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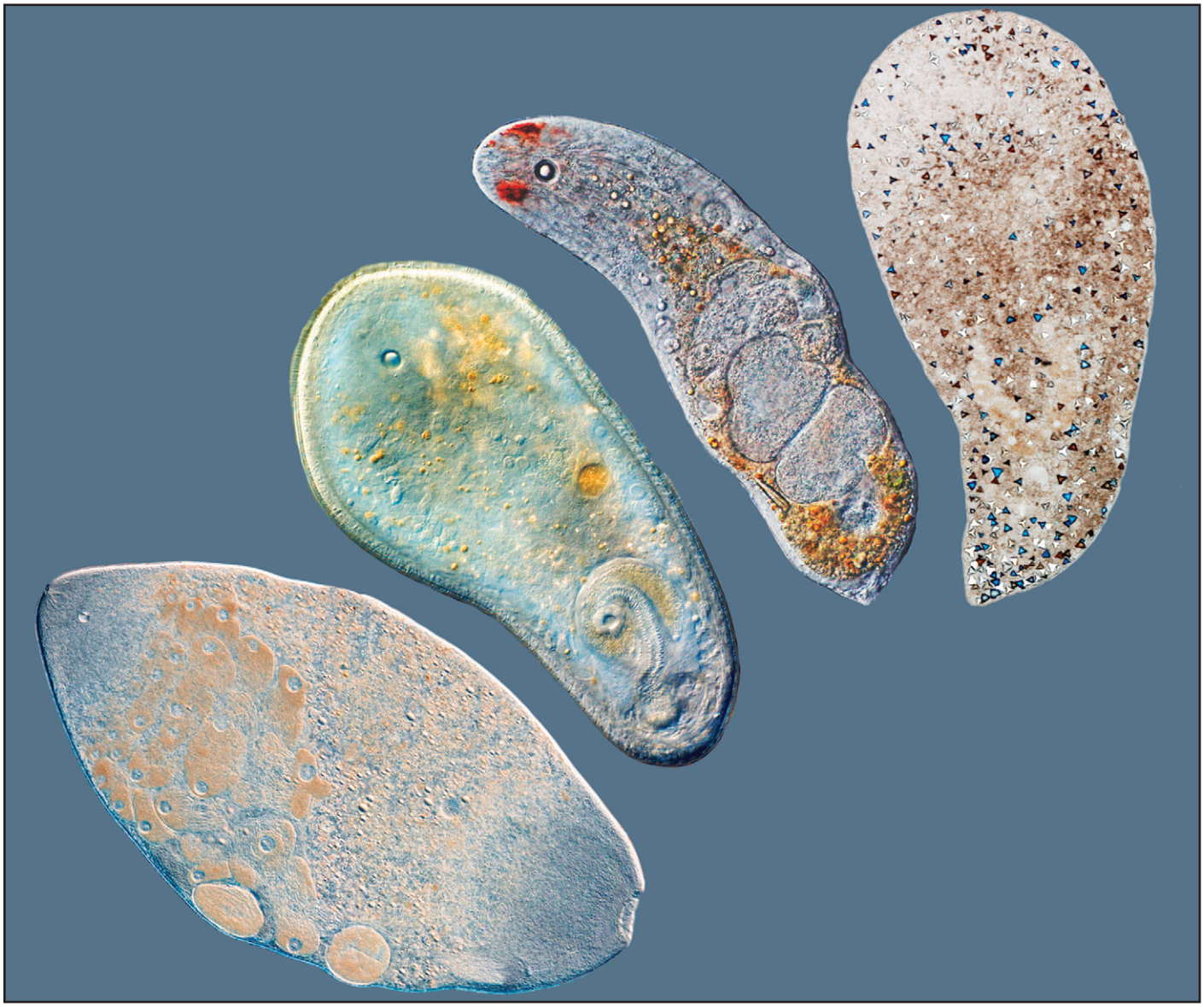

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How the Worm Got its Pharynx: Phylogeny, Classification and Bayesian Assessment of Character Evolution in Acoela

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Abstract.—Acoela are marine microscopic worms currently thought to be the sister taxon of all other bilaterians. Acoels have long been used as models in evolutionary scenarios, and generalized conclusions about acoel and bilaterian ancestral features are frequently drawn from studies of single acoel species. There is no extensive phylogenetic study of Acoela and the taxonomy of the 380 species is chaotic. Here we use two nuclear ribosomal genes and one mitochondrial gene in combination with 37 morphological characters in an analysis of 126 acoel terminals (about one-third of the described species) to estimate the phylogeny and character evolution of Acoela. We present an estimate of posterior probabilities for ancestral character states at 31 control nodes in the phylogeny. The overall reconstruction signal based on the shape of the posterior distribution of character states was computed for all morphological characters and control nodes to assess how well these were reconstructed. The body-wall musculature appears more clearly reconstructed than the reproductive organs. Posterior similarity to the root was calculated by averaging the divergence between the posterior distributions at the nodes and the root over all morphological characters. Diopisthoporidae is the sister group to all other acoels and has the highest posterior similarity to the root. Convolutidae, including several "model" acoels, is most divergent. Finally, we present a phylogenetic classification of Acoela down to the family level where six previous family level taxa are synonymized. [Acoela; ancestor; Bilateria; character evolution; divergence; posterior similarity; reconstruction signal.]

Acoela are small hermaphroditic marine worms (380 spp.) that occur commonly as part of the meiofauna of sandy or muddy sediments as well as among algae. Acoels have attracted considerable attention in recent years because analyses of ribosomal (Ruiz-Trillo et al. 1999; Telford et al. 2003; Wallberg et al. 2007) and protein coding nuclear genes (Ruiz-Trillo et al. 2002; Hejnol et al. 2009) have supported their position as one of the earliest branches within Bilateria and separate from the protostomian Platyhelminthes, in which they were previously classified. These molecular findings are consistent with their comparatively simple morphology: acoels lack excretory organs and other features of the Nephrozoa (Jondelius et al. 2002). Neurotransmitter patterns in acoels indicate a brain composed of transverse commissures and strengthened anterior parts of longitudinal cords different from the typical bilaterian pattern with a central neuropile surrounded by neurons (Raikova et al. 2001; Reuter et al. 2001). Reconstruction of acoel ancestral traits will likely provide important clues to the morphology of the last common bilaterian ancestor and the evolution of more complex bilaterians (Jondelius et al. 2002) but is restricted to inference from extant species since there is no acoel fossil record.

The study of acoels has its challenges, as the worms are small, ranging between 0.3 and 10 mm, and rather fragile. Identification of species often requires studying the reproductive organs of live specimens in a drop of sea water under a high-quality light microscope. The reproductive biology and general appearance of the worms often adds to the difficulty; interesting specimens are often immature and their soft bodies easily disintegrate under a badly applied cover slip. Moreover, many cold water specimens die in less than half an hour at room temperature. Acoela have a limited number of organs but display almost unlimited variation in their configuration. Thus, there is relatively low morphological complexity but high disparity. Such disparity could be expected from a lineage as old as Nephrozoa and likely predating the Cambrian (Budd 2008; Peterson et al. 2008). The mouth can be located almost anywhere along the ventral midline: either in the anterior as in *Proporus*, ventrally as in most Acoela or posteroterminally as in *Diopisthoporus*. A muscular pharynx exists in some taxa, but it has been considered nonhomologous among these groups due to anatomical differences (Todt 2009) implying independent appearance of a pharynx within Acoela. The acoel nervous system is highly variable in terms of position relative to the body wall and level of centralization (Kotikova and Raikova 2008). Acoela are hermaphrodites and there may be paired or single ovaries and testes or, as in *Diopisthoporus* and *Antigonaria* a single mixed gonad. Secondary female organs such as a copulatory bursa for storage of allosperm and a vagina, may be highly developed or completely absent. Male copulatory organs may take the shape of sclerotized stylets, muscular penes, or glandular structures of various shapes. One or two gonopores may be located anywhere along the ventral midline. Even the acoel spermatozoa are diverse. They have two incorporated flagella with either 9 + 2, 9 + 1 or 9 + 0 axoneme configurations and many

different arrangements of cytoplasmic microtubules and other inclusions. It is not surprising that 19th and 20th century systematists struggled with the classification of the group.

In spite of their pivotal position in animal phylogeny, there is no extensive phylogenetic study of the interrelationships within Acoela. The proliferation of family level taxa without reference to a phylogenetic hypothesis (e.g., Westblad 1948; Dörjes 1968; Kostenko 1989; Kostenko and Mamkaev 1990) has resulted in some confusion. In many cases, there are no credible synapomorphies, and the current classification has little predictive power: it is problematic to determine to which higher taxon a new specimen of an unknown species should be referred because classification into taxa such as families is more or less a matter of pigeonholing. Moreover, it is often difficult to identify a specimen belonging to a previously described species.

The largest study to date, which was based on partial 18S rDNA sequences from 32 species of 10 nominal acoel families, retrieved only 4 of these as monophyletic (Paratomellidae, Mecynostomidae, Sagittiferidae, and Anaperidae), whereas three families were reconstructed as nonmonophyletic (Actinoposthiidae, Convolutidae, and Haploposthiidae) and the status of the remaining three families represented was undetermined (Hooge et al. 2002). Hooge and coauthors observed that some features of the spermatozoa and body-wall musculature appeared to fit the topology particularly well, but without a more complete taxonomic sample it is difficult to hypothesize which states are ancestral and important for inferring the features of the last common bilaterian ancestor. There have been attempts to use some acoel species as "models" for Acoela and lower Bilateria, for example, when inferring a complex pattern with longitudinal, circular and U-shaped muscles in the acoel and bilaterian ancestors based on studies of *Isodiametra pulchra* and *Symsagittifera roscoffensis* (Semmler et al. 2008) and when inferring separate origins of the bilaterian mouth and anus based on studies of the development of *Convolutriloba longifissura* (Hejnol and Martindale 2008). The choice of species for detailed study has been based on availability or ease of identification rather than on a hypothesis spanning as much as possible of the phylogenetic diversity. Reconstructions of ancestral states must be based on thorough knowledge of character distributions and a robust phylogenetic hypothesis for the Acoela, both of which are currently lacking. Consequently, explicit hypotheses of character evolution in this group are few, restricted mainly to the evolution of the pharynx (Todt 2009), spermatozoa (Hooge et al. 2002; Achatz et al. 2010), and body-wall musculature (Hooge et al. 2002), and phylogenetic hypothesis testing has generally not been feasible.

Here we set out to reconstruct the phylogeny of Acoela using a comprehensive data set consisting of ribosomal and mitochondrial DNA sequences and morphological characters. We aim to provide a revised and simplified classification of Acoela consistent with the phylogeny, study the evolution of morphological characters, and reconstruct ancestral features of Acoela and of acoel subgroups. In particular, we compare some evolutionary properties of the body-wall musculature with those of the gonads because both organ systems have been highlighted as important for acoel phylogenetics and classification. The evolution of the acoel pharynx, as inferred by us, differs distinctly from the hypothesis put forward by Todt (2009) and we therefore evaluate alternative hypotheses on pharynx homology in more detail. We use a Bayesian framework to account for some the uncertainty of the reconstruction (Ronquist 2004) and demonstrate how this approach can be extended to provide useful tools for systematic research such as estimating the reconstruction potential of characters at deep nodes, morphological divergence among clades and help delineate taxa.

Materials and Methods

Specimens

The material represents a collecting effort spanning more than 10 years and many marine habitats and locations from all over the world (Table 1). Specimens were collected by a variety of methods ranging from beach sampling at low tide or snorkeling to scuba diving or dredging at larger depths. Live specimens were most often fully identified to species using light microscopy, but some unidentified specimens or specimens of unidentified species were only determined to genus. If available, photos of these unidentified worms can be found at the Acoela Scratchpad project (http://acoela.myspecies.info/).

Coding of Morphological Characters

General morphology such as size, shape, pigmentation, presence or absence of algal symbionts, eyes, pharynx, chordoid vacuoles, or frontal glands and most of the characters related to the gonads and genitals were coded for 104 species using original descriptions, live observations, and illustrations or photos of the actual specimen. Ultrastructural and histological information is still not available for many species, and was, with the exception of the musculature in *Diopisthoporus longitubus*, only included for those species for which these features have been described. Information about the presence or absence of interconnecting digestive cells located at the mouth or pharynx was coded for 6 species from Todt (2009); axoneme and microtubular arrangements of spermatozoa were coded for 36 species; (the 9 + 2 axoneme arrangement in *Hofstenia miamia* is our own unpublished observation); and properties of the body-wall musculature were fully coded for 46 species. The body-wall musculature of *Diopisthoporus lofolitis*, as described by Hooge and Smith (2004), was here attributed to the very similar *D. longitubus*, for which we had molecular data. See Table 2 for the full set of references used to code the morphological information. We concluded that information about the nervous system in acoels was still too fragmentary to

