25 SMALL SUBUNIT RDNA AND THE PLATYHELMINTHES: SIGNAL, NOISE, CONFLICT AND COMPROMISE

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The strategies of gene sequencing and gene characterisation in phylogenetic studies are frequently determined by a balance between cost and benefit, where benefit is measured in terms of the amount of phylogenetic signal resolved for a given problem at a specific taxonomic level. Generally, cost is far easier to predict than benefit. Building upon existing databases is a cost-effective means by which molecular data may rapidly contribute to addressing systematic problems. As technology advances and gene sequencing becomes more affordable and accessible to many researchers, it may be surprising that certain genes and gene products remain favoured targets for systematic and phylogenetic studies. In particular, ribosomal DNA (rDNA), and the various RNA products transcribed from it continue to find utility in wide ranging groups of organisms. The small (SSU) and large subunit (LSU) rDNA fragments especially lend themselves to study as they provide an attractive mix of constant sites that enable multiple alignments between homologues, and variable sites that provide phylogenetic signal (Hillis and Dixon 1991; Dixon and Hillis 1993). Ribosomal RNA (rRNA) is also the commonest nucleic acid in any cell and thus was the prime target for sequencing in both eukaryotes and prokaryotes during the early history of SSU nucleotide based molecular systematics (Olsen and Woese 1993). In particular, the SSU gene (rDNA) and gene product (SSU rRNA[1]) have become such established sources of taxonomic and systematic markers among some taxa that databanks dedicated to the topic have been developed and maintained with international and governmental funding (e.g. *The Ribosomal Database Project II*, Maidak *et al.* 1999; the *rRNA WWW Server*, van de Peer *et al.* 2000). One or more species from all metazoan phyla, except the Loricifera, have had their SSU genes characterised in part or fully and if SSU rDNA appears to be a suitable target at the outset of a phylogenetic study, these databases (including EMBL and GenBank) often provide a head start. In addition to raw sequences many of the databases include sequences aligned against models of secondary structure and/or against other sequences in the database. Addresses of the WWW pages for all these databases are given below[2]. In addition, the development of our knowledge of molecular evolution as it relates to phylogeny reconstruction has been influenced greatly by the genes chosen and SSU rRNA has certainly played its part such that features of the gene have been characterised for many phyla (e.g. Abouheif *et al.* 1998; Zrzavy *et al.* 1998).

The first flatworm species to have had its SSU sequence partly determined, in three fragments, was *Dugesia tigrina* (Field *et al.* 1988). The first fully sequenced SSU gene was, perhaps not surprisingly considering its medical importance, from *Schistosoma mansoni* (Ali

[1] *Note* – Many people are familiar with the synonymy between 18S rRNA and SSU rRNA. However, throughout the chapter we avoid referring to the sedimentation coefficient (Svedberg, or S-unit) of the SSU molecules, e.g. 18S, as these are generally estimated or assumed and infrequently determined empirically. Although the SSU molecules are homologous with one another (or indeed paralogous in the case of some triclads; Carranza *et al.* 1996, 1999) many are so large that they are unlikely to have such low S values. In this study for instance, complete SSU sequences ranged in length from 1,739 - 2,906 bps.

[2] **EMBL/EBI:** http://ebi.ac.uk
GenBank: http://www3.ncbi.nlm.nih.gov/
Ribosomal Database Project: http://www.cme.msu.edu/RDP/html/index.html
rRNA WWW Server: http://rrna.uia.be/index.html

et al. 1991). Importantly, not all the early partial fragments found their way onto public databanks (GenBank/EMBL) but many partial sequences are now being replaced by full or nearly complete sequences.

In this chapter we begin with a brief review of some of the important features of SSU molecules and how phylogenetic studies utilising the gene have affected our understanding of the phylogeny of the platyhelminths. We then take all the existing complete, or near complete, SSU data available, and add to these new sequences that were determined previously for ordinal, or at least sub-phylum level, phylogenies. Our aims are to reconstruct phylogenies based on the available SSU data and to reveal the recurring signal and underlying noise in such reconstructions, predominantly at higher taxonomic levels. At the outset we do not advocate single gene phylogenies, gene trees interpreted without reference to other phylogenetic data, the preferred use of SSU rDNA, or indeed gene sequencing as the primary means by which molecular data can add to platyhelminth phylogenetics. However, the most diverse and widely sampled gene of flatworms serves as the most suitable starting point to which new molecular data may be added to the database and from which salutary lessons may be learned. We begin with a review of some of the important features and the rationale that guided us in assembling and utilising the data set.

The monophyly of the Platyhelminthes contradicted by SSU rDNA

One of the first data sets that sampled the SSU rDNA broadly suggested that the Acoela were basal platyhelminths (Katayama *et al.* 1993), although the paper included only acoels, triclads and polyclads rooted against a species of yeast, *Saccharomyces cerevisae*, and an ascomycete fungus, *Neurospora crassa.* A more densely sampled analysis including other free-living and parasitic exemplars (Katayama *et al.* 1996) supported this finding, although the ingroup of platyhelminths was rooted against *Saccharomyces* and a collection of diploblasts. Carranza *et al.* (1997) broadened the sampling further still, largely in an attempt to test a tenet from early zoological studies, that platyhelminths are basal metazoans forming the likely sister-group of the other bilaterian phyla. Disturbingly, in these analyses of complete SSU rDNA involving various deuterostome and protostome triploblast taxa rooted against three diploblasts and a protozoan, the flatworms appeared as either paraphyletic or polyphyletic due to the placement of the catenulid and acoel species. The catenulid taxon appeared at the base of the Bilateria and the authors drew attention to the long branches exhibited by the acoels. Metazoan wide sampling, using the same data, also suggested a paraphyletic assemblage (Zrzavy *et al.* 1998). Subsequently it was felt the position of the Acoela could not be confirmed without further sampling. Denser sampling of the Platyhelminthes, including the Acoela, retained the catenulids as sister-group to all other flatworms but maintained the non-monophyly of the group with the acoels as a long-branching sister-group to all other bilaterians (Littlewood *et al.* 1999a). Most recently, a thorough analysis of metazoan taxa, including 18 species of acoels, allowed Ruiz-Trillo *et al.* (1999) to identify an acoel, *Paratomella rubra,* that was demonstrated to have evolved at a sufficiently slow rate such that long-branch effects in phylogenetic analysis (Felsenstein 1978; Siddall 1998) may be avoided. Nevertheless, analyses of SSU rDNA continued to keep the acoels apart from the other platyhelminth taxa; the catenulids, the one species of nemertodermatid and the Rhabditophora were retained as a monophyletic clade (Figure 25.1). Although denser sampling returned the catenulids as members of the Platyhelminthes, whether from a phylum-wide or Kingdom-wide perspective, the acoelomorph flatworms cannot be considered members of the Platyhelminthes *sensu stricto* based on SSU rDNA.

SSU rDNA and the position of flatworms among the Metazoa

Tyler (2000, this volume) discusses the affinities of flatworms with other phyla from broader perspectives, but here we briefly note the 'contribution' made by SSU rDNA. Historically, flatworms have been considered to represent basal tripoblasts and yet, notwithstanding the contentious basal position of the Acoela in metazoan wide analyses of SSU rDNA (Ruiz-Trillo, *et al.* 1999; Figure 25.1), the gene fails to support such a basal placement of the whole phylum. Members of the Rhabditophora, often used as representative platyhelminths, appear firmly ensconced within the Lophotrochozoa (Aguinaldo *et al.* 1997; Balavoine 1997; Adoutte *et al.* 1999; Ruiz-Trillo *et al.* 1999), or at best, when viewed conservatively, as unresolved members of the Protostomia (Abouheif *et al.* 1998). Most recently, Giribet *et al.* (in press) argue for a 'Platyzoa' clade which is sister-group to the Trochozoa. Within the 'Platyzoa' a monophyletic assemblage of flatworms (with the notable exclusion of the Nemertodermatids) is sister-group to the Gastrotricha, and together forms a clade with a monophyletic group of Gnathostomulida, Cycliophora, Monogononta, Acanthocephala and Bdelloida. Such results and the instability of these phylogenies dependent upon parameter settings, once again demonstrate that our understanding of metazoan interrelationships has a long way to go, requires new molecular evidence and a broader insight into the morphological, evolutionary and biological consequences of single gene dominated schemes. In addition, as our understanding of molecular evolution, and our ability to resolve evolutionary history from it improves, so too will our estimates of phylogeny.

History of SSU and the interrelationships of flatworms

The quest to resolve the sister-group of the Neodermata certainly gave impetus to early molecular-based studies on platyhelminth systematics, and continues to do so to this date. Establishing the sister-group allows us to discuss the origins and evolution of parasitism among the obligate parasitic groups more objectively, or at least more rigorously within a cladistic framework. Whilst the monophyly of the Neodermata is well established on both morphological and molecular grounds, differences of opinion concerning character homology has resulted in a number of candidate sister-groups. Littlewood *et al.* (1999b) reviewed some of the more popular and compelling suggestions from morphological, SSU and LSU data and concluded that SSU and LSU rejected some scenarios whilst suggesting novel ones as well. Nevertheless, identifying the sister group to the Neodermata remains a challenging task.

The first study to employ SSU rRNA to examine the interrelationships of the Platyhelminthes was that of Baverstock *et al.* (1991, Figure 25.2a). In their study of ten partial sequences rooted against man and *Artemia* (Crustacea), the small data set resulted in a reasonable degree of resolution; neodermatans were monophyletic, monogeneans were shown to be more closely related to the cestodes, and for the first time it was demonstrated that the monogeneans were not monophyletic. The data broadly supported those topologies suggested by morphologists, but accommodated conflicting topologies since the data were generally too labile to strongly contradict one or another hypothesis. Blair (1993) used the database to place the aspidogastrean *Lobatostoma manteri* but could not resolve the monophyly of the Trematoda. The apparent paraphyly of the Monogenea was noted again and in contrast to Baverstock *et al.* (1991), Blair provided strong support for the monophyly of the Cestoda. Additional taxa sampled from other platyhelminth groups allowed new questions on the phylogeny of the group to be addressed (Rohde *et al.* 1993b). These authors were cautious in their interpretation of the new data, particularly as competing hypotheses were again almost as likely to be accepted as the most parsimonious solutions offered by the data. However, these were the first indications that some key hypotheses on the interrelationships of platyhelminths founded on morphology were to be challenged by molecular data; e.g. the identity of the sister-group to the Neodermata and the apparent

similarity in flame bulb and protonephridial ultrastructure between the Rhabdocoela and the Lecithoepitheliata (see Rohde *et al.* 1993b for full discussion). Additionally, the value of the growing SSU data set was clear to those wishing to establish the phylogenetic positions of problematic taxa among the platyhelminths with data independent from morphology.

Riutort *et al.* (1992a) started to generate what is now a large SSU database on triclads (Baguñà *et al.* 2000, this volume) by considering the monophyly of selected subgenera, genera and families. Barker *et al.* (1993b) used SSU rDNA to estimate the position of the sole member of the Heronimidae within the Digenea, a topic that had been widely debated by morphologists. The debate continues even with the addition of more digenean SSU data (Cribb *et al.* 2000, this volume). The systematics and phylogenetics of other digenean taxa have also been reviewed with the addition of partial or complete SSU data (e.g. Lumb *et al.* 1993 on fellodistomids and lepocreadiids, Blair *et al.* 1998 on the Hemiuridae). The efforts of Rohde (see Rohde 2000, this volume), Watson (see Watson, 2000, this volume), Ehlers and Sopott-Ehlers (e.g. Ehlers 1995, Sopott-Ehlers 1998) among others, on the ultrastructure of various features such as protonephridia and spermatozoa, suggested new priorities for SSU sequencing in order to test controversial morphological synapomorphies. For example, the phylogenetic affinities of *Kronborgia isopodicola* was one such question, and the SSU data supported separation of the group (Fecampiida) rather than unification with any existing taxon (Rohde *et al.* 1994).

Acoels, bearing in mind their current controversial position among the Metazoa using SSU (Ruiz-Trillo *et al.* 1999), were still confidently viewed as platyhelminths when first introduced into the SSU data set (Katayama *et al.* 1993) and lived up to their expectation as basal flatworm taxa. In a search to detect the earliest divergent species of the genus *Geocentrophora* (Lecithoepitheliata) in Lake Baikal, Kuznedelov and Timoshkin (1993) pushed the SSU beyond its limits of resolution, failing to find sufficient differences between the partial SSU sequences. However, their limited results were consistent with existing taxonomic schemes. A subsequent analysis of these and additional data by the same authors (Kuznedelov and Timoshkin 1995) allowed one of the first ‘turbellarian’ based assessments to be made with SSU. Monophyly of the Seriata (Tricladida and Proseriata) was challenged, and a monophyletic clade including the Kalyptorhynchia, Proseriata and Lecithoepitheliata (rather than the strictly bifurcating topology of Ehlers (1985a)) was first suggested. Katayama *et al.* (1996) continued the focus on ‘turbellarian’ orders and provided the first comprehensive molecular based analysis of their interrelationships. Although we show here only one of the solutions provided by Katayama and her co-workers (see Figure 25.2b; original Figure 2c), it was considered that the sequences of some taxa, two proseriates and a prolecithophoran, added more noise than signal. Whether the loss of resolution was due to poor sampling, analytical or sequencing error does not appear to have been suggested. Nevertheless, even without these apparently aberrant sequences, anomalies included paraphyly of the macrostomids and trematodes, acoels were most basal and triclads were the sister-group to all other flatworms.

