Relationships within the Acanthocolpidae Lühe,1906 and their place among the Digenea

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Abstract

The phylogeny of the Acanthocolpidae, a family of marine fish parasites, was assessed using SSU and LSU rDNA and combined sequences of thirteen putative species. *Cableia pudica* is found to be a basal monorchiid, not an acanthocolpid. The remaining species form a monophylum only if the marine mammal parasite, the brachycladiid *Zalophotrema hepaticum* is included. The Acanthocolpidae comprises two clades (*Zalophotrema* (*Pleorchis, Tormopsolus*)) and (*Stephanostomum*). Some morphological similarities were detected between *Zalophotrema* and *Pleorchis*, but *Tormopsolus* is morphologically more similar to *Stephanostomum*. Nine species of the large genus *Stephanostomum* were studied, including two species from two host species each, and the relationships inferred from the analysis of sequences were poorly reflected in morphological or biological characteristics. Evidence from sister taxa indicates that the parasites of piscivorous marine mammals, the Brachycladiidae, are derived from piscivorous marine fish parasites.

Key words

Introduction

Acanthocolpid digeneans, as currently recognised, are parasites mainly of marine teleost fishes, occasionally possibly of sea snakes. They have a rather generalised plagiorchiidan (sensu Olson *et al.* 2003) morphology with a spinous tegument, no external seminal vesicle and a uterine seminal receptacle. The Acanthocolpidae was erected by Lühe (1906), as a subfamily, to include the genera *Acanthocolpus* Lühe, 1906, *Stephanochasmus* Looss, 1900 (now considered the junior synonym of *Stephanostomum* Looss, 1899) and *Deropristis* Odhner, 1902, and the species *Distomum semiarmatum* Molin, 1858. This taxon was first elevated to family rank by Lühe (1909) and since then has accumulated genera somewhat haphazardly, becoming a 'catch-all group' for worms with these generalised features and no obvious affinities to other groups. As Bray (2005a) pointed out in his key, several genera are placed in it 'with considerable lack of confidence'. Of the taxa originally placed in the family, *Deropristis* and *Distomum semiarmatum* are now considered members of the family De-

ropristidae Cable et Hunninen, 1942 (Choudhury and Dick 1998, Bray 2005b). The family is, therefore, based mainly around the fairly well known type-genus *Acanthocolpus*, and the rather better known genus *Stephanostomum*, which now has some 117 nominal species (Bray and Cribb 2003, Bray and Reimer 2004). This latter genus is also the best known in terms of the life cycle, which has been recognised as the usual pattern for the family. The first intermediate hosts are prosobranch gastropods, in which rediae develop. The oculate, caudate cercariae emerge (stylets are usually reported) and penetrate a large number of teleost species, where they encyst in the flesh. The definitive hosts are piscivorous teleosts which acquire the worms by ingestion of the second intermediate hosts (Martin 1939, Wolfgang 1955, MacKenzie and Liversidge 1975, Køie 1978, Stunkard 1961). The recent findings of *Stephanostomum* metacercariae in bivalve molluscs (Madhavi and Shaheem 1993, Pérez-Urbiola and Martínez-Díaz 2001) have shown that the pattern is not invariant. As was stated above, many genera have been added to the family, such that in Bray's (2005a) recent key 17 genera are included. This num-

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ber reflects the family's status as a 'catch-all group', and several genera were included purely for convenience of identification. In one case, *Cableia* Sogandares-Bernal, 1959, molecular evidence was already available that the genus was misplaced (Cribb *et al.* 2001, Olson *et al.* 2003). The status of the genera included in this molecular study will be discussed below, but several other genera, e.g. *Ophiotreminoides* Coil et Kuntz, 1960 (from sea snakes – Coil and Kuntz 1960), *Gissutrema* Machida et Kamegai, 1997 (from a primitive teleost – Machida and Kamegai 1997), *Pseudolepidapedon* Yamaguti, 1938 (see Bray *et al.* 1996), *Spinoplagioporus* Skrjabin et Koval, 1958 (from holocephalans – Gaevskaya 2002) and *Lepidauchen* Nicoll, 1913 (see Bray and Bartoli 1996), are equivocal acanthocolpids and are in need of further study. On the other hand, several genera not included in this study are probably genuine acanthocolpids, based on their morphological similarity to *Acanthocolpus* and *Stephanostomum*. These are *Monostephanostomum* Kruse, 1979, *Stephanostomoides* Mamaev et Oshmarin, 1966, *Manteria* Caballero, 1950, *Acaenodera* Manter et Pritchard, 1960, *Pseudacaenodera* Yamaguti, 1965 and *Venusicola* Bray et Cribb, 2000. The present study presents molecular evidence for the relationships and status of five putative acanthocolpid genera.

Some commercially important fishes, in particular flatfishes, may be affected by acanthocolpid metacercariae encysted in the soft tissues, mainly in the musculature, fins and skin (MacKenzie and Liversidge 1975, Sommerville 1981), which cause cosmetic problems for fisheries. Metacercariae also occur in the pericardial cavity of farmed rainbow trout under certain conditions causing mortality (McGladdery 1999) and in elvers they cause severe distension of the thoracic region impeding swimming (Oliveira and Campbell 1998).

