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Filling a gap in the phylogeny of flatworms: relationships within the Rhabdocoela (Platyhelminthes), inferred from 18S ribosomal DNA sequences

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The phylogeny of the Rhabdocoela, a species-rich taxon of free-living flatworms, is reconstructed based on complete 18S rDNA sequences. The analysis includes 62 rhabdocoels and 102 representatives of all major flatworm taxa. In total, 46 new sequences are used, 41 of them from rhabdocoel species, five from proseriates. Phylogenetic analysis was performed using maximum parsimony and Bayesian inference. Clade support was evaluated with parsimony jackknifing, Bremer support indices and Bayesian posterior probabilities. The resulting cladogram corroborates that the Rhabdocoela is monophyletic, but its sister group remains uncertain. The 'Dalyellioida' and the 'Typhloplanoida', both former rhabdocoel subtaxa, are polyphyletic. Within the Rhabdocoela the monophyletic Kalyptorhynchia, characterized by a muscular proboscis, forms the sister group of all other rhabdocoels. The Schizorhynchia is a monophyletic subtaxon of the Kalyptorhynchia, with the split proboscis as a synapomorphy. Except for the Dalyelliidae and the Typhloplanidae, both freshwater taxa, none of the 'families' previously included in the 'Typhloplanoida' and the 'Dalyellioida' appears to be monophyletic. As a result of this analysis, three existing and four new taxon names are formally defined following the rules of the Phylocode.

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Introduction

The first comprehensive phylogenetic analysis of the Platyhelminthes using Hennigian argumentation was made by Ehlers (1985). This analysis was mainly based on morphological (including many ultrastructural) characters. It showed that the Turbellaria is paraphyletic, but that the Cestoda (Cercomeromorpha) and the Trematoda are monophyletic, together forming the monophyletic taxon Neodermata (with a neodermis). More recent studies, using molecular data and more elaborate cladistical techniques (e.g. Katayama *et al.*

1996; Carranza *et al*. 1997; Littlewood *et al*. 1999a,b; Joffe & Kornakova 2001; Littlewood & Olson 2001; Zamparo *et al*. 2001; Lockyer *et al*. 2003; see Baguñà & Riutort 2004 for a review) confirmed the monophyly of the Neodermata, but also showed that the relationships among the various other flatworm taxa are much more complex than was suggested by Ehlers (1985). Recent molecular data even suggest that the Platyhelminthes as a whole is polyphyletic, the Acoela and Nemertodermatida being basal bilaterians (Zrzavy *et al*. 1998; Ruiz-Trillo *et al*. 1999, 2002, 2004; Telford *et al*. 2000, 2003; Baguñà *et al*. 2001a; Jondelius *et al*. 2002; but see Tyler 2001 for an alternative view).

Ehlers (1985) considered the Rhabdocoela as consisting of the 'Typhloplanoida', the 'Dalyellioida' and the parasitic Neodermata. However, since no apomorphies for either the 'Typhloplanoida' (including the Kalyptorhynchia) or the 'Dalyellioida' (including the Temnocephalida and other symbiotic taxa) could be put forward, both groups were regarded as nonmonophyletic and their exact position within the Rhabdocoela was left open. Analyses of rDNA sequences showed that the sister group of the Neodermata is included neither within the 'Dalyellioida' nor the 'Typhloplanoida', but probably consists of a large clade containing most of the neoophoran taxa (Littlewood *et al*. 1999a,b; Baguñà *et al*. 2001b; Joffe & Kornakova 2001; Littlewood & Olson 2001; Norén & Jondelius 2002; Lockyer *et al*. 2003; review in Baguñà & Riutort 2004). Therefore, the name Rhabdocoela is now commonly used by most authors for a group containing the 'Dalyellioida', 'Typhloplanoida', Kalyptorhynchia and Temnocephalida, the latter two being monophyletic. In fact, this is consistent with its most traditional use (and is a synonym of the name Neorhabdocoela of Meixner 1938).

Currently, all recent molecular studies agree on the monophyly of the Rhabdocoela, although relationships within the taxon are still unclear. The main reason for this is probably poor taxon sampling. In phylogenetic analyses based on molecular data (18S rDNA) including Rhabdocoela, only a few rhabdocoel sequences were used: five by Lockyer *et al*. (2003), 12 by Littlewood *et al*. (1999a,b), Joffe & Kornakova (2001) and Littlewood & Olson (2001), and 20 by Norén & Jondelius (2002). The main results of these studies can be summarized as follows: (1) the Kalyptorhynchia, sometimes included within the 'Typhloplanoida' (see Ehlers 1985), is monophyletic and probably forms the sister group to all other rhabdocoels; (2) neither the 'Typhloplanoida' nor the 'Dalyellioida' are monophyletic; (3) there is some evidence for a freshwater clade containing the Typhloplanidae Graff, 1905 ('Typhloplanoida') and the Dalyelliidae Graff, 1905 ('Dalyellioida') and perhaps the Temnocephalida ('Dalyellioida') (see Joffe & Kornakova 2001; Watson 2001).

The main objective of this study is to reveal the relationships within the Rhabdocoela by including a much larger number of sequences than in any previous study. With these data we aimed at resolving the following questions: (1) What are the relationships of the various typhloplanoid 'families' within the Rhabdocoela? (2) What is the position of the Kalyptorhynchia? (3) Is there in fact a 'freshwater clade' consisting of members of the 'Typhloplanoida' and the 'Dalyellioida'? Apart from the internal relationships, this study also set out to reveal possible sister group relations of the Rhabdocoela.

Materials and methods

Taxon sampling

Forty-one specimens of 39 rhabdocoel species (24 'Typhloplanoida' including *Ciliopharyngiella constricta*, 1 'Dalyellioida' and 14 Kalyptorhynchia; see Table 1) were collected in both freshwater and marine habitats. Marine specimens were extracted from the sediment or from algae using the $MgCl_2$ decantation method, while the freshwater specimens were collected by the oxygen depletion method (see Schockaert 1996). The specimen of *Mesostoma thamagai* Artois *et al*., 2004 was collected after inundating a sediment sample containing the resting propagules of this species from an ephemeral rock pool in Botswana (for details see Artois *et al*. 2004).

The animals were starved for several hours up to one day to prevent DNA contamination by gut contents. Specimens were fixed in 96% ethanol and stored at 4 °C until DNA extraction.

Five as yet undescribed species are included in the analysis, but to avoid creating *nomina nuda*, they are not given a species name here. *Promesostoma* sp. from New Caledonia clearly belongs to the taxon *Promesostoma* Graff, 1882 and its description has been submitted for publication. *Gaziella* sp. from the eastern Mediterranean is clearly identifiable as a species of *Gaziella* De Clerck and Schockaert, 1995. However, lack of material prevents its formal description. A third species, *Castrada* sp. from northern Sweden, could not be identified at the species level, but certainly belongs to the taxon *Castrada* Schmidt, 1861 (*sensu* Luther 1963). Two more species, '*stradorhynchus terminalis*' and '*arrawarria inexpectata*', belong to the kalyptorhynch taxon Polycystididae and are described in a submitted manuscript. Both species names are placed between quotation marks to stress that both are undescribed species. The sequence of '*arrawarria inexpectata*' was already used by Littlewood *et al*. (1999b) and therein named *Arrawarria* gen. nov.

Apart from the new rhabdocoel sequences, five proseriate species were sequenced (Table 1). Additional sequences (21 rhabdocoel and 97 nonrhabdocoel) were extracted from GenBank (Table 2), using BLAST search (Altschul *et al*. 1997). Species of which more than one sequence was available are numbered (e.g. *Geocentrophora baltica* 1, *G. baltica* 2).

In total, 164 sequences are included in the analyses, 62 of them from 57 different rhabdocoel species. *Paromalostomum fusculum*,

Table 1 List of all new Proseriata and Rhabdocoela sequences included in this study, with their GenBank accession number and geographical origin (§: sequences provided by Littlewood and Webster).

