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Combined large and small subunit ribosomal RNA phylogenies support a basal position of the acoelomorph flatworms

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The phylogenetic position of the phylum Platyhelminthes has been re-evaluated in the past decade by analysis of diverse molecular datasets. The consensus is that the Rhabditophora + Catenulida, which includes most of the flatworm taxa, are not primitively simple basal bilaterians but are related to coelomate phyla such as molluscs. The status of two other groups of acoelomate worms, Acoela and Nemertodermatida, is less clear. Although many characteristics unite these two groups, initial molecular phylogenetic studies placed the Nemertodermatida within the Rhabditophora, but placed the Acoela at the base of the Bilateria, distant from other flatworms. This contradiction resulted in scepticism about the basal position of acoels and led to calls for further data. We have sequenced large subunit ribosomal RNA genes from 13 rhabditophorans + catenulids, three acoels and one nemertodermatid, tripling the available data. Our analyses strongly support a basal position of both acoels and nemertodermatids. Alternative hypotheses are significantly less well supported by the data. We conclude that the Nemertodermatida and Acoela are basal bilaterians and, owing to their unique body plan and embryogenesis, should be recognized as a separate phylum, the Acoelomorpha.

Keywords: ribosomal RNA; Acoelomorpha; Acoela; Nemertodermatida; Metazoa; phylogeny

1. INTRODUCTION

One of the most striking alterations in our view of metazoan evolution brought about by analysis of molecular data involves the position of the rhabditophoran Platyhelminthes, which include almost all of the familiar turbellarian flatworms as well as the parasitic forms such as tapeworms and trematodes. Although long considered basal bilaterians owing to their lack of advanced metazoan characteristics such as a body cavity or a through gut with separate mouth and anus (e.g. Hyman (1951), although see Nielsen (1985) for an alternative morphological viewpoint), molecular studies have instead robustly linked the Rhabditophora and the closely related Catenulida to the eutrochozoan phyla such as Mollusca and Annelida, suggesting that their morphological simplicity is a secondarily derived state (Tyler 2001). For clarity we refer to the rhabditophoran + catenulid clade as the Platyhelminthes.

One other group of acoelomate worms has also traditionally been grouped with the Platyhelminthes—the Acoelomorpha, which comprises two taxa: the Acoela and the Nemertodermatida. The Acoelomorpha, however, resemble the Platyhelminthes only in being acoelomate and in lacking a through gut. Early morphological analyses were uncertain as to their relationship with the other flatworms (Ehlers 1985). One recent cladistic study, based on the morphology of these taxa, considered the flatworms to be polyphyletic, with acoels and nemertodermatids the earliest-branching bilaterian taxon and unrelated to the Platyhelminthes (Haszprunar 1996).

Early studies of partial small subunit (SSU) ribosomal RNA (rDNA) supported this basal origin of Acoela within the Bilateria (Katayama *et al.* 1993) and the comprehensive analysis of Ruiz-Trillo *et al.* (1999), who used complete SSU rDNA sequences from 18 acoels and two nemertodermatids, seemed to provide strong evidence for this result. The analyses of Ruiz-Trillo *et al.* showed all acoel sequences clustering at the base of the bilaterian metazoans, separate from all other Platyhelminthes (see also Peterson & Eernisse 2001). The major contention raised by this study, however, resulted from the position of a representative of the other acoelomorph group, the nemertodermatid *Nemertinoides*, which grouped unambiguously within the Platyhelminthes. There seems little doubt that the acoels and the nemertodermatids are related and, faced with this contradiction between the traditional position of the short-branch nemertodermatid (with other flatworms) and the more unexpected position of the relatively long-branched acoels, many zoologists preferred to believe the former. Although the sequence of *Nemertinoides* has subsequently been shown to be a contaminant (see note in Giribet *et al.* (2000) and Jondelius *et al.* (2002)), there remains widespread scepticism about the basal position of the Acoelomorpha (Erwin & Davidson 2002).

