Phylogenetic Position of Phylum Nemertini, Inferred from 18S rRNA Sequences: Molecular Data as a Test of Morphological Character Homology[1]

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Partial 18S rRNA sequence of the nemertine *Cerebratulus lacteus* was obtained and compared with those of coelomate metazoans and acoelomate platyhelminths to test whether nemertines share a most recent common ancestor with the platyhelminths, as traditionally has been implied, or whether nemertines lie within a protostome coelomate clade, as suggested by more recent morphological analyses. Maximum-parsimony analysis supports the inclusion of the nemertine within a protostome-coelomate clade that falls within a more inclusive coelomate clade. Bootstrap analysis indicates strong support for a monophyletic Coelomata composed of a deuterostome and protostome-coelomate clade. Support for a monophyletic protostome Coelomata is weak. Inference by distance analysis is consistent with that of maximum parsimony. Analysis of down-weighted paired sites by maximum parsimony reveals variation in topology only within the protostome-coelomate clade. The relationships among the protostome coelomates cannot be reliably inferred from the partial sequences, suggesting that coelomate protostomes diversified rapidly. Results with evolutionary parsimony are consistent with the inclusion of the nemertine in a coelomate clade. The molecular inference corroborates recent morphological character analyses that reveal no synapomorphies of nemertines and flatworms but instead suggest that the circulatory system and rhynchocoel of nemertines are homologous to coelomic cavities of protostome coelomates, thus supporting the corresponding hypothesis that nemertines belong within a protostome-coelomate clade. The sequence data provide an independent test of morphological character homology.

Introduction

Morphological and embryological data have contributed substantially to the elucidation of the phylogenetic relationships of animal phyla. However, the number of different phylogenies proposed for the Metazoa reflects the inability of zoologists to arrive at a consensus view of metazoan interphyletic relationships based on these characters (e.g., see Hyman 1940, p. 38; Ax 1984, pp. 285–286, 1989; Brusca and Brusca 1990, pp. 879–889). There are several reasons for the uncertainty regarding metazoan relationships. First, most phyla share few informative homologous anatomical or embryological features. Second, it is often exceedingly difficult to distinguish homologous similarity (homology) from convergent or parallel similarity (analogy),

and this is a prerequisite for inferring phylogenetic relationships. Third, the fossil record has not provided informative characters for linking most phyla, and many small and soft-bodied phyla lack a fossil record. Sequence data obtained from ribosomal RNAs offer an important new source of informative characters for inferring high-level phylogenetic relationships for many taxa and provide an independent test of hypotheses based on morphological characters, especially for metazoans (Woese 1987; Field et al. 1988; Abele et al. 1989).

We report here our investigation of the 18S rRNA of *Cerebratulus lacteus* from the phylum Nemertini or Rhynchocoela, a group of worms whose relationships have been controversial. Nemertines, or ribbon worms, are primarily epifaunal or infaunal inhabitants of the marine benthos, but marine pelagic, freshwater, and terrestrial forms also occur. The phylum comprises ~900 species (Gibson 1982). Nemertines are typically a few millimeters to several centimeters in length, but many species are much longer; there are reports in the classical literature of a species that attains a length of 30 m (Bürger 1897–1907). The major diagnostic feature of the phylum is an eversible proboscis enclosed when uneverted in a fluid-filled, cell-lined cavity, the rhynchocoel. In addition to this secondary body cavity, nemertines possess a continuous system of cell-lined channels historically referred to as a blood vascular system (Bürger 1897–1907; Hyman 1951, p. 486).

Nemertines traditionally have been considered acoelomate animals most closely related to the platyhelminths, or flatworms, on the basis of morphological and limited immunological similarity (among others, see Bürger 1897–1907; Schepotieff 1912; Hyman 1951, p. 528; fig. 1A). This hypothesis has been perpetuated by modern biology and invertebrate zoology textbooks and by more detailed accounts of the phylum (e.g., see Gibson 1972, pp. 187–191; Lutz 1986, p. 201; Curtis and Barnes 1989, p. 538; Willmer 1990). However, recent structural and ultrastructural analyses corroborate an alternative, minority hypothesis put forward by earlier zoologists (e.g., see Nusbaum and Oxner 1913; Friedrich 1935). The data suggest that the nemertine circulatory system and rhynchocoel are homologous to coelomic cavities of protostome coelomates (also referred to as schizocoelous or spiralian coelomates) and thus support the hypothesis that nemertines are most closely related to protostome coelomates such as annelids and mollusks and that they thus belong within a protostome-coelomate clade (fig. 1B; Turbeville 1986b; Turbeville 1991). The interpretation of nemertine body cavities as coelom homologues—and this alternative hypothesis of nemertine relationships—have not been accepted by all invertebrate systematists, further reflecting the difficulty of inferring the phylogenetic relationships of nemertines by use of morphological characters (e.g., see Bartolomaeus 1988). Additional informative characters are required to clarify the phylogenetic relationships of nemertines. The objective of the present investigation is to test hypotheses of nemertine relationships by using 18S rRNA sequence data with maximum-parsimony, distance-matrix, and evolutionary-parsimony methods of analysis.

