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Phylogeny of *Nematoda* and *Cephalorhyncha* Derived from 18S rDNA

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Abstract. Phylogenetic relationships of nematodes, nematomorphs, kinorhynchs, priapulids, and some other major groups of invertebrates were studied by 18S rRNA gene sequencing. Kinorhynchs and priapulids form the monophyletic Cephalorhyncha clade that is the closest to the coelomate animals. When phylogenetic trees were generated by different methods, the position of nematomorphs appeared to be unstable. Inclusion of *Enoplus brevis*, a representative of a slowly evolving nematode lineage, in the set of analyzed species refutes the tree patterns, previously derived from molecular data, where the nematodes appear as a basal bilateral lineage. The nematodes seem to be closer to the coelomate animals than was speculated earlier. According to the results obtained, nematodes, nematomorphs, tardigrades, arthropods, and cephalorhynchs are a paraphyletic association of closely related taxa.

Key words: Nematoda — Kinorhyncha — Priapulida — Molecular phylogeny — 18S rRNA gene sequences

Introduction

For many years the phylogenetic position of some groups of animals remained obscure to zoologists. Priapulids and kinorhynchs may be an example; e.g., *Priapulus caudatus* (Lamarck), was initially placed in the genus *Priapus*, together with the sea anemone, but then removed to the holothurians. Later, together with the trochophoran unsegmented worms Echiura and Sipuncula, it was included in the phylum Gephyrea, which for a long time had been thought to be the ancestor of all echinoderms. Similarly, the kinorhynchs were first described as intermediates between worms and crustaceans. Later they were proved to be dipteran neotenic larvae.

In modern systems kinorhynchs and priapulids are usually treated as independent phyla of pseudocoelomate animals, and some scholars hypothesized the existence of a close relation between kinorhynchs and priapulids, which allows including them as well as two other pseudocoelomate groups, loricifer and nematomorphs, in the phylum Cephalorhyncha (Malakhov 1980; Malakhov and Adrianov 1995). Other authors tend to include kinorhynchs, priapulids, and loricifers together with nematodes and nematomorphs in the group Introverta, considering nematomorphs as a sister group of nematodes (Nielsen 1995). Hence, Cephalorhyncha either includes kinorhynchs, priapulids loricifers, and nematomorphs (Malakhov and Adrianov 1995) or kinorhynchs, priapulids, and loricifers only (Nielsen 1995).

The latest molecular evidence supports a close relationship of *P. caudatus* (Priapulida) and coelomates (Aleshin et al. 1995; Winnepenninckx et al. 1995b). Nematodes, being represented by extremely rapidly evolving sequences of rhabtitid species, appear in the phylogenetic trees of 18S rRNA sequences as a basal branch of bilateral animals (Aleshin et al. 1995; Winnepenninckx et al. 1995b). Analysis of the cytochrome *c* gene also places the Nematoda at the base of bilateral metazoans (Vanfleteren et al. 1994). It is not improbable, however, that such a position of the Nematoda is an artifact caused by the extremely rapid nucleotide substitution rates (Swofford and Olsen 1990; Penny et al.

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1991) found in previously published rhabditid nematode 18S rRNA sequences or by the enormous differences in G + C content of 18S rRNA among species (Hasegawa and Hashimoto 1993). Taking all this into account, not only sequences of rhabditid species, but those of adenophorean species are to be analyzed. This will allow us to identify more slowly evolving species and to reexamine the relationships among nematodes, nematomorphs, kinorhynchs, and priapulids.

Therefore, the objective of this study was to assess the following questions: What concept of Cephalorhyncha is consistent with the molecular data? What place will the nematodes occupy, being represented by sequences of Adenophorea species? With these questions in mind, we sequenced complete or nearly complete sequences of a kinorhynch, a nematomorph, a nematode, an acanthocephala, and a sea spider and compared them with some other metazoan sequences taken from GenBank.

Materials and Methods

Biological Material and DNA Extraction

The animals investigated in the present study are the kinorhynch *Pycnophyes kielensis* (Zelinka 1928); the nematomorph *Gordius albopunctatus* (Müller 1827), the nematode *Enoplus brevis* (Bastian 1865), the acanthocephala *Echinorhynchus gadi* (Müller 1776), and the sea spider *Nymphon* sp.

DNA of *Gordius albopunctatus* (single specimen), *Enoplus brevis*, *Echinorhynchus gadi*, and *Nymphon* sp. (single specimen) was extracted from in 70% ethanol-fixed tissues as described by Arrighi et al. (1968). DNA of *Pycnophyes kielensis* was extracted from freshly frozen animals. Several intact animals were homogenized and incubated in a solution containing 0.5% (w/v) sodium dodecyl sulfate (SDS), 10 m*M* Tris, 5 m*M* EDTA, and 100 μg/ml proteinase K. DNA was purified by phenol/chloroform/isoamylalcohol and chloroform/isoamylalcochol extraction followed by isopropanol precipitation (Sambrook et al. 1989).

