



Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda)¹

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Abstract

Complete small subunit ribosomal RNA gene (ssrDNA) and partial (D1–D3) large subunit ribosomal RNA gene (lsrDNA) sequences were used to estimate the phylogeny of the Digenea via maximum parsimony and Bayesian inference. Here we contribute 80 new ssrDNA and 124 new lsrDNA sequences. Fully complementary data sets of the two genes were assembled from newly generated and previously published sequences and comprised 163 digenean taxa representing 77 nominal families and seven aspidogastreaan outgroup taxa representing three families. Analyses were conducted on the genes independently as well as combined and separate analyses including only the higher plagiorchiid taxa were performed using a reduced-taxon alignment including additional characters that could not be otherwise unambiguously aligned. The combined data analyses yielded the most strongly supported results and differences between the two methods of analysis were primarily in their degree of resolution. The Bayesian analysis including all taxa and characters, and incorporating a model of nucleotide substitution (general-time-reversible with among-site rate heterogeneity), was considered the best estimate of the phylogeny and was used to evaluate their classification and evolution. In broad terms, the Digenea forms a dichotomy that is split between a lineage leading to the Brachylaimoidea, Diplostomoidea and Schistosomatoidea (collectively the Diplostomida nomen novum (nom. nov.)) and the remainder of the Digenea (the Plagiorchiiida), in which the Bivesiculata nom. nov. and Transversotremata nom. nov. form the two most basal lineages, followed by the Hemiurata. The remainder of the Plagiorchiiida forms a large number of independent lineages leading to the crown clade Xiphidiata nom. nov. that comprises the Allocreadioidea, Gorgoderoidea, Microphalloidea and Plagiorchioidea, which are united by the presence of a penetrating stylet in their cercariae. Although a majority of families and to a lesser degree, superfamilies are supported as currently defined, the traditional divisions of the Echinostomida, Plagiorchiiida and Strigeida were found to comprise non-natural assemblages. Therefore, the membership of established higher taxa are emended, new taxa erected and a revised, phylogenetically based classification proposed and discussed in light of ontogeny, morphology and taxonomic history.

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Keywords: Digenea; Aspidogastrea; ssrDNA; lsrDNA; Bayesian inference

1. Introduction

Comprising ~18,000 nominal species, it has been

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argued that the Digenea represents the largest group of internal metazoan parasites (Cribb et al., 2001). They are an extraordinarily ubiquitous group, parasitising all major vertebrate groups as definitive hosts (although conspicuously absent from elasmobranchs with few exceptions), gastropods and other mollusc groups as first intermediate hosts and several phyla as second intermediate hosts. The importance of fascioliasis, schistosomiasis and other disease agents of humans and domesticated animals have long been recognised and continues to receive considerable attention from researchers across a broad range of disciplines. The

understanding of digenean biodiversity and evolution, however, has received considerably less attention and few hypotheses put forth during the last century have been subject to rigorous scrutiny. The first phylogenetic analysis providing explicit character matrices was not attempted until Cribb et al. (2001) combined a newly coded morphological matrix with new molecular data from the small subunit ribosomal RNA gene (ssrDNA). Their study (Cribb et al., 2001) utilising complete ssrDNA sequences from 75 digenean species representing 55 families, combined with 56 adult and larval morphological characters for these families, resulted in a reasonably well-resolved tree. Cribb et al. (2001) also provided a historical review of previous classification schemes and molecular phylogenetic studies on digenean groups conducted prior to their study, which remains as the current study. Other molecular phylogenetic studies of the Digenea have been more restricted in their taxonomic scope, but have provided valuable estimates of the interrelationships of constituent groups (Barker et al., 1993; Blair and Barker, 1993; Blair et al., 1998; Fernández et al., 1998a,b; Tkach and Pawlowski, 1999; Tkach et al., 2000, 2001a,b,c).

Here we build on earlier studies in order to provide additional resolution for the interpretation of their evolution and to develop a phylogenetically based higher classification. Analyses were performed on sequence data from two genes for 170 exemplar taxa representing 77 digenean and three aspidogastrea outgroup families. Following the success of combining complete ssrDNA (~1,800 bp) with partial large subunit ribosomal RNA gene (lsrDNA, variable domains D1–D3; ~1,400 bp) for the other parasitic platyhelminths, the Cestoda (Olson et al., 2001) and Monogenea (Olson and Littlewood, 2002), we continue this approach here in order to better estimate the phylogeny of the Digenea. Results are presented for analyses of both individual and combined datasets. Rather than pursuing a strict total-evidence approach, we independently estimate the molecular phylogeny for the Digenea using both parsimony and Bayesian methods and discuss the results in light of morphology, ontogeny and taxonomic history. This study follows the publication of the first of three volumes providing the most recent systematic treatment of the Trematoda (Gibson et al., 2002) and we have followed the classification found in these keys in our listing of taxa (Table 1) for reference purposes; information from the forthcoming second and third volumes were provided by the editors of those volumes (A. Jones and R.A.B., respectively, personal communication). However, a revised classification based on the results herein is presented in Section 3 and employed in Section 4 and figures.

The interrelationships of the neodermatan Platyhelminthes are somewhat controversial, relating mainly to the possible non-monophyly of the Monogenea (Justine, 1998; Littlewood et al., 2001) and of the Cercomeromorphae (Lockyer et al., 2003). However, it is well accepted that the trematodes form a monophyletic group with the Aspidogas-

tra as sister-group to the Digenea (Littlewood et al., 1999; Rohde, 2001). We have, therefore, rooted our phylogenies using a diverse sampling of aspidogastrea taxa.

2. Materials and methods

2.1. Collection of specimens and extraction of gDNA

Table 1 provides a taxonomic listing of the exemplar species analysed, their hosts and collection localities and accession numbers of the sequences. Representative voucher specimens, where available, have been deposited in major collections as indicated (Table 1). Sequences from the published studies of Cribb et al. (2001) and Littlewood and Olson (2001) based on complete ssrDNA, Lockyer et al. (2003) based on complete ssrDNA and complete lsrDNA and Tkach et al. (2000, 2001a,b,c, 2003) based on partial lsrDNA (D1–D3), provided the foundation for the present study. These and a small number of other published sequences were fully complemented with additional ssrDNA or lsrDNA as necessary, and sequences from both genes were characterised for a considerable number of additional taxa (see Table 1). Newly collected specimens were fixed live in the field using 95–100% EtOH and stored in 95% EtOH at –20°C. Ethanol in the tissue samples was replaced with 1 M Tris–EDTA (pH 8) buffer via repeated washings and the gDNA was extracted using a Qiagen® DNeasy™ tissue kit following manufacturer-recommended protocols, with the exceptions that the incubation period with proteinase-K was extended to overnight in a rotating incubator and the final elution volume was 200 µl. In some cases, the gDNA was further concentrated to a volume of ~20 µl using Millipore Microcon® columns. Alternatively, some specimens were extracted using the guanidine method of Tkach and Pawlowski (1999).

2.2. Polymerase chain reaction amplification and sequencing

Polymerase chain reaction (PCR) amplifications (25 µl) were performed using Ready-To-Go™ (Amersham Pharmacia Biotech) PCR beads (each containing ~1.5 units Taq DNA polymerase, 10 mM Tris–HCl at pH 9, 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTP and stabilisers, including BSA), 1 µl of genomic extract and 10 mM of each PCR primer using the following thermocycling profile: 3 min denaturation hold at 94°C; 40 cycles of 30 s at 94°C, 30 s at 56°C, 2 min at 72°C; and 7 min extension hold at 72°C. Near-complete ssrDNA sequences (~1,800 bp) were amplified using primers Worm-A and Worm-B (see Littlewood and Olson, 2001 for primer definitions) and partial (domains D1–D3; ~1,400 bps) lsrDNA sequences were amplified using primers LSU-5 (5'-TAG GTC GAC CCG CTG AAY TTA AGC A-3') and 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3'). PCR amplicons were either gel-

Table 1
Taxonomic listing of exemplar taxa

Classification
Class Trematoda
Subclass Aspidogastrea
Order Aspidogastrea
Family Aspidogastridae
<i>Aspidogaster conchicola</i> Ex. <i>Quadula pustulosa</i> (Freshwater mussel), Tennessee River 89, Onile, Tennessee, USA AJ287478/AY222162 ^a
<i>Cotylaspis</i> sp. Ex. <i>Pelodiscus sinensis</i> (Chinese soft-shelled turtle), Chiling, HaiDuong, Vietnam AY222083 ^a /AY222165 ^a
<i>Cotylogaster basiri</i> Ex. <i>Pogonias cromis</i> (Black drum), Gulf of Mexico, Mississippi, USA [BMNH 2003.2.11.4] AY222082 ^a /AY222164 ^a
<i>Lobatostoma manteri</i> Ex. <i>Trachinotus blochii</i> (Snubnose pompano), HI L16911/AY157177
<i>Multicotyle purvisi</i> Ex. <i>Stebenrockiella crassicolis</i> (Malaysian black mud turtle), Malaya AJ228785/AY222166 ^a
Family Multicalycidae
<i>Multicalyx elegans</i> Ex. <i>Callorhynchus milii</i> (Ghost shark), Hobart, Tasmania, Australia AJ287532/AY222163 ^a
Order Stichocotyliida
Family Rugogastridae
<i>Rugogaster hydrolagi</i> Ex. <i>Callorhynchus milii</i> (Ghost shark), Hobart, Tasmania, Australia AJ287573/AY157176
Subclass Digenea
Order Echinostomida
Superfamily Echinostomoidea
Family Atractotrematidae
<i>Atractotrema sigani</i> Ex. <i>Siganus lineatus</i> (Golden-lined spinefoot), LI AJ287479/AY222267 ^a
Family Echinostomatidae
<i>Echinostoma revolutum</i> Ex. <i>Mesocricetus auratus</i> (hamster), laboratory infection, UK AY222132 ^a /AY222246 ^a
<i>Euparyphium melis</i> Ex. <i>Nyctereutes procyonoides</i> (Raccoon dog), Kherson Region, Ukraine AY222131 ^a /AF151941
Family Fasciolidae
<i>Fasciola gigantica</i> Ex. <i>Bos taurus</i> (cattle), St. Louis, Senegal AJ011942/AY222245 ^a
<i>Fasciola hepatica</i> Ex. <i>Capra hircus</i> (goat), Saudi Arabia AJ004969/AY222244 ^a
Family Haploporidae
<i>Hapladena nasonis</i> Ex. <i>Naso unicornis</i> (Blue-spine unicornfish), LI AY222146 ^a /AY222265 ^a
<i>Pseudomegasolena ishigakiense</i> Ex. <i>Scarus rivulatus</i> (Rivulated parrotfish), HI AJ287569/AY222266 ^a
Family Haplospalanchnidae
<i>Hymenocotta mulli</i> Ex. <i>Crenimugil crenilabis</i> (Fringe-lip mullet), HI AJ287524/AY222239 ^a
<i>Schikhobalotrema</i> sp. Ex. <i>Scarus rivulatus</i> (Rivulated parrotfish), HI AJ287574/AY222238 ^a
Family Philophthalmidae
<i>Cloacitrema narrabeenensis</i> ^b Ex. <i>Batillaria australis</i> (whelk-like gastropod), Rodd Point, Iron Cove, Sydney Harbour, NSW, Australia AY222134 ^a /AY222248 ^a
Unidentified philophthalmid sp. ^b Ex. <i>Batillaria australis</i> (whelk-like gastropod), Woody Point, Moreton Bay, Queensland, Australia AY222133 ^a /AY222247 ^a
Family Psilostomidae
<i>Psilochasmus oxyurus</i> Ex. <i>Anas platyrhynchos</i> (Mallard duck), Kherson Region, Ukraine AY222135 ^a /AF151940
Superfamily Heronimoidea
Family Heronimidae
<i>Heronimus mollis</i> Ex. <i>Chelydra serpentina</i> (Snapping turtle), Pawnee County, Nebraska, USA AY222118 ^a /AY116878
Superfamily Paramphistomoidea
Family Cladorchiidae
<i>Solenorchis travassosi</i> Ex. <i>Dugong dugong</i> (Dugong), Lucinda, Queensland, Australia AY222110 ^a /AY222213 ^a
Family Diplodiscidae
<i>Diplodiscus subclavatus</i> Ex. <i>Rana ridibunda</i> (Marsh frog), Kokaljane, Bulgaria AJ287502/AY222212 ^a
Superfamily Pronocephaloidea
Family Labicolidae
<i>Labicola</i> cf. <i>elongata</i> Ex. <i>Dugong dugon</i> (Dugong), Lucinda, Queensland, Australia AY222115 ^a /AY222221
Family Notocotylidae
<i>Catatropis indicus</i> Ex. <i>Cairina moschata</i> (Muscovy duck), Laboratory infection, University of New England, Armidale, NSW, Australia AY222114 ^a /AY222220 ^a
<i>Notocotylus</i> sp. ^b Ex. <i>Lymnaea palustris</i> (gastropod), Leckford Estate, Stockbridge, UK AJ287547/AY222219 ^a
Family Opisthotrematidae
<i>Lankatrema mamarense</i> Ex. <i>Dugong dugong</i> (Dugong), Townsville, Queensland, Australia AY222116 ^a /AY222222
<i>Opisthotrema dujonis</i> Ex. <i>Dugong dugong</i> (Dugong), Townsville, Queensland, Australia AY222117 ^a /AY222223
Family Pronocephalidae
<i>Macrovestibulum obtusicaudum</i> Ex. <i>Trachemys scripta scripta</i> (Slider turtle), George County, Mississippi, USA AY222111 ^a /AY116877
Family Rhabdiopoeidae
<i>Rhabdiopoeus taylors</i> Ex. <i>Dugong dugong</i> (Dugong), Lucinda, Queensland, Australia AY222113 ^a /AY222218 ^a
<i>Taprobanelia bicaudata</i> Ex. <i>Dugong dugong</i> (Dugong), Townsville, Queensland, Australia AY222112 ^a /AY222217 ^a
Superfamily Microscaphidioidea
Family Mesometridae
<i>Mesometra</i> sp. Ex. <i>Sarpa salpa</i> (Salema), Mediterranean Sea, Fish market in Perpignan, France AJ287537/AY222216 ^a
Family Microscaphidiidae
<i>Hexangium</i> sp. Ex. <i>Siganus fuscescens</i> (Mottled spinefoot), HI AJ287522/AY222215 ^a
<i>Neohexangiotrema zebrasomatis</i> Ex. <i>Zebrasoma scopas</i> (Twotone tang), LI AJ287544/AY222214 ^a

