

# Ribbon worm relationships: a phylogeny of the phylum Nemertea

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We present the most extensive phylogenetic analysis to date, to our knowledge, of higher-level nemertean relationships, based on sequence data from four different genes (the nuclear genes for nuclear large subunit rRNA (28S rRNA) and histone H3 (H3), and the mitochondrial genes for mitochondrial large subunit rRNA (16S rRNA) and cytochrome *c* oxidase subunit I (COI)). Well-supported clades are, in general, compatible with earlier, more limited, analyses, and current classification is largely in agreement with our results, although there are some notable exceptions. Bdelonemertea (represented by *Malacobdella*) is found to be a part of Monostilifera, and Polystilifera is the monophyletic sister group to Monostilifera. Cratenemertidae is the sister group to the remaining monostiliferans (including *Malacobdella*), a group to which we apply the new name Distromatonemertea. Heteronemertea is monophyletic and forms a clade with *Hubrechtella*; for this clade we introduce the name Pilidiophora. Finally, Pilidiophora and Hoplonemertea (with *Malacobdella*) form a monophyletic group, and we introduce the name Neonemertea to refer to this group. Palaeonemertea is found to be non-monophyletic and basal among nemerteans.

**Keywords:** phylogenetic analysis; DNA sequence data; spiralian; phylogenetic taxonomy; Bayesian analysis

## 1. INTRODUCTION

Nemerteans, or ribbon worms, are unsegmented, bilaterally symmetrical and at first glance acoelomate worms with separate mouth and anus. They possess a blood vascular system, however, that most probably is homologous to a coelom (Turbeville 1986). The monophyly of Nemertea is not in doubt today and is supported morphologically by, among other things, the characteristic eversible proboscis situated in a rhynchocoel, features that are unique to the phylum. Nemertea comprises about 1150 nominal species (Gibson 1995), occupying a broad spectrum of habitats, especially in the marine environment.

The current nemertean classification (e.g. table 1) is based on a limited number of morphological characters, and comprises non-monophyletic groups (Sundberg 1993; Sundberg & Svensson 1994). Higher classification is based on Stiasny-Wijnhoff (1936) who classified the nemerteans into the subclasses Palaeonemertea, Heteronemertea, Hoplonemertea and Bdelonemertea. These in turn form Anopla, nemerteans lacking armament on the proboscis (Heteronemertea and Palaeonemertea), and Enopla, nemerteans that possess a proboscis armed with one or several stylets. Some of these higher taxa may very well be monophyletic, but apomorphies have been assigned in a *post hoc* fashion. At less inclusive levels, nemertean systematics is also in need of a review. Gibson (1985) noted that, at the time, 40% of the species were assigned to one of four 'mega-genera' (*Cerebratulus*, *Lineus*, *Amphiporus* and *Tetrastemma*). In a subsequent paper, Gibson (1995) estimates the number of valid gen-

era as 250 and the number of valid species as 1150. The taxonomic resolution has increased, but the four 'mega-genera' still comprise the lion's share of nemertean species. Recent decades have also witnessed the creation of a large number of monotypic genera, rarely accompanied by informative phylogenetic evaluation.

The phylogenetic relationships within the phylum Nemertea and its position in Metazoa have recently received some attention. Studies based on molecular and combined molecular and morphological data of Metazoa have (directly or indirectly) addressed the position within Metazoa (e.g. Turbeville 1991; Turbeville *et al.* 1992; Sundberg *et al.* 1998; Zrzavy *et al.* 1998), but there have been relatively few published studies on the relationships within the phylum based on explicit phylogenetic analyses. A few studies have focused on specific subgroups, such as reptic polystiliferans (Härlin & Sundberg 1995), *Ototyphlonemertes* (Envall & Sundberg 1998) and the palaeonemerteans (Sundberg & Hylbom 1994). Phylogenetic studies based on sequence data were applied by Sundberg *et al.* to Heteronemertea (using the mitochondrial 16S rRNA gene; Sundberg & Saur 1998) and later to the phylum (using the 18S rRNA gene; Sundberg *et al.* 2001).

Here, we report the first more extensive analysis of higher-level nemertean relationships, to our knowledge, based on sequence data from four different genes (the nuclear genes for 28S rRNA (28S) and histone H3 (H3), and the mitochondrial genes for 16S rRNA (16S) and cytochrome *c* oxidase subunit I (COI)). The data enable us to erect a phylogenetic hypothesis of the relationships within the phylum Nemertea, with good support for many clades, thus facilitating the evaluation of the higher taxa currently used. The result, when comparing well-supported clades, is compatible with the more limited analysis based on the 18S rRNA gene previously published (Sundberg *et al.* 2001). The current classification is largely

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Table 1. Current classification (mainly after Gibson 1982) and collection sites for the species used in the present study.

