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Phylogenetic relationships, host associations, and three new species of a poorly known group of "tetraphyllidean" tapeworms from elasmobranchs

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Abstract

This paper aims to expand understanding of a poorly known group of cestodes that parasitize an intriguingly diverse suite of elasmobranchs. The group's three currently described members (i.e., *Pentaloculum macrocephalum*, *Pentaloculum hoi*, and *Zyxibothrium kamienae*) parasitize an electric ray, a carpet shark, and a skate, respectively. *Pentaloculum grahami* **n. sp.** is described from a second genus of carpet shark, specifically *Parascyllium collare*, in Australia. *Zyxibothrium duffyi* **n. sp.** and *Zyxibothrium healyae* **n. sp.** are described from the deep-sea skates *Brochiraja asperula* and *Brochiraja spinifera*, respectively off New Zealand. The three new species share distinctive bothridia that bear a small number of large, circular, facial loculi and lateral bands of vitelline follicles that converge posterior to the ovary—features which are found in all other members of these genera. *Zyxibothrium healyae* **n. sp.** is unique in possessing three, rather than four or five, facial loculi. *Zyxibothrium duffyi* **n. sp.** possesses a combination of five facial loculi and vitelline follicles that stop short of the anterior margin of the proglottid. *Pentaloculum grahami* **n. sp.** is the largest member of the group with the greatest number of proglottids. Based on striking similarities in scolex morphology, *Pentaloculum* and *Zyxibothrium* have been hypothesized to belong to a distinct subgroup of "tetraphyllideans" provisionally designated as Clade 1. Based on sequence data for the D1–D3 region of the *28S rDNA* gene generated for species of *Zyxibothrium* for the first time, we confirm the reciprocal monophyly of both genera as well as the monophyly of Clade 1 and its status as a distinct lineage among the "Tetraphyllidea". This work also suggests that the presence of five facial loculi is homoplasious given this character state is found in members of both genera. The new species expand the host associations of Clade 1 to include additional skate and carpet shark genera. Moving forward we would expect to find additional members of this group parasitizing other species of parascyliid carpet sharks as well as other species of the rajid genus *Malacoraja* and the arhynchobatid genus *Brochiraja*. Here we have doubled the number of described species in the taxon referred to as Clade 1 while simultaneously expanding our understanding of the morphology and anatomy of its members. This additional information will help inform the ultimate revision of the ordinal classification of the cestodes to address the highly polyphyletic nature of the order "Tetraphyllidea" as it is currently configured.

Key words: biodiversity, phylogeny, Platyhelminthes, Cestoda, *28S rDNA*, morphology, taxonomy, Australia, New Zealand

Introduction

The genus *Pentaloculum* Alexander, 1963 is one of the 26 genera that remain in the polyphyletic cestode order "Tetraphyllidea" (Caira *et al.* 2017; Eudy *et al.* 2019). Its type species, *Pentaloculum macrocephalum* Alexander, 1963, parasitizes the blind electric ray, *Typhlonarke aysoni* (Hamilton), in New Zealand. The genus was monotypic until recently when Eudy *et al.* (2019) described a second species, *Pentaloculum hoi* Eudy, Caira, and Jensen 2019, from the Taiwan saddled carpetshark, *Cirrhoscyllium formosanum* Teng, in Taiwan, and emended the diagnosis of the genus. That work also led Eudy *et al.* (2019) to realize that the species from the collared carpetshark, *Parascyllium collare* Ramsay and Ogilby, referred to as New genus 7 n. sp. 1 in the phylogenetic analyses of Caira *et al.* (2014), is in fact an undescribed species of *Pentaloculum*, to which they assigned the provisional name *Pentaloculum* n. sp. 2. This brought the total number of known species of *Pentaloculum* to three, one of which remained undescribed.

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In the phylogenetic framework proposed by Caira *et al.* (2017), the authors suggested that the genus *Pentaloculum* was most closely related to the monotypic "tetraphyllidean" genus *Zyxibothrium* Hayden and Campbell, 1981, with *Zyxibothrium kamienae* Hayden and Campbell, 1981 parasitizing the smooth skate, *Malacoraja senta* (Garman), off the east coast of the USA. Evidence came from the striking similarity in scolex morphology between the two genera, with *Zyxibothrium* bearing bothridia with four conspicuous, large facial loculi and *Pentaloculum* bearing bothridia with five such loculi. However, the relationships between *Pentaloculum* and *Zyxibothrium* have yet to be assessed based on phylogenetic analyses of molecular data.

The association of *Z. kamienae* with a skate host prompted us to examine the cestode faunas of two species of deep-sea skates in the genus *Brochiraja* Last and McEachran that were collected off the coast of New Zealand. These skates were found to host two new cestode species with scoleces that resemble those of *Pentaloculum* and *Zyxibothrium*. Here we present descriptions of these two new cestode species as well as for *Pentaloculum* n. sp. 2 from *P. collare* in Australia. We also generated detailed ultrastructural data using scanning electron microscopy (SEM) for *P. macrocephalum* and *Z. kamienae*.

To test the hypothesized close relationship between *Pentaloculum* and *Zyxibothrium* we generated sequence data for the D1–D3 region of the *28S rDNA* gene *de novo* for four species across both genera and three outgroup species. These are the first such data available for members of the genus *Zyxibothrium*. These data were combined with comparable data for species of *Pentaloculum* and four additional outgroup species available in GenBank and phylogenetic analyses were conducted to evaluate this relationship. This analysis also allowed us to evaluate the hypothesis of Caira *et al.* (2017) that the members of these two genera belong to a distinct subgroup of "tetraphyllideans", which they provisionally designated as Clade 1.

Material and methods

Collection of hosts

Each elasmobranch specimen was assigned a unique combination of collection code and collection number. Additional information on each host specimen can be found in the Global Cestode Database (Caira *et al.* 2022) by searching for its unique collection code and number (e.g., SA-11). Host specimens examined were as follows: 23 specimens of *Parascyllium collare*, five males and 18 females (unique codes: SA-2–SA-11, SA-13–SA-16, SA-18, and SA-56–SA-63), collected by Ken Graham (New South Wales State Fisheries) in September and October of 1991 off Crowdy Head, New South Wales, Australia; eight specimens of *Brochiraja asperula* (Garrick and Paul), five males and three females (unique codes: CR-28, CR-63–CR-65, CR-74, CR-88, CR-135, and CR-154); four specimens of *Brochiraja spinifera* (Garrick and Paul), two males and two females (unique codes: CR-75, CR-76, CR-151, and CR-180); two specimens of *Typhlonarke aysoni*, one male and one female (unique codes: CR-136, CR-142), all collected by Claire Healy in January 2005 or 2007 off the Chatham Rise near New Zealand from a standard Hoki fishing trawler; one female specimen of *Malacoraja senta* (unique code: BV-3) collected by local fishermen off Gloucester, Massachusetts, USA, using a bottom trawl in May 2012. In all cases the spiral intestine was removed through a ventral incision in the body cavity and opened with a longitudinal incision. Spiral intestines were fixed in either seawater-buffered formalin or 95% ethanol. Formalin-fixed spiral intestines were subsequently transferred to 70% ethanol for storage. Cestodes were removed from spiral intestines, sorted under a dissecting microscope, and preserved in seawater-buffered formalin or 95% ethanol.

Morphological methods

Specimens prepared for light microscopy were hydrated in a graded ethanol series, stained for 30–40 min in a working solution of Delafield's hematoxylin (1:9 mixture of hematoxylin:distilled water), differentiated in tap water, destained in acidic 70% ethanol, neutralized in basic 70% ethanol, dehydrated in a graded ethanol series, cleared in methyl salicylate, mounted in Canada balsam on glass slides under glass coverslips, and left to dry in an oven set to 55°C for one week. Measurements were taken with a Zeiss Axioskop 2 Plus compound microscope (Zeiss, Thornwood, New York, USA) using a SPOT Diagnostic Instrument Digital Camera System and SPOT software (version 4.6; SPOT Imaging Solutions, Sterling Heights, Michigan, USA). Measurements are given in the text as the range (in micrometers unless stated otherwise). In instances in which more than four measurements were taken, the range is followed in parentheses by mean, standard deviation, number of specimens measured, and total number of measurements when more than one measurement was made per worm. Shape terminology follows Clopton (2004).

Scoleces prepared for scanning electron microscopy (SEM) were hydrated in a graded ethanol series, transferred to a 1% solution of osmium tetroxide overnight, dehydrated in a graded ethanol series, placed in hexamethyldisilazane in a fume hood for 30 min, and allowed to air dry. The specimens were then mounted on double-sided PELCO carbon tabs (Ted Pella Inc., Redding, California, USA) on aluminum stubs, sputter-coated with 30 nm of gold/palladium and examined with an FEI Nova NanoSEM 450 field emission SEM (FEI, Hillsboro, Oregon, USA) at the Bioscience Electron Microscopy Laboratory, University of Connecticut (Storrs, Connecticut). Microthrix terminology follows Chervy (2009).

Museum abbreviations used are as follows: LRP, Lawrence R. Penner Parasitology Collection, University of Connecticut, Storrs, Connecticut, USA.; NMNZ, Museum of New Zealand Te Papa Tongarewa, Wellington, New Zealand; QM, Queensland Museum, South Brisbane, Australia; USNM, National Museum of Natural History, Smithsonian Institution, Department of Invertebrate Zoology, Washington, D.C., USA.

Molecular methods

Sequence data were generated *de novo* for the D1–D3 region of the *28S rDNA* gene for one specimen of *Pentaloculum macrocephalum* from *T. aysoni* (hologenophore LRP No. 9826), one specimen of *Zyxibothrium kamienae* from *M. senta* (hologenophore LRP No. 9810), two specimens of the new cestode species from *B. asperula* (hologenophores LRP Nos. 9797 and 9798), and one specimen of the new cestode species from *B. spinifera* (hologenophore LRP No. 9803). In addition, sequence data for the same region were generated *de novo* for one specimen of *Barbeaucestus ralickiae* Caira, Healy, Marques and Jensen, 2017 from *Taeniura lymma* 1 (Forsskål) (hologenophore LRP No. 9832), one specimen of *Anthocephalum healyae* Ruhnke, Caira and Cox 2015 from *Neotrygon australiae* Last, White and Serét (hologenophore LRP No. 9834), and one specimen of *Stillabothrium cadenati* (Euzet, 1954) Healy and Reyda, 2016 from *Zanobatus schoenleinii* (Müller and Henle) (hologenophore LRP No. 9831) for inclusion as outgroup taxa. In all cases, the middle portion of the cestode specimen was removed and allowed to air dry for ~5 min at room temperature to be used for DNA extractions. The scolex and terminal portion of the strobila were prepared as whole mounts as described above to serve as hologenophores (sensu Pleijel *et al.* 2008) for the specimens sequenced and were deposited in LRP. The GenBank accession numbers for all newly generated sequences are provided on the phylogenetic tree.

