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The use of isozyme characters in systematic studies of turtles: preliminary data for Australian Chelids

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Abstract

In this study we explore how various treatments of a small isozyme data set may be used to generate phylogenetic hypotheses for comparison with the results of allozyme analysis involving some of the same taxa. The isozyme data were collected from various tissue samples representing eight species of four genera of Australian chelid turtles: *Chelodina*, *Elseya*, *Elusor*, and *Emydura*. Treatments of the allozyme components of these data employed a chord distance in a Distance Wagner analysis and particulate characters ("locus-as-character") for a Wagner parsimony analysis. Treatments of a tissue expression data set included coding as presence–absent in a given tissue and as tissue arrays; a maximum parsimony analysis was used for both coded data sets. The resultant phylogenetic hypotheses were then compared with the current hypotheses of relationships among these turtles arising from morphological data as well as an independent and much larger allozyme data set. Our results support the conclusions of previous studies that the genus *Elseya* is polyphyletic. However the schema as to which species-level taxa in this genus are allied to the other chelid genera vary in our study with type of data and their encoding method. The relationship of *Elseya dentata* to other chelid turtles varied considerably with respect to different data sets and different means of coding and analysis. © 1999 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Reptilia; Chelidae; Allozymes; Isozymes; Tissue specificity

1. Introduction

Of the lower vertebrate groups, turtles have been among the least studied by systematists using biochemical approaches. Studies of turtles are conspicuously

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absent from several reviews of biochemical variation in vertebrates (e.g., Avise and Aquadro, 1982; Smith et al., 1982), and the few early studies available were limited in scope as seen from today's perspective (Crenshaw, 1962; Manwell and Schlesinger, 1966). A handful of interspecific studies of slightly broader scope have since been carried out (Merkle, 1975; Vogt and McCoy, 1980; Seidel and Lucchino, 1981; Seidel et al., 1981, 1986; Sites et al., 1981, 1984; Derr et al., 1987; Seidel and Adkins, 1987), as have a very few studies of interspecific population divergence (Smith et al., 1977; Marlow and Patton, 1981; Patton, 1984; Scribner et al., 1984, 1986; Alonso-Biosca et al., 1985a, b; Busack, 1986). These studies collectively suggest that, despite their morphological conservatism, turtles may be genetically as variable as other groups of vertebrates. Electrophoretic data would thus likely be useful in resolving some systematic problems prevalent in many turtle groups. This is especially important in the light of extreme conservatism at lower taxonomic levels in chromosomal characters (see Bickham, 1981, 1984, for review of chromosome evolution in turtles) and/or the question of homology of some morphological characters (Hutchinson and Bramble, 1981). Previous studies of turtles have focused either on one or a very few blood proteins (Crenshaw, 1962; Manwell and Schlesinger, 1966), or on allozyme data collected from larger surveys of protein products (as reviewed by Buth and Rainboth, 1998). These studies typically involved various statistical treatments of "allele" or electromorph frequencies of all presumably homologous gene loci across all taxa surveyed. Such data have proven to be of considerable utility in systematics, but are unnecessarily restricted in scope and may be of limited value in intergeneric or higher category comparisons. For example, an allozyme data set reported by Sites et al. (1984) for 17 genera of turtles of the emydid subfamily Batagurinae was characterized by high levels of inferred homoplasy, and probably approaches the limits of electrophoretic resolution based on allozyme frequencies. A possible alternative is the use of various isozyme characters (Whitt, 1983, 1987) and the application to systematics problems (Buth, 1984). Buth (1984) recognized three major classes of isozyme data, including: (1) the number of loci encoding multilocus enzymes; (2) the regulation of tissue-specific expression of gene products; and (3) patterns of intralocus or interlocus heteropolymer assembly and expression. The potential for using such isozyme characters in biochemical systematics studies is virtually unexplored, but appears to be promising. Herein we explore some of these possibilities and pursue three objectives: (1) to collect both allozyme and isozyme data sets for the same group of turtle species; (2) to analyze both data sets by several tree-building algorithms, and (3) to compare the ensuing phylogenetic hypotheses for congruence among themselves and against current phyletic hypotheses for the group (those of Georges and Adams (1992) and Cann and Legler (1994)).

1.1. Australian chelid turtles

Australian representatives of the pleurodiran turtle family Chelidae comprise an odd assortment of (mostly) highly aquatic, exclusively freshwater "side-necked" turtles. Osteological studies of the various components of this fauna have been conducted by Burbidge et al. (1974), Rhodin and Mittemeir (1976), and Gaffney (1977),

and serological and karyotypic data have been reported by Burbidge et al. (1974) and Bull and Legler (1980), respectively. The rare, monotypic genus *Pseudemydura* is usually not considered related to the other Australian pleurodire genera, and likely represents an early offshoot from the ancestral chelid stock (Burbidge et al., 1974; Gaffney, 1977; Legler, 1981; Cann and Legler, 1994). The relationships among the five other Australian genera (*Chelodina*, *Elseya*, *Emydura*, *Rheodytes*, and the "short-necked alpha") were elucidated by Georges and Adams (1992), who combined the analysis of a their large allozyme data set with earlier hypotheses of relationships to provide a phylogenetic model for further testing. This synthetic proposal identifies the genus *Chelodina* as a monophyletic outgroup to the other four genera; it is apparently comprised of at least two distinct subgroups. The genus *Rheodytes* and the "short-necked alpha" (subsequently named *Elusor macrurus* by Cann and Legler, 1994) were unresolved outgroups to an interior assemblage of "side-necks" of the genera *Elseya* and *Emydura*. The allozyme data of Georges and Adams (1992, 1996), however, clearly suggest monophyly for the genus *Emydura* as currently conceived but polyphyly within *Elseya*.

Our survey of this radiation has not been ideal with respect to a cladistic approach; rather, we have made use of an opportunistically available sample of turtles. Nevertheless, from these we have obtained allozyme and isozyme data on at least eight different species representing four genera of Australian chelids. Using the phylogenies of Georges and Adams (1992) and Cann and Legler (1994) as the most current proposals available, the relationships predicted for the nine species utilized in this

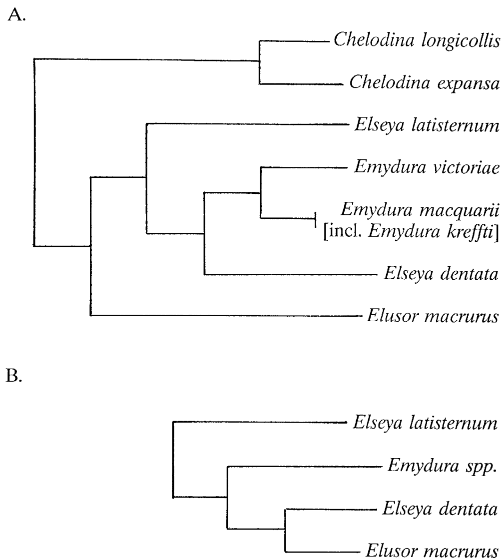


Fig. 1. Hypotheses of relationships among slected Chelid Turtles examined in common with the present study. Branching pattern only is depicted; branch lengths are not drawn to any scale. (A) Hypothesis of Georges and Adams (1992; their Fig. 4a) based on a distance-Wagner treatment of their allozymic data, and (B) Hypothesis of Cann and Legler (1994) based on a maximum parsimony analysis of morphometric, meristic, and allozymic data.

study are indicated in Fig. 1. We will use these phylogenetic hypotheses for comparison with the results of our own analyses.

