

# Phylogenetic Relationships among Higher Nemertean (Nemertea) Taxa Inferred from 18S rDNA Sequences

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**We estimated the phylogenetic relationships of 15 nemertean (phylum Nemertea) species from the four subclasses Hoplo-, Hetero-, Palaeo-, and Bdellonemertea with 18S rDNA sequence data. Three outgroup taxa were used for rooting: Annelida, Platyhelminthes, and Mollusca. Parsimony and maximum-likelihood analyses supported the monophyletic status of the Heteronemertea and a taxon consisting of hoplonemerteans and Bdellonemertea, while indicating that Palaeonemertea is paraphyletic. The monophyletic status of the two nemertean classes Anopla and Enopla is not supported by the data. The unambiguous clades are well supported, as assessed by a randomization test (bootstrapping) and branch support values.**

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## INTRODUCTION

In recent years a number of studies have addressed the phylogenetic position of the nemerteans among the Metazoa (Turbeville, 1991; Turbeville *et al.*, 1992, Sundberg *et al.*, 1998), and a few attempts to reconstruct phylogenetic relationships of particular nemertean taxa (e.g., Sundberg, 1989a,b; Härlin and Sundberg, 1995; Sundberg and Hylbom, 1994) have been published. However, the phylogenetic relationships among higher nemertean taxa have received relatively little attention. Currently, nemertean classification is nonphylogenetic (Sundberg, 1993; Sundberg and Svensson, 1994) and is based on a limited number of morphological characters. Nemerteans are currently classified into two higher-level groups, the Anopla and Enopla. The anoplans lack a proboscis armature (stylet) and the mouth is situated posterior to the brain and separated from the rhynchocoel opening. The Enopla traditionally includes nemerteans with an armed proboscis and a common mouth proboscis aperture. The mouth opening is situated anterior to the brain. These taxa are further divided into four major groups: the Hetero- and Palaeonemertea in the Anopla and the Hoplo- and Bdellonemertea in the Enopla.

These four groups are referred to as subclasses (Sundberg, 1991) or orders (e.g., Gibson, 1982) in this non-phylogenetic system. The rank is irrelevant in a truly phylogenetic classification (Sundberg and Pleijel, 1994), but we will henceforth refer to them as subclasses for convenience.

Hubrecht (1879) divided the nemerteans into three suborders, Palaeo-, Schizo-, and Hoplonemertini; the palaeonemerteans were later split by Bürger (1895, and elsewhere) into the Proto- and Mesonemertini. The Protonemertini included the genera *Carinina*, *Tubulanus*, and *Hubrechtia*, and the Mesonemertini included the genera *Cephalothrix* and *Carinoma*. Bürger divided the remaining nemerteans into the orders Heteronemertini and Metanemertini (=Hoplonemertini). Bürger discussed his classification in phylogenetic terms, but it was not based on specific phylogenetic analyses. Later, Stiasny-Wijnhoff (1936) classified the nemerteans into the subclasses Palaeo-, Hetero-, Hoplo-, and Bdellonemertea. These major taxa have not been questioned as natural groups, except in the case of the palaeonemerteans. Friedrich (1935) divided the palaeonemerteans into three "Formengruppen:" (i) *Cephalothrix*, *Cephalothrichella*, and *Procephalothrix*; (ii) *Carinoma*; and (iii) *Hubrechtia*, *Carinina* (including *Procarinina*), *Callinera*, *Carinesta*, and *Tubulanus*. Friedrich considered the second group (family Carinomidae) to have branched off from ancient heteronemerteans and mentioned what he considered a close morphological relationship between the genus *Hubrechtia* and the heteronemerteans. Hylbom (1957: textfig. 5) proposed a tentative phylogeny for the Palaeonemertea, suggesting that the two related genera *Carinoma* and *Carinomella* were linked to the lineid heteronemerteans, *Hubrechtella* was linked to the baseodiscid heteronemerteans, and *Hubrechtia* was linked to the valencinid heteronemerteans. Although this is how we would read Hylbom's tree, Hylbom himself (pers. comm.) did not question the monophyletic status of the palaeonemerteans. Both Friedrich (1935) and Hylbom (1957) based their conclusions on