Material and Methods
Organisms Analyzed

Eleven sequences representing six metazoan phyla were compared in the present study. These were *Dugesia tigrina* (Platyhelminthes), *Bothromesostoma personatum* (Platyhelminthes), *Fasciola hepatica* (Platyhelminthes), *Chaetopterus variopedatus* (Annelida), *Lumbricus* species (Annelida), *Cerebratulus lacteus* (Nemertini), *Cryptochiton stelleri* (Mollusca), *Golfingia gouldii* (Sipunculida), *Asterias forbesii* (Echi-
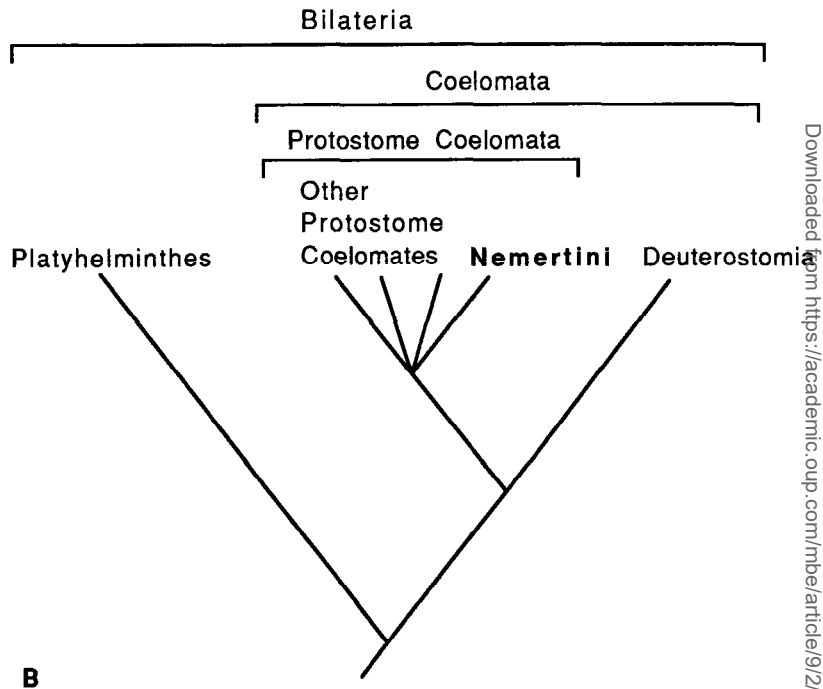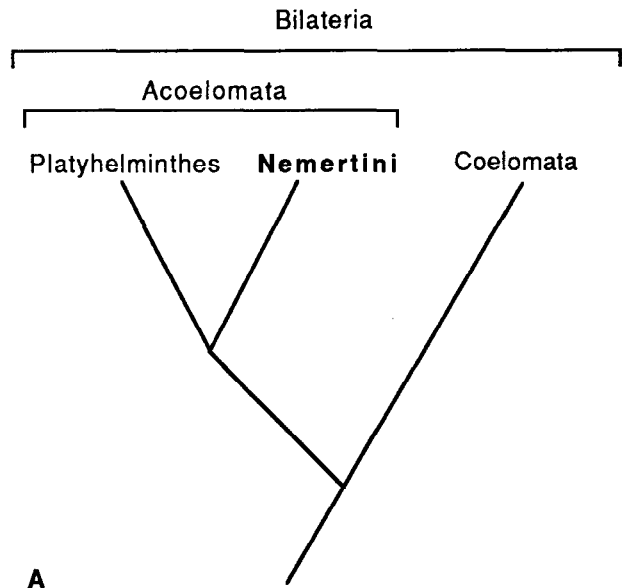
FIG. 1.—Conflicting hypotheses of nemertine relationships based on morphological characters. A, Orthodox or traditional hypothesis, indicating monophyly of Nemertini + Platyhelminthes. No unequivocal synapomorphies support this hypothesis (see text). B, Alternative hypothesis, indicating that nemertines belong within schizocoelous (see table 1) or protostome coelomate clade that is part of more inclusive coelomate clade. For an alternative hypothesis, see Brusca and Brusca (1990, pp. 331 and 882).

nodermata), and *Branchiostoma californiense* (Chordata). With the exception of the *Cerebratulus* sequence reported here and *Bothromesostoma* and *Fasciola* sequences (M. Riutort and K. G. Field, personal communication), all sequences were collected by Field et al. (1988). The nemertine and the new flatworm sequences have been submitted to EMBL.

RNA Extraction and Sequencing

Specimens of *Cerebratulus lacteus* Leidy 1851 were obtained from the Marine Biological Laboratory (Woods Hole, Mass.). Total RNA was extracted from oocytes by following a protocol adapted from Paterson and Roberts (1981) and using 8 M guanidine hydrochloride (Turbeville et al. 1991). Direct sequencing of 18S rRNA was accomplished by employing the method of Lane et al. (1985, 1988), which is a modification of the Sanger et al. (1977) dideoxynucleotide chain-terminating technique using reverse transcriptase. Six oligonucleotide primers complementary to specific conserved regions of the molecule were used in separate reactions. Sequences of the primers utilized were as follows (numbers correspond to positions on the human sequence): 445–429, 5'-TCAGGCTCCCTCTCCGG-3'; 632–615, 5'-GWAT-TACCGCGCGGCKGCTG-3'; 880–865, 5'-CCGAGGTCCTATTCCA-3'; 1009–993, 5'-TTGGCAAATGCTTTCGC-3'; 1201–1187, 5'-ATCCTTTRAGTTTC-3'; and 1708–1692, 5'-GACGGGCGGTGTGTRCA-3'.

Sequence Alignment

Sequence data were aligned by hand, beginning at universally conserved regions. Alignment gaps were inserted to account for putative length differences between the sequences (fig. 2). Alignments were refined using the secondary-structure models inferred for the bird spider *Eurypelma californica* (Hendriks et al. 1988). Positions aligned were limited to regions for which sequence data are available for all taxa. Of the 1,140 aligned positions, 946 were included in the analyses. Positions exhibiting high variability or length variation could not be reliably aligned and were excluded from the analyses. Positions analysed are indicated in figure 2.

Data Analysis

Data were analyzed using the maximum-parsimony method of the PAUP 3.0d package written by D. L. Swofford (Illinois Natural History Survey), the least-squares distance-matrix program of Olsen (1988a, 1988b), and a phylogenetic-invariants method (Lake 1987). For parsimony analysis, characters were entered unordered, and gaps were treated as missing data. The BRANCH AND BOUND option of PAUP, which guarantees the most parsimonious solutions for a given data set, was used in all analyses except bootstrapping (see below). The analysis was performed with all nucleotides and was repeated with paired positions downweighted by one-half, following the suggestion of Stahl et al. (1984) and Wheeler and Honeycutt (1988). The latter authors postulate that, the paired regions might be less reliable than unpaired regions, for inferring phylogeny, as is the case for 5S rRNA. Downweighting was accomplished in practice by assigning the unpaired positions twice the weight of the paired sites. Paired and unpaired positions were inferred by juxtaposing primary structures with the secondary-structure model proposed by Hendriks et al. (1988) for the spider *Eurypelma*. The cnidarian *Hydra* was chosen for the outgroup, on the basis of morphological and molecular analyses that suggest that the Cnidaria is the sister taxon of the bilateral metazoans (Ax 1989; authors' unpublished data). A bootstrap analysis for

placing confidence intervals on inferred phylogenies was also utilized (Felsenstein 1985), and 100 replications were run using the total data set. For bootstrapping, the HEURISTIC search was employed by using the TBR branch-swapping algorithm in combination with CLOSEST stepwise addition. Twenty trees were held at each step.