Of biological interest is the relationship between acanthocolpids and the marine mammal parasites, the Brachycladiidae Odhner, 1905 (syn. Campulidae Odhner, 1926) (see Gibson 2005). The close relationship was predicted by Cable (1974) and inferred by the molecular studies of Fernández *et al.* (1998a, b), Cribb *et al.* (2001) and Olson *et al.* (2003) and from the additional morphological studies of Gibson (2002, 2005). To further explore this relationship a brachycladiid has been included in this study.

The position of the Acanthocolpidae in the Digenea has not been well established. In the latest review, the "Keys to the Trematoda, vol. 2" (Jones *et al.* 2005), it is placed in the superfamily Lepocreadioidea Odhner, 1905. Even as the volume was in preparation it was clear to the authors that the taxon was artificial, and did not represent the inferences to be taken from molecular results (see Bray 2005c). Earlier molecular results along with the analysis described here will be discussed below.

Here the relationships of acanthocolpids among the Digenea are assessed by building on a larger data set of complete small subunit (SSU) and partial large subunit (LSU) ribosomal DNA (rDNA) sequences used recently for estimating a digenean phylogeny (Olson *et al.* 2003). New molecular sequences for 13 species have been added to the two published sequences of acanthocolpids and a broad range of available digenean sequences have been selected allowing us to investigate the monophyly of the Acanthocolpidae and the likely sister group(s) of its constituent taxa.

Materials and methods

Choice of taxa and outgroups

Table I indicates the taxa chosen, including those GenBank accession numbers for published and new sequences. Thirteen acanthocolpid species (representing four genera) were sampled and added to a data set of 62 digeneans. The sampling encompassed those taxa within a clade of the Plagiorchiida, where *Cableia* and *Stephanostomum baccatum* (Nicoll, 1907) fell in a previous molecular study (Olson *et al.* 2003). In this way, the best possible estimate of the position of these and related taxa was possible. In order to root the phylogenetic trees species of the following genera were chosen: *Paragyliauchen* Yamaguti, 1934, *Enenterum* Linton, 1910, *Koseiria* Nagaty, 1942, *Gorgocephalus* Manter, 1966, and *Preptetos* Pritchard, 1960.

DNA extraction, gene amplification and gene sequencing

Following the protocols found in Olson *et al.* (2003), 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; 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.

Twenty five μl PCR amplifications 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 $MgCl_2$, 200 μM of each dNTP and stabilizers, 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 sec at 94°C, 30 sec at 56°C, 2 min at 72°C, and 7 min extension hold at 72°C. Near-complete SSU rDNA sequences (~1,800 bps) 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-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. The ssrDNA products were sequenced in both directions using the two PCR primers and a variety of internal primers (Littlewood and Olson 2001),

and LSU rDNA 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 DQ248202-14 (SSU rDNA) and DQ248215-27 (LSU rDNA).

Gene alignment and phylogenetic analysis

New sequences of SSU and LSU rDNA were combined with previously published and aligned sequences (Olson *et al.* 2003). The new sequences were incorporated into the existing alignments with adjustments to the alignments made by eye using MacClade (Maddison and Maddison 2000). Original alignments were made with reference to secondary structure features as outlined in Olson *et al.* (2003), and new sequences did not significantly disrupt the alignment with indels. Sequences for both genes were concatenated in MacClade and regions of ambiguous alignment defined in a character exclusion set.

We have deposited the complete alignments of ssrDNA and lsrDNA with EBI and each is available by anonymous FTP from 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_000954 (SSU rDNA) and ALIGN_000955 (LSU rDNA). Exclusion sets are added as notes and the alignments may be adapted as a NEXUS file.

SSU and LSU datasets were analysed individually and combined, using Bayesian inference methods with MrBayes (Huelsenbeck 2000), and maximum parsimony with PAUP* (Swofford 2002) in order to estimate the phylogenetic placement of the acanthocolpids. Prior to Bayesian inference Modeltest (Posada and Crandall 1998) was used to estimate the best model of nucleotide substitution. In each case this was the general time reversible model, with estimates of invariant sites and gamma distributed among site rate variation (GTR+I+G). Posterior probabilities were approximated over 5,000,000 generations via four simultaneous Markov Chain Monte Carlo chains (nchains = 4) with every 1000th tree saved. Default values were used for the MCMC parameters. Consensus trees with mean branch lengths were constructed using the 'sumt' command option and ignoring the initial topologies saved during 'burn in'; the initial *n*-generations before log-likelihood values and substitution parameters plateau. For each analysis we plotted log-likelihood values against generation number and used a burn in of 1,000 for estimating sumt and sump. For combined analyses, parameter values for GTR+I+G were estimated independently for the SSU and LSU partitions. 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 unor-dered with equal weights and with gaps treated as missing data. Nodal support was estimated by bootstrapping (n = 1000).

Results

New sequences for SSU and LSU rDNA have been deposited in GenBank (see Table I). The aligned SSU dataset was comprised of 2,111 positions, of which 1,670 were unambiguously alignable and of these 1,151 were constant and 129 informative under the principles of parsimony. The aligned LSU dataset was comprised of 1,658 positions, of which 1,080 were unambiguously alignable and of these 456 were constant and 110 informative under the principles of parsimony.

