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Comparative Analysis of Glucosephosphate Isomerase, Lactate Dehydrogenase and Malate Dehydrogenase Isozymes in 9 Cyprinid Species from Italy

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ABSTRACT—The developmental and the tissue-specific expression of glucosephosphate isomerase (GPI), lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) multilocus isozymes were analyzed in samples of *Leuciscus cephalus* and the adult patterns compared with those of 8 additional Italian cyprinid species: *Alburnus alburnus alborella*, *Chondrostoma genei*, *L. lucumonis*, *L. souffia*, *Rutilus rubilio*, *R. erythrophthalmus*, *Scardinius erythrophthalmus* and *Tinca tinca*, the taxonomic status of many of them being uncertain and highly debated. The spatial and temporal patterns of expression obtained generally agree with literature data. Main exceptions are the single expression of *GPI-A** and *MDH-A** loci of the liver in *L. cephalus* and the GPI pattern of the eye in all species examined. Since delayed appearence of the subunits coded by the *GPI-B** locus and the very early ontogenetic expression of the *sMDH-B** locus were found in *L. cephalus*, the onset of expression of orthologous loci can vary in related species. Genetic structure comparisons support a high genetic divergence of *T. tinca* from all other species.

INTRODUCTION

In natural fish populations the range of variability and the genetic control of various enzyme systems have been clarified by resolving electrophoretic variants and interpreting the ensuing phenotypes in terms of Mendelian gene loci.

In vertebrates glucosephosphate isomerase (GPI), lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) enzymes are known to exist in multiple molecular forms or different isozymes, with significant variations in spatial and temporal patterns of expression (see for a review Basaglia, 1989). The three isozymes are coded by a different number of genetic loci, only some of them being homologous between tetrapods and teleosts (Fisher *et al*., 1980). Within the latter, mitochondrial and supernatant *GPI**, *LDH** and *MDH** loci have been found to be duplicated, owing to tetraploidization events that independently occurred in different phyletic lineages and were sometimes followed by functional diploidization, revealed as gene silencing (Schmidtke *et al*., 1975; Ferris and Whitt, 1977). This is a well known phenomenon in the fish family Cyprinidae and in Cypriniformes in general, in which spontaneous tandem duplications of a *GPI** locus and the existence of two *mAAT** and *FH** loci were reported (Buth, 1983; Woods and Buth, 1984; Agnese *et al*., 1990; Berrebi *et al*., 1990; Manaresi and Mantovani, 1997).

Moreover, in Cypriniformes the *LDH-C** locus has a very specific tissue expression being active only in the liver, as it happens in the order Siluriformes and in some Gadiformes (Basaglia, 1989).

In the present study the developmental and the tissue-specific expression of GPI, LDH and MDH have been determined in samples of the chub *Leuciscus cephalus* L. and the adult patterns compared with those of other northern-central Italian cyprinid species: *Alburnus alburnus alborella* De Filippi, 1844 (white bleak), *Chondrostoma genei* Bonaparte, 1839, *Leuciscus souffia* Risso, 1826, *Rutilus rubilio* Bonaparte, 1837 (Adriatic roach), *Scardinius erythrophthalmus* L. (rudd) and *Tinca tinca* L. (tench). *L. lucumonis* Bianco, 1982 (Etruscan chub) and *R. erythrophthalmus* Zerunian, 1982 (northern Italian roach), two taxa only recently erected as distinct species, have been also analyzed. According to Howes (1991), the majority of these taxa pertain to the subfamily Leuciscinae, with the exceptions of *A. alburnus* (Alburninae) and *T. tinca* (*incertae sedis*). An alternative classification includes the genus *Alburnus* in the Leuciscinae and *T. tinca* in the Cyprininae (Banarescu and Coad, 1991). The taxonomic status of most European cyprinid species is unresolved, since species definition is up to now mainly based on morphological characters, which cannot often unambiguosly be used with success. Main debated points on Italian taxa are the taxonomic position of *T. tinca* within the family Cyprinidae, the generic status of *Rutilus* and *Scardinius*, which are considered synonymous by some authors and the relationships among species actually included in the genera *Leuciscus* and *Rutilus* (Gandolfi *et al*., 1991; Howes, 1991; Bianco, 1995).

