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Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory

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We combined nearly complete sequences of large (LSU) and small (SSU) subunit rDNA from 32 flatworm species to estimate the phylogeny of the Platyhelminthes using maximum parsimony, maximum likelihood and Bayesian inference methods. Rooted against the Catenulida, combined evidence trees offered no support for the Revertospermata, which was also rejected by constraint analysis. Generally, nodal support was higher for groupings estimated from the combined data partitions and all methods of analysis provided congruent estimates of phylogeny. The Monogenea and Proseriata were resolved as monophyletic, rejecting previous suggestions of paraphyly based on SSU and partial LSU data sets and thus supporting widely accepted morphological synapomorphies. Monophyly of the Neodermata was supported and its sister group was a clade of neoophoran 'turbellarians' to the exclusion of the Proseriata which in turn was more basal. Taxa with similar spermatology to the Neodermata (*Ichthyophaga*, *Notentera*, *Urastoma* and *Kronborgia*) were the sister group to Tricladida + Prolecithophora, which in turn were sister to the Rhabdocoela. Polycladida + Macrostomida + Lecithoepitheliata was the earliest divergent offshoot of the Rhabditophora. Among the Neodermata, the Cercomeromorphae (Cestoda + Monogenea) was not supported, whereas Cestoda + Trematoda was well supported. Although there is no known synapomorphy for this latter grouping, our data highlight problems associated with the 'cercomer theory' and we reject putative homologies regarding neodermatan 'cercomers' that have been sustained in the literature without careful scrutiny. *Biological Journal of the Linnean Society*, 2003, **78,** 155–171.

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INTRODUCTION

Since the early days of modern phylogenetic methodology the resolution of flatworm (Phylum Platyhelminthes) interrelationships has been plagued by poor nodal support and conflict between independent estimates. Morphological assessments of flatworm phylogenies (e.g. see those of Ehlers, 1985; Brooks & McLennan, 1993) are founded, and often appear to founder, on interpretations of character homology. More recent studies have refined and developed morphologically based matrices (e.g. Littlewood, Rohde & Clough, 1999b), that stem from the rigorous efforts of Ehlers (1985). However, little if any consensus is apparent from morphology alone. Not only do authors differ on these assessments and interpretations (cf. Zamparo *et al.,* 2001) but, depending on the method of character coding, any set of features favoured by one author may yield a widely different range of phylogenetic estimates depending on the coding strategy employed (see general review by Hawkins, 2000). To redress the conflict, increase resolution and to test between favoured hypotheses, molecular data have been sought as an independent estimate of phylogeny. To date, the main target has been the small subunit ribosomal RNA gene (SSU rDNA) (e.g. see Katayama,

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Nishioka & Yamamoto, 1996; Carranza, Baguñà & Riutort, 1997; Campos *et al.*, 1998; Littlewood *et al.*, 1999b). The utility of this gene for resolving the Platyhelminthes was recently reviewed by Littlewood & Olson (2001), who concluded that additional genes may prove more informative than increased taxon sampling.

As with other phylogenetic problems, and particularly within the Metazoa, there has been the question of whether more data or more genes are better for increased phylogenetic resolution. Over the years SSU rDNA genes from more and more species have been characterized with few other genes evaluated for additional independent phylogenetic estimation. Currently there are over 350 published complete SSU rDNA sequences for flatworms available, and whilst partial large subunit (LSU) rDNA (variable domains D3-D6; Litvaitis & Rohde, 1999) and elongation factor 1-alpha (Berney, Pawlowski & Zaninetti, 2000) have been utilized for various levels of flatworm phylogenetics, each has been found somewhat inadequate in resolving the deeper relationships between major groups (Littlewood *et al.*, 1999a, 2001b) and neither have been much pursued for this purpose. Instead, the region incorporating the first three variable domains of LSU rDNA (D1–D3) have been used extensively in estimating relationships within the Proseriata (Littlewood, Curini-Galletti & Herniou, 2000), Monogenea (Mollaret, Jamieson & Justine, 2000; Jovelin & Justine, 2001; Olson & Littlewood, 2002), Digenea (Tkach *et al.*, 2001), Cestoda (Olson *et al.*, 2001) and Neodermata (Mollaret *et al.*, 1997), and regions encompassing domains D3-D6 have added resolution to relationships within the Proseriata (Litvaitis *et al.*, 1996) and the pseudocerotid polyclads (Litvaitis & Newman, 2001). Litvaitis & Rohde's (1999) work on partial LSU for the phylum yielded a number of results that conflicted with both morphology and SSU. For example, for a reduced data set (22 taxa) the clade (Monopisthocotylea (Cestoda (Trematoda, Polyopisthocotylea))) was consistently resolved and in the full data set (35 taxa) the Neodermata were not always monophyletic; see also comments and re-analysis in Littlewood *et al*. (1999a).

In spite of the conflict between morphological and molecular phylogenetic estimates of the flatworms there is still overwhelming support for particular taxonomic groupings that have important implications for understanding the evolution of the group (see articles in Littlewood & Bray, 2001), and in particular the evolution of parasitism within the group (Brooks, 1989; Brooks & McLennan, 1993; Littlewood *et al*., 1999a, 2001a; Zamparo *et al*., 2001). For instance, it is now well accepted that the Neodermata, a clade encompassing the Aspidogastrea, Digenea, Monogenea and Cestoda, is monophyletic and that, in addition to the synapomorphies that unite the clade (e.g. see Ehlers, 1985; Littlewood *et al*., 1999b), the adoption of parasitism as a life history strategy was a single major evolutionary event for this group. However, the interpretation of how obligate parasitism evolved in the phylum and how it radiated so successfully within the Neodermata depends on identifying the sister-group to and interrelationships within the Neodermata. Two problems stemming from incongruent morphological and molecular analysis persist, namely the identification of the neodermatan sister-group, and whether or not the Monogenea are monophyletic.

Most recently, greater phylogenetic resolution among the Metazoa has been afforded by combining complete SSU and LSU sequences (Medina *et al.*, 2001: 9707) such that the authors were encouraged to report 'continued accumulation of LSU sequences should increase our understanding of animal phylogeny'. Mallatt & Winchell (2002: 289), in a study combining LSU and SSU in order to estimate protostome interrelationships, further concluded that 'LSU adds signal, it can be used at lower taxonomic levels … [and that] molecular systematists should use LSU + SSU rRNA genes rather than SSU alone'. Certainly, among both the Cestoda and the Monogenea this holds true even with partial (D1–D3) fragments of LSU and SSU (Olson *et al*., 2001; Olson & Littlewood, 2002). Here we aim to address major competing hypotheses regarding flatworm interrelationships by providing complete sequences of both the LSU and SSU rDNA molecules, and to test the potential utility of complete LSU sequences in further resolving the phylogeny of the Platyhelminthes.

Sister-group to the Neodermata

Littlewood *et al*. (1999a) reviewed the literature concerning the neodermatan sister-group and identified eight possible scenarios supported by different authors and data sets. Those thought to be the most plausible essentially fall into three categories. The first two stem from morphologically based studies. Ehlers (1985) argued for a clade of 'dalyellioid' turbellarians that included the Temnocephalida, ectocommensals of crustaceans (Cannon & Joffe, 2001), the Fecampiidae, obligate parasites of crustaceans and annelids, and the Udonellida, ectoparasites of caligid copepods. Brooks (1989) and Brooks & McLennan (1993) took this further and placed the Temnocephalida as the sister-group to Neodermata + Udonellida with 15 putative synapomorphies, forming his Superclass Cercomeria (Brooks, 1982; Brooks, O'Grady & Glen, 1985). However, few other authors have accepted this, regarding most of the characters as apparently homoplasious or inappropriate. Some molecular evidence supported a Fecampiida + Neoder-

mata clade (Litvaitis & Rohde, 1999), but strong morphological evidence from sperm morphology and spermiogenesis collected by Joffe & Kornakova (1998; Kornakova & Joffe, 1999) found evidence that united a clade termed the Revertospermata, comprising Fecampiida (Neodermata + Urastomidae). In addition to the apparently strong ultrastructural evidence from spermatology, the clade is compelling from a parasitological perspective too, since all members of the Revertospermata are obligate parasites. Whilst commensalism and parasitism is common throughout the phylum (Jennings, 1971), uniting the obligate parasites would appear to be most parsimonious. Zamparo *et al*. (2001) have since modified the early work of Brooks and have also adopted the Revertospermata although they resolved the clade (Neodermata (Fecampiida + Urastomidae)). The third solution arises from the analysis of SSU rDNA and suggests the sister-group to the Neodermata is a large clade of neoophoran turbellarians within which the Fecampiida and Urastomidae are most closely associated with the Prolecithophora and/or the Tricladida (Littlewood *et al*., 1999a; Littlewood & Olson, 2001). Separate analyses of SSU rDNA concentrating on the interrelationships of the Prolecithophora and Tricladida have also suggested a close relationship between these clades and the urastomids and fecampiids (Baguñà *et al*. , 2001; Norén & Jondelius, 1999).

Monophyly of the Monogenea

All morphological analyses unite the Monogenea as a monophyletic group, although most coding of the constituent groups has probably assumed monophyly *a priori* (Justine, 1998). The two main subdivisions, Monopisthocotylea and Polyopisthocotylea, are united variously, depending on author, by larvae with three ciliated zones, adults and larvae with two pairs of pigmented eyes, one pair of ventral anchors and one egg filament (Boeger & Kritsky, 2001). Molecular data consistently renders the Monogenea paraphyletic, but the order of paraphyly depends on whether SSU or partial LSU (regions D1/D2) has been sampled (Mollaret *et al*., 1997; Justine, 1998; Littlewood *et al*., 1999b). Olson & Littlewood (2002) observed that the rate of divergence between the Monopisthocotylea and Polyopisthocotylea is markedly different and that this may confound their relative placement among the Neodermata in phylogenetic analyses.

