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Molecular Analysis of Ependymins from the Cerebrospinal Fluid of the Orders Clupeiformes and Salmoniformes: No Indication for the Existence of an Euteleost Infradivision

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Summary. Ependymins represent the predominant protein constituents in the cerebrospinal fluid of many teleost fish and they are synthesized in meningeal fibroblasts. Here, we present the ependymin sequences from the herring (*Clupea harengus*) and the pike (*Esox lucius*). A comparison of ependymin homologous sequences from three different orders of teleost fish (Salmoniformes, Cypriniformes, and Clupeiformes) revealed the highest similarity between Clupeiformes and Cypriniformes. This result is unexpected because it does not reflect current systematics, in which Clupeiformes belong to a separate infradivision (Clupeomorpha) than Salmoniformes and Cypriniformes (Euteleostei). Furthermore, in Salmoniformes the evolutionary rate of ependymins seems to be accelerated mainly on the protein level. However, considering these inconstant rates, neither neighbor-joining trees nor DNA parsimony methods gave any indication that a separate euteleost infradivision exists.

Key words: Evolution — Teleostei — *Clupea harengus* — *Esox lucius* — Fish — Polymerase chain reaction — Calcium binding

Ependymins, erroneously named after their first immunohistochemical localization at ependymal zones of the goldfish (reviewed in Shashoua 1985), are secretory products of meningeal cells (Königstorfer et al. 1990; Sterrer et al. 1990; Rinder et al. 1992), and they represent the predominant proteins in the cerebrospinal fluid (CSF) of many teleost fish (Hoffmann 1992, 1993).

Generally, ependymins show calcium-binding capacity (Schmidt and Makiola 1991) and they share certain characteristics with glycoproteins involved in cell-contact phenomena (Hoffmann et al. 1992). However, their molecular function is probably defined by their association with the extracellular matrix (Schwarz et al. 1993).

So far, ependymins have been characterized only in two orders of teleost fish (Cypriniformes and Salmoniformes). A comparison of these sequences revealed that ependymins are not well conserved during evolution (about 40% similarity; Müller-Schmid et al. 1992). Thus, in the past all attempts failed to detect homologous proteins in higher vertebrates. Due to their quasi-tetraploid genome, two ependymin genes have been identified in the goldfish (*Carassius auratus*) as well as in the rainbow trout (*Oncorhynchus mykiss*) which probably originated by a gene duplication about 70 million years ago (Königstorfer et al. 1989b; Müller-Schmid et al. 1992). In contrast, the zebrafish (*Brachydanio rerio*) contains only a single ependymin gene (Sterrer et al. 1990; Rinder et al. 1992).

In order to eventually characterize ependymin homologous sequences in higher vertebrates, we developed a multistep search protocol for ependymin sequences using the polymerase chain reaction

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(PCR). The search starts at very basal teleost fish, viz. the herring (order Clupeiformes), an order belonging to the monophyletic infradivision Clupeomorpha, which represents nowadays one of the four majour groups of teleost fish (Greenwood et al. 1966; Lauder and Liem 1983; Nelson 1984). In contrast, Cypriniformes and Salmoniformes were both grouped into the monophyletic infradivision Euteleostei (Lauder and Liem 1983). So far, this classification is based upon various morphological criteria, and in the past, our view concerning teleostean phylogeny has changed dramatically (review: Patterson 1976). Teleosts, which comprise about 20,000 species, constitute by far the most diverse group of vertebrates and are first known from the Upper Triassic (Carroll 1988). In the fossil record, Clupeomorpha first appeared in the Lower Cretaceous, whereas euteleost diversity arose during the Cenomanian of the Upper Cretaceous (Carroll 1988).

Based on the assumption that ependymin sequences from the same infradivision would show a higher degree of similarity than ependymins originating from different infradivisions, we expected the ependymin from the herring to be quite discernible from the sequences obtained from euteleost fish.

Materials and Methods

Herrings (*Clupea harengus*) were kindly provided by the Biologische Anstalt Helgoland. A pike (*Esox lucius*) was a gift from Dr. R. Hoffmann (University of Munich). Isolation of mRNA, cyclic thermal amplification of cDNA with Taq polymerase, cDNA cloning, and DNA sequence analysis have been described previously (Müller-Schmid et al. 1992).

For Western analysis, antisera POm-1 (against the C-terminal end of rainbow trout ependymin I; Müller-Schmid et al. 1992) and PCa-1 (against the C-terminal end of goldfish ependymins; Königstorfer et al. 1989b) were used in a 1:1,000 dilution according to Königstorfer et al. (1989b).