Many attempts have since been made to test the hypothesis that the acoels are basal metazoans rather than being related to the other flatworms. The first involved looking for so-called 'signature' peptides within their Hox genes, which should be uncontroversial indicators of a lophotrochozoan relationship. The discovery of lophotrochozoan signatures in an acoel has been mentioned (as

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'unpublished work'; Adoutte *et al.* 2000) but is contradicted by the work of C. Cook and M. Akam (personal communication). Second, Telford *et al.* (2000) looked for the presence of rare changes in mitochondrial genetic codes characteristic of the Platyhelminthes. Two such changes were shown to be present in all Rhabditophora but were not found in the catenulids, acoels or nemertodermatids, proving only that none of these three groups could have been derived from within the Rhabditophora. Finally, Berney *et al.* (2000) sequenced the elongation factor 1 (EF1)-alpha gene of one acoel, *Convoluta roscoffensis*. They found a short sequence 'signature' that seemed to link *Convoluta* to the rhabditophoran triclads in contradiction of the SSU results. This has since been discounted by Littlewood *et al.* (2001), who showed that the signature is not present in several other acoel EF1-alpha genes and is in fact very variable throughout the Metazoa and hence is an unreliable phylogenetic character.

None of these studies has been able to determine whether the acoelomorphs are closely related to the rhabditophoran Playhelminthes within the Lophotrochozoa or are truly the most basal known bilaterians. As has been pointed out (Berney *et al.* 2000; Littlewood *et al.* 2001) sequence data from further genes are essential if this conflict is to be resolved.

We have generated almost complete large subunit (LSU) rRNA sequences from 13 Platyhelminthes, three acoels and a nemertodermatid. We have combined these with other recently completed LSU sequences (Medina *et al.* 2001; Mallatt & Winchell 2002) and have added this LSU dataset to the already existing SSU data, almost tripling the length of the available dataset relevant to this problem. Our data complement and agree with recent studies of metazoan myosin heavy chain type II sequences (Ruiz-Trillo *et al.* 2002) and work by C. Cook and M. Akam (personal communication), who provide Hox sequence data from the acoel *Convoluta roscoffensis*.

2. MATERIAL AND METHODS

(a) *Specimens*

Table 1 gives the full list of species used in this study and their broader classification. All flatworm species sequenced here were originally fixed in 95% ethanol. Host details for the parasites are available from GenBank/European Molecular Biology Organization along with the sequencing details and locality where available. In GenBank there is no SSU and LSU for any single species of *Arbacia*, so we chose to concatenate the SSU from *A. lixula* with the LSU from *A. punctulata*. Similarly, for crinoids we concatenated two antedonid sequences (table 1).

(b) *DNA extraction, amplification and sequencing*

DNA was extracted from ethanol-preserved specimens using a DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol. Amplifications of 25 μl were performed with 3–5 μl genomic extract (*ca.* 10 ng) with Ready-To-Go PCR beads (Amersham Pharmacia Biotech) each containing 1.5 U of Taq Polymerase, 10 mM of Tris–HCl (pH 9.0), 50 mM of KCl, 1.5 mM of $MgCl_2$, 200 μM of each deoxyribonucleoside triphosphate (dNTP) and stabilizers including bovine serum albumin; and 0.4 μM of each PCR primer were included. The complete LSU was amplified in three overlapping sections using the primers U178/L1642, U1148/L2450 and U1846/L3449 (table 2). The PCR conditions used were: 2 min denaturation at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at 52 °C and 2 min at 72 °C; followed by 7 min at 72 °C. Where necessary to obtain a product, the stringency was reduced by adding $MgCl_2$ to reach a final concentration of 2.5 mM or reducing the annealing temperature to 50 °C.

PCR products were purified with Qiagen Qiaquick columns, cycle sequenced directly using ABI BigDye chemistry, alcohol precipitated and run on an ABI prism 377 automated sequencer. A variety of internal primers were used to obtain the full sequence on both strands (table 2). Sequences were assembled and edited using SEQUENCHER v. 3.1.1 (Genecodes Corp.) and submitted to GenBank/EMBL (see table 1 for accession numbers).

