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Orders out of chaos – molecular phylogenetics reveals the complexity of shark and stingray tapeworm relationships

Janine N. Caira[a,*], **Kirsten Jensen**[b], **Andrea Waeschenbach**[c], **Peter D. Olson**[c], and **D. Timothy J. Littlewood**[c]

[a]Department of Ecology & Evolutionary Biology, University of Connecticut, 75 N. Eagleville Rd., Storrs, Connecticut, 06269–3043, USA

[b]Department of Ecology & Evolutionary Biology and the Biodiversity Institute, University of Kansas, 1200 Sunnyside Ave., Lawrence, Kansas, 66045, USA

[c]Department of Life Sciences, Natural History Museum, Cromwell Road, London SW7 5BD, UK

Abstract

Novel molecular data are presented to resolve the long-standing issue of the non-monophyly of the elasmobranch-hosted tapeworm order Tetraphyllidea relative to the other acetabulate eucestode orders. Bayesian Inference analyses of various combinations of full *ssrDNA*, and full or partial *lsrDNA* (D1-D3), sequence data, which included 134 species representing 97 genera across the 15 eucestode orders, were conducted. New *ssrDNA* data were generated for 82 species, partial *lsrDNA* data for 53 species, and full *lsrDNA* data for 29 species. The monophyly of each of the elasmobranch-hosted orders Cathetocephalidea, Litobothriidea, Lecanicephalidea, and Rhinebothriidea was confirmed, as was the non-monophyly of the Tetraphyllidea. Two relatively stable groups of tetraphyllidean taxa emerged and are hereby designated as new orders. The Onchoproteocephalidea n. ord. is established to recognize the integrated nature of one undescribed and ten described genera of hook-bearing tetraphyllideans, previously of the family Onchobothriidae, with the members of the order Proteocephalidea. The Phyllobothriidea n. ord. is established for a subset of 12 non-hooked genera characterized by scoleces bearing four bothridia each with an anterior accessory sucker; most parasitise sharks and have been assigned to the Phyllobothriidae at one time or another. Tentative ordinal placements are suggested for 8 additional genera; placements for the remaining tetraphyllidean genera have not yet emerged. We propose these 17 genera remain in the “Tetraphyllidea”. Among these, particularly labile across analyses were *Anthobothrium*, *Megalonchos*, *Carpobothrium*, *Calliobothrium*, and *Caulobothrium*. The unique association of *Chimaerocestus* with holocephalans, rather than with elasmobranchs, appears to represent a host-switching event. Both of the non-elasmobranch hosted clades of acetabulate cestodes (i.e., Proteocephalidea and Cyclophyllidea and their kin) appear to have had their origins with elasmobranch cestodes. Across analyses, the sister group to the clade of “terrestrial” cestode orders was found to be an elasmobranch-hosted genus; as was the sister to the

[*]Corresponding author. Tel.: +1 860 486 4060; fax: +1 860 486-6364., janine.caira@uconn.edu.

Note: Supplementary data associated with this article.

Appendix A. Supplementary data

Supplementary data, table and figure associated with this article can be found, in the online version, at doi: 10.1016/j.ijpara.2013.10.004.

freshwater fish and tetrapod-hosted Proteocephalidea. Whilst further data are required to resolve outstanding nomenclatural and phylogenetic issues, the present analyses contribute significantly to an understanding of the evolutionary radiation of the entire Cestoda. Clearly, elasmobranch tapeworms comprise the backbone of cestode phylogeny.

Keywords

Elasmobranchs; *lsrDNA*; *ssrDNA*; Tetraphyllidea; Onchoproteocephalidea; Phyllobothriidea; cestode; evolution

1. Introduction

Despite global efforts to bring cestode classification into line with phylogeny, one of the most speciose of orders parasitizing elasmobranchs (i.e., the Tetraphyllidea) remains most problematic. All phylogenetic work that has included representatives of this order, whether morphological (Euzet et al., 1981; Brooks et al., 1991; Hoberg et al., 1997; Caira et al., 1999; 2001) or molecular (Mariaux, 1998; Olson and Caira, 1999; Kodedová et al., 2000; Olson et al., 2001; Waeschenbach et al., 2007, 2012), has shown the group to be paraphyletic. As a consequence, the pivotal position of the tetraphyllideans in the larger context of tapeworm evolution, and thus the importance of establishing their phylogenetic relationships, is widely recognized.

The formal dismantling of the Tetraphyllidea, as traditionally circumscribed, consisting of the speciose Onchobothriidae Braun, 1900 and Phyllobothriidae Braun, 1900, the morphologically unique Chimaerocestidae Williams and Bray, 1989 and Dioecotaeniidae Schmidt, 1969, as well as the Triloculariidae Yamaguti, 1959 (e.g., see Schmidt, 1986; Euzet, 1994), and the Serendipidae Brooks and Barriga, 1995 (see Brooks and Barriga, 1995), began almost a decade ago largely as a result of the application of molecular methods. Three suites of taxa that exhibit proglottid morphology like that of the Tetraphyllidea but bear scoleces that entirely lack acetabula characteristic of Tetraphyllidea and their derived relatives, were removed from the order. Olson and Caira (2001) resurrected the order Litobothriidea of Dailey (1969) for the members of the genus *Litobothrium* Dailey, 1969. Caira et al. (2005) resurrected the order Cathetocephalidea (of Schmidt & Beveridge, 1990) for several genera exhibiting cushion-like scoleces. In addition, most recently, Healy et al. (2009) erected the Rhinebothriidea to house the tetraphyllideans bearing stalked acetabula.

However, even with these modifications the Tetraphyllidea persists as a paraphyletic assemblage (e.g., see Waeschenbach et al., 2012). This paraphyly has particularly important implications for our understanding of the evolutionary history of the other acetabulate cestode orders (i.e., Proteocephalidea, Tetrabothriidea, Cyclophyllidea, and *Mesocestoides*), and also the non-acetabulate Nippotaeniidea, all of which parasitise vertebrate groups other than elasmobranchs. Tetraphyllidean paraphyly has manifested itself somewhat differently among molecular analyses. For example, Mariaux (1998) found tetraphyllideans to group in a ladder-like arrangement sister to a clade comprised of diphyllidean, proteocephalidean, nippotaeniidean, tetrabothriidean and cyclophyllidean exemplars. Of the four

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tetraphyllideans included by Olson and Caira (1999), three comprised a clade that also included a proteocephalidean exemplar, while the fourth grouped as sister to a clade comprised of the former clade and nippotaeniidean, tetrabothriidean and cyclophyllidean exemplars. Kodedová et al. (2000) found a similar result but with less resolution within both clades. Olson et al. (2001) reported a diversity of results depending on the data partition and method of analysis, but in general, found one of their tetraphyllidean exemplars (*Acanthobothrium* van Beneden, 1850) to group with proteocephalidean exemplars, and their remaining eight tetraphyllidean exemplars to group in a ladder-like arrangement basal to a clade comprised of the proteocephalideans and *Acanthobothrium* as well as the cyclophyllidean, tetrabothriidean, nippotaeniidean exemplars. The analyses of Waeschenbach et al. (2007; 2012) yielded similar results. Caira et al. (2005) found the nine tetraphyllidean exemplars included in their analyses to comprise a clade along with six proteocephalidean exemplars; this clade was sister to a clade comprised of the cyclophyllidean, tetrabothriidean and nippotaeniidean exemplars. Because these studies were all largely based on nuclear ribosomal gene data, the differences in topologies seem likely due to taxon sampling.

By sampling densely across the Tetraphyllidea, the present molecular study was undertaken (1) to examine the relationships among tetraphyllidean taxa to establish a foundation for systematic revision of the order, and (2) to investigate tetraphyllidean relationships with respect to the monophyly and origins of the other acetabulate cestode lineages, many of which parasitise vertebrates other than elasmobranchs. Efforts were made to include representatives of as many tetraphyllidean genera as possible so as to capture their extensive morphological variation, host associations, and the multitude of positions they appear to occupy across the cestode phylogeny. Also included, at least in some analyses, were one or more representatives of all other cestode orders, with the exception of the Amphilinidea. We build on previous studies by concentrating on large and small subunits of nuclear ribosomal RNA genes; *lsrDNA* (=28S rDNA) and *ssrDNA* (=18S rDNA), respectively.

2. Materials and methods

2.1. Sampling and study taxa

Specimens sequenced *de novo* were obtained from hosts collected around the globe over the last two decades. Sampling of elasmobranch hosts was conducted as follows: off Australia using gill nets, fish traps, and in conjunction with the commercial trawling vessels including the *FV Ocean Harvest*; in Canada off New Brunswick with local trawling vessel; off Chile in conjunction with local trawling vessels; in Malaysian Borneo from fish markets, in conjunction with trawling vessels and with gill nets; in Mexico in the Gulf of California using gill nets; off Horta in the Azores, Portugal in conjunction with Azores Fisheries; off New Zealand in conjunction with the research vessel *NZ Taicongaroga*; in Peru with hand spears and fish nets; off Senegal in conjunction with local net fishermen; off Thailand in conjuction with commercial trawlers. Within the United States: in the Gulf of Mexico using hand lines; in North Carolina with gill nets; off New York, Connecticut, and Rhode Island in conjunction with shark tournaments; off South Carolina in conjunction with the *RV Oregon II*; in the Florida Keys with hand lines. The two species of *Chimaerocestos* Williams and

Bray, 1984 were collected from holocephalans in conjuction with the NZ *Taicongaroga*. Specimens of *Proteocephalus perplexus* La Rue, 1911 were collected from Hay Bay, Ontario. All specimens were preserved in 95% ethanol. An effort was made to preserve vouchers of all specimens sequenced in this study. In most instances, whole mounts of hologenophores consisting of either the scolex and terminal proglottids, or in the cases of smaller specimens, the scolex only, were prepared following standard methods (see Pickering and Caira, 2008). In the cases of extremely tiny species, specimens were photographed and the entire specimen was then used for sequencing; in such cases photographic vouchers were deposited. Elasmobranch identities follow Naylor et al. (2012).