Here we present nearly complete LSU sequences, and complementary SSU sequences for 30 rhabditophoran taxa and two catenulids used as outgroups, generate phylogenies under different evolutionary models, evaluate phylogenetic information within and between data partitions, test between competing hypotheses of key sister-group relationships and make suggestions regarding the further use of LSU sequences within the Platyhelminthes.

MATERIAL AND METHODS

Choice of taxa and outgroup

We chose representatives of 11 major platyhelminth groups and as many fecampiids and urastomids as possible for characterizing the full LSU rDNA gene. Based on previous studies focusing on the interrelationships of each of these constituent groups, in particular the Tricladida, Proseriata, Cestoda, monopisthocotylean and polyopisthocotylean Monogenea, Digenea and Aspidogastrea (Carranza *et al.*, 1998; Littlewood *et al*., 2000; Cribb *et al*., 2001; Olson & Littlewood, 2002; and unpublished data; Olson *et al*., 2001), we chose a basal and a derived member from each group. This choice was biased only marginally by availability of specimens. The rationale for choosing a basal and a derived member of each clade was to encompass the greatest molecular diversity of a group with the fewest taxa possible. In most cases published SSU sequences were available from these and other previous studies. A full list of taxa, collecting details and EMBL/GenBank accession numbers is shown in Table 1.

Table 1. Sequences used in this study, their classification, EMBL/GenBank accession numbers, geographical origin and, where relevant, details of their host. Symbols/abbreviations: § new sequences; ¶ taxa that are members of the controversial Revertospermata; *species is undescribed but identified as a sanguinicolid (work in preparation); NS, no sequence available; † partial sequences (see text)

Species/classification	Collection locality/host of new sequences	GenBank Accession	
		LSU	SSU
PLATYHELMINTHES			
Catenulida (outgroup)			
Stenostomum leucops	lab culture, Åbo, Finland	AY157151§	AJ012519
Suomina sp.	Lake Madgwick, Armidale, Australia	AY157152§	AJ012532

Table 1. *Continued*

Species/classification	Collection locality/host of new sequences	GenBank Accession	
		LSU	SSU
Macrostomida			
Paromalostomum fusculum	Sylt, Belgium	AY157155§	AJ012531
Polycladida			
Notoplana australis	Port Phillip Bay, Victoria, Australia	AY157153§	AJ228786
Proseriata			
Monocelis lineata	Santa Marinella, Sardinia, Italy	AY157159§	U45961
Nematoplana sp.1	Shelly River, Queensland, Australia	AY157160§	AJ270160
Rhabdocoela – Temnocephalida			
Didymorchis sp.	ex *Cherax quadricarinutus*, Queensland, Australia	AY157163§	AY157182§
Temnosewellia minor	ex *Cherax destructor*, Armidale, Australia	AY157164§	AY157183§
Rhabdocoela – Dalyellida			
Pterastericola australis	ex *Patariella calcar*, Arrawarra, NSW, Australia	AY157161§	AJ012518
Provortex psammophilus	Island of Sylt, Germany	AY157162§†	NS
Provortex tubiferus	Bohuslan, Sweden	NS	AJ312269
Lecithoepitheliata			
Geocentrophora wagini	Chiwrkuy Bay, Lake Baikal, Irkutsk, Siberia	AY157156§	AJ012509
Prolecithophora			
Reisingeria hexaoculata	Kristineberg, Sweden	AY157157§	AF065426
Plicastoma cuticulata	Kristineberg, Sweden	AY157158§	AF065422
Tricladida			
Girardia tigrina		U78718	AF013157
Bdelloura candida	ex *Limulus polyphemus*, Florida, USA	AY157154§	Z99947
Urastomidae¶			
Urastoma cyprinae	ex *Mytilus galloprovincialis*, Southern France	AY157165§†	U70086
Genostomatidae¶			
Ichthyophaga sp.	ex *Siganus doliatus*, Green Island, Australia	AY157166§†	AJ012512
Fecampiida¶			
Kronborgia isopodicola	ex *Exosphaeroma obtusum,* Kaikura, New Zealand	AY157168§	AJ012513
Notentera ivanovi	ex *Nephthys ciliata*, White Sea	AY157167§†	AJ287546
NEODERMATA			
Trematoda – Aspidogastrea			
Rugogaster hydrolagi	*ex Callorhinchus milii,* Hobart, Tasmania	AY157176§	AJ287532
Lobatostoma manteri	ex *Trachinotus blochii*, Heron Island, Queensland, Australia	AY157177§	L16911
Trematoda – Digenea			
Sanguinicolid sp.*	ex *Arothron meleagris*, Moorea, French Polynesia	AY157174§	AY157184§
Lepidophyllum steenstrupi	ex *Anarhichus lupus,* North Sea	AY157175§	AJ287530
Schistosoma mansoni	lab strain. isolate NHM-3454/5/6 ex *Mus musculus*	AY157173§	X53047
Monogenea – Monopisthocotylea			
Udonella caligorum	ex caligid copepod ex *Gadus morhua,* North Sea	AY157172§	AJ228796
Dictyocotyle coeliaca	ex *Raja montagui*, North Sea	AY157171§	AJ228778
Monogenea – Polyopisthocotylea			
Diclidophora denticulata	ex *Pollachius virens*, North Sea	AY157169§	AJ228779
Polystomoides malayi	ex *Cuora amboinensis*, Kuala Lumpur	AY157170§	AJ228792
Cestoda – Amphilinidea			
Gigantolina magna	ex *Diagramma labiosum*, Heron Island, Queensland, Australia	AY157179§	AJ243681
Cestoda – Gyrocotylidea			
Gyrocotyle urna	ex *Callorhinchus milii*, Hobart, Tasmania	AY157178§	AJ228782
Cestoda – Eucestoda			
Hymenolepis diminuta	lab strain ex *Rattus norvegicus*, University Copenhagen, Denmark	AY157181§	F124475
Caryophyllaeus laticeps	ex *Rutilus rutilus*, Lake Neuchâtel, Switzerland	AY157180§	AJ287488

It is worth noting that molecular data have consistently indicated that the Udonellida, treated as separate from the Neodermata by some morphologists even today (Zamparo *et al*., 2001), are members of the Monopisthocotylea (Littlewood, Rohde & Clough, 1998b). Earlier ultrastructural studies of spermatogenesis (Rohde & Watson, 1993) and subsequent reassessments of morphology (Boeger & Kritsky, 2001) have provided additional support for this affinity and thus we include *Udonella caligorum* as a representative of the Monopisthocotylea.

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING OF COMPLETE LSU AND SSU

Total genomic DNA was extracted from ethanol-preserved specimens using DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol. 25 μL amplifications were performed with 3–5 μL of genomic extract (~10 ng) using Ready-To-Go PCR beads (Amersham Pharmacia Biotech) each containing 1.5 U Taq Polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP and stabilizers including BSA; and 0.4 μM of each PCR primer. The complete LSU was amplified in three overlapping sections using the primer combinations U178 + L1642, U1148 + L2450 and U1846 + L3449 (see Table 2). PCR conditions used were: 2 min denaturation at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at 52 °C and 2 min at 72 °C; followed by 7 min extension held at 72 °C. Where necessary to obtain a product, the stringency was reduced by adding $MgCl_2$ to a final concentration of 2.5 mM or reducing the annealing temperature to 50 °C. Complete sequencing of SSU rDNA was performed as described previously (Littlewood *et al*., 1999b).

PCR products were purified with Qiagen Qiaquick columns, cycle-sequenced directly using ABI BigDye chemistry, alcohol precipitated and run on an ABI prism 377 automated sequencer. A variety of internal primers were used to obtain the full sequence on both strands (see Table 2). In most cases we were able to sequence the full LSU and SSU. Note that, strictly speaking, these sequences are not fully complete as they are missing conserved regions at both 5′ and 3′ ends which were used for primer design; the full LSU includes variable domains D1 through D12 and we amplified D1 through D11. We were unable to sequence the full LSU for *Provortex psammophilus*, *Urastoma cyprinae*, *Notentera ivanovi* and *Ichthyophaga* sp., due to limited specimens or genomic DNA. Nevertheless, as these taxa were deemed very important (the latter three ostensibly comprise the sister taxa to the Neodermata within the Revertospermata), the data are presented here; of these non-neodermatan Revertospermata, only the fecampiid *Kronborgia isopodicola* was fully sequenced for both LSU and SSU. Sequences were assembled and edited using Sequencher v. 3.1.1 (GeneCodes Corp.) and submitted to EMBL/GenBank.

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

LSU primers	Primer sequence (5′–3′)
Amplification and sequencing	
U178	GCACCCGCTGAAYTTAAG
L1642	CCAGCGCCATCCATTTTCA
U1148	GACCCGAAAGATGGTGAA
L2450	GCTTTGTTTTAATTAGACAGTCGGA
U1846	AGGCCGAAGTGGAGAAGG
L3449	ATTCTGACTTAGAGGCGTTCA
Additional sequencing	
300F	CAAGTACCGTGAGGGAAAGTTG
300R	CAACTTTCCCTCACGGTACTTG
EDC2	CCTTGGTCCGTGTTTCAAGACGGG
900F	CCGTCTTGAAACACGGACCAAG
1200F	CCCGAAAGATGGTGAACTATGC
1200R	GCATAGTTCACCATCTTTCGG
1600F	AGCAGGACGGTGGCCATGGAAG
U2229	TACCCATATCCGCAGCAGGTCT
L2230	AGACCTGCTGCGGATATGGGT
U2562	AAACGGCGGGAGTAACTATGA
L2630	GGGAATCTCGTTAATCCATTCA
U2771	AGAGGTGTAGGATARGTGGGA
L2984	CTGAGCTCGCCTTAGGACACCT
U3119	TTAAGCAAGAGGTGTCAGAAAAGT
U3139	AAGTTACCACAGGGATAACTGGCT
LSU3–4160	GGTCTAAACCCAGCTCACGTTCCC
L3358	AACCTGCGGTTCCTCTCGTACT

ALIGNMENT

SSU and LSU sequences were each aligned with the aid of CLUSTALX (Jeanmougin *et al.*, 1998) and alignments refined by eye with MacClade (Maddison & Maddison, 2000). SSU sequences were aligned with reference to secondary structure models as described in Littlewood & Olson (2001). Although the secondary structure of the LSU of *Girardia tigrina* (Tricladida) has been modelled (Robin Gutell's comparative RNA website; http://www.rna.icmb.utexas.edu), we relied heavily on manual alignment of this gene, as only highly conserved regions of the gene were utilized. The LSU and SSU alignments were concatenated in MacClade, ambiguously aligned positions excluded and data partitions and taxa sets defined for complete LSU (subset of taxa), partial LSU (all taxa), and SSU (all taxa).

