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## THREE NEW SPECIES OF *RHINEBOTHRIUM* (CESTODA: RHINEBOTHRIIDEA) FROM THE LEOPARD WHIPRAY, *HIMANTURA LEOPARDA*, IN AUSTRALIA

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### KEY WORDS ABSTRACT

Rhinebothriidae  
New Species  
Diversity  
Host Associations  
Stingrays  
Dasyatidae

Examination of 4 specimens of the leopard whipray *Himantura leoparda*, a dasyatid stingray from northern Australia, led to the discovery of 3 new species of *Rhinebothrium*. *Rhinebothrium leopardensis* n. sp., *Rhinebothrium nandoi* n. sp., and *Rhinebothrium ruhkei* n. sp. are described, increasing the diversity of the genus to 51 species globally. All 3 new species differ from their congeners in terms of testis number, proglottid number, loculus number, and size. With respect to one another, *R. leopardensis* n. sp. has bothridia that are weakly constricted at their centers and has a greater number of proglottids than the other 2 species (93–108 vs. 11–15, and 48–78, respectively). *Rhinebothrium nandoi* n. sp. is the smallest of the 3 species found in *H. leoparda* (3.6–5 vs. 10–15 mm and 10.1–15.8 mm in total length [TL], respectively) and bears bothridia that are constricted at their centers. *Rhinebothrium ruhkei* n. sp. bears bothridia that are conspicuously constricted at their centers and has more testes than *R. leopardensis* and fewer than *R. nandoi* (7–10 vs. fewer than 7 and 21–33, respectively). Before this study, 56% (27 of 48) of *Rhinebothrium* species had been described from the freshwater river systems of South America and the marine waters surrounding South and North America. In contrast, despite the remarkably diverse nature of its batoid fauna, only 19 species were known from the Indo-Pacific region. Our work increases this number to 22, emphasizing the highly underestimated nature of *Rhinebothrium* diversity in this region of the globe. The discovery of these 3 new species was not unexpected, given the relatively poor status of our current knowledge of the cestode faunas of dasyatid stingrays in the Indo-Pacific region, and given the fact that it is common for a single batoid species to host 2 or more species of *Rhinebothrium*. Our results suggest that additional work on the cestode faunas of the batoids, especially dasyatids, from the Indo-Pacific region is likely to be highly productive in terms of contributing to the knowledge of *Rhinebothrium* diversity.

At 48 species, the global diversity of the batoid-parasitizing cestode genus *Rhinebothrium* Linton, 1890 is likely highly underestimated. More than half of these species have been described from the stingray family Dasyatidae Jordan alone, but only 18 of the 89 dasyatid species, in 10 of 19 genera, have been examined for *Rhinebothrium* (see Ruhnke et al., 2017). Furthermore, it is common to find more than 1 species of *Rhinebothrium* parasitizing the same host species. This is the case for more than half of the described species. The granulated guitarfish *Glaucostegus granulatus* (Cuvier), the filetail fanskate *Sympterygia lima* (Poeppig), the ribbontail stingray *Taeniura lymma* (Forsskål), the giant freshwater whipray *Urogymnus polylepis* (Bleeker), the

estuary stingray *Hemitrygon fluviorum* (Ogilby), the discus ray *Paratrygon aiereba* (Müller and Henle), the South American freshwater stingray *Potamotrygon motoro* (Müller and Henle), the smooth back river stingray *Potamotrygon orbignyi* (Castelnau), and the chupare stingray *Styracura schmardae* (Werner) each host 2 species of *Rhinebothrium* (see Euzet and Carvajal-Garay, 1973; Ramadan, 1984; Healy, 2006; Menoret and Ivanov, 2011; Reyda and Marques, 2011; Golestaninasab and Malek, 2015; Marques and Reyda, 2015; Trevisan and Marques, 2017; Coleman et al., 2019a, respectively). The rough-tail stingray *Bathytoshia centroura* (Mitchell), the southern stingray *Hypanus americanus* (Hildebrand and Schroeder), and the Haller's round ray *Urolophus halleri* each host 3 species (see Linton, 1890, 1924; Baer, 1948; Young, 1955; Campbell, 1970, 1975; Friggens and Duszynski, 2005).

It was therefore not unexpected that examination of specimens of the leopard whipray, *Himantura leoparda* Manjaji-Matsumoto

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and Last—a dasyatid species not previously examined for *Rhinebothrium*—led to the discovery of additional novelty. The 3 new species of *Rhinebothrium* encountered in this stingray species in northern Australia are described below.

## MATERIALS AND METHODS

### Specimen collection

In total, 3 female specimens (i.e., NT-23, NT-37, and NT-117) and 1 male specimen (NT-32) of *Himantura leoparda* were examined. The specimens were collected during a fishing trip of the fishing vessel Ocean Harvest to the Arafura Sea, east of the Wessel Islands (11°17'44"S, 136°59'48"E) in the Northern Territory, Australia between 11 and 22 November 1999. The disc width (DW) of the 3 female specimens examined was 100–136 cm and the total length (TL) of the male was 112 cm. Additional information for each specimen can be accessed in the Global Cestode Database (Caira et al., 2019) by entering their unique specimen numbers (e.g., NT-23). The spiral intestine of each stingray was removed, opened with a midventral longitudinal incision, and washed with seawater to remove some cestodes. A subset of the specimens from each stingray was preserved in 95% ethanol for future molecular work. The remaining specimens were preserved in 4% formalin-buffered seawater for several days, placed in 70% ethanol for storage, and then processed for morphological analysis. The spiral intestines of all 4 specimens were subsequently fixed in 4% formalin-buffered seawater for several days and then placed in 70% ethanol for storage.

### Morphological methods

Whole worms selected for light microscopy were hydrated in a graded ethanol series, stained with Delafield's hematoxylin (9:1 solution), destained in a 1% acid (HCl) ethanol solution, alkalized in a 1% basic (NaOH) ethanol solution, dehydrated in a graded ethanol series, and cleared in methyl salicylate. During the dehydration process, specifically in 80% ethanol, the specimens were placed on a piece of filter paper and carefully straightened using a camel-hair brush. Small pieces of glass slides were then placed on them to keep them in that position throughout the remainder of the dehydration process. The pieces of glass were removed at the transition from 100% ethanol to methyl salicylate and the specimens were mounted in Canada balsam on glass slides under coverslips.

Morphometric data and photographs were generated either with an Olympus SC30 camera with the analySIS getIT software (Olympus Soft Images Solutions, North Rhine-Westphalia, Germany), attached to an Olympus BX51 microscope (Olympus) or an Zeiss Axioskop 2 Plus compound microscope (Zeiss, Thornwood, New York) using a SPOT Diagnostic Instrument Digital Camera and SPOT version 4.6 (SPOT Imaging Solutions, Sterling Heights, Michigan). The packages Fiji/ImageJ (Schindelin et al., 2012) and WormBox (Vellutini and Marques, 2014) were used to process images and compute morphometric data, respectively. Only complete specimens with mature (i.e., with open genital pores) or further developed proglottids (e.g., with atrophied testes or vas deferens filled) were examined and measured. All measurements of reproductive structures were taken from terminal proglottids. In cases in which the terminal

proglottids contained only atrophied testes, testes data were generated from the subterminal mature proglottid. Measurements are given in the text as the range followed in parentheses by the mean, standard deviation, number of worms examined, and the total number of measurements taken if more than 1 measurement per specimen was made. Line drawings were prepared with the aid of a drawing tube attached to a Zeiss Axioskop 2.

A portion of the posterior strobila of 2 specimens of each of the 3 new species was embedded in paraffin and sectioned at 7-µm intervals using a LEICA RM 2025 (Leica Microsystems, Baden-Württemberg, Germany) retracting rotary microtome. Sections were mounted on glass slides flooded with distilled water and dried on a slide warmer for 5 min and later transferred to an oven for 30 min at 60 °C. Cross-sections were stained with Mayer's hematoxylin, counterstained with eosin, dehydrated in a graded ethanol series, cleared in xylene, and mounted under coverslips on glass slides in Entellan (Merck, Darmstadt, Germany). The anterior portion of each worm sectioned was prepared as a whole mount as described above and retained as a voucher.

Scolecites selected for scanning electron microscopy (SEM) were hydrated in a graded ethanol series, transferred to 1% osmium tetroxide overnight, dehydrated in a graded ethanol series, and placed in hexamethyldisilazane (Ted Pella Inc., Redding, California). They were allowed to air-dry for at least 1 hr in a fume hood and were subsequently mounted on aluminum stubs on double-sided PELCO carbon tabs (Ted Pella Inc.), sputter-coated with 35 nm of gold/palladium and examined with an FEI Nova NanoSEM 450 (FEI, Hillsboro, Oregon) field emission scanning electron microscope. Two or 3 specimens of each new species were examined. The strobila of the worm used for SEM was prepared as a whole mount voucher as described above. Microthrix terminology follows Chervy (2009).