Nemertea	
Enopla	
Hoplonemertea	
Monostilifera	
Amphiporidae	
<i>Amphiporus angulatus</i> (Müller, 1774)	Cobscook, ME, USA
<i>Amphiporus formidabilis</i> Griffin, 1898	San Juan Island, WA, USA
<i>Amphiporus imparispinosus</i> Griffin, 1898	San Juan Island, WA, USA
<i>Amphiporus lactifloreus</i> (Johnston, 1828)	Kandalaksha Bay, White Sea, Russia
<i>Gurjanovella littoralis</i> Ushakov, 1926	Kandalaksha Bay, White Sea, Russia
<i>Poseidonemertes collaris</i> Roe & Wickham, 1984	Bodega Bay, CA, USA
<i>Poseidonemertes</i> sp. 349	Diablo Heights, Panama City, Panama
<i>Poseidonemertes</i> sp. 508	Seto, Japan
<i>Zygonemertes simonae</i> Corrêa, 1961	Fort Pierce, FL, USA
<i>Zygonemertes vrescens</i> (Verrill, 1879)	Fort Pierce, FL, USA
Carcinonemertidae	
<i>Carcinonemertes</i> cf. <i>carcinophila imminuta</i> (Kölliker, 1845)	São Sebastião, Brazil; on <i>Callinectes danae</i>
Cratenemertidae	
<i>Nipponnemertes bimaculatus</i> (Coe, 1901)	San Juan Island, WA, USA
<i>Nipponnemertes punctatulus</i> (Coe, 1905)	Oshoro, Hokkaido, Japan
Emplectonematidae	
<i>Emplectonema buergeri</i> Coe, 1901	San Juan Island, WA, USA
<i>Emplectonema gracile</i> (Johnston, 1837)	Salcombe, UK
<i>Nemertopsis bivittata</i> (Delle Chiaje, 1841)	Fort Worth Inlet, FL, USA
<i>Paranemertes peregrina</i> Coe, 1901	San Juan Island, WA, USA
<i>Paranemertes sanjuanensis</i> Stricker, 1982	San Juan Island, WA, USA
<i>Paranemertes</i> sp. 249	San Juan Island, WA, USA
Ototyphlonemertidae	
<i>Ototyphlonemertes</i> sp. 21	Nantucket, MA, USA
Prosorhochmidae	
<i>Oerstedtia venusta</i> Iwata, 1954	Akkeshi Bay, Japan
<i>Oerstedtia zebra</i> (Chernyshev, 1993)	Akkeshi Bay, Japan
<i>Pantinonemertes</i> sp. 115	Fort Pierce, FL, USA
Tetrastemmatidae	
<i>Antarctonemertes varverae</i> Chernyshev, 1999	Ussuri Bay, Sea of Japan, Russia
<i>Nemertellina yamaokai</i> (Iwata, 1954)	Akkeshi Bay, Japan
<i>Tetrastemma elegans</i> (Girard, 1852)	Nahant, MA, USA
<i>Tetrastemma wilsoni</i> Coe, 1943	Edgewater, MD, USA
Polystilifera	
Pelagica	
<i>Nectonemertes mirabilis</i> Verrill, 1892	160 km off Point Conception, CA, USA
<i>Pelagonemertes</i> sp. 545	160 km off Point Conception, CA, USA
<i>Phallonemertes murrayi</i> (Brinkmann, 1912)	160 km off Point Conception, CA, USA
<i>Protopelagonemertes</i> sp. 544	160 km off Point Conception, CA, USA
Reptantia	
Reptant sp. 281	Carrie Bow Cay, Belize
Reptant sp. 481	Okinawa, Japan
Reptant sp. 500	Seto, Japan
Bdellonemertea	
Malacobdellidae	
<i>Malacobdella</i> cf. <i>grossa</i> (Müller, 1776)	VA, USA; on <i>Mercenaria mercenaria</i> , and White Sea, Russia; on <i>Arctica islandica</i>
Anopla	
Heteronemertea	
Riseriellidae	
<i>Riserius pugetensis</i> Norenburg, 1993	San Juan Island, WA, USA
Lineidae	
<i>Cerebratulus marginatus</i> Renier, 1804	WA, USA
<i>Lineus alborostratus</i> Takakura, 1898	Vostok Bay, Sea of Japan, Russia
<i>Lineus longissimus</i> (Gunnerus, 1770)	Anglesey, UK
<i>Lineus viridis</i> (Müller, 1774)	Manset, ME, USA
<i>Micrura alaskensis</i> Coe, 1901	San Juan Island, WA, USA
<i>Notospermus geniculatus</i> (Delle Chiaje, 1828)	Seto, Japan
<i>Parborlasia corrugatus</i> (McIntosh, 1876)	McMurdo Sound, Antarctica

Table 1. (Continued.)

<i>Parvicirrus dubius</i> (Verrill, 1879)	Georgetown, ME, USA
<i>Ramphogordius sanguineus</i> (Rathke, 1799)	Anglesey, UK
<i>Tenuilineus bicolor</i> (Verrill, 1892)	Sebastian Inlet, FL, USA
Palaeonemertea	
Carinomidae	
<i>Carinoma mutabilis</i> Griffin, 1898	San Juan Island, WA, USA
<i>Carinoma tremaphoros</i> Thompson, 1900	Fort Pierce, FL, USA
Cephalothricidae	
<i>Procephalothrix filiformis</i> (Johnston, 1828)	Akkeshi Bay, Japan
<i>Procephalothrix simulus</i> Iwata, 1952	Akkeshi Bay, Japan
<i>Procephalothrix spiralis</i> (Coe, 1930)	San Juan Island, WA, USA
Hubrechtidae	
<i>Hubrechtella dubia</i> Bergendal, 1902	Fort Pierce, FL, USA
Tubulanidae	
<i>Tubulanus punctatus</i> (Takakura, 1898)	Vostok Bay, Sea of Japan, Russia
<i>Tubulanus rhabdotus</i> Corrêa, 1954	Fort Pierce, FL, USA
<i>Tubulanus sexlineatus</i> (Griffin, 1898)	San Juan Island, WA, USA
Outgroup taxa	
Mollusca	
<i>Aplysia californica</i> Cooper, 1863	AY026366, AF192295, AF077759, AF033675
<i>Mytilus edulis</i> Linnaeus, 1758	Z29550, AF023541, U68773
Sipunculida	
Sipuncula <sup>a</sup>	AF342795, AF374337, AF374337, AF185264
Echiura	
<i>Urechis caupo</i> Fischer & MacGintie, 1928	AF342804, AF315059, U74077, X58895
Brachiopoda	
<i>Terebratalia transversa</i> (Sowerby, 1846)	AF342802, AF331161, AF331161

