MOLECULAR PHYLOGENY OF THE TREMATODE FAMILIES DIPLOSTOMIDAE AND STRIGEIDAE

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Abstract

Molecular Phylogeny of the Trematode Families

Diplostomidae and Strigeidae

Angela Rose Lapierre

Evolutionary relationships within the Strigeidae and Diplostomidae (Digenea: Diplostomoidea), which are cosmopolitan parasites of vertebrates, are poorly understood. In this study, the phylogenetic relationships of genera within these groups were studied using full small (SSU), partial large (LSU), and full internal transcribed spacer regions 1 and 2 (ITS) sequences of ribosomal DNA and partial sequences of cytochrome oxidase I (COI) from mitochondrial DNA. Sequences from nine diplostomid genera (18 species) and five strigeid genera (8 species) were analyzed using maximum parsimony and maximum likelihood methods. Markers were analyzed independently and in total evidence combinations and all molecular topologies indicated paraphyletic relationships. A maximum likelihood analysis of concatenated sequences of SSU, LSU, and COI produced a tree concordant with the fewest evolutionary changes based on a matrix of 32 morphological and life-history characters. The Strigeidae and Diplostomidae form two clades. A group comprising the diplostomids *Diplostomum*, *Tylodelphys*, *Alaria*, *Fibricola* and *Hysteromorpha* was basal to a paraphyletic clade in which the strigeids *Apharyngostrigea*, *Apatemon* and *Cotylurus,* were separated from other strigeids, *Ichthyocotylurus* and *Cardiocephaloides* by

the diplostomids *Ornithodiplostomum*, *Posthodiplostomum*, *Uvulifer* and *Bolbophorus*. Metacercariae of the two clades differ in type, encystment and limebody enclosure; however no other characters differed in the two strigeid groups. These results provide further evidence that the classification of these groups needs to be reassessed.

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Contributions of authors

This thesis has been prepared as a manuscript for submission for publication. Dr. Sean Locke contributed to the collection and identification of specimens used in this study. Dr. Sean Locke, Dr. Daniel McLaughlin and Dr. David Marcogliese contributed to the study design and manuscript preparation. The study was funded by grants to Drs. McLaughlin and Marcogliese.

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General introduction and literature review

Overview of the Digenea

Members of the Class Trematoda (Phylum Platyhelminthes) are obligate parasites. The Class is comprised of two subclasses, the Aspidogastrea, which infect marine and freshwater mollusks, fish and freshwater turtles, and the Subclass Digenea (Gibson, 2002a). The digeneans are the most speciose group of the parasitic worms (Cribb *et al*., 2001) and adult stages can be found in all classes of vertebrates (Gibson, 2002b). They have complex life cycles that require at least one intermediate host for transmission. These are normally gastropods but some digeneans use bivalves. Most digeneans also require a second intermediate host (a mollusk, annelid, arthropod or vertebrate depending on the parasite) for transmission to the vertebrate host where they reach sexual maturity.

Historically, evolutionary relationships in the Digenea were inferred from classification systems based on interpretation of morphological data. La Rue (1957) provided a comprehensive review of the development of the classification among digeneans beginning with the earliest work based on external morphology to the incorporation of internal morphology and eventually larval morphology characteristics as life cycle information became available. La Rue (1957) incorporated these features into a classification that formed the basis of the systems in use today. About the same time, Yamaguti (1958) proposed a similar classification based on morphology but also included the taxomomic affiliation of the definitive host. Subsequent

major works (e.g. Yamaguti, 1971; Gibson, 1996; Gibson *et al*., 2002; Jones *et al*., 2005; Bray *et al*., 2008) were also based on morphology and life history data. Classification systems differed slightly in these studies and in each case, the taxonomic relationships were based on the particular author's interpretation of morphological and life history data.

Brooks *et al*. (1985), Pearson (1992) and Brooks and McLennan (1993) used a cladistic approach to assess the relationships among the Digenea. Brooks *et al*. (1985) presented an updated classification of the Digenea based on adult and larval morphology and proposed the erection of two new orders and three new suborders. Pearson (1992) disagreed on the homology of some characteristics used by Brooks *et al*. (1985) and, following an analysis of a revised dataset, concluded that relationships within a number of families remained unresolved. Brooks and McLennan (1993) supplemented the database used by Brooks *et al*. (1985) and re-analyzed it. They proposed a more detailed classification that differed slightly from the earlier one with one new order, one new infra-suborder, two new families and two new subfamilies.

More recently, morphology based cladistic analyses have given way to phylogenetic studies based on DNA, sometimes in combination with morphological and biological characteristics (Cribb *et al*., 2001; Olson *et al*. 2003). Cribb *et al*. (2001) combined sequences of exemplars of 55 digenean families and 56 morphological characteristics to produce a reasonably well-resolved phylogenetic tree for the Digenea. Olson *et al*. (2003) expanded this

work to include 77 digenean families. In the process, Olson *et al*. (2003) found that a number of traditionally recognized higher taxa included "non-natural" groupings necessitating a number of emendations, revisions to certain taxa, erection of new taxa and the proposal of a new classification system. The observations by Pearson (1992) and, in particular, those by Olson *et al*. (2003) are significant because they bring into question the monophyly of several digenean groups at lower taxonomic levels. One such group in which the family relationships are being questioned is the Diplostomoidea, one of three superfamilies that form the basal clade of digeneans (Olson *et al*., 2003).

The Diplostomoidea: Diplostomidae and Strigeidae

Members of the Diplostomoidea differ morphologically from other digeneans by the presence of a unique holdfast organ (frequently referred to as the tribocytic organ) situated just posterior to the ventral sucker (Figures 1 and 2). In the most recent work on this superfamily, Niewiadomska (2002c) divides the Diplostomoidea into six families, two of which, the Diplostomidae and the Strigeidae, are considered here. Adult diplostomids and strigeids are mainly parasites of birds but species of a few genera infect mammals. Members of both families have distinctly bipartite bodies that consist of an anterior section, which includes the holdfast organ, and a posterior section that contains the hermaphroditic reproductive system. One part of the female reproductive system, the vitellaria, may extend into the forebody. The families are distinguished based on the shape of the forebody

(cup-shaped or bulbous versus foliate) and the shape of the holdfast organ (bilobed versus round or elongate with median slit) (Figures 1 and 2) (Niewiadomska, 2002a, 2002b, 2002c). Members of both families share similar three host life cycle patterns but use different species of gastropods as their first intermediate hosts. Cercariae are produced asexually in the snail host, emerge and penetrate the second intermediate host, which may be a snail, leech, fish or frog depending on the parasite, and develop into one of three morphologically distinct metacercarial types. These include the tetracotyle, neascus and diplostomulum larvae, each of which is a conserved characteristic at the generic level (Niewiadomska, 2002c) (Figure 3). In some diplostomids, e.g. *Alaria*, a mesocercariae (a developmental stage between the cercariae and the metacercariae) is also present.

Two monographs by Dubois (Strigeidae: 1968; Diplostomidae: 1970) laid the foundation for the present classification of diplostomids and strigeids (e.g., Shoop, 1989; Niewiadomska, 2002a, 2002b). Dubois (1968, 1970) provided detailed accounts of each group at all taxonomic levels, including descriptions, range of all known final and larval stage hosts and geographic distributions. Dubois (1968, 1970) inferred monophyly of the Diplostomidae and Strigeidae in his classification based on adult characteristics: the anterior segment, holdfast organ shape and on larval type and morphology. But, he also considered specificity for the definitive host as an important diagnostic character at the level of subfamily.

Dubois (1968) further divided the Strigeidae into two subfamilies, the Strigeinae and Duboisiellinae, based on the distribution of the vitellaria and final host. The Strigeinae have vitellaria in both segments and infect birds; while the Duboisiellinae have vitellaria restricted to their hindbody and infect mammals. The Diplostomidae is separated into two subfamilies, the Diplostomatinae and Alariinae, based on distribution of the vitellaria, morphology of the tribocytic organ and final host (Dubois, 1970). The Diplostomatinae have vitellaria distributed throughout their body, a small to medium size tribocytic organ and parasitize birds. The Alariinae have vitellaria confined to the forebody, a massive tribocytic organ and parasitize mammals. Within the Diplostomatinae, Dubois (1970) separated the genera among three tribes, the Diplostomatini, Crassiphialini and Codonocephalini, distinguished based on the distribution of the vitellaria and the occurrence of a progenetic (sexually mature) metacercariae in the life cycle of the Codonocephalini.

Niewiadomska (2002a, 2002b) retained much of the classification proposed for the Strigeidae by Dubois (1968) but made several changes to the Diplostomidae (Dubois, 1970). She raised two tribes: Crassiphialinae and Codonocephalinae originally proposed by Dubois (1970) to the rank of subfamily and included metacercarial type as a key diagnostic characteristic that resulted in rearrangements in the placement of some genera among the families. The nomenclature proposed by Niewiadomska (2002a, 2002b) is used throughout this thesis unless otherwise stated.

Figure 1. General external and internal morphology and structure of the genital system of a member of the Strigeidae taken from: Niewiadomska, K. 2002. Family Strigeidae Railliet, 1919. *In* Gibson, D.I., A. Jones and R.A. Bray (eds). *Keys to the Trematoda*. Volume 1. The Natural History Museum, London, UK. Pages 161 (reproductive organs) and 234 (adult). Labels for external and internal morphology have been added.

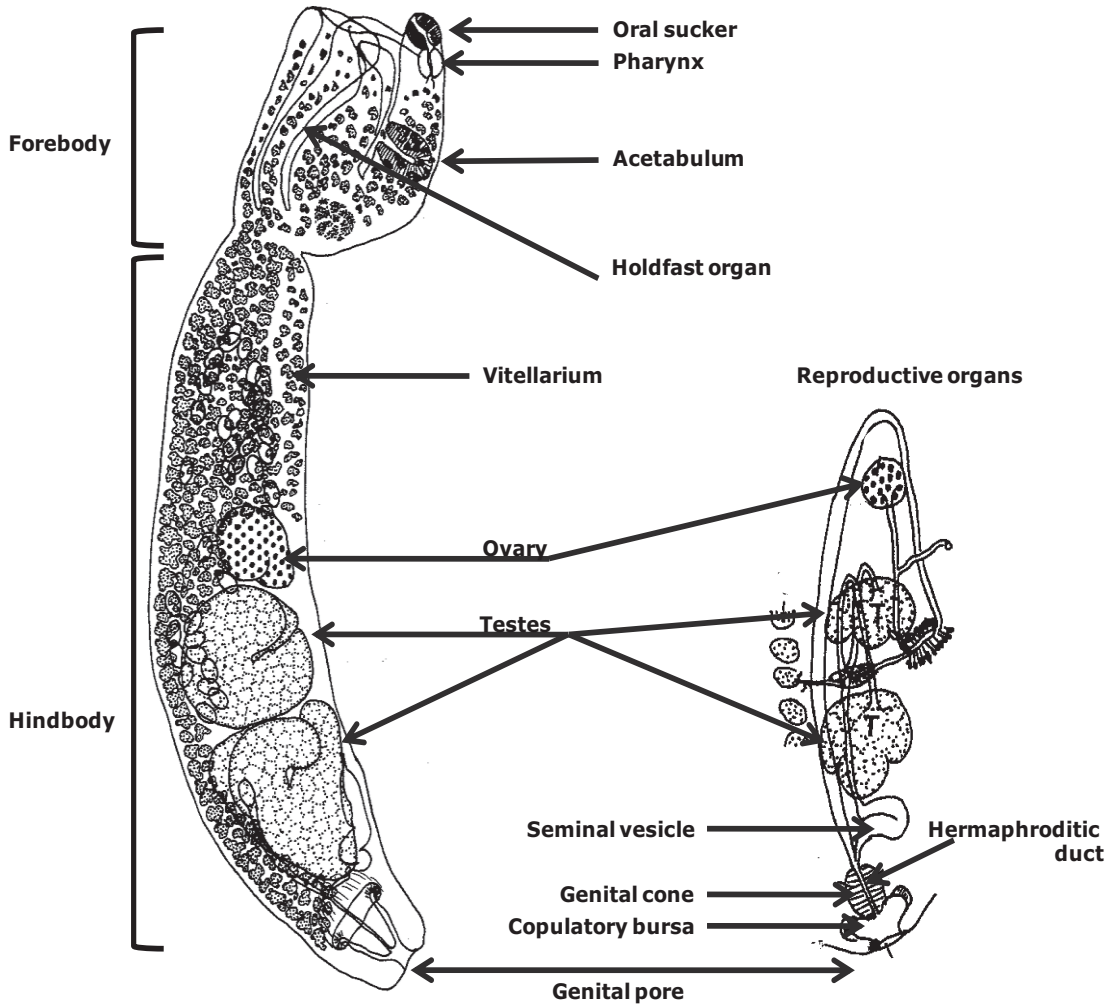


Figure 2. General external and internal morphology and structure of the genital system of a member the Diplostomidae taken from: Niewiadomska, K. 2002. Family Diplostomidae Poirier, 1886. *In* Gibson, D.I., A. Jones and R.A. Bray (eds). *Keys to the Trematoda*. Volume 1. The Natural History Museum, London, UK. Page 161. Labels for external and internal morphology have been added.

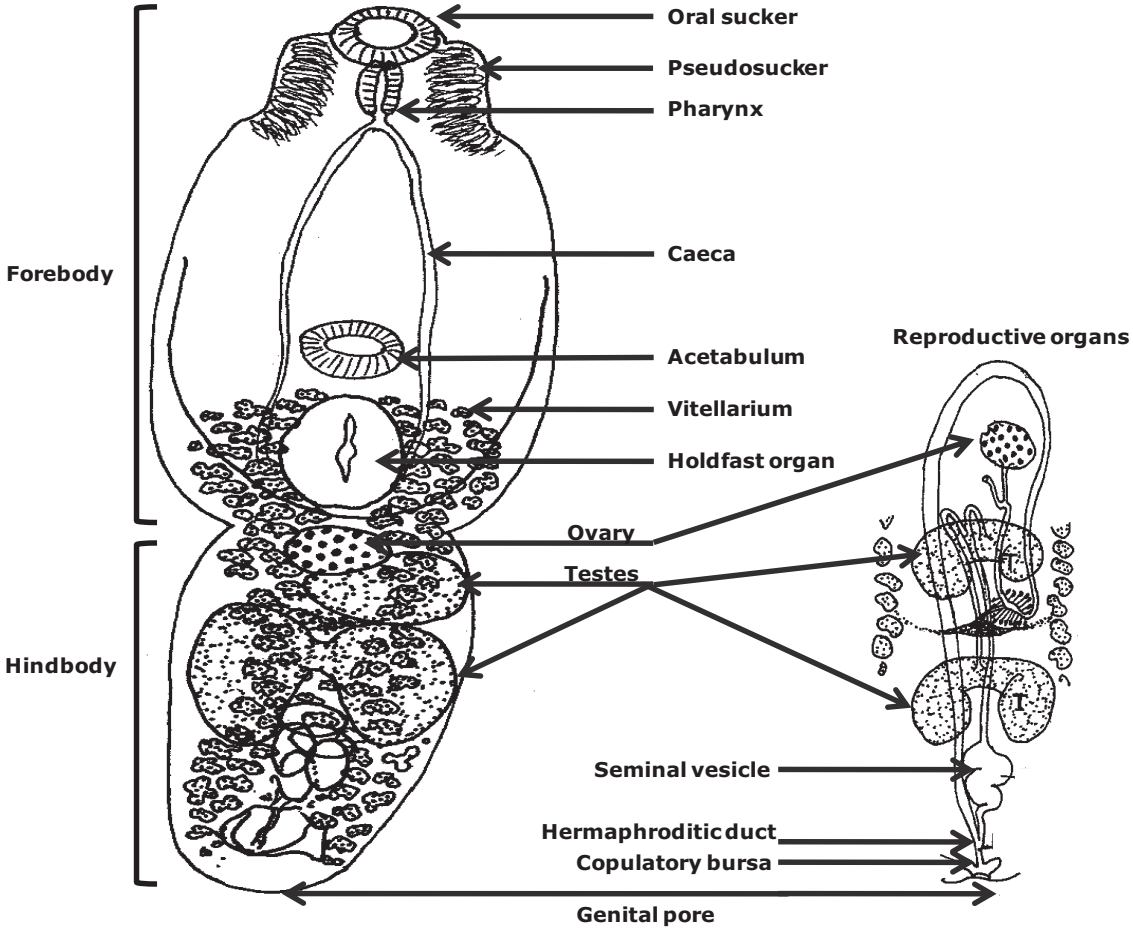
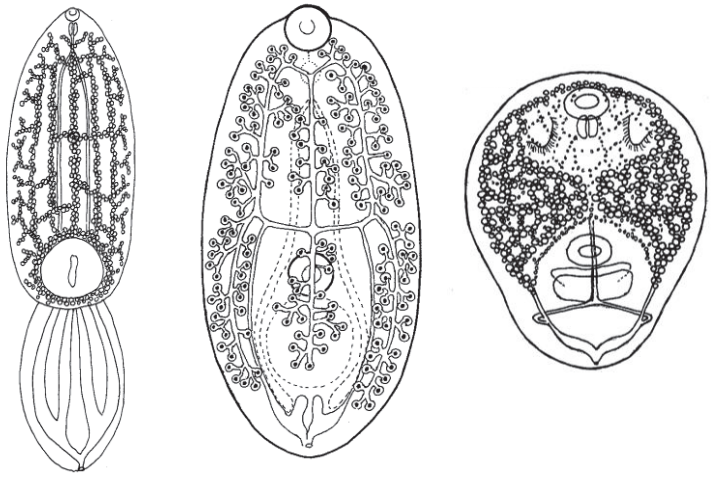


Figure 3. Metacercarial types in the Diplostomidae and Strigeidae taken from Niewiadomska, K. 2002. Superfamily Diplostomoidea Poirier, 1886. *In* Gibson, D.I., A. Jones and R.A. Bray (eds). *Keys to the Trematoda*. Volume 1. The Natural History Museum, London, UK. Page 163.

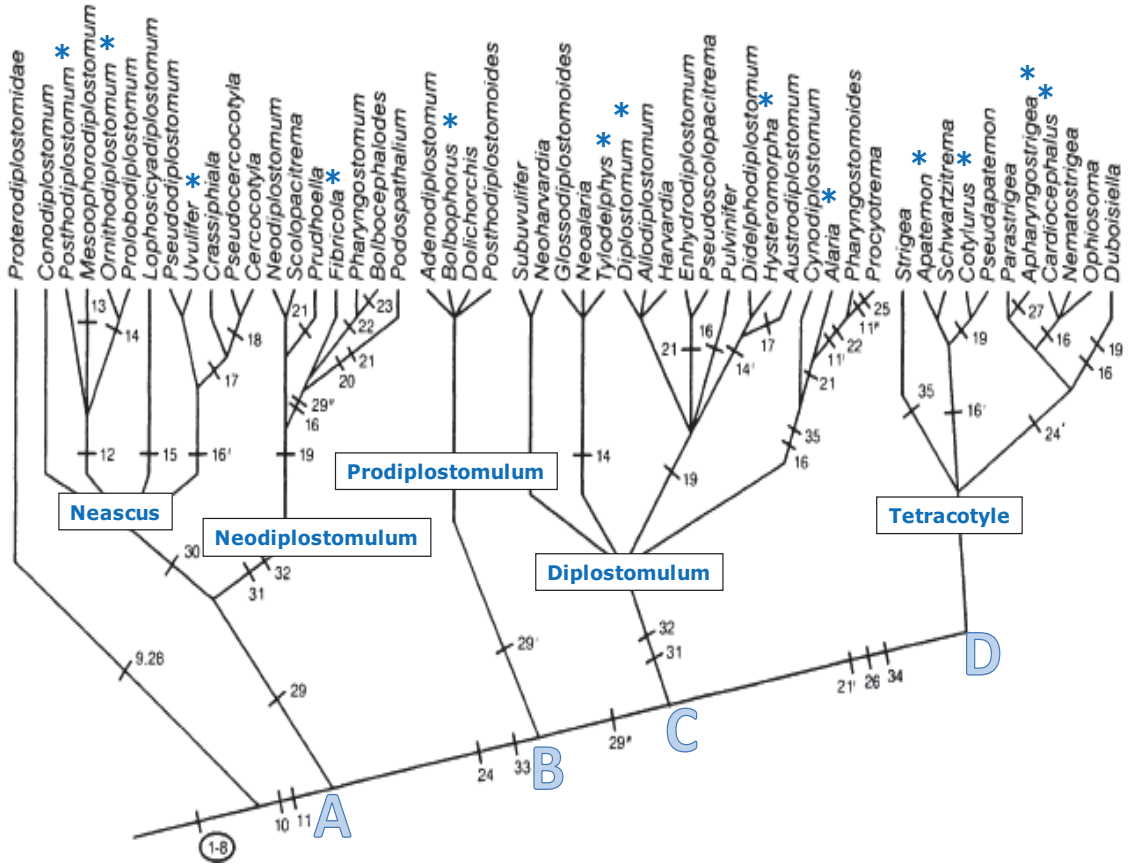


A. Neascus **B.** Diplostomulum **C.** Tetracotyle

Apart from works by Dubois (1968, 1970) and Niewiadomska (2002a, 2002b), few studies have dealt with relationships among and within the Strigeidae and Diplostomidae in any detail. The cladistic study by Shoop (1989) remains the exception. Shoop's (1989) analysis included 35 adult, larval and host characteristics of 34 of 41 nominal Diplostomidae and 11 of 13 nominal Strigeidae, one genus belonging to the Bolbocephalodidae and one genus belonging to the Proterodiplostomidae that infects reptiles. The host characteristics included infection site in the final host and whether the final host is endothermic or ectothermic. He also recognized four different metacercarial types (neascus, neodiplostomulum, diplostomulum, tetracotyle) and defined a fifth type (prodiplostomulum). Shoop (1989)

distinguished the neodiplostomulum larva which lacks pseudosuckers as a separate metacercarial type from the diplostomulum which does possesses them. He also considered the prodiplostomulum as a unique metacercariae type. The difference is based on the structure of the paranephridial plexus with an intermediate morphology between the neascus and the diplostomulum. Shoop's (1989) analysis indicated that the Strigeidae is monophyletic but the Diplostomidae is paraphyletic based on their metacercarial types (Figure 4). In Shoop's (1989) classification (Figure 4), the Diplostomidae were split into three clades. He erected two new families, the Neodiplostomidae (branch A, neascus and neodiplostomulum), Bolbophoridae (branch B, prodiplostomulum), and retained the Diplostomidae (branch C, diplostomulum) and the Strigeidae forming the last branch (branch D, tetracotyle).

Figure 4. Shoop's (1989) hypothesis based on a cladistic analysis of 51 genera in the Diplostomidae and Strigeidae with the Proterodiplostomidae as the outgroup. Adapted from Shoop, W.L. 1989. Systematic Analysis of the Diplstomidae and Strigeidae (Trematoda). *The Journal of Parasitology* 75: 21-32. Branches with metacercarial types recognized by Shoop (1989) have been identified with appropriate labels. Letters A, B, C, D indicate the main clades Shoop (1989) proposes as monophyletic families. The genera that are represented within this study are indicated with an (*) above the taxon name (as well as *Ichthyocotylurus*, which was not used in Shoop (1989).



Brooks and McLennan (1993) found support for Shoop's (1989) proposal and accepted his division of the Diplostomidae into three families. A cladistic analysis of the Strigeidae by Zarzornova and Sysoev (1993) found different internal relationships among the strigeids in comparison to Shoop (1989) and Brooks and McLennan (1993). They retained the monophyly of the family, but proposed *Pseudoapatemon* to be raised to subfamily status with the remaining genera splitting into two main clades supported by differences in the structures of the copulatory organ.

Niewiadomska (2002c) acknowledged that the evolutionary relationships within the Diplostomoidea as a whole are unclear, but retained the Diplostomidae as a monophyletic family. She cited a need for more data on the life cycles, better defined metacercarial types, and better morphological data on both the cercarial and metacercarial stages.

Most recently, Olson *et al*. (2003) illustrated a nesting of two diplostomid (*Diplostomum* and *Alaria*) within three strigeid (*Apharyngostrigea*, *Cardiocephaloides* and *Ichthyocotylurus*) representatives in a molecular study of the Digenea. Overall, a number of conflicting conclusions regarding the monophyly of each family have emerged from studies based on morphological classification (La Rue, 1957; Dubois 1968, 1970; Cable, 1974; Niewiadomska, 2002a, 2002b), cladistics (Brooks *et al*., 1985; Shoop, 1989; Brooks and McLennan, 1993) and molecular data (Olson *et al*., 2003). These hypotheses lend themselves well to a more exhaustive molecular analysis based on a larger number of taxa.

Molecular markers and methods

The advent of molecular tools provided methods for assessing phylogenetic relationships that were independent of morphology. The greatest advantage of using molecular methods is the extent of the dataset available (Hillis, 1987). This approach revolutionized the study of phylogenies (e.g. see reviews of phylogenies pertaining to vertebrates: Meyer and Zardoya, 2003; Digenea: Olson and Tkach, 2005; and Hymenoptera: Weirauch and Schuh, 2011). Molecular phylogenies examining the higher relationships within the Digenea have been based on sequences of the nuclear ribosomal DNA gene (rDNA) (Cribb *et al*., 2001; Olson *et al*., 2003).

The rDNA gene of eukaryotes is uniquely well suited for the examination of systematic questions at many taxonomic levels. This is due to presence of regions within the same gene that evolve at different rates along with an abundance of genetic material in the hundreds of tandem repeats (Nolan and Cribb, 2005). Each gene is flanked on both ends by a non-transcribed region (5’ and 3’-NTS) and is comprised of three rRNA coding regions (small [SSU], 5.8 and large [LSU] subunits) interspaced with two non-coding internal transcribed spacer regions (ITS1 and ITS2). Each coding and non-coding region of the transcription unit evolves at a different rate (i.e. SSU < 5.8S < LSU < ITS1 and ITS2) (Blair *et al*., 1996).

Depending on the systematic question, different regions will be more useful than others. Sequences in relatively conserved regions (e.g., SSU and LSU) have been used to study higher relationships among platyhelminths

(Baverstock *et al*., 1991; Littlewood and Olson, 2001; Lockyer *et al*., 2003; Olson *et al*., 2003; see also reviews Blair *et al*., 1996; Baguñà and Riutort, 2004; Olson and Tkach, 2005). The ITS regions are efficient in determining species boundaries because they are relatively conserved within a species but accumulate mutations quickly, leading to interspecific differences (Morgan and Blair, 1997; Nolan and Cribb, 2005).

Sequences from the cytochrome c oxidase subunit I (COI) a protein-coding gene in the mitochondrial genome (mDNA) have also been used to determine species boundaries in digeneans. Historically, most authors have used a 300 bp fragment beginning about 800 bp from the 5' end of COI (e.g. Bowles *et al*., 1995; Bell *et al*., 2001; Bell and Sommerville, 2002; Overstreet *et al*., 2002). Recently, Locke *et al*. (2010a, 2010b) used the first 600 bp of this gene, the DNA barcode, to discriminate diplostomid and strigeid species, among other Diplostomidae. The pattern of evolution of mDNA differs from that of the nuclear genome. Mitochondrial DNA is inherited maternally and evolves is faster than nuclear DNA, which is inherited biparentally (Bowles *et al*., 1995). There is disagreement concerning the ability of mDNA to provide a useful phylogenetic signal on its own due to its faster evolution (Olson and Tkach, 2005). However, the inclusion of mDNA may increase the level of resolution of a tree when combined with sequences of rDNA (Littlewood *et al*., 2008). For example, the relationships among the Davaineidae (Platyhelminthes, Cestoda) were better resolved when sequences of partial large subunit ribosomal RNA of mDNA

were included in an analysis of SSU and LSU rDNA (Littlewood *et al*., 2008). In addition, mitochondrial sequences can be used to determine whether small differences in more conserved gene sequences represent intraspecific variation or the presence of additional species (Locke *et al*., 2010a).

One difficulty in molecular phylogenetics is choosing molecular marker(s) that provide the best estimate of the species tree (Hypša, 2006). One way to overcome this difficulty is through the combined analysis of genes with different rates of evolution (Doyle, 1992; Page and Charleston, 1998; Littlewood *et al*., 2008). However, various authors disagree whether to combine the data in a consensus analyses (Miyamoto and Fitch, 1995), in a total evidence approach (Kluge, 1989) or only in combination if markers display a homogeneous phylogenetic signal (Bull *et al*., 1993; de Queiroz, 1993; Rodrigo *et al*., 1993). The consensus approach will analyze each molecular marker separately and then combine the hypotheses in a consensus tree (Miyamoto and Fitch, 1995). The total evidence approach will concatenate all the different sequences from the various molecular markers and analyze them as one dataset (Kluge, 1989). Huelsenbeck *et al*. (1996) reviewed the different approaches and concluded each have their advantages and disadvantages and the datasets should be combined conditionally depending on the heterogeneity of the data. If the different molecular markers are homogeneous in phylogenetic signal, then the datasets can be combined in a total evidence approach. However, if the datasets are

heterogeneous they should not be combined and the consensus approach is preferred.

Numerous methods to measure heterogeneity of datasets have been developed (Larson, 1994). One of the more popular tests, the incongruence length difference test (ILD) (Michevich and Farris, 1981; Farris *et al*., 1995) measures the degree of homoplasy in a dataset by comparing the total number of homoplastic character changes on the shortest tree with the sum of homoplastic characters in each dataset. However, a small number of phylogenetically informative sites within a large dataset of markers with different rates of evolution reduce the power of the ILD test causing it to incorrectly determine the heterogeneity in the datasets (Darlu and Lecointre, 2002).

Current analytical methods for phylogenetic analysis of morphological and molecular data have different assumptions and treat data differently. Distance methods, such as neighbor-joining (NJ), are phenetic, nonparametric algorithms that convert each pair of aligned sequences into a single similarity value. Sequences are then grouped into clusters that minimize differences. This method rapidly provides a unique solution that is often a good approximation of the correct tree, but much phylogenetic information is lost when data are reduced to pairwise distances.

