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Monophyly of Lampreys and Hagfishes Supported by Nuclear DNA–Coded Genes

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Abstract. The phylogenetic position of hagfishes in vertebrate evolution is currently controversial. The 18S and 28S rRNA trees support the monophyly of hagfishes and lampreys. In contrast, the mitochondrial DNAs suggest the close association of lampreys and gnathostomes. To clarify this controversial issue, we have conducted cloning and sequencing of the four nuclear DNA–coded single-copy genes encoding the triose phosphate isomerase, calreticulin, and the largest subunit of RNA polymerase II and III. Based on these proteins, together with the Mn superoxide dismutase for which hagfish and lamprey sequences are available in database, phylogenetic trees have been inferred by the maximum likelihood (ML) method of protein phylogeny. It was shown that all the five proteins prefer the monophyletic tree of cyclostomes, and the total log-likelihood of the five proteins significantly supports the cyclostome monophyly at the level of ±1 SE. The ML trees of aldolase family comprising three nonallelic isoforms and the complement component group comprising C3, C4, and C5, both of which diverged during vertebrate evolution by gene duplications, also suggest the cyclostome monophyly.

Key words: Cyclostomes — Gnathostomes — Glycolytic enzymes — Calreticulin — RNA polymerases — Molecular phylogeny

Introduction

The phylogenetic position of hagfishes in vertebrate evolution is currently controversial at both the morphological and molecular levels. According to the traditional view of vertebrate classification, the living jawless vertebrates, lampreys and hagfishes (cyclostomes), are more primitive than jawed vertebrates (gnathostomes) in many morphological features, and they form a monophyletic group, Cyclostomata. There is, however, a growing consensus, based on differences of morphological features of hagfishes from characteristics shared by lampreys and gnathostomes, that lampreys are more closely related to gnathostomes than to hagfishes (Janvier 1981, 1996; Helfman et al. 1997). Recently published molecular data are also controversial on this issue: The 18S and 28S rRNA sequences support the cyclostome monophyly (Stock and Whitt 1992; Mallatt and Sullivan 1998), whereas the mitochondrial DNA (mtDNA) sequences suggest the close, although statistically not significant, association of lampreys and gnathostomes (Rasmussen et al. 1998). The phylogenetic trees inferred from nuclear DNA–coded genes encoding globins and vasotocins are also controversial. In the former, hagfishes and lampreys form a monophyletic group (Goodman et al. 1988), whereas the latter united lampreys with gnathostomes (Suzuki et al. 1995).

There is still a possibility of paralogous comparison in the analyses of globins and vasotocins, each of which comprises multiple copies that were generated during vertebrate evolution by gene duplications. Extensive gene duplications are observed in the early evolution of vertebrates (Iwabe et al. 1996; Suga et al. 1997, 1999a).

The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases with accession numbers AB025322 - AB025334

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Table 1. Degenerate primers used for the cloning of cDNAs encoding cyclostome proteins

Protein	Primer			
		Name	Sequence	Amino acid sequence
TPI	sense	S1	5′-CCGGTACCAAYTGGAARATGAAYGG-3′	NWKMNG
	antisense	A1	5′-ATGGATCCCCIACIARRAAICCRTC-3′	DGFLVP
Calreticulin	sense	S1	5′-GCAGTTYWSIGTNAARCAYGARCA-3′	(S/T)VKHEQ
	antisense	A1	5′-GATIGTICCNWSYTTNACYTGCCA-3′	WQVKSGT
		A2	5′-GATAGGAGGYTCCCAYTCNCCRTC-3′	DGEW(E/T)(A/P)
RpoII and rpoIII	sense	S1	5′-CAGGATCCTTYGAYGGNGAYGARATG-3′	FDGDEM
	antisense	A1	5′-GAGAATTCAYYTGYTTNCCNGTCCA-3′	WTGKQ(I/V)
		A2	5′-GAGAATTCCATYTGNGTNSCNGGYTC-3′	EP(A/G)TQM
Aldolase	sense	S1	5′-CAGGATCCAARGGIATHYTIGCNGC-3′	KGILAA
	antisense	A1	5′-CAGAATTCGTIACCATRTTIGGYTT-3′	KPNMVT
Enolase	sense	S1	5′-CATAGCAGCAGTTCCIWSNGGNGC-3′	VPSGA
	antisense	A1	5′-CTCGGTAACISWNCCDATYTGRTT-3′	NQIG(S/T)V
		A2	5′-AGGRTTIGTIACNGTNARRTCRTC-3′	DDLTVTN

TPI, triose phosphate isomerase; rpoII and rpoIII, the largest subunits of RNA polymerase II and III, respectively. EcoRI and BamHI sites are underlined. The primer is the same for *E. burgeri* (hagfish) and *L. reissneri* (lamprey).

