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Lophotrochozoan phylogeny assessed with LSU and SSU data: Evidence of lophophorate polyphyly

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Abstract

Of the three major bilaterian clades, Lophotrochozoa has the greatest diversity and disparity of body forms and is the least understood in terms of phylogenetic history. Within this clade, small nuclear ribosomal subunit (SSU or 18S) studies have failed to provide resolution and other molecular markers have insufficient taxon sampling. To examine relationships within Lophotrochozoa, we collected and complied complete SSU data and nearly complete (>90%) large nuclear ribosomal subunit (LSU or 28S) data totaling approximately 5 kb per taxon, for 36 lophotrochozoans. Results of LSU and combined SSU + LSU likelihood analyses provide topologies more consistent with morphological data than analyses of SSU data alone. Namely, most phyla recognized on morphological grounds are recovered as monophyletic entities when the LSU data is considered (contra SSU data alone). These new data show with significant support that "Lophophorata" (traditionally recognized to include Brachiopoda, Phoronida, and Bryozoa) is not a monophyletic entity. Further, the data suggest that Platyzoa is real and may be derived within lophotrochozans rather than a basal or sister taxon. The recently discovered Cycliophora are allied to entoprocts, consistent with their initial placement based on morphology. Additional evidence for Syndermata (i.e., Rotifera + Acanthocephala) is also found. Although relationships among groups with trochophore-like larvae could not be resolved and nodal support values are generally low, the addition of LSU data is a considerable advance in our understanding of lophotrochozoan phylogeny from the molecular perspective.

Keywords: Metazoan; Phylogeny; Lophotrochozoa; Lophophorata; Trochozoa; Platyzoa; SSU; LSU

1. Introduction

Although Lophotrochozoa encompasses the greatest body plan diversity of the three major Bilaterian clades, relationships within this clade are poorly resolved, hindering our understanding of metazoan evolution. Initially identified with SSU sequences (Halanych et al., 1995), Lophotrochozoa is a well supported clade (Anderson et al., 2004; Balavoine, 1997; de Rosa et al., 1999; Halanych, 2004; Mackey et al., 1996; Mallatt and Winchell, 2002; Philippe et al., 2005) comprising the common ancestor, and all the descendents of mollusks, annelids, and the three lophophorate taxa (Brachiopoda, Phoronida, and Bryozoa). Previous studies of lophotrochozoan relationships have relied heavily on small nuclear ribosomal subunit (SSU) data, morphological cladistic analyses, or a combination of the two (Eernisse, 1997; Giribet et al., 2000; Zrzavy et al., 1998). Unfortunately, SSU data does not cluster taxa into well-recognized monophyletic units (e.g., Mollusca, Nemertea, and Brachiopoda), and applying morphological characters between recognized phyla is inherently problematic (Jenner, 1999, 2002). Herein, we examine combined SSU and large nuclear ribosomal subunit (LSU) data to address three hypothesized lophotrochozoan taxa (Lophophorata, Platyzoa, and Trochozoa) that shape our overall understanding of the group's evolution.

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Hyman (1959) grouped bryozoans, brachiopods, and phoronids together as the "Lophophorata" based on inferred homology of the ciliated feeding structure. Lophophorata monophyly has not been demonstrated and evidence suggests that not all "lophophores" are homologous (Halanych, 1996; Nielsen, 2001). Nonetheless the "Lophophorata" has been perpetuated in recent invertebrate textbooks and is commonly accepted. In particular, molecular analyses of bryozoan (a.k.a. Ectoprocta) affinities have relied upon SSU sequences, which do not recover bryozoan monophyly and place them as basal members of the Lophotrochozoa (Giribet et al., 2000; Halanych et al., 1995; Peterson and Eernisse, 2001). Hox gene data is consistent with this interpretation (Passamaneck and Halanych, 2004). Nielsen (2001) has proposed that bryozoans are most closely related to entoprocts, but this has not been evidenced by molecular data.

Platyzoa was originally diagnosed as ciliated non-segmented acoelomates or pseudocoelomates lacking a vascular system (i.e., Platyhelminthes, Rotifera, Acanthocephala, Gastrotricha, and Ganthostomulida; Cavalier-Smith, 1998). Although traditionally viewed as basal lineages within Bilateria, interpretations of platyhelminth and rotifer cleveage as spiral or "modified spiral" suggest an evolutionary relationship with spiralian lophotrochozoans such as mollusks, annelids, echiurans, sipunculans, and entoprocts (Boyer et al., 1998; Nielsen, 2001). SSU and combined SSU + morphological datasets suggest Platyzoa represents a sister clade to a Trochozoa clade (Giribet et al., 2000), or a grade which diversified basal to the last common ancestor of the Lophotrochozoa (Peterson and Eernisse, 2001). Our understanding of Platyzoa has been altered by recent analyses that place the acoelomorph playhelminthes outside Platyzoa at the base of Bilateria (Berney et al., 2000; Ruiz-Trillo et al., 2002; Telford et al., 2003). SSU analyses (Winnepenninckx et al., 1998) suggest Cycliophora, a recently discovered group hypothesized to be close to Entoprocta (Funch and Kristensen, 1995), are allied with Syndermata (acanthocephalans and rotifers; Garey et al., 1996) tying them to Platyzoa.

The term "Trochozoa" refers to taxa that have trochophore-like ciliated feeding larvae. Originally applied specifically to the annelid *Polygordius* (Hatschek, 1878), it has been loosely applied to several other protostome lineages causing confusion in the literature. Recognizing this problem, Peterson and Eernisse (2001) use several different terms to define nested clades with trochophore or trochophore-like larvae. The Neotrochozoa (i.e., annelids including echiurids, mollusks, and sipunculans) is the most restrictive clade recognized, whereas the Eutrochozoa (Nemertea and Neotrochozoa) and Trochozoa (Entoprocta and Eutrochozoa) are more inclusive. Whether these taxa are monophyletic influences our understanding of (1) the early history of larval forms and (2) the evolutionary plasticity of characters considered important to phylogeny (e.g., metatroch and apical tuft).

