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Evidence for host-specific clades of tetraphyllidean tapeworms (Platyhelminthes: Eucestoda) revealed by analysis of 18S ssrDNA☆

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Abstract

Sequence data from the V4 and V7–V9 variable regions of the 18S small subunit ribosomal DNA (ssrDNA) gene were used to examine relationships among 26 tetraphyllidean and two lecanicephalidean taxa. Newly collected specimens of 21 of the tetraphyllidean species were used to generate ssrDNA sequences that were combined with sequences previously available, including those of two diphyllidean taxa used for outgroup rooting. The sequences were aligned by eye according to secondary structural motifs of the conserved core of the molecule. Of the 1520 sites in the alignment, 874 (58%) were excluded from analysis due to alignment gaps and lack of positional homology as inferred by manual inspection. Genetic variability of the ssrDNA gene regions compared was greater than would be expected, based on the present taxonomy of the ingroup species, and the genetic divergences among tetraphyllidean 'families' and genera were comparable to that among tapeworm orders. Phylogenetic hypotheses were generated by the methods of maximum parsimony and maximum likelihood (GTR + I + Γ nucleotide substitution model). Four most parsimonious trees resulted from analysis by maximum parsimony. Strict consensus of the four trees supported the monophyly of the Tetraphyllidea, with the lecanicephalidean taxa forming a sister lineage. Among the tetraphyllidean taxa included in the analysis were three major clades: a basal clade including species of the phyllobothriid genera *Anthocephalum*, *Echeneibothrium*, *Rhinebothrium*, *Rhodobothrium* and *Spongiobothrium*; a clade uniting the phyllobothriids of the genus *Duplicibothrium* with the dioecotaeniid genus *Dioecotaenia*; and a larger sister clade to the *Duplicibothrium* + *Dioecotaenia* clade that included the phyllobothriid genera *Caulobothrium*, *Ceratobothrium*, *Clistobothrium*, *Paraorygmatobothrium* and *Prosobothrium*, the litobothriid genus *Litobothrium* and the onchobothriid genera *Acanthobothrium*, *Calliobothrium*, *Phoreiobothrium* and *Platybothrium*. Maximum likelihood analysis resulted in a topology that was congruent where nodes were strongly supported by parsimony analysis, but differed in the relative positions of the well-supported clades. In addition,

*Note: Newly generated nucleotide sequence data reported in this paper are available in the EMBL, GenBank® and DDJB databases under the accession numbers AF126064–AF126098. The full alignment has been deposited with the EMBL (accession No. DS37932) and can be accessed by anonymous FTP from ftp.ebi.ac.uk in directory /pub/databases/embl/align.

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maximum likelihood analysis grouped the lecanicephalidean taxa among the tetraphyllidean taxa, indicating paraphyly of the order Tetraphyllidea as currently defined. Relationships suggested by both methods of analysis reflected common host associations of the taxa better than their current classification, suggesting that coevolution has had a significant role in the evolution of the group.

Keywords: Eucestoda; Host–parasite cospeciation; Lecanicephalidea; Litobothriidae; Onchobothriidae; Phyllobothriidae; Platyhelminthes; ssrDNA; Tetraphyllidea

1. Introduction

The phylogenetic relationships among tetraphyllidean tapeworms are poorly understood, and this fact has resulted in considerable instability in their classification. Schmidt [1] recognised the family Onchobothriidae Braun, 1900, whose members possess hooks on their bothridia, as well as two 'non-hooked' families: Phyllobothriidae Braun, 1900 and Triloculariidae Yamaguti, 1959. In addition, he accorded ordinal status to the dioecious 'tetraphyllidean' genus *Dioecotaenia* Schmidt, 1969. More recently, Euzet [2] divided the Tetraphyllidea into eight families: Cathetocephalidae Dailey and Overstreet, 1973; Chimaerocestidae Williams and Bray, 1984; Dioecotaeniidae Schmidt, 1969; Disculiceptidae Joyeux and Baer, 1936; Litobothriidae Dailey, 1969; Onchobothriidae; Phyllobothriidae; and Prosobothriidae, Baer and Euzet, 1955. With the exception of the families Onchobothriidae and Phyllobothriidae, the remaining families recognised by Euzet [2] are species-poor, and are diagnosed by peculiar morphological features. For example, species of the Cathetocephalidae exhibit one to 24 strobilae per scolex [3]. Of the larger two tetraphyllidean families, the presence of hooks provides evidence for the monophyly of the Onchobothriidae, while no character unites all species of the Phyllobothriidae, and this family, as presently defined [2], is likely to be non-natural (see [4]).