As we have shown in the accompanying paper (Suga et al. 1999b), these gene duplications are likely to have occurred around the time of divergence of cyclostomes and gnathostomes. Furthermore, only the limited numbers of the amino acid sites are available for comparison in vasotocins. In addition, phylogenetic trees inferred from sequence data sets with large differences in evolutionary rates between different taxa are often misleading (Felsenstein 1978). In the 18S and 28S rRNA trees, unusually high evolutionary rate is observed on lineage leading to hagfishes (Stock and Whitt 1992; Mallatt and Sullivan 1998). The evolutionary rates of mtDNA-coded proteins vary greatly for different vertebrate groups (Rasmussen et al. 1998). For inferring a robust tree representing the relationship of cyclostomes and gnathostomes, it is therefore important to use many nuclear DNA–coded single copy genes with approximately constant evolutionary rates over a long evolutionary period.

In this report we have isolated and sequenced four nuclear DNA–coded single copy genes from *Eptatretus burgeri* (hagfish) and *Lampetra reissneri* (lamprey). The maximum likelihood (ML) trees inferred by these proteins, together with the Mn superoxide dismutase (SOD), support the cyclostome monophyly at the significant level of ±1 SE. In addition, the cyclostome monophyly is also supported by sets of duplicated proteins, aldolase A, B, and C, and the complement components C3, C4, and C5, as well as those of four gene groups belonging to the *src* family described in the accompanying paper (Suga et al. 1999b).

Materials and Methods

Isolation and Sequencing of Hagfish and Lamprey cDNAs

Total RNA of *E. burgeri* (hagfish) was extracted from the liver, and that of *L. reissneri* (lamprey) from the whole body using acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction method. Total RNAs of *E. burgeri* and *L. reissneri* were transcribed to cDNAs using oligo(dT) primer with reverse transcriptase (SuperScript II, Gibco BRL). These cDNAs were used as templates for PCR amplification with Expand High-Fidelity PCR System (Roche). The sense and antisense degenerate primers were designed from conserved amino acid residues as shown in Table 1.

PCR amplification was conducted as follows: 2-min denaturation step at 94°C; then 10 cycles of 94°C (20 s), 46–50°C (30 s), and 72°C (2 min); followed by 25 cycles of 94°C (20 s), 50–60°C (30 s), and 72°C (2 min). PCR amplification was carried out using primers S1 and A1 (see Table 1) for all genes shown in Table 1, followed by nested PCR with S1 and A2 for calreticulin and enolase.

The PCR products were separated in a 1.5% agarose gel containing ethidium bromide. Products of expected size were isolated as gel slices, purified by using DNA purification kit (Toyobo) and cloned into the pT7Blue vector (Novagen). More than three independent clones were isolated and sequenced by dideoxy chain termination method (Sambrook et al. 1989) with ABI DNA Sequencer 377 (Perkin-Elmer) using synthetic nucleotide as primers. The 3′ ends of aldolase, calreticulin, and enolase sequences were determined by 3′ rapid amplification of cDNA ends (Frohman et al. 1988). The sequences of the largest subunits of RNA polymerase II (rpoII) and III (rpoIII) were extended to 3′ ends by using a sequence between S1 and A1 as a primer of sense strand and A2 as that of antisense strand.

Phylogenetic Tree Inference

Optimal alignment of sequences was obtained by the methods of Needleman and Wunsch (1970) and Berger and Munson (1991), together with manual inspections. Using the amino acid sequences in regions where unambiguous alignment is possible, phylogenetic trees were inferred by the ML method of protein phylogeny (Kishino et al. 1990; Adachi and Hasegawa 1996) based on the JTT model (PROTML in Adachi and Hasegawa's program package, MOLPHY); amino acid sites where gaps exist in the alignment were excluded from the calculation. The bootstrap analysis was carried out by the methods of Felsenstein (1985) and Kishino et al. (1990). The local bootstrap probability (LBP) was calculated by the method of Adachi and Hasegawa (1996).