Sequence saturation and phylogenetic signal

The degree to which the two rDNA data partitions estimated the same relative distances among the taxa was examined by plotting the corresponding observed distances for all pairwise comparisons of the taxa from the SSU and LSU data. MacClade (Maddison & Maddison, 2000) was used to examine the distribution of site-variability in each data partition as a function of the percentage of positions in each change (step) class. Saturation of substitutions was examined by plotting observed values of C-T substitutions, as well as the percent sequence difference, for all pairwise comparisons against their corresponding patristic distances (i.e. distances based on the most parsimonious distribution of character states). Distribution of site-variability and patristic distances were based on one of five strict consensus topologies of the combined data.

We have followed one of our earlier studies (Littlewood & Olson, 2001) and Hillis & Dixon (1991), in attempting to reveal where signal lies in each molecule by plotting rescaled character-consistency index values (RC) as calculated from the MP tree and smoothed using a 5-bp sliding window technique, against character position in the full alignment. Secondary structure and regions of ambiguity, removed from the phylogenetic analyses, were also plotted and the analysis and plots for alignments representing three taxonomic levels (Platyhelminthes, Neodermata and Trematoda) repeated in order to predict future utility across these and similar taxonomic levels. Variable regions in secondary structure of SSU were determined with reference to appendix A in Olson & Caira (1999), and of LSU by aligning our *Schistosoma mansoni* sequence to *Herdmania momus* using the online alignment tool in RDP-II (Maidak *et al*., 2001) and then with reference to the models published in Degnan *et al*. (1990).

Phylogenetic analysis

Phylogenetic analyses were performed using PAUP* ver. 4.0b10 (Swofford, 2002) and the resulting networks rooted with the outgroup (Catenulida) taxa. The SSU and LSU sequence data were analysed both independently and combined using the methods of maximum parsimony (MP) and maximum likelihood (ML). Analyses by MP were performed using a heuristic search strategy (1000 search replicates), random-addition sequence and tree-bisection-reconnection (TBR) branch-swapping options. All characters were run unordered and equally weighted. Gaps were treated as missing data. Nodal support was assessed by bootstrap resampling (1000 replicates). Considering the criteria of conditional combination of independent data sets (Huelsenbeck, Bull & Cunningham, 1996; Cunningham, 1997), and using the incongruence length-difference (ILD: Farris *et al.*, 1995) test as implemented in PAUP*, we tested whether there was significant conflict between the LSU and SSU data sets prior to combining them. The test was performed with maximum parsimony, ten heuristic searches (random sequence addition, TBR branch-swapping) each for 100 homogeneity replicates on informative sites only (Lee, 2001).

Maximum likelihood analyses employed a general time reversible (GTR) model of nucleotide substitution including estimates of invariant sites (I) and among-site rate heterogeneity (G) as it was found through chi-square analysis (MODELTEST: Posada & Crandall, 1998) to provide a significantly higher likelihood score than less parameter rich models for each of the three data partitions (SSU, LSU and combined) when calculated over their corresponding single most parsimonious or strict consensus topologies. Table 3 shows the resulting parameter estimates. In calculating likelihood values, values of I and G were set to those shown in Table 3; substitution rate parameters

Table 3 Maximum likelihood parameter estimates. All estimates based on a general time reversible model of nucleotide substitution incorporating estimates of among-site rate variation (ASRV), estimated proportion of invariant sites (Inv-E), transition rates (Ts), transversion rates (Tv) and alpha shape parameter estimate of the gamma distribution (α)

	ASRV		Ts		Tv			
Data partition	α	Inv-E	AG	CT	AC	AT	GC	GT
SSU	0.622	0.271	3.104	5.599	1.350	1.618	0.780	1.000
LSU[1]	0.698	0.363	3.154	6.240	1.072	1.759	0.560	1.000
SSU+LSU[1]	0.665	0.329	3.147	5.965	1.181	1.707	0.652	1.000
SSU+LSU[2]	0.665	0.303	3.023	5.389	1.224	1.530	0.759	1.000

[1]where only complete LSU rDNA sequences were used
[2]where partial and complete LSU rDNA sequences were used

were free to vary and nucleotide frequencies used were empirical.

Bayesian inference (BI) of phylogeny was estimated using the program MrBayes (Huelsenbeck, 2000) employing the following search parameters: lset nst = 6 rates = invgamma ncat = 4 shape = estimate inferrates = yes basefreq = empirical, that approximates to a GTR + I + G model as above. Posterior probabilities were estimated over 200 000 generations, log-likelihood scores plotted and only the final 85% of trees where the log likelihood had reached a plateau used to produce the consensus tree.

RESULTS

We were able to determine or obtain complete and complementary LSU and SSU rDNA sequences for 28 species representing 15 major clades. Product length differed greatly between templates. The range in length of full LSU was 3266–5503 bp and for SSU 1651–2873 bp. For both LSU and SSU *Gigantolina magna* (Amphilinidea) was the longest sequence and is a result of large insertions in each gene, presumably representing novel secondary structure elements. Due to the lack of gDNA, only partial LSU rDNA fragments were attainable for the following: *Provortex psammophilus* (D1–D7b), *Urastoma cyprinae* (end of D3 to D6), *Ichthyophaga* sp. (D1–D6) and *Notentera ivanovi* (D1–D7a). For each of these partial fragments, complete, previously published SSU rDNA sequences were available, although in the case of *Provortex* we used the sequence from *P. tubiferus* when combining an LSU + SSU *Provortex* entry in the overall alignment.

Sequence saturation and signal

Figure 1 compares the rate and quality of signal between the two rDNA data sets; A and B show that there is little if any appreciable difference between the two genes when complete sequences are considered. A similar comparison (Olson *et al*., 2001) comparing partial (D1–D3) LSU with complete SSU sequences showed the rate of the former to be somewhat faster, whereas the complete LSU sequences considered here were comparable in rate. The plots in Figure 1C attempt to evaluate the degree of saturation seen in these gene sequences when compared among disparate members of the phylum. However, none of these show a plateau in either percent sequence difference or number of C–T transitions as pairwise comparisons are plotted according to their estimated divergences. These therefore suggest that the sequences are not saturated and should be suitable for estimates across the spectrum of taxa included in our analyses. Such estimates are limited, however, by our inability to know the 'true' genetic distances among the taxa and by the circularity of using the same data for estimating both divergence and number of substitutions. Whilst there cannot be fewer changes than we observe, estimating the actual number of changes remains a fundamental problem in molecular phylogenetics.

Figure 2 shows the results of the sliding window analysis across both the SSU and LSU rDNA for three alignments, namely those resolved for the complete data set (phylum), the Neodermata and the Trematoda. As found in a broader study based on SSU alone that included 270 platyhelminth sequences (Littlewood & Olson, 2001), there is no clear pattern in either SSU or LSU that suggests particular regions of either molecule contain more reliable, or less homoplasious, sites than do others, with the notable exception of all the variable domains (SSU: V1–V9 inclusive; LSU: D1–D11 inclusive) in which most sites had to be discarded altogether. The variability within each of these domains was very high at all taxonomic levels, and even the alignment for the Trematoda alone recovered only a few more unambiguously alignable positions, most notably among V2, V4, V5 and V8 in the SSU, and among only a small part of D2 in the LSU.

Molecular phylogenies

Results of all analyses are presented in Figures 3 and 4. In Figure 3, tree topologies are presented indicating the position of each taxon with nodal support for BI and MP. Although bootstraps were estimated for the ML tree, these values are not shown but are discussed in the text where necessary. For ML trees, the values of the model parameters are shown in Table 3. The number of trees used to estimate the BI solution is indicated, as is the number of equally parsimonious solutions for MP. The trees in Figure 4 have been redrawn to show the interrelationships of major platyhelminth groups with log likelihood values indicated for ML and tree statistics for MP.

SSU rDNA analyses (Figs 3A, 4A)

A total of 1319 unambiguously alignable characters was used, of which 685 were constant and 430 parsimony informative. For both ML and MP there was relatively poor support for the basal nodes, reflected in the short internal branches and polytomies, respectively. Nevertheless, in each analysis it was clear that polyclads, macrostomorphs and possibly lecithoepitheliates formed the basalmost clade. The two proseriates failed to be united with *Nematoplana*, the lithophoran, appearing as more basal in ML and BI. In MP the proseriates were nested in a clade with the lecithoep-