In total, 134 cestode species were analysed in this study. These are listed in Table 1 along with their hosts and collection localities. For the purposes of this study, new *ssrDNA* data were generated for 82 of these species; partial *lsrDNA* data were generated for 53 and full *lsrDNA* data for 29 species. Accession numbers for hologenophores and photographic vouchers for 82 species deposited in the Lawrence R. Penner Parasitology Collection (LRP) at the University of Connecticut, Storrs, Connecticut, USA are provided in Table 1. In the cases of 34 of the remaining 57 species, partial *lsrDNA* and *ssrDNA* data were obtained from GenBank; for 23 of the 34 species full *lsrDNA* and *ssrDNA* were obtained from GenBank. Sequences for which vouchers are available were preferred. GenBank accession numbers and sources for all species are provided in Table 1 as well as in Fig. 2 and Supplementary Fig. S5. Also indicated in Table 1 is the ordinal level placement of each species based on current cestode classification (sensu Khalil et al., 1994 as modified by Caira and Olson, 2001; Caira et al., 2005; Healy, et al., 2009), as well as its revised ordinal placement as a result of this study.

Two data sets were generated. In both cases, all acetabulate taxa were considered as members of the ingroup and, with the exception of the nippotaeniids and cathetocephalideans, all non-acetabulate taxa were considered as members of the outgroup. Inclusion of the nippotaeniids as members of the ingroup, despite their non-acetabulate nature, followed previous work (e.g., Hoberg et al., 2001; Waeschenbach et al., 2012) as did treatment of the cathetocephalideans (e.g., Caira et al. 2005). *Dataset_I* consisted of partial (i.e., D1-D3 region) *lsrDNA* and complete *ssrDNA* for 134 eucestode taxa. Informed by *Dataset_I* and to deeper explore the phylogenetic relationships, 47 of these 134 taxa were chosen for which sequence data were generated for the remaining domains of *lsrDNA* and included in *Dataset_II* (i.e., complete *ssrDNA* and complete *lsrDNA*). Outgroup taxa in *Dataset_I* consisted of exemplars of: Bothriocephalidea, Diphyllidea, Diphyllobothriidea, Litobothriidea, and Trypanorhyncha. Outgroup taxa in *Dataset_II* included exemplars of the cestode orders Bothriocephalidea, Caryophyllidea, Diphyllidea, Diphyllobothriidea, Gyrocotylidea, Litobothriidea, Spathebothriidea, and Trypanorhyncha, as well as one digenean, one aspidogastrean, and one monogenean taxon (represented by chimaeric sequences).

In order to maximise the diversity of morphological forms, representatives of 10 undescribed genera were included in one or both data sets. These consisted of the four novel genera of rhinebothriideans from Healy et al. (2009) (i.e., N. gen. 1 through N. gen. 4) and six novel genera introduced here (i.e., N. gen. 5 through N. gen. 10). The identities of these taxa are

formally anchored by voucher specimens deposited in the LRP Collection, as indicated in Table 1. Scanning electron micrographs (SEMs) of the scoleces of New genus 1 through 4 can be found in Healy et al. (2009; figs. 7–10). SEMs of the scoleces of the six remaining new genera, prepared for SEM following Healy et al. (2009), are provided in Fig. 1.

2.2. DNA extraction, gene amplification and sequencing

Total genomic DNA was extracted from ethanol-preserved specimens using the DNeasy Blood and Tissue kit or Genomic-tip 20/G (QIAGEN) following the manufacturer's instructions. PCR and sequencing primers are listed in Supplementary Table 1. Partial *lsrDNA* (1,172–1,597 bp) was amplified in one fragment using LSU5 or ZX-1 + 1500R; in the case of poor amplification, semi-nested PCRs on primary amplicons using the same forward primer and reverse primer 1200R were carried out. Complete *lsrDNA* (4,132–4,438 bp) was amplified in a number of overlapping fragments, depending on amplification success: LSU5 or ZX-1 + either L2230, LSUD6-3′ or L1642; and U1846 or 1600F + OR-2. Complete *ssrDNA* (1,912–2,260 bp) was amplified in either one fragment using WormA + WormB primers or by a number of overlapping fragments, depending on amplification success: 18S-8 + WormB or 1200R; WormA + A27′; Tet460F + Tet1420R; Tet1100F + 1200R or WormB. PCRs were carried out in 25 μl reaction volumes using Illustra PuRe Taq Ready-to-go PCR beads (GE Healthcare) and 1 μl of 10 μM of each primer. Cycling conditions for *lsrDNA* were as follows: initial denaturation for 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, 2 min at 72 °C and completed by 10 min at 72 °C. Cycling conditions for *ssrDNA* were as follows: initial denaturation for 2 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 54 °C, 2 min at 72 °C and completed by 10 min at 72 °C; in the case of nested PCRs, the annealing temperature was increased by two degrees. PCR products were purified using QIAquick Gel Extraction Kit or QIAquick PCR Purification Kit (QIAGEN). Sequencing of both strands was carried out on an Applied Biosystems 3730 DNA Analyser, using Big Dye version 1.1. Sequence identity was checked using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nih.gov/BLAST/). Contigs were assembled using Sequencher 4.8 (GeneCodes Corporation).

2.3. Sequence alignment and phylogenetic analyses

Sequences were aligned with ClustalX (Thompson et al., 1997), with default settings and penalties as follows: gap opening 10, gap extension 0.20, delay divergent sequences 30%, DNA transition weight 0.5. The alignment was improved by eye in MacClade (Maddison and Maddison, 2005). Regions, that could not be unambiguously aligned were excluded from the analysis; alignments are available from the authors on request. Modeltest version 3.7macX (Posada and Crandall, 1998) was used to select a model of evolution using the Akaike Information Criterion.

A total of six phylogenetic analyses were conducted as follows. Analysis 1: complete *lsrDNA* for the restricted set of 55 taxa (Supplementary Fig. S1; Table 1). Analysis 2: partial *lsrDNA* for the suite of 134 taxa (Supplementary Fig. S2; Table 1). Analysis 3: *ssrDNA* for the 55 taxa (Supplementary Fig. S3). Analysis 4: *ssrDNA* for the 134 taxa (Supplementary Fig. S4). Analysis 5: complete *lsrDNA* + *ssrDNA* for the 55 taxa (Figs. 2, 3). Analysis 6:

partial *lsrDNA* + *ssrDNA* for the 134 taxa (Figs. 4, 5). Thus, Analyses 1–4 were based on data from single genes; Analyses 5 and 6 were based on data from both genes.

Phylogenetic trees were constructed using Bayesian inference (BI) with MrBayes, version 3.1 (Huelsenbeck and Ronquist, 2001); likelihood settings were set to nst = 6, rates = invgamma, ngammacat = 4 (equivalent to the GTR+I+G model of evolution). In the analysis with multiple character partitions, parameters were estimated separately for each partition. Four chains (temp = 0.2) were run for 10,000,000 generations and sampled every 1,000 generations. 'Burnin' was determined as the point at which average standard deviation of split frequencies (ASDOSF) was <0.01. Maximum likelihood bootstrap values for 100 replicates were obtained using Genetic Algorithm for Rapid Likelihood Inference (GARLI) Version 0.942 (Zwickl 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Doctoral dissertation, University of Texas at Austin, TX, USA) using default settings, except setting 'Genthreshfortopoterm' to 10,000 generations. Clades were considered to have high nodal support if BI posterior probability was ≥95% and bootstrap values ≥75%.

3. Results

3.1. Tetraphyllidean relationships

Analyses of individual genes (i.e., Analyses 1–4) yielded tree topologies that differed conspicuously from one and other, and also from those resulting from the combined analyses (i.e., Analyses 5 and 6), signaling a good deal of instability in any of the topologies chosen for formal presentation here. Incongruences included not only the placement of certain taxa (e.g., *Anthobothrium* van Beneden, 1850, *Calliobothrium* van Beneden, 1850, *Carpobothrium* Shipley and Hornell, 1906, *Caulobothrium* Baer, 1948, *Megalonchos* Baer and Euzet, 1962), but also potential ordinal membership and interrelationships. As a consequence, the trees from the analysis of individual genes and also of the smaller number of taxa are presented as supplementary documents only (see Supplementary Figs. S1–S6), and we have concentrated here on the tree generated from the greater taxon sampling and data from both genes (i.e., Analysis 6). We have also provided a schematic diagram summarizing the better supported nodes for discussion purposes. The tree illustrating the phylogenetic relationships among the 55 species included in the analysis of complete *lsrDNA* and complete *ssrDNA* (i.e., Analysis 5), along with potential ordinal placements, is shown in Supplementary Fig. S5. The more robust nodes (i.e., with ≥0.95 Bayesian posterior probabilities [pp] and/or 75% Maximum Likelihood [ML] bootstrap support) are summarized in the schematic topology in Supplementary Fig. S6. Owing to its less dense taxon sampling, the results of Analysis 5 are limited with respect to their implications for the interrelationships among genera. The tree illustrating the phylogenetic relationships among the 134 cestode species included in the analysis of partial *lsrDNA* and complete *ssrDNA* data (i.e., Analysis 6), along with resulting potential ordinal placements, is shown in Fig. 2. The more robust nodes (with ≥0.95 Bayesian pp and/or 75% ML bootstrap support) are summarized in the schematic topology in Fig. 3, which has been expanded to include additional outgroups based on Waeschenbach et al. (2012). This figure also includes the major vertebrate groups hosting each cestode taxon.

Given the instability noted above, our goal of achieving breadth across tetraphyllidean genera rather than depth within tetraphyllidean genera, and the limited representation of genera of the other acetabulate orders, we have refrained from commenting on the implications of our results for the non-monophyly of individual genera (e.g., *Polypocephalus* Braun, 1878, *Echeneibothrium* van Beneden, 1849, *Rhinebothrium* Linton, 1890, *Phyllobothrium* van Beneden, 1849, *Paraorygmatobothrium* Ruhnke, 1994, *Platybothrium* Lindon, 1890, *Acanthobothrium*, *Pedibothrium* Linton, 1908, and *Proteocephalus* Weinland, 1858). We feel strongly that these issues are best addressed in analyses that include much denser taxon sampling. Furthermore, it is important to note that the outgroups employed in the analyses of *Dataset_I* (i.e., Analyses 2, 4, and 6) were much more limited than those empoyed in analyses of *Dataset_II* (i.e., Analyses 1, 3, and 5) and thus the interrelationships of the bothriate (i.e., Diphyllidea, Bothriocephalidea, Trypanorhyncha, Diphyllobothriidea) and other non-acetabulate (i.e., Caryophyllidea, Spathebothriidea, etc.) cestode orders have also not been addressed.

