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Implication of Tissue Expression of Lactate Dehydrogenase-C Gene in Phylogenetic Study of Euteleosts

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Abstract An electrophoretic investigation of the lactate dehydrogenase isozymes in twelve euteleostean species was conducted. Expression of the LDH-C locus and association of the A and B subunits in these fishes is discussed. In *Chanos chanos* this locus is found prevalent in the liver suggesting a close relation to otophysans. Presence of four iso-spaced A-B polymers in this species is a character different from otophysans which are provided with five iso-spaced A-B tetramers. Absence of tissue specificity of C4 band in all holocentrid species suggests a possible primitive phylogenetic status of this family in the Beryciformes. However, expression of the LDH-C locus provides no strong evidence for the resolution of the phylogenetic positions of the Polymixiidae and the other groups examined.

New techniques such as chromosomal analysis, DNA hybridization, DNA sequencing, amino acid sequencing and protein electrophoresis have made it possible for systematists to utilize new sets of data for phylogenetic studies (e.g., Duellman, 1985). Early studies of biochemical characters such as serum, muscle and lens proteins are well known in the areas of genetic variation and species identification (Eckroat and Wright, 1969; Feeney and Brown, 1974; Tsuyuki, 1974). However, the locus identification of these proteins is still not precise and the homologous loci among them are hard to define.

Recently, however, the research development of isozymes shows some promising results of locus identification. One of the most well documented mutilocus isozymes systems has been illustrated by the lactate dehydrogenase (Whitt et al., 1973; Markert et al., 1975). It is an isozyme system having a multigenic nature with well defined loci among the evolution of vertebrates and thus becomes an interesting model for evolutionary studies.

Lactate dehydrogenase (Lactate: NAD-oxidoreductase, ECl. 1.1.27) which has a tetrameric structure and a molecular weight of 140,000 (Darnall and Klotz, 1975) catalyzes the interconversion of lactate and pyruvate. The LDH of most higher vertebrates exists as five molecular forms resulting from the apparently random polymerization of two genetically and biochemically distinct subunits, A and B, in all possible combinations, finally yielding the following tetramers: A4, A3B, A2B2, AB3, and B4 (Cahn et al., 1962; Markert, 1963, 1968). Analysis of the vertebrate enzymes using a variety of physical kinetic, and immunochemical techniques has indicated that all LDH-A subunits are closely homologous, and, similarly, that all LDH-B subunits are closely homologous, while between the A and B subunits only a distant homology exists (Pesce et al., 1967; Bailey and Wilson, 1968; Holmes and Markert, 1969; Whitt, 1969). Generally speaking, LDH in fish is similar to the basic tetrameric structure found in other higher vertebrates. From thermostability, electrophoresis, substrate inhibition and immunochemical data, the A and B subunits of fishes appear to be homologous with the muscle subunit (M4) and heart subunit (H4), respectively, in higher vertebrates (Pesce et al., 1967; Bailey and Wilson, 1968).

The phylogenetic survey of LDH genes in fishes has been carried out by many workers. According to the early investigators, biochemical, immunological and genetic analyses of species in the major line of actinopterygians reveal the presence of three genes (A, B, C). Generally, LDH-A4 isozyme is present in tissues subjected to periods of relative anaerobiosis, e.g., skeletal muscle (Cahn et al., 1962; Dawson et al., 1964); the LDH-B4 isozyme is predominant in tissues receiving a constant supply of oxygen (heart and brain; Whitt,

1970), and it exhibits substantial activity in most other tissues.

Detailed biochemical and immunological studies have shown the similar properties of LDH-C4 isozyme to LDH-B4 in actinopterygians (Markert and Holmes, 1969; Whitt, 1970; Shaklee et al., 1973). The tissue distribution property of LDH-C4 isozyme is very similar to that of LDH-B4 in most primitive actinopterygians. The LDH-C4 appears in a variety of tissues. Whitt (1969, 1970) suggested that the LDH-C locus is clearly the result of a duplication of the LDH-B locus. It is believed that most primitive actinopterygians (i.e., Acipenseriformes, Anguilliformes, etc.) reveal their LDH-C4 isozyme in many tissues, e.g., heart, liver, eye, kidney, gill, etc., but it is quite restricted in the most advanced teleosts (Shaklee et al., 1973; Markert et al., 1975). Also, investigations employing immunological, genetic, and phylogenetic approaches have demonstrated that the eye-band LDH in many perciforms encoded in the same basic locus, even though the isozymic products have somewhat different properties in these fishes (Sensabough and Kaplan, 1972; Whitt et al., 1973; Shaklee et al., 1973; Markert et al., 1975).

