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Molecular systematics of the Acoela (Acoelomorpha, Platyhelminthes) and its concordance with morphology

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Abstract

The phylogenetic relationships of the lower worm group Acoela were investigated using newly obtained nuclear 18S rDNA sequences from 16 acoels in combination with 16 acoel sequences available on GenBank from other laboratories. Parsimony and maximum likelihood analyses of the molecular data supported the concept that the Acoela is monophyletic; however, the gene tree produced by these analyses conflicts with the current taxonomic system for the Acoela in several family-level groupings. Most notable is the apparent polyphyly of the largest family of acoels, the Convolutidae. DNA analysis grouped together species of small-bodied convolutids in one clade, while large-bodied convolutids grouped in a separate clade with other large-bodied acoels. Despite such conflicts, the branching pattern in the gene tree is well supported by morphological characters of sperm and body-wall musculature. © 2002 Elsevier Science (USA). All rights reserved.

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1. Introduction

The order Acoela is a morphologically diverse group of small (~0.5–10 mm long), soft-bodied, acoelomate worms found predominately in marine habitats. It is the major part of the Acoelomorpha (Acoela + Nemertodermatida), one of the three major clades in the phylum Platyhelminthes (see Smith et al., 1986). This clade is problematic, having no synapomorphies with the rest of the phylum, except possibly the presence of neoblasts (Rieger and Ladurner, 2001). It has been suggested (Haszprunar, 1996; Ruiz-Trillo et al., 1999) that acoels are unrelated to other flatworms and occupy a unique position as basal bilaterians. The most widely cited data supporting this claim are 18S rDNA sequence data (Carranza et al., 1997; Littlewood et al., 1999; Ruiz-Trillo et al., 1999). The same data have also been used to argue against the monophyly of the Acoelomorpha (see

Carranza et al., 1997; Ruiz-Trillo et al., 1999), despite a wealth of morphological synapomorphies that unite these taxa (Smith and Tyler, 1985; Tyler and Rieger, 1977).

In spite of the controversy surrounding the relationship of the Acoela to other bilaterians and the remarkable morphological diversity within the group (Hooge, 2001; Mamkaev, 1967, 1986; Rieger et al., 1991), its own monophyly has not been questioned. The Acoela possesses a number of autapomorphies (Ehlers, 1992; Smith et al., 1986) including epidermal ciliary rootlets with two lateral connections, an acoel-type statocyst bearing a single statolith, digestive parenchyma, spermatozoa with two incorporated axonemes, and monociliated uncolored epidermal receptor cells with specialized ciliary rootlet system. In addition, acoels display a unique duet cleavage pattern (Henry et al., 2000), although this may be a character shared with members of the Nemertodermatida. A number of morphological features of the Acoela display a great deal of plasticity, such as pharynges, gonads, and digestive systems, as do copulatory organs, which provide the primary characters used to

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divide the 332 valid acoel species into 18 families (Tyler and Bush, 2001). Eight of these families contain just a handful or fewer of species, such as the monotypic Antigonariidae, Childiidae, and Tauridae, while 90% of described acoels is classified in the remaining 10 families (Table 1). Unfortunately, many of these families appear to be “grab-bags” composed of unrelated taxa that are grouped together on the basis of morphological characters that may be homoplasious. While copulatory organs clearly contain characters that are useful for systematics, detailed ultrastructural investigations are badly needed in some families of acoels to see beyond superficial similarities and identify the phylogenetically useful elements.

Other potentially phylogenetically informative characters of acoels include structure of the digestive parenchyma (Smith and Tyler, 1985), brain, and sperm structure (Raikova et al., 2001) and patterns of body-wall musculature (Hooge, 2001). While there seems to be strong evidence for a sister-group relationship between the acoel genus *Paratomella* and all other acoels (Ehlers, 1992; Hooge, 2001; Raikova et al., 1997, 2001; Smith and Tyler, 1985), there are few proposals for resolving the relationships of the higher acoels, the Euacoela (all acoels except *Paratomella*; sensu Ehlers, 1992). An investigation of platyhelminth muscles found that patterns of body-wall musculature are useful in clarifying the systematics of the Acoela (Hooge, 2001). In particular, a distinctive pattern of muscles was found to exist in the higher acoels, to the exclusion of more basal families such as the Paratomellidae and Solenofilomorphidae; unique apomorphic muscle characters were found for both the Mecynostomidae and the Childiidae.

In an attempt to corroborate the usefulness of these newly found phylogenetic characters and to test the

validity of the presently held systematics of the Acoela, we have reconstructed a phylogeny of the Acoela using sequences of the 18S rDNA gene, a gene that has already provided meaningful data for other groups of platyhelminths, including the Proseriata (Littlewood et al., 2000) and the Prolecithophora (Norén and Jondelius, 1999). Notoriously fast rates of nucleotide substitutions in acoel 18S rDNA sequences have been a confounding factor in determining the phylogenetic position of the Acoela within the Metazoa (Carranza et al., 1997; Peterson and Eernisse, 2001; Ruiz-Trillo et al., 1999; Zrzavý, 1998). Despite the difficulty in dealing with the fast-rate sequences of the acoels, a preliminary analysis by Ruiz-Trillo et al. (1999) of 18 acoel species yielded a clearly monophyletic group, suggesting that the 18S gene might be useful for deciphering relationships of taxa within the Acoela.