Considering the usefulness of the electrophoretic analyses applied to the clarification of several taxonomic and phy-

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logenetic questions about American cyprinids (Buth *et al.*, 1991), the necessity of the biochemical approach to the systematic revision of European taxa has been pointed out (Crivelli and Dupont, 1987; Gandolfi *et al.*, 1991).

The aims of the present analysis are therefore to define the expression patterns of three multilocus enzymes in 9 representative cyprinids from Northern Italy and to throw some light on the relationships among these Italian freshwater fishes from a biochemical-genetic perspective.

MATERIAL AND METHODS

Sampling localities and species abbreviations are reported in Table 1. All samples were collected in northern-central Italy from wild population, with the exception of *T. tinca* obtained from Gavello aquaculture station.

Specimens were placed on dry ice upon capture and stored at –80°C until utilized for electrophoretic analysis. Twenty individuals of *L. cephalus* were maintained in a fish tank untill reproduction. After spawning, about 100 eggs were reared in aerated aquaria (10 l) and, at hatching, the larvae fed on natural plancton or commercial foodstuffs. Samples of fertilized eggs and larvae at the time of endogenous feeding (9-11 mm total length, 7 days) were stored at –80°C until singly analyzed. Developmental stages were scored following Economou *et al.* (1991).

A preliminary survey to check isozyme expression was carried out on fertilized eggs, larvae and samples of adult tissues (white muscle, liver, heart and eye) of *L. cephalus*; samples of white muscle or eye of adults of the following species were thereafter analyzed for genetic comparisons: *A. alburnus alborella*, *L. lucumonis*, *L. souffia*, *C. genei*, *R. rubilio*, *R. erythrophthalmus*, *S. erythrophthalmus*, *T. tinca*.

Samples were analyzed for glucosephosphate isomerase (D-glucose-6-phosphate ketol-isomerase, GPI, EC 5.3.1.9), lactate dehydrogenase (lactate: NAD-oxidoreductase, LDH, EC 1.1.1.27) and malate dehydrogenase (L-malate: NAD^+- oxidoreductase, MDH , EC 1.1.1.37). Homogenates were mechanically prepared from equal volumes of tissue and 0.1 M Tris-HCl, pH 7.5 and centrifuged at 12000 $\times$g for 10 min at room temperature; 3 μl of the supernatant were loaded on cellulose acetate membranes (Cellogel, 17 $\times$ 17 cm) previously equilibrated with TEC 0.075 run buffer (Meera Khan, 1971) for horizontal electrophoresis. Runs were carried at constant voltage (220V) for 4 hr (GPI), or 5 (LDH), or 3 (MDH). Staining procedures were after Ayala *et al.* (1972; LDH), Meera Khan *et al.* (1982; MDH) and Van Someren *et al.* (1974; GPI).

Enzyme and locus nomenclature follows Shaklee *et al.* (1990) and Buth *et al.* (1991). At each locus, the commonest allele of *L. cephalus* was reported as 100, while the other alleles were given respective figures on the basis of the relative mobility of electromorphs, adding or substracting from the 100 figure the corresponding millimeters for faster or slower electromorphs, respectively. For polymorphic loci, the 99% criterion was adopted.

RESULTS

Glucosephosphate isomerase

As for the two *GPI** loci described in fishes, the commonest electrophoretic pattern observed in the adult specimens of *L. cephalus* is represented by three equidistant bands, whose relative staining intensity varies with the analyzed tissue. The heart electromorph is the result of the binomial combination of the *GPI-A** and *GPI-B** subunits nearly equally expressed, while in the eye a predominant activity of the former is found; in the white muscle the reverse is true. Moreover, the phenotype found in eye homogenates shows that the AB heterodimer is less evident than expected on the basis of the free assembly of the A and B subunits. Finally, in liver the GPI-A homodimeric band is the only one present, as it occurs in eggs and larvae (Fig. 1a).