Carranza *et al.* (1997) utilised SSU data to question the monophyly of the Platyhelminthes and its position among major metazoan clades, as well as to infer interrelationships. Their conclusion that the phylum is not monophyletic depends largely on the anomalous position of the catenulid *Stenostomum leucops*, rather than the acoels. Macrostomids remained paraphyletic but did appear at the base of the Rhabditophora. This study showed the likelihood that the sister-group of a monophyletic Neodermata was a large clade comprised of ‘turbellarian’ taxa, although another potentially rogue sequence, this time from the acoelomorph *Nemertinoides elongatus* (Nemertodermatida) added some confusion to the otherwise ‘equitable’ phylogeny (Figure 25.2c). The same sequence continued to plague other studies (e.g. Littlewood *et al.* 1999a) until a second nemertodermatid SSU was

determined. Jondelius had been working at the same time with partial SSU sequences and with the nemertodermatid *Meara stichopi*. However, the poor signal from partial sequences apparently added more confusion to the SSU trees with his solution bearing even less resemblance to accepted or previously hypothesised schemes (Jondelius 1998; Figure 25.2d). Also, during the latter part of the 1990s Campos *et al.* (1998; Figure 25.2e) had gathered full and partial sequences from the literature and provided a more comprehensive treatment of groups. Once again, some groupings were unique, notably the grouping of Catenulida with Fecampiida and the Acoela with the Tricladida, whereas the interrelationships of the Neodermata made eminent sense in the light of morphology (e.g. Ehlers 1984). SSU alone has clearly been capable of enthralling and frustrating flatworm systematists.

The first phylum wide study to incorporate morphological evidence and combine it with SSU for a cladistic treatment was Littlewood *et al.* (1999a). SSU data alone, involving 82 sequences, reflected many patterns seen with less densely sampled analyses, but was at least allowing fewer options; e.g. a completed sequence of *Meara stichopi* provided by Ulf Jondelius tempered the saltatory behaviour of the Nemertodermatida. The treatment also highlighted the conflict between a morphological and molecular analysis with the two data sets arguing for statistically different phylogenetic solutions. Finally, prior to the present analysis, Littlewood *et al.* (1999b) added a few more taxa, adopted a refined morphological matrix from their previous study and found the SSU data to be compatible with the results based on morphology (at least in terms of passing Templeton's test) where it had failed previously (Littlewood *et al.* 1999a). The SSU data set alone provided conflicting topologies depending on the analysis performed (maximum parsimony result is shown in Figure 25.2f), but many major clades were supported as monophyletic and, combined with morphology, the data provided a working model based explicitly on much of the available evidence. The same study reviewed the influence of a molecular and combined-evidence approach in establishing the elusive sister-group to the Neodermata.

Rooting the SSU rDNA tree

Controversy regarding the position of the acoels, particularly in their distance from the acoelomorph nemertodermatids, and the placement of the latter group, are just two reasons why we have chosen not to include acoels, or indeed nemertodermatids in our present analysis. SSU rDNA sequences from acoelomorphs are notoriously difficult to align with other flatworm taxa and result in the exclusion of many more regions to maintain an ambiguity-free alignment than if they are excluded altogether. Thus, to determine the underlying phylogenetic patterns supported by SSU rDNA for the greatest number of taxa with the highest resolution, we have rooted our tree against the catenulids. Our hypotheses therefore reflect the interrelationships of the Rhabditophora (Ehlers 1984), the monophyly of which is more broadly accepted.

Why have we not chosen representatives from another phylum to root a tree of Platyhelminthes, or at least Rhabditophora + Catenulida? The sister-group to the Platyhelminthes is not certain from either morphological or molecular studies and just as there are problems with the SSU sequences of basal platyhelminth taxa, there appear to be problems with sister-group candidates. For example, both xenoturbellids (Ehlers and Sopott-Ehlers 1997b; Lundin 1998) and gnathostomulids (Haszprunar 1996) have been considered basal bilateria and/or sister-groups to the Platyhelminthes. However, SSU places xenoturbellids closer to the Mollusca (Norén and Jondelius 1997) and gnathostomulid SSU sequences have long branches and are placed variously among the Ecdysozoa (Littlewood *et al.* 1998b) or not (Zrzavy *et al.* 1998; Giribet *et al.* in press). The number of outgroups we have chosen may not be ideal for any phylogenetic reconstruction, but, following the criteria of Smith (1994), we know from previous analyses that the catenulids are suitable candidates

to root the Rhabditophora as they are monophyletic within the ingroup in larger studies of SSU (e.g. Carranza *et al.* 1997, Littlewood *et al.* 1999a,b, Ruiz-Trillo *et al.* 1999) and are the likely sister-group to the rhabditophoran flatworms.

The data set and sampling

Many partial SSU rDNA sequences are available, but to attain the highest number of variable and phylogenetically informative sites we have restricted our analysis to complete or near complete sequences. Furthermore, we have excluded certain complete sequences, despite their availability on GenBank at the time of analysis, for one or more of the following reasons: (a) SSU sequence appears more than once on GenBank for the same taxon, (b) alignment in highly conserved regions was difficult and suggested high probability of sequencing error, (c) previous phylogenetic analyses indicated sufficient error in the sequence to compromise its utility.

Whilst we will not discuss the interrelationships of the constituent major clades of flatworms sampled, it is important to highlight the diversity of taxa that underlies them. Appendix 25.1 gives a complete listing of the 270 taxa used in this study and indicates the families from which the species have been classified for each major clade. As with the majority of sequencing studies, that require access to properly fixed or fresh material that has been identified by an expert prior to fixation or molecular analysis, opportunistic collecting tends to dominate the strategy. Furthermore, in this study, our sample reflects efforts, largely by us and in collaboration with others, to sample widely for studies concentrating on smaller clades of flatworms. Readers wishing to add SSU sequences from acoelomorphs to this data set should see Ruiz-Trillo *et al.* (1999) for a listing of available sequences. An overview of the diversity of exemplar taxa follows:

Macrostomida and Haplopharyngida – perhaps more accurately grouped as Macrostomorpha (Rieger 2000, this volume) this is a small group but poorly sampled in our analysis.

Lecithoepitheliata – only three species within the same genus are represented. Campos *et al.* (1998) utilised more members of the same genus but these were the partial sequences of Kuznedelov and Timoshkin (1993).

Polycladida – although a highly diverse group we include just six sequences representing four families.

Rhabdocoela – here we include a variety of families (nine) from a variety of higher taxa that arguably should or could be treated separately (e.g. as in Littlewood *et al.* 1999a,b). However, many constituent taxa (e.g. Temnocephala) are very poorly sampled and taking the Rhabdocoela as the group of interest allows us to argue for relatively diverse sampling.

Prolecithophora – our data come largely from the studies dedicated to prolecithophoran interrelationships (Norén and Jondelius 1999, Jondelius *et al.* 2000, this volume) but include three new sequences, that in total represent five families.

Tricladida – the majority of taxa come from dense samplings of triclads by Carranza *et al.* (1998a,b) including nine families and recently reviewed by Baguñà *et al.* (2000, this volume). We add one new species.

Proseriata – although most phylum wide studies have included at least some proseriate sequences, here we provide the densest and most diverse sample that was used for a treatment on the interrelationships of the group (Littlewood *et al.* in press; see also Curini-Galletti 2000, this volume)

Fecampiida + Urastomidae – These genera represent a clade that has yet to be given a formal name. *Ichthyophaga* and *Urastoma* were each originally classified as

Prolecithophora; Watson (1997a) and Noury-Sraïri *et al.* (1989b) demonstrated differences in sperm ultrastructure in *Urastoma* and Littlewood *et al.* (1999a) showed that *Ichthyophaga* fell outside the Prolecithophora using SSU data. The fecampiid, *Kronborgia*, was shown to group with *Urastoma* and *Ichthyophaga* in Littlewood *et al.* (1999a,b). The fecampiid *Notentera ivanovi* was sequenced for this study and for another rather different perspective on flatworm phylogenetics (see Joffe and Kornakova 2000, this volume).

Monopisthocotylea – nine families including eight new sequences represent the densest sampling of SSU data for this group of monogeneans to date.

Polyopisthocotylea – 13 families including 13 new sequences represent the densest sampling of SSU data for this group to date; the majority of published monogenean sequences are from Littlewood *et al.* (1998a).

Amphilinidea – the two families of amphilinideans, each represented by a single sequence are now supplemented with an additional amphilinid.

Gyrocotylidea – two members of the single constituent family are included.

Eucestoda – 27 families representing the 12 currently recognized orders (Khalil *et al.* 1994), as well as the nominal orders Diphyllobothriidea and Litobothriidea are included from a study on cestode interrelationships (Olson *et al.* in prep.).

Aspidogastrea – three of the four families are represented and we include three new sequences.

Digenea – 55 families are sampled and include 75 new sequences generated for this study and another concentrating on digenean interrelationships (Cribb *et al.* 2000, this volume).

New sequences presented herein were determined using techniques outlined in Littlewood *et al.* (1999a) or Olson and Caira (1999). Appendix 25.2 lists primers used by the authors for PCR amplification of the complete SSU rDNA gene of platyhelminths, as well as primers for sequencing the PCR products.

Alignment

Variability of sequence lengths was extremely high, ranging from 1,739 bps in the triclad, *Girardia tigrina*, to 2,906 bps in the amphilinid tapeworm, *Gigantolina magna*. It is interesting that the neodermatan taxa possessed SSU sequences of greater length than those of the 'turbellarian' taxa without exception. In general, 'turbellarian' SSU sequences were ~1,800 bps, digenean and monogenean sequences ~1,950 bps and cestode sequences ~ 2,100 bps in length. Primarily these differences reflect modifications to variable domains of the gene (Figure 25.8), whilst the conserved core of the secondary structure model (e.g. Neefs *et al.* 1993) was alignable across the broad spectrum of taxa examined. To date only two species of flatworms, *Schistosoma mansoni* and *Spirometra erinaceieuropaei*, have had their secondary structure at least partially predicted (see Ali *et al.* 1991 and Liu *et al.* 1997, respectively), and it may be worth examining the model for other taxa. In particular, large insertions, notably among amphilinidean cestodes (Olson and Caira 1999), suggest that the mature SSU ribosomal RNA may take a wide range of forms among the flatworms.

It is well known that even small changes in alignment can have major effects on phylogeny reconstruction (e.g. Winnepenninckx and Backeljau 1996) and we have aimed to be highly conservative in our determination of positional homology. Furthermore, because the effects of missing data can have an undesirable influence on resulting trees (Barriel 1994, Wilkinson 1995), we have discarded most positions that required gaps to be inserted in the alignment for a large number of taxa. The result was that a majority (66%) of the 3,587

positions in the full alignment[3] was discarded either for lack of positional homology or for the presence of insertion/deletions unique to small numbers of taxa. In the end, 1,215 positions were included in the analyses of 270 taxa. This provided 806 variable positions of which 598 were phylogenetically informative under the criterion of parsimony (see Table 25.1). Figure 25.3a gives a diagramatic representation of the full alignment, indicating the variable domains as defined by Neefs *et al.* (1993), and the distribution of phylogenetically informative positions. Figure 25.3b shows in greater detail three regions of the alignment (*i*, *ii*, *iii*; as indicated by horizontal bars in Figure 25.3a) that together encompass all positions included the analysis. Using a 5 bp sliding window method of averaging, these three histograms depict the rescaled consistency indices (RC) of the characters (based on a maximum parsimony consensus tree) as distributed across the alignment. From this there is no clear pattern to suggest that some regions of the molecule contain more reliable, or less homoplasious, sites than do others, with the obvious exception of the variable domains in which most sites had to be discarded altogether. Instead, sites showing high RC values are scattered across the more conserved regions of the gene alignable among the 270 taxa. An effective sequencing strategy therefore requires information from the entire gene to maximize the number of such positions.

Analysis

Large data sets are not amenable to all methods of analysis. In particular, maximum likelihood analysis is not possible unless restricted, for example, to 4-taxon statements (e.g. the quartet puzzling methods of Strimmer and von Haeseler 1996, Wilson 1999). Here we restrict ourselves to minimum evolution (ME) and maximum parsimony (MP) approaches and concentrate only on the interrelationships of major clades of flatworms. We have purposefully avoided providing details of lower level interrelationships, e.g. within triclads, prolecithophorans, digeneans, cestodes etc., as these are dealt with elsewhere in this volume. Furthermore, the scope of the alignment across the Rhabditophora cannot accurately reflect the SSU signal, as many positions potentially informative within subsets of the taxa will have been excluded from the global alignment.

Numerous discussions on the philosophical merits of phylogenetic reconstruction methods exist in the literature (e.g. see the journals *Systematic Biology, Molecular Biology and Evolution, Molecular Phylogenetics and Evolution*, and *Cladistics*). Here we take two very different approaches commonly used to estimate phylogenetic patterns from nucleotide data. Maximum parsimony is a character-based approach that seeks the topological solution that incurs the fewest number of character-state changes. Minimum evolution is a distance-based algorithm that builds a topology based on pairwise distances estimated by a model of nucleotide substitution, that in turn attempts to compensate for the biases inherent to the sequence data (e.g. substitution rate variation and base-compositional bias). Considerable detail on the computational aspects of both methods can be found in Swofford *et al.* (1996). All phylogenetic analyses were conducted using PAUP* ver. 4.0 (Swofford 1998).

Treatment of gaps – Alignments of homologous genes invariably generate the need for gaps, or indels, as insertions and deletions are inferred from multiple pairwise comparisons of sequences. The inclusion of gaps as fifth state characters, available in MP analysis only, has been demonstrated to provide additional valuable statements on homology (e.g. Giribet and Wheeler 1999) and some data sets utilising SSU data rely on indels for finer phylogenetic resolution (e.g. echinoids, Littlewood and Smith 1995). In our alignment, treating gaps as fifth character states, or as missing data, had neither any effect on the number

[3] The full alignment may be obtained by anonymous FTP from FTP.EBI.AC.UK under directory pub/databases/embl/align, accession number DS*****.

of phylogenetically informative positions, nor on the topology of the MP tree. Consequently, we have restricted our analyses to working with gaps treated as missing data for both MP and ME solutions.

Minimum evolution – (Figure 25.4) The log determinant model (Lake 1991, Lockhart *et al.* 1994) of nucleotide substitution was used to estimate genetic distances that were then anlayzed by the method of minimum evolution. Tree bisection-reconnection (TBR) branch-swapping was aborted after 18 hours and > 1.5 x 10^6 topological arrangements had been evaluated.

Maximum parsimony – (Figure 25.4) Characters were run unordered and taxa were added via random addition. Not a single heuristic search using TBR branch-swapping ever reached completion before the computer ran out of memory storing trees. Thus we show the strict consensus of this same number of equally parsimonious trees (42,100) found within 13 hours of searching.