Total genomic DNA (gDNA) was extracted using the MasterPureTM DNA Purification Kit (Epicentre Technologies, Madison, Wisconsin, USA) following the manufacturer's instructions for small tissue samples. DNA was quantified using a micro-volume spectrophotometer NanoDrop 2000 (ThermoFisher Scientific, Waltham, Massachusetts, USA). Polymerase chain reaction (PCR) was used to amplify the target region. Double-stranded amplifications were generated in a 10 µl volume containing 1–3 µl DNA template, 5.0 µl GoTaq® Green Master Mix (PROMEGA, Fitchburg, Wisconsin, USA), 0.1 µl of each 10µM primer and 1.8–3.8 µl of double distilled water. Amplification was done using the primer pair LSU-5 (TAGGTCGACCCGCTGAAYTTA) (Littlewood *et al.* 2000) and LSU-1500R (GCTATCCTGGAGGGAAACTTCG) (Tkach *et al.* 2003). Sequencing was done using the primer pair LSU-55F (AACCAGGATTCCCCTAGTAACGGC) (Bueno and Caira 2017) or LSU-5 (TAGGTCGACCCGCTGAAYTTA) (Littlewood *et al.* 2000) and LSU- 1200R (GCATAGTTCACCATCTTTCGG) (Littlewood *et al.* 2000). PCR cycling conditions included an initial denaturation of 1 min at 94°C, followed by 39 cycles of denaturation for 30 sec. at 94°C, annealing for 1 min at 56°C or 59°C, extension for 1 min at 72°C, and a final extension of 5 min at 72°C. PCR product cleanup was performed by adding 1 µl of ExoSAP-IT (Affymetrix, Inc., Santa Clara, California, USA) to every 8 µl of PCR product. In some cases, stock ExoSAP-IT was diluted 1:5 in molecular grade water and 2 µl of the solution was added to 8 µl of PCR product. Clean-up conditions included 30 min at 37°C, followed by 15 min at 80°C, and a cool-down step of 10 min at 20°C. The cycle sequencing reaction protocol included an initial denaturation step of 2 min at 96°C, and 40 cycles of a denaturation step of 30 sec at 96°C, an annealing step of 30 sec at 50°C, and an extension step of 4 min at 60°C. Cycle sequencing products were then cleaned with Sephadex beads (Sigma-Aldrich, St. Louis, Missouri, USA). Sequencing of both strands was carried out on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) using ABI Big Dye dideoxy terminators version 3.1 and the sequencing primers listed above. Contigs were assembled and sequences were edited using Geneious Prime 2022 (www.geneious.com).

Alignment and phylogenetic analyses

Sequence data generated in this study were combined with comparable data available from GenBank for the following taxa: one specimen of *P. macrocephalum* from *T. aysoni* (KF685877, hologenophore LRP No. 8347; originally identified as *Pentaloculum* n. sp. 1 from *Typhlonarke tarakea* Phillipps by Caira *et al.* (2014), two specimens of *P. hoi* from *C. formosanum* (MK321593, hologenophore LRP No. 9939, and MK321594, hologenophore LRP No. 9952), and two specimens of *Pentaloculum* n. sp. 2 (as referred to by Eudy *et al.* 2019) from *P. collare* (KF685749, hologenophore LRP No. 8344, originally identified as New genus 7 n. sp. 1, and MK321595, hologenophore LRP No. 9956). In addition, sequence data for one specimen of *Echeneibothrium multiloculatum* Carvajal and Dailey, 1975 from *Dipturus chilensis* (Guichenot) (KY569549, hologenophore LRP No. 9230), one specimen of *Rhinebothrium megacanthophallus* Healy, 2006 from *Urogymnus polylepis* (Bleeker) (FJ177120, hologenophore LRP No. 3901), one specimen of *Escherbothrium* sp. from *Urotrygon rogersi* (Jordan and Starks) (KM658197, hologenophore LRP No. 8519), and one specimen of *Anthobothrium caseyi* Ruhnke and Caira, 2009 from *Prionace glauca* (Linnaeus) (KF685879, hologenophore LRP No. 8314) were also retrieved from GenBank to serve as outgroup taxa.

Sequences were aligned and trimmed using the MAFFT Multiple Alignment (Katoh and Standley 2013) plugin in Geneious Prime 2022 (www.geneious.com) using default parameter settings. They were then submitted to the Guidance2 server (Sela *et al.* 2015; guidance.tau.ac.il) for realignment with PRANK (Löytynoja 2014) with the +F flag removed; unreliable sites were removed using the Guidance2 algorithm (Sela *et al.* 2015). Maximum Likelihood (ML) and Bayesian Inference (BI) phylogenetic analyses were performed on the cluster in the Bioinformatics facility of the Institute of Systems Genomics at the University of Connecticut using IQ-TREE 1.6.10 (Nguyen *et al.* 2015) and MrBayes 3.2.7 (Ronquist and Huelsenbeck 2003), respectively. GTR+G was selected as the best-ranked model of molecular evolution according to the corrected Akaike Information Criterion (AICc) as implemented in ModelFinder (Kalyaanamoorthy *et al.* 2017) in IQ-TREE. This was followed by ML tree reconstruction and generation of 200 non-parametric bootstrap replicates using the command *iqtree -s datamatrix.nex -m MFP -merit AICc -b 200*. Following Hillis and Bull (1993), nodes with bootstrap values of ≥ 70% were considered to be strongly supported. Bayesian Inference analyses were conducted with two runs of four Markov Chain Monte Carlo (MCMC) chains each. Analyses were performed for 5 million generations with tree sampling every 1,000 generations. Summary statistics were obtained using the *sump* command; 500,000 generations were discarded as burn-in (default setting). Majority rule consensus trees were generated using the command *sumt filename=datamatrix.nex nruns=2 outputname=contree contype=halfcompat*. Nodes with posterior probability (PP) ≥ 0.95 were considered to be strongly supported (Wilcox *et al.* 2002).

Results

Phylogenetic analyses

The ML and BI analyses resulted in trees with identical topologies (Fig. 1). The phylogeny shows a well-supported clade that includes all three known species of *Pentaloculum*, *Z. kamienae*, and the two new species from deep-sea skates. There are two subclades within this clade. One of these subclades consists of *P. macrocephalum*, *P. hoi*, and the species formerly referred to as *Pentaloculum* n. sp. 2 by Eudy *et al.* (2019) but described as new below. The second subclade consists of *Z. kamienae* and the two new species from deep-sea skates. Given these relationships and similarities in proglottid anatomy, the two new species from skates are assigned to the genus *Zyxibothrium*.

Pentaloculum macrocephalum **Alexander, 1963 emended**

(Fig. 2A–F)

Type and only known host: *Typhlonarke aysoni* (Hamilton), blind sleeper ray; (Torpediniformes: Narkidae Fowler).

Type locality: New Zealand.

Site of infection: Spiral intestine.

Specimens deposited: SEM voucher LRP No. 10949 (CR-136-A); scolex prepared for SEM retained with JNC at the University of Connecticut.

Sequence data: KF685877 and OQ186458 (hologenophores, LRP Nos. 8347 [CR-136, TE-171] and LRP No. 9826 [CR-136-1, VB229]).

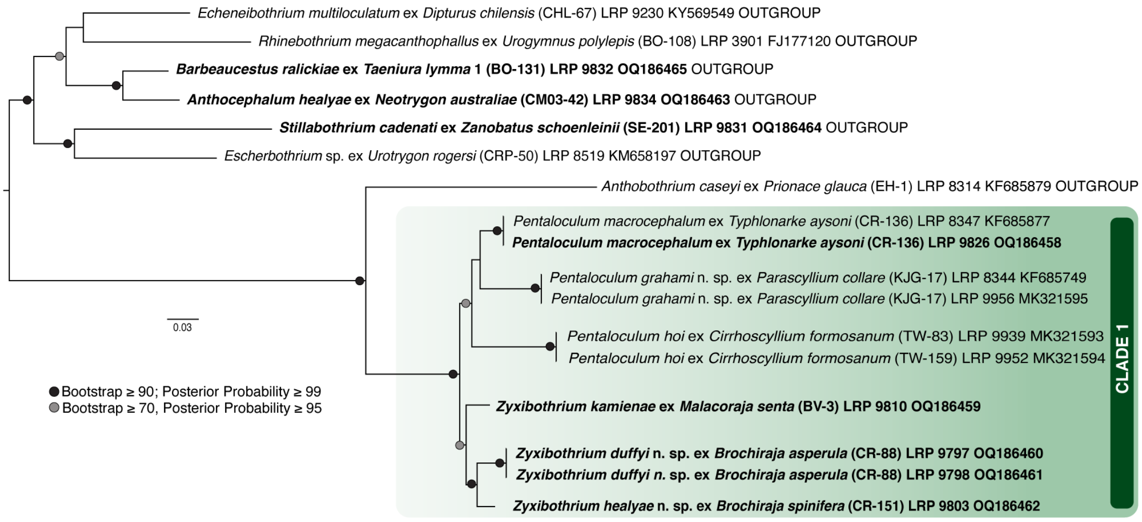

FIGURE 1. Phylogenetic tree resulting from Bayesian Inference and Maximum Likelihood analyses of the D1–D3 region of the *28S rDNA* gene for species in Clade 1 of Caira *et al.* (2017) (green box). Scale bar indicates substitutions per site. Nodes with bootstrap values ≥ 90 and posterior probabilities ≥ 99 are indicated by black dots. Nodes with bootstrap values ≥ 70 and posterior probabilities ≥ 95 are indicated by grey dots. Taxon labels are presented as cestode and host names followed by host specimen number in parentheses, Lawrence R. Penner Parasitological Collection accession number for hologenophores, and GenBank accession number. Newly generated sequences are in boldface type.

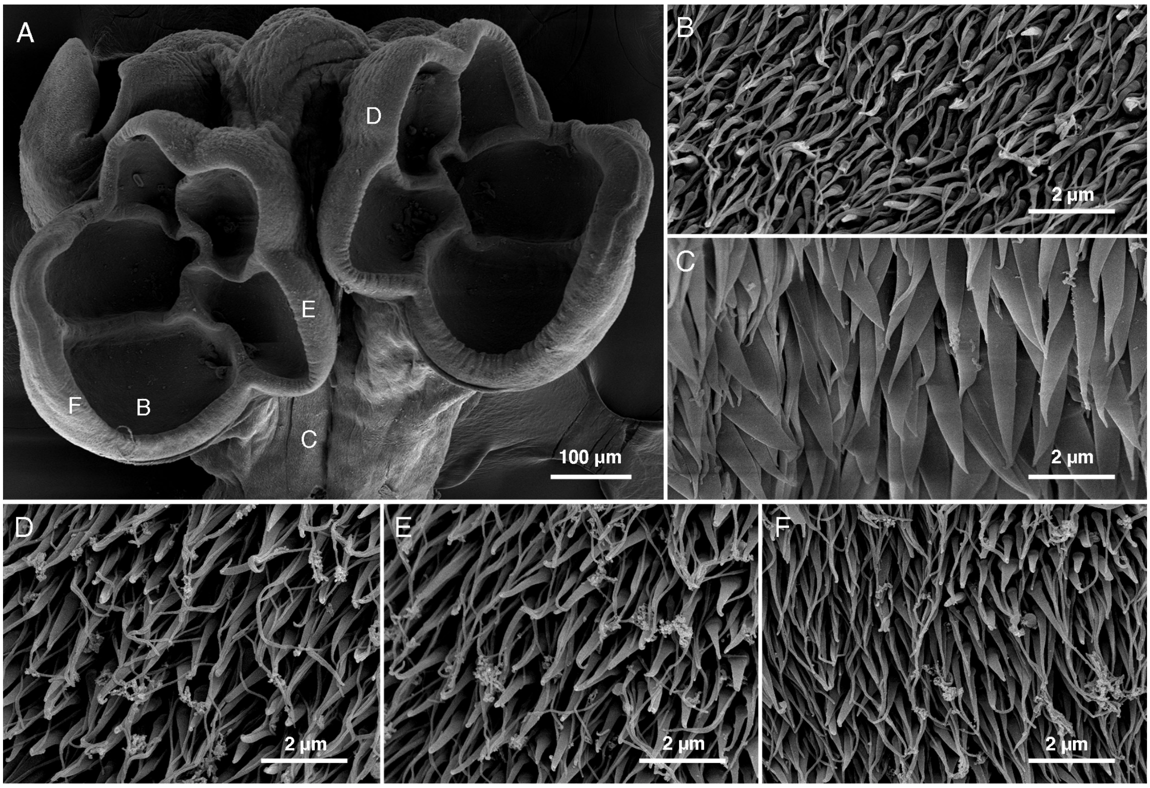

FIGURE 2. Scanning electron micrographs of *Pentaloculum macrocephalum* Alexander 1963. (A) Scolex, small letters indicate location of details in micrographs B–F. (B) Distal bothridial surface densely covered with long-tipped aristate gladiate spinitriches interspersed with gladiate spinitriches and capilliform filitriches. (C) Cephalic peduncle densely covered with large gladiate spinitriches, filitriches not observed. (D) Proximal surface of anteriormost loculus densely covered with long-tipped aristate gladiate spinitriches interspersed with gladiate spinitriches and capilliform filitriches. (E) Proximal surfaces of anterior pair of loculi densely covered with long-tipped aristate gladiate spinitriches interspersed with gladiate spinitriches and capilliform filitriches. (F) Proximal surfaces of posterior pair of loculi densely covered with long-tipped aristate gladiate spinitriches interspersed with gladiate spinitriches and capilliform filitriches.

