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

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Keywords: COI, Kinorhyncha, systematics, British Columbia, introduction

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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. kozloffii*) 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.

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

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11 **Abstract**

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13 certain kinorhynch species appears to extend over vast geographical areas. Combining
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15 by discerning cryptic species with restricted distributions from species with potentially
16 large geographical distributions. In this paper, we (1) redescribe two species of
17 kinorhynchs (*Echinoderes ohtsukai* and *E. kozloffii*) found in the northeastern Pacific
18 Ocean using molecular and morphological data and (2) provide the first evidence for a
19 disjunct geographical distribution in kinorhynchs that is consistent with the introduction
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21 Ocean (British Columbia, Canada), this species was originally described from Japan.
22 We demonstrated that specimens of *E. ohtsukai* collected from Japan and British
23 Columbia have identical DNA sequences for the mitochondrial cytochrome c oxidase
24 subunit 1 (COI) gene. These results are most consistent with a recent introduction of
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29 1. Introduction

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2 30 Kinorhynchs are a distinct group of marine permanent meiofauna, meaning that they
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4 31 spend their entire life cycle in the sediment. They are direct developers without larval
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6 32 planktonic stages, and are inferred to have a limited potential for dispersal (Giere, 2009;
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8 33 Higgins, 1988). Moreover, kinorhynchs are equipped with mucous glands and cuticular
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10 34 structures (spines and tubules) that enable them to anchor themselves within the
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12 35 sediment, preventing accidental detachments that could bring them into the water
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14 36 column and drift (Brown, 1989). However, the wide geographical distribution observed
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16 37 in some species of kinorhynchs leads to a paradox (Giere, 2009). Different hypotheses
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18 38 have been proposed to explain the wide distribution of kinorhynchs, including potential
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20 39 pathways through deep bottom currents (e.g., *Campyloderes*; Neuhaus and Sørensen,
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22 40 2013) and vicariance processes through continental drift (e.g., *Centroderes* and
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24 41 *Meristoderes*; Herranz and Pardos, 2013; Neuhaus et al., 2014). However, none of
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26 42 these hypotheses have been tested with molecular phylogeographical data, so dispersal
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28 43 mechanisms in kinorhynchs remain unclear. The only phylogeographical study focused
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30 44 on kinorhynchs was recently carried out by Yamasaki et al. (2014), who compared the
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32 45 DNA barcode region of the mitochondrial gene cytochrome c oxidase subunit I (COI) in
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34 46 two closely related species of the genus *Echinoderes* from Japan. This study
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36 47 demonstrated the occurrence of cryptic speciation in both species and suggested
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38 48 suspension, transport or rafting as possible dispersal mechanisms (Yamasaki et al.,
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40 49 2014).

41
42 50 *Echinoderes* is the largest genus of kinorhynchs with over 85 species distributed
43
44 51 worldwide and representing more than 30% of the total diversity of the group (Neuhaus,
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46 52 2013). Within *Echinoderes* the so called “*E. coulli* group” is particularly interesting in
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48 53 terms of biogeography, with most of its species being limited to intertidal brackish
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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 kozloffii* 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. kozloffii*, 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.

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79 2. Materials and methods

80 2.1. Sampling

81 Specimens of *Echinoderes ohtsukai* were collected in Mud Bay Park (Boundary Bay),
82 southeast from Vancouver, British Columbia (49°5'9.86"N; 122°51'39.95"W), in
83 November and August 2014 and November 2015 at station VAN-011 (Fig. 1A). The
84 sampled area is a brackish estuarine with a salinity ranging between 20-24‰. Samples
85 were taken from intertidal mud using a shovel to collect the uppermost, oxygenated
86 layer of sediment. Specimens of *Echinoderes kozloffii* were collected from intertidal
87 brown algae mixed with fine sediment in Clover Point, south of Victoria on Vancouver
88 Island, British Columbia (48°24'13.70"N; 123°21'3.36"W), in May 2015 at station VIC-
89 014 , salinity 32.5‰ (Fig. 1B). *E. kozloffii* was also collected intertidally from
90 Archaeology Beach on Calvert Island, north of Vancouver Island (51°39'51.88"N; 128°
91 5'50.29"W), in July 2015 at station CI-010.AB1, salinity 33‰ (Fig. 1C).

92 2.2. Microscopy

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

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

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108 2.3. DNA extraction, PCR and sequencing

109 Total genomic DNA was extracted from a single specimen of *E. ohtsukai* from Mud
110 Bay Park and two specimens of *E. kozloffii*, from Victoria and Calvert Island, all fixed in
111 99% ethanol, using a DNeasy Blood and Tissue Kit (Qiagen, Tokyo) following the
112 protocol described in Yamasaki et al. (2013). Cuticular vouchers of the specimens used
113 for the extraction were recovered from the lysis buffer and mounted in Fluoromount G®
114 using regular glass slides and deposited at the Natural History Museum of Denmark,
115 under catalogue number ZMUC KIN-968 for *E. ohtsukai* and ZMUC KIN-969 *E.*
116 *kozloffii*. DNA extraction, amplification and sequencing of the specimens were
117 performed for the mitochondrial gene COI. Polymerase chain reactions (PCR) were
118 performed using PuRe Taq Ready-To-Go PCR beads kit (GE Healthcare,
119 Buckinghamshire, UK). The primers used for COI were: LCO1490 (5'-
120 GGTCACAAATCATAAAGATATTGG-3') and HCO2198 (5'-
121 TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994). PCR cycling
122 conditions were: 95°C for 1min; 35 cycles of (95°C for 30 sec, 49°C for 1min 30 sec and
123 72°C for 3min); and 72°C for 7min. The amplified fragments were gel purified using
124 UltraClean DNA Purification Kit (MO Bio, Carlsbad, CA) and sequenced. Nucleotide
125 sequences were determined by direct sequencing with a BigDye Terminator Kit
126 (Applied Biosystems). Sequence fragments were edited and assembled using
127 Sequencher (Gene code corporations, Michigan, USA). After assembly, the sequences
128 were deposited in GenBank (NCBI) under accession numbers KU674383 for *E.*

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

133

134 **3. Results**

135 *Taxonomic account*

136 Class Cyclorhagida Zelinka, 1896

137 Order Echinorhagata Sørensen et al., 2015

138 Family Echinoderidae Zelinka, 1894

139 Genus *Echinoderes* Claparède, 1863

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141 **3.1. *Echinoderes ohtsukai* Yamasaki and Kajihara, 2012** (Figs 2-6 and Tables 1-2)

142 *3.1.1. Emended Diagnosis*

143 *Echinoderes* with a single, minute middorsal spine on segment 4, minute lateroventral
144 spines on segments 6-7; lateroventral tubes on segments 5 and 8, males furthermore
145 with well-developed tubes in laterodorsal positions on the posterior margin of segment
146 10, whereas females show much smaller laterodorsal tubes on segment 10. Small single
147 middorsal fringed tube on segment 8, subdorsal fringed tubes on segments 2 and 4,
148 laterodorsal on segments 2, 6, 8, midlateral on segment 5, ventrolateral on segment 2,
149 lateroventral on segments 3, 4 and sublateral on segments 7 and 8. Large sieve plates in
150 sublateral position on segment 9. Tergal extensions long and pointed. Males with three
151 penile spines and a long fringed area in lateroventral position; females with lateral
152 terminal accessory spines reduced to short fringed structures.

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154 *3.1.2. Material examined*

155 Three females and one male all collected from intertidal mud on November 2014, June
156 2015 and October 2015 at the same station (VAN-011) in Mud Bay Park (Boundary
157 Bay), located southeast of Vancouver (49°5'9.86"N; 122°51'39.95"W) (Figure 1A). All
158 specimens were mounted in Fluoromount G® and deposited at the Natural History
159 Museum of Denmark, under catalogue numbers ZMUC KIN-923 and ZMUC KIN-956
160 to KIN-958.

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

168 *3.1.3. DNA sequence*

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

172 *3.1.4. Description*

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

177 armature of the mouth cone formed by nine outer oral styles divided into two subunits
178 slightly alternating in size between five longer ones situated according to uneven sectors
179 of the introvert, and four shorter ones situated according to even sectors (Fig. 5B);
180 middorsal outer oral style is missing. Each outer oral style has a fringe at its base
181 showing six fringe tips and bordered by a pair of spikes (Fig. 5A, B). One of the
182 examined specimens was found to have eight instead of nine oral styles, with the same
183 appearance but distributed in a different way breaking the typical bilateral symmetry
184 showed by the specimens with nine oral styles (Fig. 5C). The exact number and
185 arrangement of the inner armature of the mouth cone could only be observed partially
186 and is therefore not plotted in the introvert diagram, but shown in Figure 5C. Three
187 rings of inner oral styles could be detected in the inner part of the mouth cone namely (-
188 03), (-02) and (-01). The ring (-01) shows tubular elongated structures with a pore in the
189 distal end. Ring (-02) shows five inner oral styles similar as those on previous ring but
190 with proximal fringed sheaths. The anteriormost ring (-03) could not be fully studied.