	Species	Accession No.	Location
PROSERIATA	*Archimonocelis oostendensis* Martens & Schockaert, 1981	AY775732	Belgium, Oostende, Mariakerke
	Cirrifera sopottehlersae Noldt & Jouk, 1988	AY775733	Belgium, North Sea sandbank
	Coelogynopora axi Sopott, 1972	AY775734	Germany, Sylt, Königshafen
	Pseudomonocelis ophiocephala (Schmidt, 1861) Meixner, 1943	AY775735	Greece, Thessaloniki, Perea
	Pseudomonocelis ophiocephala§	AY775736	Greece, Thessaloniki, Perea
RHABDOCOELA —	*Acrorhynchides robustus* Karling, 1931	AY775737	Germany, Sylt, Königshafen
Kalyptorhynchia	*Gyratrix hermaphroditus* Ehrenberg, 1831	AY775739	Sweden, Abisko
	Karkinorhynchus bruneti Schilke, 1970	AY775740	Germany, Sylt, List
	Mesorhynchus terminostylus Karling, 1956	AY775741	Sweden, Kristineberg, Gullmaren
	Phonorhynchus helgolandicus (Metschnikow, 1863) Graff, 1905	AY775742	Sweden, Kristineberg, Gullmaren
	Polycystis naegelii Kölliker, 1845	AY775743	Greece, Thessaloniki, Nea Michaniona
	Proschizorhynchus triductibus Schilke, 1970	AY775744	Belgium, Zeebrugge
	Schizochilus caecus L'Hardy, 1963	AY775745	Germany, Sylt, List
	Schizochilus choriurus Boaden, 1963	AY775746	Belgium, Oostende, Mariakerke
	Schizochilus marcusi Boaden, 1963	AY775747	Belgium, Knokke
	Schizorhynchoides caniculatus L'Hardy, 1963	AY775748	Germany, Sylt, List
	'stradorhynchus terminalis'	AY775738	Australia, NSW, Coffs Harbour
	Thylacorhynchus ambronensis Schilke, 1970	AY775749	France, Wimereux
	Zonorhynchus seminascatus Karling, 1956	AY775750	Germany, Sylt, Königshafen
RHABDOCOELA —	*Castrada lanceola* (Braun, 1885) Luther, 1904	AY775751	Sweden, Abisko
'Typhloplanoida'	*Castrada luteola* Hofsten, 1907	AY775752	Sweden, Abisko
	Castrada viridis Volz, 1898	AY775753	Sweden, Abisko
	Castrada sp.	AY775775	Sweden, Abisko
	Ciliopharyngiella constricta Martens & Schockaert, 1981	AY775754	Belgium, Oostende, Mariakerke
	Ciliopharyngiella constricta§	AY775755	Belgium, Oostende, Mariakerke
	Einarella argillophyla Luther, 1948	AY775756	Sweden, Kristineberg, Gullmaren
	Einarella argillophyla§	AY775757	Sweden, Kristineberg, Gullmaren
	Gaziella sp.	AY775776	Greece, Thessaloniki, Perea
	Litucivis serpens Ax & Heller, 1970	AY775758	Germany, Sylt, List
	Mesostoma lingua (Abildgaard, 1789) Schmidt, 1848	AY775759	Sweden, Abisko
	Mesostoma thamagae Artois *et al.*, 2004	AY775760	Botswana, Thamaga
	Olisthanella truncula (Schmidt, 1858) Luther, 1904	AY775761	Sweden, Abisko
	Phaenocora unipunctata Oersted, 1843	AY775762	Belgium, Diepenbeek, De Maten
	Promesostoma sp.§	AY775763	New Caledonia, Nouméa, Ile Nou
	Proxenetes flabellifer Jensen, 1878	AY775764	Belgium, Oostende
	Proxenetes puccinellicola Ax, 1960	AY775765	Belgium, Knokke, Zwin
	Proxenetes quadrispinosus Den Hartog, 1966	AY775766	Germany, Sylt, List
	Proxenetes simplex Luther, 1948	AY775767	Sweden, Kristineberg, Gullmaren
	Proxenetes trigonus Ax, 1960	AY775768	Germany, Sylt, List
	Ptychopera plebeia Beklemischev, 1927	AY775769	Greece, Thessaloniki, Agia Trias
	Ptychopera westbladi Luther, 1943	AY775770	Belgium, Knokke, Zwin
	Strongylostoma elongatum Hofsten, 1907	AY775771	Belgium, Diepenbeek
	Styloplanella strongylostomoides Findenegg, 1924	AY775772	Sweden, Abisko
	Trigonostomum denhartogi (Karling, 1978) Willems *et al.*, 2004	AY775773	New Caledonia, Nouméa, Anse Vata
	Trisaccopharynx westbladi Karling, 1940§	AY775774	Sweden, Kristineberg
RHABDOCOELA — 'Dalyellioida'	*Castrella truncata* (Abildgaard, 1789) Hofsten, 1907	AY775777	Sweden, Abisko

Haplopharynx rostratus and five sequences of four species of Lecithoepitheliata (*Geocentrophora* sp., *G. wagini*, *G. baltica* 1, *G. baltica* 2 and *G. sphyrocephala*) were used as outgroups.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from entire specimens using the DNeasy Tissue Kit (Qiagen) following the manufacturer's protocol.

The complete 18S rDNA gene, approximately 1800 bp long, was amplified using the primers TimA and TimB (see Table 3). Thermal cycling was started with an initial denaturation of 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s with a final extension of 8 min at 72 °C. Using nested PCR (with the same cycling profile), the 1100 bp closest to the 5′ end of the 18S rDNA gene were amplified using TimA and 18S 1100R, while the

Table 2 List of all additional flatworm species used in this study with their GenBank accession numbers and main references (*species for which a new sequence is also included, see Table 1). Numbered species names: different sequences of the same species extracted from GenBank. Species used to construct the probability model (see Materials and Methods: Alignment) are indicated with 'M'.

	Species	In model	Accession No.	Sequence reference
MACROSTOMIDA	*Paromalostomum fusculum* Ax, 1952		AJ012531	Littlewood *et al.* (1999a)
HAPLOPHARYNGIDA	*Haplopharynx rostratus* Meixner, 1938	M	AJ012511	Littlewood *et al.* (1999a)
LECITHOEPITHELIATA	*Geocentrophora baltica* 1 (Kennel, 1883)	M	AF167421	Jondelius *et al.* (2001)
	Geocentrophora baltica 2	M	AF065417	Norén & Jondelius (1999)
	Geocentrophora sp.	M	U70079	Carranza *et al.* (1997)
	Geocentrophora sphyrocephala De Man, 1876	M	D85089	Katayama *et al.* (1996)
	Geocentrophora wagini Timoshkin, 1984	M	AJ012509	Littlewood *et al.* (1999a)
PROSERIATA — Lithophora	*Archiloa rivularis* de Beauchamp, 1910	M	U70077	Carranza *et al.* (1997)
	Archimonocelis crucifera Martens & Curini-Galletti, 1993		AJ270151	Littlewood *et al.* (2000)
	Archimonocelis staresoi Martens & Curini-Galletti, 1993		AJ270152	Littlewood *et al.* (2000)
	Archotoplana holotricha Ax, 1956	M	AJ243676	Littlewood *et al.* (1999b)
	Calviria solaris Martens & Curini-Galletti, 1993		AJ270153	Littlewood *et al.* (2000)
	Coelogynopora gynocotyla Steinböck, 1924	M	AJ243679	Littlewood *et al.* (1999b)
	Monocelis lineata (Müller, 1774) Oersted, 1844	M	U45961	Carranza *et al.* (1997)
	Otoplana sp.	M	D85090	Katayama *et al.* (1996)
	Parotoplana renatae Ax, 1956	M	AJ012517	Littlewood *et al.* (2000)
PROSERIATA — Unguiphora	*Nematoplana coelogynoporoides* Meixner, 1938	M	AJ012516	Littlewood *et al.* (1999a)
	Polystylophora novaehollandiae Curini-Galletti, 1998		AJ270161	Littlewood *et al.* (2000)
BOTHRIOPLANIDA	*Bothrioplana semperi* Braun, 1881	M	AF051333	Baguñà *et al.* (2001b)
ADIAPHANIDA — Fecampiidae	*Kronborgia isopodicola* Blair & Williams, 1987	M	AJ012513	Littlewood *et al.* (1999a)
ADIAPHANIDA — Genostomatidae	*Ichthyophaga* sp.	M	AJ012512	Littlewood *et al.* (1999a)
ADIAPHANIDA — Urastomidae	*Urastoma cyprinae* 1 (Graff, 1882) Graff, 1903	M	AF065428	Norén & Jondelius (1999)
	Urastoma cyprinae 2	M	AF167422	Jondelius *et al.* (2001)
	Urastoma sp.	M	U70085	Carranza *et al.* (1997)
ADIAPHANIDA — Prolecithophora	*Allostoma neostiliferum* Karling, 1993	M	AF167420	Jondelius *et al.* (2001)
	Cylindrostoma fingalianum 1 (Claparède, 1861) Levinsen, 1878	M	AF065415	Norén & Jondelius (1999)
	Cylindrostoma fingalianum 2	M	AF051330	Baguñà *et al.* (2001b)
	Cylindrostoma gracilis Westblad, 1955	M	AF065416	Norén & Jondelius (1999)
	Euxinia baltica Meixner, 1938	M	AF167418	Jondelius *et al.* (2001)
	Plagiostomum cinctum Meixner, 1938	M	AF065418	Norén & Jondelius (1999)
	Plagiostomum ochroleucum Graff, 1882	M	AF065419	Norén & Jondelius (1999)
	Plagiostomum striatum Westblad, 1956	M	AF065420	Norén & Jondelius (1999)
	Plagiostomum vittatum 1 (Frey & Leuckart, 1847) Jensen, 1883	M	AF051331	Baguñà *et al.* (2001b)
	Plagiostomum vittatum 2	M	AF065421	Norén & Jondelius (1999)
	Plicastoma cuticulata Brandtner, 1934	M	AF065422	Norén & Jondelius (1999)
	Protomonotresis centrophora Reisinger, 1924	M	AF167419	Jondelius *et al.* (2001)
	Pseudostomum gracilis Westblad, 1955	M	AF065423	Norén & Jondelius (1999)
	Pseudostomum klostermanni (Graff, 1874) Graff, 1913	M	AF065424	Norén & Jondelius (1999)
	Pseudostomum quadrioculatum (Leuckart, 1847) Graff, 1911	M	AF065425	Norén & Jondelius (1999)
	Reisingeria hexaoculata Westblad, 1955	M	AF065426	Norén & Jondelius (1999)
	Scleraulophorus cephalatus Karling, 1940	M	AF167423	Jondelius *et al.* (2001)
	Ulianinia mollissima Levinsen, 1879	M	AF065427	Norén & Jondelius (1999)
	Vorticeros ijimai Togawa, 1918	M	D85094	Katayama *et al.* (1996)
ADIAPHANIDA — Tricladida	*Artioposthia triangulata* 1 (Dendy, 1895) Graff, 1896	M	AF033038	Carranza *et al.* (1998b)
	Artioposthia triangulata 2	M	AF033044	Carranza *et al.* (1998b)
	Artioposthia triangulata 3	M	Z99945	Carranza *et al.* (1998b)
	Australoplana sanguinea (Moseley, 1877) Winsor, 1991	M	AF033041	Carranza *et al.* (1998b)
	Australoplana sp.	M	AF050434	Carranza *et al.* (1998a)
	Baikalobia guttata (Gertsfeldt, 1858) Kenk, 1930	M	Z99946	Carranza *et al.* (1998b)
	Bdelloura candida (Girard, 1850) Girard, 1852	M	Z99947	Carranza *et al.* (1998b)
	Bipalium kewense Moseley, 1878	M	AF033039	Carranza *et al.* (1998b)
	Bipalium sp.	M	X91402	Mackey *et al.* (1996)
	Bipalium trilineatum Stimpson, 1857	M	D85086	Katayama *et al.* (1996)
	Caenoplana caerulea Moseley, 1877	M	AF033040	Carranza *et al.* (1998b)
	Caenoplana sp.	M	AF048765	Carranza *et al.* (1998a)
	Crenobia alpina (Dana, 1766) Kenk, 1930	M	M58345	Riutort *et al.* (1992)
	Cura pinguis (Weiss, 1909) Kenk, 1974	M	AF033043	Carranza *et al.* (1998a)
	Dendrocoelopsis lactea Ichikawa & Okugawa, 1958	M	D85087	Katayama *et al.* (1996)
	Dugesia iberica Gourbault & Benazzi, 1979	M	M58343	Riutort *et al.* (1992)