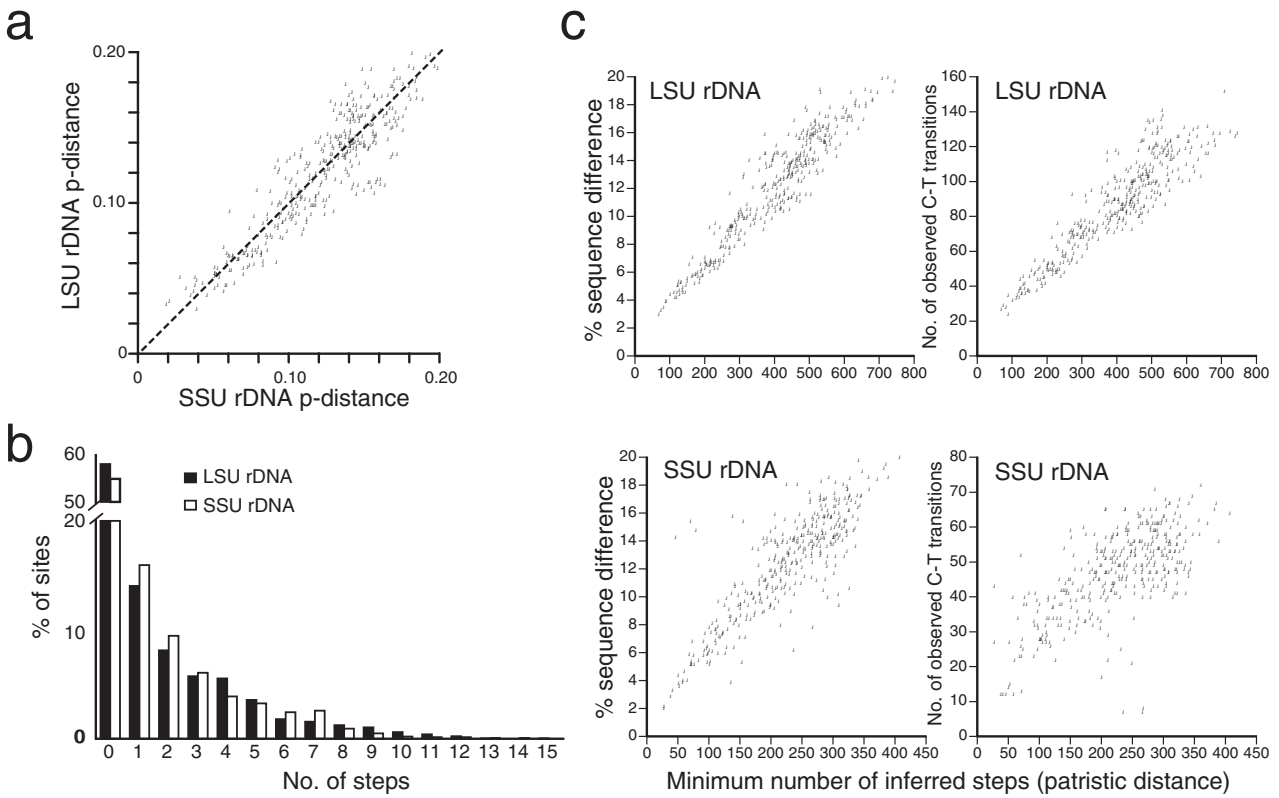

Figure 1. Comparisons of the SSU and LSU rDNA data partitions. (a) correspondence between the pairwise genetic distances estimated by SSU and LSU. Close proximity to the line of perfect correspondence (dashed) shows that both data partitions estimate the same rate of divergence among the taxa. (b) percentage of sites vs. no. of steps/site. SSU data show slightly greater variability with a smaller percentage (~55% vs. 58%) of sites being invariant (i.e. 0 steps), although both data partitions exhibit a similar pattern of among-site heterogeneity. (c) percent sequence difference and number of observed C–T transitions vs. minimum number of inferred steps (patristic distance) for each data partition. Under the assumption that point mutations were saturated in these data sets, these plots would asymptote as patristic distances grew increasingly larger. Such a pattern is difficult to detect given the inability to know the true number of changes as result of multiple substitutions. However, the C–T transition class shows the greatest number of changes (due to the interchange of cytosine and thymine bonds with guanine in the secondary structure of the mature RNA) and would therefore be expected to show the highest degree of saturation if present. As the overall percentage sequence difference as well as C–T substitutions continue to increase in relation to genetic distance, these data do not appear to be saturated, even among distantly related taxa (but see text).

itheliate. Digenea, Aspidogastrea, Trematoda, Eucestoda, Cestoda, Monopisthocotylea, Polyopisthocotylea, Rhabdocoela, Temnocephalida, Tricladida, and Prolecithophora were strongly monophyletic, i.e. where two or more exemplar taxa were sampled higher order groups were well supported. In all analyses rhabdocoels were sister group to a clade including *Kronborgia*, triclads and prolecithophorans; this well-supported turbellarian clade hereafter referred to as R(K,T,P). The Neodermata were resolved as monophyletic with Monogenea paraphyletic but with poor support (Monopisthocotylea most basal) and Cestoda sister group to the Trematoda. The sister group to the Neodermata was the R(K,T,P) clade, or unresolved. With MP the topology resolved by BI was 19 steps longer than the MP tree itself.

LSU rDNA analysis (Figs 3B, 4B)

A total of 2370 unambiguously alignable characters was used, of which 1275 were constant and 759 parsimony informative. Here the same general patterns were resolved although proseriates were monophyletic in BI, paraphyletic in ML and polyphyletic in MP. In each case the R(K,T,P) clade was resolved as above but with the inclusion of the lecithoepitheliate *Geocentrophora*. ML resolved the Monogenea as a paraphyletic taxon but both BI and MP resolved it as strongly monophyletic. Proseriates were either monophyletic

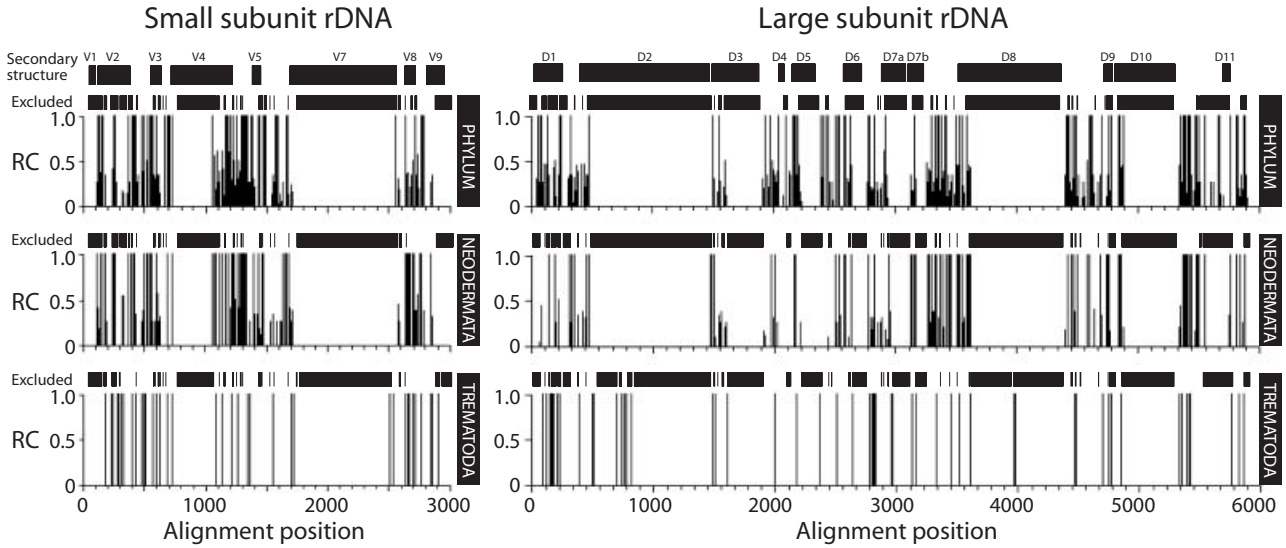

Figure 2. Graphical representations of sequence alignment indicating, for both small (SSU) and large (LSU) subunit rDNA, secondary structure variable regions (V1–V9 in SSU; D1–D11 in LSU), sites excluded and rescaled consistency index (RC) for each character in the analysis averaged over a 5-bp sliding window for three alignments: phylum (all taxa), Neodermata (Monogenea, Cestoda and Trematoda) and Trematoda. The majority of sites excluded fell within variable regions not unambiguously alignable, most notably V4, V7 and V9 in SSU and D2, D8 and D10 in LSU. As expected, alignments of more closely related taxa (Trematoda) indicated high RC values, indicating lower homoplasy in such alignments.

(ML, BI) or not. Surprisingly, the clade usually most basal (Polycladida + Macrostomida) appeared as the sister group to the Neodermata in MP, although bootstrap support was poor at all but the higher nodes. With MP the topology resolved by BI was 22 steps longer than the MP tree itself.

LSU + SSU rDNA analysis (Figs 3C,D, 4C,D)

Partition homogeneity tests (the ILD test) indicated that the LSU and SSU data partitions argued for significantly different solutions ($P < 0.02$). However, because of the fragmentary nature of the LSU data set we were also interested in the likelihood that there may be some conflict between the two halves of the LSU, and splitting it up into domains D1–D3 and D3–D6, the ILD test also suggested conflict ($P < 0.04$). Thus, despite failing the ILD test, we decided to combine the SSU and LSU data sets anyway, in a strict total evidence manner (Kluge, 1989; Littlewood, Bray & Clough, 1998a). Two combined data sets were analysed. The first included only those taxa where complete SSU and LSU sequences were available (SSU + LSU complete only). The second included those taxa where we had only partial LSU sequences, although we had complete SSU sequences for all taxa (SSU + LSU including partial LSUs).

Figures 3C and 4C show the topologies resolved for the smaller combined data set (SSU + LSU complete only). A total of 3671 unambiguously alignable characters was used, of which 1958 were constant and 1174 parsimony informative. ML and BI resolved almost identical solutions: the most basal clade comprised the macrostomid, polyclad and lecithoepitheliate. Proseriates were monophyletic. The R(K,T,P) clade was sister group to the Neodermata and within this the Monogenea were monophyletic and the Cestoda and Trematoda sister groups. In the MP solution only the interrelationships of the turbellarians differed where once again nodal support was poor. Nodal support in the BI analysis was consistently high throughout the tree. Using parsimony to estimate tree length the topology resolved by BI was 19 steps longer than the MP tree itself.