Emendation. Based on one scolex observed with SEM and its strobilar voucher.

The description of this species by Alexander (1963) should be emended to include the following information on the configuration of the microtriches of the scolex and proglottid anatomy.

Apex of scolex not observed (Fig. 2A); distal bothridial surfaces (Fig. 2B) and all proximal bothridial surfaces (Figs. 2D–F) densely covered with long-tipped aristate gladiate spinitriches, gladiate spinitriches, and capilliform filitriches. Cephalic peduncle (Fig. 2C) densely covered with large gladiate spinitriches, filitriches not observed.

Vitelline follicles arranged in two lateral bands; lateral bands confluent posterior to ovary.

Remarks. The specimen of *P. macrocephalum* examined here with SEM and light microscopy was collected from the same individual of *Typhlonarke aysoni* as the specimen provisionally identified as *Pentaloculum* **n. sp.** 1 in the molecular phylogenetic analysis of Caira *et al.* (2014), in which the host was identified as *T. tarakea*. We have followed Eudy *et al.* (2019) in referring to this cestode taxon as *P. macrocephalum* and this host taxon as *T. aysoni*. Comparison of the hologenophore of the specimen included in the analysis of Caira *et al.* (2014), which was fixed in ethanol, with the whole mount of the strobila of the specimen examined here with SEM, which was fixed in formalin, confirms this identification.

Pentaloculum grahami **n. sp.**

(Figs. 3A–E, 4A–H, 5)
ZooBank No. B8C37A6D-016A-4D85-A132-C6E7E1063BC3

Type and only known host: *Parascyllium collare* Ramsay and Ogilby, collared carpetshark; (Orectolobiformes: Parascylliidae Gill).

Type locality: Crowdy Head, New South Wales, Australia (31°50'S, 152°45'E).

Site of infection: Spiral intestine.

Type specimens: Holotype (QM No. G240343) and two paratypes (QM Nos. G240344–G240345); two paratypes and one SEM voucher (LRP Nos. 10947–10949); two paratypes (USNM Nos. 1678892–1678893). Scoleces prepared for SEM retained with JNC at the University of Connecticut.

Sequence data: KF685749 and MK321595 (hologenophores, LRP No. 8344 [KJG-17, TE-166] and LRP No. 9956 [KJG-17, JW-147]).

Etymology: This name honors fish biologist Ken Graham from the New South Wales State Fisheries and the Australian Museum who collected all specimens of *Parascyillium collare* examined in this study.

Provisional names: New genus 1 n. sp. of Caira *et al.* 2001; New genus 7 n. sp. 1 of Caira *et al.* 2014 and Ruhnke *et al.* 2015; *Pentaloculum* n. sp. 2 of Eudy *et al.* 2019.

Description. Based on one incomplete mature and six complete mature worms, and two scoleces observed with SEM.

Worms apolytic, 12.1–51.6 (28.9 ± 15.7; 6) mm long, greatest width at level of terminal proglottid; 105–174 (131 ± 29; 6) proglottids per worm. Scolex 358–460 (406 ± 38.6; 7) long by 433–593 (516.9 ± 68.9; 7) wide, consisting of four muscular sessile bothridia. Bothridia broadly ovoid, 276–405 (348.5 ± 41.3; 6; 10) long by 230–311 (254.2 ± 23.4; 5; 9) wide; each divided into five facial loculi arranged as one single anterior loculus followed by two consecutive pairs of loculi; septum dividing anterior pair of loculi slightly offset laterally from septum diving posterior pair of loculi (Figs. 3A, 4A). Cephalic peduncle lacking.

Apex of scolex (Fig. 4C) covered with gladiate spinitriches and densely packed capilliform filitriches; distal bothridial surfaces (Fig. 4B) and proximal surfaces of margins of bothridia (Fig. 4E–G) densely covered with long-tipped aristate gladiate spinitriches interspersed with gladiate spinitriches and capilliform filitriches; proximal surfaces away from bothridial margins (Fig. 4H) densely covered with short-tipped wide aristate gladiate spinitriches, filitriches not observed; scolex proper (Fig. 4D) densely covered with gladiate spinitriches interspersed with capilliform filitriches.

Proglottids craspedote. Immature proglottids 98–151 (127 ± 23.4; 6) in number, wider than long. Mature proglottids 2–11 (5.8 ± 3.8; 6) in number, wider than long (Fig. 3B), 330–830 (606.1 ± 220.6; 6; 10) long by 962–1402 (1229 ± 150.9; 6; 10) wide, length:width ratio 0.3–0.9 (0.5 ± 0.2; 6; 10):1. Gravid proglottids 1–12 (5.2 ± 3.9; 6) in number, wider than long, becoming longer than wide with maturity (Fig. 3D), 954–4881 (3056.6 ± 1554.1; 6) long by 988–2512 (1407.8 ± 581; 6) wide. Genital pores marginal, irregularly alternating, 42–57% (52 ± 6; 5) of proglottid length from posterior margin in terminal-most mature proglottid. Testes 30–58 (44.3 ± 10.5; 5; 6) in number,

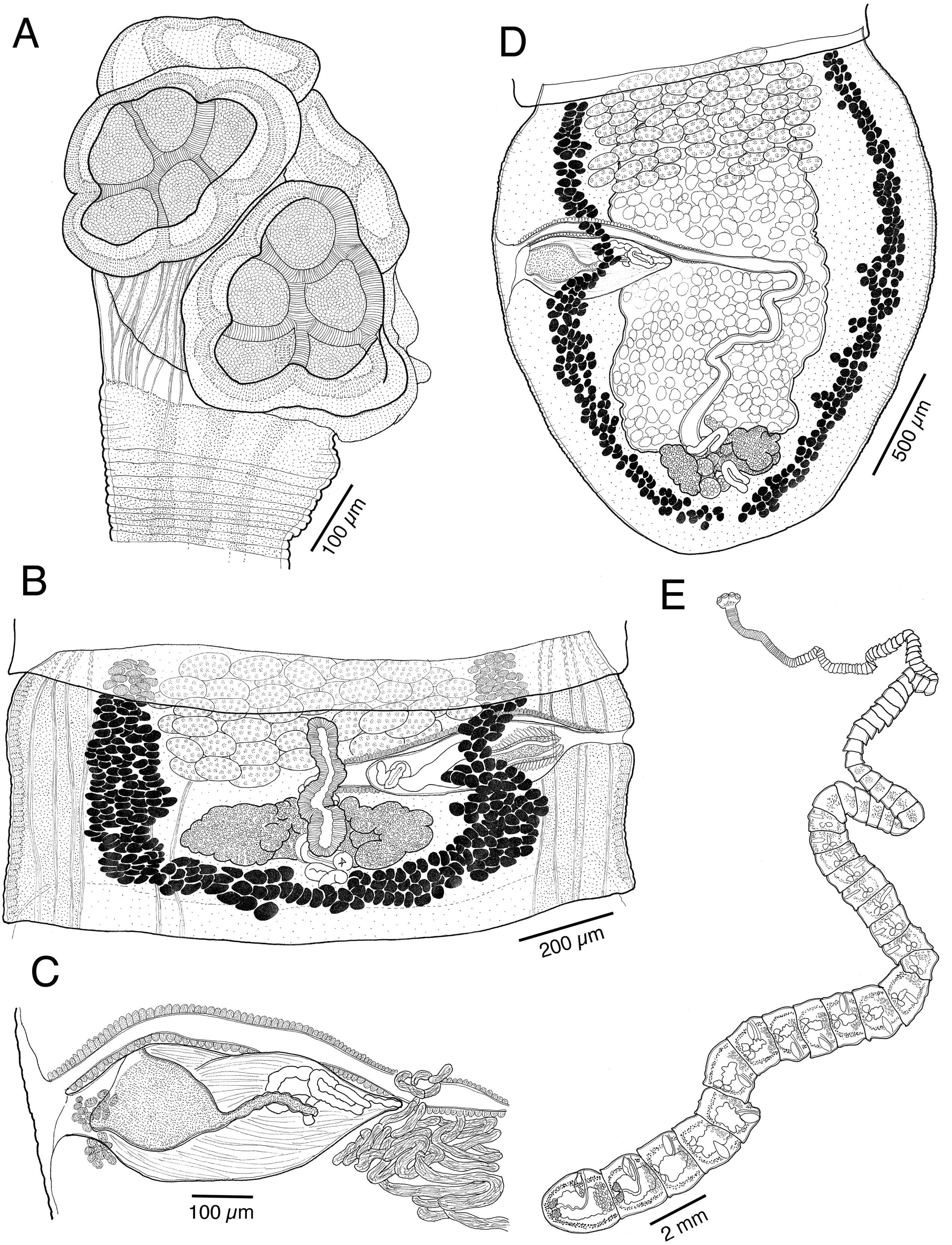

FIGURE 3. Line drawings of *Pentaloculum grahami* **n. sp.** (A) Scolex (paratype, SA-2-1, LRP No. 10947). (B) Mature subterminal proglottid (paratype, SA-16-1, USNM No. 1678892). (C) Detail of terminal genitalia (holotype, SA-16-3, QM No. G240343). (D) Gravid terminal proglottid (holotype, SA-16-3, QM No. G240343). (E) Whole worm (holotype, SA-16-3, QM No. G240343).

two layers deep, arranged in single field anterior to cirrus sac, oval in frontal view, 28–57 long (42.3 ± 9.7; 4; 12) by 74–123 (98 ± 15.6; 4; 12) wide. Vas deferens narrow, highly coiled, extending posteriorly to near level of ovary then anteriorly to enter cirrus sac at median margin. Cirrus sac (Fig. 3C) thin-walled, narrowly obovoid, containing coiled cirrus, 149–218 (181.1 ± 21.9; 5; 9) long by 333–629 (434.2 ± 86.8; 5; 9) wide; cirrus highly expanded at base, armed with small spinitriches. Vagina thick-walled, weakly sinuous, extending from ootype along medial line of proglottid to level of cirrus sac, then crossing cirrus sac ventrally and extending along anterior margin of cirrus sac to open into genital atrium anterior to cirrus; vaginal sphincter lacking; seminal receptacle lacking. Ovary near posterior end of proglottid, with slightly lobulated margins, H-shaped in frontal view, bilobed in cross-section, 446–537 (501.9 ± 29.8; 5; 9) at widest point by 78–237 (155.5 ± 52.4; 5; 18) long. Mehlis' gland posterior to ovarian isthmus. Vitellarium follicular; follicles in two lateral bands; each band consisting of multiple columns of follicles, extending throughout length of proglottid, not interrupted by cirrus sac or ovary, converging posterior to ovary; vitelline follicles oval, 22–38 (29.6 ± 5.7; 5; 15) long by 28–52 (38.7 ± 8.1; 5; 15) wide. Uterus medial, saccate, ventral to vagina, extending anteriorly from ovarian isthmus to mid-level of testicular field. Excretory vessels four, arranged in one dorsal and one ventral pair on each lateral margin of proglottid. Oncospheres organized in cocoons (Fig. 5A–B). Cocoons spherical, containing 5–7 (6.1 ± 0.6; 1; 10) oncospheres, polar filaments lacking; oncospheres spherical, 50–81 (69.7 ± 5; 1; 10) long by 63–79 (71.6 ± 4.1; 1; 57) wide.