Deciphering lophotrochozoan relationships requires critical evaluation of hypotheses such as the Lophophorata, Platyzoa, and Trochozoa, among others. However, the failure of SSU data, when used alone, to recover the monophyly of many lophotrochozoan phyla makes it unsuitable for evaluating such interphyletic relationships. A previous simulation study (Halanych, 1998) and recent phylogenetic analyses (Mallatt and Winchell, 2002; Medina et al., 2001; Passamaneck et al., 2004; Winchell et al., 2002) have suggested that combined SSU and LSU data offer more resolution than SSU data alone. To this end, we examined nearly complete sequences (>90%) of nuclear SSU and LSU rRNA genes, totaling approximately 5 kb per taxon, for 36 lophotrochozoan taxa. This is a substantial increase over the approximately 2 kb of data that 18S alone provides.

2. Materials and methods

2.1. Data collection

Thirty-six taxa were chosen to provide broad representation of extant lophotrochozoan lineages (Table 1). Two deuterostomes and three ecdysozoans were arbitrarily chosen as outgroups taking care to avoid taxa with obviously elevated rates of nucleotide substitution as judged by branch length. Genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen). Primer sequences utilized for PCR and sequencing are provided in Passamaneck et al. (2004).

Both genes were amplified using a long PCR protocol. PCRs contained 15 μl 3.3× rTth buffer, 2.5 μl of 10 μM primer, 5 μl of 2 mM dNTPs, 0.4 μl rTth (PE Applied Biosystems), 1 μl Vent polymerase (New England BioLabs) (diluted 1:100 in a buffer composed of 50% glycerol, 20 mM Hepes, 10 mM KCl, 1 mM DTT, 0.1 mM Na_2EDTA, 0.0025% Tween 20, and 0.0025% NP-40), with genomic DNA and water to a final volume of 45 μl. Following a 5-min denaturation, 5 μl of 25 mM $Mg(OAc)_2$ was added to each reaction. PCR involved 30 cycles of denaturation at 94 °C for 30 s, annealing at 45–55 °C for 1 min, and extension at 65 °C for 12 min LSU or 8 min for SSU. A final extension was carried out at 72 °C for 10 min. PCR products were cleaned with QIAquick PCR Purification Kit (Qiagen) and incubated 10 min at 70 °C with *Taq* polymerase (Promega) and 0.4 mM dATP to create adenine overhangs. Fragments were cleaned a second time and cloned using the pGEM-T Vector System (Promega).

Bidirectional sequencing used BigDye Terminator v2.0 Sequencing Reaction chemistry (Applied Biosystems) on an ABI 377 automated sequencer (Applied Biosystems). Multiple clones were sequenced.

2.2. Phylogenetic analyses

Sequences were aligned by the profile alignment function of ClustalW (Thompson et al., 1994), using existing alignments from the Ribosomal Database Project II (which

Table 1
Species and GenBank accession numbers

Species	LSU	SSU
Mollusca		
Arion silvaticus	AY145392	AY145365
Chaetopluera aplicata	AY145398	AY145370
Ilyanassa obsoleta	AY145511	AY145379
Leptochiton acellus	AY145414	AY145382
Nucalana pernula	AY145419	AY145385
Placopecten magellanicus	AF342798	X53899
Nemertea		
Amphiporus sp.	AF342786	AF119077
Cerebratulus lacteus	AY145396	AY145368
Oerstedia dorsalis	**AY210465**[a]	**AY210448**[a]
Tubulanus annulatus	**AY210473**[a]	**AYY210452**[a]
Sipuncula		
Apionsoma misakianum	**AY210454**[a]	**AY210440**[a]
Phascolion strombi	**AY210468**[a]	**AY210449**[a]
Phascolopsis gouldii	AF342795	AF342796
Bryozoa		
Alcyonidium diaphanum	**AY210453**[a]	
Alcyonidium gelatinosum		X91403
Bugula turrita	**AY210457**[a]	**AY210443**[a]
Crisia sp.	**AY210458**[a]	**AY210444**[a]
Entoprocta		
Barentsia gracilis	**AY210456**[a]	**AY210442**[a]
Brachiopoda		
Glottidia pyramidata	**AY210459**[a]	U12647
Laqueus californianus	**AY210460**[a]	U08323
Neocrania anomola	**AY210463**[a]	U08328
Terebratalia transversa	AF342802	AF025945
Phoronida		
Phoronis vanvouverensis	AF342797	**AY210450**[a]
Annelida		
Arhynchite pugettensis[b]	**AY210455**[a]	**AY210441**[a]
Eisenia fetida	AF212166	X79872
Nereis succinea	**AY210464**[a]	**AY210447**[a]
Proceraea cornuta	AF212165	AF212179
Riftia pachyptila[b]	**AY210470**[a]	AF168745
Urechis caupo[b]	AF342804	AF342805
Platyhelminthes		
Dugesia tigrina	U78718	AF013157
Stylochus zebra	AF342800	AF342801
Acanthocephala		
Oligacanthorhynchus tortuosa	**AY210466**[a]	AF064817
Oncicola sp.	**AY210467**[a]	AF064818
Rotifera		
Philodona roseola	**AY210469**[a]	AF154567
Sinantherina socialis	**AY210471**[a]	**AY210451**[a]
Cycliophora		
Symbion sp. (from *Homarus americanus*)	**AY210472**[a]	
Symbion pandora		Y14811
Myzostomida		
Myzostoma polycyclus	**AY210462**[a]	**AY210446**[a]
Ecdysozoa		
Limulus polyphemus	AF212167	U91490
Misumenops asperatus	**AY210461**[a]	**AY210445**[a]
Halicryptus spinulosus	AF342789	AF342790
Deuterostomia		
Antedon serrata		D14357
Florometra serratissima	AF212168	
Ptychodera flava	AF212176	AF278681

[a] Novel sequences in bold.
[b] *Note.* "Echiura" and "Vestimentifera" are within the Annelid radiation (see Halanych, 2004; McHugh, 1997).

employ secondary structure information; Maidak et al., 1999) as guides. Alignments were checked manually with MacClade 4 (Maddison and Maddison, 2000), and regions of questionable alignment were excluded. Alignments are available in Treebase (http://www.treebase.org). Stationarity of nucleotide frequencies was judged using a χ^2 test under the base frequencies option in PAUP.

