

Multi-Gene Analyses of the Phylogenetic Relationships among the Mollusca, Annelida, and Arthropoda

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Donald J. Colgan, Patricia A. Hutchings, and Emma Beacham (2008) Multi-gene analyses of the phylogenetic relationships among the Mollusca, Annelida, and Arthropoda. *Zoological Studies* 47(3): 338-351. The current understanding of metazoan relationships is largely based on analyses of 18S ribosomal RNA ('18S rRNA'). In this paper, DNA sequence data from 2 segments of 28S rRNA, cytochrome *c* oxidase subunit I, histone H3, and U2 small nuclear (sn)RNA were compiled and used to test phylogenetic relationships among the Mollusca, Annelida, and Arthropoda. The 18S rRNA data were included in the compilations for comparison. The analyses were especially directed at testing the implication of the Eutrochozoan hypothesis that the Annelida and Mollusca are more closely related than are the Annelida and Arthropoda and at determining whether, in contrast to analyses using only 18S rRNA, the addition of data from other genes would reveal these phyla to be monophyletic. New data and available sequences were compiled for up to 49 molluscs, 33 annelids, 22 arthropods, and 27 taxa from 15 other metazoan phyla. The Porifera, Ctenophora, and Cnidaria were used as the outgroup. The Annelida, Mollusca, Entoprocta, Phoronida, Nemertea, Brachiopoda, and Sipuncula (i.e., all studied Lophotrochozoa except for the Bryozoa) formed a monophyletic clade with maximum likelihood bootstrap support of 81% and a Bayesian posterior probability of 0.66 when all data were analyzed. The clade was also formed (including 1 arthropod, a symphylian) when only genes other than 18S rRNA were analyzed. Two molluscan genera with long branch lengths (*Nautilus* and *Philippia*) were removed from the Lophotrochozoa in the maximum-parsimony analyses of all data. The Ecdysozoa (comprised of the Kinorhyncha, Priapulida, Nematoda, Onychophora, Tardigrada, and Arthropoda) was included in a clade with the Chaetognatha (with maximum-likelihood support of 80% and a Bayesian probability of 0.57) using the total data. This clade except the symphylian had a Bayesian probability of 0.66 when 18S rDNA data were excluded. The reciprocal separation of the Annelida and Mollusca was generally supported where this could be resolved. The monophyly of the Annelida was contradicted only by the inclusion of the Sipuncula and Brachiopoda and the exclusion of *Owenia*. Molluscan monophyly was contradicted by the anomalous placement of *Nautilus* and/or *Philippia*, but these taxa were never placed in the Annelida. <http://zoolstud.sinica.edu.tw/Journals/47.3/338.pdf>

Key words: Phylogenetics, Lophotrochozoa, Eutrochozoa, Ecdysozoa, Basal Annelida.

The widespread use of 18S ribosomal RNA ("18S rRNA") data in molecular phylogenetics has presented major challenges to prevailing views of evolutionary relationships among metazoan phyla. The Arthropoda and Annelida have traditionally been regarded as being closely related based on morphological data (Willmer 1990, Wägele et al. 1999, Wägele and Misof 2001, Scholtz 2002, Nielsen 2003). Although this view

is not universally held (Ghiselin 1988, Eernisse et al. 1992, Eernisse 1997), it is widely reported in invertebrate textbooks (e.g., Brusca and Brusca 2003). Analyses of 18S rRNA data suggest 3 major changes. First, the Annelida is more closely related to the Mollusca and some other minor phyla (the Sipuncula, Nemertea, and Echiura) than to the Arthropoda. The group containing these phyla was named the Eutrochozoa (Ghiselin 1988). Second,

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the Eutrochozoa together with lophophorate phyla (Phoronida, Brachiopoda, and Ectoprocta) and the Entoprocta form a group that Halanych et al. (1995) named the Lophotrochozoa. Third, the Arthropoda forms part of a clade of molting animals including the Nematoda, Nematomorpha, Kinorhyncha, Priapulida, Gastrotricha, and Loricifera. This was proposed by Aguinaldo et al. (1997) who named it the Ecdysozoa.

Although these suggestions have been widely adopted, further study is required in some areas. As reviewed below in the "Introduction" and "Discussion", evidence for the suggestions from other molecular and morphological data is supportive, but cannot by itself be considered conclusive. Second, 18S rRNA analyses do not indicate reciprocal monophylies of the major eutrochozoan phyla. In studies including substantial numbers of both the Annelida and Mollusca, neither has been recovered as a monophyletic group (Giribet and Ribera 1998, Halanych 1998, Winnepenninckx et al. 1998, Passamanek and Halanych 2006). In the largest compilations of 18S rRNA data, the Annelida and Mollusca broadly intermingle (Giribet et al. 2000, Peterson and Eernisse 2001).

Evidence from single genes other than 18S rRNA in favor of the respective monophyly of the Eutrochozoa, Lophotrochozoa, and Ecdysozoa mostly comes from 28S rRNA. Mallat and Winchell (2002) included data comprising 2348 aligned bases of this gene from 9 lophotrochozoans and 5 ecdysozoans. Their analyses supported the monophyly of the molting clade if the (non-molting) arrow-worm *Sagitta* (Chaetognatha) was excluded. Within the Lophotrochozoa, 2 annelids and an echiuran were shown as the sister group to a clade comprising a mollusc, a phoronid, and a brachiopod. Passamanek and Halanych (2006) analyzed 2370 aligned bases from 6 molluscan, 6 annelidan, and 3 ecdysozoan taxa and found that the Ecdysozoa, Mollusca, and Annelida were respectively monophyletic, with the latter 2 phyla in a clade exclusive of the former.