Not unexpectedly, the combined analysis with broadest taxon representation (Analysis 6) confirmed the monophyly, and thus ordinal status, of the elasmobranch-hosted Lecanicephalidea, Rhinebothriidea, Litobothriidea, and Cathetocephalidea (Fig. 2). It also confirmed the monophyly of the acetabulate cestodes with one exception. Despite their non-acetabulate nature (and thus their original inclusion among the outgroup taxa), the Cathetocephalidea grouped robustly among the acetabulate taxa in both Analysis 5 (Supplemental Figs. 5, 6) and 6 (Figs. 2, 3).

In contrast, the tetraphyllideans were distributed throughout the topology of the trees resulting from both combined analyses. Taxon-dense Analysis 6 yielded the following notable affinities. Although poorly supported, the Proteocephalidea (represented by *Gangesia* Woodland, 1924, *Proteocephalus*, and *Peltidocotyle* Diesing, 1850) emerged as monophyletic, but nested with a high degree of support among a selection of mostly hooked tetraphyllidean genera (e.g., *Acanthobothrium*, *Uncibilocularis* Southwell, 1925, *Platybothrium*, *Prosobothrium* Cohn, 1902, *Phoreiobothrium* Linton, 1889, *Triloculatum* Caira and Jensen, 2009, New genus 8), with the freshwater stingray-hosted *Potamotrygonocestus* Brooks and Thorson, 1976 as its closest relative (Fig. 2). This result provides guidance for the reconfiguration of the ordinal classification of the proteocephalideans and their kin that follows below (see Section 4.2.1). Furthermore, a selection of hooked (i.e., *Spiniloculus* Southwell, 1925, *Yorkeria* Southwell, 1927, *Pedibothrium*, *Pachybothrium* Baer and Euzet, 1962, and *Balanobothrium* Hornell, 1911) and non-hooked (e.g., *Caulobothrium*, New genus 9, *Dinobothrium* van Beneden, 1889, *Ceratobothrium* Monticelli, 1892) tetraphyllidean genera grouped as sister to a clade consisting of *Mesocestoides* Vaillant, 1863 + Tetrabothriidea (i.e., *Tetrabothrius* Rudolphi, 1819) + Cyclophyllidea (i.e., *Dilepis* Weinland, 1858 and *Hymenolepis* Weinland, 1858) + Nippotaeniidea (i.e., *Nippotaenia* Yamaguti, 1939), with the tetraphyllidean *Carpobothrium* as the closest relative of the latter clade (Fig. 2).

A relatively large clade of tetraphyllideans that appears to have emerged, albeit with relatively weaker support, consists of a diversity of non-hooked genera with acetabula in the form of relatively simple bothridia, many of which parasitise sharks (i.e., New genus 10,

Phyllobothrium, Orygmatobothrium Deising, 1863, *Thysanocephalum* Linton, 1890, *Pithophorus* Southwell, 1925, *Orectolobicestus* Ruhnke, Caira and Carpenter, 2006, *Paraorygmatobothrium*, *Ruhnkecestus* Caira and Durkin, 2006, and *Scyphophyllidum* Woodland, 1927), but some of which parasitise rays (e.g., *Calyptrobothrium* Monticelli, 1893 and *Nandocestus* Reyda, 2008). *Chimaerocestos*, the only known genus of tetraphyllidean hosted by non-elasmobranch chondrichthyans (i.e., holocephalans), also nested within this clade. Within this larger clade, six genera (*Nandocestus, Paraorygmatobothrium*, *Pithophorus, Orectolobicestus*, *Ruhnkecestus*, and *Scyphophyllidum*) comprised a subclade that was particularly well supported in the tree resulting from Analysis 6 (Fig. 2).

Three additional, albeit smaller, clades of tetraphyllidean genera (i.e., *Rhoptrobothrium* Shipley and Hornell, 1906 + *Myzocephalus* Shipley and Hornell, 1906; *Calliobothrium* + *Trilocularia* Olsson, 1867 + *Crossobothrium* Linton, 1889; *Megalonchos* Baer and Euzet, 1962 + *Anthobothrium* + *Dioecotaenia* Schmidt, 1969 + *Duplicibothrium* Williams and Campbell, 1978) were found intermingled among other cestode groups in the tree resulting from Analysis 6 (Fig. 2). These taxa were found to be by far the most labile in position across the six analyses. Morphologically, the latter two clades contain a particularly puzzling assemblage of genera and the relatively low support for each raises questions about their true affinities.

3.2. Interrelationships among currently recognized cestode orders

Both Analysis 5 and 6 (i.e., analyses of both datasets) strongly support the Lecanicephalidea as the earliest divergent order of acetabulate cestodes, followed by the Rhinebothriidea, which forms the sister to a large clade consisting of the remaining orders (Supplementary Figs. S5 and S6, and Figs. 2 and 3). Also emerging, albeit with strong support only in Analysis 5, is the Cathetocephalidea as the earliest divergent order within the latter, larger clade (Supplementary Figs. S5 and S6). Interrelationships among the other members of the larger clade are less well resolved and, in some cases, also less stable across analyses. A clade comprised of the orders Cyclophyllidea, Tetrabothriidea, Nippotaeniidea and *Mesocestoides* is robustly supported in all analyses of both datasets, however their interrelationships differed between analyses.

With respect to the Tetraphyllidea, Analysis 5 and 6 are consistent in the following elements. The non-monophyly of the order is indisputable. A suite of tetraphyllidean genera are sister to the Proteocephalidea. Another suite of tetraphyllidean genera are sister to the clade comprised of the Cyclophyllidea, Tetrabothriidea, Nippotaeniidea and *Mesocestoides*. However, in both cases the closest tetraphyllidean relative is ambiguous.

4. Discussion

Phylogenetic analysis of *lsrDNA* and *ssrDNA* based on a comprehensive sampling of tetraphyllidean taxa in the context of other acetabulate cestode orders has provided support for a number of previously proposed systematic hypotheses, challenged others, and has suggested new phylogenetic scenarios. These require consideration with reference to morphology, and synapomorphies supported, challenged, or newly revealed. Such

phylogenetic hypotheses are discussed separately below in the context of their implications for cestode orders and our understanding of cestode evolution overall.

4.1. Stable elasmobranch-hosted cestode orders

4.1.1. Litobothriidea Daily, 1969—The three litobothriidean species included in our analyses consistently formed a monophyletic group distinct from the remaining major lineages. This result supports Dailey (1969) and Olson and Caira's (2001) recognition of the Litobothriidea as an independent order. The litobothriideans were found to be sister to a clade comprised of the acetabulate cestode groups. This suggests that the unusual form of the scolex seen in the seven known litobothriidean species (i.e., an apical sucker followed by a series of pseudosegments) likely represents a uniquely derived condition, rather than a modification of the acetabulate scolex form characteristic of the members of its sister clade.

4.1.2. Lecanicephalidea Baylis, 1920—The monophyly of the Lecanicephalidea and its status as an independent order were both well supported by all of the analyses conducted here. Our results suggest that this lineage of apical organ-bearing cestodes represents the sister taxon of the remaining acetabulate cestode taxa. It is interesting that *Paraberapex manifestus* Jensen, 2001, the only lecanicephalidean species included that lacks an apical organ, grouped as the sister taxon to the clade comprised of the 17 apical-organ bearing species. However the analysis included representatives of only nine of the 21 described genera and thus much remains to be explored with respect to the interrelationships within the order in general.

4.1.3. Rhinebothriidea Healy, Caira, Jensen, Webster and Littlewood, 2009—The erection of the Rhinebothriidea by Healy et al. (2009) was fully supported by the results of our analyses. In addition, *Pseudanthobothrium* Baer, 1956, not treated by Healy et al. was found to group among *Echeneibothrium* species suggesting that within the Rhinebothriidea there exists a clade of taxa the adult form of which bears an apical modification of the scolex proper (in this case a myzorhynchus). Although lacking facial loculi, *Pseudanthobothrium* exhibits bothridial stalks and on this basis was predicted by Healy et al. (2009) to belong in the Rhinebothriidea. Our results also support inclusion of *Anthocephalum* Linton, 1890 in the Rhinebothriidea as was suggested by Healy et al. (2009) despite its lack of facial bothridial loculi. In addition, a novel genus (New genus 7; Fig. 1C) and the tetraphyllidean *Pentaloculum* Alexander, 1963, not treated by Healy et al., were found to group as close relatives of the Rhinebothriidea. Although both genera exhibit facial loculi, neither is well known and their morphology and status as members of the Rhinebothriidea requires further investigation.

Although missing from our analyses and theirs, we support the suggestion of Healy et al. (2009) that the following five genera should be considered candidates for inclusion in the Rhinebothriidea based on their possession of facial loculi and possibly also bothridial stalks: *Clydonobothrium* Euzet, 1959, *Notomegarhynchus* Ivanov and Campbell, 2002, *Phormobothrium* Alexander, 1963, *Tritaphros* Lönnberg, 1889 and *Escherbothrium* Berman and Brooks, 1994. We propose that on this morphological basis *Biotobothrium* Tan, Zhou and Yang, 2009 and *Zyxibothrium* Hayden and Campbell, 1981 be added to this list.

However, the rhinebothriidan status of these seven genera remains to be confirmed with molecular data.

4.1.4. Cathetocephalidea Schmidt and Beveridge, 1990—Our analyses consistently yielded a clade comprised of three genera, two of which (*Cathetocephalus* Dailey and Overstreet, 1973 and *Sanguilevator* Caira, Mega and Ruhnke, 2005) were formally recognized in the order Cathetocephalidea by Caira et al. (2005). The inclusion of a third genus, *Disculiceps* Joyeux and Baer, 1936, in this clade, and thus this order, is a novel result and indicates that *Disculiceps* should be transferred from the Tetraphyllidea to the Cathetocephalidea. This action is fully consistent with the morphology of the scolex of *Disculiceps* which is bipartite consisting of an anterior cushion and posterior collar, and which, like the scoleces of *Cathetocephalus* and *Sanguilevator*, lacks all evidence of acetabula. Our results support recognition of the Cathetocephalidea as a distinct order at this time. However, its position relative to many of the tetraphyllidean groupings was found to be ambiguous across analyses.

4.2. Dismantling of the Tetraphyllidea Carus, 1863

The non-monophyly of the Tetraphyllidea was anticipated based on previous investigations, but among the most striking results of our analyses was the remarkably widespread intermingling of tetraphyllidean taxa among and across the other cestode orders. Also somewhat unexpected was the determination that the hooked tetraphyllideans (i.e., the Onchobothriidae) represent at least three independent lineages.