The different ependymin sequences were aligned with the program CLUSTAL 5.0 (Higgins and Sharp 1988, 1989). Using the programs SEQBOOT, DNADIST, PROTDIST (J. Felsenstein, personal communication), UPGMA, NEIGHBOR joining, and CONSENSE of the PHYLIP 3.4 package (Felsenstein 1991) the original sequences have been bootstrapped 100 times (Felsenstein 1985) and each replicate sequence set has been transformed into distance matrices of values corrected for multiple substitutions (Kimura 1980). From each of these matrices, single UPGMA or neighbor-joining trees have been calculated with a randomized input order of species. Finally, an unrooted consensus tree (R-option activated) was created with the nodal confidence values. User-defined trees have been calculated with the DNAPARS program (U-option activated) of the PHYLIP 3.4 package.

cDNA Cloning of Ependymin from C. harengus. Based on the known cDNA sequences of ependymins from *C. auratus* (Königstorfer et al. 1989a, 1989b), *B. rerio* (Sterrer et al. 1990), and *O. mykiss* (Müller-Schmid et al. 1992), the consensus oligonucleotide EPD5 d(CCCGAATTCTCGAG*AT/GTA/TCAGC/TTAT/CGACTC*) was constructed. The italicized region recognizes part of ependymin cDNAs. For cloning of the 3'-end, oligo(dT)-primed cDNA from the brain of *C. harengus* was then used as a template for polymerase chain reaction (PCR) with oligonucleotides EPD5 and PCR3' d(CCCTCGAGGATCCGAATTC$[T]_{18}$). Subcloning of the PCR product into the EcoRI site of pBluescript-II/SK$^-$ (Stratagene) resulted in cDNA clone pCh-5.3'-15. Subsequently, the 5'-end of the corresponding mRNA was cloned according to Frohman et al. (1988). Here, cDNA was primed with EPD10 d(CCCGAATTCTCG*AGAAGATGCCCTCTTCA/GAA*), dA-tailed, and amplified with PCR3' and EPD15 d(CCCGAAT*TCATCTTTGAAGTGGAG*). After subcloning the PCR product into the EcoRI site of pBluescript-II/SK$^-$, cDNA clones pCh-15.5'-5 and -9 were analyzed.

cDNA Cloning of Ependymin from E. lucius. Based on the cDNA sequences of ependymins from *C. auratus, B. rerio,* and *C. harengus,* the consensus oligonucleotide EPD11 d(CCCGAA*TTCGAAGAGGGT/CA/GTC/AT/CTC/TTATGA*) was constructed. The 3'-end of oligo(dT)-primed cDNA from the brain of *E. lucius* was then amplified with oligonucleotides EPD11 and PCR3'. Subcloning of the PCR product into the EcoRI site of pBluescript-II/SK$^-$ yielded cDNA clones pEl-57.3'-5 and -6. The sequence of the corresponding 5'-end was obtained from the same cDNA after dC-tailing, amplification with PCR5' d(CCGGATCCTCGAGAATTCTAGA$[G]_{14}$) and EPD18 d(CCCGAATT*CTCTAGAGCATGAGCGTG*), and subcloning the PCR products into the EcoRI site of pBluescript-II/SK$^-$ (cDNA clones pEl-67.5'-46 and -92).

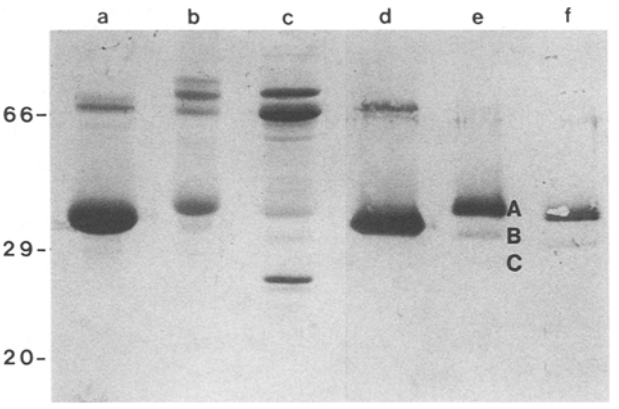

Fig. 1. SDS–polyacrylamide gel electrophoresis (13%). Coomassie staining of proteins from 0.3 µl CSF of *C. harengus* (lane a), *O. mykiss* (lane b), and *E. lucius* (lane c). Western analysis of CSF from *C. harengus* (0.08 µl, lane d), *O. mykiss* (0.005 µl, lane e), and *E. lucius* (0.01 µl, lane f) using antiserum POm-1 in a 1:1,000 dilution. *A, B,* and *C* (lane e) denote the different glycoforms of rainbow trout ependymins according to Müller-Schmid et al. (1992).