To better understand relative contributions of each rDNA gene, analyses were carried out on SSU data alone, LSU data alone, and combined data. Due to the need for brevity, we mainly focus on the combined analyses. Maximum likelihood (ML) analyses were conducted in PAUP* version 4.0 b10 (Swofford, 2002). The appropriate likelihood model for each dataset was determined by Modeltest, based upon likelihood scores calculated for an initial neighbor-joining tree (Posada and Crandall, 1998). For each dataset the likelihood model was fixed for both heuristic searches and bootstrap analyses. Details of phylogenetic reconstructions are given in the figure legends. Additionally, a partitioned Bayesian likelihood analysis was performed on the combined data set using a parallelized version of Mr. Bayes v3.0B4 (Altekar et al., 2004; Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) on a Mac computing cluster. Support for previously published lophotrochozoan hypotheses was evaluated using the Shimodaira and Hasegawa (1999) test implemented in PAUP*4.0b10 in which we considered the set of all trees that could reasonably be considered to be true.

3. Results

In the combined data set there were 6659 positions of which 3878 could be unambiguously aligned, 1966 were variable, and 1303 were parsimony informative positions. Taken individually the numbers for the LSU data set were 4611 positions, 2370 unambiguously aligned, 1183 variable, and 804 informative positions. SSU data yielded 2048 positions with 1508 unambiguously aligned, 783 variable and 499 informative positions.

Nucleotides were stationary across taxa for SSU data, but not LSU data. This observation for nuclear rDNA has been previously reported within Lophotrochozoa, specifically mollusks (Passamaneck et al., 2004), and was the reason cephalopod mollusks were not included in this analysis. The rotifer *Philodina* showed the greatest deviation from the mean frequencies of the taxa examined (means A = 0.26657, C = 0.21669, G = 0.29264, T = 0.22410; *Philodina* A = 0.29540, C = 0.19070, G = 0.25673, T = 0.25717). Noteworthy, *Philodina* also exhibited the lon-

gest branch length in the combined (Fig. 1) and LSU (Fig. 2A) analyses, but not the SSU analysis (Fig. 2B). All of the obviously long branched taxa in Fig. 2A (*Philodina*, both acanthocephalans, the flatworm *Dugesia*, the bryozoans *Bugula* and *Crisia*, and the myzostomid) displayed elevated AT frequencies relative to the other taxa. When *Philodina* was excluded with various combinations of one or more other long-branched taxa, stationarity of nucleotide frequencies was not rejected by the χ^2 test. In the ML analyses, the taxa with elevated AT frequencies do not cluster together as expected if differences in nucleotide frequencies are producing artifacts. In contrast, neighbor-joining analysis using a LogDet correction (which should be less sensitive to rate differences than other corrections) does in fact group all of the taxa with elevated AT frequencies together. Thus, in the interest of having adequate taxon sampling across Lophotrochozoa, we decided to not to remove these taxa as many of the issues herein could not be addressed.

ML trees for the combined, LSU and SSU datasets are presented in Figs. 1 and 2, respectively. Results of the partitioned Bayesian analysis on the combined data set (Fig. 3) produced an identical topology as the combined ML analysis. Note we are critical of some of the recovered posterior probability values as they likely represent clades that are not real (e.g., the numerous values of 100 splitting up brachiopods). Phylogenetic reconstructions from LSU and combined datasets recover the monophyly of the nearly all lophotrochozoan phyla. Although the bootstrap support and posterior probabilities for these nodes are generally weak, this result is a

