18S rRNA Data Indicate That Aschelminthes Are Polyphyletic in Origin and Consist of at Least Three Distinct Clades

Birgitta Winnepenninckx, Thierry Backeljau,† Laura Y. Mackey,‡ Jacqueline M. Brooks,‡ Rupert De Wachter,* Sudhir Kumar,§ and James R. Garey‡*

*Departement Biochemie, Universiteit Antwerpen (UIA); †Koninklijk Belgisch Instituut voor Natuurwetenschappen; ‡Department of Biological Sciences, Duquesne University; §Institute of Molecular Evolutionary Genetics, Pennsylvania State University

The Aschelminthes is a collection of at least eight animal phyla, historically grouped together because the absence of a true body cavity was perceived as a pseudocoelom. Analyses of 18S rRNA sequences from six Aschelminth phyla (including four previously unpublished sequences) support polyphyly for the Aschelminthes. At least three distinct groups of Aschelminthes were detected: the Priapulida among the protostomes, the Rotifera-Acanthocephala as a sister group to the protostomes, and the Nematoda as a basal group to the triploblastic Eumetazoa.

Introduction

High-level phylogenetic relationships among animals have been based upon several characters, including the number of embryonic tissue layers, early embryonic cleavage patterns, larval morphology, body symmetry, and the type of body cavity present. Three body cavity conditions are commonly recognized among the triploblastic animals: the absence of a body cavity (acoelomate), the presence of a false body cavity that appears as a persistent blastocoel (pseudocoelom), and the true body cavity that arises from within the mesoderm (eucoelom). The bilateral ancestor to the triploblastic Metazoa is often presented as a "flatworm-like" acoelomate from which the pseudocoelomate and eucoelomate animals arose following the development of the pseudocoelom and eucoloem. The body cavity presumably allowed better internal organization that enabled animals to become larger and more active (reviewed in Brusca and Brusca 1990). The eucoelomates led to the "mainstream" line of evolution that split into the protostomes and the deuterostomes. Most of the pseudocoelomate animals have been relegated to a diverse group of at least eight phyla known as the Aschelminthes (reviewed in Hyman 1951; Marcus 1958; Clark 1979; Brusca and Brusca 1990).

Ultrastructural studies have suggested that the boundaries between the three body cavity types are not well defined. These studies suggest that the body cavity conditions perceived as pseudocoelomate and acoelomate may be the result of a reduced or modified eucoelom (Rieger 1985; Ruppert 1991*a*; Rieger et al. 1991). For example, the nemerteans are traditionally allied with the acoelomate Platyhelminthes, but molecular, morphological, and embryonic studies consistently suggest that they are actually protostome coelomates (Turbeville et al. 1992). Recently, the free-living marine nematode *Anoplostoma vivipara* was described as lacking a pseudocoel (Ehlers 1994), and the author concluded that the acoelomate condition is ancestral to the nematodes. However, if acoelomates are the ancestors of modern triploblastic Metazoa, one would expect that a novel structure such as the pseudocoelom would most likely evolve only once. If so, a putative pseudocoelomate ancestor would have evolved into the modern aschelminth phyla, and the Aschelminthes would be a valid monophyletic taxon. Conversely, if eucoelomates were the ancestors of modern triploblastic Metazoa, one would expect that the pseudocoelom could have evolved any number of times by modification or partial loss of the eucoelom. This could have occurred under evolutionary pressures that favored small animals in which the eucoelom was not an advantage (Ruppert 1991*a*), in which case the aschelminth phyla would not necessarily be related to one another, and the Aschelminthes could not be considered a valid taxon.

The evolutionary relationships between animal phyla have been examined many times by a number of authors using a variety of morphological characters (for recent examples, see Brusca and Brusca 1990; Schram 1991; Eernisse et al. 1992; Backeljau et al. 1993; Nielsen 1995) with varying results concerning the polyphyly of the Aschelminthes. However, the most explicit cladistic

Key words: 18S ribosomal RNA, molecular, phylogeny, Aschelminthes, pseudocoelom, Metazoa.