Amplification and Sequencing of 18S rRNA Genes

The 18S ribosomal RNA coding regions were amplified in polymerase chain reactions using two primers complementary to the 5′ and 3′ termini of eukaryotic 16S-like rRNAs (Medlin et al. 1988). Full-length products of amplification were purified by agarose gel electrophoresis, cloned in pBluescript KS+ plasmid, and sequenced on both strands using the Sequenase Version 2.0 USB kit, a set of specific 18S rRNA internal primers, and a universal M13 sequencing primer.

Alignment and Tree Construction

Complete or nearly complete 18S rRNA sequences determined were submitted to GenBank under the following accession numbers: *Pycnophyes kielensis* (Pki), U67997; *Echinorhynchus gadi* (Ega), U88335; *Enoplus brevis* (Ebr), U88336; *Gordius albopunctatus* (Gal), U88337; and *Nymphon* sp. (Nsp), U88338. Three-letter abbreviations of binomial names used in the figures are given in parentheses. In addition to these sequences, previously published sequences representing mostly deuterostome and protostome taxa, other aschelminth taxa, acoelomates, and diploblasts were taken from GenBank and analyzed. The phylum or subphylum, binomial name, three-letter abbreviation used in the figures, and GenBank accession number of species used are as follows: Chordata, *Homo sapiens*, Hsa, M10098; Hemichordata, *Saccoglossus kowalevski*, Sko, L28054; Urochordata, *Herdmania momus*, Hmo, X53538; Echinodermata, *Stichopus japonicus*, Sja, D14364; Echinodermata, *Strongylocentrotus purpuratus*, Spu, L28055; Echinodermata, *Echinocardium cordatum*, Eco, Z37123; Mollusca, *Acanthopleura japonica*, Aja, X70210; Mollusca, *Mytilus edulis*, Med, L24489; Annelida, *Eisenia fetida*, Efe, X79872; Annelida, *Glycera americana*, Gam, U19519; Sipuncula, *Phascolosoma granulatum*, Pgr, X79874; Echiura, *Ochetostoma erythrogrammon*, Oer, X79875; Pogonophora, *Siboglinum fiordicum*, Sfi, X79876; Entoprocta *Pedicellina cernua*, Pce, U36273; Ectoprocta, *Plumatella repens*, Pre, U12649; Phoronida, *Phoronis vancouverensis*, Pva, U12648; Brachiopoda, *Lingula lingua*, Lli, X81631; Nemertini, *Lineus* sp., Lsp, X79878; Nemertini, *Prostoma eilhardi*, Pei, U29494; Arthropoda, *Artemia salina*, Asa, X01723; Arthropoda, *Tenebrio molitor*, Tmo, X07801; Arthropoda, *Eurypelma californica*, Eca, X13457; Nematoda, *Caenorhabditis elegans*, Cel, X03680; Nematoda, *Strongyloides stercoralis*, Sst, M84229; Nematoda, *Ascaris* sp., Asp (the partial 18S rRNA sequence was compiled from M58348, X06225, X05836, X06713, M74584, and M74585); Nematomorpha, *Gordius aquaticus*, Gaq, X87985; Priapulida, *Priapulus caudatus*, Pca, X87984; Acanthocephala, *Moniliformis moliniformis*, Mmo, Z19562; Acanthocephala, *Neoechinorhynchus pseudemydis*, Nps, U41400; Rotatoria, *Brachionus plicatilis*, Bpl, U29235; Rotatoria, *Philodina acuticornis*, Pac, U41281; Gastrotricha, *Lepidodermella squammata*, Lsq, U29198; Plathelminthes, *Schistosoma mansoni*, Sma, X53986; Plathelminthes, *Opisthorchis viverrini*, Ovi, X55357; Plathelminthes, *Gyrodactylus salaris*, Gsa, Z26942; Plathelminthes, *Bipalium trilineatum*, Btr, D85086; Plathelminthes, *Convoluta naikaiensis*, Cna, D83381, D17558; Orthonectida, *Rhopalura ophiocomae*, Rop, X97158; Dicyemida, *Dicyema* sp., Dsp, X97157; Myxozoa, *Hennegua dori*, Hdo, U37549; Myxozoa, *Myxidium* sp., Msp, U13829; Cnidaria, *Anemonia sulcata*, Asu, X53498; Placozoa, *Trichoplax* sp., Tsp, Z22783; Porifera, *Scypha ciliata*, Sci, L10827; Ctenophora, *Mnemiopsis leidyi*, Mle, L10826; Choanoflagellata, *Diaphanoeca* grandis, Dgr, L10824; Choanoflagellata, *Sphaeroeca volvox*, Svo, Z34900; and Ciliophora, *Paramecium tetraurelia*, Pte, X03772.