2. Methods

2.1. Taxa

The turtles used in this study were either wild-caught or progeny of wild-caught individuals that had been maintained alive in the laboratory of J. M. Legler, University of Utah. The following taxa were examined in common with Georges and Adams (1992): *Chelodina expansa*, *Chelodina longicollis*, *Elseya latisternum*, *Elseya dentata*, *Emydura victoriae*, *Emydura macquarii* (formerly *Emydura australis*; Cogger et al., 1983), and the species they referred to as "short-necked alpha", which was subsequently described as *Elusor macrurus* by Cann and Legler (1994). In addition, a third species of *Chelodina* was included that is currently under study (by Legler), which we designate herein as *Chelodina* sp. We have also included specimens identified as *Emydura krefftii*, and treat them separately; this species was recommended to be in the synonymy of *Emydura macquarii* by Georges and Adams (1996). Information on the deposition of voucher specimens may be obtained from J. M. Legler.

2.2. Tissue preparation and laboratory protocol

Turtles were euthanized with ether, and separate extracts were prepared from tissues representing all three embryonic germ layers. These included eye (ectoderm); gonad (when available), heart, kidney, skeletal muscle, and spleen (mesoderm); and intestine and liver (endoderm). Preparation and storage of tissue homogenates followed the protocol of Sites et al. (1984), and supernatant fractions of the homogenates were run on horizontal gels of 12.5% starch containing equal parts off Sigma starch (Lots 83F-0612, 44F-0169, 94F-0537) and Electrostarch (Lot 392). Products of eleven specific enzyme systems encoded by at least 14 and possibly 15 presumptive gene loci were consistently resolved for both allozyme and isozyme characters in all species, and the successful buffer system and enzyme locus combinations are listed in Table 1. Enzyme staining procedures followed Murphy et al. (1996). Enzyme nomenclature follows that recommended by the International Union of Biochemistry and Molecular Biology (1992), and locus and isozyme designations follow recommendations of Murphy and Crabtree (1985), with some modifications.

2.3. Resolution of allozyme characters

Alphabetic designation of electromorphs (allelic products) of different electrophoretic mobility consisted of assigning the letter "a" to the most common or "medium mobility" allele at a given locus, and designating successively more anodal electromorphs as "b" or "c". The "slow" alleles were assigned symbols of "d", "e", "f", etc. All such designations were based on side-by-side comparisons for all allozyme

Table 1
Enzymes studied, loci scored, and optimal electrophoretic conditions used in the study of Chelid Turtles

Enzyme	E.C. number	Locus	Time of run (h)	Buffer system[a]
Aconitate hydratase (mitochondrial)	4.2.1.3	mAcoh-A	7–8	A
Aspartate transaminase (supernatant)	2.6.1.1	sAta-A	13–14	B
Creatine kinase	2.7.3.2	Ck-A	11–12	C
Glucose-6-phosphate isomerase	5.3.1.9	Gpi-A	11–12	C
L-lactate dehydrogenase	1.1.1.27	Ldh-A and Ldh-B	7–8	A,D
Malate dehydrogenase (mitochondrial)	1.1.1.37	mMdh-A	6–7	D
Malate dehydrogenase (supernatant)	1.1.1.37	sMdh-A	6–7	D
Mannose-6-phosphate isomerase	5.3.1.8	Mpi-1[b] and Mpi-2[b]	13–14	B
Phosphogluconate dehydrogenase	1.1.1.44	Pgdh-1[c]	6–7	D
Phosphoglucomutase	5.4.2.2	Pgm-1 and Pgm-2	13–14	B
Proline dipeptidase[d]	3.4.13.9	Pep-D	9–10	B,E
Purine-nucleoside Phosphorylase	2.4.2.1	Pnp-A	7–8	A

[a](A) Tris-citrate pH 8.0, (B) Tris-HCl pH 8.5, (C) Poulik pH 8.6, (D) Tris-HCL pH 8.5, and (E) Tris- maleate pH 7.4 (all of Selander et al., 1971).
[b]Number of loci uncertain (see text).
[c]Homology of locus uncertain (see text).
[d]L-phenylalanyl-L-proline used as substrate.

variants at each locus, and all variants having mobilities similar enough to preclude consistent separation were conservatively scored as the same gene product. The BIOSYS-1 program of Swofford and Selander (1981) was used to generate a Distance Wagner cladogram (Farris, 1972) based on the clustering of Cavalli-Sforza and Edwards' (1967) chord distance coefficients (as recommended by Rogers, 1986) calculated between all pairs of taxa. BIOSYS-1 defaults were used for addition criteria, and goodness-of-fit criteria. Allozyme data were also coded for a numerical cladistic analysis using the Phylogenetic Analysis Using Parsimony (PAUP) program written by D. L. Swofford (1985). Allelic combinations were coded as character states (i.e. locus-as-character coding *sensu* Buth, 1984). All characters were treated as unordered except for two-state characters (Ldh-A, mMdh-A) and those with obvious linear transformation (Pgm-1, Pgm-2). Both cladograms were initially rooted at the midpoint to depict *Chelodina* as the outgroup, following the hypothesis of Georges and Adams (1992).

2.4. Resolution of isozyme characters

The gene regulation data set was first qualitatively analyzed by initially scoring each gene–tissue combination as follows: 0 = no scorable activity in a particular

tissues; 1 = faint or slight activity; 2 = moderate activity; and 3 = very intense activity. Because of the subjective nature of these categories, we did not emphasize this coding method. We agree completely with the objections raised by Murphy and Crabtree (1985) that: (1) electrophoretic techniques give only gross measures of relative enzyme activity; (2) differences in tissue preparation may result in differential enzyme concentration in homogenate samples; (3) impurities in lab reagents may participate in staining reactions and give spurious results; (4) amount of tissue homogenate applied to a gel covaries with wick size and density; and (5) time and conditions of tissue storage may influence enzyme activity (see also Moore and Yates, 1983). Consequently, for some of the numerical analyses, we simply considered each gene-tissue combination (mAcoh-A/heart, mAcoh-A/kidney, etc.) as a character scorable as either present (1) or absent (0) in each species.

Some of the enzyme systems resolved are both multlimeric and multilocus in several over vertebrate groups, and normally form heteropolymers as a result of interlocus recombination of different subunits. These heteropolymers are normally expressed as isozymes of intermediate electrophoretic mobility relative to the respective homopolymers, with the exact mobility and concentration of each heteropolymer being a function of the number of each kind of subunit used to form the heteropolymer (see Markert et al., 1975). One such multilocus enzyme is creatine kinase (CK), which is encoded by two loci in reptiles: Ck-A in skeletal muscle and Ck-C in stomach (Buth et al., 1985). Its use as an isozyme character was limited in this study by the fact that it was invariantly expressed only as a single isozyme (Ck-A) in skeletal muscle; we detected no Ck-C activity in any tissue. Much greater potential may be realized among patterns of heteropolymer isozyme expression of L-lactate dehydrogenase (LDH), which in reptiles is a tetramer encoded by two genes, the "A" and "B" loci whose products predominate in skeletal muscle and heart, respectively. Apparently, as in other ectothermic vertebrates, each locus codes for its own kind of subunit, and each of these usually differs in electrical charge. If both the A and B subunits are available in equal concentrations and randomly combine in a given tissue, the expected ratio of product synthesized for the A_4, A_3B_1, A_2B_2, A_1B_3, and B_4 tetramers is 1 : 4 : 6 : 4 : 1, respectively (see Murphy and Crabtree, 1985, for discussion). However, the A and B subunits are rarely expressed equivalently in a given tissue and, thus, form asymmetrical ratios of isozymes. Qualitative analysis of the expression of each isozyme in all tissues could proceed in the same manner as analyzed the gene regulation data set. Such analyses await future studies.