Rate categorization of sites – Although it is a controversial subject, there are logical reasons to justify selectively excluding positions from the analysis. One obvious reason is to reduce noise (random signal) by removing sites that are highly homoplasious based on either an *a priori* or *a posteriori* criterion. We chose to use the rescaled consistency index of the characters (based on the topology of the ME tree) as a measure by which to separate the characters into 10 categories. We then examined the effects on tree topology and resolution of removing characters with low RC values (and thus high rates) through successive maximum parsimony analyses. The result (not shown) was that the structure of the parsimony-based tree in Figure 25.4 was largely supported, but with less resolution and with the occasional spurious arrangement as more and more characters were removed from the analysis. Similar to the *a posteriori* successive approximations approach (Farris 1969), we could have *differentially* down-weighted characters in high-rate categories (rather than down-weighting them to nought by removal). However, the subjectiveness of such a weighting scheme and the lack of any striking differences in the results after the complete removal of high-rate sites suggested to us that we were unlikely to enhance the signal through further analysis.

Analysis of consensus sequences – (Figure 25.5) Because our concerns herein are focused on the interrelationships among major clades, and not interrelationships within these clades, we considered the effects of reducing the terminal taxa into representative groups (where such groups were shown to be monophyletic via previous analysis of the data, i.e., Figure 25.4), and representing these clades by a consensus of the sequences of the constituent taxa. Using GDE (Smith S.W. *et al.* 1994), it is possible to create consensus sequences whereby positions with states not common among at least 75% of the sequences considered are coded as multistate characters using the standard IUPAC code. Conversely, positions that show the same state in 75% or more of the sequences are coded as such for the group. In this way, we reduced the data set to 22 consensus sequences and analyzed them via maximum parsimony. A strict consensus of the resulting trees (Figure 25.5) provided considerably less resolution than did analyses of the complete data set (Figure 25.4), and in considering the inclusion of the Aspidogastrea within the Cercomeromorphae clade, produced highly unlikely results. Although this approach has been shown to be useful in some cases (e.g. Littlewood *et al.* 1997), it appeared to be weak in reducing conflicting signal among the taxa analyzed.

Effects of secondary structure – (Figure 25.6) Using our alignment and the inferred secondary structure of one SSU sequence (*Pseudomurraytrema* sp.; see Appendix A in Olson and Caira 1999), we classified putatively homologous base positions as being either stems or loops following the rationale of Soltis and Soltis (1998) wherein loops were defined as being four or more unpaired bases in length. Our categorisation of loops and stems is a simplification of the secondary structure features of rDNA, but essentially reflects base-

pairing regions (stems) and non-base-pairing regions (loops). Bulges and 'other' regions (*sensu* Vawter and Brown 1993) were subsumed variously into 'stem' or 'loop' categories depending on their length. Even with this simplication, it was clear that characters from both base-pairing regions and non-base-pairing regions contain phylogenetic information; although of all phylogenetically informative positions, 60.5% appeared in stems and 39.5% in loops. Table 25.1 provides a statistical summary of the different data partitions. Stem regions were slightly G-T rich, whereas loop regions were comparatively A-rich. Chi-square analysis as implemented in PAUP*, however, did not suggest that this nucleotide bias was distributed unevenly among the taxa (P = 1). Character statistics were similar between both stem and loop partitions, although the consistency index (CI) was slightly lower for loop characters suggesting a higher degree of saturation among these positions.

Because of the size of our data set and the inability to reach the end of a 'standard' heuristic search we chose not to differentially weight stem and loop positions (see eg. Dixon and Hillis 1993), but instead analyzed the data partitions separately. Maximum parsimony analysis of stem bases only indicates that these regions contribute significantly to the structure of the MP topology, and provides greater resolution than the loop positions alone. Furthermore, dubious relationships among basal 'turbellarian' groups were found when analyzing loop regions. Of course, because of the nature of the alignment and the diversity of taxa sampled, most included positions appeared in stem regions (60%).

Mutational saturation – (Figure 25.7) We attempted to examine the possibility that the antiquity of divergence events within the Platyhelminthes has resulted in saturation of the characters analyzed. In such a situation, a plot of sequence divergence vs. divergence time will become asymptotic at the time in which all sites free to vary have become saturated (see Figure 5.19 in Page and Holmes 1998). In Figure 25.7 we show a series of graphs that approximate the comparison above by plotting observed pairwise sequence substitutions (= divergence) against estimated pairwise distances (~ divergence time). Because transitions occur more frequently than do transversions, we looked at both categories of substitutions separately. Pairwise substitution ratios (transitions or transversions as a proportion of the total number of observed differences) were calculated using *Seq_db* software (authored by Richard Thomas, The Natural History Museum). We also employed two different substitution models to estimate genetic distances: Log-determinant and general time-reversible (GTR; Swofford *et al.* 1996, Waddell and Steel 1997) including estimates of invariant sites and among-site rate heterogeneity (as estimated from the ME-based topology). Differences in the assumptions of the two models generally result in quite different estimates of genetic distance (with the GTR model typically resulting in estimates of distance more disparate from observed values), and could thus lead to different conclusions regarding mutational saturation. The left column of plots in Figure 25.7 show comparisons based on all included positions, whereas plots in the right column show comparisons only from positions that change on the basal nodes (denoted by arrows in Figure 25.4) of the maximum parsimony consensus topology, where the potential for saturation would be expected to be highest due to the greater age of such early divergences. Results derived from all included positions show a tightly clumped, linear increase of divergence with distance, suggesting that neither transitions nor transversions are saturated. However, a different pattern is seen when only those positions observed to change on the basal nodes of the tree are considered in isolation. These plots show considerably greater scatter in all cases, and transitions appear to asymptote when genetic distances reach a value of ~0.1 using either model of nucleotide substitution, whereas transversions show a more linear rate of increase. Saturation of transitional substitutions along basal nodes may account in part for the instability and lack of support of these partitions in the tree.

Figure 25.7b illustrates further patterns of nucleotide substitution in the SSU data. A transition/transversion plot for pairwise comparisons of taxa demonstrates any potential bias towards one or other substitution type as well as presenting further visualisation of any saturation of substitutions as a function of time since divergence. Generally, transitions occur more frequently than transversions; in our data set the overall estimated transition:transversion ratio was 1.3:1. As in Figure 25.7a, plots that deviate from a linear relationship indicate saturation effects from multiple substitutions, erasing the record of previous changes.

Signal

SSU provides a phylogeny of the Platyhelminthes with certain relationships robust to the vagaries of reconstruction method and steadfast under the scrutiny of bootstrap analysis. It certainly seems to be the case that a denser sampling of taxa has yielded more robust phylogenies of the platyhelminths with more groups retaining monophyly than other studies to date, although some relationships have been identified even with a minimum number of taxa (e.g. see Figure 25.2). Taking the present study as the basis for discussing SSU and the platyhelminths, as it represents the most densely sampled data set and therefore the best molecular-based estimate to date (Hillis 1996, 1998; Graybeal 1998), monophyly of the following groups is found to be strongly supported: Neodermata, Trematoda, Digenea, Cestoda, Amphilinidea, Gyrocotylidea, Monopisthocotylea, and Polyopisthocotylea. The Gyrocotylidea is the sister-group to a clade comprising the eucestodes and amphilinideans. Likewise, SSU confirms the monophyly of the Tricladida, Prolecithophora, Polycladida, Lecithoepitheliata, Macrostomida+Haplopharyngida, a clade comprising the parasitic 'turbellarian' genera, *Ichthyophaga, Kronborgia, Notentera* and *Urastoma*, and the non-neodermatan rhabdocoels; namely a clade comprised of the Dalyelliida, Kalyptorhynchia, Temnocephalida and Typhloplanida. Within this latter clade Kalyptorhynchia and Dalyelliida are also monophyletic (details of relationships within the Rhabdocoela are shown in Littlewood *et al.* 1999b, Figure 3). Figure 25.6 illustrates further the strength of the signal, regarding the monophyly of the major clades in stem and loop regions of the SSU data. The signal in the solution based on stem regions is largely consistent with that in the full analyses, whereas the solution provided by loop regions offered less resolution and less congruence.

In the full analyses, the rhabdocoels and proseriates are also each monophyletic but with low bootstrap support that suggests that there is less signal in the SSU for the confident placement of these taxa. Indeed, although many clades appear to be monophyletic, few deeper branching nodes are well supported.

SSU data suggest that the sister-group to the Neodermata is a large clade comprised of all the 'turbellarian' taxa to the exclusion of the more basal macrostomids, haplopharyngids, lecithoepitheliates and polyclads. Interestingly, only with relatively dense sampling were the Proseriata both monophyletic and members of this larger 'turbellarian' sister-group clade (contrast Littlewood *et al.* 1999a,b).

Noise and Conflict

Relationships among the earliest divergent platyhelminth taxa are not well resolved with available SSU data. The two methods of analysis provide contradictory solutions with respect to the Macrostomida + Haplopharyngida and Lecithoepitheliata vying for the position of the most basal rhabditophoran. Consequently, we cannot place the Polycladida firmly either. Within the remaining 'turbellarian' groups sampled, only the interrelationships of the rhabdocoels (Kalyptorhynchia, Temnocephalida, Typhloplanida and Dalyelliida) are in conflict due to the non-monophyly of the typhloplanids sampled. The remaining conflict involves the interrelationships of the Neodermata. As the Monogenea remain to be confirmed

as truly monophyletic, we are no further in resolving the interrelationships of the cestodes, monogeneans and trematodes than we are when including morphology or LSU rDNA data (Littlewood *et al.* 1999b). This conflict is known from other ribosomal gene data (Mollaret *et al.* 1997, Justine 1998a) and in our study, in spite of denser sampling, there is conflict between the ME and MP results. Although the Monogenea are not monophyletic in either case, ME provides a more traditional scheme of neodermatan interrelationships with monogenean and cestode groups forming a clade ('Cercomeromorphae') that is the sister-group to the Trematoda (Aspidogastrea + Digenea). In contrast, MP analysis suggests that the polyopisthocotylean monogeneans are the sister-group to all other neodermatans. Considering the density of sampling so far, the problem of monogenean monophyly, which is contradicted only by ribosomal evidence, is not likely to be readily solved with the addition of more SSU data.

Influence of SSU data on combined evidence analyses – As a result of the breadth of sampling of the SSU gene among flatworms, this gene locus has been used to determine combined evidence phylogenetic solutions, usually in combination with morphologically based matrices, but occasionally in combination with other gene sequences. Combining data in phylogenetic analyses is a controversial topic (de Queiroz *et al.* 1995, Huelsenbeck *et al.* 1996) and such studies involving flatworms are few in number. A review indicates the relatively great influence SSU has on combined evidence tree topologies. We are aware of few studies of platyhelminths where SSU has been combined with other systematic evidence and analysed using cladistics (e.g. Blair et al. 1998 on hemiuroid digeneans, Olson and Caira 1999 on cestodes, and Littlewood *et al.* 1999b on the phylum). In cases where morphology alone has provided highly unresolved trees, the influence of SSU data is clearly overriding. One such example comes from the Digenea (Cribb *et al.* 2000, this volume) where an extensive morphological matrix coding many characters for numerous taxa results in a poorly resolved morphological tree when compared to that offered by SSU alone. The combined evidence solution is largely similar to the tree derived from the SSU analysis. Such scenarios not only call for more morphological characters if possible, but more characters independent of the SSU gene in order to assess the possibility of interpreting a gene phylogeny. A similar example comes from the combined evidence treatment of the Platyhelminthes (Littlewood *et al.* 1999b) where quite different scenarios are suggested each by morphology and SSU. The combined evidence solution, legitimised by the compatibility of the two data sets, appears to be more similar to the SSU tree than the morphology tree. However, some morphologically based synapomorphies (e.g. those uniting the Monogenea) persist and highlight potentially homoplastic signal in the SSU data. These studies should not be used to fuel a debate on molecules versus morphology (Hillis 1987, Patterson *et al.* 1993). Character conflict demonstrates the need for additional data and/or an understanding of where the homoplasy lies in one or more data sets (Larson 1994, Hillis 1998).

Other studies have shown that SSU data can be as much in conflict with other genes as it can with morphology. In the Proseriata both SSU and LSU rDNA suggest alternative phylogenetic solutions for the group. In the absence of sufficient morphological signal the debate turns from molecules and morphology to gene versus gene. Such results highlight deficiencies in sample size as only more taxa or more characters are likely to lead to congruence or a better estimate of phylogeny (Graybeal 1998; but see Naylor and Brown 1997).

Compromise

With a plethora of phylogenetic schemes available from SSU data in the literature, it is incumbent upon us to provide a solution that we feel reflects both the signal and the noise in the molecule. On the premise that the most densely sampled data set is best, but without

advocating one phylogenetic reconstruction method over another, we have combined the tree solutions offered by our analyses into a strict consensus, shown in Figure 25.8, where conflict between the most parsimonious trees and the topology estimated by ME are reflected as polytomies. Although a conservative estimate, a considerable amount of structure remains nonetheless.

Among the Rhabditophora the most basal clade is presently unresolved with SSU data alone, leaving us with a polytomy of macrostomorphs, lecithoepitheliates and polyclads. None of these groups is particularly well sampled in comparison to the other major groups, and if one were to rely solely on SSU data, further sequences may help resolve the polytomy. Two other major clades of platyhelminths are resolved, namely the Neodermata and a clade comprising the proseriates, rhabdocoels, Fecampiida + Urastomidae, prolecithophorans and triclads. The latter clade has not been previously reported in any study of platyhelminth interrelationships. Less densely sampled analyses have generally resulted in the exclusion of the proseriates from such a clade (e.g. Littlewood *et al.* 1999b, Baguñà *et al.* 2000, this volume). The addition of Prolecithophora and more proseriates appears to have strengthened the case for this clade, although it is poorly supported by bootstrap resampling procedures. Interestingly, the parasitic 'turbellaria' nestle within the clade and therefore refute the monophyly of the Revertospermata (but see Kornakova and Joffe 1999, Joffe and Kornakova 2000, this volume). That the triclads and prolecithophorans are sister taxa has been found by those concentrating on the interrelationships of triclads using SSU data (Baguñà *et al.* 2000, this volume), but all other relationships within this clade appear to be new hypotheses.

