

Letter to the Editor

A New Perspective on Lower Metazoan Relationships from 18S rDNA Sequences

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Despite a number of recent molecular phylogenetic analyses, the relationships between the diploblastic lower metazoan phyla are far from resolved. These molecular phylogenies, usually based on 18S rDNA sequences, often conflict with each other and with traditional views on the early evolution of the metazoa. One of the more startling hypotheses places the diploblastic phyla that have true nervous systems (Ctenophora, Cnidaria) in a clade with the sponges (Porifera). This result suggests that the nervous system may have evolved independently in diploblasts and triploblasts or has been lost in the Porifera (Christen et al. 1991; Hanelt et al. 1996; reviewed in Cavalier-Smith et al. 1996). There are many other major controversies about lower metazoan evolution. Is the former protistan phylum Myxozoa a triploblast or a member of the Cnidaria (Smothers et al. 1994; Siddall et al. 1995; Anderson, Canning, and Okamura 1998)? What are the relationships of the enigmatic diploblast *Trichoplax adhaerens* (Placozoa; Wainright et al. 1993; Bridge et al. 1995; Siddall et al. 1995; Cavalier-Smith et al. 1996)? Is the phylum Porifera monophyletic (Cavalier-Smith et al. 1996)? What are the status of the monophyly and relationships of the four Cnidarian classes (Bridge et al. 1992, 1995; Siddall et al. 1995)?

One reason for the major disagreement between these 18S rDNA studies is the large evolutionary rate differences often observed between various lineages (e.g., Maley and Marshall 1998). It is well known that long-branch attraction can confound phylogenetic analysis (Felsenstein 1978; Huelsenbeck 1997). We have adopted two methods to ameliorate the negative effect of large rate differences. First, we followed the taxon-pruning procedure advocated by Aguinaldo et al. (1997), whereby for every clearly defined monophyletic lineage, only the taxon with the slowest evolving 18S gene is retained for further analyses. While sequencing additional lineages to identify slowly evolving taxa, we increased the number of complete or nearly complete 18S rDNA sequences for the major diploblastic lineages by over 40%. Second, we used a maximum-likelihood framework to choose a model which best explains the data. Best-fit models, particularly those which incorporate among-site rate variation, have the potential to overcome the negative effects of long-branch attraction (Huelsenbeck 1997; Cunningham, Zhu, and Hillis 1998).

Key words: 18S rDNA, Metazoa, Cnidaria, Placozoa, Myxozoa, Porifera, Ctenophora, phylogenies, long-branch attraction, maximum likelihood.

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Our analysis included three steps. These were as follows: choosing an outgroup for the Metazoa, identifying major monophyletic lineages of diploblasts, and performing a higher level phylogenetic analysis using diploblast taxa selected by the Aguinaldo procedure.

In choosing an outgroup, there are two candidates for the closest outgroups to the Metazoa. These include the Fungi and the protistan Choanoflagellata. Although statistical support is not strong, most major analyses of 18S rDNA place the Choanoflagellata as the sister group to the Metazoa (Wainright et al. 1993; Cavalier-Smith et al. 1996; Kumar and Rzhetsky 1996; but see Van de Peer and De Wachter 1997). Furthermore, a slowly evolving fungal taxon (*Chytridium confervae* GB M59758, Wainright et al. 1993) was consistently farther from the Metazoa than the Choanoflagellata (fungal/metazoan logdet distance = 0.1319; mean choanoflagellate/metazoan logdet distance = 0.11624). In keeping with the Aguinaldo procedure, the choanoflagellate with the shortest branch (*Diaphanoeca grandis*) was used as the outgroup for all of our analysis. Analyses that included *Chytridium confervae* arrived at the same conclusions as those presented here (C. Cunningham, unpublished data).