3. Results

3.1. Allozyme analyses

Table 2 summarizes the genotypic arrays observed for the 15 polyallelic loci resolved among all nine turtle species examined. These genotypic data were transformed into Cavalli-Sforza and Edwards (1967) chord distance measures. A Wagner

tree generated from the Cavalli-Sforza and Edwards chord distance measures, rooted at the midpoint of the longest path, is depicted in Fig. 2A.

The allelic data that can be derived from Table 2 were coded for numerical analyses by using the locus as the character and the allelic composition as the character state. These data were ordered by linking the states to each other so as to minimize the number of gains plus losses. This method of coding conforms to the "minimum turnover model" of Mickevich and Mitter (1981, 1983; see also Matson, 1987, 1998). For example, as shown in Fig. 3, the allelic combinations of mAcoh-A locus were linked as follows: first the state "b" could be linked to the state "bc" by one loss or gain, evolution of state "a" to/from "b", "a" to/from "d," and "b" to/from "d" require one loss and one gain each if they are to be derived from one another. The sequential numbers associated with each state in Fig. 3 are arbitrary; these character-state relationships and coding for all locus characters are summarized in Fig. 3, and Table 3 presents the coded matrix for all nine species of chelids examined. The matrix in Table 3 was cladistically analyzed using the Phylogenetic Analysis Using Parsimony (PAUP) program of Swofford (1985). An exhaustive search of all possible topologies was performed and yielded 12 equally parsimonious Wagner trees. The strict consensus tree of these 12 trees is depicted in Fig. 2B.

3.2. Isozyme analyses

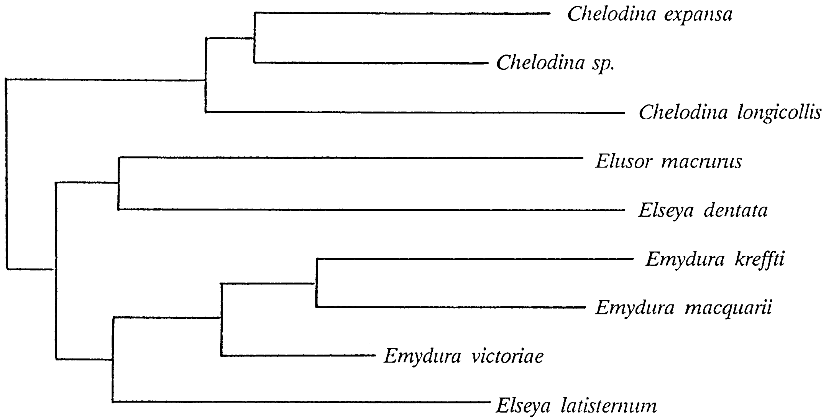
We resolved patterns of tissue expression in nine gene loci at seven of the eight tissues studied (we had spleen from only three individuals representing two species) across eight of the nine turtle species (*Emydura victoriae* is absent from isozyme analyses). With one possible exception (MPI), this preliminary study did not reveal any new isozyme loci over and above those expected in other vertebrates. Before summarizing tissue expression and heteropolymer patterns, we should comment on locus homologies so that these results may be compared to those of other reptilian groups (Dessauer and Densmore, 1983; Dessauer and Cole, 1984; Murphy and Crabtree, 1985; Murphy and Matson, 1986). For enzymes known to been encoded by mitochondrial and supernatant loci, such as aconitate hydratase (ACOH), aspartate transaminase (ATA), and malate dehydrogenase (MDH), we assumed that mitochondrial products were generally more slowly migrating and more widely expressed in different tissue types (Harris and Hopkinson, 1976). We assume the following homologies between our data and most previously published reptilian allozyme data sets: sAta-A = "Got-1", Ldh-A = "Ldh-2", Ldh-B = "Ldh-1", sMdh-A-"Mdh-1", and mMdh-A = "Mdh-2". Homologies for other loci are less certain. For example, Murphy and Crabtree (1985) reported two loci encoding phosphogluconate dehydrogenase (Pgdh-1 and Pgdh-2) in the rattlesnake *Crotalus viridis*; the Pgdh-1 locus was expressed in liver only (of 10 tissues surveyed), whereas Pgdh-2 was absent in liver but expressed in gonad, heart, kidney, lung, stomach and duodenum. We resolved only a single PGDH isozyme in chelid turtles, presumably the same one found by Georges and Adams (1992, 1996, identified as "6Pgd"), and in several species it was expressed in nearly all tissues (*Chelodina expansa*, *Chelodina* sp., *Emydura australis*, *Elseya latisternum*, and *Elseya dentata*). Dessauer and Densmore (1983) reported similar results in

Table 2
Genotypes recorded for 15 polyallelic loci resolved in selected Australian Chelid Turtles

Locus	*Genotype*	*Chelodina expansa*	*Chelodina longicollis*	*Chelodina* sp.	*Elusor macrurus*	*Emydura krefftii*	*Emydura victoriae*	*Emydura macquarii*	*Elseya latisternum*	*Elseya dentata*
mAcoh-A	aa	–	1	1	–	–	–	–	–	–
	bb	–	–	–	1	2	1	1	6	–
	bc	–	–	–	–	–	1	–	–	1
	cc	–	–	–	–	–	1	3	–	–
	dd	3	–	–	–	–	–	–	–	–
sAta-A	aa	1	–	1	–	–	3	1	6	1
	ab	1	–	–	–	–	–	–	–	–
	bb	1	–	–	–	2	–	–	–	–
	dd	–	–	–	1	–	–	1	–	–
	ae	–	1	–	–	–	–	–	–	–
	ee	–	–	–	–	–	–	1	–	–
Ck-A	aa	–	–	–	1	2	1	2	–	1
	bb	3	1	1	–	–	–	–	–	–
	dd	–	–	–	–	–	–	–	3	–
Gpi-A	aa	3	1	1	–	–	–	–	–	–
	dd	–	–	–	1	–	–	–	–	–
	ee	–	–	–	–	2	3	4	6	1
Ldh-A	aa	3	1	1	1	–	3	3	6	–
	ab	–	–	–	–	1	–	–	–	–
	gg	–	–	–	–	–	–	–	–	1
Ldh-B	aa	3	–	1	–	–	–	–	1	–
	bb	–	1	–	–	–	–	–	–	–
	dd	–	–	–	1	1	3	2	5	1
sMdh-A	aa	3	1	1	–	–	–	–	–	–
	bb	–	–	–	1	3	2	4	–	1
	bc	–	–	–	–	1	–	–	–	–
	cc	–	–	–	–	–	1	–	6	–