A study of myosin II by Ruiz-Trillo et al. (2002) found support for the Ecdysozoa (with Deuterostomia as its sister group) and Lophotrochozoa (with 17 taxa included) including the Annelida (4 taxa) as a sister group to the Mollusca (3 taxa). The Mollusca and Deuterostomia were well supported, but support for the other taxa was low. Re-analysis of an extended dataset "basically extracted" from the data of Ruiz-Trillo et al. (2002) by Giribet (2003) using direct

optimization showed support for the ecdysozoan hypothesis only under some parameter settings. Often, nematodes were placed basally in the Metazoa. Lophotrochozoans were poorly resolved into phyla and were also paraphyletic with respect to a group containing arthropods, priapulids, and chordates (Giribet 2003). The investigation of the sodium-potassium ATPase α subunit by Anderson et al. (2004) included no annelids. Sequences from this gene have subsequently been collected for a single annelid, *Hirudo medicinalis* (Kusche et al. 2005), that was found to be the sister group to the 2 flatworms rather than the 7 ecdysozoan taxa in that study.

Three multigene studies of metazoan relationships including multiple eutrochozoan and ecdysozoan taxa are available. Mallat and Winchell (2002) and Passamanek and Halanych (2006) included 18S rRNA and 28S rRNA, while Giribet (2003) included 18S rRNA, myosin II, histone H3, and elongation factor I alpha. If the Chaetognatha was excluded, the Ecdysozoa and Lophotrochozoa were both robustly supported in Mallat and Winchell's analysis (2002). The Ecdysozoa and Lophotrochozoa were both monophyletic in the maximum-likelihood and Bayesian analyses (with high support) of Passamanek and Halanych (2006) when trees were rooted on the deuterostomes. Giribet's analyses (2003) do not support the monophyly of the Lophotrochozoa or Eutrochozoa. Assuming equal costs for gap extensions, transitions, and transversions, the Mollusca plus Phoronida was the sister group to a clade containing 2 main lineages, the Ecdysozoa and a combination of the Nemertea, Sipuncula, Annelida, and Chordata. With transversion costs set to twice that of other changes, the Nematoda became basal in the Metazoa, and the Arthropoda was recovered as the sister group of a clade divided into a group containing the Nemertea, Platyhelminthes, and Mollusca, and one containing the Annelida, Phoronida, and Chordata.

A number of studies based on large numbers of genes but small taxon numbers and not including both annelid and molluscan taxa have tested implications of the Ecdysozoa hypothesis. Some, e.g., Stuart and Barry (2004) and Dopazo and Dopazo (2005), support the hypothesis while others reject it (Blair et al. 2002, Philip et al. 2005). Philippe et al. (2005) include 1 composite representative "annelid" and "mollusc" in their compiled dataset comprised of 146 genes and 49 taxa. The Ecdysozoa could be recovered

in analyses of this set if only “slowly evolving” genes were included, some taxa were omitted, and the Cnidaria was used as the sole outgroup. Otherwise, the Platyhelminthes and Nematoda were shown as sister taxa.

The investigation reported here was conducted to test whether genes other than 18S rRNA support a closer relationship between the Annelida and Mollusca than between the Annelida and Arthropoda and whether monophyletic Annelida and Mollusca can be recovered by the addition of data from other DNA sequences to 18S rRNA data. In discussing the Annelida, we follow recent opinions that the Echiura (McHugh 1997, Purschke et al. 2000, Hessling and Westheide 2002, Hessling 2003) and Pogonophora (Bartolomaeus 1995 1998, McHugh 1997, Halanych 2005) are members of this phylum. Data were compiled for two 28S rRNA segments and 3 other genes: cytochrome *c* oxidase subunit I, histone H3, and U2 small nuclear RNA. To allow comparison with 18S rDNA analyses, data from this gene were also included in many analyses. The data collected specifically for this project were added to those available from intra-phylum studies from this laboratory (Colgan et al. 1998 2000 2001 2003a b 2006 2007, Brown et al. 1999, Hall et al. 2004). Further relevant taxa were added using GenBank data. Taxa were generally included if data were available for at least 3 of the 5 gene segments other than 18S rRNA. To increase the range of these organisms, exceptions were made for 1 insect, 2 nematodes, and a poriferan, each with 2 segments plus 18S rRNA.

The data were also used to investigate a particularly difficult question in annelid phylogeny: the identification of the correct placement of the root. This has been a major problem in annelid phylogenetics (Rouse and Pleijel 2001 2003). Use of 12 molluscan taxa as outgroups previously identified the Chaetopeteridae, Amphinomidae, and Oweniidae as basal taxa with the Siboglinidae and Echiura derived (Colgan et al. 2006). Use of 5 Mollusca, 1 Brachiopoda, 3 Arthropoda, 2 Nemertea, and 3 Sipuncula as outgroups to the largest dataset yet assembled to investigate annelid phylogeny could recover neither ingroup nor outgroup monophyly (Rousset et al. 2007). When for illustration, the symphyllan genus, *Hanseniella*, was used as the outgroup, the Mollusca was shown to consist of various dispersed and derived clades in the maximum-parsimony analysis (Rousset et al. 2007). Colgan et al. (2006) and Rousset et al. (2007) both used

a relatively small number of outgroups. In the present case, there are effectively a large number of species-level outgroups for each phylum. Potentially, this should allow better estimation of character states in the phylum stem groups and a possible improvement in the placement of the root.

MATERIALS AND METHODS

Experimental methods

Polymerase chain reactions (PCRs) were performed on DNA prepared for previous studies that had been frozen at -70°C or stored in 70%-100% ethanol. The PCR was conducted according to the procedures in Colgan et al. (1998 2000) and Brown et al. (1999). DNA concentrations, annealing temperatures and times, and/or the MgCl₂ concentration were varied to obtain PCR products suitable for sequencing.

