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Phylogenetic analysis and reconfiguration of genera in the cestode order Diphyllidea

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ABSTRACT

The generic boundaries of the Diphyllidea are reassessed based on parsimony and likelihood phylogenetic analyses of 28S rDNA (ribonucleic acid large subunit), 18S rDNA (ribonucleic acid small subunit), and COI (cytochrome oxidase subunit I) sequence data for 31 species representing morphological variation across the order. Trees resulting from these analyses yielded a number of well-supported clades that are congruent with unique morphological features mandating generic revision of the order and erection of at least two new genera. Species originally assigned to *Echinobothrium* van Beneden, 1849 but bearing a corona of spines on the region of the scolex anterior to the bothria and posterior to the apical organ armature are transferred to *Coronocestus* n. gen.; members of this genus typically parasitize triakid sharks, although one report from a hemiscylliid shark exists. Species with lateral hooklets arranged in continuous bands, rather than in two distinct clusters, are transferred to *Halysioncum* n. gen.; all species parasitize batoids, mostly myliobatids and rhinopterids, but a few records also exist from arhynchobatids, rhinobatids, platyrhinids and urotrygonids. Our analyses support transfer of the five species originally assigned to *Macrobothridium* Khalil and Abdul-Salam, 1989 owing to their lack of cephalic peduncle spines to *Echinobothrium*. As a consequence, *Echinobothrium sensu stricto* includes species both with and without spines on the cephalic peduncle, but all members of the genus possess lateral hooklets arranged in clusters on either side of the dorsal and ventral apical hooks. With respect to diphyllideans parasitizing catsharks, *Ahamulina* Marques, Jensen and Caira, 2012 is unique in possessing apical hooks but lacking lateral hooklets and *Ditrachybothridium* Rees, 1959 is unique in entirely lacking scolex armature. By far the majority of species of *Echinobothrium sensu stricto* parasitize skates of the family Rajidae, guitarfish of the family Rhinobatidae, and stingrays of the dasyatid genera *Taeniura* Müller and Henle, *Dasyatis* Rafinesque, and *Himantura* Müller and Henle, although a single species each has been reported from Anacanthobatidae, Rhynchobatidae, Platyrhinidae and Myliobatidae. It now seems clear that while by far the majority of diphyllideans parasitize batoids, the diphyllideans parasitizing sharks, and catsharks in particular, remain problematic. Additional collections from these carcharhiniform hosts are likely to be particularly illuminating.

1. Introduction

Knowledge of the Diphyllidea van Beneden in Carus, 1863, one of the seven orders of cestodes parasitizing elasmobranchs, has grown substantially over the past decade with descriptions of nearly half of the 50 valid species appearing over that time. Collectively members of the order occur in a diversity of elasmobranchs, although the majority of species parasitize batoids. Unlike some of the orders found in elasmobranchs, the monophyly of the Diphyllidea is undisputed (Ivanov and Hoberg, 1999; Tyler, 2006). Its members are united by their possession of a scolex with two bothria, an apical organ that bears apical hooks and lateral hooklets, as well as a cephalic peduncle that may be armed with eight columns of spines, and a mid-ventral common genital pore—although collectively its species exhibit a wide array of configurations of scolex armature (Fig. 1). Three genera are currently recognized. At present, species of *Echinobothrium* van Beneden, 1849 possess three types of armature (apical hooks, lateral hooklets and cephalic peduncle spines), while species of *Ditrachybothridium* Rees, 1959 lack all three types of armature. The currently monotypic *Ahamulina* Marques, Jensen and Caira, 2012 possesses apical hooks but

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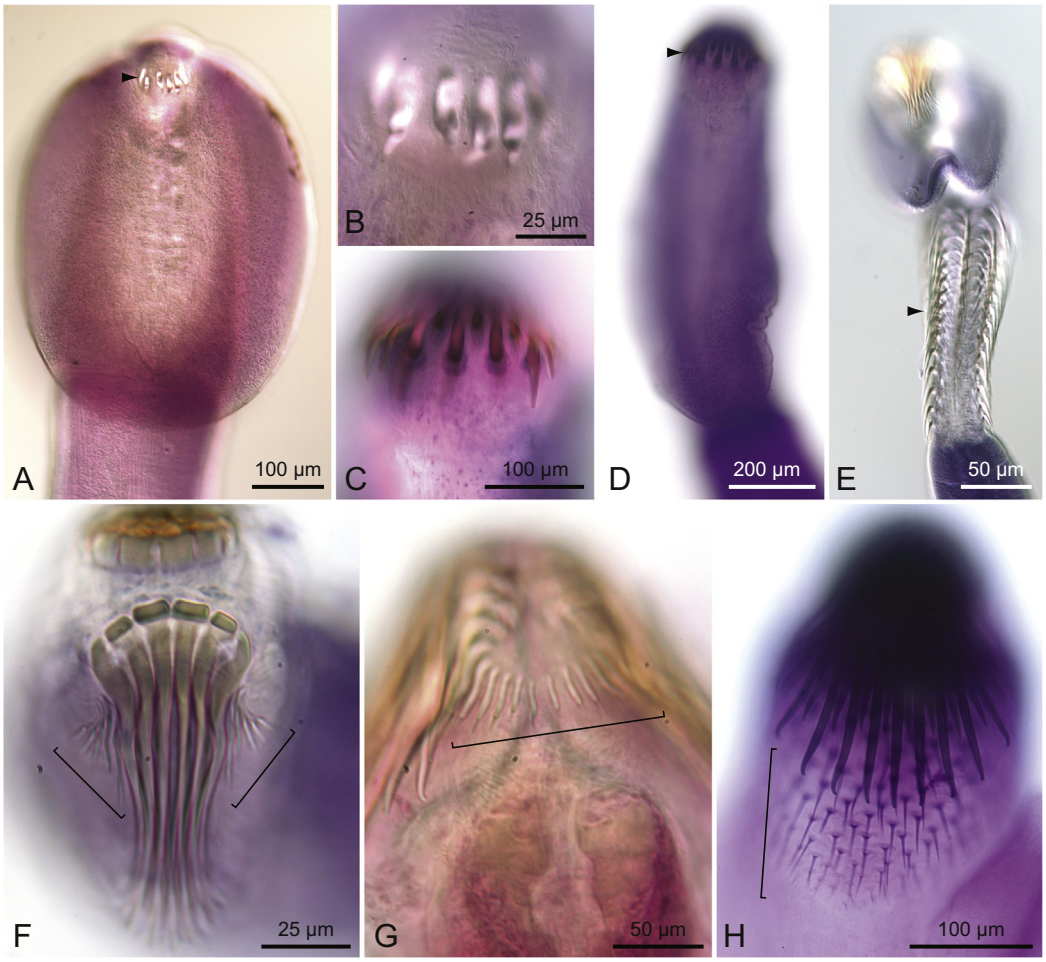

Fig. 1. Light micrographs showing diphyllidean scolex armature. (A) Scolex of *Ahamulina catarina*; arrowhead indicates the single row of apical hooks. (B) Close-up of single row of apical hooks of *A. catarina*. (C) Close-up of single row of apical hooks of *Ahamulina* n. sp. 1; note that apical hooks are unequal in length. (D) Scolex of *Ahamulina* n. sp. 1; arrowhead indicates the single row of apical hooks. (E) Scolex of *Echinobothrium dougbermani*; arrowhead indicates cephalic peduncle spines. (F) Apical organ armature of *E. dougbermani*; brackets indicate two clusters of lateral hooklets. (G) Apical organ armature of *Halysioncum mexicanum*; bracket indicates continuous band of lateral hooklets. (H) Anterior region of scolex of undescribed species of *Coronocestus* from *Iago* sp.; bracket indicates corona of spines.

lacks lateral hooklets and spines on the cephalic peduncle (Fig. 1A). Although now considered a synonym of *Echinobothrium* (see Tyler, 2006; Kuchta and Caira, 2010), *Macrobothridium* Khalil and Abdul-Salam, 1989 was erected for species that exhibit apical hooks and lateral hooklets but lack spines on the cephalic peduncle. The monophyly of all three genera remains to be comprehensively assessed. In fact, diphyllidean phylogenetic relationships have been seriously explored on only two occasions (see Ivanov and Hoberg, 1999; Tyler, 2006), in both cases based solely on morphological data. Although the interrelationships implied by these two studies were consistent in the non-monophyly of *Echinobothrium* relative to *Macrobothridium*, and in the placement of *Ditrachybothridium* as sister to that clade, they differed substantially in other aspects of their topologies. From a molecular standpoint, diphyllideans have been included as outgroups in studies focusing on phylogenetic relationships within other cestode orders (e.g., Olson and Caira, 1999; Olson et al., 1999, 2001, 2010; Littlewood and Olson, 2001; Bray and Olson, 2004; Caira et al., 2005; Brabec et al., 2006; Palm et al., 2009), or as exemplars in broad scale analyses assessing relationships among cestode orders (Waeschenbach et al., 2007, 2012). Diphyllidean interrelationships have not been addressed previously using molecular data.

The primary goals of this study were to (i) investigate the phylogenetic relationships among the diphyllideans from a molecular perspective using data from one mitochondrial (cytochrome oxidase subunit I [COI]) and two nuclear (ribonucleic acid large subunit [28S rDNA] and ribonucleic acid small subunit [18S rDNA]) genes, (ii) assess generic boundaries based on the results of the molecular analyses, (iii) explore morphological attributes that might serve to define the resulting groups, (iv) revise the generic-level classification within the order so as to be consistent with groups supported by both morphological and molecular data, and (v) examine the host associations of the order in the context of the generic-level revision.

2. Materials and methods

2.1. Study taxa

Our analyses included 31 species of diphyllideans consisting of 12 of the 50 valid species and 19 undescribed species. Fifteen of the 31 species were represented by replicates of two to five specimens each, for a total of 54 ingroup specimens. One of the greatest challenges of this study was securing molecular material representing the range of hosts and distinctive morphologies seen across the order. In many cases the only specimens available represented species new to science, many of which came from host species that had not been previously examined. In all cases hologenophores (sensu Pleijel et al., 2008) were sequenced and their associated

vouchers were prepared as whole mounts according to Olson et al. (2010). These vouchers have been deposited in the Lawrence R. Penner Parasitology Collection at the University of Connecticut, Storrs, CT, USA. These vouchers serve to ground the identities of the 19 potentially novel species until they can be formally treated. Sequence data for 50 of these 54 specimens (and 28 of 31 species) were generated de novo; data for the others were obtained from GenBank. We note that while collectively the 31 diphyllidean species included span the range of morphological variation and hosts parasitized by members of this order, 38 valid species were not represented. Detailed data on all ingroup specimens included in the analyses are provided in Table 1.

Outgroup selection was based on the molecular evidence that the Trypanorhyncha is the sister taxon of the Diphyllidea (Waeschenbach et al., 2007, 2012). Three species representing both suborders of trypanorhynchs (see Olson et al., 2010) were included as outgroups. Sequence data used for these species were those available in GenBank and thus did not include COI. Outgroups were the trypanobatoideans *Tetrarhynchobothrium* sp. (18S rDNA: DQ642960; 28S rDNA: DQ642798) and *Prochristianella clarkeae* Beveridge, 1990 (18S rDNA: DQ642947; 28S rDNA: DQ642785), and the trypanoselachoidean *Aporhynchus menezesi* Noever, Caira, Kuchta and Desjardins, 2011 (=*Aporhynchus* sp. of Olson et al., 2010) (18S rDNA: FJ572911; 28S rDNA: FJ572947).

2.2. Generation of nucleotide data

Specimens were fixed in 95% or 100% ethanol. The middle portion of each specimen was removed and allowed to air-dry for ~5 min at room temperature. Genomic DNA was extracted using an InstaGene™ DNA Extraction Kit (Bio-Rad Life Sciences, USA) following the manufacturer's instructions. Genomic DNA was quantified using a micro-volume spectrophotometer, NanoDrop 2000 (Thermo Scientific, USA). Extractions with low genomic DNA concentrations were amplified using a GenomiPhi™ DNA Amplification Kit (GE Healthcare, USA) following the manufacturer's instructions.