mMdh-A	aa	–	–	1	1	2	3	4	6	1
	dd	3	1	–	–	–	–	–	–	–
Mpi-1	aa	3	1	1	–	2	2	2	3	–
	bb	–	–	–	–	–	1	–	–	–
	dd	–	–	–	1	–	–	–	–	–
	ee	–	–	–	–	–	–	–	–	1
Mpi-2	aa	3	1	1	–	2	3	2	5	–
	dd	–	–	–	1	–	–	–	–	–
	ee	–	–	–	–	–	–	–	–	1
Pgdh-1	aa	2	1	–	1	1	2	3	–	–
	ad	1	–	1	–	–	–	–	–	–
	dd	–	–	–	–	–	–	–	–	1
	de	–	–	–	–	–	–	–	1	–
	ee	–	–	–	–	–	1	1	5	–
Pgm-1	aa	3	1	1	–	1	2	–	–	–
	ab	–	–	–	–	–	–	1	–	–
	ad	–	–	–	–	1	–	–	–	1
	dd	–	–	–	1	–	1	–	2	–
Pgm-2	aa	3	1	1	1	2	2	–	–	1
	dd	–	–	–	–	–	1	2	6	–
Pep-D	aa	3	–	1	1	–	2	–	6	–
	bb	–	–	–	–	–	–	–	–	1
	cc	–	–	–	–	2	1	2	–	–
	dd	–	1	–	–	–	–	–	–	–
Pnp-A	aa	2	–	1	–	–	1	–	–	–
	bb	1	–	–	–	2	1	4	–	–
	dd	–	–	–	1	–	–	–	–	–
	df	–	–	–	–	–	–	–	1	–
	ee	–	1	–	–	–	–	–	–	1
	ff	–	–	–	–	–	1	–	5	–

A.



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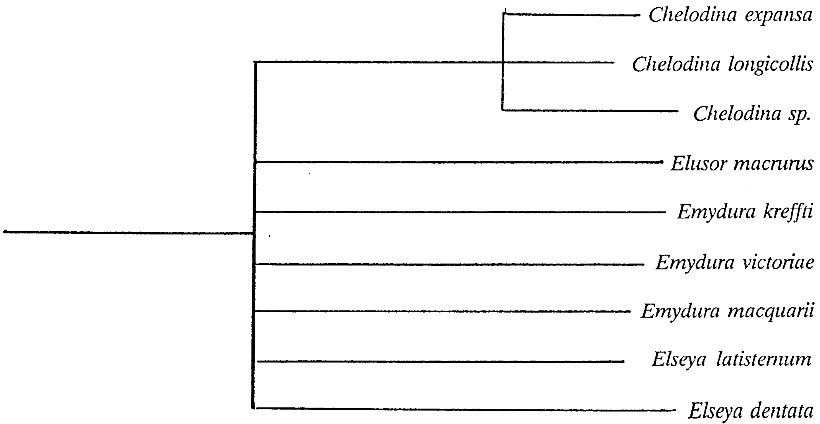


Fig. 2. Hypothesized relationships among selected Chelid Turtles. Both trees are drawn to depict *Chelodina* as the sister group to the remaining taxa, following Georges and Adams (1992). (A) Distance-Wagner tree based on Cavalli-Sforza and Edwards (1967) chord distance of the allozymic data presented in Table 2; branch lengths are drawn in proportion to the distances among taxa. The cophenetic correlation of this tree is 0.917. (B) Consensus tree of 12 equally parsimonious Wagner trees based on the allozymic data of Table 2 but coded as discrete characters (Fig. 3 and Table 3). Branching pattern only is depicted; branch lengths are not drawn to any scale. The consistency index (CI) of any of the individual trees is 0.882.

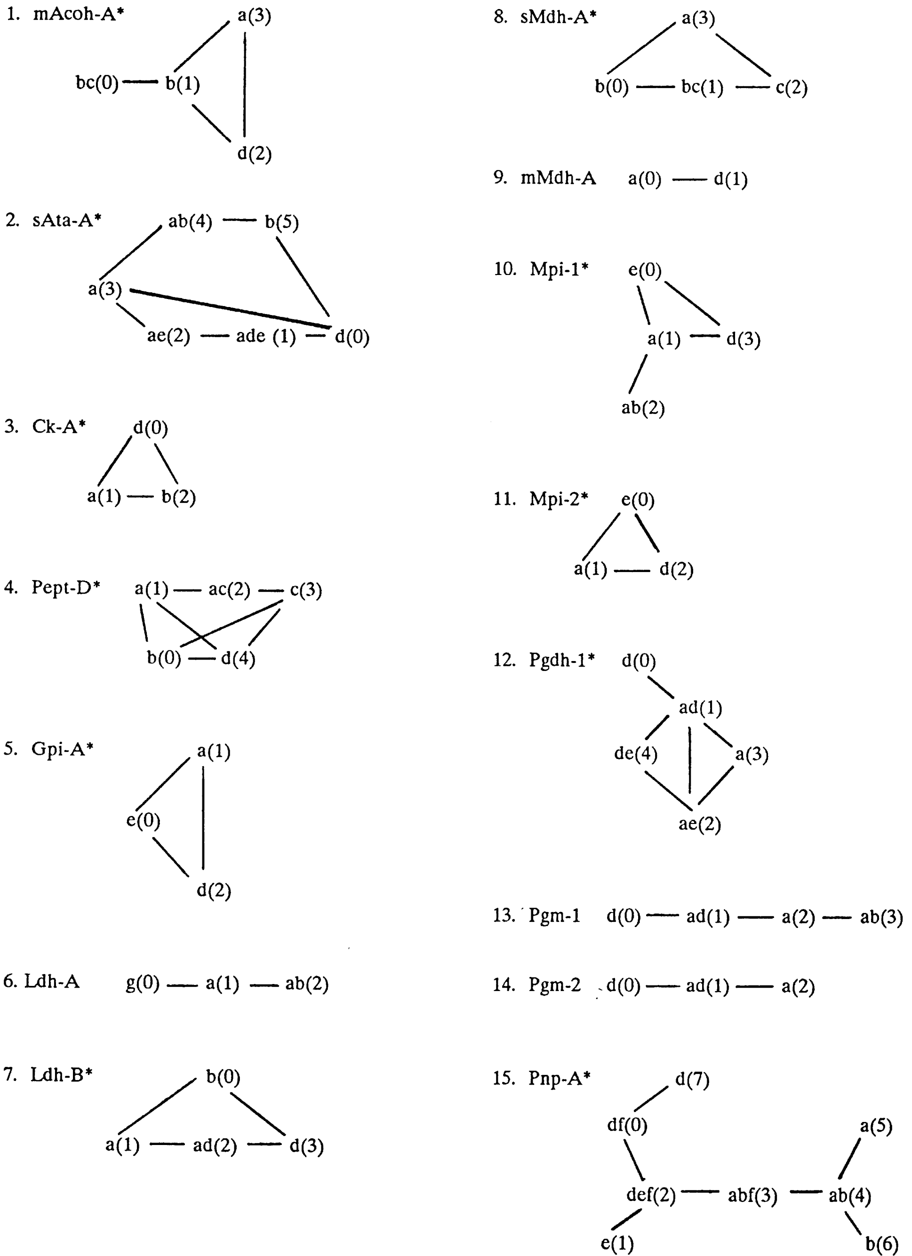


Fig. 3. Allelic data from Table 2 ordered using the "minimum turnover model" to minimize the total number of gains and losses of alleles. In cases in which transitions could not be arranged linearly, characters were treated as unordered (*). Character states are numbered in parentheses.