2. Materials and methods

2.1. Collection and DNA extraction

All 17 species sequenced (Table 2) were collected from beaches or mudflats in Maine, USA, as described by Hooge and Tyler (2002). Live animals were extracted from the sediment using magnesium-chloride anesthetization (Sterrer, 1971) and placed in 95% ethanol for preservation. DNA was extracted from whole animals using QIAamp DNA Mini Kit (Qiagen, Chatsworth, CA).

Using polymerase chain reaction (PCR); Saiki et al. (1988) the 18S gene was amplified in two equally sized fragments using the primers 1F and 5R, 5F and 9R (Carranza et al., 1997). Amplification reactions of 25 μ l were performed using 1 μ l template DNA, 0.75 units *Taq* (Invitrogen, San Diego, CA), and final concentrations of 2.5 mM MgCl₂, 1 μ M of each primer, and 0.2 mM of each deoxyribo-nucleotide triphosphate in PCR buffer (pH 9.0). Cycling conditions were 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, 45 s at 72 °C, and a final extension cycle of 15 min at 72 °C. Amplification products were visualized using agarose electrophoresis and desired bands were excised and gel-purified using MinElute Gel Extraction Kits (Qiagen, Chatsworth, CA). DNA sequences were obtained using an Applied Biosystems, Model 377 Automated Sequencer (Foster City, CA), using amplification primers and Big Dye Chemistry (Perkin-Elmer; Foster City, CA). A consensus sequence for each taxon was obtained by comparing forward and reverse sequencing reactions.

2.2. Outgroup choice, sequence analysis, and phylogeny reconstruction

Representatives of seven potential outgroups to the Acoela were included in the analyses (Table 2). By the

Table 1
Families of the Acoela

Family	Number of described species
Actinoposthiidae Dörjes, 1968	43
Anaperidae Dörjes, 1968	12
Antigonariidae Dörjes, 1968	1
Antroposthiidae Faubel, 1976	7
Childiidae Dörjes, 1968	1
Convolutidae Graff, 1905	126
Diopisthoporidae Westblad, 1940	4
Hallangidae Westblad, 1946	2
Haploposthiidae Westblad, 1948	41
Hofsteniidae Bock, 1923	6
Mecynostomidae Dörjes, 1968	33
Nadiniidae Dörjes, 1968	3
Otocelididae Westblad, 1948	18
Paratomellidae Dörjes, 1966	3
Proporidae Graff, 1882	5
Sagittiferidae Kostenko & Mamkaev, 1990	17
Solenofilomorphidae Dörjes, 1968	9
Tauridae Kostenko, 1989	1

Table 2

Taxa used in the molecular analyses, source of sequences used, and GenBank sequence Accession Nos.

Taxon	Source	GenBank Accession No.
Acoela		
Anaperidae Dörjes, 1968		
<i>Anaperus biaculeatus</i> Boguta, 1970	GenBank	AJ012527
<i>Anaperus tvaermimmensis</i> (Luther, 1912)	GenBank	AF102898
<i>Anaperus gardineri</i> Graff, 1911	Wadsworth Cove, ME	AY078365*
Actinoposthiidae Hooge, 2001		
<i>Actinoposthia beklemishevi</i> Mamkaev, 1965	GenBank	AJ012522
<i>Atriofronta polyvacuola</i> Dörjes, 1968	GenBank	AF102895
<i>Pelophila lutheri</i> (Westblad, 1946)	Wadsworth Cove, ME	AY078366*
Childiidae Dörjes, 1968		
<i>Childia groenlandica</i> (Levinsen, 1879)	Wadsworth Cove, ME	AY078367*
Convolutidae Graff, 1905		
<i>Amphiscolops</i> sp.	GenBank	D85099
<i>Aphanostoma virescens</i> Oersted, 1845	GenBank	AJ012528
<i>Aphanostoma bruscai</i> Hooge & Tyler, 2002	Wadsworth Cove, ME	AY078368*
<i>Aphanostoma sanguineum</i> Beklemishev, 1915	Crow Neck, ME	AY078369*
<i>Conaperta hortulus</i> Hooge & Tyler, 2002	Wadsworth Cove, ME	AY078370*
<i>Conaperta norvegica</i> (Westblad, 1946)	Hulls Cove, ME	AY078371*
<i>Convoluta convoluta</i> (Abildgaard, 1806)	GenBank	AJ012524
<i>Convoluta pulchra</i> Smith & Bush, 1991	Otter Cove, ME	AY078372*
<i>Praeconvoluta tigrina</i> Hooge & Tyler, 2002	Crow Neck, ME	AY078373*
<i>Praeconvoluta tornuva</i> Tyler & Hooge, 1999	Wadsworth cove, ME	AY078374*
<i>Pseudaphanostoma smithii</i> Hooge & Tyler, 2002	Wadsworth Cove, ME	AY078375*
Haploposthiidae Westblad, 1948		
<i>Haplogonaria sylvensis</i> Dörjes, 1968	GenBank	AF102900
<i>Simplicomorpha gigantorhabditis</i> Dörjes, 1968	GenBank	AF102894
Mecynostomidae Dörjes, 1968		
<i>Paedomecynostomum bruneum</i> Dörjes, 1968	GenBank	AF102896
<i>Postmecynostomum pictum</i> Dörjes, 1968	GenBank	AF102899
<i>Pseudmecynostomum phoca</i> Hooge & Tyler, 2002	Seal Harbor, ME	AY078376*
Otocelididae Westblad, 1948		
<i>Otocelis sandara</i> Hooge & Tyler, 2002	Crow Neck, ME	AY078377*
<i>Philocelis brueggemanni</i> Hooge & Tyler, 2002	Crow Neck, ME	AY078378*
Paratomellidae Dörjes, 1966		
<i>Paratomella rubra</i> Rieger & Ott, 1971	GenBank	AF102892
<i>Paratomella unichaeta</i> Dörjes, 1966	Seal Harbor, ME	AY078379*
Sagittiferidae Kostenko & Mamkaev, 1990		
<i>Praesagittifera naikaiensis</i> (Yamasu, 1982)	GenBank	D83381
<i>Symsagittifera corsicae</i> Gschwentner et al., 2002	GenBank	AJ319029
<i>Symsagittifera psammophila</i> (Beklemishev, 1957)	GenBank	AF102893
<i>Symsagittifera roscoffensis</i> (Graff, 1891)	GenBank	AJ012530
Solenofilomorphidae Dörjes, 1968		
<i>Myopea</i> sp. “callaeum”	Crow Neck, ME	AY078380*
Nemertodermatida		
<i>Meara</i> sp.	GenBank	AF051328
<i>Nemertinoides elongatus</i> Riser, 1987	Reid State Park, ME	AY078381*
Rhabditophora		
<i>Macrostomum tuba</i> Graff, 1882	GenBank	U70077
Catenulida		
<i>Stenostomum leucops aquariorum</i> Luther, 1960	GenBank	AJ012519
Gastrotricha		
<i>Chaetonotus</i> sp.	GenBank	AJ001735