The sequences obtained were manually fit into an alignment of small-subunit rRNA sequences (Van De Peer et al. 1996) and our own manually made alignments (alignments I and II, respectively). Two alignments (I and II) were analyzed to be certain that differences in alignment had little or no effect on the tree topologies. Sets of 46 sequences from these alignments were analyzed by both neighbor-joining (NJ) and maximum-parsimony (MP) methods using bootstrap resampling (Felsenstein 1985). NJ trees were inferred with the TREECON program (Van De Peer and De Wachter 1994), using the Kimura (1980) distances, modified to take gaps into account (Van De Peer et al. 1990). The substitution rates of the different alignment positions were also considered (Van De Peer et al. 1996). Confidence in NJ trees was determined by analyzing 1000 bootstrap replicates using the TREECON program and by conducting an interior branch length test (Sitnikova et al. 1995) using the PHYLTEST program (Kumar 1995). MP trees were constructed using the Dnapars program from the PHYLIP 3.572 package (Felsenstein 1993). Maximum-likelihood (ML) trees (Felsenstein 1981) were inferred using the fastDNAml program (Olsen et al. 1994) as well as the PUZZLE program (Strimmer and von Haeseler 1996). Confidence in the MP trees was determined by analyzing 1000 or more bootstrap replicates.

Results

Figure 1 shows the results of MP analysis of the set of nearly complete, manually aligned 18S rRNA sequences

