Redescription of *Echinoderes ohtsukai* Yamasaki and Kajihara, 2012 and *E. kozloffi* Higgins, 1977 from the northeastern Pacific coast, including the first report of a potential invasive species of kinorhynch.

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Abstract

Although the dispersal ability of kinorhynchs is known to be limited, the distribution of certain kinorhynch species appears to extend over vast geographical areas. Combining molecular phylogenetic data with biogeographical investigations can test this paradox by discerning cryptic species with restricted distributions from species with potentially large geographical distributions. In this paper, we (1) redescribe two species of kinorhynchs (*Echinoderes ohtsukai* and *E. kozloffi*) found in the northeastern Pacific Ocean using molecular and morphological data and (2) provide the first evidence for a disjunct geographical distribution in kinorhynchs that is consistent with the introduction of an invasive species. Although we collected *E. ohtsukai* from the northeastern Pacific Ocean (British Columbia, Canada), this species was originally described from Japan. We demonstrated that specimens of *E. ohtsukai* collected from Japan and British Columbia have identical DNA sequences for the mitochondrial cytochrome c oxidase subunit 1 (COI) gene. These results are most consistent with a recent introduction of this species into one of the habitats on the opposite side of the Pacific Ocean through human-mediated dispersal.

Key words

COI, Kinorhyncha, systematics, British Columbia, introduction

1. Introduction

Kinorhynchs are a distinct group of marine permanent meiofauna, meaning that they spend their entire life cycle in the sediment. They are direct developers without larval planktonic stages, and are inferred to have a limited potential for dispersal (Giere, 2009; Higgins, 1988). Moreover, kinorhynchs are equipped with mucous glands and cuticular structures (spines and tubules) that enable them to anchor themselves within the sediment, preventing accidental detachments that could bring them into the water column and drift (Brown, 1989). However, the wide geographical distribution observed in some species of kinorhynchs leads to a paradox (Giere, 2009). Different hypotheses have been proposed to explain the wide distribution of kinorhynchs, including potential pathways through deep bottom currents (e.g., *Campyloderes*; Neuhaus and Sørensen, 2013) and vicariance processes through continental drift (e.g., *Centroderes* and *Meristoderes*; Herranz and Pardos, 2013; Neuhaus et al., 2014). However, none of these hypotheses have been tested with molecular phylogeographical data, so dispersal mechanisms in kinorhynchs remain unclear. The only phylogeographical study focused on kinorhynchs was recently carried out by Yamasaki et al. (2014), who compared the DNA barcode region of the mitochondrial gene cytochrome c oxidase subunit I (COI) in two closely related species of the genus *Echinoderes* from Japan. This study demonstrated the occurrence of cryptic speciation in both species and suggested suspension, transport or rafting as possible dispersal mechanisms (Yamasaki et al., 2014).

Echinoderes is the largest genus of kinorhynchs with over 85 species distributed worldwide and representing more than 30% of the total diversity of the group (Neuhaus, 2013). Within *Echinoderes* the so called "*E. coulli* group" is particularly interesting in terms of biogeography, with most of its species being limited to intertidal brackish

waters but reported from very different areas of the world (Lundbye et al., 2011; Ostmann et al., 2012; Sørensen, 2013; Yamasaki and Fujimoto, 2014; Yamasaki and Kajihara, 2012). It is currently unclear whether or not *E. coulli* group species encompass uncharacterized cryptic (molecular) diversity that reflects biogeographical patterns.

Knowledge of kinorhynchs in the northeastern Pacific Ocean is scarce, limited only to the samples collected from the San Juan Archipelago (Washington, USA) and California by R.P. Higgins and collaborators several decades ago (Higgins 1960, 1961, 1977a, 1986). There are only 8 species reported from the northeastern Pacific Ocean, and only two of them belong to the genus *Echinoderes*: *Echinoderes kozloffi* Higgins, 1977 *and Echinoderes pennaki* Higgins, 1960 (Higgins, 1977a; 1960; Neuhaus, 2013). Canadian coasts, in particular, have been very poorly surveyed with a total of 6 species of kinorhynchs belonging to two different genera described so far. Sampled areas in Canada include southwest Vancouver Island with *Pycnophyes ilyocryptus* Higgins, 1961, *Pycnophyes sanjuanensis* Higgins, 1961 and *Kinorhynchus cataphractus* Higgins, 1961; the Beaufort Sea with *Pycnophyes canadensis* Higgins and Korczynski, 1989 and *Pycnophyes borealis* Higgins and Korczynski, 1989; and Nova Scotia (NW Atlantic Ocean) with *Pycnophyes frequens* Blake, 1930 (Blake, 1930; Higgins, 1961; Higgins and Korczynski, 1989).

Here we redescribe two intertidal echinoderid species, *Echinoderes ohtsukai* and *E. kozloffi*, collected off the coasts of British Columbia for the first time. We provide new morphological and molecular data for both species and demonstrate a disjunct geographical distribution for *E. ohtsukai* that might be consistent with the recent introduction of an invasive species.

2. Materials and methods

2.1. Sampling

Specimens of *Echinoderes ohtsukai* were collected in Mud Bay Park (Boundary Bay), southeast from Vancouver, British Columbia (49°5'9.86"N; 122°51'39.95"W), in November and August 2014 and November 2015 at station VAN-011 (Fig. 1A). The sampled area is a brackish estuarine with a salinity ranging between 20-24‰. Samples were taken from intertidal mud using a shovel to collect the uppermost, oxygenated layer of sediment. Specimens of *Echinoderes kozloffi* were collected from intertidal brown algae mixed with fine sediment in Clover Point, south of Victoria on Vancouver Island, British Columbia (48°24'13.70"N; 123°21'3.36"W), in May 2015 at station VIC-014 , salinity 32.5‰ (Fig. 1B). *E. kozloffi* was also collected intertidally from Archaeology Beach on Calvert Island, north of Vancouver Island (51°39'51.88"N; 128° 5'50.29"W), in July 2015 at station CI-010.AB1, salinity 33‰ (Fig. 1C).

2.2. Microscopy

Kinorhynchs were extracted from the sediment using the Higgins bubbling technique (Higgins, 1988; Neuhaus, 2003; Sørensen and Pardos, 2008) and fixed in 4% paraformaldehyde. Specimens prepared for light microscopy (LM) were dehydrated through a graded series of ethanol and transferred to glycerin prior to mounting in Fluoromount G®. The specimens were examined and photographed using a Zeiss Axioplan 2 microscope with differential interference contrast optics (DIC) equipped with a Zeiss-Axiocam 503-color camera. Measurements were made using ZEN 2 software (Zeiss, Germany). Specimens for SEM were ultrasonically cleaned by exposing them to ultrasound intervals of 5-10 seconds and posteriorly dehydrated through a graded series of ethanol and critical point dried. The dried specimens were

mounted on aluminum stubs, sputter coated with platinum-palladium and imaged with a Hitachi S4700 field emission scanning electron microscope. Coating and SEM imaging were performed at the Bioimaging Facility at UBC.

2.3. DNA extraction, PCR and sequencing

Total genomic DNA was extracted from a single specimen of *E. ohtsukai* from Mud Bay Park and two specimens of *E. kozloffi*, from Victoria and Calvert Island, all fixed in 99% ethanol, using a DNeasy Blood and Tissue Kit (Qiagen, Tokyo) following the protocol described in Yamasaki et al. (2013). Cuticular vouchers of the specimens used for the extraction were recovered from the lysis buffer and mounted in Fluoromount G® using regular glass slides and deposited at the Natural History Museum of Denmark, under catalogue number ZMUC KIN-968 for *E. ohtsukai* and ZMUC KIN-969 *E. kozloffi*. DNA extraction, amplification and sequencing of the specimens were performed for the mitochondrial gene COI. Polymerase chain reactions (PCR) were performed using PuRe Taq Ready-To-Go PCR beads kit (GE Healthcare, Buckinghamshire, UK). The primers used for COI were: LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994). PCR cycling conditions were: 95ºC for 1min; 35 cycles of (95ºC for 30 sec, 49ºC for 1min 30 sec and 72ºC for 3min); and 72ºC for 7min. The amplified fragments were gel purified using UltraClean DNA Purification Kit (MO Bio, Carlsbad, CA) and sequenced. Nucleotide sequences were determined by direct sequencing with a BigDye Terminator Kit (Applied Biosystems). Sequence fragments were edited and assembled using Sequencher (Gene code corporations, Michigan, USA). After assembly, the sequences were deposited in GenBank (NCBI) under accession numbers KU674383 for *E.*

ohtsukai and KU681520 - KU681519 for *E. kozloffi* from Victoria and Calvert Island, respectively. COI sequences from two specimens of *E. ohtsukai* from the type locality in Japan (accession numbers LC096964 and LC096965) were used for comparison with the specimens from Vancouver.

3. Results

Taxonomic account

Class Cyclorhagida Zelinka, 1896
Order Echinorhagata Sørensen et al., 2015
Family Echinoderidae Zelinka, 1894
Genus *Echinoderes* Claparède, 1863

3.1. *Echinoderes ohtsukai* Yamasaki and Kajihara, 2012 (Figs 2-6 and Tables 1-2)

3.1.1. Emended Diagnosis

Echinoderes with a single, minute middorsal spine on segment 4, minute lateroventral spines on segments 6-7; lateroventral tubes on segments 5 and 8, males furthermore with well-developed tubes in laterodorsal positions on the posterior margin of segment 10, whereas females show much smaller laterodorsal tubes on segment 10. Small single middorsal fringed tube on segment 8, subdorsal fringed tubes on segments 2 and 4, laterodorsal on segments 2, 6, 8, midlateral on segment 5, ventrolateral on segment 2, lateroventral on segments 3, 4 and sublateral on segments 7 and 8. Large sieve plates in sublateral position on segment 9. Tergal extensions long and pointed. Males with three penile spines and a long fringed area in lateroventral position; females with lateral terminal accessory spines reduced to short fringed structures.

3.1.2. Material examined

Three females and one male all collected from intertidal mud on November 2014, June 2015 and October 2015 at the same station (VAN-011) in Mud Bay Park (Boundary Bay), located southeast of Vancouver (49°5'9.86"N; 122°51'39.95"W) (Figure 1A). All specimens were mounted in Fluoromount G® and deposited at the Natural History Museum of Denmark, under catalogue numbers ZMUC KIN-923 and ZMUC KIN-956 to KIN-958.

Additional material collected at the same locality as the previous specimens includes one female, preserved in 99% ethanol and kept as a voucher after DNA extraction, mounted in Fluoromount G® and deposited at the Natural History Museum of Denmark, under catalogue number ZMUC KIN-968. Two females and one male were mounted for SEM (Figs 5-6) and stored in the authors' personal reference collection. Furthermore, eight topotypes mounted in Fluoromount G® were used for morphological comparisons.

3.1.3. DNA sequence

The mitochondrial cytochrome c oxidase subunit 1 (COI) gene (GenBank Accession number KU674383). The cuticle was kept as a voucher and deposited at the Natural History Museum of Denmark, under catalogue number ZMUC KIN-968.

3.1.4. Description

Adults with head, neck and eleven trunk segments (Figs 2, 4A, 5A, 6A). Measurements and dimensions are given in Table 1. A summary of cuticular structures positions (sensory spots, spines, sieve plates, tubes and glandular cell outlet) is provided in Table 2. The head consists of a retractable mouth cone and an introvert (Figs 3, 5A-D). Outer

armature of the mouth cone formed by nine outer oral styles divided into two subunits slightly alternating in size between five longer ones situated according to uneven sectors of the introvert, and four shorter ones situated according to even sectors (Fig. 5B); middorsal outer oral style is missing. Each outer oral style has a fringe at its base showing six fringe tips and bordered by a pair of spikes (Fig. 5A, B). One of the examined specimens was found to have eight instead of nine oral styles, with the same appearance but distributed in a different way breaking the typical bilateral symmetry showed by the specimens with nine oral styles (Fig. 5C). The exact number and arrangement of the inner armature of the mouth cone could only be observed partially and is therefore not plotted in the introvert diagram, but shown in Figure 5C. Three rings of inner oral styles could be detected in the inner part of the mouth cone namely (-03), (-02) and (-01). The ring (-01) shows tubular elongated structures with a pore in the distal end. Ring (-02) shows five inner oral styles similar as those on previous ring but with proximal fringed sheaths. The anteriormost ring (-03) could not be fully studied.

The introvert has seven rings of cuticular spinoscalids and one additional ring of trichoscalids that are associated with the placids (Figs 5A, D, E). Ring 01 with 10 primary spinoscalids consisting of a short basal sheath and a distal end piece. The basal sheath has a proximal small fringe situated medially, very close to the insertion point, with three flexible and elongated fringe tips followed by a rectangular smooth part that projects laterally into four fringed tips (Figs 5A-B, D). The distal piece of the primary spinoscalids is laterally compressed and bears a fringe composed of at least six flexible fringe tips. Ring 02 is composed of 10 laterally compressed spinoscalids, all formed by a long smooth basal part with two short, distal fringes (Figs 3, 5D). Ring 03 has 20 spinoscalids which show a well-developed sheath with a proximal flexible spine and a distal short fringe. Rings 04 and 05 consist of 10 and 20 spinoscalids respectively; all

resemble those of ring 03 but instead of a spine they have a fringed area (Fig. 5D). Ring 06 has 6 spinoscalids with the same appearance than those on previous rings but shorter. Ring 07 with 18 leaf-like scalids with a wide and hairy base from where several flexible elongations arise (Fig. 5E). See Fig. 3 for a polar diagram that summarizes the location and arrangement of oral styles, scalids and placids. Six long and hairy trichoscalids attaching to small trichoscalid plates are situated in sectors 2, 4, 5, 7, 8 and 10.

The neck consists of 16 placids numbered clockwise from the midventral 1 (Figs 3, 4B-C, 5E). Placids 2–16 are trapezoid measuring 9 μm at the base while the midventral placid is more rectangular and wider measuring 14 μm (Figs 2B, 4C, 5E). All placids articulate with the first trunk segment. Trichoscalid plates bearing trichoscalids appear dorsally on placids 6, 8, 10, 12 and ventrally on placids 2 and 16 (Fig. 3). Ventral trichoscalid plates are triangular with rounded edges, while dorsal trichoscalid plates are rounded and smaller (Figs 2A-B, 4B-C, 5D-E).