<sup>a</sup> 28S, 16S and COI sequences are from *Phascolopsis gouldii*; H3 sequence is from *Sipuncula* sp.

in agreement with our results, although there are some notable exceptions.

## 2. MATERIAL AND METHODS

### (a) Specimens and DNA extraction

The species and specimens sequenced are listed in table 1, together with the collection sites. There are 10 hitherto undescribed (or unidentified) species included in the dataset. They are identified by numbers, and reference specimens are available at the National Museum of Natural History, Smithsonian Institution. These species will be described elsewhere.

Specimens sequenced in this study were either snap frozen and kept at  $-80^{\circ}\text{C}$  or preserved in 80–95% ethanol until DNA extraction. Total DNA was extracted using a protocol modified from Winnepenninckx *et al.* (1993) as described in Tholleson (2000).

### (b) Outgroup selection

Traditionally the Nemertea have been considered to be closely related to Platyhelminthes (e.g. Hyman 1951), but recent studies strongly contradict this and indicate that closer relatives are to be found among the lophotrochozoa, such as molluscs, annelids, sipunculans and echiurans (e.g. Erber *et al.* 1998; Zrzavý *et al.* 1998; Giribet *et al.* 2000). Based on this and on sequence availability, we selected two molluscs (the bivalve *Mytilus edulis* and the gastropod *Aplysia californica*), a sipunculan (*Sipuncula*), an echiuran (*Urechis caupo*) and a brachiopod (*Terebratalia transversa*) as outgroup taxa for rooting (as discussed by Nixon & Carpenter 1993).

### (c) Amplification and sequencing

Amplification of parts of the genes coding for COI, 16S rRNA, 28S rRNA and H3 was carried out using universal pri-

mers: 16sar-L [CGCCTGTTTATCAAAAACAT] and 16sbr-H [CCGGTCTGAACTCAGATCACGT] from Palumbi *et al.* (1991) for 16S; LSU5 [ACCCGCTGAAYTTAAGCA] and LSU3 [TCCTGAGGGAACTTCGG] from Littlewood (1994) for 28S; LCO1490 [GGTCAACAAATCATAAAGAT-ATTGG] and HCO2198 [TAAACTTCAGGGTGAC-CAAAAATCA] from Folmer *et al.* (1994) for COI; and H3NF [ATGGCTCGTACCAAGCAGAC] and H3R [ATATCCTTRGGCATRATRGTGAC] from Colgan *et al.* (2000) for H3.

Each PCR was performed using a 15 ng template in a 50  $\mu\text{l}$  volume (50 mM Tris-HCl pH 9.1, 16 mM  $(\text{NH}_4)_2\text{SO}_4$ , 3.5 mM  $\text{MgCl}_2$ , 150  $\mu\text{g ml}^{-1}$  bovine serum albumin (BSA), 0.5  $\mu\text{M}$  of each primer, 160  $\mu\text{M}$  of each dNTP and 0.25  $\mu\text{l}$  of KlenTaq Taq polymerase (AB Peptides, Inc.). Thermo cycling comprised an initial 2 min denaturation at  $94^{\circ}\text{C}$ , followed by 35 cycles of 30–45 s at  $94^{\circ}\text{C}$ , 30–45 s at  $50$ – $55^{\circ}\text{C}$  (depending on the target) and 60–90 s at  $72^{\circ}\text{C}$  (the longer times are for the 28S fragment). The cycling ended with a 7 min sequence extension at  $72^{\circ}\text{C}$ .

The PCR product was purified with QIAquick (Qiagen Inc.) and used in cycle sequencing with dye-terminators using FS or BigDye chemistry (Perkin-Elmer) and standard cycles (4 min denaturation at  $96^{\circ}\text{C}$ , followed by 25 cycles of 10 s at  $96^{\circ}\text{C}$ , 5 s at  $50^{\circ}\text{C}$  and 4 min at  $60^{\circ}\text{C}$ ). The PCR primers were used for sequencing reactions, together with two additional 28S primers: D2F ([CTTTGAAGAGAGAGTTTC] Littlewood 1994) and 28z (truncated) ([CTTGGTCCGTGTTTCAAGAC] Hillis & Dixon 1991). The products were sequenced on ABI373 or ABI 377 automated sequencers (Perkin-Elmer); both strands were sequenced at least once. The sequences have been deposited with the European Molecular Biology Laboratory nucleotide sequence database (accession numbers AJ436786 to AJ436991).