TABLE 1. Species and genes sampled for phylogenetic analyses

Group/species	Voucher ID	18S	28S	COI	Source/location
Acoela					
Actinoposthiidae Hooge, 2001					
Actinoposthia beklemischevi Mamkaev, 1965	–	AJ012522	–	–	GenBank
Actinoposthia haplovata Dörjes, 1968	AWHel-30	**FR837674**	**FR837757**	**FR837838**	Offshore Kattegat, Sweden
Atriofronta polyvacuola Dörjes, 1968	–	AF102895	–	–	GenBank
Pelophila lutheri (Westblad, 1946)	–	AY078366	–	–	GenBank
Philactinoposthia coneyi Hooge and Rocha, 2006	MHSc	**FR837724**	**FR837809**	**FR837880**	São Paulo, Brazil
Philactinoposthia nsp.	MHBawiActinoTAN	**FR837726**	–	–	Zanzibar, Tanzania
Philactinoposthia nsp. 2	AWBer-26	**FR837725**	**FR837810**	–	Bermuda
Philactinoposthia saliens (Graff, 1882)	UJ07-60	**FR837727**	**FR837811**	**FR837881**	Koster Area, Swedn
Philactinoposthia sp.	UJ07-33	**FR837731**	**FR837814**	**FR837884**	Koster Area, Sweden
Philactinoposthia sp. 2	UJ08-65	**FR837728**	**FR837812**	**FR837882**	West Coast, New Caledonia
Philactinoposthia sp. 3	AWHel-35	**FR837729**	**FR837813**	**FR837883**	Offshore Kattegat, Sweden
Philactinoposthia sp. 4	UJ04-25	**FR837730**	–	–	Sardinia,Italy
Pseudactinoposthia sanguineum (Beklemischev, 1915)	–	AY078369	–	–	GenBank
Anaperidae Dörjes, 1968					
Anaperus biaculeatus Boguta, 1970	–	AJ012527	AY157602	–	GenBank
Anaperus gardineri (Graff, 1911)	MHAg	AY078365	–	**FR837840**	Maine, USA
Anaperus rubellus Westblad, 1945	UJ07-47	FN552040	FN552040	**FR837841**	Koster Area,Sweden
Anaperus singularis Hooge and Smith, 2004	AWSp-24	–	**FR837758**	**FR837842**	Galiza, Spain
Anaperus tvaerminnensis (Luther, 1912)	–	AF102898	–	–	GenBank
Childiidae Dörjes, 1968					
Childia brachyposthium (Westblad, 1942)	AWHel-27	**FR837681**	**FR837764**	**FR837847**	Offshore Kattegat, Sweden
Childia crassum (Westblad, 1942)	AWHel-08	**FR837682**	**FR837765**	–	Offshore Kattegat, Sweden
Childia cycloposthium (Westblad, 1942)	–	AF329178	AJ849494	–	GenBank
Childia groenlandica (Levinsen, 1879)	AWSp-13	AM701805	**FR837766**	–	Galiza, Spain
Childia macroposthium (Steinböck, 1931)	AWHel-28	**FR837683**	**FR837767**	**FR837848**	Offshore Kattegat, Sweden
Childia nsp. 1	UJ08-37	**FR837684**	**FR837768**	–	Noumea area, New Caledonia
Childia nsp. 2	AWSp-26	**FR837685**	**FR837769**	**FR837849**	Galiza, Spain
Childia sp.	AWHel-23	**FR837686**	**FR837770**	–	Offshore Kattegat, Sweden
Childia submaculatum (Westblad, 1942)	AWHel-02	**FR837687**	**FR837771**	**FR837850**	Offshore Kattegat, Sweden
Childia trianguliferum (Westblad, 1942)	AWHel-25	**FR837688**	**FR837772**	**FR837851**	Offshore Kattegat, Sweden
Childia vivipara Tekle et al., 2006	–	AY297954	AJ849498	-	GenBank
Convolutidae Graff, 1905					
Amphiscolops bermudensis Hyman, 1939	AWA14-Amp	FN552036	FN552037	**FR837839**	Lee Stocking Island, Bahamas
Amphiscolops sp.	–	AMB18SRNA	–	–	GenBank
Amphiscolops sp. "Hendelberg"	–	FN552041	–	–	Gullmar fiord,Sweden
Convoluta cf boehmigi (Brauner, 1920)	UJ05-25	**FR837689**	**FR837773**	–	Lee Stocking Island, Bahamas
Convoluta convoluta (Abildgaard, 1806)	MHCc	**FR837690**	–	**FR837852**	Maine, USA
Heterochaerus blumi (Achatz et al., 2007)	MHAb	FN552038	**FR837788**	**FR837864**	Carrie Bow Cay, Belize
Neochildia fusca Bush, 1975	MHNf	–	**FR837801**	**FR837876**	Maine, USA
Praesagittifera naikaiensis (Yamasu, 1982)	–	DEL18SRNAA	–	–	GenBank
Stomatricha hochbergi Hooge, 2003	MHSh	FN552046	FN552047	**FR837902**	Queensland, Australia
Waminoa brickneri Ogunlana et al., 2005	MHWa	**FR837756**	**FR837837**	–	Eilat,Israel[a]
Waminoa sp.	–	AJ875221	–	–	GenBank
Dakuidae Hooge, 2003					
Daku woorimensis Hooge, 2003	MHDw	**FR837691**	**FR837774**	**FR837854**	Queensland, Australia
Diopisthoporidae Westblad, 1940					
Diopisthoporus longitubus Westblad, 1940	UJ06-06	**FR837692**	**FR837775**	**FR837855**	Offshore Skagerrak, Sweden
Diopisthoporus nsp.	AWSp-16	**FR837693**	**FR837776**	**FR837856**	Galiza, Spain
Diopisthoporus psammophilus Dörjes, 1968	UJ04-45	**FR837694**	**FR837777**	–	Sardinia, Italy
Hallangiidae Westblad, 1946					
Hallangia proporoides Westblad, 1946	UJ06-14	**FR837698**	**FR837780**	**FR837858**	Offshore Skagerrak, Sweden
Haploposthiidae Westblad, 1948					
Haplogonaria "schillingi"	MHRr	**FR837700**	**FR837782**	**FR837859**	Maine, USA
Haplogonaria syltensis Dörjes, 1968	–	AF102900	–	–	GenBank

Continued

TABLE 1. Continued

Group/species	Voucher ID	18S	28S	COI	Source/location
Haplogonaria viridis Dörjes, 1968	UJ07-70	**FR837701**	**FR837783**	**FR837860**	Koster Area, Sweden
*Haploposthia lactomaculata*Tekle, 2004	UJ03-32	**FR837702**	**FR837784**	**FR837861**	Gullmar fiord, Sweden
Haploposthia rubra (An der Lan, 1936)	UJ03-16	**FR837703**	**FR837785**	**FR837862**	Koster Area, Sweden
Haploposthia sp.	UJ05-33	**FR837704**	**FR837786**	–	Lee Stocking Island,Bahamas
Haploposthia Vandula Hooge et al., 2002	AWBer-12	**FR837705**	**FR837787**	**FR837863**	Bermuda
Kuma albiventer (Marcus, 1954)	AWBer-34	**FR837712**	**FR837795**	**FR837872**	Bermuda
Kuma viridis (An der Lan, 1936)	UJ03-34	**FR837713**	**FR837796**	**FR837873**	Gullmar fiord, Sweden
Pseudohaplogonaria rodmani Hooge et al., 2002	MHPh	**FR837749**	**FR837830**	–	Carrie Bow Cay, Belize
Pseudohaplogonaria sp.	UJ05-02	**FR837750**	**FR837831**	**FR837898**	Lee Stocking Island, Bahamas
Simplicomorpha gigantorhabditis Dörjes, 1968	–	AF102894	–	–	
Hofsteniidae Bock, 1923					
Hofstenia miamia Correa, 1960	UJ05-34	AM701809	AM701808	**FR837865**	Lee Stocking Island, Bahamas
Hofsteniola pardii Papi, 1957	AWSp-08	AM701821	AM701822	**FR837866**	Galiza, Spain
Isodiametridae Hooge and Tyler, 2005					
Aphanostoma bruscai Hooge and Tyler, 2003	MHMa5	AY078368	**FR837759**	–	Maine, USA
Aphanostoma collinae Hooge and Tyler, 2008	MHMa14	**FR837675**	–	**FR837843**	Bocas del Toro, Panama
Aphanostoma sp.	AWHel-19	**FR837676**	**FR837760**	**FR837844**	Offshore Kattegat, Sweden
Aphanostoma virescens Oersted, 1845	–	AJ012528	–	–	GenBank
Archaphanostoma macrospiriferum (Westblad, 1946)	UJ07-18	**FR837677**	**FR837761**	**FR837845**	Koster Area, Sweden
Avagina marci Dörjes and Karling, 1975	MHAm	**FR837679**	**FR837763**	–	São Paulo, Brazil
Avagina nsp.	UJ08-14	**FR837680**	–	**FR837846**	Noumea area, New Caledonia
Faerlea glomerata Westblad, 1945	UJACOFaerlea	**FR837697**	–	–	Gullmar fiord, Sweden
Haplocelis dichona (Marcus, 1954)	MHHd	**FR837699**	**FR837781**	–	São Paulo, Brazil
Isodiametra bajaensis Hooge and Eppinger, 2005	MHIb	–	**FR837789**	**FR837867**	Baja California, Mexico
Isodiametra cuernos Hooge and Tyler, 2008	MHI1	**FR837706**	–	–	Bocas del Toro, Panama
Isodiametra divae (Marcus, 1950)	MHMa1	**FR837707**	**FR837790**	**FR837868**	São Paulo, Brazil
Isodiametra hortulus (Hooge and Tyler, 2003)	–	AY078370	–	–	GenBank
Isodiametra nicki Hooge and Tyler, 2008	MHMa16	**FR837708**	**FR837791**	**FR837869**	Bocas del Toro, Panama
Isodiametra norvegica (Westblad, 1946)	MHMa8	**FR837709**	**FR837792**	**FR837870**	Maine, USA
Isodiametra pulchra (Smith and Bush, 1991)	MHCp	**FR837710**	**FR837793**	–	Maine, USA
Isodiametra vexillaria (Marcus, 1948)	UJCove	**FR837711**	**FR837794**	**FR837871**	Belgian coast, Belgium
Otocelis erinae Hooge and Rocha, 2006	MHCo	**FR837718**	**FR837803**	–	São Paulo, Brazil
Otocelis sandara Hooge and Tyler, 2003	MHOs	**FR837719**	**FR837804**	**FR837878**	Maine, USA
Praeconvoluta bocasensis Hooge and Tyler, 2008	MHMa13	**FR837738**	**FR837820**	–	Bocas del Toro, Panama
Praeconvoluta castinea Hooge and Tyler, 2003	MHMa7	**FR837739**	**FR837821**	**FR837890**	Maine, USA
Praeconvoluta tigrina Hooge and Tyler, 2003	MHMa6	**FR837740**	**FR837822**	**FR837891**	Maine, USA
Praeconvoluta tornuva Tyler and Hooge, 1999	–	AY078374	–	–	GenBank
Proaphanostoma tenuissima (Westblad, 1946)	AWSp-22	**FR837741**	**FR837823**	**FR837892**	Galiza, Spain
Pseudaphanostoma herringi Hooge and Rocha, 2006	MHMa2	**FR837747**	**FR837828**	**FR837895**	São Paulo, Brazil
Pseudaphanostoma smithrii Hooge and Tyler, 2003	MHPs	AY078375	-	**FR837896**	Maine, USA
Raphidophallus actuosus Kozloff, 1965	MHMa12	**FR837751**	–	**FR837899**	California, USA
Mecynostomidae Dörjes, 1968					
Eumecynostomum altitudi Faubel and Regier, 1983	–	AY297955	AJ849492	–	GenBank
Mecynostomum auritum (Schultze, 1851)	–	AJ845244	AJ849493	–	GenBank
Mecynostomum sp.	AWSp-34	**FR837715**	**FR837798**	**FR837874**	Galiza, Spain
Paedomecynostomum bruneum Dörjes, 1968	AWK05-47	**FR837720**	**FR837805**	–	Gullmar fiord, Sweden
Paramecynostomum diversicolor (Oersted, 1845)	UJ07-36	**FR837721**	**FR837806**	**FR837879**	Koster Area, Sweden
Paramecynostomum sp.	UJ08-53	**FR837722**	**FR837807**	–	Noumea area, New Caledonia
Philomecynostomum lapillum Dörjes, 1968	–	AF102897	–	–	GenBank
Philomecynostomum sp.	AWSp-41	**FR837734**	**FR837817**	**FR837887**	Galiza, Spain
Postmecynostomum pictum Dörjes, 1968	–	AF102899	–	–	GenBank
Pseudmecynostomum bruneofilum Faubel, 1974	AWSp-02	**FR837748**	**FR837829**	**FR837897**	Galiza, Spain
Pseudmecynostomum phoca Hooge and Tyler, 2003	–	AY078376	–	–	GenBank
Otocelididae Westblad, 1948					
Notocelis gullmarensis (Westblad, 1946)	UJ07-37	–	**FR837802**	**FR837877**	Koster Area, Sweden
Philocelis brueggemanni Hooge and Tyler, 2003	MHPhB	**FR837732**	**FR837815**	**FR837885**	Maine, USA

Continued

TABLE 1. Continued

Group/species	Voucher ID	18S	28S	COI	Source/location
Philocelis karlingi (Westblad, 1946)	–	AJ845243	–	–	GenBank
Philocelis robrochai Hooge and Rocha, 2006	MHSa	**FR837733**	**FR837816**	**FR837886**	São Paulo, Brazil
Paratomellidae Dörjes, 1966					
Paratomella rubra Rieger & Ott, 1971	UJ04-55	**FR837723**	**FR837808**	AY228758	Sardinia, Italy
Paratomella unichaeta Dörjes, 1966	–	AY078379	–	–	GenBank
Polycanthiidae Hooge, 2003					
Polycanthus sp.	UJ08-33	**FR837736**	–	**FR837889**	Noumea area, New Caledonia
Polycanthus sp. 2	UJ08-17	**FR837735**	**FR837818**	**FR837888**	Noumea area, New Caledonia
Polycanthus torosus Hooge, 2003	MHPt	**FR837737**	**FR837819**	–	Queensland, Australia
Proporidae					
Proporus bermudensis Hooge et al., 2002	AWBer-02	**FR837742**	**FR837824**	**FR837893**	Bermuda
Proporus brochii Westblad, 1946	UJ06-18	**FR837743**	**FR837825**	–	Offshore Skagerrak, Sweden
Proporus carolinensis Hooge and Smith, 2004	MHPb	**FR837744**	**FR837826**	–	North Carolina, USA
Proporus sp.	UJ05-23	**FR837746**	**FR837827**	**FR837894**	Lee Stocking Island, Bahamas
Proporus sp. 2	UJProporussp	**FR837745**	–	–	Offshore Skagerrak, Sweden
Sagittiferidae Kostenko and Mamkaev, 1990					
Convolutriloba hastifera Winsor, 1990	–	EU710906	EU710919	EU710926	GenBank
Convolutriloba longifissura Bartolomaeus and Balzer, 1997	AWColo	FN552044	FN552045	**FR837853**	Hawaii, USA[b]
Convolutriloba macropyga Shannon and Achatz, 2007	–	EU710899	EU710916	EU710922	GenBank
Convolutriloba retrogemma Hendelberg and Åkesson, 1988	–	EU710909	EU710914	EU710924	GenBank
Symsagittifera corsicae Gschwentner et al., 2002	–	AJ319029	–	–	GenBank
Symsagittifera psammophila (Beklemischev, 1957)	AWSp-04	**FR837755**	**FR837835**	**FR837903**	Galiza, Spain
Symsagittifera roscoffensis (Graff, 1891)	AWSp-37	AJ012530	**FR837836**	**FR837904**	Galiza, Spain
Solenofilomorphidae Dörjes, 1968					
Endocincta nsp.	AWSp-33	**FR837695**	**FR837778**	**FR837857**	Galiza, Spain
Endocincta punctata Crezée, 1975	UJ07-46	**FR837696**	**FR837779**	–	Koster area, Sweden
Myopea "callaeum"	MHMc	**FR837716**	**FR837799**	**FR837875**	Maine, USA
Myopea sp.	–	AY078380	–	–	GenBank
Oligofilomorpha interstitiophilum Faubel, 1974	–	AM701823	AM701824	–	GenBank
Solenofilomorpha "crezeei"	MHSl	**FR837752**	**FR837832**	–	Maine, USA
Solenofilomorpha nsp.	UJ08-74	**FR837754**	**FR837834**	**FR837901**	Amedee island, New Caledonia
Solenofilomorpha nsp. 2	UJ08-05	**FR837753**	**FR837832**	**FR837900**	Recif Tamanou, New Caledonia
Outgroups					
Cnidaria					
Aurelia sp.	–	AY920770	EU272547	EU366144	GenBank
Montastraea franksi	–	AY026382	AY026375	AY451358	GenBank
Rosacea flaccida	–	AY937328	EU305529	AY937376	GenBank
Nemertodermatida					
Ascoparia sp.	AWHel-24	**FR837678**	**FR837762**	**FR837905**	Offshore Kattegat, Sweden
Meara stichopi	UJ03-52	**FR837714**	**FR837797**	**FR837906**	Bergen, Norway
Nemertoderma sp.	UJ06-16	**FR837717**	**FR837800**	**FR837907**	Offshore Skagerrak, Sweden
Nephrozoa					
Antalis entalis	–	DQ279936	AY145388	AY260818	GenBank
Apionsoma misakianum	–	AY210440	AY210454	EU267000	GenBank
Asterias forbesi	–	DQ077937	AF212169	AF498646	GenBank
Branchiostoma floridae	–	M97571	AF061796	AF098298	GenBank
Ciona intestinalis	–	AB013017	AF212177	AJ517314	GenBank
Nephrops norvegicus	–	FJ174918	DQ079803	FJ174945	GenBank
Priapulus caudatus	–	X80234	AY210840	DQ087502	GenBank
Ramazzottius oberhaeuseri	–	AY582122	FJ435768	FJ435800	GenBank
Terebellides stroemi	–	AY577893	DQ790066	DQ209261	GenBank
Terebratulina retusa	–	U08324	AY839244	AJ245743	GenBank

Note: new sequences produced in this study are highlighted in bold.
[a]Collected by Orit Barneah, Ben-Gurion University.
[b]Donation from Andreas Hejnol, University of Hawaii.