PCR was used to amplify the partial COI, complete 18S rDNA and the D1-D2 region of 28S rDNA. Double-stranded amplifications were performed in a 25 μl volume containing 1–10 μl of DNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 200 μM dNTPs, 1.0–3.0 mM $MgCl_2$, 0.4 μM of each primer and 1 U of Taq DNA Polymerase Recombinant (Fermentas Life Sciences, USA). Amplification and sequencing of COI was done using the primer pair nLCO 5′-TTTACTYTRGAYCATAAGCGT-3′ and Sean2 5′-AAGCAGAACCAAATTTACGAT-3′, or Sean1 5′-TTTACTTTGGATCATAAGCG-3′ and HCO2198 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (Folmer et al., 1994). PCR conditions for this fragment included initial denaturation for 2 min at 94 °C; 10 cycles of denaturation for 30 s at 94 °C, annealing for 1 min at 48 °C, and extension for 1 min 20 s at 72 °C; 25 cycles of denaturation for 30 s at 94 °C, annealing for 40 s at 50 °C, and extension for 1 min 20 s at 72 °C; followed by a final extension for 7 min at 72 °C. Amplification and sequencing of 18S rDNA was conducted using the primer pair 300F 5′-AGGGTTCGATTCCGGAG-3′ and WormB 5′-CTTGTTACGACTTTTACTTCC-3′. PCR conditions for this fragment included initial denaturation for 5 min at 95 °C, 35 cycles of denaturation for 30 s at 95 °C, annealing for 40 s at 60 °C, extension for 1 min 40 s at 72 °C and a final extension for 7 min at 72 °C. The primer 930F 5′-GCATGGAATAATGGAATAGG-3′ was also used for sequencing. Amplification and sequencing of D1-D2 region of 28S rDNA was done using the primer pair C1 5′-ACCCGCTGAATTTAAGCAT-3′ and D2 5′-TGGTCCGTGTTTCAAGAC-3′ (Hassouna et al., 1984). PCR conditions for this fragment included initial denaturation for 5 min at 95 °C, 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, extension for 1 min at 72 °C, and a final extension for 7 min at 72 °C. Some samples that did not amplify with the above primer pair were submitted to a two-step amplification process using the primer pair C1 and Rob2 5′-CACGYACTRTTTACTCTC-3′ (Chisholm et al., 2001) and the primer pair LSU-330F 5′-CAAGTACCGTGAGGGAAAGTTG-3′ and D2. PCR conditions for these primer pairs included an initial denaturation for 5 min at 94 °C, 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, extension for 40 s at 72 °C, and a final extension for 7 min at 72 °C. PCR products were purified using an Agencourt® AMPure® XP DNA Purification and Cleanup kit (Beckman Coulter Genomics, USA). Products were subsequently either re-amplified or cycle-sequenced directly from forward, reverse and, in some cases, internal strands, using ABI Big-Dye™ Sequence Terminator version 3.1, cleaned with ethanol precipitation and sequenced on an ABI Prism Genetic Analyser (3100/3700) automated sequencer.

Contiguous sequences were assembled using the package Consed/PhredPhrap (Ewing and Green, 1998; Ewing et al., 1998; Gordon et al., 1998, 2001). Sequences were initially aligned using MAFFT (Katoh et al., 2002) and visualized and edited in BioEdit (Hall, 1999). After alignment, sequences of COI were checked for stop codons using the DNA to Protein Translation online resource by Bikandi et al. (2004) and all sequences were trimmed so that the first base corresponded to the first codon position. Based on putative homologous regions within each gene, four internal blocks were created for the 18S and 28S rDNA sequences to increase computational efficiency during the dynamic homology analyses (Giribet, 2001); this step was not required for the COI data. 28S rDNA data were generated or obtained from GenBank for all 51 ingroup specimens, 18S rDNA data for all but two ingroup specimens, and COI data for all but six ingroup specimens (see Table 1). Datasets can be downloaded from http://lhe.ib.usp.br/data/.

2.3. Phylogenetic analyses

For comparative purposes, phylogenetic analyses were performed using both parsimony and likelihood methods. All analyses were conducted on the combined dataset for all three genes. These analyses are described below and summarized schematically in Supplementary Fig. S1.

2.3.1. Dynamic homology under parsimony

Initial tree searches were performed using direct optimization (DO) (Wheeler, 1996) of nucleotide sequences as implemented in POY (version 4.1.2.1; Varón et al., 2010) with parsimony as the optimality criterion. The dynamic homology approach (sensu Wheeler, 2001a,b) was chosen because it allows alignment and tree searches to be conducted simultaneously.

The numerical values for alignment parameters assigned to define cost regimes (i.e., character transformation weights) for insertion/deletion events (INDELs) and substitutions (i.e., transversions and transitions) are expressed as cost ratios. In the absence of an empirical justification for assigning any particular cost regime prior to alignment, following Wheeler (1995) we employed a number of cost ratios to define the parameter space of the analysis within which each phylogenetic inference was performed. This approach, which has been referred to as sensitivity analysis (Wheeler, 1995), allows for the identification of stable clades that prevail regardless of cost regime explored, to be distinguished from unstable clades.

Within this framework, we performed phylogenetic analyses using a two-step procedure. First, we collected candidate topologies using optimization alignment (OA) for 10 cost ratios. The first cost ratio (i.e., 0:1:1:1) assumed no penalty for opening gaps and equal costs for all three transformation types (i.e., INDELs, transversions and transitions). The remaining nine cost ratios employed gap extension costs from 1 to 8 and transformation costs from 1 to 4 with an opening gap cost twice that of the gap extension cost,

Table 1
Revised generic placements for diphyllidean species and GenBank data for specimens included in molecular analyses.

Species	Taxonomic status	Source	Type host; additional host(s)	Host family	GenBank No. (18S rDNA)	GenBank No. (28S rDNA)	GenBank No. (COI)	Molecular spcm. No.; host spcmNo. [a]	Museum voucher No.	Host of sequenced spcm.	Locality of sequenced spcm.
Ahamulina catarina Marques, Jensen and Caira, 2012	Valid	Marques et al. (2012)	*Scyliorhinus besnardi*	Scyliorhinidae III	KC860176 KC860177 KC860178 KC860179 KC860180	KC860128 KC860129 KC860130 KC860131 KC860132	KC860220 KC860221 KC860222 KC860223 KC860224	DI-60; SC09-43 DI-61; SC09-43 DI-62; SC09-63 DI-63; SC09-63 DI-64; SC09-42	LRP 7992 LRP 7993 LRP 7994 LRP 7995 LRP 7996	*Scyliorhinus besnardi*	Itajaí, Santa Catarina, Brazil, Atlantic Ocean
Ahamulina n. sp. 1	Undescribed	This study		Scyliorhinidae I	KC860165	KC860117		DI-41; AF-114	LRP 7997	*Holohalaelurus regani*	Off South Africa, Indian Ocean
Coronocestus coronatus (Robinson, 1959) n. comb.	Valid	Robinson (1959)	*Mustelus lenticulatus*	Triakidae							
Coronocestus diamanti (Ivanov and Lipshitz, 2006) n. comb.	Valid	Ivanov and Lipshitz (2006)	*Iago omanensis*	Triakidae							
Coronocestus hormozganiense (Haseli, Malek, Palm and Ivanov, 2012) n. comb.	Valid	Haseli et al. (2012)	*Mustelus mosis*								
Coronocestus musteli (Pintner, 1889) n. comb.	Valid	Pintner (1889)	"Hundshaies" (*Mustelus mustelus* by Tyler, 2006)	Triakidae							
Coronocestus notoguidoi (Ivanov, 1997) n. comb.	Valid	Ivanov (1997)	*Mustelus schmitti*	Triakidae							
Coronocestus scoliodoni (Sanaka, Vijaya Lakshmi and Hanumantha Rao, 1986) n. comb.	*Species inquirenda*	Sanaka et al. (1986)	*Chiloscyllium indicum*	Hemiscylliidae							
Coronocestus n. sp. 1	Undescribed	This study		Triakidae	KC860181 KC860182	KC860133 KC860134	KC860225 KC860226	DI-65; SO-40DI-66; SO-40	LRP 7998LRP 7999	*Mustelus* sp.	Ghizo, Solomon Islands, Pacific Ocean
Echinobothrium lateroporum Subhapradha, 1948	*Nomen nudum*	Subhapradha (1948)	*Mustelus manazo* by Tyler (2006)	Triakidae							
Ditrachybothridium macrocephalum Rees, 1959	Valid	Rees (1959)	*Raja* (=*Leucoraja*) *fullonica; Raja* (=*Leucoraja*) *circularis*, *Scyliorhinus caniculus*	Rajidae; Scyliorhinidae III							
Ditrachybothridium cf. *macrocephalum*	Undescribed?	Bray and Olson (2004); Olson et al. (2010)		Scyliorhinidae I	DQ642903[b]	AY584864[b]			BMNH 2004.1.6.1–5[c]	*Apristurus laurussonii*	Goban Spur (off Ireland), Atlantic Ocean
Ditrachybothridium piliformis Faliex, Tyler and Euzet, 2000	Valid	Faliex et al. (2000)	*Galeus* sp. (=*Galeus priapus*); *Apristurus* sp.	Scyliorhinidae I							
Echinobothrium acanthinophyllum Rees, 1961	Valid	Rees (1961)	*Raja montagui*	Rajidae							

Echinobothrium acanthocolle Wojciechowska, 1991	Valid	Wojciechowska (1991)	*Raja* (=*Amblyraja*) *georgiana*	Rajidae							
Echinobothrium affine Diesing, 1863	Valid	Diesing (1863)	*Raja asperina* (=*R. radula*)	Rajidae							
Echinobothrium benedeni Ruszkowski, 1927	Valid	Ruszkowski (1927)	*Hippolyte varians* (and immature in *Raja punctata*); *Raja asterias*	CRUSTACEAN, Rajidae							
Echinobothrium brachysoma Pintner, 1889	Valid	Pintner (1889)	"Rochenarten" (skates); *Raja clavata*, *Raja batis*	Rajidae?							
Echinobothrium chisholmae Jones and Beveridge, 2001	Valid	Jones and Beveridge (2001); Olson et al. (2001); Waeschenbach et al. (2007)	*Rhinobatos* (=*Glaucostegus*) *typus*	Rhinobatidae	AF286986	AF286922			BMNH 2000.8.3.4–7	*Rhinobatos* (=*Glaucostegus*) *typus*	Heron Island, Queensland, Australia, Pacific Ocean
Echinobothrium cf. *chisholmae*	Undescribed?	this study		Rhinobatidae	KC860146 KC860147 KC860148	KC860097 KC860098 KC860099	KC860195 KC860196 KC860197	DI-12; CM03-35 DI-13; CM03-35 DI-14; CM03-35	LRP 8000 LRP 8001 LRP 8002	*Glaucostegus typus*	Weipa, Queensland, Australia, Indian Ocean
Echinobothrium clavatum Probert and Stobart, 1989	Valid	Probert and Stobart (1989)	*Raja clavata*	Rajidae							
Echinobothrium coenoformum Alexander, 1963	Valid	Alexander (1963)	*Raja* (=*Zearaja*) *nasuta*	Rajidae							
Echinobothrium deeghai Gupta and Parmar, 1988	*Species inquirenda*	Gupta and Parmar (1988)	*Trygon* (=*Pastinachus*) *sephen*	Dasyatidae							
Echinobothrium djeddensis (Pramanik and Manna, 2005); Kuchta and Caira, 2010	*Species inquirenda*	Pramanik and Manna (2005); Kuchta and Caira (2010)	*Rhynchobatus djeddensis* (as *R. djddensis*)	Rhynchobatidae							
Echinobothrium dorothyae Caira, Pickering, Schulman and Hanessian, 2013	Valid	Caira et al. (2013)	*Raja straeleni*	Rajidae	KC860173	KC860125	KC860219	DI-49; AF-40	LRP 8003	*Raja straeleni*	Off South Africa, Indian Ocean
Echinobothrium dougbermani (Caira, Pickering, Schulman and Hanessian, 2013)	Valid	Caira et al. (2013)	*Rhinobatos annulatus*	Rhinobatidae	KC860169 KC860170	KC860121 KC860122	KC860215 KC860216	DI-45; AF-141 DI-46; AF-141	LRP 8004 LRP 8005	*Rhinobatos annulatus*	Off South Africa, Indian Ocean
Echinobothrium elegans Tyler, 2001	Valid	Tyler (2001)	*Taeniura lymma*	Dasyatidae							
Echinobothrium euterpes (Neifar, Tyler and Euzet, 2001; Tyler, 2006	Valid	Neifar et al. (2001); Tyler (2006)	*Rhinobatos rhinobatos*	Rhinobatidae	KC860150 KC860151	KC860101 KC860102	KC860199 KC860200	DI-18; SE-154 DI-19; SE-166	LRP 8006 LRP 8007	*Rhinobatos rhinobatos*	Ouakam, Senegal, Atlantic Ocean
Echinobothrium harfordi McVicar, 1976	Valid	McVicar (1976); Olson et al. (2001)	*Raja* (=*Leucoraja*) *naevus; Raja clavata*	Rajidae	AF286985	AF286921				*Raja* (=*Leucoraja*) *naevus*	United Kingdom, North Sea