Figures 3D and 4D show the topologies resolved for the larger combined data set (SSU + LSU including partial LSUs). A total of 3688 unambiguously alignable characters was used, of which 1892 were constant and 1270 parsimony informative. In this case all methods of phylogenetic analysis resolved almost identical solutions with strong support throughout. MP analysis differed in its placement of the Proseriata, non-monophyly of the Monogenea and interrelationships of the parasitic turbellarians *Ichthyophaga*, *Notentera*, *Urastoma* and *Kronborgia* (*INUK*) which were invariably resolved as a well-supported clade. Otherwise, the methods agreed on the basal placement of polyclads and macrostomids, a strongly supported clade of turbellarians resolving (Rhabdocoela

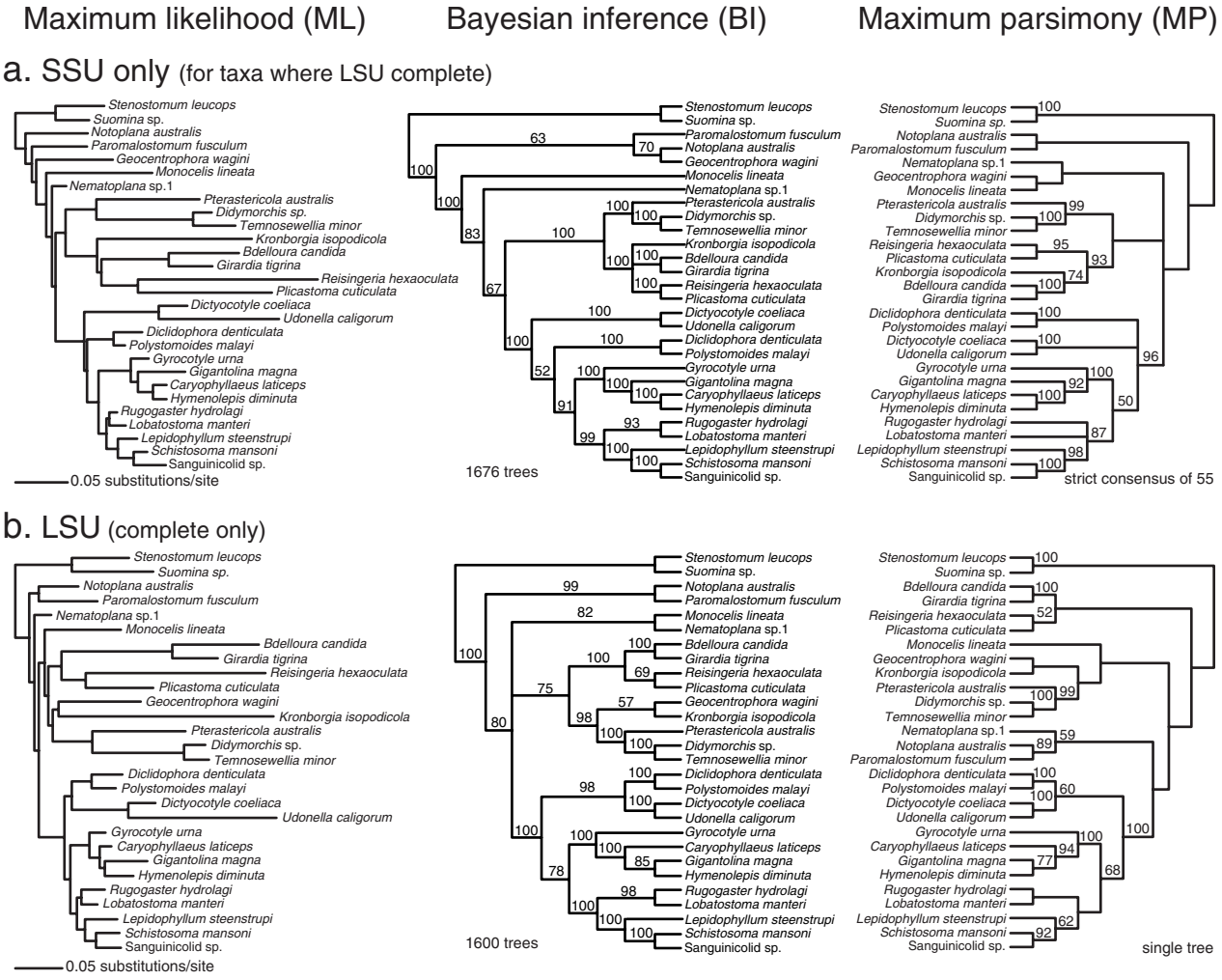

Figure 3. Phylogenetic analyses of individual and combined data sets using maximum likelihood (ML), Bayesian inference (BI) and maximum parsimony (MP). Data sets were (a) SSU only, (b) LSU (complete sequences) only, (c) SSU + LSU (complete sequences only) and (d) SSU + LSU complete sequences and partial LSU fragments. ML and BI used similar models of substitution (see text). Nodal support for BI trees and number of trees used to estimate support are shown, as are bootstrap support (%, n = 1000 replicates) and number of equally parsimonious trees for MP.

((Tricladida, Prolecithophora) (*INUK*))). The taxa with partial LSU fragments were all resolved in their expected positions, i.e. *Provortex* with *Pterastericola* (Rhabdocoela), *Ichthyophaga*, *Notentera*, *Urastoma* with *Kronborgia* although the interrelationships of these taxa are at odds with their classification and the Revertospermata hypothesis (see below). As above, the Cestoda and Trematoda were strongly supported as sister groups. With MP the topology resolved by BI was 22 steps longer than the MP tree itself.

Constraint analyses

We were interested in testing three hypotheses with our combined LSU and SSU data. We wished to test whether the Revertospermata, Monogenea and Cercomeromorphae could be held as monophyletic without resulting in trees significantly different from the unconstrained solutions found above. Each of these constraints involves constraining primarily the relationships within and between the Neodermata and rather than applying constraints with each method of analysis, we performed constraints using ML alone on the combined LSU + SSU data set (no partial data) to find the best trees, and then applied the Shimodaira–Hasegawa test (Shimodaira & Hasegawa, 1999) as implemented in PAUP* with full optimization and 1000 bootstrap replicates. Results are shown in Table 4. Constraining the Monogenea as monophyletic resulted in trees of equal likelihood as the uncon-

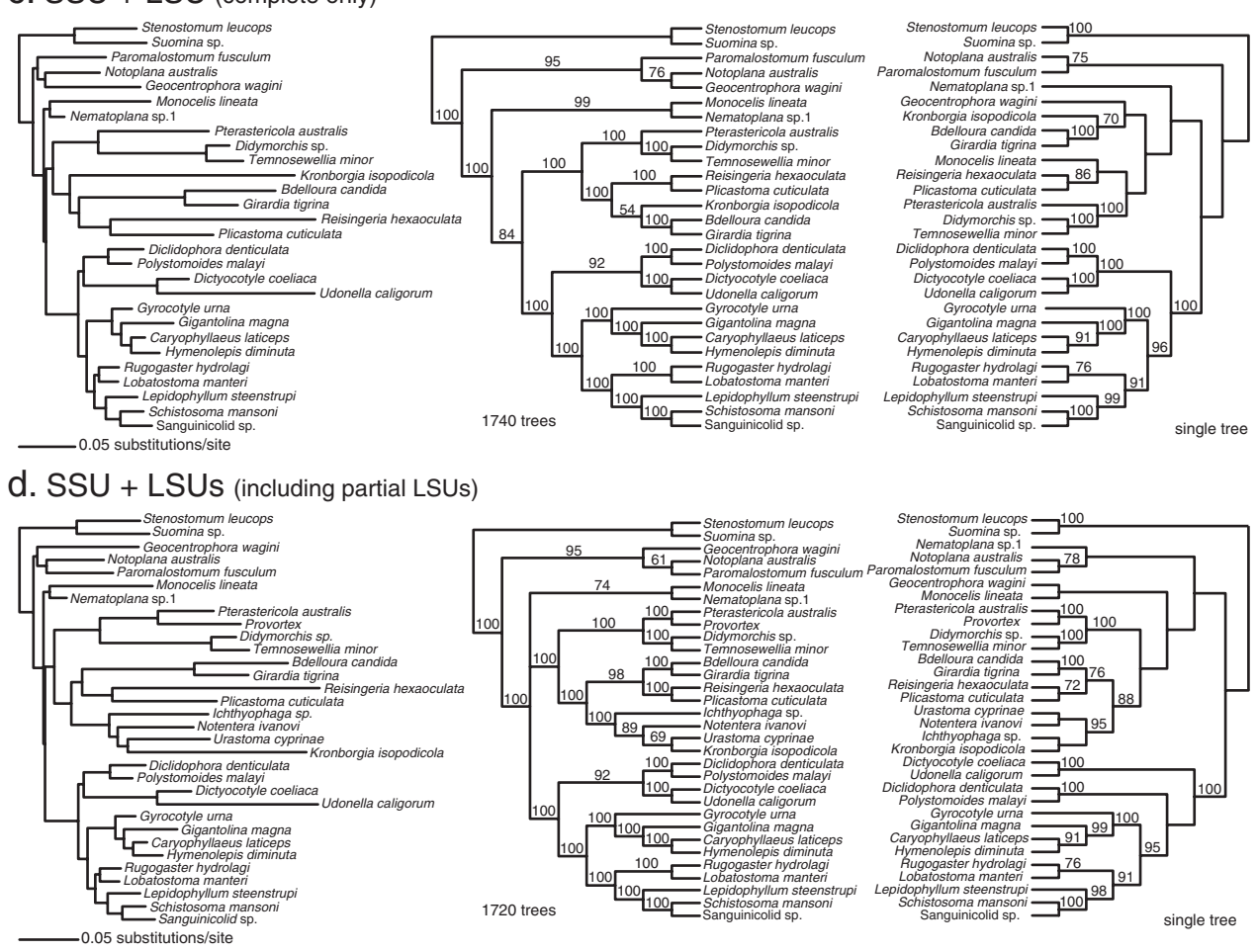

Figure 3. *Continued*

Table 4. Results of constraint analyses on molecular data testing for the likelihood of accepting monophyletic Monogenea, Revertospermata and Cercomeromorphae (Cestoda + Monogenea). Log likelihood values, their differences with respect to the unconstrained solution and the significance of the constraints tested by Shimodaira–Hasegawa as implemented in PAUP* on ML trees are indicated; $P < 0.05$ indicates a significantly different topology

LSU +SSU constraint	– lnL	diff. –lnL	*P*
Unconstrained	29 892.82		
Monophyletic Monogenea	29 892.82	0	0.769
Monophyletic Revertospermata	29 916.82	24.00	0.021
Monophyletic Cercomeromorphae	29 905.00	12.18	0.162

strained solution since ML found monophyletic Monogenea in LSU + SSU.