Markert et al. (1975), Shaklee and Whitt (1981), Buth (1984), Coopes (1984), and Kettler and Whitt (1986) emphasized the phylogenetic trends of the properties and distribution of the LDH isozymes. As these isozymes are subjected to thorough studies much more than other fish isozymes and information about these isozymes in most euteleosts remains lacking, it is one of our attempts to examine the properties and expression of these isozymes in euteleosts so that variation in these isozyme characteristics may assist us to elucidate their phylogenies.

Materials and methods

Fresh specimens of twelve euteleostean species (Table 1) were obtained from the fish markets and local aquariums. All specimens used for LDH isozyme analysis were stored frozen at −20°C before use. Ten different organs, i.e., brain, eye, gill, heart, gonad, kidney, liver, muscle, spleen, and stomach, were dissected and homogenized in two volumes of 0.1 M Tris-HCl with additional 0.001 M B-mercaptoethanol, pH 7.0 at 4°C. The homogenates were then centrifuged at 45,000×g for 45 minutes at 4°C. Extracts from the skeletal muscle, although labelled simply "muscle", are in fact extracts from white muscle containing as little red muscle as possible.

Horizontal starch gel electrophoresis was performed at 4°C for 4–8 hours in a 13% gel at 165 to 260 V. Electrophoretic buffer was Tris-citrate pH 7.0 buffer (Tsoi et al., 1987). After the electrophoresis, gels were sliced and stained according to Shaklee et al. (1973). Nomenclature of LDH isozymes, subunits, and structural genes were made in accordance to Shaklee et al. (1973). After electrophoresis, the LDH tissue distribution isozymic patterns were used to identify three LDH-A, -B, and -C loci.

Results

The zymograms of twelve euteleosts are shown in Figs. 1–8 and the characteristics of the lactate dehydrogenase isozymes are summarized in Table 1.

Each of the species studied from the different euteleost orders possesses the same three LDH loci (i.e., A, B, and C)—characteristic of most bony fishes. In all of the species examined in this study, C4 was the most anodal isozyme (Figs. 1–8, Table 1). *Chanos chanos* (Gonorynchiformes) differs from the other otophysans in the mobility of its C4 isozyme (Fig. 1); this species shows an anodal C4 isozyme in contrast to most of the cyprinids and gadids in which the C4 isozyme exhibits a relative cathodal electrophoretic mobility (Shaklee et al., 1973; Shaklee and Whitt, 1981). In Chanidae, Holocentridae, Polymixiidae, Scorpaenidae, Channidae, and Anabantidae, the A4 band shows the most cathodal electrophoretic mobility; whereas in Monocentridae, Anomalopidae, Caproidae, Dactylopteridae, Synbranchidae, and Mastacenbelidae, it is the B4 band which exhibits such a mobility (Table 1).

In all the species studied the A4 homopolymer predominated in white skeletal muscle (Figs. 1–8) while the B4 homopolymer predominated in heart, brain and stomach (Figs. 1–8). In the monocentrid, anamalopid, dactylopterid, scorpaenid, channid, synbranchid and anabantid the C4 homopolymer is primarily restricted to neural tissues such as the brain and eye (Figs. 1–8, Table 1). However, the C4 isozyme was detectable in several other tissues as well in holocentrid species (also see Tsoi et al., 1987). *Polymixia berndti* and

Antigonia rubescens (Figs. 3, 5, Table 1). It is interesting to note that the C4 isozyme in *Chanos chanos* is detectable only in the liver (Fig. 1)—a derived condition exhibited in otophysans and gadiforms (see Shaklee et al., 1973; Shaklee and Whitt, 1981).