It is beyond the scope of this study to fully revise the classification of the Tetraphyllidea so as to render it entirely monophyletic given that in some cases the relationships recovered were labile, differing across analyses and the solution for absolute monophyly is unclear. However, two new orders are erected below to accommodate the two clades of genera that were mostly stable across analyses. Although not ideal, we propose that the other genera remain in the non-monophyletic “Tetraphyllidea” until such time as their relationships can be more fully and definitively resolved based on more dense taxon sampling and additional molecular data. Ultimately, the complete dismantling of the “Tetraphyllidea” may be required to promote systematic clarity.

4.2.1. Onchoproteocephalidea n. ord.—*Synonyms:* Tetraphyllidea Carus, 1863 *pro parte*; Proteocephalidea Mola, 1928 *in toto*

4.2.1.1 Diagnosis: Cestoda: Eucestoda. Small to medium sized tapeworms. Strobila polyzoic, proglottized; simple gladiate spinitriches present posterior to scolex proper, at a minimum on cephalic peduncle, neck, and/or proliferation zone, on entire strobila in some. Proglottids hermaphroditic, generally euapolytic or apolytic, occasionally hyperapolytic. One set of reproductive organs per proglottid. Two pairs of lateral osmoregulary canals; ventral canals usually wider than dorsal canals. Scolex with four muscular bothridia. Bothridia unarmed or with one pair of hooks, facially divided or not, lacking stalks. Apical organ present in some. Metascolex present in some. Testes numerous, post-poral field present. Vas deferens convoluted. External seminal vesicle present or absent. Cirrus armed

with spinitriches. Genital pores lateral, irregularly alternating. Vagina opening anterior or posterior to cirrus sac. Vitellarium follicular; follicles generally in lateral fields. Uterus with or without lateral diverticula. Adults in freshwater fish, amphibians, reptiles and elasmobranchs. Taxa included: All proteocephalidean genera and the tetraphyllidean genera *Acanthobothrium, Acanthobothroides, Onchobothrium*, *Pinguicollum, Platybothrium, Phoreiobothrium*, *Potamotrygonocestus, Prosobothrium*, *Triloculatum, Uncibilocularis*, New genus 8; likely also *Megalonchos*.

4.2.1.2 Remarks: Within the Onchoproteocephalidea, the reciprical monophyly of the Proteocephalidea sensu de Chambrier et al. (2004) and the genera traditionally assigned to the Tetraphyllidea was either not supported (Analysis 2) or only weakly supported (Analysis 4 and 6) in all analyses with relevant representation. The establishment of this new order is thus necessary to preserve ordinal monophyly. However, it is somewhat radical in that it expands the concept of the Proteocephalidea, which heretofore has consisted solely of non-hooked taxa that primarily parasitise bony fishes and some terrestrial tetrapods, such as lizards, snakes and the occasional mammal (de Chambrier et al., 2004), to include a selection of hooked taxa that parasitise stingrays, a few skates and sharks. Nonetheless, this concept has gained substantial momentum over the past decade with *Acanthobothrium* and/or *Phoreiobothrium* being found to group with the proteocephalideans on the basis of molecular data (e.g., Olson et al., 2001; Caira et al., 2005; Waeschenbach et al., 2007; Healy et al., 2009; Waeschenbach et al., 2012). Although a morphological synapomorphy has not previously been identified to support this new order, the overall morphological resemblances between certain proteocephalideans and tetraphyllideans has been noted previously by several authors (e.g., de Chambrier et al., 1999; Scholz et al., 2013).

Our results provide definitive evidence of the integrated nature of the affinities between some members of the hook-bearing tetraphyllidean family Onchobothriidae and the order Proteocephalidea. This result is consistent with all previous molecular analyses that have included relevant representation of both groups (e.g., Olson & Caira, 1999; Olson et al., 2001; Kodedová et al., 2000; Caira et al., 2005; Waeschenbach et al., 2007; Healy et al., 2009; Waeschenbach et al., 2012). The lack of representation across the full spectrum of onchobothriids in these previous works, in part, impeded the taxonomic action required to formally recognize these affinities so as to maintain monophyly at the ordinal level. Our analyses included 13 of the 18 described and one undescribed genus of onchobothriids, and thus revision of the classification in an informed manner is now possible. The name Onchoproteocephalidea n. ord. is proposed to reflect the hybrid nature of the order.

It is much easier to identify morphological differences, rather than similarities, between genera previously assigned to the Proteocephalidea and the subset of onchobothriid genera proposed here to belong to this new order. Indeed, despite the mounting molecular evidence, formal recognition of this as a cohesive group has also been hampered by the lack of one or more morphological features uniting its members. In searching for diagnostic features we observed that among onchobothriids, genera that exhibit gladiate spinitriches throughout the length of their strobila (i.e., Phoreiobothrium, Platybothrium, Potamotrygonocestus, *Prosobothrium, Triloculatum*, and New genus 8) were included in the molecularly-defined Onchoproteocephalidea. This led us to explore this feature in proteocephalidean genera.

Remarkably, in essentially all cases for which scanning electron microscopical (SEM) data are available for body surfaces posterior to the scolex proper (i.e., proliferation zone, immature and/or mature proglottids), simple gladiate spinitriches are reported. This was true for multiple genera in both the Proteocephalidae (e.g., Scholz et al., 1999; de Chambrier, 2006; de Chambrier and de Chambrier, 2010; Ash et al., 2012; Rambeloson et al., 2012) and the Monticelliidae (e.g., Gil de Pertierra, 2002; Gil de Pertierra, 2005; de Chambrier et al., 2006; Scholz et al., 2008; Arredondo et al., 2013; Gil de Pertierra and de Chambrier, 2013). While promising, this feature requires more detailed study across genera in both families as SEM data are available only for more recently described taxa and a few exceptions may exist (e.g., possibly de Chambrier et al., 2009; Gil de Pertierra, 2009). Also worth noting is the fact that *Acanthobothrium* and *Uncibilocularis* species bear a dense covering of gladiate spinitriches restricted to the cephalic peduncle (sensu Caira et al., 1999), a region of the body immediately posterior to the scolex proper that may be homologous to the neck and/or proliferation zone.

Although not represented here, based on their scolex morphology, possession of gladiate spinitriches posterior to the bothridia, and host associations the onchobothriid genera *Acanthobothroides* Brooks, 1977, *Onchobothrium* de Blainville, 1828, and *Pinguicollum* Riser, 1955 should also be included in this order at this time. Based on these same features, and perhaps additional molecular data, we predict *Megalonchos* may ultimately also be found to belong to this new order. Tranfer of the type genus of Onchobothriidae (i.e., *Onchobothrium*) to the Onchoproteocephalidea makes it available to house at least a subset of genera of the Onchoproteocephalidea. Inclusion of a representative of *Onchobothrium* in future molecular analyses is required to confirm this action. Furthermore, the composition of the family, if retained, remains to be determined as the onchobothriids transferred to the new order do not represent a monophyletic group relative to proteocephalidean taxa.

4.2.2. Phyllobothriidea n. ord.—*Synonyms:* Tetraphyllidea Carus, 1863 *pro parte*

4.2.2.1 Diagnosis: Cestoda: Eucestoda. Small to medium sized tapeworms. Strobila polyzoic, proglottized; spinitriches restricted to bothridial surfaces, often serrate or gongylate. Neck and strobilar surfaces with filitriches arranged in scutes, or leaf-like structures in some. Proglottids hermaphroditic, euapolytic or apolytic, craspedote or acraspedote. One set of reproductive organs per proglottid. Two pairs of lateral osmoregulary canals; ventral canals usually wider than dorsal canals. Scolex with four muscular bothridia. Bothridia with anterior accessory sucker, unarmed, most without facial loculi, some with marginal loculi, extensively folded posteriorly in some, lacking stalks; accessory sucker without lateral muscular projections. Apical organ, metascolex and remi absent. Testes numerous, post-oral field present. Vas deferens convoluted. External seminal vesicle present or absent. Cirrus armed with spinitriches. Genital pores lateral, irregularly alternating. Vagina opening anterior to cirrus sac. Vitellarium follicular; follicles generally in lateral fields, occasionally circumcortical. Uterus without lateral diverticula. Adults primarily in sharks, occasionally in batoids (*Nandocestus* and *Calyptrobothrium*) and ratfish (*Chimaerocestus*). Taxa included: *Calyptrobothrium*, *Chimaerocestos, Marsupiobothrium* Yamaguti, 1952, *Nandocestus, Orectolobicestus*, Orygmatobothrium,

Paraorygmatobothrium, Phyllobothrium, *Ruhnkecestus, Scyphophyllidium, Thysanocephalum*, and New genus 10 (Fig. 1F); likely also *Alexandercestus* Ruhnke and Workman, 2013, *Bibursibothrium* McKenzie and Caira, 1998, *Cardiobothrium* McKenzie and Caira, 1998, *Clistobothrium, Crossobothrium*, *Flexibothrium* McKenzie and Caira, 1998, and *Pelichnibothrium* Monticelli, 1889.

4.2.2.2 Remarks: The second order that has emerged from among the tetraphyllideans, but admittedly less definitively, is one comprised of a subset of the non-hooked tetraphyllidean taxa most of which have at one time been assigned to the family Phyllobothriidae (see Ruhnke, 2010). With respect to morphological attributes supporting this order, all of the above genera exhibit bothridia that bear an anterior accessory sucker; most parasitize carcharhiniform or orectolobiform sharks.

We recognize that the order as circumscribed above was not consistently supported across analyses; monophyly of the representatives of these genera was not recovered in the trees resulting from Analysis 1, 2 and 5, whereas their monophyly was supported in the trees resulting from Analysis 3, 4 and, perhaps most importantly, also in Analysis 6 which included data for both genes for the full compliment of taxa. Nonetheless, we believe that establishment of a distinct order to house these taxa is justified. The name Phyllobothriidea is proposed for this new order given this clade includes *Phyllobothrium*, the type genus of the family Phyllobothriidae and current home to the majority of these genera (see Ruhnke, 2011). In fact, Ruhnke (2011) considered 10 of the above 11 described genera as confirmed or at least provisional members of the family in his monograph revising the Phyllobothriidae. While recognizing its affinities with the phyllobothriids, Ruhnke (2011) considered *Chimaerocestos*, which is unique among eucestodes in parasitizing holocephalans, in the monogeneric Chimaerocestidae. Our results have led us to include it here in the Phyllobothriidae.