Museum abbreviations used are as follows: LRP, Lawrence R. Penner Parasitology Collection, University of Connecticut, Storrs, Connecticut; QM, Queensland Museum, Brisbane, Queensland, Australia; USNM, National Museum of Natural History, Smithsonian Institution, Washington, D.C.; MZUSP, Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil.

For this work, rather than listing all 48 valid species of *Rhinebothrium*, we refer to the list of 41 species of Ruhnke et al. (2017), except for *Rhinebothrium paranaensis* Menoret and Ivanov, 2009, which was synonymized with *Rhinebothrium paratrygoni* Rego and Dias, 1976 by Reyda and Marques (2011). We also note that 8 species have been described since Ruhnke et al. (2017) generated their list. One of these was described by Trevisan and Marques (2017). The remaining 7 were described by Coleman et al. (2019a). We do not agree with the transfer of *Rhinebothrium ditesticulum* Appy and Dailey, 1977 to *Ruptobothrium* Coleman, Beveridge and Campbell, 2019b by Coleman et al. (2019b). Although the bothridia of this species are constricted at their center, they are not divided into 2 completely separate lobes, and thus this species lacks the feature used by Coleman et al. (2019b) to define their new genus. As a consequence *R. ditesticulum* is included as a species of *Rhinebothrium* in the comparisons made below for 2 of our new species that bear a similar bothridial constriction.

## DESCRIPTIONS

### *Rhinebothrium leopardensis* n. sp.

(Figs. 1–3A, B)

*Description (based on 13 complete and 4 incomplete worms, 2 scoleces observed with SEM, and cross-sections of 2 mature proglottids):* Worms craspedote, euapolytic, 10–15 ( $12 \pm 2$ ; 13) mm long; composed of 93–188 ( $151 \pm 28$ ; 10) proglottids (Fig. 1E). Scolex consisting of 4 stalked bothridia and cephalic peduncle. Bothridia 413–848 ( $637 \pm 143$ ; 10) long by 155–262 ( $216 \pm 35$ ; 10) wide, weakly constricted at center, with muscular rims, divided by 23–28 ( $26 \pm 2$ ; 13) transverse septa and 1 medial longitudinal septum into 48–58 ( $53 \pm 4$ ; 13) loculi; anteriormost and posteriormost loculi single; anteriormost loculus 27–36 ( $31 \pm 3$ ; 6) long by 40–63 ( $51 \pm 8$ ; 6) wide. Cephalic peduncle 127–241 ( $183 \pm 33$ ; 10) long by 126–172 ( $143 \pm 16$ ; 10) wide (Figs. 1A, 2A).

Proximal surface of anterior- (Fig. 2D) and posteriormost (Fig. 2G) bothridial loculus with small aristate gladiate spinitriches and capilliform filitriches; proximal surface of middle of bothridium with densely arranged, slightly aristate gladiate spinitriches, filitriches not observed (Fig. 2E). Longitudinal septum (Fig. 2F), transverse septa, and loculi on distal bothridial surface with relatively sparsely arranged, small gladiate spinitriches and capilliform filitriches. Bothridial stalks with densely arranged, small gladiate spinitriches, filitriches not observed (Fig. 2H). Cephalic peduncle with small gladiate spinitriches and capilliform filitriches (Fig. 2I).

Immature proglottids wider than long, becoming longer than wide with maturity, 74–164 ( $131 \pm 25$ ; 11) in number (Fig. 1E). Mature proglottids slightly wider than long, becoming longer than wide with maturity, 15–29 ( $21 \pm 5$ ; 13) in number (Fig. 1B, C, E). Terminal mature proglottid (Fig. 1C) 273–504 ( $407 \pm 78$ ; 10) long by 109–163 ( $137 \pm 16$ ; 9) wide, usually with extensive vas deferens and atrophied testes. Genital pores marginal, irregularly alternating, 67–78% ( $74 \pm 4$ ; 9) of proglottid length from posterior end; genital atrium conspicuous (Fig. 1D). Testes arranged in 2 irregular columns anterior to cirrus sac, oval, 23–35 ( $28 \pm 3$ ; 11; 21) long by 20–27 ( $24 \pm 2$ ; 7; 14) wide, 4–6 ( $5 \pm 1$ ; 15) in number (Fig. 1B), 1 layer deep in cross-section (Fig. 3A), postporal testes lacking. Cirrus sac in anterior third of proglottid, spherical to pyriform in shape, 64–87 ( $76 \pm 10$ ; 6) long by 61–71 ( $65 \pm 3$ ; 7) wide, bent posteriorly, containing coiled cirrus; cirrus armed with narrow spinitriches (Fig. 1D). Vas deferens coiled anteriorly, extending from anterior testes posterior to cirrus sac to near anterior lobes of ovary, then extending anteriorly to enter cirrus sac at its anterior margin. Vagina thick-walled, sinuous, extending from ootype along medial line of proglottid to anterior margin of cirrus sac then extending laterally along anterior margin of cirrus sac to open into common genital atrium anterior to cirrus sac (Fig. 1D); vaginal sphincter absent. Ovary occupying posterior half of subterminal proglottids, occupying posterior two-thirds of terminal proglottid, H-shaped in frontal view (Fig. 1C), tetralobate in cross-section (Fig. 3B), symmetrical, 103–202 ( $160 \pm 36$ ; 19) long by 30–57 ( $46 \pm 9$ ; 10) wide at isthmus. Mehlis' gland posterior to ovarian isthmus. Vitelline follicles spherical to oval, 9–16 ( $13 \pm 2$ ; 9; 18) long by 7–14 ( $10 \pm 2$ ; 9; 18) wide, arranged in 2 lateral bands; each band consisting of 1 dorsal and 1 ventral column of follicles (Fig. 3B), extending from middle

of testicular field to near posterior margin of proglottid (Fig. 1B, C), interrupted by terminal genitalia (Fig. 1D). Uterus medio-ventral, saccate, extending along median line of proglottid from near posterior margin of ovary to middle of testicular field. Excretory ducts in 2 lateral pairs. Gravid proglottids and eggs not observed.

### Taxonomic summary

*Type and only known host:* *Himantura leoparda* Manjaji-Matsumoto and Last, leopard whiplay (Myliobatiformes: Dasyatidae).

*Site of infection:* Spiral intestine.

*Type locality:* East of the Wessel Islands, Arafura Sea, Australia ( $11^{\circ}17'44''\text{S}$ ,  $136^{\circ}59'48''\text{E}$ ).

*Prevalence:* Three of 4 rays examined (75%).