In contrast, computationally intensive discrete methods like maximum parsimony and maximum likelihood are phylogenetic, rather than phenetic,

in that sequences are analyzed site-by-site in their entirety, and multiple "best" trees may be obtained. In maximum parsimony (MP), trees are built to minimize the number of evolutionary steps, with all types of changes treated as equally probable.

Maximum likelihood (ML) is a method that includes parameters that estimate the probability of the evolutionary events. These parameters, which are calculated based on the observed data, include estimates of the relative and overall rates of substitution and of base frequencies. The simplest model will consider all rates and frequencies to be equal whereas the most complex one will attribute specific parameters for each.

A gene tree chosen to represent a species tree should be biologically relevant. One way to assess and achieve this is to include morphological and other non-molecular data in gene trees in order to obtain a more comprehensive view of evolutionary patterns (Hillis, 1987). For example, given multiple plausible gene trees, the topology requiring the fewest changes in morphological character states is the most parsimonious and should therefore be the closest counterpart of the underlying species tree (Cribb *et al*., 2003). This concept was applied here to the multiple topologies obtained from molecular analyses. Life history, host range and adult and larval morphological characters were mapped onto the topologies and the tree with the most synapomorphies between sister taxa was preferred (Brooks and McLennan, 1993).

In this study, molecular data have been generated from the full SSU, partial LSU, and full ITS regions of ribosomal DNA as well as the barcode region of COI of mitochondrial DNA. Markers from the SSU and LSU have already proven to be reliable tools and are widely employed in phylogenetic studies of parasitic platyhelminths, with ITS being used when more variation is required (Olson and Tkach, 2005). The ITS and COI evolve rapidly and permit the differentiation of species (Olson and Tkach, 2005; Locke *et al*., 2010a). Sequences of COI have also been included in analyses to determine if they will increase the resolution among the taxa (Littlewood *et al*., 2008).

Research objective

The phylogenetic disparities regarding the monophyly of the Diplostomidae and Strigeidae lend themselves to an independent analysis using molecular data. This study used independent and total evidence analyses of four genetic markers (SSU, LSU, ITS, COI) with different evolutionary rates to evaluate the relationships among and within the Diplostomidae and Strigeidae. This study seeks to evaluate the conflicting hypotheses regarding the composition of the two families and resolve the phylogenetic relationship between them. Three conflicting hypotheses, regarding the families as a whole, from the literature were specifically tested:

1. The Diplostomidae and Strigeidae are monophyletic (Dubois, 1968, 1970; Brooks *et al*., 1985; Gibson, 1996; Niewiadomska, 2002a, 2002b).

2. The Diplostomidae are paraphyletic and the Strigeidae are monophyletic (Shoop, 1989; Brooks and McLennan, 1993).

3. The Diplostomidae and Strigeidae are paraphyletic (Olson *et al*., 2003).

Molecular phylogeny of the trematode families

Diplostomidae and Strigeidae

Introduction

Members of the Diplostomidae and Strigeidae (Digenea: Diplostomoidea) are common trematode parasites which infect birds and mammals (Niewiadomska, 2002a, 2002b). The two groups are distinguished on the basis of holdfast (tribocytic) organ morphology and the shape of the forebody (Niewiadomska, 2002c). The early taxonomy of the Diplostomidae and Strigeidae is summarized in extensive monographs by Dubois (1968, 1970). These works provide the foundation for the classification of these groups which, with few modifications, are still in use (Niewiadomska, 2002a, 2002b).

Studies on the relationships between the Diplostomidae and the Strigeidae have produced inconsistent results. A cladistic analysis of the Digenea by Brooks *et al*. (1985) based on 113 adult and 90 larval morphological characters from representatives of 63 families, suggested that the two groups were monophyletic. Shoop (1989) performed a detailed cladistic analysis of the two families including 34 of 41 nominal Diplostomidae and 11 of 13 nominal Strigeidae. This analysis included 25 adult and 8 larval morphological characteristics as well as two host-related characters. The results of his study indicated that Strigeidae was monophyletic but the Diplostomidae was not. These observations were

supported in later cladistic studies by Pearson (1992) and Brooks and McLennan (1993).

Morphological characters alone have been insufficient to unravel the evolutionary relationships of these families. Most recently Olson *et al*. (2003), after a molecular analysis of 77 digenean families, concluded that several currently recognized families, including the Strigeidae and Diplostomidae, are "not-natural". In their study, the Diplostomidae (represented by *Diplostomum* and *Alaria*) were nested within the Strigeidae (represented by *Apharyngostrigea*, *Ichthyocotylurus* and *Cardiocephaloides*). However, the scope of this study precluded dense sampling beyond the family level. Hence the intrafamilial relationships within many families remain unexplored.

In the present study, we use sequences from the full small (SSU), partial large (LSU), full internal transcribed regions 1 and 2 (ITS) from rDNA and the barcode region of the cytochrome oxidase I (COI) from mDNA along with adult and larval morphological characters and life history characters in a total evidence approach to explore the phylogenetic relationships among nine diplostomid genera (18 species) and five strigeid genera (8 species). The goal is to evaluate novel molecular and morphological data to assess the conflicting hypotheses regarding the relationships between the Diplostomidae and Strigeidae and contribute to a stronger classification of the Diplostomoidea. Three conflicting hypotheses from the literature were tested. The competing hypotheses are: the Diplostomidae and Strigeidae are

monophyletic (Dubois, 1968; 1970; Brooks *et al*., 1985; Gibson, 1996; Niewiadomska, 2002a, 2002b); the Diplostomidae are paraphyletic and the Strigeidae are monophyletic (Shoop, 1989; Brooks and McLennan, 1993); or the Diplostomidae are nested within the Strigeidae (Olson *et al*., 2003).

Materials and methods

Specimen collection, preservation, identification

The specimens used in this study were obtained from several sources during an ongoing survey of wildlife parasites. The host, collection data and life cycle stage for each specimen are listed in Appendix 1. All of the specimens were fixed and preserved in 95% ethanol and stored at -20°C until processed.

Two types of voucher specimens were kept. If the specimen was large enough, a small portion was removed for DNA analyses prior to staining. In the case of very small specimens it was not possible to obtain a DNA sample from the individual specimen without destroying it. Therefore a bulk lot voucher, where a sample of morphologically identical specimens from the same site in the same host individual as the DNA specimen(s), was retained. In a few cases, no voucher was kept.

Voucher specimens were stained with acetocarmine following standard procedures, mounted on slides using Canada balsam or Eukitt and left to dry naturally before being studied. Identifications were made to the lowest possible taxonomic level (subfamily, genus or species) using the available keys (Gibson, 1996; Hoffman, 1999; Niewiadomska, 2002a, 2002b) and the primary literature.

DNA extraction, amplification and sequencing

Most of the specimens (55 of 69) were sent to the Canadian Centre for DNA Barcoding (CCDB) in Guelph, Ontario. The DNA was extracted, amplified and sequenced for COI using primers and protocols developed by Moszczynska *et al*. (2009). DNA from these samples was subsequently obtained from the CCDB, diluted with 100 µl of sterilized distilled H_20 and used for further study. The DNA was extracted using a DNeasy Tissue Kit (QIAGEN, Toronto, Ontario) following the manufacturer's protocols.

In addition, all DNA was amplified to obtain sequences for SSU, LSU and ITS rDNA. DNA amplification was performed in 25 µl volumes via the polymerase chain reaction (PCR) in a 2720 ThermoCycler (Applied Biosystems, Carlsbad, California). Each PCR reaction consisted of: 17.5 µl sterilized distilled H_2O, 2.5 µl 10X (-$MgCl_2$) PCR reaction buffer, 1.25 µl $MgCl_2$ (25 mM), 0.125 µl dNTP (10 mM; Fermentas #R0191), 0.25 µl of the forward and reverse PCR primers and 0.125 µl *Taq* DNA polymerase (BioShop Canada Inc., TAQ001.1) and 3 µl of DNA template. The 10X PCR reaction buffer supplied with the *Taq* DNA polymerase consisted of 200mM Tris-HCl (pH 8.4), 200mM KCl, Tween 20 and enzyme stabilizers. The primers and protocols used to amplify SSU, LSU, and ITS regions are listed in Table 1.

Amplicons were visualized in 1% agarose gels containing ethidium bromide and viewed under ultraviolet light. The size of each DNA fragment was estimated by comparison to DNA ladder (0.5 µg/µl; Fermentas: GeneRuler^{TM} 100 bp Plus DNA Ladder #SM0321). These DNA products were

sequenced at the Genome Quebec Innovation Centre, McGill University in Montreal, Quebec. Sequencing was performed in both directions using the forward and reverse PCR primers and, when necessary, internal primers (Table 1). Sequences, chromatograms, specimen images and voucher information are available in projects ARLP, CLINO and PRIME at http://www.barcodinglife.org.

Table 1. Primers and polymerase chain reaction (PCR) protocols used to amplify and sequence the full small (SSU), partial large (LSU - variable regions D1 to D3) and full internal transcribed spacer regions 1 and 2 (ITS) of ribosomal DNA and the approximate size of the amplified fragment. Internal primers were used for sequencing only.

rDNA region	Primer name F = Forward R = Reverse	Primer sequence (5' to 3')	PCR cycling conditions	Primer reference	Amplicon length
SSU	18S-E (F)	CCGAATTCGTCGACAACCTGGTTGATCCTGCCAGT	3 min - 94°C; 10 cycles: 30 s - 94°C, 30 s - 65 to 56°C 1m30s - 72°C; 40 cycles: 30 s - 94°C, 30 s - 57°C, 1m30s - 72°C; 10 min extension at 72°C. Final hold at 4°C	Littlewood and Olson (2001)	≈ 2000
	18S-F (R)	CCAGCTTGATCCTTCTGCAGGTTCACCTAC			
	Worm-A (F)	GCGAATGGCTCATTAAATCAG			≈ 2000
	Worm-B (R)	ACGGAAACCTTGTTACGACT			
	600F (F)	GGTGCCAGCMGCCGCGGT			*Internal
	600R (R)	ACCGCGGCKGCTGGCACC			
	1270F (F)	AAACTTAAAGGAATTGACGG			
	1270R (R)	CCGTCAATTCCTTTAAGTTT			
LSU (D1-D3)	LSU-5 (F)	TAGGTCGACCCGCTGAAYTTAAGCA	3 min - 94°C; 40 cycles: 30 s - 94°C, 30 s - 56°C, 2 min - 72°C; 7 min extension at 72°C. Final hold at 4°C	Olson *et al*. (2003)	≈ 1500
	1500R (R)	GCTATCCTGAGGGAAACTTCG			
	300F (F)	CAAGTACCGTGAGGGAAAGTTG			*Internal
	ECD2 (R)	CTTGGTCCGTGTTTCAAGACGGG			
ITS	D1 (F)	AGGAATCCTGGTAAGTGCAAG	2 min - 94°C; 30 cycles: 1 min - 94°C, 1 min - 56°C, 2 min - 72°C; 5 min extension at 72°C. Final hold at 4°C	Galazzo *et al.* (2002)	≈ 1100
	D2 (R)	CGTTACTGAGGGAATCCTGGT			

Outgroup selection

Sequences were obtained from nine of 41 genera representing three of the four subfamilies (Alariinae, Crassiphialinae and Diplostominae) included in the Diplostomidae by Niewiadomska (2002a): *Alaria*, *Bolbophorus*, *Diplostomum*, *Fibricola*, *Hysteromorpha*, *Ornithodiplostomum*, *Posthodiplostomum*, *Tylodelphys* and *Uvulifer*. Five of 13 genera representing one of the two subfamilies (Strigeinae) within the Strigeidae recognized by Niewiadomska (2002b) are also represented in this study: *Apatemon*, *Apharyngostrigea*, *Cardiocephaloides*, *Cotylurus* and *Ichthyocotylurus*.

Outgroups were selected based on a molecular phylogeny of the Digenea by Olson *et al*. (2003), where the Diplostomidae and Strigeidae were bordered by two sister families, the Leucochloridiidae and the Clinostomidae. Based on their results, three genera, *Clinostomum* (Clinostomidae), *Leucochloridium* and *Urogonimus* (Leucochloridiidae), were chosen to root the phylogenies. The sequences for *Leucochloridium* were obtained from GenBank (Olson *et al*., 2003: AY222169 and AY222086; Tkach *et al*., 2001: AF184261). Sequences of *Clinostomum* and *Urogonimus* came from specimens in our own collection (Appendix 1).

Additional published data were used in the analyses to provide coverage of taxa or markers lacking in our samples. These included sequences from Cribb *et al*. (2001) (AJ287526, AJ287503), Tkach *et al*. (2001) (AF184263-4), Bell and Somerville (2002) (AJ301885, AJ301887,

AJ314760-1), Overstreet *et al*. (2002) (AF470566, AF470587, AF470610-3, AF611587, AF611610), Casey *et al*. (2003) (AY258144-5), Olson *et al*. (2003) (AY222168, AY222171-3, AY222175-6, AY222086, AY222089-92, AY222094-5), Moszczynska *et al*. (2009) (FJ469596, FJ477182, FJ477186, FJ477191, FJ477203, FJ477206, FJ477211-2, FJ477221, FJ477223), Locke *et al*. (2010a) (GQ292484, GQ292502, GQ292504, GQ292519-21, GQ292523), Locke *et al*. (2010b) (HM064618, HM064635, HM064644, HM064679-80, HM064685, HM064702, HM064711, HM064714, HM064721, HM064730, HM064752-3, HM064755, HM064775, HM064782, HM064789, HM064800-1, HM064805, HM064857-8, HM064875, HM064888, HM064911, HM06915, HM064925-6, HM064931, HM064939-40, HM064946, HM064955, HM064958-9, HM064962, HM064969).

Molecular data analysis

Contiguous sequences of SSU, LSU, ITS and COI from each specimen were created from forward and reverse chromatograms and edited using Geneious version 4.75 (Drummond *et al*., 2009). Sequences for each marker were aligned with ClustalX version 2.0.12 (Larkin *et al*., 2007) using the default settings. Alignments were trimmed using Geneious version 4.75 (Drummond *et al*., 2009).

Some specimens could only be identified to the subfamily or generic level morphologically. Sequences from the COI and ITS region from within the same species will have very high similarity (Nolan and Cribb, 2005; Locke *et al*., 2010a). Therefore each unique sequence was compared with

sequences available on GenBank using the basic local alignment search tool (BLAST). Species within the same genus that were distinguishable genetically with the COI sequences using sequence data from Moszczynska *et al*. (2009) and Locke *et al*. (2010a, 2010b), but indistinguishable morphologically were numbered as species 1, 2, 3, etc. as in Locke *et al*. (2010a, 2010b).

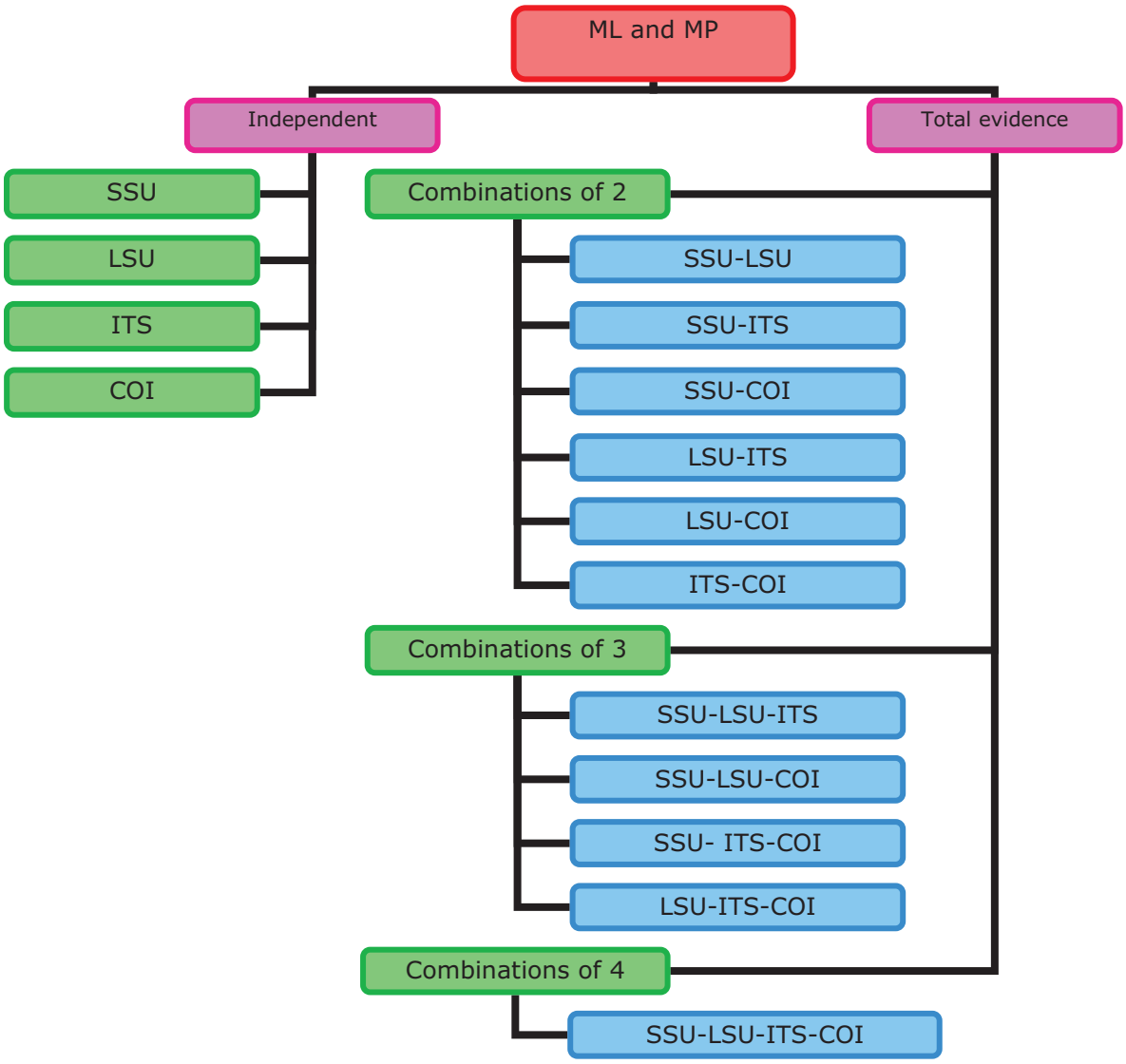
Neighbour-joining (NJ) analyses of the ITS and COI sequences for species discrimination were performed with MEGA version 4.0.2 (Tamura *et al*., 2007). The NJ algorithm was used only to view the similarity between sequences and not phylogenetic analyses. The pairwise distance dataset was created using the number of base pair differences and gaps were treated as missing data. Bootstrap values were based on 1000 replicates.

Sequences of each gene region could not be obtained from every specimen (Appendix 1) thus creating gaps in the datasets. Therefore, a single sequence was generated for each marker in each genus using Geneious version 4.75 (Drummond *et al*., 2009). Intrageneric variation was preserved by using degenerate base codes based on the International Union of Pure and Applied Chemistry (IUPAC) nucleotide codes for differences in sequences between specimens within the same genus. For example, consider an alignment of four specimens of the same genus where at a specific position in the alignment two specimens may have a cytosine (C) while the other two have a thymine (T). The character for that position in the single sequence can be replaced with a Y (IUPAC nucleotide code), signifying the character in that position for that genus could treated as be either a C or a T

during analysis. Distance tables comparing the number of nucleotide differences were generated in MEGA version 4.0.2 (Tamura *et al*., 2007) for each individual dataset using complete deletion where gaps and missing data are removed from the calculations (Appendix 4).

The single sequences for each marker were first analyzed independently and then together, in all possible combinations in a total evidence approach (Figure 5). We chose the total evidence approach because consensus trees lose resolution as trees are combined (Kluge 1989). For example, if a branch of one tree is not supported in another tree, that branch will become unresolved in consensus analysis and consequently information regarding the relationships of those taxa is lost. The total evidence approach, in contrast, provides greater resolution because the number of phylogenetically informative positions increases with each additional dataset. The total evidence datasets were concatenated in Geneious version 4.75 (Drummond *et al*., 2009).

Figure 5. Independent and total evidence analyses in both maximum likelihood (ML) and maximum parsimony (MP) of four datasets: full small (SSU), partial large (LSU), full internal transcribed spacer regions 1 and 2 (ITS) ribosomal DNA and barcode region of the cytochrome oxidase I (COI) mitochondrial DNA.



Inferred phylogenies for each dataset were obtained using two methods, maximum parsimony (MP) and maximum likelihood (ML). Both MP and ML analyses were performed with PAUP version 4.0b10 (Swofford, 2002).

The smaller number of sequences obtained using a single sequence per genus permitted use of the branch and bound algorithm rather than heuristic tree searching in the MP analyses. The branch and bound algorithm is efficient algorithm search for up to 21 taxa, greater than this is beyond the limits of PAUP (Swofford, 2002). Branch and bound search algorithms increase the efficiency in determining the most optimal solution because they subdivide the solution into several smaller groups. By doing so, they can effectively eliminate solutions with longer tree lengths and continue to search within the subdivisions for the most parsimonious tree.

Maximum parsimony branch and bound analyses were performed with all characters unordered and equally weighted. Gaps were treated as missing data. In order to increase the efficiency of the search for the most parsimonious tree, the algorithm is replicated several times. Two replication parameters affect the ability of the algorithm to find the most parsimonious tree: the agglomeration order and branch swapping. The agglomeration order is the method in which the taxa are initially added together as the tree is being constructed. There are four different methods that can be selected (in order of increasing computational complexity and exhaustiveness): as-is, simple, closest and random. Branch swapping refers to a method in which

different branches are substituted during the analysis in order to find the shortest tree. There are three different options (in order of complexity): nearest neighbour interchange, subtree pruning and regraphing or tree bisection reconnection. To obtain the most optimal solution, the most exhaustive parameters were chosen. That is, 100 search replicates were performed with random-addition taxon sampling and tree-bisection-reconnection branch-swapping. For analyses resulting in more than one most parsimonious tree, strict consensus trees were built and nodal support was estimated with 1000 bootstrap replicates for each. The choice of the best tree is based on the greatest resolution and nodal support of the branches.

The common indices to measure the fit of the characters are the consistency index (CI), retention index (RI) and rescaled consistency index (RC). These were calculated for each tree. The CI is used as a measure of homoplasy within a dataset and is calculated by dividing the sum of the steps required (m) in the tree by the sum of possible steps (s) for each character: CI = m / s. The RI is the measure of how well synapomorphies explain the tree scaled from 0 to 1. It takes into consideration the greatest number of changes (g) of each character: where RI = (g - s) / (g - m). A value of zero indicates all the characters in the dataset are homoplastic; a value of one indicates all the characters in the dataset are synapomorphic. The RC is simply the CI rescaled to zero by multiplying the CI X RI.

In the ML analyses, we calculated the Akaike Information Criterion using Modeltest version 3.7 (Posada and Crandall, 1998) to select a single

appropriate model of nucleotide evolution for each dataset (both individual and combined markers). The evolutionary model used for each analysis is shown in the statistics table for each tree (Appendix 2). For the LSU, the optimal model was HKY85 (Hasegawa *et al*., 1985), which incorporates parameters for differences in relative rates of substitutions and differences in the frequencies of the bases. For the ITS, the most complex model, the general time reversible model (GTR), with the gamma distribution was selected. The GTR model has parameters taking into account variation among the overall rate of mutation among the sites, differences in the rates of substitutions (transitions and transversions) and the frequencies of the bases in the dataset. The gamma distribution is an additional parameter that accounts for rate of mutation among the sites. For the remaining 13 of 15 trees, the GTR model with the gamma distribution and proportion of invariable sites was selected. The rate of invariable sites incorporates a specific parameter for the sites that evolve at the same rate.

Maximum likelihood analyses were performed with gaps treated as missing data. Ten heuristic search replicates were performed with random-addition taxon sampling and tree-bisection-reconnection branch-swapping (see details above in the MP analyses). The molecular clock was not enforced in these analyses as it is not known whether all of the genera evolve at the same rate. For analyses resulting in more than one most likely tree, strict consensus trees were built and nodal support was estimated with 10

bootstrap replicates. The low number of bootstrap replicates for the ML analyses was due to constraints on time.

Tree selection using mapping of morphological and life history characters

Up to 30 different topologies potentially could result from molecular analyses of the datasets (15 MP and 15 ML analyses); therefore a two-step process was used to select the most biologically plausible results. First, a subset of the 30 trees derived from molecular data was selected based on resolution of the internal branches. Trees with any internal resolution among the branches of the ingroup taxa were compared visually and grouped together if they had identical topologies. Secondly, additional data consisting of 32 characters (Appendix 3) relating to adult and larval morphology, life cycle patterns and host range of the second intermediate and adult hosts were mapped onto the resulting tree topologies using Mesquite version 2.74 (Maddison and Maddison, 2010). In some trees only a few branches were resolved; if they resembled another topology with greater resolution, they were approximated as the same topology during the character mapping. Trees with three or less nodes of internal resolution among the ingroup taxa were not mapped. The unordered characters were traced by reconstructing the most parsimonious ancestral states. The complexity of acquiring or losing a character is difficult to ascertain and thus no character was weighted. The tree with the least number of evolutionary events was chosen to represent the best proposed hypothesis.

Twenty-three of the 32 characters (Appendix 3) were obtained from Shoop (1989); 13 of 35 characters in his dataset were identical in all taxa in this study and were not used. These include: operculate eggs, absence / presence of bursal sucker, fleshy forebody, forebody base shape (concave or other), oral sucker, acetabulum, larval and adult paraprostrate gland, simple / ringed oral sucker, location of testicular and excretory pore, site of infection in and thermoregulation of final host.

Ten additional characters incorporated into the dataset used data from Dubois (1968, 1970), Shell (1970), Yamaguti (1971) and Niewiadomska (2002a, 2002b). These included: adult and metacercarial stage host range, the number of intermediate hosts, cercarial flame cell number and development, metacercarial type, adult body length, forebody shape (flattened or tubular), copulatory bursa (absence / presence and protrusibility) and genital bulb (absence / presence). The character states for *Clinostomum*, *Ichthyocotylurus, Leucochloridium*, and *Urogonimus* were attributed using descriptions provided by Dubois (1968), Kanev *et al*. (2002), Niewiadomska (2002b) and Pojmańska (2002) (see Appendix 3 for full list of characters and states).

Results

General

The present study found no support for the monophyly of either family as proposed by various authors (La Rue, 1957; Cable, 1974; Brooks *et al*., 1985; Gibson, 1996; Cribb *et al*., 2001; Niewiadomska, 2002a, 2002b). The results found not only the Diplostomidae to be paraphyletic (Shoop, 1989; Brooks and McLennan, 1993), but also the Strigeidae as proposed by Olson *et al*. (2003). Further, with the increased number of diplostomid and strigeid representatives, our findings indicate both to be nested one within the other. Evidence for this was consistently demonstrated in the molecular analyses with strigeid and diplostomid taxa separating one another. These data supported the significant systematic value of metacercarial characteristics. Two metacercarial characters, encystment and limebody morphology, supported the division of the genera studied into two main clades.

Sequences generated

Amplification of the SSU, LSU, ITS and COI markers resulted in average sequence lengths of 1722, 1147, 1225 and 468 base pairs, respectively. These yielded single sequences for each genus consisting of a minimum of 1350, 1120, 1072 and 463 nucleotides long. The differences between taxa for the SSU, LSU, ITS and COI datasets were between 0.23 – 5.17%, 1.78 – 19.14%, 2.86 – 35.11% and 10 – 30.47% respectively (Appendix 4).

ITS and COI sequences were not available for *Cardiocephaloides*, therefore this genus is not represented in trees for individual ITS (Appendix 2, Tree 5 and 6), COI (Appendix 2, Tree 7 and 8) or ITS-COI datasets (Appendix 2, Tree 19 and 20). COI sequences were not available for *Uvulifer*, therefore this genus was not represented in individual COI trees (Appendix 2, Tree 7 and 8).

Sequence comparisons

Eleven specimens (A.BC.J.Q56.04.1, A.CC.Nh.DRI.18.1, A.CC.Nh.DRI.18.2, A.CC.Nh.DRI.18.3, A.H.Ci.LBO.01.1, A.LH.Ctsp.LAE1.11.1, A.RH.Ctsp.LAE1.2.1, A.RM.Ctsp.LAE1.1.5, A.RM.Nh.BMA.17.2, A.RM.Nh.DRI.18.2 and A5.BR.Ctsp.LAE1.14.2) were only identifiable morphologically to the level of subfamily (Strigeinae). Accordingly, BLAST searches were performed to compare them with sequences on GenBank in an attempt to identify them further. ITS sequences of specimens A.CC.Nh.DRI.18.1, A.CC.Nh.DRI.18.2, A.CC.Nh.DRI.18.3 and A.RM.Nh.DRI.18.2 were within 2% of *Apatemon gracilis* reported by Bell and Somerville (2002). These high similarities in ITS sequences are sufficient to place these specimens in the genus *Apatemon* (see Nolan and Cribb, 2005).

ITS sequences from other specimens that could only be identified to genus matched published data for species of *Bolbophorus* and *Ichthyocotylurus*. BLAST searches of the ITS regions of *Bolbophorus* specimens DIB.IN.DWR08.Pe.29 and Dib.IN.DWR08.Pe.35 were within 1% of those for *Bolbophorus damnificus* published by Overstreet *et al*. (2002) and

the ITS sequences of *Ichthyocotylurus* specimens I.HT.Cc.REL.2 and I.BC.Nh.DRI.35.2 differed by less than 1% from those reported for *Icthyocotylurus erraticus* and *Ichthyocotylurus platycephalus* published by Bell *et al*. (2001).

The BLAST search along with NJ analyses gave conflicting results for one specimen. The ITS sequence for *Apatemon* specimen A.BC.J.Q56.04.1 differed by less than 1% from a *Posthodiplostomum* sequence published by Locke *et al*. (2010b) and grouped with all other *Posthodiplostomum* specimens in a NJ analysis of the ITS sequences (Figure 6). However, the morphological identification was consistent with *Apatemon* and this specimen grouped with *Apatemon* in the NJ analysis of the COI sequences (Figure 7). This discrepancy could not be resolved so the ITS sequence for this specimen was removed from further analyses.

