New species of *Tetragonocephalum* (Cestoda: Lecanicephalidea) parasitizing members of the stingray genus *Pateobatis* (Myliobatiformes: Dasyatidae) and an update of host associations of the genus

By

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The thesis committee for Isabel A. M. Pen certifies that this is the approved version of the following thesis:

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Abstract

The lecanicephalidean tapeworm genus *Tetragonocephalum* comprises 12 valid species and a *species inquirendum*. Species in this genus are easily identifiable among lecanicephalideans based on their possession of a unique combination of features: a non-retractable, muscular apical organ; a dumbbell-shaped, rather than saccate, uterus; and a large, rather than small, genital atrium. Valid species of *Tetragonocephalum* have been described from seven species of stingrays (Dasyatidae) representing the genera *Brevitrygon*, *Himantura*, *Maculabatis*, *Pastinachus*, and *Urogymnus*, all from the Indo-Pacific region. Extensive new material from Australia, the Solomon Islands, Japan, Taiwan, Vietnam, Sri Lanka, India, Egypt, Mozambique, and the island of Borneo has allowed for a more complete assessment of the diversity, host associations, and geographic distributions of *Tetragonocephalum* overall. Selected worms were prepared as whole mounts for examination with light and scanning electron microscopy and as histological sections. The new material suggests that species of *Tetragonocephalum* parasitize at least an additional 14 species of dasyatid stingrays. The new host records include, for the first time, species in the genera *Hemitrygon*, *Neotrygon*, and *Taeniura*. More detailed study of the fauna of three of the five species of *Pateobatis* indicated that *Pateobatis fai*, *P. jenkinsii*, and *P. uarnacoides* are parasitized by four, three, and two species, respectively. Four of the species found to parasitize members of the genus *Pateobatis* are formally described and included in a principal component analysis of morphological characters to determine morphological cohesion of specimens assigned to the new species. A phylogenetic analysis based on 18S, 28S, 5.8S/ITS rDNA sequence data including 25 specimens representing 23 species was conducted using Bayesian inference. Results of the phylogenetic analysis confirm all four described species as distinct from one another, as well as distinct from the three described and 16 undescribed species

included in the analysis. The phylogenetic analysis also indicates the presence of two major clades. Morphological characters to support the two clades have yet to be identified. These data corroborated published data that suggest that congeners in the same host species are the rule, not the exception in the genus *Tetragonocephalum*. Including the previously described species and published reports of undescribed species, as well as the results of this study, the estimate for the number of species in the genus *Tetragonocephalum* is significantly expanded.

Author's Disclaimer

All taxonomic actions in this work are hereby disclaimed for nomenclatural purposes, as recommended in Article 8 of the International Code of Zoological Nomenclature (Ride et al. 1999).

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INTRODUCTION

Elasmobranch tapeworms (Platyhelminthes: Cestoda) span nine orders and encompass extremely diverse morphologies. In addition, they have a cosmopolitan distribution in both marine and freshwater sharks and stingrays (e.g., Caira and Jensen, 2014). Ordinal membership can be assigned based on the morphology of the attachment structure, hereafter referred to as the scolex, and proglottid features. Members of the order Lecanicephalidea possess a scolex with four acetabula and an apical structure consisting of apical modification of the scolex proper and an apical organ used for attachment (except in species of *Aberrapex* Jensen, 2001 and *Paraberrapex* Jensen, 2001 which lack an apical structure altogether), a vagina that opens into the into the genital atrium posterior to the cirrus sac, and a substantial vas deferens that often can be found expanded into a sacciform external seminal vesicle which extends from the level of the ovarian bridge to the cirrus sac (Jensen et al., 2016). Within this order, there are several genera that possesses nearly identical scolex morphology, so examining proglottid features is critical to identifying specimens to the genus level (Jensen et al., 2016). The order is comprised of over 380 species in 24 genera (Jensen et al., 2017).

The tapeworm genus of focus for this thesis, *Tetragonocephalum* Shipley and Hornell, 1905, is the only member of the family Tetragonocephalidae Yamaguti, 1959 in the order Lecanicephalidea. This genus was erected by Shipley and Hornell in 1905 with the description of its type species *Tetragonocephalum trygonis* Shipley and Hornell, 1905. This species was described from a host species of what was believed to be *Brevitrygon walga* (Müller and Henle) (as *Trygon walga* Müller and Henle) collected in the Gulf of Mannar, off the western coast of Sri Lanka (as Ceylon). Unfortunately, the identity of the type host remains in question as *Brevitrygon walga* is now known to inhabit only the waters off the western coast of India (Last et

al., 2016a). Jensen and Guyer (2021) reasoned that, based on their distributions, including the waters surrounding Sri Lanka (Last et al., 2016a; Fernando et al., 2019), and the type locality mentioned in the original description (of Shipley and Hornell in 1905), *Brevitrygon imbricata* (Bloch and Schneider) or *B.* sp. 1 (sensu Fernando et al., 2019) is the likely type host.

Diversity and classification

Since the description of the type species *Tetragonocephalum trygonis* in 1905 by Shipley and Hornell, the taxonomic status of *Tetragonocephalum* has been addressed by a number of authors (Shipley and Hornell, 1906; Stiles and Hassall, 1912; Southwell, 1925, 1929, 1930; Poche, 1926; Pintner, 1928; Fuhrmann, 1931; Perrenoud, 1931; Baer, 1948; Wardle and McLeod, 1952; Euzet, 1952, 1954, 1959, 1994; Yamaguti, 1959; Joyeux and Baer, 1961; Euzet and Combes, 1965; Wardle et al., 1974, Freeman, 1982, 1983; Campbell and Williams, 1984; Schmidt, 1986; Brooks and McLennan, 1993; Ivanov and Campbell, 2000; Caira et al., 2001; Jensen, 2005). Similarities in scolex morphology have resulted in taxonomic confusion with another lecanicephalidean genus, *Tylocephalum* Linton, 1890, throughout the taxonomic history of *Tetragonocephalum,* with many of the aforementioned studies focused on discussing the validity of *Tetragonocephalum* and its potential synonymy with *Tylocephalum.* In short, only one year after its description, Shipley and Hornell (1906) revised their opinion on the taxonomic status of *Tetragonocephalum*, believing it to be a junior synonym of *Tylocephalum*, and creating the new combination *Tylocephalum trygonis* (Shipley and Hornell, 1905) Shipley and Hornell, 1906. Southwell (1925) expanded Linton's (1890) generic diagnosis of *Tylocephalum* to include the characteristics of *Tetragonocephalum*, and suggested that *Tylo. trygonis* be elevated to type species of the genus, as the description of the genus and its type species, *Tylocephalum pingue* Linton, 1890, was based on immature specimens. Poche (1926), while ultimately retaining

Tetragonocephalum as a junior synonym of *Tylocephalum*, stated that *Tylo. trygonis* cannot replace *Tylo. pingue* as type species of *Tylocephalum* and calls into question Southwell's interpretation of the equivalence of characters between the two genera, the vitelline follicle condition in particular. Pintner (1928) noted that two family-level groups appear within the genus *Tylocephalum*: Lecanicephalidae Braun, 1900, containing species with a non-glandular scolex, bilobed uterus, and acraspedote proglottids, and Cephalobothriidae Pintner, 1928, containing species with a glandular scolex, saccate uterus, and craspedote proglottids. Pintner (1928) placed *Tetra. trygonis* within the family Lecanicephalidae and *Tylo. pingue* within the family Cephalobothriidae, resurrecting the genus *Tetragonocephalum.* Southwell (1930) and Fuhrmann (1931) continue to treat *Tetragonocephalum* as a junior synonym of *Tylocephalum*, while Perrenoud (1931) and Baer (1948) recognized *Tetragonocephalum* as distinct, after which the validity of the genus was generally accepted (Wardle and McLeod, 1952; Euzet, 1952, 1954, 1959, 1994; Yamaguti, 1959; Joyeux and Baer, 1961; Euzet and Combes, 1965; Wardle et al., 1974; Freeman, 1982, 1983; Campbell and Williams, 1984; Schmidt, 1986; Muralidhar, 1988; Brooks and McLennan, 1993; Ivanov and Campbell, 2000; Caira et al., 2001, 2014; Jensen, 2005; Golestaninasab et al., 2014; Jensen et al., 2016, 2017; Roohi Aminjan and Malek, 2016, 2017; Jensen and Guyer, 2021).

Familial placement of the genus has also been somewhat tumultuous. Like Pintner (1928), Wardle and McLeod (1952) placed *Tetragonocephalum* within the family Lecanicephalidae. Seven years later, Yamaguti (1959) erected the family Tetragonocephalidae in the order Lecanicephalidea to include *Tetragonocephalum* with the type species as *Tetragonocephalum trygonis* and considered *Tylocephalum* a junior synonym. Euzet (1959), Euzet and Combes (1965), Campbell and Williams (1984), Schmidt (1986), and Brooks and

McLennan (1993) placed *Tetragonocephalum* in the family Lecanicephalidae while Joyeux and Baer (1961) placed *Tetragonocephalum* in Cephalobothriidae. Wardle et al. (1974) and Euzet (1994) later placed *Tetragonocephalum*, along with the since resurrected genus *Tylocephalum*, in the family Tetragonocephalidae.

While few selected species of *Tetragonocephalum* have been included in previous phylogenetic analyses based on morphological (Caira et al., 2001) and molecular sequence data (e.g., Caira et al., 2014; Jensen et al., 2016), these primarily served to place the genus among elasmobranch tapeworms. Jensen et al. (2016) included molecular data for a more extensive representation of members of *Tetragonocephalum* in a phylogenetic analysis of the order Lecanicephalidea. They found that specimens of *Tetragonocephalum* grouped together, in a clade divergent from other groups, consistent with the family-level group Tetragonocephalidae. In contrast to Yamaguti (1959) and Euzet (1994), this analysis found *Tetragonocephalum* and *Tylocephalum* to belong to different families, with *Tylocephalum* grouping away from *Tetragonocephalum*, in the family Cephalobothriidae. With respect to familial interrelationships, Jensen et al. (2016) also found, albeit with low support, that the Tetragonocephalidae placed as a sister taxon to the family Polypocephalidae Meggitt, 1924, while the family Cephalobothriidae, along with Lecanicephalidae, and Eniochobothriidae Jensen, Caira, Cielocha, Littlewood and Waeschenbach, 2016 formed a clade that was sister to the Polypocephalidae-Tetragonocephalidae clade. The fact that *Tylocephalum* and *Tetragonocephalum* are neither sister taxa, nor in fact, closely related, led Jensen et al. (2016) to emphasize the importance of using the more reliable proglottid characters, rather than those of the scolex, for classification of lecanicephalidean taxa.

A complex taxonomic history exists for species of *Tetragonocephalum*, with 35 species attributed to the genus since its erection in 1905. Though this genus might seem diverse at first glance, it has been fraught with inadequate descriptions that lead Jensen and Guyer (2021) to designate nine species as *nomina dubia*, 11 as unavailable names, and reclassify two as *incertae sedis* within the family Cephalobothriidae (likely due to the historical confusion with *Tylocephalum*) (Jensen et al., 2017; Jensen and Guyer, 2021). Of the remaining 13 species, 12 were considered by Jensen and Guyer (2021) to be valid (*Tetragonocephalum georgei* Jensen and Guyer, 2021; *Tetragonocephalum kazemii* Roohi Aminjan and Malek, 2017; *Tetragonocephalum levicorpum* Jensen and Guyer, 2021; *Tetragonocephalum mackenziei* Roohi Aminjan and Malek, 2017; *Tetragonocephalum minutum* [Southwell, 1925] Perrenoud, 1931; *Tetragonocephalum opimum* Jensen and Guyer, 2021; *Tetragonocephalum passeyi* Jensen, 2005; *Tetragonocephalum sabae* Roohi Aminjan and Malek, 2016; *Tetragonocephalum salarii* Roohi Aminjan and Malek, 2016; *Tetragonocephalum simile* [Pintner, 1928] Perrenoud, 1931; *Tetragonocephalum trygonis*, and *Tetragonocephalum uaranak* [Shipley and Hornell, 1906] Perrenoud, 1931), and one was designated as a *species inquirendum* (*Tetragonocephalum yamagutii* Muralidhar, 1988).

In addition to the 12 valid species and the *species inquirendum*, there have been reports of *Tetragonocephalum* not attributed to a species or designated as potentially new, but not formally described. These reports range from publications on comparative cestode morphology (Caira et al., 2001), a taxonomic digest of cestode systematics (Caira et al., 2014), a survey on heavy metal concentrations as an ecological indicator (Golestaninasab et al., 2014), and undescribed species used in phylogenetic analyses of the family Lecanicephalidea (Jensen et al., 2016, 2017). Caira et al. (2001) reported *Tetragonocephalum* sp. from *Himantura australis* Last,

White and Naylor (as *Himantura* sp.). Caira et al. (2014) reported *Tetragonocephalum* sp. 1 from *Urogymnus asperrimus* 1 (sensu Naylor et al., 2012). Golestaninasab et al. (2014) identified one specimen as *Tetragonocephalum* sp. from *Maculabatis* cf. *gerrardi*. Jensen et al. (2016) used three species, which they designated to be new, as *Tetragonocephalum* n. sp. 1 from *Urogymnus asperrimus* 1 (sensu Naylor et al., 2012) (which was identified to be a synonym of *Tetragonocephalum* sp. 1 of Caira et al., 2014), *Tetragonocephalum* n. sp. 2 from *Pateobatis jenkinsii* (Annandale), and *Tetragonocephalum* n. sp. 3 from *Himantura leoparda* Manjaji-Matsumoto and Last. Jensen and Guyer (2021) noted that *Tetragonocephalum* n. sp. 3 from Jensen et al. (2016) is in fact a synonym of the valid species *Tetragonocephalum passeyi*, both from *Himantura leoparda*.

Morphology

Tetragonocephalum circumscribes a group of species with fairly uniform morphology. Members of the genus *Tetragonocephalum* are generally larger than most other tapeworms parasitizing dasyatid hosts (approximately 3–40 mm in total length) and easily identifiable among lecanicephalideans by the unique combination of a scolex possessing an apical organ that is extensive, external, non-retractable, non-invaginable, and muscular with a glandular surface, along with the presence of a bisaccate (rather than saccate) uterus, an ovary that is C-shaped (rather than bilobed or tetralobed) in cross-section, and a genital atrium that is much larger than that of other genera in the order, and testes clustered at the anterior end of each proglottid (Jensen et al., 2016). The 12 valid species plus the *species inquirendum* (see Jensen et al., 2017; Jensen and Guyer, 2021) in the genus can generally be distinguished from one another by whole worm length, testes number, vitelline field count, total number of proglottids, scolex length and width, among other proglottid and scolex characters. Eight of the 12 valid species have been

examined with scanning electron microscopy (Jensen, 2005; Roohi Aminjan and Malek, 2016, 2017; Jensen and Guyer, 2021). But unlike other elasmobranch tapeworms, members of *Tetragonocephalum* appear to exhibit very little variation in the pattern of these distinctive ultrastructural surface features, called microtriches (see Chervy, 2009). Described species appear to have acicular, capilliform, or papilliform filitriches on all structures of the scolex (Jensen, 2005; Roohi Aminjan and Malek, 2016, 2017; Jensen and Guyer, 2021), and acicular or capilliform filitriches (Jensen, 2005; Jensen and Guyer, 2021) or columnar (Roohi Aminjan and Malek, 2016, 2017) spinitriches on the strobila.

Host associations and geographic distribution

Species in the genus *Tetragonocephalum* are reported to parasitize the spiral intestine of dasyatid rays. Belonging to the elasmobranch order Myliobatiformes according to Last et al. (2016a), Dasyatidae Jordan and Gilbert, the stingray family known to host *Tetragonocephalum*, contains at least 90 species in 19 genera, distributed among four subfamilies: Dasyatinae Jordan and Gilbert comprised of 34 species in eight genera (*Bathytoshia* Whitley, *Dasyatis* Rafinesque, *Hemitrygon* Müller and Henle, *Hypanus* Rafinesque, *Megatrygon* Last, Naylor and Manjaji-Matsumoto, *Pteroplatytrygon* Fowler, *Taeniurops* Garman, and *Telatrygon* Last, Naylor and Manjaji-Matsumoto); the Neotrygoninae Last, Naylor and Manjaji-Matsumoto comprised of 12 species in two genera (*Neotrygon* Castelnau and *Taeniura* Müller and Henle); the Urogymninae Gray comprised of 38 species in seven genera (*Brevitrygon* Last, Naylor and Manjaji-Matsumoto, *Fluvitrygon* Last, Naylor and Manjaji-Matsumoto, *Fontitrygon* Last, Naylor and Manjaji-Matsumoto, *Himantura* Müller and Henle, *Maculabatis* Last, Naylor and Manjaji-Matsumoto, *Pateobatis,* and *Urogymnus* Müller and Henle); and the Hypolophinae Stromer

comprised of six species in two genera (*Makararaja* Roberts and *Pastinachus* Rüppell) (Last et al., 2016a, b).

To date, the 12 valid species of *Tetragonocephalum* and the *species inquirendum* are known to collectively parasitize seven host species in five genera of dasyatid stingrays: *Brevitrygon imbricata* or *B.* sp. 1 (sensu Fernando et al., 2019), *Himantura leoparda*, *Himantura uarnak* (Gmelin), *Maculabatis randalli* (Last, Manjaji-Matsumoto and Moore), *Urogymnus asperrimus* (Bloch and Schneider) or *U. granulatus* (Macleay), *Urogymnus asperrimus* 1 (sensu Naylor et al., 2012), *Urogymnus polylepis* (Bleeker), and *Pastinachus sephen* (Forsskål) (see Jensen and Guyer, 2021). There is uncertainty in exact host identity for some of the host records for members of *Tetragonocephalum* described over a century ago; recent revisions in taxonomy for the dasyatid ray hosts have revealed that groups that were once recognized as a single, broadly-distributed species are actually comprised of multiple sister species in a species complex (Last et al., 2016a, b). The six reports of additional, only informally recognized specimens of *Tetragonocephalum* increase the number of known host species by four species and one genus (*Tetragonocephalum* sp. from *Himantura australis* by Caira et al., 2001; *Tetragonocephalum* sp. from *Maculabatis gerrardi* [Gray] by Jensen et al., 2017; *Tetragonocephalum* sp. from *Maculabatis* cf. *gerrardi* 3 (sensu Naylor et al., 2012) (as *Maculabatis* cf. *gerrardi*) by Golestaninasab et al., 2014; *Tetragonocephalum* n. sp. 2 from *Pateobatis jenkinsii* by Jensen et al., 2016; *Tetragonocephalum* n. sp. 1 from *Urogymnus asperrimus* 1 [sensu Naylor et al., 2012] by Jensen et al., 2016). To summarize, to date, *Tetragonocephalum* had been reported to parasitize at least 11 species or up to 13 species, if *Brevitrygon imbricata* and *B.* sp. 1 (sensu Fernando et al., 2019) each host different valid species, and *Tetragonocephalum uarnak* parasitizes *Himantura tutul* (Gmelin) or *H. uarnak* or *H. undulata* (Bleeker), but not *H.*

leoparda. *Tetragonocephalum* has been found to parasitize rays in six genera, representing two of the four subfamilies of dasyatid rays: Urogymninae and Hypolophinae. Species of *Tetragonocephalum* are not known to parasitize stingrays in the subfamilies Dasyatinae or Neotrygoninae.

Caira and Jensen (2014) stated that most tapeworms species hosted by elasmobranchs show a pattern of oioxenous host specificity, where each tapeworm species only parasitizes one elasmobranch species. This is a pattern that can be seen in the genus *Tetragonocephalum*, with each species only known from one species of dasyatid host (see paragraph above).

Also interesting to note is the pattern of multiple congeners of *Tetragonocephalum* in the same host species. *Pastinachus sephen* is known to host two species of *Tetragonocephalum* (Roohi Aminjan and Malek, 2017), the *Brevitrygon imbricata – B.* sp. 1 (sensu Fernando et al., 2019) species complex hosts two species (Shipley and Hornell, 1905; Pintner, 1928; Perrenoud, 1931), *Maculabatis randalli* hosts two species (Roohi Aminjan and Malek, 2016), and *Urogymnus polylepis* hosts three species (Jensen and Guyer, 2021).

Geographic information on valid species and the *species inquirendum* is limited to type localities. From this information, *Tetragonocephalum* is known to occur in the Gulf of Oman off the coast of Iran (Golestaninasab et al., 2014; Roohi Aminjan and Malek, 2016, 2017), the Gulf of Mannar, Sri Lanka (Shipley and Hornell, 1905, 1906; Southwell, 1925; Pintner, 1928; Perrenoud, 1931), the Arafura Sea near northeastern Australia (Caira et al., 2001; Jensen, 2005; Caira et al., 2014; Jensen et al., 2016), and the island of Borneo (Jensen et al., 2017; Jensen and Guyer, 2021).

Pateobatis, the only host genus with reports of *Tetragonocephalum* but no described valid species, is a member of the subfamily Urogymninae and is comprised of five species:

Pateobatis bleekeri (Blyth), *P. fai* (Jordan and Seale), *P. hortelei* (Last, Manjaji-Matsumoto and Kailola, 2006), *P. jenkinsii*, and *P. uarnacoides* (Bleeker). All species of *Pateobatis* inhabit the waters of Indo-Pacific region. Due to the amount of host diversity and specific diversity within the genus *Tetragonocephalum*, the latter portions of this thesis will focus on the fauna of *Tetragonocephalum* in *Pateobatis*.