**TABLE 1**  
**List of Nemertean Species Included in the Analyses**

Species	Accession No.	Source
Class Anopla		
Subclass Palaeonemertea		
<i>Cephalothrix rufifrons</i> (Johnston, 1837)	AY039677	Sweden
<i>Tubulanus annulatus</i> (Montagu, 1804)	AY039678	Sweden
<i>Hubrechtella dubia</i> (Bergendal, 1902)	AY039674	Sweden
<i>Carinoma tremaphoros</i> (Thompson, 1900)	AY039675	USA
Palaeonemertean 1M <sup>a</sup>	AY039676	USA
Subclass Heteronemertea		
<i>Micrura fasciolata</i> (Ehrenberg, 1831)	AY039673	Sweden
<i>Lineus ruber</i> (Müller, 1774)	AY039672	USA
<i>Zygeupolia rubens</i> (Coe, 1895)	AY039671	USA
<i>Lineus</i> sp.	X70210	Winnepenninckx <i>et al.</i> (1995)
Class Enopla		
Subclass Hoplonemertea		
<i>Amphiporus ochraceus</i> (Verrill, 1873)	AY039668	USA
<i>Prosorhochmus</i> sp.	AY039669	Italy
<i>Prostoma graecense</i> (Böhmgig, 1892)	AY039666	Sweden
<i>Prostoma eilhardi</i> (Montgomery, 1894)	U29494	Giribet <i>et al.</i> (1996)
<i>Cyanophthalma obscura</i> (Schultze, 1851)	AY039667	Sweden
Subclass Bdellonemertea		
<i>Malacobdella grossa</i> (Müller, 1776)	AY039670	Sweden

<sup>a</sup> Probable cephalothricid (*sensu* Sundberg and Hylbom, 1994).

the noncladistic view that some recent taxa are ancestors to other recent groups and that there is a progression from simple to complex taxa. The same view is evident in Iwata (1960) who suggested that the palaeonemertean family Cephalotrichidae should be placed in its own subclass, the Archinemertea, which he considered the ancestor of both the remaining palaeonemerteans and the heteronemerteans. Iwata (1960, 1985) envisioned a phylogeny in which the Archinemertea split into Palaeonemertea and Hoplonemertea, and the Palaeonemertea (*sensu* Iwata) later gave rise to the Heteronemertea. Iwata (1960) based his proposition on the view of evolution as a progression series from primitive to advanced and where a recent taxon forms the ancestor of other recent taxa, which is a flawed view in our opinion. Sundberg and Hylbom (1994) questioned the monophyletic status of Archinemertea and could not find any support for Iwata's (1960) proposal.

Here we report the first analysis of higher-level nemertean relationships using gene sequences. We have sequenced the entire 18S rRNA gene and, using appropriate outgroups, have reconstructed the phylogeny of nemerteans using parsimony and maximum-likelihood analyses. The molecular data allow a test of the monophyletic status of the nemertean classes Anopla and Enopla and the four nemertean subclasses Hoplo-, Hetero-, Palaeo-, and Bdellonemertea and the reconstruction of a phylogenetic hypothesis for these subclasses.

## MATERIALS AND METHODS

### Sample Collection and DNA Extraction

Fifteen nemertean species were included in the analyses. The nemertean taxa and collection sites are listed in Table 1. Live specimens were placed in 70–95% ethanol and stored until extracted. DNA was extracted from *Tubulanus annulatus*, *Hubrechtella dubia*, and *Prostoma graecense*, following the protocol of Crossland *et al.* (1993). For *Lineus ruber*, *Micrura fasciolata*, *Cephalothrix rufifrons*, and *Prosorhochmus* sp., DNA was extracted according to Winnepenninckx *et al.* (1993a). DNA was extracted from the *Malacobdella grossa* specimen according to the simplified procedure described in Sundberg and Andersson (1995). DNA from *Carinoma tremaphoros*, Palaeonemertean 1M, *Zygeupolia rubens*, and *Cyanophthalma obscura* was isolated with a standard protocol outlined in Strauss (1987). DNA of *Lineus ruber* and *Amphiporus ochraceus* was purified with a cesium chloride protocol (Dowling *et al.*, 1996).