Individual genes

Maximum parsimony (MP) found 456 equally parsimonious trees with SSU, and 12 equally parsimonious trees with LSU rDNA, respectively. The strict consensus topologies for SSU and LSU were congruent in topology concerning the membership of major clades (Monorchioidea, Gorgoderoidea, Plagiorchioidea, Microphalloidea, and the acanthocolpids plus other allocreadioids (sensu Olson *et al.* 2003)), both between datasets and between solutions estimated by parsimony and Bayesian inference (BI). Although these major clades were well supported with high bootstrap values (>75%) and high posterior probabilities (100%) deeper nodes in the trees were poorly supported and collapsed in the strict consensus MP solutions. Membership of the major clades was identical to that of the combined solution, as discussed below in detail. Concerning the acanthocolpids specifically, *Cableia* was sister taxon to a clade comprised of *Diplomonorchis* Thomas, 1959, *Lasiotocus* Looss in Odhner, 1911 (previously known as *Ancylocoelium* Nicoll, 1912 – see Bartoli and Bray 2004) and *Provitellus* Dove et Cribb, 1998, with *Lissorchis* Magath, 1917 sister to these taxa, for all data partitions and all methods of analysis. A monophyletic clade of *Stephanostomum* was strongly and consistently supported and the sister group to this clade was a clade comprised of the Brachycladiidae (*Zalophotrema*), *Pleorchis* and *Tormopsolus* for the LSU dataset and all analyses. With SSU, *Zalophotrema* was resolved as the sister taxon to *Stephanostomum*. Although less well resolved, the relationships found with the individual genes (trees not shown) are almost completely congruent with the combined analyses.

Combined genes

Figure 1 illustrates the tree topology found by Bayesian inference, with nodal support indicated by posterior probabilities and bootstrap percentage values (>50%) from the parsimony analysis. Maximum parsimony found a single tree (length = 5717, CI = 0.319, RI = 0.561) that was largely congruent in terms of general patterns of topology and nodal support. Almost all clades with 100% posterior probabilities were resolved with MP. Relationships within and between acantho-

Table I. List of taxa and sequences used in this study

Species and classification	Source: host/locality	GenBank accession	
		SSU	LSU
Order Plagiorchiida			
Family Enenteridae			
Enenterum aureum Linton, 1910	*Kyphosus vaigiensis*, Moorea, French Polynesia	AY222124	AY222232
Koseiria xishaense Gu et Shen, 1983	*Kyphosus vaigiensis*, HI, Australia	AY222125	AY222233
Family Gorgocephalidae			
Gorgocephalus kyphosi Manter, 1966	*Kyphosus vaigiensis*, LI, Australia	AY222126	AY222234
Family Gyliauchenidae			
Paragyliauchen arusettae Machida, 1984	*Pomacanthus sexstriatus*, Ningaloo, W. Australia	AY222127	AY222235
Family Lepocreadiidae			
Preptetos caballeroi Pritchard, 1960	*Naso vlamingi*, HI, Australia	AJ287563	AY222236
Preptetos trulla (Linton, 1907)	*Ocyurus chrysurus*, Port Royal, Jamaica	AY222128	AY222237
Family Apocreadiidae			
Homalometron armatum (MacCallum, 1895)	*Lepomis microlophus*, Pascagoula R., Mississippi, U.S.A.	AY222130	AY222241
Homalometron synagris (Yamaguti, 1953)	*Scolopsis monogramma*, HI, Australia	AJ287523	AY222243
Neoapocreadium splendens Cribb et Bray, 1999	*Scolopsis monogramma*, LI, Australia	AJ287543	AY222242
Schistorchis zancli Hanson, 1953	*Zanclus cornutus*, Opunohu Bay, Moorea	AY222129	AY222240
Family Lissorchiidae			
Lissorchis kritskyi Barnhart et Powell, 1979	*Carpioides cyprinus*, Pascagoula R., Mississippi, U.S.A.	AY222136	AY222250
Family Monorchiidae			
Lasiotocus typicus (Nicoll, 1912)	*Trachurus trachurus*, North Sea, U.K.	AJ287474	AY222254
Diplomonorchis leiostomi Hopkins, 1941	*Leiostomus xanthurus*, Gulf of Mexico, Mississippi, U.S.A.	AY222137	AY222252
Provitellus turrum Dove et Cribb, 1998	*Pseudocaranx dentex*, HI, Australia	AJ287566	AY222253
Family Opecoelidae			
Gaevskajatrema halosauropsi Bray et Campbell, 1996	*Halosauropsis macrochir*, Goban Spur, NE Atlantic	AJ287514	AY222207
Macvicaria macassarensis (Yamaguti, 1952)	*Lethrinus miniatus*, HI, Australia	AJ287533	AY222208
Peracreadium idoneum (Nicoll, 1909)	*Anarhichas lupus*, North Sea, U.K.	AJ287558	AY222209
Family Opistholebetidae			
Maculifer sp.	*Diodon hystrix*, HI, Australia	AY222109	AY222211
Opistholebes amplicoelus Nicoll, 1915	*Tetractenos hamiltoni*, SI, Australia	AJ287550	AY222210
Family Brachycladiidae			
Zalophotrema hepaticum Stunkard et Alvey, 1929	*Zalophus californianus*, California, U.S.A.	AJ224884	AY222255
Family Omphalometridae			
Rubenstrema exasperatum (Rudolphi, 1819)	*Crocidura leucodon*, Bulgaria	AJ287572	AY222275
Family Pachypsolidae			
Pachypsolus irroratus (Rudolphi, 1819)	*Lepidochelys olivacea*, Oaxaca, Mexico	AJ287554	AY222274
Family Plagiorchiidae			
Haematoloechus longiplexus Stafford, 1902	*Rana catesbeiana*, Nebraska, U.S.A.	AJ287520	AY222274
Glypthelmins quieta (Stafford, 1900)	*Rana catesbeiana*, Nebraska, U.S.A.	AJ287520	AY222280
Skrjabinoeces similis (Looss, 1899)	*Rana ridibunda*, Kokaljane, Bulgaria	AJ287575	AY222279
Family Pleurogenidae			
Pleurogenes claviger (Rudolphi, 1819)	*Rana temporaria*, Kiev Region, Ukraine	AY222152	AF151925
Pleurogenoides medians (Olsson, 1876)	*Rana lessonae*, Kiev Region, Ukraine	AY222151	AF433670
Family Prosthogonimidae			
Prosthogonimus ovatus (Rudolphi, 1803)	*Pica pica*, Chernigiv Region, Ukraine	AY222149	AF151928
Schistogonimus rarus (Braun, 1901)	*Anas querquedula*, Kherson Region, Ukraine	AY222150	AY116869
Family Telorchiidae			
Opisthioglyphe ranae (Froelich, 1791)	*Rana arvalis*, Ivano-Frankivsk Region, Ukraine	AY222157	AF151929
Telorchis assula (Dujardin, 1845)	*Natrix natrix*, Kiev Region, Ukraine	AY222156	AF151915
Family Brachycoeliidae			
Brachycoelium salamandrae (Froelich, 1789)	*Salamandra salamandra*, Zakarpatska Region, Ukraine	AY222160	AF151935
Mesocoelium sp.	*Bufo marinus*, Brisbane, Australia	AJ287536	AY222277
Family Cephalogonimidae			
Cephalogonimus retusus (Dujardin, 1845)	*Rana ridibunda*, Kokaljane, Bulgaria	AJ287489	AY222276
Family Choanocotylidae			
Choanocotyle hobbsi Platt et Tkach, 2003	*Chelodina oblonga*, Perth, Australia	AY116868	AY116865
Choanocotyle nematoides Jue Sue et Platt, 1998	*Emydura* sp., New South Wales, Australia	AY116867	AY116862
Family Eucotylidae			
Tanaisia fedtschenkoi Skrjabin, 1924	*Anas platyrhynchos*, Kherson Region, Ukraine	AY222154	AY116870
Family Zoogonidae			