Data were analyzed by the distance-matrix-analysis program of Olsen (1988a, 1988b), which is based on the method developed by Fitch and Margoliash (1967); the Jukes and Cantor (1969) correction to estimated distances was employed. In the present study, trees were rooted by the most distantly related metazoan, *Hydra*. The bootstrap algorithm was also applied to the distance analysis. One hundred replications were run. The analysis was also run with downweighted paired positions, as described above.

The method of evolutionary parsimony (Lake 1987) was applied to all positions. The version compiled for the PAUP 3.0 package was used. For evolutionary-parsimony analyses, sequences were divided into four groups, and the three possible topologies for all quartets composed of a single sequence from each group were evaluated, and the results were combined. The χ^2 values for combined trees were calculated by following the method presented by Lake (1987, Appendix). When necessary, correlation values were corrected as explained by Turbeville et al. (1991), prior to calculating the χ^2 values.

Results

Direct sequencing of the 18S rRNA molecules of *Cerebratulus lacteus* by using six primers yielded 1,208 nucleotides. Unambiguous nucleotide assignments could not be made at 53 sites, or 4.4% of the total. Ambiguities result from common artifacts of reverse-transcriptase sequencing (see Lane et al. 1985). Of the nucleotides obtained, 1,140 are shown in the alignment (fig. 2). A unique insertion (autapomorphy) 20 nucleotides in length is present between positions 200 and 202 (fig. 2).

Maximum-Parsimony Analysis

For the taxa analyzed, 253 of the reliably aligned variable sites (fig. 2) are informative for inferring phylogeny by maximum-parsimony analysis. Analysis of all positions found a single minimum-length tree of 874 steps (fig. 3A). Maximum-parsimony analysis indicates that the nemertine *Cerebratulus lacteus* falls within a protostome or spiralian coelomate clade as the sister group of *Golfingia* rather than being the sister group of the acoelomate Platyhelminthes (fig. 3A). The *Cerebratulus*-plus-*Golfingia* clade is the sister taxon of a *Cryptochiton*-plus-*Chaetopterus* clade. The oligochaete annelid *Lumbricus* is the sister group of these other protostome-coelomate taxa. Thus, monophyly of the Annelida is not supported by the partial sequence data (see Discussion). The reliability of the phylogeny was estimated by bootstrap analysis of the entire data set. Ninety-six percent of the trees from 100 bootstrap replications supported monophyly of the coelomates inclusive of the nemertine (fig. 3A). Monophyly of the spiralian coelomates is supported to a lesser extent, and relationships among the taxa of this clade are weakly supported (fig. 3A). To assess further the reliability of the maximum-parsimony inference, we considered the 124 trees saved within 1% of the length of shortest tree (874–883 nucleotide substitutions). A 50%-majority-rule consensus tree is shown in figure 3B. Variation in topology was observed within the protostome-coelomate and platyhelminth clades. When paired positions are downweighted and the total data set is analyzed, a single tree is found that has a topology that varies only within the protostome-coelomate clade (not shown). The
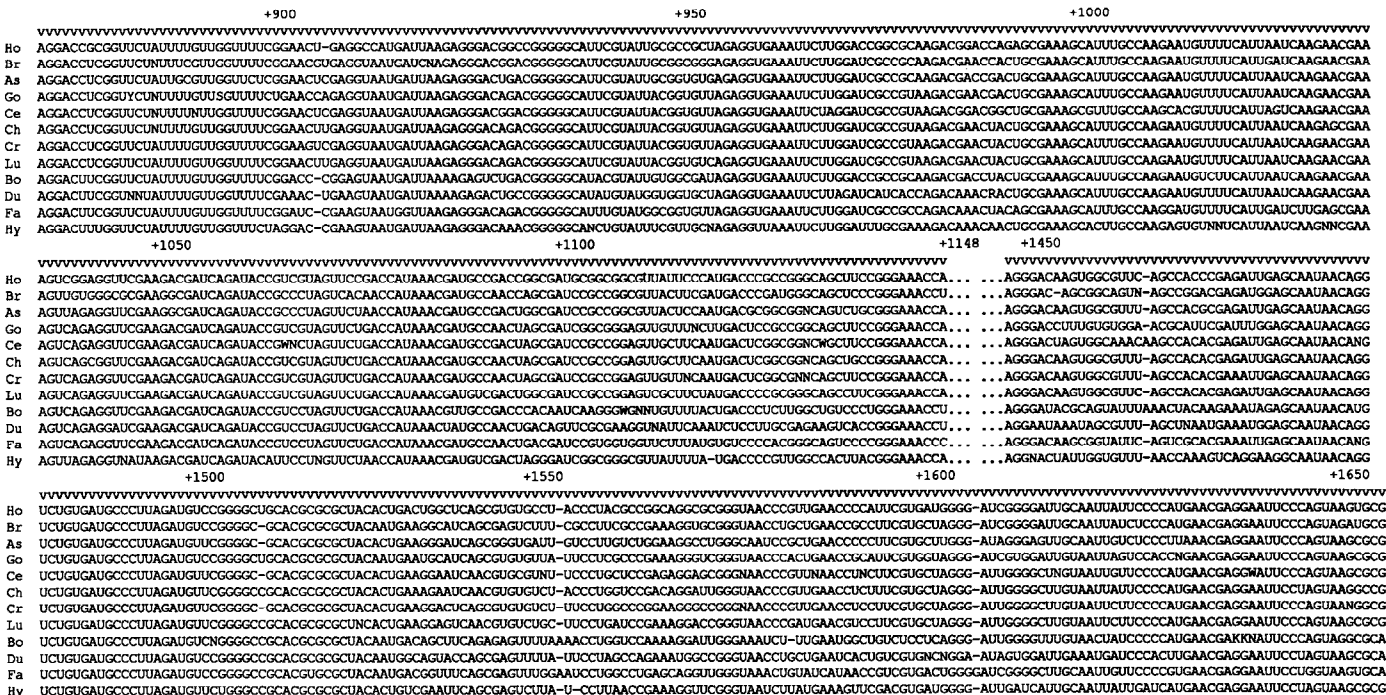
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Br  ...AUGCGAAAGAAGCGCUG-ACCUC---------------------------------ACGGUCUGGCGUGCAUUUAUCA-GACCAAGACCAACCGGGGUU--------------------------CGCCCCNGUCCCUUUGGUG-ACUCUGGAUAACCC-GAGCC
As  ...AUGCAAU-CAAGCGCUGGACCUU---------------------------------ACGG-GAGGCGUGCUCUAUUAGGAACAAGACCAACCCGGUC--------------------------CGCCCGNNUNGUUGGUGAACUCUGGAUAACCU--GGCC
Go  ...AUGCNAU-NAUGCCUUGU-RCCGG---------------------------------GGGG-GAAGAGCCGAGUUUAUUA-GAUCAAAACCAACCGCGCGC--------------------------AAGCGUCGAGCCGUUGGUG-ACUCAGAAUAACUUUGGUGU
Ce  ...AUGCUAC-AAAGCUUCCG-WCCCGGCGGCGUCGUCGUCUCACGGCGGCNNCNGWUUGGG-GAAGAGNWCUNUUAUUA--GGUCAAAACCAACGGCGGCGCUCUC--------------GCGGGCGUGUCGCUGAACAUGCUGUACG-AAUCUNGAUAACUUUGGUGCG
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Cr  ...AUGCAAC-UCCGCUCUUG-ACCUU---------------------------------UACCGG-GAAGAGNNNUUUUAUUA-GAUCAAGAUCAAUACGUGCCGC--------------------------AAGGUGCGUNCUCGUUGGUG-AUUCUNAAUAACUUUGUGCU
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Fa  GGGUGCAGCCGUGACUGGUGGGCUUGCCUGCUAGUCUUGUUGGCAUGCUCUCUUGGUGGCCUUUAAUCGGGUGU-CGGAG-GCGGA-CAGCA-CGUUUACUUUGAACAAAUUUGAGUGUUCAAAGCAGGCCUUUGU-GCCUGAAAAUUCUUGCAUGGAUAAUAAUAAAU
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Ch  AGCAGCCGGUUCGAAGACGAUCAGAUACCGUCGUAGUUCUGACCAUAAACGAUGCCGACCCUAGGGGGCAUUCGUAUUGCGGUGUUAGAGGUGAAAUUCUUGGAUCGCCGUAAGACGAACUACUGCGAAAGCAUUUGCCAAGAAUGUUUUCAUUAAUCAAGAGCGAA
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Hy  AGGACUUUGGUUCUAUUUUUGUUGGUUUUCUAGGAC-CGAAGUAAUGAUUAAGAGGGACAAACGGGGGCAUCGUAUUUCGUUGCNAGAGGUUAAAUUCUUGGAUUUUGCGAAAGACAAACAACUGCGAAAGCACUUGCCAAGAGUGUNNUCAUUAAUCAAGNNCGAA