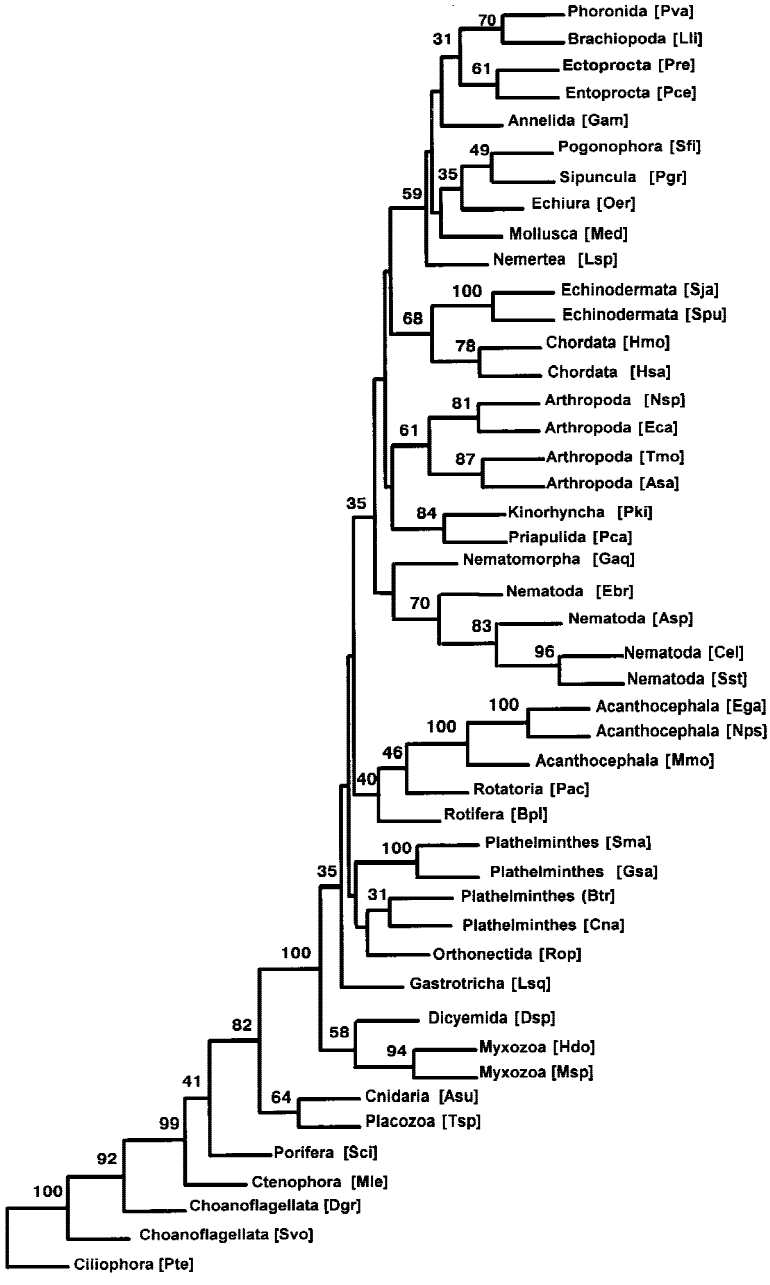

Fig. 1. Relations between cephalorhynchs and other animal groups derived from 18S rRNA gene sequences. This tree is the result of the MP analysis of our own manual alignment of nearly complete 18S rRNA gene sequences of the major animal groups. The percentages of 3000 MP bootstrap resamplings (bootstrap values) that support the corresponding topological elements are shown at the *internodes.*

of 46 species. A similar tree (not shown) was inferred from an identical set of sequences made on the basis of alignment of small-subunit rRNA sequences published by Van de Peer et al. (1996). In both trees Kinorhyncha and Priapulida form a well-supported monophyletic clade weakly linked to the arthropods. Nematomorpha (*G. albopunctatus* and *G. aquaticus*) are weakly linked to nematodes in Fig. 1, whereas in the second tree (not shown) they form a branch which is separate even though it is close to the nematodes. Nematodes in both trees form a moderately supported monophyletic group that is close to the cluster of coelomate animals, including protostome and deuterostome coelomates, arthropods, and cephalorhynchs. These results are consistent with clade Cephalorhyncha having the composition Kinorhyncha + Priapulida (Nielsen 1995) but not Kinorhyncha + Priapulida + Nematomorpha (Malakhov 1980; Malakhov and Adrianov 1995).

In an effort to investigate the effect of changes in sequence sets on the results of phylogenetic analysis, some sequences were excluded from the analysis, and the resulting sets were examined by the MP bootstrap method using the Dnapars program (Felsenstein 1993). The results of this analysis are presented in Table 1. For better visualization of the sequence set dependence of the affinity of various groups, in Table 1 the bootstrap values are shown for the clades both included and not included in consensus tree. For this reason many of the values are extremely low. However, though very low for the clades involved, they are not subject to chance. So cephalor-

Table 1. MP bootstrap support of supraphyletic groups of bilaterian animals depending on a particular set of analyzed species[a]

	Percentage					
Cluster	A	B	C	D	E	F
Cephalorhyncha and Arthropoda	11	8	27	6	—	—
Cephalorhyncha and coelomate Protostomia	6	4	5	1	—	—
Cephalorhyncha and Deuterostomia	5	5	8	20	—	—
Coelomata w/o any Aschelminthes	1	1	0	4	16	37
Cephalorhyncha and Gordius	1	1	3	17	—	—
Cephalorhyncha and N*	9	9	1	13	—	—
Gordius and Nematoda	22	22	7	23	26	7
Cephalorhyncha and Coelomata and N*	52	73	10	47	—	—
Cephalorhyncha and Coelomata	17	10	32	13	—	—
Coelomata (w/o Cephalorhyncha) and N*	0	0	0	1	42	9

[a] The set of species in Fig. 1 corresponds to column A. The secernentean nematodes (column B), *Enoplus* (column C), *Priapulus* (column D), all Cephalorhyncha (column E), and Cephalorhyncha and *Enoplus* (column F) were excluded from this set. Unconventional groups are indicated as follows: Coelomata = Deuterostomia, Arthropoda, and coelomate Protostomia (including phyla of trochophoran animals, Pogonophora, Nemertea, Entoprocta, and Lophophorata); N* = Nematoda, with varying position of Gordius. The percentage of 3000 (A) or 1000 (B–F) bootstrap replicates is shown

hynchs (consisting of priapulids and kinorhynchs) tend to cluster with coelomates (protostome and deuterostome coelomates and arthropods). Their affinity to this group increases when the most rapidly evolving sequences of secernentean nematodes are excluded and decreases when the slowly evolving sequence of *Enoplus brevis* is excluded (row 8). The affinity of cephalorhynchs and arthropods (row 1) depends on the set of species analyzed and the type of alignment (not shown) and, therefore, needs clarification. Exclusion of *Priapulus* erodes the place occupied by *Pycnophyes* (Kinorhyncha), but the affinity of the latter to coelomates still seems to be more pronounced than that to Aschelminthes (column D). Affinities of the genus *Gordius* (Nematomorpha) are not clear. They depend substantially on *Enoplus*, whose 18S rRNA sequence is among the most slowly evolving of the nematodes. An artificial but statistically significant separation of nematodes from coelomates occurs when a single sequence of *Enoplus* is excluded from the analysis. In contrast, inclusion of this sequence sharply decreases the bootstrap support of the separate clustering of the nematodes and coelomates. Moreover, their weakly supported clustering occurs when Cephalorhyncha and *Enoplus*, as representatives of Aschelminthes, are included in the analysis (rows 4 and 8). On the whole, inclusion or exclusion of the *Enoplus* sequence has a great deal to do with the order of clustering in the lower part of the 18S rRNA sequences trees, namely, with the clustering of aschelminths.