Our next step was to identify clearly monophyletic diploblastic lineages. We examined nearly all available complete (or nearly complete) diploblastic 18S rDNA sequences, seven of which were sequenced in the course of this study. Entire 18S rDNA sequences were amplified by polymerase chain reaction using primers each of which recognize conserved sequences proximal to 5' and 3' termini of eukaryotic 18S rDNAs, respectively (Medlin et al. 1988). The PCR products of *Bellonella rigida*, *Gymnanagium hians*, *Coryne pusilla*, *Obelia* sp., and *Cassiopea* sp. were inserted into T-vector (Promega) and determined on both strands by the dideoxy chain-termination method (Sanger, Nicklen, and Coulson 1977). For *Haliclystus* sp. and *Craterolophus convolvulus*, cloning of PCR products was conducted using the TA cloning kit (Invitrogen). Both strands of each sequence were obtained by cycle sequencing with ABI prism kits as per the manufacturer's instructions, read on an ABI 373 automated sequencer, and edited with Sequencher 3.0 (Genecodes Corp.). Sequences were aligned using CLUSTAL W (Thompson, Higgins, and Gibson 1994) and adjusted according to secondary structure of *Bellonella rigida* (kindly provided by R. De Wachter). The final sequence alignment consisted of 1,756 base pairs after 166 ambiguously aligned base pairs were excluded. The alignment is available from the EBI FTP server under accession code DS36208, by anonymous FTP from FTP.EBI.AC.UK in directory /pub/databases/embl/align, from the World Wide Web at ftp://ftp.ebi.ac.uk/pub/databases/embl/align/, or by send-