Table 2 *continued*

	Species	In model	Accession No.	Sequence reference
	Dugesia japonica 1 Ichikawa & Kawakatsu, 1964	M	AF013153	Carranza *et al.* (1998a)
	Dugesia japonica 2	M	D83382	Katayama *et al.* (1996)
	Dugesia ryukyuensis Kwakatsu, 1976	M	AF050433	Carranza *et al.* (1998a)
	Dugesia subtentaculata (Draparnaud, 1801) De Vries, 1986	M	AF013155	Carranza *et al.* (1998b, 1999)
	Ectoplana limuli (Ijima & Kaburaki, 1916) Kaburaki, 1917	M	D85088	Katayama *et al.* (1996)
	Girardia tigrina 1 (Girard, 1850)	M	AF013156	Carranza *et al.* (1998b, 1999)
	Girardia tigrina 2	M	AF013157	Carranza *et al.* (1998b, 1999)
	Microplana nana Mateos, Giribet & Carranza, 1998	M	AF033042	Carranza *et al.* (1998b)
	Microplana scharffi (Graff, 1896)	M	AF050435	Carranza *et al.* (1998a)
	Neppia montana (Nurse, 1950) Ball, 1974	M	AF050432	Carranza *et al.* (1998a)
	Newzealandia sp.	M	AF050431	Carranza *et al.* (1998a)
	Phagocata sibirica (Sabussow, 1903) Kenk, 1974	M	Z99948	Carranza *et al.* (1998b)
	Phagocata sp.	M	AF013150	Carranza *et al.* (1998a, 1999)
	Phagocata ullala Sluys, Ribas & Baguñà, 1995	M	AF013149	Carranza *et al.* (1998b, 1999)
	Platydemus manokwari Beauchamp, 1962	M	AF048766	Carranza *et al.* (1998a)
	Polycelis nigra (Müller, 1774)	M	AF013151	Carranza *et al.* (1998a, 1999)
	Polycelis tenuis Ijima, 1884	M	Z99949	Carranza *et al.* (1998b)
	Procerodes littoralis (Ström, 1768) Hallez, 1893	M	Z99950	Carranza *et al.* (1998b)
	Schmidtea mediterranea 1 (Benazzi *et al.*, 1975)	M	M58344	Riutort *et al.* (1992, 1993)
	Schmidtea mediterranea 2	M	U31084	Carranza *et al.* (1996)
	Schmidtea mediterranea 3	M	U31085	Carranza *et al.* (1996)
	Schmidtea polychroa 1 (Schmidt, 1861)	M	AF013152	Carranza *et al.* (1998b, 1999)
	Schmidtea polychroa 2	M	AF013154	Carranza *et al.* (1998b, 1999)
	Romankenkius libidinosus Sluys & Rohde, 1991	M	Z99951	Littlewood *et al.* (1999a)
	Uteriporus sp.	M	AF013148	Carranza *et al.* (1998b, 1999)
NEODERMATA	*Aspidogaster conchicola* Baer, 1827		AJ287478	Cribb *et al.* (2001); Littlewood & Olson (2001)
	Caryophyllaeides ergensi Scholz, 1990		AF286979	Olson *et al.* (2001)
	Dasyrhynchus pillersi Southwell, 1929		AJ287496	Littlewood & Olson (2001)
	Diphyllobothrium stemmacephalum Cobbold, 1858		AF124459	Olson & Caira (1999)
	Echinococcus granulosus (Batsch, 1786) Rudolphi, 1805		U27015	Picon *et al.* (1996)
	Echinostoma caproni Richard, 1964		L06567	Blair & Barker (1993)
	Fasciola gigantica Cobbold, 1856		AJ011942	Littlewood (1999)
	Fasciola hepatica Linnaeus, 1758		AJ004969	Fernandez *et al.* (1998)
	Gyrodactylus rhodei Zitnan, 1964		AJ567670	Matejusova *et al.* (2003)
	Multicotyle purvisi Dawes, 1941		AJ228785	Littlewood *et al.* (1999a)
	Phyllobothrium lactuca Van Beneden, 1850		AF286999	Olson *et al.* (2001)
	Troglocephalus rhinobatidis Young, 1967		AJ228795	Littlewood *et al.* (1998); Littlewood & Olson (2001)
	Udonella caligorum Johnston, 1835		AJ228796	Littlewood *et al.* (1999a)
RHABDOCOELA — 'Dalyellioida'	*Anoplodium stichopi* Bock, 1925	M	AF167424	Jondelius *et al.* (2001)
	Graffilla buccinicola Jameson, 1897	M	AJ012521	Littlewood *et al.* (1999a)
	Microdalyellia rossi (Graff, 1911) Gieysztor, 1938	M	AJ012515	Littlewood *et al.* (1999a)
	Provortex balticus (Schultze, 1851) Graff, 1882		AJ312268	Norén & Jondelius (2002)
	Provortex tubiferus Luther, 1948		AJ312269	Norén & Jondelius (2002)
	Pterastericola australis Cannon, 1986	M	AJ012518	Littlewood *et al.* (1999a)
RHABDOCOELA — 'Typhloplanoida'	*Astrotorhynchus bifidus* (McIntosh, 1874) Graff, 1905		AJ312270	Norén & Jondelius (2002)
	Bothromesostoma personatum (Schmidt, 1848) Fuhrmann, 1894	M	M58347	Turbeville *et al.* (1992); Riutort *et al.* (1992, 1993)
	Bothromesostoma sp.	M	D85098	Katayama *et al.* (1996)
	Maehrenthalia agilis (Levinsen, 1879) Graff, 1905		AJ312273	Norén & Jondelius (2002)
	Mariplanella frisia Ax & Heller, 1970	M	AJ012514	Littlewood *et al.* (1999a)
	Mesoscastrada sp.	M	U70081	Carranza *et al.* (1997)
	*Mesostoma lingua**	M	AJ243682	Littlewood *et al.* (1999b)
	Trigonostomum penicillatum Schmidt, 1857		AJ312275	Norén & Jondelius (2002)
RHABDOCOELA — Kalyptorhynchia	*'arrawarria inexpectata'*	M	AJ243677	Littlewood *et al.* (1999b)
	Cheliplana cf. *orthocirra*	M	AJ012507	Littlewood *et al.* (1999a)
	Diascorhynchus rubrus Boaden, 1963	M	AJ012508	Littlewood *et al.* (1999a)
	*Gyratrix hermaphroditus**	M	AJ012510	Littlewood *et al.* (1999a)
	*Phonorhynchus helgolandicus**		AJ312274	Norén & Jondelius (2002)
RHABDOCOELA — Temnocephalida	*Temnocephala* sp. 1	M	AJ012520	Littlewood *et al.* (1999a)
	Temnocephala sp. 2	M	AF051332	Baguñà *et al.* (2001a)

Table 3 Primers used in PCR and sequencing reactions.