Numbers of A-B tetramer in the euteleosts examined ranged from 2 to 4 (Table 1). *Chanos chanos* is unusual among these species in exhibiting a higher number of A-B tetramer (4). Holocentrid, polymixiid, caproid, synbranchid have three A-B tetramers, while monocentrid, anomalopid, dactylopterid, scorpaenid, channid, and anabantid have only two A-B tetramers (i.e., more restrictive in the association of the A and B subunits) (Table 1).

Discussion

Tissue restricted expression of the LDH-C locus

Table 1. Characteristics of euteleost lactate dehydrogenase isozymes. The numbers in parentheses indicate the number of specimens examined. RAM, relative anodal mobility. Relative quantities of LDH-C subunits: +++, most abundant; ++ and +, intermediately abundant; ±, marginally present; −, undetectable; blank, tissue not examined. Examined tissues: B, brain; E, eye; G, gill; Go, gonad; H, heart; K, kidney; L, liver; M, muscle; Sp, spleen; St, stomach.

Fish species	A-B tetramers (No.)	RAM	Expression of the LDH-C locus									
			B	E	G	Go	H	K	L	M	Sp	St
Order Gonorynchiformes												
Family Chanidae												
Chanos chanos (3)	4	C>B>A	−	−	−	−	−	−	++	−	−	−
Order Beryciformes												
Family Monocentridae												
Monocentris japonica (4)	2	C>A>B	+	+++	−	−	−	−	−	−	−	−
Family Anomalopidae												
Anomalops katoptron (1)	2	C>A>B	+	+++	−	−	−	−	−	−	−	−
Family Holocentridae												
Sargocentron caudimaculatum (1)	3	C>B>A	+	+++	±		++	++	+	±		++
Order Polymixiiformes												
Family Polymixiidae												
Polymixia berndti (2)	3	C>B>A	++	+++	±		++	++	+	±		++
Order Zeiformes												
Family Caproidae												
Antigonia rubescens (1)	3	C>A>B	++	+++		±	+++		++	−		±
Order Dactylopteriformes												
Family Dactylopteridae												
Dactyloptena orientalis (1)	2	C>A>B		+++	±		−	−	−	−	−	±
Order Scorpaeniformes												
Family Scorpaenidae												
Neosebastes entaxis (1)	2	C>B>A		+++	−		±	−	−	−	−	−
Order Synbranchiformes												
Family Synbranchidae												
Fluta alba (1)	3	C>A>B	+	−		−	−	−	−	−		−
Order Perciformes												
Family Channidae												
Channa micropeltes (1)	2	C>B>A		+++	−		±		−	−	−	−
Family Anabantidae												
Ctenopoma kingsleyae (1)	2	C>B>A		+++	−	−	±	−	−	−	−	−
Family Mastacembelidae												
Mastacembelus erythrotaenia (1)	3	C>A>B	+	+++	−		±	−	−	±		±

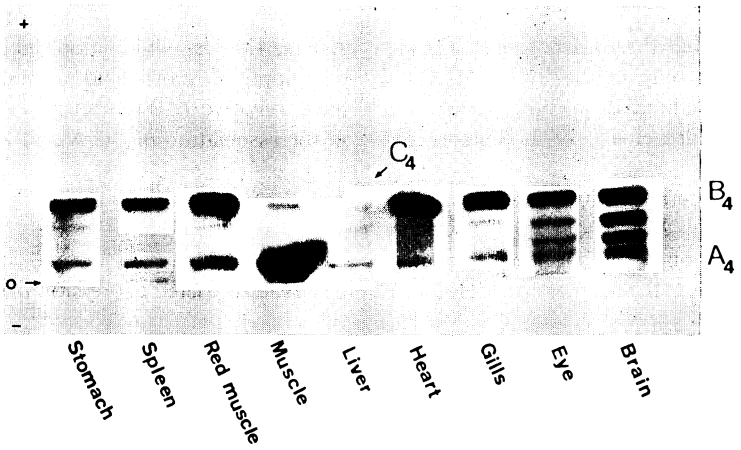

Fig. 1. LDH isozymes of the milkfish (*Chanos chanos*) (Gonorynchiformes, Gonorynchidae).