**(d) Alignment and phylogenetic inference**

The sequences were first aligned gene by gene using MEG ALIGN, v. 3.14 in the DNA\* software collection (DNASTar Inc.), using the 'CLUSTAL' option (Higgins *et al.* 1992; Thompson *et al.* 1994) with the gap-gap length penalties set to 10–5. The computer-generated alignments of the two rRNA genes were then adjusted manually based on published secondary structure models of the rRNA products (e.g. Guttell *et al.* 1993). Stem regions where only one strand was initially aligned were adjusted so that both strands were aligned, as were other conserved regions. Between these anchor points, the bases (usually loop regions) were realigned using CLUSTAL X and the original penalties. The protein coding genes (COI, H3) did not require manual intervention to produce an acceptable alignment.

Model-based phylogenetic analysis using Bayesian inference was carried out using MrBAYES, v. 2.01 (Huelsenbeck & Ronquist 2001). Each Markov chain was started from a random tree and run for  $10^6$  generations, sampling every hundredth generation from the chain. Each run comprised four (1 + 3; temp parameter = 0.2) differently heated chains. To check that the stationary phase was reached we monitored the log likelihood values of the trees in the chain, and the analysis was done in five replicates. The first  $3 \times 10^5$  generations were subsequently discarded as burn-in. The default values for priors were used: uniform priors for rate matrix (0–100), branch lengths (0–10), shape parameter for gamma distribution (0–10) and proportion of invariable sites (0–1), whereas the base frequency prior was assumed to have a Dirichlet distribution, and an uninformative prior was used for topology.

The adequate models for Bayesian inference were determined using a hierarchical likelihood ratio test ( $\eta$ LRT) approach (Huelsenbeck & Crandall 1997). We used the same test hierarchy as that implemented in the program MODELTEST (Posada & Crandall 1998) together with PAUP at  $p < 0.01$ .

Phylogenetic analyses with parsimony as the optimality criterion were done using PAUP 4.0 $\beta$ 8–10 (Swofford 2000) to enable comparisons with a method that does not use an explicit model. A heuristic search strategy (tree bisection and reconnection (TBR); random addition, 50 replicates; simple addition during bootstrap) was used with gaps treated as missing data (we had no good basis for converting them into characters). Bootstrapping (Felsenstein 1985) with 1000 replicates was used to assess sample variation and degree of support (or signal in relation to conflicting signal) in the datasets for specific clades.

Aligned sequences together with information on deleted regions and obtained trees have been deposited in TREEBASE (Sanderson *et al.* 1994; Morell 1996) with the accession number S837.

**3. RESULTS**

The data subsets, after excluding sites that could not be aligned reliably (28S and 16S sequences only), comprised 1332 bp (28S), 436 bp (16S), 666 bp (COI) and 332 bp (H3), respectively. All partitions (as well as the combined data) required modelling of rate heterogeneity, and the most adequate models according to the LRT indicated a combination of invariant sites and a gamma distribution. The 28S and COI partitions both had the general time-reversible (GTR) model as the most adequate, whereas the Tamura–Nei and the transversion (TVM) models would have been sufficient for H3 and 16S, respectively. The combined dataset had GTR as the most adequate

Table 2. Model parameter estimates from Bayesian analysis.

rates	base frequencies		rate heterogeneity		
$r_{ac}$	1.0	$\pi_A$	0.25	$\alpha$	0.39
$r_{ag}$	4.2	$\pi_C$	0.17		
$r_{at}$	1.7	$\pi_G$	0.26	SS <sub>16S</sub>	0.97
$r_{cg}$	1.8	$\pi_T$	0.33	SS <sub>28S</sub>	0.51
$r_{ct}$	5.0			SS <sub>COI</sub>	2.24
$r_{gt}$	1.0			SS <sub>H3</sub>	0.61

model. MrBAYES does not allow the Tamura–Nei and TVM submodels, and hence the GTR (as the simplest well-fitting model) was used for all partitions as well as for the combined analysis. The values for the model parameters as estimated by the Bayesian analysis are shown in table 2.

The tree in figure 1 is the summary (majority rule consensus) tree from the Bayesian analysis of the combined datasets using the GTR model with site-specific rates (i.e. each partition has its own rate) and a rate heterogeneity modelled by a (common) gamma distribution. The result of the parsimony bootstrap analysis is topologically highly congruent with the Bayesian analysis, and the bootstrap values are additionally shown on the tree in figure 1.

Nemertea is monophyletic with the posterior probability 1.0 and with a parsimony bootstrap of 98%. Enopla, Heteronemertea and Polystilifera form highly supported clades (posterior probability/parsimony bootstrap = 1.0/100%), as does Monostilifera if Bdellonemertea is considered a subtaxon (1.0/93%). *Malacobdella* (Bdellonemertea) is nested firmly within Monostilifera and appears to be the sister taxon to *Pantionemertes* sp. 115 (1.0/87%), although this specific position may be the result of inadequate taxon sampling. Within Monostilifera there are two major clades corresponding to Tetrastemmatidae (1.0/100%) and Amphiporidae + Emplectonematidae + Ototyphlonemertidae + Proserhochmididae including *Malacobdella* (1.0/64%). *Carcinonemertes* is a sister group to these two major clades, although with insignificant support. It is worth noting that *Ototyphlonemertes* forms a highly supported (1.0/100%) clade with *Poseidonemertes*, and that this clade in turn is the sister taxon to *Zygonemertes* with high posterior probability (1.0), albeit low bootstrap support (55%). Crateneimertidae (*Nipponnemertes bimaculatus* + *N. punctatulus*, 1.0/100%) is the sister group to the clade comprising all other monostiliferans + *Malacobdella* (1.0/97%).