The analysis of eye homogenates shows that both loci are polymorphic in the majority of species, but none codes for completely diagnostic alleles (Table 2). In detail, at the *GPI-A** locus *A. alburnus alborella*, *L. cephalus* and *L. lucumonis* are monomorphic for the same allele, which represents the most frequent one also in *L. souffia*, *R. erythrophthalmus* and *R. rubilio*. The two congeneric species of *Rutilus* share the same two alleles. *L. souffia* shows three alleles, two of them being shared with the *Rutilus* taxa and the third one with *Chondrostoma genei*, where it is present at a low frequency. On the other hand, the commonest allele of *C. genei* is clearly species-specific (Fig. 2a). Furthermore, *T. tinca* is monomorphic for the fastest allele also observed in *S. erythrophthalmus*, the latter species showing a second diagnostic allele in addition. Also *GPI-B** is polymorphic in all species but three, which show the same allele (*A. alburnus alborella*, *L. cephalus* and *S. erythrophthalmus*). In *L. lucumonis*, *L. souffia*, *C. genei*, *R. erythrophthalmus* and *T. tinca GPI-B** is diallelic, while in *R. rubilio* is triallelic. Moreover the commonest allele in the tench is species-specific, while *C. genei* and *L. souffia*, one side, and *L. lucumonis* and *R. erythrophthalmus*, the other, show the same allelic constitution. It is also interesting to note that

Table 1. Sampling localities of the analyzed species of Cyprinidae

Species	Abbr.	Sampling localities
Alburnus alburnus alborella	ALA	middle Reno river (Marzabotto, Bologna)
Chondrostoma genei	CHG	upper Reno river (Vergato, Bologna)
Leuciscus cephalus	LEC	upper Reno river (Vergato, Bologna)
Leuciscus lucumonis	LEL	upper Valle Tiberina (Umbértide, Perugia)
Leuciscus souffia	LES	upper Reno river (Vergato, Bologna)
Rutilus erythrophthalmus	RUE	upper Reno river (Vergato, Bologna)
Rutilus rubilio	RUR	upper Valle Tiberina (Umbértide, Perugia)
Scardinius erythrophthalmus	SCE	Emilia-Romagna canal (Bubano, Bologna)
Tinca tinca	TIT	Gavello (Modena)