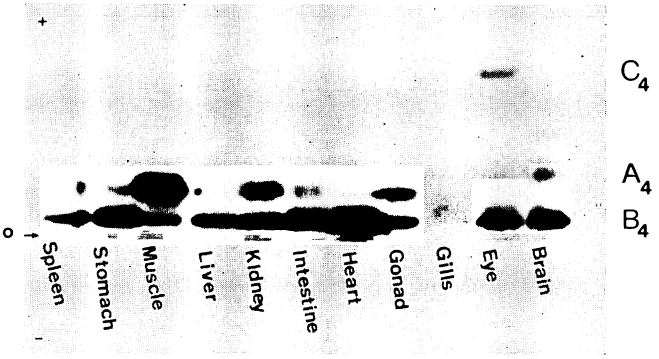

Fig. 2. LDH isozymes of the pine-cone fish (*Monocentris japonica*) (Beryciformes, Monocentridae).

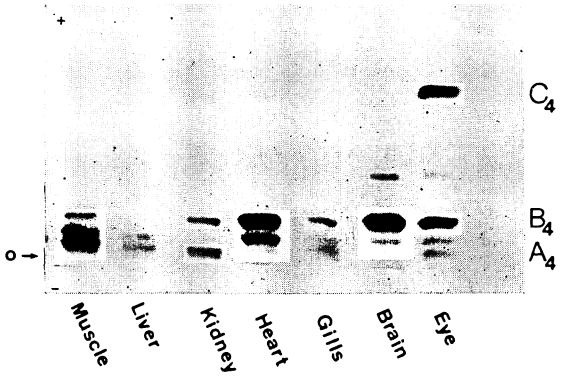

Fig. 3. LDH isozymes of the squirrelfish (*Sargocentron caudimaculatum*) (Beryciformes, Holocentridae). The C isozyme is observed in nearly all tissues with high activities in exracts from the eye, heart and kidney.

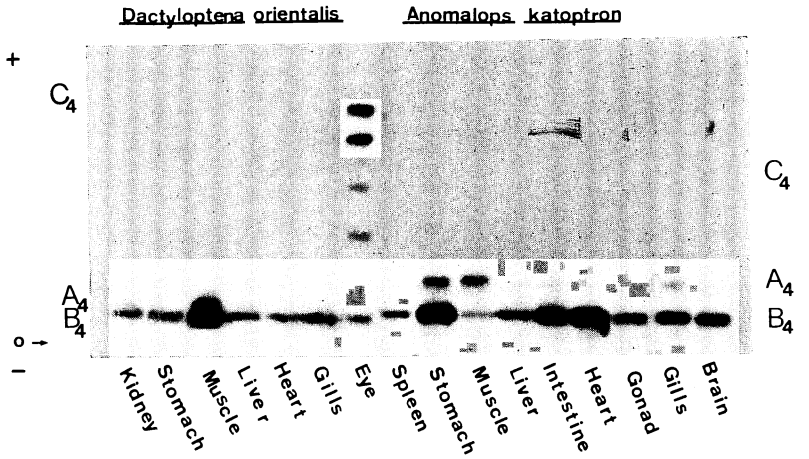

Fig. 4. LDH isozymes of the lanterneye (*Anomalops katoptron*) (Beryciformes, Anomalopidae) on the right and the flying gurnard (*Dactyloptena orientalis*) (Dactylopteriformes, Dactylopteridae) on the left. Note the unique highly-anodal isozymes in eye extracts.