(c) *Alignment*

SSU sequences were downloaded already aligned according to secondary structure from the ribosomal database project II (http://rdp.cme.msu.edu/html/). Ten appropriate metazoan LSU sequences were available aligned according to secondary structure on the rRNA server (http://oberon.rug.ac.be:8080/rRNA). Other LSU sequences were taken from GenBank and, along with our new sequences, were aligned to the previously assembled LSU sequences using CLUSTAL X (Jeanmougin *et al.* 1998) and the profile alignment option. Alignments were refined by eye using MACCLADE v. 4.03 (Maddison & Maddison 2000). SSU and LSU sequences were concatenated in MACCLADE.

(d) *Exclusion of unreliably aligned positions*

In an attempt to avoid subjectivity in excluding unreliably aligned positions from phylogenetic analyses, the program GBLOCKS v. 0.73b (Castresana 2000) was used with the standard RNA settings. This gave 1213 positions within the SSU and 2263 within the LSU.

(e) *Relative-rate tests*

Relative-rate tests on all ingroup sequences (bilaterian combined SSU + LSU) were conducted with reference to 13 outgroup taxa (Choanoflagellata, Porifera, Cnidaria and Ctenophora) using the RRTREE software (Robinson *et al.* 1998). Each ingroup species was considered as a separate lineage and no topology was specified; Kimura two-parameter rate correction was used. The mean and standard deviation of the rate of change within the Bilateria were calculated using Microsoft EXCEL. Species with rates more than one standard deviation above or below the mean rate for Bilateria were excluded from further analyses in an attempt to avoid artefacts caused by unequal rates of substitution. Importantly, according to this procedure, an acoel, *Paratomella rubra*, and a nemertodermatid, *Meara stichopi*, both have 'normal' rates of evolution, leading us to expect inference of their phylogenetic position to be unbiased by unequal rates.

(f) *Phylogenetic reconstruction*

Maximum-likelihood (ML) tree estimation used PAUP* v. 4.0b10 (Swofford 2002). Initial tree inferences were determined using the neighbour-joining (NJ) method with ML estimation of the rate matrix (NJML) using default parameters. This primary tree was swapped using nearest-neighbour interchange (NNI) branch swapping. ML estimates of the gamma shape parameter (α) with eight rate categories, proportion of invariant sites (p_{inv}), general time-reversible substitution-rate matrix (r_{mat}) and nucleotide frequencies (base) were calculated on this sec-

Table 1. List of species, their classification and GenBank/EMBL accession numbers, used in this study. (Asterisk indicates sequence is new.)

classification	SSU	LSU
outgroups (diploblasts)		
Cnidaria		
Montastrea franksi	AY026382	AY026375
Atolla vanhoeffeni	AF100942	AY026368
Nectopyramis sp.	AF358068	AY026377
Hydra circumcincta	AF358080	AY026371
Choanoflagellida		
Salpingoeca infusionum	AF100941	AY026380
Monosiga brevicollis	AF174375	AY026374
Porifera		
Rhabdocalyptus dawsoni	AF100949	AY026379
Suberites ficus	AF100947	AY026381
Mycale fibrexilis	AF100946	AY026376
Leucosolenia sp.	AF100945	AY026372
Ctenophora		
Pleurobrachia pileus	AF293678	AY026378
Mnemiopsis leidyi	AF293700	AY026373
Beroe sp.	AF293694	AY026369
ingroups (triploblasts)		
Platyhelminthes (Acoelomorpha)		
Acoela		
Anaperus biaculeatus	AJ012527	AY157602*
Childia groenlandica	AJ012529	AY157603*
Paratomella rubra	AF102892	AY157604*
Nemertodermatida		
Meara stichopi	AF051328	AY157605*
Platyhelminthes (Catenulida)		
Suomina sp.	AJ012532	AY157152*
Stenostomum leucops	AJ012519	AY157151*
Platyhelminthes (Rhabditophora)		
Bdelloura candida	Z99947	AY157154*
Girardia tigrina	AF013157	U78718
Notoplana australis	AJ228786	AY157153*
Stylochus zebra	AF342801	AF342800
Polystomoides malayi	AJ228792	AY157170*
Diclidophora denticulata	AJ228779	AY157169*
Dictyocotyle coeliaca	AJ228778	AY157171*
Udonella caligorum	AJ228796	AY157172*
Gyrocotyle rugosa	AF124455	AY157178*
Hymenolepis diminuta	AF124475	AY157181*
Lepidophyllum steenstrupi	AJ287530	AY157175*
Rugogaster hydrolagi	AJ287573	AY157176*
Lobatostoma manteri	L16911	AY157177*
Mollusca		
Placopecten magellanicus	X53899	AF342804
Aplysia californica	AY039804	AY026366
Brachiopoda		
Terebratalia transversa	AF025945	AF342802
Phoronis vancouverensis	U12648	AF342797
Sipunculida		
Phascolopsis gouldii	AF342796	AF342795
Echiura		
Urechis caupo	AF342790	AF342789
Annelida		
Eisenia fetida	AB076887	AF212166
Proceraea cornuta	AF212179	AF212165
Nemertea		
Amphiporus sp.	AF119077	AF342786
Onycophora		
Peripatoides novaezealandiae	AF342794	AF342791–3