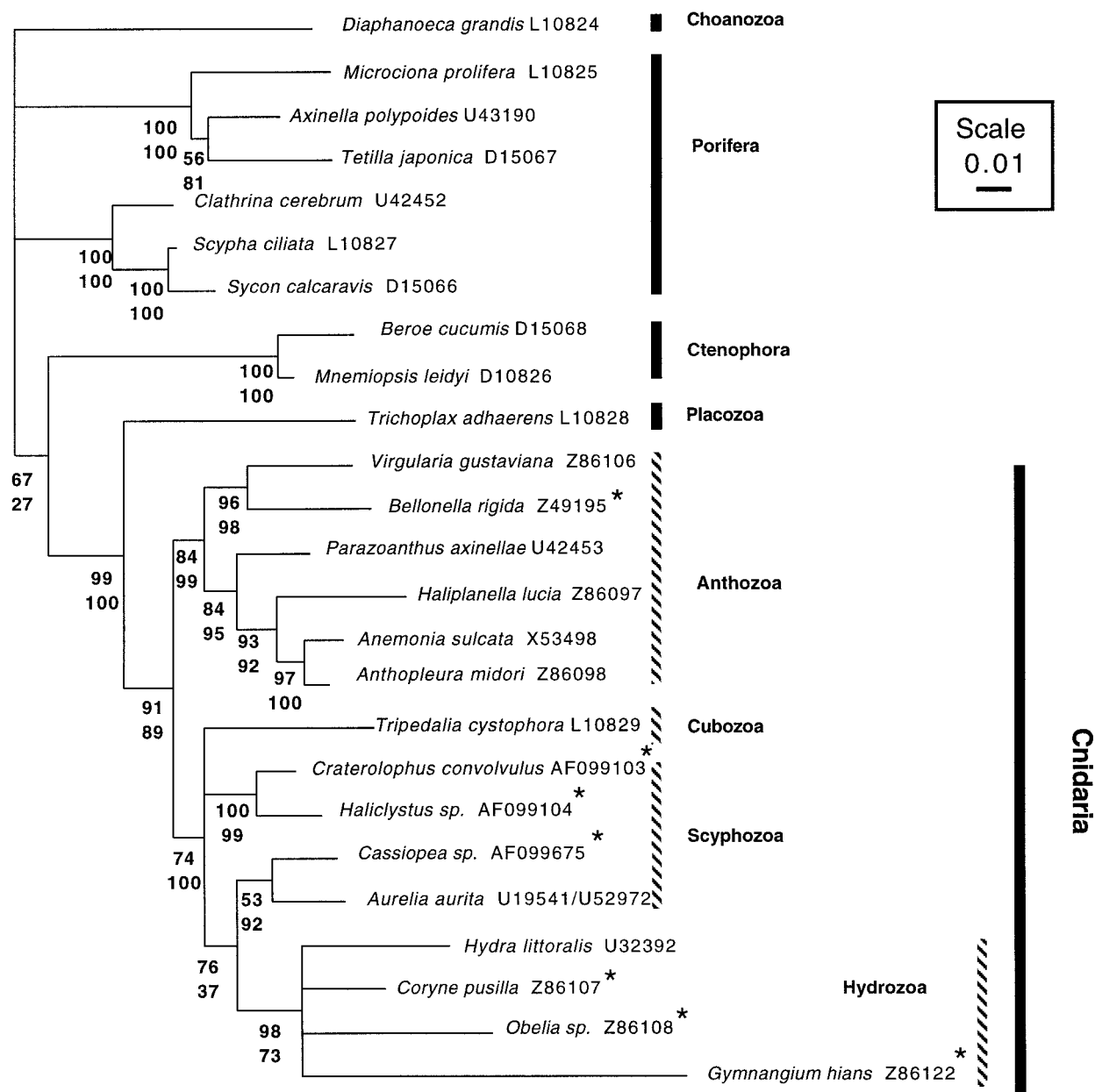


FIG. 1.—Phylogenetic analysis of 18S rDNA sequences for the diploblastic taxa. Numbers near taxa refer to GenBank accession numbers, and asterisks refer to taxa sequenced in the course of this study. The scale is in substitutions per site as calculated in our ML analysis. All branches were collapsed with ML bootstrap support <50%. The top number at each node refers to 100 bootstrap pseudoreplicates with the best-fit ML model. The best-fit model was found as described by Yang (1996) and Cunningham, Zhu, and Hillis (1998) and was the general time-reversible model (Lanave et al. 1984), with equal base frequencies and with among-site rate variation approximated by estimating the number of invariable sites. A discrete gamma distribution was not estimated because the increase in computational time was prohibitive (see Cunningham, Zhu, and Hillis 1998). The bottom number at each node represents a minimum-evolution distance with logdet/paralinear distances (Lake 1994; Lockhart et al. 1994). Both methods were applied by heuristic searches with tree-bisection-reconnection branch swapping with PAUP*, version 4.0d63 (written by D. L. Swofford).

ing an e-mail message to netserv@ebi.ac.uk including the line GET ALIGN:DS36208.DAT.

We surveyed a reasonably comprehensive sample of diploblastic taxa. According to the classification in Brusca and Brusca (1990), our study includes two out of three classes of Porifera, both classes of Ctenophora, the only living representative of the Placozoa, and all four classes of Cnidarians. The Cnidarian classes were particularly well represented, including three out of four

orders of scyphozoans and two orders from each of the two subclasses of anthozoans. The Hydrozoa were less well sampled, but their monophyly (with the possible exception of the narcomedusan *Polypodium hydriforme*, see below) has been strongly supported by a much broader survey using partial 18S rDNA sequences (Bridge et al. 1995).

Our first analysis identified a number of strongly supported monophyletic groups (fig. 1). For each mono-