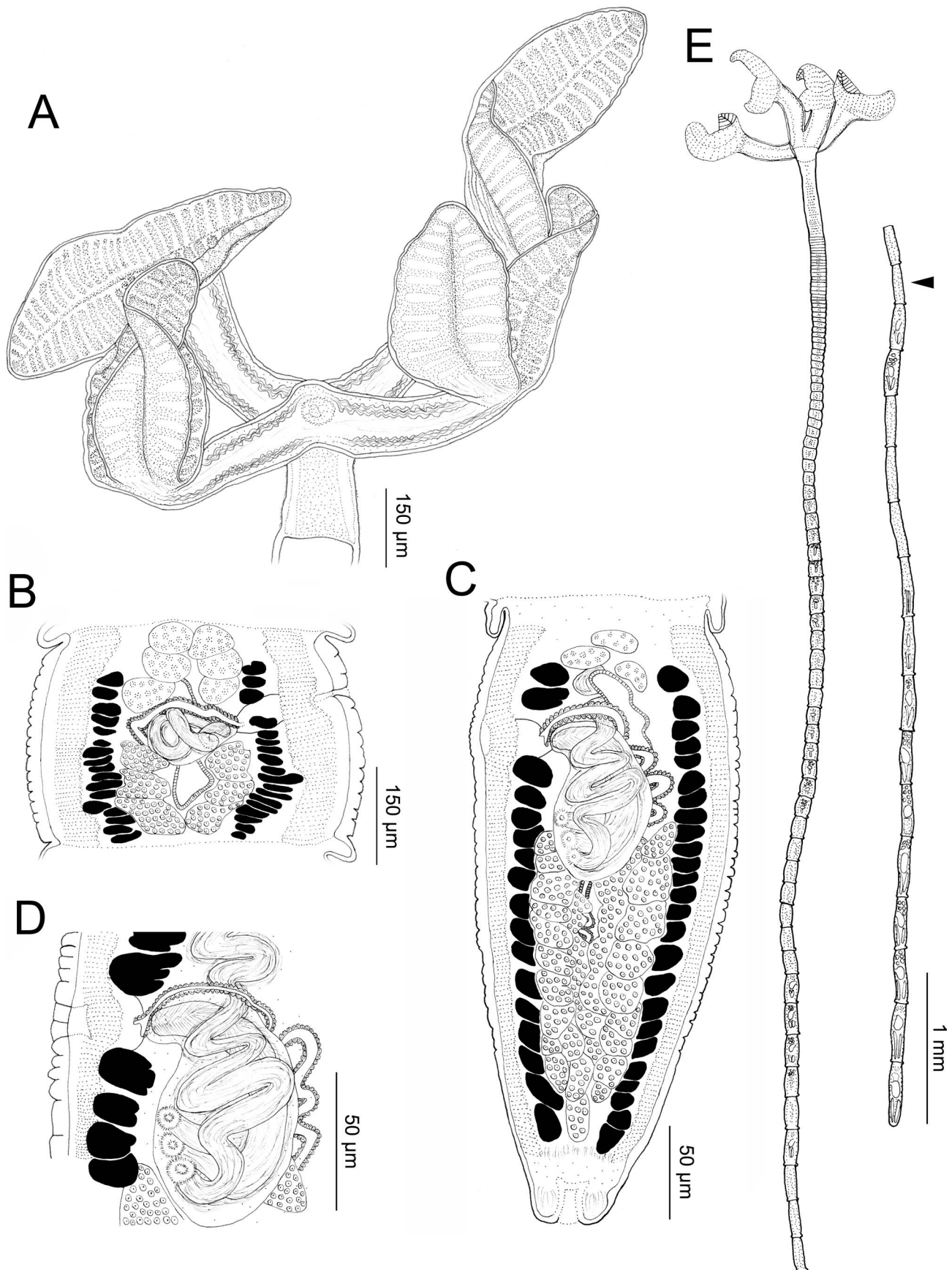
*Specimens deposited:* Holotype (QM G238337) and 5 paratypes (QM G238338–G238342); 5 paratypes (USNM 1618778–1618782); 6 paratypes (LRP 10151–10156), 2 SEM vouchers (LRP 10177–10178), scoleces examined with SEM retained with JNC at the University of Connecticut; 10 cross-section series of 2 specimens and their whole-mounted vouchers (MZUSP 7973a–7973e, 7974a–7974g).

*ZooBank registration:* urn:lsid:zoobank.org:act:A3D9EE82-EE31-4415-A99E-260793342F72.

*Etymology:* This species is named for its type host, *Himantura leoparda*.

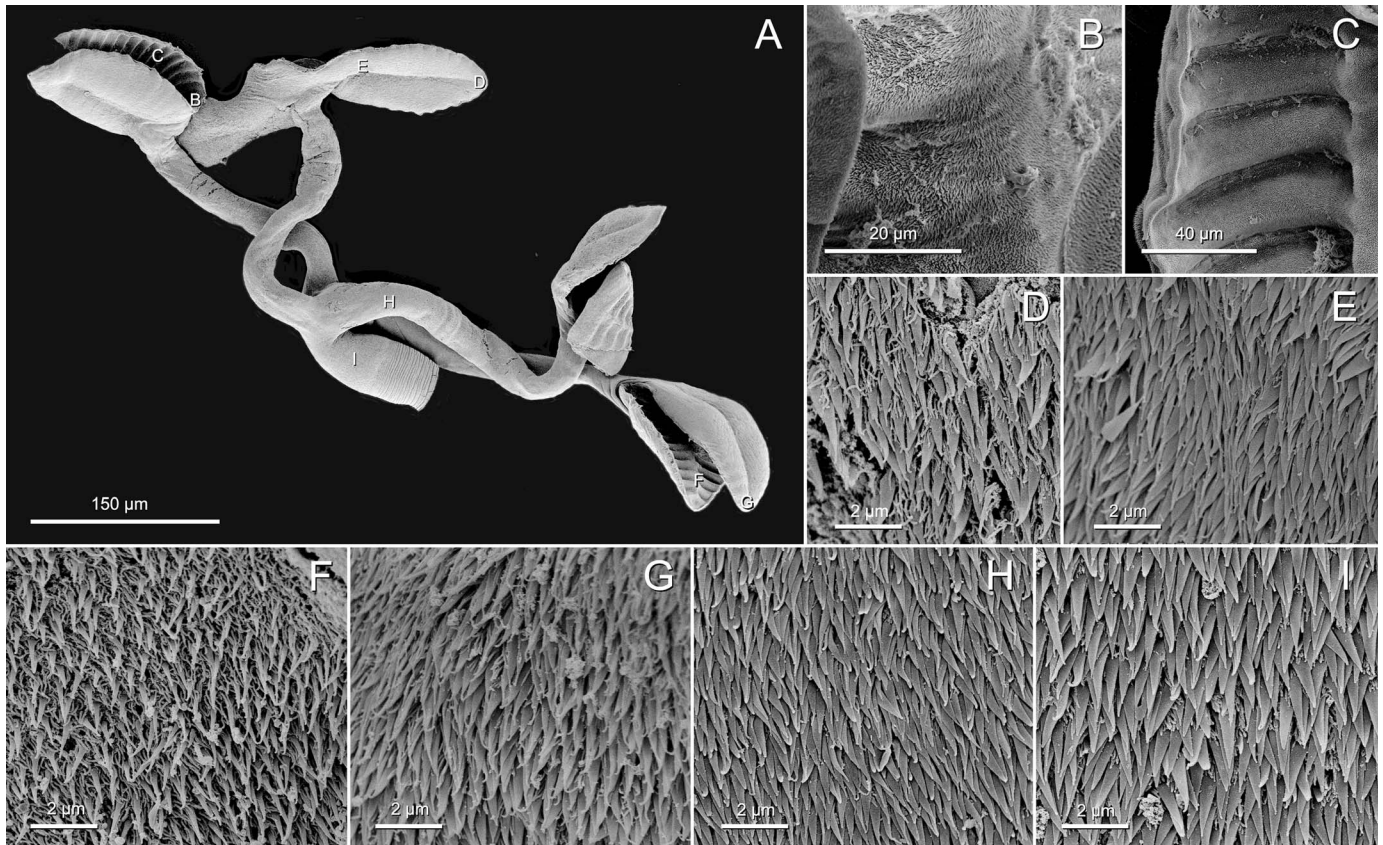
### Remarks

*Rhinebothrium leopardensis* n. sp. differs from all but 18 of the 48 valid species of *Rhinebothrium* in its possession of fewer than 7 testes. With respect to the remaining 18 species, it has a greater number of proglottids than *Rhinebothrium biorchidum*; *Rhinebothrium bunburyense* Coleman, Beveridge and Campbell, 2019; *Rhinebothrium dasyatidis* Coleman, Beveridge and Campbell, 2019; *Rhinebothrium kruppi* Golestaninasab and Malek, 2015; *Rhinebothrium rhinobati* Dailey and Carvajal, 1976; and *Rhinebothrium spinicephalum* (93–188 vs. 15–16, 55, 34–46, 12–17, 18–33, 36–49, respectively); it has fewer proglottids than *Rhinebothrium copianullum* Reyda, 2008 and *Rhinebothrium vandiemeni* Coleman, Beveridge and Campbell, 2019 (93–188 vs. 304 and 456–880, respectively). It is a longer worm than *Rhinebothrium chollaensis* Friggens and Duszynski, 2005 and *Rhinebothrium margaritense* Mayes and Brooks, 1981 (10–15 vs. 1.3–5.1 and less than 5.8 mm, respectively). It has a greater number of loculi per bothridium than *Rhinebothrium maccallumi* Linton, 1924 and *Rhinebothrium fungiforme* Coleman, Beveridge and Campbell, 2019 (48–58 vs. 29–31 and 30–34, respectively) and fewer loculi than *Rhinebothrium corbatai* Menoret and Ivanov, 2011 (48–58 vs. 71–75). It differs from *R. ditesticulum*; *Rhinebothrium fulbrighti* Reyda and Marques, 2011; and *Rhinebothrium tetralobatum* Brooks, 1977 in its possession of 4–6, rather than 2 testes. It differs from *Rhinebothrium urolophi* Coleman, Beveridge and Campbell, 2019 in that its bothridia are only weakly constricted at their centers rather than being conspicuously hinged, and in that the 2 pairs of loculi adjacent to this region of the bothridium are not reduced in size. This new species differs from *Rhinebothrium reydai* Trevisan and Marques, 2017 by possessing, rather than lacking, a cephalic peduncle, and in that its cirrus sac is bent posteriorly, rather than anteriorly. *Rhinebothrium leopardensis*



**Figure 1.** Line drawings of *Rhinebothrium leopardensis* n. sp. (A) Scolex (paratype Lawrence R. Penner Parasitology Collection 10153). (B) Subterminal mature proglottid (paratype National Museum of Natural History [USNM] 1618782). (C) Terminal mature proglottid (paratype USNM 1618782). (D) Detail of terminal genitalia (paratype USNM 1618782). (E) Whole worm (holotype Queensland Museum G238337); arrow indicates anteriormost mature proglottid.