The NJ tree (not shown) of the same set of sequences was inferred from the distances calculated by Van de Peer et al. (1996) method. It differs from MP trees in that nematodes, nematomorphs, cephalorhynchs, and arthropods comprise a weakly supported (47% of bootstrap replicates) clade within coelomate animals, which is a sister to deuterostome coelomates. Thus, the results of MP and NJ analysis of the relatively large set of sequences are somewhat controversial.

With this in mind, an attempt has been made to resolve the problem of relationships among these groups by means of detailed analysis of a more restricted set of sequences, using not only MP and NJ but also ML methods. Because analyses of the large sequence sets from two alignments produced similar tree topologies, further phylogenetic reconstruction was performed using alignment II alone. Based on the results of the NJ analysis, the most rapidly evolving sequences were excluded from the sets to be analyzed, and some sequences were replaced by more slowly evolving ones. For example, the sequences from *Strongylocentrotus purpuratus* (Spu), *Herdmania momus* (Hmo), and *Bipalium trilineatum* (Btr) were replaced by sequences from *Echinocardium cordatum* (Eco), *Saccoglossus kowalevski* (Sko), and *Dugesia japonica* (Dja), respectively. The sequences from *Acanthopleura japonica* (Aja), *Eisenia fetida* (Efe), and *Prostoma eilhardi* (Pei) were added to the sequences from *Mytilus edulis*, *Glycera americana*, and *Lineus* sp. so that corresponding groups (Mollusca, Annelida, and Nemertini) would be represented by two species. Figure 2A demonstrates a NJ tree constructed basing on Kimura (1980) distances modified to take gaps into account (Van de Peer et al. 1990) for such a set of 18S rRNA sequences. In this tree nematodes, nematomorphs, tardigrades, cephalorhynchs, and arthropods comprise a weakly supported (20% bootstrap replicates) monophyletic clade. *Enoplus brevis*, the most slowly evolving representative of nematodes, forms the clade with tardigrades, which in turn is a sister group of arthropods. Similar results (not shown) were obtained by analysis of the same set of sequences by the minimum-evolution tree method (Rzhetsky and Nei 1992) using the METREE program (Rzhetsky and Nei 1994). These results differ from those in Fig. 2A in that the clade of nematodes, tardigrades, cephalorhynchs, nematomorphs, and arthropods is more strongly supported by the bootstrap analysis (60% of bootstrap replicates) and in that nematomorphs