Results

Figure 1 represents an electrophoretic analysis of CSF from the herring in comparison with CSFs from two salmoniform fish, viz. the rainbow trout (family Salmonidae) and the pike (family Esocidae). The predominant protein from the herring, with an

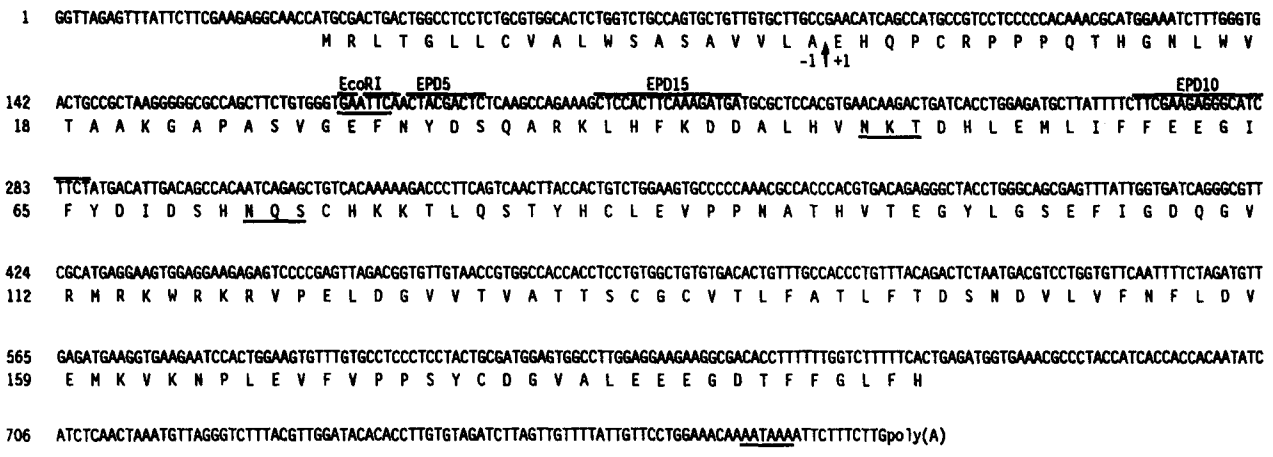

Fig. 2. Nucleotide sequence and translation of the ependymin transcript from *C. harengus* as deduced from cDNA clones pCh-15.5′-9 (positions 1–180), pCh-15.5′-5 (positions 6–221), and pCh-5.3′-15 (positions 176–802). Position 46 is changed to T in pCh-15.5′-5. The potential cleavage site for signal peptidase is indicated by an *arrow*. The polyadenylation signal, restriction sites, and potential N-glycosylation sites are *underlined*. Also marked are the positions of the synthetic oligonucleotides. This nucleotide sequence has been submitted to GenBank with accession number L09065.