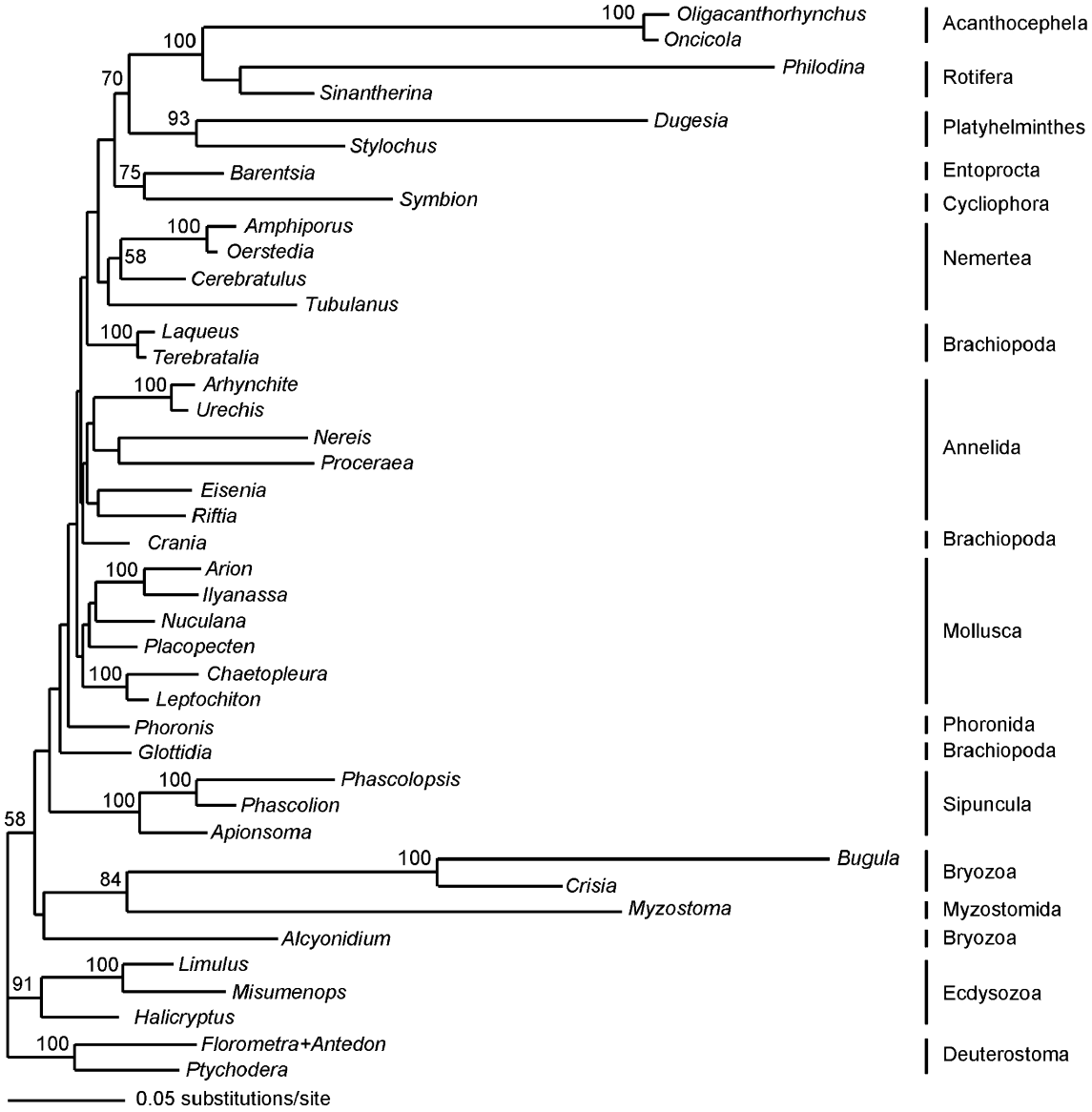

Fig. 1. ML tree for the combined LSU + SSU dataset. One hundred heuristic replicates were performed using the SYM model with equal base frequencies and estimation of γ parameter shape distribution ($\alpha = 0.5750$) and proportion of invariant sites ($I = 0.3234$). ML bootstrap (100 replicates, faststep sequence addition) values are shown above nodes with values >50%. Bayesian likelihood produced the same topology; see text for details. Note "Echiura" and "Vestimentifera" are within the Annelid radiation (see Halanych, 2004; McHugh, 1997).

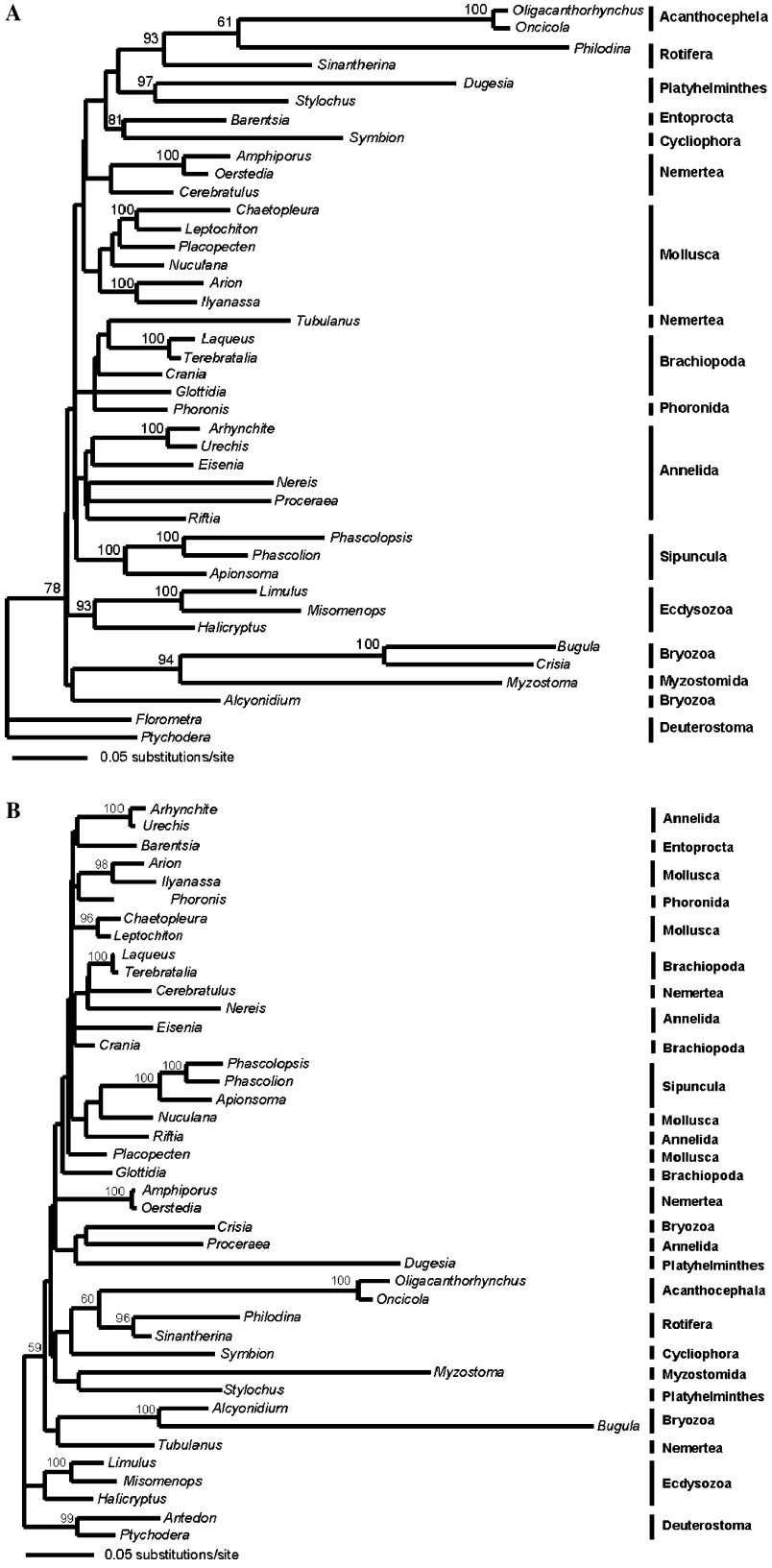

Fig. 2. (A) ML tree for the LSU dataset. One hundred heuristic replicates were performed using the TIM model with equal base frequencies and estimation of gamma parameter shape distribution ($\alpha = 0.5229$) and proportion of invariant sites ($I = 0.3228$). (B) ML tree for the SSU dataset. One hundred heuristic replicates were performed using the TrN model with equal base frequencies and estimation of gamma parameter shape distribution ($\alpha = 0.6199$) and proportion of invariant sites ($I = 0.3112$). For both, ML bootstrap (100 replicates, faststep sequence addition) values are shown above nodes with values $> 50\%$. Note "Echiura" and "Vestimentifera" are within the Annelid radiation (see Halanych, 2004; McHugh, 1997).