(*continued on next page*)

Table 1 (*continued*)

Species	Taxonomic status	Source	Type host; additional host(s)	Host family	GenBank No. (18S rDNA)	GenBank No. (28S rDNA)	GenBank No. (COI)	Molecular spcm. No.; host spcmNo. [a]	Museum voucher No.	Host of sequenced spcm.	Locality of sequenced spcm.
Echinobothrium helmymohamedi Saoud, Ramadan and Hassan, 1982	Valid	Saoud et al. (1982)	*Taeniura lymma*	Dasyatidae							
Echinobothrium heroniense Williams, 1964	Valid	Williams (1964)	*Taeniura lymma*	Dasyatidae	KC860160 KC860161 KC860164	KC860112 KC860113 KC860116	KC860207 KC860208 KC860211	DI-32; NT-9 DI-33; NT-9 DI-36; NT-9	LRP 8008 LRP 8009 LRP 8010	*Taeniura lymma* 2	Nhulunbuy (Gove), Northern Territory, Australia, Pacific Ocean
Echinobothrium cf. *heroniense*	Undescribed?	This study		Dasyatidae	KC860159 KC860162 KC860163	KC860111 KC860114 KC860115	KC860206 KC860209 KC860210	DI-31; NT-9 DI-34; NT-9 DI-35; NT-9	LRP 8011 LRP 8012 LRP 8013	*Taeniura lymma* 2	Nhulunbuy (Gove), Northern Territory, Australia, Pacific Ocean
Echinobothrium joshuai Rodriguez, Caira and Pickering, 2011	Valid	Rodriguez et al. (2011)	*Cruriraja hulleyi*	Anacanthobatidae	KC860166 KC860167	KC860118 KC860119	KC860212 KC860213	DI-42; AF-17DI-43; AF-17	LRP 8014 LRP 8015	*Cruriraja hulleyi*	Off South Africa, Indian Ocean
Echinobothrium levicolle Lespés, 1857	*Species inquirenda*	Lespés (1857)	*Nassa reticulata*	MOLLUSC							
Echinobothrium longicolle Southwell, 1925	Valid	Southwell (1925)	*Trygon* (=*Neotrygon*) *kuhlii*	Dasyatidae							
Echinobothrium mathiasi Euzet, 1951	Valid	Euzet (1951)	*Leiobatis* (=*Myliobatis*) *aquila*	Myliobatidae							
Echinobothrium minutamicum Twohig, Caira and Fyler, 2008	Valid	Twohig et al. (2008)	*Himantura walga*	Dasyatidae							
Echinobothrium nagabhushani Chincholikar and Shinde, 1976) Tyler, 2006	*Species inquirenda*	Chincholikar and Shinde (1976); Tyler (2006)	"*Trygon* sp."	Dasyatidae							
Echinobothrium nigracanthum Reimer, 1975	*Species inquirenda*	Reimer (1975)	*Bullia melanoides*	MOLLUSC							
Echinobothrium persiense Haseli, Malek, Palm and Ivanov, 2012	Valid	Haseli et al. (2012)	*Rhinobatos punctifer*	Rhinobatidae							
Echinobothrium raji Heller, 1949	Valid	Heller (1949)	*Raja scabrata* (=*Amblyraja radiata*?)	Rajidae							
Echinobothrium reesae Ramadevi, 1969	Valid	Ramadevi (1969)	*Himantura walga*	Dasyatidae							
Echinobothrium rhynchobati (Khalil and Abdul-Salam, 1989) Tyler, 2006	Valid	Khalil and Abdul-Salam (1989); Tyler (2006)	*Rhynchobatus* [sic] (=*Glaucostegus*) *granulatus*	Rhinobatidae							
Echinobothrium cf. *rhynchobati* 1	Undescribed?	This study		Rhinobatidae	KC860138 KC860139 KC860140	KC860088 KC860089 KC860090	KC860186 KC860187 KC860188	DI-1; BO-120 DI-2; BO-120 DI-3; BO-120	LRP 8016 LRP 8017 LRP 8018	*Glaucostegus* cf. *typus*	off Sabah, Malaysia, Sulu Sea, Pacific Ocean
Echinobothrium cf. *rhynchobati* 2	Undescribed?	Olson and Caira (1999); Bray and Olson (2004)		Rhinobatidae	AF124463[d]	AY584861[e]			LRP 2149[f]; BMNH 2004.3.18.101[g]	*Glaucostegus typus* (partly as *Rhinobatos typus*)	Darwin, Northern Territory, Australia, Timore Sea and Yorkey's Knob, Queensland, Australia, Pacific Ocean;

Echinobothrium sematanense Ivanov and Caira, 2012	Valid	Ivanov and Caira (2012)	*Glaucostegus thouin*	Rhinobatidae	KC860155	KC860106	KC860203	DI-24; CM03-35	LRP 8019		Weipa, Queensland, Australia, Indian Ocean
Echinobothrium sinensis (Li and Wang, 2007) Kuchta and Caira, 2010	Valid	Li and Wang (2007); Kuchta and Caira (2010)	*Platyrhina sinensis*	Platyrhinidae							
Echinobothrium syrtensis (Neifar, Tyler and Euzet, 2001) Tyler, 2006	Valid	Neifar et al. (2001); Tyler (2006)	*Rhinobatos cemiculus*	Rhinobatidae							
Echinobothrium tetabuanense Ivanov and Caira, 2012	Valid	Ivanov and Caira (2012)	*Glaucostegus* cf. *typus*	Rhinobatidae	KC860141 KC860142	KC860091 KC860092	KC860189 KC860190	DI-04; BO-120 DI-05; BO-120	LRP 8020 LRP 8021	*Glaucostegus* cf. *typus*	Kampung Tetabuan, Sabah, Malaysia, Sulu Sea, Pacific Ocean
Echinobothrium typus van Beneden, 1849	Valid	van Beneden (1849)	"raie bouclée" (=*Raja clavata* by Tyler, 2006)	Rajidae							
Echinobothrium weipaense Ivanov and Caira, 2012	Valid	Ivanov and Caira (2012)	*Glaucostegus typus*	Rhinobatidae							
Echinobothrium n. sp. 1	Undescribed	This study		Dasyatidae	KC860154	KC860105		DI-23; BO-477	LRP 8022	*Dasyatis* cf. *zugei*	Mukah, Malaysia, Pacific Ocean
Echinobothrium n. sp. 2	Undescribed	This study		Rajidae	KC860184	KC860136	KC860228	DI-68; AF-129	LRP 8023	*Leucoraja wallacei*	Off South Africa, Indian Ocean
Echinobothrium n. sp. 3	Undescribed	This study		Rajidae	KC860175	KC860127		DI-59; AF-148	LRP 8024	*Raja* cf. *miraletus* 1	Off South Africa, Indian Ocean
Echinobothrium n. sp. 4	Undescribed	This study		Dasyatidae		KC860095	KC860193	DI-10; BO-336	LRP 8025	*Neotrygon kuhlii* 1	Off Sarawak, Malaysia, South China Sea, Pacific Ocean
Echinobothrium n. sp. 5	Undescribed	This study		Dasyatidae	KC860174	KC860126		DI-56; KA-255	LRP 8026	*Himantura oxyrhyncha*	Jungkat, Indonesia, Pacific Ocean
Echinobothrium n. sp. 6	Undescribed	This study		Rhinobatidae	KC860168	KC860120	KC860214	DI-44; AF-141	LRP 8027	*Rhinobatos annulatus*	Off South Africa, Indian Ocean
Halysioncum boisii (Southwell, 1911) n. comb.	*Species inquirenda, incertae sedis*	Southwell (1911)	*Aetobatis* (=*Aetobatus*) *narinari*	Myliobatidae							
Halysioncum bonasum (Williams and Campbell, 1980) n. comb.	Valid	Williams and Campbell (1980)	*Rhinoptera bonasum*	Rhinopteridae	KC860143 KC860144	KC860093 KC860094	KC860191 KC860192	DI-06; SSC-4 DI-07; SSC-4	LRP 8028 LRP 8029	*Rhinoptera* cf. *steindachneri*	Mississippi, USA, Gulf of Mexico
Halysioncum californiense (Ivanov and Campbell, 1998) n. comb.	Valid	Ivanov and Campbell (1998a)	*Platyrhinoidis triseriata*	Platyrhinidae							
Halysioncum euzeti (Campbell and Carvajal, 1980) n. comb.	Valid	Campbell and Carvajal (1980)	*Psammobatis* (=*Sympterygia*) *lima*	Arhynchobatidae							
Halysioncum fautleyae (Tyler and Caira, 1999) n. comb.	Valid	Tyler and Caira (1999)	*Myliobatis longirostris; Myliobatis californica*	Myliobatidae							
Halysioncum hoffmanorum (Tyler, 2001) n. comb.	Valid	Tyler (2001)	*Urobatis maculatus; Urobatis halleri, Urobatis concentricus*	Urobatidae							

(*continued on next page*)

Table 1 (*continued*)

Species	Taxonomic status	Source	Type host; additional host(s)	Host family	GenBank No. (18S rDNA)	GenBank No. (28S rDNA)	GenBank No. (COI)	Molecular spcm. No.; host spcmNo. [a]	Museum voucher No.	Host of sequenced spcm.	Locality of sequenced spcm.
Halysioncum mexicanum (Tyler and Caira, 1999) n. comb.	Valid	Tyler and Caira (1999)	*Rhinoptera steindachneri; Myliobatis californica*	Rhinopteridae; Myliobatidae	KC860153 KC860152	KC860104 KC860103	KC860202 KC860201	DI-22; BJ-626 DI-21; BJ-626	LRP 8030LRP 8031	*Myliobatis californica*	Bahia de Los Angeles, Mexico, Pacific Ocean
Halysioncum megacanthum (Ivanov and Campbell, 1998) n. comb.	Valid	Ivanov and Campbell (1998b)	*Myliobatis goodei*	Myliobatidae							
Halysioncum nataliae (Kuchta and Caira, 2010) n. comb.	Valid	Kuchta and Caira (2010)	*Pastinachus solocirostris*	Dasyatidae	KC860145	KC860096	KC860194	DI-11; BO-464	LRP 8032	*Pastinachus solocirostris*	Mukah, Malaysia, Pacific Ocean
Halysioncum pigmentatum (Ostrowski de Núñez, 1971) n. comb.	Valid	Ostrowski de Núñez (1971)	*Zapteryx brevirostris*	Rhinobatidae							
Halysioncum raschii (Campbell and Andrade, 1997) n. comb.	Valid	Campbell and Andrade (1997)	*Rhinoraja longi*	Arhynchobatidae							
Halysioncum rayallemangi (Tyler, 2001) n. comb.	Valid	Tyler (2001)	*Rhinobatos leucorhynchus*	Rhinobatidae							
Halysioncum reginae (Kuchta and Caira, 2010) n. comb.	Valid	Kuchta and Caira (2010)	*Pastinachus* cf. *atrus*	Dasyatidae							
Halysioncum rhinoptera (Shipley and Hornell, 1906) n. comb.	*Species inquirenda, incertae sedis*	Shipley and Hornell (1906)	*Rhinoptera javanica*	Rhinopteridae							
Halysioncum vojtai (Kuchta and Caira, 2010) n. comb.	Valid	Kuchta and Caira (2010)	*Pastinachus gracilicaudus* (as *Pastinachus* sp.)	Dasyatidae							
Halysioncum n. sp. 1	Undescribed	This study		Rhinopteridae		KC860107	KC860204	DI-25; CM03-43	LRP 8033	*Rhinoptera neglecta*	Weipa, Queensland, Australia, Indian Ocean
Halysioncum n. sp. 2	Undescribed	This study		Rhinopteridae	KC860156 KC860157	KC860108 KC860109		DI-26; SE-249 DI-27; SE-249	LRP 8034 LRP 8035	*Rhinoptera* cf. *bonasus*	Diogue, Senegal, Atlantic Ocean
Halysioncum n. sp. 3	Undescribed	This study		Myliobatidae	KC860158	KC860110	KC860205	DI-28; SE-257	LRP 8036	*Pteromylaeus bovinus*	Diogue, Senegal, Atlantic Ocean
Halysioncum n. sp. 4	Undescribed	This study		Myliobatidae	KC860183	KC860135	KC860227	DI-67; AF-74	LRP 8037	*Myliobatis aquila*	Off South Africa, Indian Ocean
Halysioncum n. sp. 5	Undescribed	This study		Dasyatidae	KC860149	KC860100	KC860198	DI-17; NT-105	LRP 8038	*Pastinachus atrus*	Northern Territory, Australia, Arafura Sea Pacific Ocean
New genus n. sp. 1	Undescribed	This study		Rajidae	KC860171 KC860172 KC860185	KC860123 KC860124 KC860137	KC860217 KC860218 KC860229	DI-47; AF-29 DI-48; AF-29 DI-70; AF-127	LRP 8039 LRP 8040 LRP 8041	*Leucoraja wallacei*	Off South Africa, Indian Ocean