The abbreviations for the amplified gene segments used here are 28S D1 for the D1 expansion region of the 28S rRNA gene, 28S VIX for the region of 28S rRNA amplified using the VI and X primers of Hillis and Dixon (1991), COI for cytochrome *c* oxidase subunit I, H3 for histone H3, and U2 for the U2 snRNA, while 18S rRNA was not further abbreviated. Primers used for new data collection are listed in table 1. Generally, all sequence data between these primers were used in the dataset. However, for COI only bases in the overlap of the product amplified by Folmer et al.'s (1994) “universal” primers and arthropod COI sequences collected by G. Thampapillai (unpubl. data) were included. Sequencing methods generally followed Colgan et al. (2006), using AMPURE magnetic beads (Agencourt, Beverly, MA, USA) to purify the PCR products. Purified products other than 18S rRNA were sequenced in both directions with an ABI® 310 DNA automatic capillary sequencer (Applied Biosystems, Foster City CA, USA) using the DyeDeoxy™ Terminator sequencing method (Big Dye™, vers. 1.0 or 2.0) with the amount of Big Dye generally reduced to 2 µl. Consensus sequences for each taxon were generated using Sequence Navigator (Applied Biosystems 1994) or Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, MI, USA). For new 18S rRNA data, sequences were collected using ET (General Electric (GE), Piscataway, NJ, USA) sequencing chemistry according to the manufacturer's protocols except that sequencing buffer (1 M Tris-HCl and 1 M MgCl₂; pH 9.0) (4 µl)

was used and the amount of ET was reduced to 4 μ l in a final reaction of 20 μ l. Sequencing reactions were cleaned by ethanol precipitation and run on a GE MEGABACE automatic capillary sequencer.

Sequences analyzed

Accession numbers for the new sequences collected for this paper are included in the electronic supplementary material. Additional published sequence data from GenBank from this or other laboratories are also indicated and referenced in this table. The alignment is available (as accession S1953) in Treebase (<http://www.treebase.org/treebase/index.html>).

Phylogenetic analyses

Sequences were aligned using the default values for parameters in CLUSTAL X (Thompson et al. 1997). MacCLADE (Maddison and Maddison 1992) was used for data manipulations such as joining files for individual genes and specifying character sets. The conventions were adopted that (a) a comma (,) separates monophyletic groups within clades specified by parentheses; (b) a plus (+) sign indicates that the group before the sign is paraphyletic with respect to the group following

the sign; and (c) a minus (-) sign indicates that the group after the sign is missing from the clade before the sign. If the paraphyletic taxon of convention (b) anomalously includes multiple non-sister taxa, then this is described by “+ ... and ...”.

Many analyses were conducted but only the 4 most comprehensive are reported here. These are Bayesian-inference (ABY), maximum-likelihood (AML), and maximum-parsimony (AMP) of the complete data and Bayesian-inference of genes other than 18S rRNA (BYno18S). Topologies were rooted using the Porifera, Ctenophora, and Cnidaria as an outgroup. Parsimony analyses were conducted using heuristic searches in PAUP* 4.0 vers. beta 10 (Swofford 2001) with the tree-bisection-reconnection branch-swapping algorithm for 1000 replications of random stepwise addition of taxa.

Maximum-parsimony analyses of individual genes were conducted to search for possible PCR artifacts such as contamination. Contamination by other organisms used in this study was suspected if the sequences from 2 taxa that were not closely related were found to be identical. Contamination from amplification of DNA from unintended targets (e.g., parasites) was revealed by taxa with very long branch lengths or unexpected placement in the single gene analyses. Individual BLAST

Table 1. Primers used to collect new sequences for this investigation. Positions of the 28S rDNA primers are given for the 3' end of the oligonucleotide in the GenBank mouse 28S rDNA sequence (X00525) (Hassouna et al. 1984). The most frequent annealing temperature is indicated, together with the range used for collecting the new data

Gene	Primer	Sequence	Annealing temperature	Reference
Cytochrome c oxidase	COI1490F	GGTCAACAAATCATAAAGATATTGG	45° (40°-52°)	Folmer et al. 1994
	COI2198R	TAAACTTCAGGGTGACCAAAAAATCA		
Histone H3	H3F	ATGGCTCGTACCAAGCAGACVGC	50° (48°-53°)	Colgan et al. 1998
	H3R	ATATCCTTRGGCATRATRGTGAC		
U2 snRNA	U2F	TCT CGGCCT (AT)(AT) T GGC TAA	50° (48°-53°)	Colgan et al. 1998
	U2R	G(AC)G GTA (GC)TG CAA TAC CGG		
28S rRNA	28s D1F	ACCCSCTGAAYTTAAGCAT (43)	50° (47°-50°)	McArthur and Koop 1999
	28s D1R	AACTCTCTCMTTCARAGTTC (406)		
	28S VI	AAGGTAGCCAAATGCCTCATC (2565)	54°	Hillis and Dixon 1991
	28S X	GTGAATTCTGCTTCATCAATGTAGGAAGAGCC(3161)		Hillis and Dixon 1991
18S rRNA	1F	TACCTGGTTGATCCTGCCAGTAG	49°	Giribet et al. 1996
	4r	GAATTACCGCGGCTGCTGG		Giribet et al. 1996
	18Sbi	GAGTCTCGTTCGTTATCGGA	49°	Whiting et al. 1997
	3F	GTTTCGATCCGGAGAGGGA		Giribet et al. 1996
	a2.0	ATGGTTGCAAAGCTGAAAC	49°	Whiting et al. 1997
	9r	GATCCTCCGAGGTTACCTAC		Giribet et al. 1996

searches of GenBank were made for sequences falling into this category in order to test whether they were truly derived from the phylum of their supposed source. Most such sequences were most similar to related taxa in GenBank. The few anomalous sequences that were discovered were all revealed by long branch lengths, and BLAST searches identified them as non-target taxa, principally algae. A 28S rRNA DVIX sequence prepared in this laboratory, supposedly *Amphicteis dalmatica*, was identified as a contaminant by this procedure and removed from GenBank. Any sequences suspected of doubtful provenance following such searches were removed from the data.

For maximum parsimony of the complete data set, all characters were unordered and unweighted. The steepest descent option was not enforced. Zero-length branches were collapsed to give polytomies. Gaps were treated as unknowns. For bootstrap pseudo-sampling, heuristic searches were conducted for 1000 bootstrap replicates, each with 20 random-addition iterations.

The AML analyses were conducted with RaxML vers. 2.2.3 (Stamatakis, 2006) using the RaxML “black box” at <http://phylobench.vital-it.ch/raxml-bb/> assuming a partitioned model with an independent branch length estimation for each gene segment. No sites were assumed to be invariant in this analysis. One hundred replicates were analyzed for bootstrapping.