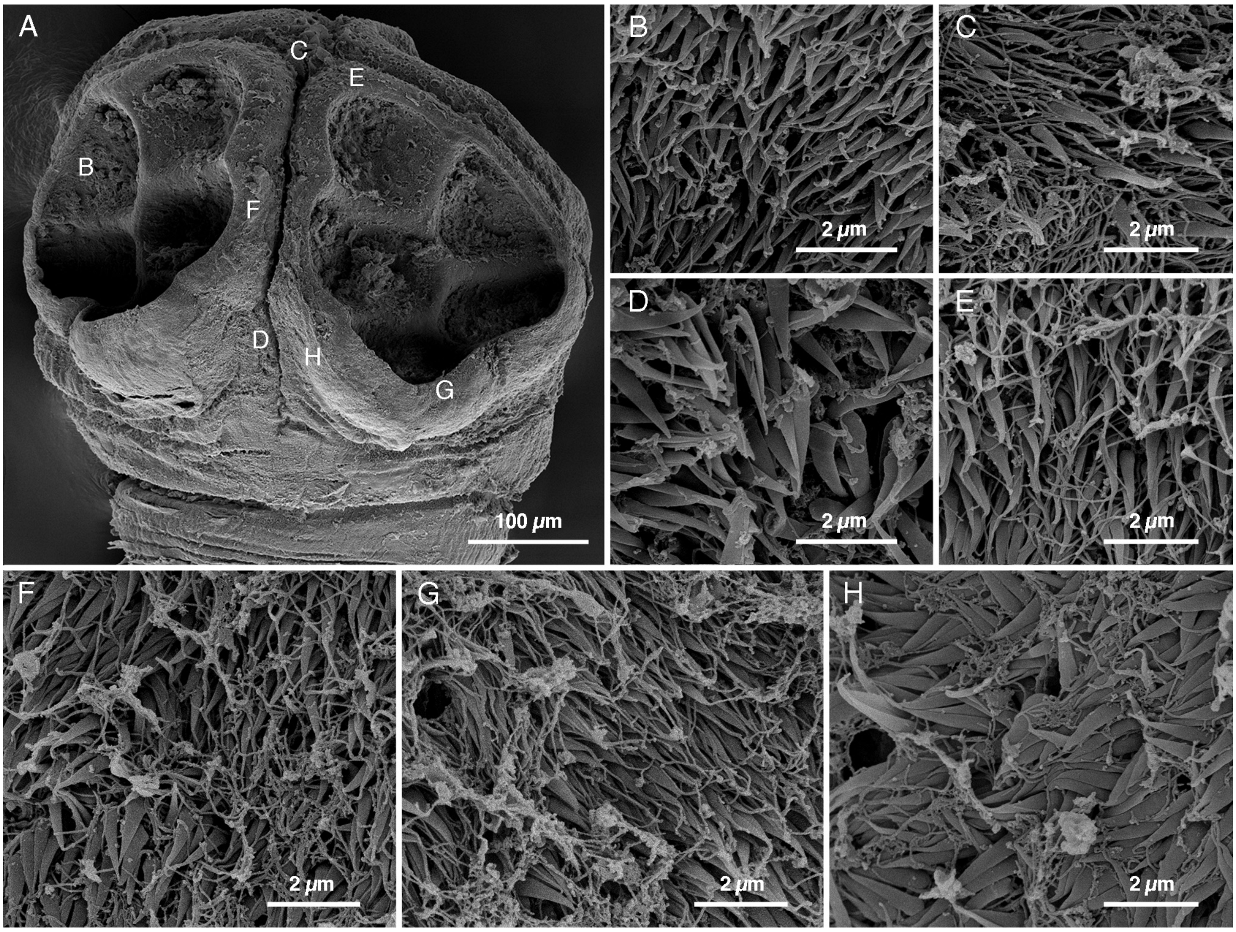

FIGURE 4. Scanning electron micrographs of *Pentaloculum grahami* **n. sp.** (A) Scolex, small letters indicate location of details in micrographs B–H. (B) Distal bothridial surface densely covered with long-tipped aristate gladiate spinitriches interspersed with gladiate spinitriches and capilliform filitriches. (C) Apex of scolex covered with gladiate spinitriches and densely packed capilliform filitriches. (D) Scolex proper densely covered with gladiate spinitriches interspersed with capilliform filitriches. (E) Proximal surface of margin of anteriormost loculus densely covered with long-tipped aristate gladiate spinitriches interspersed with gladiate spinitriches and capilliform filitriches. (F) Proximal surfaces of margins of anterior pair of loculi densely covered with long-tipped aristate gladiate spinitriches interspersed with gladiate spinitriches and capilliform filitriches. (G) Proximal surfaces of margins of posterior pair of loculi densely covered with long-tipped aristate gladiate spinitriches interspersed with gladiate spinitriches and capilliform filitriches. (H) Proximal surfaces away from margins of posterior pair of loculi densely covered with short-tipped wide aristate gladiate spinitriches, filitriches not observed.

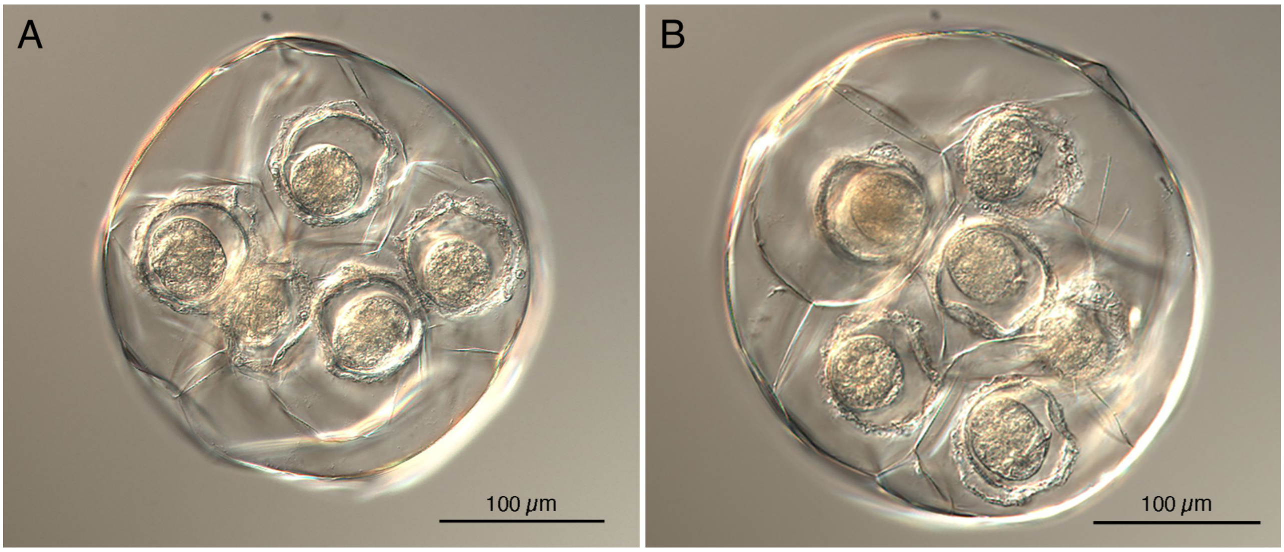

FIGURE 5. Photomicrographs of cocoons of *Pentaloculum grahami* **n. sp.** showing variation in number of oncospheres. (A) Cocoon containing five oncospheres. (B) Cocoon containing six oncospheres.

Remarks. *Pentaloculum grahami* **n. sp.** differs from *P. macrocephalum* and *P. hoi* in total worm length (12.08 – 51.62 vs. 8 and 1.7–3.5 mm, respectively) and total number of proglottids (105–174 vs. 23 and 16–26, respectively). It can be further distinguished from *P. macrocephalum* and *P. hoi* in that its mature proglottids are wider than long, as opposed to longer than wide, and in that the ovary is H-shaped rather than inverted A-shaped. It further differs from *P. hoi* in that its cocoons lack, rather than possess, bipolar filaments. It is of note that Hayden and Campbell (1981) described *Zyxibothrium kamienae* as possessing eggs that were contained in a membranous intrauterine tube, rather than arranged in cocoons.

Genus *Pentaloculum* Alexander, 1963 emended

Emendation. The revised diagnosis of this genus presented by Eudy *et al.* (2019) should be emended as follows to accommodate this new species. Additions are indicated in bold.

"Tetraphyllidea": Clade 1 of Caira *et al.* (2017). Scolex with four bothridia; each bothridium divided into five large facial loculi approximately equal in size, arranged as one anterior loculus followed by two consecutive pairs of loculi; septum dividing anterior pair of loculi slightly offset laterally from septum dividing posterior pair of loculi. Myzorhynchus lacking; cephalic peduncle present **or absent**. Strobila craspedote, hyperapolytic **or apolytic**. Genital pores lateral, irregularly alternating. Testes numerous, entirely anterior to ovary; postporal testes absent. Ovary posterior, inverted-A **or H-shaped** in frontal view, bilobed in cross-section. Vagina opening into genital atrium anterior to cirrus sac. Vitelline follicles in two lateral bands; bands consisting of two or more columns of follicles, **confluent posterior to the ovary**. Uterus saccate. Parasites of *Typhlonarke* and Parascylliidae; Pacific Ocean. Type species: *Pentaloculum macrocephalum* Alexander, 1963. Additional species: *P. hoi* Eudy, Caira and Jensen, 2019, *P. grahami* **n. sp.**

Zyxibothrium kamienae Hayden and Campbell, 1981 emended

(Fig. 6A–H)

Type and only known host: *Malacoraja senta* (Garman), smooth skate; (Rajiformes: Rajidae Bonaparte).
Type locality: Gulf of Maine, Maine, USA.
Site of infection: Spiral intestine.
Specimens examined: Scolex prepared for SEM retained with JNC at the University of Connecticut.
Sequence data: OQ186459 (hologenophore, LRP No. 9810 [BV-3-1, VB187])

Emendation. The description of this species presented by Hayden and Campbell (1981) should be emended to include the following information on the configuration of the microtriches of the scolex obtained from examination of one scolex with SEM (Fig. 6A).