The trunk is divided into 11 segments (Figs 2A-B, 4A, 6A). Segments 1 and 2 consist of a closed cuticular ring (Figs 2A-B, 4A-C, 6A, C) while segments 3-11 are composed of one tergal and two sternal plates (Figs 2A-B, 4A, 6A). Glandular cell outlets type 1 consist of numerous minute pores and are situated in the anterior part of the segments usually hidden under the posterior part of the previous segment (Figs 2A-B). Dorsal outlets are unpaired middorsally on segments 1- 3 and 11, and paired paradorsally on segments 4-9 (Figs 2A, 4B). Segment 10 with two glandular cell outlets aligned in middorsal position (Fig. 2A). Ventral outlets are lateroventral on segment 1 and ventromedial on segments 2-10 (Figs 2B, 4C-E). Primary pectinate fringes well developed in all segments, showing very short fringe tips on segments 1 and 2 (Figs 2A-B, 6A, C) and long and flexible tips on remaining segments (Figs 2A-B, 6A-B, E). Secondary pectinate fringe absent on segment 1. Secondary fringes of segments 2-11

consisting of a single belt of minute and regular teeth usually hidden under the primary pectinate fringe of previous segment (Fig. 6B, E).

Segment 1 consists of a closed cuticular ring. Sensory spots are rounded with a collar of short papillae surrounding at least one pore. There are three pairs of sensory spots located very close to the anterior segment margin in subdorsal and laterodorsal positions and more posteriorly in ventromedial position (Figs 2A-B, 6A, C). Cuticular hairs are abundant and distributed forming a wide belt covering most of the dorsal surface of the segment, and narrowing towards the ventral side (Figs 2A-B, 4B-C, 6A, C). All cuticular hairs emerging from round perforation sites in this and the following nine segments. The posterior segment margin is straight along the dorsal and lateral sides, but extends more posteriorly in the ventromedial and midventral areas (Figs 2B, 6A).

Segment 2 consists of a closed cuticular ring. Three pairs of short, fringed tubes (ca. 4 μm from SEM) are located anteriorly in subdorsal, laterodorsal and ventrolateral positions, sometimes partially hidden under the previous segment and therefore difficult to spot (Figs 2A-B, 4B-C). These fringed tubes are composed of a short basal part attached to the trunk cuticle and a distal fringed end (see Fig. 6D for similar structure from segment 5). Three pairs of sensory spots are located in laterodorsal (two pairs) and ventromedial positions (one pair) and a single sensory spot in middorsal position. Sensory spots on this and the following segments are more elongated showing longer papillae in their posteriormost parts (see Fig. 6D for similar structure from segment 5). Hairs densely distributed in a belt covering the segment showing hairless areas in ventromedial position in this and the following segments (Fig. 2B). The posterior segment margin extends more posteriorly in midventral and paraventral areas (Figs 2B, 6A).

Segment 3 and the following segments consist of one tergal and two sternal plates. A pair of fringed tubes is located in lateroventral position. Two pairs of sensory spots are present in subdorsal and midlateral positions (Figs 2A-B, 4A-C). Dorsal hair pattern as described on previous segment. Cuticular hairs on the ventral side are densely covering the sternal plates except for a narrow hairless patch in the ventrolateral/ventromedial position.

Segment 4 with a minute middorsal acicular spine (6 μm) (Figs 2A, 6H). Two pairs of fringed tubes are located in subdorsal and lateroventral positions. No sensory spots present. Other characters similar to previous segment.

Segment 5 with a pair of long and thin lateroventral tubes (Figs 2B, 4C, 6B). Each tube consists of a short and smooth basal part, and a longer distal part with two small wing-like lateral projections. A pair of fringed tubules is present in midlateral position (Figs 2A, 4C, 6D). Three pairs of sensory spots are present in subdorsal, laterodorsal and ventromedial positions (Figs 2A-B). Other characters similar to previous segment.

Segment 6 with a pair of very reduced lateroventral spines measuring ca. 5 μm from SEM (Figs 2B, 6B) and easily confused with cuticular hairs. A pair of fringed tubes is present in laterodorsal position (Fig. 2A). Three pairs of sensory spots are present in subdorsal, laterodorsal and ventromedial positions (Figs 2A-B). Remaining characters as on previous segments.

Segment 7 with a pair of very short acicular spines slightly longer (ca. 6 μm from SEM) than those on segment 6 (Figs 2B, 6B). A pair of fringed tubes is present in sublateral position above the insertion line of the lateroventral spines (Figs 2B, 6B). Three pairs of sensory spots located in the same positions as on segment 6 (Figs 2A-B, 6A). Remaining characters as on previous segments.

Segment 8 with a pair of lateroventral tubes similar to those described on segment 5 (Figs 2B, 4E, 6I). Two pairs of fringed tubes are located in laterodorsal and sublateral positions plus an unpaired fringed tube in middorsal position (Figs 2A-B, 6I). A single pair of sensory spots is present in subdorsal position. Other characters similar to previous segments.

Segment 9 without acicular spines or tubes. Four pairs of sensory spots are present in paradorsal, subdorsal, midlateral and ventrolateral positions (Figs 2A-B, 6A, E). A par of large and very elongated sieve plates (c.a 15 μm length from LM) is present in sublateral position. These sieve plates consist of an oval perforated field with a posterior round pore (Figs 2B, 4E, 6E, E'). Other characters similar to previous segments.

Segment 10 with a pair of laterodorsal tubes at or near posterior segment margin. In males the tubes are long and similar of those described on segments 5 and 8 located in small indentations in the posterior segment margin (Figs 2C-D, 4D, F, 6F). In females the tubes lack the basal part, showing just a flexible and short tube-like structure (Figs 2A-B, 4H, 6E). Two pairs of elongated sensory spots are present in subdorsal and ventrolateral positions (Figs 2A-B, 6E-G). The posterior segment margin of the tergal plate is straight and with a small but well-developed pectinate fringe (Fig. 6E), whereas the margins of the sternal plates are concave, extending posteriorly near the midventral junction, and with well-developed fringe tips (Figs 2B, 6 E-G).

Segment 11 with lateral terminal spines (Figs 2A-D, 4A, F-H, 6A, E-G). Males with three pairs of penile spines, two of them flexible and elongated (ca 20 μm from LM) and one short and stout (Figs 2 C-D, 4F, 6F). Additionally, males show a modified pectinate fringe of the sternal plates forming a long and flexible fringed tuft close to the insertion of the lateral terminal spines (Figs 2D, 6F). Females with a pair of extremely

reduced lateral terminal accessory spines with fringed ends (Figs 2A-B, 4G-H, 6E, G). One pair of sensory spots is present in paradorsal and two pairs in ventromedial positions (Figs 2A-B, 6 E, F-G). Paradorsal sensory spots are larger, situated adjacent to the middorsal fringed area of the tergal plate. Ventromedial sensory spots are round and small, and situated at the posterior edge of the segment (Figs 2A-B, 6F-G). The segment is completely devoid of cuticular hairs but has hair-like extensions and fringes covering the margins of the tergal plate (Figs 6A, F-G). Tergal extensions are long and pointed and ventrally hairy with a distinct notch. (Figs 2A-B, 6F-G). Sternal plates with a straight posterior margin and a ventromedial projection (Figs 2B, 6F-G).

3.2. *Echinoderes kozloffi* Higgins, 1977 (Figs 7-12, Tables 3-4)

3.2.1. Emended diagnosis

Echinoderes with four middorsal spines on segments 4-8. Spines from segments 4-7 slightly increasing in length while the middorsal spine on segment 8 is twice as long as the previous. Lateroventral spines present on segments 6-9. Tubes present in lateroventral position on segments 2 and 5 and in laterodorsal position on segment 10. Glandular cell outlets type 2 in laterodorsal position on segment 8, females furthermore with ventrolateral cuticular papillae on segments 6-7 and ventromedial on segment 8. Segments 2-3 showing strongly developed pectinate fringe on the ventral side while weakly developed in the dorsal side.

3.2.2. Material examined

Female holotype (USNM 53337) and male allotype (USNM 53338) were loaned from the Smithsonian Institution, United States National Museum, and examined with light microscope equipped with DIC optics. The type specimens originate from North

Bay, San Juan Island, Washington, USA. Additional material includes 14 specimens: two of them collected intertidally at the type locality in San Juan Island, 10 collected intertidally from brown algae in Clover Point, Victoria, British Columbia, Canada and 2 collected in Archaeology Beach in Calvert Island, BC (Fig 1B-C). Seven specimens were mounted for LM, of them 3, one male from Victoria and two females (one from Victoria and one from Calvert Island) are deposited at the Natural History Museum of Denmark under catalogue numbers ZMUC KIN-959 to KIN-960 (Victoria) and KIN-960 (Calvert Island). Remaining LM and SEM specimens are stored in the authors' personal reference collection.

3.2.3. DNA sequence

The mitochondrial cytochrome c oxidase subunit 1 (COI) gene form two specimens, one from Calvert Island and one from Victoria (GenBank Accession numbers KU681519 - KU681520, respectively). The cuticle could be recovered just from the specimen from Calvert Island and deposited as a voucher at the Natural History Museum of Denmark, under catalogue number ZMUC KIN-969.

3.2.4. Description

Adults with head, neck and eleven trunk segments (Figs 7A-B, 10A-B, 11A). Measurements and dimensions are given in Table 3. A summary of sensory spots, spines, papillae, sieve plates, tubes and glandular cell outlet positions is provided in Table 4. The head consists of a retractable mouth cone and an introvert (Figs 8, 11C-F). Outer armature of the mouth cone formed by nine outer oral styles divided into two subunits, slightly alternating in size between 5 longer ones situated according to uneven sectors of the introvert, and 4 shorter ones situated according to even sectors (Figs 8, 11C-D); middorsal outer oral style is missing. Outer oral styles with a short fringe at

their bases composed of 15-20 small and regular tips and an additional distal fringe composed of 3-4 elongated and flexible tips. Inner armature of the mouth cone could not be studied.

The introvert has seven rings of cuticular spinoscalids and one additional ring of trichoscalids that are associated with the placids (Figs 8, 11C). Ring 01 with 10 primary spinoscalids consisting of a short basal sheath and a distal end piece. The basal sheath has a small proximal fringe, situated very close to the insertion point, showing just two lateral tips, followed by a smooth part that projects into 6-8 flexible fringed tips (Fig. 11D-F). The distal pieces of the primary spinoscalids are laterally compressed and each bears a fringe composed of at least ten elongated flexible fringe tips. Ring 02 is composed of 10 laterally compressed spinoscalids, all formed by a long smooth basal which ends into a long fringe (Fig. 11E-F). Rings 03, 04 and 05 consist of 20, 10 and 20 spinoscalids respectively; all resemble those of ring 02 (Fig. 11E-F). Ring 06 has 6 spinoscalids with the same appearance but shorter than those on previous rings. The number and arrangement of spinoscalids from rings 6-7 could not be assessed with certainty in all sectors due to the partial eversion of the introvert in the specimens studied, but plotted in the polar diagram when visible (Fig 8). Six long and hairy trichoscalids attached to trichoscalid plates are situated ventrally in sectors 2, 10 and dorsally in sectors 4, 5, 7, 8 (Figs 8, 9A-B, 11B).

The neck is composed of 16 elongated placids with the midventral placid being rectangular and wider, measuring ca. 15 μm at the base, while the remaining ones are more trapezoid, measuring ca. 10 μm at the base (from LM) (Figs 9A-B, 11B). All placids articulate with the first trunk segment. Trichoscalid plates bearing trichoscalids appear dorsally on placids 6, 8, 10, 12 and ventrally on placids 2 and 16 (Fig. 8).

Ventral trichoscalid plates are triangular-shaped with rounded edges, while dorsal trichoscalid plates are more oval and smaller (Figs 7A-B, 9A-B, 10 B, 11B).

The trunk is divided into 11 segments (Figs 7A-B, 10A-B, 11A). Segments 1-2 are composed of a closed ring (Figs 7A-B, 9A-B, 10A-B, 12A) while segments 3-11 are composed of one tergal and two sternal plates (Figs 7A-B, 10A-B, 12A). Glandular cell outlets type 1 situated in the anterior part of the segments usually hidden under the posterior part of the previous segment. Dorsal outlets are single and middorsal on segments 1- 3 and 11, appearing as two middorsal outlets on segment 10, and paired and subdorsal on segments 4-10 (Figs 7A-B, 10 A-B). Ventral outlets are lateroventral on segment 1 and ventromedial on segments 2-10 (Figs 7A-B, 10A-B). Primary pectinate fringe well developed on all segments, showing very short fringe tips on segment 1 and dorsally on segments 2-3 (Figs 7A-B, 12A, D) but with long and flexible tips ventrally on segments 2-3. Transition from long to short pectinate fringes in segments 2-3 occurs at midlateral position (Fig. 12D). Remaining segments with uniform long pectinate fringes (Figs 7A-B, 12A). Secondary pectinate could not be studied due to the degree of contraction in the specimens. Cuticular hairs abundant in all segments, emerging from round perforation sites on segment 1 (Fig. 12A) while from horizontal flat perforation sites in remaining segments (Fig. 12B).

Segment 1 consists of a closed cuticular ring (Figs 7A-B, 9A-B, 10A-B, 12A). Three pairs of sensory spots are located in subdorsal, midlateral and ventrolateral positions. Subdorsal and midlateral sensory spots located closed to the anterior segment margin than the ventrolateral ones, all of them small and round, composed of short papillae surrounding a cilium (Figs 7A-B, 11B, 12A). Cuticular hairs very long, distributed forming a continuous belt of 3-5 rows that narrows towards the ventral side and widens again in midventral position (Figs 7A-B, 12A).