Bayesian analyses were conducted with vers. 3.1.2 of MrBayes (Huelsenbeck and Ronquist 2001). In each Bayesian analysis, 2 simultaneous runs were conducted for 1×10^6 generations

each with 4 differentially heated, Metropolis-coupled, Monte Carlo Markov chains. Topologies were sampled every 100 generations. A discrete gamma distribution was assumed for variations in the rate of substitution between nucleotide positions in the alignment. The shape parameter of this distribution, and base frequencies and rates for the 6 substitution types were estimated during the run. Parameters were estimated separately for each gene (and each codon position within coding sequences) using a character partition and the “unlink” command in MrBayes. Multiple series of Bayesian analyses were run with very similar results, except for a notable variation in the number of generations required to reach a plateau in the plot of tree likelihoods. The reported results are from the run in which the Markov Chain converged more quickly to a stable posterior distribution as judged by the criterion that sample log likelihoods were no more than 0.2% worse than the trees at the end of the simulation. The node support values (abbreviated as PP) reported here are the posterior probabilities.

RESULTS

Numbers of various categories of bases in the combined alignment and individual segments are shown in table 2.

The majority rule consensus tree of the 8250 trees sampled after the cutoff for the more rapidly convergent of the Monte Carlo simulations of the ABY analysis is shown in figure 1. These likelihood values converged to a figure of around

Table 2. Numbers of bases in the alignment of individual segments and the combined data. The columns show, respectively, the number of bases without variation (Constant), the numbers that are variable but parsimony-uninformative (Variable), the number of parsimony-informative sites (Informative), the total number of sites (Total), and the probability for the χ^2 test of homogeneity of base composition frequencies (Probability)

Segment	Constant	Variable	Informative	Total	Probability
COI	102	83	368	553	0.0000
Histone H3	45	29	199	273	0.1139
U2 snRNA	37	21	71	129	1.0000
28S D1	82	48	252	382	0.9022
28S VIX	186	211	453	850	0.5890
18S rRNA	770	811	1291	2872	0.0000
Combined	1222	1203	2634	5059	0.0000

-138,213, so that the trees included in the majority rule consensus have a likelihood of $> -138,490$. The Chaetognatha + Bryozoa and almost all the Ecdysozoa (the Arthropoda except the symphylian *Hanseniella*, and Tardigrada, Onychophora, Kinorhyncha, Priapulida, and Nematoda) was shown to be the monophyletic sister group to all other ingroup taxa with moderate support (a PP of 0.66). The latter group corresponds to the Lophotrochozoa except for the Bryozoa and with the anomalous inclusion of *Hanseniella*. Within this group, *Owenia* (Annelida) and *Nautilus* (Mollusca) were removed from their traditional phylum. Otherwise these 2 phyla were resolved into distinct clades. The annelid clade also included the Brachiopoda and Sipuncula, and the molluscan clade was not resolved from the Phoronida.

In the majority-rule consensus tree of the AML bootstrap replicates, there was support for the monophyly of the clades (Ecdysozoa + Chaetognatha) and (Lophotrochozoa - Bryozoa) with respective bootstrap percentages of 80% and 81% (Fig. 2). There were notable clades within these 2 major groups. Within the Ecdysozoa, the Kinorhyncha and Priapulida were shown to be sister groups in all bootstrap replicates. (Chaetognatha, Onychophora) had bootstrap support of 94%. There was less resolution among the Lophotrochozoa in the consensus tree. All genera of the Annelida except *Owenia* were included in a clade with bootstrap support of 61% that also included the Brachiopoda and Sipuncula. One large group of Mollusca (the Apogastropoda sensu Ponder and Lindberg (1997) + *Austrocochlea*) showed bootstrap support of 62%, but the other members of this phylum were not resolved within the Lophotrochozoa.

The actual topology of the best tree from the AML placed the Entoprocta as the sister group to all other Lophotrochozoa except the Bryozoa, with the Nemertea as the sister group to the remaining members of the group. *Owenia* was the sole member of the next branching clade, forming the sister group of all Lophotrochozoa except the Bryozoa, Entoprocta, and Nemertea. All molluscan genera except *Nautilus* were included in a single clade which included no members of any other phylum. The Phoronida was the sister group of (Mollusca - *Nautilus*). All annelids except *Owenia* were included in a clade that also contained *Nautilus*, both brachiopods (not monophyletic), and the sipunculan.

There were 6 trees with a length of 35,001

and a consistency index (CI) of 0.213 resulting from the AMP analysis. In the strict consensus of all 6 AMP trees, (Nematoda, Bryozoa) was basal, with the deuterostomes (Echinodermata, Chordata) shown as the sister group to the remaining Ecdysozoa and Lophotrochozoa. *Nautilus* and *Philippia* (both relatively long-branched taxa) were removed from the Lophotrochozoa and included in the Arthropoda (together with the Onychophora, Tardigrada, and Chaetognatha). The Ecdysozoa was shown to be paraphyletic with respect to the remaining Lophotrochozoa as this clade was shown to be the sister group of the pair (Priapulida, Kinorhyncha). Within the Lophotrochozoa, the main molluscan clade was shown to be paraphyletic to the clade comprising (Annelida + Brachiopoda, and Sipuncula and Phoronida). Only 3 phyla, Onychophora (77%), Nematoda (95%), and Chordata (100%), were supported in the bootstrap consensus tree. Three relationships between phyla were supported. These were 99% support for the sister pairing of the Priapulida and Kinorhyncha, 83% for the sister pairing of the Echinodermata and Chordata, and 100% for the clade comprising all taxa except the outgroups.