With respect to potential other members of the order, *Crossobothrium* and *Clistobothrium* Dailey and Vogelbein, 1990 are candidates. They were treated as confirmed members of the Phyllobothriidae by Ruhnke (2011); both also bear an anterior accessory sucker and parasitize sharks. However, they were either labile in position across analyses and/or their association with the other members of the order was only weakly supported thus we have refrained from formally including them in the order at this time. Although not represented here, three other confirmed or provisional members of the Phyllobothriidae sensu Ruhnke (2011) (i.e., *Bibursibothrium* McKenzie and Caira, 1998, *Cardiobothrium* McKenzie and Caira, 1998, *Flexibothrium* McKenzie and Caira, 1998) and one erected since (i.e., *Alexandercestus* Ruhnke and Workman, 2013) are worth consideration. Although still poorly known, *Pelichnibothrium* may also ultimately be found to belong in this order. These genera all bear anterior accessory suckers and also parasitize sharks.

The presence of an anterior accessory sucker in the rhinebothriidean genera *Anthocephalum* and *Escherbothrium* Berman and Brooks, 1994 is likely a homoplasious occurrence of this feature. Among the other tetraphyllidean genera included in our analyses but not formally assigned to this order, only *Caulobothrium* and *Dinobothrium* appear to exhibit accessory suckers. At this time we have refrained from transferring either for there is little other

evidence to support their inclusion in the Phyllobothriidea. In the former case, its placement relative to the other genera among analyses was particularly unstable (e.g., Fig. 2 vs. Supplementary Fig. S5) and the presence of an anterior accessory sucker on its otherwise rhinebothriidean-like loculated bothridia remains to be confirmed. *Dinobothrium* failed to group with the above genera in any of the analyses and exhibits anterior lappets that support its affiliation with *Ceratobothrium*.

4.2.3. "Tetraphyllidea"—While adoption of the proposed two new orders will do much to reduce the polyphyletic nature of the Tetraphyllidea by providing new or provisional ordinal placements for 31 genera, the relationships and appropriate ordinal homes for the remaining described genera (see Table 1) are unresolved. The remaining genera clearly do not represent a monophyletic assemblage and in most cases their hypothesized affinities differed across analyses and support for their inclusion in any larger clade was generally low. We propose they remain in the order in its revised sense (i.e., "Tetraphyllidea") until such time as additional sampling and data can be generated to explore their phylogenetic affinities more fully. So as to guide future work, issues with each suite of taxa are treated below.

Among the cestodes remaining in the "Tetraphyllidea" are two independent clades of hooked taxa. The most diverse is the clade *Pachybothrium* + *Pedibothrium* + *Balanobothrium* + *Spiniloculus* + *Yorkeria*. These genera are united by their lack of post-poral testes and by their association with orectolobiform sharks. They differ from most other hooked taxa in that their hooks are either bipronged with internal channels that open separately in each prong (rather than via a single pore), or are unipronged. It is puzzling that this clade consistently grouped with subsets of the following taxa: *Ceratobothrium* + *Dinobothrium*, *Caulobothrium*, New gen. 9, and in one instance (Analysis 2) *Carpobothrium*. This fact is difficult to reconcile with morphology (all four of the latter genera lack hooks) or host associations (the first two parasitise lamniform sharks, the second two batoid rays and the latter orectilobiform sharks). Furthermore, the interrelationships between the various unhooked taxa and the hooked clade are unstable across analyses.

The second clade of hooked taxa, consisting of two species of *Calliobothrium* that parasitise triakid sharks, was similarly problematic. This genus consistenly grouped well away from all other hooked genera, a fact that is reconcilable with its possession of unique armature in the form of two pairs of hooks per bothridium, rather than a single pair. However, its hypothesized affinities varied substantially across analyses and in all cases support for these affinities was weak. Although not included in the present study, we anticipate that *Biloculuncus* Nasin, Caira and Euzet, 1997 and *Erudituncus* Healy, Scholz and Caira, 2001 will be found to be closely allied with *Calliobothrium* based on their association with triakid sharks and possession of multiple pairs of hooks. Their inclusion in future analyses may serve to improve resolution of the affinities of what is likely a third lineage of hooked tetraphyllideans.

In terms of reconciling morphology, host associations and stability of their overall relationships, by far the most problematic of the non-hooked genera remaining in the "Tetraphyllidea" (apart from *Megalonchos* which was treated above with the Onchoproteocephalidea) are *Dioecotaenia*, *Duplicibothrium, Rhoptrobothrium,*

Myzocephalus, *Ceratobothrium, Dinobothrium*, and *Anthobothrium*. Among these genera three robust pairs of sister-taxa have emerged: (1) *Duplicibothrium* + *Dioecotaenia*, both of which parasitise cownose rays and bear facial bothridial loculi, was strongly supported in all six analyses. (2) *Rhoptrobothrium* + *Myzocephalus*, both of which parasitise myliobatid stingrays and share their possession of a bipartite scolex bearing an elongate cephalic peduncle equipped with four flap-like remi, was strongly supported in all three analyses that included both genera (i.e., Analyses 2, 4 and 6). Based on morphology and host associations, we predict that *Myzophyllobothrium* Shipley and Hornell, 1906, if valid, will ultimately be found to belong to this clade. (3) *Ceratobothrium* + *Dinobothrium*, both of which infect lamniform sharks and bear lateral muscular lappets on the apical region of each bothridium, was highly supported in all three analyses that included both genera (i.e., Analyses 2, 4 and 6). Based on its morphology and host associations we believe *Dinobothrium* will ultimately also be found to belong in this clade. Nonetheless, these three clades and *Anthobobothrium* were by far the most labile of tetraphyllidean taxa in that their phylogenetic positions differed conspicuously across analyses and they generally grouped with taxa with which they bear no obvious morphological and/or host similarities. Curiously, *Anthobothrium* which is essentially unique among tetraphyllideans in lacking apical bothridial suckers, failed to group consistently with any genus or clade but was most commonly found allied, with only weak support, with the hooked *Megalonchos*.

4.3. Cyclophyllidea and their kin

An intriguing result of our analyses was the fact that the sister taxon to the clade composed of the primarily terrestrial, tetrapod-parasitizing Cyclophyllidea + Mesocestoides + Tetrabothriide + Nippotaeniidea was consistently found to be a member of the "Tetraphyllidea". The tetraphyllideans comprising the sister group to this clade could not, however, be determined unambiguously because the specific tetraphyllidean taxon (or suite of taxa) and the level of support differed across analyses. Candidates are: New genus 9 (Analysis 1 and 5), New genus 9 + *Caulobothrium* (Analysis 2), *Carpobothrium* (Analysis 3 and 6), and *Carpobothrium* + *Caulobothrium* (Analysis 4). This result is generally inconsistent with previous works (e.g., Olson and Caira, 1999; Olson et al., 2001; Hoberg et al., 2001; Waeschenbach et al., 2007; 2012), all of which have found candidate sister taxa to the tetrapod-parasitizing cestodes to include tetraphyllidean genera we have referred to the Onchoproteocephalidea here. We would attribute this to the fact that none of these previous studies included any of the tetraphyllidean candidate sister taxa identified here. Thus, these new results are of wider taxonomic and evolutionary significance.

4.4. Evolution and host associations of major eucestode lineages

Based on the new configuration of orders proposed here, the Eucestoda, or true tapeworms, should be considered to consist of the following 17 orders: Bothriocephalidea, Caryophyllidea, Cathetocephalidea, Cyclophyllidea, Diphyllidea, Diphyllobothriidea, Haplobothriidea, Lecanicephalidea, Litobothriidea, Nippotaeniidea, Onchoproteocephalidea n. ord., Phyllobothriidea n. ord., Rhinebothriidea, Spathebothriidea, Tetrabothriidea, Trypanorhyncha, and "Tetraphyllidea". This brings the total number of orders in the class Cestoda to 19 (i.e., including the Gyrocotylidea and Amphilinidea). It is interesting that nine of these orders (i.e., ~50%) are associated with elasmobranchs. But, truly remarkable is the

key role that elasmobranch-hosted taxa appear to have played in the evolution of cestodes overall. Mapping of major vertebrate groups on the schematic diagram of the tree from Analysis 6 shown in Fig. 3 emphasizes elasmobanch-hosted cestodes comprise the backbone of the cestode phylogeny overall. Our results suggest that both of the non-elasmobranch hosted clades of acetabulate cestodes had their origins in elasmobranch-hosted taxa. Depending on resolution of the relationships among bothriate taxa, this is possibly also true for the Bothriocephalidea relative to the Diphyllidea and Trypanorhyncha. Futhermore, several additional elasmobranch-hosted cestode lineages will likely need to be recognized among the “Tetraphyllidea” once these relationships are more fully understood.

With respect to the taxa previously assigned to the Proteocephalidea, the majority of which parasitise freshwater teleosts (see de Chambrier et al., 2004), their candidate elasmobranch-hosted sister taxa are illuminating for all have some affinity with freshwater habitats. *Potamotrygonocestus* parasitises freshwater stingrays of the family Potamotrygonidae Garman, 1877; New genus 8 parasitises sawfish of the genus *Pristis* Linck, 1790 and *Uncibilocularis* parasitises stingrays of the genus *Pastinachus* Rüppell, 1829, both of which frequent coastal euryhaline habitats and in some instances even freshwaters (Martin, 2005). This suggests that teleost-parasitizing cestodes may have had their origin with freshwater, or at least euryhaline, elasmobranchs. The final determination awaits definitive resolution of the sister taxon to the clade.

The fact that marine elasmobranchs may also constitute the sister taxon to what is generally referred to as the “terrestrial” clade (i.e., Cyclophyllidea + Tetrabothriidea + Nippotaeniidea + *Mesocestoides*) clearly invites further investigation. Much denser taxon sampling of the thousands of species and hundreds of genera in this “terrestrial” clade (i.e., beyond the 6-8 species employed here), of the Cyclophyllidea in particular, is required to resolve these interrelationships with confidence. Inclusion of more dense taxon sampling of the three elasmobranch-hosted candidate sister genera (i.e., *Carpobothrium*, New genus 9, and *Caulobothrium*) would also be informative. These three genera offer remarkably different evolutionary scenarios with respect to the potential origins of the “terrestrial” clade. *Carpobothrium* parasitises bamboosharks of the family Hemiscylliidae Gill, 1892 (order Orectolobiformes) and is currently restricted to Indo-Pacific waters; *Caulobothrium* parasitises stingrays of the family Dasyatidae Jordan, 1888 (order Myliobatiformes) and occurs in essentially a circumtropical band; New genus 9 parasitises stingrays of the family Dasyatidae and guitarfish of the family Rhinobatidae Müller and Henle, 1837 (Rhinopristiformes) and also is restricted to Indo-Pacific waters.