Table 3
Multistate allozyme character matrix from Fig. 3 coded for all species of Chelid Turtles examined

Locus	*Chelodina expansa*	*Chelodina longicollis*	*Chelodina* sp.	*Elusor macrurus*	*Emydura krefftii*	*Emydura victoriae*	*Emydura macquarii*	*Elseya latisternum*	*Elseya dentata*
mAcoh-A[a]	2	3	3	1	1	0	0	1	0
sAta-A[a]	4	2	3	0	5	3	1	3	3
Ck-A[a]	2	2	2	1	1	1	1	0	1
Gpi-A[a]	1	1	1	2	0	0	0	0	0
Ldh-A	1	1	1	1	2	1	1	1	0
Ldh-B[a]	1	0	1	3	3	3	3	2	3
sMdh-A[a]	3	3	3	0	1	1	0	2	0
mMdh-A	1	1	0	0	0	0	0	0	0
Mpi-1[a]	1	1	1	3	1	2	1	1	0
Mpi-2[a]	1	1	1	2	1	1	1	1	0
Pgdh-1[a]	1	3	1	3	3	2	2	4	0
Pgm-1	2	2	2	0	1	1	3	0	1
Pgm-2	2	2	2	2	2	1	0	0	2
Pep-D[a]	1	4	1	1	3	2	3	1	0
Pnp-A[a]	4	1	5	7	6	3	6	0	1

[a]Unordered species.

their study of *Alligator* (i.e., widespread tissue expression of a single locus). Similar tissue distributions suggest homology between *Alligator* and the chelids for Pgdh-2, but until more taxa have been surveyed, we refrain from assigning a specific locus to the PGDH locus we observed (provisionally numbered Pgdh-1).

Similar uncertainties are also apparent in two other enzymes. Phosphoglucomutase (PGM) was expressed as a single locus in *Crotalus viridis* (Murphy and Crabtree, 1985), but two loci were found in several species of the colubrid snake genus *Thamnophis*, (Lawson and Dessauer, 1979). Two PGM loci with unequal tissue distribution patterns were found in *Alligator* (Dessauer and Densmore, 1983), in liver homogenates for the iguanid lizard *Sceloporus grammicus* (Sites and Greenbaum, 1983), in both liver and white muscle homogenates in the teiid lizard *Cnemidophorus tigris* (Dessauer and Cole, 1984), the gekkonids *Phyllodactylus unctus* and *P. paucituberculatus* (Murphy and Papenfuss, 1980), and possibly two loci in liver in the skink genus *Eumeces* (Murphy et al., 1983). Earlier turtle studies show examples of both; a single PGM locus was resolved in species studied by Seidel et al. (1981), Scribner et al. (1984, 1986), and Sites et al. (1984), but two loci were resolved in the genus *Rhinoclemmys* (Sites et al., 1981). Significantly, Georges and Adams (1992, 1996) report only a single PGM locus in their large series of Australian chelids. Until homologies are certain, we simply label the two loci resolved in our chelid samples as Pgm-1 and Pgm-2, in order of decreasing anodal mobility of their products.

The isozyme pattern for mannose-6-phosphate isomerase (MPI) was interesting because of tissue-specific variation in isozyme number and mobility. This enzyme is a monomer in human beings, encoded by a single autosomal locus (Harris and Hopkinson, 1976). *Alligator* (Dessauer and Densmore, 1983), the lizard *Cnemidophorus tigris* (Dessauer and Cole, 1984), and the rattlesnake *Crotalus viridis* (Murphy and Crabtree, 1985) also express only a single locus. To our knowledge this enzyme has not received detailed study for patterns of tissue expression in turtles. Georges and Adams (1996) reported a single locus with three electromorphs in their chelid study; in all chelids examined by us there were two isozymes expressed in some tissues and a single band in others. In *Chelodina expansa*, for example, two highly anodal bands of equal intensity were expressed in muscle, spleen, and testis, while the most anodal of these was more intensely expressed in eye, and was almost the only isozyme detectable in intestine. Only the least anodal of these two bands was expressed in liver and heart, whereas in kidney this isozyme and a third slower band were intensely expressed. Kidney tissue was unique in that the most anodal isozyme was never expressed in any species, whereas the adjacent enzyme of slower electrophoretic mobility was always present. An electrophoretically slower enzyme was also expressed in both kidney and eye of *Elseya dentata*. Multiple isozyme patterns could result from individuals either (1) being heterozygous for different mobility alleles at a single locus, which would produce a two-banded pattern in a monomeric protein, (2) MPI being encoded by multiple loci, or (3) some form of epigenetic or posttranslational modification resulting in the production of conformational isozymes or "subbands" (Harris and Hopkinson, 1976). We do not think allozyme variation is a likely explanation for at least two reasons. First, one would expect to see the heterozygous pattern in all tissues of the same individual if such a pattern was due to allelic

Table 4
Comparison of tissue-specific distributional patterns of nine variable loci scored for eight species of Chelid Turtles

Locus	*Chelodina expansa*	*Chelodina longicollis*	*Chelodina* sp.	*Elusor macrurus*	*Emydura krefti*	*Emydura macquarii*	*Elseya latisternum*	*Elseya dentata*
mAcoh-A	–	–	–	–	–	Eye	–	Eye
	Gonad	?	Gonad	–	Gonad	Gonad	Gonad	Gonad
	Heart	Heart	Heart	–	–	Heart	Heart	–
	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney
	Muscle	Muscle	Muscle	Muscle	Muscle	Muscle	Muscle	Muscle
	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine
	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver
sAta-A	–	–	–	–	–	–	–	–
	–	?	–	–	–	–	–	–
	Heart	–	Heart	–	–	–	–	–
	Kidney	–	–	Kidney	Kidney	Kidney	Kidney	Kidney
	–	–	–	–	Muscle	–	–	–
	–	–	–	–	–	–	Intestine	Intestine
	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver
Mpi-1	Eye	Eye	Eye	Eye	Eye	Eye	Eye	–
	Gonad	?	Gonad	–	Gonad	Gonad	Gonad	?
	Heart	Heart	Heart	–	–	Heart	Heart	Heart
	–	–	–	–	–	–	–	–
	Muscle	–	–	Muscle	Muscle	Muscle	–	–
	Intestine	Intestine	–	Intestine	Intestine	Intestine	Intestine	Intestine
	–	Liver	–	–	Liver	Liver	Liver	Liver
Mpi-2	–	–	Eye	Eye	Eye	Eye	–	Eye
	Gonad	?	Gonad	–	–	Gonad	Gonad	?
	Heart	–	Heart	–	–	–	–	Heart
	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney
	Muscle	Muscle	Muscle	Muscle	Muscle	–	–	–
	Intestine	–	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine
	Liver	Liver	Liver	–	Liver	Liver	Liver	Liver
Pgdh-1	Eye	Eye	Eye	–	Eye	Eye	Eye	Eye
	Gonad	?	Gonad	Gonad	–	Gonad	Gonad	?

