The Placement of Chondrichthyes within the Vertebrate Phylogenetic Tree

Nadine Harmel[1,3], Maja Djurisic[2,3], and Biff Forbush[3]

[1]Department of Biophysics, University of Konstanz, Konstanz, Germany, 78457
[2]Deptartment of Neurobiology, Harvard Medical School, Boston, MA, 02115
[3]Department of C & M Physiology, Yale University School of Medicine, New Haven, CT, 06510

The Chondrichthyes, including sharks, skates, rays, and chimeras, diverged from other vertebrates over 400 million years ago. The traditional version of the tree of jawed vertebrates (Gnathostomata) places the Chondrichthyes in a basal position, with two clades of Osteichthyes or bony fish branching off subsequently: the Actinopterygii (including Teleostei) and Sarcopterygii (lobe-finned fish, including lungfish and coelacanth, and terrestrial vertebrates). A simplified version of the traditional tree is given in Figure 1A. There are numerous morphologic features that separate the Chondrichthyes from most other fish, including lack of swim bladder and bony skeleton, individual gill slits, rectal gland, tiny scales, and jaw structure. It has however been debated whether these features establish chondrichthyians as basal to other gnathostomes.

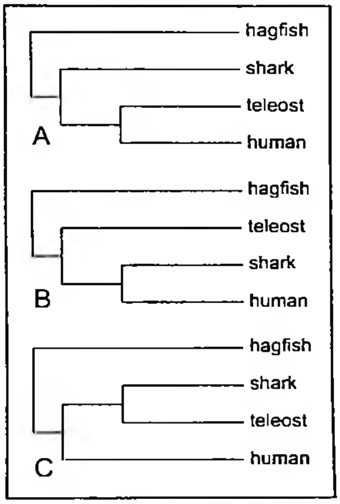

Figure 1. Phylogenetic trees

Since cloning NKCCs from sharks and humans we have been struck by the high degree of interspecies homology in these transport proteins, and the corresponding lower homology of teleost genes; the NKCC tree has the topology shown in Fig. 1B. Such differences are often discussed as reflective of an unusually rapid clock rate in teleosts that has created greater divergence over evolutionary time. Indeed, because it is generally recognized that clock rates do vary among organisms, meaningful phylogenetic analyses can only be carried out with the inclusion of a suitable outgroup: for examination of gnathostome relationships the closest available outgroup candidates are the cyclostomes, lampreys and hagfish.

The phylogenetic placement of Chondrichthyes has been largely taken for granted during the development of molecular phylogenetic methods, with only a few studies addressing the issue directly. The cumulative results of these studies are conflicting. In analyses of the complete mitochondrial genome, chondrichthyians are found to cluster with other fish[1], both Actinopterygii and Sarcopterygii (Figure 1C), whereas studies of a small number of highly-conserved nuclear-coded genes[2] have tended to support the conventional tree (Figure 1A).

We are addressing this issue ourselves by significantly increasing the amount of data that is available for analysis. We are cloning cDNAs for hagfish (*Myxine glutinosa*) and shark (*Squalus acanthias*) genes, focusing first on ion transport-related proteins that are related to our other interests, and on a few genes for which sequence is already available for either hagfish or shark; these include NKCCs, NKAs, Gluts, NCXs, KvLQT, actin, Hsp90, and gp96, among others. In addition we are able to extend the study using *Squalus* and *Leucoraja* EST sequences and clones made available through the efforts of Chris Smith and David Towle at MDIBL. Finally, we are also cloning NKCCs and NKAs from *Polypterus* (bichir) and *Protopterus* (lungfish), two genera that occupy very important positions in the vertebrate tree.

Altogether we are working on 34 new cDNAs representing 16 genes; 27 cDNAs have been identified by degenerate PCR. We are planning to obtain full-length sequences using 5'RACE and 3'RACE whenever possible, and have completed 16 full-length sequences. Even at this stage, the amount of new sequence is greater than that which has been used to address the "Chondrichthyes" question in the above studies.