Deretrema nahaense Yamaguti, 1942	*Thalassoma lunare*, LI, Australia	AJ287498	AY222273
Diphterostomum sp.	*Scolopsis monogramma*, HI, Australia	AY222153	AY222272
Lepidophyllum steenstrupi Odhner, 1902	*Anarhichas lupus*, North Sea, U.K.	AJ287530	AY157175
Zoogonoides viviparus (Olsson, 1868)	*Callionymus lyra*, North Sea, U.K.	AJ287590	AY222271
Family Auridistomidae			
Auridistomum chelydrae (Stafford, 1900)	*Chelydra serpentina*, Mississippi, U.S.A.	AY222159	AY116872
Family Macroderoididae			
Macroderoides typicus (Winfield, 1929)	*Lepisosteus platostomus*, Reelfoot Lake, TN, U.S.A.	AY222158	AF433673
Family Faustulidae			
Antorchis pomacanthi (Hafeezullah et Siddiqi, 1970)	*Pomacanthus sexstriatus*, HI, Australia	AJ287476	AY222268
Bacciger lesteri Bray, 1982	*Selenotoca multifasciata*, Moreton Bay, Australia	AJ287482	AY222269
Trigonocryptus conus Martin, 1958	*Arothron nigropunctatus*, HI, Australia	AJ287584	AY222270
Family Lecithodendriidae			
Lecithodendrium linstowi Dollfus, 1931	*Nyctalus noctula*, Sumy Region, Ukraine	AY222147	AF151919
Prosthodendrium longiforme (Bhalerao, 1926)	*Myotis daubentoni*, Kiev Region, Ukraine	AY222148	AF151921
Family Microphallidae			
Maritrema oocysta Lebour, 1907	*Hydrobia ulvae*, Belfast Lough, Northern Ireland	AJ287534	AY220630
Microphallus fusiformis Reimer, 1963	*Hydrobia ulvae*, Belfast Lough, Northern Ireland	AJ287531	AY220633
Microphallus primas (Jägerskiöld, 1908)	*Carcinus maenus*, Belfast Lough, Northern Ireland	AJ287541	AY220627
Family Haploporidae			
Hapladena nasonis Yamaguti, 1970	*Naso unicornis*, LI, Australia	AY222146	AY222265
Pseudomegasolena ishigakiensis Machida et Kamiya, 1976	*Scarus rivulatus*, HI, Australia	AJ287569	AY222266
Family Atractotrematidae			
Atractotrema sigani Durio et Manter, 1969	*Siganus lineatus*, LI, Australia	AJ287479	AY222267
Family Troglotrematidae			
Nanophyetus salmincola Chapin, 1927	*Oncorhynchus mykiss*, Benton Co., Oregon, U.S.A.	AY222138	AY116873
Nephrotrema truncatum (Leuckart, 1842)	*Neomys anomalus*, Zakarpatska Region, Ukraine	AY222139	AF151936
Family Paragonimidae			
Paragonimus iloktsuenensis Chen, 1940	*Rattus norvegicus*, Amami Island, Japan	AY222141	AY116875
Paragonimus westermani (Kerbert, 1878)	*Canis familiaris*, Hyogo, Japan	AY222140	AY116874
Family Orchipedidae			
Orchipedum tracheicola Braun, 1901	*Cygnus olor*, Drumpellier Loch, Scotland	AJ287551	AY222258
Family Encyclometridae			
Encyclometra colubrimurorum (Rudolphi, 1819)	*Natrix natrix*, Kiev Region, Ukraine	AY222142	AF184254
Family Dicrocoeliidae			
Brachylecithum lobatum (Railliet, 1900)	*Corvus corone*, Záhlinice, Czech Republic	AY222144	AY222260
Dicrocoelium dendriticum (Rudolphi, 1819)	*Ovis aries*, Spain	Y11236	AY222261
Lyperosomum collurionis (Skrjabin et Issaitschikoff, 1927)	*Sylvia atricapilla*, Záhlinice, Czech Republic	AY222143	AY222259
Family Gorgoderidae			
Degeneria halosauri (Bell, 1887)	*Halosauropsis macrochir*, NE Atlantic Ocean	AJ287497	AY222257
Gorgodera cygnoides (Zeder, 1800)	*Rana ridibunda*, Sofia, Bulgaria	AJ287518	AY222257
Nagmia floridensis Markell, 1953	*Rhinoptera bonasus*, East Ship Island, Mississippi, U.S.A.	AY222145	AY222262
Xystretrum sp.	*Sufflamen chrysopterus*, LI, Australia	AJ287588	AY222263
Family Acanthocolpidae			
Cableia pudica Bray, Cribb et Barker, 1996	*Cantherines pardalis*, HI, Australia	AJ287486	AY222251
Pleorchis polyorchis (Stossich, 1889)	*Sciaena umbra*, Scandola, Corsica	§DQ248202	§DQ248215
Pleorchis uku Yamaguti, 1970	*Aprion virescens*, LI, Australia	§DQ248203	§DQ248216
Tormopsolus orientalis (Yamaguti, 1934)	*Seriola dumerili*, Scandola, Corsica	§DQ248204	§DQ248217
Stephanostomum baccatum (Nicoll, 1907)	*Eutrigla gurnardus*, North Sea, U.K.	AJ287577	AY222256
Stephanostomum baccatum (Nicoll, 1907)	*Hippoglossus hippoglossus*, North Sea, U.K.	§DQ248205	§DQ248218
Stephanostomum cf. *uku* Yamaguti, 1970	*Aprion virescens*, LI, Australia	§DQ248206	§DQ248219
Stephanostomum tantabiddii Bray et Cribb, 2004	*Carangoides fulvoguttatus,* Ningaloo, Australia	§DQ248207	§DQ248220
Stephanostomum gaidropsari Bartoli et Bray, 2001	*Gaidropsarus mediterraneus*, Marseille, France	§DQ248208	§DQ248221
Stephanostomum pristis (Deslongchamps, 1824)	*Phycis phycis*, Scandola, Corsica	§DQ248209	§DQ248222
Stephanostomum interruptum Sparks et Thatcher, 1958	*Menticirrhus americanus*, Gulf of Mexico, Mississippi, U.S.A.	§DQ248210	§DQ248223
Stephanostomum minutum (Looss, 1901)	*Uranoscopus scaber*, Scandola, Corsica	§DQ248211	§DQ248224
Stephanostomum bicoronatum (Stossich, 1883)	*Sciaena umbra*, Scandola, Corsica	§DQ248212	§DQ248225
Stephanostomum cesticillum (Molin, 1858)	*Lophius piscatorius*, Scandola, Corsica	§DQ248213	§DQ248226
Stephanostomum cf. *cesticillum* (Molin, 1858)	*Zeus faber*, Scandola, Corsica	§DQ248214	§DQ248227