[a] See http://elasmobranchs.tapewormdb.uconn.edu for host specimen details.
[b] As *Ditrachybothridium macrocephalum.*
[c] Voucher number for AY584864.
[d] As *Macrobothridium* sp.Please cite footnote “d” in Table 1.
[e] As *Macrobothridium rhynchobati.*
[f] Voucher number for AF124463.
[g] Voucher number for AY584861.

specifically: 2:1:1:1, 2:1:1:2, 2:1:2:1, 2:2:1:1, 2:2:1:2, 2:2:2:1, 2:4:1:1, 2:4:1:2 and 2:4:2:1. For each cost ratio, we ran five iterations; in each we searched for optimal solutions by performing three sequential searches of 4 h each (POY command "search(max_time:0:4:0)"), after which the best and unique trees were selected. All tree searches under optimization alignment were performed on a 16 × 2.83 GHz Q9550 Intel® Core™2 Quad CPU cluster at the Department of Zoology-IB, University of São Paulo, Brazil. During each tree search, tree length was calculated as the sum of the costs for all hypothesized substitutions and INDELs via optimization of Sankoff characters (Sankoff and Rousseau, 1975). Upon completion of the first step, we re-diagnosed unique trees to reduce alignment lengths, and hence tree lengths, using iterative pass (IP) optimization (Wheeler, 2003) for all 10 cost ratios. All trees were rooted using the trypanorhynch *P. clarkeae*.

Upon completion of the search and refinement steps described above, we selected the tree generated by the cost ratio that assumed equal weight for all transformations as our working hypothesis. To evaluate the dependence of our results (i.e., clades) using different cost ratios, we performed a sensitivity analysis for most clades on the best topology using Cladescan (version 1.0; Sanders, 2009). Nodal support was evaluated using the Goodman–Bremer support metric (Goodman et al., 1982; Bremer, 1988; see Grant and Kluge, 2008). To obtain this metric in POY, we diagnosed the selected tree under IP, transformed the implied alignment into static homology characters and exported the matrix in the Hennig86 (Farris, 1989) format. We then performed Goodman–Bremer support calculations (command calculate_support) in POY considering 1,000 tree constructions retaining up to two of the best trees generated during branch swapping on each Wagner tree (e.g., command "calculate_support (bremer, build (trees:1000), swap(trees:2))"). Scripts illustrating each step of our analyses can be downloaded from http://lhe.ib.usp.br/data/.

2.3.2. Maximum likelihood analyses

In total 70 maximum likelihood (ML) analyses were conducted with GARLI (version 2.0; 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; Zwickl, 2006–2011. GARLI – Genetic Algorithm for Rapid Likelihood Inference Available: https://www.nescent.org/wg_garli/Main_Page. Accessed November 2012) on the seven data partition models (Partition Models 1–7 in Supplementary Fig. S1) for the implied alignments generated by POY/IP for each of the 10 cost ratios (i.e., opening gaps:gap extension:transversion:transition). If the POY/IP analysis for a cost ratio returned more than a single optimum alignment, the first was selected for ML analysis. The seven data partition models differed with respect to whether the genes were treated as separate partitions or combined, and also with respect to partitioning of the three codon positions of COI (see Supplementary Fig. S1).

For each partition within each partition model the best fitting substitution model was selected based on the corrected Akaike Information Criterion (AICc) (see Posada and Buckley, 2004) using GModeltest.pl (PERL script that calculates AICcbased on GARLI [version 2.0; 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; Zwickl, 2006–2011. GARLI – Genetic Algorithm for Rapid Likelihood Inference Available: https://www.nescent.org/wg_garli/Main_Page. Accessed November 2012]; runs available upon request). In total 88 substitution models were evaluated for the five partitions of COI (i.e., the whole fragment, each codon position individually, and first and second codon partitions only), and for each implied alignment of 28S rDNA, 18S rDNA, 28S rDNA + 18S rDNA and 28S rDNA + 18S rDNA + COI. Elements evaluated with the substitution models included 11 substitution schemes, unequal/equal base frequencies, proportion of invariable sites and four categories of variable rates.

Tree searches were performed using the parallel implementation of GARLI (version 2.0; Zwickl, 2006–2011). For each of the 70 analyses, 100 independent search replicates (searchreps = 10 in 10 CPUs) were conducted using different subset rates (linkmodels = 0 and subsetspecificrates = 1), and the remaining default parameters of the GARLI configuration file. We selected the optimal topology for each implied alignment/partition model/substitution model based on likelihood scores. The selection of our working hypothesis using this optimality criterion was based on AICc scores of those implied alignment/partition model/substitution models. For each implied alignment, the analysis using the selected partition model/substitution model combination was then re-run using a more aggressive search strategy considering a total of 1,000 independent search replicates (searchreps = 100 in 10 CPUs). Nodal support was inferred by bootstrap proportions after 2,000 bootstrap replicates with one independent search replicate each (bootstrapreps = 100 and searchreps = 1 in 10 CPUs). Bootstrap results were compiled using SUMTREES (version 3.1.0; Sukumaran and Holder, 2010). All ML analyses were performed on a 10 × 2.83 GHz Q9550 Intel® Core™2 Quad CPU cluster. Dataset and an example of a configuration file for GARLI can be downloaded from http://lhe.ib.usp.br/data/.

2.4. Material examined

In combination, the authors of this paper have described or contributed to the descriptions of 22 of the 50 valid species of diphyllideans and thus have intimate knowledge of the morphology of these taxa. With respect to the remaining 28 species the following material was examined for the purposes of this project: *Ditrachybothridium macrocephalum* Rees, 1959 (paratype: BMNH 1959.8.4.196); *Echinobothrium typus* van Beneden, 1849 (eight specimens from L. Euzet from *Raja clavata* L.); *Echinobothrium acanthinophyllum* Rees, 1961 (holotype: BMNH No. 1962.28.14); *E. acanthinophyllum* (15 specimens from G. Rees, MHNG, ex. *Raja montagui* Fowler), *Echinobothrium affine* Diesing, 1863 (six specimens from L. Euzet from *R. clavata*); *Echinobothrium acanthocolle* Wojciechowska, 1991 (holotype: No. 1237 Polish Academy of Sciences; *E. affine* (neotype: BMNH No. 1976.4.13.32); *E. bonasum* Williams and Campbell, 1980 (holotype: USNPC No. 75770); *Echinobothrium chisholmae* Jones and Beveridge, 2001 (paratype: BMNH No. 2000.8.3.4); *Echinobothrium clavatum* Probert and Stobart, 1989 (lectotype: BMNH No. 1988.6.1.1-3); *Echinobothrium coronatum* Robinson, 1959 (holotype: ZW No. 202a, 202b); *Echinobothrium euzeti* Campbell and Carvajal, 1980 (holotype: USNPC No. 75774); *Echinobothrium harfordi* McVicar, 1976 (paratype: BMNH No. 1975.9.16.2); *Echinobothrium helmymohamedi* Saoud, Ramadan and Hassan, 1982 (paratype: BMNH No. 1998.10.19.113); *Echinobothrium longicolle* Southwell, 1925 (four syntypes: L. Euzet); *Echinobothrium mathiasi* Euzet, 1951 (holotype and three paratypes: L. Euzet); *Echinobothrium pigmentatum* Ostrowski de Núñez, 1971 (holotype: personal collection Ostrowski de Núñez); *Echinobothrium raschii* Campbell and Andrade, 1997 (paratype: USNPC No. 86770); *Echinobothrium rhynchobati* (Khalil and Abdul-Salam, 1989) Tyler, 2006 (one paratype: BMNH Nos. 1998.11.20.319–323). *Macrobothridium* sp. ex. *Glaucostegus typus* (Anonymous [Benett]) as *R. typus*, Coll. I. Beveridge, Yorkey's Knob, Qld (mol. voucher: BMNH 2004.3.18.101). *Ditrachybothridium macrocephalum* (one gravid specimen, ex. *Galeus melastomus* Rafinesque, N. North Sea, BMNH 1973.6.11.11–13); *D. macrocephalum* (six excysted specimens, ex. "*Raja*? *bigelowi*", Porcupine Sea Bight, BMNH 2004.1.6.6–11); *D. macrocephalum* (two encysted specimens, ex. *Apristurus laurussonii* (Saemundsson), Goban Spur, BMNH

2004.1.6.1–5); *D. macrocephalum* (one excysted specimen, ex. *Apristurus* sp., Porcupine Bight, BMNH 2001.19.5).

Museum abbreviations used are as follows: BMNH, The Natural History Museum, London, United Kingdom; LRP, Lawrence R. Penner, Parasitology Collection, University of Connecticut, Storrs, CT, USA; USNPC, U.S. National Parasite Collection, Beltsville, MD, USA; ZW, Museum of New Zealand, Wellington, New Zealand. Host taxonomy and classification follow Naylor et al. (2012a,b respectively).

3. Results

3.1. Phylogenetic analyses

The total number of unaligned base pairs for COI was 504–507, for 28S rDNA was 797–858, and for 18S rDNA was 1,524–1,565. The total lengths of the POY optimal alignments for each of the 10 cost ratios are given in Table 2 (see Supplementary Data S1 for more detail); including the COI data these ranged from 2,980 (cost ratio 2:4:1:2) to 3,099 (cost ratio 0:1:1:1). The parameter set with a cost ratio of 0:1:1:1 yielded the two shortest tree topologies, each with a cost of 3,663 steps. These trees proposed ambiguous sister-group relationships for terminals with zero or near zero branch lengths; the tree presented in Fig. 2 represents the strict consensus of these two POY trees. Taxon names shown incorporate generic-level taxonomic actions proposed below. Bootstrap support values (ML analyses) and Goodman–Bremer support (POY analyses) are given above and below branches, respectively for each node. The optimal topology resulting from ML was derived from the implied alignment based on the cost ratio 0:1:1:1 under Partition Model 3 (see Supplementary Data S1 for more detail), which considered a distinct unlinked substitution model for each non-coding region and for each codon position of COI (i.e., Partition Model 3, Table 3) and had a likelihood score of −18930.1759. The results of all implied alignment/partition model/substitution model combinations are given in Supplementary Table S1. This ML topology differed from the tree in Fig. 2 in only three respects: (i) placement of *Echinobothrium euterpes* as sister to the clade consisting of *E. heroniense*, *E.* cf. *heroniense*, *Echinobothrium* n. sp. 4, *E. harfordi*, *Echinobothrium dougbermani*, *Echinobothrium* n. sp. 5, and *Echinobothrium* n. sp. 6, (ii) placement of *Halysioncum nataliae* as sister to *Halysioncum* n. sp. 3 + *Halysioncum* n. sp. 4, (iii) *Halysioncum* n. sp. 1 as sister to *Halysioncum* n. sp. 2. Sensitivity plots are shown for all nodes of consequence in considerations of generic boundaries. Each plot indicates presence (black squares) or absence (white squares) of support of the clade in the parsimony analyses (upper 10 squares) and ML analyses (lower 10 squares) of the 10 implied alignments based on distinct cost ratios. Plots suggest that most nodes were insensitive to the array of alignment parameters explored in the analyses.