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

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202 resemble those of ring 03 but instead of a spine they have a fringed area (Fig. 5D). Ring
203 06 has 6 spinoscalids with the same appearance than those on previous rings but shorter.
204 Ring 07 with 18 leaf-like scalids with a wide and hairy base from where several flexible
205 elongations arise (Fig. 5E). See Fig. 3 for a polar diagram that summarizes the location
206 and arrangement of oral styles, scalids and placids. Six long and hairy trichoscalids
207 attaching to small trichoscalid plates are situated in sectors 2, 4, 5, 7, 8 and 10.
208 The neck consists of 16 placids numbered clockwise from the midventral 1 (Figs 3, 4B-
209 C, 5E). Placids 2–16 are trapezoid measuring 9 μm at the base while the midventral
210 placid is more rectangular and wider measuring 14 μm (Figs 2B, 4C, 5E). All placids
211 articulate with the first trunk segment. Trichoscalid plates bearing trichoscalids appear
212 dorsally on placids 6, 8, 10, 12 and ventrally on placids 2 and 16 (Fig. 3). Ventral
213 trichoscalid plates are triangular with rounded edges, while dorsal trichoscalid plates are
214 rounded and smaller (Figs 2A-B, 4B-C, 5D-E).
215 The trunk is divided into 11 segments (Figs 2A-B, 4A, 6A). Segments 1 and 2 consist of
216 a closed cuticular ring (Figs 2A-B, 4A-C, 6A, C) while segments 3-11 are composed of
217 one tergal and two sternal plates (Figs 2A-B, 4A, 6A). Glandular cell outlets type 1
218 consist of numerous minute pores and are situated in the anterior part of the segments
219 usually hidden under the posterior part of the previous segment (Figs 2A-B). Dorsal
220 outlets are unpaired middorsally on segments 1- 3 and 11, and paired paradorsally on
221 segments 4-9 (Figs 2A, 4B). Segment 10 with two glandular cell outlets aligned in
222 middorsal position (Fig. 2A). Ventral outlets are lateroventral on segment 1 and
223 ventromedial on segments 2-10 (Figs 2B, 4C-E). Primary pectinate fringes well
224 developed in all segments, showing very short fringe tips on segments 1 and 2 (Figs 2A-
225 B, 6A, C) and long and flexible tips on remaining segments (Figs 2A-B, 6A-B, E).
226 Secondary pectinate fringe absent on segment 1. Secondary fringes of segments 2-11

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

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

238 Segment 2 consists of a closed cuticular ring. Three pairs of short, fringed tubes (ca. 4
239 μm from SEM) are located anteriorly in subdorsal, laterodorsal and ventrolateral
240 positions, sometimes partially hidden under the previous segment and therefore difficult
241 to spot (Figs 2A-B, 4B-C). These fringed tubes are composed of a short basal part
242 attached to the trunk cuticle and a distal fringed end (see Fig. 6D for similar structure
243 from segment 5). Three pairs of sensory spots are located in laterodorsal (two pairs) and
244 ventromedial positions (one pair) and a single sensory spot in middorsal position.

245 Sensory spots on this and the following segments are more elongated showing longer
246 papillae in their posteriormost parts (see Fig. 6D for similar structure from segment 5).

247 Hairs densely distributed in a belt covering the segment showing hairless areas in
248 ventromedial position in this and the following segments (Fig. 2B). The posterior
249 segment margin extends more posteriorly in midventral and paraventral areas (Figs 2B,
250 6A).

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

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

260 Segment 5 with a pair of long and thin lateroventral tubes (Figs 2B, 4C, 6B). Each tube
261 consists of a short and smooth basal part, and a longer distal part with two small wing-
262 like lateral projections. A pair of fringed tubules is present in midlateral position (Figs
263 2A, 4C, 6D). Three pairs of sensory spots are present in subdorsal, laterodorsal and
264 ventromedial positions (Figs 2A-B). Other characters similar to previous segment.

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

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

274 Remaining characters as on previous segments.

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

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

285 Segment 10 with a pair of laterodorsal tubes at or near posterior segment margin. In
286 males the tubes are long and similar of those described on segments 5 and 8 located in
287 small indentations in the posterior segment margin (Figs 2C-D, 4D, F, 6F). In females
288 the tubes lack the basal part, showing just a flexible and short tube-like structure (Figs
289 2A-B, 4H, 6E). Two pairs of elongated sensory spots are present in subdorsal and
290 ventrolateral positions (Figs 2A-B, 6E-G). The posterior segment margin of the tergal
291 plate is straight and with a small but well-developed pectinate fringe (Fig. 6E), whereas
292 the margins of the sternal plates are concave, extending posteriorly near the midventral
293 junction, and with well-developed fringe tips (Figs 2B, 6 E-G).

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

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

308 **3.2. *Echinoderes kozloffi* Higgins, 1977** (Figs 7-12, Tables 3-4)

309 *3.2.1. Emended diagnosis*

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

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319 *3.2.2. Material examined*

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

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323 Bay, San Juan Island, Washington, USA. Additional material includes 14 specimens:
324 two of them collected intertidally at the type locality in San Juan Island, 10 collected
325 intertidally from brown algae in Clover Point, Victoria, British Columbia, Canada and 2
326 collected in Archaeology Beach in Calvert Island, BC (Fig 1B-C). Seven specimens
327 were mounted for LM, of them 3, one male from Victoria and two females (one from
328 Victoria and one from Calvert Island) are deposited at the Natural History Museum of
329 Denmark under catalogue numbers ZMUC KIN-959 to KIN-960 (Victoria) and KIN-
330 960 (Calvert Island). Remaining LM and SEM specimens are stored in the authors'
331 personal reference collection.

332 3.2.3. *DNA sequence*

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

338 3.2.4. *Description*

339 Adults with head, neck and eleven trunk segments (Figs 7A-B, 10A-B, 11A).
340 Measurements and dimensions are given in Table 3. A summary of sensory spots,
341 spines, papillae, sieve plates, tubes and glandular cell outlet positions is provided in
342 Table 4. The head consists of a retractable mouth cone and an introvert (Figs 8, 11C-F).
343 Outer armature of the mouth cone formed by nine outer oral styles divided into two
344 subunits, slightly alternating in size between 5 longer ones situated according to uneven
345 sectors of the introvert, and 4 shorter ones situated according to even sectors (Figs 8,
346 11C-D); middorsal outer oral style is missing. Outer oral styles with a short fringe at

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

350 The introvert has seven rings of cuticular spinoscalids and one additional ring of
351 trichoscalids that are associated with the placids (Figs 8, 11C). Ring 01 with 10 primary
352 spinoscalids consisting of a short basal sheath and a distal end piece. The basal sheath
353 has a small proximal fringe, situated very close to the insertion point, showing just two
354 lateral tips, followed by a smooth part that projects into 6-8 flexible fringed tips (Fig.
355 11D-F). The distal pieces of the primary spinoscalids are laterally compressed and each
356 bears a fringe composed of at least ten elongated flexible fringe tips. Ring 02 is
357 composed of 10 laterally compressed spinoscalids, all formed by a long smooth basal
358 which ends into a long fringe (Fig. 11E-F). Rings 03, 04 and 05 consist of 20, 10 and 20
359 spinoscalids respectively; all resemble those of ring 02 (Fig. 11E-F). Ring 06 has 6
360 spinoscalids with the same appearance but shorter than those on previous rings. The
361 number and arrangement of spinoscalids from rings 6-7 could not be assessed with
362 certainty in all sectors due to the partial eversion of the introvert in the specimens
363 studied, but plotted in the polar diagram when visible (Fig 8). Six long and hairy
364 trichoscalids attached to trichoscalid plates are situated ventrally in sectors 2, 10 and
365 dorsally in sectors 4, 5, 7, 8 (Figs 8, 9A-B, 11B).

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

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

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

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

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

403 Segment 3 and remaining trunk segments consist of a tergal and two sternal plates (Figs
404 7A-B, 9A, 10 A-B, 11A). Three pairs of sensory spots with the same appearance as on
405 previous segments present in subdorsal, midlateral and ventromedial positions.

406 Cuticular hairs as described in previous segments.

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

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

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

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

423 Segment 8 with a middorsal acicular spine almost twice as long as the spines of
424 segments 4-7 (41 μm vs. 20-26 μm) flanked by a pair of paradorsal oval sensory spots
425 (Figs 7A, 9E, 10A, 12G). A pair of acicular spines with the same appearance as those
426 on previous segments present in lateroventral position. Additional paired sensory spots
427 present in subdorsal and laterodorsal positions. A pair of glandular cell outlets type 2 is
428 present in laterodorsal position slightly anterior to the sensory spots (Figs 7A, 9H, 12B).

429 Females furthermore with ventromedial papillary structures as described for segments
430 6-7 (Figs 7B, 9D, 10C). Cuticular hairs as described in previous segments.

431 Segment 9 with a pair of lateroventral acicular spines (Figs 7B, 9F-G, 10B-C). Paired
432 sensory spots present in paradorsal, subdorsal, laterodorsal and ventrolateral positions
433 (Figs 7A-B, 12G). A pair of round sieve plates (c.a. 2 μm from SEM) with 20-30 holes
434 plus an additional posterior round pore present in lateral accessory position (Figs 7B,
435 9G, 10C). Cuticular hairs as described in previous segments.

436 Segment 10 with a pair of long tubes in laterodorsal position near the posterior margin
437 of the segment in both females and males (Figs 7A-D, 9F-G, 10B, D, 12G-H). Two
438 pairs of oval sensory spots present in subdorsal and ventrolateral positions (Figs A-B,
439 12F-G). Posterior edge of the segment straight in the tergal plate whereas extending
440 posteriorly in a V-shape towards the paraventral/ventromedial area in the sternal plates
441 (Figs 7B, 12F). Cuticular hairs as described in previous segments.

442 Segment 11 with lateral terminal spines (Figs 7A-B, 9F-G, 10B, D, 11A, 12F-H).

443 Females with a pair of lateral terminal accessory spines (Figs 7A-B, 9F, 10B, 12H) and

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444 males with three pairs of long penile spines (Figs 7C-D, 9G, 10D, 12F). Two of the
445 penile spines (p1 and p3) are flexible and elongated while p2 is shorter and wider (Fig.
446 12F). Two pairs of sensory spots are present on the tergal plate, in subdorsal position,
447 and one pair in ventromedial position in the sternal plate (Fig 12G). Tergal extensions
448 are short and pointy projecting slightly longer than the sternal plates (Figs 7A, D, 10D,
449 12G-H). Sternal plates triangular shaped projecting at the ventromedial position (Figs
450 7B, D, 12F). Segment densely covered with cuticular hairs without perforation sites
451 (Fig 12 F-G).