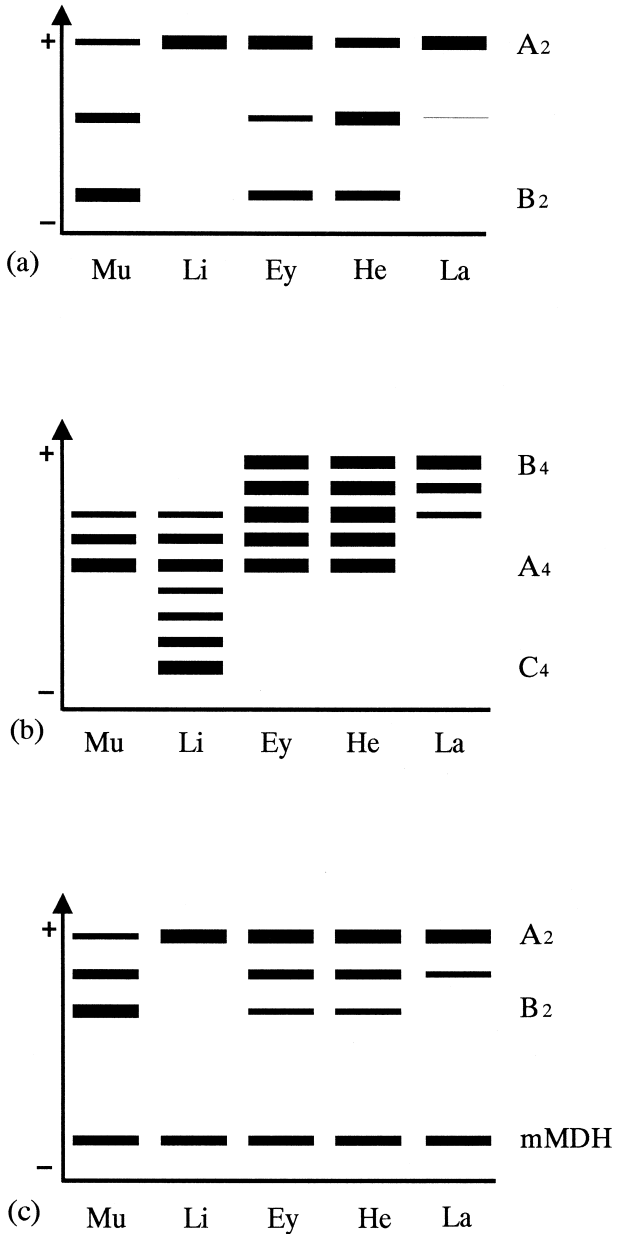

Fig. 1. Schematic representation of differential expression of GPI (**a**), LDH (**b**) and MDH (**c**) isozymes in adult tissues (Mu, white muscle; Li, liver; Ey, eye; He, heart) and larvae (La) of *L. cephalus*. Egg pattern is identical to the larval one. Differences in line thickness refer to different staining intensities.

R. rubilio differs from the congeneric species, *R. erythrophthalmus*, in having an additional allele (*GPI-B* 104*).

Lactate dehydrogenase

Tissue analysis in *L. cephalus* shows that eye and heart electrophoretic patterns consist of five isozymes derived by the contemporary expression of the two isoloci *LDH-A** and *LDH-B**, with a comparable homo- and heterotetrameric activity, except for the most anodal band, which is less intense in the heart. It should be noted that, when the run is prolonged, the central heterotetrameric band A_2B_2 appears to be split, as commonly observed in fishes, owing to the formation of conformational isomers (Frankel, 1987). In the muscle, the pattern is characterized by bands of decreasing staining intensity with the slowest one of the A_4 homopolymer more expressed; the most anodal isozymes could not be detected. In the liver we found the expression of a third locus (*LDH-C**), whose product is the slowest (Fig. 1b). It is important to note that the most intense bands in liver phenotypes are the homotetramer C_4 and A_4; the heterotetramers from the *LDH-C** and *LDH-A** loci are also expressed, but they show a decreasing intensity from the C_3A_1 to the C_1A_3 heteropolymer. Finally, the heteropolymer A_3B_1 and A_2B_2, with a decreasing intensity in this order, are the only evidence on the activity of *LDH-B**.

Eggs and larvae electromorphs in *L. cephalus* show a decreasing staining intensity from the most anodal bands, thus indicating that the *LDH-B** locus is the most active, while the A_4 homopolymer is clearly missing (Fig. 1b).

For population analysis, only the *LDH-A** and *LDH-B** were studied extensively in eye homogenates (Fig. 2b and Table 2). The former locus is monomorphic for the same allele (*LDH-A* 100*) in all species but *R. rubilio*. Only *T. tinca* can be discriminated on the basis of *LDH-A**. A quite different situation emerges from the *LDH-B** locus. *L. souffia*, *C. genei*, *R. rubilio* and *T. tinca*, one side, and *L. lucumonis* and *S. erythrophthalmus*, the other, show fixed alternative alleles. The commonest alleles of *R. erythrophthalmus* and of *A. alburnus alborella* and *L. cephalus* match those found in the former and in the latter group, respectively.

Malate dehydrogenase

Three loci are known to exist in fishes; the product from the mitochondrial locus (*mMDH**) forms a homodimer and never assembles with those from the two supernatant loci (*sMDH-A** and *sMDH-B**). On the contrary, the heterodimer is always formed when both *sMDH-A** and *sMDH-B** loci are active. The mitochondrial and the supernatant electrophoretic zone of MDH activity are therefore easily recognized.

The *mMDH** appears almost equally expressed in all analyzed adult tissues and in eggs and larvae of *L. cephalus*, whereas the products of the two cytoplasmic loci are differently expressed during the developmental stages and in the adult tissues. The only *sMDH-A** locus is expressed in the liver and it is also predominant in the eye and heart. Both *sMDH-A** and *sMDH-B** loci are expressed in the muscle, but the homodimeric activity of the latter is predominant. It should be noted that in developing eggs and larvae electromorphs expressing both supernatant genes are found; they show a clearcut difference in activity, no B_2 band being detectable (Fig. 1c).