Whilst SSU provides excellent resolution of and within the Neodermata, the monophyly of the Monogenea remains uncertain. Campos *et al.* (1998) suggested monophyly with SSU but greater sampling leaves the group paraphyletic (Littlewood *et al.* 1999a,b, this study). The compromise solution does, however, support traditional relationships among the gyrocotylideans, amphilinideans and eucestodes.

Are these relationships correct? We can only hope to have demonstrated the signal that SSU data provides. As with any phylogeny, we can impose subjective decisions as to the value of particular nodes, or hopefully, judge the signal against additional apomorphies from independent data sets (e.g. the reader may look elsewhere in this volume). It is also important to note that our compromise topology is probably the best estimate of what the SSU data presently provides and, among the resolved nodes including those with low bootstrap support, it may truly reflect the evolution of the gene whilst not necessarily reflecting the evolutionary history of the species. Conflict between species trees and gene trees are well known (e.g. Page and Charleston 1997, Slowinski and Page 1999) and it is important to bear this in mind when evaluating or utilising single gene phylogenies.

Clearly, there is need for greater SSU sampling of many of the 'turbellarian' groups, in particular the Macrostomida, Lecithoepitheliata, Temnocephalida, Kalyptorhynchia, Dalyelliida, and Typhloplanida, not only for the placement of these taxa, but in order to evaluate the interrelationships within these groups. Whilst we have not covered the utility of SSU within some of the major clades in this chapter, the gene has clearly demonstrated great utility among constituent platyhelminth taxa; e.g. the triclads (Baguñà et al 2000, this volume), the prolecithophorans (Jondelius *et al.* 2000, this volume), the monogeneans (Littlewood *et al.* 1998a), the cestodes (Mariaux and Olson 2000, this volume), and the digeneans (Cribb *et al.* 2000, this volume). As regards the broader relationships, the SSU data set is now generally well sampled, and attention spent on other genes and molecular markers will probably be more profitable.

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Table 25.1. Statistical summary of SSU rDNA datasets analyzed.

	Number of positions*:									Averages†		
Dataset	**Included**	**Constant**	**Uninform.**	**Gapped**	**Inform.**	**%G**	**%A**	**%T**	**%C**	**CI**	**RI**	**RC**
Complete	1,215	409	208	363	598	26.6	27.6	25.5	20.3	0.57	0.57	0.24
Stems	727	237	128	213	362	29.8	22.7	25.8	21.6	0.57	0.58	0.27
Loops	488	172	80	150	236	21.7	34.8	25.1	18.4	0.52	0.57	0.20

* Total number of positions in alignment = 3,587. Numbers of uniformative and informative positions based on parsimony.
† Values represent means of the character consistency index (CI), retention index (RI) and rescaled consistency index (RC) for all positions included in the dataset analyzed.

FIGURE LEGENDS

Figure 25.1. Simplified diagrammatic representation of an SSU rDNA-based maximum likelihood tree of 61 metazoan species published by Ruiz-Trillo *et al.* (1999). Acoels were consistently placed at the base of the Bilateria and never grouped with other platyhelminths, including nemertodermatid acoelomorphs.

Figure 25.2. Recent hypotheses on the interrelationships of the Platyhelminthes based on SSU rDNA sequence data with the number of taxa representing each higher group indicated parenthetically. Filled triangles represent non-monophyletic groupings of constituent taxa. Abbreviations: ML, maximum likelihood; MP, maximum parsimony.

Figure 25.3. Graphical representations of the sequence alignment consisting of 270 platyhelminth SSU sequences. **a.** Complete alignment indicating the distribution of parsimony-informative positions (black columns), variable domains as defined by Neefs *et al.* (1993; dotted boxes) and the three alignment regions (*i, ii, iii*) shown in b (horizontal bars). **b.** Rescaled consistency-index for each character included in the analysis averaged over a 5 bp sliding window. Variable domains indicated by dashed boxes. Black columns below the x-axis indicate positions excluded from the analyses; note, however, that the method of averaging employed yields values even for excluded positions so long as they are within 4 bps of an adjacent position with a value > 0.

Figure 25.4. Results of minimum evolution (ME) and maximum parsimony (MP) analyses (1,215 characters). Catenulids designated as outgroup (**OG**) taxa. Left topology depicts the minimal tree based on a distance matrix (LogDet model of nucleotide substitution); right topology depicts the majority-rule (maj-rule) consensus of 42,100 equally parsimonious trees (EPTs; 5,185 steps, CI = 0.26, RI = 0.77, RC = 0.2); heuristic search aborted after examining > 2×10^9 topological arrangements. A vast majority of nodes were common among all EPTs; those found in less than 95% of the EPTs are indicated with open diamonds. Bootstrap support based on 26,973 replicates using a fast heuristic search algorithm. Arrows indicate the basal nodes used for examining the potential saturation of character state substitutions as shown in Figure 25.6B (see text).

Figure 25.5. Results of maximum parsimony analysis of 75% consensus sequences representing 22 clades shown to be monophyletic by prior analysis. Catenulida designated as the outgroup (**OG**) taxon. Topology based on a strict consensus of 612 equally parsimonious trees (466 steps, CI = 0.7, RI = 0.66, RC = 0.46).

Figure 25.6. Results of separate maximum parsimony analyses of ‘stem’ (717 characters) and ‘loop’ (488 characters) positions as defined in the text. Catenulids designated as outgroup (**OG**) taxa. Left topology depicts the strict consensus of 32,200 equally parsimonious trees (EPTs) (2,763 steps, CI = 0.3, RI = 0.79, RC = 0.24), and the right topology depicts the strict consensus of 32,000 EPTs (2,336 steps, CI = 0.24, RI = 0.75, RC = 0.18).

Figure 25.7. Scatter plots of character state substitutions based on all possible pairwise comparisons of the taxa (N = 36,316). **a.** Observed transitions or transversions vs. genetic distance as estimated by either of two nucleotide substitution models: log determinant (LogDet) or general-time reversible, including estimates of invariant sites and among-site rate variation (GTR+I+G). **b.** Scatter plots of observed transitions vs. transversions. Plots in the left column are calculated from all included positions; those of the right column are calculated from only those characters observed to change along the basal nodes of the consensus tree (see arrows in Figure 25.4). For each pairwise comparison, the substitution value (transition or transversion) is relative to the total number of changes observed between the two taxa.

Figure 25.8. Compromise — phylogenetic relationships among the major clades of platyhelminths based on a strict consensus of the results of minimum evolution and maximum parsimony analyses shown in Figure 25.4.

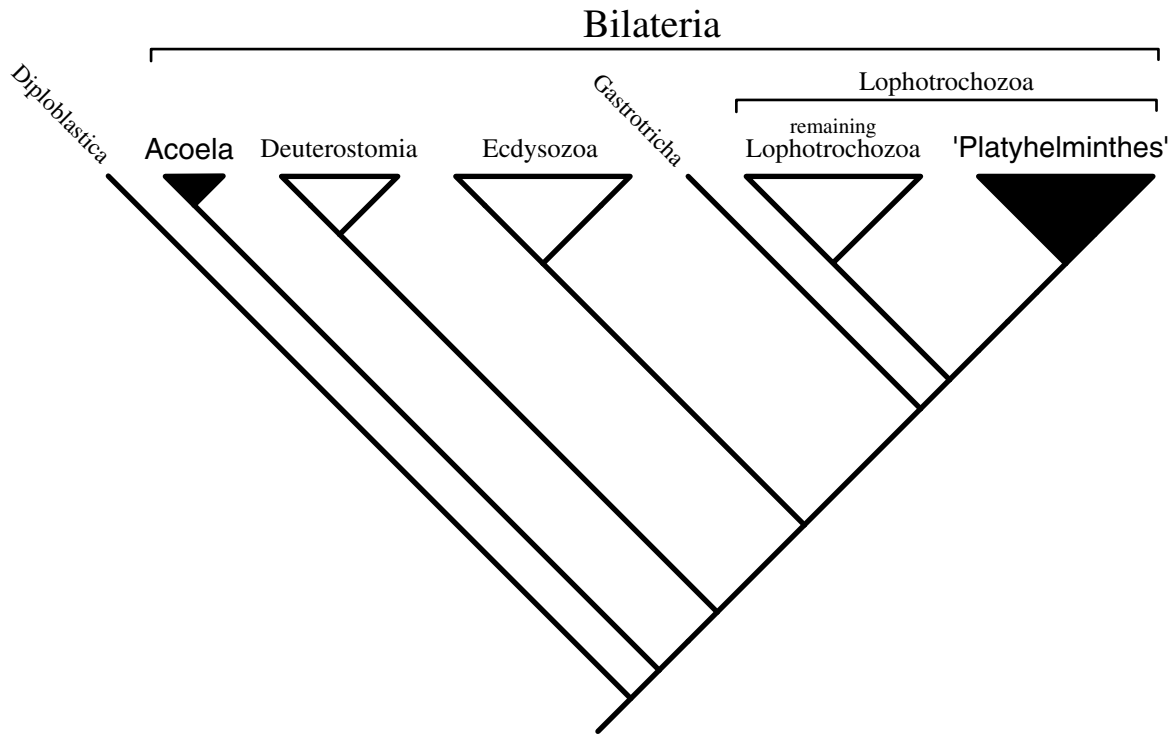
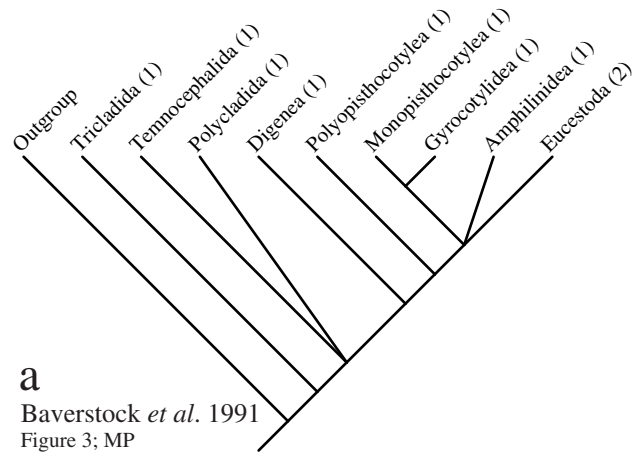
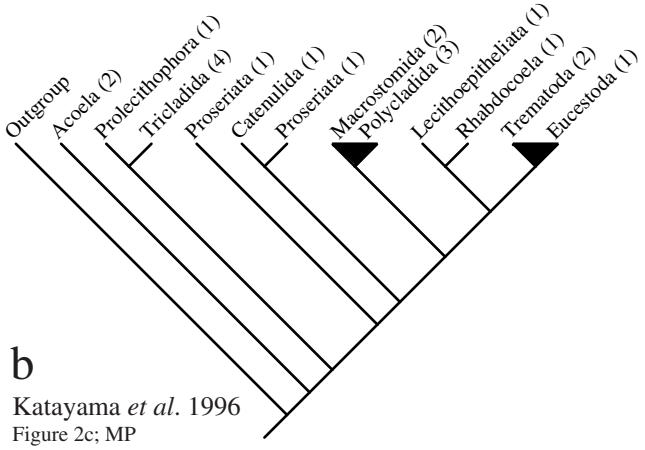


Figure 25.1 Littlewood & Olson [25.1.ill]



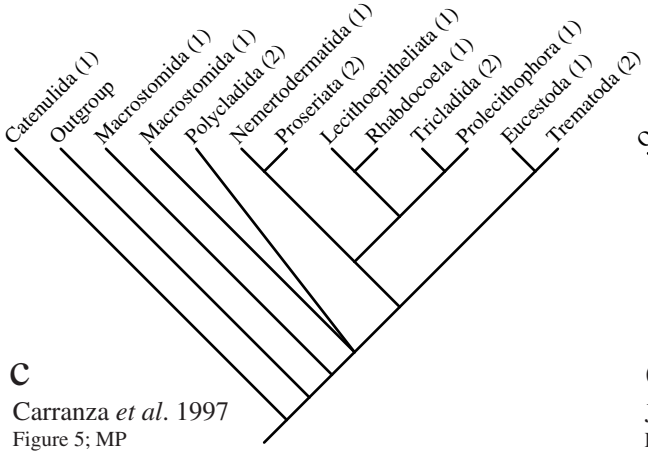
a

Baverstock *et al*. 1991
Figure 3; MP
partial SSU



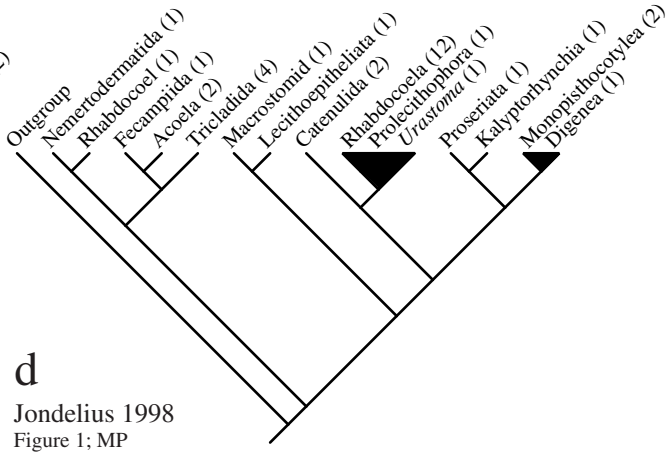
b

Katayama *et al*. 1996
Figure 2c; MP
complete SSU; (see Figure 3 also)



