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Expression of LDH-C Isozyme among Lizard Taxa: Evolutionary Implications for the Vertebrate Lactate Dehydrogenase Gene Family

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Chien-Hsien Kuo, San Kao, Ching-Feng Weng and Sin-Che Lee (1999) Expression of LDH-C isozyme among lizard taxa: evolutionary implications for the vertebrate lactate dehydrogenase gene family. *Zoological Studies* **38**(3): 344-349. In order to more completely understand the complete basis for the multiple LDH isozymes in lizards, seven species of Taiwanese lizards belonging to 4 families and 7 genera were sampled. Starch gel electrophoretic patterns of lizard LDH isozymes from brain, eye, heart, liver, muscle, and testis were analyzed. Like all other vertebrates, lizards possess the 2 fundamental LDH loci—A and B. A 3rd locus, LDH-C, was detected only in testes of *Hemidactylus frenatus, Eumeces elegans*, and *Mabuya longicaudata*. This testis-specific product, designated according to tissue-specific expression exhibited, a faster anodal migration than did the other LDH isozymes. These findings suggest that the testis-specific LDH isozyme was derived from ancestor amniote LDH-A.

Key words: Lizard, Lactate dehydrogenase, Evolution, Amniote.

Isozymes are multiple molecular forms of enzymes (Markert and Möller 1959). Their biological significance and function have proved to be very important in research on biochemical and genetic mechanisms during the development and evolution of vertebrates (Markert 1983). The L-lactate dehydrogenase (LDH, EC 1.1.1.27) isozyme system is one of the most extensively studied models used to investigate the origin and evolution of isozymes and regulation of multigene families (Holmes 1972, Markert et al. 1975, Li 1990). Of the 2 isozymes present in almost all vertebrate species examined, the LDH-A isozyme is better known for pyruvate reduction in anaerobic tissues (muscle), whereas LDH-B is better for L-lactate oxidation in aerobic tissues (heart and brain) (Holbrook et al. 1975, Markert et al. 1975). An additional locus encoding an isozyme with more variable kinetic properties (LDH-C) is expressed in a variety of tissues in vertebrates. In lower teleost fish (Acipenseriformes, Amiiformes, Anguilliformes, etc.), the 3rd LDH has a generalized tissue distribution, but in advanced teleost fish (Salmoniformes, Myctophiformes, Perciformes, etc.), it is found either in liver (e.g., cod) or in the eye (e.g., salmon) (Almeida-Val and Val 1993). In mammals and columbid birds, a 3rd LDH isozyme is expressed in mature testes (Matson 1989, Wheat and Goldberg 1983). However, only the LDH-A and LDH-B isozymes are present in other birds (Matson 1989). Surprisingly, there were no reports related to the 3rd LDH in reptiles (Mannen et al. 1997). The major group of amniotes diverged from a common ancestor during a short period about 250-300 Mya (Laurin and Reisz 1995). The classical phylogenetic relationships among the amniotes based on paleontological and morphological evidence (Carroll 1987) have recently been judged using 18S and 28S rRNA genes and protein sequences (Hedges et al. 1990, Eemisse and Kluge 1993). Nevertheless, evolutionary relationships among these LDH isozymes of vertebrates have not been completely resolved.

Here, we report on the expression of LDH-C isozyme in testes of some Taiwanese lizard species. We also discuss the evolution of vertebrate LDH isozymes.

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MATERIALS AND METHODS

Seventeen individuals of 7 lizard species, belonging to 4 families and 7 genera, were tested in this study: *Hemidactylus frenatus, Japalura mitsukurii, Takydromus formosanus, Eumeces elegans, Mabuya longicaudata, Sphenomorphus indicus*, and *Scincella formosensis*. The brain, eye, heart, liver, skeletal muscle, and gonad (testes or ovaries) of each individual were dissected and homogenized separately in 2 volumes of chilled Tris buffer (0.1 mM Tris-HCL, pH 7.0, with 1 mM EDTA and 0.05 mM NADP). Homogenates were centrifuged at 13 500 g for 40 min at 4 °C. Supernatants were stored at –70 °C until electrophoresis. A 12% (w/v) starch gel was used to perform the isozyme assay. The Tris-citrate pH 8.0 buffer system or Tris-citrate/borate, pH 7.7 ("Poulik") buffer system (Selander 1971) was used and run at 14 V/cm for 5-6 h at 4 °C. After electrophoresis, the gel was sliced into several thin layers and stained with LDH-specific enzyme following Shaklee et al. (1973).