NJ analyses

The NJ analyses of ITS (Figure 6) and COI (Figure 7) sequences confirmed the presence of single species of *Apharyngostrigea*, *Fibricola*, *Hysteromorpha* and *Uvulifer*, two species of *Alaria*, *Bolbophorus*, *Cotylurus*, *Ichthyocotylurus*, and *Tylodelphys,* and at least three species of *Apatemon*, *Diplostomum*, *Ornithodiplostomum* and *Posthodiplostomum* in the database. In all cases except the aberrant *Apatemon* ITS sequence discussed above, sequences of the same genus grouped into strongly supported clusters. Collectively these represented 18 species from the Diplostomidae and 8 species from the Strigeidae.

Figure 6. Neighbour-joining tree of 67 Diplostomoidea samples using ITS data (gaps treated as missing data). Bootstrap values based on 1000 replicates are shown using the symbols identified in the legend for branch for values >60%. Tree is drawn to scale, branch length scale is based on number of nucleotide differences in a total of 143 informative positions within the dataset. Sequences with an (*) were not included in the single sequence creation for each genus.

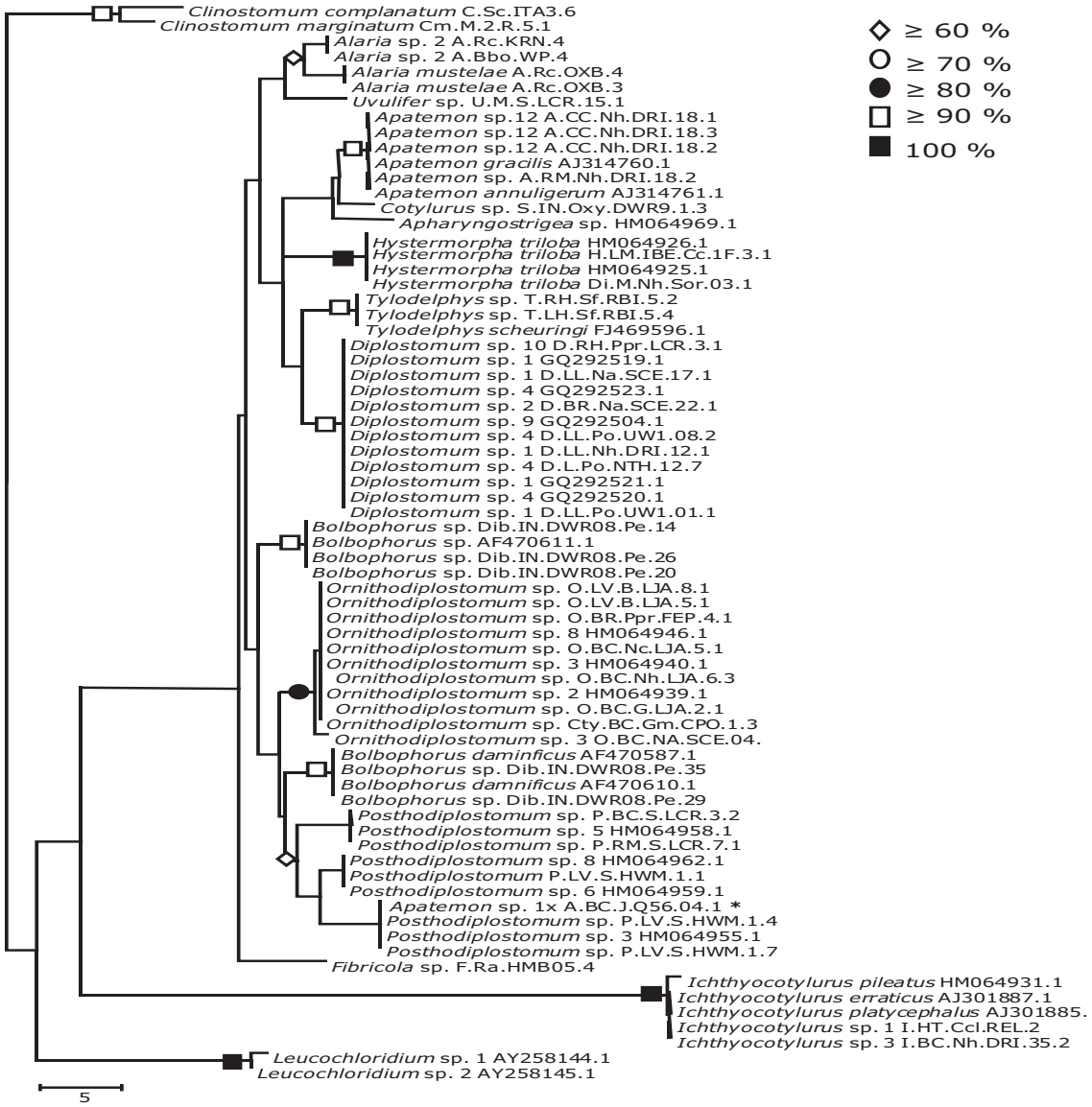
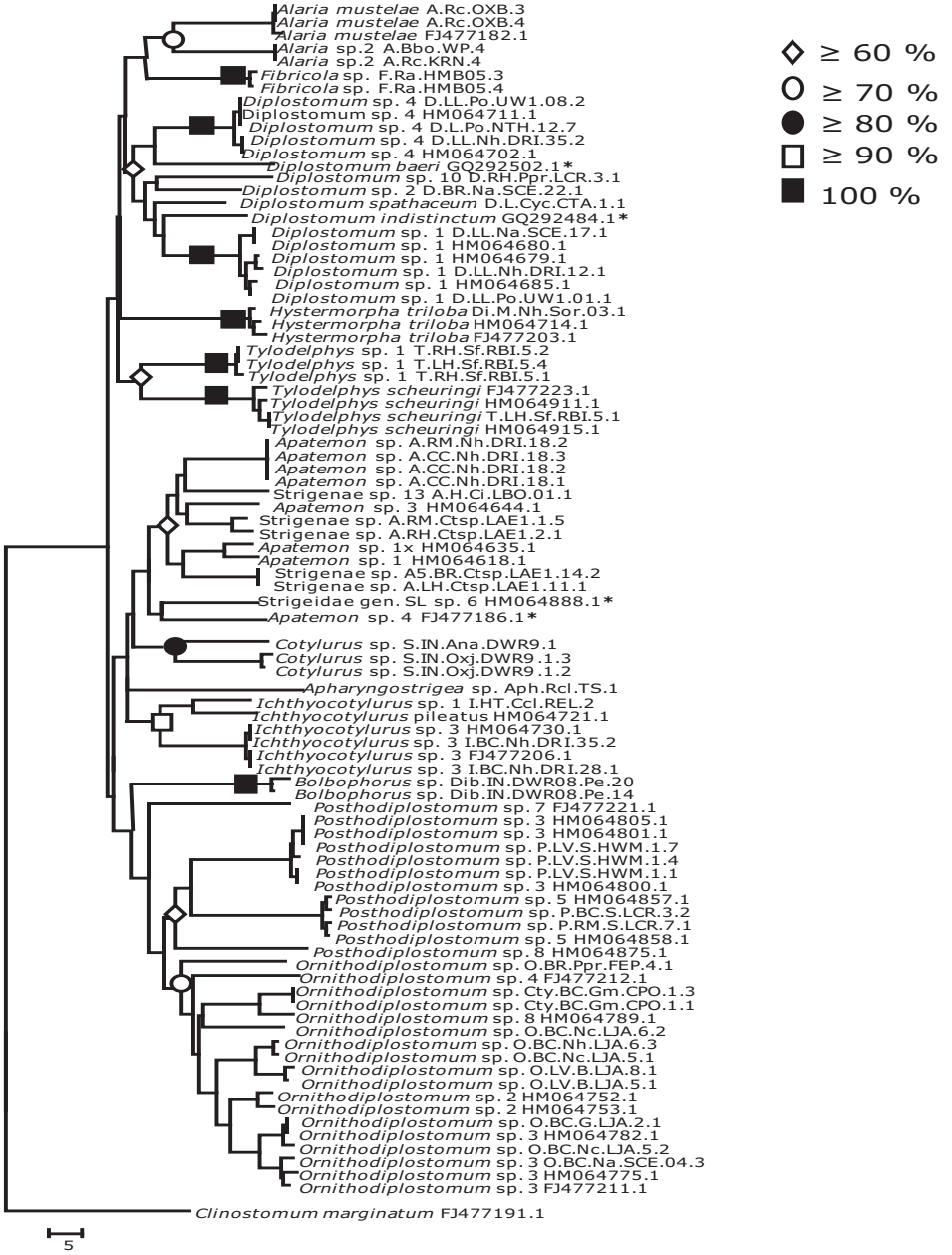


Figure 7. Neighbour-joining tree of 89 Diplostomoidea samples using COI data (gaps treated as missing data). Bootstrap values based on 1000 replicates shown using the symbols identified in the legend for branch for values >60%. Tree is drawn to scale, branch length scale is based on number of nucleotide differences in a total of 269 informative positions within the dataset. Sequences with an (*) were not included in the single sequence creation for each genus.



Phylogenetic analyses

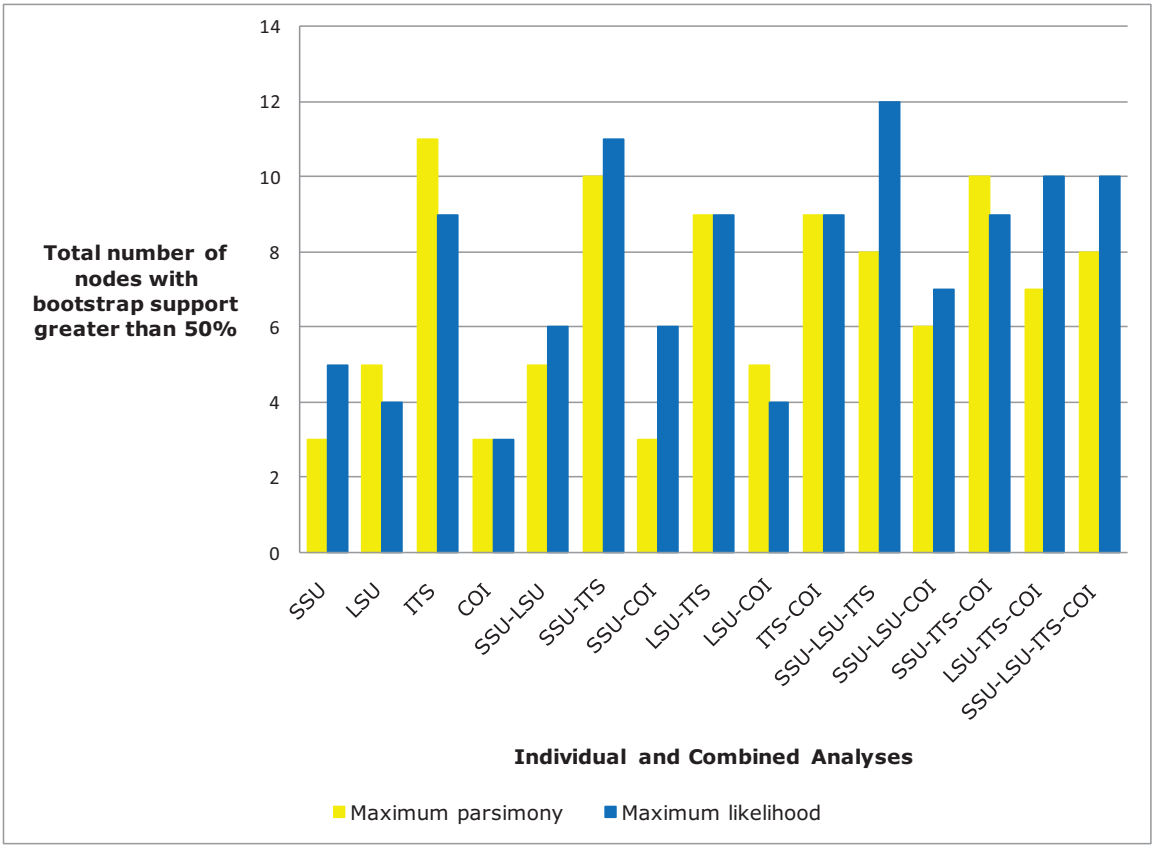
Thirty analyses were performed based on the 15 datasets generated from the individual and total evidence DNA datasets and the two analytical methods of MP and ML (Figure 5, Appendix 2). All 30 analyses strongly support one monophyletic clade that includes all genera within both families (Appendix 2). However, the internal tree topology among the taxa differed depending on the marker / combination of markers and method of analysis. The number and list of characters are not equal in the different datasets; therefore using the tree length (MP) or likelihood values (ML) are not analogous and cannot be used to evaluate the different tree topologies.

The overwhelming majority of the analyses indicated members of both the Strigeidae and Diplostomidae were separate (29 of 30 analyses). The ML analysis of the COI dataset was the only analysis to group the strigeids together (Appendix 2, Tree 7). The strigeids *Apatemon*, *Cotylurus* and *Apharyngostrigea* were consistently separated from *Ichthyocotylurus* and *Cardiocephaloides* by a branch with various Diplostomidae taxa. Two nodes with consistently strong support were those of *Apatemon*, *Cotylurus* and *Apharyngostrigea*, and *Ornithodiplostomum* and *Posthodiplostomum*.

The ITS dataset was the only individual analysis with complete resolution and strong nodal support. Overall, the individual ITS dataset (as well as any combinations that included the ITS dataset) had the most nodal support in both the MP and ML analyses (Figure 8). Except for the ITS, the

combined datasets had stronger nodal support than the individual markers (Figure 8).

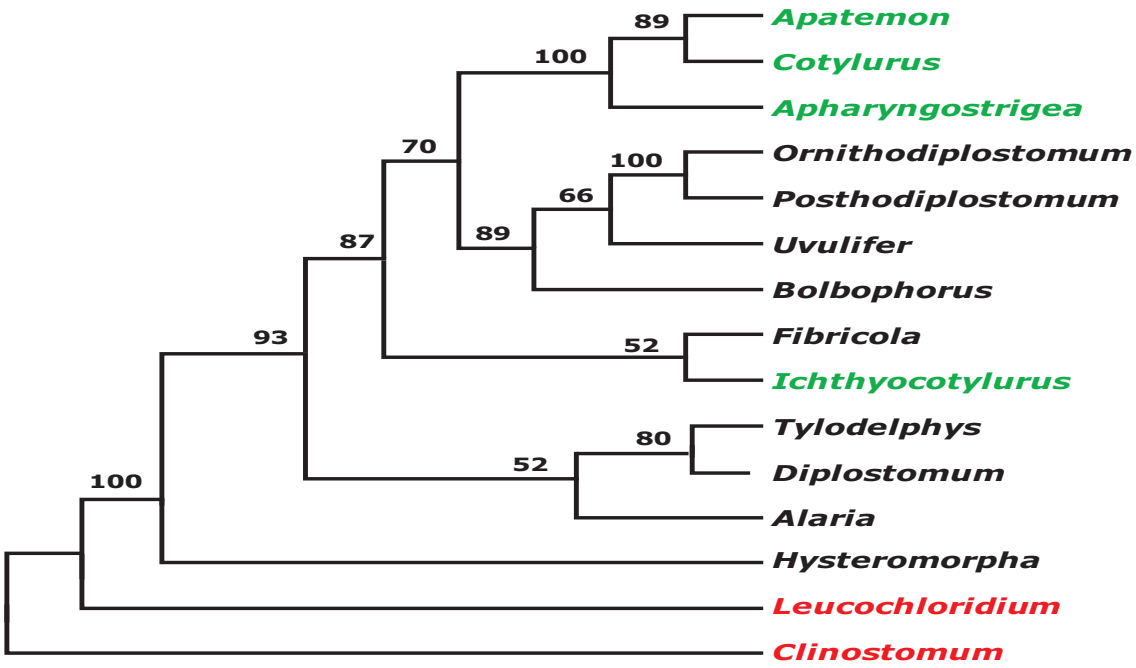
Figure 8. Total number of nodes with bootstrap support greater than 50% for 1000 replicates in the maximum parsimony and 10 replicates in the maximum likelihood analyses of the individual and combined datasets of the full small subunit (SSU), partial large subunit (LSU), full internal transcribed spacer regions 1 and 2 (ITS) of ribosomal DNA and the barcode region of cytochrome oxidase I (COI) of mitochondrial DNA.



Maximum parsimony

The number of phylogenetically informative sites differed among the individual molecular markers as follows: ITS (383 bp), LSU (267 bp), SSU (162 bp) and COI (154 bp). The branch lengths are tabulated and presented for the individual analyses in Appendix 5. The MP tree with the most resolved branches and strongest nodal support was the individual analysis of the ITS dataset (Figure 9). The results for this tree were two equally parsimonious trees with a tree length of 1444 steps for both with a CI of 0.68, RI of 0.52 and RC of 0.35. All branches were resolved in the strict consensus except for the placement of *Alaria*. Bootstrapping results (52%) resolve the placement of *Alaria* as sister to *Diplostomum* and *Tylodelphys* (Figure 9). The monophyly of the taxa is well supported with *Hysteromorpha* forming the first branch sister to all other taxa. The remainder of the taxa form four main branches. *Alaria*, *Diplostomum* and *Tylodelphys* form one clade sister to the three other branches. *Fibricola* and *Ichthyocotylurus* group together on the next branch. The two upper branches form two sister clades, one composed of *Ornithodiplostomum*, *Posthodiplostomum*, *Uvulifer* and *Bolbophorus*. The last clade consists of *Apatemon*, *Cotylurus* and *Apharyngostrigea*. No ITS datum was available for *Cardiocephaloides* therefore this taxon is not represented on this tree.

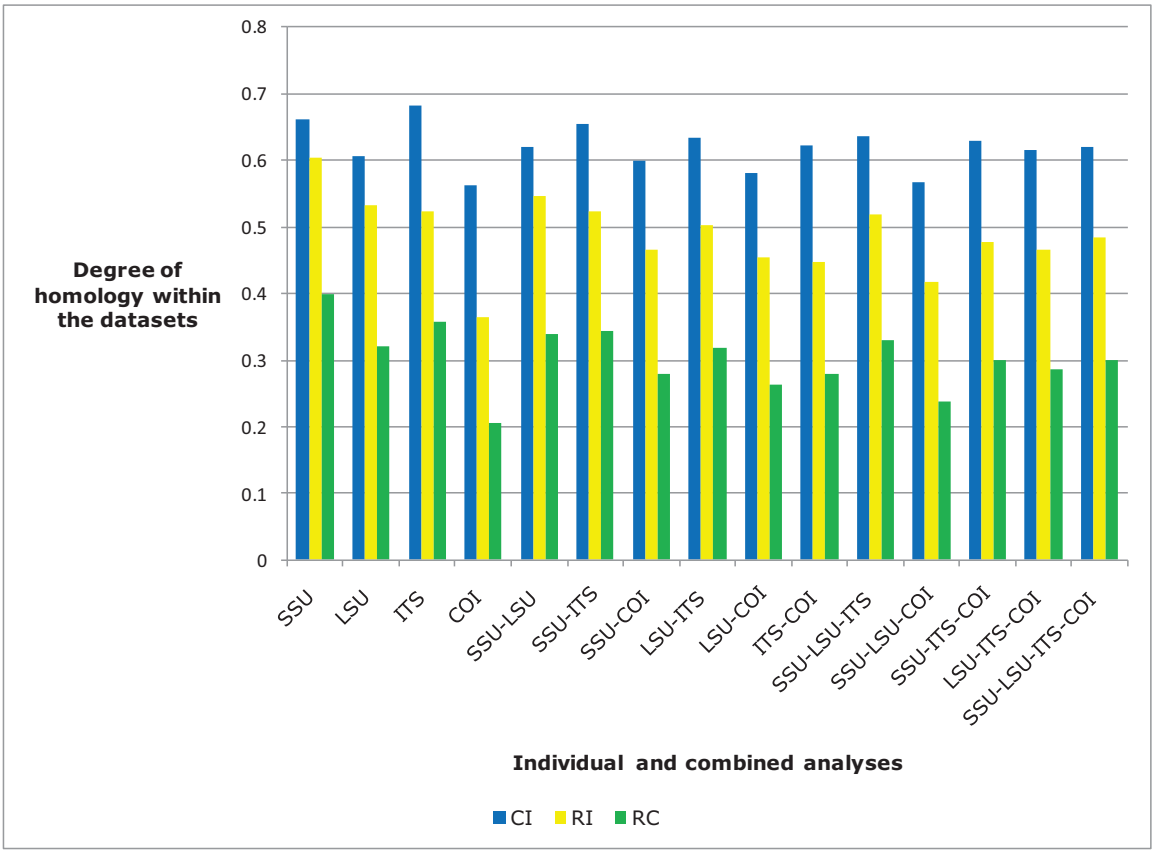
Figure 9. The strict consensus tree of two equally parsimonious trees of a maximum parsimony (MP) branch and bound analysis of the full internal transcribed spacer regions 1 and 2 (ITS) of ribosomal DNA of the Diplostomidae and Strigeidae. Bootstrap values based on 1000 replicates shown above branch. Outgroup taxa are red, strigeid taxa are green and diplostomid taxa are black. See also Appendix 2, Tree 5. The branch for *Alaria* was unresolved in the strict consensus, but had a bootstrap value of greater than 50%; the resolved branch is shown here.



ITS MP Results:	
Ingroup taxa	13
Outgroup taxa	2
Total number of characters	1225
Constant	572
Parsimony informative	383
Parsimony uninformative	270
Number of equally parsimonious trees	2
Length	1444
Consistency index	0.6820
Retention index	0.5234
Rescaled consistency index	0.3570

The tree statistics for the MP analyses showed the greatest homology based on CI within the ITS dataset and based on RC within the SSU dataset (Figure 10). The RI showed the greatest synapomorphies among the SSU dataset (Figure 10). The dataset with the least homology within all three statistics was the COI dataset (Figure 10).

Figure 10. Maximum parsimony tree statistics, consistency index (CI), retention index (RI) and rescaled consistency index (RC), for each individual and combined datasets of the full small subunit (SSU), partial large subunit (LSU), full internal transcribed spacer regions 1 and 2 (ITS) of ribosomal DNA and the barcode region of cytochrome oxidase I (COI) of mitochondrial DNA. For each statistic the higher the value (0 to 1) indicates greater homology within the dataset.



In general the MP analyses added the branches in a stepwise pattern. The ingroup taxon occupying the basal position among the other individual and total evidence analyses was not consistent. In the various MP analyses, the basal genus was *Bolbophorus* (Appendix 2: Tree 7, 17, 23), *Cardiocephaloides* (Appendix 2: Tree 15, 21, 27, 29)*, Diplostomum* (Appendix 2: Tree 1), *Fibricola* (Appendix 2: Tree 3, 9) or *Hysteromorpha* (Appendix 2: Tree 5, 11, 19, 25).

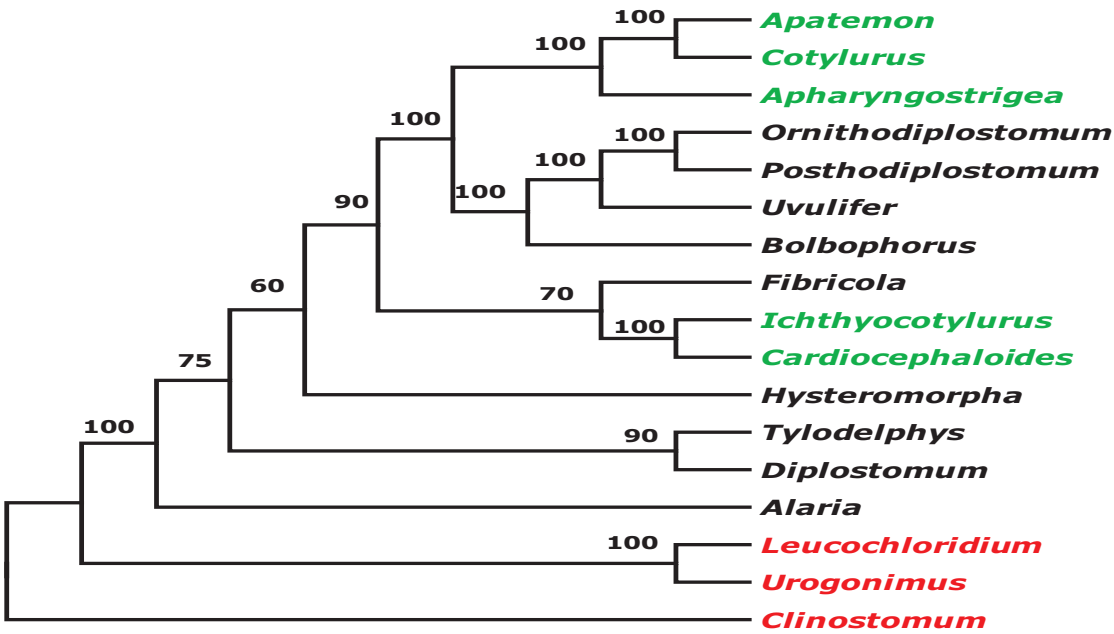
The only total evidence MP analysis with no resolution (other than the consistently supported nodes mentioned above) was that of the SSU-COI dataset. Further, the MP analyses of the LSU-COI and SSU-LSU-COI were the least consistent with the other topologies. These were the only datasets resulting with the Strigeidae taxa separated by *Alaria*, *Fibricola*, *Diplostomum* and *Tylodelphys* (Appendix 2: Tree 17 and 23).

Maximum likelihood

The ML tree with the most resolved branches and strongest nodal support was the total evidence analysis of the SSU-LSU-ITS dataset (Figure 11). This tree was also the most robust of all the 30 analyses. All branches were resolved. The monophyly of the taxa is well supported with *Alaria* forming the first branch sister to all other taxa. The second branch clusters *Diplostomum* and *Tylodelphys*, followed by a branch with *Hysteromorpha*. The remaining taxa form three clades, one consisted of *Fibricola* and *Ichthyocotylurus* and *Cardiocephaloides* sister to the next two branches. The two upper branches form two sister clades, one composed of

Ornithodiplostomum, *Posthodiplostomum*, *Uvulifer* and *Bolbophorus*. The last clade consists of *Apatemon*, *Cotylurus* and *Apharyngostrigea*.

Figure 11. The maximum likelihood (ML) total evidence analysis of full small subunit (SSU), partial large subunit (LSU) and full internal transcribed spacer regions 1 and 2 (ITS) of ribosomal DNA of the Diplostomidae and Strigeidae. Bootstrap values based on 10 replicates shown above the branch. Outgroup taxa are red, strigeid taxa are green and diplostomid taxa are black. See also Appendix 2, Tree 22.



SSU-LSU-ITS ML Results:	
Ingroup taxa	14
Outgroup taxa	3
Total number of characters	4094
Model	GTR+G+I model
Likelihood (-ln)	18174.36943
Number of equally likely trees	1

In general the ML topologies arranged the taxa in either a stepwise pattern of evolution with a basal taxon (Appendix 2: Tree 2, 6, 8, 12, 16, 22), a basal clade (Appendix 2: Tree 4, 14, 20) or dividing the taxa into two clades from the primary node (Appendix 2: Tree 10, 18, 24, 26, 28, 30). In the stepwise pattern, as in the MP analyses, the taxon occupying the basal position was not consistent. In the various ML analyses, the basal genus was *Alaria* (Appendix 2: Tree 2, 12, 22), *Bolbophorus* (Appendix 2: Tree 8) or *Hysteromorpha* (Appendix 2: Tree 6, 16). In the trees forming a basal clade, the taxa consisted of *Ornithodiplostomum*, *Posthodiplostomum* and *Uvulifer* (Appendix 2: Tree 4), *Diplostomum* and *Tylodelphys* (Appendix 2: Tree 14) or *Diplostomum*, *Tylodelphys* and *Hysteromorpha* (Appendix 2: Tree 20). The trees which clustered the taxa into two clades followed one of three different patterns. One topology grouped *Alaria*, *Diplostomum*, *Cardiocephaloides*, *Fibricola*, *Ichthyocotylurus*, and *Tylodelphys* (Appendix 2: Tree 10). The other two included *Alaria*, *Diplostomum*, *Hysteromorpha* and *Tylodelphys* with or without *Fibricola* (Appendix 2, Tree 18 or Appendix 2, Tree 26, 28, 30 respectively).

The ML analyses of the LSU and SSU-LSU were the least consistent with the other topologies. The LSU dataset resulted in the Strigeidae separated by *Alaria*, *Fibricola*, *Diplostomum* and *Tylodelphys* (Appendix 2, Tree 4). The SSU-LSU dataset separated the branch of *Apatemon*, *Cotylurus* and *Apharyngostrigea* from *Cardiocephaloides* and *Ichthyocotylurus* which

joined a clade comprised of *Alaria*, *Fibricola, Diplostomum* and *Tylodelphys* (Appendix 2, Tree 10).

Character mapping

All trees were compared visually for similar topologies. Twenty-seven of the 30 analyses assembled into one of 15 different topologies (Appendix 6). Nodal support differed with regards to the marker(s) and method of analysis. No distinction was made between the topologies based on differences of nodal support. Within these 27 trees, 23 were fully resolved. The other four resembled a fully resolved tree in all other branch placements and were approximated as the same topology (Appendix 2, Trees 6, 9, 11, 19; Appendix 6).

Two trees (ML of COI, Appendix 2, Tree 7 and MP of SSU-COI, Appendix 3, Tree 9) had no internal resolution apart from the branches of *Apharyngostrigea*, *Cotylurus* and *Apatemon* and *Ornithodiplostomum* and *Posthodiplostomum* common to all trees. The ML analysis of the COI dataset (Appendix 2, Tree 8) was missing sequence data for *Cardiocephaloides* and *Uvulifer*. These three trees were not mapped.

All 15 topologies strongly support the monophyly of the taxa among the Strigeidae and the Diplostomidae represented here. Eleven out of 15 topologies (Appendix 6, Topology B, E-I, K-O) strongly support a branch splitting off into two sister clades, one clade consisting of *Apatemon*, *Cotylurus* and *Apharyngostrigea* and the other clade containing

Ornithodiplostomum, *Posthodiplostomum*, *Uvulifer* and *Bolbophorus*. Eight out of 15 topologies cluster *Fibricola* with *Ichthyocotylurus* (Appendix 6, Topology B, E-H, L, N-O). Eight out of 15 topologies place *Diplostomum*, *Tylodelphys*, *Apatemon* and *Hysteromorpha* as basal taxa among the ingroup (Appendix 6, Topology E, H-I, K-O).