**Figure 2.** Scanning electron micrographs of *Rhinebothrium leopardensis* n. sp. (A) Scolex; small letters indicate locations of details in micrographs B–I. (B) Distal surface of bothridium at level of central constriction. (C) Detail of loculi on distal bothridial surface. (D) Proximal surface of anteriormost loculus of bothridium. (E) Proximal surface of middle of bothridium. (F) Distal surface of loculus. (G) Proximal surface of posteriormost loculus of bothridium. (H) Surface of bothridial stalk. (I) Cephalic peduncle.

further differs from *R. reydai* in its possession of a greater number of transversal septa (23–28 vs. 17–22) and loculi (48–58 vs. 33–44). This new species is slightly larger than the single specimen of *Rhinebothrium walga* (Shiple and Hornell, 1906) Euzet 1953 on which the brief description of this species by Shiple and Hornell (1906) was based (10–15 vs. 7 mm). Also, we would not characterize it as a “very delicate little tapeworm” (Shiple and Hornell, 1906; p. 81), rather it is a moderately robust worm. Furthermore, the characterization by Shiple and Hornell (1906; p. 81) of the bothridia of *R. walga* as “Each half of the bothridia faces the other and they somewhat resemble the clasping appearance of a Gecko’s toes” suggests that the bothridia of *R. walga* are more constricted at their center than are those of the new species.

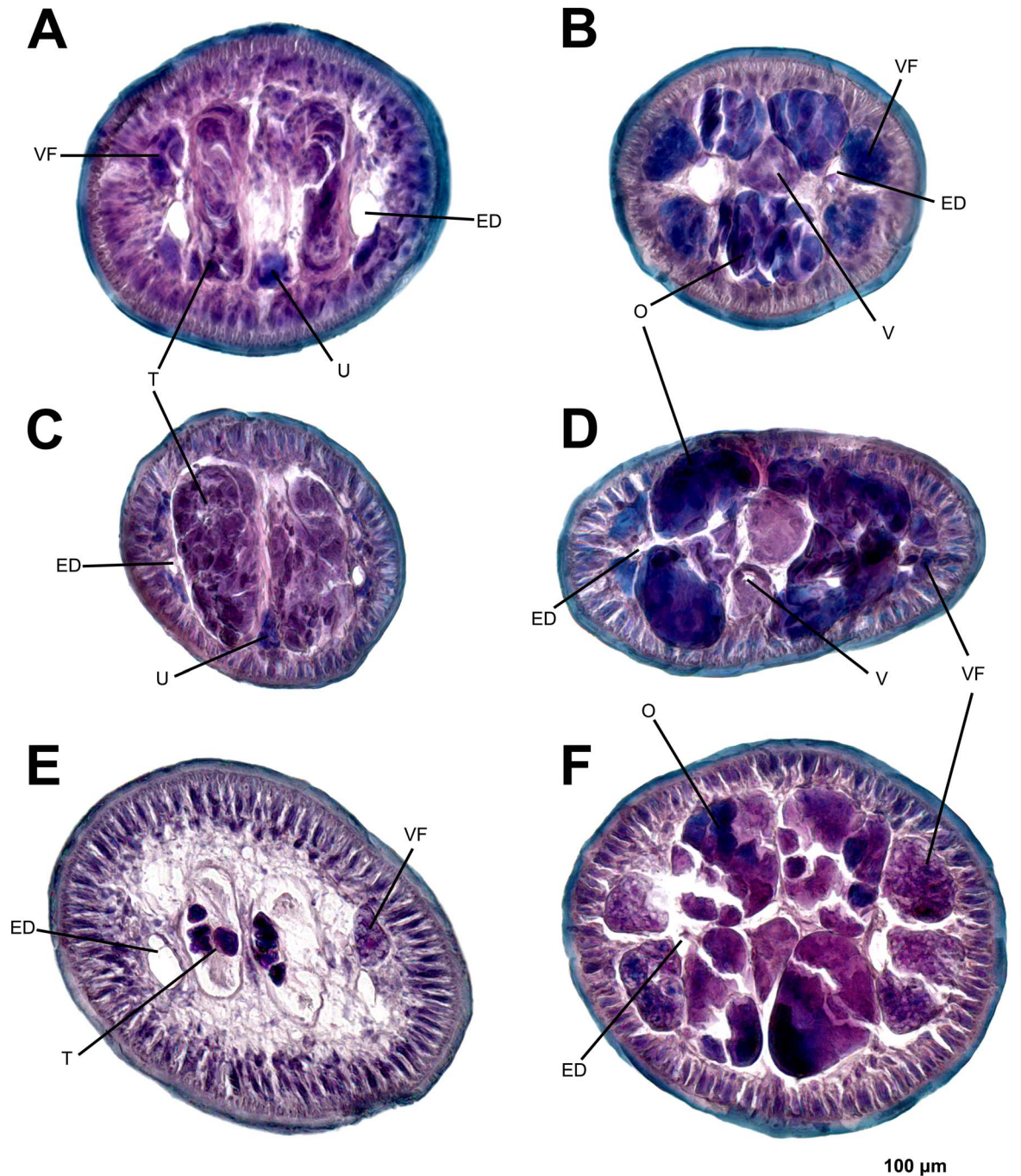
***Rhinebothrium nandoi* n. sp.**  
(Figs. 3C, D–5)

**Description** (based on 10 complete and 3 incomplete worms, 2 scoleces observed with SEM, and cross-sections of 2 mature proglottids): Worms acraspedote, euapolytic 3.6–5.0 ( $4.1 \pm 0.5$ ; 10) mm long; composed of 10–15 ( $13 \pm 1.2$ ; 10) proglottids (Fig. 4B). Scolex consisting of 4 stalked bothridia and cephalic peduncle. Bothridia 649–953 ( $806 \pm 112$ ; 10) long by 121–217 ( $168 \pm 30$ ; 12) wide, constricted at center, with muscular rims, divided by 31–36 ( $33 \pm 2$ ; 10) transverse septa and 1 medial

longitudinal septum into 64–74 ( $68 \pm 3$ ; 10) loculi; anteriormost and posteriormost loculi single; anteriormost loculus 27–41 ( $35 \pm 4$ ; 12) long by 35–53 ( $45 \pm 6$ ; 12) wide (Figs. 4A, 5A). Cephalic peduncle inconspicuous, 35–62 ( $48 \pm 9$ ; 9) long by 68–89 ( $81 \pm 7$ ; 8) wide (Fig. 4A).

Proximal surface of anteriormost bothridial loculus with small gladiate spinitriches and capilliform filitriches (Fig. 5D); proximal surface of middle of bothridium with gladiate spinitriches and capilliform filitriches (Fig. 5E); proximal surface of posteriormost loculus with small gladiate spinitriches and acicular to capilliform filitriches (Fig. 5G). Longitudinal septum (Fig. 5F), transverse septa, and loculi on distal bothridial surface with relatively sparsely arranged, small gladiate spinitriches and capilliform filitriches. Bothridial stalks (Fig. 5H) and cephalic peduncle (Fig. 5I) with capilliform filitriches only.