Purpose of study

This study has six major goals: (1) expand the knowledge of host associations for members of the genus *Tetragonocephalum*; (2) characterize the diversity of species of *Tetragonocephalum* in dasyatid rays of the genus *Pateobatis*; (3) assess the degree of novelty of species of *Tetragonocephalum* in *Pateobatis*; (4) formally describe selected species of *Tetragonocephalum* from *Pateobatis fai*, *P. jenkinsii*, and *P. uarnacoides*; (5) discuss the phylogenetic relationships between species of *Tetragonocephalum* based on 18S, 28S, 5.8S/ITS rDNA sequence data; and (6) use morphological characters to determine the morphological cohesion of a group of selected specimens of *Tetragonocephalum* in a principle component analysis of morphological characters.

MATERIALS AND METHODS

Host specimens

Over the last several decades, extensive, global collections of tapeworms that parasitize elasmobranchs have resulted in an expansive assemblage of material fixed for morphological and molecular work in the lab of Dr. Kirsten Jensen. Vialed and mounted specimens of *Tetragonocephalum* had been preliminarily identified to genus by previous researchers, and were newly catalogued for this study. Collectively, material used in this study was collected from

August 15th, 1997 to March 10th, 2018. Species, size, and locality data for the 75 elasmobranch specimens found to host *Tetragonocephalum* in this study can be found in Table I. Host species were identified in the field and identifications confirmed using molecular sequence data (Naylor et al., 2012; Fernando et al., 2019). Host locality data are visualized in Figure 1. Host specimens were captured using drift net, trawl, gill net, hand spear, or hand line. Additional host specimen information can be found by searching by unique host code in the Global Cestode Database (https://tapewomdb.uconn.edu/index.php/hosts/specimen_search/elasmobranch). Spiral intestines of hosts containing worms were either fixed in 10% seawater-buffered formalin and later transferred to 70% ethanol, or stored in 95% ethanol for molecular sequencing. In total, 398 specimens of *Tetragonocephalum* from 75 host specimens representing 21 host species were examined for this study.

Specimen preparation

Selected formalin-fixed specimens were prepared as whole mounts for examination with light microscopy as follows. Specimens were removed from permanent storage in 70% ethanol and hydrated in distilled water, stained in a diluted Delafield's hematoxylin, washed in distilled water, differentiated in tap water, and placed in 70% ethanol. Worms were then destained in acidic ethanol, neutralized in basic ethanol, rinsed in 70% ethanol, flattened and straightened with glass shards, dehydrated in a graded ethanol series, cleared in methyl salicylate, and mounted on glass slides in Canada balsam.

Scoleces were prepared for examination using scanning electron microscopy as follows. Selected specimens were cleaned of debris with forceps and insect pins, given unique identifying specimen numbers according to their associated unique host code, and photographed with

Table I. Host species sex, size, and locality data for the 75 host specimens in the family Dasyatidae examined in this study. Host specimen identifications follow Naylor et al. (2012) and Fernando et al. (2019).

Host Species	Unique Host Code	Capture Locality	Coordinates	Date of Collection	Sex	Disk Width (cm)
Hemitrygon akajei	JN-22*	Oga City, Japan, Pacific Ocean	39° 46.93' N, 139° 51.82' E	10/19/99	Female	39
	JN-53*	Oga City, Japan, Pacific Ocean	Unknown	10/20/99	Female	42.5
	TW-147*	Taiwan Strait, Taiwan, Pacific Ocean	22¼43'34"N, 120¼15'10"E	1/7/17	Male	28.4
Hemitrygon cf. bennetti	TW-25*	Taiwan Strait, Taiwan, Pacific Ocean	23°29'12"N, 120° 9'27"E	5/14/05	Female	28
Neotrygon kuhlii 3	MZ-18	Mozambique Channel, Mozambique, Indian Ocean	12°17'3.44"S, 40°35'10.83"E	6/29/16	Female	36.5
	MZ-26	Mozambique Channel, Mozambique, Indian Ocean	12°17'3.44"S, 40°35'10.83"E	6/29/16	Male	33
Neotrygon varidens	VN-107	Phan Thiet, Vietnam, Pacific Ocean	10°55'22.21"N, 108°06'16.61"E	3/19/10	Female	23.6
Taeniura lymma 1	BO-130	Celebes Sea, Malaysian Borneo, Pacific Ocean	04°14'44.02"N, 118°37'53.32"E	5/5/03	Male	22.5
Himantura australis	AU-139	Buffalo Creek, Timor Sea, Australia, Indian Ocean	12°20'11"S, 130°54'39"E	8/15/97	Male	34
	CM03-3	Gulf of Carpentaria, Australia, Indian Ocean	12°35'11"S, 141°42'34"E	6/6/03	Male	53
	CM03-10	Gulf of Carpentaria, Australia, Indian Ocean	12°35'11"S, 141°42'34"E	6/6/03	Male	55
	CM03-13	Gulf of Carpentaria, Australia, Indian Ocean	12°35'11"S, 141°42'34"E	6/6/03	Male	39
	CM03-24	Gulf of Carpentaria, Australia, Indian Ocean	12°35'11"S, 141°42'34"E	6/7/03	Female	49.5
	CM03-25	Gulf of Carpentaria, Australia, Indian Ocean	12°35'11"S, 141°42'34"E	6/7/03	Female	45
	SO-4	Solomon Sea, Vonavona, Solomon Islands, Pacific Ocean	08°12'56.5"S, 156°59'59.5"E	3/16/12	Male	89.5
	SO-13	Solomon Sea, Vonavona, Solomon Islands, Pacific Ocean	08°13'23.8"S, 157°0'2.4"E	3/22/12	Female	74
	SO-16	Solomon Sea, Vonavona, Solomon Islands, Pacific Ocean	08°13'23.8"S, 157°0'2.4"E	3/22/12	Male	87
Himantura leoparda	NT-32*	Arafura Sea, Australia, Pacific Ocean	11°17'44"S, 136°59'48"E	11/12/99	Male	112
	NT-37*	Arafura Sea, Australia, Pacific Ocean	11°17'44"S, 136°59'48"E	11/13/99	Female	136
	NT-117*	Arafura Sea, Australia, Pacific Ocean	11°17'44"S, 136°59'48"E	11/22/99	Female	100
Himantura tutul	KA-48	Makassar Strait, Indonesian Borneo, Pacific Ocean	01°45'58.92"S, 116°23'36.09"E	11/26/06	Male	40.5
	SL-10*	Pukalam Landing Site, Sri Lanka, Indian Ocean	08°33'35"N, 79°55'11"E	3/8/18	Female	76.5
	SL-42*	Munnai Market, Point Pedro, Sri Lanka, Indian Ocean	09°49'42.11"N, 80°14'40.22"E	3/10/18	Female	117
Himantura uarnak	EG-2	Abu Malh Reef, off Hurghada, Egypt, Red Sea	27°35.53000' N, 33°48.95833' E	1/8/15	Female	121
Himantura undulata	KA-43	Makassar Strait, Indonesian Borneo, Pacific Ocean	01°45'58.92"S, 116°23'36.09"E	11/26/06	Male	90
	KA-326	South China Sea, Indonesian Borneo, Pacific Ocean	00°42'43.70"N , 108°53'14.90"E	7/28/07	Male	54.6
Maculabatis bineeshi	IN-22	Bay of Bengal, India, Indian Ocean	21°37'49.4"N, 87°32'38.9"E	6/7/13	Male	42.5
	IN-31	Bay of Bengal, India, Indian Ocean	21°37'49.4"N, 87°32'38.9"E	6/8/13	Female	40.5
	IN-55	Bay of Bengal, India, Indian Ocean	21°37'49.4"N, 87°32'38.9"E	6/10/13	Male	66
Maculabatis gerrardi	BO-23	South China Sea, Malaysian Borneo, Pacific Ocean	01°48'15.45"N, 109°46'47.17"E	6/3/02	Male	61.7
	BO-49	South China Sea, Malaysian Borneo, Pacific Ocean	02°53'52.16"N, 112°05'44.12"E	6/10/02	Male	62
	BO-88	Celebes Sea, Malaysian Borneo, Pacific Ocean	04°14'34.25"N, 117°53'00.57"E	6/28/02	Female	62
	BO-138	Celebes Sea, Malaysian Borneo, Pacific Ocean	04°14'44.02"N, 118°37'53.32"E	5/6/03	Male	62
	BO-466	South China Sea, Malaysian Borneo, Pacific Ocean	02°53'52.16"N, 112°05'44.12"E	4/28/04	Female	25
	KA-75	Makassar Strait, Indonesian Borneo, Pacific Ocean	03°36'36.00"S, 115°54'59.40"E	11/29/06	Male	54
	KA-211	Java Sea, Indonesian Borneo, Pacific Ocean	01°21'45.20"S, 110°04'10.30"E	7/16/07	Female	65.5
	KA-207	Java Sea, Indonesian Borneo, Pacific Ocean	02°14'13.36"S, 110°05'48.95"E	7/16/07	Female	69
	KA-394	Sulawesi Sea, Indonesian Borneo, Pacific Ocean	03°17'47.04"N, 117°34'57.26"E	7/20/08	Female	53
Maculabatis cf. gerrardi 5	VN-87*	Vung Tau, Vietnam, Pacific Ocean	10°22'52.9"N, 107°04'03.9"E	3/18/10	Male	66.4
	VN-88	Vung Tau, Vietnam, Pacific Ocean	10°22'52.9"N, 107°04'03.9"E	3/19/10	Female	59.5
Maculabatis cf. gerrardi 6	MZ-12	Mozambique Channel, Mozambique, Indian Ocean	12°19'41.25"S, 40°34'17.05"E	6/28/16	Female	98
Maculabatis macrura	BO-337	South China Sea, Malaysian Borneo, Pacific Ocean	02°30'07.34"N, 110°40'16.82"E	4/28/04	Unknown	Unknown
	BO-400	South China Sea, Malaysian Borneo, Pacific Ocean	02°00'00.00"N, 110°37'60.00"E	4/19/04	Male	49.5
	KA-86	Makassar Strait, Indonesian Borneo, Pacific Ocean	03°54'15.40"S, 115°15'31.80"E	11/30/06	Male	34.5
	KA-293	South China Sea, Indonesian Borneo, Pacific Ocean	00°12'51.60"S, 109°05'00.30"E	7/25/07	Male	31.2
Maculabatis pastinacoides	BO-12	South China Sea, Malaysian Borneo, Pacific Ocean	01°48'15.45"N, 109°46'47.17"E	6/1/02	Female	42
	BO-61*	South China Sea, Malaysian Borneo, Pacific Ocean	02°53'52.16"N, 112°05'44.12"E	6/12/02	Male	46
	BO-76	Sulu Sea, Malaysian Borneo, Pacific Ocean	06°01'10.32"N, 117°42'14.76"E	6/21/02	Female	55
	BO-79	Sulu Sea, Malaysian Borneo, Pacific Ocean	05°50'20.17"N, 118°07'15.78"E	6/22/02	Unknown	Unknown
	BO-98	Sulu Sea, Malaysian Borneo, Pacific Ocean	06°01'10.32"N, 117°42'14.76"E	4/28/03	Female	58.5
	BO-100	Sulu Sea, Malaysian Borneo, Pacific Ocean	06°01'10.32"N, 117°42'14.76"E	4/28/03	Male	51
	BO-168	South China Sea, Malaysian Borneo, Pacific Ocean	01°48'15.45"N, 109°46'47.17"E	5/14/03	Female	19.5
	KA-95	Java Sea, Indonesian Borneo, Pacific Ocean	03°19'13.70"S, 114°33'41.70"E	12/1/06	Female	48
	KA-203*	Java Sea, Indonesian Borneo, Pacific Ocean	01°54'26.80"S, 110°00'47.50"E	7/15/07	Female	85.5
Pateobatis fai	NT-33	Arafura Sea, Australia, Pacific Ocean	11°17'44"S, 136°59'48"E	11/12/99	Male	139.5
	SO-20	Solomon Sea, Vonavona, Solomon Islands, Pacific Ocean	8°13'23.8"S, 157°0'2.4"E	3/22/12	Male	88
	SO-43	Solomon Sea, Nusa Alana, Solomon Islands, Pacific Ocean	8°14'13.4"S, 157°1'53.7"E	3/26/12	Male	81
	SO-44	Solomon Sea, Nusa Alana, Solomon Islands, Pacific Ocean	8°14'13.4"S, 157°1'53.7"E	3/26/12	Unknown	74.5
Pateobatis jenkinsii	NT-38*	Arafura Sea, Australia, Pacific Ocean	11°17'44"S, 136°59'48"E	11/12/99	Female	138
	NT-106	Arafura Sea, Australia, Pacific Ocean	11°17'44"S, 136°59'48"E	11/19/99	Female	84
	NT-107	Arafura Sea, Australia, Pacific Ocean	11°17'44"S, 136°59'48"E	11/19/99	Female	97
	BO-339	South China Sea, Malaysian Borneo, Pacific Ocean	02°30'07.34"N, 110°40'16.82"E	4/28/04	Male	Unknown
	SL-43	Munnai Market, Point Pedro, Sri Lanka, Indian Ocean	09°49'42.11"N, 80°14'40.22"E	3/10/18	Female	101
Pateobatis uarnacoides	BO-77*	Sulu Sea, Malaysian Borneo, Pacific Ocean	05°50'20.17"N, 118°07'15.78"E	6/22/02	Unknown	Unknown
	BO-78	Sulu Sea, Malaysian Borneo, Pacific Ocean	05°50'20.17"N, 118°07'15.78"E	6/23/02	Unknown	Unknown
	BO-118	Sulu Sea, Malaysian Borneo, Pacific Ocean	06°01'10.32"N, 117°42'14.76"E	5/4/03	Male	56
	BO-167	South China Sea, Malaysian Borneo, Pacific Ocean	01°48'15.45"N, 109°46'47.17"E	5/14/03	Female	25
	KA-81	Makassar Strait, Indonesian Borneo, Pacific Ocean	03°36'46.10"S, 115°55'05.10"E	11/30/06	Female	63.5
	KA-206	Java Sea, Indonesian Borneo, Pacific Ocean	02°14'13.36"S, 110°05'48.95"E	7/16/07	Female	75
	KA-210	Java Sea, Indonesian Borneo, Pacific Ocean	01°21'45.20"S, 110°04'10.30"E	7/16/07	Female	46.5
	KA-386	South China Sea, Indonesian Borneo, Pacific Ocean	01°14'33.10"S, 109°57'00.20"E	7/17/08	Female	31
Urogymnus asperrimus 1	CM03-53	Gulf of Carpentaria, Australia, Indian Ocean	12°35'11"S, 141°42'34"E	6/11/03	Female	46
	SO-32	Solomon Sea, Ghizo, Solomon Islands, Pacific Ocean	8°2'15.1"S, 156°45'57.1"E	3/24/12	Male	89
Urogymnus polylepis	BO-108*	Kinabatangan River, Malaysian Borneo	05°41'10.81"N, 118°23'08.35"E	4/30/03	Male	90.5
	KA-393	Sulawesi Sea, Indonesian Borneo, Pacific Ocean	03°17'47.04"N, 117°34'57.26"E	7/20/08	Female	99.2

*species identity unconfirmed

millimeter ruler under a dissection scope. Scoleces were then cut from strobila using tweezer scissors, and the strobila was retained as a voucher and prepared as a permanent whole mount according to the above procedure. The scoleces were then hydrated with distilled water, osmicated in a 1% OsO_4 solution, and left refrigerated overnight. The following day, scoleces were rinsed in distilled water, dehydrated using a graded ethanol series, placed in hexamethyldisilazane (Ted Pella, Inc., Redding, California, USA) for 30 minutes, allowed to air-dry, and mounted on an aluminum stub covered with double-sided adhesive carbon tape. This stub was then sputter-coated with 35 nm of gold and examined using Hitachi S4700 scanning

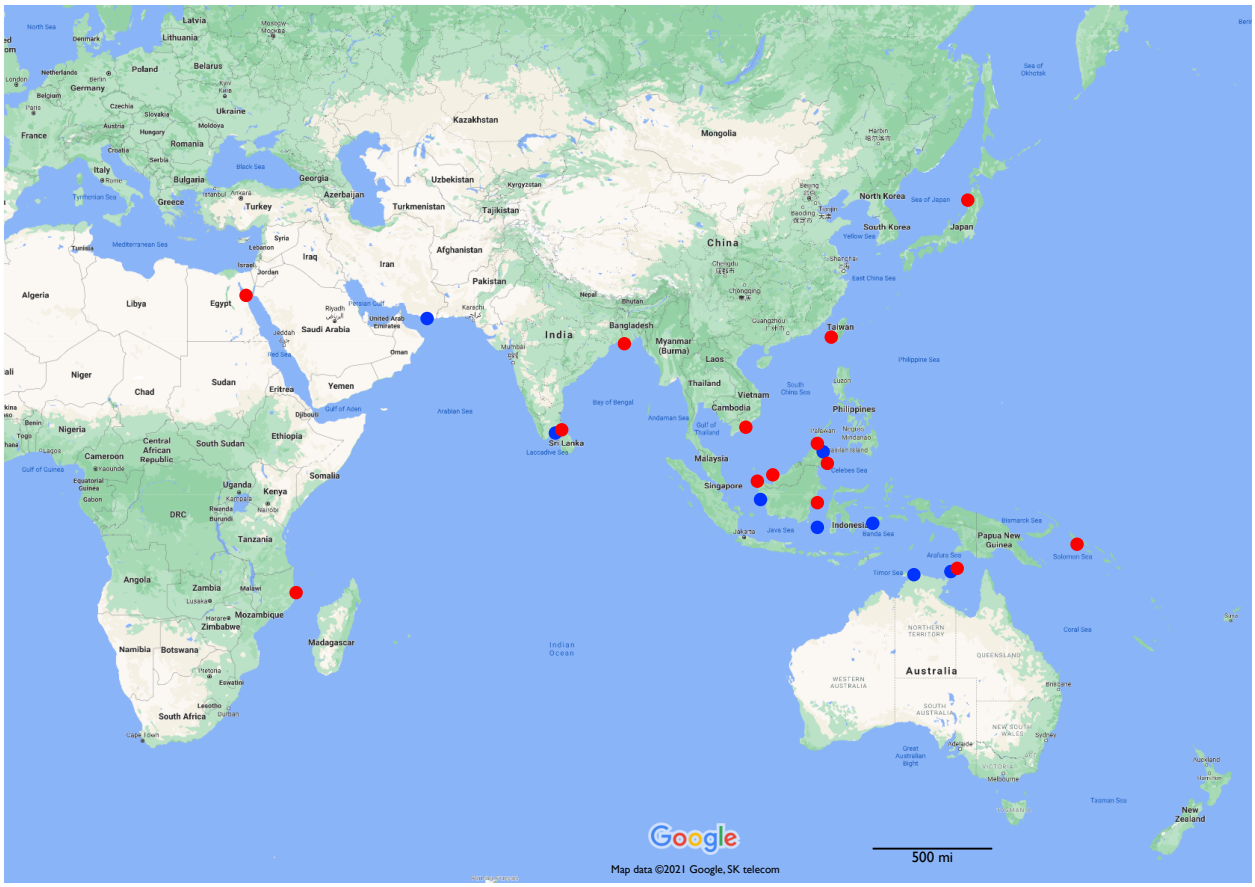

Figure 1. Localities for dasyatid rays known to host species of *Tetragonocephalum*.Blue dots indicate localities of rays from which valid species and previous records of *Tetragonocephalum* that have been reported; red dots indicate localities of rays found to *Tetragonocephalum* in this study.

electron microscope at the Microscopy and Analytical Imaging Laboratory, University of Kansas, Lawrence, Kansas, USA.

Specimens prepared as histological sections were either embedded in paraffin or plastic resin. In both cases, selected specimens were given unique identifying specimen numbers according to their associated unique host code and photographed with millimeter ruler under dissection scope. Scoleces or proglottids of interest were then cut from strobila using tweezer scissors, and the remainder of each worm was retained and prepared as whole mount according to the previously mentioned whole mount procedure. Proglottids sectioned in paraffin were prepared as follows. Scoleces or proglottids of interest were dehydrated using a graded ethanol series, stained to differentiate from paraffin color in fast green, cleared in xylene, transferred to 1:1 paraffin-xylene solution, transferred to paraffin, and embedded in a paraffin sectioning mold for histological sectioning. Serial sections were cut at 4 μm intervals using an Olympus TBS CUT 4060 microtome (Triangle Biomedical Sciences, Durham North Carolina, USA). Resulting serial sections were floated on 3% sodium silicate on glass slides placed on a slide warmer. Slides were stained by first dissolving the paraffin in xylene, then rehydrating slides in a graded ethanol series, stained with Delafield's hematoxylin, counterstained in eosin, differentiated in Scott's solution, dehydrated in a graded ethanol series, and cleared in xylene. Sections were then mounted under cover slips in Canada balsam.

Scoleces and proglottids were prepared as plastic resin histological sections as follows. Scoleces and proglottids of interest were dehydrated in a graded ethanol series, transferred to a 1:1 solution of 100% ethanol and Technovit® H7100 infiltrating resin (Heraeus Kluzer GmbH, Wehrheim, Germany) for two hours, then transferred to infiltrating resin and refrigerated overnight. Scoleces and proglottids were then embedded in Technovit® H7100 embedding

solution in plastic block holders. Individual sections were cut at 4 μm intervals using an Olympus TBS CUT 4060 microtome and floated on drops of distilled water on Fisherbrand® Superfrost Plus charged microscope slides (Fisherbrand; Fisher, Pittsburgh, Pennsylvania, USA) placed on a slide warmer. Slides were stained with Delafield's hematoxylin, differentiated in Scott's solution, dried in a slide oven at 60°C until dry, counterstained in eosin, dehydrated in graded ethanol series, and mounted under cover slips in Canada balsam.