The mapping of the morphological characters on the topologies resulting from the various individual and total evidence datasets of the SSU, LSU, ITS and COI sequences showed a range between 82 – 100 character state changes among the different topologies (Appendix 6). The most plausible tree with the least amount of steps was the ML analysis of the SSU-LSU-COI (Figure 12; Appendix 2, Tree 24; Appendix 6, Topology K). There were 82 changes in the mapped characters (Appendix 6, Topology K) in this tree. While this is not a great difference in comparison to the other topologies, it was the tree with the greatest overall number of non-molecular synapomorphies between sister taxa, therefore the most biologically plausible (Brooks and McLennan, 1993).

Five synapomorphic characters (Appendix 3) supported the monophyly of all the Diplostomidae and Strigeidae (Figure 12). These include: sporocyst cercariae development (2), presence of a holdfast (tribocytic) organ (17), presence of a hermaphroditic duct (20), absence of a cirrus sac (24) and a pretesticular ovary (29).

The taxa clustered into two major clades. One clade consisted of *Diplostomum* and *Tylodelphys*, *Hysteromorpha*, *Alaria* and *Fibricola*. The

other clade consisted of three branches, *Ichthyocotylurus* and *Cardiocephaloides* sister two branches: one composed of *Bolbophorus*, *Uvulifer*, *Ornithodiplostomum* and *Posthodiplostomum*, the other branch comprised of *Apharyngostrigea*, *Cotylurus* and *Apatemon*. The characters supporting the division of the basal branch from all other taxa are the metacercarial characters of encystment (6) and free or enclosed limebodies (10).

The following characters also support the branching of the taxa into two distinct clades: metacercarial type (4), metacercarial forebody shape (7), adult forebody shape (13) and morphology (14), holdfast organ shape (18) and copulatory bursa (26). Members of the clade comprised of *Diplostomum*, *Tylodelphys*, *Hysteromorpha*, *Alaria* and *Fibricola* all have a diplostomulum metacercaria, a flattened-spatulate adult and metacercarial forebody, a spherical holdfast organ and a non-protrusible copulatory bursa. The upper clade has a tetracotyle metacercaria type, a tubular cup-shaped adult and metacercarial forebody, a bilobed holdfast organ and a protrusible copulatory bursa. However, the internal branch comprised of *Bolbophorus*, *Uvulifer*, *Ornithodiplostomum* and *Posthodiplostomum* has evolved a neascus metacercaria. This internal branch also has evolved to parallel the phenotype of the basal clade and similarly have a flattened-spatulate adult and metacercarial forebody and a spherical holdfast organ. *Bolbophorus* is the only taxon in the upper clade with a non-protrusible copulatory bursa. There

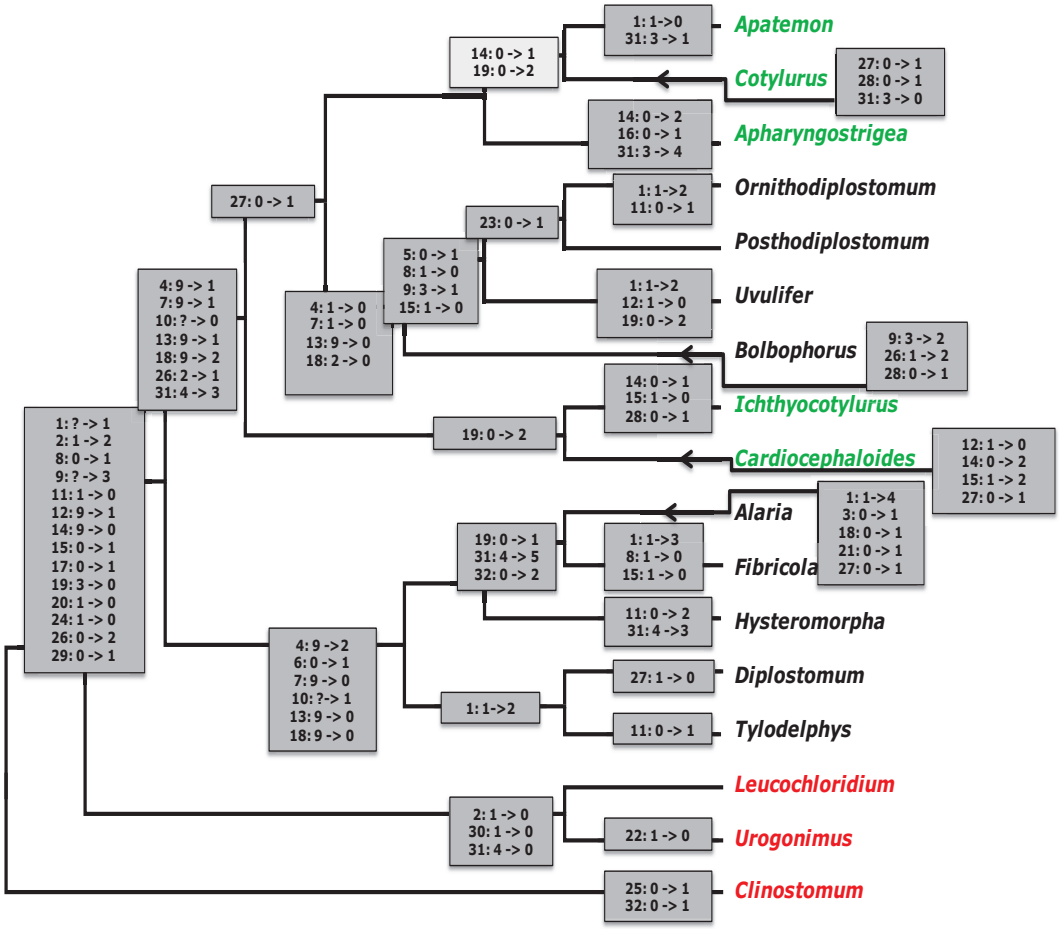
were no non-molecular characters supporting the splitting of the strigeid taxa.

Characters which were informative regarding internal clusters but not the overall interfamilial relationships within the topology included: characters of body segmentation (5), metacercarial presence / absence of pseudosuckers (8), paranephridial plexus (9), vitelline distribution (19), presence / absent genital prepuce (23), number of intermediate hosts (30), and final host (32).

Several non-molecular characters were uninformative due to autapomorphy or multiple changes among the taxa. The following adult and larval characters were autapomorphic: presence / absence of a mesocercariae stage (3), presence / lost pharynx (16), testicular position (21), testicular shape (22) and genital pore location (25).

Adult, larval and life cycle characters belonging only to a few genera or with multiple changes among the taxa and thus uninformative included: adult body shape (11), body length (12), lost / present / absent pseudosuckers (15), presence / absence of a genital cone (27) or genital bulb (28), cercarial character of flame cell number (1), and life cycle characters of metacercarial host (31).

Figure 12. Most parsimonious topology illustrated by the character mapping of 32 adult and larval morphological characteristics, life history traits and range of final and second intermediate hosts (Appendix 3). Total number of character state changes is 82. Changes in character states are given inside the grey boxes at the branches. Characters and character states as numbered in Appendix 3. The topology was supported by the maximum likelihood total evidence analyses of the full small (SSU) and partial large (LSU) subunit of ribosomal DNA and the barcode region (COI) of cytochrome oxidase I of mitochondrial DNA (See also Appendix 2, Tree X; Appendix 6, Topology K). Outgroup taxa are red, strigeid taxa are green and diplostomid taxa are black.



Discussion

Phylogenetic analyses of four molecular markers of varying evolutionary rates (SSU, LSU, ITS and COI) in independent and total evidence analyses revealed that genera currently included in the Diplostomidae and Strigeidae cluster into a single monophyletic clade. This clade was strongly supported in all analyses (bootstrap 100%), except for the COI dataset where bootstrap support in the MP and ML searches was 67% and 60%, respectively. As the genera from both families formed one clade, it would be justifiable to consider the clade as a single family. If this were to occur, the older family name Diplostomidae Poirier, 1886, would prevail based on the rules of priority.

In contrast with the most recent taxonomic treatment of the group (Niewiadomska, 2002a, 2002b), the overwhelming majority of the analyses (the single exception being the ML of the COI dataset), our data indicated that genera belonging to the Diplostomidae and Strigeidae were paraphyletic. An earlier molecular study, based on analyses of SSU and LSU sequences, also suggested that these two families were paraphyletic (Olson *et al*., 2003). Their analyses indicated that the diplostomid genera *Diplostomum* and *Alaria* were nested among strigeid genera *Apharyngostrigea*, *Cardiocephaloides* and *Ichthyocotylurus*; however, the relationships were not described in detail. Olson *et al*. (2003) reported similar situations in eight other pairs of digenean families; hence, this situation is not unique to the Diplostomoidea.

The most robust trees obtained from molecular data were MP and ML analyses of ITS and SSU-LSU-ITS datasets, respectively. Both of these molecular topologies were similar to the ML analysis of the SSU-LSU-COI dataset selected by character mapping. The higher branches were consistent for all three trees and included a branch grouping *Apatemon*, *Cotylurus* and *Apharyngostrigea* sister to a branch composed of *Ornithodiplostomum*, *Posthodiplostomum*, *Uvulifer* and *Bolbophorus.* These were sister to an outer branch that consisted of *Ichthyocotylurus* and *Cardiocephaloides.* Inconsistencies occurred among the lower branches, particularly the relationship of *Fibricola, Alaria* and *Hysteromorpha* to *Diplostomum* and *Tylodelphys*.

Both of the robust molecular topologies included *Fibricola* (Alariinae) as a sister to *Ichthyocotylurus* (Strigeinae) but the tree selected by mapping analysis grouped it with *Alaria* (Alariinae), which is consistent with current taxonomic practise (Niewiadomska, 2002a). Morphologically and biologically, *Fibricola* and *Ichthyocotylurus* are quite different. Based on the mapping analysis they differ in the following characters: metacercarial type (including: presence / absence of encystment, forebody shape, presence / absence of pseudosuckers and morphology of the paranephridial plexus and limebodies) and adult features including: a non-protrusible copulatory bursa, distribution of vitellaria and differences in the morphology of the forebody and tribocytic organ. Based on morphology, pairing of *Fibricola* with *Ichthyocotylurus* would require 10 evolutionary changes compared to three if it is paired with *Alaria*.

The sequence used in this study was obtained from a metacercaria identified as *Fibricola*. Unfortunately, the relationship of this specimen cannot be satisfactorily resolved with molecular data because it groups with *Ichthyocotylurus* in some trees and with *Alaria* in others. Grouping of *Fibricola* with *Alaria* is consistent with morphological and biological data and current taxonomic practice. Likewise, the relationships between *Alaria* and *Hysteromorpha* with *Diplostomum* and *Tylodelphys* are inconclusive. Whether these relationships can be resolved by denser taxon sampling of their respective subfamilies remains to be determined.

Excluding *Fibricola* (see above), the internal relationships within each of the main branches generally reflect the subfamily relationships of the Diplostomidae proposed in the most recent classification (Niewiadomska, 2002a). The diplostomids represented within this study belonging to two subfamilies, Crassiphialinae and Diplostominae, each cluster into their own distinct clades. Those genera included in the Diplostominae (*Diplostomum*, *Hysteromorpha* and *Tylodelphys*) grouped together forming the basal branches. Genera represented from the Alariinae (*Alaria* [and *Fibricola* in the mapping study]) formed a branch within the Diplostominae. All of these have a diplostomulum type metacercariae that, upon further analysis, may preclude recognition of Alariinae as a separate subfamily. Genera from the Crassiphialinae (*Bolbophorus, Ornithodiplostomum*, *Posthodiplostomum*, and *Uvulifer*) all have a neascus type metacercariae and formed a strongly supported branch in our topology (70% bootstrap support).

Our results were not consistent with current taxonomic view of the Strigeidae. Our sample included five of the 12 genera currently included in the Strigeinae (Niewiadomska, 2002b). These formed two well supported clades separated by the crassiphialinids. Interestingly, no morphological characters within our dataset differentiated *Apatemon*, *Apharyngostrigea* and *Cotylurus* from *Cardiocephaloides* and *Ichthyocotylurus*.

The most plausible molecular tree according to the mapping analysis, the ML analysis of the SSU-LSU-COI dataset, was chosen as the best approximation of the species tree. Previous molecular analyses of higher level relationships in the Digenea have been based on various ribosomal and mitochondrial DNA markers, used independently or in combination, to infer phylogenetic relationships (see review Olson and Tkach, 2005; Bray *et al*., 2009). In one study, Littlewood *et al.* (2008) suggested neither SSU nor LSU datasets, analyzed alone or in combination, had enough resolving power to produce a robust tree. This was consistent with observations in this study. Similarly, COI sequences alone were inadequate in resolving the evolutionary relationships due to the small number of phylogenetically informative sites (Olson and Tkach, 2005). However COI was informative for species delineation (Hajibabaei *et al*., 2007; Locke *et al*., 2010a, 2010b) and the addition of COI to the SSU-LSU dataset proved useful in generating the most plausible topology. The ITS phylogeny was the most robust of those obtained with individual markers, but character mapping indicated it did not represent the species tree. The inconsistency of this marker may perhaps be explained

by its high degree of divergence, compared to SSU and LSU sequences, causing a greater number of possible alignments (Hillis and Dixon, 1991).

Three hypotheses regarding the relationships of two of six families, within the Diplostomoidea, the Diplostomidae and the Strigeidae, were examined. First, no support was found for the monophyly of either family proposed by various authors (La Rue, 1957; Cable, 1974; Brooks *et al*., 1985; Gibson, 1996; Cribb *et al*., 2001; Niewiadomska, 2002a, 2002b). Second, the paraphyly of the Diplostomidae and monophyly of the Strigeidae as proposed by Shoop (1989) and Brooks and McLennan (1993) was partially supported by these data. This study not only found the Diplostomidae to be paraphyletic, but also the Strigeidae, with both families nested one within one another. Further, this study supported the significant systematic value of metacercarial characteristics, an important conclusion of Shoop (1989). Here, two metacercarial characters, encystment and limebody morphology, supported the division of the genera studied into two main clades. Lastly, the nesting of diplostomid taxa within strigeid taxa in a paraphyletic relationship, as proposed by Olson *et al*. (2003), is supported by these data. Evidence for this was consistently demonstrated in the molecular analyses with strigeid and diplostomid taxa separating one another.

While questions remain regarding the intrafamilial relationships among these genera, our molecular data points towards the members of Diplostomidae and Strigeidae having a single common ancestor. At this level the monophyly of these genera is also supported by numerous morphological

and life cycle characters. A more complete tree, including representatives from the Codonocephalinae (Diplostomidae) and Duboisiellinae (Strigeidae) will be necessary for a more comprehensive understanding of the evolution of the group.

General conclusions

This thesis provides an initial step towards a comprehensive classification of the Diplostomidae and Strigeidae reflecting their phylogenetic relationships. Maximum likelihood and maximum parsimony analyses were conducted on sequences of one mitochondrial and four ribosomal DNA markers with varying evolutionary rates, with genes analyzed in combination and individually. All results show that fourteen genera in the Diplostomidae and Strigeidae form a monophyletic family. Mapping of adult and larval morphological characters and life history traits indicate a molecular topology closely resembling the subfamily divisions of Niewiadomska (2002) is more biologically plausible than two other well supported trees based on nodal support alone. In contrast, the monophyly of the two families proposed by various authors (La Rue, 1957; Cable, 1974; Brooks *et al*., 1985; Gibson, 1996; Cribb *et al*., 2001; Niewiadomska, 2002a, 2002b) was not supported. The division of the Diplostomidae into three families, with the Strigeidae remaining monophyletic (Shoop, 1989; Brooks and McLennan, 1993) was also not supported. The results indicate a paraphyletic relationship of the Diplostomidae and Strigeidae, similar to that obtained in a molecular phylogeny of fewer diplostomid and strigeid taxa by Olson *et al*. (2003). Further studies are needed to expand the new framework presented here, i.e., that the Strigeidae and Diplostomidae be collapsed into the Diplostomidae Poirier, 1886.

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Appendices

Appendix 1. Specimens used in this study, including their host, collection data, life cycle stage and molecular markers sequenced. The molecular markers sequenced are the full small subunit (SSU), partial large subunit (LSU), full internal transcribed spacer regions 1 and 2 (ITS) of ribosomal DNA and the barcode region of cytochrome oxidase 1 (COI) of mitochondrial DNA. Subfamily divisions according to Niewiadomska (2002a, 2002b) are coded as: A: Alariinae, C: Crassiphialinae, Cl: Clinostominae, D: Diplostominae and S: Strigeinae. Families are coded as: C: Clinostomidae, D: Diplostomidae, L: Leucochloridiidae and S: Strigeidae. Life cycles are coded as: M: metacercariae, A: adult. Samples sent to CCDB for DNA extraction, amplification and sequencing of the barcode COI region are indicated with ß. Samples used in the Locke *et al*. (2011) study are indicated with (*). Samples used in the Caffara *et al*. (2011) study are indicated with (**).

Genus and species	Sub-family	Family	BOLD process ID	Sample ID	Molecular markers SSU	LSU	ITS	COI	Life stage	Host info	Collected (dd/mm/yy)	Country
Alaria mustelae	A	D	TREMA2448-10	A.Rc.OXB.3 ß**			**1047**	**621**	M	*Lithobates clamitans*	24/8/09	USA
Alaria mustelae	A	D	TREMA2449-10	A.Rc.OXB.4 ß**	**1335**	**1179**	**988**	**621**	M	*L. clamitans*	24/8/09	USA
Alaria sp. 2	A	D	TREMA2439-10	A.Bbo.WP.4 ß**	**651**	**1195**	**1044**	**618**	M	*Anaxyrus boreas*	29/7/09	USA
Alaria sp. 2	A	D	TREMA2447-10	A.Rc.KRN.4 ß**	**1055**	**1196**	**1000**	**621**	M	*Lithobates catesbeiana*	30/7/09	USA
Strigenae	S	S	TREMA2246-10	A.BC.J.Q56.04.1 ß*				**559**	M	*Etheostoma nigrum*	17/6/09	Canada
Apatemon	S	S	TREMA2264-10	A.CC.Nh.DRI.18.1 ß*			**1162**	**624**	M	*Notropis hudsonius*	25/9/08	Canada
Apatemon	S	S	TREMA2265-10	A.CC.Nh.DRI.18.2 ß*			**1170**	**624**	M	*N. hudsonius*	25/9/08	Canada
Apatemon	S	S	TREMA2266-10	A.CC.Nh.DRI.18.3 ß*			**1194**	**624**	M	*N. hudsonius*	25/9/08	Canada
Strigenae	S	S	TREMA2618-10	A.H.Ci.LBO.01.1 ß*	**1032**	**1120**		**622**	M	*Culea inconstans*	7/9/06	Canada
Strigenae	S	S	TREMA2501-10	A.LH.Ctsp.LAE1.11.1 ß*	**965**			**621**	M	*Cottus* sp.	28/7/09	USA
Strigenae	S	S	TREMA2482-10	A.RH.Ctsp.LAE1.2.1 ß*	**1372**	**1193**		**461**	M	*Cottus* sp.	28/7/09	USA
Strigenae	S	S	TREMA2480-10	A.RM.Ctsp.LAE1.1.5 ß*	**1043**	**1173**		**621**	M	*Cottus* sp.	28/7/09	USA
Strigenae	S	S	FLUKE641-11	A.RM.Nh.BMA.17.2	**1667**	**1120**			M	*N. hudsonius*	2/10/08	Canada
Apatemon	S	S	TREMA2270-10	A.RM.Nh.DRI.18.2 ß*	**951**	**1204**	**1198**	**624**	M	*N. hudsonius*	25/9/08	Canada
Strigenae	S	S	TREMA2515-10	A5.BR.Ctsp.LAE1.14.2 ß*				**621**	M	*Cottus sp.*	28/7/09	USA
Apharyngostrigea	S	S	TREMA2457-10	Aph.Rcl.TS.1 ß*				**602**	M	*Rana clamitans*	15/6/09	USA
Bolbophorus damnificus	C	D	FLUKE631-11	Dib.IN.DWR08.Pe.29	**1771**	**1138**	**1185**		A	*Pelecanus erythrhynchos*	3/10/08	Canada
Bolbophorus damnificus	C	D	FLUKE632-11	Dib.IN.DWR08.Pe.35	**1761**	**1109**	**1168**		A	*P. erythrhynchos*	3/10/08	Canada
Bolbophorus sp.	C	D	TREMA2460-10	Dib.IN.DWR08.Pe.14 ß*	**1508**	**1208**	**598**	**616**	A	*P. erythrorhynchos*	3/10/08	Canada
Bolbophorus sp.	C	D	TREMA2466-10	Dib.IN.DWR08.Pe.20 ß*	**1779**	**1196**	**1077**	**605**	A	*P. erythrorhynchos*	3/10/08	Canada
Bolbophorus sp.	C	D	FLUKE630-11	Dib.IN.DWR08.Pe.26		**1161**	**1093**		A	*P. erythrhynchos*	3/10/08	Canada
Clinostomum	Cl	C	FLUKE629-11	C.LM.G.RTO.1.4.1	**1758**	**1162**			M	Gobidae	3/3/10	Mexico
Clinostomum complanatum	Cl	C	CLINO036-10	C.Sc.ITA3.6 ß***			**1027**	**610**	M	*Squalius cephalus*	1/1/98	Italy
Clinostomum marginatum	Cl	C	CLINO013-10	Cm.M.2.R.5.1 ß***			**1042**	**619**	M	*Ambloplites rupestris*	1/6/06	Canada
Cotylurus	S	S	TREMA2613-10	S.IN.Ana.DWR9.1 ß*	**1291**	**1114**		**618**	A	*Anas acuta*	1/9/09	Canada
Cotylurus	S	S	TREMA2606-10	S.IN.Ao.EMR.1.3 ß*	**787**				A	*Asio otus*	unknown	Italy
Cotylurus	S	S	TREMA2616-10	S.IN.Oxj.DWR9.1.2 ß*	**1355**	**1303**		**623**	A	*Oxyura jamaicensis*	1/9/09	Canada
Cotylurus	S	S	TREMA2617-10	S.IN.Oxj.DWR9.1.3 ß*			**1192**	**590**	A	*O. jamaicensis*	1/9/09	Canada
Diplostomum	D	D	TREMA2772-10	D.L.Cyc.CTA.1.1	**1771**	**1119**		**492**		*Cyprinus carpio*	unknown	Croatia

Appendix 1. Continued

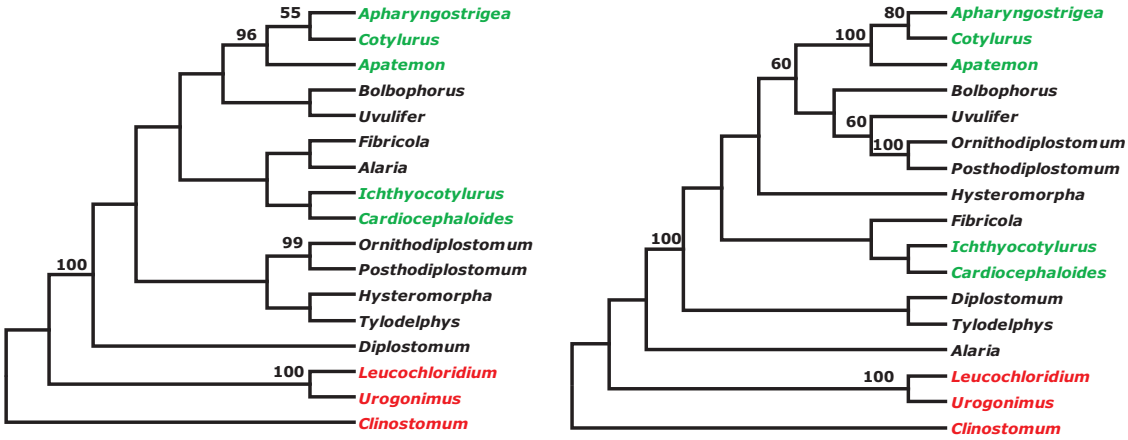
Genus and species	Sub-family	Family	BOLD process ID	Sample ID	Molecular markers SSU	LSU	ITS	COI	Life stage	Host info	Collected (dd/mm/yy)	Country
Diplostomum	D	D	TREMA2772-10	D.L.Cyc.CTA.1.1	**1771**	**1119**		**492**		*Cyprinus carpio*	unknown	Croatia
Diplostomum	D	D	FLUKE640-11	D.LL.Cc.IBE8.2F.1	**1724**				M	*Catostomus commersonii*	13/6/08	Canada
Diplostomum sp. 1	D	D	TREMA2346-10	D.LL.Na.SCE.17.1 **ß***	**1060**	**1204**	**1064**	**463**	M	*Notropis atherinoides*	12/6/09	Canada
Diplostomum sp. 1	D	D	TREMA2244-10	D.LL.Nh.DRI.12.1 **ß***			**1111**	**430**	M	*N. hudsonius*	25/9/08	Canada
Diplostomum sp. 1	D	D	TREMA2215-10	D.LL.Po.UW1.01.1 **ß***			**1036**	**463**	M	*Percopsis omiscomaycus*	21/9/09	Canada
Diplostomum sp. 10	D	D	TREMA2185-10	D.RH.Ppr.LCR.3.1 **ß***	**1245**	**1148**	**1147**	**363**	M	*Pimephales promelas*	12/9/09	Canada
Diplostomum sp. 2	D	D	TREMA2340-10	D.BR.Na.SCE.22.1 **ß***	**934**	**1204**	**1011**	**463**	M	*N. atherinoides*	12/6/09	Canada
Diplostomum sp. 4	D	D	TREMA2409-10	D.L.Po.NTH.12.7 **ß***			**1015**	**463**	M	*P. omiscomaycus*	23/9/09	Canada
Diplostomum sp. 4	D	D	TREMA2240-10	D.LL.Nh.DRI.35.2 **ß***		**1211**		**463**	M	*N. hudsonius*	25/9/08	Canada
Diplostomum sp. 4	D	D	TREMA2412-10	D.LL.Po.UW1.08.2 **ß***	**938**	**1193**	**1077**	**463**	M	*P. omiscomaycus*	21/9/09	Canada
Fibricola sp. 1	A	D	TREMA2435-10	F.Ra.HMB05.3 **ß****				**577**	M	*Lithobates aurora*	25/8/09	USA
Fibricola sp. 1	A	D	TREMA2436-10	F.Ra.HMB05.4 **ß****	**1700**	**1194**	**1118**	**602**	M	*L. aurora*	25/8/09	USA
Hysteromorpha triloba	D	D	TREMA2419-10	Di.M.Nh.Sor.03.1 **ß****	**888**	**1195**	**1017**	**463**	M	*N. hudsonius*	11/6/07	Canada
Hysteromorpha triloba	D	D	FLUKE634-11	H.LM.IBE.Cc.1F.3.1	**1840**	**1146**	**1147**		M	*C. commersonii*	13/6/08	Canada
Ichthyocotylurus erraticus	S	S	TREMA2597-10	I.HT.Ccl.REL.2 **ß***	**1656**	**1144**	**419**	**606**	M	*Coregonus clupeaformis*	1/9/09	Canada
Ichthyocotylurus platycephalus	S	S	TREMA2260-10	I.BC.Nh.DRI.28.1 **ß***	**985**	**1229**		**617**	M	*N. hudsonius*	25/9/08	Canada
Ichthyocotylurus platycephalus	S	S	TREMA2272-10	I.BC.Nh.DRI.35.2 **ß***	**1323**	**1198**	**353**	**553**	M	*N. hudsonius*	25/9/08	Canada
Ornithodiplostomum	C	D	TREMA2610-10	Cty.BC.Gm.CPO.1.1 **ß***	**1559**	**1148**		**438**	M	*Gambusia affinis*	unknown	USA
Ornithodiplostomum	C	D	TREMA2612-10	Cty.BC.Gm.CPO.1.3 **ß***			**1129**	**438**	M	*G. affinis*	unknown	USA
Ornithodiplostomum sp. 3	C	D	TREMA2588-10	O.BC.G.LJA.2.1 **ß***			**1169**	**431**	M	*Notemigonus crysoleucas*	20/7/09	Canada
Ornithodiplostomum	C	D	TREMA2593-10	O.BC.Nc.LJA.5.1 **ß***	**1557**	**1146**	**1182**	**437**	M	*Notropis cornutus*	20/7/09	Canada
Ornithodiplostomum sp. 3	C	D	TREMA2594-10	O.BC.Nc.LJA.5.2 **ß***	**1650**	**1141**		**550**	M	*N. cornutus*	20/7/09	Canada
Ornithodiplostomum	C	D	TREMA2592-10	O.BC.Nc.LJA.6.2 **ß***	**1649**	**1150**		**436**	M	*N. cornutus*	20/7/09	Canada
Ornithodiplostomum	C	D	TREMA2578-10	O.BC.Nh.LJA.6.3 **ß***	**1752**	**1219**	**1169**	**269**	M	*N. hudsonius*	20/7/09	Canada
Ornithodiplostomum	C	D	TREMA2599-10	O.BR.Ppr.FEP.4.1 **ß***	**1366**	**1150**	**1156**	**436**	M	*Pimephales promelas*	1/9/09	Canada
Ornithodiplostomum	C	D	TREMA2584-10	O.LV.B.LJA.5.1 **ß***	**1763**	**1205**	**1190**	**437**	M	*Pimephales notatus*	20/7/09	Canada
Ornithodiplostomum	C	D	TREMA2585-10	O.LV.B.LJA.8.1 **ß***	**1767**	**1215**	**1181**	**436**	M	*P. notatus*	20/7/09	Canada
Ornithodiplostomum sp. 3	C	D	TREMA2281-10	O.BC.Na.SCE.04.3 **ß***	**745**	**1184**	**1166**	**591**	M	*N. atherinoides*	12/6/09	Canada
Posthodiplostomum	C	D	FLUKE636-11	P.BC.Nh.FAC.26.1.1	**1776**				M	*N. hudsonius*	22/9/08	Canada
Posthodiplostomum sp. 3	C	D	TREMA2248-10	P.LV.S.HWM.1.1 **ß***			**1151**	**542**	M	*Lepomis gibbosus*	24/9/09	Canada
Posthodiplostomum sp. 3	C	D	TREMA2251-10	P.LV.S.HWM.1.4 **ß***			**993**	**527**	M	*L. gibbosus*	24/9/09	Canada
Posthodiplostomum sp. 3	C	D	TREMA2254-10	P.LV.S.HWM.1.7 **ß***			**1094**	**543**	M	*L. gibbosus*	24/9/09	Canada
Posthodiplostomum sp. 5	C	D	TREMA2561-10	P.BC.S.LCR.3.2 **ß***	**1056**	**1169**	**1094**	**590**	M	*L. gibbosus*	12/9/09	Canada
Posthodiplostomum sp. 5	C	D	TREMA2567-10	P.RM.S.LCR.7.1 **ß***			**1071**	**590**	M	*L. gibbosus*	12/9/09	Canada
Tylodelphys	D	D	FLUKE633-11	H.IN.Pa.OSP.1.1	**1783**	**1125**			A	*Phalacrocorax auritus*	unknown	USA
Tylodelphys	D	D	FLUKE637-11	T.H.On.Ky.X.1.1	**1471**	**1136**			M	*Oreochormis niloticus niloticus*	unknown	Kenya
Tylodelphys scheuringi	D	D	TREMA2181-10	T.LH.Sf.RBI.5.1 **ß***				**463**	M	*Salvelinus fontinalis*	17/7/09	Canada

Appendix 2. Individual and total evidence analyses of the full small subunit (SSU), partial large subunit (LSU) and full internal transcribed spacer regions 1 and 2 (ITS) of ribosomal DNA and the barcode region of cytochrome oxidase I (COI) of mitochondrial DNA using methods of maximum parsimony (MP) and maximum likelihood (ML). Bootstrap values greater than 50% are shown above the branches and are based on 1000 replicates for the MP and 10 replicates for the ML. In cases when more than one tree was equally parsimonious or likely, the tree presented is a strict consensus tree. Conflicting branches in the strict consensus were shown as resolved if the bootstrap support was greater than 50%. Outgroup taxa are red, strigeid taxa green and diplostomid taxa black.