	Heart	Heart	Heart	–	Heart	Heart	–	Heart
	Kidney	–	Kidney	–	Kidney	Kidney	–	Kidney
	–	–	–	–	–	Muscle	Muscle	Muscle
	Intestine	Intestine	–	Intestine	–	–	Intestine	Intestine
	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver
Pgm-1	Eye	Eye	Eye	Eye	Eye	Eye	Eye	Eye
	Gonad	?	Gonad	–	–	–	–	–
	Heart	Heart	Heart	–	–	–	–	–
	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney
	Muscle	–	–	Muscle	Muscle	–	–	Muscle
	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine
	Liver	Liver	Liver	–	–	–	–	–
Pgm-2	Eye	Eye	Eye	–	Eye	Eye	Eye	Eye
	Gonad	?	Gonad	–	Gonad	Gonad	Gonad	?
	Heart	Heart	Heart	–	Heart	Heart	Heart	Heart
	Kidney	–	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney
	Muscle	Muscle	Muscle	Muscle	Muscle	Muscle	Muscle	Muscle
	Intestine	–	Intestine	–	–	–	Intestine	Intestine
	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver
Pep-D	Eye	Eye	–	–	Eye	Eye	Eye	Eye
	Gonad	?	Gonad	–	Gonad	Gonad	Gonad	?
	Heart	–	–	–	–	Heart	Heart	Heart
	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney
	Muscle	–	Muscle	–	–	Muscle	–	–
	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine
	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver
Pnp-A	Eye	Eye	Eye	–	Eye	–	–	Eye
	Gonad	?	Gonad	Gonad	Gonad	Gonad	Gonad	Gonad
	Heart	Heart	Heart	Heart	–	Heart	–	Heart
	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney
	Muscle	Muscle	Muscle	Muscle	Muscle	–	–	Muscle
	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine
	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver

Note: Hyphens indicate the absence of enzyme expression in a particular tissue, whereas question marks indicate the absence of a tissue sample.

heterozygosity, and second, it seems unlikely to us that single individuals or very small samples of all species studied would be heterozygous at the same locus. We also suggest that the independent pattern of isozyme expression seen in some species, such as the mutually exclusive expression of these isozymes in different tissues of the same individuals (*Chelodina longicollis*, *Emydura australis*, *Elseya latisternum*; see Table 4) is inconsistent with a diallelic, single-locus hypothesis. This pattern also seems unlikely to us if the fastest isozyme was strictly conformational to the slower one (but see Murphy and Crabtree, 1985, for an example of post-translational modification of MPI in *Crotalus viridis*), so we have tentatively identified the most anodal isozyme as the product of Mpi-1, and the one immediately cathodal to it as Mpi-2. This hypothesis is provisionally confirmed by the independent pattern of variation observed in the two MPI isozymes in *Emydura victoriae* (Table 2). Allozyme variation present in Mpi-1 is absent in Mpi-2, and such independent behavior suggests a two-locus system. Unfortunately, this is the species for which we did not have an array of different tissues, and consequently cannot evaluate the tissue-specific nature of this observation. We also have little information on the slower isozyme seen in kidney tissues of *Chelodina expansa*, and omit it from our analyses. Nevertheless, the possibility of a two- or even three-locus MPI system in turtles warrants further investigation.

Patterns of tissue-specific gene expression were quite variable and ranged from products of gene loci that were expressed with almost equal intensity in virtually all tissues in all species, such as Gpi-A, mMdh-A, and sMdh-A, to those whose products were expressed in only a single tissue, such as Ck-A in skeletal muscle. The remaining loci fall somewhere between these extremes, and their tissue-specific patterns of expression are summarized in Table 4. Note that LDH is not included in Table 4, because the distribution of Ldh-A and Ldh-B products among heterotetramers made the scoring process more difficult.

Among the loci listed in Table 4, the most restricted in its expression was sAta-A. It was entirely absent from eye and gonad in all species, weakly expressed in skeletal muscle only in *Emydura krefftii*, and expressed in only two species in heart (*Chelodina expansa* and *Chelodina* sp.) and intestine (both species of *Elseya*). Mpi-1 was not expressed in kidney of any species, although it was expressed to some degree in all other tissues in at least some species. Pgm-1 was diagnostic for *Chelodina* to the extent that it was weakly expressed in gonad, heart, and liver, and absent in those same tissues in all of the other "side-necked" species. We calculated the "percentage of expression" both for tissues across all species and for each species across all tissues by counting the total number of positive scores (1) for each tissue-locus-individual combination and dividing by the total number possible for each. For example, the total number of positive scores for heart was 55 and the total number possible was 99 (number of loci $\times$ number of individuals), which gives a proportion of 0.555. Thus, across all species, heart tissue was most specialized with respect to degree of restriction of gene product expression. The remaining six tissues, in order from most to least restrictive are: eye (0.586), skeletal muscle (0.619), gonad (0.706), intestine (0.745), kidney (0.788), and liver (0.856). The species also varied considerably in the proportion of gene product expression across all tissues. Expression in *Elusor macrurus* was most

restricted, showing activity at just under half of all enzyme-tissue characters (0.492). The remaining species showed increases as follows: *Emydura krefftí* (0.624), *Chelodina longicollis* (0.679), *Elseya latisternum* (0.683), *Chelodina expansa* (0.745), *Chelodina* sp. and *Emydura australis* (both with 0.746), and *Elseya dentata* (0.780).

The data summarized in Table 4 are amenable to several types of numerical analysis, depending on methods of coding. First, we coded the expression of a locus as present (1) or absent (0) in each tissue; those locus/tissue combinations that varied among taxa are listed in Table 5. These data were subjected to a parsimony analysis that employed an exhaustive search of all possible tree topologies (Swofford, 1985). Next, we used each locus as a character and the tissue array as the state, and again arranged transformations so as to minimize the number of gains and losses. For example, the product of the mAcoh-A locus is expressed in all tissues in *Emydura macquarii* and in all tissues except eye in the outgroup taxa (taken collectively), but has a different and somewhat more restricted distribution in the other species (Table 4). These states were then ordered in the same manner as the allozyme character states (= minimum turnover model of Mickevich and Mitter, 1981, 1983) and linked to minimize the number of gains plus losses. Returning to mAcoh-A as an example as shown in Fig. 4, we could start with expression of this locus over all tissues (EHKMIL), and link it to the EKMIL and HKMIL arrays by the loss/gain of expression in H and E, respectively. The remaining state of KMIL could be linked to HKMIL with the loss/gain of H. This most parsimonious linkage can be arranged in a linear pattern (Fig. 4) and treated as an ordered character. The character state transformations of gene expression patterns from the nine variable loci are summarized in Fig. 4; the coded matrix for these data is shown in Table 6. This matrix was subjected to the same PAUP algorithm as the previous data set.

The maximum parsimony analyses produced two trees each for the tissue-expression presence-absence data set (Table 5) and the multistate tissue-expression data set (Table 6). Strict consensus trees for both are shown in Fig. 5.