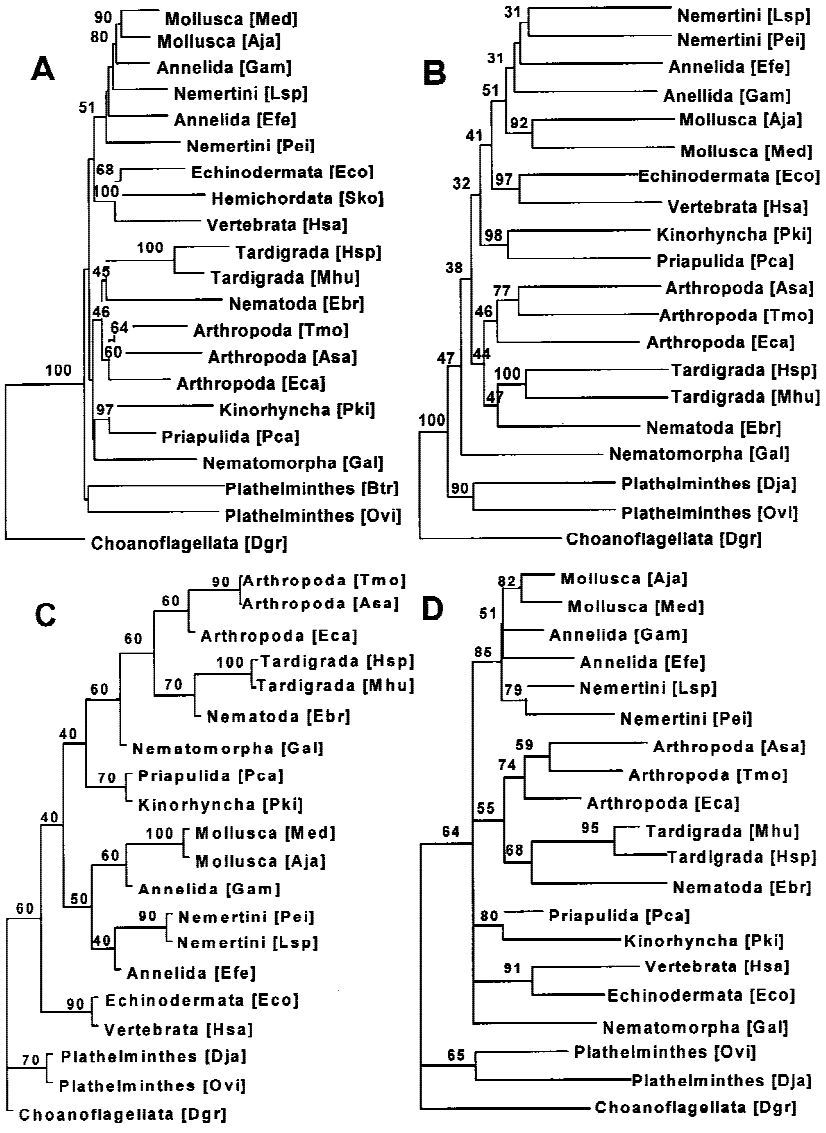

Fig. 2. Phylogenetic trees of a set of 18S rRNA sequences selected by short NJ distances. Percentage bootstrap values are shown *at the internodes.* Three-letter binomial abbreviations are shown *in brackets.* See text for their definitions. **A** NJ tree derived with the TREECON program. **B** MP tree derived with the DNApars program. **C** ML tree derived with the fastDNAml program. **D** ML tree derived with the PUZZLE program. Though the support values provided by PUZZLE are not equivalent to the bootstrap values, they have the same practical meaning as the latter (Strimmer and von Haeseler 1997).

do not form a cluster with cephalorhynchs but are weakly linked to the cluster of nematodes, tardigrades, and arthropods.

Somewhat different results were obtained by the MP analysis of the slightly modified set of 18S rRNA sequences [the sequence of *Saccoglossus kowalevski* (Sko) was excluded so that deuterostomes would be represented by two species, and the sequence of *Bipalium trilineatum* (Btr) was replaced by the more slowly evolving sequence of *Dugesia japonica* (Dja)]. In this analysis (Fig. 2B), nematodes, nematomorphs, tardigrades, arthropods, and cephalorhynchs form a paraphyletic rather than a monophyletic group. Nematomorphs branch off separately at the base of this paraphyletic group, whereas cephalorhynchs are weakly linked to the cluster of deuterostome and protosome coelomates. Relationships among nematodes, tardigrades, and arthropods are the same as in the NJ trees.

The same set of 18S rRNA sequences was also analyzed by the ML method using the programs fastDNAml (Olsen et al. 1994) and PUZZLE (Strimmer and von Haeseler 1996). Trees generated by both the fastDNAml (Fig. 2C) and the PUZZLE (Fig. 2D) programs are similar to those obtained by the MP and NJ methods in that nematodes (*Enoplus brevis*), tardigrades, and arthropods form a cluster in this case and in that nematodes share the most recent common ancestor with tardigrades.

To summarize briefly, these results suggest that nematodes, being represented by the most slowly evolving species (*Enoplus brevis*), are closely related to tardigrades, comprising with them a clade which is sister to athropods. These groups form a cluster with cephalorhynchs and nematomorphs which was observed in the NJ and ML, but not in the MP, trees. Due to the variable position of nematomorphs in the trees generated by different methods, these results show the best correlation with the cephalorhyncha clade consisting of priapulids and kinorhynchs (Nielsen 1995). As a whole, these data can be generalized best by the tree which was generated by the PUZZLE program using 1000 puzzling steps and

Hasegawa et al. (1985) as well as Schoeniger–von Haeseler (1994) models of nucleotide substitution. Both analyses gave identical tree topologies. In the resulting tree (Fig. 2D), five relatively well-supported (quartet puzzling reliability, 55–85%) monophyletic groups (deuterostome coelomates, protostome coelomates, cephalorhynchs, arthropods + tardigrades + nematodes, and nematomorphs) form an unresolved multifurcation.