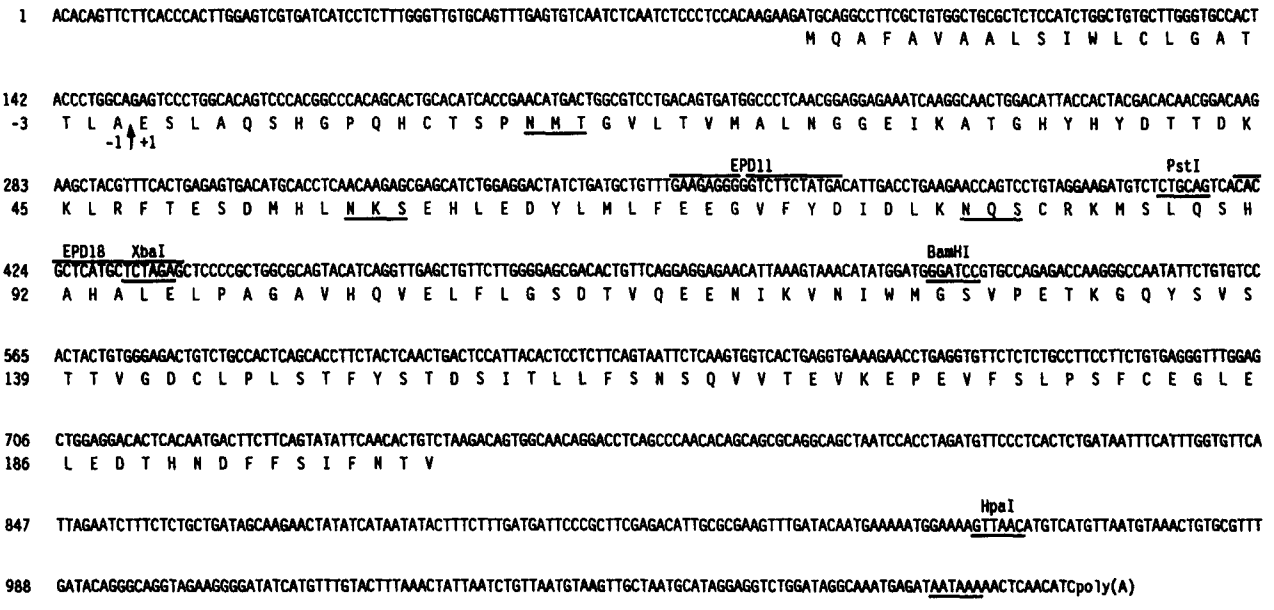

Fig. 3. Nucleotide sequence and translation of the ependymin transcript from *E. lucius* as deduced from cDNA clones pEl-67.5′-46 (positions 1–438) and pEl-57.3′-5 (positions 352–1,106). The potential cleavage site for signal peptidase is indicated by an *arrow*. The polyadenylation signal, restriction sites, and potential N-glycosylation sites are *underlined*. Also marked are the positions of the synthetic oligonucleotides. This nucleotide sequence has been submitted to GenBank with accession number L09066.

MW of about 34k, shows cross-reactivity with antiserum POm-1. Also, weak immunostaining with antiserum PCa-1 is observed (data not illustrated). Thus, this band has to be considered as the ependymin from *C. harengus*. In contrast, immunoreactive bands in the CSF from the pike show the same pattern as in the trout, viz. three different glycosylation variants termed A, B, and C (Müller-Schmid et al. 1992). Generally, ependymins from the pike show a slightly diminished MW when compared with the trout.

In Fig. 2, the complete ependymin cDNA sequence from *C. harengus* is shown. The encoded precursor consists of 212 amino acid residues with a calculated MW of 23,678, which is reduced after cleavage by signal peptidase to 21,622. Two potential N-glycosylation sites are present. In analogy to ependymins from Cypriniformes and Salmoniformes, N-glycosylation presumably accounts for the difference compared to the MW observed (34k).

The ependymin cDNA sequence from *E. lucius* encodes a precursor comprised of 221 amino acid residues (MW of 24,479), which is reduced to 22,345 by signal peptidase (Fig. 3). Similarly, as in the trout, three potential N-glycosylation sites are observed.