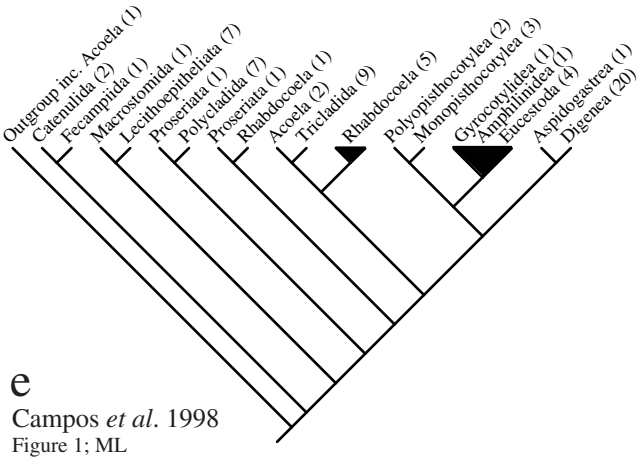
c

Carranza *et al*. 1997
Figure 5; MP
complete SSU



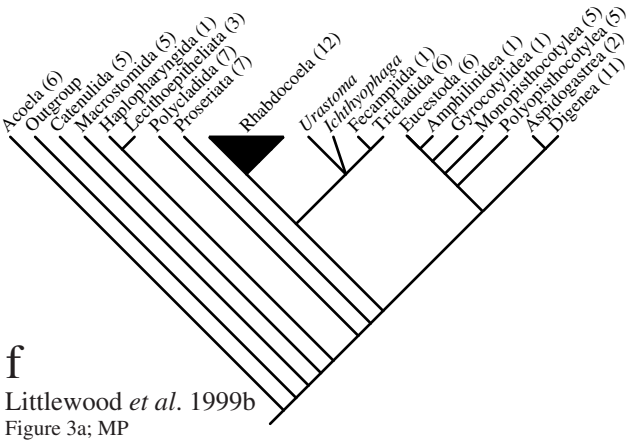
d

Jondelius 1998
Figure 1; MP
partial SSU



e

Campos *et al*. 1998
Figure 1; ML
complete and partial SSU



f

Littlewood *et al*. 1999b
Figure 3a; MP
complete SSU

Figure 25.2 Littlewood & Olson [25.2.ill]

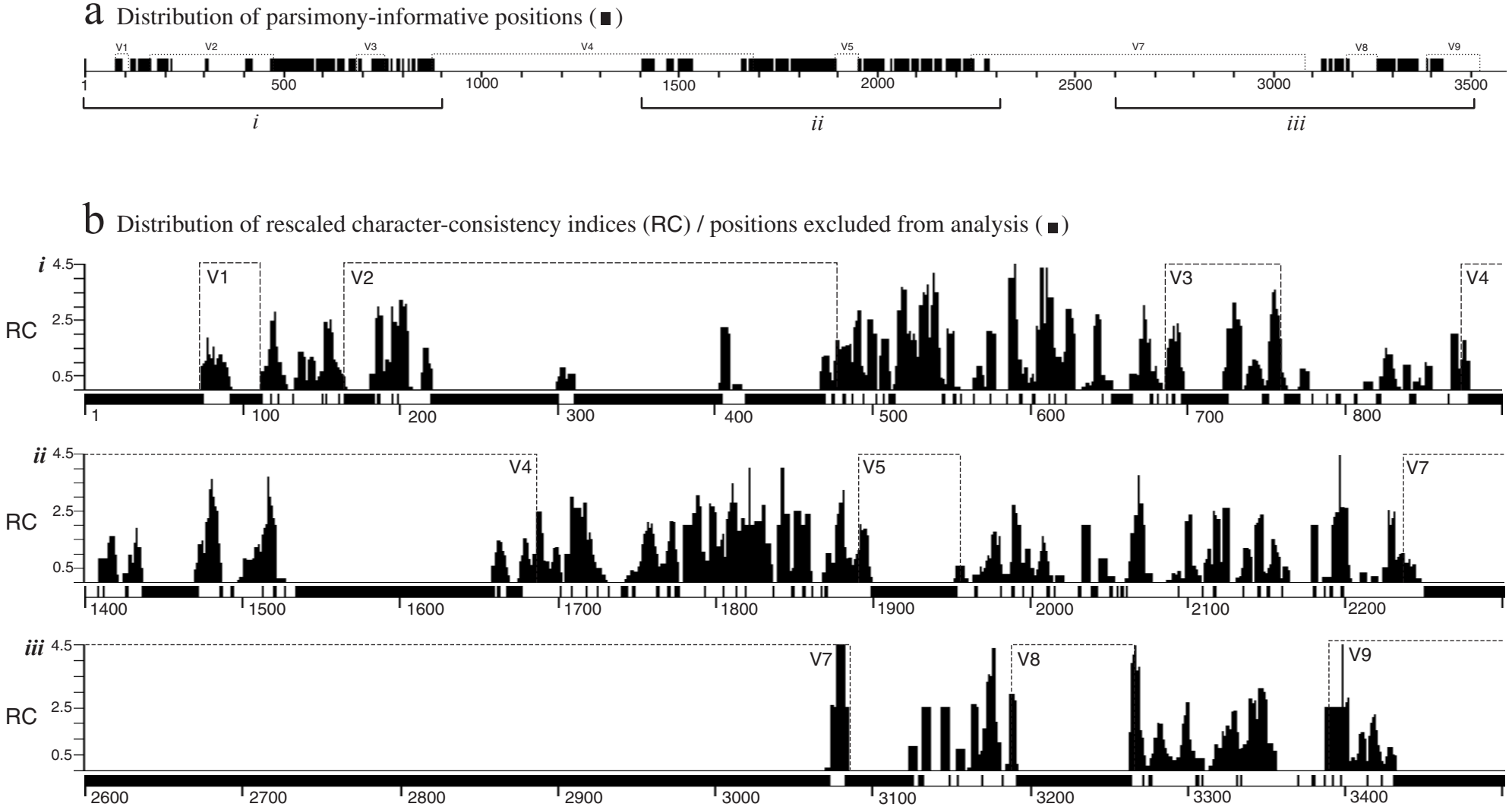


Figure 25.3 Littlewood & Olson [25.3.ill]

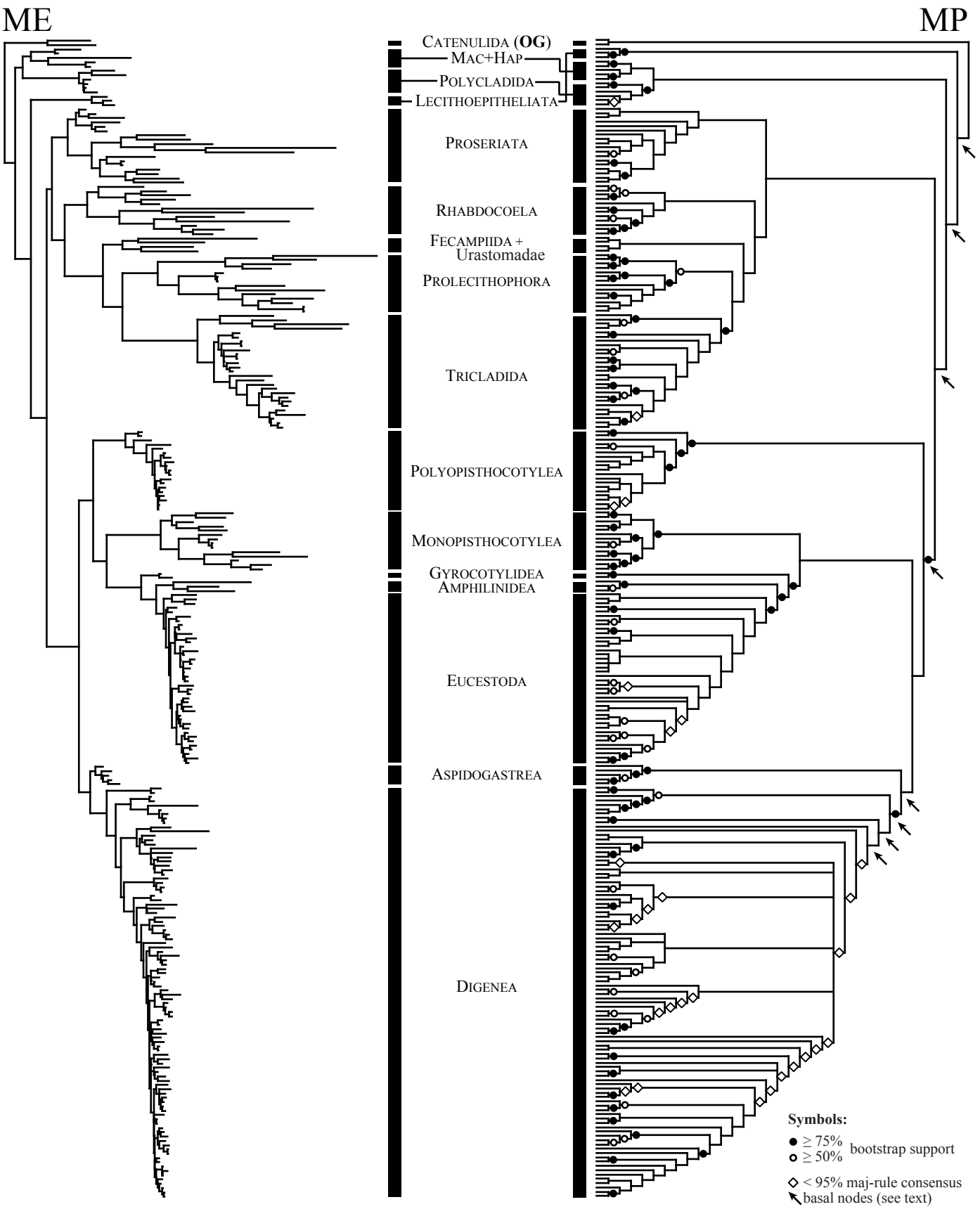


Figure 25.4 Littlewood & Olson [25.4.ill]

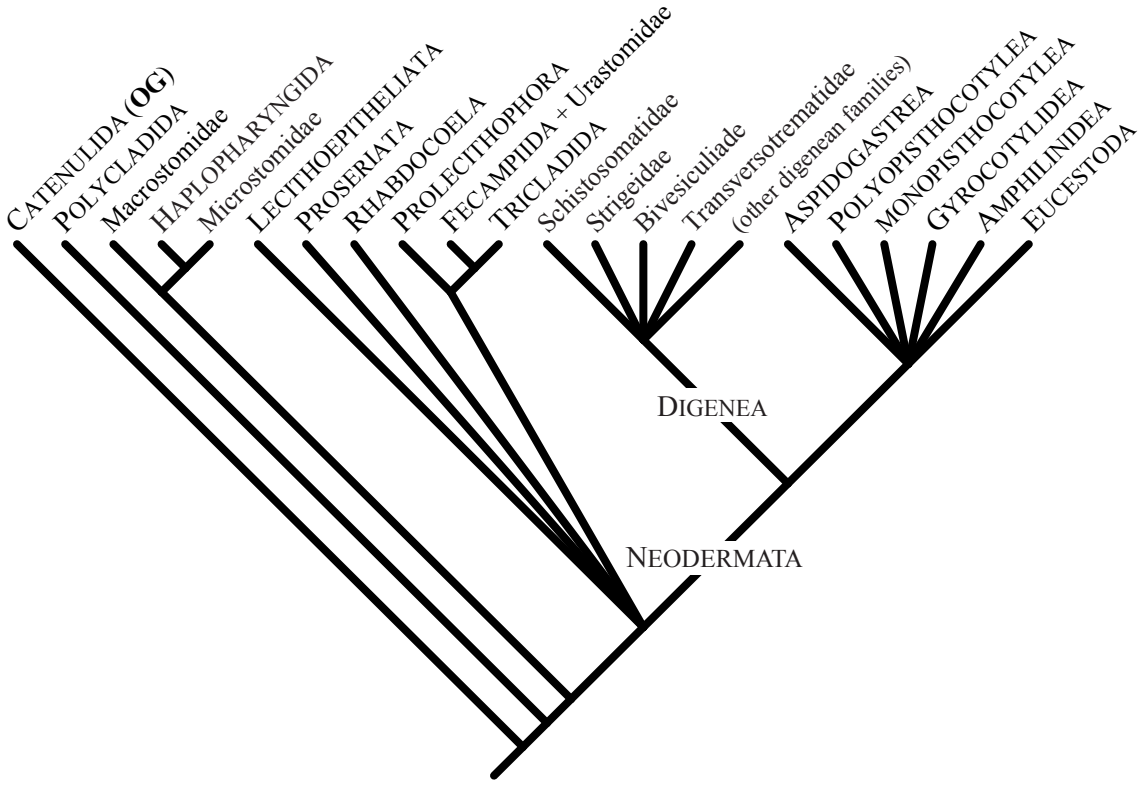


Figure 25.5 Littlewood & Olson [25.5.ill]