We have not yet carried out a thorough statistical analysis of our data set. However, preliminary examination suggests that even with our expanded list of sequences, the issue of the base of the gnathostome tree may remain controversial. The phylogenetic tree for the Na-K-Cl cotransporters (Figure 2) strongly supports (with bootstrap values >98) our original counter-hypothesis, that Actinopterygii (including teleosts) are at the base of the gnathostome tree and that Chondrichthyes have a branch point closer to mammals (Figure 1B). This result is seen both for NKCC1 and NKCC2, two isoforms that arose from a duplication event that appears to have occurred shortly after the cyclostome branch point, but before the major vertebrate radiation.

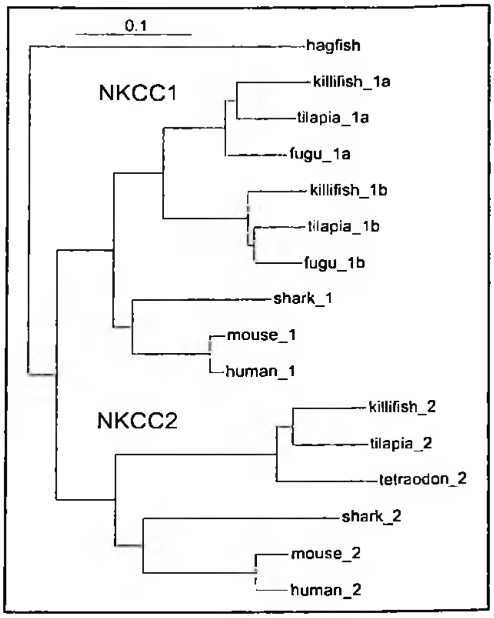

Fig. 2. Phylogenetic tree of NKCCs, constructed using the Neighbor-Joining algorithm. Scale: substitutions per site

Although the majority of genes in our data set exhibit preliminary trees similar to those of the NKCCs, others exhibit tree topologies shown in Figures 1A and 1C, albeit with less statistical power. The tree for Na,K-ATPase alpha subunits (Figure 3) is illustrative of a 1A- and 1C-supporting family. The molecular distances are substantially less than for NKCCs, and with less distance separating branch points compared to the evolutionary distances in each branch; this characteristic appears to be common to the trees for very highly-conserved genes in this and other studies. Interestingly, and similar to the result for NKCCs, hagfish appear to have a single member of the NKA family corresponding to the three NKAs of gnathostomes.

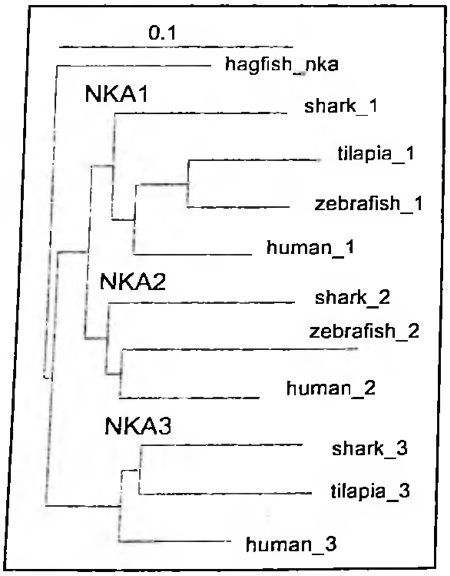

Fig. 3. Phylogenetic tree of NKAs, constructed using the N-J algorithm. Scale:substitutions per site

We anticipate that taken together our results will support the tree in Figure 1B, arguing that Chondrichthyes are not at the base of the tree of jawed vertebrates. It is already clear however that even with this expanded dataset, the problem will not be solved unambiguously or without controversy. A final resolution of the problem should be attained with the sequencing of the skate genome in conjunction with that of a suitable outgroup. Hagfish or lamprey would be optimal as an outgroup, but the urchin genome, now largely completed, may also suffice for this purpose.

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1. **Arnason, U., A. Gullberg, A. Janke, J. Joss, and C. Elmerot.** Mitogenomic analyses of deep gnathostome divergences: a fish is a fish. *Gene* 333: 61-70, 2004.
2. **Kikugawa, K., K. Katoh, S. Kuraku, H. Sakurai, O. Ishida, N. Iwabe, and T. Miyata.** Basal jawed vertebrate phylogeny inferred from multiple nuclear DNA-coded genes. *BMC Biology* 2: 1-11, 2004.