TABLE 2. Primary literature sources used for coding acoel morphology

General morphology

Abildgaard (1806)
Achatz et al. (2007)
An der Lan (1936)
Bartolomaeus and Balzer (1997)
Beklemischev (1915)
Beklemischev (1957)
Boguta (1970)
Brauner (1920)
Bush (1975)
Correa (1960)
Crezée (1975)
Dörjes (1966)
Dörjes (1968)
Dörjes and Karling (1975)
Faubel (1974)
Faubel and Regier (1983)
Graff (1882)
Graff (1891)
Graff (1905)
Graff (1911)
Gray and Rieger (1971)
Gschwentner et al. (2002)
Hendelberg and Åkesson (1988)
Hooge (2003)
Hooge and Eppinger (2005)
Hooge and Rocha (2006)
Hooge and Smith (2004)
Hooge and Tyler (1999)
Hooge et al. (2002)
Hooge and Tyler (2003)
Hooge and Tyler (2005)
Hooge and Tyler (2008)
Hyman (1939)
Kozloff (1965)
Levinsen (1879)
Luther (1912)
Mamkaev (1965)
Marcus (1948)
Marcus (1950)
Marcus (1954)
Oersted (1845)
Ogunlana et al. (2005)
Papi (1957)
Schultze (1851)
Shannon and Achatz (2007)
Smith and Bush (1991)
Steinböck (1931)
Tekle (2004)
Tekle et al. (2006)
Tekle et al. (2007)
Uljanin (1870)
Westblad (1940)
Westblad (1942)
Westblad (1945)
Westblad (1946)
Winsor (1990)
Yamasu (1982)

Body-wall musculature

Gschwentner et al. (2002)
Gschwentner et al. (2003)
Hooge (2001)
Hooge (2003)
Hooge and Eppinger (2005)
Hooge and Rocha (2006)
Hooge and Smith (2004)
Hooge and Tyler (2003)
Shannon and Achatz (2007)
Tekle et al. (2005)
Todt (2009)
Tyler and Hooge (1999)
Tyler and Rieger (1999)

Sperm ultrastructure

Afzelius (1966)
Afzelius (1982)
Costello et al. (1969)
Crezée (1975)
Hendelberg (1969)
Hendelberg (1974)
Hendelberg (1975)
Hendelberg (1977)
Henley and Costello (1969)
Henley (1968)
Henley (1974)
Henley et al. (1968)
Hooge and Tyler (2005)
Mamkaev and Ivanov (1970)
Petrov et al. (2004)
Raikova and Justine (1994)
Raikova et al. (1997)
Raikova et al. (1998)
Raikova et al. (2001)
Raikova and Justine (1999)
Silveira (1967)
Silveira (1969)
Tekle et al. (2007)

be included in the analysis. Coding and management of the data matrix was done with the web-based MX content management system (Yoder et al. 2006). Both binary and multistate characters were coded. The core data set consisted of 37 characters coded for 104 acoel species. Seven additional characters were coded as multistate versions of some binary characters to provide additional details at a few nodes. Three Nemertodermatida species were coded specifically to assess how inclusion of morphological information from this taxon would influence our inference. See Table 3 for the morphological characters and states and the Supplementary Data for the morphological matrix (available from http://www.sysbio.oxfordjournals.org).

Molecular Sequence Assembly and Alignment

We used the 18S rRNA, 28S rRNA, and COI genes as molecular markers for the phylogenetic analyses. Eighty-seven new sequences of about 1780 bp of the 18S rRNA gene and 82 new sequences of about 3200 bp of the 28S rRNA gene were produced for acoels according to the PCR protocols described in Wallberg et al. (2007). A few additional 28S primers were used: F2093 (alternative to U1846) 5″-CCCAYAACCGCATCAGGT-3″, R3182 (alternative to L3449) 5″-AACACTTTGCGAATTCTGCTTCGC-3″, and R3239 (alternative to L3449) 5″-CCTGTCT CACGACGGTCTAAACCC-3″. Sixty-seven new acoel sequences of about 660 bp of the COI gene were produced using the universal LCO/HCO primers and protocols from Folmer et al. (1994) and with minor variations of the annealing temperature around 50 °C. Contigs were produced with Staden v1.6.0 (Staden 1996) and the Phred/Phrap base calling and assembly modules (Ewing and Green 1998; Ewing et al. 1998). The three genes were also sequenced in the three nemertodermatids used as outgroups. Additional acoel and outgroup sequences were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/). See Table 1 for the complete list of sequences included in the analyses. Both ribosomal genes and COI were aligned with Multiple Alignment using Fast Fourier Transform (MAFFT) (Katoh and Toh 2008; Katoh et al. 2009). The COI data were aligned using the nonstructural global Needleman–Wunsch algorithm. Because acoels are known to have unusually variable ribosomal sequences compared with most metazoans (Ruiz-Trillo et al. 1999), 18S and 28S sequences were aligned with alternative algorithms in MAFFT, using either the nonstructural method above or a structural option that aims to incorporate folding properties of the ribosomal genes

TABLE 3. Morphological characters

No.	Character	States
1	Size	0 = small, 1 = conventional, 2 = large
2	Body shape	0 = cylindrical, 1 = convoluted, 2 = flattened
3	Threadlike (slender)	0 = no, 1 = yes
4	Chordoid vesicles (large vacuoles)	0 = absent, 1 = present
5	Level of chordoid vesicles	0 = chordoid vesicles absent, 1 = weakly developed, 2 = well developed
6	Frontal glands	0 = absent, 1 = present
7	Level of frontal glands[a]	0 = frontal glands absent, 1 = weakly developed, 2 = well developed
8	Symbiotic algae	0 = absent, 1 = present
9	Sagittocysts	0 = absent, 1 = present
10	Pigmentation	0 = absent, 1 = present
11	Eyes	0 = absent, 1 = ocelli with platelets, 2 = pigmented eyespots
12	Mouth position	0 = anteroterminal or subterminal, 1 = ventral, 2 = posteroterminal or subterminal
13	Pharynx	0 = absent, 1 = present
14	Interconnecting cells (glandular cells at the pharynx epithelium)	0 = absent, 1 = present
15	Testis	0 = unpaired, 1 = paired
16	Seminal vesicle	0 = absent, 1 = present
17	Glandomuscular eversible copulatory organ	0 = absent, 1 = present
18	Copulatory organ partly inside seminal vesicle	0 = no, 1 = yes
19	Copulatory organ invaginated into seminal vesicle	0 = no, 1 = yes
20	Shape of glandomuscular copulatory organ[a]	0 = glandomuscular copulatory organ absent, 1 = isodiametric tube, 2 = conical tube, 3 = spherical, 4 = globular papilla, 5 = multilayered muscular seminal vesicle
21	Penis stylet (sclerotized copulatory organ)	0 = absent, 1 = present
22	Accessory penetrating hard structures	0 = absent, 1 = adenodactyls, 1 = needles
23	Antrum	0 = absent, 1 = present
24	Antrum ciliation[a]	0 = antrum absent, 1 = ciliated, 2 = not ciliated, 3 = shallow
25	Antrum shape[a]	0 = antrum absent, 1 = long and tubular, 2 = irregular or saclike
26	Male opening position	0 = anteroterminal or subterminal, 1 = midbody, 2 = posteroterminal or subterminal
27	Ovary	0 = unpaired, 1 = paired
28	Bursa seminalis (receptacle for allosperm)	0 = absent, 1 = present
29	Number of bursae[a]	0 = bursa absent, 1 = one, 2 = several
30	Bursal nozzle (sclerotized proximal opening of bursa)	0 = absent, 1 = present
31	Number of bursal nozzles[a]	0 = nozzle absent, 1 = one, 2 = several
32	Vagina	0 = absent, 1 = present
33	Separate female gonopore	0 = absent, 1 = present
34	Ovary positioned or reaches behind testes	0 = no, 1 = yes
35	Hermaphrodite gonad (testis and ovary mixed)	0 = no, 1 = yes
36	Asexual reproduction	0 = absent, 1 = present
37	Sperm axonemes	0 = 9 + 0, 1 = 9 + 1, 2 = 9 + 2
38	Sperm cytoplasmic microtubules	0 = cortical, 1 = axial, 2 = distal only, 3 = corticoaxial
39	Longitudinal muscles enwrap mouth	0 = absent, 1 = present
40	Ventral diagonal muscles	0 = absent, 1 = present
41	Ventral crossover muscles	0 = absent, 1 = present
42	Dorsal diagonal muscles	0 = absent, 1 = present
43	Longitudinal muscles outside of circular muscles	0 = absent, 1 = present
44	Longitudinal muscles between mouth and frontal pore	0 = absent, 1 = present

[a]Character not included in posterior similarity and reconstruction signal computations for nodes.

to assess position homology by using the Vienna RNA package (Hofacker et al. 2002) and MXSCARNA (Tabei et al. 2008) from within MAFFT. To further assess the influence of ambiguously aligned regions in the ribosomal data on downstream phylogenetic analyses, these alignments were filtered using Aliscore (Misof K. and Misof A. 2009), treating gaps as ambiguous characters and using a sliding window size of 4. Thus, four different concatenated data sets were produced and analyzed, with respect to the ribosomal data: nonstructural unfiltered versus filtered alignments; and structural unfiltered versus filtered alignments, respectively. These data sets spanned 126 acoels and the taxon sampling coverage of the 18S, 28S, and COI genes were 95%, 72% and 55%, respectively.

Substitution Models and Phylogenetic Analysis

We used Bayesian, maximum likelihood (ML), and cladistic methods of phylogenetic inference. For the model-based analyses of the genes, we used MrAIC (Nylander 2004) and PhyML v2.4.4 (Guindon and Gascuel 2003) to help select among commonly implemented substitution models. We used GTR + G for phylogenetic analysis of 18S, HKY + G for 28S, and GTR + G for COI. Phylogenies were inferred from the combined

data using either nemertodermatids or additional bilaterian and cnidarian taxa as the outgroup. Bayesian phylogenetic inference was performed using a CVS version of MrBayes v3.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). We performed the analysis using the concatenated molecular data with and without morphological data and used a G model to allow variation in rates among the morphological characters. The default prior settings were used to estimate parameters such as nucleotide state frequencies, substitution rates, and the shape of the site-to-site rate distribution for each gene. We also analyzed the COI gene separately. For the concatenated data we ran two parallel runs of eight Markov chain Monte Carlo (MCMC) chains using a melting temperature of 0.1 because we found chain mixing to be very low and convergence among runs to be insufficient using the default MCMC settings of four chains and a temperature of 0.2. The combination of additional chains and lower temperature make the chains cover approximately the same range of temperatures as the default settings, but subdivided into more intervals, which in our case improved chain mixing and convergence. The COI analyses used the default settings. The chains ran for 10 million generations and trees and parameters were sampled every 1000 generation. We used AWTY (Nylander et al. 2008), LnL, the potential scale reduction factor, and the average standard deviation of split frequencies to monitor and infer convergence. At 10 million generations, the latter measure was always less than 1.5%. Rapid ML bootstrapping of the molecular data was done with the Pthreads version of RAxML v7.0.4 (Stamatakis 2006; Stamatakis et al. 2008). We ran 500 bootstrap replications using the default "fast and easy" settings and a separate GTR + G model for each data partition. Five-hundred parsimony jackknife replications were run in the software TNT (Goloboff et al. 2008), each of which used five random additions holding up to five trees, sectorial search, and a round of tree fusing (Goloboff 1999). We found that the implied weights algorithm generally produced majority-rule consensus trees that were more congruent with the model-based analysis. The ML bootstrap and parsimony jackknife sets of trees were compared with the Bayesian consensus tree.

Reconstruction of Ancestral Morphology

Reconstructions of ancestral morphological states were performed with BayesTraits (Pagel et al. 2004), and the inference of states at the root were compared with a cladistic reconstruction based on the majority-rule consensus tree in MacClade v4 (Maddison D.R. and Maddison W.P. 2001). We used the trees sampled from the stationary distribution in the Bayesian molecular phylogenetic analysis to represent the current best estimate of the interrelationships and evolutionary distances among taxa. To study the extent to which topological differences related to alignment ambiguities in the molecular data influenced our conclusions about morphological evolution in acoels, we analyzed the posterior distribution of trees produced from all four alternative alignments in BayesTraits. Three aspects of morphological evolution were compared among the analyses: the inferred evolutionary rates and reconstruction signals (see below) of each morphological character, as well as the states inferred at ancestral nodes. For each analysis, the original sample of 10,000 trees was thinned to 2000 trees due to computational memory constraints, using an in-house Perl script. The outgroup terminals and uncoded acoel species were pruned and the trees were rooted on the first split inside Acoela using Phyutility (Smith and Dunn 2008). Ancestral states were specified to be estimated for nodes of particular interest using the AddMRCA option in BayesTraits and the Multistate version of the program. Two acoel specifiers were used for each node. We focused on the deep nodes in the tree, those nodes roughly corresponding to the traditional families and a few within-family nodes of particular interest. All in all, 31 such nodes were specified. We used the reversible-jump MCMC algorithm (Pagel and Meade 2006) and tried both the exponential and gamma hyperpriors to seed the prior distribution of rates. An MCMC analysis of 101 million generations was run separately for each character. The first million generations were considered burn-in and discarded. Posterior probabilities of states at all nodes were then sampled every 20,000 generation to avoid autocorrelation among samples, amounting to 5000 samples per character-state and node. The analyses were repeated several times but we noted very little difference in the posterior probabilities between runs using the exponential or gamma hyperprior. The final analyses used the default reversible jump setting (rjhp gamma 0 10 0 10). We repeated the analyses without *Hallangia proporoides* because this taxon was recovered at an unexpected part of the tree. To evaluate alternative hypotheses on the evolution and homology of the pharynx among acoel families, we constrained the states of various basal nodes and took the difference between the harmonic means of the marginal likelihoods among different MCMC runs (Bayes factors) as indications for how well different hypotheses fit our phylogenetic evidence. A difference of 2 indicates "positive" evidence to reject the worse hypothesis, whereas a difference of >5 can be taken as strong evidence (Kass and Raftery 1995). BTParser, a cross-platform Perl program, was written specifically to automate running BayesTraits and summarizing the output and can be downloaded from http://code.google.com/p/btparser/.

Morphological Divergence and Reconstruction Potential

We implemented a posterior similarity index that compares the posterior probabilities of states estimated at each control node in a set of trees with those of the other control nodes to study morphological divergence. The maximum difference between two posterior probability distributions is 2 and it is recovered when the nodes have full support for different states,

whereas minimal difference is 0, which is recovered when the nodes have identical posterior distributions among states. We inverted and normalized the scale between 0 (maximal divergence) and 1 (maximal similarity) to harmonize this index with other commonly used phylogenetic indexes. A benefit of this approach, compared with analyzing averaged branch lengths in a single morphological tree, is that divergence can be directly analyzed for individual characters or arbitrary groups of characters, even when a well-supported morphological tree is difficult to reconstruct with that data or incongruent with the genealogy. Overall posterior similarity between nodes representing acoel families and the root of the tree was computed by averaging the posterior similarities between the nodes and the root for the core set of morphological characters.