Apex of scolex (Fig. 6B) and distal bothridial surfaces (Fig. 6C) densely covered with very long slender aristate gladiate spinitriches interspersed with gladiate spinitriches, filitriches not observed; proximal surfaces of anterior loculus (Fig. 6F) and margins of paired loculi (Fig. 6G) densely covered with very long slender aristate gladiate spinitriches interspersed with gladiate spinitriches, filitriches not observed; proximal surface of posterior loculus (Fig. 6H) densely covered with long slender gladiate spinitriches interspersed with acicular filitriches; proximal surface of bothridium away from locular margins (Fig. 6E) and scolex proper (Fig. 6D) densely covered with gladiate spinitriches, filitriches not observed.

Remarks. The scolex examined with SEM here is the one examined by Caira *et al.* (2001). However, after adding an additional 10 nm of gold/palladium to the specimen, we were able to characterize the microtriches in more detail and have followed the unified terminology for microtriches advanced by Chervy (2009). In addition to providing more detailed descriptions of the microtriches on the proximal and distal bothridial surfaces, we have now characterized the microtriches on the surfaces of the apex of the scolex and the scolex proper.

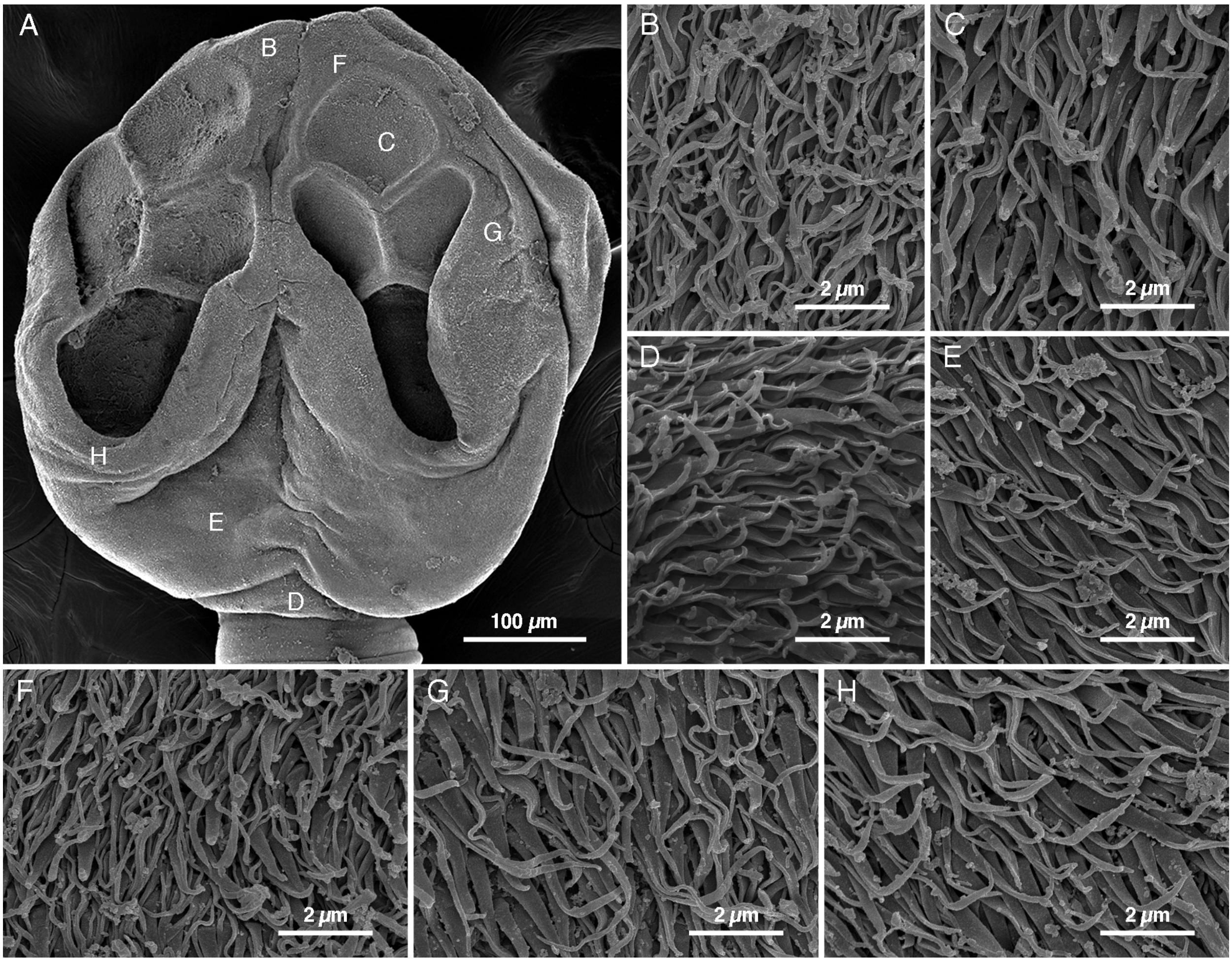

FIGURE 6. Scanning electron micrographs of *Zyxibothrium kamienae* Hayden and Campbell 1981. (A) Scolex, small letters indicate location of details in micrographs B–H. (B) Apex of scolex densely covered with very long slender aristate gladiate spinitriches interspersed with gladiate spinitriches, filitriches not observed. (C) Distal bothridial surface densely covered with very long slender aristate gladiate spinitriches interspersed with gladiate spinitriches, filitriches not observed. (D) Posterior region of scolex proper densely covered with gladiate spinitriches, filitriches not observed. (E) Proximal bothridial surface away from locular margins densely covered with gladiate spinitriches, filitriches not observed. (F) Proximal surface of anterior loculus densely covered with very long slender aristate gladiate spinitriches interspersed with gladiate spinitriches, filitriches not observed. (G) Proximal surface of paired loculi densely covered with very long slender aristate gladiate spinitriches interspersed with gladiate spinitriches, filitriches not observed. (H) Proximal surface of posterior loculus densely covered with long slender gladiate spinitriches interspersed with acicular filitriches.

Zyxibothrium duffyi n. sp.

(Figs. 7A–*D*, 8A–G)

ZooBank No. A0050BAE-FA9B-439D-B546-66F6BBFB2883

Type and only known host: *Brochiraja asperula* (Garrick and Paul), smooth deep-sea skate; (Rajiformes: Arhynchobatidae Fowler).

Type locality: Chatham Rise, New Zealand (44°0'S, 174°05'E).

Site of infection: Spiral intestine.

Type specimens: Holotype (NMNZ No. W.003925) and three paratypes (NMNZ Nos. W.003926–W.003928); three paratypes (LRP Nos. 9785, 9990, 1045) and two SEM vouchers (LRP Nos. 9796, 10946); two paratypes (USNM Nos. 1678894–1678895). Scoleces prepared for SEM retained with JNC at the University of Connecticut.

Sequence data: OQ186460 and OQ186461 (hologenophores LRP No. 9797 [CR-88-1, VB213] and LRP No. 9798 [CR-88-2, VB214]).

Etymology: This name honors elasmobranch biologist Clinton Duffy from the Department of Conservation, Wellington, New Zealand for his enthusiasm for elasmobranch parasites and for providing us with elasmobranch samples and their cestodes from New Zealand.

Description. Based on eight complete mature worms, and one scolex observed with SEM.

Worms euapolytic, 2.9–5.2 (4.2 ± 0.9; 6) mm long greatest width at level of scolex; 8–18 (14.4 ± 3.5; 8) proglottids per worm. Scolex consisting of four muscular bothridia and short cephalic peduncle. Scolex proper 374–805 (640.1 ± 147.4; 7) long by 556–737 (648.9 ± 63.6; 7) wide. Bothridia broadly ovoid, 342–629 (480.3 ± 90.5; 14; 7) long by 187–365 (290.5 ± 59.7; 6; 12) wide; each divided into five facial loculi arranged as one single anterior loculus followed by two consecutive pairs of loculi; septum dividing anterior pair of loculi slightly offset laterally from septum dividing posterior pair of loculi (Figs. 7A, 8A). Cephalic peduncle 65–248 (149.7 ± 73.8; 6) long by 218–274 (253.1 ± 20.5; 6) wide.

Apex of scolex (Fig. 8B) densely covered with capilliform filitriches; distal bothridial surfaces (Fig. 8F) densely covered with gladiate spinitriches interspersed with long slender aristate gladiate spinitriches and capilliform filitriches; proximal surfaces of anteriormost loculus (Fig. 8C) and anterior pair of loculi (Fig. 8D) densely covered with long slender aristate gladiate spinitriches interspersed with gladiate spinitriches and capilliform filitriches; proximal surfaces of posterior pair of loculi (Fig. 8E) densely covered with long slender aristate gladiate spinitriches interspersed with gladiate spinitriches, filitriches not observed; cephalic peduncle (Fig. 8G) densely covered with long slender gladiate spinitriches interspersed with long slender aristate gladiate spinitriches, filitriches not observed.

Proglottids slightly craspedote. Immature proglottids 7–16 (13.1 ± 3.4; 8) in number, wider than long, becoming longer than wide with maturity (Fig. 7C). Mature proglottids 1– 2 (1.3 ± 0.5; 8) in number, longer than wide (Fig. 7D), 1031–1597 (1247.3 ± 182.7; 8) long by 310–564 (413.4 ± 77.1; 8) wide, length:width ratio 2.5–3.9 (3.1 ± 0.6; 8):1; gravid proglottids not observed. Genital pores marginal, irregularly alternating, 94–97% (96 ± 1; 8) of proglottid length from posterior margin in terminal mature proglottid. Testes 32–51 (39.6 ± 7.6; 6; 7) in number, two layers deep, arranged in single dorsal field extending from mid-level of proglottid to anterior margin of proglottid, oval in frontal view, 30–53 long (41.5 ± 6.8; 23; 8) by 45–82 (64.1 ± 11; 8; 23) wide. Vas deferens highly coiled posterior to cirrus sac, extending posteriorly to level of ovarian bridge and then anteriorly to enter cirrus sac at medial margin. Cirrus sac (Fig. 7B) thin-walled, pyriform, tilted posteriorly, containing coiled cirrus, 276–355 (318.1 ± 26.1; 7) long by 134–182 (157.7 ± 16.3; 7) wide; cirrus armed with small spinitriches. Vagina thick-walled, weakly sinuous, extending from ootype along medial line of proglottid to level of cirrus sac, crossing cirrus sac ventrally and extending along anterior margin of cirrus sac to open into genital atrium anterior to cirrus; vaginal sphincter lacking; seminal receptacle lacking. Ovary near posterior end of proglottid, with smooth margins, H-shaped in frontal view, bilobed in cross-section, symmetrical; 168–266 (207.3 ± 30.2; 8) at widest point, 243–488 (328.4 ± 62.1; 8; 16) long. Mehlis' gland posterior to ovarian isthmus. Vitellarium follicular; follicles in two lateral bands; each band consisting of multiple columns of follicles, extending anteriorly to midlevel of cirrus sac, converging posterior to ovary; vitelline follicles oval, 17–33 (24.7 ± 64.4; 8; 24) long by 16–36 (24.3 ± 5.8; 8; 24) wide. Uterus medial, saccate, ventral to vagina, extending anteriorly from ovarian isthmus to posterior margin of cirrus sac. Excretory vessels four, arranged in one dorsal and one ventral pair on each lateral margin of proglottid. Oncospheres not observed.