452 **4. Discussion**

453 *4.1. Notes on diagnostic features in E. ohtsukai*

454 *E. ohtsukai* was recently described from the Seto Inland Sea in Japan by Yamasaki and
455 Kajihara (2012). Despite the high quality of morphological data from both LM and
456 SEM, the description lacks several important diagnostic features that were added in the
457 present redescription. These new characters include the presence of minute lateroventral
458 acicular spines on segments 6-7, extremely reduced LTAS in females, and extra fringed
459 tubes and sensory spots on certain segments. These structures are very small and easy to
460 overlook, sometimes hidden under either the dense cuticular hairs or the free flap from
461 the previous segment. Furthermore, some of these structures are only visible using
462 SEM, such as the acicular spines of segments 6-7, which could be easily confused with
463 cuticular hairs using LM. Nonetheless, this pattern of spines and tubes distinguishes *E.*
464 *ohtsukai* from other species of *Echinoderes*.
465 *E. ohtsukai* plus eight additional species belong to the so-called "Echinoderes coulli
466 group" (Ostmann et al., 2012). This group of species is found in intertidal and estuarine
467 environments and is characterized by (1) the presence of enlarged sieve plates, which is
468 inferred to be functionally associated with brackish environments; (2) the reduction or

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469 absence of acicular spines in the middorsal and lateroventral areas; and (3) the reduction
470 or absence of the LTAS in females [see Table 5 for updated diagnostic characters in
471 species of the *Echinoderes coulli* group based on Sørensen (2014) and Yamasaki and
472 Fujimoto (2014)]. Within this group, *E. ohtsukai* shares the presence of a middorsal
473 spine on segment 4 with *E. maxwelli* Omer-Cooper, 1957; *Echinoderes rex* Lundbye et
474 al., 2011; and *E. teretis* Brown, 1985 in Adrianov and Malakhov, 1999 (see Lundbye et
475 al., 2011 for *E. maxwelli*) (Adrianov and Malakhov, 1999; Lundbye et al., 2011; Omer-
476 Cooper, 1957). The species that most resembles *E. ohtsukai* is *E. rex*, because they both
477 share a minute middorsal spine on segment 4, lateroventral spines/tubes on segments 5-
478 8 and dorsoventral tubes on segment 10 (Table 5). Both species are also distinctive
479 within the *Echinoderes coulli* group by sharing fringed tubes also named “modified
480 glandular cell outlets type 2” (Yamasaki and Kajihara, 2012). However, it is possible
481 that these modified glandular outlets are present in *E. maxwelli* as well (Lundbye et al.
482 2011); this study also confirmed the presence of minute lateroventral spines on segment
483 s 6-7 (Table 5). Although the trunk length of *E. rex* is considerably longer than in *E.*
484 *ohtsukai* (482-528 μm vs. 330-410 μm), the lateral terminal spines of *E. rex* are much
485 shorter than those of *E. ohtsukai* (20-24 μm vs. 178-181 μm). *E. rex* has two pairs of
486 penile spines in males whereas three well-developed penile spines are present in *E.*
487 *ohtsukai*. The presence of very reduced lateral accessory spines was also reported for
488 *Echinoderes hwiizaa* Yamasaki and Fujimoto, 2014 and *Echinoderes komatsui*
489 Yamasaki and Fujimoto, 2014 (Yamasaki and Fujimoto, 2014). Despite the enlarged
490 sieve plate exhibited by *E. rex* it is the only of the *E. coulli* group species found
491 subtidally and therefore considered fully marine (Lundbye et al., 2011).
492 Outside of the *Echinoderes coulli* group, the only other species with a spine/tube pattern
493 that is similar to *E. ohtsukai* is *Echinoderes cantabricus* Pardos et al., 1998. These two

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

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

507

508 4.2. Notes on epibiontic growth in *E. ohtsukai*

509 Epibiontic filamentous bacteria were found in almost all specimens collected. Some of
510 the specimens had a heavy coverage of these bacteria attaching to different places on
511 the head (Fig. 5C-E) and trunk regions (Figs 6C, I, 13A-C), but epibionts were mostly
512 concentrated in the posteriormost segments or in areas restricted to the glandular
513 openings and sensory spots (Fig. 13A, C). Live animals looked and behaved normally,
514 so the presence of the epibionts did not appear to affect the health of the kinorhynchs.
515 Epibiontic assemblages like these have been described on kinorhynchs before (Neuhaus,
516 2013), including *Echinoderes* species such as *E. spinifurca* reported from the tropical
517 Atlantic Ocean (Neuhaus, 2013) and *E. applicitus* from Java (Ostmann et al., 2012).
518 Different kinds of epibionts were described on *E. applicitus*, including ciliates and

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

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528 4.3. Notes on diagnostic features in *E. kozloffii*

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

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544 (examined with LM and SEM) provided new diagnostic characters that were previously
545 overlooked or only briefly mentioned in the original description. These characters are:
546 (1) the presence of a pair of laterodorsal glandular cell outlets type 2 on segment 8; (2)
547 irregular and very conspicuous pectinate fringe on segments 2-3, well-developed on the
548 ventral side while very short and thin on the dorsal side; (3) and dimorphic papillae or
549 modified glands in females situated ventrolaterally on segments 6-7 and midventrally on
550 segment 8. The presence of the papillae was briefly mentioned in the original
551 description as “prominent scars” but never identified as a dimorphic feature (Higgins,
552 1977a). The SEM observations of the new material allowed the inclusion of the
553 introvert description (Fig. 8) and mapping of the sensory spots of the trunk (see Table
554 4).

555 The combination of the old and new diagnostic characters enables straightforward
556 differentiation of *E. kozloffii* from relatively similar species of *Echinoderes*. The pattern
557 of middorsal spines combined with the presence of ventrolateral spines/tubes on
558 segments 5-9 and ventrolateral tubes on segment 2 is shared by 21 other species of
559 *Echinoderes*, but the addition of a pair of laterodorsal glandular cell outlets type 2 in
560 segment 8 reduces the number to only 2 other species: *Echinoderes gerardi* Higgins,
561 1978 and *E. microaperturus* Sørensen et al., 2012 (Higgins, 1978; Sørensen et al.,
562 2012b). The description of *E. gerardi* represents another one of the early contributions
563 by Higgins, described just one year after *E. kozloffii*. *E. gerardi* was subsequently
564 collected and revised by other researchers, who described the presence of paired
565 glandular cell outlets type 2 on segments 8-9 (see contribution of Sørensen et al. in this
566 special issue). *E. gerardi* differs from *E. kozloffii* by the presence of two pairs of
567 glandular cell outlets type 2, instead of one pair, and by the presence of lateral accessory

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568 tubes on segment 8. *E. gerardi* can be distinguished further by its extraordinary short
569 middorsal spines measuring 8-10 μm compared to 17-44 μm in *E. kozloffii*.
570 *E. microaperturus* differs from *E. kozloffii* by the presence of extra subdorsal glandular
571 cell outlets type 2 on segment 2 and laterodorsal spines on segment 9; and the presence
572 of long and pointy tergal extensions instead of the short and round ones found in *E.*
573 *kozloffii*. Both *E. gerardi*, *E. microaperturus* and *E. kozloffii* share dimorphic ventral
574 glands (or papillae) in females. In case of *E. gerardi*, the glands are positioned in the
575 same segments as *E. kozloffii* (Higgins, 1978) while *E. microaperturus* lacks the pair on
576 segment 6. The presence of these papillae or glands (also described as brace-shaped,
577 bracket-shaped or prominent scars) was also investigated by Thormar and Sørensen
578 (2010) reporting their potential presence in several other *Echinoderes* spp (see Thormar
579 and Sørensen 2010 for the complete list of species) but confirming it for *Echinoderes*
580 *collinae* Sørensen, 2006, *Echinoderes spinifurca* Sørensen, 2006 and *E. gizoensis*
581 Thormar and Sørensen, 2010. These glands also appear to be present in the females of
582 other echinoderid genera such as *Fissuroderes* and *Cephalorhyncha* (Thormar and
583 Sørensen, 2010), therefore, it seems that the presence of dimorphic glands/papillae may
584 be way more widespread than originally thought but just overlooked in the original
585 descriptions.
586 The glandular cell outlets type 2 and the fringed tubes were also very important features
587 in the present redescriptions of *E. kozloffii* and *E. ohtsukai*. These traits were either not
588 observed or poorly described in the original descriptions of these two species. Sørensen
589 et al. (this issue) reexamined several “old *Echinoderes*” species from the Smithsonian
590 Natural History Museum collections and found undescribed glandular cell outlets type 2
591 in most of them (*E. abbreviatus* Higgins, 1983, *E. andamanensis* Higgins and Rao,
592 1979, *E. bookhouti*, *E. imperforatus* Higgins, 1983, *E. kristenseni* Higgins, 1985, *E.*

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593 *pennaki* Higgins, 1960, *E. truncatus* Higgins, 1983, and *E. wallaceae* Higgins, 1983).
594 Outside *Echinoderes*, these glands also play an important role as a taxonomic character
595 in the genera *Meristoderes* and *Fissuroderes* (see Herranz and Pardos, 2013; Neuhaus
596 and Blasche, 2006; Sørensen et al., 2013).
597 In the present publication, we generated DNA sequences of the barcode region of the
598 mitochondrial gene cytochrome c oxidase subunit I (COI) for both *E. ohtsukai* and *E.*
599 *kozloffii* to complement the morphological descriptions. We think it is important that
600 new descriptions and redescriptions of kinorhynch species include molecular
601 phylogenetic data to complement the comparative morphological data. Future
602 identifications of kinorhynch species will be greatly facilitated by the availability of
603 DNA sequences with adequate variation, especially when studying problematic species
604 and potential cryptic species where the diagnostic morphological traits are
605 inconspicuous.