Table 2 (continued)

Taxon	Source	GenBank Accession No.
Gnathostomulida		
<i>Gnathostomula</i> sp.	GenBank	AF119083
Cnidaria		
<i>Anemonia sulcata</i> (Pennant, 1776)	GenBank	X53498

Note. The asterisks beside the accession numbers indicate original sequences.

current taxonomy, the Nemertodermatida is sister-group to the Acoela (Rieger et al., 1991), and so we used a newly obtained sequence of *Nemertinoidea elongatus* [acquired using the procedures described above; a previously obtained sequence of *N. elongatus* (GenBank Accession No. U70083), which has been reported to be a sequence artifact (Giribet et al., 2000), is approximately 30% different from our newly obtained sequence], as well as a sequence from GenBank for *Meara* sp., to represent this group; all of the other outgroups were represented in our analyses with sequences from GenBank. Because 18S rDNA has been claimed to show the Acoela to be the most basal bilaterians and so sister-group to the diploblasts (Ruiz-Trillo et al., 1999), sequence for the cnidarian *Anemonia sulcata* was chosen as representative; the same data have been used to claim no relationship between Acoela and Nemertodermatida and so the other two major clades in the Platyhelminthes (Rhabditophora and Catenulida) were chosen to be represented by *Macrostomum tuba* and *Stenostomum leucops*, respectively. The Gnathostomulida has been proposed (Ax, 1996) as the outgroup to the Platyhelminthes as a whole (with the Acoela basal-most in the phylum) and so a sequence for a *Gnathostomula* species was taken as representative. The Gastrotricha has been tied with the Platyhelminthes in a group called the Platyzoa (Giribet et al., 2000) and so this phylum was chosen to be represented by sequence from a species of *Chaetonotus*.

The newly acquired sequences were added to other acoel and outgroup sequences from GenBank (Table 2) and aligned using CLUSTAL W (Thompson et al., 1994). Three gap opening penalty/gap extension penalty schemes were examined: 25/5, 10/10, and 10/5. Likewise, three transition weighting schemes were evaluated with respect to transversions (1:1, 2:1, and 3:1). Minor adjustments of the alignment were made as judged by eye using Sequence Navigator (Applied Biosystems, version 1.0, 1994, Foster City, CA). To remove ambiguous sites, both ends of the sequences were truncated, and the middle region, where the two fragments joined, was removed. The sequence alignment used in these analyses is available from <http://devbio.umesci.maine.edu/styler/alignment>.

To assess the amount of saturation of the sequences potentially due to multiple substitutions, the ratio of transversions to transitions (Tv:Ti) was calculated for every pair of taxa and a linear regression was performed

versus Jukes–Cantor genetic distances (Jukes and Cantor, 1969). A Mantel test (Mantel, 1967) was used to evaluate the level of association between the distance and Tv:Ti ratio matrices.

Ruiz-Trillo et al. (1999) found that among the acoel taxa included in their study, only a single species, *Paratomella rubra*, passed a relative-rate test. Given this, we performed relative-rate tests (e.g., Takezaki et al., 1995, Tyler and Rieger, 1999) using PHYLTEST (version 2.0, Kumar, 1996) and Hy-Phy (Muse and Pond, 2000) to detect the presence of rate heterogeneity among our acoel taxa.