The few phylogenetic studies of tetraphyllideans that have been published differ substantially in completeness. Brooks and McLennan [5] provided a phylogenetic summary of tetraphyllideans at the 'generic' level, in addition to species-level phylogenies of the genera *Rhinebothrium* Linton, 1890 and *Rhinebothroides* Mayes, Brooks and Thorson, 1981. Their trees were based on a small suite of morphological characters and the methodological approach was not explicitly cladistic. Caira et al. [4] recently completed a more comprehensive cladistic analysis of selected species representing the majority of known diphyllidean, lecanicephalidean and tetraphyllidean genera. In total, their study included 56 ingroup and seven outgroup species, and was based on 120 morphological characters. Results of their analysis supported the monophyly of the Onchobothriidae. The Phyllobothriidae, however, was found to be paraphyletic and consisted of two main lineages (in addition to a large polytomy of unresolved taxa), one of which formed a trichotomy with the lecanicephalidean and onchobothriid clades. Although many relationships remained unresolved, their work represents a rigorous initial hypothesis of the phylogenetic relationships of tetraphyllidean genera, and provides a comprehensive suite of morphological characters that may be later compared and combined with other classes of data for 'total evidence' analysis.

Molecular systematic studies of cestodes are currently few in number. Olson and Caira [6] and Mariaux [7] have addressed relationships at the ordinal level. However, most of the lower-level taxonomic studies have focused on species of the order Cyclophyllidea; typically being comparisons between species within a genus of medical or economic importance (e.g. [8, 9]). The primary purpose of the present study was to evaluate the phylogenetic utility of the two most variable regions of the 18S small subunit ribosomal DNA gene (ssrDNA), the V4 and V7, for resolving relationships among members of the order

Tetraphyllidea. Although both the analyses by Olson and Caira [6] as well as Mariaux [7] included at least some representatives of the order Tetraphyllidea, the current investigation represents the first molecular systematic study to focus specifically on this group of elasmobranch parasites. The phylogenetic information generated by this study is useful for examining the naturalness of the present familial-level classification of the order [2]. In addition, because of the strict host-specificity common among many tetraphyllidean species [10, 11], examination of their phylogenetic relationships has the potential to uncover interesting patterns of host–parasite associations. Revealing such patterns is the first step toward a broader understanding of the evolutionary history between the parasites and their hosts, and can be used as a guide for future investigations.

2. Materials and methods

2.1. Collection of specimens

Fresh specimens of 21 previously collected tetraphyllidean tapeworm species were used to generate new ssrDNA sequences. Table 1 shows a taxonomic listing of these species, their hosts and collection localities, as well as the other species included in the analyses for which ssrDNA sequences were available previously. In addition to the tetraphyllidean taxa, two lecanicephalidean species were included in the analyses to examine the position of the Lecanicephalidea relative to the Tetraphyllidea. All specimens were fixed and stored in 95% EtOH prior to DNA extraction. Some of the species analysed in the present study were determined to be new to science, and taxonomic descriptions are currently in preparation (T.R. Ruhnke). Voucher specimens of the new material have been deposited at the Connecticut State Museum of Natural History.

2.2. DNA isolation, PCR amplification and gene sequencing

Genomic DNA of whole worms was extracted following the method of either Coen et al. [12] or Gustincich et al. [13]. Prior to extraction, all specimens were rinsed thoroughly in 95% EtOH and lyophilised to facilitate grinding of the tissue. The entire ssrDNA gene was amplified by PCR in two overlapping fragments: a 1100 bp fragment using primers 18S-E (5′-CCGAATTCGACAACCTGGTTGATCCTGCCAGT-3′) and 18S-A27′ (5′-CCATACAAACGTCCCCGCCTG-3′), and a 1500 bp fragment using primers 18S-8 (5′-GCAGCCGCGGTAATTCCAGC-3′) and 18S-Cestode-6 (5′-ACGGAAACCTTGTTACGACT-3′). Primers A27′ and Cestode-6 were designed previously to better match the tapeworm ssrDNA gene [6]. Robust, high-fidelity double-stranded amplifications were obtained with a Perkin-Elmer 2400 thermocycler using 2.5 mM MgCl buffer [14] and the following thermocycling profile: 3 min denaturation hold at 97°C; 36 cycles of 1 min at 96°C, 1 min at 54°C, 1 min at 72°C; and 7 min extension hold at 72°C.

Unincorporated PCR primers and nucleotides were removed from the PCR products prior to sequencing by use of Qiagen® QIAQuick™ spin columns. Nucleotide sequences were determined directly from cleaned PCR products by automated sequencing using ABI BigDye™ Terminator Cycle Sequencing Ready Reaction Mix and an ABI PRISM™ 377 automated sequencer. Sequencing reactions were cleaned using Sephadex-filled Centrisep™ spin columns (Princeton Separations). Sequences were determined for two non-contiguous regions of the ssrDNA gene: from stems 20–22 (encompassing the V4 region) and stems 40–47 (encompassing the V7–V9 regions). Sequencing primers used were 18S-8, 18S-A-27′, 18S-11F (5′-GGGTGGTGGTGCATGGCCGTT-3′) and 18S-Cestode-6. The sequences were determined for the majority of sites from both the sense and anti-sense strands.