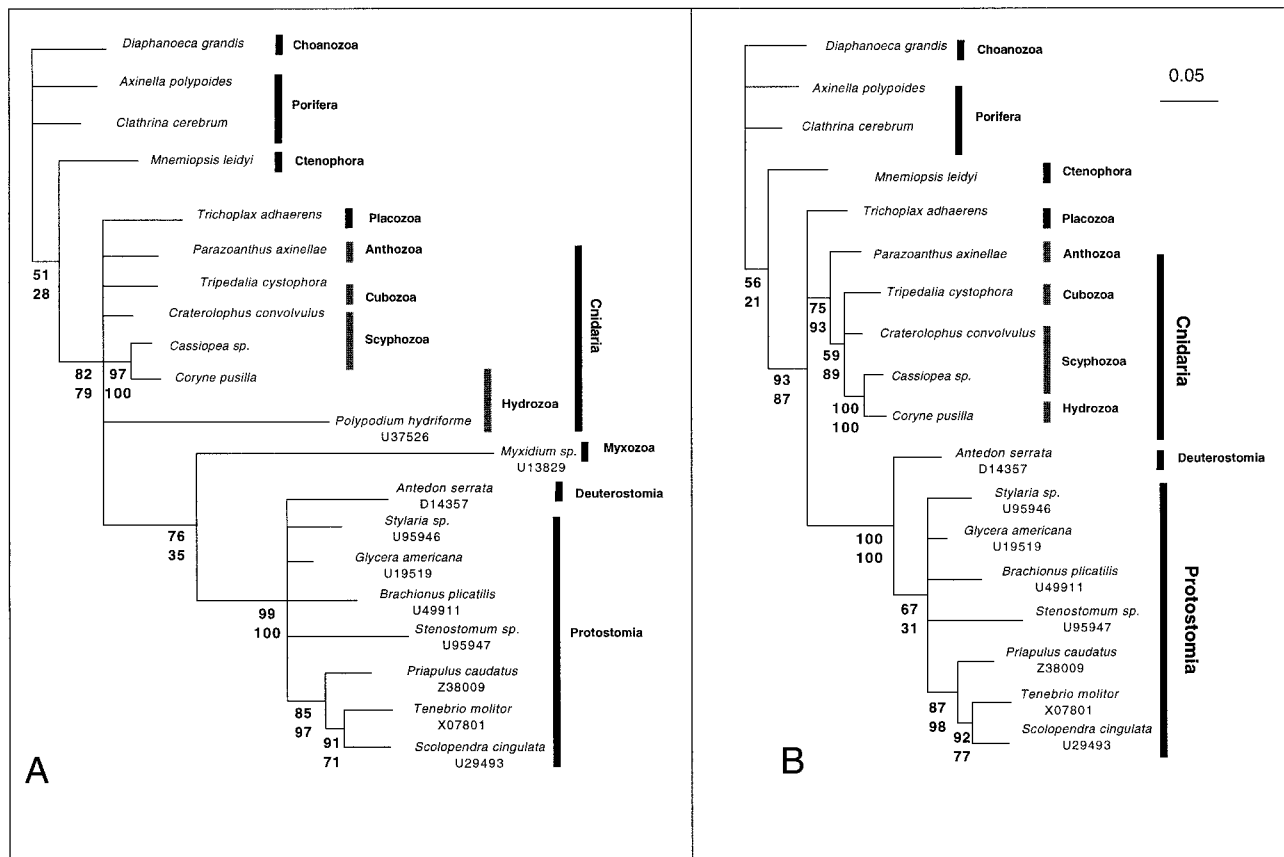


FIG. 2.—(A) Phylogenetic analysis of pruned data set from figure 1 together with eight taxa from the Aguinaldo et al. (1997) study and two enigmatic taxa (*Myxozoa* and *P. hydriforme*) that were excluded from the preliminary analysis. Top numbers refer to bootstrap support from ML, and bottom numbers refer to bootstrap support from minimum-evolution analysis. All analyses performed as described in figure 1. (B) Phylogenetic analysis as in figure 2A, excluding two taxa with very long branches (*Myxozoa* and *P. hydriforme*).

phylogenetic group with multiple representatives, the species with the shortest branch length to the ancestral node for that group was retained for further analysis (Aguinaldo et al. 1997). The strongly supported monophyletic groups with multiple representatives were two orders of Porifera, the Ctenophora, the Anthozoa, and the Hydrozoa. Although one of the two scyphozoan lineages was weakly supported in the maximum-likelihood (ML) analysis (fig. 1), *Aurelia aurita* was left out of the final analysis because it had by far the largest proportion of missing data of the taxa we analyzed. All told, 10 of the 25 taxa in figure 1 were retained for further analysis. From the alignment of Aguinaldo et al. (1997; their fig. 2), we chose seven representative taxa. Because this alignment excluded the important Platyhelminthes (flatworms), we aligned *Stenostomum* sp. (from Aguinaldo et al. [1997], their fig. 3) to the first seven taxa using the default parameters in CLUSTAL W (Thompson, Higgins, and Gibson 1994) and then removed the regions of ambiguous alignment that were excluded by Aguinaldo et al. (1997). This procedure was repeated three times: with the 10 diploblastic sequences retained from our preliminary analysis; with the myxozoan *Myxidium* sp., the myxozoan with the shortest branch (Siddall et al. 1995); and with the hydrozoan, *P. hydriforme* (Siddall et al. 1995). The final aligned matrix consisted

of 20 taxa with 1,337 base pairs of unambiguously aligned sequence.