The three *MDH** loci are monomorphic for the same allele in muscle homogenates of all analyzed species, with the exceptions of *T. tinca*, which has a fixed diagnostic allele at each *MDH** locus, and of *R. erythrophthalmus*, which is polymorphic at the *sMDH-B** locus with a low frequency allele (*sMDH-B* 86*) (Table 2).

Table 2. Allelic frequencies at the *GPI**, *LDH** and *MDH** loci in 9 cyprinid species. Abbreviations as in Table 1 (n° = sample size).

Locus Alleles	ALA	CHG	LEC	LEL	LES	RUE	RUR	SCE	TIT
*GPI-A**									
91	0	0.038	0	0	0.154	0	0	0	0
94	0	0.962	0	0	0	0	0	0	0
100	1	0	1	1	0.731	0.821	0.714	0	0
104	0	0	0	0	0.115	0.179	0.286	0	0
107	0	0	0	0	0	0	0	0.25	0
114	0	0	0	0	0	0	0	0.75	1
n°	11	13	12	15	13	28	14	12	36
*GPI-B**									
95	0	0	0	0.033	0	0.091	0.269	0	0
100	1	0.923	1	0.967	0.833	0.909	0.539	1	0.083
102	0	0	0	0	0	0	0	0	0.917
104	0	0	0	0	0	0	0.192	0	0
106	0	0.077	0	0	0.167	0	0	0	0
n°	12	13	14	15	15	33	13	13	36
*LDH-A**									
100	1	1	1	1	1	1	0.923	1	0
103	0	0	0	0	0	0	0.077	0	0
105	0	0	0	0	0	0	0	0	1
n°	14	14	17	9	13	33	13	13	35
*LDH-B**									
85	0	1	0	0	1	0.955	1	0	1
90	0	0	0.029	0	0	0	0	0	0
92	0	0	0	0	0	0.045	0	0	0
94	0.25	0	0	0	0	0	0	0	0
100	0.75	0	0.971	1	0	0	0	1	0
n°	14	14	17	9	13	33	13	13	35
*mMDH**									
98	0	0	0	0	0	0	0	0	1
100	1	1	1	1	1	1	1	1	0
n°	11	11	13	9	13	32	10	10	36
*sMDH-A**									
97	0	0	0	0	0	0	0	0	1
100	1	1	1	1	1	1	1	1	0
n°	11	11	13	9	13	32	10	10	36
*sMDH-B**									
86	0	0	0	0	0	0.047	0	0	0
97	0	0	0	0	0	0	0	0	1
100	1	1	1	1	1	0.953	1	1	0
n°	11	11	13	9	13	32	10	10	36

DISCUSSION

The presence of two *GPI**, three *LDH** and three *MDH** loci supports, for all taxa including *L. lucumonis* and *R. erythrophthalmus*, a diploid constitution in line with their karyotypic characterization (Fontana *et al.*, 1970; Cataudella *et al.*, 1977; Manaresi, 1996).

Present data in *L. cephalus* show that, as in other cyprinids and advanced teleosts, *GPI-A** is the only locus expressed during the earliest stages of development and significantly expressed in all tissues with the exception of the white muscle, where *GPI-B** predominates (Schmidtke *et al.*, 1975; Basaglia, 1989; Basaglia *et al.*,1990). The appearence of the subunits coded by the *GPI-B** locus seems to be delayed when compared to the condition described in other cypriniforms (Shaklee *et al.*, 1974), a noticeable level of AB isozyme being reached only some days after hatching. Our results support the observation that the onset of the developmental expression of orthologous loci can vary in related fish species (Philipp *et al.*, 1983).

At variance with literature data (Buth, 1984), we observed the expression of the only *GPI-A** locus in the liver of *L. cephalus* and the unbinomial GPI isozymes pattern in the eye of all species examined.