2.3. Selection of outgroup taxa and sequence alignment

Two diphyllidean (Platyhelminthes: Eucestoda) taxa were selected as outgroups, based on the results of Olson and Caira [6] which showed

Table 1
List of taxa analysed, their hosts and collection localities. Classification follows that of Euzet [2]

Classification Species [GenBank No.]	Host (common name)	Collection locality
	OUTGROUP	
Or. DIPHYLLIDEA		
Echinobothrium fautleyae [AF124464]	*Rhinoptera steindachneri* (Pacific cownose ray)	Gulf of California, Santa Rosalia, Mexico
Macrobothridium sp. [AF124463]	*Rhinobatos typus* (Giant shovelnose ray)	Timor Sea, Shoal Bay, Darwin, NT Australia
	INGROUP	
Or. LECANICEPHALIDEA		
Cephalobothrium cf. aetobatidis [AF124464]	*Aetobatus narinari* (Spotted eagle ray)	Gulf of Thailand, Bungsaray, Thailand
Eniochobothrium gracile [AF124465]	*Rhinoptera* sp. (Cownose ray)	Timor Sea, Fog Bay, NT Australia
Or. TETRAPHYLLIDEA		
Fm. Litobothriidae		
Litobothrium alopias [AF124468]	*Alopias superciliosus* (Bigeye thresher shark)	Gulf of California, Santa Maria, Mexico
Litobothrium amplifica [AF124467]	*Alopias pelagicus* (Pelagic thresher shark)	Gulf of California, Santa Maria, Mexico
Fm. Prosobothriidae		
Prosobothrium armigerum [AF126068]	*Prionace glauca* (Blue shark)	Atlantic Ocean, Montauk, New York, USA
Fm. Dioecotaeniidae		
Dioecotaenia cancellata [AF126074]	*Rhinoptera bonasus* (Cownose ray)	Chesapeake Bay, Cambridge, Maryland, USA
Fm. Onchobothriidae		
Acanthobothrium sp. 1 [AF126065–66]	*Dasyatis longus* (Longtail stingray)	Gulf of California, La Paz, Mexico
Acanthobothrium sp. 2 [AF126067]	*Dasyatis brevis* (Whiptailed stingray)	Gulf of California, Bahia de Los Angelos, Mexico
Calliobothrium sp. [AF124469]	*Mustelus canis* (Dusky smooth-hound)	Long Island Sound, Connecticut, USA
Calliobothrium violae [AF126064]	*Mustelus canis* (Dusky smooth-hound)	Long Island Sound, Connecticut, USA
Platybothrium auriculatum [AF124470]	*Prionace glauca* (Blue shark)	Atlantic Ocean, Montauk, New York, USA
Phoreiobothrium sp. [AF126095–96]	*Sphyrna mokarran* (Great hammerhead)	Gulf of Mexico
Fm. Phyllobothriidae		
SbFm. Echeneibothriinae		
Echeneibothrium vernetae [AF126083–84]	*Raja erinacea* (Little skate)	Passamoquaddy Bay, New Brunswick, Canada
SbFm. Phyllobothriinae		
Anthocephalum alicae [AF126091–92]	*Dasyatis americana* (Southern stingray)	Atlantic Ocean, Florida Bay, Florida, USA
Anthocephalum n. sp. 1 [AF126087–88]	*Dasyatis longus* (Longtail stingray)	Gulf of California, La Paz, Mexico
Anthocephalum n. sp. 2 [AF126089–90]	*Dasyatis brevis* (Whiptailed stingray)	Gulf of California, Bahia de Los Angelos, Mexico
Ceratobothrium xanthocephalum [AF126085–86]	*Isurus oxyrinchus* (Shortfin mako shark)	Atlantic Ocean, Montauk, New York, USA

Table 1 (*continued*)