                    +1050                        +1100                    +1148         +1450
          vvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvv          vvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvvv
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FIG. 2.—Partial 18S rRNA sequences aligned to corresponding regions of *Homo sapiens* (Chordata; Torczynski et al. 1983) sequence for reference. Only regions for which sequence data are available for all taxa are included. V = Position used in analyses. Ho = *Homo*; Br = *Branchiostoma*; As = *Asterias*; Go = *Golfingia*; Ce = *Cerebratulus*; Ch = *Chaetopterus*; Cr = *Cryptochiton*; Lu = *Lumbricus*; Bo = *Bothromesostoma*; Du = *Dugesia*; Fa = *Fasciola*; and Hy = *Hydra*. Accession numbers for unpublished sequences are as follows: Ce, M81167; Bo, M58347; and Fa, X56041.
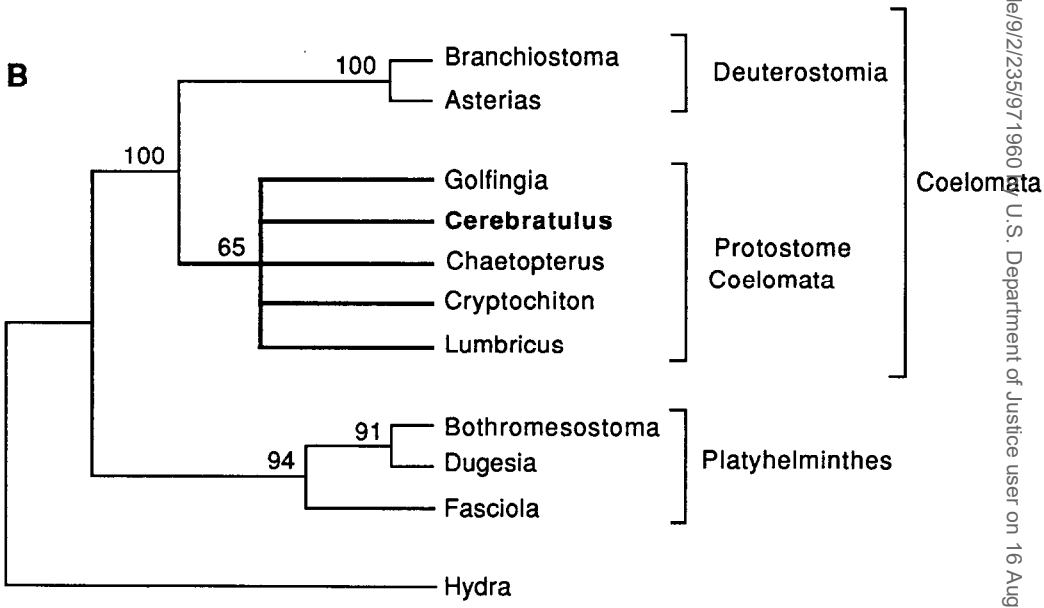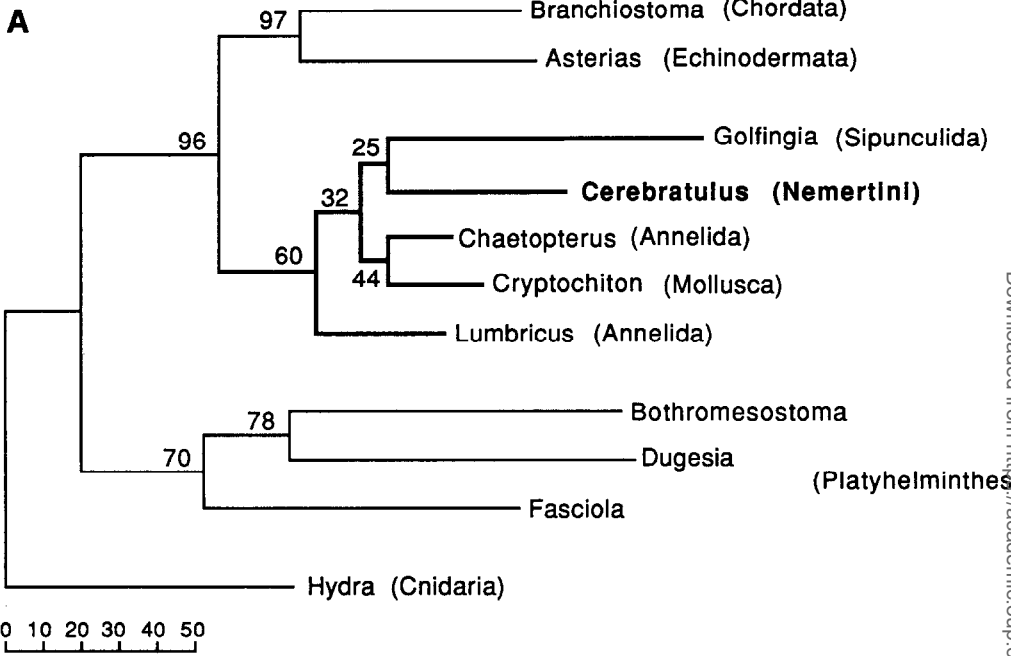
FIG. 3.—Trees inferred by maximum-parsimony analysis. A, Shortest tree (874 nucleotide substitutions). The overall consistency index is 0.708. The consistency index when uninformative positions are excluded is 0.605. Numbers at nodes indicate the frequency with which the clade descending from that node was found by bootstrapping. Branch lengths are proportional to the number of nucleotide substitutions. The scale bar indicates the approximate number of nucleotide substitutions. B, Fifty-percent-majority rule consensus tree of 124 trees saved within 1% length of most parsimonious tree.