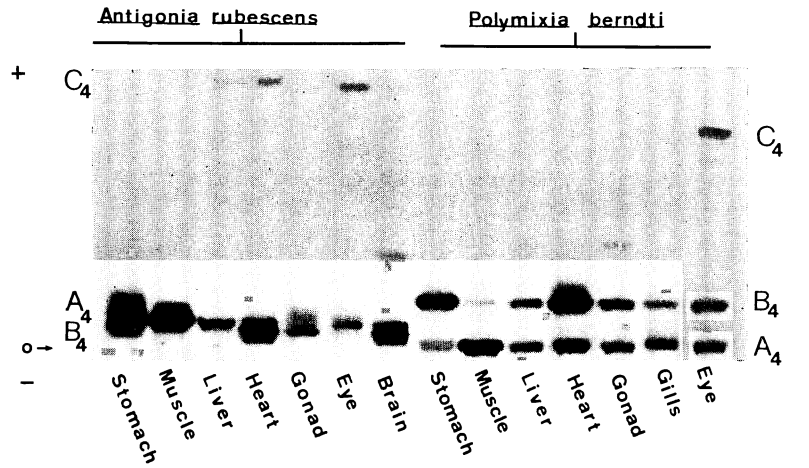

Fig. 5. LDH isozymes of the beardfish (*Polymixia berndti*) (Polymixiiformes, Polymixiidae) on the right and the boarfish (*Antigonia rubescens*) (Zeiformes, Caproidae) on the left.

takes two forms. One is predominating in the eye and/or brain and the other in the liver. The first form has been noted in clupeiforms and acanthopterygians, while the second form has been documented primarily in otophysans and gadiforms (Markert et al., 1975; Shaklee et al., 1981). Winans (1980) reported two loci for the gonorynchiform *Chanos chanos* lactate dehydrogenase isozymes in muscle, heart, liver and intestine. However, our zymogram of this species reveals a LDH-C isozyme in liver with a more anodal electrophoretic mobility. Gosline (1973) adopted the order Cypriniformes to include most of the ostariophysans except the Gonorynchiformes which he had placed under the Clupeiformes. His conclusion on the phylogenetic position of his order Cypriniformes was that it is related to the gonorynchoids and clupeoids, and possibly closer to the former. Rosen (1973) treated the Ostariophysi (including the Gonorynchiformes) as the most primitive euteleostean group. Fink and Weitzman (1982), on the other hand, noted that

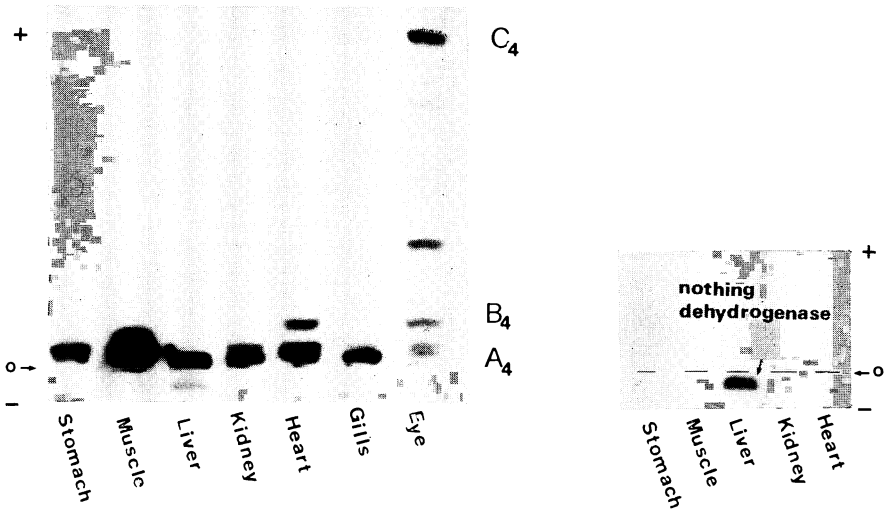

Fig. 6. LDH and "nothing dehydrogenase" isozymes of the scorpionfish (*Neosebastes entaxis*) (Scorpaeniformes, Scorpaenidae). Right side: gel stained for "nothing dehydrogenase" without substract lithium-DL-lactate.

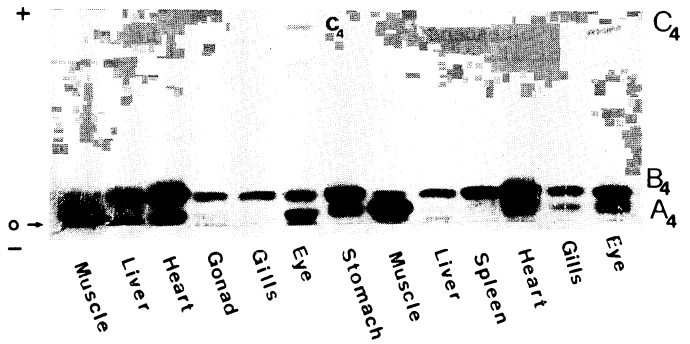

Fig. 7. LDH isozymes of the snakehead (*Channa micropeltes*) (Perciformes, Channidae) on the right side and the climbing gouramy (*Ctenopoma kingsleyae*) (Perciformes, Anabantidae) on the left side.