### DNA Amplification and Sequencing

The entire 18S rRNA gene was amplified by the polymerase chain reaction (PCR) with eukaryotic specific primers (Medlin *et al.*, 1988) and a Perkin-Elmer DNA Thermal Cycler or a PTC-100 (MJ Research, Inc.). An approximately 1900-bp region of the 18S rRNA gene was amplified by the polymerase chain reaction. PCRs were carried out in a final volume of 50

$\mu$ l. The final concentrations of the components were template DNA 1  $\mu$ l (around 100 ng/ $\mu$ l), TMAC 0.001%, Mg<sup>2+</sup> 2.0 mM, primer (PCRA and PCRB) 0.3  $\mu$ m, 1 $\times$  reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), [dNTP] 0.4 mM, *Taq* polymerase (Perkin-Elmer) 2.0 U. The PCR temperature profile was as follows: 94°C for 1.5 min; 35 cycles with 94°C for 1 min, 50–60°C for 1 min, and 72°C for 2 min; ending at 72°C for 7 min. Amplification products were purified with Promega Wizard PCR preps or gel-purified with Nusieve Agarose (FMC, Inc.) in conjunction with a silica-binding recovery technique.

DNAs were sequenced by radioactive cycle sequencing or fluorescent cycle sequencing with the Fmol DNA cycle sequencing kit (Promega) or CY5 Autocycle (Pharmacia) for most species. In some cases sequencing of single-stranded DNA generated from single primer amplification (see Palumbi, 1996) of original double-stranded products was carried out with a sequenase kit (USB). Both strands were sequenced for all species.

Sequencing was performed manually using standard techniques or on an ALFexpress Automated DNA Sequencer (Pharmacia).

#### *Outgroup, Sequence Analysis, and Phylogeny Reconstruction*

Nemerteans are traditionally placed close to the Platyhelminthes (e.g., Hyman, 1951), a conclusion also reached by the cladistic analysis of Nielsen *et al.* (1996). But this view has been questioned (e.g., Norenburg, 1985; Turbeville, 1991) and is not supported by alternative cladistic analyses of morphology (Eernisse *et al.*, 1992; Schram, 1991; Zrzavy *et al.*, 1998). The latter analyses suggest that the nemerteans are most closely related to spiralian (Protostome) coelomates. Analyses of 18S rRNA gene sequences also support a close relationship of nemerteans and spiralian coelomates (e.g., Turbeville *et al.*, 1992; Winnepeninckx *et al.*, 1995; Winnepeninckx *et al.*, 1996; Carranza *et al.*, 1997; Giribet *et al.*, 2000). Given these findings, we have used representatives of three spiralian taxa as outgroups: four molluscs, *Nerita albicilla* (GenBank Accession No. X91971), *Siphonaria algesirae* (X91973), *Littorina littorea* (X91970) (all from Winnepeninckx *et al.*, 1996), and *Acanthopleura japonica* (X70210) (Winnepeninckx *et al.*, 1993b); three Platyhelminthes, *Convoluta naikaiensis* (D83381), *Planocera multitentaculata* (D83383), and *Dugesia japonica* (D83382) (all from Katayama *et al.*, 1995); and three annelids, *Haemopsis sanguisuga* (X91401), *Lanice chiliega* (X79873), and *Eisenia fetida* (X79872) (all from Winnepeninckx *et al.*, 1996). Additional analyses were conducted with the annelids alone as the outgroup. Although the selection of annelids is somewhat arbitrary, it is important to note that analyses with alternate outgroups (molluscs or platyhelminths) did

not alter the overall topology. Based on current evidence (e.g., compare Eernisse *et al.*, 1992; Zrzavy *et al.*, 1998; Giribet *et al.*, 2000), a preferred outgroup cannot be reliably determined, although annelids and molluscs can be inferred as more appropriate than platyhelminthes from 18S rDNA analyses (e.g., Giribet *et al.*, 2000; Winnepeninckx *et al.*, 1995).

Sequences were edited with Lasergene (DNASTAR Inc.), and aligned using CLUSTAL-V (Higgins *et al.*, 1992) algorithm. Three different gap penalty schemes were applied (number of gaps/length of gaps): 10/10, 15/5, 20/5. A combination of penalties was used for the alignment including annelids as outgroup. The number of gaps/length of gap penalties was set to 20/5 for the bases up to position 1786; from there the ratio was set to 10/10, which improved similarity as judged by eye. Thus, in general the number of gaps was penalized more than the size of gaps. This weighting scheme was employed because it gave a better similarity match between sequences and because there is no a priori reason to assume that insertion/deletions are more likely to involve short sequence stretches. The alignment including annelids as outgroup was further adjusted with a secondary structure model. The alignment (deposited in EMBL alignment bank as ALIGN000139) position 1 corresponds to position 177 in *Homo sapiens* (GenBank Accession No. M 10098).