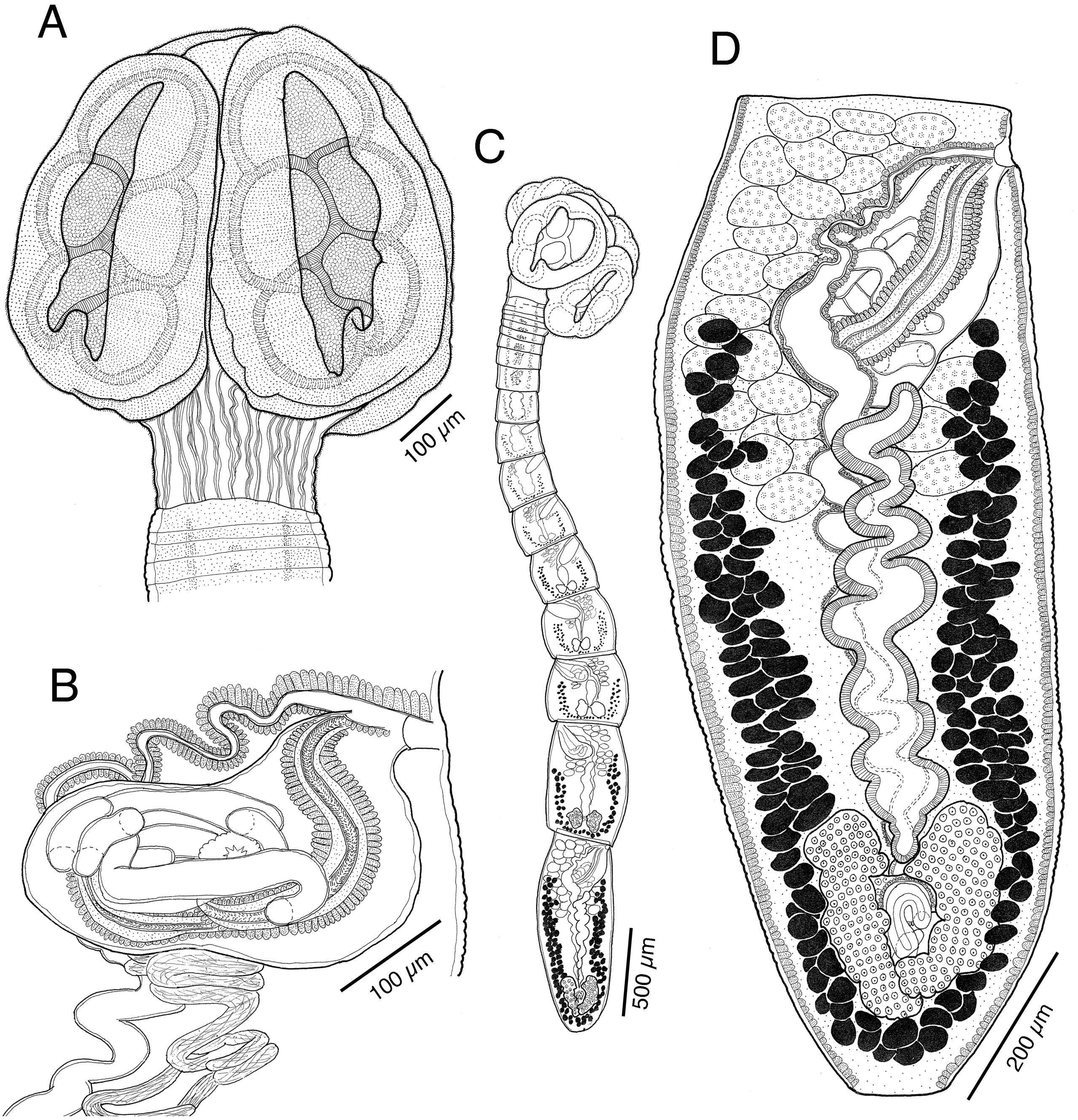

FIGURE 7. Line drawings of *Zyxibothrium duffyi* **n. sp.** (A) Scolex (paratype, CR-65-5, LRP No. 9990). (B) Detail of terminal genitalia (paratype, CR-28-1, LRP No. 9785). (C) Whole worm (holotype, CR-65-1, NMNZ No. W.003925). (D) Mature proglottid (holotype, CR-65-1, NMNZ No. W.003925).

Remarks. *Zyxibothrium duffyi* **n. sp.** differs from *Z. kamienae* most conspicuously in having five, rather than four, loculi on each bothridium. It can be further distinguished from *Z. kamienae* in that it is euapolyic rather than apolytic, possesses a greater number of testes (32–51 vs 19–24), and has a greater number of proglottids (8–18 vs 5–8).

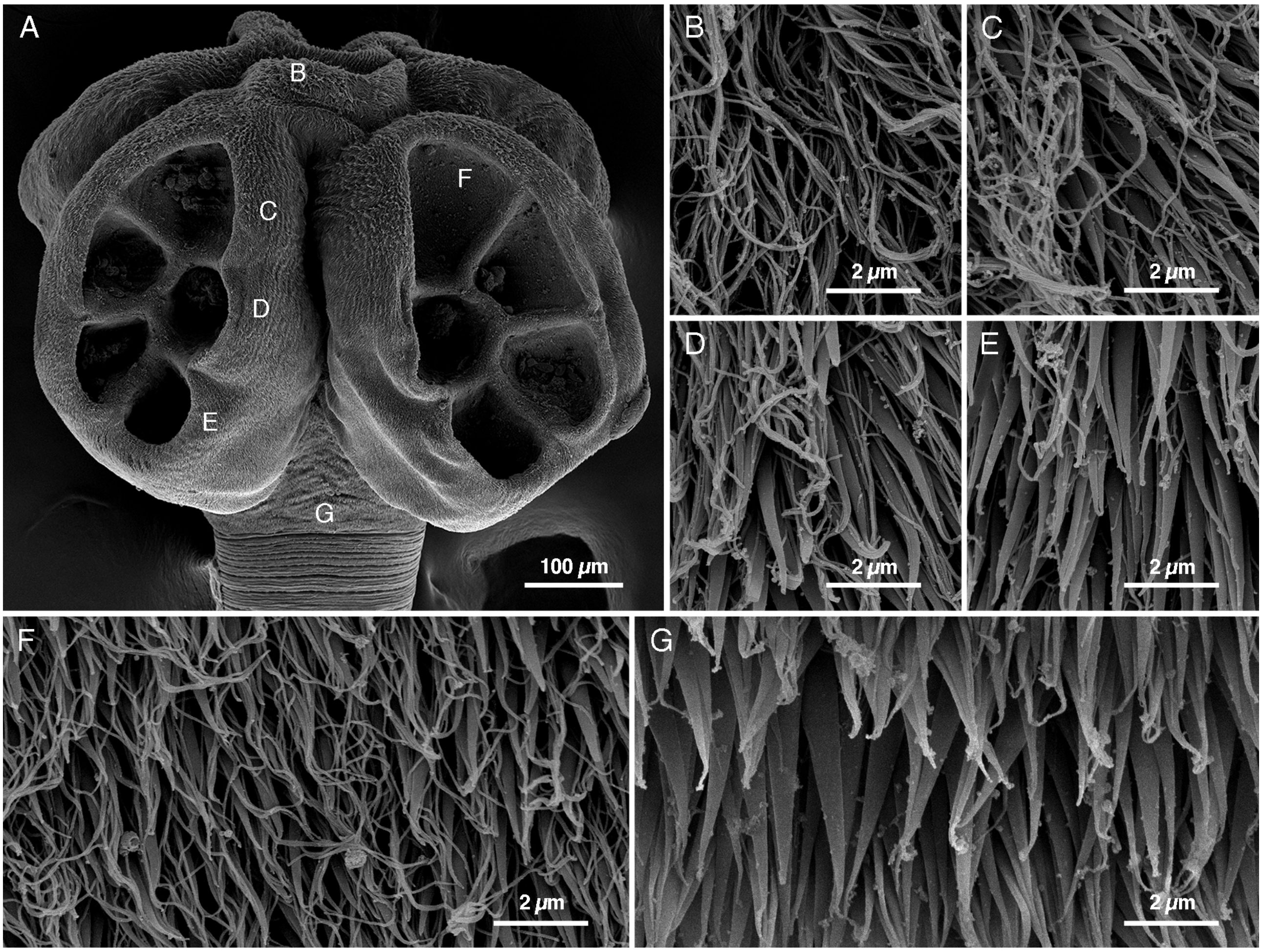

FIGURE 8. Scanning electron micrographs of *Zyxibothrium duffyi* **n. sp.** (A) Scolex, small letters indicate location of details in micrographs B–G. (B) Apex of scolex densely covered with capilliform filitriches. (C) Proximal surface of anteriormost loculus densely covered with long slender aristate gladiate spinitriches interspersed with gladiate spinitriches and capilliform filitriches. (D) Proximal surfaces of anterior pair of loculi densely covered with long slender aristate gladiate spinitriches interspersed with gladiate spinitriches and capilliform filitriches. (E) Proximal surfaces of posterior pair of loculi densely covered with long slender aristate gladiate spinitriches interspersed with gladiate spinitriches, filitriches not observed. (F) Distal bothridial surface densely covered with long slender aristate gladiate spinitriches interspersed with gladiate spinitriches and capilliform filitriches. (G) Cephalic peduncle densely covered with long slender gladiate spinitriches interspersed with long slender aristate gladiate spinitriches, filitriches not observed.

Zyxibothrium healyae n. sp.
(Figs. 9A–D, 10A–G)
ZooBank No. 024AF7D4-85BF-47C2-8310-4C7BF5F0151F

Type and only known host: *Brochiraja spinifera* (Garrick and Paul), prickly deep-sea skate; (Rajiformes: Arhynchobatidae Fowler).

Type locality: Chatham Rise, New Zealand (43°08’S, 174°19’E).

Site of infection: Spiral intestine.

Type specimens: Holotype (NMNZ No. W.003930) and two paratypes (NMNZ Nos. W.003929 and W.003931); two paratypes (LRP Nos. 9799 and 9802); one paratype (USNM No. 1678896). Scolex prepared for SEM retained with JNC at the University of Connecticut.

Sequence data: OQ186462 (hologenophore LRP No. 9803 [CR-151-3, VB232]).

Etymology: This name honors parasitologist Claire Healy who collected the specimens of this and several other cestode species used in this study from elasmobranchs in New Zealand.

Description. Based on six complete mature worms, and one scolex observed with SEM.

Worms euapolytic, 4.6–7.4 (6.1 ± 1.1; 6) mm long, greatest width at level of scolex; 14–17 (15.1 ± 1.1; 6) proglottids per worm. Scolex consisting of four muscular bothridia and short cephalic peduncle. Scolex proper 516–978 (770.3 ± 180.6; 6) long by 504–684 (624.8 ± 71.9; 6) wide. Bothridia narrowly dolioform, 446–768 (622.8 ± 112.8; 6; 12) long by 213–341 (283.1 ± 37.6; 6; 12) wide; each divided into three facial loculi arranged in one column (Figs. 9A, 10A). Cephalic peduncle 77–244 (157.8 ± 71.6; 6) long by 191–338 (262.5 ± 53.8; 6) wide.