The Bayesian analysis for all data except 18S rRNA is shown in figure 3. The bryozoan was again removed from the Lophotrochozoa. The Deuterostomia was shown to be the sister group to a clade including both (Ecdysozoa + Chaetognatha) and (Lophotrochozoa - Bryozoa). The symphylian genus, *Hanseniella*, was found in the main Lophotrochozoan clade, but with this exception, all other members of the Ecdysozoa were found in a clade that otherwise included only the chaetognath with a PP of 0.57. The Lophotrochozoa was monophyletic except for the inclusion of *Hanseniella* and the exclusion of the Bryozoa. This analysis did not resolve relationships between the Mollusca and Annelida. All Annelida except *Owenia* were included in a clade with a PP of 0.80. This clade also included the Sipuncula and Brachiopoda.

All analyses of the total data and the total data except 18S rRNA resolved a clade that included all Annelida with the exception of *Owenia*. This clade showed PP support of 0.66 in ABY and 0.80 in BYno18S, and bootstrap support of 61% in AML. Other taxa (the Brachiopoda and Sipuncula) were also included in the clade (Figs. 1-3). In AMP, *Owenia* was the sister group of the entoproct, and this pair was the sister group of (*Phoronis*, Annelida - *Owenia*, + Sipuncula and Brachiopoda). These topologies formally place

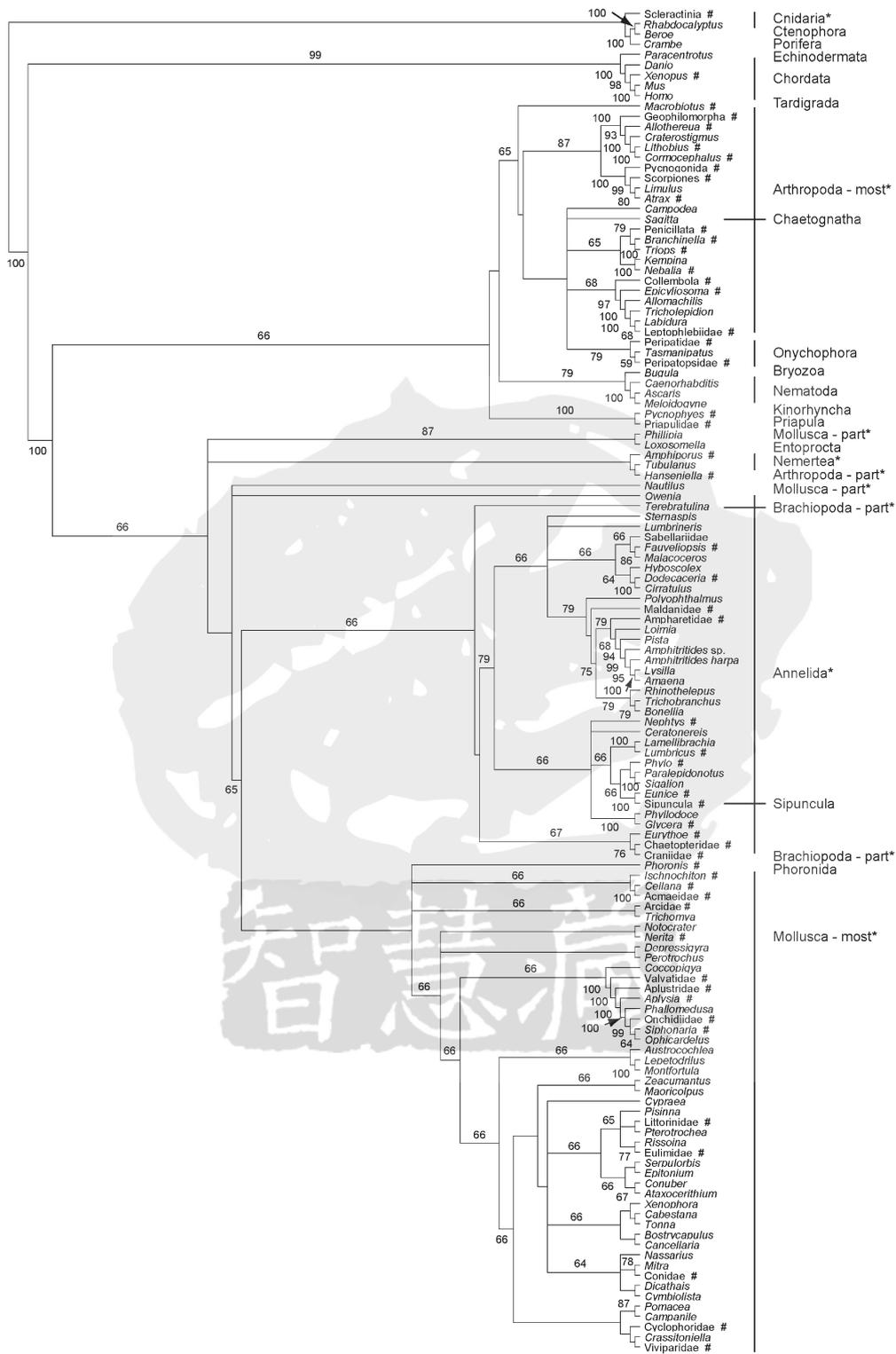


Fig. 1. Majority rule consensus of the last 8250 trees sampled from the Metropolis-Coupled Monte Carlo Markov chain simulation for the Bayesian analysis. Numbers near the branches (sometimes with a clarifying arrow) are posterior probabilities (above 0.65) multiplied by 100 to aid legibility. Lines at the right of the figure link members of the same taxon. Asterisks on these names indicate that the taxon was not resolved to be monophyletic. Where a horizontal line points to a taxon with fewer representatives by crossing a vertical line, the taxon indicated by the vertical line is paraphyletic. Hatch marks after a terminal name indicate that data were collected from more than 1 species. Names of these terminals are the smallest taxon including all species providing data. Details are given in the electronic supplementary material.