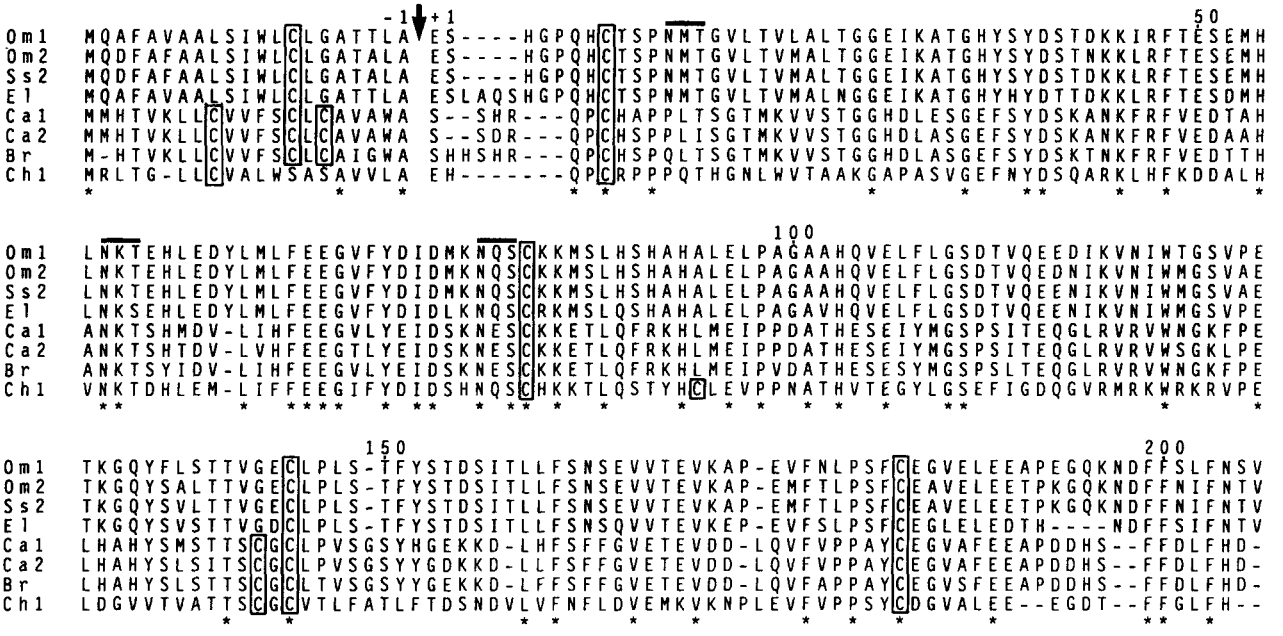

Fig. 4. Comparison of all ependymin homologous sequences known so far using the CLUSTAL 5.0 program. *Gaps* are introduced to maximize homologies and *asterisks* indicate invariant positions in all amino acid sequences. The *arrow* represents the cleavage site for signal peptidase. Potential N-glycosylation sites are indicated by *horizontal bars* and cysteine residues are enclosed in *boxes*. Om, *O. mykiss* (Müller-Schmid et al. 1992); Ss, *S. salar* (Müller-Schmid et al. 1992); El, *E. lucius;* Ca, *C. auratus* (Königstorfer et al. 1989a,b); Br, *B. rerio* (Sterrer et al. 1990); Ch, *C. harengus*.

Discussion

Comparison of Ependymins from Different Orders of Teleosts

In Fig. 4 all known ependymin homologous sequences were compiled. These sequences were obtained from three different orders of teleost fish (Salmoniformes, Cypriniformes, Clupeiformes). Within these three orders, six species (*O. mykiss, Salmo salar, E. lucius, C. auratus, B. rerio, C. harengus*) from four different families were compared (Salmonidae, Esocidae, Cyprinidae, Clupeidae). Generally, ependymins are highly divergent glycoproteins. Even the cysteine residues are not completely conserved. Mature salmoniform sequences (*O. mykiss, S. salar,* and *E. lucius*) contain only four cysteine residues, whereas in cypriniform (*C. auratus, B. rerio*) and clupeiform ependymins (*C. harengus*) five or six such residues are found, respectively. The cysteine residue at position 142 is probably responsible for formation of an intermolecular disulfide bridge, causing dimerization of cypriniform and clupeiform ependymins. Ependymins also vary by short deletions/insertions mainly at their very N- and C-terminal ends. The most-conserved region is located at positions 68–77 (sequence F-E-E-G-x-x-Y-D/E-I-D) between the two invariant potential N-glycosylation sites (positions 56–58 and 80–82). Here, very well-defined sugar moieties are added which terminate with sialic acid residues probably responsible for the calcium-binding capacity of ependymins (Ganß and Hoffmann, in preparation). Thus, this region between the two N-glycosylation sites seems to represent a crucial portion of ependymins. Interestingly, this region is encoded by two different exons (Rinder et al. 1992; Müller-Schmid et al. 1992). Another hallmark of all sequences is the invariant tryptophan residue at position 124. Here, a calcium-induced conformational change of ependymins can be monitored—e.g., by measuring the tryptophan fluorescence (Ganß and Hoffmann, in preparation).

Despite their high divergence, the hydropathic profiles of ependymins are remarkably similar (Fig. 5). There is only one characteristic difference observed in the sequence of *C. harengus*. Here, a pronounced hydrophobic stretch is present at positions 125–155 which is lacking in the other sequences. Currently, the significance of this region is unclear and interaction with cell membranes cannot be excluded.