(continued on next page)

Table 1 (continued)

Classification
Order Plagiorchiida
Superfamily Allocreadioidea
Family Opecoelidae
<i>Gaevskejatrema halosauropsi</i> Ex. <i>Halosauropsis macrochir</i> (Abyssal halosaur), Goban Spur, NE Atlantic Ocean, UK [BMNH 1995.5.30.20–21] AJ287514/AY222207 ^a
<i>Macvicaria macassarensis</i> Ex. <i>Lethrinus miniatus</i> (Sweetlips), HI AJ287533/AY222208 ^a
<i>Peracreadium idoneum</i> Ex. <i>Anarhichas lupus</i> (Wolf-fish), North Sea, UK AJ287558/AY222209 ^a
Family Opistholebetidae
<i>Maculifer</i> sp. Ex. <i>Diodon hystrix</i> (Porcupine fish), HI AY222109 ^a /AY222211 ^a
<i>Opistholebes amplicolus</i> Ex. <i>Tetractenos hamiltoni</i> (Common toadfish), Stradbroke Island, Queensland, Australia AJ287550/AY222210 ^a
Superfamily Lepocreadioidea
Family Acanthocolpidae
<i>Cableia pudica</i> Ex. <i>Cantherines pardalis</i> (Honeycomb filefish), HI AJ287486/AY222251 ^a
<i>Stephanostomum baccatum</i> Ex. <i>Eutrigla gurnardus</i> (Grey gurnard), North Sea, UK [BMNH 1993.9.17.10] AJ287577/AY222256 ^a
Family Apocreadiidae
<i>Homalometron armatum</i> Ex. <i>Lepomis microlophus</i> (Redear sunfish), Pascagoula River, Wilkerson's Ferry, Mississippi, USA [BMNH 2002.4.9.39] AY222130 ^a /AY222241 ^a
<i>Homalometron synagris</i> Ex. <i>Scolopsis monogramma</i> (Monogrammed monocle bream), HI AJ287523/AY222243 ^a
<i>Neapocreadium splendens</i> Ex. <i>Scolopsis monogramma</i> (Monogrammed monocle bream), LI AJ287543/AY222242 ^a
<i>Schistorchis zancli</i> Ex. <i>Zanclus cornutus</i> (Moorish idol), East Opunohu Bay, Moorea, French Polynesia AY222129 ^a /AY222240 ^a
Family Brachycladiidae
<i>Zalophotrema hepaticum</i> Ex. <i>Zalophus californianus</i> (California sealion), California, USA AJ224884/AY222255 ^a
Family Enenteridae
<i>Enenterum aureum</i> Ex. <i>Kyphosus vaigiensis</i> (Brassy chub), Fish market, Moorea, French Polynesia [BMNH 2002.7.17.1–7] AY222124 ^a /AY222232 ^a
<i>Koseiria xishaense</i> Ex. <i>Kyphosus vaigiensis</i> (Brassy chub), HI [BMNH 2002.7.17.22–24] AY222125 ^a /AY222233 ^a
Family Gorgocephalidae
<i>Gorgocephalus kyphosi</i> Ex. <i>Kyphosus vaigiensis</i> (Brassy chub), LI [BMNH 2003.1.21.1–6] AY222126 ^a /AY222234 ^a
Family Gyliauchenidae
<i>Paragyliauchen arusettae</i> Ex. <i>Pomacanthus sexstriatus</i> (Sixbar angelfish), Ningaloo, Western Australia AY222127 ^a /AY222235 ^a
Family Lepocreadiidae
<i>Preptetos caballeroi</i> Ex. <i>Naso vlamingi</i> (Bignose unicornfish), HI AJ287563/AY222236 ^a
<i>Preptetos trulla</i> Ex. <i>Ocyurus chrysurus</i> (Yellow-tail snapper), Port Royal, Kingston, Jamaica [BMNH 1995.9.26.1–5] AY222128 ^a /AY222237 ^a
Superfamily Microphalloidea
Family Microphallidae
<i>Maritrema oocysta</i> ^b Ex. <i>Hydrobia ulvae</i> (Laver spire shell), Belfast Lough, Northern Ireland AJ287534/AY220630
<i>Microphallus fusiformis</i> ^b Ex. <i>Hydrobia ulvae</i> (Laver spire shell), Belfast Lough, Northern Ireland AJ287531 ^c /AY220633
<i>Microphallus primas</i> ^b Ex. <i>Carcinus maenus</i> (Shore crab), Belfast Lough, Northern Ireland AJ287541/AY220627
Superfamily Opisthorchioidea
Family Cryptogonimidae
<i>Caecincola parvulus</i> Ex. <i>Micropterus salmoides</i> (Largemouth bass), Pascagoula River, Wilkerson's Ferry, Mississippi, USA [BMNH 2002.4.9.40] AY222123 ^a /AY222231 ^a
<i>Siphodera vinalwardsii</i> sp. Ex. <i>Sciaenops ocellatus</i> (Red drum), Gulf of Mexico, South of Horn Island, Mississippi, USA [BMNH 2003.2.11.3] AY222122 ^a /AY222230 ^a
<i>Mitotrema anthostomatium</i> Ex. <i>Cromileptes altivelis</i> (Barramundi cod), HI AJ287542/AY222229 ^a
Family Heterophyidae
<i>Cryptocotyle lingua</i> Ex. <i>Littorina littorea</i> (Edible periwinkle), Isle of Sylt, North Sea, Germany AJ287492/AY222228 ^a
<i>Galactosomum lacteum</i> Ex. <i>Phalacrocorax carbo</i> (Great cormorant), Kherson Region, Ukraine AY222120 ^a /AY222227 ^a
<i>Haplorchoides</i> sp. Ex. <i>Arius graeffei</i> (Lesser salmon catfish), Lake Wivenhoe, Queensland, Australia AJ287521/AY222226 ^a
Family Opisthorchiidae
<i>Amphimerus ovalis</i> Ex. <i>Trionyx muticus</i> (Softshell turtle), George County, Mississippi, USA AY222121 ^a /AY116876
Superfamily Plagiorchioidea
Family Auridistomidae
<i>Auridistomum chelydrae</i> Ex. <i>Chelydra serpentina</i> (Snapping turtle), Jackson County, Mississippi, USA AY222159 ^a /AY116872
Family Brachycoeliidae
<i>Brachycoelium salamandrae</i> Ex. <i>Salamandra salamandra</i> (salamander), Zakarpatska Region near Rakhiv, Ukraine AY222160 ^a /AF151935
<i>Mesocoelium</i> sp. Ex. <i>Bufo marinus</i> (Cane toad), Brisbane, Queensland, Australia AJ287536/AY222277 ^a
Family Cephalogonimidae
<i>Cephalogonimus retusus</i> Ex. <i>Rana ridibunda</i> (Marsh frog), Kopaljane, near Sofia, Bulgaria AJ287489/AY222276 ^a
Family Choanocotylidae
<i>Choanocotyle hobbsi</i> Ex. <i>Chelodina oblonga</i> (Oblong turtle), Murdoch University Veterinary School Campus, Perth, Western Australia AY116868/AY116865
<i>Choanocotyle nematoides</i> Ex. <i>Emydura</i> sp. (turtle), New South Wales, Australia AY116867/AY116862
Family Dicrocoeliidae
<i>Brachylecithum lobatum</i> Ex. <i>Corvus corone</i> (Carrion crow), Záhlinice, Czech Republic AY222144 ^a /AY222260 ^a
<i>Dicrocoelium dendriticum</i> Ex. <i>Ovis aries</i> (Domestic sheep), Spain Y11236/AY222261 ^a
<i>Lyperosomum collurionis</i> Ex. <i>Sylvia atricapilla</i> (Blackcap), Záhlinice, Czech Republic AY222143 ^a /AY222259 ^a
Family Encyclometridae
<i>Encyclometra colubrimurorum</i> Ex. <i>Natrix natrix</i> (Grass snake), Kiev Region, Ukraine AY222142 ^a /AF184254
Family Gorgoderidae
<i>Degeneria halosauri</i> Ex. <i>Halosauropsis macrochir</i> (Abyssal halosaur), NE Atlantic Ocean [BMNH 1995.3.30.25–28] AJ287497/AY222257 ^a

Table 1 (continued)