Clearly, the Revertospermata hypothesis was significantly challenged and rejected by the test, the results do not argue against the Monogenea as a clade, and although the Cercomeromorphae hypothesis was compatible with our data, in none of our ML, BI or MP analyses and in none of the data partitions was this clade resolved.

DISCUSSION

We set out to test the utility of adding complete LSU rDNA sequences to complete SSU data in resolving the interrelationships of the Platyhelminthes. In particular we were interested in whether we could better resolve the identity of the sister group to the Neodermata and test further whether the Monogenea are a

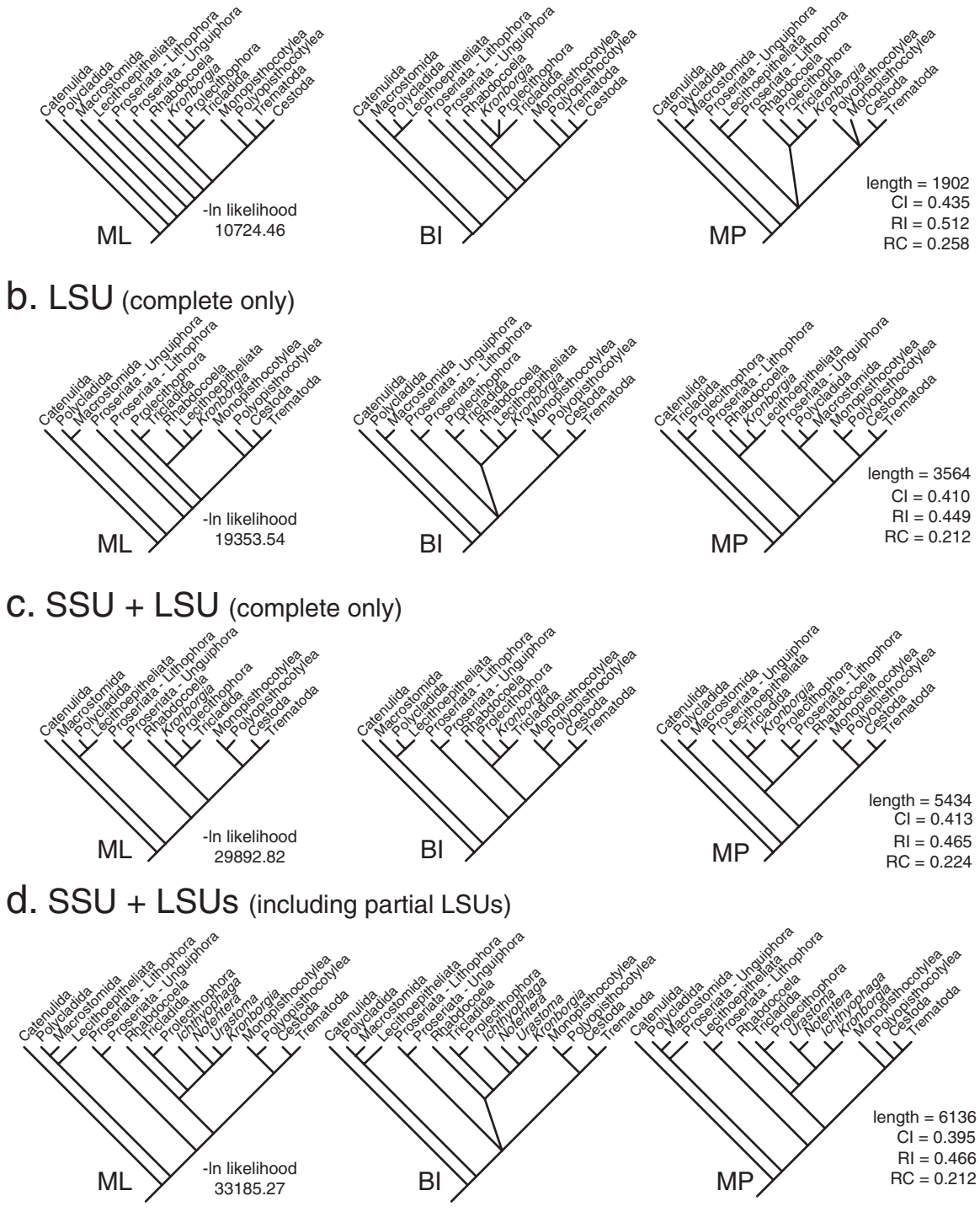

Figure 4. Summary of results shown in Fig. 3, using higher order taxon names where possible to indicate monophyletic clades, tree statistics for maximum parsimony trees.

truly monophyletic group or representing two independent lineages. In contrast to previous analyses, we also used Bayesian inference methods to estimate phylogenies.

SSU rDNA data alone resolved phylogenetic estimates very similar in topology to other SSU based trees, whether from sparsely or densely sampled analyses. Our trees are compatible with those previously published (Littlewood *et al*., 1999b; Littlewood & Olson, 2001) with the notable exception that the Cestoda and Trematoda were sister taxa in this analysis. The Cercomeromorphae (Monogenea + Cestoda) was not supported, although with MP it was not significantly different from a tree in which the monophyly of the Cercomeromorphae was constrained. Differences between results obtained from alternative methods of reconstruction, the mono/paraphyly of the Proseriata and the identity of the basal most flatworm group all follow patterns discussed elsewhere in an in-depth analysis of SSU (Littlewood & Olson, 2001). Bayesian inference provided a somewhat intermediate topology between those provided by ML and MP.

LSU rDNA alone provided poor resolution for deeper nodes regardless of tree reconstruction method. The general topologies of the ML and BI trees were compatible and followed the major patterns revealed by our new and previously published SSU analyses. BI resolved the Proseriata as monophyletic, possibly indicating further the strength of this method; see Curini-Galletti (2001) for a discussion on the monophyly of this group and the problems from and SSU and partial LSU data. At first sight the MP analysis provided some confusing results with respect to the turbellarian groups; again, however, there was little nodal support for the deeper branches, leading us to conclude that overall the complete LSU is less useful than SSU for resolving relationships throughout the phylum. Previous studies utilizing partial LSU alone have failed to provide convincing phylogenetic estimates for the Platyhelminthes (Litvaitis & Rohde, 1999), largely because of the poor resolution at deeper nodes, although these same fragments remain very useful for resolving more derived taxonomic groups, such as the Proseriata, Monogenea and Cestoda (Litvaitis *et al*., 1996; Mollaret *et al*., 1997, 2000; Littlewood *et al*., 2000; Olson *et al*., 2001; Olson & Littlewood, 2002). The value of sequencing complete LSU for resolving relationships within high order clades such as these remains to be seen. Nevertheless, considering the relative cost of sequencing each gene, the SSU proved more cost-effective than LSU. LSU provided an additional 1051 alignable positions than SSU (1319); an increase of almost 80% of which an additional 329 positions were parsimony informative.

The fact that our analyses suggest the LSU and SSU genes argue for different topologies, and do not pass the partition homogeneity test, is of some concern. As parts of the same tandemly repeated array, small and large ribosomal units are physically linked within the genome and it seems unlikely that each gene has undergone a different evolutionary radiation. For this reason, it seems prudent to take a strict 'total evidence' approach, as prescribed by Kluge (1989; see also Littlewood *et al*., 1998a), in which we consider only the solutions presented in Figures 3C,D and 4C,D.

As suggested by Mallatt & Winchell (2002: 299), combining the complete LSU and SSU rDNA genes 'could help to free systematists who now use SSU from the frustrating cycle that many are experiencing, i.e. a cycle of adding progressively more SSU sequences without much improvement in taxonomic resolution.' Additionally, we have found that Bayesian inference and maximum likelihood appear to give more congruent trees than maximum parsimony with respect to traditional concepts. Further still, the nodal support with Bayesian inference improves at all nodes with the combination of SSU and LSU. We suggest that the Bayesian trees of LSU + SSU, of both fully complementary sequences (Figs 3C,4C) and those including partial sequences (Figs 3D,4D), are good starting points for discussing the morphological implications of accepting the combined rDNA trees. The major conclusions are congruent with some earlier studies on SSU alone (see review in Littlewood & Olson, 2001) but nodal support is stronger. The most basal Rhabditophora is a clade comprising the Macrostomorpha, Polycladida and Lecithoepitheliata. With the exception of the Lecithoepitheliata, the remaining flatworms include the Neoophora. Of the neoophoran species, the Proseriata are basal, monophyletic and sister group to a clade comprising other turbellarians (Rhabdocoela ((Tricladida, Prolecithophora), *INUK*)) and the Neodermata. Importantly, the Revertospermata (Kornakova & Joffe, 1999) is not supported and those turbellarians expected to group with the Neodermata in this scheme do not, i.e. *Ichthyophaga* (Genostomatidae), *Notentera* (Notenteridae), *Urastoma* (Urastomidae), *and Kronborgia* (Fecampiidae). This suggests that in spermiogenesis, the evolution of axonemes fusing in a proximo-distal direction and the median cytoplasmic process arose twice (see also Joffe & Kornakova, 2001). The sister group to the Neodermata does not include the Proseriata, as suggested with SSU alone in larger analyses (Littlewood & Olson, 2001), but includes the Rhabdocoela, Tricladida, Prolecithophora, Genostomatidae, Notenteridae, Urastomidae and Fecampiidae. Single, smaller clades or groups hypothesized to be sister taxa to the Neodermata, such as the Temnocephalida (Brooks & McLennan, 1993) or Dalyellidae + Temnocephalida + Typhloplanida (Zamparo *et al*., 2001), are not sup-

ported by any molecular data and have been also challenged by other interpretations of morphology, e.g. Littlewood *et al*. (1999b). The fact that with both molecular and morphological data (Littlewood *et al*., 1999b) the Temnocephalida resolutely fall within the Rhabdocoela means that it is not most parsimonious to suggest that the common ancestor of the Neodermata had a vertebrate-arthropod two-host life cycle (Zamparo *et al*., 2001), but it does remain the case that it must have had a vertebrate host (Littlewood *et al*., 1999a).