Another surprising result of this work was the relatively derived phylogenetic position occupied by the two species of *Chimaerocestos*. Given their association with holocephalans, rather than elasmobranchs, we had anticipated they would occupy a much earlier divergent position on the tree relative to species parasitizing elasmobranchs. Instead, both species consistently and robustly grouped among the members of the new order Phyllobothriidea. This suggests that the association of this genus with holocephalans likely represents a host-switching event from elasmobranchs (most likely sharks) to holocephalans, rather than vice versa.

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4.5. Morphological evolution

The spectacular diversity of scolex forms seen among the genera that have been assigned to the Tetraphyllidea over time (e.g., see Euzet, 1994; Caira et al., 2001) is essentially without parallel in any other cestode order. Scolex elaborations seen in these elasmobranch-hosted cestodes include, for example, hooks, suckers, bothridia, pedicels, facial loculi, marginal loculi both with or without external septa, anterior pads, fusion of portions resulting in pouch-like structures, circular muscle bands, extensions on lateral margins of bothridia, stalked remi with primary and secondary areolae, etc. Several preliminary attempts to place this diversity of forms into a phylogenetic context have been made. For example, Caira et al. (1999) circumscribed 120 morphological characters, nearly 80 of which involved scolex features; Caira et al. (2001) expanded this list to 157 characters, over 100 of which treated scolex features. Unfortunately, in both cases the phylogenetic trees resulting from analyses of these data were relatively unresolved, and the phylogenetic relationships implied were relatively incongruent. However, in both studies the non-monophyly of the Tetraphyllidea relative to other cestode orders was apparent. Given the results presented here, it now seems clear that much of the remarkable morphological diversity is a result of multiple radiations that represent independent lineages. Our proposal of two new orders resolves issues with a subset of the genera. However, the phylogenetic relationships and thus proper ordinal placement of many genera remain to be determined. It is intriguing that in most cases these genera are among the most morphologically enigmatic tetraphyllidean taxa. Unlike all other elasmobranch-hosted cestodes, *Dioecotaenia* bears proglottids that are dioecious rather than monoecious. Whether this represents sequential or simultaneous dioecy across the strobila remains to be determined. Similarly, unlike essentially all other “tetraphyllidean” taxa, *Anthobothrium* bears bothridia that lack, rather than possess, an apical orientiation and/or apical modificiation of the bothridia. *Rhoptrobothrium* and *Myzocephalus* are unique in their possession of a scolex with four stalked remi extending from their cephalic peduncle, each of which bears primary and secondary areoli.

Even further dismantling of the “Tetraphyllidea” is inevitable and likely desirable, perhaps even to its final destruction. Our results suggest this may ultimately result in recognition of even a greater number of elasmobranch-hosted orders but if monophyly of cestode orders is to be maintained will likely be necessary. The application of data from other molecular markers to further explore cestode interrelationsips is now indicated. Only in this way can we achieve taxonomic clarity and reveal further the complex evolutionary histories of cestodes and their elasmobranch hosts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Arredondo NJ, de Chambrier A, Gil de Pertierra AA. A new genus and species of Monticelliinae (Eucestoda: Proteocephalidea), a parasite of *Pseudoplatystoma fasciatum* (Pisces: Siluriformes) from the Paraná River basin (Argentina), with comments on microtriches of proteocephalideans. Folia Parasitologica. 2013; 60:248–256. [PubMed: 23951932]

Ash A, Scholz T, de Chambrier A, Brabec J, Oros M, Kar PK, Chavan SP, Mariaux J. Revision of *Gangesia* (Cestoda: Proteocephalidea) in the Indomalayan region: morphology, molecules and surface ultrastructure. PLoS ONE. 2012; 7:e46421. [PubMed: 23056306]

Brooks DR, Barriga R. *Serendip deborahae* n. gen. and n. sp. (Eucestoda: Tetraphyllidea: Serendipidae n. fam.) in *Rhinoptera steindachneri* Evermann and Jenkins, 1891 (Chondrichthyes: Myliobatiformes: Myliobatidae. Journal of Parasitology. 2005; 81:80–84. [PubMed: 7876984]

Brooks DR, Hoberg EP, Weekes PJ. Preliminary phylogenetic systematic analysis of the major lineages of the Eucestoda (Platyhelminthes: Cercomeria). Proceedings of the Biological Society of Washington. 1991; 104:651–668.

Caira JN, Jensen K, Healy CJ. On the phylogenetic relationships among tetraphyllidean, lecanicephalidean and diphyllidean tapeworm genera. Systematic Parasitology. 1999; 42:77–151. [PubMed: 10612438]

Caira, JN.; Jensen, K.; Healy, CJ. Interrelationships among tetraphyllidean and lecanicephalidean cestodes. In: Littlewood, DTJ.; Bray, RA., editors. Interrelationships of the Platyhelminthes. Taylor & Francis; London: 2001. p. 135-158.

Caira JN, Mega J, Ruhnke TR. An unusual blood sequestering tapeworm (*Sanguilevator yearsleyi* n. gen., n. sp.) from Borneo with description of *Cathetocephalus resendezi* n. sp. from Mexico and molecular support for the recognition of the order Cathetocephalidea (Platyhelminthes: Eucestoda). International Journal for Parasitology. 2005; 35:1135–1152. [PubMed: 16019004]

Dailey MD. *Litobothrium alopias* and *L. coniformis*, two new cestodes representing a new order from elasmobranch fishes. Proceedings of the Helminthological Society of Washington. 1969; 36:218–224.

de Chambrier A. Redescription of *Kapsulotaenia sandgroundi* (Carter, 1943) (Eucestoda: Proteocephalidea: Acanthotaeniinae), a parasite of *Varanus komodoensis* (Veranoidea: Varanidae) from Indonesia. Sytematic Parasitology. 2006; 63:83–93.

de Chambrier A, Coquille SC, Mariaux J, Tkach V. Redescription of *Testudotaenia testudo* (Magath, 1924) (Eucestoda: Proteocephalidea), a parasite of *Apalone spinifera* (Le Sueur) (Reptilia: Trionychidae) and *Amia calva* L. (Pisces: Amiidae) in North America and erection of the Testudotaeniinae n. subfam. Systematic Parasitology. 2009; 73:49–64. [PubMed: 19337859]

de Chambrier A, Takemoto RM, Pavanelli GC. *Nomimoscolex pertierrae* n. sp. (Eucestoda: Proteocephalidea), a parasite of *Pseudoplatystoma corruscans* (Siluriformes: Pimelodidae) in Brazil and redescription of *N. sudobim* Woodland, 1935, a parasite of *P. fasciatum*. Systematic Parasitology. 2006; 64:191–202. [PubMed: 16786284]

de Chambrier A, Zehnder M, Vaucher C, Mariaux J. The evolution of the Proteocephalidea (Platyhelminthes, Eucestoda) based on an enlarged molecular phylogeny, with comments on their uterine development. Systematic Parasitology. 2004; 57:159–171. [PubMed: 15010590]

de Chambrier S, de Chambrier A. Two new genera and two new species of proteocephalidean tapeworms (Eucestoda) from reptiles and amphibians in Australia. Folia Parasitologica. 2010; 57:263–279. [PubMed: 21344839]

Euzet, L. Order Tetraphyllidea Carus, 1863. In: Khalil, LF.; Jones, A.; Bray, RA., editors. Keys to the cestode parasites of vertebrates. CAB International; Wallingford: 1994. p. 149-194.

Euzet L, Swiderski Z, Mokhtar-Maamouri F. Ultrastructure comparée du spermatozoide des cestodes. Relations avec la phylogenèse. Annales de Parasitologie Humaine et Comparée. 1981; 56:247–259.

Europe PMC Funders Author Manuscripts

Gil de Pertierra AA. *Nomimoscolex semanasae* n. sp. (Proteocephalidea: Monticelliidae), a cestode parasite of *Diplomystes viedmensis* (Pisces: Siluriformes) from the Patagonian region of Argentina. Systematic Parasitology. 2002; 53:183–190. [PubMed: 12510163]

Gil de Pertierra AA. Comparative study of microtriches of adult Cestodes (Proteocephalidea: Monticelliidae), and some comments on their systematic value. Zoologischer Anzeiger. 2005; 243:295–304.

Gil de Pertierra AA. *Luciaella ivanovae* n. g., n. sp. (Eucestoda: Proteocephalidea: Peltidocotylinae), a parasite of *Ageneiosus inermis* (L.) (Siluriformes: Auchenipteridae) in Argentina. Systematic Parasitology. 2009; 73:71–80. [PubMed: 19337861]

Gil de Pertierra AA, de Chambrier A. *Harriscolex nathaliae* n. sp. (Cestoda: Proteocephalidea) from *Pseudoplatystoma corruscans* (Siluriformes: Pimelodidae) in the Paraná River Basing, Argentina. Journal of Parasitology. 2013; 99:480–486. [PubMed: 23146094]

Healy CJ, Caira JN, Jensen K, Webster BL, Littlewood DTJ. Proposal for a new tapeworm order, Rhinebothriidea. International Journal for Parasitology. 2009; 39:497–511. [PubMed: 18929566]

Hoberg EP, Mariaux J, Brooks DR. Phylogeny among the orders of Eucestoda (Cercomeromorphae): Integrating morphology, molecules and total evidence. Systematics Association Special Volume Series. 2001; 60:112–126.

Hoberg EP, Mariaux J, Justine JL, Brooks DR, Weekes PJ. Phylogeny of the orders of the Eucestoda (Cercomeromorphae) based on comparative morphology: historical perspectives and a new working hypothesis. Journal of Parasitology. 1997; 83:1128–1147. [PubMed: 9406791]

Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics. 2001; 17:754–755. [PubMed: 11524383]

Khalil, LF.; Jones, A.; Bray, RA. Keys to the Cestode Parasites of Vertebrates. CAB International; Wallingford, U.K.: 1994.

Kodedova I, Dolezel D, Brouckova M, Jirku M, Hypsa V, Lukes J, Scholz T. On the phylogenetic positions of the Caryophyllidea, Pseudophyllidea and Proteocephalidea (Eucestoda) inferred from 18S rRNA. International Journal for Parasitology. 2000; 30:1109–1113. [PubMed: 10996329]

Maddison, DR.; Maddision, WP. MacClade 4: Analysis of phylogeny and character evolution. Version 4.08. Sinauer Associates, Inc.; Sunderland, Massachusetts, USA: 2005.