Photomicrographs of whole mounts and histological sections were taken using a Lumenera Infinity 3 camera (Lumenera Corporation, Ottawa, Ontario, Canada) attached to a Zeiss Axioskop 2 plus (Carl Zeiss Microscopy, LLC, Thornwood, New York, USA). Measurements were taken with the image analysis software INFINITY ANALYZE (Lumenera Corporation) for Mac. Measurements are reported in micrometers unless noted otherwise and given as follows: Range followed by mean ± standard deviation; number of individuals measured; number of measurements taken [if more than one measurement was taken per specimen]. Scolex measurements taken are illustrated in Figure 2; note that scolex proper length measurement was taken for the posterior region of the scolex and does not include the apical modification of the scolex proper. Line drawings were made using a camera lucida using the aforementioned microscope. Terminology for microthrix description follows Chervy (2009).

Molecular and phylogenetic methods

Molecular sequence data of the 18S, 28S, 5.8S/ITS rDNA genes were generated for all specimens used in phylogenetic analysis by colleagues at the University of Connecticut as a part of a larger project. Worm specimens used for the phylogenetic analysis were each given unique specimen codes (e.g., JW226 or KW12). For each specimen, a hologenophore (sensu Pleijel et al., 2008) was prepared as a whole mount to confirm identification.

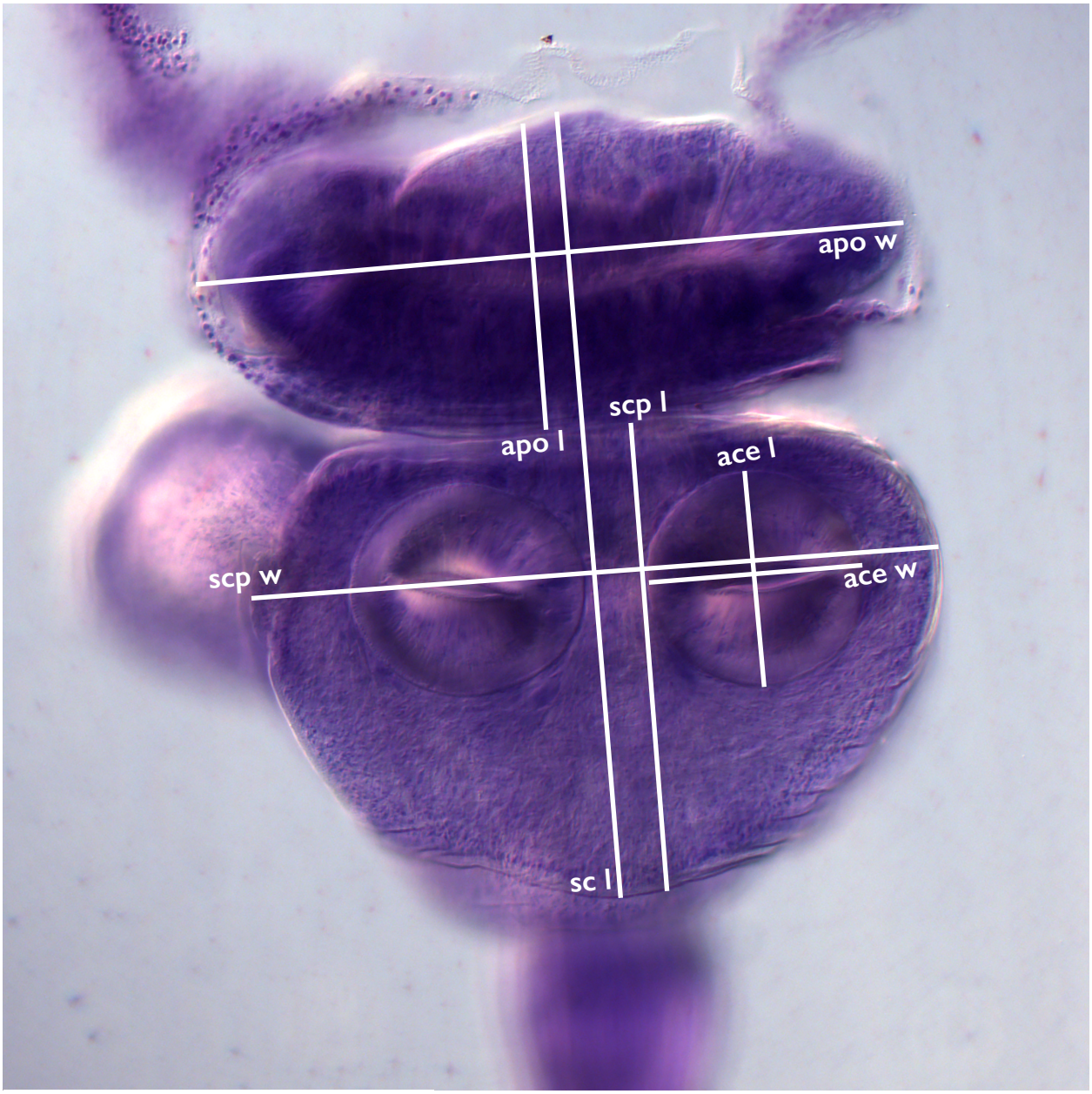

Figure 2. Scolex measurements taken. *Abbreviations:* ace l, acetabulum length; ace w, acetabulum width; apo l, apical organ length; apo w, apical organ width; sc l, scolex length; scp l, scolex proper length; scp w, scolex proper width.

A dataset of 25 specimens identified by their hologenophore to belong to the genus *Tetragonocephalum* was assembled. These 25 specimens represent 23 species, including three known species (i.e., *Tetragonocephalum georgei*, *Tetragonocephalum levicorpum*, and *Tetragonocephalum passeyi*), four species described in this study (i.e., KW26 as

Tetragonocephalum n. sp. 4, KW16 as *Tetragonocephalum* n. sp. 5, KW15 as *Tetragonocephalum* n. sp. 6, KW484 and KW485 as *Tetragonocephalum* n. sp. 7), and 16 species that remain undescribed. Two of the species, *Tetragonocephalum* n. sp. 7 (KW484 and KW485) and *Tetragonocephalum* sp. 13 (KW13 and KW14), were represented by replicate specimens. In addition, a total of two lecanicephalidean specimens, one specimen each of the genera *Polypocephalus* Braun, 1878 and *Zanobatocestus* Jensen, Mojica and Caira, 2014 were included in the analysis as outgroups.

Sequence data were aligned with ClustalW (Thompson et al., 1994) in R (R Core Team, 2020) using the msa function from the package msaR: Multiple Sequence Alignment for R Shiny version 0.3.0 (available at https://cran.microsoft.com/snapshot/2020-04-24/web/packages/msaR/index.html). A general time reversible model of evolution with invariable sites and gamma distribution (GTR + I + Γ) was determined to be the scheme with the lowest AICc score using PartitionFinder 2 (Lanfear et al., 2016) with model set to “best”. A Bayesian analysis was conducted as follows. A MrBayes (Ronquist et al., 2012) script was run on XSEDE using CIPRES (Miller et al., 2010) in two Markov chain Monte Carlo (MCMC) runs of four chains each, for 100 million generations to ensure run convergence. The program Tracer (version 1.7.1, Rambaut et al., 2018) was used to obtain effective sample sizes of all parameters and to ensure that the effective sample sizes were all above 200. The maximum standard deviation of split frequencies was checked to ensure that the runs had converged for well-supported nodes on the tree. The average standard deviation of split frequencies was 0.001931, with the maximum being 0.006929. Nodal support was estimated based on posterior probabilities.

Principal component analysis

The principal component analysis was generated in the software R with the rda function from the VEGAN package (Dixon, 2003). Specimens that were incomplete or were missing one or more of the character measurements were excluded from this principal component analysis. Certain characters that were consistently missing measurements for multiple specimens were also excluded from this analysis. A total of 30 specimens were used in this principal component analysis: seven specimens of *Tetragonocephalum* n. sp. 4, 11 specimens of *Tetragonocephalum* n. sp. 5, seven specimens of *Tetragonocephalum* n. sp. 6, and five specimens of *Tetragonocephalum* n. sp. 7. The 24 characters used in this analysis, taken as a subset of the characters used in species descriptions, were as follows: worm length, worm length anterior to first gravid proglottid, total proglottid count, gravid proglottid count, mature proglottid count, immature proglottid count, whole scolex width, scolex proper width, acetabula length, acetabula width, apical organ width, posterior-most immature proglottid length, posterior-most immature proglottid width, posterior-most mature proglottid length, posterior-most mature proglottid width, anterior-most gravid proglottid length, anterior-most gravid proglottid width, terminal gravid proglottid length, terminal gravid proglottid width, testes count, testes length, testes width, vitelline field number, and genital pore distance from posterior end of proglottid. Ellipses were used to indicate 95% confidence intervals for each respective group of specimens representing each of the new species described in this study.

RESULTS

Novel host records

For this study, 75 host specimens of 21 species representing seven genera from three of the four subfamilies in the family Dasyatidae were examined and found to host specimens of *Tetragonocephalum* (see Table II). These records represent two previously unreported subfamilies (Dasyatinae and Neotrygoninae), indicating that *Tetragonocephalum* parasitizes members of every subfamily of dasyatid rays. Reported host records include *Neotrygon kuhlii* 3, *Taeniura lymma* 1, *Maculabatis* cf. *gerrardi* 3, *Maculabatis* cf. *gerrardi* 5, *Maculabatis* cf. *gerrardi* 6, and *Urogymnus asperrimus* 1, which are informal designations for host species which are molecularly distinct and that exist in species complexes and are likely new to science but have yet to be described (Naylor et al., 2012). These new records also add three genera (*Hemitrygon*, *Neotrygon*, and *Taeniura*) to the list of known hosts of *Tetragonocephalum*, bringing the number genera known to host *Tetragonocephalum* to nine. With the addition of these 16 species, there are now 24 species known to host *Tetragonocephalum*.

New locality records

Locality reports of *Tetragonocephalum* from this study significantly expand the known range of *Tetragonocephalum*. The previous six countries in which *Tetragonocephalum* can be found has been expanded include 18 localities in 12 countries (Australia, Egypt, India, Indonesia, Iran, Japan, Taiwan, Malaysia, Mozambique, the Solomon Islands, Sri Lanka, and Vietnam) (see Table I). The range of *Tetragonocephalum* remains restricted to the Indo-Pacific region (Fig. 1).

Table II. Host, species, and locality records for species of *Tetragonocephalum* by host subfamily. New host and species records in bold.

Host Subfamily	Host Species	Reports	Locality
Dasyatinae	***Hemitrygon akajei***	***Tetragonocephalum* sp. 8 (this study)**	**Northwestern Japan; Taiwan Strait, Taiwan**
Dasyatinae	***Hemitrygon* cf. *bennetti***	***Tetragonocephalum* sp. 6 (this study)**	**Taiwan Strait, Taiwan**
Hypolophinae	*Pastinachus sephen*	*Tetragonocephalum kazemii*, *T. mackenziei*	Gulf of Oman, Iran
Neotrygoninae	***Neotrygon kuhlii* 3**	***Tetragonocephalum* sp. 22 (this study)**	**Mozambique Channel, Mozambique**
Neotrygoninae	***Neotrygon varidens***	***Tetragonocephalum* sp. 23 (this study)**	**Phan Thiet, Vietnam**
Neotrygoninae	***Taeniura lymma* 1**	***Tetragonocephalum* sp. 24 (this study)**	**Celebes Sea, Malaysian Borneo**
Urogymninae	*Brevitrygon imbricata* or *B.* sp. 1	*Tetragonocephalum simile*, *T. trygonis*, *T. yamagutii*	Gulf of Mannar, Sri Lanka (as Ceylon); eastern coast of India
Urogymninae	*Himantura australis*	*T.* sp. of Caira et al. (2001), ***Tetragonocephalum* sp. 11 (this study)**	Northern Territory, Australia; **Timor Sea, Australia**; **Gulf of Carpentaria, Australia**; **Solomon Sea, Solomon Islands**
Urogymninae	*Himantura leoparda* or *H. tutul* or *H. uarnak* or *H. undulata*	*Tetragonocephalum uarnak*	Gulf of Mannar, Sri Lanka (as Ceylon)
Urogymninae	*Himantura leoparda*	*Tetragonocephalum passeyi*, *T.* n. sp. 3 of Jensen et al. (2016), ***Tetragonocephalum* sp. 6 (this study)**	Arafura Sea, Australia; Wessel Islands, Northern Territory, Australia; **Anafura Sea, Australia**
Urogymninae	***Himantura tutul***	***Tetragonocephalum* sp. 10 and *Tetragonocephalum* sp. 19 (this study)**	**Makassar Strait, Indonesian Borneo**
Urogymninae	***Himantura uarnak***	***Tetragonocephalum* sp. 12 (this study)**	**Red Sea, Egypt**
Urogymninae	***Himantura undulata***	***Tetragonocephalum* sp. 9 and *Tetragonocephalum* sp. 21 (this study)**	**Makassar Strait and South China Sea, Indonesian Borneo**
Urogymninae	***Maculabatis bineeshi***	***Tetragonocephalum* sp. 18 (this study)**	**Bay of Bengal, India**
Urogymninae	*Maculabatis gerrardi*	*Tetragonocephalum* sp. of Caira and Jensen (2017), ***Tetragonocephalum* sp. 7 (this study)**	Java Sea, Indonesian Borneo; **Celebes Sea and South China Sea, Malaysian Borneo**; **Java Sea, Makassar Strait, and Sulawesi Sea, Indonesian Borneo**
Urogymninae	*Maculabatis* cf. *gerrardi* 3	*Tetragonocephalum* sp. of Golestaninasab et al. (2014) (as *M.* cf. *gerrardi*)	Gulf of Oman, Iran
Urogymninae	***Maculabatis* cf. *gerrardi* 5**	***Tetragonocephalum* sp. 14 and *Tetragonocephalum* sp. 20 (this study)**	**Vung Tau, Vietnam**
Urogymninae	***Maculabatis* cf. *gerrardi* 6**	***Tetragonocephalum* sp. 16 (this study)**	**Mozambique Channel, Mozambique**
Urogymninae	***Maculabatis macrura***	***Tetragonocephalum* sp. 13 (this study)**	**South China Sea, Malaysian Borneo**; **Makassar Strait and South China Sea, Indonesian Borneo**
Urogymninae	*Maculabatis randalli*	*Tetragonocephalum sabae*, *T. salarii*	Gulf of Oman, Iran
Urogymninae	***Maculabatis pastinacoides***	***Tetragonocephalum* sp. 15 (this study)**	**South China Sea and Sulu Sea, Malaysian Borneo**; **Java Sea, Indonesian**
Urogymninae	***Pateobatis fai***	***Tetragonocephalum* n. sp. 4 (this study)**	**Arafura Sea, Australia**; **Soloman Sea, Solomon Islands**
Urogymninae	*Pateobatis jenkinsii*	*Tetragonocephalum* n. sp. 2 of Jensen et al. (2016), ***Tetragonocephalum* n. sp. 5 and *Tetragonocephalum* n. sp. 6 (this study)**	Wessel Islands, Northern Territory, Australia; **Anafura Sea, Australia**; **South China Sea, Malaysian Borneo**; **Northern Sri Lanka**
Urogymninae	***Pateobatis uarnacoides***	***Tetragonocephalum* n. sp. 7 (this study)**	**South China Sea and Sulu Sea, Malaysian Borneo**; **Java Sea, Makassar Strait, and South China Sea, Indonesian Borneo**
Urogymninae	*Urogymnus asperrimus* or *U. granulatus*	*Tetragonocephalum minutum*	Gulf of Mannar, Sri Lanka (as Ceylon)
Urogymninae	*Urogymnus asperrimus* 1	*Tetragonocephalum* n. sp. 1 of Jensen et al. (2016) (= *T.* sp. 1 of Caira et al., 2014), ***Tetragonocephalum* sp. 17 (this study)**	Weipa, Queensland, Australia; **Gulf of Carpentaria, Australia**; **Solomon Sea, Solomon Islands**
Urogymninae	*Urogymnus polylepis*	*Tetragonocephalum georgei*, *T. levicorpum*, *T. opimum*	Celebes Sea, Indonesian Borneo; Kinabatangan River, Malaysian Borneo

Fauna of *Tetragonocephalum* in *Pateobatis*

The host genus of focus, *Pateobatis*, was found to host nine unique species of *Tetragonocephalum*: four species in *Pateobatis fai*, three in *Pateobatis jenkinsii*, and two in *Pateobatis uarnacoides*. It is likely that, all of these species are new to science given the host specificity observed in known species of *Tetragonocephalum*, and since no species of *Tetragonocephalum* has been described from the host genus *Pateobatis*; a formal description of each of these nine species is outside the scope of this project. Based on molecular sequence data (see Fig. 14), in addition to comparisons to valid species of *Tetragonocephalum*, we were able to determine that four of these nine species as new to science: *Tetragonocephalum* n. sp. 4 from *Pateobatis fai*, *Tetragonocephalum* n. sp. 5 and *Tetragonocephalum* n. sp. 6 from *Pateobatis jenkinsii*, and *Tetragonocephalum* n. sp. 7 from *Pateobatis uarnacoides*. The naming scheme begins at *Tetragonocephalum* n. sp. 4 to account for the three undescribed new species designated by Jensen et al. (2016). We have verified, by comparison of vouchers, that the species designated as *Tetragonocephalum* n. sp. 2 by Jensen et al. (2016) is a synonym of *Tetragonocephalum* n. sp. 4 from this study, and we herein refer to this species as *Tetragonocephalum* n. sp. 4. The remainder of the species found to parasitize *Pateobatis* are *Tetragonocephalum* sp. 1, *Tetragonocephalum* sp. 2, and *Tetragonocephalum* sp. 3 from *P. fai*, *Tetragonocephalum* sp. 4 from *P. jenkinsii*, and *Tetragonocephalum* sp. 5 from *P. uarnacoides*. Though there is preliminary evidence to suggest that all of these species are new, only a small subset of measurements are presented to be able to distinguish congeners in the same host species. A characterization, or species description where appropriate, of each of these species is below.

Species of *Tetragonocephalum* in *Pateobatis fai*

Pateobatis fai was found to host four species of *Tetragonocephalum*: *Tetragonocephalum* sp. 1, *Tetragonocephalum* sp. 2, *Tetragonocephalum* n. sp. 4, and *Tetragonocephalum* sp. 3. *Tetragonocephalum* n. sp. 4 is the only species included in the phylogenetic analysis; *Tetragonocephalum* n. sp. 4 is formally described.

***Tetragonocephalum* sp. 1** (Figs. 3, 5a and e)

Recognition of *Tetragonocephalum* sp. 1 is based on three specimens from a single host specimen collected in the Arafura Sea off the Northern Australian coast (NT-33). This cestode species is 28.2–64.6 mm (50.5 ± 19.5; 3) in total length, with a terminal gravid proglottid 2,743–4,904 (4,008 ± 1,127; 3) long and 646–903 (759 ± 131; 3) wide. *Tetragonocephalum* sp. 1 possesses a scolex that is 421 (n=1) long and 480–572 (528 ± 46; 3) wide, bearing acetabula 79–86 (83 ± 2; 3; 6) long and 76–87 (82 ± 5; 3; 6) wide.

***Tetragonocephalum* sp. 2** (Figs. 3, 5b and f, and 6)

Tetragonocephalum sp. 2 is known from seven whole mounts from a specimen of *Pateobatis fai* collected in the Arafura Sea off the Northern Australian coast (NT-33). This species is 6.3–21.7 mm (12.6 ± 6.1; 7) in total length, with a terminal gravid proglottid 1,782–3,373 (2,648 ± 567; 7) long and 240–431 (328 ± 62; 7) wide. *Tetragonocephalum* sp. 2 possesses a scolex that is 567–624 (596 ± 40; 2) long and 458–749 (626 ± 98; 7) wide, bearing acetabula 130–201 (159 ± 21; 7; 14) long and 143–219 (169 ± 20; 7; 14) wide. Surface of the scolex proper is covered with acicular filitriches.

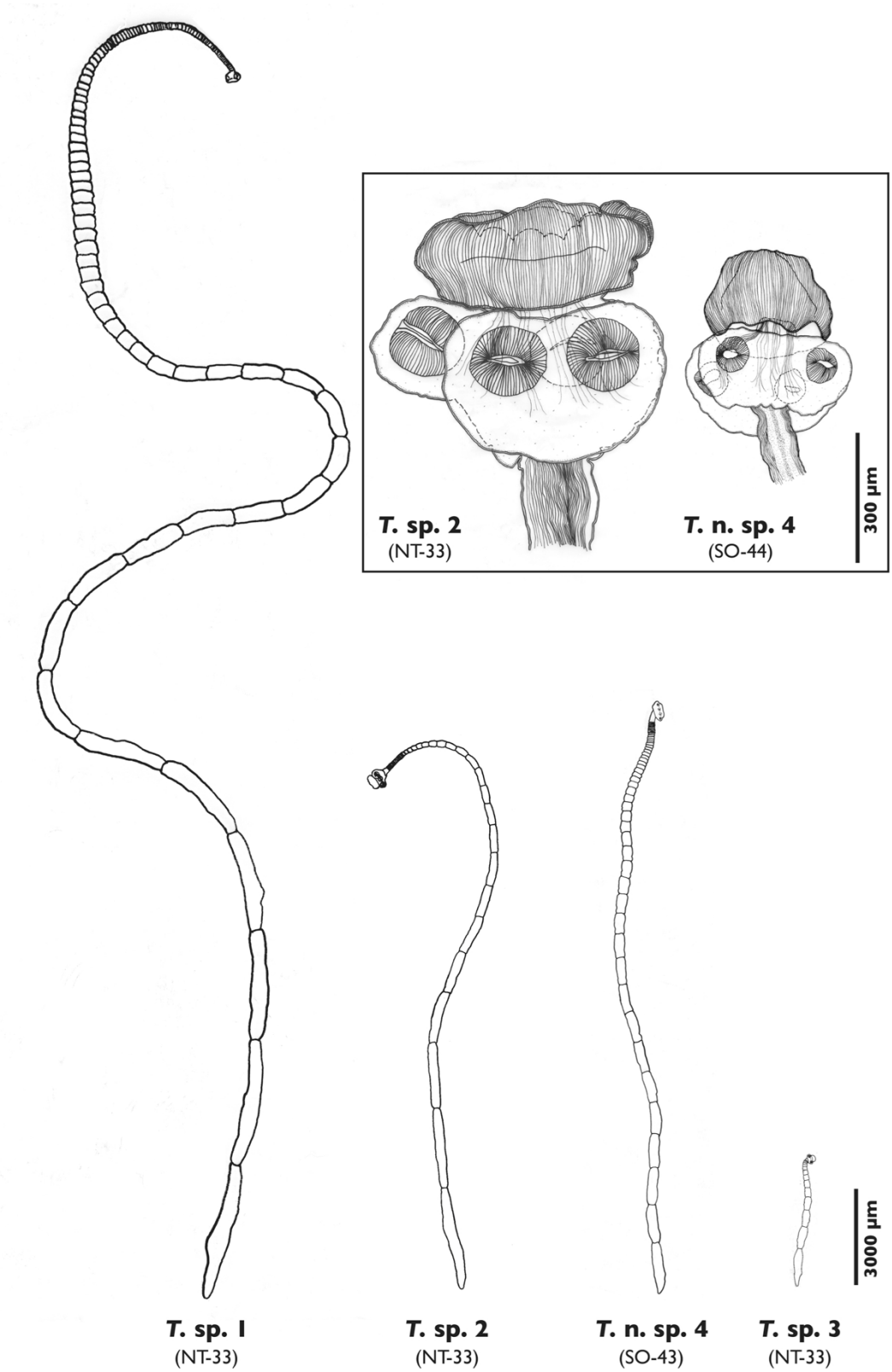

Figure 3. Line drawings of species of *Tetragonocephalum* from *Pateobatis fai*. Species designations and unique host codes given. Whole worms and scoleces drawn to scale.