The aligned sequences were used both with the variable regions included in one set of analyses and with these regions excluded in another set. Ambiguous regions were excluded in the second set based on a comparison of the three alignments with penalty ratios as above; in the final analysis with annelids as outgroup the decision to exclude regions was based on the alignment with penalty schemes combined with the secondary structure interpretation. In the final analysis with the annelids, the following regions were excluded: 129–154, 188–243, 694–699, 723–727, 736–741, 745–747, 836–838, 906–907, 1432–1442, 1449–1450, 1456–1483, 1491–1499, 1618–1623, 1816–1817, 1829–1835, 1857–1868, and 1907–1910. The alignment ends at position 1930. The exclusion of ambiguous regions is based on the reasoning that positions that do not align consistently over the chosen alignment parameters are considered unreliable positional homologues relative to sites that are alignment invariant (Gates *et al.*, 1993).

Phylogeny was reconstructed with parsimony (branch-and-bound search) and maximum-likelihood (ML). Maximum-likelihood analysis requires an explicit model of base substitution (see Steel and Penny (2000) for a recent review). Since the model parameters are unknown and have to be estimated from the data, maximum-likelihood analysis has been criticized for not only explaining changes in the observed data along a phylogenetic tree but also trying to estimate the most probable changes, whereby uncertainties are added to

TABLE 2

Parsimony Tree Statistics for Different Outgroups<sup>a</sup>

Outgroup	Alignment-ambiguous regions	No. trees	CI	RI
Mollusca	Incl	4	0.66	0.69
	Excl	2	0.69	0.73
Platyhelminthes	Incl	2	0.72	0.67
	Excl	4	0.60	0.69
Annelida <sup>b</sup>	Incl	5	0.67	0.67
	Excl	2	0.67	0.71

<sup>a</sup> See also Fig. 1. Analyses were conducted with (Incl) and without (Excl) ambiguous regions of the alignment. No. trees, number of most parsimonious trees obtained; CI, consistency index; RI, retention index.

<sup>b</sup> See Materials and Methods for details.

the analysis (e.g., Siddall, 1998). Despite these criticisms, ML has been shown in computer simulations to be more robust than parsimony under numerous models of sequence evolution (e.g., Huelsenbeck, 1995; but see, e.g., Siddall (1998) and Siddall and Kluge (1999) for a different view), and we therefore consider it worthwhile to include ML analysis in this study for comparative purposes.

The ML analysis was based on the HKY85 distance (Hasegawa *et al.*, 1985) which allows for unequal base frequencies and different rates of transitions and transversions. The two most parsimonious trees were used as starting trees in the maximum-likelihood heuristic search and model parameters were estimated from a likelihood analysis of these trees. The ML analysis included a nucleotide model with six substitution types (a GTR model) and among-sites rate heterogeneity using a gamma distribution with shape parameter of 0.25. Nucleotide frequencies were set to empirical values. Clade support was assessed by bootstrapping (Felsenstein, 1985) and by calculation of branch support values (Bremer, 1994). All phylogenetic analyses were carried out with PAUP\* ver 4.0b2a (PPC) (Swofford, 1998).

## RESULTS AND DISCUSSION

The total result of parsimony analyses with the three outgroups and including/excluding ambiguous regions resulted in 19 most parsimonious trees (Table 2). The strict consensus of these trees (Fig. 1) indicates monophyly of Heteronemertea and monophyly of a taxon composed of the hoplonemerteans and *Malacobdella* (representing Bdeilonemertea). Monophyly of the Palaeonemertea is not supported.

In the final analysis with Annelida as the outgroup and exclusion of ambiguous regions as described above, the multiple alignment of the 18S sequences resulted in 1955 nucleotide positions when ends were pruned. Of these, 301 positions were phylogenetically informa-