(*Continued.*)

Table 1. (*Continued.*)

classification	SSU	LSU
Chaetognatha		
Sagitta elegans	Z19551	AF342799
Priapulida		
Halicryptus spinulosus	AF342790	AF342789
Nematoda		
Trichinella spiralis	U60231	AF342803
Caenorhabditis elegans	X03680	X03680
Nematomorpha		
Chordodes morgani	AF036639	AF342787
Arthropoda		
Limulus polyphemus	U91490	AF212167
Triops longicaudatus	AF144219	AY157606*
Drosophila melanogaster	M21017	M21017
Echinodermata		
Antedon serrata (Antedonidae)	D14357	—
Florimetra serratissima (Antedonidae)	—	AF212168
Arbacia lixula	Z37514	—
Arbacia punctulata	—	AY026367
Strongylocentrotus purpuratus	L28056	AF212171
Hemichordata		
Balanoglossus sp.	D14359	AF278684
Ptychodera flava	AF278681	AF212176
Harrimania sp.	AF236799	AF212173
Saccoglossus kowalevskii	L28054	AF212175
Cephalodiscus gracilis	AF236798	AF212172
Chordata, Urochordata		
Ciona intestinalis	AB013017	AF212166
Thalia democratica	D14366	AF158725
Styela plicata	L12444	AF158724
Oikopleura sp.	D14360	AF158726
Chordata: Cephalochordata		
Branchiostoma floridae	M97571	AF061796
Chordata, Vertebrata		
Triakis semifasciata	AF212180	AF212182
Raja schmidti	AF278682	AF278683
Petromyzon marinus	M97575	AF061798
Oncorhynchus mykiss	AF308735	AF061801
Homo sapiens	K03432	J01866

ondary NNI tree. These parameter estimates and tree topology were then used to conduct a further and much more thorough search using tree bisection and reconnection (TBR) branch swapping. The ML parameters were recalculated on this secondary TBR tree and this procedure was repeated until the likelihood score became stationary.

We also used the MRBAYES software (Huelsenbeck & Ronquist 2001) to estimate the best tree using Bayesian inference of phylogeny. The parameters for estimation were the same as for the NJML tree (α with eight rate categories, p_{inv}, GTR matrix and nucleotide frequencies). Four chains were run with 100 000 generations. Every hundredth tree was stored and graphing their likelihoods showed this to have plateaued after *ca.* 75% of the run. The last 100 of the stored trees (the final 10%) were used to make a consensus tree in which the frequency of each clade gives some indication of the support for the clade. The topology of this consensus tree 'BI' was identical to our ML tree.

(g) *Non-parametric bootstraps*

NJML non-parametric bootstrapping (NJMLBP) was used to gauge support for relationships. For 1000 bootstrapped datasets, ML distances were calculated using the gamma parameter (α) and the proportion of invariant sites (p_{inv}) set as calculated for the original dataset based on the ML tree. Base frequencies and rate-matrix values were estimated for each bootstrap replicate. An NJ tree was calculated for each of these distance matrices and a majority rule consensus tree was created from the 1000 NJ trees.

(h) *Testing of alternative hypotheses with likelihood ratio tests*

To see whether our ML tree is significantly better supported by our dataset than alternative ideas of phylogeny we used likelihood ratio tests (LRTs) that were carried out as follows. To find the best tree that is consistent with an alternative hypothesis we used PAUP* in exactly the same way as before but with the appropriate constraint enforced (monophyletic Platyhelminthes + Acoelomorpha or a monophyletic Acoelomorpha).