Implications for the Evolution of Ependymins in Teleost Fish

Euteleosts possess characteristic morphological features unique to this infradivision—e.g., the presence of an adipose fin (Lauder and Liem 1983). Salmoniformes are documented starting from the early Upper Cretaceous (Carroll 1988; Benton 1990). However, Esocidae are considered to be

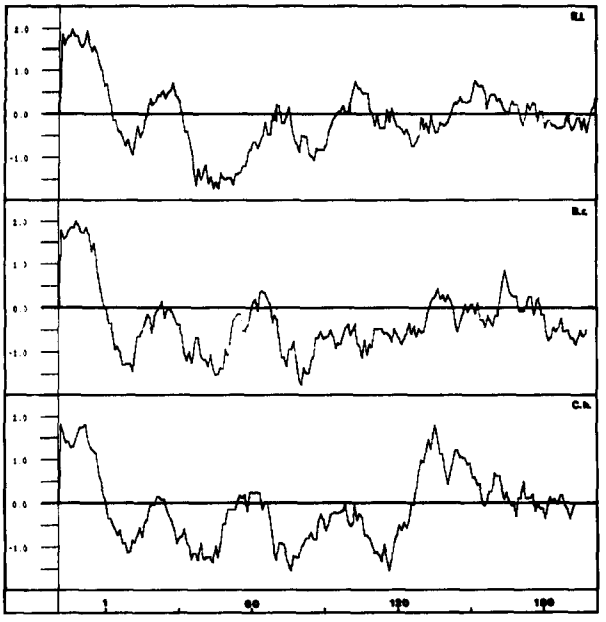

Fig. 5. Comparison of the hydropathic profiles of the ependymin precursors from *E. lucius, B. rerio* (Sterrer et al. 1990), and *C. harengus* according to the algorithm of Kyte and Doolittle (1982). Span: 19 residues. Clearly visible is the hydrophobic N-terminal cleavable signal sequence in every precursor.

more primitive than other euteleosts and they lack an adipose fin. Well-conserved esocoids are known from the Paleocene (Carroll 1988). Cypriniformes and members of its largest family, Cyprinidae, are known starting from the Eocene (Carroll 1988). In contrast, the members of the infradivision Clupeomorpha are distinguished from other teleosts—e.g., in having a specialized connection between the swim bladder and the ear (Nelson 1973; Lauder and Liem 1983). This characteristic was already developed in the Lower Cretaceous and recent *Clupea* species are known starting from the Eocene (Müller 1985).

Table 1 represents a numerical comparison (percent difference) of the various ependymin sequences based on their alignment according to the program CLUSTAL 5.0. The values in parentheses were corrected according to the two-parameter model (Kimura 1980) in order to compensate for multiple substitutions. The different sequences from the quasi-tetraploid species *C. auratus* and *O. mykiss* represent paralogous sequences (Königstorfer et al. 1989b; Müller-Schmid et al. 1992) whereas ependymins from the diploid species *B. rerio, E. lucius,* and *C. harengus* are orthologous (Sterrer et al. 1990; Svärdson and Wickbom 1939; Klose et al. 1968). However, the gene duplications in goldfish and trout probably occurred as independent events after the genera separated, because there is neither a preferential similarity by pairs observed between ependymins of these two species nor a particular similarity of one ependymin gene to the single ependymin gene of the diploid species from the same order. Therefore, we consider the relationship of ependymins between all genera orthologous. Generally, at the amino acid level cypriniform and clupeiform sequences are more related (52.8% difference) than cypriniform and salmoniform (60.7% difference) or salmoniform and clupeiform ependymins (61.5% difference; Table 2). These interordinal differences are more pronounced on the protein level than on the nucleic acid level (Table 2). In contrast, if one compares intraordinal differences (viz. within the orders Cypriniformes and Salmoniformes, respectively), DNA sequences differ more than the corresponding protein sequences (Table 2). This may be due to the following two reasons: (1) The gaps excluded in common-difference alignments (e.g., CLUSTAL) are relatively more pronounced on the DNA than on the protein level. In agreement with that, using an all-difference alignment (e.g., ALNED), the values on the DNA and protein levels do not differ so much any more (data not illustrated). (2) In rapidly evolving sequences multiple hits and the saturation at many nucleotide sites could account for an underestimation of differences on the DNA level. Using the corrected distance values according to Kimura (1980), such as effect can indeed be demonstrated (data not shown).

Also clearly shown in Table 2 is the close relationship of the two different families of Salmoniformes, viz. Salmonidae and Esocidae, which is in the same range as members of the family Cyprinidae. In the past, this close relation was debatable (Fink and Weitzman 1982), but nowadays it seems to be widely accepted (Nelson 1984). In accordance with previous reports (Kocher et al. 1989; Thomas and Beckenbach 1989), the ratio of transitions to transversions increases the closer the species are related (Table 2).