§Indicates sequences newly characterised for this study; HI – Heron Island, Great Barrier Reef; LI – Lizard Island, Great Barrier Reef; SI – Stradbroke Island, Queensland. Further details in text and associated with accession numbers on GenBank.

Fig. 1. Phylogeny of selected Digenea estimated from combined complete SSU and partial LSU rDNA, indicating the position of the Acanthocolpidae. The tree is derived from Bayesian inference, employing the GTR+I+G model of substitution for each data partition, with nodal support provided by posterior probabilities (number above line or only number) and maximum parsimony bootstrap percentages (not shown if <50%); see text for further details

colpids and their sister taxa were identical. As with the individual analyses, *Cableia* failed to be resolved with the other putative acanthocolpids. *Stephanostomum* is a strongly monophyletic clade with *S. baccatum* sister species to all other members of the genus. The two samples of *S. baccatum* were slightly different in sequence for SSU (0.4%) only. The samples of *S. cesticillum* (Molin, 1858) and *S.* cf. *cesticillum* were identical suggesting they are in fact the same species. *Stephanostomum* was resolved as the sister group to a clade consisting of *Zalophotrema*, *Tormopsolus* and *Pleorchis*. Relationships among species of *Stephanostomum* were reasonably well supported and tree topologies were the same between methods of analysis.