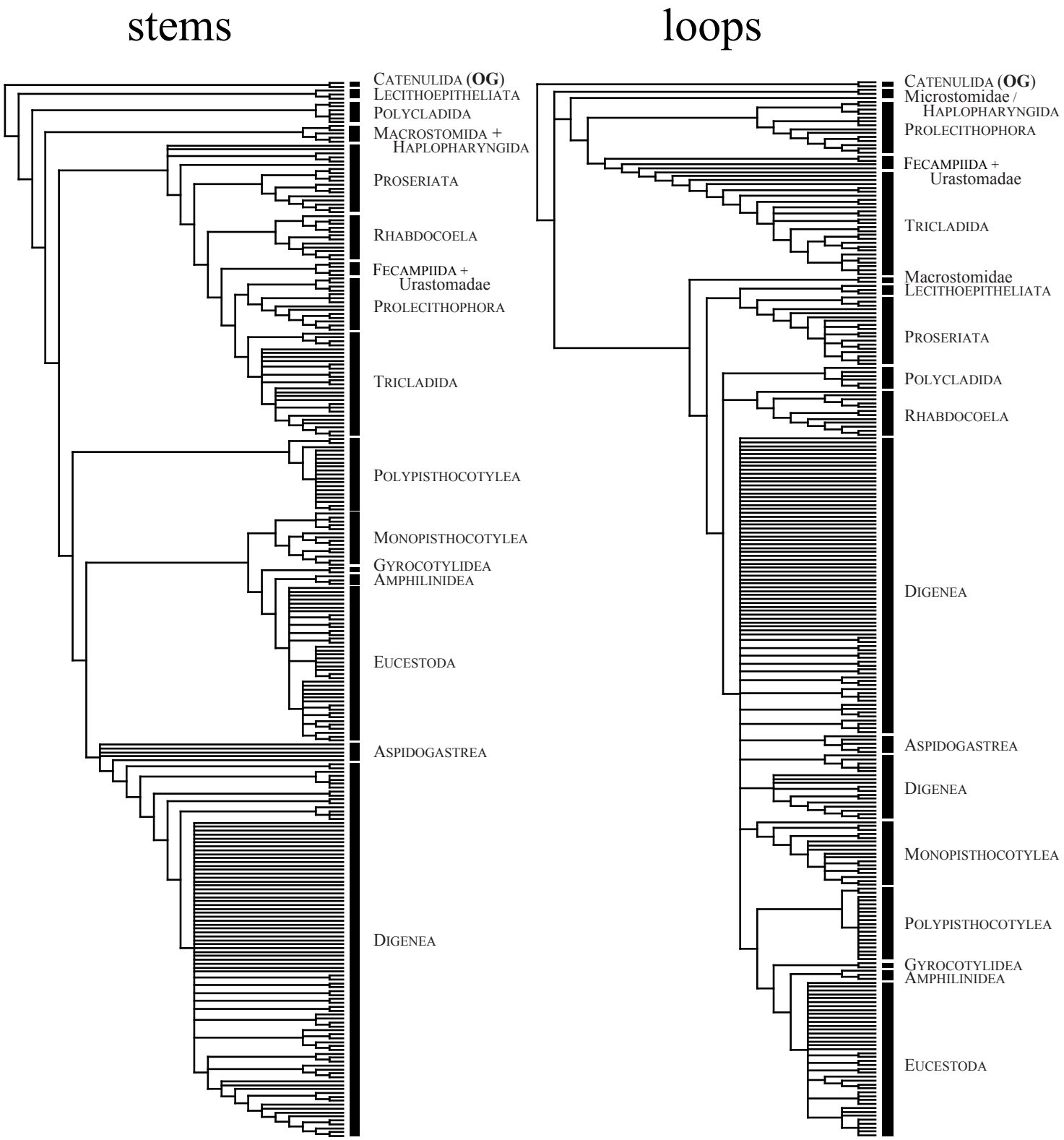


Figure 25.6 Littlewood & Olson [25.6.ill]

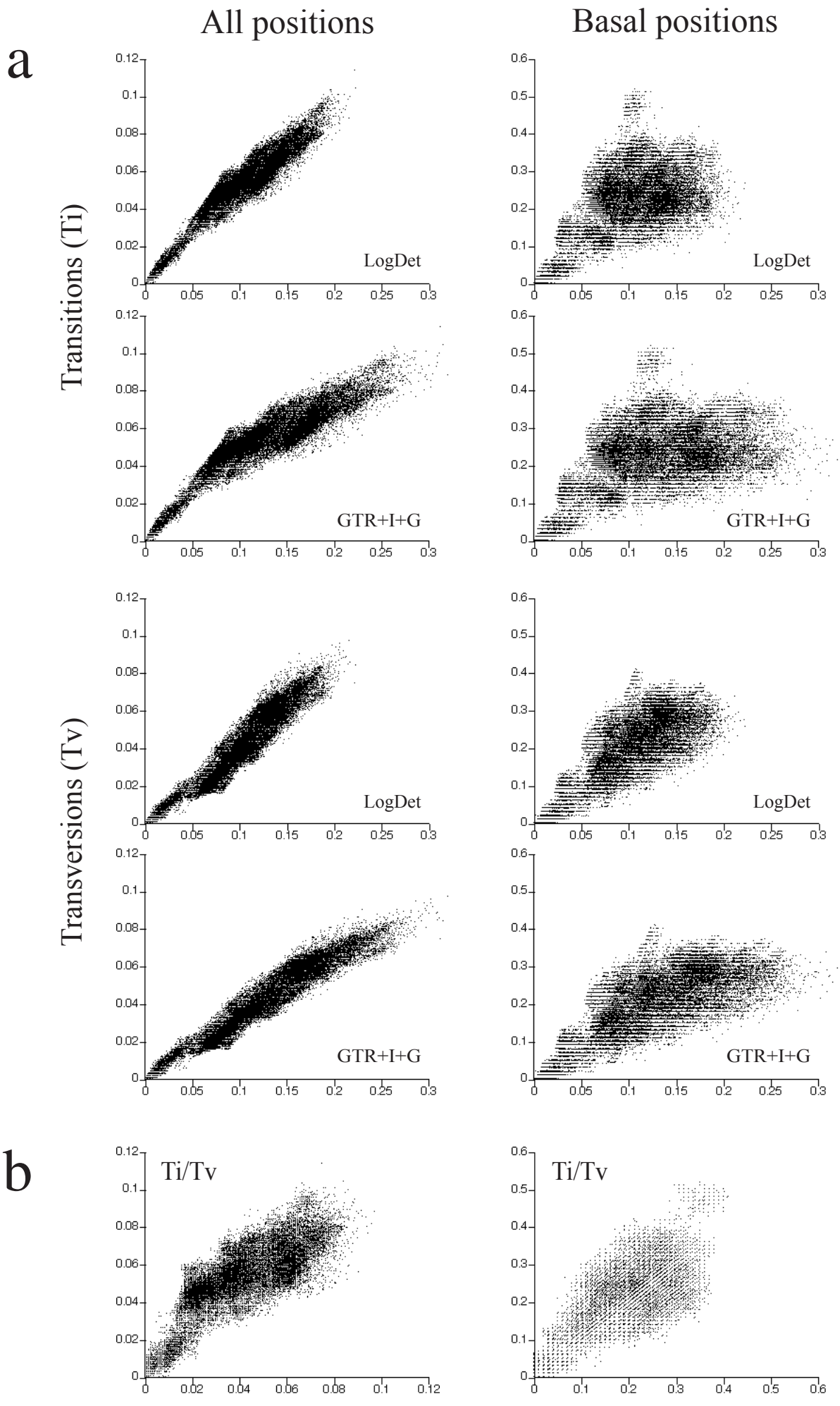


Figure 25.7 Littlewood & Olson [25.7.ill]

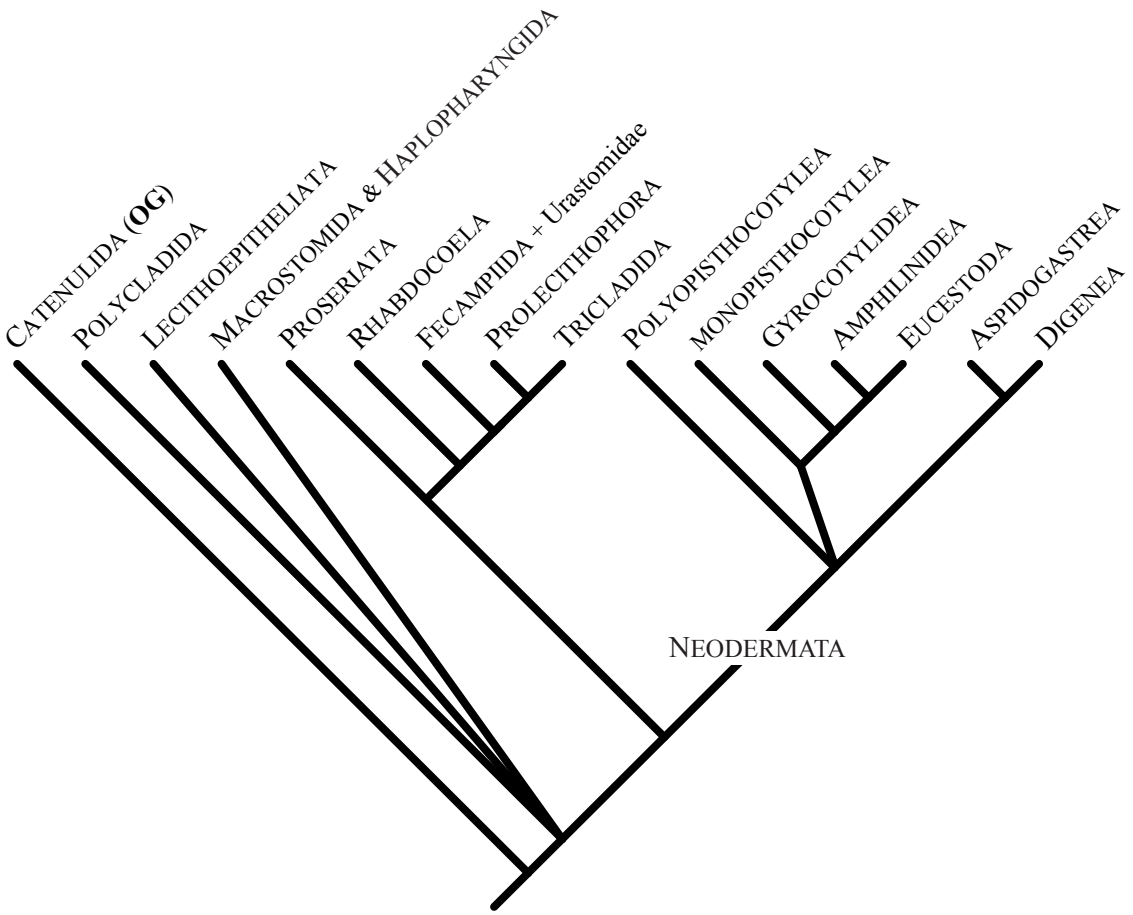


Figure 25.8 Littlewood & Olson [25.8.ill]

Appendix 25.1. Taxonomic listing of platyhelminth species analyzed and the GenBank/EMBL accession numbers of their complete SSU rDNA sequences. Accession numbers followed by '§' are new to this study.

CATENULIDA		
Stenostomidae	*Stenostomum leucops aquariorum*	AJ012519
	Suomina sp.	AJ012532
MACROSTOMIDA		
Macrostomidae	*Macrostomum tuba*	U70082
	Macrostomum tuba	D85092
Microstomidae	*Microstomum lineare*	U70081
	Microstomum lineare	D85091
HAPLOPHARYNGIDA		
Haplopharyngidae	*Haplopharynx rostratus*	AJ012511
LECITHOEPITHELIATA		
Prorhynchidae	*Geocentrophora sphyrocephala*	D85089
	Geocentrophora sp.	U70080
	Geocentrophora wagini	AJ012509
POLYCLADIDA		
Leptoplanidae	*Notoplana koreana*	D85097
	Notoplana australis	AJ228786
Planoceridae	*Planocera multitentaculata*	D83383/D17562
Discocoelidae	*Discocelis tigrina*	U70079
Pseudocerotidae	*Thysanozoon brocchii*	D85096
	Pseudoceros tritriatus	AJ228794
RHABDOCOELA		
Dalyellidae	*Microdalyellia rossi*	AJ012515
Graffilidae	*Graffila buccinicola*	AJ012521
Pterastericolidae	*Pterastericola australis*	AJ012518
Temnocephalidae	*Temnocephala* sp.	AJ012520
Trigonostomidae	*Mariplanella frisia*	AJ012514
Typhloplanidae	*Bothromesostoma* sp.	D85098
	Mesocastrada foremani	U70082
	Mesostoma lingua	AJ243682
Polycystidae	*Gyratrix hermaphroditus*	AJ012510
	Arrawaria sp.	AJ243677
Diascorhynchidae	*Diascorhynchus rubrus*	AJ012508
Karkinorhynchidae	*Cheliplana* cf. *orthocirra*	AJ012507
PROLECITHOPHORA		
Baicalarctiidae	*Baicalarctica gulo*	AJ287483§
	Friedmaniella karlingi	AJ287513§
	Friedmaniella sp. (rufula?)	AJ287512§
Pseudostomidae	*Pseudostomum gracilis*	AF065426
	Pseudostomum klostermanni	AF065424
	Pseudostomum quadrioculatum	AF065425
	Reisingeria hexaoculata	AF065426
Cylindrostomidae	*Cylindrostoma fingalianum*	AF051330
	Cylindrostoma gracilis	AF065416
Plagiostomidae	*Plagiostomum cinctum*	AF065418
	Plagiostomum vittatum	AF051331

	Plicastoma cuticulata	AF065422
	Vorticeros ijimai	D85094
Ulianinidae	*Ulianinia mollissima*	AF065427

TRICLADIDA

Procerodidae	*Ectoplana limuli*	D85088
	Procerodes littoralis	Z99950
Bdellouridae	*Bdelloura candida*	Z99947
Uterioporidae	*Uterioporus* sp.	AF013148
Geoplanidae	*Artioposthia triangulata*	AF033038
	Cenoplana caerulea	AF033040
	Australoplana sanguinea	AF033041
Bipaliidae	*Bipalium kewense*	AF033039
Rhynchodemidae	*Microplana nana*	AF033042
Planariidae	*Crenobia alpina*	M58345
	Polycelis nigra	AF013151
	Polycelis tenuis	Z99949
	Phagocata ullala	AF013149
	Phagocata sp.	AF013150
	Phagocata sibirica	AJ287559§
Dendrocoelidae	*Dendrocoelum lacteum*	M58346
	Dendrocoelopsis lactea	D85087
	Baikalobia guttata	Z99946
Dugesiidae	*Schmidtea mediterranea*	U31084
	Schmidtea polychroa	AF013152
	Romankenkius lidinosus	Z99951
	Cura pinguis	AF033043
	Dugesia subtentaculata	M58343
	Dugesia japonica	AF013153
	Girardia tigrina	AF013157

PROSERIATA

Archimonocelididae	Archimonocelidinae n.gen.sp.1	AJ270150
	Archimonocelis crucifera	AJ270151
	Archimonocelis staresoi	AJ270152
	Calviria solaris	AJ270153
Coelogynoporidae	*Cirrifera dumosa*	AJ270154
	Coelogynopora gynocotyla	AJ243679
	Vannuccia sp.	AJ270162
Monocelididae	*Archiloa rivularis*	U70077
	Monocelis lineata	U45961
Monotoplanidae	*Monotoplana* cf. *diorchis*	AJ270159
Otoplanidae	*Archotoplana holotricha*	AJ243676
	Monostichoplana filum	AJ270158
	Otoplana sp.	D85090
	Paratoplana renatae	AJ012517
	Xenotoplana acus	AJ270155
Unguiphora	*Nematoplana coelogynoporoides*	AJ012516
	Nematoplana sp.	AJ270160
	Polystyliphora novaehollandiae	AJ270161