The predominant activity of the *LDH-B** locus during early developmental stage has been ascertained in other fishes and in other vertebrate classes (Shaklee *et al.*, 1974), the only exception so far evidenced being two *Micropterus* species

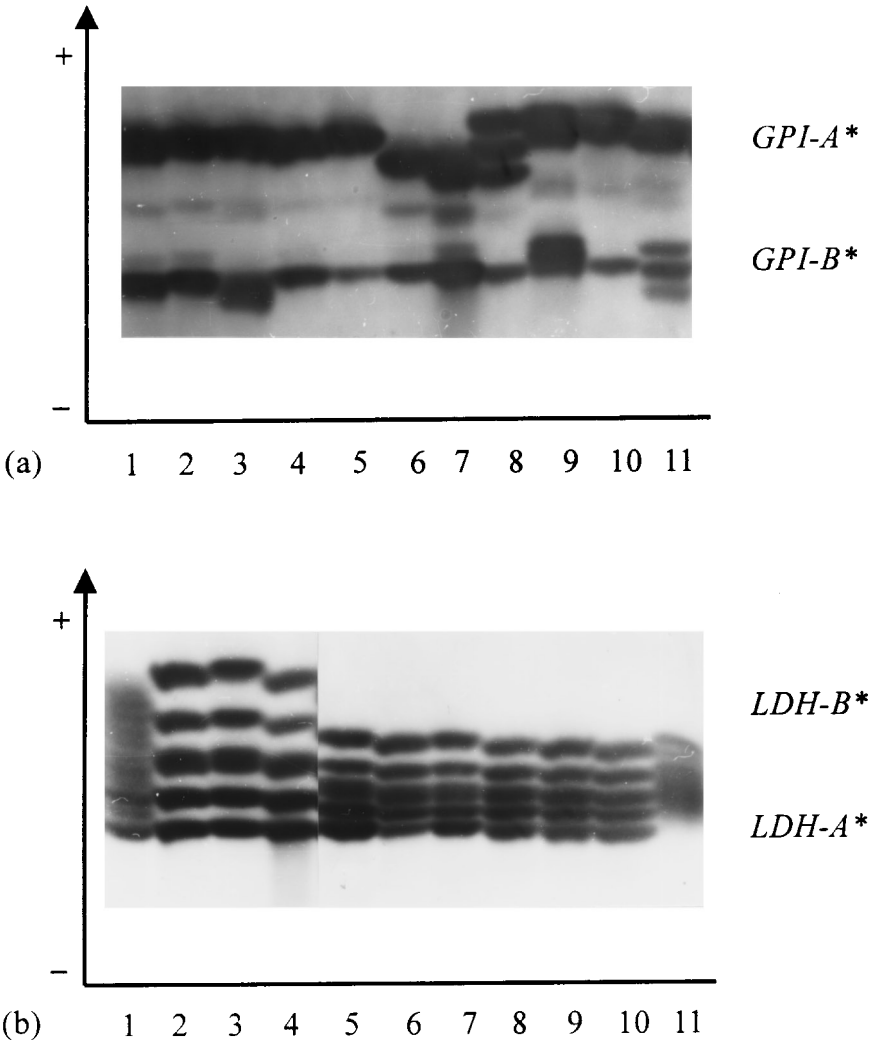

Fig. 2. GPI (**a**) and LDH (**b**) multilocus enzyme expression in eye. (**a**) 1-2, ALA; 3, LEL; 4-5, LEC; 6-7, CHG; 8-9, LES; 10, RUE; 11, RUR; all electromorphs are *GPI-A* 100/100* and *GPI-B* 100/100*, with the exception of specimens 3 (*GPI-B* 95/100*), 6 (*GPI-A* 94/94*), 7 (*GPI-A* 91/94*), 8 (*GPI-A* 91/104*), 9 (*GPI-A* 100/104* and *GPI-B* 100/106*), 10 (*GPI-A* 100/104*), 11 (*GPI-B* 95/104*). (**b**) 1, ALA; 2, LEC; 3, LEL; 4, SCE; 5-6, LES; 7, CHG; 8-9, RUE; 10-11, RUR; all electromorphs are *LDH-A* 100/100* and *LDH-B* 100/100*, with the exception of specimens 1 (*LDH-B* 94/100*); 5-10 (*LDH-B* 85/85*) and 11 (*LDH-A* 100/103* and LDH-B* *85/85*). Abbreviations as in Table 1.