Address for correspondence and reprints: James R. Garey, Department of Biological Sciences, Duquesne University, Pittsburgh, Pennsylvania 15282; E-mail: garey@next.duq.edu.

Mol. Biol. Evol. 12(6):1132–1137. 1995.

0737-4038/95/1206-0016$02.00

analysis that focused specifically on aschelminth evolutionary relationships is that of Lorenzen (1985). He considered the phyla Rotifera, Acanthocephala, Nematoda, Nematomorpha, Gastrotricha, Kinorhyncha, and Priapulida and concluded that the pseudocoelomates are polyphyletic, forming a number of distinct clades (Rotifera-Acanthocephala, Nematoda-Nematomorpha-Gastrotricha, and Priapulida-Kinorhyncha).

Molecular data can complement morphological data and are useful when comparing distantly related organisms that have few common characters. The small ribosomal subunit RNA gene (18S rRNA) has proven useful with distantly related organisms because it is reasonably large (about 1,800 nucleotides in length), highly conserved, and data are available from a large number of organisms (Hillis and Dixon 1991; Larsen et al. 1993; Neefs et al. 1993). The 18S rRNA gene has been used to investigate the origin of the animal kingdom (Field et al. 1988; Wainright et al. 1993) and several other problems in animal phylogeny (see, e.g., Nadler 1992; Turbeville et al. 1992; Telford and Holland 1993; Halanych et al. 1995).

We have chosen to test the hypothesis that the Aschelminthes is made up of several clades and to determine the location of these clades among the Eumetazoa by analysis of the 18S rRNA gene. This analysis includes new unpublished sequences from one representative of the phyla Gastrotricha, Nematomorpha, Priapulida, and Rotifera (Monogononta) as well as previously published sequences of representatives of the phyla Acanthocephala and Nematoda (Ellis et al. 1986; Telford and Holland 1993; Fitch et al. 1995) that have historically been included in the Aschelminthes.

Material and Methods

Rotifer (*Brachionus plicatilis)* cysts were obtained commercially (Aquaculture Supply; Dade City, Fla.) and cultured in sea water (Snell et al. 1987). Rotifers were separated from feeder algae and concentrated by sieving through plankton netting and starved for several hours to clear algae from the gut. Gastrotrichs (*Lepidodermella squammata*) were purchased from a commercial supplier (Carolina Biological Supply Company; Burlington, N.C.), and several hundred individuals were isolated from the culture with a mouth micropipette and starved for several hours. DNA was prepared (Hempstead et al. 1990) and 0.1 μg used as template for polymerase chain reaction (PCR) amplification. Four primers were used that resulted in two overlapping fragments representing a nearly complete fragment of the 18S rRNA gene corresponding to nucleotides 130–1,965 of the human sequence (GenBank accession M10098). PCR amplification was carried out for 35 cycles with 30 s at 94° denaturing, 90 s at 55° annealing, and 120 s at 72° extension (Ausubel et al. 1995). The primers contained restriction endonuclease site containing tails for cloning purposes (Garey et al. 1992). The PCR products were cloned into M13 mp18 nondirectionally using an appropriate restriction enzyme. M13 clones containing inserts in opposite orientation were identified by complement testing. DNA sequencing of single cloned fragments was carried out completely in both directions from the M13 templates with the chain termination method using Sequenase (US Biochemical; Cleveland, Ohio), commercial M13 primers, and conserved internal primers. Additional sequencing reactions were carried out using inosine mixes as needed to resolve some sequencing artifacts. Nematomorphs (*Gordius aquaticus*) were collected in the Pyrenees (France). A priapulid (*Priapulus caudatus*) was found in the coastal waters of Kristineberg (Sweden). DNA was extracted (Winnepenninckx et al. 1993) from a single nematomorph specimen and from the skin tissue of the priapulid. The 18S rRNA genes were PCR amplified in two overlapping fragments using two primers complementary to the 5′ and 3′ ends of the 18S rRNA gene and two primers complementary to a conserved part of the 18S rRNA gene and the 5′ end of the 28S rRNA gene. PCR amplification was carried out on 10 ng DNA template for 30 cycles of 60 s at 94°, 60 s at 55°, and 120 s at 72°. PCR fragments were ligated into T-tailed PSK+ vector (BioRad; Richmond, Calif.), and DNA from a pool of 10 clones was sequenced using a variety of primers (Winnepenninckx et al. 1994). Recently, an unpublished sequence of the 18S rRNA gene of *Priapulus caudatus,* appeared in GenBank (GenBank accession number Z38009) and differs from the sequence reported here at 15 different nucleotide sites. It did not affect the topology of our trees when substituted for the priapulid sequence reported here.