To assess how clearly characters were reconstructed over all control nodes and how well the ancestral worms were reconstructed at each control node, we used the Shannon index to capture the shape of the posterior distributions. Whereas posterior probabilities are mainly used to highlight those states that dominate the posterior, the Shannon index can be used to measure the full shape of the posterior and further help distinguish signal from noise. We here computed the reconstruction signal R to capture the ratio of signal to noise in the posterior distributions by taking 1 minus the evenness among the posterior probabilities $(p_1, p_2, \ldots, p_N)$ of all states $(1, 2, \ldots, N)$ for a given character at a given node:

$$R = 1 - \sum_{i=1}^{N} \left(-p_i \times \frac{\ln p_i}{\ln N} \right)$$

A flat posterior where states are recovered with nearly the same probabilities has no signal, whereas a highly unbalanced posterior distribution favoring a particular state has a strong signal (Fig. 1). The main benefit of this approach, compared with evaluating the posterior probabilities of only the dominating states at various nodes, is that the reconstruction signal can be computed for characters with an arbitrary set of states and therefore more naturally lends itself to summation over heterogeneous multistate characters to provide an approximation of how well a particular ancestor is reconstructed and understood. We calculated the overall signal at nodes by averaging over the core set of morphological characters and plotted the estimates on the Bayesian consensus tree. We used the Wilcoxon rank-sum test as implemented in R to test whether a group of nodes or characters appeared to be reconstructed with significantly higher signal than another group, given a common set of trees. Two comparisons were done. We first compared the reconstruction signals at 9 control nodes corresponding to families (Fig. 8a,b; gray nodes) with those of 11 deeper parent nodes (Fig. 8a,b; black nodes), most of which were strongly supported in the phylogenetic analyses. We then compared the reconstruction signals of the characters related to the body-wall musculature of the worms with those related to the reproductive system. BayesTraits draws

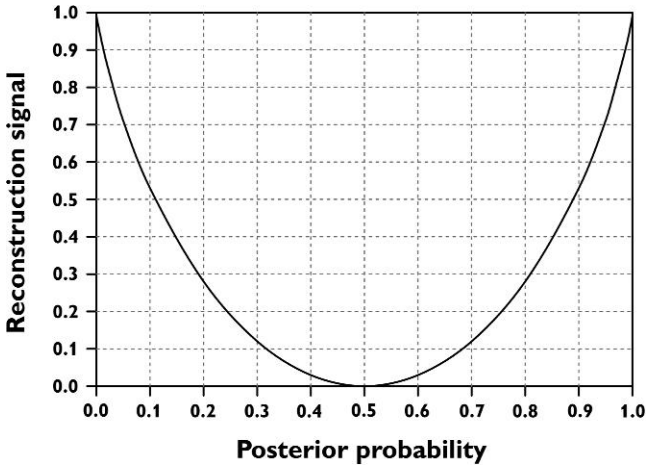

FIGURE 1. Relationship between reconstruction signal and posterior probabilities of states in a binary character. A flat posterior distribution among the two states with $P = 0.5$ has no signal, whereas a highly skewed posterior has a strong signal.

trees randomly from the pool of input trees, so the sequence of sampled trees differs among the MCMC chains of each character. When samples were structured to compare the overall signals of families with their parent nodes, the pattern of sequenced trees were identical among the two sets and estimates generated for all 2000 input trees were compared. To facilitate statistical evaluation between the musculature (six characters, fully sampled in 1109 trees) and reproductive signals (19 characters, fully sampled in 106 trees), we filtered the BayesTraits output to find trees that had been sampled by all 25 MCMC chains, resulting in 55 fully sampled trees. Statistical tests were conducted on a tree-by-tree basis or by pooling the reconstruction signals of each group of samples. In addition to using the full set of taxa in the comparison, we repeated the BayesTraits analyses keeping only those taxa for which the body-wall musculature was coded in the trees.

RESULTS

Phylogenetic Analyses of Molecular Data

The results of the phylogenetic analyses of the concatenated molecular data are summarized on a Bayesian majority-rule consensus tree inferred from the filtered structural alignment in Figure 2. The deep nodes are generally recovered with high support across Bayesian inference, ML bootstrapping, and parsimony jackknifing. Compared with this phylogeny, we detected only minor discrepancies in topologies and clade support among the sets of Bayesian and ML trees inferred from the alternative alignments (see Supplementary Data). Ribosomal sequence alignments are often rich in gaps due to insertions and deletions. For the structural 18S alignment, Aliscore filtered out about 15% of the original positions, yet purging only about 5% of the original sequence data. About 18% of the sequence data were removed from the more variable structural 28S

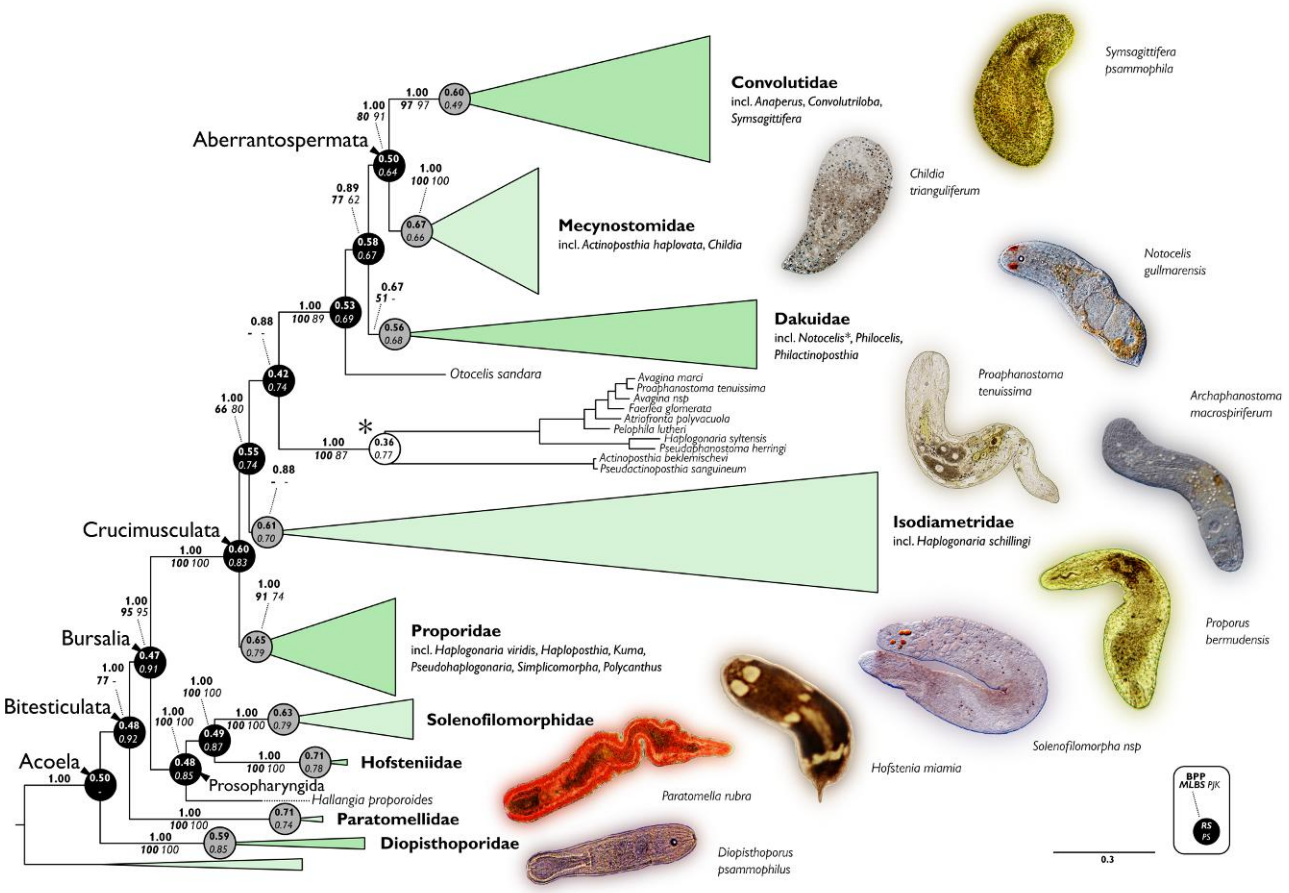

FIGURE 2. Overview of acoel phylogeny. Total-evidence Bayesian majority-rule consensus tree inferred from 18S, 28S, and COI sequences. Bayesian posterior probabilities are shown in bold above branches. ML bootstrap (bold italics) and parsimony jackknife (italics) support below branches. Deep nodes are marked with black circles, nodes corresponding to recognized families are gray. The white control node represents a poorly sampled clade with unstable position. The reconstruction signal (RS) (bold) and posterior similarity to the root (PS) (italics) is displayed on the nodes. A mixed clade with actinoposthiids and isodiametriids with unstable position among analyses of different alignments is marked with an asterisk. Diopoisthoporidae is the sister taxon to all other acoels and has the highest similarity to the features reconstructed at the root. Convolutidae is the most derived taxon.

alignment. For the nonstructural alignments, 6% and 14% of the data were removed, respectively. For both the 18S and 28S genes, the structural alignment approach produced the longest filtered alignments (1796 positions and 2811 positions, respectively). However, the only discernible effect of the different data treatments is a slight escalation of topological uncertainties regarding two nodes in the tree: (i) a clade of actinoposthiids and isodiametrids grouping either inside (nonstructural alignments) or outside of Isodiametridae (structural alignments; Fig. 2; clade marked with an asterisk); and (ii) the unresolved placement of *Notocelis gullmarensis* either inside Dakuidae or basal to it; both of which are based on partially sequenced taxa lacking one of the two ribosomal genes (see further results and discussion below).

In view of the nearly identical topologies produced from the analyses of the alternative alignments, we here chose to focus on the results of the phylogenetic analyses of the longer and potentially least ambiguous filtered versions of the structural data sets. In the phylogeny, *Childia* is nested inside Mecynostomidae. *Anaperus* and those sagittiferid genera included in the analysis are nested inside Convolutidae. Most of the haploposthiid species form a group with *Proporus* and *Polycanthus* but some group within Isodiametridae. Members of the Actinoposthiidae are distributed across Mecynostomidae, Dakuidae, and Isodiametridae. Hofsteniidae and Solenofilomorphidae are recovered as sister taxa, as are Mecynostomidae and Convolutidae. Diopisthoporidae is found to be the sister taxon to all other acoels and further shortens the branch between acoels and other bilaterians. When cnidarians and other bilaterians are included as outgroups, acoels and the included nemertodermatids do not form a monophylum (see Supplementary Material), as was also reported by Wallberg et al. (2007). The molecular tree is presented in more detail in Figure 8a,b.

The Bayesian analysis of the mitochondrial COI gene alone results in a less well-supported consensus

TABLE 4. Reconstructions of ancestors at deep nodes (BPP cut-off = 0.6)

No.	Character	Acoela	BPP	Bitesticulata	BPP	Bursalia	BPP	Crucimusculata	BPP	Prosopharyngida	BPP	Aberrantospermata	BPP
1	Size	?	–	?	–	?	–	Small	0.75	?	–	?	–
2	Body shape	Cylindrical	0.93	Cylindrical	0.95	Cylindrical	0.95	Cylindrical	1	ylindrical	0.96	?	–
3	Threadlike	No	0.76	No	0.71	No	0.76	No	0.8	No	0.76	No	0.78
4	Chordoid vacuoles	Absent	0.98	Absent	0.98	Absent	0.98	Absent	0.89	Absent	0.99	Absent	0.96
5	Level of chordoid vacuoles	Xhordoid vesicles absent	0.86	Chordoid vesicles absent	0.85	Chordoid vesicles absent	0.83	Chordoid vesicles absent	0.71	Chordoid vesicles absent	0.95	Chordoid vesicles absent	0.83
6	Frontal glands	Present	0.93	Present	0.85	Present	0.95	Present	0.96	Present	0.95	Present	0.95
7	Level of frontal glands	?	–	?	–	Well developed	0.76	Well developed	0.97	?	–	?	—
8	Symbiotic algae	Absent	1	Absent	1	Absent	1	Absent	1	Absent	1	Absent	0.68
9	Sagittocysts	Absent	1	Absent	1	Absent	1	Absent	1	Absent	1	Absent	1
10	Pigmentation	Absent	0.81	Absent	0.73	Absent	0.8	Absent	0.7	Absent	0.82	Absent	0.62
11	Eyes	Absent	0.99	Absent	0.99	Absent	0.99	Absent	1	Absent	0.99	Absent	0.7
12	Mouth position	postero-terminal or sub-terminal	0.69	Antero-terminal or sub-terminal	0.75	Antero-terminal or sub-terminal	0.75	Antero-terminal or sub-terminal	0.82	Antero-terminal or sub-terminal	0.78	Ventral	0.9
13	Pharynx	Present	0.98	Present	0.93	Present	0.97	Present	0.92	Present	1	Absent	0.97
14	Interconnecting cells	?	–	?	–	?	–	?	–	?	–	?	–
15	Testis	?	–	Paired	0.92	Paired	0.83	Paired	0.98	?	–	Paired	0.99
16	Seminal vesicle	?	–	?	–	?	–	?	–	?	–	?	–
17	Glandomuscular eversible copulatory organ	absent	0.75	?	–	?	–	Absent	0.76	Present	0.72	Present	0.8
18	Copulatory organ partly inside seminal vesicle	No	0.91	No	0.88	No	0.84	No	0.95	No	0.76	No	0.95
19	Copulatory organ invaginated into seminal vesicle	No	0.99	No	0.97	No	0.95	No	0.98	No	0.91	No	0.99
20	Shape of glandomuscular copulatory organ	?	–	?	–	?	–	?	–	Spherical	0.72	?	–
21	Penis stylet	Present	0.95	Present	0.96	Present	0.95	Present	0.95	Present	0.95	Present	0.97
22	Accessory penetrating hard structures	Absent	0.7	Absent	0.72	Absent	0.74	Absent	0.87	Absent	0.74	Absent	0.63
23	Antrum	present	0.72	Present	0.72	Present	0.69	Present	0.97	?	–	Pbsent	0.92
24	Antrum ciliation	?	–	?	–	?	–	Ciliated	0.76	?	–	Antrum absent	0.85
25	Antrum shape	Long and tubular	0.95	Long and tubular	0.96	Long and tubular	0.94	Long and tubular	0.96	Long and tubular	0.86	Antrum absent	0.93