Results and Discussion

Phylogenetic Relationship of Cyclostomes and Gnathostomes Based on Single-Copy Genes

To exclude a possibility of paralogous comparison, we selected four nuclear DNA–coded genes encoding the

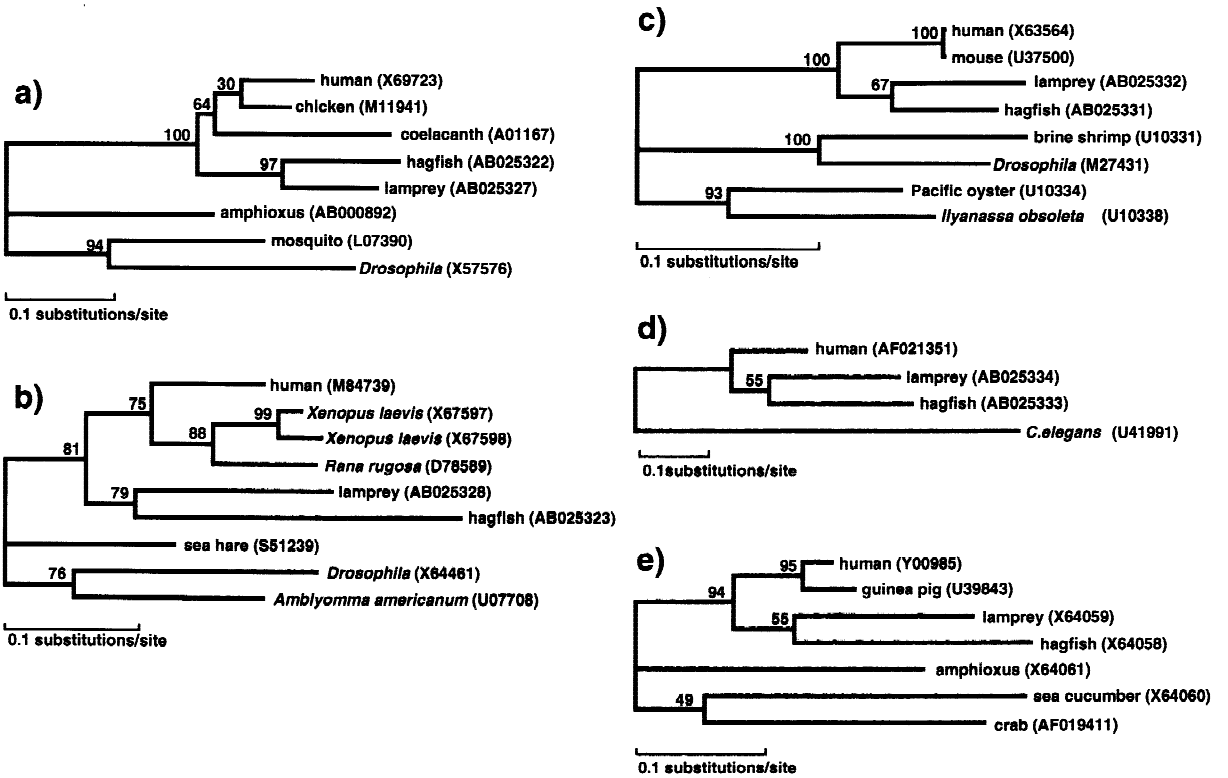


Fig. 1. Vertebrate phylogeny inferred from **a,** triose phosphate isomerase, **b,** calreticulin, **c,** RNA polymerase II largest subunit, **d,** RNA polymerase III largest subunit, and **e,** Mn superoxide dismutase. The trees were inferred by the ML method of protein phylogeny (Kishino et al. 1990; Adachi and Hasegawa 1996). The following species were used for outgroups: amphioxus, mosquito, and *Drosophila* in **a;** sea hare, *Drosophila,* and *Amblyomma americanum* in **b;** brine shrimp, *Drosophila,* Pacific oyster, and *Ilyanassa obsoleta* in **c;** *C. elegans* in **d;** and amphioxus, sea cucumber, and crab in **e.** The number at each node represents the local bootstrap probability calculated by the method of Adachi and Hasegawa (1996). The branch length is proportional to the number of amino acid substitutions. Sequence data were taken from GenBank release 110, except for hagfish and lamprey sequences in **a–d.** The accession numbers of sequences used are shown in parentheses.