606 607 4.4. Notes on the geographical distribution of *E. ohtsukai* and *E. kozloffii*

608 Both *E. ohtsukai* and *E. kozloffii* are part of the largest and most diverse genus within
609 kinorhynchs, which extends worldwide from the intertidal zone to the abyss (Neuhaus,
610 2013; Sørensen and Pardos, 2008). The species with the widest geographical
611 distribution among *Echinoderes* is *E. tchefouensis* Low, 1934, which has been recorded
612 from China, Korea, Philippines, Malaysia, Marianas and Singapore (Sørensen et al.,
613 2012b; Sørensen et al., 2016).

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

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

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

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643 British Columbian coastal environments. The first hypothesis comes with additional
644 uncertainties such as how an exclusively intertidal (brackish) species extended its
645 population across the Pacific Ocean within the context of the limited reproductive and
646 dispersive potential that characterizes kinorhynchs. If this hypothesis is correct, then
647 future studies should be able to demonstrate the presence of *E. ohtsukai* in different
648 areas and depths across the Pacific Ocean. Current biogeographical evidence, however,
649 challenges this hypothesis because the coasts of Japan and Korea have been intensively
650 surveyed for kinorhynchs from deep sea sediments to the intertidal zone, and so far *E.*
651 *ohtsukai* has only been reported from the type locality (e.g. Sørensen et al., 2010,
652 2012a, 2012b, 2013; Thomsen et al., 2013; Yamasaki, 2015; Yamasaki and Fujimoto,
653 2014; Yamasaki et al., 2014; Yamasaki and Kajihara, 2012).

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

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667 It is difficult to explain how specimens from a single population that spans across the
668 world's largest ocean share exactly the same DNA sequence for the mitochondrial COI
669 gene. This result is best explained by human-mediated transportation of one population
670 on one side of the Pacific Ocean to a similar environment on the other side. The coast of
671 British Columbia has historically had a high level of maritime traffic connecting to the
672 coast of Japan. We strongly doubt that ship ballast water and bio-fouling are likely
673 invasion vectors, and suspect that aquacultural activities played a major role in
674 transporting kinorhynchs from one coast of the Pacific Ocean to the other.
675 The sampled area in British Columbia (Boundary Bay) is well known for its high
676 numbers of benthic exotic species originating from Japan (e.g., six species of bivalves,
677 seven species of snails and four species of polychaete worms) (see Klinkenberg, 2015
678 for a list of species). Almost all of these exotic species were related to the introduction
679 and maintenance of Japanese and Atlantic oysters for commercial production. The
680 transplant of the so-called Pacific oyster (also known as the giant or Japanese oyster
681 *Crassostrea gigas*) was initiated around 1914 and became the mainstay of the oyster
682 industry in British Columbia (Carlton, 1979; Waldichuk et al., 1994). However, because
683 this species was not able to reproduce in the low temperatures of BC waters it was
684 regularly imported from Japan, increasing the risk of introduction of exotic fauna. This
685 practice was regulated in 1940, but by that time most of the introductions might had
686 already occurred. Apparently most of the known introduced species have remained
687 localized in a very restricted area (Waldichuk et al., 1994). If this was the case for *E.*
688 *ohtsukai*, then we should not be able to find it in nearby localities with the same
689 conditions.
690 Nonetheless, the aim of this paper was not to accomplish a phylogeographical analysis
691 of the disjunct populations of *E. ohtsukai*, but to report this interesting finding in order to

692 motivate future investigations on this topic. These results will certainly affect the way

693 we interpret the distribution of kinorhynch species and other meiofaunal groups,

694 opening new debate and areas of research.

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853 **Figure legends**

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

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

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

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

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

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

892 **Fig. 6.** Scanning electron micrographs (SEM) showing overviews and details of
893 *Echinoderes ohtsukai*. A. Male, ventral overview. B. Detail of segments 6-7
894 lateroventral view. C. Detail of neck and segment 1, dorsal view. D. Detail of segment
895 5, showing a fringed tube and a sensory spot, laterodorsal view. E. Details segments 8-
896 11, laterodorsal view. Inset (E') shows a close up of the sieve plate. F. Male, detail of
897 segments 10-11, ventral view. G. Female, detail of segments 10-11, ventral view. H.
898 Detail of middorsal spine on segment 4, dorsal view. I. Detail of tube and fringed tube
899 of segment 8, lateroventral view. Abbreviations: fb, filamentous bacteria; ft, fringed
900 tube; ldss, laterodorsal sensory spot; ldt, laterodorsal tube; lts, lateroterminal spine; ltas,
901 lateral terminal accessory spine; lvs, lateroventral spine; lvt, lateroventral tube; md,

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

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

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

913 **Fig. 9.** Light micrographs (DIC) showing details in female holotype, USNM 53337 (A,
914 D, E, F, H) and male allotype USNM 53338 (B, C, G) of *Echinoderes kozloffi* from the
915 northeast Pacific described by Higgins (1977). A. Detail of the neck and segments 1-3,
916 ventral view. B. Detail of the neck and the first trunk segment, dorsal view. C. Detail of
917 right half of segments 5-8 in male allotype, ventral view. D. Female, detail of left half
918 of segments 5-8 in, ventral view. E. Male, detail of segments 4-8, dorsal view. F.
919 Female, detail of segments 9-11, ventral view. G. Male, detail of segments 9-11, ventral
920 view. H. Detail of left glandular cell outlet type 2 on segment 8, dorsal view.

921 Abbreviations: gco2, glandular cell outlet type 2; ldt, laterodorsal tube; lvt, lateroventral
922 tube; lvs, lateroventral spine; ltas, lateral terminal accessory sipine; lts, lateral terminal
923 spine; md, middorsal spine; pa, papillae; pl, placid; ps, penile spines; si, sieve plate; tp,
924 trichoscalid plate. Circles indicate the presence of glandular cell outlets type 1. Digits
925 after abbreviations refer to segment number.

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926 **Fig. 10.** Light micrographs (DIC) showing traits in *Echinoderes kozloffii*, female KIN-
927 961 (A), female KIN-960 (B-C) and male KIN-959 (D). A. Dorsal overview segments
928 1-10. B. Ventral overview. C. Detail of left sternal plate of segments 6-9 focused in
929 order to visualize the papillae. D. Segments 10-11, ventral view. Abbreviations: gco1,
930 glandular cell outlet type 1; ldt, laterodorsal tube; lvt, lateroventral tube; lvs,
931 lateroventral spine; ltas, lateral terminal accessory sipine; lts, lateral terminal spine; md,
932 middorsal spine; pa, papillae; ps, penile spines; si, sieve plate; te, tergal extensions.
933 Dashed circles indicate the position of sensory spots, solid line circles indicates the
934 presence of glandular cell outlets type 1. Digits after abbreviations refer to segment
935 number.

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

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

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

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

Table 1. Measurements (in μm) of adult *Echinoderes ohtsukai*. Lateroventral spine of segment 6 could not be measured in any of the specimens 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; gcol, 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	gcol		ss	ss				gcol		ss
2	ss, gcol		ft	ss, ss, ft					ft	ss, gcol
3	gcol		ss		ss			ft		gcol
4	ac	gcol	ft					ft		gcol
5		gcol	ss	ss	ft			tu		ss, gcol
6		gcol	ss	ss, ft				ac		ss, gcol
7		gcol	ss	ss		ft		ac		ss, gcol
8	ft	gcol	ss	ft		ft		tu		gcol
9		ss, gcol	ss		ss	si			ss	gcol
10	gcol, gcol		ss	tu					ss	gcol
11	gcol	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.