Mariaux J. A molecular phylogeny of the Eucestoda. Journal of Parasitology. 1998; 84:114–124. [PubMed: 9488348]

Martin RA. Conservation of freshwater and euryhaline elasmobranchs: a review. Journal of the Marine Biological Association of the United Kingdom. 2005; 85:1049–1073.

Naylor GJP, Caira JN, Jensen K, Rosana KAM, White WT, Last PR. A DNA sequence-based approach to the identification of shark and ray species and its implications for global elasmobranch diversity and parasitology. Bulletin of the American Museum of Natural History. 2012; 367:1–262.

Olson PD, Caira JN. Evolution of the major lineages of tapeworms (Platyhelminthes: Cestoidea) inferred from 18S ribosomal DNA and elongation factor-1α. Journal of Parasitology. 1999; 85:1134–1159. [PubMed: 10647048]

Olson PD, Caira JN. Two new species of *Litobothrium* Dailey, 1969 (Cestoda: Litobothriidea) from thresher sharks in the Gulf of California, Mexico, with redescriptions of two species in the genus. Systematic Parasitology. 2001; 48:159–177. [PubMed: 11302614]

Olson PD, Littlewood DTJ, Bray RA, Mariaux J. Interrelationships and evolution of the tapeworms (Platyhelminthes: Cestoda). Molecular Phylogenetics and Evolution. 2001; 19:443–467. [PubMed: 11399152]

Pickering M, Caira JN. *Calliobothrium schneiderae* n. sp. (Cestoda: Tetraphyllidea) from the spotted estuary smooth-hound shark, *Mustelus lenticulatus*, from New Zealand. Comparative Parasitology. 2008; 75:174–181.

Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. Bioinformatics. 1998; 14:817–818. [PubMed: 9918953]

Rambeloson VR, Ranaivoson HC, de Chambrier A. *Ophiotaenia lapata* sp n. (Eucestoda: Proteocephalidea) from Madagascar: a parasite of the endemic snake *Madagascarophis colubrinus* (Colubridae). Revue Suisse de Zoologie. 2012; 119:547–559.

Ruhnke TR. Tapeworms of elasmobranchs (Part III). A monograph on the Phyllobothriidae (Platyhelminthes, Cestoda). Bulletin of the University of Nebraska State Museum. 2011; 25:i–xii. 1–208.

Schmidt, GD. CRC Handbook of tapeworm identification. CRC Press Inc.; Florida: 1986.

Schmidt GD, Beveridge I. *Cathetocephalus australis* n. sp. (Cestoidea: Cathetocephalidae) from Australia, with a proposal for Cathetocephalidea n. ord. Journal of Parasitology. 1990; 76:337–339.

Scholz T, de Chambrier A, Kuchta R. Redescription of the tapeworm *Monticellia amazonica* de Chambrier et Vaucher, 1997 (Cestoda, Proteocephalidea), a parasite of *Calophysus macropterus* (Siluriformes, Pimelodidae) from the Amazon River. Acta Parasitologica. 2008; 53:30–35.

Scholz T, de Chambrier A, Kuchta R, Littlewood DTJ, Waeschenbach A. *Macrobothriotaenia ficta* (Cestoda: Proteocephalidea), a parasite of sunbeam snake (*Xenopeltis unicolor*): example of convergent evolution. Zootaxa. 2013; 3640:485–499. [PubMed: 26000432]

Scholz T, Žd'árská Z, de Chambrier A, Drabek R. Scolex morphology of the cestode *Silurotaenia siluri* (Batsch, 1786) (Proteocephalidae: Gangesiinae), a parasite of European wels (*Silurus glanis*). Parasitology Research. 1999; 85:1–6. [PubMed: 9950220]

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research. 1997; 25:4876–4882. [PubMed: 9396791]

Waeschenbach A, Webster BL, Bray RA, Littlewood DTJ. Added resolution among ordinal level relationships of tapeworms (Platyhelminthes: Cestoda) with complete small and large subunit nuclear ribosomal RNA genes. Molecular Phylogenetics and Evolution. 2007; 45:311–325. [PubMed: 17485227]

Waeschenbach A, Webster BL, Littlewood DTJ. Adding resolution to ordinal level relationships of tapeworms (Platyhelminthes: Cestoda) with large fragments of mtDNA. Molecular Phylogenetics and Evolution. 2012; 63:834–847. [PubMed: 22406529]

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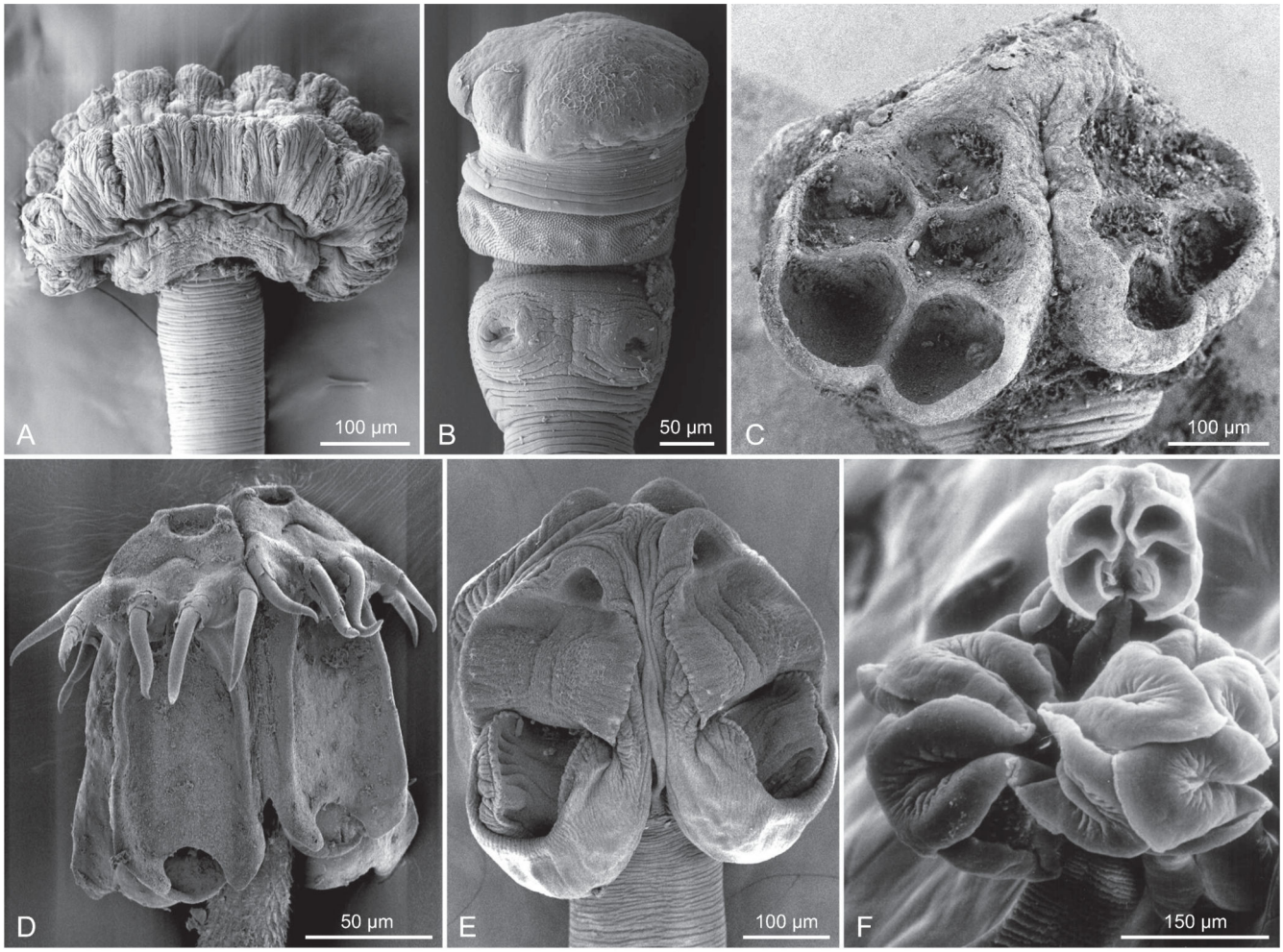

Fig. 1.

Scanning electron micrographs of scoleces of undescribed genera included in molecular analyses. (A) New genus 5. (B) New genus 6. (C) New genus 7. (D) New genus 8. (E) New Genus 9. (F) New genus 10.