nemertines become the sister group of the rest of the protostome coelomates. Within the subordinate clade, the polychaete *Chaetopterus* is placed between the chiton and a *Lumbricus*-plus-*Golfingia* clade. If maximum-parsimony analyses were run with the nucleotides excluded from the analyses, two equally parsimonious trees were found with variation only within the protostome-coelomate clade (authors' unpublished results).

Distance Analysis

Distance-matrix results are consistent with those of the maximum-parsimony analyses, indicating that nemertines lie within a protostome-coelomate group and that the nemertine RNA molecule has accumulated a somewhat greater number of substitutions than have those of the other protostome coelomates included in the analysis (fig. 4). The topology of the tree inferred from all positions agrees with the parsimony analysis, except among the protostome coelomates. The sipunculid is the sister group of the rest of the protostome-coelomate taxa, and the nemertine is placed between the oligochaete *Lumbricus* and a *Cryptochiton*-plus-*Chaetopterus* group. The distance tree inferred from downweighting paired positions is identical in topology to the tree inferred from unweighted data. Bootstrap analysis of the total data set indicates support both for the coelomate lineage in 100% of the outcomes and for a protostome-coelomate group in 85% (fig. 4). Support for relationships among the protostome coelomates is
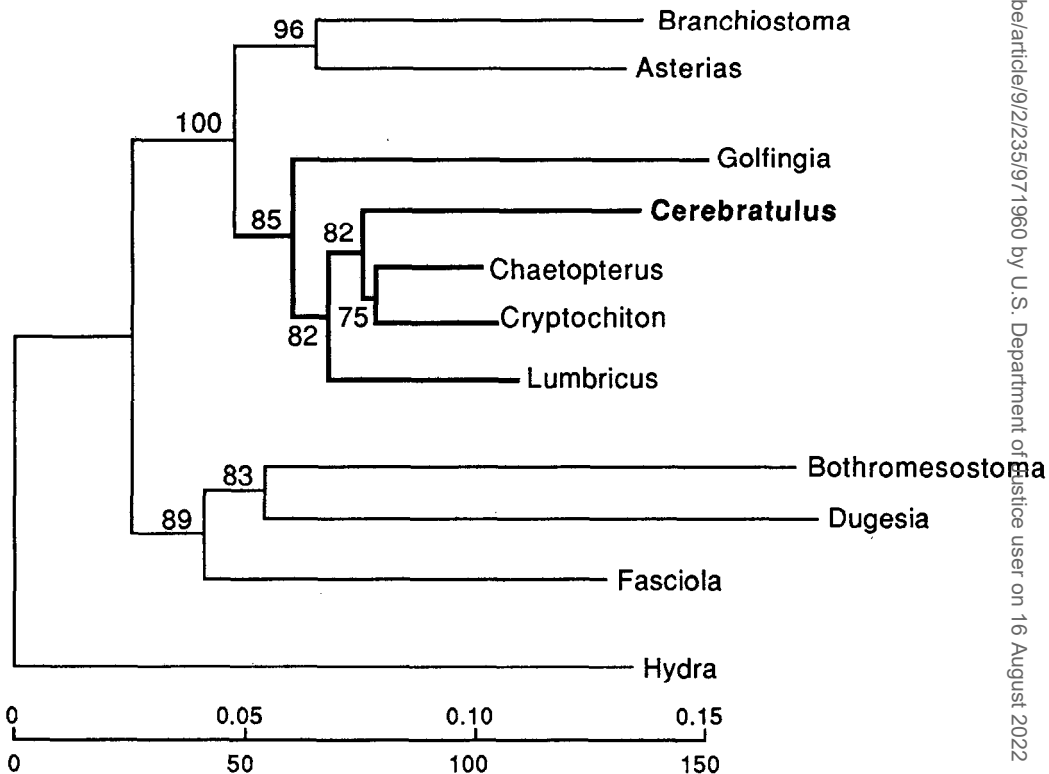
FIG. 4.—Distance tree constructed by least-squares distance-matrix program. The scale bars indicate the average number of substitutions per sequence position (upper numbers) and the average number of substitutions (lower numbers). Numbers at nodes represent bootstrap percentiles.

somewhat weaker (fig. 4). No variation in tree topology was observed (authors' unpublished results) when unreliably aligned positions were included in the analysis.

Evolutionary Parsimony

For evolutionary parsimony, the total number of informative transversion positions (parsimony counts minus background counts) per quartet was between zero and nine. Figure 5 illustrates inferences of nemertine relationships by evolutionary parsimony. The results are a subset of all possible combinations. The first set of analyses tests whether the nemertine is most closely related to protostome coelomates, deuterostomes, or flatworms. The favored tree ($P \sim 0.3$) in the first set of analyses (fig. 5A) indicates monophyly of nemertine + protostome coelomates, although support is not significant (fig. 5A). The results are also dependent on which taxa are included in the four groups (see Turbeville et al. 1991). The P values of the two alternative topologies, one linking the nemertines and the flatworms and the other linking the nemertine and deuterostomes, are $P = 1$ and $P \sim 0.5$, respectively. The second set of analyses was designed to test monophyly of the coelomates and the nemertine (fig. 5B). The favored tree links the coelomate taxa and the nemertine, but support for this topology is also not significant ($P \sim 0.07$).