Phylogenetic analyses were performed with parsimony (branch-and-bound search) and maximum likelihood optimality criteria using PAUP* (version 4.0b8; Swofford, 1998) with random sequence addition. For maximum likelihood analyses, the program Modeltest (version 3.06, Posada and Crandall, 1998) was employed to select the appropriate model of molecular evolution for the 18S rDNA data set.

Testing for base frequency homogeneity was performed using a χ^2 -test from PAUP*. The skewness statistic (g_1) was obtained from the tree length frequency distribution using the exhaustive parsimony search from PAUP* (Hillis and Huelsenbeck, 1992; Huelsenbeck, 1991). Clade support was evaluated by bootstrapping (1000 replicates; Felsenstein, 1985) and with Bremer support values (Bremer, 1994) which were obtained in PAUP* using a command file created by AutoDecay (version 4.0, Eriksson, 1999).

Alternative maximum parsimony hypotheses were evaluated where the Convolutidae, Haploposthidae, and Otocelidae were each constrained into monophyletic clades. The non-parametric ranked sign test of Templeton (Larson, 1994) was used at $a = 0.5$ to show statistical differences.

3. Results

Only minor differences were detectable between the alignments generated using different gap opening penalty/gap extension penalty schemes and transition weighting schemes. The 10/10 ratio was arbitrarily employed so that gap openings were penalized by the same gap extensions. Base substitutions were weighted equally.

The final truncated-sequence data set consisted of an 1853 basepair (bp) segment of the 18S rDNA gene, of which 1028 bp was parsimony-informative.

The linear regression for the plot of the transversions to transitions ratio (Tv:Ti) versus Jukes–Cantor genetic distance ($y = 0.76x + 0.92$) had a significantly positive slope ($p < 0.05$), indicating that the 18S rDNA gene sequences are not completely saturated with mutations, and are thus potentially phylogenetically informative.

The relative-rate tests revealed three different sequence rates within the data set that were associated with short, medium, and long branches of the parsimony analysis phylogram (Fig. 1). The medium-branch group included the majority of the taxa, while the short-branch group was composed of several acuels belonging to the family Convolutidae that grouped together in a distinct clade in our analyses (Fig. 1). The final sequence rate group, which had long branches, was composed of