The Allocreadioidea, as recognised by Olson *et al*. (2003), comprising the Opecoelidae, Opistholebetidae, Brachycladiidae and Acanthocolpidae was not resolved as a monophyletic clade. A paraphyletic Opecoelidae was sister group to the Opistholebetidae, but these in turn were poorly resolved in relation to other taxa. Indeed, the poor nodal support between the major clades of allocreadioids suggests the clade requires greater sampling of constituent families beyond just the acanthocolpids. Relationships within the superfamilies were much the same as was resolved by Olson *et al*. (2003).

Discussion

As stated above, the position of the Acanthocolpidae in the Digenea has not been well established. The revised classification of the Digenea presented by Olson *et al*. (2003) indicated that the Acanthocolpidae + Brachycladiidae is the sister group to the Opecoelidae + Opistholebetidae (forming the superfamily Allocreadioidea), based on Bayesian inference of combined ssrDNA and lsrDNA. In the classification of Olson *et al*. this superfamily is flanked, basally, by the Gorgoderoidea and the sister taxon is Plagiorchioidea + Microphalloidea. On the detailed species level phylogram, however, the group is flanked, basally, by the Lepocreadioidea (furthest) and Monorchioidea (closest) and, as the sister taxon, by the remainder of the Xiphidiata. The result of the analysis presented here (Fig. 1) is rather different in that the sister group of the Acanthocolpidae + Brachycladiidae is the group (Opecoelidae + Opistholebetidae) + Plagiorchioidea + Microphalloidea (all sensu Olson *et al*. 2003), i.e. the Acanthocolpidae is not in the Allocreadioidea. There are two unresolved sister groups to the clade containing the Acanthocolpidae, most of the Gorgoderoidea sensu Olson *et al*. (2003) and the Haploporidae + Atractotrematidae. The latter group was included within the Gorgoderoidea by Olson *et al*. (2003). Implications of this topology are discussed below, in particular in its relevance to the evolution of host relationships.

Only one of the putative acanthocolpid genera in this study is clearly misplaced. *Cableia* was originally placed in the Lepocreadiidae (see Sogandares-Bernal 1959) and was moved to the Opecoelidae (see Yamaguti 1971) and the Enenteridae (see Gibson and Bray 1982), before being placed 'pro

tem' in the Acanthocolpidae by Bray *et al*. (1996). It is now clear that molecular evidence points to it being close to the Monorchiidae (see Cribb *et al*. 2001) and, indeed, closer to the Monorchiidae than is the Lissorchiidae (see Olson *et al*. 2003, present study) (Fig. 1). There is no evidence that *Cableia* has the elaborate, often viciously spined male and female terminal genitalia, nor the specialised terminal organ as found in the female system of monorchiids. Despite the name, some monorchiids have two testes and members of this family share with *Cableia* a spiny tegument and a uterine seminal receptacle (see Yamaguti 1971, Dove and Cribb 1998). The monorchiid vitellarium is usually less extensive than that of *Cableia*. Thus, morphologically *Cableia* cannot be considered to fit comfortably into the current concept of the Monorchiidae. Lissorchiids are parasites of freshwater fishes, mainly Cypriniformes, and are reported only from North America, the Palaearctic region, India and South-eastern Asia. They usually have a spinous tegument, spines in the ejaculatory duct and metraterm, a lateral genital pore and a restricted follicular vitellarium. Many species have a single testis, but with two vasa efferentia clearly indicating a reduction from two (Shimazu 1992). *Cableia*, therefore, shows both morphological and biological distinction from the Lissorchiidae. Only two species are known in *Cableia*, the type-species *C. trigoni* Sogandares-Bernal, 1959 and the species used in this study, *C. pudica* Bray, Cribb et Barker, 1996. Including the present study, three molecular analyses have now placed *Cableia* in the same position relative to the Monorchiidae, providing strong evidence that it is neither a lepocreadiid, opecoelid, enenterid nor acanthocolpid. Using the molecular phylograms as evidence for taxonomic decisions, there are two alternative solutions to the placement of *Cableia*. It can either be considered a basal monorchiid or it can be the type-genus of a new family. At present we consider the former solution to be preferable, particularly as monogeneric families say little useful about relationships. We are, therefore, considering *Cableia* a basal and morphologically highly atypical, monorchiid. *Cableia* species are known only from tetradontiform fishes. The host of the specimens sequenced is the monacanthid *Cantherhines pardalis*, a benthic feeder (Froese and Pauly 2005), further evidence that this form is not an acanthocolpid.