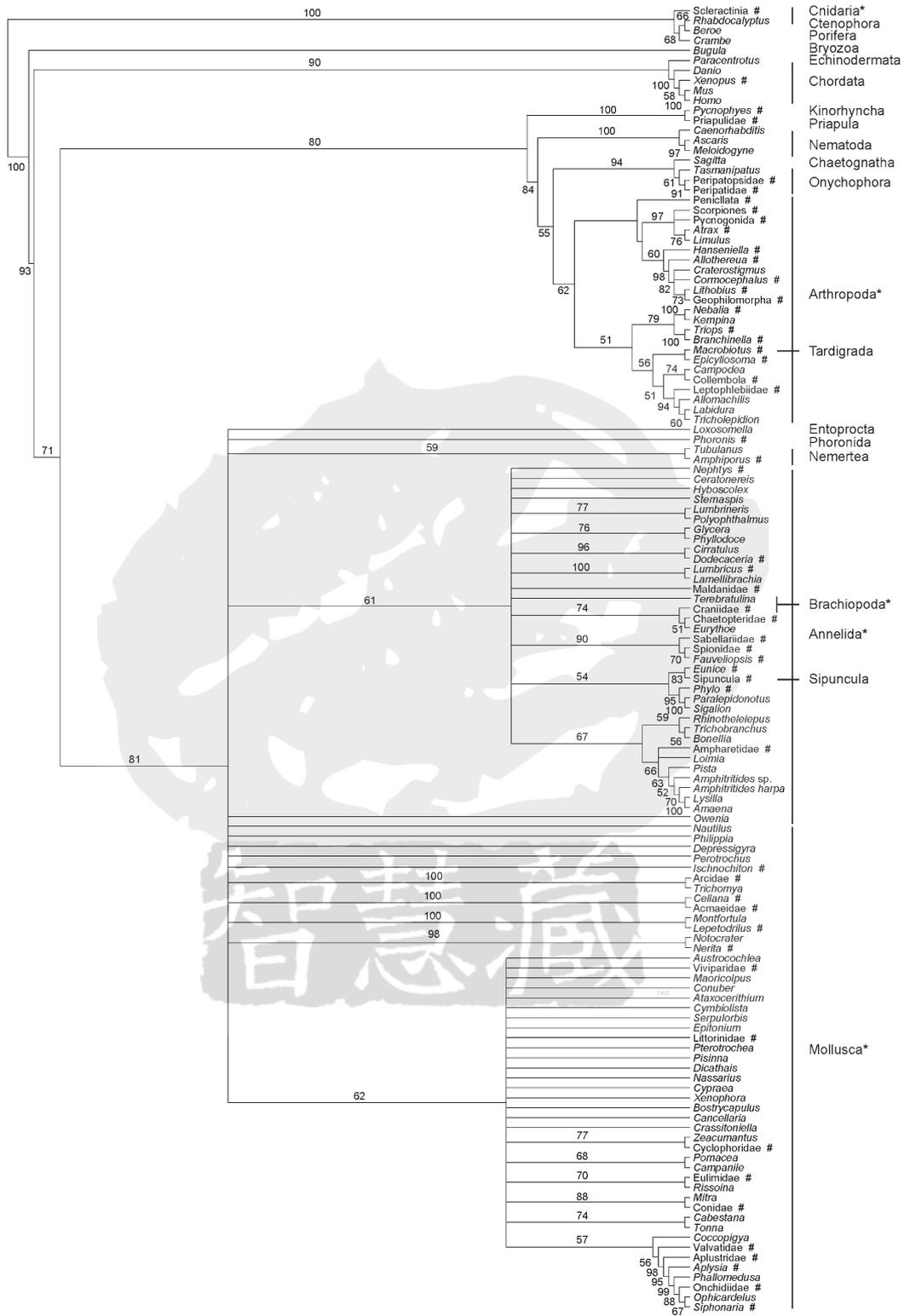


Fig. 2. Consensus tree of the maximum-likelihood bootstrap analysis performed by RAxML. Figures indicate the number of times the indicated branch was seen in 100 bootstrap replicates (only shown when this was > 50%). Lines at the right of the figure link members of the same taxon. Asterisks on these names indicate that the taxon was not resolved as being monophyletic. Where a horizontal line points to a taxon with fewer representatives by crossing a vertical line, the taxon indicated by the vertical line is paraphyletic. Hatch marks after a terminal name indicate that the data were collected from more than 1 species. Names of these terminals are the smallest taxon including all species providing data. Details are given in the electronic supplementary material.