In Fig. 6, the numerical results on the DNA and protein level from Table 1 are presented graphically. Here, we used the UPGMA method, where (in contrast to all other algorithms) the accuracy of the reconstructed tree is not impaired by lack of an a priori defined outgroup (Saitou and Nei 1986). Furthermore, the robustness of these relationships was tested by bootstrapping (Felsenstein 1985). The general conclusion is that certain members of Euteleostei appear more closely related to Clupeomorpha than to members of their own infradivision. This result is unexpected because it does not reflect precisely the current taxonomy of these three orders of teleost fish (Lauder and Liem 1983). Thus, one possible consequence could be a reevaluation of the established taxonomy of fish. Based on just a single set of molecular data, at present this would not be appropriate.

Table 1. Similarities of ependymin homologous sequences from different species[a]

DNA / pro	Om1	Om2	El	Ca1	Ca2	Br	Ch
Om1		9.5 (10.2)	11.5 (12.5)	59.2 (108.7)	58.3 (105.2)	58.3 (105.2)	60.5 (113.3)
Om2	8.9 (9.5)		12.4 (13.6)	61.1 (115.9)	60.7 (114.0)	61.1 (115.9)	62.4 (120.9)
El	13.7 (15.2)	14.1 (15.7)		62.9 (123.0)	62.4 (120.9)	62.6 (121.7)	61.7 (117.9)
Ca1	43.6 (66.3)	44.6 (69.2)	46.5 (74.1)		6.9 (7.3)	8.4 (8.9)	51.7 (84.4)
Ca2	43.2 (66.9)	43.9 (69.0)	46.0 (74.3)	8.1 (8.6)		10.7 (11.6)	52.2 (85.8)
Br	42.3 (63.8)	42.6 (64.7)	44.4 (68.9)	11.7 (12.8)	12.1 (13.4)		54.6 (92.9)
Ch	47.4 (76.2)	47.2 (76.1)	48.5 (79.6)	41.1 (61.2)	41.2 (62.2)	41.4 (62.5)	

[a] Shown are the percent differences of the various pairs using the CLUSTAL 5.0 program. Positions which contain gaps in any comparison by pairs have been excluded (common-difference comparison). The corrected values according to the two-parameter model (Kimura 1980) are shown in parentheses. Lower left: differences on nucleic acid level; upper right: differences on protein level. Om, *O. mykiss* (Müller-Schmid et al. 1992); El, *E. lucius;* Ca, *C. auratus* (Königstorfer et al. 1989a, 1989b); Br, *B. rerio* (Sterrer et al. 1990); Ch, *C. harengus*

Table 2. Inter- and intraordinal comparison of ependymins from the teleost orders Cypriniformes (Cyp), Clupeiformes (Clu), and Salmoniformes (Sal)[a]

Order	Prot.	DNA	ti/tv	RRV_{pro}	RRV'_{pro}	RRV_{DNA}	RRV'_{DNA}	Outgroup
Cyp:Clu	52.8%	41.2%	1.0945	1:1.02–1.04	1:1.04–1.10	1:1.14–1.25	1:1.26–1.45	Sal
Cyp:Sal	60.7%	44.1%	0.9398	1:1.23–1.42	1:1.48–1.92	1:1.31–1.38	1:1.51–1.66	Clu
Clu:Sal	61.5%	47.7%	0.8173	1:1.21–1.38	1:1.40–1.82	1:1.08–1.20	1:1.10–1.30	Cyp
IntraCyp	8.7%	10.6%	1.5152	1:1.46–2.20	1:1.76–39.7	1:1.02–1.08	1:1.07–1.21	Clu
IntraSal	11.2%	12.2%	1.1753	1:1.08–1.35	1:1.20–2.51	1:1.02–1.20	1:1.05–1.60	Clu

[a] All values were obtained from the set of data presented in Table 1. Compared are the mean percent differences on the protein and DNA levels. The ratios of the mean number of transitions to tranversions are also given. The range of the relative rate values on the protein and on the DNA level was calculated for each pair according to Sarich and Wilson (1973) using either the uncorrected values (RRV) or the corrected values (RRV') of Table 1. For the interordinal relative rate values three OTUs were used whereas the calculation of intraordinal relative rate values was based upon a four-OTU model

Alternatively, it would certainly be worthwhile to test one basic assumption of the molecular clock hypothesis (Kimura and Ohta 1974; Wilson et al. 1977; Kimura 1987; Zuckerkandl 1987)—namely, the relatively constant rate of evolution. If this criterion is not fulfilled, this could also explain our paradoxical results, because the UPGMA method is based upon a constant evolutionary rate. Therefore, from the percent difference data shown in Table 1 the relative rate values (RRV and RRV', respectively) were calculated according to Sarich and Wilson (1973) using either three or four operational taxonomic units (OTUs; Table 2). As a conclusion of these calculations, the following rate order is determined: protein (Cypriniformes ≈ Clupeiformes ≪ Salmoniformes); DNA (Cypriniformes < Clupeiformes < Salmoniformes). Thus, evolution of ependymins seems to be relatively accelerated mainly on the protein level within Salmoniformes.