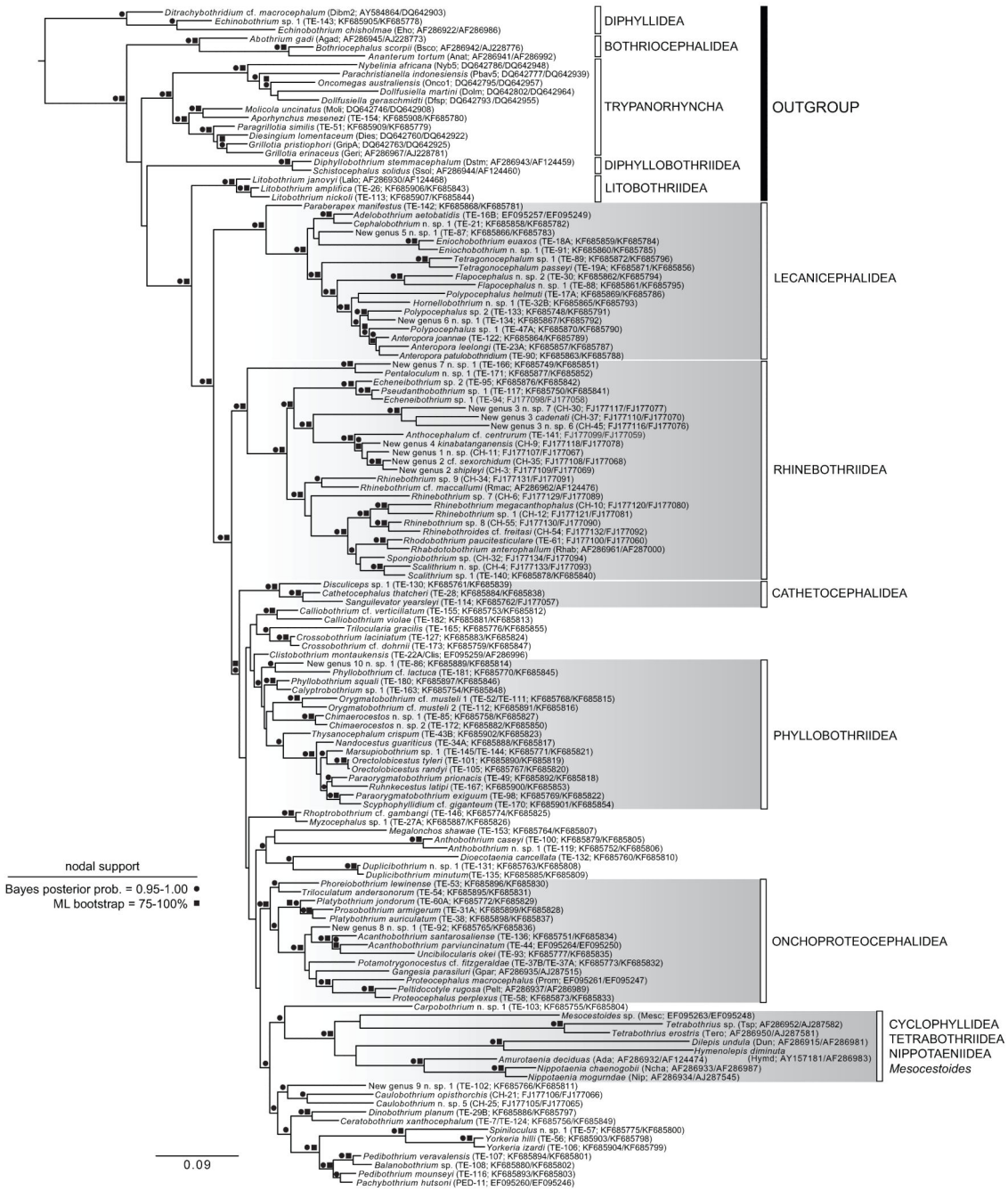

Fig. 2.

Phylogenetic tree: Analysis 6. Bayesian analysis of the concatenated partial *lsrDNA* (D1-D3) + complete *ssrDNA* data of 134 taxa (*Dataset_1*). Constructed using MrBayes under the GTR+I+G model. Analysis was run for 10,000,000 generations, with 4,000,000 generations discarded as burn-in. Branch length scale bar indicates number of substitutions per site.

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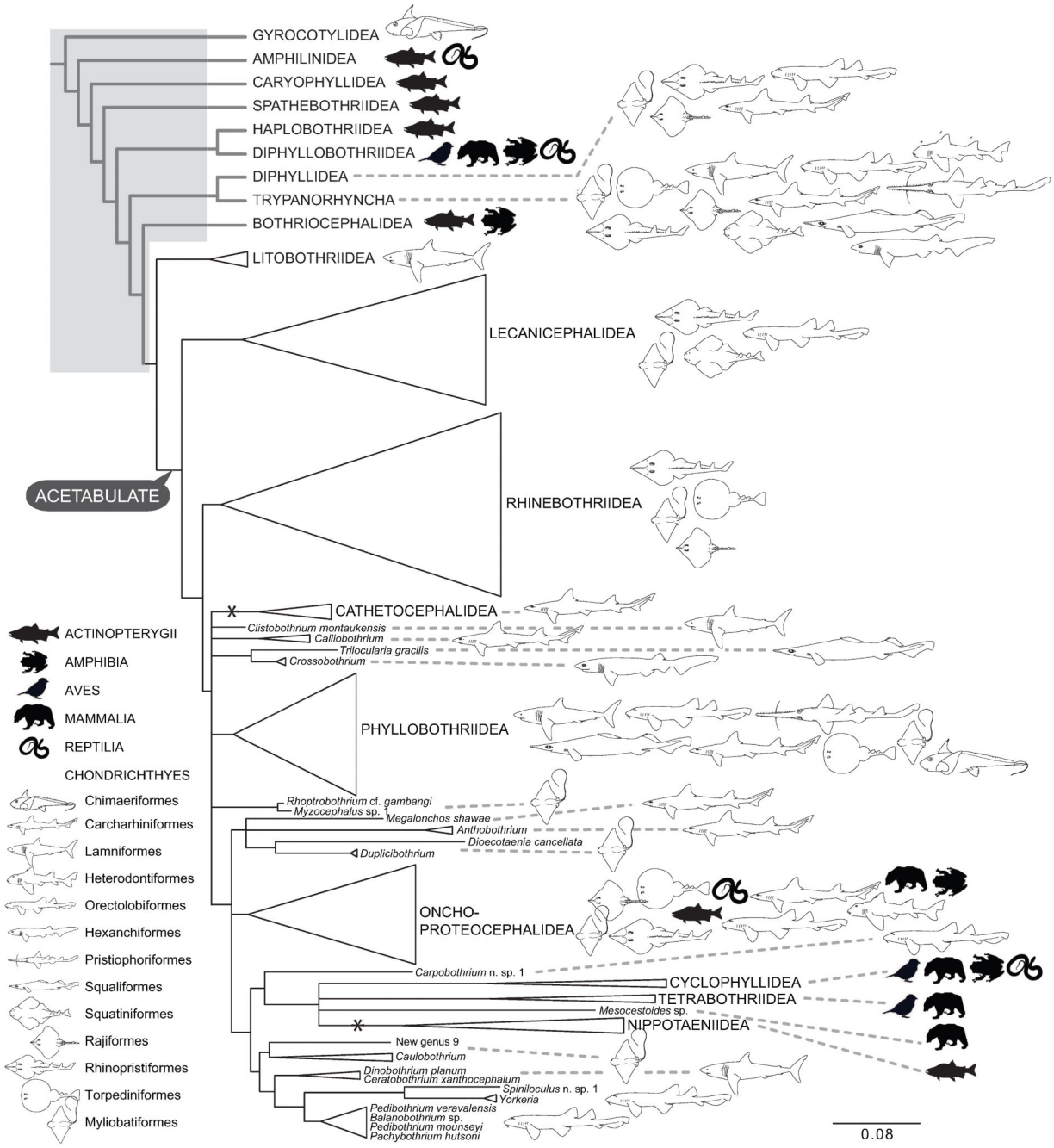

Fig. 3.

Schematic diagram of phylogenetic tree from Analysis 6 (Fig. 2) expanded to include additional outgroups based on Waeschenbach et al. (2012). Major vertebrate host groups are indicated. Nodes which were supported by <0.95 Bayesian posterior probability and/or <75% Maximum Likelihood bootstrap support were collapsed. Asterisks indicate loss of acetabulae. Branch length scale bar indicates number of substitutions per site.

Table 1

List of ingroup (IG) and ougroup (OG) taxa included in the analyses with their ordinal placement, sequences generated as part of this study.

Ingroup (IG)/ Outgroup (OG)	Taxa in analyses 1, 3, & 5	Taxa in analyses 2, 4, & 6	Taxon	Specimen ID	Current Order	Revised Order	Voucher Acc. No.	GenBank Acc. No. (*IsrDNA*)	GenBank Acc. No. (*IsrDNA* [D1-D3])
IG	**yes**	**yes**	*Disculiceps* sp. 1	TE-130	Tet	Cat	LRP 8328	**KF685761**	
IG		**yes**	*Cathetocephalus thatcheri*	TE-28	Cat	Cat	LRP 8281		**KF685884**
IG	**yes**	**yes**	*Sanguilevator yearsleyi*	TE-114	Cat	Cat	LRP 4218	**KF685762**	
IG	**yes**	**yes**	*Dilepis undula*	Dun	Cyc	Cyc		AF286915	
IG	**yes**	**yes**	*Hymenolepis diminuta*	Hymd	Cyc	Cyc		AY157181	
IG	**yes**	**yes**	*Mesocestoides* sp.	Mesc	Cyc	Cyc		EF095263	
IG	**yes**	**yes**	*Adelobothrium aetobatidis*	TE-16B	Lec	Lec	LRP 8272	EF095257	
IG		**yes**	*Anteropora joannae*	TE-122	Lec	Lec	LRP 8326		**KF685864**
IG		**yes**	*Anteropora leelongi*	TE-23A	Lec	Lec	LRP 8278		**KF685857**
IG		**yes**	*Anteropora patulobothridium*	TE-90	Lec	Lec	LRP 8307		**KF685863**
IG		**yes**	*Cephalobothrium* n. sp. 1	TE-21	Lec	Lec	LRP 8275		**KF685858**
IG		**yes**	*Eniochobothrium euaxos*	TE-18A	Lec	Lec	LRP 8274		**KF685859**
IG		**yes**	*Eniochobothrium* n. sp. 1	TE-91	Lec	Lec	LRP 8308		**KF685860**
IG		**yes**	*Flapocephalus* n. sp. 1	TE-88	Lec	Lec	LRP 8302		**KF685861**
IG		**yes**	*Flapocephalus* n. sp. 2	TE-30	Lec	Lec	LRP 8283		**KF685862**
IG		**yes**	*Hornellobothrium* n. sp. 1	TE-32B	Lec	Lec	LRP 8285		**KF685865**
IG		**yes**	New genus 5 n. sp. 1	TE-87	Lec	Lec	LRP 8305		**KF685866**
IG		**yes**	New genus 6 n. sp. 1	TE-134	Lec	Lec	LRP 8331		**KF685867**
IG		**yes**	*Paraberapex manifestus*	TE-142	Lec	Lec			**KF685868**
IG		**yes**	*Polypocephalus helmuti*	TE-17A	Lec	Lec	LRP 8273		**KF685869**
IG		**yes**	*Polypocephalus* sp. 1	TE-47A	Lec	Lec	LRP 8292		**KF685870**
IG	**yes**	**yes**	*Polypocephalus* sp. 2	TE-133	Lec	Lec	LRP 8330	**KF685748**	
IG		**yes**	*Tetragonocephalum passeyi*	TE-19A	Lec	Lec	LRP 7276		**KF685871**
IG		**yes**	*Tetragonocephalum* sp. 1	TE-89	Lec	Lec	LRP 8306		**KF685872**
IG		**yes**	*Amurotaenia deciduas*	Ada	Nip	Nip			AF286932
IG		**yes**	*Nippotaenia chaenogobii*	Ncha	Nip	Nip	BMNH 2000.3.7.11	-12	AF286933