We have concentrated on generic- rather than specific-level inferences because our taxon sampling focused on maximizing representation of major morphological differences, rather than number of species. Given that 38 of the 50 valid diphyllidean species are not represented in our analyses any interspecific relationships implied by the analyses require confirmation in the context of more dense taxon sampling.

3.2. Classification and generic boundaries

Our results revealed a substantial amount of well-supported phylogenetic structure within the Diphyllidea. A number of taxonomic actions are required if diphyllidean generic-level classification is to be congruent with these relationships. Most conspicuously, there is no support for the monophyly of *Echinobothrium* as it stands unless the Diphyllidea is considered to consist solely of this single genus. As an alternative to this impractical solution we propose that generic status be assigned to six clades/lineages supported by our analyses. To validate this proposition we have identified morphological features to diagnose five of these genera. Overall, the revised generic classification involves: (i) narrowing of the concept of *Echinobothrium*, (ii) recognition of three new genera, two of which are formally erected here, (iii) slight modifications of the diagnoses of *Ditrachybothridium* and *Ahamulina*, and confirmation of *Macrobothridium* as a synonym of *Echinobothrium sensu stricto*. Table 1 provides revised generic assignments, establishing new combinations as needed, for all nominal diphyllidean species regardless of whether they were included in our molecular analyses; these placements were based on the diagnostic morphological features identified below for each genus.

3.2.1. Echinobothrium van Beneden, 1849 sensu stricto (Figs. 1E, F and 4C)

The following diagnosis narrows the concept of *Echinobothrium* from those of Khalil (1994) and Tyler (2006).

Diagnosis: Scolex with one dorsal and one ventral bothrium, armed apical organ, and cephalic peduncle. Bothria free posteriorly for some of their length, covered with palmate, pectinate and/or trifid spinitriches on proximal surfaces, with trifurcate spinitriches on distal surfaces. Apical organ with one dorsal and one ventral group of solid hooks; hooks in each group arranged in two regular rows consisting of A hooks (anterior row) alternating with B hooks (posterior row); adjacent hooks articulating with one another. Lateral hooklets arranged in distinct clusters on either side of dorsal and ventral group of apical hooks (Fig. 1F). Corona of spines between apical organ armature and bothria lacking. Cephalic peduncle with or without eight columns of posteriorly directed spines with triradiate or rarely multi-lobed bases, acraspedote in most species. Worms apolytic or euapolytic. Common genital pore mid-ventral. Cirrus sac unipar-

Table 2
Summary of tree lengths and number of trees obtained during OA/IP analyses for 10 cost ratios. Row in bold indicates cost ratio resulting in shortests tree(s).

Cost ratio	Cost range OA	Number of compiled trees	Number of unique/best trees	Cost IP	Final MPTs
0:1:1:1	3671	10	2	3663	2
2:1:1:1	3985	10	2	3977	2
2:1:1:2	6349	52	18	6341	18
2:1:2:1	5873	58	12	5866	6
2:2:1:1	4480	20	4	4474	4
2:2:1:2	7269–7270	49	45/15	7228	27
2:2:2:1	6841	37	9	6835	9
2:4:1:1	5331	27	10	5322	10
2:4:1:2	8803	53	32	8763	32
2:4:2:1	8514–8516	46	32/15	8505	9

IP, iterative pass; MPT, most parsimonious tree; OA, optimization alignment.

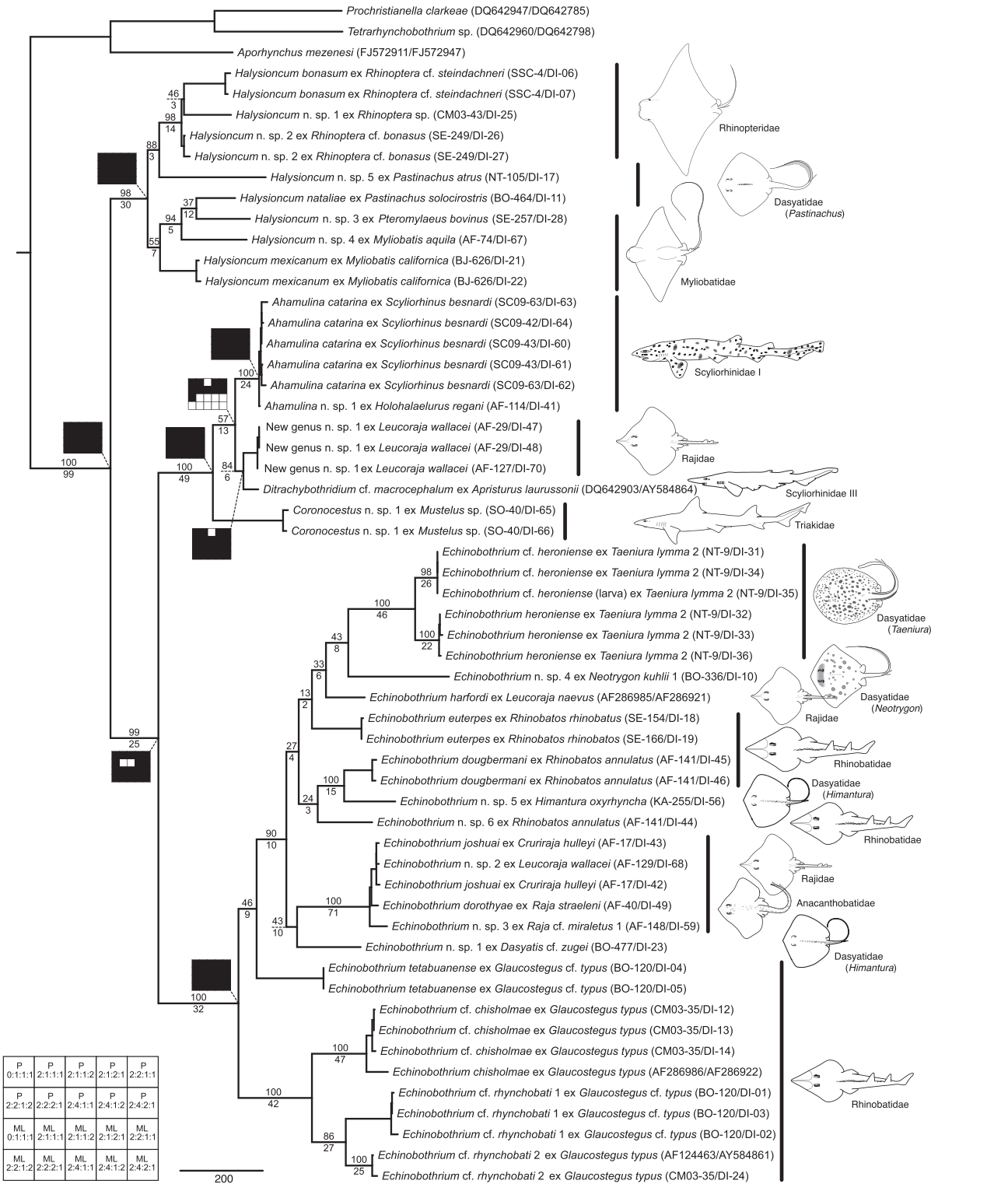

Fig. 2. Strict consensus of two most parsimonious trees based on combined ribosomal nucleic acid small subunit (18S rDNA), partial ribosomal nucleic acid large subunit (28S rDNA) and partial cytochrome oxidase subunit I (COI) data partitions resulting from an analysis using direct optimization under the cost ratio of 0:1:1:1 (i.e., no penalty for opening gaps and equal costs for all three transformation types); nodal support is given as bootstrap values (above the line) and Goodman-Bremer values (below the line); sensitivity plots are shown for selected nodes indicating presence (black square) or absence (white square) of support for that node in the parsimony (P) analyses (top two rows) and maximum likelihood (ML) analyses (bottom two rows) for each of the 10 cost ratio alignments; elasmobranch icons represent the host families (and genera for Dasyatidae) parasitized by the respective diphyllidean taxon.

tite; cirrus armed with spinitriches. Testes in one to many columns anterior to ovary. Vagina opening posterior to cirrus sac. Ovary H-shaped in frontal view, bilobed in cross-section. Vitellarium follicular; vitelline follicles in two lateral bands or circumcortical. Uterus saccate, ventral. Eggs unembryonated when laid. Primarily parasites of Rajidae, Rhinobatidae, Dasyatidae; some in Anacanthobatidae, Platyrhinidae, Rhynchobatidae and Myliobatidae.

Table 3
Maximum likelihood (nIL) and corrected Akaike Information Criterion (AICc) scores for maximum likelihood analyses using Partition Model 3 for 10 cost ratios. Row in bold indicates cost ratio of iterative pass (IP)-implied alignment resulting in lowest AICc score.

Cost ratio	Substitution models for Partition Model 3: [28S rDNA][18S rDNA][COI 1st][COI 2nd][COI 3rd]	nIL	AICc
0:1:1:1	[JC][GTR+I+G][TrN+G][F81+G][TrN+I+G]	−18930.1759	38162.48856
2:1:1:1	[GTR+I+G][TIM2+I+G][TrN+G][F81+G][TrN+I+G]	−19379.3008	39067.80491
2:1:1:2	[TPM3uf+I+G][TIM2+I+G][TrN+G][F81+G][TrN+I+G]	−19852.879	40008.37993
2:1:2:1	[TIM3+I+G][TIM2+I+G][TrN+G][F81+G][TrN+I+G]	−19414.6684	39134.14106
2:2:1:1	[TPM3uf+I+G][TIM2+I+G][TrN+G][F81+G][TrN+I+G]	−19700.9324	39704.50210
2:2:1:2	[TPM3uf+I+G][TIM2+I+G][TrN+G][F81+G][TrN+I+G]	−20309.8061	40922.29067
2:2:2:1	[TPM3uf+I+G][TIM2+I+G][TrN+G][F81+G][TrN+I+G]	−19677.0274	39656.70739
2:4:1:1	[TPM3uf+I+G][TIM3+I+G][TrN+G][F81+G][TrN+I+G]	−20399.0647	41100.81813
2:4:1:2	[TVM+G][TIM3+I+G][TrN+G][F81+G][TrN+I+G]	−20929.3152	42163.57045
2:4:2:1	[TPM3uf+I+G][TrN+I+G][TrN+G][F81+G][TrN+I+G]	−20290.3178	40881.15230

Type species: Echinobothrium typus van Beneden, 1849, ex "raie bouchlée" (probably = *R. clavata*).

Additional species: The additional 34 described members (28 of which are valid) and 10 undescribed members of *Echinobothrium sensu stricto* included in our molecular analyses are listed in Table 1.

Remarks: The above diagnosis revises the current concept of *Echinobothrium* (e.g., Khalil, 1994; Tyler, 2006) to include only taxa possessing apical hooks and lateral hooklets arranged in two clusters, with or without cephalic peduncle spines. Although the type of the genus, *E. typus*, was not included in our molecular analyses the above revised generic diagnosis is fully consistent with Tyler's (2006) redescription of this species. Species lacking cephalic peduncle spines (e.g., *E.* cf. *rhynchobati* 1, *E.* cf. *rhynchobati* 2, and *E. euterpes*) were found to cluster robustly among species of *Echinobothrium* bearing cephalic peduncle spines, reinforcing the synonymy between *Echinobothrium* and *Macrobothridium*.

3.2.2. Ahamulina Marques, Jensen and Caira, 2012 (Figs. 1A–D, 4B)

The following diagnosis is slightly emended from that of Marques et al. (2012) to include the undescribed species used in this study, as well as features that aid in distinguishing *Ahamulina* from the two new genera erected below.

Diagnosis: Scolex with one dorsal and one ventral bothrium, armed apical organ, and cephalic peduncle. Bothria free posteriorly for much of their length, with trifurcate spinitriches on proximal and distal surfaces. Apical organ bearing one dorsal and one ventral group of solid hooks; hooks in each group arranged in single row; adjacent hooks articulating or not with one another. Lateral hooklets absent. Corona of spines between apical organ and bothria lacking. Cephalic peduncle short, unarmed, craspedote. Worms apolytic. Common genital pore mid-ventral. Cirrus sac bipartite, consisting of spherical proximal portion and tubular distal portion; cirrus armed with spinitriches. Testes in multiple columns anterior to ovary. Vagina opening posterior to cirrus sac. Ovary inverted-A shaped in frontal view, bilobed in cross-section. Vitellarium follicular; vitelline follicles circumcortical, anterior to ovary. Uterus saccate, ventral; uterine duct extensive, sinuous. Eggs unembryonated when laid. Parasites of catsharks (Scyliorhinidae I and III sensu Naylor et al., 2012b).