However, it seems unlikely that this accelerated rate can indeed account for the discrepancy between molecular data and taxonomic classification since all the interordinal relative rates have values below 2.0. Thus, we tested three different user-defined trees representing the three theoretically possible relationships, viz. Clu(Cyp-Sal), Sal(Clu-Cyp), and Cyp(Sal-Clu), with a DNA parsimony algorithm. This algorithm seems to provide valid results as long as the relative rate values are below 2.0 (Felsenstein 1991). Interestingly, all topologies tested resulted in exactly the same number of nucleotide substitutions—i.e., 1,451. This result indicates a trichotomy between Cypriniformes, Salmoniformes, and Clupeiformes—i.e., all three orders of teleost fish are equally distantly related to each other. Thus, there is no indication of the existence of a separate cohort Euteleostei combining Salmoniformes and Cypriniformes.

Using the corrected values according to Kimura (1980), a similar result has been obtained on the

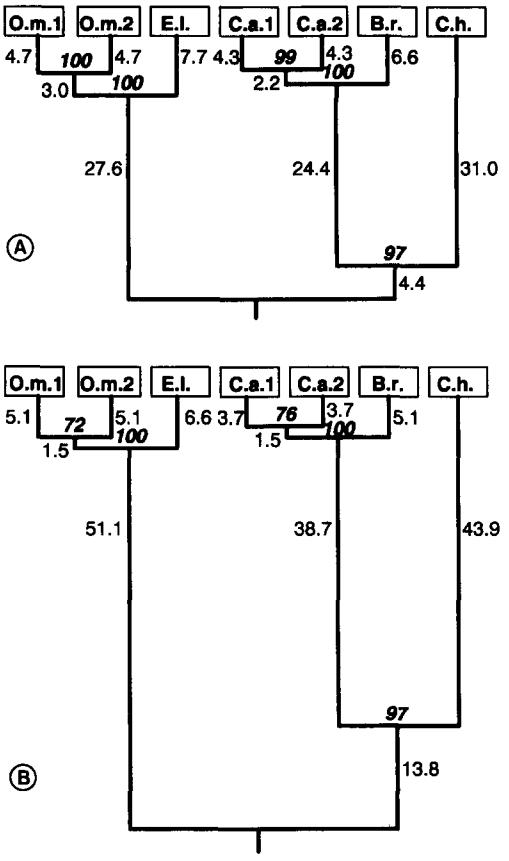

Fig. 6. Consensus phenogram illustrating the relationships between the different ependymin sequences on DNA (**A**) and protein level (**B**). The UPGMA method of the PHYLIP 3.4 package has been employed with a correction for multiple substitutions (Kimura 1980). The branch lengths were calculated from the set of data presented in Table 1. The confidence values of the branch points in the consensus tree (*numbers in italics*) were computed by bootstrapping (Felsenstein 1985). Om, *O. mykiss* (Müller-Schmid et al. 1992); El, *E. lucius;* Ca, *C. auratus* (Königstorfer et al. 1989a, 1989b); Br, *B. rerio* (Sterrer et al. 1990); Ch, *C. harengus*.

protein as well as on the DNA level by neighbor joining, which also accounts for inconstant rates. In both consensus trees obtained after bootstrapping, Salmoniformes were significantly grouped together with *C. harengus* but not with Cypriniformes (data not illustrated). This again does not support the concept of Euteleostei.

Remarkably, in the past there have been some reports which discussed interrelationships similar to those observed here. For example, based on fusion patterns of the lower jaw, Nelson (1973) suggested a relationship between Clupeomorpha and Euteleostei, and Patterson and Rosen (1977) proposed a new scheme of teleostean interrelationships in which the Euteleostei and the Clupeomorpha would be sister groups. However, this did not result in unification of these two cohorts of teleosts. Furthermore, Nelson (1984) stated that "there is no convincing evidence that Euteleostei are monophyletic" and that "much more work remains to be done before a sound classification of euteleosteans can be erected." Our results basically support this view.

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