Within Polystilifera, the reptants are not monophyletic in the Bayesian analysis, with reptant species 481 being closer to the pelagics (insignificant support, 0.86). The parsimony analysis, however, renders Reptantia monophyletic with 81% bootstrap support. Pelagica is monophyletic (1.0/98%) in both analyses.

Within Heteronemertea, *Riserius pugetensis* is the sister to the remaining heteronemerteans (Lineidae). The high support for this (1.0) in the Bayesian analysis is lost in the parsimony analysis (57%). Furthermore, it is noted that *Hubrechtella* is a sister taxon to Heteronemertea *sensu stricto* with a posterior probability of 1.0 for the clade, but with no parsimony bootstrap support. The parsimony analysis places *Hubrechtella* together with *Carinoma*, but

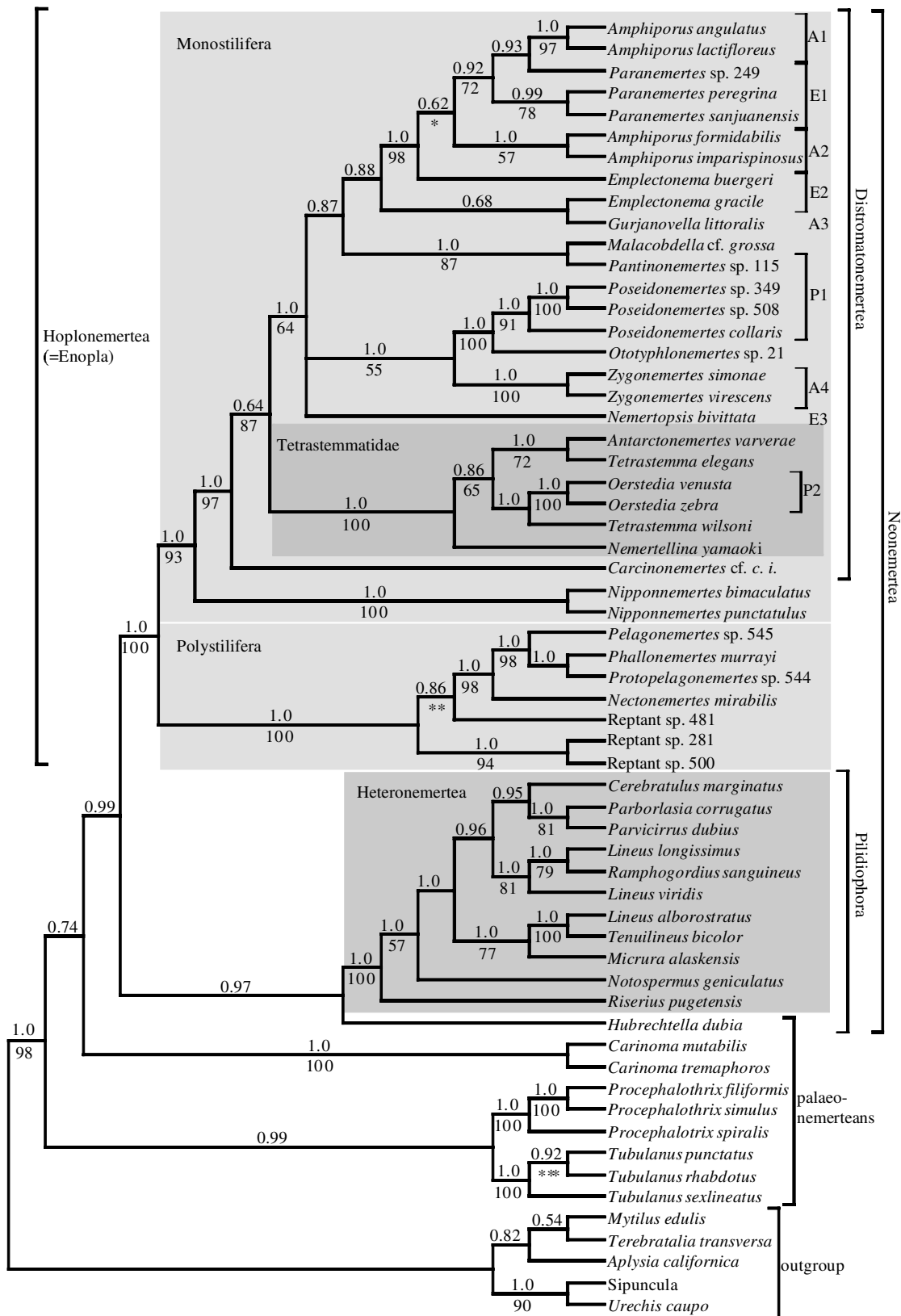


Figure 1. Summary (majority rule consensus) tree for the Bayesian analysis, using the GTR model with site-specific + gamma rates. Numbers above branches are posterior probabilities; numbers below branches are bootstrap percentages from a parsimony analysis. Asterisks indicate bootstrap support for clades (\* 50–70%, \*\* 71–90%, \*\*\* > 90%) incompatible with the marked clades in the Bayesian tree; none of these clades in the Bayesian tree has a significant support (posterior probability  $\geq 0.95$ ), however, and incongruence might simply be the result of insufficient character sampling. Clades indicated by A and a number are taxa hitherto placed in Amphiporidae; clades with E and a number are taxa hitherto in Emplectonematidae; and taxa in clades with P and a number were assigned to Prosorhochmididae. In this paper we introduce three new names (written vertically): Neonemertea, Piliophora and Distromatonemertea.

with no bootstrap support. The genus *Lineus* does not form a monophyletic group.