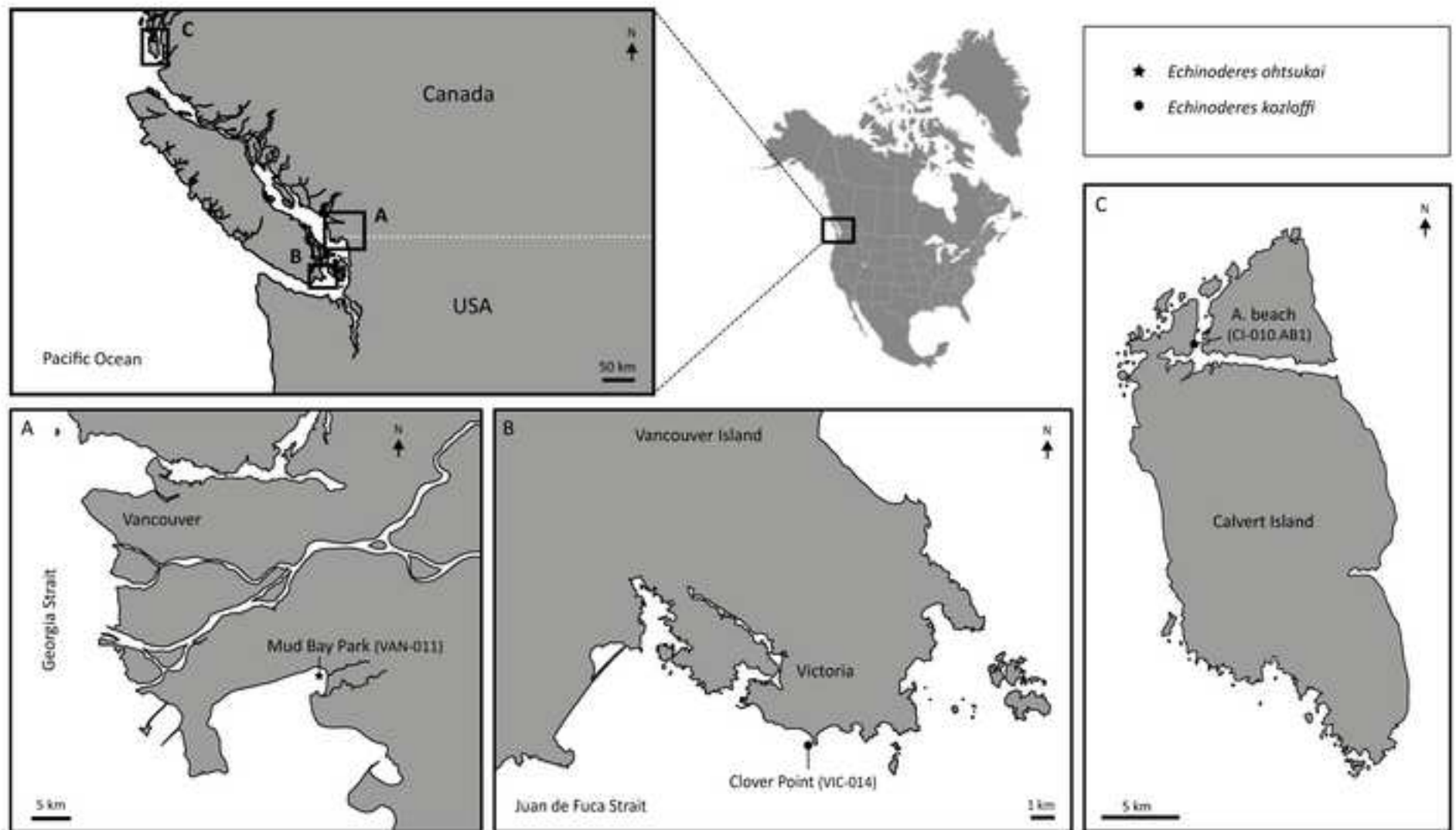
Character	n	Range	Mean	SD
TL	4	334-383	349	29,07
MSW (8)	4	72-78	75	3,76
MSW/TL (%)	4	20% - 22%	21%	1%
SW	4	64-73	68	3,89
SW/TL (%)	4	19% - 21%	20%	1%
S1	4	39-46	43	4,62
S2	4	32-48	37	7,87
S3	4	27-35	31	3,29
S4	4	30-33	31	2,13
S5	4	31-36	33	2,69
S6	4	34-39	37	3,10
S7	4	38-39	39	1,36
S8	4	39-44	42	2,19
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 kozloffii*. 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.

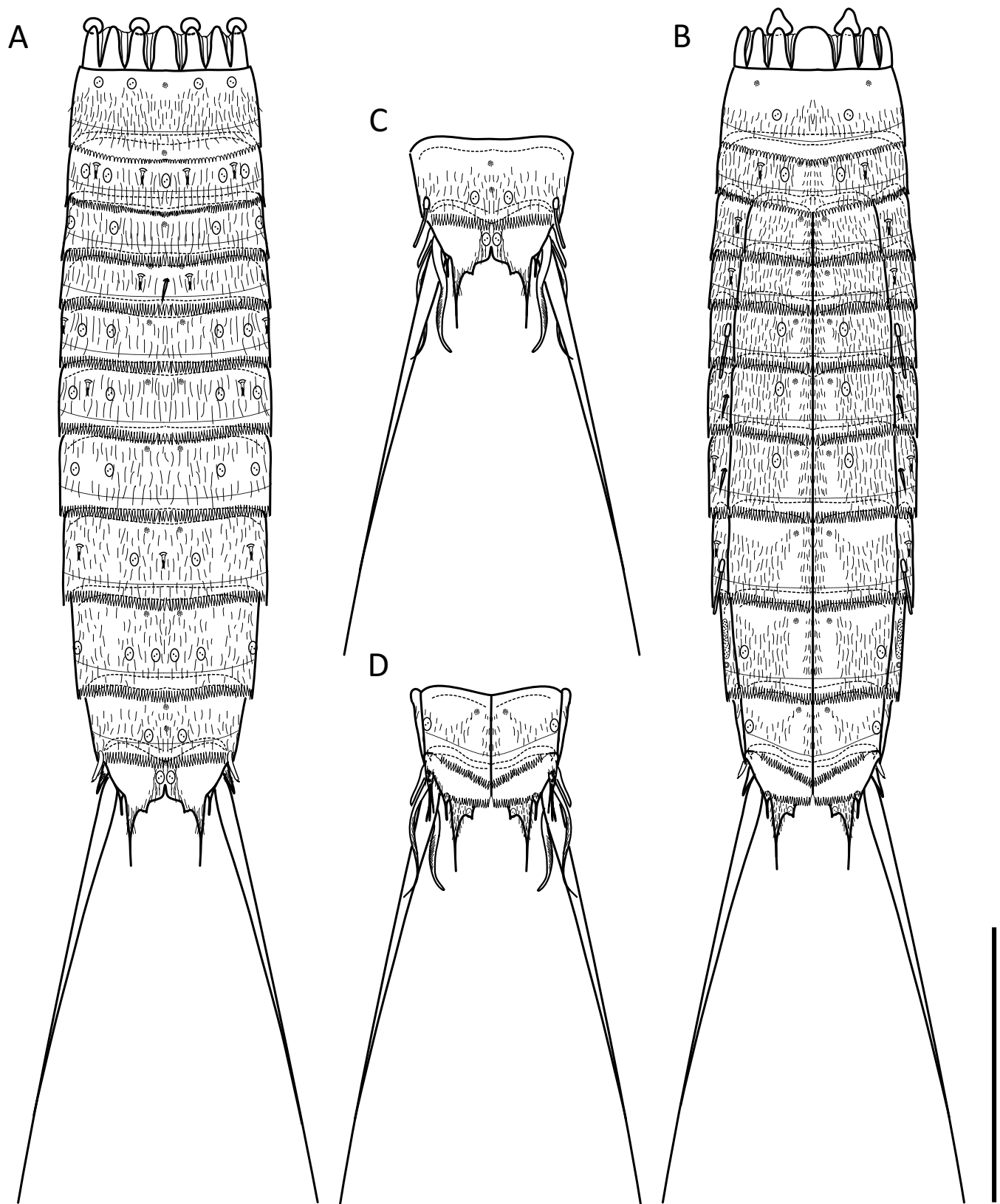
Position Segment	MD	PD	SD	LD	ML	SL	LA	LV	VL	VM
1	gco1		ss		ss			gco1	ss	
2	gco1		ss	ss				tu		ss, gco1
3	gco1		ss		ss					ss, gco1
4	ac		gco1	ss						ss, gco1
5	ac		ss, gco1	ss				tu		ss, gco1
6	ac	ss	ss, gco1	ss				ac	pa(♀)	ss, gco1
7	ac	ss	ss, gco1	ss				ac	pa(♀)	ss, gco1
8	ac	ss	ss, gco1	ss, gco2			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

Figure

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Figure







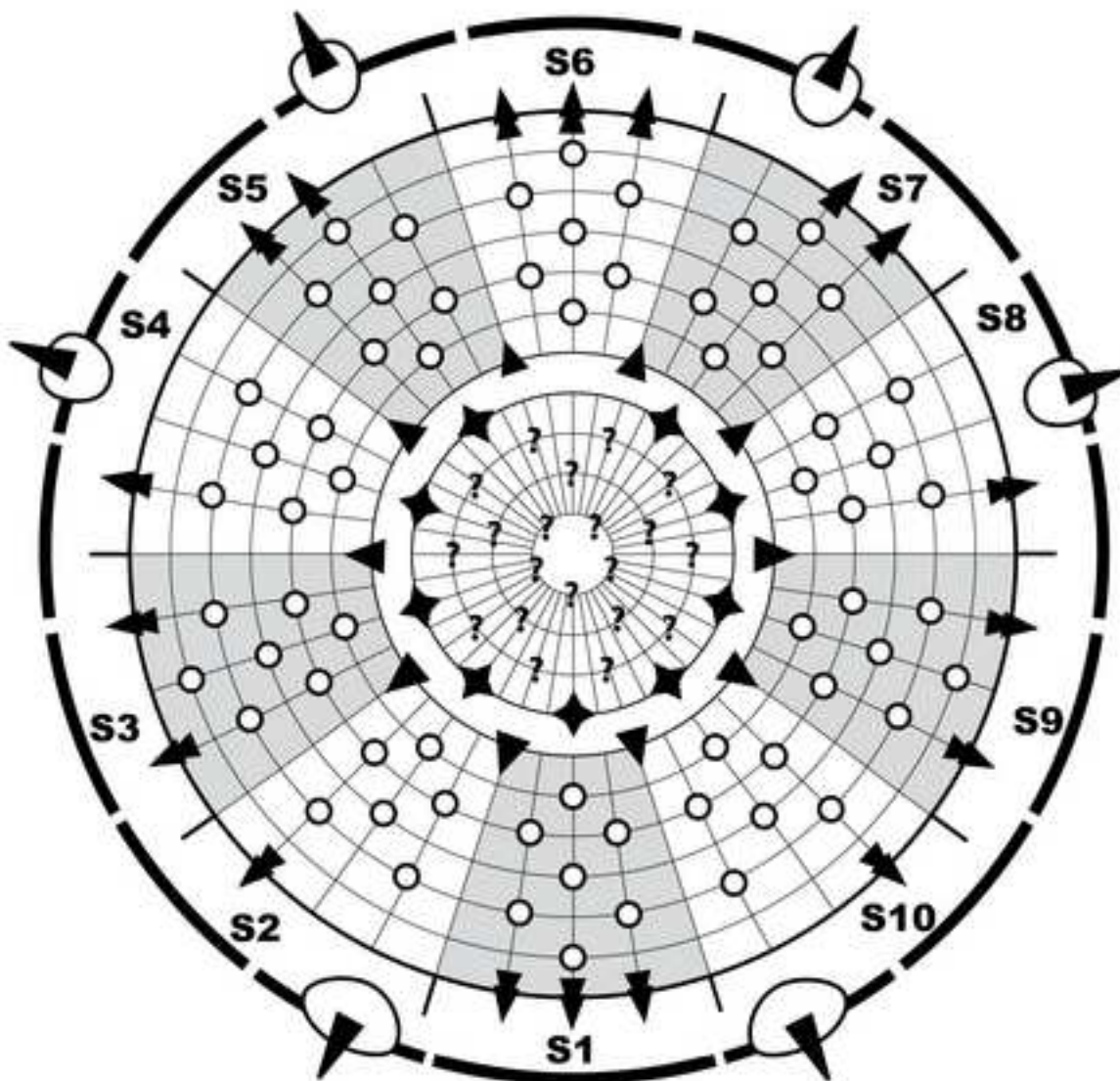
	Glandular cell outlet type 1		Fringed tube
	Sensory spot		Sieve plate