Classification Species [GenBank No.]	Host (common name)	Collection locality
Clistobothrium montaukensis [AF126069]	*Isurus oxyrinchus* (Shortfin mako shark)	Atlantic Ocean, Montauk, New York, USA
Paraorygmatobothrium sp. [AF126081–82]	*Mustelus californicus* (Grey smoothhound)	Gulf of California, Puertecitos, Mexico
Rhodobothrium sp. [AF126097–98]	*Rhinoptera bonasus* (Cownose ray)	Gulf of Mexico, Ocean Springs, Mississippi, USA
Spongiobothrium sp. [AF126079–80]	*Dasyatis brevis* (Whiptailed stingray)	Gulf of California, Puertecitos, Mexico
SbFm. Rhinebothriinae		
Caulobothrium sp. [AF126093–94]	*Myliobatis californicus* (Bat eagle ray)	Gulf of California, Bahia de Los Angelos, Mexico
Duplicibothrium minutum [AF126070–71]	*Rhinoptera bonasus* (Cownose ray)	Gulf of Mexico, Ocean Springs, Mississippi, USA
Duplicibothrium n. sp. 1 [AF126072]	*Rhinoptera steindachneri* (Pacific cownose ray)	Gulf of California, Puertecitos, Mexico
Duplicibothrium n. sp. 2 [AF126073]	*Rhinoptera steindachneri* (Pacific cownose ray)	Gulf of California, Puertecitos, Mexico
Rhinebothrium sp. [AF126075–76]	*Dasyatis americana* (Southern stingray)	Atlantic Ocean, Florida Bay, Florida, USA
Rhinebothrium maccallumi [AF124476]	*Dasyatis americana* (Southern stingray)	Gulf of Mexico
Rhinebothrium n. sp. [AF126077–78]	*Dasyatis longus* (Longtail stingray)	Gulf of California, La Paz, Mexico

them to be close, but clearly outside of the Tetraphyllidea. Newly generated sequences were assembled using Sequencher 3.0 (GeneCodes). Contiguous sequences, together with ssrDNA sequences retrieved from GenBank, were aligned by reference to the conserved core of the ssrDNA secondary structural model of Neefs et al. [15]. Application of this model to the ssrDNA gene of cestodes can be found in Olson and Caira ([6], their Appendix A). Alignments were handled using GDE [16] for a SUN workstation and exported to a Macintosh personal computer for analysis. Regions in which homologies could not be unambiguously determined or where gaps exceeded 2 bp in length were excluded from analyses. This resulted in 874 (58%) of the 1520 total sites in the alignment (including alignment gaps) being excluded. The sites included in the analysis encompassed the conserved 3′ end of the V4 region combined with the last quarter of the gene (from stem 40 to 47, encompassing the conserved 3′ end of the V7 region as well as the V8 and V9 regions). The full alignment is available by anonymous FTP (accession No. DS37932) from ftp.ebi.ac.uk in directory/pub/databases/embl/align.

2.4. Phylogenetic analyses

All analyses were performed with PAUP* (DL Swofford, 1998, version 4.0b1a), and the resulting networks were rooted with the outgroup taxa. Analysis by maximum parsimony (MP) was performed using the heuristic search (1000 search replicates), random-addition sequence and TBR branch-swapping options. All characters were run unordered and with equal weights. Gaps were treated as missing data. Nodal support was assessed by bootstrap resampling (1000 bootstrap replicates with three heuristic searches/replicate), and by decay analysis [17] using AutoDecay (T Eriksson, N Wikström, version 3.0.3).

Alternative topologies consistent with current classification [2] or strict patterns of host association were analysed in order to compare their differences in tree length. Nexus tree files were

generated with MacClade (WP Maddison, DR Maddison, 1997, version 3.07) such that different groups of taxa (i.e. Onchobothriidae, Phyllobothriidae, Phyllobothriinae, Rhinebothriinae, Lecanicephalidea within the Tetraphyllidea, ray-hosted taxa, and shark-hosted taxa) were constrained to form monophyletic, but unresolved clades. Maximum parsimony analyses were performed using these constraints and the significance of the difference in the lengths of the resulting strict consensus trees evaluated by comparison to the unconstrained most parsimonious trees (MPTs) using the Kishino–Hasegawa and Templeton compatibility tests implemented in PAUP* (Table 2).

Sequences were also analysed by the method of maximum likelihood (ML) in order to examine the potentially misleading effect of multiple substitutions on analysis by MP. A model of nucleotide substitution was chosen in the following way: each model implemented by PAUP* [Jukes–Cantor, Kimura two-parameter, Felsenstein, 84/Hasegawa, Kishino and Yano, 85 and General Time-Reversible (GTR)] was used to generate a log-likelihood value based on the strict consensus topology from analysis by parsimony. In addition to testing each of the four models alone, estimates of among-site rate variation were incorporated using: (1) an invariant sites model (I), (2) a gamma model (Γ), and (3) both an invariant sites and a gamma model ($I + \Gamma$). Log-likelihood scores for the 16 possible combinations were compared by chi-square analysis (see [18]). Because it was computationally infeasible to use even a heuristic search strategy for the ML analysis, it was necessary to limit the tree space in which topologies were evaluated under the optimality criterion of ML. This was achieved by saving all trees one step longer than the most parsimonious tree, and using the most likely of these as the starting tree for the ML analysis. Saving all trees at this length resulted in the evaluation of topologies which differed among all basal nodes subtending the ingroup taxa, as evident by Bremer support values (Fig. 1A). The possibility of a more likely topology was then examined by re-arranging the starting tree via TBR branch swapping.