Aschelminth sequences were aligned with those of other animals and yeast (see below) according to a secondary structure model (Neefs et al. 1993). Sites containing gaps were excluded from phylogenetic analyses to reduce systematic errors. Alignments were analyzed with the MEGA program (Kumar et al. 1994) to produce neighbor-joining (NJ) trees using the Kimura two-parameter model in which substitution rates follow a gamma distribution with shape parameter $a = 0.72$ to correct for multiple substitutions at the same site (Jin and Nei 1990). The gamma parameter of 0.72 was estimated from the distribution of the number of nucleotide substitutions across different sites as obtained in a parsimony analysis of 202 diverse eukaryotic 18S rRNA sequences (S. Kumar and A. Rzhetsky, unpublished data). Confidence in NJ trees was determined by analyzing 1,000 bootstrap replicates using the MEGA pro-

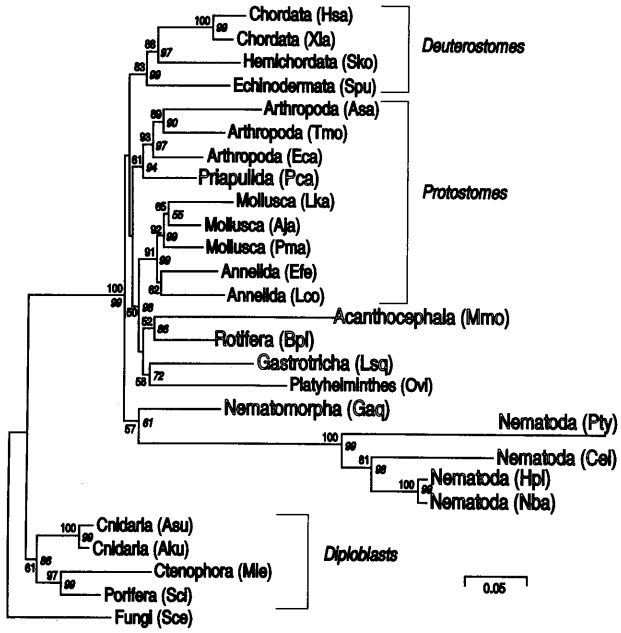

FIG. 1.—The neighbor-joining tree. The aschelminth phyla names are indicated with hollow letters. Note that the aschelminth "pseudocoelomates" cannot be considered monophyletic because the Priapulida form a clade with the arthropods, the acoelomate Platyhelminthes are buried within a number of aschelminth phyla, and the Nematoda-Nematomorpha clade branches before any other eumetazoans. Numbers to the left of each fork are percentages of 1,000 bootstrap replicates that support the branch, and italicized numbers to the right are the confidence probabilities (see text) except for the branch leading to the triploblasts where the bootstrap number is above and the confidence probability is below the fork. Values are shown only if over 50%. All branch lengths are drawn to scale. See text for definitions of the three-letter binomial abbreviations shown in parentheses.

gram and by conducting an interior branch-length test (Sitnikova et al. 1995) to compute the confidence probability that the branch lengths are significantly different from zero (confidence probability test; Rzhetsky and Nei 1992) using the PHYLTEST program (Kumar 1995). Bootstrapped maximum-parsimony (MP) trees were produced using DNAPARS and associated programs from the PHYLIP package (Felsenstein 1993). Confidence in the MP trees was determined by analyzing 1,000 bootstrap replicates. An additional parameter used to determine tree reliability was the congruence of trees produced using different tree-making algorithms (NJ and MP).