FECAMPIIDA

Fecampiidae	*Kronborgia isopodicola*	AJ012513
'Fecampiid'	*Notentera ivanovi*	AJ287546§

'*TURBELLARIA*' INCERTAE SEDIS

Urastomidae	*Urastoma cyprinae*	U70086

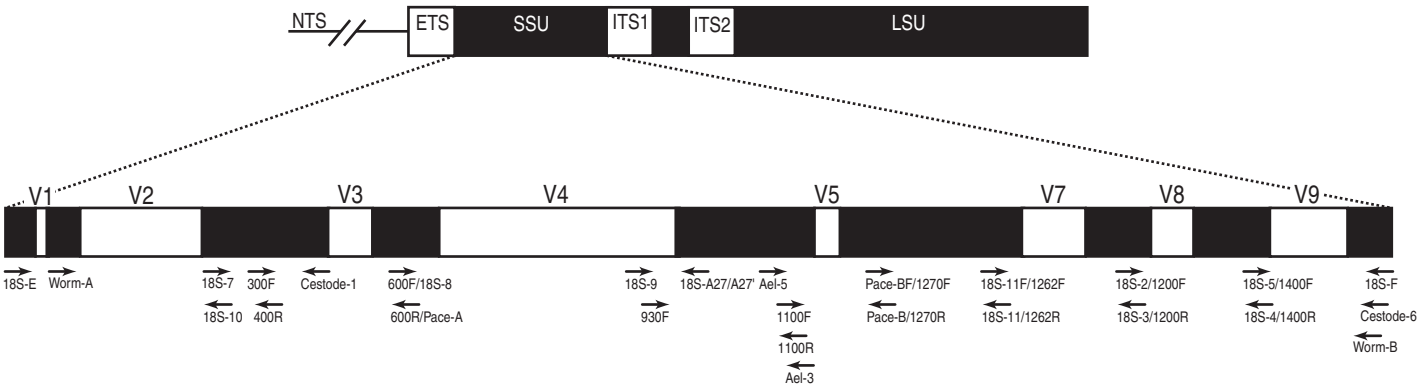
	Ichthyophaga sp.	AJ012512
MONOGENEA – MONOPISTHOCOTYLEA		
Monocotylidae	*Calicoctyle affinis*	AJ228777
	Dictyocotyle coeliaca	AJ287499§
	Troglocephalus rhinobatidis	AJ287585§
Capsalidae	*Encotyllabe chironemi*	AJ287506§
	Benedenia sp.	AJ287484§
	Capsala martinieri	AJ276423§
Gyrodactylidae	*Gyrodactylus salaris*	Z26942
Anoplodiscidae	*Anoplodiscus cirrusspiralis*	AJ287475§
Udonellidae	*Udonella caligorum*	AJ228796
Dactylogyridae	*Pseudohaliotrema sphincteroporus*	AJ287568§
	Pseudodactylogyrus sp.	AJ287567§
Pseudanonchidae	*Sundanonchus micropeltis*	AJ287579§
Pseudomurraytrematidae	*Pseudomurraytrema* sp.	AJ228793
Microbothriidae	*Leptocotyle minor*	AJ228784
MONOGENEA – POLYOPISTHOCOTYLEA		
Polystomatidae	*Neopolystoma spratti*	AJ228788
	Polystomoides malayi	AJ228792
Diclybothriidae	*Pseudohexabothrium taeniurae*	AJ228791
Plectanocotylidae	*Plectanocotyle gurnardi*	AJ287561§
Mazocraeidae	*Kuhnia scombri*	AJ228783
Allodiscocotylidae	*Metacamopia oligoplites*	AJ287538§
Neothoracocotylidae	*Paradawesia* sp.	AJ287555§
	Mexicotyle sp.	AJ287539§
Gotocotylidae	*Gotocotyla bivagina*	AJ276424§
	Gotocotyla secunda	AJ276425§
Diclidophoridae	*Diclidophora merlangi*	AJ228779
Discocotylidae	*Discocotyle sagittata*	AJ287504§
Diplozoidae	*Eudiplozoon nipponicum*	AJ287510§
Microcotylidae	*Bivagina pagrosomi*	AJ228775
	Cynoscionicola branquias	AJ287495§
	Microcotyle sebastis	AJ287540§
	Neomicrocotyle pacifica	AJ228787
Axinidae	*Zeuxapta seriolae*	AJ287589§
Heteraxinidae	*Probursata brasiliensis*	AJ276426
CESTODA – AMPHILINIDEA		
Amphilinidae	*Austramphilina elongata*	AJ287480§
	Gigantolina magna	AJ243681
Schizochoeridae	*Schizochoerus liguloideus*	AF124454
CESTODA – GYROCOTYLIDEA		
Gyrocotylidae	*Gyrocotyle urna*	AJ228782
	Gyrocotyle rugosa	AF124455
CESTODA – EUCESTODA		
Caryophyllaeidae	*Caryophyllaeus laticeps*	AJ287488§
	Hunterella nodulosa	AF124457
Hymenolepididae	*Hymenolepis diminuta*	AF124475
	Hymenolepis microstoma	AJ287525§
	Wardoides nyrocae	AJ287587§
Echinobothriidae	*Echinobothrium fautleyi*	AF124464
Macrobothrididae	*Macrobothridium* sp.	AF124463
Diphyllobothriidae	*Diphyllobothrium stemmacephalum*	AF124459
	Schistocephalus solidus	AF124460
Haplobothriidae	*Haplobothrium globuliforme*	AF124458

Lecanicephalidae	*Cephalobothrium cf aetobatidis*	AF124466
	Eniochobothrium gracile	AF124465
Tetragonocephalidae	*Tylocephalum* sp.	AJ287586§
Litobothriidae	*Litobothrium* sp.	AF124468
	Litobothrium amplifica	AF124467
Nippotaeniidae	*Amurotaenia decidua*	AF124474
	Nippotaenia mogurndae	AJ287545§
Monticellidae	*Gangesia parasiluri*	AJ287515§
Proteocephalidae	*Proteocephalus perplexus*	AF124472
Bothriocephalidae	*Bothriocephalus scorpii*	AJ228776
Triaenophoridae	*Abothrium gadi*	AJ228773
	Anchistrocephalus microcephalus	AJ287473§
	Eubothrium crassum	AJ287509§
Acrobothriidae	*Cyathocephalus truncatus*	AJ287493§
Spathebothriidae	*Spathebothrium simplex*	AF124456
Tetrabothriidae	*Tetrabothrius erostris*	AJ287581§
	Tetrabothrius forsteri	AF124473
	Tetrabothrius sp.	AJ287582§
Onchobothriidae	*Calliobothrium cf verticillatum*	AF124469
	Platybothrium auriculatum	AF124470
Dasyrhynchidae	*Dasyrhynchus pillersi*	AJ287496§
Gilquiniidae	*Gilquinia squali*	AJ287516§
Grillotiidae	*Grillotia erinaceus*	AJ228781
	Grillotia heronensis	AJ287519§
Hepatoxylidae	*Hepatoxylon* sp.	AF124462
Lacistorhynchidae	*Callitetrarhynchus gracilis*	AJ287487§
Otobothriidae	*Otobothrium dipsacum*	AJ287552§
Pterobothriidae	*Pterobothrium lintoni*	AJ287570§
Sphyriocephalidae	*Sphyriocephalus* sp.	AJ287576§
Tentaculariidae	*Tentacularia* sp.	AF124461
ASPIDOGASTREA		
Aspidogastridae	*Aspidogaster conchicola*	AJ287478§
	Lobatostoma manteri	L16911
Multicalycidae	*Multicalyx* sp.	AJ287532§
Multicotylidae	*Multicotyle purvisi*	AJ228785
Rugogastridae	*Rugogaster* sp.	AJ287573§
DIGENEA		
Accacoeliidae	*Accacoelium contortum*	AJ287472§
Acanthocolpidae	*Cableia pudica*	AJ287486§
	Stephanostomum baccatum	AJ287577§
Angiodictyidae	*Neohexangitrema zebrasomatis*	AJ287544§
	Hexangium sp.	AJ287522§
Apocreadiidae	*Homalometron synagris*	AJ287523§
	Neoapocreadium splendens	AJ287543§
Atractotrematidae	*Atractotrema sigani*	AJ287479§
Azygiidae	*Otodistomum cestoides*	AJ287553§
Bivesiculidae	*Bivesicula claviformis*	AJ287485§
	Paucivitellosus fragilis	AJ287557§
Brachycoelidae	*Mesocoelium* sp.	AJ287536§
Bucephalidae	*Prosorhynchoides gracilescens*	AJ228789
Bunocotylidae	*Opisthadena* sp.	AJ287549§
Campulidae	*Nasitrema globicephalae*	AJ004968
Cephalogonimidae	*Cephalogonimus retusus*	AJ287489§
Cryptogonimidae	*Mitotrema anthostomatum*	AJ287542§
Cyclocoelidae	*Cyclocoelum mutabile*	AJ287494§
Derogenidae	*Derogenes varicus*	AJ287511§
Dicrocoelidae	*Dicrocoelium dendriticum*	Y11236
Didymozoidae	*Didymozoon scombri*	AJ287500§

Diplodiscidae	*Diplodiscus subclavatus*	AJ287502§
Diplostomidae	*Diplostomum phoxini*	AJ287503§
Echinostomatidae	*Echinostoma caproni*	L06567
Enenteridae	Enenterid sp.1	AJ287507§
	Enenterid sp.2	AJ287508§
Fasciolidae	*Fasciola gigantica*	AJ011942
	Fasciola hepatica	AJ004969
	Fasciolopsis buski	L06668
Faustulidae	*Antorchis pomacanthi*	AJ287476§
	Bacciger lesteri	AJ287482§
	Trigonocryptus conus	AJ287584§
Fellodistomidae	*Fellodistomum fellis*	Z12601
	Tergestia laticollis	AJ287580§
	Steringophorus margolisi	AJ287578§
	Olssonium turneri	AJ287548§
Gorgoderidae	*Degeneria halosauri*	AJ287497§
	Gorgodera sp.	AJ287518§
	Xystretrum sp.	AJ287588§
Gyliauchenidae	*Robphildollfusium fractum*	AJ287571§
	Gyliauchen sp.	L06669
Haploporidae	*Pseudomegasolena ishigakiense*	AJ287569§
Haplosplanchnidae	*Hymenocotta mulli*	AJ287524§
	Schickhobalotrema sp.	AJ287574§
Hemiuridae	*Dinurus longisinus*	AJ287501§
	Lecithochirium caesionis	AJ287528§
	Lecithocladium excisum	AJ287529§
	Merlucciotrema praeclarum	AJ287535§
	Plerurus digitatus	AJ287562§
Heronimidae	*Heronimus mollis*	L14486
Heterophyidae	*Cryptocotyle lingua*	AJ287492§
	Haplorchoides sp.	AJ287521§
Lecithasteridae	*Lecithaster gibbosus*	AJ287527§
Lepocreadiidae	*Austroholorchis sprenti*	AJ287481§
	Lepidapedon rachion	Z12607
	Lepidapedon elongatum	Z12600
	Preptetos caballeroi	AJ287563§
	Tetracerasta blepta	L06670
Mesometridae	*Mesometra* sp.	AJ287537§
Microphallidae	*Levenseniella minuta*	AJ287531§
	Maritrema oocysta	AJ287534§
	Microphallus primas	AJ287541§
	unidentified	AJ001831
Monorchiidae	*Ancylocoelium typicum*	AJ287474§
	Provitellus turrum	AJ287566§
Nasitrematidae	*Zalophotrema hepaticum*	AJ224884
Notocotylidae	*Notocotylus* sp.	AJ287547§
Opecoelidae	*Gaevskajatrema halosauropsi*	AJ287514§
	Macvicaria macassarensis	AJ287533§
	Peracreadium idoneum	AJ287558§
Opisthorchiidae	*Opisthorchis viverrini*	X55357
Opistholebetidae	*Opistholebes amplicoelus*	AJ287550§
Orchipedidae	*Orchipedum tracheicola*	AJ287551§
Pachypsolidae	*Pachypsolus irroratus*	AJ287554§
Paramphistomidae	*Calicophoron calicophorum*	L06566
Paragonimidae	*Paragonimus westermani*	AJ287556§
Philophthalmidae	philophthalmid sp.	AJ287560§
Plagiorchiidae	*Glypthelmins quieta*	AJ287517§
	Haematolechus longiplexus	AJ287520§
	Rubenstrema exasperatum	AJ287572§
	Skrjabinoeces similis	AJ287575§

Sanguinicolidae	*Aporocotyle spinosicanalis*	AJ287477§
Schistosomatidae	*Schistosoma haematobium*	Z11976
	Schistosoma japonicum	Z11590
	Schistosoma mansoni	X53017
	Schistosoma spindale	Z11979
Sclerodistomidae	*Prosogonotrema bilabiatum*	AJ287565§
Strigeidae	*Ichthyocotylurus erraticus*	AJ287526§
Syncoelidae	*Copiatestes filiferus*	AJ287490§
Tandanicolidae	*Prosogonarium angelae*	AJ287564§
Transversotrematidae	*Crusziella formosa*	AJ287491§
	Transversotrema haasi	AJ287583§
Zoogonidae	*Deretrema nahaense*	AJ287498§
	Lepidophyllum steenstrupi	AJ287530§
	Zoogonoides viviparus	AJ287590§

Appendix 25.2a FIGURE LEGEND: Ribosomal array showing relative positions of primers for the SSU gene locus. Abbreviations: ETS, external transcribed spacer; ITS1-2, internal transcribed spacers; LSU, large subunit; NTS, non-transcribed spacer; SSU, small subunit; V1-9, variable domains.]