Type species: Ahamulina catarina Marques, Jensen and Caira, 2012.

Additional species: Although undescribed, *Ahamulina* n. sp. 1 from *Holohalaelurus regani* (Gilchrist) in the Indian Ocean off South Africa appears to represent a second species based on morphological and molecular data.

Remarks: As noted by Marques et al. (2012), the armature of *Ahamulina catarina* is very rudimentary. The apical hooks are arranged in a single irregular row and adjacent hooks do not articulate with one another (Fig. 1B). Furthermore, the hooks are only tenuously attached to the scolex. The second species (included here as *Ahamulina* n. sp. 1), which was almost identical in sequence with *A. catarina*, shares some, but not all of these diagnostic features. Like *A. catarina*, it lacks spines on the cephalic peduncle (Fig. 1D), the armature of its apical organ consists of apical hooks arranged in a single row and lateral hooklets are lacking. However, unlike those of *A. catarina*, adjacent apical hooks articulate with one another and consist of two sizes of hooks (small and large) that alternate (Fig. 1C) but begin at the same level on the scolex. Thus, the resemblance to the two-row arrangement consisting of A hooks (anterior row) alternating with B hooks (posterior row) seen in other genera is only superficial.

3.2.3. Ditrachybothridium Rees, 1959 (Fig. 4A)

The following diagnosis combines information from Rees (1959) and Tyler (2006), and is slightly emended to include features that aid in distinguishing *Ditrachybothridium* from the two new genera erected below.

Diagnosis: Scolex with one dorsal and one ventral bothrium, weakly developed apical organ and unarmed cephalic peduncle; apical organ lacking apical hooks and lateral hooklets. Bothria free posteriorly for much of their length, with coniform spinitriches on proximal surfaces and trifurcate, palmate or pectinate spinitriches on distal surfaces. Corona of spines between apical organ armature and bothria lacking. Cephalic peduncle unarmed, short, craspedote. Worms apolytic. Common genital pore mid-ventral. Cirrus sac unipartite. Cirrus armed with spinitriches. Testes in multiple columns anterior to ovary. Vagina opening posterior to cirrus sac. Ovary H-shaped in frontal view, bilobed in cross-section. Vitellarium follicular; vitelline follicles in two lateral bands. Uterus saccate, ventral. Eggs unembryonated when laid. Primarily parasites of catsharks (Scyliorhinidae I and III sensu Naylor et al., 2012b) and Rajidae.

Type species: Ditrachybothridium macrocephalum Rees, 1959.

Additional species: Ditrachybothridium piliformis Faliex, Tyler and Euzet, 2000.

Remarks: Examination of museum material identified as *D. macrocephalum* taken from a diversity of hosts suggests that *Ditrachybothridium* may be more speciose than currently recognized. Not only do these specimens collectively differ in bothrial length and width, but they also exhibit a diversity of forms of "spines", referred to herein as coniform spinitriches, covering the proximal bothrial surfaces and we are fairly confident they represent several distinct species. Unfortunately, in no case was sufficient material available for the description of novel taxa. Furthermore, in several cases the specimens are excysted juveniles bearing only rudimentary reproductive tissue. Nonetheless, this calls into question the breadth of the host associations reported for *D. macrocephalum*. It also causes us to question the identity of Bray and Olson's (2004) specimens (DQ642903; AY584864), taken from the catshark *A. laurussonii*, used here. In order to call attention to this potential issue we have referred to these specimens as *D.* cf. *macrocephalum*.

3.2.4. Halysioncum n. gen. (Figs. 1G and 4D)

Diagnosis: Scolex with one dorsal and one ventral bothrium, armed apical organ and cephalic peduncle. Bothria free posteriorly for part of their length, covered with palmate, pectinate and/or trifid spinitriches on proximal surfaces, with palmate, trifid, or trifurcate spinitriches on distal surfaces. Apical organ with one dorsal and one ventral group of solid hooks; hooks in each group arranged in two regular rows consisting of A hooks (anterior row) alternating with B hooks (posterior row); adjacent hooks articulating with one another. Lateral hooklets arranged in single continuous band flanking dorsal and ventral groups of apical hooks on each side (Fig. 1G). Corona of spines between apical organ armature and bothria lacking. Cephalic peduncle armed with eight columns of posteriorly directed spines with triradiate bases, acraspedote. Worms apolytic or euapolytic. Common genital pore mid-ventral. Cirrus sac unipartite; cirrus armed with spinitriches. Testes in one to many columns anterior to ovary. Vagina opening posterior to cirrus sac. Ovary H-shaped in frontal view, bilobed in cross section. Vitellarium follicular; vitelline follicles in two lateral bands. Uterus saccate, ventral. Eggs unembryonated when laid. Primarily parasites of Myliobatidae and Rhinopteridae, some in Dasyatidae, Urotrygonidae, and Arhynchobatidae, occasionally in Platyrhinidae and Rhinobatidae.

Type species: Halysioncum mexicanum (Tyler and Caira, 1999) n. comb.

Additional species: Halysioncum boisii (Southwell, 1911) n. comb.; *Halysioncum bonasum* (Williams and Campbell, 1980) n. comb.; *Halysioncum californiense* (Ivanov and Campbell, 1998) n. comb.; *Halysioncum euzeti* (Campbell and Carvajal, 1980) n. comb.; *Halysioncum fautleyae* (Tyler and Caira, 1999) n. comb.; *Halysioncum hoffmanorum* (Tyler, 2001) n. comb., *Halysioncum megacanthum* (Ivanov and Campbell, 1998) n. comb.; *Halysioncum nataliae* (Kuchta and Caira, 2010) n. comb.; *Halysioncum pigmentatum* (Ostrowski de Núñez, 1971) n. comb.; *Halysioncum raschii* (Campbell and Andrade, 1997) n. comb.; *Halysioncum rayallemangi* (Tyler, 2001) n. comb.; *Halysioncum reginae* (Kuchta and Caira, 2010) n. comb.; *Halysioncum rhinoptera* (Shipley and Hornell, 1906) n. comb.; and *Halysioncum vojtai* (Kuchta and Caira, 2010) n. comb.

Etymology: Halysioncum (*halysion*, Gr. n. diminutive, chain; *onkos*, Gr. hook) refers to the continuous configuration of the lateral hooklets in members of this genus.

Remarks: Halysioncum n. gen. conspicuously differs from *Ditrachybothridium* in its possession of armature on its apical organ and cephalic peduncle. It is readily distinguished from *Ahamulina* in its possession of lateral hooklets and cephalic peduncle armature. It most closely resembles *Echinobothrium sensu stricto* but differs in that its lateral hooklets are arranged in a continuous band rather than in distinct clusters on either side of the dorsal and ventral groups of hooks (Fig. 1G).

Based on their possession of a continuous band of lateral hooklets we have formally transferred 15 species (13 valid) from *Echinobothrium* to this new genus (see Table 1). In addition to three of these described species (i.e., *H. bonasum*, *H. nataliae*, and *H. mexicanum*), our molecular analyses also included five undescribed species. The host associations of the 20 species recognized here, suggest that *Halysioncum* has a particular affinity for batoids of the families Myliobatidae (eagle rays) and Rhinopteridae (cownose rays). However, it also includes a few species that parasitize Arhynchobatidae, Dasyatidae (specifically *Pastinachus* Rüppell), as well as Platyrhinidae, Rhinobatidae, and Urotrygonidae.

Although they were among the species transferred to this new genus, *H. boisii* and *H. rhinoptera* are somewhat problematic given that the configuration of their lateral hooklets is unclear. Both species were described from a paucity of material and Southwell (1911) made no mention of lateral hooklets in the former species; Shipley and Hornell (1906) described the latter species as lacking both apical hooks and lateral hooklets. We suspect the specimens of these species may have been in poor condition. Unfortunately the location of their type material is unknown. A specimen (LRP 8043) available to us from the type host of *H. boisii* (as *Aetobatus ocellatus*) appears to bear continuous bands of lateral hooklets. Given this morphological evidence and the fact that both species were reported from myliobatid or rhinopterid batoids, we believe *Halysioncum* is the most appropriate genus for them at this time. Following Tyler (2006) and Kuchta and Caira (2010) both should remain *species inquirendae*; they should also be considered *incertae sedis*.

3.2.5. Coronocestus n. gen. (Figs. 1H and 4E)

Diagnosis: Scolex with one dorsal and one ventral bothrium, armed apical organ, and cephalic peduncle. Bothria free posteriorly for part of their length, covered with palmate, pectinate and/or trifid spinitriches on proximal surfaces, with trifurcate spinitriches on distal surfaces. Apical organ with one dorsal and one ventral group of solid hooks; hooks in each group arranged in two regular rows consisting of A hooks (anterior row) alternating with B hooks (posterior row); adjacent hooks articulating with one another. Lateral hooklets arranged in distinct clusters on either side of dorsal and ventral groups of apical hooks. Corona of spines between apical organ armature and bothria present (Fig. 1H). Cephalic peduncle armed with eight columns of posteriorly directed spines with triradiate bases, acraspedote. Worms apolytic or euapolytic. Common genital pore mid-ventral. Cirrus sac unipartite; cirrus armed with spinitriches. Vagina opening posterior to cirrus sac. Ovary H-shaped in frontal view, bilobed in cross section. Vitellarium follicular; vitelline follicles circum-medullary or in two lateral bands. Uterus saccate, ventral. Eggs unembryonated when laid. Parasites of houndsharks (Triakidae), possibly also bamboo sharks (Hemiscyllidae).

Type species: Coronocestus diamanti (Ivanov and Lipshitz, 2006) n. comb.

Additional species: Coronocestus coronatus (Robinson, 1959) n. comb.; *Coronocestus hormozganiensis* (Haseli, Malek, Palm and Ivanov, 2012) n. comb.; *Coronocestus musteli* (Pintner, 1889) n. comb.; *Coronocestus notoguidoi* (Ivanov, 1997) n. comb.; *Coronocestus scoliodoni* (Sanaka, Vijaya Lakshmi and Hanumantha Rao, 1986) n. comb.

Etymology: Coronocestus (*coron*, L., corona, or ring around; *cestus*, L., worm) refers to the corona of spines present between the apical organ and bothria.

Remarks: The presence of a corona of spines between the apical organ armature and bothria distinguishes *Coronocestus* n. gen. from *Ahamulina*, *Ditrachybothridium*, *Echinobothrium sensu stricto*, and *Halysioncum*. It can be further distinguished from *Ditrachybothridium* in that it bears (rather than lacks) apical hooks, lateral hooklets and spines on the cephalic peduncle, and from *Ahamulina* in its possession (rather than lack) of spines on the cephalic peduncle and lateral hooklets. It further differs from *Halysioncum* in that its lateral hooklets are arranged in distinct clusters on either side of the dorsal and ventral groups of apical hooks, rather than in a continuous band.

We have formally transferred six species to this new genus; all six parasitize sharks. Five of these occur in sharks of the family Triakidae: *C. diamanti* was described from *Iago omanensis* (Norman) by Ivanov and Lipshitz (2006); *C. musteli* was described from a host identified as "Hundshaie" by Pintner (1889), which Tyler (2006) determined was most likely *Mustelus mustelus* Bonaparte; *C. notoguidoi* was described by Ivanov (1997) from *Mustelus schmitti* Springer; *C. hormozganiensis* was described by Haseli et al. (2012) from *Mustelus mosis* Hemprich and Ehrenberg. *Coronocestus coronatus* was originally described by Robinson (1959) from *Mustelus*

lenticulatus Phillipps, but the description was based on a single specimen, which Tyler (2006) found to be in very poor condition. Although Robinson (1959, p. 384) did not specifically mention the presence of a corona of spines, he described the bothria as "armed with small spines on their outer surface; spines do not cover the entire bothridial surfaces, but only occur near the base." We believe this is sufficient evidence to support transfer of this species to this new genus. However, this action will require confirmation by examination of material from the type host and locality.