Classification
<i>Gorgodera cygnoides</i> Ex. <i>Rana ridibunda</i> (Marsh frog), Kokaljane, near Sofia, Bulgaria AJ287518/AY222264 ^a
<i>Nagnia floridensis</i> Ex. <i>Rhinoptera bonasus</i> (Cownose ray), Gulf of Mexico, East Ship Island, Mississippi, USA AY222145 ^a /AY222262 ^a
<i>Xystretrum</i> sp. Ex. <i>Sufflamen chrysopterus</i> (Halfmoon triggerfish), LI AJ287588/AY222263 ^a
Family Lecithodendriidae
<i>Lecithodendrium linstowi</i> Ex. <i>Nyctalus noctula</i> (Noctule bat), Sumy Region, Ukraine AY222147 ^a /AF151919
<i>Prosthodendrium longiforme</i> Ex. <i>Myotis daubentonii</i> (Daubenton's bat), Kiev Region, Ukraine AY222148 ^a /AF151921
Family Macroderoididae
<i>Macroderoides typicus</i> Ex. <i>Lepisosteus platostomus</i> (Alligator gar), Reelfoot Lake, Tennessee, USA AY222158 ^a /AF433673
Family Omphalometridae
<i>Rubenstrema exasperatum</i> Ex. <i>Crociodura leucodon</i> (White-toothed shrew), Bulgaria AJ287572/AY222275 ^a
Family Pachypsolidae
<i>Pachypsolus irroratus</i> Ex. <i>Lepidochelys olivacea</i> (Olive Ridley's turtle), Oaxaca, Mexico AJ287554/AY222274 ^a
Family Plagiorchidae
<i>Haematoloechus longiplexus</i> Ex. <i>Rana catesbeiana</i> (North American bullfrog), Keith County, Nebraska, USA AJ287520/AY222280 ^a
<i>Glypthelminis quieta</i> Ex. <i>Rana catesbeiana</i> (North American bullfrog), Keith County, Nebraska, USA AJ287517/AY222278 ^a
<i>Skrjabinoeces similis</i> Ex. <i>Rana ridibunda</i> (Marsh frog), Kokaljane, near Sofia, Bulgaria AJ287575/AY222279 ^a
Family Pleurogenidae
<i>Pleurogenes claviger</i> Ex. <i>Rana temporaria</i> (Common frog), Kiev Region, Ukraine AY222152 ^a /AF151925
<i>Pleurogenoides medians</i> Ex. <i>Rana lessonae</i> (Pool frog), Kiev Region, Ukraine AY222151 ^a /AF433670
Family Prosthogonimidae
<i>Prosthogonimus ovatus</i> Ex. <i>Pica pica</i> (Magpie), Chernigiv Region, Ukraine AY222149 ^a /AF151928
<i>Schistogonimus rarus</i> Ex. <i>Anas querquedula</i> (Little puddle duck), Kherson Region, Ukraine AY222150 ^a /AY116869
Family Telorchidae
<i>Opisthioglyphe ranae</i> Ex. <i>Rana arvalis</i> (Moor frog), Ivano-Frankivsk Region, Ukraine AY222157 ^a /AF151929
<i>Telorchis assula</i> Ex. <i>Natrix natrix</i> (Grass snake), Kiev Region, Ukraine AY222156 ^a /AF151915
Superfamily Renicoloidea
Family Renicolidae
<i>Renicola</i> sp. Ex. <i>Numenius arquata</i> (Curlew), Kherson Region, Ukraine AY222155 ^a /AY116871
Superfamily Troglotrematoidea
Family Orchipidae
<i>Orchipedum tracheicola</i> Ex. <i>Cygnus olor</i> (Mute swan), Drumpellier Loch, Scotland [BMNH 1996.4.25.19–38] AJ287551/AY222258 ^a
Family Paragonimidae
<i>Paragonimus iloktsuenensis</i> Ex. <i>Rattus norvegicus</i> (Norway rat), Amami Island, Japan AY222141 ^a /AY116875
<i>Paragonimus westermani</i> Ex. <i>Canis familiaris</i> (Domestic dog), Hyogo, Japan AY222140 ^a /AY116874
Family Troglotrematidae
<i>Nanophyetus salminicola</i> Ex. <i>Oncorhynchus mykiss</i> (Rainbow trout), Alsea hatchery, Benton County, Oregon, USA AY222138 ^a /AY116873
<i>Nephrotrema truncatum</i> Ex. <i>Neomys anomalus</i> (Lesser water shrew), Zakarpatska Region, Ukraine AY222139 ^a /AF151936
Superfamily Zoogonoidea
Family Faustulidae
<i>Antorchis pomacanthi</i> Ex. <i>Pomacanthus sexstriatus</i> (Sixbar angelfish), HI, AJ287476/AY222268 ^a
<i>Bacciger lesteri</i> Ex. <i>Selenotoca multifasciata</i> (Spotbanded scat), Moreton Bay, Brisbane, Queensland, Australia AJ287482/AY222269 ^a
<i>Trigonocryptus conus</i> Ex. <i>Arothron nigropunctatus</i> (Black-spotted puffer), HI, AJ287584/AY222270 ^a
Family Lissorchiidae
<i>Lissorchis kritskyi</i> Ex. <i>Carpiodes cyprinus</i> (Quillback carpsucker), Pascagoula River, Wilkerson's Ferry, Mississippi, USA AY222136 ^a /AY222250 ^a
Family Monorchiidae
<i>Ancylucoelium typicum</i> Ex. <i>Trachurus trachurus</i> (Horse mackerel), North Sea, UK AJ287474/AY222254 ^a
<i>Diplomonorchis leiostomi</i> Ex. <i>Leiostomus xanthurus</i> (Spot), Gulf of Mexico, Ocean Springs, Mississippi, USA [BMNH 2003.2.11.1–2] AY222137 ^a /AY222252 ^a
<i>Provitellus turrum</i> Ex. <i>Pseudocaranx dentex</i> (White trevally), HI AJ287566/AY222253 ^a
Family Zoogonidae
<i>Deretrema nahaense</i> Ex. <i>Thalassoma lunare</i> (Moon wrasse), LI AJ287498/AY222273 ^a
<i>Diphtherostomum</i> sp. Ex. <i>Scolopsis monogramma</i> (Monogrammed monocle bream), HI AY222153 ^a /AY222272 ^a
<i>Lepidophyllum steenstrupi</i> Ex. <i>Anarhichas lupus</i> (Wolf-fish), North Sea, UK AJ287530/AY157175
<i>Zoogonoides viviparus</i> Ex. <i>Callionymus lyra</i> (Dragonet), North Sea, UK AJ287590/AY222271 ^a
Order Strigeida
Superfamily Azygioidea
Family Azygiidae
<i>Otodistomum cestoides</i> Ex. <i>Raja montagui</i> (Spotted ray), North Sea, UK AJ287553/AY222187 ^a
Superfamily Bivesiculoidea
Family Bivesiculidae
<i>Bivesicula claviformis</i> Ex. <i>Epinephelus quoyanus</i> (Longfin grouper), LI AJ287485/AY222182 ^a
<i>Bivesicula unexpecta</i> Ex. <i>Acanthochromis polyacanthus</i> (Spiny chromis), HI AY222099 ^a /AY222181 ^a
<i>Bivesiculoides fusiformis</i> Ex. <i>Atherinomorus capricornensis</i> (Hardyhead), HI AY222100 ^a /AY222183 ^a
Superfamily Brachylaimoidea
Family Brachylaimidae
<i>Brachylaima</i> sp. Ex. <i>Mus musculus</i> (mouse), laboratory infection, Queensland, Australia AY222084 ^a /AY222167 ^a
<i>Brachylaima thompsoni</i> Ex. <i>Blarina brevicaudata</i> (Musk shrew), Wisconsin, USA AY222085 ^a /AF184262
<i>Zeylanurotrema spearei</i> Ex. <i>Bufo marinus</i> (Cane toad), Daintree region, Queensland, Australia AY222088 ^a /AY222170 ^a
Family Leucochloridiidae
<i>Leucochloridium perturbatum</i> Ex. <i>Turdus merula</i> (Blackbird), Záhlinice, Czech Republic AY222087 ^a /AY222169 ^a

(continued on next page)

Table 1 (continued)

Classification
<i>Urogonimus macrostomus</i> Ex. <i>Anas platyrhynchos</i> (Mallard duck), laboratory infection, Ukraine AY222086 ^a /AY222168 ^a
Superfamily Bucephaloidea
Family Bucephalidae
<i>Prosorhynchoides gracilescens</i> Ex. <i>Lophius piscatorius</i> (Anglerfish), North Sea, UK [BMNH 1997.10.28.15–50] AJ228789/AY222224 ^a
<i>Rhipidocotyle galeata</i> Ex. <i>Eutrigla gurnardus</i> (Grey gurnard), North Sea, UK AY222119 ^a /AY222225 ^a
Superfamily Clinostomoidea
Family Clinostomidae
<i>Clinostomum</i> sp. ^b Ex. <i>Hypseleotris galii</i> (Firetailed gudgeon), Moggil Creek, Queensland, Australia AY222094 ^a /AY222175 ^a
<i>Clinostomum</i> sp. ^b Ex. <i>Rana catesbeiana</i> (North American bullfrog), Reelfoot Lake, Tennessee, USA AY222095 ^a /AY222176 ^a
Superfamily Cyclocoeloidea
Family Cyclocoelidae
<i>Cyclocoelum mutabile</i> Ex. <i>Calidris canutus</i> (Knot), Fair Isle Bird Observatory, Fair Isle, Scotland [BMNH 1997.1.3.1] AJ287494/AY222249 ^a
Family Eucotyliidae
<i>Tanaisia fedtschenkoi</i> Ex. <i>Anas platyrhynchos</i> (Mallard duck), Kherson Region, Ukraine AY222154 ^a /AY116870
Superfamily Diplostomoidea
Family Diplostomidae
<i>Alaria alata</i> Ex. <i>Nyctereutes procyonoides</i> (Raccoon dog), Kherson Region, Ukraine AY222091 ^a /AF184263
<i>Diplostomum phoxini</i> ^b Ex. <i>Phoxinus phoxinus</i> (Common minnow), Aberystwyth, Wales AY222090 ^a /AY222173 ^a
Family Strigeidae
<i>Apharyngostrigea cornu</i> Ex. <i>Ardea cinerea</i> (Grey heron), Kherson Region, Ukraine AY222092 ^a /AF184264
<i>Cardiocephaloides longicollis</i> Ex. <i>Larus ridibundus</i> (Black-headed gull), Kherson Region, Ukraine AY222089 ^a /AY222171 ^a
<i>Ichthyocotylurus erraticus</i> Ex. <i>Coregonus autumnalis</i> (Arctic cisco), Lough Neagh, Northern Ireland, UK AJ287526/AY222172 ^a
Superfamily Gymnophalloidea
Family Callodistomidae
<i>Prosthenhystera obesa</i> Ex. <i>Hoplias</i> sp. (Trahira), Rio Itaya, 50 km from Iquitos, Peru AY222108 ^a /AY222206 ^a
Family Fellodistomidae
<i>Fellodistomum fellis</i> Ex. <i>Anarhichas lupus</i> (Wolf-fish), North Sea, UK Z12601/AY222282 ^a
<i>Olssonium turneri</i> Ex. <i>Alepocephalus agassizi</i> (Agassiz' slickhead), Porcupine Seabight, NE Atlantic [BMNH 1997.10.28.102] AJ287548/AY222283 ^a
<i>Proctoeces maculatus</i> Ex. <i>Archosargus probatocephalus</i> (Sheepshead), Gulf of Mexico, Mississippi, USA [BMNH 2002.4.9.35–38] AY222161 ^a /AY222284 ^a
<i>Steringophorus margolisi</i> Ex. <i>Spectrunculus grandis</i> (Pudgy cuskeel), Rockall Trough, NE Atlantic [BMNH 1992.3.24.10–14] AJ287578/AY222281 ^a
Family Tandanicolidae
<i>Prosogonarium angelae</i> Ex. <i>Euristhmus lepturus</i> (Long-tailed catfish), Moreton Bay, Brisbane, Queensland, Australia AJ287564/AY222285 ^a
Superfamily Hemiuroidea
Family Accacoeliidae
<i>Accacoelium contortum</i> Ex. <i>Mola mola</i> (Ocean sunfish), North Sea, UK [BMNH 1999.2.4.7–36] AJ287472/AY222190 ^a
Family Derogenidae
<i>Derogenes varicus</i> Ex. <i>Hippoglossoides platessoides</i> (Long rough dab), North Sea, UK AJ287511/AY222189 ^a
<i>Hemiperina manteri</i> Ex. <i>Latridopsis forsteri</i> (Bastard trumpeter), Tasmania, Australia AY222105 ^a /AY222196 ^a
Family Didymozoidae
Unidentified didymozoid sp. 1 Ex. <i>Epinephelus cyanopodus</i> (Speckled blue grouper), HI AY222103 ^a /AY222193 ^a
Unidentified didymozoid sp. 2 Ex. <i>Taeniura lymma</i> (Blue-spotted stingray), HI AY222102 ^a /AY222192 ^a
Unidentified didymozoid sp. 3 Ex. <i>Apogon cookii</i> (Cook's cardinalfish), HI AY222104 ^a /AY222194 ^a
<i>Didymozoon scombr</i> Ex. <i>Scomber scombrus</i> (Mackerel), North Sea, UK AJ287500/AY222195 ^a
Family Hemiuridae
<i>Dinurus longisinus</i> Ex. <i>Coryphaena hippurus</i> (Dolphin fish), Port Royal, Kingston, Jamaica [BMNH 1996.8.19.4–5] AJ287501/AY222202 ^a
<i>Lecithochirium caesionis</i> Ex. <i>Caesio cuning</i> (Red-belly yellowtail fusilier), HI AJ287528/AY222200 ^a
<i>Lecithocladium excisum</i> Ex. <i>Scomber scombrus</i> (Mackerel), North Sea, UK AJ287529/AY222203 ^a
<i>Machidatrema chilotoma</i> Ex. <i>Kyphosus vaigiensis</i> (Brassy chub), Moorea, French Polynesia AY222106 ^a /AY222197 ^a
<i>Merluccioretrema praeclarum</i> Ex. <i>Cataetx laticeps</i> (Viviparous brotula), Goban Spur, NE Atlantic [BMNH 1995.7.25.4–7] AJ287535/AY222204 ^a
<i>Opisthadena dimidia</i> Ex. <i>Kyphosus cinerascens</i> (Blue seachub), HI [QM G217866-7 and BMNH 2002.4.18.7] AJ287549/AY222198 ^a
<i>Plerurus digitatus</i> Ex. <i>Scomberomorus commerson</i> (Narrow-barred Spanish mackerel), HI AJ287562/AY222201 ^a
Family Lecithasteridae
<i>Lecithaster gibbosus</i> Ex. <i>Merlangius merlangus</i> (Whiting), North Sea, UK AJ287527/AY222199 ^a
<i>Lecithophyllum botryophorum</i> Ex. <i>Alepocephalus bairdii</i> (Baird's smoothhead), Goban Spur, NE Atlantic [BMNH 1997.10.30.4–8] AY222107 ^a /AY222205 ^a
Family Sclerodistomidae
<i>Prosogonotrema bilabiatum</i> Ex. <i>Caesio cuning</i> (Red-belly yellowtail fusilier), HI AJ287565/AY222191 ^a
Family Syncoeliidae
<i>Copiatestes filiferus</i> Ex. <i>Trachurus murphyi</i> (Inca scad), New Zealand [BMNH 1993.10.7.2–4] AJ287490/AY222188 ^a
Superfamily Schistosomatoidea
Family Sanguinicolidae
Unidentified sanguinicolid sp. Ex. <i>Arothron meleagris</i> (Guineafowl puffer), Moorea, French Polynesia [BMNH 2003.1.17.1–8] AY157184/AY157174
<i>Aporocotyle spinosicanalis</i> Ex. <i>Merluccius merluccius</i> (Hake), Off Orkney Islands, NE Atlantic Ocean AJ287477/AY222177 ^a
<i>Chimaerohemecus trondheimensis</i> Ex. <i>Chimaera monstrosa</i> (Chimaera), Korsfjorden, Bergen, Norway [BMNH 2002.9.27.1] AY157213/AY157239
<i>Neoparacardicola nasonis</i> Ex. <i>Naso unicornis</i> (Bluespine unicornfish), LI AY222097 ^a /AY222179 ^a
<i>Plethorchis acanthus</i> Ex. <i>Mugil cephalus</i> (Flathead mullet), Brisbane River, Queensland, Australia AY222096 ^a /AY222178 ^a
<i>Sanguinicola</i> cf. <i>inermis</i> ^b Ex. <i>Lymnaea stagnalis</i> (gastropod), Warminia-Mazury Region, Poland AY222098 ^a /AY222180 ^a
Family Schistosomatidae
<i>Austroilharzia terrigalensis</i> ^b Ex. <i>Batillaria australis</i> (whelk-like snail), Rodd Point, Iron Cove, Sydney Harbour, NSW, Australia AY157223/AY157249
<i>Bilharziella polonica</i> Ex. <i>Anas platyrhynchos</i> (Mallard duck), Kherson Oblast, Ukraine AY157214/AY157240
<i>Dendritobilharzia pulverulenta</i> Ex. <i>Gallus gallus domesticus</i> (chicken), Bernalillo County, New Mexico, USA AY157215/AY157241