Previously published sequences used for phylogenetic analyses were obtained from GenBank. They were chosen to represent the major deuterostome and protostome taxa, other aschelminth groups, acoelomates, and diploblasts. The following lists the phylum or subphylum, common name if any, binomial name, three-letter abbreviation used in figures 1 and 2, and the GenBank accession number of species used: Chordata, human, *Homo sapiens,* Hsa, M10098; Chordata, frog, *Xenopus laevis,* Xla, X02995; Hemichordata, acorn worm, *Saccoglossus kowalevskii,* Sko, L28054; Echinodermata, sea urchin, *Strongylocentrotus purpuratus,* Spu, L28056; Arthropoda, brine shrimp, *Artemia salina,* Asa, X01723; Arthropoda, beetle, *Tenebrio molitor,* Tmo, X07801; Arthropoda, spider, *Eurypelma californica,* Eca, X13457; Priapulida, *Priapulis caudatus,* Pca, X87984; Mollusca, snail, *Limicolaria kambeul,* Lka, X66374; Mollusca, chiton, *Acanthopleura japonica,* Aja, X70210; Mollusca, scallop, *Placopecten magellanicus,* Pma, X53899; Annelida, earthworm, *Eisenia fetida,* Efo, X79872; Annelida, Polychaete, *Lanice conchilega,* Lco, X79873; Acanthocephala, *Moniliformis moniliformis,* Mmo, Z19562; Rotifera, *Brachionus plicatilis,* Bpl, U29235; Gastrotricha, *Lepidodermella squammata,* Lsq, U29198; Platyhelminthes, fluke, *Opisthorchis viverrini,* Ovi, X55357; Nematomorpha, *Gordius aquaticus,* Gaq, X87985; Nematoda, *Pellioditis typica,* Pty, U13933; Nematoda, *Caenorhabditis elegans,* Cel, X03680; Nematoda, *Haemonchus placei,* Hpl, L04154; Nematoda, *Nematodirus battus,* Nba, U01230; Cnidaria, sea anemone, *Anemonia sulcata,* Asu, X53498; Cnidaria, sea anemone, *Anthopleura kurogane,* Aku, Z21671; Ctenophora, comb jelly, *Mnemiopsis leidyi,* Mle, L10826; Porifera, sponge, *Scypha ciliata,* Sci, L10827; Fungi, yeast, *Saccharomyces cerevisiae,* Sce, M27607.

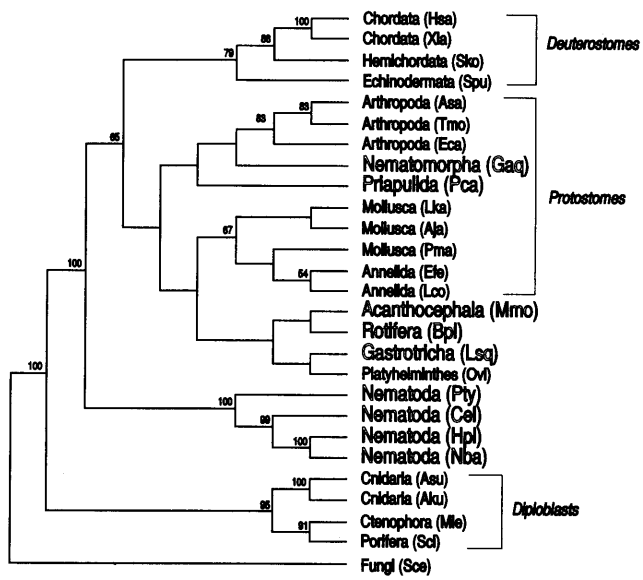

FIG. 2.—The maximum-parsimony tree. The topology is very similar to the neighbor-joining tree in fig. 1 except for the location of the Nematomorpha. Aschelminth phyla names are indicated with hollow letters, and numbers above each fork are percentages of 1,000 bootstrap replicates that support the branch and are shown only for values over 50%. Branch lengths shown are arbitrary and unrelated to evolutionary distance. See text for definitions of the three-letter binomial abbreviations shown in parentheses.