Tree 1

SSU	
Ingroup taxa	14
Outgroup taxa	3
Total number of characters	1722

Tree 2



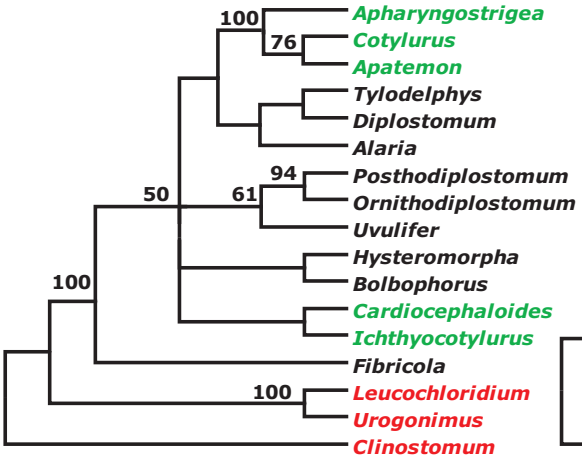
MP Results:	
Constant	1467
Parsimony informative	162
Parsimony uninformative	93
Number of equally parsimonious trees	1
Length	468
Consistency index	0.6624
Retention index	0.6040
Rescaled consistency index	0.4001

ML Results:	
Model	GTR+G+I model
Likelihood (-ln)	4840.14361
Number of equally likely trees	1

Appendix 2. Continued

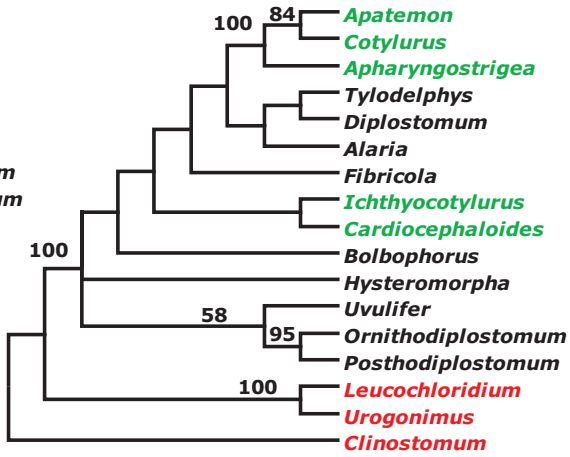
Tree 3

LSU	
Ingroup taxa	14
Outgroup taxa	3
Total number of characters	1147



MP Results:	
Constant	744
Parsimony informative	267
Parsimony uninformative	136
Number of equally parsimonious trees	4
Length	811
Consistency index	0.6054
Retention index	0.5315
Rescaled consistency index	0.3218

Tree 4



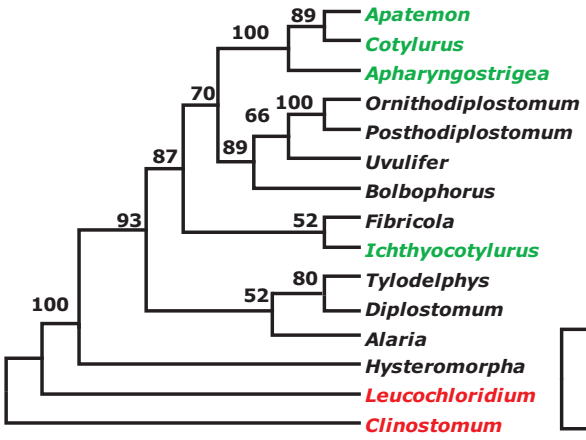
ML Results:	
Model	HKY85 model
Likelihood (-ln)	5328.1932
Number of equally likely trees	1

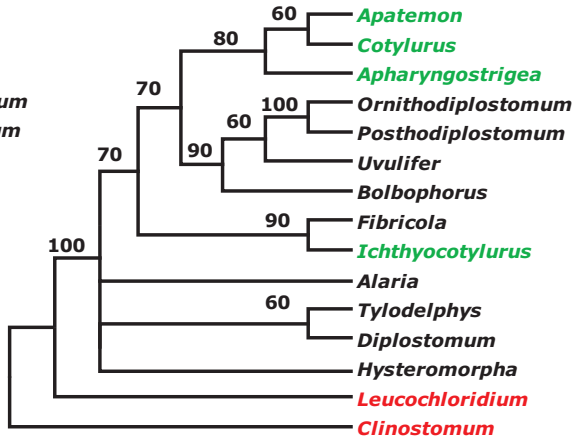
Appendix 2. Continued

Tree 5

ITS	
Ingroup taxa	13
Outgroup taxa	2
Total number of characters	1225

Tree 6





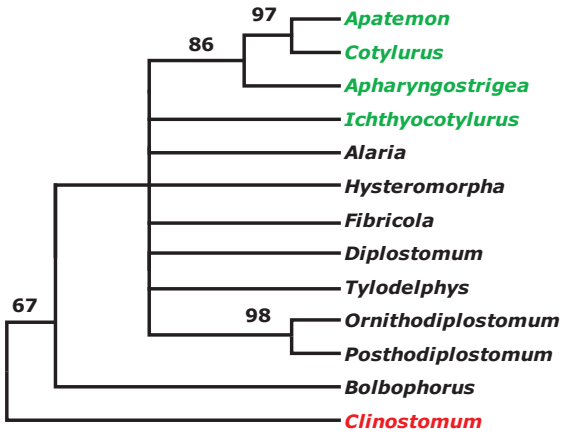
MP Results:	
Constant	572
Parsimony informative	383
Parsimony uninformative	270
Number of equally parsimonious trees	2
Length	1444
Consistency index	0.6820
Retention index	0.5234
Rescaled consistency index	0.3570

ML Results:	
Model	GTR+G model
Likelihood (-ln)	7513.84035
Number of equally likely trees	2

Appendix 2. Continued

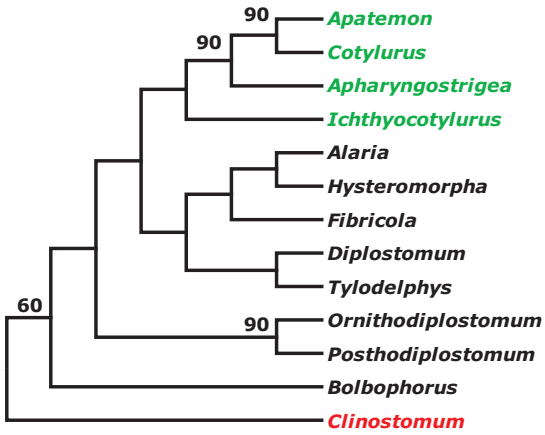
Tree 7

COI	
Ingroup taxa	12
Outgroup taxa	1
Total number of characters	468



MP Results:	
Constant	241
Parsimony informative	154
Parsimony uninformative	73
Number of equally parsimonious trees	5
Length	620
Consistency index	0.5613
Retention index	0.3645
Rescaled consistency index	0.2046

Tree 8

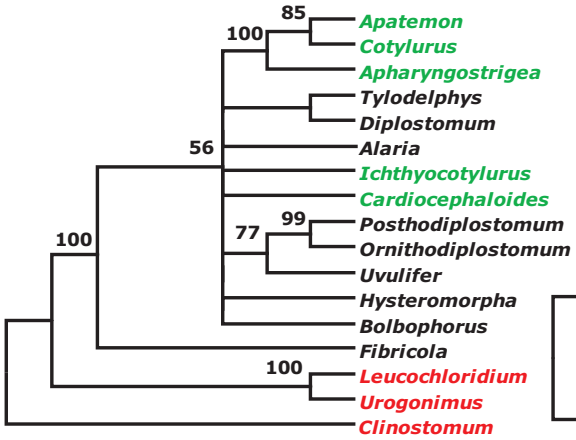


ML Results:	
Model	GTR+G+I model
Likelihood (-ln)	3058.87013
Number of equally likely trees	1

Appendix 2. Continued

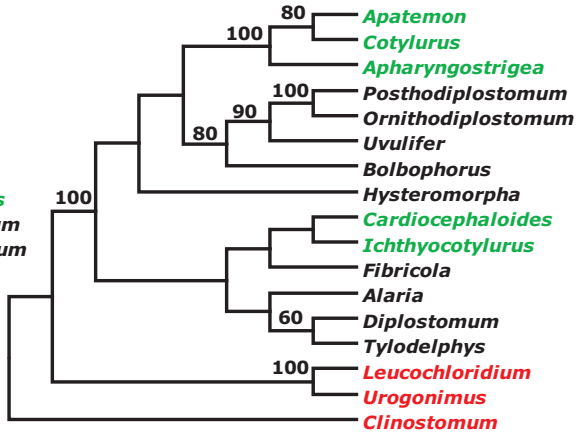
Tree 9

SSU-LSU	
Ingroup taxa	14
Outgroup taxa	3
Total number of characters	2869



MP Results:	
Constant	2211
Parsimony informative	429
Parsimony uninformative	229
Number of equally parsimonious trees	9
Length	1291
Consistency index	0.6204
Retention index	0.5471
Rescaled consistency index	0.3395

Tree 10



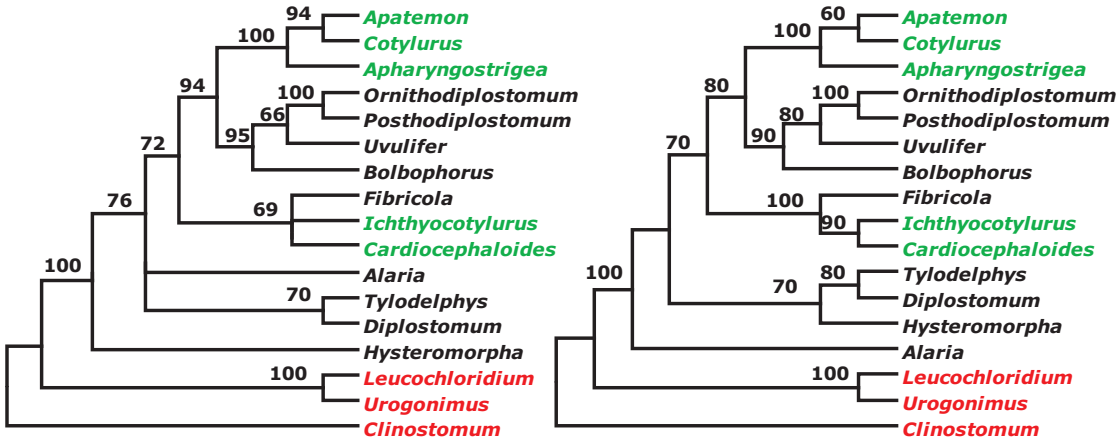
ML Results:	
Model	GTR+G+I model
Likelihood (-ln)	10308.25745
Number of equally likely trees	1

Appendix 2. Continued

Tree 11

SSU-ITS	
Ingroup taxa	14
Outgroup taxa	3
Total number of characters	2947

Tree 12



MP Results:	
Constant	2039
Parsimony informative	545
Parsimony uninformative	363
Number of equally parsimonious trees	2
Length	1919
Consistency index	0.655
Retention index	0.5231
Rescaled consistency index	0.3426

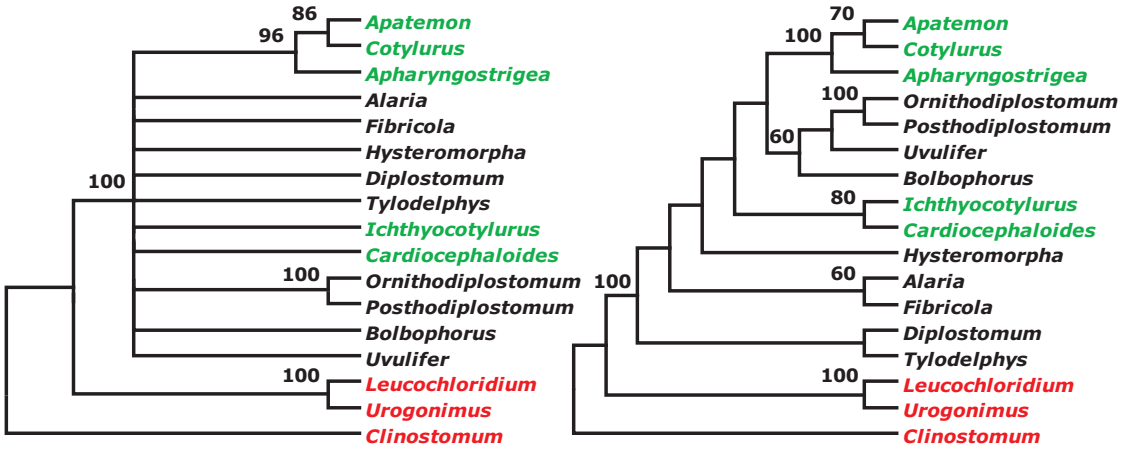
ML Results:	
Model	GTR+G+I model
Likelihood (-ln)	12740.88108
Number of equally likely trees	1

Appendix 2. Continued

Tree 13

SSU-COI	
Ingroup taxa	14
Outgroup taxa	3
Total number of characters	2190

Tree 14



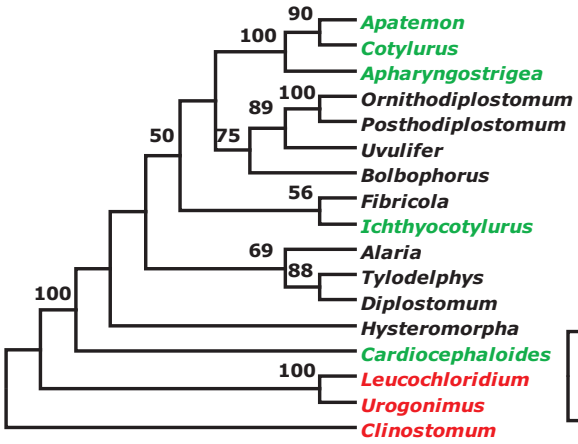
MP Results:	
Constant	1708
Parsimony informative	316
Parsimony uninformative	166
Number of equally parsimonious trees	6
Length	1100
Consistency index	0.5982
Retention index	0.4655
Rescaled consistency index	0.2785

ML Results:	
Model	GTR+G+I model
Likelihood (-ln)	8213.11644
Number of equally likely trees	1

Appendix 2. Continued

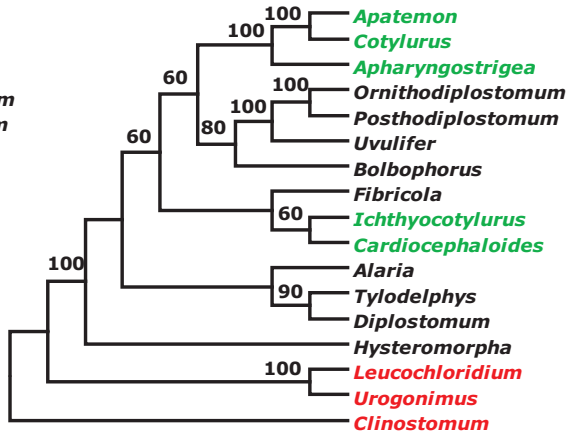
Tree 15

LSU-ITS	
Ingroup taxa	14
Outgroup taxa	3
Total number of characters	2372



MP Results:	
Constant	1316
Parsimony informative	650
Parsimony uninformative	406
Number of equally parsimonious trees	1
Length	2269
Consistency index	0.6338
Retention index	0.5030
Rescaled consistency index	0.3188

Tree 16

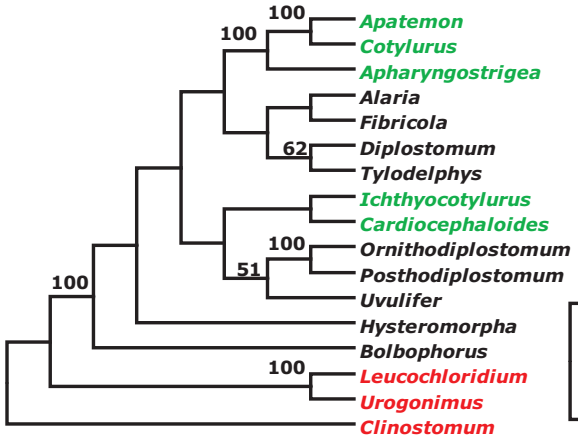


ML Results:	
Model	GTR+G+I model
Likelihood (-ln)	13027.05399
Number of equally likely trees	1

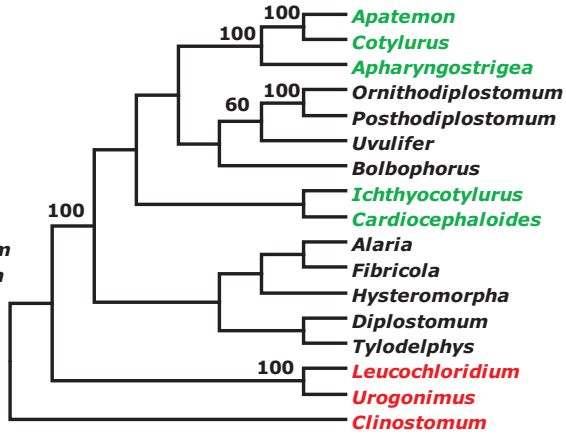
Appendix 2. Continued

Tree 17

LSU-COI	
Ingroup taxa	14
Outgroup taxa	3
Total number of characters	1615



Tree 18



MP Results:	
Constant	985
Parsimony informative	421
Parsimony uninformative	209
Number of equally parsimonious trees	1
Length	1445
Consistency index	0.5806
Retention index	0.4545
Rescaled consistency index	0.2639

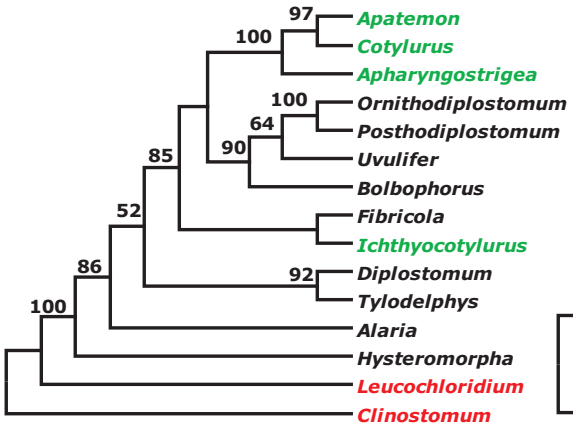
ML Results:	
Model	GTR+G+I model
Likelihood (-ln)	8616.40413
Number of equally likely trees	2

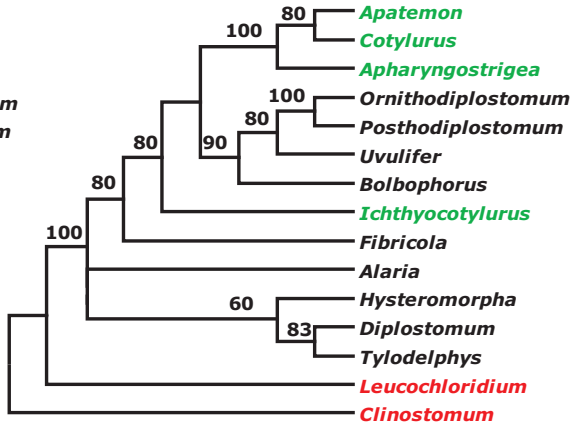
Appendix 2. Continued

Tree 19

ITS-COI	
Ingroup taxa	13
Outgroup taxa	2
Total number of characters	1693

Tree 20





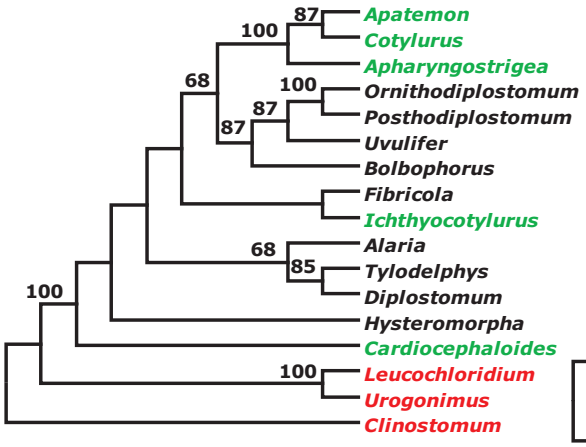
MP Results:	
Constant	843
Parsimony informative	537
Parsimony uninformative	343
Number of equally parsimonious trees	1
Length	2078
Consistency index	0.6232
Retention index	0.4474
Rescaled consistency index	0.2788

ML Results:	
Model	GTR+G+I model
Likelihood (-ln)	10738.10398
Number of equally likely trees	3

Appendix 2. Continued

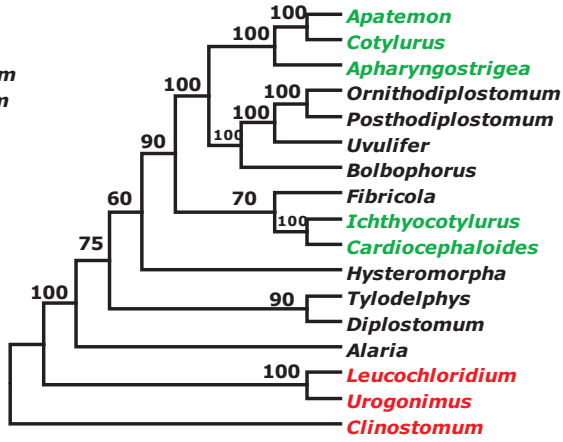
Tree 21

SSU-LSU-ITS	
Ingroup taxa	14
Outgroup taxa	3
Total number of characters	4094



MP Results:	
Constant	2783
Parsimony informative	812
Parsimony uninformative	499
Number of equally parsimonious trees	1
Length	2747
Consistency index	0.6363
Retention index	0.5176
Rescaled consistency index	0.3294

Tree 22



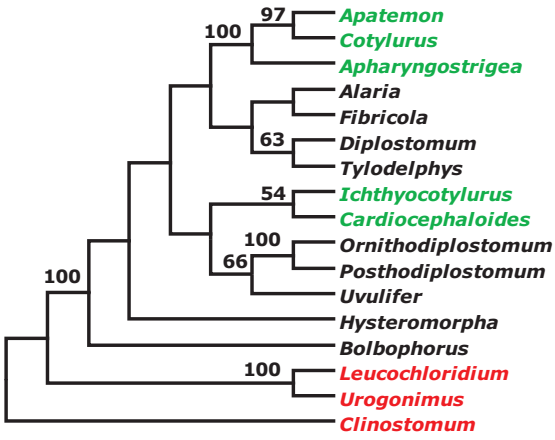
ML Results:	
Model	GTR+G+I model
Likelihood (-ln)	18174.36943
Number of equally likely trees	1

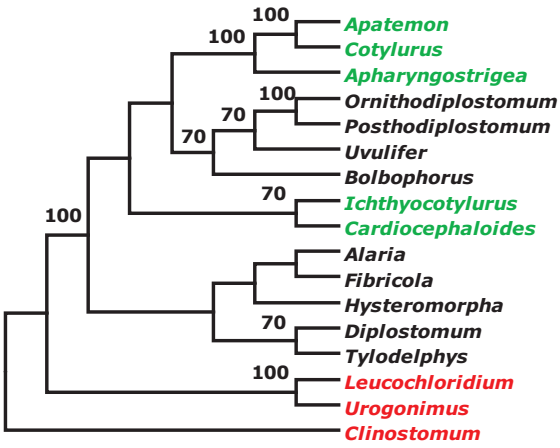
Appendix 2. Continued

Tree 23

SSU-LSU-COI	
Ingroup taxa	14
Outgroup taxa	3
Total number of characters	3337

Tree 24





MP Results:	
Constant	2452
Parsimony informative	583
Parsimony uninformative	302
Number of equally parsimonious trees	1
Length	1927
Consistency index	0.5668
Retention index	0.4185
Rescaled consistency index	0.2373

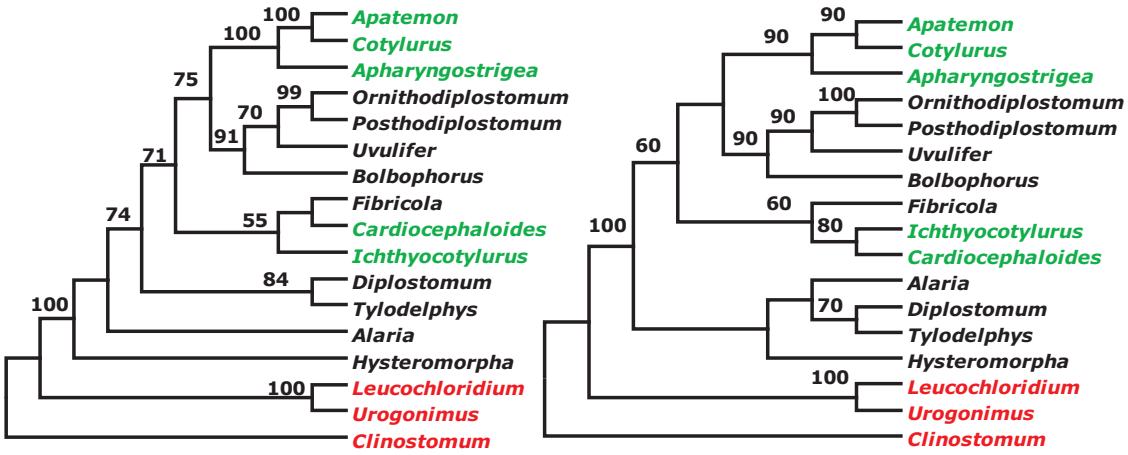
ML Results:	
Model	GTR+G+I model
Likelihood (-ln)	13696.74354
Number of equally likely trees	1

Appendix 2. Continued

Tree 25

SSU-ITS-COI	
Ingroup taxa	14
Outgroup taxa	3
Total number of characters	3415

Tree 26



MP Results:	
Constant	2280
Parsimony informative	699
Parsimony uninformative	436
Number of equally parsimonious trees	2
Length	2554
Consistency index	0.6284
Retention index	0.4774
Rescaled consistency index	0.3000

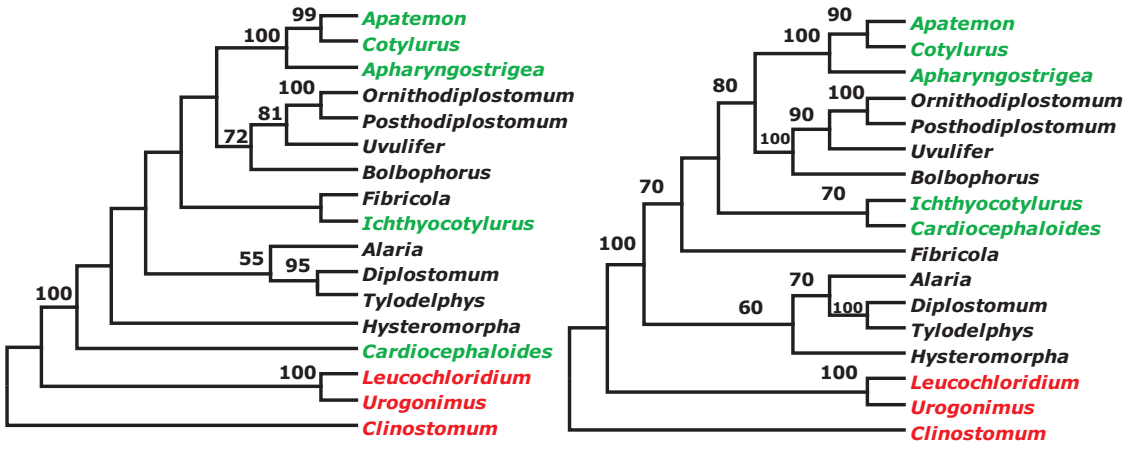
ML Results:	
Model	GTR+G+I model
Likelihood (-ln)	16139.51087
Number of equally likely trees	1

Appendix 2. Continued

Tree 27

LSU-ITS-COI	
Ingroup taxa	14
Outgroup taxa	3
Total number of characters	2840

Tree 28



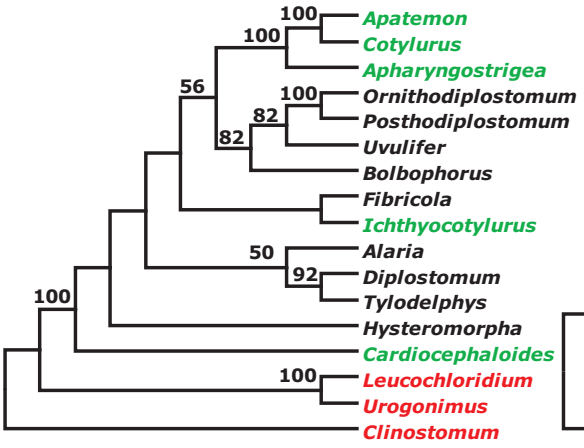
MP Results:	
Constant	1557
Parsimony informative	804
Parsimony uninformative	479
Number of equally parsimonious trees	2
Length	2906
Consistency index	0.6146
Retention index	0.4667
Rescaled consistency index	0.2868

ML Results:	
Model	GTR+G+I model
Likelihood (-ln)	16294.45095
Number of equally likely trees	1

Appendix 2. Continued

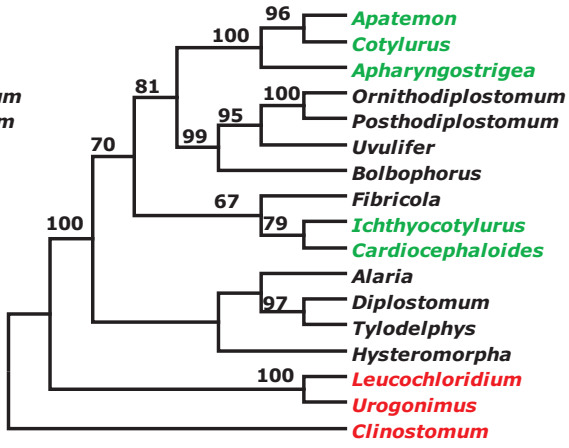
Tree 29

SSU-LSU-ITS-COI	
Ingroup taxa	14
Outgroup taxa	3
Total number of characters	4562



MP Results:	
Constant	3024
Parsimony informative	966
Parsimony uninformative	572
Number of equally parsimonious trees	1
Length	3384
Consistency index	0.6194
Retention index	0.4846
Rescaled consistency index	0.3002

Tree 30



ML Results:	
Model	GTR+G+I model
Likelihood (-ln)	21503.85403
Number of equally likely trees	2

Appendix 3. Adult and larval morphological characters, life cycle and host specificity data based on Shoop's (1989) cladistic analysis and Niewiadomska's (2002a, 2002b) classification used in the character mapping analyses. Outgroups are outlined in blue.