3. Results

3.1. Analysis by parsimony

Of the 663 sites included, 440 (66%) were constant, 62 (10%) were autapomorphic and 161 (24%) were parsimony informative. Empirical nucleotide frequencies showed a bias toward guanine and thymine relative to adenine and cytosine (29%, 26%, 21% and 24%, respectively).

Table 2
Tree scores and tests of taxonomic and host-association constraint clades

Constraint clade	N[a]	Tree length	No. of trees	Steps longer	Kishino–Hasegawa	Templeton's
Taxonomic						
Lecanicephlidea within Tetraphyllidea[b]	19	730	16	39	< 0.0001*	< 0.0001*
Fm. Onchobothriidae	6	714	8	23	0.0004*	0.0007*
Fm. Phyllobothriidae	16	696	2	5	0.6686	0.6353
SbFm. Phyllobothriinae	8	717	30	26	0.0019*	0.0087*
SbFm. Rhinebothriinae	7	710	6	19	0.0172*	0.0171*
Host association						
Ray-hosted tetraphyllideans	14	698	6	7	0.2975	0.2632
Shark-hosted tetraphyllideans	12	694	4	3	0.7817	1.000

[a]Number of taxa in constraint clade.
[b]Constrained node indicated by an asterisk in Fig. 1B.
*Indicates a significant difference ($P < 0.05$) in tree length between the strict consensus topologies of the constrained and most parsimonious trees.