***Tetragonocephalum* n. sp. 4** (Figs. 3, 4, 5c and g, and 7)

(Synonym; *Tetragonocephalum* n. sp. 2 of Jensen et al. [2016])

Description (based on 10 specimens: 7 complete gravid worms and 3 specimens prepared for SEM and their whole-mounted vouchers): Worms 14.7–26.4 mm (18.7 ± 4.2; 7) long, apolytic, maximum width at level at scolex; proglottids 68–106 (85 ± 15.2; 7) in total number, acraspedote. Scolex 162–355 (234 ± 105.5; 3) long by 334–543 (425 ± 76.3; 7) wide, consisting of scolex proper, apical modification of scolex proper, and apical organ, widest at level of scolex proper. Scolex proper 65–171 (134 ± 59.6; 3) long by 334–544 (430 ± 74.4; 7) wide, bearing four acetabula. Acetabula sucker-like in form, 50–81 (64 ± 10.1; 7) long by 60–80 (69 ± 7.6; 7) wide. Apical modification of scolex proper cylindrical, narrower than scolex proper, bearing apical organ. Apical organ large, globular in form, 102–190 (137 ± 46.8; 3) long by 192–350 (296 ± 51.4; 7) wide, muscular, non-invaginable, non-retractable, with glandular surface. Cephalic peduncle absent.

Surface of apical organ covered with narrowly gladiate spinitriches. Surface of scolex proper and acetabula covered with acicular to capilliform filitriches (Fig. 7).

Immature proglottids generally wider than long, 49–75 (62 ± 10.1; 7) in number, posterior-most immature proglottid 157–275 (210 ± 46.5; 7) long by 161–270 (223 ± 32.1; 7) wide. Mature proglottids 4–7 (5 ± 1; 7) in number, posterior-most mature proglottid 345–448 (399 ± 37.6; 7) long by 204–297 (235 ± 32.8; 7) wide. Worm length to first gravid proglottid 3,601–7,520 (5,435 ± 1,452; 7). Gravid proglottids 11–30 (18 ± 6; 7) in number, first gravid proglottid 336–501 (401 ± 62.9; 7) long by 212–267 (243 ± 17.6; 7) wide, terminal gravid proglottid 1,277–2,150 (1,567 ± 316; 7) long by 232–364 (305 ± 48; 7) wide. Testes 15–20 (17 ±

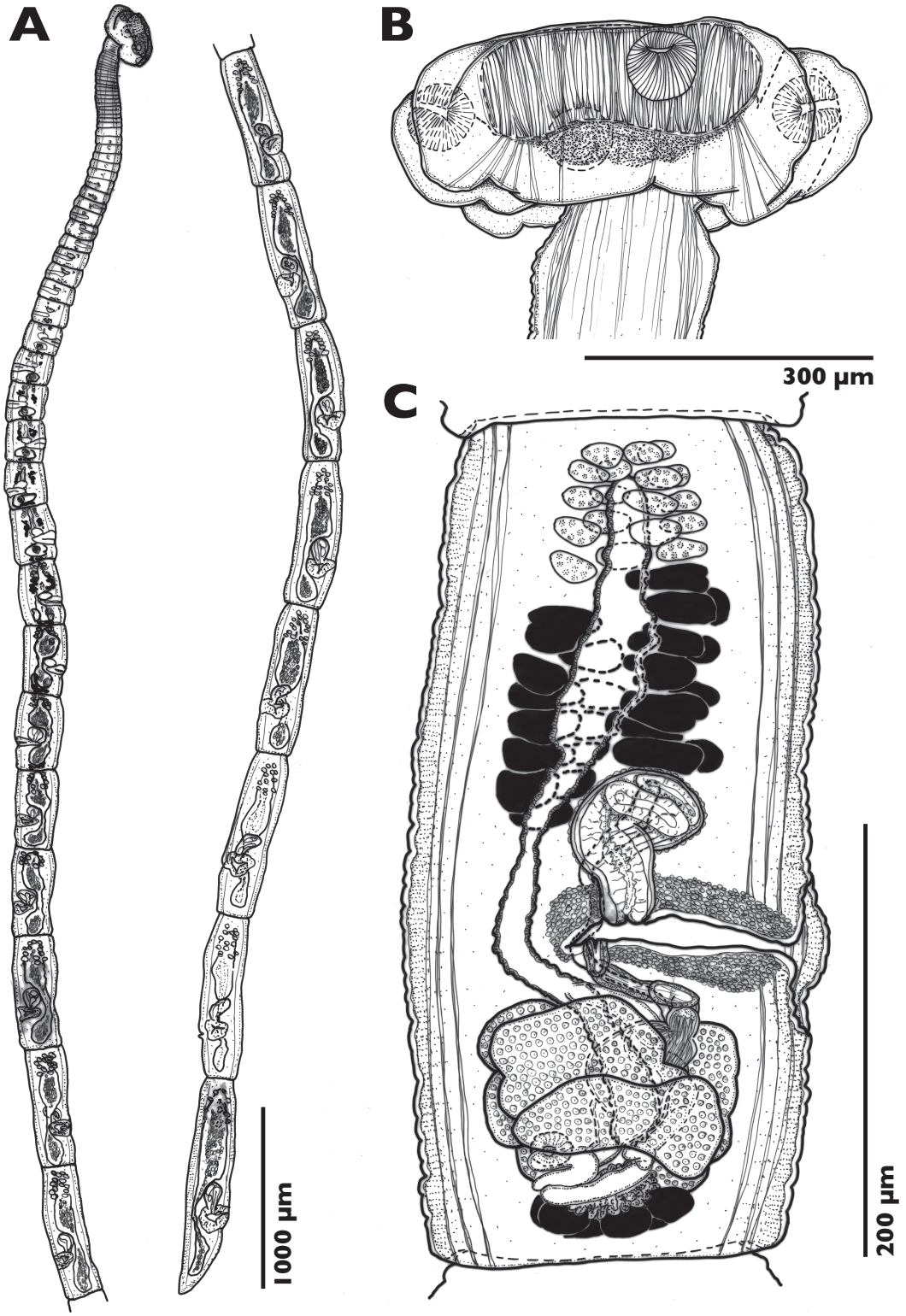

Figure 4. Line drawings of *Tetragonocephalum* n. sp. 4 from *Pateobatis fai*. Drawn by K. Jensen. (A) Whole worm from SO-43. (B) Scolex from SO-43. (C) Subterminal mature proglottid from SO-20.

1.3; 7; 21) in number, 9–23 (15 ± 3.9; 7; 21) long by 18–38 (28 ± 5.3; 7 ; 21) wide, in field extending from anterior margin of proglottid to anterior margin of anterior-most vitellarian field, arranged in two irregular columns in dorso-ventral view, two layers deep in cross-section. Vas deferens extending from region anterior to ovary to enter cirrus sac at anterior margin; external seminal vesicle absent. Internal seminal vesicle absent. Cirrus sac shallowly deltoid (sensu Clopton, 2004), oriented anteriorly, 68–98 (79 ± 10.8; 7) long by 42–66 (50 ± 7.6; 7) wide, containing coiled cirrus. Cirrus armed. Genital pores lateral, irregularly alternating, 35–42% (37 ± 2.2; 7) of proglottid length from posterior end. Genital atrium extensive, extending past midline of proglottid. Ovary very broadly dolioform (sensu Clopton, 2004), C-shaped in cross-section, 68–125 (100 ± 22.1; 7) long by 130–166 (150 ± 13.1; 7) wide; ovicapt in posterior third of proglottid; Mehlis' gland at posterior margin of ovary. Vagina thick walled, medial, extending from ootype to genital atrium, opening into genital atrium at medial end posterior to cirrus sac; vaginal sphincter absent. Vitellarium follicular; vitelline follicles 10–33 (16 ± 5.7; 19; 6) long by 28–69 (48 ± 11.9; 19; 6) wide, in two regions: arranged in single compact field along lateral margins anterior to genital atrium and in field posterior to ovary. Uterus bisaccate when gravid, constricted at level of genital atrium, medial, extending from near anterior extent of field of testes to near posterior margin of ovary. Excretory ducts in two lateral pairs.

Taxonomic summary

Type and only known host: Pateobatis fai (Jordan and Seale), pink whipray (Myliobatiformes: Dasyatidae).

Type locality: Nusa Alana (8°14'13.4"S, 157°1'53.7"E), Solomon Islands, Solomon Sea, Pacific Ocean.

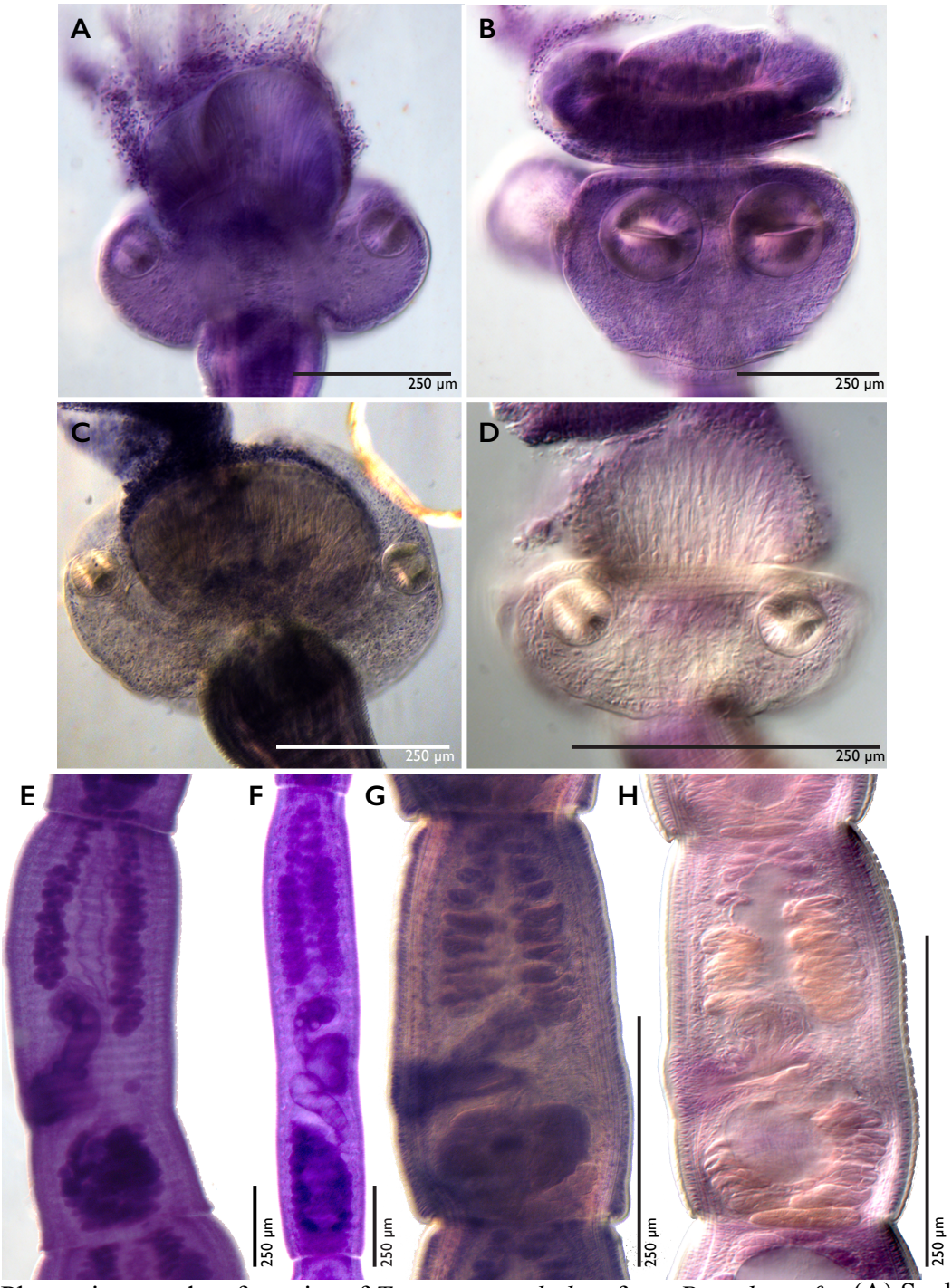

Figure 5. Photomicrographs of species of *Tetragonocephalum* from *Pateobatis fai.* (A) Scolex of *Tetragonocephalum* sp. 1 from NT-33. (B) Scolex of *Tetragonocephalum* sp. 2 from NT-33. (C) Scolex of *Tetragonocephalum* n. sp. 4 from SO-43. (D) Scolex of *Tetragonocephalum* sp. 3 from NT-33. (E) Posterior-most mature proglottid of *Tetragonocephalum* sp. 1 from NT-33. (F) Posterior-most mature proglottid of *Tetragonocephalum* sp. 2 from NT-33. (G) Posterior-most mature proglottid of *Tetragonocephalum* n. sp. 4 from SO-43. (H) Posterior-most mature proglottid of *Tetragonocephalum* sp. 3 from NT-33.

Additional localities: Vonavona (8°13'23.8"S, 157°0'2.4"E), Solomon Islands, Solomon Sea, Pacific Ocean; east of Wessel Islands (11°17'44"S, 136°59'48"E), Australia, Arafura Sea, Pacific Ocean.

Site of infection: Spiral intestine.

Specimen deposited: Holotype (SO-43) and 6 paratypes (NT-33, SO-20, SO-44).

Sequence data: KW26 (SO-20).

Remarks

Tetragonocephalum n. sp. 4 is a member of *Tetragonocephalum* due to its possession of a scolex possessing four acetabulate suckers and an apical organ that is extensive, external, non-retractable, non-invaginable, and muscular with a glandular surface, along with the presence of a bisaccate (rather than saccate) uterus, an ovary that is C-shaped (rather than bilobed or tetralobed) in cross-section, and an expansive genital atrium. Its possession of only two fields of vitelline follicles (i.e., absence of field posterior to genital pore and anterior to ovary) differentiates this species from all valid congeners except *T. opimum* (see Jensen and Guyer, 2021). In addition, *T.* n. sp. 4 possesses fewer testes than *T. kazemii*, *T. passeyi*, *T. sabae*, *T. salarii*, *T. minutum*, and *T. georgei* (15–20 vs. 30–42, 54–73, 42–50, 30–38, 38–63, and 27–45, respectively). This species has a greater number of proglottids than *T. mackenziei*, *T. trygonis*, *T. uarnak*, *T. levicorpum*, and *T. opimum* (68–106 vs. 34–49, approx. 60 [based on fig. 3 of Shipley and Hornell, 1905; pg. 54], 30–40, 15–32, and 34, respectively). Furthermore, *T.* n. sp. 4 is longer than *T. opimum* (14.7–26.5 mm vs. 6.9). *Tetragonocephalum* n. sp. 4 also possesses a scolex that is shorter than *T. simile* (162–355 vs. approx. 463 [based on fig. 38 of Pintner, 1928; pg. 92]). This new species also differs from the *species inquirendum*, *T. yamagutii*, in possessing fewer testes number (15–20 vs. 34–60) and a longer total length (4.7–26.5 mm vs. 20–25 mm).

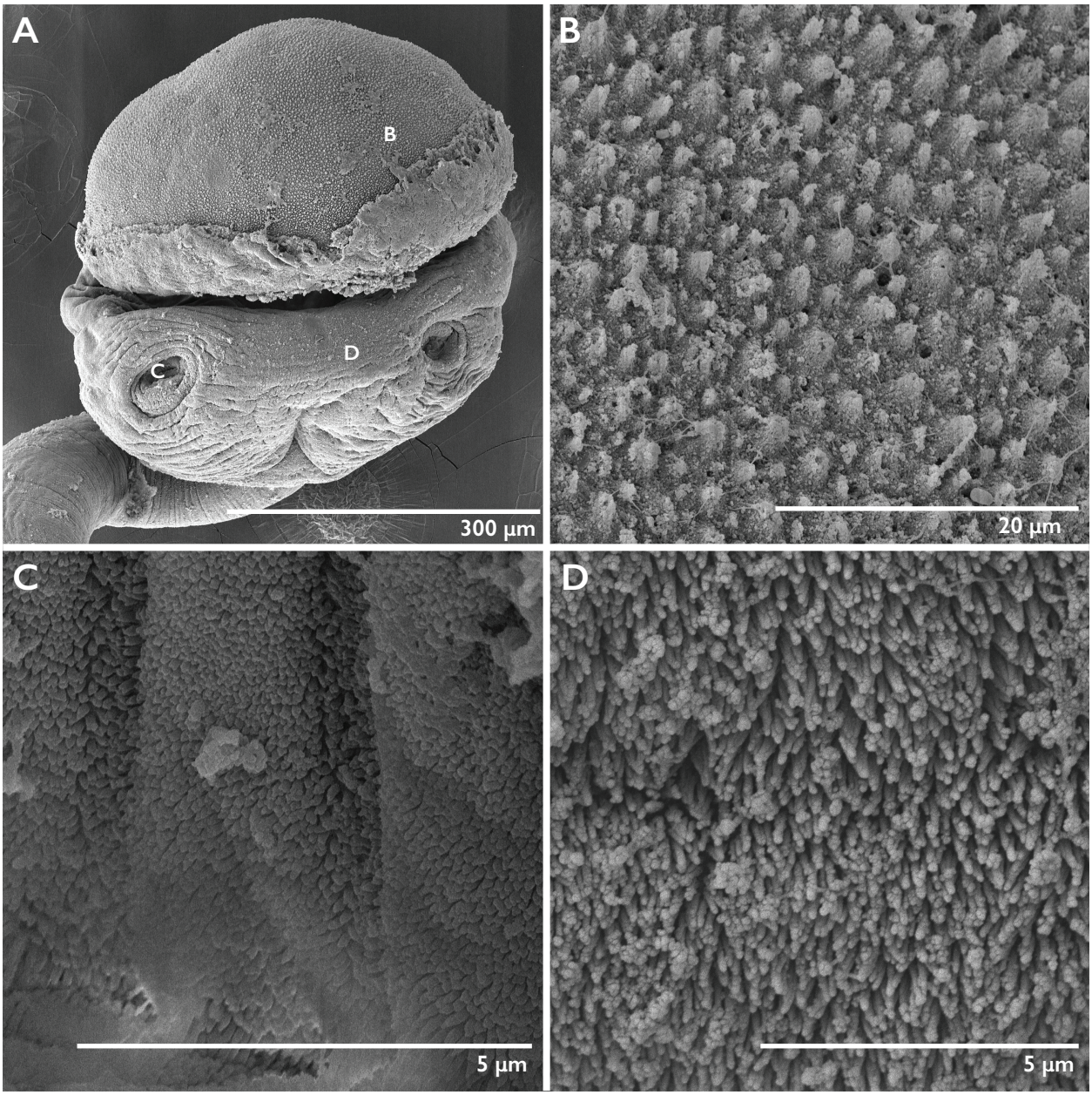

Figure 6. Scanning electron micrographs of *Tetragonocephalum* sp. 2 from *Pateobatis fai* (NT-33). (A) Scolex. (B) Surface of apical organ. (C) Surface of acetabulum. (D) Surface of scolex proper covered with acicular to capilliform filitriches.