Table 1 (continued)

Classification
<i>Gigantobilharzia huronensis</i> Ex. <i>Agelaius phoeniceus</i> (Red-winged blackbird), Wisconsin, USA AY157216/AY157242
<i>Heterobilharzia americana</i> Ex. <i>Mesocricetus auratus</i> (hamster), laboratory infection, UK AY157220/AY157246
<i>Ornithobilharzia canaliculata</i> Ex. <i>Larus delawarensis</i> (Delaware gull), Donley County, Texas, USA AY157222/AY157248
<i>Schistosoma haematobium</i> Ex. <i>Mesocricetus auratus</i> (hamster), laboratory infection, UK Z11976/AY157263
<i>Schistosoma japonicum</i> Ex. <i>Mus musculus</i> (mouse), laboratory infection, UK AY157226/AY157607
<i>Schistosoma mansoni</i> Ex. <i>Mus musculus</i> (mouse), laboratory infection, UK M62652/AY157173
<i>Schistosomatium douthitti</i> Ex. <i>Mesocricetus auratus</i> (hamster), laboratory infection, Indiana, USA AY157221/AY157247
Family Spirorchidae
<i>Spirorchis scripta</i> Ex. <i>Trachemys scripta scripta</i> (Slider turtle), Van Cleave, Mississippi, USA AY222093 ^b /AY222174 ^b
Superfamily Transversotrematoidea
Family Transversotrematidae
<i>Crusziella formosa</i> Ex. <i>Crenimugil crenilabis</i> (Fringelip mullet), HI AJ287491/AY222185 ^a
<i>Prototransversotrema steeri</i> Ex. <i>Acanthopagrus australis</i> (Surf seabream), Iluka, Queensland, Australia AY222101 ^a /AY222184 ^a
<i>Transversotrema haasi</i> Ex. <i>Caesio cuning</i> (Redbelly yellowtail fusilier), HI AJ287583/AY222186 ^a

Taxon Ex. *Host species* (common name), collection locality [voucher specimen accession number] ssrDNA/lsrDNA sequence accession numbers. BMNH, Parasitic Worms Division, Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK; HI, Heron Island, Coral Sea, Great Barrier Reef, Queensland, Australia; LI, Lizard Island, Coral Sea, Great Barrier Reef, Queensland, Australia; QM, Queensland Museum, South Brisbane, Queensland 4101, Australia.

^a Previously unpublished sequences (new sequences).

^b Identification and sequence based on larval worm (redia, sporocyst, cercaria, metacercaria or shistosomula).

excised or purified directly using Qiagen Qiaquick™ columns, cycle-sequenced from both strands using ABI BigDye™ chemistry, alcohol-precipitated and run on an ABI Prism 377™ automated sequencer. ssrDNA products were sequenced in both directions using the two PCR primers and a variety of internal primers (Littlewood and Olson, 2001 provide a complete listing of ssrDNA primers designed or modified for platyhelminths), and lsrDNA products were sequenced using the two PCR primers and internal primers 300F (5'-CAA GTA CCG TGA GGG AAA GTT G-3') and ECD2 (5'-CTT GGT CCG TGT TTC AAG ACG GG-3'), as well as primers 400R (5'-GCA GCT TGA CTA CAC CCG-3') and 900F (5'-CCG TCT TGA AAC ACG GAC CAA G-3') in some cases. Contiguous sequences were assembled and edited using Sequencher™ (GeneCodes Corp., ver. 3.1.1) and submitted to GenBank under accession numbers AY222082–161 (ssrDNA) and AY222162–285 (lsrDNA, see also Table 1).

2.3. Alignments

Newly generated ssrDNA and lsrDNA sequences were combined with sequences previously published and aligned by eye using MacClade (Maddison and Maddison, 2002, ver. 4). lsrDNA sequences were concatenated with ssrDNA sequences in MacClade and regions of ambiguous alignment defined in a character exclusion set. Regions containing gaps in a majority of taxa were also excluded from analyses even if these regions were alignable among the minority of taxa possessing the insertions. Two alignments were constructed in order to maximise the alignable positions whilst maintaining the most inclusive sets of taxa: a 'full' alignment of 170 taxa (Table 1) with 2,648 included positions and the aspidogastrea taxa designated as an outgroup, and a reduced 'higher plagiorchidians' alignment of 108 taxa with 2,950 included positions and

the Bucephalidae designated as the functional outgroup (Watrous and Wheeler, 1981), based on the results of the combined analyses including all taxa (see below). We have deposited the complete alignments of ssrDNA and lsrDNA with EBI and each is available by anonymous FTP from <http://ftp.ebi.ac.uk> in directory/pub/databases/embl/align and via the EMBLALIGN database via SRS at <http://srs.ebi.ac.uk>, under the following accessions ALIGN_000525 (ssrDNA) and ALIGN_000526 (lsrDNA). Exclusion sets are added as notes and the alignments may be adapted as a NEXUS file.

2.4. Phylogenetic analyses

Individual phylogenetic analyses by the methods of maximum parsimony and Bayesian inference were conducted on the lsrDNA and ssrDNA data partitions, as well as the combined data for the full complement of taxa. Analyses of the reduced 'higher plagiorchidians' alignment were conducted with the combined data only. Maximum parsimony analyses were conducted with PAUP* (Swoford, 2001, ver. 4.0b10) and Bayesian inference analyses with MrBayes (Huelsenbeck and Ronquist, 2001, ver. 2.01). Maximum parsimony analyses were conducted using a heuristic search strategy with 100 search replicates, random-addition taxon sampling, tree-bisection-reconnection branch-swapping, with all characters run unordered with equal weights and with gaps treated as missing data. Models of nucleotide substitution were evaluated for each data partition independently using ModelTest (Posada and Crandall, 1998, ver. 3.06), and for each partition the most parameter rich model (i.e. general-time-reversible including estimates of invariant sites and gamma distributed among-site rate variation) was found to provide the best fit to the data. This was true when evaluating the models over a neighbour-joining topology (as implemented in ModelTest)

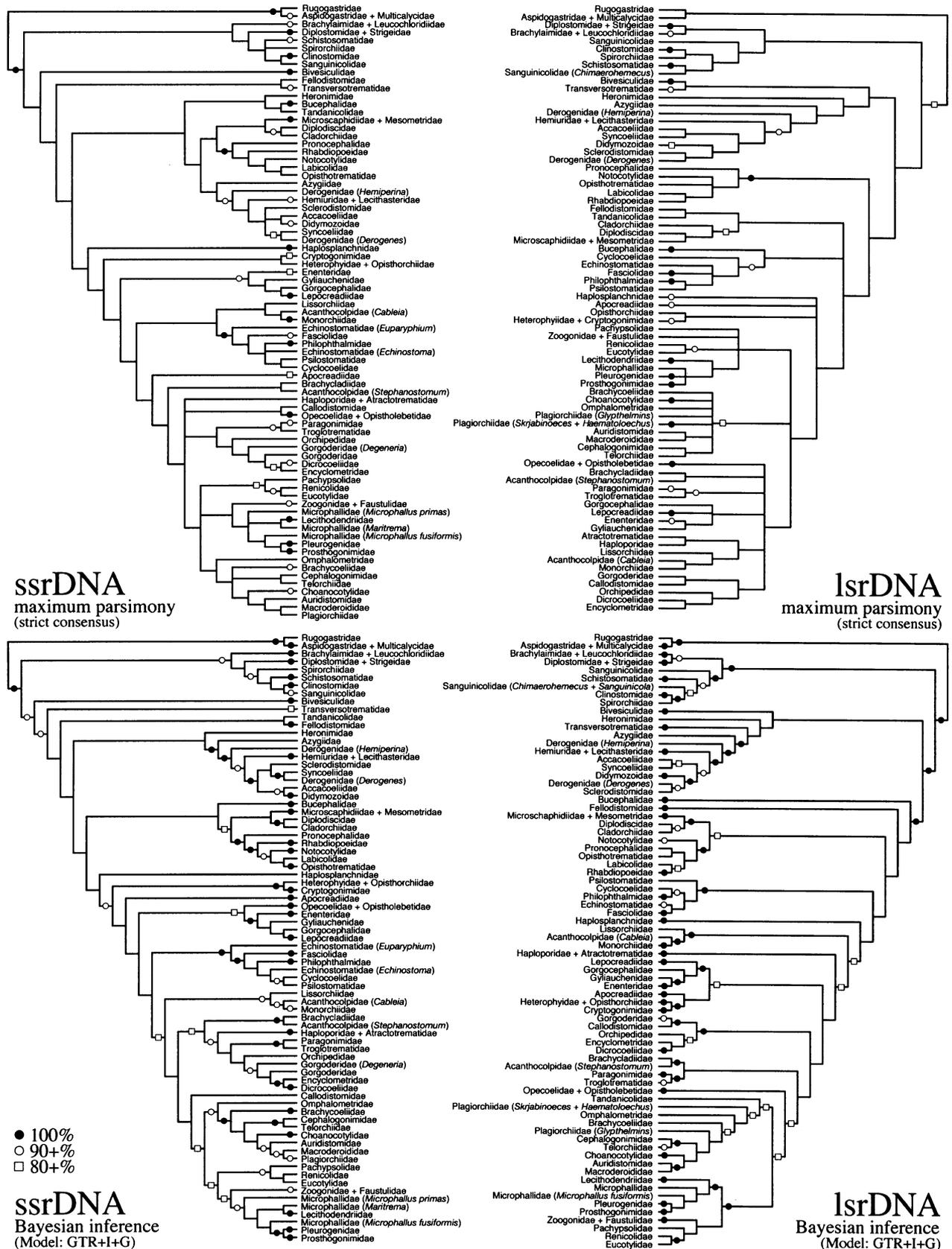


Fig. 1. Comparison of independent analyses of *ssrDNA* and *lsrDNA* using maximum parsimony and Bayesian inference for the Trematoda. Nodal support based on bootstrapping (maximum parsimony) and posterior probabilities (Bayesian inference). Families shown together as terminal taxa contain one or more paraphyletic groups. Families shown together as terminal taxa contain one or more paraphyletic groups.

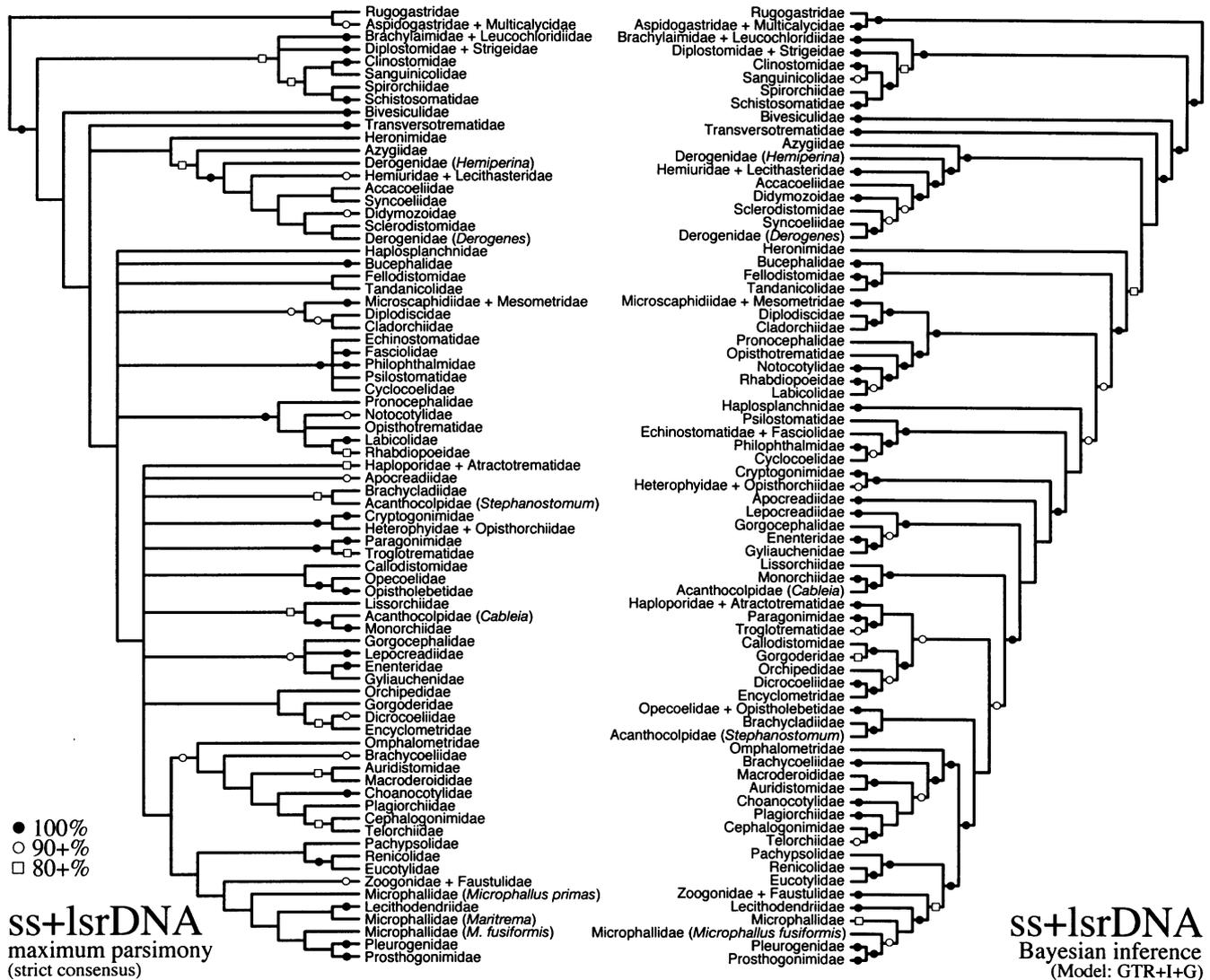


Fig. 2. Comparison of maximum parsimony and Bayesian inference on the combined data (ssrDNA + lsrDNA) for the Trematoda. Nodal support based on bootstrapping (maximum parsimony) and posterior probabilities (Bayesian inference). Families shown together as terminal taxa contain one or more paraphyletic groups.