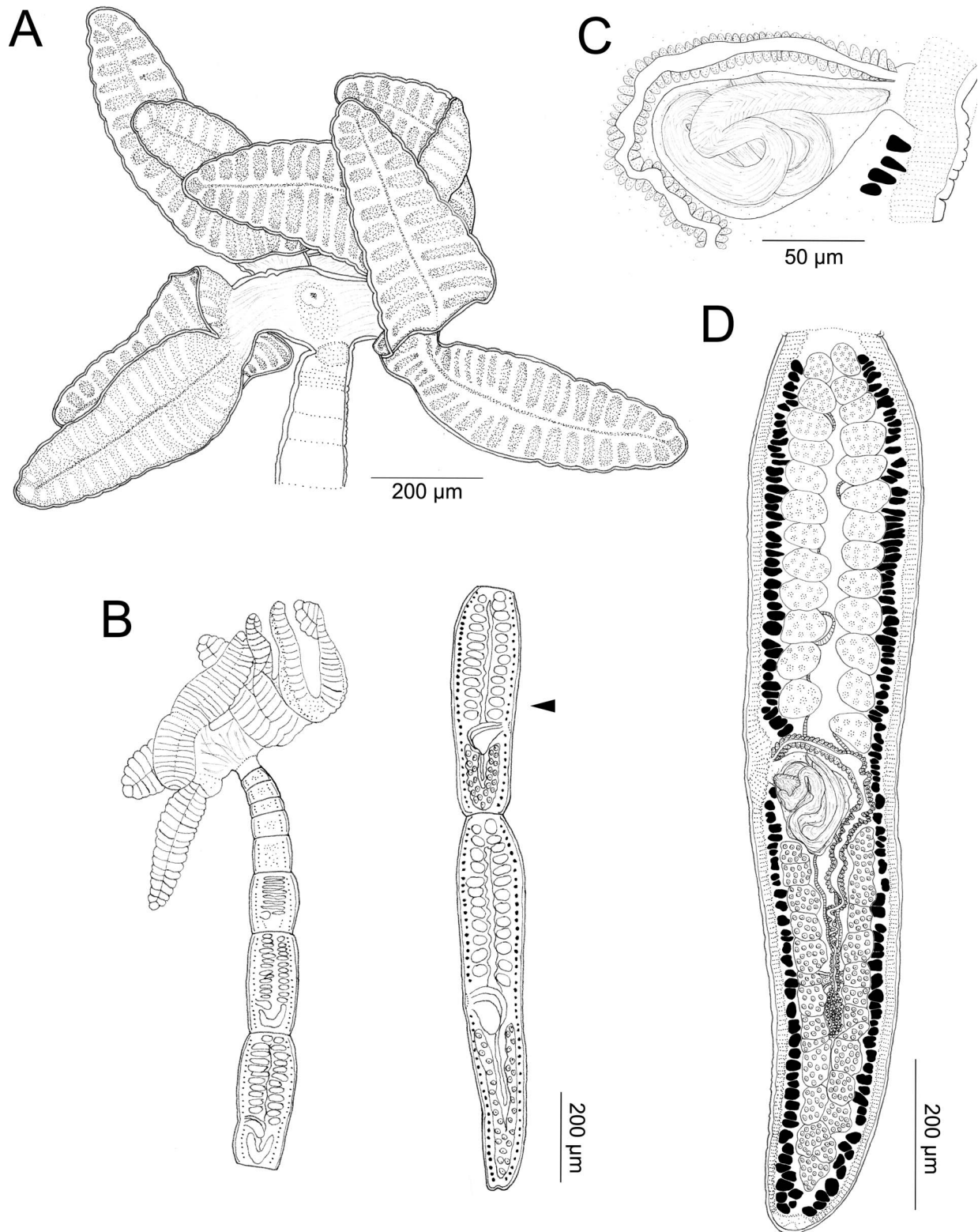
Immature proglottids wider than long, becoming longer than wide with maturity, 9–13 ( $11 \pm 1$ ; 10) in number (Fig. 4B). Mature proglottids longer than wide, 2–4 ( $2 \pm 1$ ; 10) in number (Fig. 4D). Terminal mature proglottid (Fig. 4D) 1,216–1,839 ( $1,465 \pm 193$ ; 8) long by 167–254 ( $215 \pm 27$ ; 8) wide. Genital pores marginal, irregularly alternating, 49–60% ( $51 \pm 4$ ; 8) of proglottid length from posterior end; genital atrium shallow (Fig. 4C). Testes arranged in 2 regular columns anterior to cirrus sac, spherical to oval, 44–68 ( $54 \pm 7$ ; 5; 10) long by 44–64 ( $54 \pm 6$ ; 5; 10) wide, 21–33 ( $26 \pm 4$ ; 13) in number (Fig. 4B), 1 layer deep in



**Figure 3.** Cross-sections through mature proglottids of *Rhinebothrium leopardensis* n. sp., *Rhinebothrium nandoi* n. sp., and *Rhinebothrium ruhnei* n. sp. (A) Section at level of testes of *R. leopardensis*. (B) Section at level of ovary of *R. leopardensis*. (C) Section at level of testes of *R. nandoi*. (D) Section at level of ovary of *R. nandoi*. (E) Section at level of testes of *R. ruhnei*. (F) Section at level of ovary of *R. ruhnei*. Abbreviations: ED, excretory duct; O, ovary; T, testis; U, uterus; V, vagina; VF, vitelline follicle. Color version available online.

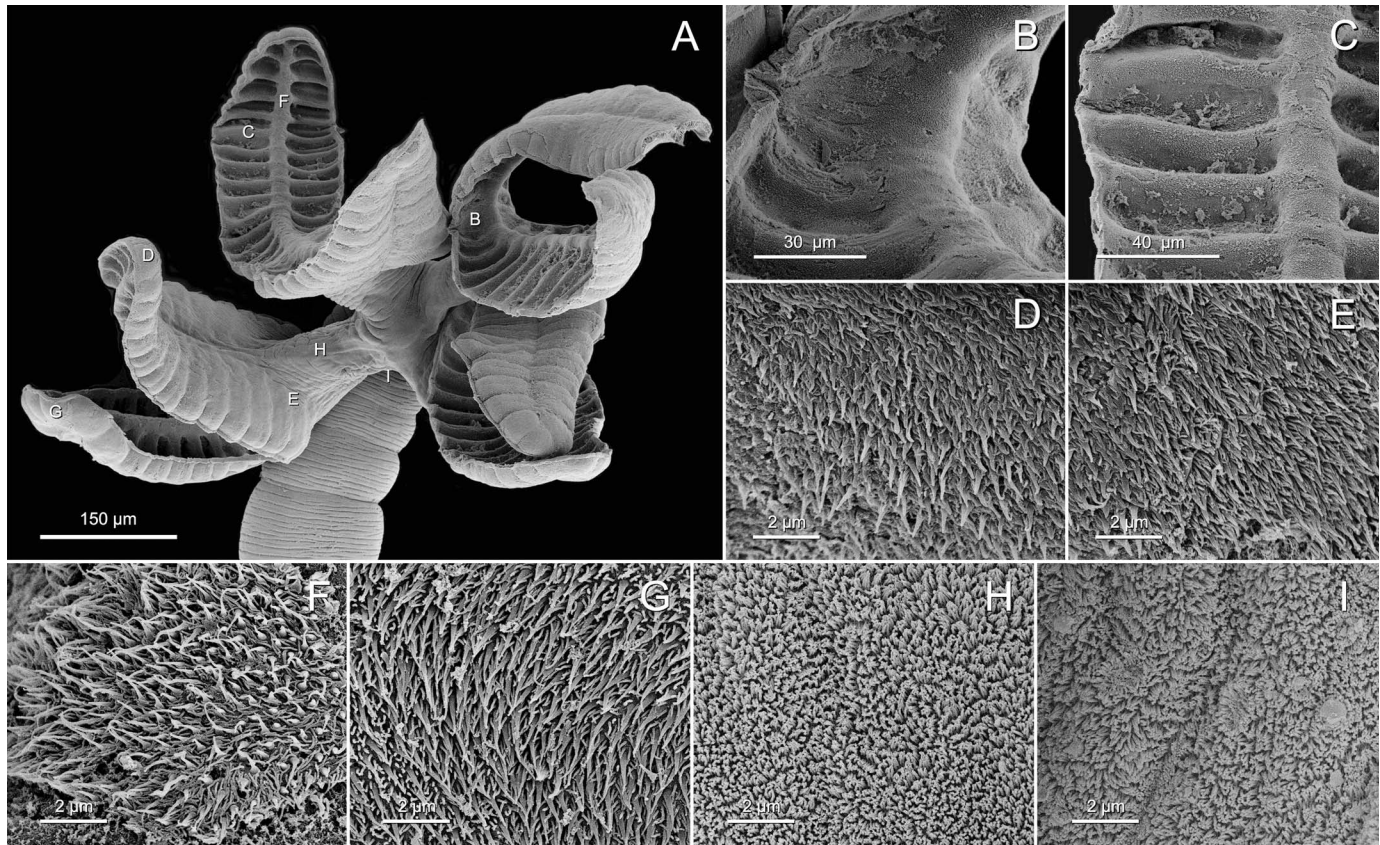
cross-section (Fig. 3C), postporal testes lacking. Cirrus sac in posterior half of proglottid, spherical to pyriform in shape, 120–185 ( $145 \pm 22$ ; 7) long by 90–130 ( $104 \pm 14$ ; 7) wide, tilted posteriorly, containing coiled cirrus; cirrus armed with narrow spinitriches (Fig. 4C). Vas deferens coiled anteriorly, anterior testes posterior to cirrus sac to near anterior lobes of ovary, then narrowing and extending anteriorly to enter cirrus sac at its anterior margin. Vagina thick-walled, weakly sinuous, extending

from ootype along medial line of proglottid to anterior margin of cirrus sac then extending laterally along anterior margin of cirrus sac to open into common genital atrium anterior to cirrus sac (Fig. 4C); vaginal sphincter absent. Ovary occupying posterior third of subterminal proglottids, occupying approximately posterior half of terminal proglottid, H-shaped in frontal view (Fig. 4D), tetralobate in cross-section (Fig. 3D), symmetrical, 417–615 ( $511 \pm 70$ ; 7) long by 72–100 ( $89 \pm 10$ ; 7) wide at