Segment 2 consists of a closed cuticular ring (Figs 7A-B, 9A-B, 10A-B, 12A) with a pair of long lateroventral tubes with a typical configuration (basal short piece and distal wing-like piece). Three pairs of oval sensory spots with the same appearance of those on segment 1 present in subdorsal, laterodorsal and ventromedial positions. Cuticular hairs covering the surface forming a continuous belt interrupted in ventromedial position. Paraventral areas showing a patch of cuticular hairs without perforation sites (Figs 7B, 12A).

Segment 3 and remaining trunk segments consist of a tergal and two sternal plates (Figs 7A-B, 9A, 10 A-B, 11A). Three pairs of sensory spots with the same appearance as on previous segments present in subdorsal, midlateral and ventromedial positions. Cuticular hairs as described in previous segments.

Segment 4 with a short, middorsal acicular spine (Figs 7A, 9E, 10A). Two pairs of sensory spots are located in laterodorsal and ventromedial positions. Cuticular hairs as described in previous segments.

Segment 5 with a middorsal acicular spine slightly longer (2-3 μm) than the one on previous segment (Figs 7A, 9E, 10A). A pair of long tubes as described for segment 2 is present in lateroventral positions (Figs 7B, 9C-D, 10B, 12A). Three pairs of sensory spots present in subdorsal, laterodorsal and ventromedial positions (Figs 7A-B, 12A). Cuticular hairs as described in previous segments.

Segments 6 and 7 with the same composition showing a middorsal acicular spine slightly longer than the one on previous segments, flanked by a pair of oval sensory spots (Figs 7A, 9E, 10A), and a pair of lateroventral acicular spines with fringed edges (Figs 7B, 9C-E, 10A-C, 12A, C, E). Additional pairs of sensory spots in subdorsal, laterodorsal and ventromedial positions. Females furthermore with a pair of small

cuticular openings surrounded of thin papillae in ventrolateral position visible both in LM and SEM (Figs 7B, 9D, 10C, 12C). Cuticular hairs as described in previous segments.

Segment 8 with a middorsal acicular spine almost twice as long as the spines of segments 4-7 (41 μm vs. 20-26 μm) flanked by a pair of paradorsal oval sensory spots (Figs 7A, 9E, 10A, 12G). A pair of acicular spines with the same appearance as those on previous segments present in lateroventral position. Additional paired sensory spots present in subdorsal and laterodorsal positions. A pair of glandular cell outlets type 2 is present in laterodorsal position slightly anterior to the sensory spots (Figs 7A, 9H, 12B). Females furthermore with ventromedial papillary structures as described for segments 6-7 (Figs 7B, 9D, 10C). Cuticular hairs as described in previous segments.

Segment 9 with a pair of lateroventral acicular spines (Figs 7B, 9F-G, 10B-C). Paired sensory spots present in paradorsal, subdorsal, laterodorsal and ventrolateral positions (Figs 7A-B, 12G). A pair of round sieve plates (c.a. 2μm from SEM) with 20-30 holes plus an additional posterior round pore present in lateral accessory position (Figs 7B, 9G, 10C). Cuticular hairs as described in previous segments.

Segment 10 with a pair of long tubes in laterodorsal position near the posterior margin of the segment in both females and males (Figs 7A-D, 9F-G, 10B, D, 12G-H). Two pairs of oval sensory spots present in subdorsal and ventrolateral positions (Figs A-B, 12F-G). Posterior edge of the segment straight in the tergal plate whereas extending posteriorly in a V-shape towards the paraventral/ventromedial area in the sternal plates (Figs 7B, 12F). Cuticular hairs as described in previous segments.

Segment 11 with lateral terminal spines (Figs 7A-B, 9F-G, 10B, D, 11A, 12F-H). Females with a pair of lateral terminal accessory spines (Figs 7A-B, 9F, 10B, 12H) and

males with three pairs of long penile spines (Figs 7C-D, 9G, 10D, 12F). Two of the penile spines (p1 and p3) are flexible and elongated while p2 is shorter and wider (Fig. 12F). Two pairs of sensory spots are present on the tergal plate, in subdorsal position, and one pair in ventromedial position in the sternal plate (Fig 12G). Tergal extensions are short and pointy projecting slightly longer than the sternal plates (Figs 7A, D, 10D, 12G-H). Sternal plates triangular shaped projecting at the ventromedial position (Figs 7B, D, 12F). Segment densely covered with cuticular hairs without perforation sites (Fig 12 F-G).

4. Discussion

4.1. Notes on diagnostic features in E. ohtsukai

E. ohtsukai was recently described from the Seto Inland Sea in Japan by Yamasaki and Kajihara (2012). Despite the high quality of morphological data from both LM and SEM, the description lacks several important diagnostic features that were added in the present redescription. These new characters include the presence of minute lateroventral acicular spines on segments 6-7, extremely reduced LTAS in females, and extra fringed tubes and sensory spots on certain segments. These structures are very small and easy to overlook, sometimes hidden under either the dense cuticular hairs or the free flap from the previous segment. Furthermore, some of these structures are only visible using SEM, such as the acicular spines of segments 6-7, which could be easily confused with cuticular hairs using LM. Nonetheless, this pattern of spines and tubes distinguishes *E. ohtsukai* from other species of *Echinoderes*.

E. ohtsukai plus eight additional species belong to the so-called ”*Echinoderes coulli* group” (Ostmann et al., 2012). This group of species is found in intertidal and estuarine environments and is characterized by (1) the presence of enlarged sieve plates, which is inferred to be functionally associated with brackish environments; (2) the reduction or

absence of acicular spines in the middorsal and lateroventral areas; and (3) the reduction or absence of the LTAS in females [see Table 5 for updated diagnostic characters in species of the *Echinoderes coulli* group based on Sørensen (2014) and Yamasaki and Fujimoto (2014)]. Within this group, *E. ohtsukai* shares the presence of a middorsal spine on segment 4 with *E. maxwelli* Omer-Cooper, 1957*; Echinoderes rex* Lundbye et al., 2011; and *E. teretis* Brown, 1985 in Adrianov and Malakhov, 1999 (see Lundbye et al., 2011 for *E. maxwelli*) (Adrianov and Malakhov, 1999; Lundbye et al., 2011; Omer-Cooper, 1957). The species that most resembles *E. ohtsukai* is *E. rex*, because they both share a minute middorsal spine on segment 4, lateroventral spines/tubes on segments 5-8 and dorsoventral tubes on segment 10 (Table 5). Both species are also distinctive within the *Echinoderes coulli* group by sharing fringed tubes also named "modified glandular cell outlets type 2" (Yamasaki and Kajihara, 2012). However, it is possible that these modified glandular outlets are present in *E. maxwelli* as well (Lundbye et al. 2011); this study also confirmed the presence of minute lateroventral spines on segment s 6-7 (Table 5). Although the trunk length of *E. rex* is considerably longer than in *E. ohtsukai* (482-528 µm vs. 330-410 µm), the lateral terminal spines of *E. rex* are much shorter than those of *E. ohtsukai* (20-24 µm vs. 178-181 µm). *E. rex* has two pairs of penile spines in males whereas three well-developed penile spines are present in *E. ohtsukai.* The presence of very reduced lateral accessory spines was also reported for *Echinoderes hwiizaa* Yamasaki and Fujimoto, 2014 and *Echinoderes komatsui* Yamasaki and Fujimoto, 2014 (Yamasaki and Fujimoto, 2014). Despite the enlarged sieve plate exhibited by *E. rex* it is the only of the *E. coulli* group species found subtidally and therefore considered fully marine (Lundbye et al., 2011).

Outside of the *Echinoderes coulli* group, the only other species with a spine/tube pattern that is similar to *E. ohtsukai* is *Echinoderes cantabricus* Pardos et al., 1998. These two

species share a middorsal spine on segment 4 and ventrolateral tubes/spines on segments 5-8 (Pardos et al., 1998). However, *E. cantabricus* differs from *E. ohtsukai* in many other characters: presence of a pair of midlateral tubes on segment 1, presence of four pairs of tubes on segment 2, no fringed tubes, presence of small and rounded sieve plates, and an overall barrel-shape. *E. cantabricus* also has relatively short tergal extensions when compared to those in *E. ohtsukai* and relatively long lateral terminal accessory spines in females compared to those in *E. ohtsukai*.

Our reexamination of *E. ohtsukai* and inclusion of new diagnostic features confirms its placement within the *E. coulli* group; however the fact that it has very reduced lateroventral spines suggest a closer relationship with *E. rex* and *E. maxwelli* and with the recently described *E. hwiizaa* and *E. komatsui* for the presence of very small lateral accessory terminal spines in females. The generation of molecular phylogenetic data is expected to resolve the internal relationships within the *E. coulli* group.

4.2. Notes on epibiontic growth in E. ohtsukai

Epibiontic filamentous bacteria were found in almost all specimens collected. Some of the specimens had a heavy coverage of these bacteria attaching to different places on the head (Fig. 5C-E) and trunk regions (Figs 6C, I, 13A-C), but epibionts were mostly concentrated in the posteriormost segments or in areas restricted to the glandular openings and sensory spots (Fig. 13A, C). Live animals looked and behaved normally, so the presence of the epibionts did not appear to affect the health of the kinorhynchs. Epibiontic assemblages like these have been described on kinorhynchs before (Neuhaus, 2013), including *Echinoderes* species such as *E. spinifurca* reported from the tropical Atlantic Ocean (Neuhaus, 2013) and *E. applicitus* from Java (Ostmann et al., 2012). Different kinds of epibionts were described on *E. applicitus*, including ciliates and

filamentous bacteria that were very similar in appearance to the epibionts we observed on *E. ohtsukai*. Ostmann et al. (2012) tentatively identified them as sulfur bacteria from the genus *Thiothrix*, which have also been reported on other intertidal marine animals, such as amphipods (Gillan and Dubilier, 2004). *Thiothrix* is present in environments with high sulfur content and a wide range of salinities, which corresponds with the conditions of the sampling locality of *E. ohtsukai* in Boundary Bay. However, the precise identity of the epibionts on *E. ohtsukai* will require information from molecular sequence data.

4.3. Notes on diagnostic features in E. kozloffi

Echinoderes kozloffi was described by R.P Higgins in 1977 from San Juan Island in Washington, USA, but previously collected by Kozloff in 1972 (named *Echinoderes* sp.) and Merriman and Corwin in 1973 who mistakenly identified it as *Echinoderes dujardinii* (Kozloff, 1972; Merriman and Corwin, 1973). The description of *E. kozloffi* is based on traditional light microscopy observations providing a line art illustration but lacking (1) any images produced from LM or SEM and (2) molecular phylogenetic data. *E. kozloffi* also belongs to a group of around 35 *Echinoderes* that share the same spine/tube pattern consisting of 5 middorsal spines and lateroventral tubes/spines on segments 5-9. The reexamination and redescription of *E. kozloffi* was necessary in order to complete and provide new diagnostic characters to facilitate its identification among the remaining 34 species of similar *Echinoderes*. Over the years kinorhynch taxonomy has evolved, providing more detailed descriptions that focus on different characters beyond the distribution of spines and tubes. Because *E. kozloffi* is a widespread species along the northeastern Pacific coast, we were able to collect enough material to pursue this redescription. The examination of the type specimens together with new specimens

(examined with LM and SEM) provided new diagnostic characters that were previously overlooked or only briefly mentioned in the original description. These characters are: (1) the presence of a pair of laterodorsal glandular cell outlets type 2 on segment 8; (2) irregular and very conspicuous pectinate fringe on segments 2-3, well-developed on the ventral side while very short and thin on the dorsal side; (3) and dimorphic papillae or modified glands in females situated ventrolaterally on segments 6-7 and midventrally on segment 8. The presence of the papillae was briefly mentioned in the original description as "prominent scars" but never identified as a dimorphic feature (Higgins, 1977a). The SEM observations of the new material allowed the inclusion of the introvert description (Fig. 8) and mapping of the sensory spots of the trunk (see Table 4).

The combination of the old and new diagnostic characters enables straightforward differentiation of *E. kozloffi* from relatively similar species of *Echinoderes*. The pattern of middorsal spines combined with the presence of ventrolateral spines/tubes on segments 5-9 and ventrolateral tubes on segment 2 is shared by 21 other species of *Echinoderes*, but the addition of a pair of laterodorsal glandular cell outlets type 2 in segment 8 reduces the number to only 2 other species: *Echinoderes gerardi* Higgins, 1978 and *E. microaperturus* Sørensen et al., 2012 (Higgins, 1978; Sørensen et al., 2012b). The description of *E. gerardi* represents another one of the early contributions by Higgins, described just one year after *E. kozloffi*. *E. gerardi* was subsequently collected and revised by other researchers, who described the presence of paired glandular cell outlets type 2 on segment s 8-9 (see contribution of Sørensen et al. in this special issue). *E. gerardi* differs from *E. kozloffi* by the presence of two pairs of glandular cell outlets type 2, instead of one pair, and by the presence of lateral accessory

tubes on segment 8. *E. gerardi* can be distinguished further by its extraordinary short middorsal spines measuring 8-10 μm compared to 17-44 μm in *E. kozloffi*.

E. microaperturus differs from *E. kozloffi* by the presence of extra subdorsal glandular cell outlets type 2 on segment 2 and laterodorsal spines on segment 9; and the presence of long and pointy tergal extensions instead of the short and round ones found in *E. kozloffi*. Both *E. gerardi*, *E. microaperturus* and *E. kozloffi* share dimorphic ventral glands (or papillae) in females. In case of *E. gerardi*, the glands are positioned in the same segments as *E. kozloffi* (Higgins, 1978) while *E. microaperturus* lacks the pair on segment 6. The presence of these papillae or glands (also described as brace-shaped, bracket-shaped or prominent scars) was also investigated by Thormar and Sørensen (2010) reporting their potential presence in several other *Echinoderes* spp (see Thormar and Sørensen 2010 for the complete list of species) but confirming it for *Echinoderes collinae* Sørensen, 2006, *Echinoderes spinifurca* Sørensen, 2006 and *E. gizoensis* Thormar and Sørensen, 2010. These glands also appear to be present in the females of other echinoderid genera such as *Fissuroderes* and *Cephalorhyncha* (Thormar and Sørensen, 2010), therefore, it seems that the presence of dimorphic glands/papillae may be way more widespread than originally thought but just overlooked in the original descriptions.