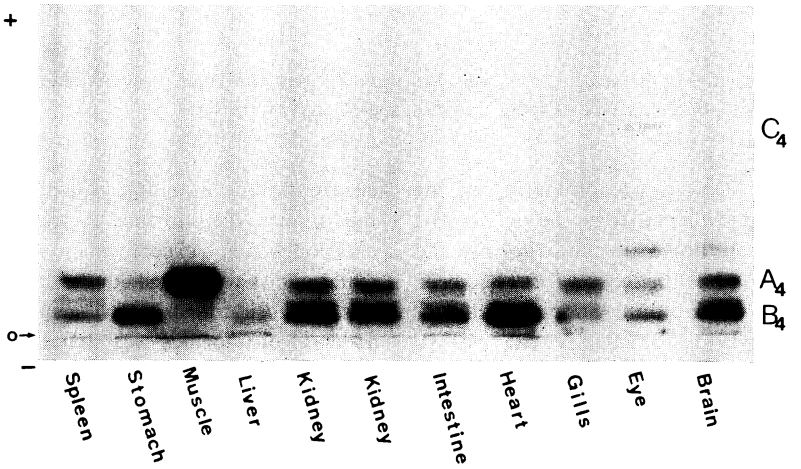

Fig. 8. LDH isozymes of the spiny eel (*Mastacembelus erythrotaenia*) (Perciformes, Mastacembelidae).

both ostariophysan and all non-esocoid euteleosts lack a toothplate over the fourth basibranchial. This evidence has led to a lower euteleostean status for esocoids and the Ostariophysi affiliated to either (1) Argentinoidei+Osmeridei, (2) Salmonidae, (3) Neoteleostei including the Stomiiformes, Aulopiformes, Myctophiformes, and Acanthomorpha (Fink and Weitzman, 1982). Though data of the LDH in other gonorynchiforms (i.e., gonorynchids and knerioids) are lacking, restriction of the LDH-C locus to the liver in chanids is evidence to (1) support the hypothesis that the Anotophysi and Otophysi are sister groups and (2) falsify the monophyly of the Gonorynchiformes and Clupeiformes.

Degree of tissue restriction and the tissue(s) in which the C homopolymer is predominant could vary within a single family. Take the Umbridae as an example (Kettler and Whitt, 1986), congeneric *Umbra* species, i.e., *Umbra limi* and *U. pygmaes*, share a character state that C homopolymer was highly restricted and was solely detectable in the brain and eye. It was present in almost all the examined tissues (i.e., brain, eye, heart, muscle, and stomach) in *Dallia pectoralis* and *Novumbra hubbsi*, but it was predominant in the eye and liver.

Similarly, expression of the C locus follows at least two evolutionary trends in the Paracanthopterygii (including the Gadiformes, Percopsiformes, and Batrachoidiformes); LDH-C locus is predominant in the liver in gadiform, while it is predominant in neural tissues in percopsiform. Data on the batrachoidiform LDH, however, is not available.

Information on the tissue-restricted expression of the LDH-C locus in Percopsiformes and Acanthopterygii is still limited (e.g., Markert et al., 1975; Coopes, 1984). However, on the basis of the available data, it is observable that there are scattered species in these acanthopterygian groups where tissue-restricted expression of the LDH-C locus does not take place—a character state resembling the lower teleosts (e.g., osteoglossiforms and elopiforms; Markert et al., 1975). This character state has been suggested by previous researchers as a character state associated with the primitive member of a group (e.g., Kettler and Whitt, 1986). Resemblance in the LDH-C locus expression in Ostariophysi-Gadiformes and Clupeiformes-Percopsiformes-Acanthopterygii may either be convergent or synapomorphic. In view of the distribution of the LDH-C locus in euteleosts (Markert et al., 1975; Table 1), it is more plausible that the common ancestors along the evolutionary lineage in the Teleostei should have the C locus broadly expressed in various tissues (at least in eye, brain and liver) and from these ancestors various descendants including those with this locus predominating in eye-brain and liver were diverged. Though either a broad or restricted expression of this locus may be treated as a primitive character state for some acanthopterygian groups (e.g., Perciformes), the primitive status of a broad-tissue expression may be more possible because the LDH-C locus in most members of these groups occur solely in the eye or brain.