Primer	Used in	Primer sequence	Reference
Tim A	PCR/Sequencing	5′-AMCTGGTTGATCCTGCCAG-3′	Norén & Jondelius (1999)
Tim B	PCR/Sequencing	5′-TGATCCATCTGCAGGTTCACCT-3′	Norén & Jondelius (1999)
600F	PCR	5′-GGTGCCAGCAGCCGCGGT-3′	Modified after Norén & Jondelius (1999)
1100R	PCR	5′-GATCGTCTTCGAACCTCTG-3′	Norén & Jondelius (1999)
18S4FB	Sequencing	5′-CCAGCAGCCGCGGTAATTCCAG-3′	Norén & Jondelius (1999)
18S4FBK	Sequencing	5′-CTGGAATTACCGCGGCTGCTGG-3′	Norén & Jondelius (1999)
18S5F	Sequencing	5′-GCGAAAGCATTTRYCHAGDA-3′	Modified after Norén & Jondelius (1999)
18S5FK	Sequencing	5′-THCTDGRYAAATGCTTTCGC-3′	Modified after Norén & Jondelius (1999)
18S7F	Sequencing	5′-GCAATAACAGGTCTGTGATGC-3′	Norén & Jondelius (1999)
18S7FK	Sequencing	5′-GCATCACAGACCTGTTATTGC-3′	Norén & Jondelius (1999)

1200 bp closest to the 3′ end were amplified with the primers 18S 600F and TimB, which gave approximately a 500 bp overlap. All PCR reactions were performed in 25 μL, using Promega PCR Core System I. These reactions, containing 0.2 μM of the respective forward and reverse primer, 1 mM of each dNTP, one-tenth volume of *Taq* DNA Polymerase buffer 10X, 1.5 mM $MgCl_2$ and 1.25 U of *Taq* DNA Polymerase, were carried out on an Eppendorf Mastercycler Gradient. The PCR results (5 μL) were verified on a 1% agarose gel, stained with ethidium bromide. PCR products were purified with the Qiaquick PCR Purification Kit from Qiagen and stored at 4 °C.

Sequencing was performed by the Genetic Service Facility of VIB (Flanders Interuniversity Institute for Biotechnology), using TimA, TimB and six internal primers (see Table 3) on an ABI 3730 DNA Analyser (Applied Biosystems) with the ABI PRISM BigDye Terminator cycle sequencing kit. The sequences were verified by forward and reverse comparisons using Chromas v. 1.45 (freeware from http://www.technelysium.com.au/index.html).

Three rhabdocoel species for which 18S rDNA sequences are available in GenBank (*Gyratrix hermaphroditus*, *Mesostoma lingua* and *Phonorhynchus helgolandicus*; see asterisked entries in Table 2) are also represented by a new sequence as an additional check on the sequence's quality. For some of the specimens, both PCR reactions and sequencing were performed in DTJL's laboratory by Bonnie Webster (§ in Table 1) using protocols outlined in Littlewood *et al*. (2000).

All new sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov). Their accession numbers can be found in Table 1.

Alignment

A prior alignment of 94 sequences of rhabditophoran flatworms (M column in Table 2) based on secondary structures was downloaded from the SSU rRNA database (http://www.psb.ugent.be/rRNA; see also Wuyts *et al*. 2004). This initial alignment was used to create a hidden Markov model profile with the hmmbuild option in HMMER 2.3.2 (http://hmmer.wustl.edu/; see also Eddy 1998). This model contains probability parameters, which are estimated from the observed frequencies of residues and transitions in the initial multiple sequence alignment. With this probability model, HMMER was used to create an alignment (hmmalign option) of the new sequences (see Table 1) and 24 additional sequences from GenBank, mainly Neodermata sequences (see Table 2). This resulted in an alignment of 3522 base positions, which was edited using MacClade 4.06 (Maddison & Maddison 2003). In total, 1729 positions of the alignment were deleted because they had a gap in all except for one or two of the species. These positions would not have influenced the parsimony analysis, but would have required a larger amount of computational effort in the Bayesian analyses. Longer blocks of deleted nucleotides only appeared in the neodermatan species.

Phylogenetic analyses

The final data matrix consists of 1793 unambiguously alignable base positions for 164 species and was analysed using maximum parsimony and Bayesian inference. Prior to the analyses, base composition (% GC content) was calculated using PAUP* 4.0b10 (Swofford 2003) to account for possible base compositional bias.

Parsimony analysis (with gaps treated as missing data) was performed using both PAUP* and TNT 1.0 (Goloboff *et al*. 2001), the former in combination with PAUPRat (Sikes & Lewis 2001). PAUPRat implements the parsimony ratchet (Nixon 1999), making the tree search more efficient. In PAUP* the Rat-search was performed once and the settings were as follows: nchar = 1793, random seed = 0, nreps = 200, pct = 15 (default; ideally between 5 and 25%, see Nixon 1999), wtmode = uniform, terse. For all the heuristic searches performed by PAUPRat, the default settings were used. In TNT the Rat-search was repeated 20 times (as recommended in the PAUPRat manual: Sikes & Lewis 2001), each with 500 iterations and about the same deletion frequency as above.

We employed two approaches to control for rate heterogeneity effects that may affect the results of a parsimony analysis: taxa that had the largest pairwise distances, and thus may constitute long branches, were removed from the dataset and a separate parsimony analysis was run. In addition, our Bayesian analysis was designed to take unequal rates into account.

Bayesian inference (Rannala & Yang 1996; Mau & Newton 1997; Yang & Rannala 1997; Mau *et al.* 1999; Larget & Simon 1999) was performed in MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001) under the general time-reversible model (GTR; Rodríguez *et al.* 1990), with discrete gamma-distributed rate variation among sites (Yang 1993, 1994) with four categories, and allowing for invariant sites (Gu *et al.* 1995; Waddell & Steel 1997). This model was chosen by MODELTEST 3.06 (Posada & Crandall 1998) as the model of DNA evolution that best fitted the data. Five independent runs were performed, each with 2 million generations and four chains (default temperature), sampled every 100 generations. Branch lengths were saved. An additional run with 10 million generations was also done to ensure that the analysis was running long enough to converge on a stable LnL value. Different parameters can converge at different rates (Huelsenbeck *et al.* 2002). Therefore both log-likelihood and tree length values were plotted against the generation number. The burn-in value was chosen in function of both parameters converging on a stable value. After discarding the trees sampled during the burn-in, the results were summarized in 95% majority rule consensus trees. All analyses were run on two 86 AMD 2800+ CPUs.

The occurrence of long-branch attraction (see Felsenstein 1978) was additionally tested by omitting the four taxa with the largest mean pairwise distance compared to all other taxa from the parsimony analysis in PAUP*: *Graffilla buccinicola* Jameson, 1897, *Plagiostomum ochroleucum* Graff, 1882, *Udonella caligorum* Johnston, 1835 and *Vorticeros ijimai* Graff, 1899.

Clade support

Clade support was assessed by calculating jackknife values (Lanyon 1985; Siddall 1995), Bremer support values (Bremer 1988, 1994) and Bayesian posterior probabilities (see above for references).

Bremer support (Bremer 1988, 1994) was calculated using TreeRot v2 (Sorenson 1999). This program generates a command file for PAUP*, which consists of the constraint statement for each node and the commands to search for the shortest tree incompatible with this node. The constrained searches to determine the Bremer support indices were done using the parsimony ratchet.

For estimating nodal support in the parsimony analysis, character jackknifing was preferred to bootstrapping for its computational efficiency (see Farris 1998), and was performed with Xac (Farris 1997); character deletion frequency e^{-1}, 1000 replicates, 3 random additions and branch swapping enabled. For Bayesian analysis nodal support was estimated by determining posterior probabilities with MrBayes.

Monophyly of the Typhloplanoida and Typhloplanoida *sensu lato* (Typhloplanoida + Kalyptorhynchia; see Ehlers 1985) was tested by constructing two constraint trees in MacClade, one with a monophyletic Typhloplanoida, the other with a monophyletic Typhloplanida. A PAUPRat search in PAUP* with 200 iterations was run and Templeton (Templeton 1983) and Winning sites (Prager & Wilson 1988) tests were performed with PAUP* to determine whether these constraint solutions were significantly different from the most parsimonious solution. The constraint trees were also used to filter the trees sampled in the Bayesian analysis.