or when using a strict consensus topology of the equally parsimonious trees resulting from prior MP analyses. Thus all Bayesian inference analyses used the following parameters: nst = 6, rates = invgamma, ncat = 4, shape = estimate, inferrates = yes and basefreq = empirical, that corresponds to the model estimated (general-time-reversible including estimates of invariant sites and gamma distributed among-site rate variation). Posterior probabilities were approximated over 300,000 generations (ngen = 300,000) via four simultaneous Markov Chain Monte Carlo (MCMC) chains (nchains = 4) with every 100th tree saved (samplefreq = 100). Default values were used for the MCMC parameters. Consensus trees with mean branch lengths were constructed using the ‘sumt’ command with the ‘contype = allcompat’ option and ignoring the initial topologies saved during ‘burn in’; the initial *n*-generations before log-likelihood values and substitution parameters

plateau (see Huelsenbeck and Ronquist, 2001). Maximum parsimony nodal support was estimated by bootstrap analysis (fast-heuristic, 10,000 replicates), and as posterior probabilities in the Bayesian inference analyses (Huelsenbeck et al., 2001).

3. Results

3.1. Presentation of results

Figs. 1–5 depict the results of the individual analyses and Fig. 6 depicts a revised classification based on the results of Bayesian inference of lsrDNA and ssrDNA combined (Fig. 3). Figs. 1, 2, 4 and 6 show reduced trees that depict interrelationships of the trematode families as currently defined (Table 1). In cases of paraphyly, the families are



Fig. 3. Species-level phylogram (170 taxa) of the Trematoda based on Bayesian inference of the combined data (ssrDNA + IsrDNA) using a general-time-reversible model of nucleotide substitution incorporating among-site rate variation.

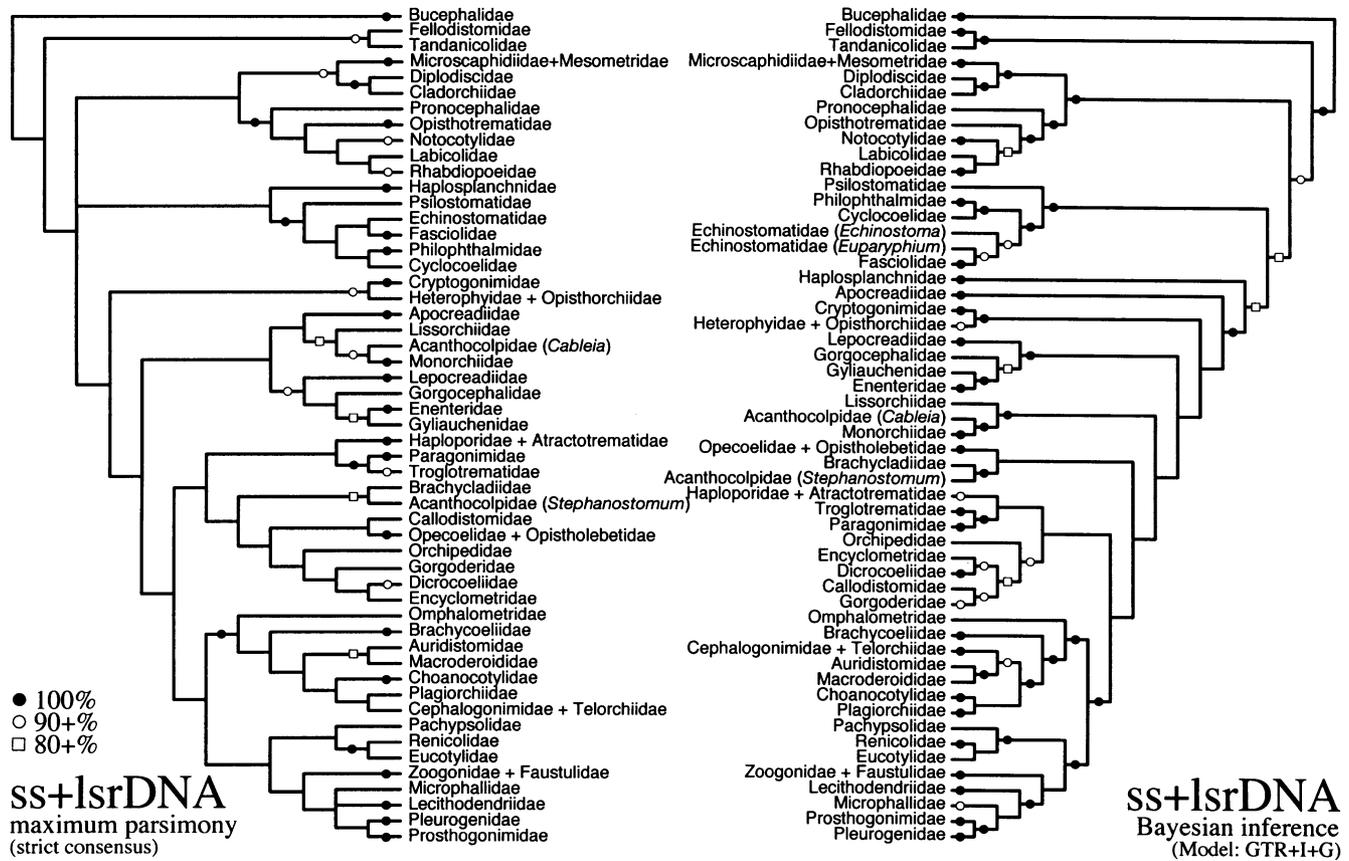


Fig. 4. Comparison of maximum parsimony and Bayesian inference on the combined data (ssrDNA + lsrDNA) for the higher plagiogrihids (see text). Nodal support based on bootstrapping (maximum parsimony) and posterior probabilities (Bayesian inference). Families shown together as terminal taxa contain one or more paraphyletic groups.

represented by a single branch (e.g. Aspidogastridae + Multicalycidae) indicating that members of one or both families are nested one within the other, whereas in cases of polyphyly, the individual exemplar species are shown as separate terminal branches in their respective parts of the tree (e.g. members of the Acanthocolpidae). All maximum parsimony results are shown as strict consensus trees of the equally parsimonious trees; see Table 2 for the numbers of trees and other statistics resulting from the individual maximum parsimony analyses. Figs. 3 and 5 depict species-level phylograms of the Trematoda and ‘higher plagiogri-

iids’, respectively, showing relative branch lengths based on the results of Bayesian analyses, allowing for a visual comparison of the relative rates of evolution (nucleotide substitution) among the clades and terminal branches. Branch lengths were calculated as means of the branch lengths in the individual topologies saved during Bayesian analysis and summarised using the ‘sumt’ command of MrBayes. Named clades (including families and superfamilies) discussed below are shown in Fig. 6 and differ in composition in most cases to what is listed in Table 1, based on the classification of Gibson et al. (2002).

Table 2
Data partitions and tree statistics

Data partition	No. of ingroup (outgroup) taxa	No. of characters (%)				Tree statistics	
		Included	Constant	Gapped	Parsimony informative sites	No. equally parsimonious trees	Length (steps)
Full Digenea							
ssrDNA	163 (7)	1,668	888	139	598	1,057	5,299
lsrDNA	163 (7)	980	316	253	566	2,964	8,238
ssrDNA + lsrDNA	163 (7)	2,648	1,204	392	1,164	198	13,635
‘Plagiogrihida’							
ssrDNA + lsrDNA	106 (2)	2,950	1,505	358	1,179	4	11,074



Fig. 5. Species-level phylogram (108 taxa) of the higher plagiorchids based on Bayesian inference of the combined data (ssrDNA + lsrDNA) using a general-time-reversible model of nucleotide substitution incorporating among-site rate variation.

3.2. Analyses of the Trematoda

In all analyses, the aspidogastreaan outgroup taxa formed a monophyletic group with *Rugogaster hydrolagi* (Rugogasteridae) as the sister to a clade in which *Multicalyx elegans* (Multicalycidae) was placed within a paraphyletic Aspidogasteridae. The Digenea consistently formed two

major clades with strong nodal support: the Diplostomida nomen novum and Plagiorchida La Rue, 1957 (Figs. 1–3). The Diplostomida comprised three main lineages: (i) the Brachylaimoidea, in which the Leucochloridiidae was placed within the Brachylaimidae and (ii) the Diplostomoidae, in which members of both the Diplostomidae and Strigeidae were intermingled. The two superfamilies

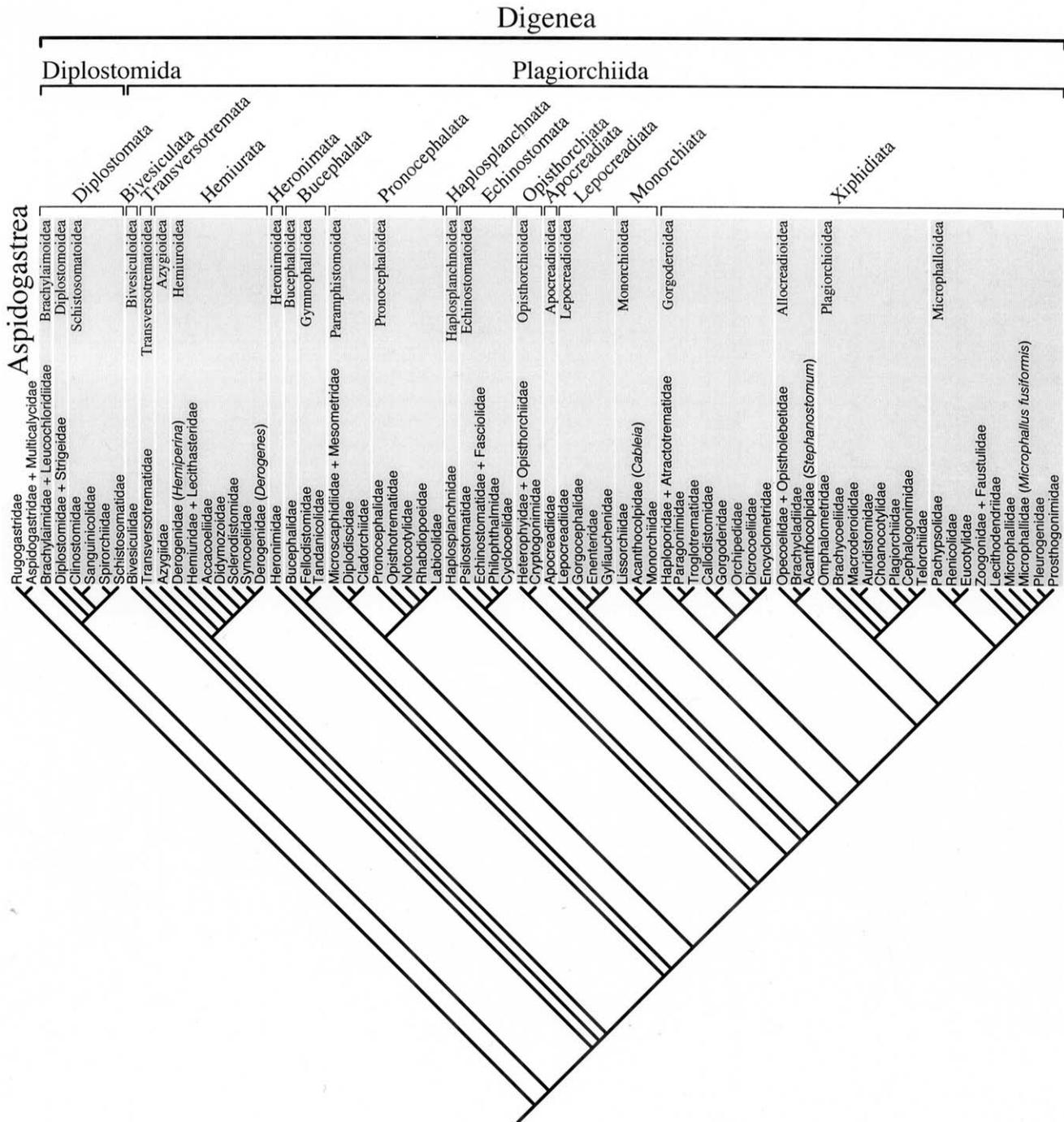


Fig. 6. Revised classification of the Digenea based on the results of Bayesian inference of lsrDNA and ssrDNA combined (see Fig. 3).

formed either sister clades (lSrDNA) or separate lineages (ssrDNA). The third lineage comprised the blood-fluke groups (iii) the Sanguinicolidae, Schistosomatidae and Spirorchidae, together with the Clinostomidae. Analyses of lsrDNA alone failed to support monophyly of the Sanguinicolidae, although the high rate of divergence of the sanguinicolids (Fig. 3) may account for this, and for the differences in the relative positions of the four families among the analyses (Fig. 1). In the combined analyses (Fig. 2), however, both methods resolved the monophyly of the

Sanguinicolidae and yielded congruent, fully resolved topologies with strong nodal support.