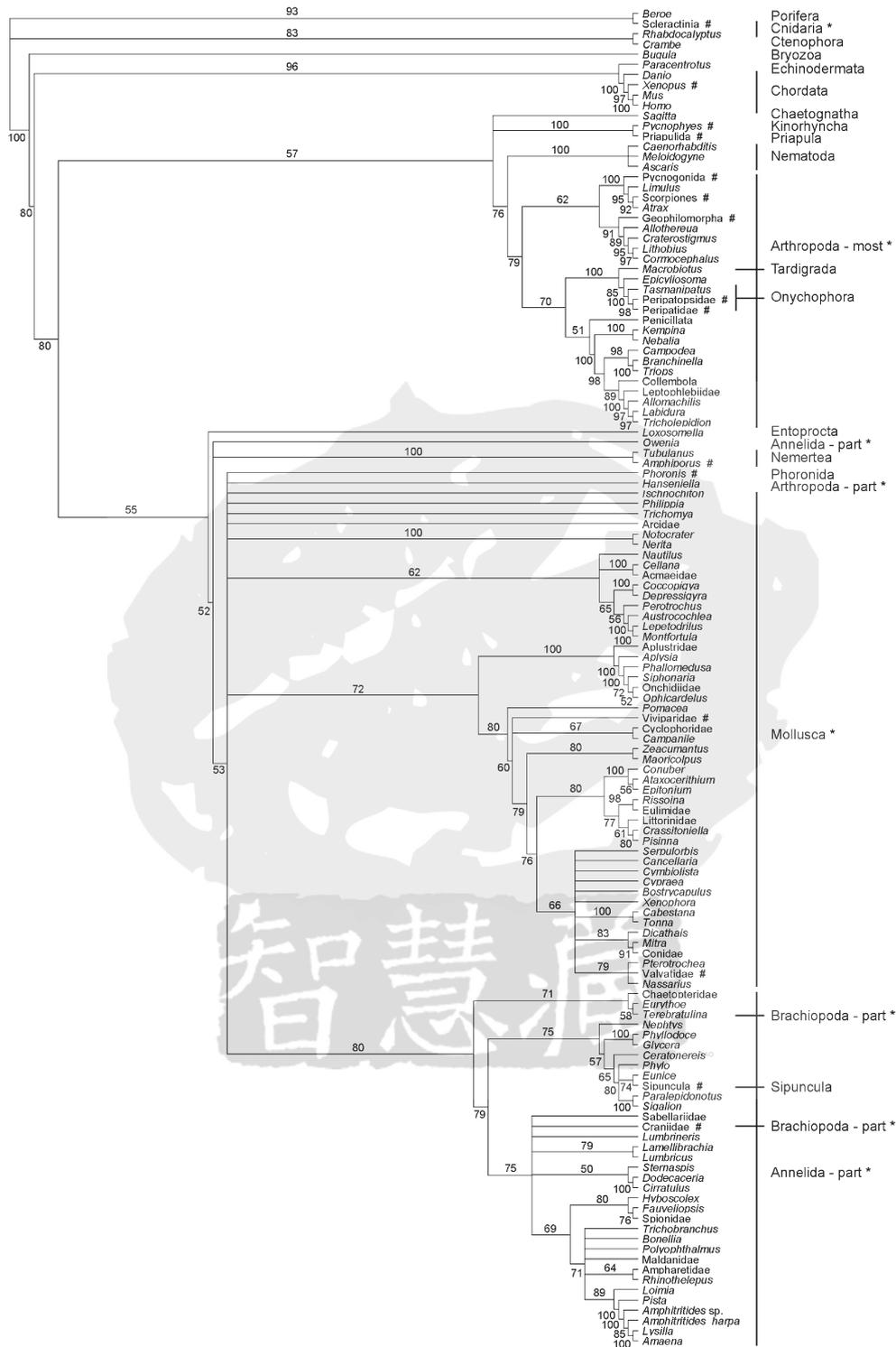


Fig. 3. Majority rule consensus of the last 8800 trees sampled from the Metropolis-Coupled Monte Carlo Markov chain simulation for the Bayesian analysis of all genes except 18S rRNA. Numbers near the branches are posterior probabilities (above 0.65) multiplied by 100 to aid legibility. Lines at the right of the figure link members of the same taxon. Asterisks on these names indicate that the taxon was not resolved as being monophyletic. Where a horizontal line points to a taxon with fewer representatives by crossing a vertical line, the taxon indicated by the vertical line is paraphyletic. Hatch marks after a terminal name indicate that the data were collected from more than 1 species. Names of these terminals are the smallest taxon including all species providing data. Details are given in the electronic supplementary material. The same taxon names are used as in figures 1 and 2, although with the exclusion of 18S rRNA data, some taxa that previously included sequences from multiple species now include data from 1 species only.

the root in the Annelida between *Owenia* and the other taxa. Two other taxa, *Mesochaetopterus* (Chaetopteridae) and *Eurythoe* (Amphinomidae), that Colgan et al. (2006) suggested might be basal in Annelida were also generally basal in these analyses. A triplet including this pair and one of the brachiopods (*Terebratulina* in ABY and the Craniidae in BYno18S) formed a monophyletic clade that was the sister group to (other Annelida - *Owenia*, + Sipuncula and 1 brachiopod). This large clade received a PP of 0.79 in both the ABY and BYno18S analyses. The triplet of *Eurythoe*, Chaetopteridae, and Craniidae was not resolved within the grouping of (Annelida - *Owenia*, + Sipuncula and Brachiopoda) in AML, although the group comprising *Eurythoe*, the Chaetopteridae, and both brachiopods was shown to be the sister group to (other annelids - *Owenia*, + Sipuncula) in the actual maximum-likelihood topology. In AMP, Chaetopteridae was the sister group of Craniidae and *Eurythoe*, the sister group of *Terebratulina*, but the pairs were topologically distant within the Annelida and not basal.

DISCUSSION

All scored lophotrochozoan phyla except the Bryozoa (i.e., the Annelida, Mollusca, Nemertea, Entoprocta, Sipuncula, and Phoronida) were grouped to the exclusion of all ecdysozoan phyla in the ABY and AML bootstrap analyses when topologies were rooted on the outgroup comprised of the Porifera, Cnidaria, and Ctenophora. The grouping received PP support of 0.66 in ABY, and bootstrap support of 81% in AML. The ecdysozoan phyla (Arthropoda, Onychophora, Tardigrada, Nematoda, Kinorhyncha, and Priapulida) were included in a clade that also included the Chaetognatha and received PP support of 0.66 in ABY and bootstrap support of 80% in AML. These relationships were generally similar for BYno18S except that the arthropod *Hanseniella* was removed to the lophotrochozoan clade (with a PP of 0.55). The Ecdysozoa, except *Hanseniella*, had a PP of 0.57 in BYno18S. There were some differences from these topologies in AMP, principally due to the long-branch taxa (see "Results") but no significant contradictions to the general support for the Ecdysozoa (Aguinaldo et al. 1997) and Lophotrochozoa (Halanych et al. 1995). This support is based on a wider taxonomic sampling within the 3 major phyla than has previously been available for multi-gene

studies of metazoan phylogeny. Additionally, the data were collected from genes other than 18S rRNA, allowing an independent assessment of relationships and reinforcing the results of 18S rRNA analyses. Moreover, the results were obtained without resort to arbitrarily excluding data classified as "uncertain" in alignment.

Resolution of the Annelida and Mollusca into reciprocally monophyletic clades was better than in previous analyses (Giribet et al. 2000, Peterson and Eernisse 2001), although a few exceptions remain (*Nautilus* and *Philippia* in AMP, *Nautilus* and *Owenia* in ABY, and *Owenia* in BYno18S). The phyla were reciprocally monophyletic in the AML topology although not resolved in the bootstrap consensus. The monophyly of the Annelida was contradicted in the analyses only by the exclusion of *Owenia* and the inclusion of the Sipuncula and Brachiopoda.