Results

Sequence data

Within the alignment the length of the 18S rDNA fragment varied between 1308 bp (*Dugesia iberica*) and 1792 bp (*Archimonocelis crucifera*, *Cirrifera sopottehlersae* and *Coelogynopora axi*). The GC content varied between 40.4% (*Dugesia mediterranea* 3) and 51.7% (*Echinococcus granulosus*) with an average of 45.3%. Remarkably, all neodermatans have a high GC content, ranging from 47.1% to 51.7%, whereas most of the triclads have a rather low value ranging between 40.7% and 47.4%. Representatives of the Rhabdocoela had a GC content which is scattered over almost the whole range, from 42.3% in *Astrotorhynchus bifidus* to 48% in *Olisthanella truncula*. The difference between these extremes (within the Rhabdocoela) was lower than the 8–10% value, which is often assumed to be the maximum value at which a biasing effect of compositional heterogeneity can occur (e.g. Galtier & Gouy 1995). Therefore, it is unlikely that GC bias consitutes a problem in our data set.

Parsimony analysis

Of the 1793 unambiguously alignable base positions in the final alignment, 468 sites were constant and 245 were parsimony-uninformative, resulting in 1080 parsimony-informative characters. The analysis in PAUP* combined with PAUPRat generated seven topologically distinct MP trees (length = 14 822 steps; CI = 0.180; RI = 0.678; RC = 0.122). The strict consensus of these trees is depicted in Figs 1–2. TNT ratchet analyses yielded trees of the same length and topology as PAUPRat.

The analysis in which the taxa with the largest mean pairwise distance were excluded resulted in a tree of 14 022 steps with the same overall topology as that of the tree depicted in Figs 1–2.

Bayesian inference

Four out of five independent runs of 2 million generations converged after 110 000–250 000 generations, although with

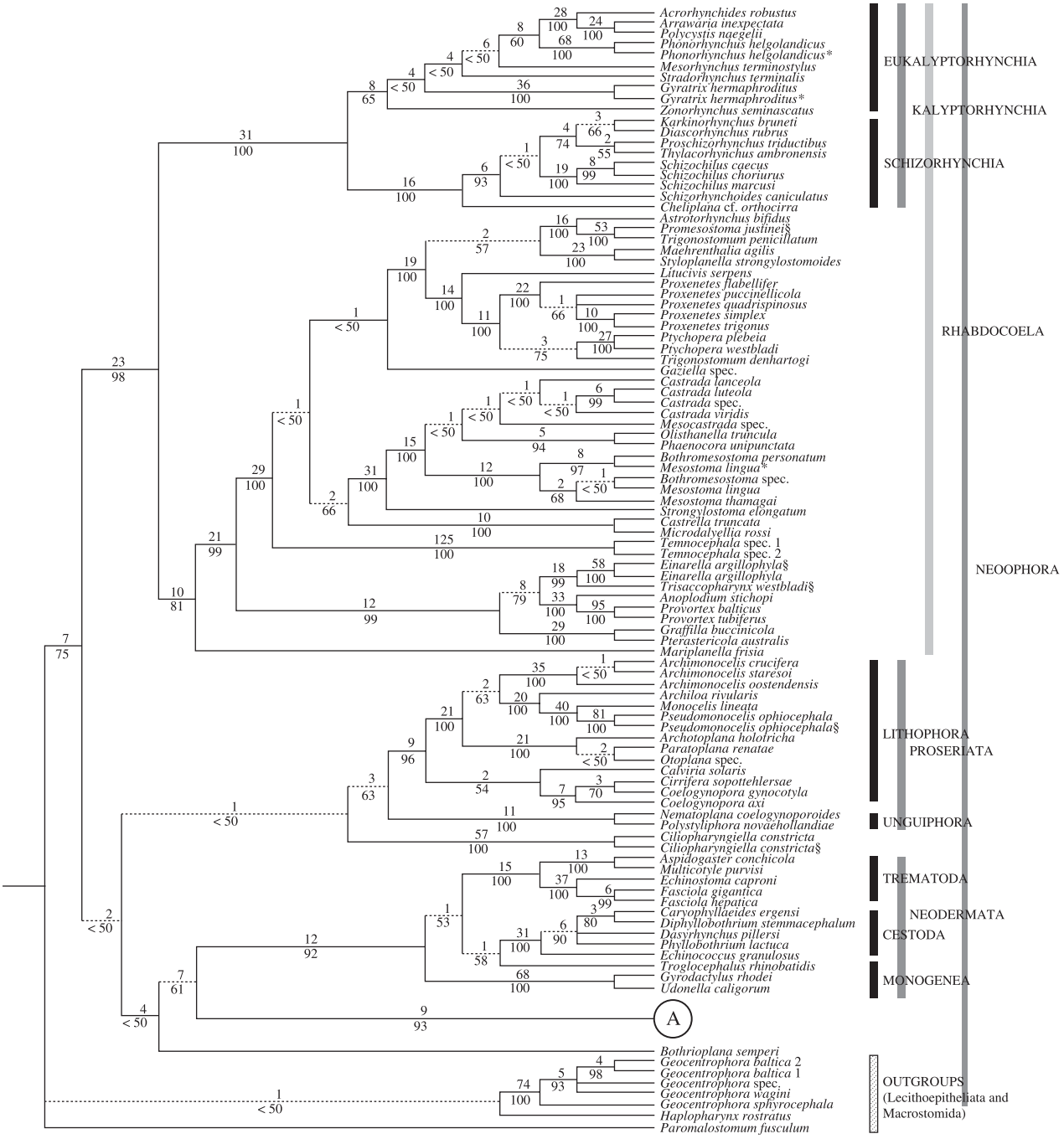

Fig. 1 Strict consensus of seven most parsimonious trees of 14 822 steps (CI = 0.180; RI = 0.678; RC = 0.122) obtained in PAUP* combined with PAUPRat (200 iterations). Bremer support values are indicated above each clade, jackknife values beneath. Clades with a posterior probability of ≤ 95% in the Bayesian analysis are indicated with dashed lines. Present taxonomic positions are indicated on the right. (§ and *: see Tables 1 and 2). Adiaphanida (A) is depicted in Fig. 2.

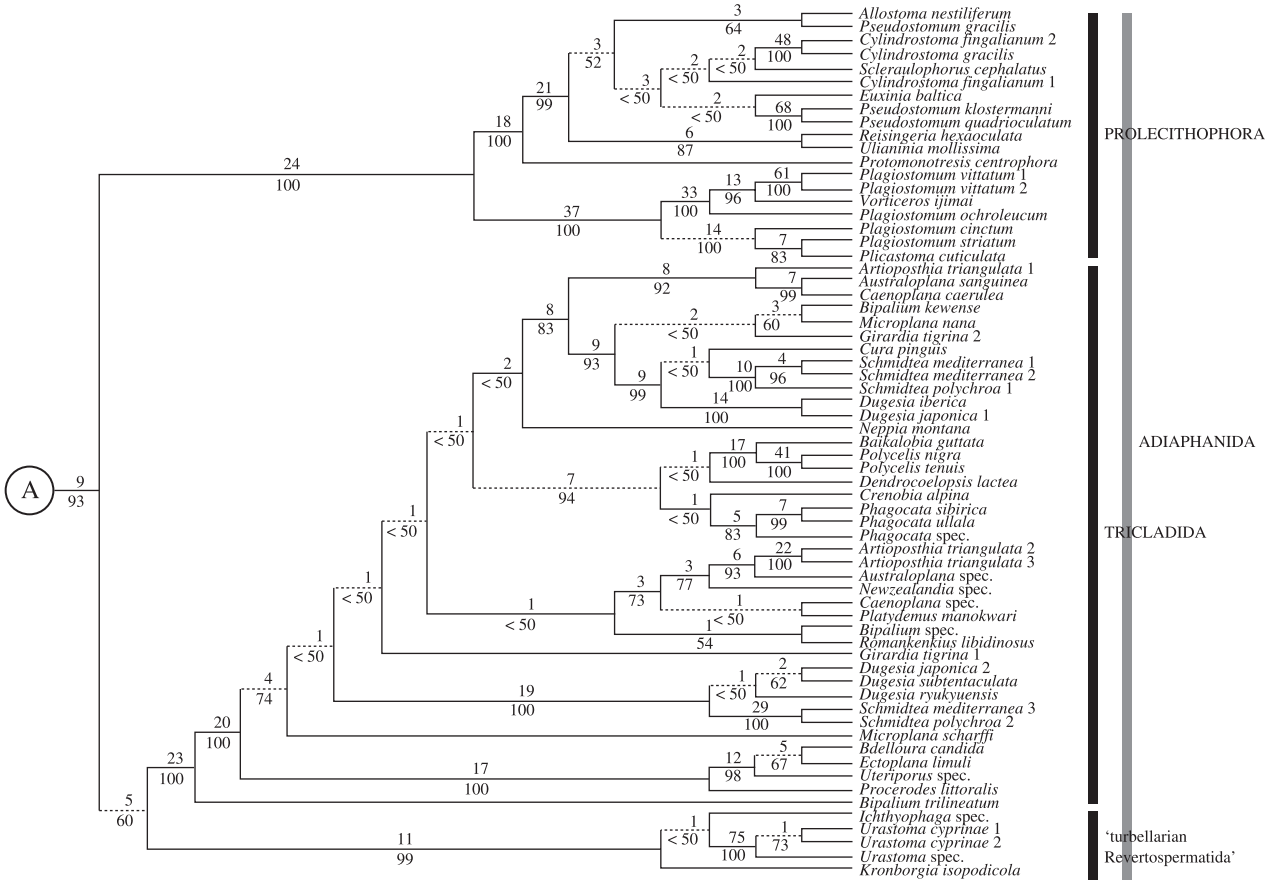

Fig. 2 Adiaphanida. Part of the tree in Fig. 1, therein abbreviated as (A).