triose phosphate isomerase (TPI), calreticulin, which modulates both integrin adhesive functions and integrin-initiated signaling (Coppolino et al. 1997), and the largest subunit of RNA polymerase II (rpoII) and III (rpoIII), for which no gene duplication on vertebrate lineage has been reported to date, except for calreticulin, in which a recent gene duplication on *Xenopus* lineage was observed. In addition, the evolutionary rates of TPI and calreticulin are approximately constant over a wide evolutionary distance (Iwabe et al. 1995; Nikoh et al. 1997; Kuraku et al. unpublished data). Cloning of these cDNAs from *L. reissneri* and *E. burgeri* has been conducted by the method described in Materials and Methods. For each of TPI, calreticulin, rpoII and rpoIII, we obtained one cDNA from each of *L. reissneri* and *E. burgeri.* The deduced amino acid sequences showed apparent similarity to those from vertebrates and invertebrates, and they contain signature motifs at the precise position (alignment not shown). It is therefore highly likely that the isolated cDNAs are the products of the respective genes. In addition, from database searches, we have sampled the data set of Mn superoxide dismutases (SODs) for which hagfish and lamprey sequences are available (Smith and Doolittle 1992).

Unambiguous alignment is possible for regions (comprising 206 amino acid positions in total) corresponding to the amino acid positions 18–33, 36–56, and 59–227 of human sequence for TPI; those corresponding to 102–346, and 348–366 for calreticulin (264 amino acids), those corresponding to 502–1096 for rpoII (595 amino acids); those corresponding to 506–626, 628–937, 939–954, and 956–1029 for rpoIII (521 amino acids); and those corresponding to 41–106, 108–155, and 157–184 (142 amino acids) for Mn SOD. On the basis of the alignments and using invertebrate sequences as outgroups, phylogenetic trees of vertebrates have been inferred by the following procedures: First, for each protein, the phylogenetic tree was inferred by the method described in the accompanying paper (Suga et al. 1999b); this method consists of performing rearrangement of tree topology for a limited number of initial trees by the methods of nearest-neighbor interchange (NNI; Swofford et al. 1996; Adachi and Hasegawa 1996) and subtree pruning and regrafting (SPR; Swofford et al. 1996). All trees inferred by the five proteins showed an independent cluster of gnathostomes. Next, assuming the phylogenetic relationship ([(mammals, [birds, reptile]), amphibians], fishes) among gnathostomes, the ML method of

Table 2. Phylogenetic relationships of gnathostomes (G), hagfishes (H), and lampreys (L) inferred from five different single-copy proteins

	Tree 1 ((H,L),G)		Tree 2 ((G,L),H)		Tree 3 ((G,H),L)	
Protein	$\Delta/_1 \pm$ SE	P_1	$\Delta/_2 \pm$ SE	P_2	$\Delta/_3 \pm$ SE	P_3
TPI	ML	0.96	−14.8 ± 7.9	0.02	−14.3 ± 8.1	0.03
Calreticulin	ML	0.82	−5.4 ± 5.8	0.12	−6.2 ± 5.5	0.06
RpoII	ML	0.69	−10.7 ± 6.5	0.005	−4.4 ± 8.4	0.30
RpoIII	ML	0.55	−6.6 ± 4.7	0.008	−0.9 ± 6.6	0.45
SOD	ML	0.57	−5.0 ± 4.2	0.007	−0.7 ± 6.4	0.43
Total	ML	0.92	−33.5 ± 13.2	0.001	−22.7 ± 15.0	0.08

The trees were inferred by the ML method of protein phylogeny (Kishino et al 1990; Adachi and Hasegawa 1996). For outgroups used, see legends of Fig. 1. ((H,L),G), for example, represents a tree that gnathostomes are outgroup to hagfish–lamprey clade. $\Delta/_i$ represents the difference of log-likelihood of tree i (i = 1, 2, or 3) from that of the ML tree. P_i represents the bootstrap probability that tree i is realized. TPI, triose phosphate isomerase; rpoII and rpoIII, the largest subunits of the RNA polymerase II and III, respectively; SOD, Mn superoxide dismutase.

protein phylogeny (Kishino et al. 1990; Adachi and Hasegawa 1996) has been carried out for four OTUs (gnathostome group, lamprey, hagfish, and outgroup); the following invertebrates were used as outgroups; amphioxus, mosquito, and *Drosophila* in TPI tree; sea hare, *Drosophila,* and *Amblyomma americanum* in calreticulin tree; brine shrimp, *Drosophila,* Pacific oyster, and *Ilyanassa obsoleta* in rpoII tree; *C. elegans* in rpoIII tree; and amphioxus, sea cucumber, and crab in SOD tree.