		Alaria	*Apatemon*	*Apharyngostrigea*	*Bolbophorus*	*Cardiocephaloides*	*Clinostomum*	*Cotylurus*	*Diplostomum*	*Fibricola*	*Hysteromorpha*	*Ichthyocotylurus*	*Leucochloridium*	*Ornithdiplostomum*	*Posthodiplostomum*	*Tylodelphys*	*Urogonimus*	*Uvulifer*
Cercaria	**Flame cell**	4	0	1	1	1	?	1	2	3	1	1	?	2	1	2	?	2
1	0: 10 1: 20 2: 16 3: 12 4: 24 ?: unknown																	
2	**Develop in:** 0: branched sporocysts 1: redia 2: sporocysts	2	2	2	2	2	1	2	2	2	2	2	0	2	2	2	0	2
Mesocercariae	**Mesocercaria**	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0: absent 1: present																	
Metacercariae	**Type:**	2	1	1	0	1	9	1	2	2	2	1	9	0	0	2	9	0
4	0: neascus 1: tetracotyle 2: diplostomulum 9: unapplicable																	
5	**Body shape** 0: unsegmented 1: bisegmented	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1
6	**Encystment** 0: present 1: lost	1	0	0	0	0	0	0	1	1	1	0	0	0	0	1	0	0
7	**Forebody** 0: spathulate 1: cup-shaped 9: unapplicable	0	1	1	0	1	9	1	0	0	0	1	9	0	0	0	9	0
8	**Pseudosuckers** 0: absent 1: present	1	1	1	1	1	0	1	1	0	1	1	0	0	0	1	0	0
9	**Paranephridial plexus** 0: 3 logitudinal vessels with many anastomoses 1: 3 longitudinal vessels, but some anastomoses form numerous distinct transverse commissures 2: 3 longitudinal vessels, transverse commissures reduced to 3 or fewer, some small anastomoses still present 3: 3 longitudinal vessels, 3 or fewer transverse commissures, but other anastomises lost ?: unknown	3	3	3	2	3	?	3	3	3	3	3	?	1	1	3	?	1
10	**Limebodies** 0: free 1: enclosed ?: Unknown	1	0	0	0	0	?	0	1	1	1	0	?	0	0	1	?	0

Appendix 3. Continued

		Alaria	*Apatemon*	*Apharyngostrigea*	*Bolbophorus*	*Cardiocephaloides*	*Clinostomum*	*Cotylurus*	*Diplostomum*	*Fibricola*	*Hysteromorpha*	*Ichthyocotylurus*	*Leucochloridium*	*Ornithdiplostomum*	*Posthodiplostomum*	*Tylodelphys*	*Urogonimus*	*Uvulifer*
Adult	**Body shape**	0	0	0	0	0	1	0	0	0	2	0	1	1	0	1	1	0
11	0: bipartite 1: linguiform 2: pyriform (pear-shaped)																	
12	**Hindbody length** 0: very long (5-25 times longer than forebody, long, slender neck region) 1: moderate length («6 times longer than forebody, neck region small or absent) 9: unapplicable	1	1	1	1	0	9	1	1	1	1	1	9	1	1	1	9	0
13	**Forebody:** 0: flattened 1: tubular 9: Unapplicable	0	1	1	0	1	9	1	0	0	0	1	9	0	0	0	9	0
14	**Forebody morphology** 0: spathulate 1: cup-shaped 2: pear-shaped 9: Unapplicable	0	1	2	0	2	9	1	0	0	0	1	9	0	0	0	9	0
15	**Pseudosuckers** 0: absent 1: present 2: vestigial or lost	1	1	2	1	2	0	1	1	0	1	0	0	0	0	1	0	0
16	**Pharynx** 0: present 1: lost	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	**Tribocytic organ** 0: present 1: absent	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
18	**Tribocytic organ shape** 0: spherical 1: lingual (oval) 2: bilobate 9: Unapplicable	1	2	2	0	2	9	2	0	0	0	2	9	0	0	0	9	0
19	**Viteline distribution** 0: whole body 1: forebody 2: hindbody 3: other	1	2	0	0	2	3	2	0	1	0	2	3	0	0	0	3	2
20	**Hermaphroditic duct** 0: present 1: other	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
21	**Testicular position** 0: tandem 1: opposite	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	**Testicular shape:** 0: spherical 1: lobate 2: secondarily spherical 3: elongate	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1

Appendix 3. Continued

		Alaria	*Apatemon*	*Apharyngostrigea*	*Bolbophorus*	*Cardiocephaloides*	*Clinostomum*	*Cotylurus*	*Diplostomum*	*Fibricola*	*Hysteromorpha*	*Ichthyocotylurus*	*Leucochloridium*	*Ornithdiplostomum*	*Posthodiplostomum*	*Tylodelphys*	*Urogonimus*	*Uvulifer*
23	**Genital prepuce** 0: absent 1: present	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
24	**Cirrus sac** 0: absent 1: other	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
25	**Genital pore** 0: subterminal 1: other	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
26	**Copulatory bursa:** 0: absent 1: protrusible 2: not protrusible	2	1	1	2	1	0	1	2	2	2	1	0	1	1	2	0	1
27	**Genital cone** 0: absent 1: present	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	0	1
28	**Genital bulb** 0: absent 1: present	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0
29	**Ovarian location** 0: intertesticular 1: pretesticular	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1
Host 30	**Number of intermediate hosts:** 0: 1 1: 2 or more	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1
31	**Metacercarial host:** 0: snails and leeches 1: fish and leeches 3: fish 4: fish and amphibians 5: amphibians	5	1	4	3	3	4	0	4	5	3	3	0	3	3	4	0	3
32	**Final host** 0: birds 1: birds and mammals 2: mammals	2	0	0	0	0	1	0	0	2	0	0	0	0	0	0	0	0

Appendix 4. Base pair differences between taxa for the molecular markers of the full small subunit (SSU), partial large subunit (LSU) and full internal transcribe spacer regions 1 and 2 (ITS) of ribosomal DNA and the barcode region of cytochrome oxidase I (COI) of mitochondrial DNA calculated in MEGA version 4.0.2 (Tamura *et al*., 2007). Distances in the lower left are number of nucleotide differences based on complete deletion. Distances in the upper right are percentages. The final data set (with gaps and missing data removed from the calculation) for the SSU, LSU, ITS and COI datasets are 1722, 1066, 524 and 430 respectively. Outgroup taxa are red, strigeid taxa green and diplostomid taxa black.

A. SSU dataset

	Alaria	*Apatemon*	*Apharyngostrigea*	*Bolbophorus*	*Cardiocephaloides*	*Clinostomum*	*Cotylurus*	*Diplostomum*	*Fibricola*	*Hysteromorpha*	*Ichthyocotylurus*	*Leucochloridium*	*Ornithodiplostomum*	*Posthodiplostostomum*	*Tylodelphys*	*Urogonimus*	*Uvulifer*
Alaria	-	0.81	1.10	1.05	0.99	4.76	1.16	0.75	1.10	1.22	0.52	4.70	0.93	1.16	0.99	4.30	1.28
Apatemon	14	-	0.75	1.22	1.51	4.59	0.70	1.05	1.63	1.34	0.93	4.82	1.10	1.22	1.16	4.41	1.39
Apharyngostrigea	19	13	-	1.39	1.80	4.76	1.05	1.28	1.63	1.63	1.22	4.82	1.28	1.28	1.57	4.65	1.68
Bolbophorus	18	21	24	-	1.57	4.59	1.63	1.22	1.92	1.45	0.87	4.70	1.05	1.16	0.99	4.41	1.45
Cardiocephaloides	17	26	31	27	-	4.82	1.97	1.39	1.97	1.74	0.81	4.82	1.28	1.51	1.39	4.30	1.51
Clinostomum	82	79	82	79	83	-	5.11	4.59	4.47	4.88	4.47	4.94	4.70	4.76	4.65	4.65	4.70
Cotylurus	20	12	18	28	34	88	-	1.51	1.92	1.92	1.39	5.17	1.57	1.68	1.74	4.76	1.86
Diplostomum	13	18	22	21	24	79	26	-	1.45	1.22	0.81	4.76	1.10	1.22	1.05	4.24	1.39
Fibricola	19	28	28	33	34	77	33	25	-	2.03	1.39	4.94	1.80	1.92	1.74	4.30	1.80
Hysteromorpha	21	23	28	25	30	84	33	21	35	-	1.16	4.76	1.22	1.34	1.16	4.59	1.80
Ichthyocotylurus	9	16	21	15	14	77	24	14	24	20	-	4.65	0.87	0.99	0.70	4.12	1.22
Leucochloridium	81	83	83	81	83	85	89	82	85	82	80	-	4.70	4.70	4.65	1.16	4.65
Ornithodiplostomum	16	19	22	18	22	81	27	19	31	21	15	81	-	0.23	1.10	4.47	0.99
Posthodiplostostomum	20	21	22	20	26	82	29	21	33	23	17	81	4	-	1.22	4.47	1.22
Tylodelphys	17	20	27	17	24	80	30	18	30	20	12	80	19	21	-	4.36	1.51
Urogonimus	74	76	80	76	74	80	82	73	74	79	71	20	77	77	75	-	4.12
Uvulifer	22	24	29	25	26	81	32	24	31	31	21	80	17	21	26	71	-

Appendix 4. Continued

B. LSU dataset

	Alaria	*Apatemon*	*Apharyngostrigea*	*Bolbophorus*	*Cardiocephaloides*	*Clinostomum*	*Cotylurus*	*Diplostomum*	*Fibricola*	*Hysteromorpha*	*Ichthyocotylurus*	*Leucochloridium*	*Ornithodiplostomum*	*Posthodiplostostomum*	*Tylodelphys*	*Urogonimus*	*Uvulifer*
Alaria	-	4.78	4.32	4.03	3.38	16.23	5.35	3.47	4.88	4.32	4.41	17.64	5.91	6.19	4.22	15.38	4.78
Apatemon	51	-	1.78	5.44	5.35	17.35	2.63	5.16	6.47	6.00	5.63	17.54	7.69	7.41	6.00	17.07	5.91
Apharyngostrigea	46	19	-	5.25	4.97	17.35	3.38	5.16	6.29	5.82	5.63	17.54	7.22	7.04	5.53	16.70	5.91
Bolbophorus	43	58	56	-	3.75	16.89	6.10	4.88	6.00	3.94	4.69	17.92	6.29	6.38	5.44	16.32	4.97
Cardiocephaloides	36	57	53	40	-	16.04	6.19	4.41	5.16	4.50	3.66	18.01	6.10	6.38	4.97	16.23	4.60
Clinostomum	173	185	185	180	171	-	17.73	16.98	16.23	17.07	17.07	17.35	16.51	17.26	16.70	16.04	16.89
Cotylurus	57	28	36	65	66	189	-	6.19	7.22	6.47	5.91	18.11	7.97	7.69	6.75	17.64	6.47
Diplostomum	37	55	55	52	47	181	66	-	6.00	4.69	4.60	18.39	6.57	6.19	4.41	16.60	4.60
Fibricola	52	69	67	64	55	173	77	64	-	6.10	6.00	18.76	8.35	8.16	6.38	17.45	6.75
Hysteromorpha	46	64	62	42	48	182	69	50	65	-	4.78	18.67	6.47	7.13	5.91	16.32	4.97
Ichthyocotylurus	47	60	60	50	39	182	63	49	64	51	-	18.29	6.66	7.04	5.35	16.51	5.35
Leucochloridium	188	187	187	191	192	185	193	196	200	199	195	-	18.48	19.04	18.67	8.72	19.14
Ornithodiplostomum	63	82	77	67	65	176	85	70	89	69	71	197	-	4.50	6.85	16.51	5.82
Posthodiplostostomum	66	79	75	68	68	184	82	66	87	76	75	203	48	-	7.41	17.07	6.19
Tylodelphys	45	64	59	58	53	178	72	47	68	63	57	199	73	79	-	16.98	6.10
Urogonimus	164	182	178	174	173	171	188	177	186	174	176	93	176	182	181	-	17.82
Uvulifer	51	63	63	53	49	180	69	49	72	53	57	204	62	66	65	190	-

C. ITS dataset

	Alaria	*Apatemon*	*Apharyngostrigea*	*Bolbophorus*	*Clinostomum*	*Cotylurus*	*Diplostomum*	*Fibricola*	*Hysteromorpha*	*Ichthyocotylurus*	*Leucochloridium*	*Ornithodiplostomum*	*Posthodiplostostomum*	*Tylodelphys*	*Uvulifer*
Alaria	-	11.64	12.79	12.60	31.11	12.40	8.59	10.50	13.17	12.21	33.78	13.93	12.60	8.21	12.79
Apatemon	61	-	4.96	11.83	32.06	4.01	11.26	10.69	15.27	11.26	34.16	12.79	11.83	11.26	11.83
Apharyngostrigea	67	26	-	14.12	32.44	5.92	13.36	11.83	16.60	11.83	34.92	12.60	12.60	12.40	13.17
Bolbophorus	66	62	74	-	33.78	13.17	13.93	12.21	17.18	12.98	34.16	11.45	9.35	13.74	13.74
Clinostomum	163	168	170	177	-	32.63	30.53	33.21	29.96	32.44	33.59	33.40	33.78	29.58	31.11
Cotylurus	65	21	31	69	171	-	11.83	11.45	14.89	12.02	33.97	12.79	12.60	12.21	12.79
Diplostomum	45	59	70	73	160	62	-	11.83	11.26	12.40	34.73	14.31	13.74	6.87	13.36
Fibricola	55	56	62	64	174	60	62	-	14.69	9.92	33.02	11.26	10.69	11.64	12.79
Hysteromorpha	69	80	87	90	157	78	59	77	-	15.08	32.44	15.84	16.41	11.45	14.89
Ichthyocotylurus	64	59	62	68	170	63	65	52	79	-	32.25	12.79	12.02	11.64	13.17
Leucochloridium	177	179	183	179	176	178	182	173	170	169	-	34.16	34.16	35.11	33.78
Ornithodiplostomum	73	67	66	60	175	67	75	59	83	67	179	-	2.86	15.46	12.79
Posthodiplostostomum	66	62	66	49	177	66	72	56	86	63	179	15	-	14.50	12.02
Tylodelphys	43	59	65	72	155	64	36	61	60	61	184	81	76	-	12.79
Uvulifer	67	62	69	72	163	67	70	67	78	69	177	67	63	67	-

Appendix 4. Continued

D. COI dataset

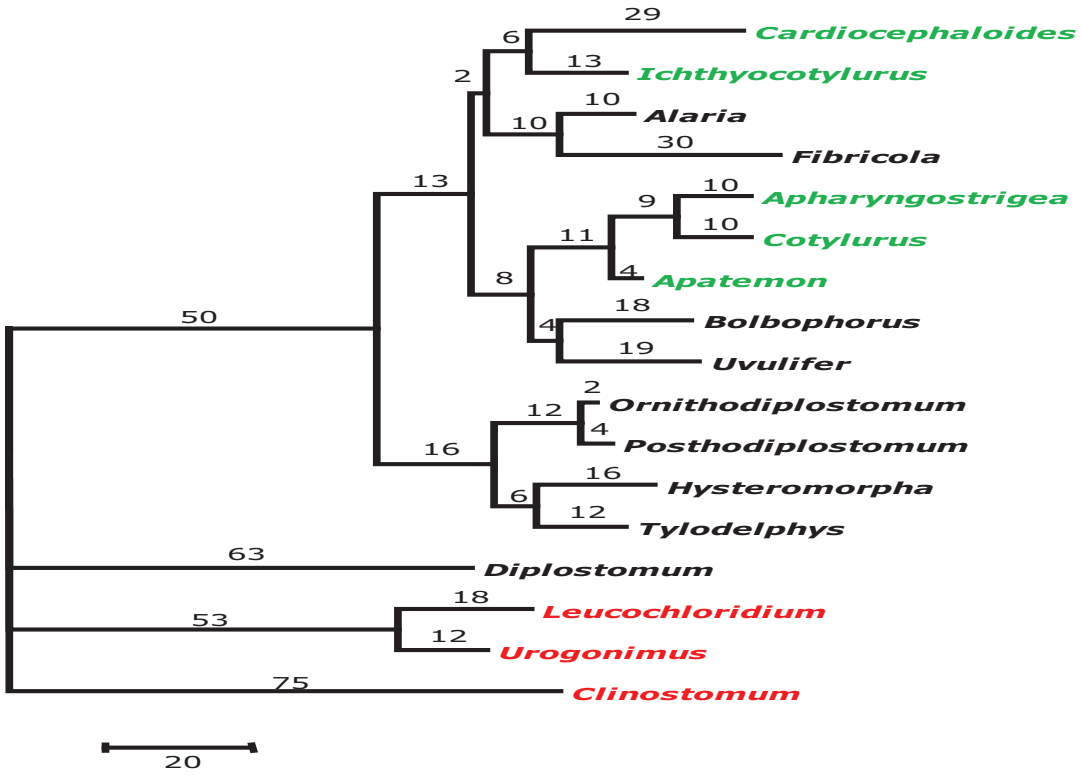
	Alaria	*Apatemon*	*Apharyngostrigea*	*Bolbophorus*	*Clinostomum*	*Cotylurus*	*Diplostomum*	*Fibricola*	*Hysteromorpha*	*Ichthyocotylurus*	*Ornithodiplostomum*	*Posthodiplostostomum*	*Tylodelphys*
Alaria	-	16.98	18.14	16.98	27.67	17.91	11.40	10.70	11.86	17.21	18.84	16.28	13.02
Apatemon	73	-	13.49	16.28	27.67	10.00	13.49	14.42	15.81	14.88	19.77	15.81	14.65
Apharyngostrigea	78	58	-	19.77	27.44	13.72	16.74	15.12	15.81	16.28	20.47	19.30	17.21
Bolbophorus	73	70	85	-	26.28	16.51	13.26	15.81	16.05	17.67	16.74	16.05	15.81
Clinostomum	119	119	118	113	-	28.60	27.21	27.44	27.44	30.47	30.00	28.84	27.21
Cotylurus	77	43	59	71	123	-	16.05	16.98	18.14	16.98	19.07	18.84	16.05
Diplostomum	49	58	72	57	117	69	-	11.63	11.63	12.79	14.88	13.72	10.00
Fibricola	46	62	65	68	118	73	50	-	11.63	13.02	16.05	15.35	13.26
Hysteromorpha	51	68	68	69	118	78	50	50	-	14.19	17.91	16.51	12.56
Ichthyocotylurus	74	64	70	76	131	73	55	56	61	-	15.81	17.21	14.65
Ornithodiplostomum	81	85	88	72	129	82	64	69	77	68	-	11.63	17.21
Posthodiplostostomum	70	68	83	69	124	81	59	66	71	74	50	-	14.65
Tylodelphys	56	63	74	68	117	69	43	57	54	63	74	63	-

Appendix 5. Branch lengths for the maximum parsimony analyses of the independent datasets of the full small subunit (SSU), partial large subunit (LSU) and full internal transcribe spacer regions 1 and 2 (ITS) of ribosomal DNA and the barcode region of cytochrome oxidase I (COI) of mitochondrial DNA of 14 genera among the Diplostomidae and Strigeidae. The numbers above the branches on each tree correspond to the branch lengths. Outgroup taxa are red, strigeid taxa green and diplostomid taxa black.

Appendix 5. Continued

A. Branch lengths and maximum parsimony tree 1 of 1 for SSU dataset.

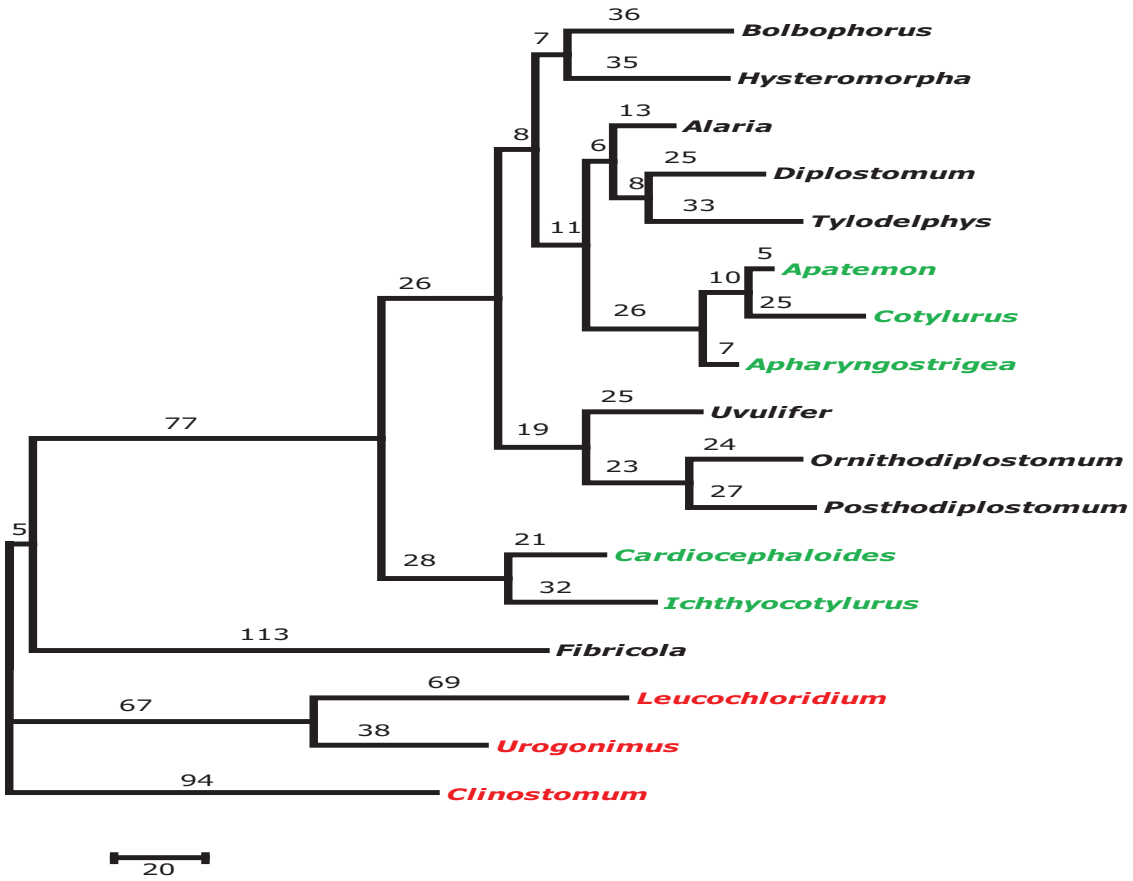
SSU	Assigned branch length	Minimum possible branch length	Maximum possible branch length
Alaria	10	10	14
Apatemon	4	4	6
Apharyngostrigea	10	7	17
Bolbophorus	18	16	19
Cardiocephaloides	29	21	23
Clinostomum	75	61	84
Cotylurus	10	9	13
Diplostomum	63	7	13
Fibricola	30	26	30
Hysteromorpha	16	14	19
Ichthyocotylurus	13	4	7
Leucochloridium	18	14	26
Ornithodiplostomum	2	2	2
Posthodiplostosmum	4	4	4
Tylodelphys	12	11	14
Urogonimus	12	6	16
Uvulifer	19	18	21



Appendix 5. Continued

B. Branch lengths and maximum parsimony tree 1 of 4 for LSU dataset.

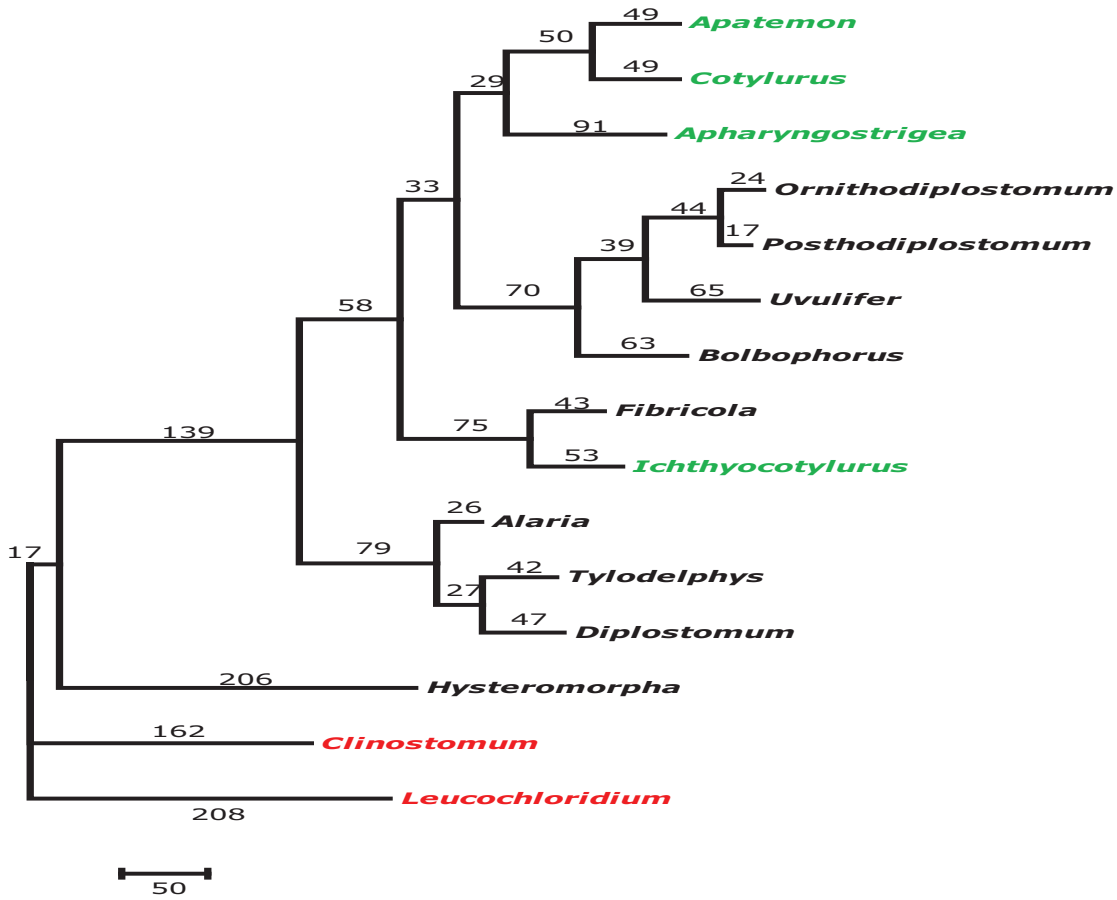
LSU	Assigned branch length	Minimum possible branch length	Maximum possible branch length
Alaria	13	12	17
Apatemon	5	3	10
Apharyngostrigea	7	7	15
Bolbophorus	36	20	27
Cardiocephaloides	21	20	25
Clinostomum	94	71	114
Cotylurus	25	20	27
Diplostomum	25	20	25
Fibricola	113	25	43
Hysteromorpha	35	24	31
Ichthyocotylurus	32	28	33
Leucochloridium	69	57	79
Ornithodiplostomum	24	21	29
Posthodiplostomum	27	24	30
Tylodelphys	33	33	38
Urogonimus	38	28	50
Uvulifer	25	22	27