Within the Ecdysozoa there was strong support for the pairing of Priapulida and Kinorhyncha as previously observed for 18S rRNA (Peterson and Eernisse 2001). The Panarthropoda (including the Tardigrada and Onychophora) was monophyletic only in the BYno18S analyses (with a PP of 0.79) with the Chaetognatha usually being included in the panarthropod grouping, as the sister to the Onychophora in AML (with bootstrap support of 94%) and AMP (with bootstrap support of < 50%). The Nematoda was closer to the panarthropods in all analyses than was (Kinorhyncha, Priapulida).

Data from more sipunculans are required before a derived position for the phylum within the Annelida can seriously be hypothesized. Currently there is general agreement that the Sipuncula is a protostome group belonging to the Lophotrochozoa with affinities to annelids and/or molluscs (Zrzavý et al. 1998, Giribet et al. 2000, Nielsen 2003), although its precise position remains unresolved (Schulze et al. 2005). Although not based on formal analyses, mitochondrial (mt)DNA evidence suggests a very close relationship between the Annelida and Sipuncula. Approximately 1/2 of the mtDNA sequence of the sipunculan, *Phascalopsis gouldii*, has been determined (Boore and Staton 2002). The gene order there differs from that of the oligochaete, *Lumbricus terrestris* (Boore and Brown 1995), only by 1 inversion and 1 transposition.

The Phoronida was included in the Lophotrochozoa in all analyses contradicting Nielsen's (2001) hypothesis that the phoronids are deuterostomes rather than protostomes. The

group's representative was not associated with deuterostomes in the present analyses or other molecular studies, including DNA sequences (Giribet et al. 2000, Cohen and Weydmann 2005) and the mitochondrial gene order (Helfenbein and Boore 2004). Those data support a close relationship between the Phoronida and Brachiopoda within the Lophotrochozoa, but that was not observed here.

Changes in the understanding of metazoan evolution arising from 18S rRNA studies have led to a reconsideration of morphology (e.g., Schmidt-Rhaesa et al. 1998) including a notable trend to emphasize the complexity of the common ancestor of the Bilateria. Adoutte et al. (2000) for example suggested that morphological innovation relied more on "tinkering with an already existing array rather than the generation of new genes. The distribution of some of the principal suggested synapomorphies within the major phyla suggests that taxa very early in the bilaterian stem group lineage already possessed a complex body plan. Such early taxa may have shown many of the characters, such as metameric segmentation (Balavoine and Adoutte 2003), thought to be synapomorphies of super-phylum clades within the Bilateria. If this were the case, then much of the complexity in the early Bilateria may have been lost in some phyla or even lineages within phyla. "

A possible example of character loss/reduction is ecdysis. This has now been observed in 2 distinct groups in the Annelida: in the Clitellata (Sauber et al. 1983) and Onuphidae (Paxton 2005). Paxton (2005) suggested that molting may have been present in the last common ancestor of the Lophotrochozoa and Ecdysozoa, rather than arising independently in the Annelida. If this were the case, homologous processes or vestigial adaptations of them may be discovered in more lineages, including other Annelida, than those presently known. For example, unpublished data of Hutchings indicate that replacement of hook-like structures in *Melinna* (family Ampharetidae) occurs throughout life. This might even be the case in the Mollusca. Paxton (2005) mentions the radula of Mollusca as a sclerotized cuticular structure in relation to the evolution of pharyngeal molting in the Annelida but does not speculate about detailed relationships between the erosion of radular teeth and ecdysis. Elucidating such a relationship or demonstrating other ecdysis-related functions in the Mollusca would be a fruitful area for further research.

Segmentation is another possible area of character loss/reduction. Metameric segmentation in the Annelida and Arthropoda may be homologous, as recently argued, for example, by Scholtz (2002). If this is true, the results of the present analyses, concurring as they do with other molecular studies, imply that segmentation must have been present in the stem lineage of the Mollusca after its split from the Annelida. Jacobs et al. (2005) suggested that telomeric addition as a means of growth did occur in fossil species of Mollusca. They suggested that there has been an independent reduction in multiple molluscan lineages in both the number of serially organized units and the degree to which these are apparent morphologically. However, recent work, particularly Okusu's study (2002) of early post-metamorphic growth of the aplacophoran mollusc, *Epimenia babai*, and Friedrich et al.'s investigation (2002) of neurogenesis of the polyplacophoran, *Mopalia muscosa*, found no evidence of true metameric segmentation in extant Mollusca. Homology of the segmentation in the Annelida and Arthropoda will remain doubtful unless such evidence is found.

Nielsen (2003) raised another possibility for the relationship of segmentation in the Annelida and Arthropoda, suggesting that the Ecdysozoa is the sister group of the Annelida. This supposes the loss of characters such as ciliated larvae (in the Ecdysozoa), mesodermal segmentation (in the Cycloneuralia, comprising the Priapula, Kinorhyncha, and Nematoda), and ectodermal segmentation (in the Priapulida and Nematoda). The present analyses cannot however be reconciled with Nielsen's (2001) hypothesis of the sister group relationship of the Ecdysozoa and Annelida.

Placing the root of the annelid tree is one of the major problems in the phylogeny of this extremely problematic group (Rouse and Pleijel 2001 2003, Colgan et al. 2006, Rousset et al. 2007). The use of a large number of outgroups here suggested that the root should be placed between *Owenia* and the remainder of the Annelida with *Eurythoe* and the Chaetopteridae being members of the next branch to split from the main annelid lineage. This is consistent with the placement of the annelid root in Colgan et al. (2006).

It is notable that the use of all of the sequence data, including possible "regions of uncertain alignment" and 3rd base codon positions, showed very good agreement with the morphological allocations of the taxa to the various phyla. In

particular, COI data were included in the present analyses although reservations about the use of the gene in higher-order phylogenetics have been raised owing to the high rates of evolution and synonymous changes in its sequence (e.g., Carlini and Graves 1999, Nylander et al. 1999). Similar points could be argued regarding the H3 data, especially for 3rd codon positions. The present results suggest that the accumulation of data from increasing numbers of taxa can overcome phylogenetic noise, so that even variable sequences may be useful at higher taxonomic levels.

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