Continued

TABLE 4. Continued

No.	Character	Acoela	BPP	Bitesticulata	BPP	Bursalia	BPP	Crucimusculata	BPP	Prosopha-ryngida	BPP	Aberranto-spermata	BPP
26	Male opening position	Posteroterminal or sub-terminal	0.7	Midbody	0.64	?	-	Posteroterminal or sub-terminal	1	midbody	0.83	Posteroterminal or subter-minal	0.88
27	Ovary	Unpaired	0.74	Unpaired	0.63	?	–	Unpaired	0.67	?	–	Paired	0.89
28	Bursa seminalis	Absent	0.64	?	–	Present	0.65	?	–	Present	0.74	Present	0.87
29	Number of bursae	?	–	?	–	?	–	?	–	?	–	?	–
30	Bursal nozzle	Absent	0.98	Absent	0.99	Absent	0.99	Absent	0.99	Absent	0.99	Absent	0.65
31	Number of bursal nozzles	Absent	0.97	Absent	0.98	Absent	0.98	Absent	1	Absent	0.98	?	–
32	Vagina	?	–	?	–	?	–	?	–	?	–	?	–
33	Separate female gonopore	Absent	0.78	Absent	0.75	Absent	0.71	Absent	0.98	?	–	Present	0.88
34	Ovary positioned or reaches behind testes	Yes	0.97	Yes	0.98	Yes	0.98	No	1	Yes	0.98	No	0.97
35	Hermaphrodite gonad	?	–	No	0.89	No	0.9	No	0.91	No	0.83	No	0.99
36	Asexual reproduc-tion	Absent	1	Absent	0.99	Absent	1	Absent	1	Absent	1	Absent	1
37	Sperm axonemes	9 + 2	1	9 + 2	1	9 + 2	1	9 + 2	1	9 + 2	0.98	?	–
38	Sperm cytoplas-mic micro tubules	Cortical	0.96	Cortical	0.95	Cortical	0.86	Cortical	0.97	?	–	?	–
39	Longitudinal muscles enwrap mouth	Absent	0.88	Absent	0.68	Absent	0.81	?	–	Absent	0.98	?	–
40	Ventral diagonal muscles	Present	1	Present	1	Present	1	Present	1	Absent	0.69	Present	1
41	Ventral crossover muscles	Absent	0.96	Absent	0.89	?	–	Present	1	Absent	0.97	Present	0.99
42	Dorsal diagonal muscles	Absent	0.83	Absent	0.69	Absent	0.79	Absent	0.64	Absent	0.99	Present	0.95
43	Longitudinal muscles outside of circular muscles	Absent	1	Absent	1	Absent	1	Absent	1	Absent	0.99	Absent	099
44	Longitudinal muscles between mouth and frontal pore	Absent	0.6	Absent	0.6	Absent	0.6	?	–	Present	0.68	Absent	0.75

TABLE 5. Reconstruction of family ancestors (BPP cut-off = 0.6)

Nr	Character	Diopisthoporidae	BPP	Paratomellidae	BPP	Hofsteniidae	BPP	Solenofilomorphidae	BPP	Proporidae	BPP	Isodiametridae	BPP	Dakuidae	BPP	Convolutidae	BPP	Mecynostomidae	BPP
1	Size	?	0	Conventional	0.72	Large	0.7	?	0	Small	0.74	?	0	?	0	?	0	Conventional	0.64
2	Body shape	Cylindrical	0.94	Cylindrical	0.99	Cylindrical	0.99	Cylindrical	0.98	Cylindrical	0.99	cylindrical	0.99	Cylindrical	0.92	Convoluted	0.79	Cylindrical	0.99
3	Threadlike	No	0.77	Yes	0.91	No	0.84	No	0.6	No	0.8	No	0.8	No	0.78	No	0.79	No	0.79
4	Chordoid vesicles	Absent	0.98	Absent	1	Absent	1	Absent	1	Absent	0.98	Absent	0.78	Present	0.79	Absent	1	Absent	0.81
5	Level of chordoid vesicles	Chordoid vesicles absent	0.93	Chordoid vesicles absent	0.99	Chordoid vesicles absent	0.99	Chordoid vesicles absent	0.98	Chordoid vesicles absent	0.78	?	0	?	0	Chordoid vesicles absent	0.99	Chordoid vesicles absent	0.66
6	Frontal glands	Present	0.97	Absent	1	Present	0.99	Present	0.81	Present	0.99	Present	0.88	Present	0.97	Present	0.95	Present	0.99
7	Level of frontal glands	?	0	Frontal glands absent	0.99	Weakly developed	0.98	?	0	Well developed	0.99	?	0	Well developed	0.9	Weakly developed	0.95	Well developed	0.85
8	Symbiotic algae	Absent	1	Absent	1	Absent	1	Absent	1	Absent	1	Absent	1	Absent	1	Present	0.96	Absent	1.00
9	Sagittocysts	absent	1	Absent	1	Absent	1	Absent	1	Absent	1	Absent1	Absent	1	Absent	1	Absent	1.00	
10	Pigmentation	Absent	0.88	Present	0.61	Present	0.99	Absent	0.92	Present	0.75	Absent	0.89	Absent	0.85	Present	0.97	Pbsent	0.95
11	Eyes	Absent	0.99	Absent	1	Absent	1	Absent	0.99	Absent	1	Absent	0.99	Absent	0.94	Ocelli with platelets	0.75	Absent	0.99
12	Mouth position	Posteroterminal or subterminal	0.88	Ventral	0.96	Anteroterminal or subterminal	0.97	Ventral	0.93	Anteroterminal or subterminal	0.85	Ventral	0.95	Ventral	0.86	Ventral	0.95	Ventral	0.95
13	Pharynx	Present	1	Absent	0.99	Present	1	Present	1	Present	0.92	Absent	0.99	Absent	0.95	Absent	0.99	Absent	0.99
14	Interconnecting cells	?	0	Absent	0.62	Present	0.68	Present	0.61	?	0	?	0	?	0	?	0	?	0.00
15	Testis	Unpaired	0.98	Paired	1	Unpaired	0.68	Unpaired	1	Paired	0.75	Paired	0.99	Paired	0.98	Paired	0.99	Paired	0.99
16	Seminal vesicle	?	0	Present	0.67	Present	0.7	?	0	?	0	Present	0.61	?	0	Absent	0.62	?	0.00
17	Glandomuscular eversible copulatory organ	Absent	0.89	Absent	0.98	Present	0.99	Absent	0.77	Absent	0.97	Present	0.85	Absent	0.77	Present	0.86	Present	0.67
18	Copulatory organ partly inside seminal vesicle	No	0.95	No	0.99	No	0.88	No	0.91	No	1	No	0.65	No	0.95	No	0.85	No	0.98
19	Copulatory organ invaginated into seminal vesicle	No	0.99	No	1	No	1	No	0.96	No	1	No	0.84	No	0.99	No	0.94	No	1.00
20	Shape of glandomuscular copulatory organ	glandomuscular copulatory organ absent	0.65	Glandomuscular copulatory organ absent	0.92	Spherical	0.98	?	0	Glandomuscular copulatory organ absent	0.85	Isodiametric	0.79	?	0	Conical	0.98	Globular papilla	0.87
21	Penis stylet	Absent	0.87	Present	1	Present	1	Absent	0.96	Absent	0.99	Absent	0.97	Present	0.99	Absent	0.97	Present	0.99
22	Accessory penetrating hard structures	Absent	0.74	Absent	0.88	Absent	0.9	Absent	0.82	Absent	0.84	Absent	0.85	Absent	0.74	?	0	Absent	0.85

Continued

TABLE 5. Continued

Nr	Character	Diopistho-poridae	BPP	Paratomellidae	BPP	Hofsteniidae	BPP	Solenofilo-morphidae	BPP	Proporidae	BPP	Isodiametridae	BPP	Dakuidae	BPP	Convolutidae	BPP	Mecynostomidae	BPP
23	Antrum	Present	0.74	Present	0.97	Present	0.98	Present	0.94	Present	0.98	Present	0.84	Absent	0.88	Absent	0.93	Absent	0.96
24	Antrum ciliation	?	0	Ciliated	0.8	Ciliated	0.83	Ciliated	0.72	Ciliated	0.78	Ciliated	0.65	Antrum absent	0.8	Antrum absent	0.83	Antrum absent	0.91
25	Antrum shape	Long and tubular	0.89	Long and tubular	1	Long and tubular	1	Long and tubular	0.99	Long and tubular	1	Long and tubular	0.69	Antrum absent	0.91	Antrum absent	0.89	Antrum absent	0.98
26	Male opening position	Postero-terminal or Sub-terminal	0.94	Midbody	0.99	Anteroterminal or subter-minal	1	Midbody	0.93	Posteroterminal or subter-minal	1	Posteroterminal or subter-minal	0.98	Posteroterminal or subter-minal	0.91	?	0	Posteroterminal or subter-minal	0.95
27	Ovary	Unpaired	0.85	Unpaired	0.97	Unpaired	0.71	Unpaired	0.85	Unpaired	0.95	Paired	0.83	Paired	0.82	Paired	0.94	Paired	0.86
28	*Bursa seminalis*	Absent	0.81	Absent	0.93	Absent	0.94	Present	0.84	Absent	0.83	Present	0.74	Present	0.84	Present	0.9	Present	0.91
29	Number of bursae	?	0	Absent	0.68	Absent	0.71	?	0	?	0	?	0	?	0	?	0	One	0.61
30	Bursal nozzle	Absent	0.98	Absent	1	Absent	1	Absent	1	Absent	1	Absent	0.88	Present	0.97	Present	1	Absent	1.00
31	Number of bursal nozzles	Absent	0.97	Absent	1	Absent	1	Absent	0.99	Absent	1	Absent	0.95	One	0.81	Several	0.71	Absent	1.00
32	Vagina	?	0	?	0	Absent	0.62	?	0	?	0	?	0	?	0	?	0	?	0.00
33	Separate female gonopore	Absent	0.85	Absent	0.98	Absent	0.98	Absent	0.89	Absent	0.97	Absent	0.96	Present	0.83	Present	0.87	Present	0.87
34	Ovary positioned or reaches behind testes	No	0.93	Yes	1	Yes	1	Yes	1	No	0.99	No	0.98	No	0.95	No	0.99	No	0.98
35	Hermaphrodite gonad	Yes	0.99	No	0.69	No	1	Yes	1	No	0.89	No	1	No	0.99	No	1	No	1.00
36	Asexual reproduc-tion	absent	1	Absent	0.8	Absent	1	Absent	1	Absent	1	Absent	1	Absent	1	Absent	1	Absent	1.00
37	Sperm axonemes	9 + 2	0.97	9 + 2	0.99	9 + 2	0.99	9 + 2	0.96	9 + 2	0.95	9 + 2	0.99	9 + 2	0.97	9 + 0	1	9 + 1	0.98
38	Sperm cytopla-smic micro-tubules	Cortical	0.9	Cortical	0.96	?	0	?	0	Cortical	0.85	Cortical	0.98	Axial	0.64	Axial	0.93	Distal only	0.88
39	Longitudinal muscles enwrap mouth	absent	0.97	Present	0.87	Absent	0.99	Absent	0.98	Absent	0.65	Present	0.92	Present	0.77	Present	0.91	?	0.00
40	Ventral diagonal muscles	Absent	0.71	Present	1	Absent	0.9	Absent	0.86	present	1	Ppresent	0.99	Present	1	Absent	0.86	Present	1.00
41	Ventral crossover muscles	Absent	0.96	Absent	0.98	Absent	0.99	Absent	1	Present	1	Present	0.99	Present	0.99	Present	0.99	Present	0.96
42	Dorsal diagonal muscles	Absent	0.98	Present	0.89	Absent	0.99	Absent	1	Absent	0.65	Present	0.93	Present	0.91	Present	0.93	Present	0.97
43	Longitudinal muscles outside of circular muscles	Absent	0.99	Absent	0.99	Absent	1	Absent	1	Absent	1	Absent	1	Absent	1	Absent	1	Absent	0.98
44	Longitudinal muscles between mouth and frontal pore	Present	0.73	Present	0.8	?	0	Present	0.82	Present	0.79	Present	0.83	Present	0.63	Absent	0.6	Absent	0.88

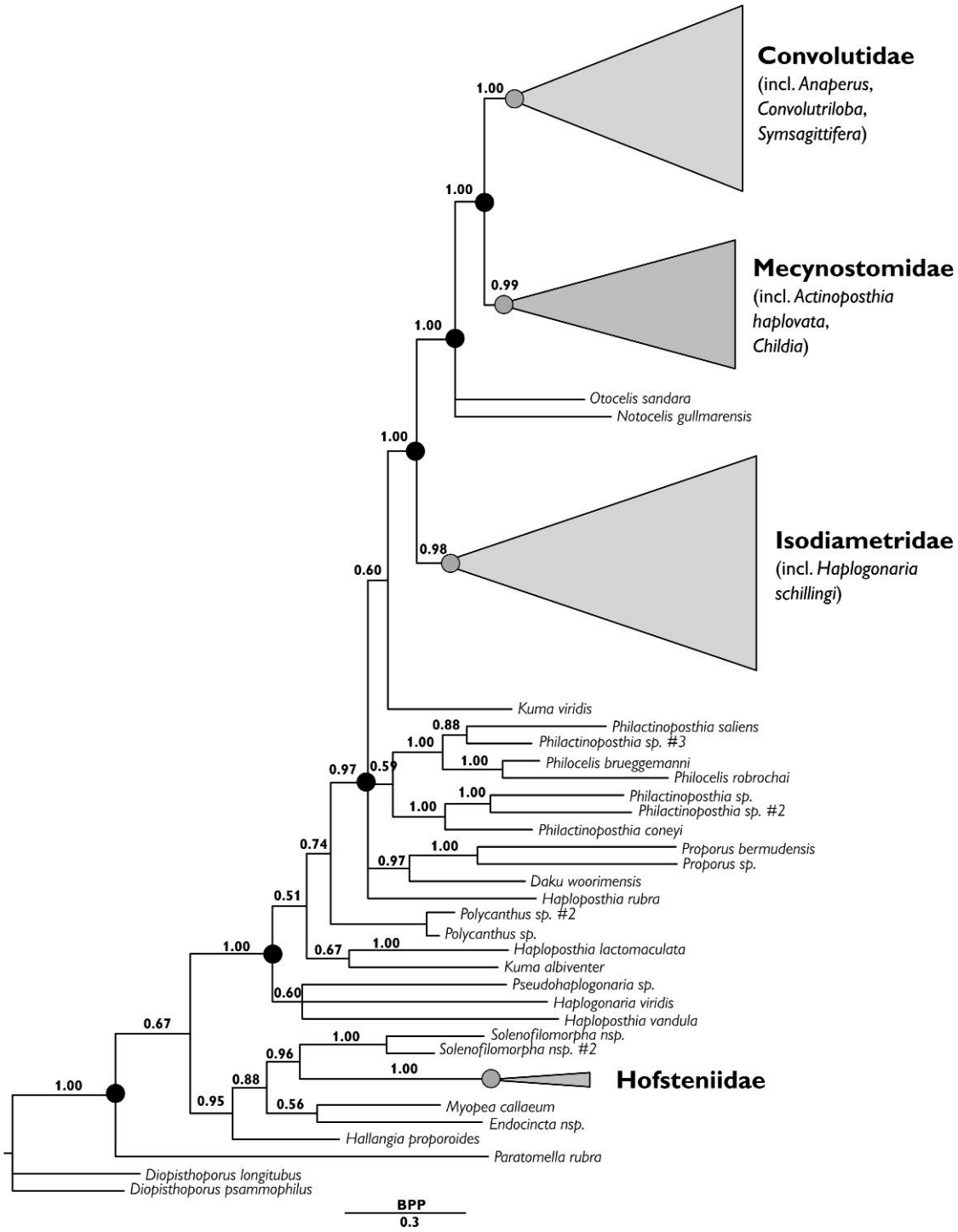

FIGURE 3. Bayesian majority-rule consensus tree inferred from the mitochondrial COI gene. Bayesian posterior probabilities are shown in bold above branches. Some families (gray nodes) and some deep nodes (black) are recovered as monophyletic clades, but others are unresolved. There is no conflict with the total evidence tree, although the COI tree is less well resolved.

tree (Fig. 3), particularly close to the root. Whereas some clades such as Mecynostomidae, Convolutidae, Isodiametridae, and Hofsteniidae are still recovered as monophyletic with high support, other taxa such as Dakuidae and Proporidae are not well resolved. There is however still a strongly supported basal split between the clade uniting *Hallangia*, Hofsteniidae and the solenofilomorphiids and the "higher" acoels, just as in the analysis of all molecular data. Alignments and trees can be downloaded from TreeBASE using the accession URL: http://purl.org/phylo/treebase/phylows/study/TB2:S11235.