Appendix 5. Continued

C. Branch lengths and maximum parsimony tree 1 of 2 for ITS dataset.

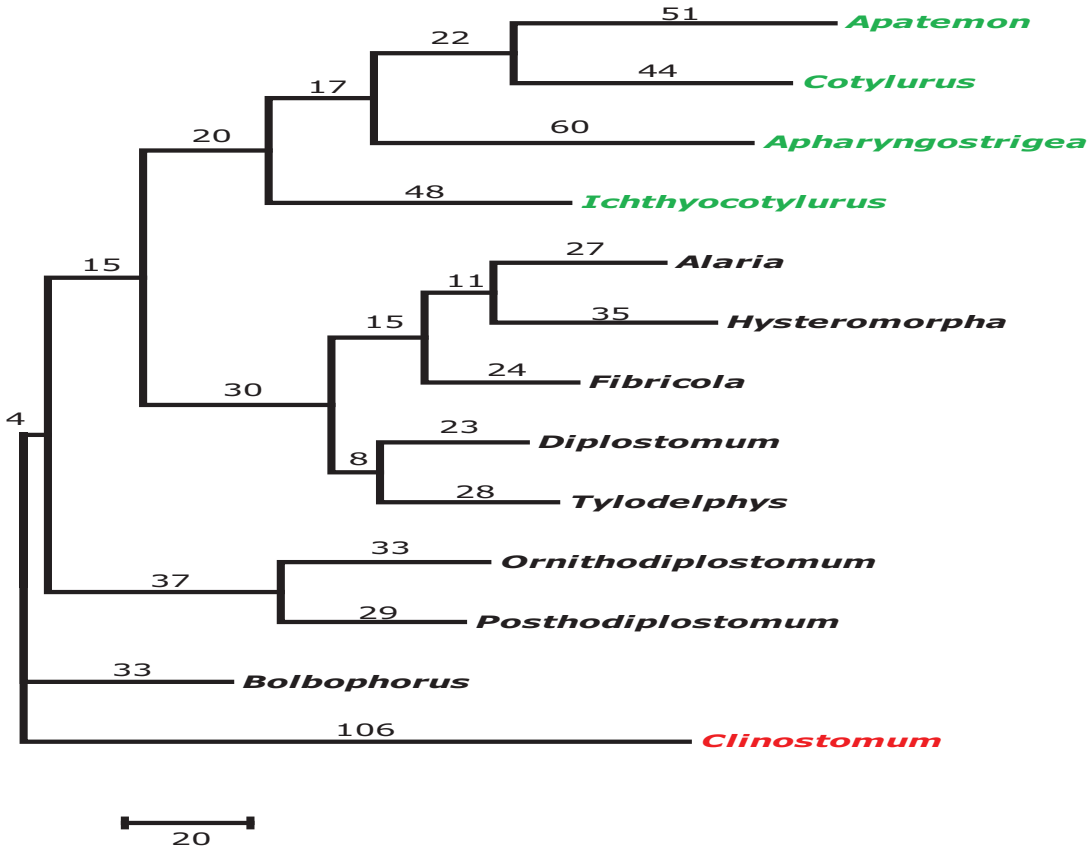
ITS	Assigned branch length	Minimum possible branch length	Maximum possible branch length
Alaria	23	24	46
Apatemon	48	12	22
Apharyngostrigea	87	27	46
Bolbophorus	47	43	81
Clinostomum	81	50	102
Cotylurus	47	21	32
Diplostomum	38	32	48
Fibricola	36	25	52
Hysteromorpha	163	38	74
Ichthyocotylurus	44	42	57
Leucochloridium	97	51	134
Ornithodiplostomum	21	16	25
Posthodiplostomum	13	9	20
Tylodelphys	39	32	46
Uvulifer	57	44	80



Appendix 5. Continued

D. Branch lengths and maximum parsimony tree 1 of 5 for COI dataset.

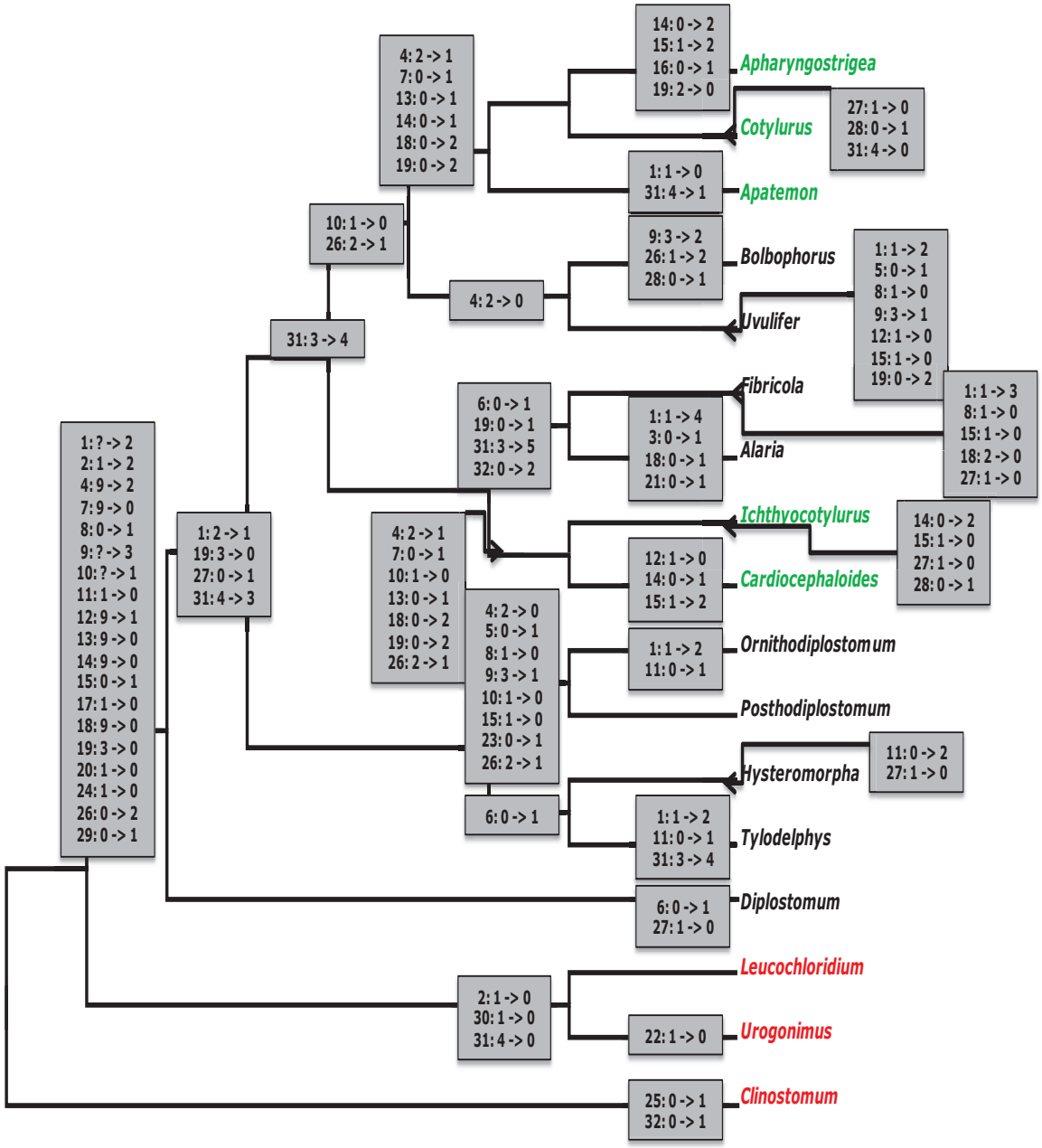
COI	Assigned branch length	Minimum possible branch length	Maximum possible branch length
Alaria	27	24	39
Apatemon	51	18	30
Apharyngostrigea	60	33	50
Bolbophorus	33	25	38
Clinostomum	33	25	47
Cotylurus	44	21	34
Diplostomum	23	17	28
Fibricola	24	20	34
Hysteromorpha	35	24	38
Ichthyocotylurus	48	28	44
Ornithodiplostomum	33	25	37
Posthodiplostomum	29	25	38
Tylodelphys	28	24	35



Appendix 6. Mapping of adult and larval morphological and life history traits onto 15 topologies retained from thirty phylogenetic analyses of four molecular markers with the numbers in the grey boxes referring the number of the mapped characters listed in Appendix 3. Pleisiomorphic character states that were unknown (?) were not included in the count of the total number of steps. The molecular markers consisted of the full small subunit (SSU), partial large subunit (LSU) and full internal transcribe spacer regions 1 and 2 (ITS) of ribosomal DNA and the barcode region of cytochrome oxidase I (COI) of mitochondrial DNA of 14 genera among the Diplostomidae and Strigeidae. The datasets were analyzed independently and in total evidence combinations in both maximum parsimony (MP) and maximum likelihood (ML) analyses. Outgroup taxa are red, strigeid taxa green and diplostomid taxa black.

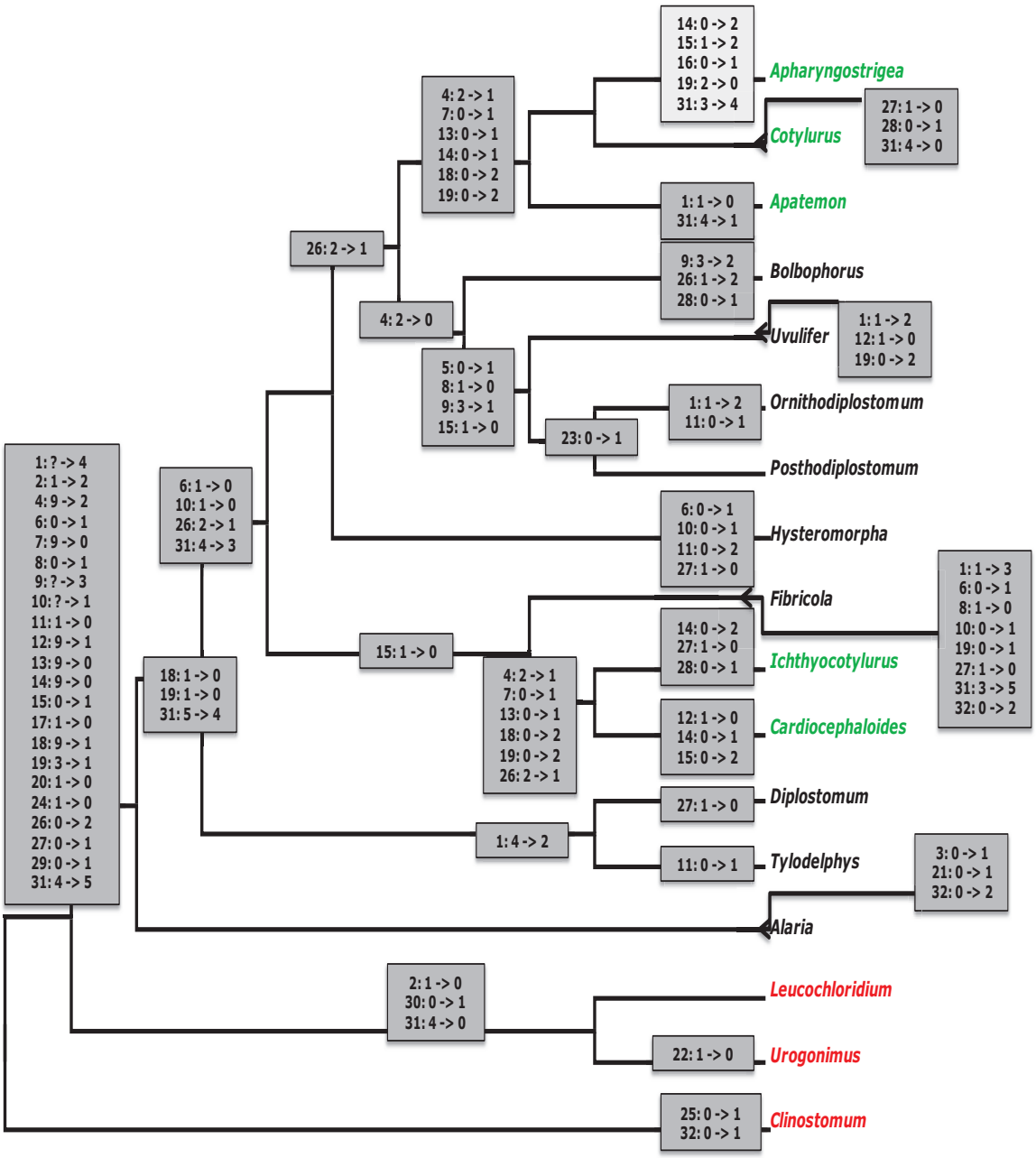
Appendix 6. Continued

Topology A. Supported by the MP analyses of the SSU dataset. Character mapping results in a total of 100 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 90.



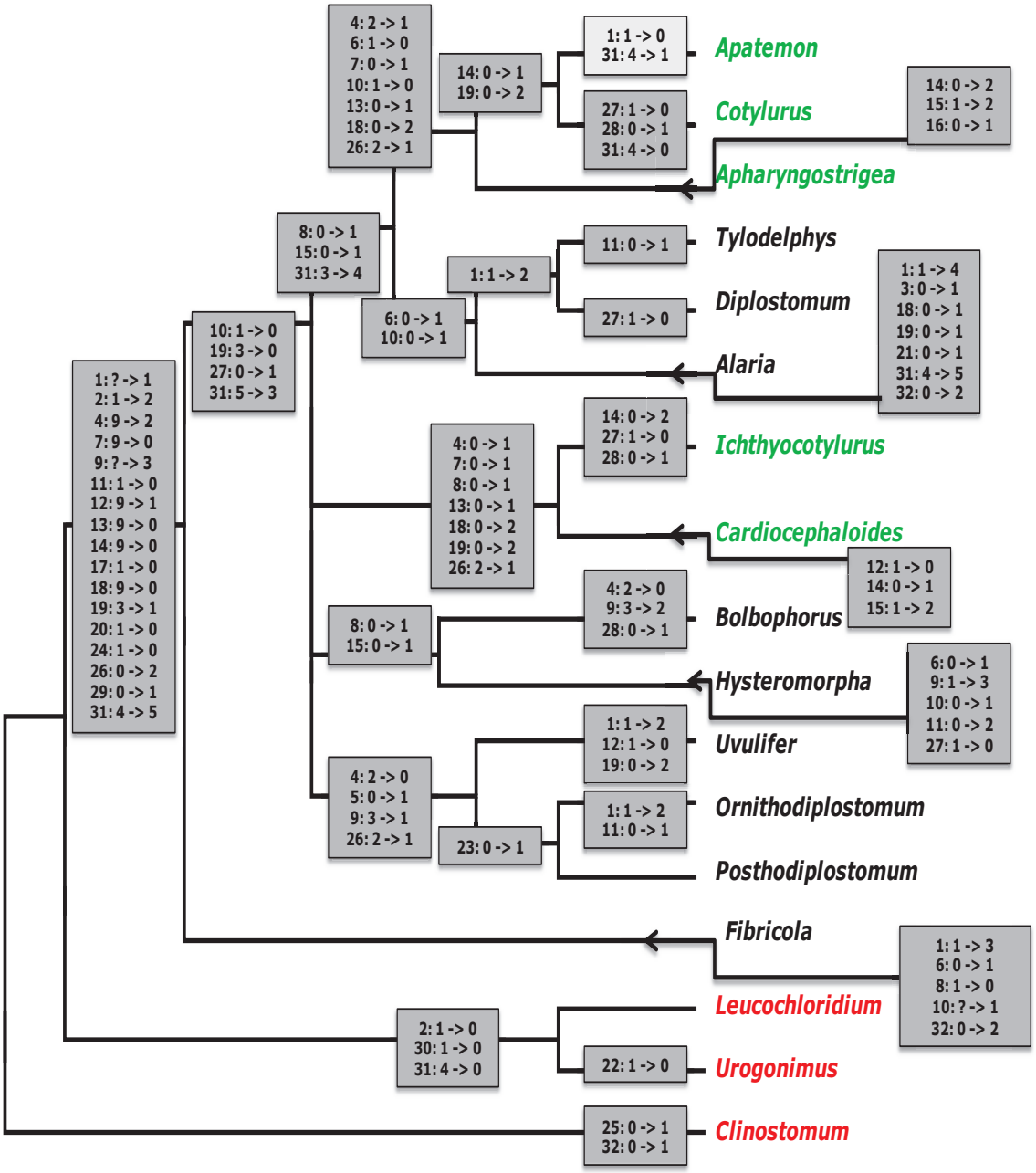
Appendix 6. Continued

Topology B. Supported by the ML analyses of the SSU dataset. Character mapping results in a total of 94 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 82.



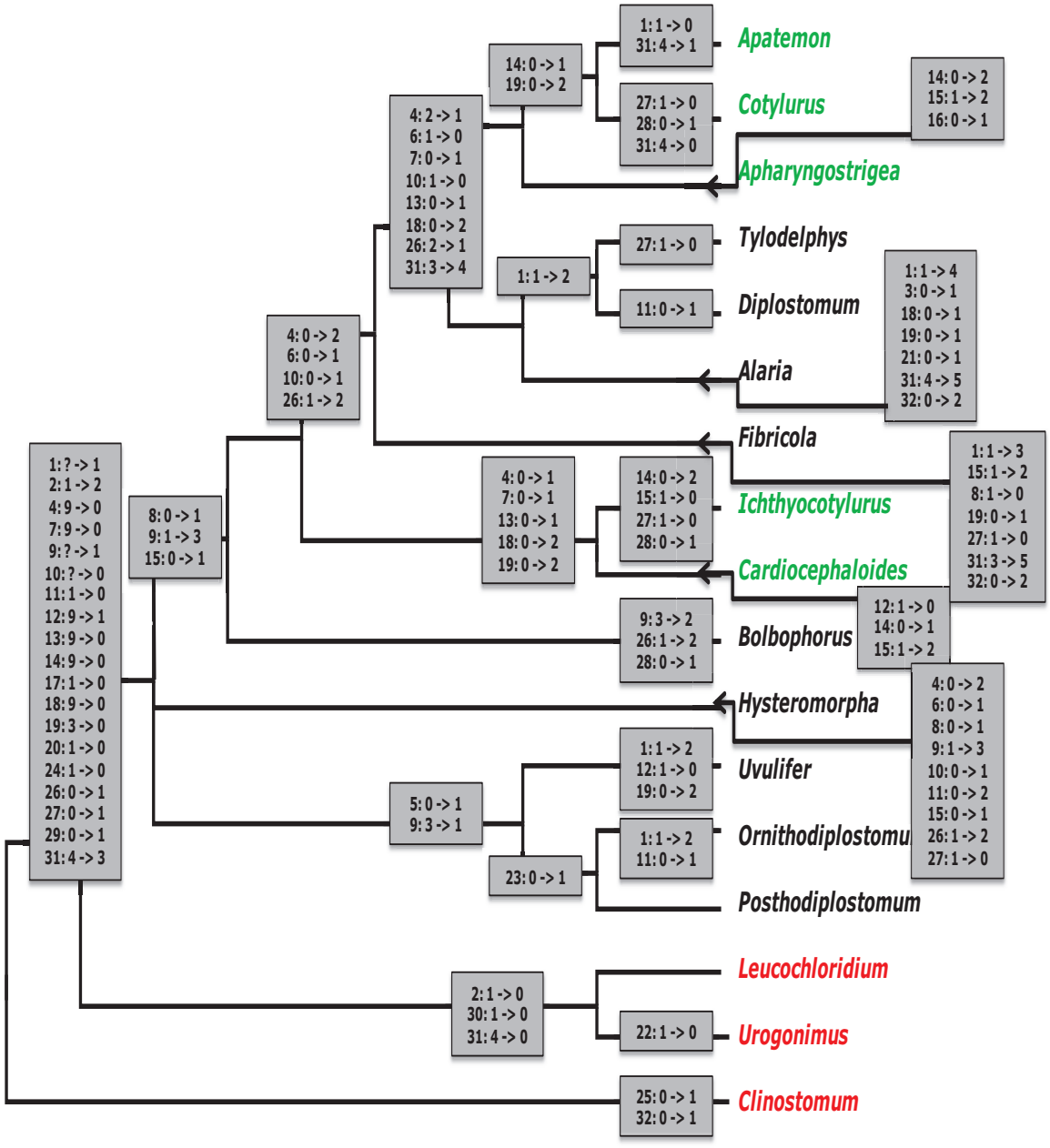
Appendix 6. Continued

Topology C. Supported by the MP analyses of the LSU and SSU–LSU datasets. The SSU-LSU dataset has no resolution other than the branch of *Fibricola*. Character mapping results in a total of 94 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 83.



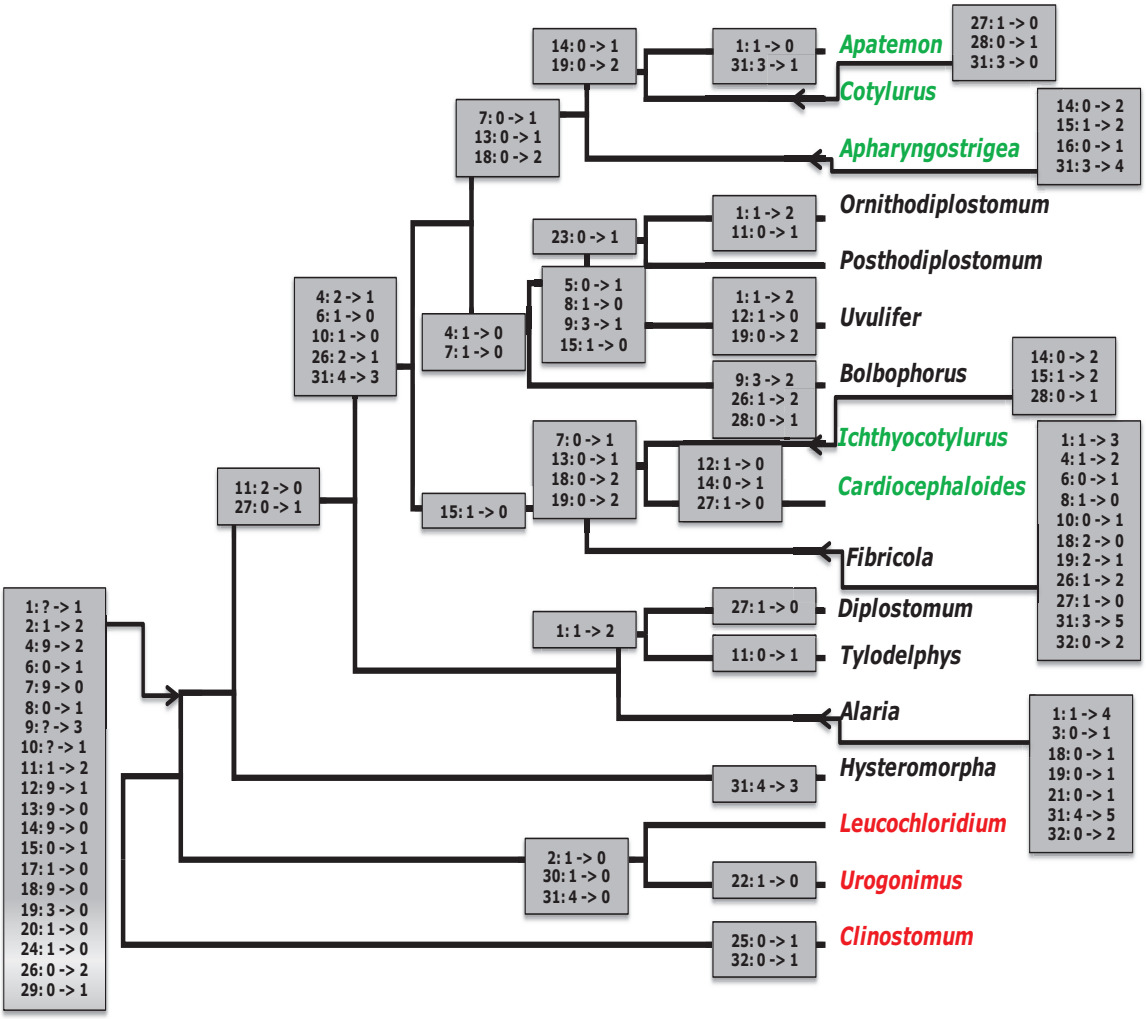
Appendix 6. Continued

Topology D. Supported by the ML analyses of the LSU dataset. Character mapping results in a total of 96 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 87.



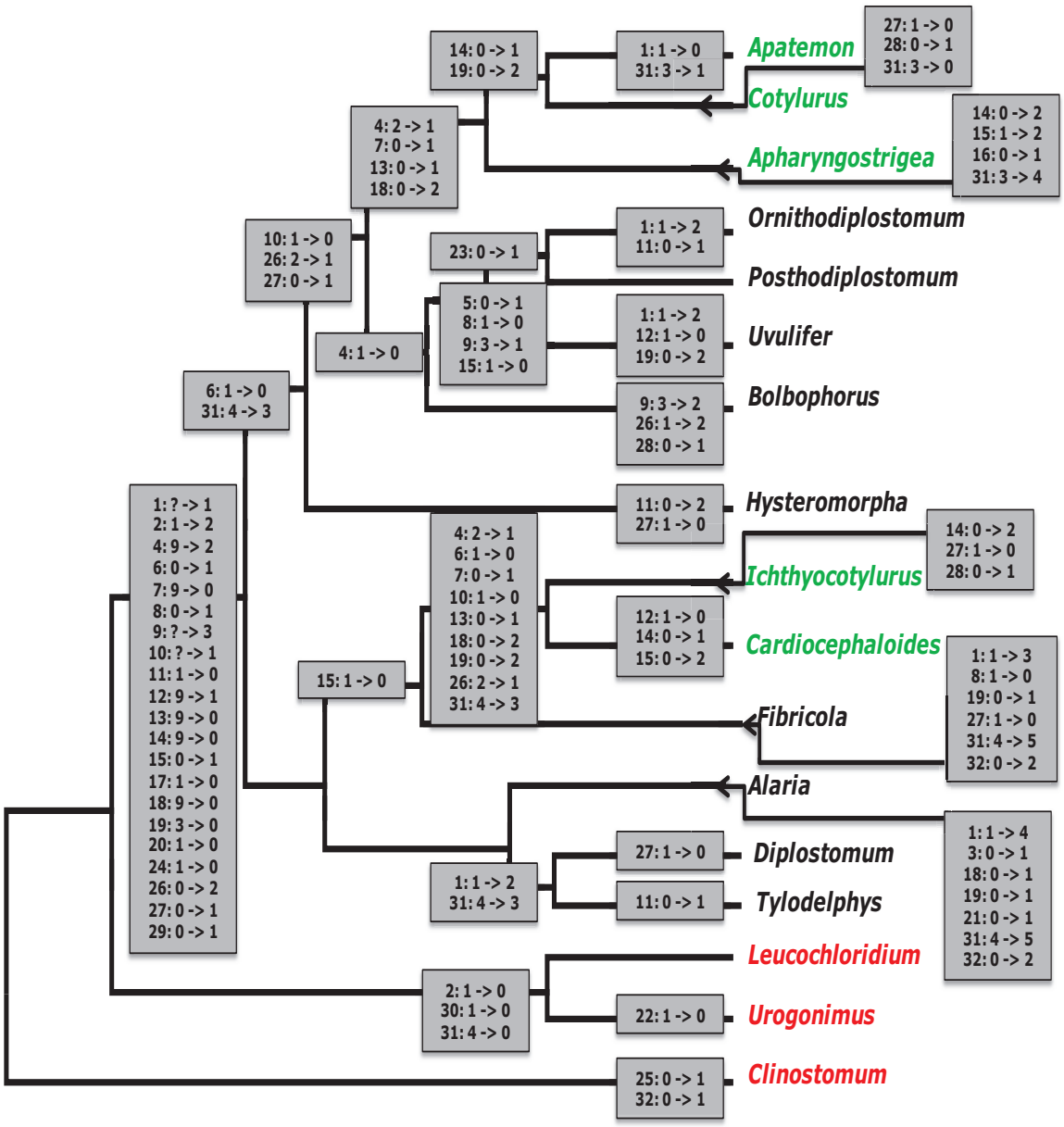
Appendix 6. Continued

Topology E. Supported by the MP analyses of the ITS dataset and the ML analyses of the ITS and LSU-ITS dataset. Note that for the ITS there was no *Cardiocephaloides* sequence available, and *Alaria* was unresolved in the MP analysis but did not change the total number of steps. Character mapping results in a total of 92 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 80.



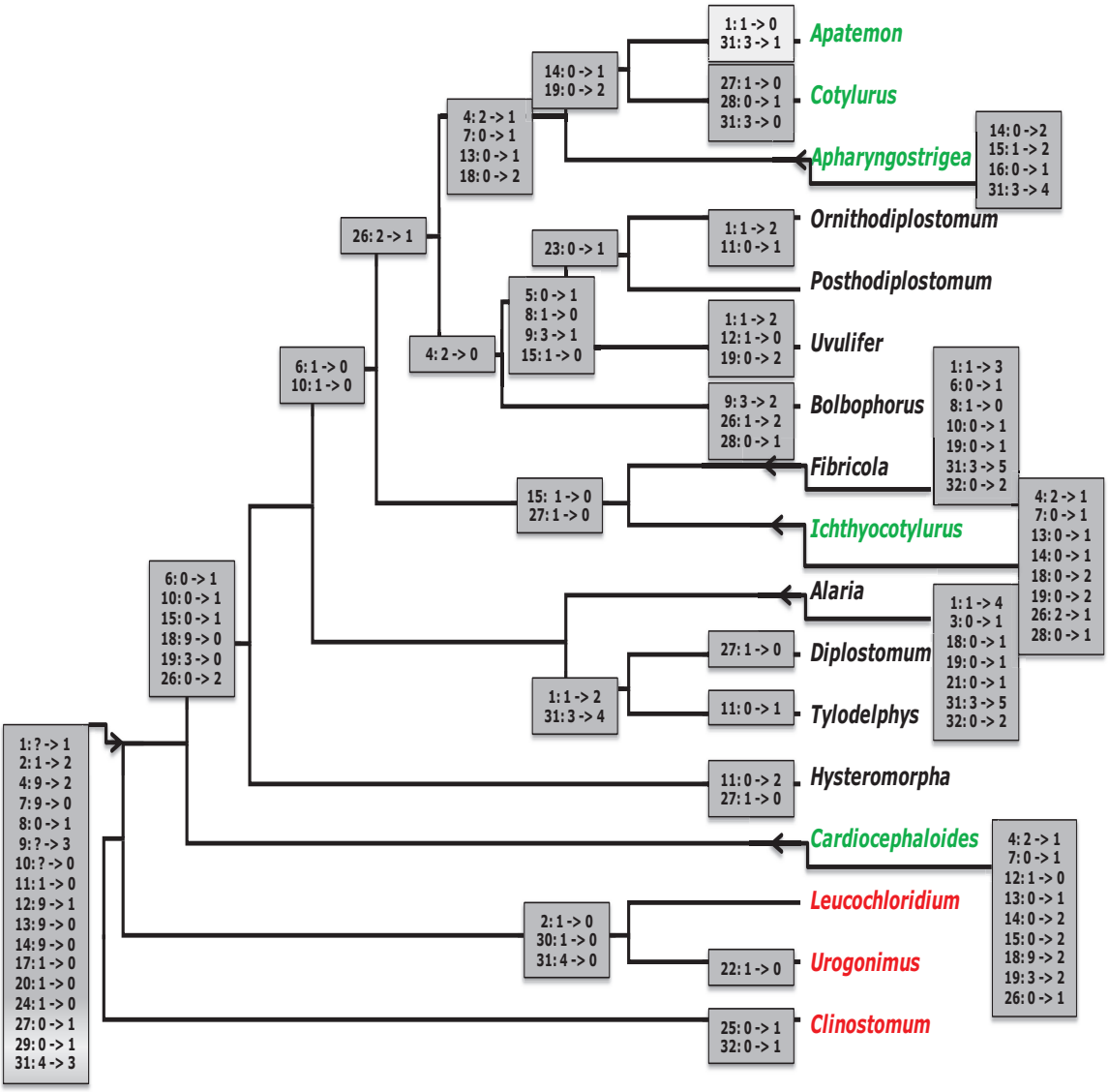
Appendix 6. Continued

Topology F. Supported by the ML analyses of the SSU-LSU dataset. Character mapping results in a total of 93 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 80.