Discussion

The results support the hypothesis that nemertines are coelomate animals that belong within a protostome-coelomate clade. Measures of reliability, including bootstrap analyses and consideration of all trees within 1% of the length of the most parsimonious solution, firmly support coelomate monophyly and, to a lesser extent, protostome-coelomate monophyly. Results with evolutionary parsimony are less robust, perhaps owing to the small number of informative transversion positions (between
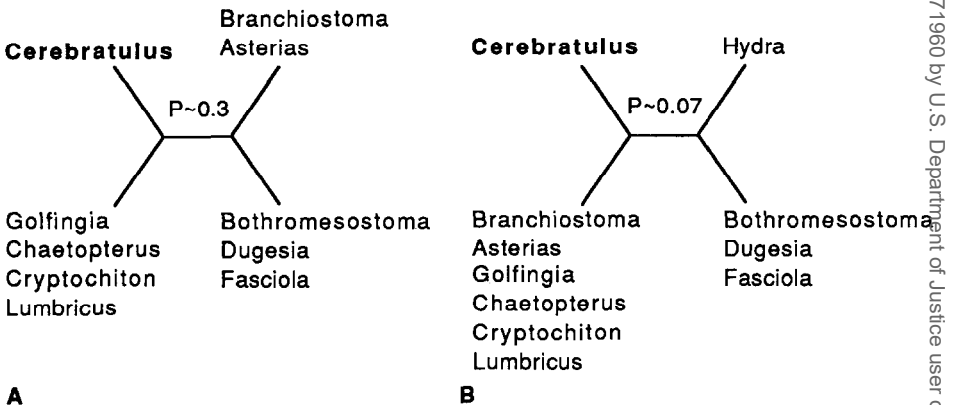
FIG. 5.—Summary of analyses with evolutionary parsimony. A, Test of nemertine+protostome-coelomate monophyly. The favored topology resulting from combining results of 24 quartets ($1 \times 2 \times 3 \times 4$) is illustrated. One taxon from each group was compared in turn. The favored tree links the nemertine (*Cerebratulus*) to the protostome coelomates *Lumbricus, Chaetopterus, Golfingia,* and *Cryptochiton* ($P \sim 0.3$) rather than to the platyhelminths *Dugesia, Bothromesostoma,* and *Fasciola* ($P = 1$) or to the deuterostomes *Asterias* and *Branchiostoma* ($P \sim 0.5$). B, Test of nemertine+coelomate monophyly. Eighteen quartets ($1 \times 1 \times 3 \times 6$) were evaluated and combined. The favored topology is shown and links the nemertine and coelomates. P values for the tree linking the nemertine and flatworms and for the tree linking the nemertine and cnidarian are, respectively, $P \sim 0.8$ and $P \sim 0.4$. The χ^2 test for correlated data presented by Lake (1987) was used for both trees.

zero and nine) in the data set. All analyses support monophyly of coelomates and are consistent, in part, with the studies of Field et al. (1988), Ghiselin (1988), and Lake (1990). The coelomates or "Coelomata" comprise the deuterostome and protostome-coelomate clades (figs. 1B–4). Monophyly of Platyhelminthes is also firmly supported by the partial sequence data. The present study and those by Field et al. (1988) and Ghiselin (1988) suggest that Platyhelminthes is the most primitive bilateral metazoan taxon and thus constitutes the sister group of the remaining Bilateria or Eubilateria. These preliminary molecular analyses support, in part, one of two equally plausible hypotheses based on a cladistic analysis of morphological features (Ax 1984, pp. 258–286, 1989).

Downweighting paired sites of 18S rRNA altered topology within the protostome-coelomate clade only when they were analyzed by maximum parsimony, suggesting that the paired sites contain some noise. However, the utility of this procedure is uncertain (see Smith 1989; Hedges et al. 1990).

Measures of reliability indicate that relationships among the protostome-coelomate taxa cannot be confidently inferred by the partial sequence data; even the Annelida is not inferred as monophyletic. These results may be attributable, at least in part, to the fact that the regions of the molecule sequenced and compared are the most highly conserved. For example, the polychaete annelid (*Chaetopterus*) sequence and the nemertine sequence are ~90% identical. It is reasonable to assume that relatively few substitutions have occurred in the molecule after the protostome-coelomate clade became distinct and before the taxa of the protostome-coelomate clade diverged, and this situation limits the number of potentially informative sites available for inferring their precise relationships. This suggests *rapid* diversification of these taxa after the initial radiation of the coelomates. When one is inferring relationships at great evolutionary distances, it is important to limit analyses to well-conserved (i.e., slowly evolving) molecules or regions of a molecule, in order to minimize the amount of homoplasy in the data set. Sequence data from the rest of the 18S rRNA molecule and from other more rapidly evolving genes should offer a greater number of informative positions for inferring more recent divergences.

There exist two dominant hypotheses of nemertine relationships based on morphological characters. The first, or orthodox, view states that nemertines are most closely related to platyhelminths (flatworms), the implication being that nemertines and flatworms shared a most recent common ancestor (fig. 1A). The second hypothesis holds that nemertines are coelomate worms that belong within a protostome-coelomate clade (fig. 1B). Body organization, specifically the organization of the space between the body wall and gut, is of central importance for understanding the conflicting hypotheses of nemertine relationships and must first be considered.

Historically, nemertines have been regarded as acoelomate in body organization, a condition considered homologous to that of the Platyhelminthes. This shared similarity has been considered support for the hypothesis that nemertines and platyhelminths shared a most recent common ancestor. However, it was also known that nemertines possessed both a cell-lined cavity enclosing an eversible proboscis, the rhynchocoel, and an independent system of cell-lined cavities forming a continuous loop, or circulatory system. Proponents of the orthodox hypothesis (e.g., see Bürger 1897–1907; Hyman 1951, pp. 486–490; Gibson 1972, pp. 71–75) implicitly considered the rhynchocoel to be a unique coelom analogue and apparently viewed the circulatory vessels as blood-vessel homologues. However, a minority of investigators suggested that these spaces are coelom homologues and that nemertines thus are actually coelo-

mate organisms (e.g., see Nusbaum and Oxner 1913; Nawitzki 1931; Friedrich 1935). The latter hypothesis has, for the most part, been ignored in the English zoological literature, perhaps because of the language barrier (the articles are in German) and because limited and sometimes conflicting data on vessel ontogeny did not clearly corroborate anatomical and histological evidence of vessel and coelom homology (for extensive review, see Turbeville 1986a, 1986b). The hypothesis that nemertine vessels are coelom homologues has been tested recently by utilizing transmission-electron microscopy, which enhances the evaluation of characters and thereby allows for more critical tests of hypotheses of homology by following established criteria of homology recognition (table 1; Turbeville 1986a, 1986b, 1991).