Two sets of analyses were performed with this final data matrix. The first included *Myxidium* sp. and the enigmatic parasitic hydroid *Polypodium hydriforme* (fig. 2A). The overall resolution of this phylogeny is rather poor compared to figure 1. A second analysis was performed without the two taxa with extraordinarily long branches: *Myxidium* sp. and *P. hydriforme* (fig. 2B). In the discussion that follows, we focus on the results of ML. In cases where distance-based minimum-evolution methods (Kidd and Sgaramella-Zonta 1971) lead to different conclusions, these are discussed, although all figures show the resolution favored by ML.

All three of our analyses strongly support the hypothesis that Porifera (sponges) and Ctenophora (comb jellies) branched off before the remaining metazoa (figs. 1–2). The basal placement of the Porifera, which lack a nervous system or even tissues (Hyman 1940; Brusca and Brusca 1990), agrees with most earlier 18S rDNA studies (Wainright et al. 1993; Cavalier-Smith et al. 1996; Kumar and Rzhetsky 1996) and is in accord with traditional hypotheses of metazoan evolution (Hyman 1940; Brusca and Brusca 1990). Our results contradict some earlier 18S rDNA studies that supported the monophyly of the diploblast taxa (Porifera, Ctenophora, Cni-

daria, and Placozoa; Hanelt et al. 1996; Van de Peer and De Wachter 1997). Under ML, there is also weak support in all three analyses for the hypothesis that the Porifera are basal relative to the Ctenophora (51–67%). In any case, our results strongly suggest that the Ctenophora are the most basal living group with a true nervous system and a tissue grade of organization (Hyman 1940; Brusca and Brusca 1990).

Under ML, there is no support for a monophyletic Porifera (figs. 1–2), although distance methods do support a monophyletic Porifera in the higher level analyses shown in figure 2 (78%–79%). In all of our analyses, there is strong support for the hypothesis that the Porifera and Ctenophora are basal relative to the monotypic phylum Placozoa, which, like the Porifera, lacks a nervous system or a tissue grade of organization (Hyman 1940; Brusca and Brusca 1990). Our results suggest that the Placozoa are secondarily simplified and contradict the long-held hypothesis that the Placozoa are relictual descendants of the earliest metazoans (Hyman 1940; Brusca and Brusca 1990).

Within the Cnidaria, two of our three analyses (figs. 1 and 2B) strongly support a basal placement for the Anthozoa (corals and sea anemones). The third analysis, which was confounded by two taxa with very long branches (*Polypodium* and *Myxidium*), could not resolve Cnidarian relationships (fig. 2A). Our conclusion that the Anthozoa are the basal Cnidarian class agrees not only with an earlier study of partial 18S rDNA sequences (Bridge et al. 1995), but with mitochondrial conformation data. Of the four Cnidarian classes, only the Anthozoa has circular mtDNA; all three classes sharing a medusoid (jellyfish) stage have linear mtDNA (Bridge et al. 1992). Interestingly, however, neither the partial 18S rDNA sequences nor mitochondrial conformation data could rule out the possibility of a paraphyletic Anthozoa. Our study is therefore the first to show strong molecular support for a monophyletic Anthozoa (fig. 1).

Like the earlier study of partial 18S rDNA sequences (Bridge et al. 1995), our study finds no support for a monophyletic Scyphozoa. In fact, two of our ML analyses show varying degrees of support for a sister taxon relationship between two scyphozoan groups (*Cassiopea* and *Aurelia*) and the class Hydrozoa (figs. 1 and 2B). More data and a broader taxonomic sampling are necessary to resolve the monophyly of the Scyphozoa and the relationships of the three medusoid Cnidarian classes.

The relationships of two enigmatic taxa have been controversial: the endoparasitic phylum Myxozoa and the bizarre parasitic hydrozoan *P. hydriforme* (Raikova 1994). Although originally considered protists, myxozoans fall within the metazoans in all previous 18S rDNA studies (Smothers et al. 1994; Siddall et al. 1995; Cavalier-Smith et al. 1996). But all of these studies disagreed about the myxozoans' placement within the Metazoa. The most provocative study placed the Myxozoa within the Cnidaria (Siddall et al. 1995). A Cnidarian ancestry for the Myxozoa is supported by a number of interesting morphological similarities between the endoparasitic myxozoans and Cnidarian cnidocytes, or

stinging cells (Siddall et al. 1995). Other 18S rDNA studies (Smothers et al. 1994; Siddall et al. 1995; Cavalier-Smith et al. 1996) and one study of Hox genes (Anderson, Canning, and Okamura 1998) suggest that the Myxozoa are more closely allied with triploblast metazoans.