(Philipp *et al*., 1979). The presence of the heterotetrameric bands indicates that in larvae of *L. cephalus*, the gene product of *LDH-A** locus is also expressed during early ontogenetic stages, even if to a low extent. The appearence of the homotetramer A_4 could be related to the differentiation of the muscle cells (Frankel, 1987; Basaglia, 1989). A correlation between isozyme patterns and morphogenetic events, leading to the differentiation of specific tissues and organs, is also suggested by present observation concerning the lack of LDH-C isozymes during the early stages of development and their expression in the adult liver of *L. cephalus* (Shaklee *et al*., 1974; Basaglia, 1989).

The tissue-specific expression of LDH loci in adults of *L. cephalus* supports the existence of the cyprinid liver-specific *LDH-C** locus, with the slowest migrating C_4 homotetramer (Shaklee *et al*., 1974, Frankel, 1987; Basaglia, 1989). While the simultaneous presence of A, B and C subunits in liver extracts is not unusual in the family Cyprinidae (Frankel, 1987; Basaglia, 1989), it should be noted that *LDH-C** appears here the most active, followed by *LDH-A** and *LDH-B**. The non-binomial staining intensity of the C and A heterotetrames is probably due to the instability of the A-rich heterotetramers in the hepatic tissue, as in AB heterotetramers in the liver of other teleosts (Basaglia, 1989).

The presence of A_3B_1 and A_2B_2 heterotetramers in the white skeletal muscle of *A. alburnus alborella* and *S. erythrophthalmus* significantly differs from literature data (Basaglia, 1989). Moreover, differently from several cyprinids (Rainboth and Whitt, 1974; Frankel, 1987), all species examined completely lack the fastest muscle isozymes. The polymorphism found at *LDH-B** in *A. alburnus alborella* well agrees with literature data (Callegarini and Basaglia, 1982), whereas in *T. tinca* we did not found any variation at *LDH-A** locus (Basaglia and Callegarini, 1981). The lower variability of *LDH-*

*A** locus appears to be a general feature of cyprinids and teleosts (Rainboth and Whitt, 1974).

Comparing the expression patterns of the *sMDH-A** and *sMDH-B** loci with those in literature data, the A_2 homodimer is more anodal (and consequently the fastest MDH form) than the B_2 band in this study and vice versa in literature. Otherwise, the MDH patterns observed during development and in adult tissues well agree with previous data on cypriniformes and other teleosts (Philipp *et al.*,1979; Buth, 1984; Basaglia, 1989), with the exception of the liver in which only the activity of the *sMDH-A** locus is evident. A further interesting finding is the existence of the AB heterodimer in *L. cephalus* eggs a few hours after fertilization, indicating a very early ontogenetic expression of the *sMDH-B** locus. This is an additional example of variation in the timing of expression of homologous loci among different fish species (Philipp *et al.*, 1983).

It is evident that the three *MDH** gene loci are evolutionally more conservative than *GPI** and *LDH** loci, 8 species being virtually identical and only *T. tinca* showing a diagnostic allele at each locus. Intraspecific polymorphism is also very low, *R. erythrophthalmus* only being diallelic. *T. tinca* is well separated from other species at the *LDH-A** locus as well. On the other hand, the *LDH-B** locus defines two groups: the former, sharing the fastest migrating allele, includes *A. alburnus alborella*, *L. cephalus*, *L. lucumonis* and *S. erythrophthalmus*, while the latter, with the slowest migrating allele, is formed by *C. genei*, *L. souffia*, *R. rubilio*, *R. erythrophthalmus* and *T. tinca*. Considering present data together, the tench is clearly differentiated from the other species, supporting the hypothesis that *T. tinca* should be considered taxonomically apart from the other cyprinid species belonging to the subfamily Leuciscinae (Kryzhanovskii, 1947; Bogutskaya, 1986; Banarescu and Coad, 1991). On the contrary, *A. alburnus alborella*, which is assigned to the subfamily Alburninae by some authors (Howes, 1991), is not so different from Leuciscinae species by preliminary pairwise comparison of present data. In particular, *L. cephalus* and *L. lucumonis* appear very similar to this species. From present analysis, therefore, the retention of *A. alburnus alborella* in the subfamily Leuciscinae seems reliable, in accordance with Banarescu and Coad (1991).

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