**Figure 4.** Line drawings of *Rhinebothrium nandoi*. (A) Scolex (paratype Lawrence R. Penner Parasitology Collection 10160). (B) Whole worm (holotype Queensland Museum [QM] G238343); arrow indicates anteriormost mature proglottid. (C) Detail of terminal genitalia (paratype National Museum of Natural History 1618784). (D) Terminal mature proglottid (holotype QM G238343).





**Figure 5.** Scanning electron micrographs of *Rhinebothrium nandoi*. (A) Scolex; small letters indicate locations of details in micrographs B–I. (B) Distal surface of bothridium at level of central constriction. (C) Detail of loculi on distal bothridial surface. (D) Proximal surface of anteriormost loculus of bothridium. (E) Proximal surface of middle of bothridium. (F) Distal surface of longitudinal septum. (G) Proximal surface of posteriormost loculus of bothridium. (H) Surface of bothridial stalk. (I) Cephalic peduncle.

isthmus (Fig. 4D). Mehlis's gland posterior to ovarian isthmus. Vitelline follicles spherical to oval, 10–17 ( $14 \pm 2$ ; 6; 12) long by 8–16 ( $12 \pm 2$ ; 6; 12) wide, arranged in 2 lateral bands; each band consisting of 1 dorsal and 1 ventral column of follicles (Fig. 3D), extending throughout length of proglottid, interrupted by terminal genitalia (Fig. 4D). Uterus medioventral, saccate, extending along median line of proglottid from level of ovarian bridge to near anterior margin of proglottid. Excretory ducts in 2 lateral pairs. Gravid proglottids and eggs not observed.

### Taxonomic summary

*Type and only known host:* *Himantura leoparda* Manjaji-Matsumoto and Last, leopard whray (Myliobatiformes: Dasyatidae).

*Site of infection:* Spiral intestine.

*Type locality:* East of the Wessel Islands, Arafura Sea, Australia (11°17'44"S, 136°59'48"E).

*Prevalence:* Three of 4 rays examined (75%).

*Specimens deposited:* Holotype (QM G238343) and 4 paratypes (QM G238344–G238347); 4 paratypes (USNM 1618783–1618786); 4 paratypes (LRP 10157–10160), 2 SEM vouchers (LRP 10175–10176), scoleces examined with SEM retained with JNC at the University of Connecticut; 7 cross-section series of 2 specimens and their whole-mounted vouchers (MZUSP 7975a–7975b, 7976a–7976e).

*ZooBank registration:* urn:lsid:zoobank.org:act:70AB6961-7FBD-4CE6-887B-7327BB825AE9.

*Etymology:* This species honors Dr. Fernando Marques, who has contributed greatly to the knowledge of rhinebothriidean systematics, and who although he lost a genus as a result of the work of Caira et al. (2020), has gained a species here.

### Remarks

*Rhinebothrium nandoi* differs from all 49 valid members of the genus, including *R. leopardensis*, as follows. It has fewer testes than *Rhinebothrium chilensis* Euzet and Carvajal, 1973, *Rhinebothrium leiblei* Euzet and Carvajal, 1973, and *Rhinebothrium monodi* Euzet, 1954 (21–33 vs. 35–45, 36–46, and 60–80, respectively). It has a greater number of testes (21–33 vs. less than 20) than all but the following 7 remaining species: *Rhinebothrium baeri* Euzet, 1959; *Rhinebothrium burgeri* Baer, 1948; *Rhinebothrium devaneyi* Brooks and Deardorff, 1988; *Rhinebothrium nickoli* Coleman, Beveridge and Campbell, 2019; *Rhinebothrium pearsoni* Butler, 1987; *Rhinebothrium persicum* Golestaninasab and Malek, 2015; and *Rhinebothrium setiensis* Euzet, 1955. It differs from all of the 7 above species except *R. nickoli* in its possession of fewer proglottids (11–15 vs. greater than 20). It conspicuously differs from the latter species in its possession of a much greater number of bothridial loculi per bothridium (64–74 vs. 12).

***Rhinebothrium ruhnei* n. sp.**

(Figs. 3E, F, 6A–D, 7)

**Description** (based on 20 complete and 12 incomplete worms, 3 scoleces observed with SEM, and cross-sections of 2 mature proglottids): Worms acraspedote, euapolytic 10.1–15.8 ( $12.2 \pm 1.6$ ; 20) mm long; composed of 48–78 ( $64 \pm 8$ ; 21) proglottids (Fig. 6D). Scolex consisting of 4 stalked bothridia and cephalic peduncle. Bothridia 990–1,338 ( $1,194 \pm 101$ ; 23) long by 171–298 ( $227 \pm 38$ ; 20) wide, conspicuously constricted at center (Fig. 7A, B), with muscular rims, divided by 34–39 ( $37 \pm 2$ ; 16) transverse septa and 1 medial longitudinal septum into 68–78 ( $74 \pm 3$ ; 16) loculi, lacking loculi from central constricted region; anteriormost and posteriormost loculi single; anteriormost loculus 30–45 ( $35 \pm 4$ ; 16) long by 43–63 ( $52 \pm 8$ ; 14) wide (Figs. 6A, 7A). Cephalic peduncle inconspicuous, 74–93 ( $82 \pm 8$ ; 5) long by 74–103 ( $88 \pm 12$ ; 5) wide (Fig. 6D).

Proximal surfaces of anteriormost bothridial loculus, middle of bothridium and distal surface of constriction with papilliform filitriches only (Fig. 7E, F, B, respectively); proximal surface of margins of bothridium with small sparsely arranged gladiate spinitriches and papilliform filitriches (Fig. 7D). Longitudinal septum (Fig. 7G), transverse septa, loculi on distal bothridial surface and proximal surface of posteriormost loculus (Fig. 7H) with acicular filitriches. Bothridial stalks with papilliform filitriches only (Fig. 7I). Cephalic peduncle with capilliform filitriches only (Fig. 7J).

Immature proglottids wider than long, becoming longer than wide with maturity, 40–66 ( $55 \pm 7$ ; 21) in number (Fig. 6D). Mature proglottids longer than wide, 5–15 ( $10 \pm 3$ ; 20) in number. Terminal mature proglottid (Fig. 6C) 886–1,210 ( $1,035 \pm 116$ ; 14) long by 164–267 ( $217 \pm 31$ ; 14) wide, usually with extensive vas deferens and atrophied testes. Genital pores marginal, irregularly alternating, 58–64% ( $62 \pm 2$ ; 14) of proglottid length from posterior end; genital atrium shallow (Fig. 6B). Testes arranged in 2 regular columns anterior to cirrus sac, spherical to oval, 40–65 ( $50 \pm 6$ ; 11; 22) long by 34–57 ( $42 \pm 7$ ; 12; 24) wide, 7–10 ( $9 \pm 1$ ; 26) in number (Fig. 6C), 1 layer deep in cross-section (Fig. 3E), postporal testes lacking. Cirrus sac in the middle of proglottid, pyriform in shape, 95–134 ( $113 \pm 13$ ; 7) long by 103–133 ( $116 \pm 11$ ; 13) wide, tilted posteriorly, containing coiled cirrus; cirrus armed with narrow spinitriches (Fig. 6B). Vas deferens coiled anteriorly, extending from medial testes posterior to cirrus sac to near anterior lobes of ovary, then extending anteriorly to enter cirrus sac at its anterior margin. Vagina thick-walled, weakly sinuous, extending from ootype along medial line of proglottid to anterior margin of cirrus sac then extending laterally along anterior margin of cirrus sac to open into common genital atrium anterior to cirrus sac (Fig. 6B, C); vaginal sphincter absent. Ovary occupying posterior one-third to two-thirds of subterminal proglottids, occupying posterior one-half to two-thirds of terminal proglottid, H-shaped in frontal view (Fig. 6C), tetralobate in cross-section (Fig. 3F), weakly asymmetrical, 347–531 ( $427 \pm 58$ ; 17) long by 62–95 ( $77 \pm 11$ ; 10) wide at isthmus. Mehli's gland posterior to ovarian isthmus. Vitelline follicles spherical to oval, 17–35 ( $26 \pm 5$ ; 18; 36) long by 15–26 ( $19 \pm 3$ ; 18; 36) wide, arranged in 2 lateral bands; each band consisting of 1 dorsal and 1 ventral column of follicles (Fig. 3F), extending from near anterior margin of testicular field to near posterior margin of proglottid, interrupted by terminal genitalia (Fig. 6C).