Figure 1 shows the ML trees inferred from the five protein species. According to Fig. 1, all the trees support the cyclostome monophyly. Particularly in the TPI tree, the monophyletic relationship of hagfishes and lampreys is significant at the level of ±1 SE. In the calreticulin, rpoII, and rpoIII, and SOD trees, the cyclostome monophyly is not statistically significant. The total log-likelihood of the five protein species unambiguously supports the cyclostome monophyly at the level of ±1 SE (Table 2). This result is consistent with that of 18S and 28S rRNA trees (Stock and Whitt 1992; Mallatt and Sullivan 1998). The present result that in each protein except for TPI, the cyclostome monophyly is not statistically significant may suggest close divergence times of three lineages leading to hagfishes, lampreys, and gnathostomes.

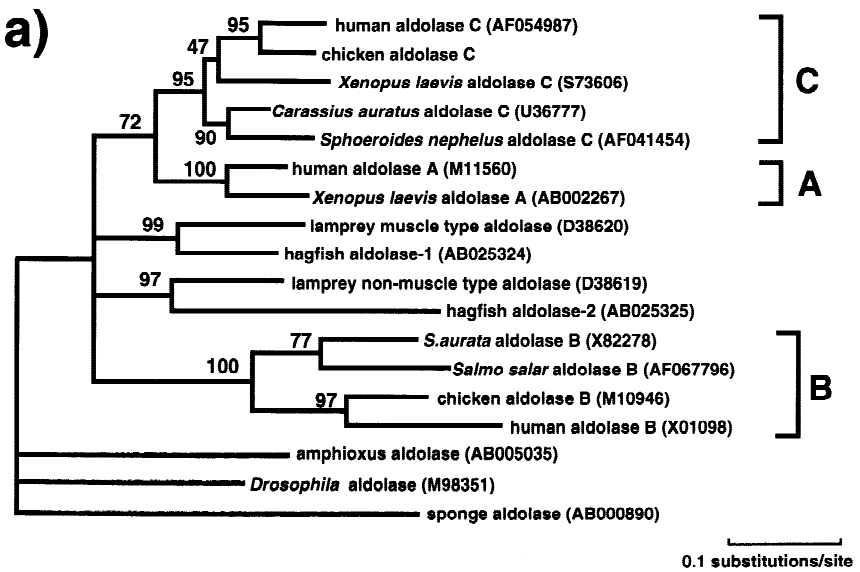
Phylogenetic Relationship of Cyclostomes and Gnathostomes Inferred from Duplicated Genes

As we have shown in the accompanying paper (Suga et al. 1999b), vertebrates have multiple isoform genes that have been generated in the early evolution of vertebrates around the time of divergence of cyclostomes and gnathostomes. Thus, a careful analysis is necessary for understanding the phylogenetic relationship of cyclostomes and gnathostomes based on the set of duplicated genes. In this report, phylogenetic analyses have been carried out by two enzymes, aldolase and enolase, each of which has different tissue-specific isoforms generated by gene duplication during vertebrate evolution. In addition, from database searches, we obtained the set of sequence data of the complement components C3, C4, and C5, for which hagfish and lamprey sequences are available.

Gnathostomes have three duplicated aldolases, aldolase A, B, and C, which differ from each other in tissue distribution. Also, lampreys have two nonallelic aldolases, muscle- and nonmuscle-type aldolase (Zhang et al. 1995). Because no hagfish aldolase sequence has been reported to date, we have conducted isolation and sequencing of hagfish aldolase cDNAs by the method described in Materials and Methods. We obtained two cDNAs, aldolase-1 and aldolase-2, whose amino acid sequences show close similarity to the muscle- and nonmuscle-type aldolases from lampreys, respectively.

The amino acid sequences of these cyclostome aldolases were compared with those of gnathostome and invertebrate aldolases. Unambiguous alignment is possible for regions corresponding to amino acid positions 34–68, 72–239, 241–343, and 352–364 of human aldolase C (319 amino acid sites total). Based on the alignment and using amphioxus, *Drosophila,* and sponge aldolases as an outgroup, we first inferred a phylogenetic tree by an approximate method for inferring the ML tree described by the accompanying paper (Suga et al. 1999b). Because the inferred ML tree showed five independent clusters comprising gnathostome aldolase A, B, C, and cyclostome aldolase-1 and -2, a further tree analysis based on the ML method of protein phylogeny (Kishino et al. 1990; Adachi and Hasegawa 1996) was carried out for eight OTUs comprising three gnathostome aldolase A, B, and C groups (tree topology in each group was assumed), four cyclostome aldolases (i.e., hagfish aldolase-1 and -2, and lamprey muscle- and nonmuscle-type aldolases) and the outgroup.