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Scalid and style arrangement

Ring/Sector	1	2	3	4	5	6	7	8	9	10	Total
00 oos \blacklozenge	1	1	1	1	1	0	1	1	1	1	9
01 psp \blacktriangledown	1	1	1	1	1	1	1	1	1	1	10
02 sp \circ	1	1	1	1	1	1	1	1	1	1	10
03 sp \circ	2	2	2	2	2	2	2	2	2	2	20
04 sp \circ	1	1	1	1	1	1	1	1	1	1	10
05 sp \circ	2	2	2	2	2	2	2	2	2	2	20
06 sp \circ	1	0	1	0	1	1	1	0	1	0	6
07 ls \blacktriangledown	3	1	2	1	2	3	2	1	2	1	18
08 tr \odot	0	1	0	1	1	0	1	1	0	1	6
Total scalds	8	8	9	8	10	10	10	8	9	8	90

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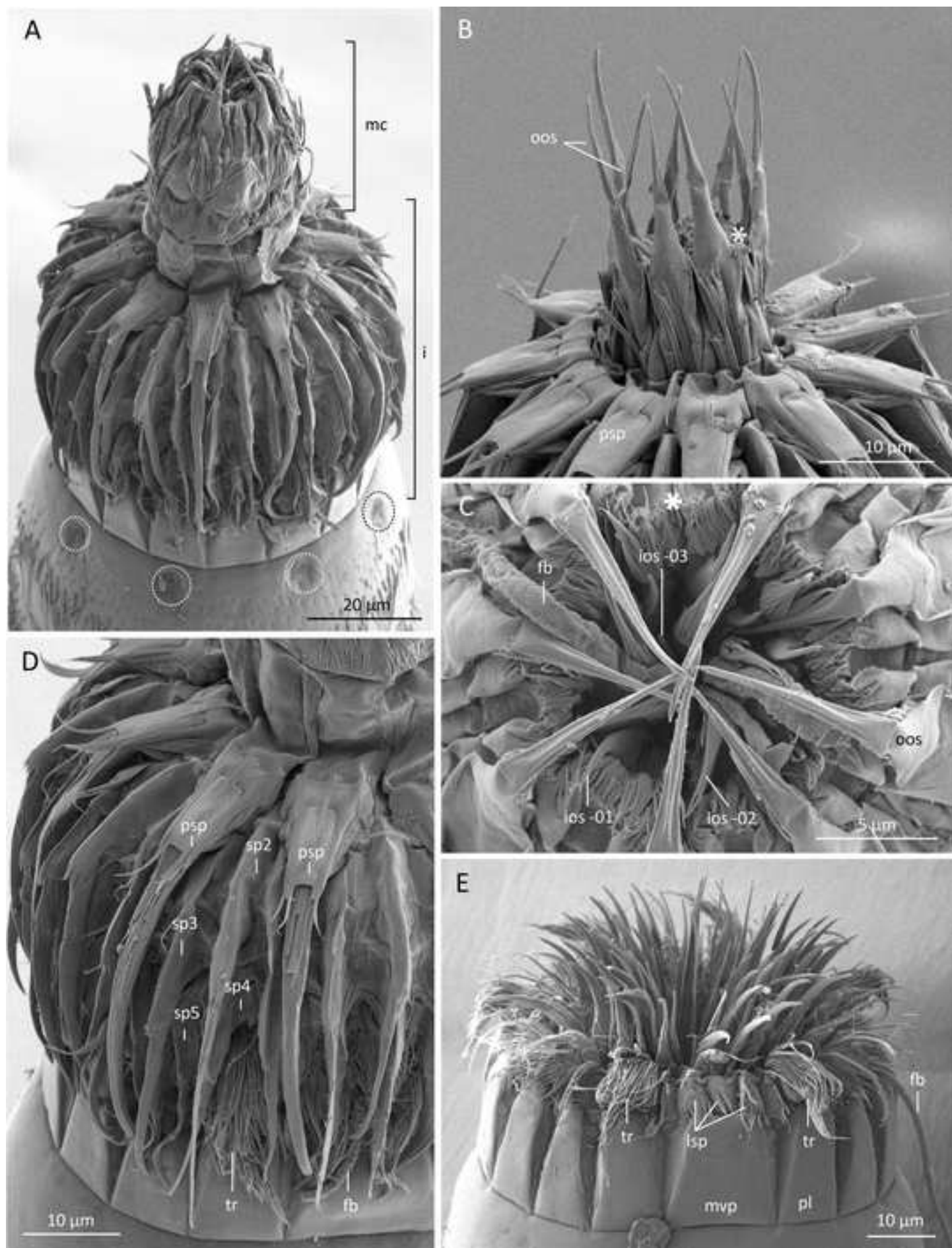
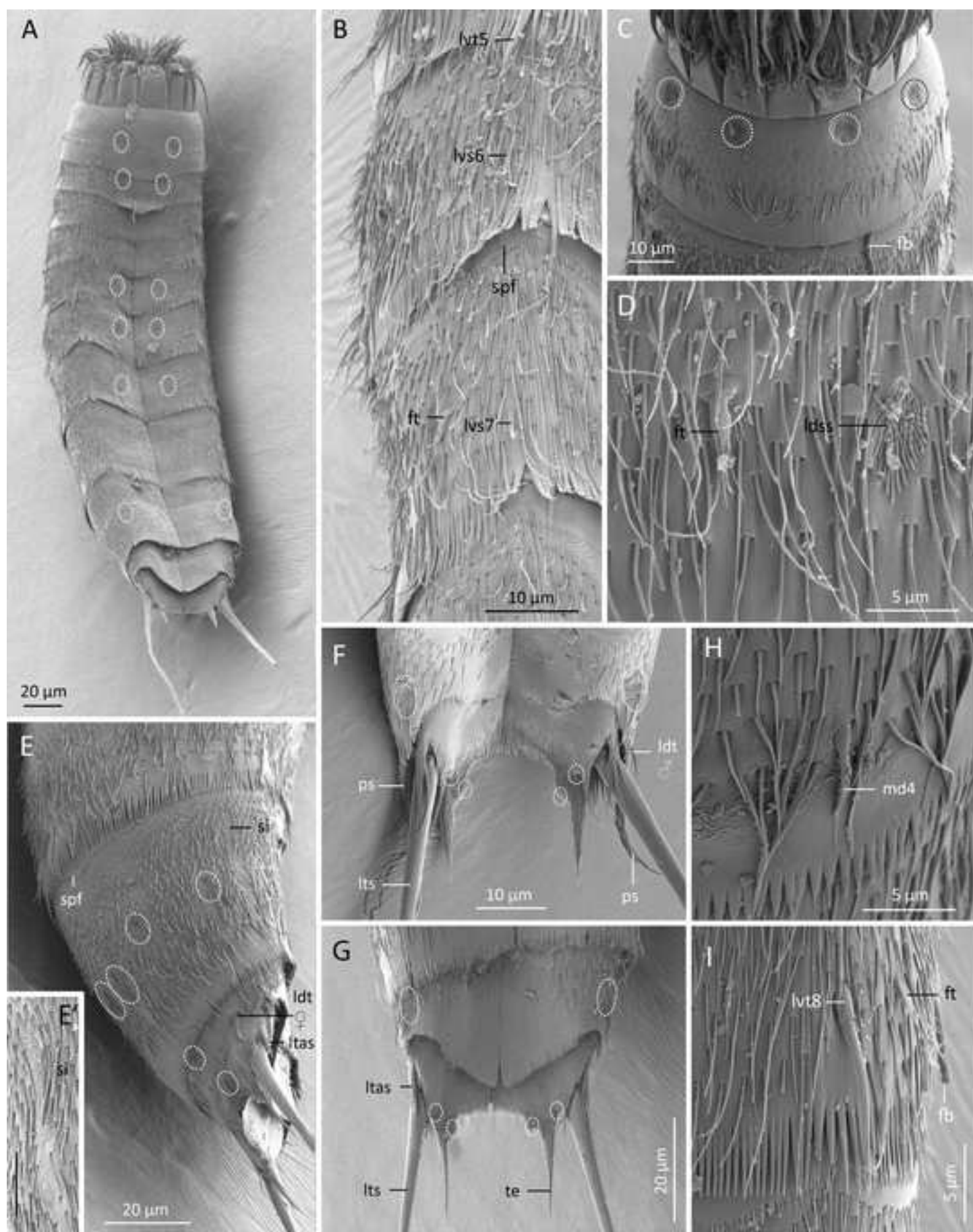