Uterus medioventral, saccate, extending along median line of proglottid from near posterior margin of proglottid to level of anteriormost pair of testes (Fig. 6C). Excretory ducts in 2 lateral pairs. Gravid proglottids and eggs not observed.

**Taxonomic summary**

**Type and only known host:** *Himantura leoparda* Manjaji-Matsumoto and Last, leopard whray (Myliobatiformes: Dasyatidae).

**Site of infection:** Spiral intestine.

**Type locality:** East of the Wessel Islands, Arafura Sea, Australia ( $11^{\circ}17'44''\text{S}$ ,  $136^{\circ}59'48''\text{E}$ ).

**Prevalence:** Three of 4 rays examined (75%).

**Specimens deposited:** Holotype (QM G238348) and 10 paratypes (QM G238349–G238358); 10 paratypes (USNM 1618787–1618796); 11 paratypes (LRP 10161–10171), 3 SEM vouchers (LRP 10172–10174), scoleces examined with SEM retained with JNC at the University of Connecticut; 19 cross-section series of 2 specimens and their whole-mounted vouchers (MZUSP 7977a–7977i, 7978a–7978l).

**ZooBank registration:** urn:lsid:zoobank.org:act:985F9556-80C2-4A10-ABE6-20BF0E44B7CB.

**Etymology:** This species honors Dr. Tim Ruhnke for his contributions to rhinebothriidean taxonomy and systematics, and who although he lost a genus as a result of the work of Caira et al. (2020), has gained a species here.

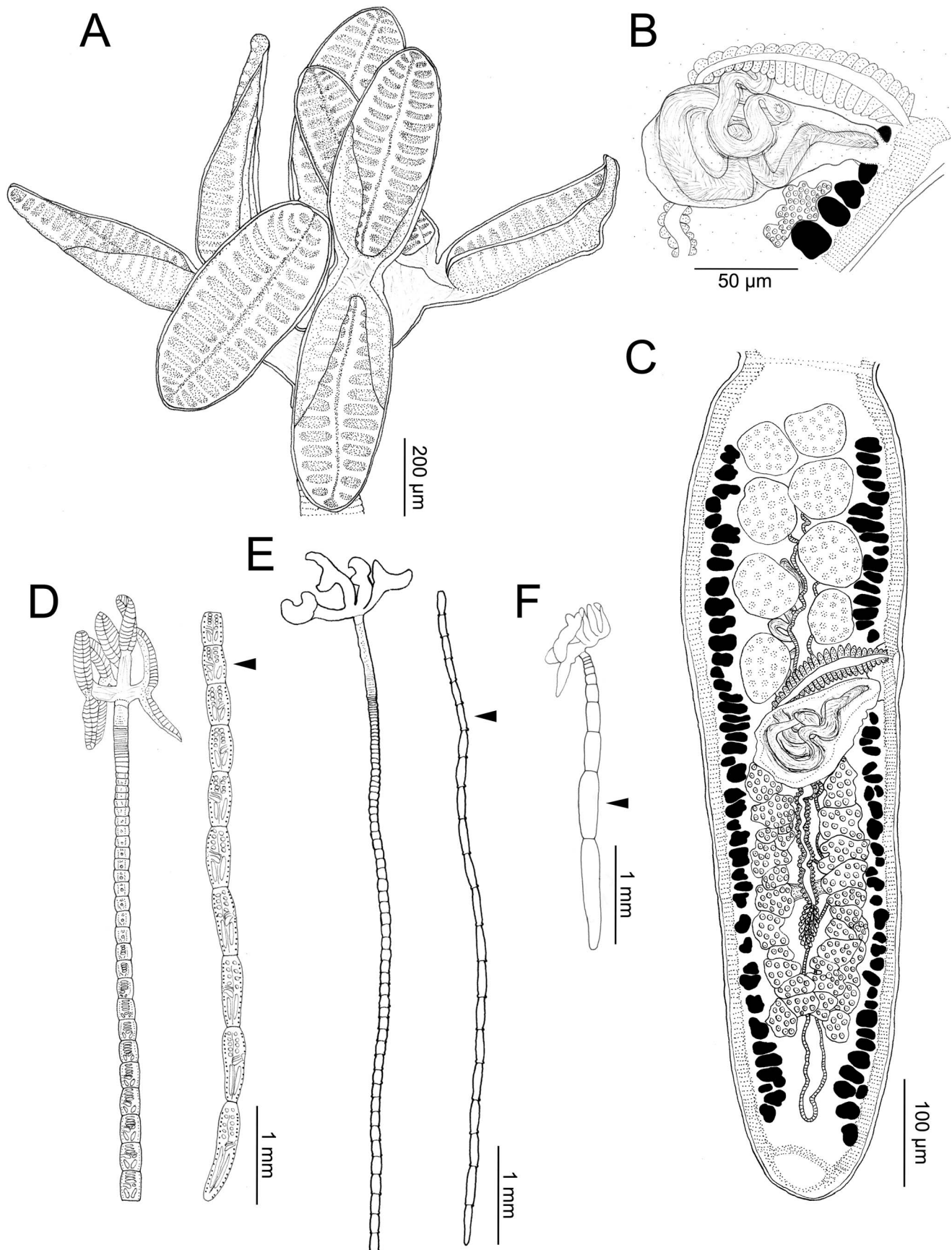
**Remarks**

*Rhinebothrium ruhnei* n. sp. differs from all 50 valid members of the genus (including *R. leopardensis* and *R. nandoi*) as follows. With 7–11 testes, it has fewer or more testes than all but 13 of its 50 valid congeners. With respect to these 13 species, it has more proglottids than *R. fluviorum* Coleman, Beveridge and Campbell, 2019; *R. gravidum* Friggens and Duszynski, 2005; and *R. hawaiiensis* Cornford, 1974 (48–78 vs. 11–18, 9–21, and 13, respectively). It has fewer proglottids than *R. copianullum*, *R. mistyae* Menoret and Ivanov, 2011, and *R. paratrygoni* (48–78 vs. 456–880, 353–974, and 266–1,060, respectively). In addition, it is a longer worm than *R. urobatidium* (Young, 1955) Appy and Dailey, 1977 (10.1–15.8 vs. 3.1–3.4 mm). It has fewer bothridial loculi than *R. jamei* Marques and Reyda, 2015; *R. lintoni* Campbell, 1970, *R. megacanthophallus* Healy, 2006; *R. taeniuri* Ramadan, 1984; and *R. xiamensis* Wang and Yang, 2001 (68–78 vs. 49–55, 54–56, 50–58, 8–22, 38–42, and 36–42, respectively). The new species differs from *R. brooksi* Reyda and Marques, 2011 in that the posterior region of each of its bothridia bears only a single, rather than 2, loculi.