Figure 2 shows the result. The tree b in Fig. 2 is ML



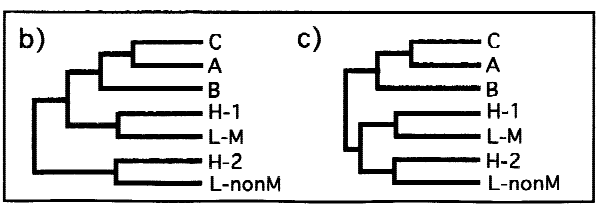


Fig. 2. The ML tree of vertebrate aldolases. Initially a phylogenetic tree was inferred by an approximate method for inferring the ML tree described by the accompanying paper (Suga et al. 1999b) using amphioxus, *Drosophila*, and sponge aldolases as an outgroup. Based on the ML method of protein phylogeny (Kishino et al. 1990; Adachi and Hasegawa 1996), a further ML analysis was carried out for eight OTUs comprising three gnathostome aldolase A, B, and C groups (tree topology in each group was assumed); four cyclostome aldolases (i.e., hagfish aldolase-1 and -2, and lamprey muscle- and nonmuscle-type aldolases), and the outgroup. The tree **b** is ML tree. The tree **c,** however, is also likely; the difference of log-likelihood value of tree **c** from that of tree **b** is −0.6 ± 3.6. Thus, assuming the star phylogeny of five groups, gnathostome aldolase A, B, and C, and cyclostome aldolase-1 and -2, tree **a** was inferred. The number at each node represents the local bootstrap probability estimated by the method of Adachi and Hasegawa (1996). A, B, and C in **a–c,** aldolase A, B, and C, respectively; L-M and L-nonM in **b** and **c,** muscle- and nonmuscle-type aldolases of lamprey; H-1 and H-2, aldolase-1 and -2 of hagfish. Accession number of each sequence is shown in parentheses, except for chicken aldolase C, which was taken from Ono et al. (1990).

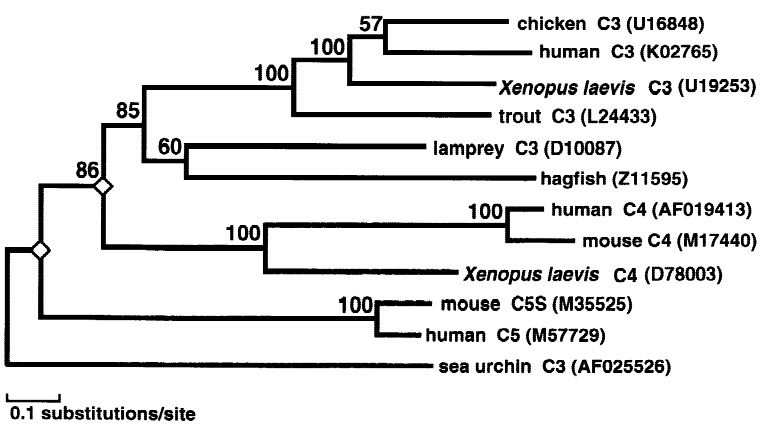


Fig. 3. The ML tree of vertebrate complement components C3, C4, and C5. The tree was inferred as follows: On the basis of the alignment of the highly conserved region corresponding to amino acid positions 122–1636 of human C3 (1,039 amino acid sites in total, excluding gaps) and using sea urchin C3 as an outgroup, a phylogenetic tree was inferred by the same method as in Fig. 2. The number at each node represents the local bootstrap probability estimated by the method of Adachi and Hasegawa (1996). Rhombuses, gene duplications. Accession number of each sequence is shown in parentheses.

tree, but an alternative tree, tree c, is equally likely; the difference of log-likelihood value of tree c from that of tree b is only −0.6 ± 3.6. In tree b, the bootstrap probability that the hagfish aldolase-1 and the lamprey muscle-type aldolase forms a cluster is very high (98%). For the pair of the hagfish aldolase-2 and the lamprey nonmuscle-type aldolase, the corresponding probability is also high (86%). Assuming the star phylogeny of four groups, the gnathostome aldolase A/C, and B, and the cyclostome aldolase-1 and -2, tree a in Fig. 2 was inferred. Although we do not know at present whether the cognate gene of gnathostome aldolase A/C (or A/B/C) is aldolase-1 or aldolase-2, the aldolase tree strongly suggests the cyclostome monophyly.