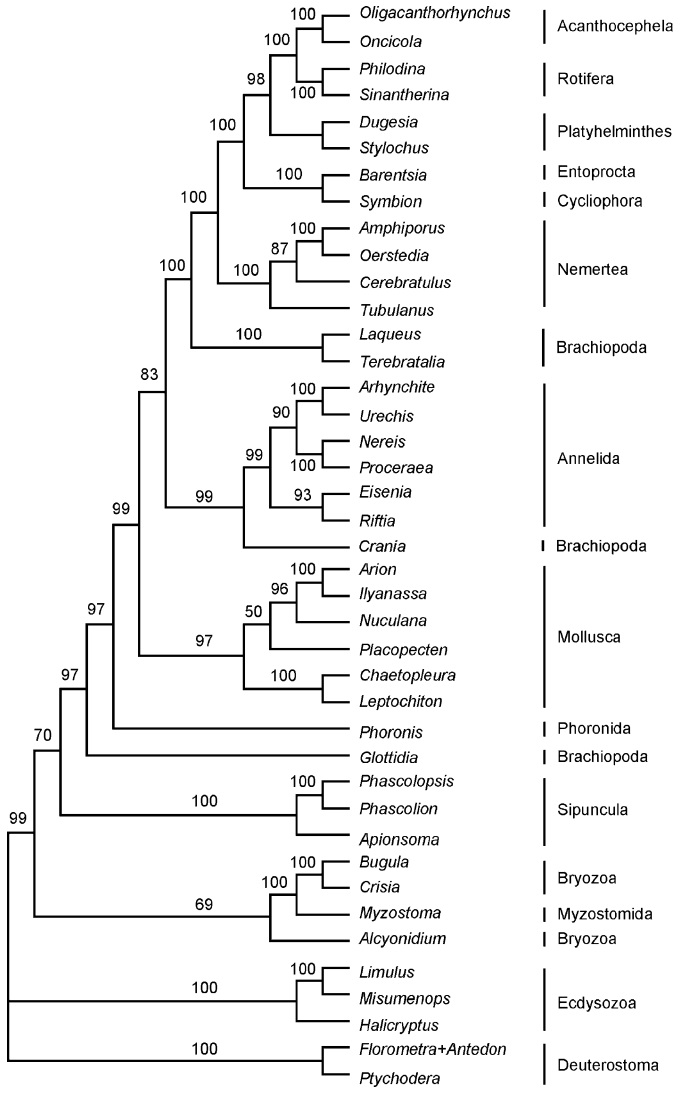

Fig. 3. Bayesian analysis of combined data using a GTR + Γ + I model for each partition using a mixed partition, unlinked search. Individual parameters for each partition were estimated during the search. Twenty million generations were run and sampled every 200 generations retaining generations for which average standard deviation of split frequencies was <0.002 (i.e., first 89,020 out of 100,000 discarded as burnin because it took a long time to reach stationarity). Posterior probabilities (100×) are shown on the relevant node.

substantial improvement over the situation with SSU data alone (compare Figs. 1 and 2B). This boost in signal is clearly due to the LSU data, which also found a tree (Fig. 2A) much more consistent with our morphological understanding of animal relationships (i.e., major phyla monophyletic) than the SSU topology. SSU reconstructions have also been maligned because of the potential for long-branch attraction (Abouheif et al., 1998; Maley and Marshall, 1998). Interestingly, most of the long branches clump together in the SSU tree, but not in the LSU or combined trees, suggesting that rate effects may be less severe in these datasets.

Table 2 gives results of the Shimodaira–Hasegawa tests for combined data, LSU, and SSU data sets. Monophyly of the Lophophorata was significantly rejected by both LSU and combined datasets. In all analyses, Bryozoa consistently fell out basal to other lophotrochozoans, including brachiopods and phoronids. The resultant non-monophyly of Brachiopoda in the combined dataset bears further investigation. Additionally, the hypothesis that Bryozoa is sister to Entoprocta was rejected for both the LSU and combined data sets. Neither result appeared to be affected by the presence of *Myzostoma* within the Bryozoa, as bryozoan monophyly was not significantly rejected under either data set.

Table 2
Shimodaira–Hasegawa test results

	SSU	LSU	LSU + SSU
Lophophorata monophyly	0.128	0.005[a]	0.041[a]
Bryozoa + Entoprocta monophyly	0.173	0.013[a]	0.017[a]
Bryozoa monophyly	0.283	0.260	0.275
Parenchyma monophyly	0.050	0.362	0.011[a]
Neotrochozoa monophyly	0.164	0.443	0.269
Eutrochozoa monophyly	0.056	0.220	0.165
Trochozoa monophyly	0.066	0.114	0.269
Platzoa as a monophyletic sister group to Trochozoa	0.212	0.059	0.133

[a] Monophyly significantly rejected with $P < 0.05$.

LSU and combined analyses recovered a clade including Entoprocta, Cycliopohora, Platyhelminthes, Syndermata (Rotifera + Acanthocephala), and Nemertea. Within this clade the Entoprocta and Cycliophora appear as each other's closest relatives and form a sister group to the Platyzoa. Although the nemertean *Tubulanus* branches within the Brachiopoda in the LSU tree, the Nemertea are recovered as monophyletic in the combined analysis. The combined analysis uniting Platyhelminthes and Syndermata had a likelihood score significantly better than a tree including Platyhelminthes and Nemertea are sister taxa (a.k.a. Parenchyma hypothesis; Nielsen, 2001).