RESULTS

Electrophoretic patterns of LDH isozymes scored from brain, eye, heart, liver, muscle, and testis of the lizard, *Hemidactylus frenatus* were analyzed by tissue-specific scanning (Fig.1). As shown in table 1, tissues of lizard, like those of virtually all other vertebrates, possess 2 fundamental LDH loci–A and B. In skeletal muscle, a major band with the slowest mobility toward the cathode was likely to be the homotetrameric LDH-A_4 isozyme. In heart, the predominant band with the fastest mobility toward the anode was likely to be the homotetrameric LDH-B_4 isozyme. The number and distribution of LDHs in the tissues of lizards indicated the main isozymes: LDH-A_4 and LDH-B_4. Three other bands

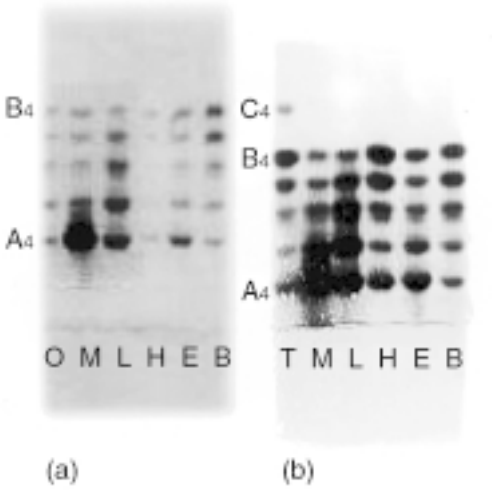

Fig. 1. Lactate dehydrogenase isozymes of *Hemidactylus frenatus*. A_4 predominates in skeletal muscle, B_4 in heart, and C_4 in testes. Note that the C_4 homopolymer is highly anodal and was only detected in testes. (a) Gel run using extracts of female lizard. (b) Gel run using extracts of male lizard.
B, brain; E, eye; H, heart; L, liver; M, skeletal muscle; O, ovary; T, testis.

Table 1. Characteristics of lizard lactate dehydrogenase isozymes. Numbers in parentheses indicate the number of specimens examined

Species		Snout-vent Length (mm)	Collection month	A-B Tetramers (N)	RAM	Expression of the LDH-C locus						
						B	E	H	L	M	O	T
Family Gekkonidae												
Hemidactylus frenatus	♂ (2)		June	3	C > B > A	–	–	–	–	–		±
	♀ (2)					–	–	–	–	–	–	
Family Agamidae												
Japalura mitsukurii	♂ (1)		June	4	B > A	–	–	–	–	–		–
	♀ (2)					–	–	–	–	–	–	
Family Lacertidae												
Takydromus formosanus	♂ (2)		Mar.	1	B > A	–	–	–	–	–		–
Family Scincidae												
Eumeces elegans	♂ (3)		Mar.	2	C > B > A	–	–	–	–	–		+
Mabuya longicaudata	♂ (2)	95	Jan.	2	C > B > A	–	–	–	–	–		+
Sphenomorphus indicus	♂ (1)	60	Sept.	3	B > A	–	–	–	–	–		–
Scincella formosensis	♂ (1)	35	Oct.	2	B > A	–	–	–	–	–		–

RAM, relative anodal mobility. Relative quantities of LDH-C subunits: +, mediately abundant; ±, marginally present; –, undetectable. Examined tissue: B, brain; E, eye; H, heart; L, liver; M, skeletal muscle; O, ovary; T, testis.