Our combined data strongly support the monophyly of the Monogenea, thus lending molecular support to at least four morphological synapomorphies proposed for the clade; larvae with three ciliated zones, larvae and adults with two pairs of pigmented eyes, one pair of ventral anchors and one egg filament (Boeger & Kritsky, 2001). This scenario is therefore better supported than solutions previously suggested by SSU or partial LSU alone (see reviews by Justine, 1998; Littlewood *et al*., 2001a). Moreover, these results further suggest that problems of rate heterogeneity between the Monopisthocotylea and Polyopisthocotylea account for their non-monophyly in analyses based solely on SSU or partial (D1–D2) LSU rDNA data (Olson & Littlewood, 2002). Although morphology has consistently suggested a stable topology within the Neodermata, molecular data have provided two alternatives, both of which result from the non-monophyly of the Monogenea. Almost without exception, SSU rDNA has resolved the Monogenea as a paraphyletic assemblage with the Polyopisthocotylea sister group to Monopisthocotylea + Cestoda. In contrast, partial LSU (D1–D2) resolved a paraphyletic Monogenea with the Monopisthocotylea sister group to all other Neodermata. Only SSU maintained the Cercomeromorphae (Monogenea + Cestoda) clade. Our new data combining LSU + SSU support a monophyletic Monogenea, but the Cercomeromorphae is categorically not supported. With such robust new results for relationships within the Neodermata, the cercomermorph hypothesis requires re-evaluation.

The Cercomeromorphae revisited

The subclass Cercomerophora was erected by Janicki (1920) whose 'cercomer' theory suggested that the digenean cercarial tail, the monogenean opisthaptor and the posterior, hooked end of the larval cestode (i.e. 'cercomer': specifically that of the procercoid of pseudophyllidean cestodes) were homologues, and wherein the Cestoda were derived from the Digenea. The hypothesis neatly separated the obligate parasitic flatworms from the free-living 'Turbellaria' but the alleged homology has proved highly contentious. That the obligate parasites do indeed form a monophyletic clade is of little doubt, but this is based on the shared characters defining the Neodermata, and not on the cercomer. Subsequent to Janicki, and largely due to arguments concerning the definition of the cercomer, membership of the clade and the cercomer theory has evolved. Bychowsky (1937) used the term Cercomeromorphae to represent a clade comprising the Cestoda and the Monogenea, homologising only the posterior end of the larval cestode that contains oncospheral hooks (the 'cercomer') and the opisthaptor of monogeneans. He excluded the Trematoda and those monogenean families without hooks. Currently, the synapomorphy for the Cercomeromorphae (Cestoda + Monogenea) concerns the cercomer, and may be best defined as the posterior end of the larva bearing hooks, of which some may be retained in adults. This remains the only synapomorphy for the grouping Cestoda + Monogenea, and yet the cercomer of cestodes and suggested homologies with organs among other platyhelminths has certainly caused much debate. Freeman (1973) followed the traditional usage of the term and defined the cercomer as the 'tail' forming at the posterior end of a larval cestode, usually containing the oncospheral hooks, and although it has been defined differently elsewhere (e.g. Jarecka, Michajlow & Burt, 1981), this is the most widely used definition. Brooks (Brooks, 1982; Brooks *et al*., 1985) suggested that all 'posterior adhesive organs' among the Trematoda, Cestoda, Monogenea and Temnocephalida are homologous, uniting these taxa in the so-called Cercomeria. This hypothesis has been convincingly rejected by morphological, embryological, ultrastructural and molecular data (e.g. Lebedev, 1987; Rohde & Watson, 1995; Rohde *et al*., 1995; Littlewood *et al*., 1999b) and yet the coding of what Brooks terms 'posterior adhesive organs' as homologous features among these taxa is still maintained and even proliferated through methods of character coding in the latest of Brooks' assessments (Zamparo *et al*., 2001). As regards the Cercomeromorphae, the clade also has its critics. Gulyaev (1996), among others, concluded that the 'armed muscular pads' of second stage larval eucestodes (procercoids), lycophore larvae of Amphilinidea and Gyrocotylidea, and larval Monogenea (oncomiracidia), are not at all homologous with one another, let alone with trematode or other platyhelminth posterior adhesive organs. Our data, and previous molecular analyses (Rohde *et al*., 1995; Littlewood *et al*., 1999b) support this and the rejection of the Cercomeromorphae. Unless a convincing synapomorphy can be found, it seems unlikely that all higher groupings dependent upon 'cercomers', beyond the Cestoda (wherein there is also some debate, e.g. Gulyaev, 1996; Mackinnon & Burt, 1984) should be abandoned. In a recent report, Chervy (2002) provides an excellent review of cestode 'cercomers', in which the author con-

cludes that 'presence or absence of a cercomer remains to be determined in several cestode groups' (p. 17, op. cit.) and that the loss of the cercomer in many cestode taxa may be a homoplasious condition (see also Hoberg *et al.*, 1999; Beveridge, 2001), thus in our opinion rendering the feature as even less useful in resolving the interrelationships of flatworms at any taxonomic level.

CONCLUSION

By adding complete LSU rDNA we have increased the phylogenetic resolution afforded by SSU rDNA alone. Based on sequencing effort, however, the SSU gene appears to contain marginally more phylogenetically informative positions and therefore is perhaps the first ribosomal gene of choice in resolving the interrelationships of the Platyhelminthes. Our data and analyses reject the Revertospermata hypothesis. The sister group to the Neodermata was a well-supported clade of predominantly neoophoran turbellarians and within the Neodermata the Monogenea (Monopisthocotylea + Polyopisthocotylea) were strongly monophyletic, supporting a number of morphologically based synapomorphies. In contrast to previous studies using SSU alone, our combined LSU + SSU analysis rejected, though not significantly, the Cercomeromorphae and instead resolved the Cestoda as sister group to the Trematoda for each data partition and analysis. However, no morphological synapomorphy is known to support this clade. All other groupings that were indicated by previous SSU studies were even more strongly supported in the current analyses. LSU alone was poorer at resolving deeper nodes and was incapable of resolving the Proseriata as monophyletic. We advocate the combination of complete LSU and SSU in resolving the interrelationships between the major flatworm clades, but still there appears to be a need for additional independent evidence to fully clarify the interrelationships of this phylum.

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REFERENCES

Baguñà J, Carranza S, Paps J, Ruiz-Trillo I, Riutort M. 2001. Molecular taxonomy and phylogeny of the Tricladida. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, 49–56.

Berney C, Pawlowski J, Zaninetti L. 2000. Elongation factor 1-alpha sequences do not support an early divergence of the acoela. *Molecular Biology and Evolution* **17:** 1032–1039.

Beveridge I. 2001. The use of life-cycle characters in studies of the evolution of the cestodes. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, 250–256.

Boeger WA, Kritsky DC. 2001. Phylogenetic relationships of the Monogenoidea. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, 92–102.

Brooks DR. 1982. Higher level classification pf parasitic platyhelminths and fundamentals of cestode classification. In: Mettricks DF, Desser SS, eds. *Parasites: their world and ours*. Amsterdam: Elsevier, 189–193.

Brooks DR. 1989. A summary of the database pertaining to the phylogeny of the major groups of parasitic platyhelminths, with a revised classification. *Canadian Journal of Zoology* **67:** 714–720.

Brooks DR, McLennan DA. 1993. *Parascript: parasites and the language of evolution*. Washington: Smithsonian Institution Press.

Brooks DR, O'Grady RT, Glen DR. 1985. The phylogeny of the Cercomeria Brooks, 1982 (Platyhelminthes). *Proceedings of the Helminthological Society of Washington* **52:** 1–20.

Bychowsky BE. 1937. Ontogenesis and phylogenetic interrelations of parasitic flatworms. *News of the Academy of Sciences, USSR Department of Mathematics and Natural Sciences* **4:** 1353–1383.

Campos A, Cummings MP, Reyes JL, Laclette JP. 1998. Phylogenetic relationships of Platyhelminthes based on 18S ribosomal gene sequences. *Molecular Phylogenetics and Evolution* **10:** 1–10.

Cannon LRG, Joffe BI. 2001. The Temnocephalida. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, 83–91.

Carranza S, Baguñà J, Riutort M. 1997. Are the Platyhelminthes a monophyletic primitive group? An assessment using 18S rDNA sequences. *Molecular Biology and Evolution* **14:** 485–497.

Carranza S, Littlewood DTJ, Clough KA, Ruiz-Trillo I, Baguñà J, Riutort M. 1998. A robust molecular phylogeny of the Tricladida (Platyhelminthes: Seriata) with a discussion on morphological synapomorphies. *Proceedings of the Royal Society of London, Series B* **265:** 631–640.

Chervy L. 2002. The terminology of larval cestodes or metacestodes. *Systematic Parasitology* **52:** 1–33.

Cribb TH, Bray RA, Littlewood DTJ, Pichelin SP, Herniou EA. 2001. The Digenea. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, 168–185.

Cunningham CW. 1997. Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution* **14:** 733–740.

Curini-Galletti M. 2001. The Proseriata. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, 41–48.

Degnan B, Yan J, Hawkins CJ, Lavin MF. 1990. rRNA genes from the lower chordate *Herdmania momus*: structural similarity with higher eukaryotes. *Nucleic Acids Research* **18:** 7063–7070.

Ehlers U. 1985. *Das Phylogenetische System der Platyhelminthes*. Gustav Fischer, Stuttgart.

Farris JS, Kallersjo M, Kluge AG, Bult C. 1995. Constructing a significance test for incongruence. *Systematic Biology* **44:** 570–572.

Freeman RS. 1973. Ontogeny of cestodes and its bearing on their phylogeny and systematics. In: Dawes B, ed. *Advances in parasitology*. London: Academic Press, 481–557.