The phylogenetic tree of the complement components C3, C4, and C5 also provides supporting evidence for the cyclostome monophyly. The ML tree of the complement components was inferred by the same method as in the case of aldolases (Fig. 3). According to Fig. 3, the hagfish and lamprey lineages show a monophyletic group, although the bootstrap probability is not high enough, and gene duplications that gave rise to C3, C4, and C5 antedate the cyclostome–gnathostome split. In addition, as we have shown in the accompanying paper, the phy-

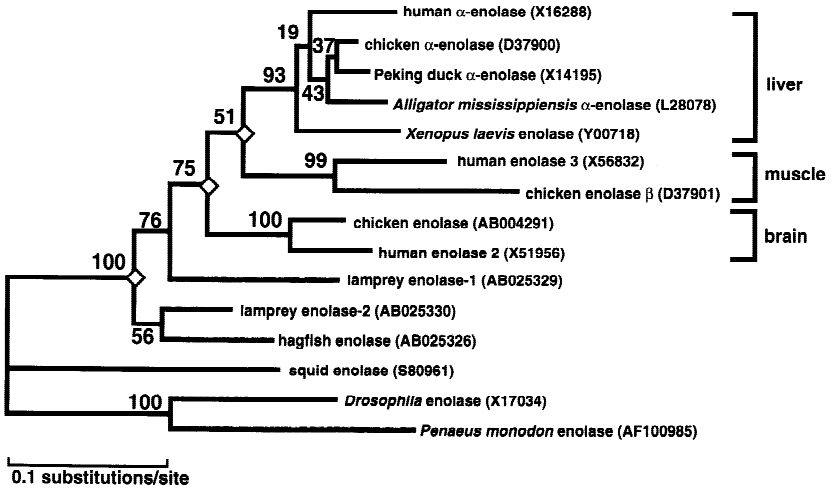


Fig. 4. The ML tree of vertebrate enolases. The tree was inferred as follows: On the basis of the alignment of the highly conserved region corresponding to amino acid positions 40–417 of human enolase α, and using squid, *Drosophila,* and *Penaeus monodon* enolases as an outgroup, a phylogenetic tree was inferred by the same method as in Fig. 2. The number at each node represents the local bootstrap probability estimated by the method of Adachi and Hasegawa (1996). Rhombuses, gene duplications. Accession number of each sequence is shown in parentheses.

logenetic trees of four subfamilies belonging to the protein tyrosine kinase family provide four more examples of duplicated genes that suggest the cyclostome monophyly. These include EphB in Eph subfamily, fibroblast growth factor receptor (FGFR) 3/4 in FGFR subfamily, *src* in *src* subfamily, and CSF-1R/*c-kit* in platelet-derived growth factor receptor subfamily.

Missing one of the duplicated genes on one lineage of cyclostomes due to deletion event during evolution or due to unsuccessful cloning might mislead one into assuming an incorrect relationship between cyclostomes and gnathostomes. By cloning and sequencing procedures described in Materials and Methods, we have isolated two cDNAs encoding enolase isoforms, enolase-1 and enolase-2, from *L. reissneri.* We have also isolated one enolase cDNA from *E. burgeri.* Including these sequences, a phylogenetic tree was inferred by the same method as in the case of aldolases (Fig. 4). According to Fig. 4, the lamprey enolase-1 might be the homolog of gnathostome enolases (i.e., liver, muscle, and brain types), which are likely to have diverged by two gene duplications in the early evolution of gnathostome split. If we did not successfully isolated enolase-2 from *L. reissneri,* the enolase tree might mislead into the currently prevailing belief based on morphological features that lampreys are more closely related to gnathostomes than to hagfishes.