Phylogenetic Analysis of Morphological Data

The Bayesian phylogenetic analysis of the morphological data is presented as a majority-rule consensus tree in Figure 4. A few traditional clades are recovered, but families such as Isodiametridae, Mecynostomidae, and Proporidae are unresolved at the 0.5 posterior probability cutoff level. In a consensus tree where all compatible clades are resolved, Mecynostomidae groups with *Childia* and *Anaperus* groups with Convolutidae

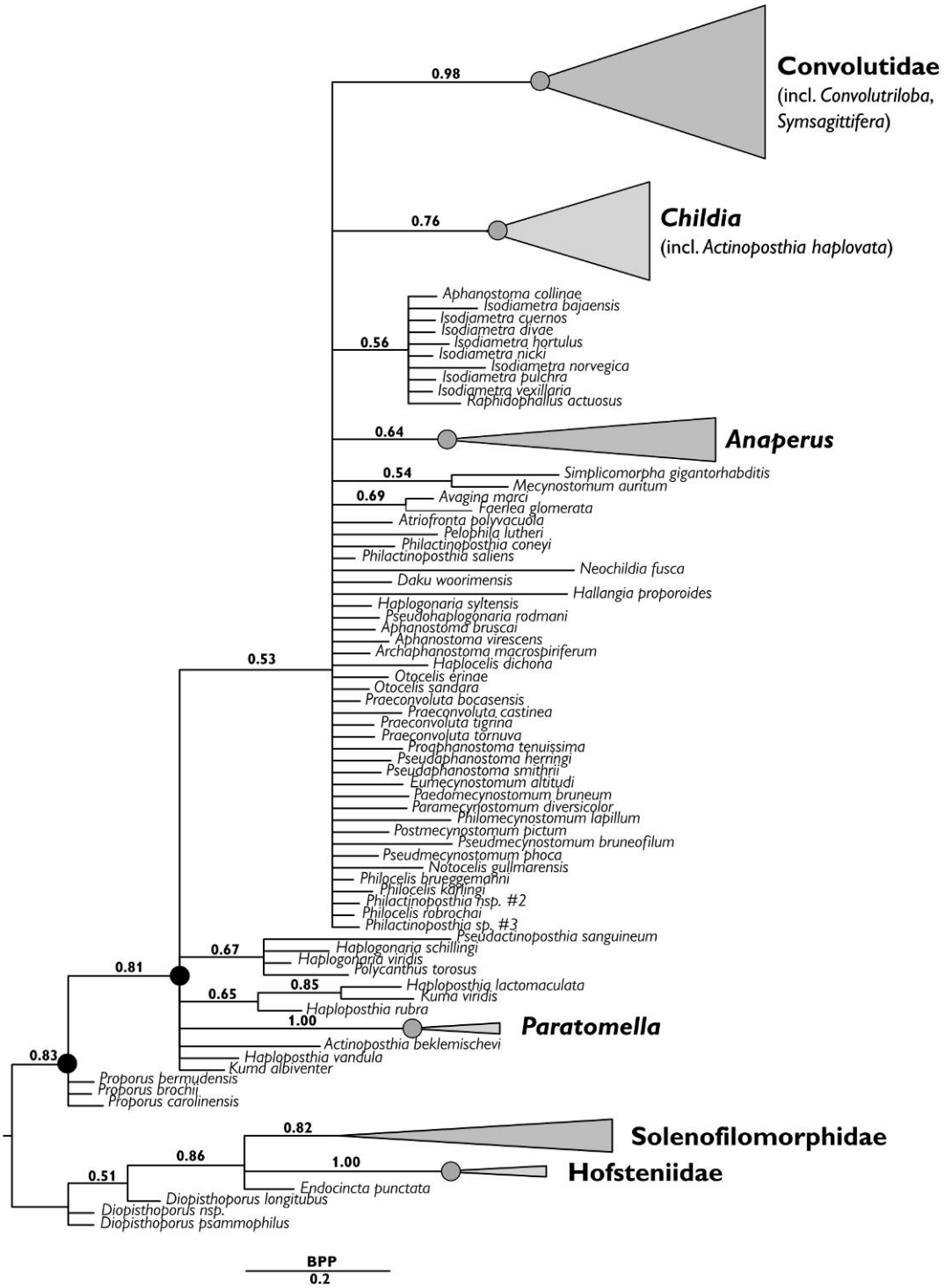

FIGURE 4. Bayesian majority-rule consensus tree inferred from morphological characters. Bayesian posterior probabilities are shown in bold above branches. Some families and genera are monophyletic, whereas the relationships among isodiametriids, dakuiids, and mecynostomiids are completely unresolved. *Paratomella* and *Hallangia* appear more deeply nested in the tree than in the molecular analyses.

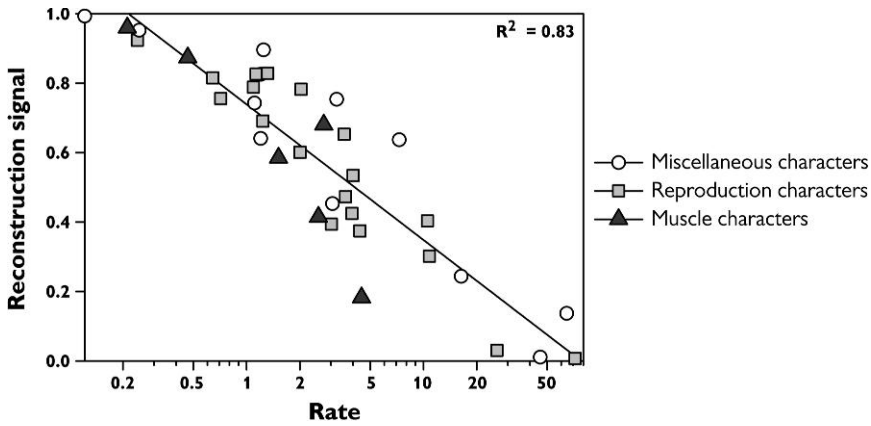

FIGURE 5. The correlation between reconstruction signal and evolutionary rates of morphological characters. There is a relationship between the rate of a character and how clearly it is reconstructed at the control nodes. Characters related to the reproductive organs cover a wider range of rates and signals than those of the body-wall musculature.

(see Supplementary Material) similar to the molecular data. Apart from the nested positions of *Paratomella* and *Hallangia*, there is no strongly supported conflict with the molecular data. It is evident that those morphological characters that we were able to score across all of Acoela are not sufficiently informative to recover a fully resolved tree alone.

Reconstruction of Ancestral Morphology

The insignificant topological disagreements among the alternative sets of posterior distributions of trees produced from the different alignments naturally result in striking similarities among the features of morphological evolution selected for comparison: (i) the rates and reconstruction signals of all characters are highly concordant among the sets (see Supplementary Data); and (ii) only 2 characters out of 44 are reconstructed with alternative states with posterior probabilities of 0.70 or higher, which occurs at only a single control node out of 30 (hence conflicts are restricted to two cells in a 1320-cell matrix). This ambiguity relates to details of the copulatory organ inferred at a node inside Isodiametridae depending on whether or not the clade containing many actinoposthiids groups inside Isodiametridae with high support (Figs. 2 and 8a,b and the Supplementary Data). As a consequence of these uniform results, we again chose to focus on the results inferred from the Bayesian trees generated from the filtered structural

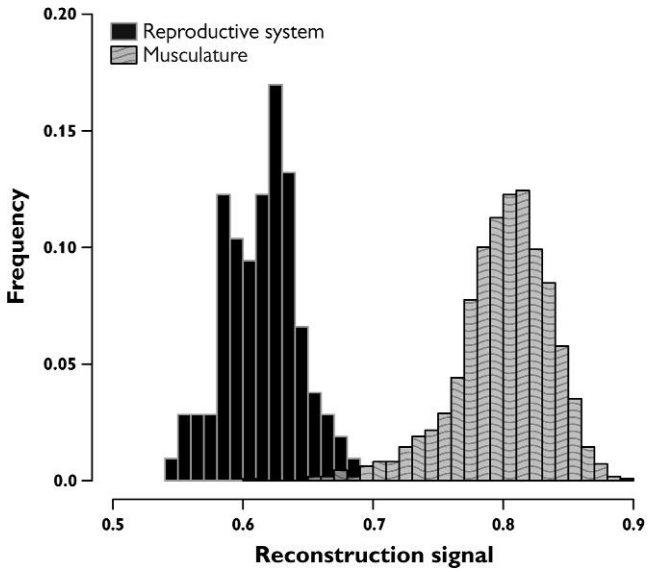

FIGURE 6. The reconstruction signals of the reproductive system and musculature. The characters of the body-wall musculature are reconstructed with significantly stronger signals than those of the reproductive system (Wilcoxon rank-sum test; pooled data: $p(H0) < 2.2e^{-16}$). The histograms represent the distribution of mean reconstruction signals estimated for the reproductive system (19 characters) on 106 fully sampled trees and the musculature (six characters) on 1109 fully sampled trees, respectively, out of 2000 Bayesian input trees.

TABLE 6. Proportion of successfully reconstructed states at different nodes and confidence levels

Node	Confidence level				
	0.5	0.6	0.7	0.8	0.9
Higher taxon					
Acoela	0.93	0.77	0.70	0.55	0.45
Bitesticulata	0.91	0.77	0.66	0.52	0.41
Bursalia	0.91	0.75	0.68	0.52	0.41
Prosopharyngida	0.91	0.73	0.68	0.52	0.43
Hofsteniidae + Solenofilomorphidae	0.91	0.75	0.68	0.52	0.45
Crucimusculata	1.00	0.81	0.70	0.65	0.57
((Abberantospermata + Dakuidae) + Otocelis) + Isodiametridae + "Avaginaclade"	0.89	0.73	0.52	0.41	0.32
((Abberantospermata + Dakuidae) + Otocelis) + Isodiametridae	0.91	0.77	0.66	0.59	0.45
(Abberantospermata + Dakuidae) + Otocelis	0.91	0.80	0.68	0.59	0.48
Abberantospermata + Dakuidae	0.93	0.77	0.70	0.61	0.52
Abberantospermata	0.93	0.73	0.61	0.57	0.41
Average	0.92	0.76	0.66	0.55	0.45
Family					
Diopisthoporidae	0.98	0.84	0.82	0.70	0.52
Paratomellidae	1.00	0.98	0.86	0.82	0.70
Hofsteniidae	0.95	0.95	0.86	0.80	0.68
Solenofilomorphidae	0.95	0.84	0.80	0.75	0.59
Proporidae	1.00	0.91	0.86	0.73	0.57
Isodiametridae	0.93	0.86	0.77	0.70	0.48
Dakuidae	0.98	0.84	0.80	0.68	0.48
Convolutidae	0.95	0.86	0.82	0.73	0.59
Mecynostomidae	0.98	0.91	0.82	0.80	0.61
Average	0.97	0.93	0.86	0.78	0.66

data set. The overall reconstruction signals of all characters are plotted as a function of their evolutionary rates in Figure 5 (see Supplementary Material for the corresponding data table and detailed transition rates among states). The characters span a wide range of rates and, as expected, more rapidly evolving characters have less reconstruction signal at the control nodes. Although the characters of the body-wall musculature generally have lower rates, the rates of characters related to the reproductive system range across the whole spectrum. Some features of the reproductive organs, such as the absence or presence of a vagina or seminal vesicle are reconstructed with extremely high rates to fit their patchy distribution in extant taxa and provide virtually no signal at all as to what state would be most probable in the deeper parts of the tree. The characters of the muscle system are significantly more clearly reconstructed than those of the reproductive system at the control nodes (pooled data: $p(H0) < 2.2e^{-16}$; tree-by-tree: 55 out of 55 trees $p(H0) < 0.05$; see also Fig. 6), but the difference appears to be smaller at the family nodes than the deeper backbone nodes (see Supplementary Material for a more detailed breakdown of all data samples). Some of these family nodes, such as the Mecynostomidae and the Proporidae, show the opposite pattern in that the genitals are reconstructed with a slightly stronger signal than the muscles (Fig. 8a,b). The reconstructions of the acoel root and other deep nodes are presented in Table 4 and the reconstructions of the family ancestors are presented in Table 5 (see Supplementary Material for the reconstructions at all control nodes). The hypothetical common ancestor of a recognized family, such as that of Mecynostomidae or Convolutidae, is typically more clearly reconstructed than that of a deeper node (pooled data: $p(H0) < 2.2e^{-16}$; tree-by-tree: 1247 out of 2000 trees $p(H0) < 0.05$; no tree with significant support for the opposite pattern; see also Table 6 and Fig. 7), such as Abberrantospermata, the parent node of both Mecynostomidae or Convolutidae (Fig. 8a,b). There is a clear relationship between cutoff levels of credibility

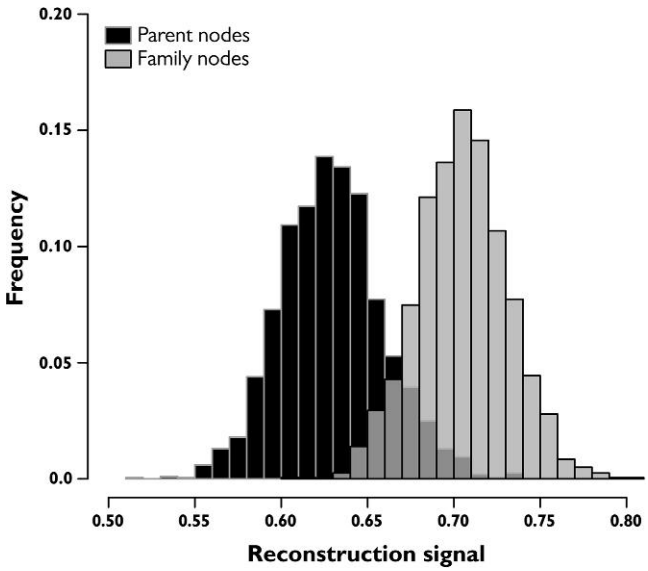

FIGURE 7. The reconstruction signals of all characters at different sets of nodes. Nodes corresponding to families are reconstructed with significantly stronger signals than their deeper parent nodes (Wilcoxon rank-sum test; pooled data: $p(H0) < 2.2e^{-16}$). The histograms represent the distribution of mean reconstruction signals estimated for families (9 nodes) and parent nodes (11 nodes) on the same set of 2000 Bayesian input trees.

in the reconstructions and the proportion of characters that are successfully reconstructed at that level (Table 6). In the Bayesian analysis, the pharynx was inferred to be homologous among all families with high posterior probabilities (Tables 4 and 5) and alternative hypotheses were associated with likelihood values orders of magnitudes lower (Table 7).