The Shimodaira–Hasegawa test as implemented in PAUP* was used to test whether these alternative topologies were significantly less well supported by the data than those found in our ML tree and could therefore be confidently rejected. The resampling estimated log-likelihood (RELL) approximation method was used with 1000 bootstrap replicates. The much slower

Table 2. Primers used for PCR amplification and sequencing of complete LSU rDNA.

LSU primers	primer sequence (5′–3′)
amplification and sequencing	
U178	GCACCCGCTGAAYTTAAG
L1642	CCAGCGCCATCCATTTTCA
U1148	GACCCGAAAGATGGTGAA
L2450	GCTTTGTTTTAATTAGACAGTCGGA
U1846	AGGCCGAAGTGGAGAAGG
L3449	ATTCTGACTTAGAGGCGTTCA
additional sequencing	
300F	CAAGTACCGTGAGGGAAAGTTG
300R	CAACTTTCCCTCACGGTACTTG
EDC2	CCTTGGTCCGTGTTTCAAGACGGG
900F	CCGTCTTGAAACACGGACCAAG
1200F	CCCGAAAGATGGTGAACTATGC
1200R	GCATAGTTCACCATCTTTCGG
1600F	AGCAGGACGGTGGCCATGGAAG
U2229	TACCCATATCCGCAGCAGGTCT
L2230	AGACCTGCTGCGGATATGGGT
U2562	AAACGGCGGGAGTAACTATGA
L2630	GGGAATCTCGTTAATCCATTCA
U2771	AGAGGTGTAGGATARGTGGGA
L2984	CTGAGCTCGCCTTAGGACACCT
U3119	TTAAGCAAGAGGTGTCAGAAAAGT
U3139	AAGTTACCACAGGGATAACTGGCT
LSU3_4160	GGTCTAAACCCAGCTCACGTTCCC
L3358	AACCTGCGGTTCCTCTCGTACT

FULL optimization also used 100 bootstrap replicates. The results from the two approaches were congruent in all cases and we cite only the results from the FULL optimization.

3. RESULTS

(a) *Phylogenetic position of the Acoelomorpha*

ML and Bayesian tree reconstruction place the acoelomorph sequences at the base of the Bilateria in a situation identical to that found by analyses based on SSU alone (Ruiz-Trillo *et al.* 1999). The ML and BI trees are shown in figure 1 with non-parametric support indicated. Support for the Bilateria excluding the Acoelomorpha is 68% in the NJMLBP and this is seen in 100% of the best BI trees.

The LRT was used to see whether the alternative hypothesis of a sister-group relationship between the Acoelomorpha and Platyhelminthes was supported or rejected by the data. The traditional grouping of a monophyletic Platyhelminthes including the Acoelomorpha was significantly worse than our BI tree with a basal Acoelomorpha ($\Delta - \ln L = 61$, $p = 0.01$). The basal position of the Acoelomorpha is very strongly supported.

We also reanalysed the data after removing all of the outgroup taxa to rule out the possibility that the acoelomorphs were found basal owing to long branch attraction (LBA) to the outgroups. Their position on this unrooted tree, between the deuterostomes and protostomes, was as predicted for the most basally branching bilaterians, suggesting that LBA was not the cause of their basal position. We also assessed whether both the acoel and the nemertodermatid branched at the base of the Bilateria when the other was omitted from the analysis and in both cases this was found to be true. These two results were tested with the LRT and in each case the optimal solution (basal acoelomorph) was significantly better than grouping either acoelomorph with the Platyhelminthes (*Paratomella* basal $\Delta - \ln L = 39$, $p = 0.00$; *Meara* basal $\Delta - \ln L = 21$, $p = 0.04$).