Results and Discussion

The alignment produced NJ and MP trees that are generally consistent with what is known of animal evolution (figs. 1 and 2). For example, the deuterostome phyla form a single clade, while the protostome groups form an arthropod clade and an annelid-mollusk clade, reminiscent of the Eutrochozoa taxon (Eernisse et al. 1992). However, the analyses do not lend support as to the monophyly of protostomes because they appear as a statistically unsupported paraphyletic group in the NJ and MP trees as do the mollusks in the MP tree. As expected, the four diploblastic taxa branch immediately after yeast but before all triploblastic groups.

The aschelminth groups fall into several regions of the trees. The Priapulida are clearly associated with the arthropods. This relationship is supported by the congruence of both MP and NJ trees and in moderate bootstrap and strong confidence probability support in the NJ tree. The Acanthocephala and Rotifera form a clade in both trees, with weak bootstrap but moderate confidence probability support in the NJ tree. Similarly, the Gastrotricha and Platyhelminthes form a clade in both trees, with weak bootstrap and confidence probability support in the NJ tree. The association of Gastrotricha + Platyhelminth with Acanthocephala + Rotifera is seen in both trees but is not supported statistically. The Rotifera, Acanthocephala, Gastrotricha, and Platyhelminthes appear to be loosely associated with the protostomes in both trees, but with low bootstrap support in the NJ tree and no bootstrap support in the MP tree. The nematodes appear as a basal triploblastic lineage strongly supported by bootstrap analyses and congruence between the trees.

The branches leading to the nematode taxa are long, and the Nematoda form a basal lineage to the triploblastic Eumetazoa in the trees (figs. 1 and 2). Although we cannot eliminate the possibility that the position of the Nematoda is an artifact due to the long branch length, we examined topologies where the Nematoda was not placed as a basal lineage using four-cluster analysis (Rzhetsky et al.1995). These analyses showed that the placement of the Nematoda as a basal lineage of the triploblastic Eumetazoa led to the smallest (minimum-evolution) tree and that the Nematoda are closer to Cnidaria and yeast than to any bilateral metazoan with the exception of the Nematomorpha. A recent analysis of the cytochrome *c* gene also places the Nematoda as a basal triploblastic eumetazoan (Vanfleteren et al. 1994). However, the extremely long branches leading to the nematode taxa makes their placement among the Metazoa uncertain.

The NJ and MP trees (figs. 1 and 2) appear to support at least three separate aschelminth clades: the Priapulida with the arthropod protostomes, the Acanthocephala + Rotifera clade, and the Nematoda. The placement of the Gastrotricha and Nematomorpha is more uncertain. The Gastrotricha do not appear to be associated with other aschelminth taxa but may be associated with the Platyhelminthes, and there is some bootstrap and confidence probability support for the inclusion of the Nematomorpha with the Nematoda in the NJ tree (fig. 1). It is of interest to note that the Gastrotricha, although historically considered pseudocoelomate, have recently been described as being acoelomate (reviewed in Brusca and Brusca 1990; Ruppert 1991*b*). The Nematomorpha appear in a clade with the nematodes in the NJ tree with some statistical support but appear among the arthropods with no bootstrap support in the MP tree. The position of the Nematomorpha is the only major disagreement between the MP and NJ tree topologies, possibly due to the rapid evolutionary rate of nematodes.

Our analyses strongly support the hypothesis that the Aschelminthes are polyphyletic in origin and consist of several distinct clades. Polyphyly is supported by the location of "pseudocoelomate" aschelminths in three distinct branches of animal phylogeny (figs. 1 and 2): the Priapulida fall clearly among the protostomes; the Gastrotricha, Rotifera, and Acanthocephala form a loose "group" just outside the protostomes with the acoelomate nonaschelminth Platyhelminthes buried within; and the Nematoda and possibly Nematomorpha branch before all other triploblastic Eumetazoa. Many of these results are in agreement with independent morphological analyses. Authors have most commonly included the Priapulida either among the protostomes or among the Aschelminthes in recent years (Van Der Land and Nørrevang 1985; Conway Morris 1993). Lorenzen (1985) concluded that the phyla Nematomorpha, Nematoda, and Gastrotricha form a clade based on similarities in early development patterns, pharynx structure, nervous system, and body muscle innervation. Our data show some support for a Nematoda-Nematomorpha clade and are inconclusive with respect to the location of the Gastrotricha. Lorenzen also suggested that the Acanthocephala are closely related to Rotifera, based on similarities of the epidermis and proboscis and the presence of lemnisci in both groups. This relationship is also supported by ultrastructural studies of spermatozoa (Melone and Ferraguti 1994) and other morphological characters (Remane 1963). Our molecular data provide additional support for this relationship. The close relationship between Acanthocephala and Rotifera is also supported by a recent cladistic analysis (Neuhaus 1994) which also proposes that the phyla Gastrotricha, Nematoda, Nematomorpha, Priapulida, Loricifera, and Kinorhyn-