Discussion

Phylogeny

We were able to reconstruct a comprehensive and well-resolved phylogeny of Acoela using new and previously published ribosomal and mitochondrial sequence data from over 120 acoel species, about one-third of the described species. The tree recovered from analysis of the mitochondrial COI gene is less well resolved than the ribosomal gene trees but is generally compatible with the tree of all combined data. In addition to what was previously understood about acoel interrelationships, there is now strong evidence for: (i) Diopisthoporidae being the sister taxon to all other acoels; (ii) a clade uniting *Hallangia*, Hofsteniidae, and Solenofilomorphidae; (iii) the close relationship between *Proporus*, most haploposthiids and *Polycanthus*; (iv) the polyphyly of Actinoposthiidae with taxa distributed among Dakuidae and Mecynostomidae and possibly inside Isodiametridae; (v) a clade uniting *Daku*, *Notocelis*, *Philactinoposthia*, and *Philocelis*; (vi) *Anaperus*, *Symsagittifera*, and *Convolutriloba* being part of Convolutidae; (vii) *Childia* being nested inside Mecynostomidae; and (viii) Mecynostomidae and Convolutidae being

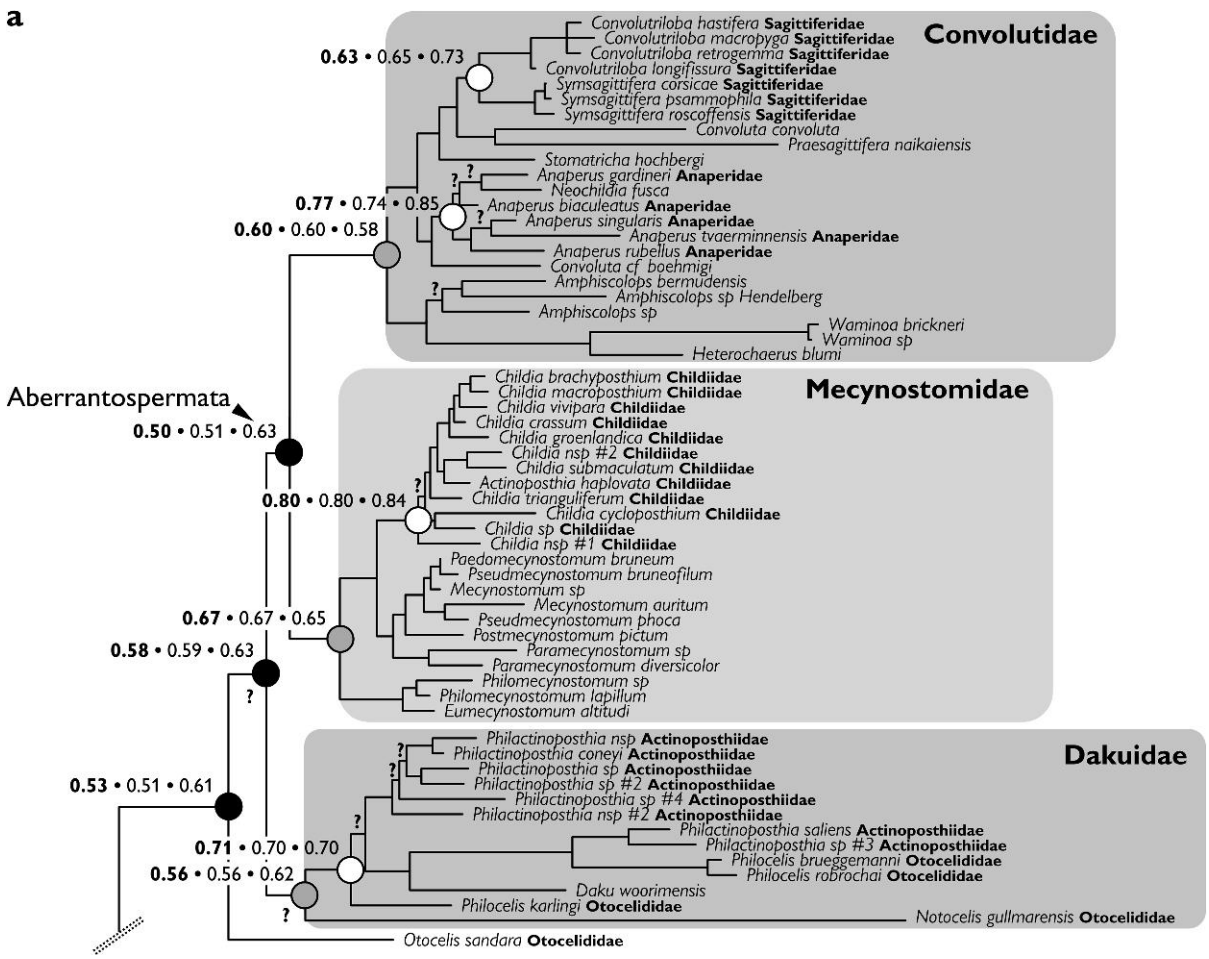

FIGURE 8. Detailed presentation of the acoel phylogeny. Total-evidence Bayesian majority-rule consensus tree inferred from 18S, 28S, and COI. All control nodes are displayed. Nodes corresponding to families are highlighted as gray, their deep parent nodes are black, and other control nodes are white. Nodes for which Bayesian posterior probabilities are below 0.90 are marked with a question mark. The overall reconstruction signals and the averaged reconstruction signals of the reproductive organs and body-wall musculature are displayed at all control nodes. Families in dotted boxes. Species transferred from other families or recovered outside their expected family clade are highlighted with their old family names in bold. Family nodes generally have a higher overall reconstruction signal than their parent nodes and body-wall musculature is generally reconstructed with stronger signals than the reproduction system. A mixed clade with actinoposthiids and isodiametriids with unstable position among analyses of different alignments is marked with an asterisk. The positions of *Hallangia proporoides* and *Actinoposthia beklemischevi* + *Pseudactinoposthia sanguineum* are associated with drops in the reconstruction signals of the reproductive organs.

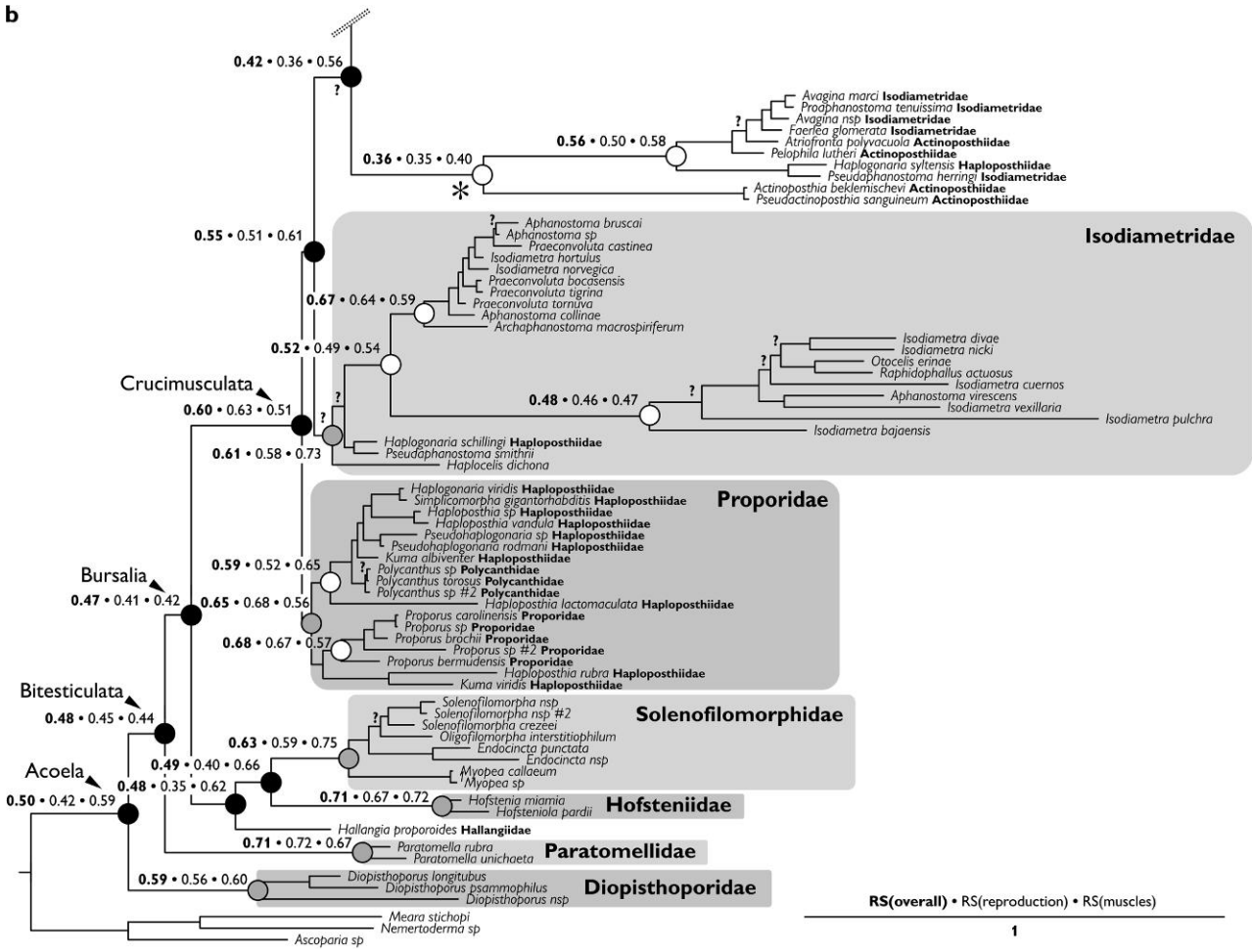

FIGURE 8. Continued

sister taxa. Although a few monotypic families are missing from our analysis, we believe that it is now possible to trace the evolution of morphological traits in Acoela with some accuracy.

Reconstruction of Ancestral Morphology

By using a Bayesian approach to character evolution, we aim to accommodate uncertainties in topological relationships and evolutionary distances among nodes and species when inferring ancestral states. Such uncertainties appear to be low in our phylogenetic inferences, given the currently available molecular data, alignment techniques, and the substitution models that we applied. The Bayesian approach to inference does not assign fixed synapomorphies to all clades. It instead assumes that all states are possible at all nodes and that their posterior probabilities are proportional to how well they fit the data and the model. Although this aims for more realistic inferences of evolutionary properties of the data when the true genealogy and evolutionary processes are not known, it leaves the job of assigning character states and changes to the observer. In the end, only one of several mutually exclusive states was actually present in an ancestral organism. To decide if variations in posterior probabilities of states among adjacent nodes represent different states at these nodes, we have to find a level of confidence that balances credibility and information content. As we show, there is a correlation between the level of confidence and the number of characters available for discussion. We here focus our discussion of ancestral morphological features on characters with states reconstructed with average posterior probabilities of close to 0.7 or higher. This corresponds to a reconstruction signal of 0.12 or higher in a binary character.

The ancestral acoel was likely a cylindrical worm with frontal glands and a pharynx located in the posterior end of the body (Fig. 9 and Table 4). Chordoid vacuoles were absent. The body-wall musculature consisted of an orthogonal muscle grid with additional ventral diagonal fibers. It had an unpaired ovary, which may have formed a diffuse mixed gonad with the testis. There was likely a tubular antrum through which the sperm was transported to a posteroterminal or subterminal male opening. The spermatozoa had a 9 + 2 arrangement of axonemes and cortical microtubules. Copulation may have been assisted with a stylet but other accessory penetrating muscular or hard structures

TABLE 7. Evaluation of alternative hypotheses on the evolution of the acoel pharynx

Node constraint(s)	Mean of log Lh	Harmonic mean (H) of log Lh	Test statistic
None	−13.51	−14.59	–
Proporidae = absent	−14.80	−16.12	3.07
Acoela = absent	−16.32	−17.10	5.03
Bursalia = absent	−16.00	−17.28	5.39
H + S = absent, Prosopharyngida = absent	−21.25	−22.09	15
H + S = absent	−21.22	−22.09	15
Acoela, Bitesticulata, Bursalia, Sphinctostomata, Prosopharyngida, H + S = absent	−21.58	−22.85	16.53

were absent. Female accessory organs such as a separate female gonopore, bursa, and bursal nozzle were absent. Traits mainly associated with Convolutidae such as symbiotic algae, sagittocysts, adenodactyls, pigmentation, and eyes were absent. Extant species of *Diopisthoporus* possess many of these ancestral traits and of all the families included in the analysis, the node corresponding to Diopisthoporidae has the posterior distribution of states that is the most similar to the posterior distributions inferred at the root of the tree. Our parsimony-based reconstruction of unambiguous changes in the ancestral acoel (morphological characters optimized on Bayesian molecular tree) implies a cylindrical body with frontal glands, without chordoid vacuoles, orthogonal body-wall musculature without ventral diagonal fibres, an unpaired ovary, a ciliated male antrum, and 9 + 2 arrangement of axonemes and cortical microtubules in the spermatozoa. The parsimony reconstruction further entails absence of a copulatory stylet, absence of vagina, bursa and bursal nozzles, and absence of pigmentation, eyes, or symbionts. In contrast to the Bayesian reconstruction, the presence or absence of a pharynx, position of the mouth, and existence of a mixed gonad could not be determined under the parsimony framework. Except for the presence of a muscular pharynx, our Bayesian estimate of character states at the acoel root is largely consistent with Westblad's list of ancestral acoel features: a mixed gonad, lack of accessory female structures, and posteroterminal gonopore were all regarded as "primitive" by Westblad (1948). Many of the ancestral features, including the cylindrical body with frontal glands, pharynx, the indistinct division between testes and ovary (a hermaphrodite gonad), tubular antrum opening into a posterior gonopore, penis stylet, and 9 + 2 sperm axoneme structure with cortical microtubules occur in deep nodes and "basal" families. In this context, the basal position of *Hallangia proporoides* is unexpected. This species stands out as it has paired ovaries that do not mix with the testes and its copulatory organ and chordoid vesicles are similar to those of Isodiametridae. Consequently, some of the ancestral features shared among many deep nodes cannot be assumed to be homologous without recognizing some level of ambiguity in this region of the tree (see Supplementary Data for reconstructions with stronger signals without this species). The reconstruction of the ancestral acoel is nearly identical in the presence of Nemertodermatida (see Supplementary Data), with the exception that the position of the mouth opening is unresolved and that the testis may have been paired structures (BPP = 0.65). However, the sister group relationship between Acoela and Nemertodermatida (Ehlers 1985) was recently challenged (Wallberg et al. 2007) and we here prefer to infer ancestral features of Acoela from extant acoel species.