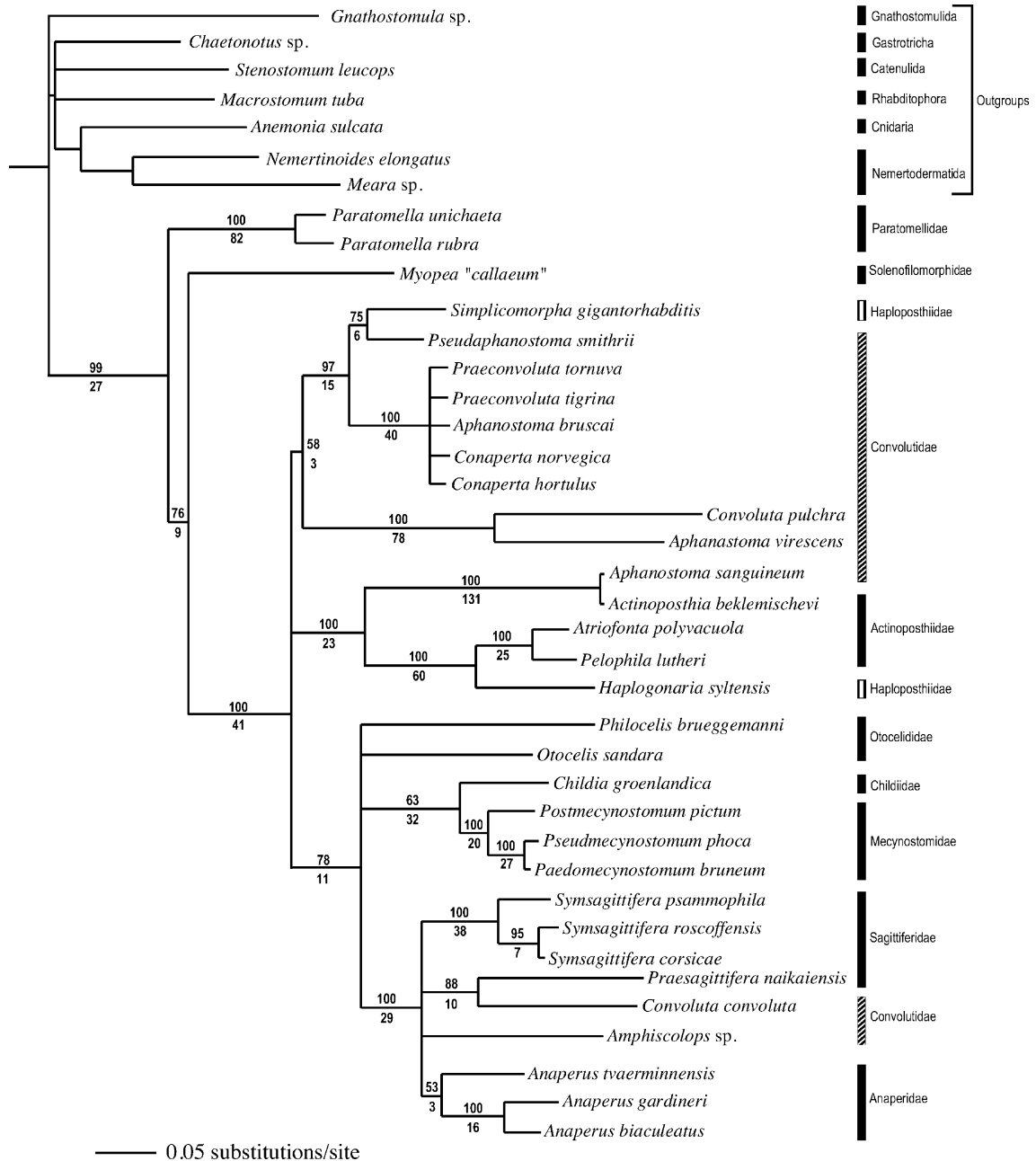


Fig. 1. Bootstrap consensus tree based on maximum parsimony for 18S rDNA sequences from 32 acuels (length 6976 steps, $g_1 = -0.60$ ($p < 0.01$); CI=0.37; RI=0.57; RC=0.21). Topology for this tree was also supported by maximum likelihood analysis (fast heuristic search, distance measure=GTR+I+G). Numbers above branches are bootstrap percentages from 1000 replicates (branch-and-bound, with resampling). Numbers below branches are Bremer support values.

four taxa: *Aphanostoma virescens*, *Convoluta pulchra*, *C. convoluta*, and *Praesagittifera naikaiensis*. Excluding either the short-branch group or the long-branch group did not change the topology of the remaining taxa, and thus, no taxa were removed from the final analyses.

No significant base frequency homogeneity was found among the taxa in our data set. For maximum likelihood analyses, the chosen model was the General Time Reversible model (Rogers, 2001) of base substitutions (GTR; base frequencies: A = 0.2490, C = 0.2027, G = 0.2604, T = 0.2879; substitution model rate matrix: [A–C] = 1.252, [A–G] = 2.898, [A–T] = 1.519, [C–G] = 1.129, [C–T] = 4.023, [G–T] = 1.000) with among-site rate variation (I; 0.2457) and a Gamma (G) distribution shape parameter (α) of 0.89; i.e., GTR + I + G. Tree topology was not altered by the differential weighting of transitions and transversions, or stems and loops (data not shown).

Parsimony analysis produced a bootstrap consensus tree of 6976 steps (Fig. 1; $g_1 = -0.60$ ($p < 0.01$); CI = 0.37; HI = 0.63; RI = 0.57; RC = 0.21); the topology of this tree was also supported by maximum likelihood analysis ($-\ln$ likelihood = 29207.34). Bootstrap and Bremer-support values provide strong support for the monophyly of the Acoela (Fig. 1). All major nodes had bootstrap support exceeding 50% (Fig. 1). Treating gaps as missing data or as a fifth base did not alter the tree topology.

Species of the Paratomellidae and Solenofilomorphidae grouped basally relative to the other acoel species; the others grouped in a polytomy comprising three clades, which are, in general, (1) small-bodied convolutids, (2) actinoposthiids, and (3) large-bodied convolutids + sagittiferids + anaperids + mecynostomids. Several families grouped polyphyletically, most notably the Convolutidae and Haploposthiidae. The clade with large-bodied convolutids, sagittiferids, and anaperids was well supported as was the actinoposthiid clade. That with the small-bodied convolutids was adequately supported but, with comparatively weaker Bremer and bootstrap values. *Childia groenlandica* (Childiidae) was sister-group to the Mecynostomidae and two species of Otocelididae formed two branches of a four-part polytomy within the clade (Fig. 1).

Either constraining a monophyletic Convolutidae or a monophyletic Haploposthiidae resulted in trees that were statistically longer than the maximum parsimony trees ($p < 0.0001$). Constraining a monophyletic Otocelididae though, only added five additional steps which was not significantly longer.

4. Discussion

While the gene tree produced by our analyses groups together species in certain families as would be expected

from the current taxonomic system of the Acoela, its groupings conflict with the current system for four other families. In particular, the Convolutidae, Haploposthiidae, Otocelididae, and the Childiidae *sensu* Dörjes, 1968 (Actinoposthiidae + Childiidae, see Hooge, 2001), all appear to be polyphyletic. Such polyphyly in acoel families has been suspected (Hooge, 2001; Raikova et al., 2001), and in any event, the current classification system does not represent relationships of the Acoela so much as subdivide them into conveniently recognizable family-level groupings on the basis of characters of the male copulatory organ. The branching pattern of the gene tree (Fig. 1) is, however, substantiated by morphological characters of sperm and body-wall musculature (Fig. 2).