The glandular cell outlets type 2 and the fringed tubes were also very important features in the present redescriptions of *E. kozloffi* and *E. ohtsukai*. These traits were either not observed or poorly described in the original descriptions of these two species. Sørensen et al. (this issue) reexamined several "old *Echinoderes*" species from the Smithsonian Natural History Museum collections and found undescribed glandular cell outlets type 2 in most of them (*E. abbreviatus* Higgins, 1983, *E. andamanensis* Higgins and Rao, 1979, *E. bookhouti*, *E. imperforatus* Higgins, 1983, *E. kristenseni* Higgins, 1985, *E.*

pennaki Higgins, 1960, *E. truncatus* Higgins, 1983, and *E. wallaceae* Higgins, 1983). Outside *Echinoderes*, these glands also play an important role as a taxonomic character in the genera *Meristoderes* and *Fissuroderes* (see Herranz and Pardos, 2013; Neuhaus and Blasche, 2006; Sørensen et al., 2013).

In the present publication, we generated DNA sequences of the barcode region of the mitochondrial gene cytochrome c oxidase subunit I (COI) for both *E. ohtsukai* and *E. kozloffi* to complement the morphological descriptions. We think it is important that new descriptions and redescriptions of kinorhynch species include molecular phylogenetic data to complement the comparative morphological data. Future identifications of kinorhynch species will be greatly facilitated by the availability of DNA sequences with adequate variation, especially when studying problematic species and potential cryptic species where the diagnostic morphological traits are inconspicuous.

4.4. Notes on the geographical distribution of E. ohtsukai and E. kozloffi

Both *E. ohtsukai* and *E. kozloffi* are part of the largest and most diverse genus within kinorhynchs, which extends worldwide from the intertidal zone to the abyss (Neuhaus, 2013; Sørensen and Pardos, 2008). The species with the widest geographical distribution among *Echinoderes* is *E. tchefouensis* Low, 1934, which has been recorded from China, Korea, Philippines, Malaysia, Marianas and Singapore (Sørensen et al., 2012b; Sørensen et al., 2016).

E. kozloffi was mainly recorded from intertidal localities in the Pacific coast of the United States (Higgins, 1977a; Kozloff, 1972; Merriman and Corwin, 1973) but also tentatively reported from subtidal areas in Hawaii (see Thormar and Sørensen, 2010). The specimens we collected came from two different intertidal localities situated 500

km apart (Calvert Island and Victoria, British Columbia) (see Fig.1). The sampling area in Victoria is relatively close (20-30 km) to the type locality in San Juan Island, Washington, but the second locality (Calvert Island) is farther north and enlarges the known distribution area for the species. Interestingly, the COI sequence for these isolates was identical in specimens collected from both sampling sites (Victoria and Calvert Island), meaning that they could belong to the same population. Additional studies using DNA barcodes (e.g., COI) from specimens isolated from different sampling sites will be able to provide a more complete picture of the distributional boundaries for this species.

Species within the *Echinoderes coulli* group show restricted distributions and are, except for *E. rex* which is fully marine, always associated with brackish environments (Lundbye et al., 2011; Ostmann et al., 2012; Yamasaki et al., 2014; Yamasaki and Fujimoto, 2014; Yamasaki and Kajihara, 2012). The species with the widest reported distribution in this group is *Echinoderes coulli* Higgins, 1977, which was described from the northwestern Atlantic Ocean extending along the coasts of North and South Carolina (Coull and Wells, 1981; Higgins, 1977b; Higgins and Fleeger, 1980). *E. ohtsukai* also belongs to the *E. coulli* group and was originally reported from a brackish mud flat in the Seto Inland Sea in Japan (Yamasaki and Kajihara, 2012). Surprisingly, and contrary to the known restricted distributions of other species within the *E. coulli* group, we found *E. ohtsukai* on the opposite side of the Pacific Ocean (i.e., the British Columbian coastline), in an environment that resembles the conditions of the Japanese type locality. This result could be explained by two alternative hypotheses: (1) the distribution area of *E. ohtsukai* is the largest so far for any known species of *Echinoderes* and extends beyond brackish habitats into subtidal environments as in *E. rex* (Lundbye et al., 2011) or (2) *E. ohtsukai* is an invasive species in either Japanese or

British Columbian coastal environments. The first hypothesis comes with additional uncertainties such as how an exclusively intertidal (brackish) species extended its population across the Pacific Ocean within the context of the limited reproductive and dispersive potential that characterizes kinorhynchs. If this hypothesis is correct, then future studies should be able to demonstrate the presence of *E. ohtsukai* in different areas and depths across the Pacific Ocean. Current biogeographical evidence, however, challenges this hypothesis because the coasts of Japan and Korea have been intensively surveyed for kinorhynchs from deep sea sediments to the intertidal zone, and so far *E. ohtsukai* has only been reported from the type locality (e.g. Sørensen et al., 2010, 2012a, 2012b, 2013; Thomsen et al., 2013; Yamasaki, 2015; Yamasaki and Fujimoto, 2014; Yamasaki et al., 2014; Yamasaki and Kajihara, 2012).

Molecular fingerprints strongly support the hypothesis that *E. ohtsukai* is an invasive species in one of the opposite coasts of the Pacific Ocean. The COI sequences generated from specimens of *E. ohtsukai* isolated from the type locality in Japan and from specimens of *E. ohtsukai* isolated from British Columbia were identical, which is corroborated by their identical morphologies. These results are particularly striking if we compare them with the results of the only and most recent phylogeographical study on two intertidal species of *Echinoderes* from Japan. This study showed low connectivity between two relatively nearby populations having intraspecific divergences in the COI DNA sequences ranging between 0.44% to 0.85% and 0.05% to 1.12% (Yamasaki et al., 2014). Moreover, the COI sequences generated from the Japanese and British Columbian isolates of *E. ohtsukai* were sequenced in different laboratories (in different years by different researchers using slightly different methods; this eliminates contamination issues.

It is difficult to explain how specimens from a single population that spans across the world's largest ocean share exactly the same DNA sequence for the mitochondrial COI gene. This result is best explained by human-mediated transportation of one population on one side of the Pacific Ocean to a similar environment on the other side. The coast of British Columbia has historically had a high level of maritime traffic connecting to the coast of Japan. We strongly doubt that ship ballast water and bio-fouling are likely invasion vectors, and suspect that aquacultural activities played a major role in transporting kinorhynchs from one coast of the Pacific Ocean to the other.

The sampled area in British Columbia (Boundary Bay) is well known for its high numbers of benthic exotic species originating from Japan (e.g., six species of bivalves, seven species of snails and four species of polychaete worms) (see Klinkenberg, 2015 for a list of species). Almost all of these exotic species were related to the introduction and maintenance of Japanese and Atlantic oysters for commercial production. The transplant of the so-called Pacific oyster (also known as the giant or Japanese oyster *Crassostrea gigas*) was initiated around 1914 and became the mainstay of the oyster industry in British Columbia (Carlton, 1979; Waldichuk et al., 1994). However, because this species was not able to reproduce in the low temperatures of BC waters it was regularly imported from Japan, increasing the risk of introduction of exotic fauna. This practice was regulated in 1940, but by that time most of the introductions might had already occurred. Apparently most of the known introduced species have remained localized in a very restricted area (Waldichuk et al., 1994). If this was the case for *E. ohtsukai*, then we should not be able to find it in nearby localities with the same conditions.

Nonetheless, the aim of this paper was not to accomplish a phylogeographical analysis of the disjunt populations of *E. ohtsukai*, but to report this interesting finding in order to

motivate future investigations on this topic. These results will certainly affect the way we interpret the distribution of kinorhynch species and other meiofaunal groups, opening new debate and areas of research.

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Figure legends

Fig. 1. Maps showing the sampling locations in the northeastern Pacific coast. Sampling areas marked as A, B, C. A. Vancouver area, showing the sampling locality of *Echinoderes ohtsukai* at Mud Bay Park. B. Victoria area, showing the sampling locality of *Echinoderes kozloffi* at Clover Point. C. Calvert Island area, showing the sampling locality of *Echinoderes kozloffi* at Archaeology Beach. Station numbers are given in brackets.

Fig. 2. Line art illustrations of *Echinoderes ohtsukai*. A. Female, dorsal view. B. Female, ventral view. C. Male, detail of segments 10-11, dorsal view D. Male, detail of segments 10-11, ventral view. The legend shows all the cuticular characters represented in the line art excluding spines and tubes. Scale bar, 100 μm.

Fig. 3. Diagram of mouth cone, introvert and placids showing the distribution of oral styles, scalids and trichoscalid plates in *Echinoderes ohtsukai*. The table below shows the scalid arrangement by sector and summarizes scalid numbers by rings and sectors. "Double diamonds" are marked in the table with double lines and quincunxes are marked with dotted lines. Abbreviations: ls, leaf-like scalid; oos, outer oral style; psp, primary spinoscalid; sp, spinoscalid; ts, trichoscalid. Question marks indicate uncertain positions.

Fig. 4. Light micrographs (DIC) showing traits in *Echinoderes ohtsukai*. Male KIN-923 (A, B, D, F), female KIN-956 (G), female KIN-957 (C, E) and (H) female KIN-958. A. Male overview, ventral view. B. Detail of segments 1-4, dorsal view. C. Detail of segments 1-5, ventral view. D. Detail of segments 8-10, dorsal view. E. Detail of segments 8-9, lateroventral view. F. Male, detail of segments 9-11, focus is on the penile spines. G. Female, detail of segments 9-11, ventral view. H. Female, detail of

segments 9-11 lateral view. Abbreviations: ldt, laterodorsal tube; lts, lateroterminal spine; ltas, lateral terminal accessory spine; lvt, lateroventral tube; pl, placid; ps, penile spines; si, sieve plate; te, tergal extension; tp, trichoscalid plate. Circles indicate the position of glandular cell outlets type 1. Arrowheads indicate the position of fringed tubes. Digits after abbreviations refer to segment number.

Fig. 5. Scanning electron micrographs (SEM) showing introvert and mouth cone morphology of *Echinoderes ohtsukai*. A. Extended head, dorsal view. B. Detail of the mouth cone, lateral view. C. Detail of the inner armature of the mouth cone, apical view (Note that in this specimen there are only 8 outer oral styles, most likely a mutation). D. Introvert showing sector 2, lateroventral view. E. Detail of the neck showing the placids and trichoscalids, head partially extended, ventral view. Abbreviations: fb, filamentous bacteria; i, introvert; ios -01-03, inner oral styles; lsp, leaf-like spinoscalid; mc, mouth cone; mvp, midventral placid; oos, outer oral styles; psp, primary spinoscalids; sp1-5, spinoscalids, number refers to the rows; tr, trichoscalid. Asterisks mark the middorsal position. Dashed circles indicate the position of sensory spots.

Fig. 6. Scanning electron micrographs (SEM) showing overviews and details of *Echinoderes ohtsukai*. A. Male, ventral overview. B. Detail of segments 6-7 lateroventral view. C. Detail of neck and segment 1, dorsal view. D. Detail of segment 5, showing a fringed tube and a sensory spot, laterodorsal view. E. Details segments 8-11, laterodorsal view. Inset (E') shows a close up of the sieve plate. F. Male, detail of segments 10-11, ventral view. G. Female, detail of segments 10-11, ventral view. H. Detail of middorsal spine on segment 4, dorsal view. I. Detail of tube and fringed tube of segment 8, lateroventral view. Abbreviations: fb, filamentous bacteria; ft, fringed tube; ldss, laterodorsal sensory spot; ldt, laterodorsal tube; lts, lateroterminal spine; ltas, lateral terminal accessory spine; lvs, lateroventral spine; lvt, lateroventral tube; md,

middorsal spine; ps, penile spines; si, sieve plate, spf, secondary pectinate fringe; te, tergal extension. Dashed circles indicate the position of sensory spots. Digits after abbreviations refer to segment number.

Fig. 7. Line art illustrations of *Echinoderes kozloffi*. A. Female, dorsal view. B. Female, ventral view. C. Male, detail of segments 10-11, dorsal view D. Male, detail of segments 10-11, ventral view. The legend shows all the cuticular characters represented in the line art excluding spines and tubes. Scale bar, 100 μm.

Fig. 8. Diagram of mouth cone, introvert and placids showing the distribution of oral styles, scalids and trichoscalid plates in *Echinoderes kozloffi.* The table present in Figure 3 was omitted here due to the lack of information on the number and arrangement of the last scalid rows. Question marks indicate uncertain positions.

Fig. 9. Light micrographs (DIC) showing details in female holotype, USNM 53337 (A, D, E, F, H) and male allotype USNM 53338 (B, C, G) of *Echinoderes kozloffi* from the northeast Pacific described by Higgins (1977). A. Detail of the neck and segments 1-3, ventral view. B. Detail of the neck and the first trunk segment, dorsal view. C. Detail of right half of segments 5-8 in male allotype, ventral view. D. Female, detail of left half of segments 5-8 in, ventral view. E. Male, detail of segments 4-8, dorsal view. F. Female, detail of segments 9-11, ventral view. G. Male, detail of segments 9-11, ventral view. H. Detail of left glandular cell outlet type 2 on segment 8, dorsal view. Abbreviations: gco2, glandular cell outlet type 2; ldt, laterodorsal tube; lvt, lateroventral tube; lvs, lateroventral spine; ltas, lateral terminal accessory sipine; lts, lateral terminal spine; md, middorsal spine; pa, papillae; pl, placid; ps, penile spines; si, sieve plate; tp, trichoscalid plate. Circles indicate the presence of glandular cell outlets type 1. Digits after abbreviations refer to segment number.