Interrelationships of the Plagiorchiida were less consistent among the different genes and methods of analysis (Fig. 1), but as described above, showed greater consistency between methods of analysis when both genes were combined (Fig. 2). In combined analyses, the Bivesiculidae formed the basal lineage of the clade followed by the Transversotrematidae (BI; Figs. 2 and 3) or by all remaining taxa (maximum parsimony; Fig. 2). As with the sanguini-

colids, the lack of strong support for the position of the Transversotrematidae may have been due to a high rate of divergence (see [branch lengths in Fig. 3](#)). The remaining plagiiorchiidans were split between the Hemiurata and the ‘higher plagiiorchiidans’, with the Heronimidae allied with either the former (maximum parsimony) or latter (Bayesian inference) clade. The Hemiurata exhibited unique differences in their primary sequences including large indels, and together with the sanguinicolids and transversotrematids, showed the highest rates of divergence ([Fig. 3](#)). These differences accounted for a large proportion of positions that were unalignable in both genes when considering all exemplar taxa simultaneously and thus prompted realignment and analysis of the ‘higher plagiiorchiid’ taxa alone ([Figs. 4 and 5](#), discussed below). Nodal support for the Hemiurata and most relationships within it were strong.

3.3. Analyses of the higher plagiiorchiidans

Relationships within the ‘higher plagiiorchiidans’ clade were inconsistent among the analyses including all taxa ([Figs. 1–3](#)), although the Bucephalidae (sometimes together with one or both gymnophalloid families; Fellodistomatidae and Tandanicolidae) generally formed the basal branch in the clade and was thus used as a functional outgroup for the restricted analyses including only these higher taxa ([Figs. 4 and 5](#)). Realignment of the two genes for the 108 taxa in the analyses allowed the inclusion of 302 additional characters ([Table 2](#)), but made only marginal difference to the robustness of the results in comparison to the full analyses described above (compare [Figs. 2 and 4](#)). The most labile taxa included the Haplospilachnidae, Haploporidae + Atractotrematidae and Apocreadiidae and strong nodal support was restricted largely to the nodes subtending families and in some cases superfamilies, but generally not those subtending more inclusive groupings of taxa ([Fig. 4](#)). The Acanthocolpidae, represented by *Cableia pudica* and *Stephanostomum baccatum*, was found to be polyphyletic, with *C. pudica* grouping together with the Monorchiiidae and *S. baccatum* together with the Brachycladiidae. Many groups were found to be paraphyletic: Microscephaliidae + Mesometridae, Cephalogonimidae + Telorchiiidae, Haploporidae + Atractotrematidae, Heterophyidae + Opisthorchiidae, Opecoelidae + Opistholebetidae and Zoogonidae + Faustulidae. Nevertheless, clades above the level of family were consistently recovered and corresponded to those recovered by analyses including all taxa.

3.4. Classification of the Digenea

The most recent classification of the Digenea, followed in [Table 1](#) for reference, was supported by molecular phylogenetic analyses at the level of family in most cases, and to a lesser extent at the level of superfamily. However, larger subdivisions of the Digenea Carus, 1863 and the

traditional compositions of the orders Echinostomida La Rue, 1957, Plagiiorchiida La Rue, 1957 and Strigeida Poche, 1926, were found to reflect non-natural groupings of taxa. The Diplostomida (*sensu* [Fig. 6](#)) comprises some of the members of the Strigeida, whilst the remaining strigeid groups formed the basal lineages of the sister clade to the Diplostomida (i.e. Plagiiorchiida in [Fig. 6](#)). The Echinostomida was found to represent a polyphyletic assemblage with its members scattered throughout the Plagiiorchiida (as defined in [Fig. 6](#)). Using results from Bayesian analysis of the combined data ([Fig. 4](#)), we have proposed a phylogenetically based classification of the Trematoda, emending the membership of currently recognised superfamilies where necessary, and recognising new taxa as shown in [Fig. 6](#) and elaborated below:

Class Trematoda Rudolphi, 1808

Subclass Aspidogastrea Faust and Tang, 1936

Subclass Digenea Carus, 1863

Order Diplostomida *nom. nov.*

Suborder Diplostomata *nom. nov.*

Superfamily Brachylaimoidea Joyeux and Foley, 1930

Superfamily Diplostomoidea Poirier, 1886

Superfamily Schistosomatoidea Stiles and Hassall, 1898

Order Plagiiorchiida La Rue, 1957

Suborder Apocreadiata *nom. nov.*

Superfamily Apocreadioidea Skrjabin, 1942

Suborder Bivesiculata *nom. nov.*

Superfamily Bivesiculoidea Yamaguti, 1934

Suborder Bucephalata La Rue, 1926

Superfamily Bucephaloidea Poche, 1907

Superfamily Gymnophalloidea Odhner, 1905

Suborder Echinostomata La Rue, 1926

Superfamily Echinostomoidea Looss, 1902

Suborder Haplospilachnata *nom. nov.*

Superfamily Haplospilachnoidea Poche, 1925

Suborder Hemiurata Skrjabin and Guschanskaja, 1954

Superfamily Azygioidea Lühe, 1909

Superfamily Hemiuroidea Looss, 1899

Suborder Heronimata Skrjabin and Schulz, 1937

Superfamily Heronimoidea Ward, 1918

Suborder Lepocreadiata *nom. nov.*

Superfamily Lepocreadioidea Odhner, 1905

Suborder Monorchhiata *nom. nov.*

Superfamily Monorchioidea Odhner, 1911

Suborder Opisthorchiata La Rue, 1957

Superfamily Opisthorchioidea Braun, 1901

Suborder Pronocephalata *nom. nov.*

Superfamily Pronocephaloidea Looss, 1899

Superfamily Paramphistomoidea Fiscoeder, 1901

Suborder Transversotremata *nom. nov.*

Superfamily Transversotrematoidea Witenberg, 1944
 Suborder Xiphidiata nom. nov.
 Superfamily Allocreadioidea Looss, 1902
 Superfamily Gorgoderoidea Looss, 1901
 Superfamily Microphalloidea Ward, 1901
 Superfamily Plagiorchioidea Lühe, 1901

4. Discussion

By combining data from two nuclear ribosomal RNA genes, we have been able to provide phylogenetic resolution and a good estimate of the interrelationships among 77 digenean families from a broad spectrum of hosts and localities (Table 1), including the positions of medically and economically important taxa (e.g. *Schistosoma*, *Fasciola* and *Paragonimus* spp.). Individual gene estimates provided by maximum parsimony and Bayesian inference analyses are less well-resolved but are largely compatible with the combined evidence solutions. Increased taxon sampling and the addition of *lsrDNA* has much improved earlier phylogenetic estimates based on *ssrDNA* alone (Cribb et al., 2001), as it has done with the Cestoda (Olson et al., 2001) and Monogenea (Olson and Littlewood, 2002).

We base our discussion and revised classification on the results stemming from Bayesian analysis of the combined data (Figs. 3 and 6) as this approach takes advantage of all available taxa and data, and utilises a more realistic model of nucleotide substitution than can be practically applied to analysis by parsimony. Moreover, as the two methods of analysis differed primarily in resolution and were otherwise largely compatible (see Fig. 2), the Bayesian solution provides a more complete hypothesis for examining the evolution of the Digenea. The implications of this hypothesis are discussed in the context of their ontogeny, morphology and taxonomic history, with particular regard given to characters described by Cribb et al. (2001) that bear on the present results.

4.1. Diplostomida

The Diplostomida represents one of the two fundamental lineages from which extant digeneans have diversified. Included as its hallmark are the unique blood-dwelling groups, Sanguinicolidae, Schistosomatidae and Spirorchidae, together with the Clinostomidae, for which a close relationship has long been recognised (La Rue, 1957). Our results show that the three blood-dwelling families do not form a monophyletic group without the inclusion of the non-blood-dwelling family, Clinostomidae and we, thus, include it within the Schistosomatoidea (cf. Gibson et al., 2002). The Strigeidae is paraphyletic, with the Diplostomidae nested within it, and similarly, the Brachylaimidae is paraphyletic, with the Leucochloridiidae nested. If, however, *Zeylanuratrema* deserves its own family within the

Brachylaimoidea, as suggested by Pojmanska (2002), then Brachylaimidae and Leucochloridiidae are sister taxa. A striking feature of the diplostomidans as a whole is that only the Sanguinicolidae and perhaps some Urotrematidae, which were not available for analysis, represent them in fishes and the diplostomidans are thus predominately parasites of tetrapods.

When present, the adult genital pore position (character 11 of Cribb et al., 2001) of diplostomidans is always posterior to the ventral sucker, and in all but the schistosomes, at or close to the posterior extremity of the body. This appears to be a synapomorphy uniting the group, as in the aspidogastrean outgroup and in the basal plagiorchiidans, the genital pore is in the forebody. The sole plagiorchiidan group with a posteriorly situated genital pore is the Bucephalidae, in which it may be argued that the normal digenean *bauplan* is so altered (with the mouth on the mid-ventral surface and the anteriorly placed blind sucker or rhynchus) that the posterior position of the genital pore could not be considered homologous with the condition in the Diplostomida. No molecular data are available on the Urotrematidae, which also have a posterior genital pore and have been considered plagiorchioids (La Rue, 1957; Brooks et al., 1985). Yamaguti (1971), however, considered that they resembled the Leucochloridiidae. Nothing is known of the life-cycle of members of the family Urotrematidae, which has been recently extended to encompass species from freshwater fishes in addition to lizards, bats and rodents (Bray et al., 1999). Their phylogenetic affinities thus remain an important question to be addressed.

A dorsal body fin-fold in the cercaria (character 35 of Cribb et al., 2001) may be a synapomorphy of the Schistosomatoidea, albeit reduced in the Spirorchidae and apparently secondarily absent in the Schistosomatidae (Pearson, 1992). Its presence in the Clinostomidae is particularly interesting in that it supports the affinities of this non-blood-dwelling group within the clade. Cercarial penetration glands that penetrate the oral sucker (character 32 of Cribb et al., 2001) unites the Diplostomoidea + Schistosomatoidea.

4.2. Basal Plagiorchiida

The other fundamental branch of the digenean tree gives rise to, what we have redefined as, the Plagiorchiida, and comprises a vast diversity of forms. Within it, we recognise 13 independent lineages (Fig. 6) that we classify as suborders (Section 3.3). The Bivesiculata, followed by the Transversotremata, form the basal lineages of the clade. The most basal lineage is the Bivesiculata, consisting only of the Bivesiculidae. Despite its position, it appears that the apparently primitive absence of suckers must, in fact, be a derived condition, or it must be postulated that the development of suckers in the Diplostomida and the remainder of the Plagiorchiata occurred independently.

Like the Bivesiculidae, the unusual biology of the

Transversotrematidae offers little evidence of their affinities within the Digenea, and although their placement is somewhat unstable among the individual analyses, they are clearly basal members of the Plagiorchiida. Morphologically, they are highly adapted to their adult habitat beneath the scales of fishes and are the only digeneans known from this site. Interestingly, transversotrematids may also lack oral suckers, although some taxa have possible analogues. If this is the case, then it provides some indirect support for the possibility that oral suckers arose independently in the Diplostomida and the Plagiorchiida. It is worth noting the high divergence rate among transversotrematids (see branch lengths in Fig. 3), and speculating whether they may be correlated with their unique biology. Elevated rates in molecular evolution are known to be associated with a number of factors including metabolic rate, body mass and generation time (Martin and Palumbi, 1993), but it is unclear as to whether these or other factors are the cause of an elevated rate among the Transversotrematidae.

The Hemiurata is a strongly supported clade within which we recognise the Azygioidea and Hemiuroidea as separate superfamilies in accordance with Gibson et al. (2002) (but also see Blair et al., 1998). Although Gibson and Bray (1979) felt the Azygiidae were best considered basal hemiuroids, we regard their uniqueness merits superfamily status. Within the Hemiuroidea, it is apparent that the Derogenidae is polyphyletic and the Hemiuridae and Lecithasteridae are not distinct; lecithasterids are nested within the Hemiuridae as found by Cribb et al. (2001). Unfortunately, the lack of ptychogonimids in our analyses is an important omission as their use of scaphopods as first intermediate hosts, the sole use of elasmobranchs as definitive hosts (the only digenean family for which this can be said) and the motile free-living sporocysts are most unusual digenean features that have been argued to reflect a primitive condition (Gibson and Bray, 1994).