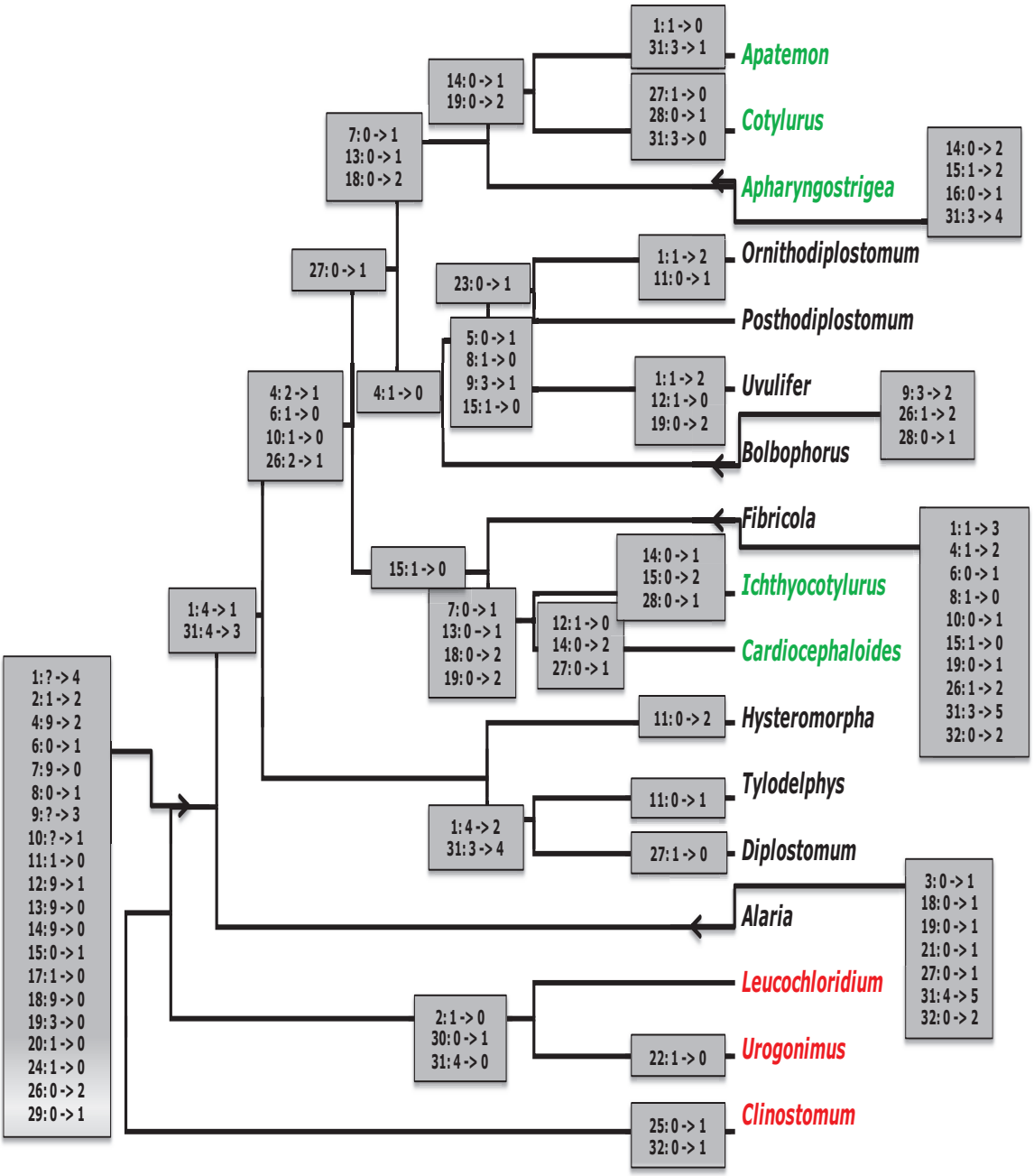
Appendix 6. Continued

Topology G. Supported by the MP analyses of the SSU–ITS, LSU-ITS, SSU-LSU-ITS, LSU-ITS-COI and SSU-LSU-ITS-COI datasets. Note that within the SSU-ITS topology the following branches were unresolved: *Alaria* and the branch composed of *Fibricola*, *Ichthyocotylurus* and *Cardiocephaloides*. Character mapping results in a total of 97 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 85.



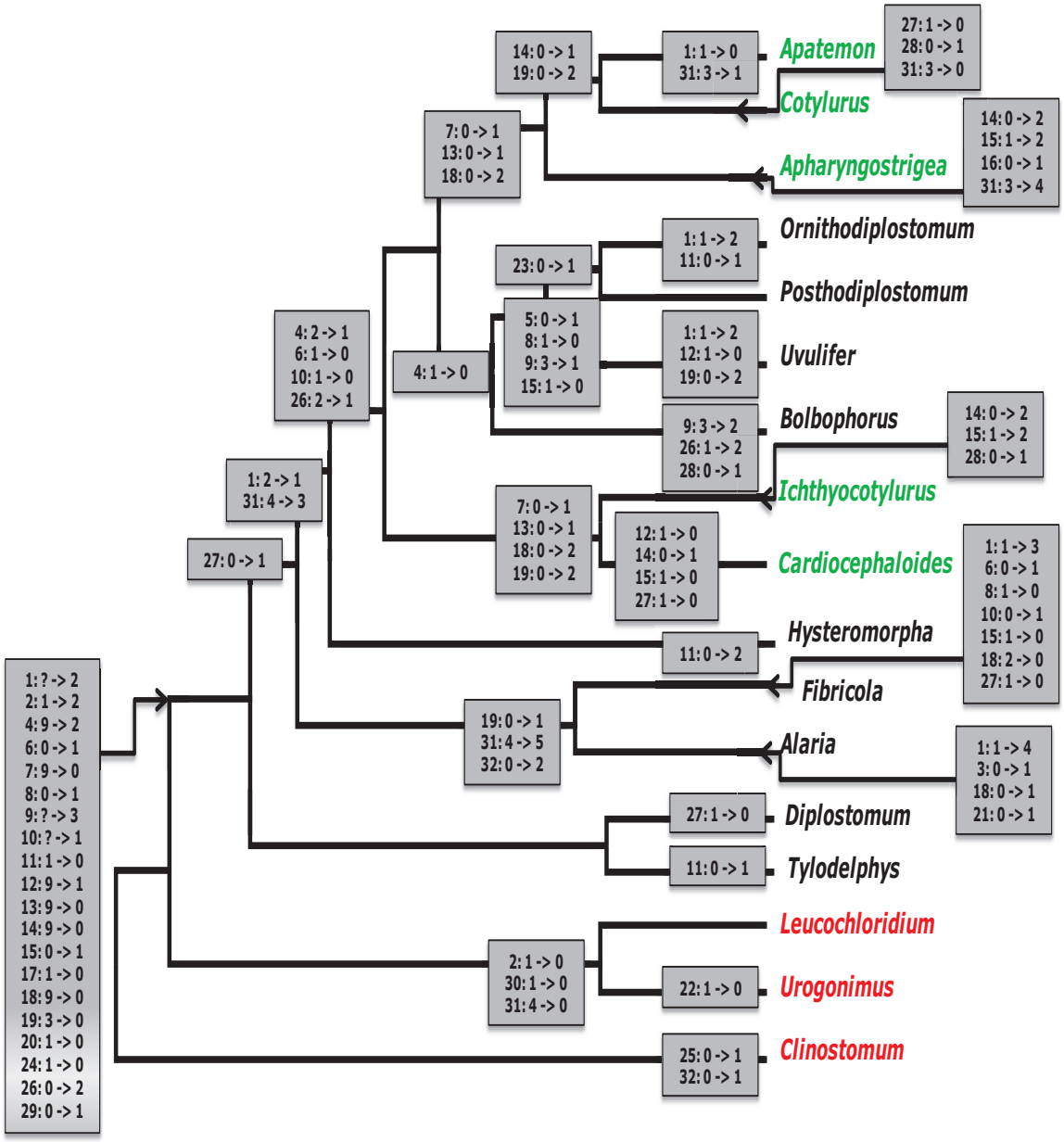
Appendix 6. Continued

Topology H. Supported by the ML analyses of the SSU-ITS dataset. Character mapping results in a total of 88 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 76.



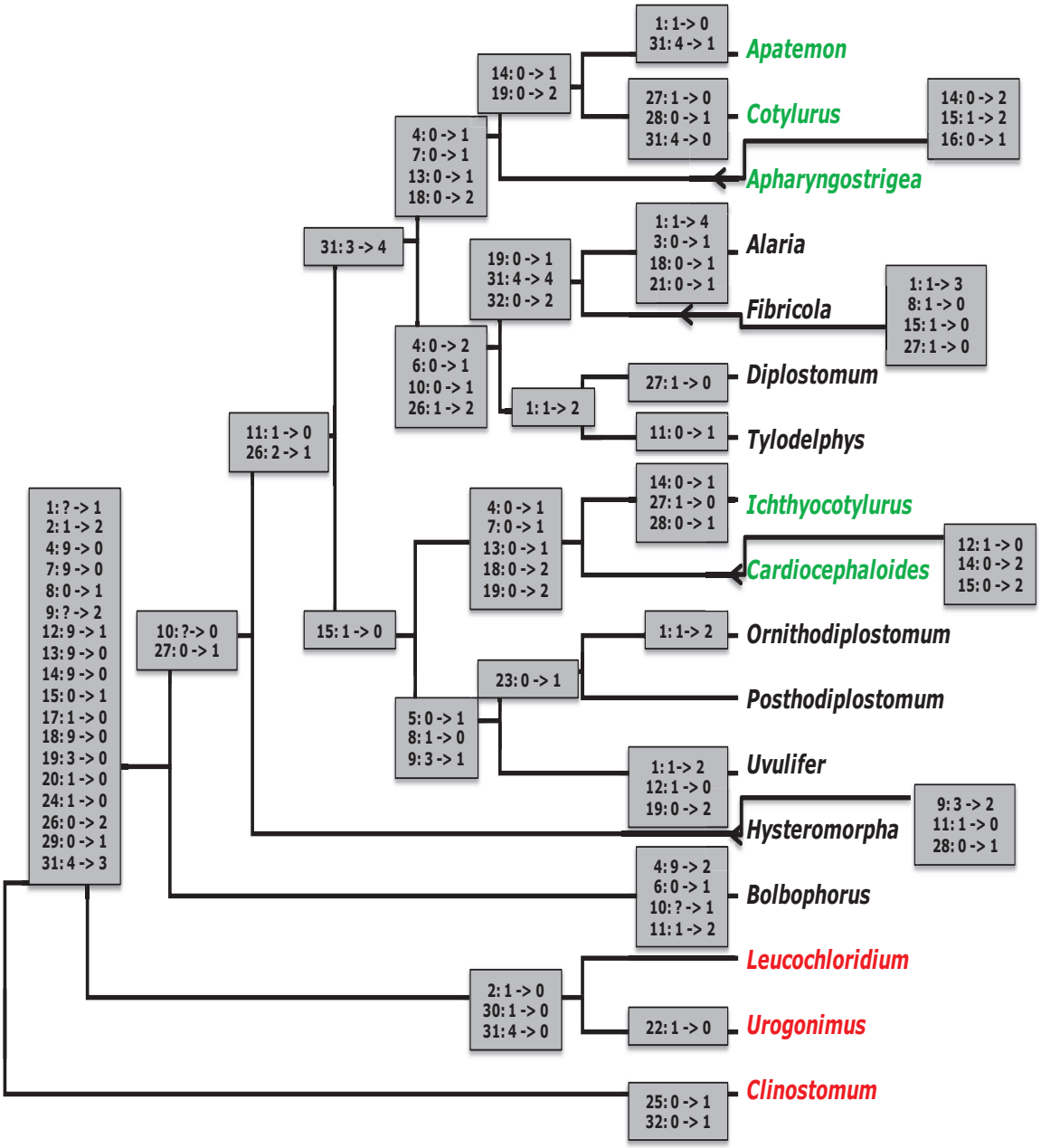
Appendix 6. Continued

Topology I. Supported by the ML analyses of the SSU-COI dataset. Character mapping results in a total of 86 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 78.



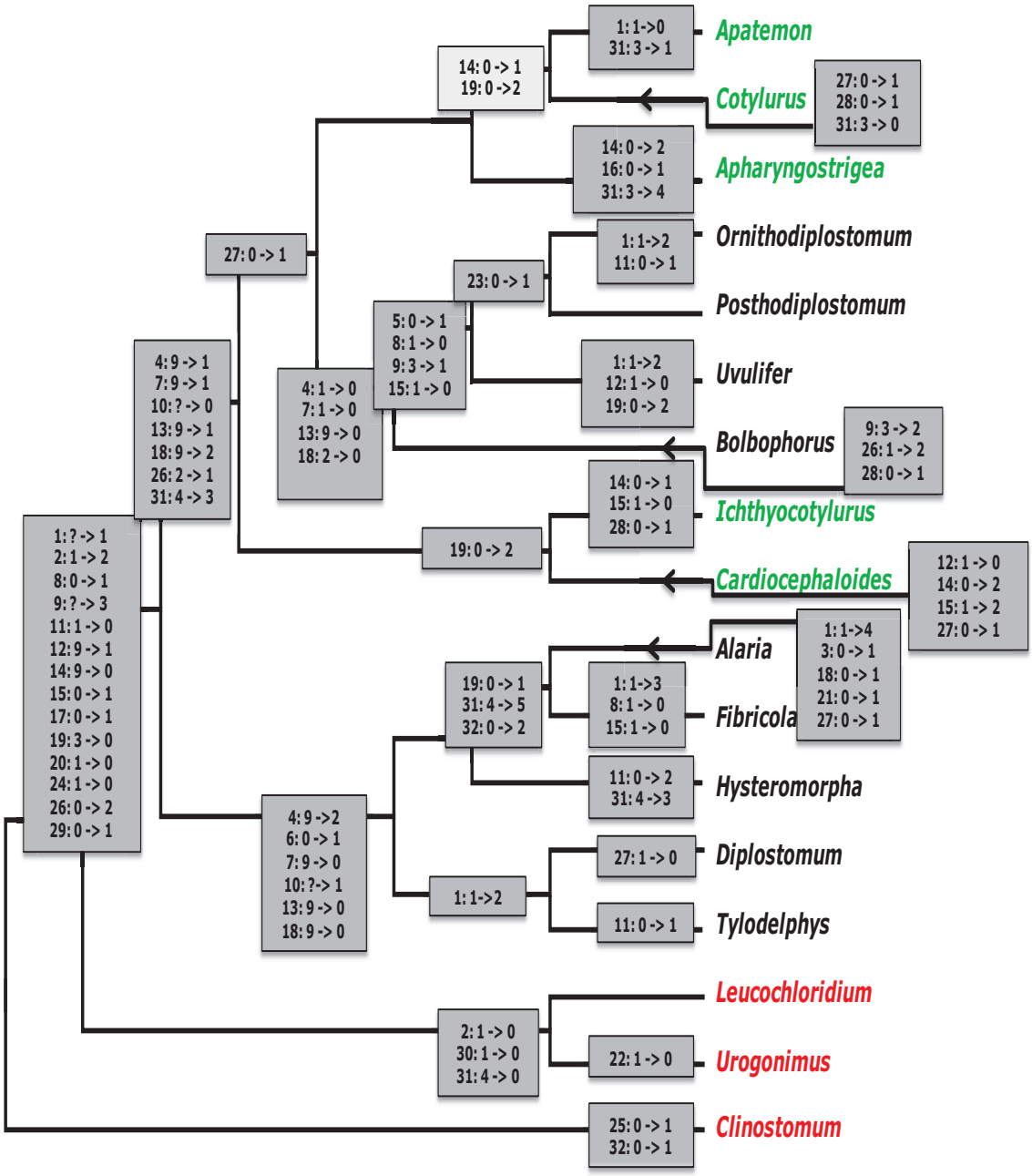
Appendix 6. Continued

Topology J. Supported by the MP analyses of the LSU-COI and SSU-LSU-COI datasets. Character mapping results in a total of 85 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 77.



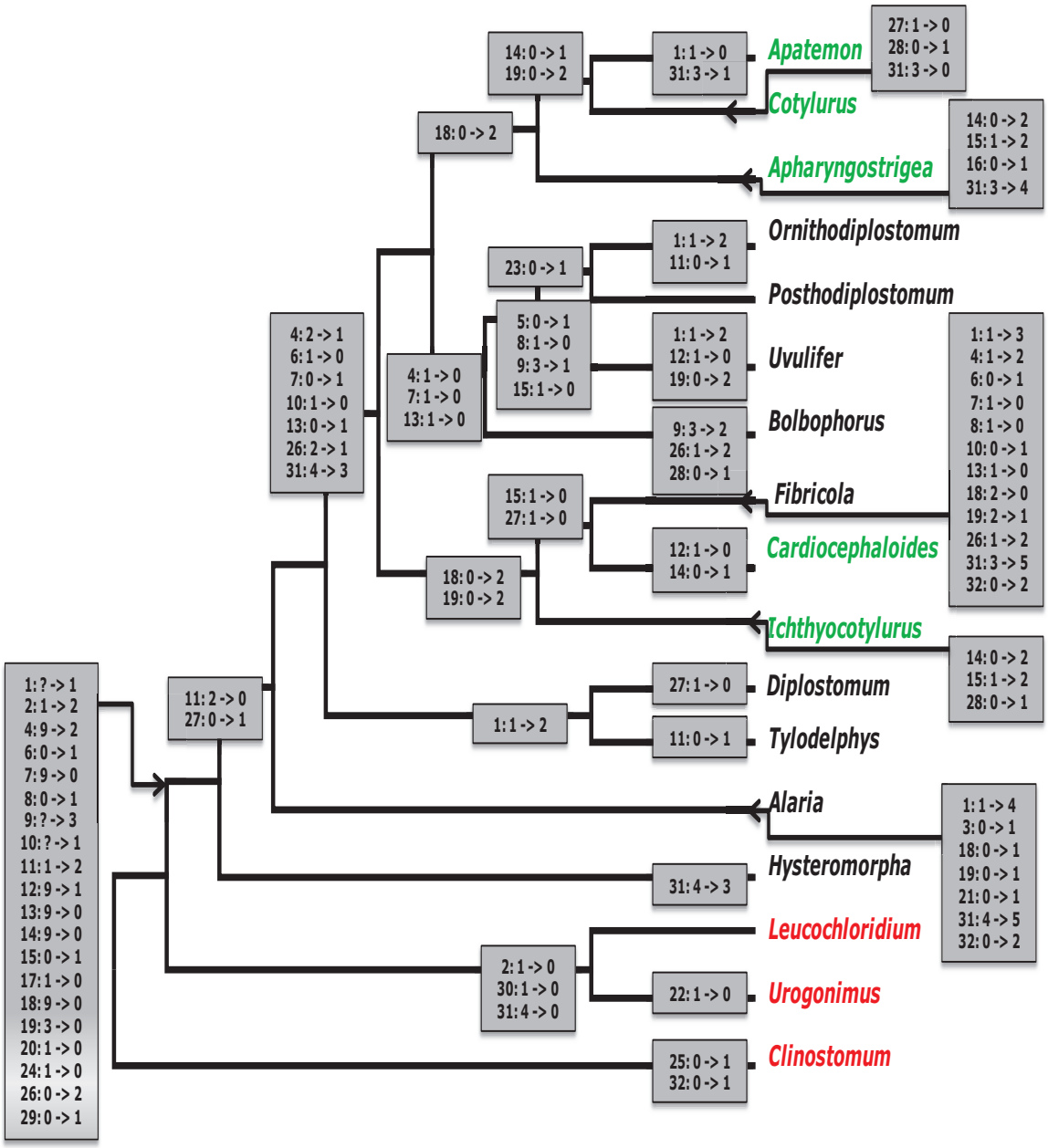
Appendix 6. Continued

Topology K. Supported by the ML analyses of the LSU-COI and SSU-LSU-COI datasets. Character mapping results in a total of 82 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 76.



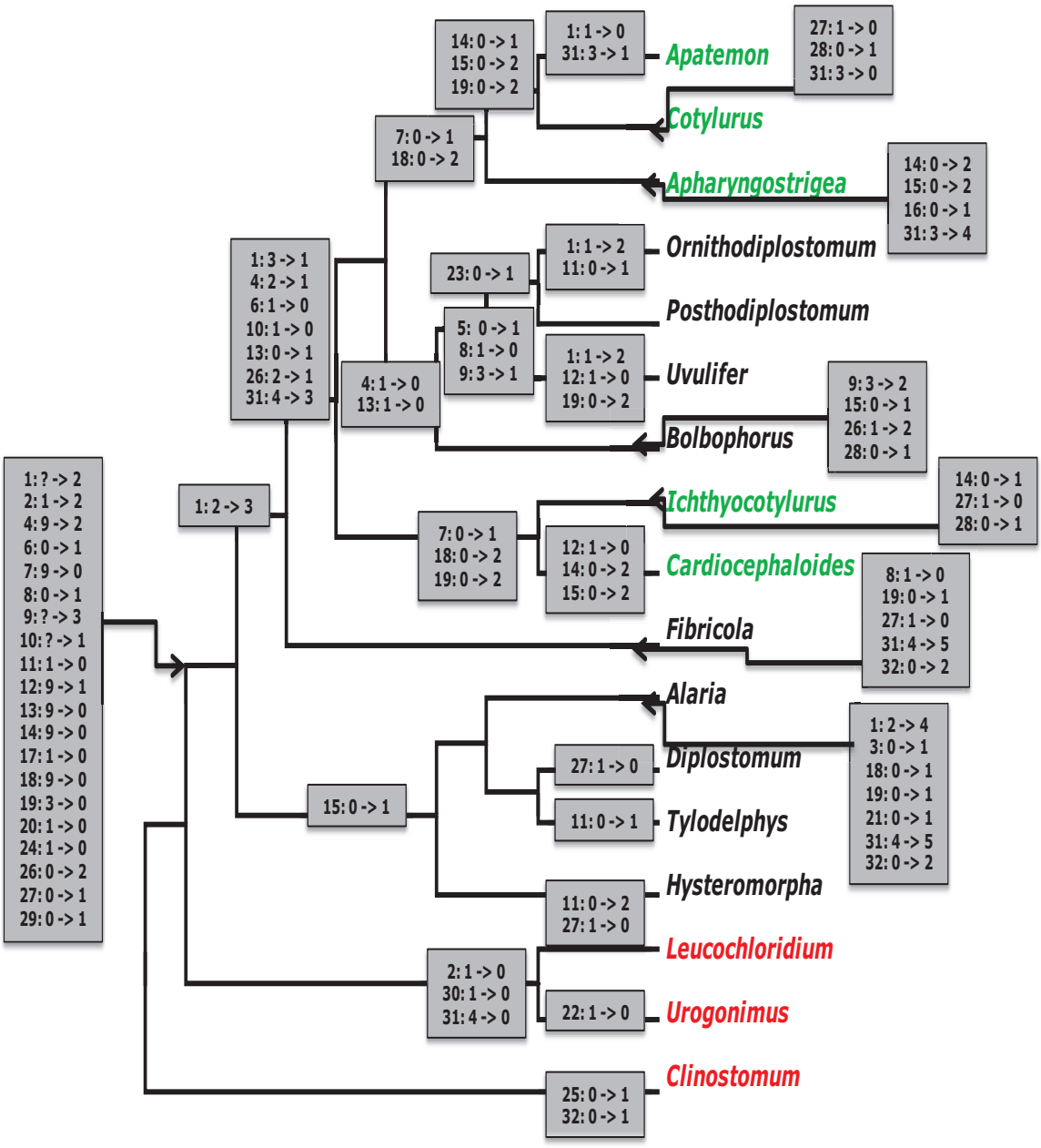
Appendix 6. Continued

Topology L. Supported by the MP analyses of the ITS-COI and SSU-ITS-COI datasets. Character mapping results in a total of 92 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 80.



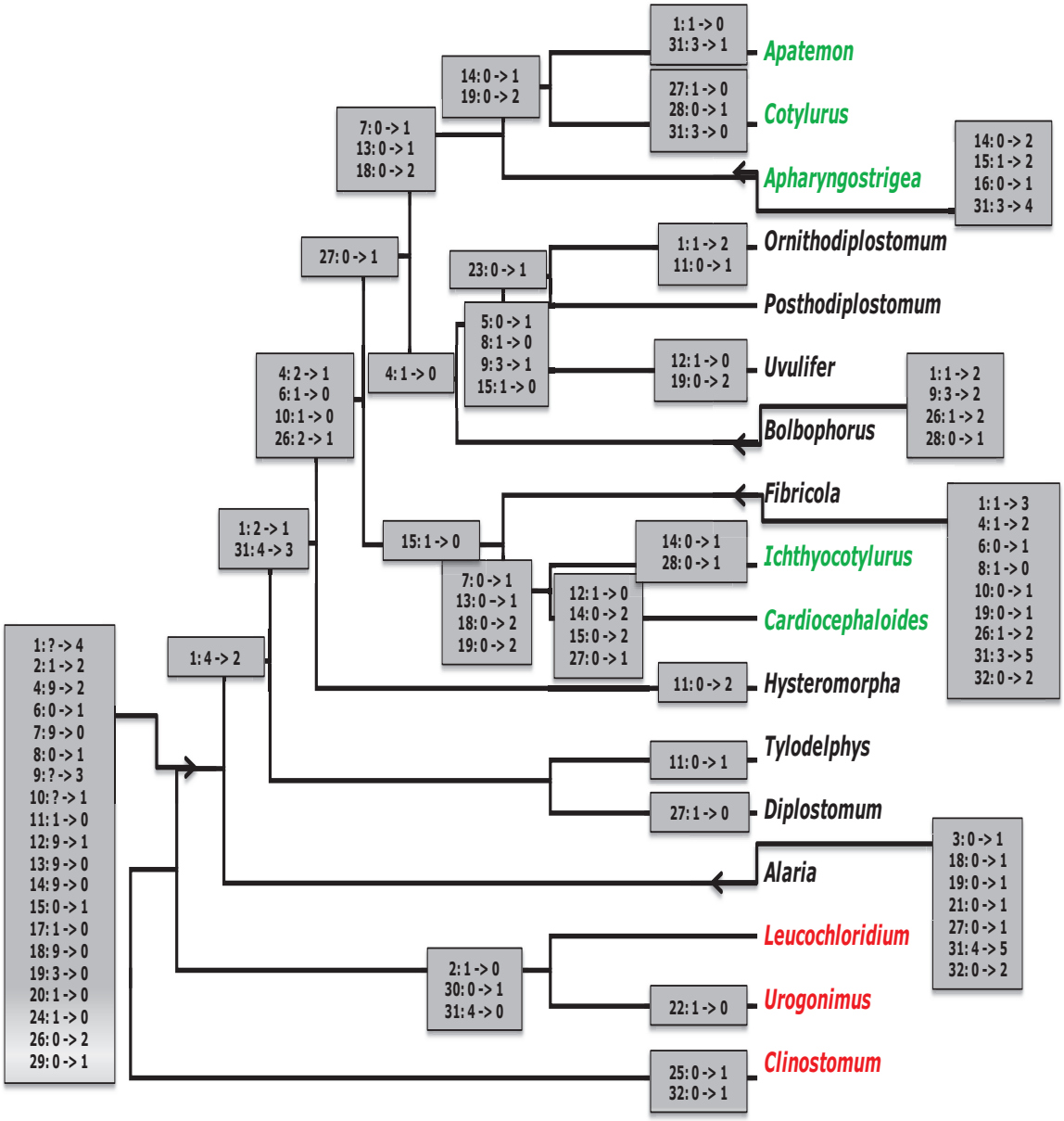
Appendix 6. Continued

Topology M. Supported by the ML analyses of the ITS-COI and LSU-ITS-COI datasets. Character mapping results in a total of 86 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 75.



Appendix 6. Continued

Topology N. Supported by the ML analyses of the SSU-LSU-ITS dataset. Character mapping results in a total of 85 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 74.



Appendix 6. Continued

Topology O. Supported by the ML analyses of the SSU-ITS-COI, LSU-ITS-COI and SSU-LSU-ITS-COI datasets. Character mapping results in a total of 92 steps for this topology. Removing the characters of host information 30-32 (Appendix 3), the total steps are equal to 80.