cha form a monophyletic group (Nemathelminthes), in disagreement with our molecular analysis. A more recent morphological study (Rieger and Tyler 1995) has proposed that the Acanthocephala-Rotifera clade be extended to include the phylum Gnathostomulida, while Nielsen (1995) still views the Aschelminthes as a monophyletic group.

The most fundamental evolutionary implication of multiple origins of pseudocoelomates is that the body cavity type is of less phylogenetic significance than previously considered (Remane 1963; Rieger 1985; Ruppert 1991*a*; Rieger et al. 1991) and implies that body cavities that appear to be pseudocoeloms could easily be derived by the modification of existing eucoeloms. This hypothesis has been suggested numerous times and is supported by our data. Further, our analysis rejects the Aschelminthes as a valid phylogenetic grouping.

Acknowledgments

This research was supported by U.S. Department of Agriculture grants 9304072 to J.R.G., FJBR 2.0030.93 to R.D.W., and FJBR 2.0023.94 to T.B. S.K. was supported by National Science Foundation grant BSR 9119802 to M. Nei. B.W. was supported by an IWT scholarship. The authors thank Terry Snell at Georgia Tech University for help with the rotifer cultures and Margaret Hummon at Ohio University and David King at Carolina Biological for assistance with gastrotrich cultures. We also thank W.H.O. De Smet at the University of Antwerp for providing the nematomorph sample and M. Thollesson at the University of Göteborg for providing the priapulid DNA. We thank Edward Ruppert and Peter Emschermann for their helpful comments on the manuscript.

LITERATURE CITED

AUSUBEL, F. M., R. BRENT, R. E. KINGSTON, D. D. MOORE, J. G. SEIDMAN, J. A. SMITH, and K. STRUHL. 1995. Current protocols in molecular biology. Greene, New York.

BACKELJAU, T., B. WINNEPENNINCKX, and L. DE BRUYN. 1993. Cladistic analysis of metazoan relationships: a reappraisal. Cladistics **9**:167–181.

BRUSCA, R. C., and G. J. BRUSCA. 1990. Invertebrates. Sinauer, Sunderland, Mass.

CLARK, R. B. 1979. Radiation of the Metazoa. Pp. 55–101 *in* M. R. HOUSE, ed. The origins of major invertebrate groups. Academic Press, New York.

CONWAY MORRIS, S. 1993. The fossil record and the early evolution of the Metazoa. Nature **361**:219–225.

EERNISSE, D. J., J. S. ALBERT, and F. E. ANDERSON. 1992. Annelida and Arthropoda are not sister taxa: a phylogenetic analysis of spiralian metazoan morphology. Syst. Biol. **41**: 305–330.

EHLERS, U. 1994. Absence of a pseudocoel or pseudocoelom in *Anoplostoma vivipara* (Nematodes). Microfauna Marina **9**:345–350.

ELLIS, R. E., J. E. SULSTON, and A. R. COULSON. 1986. The rDNA of *C. elegans:* sequence and structure. Nucleic Acids Res. **14**:2345–2364.

FELSENSTEIN, J. 1993. PHYLIP: phylogeny inference package, version 3.5. University of Washington, Seattle.

FIELD, K. G., G. J. OLSEN, D. J. LANE, S. J. GIOVANNONI, M. T. GHISELIN, E. C. RAFF, N. R. PACE, and R. A. RAFF. 1988. Molecular phylogeny of the animal kingdom. Science **239**:748–753.