The Acanthocolpidae is recovered as a monophyletic group in our study only if the Brachycladiidae is included within it. The clade (*Zalophotrema* (*Tormopsolus*, *Pleorchis*)) is recovered as a sister group to the genus *Stephanostomum*. More evidence would be needed before splitting this clade from the Acanthocolpidae, particularly as *Tormopsolus*, represented here by *T. orientalis* Yamaguti, 1934 (see Bartoli *et al*. 2004a) is morphologically similar to *Stephanostomum*. As the type-genus of the Acanthocolpidae is not represented in our study, the family is represented mainly by *Stephanostomum* spp.; therefore, it is quite conceivable that *Acanthocolpus* is closer to the clade (*Zalophotrema* (*Tormopsolus*, *Pleorchis*)), forming taxa which would reflect the subfamilies Acanthocolpinae and Stephanostominae as recognised by Yamaguti (1958). An alternative is to consider the Brachycladi-

idae as a synonym of the Acanthocolpidae. *Zalophotrema hepaticum* Stunkard et Alvey, 1929, the brachycladiid representative in this study, is found in the Californian sealion *Zalophus californianus*. It is a large, robust worm (up to 21 mm long) (Stunkard and Alvey 1930, Pérez-Ponce de León and Ramírez Lezama 1991), but in general morphology is similar to the Acanthocolpidae, with a spinous tegument, no external seminal vesicle and a uterine seminal receptacle. It shares with the *Pleorchis* spp. an H-shaped intestine, but not the numerous testes, although the testes are deeply lobed. Other brachycladiids may lack the H-shaped intestine (the two nasitrematine genera), but all eight brachycladiine genera have this, or a similar, character (Gibson 2005). Virtually nothing is known of the life cycle of brachycladiids, and nothing is known of intermediate hosts. Cable (1974), who recognised the relationship between acanthocolpids and brachycladiids (as campulids), indicated that he had unpublished information on the life history of *Orthosplanchnus fraterculus* Odhner, 1905, but all he said was that the cercaria was biocellate, as is found in acanthocolpids. A larger sample of brachycladiids would be needed before decisions should be made on the amalgamation of the families, and priority would be with Brachycladiidae, but the discussion below on the evolution of the group is predicated on the families being related in the topology shown in the phylogram.

The clade (*Tormopsolus*, *Pleorchis*) is not one that would have been predicted by morphology. *Tormopsolus* is morphologically similar to *Stephanostomum* but lacking the enlarged circum-oral spine array characteristic of the latter genus. The life cycle of *Pleorchis* is not known, but a few data on cercariae postulated to belong to *Tormopsolus* are available (see Bartoli and Gibson 1998). The cercariae in question are *Cercaria itoi* Shimura, 1984 (Shimura 1984) and the unnamed cercariae described by Bartoli and Gibson (1998). Both are found in rediae in buccinid prosobranchs, are biocellate and lack both a stylet and enlarged circum-oral spines. They are considered by Bartoli and Gibson (1998), however, to have 'numerous morphological similarities' to other acanthocolpids. Could it be that these cercariae are, in fact, brachycladiids?

Pleorchis is a well defined genus, with distinct autapomorphies (acinous ovary, numerous testes, possibly H-shaped intestine), which some authors have used to differentiate the family Pleorchiidae Poche, 1926. The two species used in this study form a clade. One is the type-species *P. polyorchis* (Stossich, 1889), from the sciaenid *Sciaena umbra* (see Bartoli *et al.* 2004b). Members of the genus are mainly in sciaenid fishes, but some species are reported in other perciforms, including the other species studied which was from a lutjanid (*Pleorchis uku* Yamaguti, 1970 in *Aprion virescens*).

Stephanostomum is also a well defined genus and the forms studied here, from eleven host species, comprise a mo-

nophylum of, probably, nine species. The subclades within *Stephanostomum* exhibit few biological or morphological apomorphies. This may not be surprising as the sample size is relatively small in relation to the size of the genus. The sister to the remainder of the genus (the most basally derived group) is the group of two forms considered to be members of the species *Stephanostomum baccatum* (Nicoll, 1907) from a gurnard and a flatfish (the type-host) from the North Sea. Gene sequences for these two specimens are identical for LSU and differ by only 7 bp between SSU rDNA sequences, suggesting strongly that these are in fact the same species; the differences are in one small variable loop region. The most basally derived species in the remainder of the genus is the lutjanid parasite *Stephanostomum*? *uku* Yamaguti[1], 1970, from the Great Barrier Reef. Moving through the tree, the next three species are derived in stepwise fashion with no monophyletic groups indicated. Next is the carangid parasite *S. tantabiddii* Bray et Cribb, 2004 from northern Western Australia. The next two species, although not forming a clade, are both from gadiform families, with *S. pristis* (Deslongchamps, 1924) from a phycid (this species is mostly reported from gadids with 89% of records – Bray and Cribb 2003) and *S. gaidropsari* Bartoli et Bray, 2001 from a lotid, both from the western Mediterranean. The remainder of the species form two clades. One is the pairing of *S. interruptum* Sparks et Thatcher, 1958, from a sciaenid in the Gulf of Mexico and *S. minutum* (Looss, 1901) from a uranoscopid in the western Mediterranean. The other clade is a group of three forms, two of which have identical sequences and must be considered conspecific. The basal singleton is *S. bicoronatum* (Stossich, 1883) from a sciaenid in the western Mediterranean. This is a widespread species, mainly of sciaenids (80% of records – Bray and Cribb 2003). Remaining are the two forms of *S. cesticillum* (Molin, 1858) from the anglerfish (type-host and with 67% of records – Bray and Cribb 2003) and from the zeid, *Zeus faber*. This fish has occasionally been reported as host of this species (see Bartoli and Bray 2001). As can be seen, there are no discernable patterns of host-relationships or distribution associated with the *Stephanostomum* species studied. The sample is less than 10% of the nominal species and just this small sample of eleven forms are from ten host families and six host orders. Two sciaenid parasites were studied, but these are in different clades. Two gadiform parasites were studied, and these form a paraphyletic group, which might conceivably be significant. The five perciform parasites do not form a clade. Of the clades, only one (*interruptum*, *minutum*) is reported from a single order of fishes, in this case the Perciformes. All the hosts are piscivores, mostly benthic feeders which also take other benthic organisms such as crustaceans. Two hosts, the lutjanid and the carangid, probably take more pelagic organisms. It seems likely that at the parasite species level the host assemblage is related closely to host diet. The two hosts of *S. bac-*

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[1]All the available specimens of this species are immature, so a definitive identification is not possible.

catum are benthic feeders with much other benthos in their diet, and the two hosts of *S. cesticillum* are the only ones with a predominantly piscivorous diet.