Characters of body-wall musculature (Fig. 3) identified by Hooge (2001) include five that are synapomorphies for clades in the gene tree (Fig. 2). Possession of “U-shaped muscles,” i.e., muscles that loop behind the mouth, appears to define the entire clade Acoelomorpha and is, in this cladogram, a symplesiomorphy shared with the Nemertodermatida. Possession of “cross-over muscles” in both the dorsal and ventral body wall, i.e., muscles that originate in the anterior end of the body as longitudinal fibers but cross diagonally behind the mouth, characterizes the clade encompassing all but the paratomellids and solenofilomorphids. The Mecynostomidae clade is supported by the characters “sigmoid-shaped ventral diagonal muscles” and “gap in straight longitudinal muscles anterior to the mouth.” Muscle characters also support the monophyly of the Childiidae. *Childia groenlandica* has a unique pattern of musculature, with reversed U-shaped muscles anterior to the mouth (and apparently secondary loss of the other U-shaped and cross-over muscles) and with longitudinal muscles that are positioned to the outside of the circular muscles. The unique nature of *C. groenlandica* led Hooge (2001) to remove the other species in the Childiidae to a new family, the Actinoposthiidae. The gene tree supports this taxonomic revision; however, some further changes may be necessary, inasmuch as members of the actinoposthiid genus *Paraphanostoma* also have longitudinal muscles positioned outside of their circular muscles (Westblad, 1942).

Whereas the Mecynostomidae appeared to have characters of musculature that implied a derived condition relative to the Convolutidae, with perhaps *Pseudaphanostoma smithrii* representing a transition between the two (Hooge, 2001), the key muscular character defining it, “sigmoid-shaped ventral diagonal muscles,” may be convergent, with *P. smithrii*, a taxon well separated from the Mecynostomidae in the 18S rDNA tree. Additional types of ventral diagonal muscles were found inconsistently in species belonging to other families of acoels (Hooge, 2001).