***Tetragonocephalum* sp. 3** (Figs. 3, 5d and h)

Tetragonocephalum sp. 3 is known from two whole mounts from a specimen of *Pateobatis fai* collected in the Arafura Sea off the Northern Australian coast (NT-33). This species is 4.2–5.9 mm (5.0 ± 1; 2) in total length, with a terminal gravid proglottid 1,157–1,672

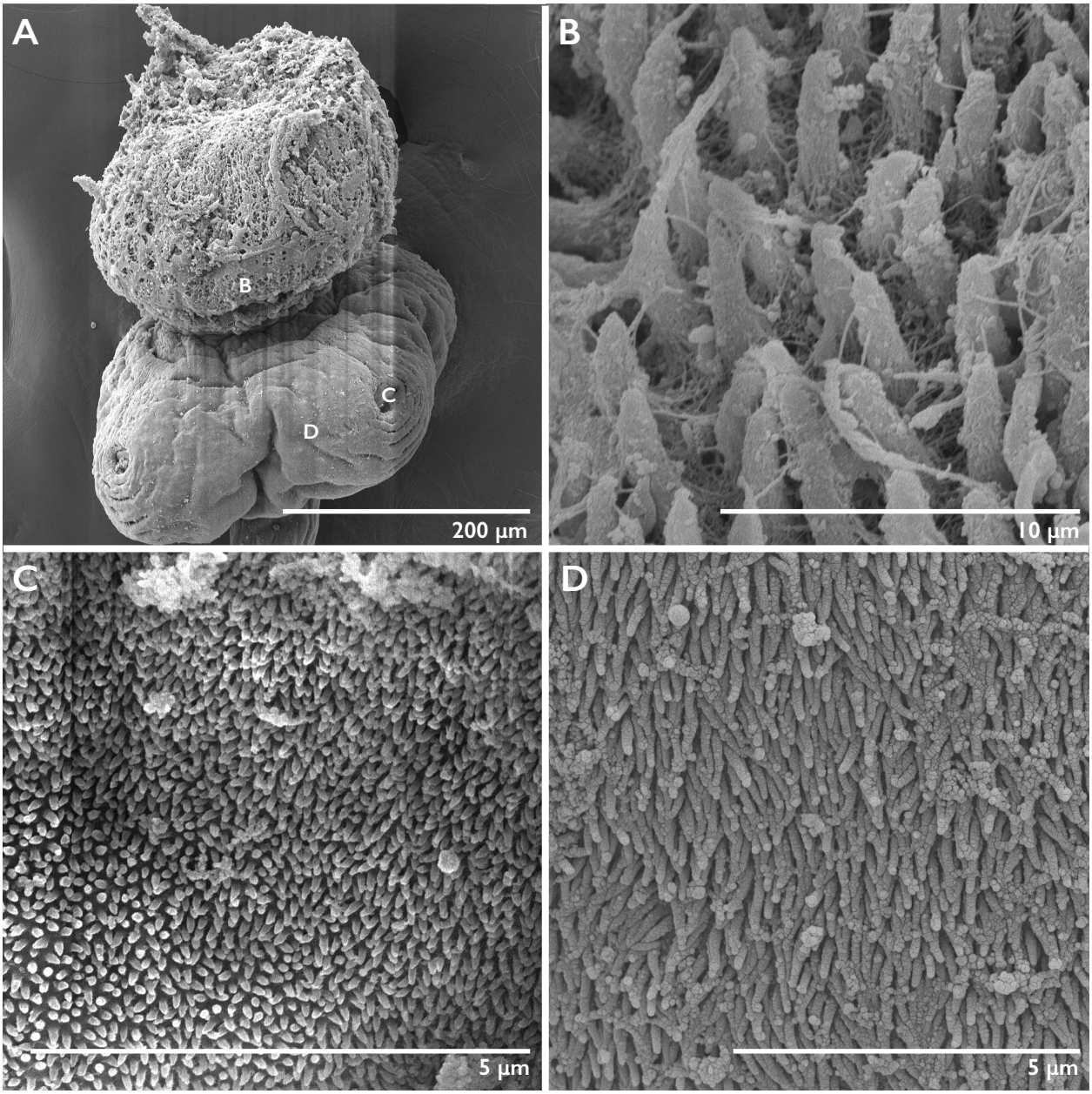

Figure 7. Scanning electron micrographs of *Tetragonocephalum* n. sp. 4 from *Pateobatis fai* (SO-44). (A) Scolex. (B) Surface of apical organ. (C) Surface of acetabulum. (D) Surface of scolex proper covered with acicular to capilliform filitriches.

(1,415 ± 364; 2) long and 225–254 (240 ± 21; 2) wide. *Tetragonocephalum* sp. 3 possesses a scolex that is 239–289 (264 ± 35; 2) long and 318–347 (333 ± 21; 2) wide, bearing acetabula 54–66 (59 ± 5; 2; 4) long and 52–66 (60 ± 7; 2; 4) wide.

Distinguishing species of *Tetragonocephalum* parasitizing *Pateobatis fai*

Of the species of *Tetragonocephalum* that parasitize *P. fai*, *Tetragonocephalum* sp. 1 and *Tetragonocephalum* sp. 3 can be easily distinguished from one another and from *Tetragonocephalum* sp. 2 and *Tetragonocephalum* n. sp. 4 by total length. *Tetragonocephalum* sp. 1 is longer and *Tetragonocephalum* sp. 3 is much shorter than both *Tetragonocephalum* sp. 2 and *Tetragonocephalum* n. sp. 4 (28.2–64.5 mm and 4.2–5.9 mm vs. 14.7–26.5 mm and 6.3–21.7 mm, respectively). *Tetragonocephalum* sp. 2 and *Tetragonocephalum* n. sp. 4, which overlap in their total lengths, can further be distinguished by acetabula size; *Tetragonocephalum* sp. 2 possesses acetabula that are much wider (130–201 vs. 50–81) and longer (143–219 vs. 60–80) than those of *Tetragonocephalum* n. sp. 4.

Species of *Tetragonocephalum* in *Pateobatis jenkinsii*

Pateobatis jenkinsii was found to be parasitized by three species of *Tetragonocephalum*: *Tetragonocephalum* n. sp. 5, *Tetragonocephalum* n. sp. 6, and *Tetragonocephalum* sp. 4. *Tetragonocephalum* n. sp. 5 and *Tetragonocephalum* n. sp. 6 were both included in the phylogenetic analysis; both are formally described below.

***Tetragonocephalum* n. sp. 5** (Figs. 8, 9a and 9e, and 10d)

Description (based on 13 specimens: 12 complete gravid worms; 1 cross-section series of mature proglottids): Worms 7.5–24.9 mm (14.1 ± 5.4; 12) long, apolytic, maximum width at level at scolex; proglottids 51–117 (80 ± 22.4; 12) in total number, acraspedote. Scolex 246–356 (292 ± 32.9; 9) long by 306–519 (386 ± 77.7; 11) wide, consisting of scolex proper, apical modification of scolex proper, and apical organ, widest at level of scolex proper. Scolex proper

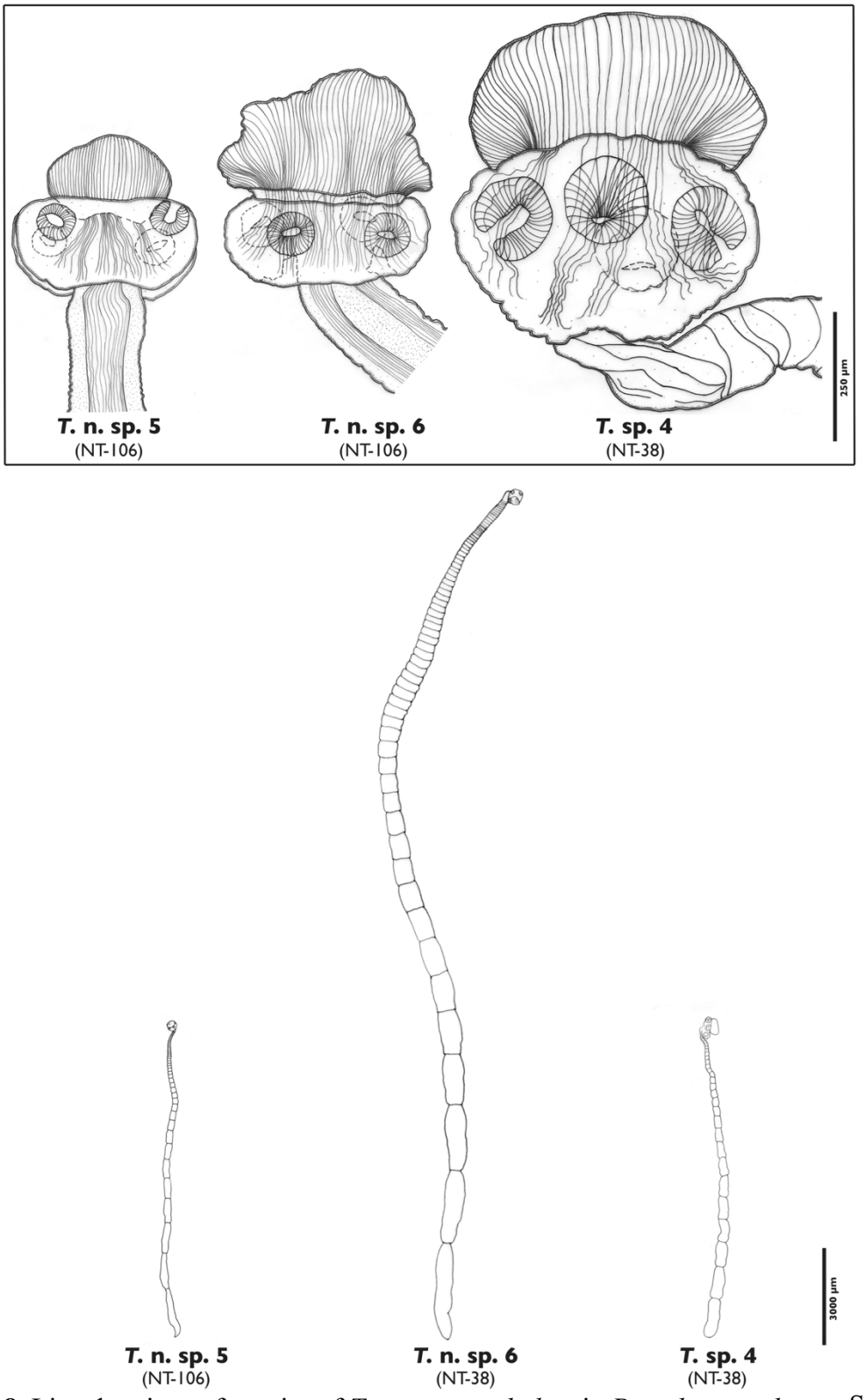

Figure 8. Line drawings of species of *Tetragonocephalum* in *Pateobatis jenkinsii*. Species designations and unique host codes given. Whole worms and scoleces drawn to scale.

138–222 (170 ± 24.5; 9) long by 306–519 (386 ± 77.7; 11) wide, bearing four acetabula. Acetabula sucker-like in form, 54–88 (65 ± 8.5; 12; 19) long by 52–74 (67 ± 6.6; 12; 18) wide. Apical modification of scolex proper cylindrical, narrower than scolex proper, bearing apical organ. Apical organ large, globular in form, 91–183 (146 ± 31.5; 10) long by 193–327 (260 ± 41.6; 12) wide, muscular, non-invaginable, non-retractable, with glandular surface. Cephalic peduncle absent.

Immature proglottids generally wider than long, 39–88 (59 ± 15.6; 12) in number, posterior-most immature proglottid 157–219 (181 ± 18.9; 12) long by 163–335 (241± 55.1; 12) wide. Last mature proglottids 2–8 (4 ± 1.4; 12) in number, posterior-most mature proglottid 178–322 (266 ± 41.3; 12) long by 173–343 (252 ± 47.8; 12) wide. Worm length to first gravid proglottid 3,102–5,340 (4,012 ± 812; 12). Gravid proglottids 7–27 (16 ± 6.8; 12) in number, first gravid proglottid 253–377 (306 ± 37.7; 12) long by 200–352 (267 ± 45.6; 12) wide, terminal gravid proglottid 1,037–1,942 (1,387 ± 235; 12) long by 250–414 (325 ± 53; 12) wide. Testes 13–22 (17 ± 1.9; 12; 36) in number, 6–16 (12 ± 2.4; 12; 26) long by 19–48 (31 ± 7.2; 12 ; 36) wide, extending from anterior margin of proglottid to anterior margin of anterior-most vitellarian field, arranged in two irregular columns in dorso-ventral view, two layers deep in cross-section. Vas deferens extending from region anterior to ovary to enter cirrus sac at anterior margin; external seminal vesicle present. Internal seminal vesicle absent. Cirrus sac very shallowly deltoid (sensu Clopton, 2004), oriented anteriorly, 36–95 (63 ± 18.1; 10) long by 34–94 (53 ± 20.2; 9) wide, containing coiled cirrus. Cirrus armed. Genital pores lateral, irregularly alternating, 33–47% (41 ± 4.5; 12) of proglottid length from posterior end. Genital atrium extensive, extending past midline of proglottid. Ovary very broadly dolioform (sensu Clopton,

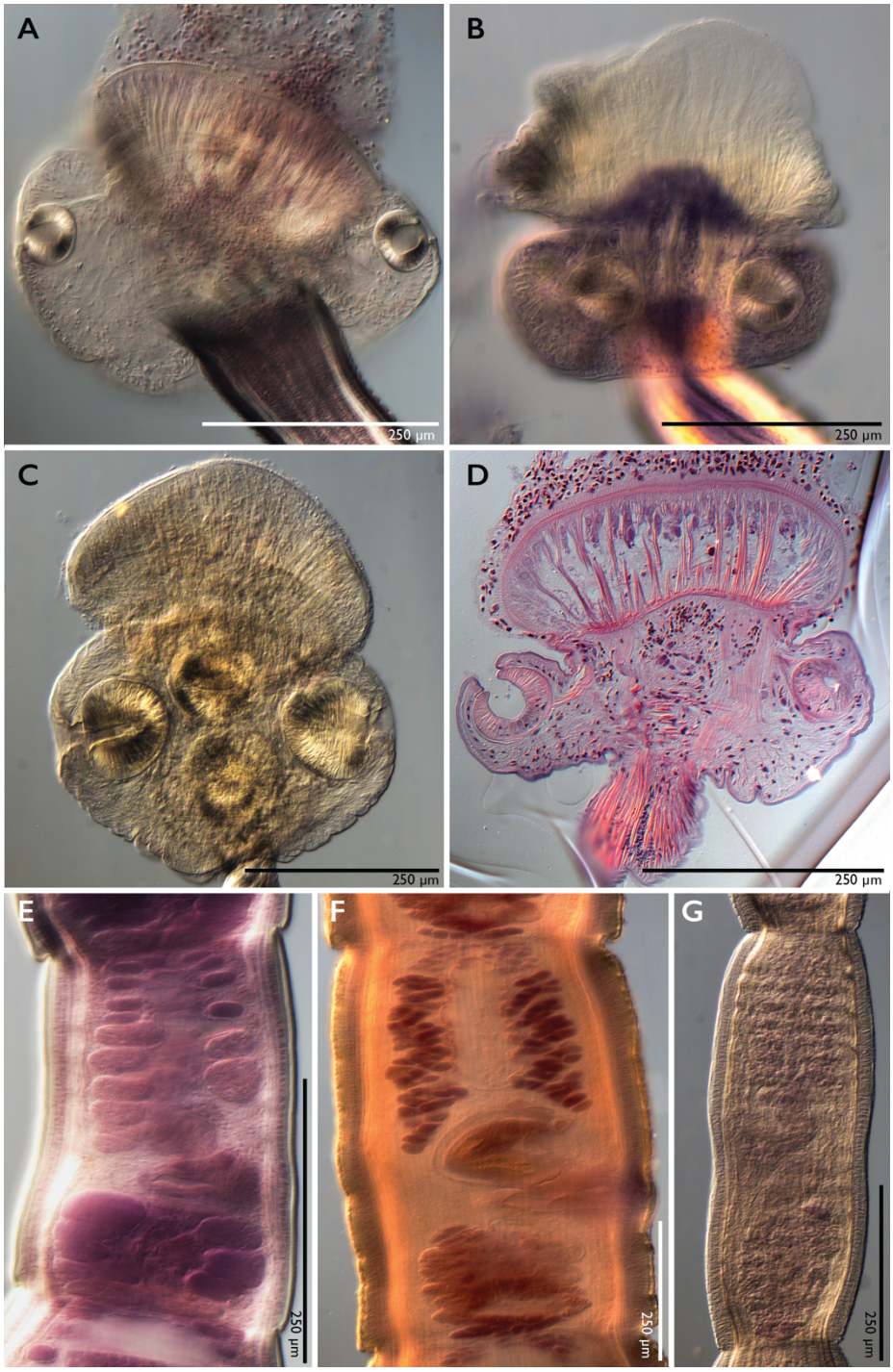

Figure 9. Photomicrographs of species of *Tetragonocephalum* from *Pateobatis jenkinsii.* (A) Scolex of *Tetragonocephalum* n. sp. 5 from NT-106. (B) Scolex of *Tetragonocephalum* n. sp. 6 from NT-106. (C) Scolex of *Tetragonocephalum* sp. 4 from NT-38. (D) Scolex longitudinal-section of *Tetragonocephalum* n. sp. 6 from NT-106. (E) Posterior-most mature proglottid of *Tetragonocephalum* n. sp. 5 from NT-106. (F) Posterior-most mature proglottid of *Tetragonocephalum* n. sp. 6 from NT-38. (G) Posterior-most mature proglottid of *Tetragonocephalum* sp. 4 from NT-38.

2004), C-shaped in cross-section (Fig. 10d), 73–108 (85 ± 9.8; 12) long by 135–220 (171 ± 31.2; 12) wide; ovicapt in posterior third of proglottid; Mehlis' gland at posterior margin of ovary. Vagina thick walled, medial, extending from ootype to genital atrium, opening into genital atrium at medial end posterior to cirrus sac; vaginal sphincter absent. Vitellarium follicular; vitelline follicles 11–44 (20 ± 6.4; 12; 36) long by 36–81 (53 ± 10.3; 12; 36) wide, in three regions: arranged in single compact field along lateral margins anterior to genital atrium, in field between genital pore and ovary, and in field posterior to ovary. Uterus bisaccate when gravid, constricted at level of genital atrium, medial, extending from near posterior margin of ovary to near anterior extent of field of testes. Excretory ducts in two lateral pairs.

Taxonomic summary

Type and only known host: Pateobatis jenkinsii, Jenkins' whipray (Myliobatiformes: Dasyatidae).

Type and only known locality: east of Wessel Islands (11°17'44"S, 136°59'48"E), Australia, Arafura Sea, Pacific Ocean.

Site of infection: Spiral intestine.

Specimen deposited: Holotype (NT-106) and 11 paratypes.

Sequence data: KW16 (NT-106).

Remarks

Tetragonocephalum n. sp. 5 is a member of *Tetragonocephalum* due to its possession of a scolex possessing four acetabulate suckers and an apical organ that is extensive, external, non-retractable, non-invaginable, and muscular with a glandular surface, along with the presence of a bisaccate (rather than saccate) uterus, an ovary that is C-shaped (rather than bilobed or

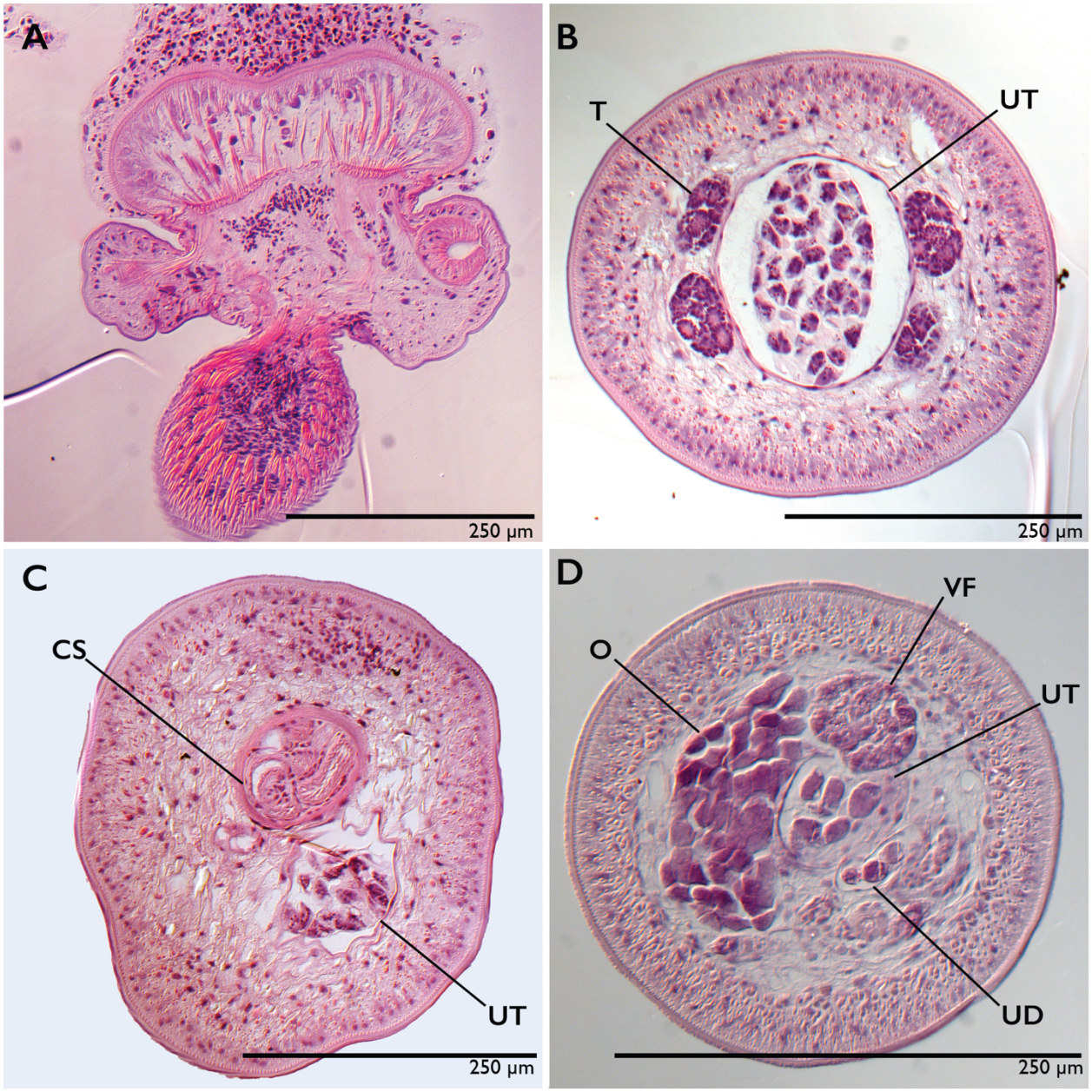

Figure 10. Photomicrographs of sections of *Tetragonocephalum* from *Pateobatis jenkinsii.* (A) Longitudinal-section of scolex of *Tetragonocephalum* n. sp. 6 from BO-339. (B) Cross-section of *Tetragonocephalum* n. sp. 6 from BO-339. showing two columns of testes in two rows. (C) Cross-section at level of cirrus sac of *Tetragonocephalum* n. sp. 6 from BO-339. (D) Cross-section of *Tetragonocephalum* n. sp. 5 from NT-106 at level of ovary. *Abbreviations:* T, testis; UT, uterus; CS, cirrus sac; O, ovary; VF, vitelline follicle; UD, uterine duct.

tetralobed) in cross-section, and an expansive genital atrium. *Tetragonocephalum* n. sp. 5 possesses fewer testes than *T. kazemii*, *T. passeyi*, *T. sabae*, *T. salarii*, *T. minutum*, and *T. georgei* (13–22 vs. 30–42, 54–73, 42–50, 30–38, 38–63, and 27–45, respectively). This new species has a greater number of proglottids than *T. mackenziei*, *T. passeyi*, *T. uarnak*, *T. minutum*, *T. georgei*, *T. levicorpum*, and *T. opimum* (51–117 vs. 34–49, 18–33, 30–40, 11–20, 22–39, 15–32, and 34, respectively). Furthermore, *T.* n. sp. 5 is shorter than *T. kazemii* (7.5–24.9 mm vs. 28.8–36.6) and longer than *T. opimum* (7.5–24.9 mm vs. 6.9). *Tetragonocephalum* n. sp. 5 also possesses a shorter scolex than *T. sabae*, *T. salarii*, *T. simile*, *T. uarnak*, and *T. levicorpum* (246–356 vs. 401–453, 453–501, approx. 463 [based on fig. 38 of Pintner, 1928; pg. 92], approx. 786 [based on fig. 86 of Shipley and Hornell, 1906; pg. 101], and 542–752, respectively).