FITCH, D. H. A., B. BUGAJ-GAWEDA, and S. W. EMMONS. 1995. 18S ribosomal RNA gene phylogeny for some Rhabditidae related to *Caenorhabditis.* Mol. Biol. Evol. **12**:346–358.

GAREY, J. R., R. LABBE-BOIS, A. CHELTOWSKA, J. RYTKA, L. M. HARRISON, J. P. KUSHNER, and P. LABBE. 1992. Heme biosynthesis in *Saccharomyces cerevisiae: HEM12* gene sequence and evidence for two conserved glycines essential for enzymatic activity. Eur. J. Biochem. **205**:1011–1016.

HALANYCH, K. M., J. D. BACHELLER, A. M. A. AGUINALDO, S. M. LIVA, D. M. HILLIS, and J. A. LAKE. 1995. Evidence from 18S ribosomal DNA that the lophophorates are protostome animals. Science **267**:1641–1643.

HEMPSTEAD, P. G., S. C. REGULAR, and I. R. BALL. 1990. A method for the preparation of high-molecular-weight DNA from marine and freshwater triclads. DNA Cell Biol. **9**: 57–61.

HILLIS, D. M., and M. T. DIXON. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Quart. Rev. Biol. **66**:411–446.

HYMAN, L. B. 1951. The invertebrates. Vol. **3**. Pseudocoelomate groups. McGraw-Hill, New York.

JIN, L., and M. NEI. 1990. Limitations of the evolutionary parsimony method of phylogenetic analysis. Mol. Biol. Evol. **7**:82–102.

KUMAR, S. 1995. PHYLTEST: phylogeny hypothesis testing using the minimum evolution method. Pennsylvania State University, State College.

KUMAR, S., K. TAMURA, and M. NEI. 1994. MEGA: molecular evolutionary genetics analysis software for microcomputers. Comput. Appl. Biosci. **10**:189–191.

LARSEN, N., G. J. OLSEN, B. L. MAIDAK, M. J. MCCAUGHEY, R. OVERBEEK, T. J. MACKE, T. L. MARSH, and C. R. WOESE. 1993. The ribosomal database project. Nucleic Acids Res. **21**:3021–3023.

LORENZEN, S. 1985. Phylogenetic aspects of pseudocoelomate evolution. Pp. 210–223 *in* S. CONWAY MORRIS, J. D. GEORGE, R. GIBSON, and H. M. PLATT, eds. The origins and relationships of lower invertebrates. Systematics Association Vol. **28**. Clarendon, Oxford.

MARCUS, E. 1958. On the evolution of the animal phyla. Quart. Rev. Biol. **33**:24–58.

MELONE, G., and M. FERRAGUTI. 1994. The spermatozoa of *Brachionus plicatilis* (Rotifera, Monogononta) with some

notes on sperm ultrastructure in Rotifera. Acta Zool. **75**: 81–88.

NADLER, S. A. 1992. Phylogeny of some ascaridoid nematodes, inferred from comparison of 18S and 28S rRNA sequences. Mol. Biol. Evol. **9**:932–944.

NEEFS, J.-M., Y. VAN DE PEER, P. DE RIJK, S. CHAPELLE, and R. DE WACHTER. 1993. Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res. **21**:3025–3049.

NEUHAUS, B. 1994. Ultrastructure of alimentary canal and body cavity, ground pattern, and phylogenetic relationships of the Kinorhyncha. Microfauna Marina **9**:61–156.

NIELSEN, C. 1995. Animal evolution, interrelationships of the living phyla. Oxford University Press, Oxford.

REMANE, A. 1963. The systematic position and phylogeny of the pseudocoelomates. Pp. 247–255 *in* E. C. DOUGHERTY, ed. The lower Metazoa. University of California Press, Berkeley.

RIEGER, R. M. 1985. The phylogenetic status of the acoelomate organization within the Bilateria: a histological perspective. Pp. 101–122 *in* S. CONWAY MORRIS, J. D. GEORGE, R. GIBSON, and H. M. PLATT, eds. The origins and relationships of lower invertebrates. Systematics Association Vol. **28**. Clarendon, Oxford.