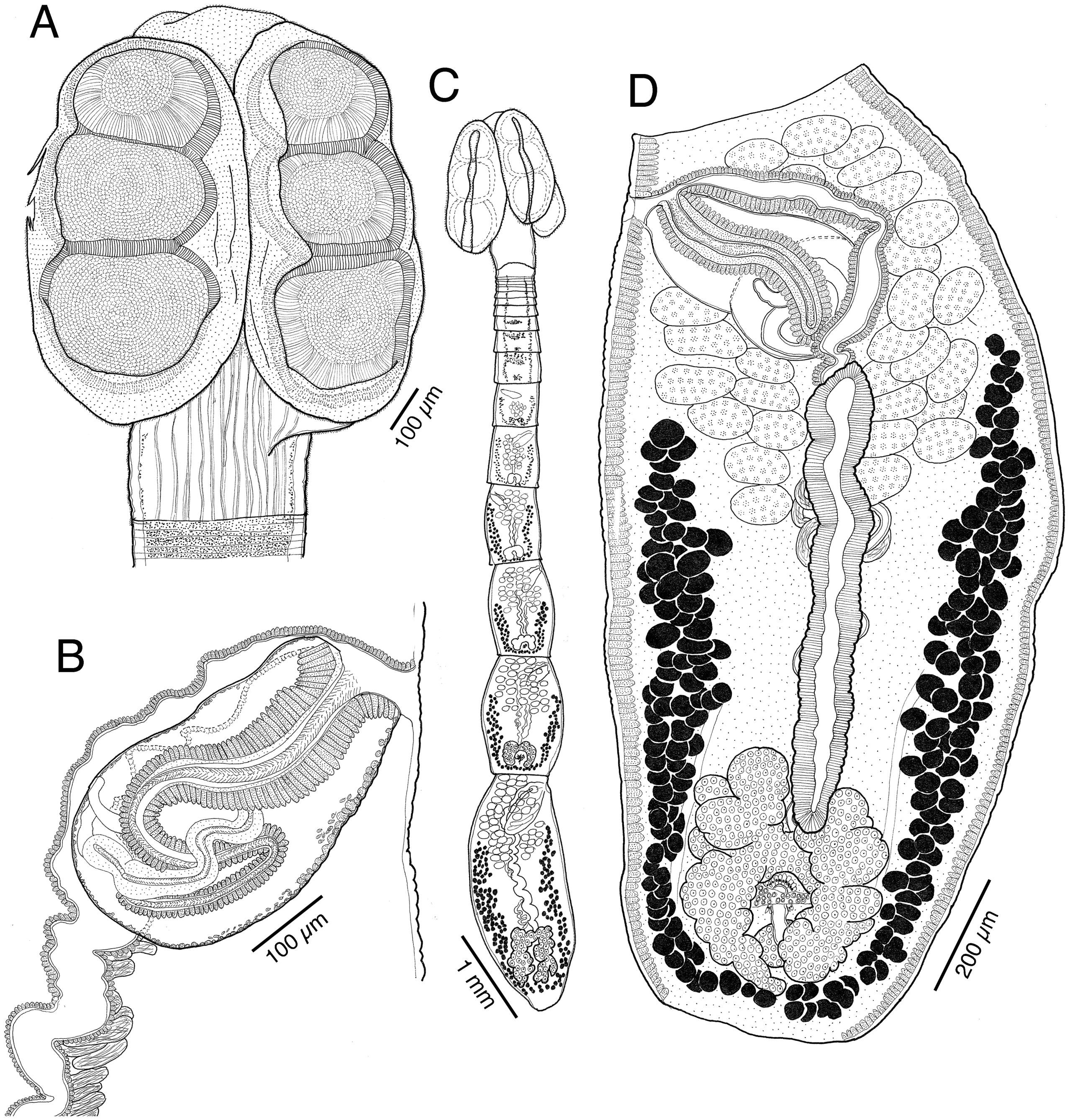

FIGURE 9. Line drawings of *Zyxibothrium healyae* **n. sp.** (A) Scolex (paratype, CR-76-1, NMNZ No. W.003931). (B) Detail of terminal genitalia (holotype, CR-75-2, NMNZ No. W.003930). (C) Whole worm (holotype, CR-75-2, NMNZ No. W.003930). (D) Mature proglottid (paratype, CR-75-1, LRP No. 9799).

Apex of scolex (Fig. 10B) sparsely covered with long slender aristate gladiate spinitriches and densely packed capilliform filitriches; distal bothridial surfaces (Fig. 10F) densely covered with long slender aristate gladiate spinitriches interspersed with capilliform filitriches; proximal bothridial surfaces of anterior loculus (Fig. 10C) and middle loculus (Fig. 10D) densely covered with long slender aristate gladiate spinitriches interspersed with gladiate spinitriches, filitriches not observed; proximal surface of posterior loculus (Fig. 10E) densely covered with long slender gladiate spinitriches, filitriches not observed; cephalic peduncle (Fig. 10G) densely covered with large gladiate spinitriches interspersed with small gladiate spinitriches, filitriches not observed.

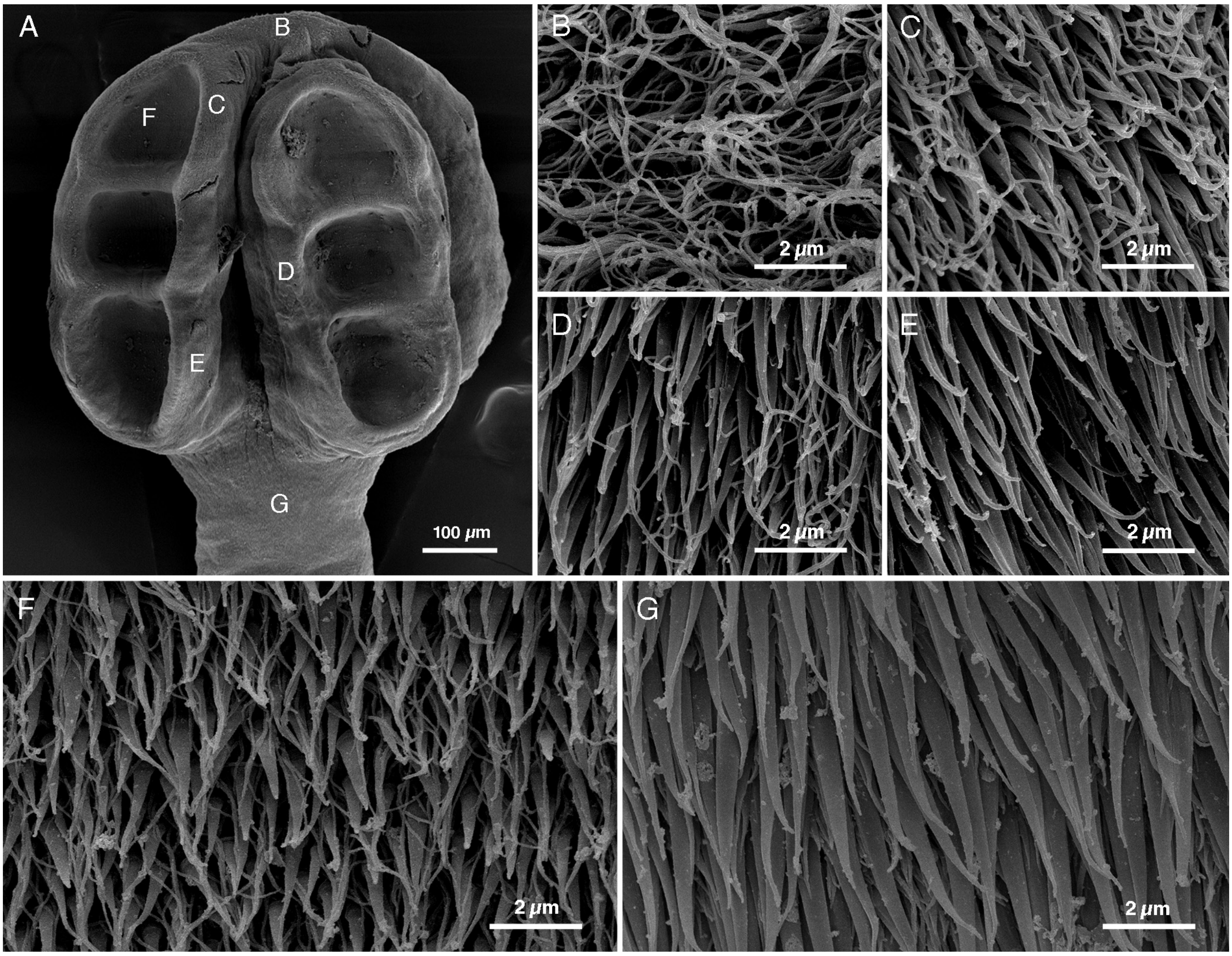

FIGURE 10. Scanning electron micrographs of *Zyxibothrium healyae* **n. sp.** (A) Scolex, small letters indicate location of details in micrographs B–G. (B) Apex of scolex sparsely covered with long slender aristate gladiate spinitriches and densely packed capilliform filitriches. (C) Proximal surface of anterior loculus densely covered with long slender aristate gladiate spinitriches interspersed with gladiate spinitriches, filitriches not observed. (D) Proximal surface of middle loculus densely covered with long slender aristate gladiate spinitriches interspersed with gladiate spinitriches, filitriches not observed. (E) Proximal surface of posterior loculus densely covered with long slender gladiate spinitriches, filitriches not observed. (F) Distal bothridial surface densely covered with long slender aristate gladiate spinitriches interspersed with capilliform filitriches. (G) Cephalic peduncle densely covered with large gladiate spinitriches interspersed with small gladiate spinitriches, filitriches not observed.

Proglottids slightly craspedote. Immature proglottids 12–16 (13.8 ± 1.5; 6) in number, wider than long, becoming longer than wide with maturity (Fig. 9C). Mature proglottids (Fig. 9D) 1–2 (1.3 ± 0.5; 6) in number, 1229–1601 (1435.7 ± 138.9; 6) long by 328–717 (514.5 ± 162.9; 6) wide, length:width ratio 2.1–4.5 (3 ± 0.9; 6):1; gravid proglottids not observed. Genital pores marginal, irregularly alternating, 91–94% (93 ± 1; 6) of proglottid length from posterior margin in terminal mature proglottid. Testes 24–31 (26.1 ± 2.2; 6; 7) in number, two layers deep, dorsal to cirrus sac, arranged in single dorsal field extending from mid-level of proglottid to anterior margin of proglottid, oval in frontal view, 33–77 long (50.8 ± 12.5; 6; 17) by 49–114 (71.3 ± 19.3; 6; 17) wide. Vas deferens coiled posterior to cirrus sac, extending posteriorly to the level of ovarian bridge and then anteriorly to enter cirrus sac at medial margin. Cirrus sac (Fig. 9B) thin-walled, pyriform, tilted posteriorly, containing coiled cirrus, 223–384 (309.2 ± 59.9; 5) long by 162–212 (185 ± 19.7; 5) wide; cirrus armed with small spinitriches. Vagina thick-walled, weakly sinuous, extending from ootype along medial line of proglottid to level of cirrus sac, crossing cirrus sac ventrally and extending along anterior margin of cirrus sac to open into genital atrium anterior to cirrus;

vaginal sphincter lacking; seminal receptacle lacking. Ovary near posterior end of proglottid, with weakly lobulated margins, H-shaped in frontal view, bilobed in cross-section, symmetrical; 157–311 (215.3 ± 66.6; 6; 7) at widest point, 319–422 (360 ± 39; 4; 8) long. Mehlis' gland posterior to ovarian isthmus. Vitellarium follicular; follicles in two lateral bands; each band consisting of multiple columns of follicles, extending anteriorly to posterior margin of cirrus sac, converging posterior to ovary; vitelline follicles oval, 16–40 (27± 6.5; 5; 15) long by 22–43 (30.6 ± 7.6; 5; 15) wide. Uterus medial, saccate, ventral to vagina, extending anteriorly from ovarian isthmus to posterior margin of cirrus sac. Excretory vessels four, arranged in one dorsal and one ventral pair on each lateral margin of proglottid. Oncospheres not observed.