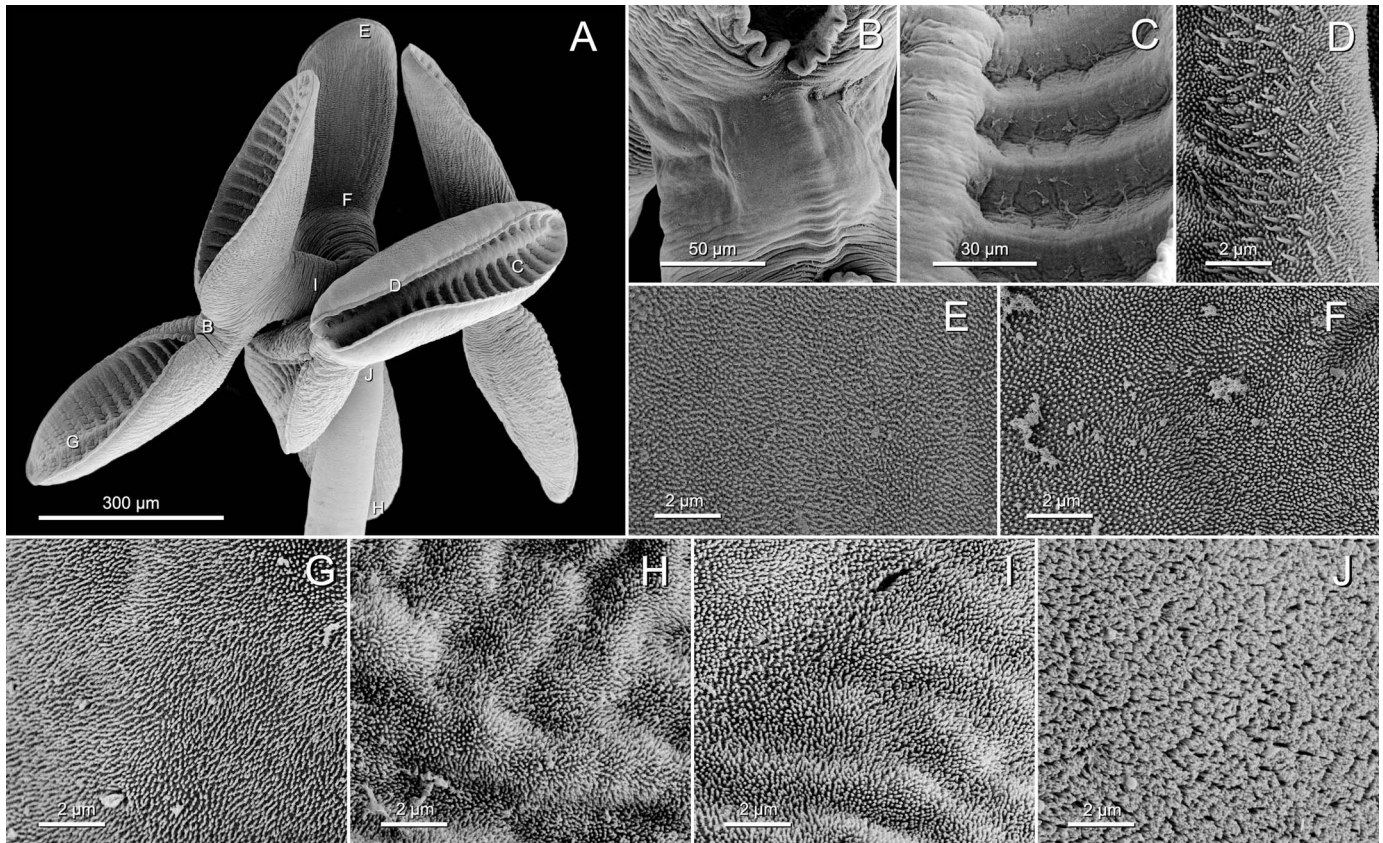
In addition to the above features, *R. ruhnei* can be differentiated from all of its congeners except *R. ditesticulum*, in that it lacks, rather than possesses, facial loculi on the central constricted region of its bothridia (Fig. 6A, 7B). But, with 7–11 testes it is easy to distinguish from *R. ditesticulum*, which possesses only 2 testes per proglottid.

**DISCUSSION**

The discovery of 3 new species of *Rhinebothrium* parasitizing the leopard stingray (*H. leoparda*) was not unexpected given the relatively poor status of our current knowledge of the rhine-







**Figure 7.** Scanning electron micrographs of *Rhinebothrium ruhnei*. (A) Scolex; small letters indicate locations of details in micrographs B–J. (B) Distal surface of bothridium at level of central constriction. (C) Detail of loculi on distal bothridial surface. (D) Proximal surface of bothridial rim. (E) Proximal surface of anteriormost loculus of bothridium. (F) Proximal surface of middle of bothridium. (G) Distal surface of longitudinal septum. (H) Proximal surface of posteriormost loculus of bothridium. (I) Surface of bothridial stalk. (J) Cephalic peduncle.

bothriids fauna of dasyatid stingrays in general and, also as noted above, how common it is for a single batoid species to host 2 or more members of this genus. The degree to which species co-occurring in the same host species are related to one another will be interesting to investigate once molecular data become available for a larger number of species. Unfortunately, of the 51 valid species of *Rhinebothrium*, sequence data are currently available for only the following 8 species: *R. brooksi*, *R. copianullum*, and *R. fulbrighti* (see Reyda and Marques, 2011); *R. maccallumi* (see Olson and Caira, 1999; Olson et al., 2001); *R. megacanthophallus* Healy, 2006 (see Healy et al., 2009); *R. paratrygoni* Rego and Dias, 1976 (see Reyda and Marques, 2011); *R. reydei* (Trevisan et al., 2019); and *R. walga* (see GenBank: MH000370.1). Furthermore, in many cases, the data available for these species are from different genes.

Before this study, 56% (27 of 48) of *Rhinebothrium* species had been described from the freshwater river systems of South America and the marine waters around both South and North

America. In contrast, despite the remarkably diverse nature of its batoid fauna, only 19 species were known from the Indo-Pacific region (Williams, 1964; Butler, 1987; Healy, 2006; Coleman et al., 2019a). Our work increases the number of species known from the Indo-Pacific to 22. It also helps to emphasize the highly underestimated nature of *Rhinebothrium* diversity in this region of the globe. The current host associations of *Rhinebothrium* suggest that the batoid family Dasyatidae is likely to be an especially productive source of *Rhinebothrium* novelty in this region. In the former family alone, 9 genera and approximately 70 species, many of which have yet to be examined, are not known to host the genus. Additional work on the cestode faunas of dasyatids of this region is especially likely to be highly productive with respect contributing to the knowledge of *Rhinebothrium* diversity because a large proportion of these stingray species occur only in the waters of the Indo-Pacific region.

The morphology of *R. ruhnei* has some bearing on the newly erected genus *Ruptobothrium* Coleman, Beveridge and Campbell,

**Figure 6.** Line drawings of *Rhinebothrium ruhnei*, *Rhinebothrium leopardensis*, and *Rhinebothrium nandoi*. (A) Scolex of *R. ruhnei* (paratype Lawrence R. Penner Parasitology Collection 10161). (B) Detail of terminal genitalia of *R. ruhnei* (paratype National Museum of Natural History [USNM] 1618789). (C) Terminal mature proglottid of *R. ruhnei* (paratype USNM 1618789). (D) Whole worm of *R. ruhnei* (holotype Queensland Museum G238348); arrow indicates anteriormost mature proglottid. Additional line drawings of whole worms of other 2 new species of *Rhinebothrium* are presented at same scale as *R. ruhnei* to illustrate their comparative sizes and morphologies; arrows indicate anteriormost mature proglottids. (E) Whole worm of *R. leopardensis*. (F) Whole worm of *R. nandoi*.



2019. When Coleman et al. (2019b) erected this genus, among the distinguishing characteristics they identified for the genus were 50 loculi per bothridium, small size, 12–23 proglottids, 9–13 testes, vitelline bands that are interrupted by the genital pore, an armed cirrus, near-equatorial genital pores, and an H-shaped ovary. However, all of these features are found in various subsets of *Rhinebothrium* species and thus are not unique to *Ruptobothrium*. The feature that holds some promise for distinguishing this genus is the reported distinct separation of the 2 lobes of each bothridium—a feature that is supported by their figure 1A of *Ruptobothrium louiseuzeti* Coleman, Beveridge and Campbell, 2019. What undermines the distinct nature of this feature is their transfer of *R. ditesticulum* to *Ruptobothrium*. Examination of stacked images of the bothridia of the type specimens (USNPC-73100/73101) of *R. ditesticulum*, kindly provided to us by F. Marques, showed that the bothridia of this species are not divided into 2 separate lobes; rather, their configuration is like that seen in the bothridia of *R. ruhnei*, which consists merely of a central constriction of each bothridium that lacks loculi, rather than a distinct separation of the anterior and posterior lobes. At this point, we are inclined to question the validity of *Ruptobothrium* as a taxon independent from *Rhinebothrium*, especially given the existence of yet other members of the latter genus that bear bothridia with central constrictions that lack loculi (e.g., *Rhinebothrium biorchidum* Huber and Schmidt, 1985; *Rhinebothrium bunburyense* Coleman, Beveridge, and Campbell, 2019; *Rhinebothrium ditesticulum* Appy and Dailey, 1977; *Rhinebothrium urolophi* Coleman, Beveridge, and Campbell, 2019; and *Rhinebothrium vandiemeni* Coleman, Beveridge, and Campbell, 2019). It will be extremely interesting to investigate the monophyly of *Ruptobothrium* relative to *Rhinebothrium* in the context of a molecular phylogenetic analysis.

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