Ultrastructural analyses of adult vessel morphology and an analysis of vessel ontogeny provided evidence supporting the alternative hypothesis that the circulatory-system vessels are coelom homologues, thus suggesting that nemertines belong within a protostome-coelomate clade. In anatomical position, histology, cytology, and mode of formation, the vessels correspond to coelomic cavities of coelomate protostomes, whereas the position, composition, and formation of invertebrate blood vessels are considerably different (table 1; Turbeville 1986b, 1991). Cell-lined vertebrate vessels also are structurally and developmentally unlike nemertine vessels (for discussion, see Turbeville 1986b). Data on the rhynchocoel also are consistent with its interpretation as a modified coelom homologue (Turbeville 1991). The molecular data corroborate the morphological analysis of nemertine vessels and rhynchocoel, supporting the alternative hypothesis. Some invertebrate systematists (e.g., see Ax 1984, p. 271; Bartolomaeus 1988) interpret the correspondence of structure and ontogeny of nemertine vessels and coelomic cavities as convergent or parallel similarity and consider the vessel system in nemertines to be a unique feature (autapomorphy) of the phylum, thus rejecting the hypothesis of a close relationship between nemertines and coelomates. However, the molecular data do not support this interpretation.

In addition to a shared acoelomate condition that is unsupported by the data (see above), the orthodox hypothesis—i.e., that nemertines are most closely related

Table 1
Summary of Homology Analysis

	Nemertine Vessels	Coelomic Cavities	Invertebrate Blood Vessels
Anatomical position ...	Lateral	Lateral	Dorsal and ventral
Lining	Continuous mesothelium with intercellular junctions, cilia, and myofilaments[a]	Continuous mesothelium with intercellular junctions, cilia, and myofilaments	Lined by extracellular matrix or discontinuous cell layer, lacking junctions, cilia and myofilaments.
Tissues separating during ontogeny	Mesoderm	Mesoderm[b]	Extracellular matrices

NOTE.—Homology analysis follows established criteria for testing hypotheses of morphological character homology (see Remane 1956, pp. 30–60; Riedl 1978, pp. 33–36; Wiley 1981, pp. 130–138; Rieger and Tyler 1985).

SOURCE.—Turbeville (1986a, 1986b, 1991).

[a] One unconfirmed report of myofilaments (see Turbeville 1986a).

[b] This mode of coelom formation is referred to as schizocoely.

to flatworms—has been based on their common possession of several other presumably homologous characters, including the multiciliated epidermis, special secretory bodies termed "rhabdites," ocelli, frontal organs, and nephridia (=excretory organs). Recent detailed analyses of these characters do not support their interpretation as shared derived homologues (synapomorphies; Turbeville 1991). Some of these characters (e.g., epidermis and nephridia) are symplesiomorphies, and the homology of others (e.g., frontal organs and rhabdites) is unsupported. Therefore, these characters do not support the hypothesis of a most recent common ancestry of platyhelminths and nemertines (see Bartolomaeus 1985, 1988; Turbeville and Ruppert 1985; Turbeville 1991). The molecular data are in full accord with this conclusion (figs. 3 and 4).

The microcomplement-fixation analysis of Schepotieff (1912) revealed immunological similarity between a single nemertine and a polyclad flatworm, rather than between the nemertine and a polychaete annelid, suggesting a closer relationship between nemertines and flatworms. However, both morphological and sequence analyses suggest that this shared similarity, if homologous, should be interpreted as a symplesiomorphy.

The molecular data support the hypothesis that nemertines are coelomate animals that belong within a protostome-coelomate clade (figs. 1B, 3 and 4). Thus, these data also affirm the interpretation of nemertine body cavities as coelom homologues rather than as independently derived coelom analogues. The sequence data have provided an independent but indirect test of morphological character homology. The results of both morphological and sequence analyses necessitate rejection of the hypothesis that nemertines represent the sister group of the flatworms. Furthermore, the molecular data refute the recently revived hypothesis that nemertines share a most recent common ancestry with vertebrates or chordates (Jensen 1988), a hypothesis also untenable on morphological grounds. The sequence data in the present paper thus provide informative characters allowing for the resolution of controversies resulting from differing interpretations of the same morphological characters. This study illustrates the importance of considering both molecular and morphological data when one is evaluating high-level evolutionary relationships of metazoans.

Acknowledgments

LITERATURE CITED

ABELE, L. G., W. KIM, and B. E. FELGENHAUER. 1989. Molecular evidence for inclusion of the phylum Pentastomida in the Crustacea. Mol. Biol. Evol. 6:685–691.

AX, P. 1984. Das phylogenetische System. Gustav Fischer, Stuttgart.

———. 1989. Basic phylogenetic systematization of the Metazoa. Pp. 229–245 in B. FERNHOLM, K. BREMER, and H. JÖRNVALL, eds. The hierarchy of life. Elsevier Science, Amsterdam.

BARTOLOMAEUS, T. 1985. The ultrastructure and development of the protonephridia of Lineus viridis (Nemertini). Microfauna Marina 2:61–83.

————. 1988. No direct contact between the excretory system and the circulatory system in *Prostomatella arenicola* Friedrich (Nemertini). Hydrobiologia **156**:175–181.

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

BÜRGER, O. 1897–1907. Nemertini. *In* H. G. BRONN, ed. Klassen und Ordnungen des Tierreichs. Vol. **4**, Suppl. Akademische Verlagsgesellschaft, Leipzig.

CURTIS, H., and N. S. BARNES. 1989. Biology, 5th ed. Worth, New York.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**:783–791.

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, W. M., and E. MARGOLIASH. 1967. Construction of phylogenetic trees. Science **155**: 279–284.

FRIEDRICH, H. 1935. Studien zur Morphologie, Systematik und Ökologie der Nemertinen der Kieler Bucht. Archiv. Naturgeschichte N. F. **4**:293–374.