The palaeonemerteans, even with *Hubrechtella* excluded, do not form a monophyletic group, but are found basally on the tree as paraphyletic to the remaining nemerteans. The Bayesian analysis yields a clade corresponding to the monophyletic groups Tubulanidae and Cephalothricidae (both 1.0/100%) with a posterior probability of 0.99. In the parsimony bootstrap analysis, however, there is no support for this, but the four palaeonemertean families (Tubulanidae, Cephalothricidae, Carinomidae, Hubrechtidae) form a hexachotomy with Heteronemertea and Enopla.

A  $\chi^2$  test indicated that there are significant differences in base frequencies between different taxa, and thus the GTR model and parsimony may give misleading results. A minimum-evolution analysis using LOG<sub>DET</sub> distances, however, gave essentially the same result as the Bayesian analyses (tree not shown), and an analysis using a non-reversible (12-parameter) model in a Bayesian analysis gave a congruent result (tree not shown). The only difference is that the clade with *Carinoma mutabilis* and *C. tremaphoros*, which for the GTR model is the sister group to Heteronemertea + Hoplonemertea (with the low probability of 0.74), is the sister group to the Cephalothricidae + Tubulanidae with a negligible 0.56 posterior probability under the non-reversible model.

Analysing the genes separately markedly reduces the number of clades with significant posterior probabilities, with 28S and 16S having the most and H3 the fewest. There are no obviously incongruent clades with significant posterior probabilities, with one exception. For the 16S gene, Monostilifera is not monophyletic: Cratenemertidae is a sister taxon to Polystilifera with Tetrastemmatidae as a sister to the entire clade. This group has a posterior probability of 0.98, whereas the remaining monostiliferan clade has a posterior probability of 0.95. However, this incongruence is highly affected by the 16S sequence of *Hubrechtella dubia* (found in a basal trichotomy with Hoplonemertea and Heteronemertea), and when *Hubrechtella* is excluded from the analysis the resulting phylogeny is congruent with the other genes and with the combined analysis.

#### 4. DISCUSSION

The nemertean classification has been relatively stable since Stiasny-Wijnhoff (1923), although some new revisions, mostly at lower levels, have been published during the last 20 years. There has been an implicit consensus about the monophyly of Heteronemertea and Hoplonemertea. The third large group, Palaeonemertea, has been regarded as a basal group and the font of nemertean diversification since Hubrecht (1879), and hence is implicitly paraphyletic. Our analysis confirms this consensus: Heteronemertea and Hoplonemertea are monophyletic with good support, the latter, however, only with the provision that the commensal *Malacobdella*, hitherto treated as a taxon separate from Hoplonemertea, is included. Furthermore, our study indicates that Palaeonemertea is non-monophyletic.

Sundberg *et al.* (2001) presented a phylogenetic study of Nemertea based on the 18S rRNA gene and 15 species;

only three of their species were included in the present study. In terms of the general pattern, their study is completely congruent with ours in supporting a monophyletic Heteronemertea and a monophyletic Hoplonemertea, including Bdellonemertea, with no support for monophyly of Palaeonemertea. It is worth noting that *Malacobdella* was found together with a prosorhochmid monostilifer (*Prosorhochmus* sp., with 61% bootstrap support) by Sundberg *et al.* (2001). In the present study, it is also found with a prosorhochmid, albeit another genus and species (*Pantinonemertes* sp.).

#### (a) *Hoplonemertea*

Brinkmann (1917) divided Hoplonemertea into Monostilifera and Polystilifera based on the armament of the proboscis. He further subdivided Polystilifera into Reptantia and Pelagica. This classification was challenged by Gibson (1988) who transferred the monostiliferan family Cratenemertidae to the taxon Paramonostilifera and considered it as a sister group to the Polystilifera *sensu stricto*, comprising the reptants. This group in turn was the sister group to Pelagica. Sundberg (1990) rejected this in an analysis of morphological data, and placed Cratenemertidae as the sister group to the remaining Monostilifera. This is the exact position found in the present study with high posterior probability, thereby affirming rejection of the taxon Paramonostilifera.

Polystilifera, however, was not monophyletic in Sundberg's (1990) analysis, rather Pelagica was a sister group to a clade with Monostilifera and the reptants (which were paraphyletic). Stiasny-Wijnhoff (1936) divided the Reptantia Eureptantia into Aequifurcata and Inaequifurcata based on several implicit synapomorphies. However, cladistic analyses by Härlin & Sundberg (1995) and Härlin & Härlin (2001) indicate that neither subgroup is monophyletic. Our results for reptants are consistent with the relatively 'unbalanced' trees for reptants presented by Härlin & Härlin (2001). In the present study there is very strong support for a monophyletic Polystilifera as a sister group to Monostilifera. This has important bearings on the interpretation of the evolution of proboscis armament: in Sundberg's (1990) phylogeny, the plesiomorphic state is many stylets, whereas in our phylogeny either one or many stylets can be plesiomorphic. However, the taxonomic sampling of Reptantia and Pelagica needs to be extended before a non-monophyletic Polystilifera can be ruled out.