Gulyaev VD. 1996. Origin of basic characters in morphology and ontogenesis of tapeworms (Cestoda). 2. Cercomere homology in cestodes and origin of intercalary parasitic larva-cercoid. *Zoologichesky Zhurnal* **75:** 961–971.

Hawkins JA. 2000. A survey of primary homology assessment: different botanists perceive and define characters in different ways. In: Scotland R, Pennington RT, eds. *Homology and systematics*. London: Taylor & Francis, 22–53.

Hillis DM, Dixon MT. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Quarterly Review of Biology* **66:** 411–453.

Hoberg EP, Jones A, Bray RA. 1999. Phylogenetic analysis among the families of the Cyclophyllidea (Eucestoda) based on comparative morphology, with new hypotheses for coevolution in vertebrates. *Systematic Parasitology* **42:** 51–73.

Huelsenbeck JP. 2000. *MrBayes: Bayesian inference of phylogeny*. Rochester, NY: Department of Biology. University of Rochester.

Huelsenbeck JP, Bull JJ, Cunningham CW. 1996. Combining data in phylogenetic analysis. *Trends in Ecology and Evolution* **11:** 152–158.

Janicki Cv. 1920. Grundinien einer 'Cercomer Theorie' zur Morphologie der Trematoden und Cestoden. *Festschrift für Zschokke* **30:** 1–22.

Jarecka L, Michajlow W, Burt MDB. 1981. Comparative ultrastructure of cestode larvae and Janicki's cercomer theory. *Acta Parasitologica Polonica* **28:** 65–72.

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. 1998. Multiple sequence alignment with ClustalX. *Trends in Biochemical Sciences* **23:** 403–405.

Jennings JB. 1971. Parasitism and commensalism in the Turbellaria. *Advances in Parasitology* **9:** 1–32.

Joffe BI, Kornakova EE. 1998. *Notentera ivanovi* Joffe *et al.* 1997: a contribution to the question of phylogenetic relationships between 'turbellarians' and the parasitic Plathelminthes (Neodermata). *Hydrobiologia* **383:** 245–250.

Joffe BI, Kornakova EE. 2001. Flatworm phylogeneticist: between molecular hammer and morphological anvil. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, 279–291.

Jovelin R, Justine JL. 2001. Phylogenetic relationships within the polyopisthocotylean monogeneans (Platyhelminthes) inferred from partial 28S rDNA sequences. *International Journal for Parasitology* **31:** 393–401.

Justine J-L. 1998. Non-monophyly of the monogeneans? *International Journal for Parasitology* **28:** 1653–1657.

Katayama T, Nishioka M, Yamamoto M. 1996. Phylogenetic relationships among turbellarian orders inferred from 18S rDNA sequences. *Zoological Science* **13:** 747–756.

Kluge AG. 1989. A concern for evidence and a phylogenetic hypothesis of relationships among *Epicrates* (Boidae, Serpentes). *Systematic Biology* **38:** 7–25.

Kornakova EE, Joffe BI. 1999. A new variant of the neodermatan-type spermiogenesis in a parasitic 'turbellarian', *Notentera ivanovi* (Platyhelminthes) and the origin of the Neodermata. *Acta Zoologica (Stockholm)* **80:** 135–151.

Lebedev BI. 1987. Is the trematode acetabulum a cercomer – (about one platyhelminth's classification). *Journal of Parasitology* **73:** 1250–1251.

Lee MSY. 2001. Uninformative characters and apparent conflict between molecules and morphology. *Molecular Biology and Evolution* **18:** 676–680.

Littlewood DTJ, Bray RA, eds. 2001. *Interrelationships of the Platyhelminthes*. London: Taylor & Francis.

Littlewood DTJ, Bray RA, Clough KA. 1998a. A phylogeny of the Platyhelminthes: towards a total-evidence solution. *Hydrobiologia* **383:** 155–160.

Littlewood DTJ, Cribb TH, Olson PD, Bray RA. 2001a. Platyhelminth phylogenetics – a key to understanding parasitism? *Belgian Journal of Zoology* **131:** 35–46.

Littlewood DTJ, Curini-Galletti M, Herniou EA. 2000. The interrelationships of Proseriata (Platyhelminthes: Seriata) tested with molecules and morphology. *Molecular Phylogenetics and Evolution* **16:** 449–466.

Littlewood DTJ, Olson PD. 2001. Small subunit rDNA and the Platyhelminthes: signal, noise, conflict and compromise. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, 262–278.

Littlewood DTJ, Olson PD, Telford MJ, Herniou EA, Riutort M. 2001b. Elongation factor 1-alpha sequences alone do not assist in resolving the position of the Acoela within the Metazoa. *Molecular Biology and Evolution* **18:** 437–442.

Littlewood DTJ, Rohde K, Bray RA, Herniou EA. 1999a. Phylogeny of the Platyhelminthes and the evolution of parasitism. *Biological Journal of the Linnean Society* **68:** 257–287.

Littlewood DTJ, Rohde K, Clough KA. 1998b. The phylogenetic position of *Udonella* (Platyhelminthes). *International Journal for Parasitology* **28:** 1241–1250.

Littlewood DTJ, Rohde K, Clough KA. 1999b. The interrelationships of all major groups of Platyhelminthes: phylogenetic evidence from morphology and molecules. *Biological Journal of the Linnean Society* **66:** 75–114.

Litvaitis MK, Curini-Galletti M, Martens PM, Kocher TD. 1996. A reappraisal of the systematics of the Monocelididae (Platyhelminthes, Proseriata): inferences from rDNA sequences. *Molecular Phylogenetics and Evolution* **6:** 150–156.

Litvaitis MK, Newman LJ. 2001. A molecular framework for the phylogeny of the Pseudocerotidae (Platyhelminthes, Polycladida). *Hydrobiologia* **444:** 177–182.

Litvaitis MK, Rohde K. 1999. A molecular test of platyhelminth phylogeny: inferences from partial 28S rDNA sequences. *Invertebrate Biology* **118:** 42–56.

Mackinnon BM, Burt MDB. 1984. The development of the tegument and cercomer of the polycephalic larvae (cercoscolices) of *Paricterotaenia paradoxa* (Rudolphi, 1802) (Cestoda, Dilepididae) at the ultrastructural level. *Parasitology* **88:** 117–&.

Maddison WP, Maddison DR. 2000. *MacClade*, *Version 4.* Sunderland, MA: Sinauer Associates.

Maidak BL, Cole JR, Lilburn TG, Parker CT, Saxman PR, Farris RJ, Garrity GM, Olsen GJ, Schmidt TM, Tiedje JM. 2001. The RDP-II (Ribosomal Database Project). *Nucleic Acids Research* **29:** 173–174.

Mallatt J, Winchell CJ. 2002. Testing the new animal phylogeny: first use of combined large-subunit and small-subunit rRNA gene sequences to classify the Protostomes. *Molecular Biology and Evolution* **19:** 289–301.

Medina M, Collins AG, Silberman JD, Sogin ML. 2001. Evaluating hypotheses of basal animal phylogeny using complete sequences of large and small subunit rRNA. *Proceedings of the National Academy of Sciences, USA* **98:** 9707–9712.

Mollaret I, Jamieson BGM, Adlard RD, Hugall A, Lecointre G, Chombard C, Justine J-L. 1997. Phylogenetic analysis of the Monogenea and their relationships with Digenea and Eucestoda inferred from 28S rDNA sequences. *Molecular and Biochemical Parasitology* **90:** 433–438.

Mollaret I, Jamieson BGM, Justine J-L. 2000. Phylogeny of the Monopisthocotylea and Polyopisthocotylea (Platyhelminthes) inferred from 28S rDNA sequences. *International Journal for Parasitology* **30:** 171–185.

Norén M, Jondelius U. 1999. Phylogeny of the Prolecithophora (Platyhelminthes) inferred from 18S rDNA sequences. *Cladistics* **15:** 103–112.

Olson PD, Caira JN. 1999. Evolution of the major lineages of tapeworms (Platyhelminthes: Cestoidea) inferred from 18S ribosomal DNA and elongation factor-1 alpha. *Journal of Parasitology* **85:** 1134–1159.

Olson PD, Littlewood DTJ. 2002. Phylogenetics of the Monogenea – evidence from a medley of molecules. *International Journal for Parasitology* **32:** 233–244.

Olson PD, Littlewood DTJ, Bray RA, Mariaux J. 2001. Interrelationships and evolution of the tapeworms (Platyhelminthes: Cestoda). *Molecular Phylogenetics and Evolution* **19:** 443–467.

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

Rohde K, Johnson AM, Baverstock PR, Watson NA. 1995. Aspects of the phylogeny of Platyhelminthes based on 18S ribosomal DNA and protonephridial ultrastructure. *Hydrobiologia* **305:** 27–35.

Rohde K, Watson NA. 1993. Spermatogenesis in *Udonella* (Platyhelminthes, Udonellidea) and the phylogenetic position of the genus. *International Journal for Parasitology* **23:** 725–735.

Rohde K, Watson NA. 1995. Comparative ultrastructural study of the posterior suckers of four species of symbiotic Platyhelminthes, *Temnocephala* sp. (Temnocephalida), *Udonella caligorum* (Udonellidea), *Anoplodiscus cirrusspiralis* (Monogenea, Monopisthocotylea), and *Philophthalamus* sp. (Trematoda, Digenea). *Folia Parasitologica* **42:** 11–28.

Shimodaira H, Hasegawa M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution* **16:** 1114–1116.

Swofford DL. 2002. *PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods).* 4th ed. Sunderland, MA: Sinauer Associates, Inc.

Tkach VV, Pawlowski J, Mariaux J, Swiderski Z. 2001. Molecular phylogeny of the suborder Plagiorchiata and its position in the system of Digenea. . In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor & Francis.

Zamparo D, Brooks DR, Hoberg EP, McLennan DA. 2001. Phylogenetic analysis of the Rhabdocoela (Platyhelminthes) with emphasis on the Neodermata and relatives. *Zoologica Scripta* **30:** 59–77.