GHISELIN, M. T. 1988. The origin of molluscs in the light of molecular evidence. Pp. 66–95 *in* P. HARVEY and L. PARTRIDGE, eds. Oxford surveys in evolutionary biology. Oxford University Press, Oxford.

GIBSON, R. 1972. Nemerteans. Hutchinson, London.

————. 1982. Nemertea. Pp. 823–846 *in* S. P. PARKER, ed. Synopsis and classification of living organisms. McGraw-Hill, New York.

HEDGES, S. B., K. D. MOBERG, and L. R. MAXSON. 1990. Tetrapod phylogeny inferred from 18S and 28S ribosomal RNA sequences and a review of the evidence for amniote relationships. Mol. Biol. Evol. **7**:607–633.

HENDRIKS, L., C. VAN BROEKHOVEN, A. VANDENBERGHE, Y. VAN DE PEER, and R. DE WACHTER. 1988. Primary and secondary structure of the 18S ribosomal RNA of the bird spider *Eurypelma californica* and evolutionary relationships among eukaryotic phyla. Eur. J. Biochem. **177**:15–20.

HYMAN, L. H. 1940. The invertebrates. Vol. **1**: Protozoa through Ctenophora. McGraw-Hill, New York.

————. 1951. The invertebrates. Vol. **2**: Platyhelminthes and Rhynchocoela. McGraw-Hill, New York.

JENSEN, D. 1988. HUBRECHT, MACFARLANE, JENSEN and WILLMER: on the nature and testability of four versions of the nemertean theory of vertebrate origins. Hydrobiolgia **156**:99–104.

JUKES, T. H., and C. R. CANTOR. 1969. Evolution of protein molecules. Pp. 21–132 *in* H. N. MUNRO, ed. Mammalian protein metabolism. Academic Press, New York.

LAKE, J. A. 1987. A rate-independent technique for analysis of nucleic acid sequences: evolutionary parsimony. Mol. Biol. Evol. **4**:167–191.

————. 1990. Origin of the metazoa. Proc. Natl. Acad. Sci. USA **87**:763–766.

LANE, D. J., K. G. FIELD, G. J. OLSEN, and N. R. PACE. 1988. Reverse transcriptase sequencing of ribosomal RNA for phylogenetic analysis. Methods Enzymol. **167**:138–144.

LANE, D. J., B. PACE, G. J. OLSEN, D. A. STAHL, M. L. SOGIN, and N. R. PACE. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. Proc. Natl. Acad. Sci. USA **82**:6955–6959.

LUTZ, P. E. 1986. Invertebrate zoology. Addison-Wesley, Reading, Mass.

NAWITZKI, W. 1931. *Procarinina remanei:* eine neue Paläonemertine der Kieler Förde. Zool. Jahrb. Anat. **54**:159–234.

NUSBAUM, J., and M. OXNER. 1913. Die Embryonalentwicklung des *Lineus ruber* Müll: ein Beitrag zur Entwicklungsgeschichte der Nemertinen. Z. Wiss. Zool. **107**:78–197.

OLSEN, G. J. 1988*a*. The earliest phylogenetic branchings: comparing rRNA-based evolutionary trees inferred with various techniques. Cold Springs Harbor Symp. Quant. Biol. **52**:825–837.

————. 1988*b*. Phylogenetic analysis using ribosomal RNA. Methods Enzymol. **164**:793–812.

PATERSON, B. M., and B. E. ROBERTS. 1981. Structural gene identification utilizing cell-free

translational systems. Pp. 417–437 *in* J. G. CHIRIKIJIAN and T. S. PAPAS, eds. Gene amplification and analysis. Vol. 2: Structural analysis of nucleic acids. Elsevier, New York.

REMANE, A. 1956. Die Grundlagen des natürlichen Systems, der vergleichenden Anatomie und der Phylogenetik, 2d ed. Akademische Verlagsgesellschaft Geest & Portig K.-G., Leipzig. (Reprint 1971. Otto Koeltz, Koenigstein).

RIEDL, R. 1978. Order in living systems. John Wiley, New York.

RIEGER, R. M., and S. TYLER. 1985. Das Homologietheorem in der Ultrastrukturforschung. Pp. 21–36 *in* J. A. OTT, G. P. WAGNER, and F. M. WUKETITS, eds: Evolution, Ordnung und Erkenntnis. Paul Parey, Berlin and Hamburg.

SANGER, F., S. NICKELN, and A. R. COULSEN. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5468.

SCHEPOTIEFF, A. 1912. Über die Bedeutung der Wassermannschen Reaktion für die biologische Forschung. Zool. Anz. **41**:49–54.

SMITH, A. B. 1989. RNA sequence data in phylogenetic reconstruction: testing the limits of its resolution. Cladistics **5**:321–344.

STAHL, D. A., D. J. LANE, G. J. OLSEN, and N. R. PACE. 1984. Analysis of hydrothermal vent-associated symbionts by ribosomal RNA sequences. Science **224**:409–411.

TORCZYNSKI, R. M., M. FUKE, and A. P. BOLLON. 1985. Cloning and sequencing of a human 18S ribosomal RNA gene. DNA **4**:283–291.

TURBEVILLE, J. M. 1986*a*. Comparative ultrastructure and ontogeny of the nemertine circulatory system. Ph.D. diss., Department of Biological Sciences, Clemson University, Clemson, S.C.

———. 1986*b*. An ultrastructural analysis of coelomogenesis in the hoplonemertine *Prosorhochmus americanus* and the polychaete *Magelona* sp. J. Morphol. **187**:51–60.

———. 1991. Nemertinea. Pp. 285–328 *in* F. W. HARRISON and B. J. BOGITSH, eds. Microscopic anatomy of invertebrates. Vol. 3: Platyhelminthes and Nemertinea. Wiley-Liss, New York.

TURBEVILLE, J. M., D. M. PFEIFER, K. G. FIELD, and R. A. RAFF. 1991. The phylogenetic status of arthropods, as inferred from 18S rRNA sequences. Mol. Biol. Evol. **8**:669–686.

TURBEVILLE, J. M., and E. E. RUPPERT. 1985. Comparative ultrastructure and the evolution of nemertines. Am. Zool. **25**:53–71.

WHEELER, W. C., and R. L. HONEYCUTT. 1988. Paired sequence difference in ribosomal RNAs: evolutionary and phylogenetic implications. Mol. Biol. Evol. **5**:90–96.

WILEY, E. O. 1981. Phylogenetics. John Wiley, New York.

WILLMER, P. 1990. Invertebrate relationships. Cambridge University, Cambridge.

WOESE, C. R. 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.

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