present between A_4 and B_4 in *Hemidactylus frenatus* and *Sphenomorphus indicus* were likely the A_3B, A_2B_2, and AB_3 heterotetrameric LDH isozymes. In *Eumeces elegans, Mabuya longicaudata*, and *Scincella formosensis*, 2 additional bands were present between A_4 and B_4; while *Japalura mitsukurii* presented several extra bands between A_4 and B_4 (Figs. 2, 3). *Takydromus formosanus* presented 1 additional band, A_2B_2, between A_4 and B_4 (Fig. 3a). The result was similar to that shown in a lacertid lizard by Buth (1984)and in Tropiduridae as described by Martins (1995). *Takydromus formosanus* lacked the asymmetric heterotetrameric AB_3 and A_3B. The 3rd locus, LDH-C, was only detected in the testes of *H. frenatus, E. elegans*, and *M. longicaudata*. This latter locus was identified as a unique form of LDH based on tissue-specific expression and the following 2 characteristics. First, this testis-specific product exhibited a faster anodal migration than did other isozyme LDHs. This product migrated faster than the A_4 homotetramer (Figs. 1b, 2). Second, the product of this locus expressed low enzyme activity. As shown in figures 1b, 2a, and 2b, the product of *Ldh*-C had a lower band intensity.

In females of all species examined, no products of the 3rd presumptive *Ldh* locus were found.

DISCUSSION

The data presented herein represent the 1st documentation revealing the occurrence of a testis-specific LDH (LDH-C) in lizards, thus indicating its wide distribution among reptile taxa. There is no previously conclusive evidence for the existence of a testis-specific LDH in any amphibians or reptiles (Fisher et al. 1980, Murphy and Crabtree 1985, Murphy and Matson 1986). Citing from Qureshi et al. (1978), Rehse and Davidson (1986) claim that LDH-C is present in reptilian testes, i.e., testicular extracts of the lizard, *Uromastix hardwickii* (Qureshi et al. 1978). However, Qureshi et al. (1978) showed

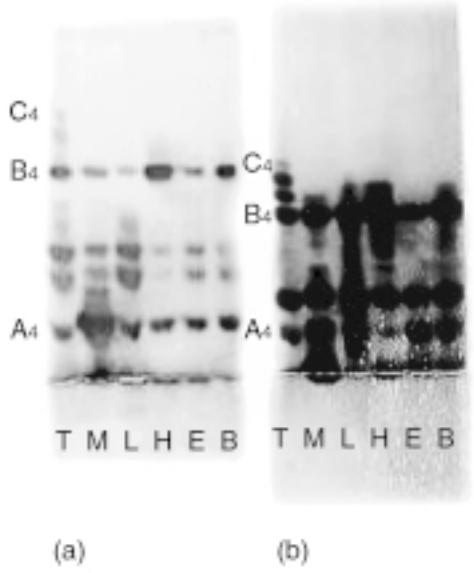

Fig. 2. Lactate dehydrogenase isozymes of (a) *Eumeces elegans* and (b) *Mabuya longicaudata*. C_4 homopolymer is highly anodal and was only detected in testes. For abbreviations see figure 1.

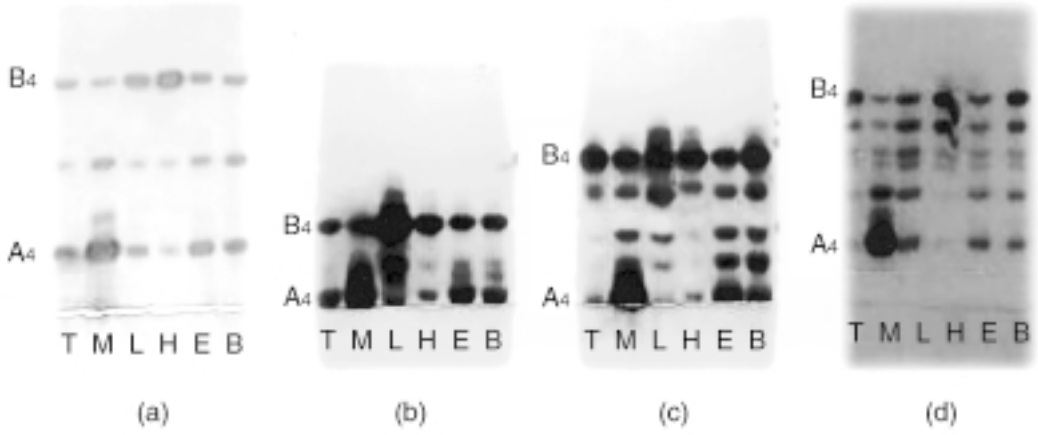

Fig. 3. Lactate dehydrogenase isozymes of (a) *Takydromus formosanus*, (b) *Scincella formosensis*, (c) *Sphenomorphus indicus*, and (d) *Japalura mitsukurii*. For abbreviations see figure 1.

no testis-specific products on a zymogram and did not well separate LDH-C from other LDH isozymes. Contrarily, our result fully distinguishes LDH-C expression from that of other LDH isozymes through an electrophoretic analysis.