tive. Mean distance (HKY85, calculated in PAUP\*) between ingroup and outgroup taxa ranged from 0.05 to 0.14 and among ingroup taxa from 0.004 (between the two *Prostoma* species) to 0.15. The parsimony analysis resulted in two most parsimonious trees (Fig. 2) differing only in the position of *Cyanophthalma obscura* and *Amphiporus ochraceus*. Support for the Hoplonemertea + Bdeilonemertea and Heteronemertea as monophyletic taxa is high judged both from support values and from bootstrapping (Fig. 2). The Palaeonemertea is paraphyletic and the Bdeilonemertea (*Malacobdella grossa*) is a sister taxon to the hoplonemerteans in this analysis. However, support for a monophyletic Hoplonemertea exclusive of *Malacobdella* is weak (60% of the bootstrap trees, and a corresponding support index of 1). Furthermore, maximum-likelihood analysis (Fig. 3) shows no support for this sister taxon relationship between *Malacobdella* and the other hoplonemerteans, whereas the clade composed of the hoplonemerteans and the bdeilonemertean is well supported in this analysis. In the ML analysis, *Malacobdella* forms a weakly supported clade with *Prosorhochmus*, as judged by its bootstrap value of 60%. Taken together, we consider it premature to make a decision about the exact relationship of *M. grossa* vis-a-vis the hoplonemerteans but prefer to include this bdeilonemertean species among the Hop-

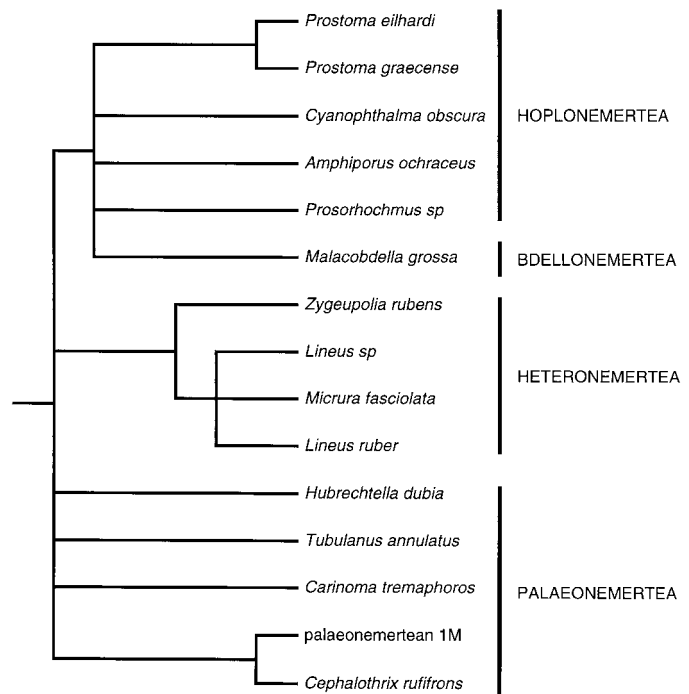
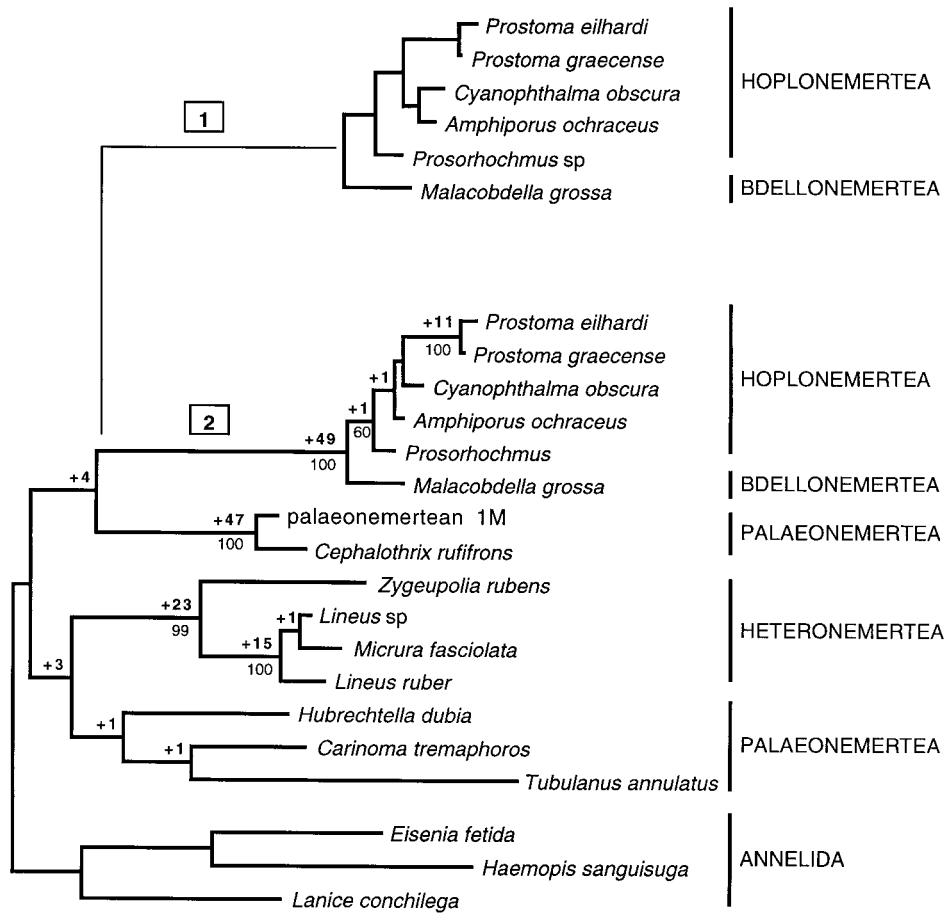