Tetragonocephalum n. sp. 5 possesses acetabula that are longer than those of *T. trygonis* (58–88 vs. approx. 16 [based on fig. 3 of Shipley and Hornell, 1905; pg. 54]). Also, *T.* n. sp. 5 possesses acetabula that are more narrow than those of *T. simile* (52–74 vs. approx. 77 [based on fig. 38 of Pintner, 1928; pg. 92]) and wider than those of *T. trygonis* (52–74 vs. approx. 23 [based on fig. 3 of Shipley and Hornell, 1905; pg. 54]). *Tetragonocephalum* n. sp. 5 also possess mature proglottids that are shorter than those of *T. mackenziei* and *T.* n. sp. 4 (178–322 vs. 337–516 and 345–448). *Tetragonocephalum* n. sp. 5 differs from *T.* n. sp. 4 in vitelline field number (3 vs. 2). *Tetragonocephalum* n. sp. 5 differs from the *species inquirendum*, *T. yamagutii*, in possessing fewer testes (13–22 vs. 54–56) and a greater number of proglottids (51–117 vs. 20–25).

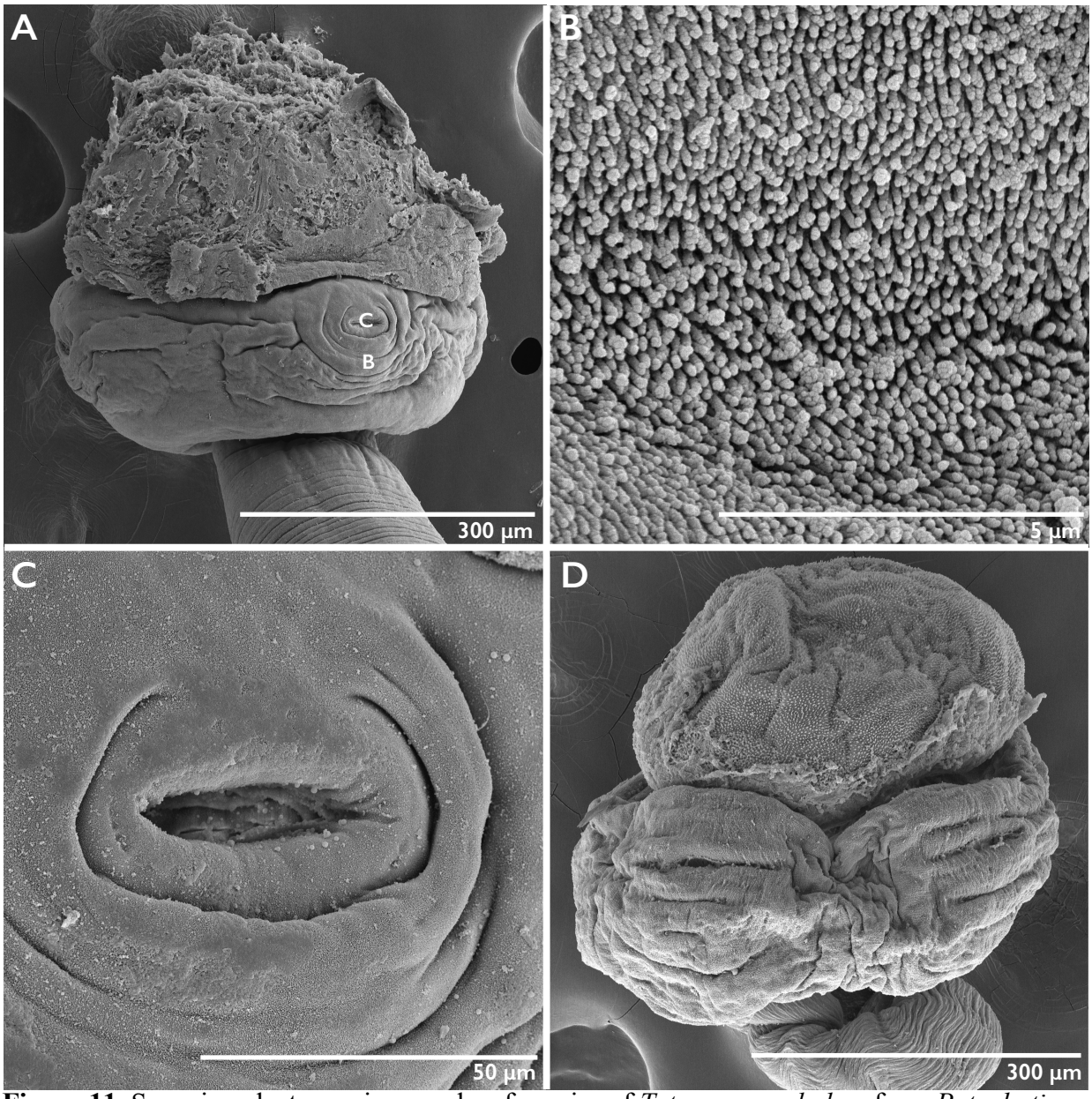

Figure 11. Scanning electron micrographs of species of *Tetragonocephalum* from *Pateobatis jenkinsii*.(A) Scolex of *Tetragonocephalum* n. sp. 6 from NT-106. (B) Surface of scolex proper of *Tetragonocephalum* n. sp. 6 from NT-106 covered with acicular to capilliform filitriches. (C) Close-up of acetabulum of *Tetragonocephalum* n. sp. 6 from NT-106. (D) Scolex of *Tetragonocephalum* sp. 4 from NT-38.

***Tetragonocephalum* n. sp. 6** (Figs. 8, 9b, 9d, 9f, 10a–c, and 11a–c)

Description (based on 12 specimens: 8 complete gravid worms; 2 incomplete gravid worms; and 2 specimens prepared for SEM and their whole-mounted vouchers): Worms 18.1–35.2 mm (29.1 ± 5.8; 8) long, apolytic, maximum width at level at scolex; proglottids 61–102 (81 ± 14.5; 8) in

total number, acraspedote. Scolex 366–468 (405 ± 34.6; 7) long by 347–564 (457 ± 69.2; 10) wide, consisting of scolex proper, apical modification of scolex proper, and apical organ, widest at level of scolex proper. Scolex proper 172–258 (223 ± 26.3; 7) long by 347–564 (458 ± 68.1; 10) wide, bearing four acetabula. Acetabula sucker-like in form, 74–101 (84 ± 7.3; 10; 18) long by 74–103 (89 ± 8.5; 10; 19) wide. Apical modification of scolex proper cylindrical, narrower than scolex proper, bearing apical organ. Apical organ large, globular in form, 184–218 (200 ± 14.9; 6) long by 232–422 (351 ± 68.6; 9) wide, muscular, non-invaginable, non-retractable, with glandular surface. Cephalic peduncle absent.

Surface of scolex proper covered with acicular to papilliform filitriches. Surface of apical organ and acetabula not observed (Fig. 11a–c).

Immature proglottids initially wider than long, 48–87 (63 ± 12.8; 10) in number, posterior-most immature proglottid 435–784 (550 ± 97.2; 11) long by 304–590 (437± 102.4; 11) wide. Last mature proglottids 4–8 (5 ± 1.3; 11) in number, posterior-most mature proglottid 632–848 (716 ± 60.4; 10) long by 386–565 (494 ± 62.7; 10) wide. Worm length to first gravid proglottid 8,670–13,410 (11,388 ± 1,604; 10). Gravid proglottids 9–17 (13 ± 2.9; 9) in number, first gravid proglottid 658–876 (750 ± 80.3; 11) long by 365–614 (493 ± 74.8; 11) wide, terminal gravid proglottid 1,716–29,70 (2,455 ± 434.8; 9) long by 410–603 (523 ± 59.5; 9) wide. Testes 37–58 (50 ± 5.1; 9; 23) in number, 10–34 (20 ± 6.4; 9; 27) long by 27–80 (53 ± 13.5; 9 ; 27) wide, extending from anterior margin of proglottid to well into anterior-most vitellarian field, arranged in two irregular columns in dorso-ventral view, two layers deep in cross-section (Fig. 10b). Vas deferens extending from region anterior to ovary to enter cirrus sac at proximal

margin; external seminal vesicle absent. Internal seminal vesicle absent. Cirrus sac very shallowly deltoid (sensu Clopton, 2004), oriented anteriorly, 64–169 (104 ± 35.9; 10) long by 155–264 (211 ± 41.2; 10) wide, containing coiled cirrus (Fig. 10c). Cirrus armed. Genital pores lateral, irregularly alternating, 30–47% (40 ± 4.5; 11) of proglottid length from posterior end. Genital atrium extensive, extending past midline of proglottid. Ovary shallowly dolioform (sensu Clopton, 2004), C-shaped in cross-section, 130–254 (174 ± 34.7; 11) long by 221–330 (279 ± 34.1; 11) wide; ovicapt in posterior third of proglottid; Mehlis' gland at posterior margin of ovary. Vagina thick walled, medial, extending from ootype to genital atrium, opening into genital atrium at medial end posterior to cirrus sac; vaginal sphincter absent. Vitellarium follicular; vitelline follicles 15–59 (31 ± 11.1; 7; 21) long by 45–99 (71 ± 17.7; 7; 21) wide, in three regions: arranged in single compact field along lateral margins anterior to genital atrium, in field between genital pore and ovary, and in field posterior to ovary. Uterus bisaccate when gravid, constricted at level of genital atrium, medial, extending from near posterior margin of ovary to near anterior extent of field of testes. Excretory ducts in two lateral pairs.

Taxonomic summary

Type and only known host: Pateobatis jenkinsii, Jenkins' whipray (Myliobatiformes: Dasyatidae).

Type and only known locality: east of Wessel Islands (11°17'44"S, 136°59'48"E), Australia, Arafura Sea, Pacific Ocean.

Site of infection: Spiral intestine.

Specimen deposited: Holotype (NT-38) and 9 paratypes (NT-38 and NT-106).

Sequence data: KW15 (NT-106).

Remarks

Tetragonocephalum n. sp. 6 is a member of *Tetragonocephalum* due to its possession of a scolex possessing four acetabulate suckers and an apical organ that is extensive, external, non-retractable, non-invaginable, and muscular with a glandular surface, along with the presence of a bisaccate (rather than saccate) uterus, an ovary that is C-shaped (rather than bilobed or tetralobed) in cross-section, and an expansive genital atrium. *Tetragonocephalum* n. sp. 6 has a greater number of proglottids than *T. mackenziei*, *T. passeyi*, *T. sabae*, *T. uarnak*, *T. minutum*, *T. georgei*, *T. levicorpum*, and *T. opimum* (61–102 vs. 34–49, 18–33, 42–53, 30–40, 11–20, 22–39, 15–32, and 34, respectively). This species possesses terminal gravid proglottids that are longer than those of *T. salarii*, *T. simile*, and *T. opimum* (1,716–2,970 vs. 1,036–1,482, approx. 1,350 [fig. 56 of Pintner, 1928; pg. 102], and 1,029, respectively), and shorter those of than *T. uarnak* and *T. minutum* (1,716–2,970 vs. 5,000 and 4,500). Furthermore, *T.* n. sp. 6 possesses a longer scolex than does *T. mackenziei*, *T. trygonis*, *T.* n. sp. 4 from *Pateobatis fai* and *T.* n. sp. 5 from *Pateobatis jenkinsii* (366–468 vs. 228–315, approx. 353 [based on fig. 3 of Shipley and Hornell, 1905; pg. 54], 162–355, and 246–356) and a shorter scolex than *T. levicorpum* (366–468 vs. 542–752). This new species also possesses more immature proglottids than *T. passeyi* and *T. georgei* (48–87 vs. 12–25 and 20–38). *Tetragonocephalum* n. sp. 6 possesses a greater number of testes than *T.* n. sp. from *Pateobatis fai* and *T.* n. sp. 5 from *Pateobatis jenkinsii* (37–58 vs. 15–20 and 13–22). *Tetragonocephalum* n. sp. 6 possesses acetabula that are greater in length than *T. simile* and *T. trygonis* (74–101 vs. approx. 67 [based on fig. 38 of Pintner, 1928; pg. 92] and 16 [based on fig. 3 of Shipley and Hornell, 1905; pg. 54]). *Tetragonocephalum* n. sp. 6 is also possesses fewer gravid proglottids than *T. salarii* (7–17 vs. 18–20). *Tetragonocephalum* n. sp. 6 posesses immature proglottids that are wider than *T. kazemii* (304–590 vs. 166–219) and mature

proglottids that are wider than *T. kazemii* and *T. sabae* (386–565 vs. 187–256 and 226–336). This new species differs from the *species inquirendum*, *T. yamagutii*, in possessing a greater number of proglottids (61–102 vs. 20–25) and a longer total length (18–35 mm vs. 6.05–7 mm).

***Tetragonocephalum* sp. 4**

Tetragonocephalum sp. 4 is known from nine whole mounts from one specimen of *Pateobatis jenkinsii* collected in the Arafura Sea off the Northern Australian coast (NT-38). This species is 3.6–11.8 mm (7.9 ± 2.7; 9) in total length, with a terminal gravid proglottid 522–1,769 (1,258 ± 400; 8) long and 285–411 (368 ± 40; 8) wide. *Tetragonocephalum* sp. 4 possesses a scolex that is 510–673 (602 ± 64; 7) long and 443–700 (579 ± 87; 9) wide, bearing acetabula 111–180 (156 ± 18; 9; 18) long and 121–187 (161 ± 18; 9; 18) wide.

Distinguishing species of *Tetragonocephalum* parasitizing *Pateobatis jenkinsii*

The species of *Tetragonocephalum* known to parasitize *P. jenkinsii* can be distinguished from each other based on total length, scolex length, and size of acetabula. *Tetragonocephalum* n. sp. 6, with a total length of 18.1–35.2 mm, is much longer than *Tetragonocephalum* sp. 4 at 3.6–11.8 mm. *Tetragonocephalum* n. sp. 6 can be differentiated from *Tetragonocephalum* n. sp. 5 based on scolex length (366–468 vs. 246–356). *Tetragonocephalum* n. sp. 5 and *Tetragonocephalum* sp. 4 can be distinguished based on acetabula size, with *Tetragonocephalum* n. sp. 5 possessing acetabula that are narrower in length (54–88 vs. 111–180) and width (52–74 vs. 121–187) than *Tetragonocephalum* sp. 4.

Species of *Tetragonocephalum* in *Pateobatis uarnacoides*

Pateobatis uarnacoides was found to host two species of *Tetragonocephalum*: *Tetragonocephalum* n. sp. 7 and *Tetragonocephalum* sp. 5. The former species was included in the phylogenetic analysis and is formally described below.

***Tetragonocephalum* n. sp. 7** (Figs. 12, 13a and c)

Description (based on 6 specimens: 5 complete mature gravid worms; 1 incomplete gravid worm): Worms 6.7–14.3 mm (9.4 ± 3.1; 5) long, apolytic, maximum width at level at scolex; proglottids 26–51 (37 ± 9.7; 5) in total number, acraspedote. Scolex 285–470 (354 ± 73.2; 5) long by 330–339 (366 ± 25.2; 6) wide, consisting of scolex proper, apical modification of scolex proper, and apical organ, widest at level of scolex proper. Scolex proper 153–198 (172 ± 16.8; 5) long by 330–399 (366 ± 25.2; 6) wide, bearing four acetabula. Acetabula sucker-like in form, 59–76 (69 ± 5.9; 6; 12) long by 63–79 (72 ± 4.9; 6; 12) wide. Apical modification of scolex proper cylindrical, narrower than scolex proper, bearing apical organ. Apical organ large, globular in form, 105–277 (179 ± 65.9; 5) long by 240–332 (297 ± 34.5; 6) wide, muscular, non-invaginable, non-retractable, with glandular surface. Cephalic peduncle absent.

Immature proglottids initially wider than long, 22–43 (33 ± 6.9; 6) in number, posterior-most immature proglottid 217–652 (453 ± 142.2; 6) long by 179–233 (211± 20.3; 6) wide. Last mature proglottids 2–4 (3 ± 0.8; 6) in number, posterior-most mature proglottid 549–1065 (808 ± 175.5; 6) long by 208–292 (248 ± 31.9; 6) wide. Gravid proglottids 2–5 (3 ± 1.2; 5) in number, 683–1142 (942 ± 166.8; 6) long by 240–378 (291 ± 50.4; 6) wide, worm length to

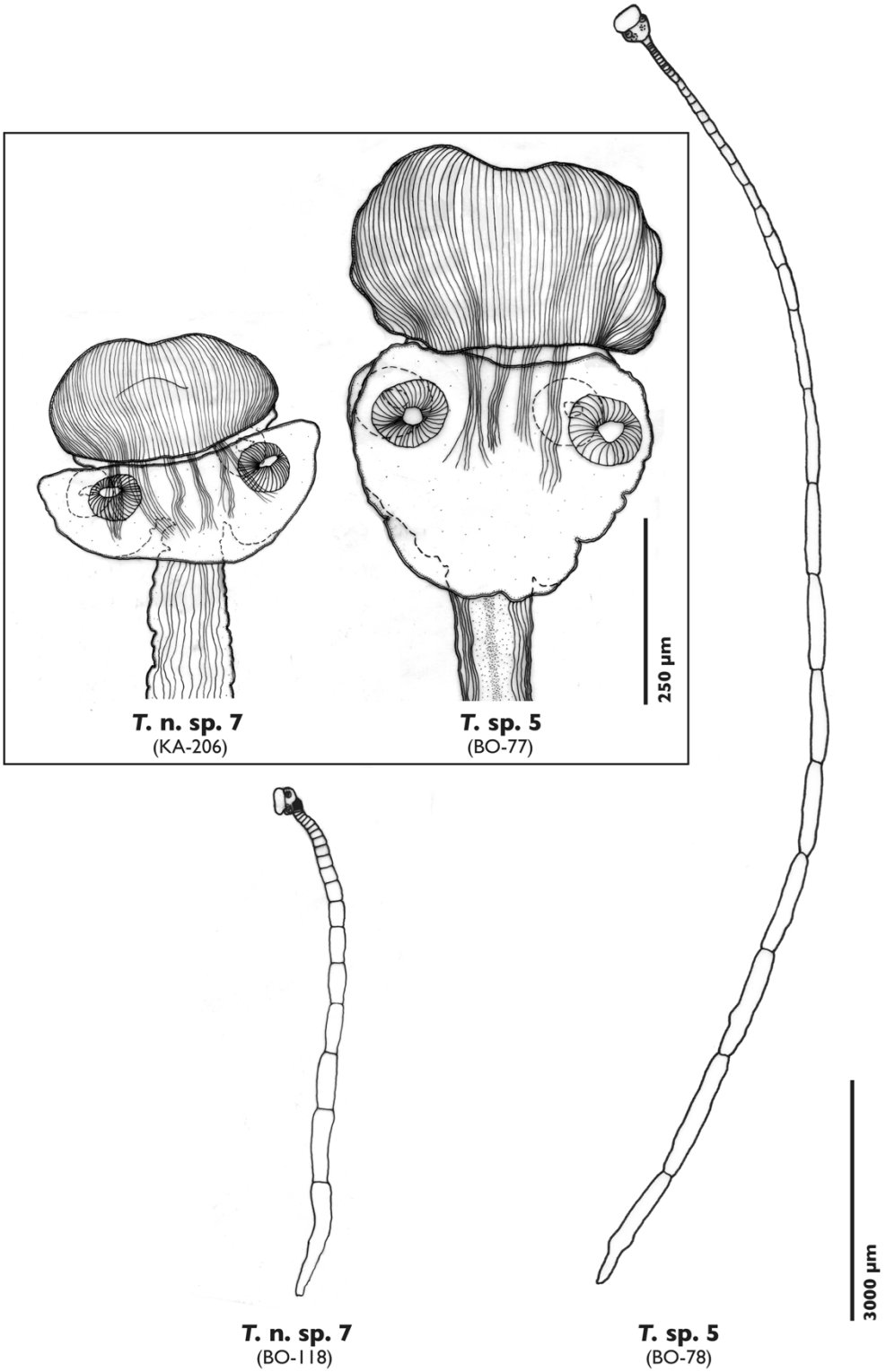

Figure 12. Line drawings of species of *Tetragonocephalum* in *Pateobatis uarnacoides*. Species designations and unique host codes given. Whole worms and scoleces drawn to scale.

first gravid proglottid 4,130–7,460 (5,490 ± 1300; 6), terminal gravid proglottid 1,146–1,630 (1,451 ± 187.9; 5) long by 255–369 (300 ± 53.9; 5) wide. Testes 31–47 (38 ± 6.4; 6; 12) in number, 7–25 (16 ± 4.7; 6; 18) long by 24–61 (43 ± 9.4; 6; 18) wide, extending from anterior margin of proglottid to well into anterior-most vitellarian field, arranged in two irregular columns in dorso-ventral view, two layers deep in cross-section. Vas deferens extending from region anterior to ovary to enter cirrus sac at proximal margin; external seminal vesicle absent. Internal seminal vesicle absent. Cirrus sac very shallowly ovoid (sensu Clopton, 2004), oriented anteriorly, 82–131 (95 ± 18.3; 6) long by 113–144 (132 ± 12.6; 6) wide, containing coiled cirrus. Cirrus armed. Genital pores lateral, irregularly alternating, 38–43% (41 ± 1.8; 6) of proglottid length from posterior end. Genital atrium extensive, extending past midline of proglottid. Ovary broadly dolioform (sensu Clopton, 2004), C-shaped in cross-section, 164–291 (199 ± 46.4; 6) long by 122–184 (163 ± 23.6; 6) wide; ovicapt in posterior third of proglottid; Mehlis' gland at posterior margin of ovary. Vagina thick walled, medial, extending from ootype to genital atrium, opening into genital atrium at medial end posterior to cirrus sac; vaginal sphincter absent. Vitellarium follicular; vitelline follicles 12–28 (18 ± 4.9; 4; 12) long by 38–50 (44 ± 3.5; 4; 12) wide, in three regions: arranged in single compact field along lateral margins anterior to genital atrium, in field between genital pore and ovary, and in field posterior to ovary. Uterus bisaccate when gravid, constricted at level of genital atrium, medial, extending from near posterior margin of ovary to near anterior extent of field of testes. Excretory ducts in two lateral pairs.