LDH-C was detected in 3 of 7 lizard species examined. The absence of LDH-C in certain lizard taxa may be explained in the following ways. First, as is known, LDH-C is a molecule which is found in spermatozoa and spermatogenic cells of many mammalian and avian species (Blanco 1991). These species were presumably all at sexual maturity when seasonal changes of enzyme activity is well correlated with germinal activity of testes (Grimalt et al. 1995). The specimens used in this study were not at full sexual maturity, suggesting that they had not yet entered the reproductive season. However, LDH-C has been detected in mature testes of some amphibians and reptiles (Rehse and Davidson 1986). Testes of non-reproductive reptiles show very low enzyme activity (Grimalt et al. 1995). *Sphenomorphus indicus* has a non-continuous reproductive cycle, described as single-brooded and late-maturing viviparous. The male spermatogenesis proceeds during summer and autumn, with the smallest mature body size at 63 mm (snout-vent length). Peak spermatogenesis occurs during spring at the time when testis somatic indices reach a maximum. Testicular regression occurs during summer, with seminiferous tubule diameters at their minimum (Huang 1996). One male *Sphenomorphus indicus* specimen of 60-mm snout-vent length was collected in September, when the species had not yet entered reproductive seasonality with the testes in a regressed condition. Adult *Japalura mitsukurii* shows reproductive activity from early March to late November, with the smallest size of mature males at 54 mm. Its gonadal regression lasts from June until October (Lin and Cheng 1986). Our materials collected in June correspond to the testis condition which is supposedly in regression. Second, the small body size of our examined materials may have influenced the occurrence of LDH-C in testes to some extent. Our results show a very low level of LDH-C detectable in the testes of *Hemidactylus frenatus* and *Eumeces elegans*, and show a moderate level in the testes of *Mabuya longicaudata*, in contrast to the absence of that locus in *Takydromus formosanus* and *Scincella formosensis*. Small body size may indicate immaturity of the animals, which cannot express high enzyme activity in association with their reproductive season. The smallest mature sizes (snout-vent length) of males in *T. formosanus* and *S. formosensis* were 40 and 38 mm, respectively (Lin and Cheng 1990). Since LDH-C products have been detected in taxa ranging from teleosts to columbid birds and mammals, reptiles, phylogenetically positioned between them, would be expected to show LDH-C activity. It is expected that LDH-C product in some taxa examined should have a mobility equivalent to that of other LDH isozymes. The last explanation for this matter is that species specificity in LDH-C expression may be found in some lizards we examined. Extensive examples in certain reptiles examined by Qureshi et al. (1978) may further support this hypothesis. In conclusion, the expression of the LDH-C locus is distributed extensively among reptilian taxa, and the presence of the LDH-C may be, in a broad sense, an amniote character.

The traditional model for the evolution of vertebrate LDH suggests that the duplication of an ancestral locus took place in earlier vertebrates, which gave rise to *Ldh*-A and *Ldh*-B (Markert et al. 1975). Subsequent duplication of the *Ldh*-B locus gave rise to 3 different types of 3rd LDH genes with different mobilities, found respectively in actinopterygian fishes, columbid birds, and mammals (Holmes 1972). This traditional hypothesis has been refined somewhat with the suggestion that fish and pigeon *Ldh*-C loci are 2 independent duplicates originating from the *Ldh*-B locus, while mammalian *Ldh*-C is probably a derivative of the *Ldh*-A locus (Markert 1987). This traditional hypothesis has been challenged on the basis of amino acid sequences and immunological similarities of the 3 LDH isozymes (Li et al. 1983, Rehse and Davidson 1986, Baldwin and Lake 1987 Baldwin et al. 1987, Crawford et al. 1989). They suggest that the primordial LDH gene was duplicated to form *Ldh*-C and the other locus that later gave rise to *Ldh*-A and *Ldh*-B. In other words, LDH-C isozymes in fish, birds, and mammals are orthologous, while the LDH-A and LDH-B isozymes are more recently derived. Alternately, Goldberg (1971 1977) suggests that all 3 LDH genes evolved from a single ancestral gene. Results of amino acid sequencing strongly support the grouping of testicular isozymes in all mammals (Stock et al. 1997), assuming that mammalian testicular LDH-C was derived from duplication of mammalian LDH-A. This evidence arose from amino acid sequence data when analyzed with the most parsimonious and neighbor-joining trees.