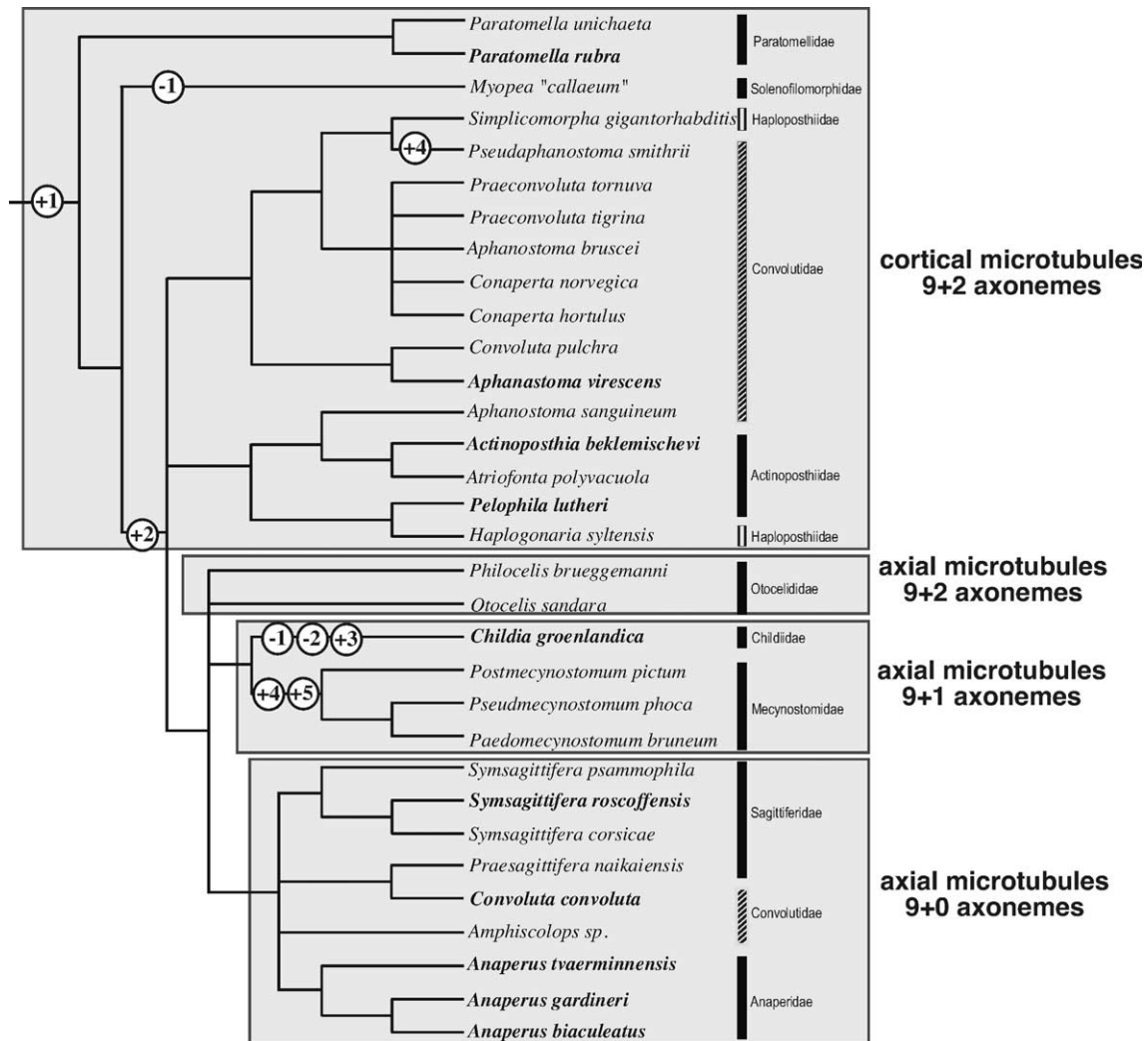


Fig. 2. The distribution of two morphological characters superimposed on an 18S rDNA tree (Fig. 1). Structure of spermatozoa is indicated by labeled shaded areas. Species names in bold denote those taxa for which sperm morphology is known. For complete list of acoels with known sperm morphology refer to Raikova et al. (2001). Character state changes of body-wall musculature are denoted by circled numbers on tree branches. For acoel body-wall musculature characters: (+) new character state, (–) loss of character, (1) U-shaped muscles—longitudinal muscles that wrap around posterior rim of mouth, (2) dorsal cross-over muscles and ventral cross-over muscles, (3) Childiidae musculature—longitudinal muscles positioned outside of circular muscles, inverted U-shaped muscles anterior to mouth, sigmoid-shaped dorsal diagonal muscles positioned inside longitudinals and circulars, (4) sigmoid-shaped ventral diagonal muscles, and (5) gap (absence) in straight longitudinal muscles anterior to mouth.

A remarkable association between the gene tree and characters in sperm ultrastructure provides further concordance of the gene tree (Fig. 2). Acoel spermatozoa have two incorporated axonemes with microtubule patterns of 9 + 2, 9 + 1, or 9 + 0, i.e., either two central singlets, one central core, or no core at all, and they have accessory microtubules in either cortical or axial positions within the sperm. Raikova et al. (2001) showed how these characters fall into four combinations in the Acoela (Figs. 3C–F), and they investigated these combinations for reconstructing acoel phylogeny. In their list of over 30 species of acoels for which sperm morphology is known, Raikova et al. (2001) found that the distribution of the four types of sperm structure presented general paradoxes given the current taxonomy of

the Acoela. Within the Convolutidae, two species, *Aphanostoma virescens* and *Archaphanostoma agile*, have cortical microtubules and 9 + 2 axonemes, while several other species including *Convoluta convoluta* and a species of *Amphiscolops* have axial microtubules and 9 + 0 axonemes. However, this discrepancy can be explained if the Convolutidae is polyphyletic, an idea consistent with the gene tree (Fig. 2). The four patterns of microtubules can be arranged into four distinct groupings in the gene tree for the 10 species in the tree for which the patterns are known; two of these groups correspond to distinct clades in the gene tree, while the other two are paraphyletic assemblages.

We expected the two species of Otocelididae to cluster closer to the small convolutids, with which they share a

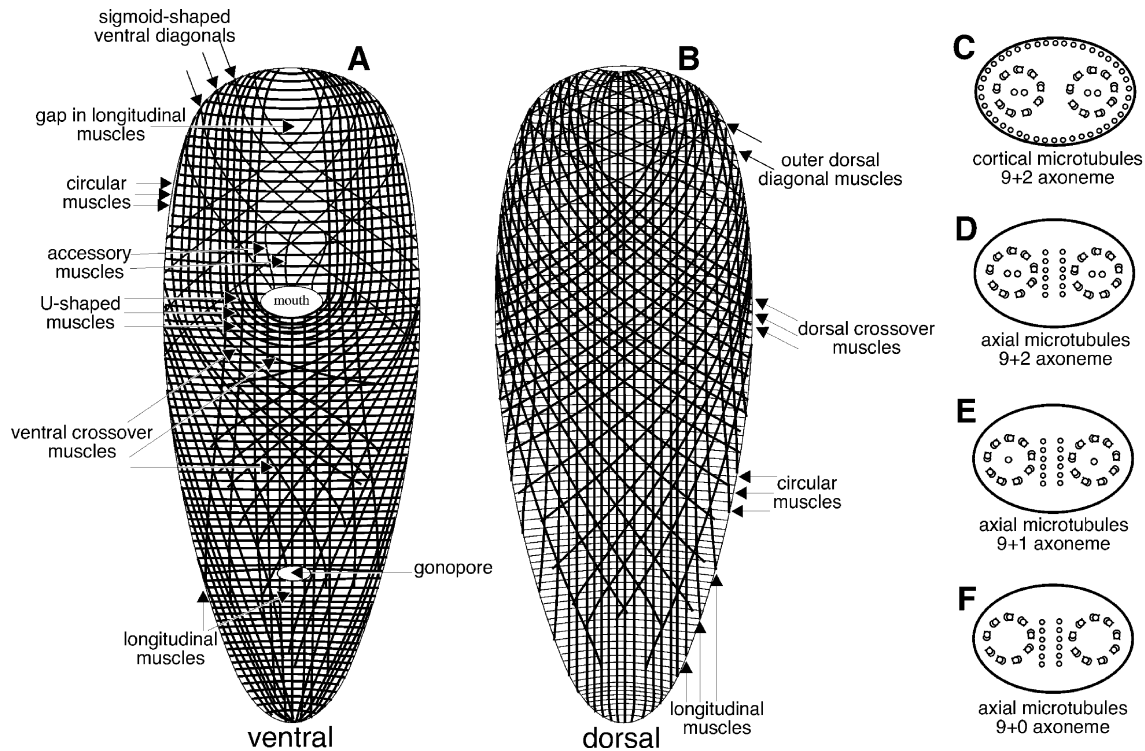


Fig. 3. Acoel morphology. Scheme of arrangement of ventral (A) and dorsal (B) body-wall musculature in mecynostomid acoels (modified from Hooge, 2001). (C)–(F) Diagram of arrangement of cortical microtubules, axial microtubules, and axonemes in acoel sperm (modified from Raikova et al., 2001).