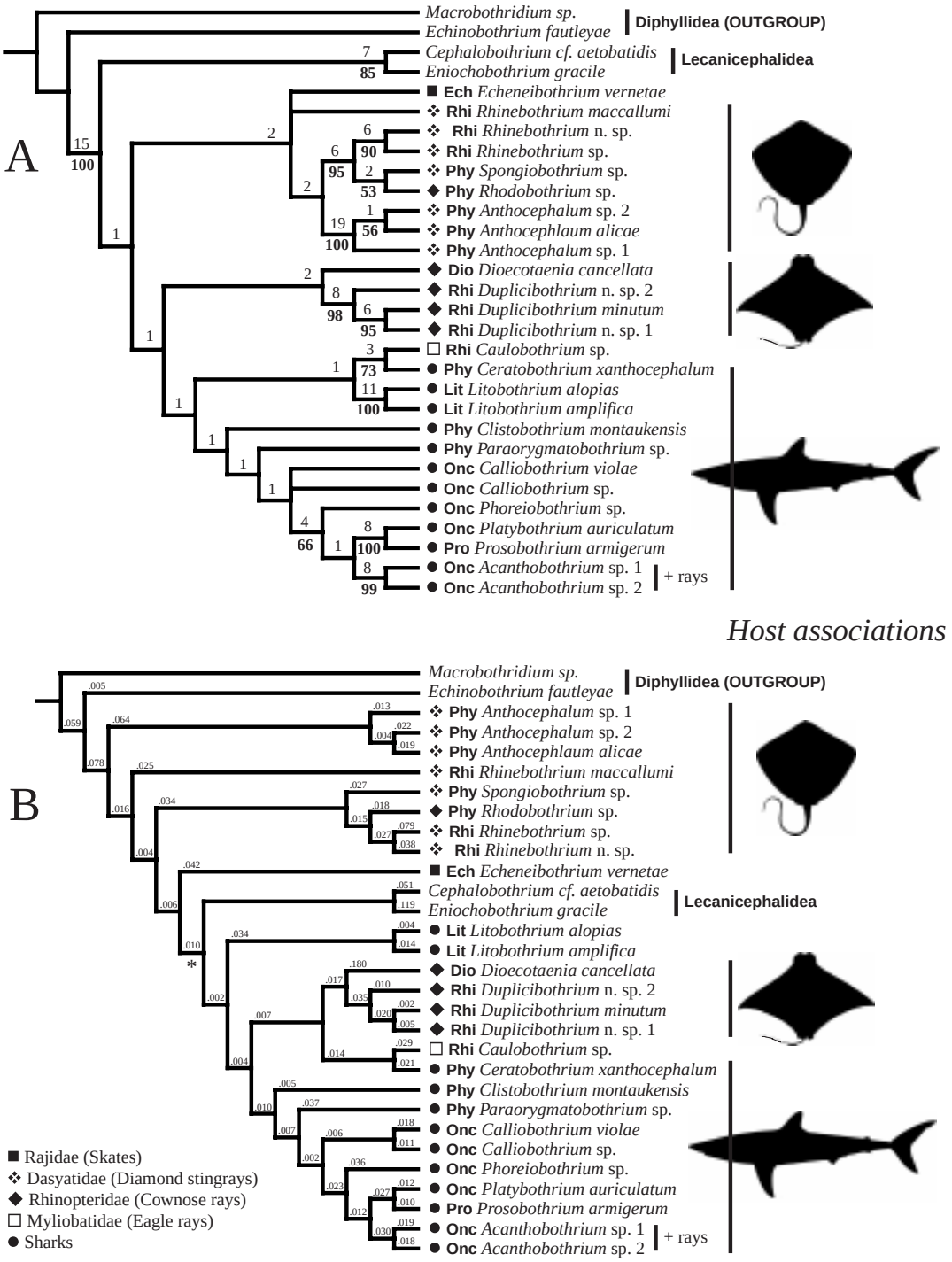

Fig. 1. Results of phylogenetic analysis by maximum parsimony (A) and maximum likelihood (B) showing patterns of host association. (A) Strict consensus of four most parsimonious trees. Tree-length = 691 steps (max./min. steps = 347/1113), CI = 0.5, RI = 0.55, RC = 0.23, HI = 0.5. Numbers above nodes are decay indices; numbers below nodes are bootstrap indices $\geqslant$ 50% (1000 replicates). (B) Most likely topology based on a GTR + I + Γ nucleotide substitution model. Numbers show estimates of genetic distance. An asterisk (*) indicates the constraint node used to test the position of the Lecanicephalidea (see text). Dio, Dioecotaniidae; Ech, Echeneibothriinae; Lit, Litobothriidae; Phy, Phyllobothriinae; Pro, Prosobothriidae; Onc, Onchobothriidae; Rhi, Rhinebothriinae.

However, χ^2 analysis did not reveal significant heterogeneity in base frequencies among taxa, and these data were thus not subject to this potential source of systematic error. Four MPTs resulted from the analysis (691 steps, CI = 0.5, RI = 0.55, RC = 0.23 and HI = 0.5). A strict consensus of the MPTs is shown in Fig. 1A. Strong nodal support was found for most of the distal nodes of the tree, while deeper-level nodes were generally supported by decay indices of 1 and bootstrap values below 50%. The lecanicephalidean taxa were found to be the sister to the tetraphyllidean species, and monophyly of the Tetraphyllidea was thus supported. Within the Tetraphyllidea, three major clades were recovered: (1) a basal clade including species in the phyllobothriid genera *Anthocephalum*, *Echeneibothrium*, *Rhinebothrium*, *Rhodobothrium* and *Spongiobothrium*; (2) a clade uniting the phyllobothriid genus *Duplicibothrium* with the dioecotaeniid genus *Dioecotaenia*; and (3) a larger sister clade to the *Duplicibothrium* + *Dioecotaenia* clade that included the phyllobothriid genera *Caulobothrium*, *Ceratobothrium*, *Clistobothrium*, *Paraorygmatobothrium*, the prosobothriid genus *Prosobothrium*, the litobothriid genus *Litobothrium* and the onchobothriid genera *Acanthobothrium*, *Calliobothrium*, *Phoreiobothrium* and *Platybothrium*.

Based on current concepts of tetraphyllidean classification [2], the family Phyllobothriidae was found to be paraphyletic and monophyly of the family Onchobothriidae was brought into question by the position of the prosobothriid species, *Prosobothrium armigerum*, as the sister taxon to *Platybothrium auriculatum*. Of the genera for which multiple species were included in the analysis, support of monophyly was found for *Acanthobothrium*, *Anthocephalum*, *Duplicibothrium* and *Litobothrium*. Species in the genus *Rhinebothrium* were found to be paraphyletic, and monophyly of the two species in the genus *Calliobothrium* was ambiguous. Constraint analyses (Table 2) showed that forcing the family Onchobothriidae, and subfamilies Phyllobothriinae and Rhinebothriinae to be monophyletic, resulted in significantly longer tree lengths in each case. However, forcing the family Phyllobothriidae to be monophyletic did not result in a significantly longer tree length.

3.2. Analysis by maximum likelihood

Chi-square analysis showed the GTR + I + Γ model of nucleotide substitution to be a significantly better fit to the data than the less-complex models, based on a strict consensus topology of the MPTs. The most likely topology (Fig. 1B) had a log-likelihood of 4201.0417; the proportion of sites estimated to be invariable (I) was 53% (observed proportion = 66%) and the estimate of Γ was 0.74. Estimated branch lengths among internal ingroup nodes ranged from 0.002 to 0.064, although branch lengths of deep-level internal nodes ranged from 0.002 to only 0.016. Terminal branch lengths ranged from 0.002 to 0.18. The topology based on ML analysis differed from the MPTs, primarily in that the lecanicephalidean taxa were placed within the Tetraphyllidea, and some members of basal clades were 'ladderised' (Fig. 1B). The three *Anthocephalum* species formed the most basal clade in the ingroup.