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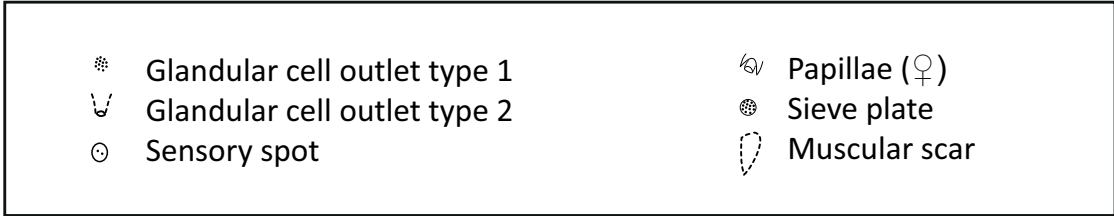
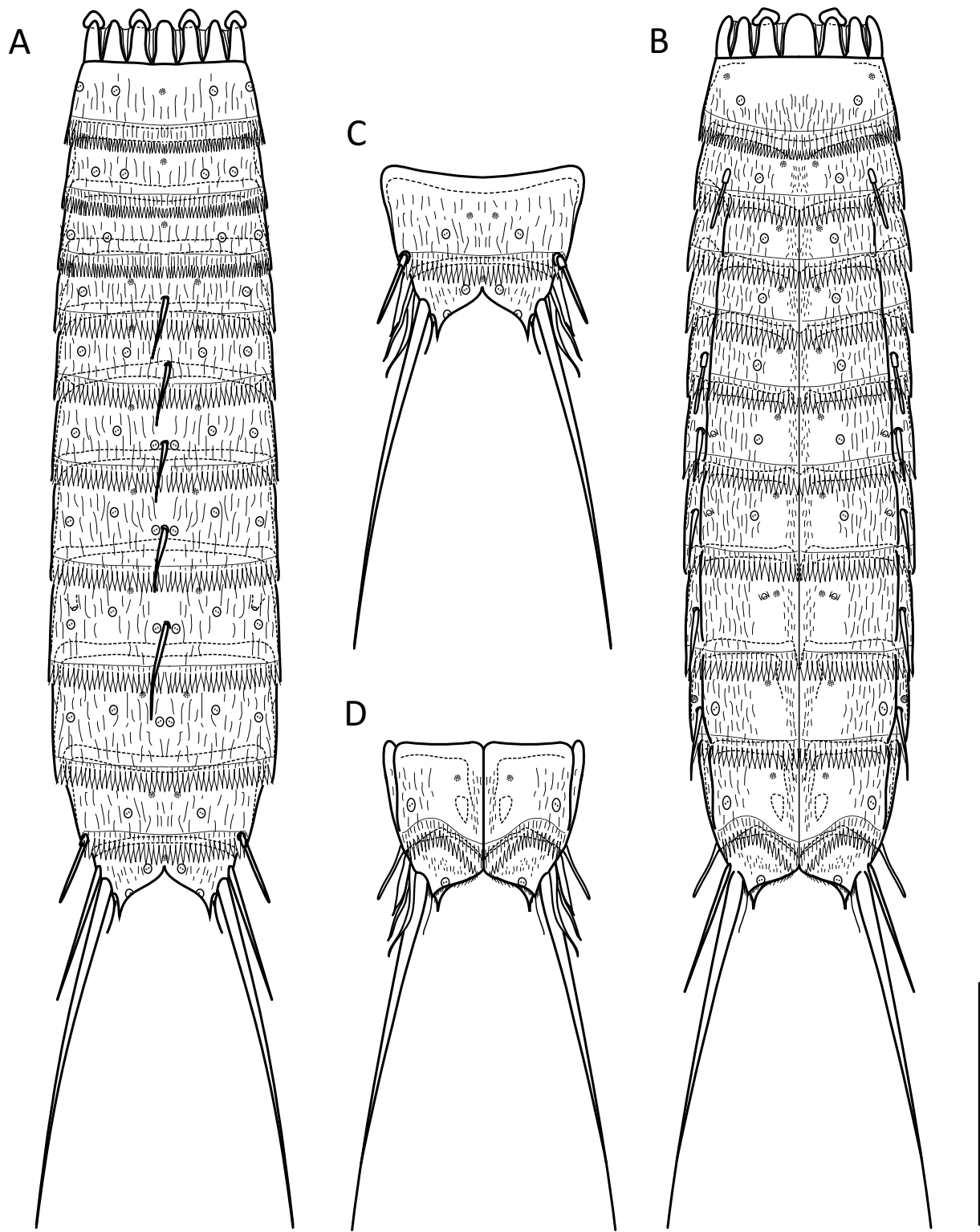
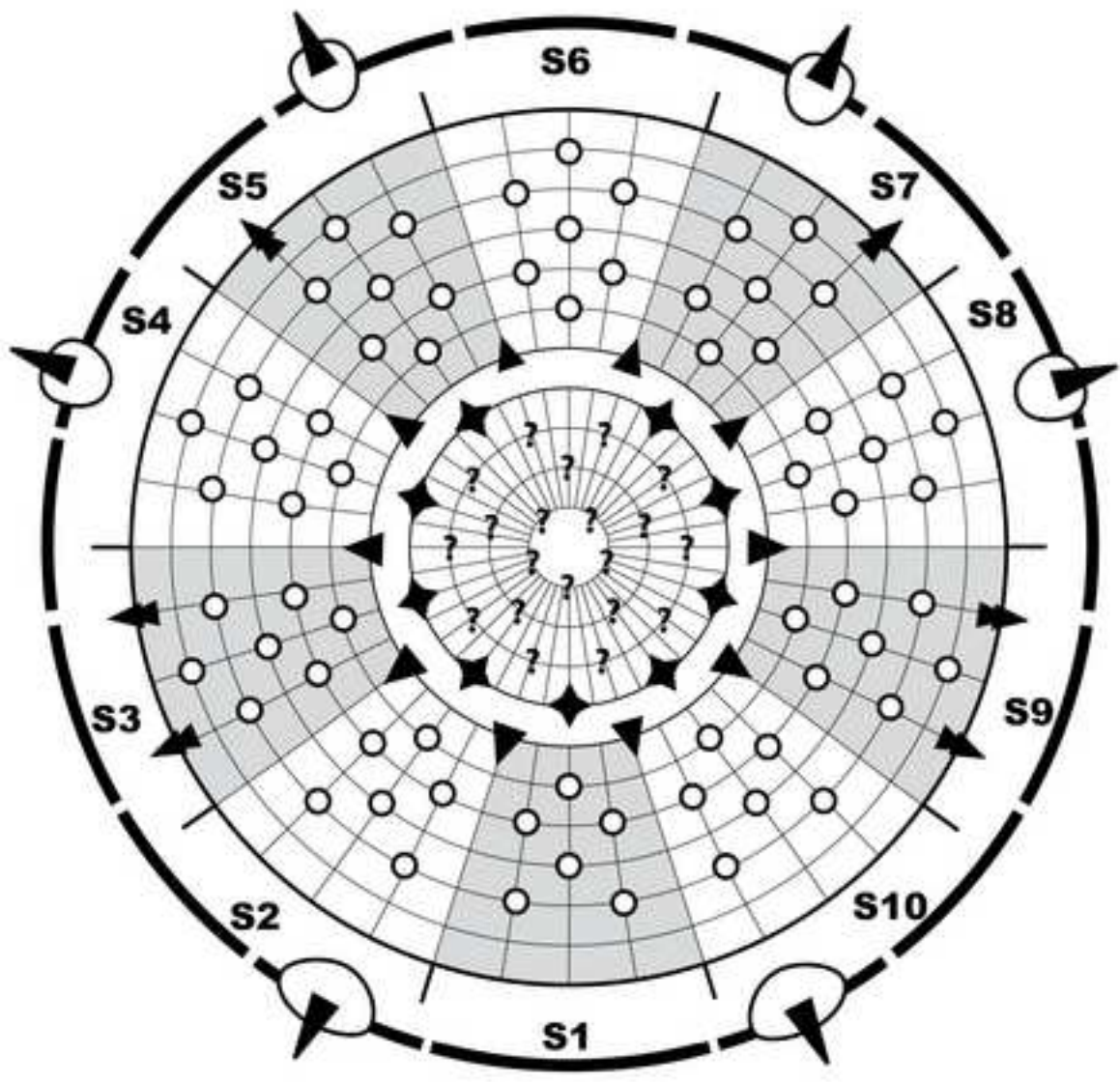


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- | | |
|---------------------|-------------------|
| ◆ Outer oral styles | ▼ Fringed scalids |
| ▼ Spinoscalids | ⊕ Trichoscalids |
| ○ Scalids | |