In our Bayesian reconstruction, the pharynx of Diopisthoporidae, *Hallangia*, Solenofilomorphidae, Hofsteniidae, and *Proporus* is homologous. This provides an alternative to the hypothesis on the origin the acoel pharynx put forward by Todt (2009). Todt argued, based on anatomical differences in extant species, that the pharynges of different families had evolved independently, and she found it unlikely that a pharynx would have

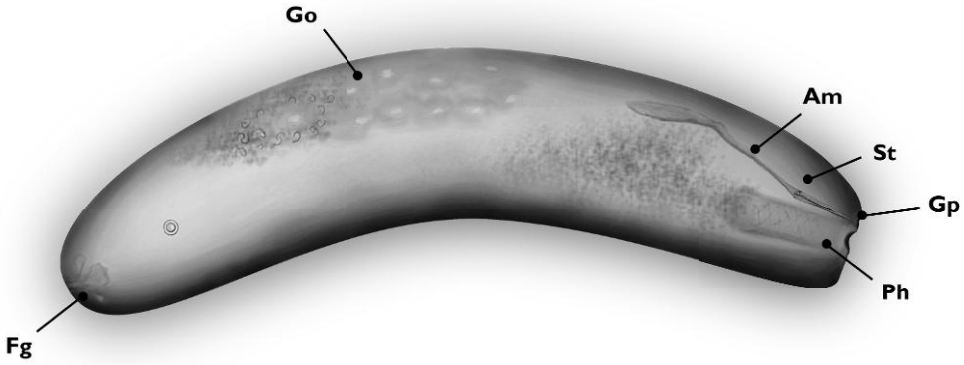

FIGURE 9. A representation of the ancestral acoel worm as inferred from molecular and morphological analyses. Fg = frontal glands; Go = mixed hermaphroditic gonads; Am = tubular antrum,; St = copulatory stylet; Gp = posteroterminal gonopore; Ph = posteroterminal muscular pharynx. Illustration: Karolina Larsson. This figure is available in black and white in print and in color at *Systematic Biology* online.

been present in the acoel ancestor. Our phylogenetic analyses suggest the opposite: the pharynx is a feature that likely evolved only once ($q0 \rightarrow 1 = 0.015$) but was lost multiple times ($q1 \rightarrow 0 = 1.19$): once in *Paratomella*, twice in the clade comprising *Proporus* and various haploposthiids and once in the ancestor of Isodiametridae and its sister taxon (Tables 4and 5; Fig. 8a,b). Multiple independent origins of the pharynx is a far less likely hypothesis (Table 7). To accommodate fully independent origins of pharynges among families, the relative transition rates between the absence and presence of a pharynx need to be reversed ($q0 \rightarrow 1 = 0.218$ and $q1 \rightarrow 0 = 0.083$, respectively). Under our reconstruction, the variation in pharynx tube muscle layers and associated tissues among the groups are better interpreted as autapomorphies. Most extant acoels lack a pharynx, but this condition is often associated with the presence of additional ventral muscles oriented in such a way that they could pull the posterior part of the body and mouth forward, forcing food items into the mouth. Thus, ventral longitudinal muscles crossing over from one side to the other just behind the mouth or U-shaped fibers enwrapping the mouth may substitute for a specialized pharynx (Tyler and Rieger 1999). Is the gain of such muscles correlated with the loss of the pharynx in our reconstruction? U-shaped fibers have been found in the basal but pharynx-less *Paratomella* and most Crucimusculata species studied to date, but not in any acoel with a pharynx (Todt 2009). Loss of the pharynx represents independent events in these lineages and the appearance of U-shaped fibers are accordingly inferred to be convergent in our reconstructions. Todt (2009) noted that the U-shaped muscles of *Paratomella unichaeta* form a distinct layer of fibers not connected to the ventral longitudinal fibers, which is otherwise the case in Crucimusculata; a difference that could be explained by independent origins of these fibers among these lineages. Ventral crossover muscles are not present in the basal and pharyngeate Diopisthoporidae, Solenofilomorphidae, and Hofstenidae and is here inferred to have evolved only once along the lineage leading to Crucimusculata. The Bayesian inference reconstructs the pharynx of *Proporus* as homologous to that of the other acoels, whereas the parsimony analysis infers a separate origin. Although the latter hypothesis is not the preferred explanation in the Bayesian analysis, it is the best-fitting alternative solution among those explored here (Table 7). It should be noted that the pharynx in *Proporus* appears different from other acoel pharynges when observed in live specimens and some unique features such as the presence of pharynx retractors and the absence of oral sphincter muscles have been reported from *Proporus bermudensis* (Todt 2009), the single species of the genus that has been studied in detail. Although *P. bermudensis* does have the ventral crossover musculature associated with Crucimusculata, other ventral fibers of the orthogonal muscle grid shared among most acoels appear to be secondarily reduced (Hooge 2001). The pharynx and elaborate ventral muscle fibres seem to be coevolving and mutually exclusive. The pharynx is likely an ancestral feature of the acoel feeding apparatus, but a separate origin of the divergent pharynx in *Proporus* cannot be completely ruled out, especially as it is consistent with the parsimony-based reconstruction. Comparative studies of the developmental program as well as the feeding mechanics and ecology in *Diopisthoporus*, *Hofstenia*, *Proporus*, and other acoels are needed to further elucidate the evolution of the acoel pharynx.

From our attempts to evaluate the reconstruction performance of characters related to the body-wall musculature relative to those of the reproductive system, we conclude that the musculature is generally easier to reconstruct at deep nodes than many features of the reproductive system, that is, they make up a more conservative set of characters. The difference was significant also when only species completely coded for both organ systems were included in the analyses (Supplementary Material). The higher evolutionary rates of reproductive system characters are consistent with the results of Achatz et al. (2010), who found indications of a high rate of coevolution between sperm morphology, secondary female reproductive organs and speciation in Convolutidae. When averaged over all characters, deeper nodes tend to have a weaker reconstruction signal than nodes at the family level. The family level nodes can be regarded as "outpost" nodes for which many aspects of the ancestor can still be reconstructed with high confidence. These nodes typically contain all sampled representatives of the family according to the traditional classification but in some cases also additional species, or in the case of Dakuidae constitute a completely new configuration of species. Although recovered in a nested position, Proporidae retains many plesiomorphic states and therefore has a high posterior similarity to the ancestral acoel (PS(non-structural) = 0.82; PS(structural) = 0.79), second only to Diopisthoporidae. In the analysis of only the morphological data sets, *Proporus* was basal even to *Paratomella*, even though the latter taxon holds a basal position in the molecular tree. Convolutidae (including *Anaperus* and Sagittiferidae) on the other hand are the acoels that diverged the most from the ancestral acoel with a posterior similarity score of only 0.50.

Our results appear to be robust with respect to the small differences in the posterior distributions of trees inferred from molecular data sets aligned with different algorithms and optional filtering of ambiguous regions. Furthermore, they underscore the importance of broad taxonomic sampling and an informed choice of "models" for in-depth studies of for example, developmental biology in Acoela. Inferences about ancestral properties of Acoela should not be drawn from those species that share the fewest features with the common ancestor of Acoela. It is ironic to note that recent studies drawing conclusions about the developmental origin of the mouth and gonopores in acoels (Hejnol and Martindale 2008), the architecture of the ancestral acoel muscular system (Semmler et al. 2008), the phylogenetic significance of serotonergic nerve chords in Acoela (Gaerber et al. 2007), and the anatomy of the

TABLE 8. Family level classification of Acoela

Name and author	Nomenclatorial acts in this paper	Specifiers for recognized families	Emended diagnoses
Actinoposthiidae, Hooge 2001	*Philactinoposthia* is transferred *to Dakuidae. Actinoposthia haplovata* is transferred to *Childia*	No specifiers can be provided as the taxon was insufficiently sampled	No changes
Anaperidae, Dörjes 1968	Anaperidae is synonymized with Convolutidae	–	Synonymized
Anthroposthiidae, Faubel 1976	none	No specifiers can be provided. This monotypic taxon was not available for study	No changes
Antigonariidae, Dörjes 1968	None	No specifiers can be provided. This monotypic taxon was not available for study	No changes
Childiidae, Dörjes 1968	Childiidae is synonymized with Mecynostomidae	–	Synonymized
Convolutidae, Graff 1905	Anaperidae and Sagittiferidae are synonymized with Convolutidae	The most inclusive clade that contains *Amphiscolops bermudensis* and *Convolutriloba longifissura* but not *Childia macroposthium*	Spermatozoa with 9+0 axonemes. Pigmentation, algal symbionts and eye spots often present. Male antrum may have numerous stimulatory organs. Sagittocysts may be present. Includes large and active worms
Dakuidae, Hooge 2003	*Philocelis*, *Philactinoposthia*, and *Notocelis* are transferred to this family.	The most inclusive clade that contains *Daku woorimensis* and *Philactinoposthia coneyi* but not *Convoluta convoluta*	Penis with sclerotized needles, muscular, or absent. Seminal vesicle may be highly muscular. Bursal nozzle present. Sperm with axial microtubules and 9+2 axonemes
Diopisthoporidae, Westblad 1940	None	The most inclusive clade that contains *Diopisthoporus longitubus* and *D. psammophilus* but not *Paratomella rubra*	No changes
Hallangiidae, Westblad 1946	None	No specifiers can be provided as this taxon was insufficiently sampled in this study	No changes
Haploposthiidae, Westblad 1948	Haploposthiidae is synonymized with Proporidae. *Haplogonaria schillingi* and *Haplogonaria syltensis* are transferred to Pseudoaphanostoma (Isodiametridae).	–	No changes
Hofsteniidae, Papi 1957	none	The most inclusive clade that contains *Hofstenia miamia* and *H. pardii* but not *Myopea callaeum*.	No changes
Isodiametridae, Hooge and Tyler 2005	*Haplogonaria schillingi* is transferred to *Pseudaphanostoma* within this family.	The most inclusive clade that contains *Isodiametra norvegica* and *Haplocelis dichona*, but not *Philactinoposthia saliens*	Body-wall musculature with circular, longitudinal, and longitudinal crossover muscle fibers in both the dorsal and ventral body wall, and U-shaped fibers in the ventral body wall. Male copulatory organ invaginated into muscular seminal vesicle, usually a tubular muscular isodiametric penis, often glandular. Penis musculature with inner circular and outer nonanastomosing longitudinal fibers. Never with symbiotic algae. Ocelli, when present, do not contain platelets in the pigment cell
Mecynostomidae, Dörjes 1968	Childiidae is synonymized with Mecynostomidae	The most inclusive clade that contains *Paramecynostomum diversicolor* and *Childia macroposthium*, but not *Convolutriloba longifissura*	Spermatozoa with 9+1 axonemes and distal microtubules only. Four layers of dorsal body-wall muscles
Nadinidae, Dörjes 1968	None	No specifiers can be provided. This monotypic taxon was not available for study	No changes
Otocelididae	*Philocelis* and *Notocelis* are transferred to Dakuidae	No specifiers can be provided, as this taxon was insufficiently sampled in this study	No changes
Paratomellidae, Dörjes 1966	None	The most inclusive clade that contains *Paratomella rubra* and *P. unichaete* but not *Hofstenia miamia*	No changes
Polycanthidae, Hooge 2003	Polycanthidae is synonymized with Proporidae. *Polycanthus* is transferred to Proporidae.	–	Synonymized
Proporidae, Graff 1882-kolla	Haploposthiidae and Polycanthiidae are synonymized with Proporidae, *Haplogonaria viridis* is transferred to *Simplicomorpha*	The most inclusive clade that contains *Proporus brochii* and *Polycanthus torosus*, but not *Haplocelis dichona*	Male copulatory organ posteroterminal, seminal vesicle present. Pharynx, when present located anteriorly. Ovaries never reaching behind testes
Sagittiferidae, Kostenko and Mamkaev 1990	Sagittiferidae is synonymized with Convolutidae.	–	synonymized
Solenofilomorphidae, Dörjes 1968	None	The most inclusive clade that contains *Oligofilomorpha interstitiophilum* and *Myopea callaeum* but not *Hofstenia miamia*	No changes
Tauridiidae, Kostenko 1989	None	No specifiers can be provided. This monotypic taxon was not available for study	No changes

brain (Bery et al. 2010) are all based on single species of the Sagittiferidae. Likewise, Ramachandra et al. (2002) made general inferences about acoel and early bilaterian morphogenesis based on observations restricted to *Neochildia fusca*. The stem cell system in Acoela and its phylogenetic implications were studied by Egger et al. (2009), who improved sampling by studying two species of Isodiametridae and one species of Sagittiferidae. Egger and coauthors suggested that the stem cell system in their acoel species and stem cells in Platyhelminthes constitute a synapomorphy conflicting with molecular phylogenies that place Acoela at the base of Bilateria (e.g., Telford et al. 2003; Wallberg et al. 2007; Dunn et al. 2008, Hejnol et al. 2009). Unfortunately, Isodiametridae does not appear to be a particularly plesiomorphic family. Some species of Sagittiferidae are among the few Acoela reported to reproduce asexually through fission (Åkesson et al. 2001), a process that requires considerable regenerative powers. This is a highly derived feature in our reconstruction. Thus, in addition to being part of the clade that diverged the most from the acoel ancestor, sagittiferids have special adaptations for regeneration. We propose that the similarity between stem cells in Platyhelminthes and stem cells in the Acoela studied by Egger and coworkers is best explained as convergence in view of the strong support for Acoela as a basal bilaterian clade in the DNA sequence–based studies, the specialized nature of sagittiferids, and the uncertainties regarding reconstruction of ancestral isodiametrid characters. We recommend that species from less divergent clades with high reconstruction scores should be considered when choosing material for in-depth studies seeking to draw generalized conclusions about acoel biology and that such conclusions should be based on more than one species, spanning as much phylogenetic diversity as possible.

A formal reclassification of Acoela at the family level, consistent with the phylogenetic hypothesis presented here is given in Table 9. In order to achieve the goal of monophyletic family level groups, some reassignments of species and genera are necessary: all the formal nomenclatorial acts are reported in Table 8. We have kept the reclassifications to the minimum necessary for obtaining monophyletic family level taxa. Generally, the family level clades are strongly supported in the Bayesian, ML, and parsimony analyses. We have chosen not to reclassify the species of Actinoposthiidae that group within the Isodiametridae clade (Fig. 8a,b) as our sequence data for some of these species are incomplete. Their position is based solely on 18S rRNA sequences from GenBank and depending on underlying alignment algorithms of the molecular data, there are competing solutions indicating either inclusion of actinoposthiids in Isodiametridae or paraphyly of Isodiametridae with regard to a few isodiametrid species grouping with the actinoposthiids. Better sequence coverage and more thorough sampling preferably involving type species of the genera is required to determine the phylogenetic status of Actinoposthiidae, which we regard as unresolved at this point. A comprehensive phylogenetic system is essential for further studies of acoel biodiversity and evolution. Informal names for

TABLE 9. Phylogenetic classification of Acoela with diagnostic features and specifiers of suprafamilial clades

Clade
Acoela (digestive parenchyma, biflagellate spermatozoa)
Diopisthoporidae Westblad, 1940
Bitesticulata (Paired or follicular testes, ventral gonopore. The most inclusive clade that contains
Paratomella rubra and Symsagittifera roscoffensis but not Diopisthoporus longitubus)
Paratomellidae Dörjes, 1966
Bursalia (Copulatory bursa often present. The most inclusive clade that contains
Oligofilomorpha interstitiophilum and Childia groenlandica but not Paratomella rubra)
Prosopharyngida (Muscular pharynx in anterior part of body. The most inclusive clade that contains
Hofstenia miamia and Oligofilomorpha interstitiophilum but not Haploposthia rubra)
Hallangidae Westblad, 1946
Hofsteniidae Papi, 1957
Solenofilomorphidae Dörjes, 1968
Crucimusculata (Ventral crossover muscle fibres). The most inclusive clade that contains
Actinoposthia beklemischevi and Childia groenlandica but not Haploposthia rubra)
Dakuidae Hooge 2003
Isodiametridae Hooge and Tyler 2005
Otocelididae Westblad, 1948
Proporidae Graff, 1882
Aberrantospermata (Spermatozoa with 9 + 0 or 9 + 1 axonemes. The most inclusive clade that contains
Neochildia fusca and Childia groenlandica but not Actinoposthia beklemischevi)
Convolutidae Graff, 1905
Mecynostomidae Dörjes, 1968
Acoela Incertae cedis, not sampled in this study
Actinoposthiidae (see discussion)
Anthroposthiidae (single species)
Antigonariidae (single species)
Nadinidae (single species)
Tauridiidae (single species)

strongly supported higher level groups are introduced in Table 9, thereby completing a phylogenetic classification of Acoela down to the family level.

Supplementary Material

Supplementary material, including data files and/or online-only appendices, can be found at http://www.sysbio.oxfordjournals.org/.

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