Taxonomic summary

Type and only known host: Pateobatis uarnacoides (Bleeker), whitenose whipray (Myliobatiformes: Dasyatidae).

Type locality: Kampunng Tetabuan (06°01'10.32"N, 117°42'14.76"E), Malaysian Borneo, Sulu Sea, Pacific Ocean.

Additional localities: Pagarantimum (02°14'13.36"S, 110°05'48.95"E) and Sidu (01°21'45.20"S, 110°04'10.30"E), Indonesian Borneo, Java Sea, Pacific Ocean.

Site of infection: Spiral intestine.

Specimen deposited: Holotype (BO-118) and 5 paratypes (BO-118, KA-206, and KA-210).

Sequence data: KW484 and KW485 (KA-81).

Remarks

Tetragonocephalum n. sp. 7 is a member of *Tetragonocephalum* due to its possession of a scolex possessing four acetabulate suckers and an apical organ that is extensive, external, non-retractable, non-invaginable, and muscular with a glandular surface, along with the presence of a bisaccate (rather than saccate) uterus, an ovary that is C-shaped (rather than bilobed or tetralobed) in cross-section, and an expansive genital atrium. *Tetragonocephalum* n. sp. 7 is shorter in total length than *T. kazemii*, *T. sabae*, *T. salarii*, *T. simile*, *T. trygonis*, *T. minutum*, *T.* n. sp. 4, and *T.* n. sp. 6 (6.7–14.3 mm vs. 28.8–36.6, 23.2–32.4, 23.5–35.9, approx. 21 [based on fig. 37 of Pintner, 1928; pg. 91], 20–40, 20, 14.7–26.5, and 18.1–35.2, respectively). This species possesses fewer gravid proglottids than *T. kazemii*, *T. mackenziei*, *T. sabae*, *T. salarii*, *T. opimum*, *T.* n. sp. 4, *T.* n. sp. 5, and *T.* n. sp. 6 (2–5 vs. 9–15, 7–10, 8–10, 18–20, 11, 11–30, 7–27, and 7–17, respectively). Furthermore, *T.* n. sp. 7 possesses fewer proglottids than *T. simile*, *T. trygonis*, and *T.* n. sp. 5 (26–51 vs. approx. 75 [based on fig. 37 of Pintner, 1928; pg. 91], approx. 60 [based on fig. 3 of Shipley and Hornell, 1905; pg. 54], and 51–117) and more proglottids than *T. minutum* (26–51 vs. 11–20). *Tetragonocephalum* n. sp. 7 also possesses a narrower scolex than *T. passeyi* and *T. uarnak* (330–339 vs. 379–672 and approx. 933 [based on fig. 86 of

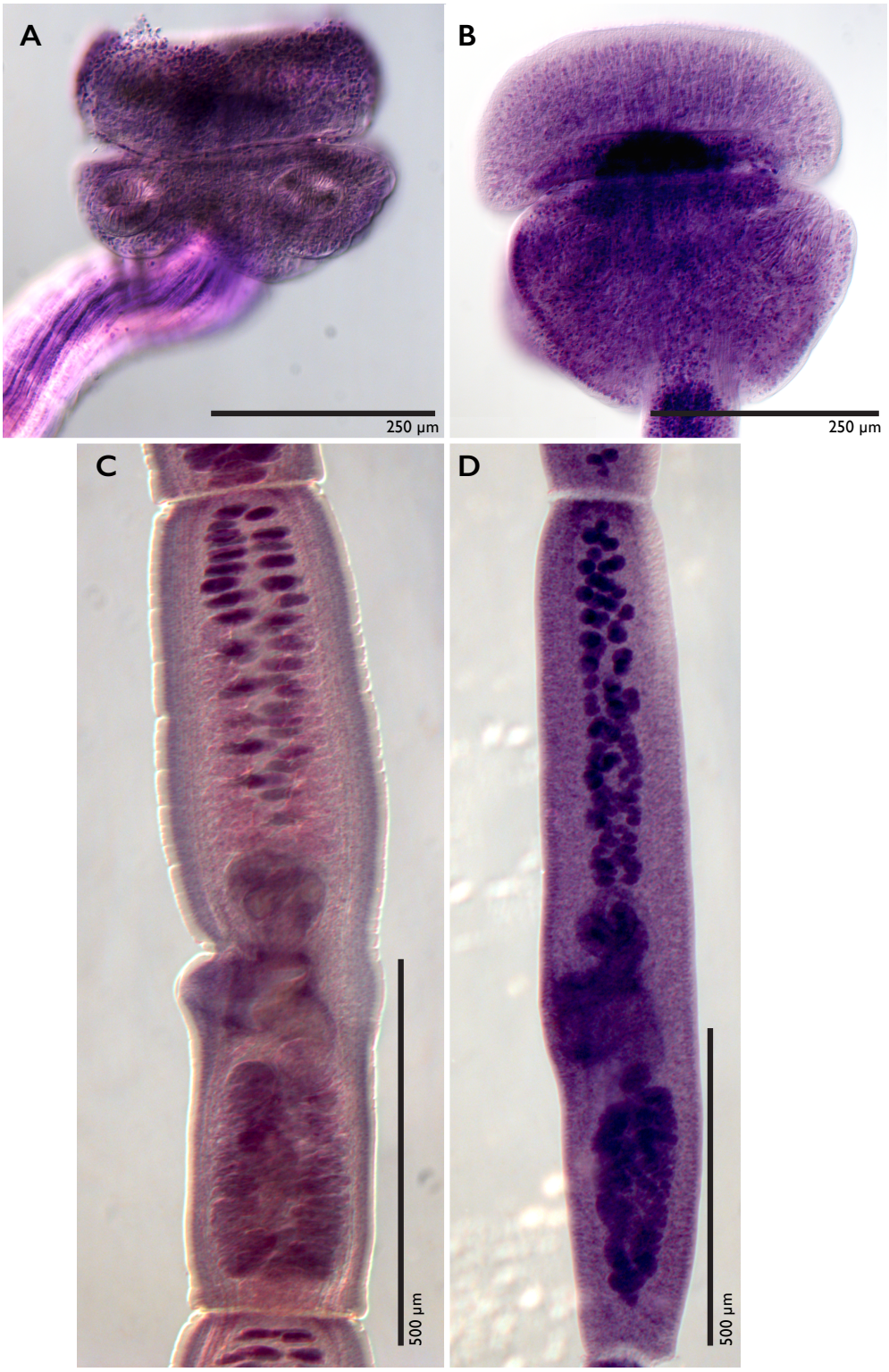

Figure 13. Photomicrographs of species of *Tetragonocephalum* from *Pateobatis uarnacoides.* (A) Scolex of *Tetragonocephalum* n. sp. 7 from BO-118. (B) Scolex of *Tetragonocephalum* sp. 5 from BO-78. (C) Posterior-most mature proglottid of *Tetragonocephalum* n. sp. 7 from KA-206. (D) Posterior-most mature proglottid of *Tetragonocephalum* sp. 5 from BO-78.

Shipley and Hornell, 1906; pg. 101]). *Tetragonocephalum* n. sp. 7 possesses a shorter scolex than *T. uarnak* (285–470 vs. approx. 786 [based on fig. 86 of Shipley and Hornell, 1906; pg. 101]). *Tetragonocephalum* n. sp. 7 possesses fewer testes than *T. passeyi* (31–47 vs. 54–73) and more testes than *T. mackenziei* and *T. opimum* (31–47 vs. 10–14 and 17). *Tetragonocephalum* n. sp. 7 is apolytic while both *T. georgei* and *T. levicorpum* are euapolytic. This new species differs from the *species inquirendum*, *T. yamagutii*, in possessing fewer testes (31–47 vs. 54–56) and larger acetabula (59–76 long by 63–76 wide vs. 30–40 long by 40–50 wide).

***Tetragonocephalum* sp. 5**

Tetragonocephalum sp. 5 is known from seven whole mounts from two specimens of *Pateobatis uarnacoides* collected from the Sulu Sea off the coast of Malaysian Borneo (BO-77 and BO-78). This species has is 14.0–46.3 mm (22.4 ± 12.3; 6) in total length, with terminal gravid proglottid 1,309–3,254 (2,219 ± 717; 6) long and 269–413 (308 ± 54; 7) wide. *Tetragonocephalum* sp. 5 possesses a whole scolex that is 403–590 (486 ± 59; 7) long and 386–506 (430 ± 43; 7) wide, bearing acetabula 58–114 (87 ± 14; 7; 14) long and 54–101 (82 ± 14; 7; 14) wide.

Distinguishing species of T*etragonocephalum* parasitizing *Pateobatis uarnacoides*

Though these two species of *Tetragonocephalum* that parasitize *Pateobatis uarnacoides* have lengths that are only narrowly overlapping, with *Tetragonocephalum* n. sp. 7 being generally a shorter worm than *Tetragonocephalum* sp. 5 (6.7–14.3 mm vs. 14.0–46.3 mm), they can be further distinguished by scolex width, with *Tetragonocephalum* n. sp. 7 narrower scolex than does *Tetragonocephalum* sp. 5 (330–339 vs. 386–506).

Phylogenetic analysis

The alignment generated was 5,098 base pairs in length. The tree resulting from this analysis shows the presence of two major clades; one containing 17 species and the other containing six species (Clades I and II in Fig. 14). The two clades recovered in this analysis received strong support (Erixon et al., 2003), each with posterior probabilities of 1. As expected, the two specimens *Tetragonocephalum* n. sp. 7 (KW484 and KW485) grouped together and exhibited low sequence divergence. This was not the case for the two specimens of *Tetragonocephalum* sp. 13 (KW13 and KW14), which did not group together. Instead, one of the specimens (KW13) grouped more closely with a long-branched taxon, *Tetragonocephalum* sp. 14 (KW141). However, given the short internode uniting *Tetragonocephalum* sp. 13 (KW13) and *Tetragonocephalum* sp. 14 (KW141) and, thus, the resulting low branch support (0.6836), the morphological data support the conspecificity of KW13 and KW14 as distinct from KW141.

These 23 species are collectively hosted by 17 species of dasyatids. Represented in the analysis were a single species of *Tetragonocephalum* from each of 11 host species, and two species each of *Tetragonocephalum* from the following six host species: *Maculabatis bineeshi* Manjaji-Matsumoto and Last, *Urogymnus polylepis*, *Himantura undulata*, *Himantura tutul*, *Maculabatis* cf. *gerrardi* 5 (sensu Naylor et al., 2012), and *Pateobatis jenkinsii*. In five of the six cases in which two congeners were included for the same host species (i.e., *Maculabatis bineeshi*, *Himantura undulata*, *Himantura tutul*, *Maculabatis* cf. *gerrardi* 5 [sensu Naylor et al., 2012], and *Pateobatis jenkinsii*), one of the species in the pair was a member of Clade I while the others was a member of Clade II. Only for the species of *Tetragonocephalum* from *Urogymnus polylepis* did both species appear in the same clade, Clade I.

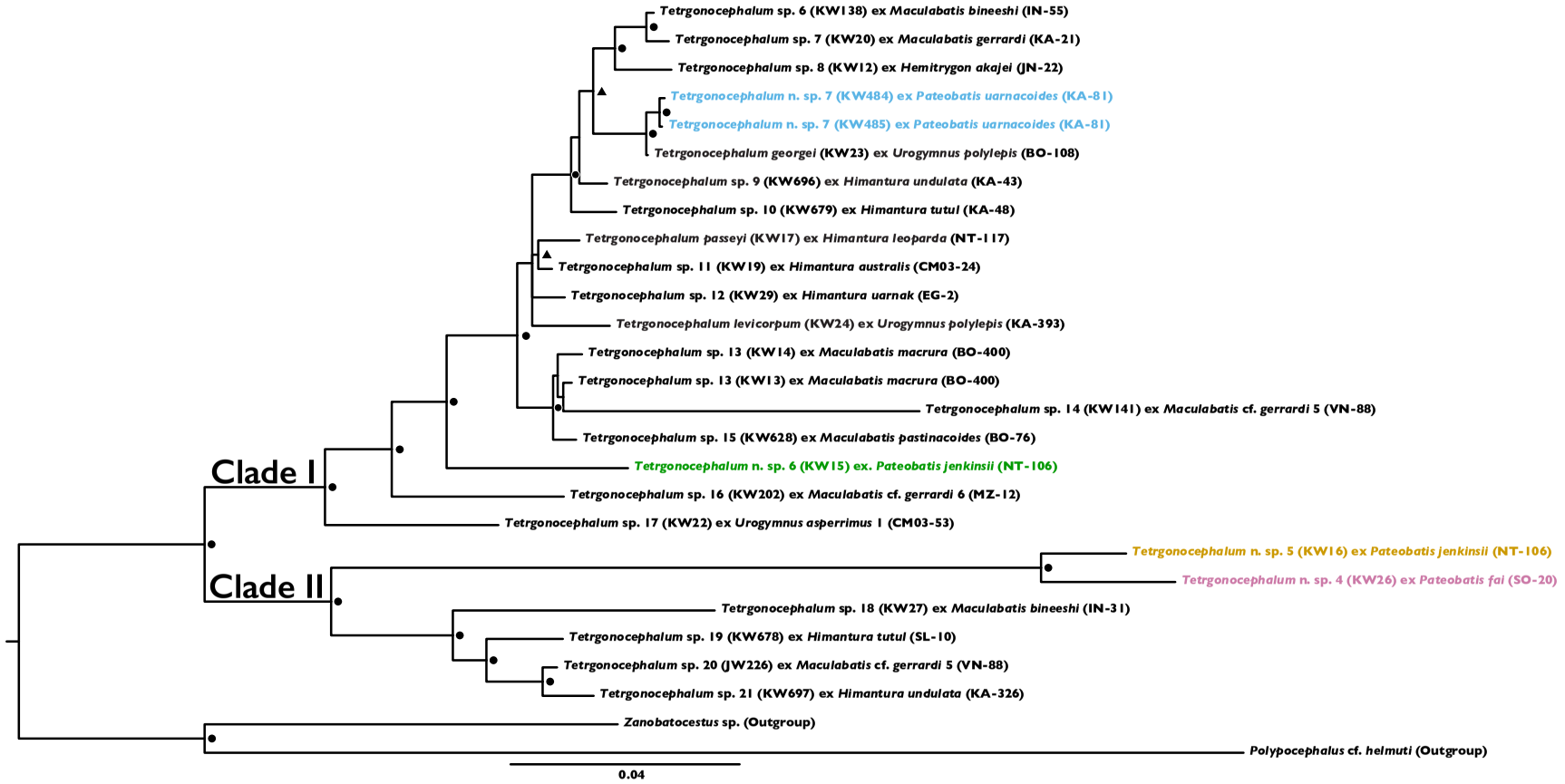

Figure 14. Tree resulting from Bayesian analysis of species of *Tetragonocephalum*. Based on sequence data of 18S, 28S, 5.8S/ITS rDNA (5,098 base pairs in length). Circles on nodes indicate posterior probabilities of 0.95 or higher. Triangles on nodes indicate posterior probabilities of between 0.95 and 0.90. Taxon labels include species name, unique molecular specimen number, host species, and unique host code. Species confirmed to be new to science in this study indicated in color. Branch length scale bar indicates number of substitutions per site.

The molecular sequence data supported independent species status for all 23 species. This included specimens representing the three known species and the four new species described herein. Thus these data suggest that this analysis includes sequence data for an additional 16 likely new species.

Principal Component Analysis of novel species

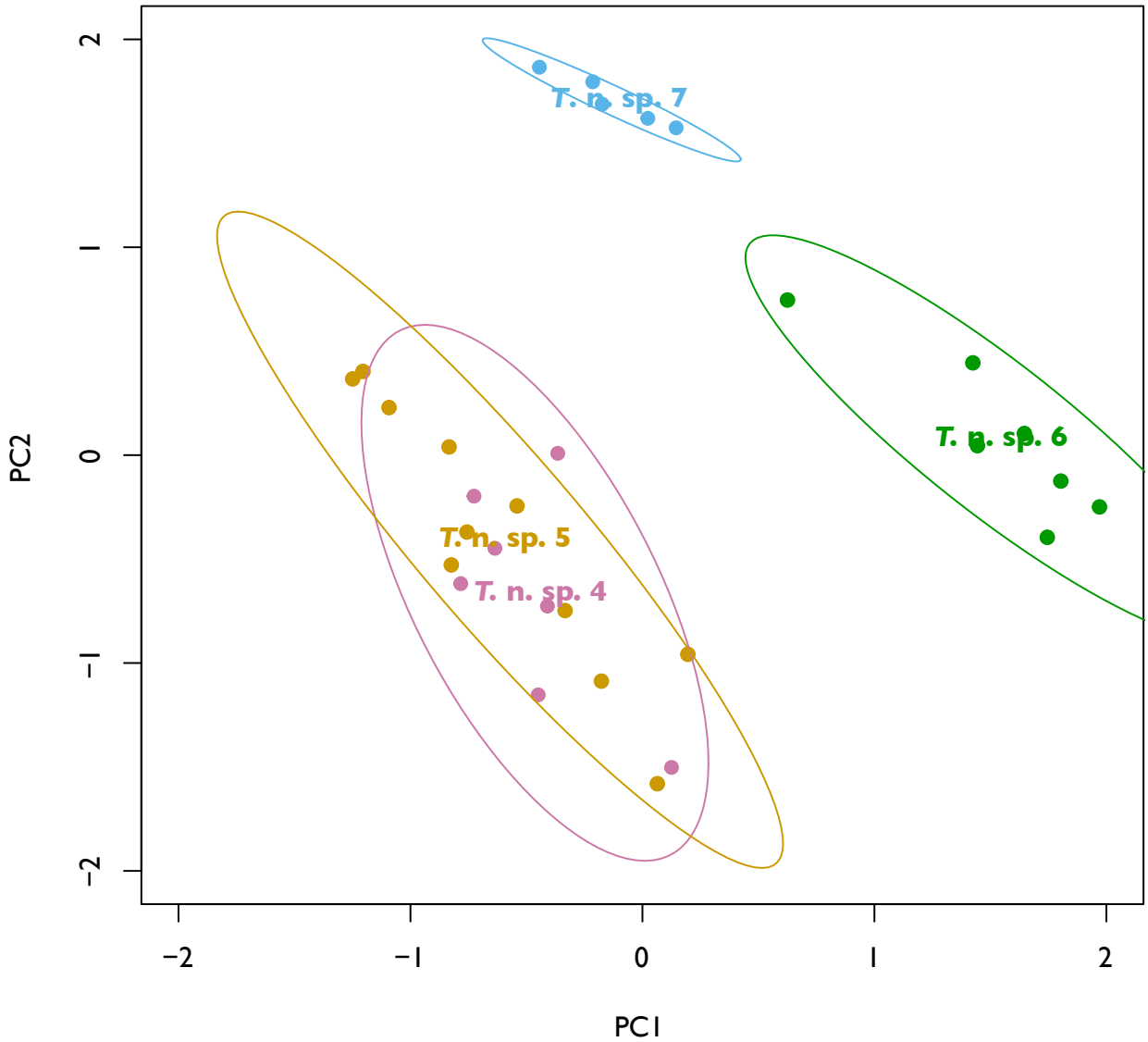

Figure 15. Plot of the first two principal components (collectively explaining 67.9% of variance in the data) from principal component analysis of measurement data of morphological characters for species of *Tetragonocephalum* described in this study. Specimens of the same species in the same color dot, enclosed by ellipse representing 0.95 confidence interval.