4. Discussion

Previous hypotheses of phylogenetic relationships of the eight taxa examined in this study are presented in Fig. 1. Fig. 1A follows the phylogenetic model of Georges and Adams (1992), produced by combining their large allozyme data set with previously accepted phylogenetic hypotheses. Fig. 1B represents the cladistic relationships of Australian short-necked turtles presented by Cann and Legler (1994), based upon a combination of morphological characters and a selection of the allozyme data set of Georges and Adams (1992). Both phylogenies are similar in that they suggest the genus *Elseya* is paraphyletic; they differ in the placement of *Elusor macrurus* (the “short-necked alpha” of Georges and Adams, 1992, and other works). Georges and Adams (1992) place *Elusor* as a sister taxon to the entire *Elseya/Emydura* clade. The phylogeny of Cann and Legler (1994) suggests *Elusor* is a within-clade derived taxon whose closest relative is *Elseya dentata*. The latter conclusion is foreshadowed by one of the cladograms produced by Georges and Adams strictly from allozyme data from

Table 5
Presence–absence (1 and 0, respectively) coding of enzyme expression by tissue for eight species or Chelid Turtles

Locus	*Chelodina expansa*	*Chelodina longicollis*	*Chelodina* sp.	*Elusor macrurus*	*Emydura kreffti*	*Emydura macquarii*	*Elseya latisternum*	*Elseya dentata*
mAcoh-A/eye	0	0	0	0	0	1	0	1
mAcoh-A/gonad	1	?	1	0	1	1	1	1
mAcoh-A/heart	1	1	1	0	0	1	1	0
sAta-A/heart	1	0	1	0	0	0	0	0
sAta-A/kidney	1	0	0	1	1	1	1	1
sAta-A/muscle	0	0	0	0	1	0	0	0
sAta-A/intestine	0	0	0	0	0	0	1	1
Mpi-1/eye	1	1	1	1	1	1	1	0
Mpi-1/gonad	1	?	1	0	1	1	1	?
Mpi-1/heart	1	1	1	0	0	1	1	1
Mpi-1/muscle	1	0	0	1	1	1	0	0
Mpi-1/intestine	1	1	0	1	1	1	1	1
Mpi-1/liver	0	1	0	0	1	1	1	1
Mpi-2/eye	0	0	1	1	1	1	0	1
Mpi-2/gonad	1	?	1	0	0	1	1	?
Mpi-2/heart	1	0	1	0	0	0	0	1
Mpi-2/muscle	1	1	1	1	1	0	0	0
Mpi-2/intestine	1	0	1	1	1	1	1	1
Mpi-2/liver	1	1	1	0	1	1	1	1

Pgdh-1/eye	1	1	1	0	1	1	1	1
Pgdh-1/gonad	1	?	1	1	0	1	1	?
Pgdh-1/heart	1	1	1	0	1	1	0	1
Pgdh-1/kidney	1	0	1	0	1	1	0	1
Pgdh-1/muscle	0	0	0	0	0	1	1	1
Pgdh-1/intestine	1	1	0	1	0	0	1	1
Pgm-1/gonad	1	?	1	0	0	0	0	0
Pgm-1/heart	1	1	1	0	0	0	0	0
Pgm-1/muscle	1	0	0	1	1	0	0	1
Pgm-1/liver	1	1	1	0	0	0	0	0
Pgm-2/eye	1	1	1	0	1	1	1	1
Pgm-2/gonad	1	?	1	0	1	1	1	?
Pgm-2/heart	1	1	1	0	1	1	1	1
Pgm-2/kidney	1	0	1	1	1	1	1	1
Pgm-2/intestine	1	0	1	0	0	0	1	1
Pep-D/eye	1	1	0	0	1	1	1	1
Pep-D/gonad	1	?	1	0	1	1	1	?
Pep-D/heart	1	0	0	0	0	1	1	1
Pep-D/muscle	1	0	1	0	0	1	0	0
Pnp-A/eye	1	1	1	0	1	0	0	1
Pnp-A/heart	1	1	1	1	0	1	0	1
Pnp-A/muscle	1	1	1	1	1	0	0	1

All "single state" characters (those expressed equivalently in the same tissue in all species) were omitted; see Table 4. Question marks indicate the absence of a tissue sample, i.e. missing data.

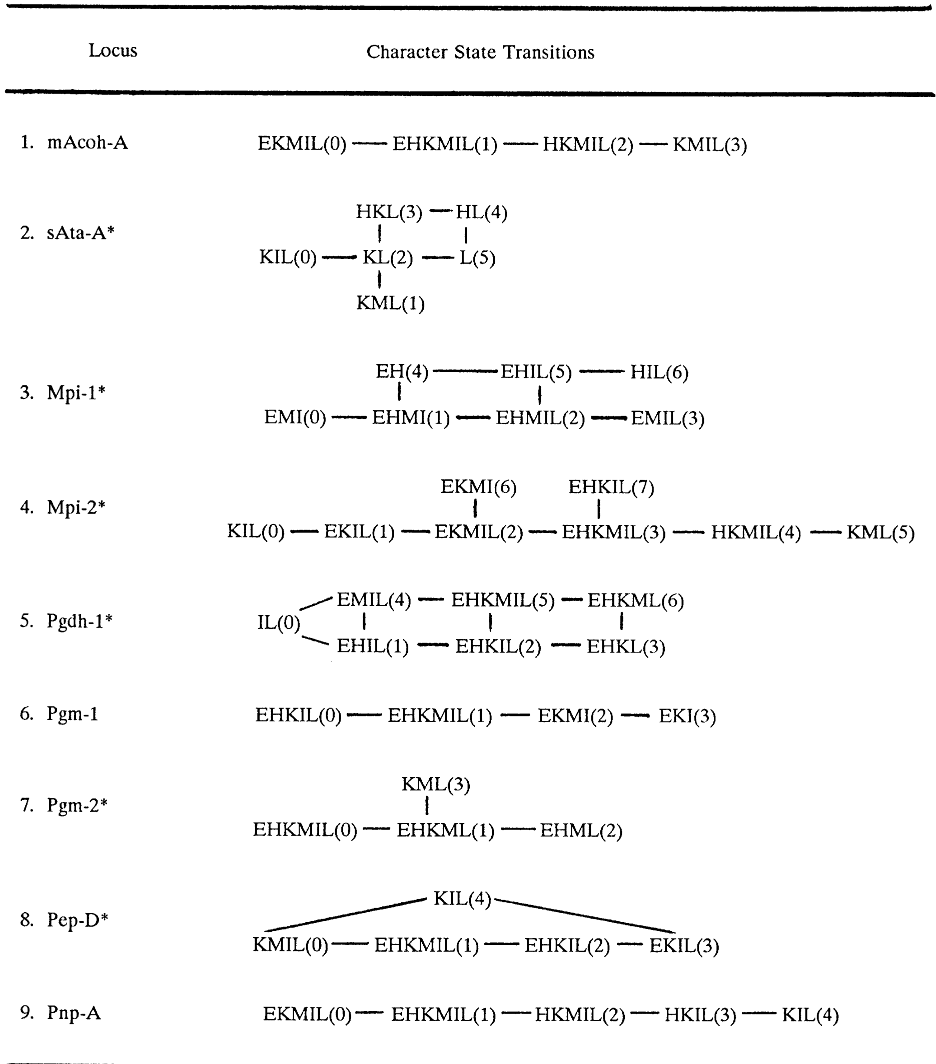


Fig. 4. Patterns of tissue distributions from Table 4 ordered using the "minimum turnover model to minimize the total number of gains and losses of expression. In cases in which transitions could not be arranged linearly, characters were treated as unordered (*). Character states are numbered in parentheses.

which polyallelic and uninformative loci had been removed; that branching pattern for the taxa utilized in this study is identical to that of Cann and Legler (Fig. 1B).