We are less confident about transfer of the sixth species (i.e., *C. scoliodoni*). The description and illustrations presented by Sanaka et al. (1986) are somewhat superficial and no mention is made of type material—deficiencies that led Tyler (2006) and Kuchta and Caira (2010) to consider this a *species inquirenda*. Nonetheless, it appears this species bears a corona of spines between its bothria and apical organ (see their Fig. 1) and thus it too has been transferred to *Coronocestus* here. Its host, however, remains to be verified owing to the conflict between its type host of record (the hemiscylliid shark *Chiloscyllium indicum* [Gmelin]) and its specific epithet ("*scoliodoni*"), which presumably refers to the carcharhinid genus *Scoliodon* Müller and Henle. At this point, the majority of *Coronocestus* species parasitize carcharhiniform sharks of the family Triakidae. The purported association of *C. scoliodoni* with orectolobiform sharks of the genus *Chiloscyllium* Müller and Henle requires further investigation. Despite its report from *Mustelus manazo* Bleeker, the description of *Echinobothrium lateroporum* appeared only in an unpublished thesis (Subhapradha, 1948. Helminth parasites of the economic fishes of the Madras coast. M. Sc. thesis. University of Madras, India) and although the name was formally published by Anantaraman in 1963 it was not accompanied by a description. It thus represents a *nomen nudum* (see also Campbell and Andrade, 1997; Tyler, 2006) and we have left this name as it stands.

Unfortunately, the only material of a diphyllidean bearing the corona of spines available to us for this study consisted of two specimens of an undescribed species taken from an unidentified specimen of *Mustelus* Linck collected in the Solomon Islands. This species has been referred to here as *Coronocestus* n. sp. 1, until such time as it can be studied in more detail.

3.3. Key to diphyllidean genera

1a. Scolex with dorsal and ventral group of apical hooks..... 2
1b. Scolex without dorsal and ventral group of apical hooks..... *Ditrachybothridium* (Fig. 4A)
2a. Scolex with lateral hooklets..... 3
2b. Scolex without lateral hooklets..... *Ahamulina* (Fig. 4B)
3a. Lateral hooklets arranged in single, continuous band on either side of apical hooks..... Halysioncum n. gen. (Fig. 4D)
3b. Lateral hooklets arranged in two clusters on either side of apical hooks..... 4
4a. Scolex with corona of spines between bothria and apical organ armature..... Coronocestus n. gen. (Fig. 4E)
4b. Scolex without corona of spines between bothria and apical organ armature..... *Echinobothrium s.s.* (Fig. 4C)

3.4. Diphyllidean families

Two family-level classifications of diphyllideans have been proposed since the description of *Macrobothridium*. Three monogeneric families (Echinobothriidae Perrier, 1897, Ditrachybothriidae Schmidt, 1970, and Macrobothriidae Khalil and Abdul-Salam, 1989) have been recognized by some authors (e.g., Ivanov and Hoberg, 1999; Khalil and Abdul-Salam, 1989; Khalil, 1994). In contrast, Tyler (2006) recognized only Ditrachybothriidae and Echinobothriidae, given that he considered *Macrobothrium* to be a junior synonym of *Echinobothrium*. Marques et al. (2012), in fact, refrained from identifying a family placement for *Ahamulina*. Unfortunately, our results are inconsistent with these previous classifications and among the three major clades recovered by our analyses, only one (the *Halysioncum* clade), is supported by a diagnosable feature. In the absence of a more complete taxon sampling, we would advocate that all five genera be considered members of the single family Echinobothriidae with characteristics of the order. This will at least serve to provide family placements for the orphans *Ahamulina* and *Halysioncum*.

4. Discussion

The utility of our results for understanding the interspecific relationships within the order overall is limited and we have refrained from discussion of this topic at this time. We note that our analyses included only 12 of the 50 valid diphyllidean species, representing only a subset of described members in each genus. In summary, taxa included were as follows: the single described and one undescribed species of *Ahamulina*; eight of 20 species of *Halysioncum* (consisting of three of the 15 described species and five undescribed species); none of the five valid described species of *Coronocestus*, but one undescribed species; potentially one of the two described species of *Ditrachybothridium*; nine of the 38 described species of *Echinobothrium sensu stricto* (only 29 of which are valid) as well as seven undescribed species and four species that resemble existing taxa, but whose identities remain to be confirmed (i.e., *E.* cf. *heroniense*, *E.* cf. *chisholmae*, *E.* cf. *rhynchobati* 1, and *E.* cf. *rhynchobati* 2), and a new genus that remains to be formally characterized.

Our molecular results have provided a concrete framework for the generic reclassification of the Diphyllidea. As can be seen in Fig. 2, the monophyly of each of the three genera represented by more than a single exemplar in our analyses (i.e., *Halysioncum*, *Ahamulina* and *Echinobothrium sensu stricto*) was well supported. *Ditrachybothridium* and *Coronocestus*, although each represented by only a single species, placed outside of each of the above genera. All five genera are supported by putative morphological synapomorphies involving character states for various configurations of the armature of the scolex. These putative morphological synapomorphies provide diagnostic features for each genus (see schematic tree in Fig. 3) and serve as the foundation for the generic key provided above. In instances of equally parsimonious solutions for character state mappings we have chosen losses over gains. We note that these character mappings reveal some interesting trends in the evolution of diphyllidean armature which, given the robust grouping of the relatively naked genus *Ahamulina* and fully naked genus *Ditrachybothridium* among wholly armed taxa (i.e., *Coronocestus* and the new genus), suggest the parallel loss of both cephalic peduncle spines and lateral hooklets in the former two genera.

With respect to relationships among diphyllidean genera, the three genera that primarily parasitize sharks (i.e., *Coronocestus*, *Ditrachybothridium* and *Ahamulina*) were found to comprise a clade. *Echinobothrium sensu stricto* is sister to this primarily shark-hosted clade, and *Halysioncum* is their sister. The latter two, batoid-hosted genera, are the most speciose of the order. Overall, these relationships suggest that, within the Diphyllidea, the association with sharks is a derived feature.

By far the most puzzling result with respect to diphyllidean generic boundaries, however, was the robust grouping of new genus n. sp. 1 from the skate *Leucoraja wallacei* (Hulley) among the shark-hosted diphyllidean taxa, most closely allied with taxa that entirely lack (*Ditrachybothridium*) or exhibit reduced (*Ahamulina*) scolex armature. This placement seems somewhat anomalous because the specimens from *L. wallacei* bear full scolex armature

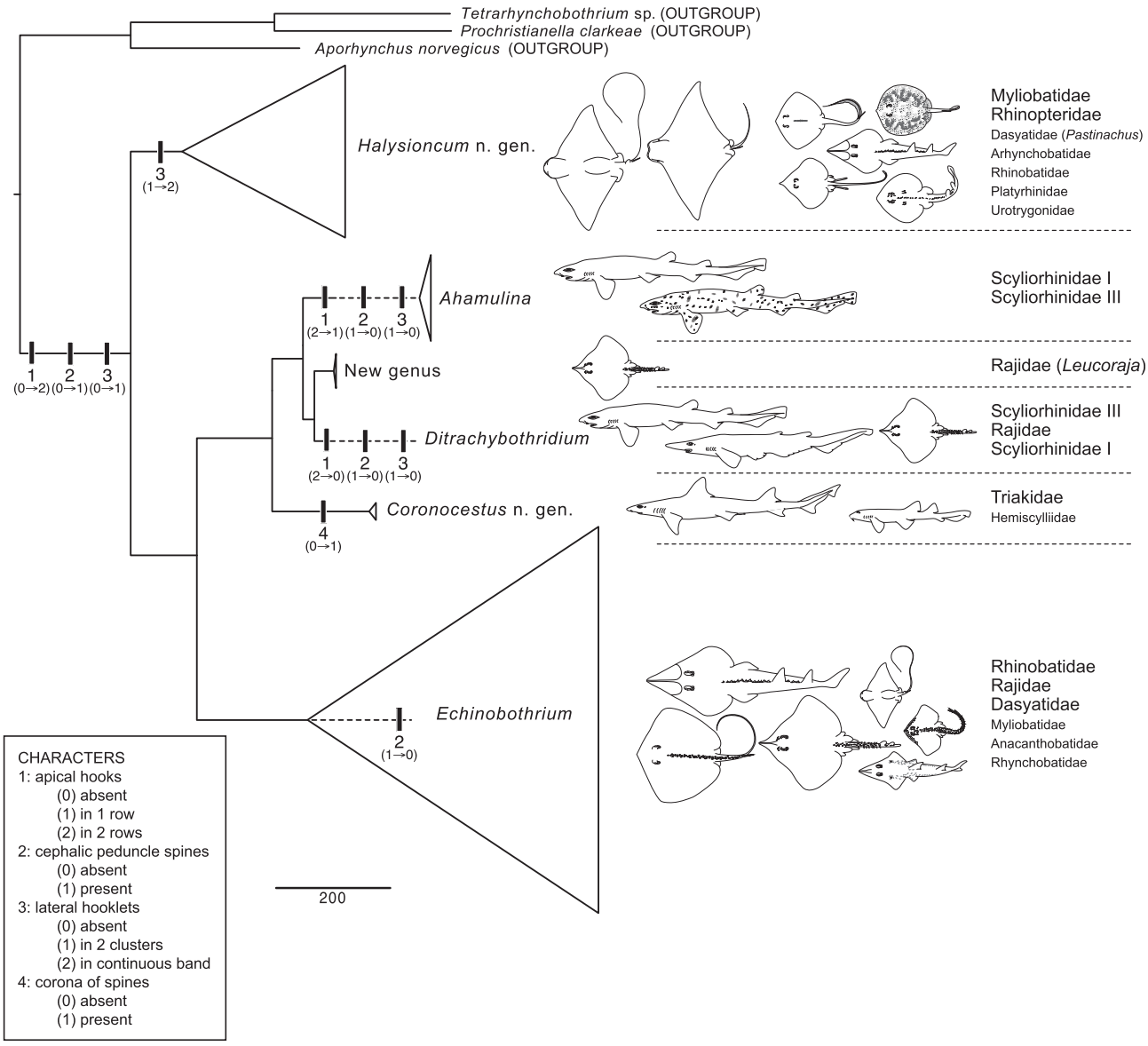

Fig. 3. Schematic representation of Fig. 2 with diphyllidean genera collapsed and represented by triangles; scolex armature characters are mapped on the branches; elasmobranch icons represent the host families parasitized by the respective diphyllidean genus; size of elasmobranch icons and host family name indicated major (larger size) and minor (smaller size) host group.

(i.e., apical hooks, lateral hooklets, and cephalic peduncle spines) much like the other batoid-hosted species of *Echinobothrium sensu stricto* and *Halysioncum*, but group well away from members of both genera. The inclusion of three replicate specimens from two different host individuals sequenced at different times eliminated some of the possible sources of error associated with this result. However, close examination of the scolex vouchers reveals that these specimens bear an unusual configuration of lateral hooklets relative to all other diphyllideans and in fact likely represent a distinct genus. At this point we have referred to this taxon as new genus n. sp. 1 until a thorough morphological investigation of multiple specimens has been completed.

Our molecular results confirm that *Macrobothridium* is not a valid monophyletic group. Its species (e.g., *M. euterpes*, *M.* cf. *rhynhobati*) are more appropriately considered members of *Echinobothrium sensu stricto*, as has already been suggested by a number of authors (Ivanov and Hoberg, 1999; Tyler, 2006; Kuchta and Caira, 2010) based on analyses of morphological data. Species lacking spines on the cephalic peduncle were found to be distributed throughout the clade *Echinobothrium sensu stricto*. Across the order, our results suggest that the spines of the cephalic peduncle could have been lost at least four times: once in *Ditrachybothridium*, once in *Ahamulina* and at least twice within *Echinobothrium sensu stricto* (i.e., once in *E. euterpes* and once in the clade consisting of the specimens of *E.* cf. *rhynchobati* 1 + *E.* cf. *rhynchobati* 2). However, since our molecular analyses included only a subset of species lacking spines on the cephalic peduncle, the number of times this feature has been lost within *Echinobothrium sensu stricto* may be found to be even greater.