The visualization of the principal component analysis (Fig. 15) is based on 24 measurements from 30 specimens from the four species described in detail earlier in this study. The visualization plots principal components 1 and 2, which collectively account for 67.9% of variance in the data. The results of this principal component analysis show that the specimens of *Tetragonocephalum* n. sp. 6 and *Tetragonocephalum* n. sp. 7 are clearly grouping among their respective species groups, away from other groups. Specimens of *Tetragonocephalum* n. sp. 4 and *Tetragonocephalum* n. sp. 5 were overlapping in range, but away from the other two groups. Ellipses indicate 95% confidence intervals.

DISCUSSION

In this study, multiple facets of the *Tetragonocephalum*-dasyatid parasite-host system are examined: host associations, species diversity, novelty of species in the host genus *Pateobatis*, formal descriptions of novel species, geographic distributions, phylogenetic interrelationships, and the use of morphological characters to determine the morphological cohesion of a group of specimens. These aspects have been investigated to gain a better understanding of the true scale and breadth of this genus.

Species diversity in *Tetragonocephalum*

The four new species described in this study increase the number of valid species of *Tetragonocephalum* from 12 to 16, in addition to one *species inquirendum*. Including the 16 reports of undescribed species of *Tetragonocephalum* represented in the phylogenetic analysis in this study, five species of *Tetragonocephalum* parasitizing species of the whipray genus *Pateobatis* left undescribed in this study, and reports of undescribed species from previous studies (Caira et al., 2001; Golestaninasab et al., 2014; Jensen et al., 2016, 2017), there may be

as many as 41 species in this genus. Additionally, there are specimens of *Tetragonocephalum* identified from novel host species in this study but not included in the phylogenetic analysis (*Tetragonocephalum* sp. 22–24; see Table II) that were not named or characterized further than a report of their presence, which could ultimately increase the number of species in this genus to 44.

To date, every species of *Tetragonocephalum* has only been described from a single host species: *Tetragonocephalum georgei*, *T. levicorpum*, *T. opimum* and from *Urogymnus polylepis* (see Jensen and Guyer, 2021), *T. kazemii* and *T. mackenziei* from *Pastinachus sephen* (see Roohi Aminjan and Malek, 2017), *T. minutum* from *U. asperrimus* or *U. granulatus* (see Southwell, 1925; Perrenoud, 1931), *T. passeyi* from *Himantura leoparda* (see Jensen, 2005), *T. sabae* and *T. salarii* from *Maculabatus randalli* (see Roohi Aminjan and Malek, 2016), *T. simile*, *T. trygonis*, and the *species inquirendum T. yamagutii* from *Brevitrygon imbricata* or *B.* sp.1 (see Pintner, 1928; Perrenoud, 1931; Shipley and Hornell, 1905; Muralidhar, 1988), and *T. uarnak* from *H. leoparda* or *H. tutul* or *H. uarnak* or *H. undulata* (see Shipley and Hornell, 1906; Perrenoud, 1931; Jensen and Guyer, 2021). The species formally described in this study also follow this pattern: *Tetragonocephalum* n. sp. 4 was only found to parasitize *Pateobatis fai*, both *Tetragonocephalum* n. sp. 5 and *Tetragonocephalum* n. sp. 6 were only found to parasitize *P. jenkinsii*, and *Tetragonocephalum* n. sp. 7 was only found to parasitize *P. uarnacoides*. Thus, all these species follow the oioxenous pattern of host specificity observed in the majority of elasmobranch tapeworms (Caira and Jensen, 2014), where one species of tapeworm is only found to parasitize one species of elasmobranch host.

Although not all of the nine species of *Tetragonocephalum* characterized in this study were formally described, multiple congeners in the same host species were documented: four

species in *Pateobatis fai*, three in *Pateobatis jenkinsii*, and two in *Pateobatis uarnacoides*. This phenomenon has also been observed among the five host species of valid species of *Tetragonocephalum* (see above paragraph): three species from *Urogymnus polylepis* (see Jensen and Guyer, 2021), two from *Pastinachus sephen* (see Roohi Aminjan and Malek, 2017), two from *Maculabatus randalli* (see Roohi Aminjan and Malek, 2016), and two species and the *species inquirendum* from *Brevitrygon imbricata* or *B.* sp.1 (sensu Naylor et al., 2012) (see Pintner, 1928; Perrenoud, 1931; Shipley and Hornell, 1905; Muralidhar, 1988; see also Jensen and Guyer, 2021). In addition, congeners were documented from six of the 17 host species represented in the phylogenetic analysis. This plethora of examples of this pattern is strong evidence that this pattern is likely to be common within the genus. It further suggests that if, to date, only a single species of *Tetragonocephalum* has been reported from a dasyatid host species, it is likely that, given additional sampling, more than one species of *Tetragonocephalum* will be found to parasitize that particular host species. Future research in this aspect will likely expand the number of species in this genus past the hypothesized 44 species here.

While there is ample evidence that the presence of multiple congeners in a host species is pervasive in the genus *Tetragonocephalum*, it may not be only the lecanicephalidean genus in which the pattern can be found. Of the 17 other non-monotypic genera of lecanicephalideans, 12 are known to have congeners parasitizing the same host species (Jensen et al., 2017): *Aberrapex*; *Anteropora* Subhapradha, 1955; *Corollapex* Herzog and Jensen, 2017; *Elicilacunosis* Koch, Jensen and Caira, 2012; *Flapocephalus* Deshmukh, 1979; *Healyum* Jensen, 2001; *Hornellobothrium* Shipley and Hornell, 1906; *Lecanicephalum* Linton, 1890; *Polypocephalus*; *Stoibocephalum* Cielocha and Jensen, 2013; *Tylocephalum*; *Zanobatocestus*. While in *Tetragonocephalum* and most of the other lecanicephalidean genera, congeners in the same host

species range from 2–3 or 4 (e.g., Koch et al., 2012; Mojica et al., 2013; Herzog and Jensen, 2017), *Hornellobothrium* is the exception with six species reported from the spotted eagle ray, *Aetobatus ocellatus* (Kuhl) (see Shipley and Hornell, 1906; Jensen, 2005; Mojica et al., 2014).

Phylogenetic analysis of *Tetragonocephalum*

This is the most comprehensive phylogenetic analysis of *Tetragonocephalum* to date, and, in fact, the first to address the interrelationships of species in the genus. The next most comprehensive analysis, in terms of taxon sampling for species of *Tetragonocephalum*, only included three species with the purpose of placing this genus in a phylogenetic context in the order Lecanicephalidea (see Jensen et al., 2016). The tree recovered from the analysis in this study indicates high number of currently undescribed species of *Tetragonocephalum*. Given the minimally overlapping nature of sampling between these two analyses, we are unable to compare topologies.

The phylogenetic analysis was restricted to specimens of *Tetragonocephalum* hosted by members of the subfamily Urogymninae and a subset of members of the subfamily Dasyatinae now known to host *Tetragonocephalum*. Specimens of *Tetragonocephalum* from the dasyatine *Hemitrygon* cf. *bennetti* (Müller and Henle) were not included in the phylogenetic analysis due to lack of material preserved in ethanol for sequencing. Similarly, sequence data remain unavailable for species of *Tetragonocephalum* from the host family Neotrygoninae (see Table II). In addition, sequence data are unavailable for the two species of *Tetragonocephalum* described by Roohi Aminjan and Malek (2017) from the Hypolophinae from Iran.

The pattern of congeners from the same host species placing on both clades is intriguing; again the exception to this is the two species from *Urogymnus polylepis* both placing in Clade I. Given that Jensen and Guyer (2021) described three species of *Tetragonocephalum* from that

host species, we might predict that the third species would perhaps place as a member of Clade II. Only a single species of *Tetragonocephalum* was included for 11 of the 17 host species (see Fig. 14). Preliminary specimen data from this study indicates that at least some of these host species, like species in the genus *Pateobatis*, are parasitized by multiple congeners. Adding these additional congeners to a phylogenetic analysis would allow this two-clade pattern to be tested. Morphological characters to support these two major clades have unfortunately yet to be identified.

Principal component analysis of novel species

The specimens included in this principal component analysis of morphological data grouped into their respective species groups based on the 24 characters used for this analysis (Fig. 15). Species of *Tetragonocephalum* n. sp. 6 (parasitizing *P. jenkinsii*) and *Tetragonocephalum* n. sp. 7 (parasitizing *P. uarnacoides*) both cluster more closely with one another than with specimens from other species. This pattern supports their validity as distinct species based on features not captured by the principal component analysis. Such clear conclusions are not the case for the two other novel species; while *Tetragonocephalum* n. sp. 4 (parasitizing *P. fai*) and *Tetragonocephalum* n. sp. 5 (parasitizing *P. jenkinsii*) did group away from the two species mentioned previously, their groups are largely overlapping. These two species are both considered distinct, as discussed in the remarks section of the description of *Tetragonocephalum* n. sp. 5; *Tetragonocephalum* n. sp. 4 only possesses two fields of vitelline follicles (as opposed to three found in *Tetragonocephalum* n. sp. 5), in addition to possessing longer mature proglottids than *Tetragonocephalum* n. sp. 5. The validity of these two species is also supported by molecular sequence divergence (see Fig. 14). Interestingly, these two species appear to be each other's closest relatives.

Host associations and distribution

Tetragonocephalum is now known to parasitize 27 species in nine genera, representing all four subfamilies of the family Dasyatidae (Dasyatinae, Hypolophinae, Neotrygoninae, and Urogymninae) (Table II). *Tetragonocephalum* is not known to parasitize any other family of elasmobranch. While there are valid species from other genera in the order Lecanicephalidea that are known to parasitize dasyatid rays (i.e., *Aberrapex*, *Anteropora*, *Corollapex*, *Flapocephalus*, *Lecanicephalum*, New genus 12 [sensu Jensen et al., 2016], *Polypocephalus*, and *Seussapex* Jensen and Russell, 2014), only *Corollapex* (with two valid species, only known from one host species) is known to parasitize only dasyatid rays (Jensen et al., 2016; Herzog and Jensen, 2017). Some of the other genera parasitize several families of myliobatiform rays (e.g., *Aberrapex*, *Seussapex*), several orders of batoids (e.g., *Polypocephalus*), or even batoids and sharks (e.g., *Anteropora*, *Tylocephalum*) (Jensen et al., 2017).

These new records also expand the geographic range of *Tetragonocephalum* from Iran, Sri Lanka (as Ceylon), India, Australia, Indonesian Borneo, and Malaysian Borneo (all from described species or other previous records) to include Japan, Taiwan, Vietnam, Mozambique, Egypt, and the Solomon Islands (see Table I and Fig. 1). These data intimate that *Tetragonocephalum* is restricted to the Indo-Pacific region. Though other lecanicephalidean families are known to parasitize elasmobranchs from the Indo-Pacific region (Aberrapecidae Jensen, Caira, Cielocha, Littlewood, and Waeschenbach, 2016, Cephalobothriidae, Eniochobothriidae, Lecanicephalidae, and Polypocephalidae), these families can also be found in the some part of the Atlantic Ocean as well (Jensen et al., 2016). Similar to the Tetragonocephalidae, members of the lecanicephalidean family Zanobatocestidae Jensen, Caira, Cielocha, Littlewood, and Waeschenbach, 2016 also possess a geographic range that is restricted

to one ocean, albeit, in this case, the eastern Atlantic Ocean (Jensen et al., 2016). This would indicate that the family Tetragonocephalidae is unique among lecanicephalidean families with a geographic distribution that is restricted to the Indo-Pacific region.

Although *Tetragonocephalum* is now known to parasitize all four subfamilies in Dasyatidae (Dasyatinae, Hypolophinae, Neotrygoninae, and Urogymninae), their diversity is not evenly distributed among hosts at the generic level. The subfamily Dasyatinae, comprised of the eight genera (*Bathytoshia*, *Dasyatis*, *Hemitrygon*, *Hypanus*, *Megatrygon*, *Pteroplatytrygon*, *Taeniurops*, and *Telatrygon*), is known to host *Tetragonocephalum* from only one genus (*Hemitrygon*). If the geographic distribution of *Tetragonocephalum* is in fact restricted to the Indo-Pacific region, then the absence of *Tetragonocephalum* in remaining seven genera can perhaps be explained by a combination of lack of sampling effort and geographic distribution. While both *Hemitrygon akajei* (Bürger) and *H.* cf. *bennetti* were found to host *Tetragonocephalum*, the remaining eight species in the genus *Hemitrygon* (*Hemitrygon fluviorum* [Ogilby], *Hemitrygon izuensis* [Nishida and Nakaya], *Hemitrygon laevigata* [Chu], *Hemitrygon laosensis* [Roberts and Karnasuta], *Hemitrygon longicauda* [Last and White], *Hemitrygon. navarrae* [Steindachner], *Hemitrygon parvonigra* [Last and White], and *Hemitrygon sinensis* [Steindachner]), two species in the genus *Bathytoshia* (*Bathytoshia brevicaudata* [Hutton] and *Bathytoshia lata* [Garman]), one species in the genus *Taeniurops* (*Taeniurops meyeni* [Müller and Henle]), as well as species in the monotypic genera *Megatrygon* and *Pteroplatytrygon* have been reported from the Indo-Pacific region (Last et al., 2016a), but have not been sampled well enough to draw conclusions about the presence or absence of *Tetragonocephalum* (Caira et al., 2020). The two species in the paraphyletic genus *Telatrygon* (*Telatrygon biasa* Last, White and Naylor and *Telatrygon zugei* [Bürger]) have been sampled

extensively (see Caira et al., 2020) and are found within the range of *Tetragonocephalum* (Last et al., 2016a). Some other explanation is needed to explain the absence of *Tetragonocephalum* from these two species. Members of the genera *Dasyatis*, *Hypanus*, and the other species in the genus *Taeniurops* (*Taeniurops grabatus* [Geoffroy Saint-Hilaire]) occur only in regions outside of the Indo-Pacific (Last et al., 2016a).

The subfamily Hypolophinae is comprised of only two genera: *Makararaja* and *Pastinachus*. The monotypic genus *Makararaja* (*Makararaja chindwinensis* [Roberts]) can be found within the range of *Tetragonocephalum* (Last et al., 2016a), however no specimens of this species have been collected to examine the tapeworm fauna of this host (Caira et al., 2020). The genus *Pastinachus* is comprised of five species, one of which (*P. sephen*) has been reported to host *Tetragonocephalum*. The remaining four species can all be found in the Indo-Pacific region (Last et al., 2016a). Three of these species (*Pastinachus ater* [Macleay], *Pastinachus gracillicaudus* Last and Manjaji-Matsumoto, and *Pastinachus solocircostris* Last, Manjaji and Yearsley) have been densely sampled and are known to host a plethora of tapeworms (Campbell and Beveridge, 2002; Kuchta and Caira, 2010; Schaeffner and Beveridge, 2012, 2013; Caira et al., 2020), they are not known to host *Tetragonocephalum.*

The subfamily Neotrygoninae is only comprised of the two genera *Neotrygon* and *Taeniura*, both of which contain species (*Neotrygon kuhlii* 3 [sensu Naylor et al., 2012], *Neotrygon varidens* [Garman], and *Taeniura lymma* 1 [sensu Naylor et al., 2012] in this study) that have been found to host *Tetragonocephalum*. The remaining eight valid species of *Neotrygon* and two valid species of *Taeniura* all occur within the range of *Tetragonocephalum* (Last et al., 2016a), however all but *Neotrygon orientalis* Last, White & Serét have not been sampled well enough to draw conclusions about the presence or absence of *Tetragonocephalum*

(Caira et al., 2020). Tens of specimens of *Neotrygon orientalis* have been collected (Caira et al., 2020), however to date, only a single species of tapeworm, *Anthocephalum odonnellae*, has been described from this host (Ruhnke et al., 2015). More work on the tapeworm fauna of *N. orientalis* is needed to explore why this species has not been found to host species of *Tetragonocephalum.*

The fourth subfamily of Dasyatidae, Urogymninae, is comprised of seven genera, five of which (*Brevitrygon*, *Himantura*, *Maculabatis*, *Pateobatis*, and *Urogymnus*) are known to host *Tetragonocephalum*. This is the subfamily wherein the greatest amount of species diversity of *Tetragonocephalum* lies. Despite reports that all members of the genus *Brevitrygon* occur in the Indo-Pacific region (Last et al., 2016a), *Tetragonocephalum* has only been found in at least one, possibly two, species in the genus *Brevitrygon*, *B. imbricata* and *B.* sp. 1 (sensu Fernando et al., 2019). The uncertainty of the status of these two species as hosts of *Tetragonocephalum* is due to updates in the specific identities of rays in the genus *Brevitrygon* from the type localities listed in the descriptions of *T. simile*, *T. trygonis*, and *T. yamagutii* (see Jensen and Guyer, 2021). Of the remaining three species in this genus, *Brevitrygon javaensis* (Last and White) has not been sampled, while *Brevitrygon heterura* (Bleeker) and *Brevitrygon walga* (Müller and Henle) have both been extensively sampled but not found to host *Tetragonocephalum* (Caira et al., 2020). More investigation is needed to explain this absence. All three members of the genus *Fluvitrygon* can be found in the range of *Tetragonocephalum* (Last et al., 2016a), however despite the somewhat extensive sampling of *Fluvitrygon oxyrhynchus* (Sauvage) and *Fluvitrygon signifer* (Compagno and Roberts), these two species have not been found to host *Tetragonocephalum* (see Caira et al., 2020). *Fluvitrygon kittipongi* (Vidthayanon and Roberts) has not been sampled well enough to draw conclusions about the presence or absence of *Tetragonocephalum.* All six

species in the genus *Fontitrygon* can be found only outside of the range of *Tetragonocephalum* (Last et al., 2016a). Though *Himantura* was known to host *Tetragonocephalum* from two previous reports (*H. australis* known from Caira et al., 2001 and *H. leoparda* from Jensen, 2005), this study has confirmed that *Tetragonocephalum* parasitizes the remaining three species in this genus (*H. tutul*, *H. uarnak*, and *H. undulata*). In the genus *Maculabatis*, all members of which can be found in the Indo-Pacific region (Last et al., 2016a), *Tetragonocephalum* has been found to parasitize five of the nine species (*Maculabatis bineeshi*, *Maculabatis gerrardi*, *Maculabatis macrura* [Bleeker], *Maculabatis pastinacoides* [Bleeker], and *Maculabatis randalli*). Of the remaining four species, three have not been sampled at all or enough to draw conclusions (*Maculabatis ambigua* Last, Bogorodsky and Alpermann, *Maculabatis arabica* Manjaji-Matsumoto and Last, and *Maculabatis toshi* [Whitley]) (Caira et al., 2020), while there is not a satisfactory explanation as to why the other species (*Maculabatis astra* [Last, Manjaji-Matsumoto and Pogonoski]) has not been found to host *Tetragonocephalum*. All members of the genus *Pateobatis* can be found within the Indo-Pacific region (Last et al., 2016a). Of the five species within the genus *Pateobatis*, on which this study focuses, three were found to host -and *P. hortlei*) were not sampled enough to draw conclusions about the presence or absence of *Tetragonocephalum* from those two species in question (Caira et al., 2020). Only two members of the genus *Urogymnus*, all species of which can be found within the Indo-Pacific region (Last et al., 2016a), have been found to host species of *Tetragonocephalum* (*U. asperrimus* 1 [sensu Naylor et al., 2012) and *U. polylepis*). There are two species of *Urogymnus* (*U. asperrimus* and *U. granulatus*) which could be the type species of *Tetragonocephalum minutum*. Southwell (1925) did not specify between these two ray species and no host material was collected for later identification. The true identity of the type host for this species remains uncertain as the type

specimen of *T. minutum* has not been matched to a specimen collected from either of these two hosts. Of the three remaining species, two (*Urogymnus acanthobothrium* Last, White, and Kyne and *Urogymnus dalyensis* [Last and Manjaji-Matsumoto]) have not been sampled enough to draw conclusions about the presence or absence of *Tetragonocephalum* (Caira et al., 2020), although tapeworms have been described from *U. acanthobothrium* (Fyler et al., 2009). There is not a satisfying reason to explain the absence of *Tetragonocephalum* in *Urogymnus lobistoma* (Manjaji-Matsumoto and Last).

Interesting to note is the association of *Tetragonocephalum* with freshwater members of the family Dasyatidae, given that three of the 16 valid species of *Tetragonocephalum* were described from the freshwater stingray *Urogymnus polylepis*. The three members of the genus *Fluvitrygon* (*F. kittipongi*, *F. oxyrhynchus*, and *F. signifer*), in addition to *Hemitrygon laosensis*, can be found in rivers in Borneo and southeast Asia, a range that overlaps with that of *U. polylepis* (Last et al., 2016). The absence of *Tetragonocephalum* from the two freshwater members the genus *Fontitrygon* can be explained by geography. These rays inhabit the Atlantic ocean or rivers adjacent to the Atlantic ocean, near the coast of Nigeria and Cameroon (*Fontitrygon garouaensis* [Stauch and Blanc] and *Fontitrygon ukpam* [Smith]) (Last et al., 2016a).

Future directions

Though only four species of *Tetragonocephalum* were described in this study, there are at least five additional species from the species of *Pateobatis* examined. Given the high degree of undescribed species diversity in this genus, there is much work to be done on descriptions. Though material from 21 host species was examined for this study, descriptions for each of the novel taxa encountered was outside the scope of this project. Based on the phylogenetic analysis

and the host material examined, *Himantura* and *Maculabatis* are ideal candidate genera for continuing the process of species discovery and novel species description.

The identity of the type hosts for four valid species of *Tetragonocephalum* (*T. minutum*, *T. simile*, *T. trygonis*, and *T. uarnak*) that were described almost a century ago and the *species inquirendum* (*T. yamagutii*) are still in question. Collections from the type localities of these valid species are needed to confirm the identity of the type hosts, and possibly could contribute to an updated description to include additional measurements, as well as examination of species with scanning electron microscopy for classification of the microthrix patterns, which are all standards for modern descriptions.

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