The other enigmatic taxon is the parasitic hydrozoan *P. hydriforme* (Raikova 1994). Although traditionally members of the Hydrozoa (Hyman 1940), it is by no means clear whether the adult stage of *Polypodium* is homologous with the polyp or the medusoid stage of other Cnidarians (Hyman 1940, Raikova 1994). The 18S rDNA study of Siddall et al. (1995) identifies *Polypodium* as the sister taxon to the Myxozoa, and places both groups within the Cnidaria. Based on these results, Siddall et al. (1995) proposed that the phylum Myxozoa be abandoned.

Both *Polypodium* and the myxozoan *Myxidium* have very long branches (fig. 2A), and their addition to the data matrix has a detrimental effect on overall phylogenetic resolution (compare with fig. 2B). Our distance analyses weakly support a sister taxon relationship between *Polypodium* and *Myxidium* (64% bootstrap support). Although this sister taxon relationship agrees with Siddall et al. (1995), according to our distance these taxa do not fall within the remaining Cnidaria but are the sister group to the triploblastic metazoa. When we analyzed the matrix by ML with the best-fit model, the long branches of *Polypodium* and *Myxidium* were no longer attracted together (fig. 2A). In the ML analysis, only *Myxidium* was the sister taxon to the triploblastic Metazoa (76% bootstrap support). Because the parsimony-based analysis placing the Myxozoa within the Cnidaria appears to have resulted from long-branch attraction (Siddall et al. 1995), we cannot support the dismantling of the phylum Myxozoa.

Our approach of combining the Aguinaldo taxon-pruning strategy with a best-fit model chosen under an ML framework has arrived at a satisfying perspective on lower metazoan relationships. In some respects, our conclusions are very traditional: a basal position for the Porifera (sponges) and the first molecular evidence for a monophyletic Anthozoa (corals and sea anemones). In other respects, our conclusions are less traditional. We find the strongest support to date for the hypothesis that the Ctenophora are the most basal living metazoans with a nervous system and a tissue grade of organization. Although often grouped with the Cnidaria under the name Coelenterata, the Ctenophora lack many Cnidarian features, such as cnidocytes and a benthic polyp stage. Closer examination of the Ctenophora may offer clues to the origin of the tissue grade of organization. Finally, our placement of the Myxozoa is more consistent with other molecular and morphological information than several proposed hypotheses. As in an earlier Hox gene study (Anderson, Canning, and Okamura 1998), the Myxozoa are closely allied with triploblast metazoans. Morphologically, this placement also makes sense, as long as the Cnidarian characteristics of the Myxozoa are considered symplesiomorphies.

The Aguinardo taxon-pruning strategy is attractive because it helps to ameliorate the effects of long branches and helps bring the number of taxa down to the point where ML methods are tractable. Our study suggests that combining these approaches may help resolve difficult phylogenetic questions.