4. Discussion

4.1. Genetic variability and phylogenetic signal within the ssrDNA gene

Variability in the V4 and V7 regions of the ssrDNA gene observed among the tetraphyllidean genera and families was comparable to that among at least some of the orders of tapeworms (cf. [6]). Such variation was greater than would be expected for comparisons at these levels of taxonomic inference [19], suggesting that the taxa involved are indeed very old. As a result, most of the sites within these regions had to be removed prior to analysis; the divergence among sequences was too great to reliably infer their positional homologies. How the tetraphyllideans compare with other orders of tapeworms with regard to intra-ordinal variability of the ssrDNA gene cannot be readily determined at this time. In contrast to the present study, Mariaux's ordinal-level

molecular analysis of tapeworms [7] targeted regions of the ssrDNA gene that show high levels of conservation. Mariaux analysed nucleotide positions from stem 6 to stem 16, encompassing the V1–V2 regions, the conserved 3′ end of the V4 region to stem 35, encompassing the V5 region, and the highly conserved V8–V9 regions of the 3′ end of the ssrDNA gene. These areas are adjacent to the regions examined herein, with the exception of the short V8–V9 regions common to both studies. He found almost no intra-ordinal variability among species of proteocephalideans, while the same conserved regions were found to be informative for inferring relationships among species of cyclophyllideans [7]. Thus, we can only draw the conclusion that the relative ages of the currently recognised higher taxa (i.e. orders) may be more disparate than their equal taxonomic ranking [20] would imply.

Estimates of branch lengths, based on both MP and ML analyses, showed that most terminal branches were approximately equivalent in length. However, the tetraphyllidean taxon, *Dioecotaenia cancellata*, was found to be highly divergent relative to the other study taxa (with a branch length of 0.18 as estimated by the GTR + I + Γ nucleotide substitution model). The next terminal branch closest in length was that of the lecanicephalidean, *Eniochobothrium gracile*, which had an estimated branch length of 0.12. Lengths of nearly all other terminal branches were well below 0.05. This is notable because the genus *Dioecotaenia* is one of the only groups of tapeworms that exhibits strobila of separate sexes (dioecy). As a rule, tapeworms are strictly hermaphroditic and true dioecy is known only among the members of one other unrelated group, the cyclophyllidean family Dioecocestidae [21]. The possibility that the unique sexual dimorphism of *D. cancellata* may be coupled with an increased rate of genetic divergence is intriguing and warrants further investigation. Furthermore, it is interesting that the phylogenetic position of *D. cancellata* was apparently not affected by either long-branch attraction [22] or repulsion [23], as it was found by both MP and ML analyses to be closest to species in the genus *Duplicibothrium* which were not found to have either particularly long or short terminal branches relative to the other taxa (Fig. 1B).

Evaluation of the tree lengths of 1 000 000 random topological arrangements of the taxa resulted in a g1-statistic of −0.878; a highly left-skewed distribution indicating a strong degree of hierarchical structure in the data. However, left-skewness in the distribution of cladogram lengths is influenced, among other things, by characters that strongly support small clades of taxa within a larger, and potentially poorly supported, tree [24]. Thus, the value of the g1 statistic in this case was likely influenced by the support for well-resolved terminal clades, rather than strong signal for the overall topology.

4.2. Phylogenetic implications

The multiloculate bothridial morphology exhibited by species in the genera *Caulobothrium*, *Dioecotaenia*, *Duplicibothrium*, *Echeneibothrium*, *Rhinebothrium* and *Spongiobothrium* (e.g. see figures in [2]) was found to be a symplesiomorphic condition of tetraphyllideans, rather than a synapomorphy of these taxa. However, the phylogenetic position of *Caulobothrium* sp. as the sister taxon to *Ceratobothrium xanthocephalum* was unexpected based on morphological differences between these two species. In addition to bothridial homologies, species in the genus *Caulobothrium* share derived morphological features of the proglottid with species in the genera *Dioecotaenia*, *Duplicibothrium*, *Glyphobothrium* and *Serendip* (see [25]). For example, members of these genera have testes distributed in the ovarian field, and marked protandric segment development, which are features not found in *C. xanthocephalum*. Nevertheless, the close affinity of *Caulobothrium* sp. to *C. xanthocephalum* was supported by both methods of analysis, and showed strong nodal support (Fig. 1A).

The placement of *Duplicibothrium* spp. with *D. cancellata* supports the contention of Brooks and Barriga [25] that these genera are closely related. These species share the presence of bothridia that are fused dorso-ventrally as well as the absence of the large class of microtriches on all bothridial

surfaces (T.R. Ruhnke, personal observation). In addition, the three species of *Duplicibothrium* and the species of *Dioecotaenia* are found exclusively in cownose rays (*Rhinoptera* spp.).

The cruciform bothridial morphology of the Litobothriidae is sufficiently unique among all known tapeworms to have been used as a basis for the recognition of ordinal status of the group [26]. The position of these species in the present analysis, however, is consistent with their more recent taxonomic placement within the order Tetraphyllidea by Euzet [2], but not with the molecular analysis of Olson and Caira [6] in which these same two representative species were found to be the sister group of the lecanicephalidean, proteocephalidean and tetraphyllidean taxa. Similarly, Caira et al. [4] showed the genus *Litobothrium* to be the sister to all of the lecanicephalidean, proteocephalidean and tetraphyllidean taxa in their study. Although the position of the Litobothriidae within the Tetraphyllidea was supported by both methods of analysis, its exact position differed between the methods and did not show a high level of nodal support.