Several observations can be made with respect to how our results compare to those of the two previous morphological phylogenetic analyses of the order that included a representative number of taxa (i.e., greater than 20 identified species). The recognition of *Halysioncum* is fully consistent with the results of Tyler (2006), who, in the strict consensus tree resulting from his analysis of 30 diphyllidean species recovered a clade comprised solely of the 10

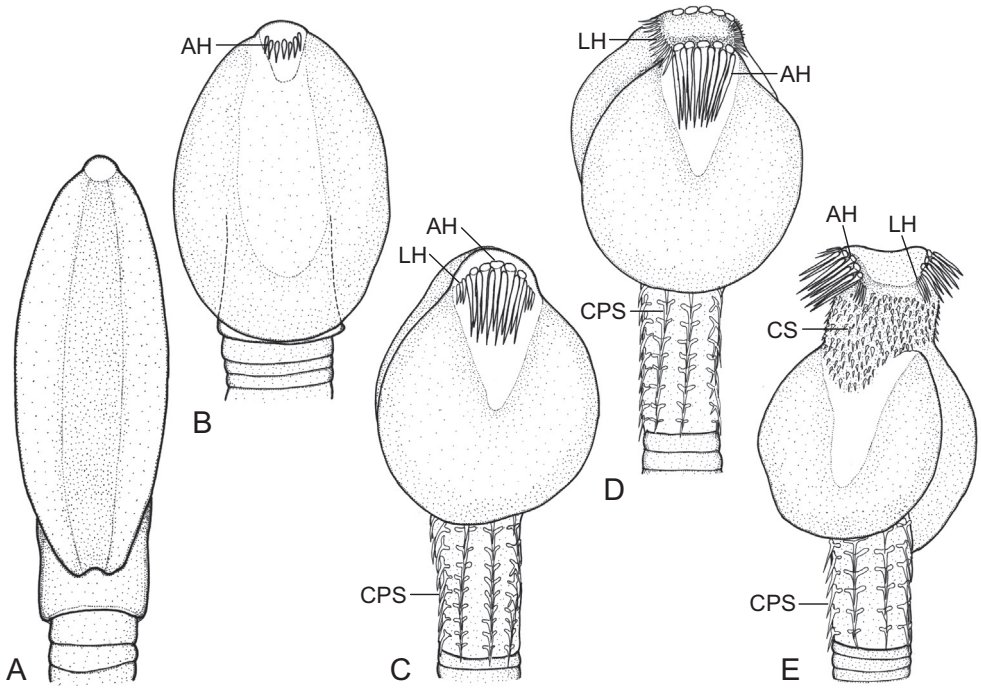

Fig. 4. Generalized line drawings of scoleces of named diphyllidean genera. (A) *Ditrachybothridium*. (B) *Ahamulina*. (C) *Echinobothrium sensu stricto*. (D) *Halysioncum*. (E) *Coronocestus*. AH, apical hooks; CPS, cephalic peduncle spines; CS, corona of spines; LH, lateral hooklets.

species bearing lateral hooklets that are arranged in a single band on either side of the apical hooks. The analysis of Ivanov and Hoberg (1999) yielded a clade consisting of five of the six species bearing lateral hooklets in a continuous band included in their study. In contrast, neither study found convincing support for *Coronocestus*. Ivanov and Hoberg (1999) recovered a group consisting of only two of the three species bearing a corona of spines included in their analysis; Tyler's (2006) study included only a single species bearing a corona of spines. Tyler (2006) found *Ditrachybothridium* to be monophyletic, albeit as a sister group to all other diphyllideans and Ivanov and Hoberg (1999) obtained a similar result. This result differs substantially from our work in which *Ditrachybothridium* nested among diphyllidean taxa. Neither study included representatives of the recently described *Ahamulina*.

Specimens included here have expanded the host range of the Diphyllidea to include two genera (*Pteromylaeus* and *Holohalaelurus*) and 10 species of elasmobranchs from which diphyllideans had not previously been reported. In total, the order is now known from 70 species in 30 genera and 14 families of elasmobranchs. The breakdown between the two major clades of elasmobranchs (i.e., Batoidea and Selachii) is 56 species in 23 genera in 10 families of batoids (i.e., rays, guitarfishes, etc.) and 14 species in seven genera and four nominal families of selachians (sharks). Species of *Coronocestus* and *Ahamulina* parasitize sharks, and species of *Halysioncum* and *Echinobothrium sensu stricto* parasitize rays and their kin. At this point it appears that *Ditrachybothridium* includes species that parasitize both sharks and rays (e.g., Rees, 1959).

Nearly half of the 20 known (but not necessarily described) species assigned to *Halysioncum* parasitize members of the closely related families Myliobatidae and Rhinopteridae. One species, *H. fautleyae*, has been reported from hosts in both families (Tyler and Caira, 1999). Assuming the revised generic assignments for *H. rhinoptera* and *H. boisii* can be confirmed (i.e., if they are found to exhibit lateral hooklets arranged in a continuous band), *Halysioncum* is the only genus of diphyllidean that parasitizes the Rhinopteridae. With the exception of *E. mathiasi*, *Halysioncum* also appears to be the only diphyllidean genus that parasitizes rays of the family Myliobatidae. *Echinobothrium mathiasi* was reported by Euzet (1951) from *Myliobatis aquila* (L.) (as *Leiobatis aquila*) off the coast of France. Examination of type material of this species provides fairly convincing evidence to confirm that the lateral hooklets are indeed arranged in two distinct groups. However, newly collected specimens from *M. aquila* off the coast of South Africa (*Halysioncum* n. sp. 4) exhibit lateral hooklets arranged in a continuous band. They also differ in a number of other respects and are thus unlikely to be conspecific with *E. mathiasi*. As it stands, *E. mathiasi* is the only diphyllidean outside of the genus *Halysioncum* known to parasitize a myliobatid host.

The batoid hosts of *Halysioncum* extend well beyond these two families. Three species of *Halysioncum* parasitize the dasyatid genus *Pastinachus*, one parasitizes the urobatid genus *Urobatis*, one species each is known from the arynchobatid skate genera *Psammobatis* Günther and *Rhinoraja* Ishiyama, one species parasitizes the platyrhinid genus *Platyrhinoidis* Garman, and finally, one species each has been reported from the rhinobatid genera *Rhinobatos* Linck and *Zapteryx* Jordan and Gilbert. Thus, among the four orders of batoids, the hosts of *Halysioncum* emphasize the Myliobatiformes over the Rhinopristiformes (sensu Naylor et al., 2012b) and Rajiformes; no members of the Torpediniformes have been found to host this genus.

By far the majority (i.e., six of seven) species of *Coronocestus* parasitize sharks of the carcharhiniform family Triakidae. As noted above, *C. scoliodoni* is the exception in that it has been reported from a species in the orectilobiform shark family Hemiscylliidae (i.e., *C. indicum*). However, this diphyllidean species is poorly known. Our efforts to verify the report of specimens by Sanaka et al. (1986) bearing a corona of spines from *Chiloscyllium* were unsuccessful. A diphyllidean we obtained from a newly collected specimen of *Chiloscyllium* cf. *punctatum* (LRP 8042) although potentially novel, appears to be a member of *Echinobothrium sensu stricto*.

Our work provides some insight into reports of *Ditrachybothridium* from hosts representing both of the major lineages of elasmobranchs. When Rees (1959) erected the genus, she reported its type species, *D. macrocephalum*, from the skates *Raja fullonica* L. (= *Leucoraja fullonica*) and *R. circularis* Couch (= *Leucoraja circularis*), as well as the catshark *Scyliorhinus caniculus* (L.). All but two of the 12 specimens in the type series (both from *L. fullonica*) were immature. The other member of the genus, *Ditrachybothridium piliformis*, was described from mature and gravid worms taken from the catshark *Galeus priapus* Last and Séret (as *Galeus* sp.) by Faliex et al. (2000). At that time, Faliex et al. (2000) made a case that deep water scyliorhinids (rather than skates or shallow water catsharks such as *Scyliorhinus*) were likely the "natural" definitive hosts for *Ditrachybothridium*, hypothesizing that the immature condition of worms taken from the latter hosts attest to their representing accidental infections. Bray and Olson (2004) subsequently reported encysted larvae they identified as *D. macrocephalum* from the spiral intestines of both the skate "cf. *Rajella bigelowi* (Stehmann)" and the catshark *A. laurussonii*. Based on a mature specimen identified as *D. macrocephalum* at the Natural History Museum in London (BMNH 1973.6.11.11–13) they expanded the hosts of this species to include the catshark *G. melastomus*, and based on a specimen consisting of a scolex and strobilar fragment (BMNH 1982.4.26.261) also added *Raja fyllae* Lütken. The specimen from *G. melastomus* was the basis on which Tyler (2006) emended the description of *D. macrocephalum*. Both Bray and Olson (2004) and Tyler (2006) generally supported the notion of Faliex et al. (2000) regarding the natural hosts of the genus. However, as noted above, we believe that, collectively, these specimens actually represent more than a single species. Thus, while the genus appears to parasitize both batoids and selachians, that may not be true for individual species. Although our results show *Ditrachybothridium* to group robustly among the other diphyllideans that parasitize sharks, it is sister to new genus n. sp. 1, which curiously, parasitizes the skate *L. wallacei*. We would argue that this result returns skates to the list of viable definitive hosts for members of the genus, particularly given Rees' (1959) finding of specimens she considered to be mature in *L. fullonica*. Of course, this issue can only be firmly resolved through collection of additional material from both catsharks and skates, specifically of the genus *Leucoraja* Malm.

Both of the known species of *Ahamulina* parasitize catsharks—at this point only species of *Scyliorhinus* and *Holohalaelurus*. However, the scyliorhinids remain one of the most poorly sampled groups of sharks (see Caira and Jensen, 2001) and we suspect will be found to harbor a much greater diversity of interesting (potentially hookless) diphyllideans. Evidence is mounting to suggest that the Scyliorhinidae is not monophyletic (e.g., Iglésias et al., 2005; Human et al., 2006). In fact, the molecular phylogenetic work of Naylor et al. (2012b) yielded three distinct lineages of catsharks (Scyliorhinidae I through III). The hosts of *Ditrachybothridium* and *Ahamulina* include species in both Scyliorhinidae I and III. Cestodes have yet to be reported from members of Scyliorhinidae II.

Species of *Echinobothrium sensu stricto* are restricted to batoids. Members of the families Rhinobatidae, Rajidae and Dasyatidae host all but four of the 43 species (i.e., 13, 14 and 12, respectively) for which definitive hosts are known (see Table 1). The exceptions are *E. joshuai*, *E. djeddensis*, *E. sinensis*, and *E. mathiasi*, which have been reported from the families Anacanthobatidae, Rhynchobatidae, Platyrhinidae and Myliobatidae, respectively. Again, no clear picture emerges from these associations because the three primary host families belong to three different batoid orders (Rajiformes, Myliobatiformes and Rhinopristiformes). *Echinobothrium sensu stricto* does share some host families with *Halysioncum* (i.e., Dasyatidae, Rhinobatidae and Myliobatidae). However, within a family, there is little overlap among host genera parasitized by these two diphyllidean taxa. For example, whereas *Echinobothrium sensu stricto* parasitizes dasyatids of the genera *Dasyatis*, *Himantura*, and *Taeniura*, species of *Halysioncum* parasitize members of *Pastinachus*.

Despite the number of new hosts and diphyllideans examined here, host specificity of diphyllideans remains essentially oioxenous. Historical reports of individual species from a broad spectrum of hosts (e.g., see hosts for *E. typus* and *E. affine* in Tyler, 2006) are worthy of closer scrutiny. We are convinced that, as discussed above for the situation with *D. macrocephalum*, careful morphological (and molecular) work is likely to reveal complexes of species in such instances.

Clearly much diphyllidean diversity remains to be discovered beyond the 19 potentially novel species included here. Our work suggests that, among sharks, the many species of catsharks and hound sharks not yet examined for cestodes will be productive sources of additional novel diphyllideans. Unexamined myliobatids, rhinopterids, rhinobatids, dasyatids and rajids would be best to target for additional novel batoid diphyllideans.

Formal assessment of co-phylogeny between diphyllideans and their elasmobranch hosts is approaching accessibility. A relatively comprehensive phylogeny of elasmobranch species is now available (see Naylor et al., 2012b). What is currently lacking is a broad species-level phylogeny for the diphyllideans. A concerted effort to obtain material of the 38 species not included here in a comprehensive molecular analysis is required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2013.03.001.

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