(b) *Monophyly of the Acoelomorpha*

In our ML tree the acoel and the nemertodermatid do not form a monophyletic group as suggested by their similar morphology but rather *Meara* is closer to the other Bilateria than is *Paratomella*; this result concurs with recent findings based on SSU sequences (Jondelius *et al.* 2002). If shown to be true, this result would suggest that any ultrastructural and morphological characters shared by the nemertodermatids and acoels (Littlewood *et al.* 1999) are not autapomorphies of a monophyletic Acoelomorpha but are actually plesiomorphies of all Bilateria. The huge potential significance of this result led us to test it using the LRT. As our null hypothesis, we constrained the acoels and nemertodermatids to group together. We found that this null tree was not significantly worse than our ML tree ($\Delta - \ln L = 18$, $p = 0.44$) and we cannot reject the hypothesis that the acoels and nemertodermatids are sister groups as their morphology would lead us to believe.

(c) *Position of the Catenulida*

According to our BI tree the catenulids are the sister group to all of the Rhabditophora. This result is in agreement with previous analyses based on SSU sequences alone (Littlewood *et al.* 1999) and is supported by high non-parametric bootstrap values: Rhabditophora + Catenulida are grouped with 96% NJMLBP and the Rhabditophora were monophyletic excluding the Catenulida with 100% NJMLBP support.