Fig. 10. Light micrographs (DIC) showing traits in *Echinoderes kozloffi*, female KIN-961 (A), female KIN-960 (B-C) and male KIN-959 (D). A. Dorsal overview segments 1-10. B. Ventral overview. C. Detail of left sternal plate of segments 6-9 focused in order to visualize the papillae. D. Segments 10-11, ventral view. Abbreviations: gco1, glandular cell outlet type 1; ldt, laterodorsal tube; lvt, lateroventral tube; lvs, lateroventral spine; ltas, lateral terminal accessory sipine; lts, lateral terminal spine; md, middorsal spine; pa, papillae; ps, penile spines; si, sieve plate; te, tergal extensions. Dashed circles indicate the position of sensory spots, solid line circles indicates the presence of glandular cell outlets type 1. Digits after abbreviations refer to segment number.

Fig. 11. Scanning electron micrographs (SEM) showing traits of the introvert and mouth cone of *Echinoderes kozloffi.* A. Female overview, lateral view. B. Detail of the neck and segment 1, apical view, head retracted. C. Overview of the mouth cone introvert and neck, dorsal view. C. Detail of the mouth cone showing the outer oral styles. D. Detail of introvert sector 5. E. Detail of introvert sector 4. Abbreviations: i, introvert; mc, mouth cone; mdp, middorsal placid; mvp, midventral placid; oos, outer oral styles; psp, primary spinoscalids; sp1-5, spinoscalids rows 1-5; tr, trichoscalid; trp, trichoscalid plate. Dashed circles indicate the position of sensory spots.

Fig. 12. Scanning electron micrographs showing details of the trunk of *Echinoderes kozloffi.* A. Detail of segments 1-7, ventral view. B. Detail of glandular cell outlet type 2 and sensory spot of segment 8, laterodorsal view. C. Detail of the lateroventral spine and papillae of segment 6 in a female, lateroventral view. D. Detail of the transition from long primary pectinate fringe to short one on segments 2-3, lateral view. E. Detail of middorsal spine and sensory spots on segment 7. F. Male, detail of segments 10-11, ventral view. G. Male, detail of segments 8-11, dorsal view. H. Female, detail of half

tergal plate of segments 10-11, dorsal view. Abbreviations: ldss, laterodorsal sensory spot; gco2, glandular cell outlet type 2; ldt, laterodorsal tube; lts, laterоterminal spine; ltas, laterоterminal accessory spine; lvs, lateroventral spine; lvt, lateroventral tube; md, middorsal spine; mlss, midlateral sensory spot; pa, papillae; pdss, paradorsal sensory spot; ps1-3, penile spines 1-3; te, tergal extesion. Dashed circles indicate the position of sensory spots. Digits after abbreviations refer to segment number.

Fig. 13. Scanning electron micrographs showing epibiontic filamentous bacteria on *Echinoderes ohtsukai.* A. Detail of segments 8-11, ventral view. B. Detail of filamentous bacteria attached to segment 2, ventral view. C. Detail of sternal plate of segment 11, left half, ventral view.

Table

Table 1. Measurements (in µm) of adult *Echinoderes ohtsukai*. Lateroventral spine of segment 6 could not be measured in any of the speciens in LM and therefore not included in the present table. Abbreviations: LDT, laterodorsal tubule; LTAS, lateral terminal accessory spine; LTS, lateral terminal spine; LVS, lateroventral spine; LVT, lateroventral tubule; MD, middorsal spine; MSW, maximum sternal width; n, number of specimens; SD, standard deviation; SW, standard width; S1–S11, segment lengths of trunk segments 1–11; TL, trunk length; VLT, ventrolateral tubule. Numbers, where inserted, indicate segment number.

Character	n	Range	Mean	SD
TL	5	330-410	379	29,07
MSW (8)	5	73-81	75	3,76
MSW/TL (%)	5	18% - 22%	20%	1%
SW	5	62-70	66	3,89
SW/TL (%)	5	16% - 21%	17%	1%
S1	5	34-39	37	4,62
S2	5	29-34	31	7,87
S3	5	30-34	32	3,29
S4	5	30-42	35	2,13
S5	5	33-45	40	2,69
S6	5	36-47	41	3,10
S7	5	40-49	45	1,36
S8	5	49-56	52	2,19
S9	5	50-60	54	2,00
S10	5	40-45	43	3,12
S11	5	43-48	46	1,94
MD4	1	6	-	-
LVT5	4	20-25	22	1,64
LVS7	1	6	-	-
LVT8	4	16-21	19	1,05
LDT10	4	19-21	20	2,08
LTS11	2	178-181	180	4,52

Table 2. Summary of nature and location of sensory spots, glandular cell outlets, tubes and spines arranged by series in *Echinoderes ohtsukai*. Abbreviations: LA: lateral accessory; LD: Laterodorsal; LV: lateroventral; MD: middorsal; ML: midlateral; PD: paradorsal; SD: subdorsal; SL: sublateral; VL: ventrolateral; VM: ventromedial; ac, acicular spine; ft, fringed tube; gco1, glandular cell outlet type 1; ltas, lateral terminal accessory spine; lts, lateral terminal spine; pe, penile spines; si, sieve plate; ss, sensory spot; tu, tube; (♀), female and (♂), male conditions of sexually dimorphic characters.

Position Segment	MD	PD	SD	LD	ML	SL	LA	LV	VL	VM
1	gco1		ss	ss				gco1		ss
2	ss, gco1		ft	ss, ss, ft					ft	ss, gco1
3	gco1		ss		ss			ft		gco1
4	ac	gco1	ft					ft		gco1
5		gco1	ss	ss	ft			tu		ss, gco1
6		gco1	ss	ss, ft				ac		ss, gco1
7		gco1	ss	ss		ft		ac		ss, gco1
8	ft	gco1	ss	ft		ft		tu		gco1
9		ss, gco1	ss		ss	si			ss	gco1
10	gco1, gco1		ss	tu					ss	gco1
11	gco1	ss			pe(♂)		ltas(♀)	lts		ss, ss

Table 3. Measurements (in µm) of adult *Echinoderes kozloffi*. Abbreviations: LDT, laterodorsal tubule; LTAS, lateral terminal accessory spine; LTS, lateral terminal spine; LVS, lateroventral spine; LVT, lateroventral tubule; MD, middorsal spine; MSW, maximum sternal width; n, number of specimens; SD, standard deviation; SW, standard width; S1–S11, segment lengths of trunk segments 1–11; TL, trunk length; VLT, ventrolateral tubule. Numbers, where inserted, indicate segment number.

Character	n	Range	Mean	SD
TL	4	334-383	349	29,07
MSW (8)	4	72-78	75	3,76
MSW/TL (%)	4	20% - 22%	21%	1%
SW	4	64-73	68	3,89
SW/TL (%)	4	19% - 21%	20%	1%
S1	4	39-46	43	4,62
S2	4	32-48	37	7,87
S3	4	27-35	31	3,29
S4	4	30-33	31	2,13
S5	4	31-36	33	2,69
S6	4	34-39	37	3,10
S7	4	38-39	39	1,36
S8	4	39-44	42	2,19
S9	4	41-44	43	2,00
S10	4	48-53	51	3,12
S11	4	34-38	37	1,94
MD4	4	17-23	20	2,49
MD5	3	21-23	22	1,80
MD6	4	22-26	24	2,84
MD7	3	22-29	26	3,57
MD8	4	35-44	41	5,61
VLT2	4	27-28	28	0,61
LVT5	4	26-28	27	1,64
LVS6	4	21-22	22	0,98
LVS7	4	26-28	27	0,90
LVS8	4	27-29	28	1,05
LVS9	4	24-28	26	2,50
LDT10	4	21-26	25	2,08
LTS11	4	176-185	181	4,52
LTAS11	2	56-65	61	6,61

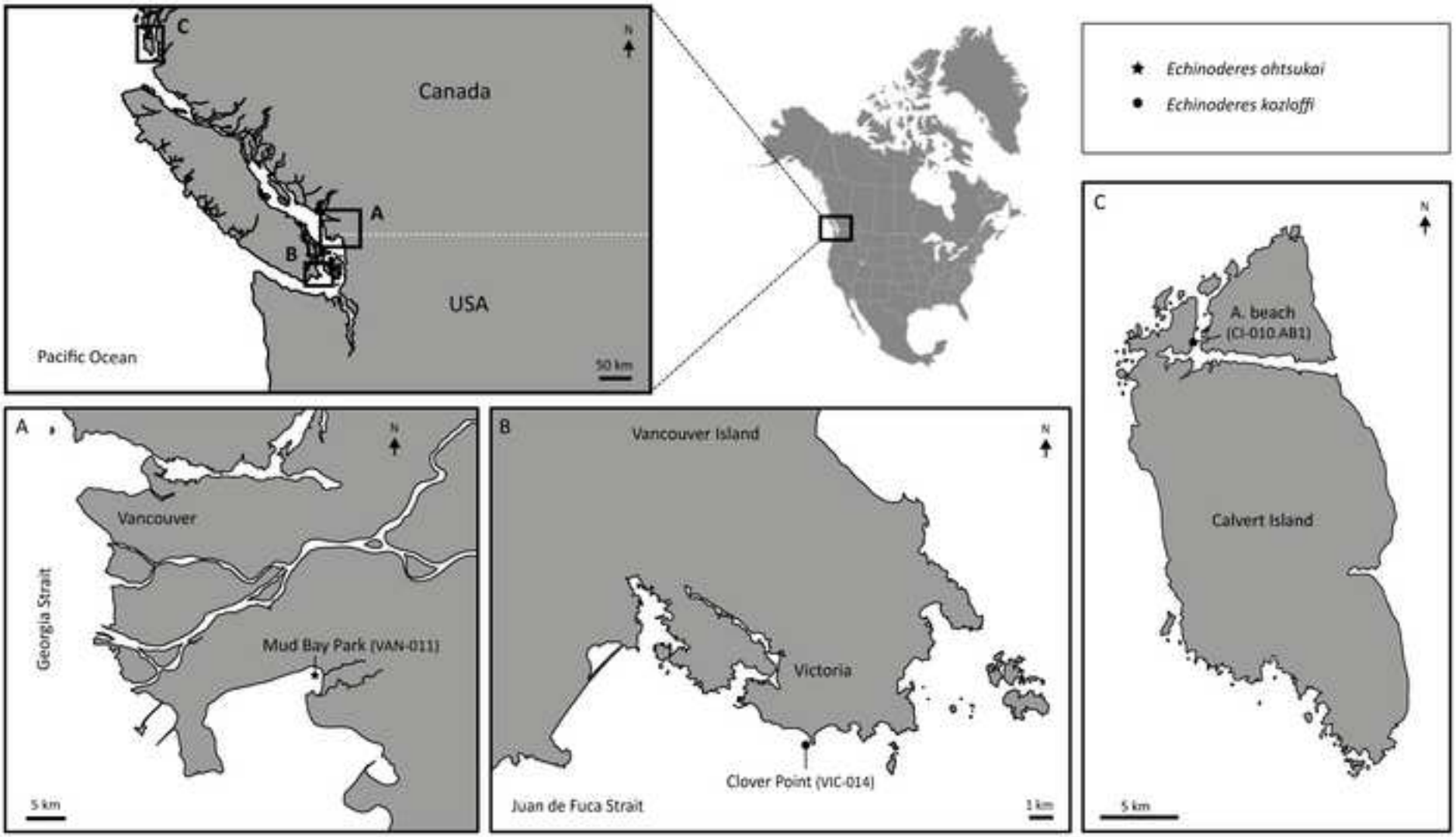
Table 4. Summary of nature and location of sensory spots, glandular cell outlets, tubes and spines arranged by series in *Echinoderes kozloffi*. Abbreviations: LA: lateral accessory; LD: Laterodorsal; LV: lateroventral; MD: middorsal; ML: midlateral; PD: paradorsal; SD: subdorsal; SL: sublateral; VL: ventrolateral; VM: ventromedial; ac, acicular spine; gco 1/2, glandular cell outlet type 1/2; ltas, lateral terminal accessory spine; lts, lateral terminal spine; pa, papillae; pe, penile spines; si, sieve plate; ss, sensory spot; tu, tube; (♀), female and (♂), male conditions of sexually dimorphic characters.

Position Segment	MD	PD	SD	LD	ML	SL	LA	LV	VL	VM
1	gco1		ss		ss			gco1	ss	
2	gco1		ss	ss				tu		ss, gco1
3	gco1		ss		ss					ss, gco1
4	ac		gco1	ss						ss, gco1
5	ac		ss, gco1	ss				tu		ss, gco1
6	ac	ss	ss, gco1	ss				ac	pa(♀)	ss, gco1
7	ac	ss	ss, gco1	ss				ac	pa(♀)	ss, gco1
8	ac	ss	ss, gco1	ss, gco2			ac			pa(♀), gco1
9		ss	ss, gco1	ss			si	ac	ss	gco1
10	gco1, gco1		ss	tu					ss	gco1
11	gco1		ss, ss		pe(♂)		ltas(♀)	lts		ss

Table 5. Summary of diagnostic charcaters in species among the *Echinoderes coulli* group, modified and updated from Sørensen (2014) and Yamasaki and Fujimoto (2014). Abbreviations: Gco2, glandular cell outlet type 2; Ldt, laterodorsal tubule; Ltas, lateral terminal accessory spine; Lts, lateral terminal spine; Lvs, lateroventral spine; Lvt, lateroventral tubule; Md, middorsal spine; S, segments; Sdt, subdorsal tube. Numbers, where inserted, indicates segment number. (+) means presence and (–) absence

	E. applicitus	***E. coulli***	***E. hwiizaa***	***E. komatsui***	***E. marthae***	***E. maxwelli***	***E. ohtsukai***	***E. rex***	***E. teretis***
Md (4)	-	-	-	-	-	+	+	+	+
Lvt (5)	+	+	+	+	+	+	+	+	+
Lvs (6)	-	-	-	-	-	+	+	+	+
Lvs (7)	-	-	+	-	-	+	+	+	+
Lvt (8)	-	+	+	+	+	-	+	+	-
Lat (8)	+	-	-	-	-	+	-	-	+
Ldt (8)	-	-	+	-	+	-	-	-	-
Mlt (8)	-	-	+	-	-	-	-	-	-
Lvt (9)	-	-	+	-	-	-	-	-	-
Sdt (10)	-	-	-	-	+	-	-	-	-
Ldt (10)	+	-	+	+	-	+	+	+	+
Lts short	-	-	+	-	-	-	-	+	-
Ltas	-	-	+	+	-	-	+	-	-
Gco2/Fringed tubes present	-	+	+	+	-	-	+	+	-
S10 projecting over S11	+	-	-	+	-	+	-	-	-
Anterior part of the trunk much thicker than the posterior part	-	-	-	-	-	-	-	-	+
Intertidal	+	+	+	+	+	+	+	-	+