Our Distance Wagner tree (Fig. 2A) is concordant with the phylogenetic hypotheses of both Georges and Adams (1992) and Cann and Legler (1994) with respect to suspected polyphyletic nature of the genus *Elseya*, and follows the former insofar as

Table 6
Multistate tissue-expression matrix from Fig. 4 coded for nine variable loci scored for eight species of Chelid Turtles

Locus	*Chelodina expansa*	*Chelodina longicollis*	*Chelodina* sp.	*Elusor macrurus*	*Emydura krefftii*	*Emydura macquarii*	*Elseya latisternum*	*Elseya dentata*
mAcoh-A	2	2	2	3	3	1	2	0
sAta-A[a]	3	5	4	2	1	2	0	0
Mpi-1[a]	1	5	4	0	3	2	5	6
Mpi-2[a]	4	5	3	6	2	1	0	7
Pgdh-1[a]	2	1	3	0	3	6	4	5
Pgm-1	1	0	0	2	2	3	3	2
Pgm-2[a]	0	2	0	3	1	1	0	0
Pep-D[a]	1	3	0	4	3	1	2	2
Pnp-A	1	1	1	2	0	3	4	1

[a]Non-linear characters were treated as unordered.

the outgroup nature of the turtle genus *Chelodina*. *Elusor macrurus*, however, is clearly allied to *Elseya dentata*, as suggested directly by Cann and Legler (1994) and indirectly through the aforementioned cladogram of Georges and Adams that was based solely on a subset of their allozyme data. Additionally, *Emydura kreffti* appears as fairly distinctive from *E. macquarii*, in contradiction to the findings of Georges and Adams (1992, 1996) who could not distinguish them. Our sample sizes of *E. kreffti* ($n = 2$) and *E. macquarii* ($n = 4$) may have been too small to pick up the full range of variation and thus the lack of fixed differences that was observed by these authors ($n = 19$ for *E. kreffti* and $n = 23$ for *E. macquarii* in the 1996 paper), and thus the difference we measure may be artificial. However, we do not necessarily subscribe to their "yard-stick" argument that the lack of allozymic divergence is a major consideration for deciding the two taxa as conspecific.

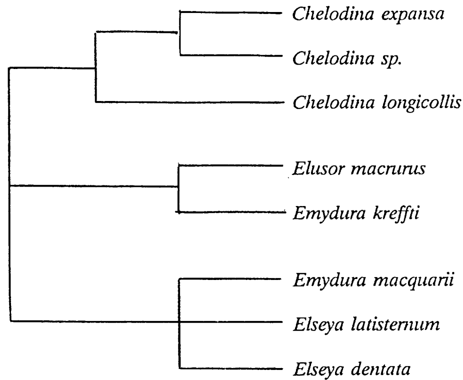
The cladistic analysis of our allozyme data set produced 12 equally parsimonious trees; the consensus tree is shown in Fig. 2B. Unfortunately, the coding of these data in particulate form yielded no resolution of the functional ingroup taxa (*Chelodina* was defined as the outgroup). Each of the 12 equally parsimonious trees yielded a relatively high consistency index of 0.882 so the lack of resolution was not due to excessive homoplasy. The coded characters can be arranged in several ways in support of many different hypotheses of relationship.

The results two different coding methods for the tissue expression data are compared to one another, and to the two treatments of the allozyme data in Table 7. Presence-absence coding of the tissue expression data yielded the largest number of characters and a reasonable degree of resolution (Fig. 5A) but a lower consistency index compared to data coded as tissue arrays (Fig. 5B). What is most interesting about these cladograms is not necessarily what they reveal in common but what they show as differences. *Elseya dentata* was shown to be a problematic taxon. Equivalent treatments of different allozyme data sets have shown this species to be related to *Emydura* spp. (our Fig. 1A; Georges and Adams, 1992) or to *Elusor macrurus* (Fig. 2A). The tissue expression data demonstrate a relationship of *E. dentata* with *Emydura macquarii* and *Elseya latisternum* (Fig. 5A). Further study is necessary to assess the

Table 7
Comparison of variables and results of analyses

Number of taxa	Nature of data	Number of characters	Coding	Number of equally parsimonious trees (figure)	Cophenetic correlation coefficient or consistency index
9	Allozymes	15	Not applicable in distance Wagner	1 (Fig. 2A)	CC = 0.917
9	Allozymes	15	Locus as character	12 (Fig. 2B)	CI = 0.882
8	Tissue expression	41	Presence–absence	2 (Fig. 5A)	CI = 0.631
8	Tissue expression	9	Tissue expression arrays	2 (Fig. 5B)	CI = 0.890

A.



B.

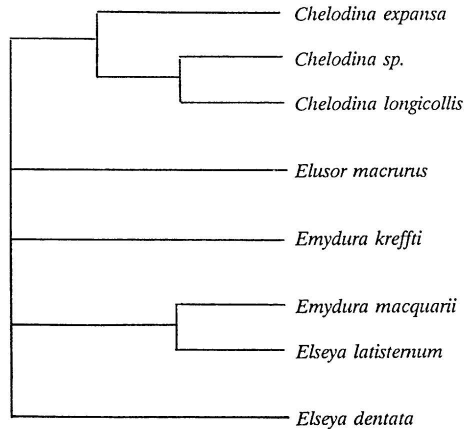


Fig 5. Relationships among selected chelid turtles based on maximum parsimony analyses of (A) presence-absence tissue expression from Table 5 and (B) Tissue-expression arrays from Table 6. Both trees are strict consensus trees of two trees generated in each analysis. Consistency indices of primary trees: $A = 0.631$, $B = 0.890$.

relationship of *E. dentata* to the rest of the Chelidae, and such a resolution is required before we can fully evaluate the information content of the different data sets and the effect of the different coding methods.

Tissue expression data do provide another perspective when estimating phylogenetic relationships. We recommend that their use be considered and that additional coding methods be tested in future studies.

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Appendix A

Representative voucher specimens for tissue samples deposited with the University of Utah (UU).

Chelodia expansa Victoria: Turner's Lagoon (UU 14348); *Chelodian longicollis* Queensland: Fitzroy River, 63 km N & 25 km E Duaringa (UU 16839); *Chelodina* sp. Western Australia: Campbell Creek, 4 km SW Ellenbrae Hstd. (UU 18832); *Elseya dentata* Queensland: Raglan Creek, near Raglan (UU 18514); *Elseya latisternum* New South Wales: Hanging Rock Creek, 11.8 km E & 1.0 km S Kyogle (UU 17080); Richmond River, 2.5 km N & 2.5 km W Wiangaree (UU 15025); Queensland: Einasleigh River, Carpentaria Downs Sta. (UU 18862-63); *Elusor marcurus* Queenland: Mary River (no specific voucher associated with tissue sample); *Emydura kreffti* Queensland: Baramba Creek, 7.8 km S & 9.2 km E Gayndah (UU 17001); Gladys Lagoon, 21 mi. SW Home Hill (UU 15791); *Emydura macquarii* Northern Territory: Barramundie Creek, 9 km S & 7 km W Spring Peak (UU 186112, catalogued as E. *australis*); Queensland: Mitchell River, 16 mi. W & 1.5 mi. S Mt. Carbine (UU 15404, catalogued as E. *australis*) *Emydura victoriae*: Western Australia: Campbell Creek, 4 km SW Ellenbrae Hstd. (UU 18736); Drysdale River, 4 km N Drysdale River Hstd. (UU 18712, 18721).

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