Few morphological characters are reflected in the putative phylogeny. Bray and Cribb (2003) summarised some major characters of *Stephanostomum* species, initially dividing the genus by vitellarium type. All the species studied belong in type 1 (<10% of hindbody devoid of follicles) and 2 (>10% of hindbody devoid of follicles). Apart from the forms considered conspecific, none of the clades recovered shared the same vitelline distribution. Circum-oral spine number is an important diagnostic character of *Stephanostomum* species. The basal species, *S. baccatum*, the only North Sea species, stands apart from the other species studied in the large number of circum-oral spines (44–58 spines). Only *S. tantabiddii* approaches this number (38–45). All other species have a spine number between 30 and 36, except for *S. interruptum* with 22 to 23 spines. Relatively few *Stephanostomum* species have over 50 circum-oral spines, but the separation of *S. baccatum* may indicate that the genus includes two clades with distinct numbers of circum-oral spines. A distinct ventral hiatus in the circum-oral spine rows appears to be a consistent diagnostic character. This condition is found scattered on the tree, being found in *S. tantabiddii*, *S. interruptum* and the clade (*bicoronatum*, *cesticillum*). Some *Stephanostomum* species have a uroproct (i.e. the intestinal caeca open into the posterior part of the excretory system) and it has been suggested (Bray 1985) that this character would be useful in splitting this large genus. This character is found in *S. gaidropsari*, *S. minutum* and *S. bicoronatum* and probably in *S. tantabiddii* and, therefore, Bartoli and Bray's (2001) conclusion that it was not an indicator of relationships, is borne out by these molecular results.

Unfortunately, it was not possible to extract DNA from specimens of *Neophasis anarrhichae* (Nicoll, 1909) from *Anarhichas lupus* (Halibut Bank, off Shetland Islands, U.K.), so the status of *Neophasis* Stafford, 1904, morphologically similar to *Spinoplagioporus*, cannot be further explored here. Bray and Gibson (1991) produced morphological and life cycle evidence for its inclusion in the Acanthocolpidae. *Neophasis* metacercariae encyst in both teleosts and bivalves, as we now know also happens in *Stephanostomum* (see Madhavi and Shaheem 1993, Pérez-Urbiola and Martínez-Díaz 2001).

Conclusions

There seems little doubt now that the Brachycladiidae and Acanthocolpidae are closely related and have, at least, a common ancestor. It is not surprising that two groups of piscivorous marine hosts should have similar parasites. The question is, were brachycladiids derived from fish parasites, or were acanthocolpids derived from marine mammal parasites? The topology of the Bayesian trees reproduced in Olson *et al.* (2003) suggests the former, with the acanthocolpid/brachy-

cladiid clade being a sister to the opecoelid/opistholebetid fish parasite clade. The topology found in this study (Fig. 1) also suggests that the brachycladiid host group (marine mammals) is derived from a fish (teleost) host. The basal groups in the tree, the Lepocreadioidea, Apocreadioidea and Monorchioidea, are all fish parasites, predominantly marine. Of the two sister groups of the clade including the Acanthocolpidae + Brachycladiidae, one, the Haploporidae + Atractotrematidae, consists solely of fish parasites. The other, the remainder of the Gorgoderoidea sensu Olson *et al.* (2003) has tetrapod hosts as basal, although fish parasites (including elasmobranch parasites) have, apparently, been derived within it. Within the sister group, the most basal clade, the Allocreadioidea, are fish parasites, whilst the remainder of the sister group are mainly tetrapod parasites, with fish parasites derived within (i.e. Zoogonidae + Faustulidae). Thus, it appears that tetrapod parasites have been derived from fish parasites at the base of the Gorgoderoidea (minus the Haploporidae), the base of the Brachycladiidae (an assumption based on one species) and the base of the higher Xiphidiata (Plagiorchioidea + Microphalloidea). Within these 'higher' digeneans (as elsewhere in the group) host switching has occurred readily and neither in detail, nor in overview, can co-evolution be readily detected (Cribb *et al.* 2003). Even within the Brachycladiidae the hosts are from two distinct, unrelated, mammalian groups, the Cetacea and the Pinnipedia. Recent studies indicate that cetaceans are related to artiodactyls, particularly hippopotami, and pinnipeds are related to or within the Carnivora (O'Leary *et al.* 2004). The sequencing of complete SSU and partial LSU rDNA continues to provide valuable phylogenetic information for the Digenea as more taxa are sampled and the monophyly of problematic families is tested. Denser sampling of all groups will provide stronger evidence on the evolution of host-parasite relationships.

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