Discussion

Both the NJ and the MP, as well as the ML and PUZZLE, analyses support unambiguously the monophyly of the Kinorhyncha + Priapulida clade. The monophyly of this clade is apparent regardless of the analysis method, sequence alignment, or species set. Such a case is rather unusual where groups of supraphylum rank are analyzed. Typically, the monophyly of the Cephalorhyncha clade (Kinorhyncha + Priapulida) is supported by 70–98% of bootstrap replicates. This level of support is higher than that for such good phyla as Chordata and Arthropoda. No bilaterian taxon of supraphylum rank has bootstrap support as high as does Cephalorhyncha. Other groups within Bilateria (Field et al. 1988; Wainright et al. 1993; Kobayashi et al. 1993; Vladychenskaya et al. 1995) have a lower level of bootstrap support, which is the highest in Deuterostomia (Wada and Sato 1994a,b), the ''coelomate Protostomia'' [including phyla of trochophoran animals, Lophophorata (Halanych et al. 1995; Conway Morris et al. 1996; Cohen and Gawthrop 1996), Pogonophora (Winnepenninckx et al. 1995a), Nemertea (Turbeville et al. 1992), and Entoprocta (Mackey et al. 1995)], the Brachiopoda + Phoronida clade (Halanych et al. 1995; Conway Morris et al. 1996; Cohen and Gawthrop 1996), and the Rotifera + Acanthocephala clade (Winnepenninckx et al. 1995b).

In addition to the monophyly of the Priapulida + Kinorhyncha clade, the analysis of 18S rRNA sequences and trees demonstrates its proximity to coelomates. In general, three monophyletic groups could be distinguished within the coelomate animals in the phylogenetic trees of 18S rRNA sequences (Fig. 1): Deuterostomia, Arthropoda, and coelomate Protostomia. Cephalorhynchs appear to be the fourth lineage within coelomates and the only representative of the true primary body cavity animals in this clade. Indeed, recent electron microscopy studies destroyed the foundation of earlier views on the existence of a coelomic cavity in cephalorhynchs, though certain features of mesenchyme differentiation in some cephalorhynch species, for example, the epithelial lining in the mouth cone of *Meiopriapulus fijiensis* (Storch et al. 1989) and the inner intestinal longitudinal musculature of *Priapulus caudatus* (Malakhov and Adrianov 1995), are not typical for Aschelminthes. On the other hand, according to electron microscopy data, many true coelomate animals have no conventional coelom (Zavarzina and Tsetlin 1990; Ruppert 1991).

In the phylogenetic analysis of 18S rRNA gene sequences, when the set of species analyzed was subjected to changes (Table 1), cephalorhynchs mostly demonstrated a slightly closer affinity to Arthropoda than to other coelomate groups. Affinity of the Priapulida to Arthropoda was proposed earlier on the basis of 18S rRNA sequence data (Garey et al. 1996). In the NJ analysis of the large sequence set and in the NJ, ME, and ML analyses of the restricted sequence set, the cephalorhynchs tend to form a cluster with nematomorphs, nematodes, arthropods, and tardigrades, supported by 20–60% of bootstraps. In the ML tree derived with PUZZLE, these groups form three unlinked clades within coelomate animals. Thus, the proximity of cephalorhynchs to arthropods is poorly supported by the molecular data. The morphological and paleontological data on this subject are as poor as the molecular data. Like arthropods, kinorhynchs have a metamery enclosing the integument, muscle, and nerve systems. The presence of a cuticle necessitates a molting cycle and loss of the ciliated larva. Although many animal lineages including aschelminths have chitin, outside cephalorhynchs, only arthropods, onychophores, and tardigrades have a chitinized body cuticle (Jeuniaux 1975; Cox Kusch and Edgar 1981; Bird and Bird 1991; Lemburg 1995). In addition, some paleontological evidence points to the probable relationship of cephalorhynchs and arthropods. *Xenusion*, the controversial onychophore-like Cambrian creature, was recently redefined as a link among arthropods, onychophores, and priapulids (Dzik and Krumbiegel 1989). Priapulids and kinorhynchs were considered the closest outgroup for arthropods in cladistic analysis of fossil and recent forms of Arthropoda (Waggoner 1996).

Other patterns of clustering of cephalorhynchs were also proposed based on the fossil records (Conway Morris 1993). Though they are weakly supported by molecular evidence, these patterns should not be ruled out. The cuticular sclerites in Palaeoscolecida, possibly related to Cephalorhyncha, are similar to those in one other group of coelomates, Deuterostomia (Müller and Hinz-Schallreuter 1993).

Taking all these considerations into account, it becomes possible to represent Bilateria evolution as follows: lower Bilateria → Cephalorhyncha → Coelomata.

Such a position of cephalorhynchs in 18S rRNA trees proves conclusively the artificial nature of the Aschelminthes taxon. However, 18S rRNA gene sequences of the aschelminths are known to be the source of a lot of problems for reconstruction of phylogenetic trees. Indeed, the extremely high evolution rate of this gene in secernentean nematodes (e.g., *Caenorhabditis elegans* and many parasitic roundworms) have resulted in artificial reconstruction (Swofford Olsen 1990; Penny et al.