The development of the sinus-sac (character 19 of Cribb et al., 2001) is a synapomorphy of the hemiuroid families. Gibson and Bray (1979) suggested a sequence of evolution of the sinus-sac within the group, but Brooks et al. (1985) postulated that the hemiuroid sinus-sac was derived from the bivesiculid cirrus-sac. The combined evidence study of Blair et al. (1998) indicated that the cirrus-sac was lost prior to the acquisition of the sinus-sac, in that the taxa basal to the Hemiuroidea in their analysis (Ptychogonimidae and Azygiidae) lack cirrus-sacs. Although we do not have data for the Ptychogonimidae, our results also support this view. The sinus-sac was defined as ‘a muscular sac which surrounds the base of the genital atrium, if present, and encloses the hermaphroditic duct and/or the terminal portions of the ejaculatory duct and uterus’ (Gibson and Bray, 1979). This definition also covers the ‘hermaphroditic-sac’ of haploporids and atractotrematids, except that the terminal parts of the male and female ducts are always, rather than occasionally, internal. The Hemiuroidea and Haploporidae + Atractotrematidae are not closely related,

and thus the similarity in these structures must be considered convergent.

A reduced vitellarium (character 30 of Cribb et al., 2001), i.e. a condensation from the follicular condition, to one or a few masses or filaments, is characteristic of the non-azygiid hemiuroids, or the Hemiuroidea (*sensu* Gibson et al., 2002). The vitellarium of the vast majority of Digenea is follicular and widespread, with a large number of follicles as found among the outgroup. A similar reduction of the vitellarium in the Gorgoderidae, Heronimidae and Zoogoninae is inferred to be homoplasious. In the Gorgoderidae, the vitellarium is usually condensed into a pair of compact or lobed masses, but in some anaporrhutine gorgoderid genera, the vitellarium is distinctly follicular (e.g. *Probolitrema*, see Gibson, 1976), suggesting that the normal gorgoderid condition is homoplasious with the hemiuroid condition. The vitellarium in the zoogonid subfamily Zoogoninae is also reduced, in parallel with the loss of the tanned egg-capsule (Bray, 1987). Intermediate conditions can be seen in the subfamily and the vitellarium is fully follicular (and the eggs tanned) in the other subfamily Lepidophyllinae and the related Faustulidae. A similar parallel reduction in vitelline size and egg-capsule tanning can be seen within the Transversotrematidae (Cribb et al., 1992). The reduction of the vitellarium is, therefore, a synapomorphy for the ‘higher’ Hemiurata and its occurrence elsewhere, except possibly the Heronimidae, can be argued convincingly as homoplasious.

4.3. Higher Plagiorchiida

Within the Plagiorchiida, the more basal bivesiculid, transversotrematid and hemiuratan lineages were considerably divergent from the more derived plagiorchiid groups (Fig. 3), which thus form a ‘higher’ clade (all taxa to the right of the Heronimidae in Fig. 6). Indeed, 1srDNA analyses alone (Fig. 1) grouped the bivesiculid, transversotrematid and hemiuratan taxa, together with the Heronimidae, in a single clade, whereas ssrDNA alone and the combined solutions did not. Resolution within the higher Plagiorchiida was limited among maximum parsimony analyses and the short and often poorly supported internal nodes were only marginally improved by the addition of more characters made possible by the removal of hemiuratan taxa from the alignment (Figs. 4 and 5). The largely pectinate topology resulting from Bayesian analysis (Fig. 6) allows for the recognition of many superfamilies, but rather few groupings among them, unless a more Hennigian classification scheme is desired in which all nested clades are accorded formal recognition. Rejecting the latter approach as an unnecessary and cumbersome approach to classification, we designate nine suborders each containing a single superfamily (albeit that some superfamilies are now more inclusive than as defined previously): the Apocreadiata, Bivesiculata, Echinostomata, Haplospilochnata, Heronimata, Lepocreadiata, Monorchhiata, Opisthorchiata and

Transversotremata; and four suborders comprising multiple superfamilies: Bucephalata including the Bucephaloidea and Gymnophalloidea, Hemiurata including the Azygioidea and Hemiuroidea, Paramphistomata including the Paramphistomoidea and Pronocephaloidea and the Xiphidiata including the Allocreadioidea, Gorgoderoidea, Microphalloidea and Plagiorchioidea. Our decisions to both formally recognise, as well as to not recognise, clades revealed by our analyses have been based on the strength of the results, historical continuity and our ability to recognise putative synapomorphies that add morphological or ontological support to the molecular data. With the exceptions of the distinctive Bucephalata and Paramphistomata, most of the higher plagiorchiid taxa are variations on the same morphological theme and it is difficult to find many striking characteristics that define them uniquely. Indeed, many aspects of the phylogeny make most sense when knowledge of morphology is combined with knowledge of life-cycles, and this approach is more broadly applied in the recent work of Cribb et al. (2003).

The most basal taxon of the higher plagiorchiid clade is the Heronimidae, although its affinities have been controversial and its position within the Plagiorchiiida showed instability among our analyses. The latter fact prevented us from using this taxon as a functional outgroup. Gibson (1987) and Pearson (1992) discussed in detail the opinions of Poche (1926) and Brooks et al. (1985) who considered *Heronimus* as the most primitive or one of the most primitive extant digeneans and rejected this viewpoint, concluding that it is simply an aberrant form adapted to peculiar conditions. Barker et al. (1993) devoted their study to testing the above hypothesis based on ssrDNA sequences and found no support for the hypothesis that *Heronimus mollis* is the sister taxon of the remaining digeneans. Cribb et al. (2001) included an ssrDNA sequence of *H. mollis* into their much larger data set and confirmed that *Heronimus* cannot be considered a candidate for the most primitive of digeneans. The present study based on two genes, also did not support this hypothesis, nor the suggestions of Crandall (1960) and Cable (1965) who considered *Heronimus* as a close relative of the Paramphistomidae.

Following the Heronimata is the Bucephalata, uniting the superfamilies Bucephaloidea and Gymnophalloidea. The lack of a gymnophallid in our analyses is a significant omission in that the Gymnophallidae is the type-family of the Gymnophalloidea and the only one found in birds. However, two of the widely recognised gymnophalloid families (see Bray, 2002), the Fellodistomidae and Tandanicolidae, are recovered as sister taxa. The much smaller molecular data set of Hall et al. (1999) also supports the Bucephalata as defined herein. The union of Bucephaloidea and Gymnophalloidea is also supported by the possession of a sporocyst stage in bivalve molluscs (Cribb et al., 2003).

The Paramphistomoidea and Pronocephaloidea are sister taxa and are here united as the Paramphistomata. This grouping is supported by the absence of the oral sucker (or

pharynx); all paramphistomates have a single muscular structure at the opening to their gut instead of two, the latter condition being characteristic of most other digeneans. Whether this structure is a pharynx or an oral sucker has been the subject of considerable debate (Pearson, 1992). Present views tend to favour that it is a pharynx but the matter cannot be considered finalised. With the exception of the lineage leading to the Diplodiscidae and Cladorchiidae, members of the Paramphistomata are also characterised by the lack of a ventral sucker. Both superfamilies have radiated more extensively among tetrapods than in fishes and both are uncommon in marine fishes. These distributions suggest a possible freshwater origin, perhaps in association with the appearance of tetrapods.

Within the Paramphistomoidea are the paraphyletic Microscophiidae + Mesometridae and its sister clade including the Diplodiscidae and Cladorchiidae. The Paramphistomoidea incorporates major radiations in fishes, amphibians, reptiles and mammals. The Mesometridae is a tiny family found primarily in herbivorous sparid fishes. Our results suggest they should be considered part of the larger Microscophiidae, which occur both in marine reptiles and other herbivorous fishes.

The pronocephaloids form a well-supported clade, but are noticeably distinct from the Paramphistomoidea only in their tiny, filamented eggs that must be eaten by the molluscan intermediate host, whereas those of the Paramphistomoidea hatch to penetrate their hosts externally. The group of strange pronocephaloids inhabiting sirenians (Opisthotrematidae, Rhabdiopoeidae, Labicolidae) do not form a monophyletic group within the Pronocephaloidea. The latter two families are sister taxa, and jointly are sister to the Notocotylidae and the most basal pronocephaloid is the namesake of the superfamily, Pronocephaloidea. A majority of taxa belonging to Pronocephaloidea are represented by parasites of marine and freshwater turtles, one of the most ancient groups of tetrapods and thus the basal position of this family within the Pronocephaloidea clade is not surprising.

The Haplospalchnidae was among the most unstable taxa in this study, shifting relative position dependent upon data set and analysis, and has, as far as we are aware, not been considered a distinct group at higher levels before. La Rue (1957) considered it an echinostomatoid, but Brooks et al. (1985) included it with the Haploporidae and Megaperidae in their new order Haploporiformes. Our results show the Haplospalchnoidea diverging immediately before the Echinostomata and well separated from the Haploporidae. We thus recognise the Haplospalchnata as a distinct lineage. This group is unremarkable except in possessing apparently simplified terminal genitalia and a single intestinal caecum. All records of adults of this small suborder are from marine teleosts and overwhelmingly from herbivores.

The Echinostomata as circumscribed here is a smaller group than has been traditionally recognised, containing the

Cyclocoelidae, Echinostomatidae, Fasciolidae, Philophthalmidae and Psilostomidae. The ‘Echinostomida’ of La Rue (1957) contained 23 families and putative families, including paramphistomoids, pronoccephaloids and renicolids. In ssrDNA analyses (Fig. 1) and the combined Bayesian analysis (Figs. 2 and 3), *Echinostoma* and *Euparyphium* formed separate lineages leading to the fasciolid taxa, and thus paraphyly of Echinostomidae (see also Kostadinova et al., 2003). In lsrDNA analyses (Fig. 1), however, the Echinostomidae was recovered as a sister clade to the Fasciolidae. La Rue (1957) considered the cyclocoeloids as strigeatoids, and this latter opinion was retained by Kanev et al. (2002). Both authors also included the Eucotyliidae in the Cyclocoeloidea, a placement not supported by this study, where the eucotyliids are microphalloids close to the Renicolidae (see also Tkach et al., 2001b). The Echinostomata is a clade characterised by parasitism of tetrapods.

The Opisthorchioidea is resolved, as is one of its constituent families, the Cryptogonimidae. However, the Heterophyidae is paraphyletic with the Opisthorchiidae nested within it. The only representative of opisthorchiids used in our study, *Amphimerus ovalis*, is a parasite of turtles whilst a majority of opisthorchiids and heterophyids are parasites of birds and mammals. Until more representatives of these groups are examined, the possibility that the evolutionary history of turtle opisthorchiids may differ from that of the members of the family parasitic in warm-blooded vertebrates cannot be dismissed.

The Apocreadiata forms a separate lineage including mainly species that before Cribb and Bray (1999) were included in the Homalometridae. This group has usually been considered close to or has been included within the Lepocreadiidae (Cable and Hunninen, 1942; Overstreet, 1970; Cribb and Bray, 1999), and as far as we are aware, has never been considered distinct at the level our results suggest. Adults are known from marine and freshwater fishes and possibly chameleons (Cribb and Bray, 1999).

The Lepocreadiata unites four families, the Enenteridae, Gorgocephalidae, Gyliachenidae and Lepocreadiidae. Enenterids are widely recognised as close to the lepocreadiids (Bray and Cribb, 2001) or as lepocreadiids (Brooks et al., 2000). The gyliachenids and gorgocephalids are also widely considered to be close to the lepocreadiids, despite their apparently very different morphologies. *Gorgocephalus* was originally placed in its own subfamily within the Lepocreadiidae by Manter (1966), but due to its highly unusual morphology (e.g. oral sucker with tentacles, single caecum with non-terminal ventral opening in the forebody and huge pocketed genital atrium opening dorsally) a separate family status is justifiable. Blair and Barker (1993) discussed the competing hypotheses for the relationships of the Gyliachenidae, i.e. whether they are close to the paramphistomids or the lepocreadiids. Morphological arguments are equivocal, but all molecular evidence from theirs to the present place the gyliachenids close to the lepocreadiids.

The Monorchiata includes the Monorchiidae, Lissorchiidae and the genus *Cableia*. *Cableia* was most recently placed in the Acanthocolpidae, but repeatedly associates with the monorchiids in molecular phylogenies (Cribb et al., 2001), suggesting its status needs closer examination. A more reasonable estimate of the position of the Acanthocolpidae is probably reflected by the position of *Stephanostomum*, as sister-group to the Brachycladiidae within the Allocreadioidea. Many lissorchiid genera have been considered monorchiids until the recent work by Shimazu (1992) who delineated the characters differentiating the families and pointed out that lissorchiids are freshwater and monorchiids marine, almost exclusively. The sister relationship of these families suggested by our results (including the enigmatic *Cableia* in the Monorchiidae) is, therefore, a predictable result seeing that the families have only been satisfactorily delineated recently.