We also note that Reptantia is not monophyletic in the Bayesian analysis, but it is in the parsimony analysis. The posterior probability for the offending species (sp. 481), placed together with Pelagica in the Bayesian analysis, is very low (0.86), whereas the bootstrap support for a monophyletic Reptantia in the parsimony analysis is moderate (81%). Thus, we treat this incongruence as the result of insufficient sampling for the time being, and see no reason to reject the hypothesis of a monophyletic Reptantia.

#### (b) *Monostilifera*

The monostiliferous hoplonemerteans are monophyletic, and there are two well-supported clades roughly corresponding to Amphiporidae and Tetrastemmatidae, respectively, but overall our results challenge the monophyly of the taxon-rich traditional families Amphiporidae,

Emplectonematidae and Tetrastemmatidae, as well as the more circumscribed Prosohochmididae (based on the position of *Oerstedtia*). The pairing of *Amphiporus formidabilis* and *A. imparispinosus*, with high posterior probability but low bootstrap support, is notable in that these two sympatric forms are notoriously difficult to distinguish. They differ here by 10% and 13% for 16S and COI, respectively.

The strong support for the clade *Poseidonemertes* + *Ototyphlonemertes* is intriguing, as members of both groups are recognized as morphologically specialized and conspicuously set apart from other monostiliferans by habitat. The recognized *Poseidonemertes* are relatively large-bodied very muscular worms with a pointed head that burrow actively through sandy habitats (unlike *Paranemertes peregrina*, which appears to occupy relatively static burrows or crawl through soft surface mud). By contrast, *Ototyphlonemertes* are among the thinnest nemerteans and occupy almost exclusively relatively coarse sediments where they are able to penetrate the aqueous pore space without burrowing.

It is also worth noting here that although the two *Malacobdella* cf. *grossa* individuals that were used in this study (table 1) are from two geographically widely separated host species these specimens were identical for the common segments that were sequenced. This begins to address Kozloff's (1991) reasonable concern about host specificity and scepticism about the reported wide geographical range of *M. grossa*.

#### (c) *Palaeonemerteans*

The prevalent notion that palaeonemerteans are a basal group in Nemertea was contested by Sundberg & Hylbom (1994). In their analysis to find an outgroup to Palaeonemertea, they found Heteronemertea to be this outgroup and Hoplonemertea to be their basal sister group. This is probably the result of too few characters and the use of the turbellarian *Haplopharynx rostratus* as the single outgroup, together with some miscoding in the matrix. Our analysis moves the root, and the palaeonemerteans are found basally among the nemerteans. Sundberg & Hylbom (1994), furthermore, assumed monophyly of Palaeonemertea. This has been (implicitly) contested for a long time; for example an interpretation of Bürger's (1895) view places *Hubrechtia* as a sister group to Heteronemertea, *Cephalothrix* and *Carinoma* as a sister group to Hoplonemertea, and *Carinina* as a sister group to all other nemerteans. Subsequent authors (e.g. Friedrich 1935; Hylbom 1957; Iwata 1960) have moved parts of the Palaeonemertea around, but have essentially maintained the closer relationship between some palaeonemerteans and other nemertean taxa (i.e. non-monophyly).

The present study also indicates that Palaeonemertea is non-monophyletic. The Bayesian analysis shows a significant probability for *Hubrechtella dubia* as the sister taxon to Heteronemertea *sensu stricto*. This was suggested by Norenburg (1985, 1988) because *Hubrechtella* has true pilidium larvae (Cantell 1969), otherwise a unique feature of Heteronemertea, and others have considered other members of Hubrechtidae as close relatives to Heteronemertea (e.g. Bürger 1895; Friedrich 1935).

Iwata (1960) proposed a split of the palaeonemerteans into Archinemertea, comprising Cephalothricidae, on the

one hand and Palaeonemertea *sensu stricto* on the other, where he considered the former to be the ancestor of all other Nemertea. This was questioned by Sundberg & Hylbom (1994), who did not find any morphological support for Iwata's hypothesis. Sundberg *et al.* (2001) found two clades, corresponding to Archinemertea and Palaeonemertea *sensu stricto* in their parsimony analysis, although without bootstrap support. In the present study, we, respectively, found a clade comprising Cephalothricidae and Tubulanidae, and hence we must refute Archinemertea. Furthermore, even though Carinomidae is the sister group to Hoplonemertea + Heteronemertea + *Hubrechtella*, this has no significant probability and—with the exclusion of *Hubrechtella*—the monophyly of Palaeonemertea still cannot be firmly rejected. The present results argue strongly that a well-developed cerebral sensory organ, a structure apparently unique to nemerteans, was present in the ancestor of the analysed clades, unless one wishes to argue for two origins and a remarkable degree of convergence.