1991) where these rather highly specialized animals were represented as basal branches of the bilaterian tree. This artificial clustering was reproduced for nearly two dozen secernentean nematode sequences found in GenBank and EMBL. The situation changes drastically when *Enoplus brevis* (Enoplida) is included in the set of species analyzed. The genetic distance between the 18S rRNA gene sequences of Cephalorhyncha and *Enoplus brevis* is about half the distance between Cephalorhyncha and *Caenorhabditis elegans.* The 18S rRNA gene of *Enoplus brevis* may be considered as retaining a state ancestral to all nematodes, since the monophyly of this group is beyond question. Exclusion of secernentean nematodes from the set of species analyzed results in a significantly different position of the nematodes in 18S rRNA gene sequence trees, converging Cephalorhyncha, Coelomata, and Nematoda. The clustering of cephalorhynchs with coelomates has a higher level of bootstrap support than that of cephalorhynchs with nematodes (Table 1). From this standpoint, the association of the latter taxa could be paraphyletic. In any case, the sequence of 18S rRNA in *Enoplus brevis* does not suggest that nematodes are the first branch of Bilateria. In contrast, the Nematoda could be the closest line to coelomate animals, after the Cephalorhyncha.

Relationships between cephalorhynchs and other aschelminth groups were also investigated. Their affinity to the Rotifera + Acanthocephala clade escaped detection by analysis of 18S rRNA gene sequences. Attempts to confirm the clade Cephalorhyncha consisting of Kinorhyncha + Priapulida + Nematomorpha fared poorly regardless of which set of species was analyzed. Two species of *Gordius* were shown to be very close to each other but far apart from all other phyla (Table 1). By these criteria, their relations to the other phyla remain unclear.

Summing up, the following view on bilaterian 18S rRNA gene evolution can be suggested, stemming from the fact that a major group of bilaterian phyla is clustered in a close core of ‘‘coelomate Protostomia.’’ All the 18S rRNA sequences of this core have a large set of synapomorphic characters and are characterized by short genetic distances between them. Consequently, one may hypothesize that all Bilateria have a common set of synapomorphic characters which is more or less altered in arthropods, deuterostomes, cephalorhynchs, and all lower bilaterian lineages. This hypothetical pattern of evolution could be in accordance with the classical Procoelomata concept of a Bilateria origin from a common annelid-like coelomic segmented ancestor (Sedgwick 1884; Van Beneden 1891). In this context, some phylogenetic conclusions derived from 18S rRNA comparisons could be an artifact not only for the secernentean nematodes, but also for some other groups of lower bilaterians (e.g., flatworms).

However, the suggested hypothesis is in conflict with at least four facts. (i) It is strange that, of all the animals studied, the highest rate of 18S rRNA sequence evolution is observed in lower bilaterians. (ii) The genetic distances to *Priapulus* are not larger than those within the Bilateria core, but *Priapulus* tends to be linked to the core rather than included in it. (iii) Although the distances to *Enoplus brevis* (Nematoda) and *Brachionus plicatilis* (Rotifera) are not significantly larger than those within the coelomate core, the two species tend to be placed in the lower part of the 18S rRNA sequences tree. (iv) Although the distances to *Pycnophyes kielensis* (Kinorhyncha) are not smaller than those to many lower bilaterians, this species tends to cluster with coelomate animals even if *Priapulus* is excluded from the set of species analyzed. Nevertheless, such a pattern should not be ruled out altogether. It is possible that some of the clades branching off separately would be closer to the coelomate core if their less divergent representatives were included in the study. The case of *Enoplus brevis* clearly demonstrates the inequality of different representatives of monophyletic clades for phylogenetic analysis of 18S rRNA gene sequences.

Similar results concerning the phylogenetic relationships among nematodes, nematomorphs, kinorhynchs, priapulids, tardigrades, and arthropods have been published by Aguinaldo et al. (1997). They closely resemble our results in the phylogenetic position of nematodes represented by one adenophorean species, *Trichinella spiralis,* within higher animals but differ in that nematodes nematomorphs, kinorhynchs, priapulids, tardigrades, and arthropods form a well-supported monophyletic group of molting animals, named Ecdysozoa. However, our phylogenetic analysis of 18S rRNA genes failed to support the monophyly of this group definitively. Therefore, it is our opinion that the monophyly of molting animals needs additional support, and new molecular data from other genes and/or more sophisticated methods of analysis are necessary to understand the phylogenetic relationships among these animals groups.

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