Rosen and Patterson (1969) suggested that polymixioids are the closest relatives of the paracanthopterygians and erected a new series Polymixiomorpha in the superorder Paracanthopterygii largely on the basis of the similarities in the caudal skeleton. This view was later rejected by Rosen (1973) and Fraser (1972). These authors placed it under the Beryciformes as did Berg (1940), Greenwood et al. (1966), Gosline (1971), Woods and Sonoda (1973), and Zehren (1975). Zehren (1975) treated the Beryciformes as a monophyletic group in which Polymixiidae is not included. Monophyly of the Beryciformes in which Polymixiidae is excluded is supported by pelvic bone morphology (personal data to be published elsewhere). A broad tissue expression of the LDH-C locus—a generalized teleost character state—while occurring in the small family Polymixiidae (one living genus with five species; Nelson, 1984) is not evident to neither falsify the validity of the ordinal status nor the membership in Beryciformes of the Polymixiidae. It is, however, plausible that this family is a rather primitive euteleostean group if the remainders of the Polymixiidae also show this generalized character state of the LDH-C locus.

In almost all acanthopterygians studied, only a few in a particular group (e.g., a family) retain the primitive LDH-C locus expression (i.e., detectable in many tissues), while the majority show significant tissue restriction. Holocentridae is an exceptional family; LDH-C locus in all the species studied by Tsoi et al. (1987) was not restricted. The LDH characteristic suggests a possible primitive position of this family in the Beryciformes. Monocen-

tridae and Anomalopidae are derived beryciform families in having restricted-tissue expression of the LDH-C locus (Table 1).

Restricted tissue expression of the C locus takes place in caproid (*Antigonia rubescens*), Dactylopteriformes, Scorpaeniformes, Synbranchiformes and most Perciformes (Table 1). Since this character state is plesiomorphic for Percomorpha, such information does not help resolve the phylogenetic relationships of these groups.

Markert et al. (1975) noted that though many fishes do exhibit unrestricted association of A and B subunits of the LDH, the majorities do not. These authors assumed that the intersubunit binding sites of the A and B subunits have changed during evolution and this structural change leads to the prevention of certain specific subunit associations, i.e., reduction in number of tetramers. In other words, a low number of tetramers indicates an evolutionary advancement. According to the data of Markert et al. (1975), only in Squaliformes, Acipenseriformes, Elopiformes, and Cypriniformes that unrestricted combination of the A and B subunits (i.e., higher number of tetramers) is a generalized character for the group. Occurrence of this generallized character state in *Chanos chanos* does not cause character conflict to the hypothesis of its close relationship to the Cypriniformes.

Acknowledgments

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乳酸脱水素酵素C遺伝子組織特異性の真骨類系統学的研究における意義

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真骨類 12 種の乳酸脱水素酵素 (LDH) を電気泳動法により分析した．これらの魚種における LDH-C 遺伝子座の発現組織，サブユニット A および B の結合性について論議した．サバヒーでは，LDH-C 遺伝子座は肝臓で特異的に発現し，コイ目との近縁性が示唆された．サバヒーの A-B ポリマーが等間隔の 4 本バンドとして発現することは等間隔の 5 本バンドを形成するコイ目と異なる特徴である．また，イットウダイ科魚類の C4 バンドは組織特異性が弱いという事実は，これらがキンメダイ目のなかで原始的位置関係にあることを示唆している．しかし，C4 バンドの出現状態はギンメダイ科とキンメダイ目のその他のグループの系統的位置関係を解明するための強力な証拠を与えなかった．