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LITERATURE CITED

- AGUINALDO, A. M., J. M. TURBEVILLE, L. S. LINFORD, M. C. RIVERA, J. R. GAREY, R. A. RAFF, and J. A. LAKE. 1997. Evidence for a clade of nematodes, arthropods, and other moulting animals. *Nature* **387**:489–493.
- ANDERSON, C. R., E. U. CANNING, and B. OKAMURA. 1998. A triploblast origin for Myxozoa? *Nature* **392**:346–347.
- BRIDGE, D., C. W. CUNNINGHAM, L. BUSS, and R. DESALLE. 1995. Class-level relationships in the phylum Cnidaria: molecular and morphological evidence. *Mol. Biol. Evol.* **12**:679–689.
- BRIDGE, D., C. W. CUNNINGHAM, B. SCHIERWATER, R. DESALLE, and L. W. BUSS. 1992. Mitochondrial DNA structure and Cnidarian phylogeny. *Proc. Natl. Acad. Sci. USA* **89**:8750–8753.
- BRUSCA, R. C., and G. J. BRUSCA. 1990. *Invertebrates*. Sinauer Associates, Sunderland, Mass.
- CAVALIER-SMITH, T., M. T. E. P. ALLSOPP, E. E. CHAO, E. N. BOURY, and J. VACELET. 1996. Sponge phylogeny, animal monophyly, and the origin of the nervous system: 18S rRNA evidence. *Can. J. Zool.* **74**:2031–2045.
- CHRISTEN, R., A. RATTO, A. BAROIN, R. PERASSO, K. G. GRELL, and A. ADOUETTE. 1991. An analysis of the origin of metazoans, using comparisons of partial sequences of the 28S RNA, reveals an early divergence of triploblasts. *EMBO J.* **10**:499–503.
- CUNNINGHAM, C. W., H. ZHU, and D. M. HILLIS. 1998. Best-fit maximum likelihood models for phylogenetic inference: empirical tests with known phylogenies. *Evolution* **52**:978–987.
- FELSENSTEIN, J. 1978. Cases in which parsimony or compatibility methods will be positively misleading. *Syst. Zool.* **27**:401–410.
- HANELT, B., D. VANSCHYNDEL, C. M. ADEMA, L. A. LEWIS, and E. S. LOKER. 1996. The phylogenetic position of rhopalura-ophiocomae (orthonectida) based on 18S ribosomal DNA-sequence analysis. *Mol. Biol. Evol.* **13**:1187–1191.
- HUELSENBECK, J. P. 1997. Is the Felsenstein zone a fly trap? *Syst. Biol.* **46**:69–74.
- HYMAN, L. H. 1940. *The Invertebrates*, Vol. 1. Protozoa through Ctenophora. McGraw Hill, New York.
- KIDD, K. K., and L. A. SGARAMELLA-ZONTA. 1971. Phylogenetic analysis: concepts and methods. *Am. J. Hum. Genet.* **23**:235–252.
- KUMAR, S., and A. RZHETSKY. 1996. Evolutionary relationships of eukaryotic kingdoms. *J. Mol. Evol.* **42**:183–193.
- LAKE, J. A. 1994. Reconstructing evolutionary trees from DNA and protein sequences; paralogous distances. *Proc. Natl. Acad. Sci. USA* **91**:1455–1459.
- LANAVE, C., G. PREPARATA, C. SACCONI, and G. SERIO. 1984. A new method for calculating evolutionary substitution rates. *J. Mol. Evol.* **20**:86–93.
- LOCKHART, P. J., M. A. STEEL, M. D. HENDY, and D. PENNY. 1994. Recovering evolutionary trees under a more realistic model of sequence evolution. *Mol. Biol. Evol.* **11**:605–612.
- MALEY, L. E., and C. R. MARSHALL. 1998. The coming of age of molecular systematics. *Science* **279**:505–506.
- MEDLIN, L., H. J. ELWOOD, G. J. OLSEN, and M. L. SOGIN. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA coding regions. *Gene* **71**:491–499.
- RAIKOVA, E. 1994. Life cycle, cytology, and morphology of *Polypodium hydriforme*, a coelenterate parasite of the eggs of Acipenseriform fishes. *J. Parasitol.* **80**:1–22.
- SANGER, F., S. NICKLEN, and A. R. COULSON. 1977. DNA sequencing with chain-terminating inhibitor. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
- SIDDALL, M. E., D. S. MARTIN, D. BRIDGE, S. DESSER, and D. CONE. 1995. The demise of a phylum of protists: phylogeny of Myxozoa and other parasitic Cnidaria. *J. Parasitol.* **8**:961–967.
- SMOTHERS, J. F., C. D. VON DOHLEN, H. S. J. SMITH, and R. D. SPALL. 1994. Molecular evidence that the myxozoan protists are Metazoans. *Science* **265**:1719–1721.
- THOMPSON, J. D., D. G. HIGGINS, and T. J. GIBSON. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.
- VAN DE PEER, Y., and R. DE WACHTER. 1997. Evolutionary relationships among the eukaryotic crown taxa taking into account site-to-site rate variation in 18S rRNA. *J. Mol. Evol.* **45**:619–630.
- WAINRIGHT, P. O., G. HINKLE, M. SOGIN, and S. K. STICKEL. 1993. Monophyletic origins of the Metazoa: an evolutionary link with Fungi. *Science* **260**:340–342.
- YANG, Z. 1996. Among-site rate variation and its impact on phylogenetic analyses. *Trends Ecol. Evol.* **11**:367–372.

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