The crown clade of the Digenea is the Xiphidiata which comprises four superfamilies: Gorgoderioidea, Allocreadioidea, Plagiorchioidea and Microphalloidea; the latter two being sister taxa (Fig. 6). The union of these superfamilies is supported by the presence of a penetrating stylet in the cercariae, reflected in the name ‘Xiphidiata’. The significance of the stylet appears to be in conferring the ability of the cercariae to penetrate arthropod cuticle or membranes. This unique character, found nowhere else in the Digenea, is absent in the Haploporidae and perhaps some Acanthocolpidae. Absence may relate to secondary loss (a likely explanation for the Acanthocolpidae) or, in the case of the Haploporidae, may point to a phylogenetic misplacement. Similar to the Haplosporididae (see above), the Haploporidae showed considerable instability in its placement in the separate analyses (Fig. 1). In the combined analyses (Figs. 2 and 4), however, it was consistently recovered in the position shown in Fig. 6. Several groups of Xiphidiata (Telorchidae, Ochetsomatidae, Leptophallidae, etc.) use, perhaps secondarily, amphibians instead of arthropods as second intermediate hosts. Their cercariae are nevertheless armed with stylets similar to their relatives that must penetrate the arthropod cuticle.

The Gorgoderioidea includes a group of taxa that have not all been considered related in the past. There are two major clades: the first includes the Haploporidae with the Atractotrematidae nested within it and the Paragonimidae + Troglotrematidae as the sister lineage. The second includes the Callodistomidae + Gorgoderidae and its sister lineage including the Orchipidae and Dicrocoelidae + Encyclometridae. This assemblage of parasites exhibits a remarkable range of hosts. The type-family, Gorgoderidae, incorporates forms that have apparently host-switched into elasmobranchs as well as significant radiations into tetrapods. Some taxa, such as the Orchipidae and Troglotrematidae, have aquatic molluscan hosts but life-cycles that lead to the infection of terrestrial tetrapods. Finally, the Dicrocoeliidae are (along with some of the diplostomidan

Brachylaimoidea) the only trematodes to have completely terrestrial life-cycles.

The Allocreadioidea, somewhat surprisingly, includes the Opecoelidae + Opistholebetidae, the Brachycladiidae (syns. Campulidae, Nasitrematidae) and the Acanthocolpidae. The relationship of the former with the two latter has not, as far as we are aware, been postulated before. On the other hand, the sister taxon relationship of the Brachycladiidae and Acanthocolpidae was predicted by Cable (1974) and has been previously recovered in molecular phylogenies (see Fernández et al., 1998a,b; Cribb et al., 2001). The lack of exemplar taxa of the Allocreadiidae is another important omission, as they are the namesakes of the superfamily. The Opecoelidae + Opistholebetidae and Acanthocolpidae are exclusively parasites of fishes and the Brachycladiidae are from marine mammals. The position of the Brachycladiidae is intriguing in that it implies a host-switch from fishes to cetaceans.

The present analysis based on two different genes, has generally confirmed the results of Tkach et al. (2000, 2001a, b) obtained using partial *lsrDNA* sequences, regarding the composition and interrelationships of the main groups of the Plagiorchioidea and Microphalloidea, as well as the conclusion that these groups belong to the crown clade of the Digenea. On the other hand, inclusion of new important taxa into the current analysis revealed some interrelationships that had not been considered previously.

The Plagiorchioidea is represented in our data set by eight families. The topology within the clade was labile depending on the method of analysis and taxon set. However, there were two particularly stable associations: Telorchiiidae + Cephalogonimidae and Macroderoididae + Auridistomidae. Cephalogonimids have not generally been considered close relatives to the Telorchiiidae in the literature. Many authors (Mehra, 1937; Prudhoe and Bray, 1982; Brooks et al., 1985; Sharpilo and Iskova, 1989) either considered the Cephalogonimidae as related to the Plagiorchiidae or placed Cephalogonimidae as a subfamily of the Plagiorchiidae. Odening (1964) could not find a proper superfamilial allocation for Cephalogonimidae due to their protonephridial formula which differed from those in putative related digenean groups. However, Grabda-Kazubska's (1971) classification of the xiphidiocercariae armatae group attributed the cercariae of *Cephalogonimus* to the 'Opisthioglyphe' type; in other words, indicated close relationships among representatives of Cephalogonimidae and Telorchiiidae. These groups share a similar life-cycle involving anuran amphibians as second intermediate hosts. Brooks et al. (1989), however, did suggest the monophyly of the Telorchioidea, the Cephalogonimidae and the Auridistomidae. The inclusion of the latter is contrary to the present analyses.

A close affinity between the families Macroderoididae and Auridistomidae would not be readily predicted. The former family was represented in our study by *Macroderoides typicus* and the latter by *Auridistomum chelydrae*,

both collected in North America. These taxa have a similar body plan and use amphibians as second intermediate hosts. The Auridistomidae is a small and enigmatic group of digeneans parasitic in freshwater turtles whose phylogenetic affinities were unclear and were rarely considered in the literature. The Macroderoididae, as presently defined, is an obviously heterogeneous group that needs more comprehensive study (Yamaguti, 1971; Smythe and Font, 2001; Tkach et al., 2001c). As it is the only group in the Plagiorchioidea that includes a number of fish parasites, this family may be key in the determination of the origin and radiation of the plagiorchioideans.

The Microphalloidea includes two clades. The first includes the Pachypsolidae, the Rencolidae and the Eucotyliidae. The second includes the Zoogonidae + Faustulidae as the most basal taxon with the Lecithodendriidae, Microphallidae, Pleurogenidae and Prosthogonimidae as progressively more derived. The present analysis further corroborates the conclusions of Tkach et al. (2001b) regarding the close relationships of Rencolidae and Eucotyliidae and their affinities with the Microphalloidea. This rejects the viewpoint of those authors who assigned renicolids to a higher taxonomic rank such as La Rue (1957), who established a separate order solely to house this family. The phylogenetic affinities and systematic position of the Eucotyliidae have long been uncertain and its position as a sister-group of the Rencolidae was considered novel by Tkach et al. (2001b) as these taxa had never been considered closely related. However, this relationship is strongly supported by the present study and was not affected by gene choice, analysis or taxon sampling. Despite differences in body shape, both renicolids and eucotyliids (at least the members of the subfamily Tanaisiinae used in our study) share many morphological features. Moreover, both groups are parasites of bird kidneys, which is a specialised niche in comparison with enteric parasitism. However, the Eucotyliidae itself may be a polyphyletic group; Brooks et al. (1985) listed numerous morphological and life-cycle differences between the Eucotyliinae and the Tanaisiinae and concluded that these subfamilies may belong to quite different phylogenetic lineages. According to the diagnoses of Yamaguti (1971), the Tanaisiinae (represented by *Tanaisia fedtschenkoi* in our data) have even more morphological similarities with the Rencolidae than with the Eucotyliinae, such as the absence of the cirrus-sac (present in Eucotyliinae) and presence of a seminal receptacle (absent in Eucotyliinae). Unfortunately, exemplars of more typical Eucotyliinae were not available for the present study and thus the potential paraphyly of the Eucotyliidae was not addressed.

The systematic position of the relatively recently established family Pachypsolidae, an enigmatic group of digeneans with unknown life-cycles and parasitic in marine turtles and neotropical caimans, has never been clear (Yamaguti, 1971; Brooks et al., 1985). An affinity of this group with the Rencolidae and Eucotyliidae has no

immediate explanation and should be verified by further analysis.

The second large clade of the Microphalloidea includes two sub-clades: the Zoogonidae + Faustulidae and another comprising the same four families that represented the Microphalloidea in the studies of Tkach et al. (2001b, 2003). This is, as far as we are aware, the first time this relationship has been proposed. Most genera now housed in the Faustulidae have hitherto been considered fellodistomes, usually as belonging to the subfamily Baccigerinae (Bray, 1988). Hall et al. (1999) found a close relationship of faustulids to the Zoogonidae in their molecular phylogeny. The original description of *Faustula* by MacCallum (1919) was erroneous in describing paired lateral vaginae (similar to the situation in some Monogenea) and led to the erection of the 'supersuperfamilie' Faustulida by Poche (1926). The redescription of *Faustula* by Price (1938) showed that there was no supporting evidence for the erection of a new major taxon. The rest of the families in this clade have traditionally been considered closely related, although their systematic position and taxonomic status varied. For instance, they were grouped within the superfamilies Prosthogonimoidea and Microphalloidea in the system of Odening (1964) and Lecithodendrioidae and Microphalloidea in the system of Brooks et al. (1985, 1989).

The distinct separation of the Lecithodendriidae and Pleurogenidae by present molecular data supports the systematic arrangement proposed by Odening (1959) who removed the subfamily Pleurogeninae from the Lecithodendriidae and raised it to the family level. Odening's viewpoint has been variously accepted (e.g. Sharpilo and Iskova, 1989) or rejected (Yamaguti, 1971; Prudhoe and Bray, 1982), but has been supported by molecular data (e.g. Tkach et al., 2001b, 2003 and herein).

In some analyses (Figs. 1–3), members of two subfamilies of the Microphallidae, Microphallinae and Maritreminae, were split among different clades. In some cases *Maritrema* was closer to the Lecithodendriidae than to *Microphallus* which fits the hypothesis of Bayssade-Dufour et al. (1993) based on the comparative analysis of cercarial chaetotaxy. However, the recent molecular phylogenetic investigation of the Microphalloidea by Tkach et al. (2003) supports the branch topology presented on Figs. 5 and 6 in which the Microphallidae is monophyletic.

Opinions on the taxonomic status of Prosthogonimidae have varied considerably and Mehra (1937) regarded it a subfamily of the Plagiorchiidae (= Lepodermatidae). However, the majority of authors have considered it a separate family, and Odening (1964) went as far as to erect the superfamily Prosthogonimoidea. Results of the present analysis support the viewpoint of Brooks et al. (1989) who placed them in the superfamily Microphalloidea, although it is unclear how they reached this conclusion based on their morphological phylogenetic analysis (see Cribb et al., 2001).

Cercarial penetration gland openings dorsally to the oral sucker (character 31 of Cribb et al. (2001) is a feature uniformly exhibited, as far as we know, among members of the Apocreadiata, Lepocreadiata, Monorchhiata, Opisthorchioidea and Xiphidiata, which form a nested clade within the higher Plagiorchiida (Fig. 6). As this condition is also found in many echinostomatoids, it may well be a synapomorphy of the larger nested clade including also the Echinostomata.

4.4. Need for taxonomic revision

The present study shows that revision of the classification of the Digenea is warranted in order to better reflect the phylogenetic affinities of the taxa that are consistently supported by this and previous molecular phylogenetic studies, as well as recent morphological estimates (e.g. Cribb et al., 2001). The traditional order Echinostomida is clearly a polyphyletic assemblage formed for taxonomic convenience, whilst the traditional orders Plagiorchiida and Strigeida are paraphyletic. Such a trichotomous scheme cannot be maintained if we want digenean classification to reflect their phylogeny, and our results necessitate the recognition of a greater number of independent lineages.

Several families have been found to be paraphyletic and consideration must be made at some point as to the advisability of making those families that are nested, synonyms of the paraphyletic ones, or of redefining the paraphyletic families. The pairs of taxa in question are the Brachylaimidae + Leucochloridiidae, Diplostomidae + Strigeidae, Hemiuridae + Lecithasteridae, Microscoaphidiidae + Mesometridae, Echinostomidae + Fasciolidae, Heterophyidae + Opisthorchiidae, Haploporidae + Atractotrematidae, Opecoelidae + Opistholebetidae and Zoogonidae + Faustulidae. In some cases, these actions would amount to the reinstatement of earlier classifications and in other cases the synonymies have been mooted before. Sinking of certain taxa (e.g. Atractotrematidae) might be warranted immediately, whereas other taxa warrant additional evidence prior to any taxonomic revision.

More significant are the families found to be polyphyletic, Acanthocolpidae and Deroegenidae. Again, greater taxon sampling is needed to initiate major changes. However, the most obvious taxon for immediate scrutiny is probably the Acanthocolpidae, where it now appears clear that *Cableia* is a basal monorchiid and not an acanthocolpid, lepecreadiid, opecoelid or enenterid as variously suggested (Bray et al., 1996). Attempts should thus be made to assess its morphology as a putative monorchiid.

4.5. Missing taxa and unresolved questions

Although the present analyses represent the broadest sampling of Digenea to date, a group comprising over 140 families (Gibson et al., 2002), a number of important omissions remain, including the Allocreadiidae, Gymnophallidae, Liolopidae, Mesotretidae, Paramphistomidae,

Ptychogonimidae, Rhytidodidae and Urotrematidae. Some of these omissions are likely to be crucial to the full elucidation of digenean phylogeny. The position of the Ptychogonimidae, for example, could have major implications for the estimation of the sequence of first intermediate host acquisition in the group. The Urotrematidae, known now from fishes, could also be pivotal in our understanding of the sequence of acquisition of definitive vertebrate hosts in the group. The systematics of the Digenea is still riddled with puzzles and inconsistencies at all levels that morphology has failed to resolve, but which may yield to molecular techniques in due course.

The extraordinary diversity of the Digenea has required us to restrict our analyses and discussion largely to topics concerning their phylogeny and classification. We appreciate that other aspects of their biology can be better understood in a historical context as well, and a separate paper gives consideration solely to the evolution of digenean life-cycles and their host associations in light of the results herein (see Cribb et al., 2003).

Author's note:

Cotylogaster dinosoides should read *cotylogaster basiri* on Fig. 3. *Metadena* sp. should read *Siphodera vinaledwardsii* on Figs. 3 and 5.

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