Figure 1


C
N
Canada
A
B
USA
Pacific Ocean
50 km
Echinoderes ahtsukai
Echinoderes kozloffi
C
N
A. beach
(CI-010 AB1)
Calvert Island
5 km
A
N
Vancouver
Georgia Strait
Mud Bay Park (VAN-011)
5 km
B
Vancouver Island
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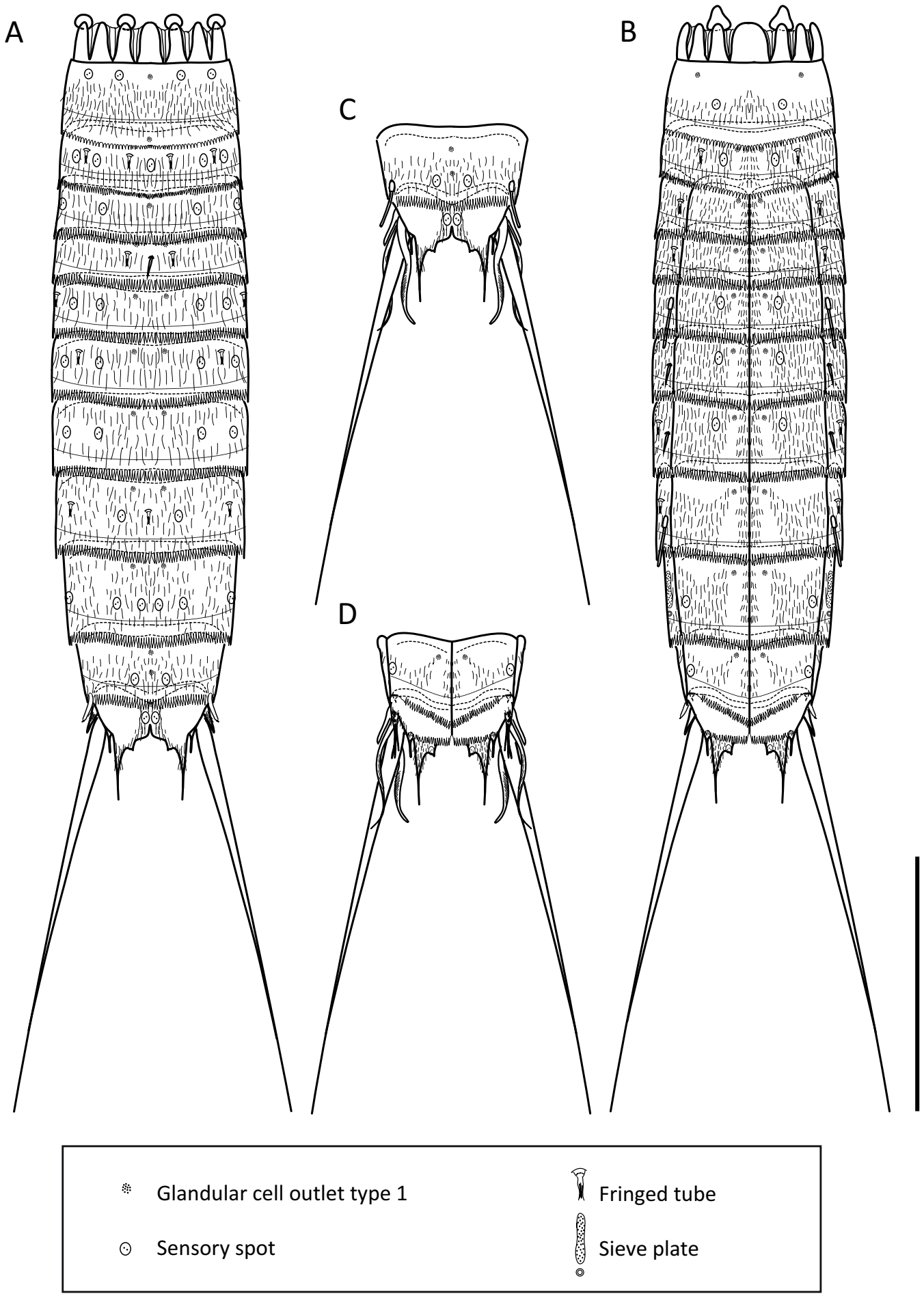
Victoria
Clover Point (VIC-014)
Juan de Fuca Strait
1 km

Figure 2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B
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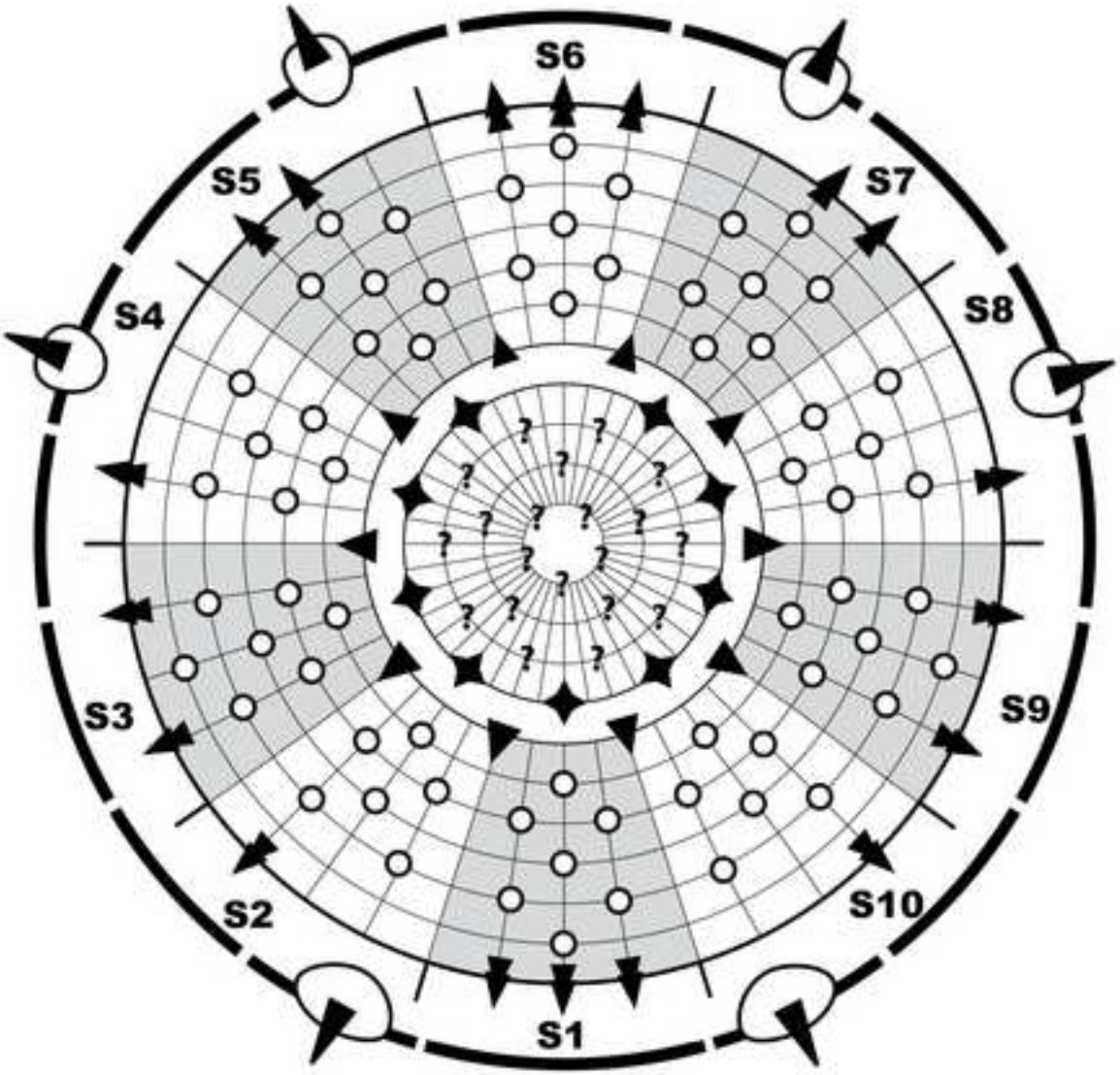
D
Glandular cell outlet type 1
Sensory spot
Fringed tube
Sieve plate

Figure 3



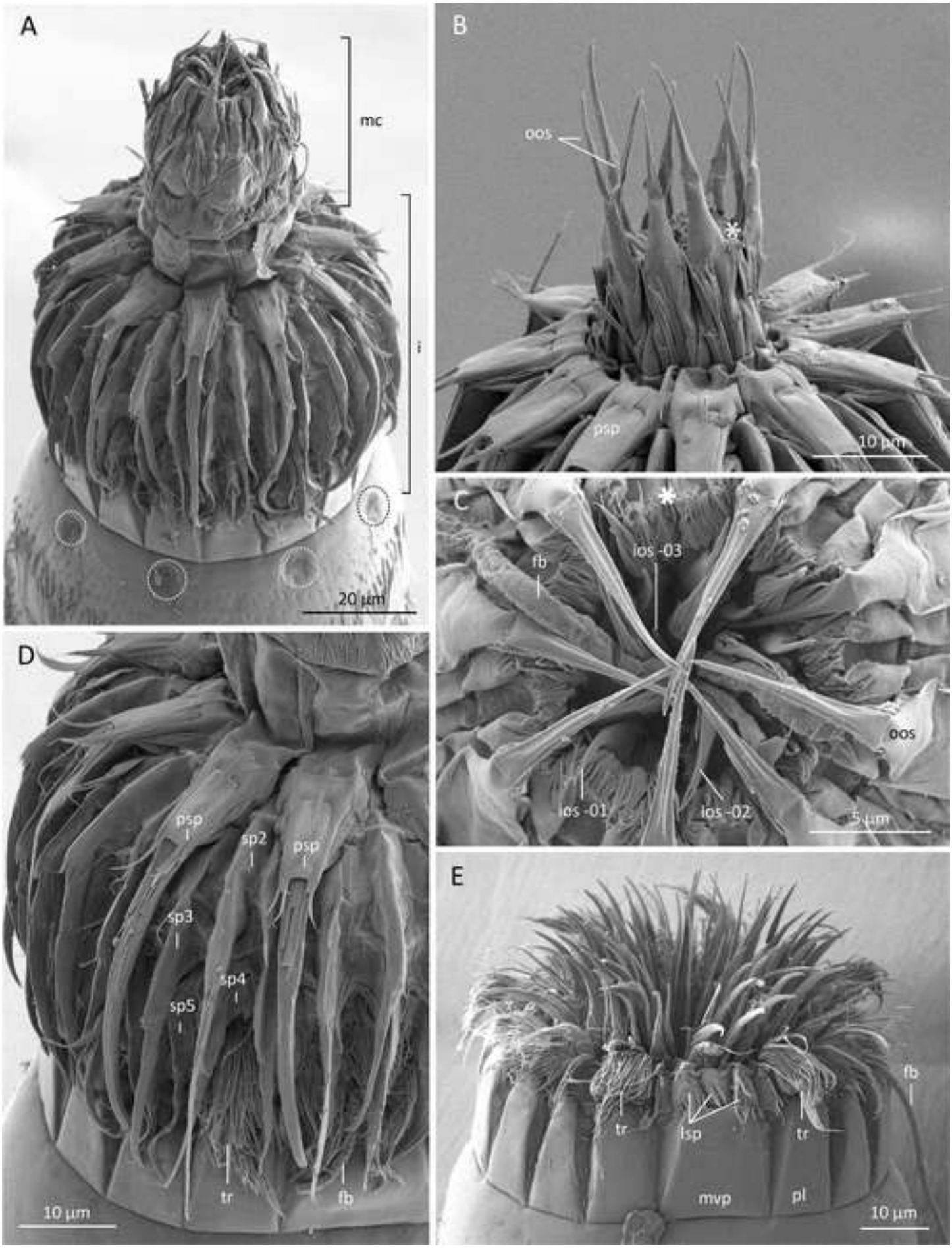
Scalid and style arrangement

Ring/Sector	1	2	3	4	5	6	7	8	9	10	Total
00 oos ✦	1	1	1	1	1	0	1	1	1	1	9
01 psp ▼	1	1	1	1	1	1	1	1	1	1	10
02 sp ○	1	1	1	1	1	1	1	1	1	1	10
03 sp ○	2	2	2	2	2	2	2	2	2	2	20
04 sp ○	1	1	1	1	1	1	1	1	1	1	10
05 sp ○	2	2	2	2	2	2	2	2	2	2	20
06 sp ○	1	0	1	0	1	1	1	0	1	0	6
07 ls	3	1	2	1	2	3	2	1	2	1	18
08 tr	0	1	0	1	1	0	1	1	0	1	6
Total scalids	8	8	9	8	10	10	10	8	9	8	90

Figure 4


A
B
tp
pl
20 µm
C
tp
pl
lvt5
20 µm
D
ldt
20 µm
E
lvt8
si
20 µm
F
ps
ldt
te
ps
lts
20 µm
G
ltas
lts
20 µm
H
ldt
ltas
te
lts
20 µm