Table 4 Burn-in (with respect to log-likelihood and tree length values) and mean log-likelihood values of six independent MrBayes runs. Run III failed to converge: LnL plot showed plateaus after 150 000, 850 000, 1 060 000 and 1 400 000 generations; TL plot after 250 000, 870 000, 1 030 000 and 1 370 000 generations (I–V: 2 M generations, VI: 10 M generations; burn-in value used in computing consensus tree is indicated in bold).

Run	Burn-in (LnL)	Burn-in (TL)	LnL
I	110 000	**250 000**	–67346.772
II	**150 000**	**150 000**	–67347.648
III	1 400 000	1 370 000	/
IV	**240 000**	200 000	–67343.042
V	140 000	**165 000**	–67344.184
VI	100 000	**150 000**	–67345.293

different values for the burn-in according to log-likelihood and treelength values (see Table 4). The third run (no. III, see Table 4) did not converge, but the majority rule consensus tree (burn-in chosen at 250 000) was identical to the strict consensus of the MP trees.

The 95% majority rule consensus trees were identical to the strict consensus of the parsimonious trees, depicted in Figs 1–2.

Tree topology and clade support

The tree in Figs 1–2 shows the results of both the parsimony and the Bayesian analysis. This tree is simplified in Fig. 3 by collapsing clades with jackknife ≤ 90% and by considering only the major taxa. The sister group relations of the major (ingroup) neoophoran taxa are far from resolved, which is evident from the tree in Figs 1–2 and 3. They form a large polytomy in a poorly supported clade (Bremer support 7; jackknife 75%; Bayesian posterior probability 100%), formed by the Rhadocoela, Lithophora, Unguiphora, Neodermata and Adiaphanida (= Prolecithophora + Tricladida + 'turbellarian Revertospermatida') and two isolated species, *Ciliopharyngiella constricta* and *Bothrioplana semperi*. It is beyond the scope of this contribution to discuss any of the other supported clades within these taxa. There is strong support for a monophyletic Rhabdocoela (BS 23; jackknife 98%; BPP 100%). Its sister group, however, cannot be indicated as yet.

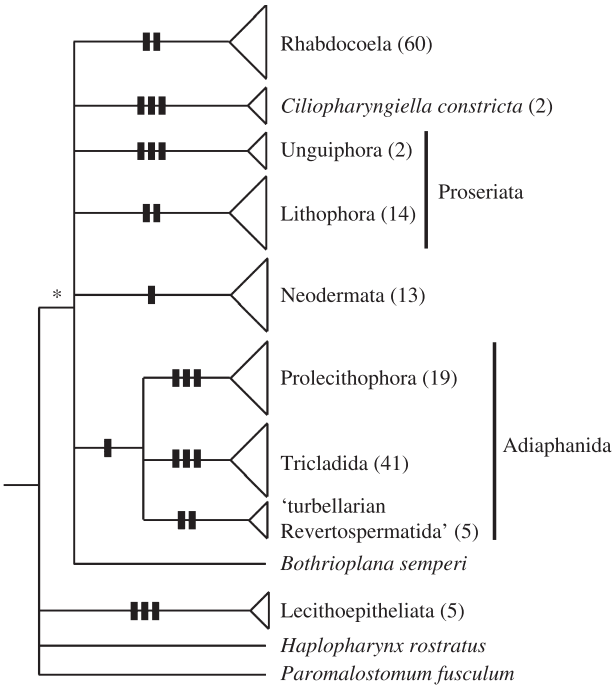

Fig. 3 Summary of results based on strict consensus of seven most parsimonious trees with the major taxa under consideration. Only clades with jackknife values of > 90% are indicated. One rectangle represents > 90% jackknife support, two represent > 95% and three, 100%; * clade with 75% support. Number of sequences used is indicated in parentheses following taxon names.

Figure 4 shows the relationships within the Rhabdocoela. All clades except two (see Fig. 4: *) have jackknife support of 99–100% and all collapsed clades have jackknife support of ≤ 65%. The Rhabdocoela shows a trichotomy formed by *Mariplanella frisia*, the monophyletic Kalyptorhynchia and a clade containing all 'Dalyellioida' and all 'Typhloplanoida' (excl. *Mariplanella frisia* and *Ciliopharyngiella constricta*), hence referred to as Dalytyphloplanida (see Nomenclatural implications). There is support for a clade formed by the Dalytyphloplanida and *M. frisia* (BS 10; jackknife 81%; BPP 99% — see Figs 1–2).

Within the Kalyptorhynchia there is strong support for a monophyletic Schizorhynchia, but not for a monophyletic Eukalyptorhynchia. None of the 'families' within the Schizorhynchia nor the Eukalyptorhynchia can be recognized.

Within the Dalytyphloplanida there are two highly supported clades, the Neodalyellida and the Neotyphloplanida (see Nomenclatural implications). In the Neodalyellida we find all marine dalyellioids (including the symbionts) and two marine typhloplanoids: *Trisaccopharynx westbladi* (Solenopharyngidae) and *Einarella argillophyla* (Promesostomidae). The Neotyphloplanida consists of a polytomy with *Gaziella* sp. and four clades: the (freshwater) Temnocephalida Blanchard, 1849, the (freshwater) Dalyelliidae Graff, 1905, the (freshwater) Typhloplanidae Graff, 1905, and a clade with all marine typhloplanoids (+ the freshwater *Styloplanella strongylostomoides*, so far included in the Typhloplanidae). This last taxon, with all marine typhloplanoids, receives the name Thalassotyphloplanida (see Nomenclatural implications). There is evidence for the monophyly of the Dalyelliidae and the Typhloplanidae but not for other taxa of the family level.

The Templeton and Winning sites tests both found the 14 distinct MP trees to be significantly different at the 95% confidence level from the 'Typhloplanoida' and 'Typhloplanoida' *s.l.* (= Typhloplanoida + Kalyptorhynchia) constraint trees ($P < 0.0001$). Moreover, filtering all trees resulting from the Bayesian analyses, including the trees from run III (excluding 240 000 generations as burn-in), did not resolve any tree compatible with a monophyletic Typhloplanoida and Typhloplanoida *s.l.* Thus all our tests rejected the monophyly of Typhloplanoida *s.l.* and Typhloplanoida.

In Fig. 5 all three measures of clade support are compared. Clades with jackknife and BPP values < 50% are not included in the graphs. From Fig. 5A, it appears that for clades with Bremer support values higher than > 10, the jackknife support was > 80%. The posterior probabilities were exceptionally high compared to the jackknife (Fig. 5B) and the Bremer support (Fig. 5C) values. These results confirm that BPP values may be misleadingly high, even with low jackknife support (see also Simmons *et al*. 2004).

Discussion

The reconstruction of the 18S rDNA gene tree for the taxa under consideration seems to be robust. The three different support values calculated here (Bremer support, jackknife and posterior probabilities) agreed in most cases (see Fig. 5). However, based on simulation studies, Bayesian posterior probabilities are said to be misleadingly high in comparison with jackknife values (Simmons *et al*. 2004) and bootstrap values (Suzuki *et al*. 2002; Alfaro *et al*. 2003; Douady *et al*. 2003; Erixon *et al*. 2003). From Fig. 5C, it is clear that in our empirical study Bayesian posterior probabilities are indeed considerably higher than the jackknife values. However, in the preferred tree (see Figs 1–2) clades with high jackknife values (> 90%) in most cases also have a high Bremer support value (> 10) and a very high posterior probability (> 95% and even 100% in most cases).