The data are equivocal about the reality of various "trochozoan" hypotheses. LSU data place sipunculans as the sister to annelids, which includes echiurids and siboglinids (a.k.a. pogonophorans including vestimentiferans). The placement of mollusks relative to this clade still is not clear.

4. Discussion

The addition of nearly full-length LSU data to SSU data provides a substantially more resolved topology than SSU alone. In addition to supporting the monophyly of several lophotrochozoan phyla, this study provides evidence for

- the polyphyly of Lophophorata
- a derived monophyletic Platyzoa clade
- a sister relationship between Cycliophora and Entoprocta
- and Syndermata (Rotifera and Acnathocephala) monophyly.

Each of these will be discussed in turn.

The "Lophophorata" hypothesis which unites bryozoans with brachiopods and phoronids, is rejected under SH tests of both the LSU and combined datastets. Likewise, grouping of the Bryozoa and Entoprocta as sister taxa is not supported. These results confirm previous arguments (Halanych, 1996; Nielsen, 2001) that the similarities in feeding mechanics, ciliation patterns, and gross morphology in bryozoans, brachiopods, phoronids, and other tentacular suspension feeders (e.g., pterobranch hemichordates) are the product of convergent evolution rather than common ancestry. Independent origins of bryozoans and brachiopods + phoronids is also supported by recent analyses of sodium–potassium ATPase α-subunit sequence data (Anderson et al., 2004). This recognition renders the term "lophophorates" descriptive of function rather than history.

While the position of Bryozoa differs between the LSU and combined trees, both reconstructions place bryozoans basal to other lophotrochozoans. These results suggest that Bryozoa diverged by at least the early Cambrian period, when other lophotrochozoans such as brachiopods and mollusks first appear in the fossil record. Such a result contrasts with the available paleontological data, given that fossil Bryozoa have not been found from before the Ordovician, despite being well preserved in later sediments (Lehmann and Hillmer, 1983). We therefore hypothesize that Bryozoa went through a period of cryptic evolution, unrecorded in the fossil record. A late evolution of a calcified skeleton is one possible explanation for this discrepancy between the molecular data and the fossil record.

Analyses of both the LSU and combined data sets placed the myzostomid within the Bryozoa. The parasitic myzostomids have traditionally been viewed as derived annelids (e.g., Fauchald and Rouse, 1997; Muller and Westheide, 2000). However, molecular phylogenetic analyses of SSU rRNA and elongation factor-1alpha have suggested a possible link with platyhelminthes (Eeckhaut et al., 2000). Given the long branch lengths and nucleotide frequencies of *Myzostoma* and the bryozoans *Bugula* and *Crisia* in the LSU and combined dataset trees, the weak support for the result, and morphological and other molecular evidence concerning myzostomid origins, we are cautiously skeptically about the bryozoan/myzostomid result. Although an interesting result, more information needs to be gathered to determine its accuracy.

Analyses of both the LSU and combined datasets support Platyzoa monophyly, and place the group well within the Lophotrochozoa. To our knowledge, herein is the strongest, soley molecular, evidence for the existence of Platyzoa. Admittedly, nodal support values are low and addition evidence is needed. Previous studies of combined SSU and morphology datasets have found Platyzoa to be a sister clade to Trochozoa (Giribet et al., 2000) or a paraphyletic grade at the base of the Lophotrochozoa (Peterson and Eernisse, 2001), each supporting, in a general sense, that bilaterians evolved from simple to complex. In contrast, LSU and combined data suggests that the morphology of Platyzoans represent a secondary simplification of body form. Such a secondary simplification could be explained by Rieger's (1986) hypothesis of a progenetic origin of acoelomate adults from the acoelomate larvae, as has been proposed for myzostomids (Eeckhaut et al., 2003). Although the hypothesis of Platyzoa as a sister group to the Trochozoa could not be rejected, placement of the Platyzoa as a derived clade within the Lophotrochozoa provides a markedly different interpretation of bilaterian evolution and warrants further investigation. In particular, sampling of gnathostomulids and gastrotrichs will be required to validate the monophyly of the Platyzoa as defined by Cavalier-Smith (1998).

One putative member of Platyzoa whose evolutionary affinities are drawn into question is the cycliophoran *Symbion*. Analyses of SSU data, including those presented here, have suggested cycliophorans are closely related to rotifers and acanthocephelans. In contrast, the recovery of Cycliophora and Entoprocta as sister taxa in the LSU and combined analyses is consistent with the evolutionary relationship hypothesized when this enigmatic taxon was first described (Funch and Kristensen, 1995), as well as with the results of morphological cladistic analyses (Sørensen et al., 2000; Zrzavy et al., 1998). However, all analyses, to date, have lacked sufficient taxon sampling to thoroughly elucidate the relationship between these taxa. Given that gross morphology is evolutionarily plastic to the point of being misleading (e.g., acanthocephalans are derived rotifers; echiurids are annelids), we should be open to the possibility that cycliophorans are derived entoprocts. The fact that entoprocts are commonly found living on the epidermis or cuticle of other animals is noteworthy given that cycliophorans are found on the mouth parts of some decapod crustaceans.

The idea that rotifers and acanthocephalan formed a monophyletic clade, Syndermata, was put forth by Garey and colleagues (Garey et al., 1996; Garey and Schmidt-Rhaesa, 1998) based on SSU data. Syndermata has also received morphological support (e.g., Zrzavy et al., 1998; Giribet et al., 2000 reanalysis of same data). More specifically acanthocephalans are thought to be within Rotifera (Garey et al., 1996; Herlyn et al., 2003). Interestingly, analysis of an acanthocephalan mitochondrial genome suggests an affinity to platyhelminthes (Steinauer et al., 2005; acoelomorphs were not considered). However, this analysis did not include a rotifer mitochondrial genome. Herein, not only does LSU data support Syndermata, but support is stronger than that from SSU data (LSU bootstrap = 93%, SSU bootstrap = 60%).