Figure 5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B
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fb
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D
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sp2
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sp5
tr
fb
10 µm
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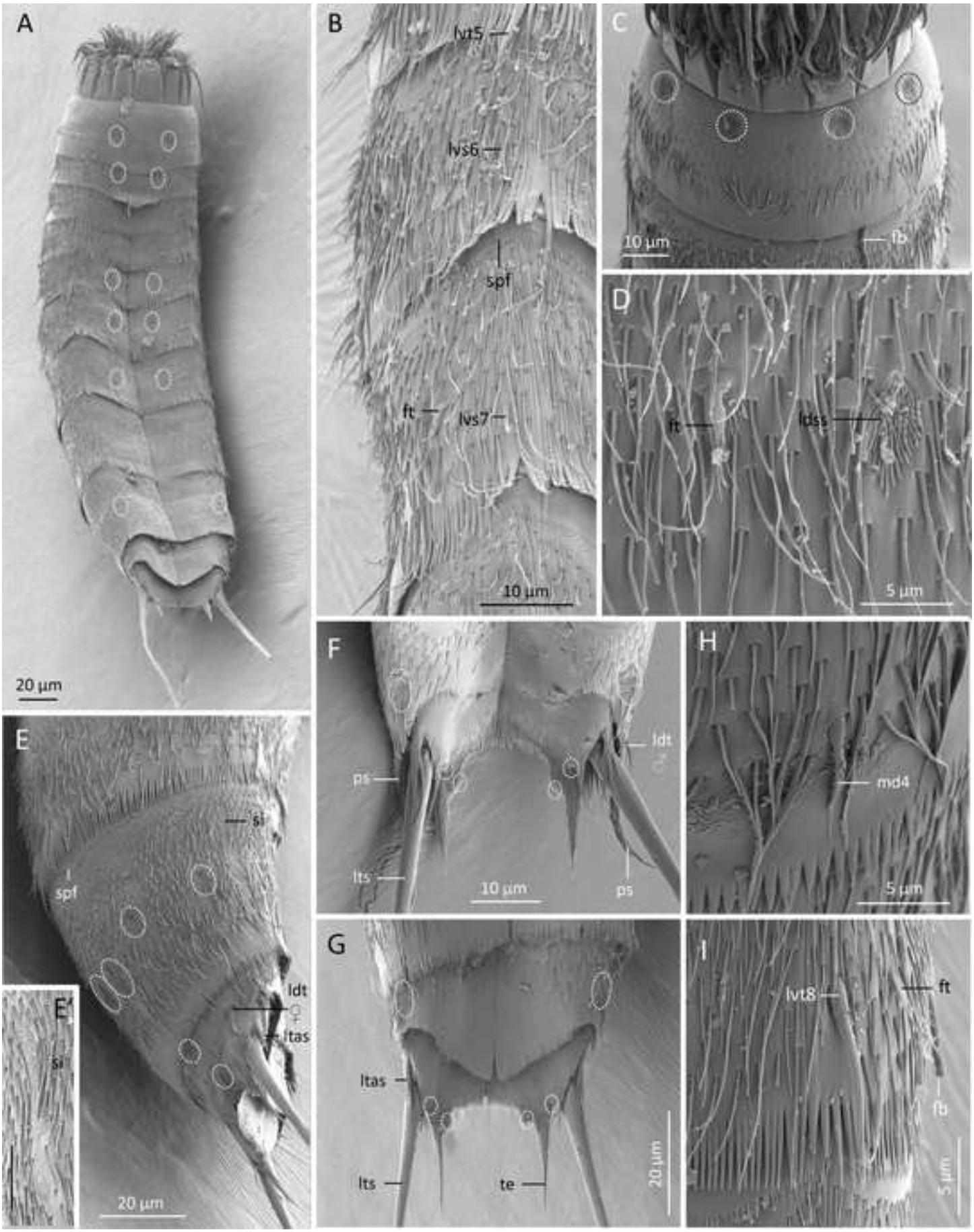
fb
tr
lsp
tr
mvp
pl
10 µm

Figure 6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20 µm
B
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lvs7
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C
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fb
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si
spf
ldt
ltas
20 µm
E'
si
F
ldt
ps
lts
10 µm
ps
G
ltas
lts
te
20 µm
H
md4
5 µm
I
lvt8
ft
fb
5 µm

Figure 7

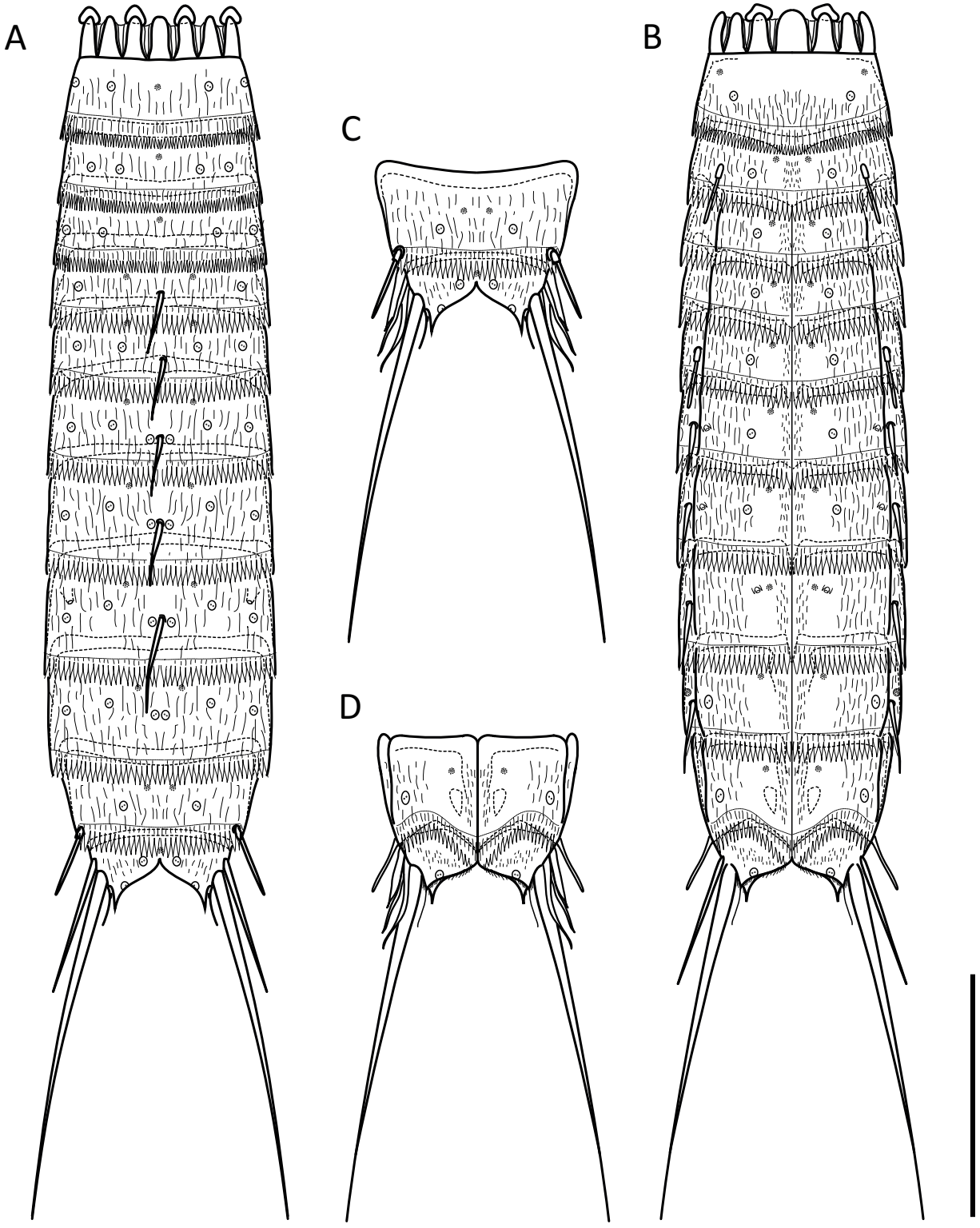
A
B
C
D
Glandular cell outlet type 1
Glandular cell outlet type 2
Sensory spot
Papillae (♀)
Sieve plate
Muscular scar

Figure 8

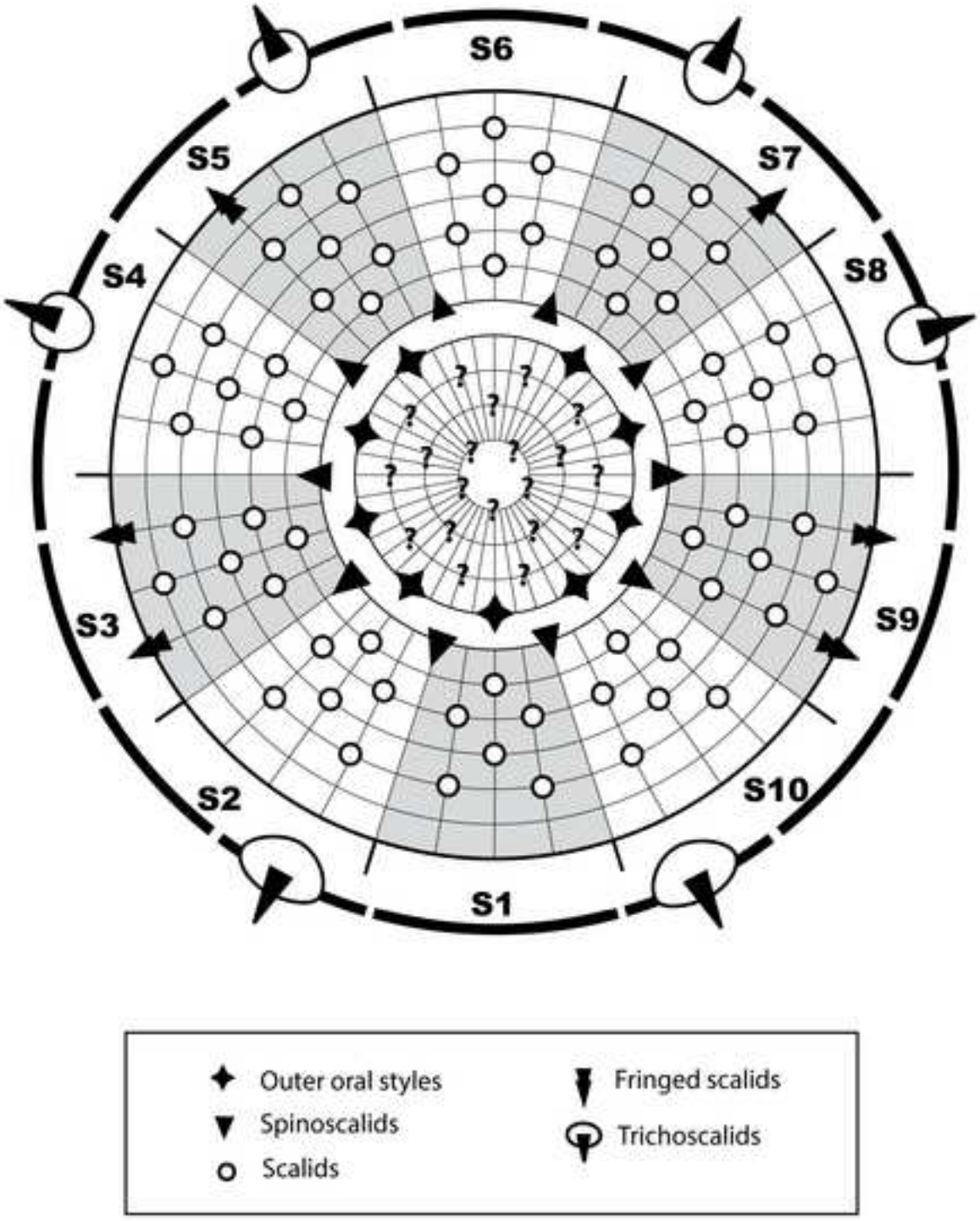
S6
S5
S7
S4
S8
S3
S9
S2
S10
S1
Outer oral styles
Spinoscalids
Scalids
Fringed scalids
Trichoscalids

Figure 9

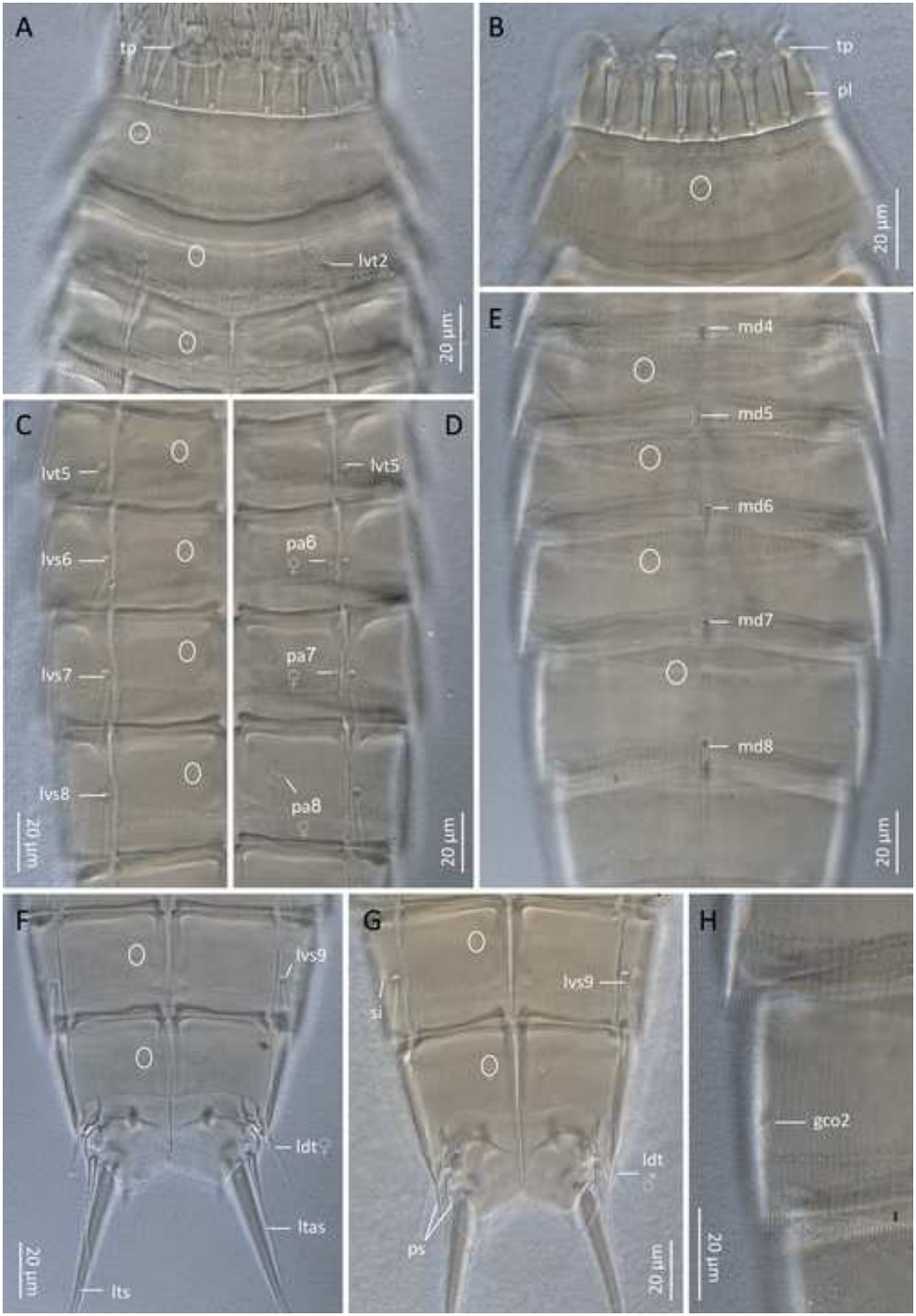
A
tp
lvt2
20 µm
B
tp
pl
20 µm
C
lvt5
lvs6
lvs7
lvs8
20 µm
D
lvt5
pa6
pa7
pa8
20 µm
E
md4
md5
md6
md7
md8
20 µm
F
lvs9
ldt
ltas
lts
20 µm
G
lvs9
si
ldt
ps
20 µm
H
gco2
20 µm