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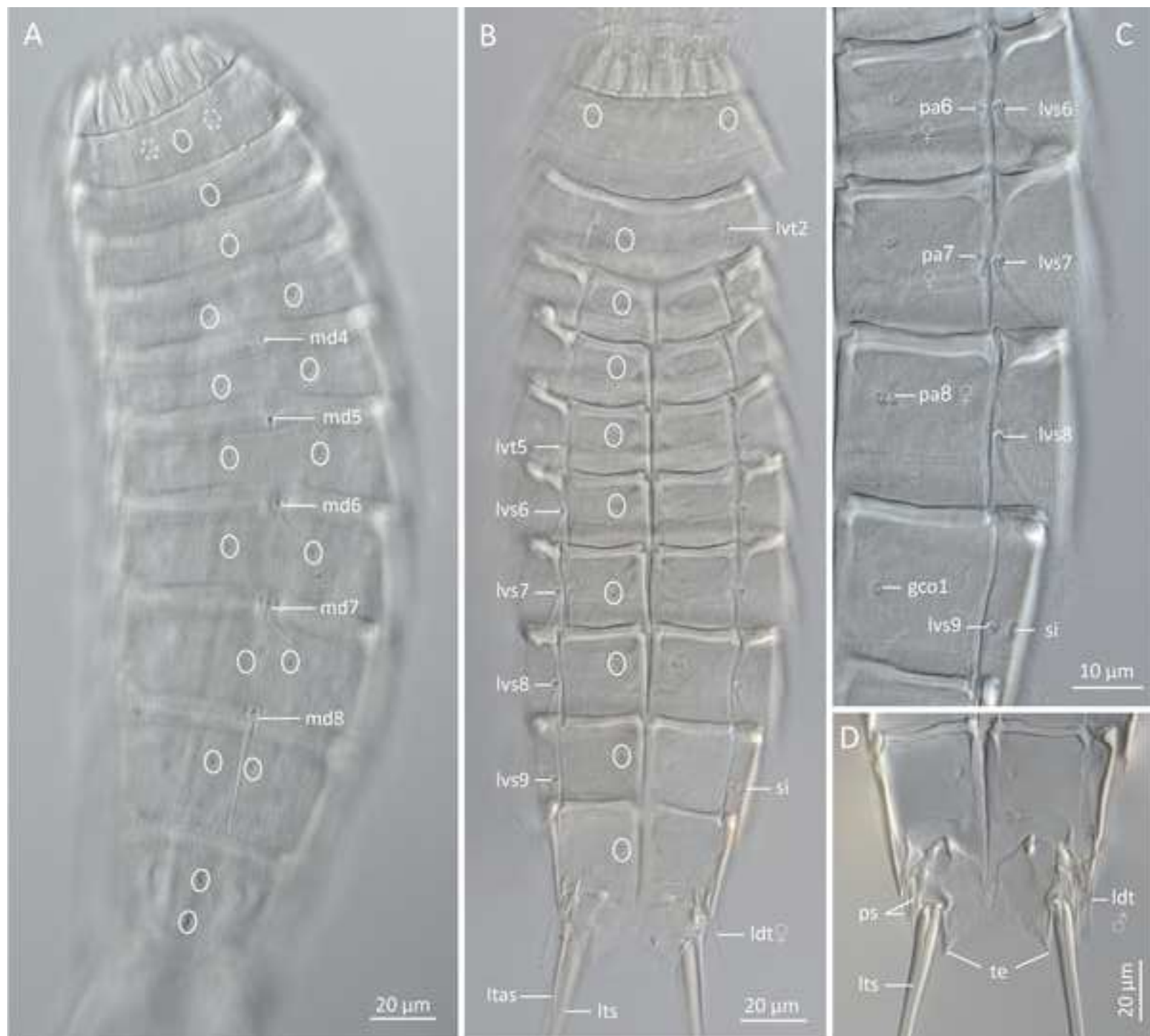
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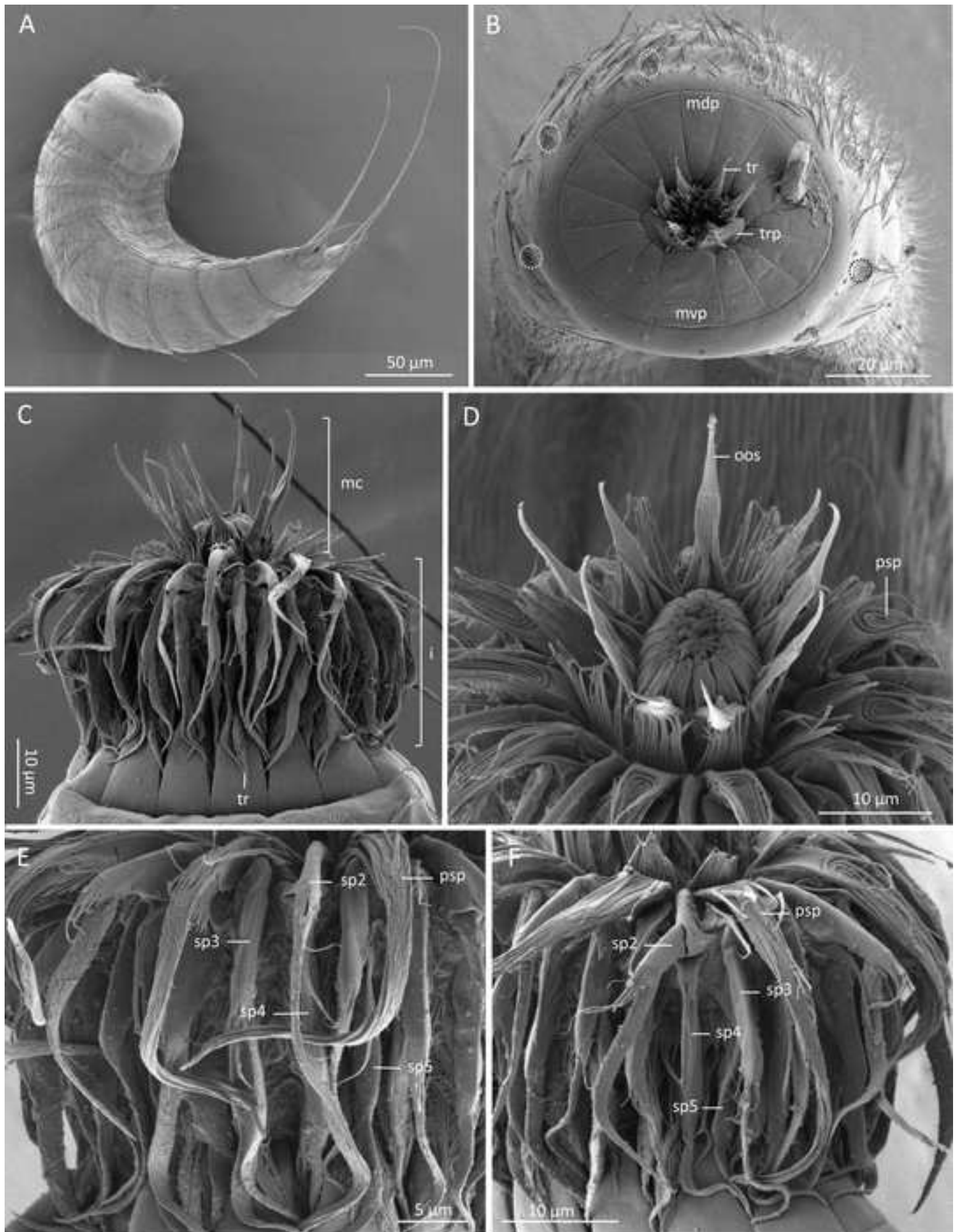


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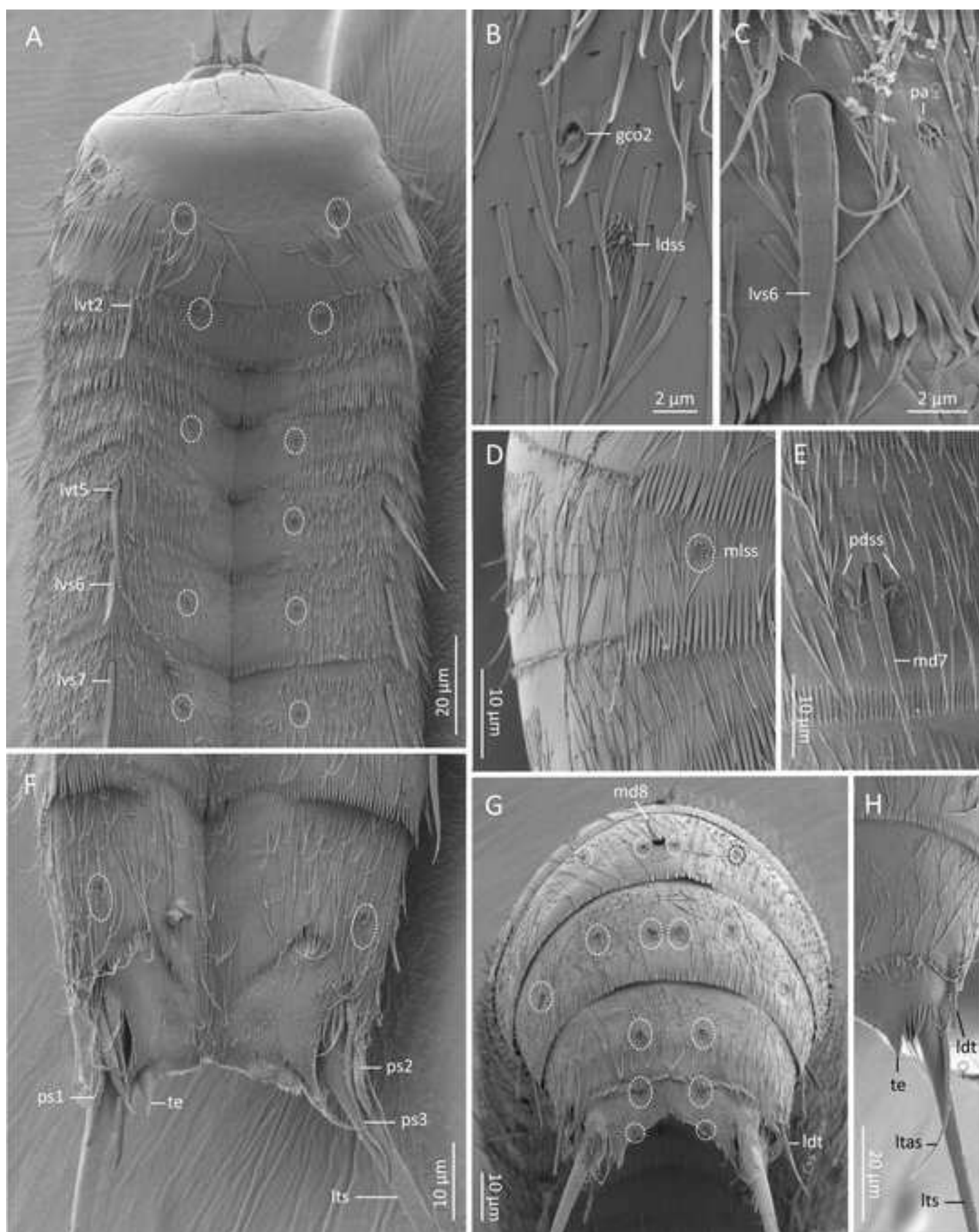


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