Most of the deeper branches, i.e. the relationships between the major neoophoran taxa, are only weakly supported (see Figs 1–3), which could be an indication that the relationships between these taxa cannot be properly revealed with 18S rDNA alone. It highlights the necessity of searching for other molecular markers to sort out the relationships within the Platyhelminthes. In contrast, lower level clades within the Rhabdocoela (and within the other taxa) are strongly supported. The support values for the Rhabdocoela and well

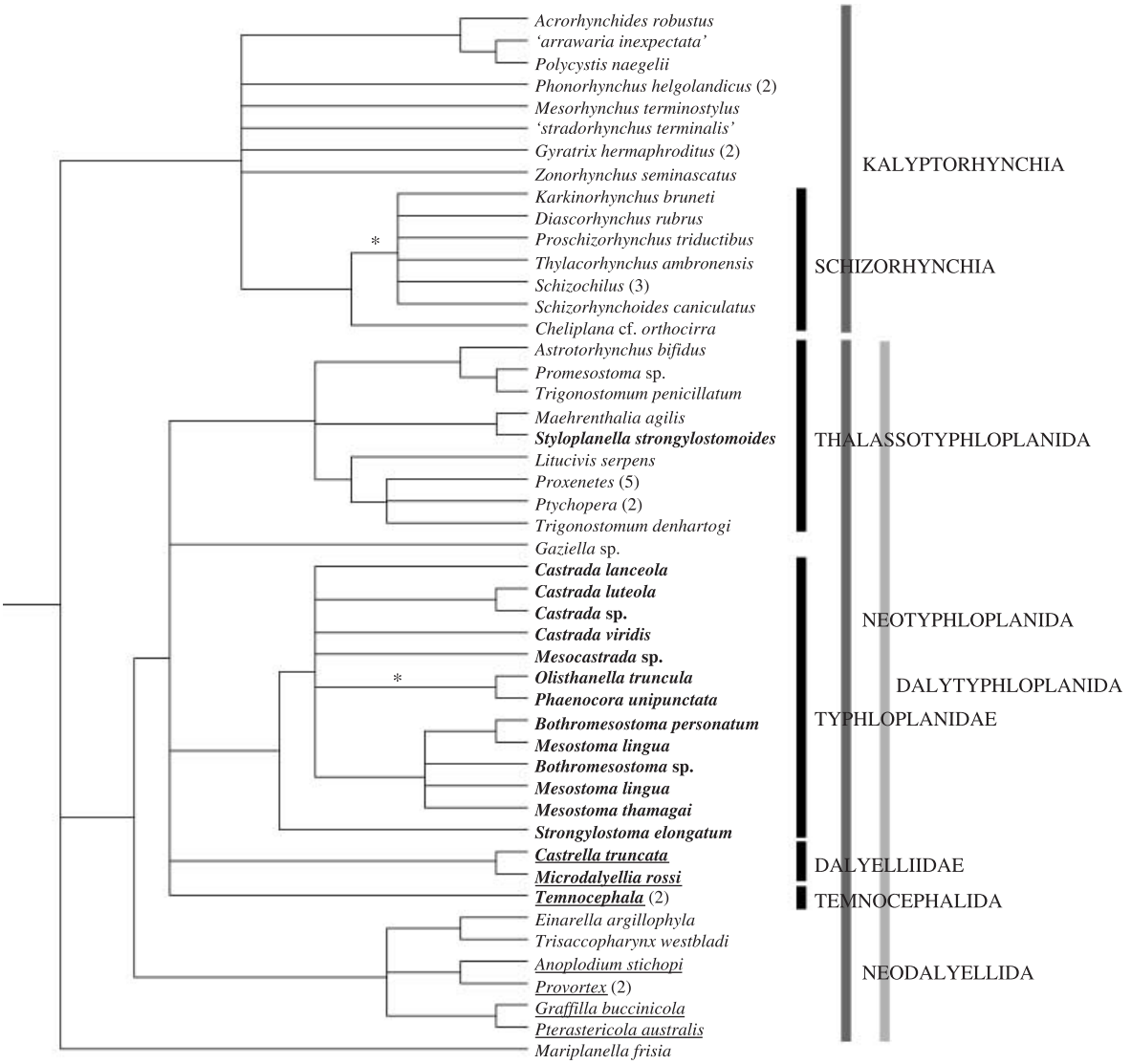

Fig. 4 Rhabdocoela. Strict consensus of seven most parsimonious trees obtained in PAUP* combined with PAUPRat. Clades with jackknife values of < 90% are collapsed. Named (and unnamed) monophyletic taxa are on the right. The number of species in a terminal taxon is indicated in parentheses. Freshwater species are in bold, 'Dalyellioida' are underlined. All collapsed clades have < 65%, jackknife support, most remaining clades have 99–100%; those indicated with an asterisk have 94% support.

supported clades within this taxon are given in Table 5, together with possible morphological apomorphies (see below).

Another indication of the robustness of the tree is the congruence between the MP tree and the Bayesian tree. As Bayesian inference accounts for rate heterogeneity across sites (if the used model is corrected for this), the congruence between both methods indicates absence of long-branch attraction artifacts. This view is further supported by the fact that the same overall tree topology is found when taxa with the largest main pairwise distances in comparison to the other taxa are excluded from the parsimony analysis.

Robustness of the rhabdocoelan part of the tree was further tested by enforcing the monophyly of the 'Typhloplanoida' and a monophyletic 'Typhloplanoida + Kalyptorhynchia'. The results of the tests clearly showed that both groups are not monophyletic.

At present, no molecular phylogenetic study deals excusively with the Rhabdocoela, which nevertheless is one of the largest taxa of free-living flatworms. Earlier molecular analyses that included more than one rhabdocoel sequence (e.g. Littlewood *et al*. 1999a,b; Baguñà *et al*. 2001b; Joffe & Kornakova 2001; Littlewood & Olson 2001; Norén & Jondelius

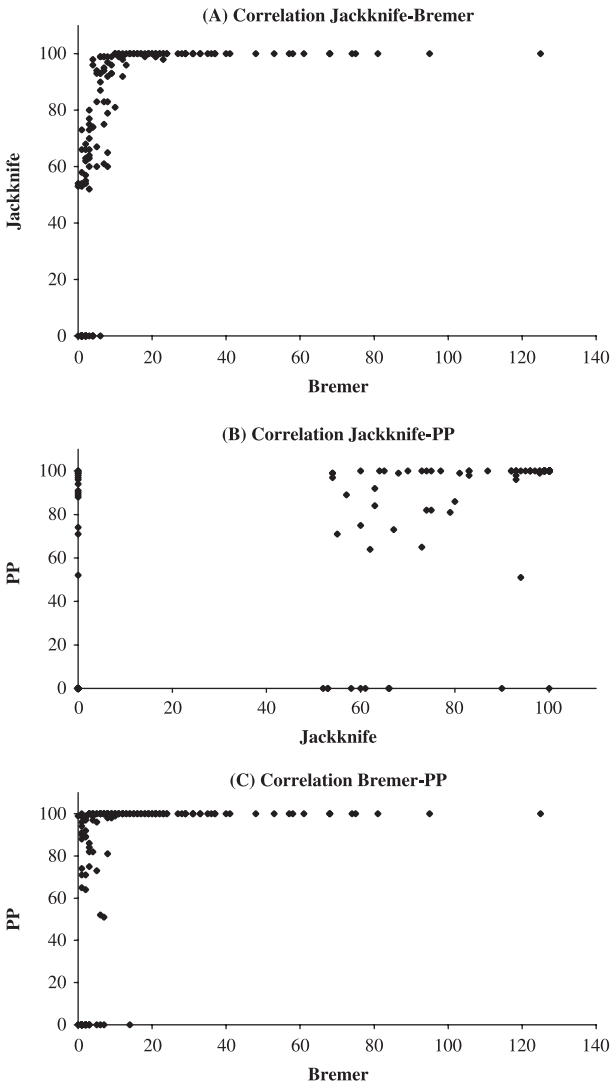

Fig. 5 A–C. Comparison of Bremer support, jackknife and posterior probability (PP) values. Correlation between jackknife and Bremer support values (A), jackknife values and posterior probabilities (B), and Bremer support values and posterior probabilities (C).

2002; Lockyer *et al*. 2003) paid little attention to the relationships within the Rhabdocoela. All these studies are based on 18S rDNA, except for those by Littlewood *et al*. (1999b), Norén & Jondelius (2002) and Lockyer *et al*. (2003), which also include data on 28S rDNA.

All the above studies report a monophyletic Rhabdocoela, albeit with varying nodal support (bootstrap values of < 50–77% in Littlewood *et al*. 1999a,b; Baguñà *et al*. 2001b; Joffe & Kornakova 2001; Littlewood & Olson 2001; jackknife value of 98–99% in the present study and in Norén & Jondelius 2002). With the present results the sister group of the Rhabdocoela cannot be identified as it forms part of a polytomy (see Fig. 3). Former studies found some support for a sister group relationship of the Rhabdocoela with the Adiaphanida (Littlewood *et al*. 1999a,b; Baguñà *et al*. 2001b; Joffe & Kornakova 2001; Littlewood & Olson 2001; Norén & Jondelius 2002; Lockyer *et al*. 2003). However, with bootstrap or jackknife values around 50%, none of these studies is convincing and with poorly supported clades collapsed, all cladograms would show the same polytomy as in our study. In our study, one of the members of the polytomy is *Ciliopharyngiella constricta* Martens & Schockaert, 1981. The taxon *Ciliopharyngiella* Ax, 1952 was formerly placed within the 'Typhloplanoida' (see Ehlers 1972), but its taxonomic position has been heavily debated based on morphological (see Ax 1952; Ehlers 1972) and ultrastructural data (see Brüggeman 1985; Sopott-Ehlers 1997, 1999, 2001) and now it appears that molecular data do not completely solve the problem either.