FIG. 1. Strict consensus tree of 19 different phylogenetic hypotheses (Table 2) resulting from parsimony analyses incorporating three different outgroup taxa: Mollusca, Annelida, and Platyhelminthes.



**FIG. 2.** Parsimony tree for the 15 nemertean species based on 18S DNA sequences with regions of ambiguous alignment excluded (see text). There are two equally most parsimonious trees (length 1052, CI = 0.67, RI = 0.71) differing in the position of *Cyanophthalma* and *Amphiporus* (1 and 2 in boxes). The outgroup is Annelida. Numbers above branches are support values or decay indices. Numbers below nodes are bootstrap percentiles from 5000 replicates (heuristic search, random addition, 10 replicates).

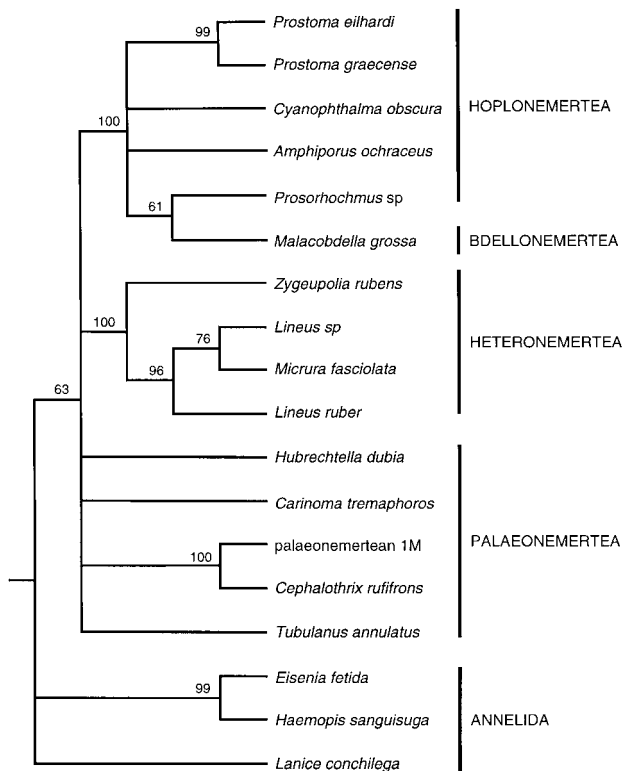
lonemertea, whereby this taxon is monophyletic in all analyses.

The Heteronemertea is also monophyletic in all analyses, but the two taxa Enopla and Anopla are not monophyletic. Monophyly of Heteronemertea is also supported by at least one morphological synapomorphy, the presence of an outer longitudinal muscle layer between the epidermis and the central nervous system (Norenburg, 1993). The results also support monophyly of the lineid heteronemerteans (*Micrura fasciolata*, *Lineus ruber*, and *Lineus* sp.); horizontal cephalic grooves are a potential morphological synapomorphy of these lineid taxa (Norenburg, 1993). The position of the palaeoneurtean species, however, is unsettled, although the analysis unequivocally shows that the taxon is nonmonophyletic.

Long branches may lead to spurious results when phylogeny is reconstructed by parsimony (Felsenstein, 1978). Since long branches may be expected especially between the outgroup species and the ingroup (see Smith, 1994), the analysis was done without the out-

group, but this did not change the topology in any significant way.