#### (d) *Heteronemertea*

With the exception of *Riserius pugetensis*, all heteronemerteans in the study are classified in the family Lineidae. The type genus of this family, *Lineus*, is one of the four 'mega-genera', and some recent taxonomic revisions have been presented, mainly by Riser (1991, 1993, 1994), but have not found practical acceptance yet. In our analysis *Lineus* is, however, still non-monophyletic even with the application of Riser's new genera. Riser (1994) diagnosed the new genus *Myoisophagus*, later (Riser 1998) recognized as a junior synonym of *Ramphogordius*. Riser (1994) furthermore concluded that *Lineus viridis* should not be part of *Lineus*, although he did not place it formally in any other genus. In our analysis *Ramphogordius sanguineus* is the sister taxon to *L. longissimus* (type species of *Lineus*), and *L. viridis* is their sister taxon. We also note that *L. alborostratus* forms a clade with *Tenuilineus bicolor*, and thus is better considered as part of *Tenuilineus* (Riser 1993), and that the erection of *Notospermus* (Riser 1991) for *L. geniculatus* seems justified. Considering the different taxon sampling, this is in agreement with the results of Sundberg & Saur (1998) and a strong indication that the genus *Lineus sensu lato* is in need of extensive phylogenetic revision.

#### (e) *Taxonomic implications*

Bdellonemertea, represented here by *Malacobdella* cf. *grossa*, has traditionally been grouped together with, and with the same rank as, Hoplonemertea in the taxon Enopla; a notable exception is a lengthy treatise by Senz (1997) in which he considers it to be 'a specialized offshoot from the nemertean stock'. Our analysis shows unequivocally that Bdellonemertea is a specialized monostiliferous hoplonemertean group; they are commensal and many of their unique features can probably be attributed to lifestyle. This position has already been indicated by Friedrich (1936), who argued that Bdellonemertea was derived from a monostiliferous hoplonemertean, even though he treated it as a taxon of the same rank as Hoplonemertea. Thus, the taxon Bdellonemertea should be abandoned and Malacobdellidae treated as a group in Monostilifera pending further analyses of Monostilifera. This also means that Enopla and Hoplonemertea become

synonymous. Although Enopla was introduced by Schultze (1851) and is the older name, we prefer to keep Hoplonemertea (Hubrecht 1879) as the name for this clade.

There are a few clades that currently lack formal names, but where names are warranted. We here name these clades in a phylogenetic framework as outlined by de Queiroz & Gauthier (1990, 1992) and others (e.g. Schander & Tholleson 1995), an approach that has already been applied within Nemertea (to Reptantia by Härlin & Sundberg 1995; Härlin & Härlin 2001).

The evident morphological discontinuity between cratenemertids and the remaining monostiliferans (reviewed by Crandall 1993) is well supported in the present study. We believe that effective communication is served by providing a name to the sister clade of cratenemertids, which we propose renaming as Cratenemertea, while applying the name Distromatonemertea (after the ill-fated Distromatorhynchocoelomia of Gibson (1988), which has roughly the same composition) to its sister clade. Formally, Distromatonemertea refers to the most inclusive clade comprising all monostiliferous nemertea except Cratenemertea.

Even though the relationships between all palaeonemertean taxa are not yet resolved, we think that the clade comprising Hoplonemertea and Heteronemertea + former Palaeonemertea with pilidium larvae (represented here by *Hubrechtella*) is such a divergence from past nomenclature and tree-thinking that it warrants a new name. Thus, we propose Neonemertea (Gr. 'neo', new) to refer to the least inclusive clade comprising Hoplonemertea and Heteronemertea.

Finally, the clade comprising Heteronemertea and *Hubrechtella* (and its relatives) also needs a name. One option would be simply to include the *Hubrechtella* clade in Heteronemertea. However, as Heteronemertea in its present use refers to a monophyletic group, we prefer to keep the name referring to a clade with the same content. The Heteronemertea + *Hubrechtella* clade has a unique feature in the pilidium larva, and we thus propose the name Pilidiophora (Gr. 'pilios', cap; 'phora', bearing) to refer to the most inclusive clade comprising Heteronemertea and *Hubrechtella* but not Hoplonemertea or *Carinoma*.

Several people have contributed specimens and material help to this study; in particular the help of Alexei Chernyshev, Tomas Dahlgren, Joan Ferraris, Hiroshi Kajihara, Svetlana Maslakova, Nathan Riser, Pamela Roe, Alex Rogers, Cynthia Santos, Megan Schwartz, Jeffrey Shields, Stephen Stricker, Shichun Sun and Martin Thiel is gratefully acknowledged, as is Joan Ferraris for significant support and advice in the molecular work. Computational resources were provided by Hygd Beowulf cluster at the Linnaeus Centre of Bioinformatics, and two anonymous reviewers gave valuable comments on the manuscript. This work was supported in large part by a Smithsonian Institution Scholarly Studies Award. Significant parts of the collecting were supported by the National Science Foundation under PEET grant no. DEB 9712463. This is contribution 537 from the Smithsonian Marine Station at Fort Pierce and contribution 644 from the Caribbean Coral Reef Ecosystems programme. We are grateful for financial support and staff assistance provided by these entities, as well as by the staffs of the Smithsonian Institution Laboratory for Molecular Systematics, the University of the Ryukus Sesoko Marine Science Centre, the Kyoto University Seto Marine Laboratory

and the Hokkaido University Oshoro and Akkeshi Marine Laboratories.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.