Unfortunately, the current analyses still do not offer much resolution among trochozoan relationships. LSU data indicates a sister relationship between annelids and sipunculids, but it is weakly supported due to a very short internal branch length,. This relationship has also recently been reported based on mitochondrial genome data (Boore and Staton, 2002; Jennings and Halanych, 2005). If correct,

this hypothesis would have profound implications for our understanding of the evolution of segmentation in metazoans.

LSU data greatly improves the phylogenetic signal recovered for lophotrochozoan interphyletic relationships over SSU data alone. LSU sequences recover monophyly of nearly all recognized phyla sampled, including mollusks and annelids, which have consistently appeared as polyphyletic in studies using SSU alone. This increase in resolution provides a tool by which we can begin to decipher deep-level relationships within Lophotrochozoa.

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References

Abouheif, E., Zardoya, R., Meyer, A., 1998. Limitations of metazoan 18S rRNA sequence data: implications for reconstructing a phylogeny of the animal kingdom and inferring the reality of the Cambrian explosion. J. Mol. Evol. 47, 394–405.

Altekar, G., Dwarkadas, S., Huelsenbeck, J.P., Ronquist, F., 2004. Parallel Metropolis-coupled Markov chain Monte Carlo for Bayesian phylogenetic inference. Bioinformatics 20, 407–415.

Anderson, F.E., Cordoba, A.J., Thollesson, M., 2004. Bilaterian phylogeny based on analyses of a region of the sodium-potassium ATPase beta-subunit gene. J. Mol. Evol. 58, 252–268.

Balavoine, G., 1997. The early emergence of platyhelminths is contradicted by the agreement between 18S rRNA and Hox genes data. C. R. Acad. Sci. 320, 83–94.

Berney, C., Pawlowski, J., Zaninetti, L., 2000. Elongation Factor 1-alpha sequences do not support an early divergence of the Acoela. Mol. Biol. Evol. 17, 1032–1039.

Boore, J.L., Staton, J.L., 2002. The mitochondrial genome of the Sipunculid *Phascolopsis gouldii* supports its association with Annelida rather than Mollusca. Mol. Biol. Evol. 19, 127–137.

Boyer, B.C., Henry, J.Q., Martindale, M.Q., 1998. The cell lineage of a polyclad turbellarian embryo reveals close similarity to coelomate spiralians. Dev. Biol. 204, 111–123.

Cavalier-Smith, T., 1998. A revised six-kingdom system of life. Biol. Rev. 73, 203–266.

de Rosa, R., Grenier, J.K., Andreeva, T., Cook, C.E., Adoutte, A., Akam, M., Carroll, S.B., Balavoine, G., 1999. HOX genes in brachiopods and priapulids and protostome evolution. Nature 399, 772–776.

Eeckhaut, I., Fievez, L., Muller, M.C., 2003. Larval development of *Myzostoma cirriferum* (Myzostomida). J. Morphol. 258, 269–283.

Eeckhaut, I., McHugh, D., Mardulyn, P., Tiedemann, R., Monteyne, D., Jangoux, M., Milinkovitch, M.C., 2000. Myzostomida: a link between trochozoans and flatworms? Proc. Biol. Sci. 267, 1383–1392.

Eernisse, D.J., 1997. Arthropod and annelid relationships re-examined. In: Fortey, R.A., Thomas, R.H. (Eds.), Arthropod Relationships. Chapman and Hall, London, pp. 43–56.

Fauchald, K., Rouse, G.W., 1997. Polychaete systematics: past and present. Zoolog. Sci. 26, 71–138.

Funch, P., Kristensen, R.M., 1995. Cycliophora is a new phylum with affinities to Entoprocta and Ectoprocta. Nature 378, 711–714.

Garey, J.R., Near, T.J., Nonnemacher, M.R., Nadler, S.A., 1996. Molecular evidence for Acanthocephala as a subtaxon of Rotifera. J. Mol. Evol. 43, 287–292.

Garey, J.R., Schmidt-Rhaesa, A., 1998. The essential role of "minor" phyla in molecular studies of animal evolution. Am. Zool. 38, 907–917.

Giribet, G., Distel, D.L., Polz, M., Sterrer, W., Wheeler, W., 2000. Triploblastic relationships with emphasis on the Acoelomates and the position of Gnathostomulida, Cycliophora, Plathelminthes, and Chaetognatha: a combined approach of 18S rDNA sequences and morphology. Syst. Biol. 49, 539–562.

Halanych, K.M., 1996. Convergence in the feeding apparatuses of lophophorates and pterobranch hemichordates revealed by 18S rDNA: an interpretation. Biol. Bull. 190, 1–5.

Halanych, K.M., 1998. Phylogenetic considerations for metazoans. Am. Zool. 38, 145–155.

Halanych, K.M., 2004. The new view of animal phylogeny. Ann. Rev. Ecol. Evol. Syst. 35, 229–256.

Halanych, K.M., Bacheller, J.D., Aguinaldo, A.M.A., Liva, S.M., Hillis, D.M., Lake, J.A., 1995. Evidence from 18S ribosomal DNA that the lophophorates are protostome animals. Science 267, 1641–1643.

Hatschek, B., 1878. Studien über die Entwicklungsgeschichte der Anneliden. Ein Beitrag zur Morphologie der Bilaterien. Arb. Zool. Inst. Univ. Wien 1: 277 404. Arb. Zool. Inst. Univ. Wien 1, pp. 277–404.

Herlyn, H., Piskurek, O., Schmitz, J., Ehlers, U., Zischler, H., 2003. The syndermatan phylogeny and the evolution of acanthocephalan endoparasitism as inferred from 18S rDNA sequences. Mol. Phylogenet. Evol. 26, 155–164.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.