If testis-specific *Ldh* did evolve from a common reptilian ancestor to birds and mammals and is therefore orthologous, a more widespread distribution of this presumed character among reptile taxa would be expected. The presence of LDH-C in Gekkonidae and Scincidae show in our results and in

the lizard, *Uromastix hardwickii*, by Qureshi et al. (1978), suggests that *Ldh*-C has a very broad distribution in lizards. Other data available in the literature may further support the hypothesis of an orthologous origin of mammalian testis-specific LDH.

The suggestion of an independent evolutionary origin for avian LDH-C is supported by the fact that only columbid birds have testis-specific LDH expression (Matson 1989), which is otherwise not found in amphibians or some reptiles (Fisher et al. 1980, Murphy and Crabtree 1985, Murphy and Maston 1986, Maston 1989). On the contrary, we assume that testis-specific LDH is derived from ancestal amniote LDH-A. Based on evidence of the presence of the *Ldh* locus in reptiles, broad expression of testis-specific LDH in avian taxa, the evolutionary position of reptiles as one of the amniotes, and additional evidence of mammalian testis-specific LDH, amino acid sequences (Mannen et al. 1997) further support its evolution from earlier vertebrates. Stock et al. (1997) also assessed an LDH sequence and proposed a phyletic relationship of vertebrate and invertebrate LDHs. Their results provide strong support for duplication giving rise to multiple vertebrate LDHs which occurred after vertebrates diverged from protochordates. The timing of these LDH duplications is consistent with data from a number of other gene families, suggesting widespread gene duplication at the time of the origin of vertebrates. With respect to the relationships among vertebrate LDHs, their data reveal that the duplication of an ancestral *Ldh* locus gave rise to *Ldh*-A and *Ldh*-B in the earliest divergence. Subsequently, 2 independent duplications occurred: actinopterygian fish *Ldh*-C originated from the *Ldh*-B locus, while mammalian *Ldh*-C was derived from the *Ldh*-A locus. Based on parsimonious evolutionary steps, we suggest that amniote *Ldh*-C was derived from *Ldh*-A. In other words, all amniote *Ldh*-C originated from a common ancestor. In conclusion, the results of the present study, and data available in the literature, support the assumption that testis-specific LDH was derived from ancestal amniotes. However, the hypothesis remains to to domonstrated by sequencing cDNAs of all LDH-A, LDH-B, and LDH-C from a given reptile.

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C 型乳酸去氫酶(LDH-C)在蜥蜴之表現：對脊椎動物乳酸去氫酶基因族在演化上之涵義

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為了更完整的瞭解蜥蜴類之乳酸去氫酶同功酶的表現，以澱粉凝膠電泳法進行實驗，共分析 *Hemidactylus frenatus, Japalura mitsukurii, Takydromus formosanus, Eumeces elegans, Mabuya longicaudata, Sphenomorphus indicus*, 及 *Scincella formosensis* 等四科七屬之蜥蜴。分別就蜥蜴的腦、眼、心臟、肝臟、骨骼肌和生殖腺分析乳酸去氫酶同功酶表現的型式。發現蜥蜴除了具有與其他脊椎動物相同的 A 、 B 兩個基本的酵素外，在 *H. frenatus, E. elegans* 及 *M. longicaudata* 三種蜥蜴的睪丸中發現第三種型式的乳酸去氫酶(LDH-C)的表現。此睪丸專一的酵素產物在陽極的移動上較其他乳酸去氫酶同功酶快。由此一發現我們推斷睪丸專有的乳酸去氫酶(LDH-C)是由有羊膜類祖先之乳酸去氫酶 A 型(LDH-A)所衍生出來的。

關鍵詞：蜥蜴，乳酸去氫酶，演化，有羊膜類。

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