*M. grossa* represents the taxon Bdellonemertea, which traditionally is grouped together with the hoplonemerteans at the same rank in the taxon Enopla (e.g., Hyman, 1951; Gibson, 1994). Bdellonemertea comprises commensal animals with several morphological characteristics that probably can be assigned to its life style, and its systematic position is unclear from morphology. Friedrich (1936, IV. d 49) argued based on characters such as proboscis morphology that the taxon probably is derived from a monostiliferous hoplonemertean; that is, it is most closely related to these hoplonemerteans. Still, Friedrich grouped both poly- and monostiliferous hoplonemerteans in the same taxon Hoplonemertea and considered it of the same rank as the Bdellonemertea. A phylogenetic interpretation of Friedrich would instead be that the Bdellonemertea should be contained in the Hoplonemertea. Bürger (1895) also recognized the Bdellonemertea as a family within the Hoplonemertea, whereas Crandall



**FIG. 3.** Majority rule consensus tree of 2000 BOOTSTRAP replicates of maximum-likelihood analysis (fast heuristic search, distance measure = HKY85, with parameters as described in the text). Numbers above branches refer to the percentage of the replicates in which the clade is supported. The outgroup is Annelida.

(1993) concluded that Bdello- and Hoplonemertea shared a common ancestor but placed them in different taxa based on morphological divergence. Our analysis shows that, irrespective of outgroup and phylogeny algorithm, *Malacobdella* and the hoplonemerteans form a clade and we suggest that the monotypic taxon Bdellonemertea be removed. A putative morphological synapomorphy of this clade is the position of the brain posterior to the mouth.

Our analysis, irrespective of phylogeny reconstruction algorithm or outgroup utilized, shows that (i) *Malacobdella* forms a clade with the hoplonemerteans (*Prostoma*, *Amphiporus*, *Prosorhochmus*, and *Cyanophthalma*), (ii) the three heteronemertean species (*Lineus*, *Zygeupolia*, and *Micrura*) form a monophyletic group, and (iii) the palaeonemertean species (*Tubulanus*, *Hubrechtella*, *Carinoma*, palaeonemertean 1M, and *Cephalothrix*) do not constitute a monophyletic taxon. The heteronemertean clade and the clade containing *Malacobdella* and the stylet-bearing hoplonemerteans are well supported as measured by branch support (Bremer, 1994) and bootstrapping (Felsenstein, 1985).

Many nemertean systematists have regarded Palaeonemertea the most basal taxon within the phylum,

but our analysis is inconclusive in this respect. It shows unequivocally, though, that the Palaeonemertea is nonmonophyletic, which also has been alluded to by other authors. Hylbom (1957), for example, argues that some palaeonemertean species may have a closer relationship with the heteronemerteans. Turbeville (1991) mentions a number of characters indicating that at least certain heteronemerteans and palaeonemerteans may share a recent common ancestor. For example, the mesodermal muscle processes in the epidermis are reported only from *Carinoma* and *Carinomella* species (Turbeville and Ruppert, 1983) and the heteronemertean *Lineus socialis* (now *Myoisophagus sanguineus*) (Turbeville, 1991). Turbeville (1991) furthermore interprets the presence of unique secretory bodies (rhabdoids or pseudocnids) in palaeo- and heteronemerteans as a possible synapomorphy. Also, Norenburg (1985, 1993) concluded that the palaeonemerteans form a nonmonophyletic group and that some palaeonemertean taxa (e.g., *Hubrechtella*) were more closely related to heteronemerteans than to other palaeonemerteans. We, however, have not been able to establish any well-supported sister group relationships between the palaeonemertean and the heteronemertean species included using 18S rDNA sequences. A clade containing the palaeonemerteans *Hubrechtella*, *Carinoma*, and *Tubulanus* is the sister taxon to the heteronemertean clade in the parsimony analysis with annelids as outgroups (Fig. 2), but this result is weakly supported by the data and does not hold when alternative outgroups are used.

We cannot draw any conclusions about phylogenetic relationships within the higher nemertean taxa based on our analysis, but the results show that while the Hetero- and the Hoplonemertea (inclusive of Bdellonemertea) are monophyletic, the Palaeonemertea is not. The results also imply that taxon Anopla (traditionally comprising the Palaeo- and Heteronemertea) is nonmonophyletic. Further clarification of nemertean relationships will require analyses of additional molecular and morphological characters.

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