Of all above mentioned studies, the one of Norén & Jondelius (2002), which includes 20 rhabdocoel species, has the most extensive taxonomic sampling within the Rhabdocoela, whereas the others include five (Lockyer *et al*. 2003) or 12 (Littlewood *et al*. 1999a,b; Joffe & Kornakova 2001; Littlewood & Olson 2001) species. Therefore the results of our analyses, which include 62 sequences of 57 different rhabdocoel species currently included within the Rhabdocoela, are best compared with those of Norén & Jondelius (2002). The tree topology within the Rhabdocoela is identical and

Table 5 Overview of support values and possible morphological apomorphies for the Rhabdocoela and newly defined clades within this taxon.

	Jackknife (%)	Bremer support	BPP (%)	Possible apomorphies
Rhabdocoela	98	23	100	pharynx bulbosus (?); terminal cell of protonephidia with single row of ribs (?); type C protonephridia (?); dense heel in sperm (?)
Kalyptorhynchia	100	31	100	proboscis; incorporation of axonemes in sperm
Schizorhynchia	100	16	100	split proboscis; loss of one axoneme in sperm
Dalytyphloplanida	99	21	100	presence of small dense granules, an axonemal spur, a group of longitudinal microtubules in the sperm and a fine connection between nuclear and plasma membranes (?)
Neodalyellida	99	12	100	none
Neotyphloplanida	100	29	100	none
Thalassotyphloplanida	100	19	100	none

clades that coincide with our Dalytyphloplanida, as well as with our Neodalyellida and Neotyphloplanida are apparent (see Norén & Jondelius 2002: Fig. 1).

Previous morphological analyses dealing with the Rhabdocoela (Jondelius & Thollesson 1993; Zamparo *et al*. 2001) are difficult to compare with, since the taxonomic composition is very different and because they include only a small number of terminals, almost all of them of the 'family'-level. Nevertheless, it is possible to indicate morphological apomorphies for some of the rhabdocoelan clades (see Table 5). However, a large number of morphological aspects concern ultrastructural data, which should be interpreted with caution, as few taxa have been sampled and therefore these data are highly fragmentary. Good reviews of current knowledge on the ultrastructure of sperm and protonephridia can be found in Watson (2001) and Rohde (2001), respectively. Several contributions on the ultrastructure of other organs (e.g. male and female atrial system, eyes, and epidermis) exist (e.g. Brüggeman 1985; Rohde *et al*. 1987; Sopott-Ehlers 1996, 1997; Sopott-Ehlers & Ehlers 1997), but are even more fragmentary than those on spermatology and protonephridia.

The Rhabdocoela is still not supported by a clear morphological apomorphy. All rhabdocoels have a 'pharynx bulbosus', which they share with the parasitic neodermatans, some prolecithophorans and lecithoepithelians. Therefore it is possible that the 'pharynx bulbosus' has originated more than once, and that the homologies should be reconsidered carefully (as suggested by Joffe 1987). A first possible apomorphy could be found in the ultrastructure of the protonephridial system. In all rhabdocoels the terminal cell has a single row of longitudinal ribs (see Rohde 2001). However, this construction is also found in some other taxa (Lecithoepitheliata and Prolecithophora; see Littlewood *et al*. 1999a; Rohde 2001). A second possible apomorphy based on protonephridial ultrastructure is the lack of a terminal perikaryon, whereas the flame bulb is continuous with the proximal canal cell and without a junction (Type C of Watson & Schockaert 1997; see also Rohde 2001). Moreover, Watson (2001) also proposed the presence of a dense heel on the basal bodies during spermiogenesis as a possible apomorphy for the Rhabdocoela. However, this feature is lost in several taxa within the Rhabdocoela (all kalyptorhynchs, except for some schizorhynchs).

For the Kalyptorhynchia two clear apomorphies can be indicated: the presence of a muscular proboscis and the incorporation of the axonemes within the sperm body during spermiogenesis (see Ehlers 1985; Watson 2001). Within the Kalyptorhynchia, a split proboscis and the loss of one axoneme during spermiogenesis (see Watson 2001) characterizes all representatives of the Schizorhynchia. The sister taxon of the Kalyptorhynchia is still unclear, as it forms a polytomy with the Dalytyphloplanida and *Mariplanella frisia* in all phylogenetic studies based on 18S rDNA. However, the present study shows a relatively high support for a clade uniting *M. frisia* with the Dalytyphloplanida (see Fig. 1: jackknife 81%). Therefore, this clade most probably is the sister group of the Kalyptorhynchia. The presence of small dense granules, an axonemal spur and a group of longitudinal microtubules (originating from a particular manner of flagellar rotation) in the sperm (see Littlewood *et al*. 1999a; Watson 2001) are possible apomorphies for the Dalytyphloplanida. However, these features are secondarily lost within some thalassotyphloplanids (in all species formerly included in the Trigonostomidae, which were studied by Watson (2001)) and within the neodalyellids. It is possible that the loss of these features is synapomorphic for a subclade of the Thalassotyphloplanida and, independently, also for a subclade of the Neodalyellida.

The Neotyphloplanida consists of a polytomy, including three large freshwater taxa (Temnocephalida, Typhloplanidae, Dalyelliidae), *Gaziella* sp. and the Thalassotyphloplanida. Littlewood *et al*. (1999a,b) and Joffe & Kornakova (2001) suggest that a freshwater rhabdocoel clade may exist (Typhloplanidae + Dalyelliidae + Temnocephalida). We did not find such a clade, but there is some support for a clade consisting of the Typhloplanidae and the Dalyelliidae (see Fig. 1: jackknife: 66%). However, with only two species included in the analysis, the dalyelliids (as with the temnocephalids) are very poorly sampled. Based only on our molecular data, all we can say is that the Typhloplanidae (excl. *Styloplanella strongylostomoides*) and the Dalyelliidae, and probably the Temnocephalida, are each monophyletic freshwater taxa.

Nomenclatural implications

In this section several new clade names are defined, following phylogenetic nomenclature (De Queiroz & Gauthier 1990, 1992, 1994) and the rules of the draft Phylocode (available at http://www.ohio.edu/phylocode). However, only clades for which strong support (jackknife > 95%) was found (see also Table 5), and for which we think naming is useful, are defined. To avoid nomenclatural instability, taxa do not receive a converted name when the original nomenclatural types were not included in the analysis.

Converted names are indicated with n.c.c. (*nomen cladi conversum*), whereas completely new clade names receive the indication n.c.n. (*nomen cladi novum*) following article 9.3 of the Phylocode.

Rhabdocoela Ehrenberg, 1831 n.c.c. (stem-based): the most inclusive clade containing *Polycystis naegelii* Kölliker, 1845 but not *Ciliopharyngiella constricta* Ax, 1952; *Monocelis lineata* (Müller, 1774) Oersted, 1844, *Nematoplana coelogynoporoides* Meixner 1938, *Fasciola hepatica* Linnaeus, 1758 and *Pseudostomum quadrioculatum* (Leuckart, 1847) Graff, 1911.

Kalyptorhynchia Graff, 1905 n.c.c. (stem-based): the most inclusive clade containing *Polycystis naegelii* Kölliker,

1845 but not *Mariplanella frisia* Ax & Heller, 1970 and *Provortex balticus* (Schultze, 1851) Graff, 1882.

Schizorhynchia Meixner, 1928 n.c.c. (stem-based): the most inclusive clade containing *Schizochilus marcusi* Boaden, 1963 but not *Polycyctis naegelii* Kölliker, 1845.

Dalytyphloplanida n.c.n. (stem-based): the most inclusive clade containing *Provortex balticus* (Schultze, 1851) Graff, 1882 but not *Mariplanella frisia* Ax & Heller, 1970 and *Polycystis naegelii* Kölliker, 1845.

Neodalyellida n.c.n. (stem-based): the most inclusive clade containing *Provortex balticus* (Schultze, 1851) Graff, 1882 but not *Proxenetes flabellifer* Jensen, 1878.

Neotyphloplanida n.c.n. (stem-based): the most inclusive clade containing *Proxenetes flabellifer* Jensen, 1878 but not *Provortex balticus* (Schultze, 1851) Graff, 1882.

Thalassotyphloplanida n.c.n. (stem-based): the most inclusive clade containing *Proxenetes flabellifer* Jensen, 1878 but not *Castrada lanceola* (Braun, 1885) Luther, 1904 and *Castrella truncata* (Abildgaard, 1789) Hofsten, 1907.

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