Hyman, L.H., 1959. The Invertebrates: Smaller Coelomate Groups, Chaetognatha, Hemichordata, Pogonophora, Phoronida, Ectoprocta, Brachiopoda, Sipunculida, the coelomate Bilateria. McGraw-Hill, New York.

Jenner, R.A., 1999. Metazoan phylogeny as a tool in evolutionary biology: current problems and discrepancies in application. Belg. J. Zool. 129, 245–262.

Jenner, R.A., 2002. Boolean logic and character state identity: pitfalls of character coding in metazoan cladistics. Contri. Zool. 71, 67–91.

Jennings, R.M., Halanych, K.M., 2005. Mitchondrial genomes of *Clymenella torquata* (Maldanidae) and *Riftia pachyptila* (Siboglinidae): Evidence for conserved gene order in Annelida. Mol. Biol. Evol. 22, 210–222.

Lehmann, U., Hillmer, G., 1983. Fossil Invertebrates. Cambridge University Press, Cambridge.

Mackey, L.Y., Winnepennickx, B., De Wachter, R., Beckeljau, T., Emschermann, P., Garey, J.R., 1996. 18S rRNA suggests that Entoprocta are protostomes, unrelated to Ectoprocta. J. Mol. Evol. 42, 552–559.

Maddison, W.P., Maddison, D.R., 2000. MacClade: Analysis of Phylogeny and Character Evolution version 4.0. Sinauer Associates, Inc., Sunderland.

Maidak, B.L., Cole, J.R., Parker Jr., C.T., Garrity, G.M., Larsen, N., Li, B., Lilburn, T.G., McCaughey, M.J., Olsen, G.J., Overbeek, R., Pramanik, S., Schmidt, T.M., Tiedje, J.M., Woese, C.R., 1999. A new version of the RDP (Ribosomal Database Project). Nucleic Acids Res. 27, 171–173.

Maley, L.E., Marshall, C.R., 1998. The coming of age of molecular systematics. Science 279, 505–506.

Mallatt, J., Winchell, C.J., 2002. Testing the new animal phylogeny: first use of combined large-subunit and small-subunit rRNA gene sequences to cassify the protostomes. Mol. Biol. Evol. 19, 289–301.

McHugh, D., 1997. Molecular evidence that echiurans and pogonophorans are derived annelids. Proc. Natl. Acad. Sci. USA 94, 8006–8009.

Medina, M., Collins, A.G., Silberman, J.D., Sogin, M.L., 2001. Evaluating hypotheses of basal animal phylogeny using complete sequences of large and small subunit rRNA. Proc. Natl. Acad. Sci. USA 98, 9707–9712.

Muller, M.C., Westheide, W., 2000. Structure of the nervous system of Myzostoma cirriferum (Annelida) as revealed by immunohistochemistry and cLSM analyses. J. Morphol. 245, 87–98.

Nielsen, C., 2001. Animal Evolution: Interrelationships of the Living Phyla, Second Ed. Oxford University Press, Oxford.

Passamaneck, Y.J., Halanych, K.M., 2004. Evidence from Hox genes that bryozoans are lophotrochozoans. Evol. Dev. 6, 275–281.

Passamaneck, Y.J., Schander, C., Halanych, K.M., 2004. Investigation of molluscan phylogeny using large-subunit and small-subunit nuclear rRNA sequences. Mol. Phylogenet. Evol. 32, 25–38.

Peterson, K.J., Eernisse, D.J., 2001. Animal phylogeny and the ancestry of bilaterians: inferences from morphology and 18S rDNA gene sequences. Evol. Dev. 3, 170–205.

Philippe, H., Lartillot, N., Brinkmann, H., 2005. Multigene analyses of bilaterian animals corroborate the monophyly of Ecdysozoa, Lophotrochozoa, and Protostomia. Mol. Biol. Evol. 22, 1246–1253.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Rieger, R.M., 1986. ber den Ursprung der Bilateria: die Bedeutung der Ultrastrukturforshung für ein neues Verstehen der Metazoenevolution. Verh. Dtsch. Zool. Ges. 79, 31–50.

Ronquist, F., Huelsenbeck, J.P., 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Ruiz-Trillo, I., Paps, J., Loukota, M., Ribera, C., Jondelius, U., Baguña, J., Riutort, M., 2002. A phylogenetic analysis of myosin heavy chain type II sequences corroborates that Acoela and Nemertodermatida are basal bilaterians. Proc. Natl. Acad. Sci. USA 99, 11246–11251.

Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114–1116.

Steinauer, M.L., Nickol, B.B., Broughton, R., Orti, G., 2005. First sequenced mitochondrial genome from the phylum Acanthocephala (*Leptorhynchoides thecatus*) and its phylogenetic position within Metazoa. J. Mol. Evol. 60, 706–715.

Swofford, D.L., 2002. PAUP* 4.0 (Phylogenetic Analysis Using Parsimony). Sinauer, Sunderland, Massachusettes.

Sørensen, M.V., Funch, P., Willerslev, E., Hansen, A.J., Olesen, J., 2000. On the phylogeny of the metazoa in the light of Cycliophora and Micrognathozoa. Zool. Anz. 239, 297–318.

Telford, M.J., Lockyer, A.E., Cartwright-Finch, C., Littlewood, D.T., 2003. Combined large and small subunit ribosomal RNA phylogenies support a basal position of the acoelomorph flatworms. Proc. R. Soc. Lond. B 270, 1077–1083.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Winchell, C.J., Sullivan, J., Cameron, C.B., Swalla, B.J., Mallatt, J., 2002. Evaluating hypotheses of deuterostome phylogeny and chordate evolution with new LSU and SSU ribosomal DNA data. Mol. Biol. Evol. 19, 762–776.

Winnepenninckx, B.M.H., Backeljau, T., Kristensen, R.M., 1998. Relations of the new phylum Cycliophora. Nature 393, 636–638.

Zrzavy, J., Milhulka, S., Kepka, P., Bezdek, A., Tietz, D.F., 1998. Phylogeny of the Metazoa based on morphological and 18S ribosomal DNA evidence. Cladistics 14, 249–285.