraction, the other, with slower mobility, as the mithocondrial form. In no sample the supernatant isozymes were associated with the mitochondrial ones to form heterodimers.

In highly concentrated homogenates of all tissues, mAAT showed a three banded phenotype, with the slower migrating band of higher intensity in liver and heart and the faster one only faintly stained in all samples; in larvae homogenates these two bands are of equal intensity (Fig. 2). While mAAT is known to be coded by a single locus in diploid fishes, namely *mAAT-A** in cyprinids (Buth *et al.*, 1991), present data suggest the existence of two distinct mitochondrial loci, here defined as *mAAT-1** and *mAAT-2** starting from the most anodal one. Their proteinic products give heterodimers during larval stages and in all analyzed adult tissues. The two loci show a differential pattern of expression, being equally active in the larvae, while in the adults the *mAAT-2** locus is predominantly expressed, even with a certain degree of tissue-specificity, *mAAT-2** showing a higher expression in liver and heart, *mAAT-1** being only slightly expressed in all tissues.

The correctness of this interpretation is clearly supported by the three banded phenotype evidenced in larvae, in which both homodimers mAAT-1 and mAAT-2 are of similar intensity, whereas the central heterodimer shows a double staining intensity (Fig. 2).

For the two mitochondrial loci, the evolution of a regulatory mechanism can be postulated. On the basis of present observations it can also be suggested that *mAAT-2** is homologous to the unique *mAAT-A** locus previously described in cyprinids.

Fumarate hydratase constantly showed a two banded phenotype in muscle extracts, with the faster band slightly less stained. A comparable phenotype could be observed in all tissues and larvae, the two bands being equally stained in liver, heart and larvae homogenates (Fig. 3). Owing to its tetrameric structure, these phenotypes could be interpreted as due to two distinct loci, here defined as *FH-1** and *FH-2**, whose homotetrameric products were the only isozymes evidenced. The two detected bands could either correspond to the homotetrameric products with none of the three expected FH-1 and FH-2 heterotetramers expressed or to a homotetrameric plus one heterotetrameric band, the faster locus being less active.

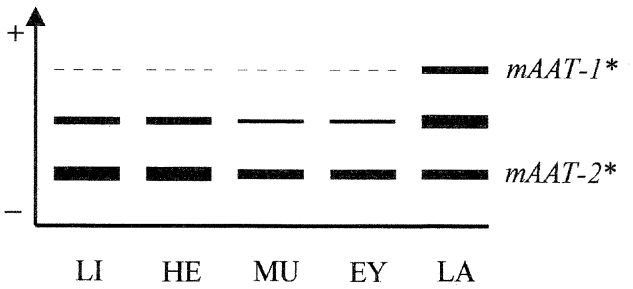

Fig. 2. Schematic representation of differential expression of mAAT enzyme. The electrophoretic patterns observed in larvae (LA) and different adult tissues (LI: liver; HE: heart; MU: white muscle; EY: eye) of *Leuciscus cephalus* are shown. Differences in line thickness refer to different staining intensities.

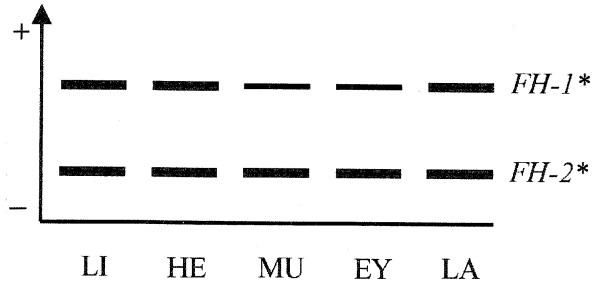

Fig. 3. Schematic representation of FH multilocus enzyme expression. Various adult tissues of *Leuciscus cephalus* are presented (captions as in Fig. 2). Differences in line thickness refer to different staining intensities.

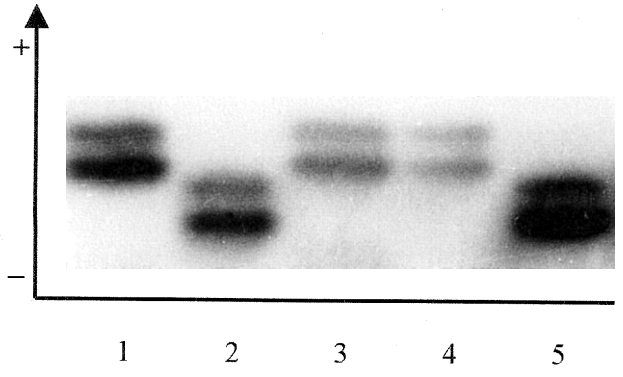

Fig. 4. Zymogram showing FH phenotype in white muscle of *Leuciscus souffia* (lane 1), *Alburnus alburnus alborella* (lanes 2,5), *L. cephalus* (lane 3) and *L. lucumonis* (lane 4). All specimens are *FH-1** and *FH-2* 100/100* with the exception of *A. alburnus alborella* (*FH-1** and *FH-2* 91/91*).

Of the two loci coding for fumarate hydratase here designed as *FH-1** and *FH-2**, the latter should correspond to *Fum-A**, which appears to be the only one so far detected in brain and muscle of North American cyprinids (Buth, 1984; Buth *et al.,* 1991).

It is to be noted that the existence of *mAAT-1** and *FH-1** has received support from the 8 Italian cyprinid taxa, whose genetic characterization has been currently undertaken: *Alburnus alburnus alborella, Leuciscus lucumonis, L. souffia, Chondrostoma genei, Rutilus rubilio, R. erythrophthalmus, Scardinius erythrophthalmus, Tinca tinca.* (unpublished; Fig. 4).

The widespread occurrence of double system in diploid fishes could be a consequence either of an ancestral duplication event followed by a divergent evolution or several indipendent tandem duplications in different lineages. The former hypothesis, more parsimonious, has been forwarded to explain the evolution of many paralogous isozyme loci among the most studied multilocus enzyme systems, such as creatine kinase, glucose-6-phosphate isomerase, glycerol-3-phosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase in chordates (Fisher *et al.*, 1980).

Finally our results strengthen the possibility that a different number of loci are coding for the same enzyme in differently related species, as evidenced in diploid cyprinids, for which a second locus of isocitric dehydrogenase has been described only in some European and north American cyprinids (Dowling and Moore, 1985; Buth *et al.*, 1991) and in other teleost fishes (Kuhl *et al.*, 1976; Turner *et al.*, 1980).

ACKNOWLEDGMENTS

We would like to thank Prof. V. Scali and Prof. F. Zaccanti for encouragement and critical reading of the manuscript.

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(Received September 11, 1996 / Accepted February 4, 1997)