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References

Adachi J, Hasegawa M (1996) Computer science monographs, no. 28, MOLPHY: programs for molecular phylogenetics. Institute of Statistical Mathematics, Tokyo

Berger MP, Munson PJ (1991) A novel randomized iterative strategy for aligning multiple protein sequences. CABIOS 7:479–484

Coppolino MG, Woodside MJ, Demaurex N, Grinstein S, St-Arnaud R, Dedhar S (1997) Calreticulin is essential for integrin-mediated calcium signalling and cell adhesion. Nature 386:843–847

Felsenstein J (1978) Cases in which parsimony and compatibility methods will be positively misleading. Syst Zool 27:401–410

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Frohman MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85: 8998–9002

Goodman M, Pedwaydon J, Czelusniak J, Suzuki T, Gotoh T, Moens L, Shishikura F, Walz D, Vinogradov S (1988) An evolutionary tree for invertebrate globin sequences. J Mol Evol 27:236–249

Helfman GS, Collette BB, Facey DE (1997) The diversity of fishes. Blackwell Science Press, Malden, MA

Iwabe N, Kuma K, Nikoh N, Miyata T (1995) Molecular clock for dating of divergence between animal phyla. Jpn J Genet 70:687–692

Iwabe N, Kuma K, Miyata T (1996) Evolution of gene families and relationship with organismal evolution: rapid divergence of tissue-specific genes in the early evolution of chordates. Mol Biol Evol 13:483–493

Janvier P (1981) The phylogeny of the Craniata, with particular reference to the significance of the fossil agnathans. J Vert Paleontol 1:121–142

Janvier P (1996) Early vertebrates. Oxford University Press, Oxford

Kishino H, Miyata T, Hasegawa M (1990) Maximum likelihood inference of protein phylogeny and the origin of chloroplasts. J Mol Evol 30:151–160

Mallatt J, Sullivan J (1998) 28S and 18S rDNA sequences support the monophyly of lampreys and hagfishes. Mol Biol Evol 15:1706–1718

Needleman SB, Wunsch CD (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. J Mol Biol 48:443–453

Nikoh N, Iwabe N, Kuma K, Ohno M, Sugiyama T, Watanabe Y, Yasui K, Shicui Z, Hori K, Shimura Y, Miyata T (1997) An estimate of divergence time of Parazoa and Eumetazoa and that of Cephalochordata and Vertebrata by aldolase and triose phosphate isomerase clocks. J Mol Evol 45:97–106

Ono K, Tsutsumi K, Ishikawa K (1990) Structure of chicken aldolase C mRNA and its expression in the liver and brain during development. Biochem Int 20:921–929

Rasmussen A, Janke A, Arnason U (1998) The mitochondrial DNA molecule of the hagfish (*Myxine glutinosa*) and vertebrate phylogeny. J Mol Evol 46:382–388

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2d ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Smith MW, Doolittle RF (1992) A comparison of evolutionary rates of the two major kinds of superoxide dismutase. J Mol Evol 34:175–184

Stock DW, Whitt GS (1992) Evidence from 18S ribosomal RNA sequences that lampreys and hagfishes form a natural group. Science 257:787–789

Suga H, Kuma K, Iwabe N, Nikoh N, Ono K, Koyanagi M, Hoshiyama D, Miyata T (1997) Intermittent divergence of the protein tyrosine kinase family during animal evolution. FEBS Lett 412:540–546

Suga H, Koyanagi M, Hoshiyama D, Ono K, Iwabe N, Kuma K, Miyata T (1999a) Extensive gene duplication in the early evolution of animals before the parazoan-eumetazoan split demonstrated by G protein and protein tyrosine kinases from sponge and hydra. J Mol Evol 48:646–653

Suga H, Hoshiyama D, Kuraku S, Katoh K, Kubokawa K, Miyata T (1999b) Protein tyrosine kinase cDNAs from amphioxus, hagfish, and lamprey; isoform duplications around the divergence of cyclostomes and gnathostomes. J Mol Evol 49:729–735

Suzuki M, Kubokawa K, Nagasawa H, Urano A (1995) Sequence analysis of vasotocin cDNAs of the lamprey, *Lampetra japonica*, and the hagfish, *Eptatretus burgeri:* evolution of cyclostome vasotocin precursors. J Mol Endocrinol 14:67–77

Swofford DL, Olsen GJ, Waddell PJ, Hillis DM (1996) Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK (eds) Molecular systematics, 2d edition. Sinauer Associates, Sunderland, MA, pp 407–514

Zhang R, Yatsuki H, Kusakabe T, Iwabe N, Miyata T, Imai T, Yoshida M, Hori K (1995) Structures of cDNAs encoding the muscle-type and non-muscle-type isozymes of lamprey fructose bisphosphate aldolases and the evolution of aldolase genes. J Biochem 117:545–553