Similar to the Litobothriidae, ordinal status of the Lecanicephalidea has not been universally accepted. An alternative position of the order was the primary difference between the results of the two methods of analysis; MP (Fig. 1A) supported their position outside of a monophyletic Tetraphyllidea, while ML (Fig. 1B) supported their position within the order. Constraining the node subtending the position of the Lecanicephalidea in the ML analysis (denoted by an asterisk in Fig. 1B) resulted in a significantly longer tree length via MP analysis (Table 2). It is interesting that while the ML analysis was consistent with the results of Caira et al. [4] in the inclusion of the Lecanicephalidea within the Tetraphyllidea, in their analysis the lecanicephalidean taxa formed a clade together with *Echeneibothrium vernetae*, which bears an apical structure of the scolex potentially homologous with those of lecanicephalideans. However, in the present study, the positions of both the lecanicephalidean taxa and *E. vernetae* moved together between the two methods of analysis, and in the case of ML were separated by only a single node.

The unexpected grouping of the prosobothriid species *Prosobothrium armigerum* with the onchobothriid species *Platybothrium auriculatum* was supported by both methods of analysis and showed among the highest levels of nodal support. This indicates that the Onchobothriidae, a tetraphyllidean family supported by several derived morphological features, and shown to be monophyletic by Caira et al. [4], may include at least one non-hooked species within it. An affinity between these two taxa, albeit difficult to explain morphologically, is consistent with the fact that both are parasites of blue sharks (*Prionace glauca*).

4.3. Host–parasite associations

Rigorous comparisons of the congruence between independently derived host and parasite phylogenies (e.g. [27–29]) are few in number, and have yet to be performed for members of the order Tetraphyllidea. However, tetraphyllidean species typically show a high degree of host-specificity [4, 10]. For example, species in the genus *Paraorygmatobothrium* show a strong degree of specificity with their shark hosts (see [30, 31]), and species in the genus *Calliobothrium* are specific to various species of the shark genus *Mustelus* (e.g. [32]). Additionally, species of the genus *Echeneibothrium* seem in general to be specific to species of the skate genus *Raja* (see [1]). Species of the genus *Potamotrygonocestus* are specific to their freshwater stingray hosts species (*Potamotrygon* spp.) and may have co-speciated with them [33]. These and other examples indicate that parallel speciation between tetraphyllideans and their elasmobranch hosts is a feature common to the evolution of these tapeworms, particularly at lower taxonomic levels (i.e. families and genera). This pattern could be spurious, however, if the morphological variation among worms is a result of host-induced variation. If such a phenomenon occurred, then parasite species within a genus could be distributed among several host species. The ssrDNA nucleotide variation among the congeneric species sequenced in this study, however,

does not support the idea that morphological differences among parasite taxa is a result of such host induced variation. For example, *Duplicibothrium minutum* and *Duplicibothrium* n. sp. 1 differ primarily in bothridial morphology, which could reasonably be postulated to be the result of adaptation to differences in the mucosal lining of the host intestine. However, these two species showed four nucleotide differences even in the highly conserved regions of the ssrDNA gene and many more in the variable regions excluded from the analysis.

Results of both methods of analysis showed that the relationships among the tetraphyllidean taxa included in the present study reflect common host associations better than their current classification (Fig. 1). Indeed, forced monophyly of taxa based on strict patterns of host association did not result in significantly longer tree lengths than the unconstrained MPTs, whereas most constraints based on their present classification did (Table 2). In the MP analysis, the phyllobothriid parasites of sharks formed a clade together with the litobothriid, onchobothriid and prosobothriid parasites of sharks, rather than a clade with the other phyllobothriid taxa included in the analysis, all of which parasitise rays. Similarly, a more restricted clade of shark parasites was observed in the ML analysis, including both phyllobothriid and onchobothriid taxa. Both methods of analysis united *D. cancellata* (Dioecotaniidae) with the three species of *Duplicibothrium* (Rhinebothriinae); all of which parasitise cownose rays (*Rhinoptera* spp.). The other members of the subfamily Rhinebothriinae (*Rhinebothrium* spp.) were grouped with members of the subfamily Phyllobothriinae, in a clade (MP analysis), or set of clades (ML analysis), in which their host association (diamond stingrays; *Dasyatis* spp.) was the common feature. Barring evidence of other influences, such host-specific phylogenetic patterns are best explained by a co-evolutionary history between the parasites and their hosts. Whether or not the patterns observed herein are robust to the inclusion of additional taxa and/or data awaits further investigation.

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