similar morphology. However, in our molecular analyses the otocelidids lie in the clade with the large-bodied convolutids and this position bears support from sperm morphology. Acoels in this clade for which sperm have been studied have axial microtubules; this also applies to a member of the Otocelididae, *Philocelis karlingi* (see Hendelberg, 1977). (The condition in the otocelidids used in our molecular analyses is not known.) Retention of the 9 + 2 axoneme in this otocelidid appears to be a plesiomorphy, perhaps one characteristic of the family.

Possession of a 9 + 1 axoneme and axial microtubules in the sperm appears to be a promising synapomorphy for the Childiidae + Mecynostomidae, judging from the associations evident in our tree (Fig. 2). This pattern of spermatozoa has been found in three species of mecynostomids (Raikova et al., 2001), as well as in *Childia groenlandica* (Hendelberg, 1977), and *Paraphanostoma cycloposthium* (see Raikova et al., 2001).

Spermatozoa with axial microtubules and 9 + 0 axoneme are found in several species that fall in the clade of large-bodied acoels on our tree, namely anaperids, *Convoluta convoluta*, and sagittiferids (Raikova et al., 2001). This sperm morphology may be a robust synapomorphy for these taxa (Fig. 2). The species in this Anaperidae + Convolutidae + Sagittiferidae clade are mostly large animals, typically greater than 2 mm in length; among such convolutids are *C. convoluta*, *Polychoerus carmelensis*, *Polychoerus caudatus*, *Amphiscolops* sp., *Oligochoerus limnophilus*, all of which are known to have

this type of spermatozoa. Smaller convolutids with this type include *Convoluta boyeri* and *Convoluta philippinensis*; another exception in body size is *Symsagittifera corsicae*, which is approximately 850 μ m long (sperm type unknown). The presence of endosymbiotic algae (zooxanthellae and zoochlorellae) is common among the large-bodied acoels in our tree and those known to have axial microtubules and 9 + 0 axoneme, including species of *Amphiscolops*, *Polychoerus*, *Symsagittifera*, and *C. convoluta*. We hypothesize that other large (> 2 mm long) acoels and those with endosymbionts might also group in this clade, including species of the genera *Heterochoerus*, *Haplodiscus*, and *Wulguru*.

Despite the notable association between the gene tree and characters of sperm morphology, several of the acoels listed by Raikova et al. (2001) have sperm morphology different than expected. Three species of the Mecynostomidae, *Eumecynostomum westbladi*, *Mecynostomum auritum*, and *Paramecynostomum diversicolor*, have axial microtubules and 9 + 1 axonemes, but a fourth undescribed species of mecynostomid (“*Mecynostomum* sp.”) is reported to have axial microtubules and 9 + 2 axonemes (Raikova et al., 2001). While the described members of the Actinoposthiidae have 9 + 2 axonemes and cortical microtubules (Fig. 2), one undescribed species of *Pseudactinoposthia* was reported to have axial microtubules and 9 + 1 axonemes (see Rohde et al., 1988) and another undescribed congener in this family was reported with axial microtubules and 9 + 0

axonemes (see Raikova et al., 2001). Confirmation of the affinities of these undescribed species is needed. Finally, because of a misidentification, *Childia groenlandica*, which has 9 + 1 axonemes, has also been reported to have 9 + 0 axonemes (Costello et al., 1969; Henley, 1968, 1974; Henley et al., 1968), but the animal used for these reports is actually *Neochildia fusca*, a large-bodied member of the family Convolutidae (see Bush, 1975).

In at least two cases in which our gene tree appears to conflict with current taxonomy, support for the molecular hypothesis is provided by other morphological characters in addition to sperm and musculature. One case is *Aphanostoma sanguineum*, presently positioned in the family Convolutidae, a placement that is tenuous given its indistinct (and therefore systematically uninformative) copulatory organ (see Hooge, 2001). Its small penis is composed of several thin strands of muscle that resemble the needles in the penes common among actinoposthiids; its position in the gene tree with actinoposthiids lends weight to that similarity. Second is the case of *Convoluta convoluta*, which grouped more closely to a sagittiferid, *Praesagittifera naikaiensis*, than it did to any other convolutid in our analysis. Although these two species differ in general body shape, both taxa possess penes with completely ciliated lumens, a feature uncommon among other acoels.

The remarkable association between our gene tree and the morphological characters of body-wall musculature and sperm suggest that we have made a significant breakthrough in identifying characters that can be used to reorganize the systematics of the Acoela into natural groupings. Extensive collection of data on sperm and muscle morphology, as well as on informative gene sequences, will be necessary to fill in the gaps, especially for the seven acoel families for which we have yet to collect any such information. Compilation of a matrix of other morphological characters that can be used in phylogenetic analyses will also be needed. We foresee making major revisions to the systematics of the Acoela, the first of which will involve splitting the family Convolutidae as well as the genus *Convoluta*.

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