RIEGER, R. M., G. HASZPRUNAR, and P. SCHUCHERT. 1991. On the origin of the Bilateria: traditional views and recent alternative concepts. Pp. 107–112 *in* A. M. SIMONETTA and S. CONWAY MORRIS, eds. The early evolution of Metazoa and the significance of problematic taxa. Cambridge University Press, New York.

RIEGER, R. M., and S. TYLER. 1995. Sister-group relationship of Gnathostomulida and Rotifera-Acanthocephala. Invert. Biol. **114**:186–188.

RUPPERT, E. E. 1991*a*. Introduction to the aschelminth phyla: a consideration of mesoderm, body cavities, and cuticle. Pp. 1–17 *in* F. W. HARRISON and E. E. RUPPERT, eds. Microscopic anatomy of invertebrates. Vol. **4**. Aschelminthes. Wiley-Liss, New York.

———. 1991*b*. Gastrotricha. Pp. 41–109 *in* F. W. HARRISON and E. E. RUPPERT, eds. Microscopic anatomy of Invertebrates. Vol. **4**. Aschelminthes. Wiley-Liss, New York.

RZHETSKY, A., S. KUMAR, and M. NEI. 1995. Four-cluster analysis: a simple method to test phylogenetic hypotheses. Mol. Biol. Evol. **12**:163–167.

RZHETSKY, A., and M. NEI. 1992. A simple method for estimating and testing minimum-evolution trees. Mol. Biol. Evol. **9**:945–967.

SCHRAM, F. R. 1991. Cladistic analysis of metazoan phyla and the placement of fossil problematica. Pp. 35–46 *in* A. M. SIMONNETTA and S. CONWAY MORRIS, eds. The early evolution of Metazoa and the significance of problematic taxa. Cambridge University Press, Cambridge.

SITNIKOVA, T., A. RZHETSKY, and M. NEI. 1995. Interior-branch and bootstrap tests of phylogenetic trees. Mol. Biol. Evol. **12**:319–333.

SNELL, T. A., M. J. CHILDRESS, and E. M. BOYER. 1987. Assessing the status of rotifer mass cultures. J. World Aquacult. Soc. **18**:270–277.

TELFORD, M. J., and P. W. H. HOLLAND. 1993. The phylogenetic affinities of the Chaetognaths: a molecular analysis. Mol. Biol. Evol. **10**:660–676.

TURBEVILLE, J. M., K. G. FIELD, and R. A. RAFF. 1992. Phylogenetic position of phylum Nemertini, inferred from 18S rRNA sequences: molecular data as a test of morphological character homology. Mol. Biol. Evol. **9**:235–249.

VAN DER LAND, J., and A. NØRREVANG. 1985. Affinities and intraphyletic relationships of the Priapulida. Pp. 261–273 *in* S. CONWAY MORRIS, J. D. GEORGE, R. GIBSON, and H. M. PLATT, eds. The origins and relationships of lower invertebrates. Systematics Association Vol. **28**. Clarendon, Oxford.

VANFLETEREN, J. R., Y. VAN DE PEER, M. L. BLAXTER, S. A. R. TWEEDIE, C. TROTMAN, L. LU, M.-L. VAN HAUWAERT, and L. MOENS. 1994. Molecular genealogy of some nematode taxa as based on cytochrome *c* and globin amino acid sequences. Mol. Phylogen. Evol. **3**:92–101.

WAINRIGHT, P. O., G. HINKLE, M. L. SOGIN, and S. K. STICKEL. 1993. Monophyletic origins of the Metazoa: an evolutionary link with fungi. Science **260**:340–342.

WINNEPENNINCKX, B., T. BACKELJAU, and R. DE WACHTER. 1993. Extraction of high molecular weight DNA from molluscs. Trends Genet. **9**:407.

———. 1994. Small ribosomal subunit RNA and the phylogeny of molluscs. Nautilus, Suppl. **2**:98–110.

MITCHELL SOGIN, reviewing editor

Received February 24, 1995

Accepted July 5, 1995