NTS
ETS
SSU
ITS1
ITS2
LSU
V1
V2
V3
V4
V5
V7
V8
V9
18S-E
Worm-A
18S-7
300F
Cestode-1
600F/18S-8
18S-9
18S-A27/A27'
Ael-5
Pace-BF/1270F
18S-11F/1262F
18S-2/1200F
18S-5/1400F
18S-F
18S-10
400R
600R/Pace-A
930F
1100F
Pace-B/1270R
18S-11/1262R
18S-3/1200R
18S-4/1400R
Cestode-6
1100R
Worm-B
Ael-3

Appendix 25.2b. SSU rDNA Primers. Conserved PCR/sequencing primers for the SSU rDNA gene used by the authors are listed below (format follows Simon *et al.* 1994) showing discrepancies observed among 22 platyhelminth exemplar sequences (listed below). Primer names and aliases are given followed by the direction of priming (→, 5'-3'; ←, 3'-5'). The following line shows the size, definition, and annealing location of the primer based on the complete 1,932 bp SSU sequence of *Pseudomurraytrema* sp. [GenBank No. AJ228793].

ID:	**Classification (exemplar taxon):**
"Turbellaria"	
Cate	Catenulida (*Stenostomum leucops*)
Macr	Macrostomida (*Macrostomum tuba*)
Leci	Lecithoepitheliata (*Geocentrophora wagini*)
Poly	Polycladida (*Discocelis tigrina*)
Pros	Proseriata (*Polystyliphora novaehollandiae*)
Kaly	Rhabdocoela: Kalyptorhynchia (*Cheliplana orthocirra*)
Daly	Rhabdocoela: Dalyelliida (*Graffila buccinicola*)
Typh	Rhabdocoela: Typhloplanida (*Mesocastrada foremani*)
Note	Fecampiida (*Notentera ivanovi*)
Prol	Prolecithophora (*Cylindrostoma gracilis*)
Tric	Tricladida (*Phagocata ullala*)
Neodermata	
Monp	Monogenea: Polypisthocotylidea (*Neomicrocotyle pacifica*)
Monm	Monogenea: Monopisthocotylidea (*Dictyocotyle coeliaca*)
Gyro	Cestoda: Gyrocotylidea (*Gyrocotyle urna*)
Amph	Cestoda: Amphilinidea (*Gigantolina magna*)
Spat	Eucestoda: Spathebothriidea (*Spathebothrium simplex*)
Tetr	Eucestoda: Tetraphyllidea (*Calliobothrium* cf. *verticillatum*)
Cycl	Eucestoda: Cyclophyllidea (*Hymenolepis diminuta*)
Aspi	Aspidobothrea (*Aspidogaster conchicola*)
Schi	Digenea: Schistosomatidae (*Schistosoma mansoni*)
Fasc	Digenea: Fasciolidae (*Fasciolopsis buski*)
Hemi	Digenea: Hemiuridae (*Merlucciotrema praeclarum*)

18S-E (alias 18S-A) (→)

```
(35mer)     5′ CCGAATTCGTCGACAACCTGGTTGATCCTGCCAGT 3′
```

Comments: It is not informative to check this 'universal' 5'-end primer as it was itself used to amplify the SSU gene in a majority of the taxa above (and was thus incorporated into the PCR products sequenced).

WormA (→)

```
(21mer)     5′ GCGAATGGCTCATTAAATCAG 3′          [67-87]
Leci           .....A.........G.....
Daly           ..AT...........T.A...
Tric           ...G...........T.A...
Monp           A...................
```

18S-7 (→)

```
(22mer)     5′ GCCCTATCAACTGTCGATGGTA 3′         [295-316]
Cate           .A..........A........
Macr           .A..........A........
Leci           .A...................
Poly           ............TA..T.....
Pros           ............TA........
```

```
Kaly            .AA.........A.G..A....
Daly            ............--A......G
Typh            .A.......T...........G
Note            ............CA.T.......
Prol            .A.......G..A....C....
Tric            .A..........T.........
Monp            ............TA........
Gyro            ............T.........
Amph            ............A.........
Spat            ............T.........
Tetr            ............T.........
Cycl            ............T.........
Aspi            ............TA........
Schi            ..........T.T--.T.....
Fasc            ..........T.T.........
Hemi            ..........T...........
```

18S-10 (←)

```
(22mer)     5′ TACCATCGACAGTTGATAGGGC 3′        [316-295]
```

Comments: Reverse complement of 18S-7 above.

300F (→)

```
(17mer)     5′ AGGGTTCGATTCCGGAG 3′             [358-374]
Cate            .........T.......
Macr            .........C.......
Typh            ..T..............
```

400R (alias 300R) (←)

```
(18mer)     5′ TCAGGCTCCCTCTCCGGA 3′            [385-368]
Cate            .......-.........
Poly            ...T..............
Kaly            .A................
Daly            .A................
Typh            .A................
Note            .......AA.........
```

Comments: 3’ end partially overlaps with 300F.

Cestode-1 (←)

```
(20mer)     5′ TTTTTCG-TCACTACCTCCCC 3′         [463-444]
Cate            .......-...........T.
Macr            .......-...........T.
Poly            .......T............
Kaly            .......-...........T.
Daly            ..C.CT.T.GG...TT..A.T
Typh            .......-...........T.
Prol            .....T.-............
Tric            ...A.T.-..........A..
Monm            ...G..T-.T.........T.
Amph            .......-....C........
Spat            .......-....C........
Tetr            .......-....C........
Cycl            .......-....C........
Hemi            .......-..........A..
```

600F /

18S-8 (→)

```
(18mer)     5′ GGTGCCAGCMGCCGCGGT          3′   [549-566]
(20mer)     5′        GCAGCCGCGGTAATTCCAGC 3′   [556-575]
Leci           ..............-.........
Poly           ..............-.........
Kaly           ..................C......
Monp           ..................C......
Gyro           ..................C......
Amph           ..................C......
Spat           ..................C......
Tetr           ..................C......
Cycl           ..............-...C......
Aspi           ..................C......
Schi           ..................C......
Fasc           ..................C......
Hemi           ..................C......
```

600R (←)

```
(18mer)     5′ ACCGCGGCKGCTGGCACC 3′           [566-549]
```

Comments: Reverse complement of 600F.

Pace-A (←)

```
(18mer)     5′ GTGTTACCGCGGCTGCTG 3′           [571-554]
Cate           .AA.............
Macr           .AA.............
Leci           .AA...-.........
Poly           .AA.............
Pros           .AA.............
Kaly           .A..............
Daly           .AA.............
Typh           .AA.............
Note           .AA.............
Prol           .AA.............
Tric           .AA.............
Monp           .A..............
Monm           .A..............
Gyro           .A..............
Amph           .A..............
Spat           .A..............
Tetr           .A..............
Cycl           .A....-.........
Aspi           .A..............
Schi           .A..............
Fasc           .A..............
Hemi           .A..............
```

18S-9 (→)

```
(18mer)     5′ TTTGAGTGCTCAAAGCAG 3′           [863-880]
Cate           ..A.............
Macr           ..A.............
Leci           ..A.......T......A
Poly           ..G..........T...
Pros           ..A.............
Kaly           ..A.............
Daly           ..A.............
Typh           ..AA............
```

```
Note            ..A.......T.......
Prol            ..G...............
Tric            ..A.......T.......
Gyro            ..............C...
Amph            ..............A...
Spat            ..............C...
Tetr            ..............C...
Cycl            ..............T...
```

930F (→)

```
(20mer)     5′ GCATGGAATAATGGAATAGG 3′          [904-923]
Leci            ............A.......
Poly            ................A...
Daly            ................C...
Note            ............A......
Prol            ............A....A.
Tric            ............A.......
Monp            ............A.......
Amph            ............A.......
Schi            ............A......
Hemi            ............A......
```

18S-A27 (←)

```
(21mer)     5′ CCATACAAATGCCCCCGTCTG 3′         [997-977]
Cate            AA....G..........A.C.
Macr            AA....G..........A.C.
Leci            .A....G..........C...
Poly            .A....G.............
Kaly            .......G............
Daly            ......TTT........G.A.
Typh            ................G.A.
Note            G......-...-........
Prol            GT.....G..........A.
Tric            G......T.........G.A.
Gyro            .........C.T.........
Amph            .........C.T.....C.C.
Spat            .........C.T.....C...
Tetr            .........C.T.....C...
Cycl            .........C.T.....C...
Hemi            ................T..

(A27′)          .........C.T.....C...
```

Comments: A27′ (Olson and Caira 1999) was a modification to match eucestodes.

Ael-5 (→)

```
(20mer)     5′ TGTTTTCATTGACCATGAGC 3′          [1063-1082]
Cate            C..C.C....A.T..A..A.
Macr            C..C.C....A.T..A..A.
Leci            .....-.G..A.T..A..A.
Poly            .....C....A.T..A..A.
Pros            ..........A.T..A..A.
Kaly            ..........A.T..A..A.
Daly            ..C.C.....A.T..A..A.
Typh            ...C......A.T..A..A.
Note            ...C......A.T..A..A.
Prol            ..........A.T..A..A.
Tric            ..........A.T..A..A.
```

```
Monp            ............A.A....
Monm            ..........T....G....
Tetr            ...........G........
Cycl            ...........G........
Aspi            ............T..G....
Schi            ............T..G....
Fasc            ............T.T.....
Hemi            ............T.TG....
```

Comments: Design (DTJL) based on the sequence of *Austramphilina elongata* (Cestoda: Amphilinidea).

1100F (→)

```
(19mer)     5′ CAGAGATTCGAAGACGATC 3′           [1089-1107]
Cate            .....G.............
Macr            .....G.............
Leci            .....G......NN....T
Poly            .....G.............
Pros            .....G.............
Kaly            .....G........G....
Daly            T....G.............
Typh            .....G.............
Note            .....G.............
Prol            .....G.............
Tric            .....GA............
Monp            .....G.............
Monm            .....G.............
Gyro            G....GC............
Amph            .....GC............
Spat            .....GC............
Tetr            .....GC............
Cycl            .....GC............
Aspi            .....G......T......
Schi            .....T.............
Fasc            .....G.............
Hemi            .....GA............
```

1100R (←)

```
(18mer)     5′ GATCGTCTTCGAACCTCTG 3′           [1107-1089]
```

Comments: Reverse complement of 1100F.

Ael-3 (←)

```
(20mer)     5′ GTATCTGATCGTCTTCGAGC 3′          [1113-1094]
Cate            ..G..............A.
Macr            ..G..............A.
Leci            ......A....NN.....A.
Poly            ..................A.
Pros            ..................A.
Kaly            ..........C.......A.
Daly            ..................A.
Typh            ..................A.
Note            ..................A.
Prol            ..................A.
Tric            ..................T.
Monp            ..................A.
Monm            ..................A.
Aspi            ............A.....A.
Schi            ..................AA
```

```
Fasc            .................A.
Hemi            .................T.
```

Comments: Design (DTJL) based on the sequence of *Austramphilina elongata* (Cestoda: Amphilinidea).

Pace-B /
1270R (←)

```
(20mer)     5′ CCGTCAATTCCTTTAAGTTT 3′          [1260-1241]
(18mer)     5′ CCGTCAATTCCTTTAAGT   3′          [1260-1243]
Leci            ...C..............
```

Comments: Highly conserved reverse primer.

Pace-BF /
1270F (→)

```
(20mer)     5′ AAACTTAAAGGAATTGACGG 3′          [1241-1260]
(18mer)     5′   ACTTAAAGGAATTGACGG 3′          [1243-1260]
```

Comments: Reverse complements of Pace-B/1270R.

18S-11 /
1262R (alias 1055R) (←)

```
(21mer)     5′ AACGGCCATGCACCACCACCC 3′         [1393-1373]
(15mer)     5′   CGGCCATGCACCACC     3′         [1391-1377]
Cate            .................T..
Macr            .................T..
Daly            .................TT.
Note            .....A............
Monp            .................A..
Monm            ...............T.A..
Gyro            .................A..
Amph            .................A..
Spat            .................A..
Tetr            .................A..
Cycl            .................A..
Aspi            .................A..
Fasc            .................A..
Hemi            .................T..
```

18S-11F /
1262F (alias 1055F) (→)

```
(21mer)     5′ GGGTGGTGGTGCATGGCCGTT 3′         [1373-1393]
(15mer)     5′     GGTGGTGCATGGCCG   3′         [1377-1391]
```

Comments: Reverse complements of 18S-11/1262R.

18S-2 /
1200F (→)

```
(25mer)     5′ ATAACAGGTCTGTGATGCCCTTAGA 3′     [1579-1603]
(16mer)     5′     CAGGTCTGTGATGCCC      3′     [1583-1598]
Tric            ......................A.
Hemi            ..................C...
```

18S-3 /
1200R (←)

```
(25mer)     5′ TCTAAGGGCATCACAGACCTGTTAT 3′     [1603-1579]
(16mer)     5′      GGGCATCACAGACCTG     3′     [1598-1583]
```

Comments: Reverse complements of 18S-2 / 1200F.

18S-5 /
1400F (→)

```
(25mer)     5′ CCCTTTGTACACACCGCCCGTCGCT 3′     [1779-1807]
(17mer)     5′      TGYACACACCGCCCGTC    3′     [1788-1804]
```

18S-4 /
1400R (←)

```
(19mer)     5′ AGCGACGGGCGGTGTGTAC 3′           [1807-1789]
(15mer)     5′     ACGGGCGGTGTGTAC 3′           [1803-1789]
```

Comments: Truncated reverse complements of 18S-5 / 1400F.

Cestode-6 /
WormB (←)

```
(20mer)     5′ ACGGAAACCTTGTTACGACT          3′ [1932-1913]
(21mer)     5′          CTTGTTACGACTTTTACTTCC 3′ [1924-1904]
```

Comments: 3’ end primers designed to avoid misannealing of 18S-F in platyhelminth taxa.

18S-F (alias 18S-B) (←)

```
(30mer)     5′ CCAGCTTGATCCTTCTGCAGGTTCACCTAC 3′
```

Comments: ‘Universal’ 3’-end primer. Mis-annealing in cestode taxa results in a ~400 bp PCR product when used in conjunction with 18S-E.