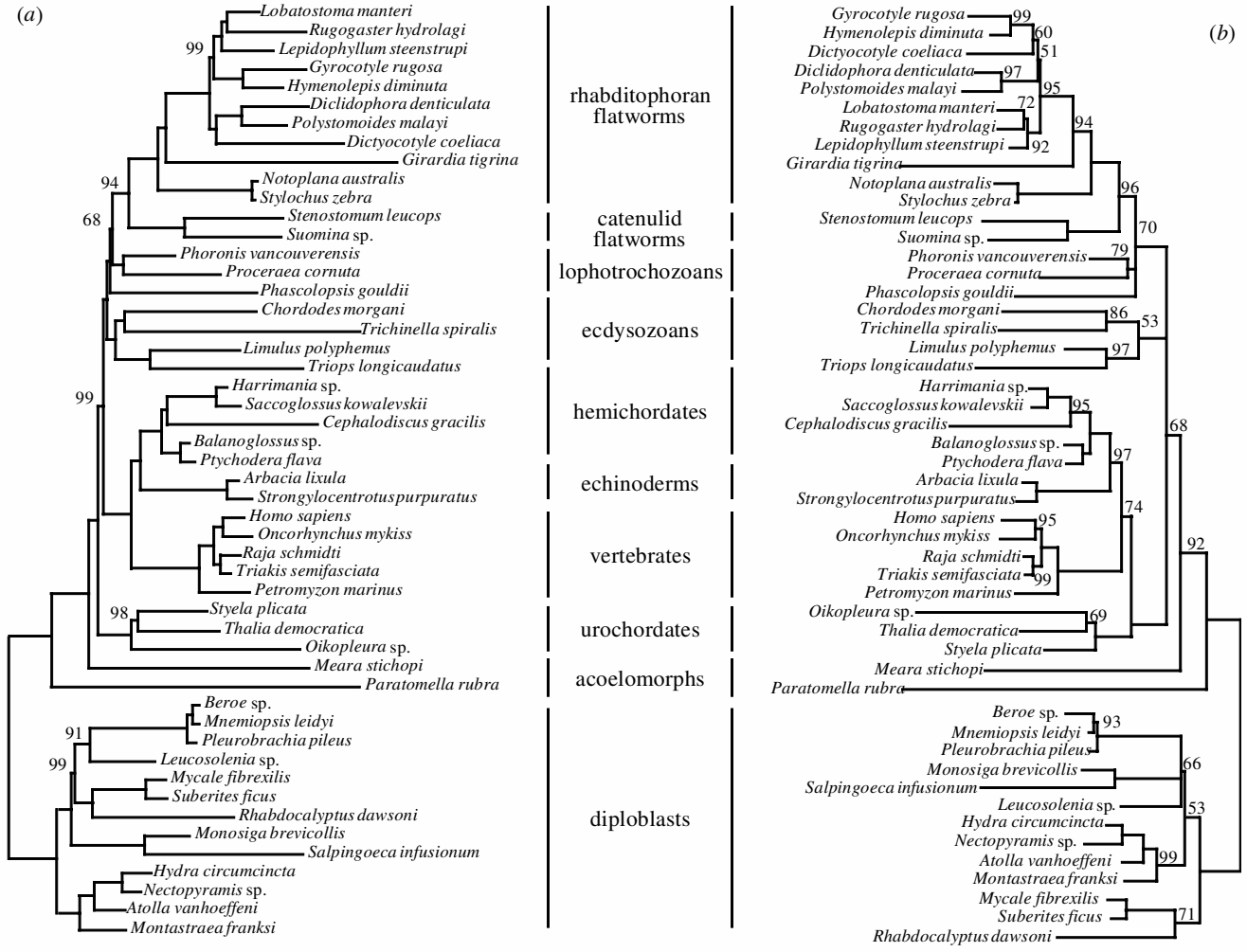

Figure 1. Comparison of the ML tree and the non-parametric bootstrap tree. (*a*) Best ML/BI tree found as described in § 2f. Both Acoelomorphs are basal to all other Bilateria. The urochordates do not group with the other chordates; a result also seen in previous studies of rRNA genes. A tree in which chordates including urochordates are forced to be monophyletic is not significantly worse than the ML tree shown (data not shown). Numbers are the percentages of the optimal trees in which the particular clade was found. All values were 100% unless shown. (*b*) NJML bootstrap tree found as described in § 2g. All bootstraps are 100% unless indicated on the tree.

4. DISCUSSION

(a) *The Acoelomorpha are basal within the triploblastic Metazoa and are probably monophyletic*

Our analyses of combined SSU and LSU rRNA gene sequences give strong support to the finding that the Acoelomorph flatworms are the most basal triploblastic bilaterian animals, as previously shown (at least for the Acoela) by Ruiz-Trillo *et al.* (1999) and most recently by Ruiz-Trillo *et al.* (2002). We have applied LRTs to evaluate alternative hypotheses and we strongly reject the alternative (traditional) hypothesis of a close relationship between Acoelomorpha and the Platyhelminthes. We also replicated the other striking finding of Jondelius *et al.* (2002) of a paraphyletic relationship between Acoela and Nemertodermatida (both basal to the triploblasts) but our LRTs failed to show that this is significantly better supported than an acoelomorph clade with Acoela and Nemertodermatida as sister groups; we therefore reject this finding in favour of an Acoelomorpha clade.

(b) *Rhabditophora and Catenulida*

Our complete concatenated SSU and LSU sequences also support the consensus view of Platyhelminthes (minus Acoelomorpha) as members of the Lophotrochozoan clade rather than basal triploblasts as had been inferred from their morphological simplicity. Our analysis of complete SSUs and LSUs, including non-parametric bootstrapping, also gave strong support for the sister-group relationship between the Catenulida and the Rhabditophora. Exclusion of the Catenulida from within the Rhabditophora was also supported by these analyses, as had been shown by SSU studies and as had been indicated by the lack of the synapomorphic rhabditophoran mitochondrial genetic code changes in the Catenulida (Telford *et al.* 2000), and this result seems extremely robust. We conclude that the Catenulida are the closest outgroups to the Rhabditophora and, practically speaking, should be used in future to root the phylogeny of the Rhabditophora and to polarize the evolution of all rhabditophoran characteristics through outgroup comparison. The relationships within the Rhabditophora based on complete combined

SSUs and LSUs are analysed in more detail in Lockyer *et al.* (2003).

5. CONCLUSIONS

Our combined dataset of SSU and LSU rRNA gene sequences is, to our knowledge, the largest yet used to test the position of the acoelomorph flatworms. We show that the Acoelomorpha are the most basal known triploblastic Bilateria and we provide statistical evidence of strong support for this result over alternative hypotheses. Considerable morphological evidence supports the monophyly of the Acoelomorpha. Their unique characteristics include a complete lack of protonephridia, the presence of which unites all other Bilateria, their lack of a true brain with neuropile, a unique pattern of neurotransmitter activity (Raikova *et al.* 2001) and unique body-wall musculature (Hooge 2001) and mode of embryonic development (Henry *et al.* 2000). These merit the establishment of their own phylum. The phylum Aceolomorpha contains the earliest-branching bilaterians currently known and is therefore of great significance to our understanding of the evolution of all aspects of bilaterian biology.

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