Remarks. *Zyxibothrium healyae* **n. sp.** differs from *Z. kamienae* and *Z. duffyi* most conspicuously in having three, rather than four or five, loculi on each bothridium, respectivel*y*. It can be further distinguished from *Z. kamienae* and *Z. duffyi* in testes number (24–31 vs. 19–24 and 32–51, respectively). It also possesses a greater number of proglottids than *Z. kamienae* (14–17 vs 5–8).

Genus *Zyxibothrium* Hayden and Campbell, 1981 revised

Revision. Hayden and Campbell's (1981) original diagnosis of this genus is relatively brief. The following expanded diagnosis accommodates both of the new species described here and also facilitates comparisons between genera in Clade 1.

"Tetraphyllidea": Clade 1 of Caira *et al.* (2017). Scolex with four bothridia; each bothridium divided into three, four, or five large facial loculi, equal or unequal in size, arranged as one anterior loculus followed by two consecutive pairs of loculi, or a single pair of loculi and one posterior loculus, or two single loculi. Myzorhynchus lacking; cephalic peduncle present or absent. Strobila craspedote, euapolytic or apolytic. Genital pores lateral, irregularly alternating. Testes numerous, entirely anterior to ovary, distributed in single field dorsal to cirrus sac. Ovary posterior, H-shaped, bilobed in cross-section. Vagina opening into genital atrium anterior to cirrus sac. Vitelline follicles in two lateral bands, each band consisting of two or more columns of follicles; bands confluent posterior to the ovary. Uterus saccate. Parasites of *Malacoraja* and *Brochiraja*; Atlantic and Pacific Oceans. Type species: *Zyxibothrium kamienae* Hayden and Campbell, 1981. Additional species: *Z. duffyi* **n. sp.**, *Z. healyae* **n. sp.**

Discussion

The phylogenetic tree presented here (Fig. 1) supports the hypothesis advanced by Caira *et al.* (2017) that *Pentaloculum* and *Zyxibothrium* are closely related genera and potentially represent an independent lineage among cestodes of elasmobranchs, which they referred to provisionally as Clade 1. Although the position of this lineage relative to other cestode groups remains unresolved, its monophyly is well supported by both morphological and molecular data. The possession of a scolex with a small number (i.e., 3–5) of large, circular loculi on each bothridium appears to be the main diagnostic feature for species that belong to Clade 1. Another distinguishing feature for these species is the distribution of the vitelline follicles. In many groups of cestodes that parasitize elasmobranchs, these structures are distributed in two separate lateral bands. In contrast, in species of Clade 1 these lateral bands converge posterior to the ovary, creating a single continuous U-shaped band. We would note that this interpretation of the distribution of the vitelline follicles differs from both the description and illustrations of *P. macrocephalum* given by Alexander (1963) and also from the reinterpretation of the vitelline follicle distribution provided by Eudy *et al.* (2019) following their examination of two hologenophores fixed in ethanol. The revised interpretation presented here was based on examination of the formalin-fixed voucher of the specimen we examined with SEM. The mature proglottids of this specimen clearly show that the lateral bands of vitelline follicles are confluent posterior to the ovary, as observed in the five other species in Clade 1.

The topology of the tree also shows that *Pentaloculum* and *Zyxibothrium* are reciprocally monophyletic subgroups within Clade 1. In addition, there is strong anatomical evidence to support the mutual monophyly of the two genera. Species can be identified to genus based on the position of the genital pore and the configuration of the lateral bands of vitelline follicles. All three species of *Pentaloculum* have genital pores that are located either in the middle or slightly toward the posterior end of the proglottid. In contrast, all three species of *Zyxibothrium* bear genital pores that are located near the anterior margin of the proglottid. Furthermore, in all species of *Pentaloculum* the lateral bands of vitelline follicles extend throughout the length of the proglottid almost to its anterior margin, whereas in species of Zyxi*bothrium*, the lateral bands of vitelline follicles extend only to the level of the posterior margin or middle of the cirrus sac.

The number of bothridial loculi, however, is not suitable for diagnosis at the genus level. Although, as their generic name implies, all three species of *Pentaloculum* have bothridia with five large facial loculi, so too does *Z. duffyi.* Given the two other species of *Zyxibothrium* have three or four loculi, the presence of five loculi appears to be a homoplasious character state in this clade.

Our current knowledge of the host associations of both *Pentaloculum* and *Zyxibothrium* can help guide future field work targeting elasmobranchs that are most likely to host additional species in Clade 1. We echo the suggestion of Eudy *et al.* (2019) that the presence of *P. macrocephalum* in an electric ray is exceptional and thus examination of the cestode faunas of carpet sharks in the family Parascylliidae beyond *C. formosanum* and *P. collare* is likely to be the most productive strategy for discovering additional novelty in *Pentaloculum*. However, we predict that this work is unlikely to yield a large number of new species because the number of potential hosts is relatively small with only two species of *Cirrhoscyllium* Smith and Radcliff and five species of *Parascyllium* Gill remaining to be investigated.

Prior to the present work, the strategy for discovering additional novelty in *Zyxibothrium* would have mainly included examination of the cestode faunas of the six species of the skate genus *Malacoraja* Stehmann (see Last *et al.* 2016) that remain to be sampled for cestodes. Discovery of two additional species of *Zyxibothrium* in members of the skate genus *Brochiraja* suggests that expanding sampling to include the six species of *Brochiraja* that have not yet been examined for cestodes is also in order. In some respects, this host distribution is similar to the situation seen in *Pentaloculum* because these two skate genera are only distantly related; *Malacoraja* belongs to the family Rajidae Bonaparte and *Brochiraja* belongs to the family Arhynchobatidae Fowler. Nonetheless, it might also be productive to expand sampling to include the close relatives of these two skate genera. According to Naylor *et al.* (2012), *Malacoraja* is the sister taxon of *Neoraja* Ishiyama, all five species of which have yet to been examined for cestodes. Naylor *et al.* (2012) also found *Brochiraja* to be the sister taxon of *Pavoraja* Whitley, and that subclade was sister to a subclade consisting of *Insentiraja* Yearsley and Last and *Notoraja* Ishiyama. This suggests that the six species of *Pavoraja*, two species of *Insentiraja*, and 15 species of *Notoraja* (*sensu* Last *et al.* 2016; Concha *et al.* 2016; White *et al.* 2017; Weigmann *et al.* 2021) that have not previously been examined for cestodes are also candidate hosts. If the oioxenous nature of members of species of *Zyxibothrium* persists, the total number of potential species of *Zyxibothrium* may be as high as 36.

However, records show that many skate genera do not host *Zyxibothrium*. The clade consisting of *Brochiraja*, *Pavoraja*, *Insentiraja* and *Notoraja* is sister to a large clade containing species in the genera *Bathyraja* Ishiyama and *Rhinoraja* Ischiyama (Naylor *et al.* 2012). Of the 58 species in those two genera, 10 species of *Bathyraja* have been examined for cestodes. That work (Campbell 1977; Wojciechowska 1990a, 1990b, 1991a, 1991b; Campbell and Andrade 1997; Rocka and Zdzitowiecki 1998; Keeney and Campbell 2001; Ivanov 2006; Franzese and Ivanov 2020; Caira *et al.* 2021; Menoret and Ivanov 2021; Franzese *et al.* 2022) has resulted in the description of 18 cestode species belonging to five orders—the Rhinebothriidea, the Onchoproteocephalidea, the Diphyllidea, the Phyllobothriidea, and the Trypanorhyncha—but no species of *Zyxibothrium* have yet been found. Representatives of the same five cestode orders have been reported from skate species that are closely related to *Malacoraja* and *Neoraja*. In the phylogeny of Naylor *et al.* (2012), the subclade that includes *Malacoraja* and *Neoraja* is sister to a large subclade that consists of 51 species in five genera—*Amblyraja* Malm, *Rajella* Stehmann, *Leucoraja* Malm, *Orbiraja* Last, Weigmann and Dumale, and *Rostroraja* Hulley (Ebert and Leslie 2019; Last *et al.* 2016; Naylor *et al.* 2012). Examination of the cestode fauna of nine species in this subclade has yielded 26 cestode species (Abbott and Caira 2014; Baer 1956; Beveridge and Campbell 2007; Campbell 1975, 1977; Euzet 1956; Hassan 1982; Heller 1949; Keeling and Burt 1996; McVicar 1976; Olsson 1886; Randhawa *et al.* 2008; Rees 1959; Schmidt 1986; Van Der Spuy *et al.* 2022; Williams 1966, 1968a; 1968b, 1969; Wojciechowska 1990b, 1991a, 1991b), none of which are species of *Zyxibothrium*. These records lead us to believe that none of these skate genera are suitable hosts for *Zyxibothrium*.

The current members of Clade 1 exhibit an interesting geographic distribution in that all species of *Pentaloculum* and all but one species of *Zyxibothrium* occur only in the waters of the western Pacific Ocean (i.e., off of Taiwan, eastern Australia, and New Zealand). The exception is *Z. kamienae* which occurs only in the northeastern Atlantic Ocean. The obvious explanation for these distributions is that each of these cestode species exhibits oioxenous specificity for its elasmobranch host and thus occurs only in regions in which its host species occurs. The host of *Z. kamienae* occurs in the northeastern Atlantic Ocean and those of all of the other members of Clade 1 occur in the western Pacific Ocean. Even if our predictions about other potential hosts of members of Clade 1 are confirmed, these distributions are unlikely to change too dramatically because all other species of *Brochiraja* and all species of the closest relatives of this genus (i.e., *Pavoraja*, *Insentiraja*, and *Notoraja*) are found either in the western Pacific Ocean or in the coastal waters around Australia. The exception is *Notoraja martinezi* Concha, Ebert and Long, which is found off the coast of Ecuador. This is also the case for all of the species of *Cirrhoscyllium* and *Parascyllium* that remain to be examined for cestodes. Similarly, all species of *Malacoraja* and *Neoraja* that remain to be examined, occur in the Atlantic Ocean. That does not, however get at the more interesting question of why these host groups have such a disjunct distribution.

Among the most important contributions of this work are the facts that we have doubled the number of described species in the taxon referred to as Clade 1 among the "tetraphyllidean" relics by Caira *et al.* (2017), while simultaneously expanding our understanding of the morphology and anatomy of its members. Moreover, we have confirmed the monophyly of the group that consists of *Pentaloculum* and *Zyxibothrium*. It is our hope that this additional information will help inform the ultimate revision of the ordinal classification of the cestodes to address the highly polyphyletic nature of the "Tetraphyllidea" as the order is currently configured.

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