Figure 10

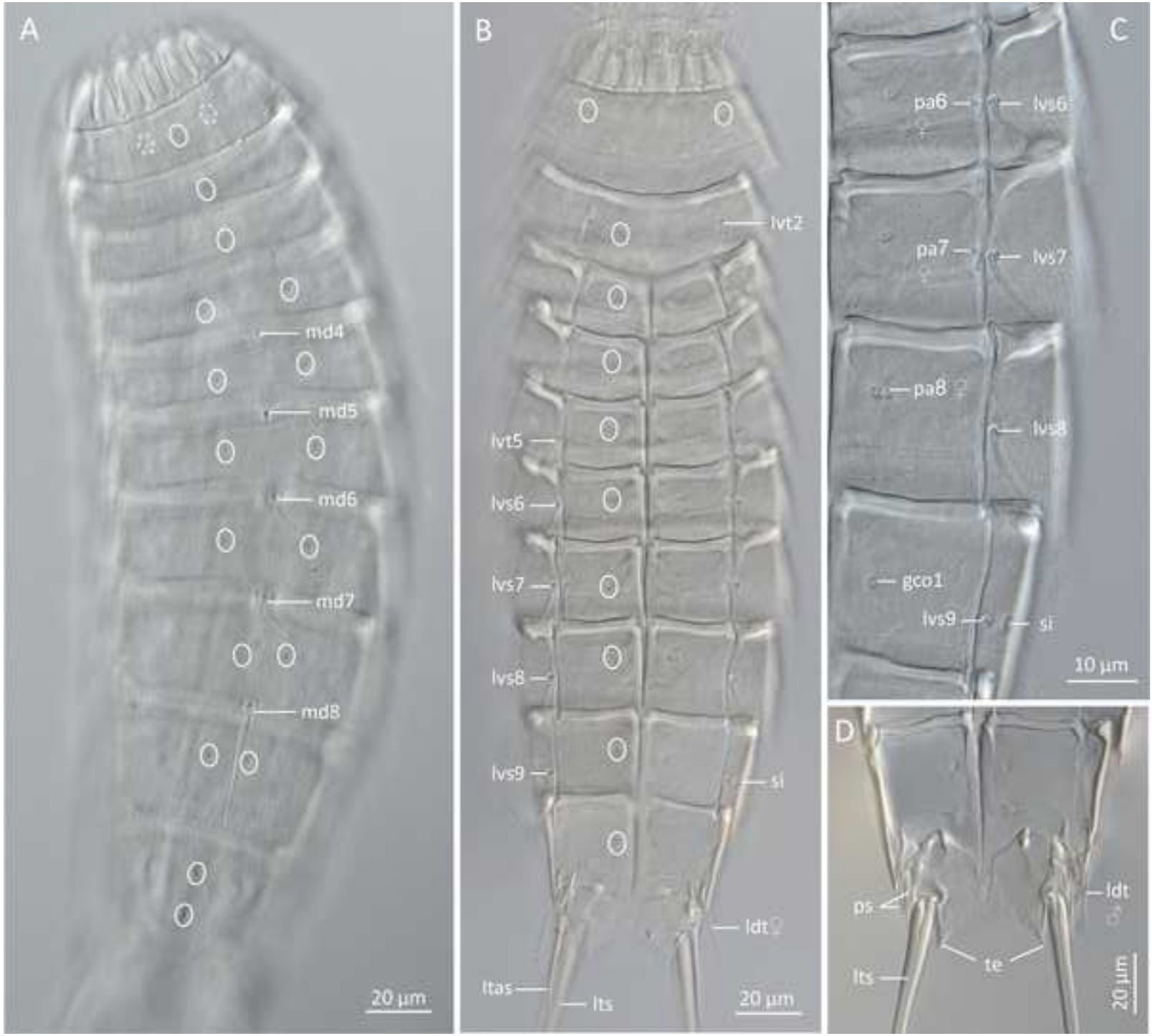
A
md4
md5
md6
md7
md8
20 µm
B
lvt2
lvt5
lvs6
lvs7
lvs8
lvs9
si
ldt
ltas
lts
20 µm
C
pa6
lvs6
pa7
lvs7
pa8
lvs8
gco1
lvs9
si
10 µm
D
ldt
ps
te
lts
20 µm

Figure 11

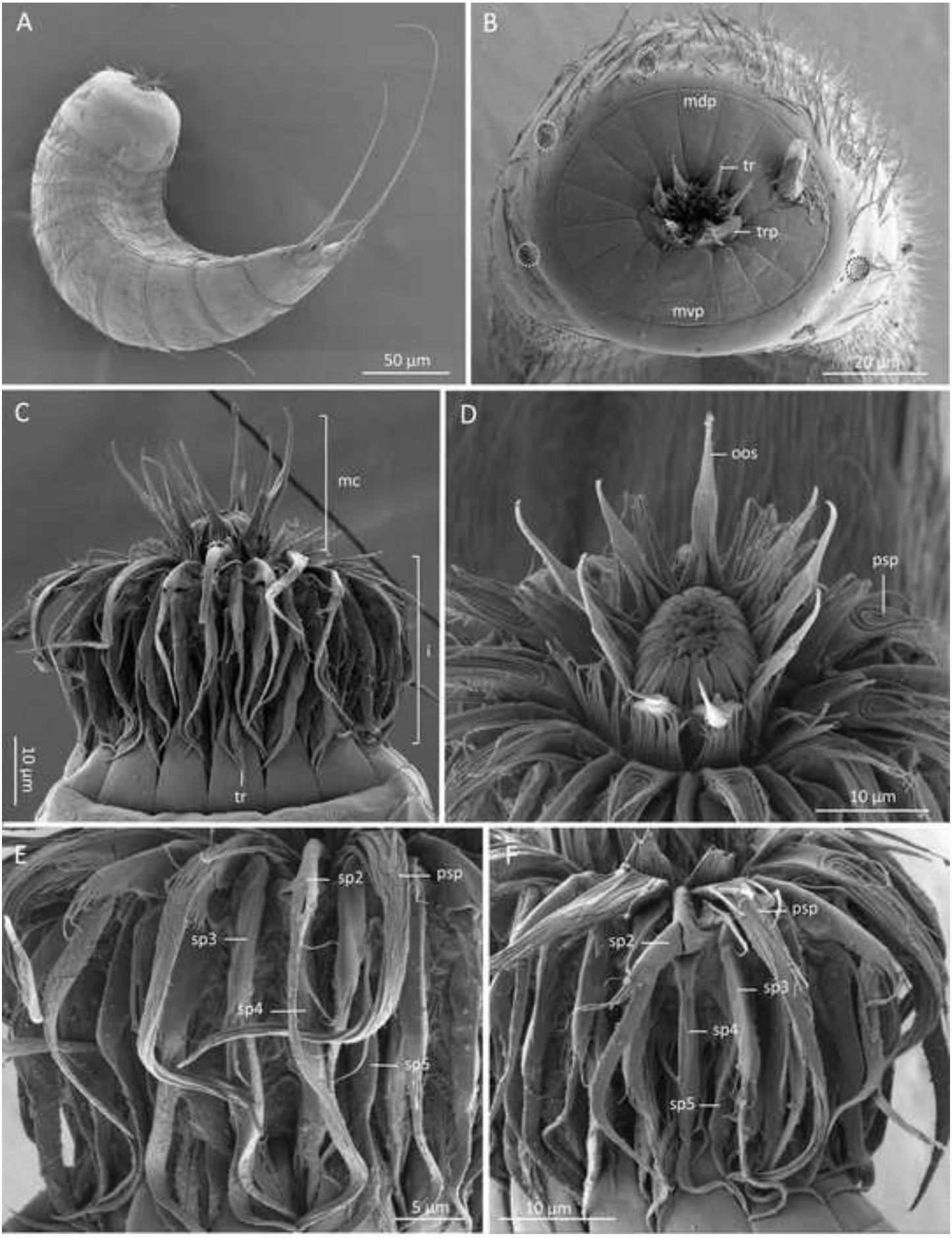
A
50 µm
B
mdp
tr
trp
mvp
20 µm
C
mc
l
10 µm
tr
D
oos
psp
10 µm
E
sp2
psp
sp3
sp4
sp5
5 µm
F
psp
sp2
sp3
sp4
sp5
10 µm

Figure 12

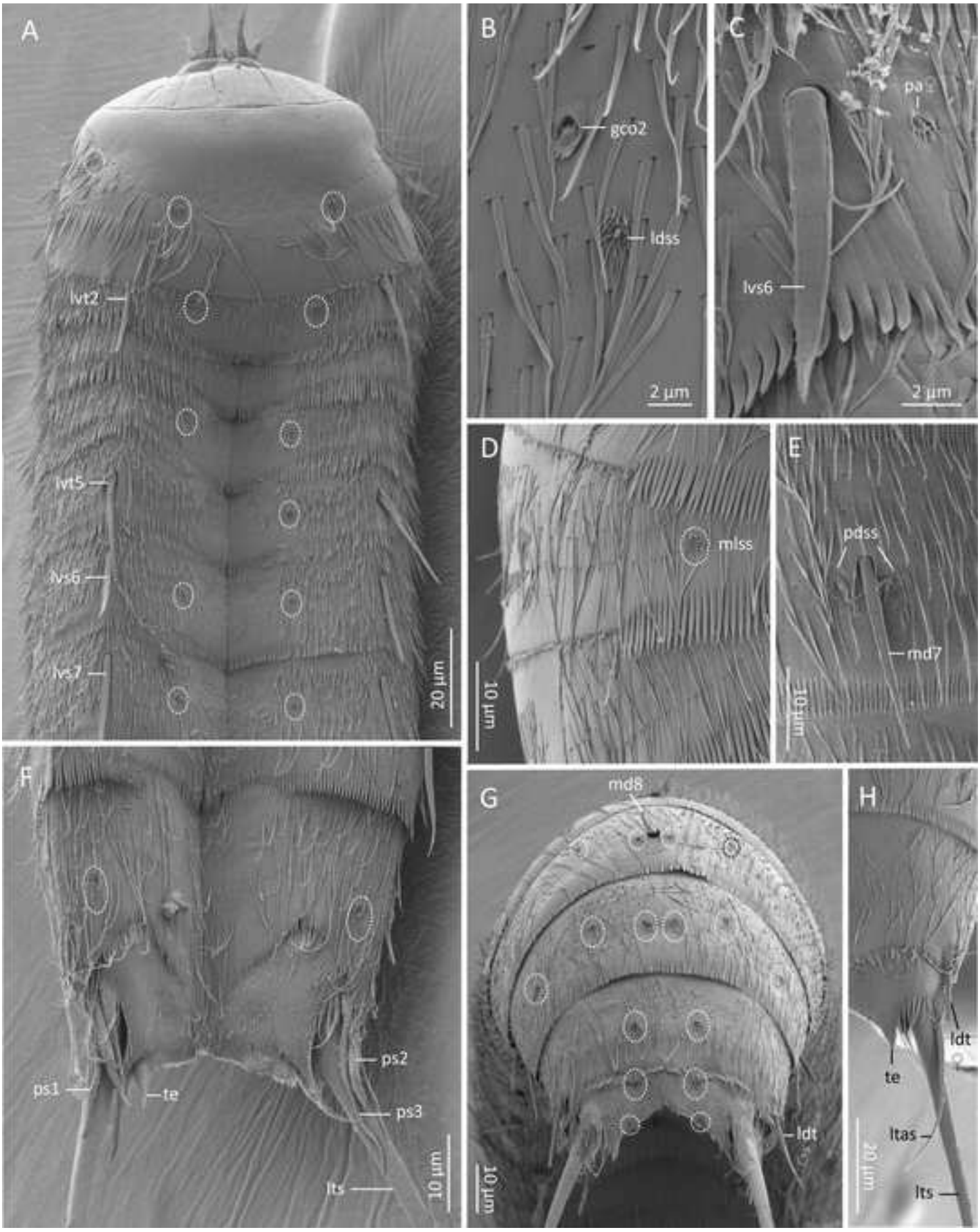
A
lvt2
lvt5
lvs6
lvs7
20 µm
B
gco2
ldss
2 µm
C
pa1
lvs6
2 µm
D
mlss
10 µm
E
pdss
md7
10 µm
F
ps1
te
ps2
ps3
lts
10 µm
G
md8
ldt
10 µm
H
ldt
te
ltas
lts
20 µm

Figure 13