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A new species of *Eoperipatus* (Onychophora) from Vietnam reveals novel morphological characters for the South-East Asian Peripatidae

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ABSTRACT

Although representatives of Peripatidae are widely distributed in South-East Asia, only three valid species of *Eoperipatus* and one species of *Typhloperipatus* have been described from this region. According to previous reports, the three species of *Eoperipatus* show little morphological variation and are difficult to distinguish from each other. In this study, we describe a new species of *Eoperipatus* from Vietnam, *E. totoro* **sp. nov.**, using morphological (light and scanning electron microscopy) and molecular data (mitochondrial *COI* and *12S rRNA* sequences). A comparison with specimens of an undescribed species of *Eoperipatus* from Thailand revealed novel species-specific characters, including the characteristics of male crural complexes, distinct types of scales on the ventral body surface, the inner structure of the circular pits on the male genital pad, and the position and size of the anal gland pads in males. The results of our molecular analyses correspond with those of morphological studies. In contrast to previous assumptions, our findings suggest a high diversity of the South-East Asian Peripatidae, which requires further exploration.

1. Introduction

Only four valid species of Peripatidae (Onychophora, velvet worms) have been described from South-East Asia, the last description being published 100 years ago (Kemp, 1913; Oliveira et al., 2012a). The valid species include *Eoperipatus butleri* Evans, 1901a, *Eoperipatus horsti* Evans, 1901b, *Eoperipatus weldoni* Evans, 1901b and *Typhloperipatus williamsoni* Kemp, 1913, whereas *Eoperipatus sumatranus* (Sedgwick, 1888) is a *nomen dubium*, as neither the holotype has been designated nor the type locality is known for this species (Horst, 1886; Oliveira et al., 2012a; Sedgwick, 1888; van der Lande and Holthuis, 1986). Representatives of *Eoperipatus* were reported from numerous localities in Malaysia (including the Malaysian part of Borneo), but also from Singapore and, more recently, from Thailand and Vietnam, thus considerably extending the north-eastern distribution range of this taxon (Fig. 1A; Baer and Mayer, 2012; Bai and Anh, 2010; van der Lande and Holthuis, 1986).

Despite numerous records from a large geographic area occupying thousands of square kilometres, ranging in altitude and separated by the sea, representatives of *Eoperipatus* from different localities apparently show only little morphological variation, which has led to subsequent synonymisations (e.g., Bouvier, 1905; Dover, 1927; Gravier and Fage, 1926; Hendrickson, 1957; Kloss, 1926; Smedley, 1932; Sundara Rajulu and Singh, 1969; van der Lande, 1988). The most extreme case of synonymisation was the inclusion of all described species of *Eoperipatus* into a single species, *E. sumatranus* (e.g., Smedley, 1932; van der Lande and Holthuis, 1986). However, this inclusion might be unjustified, given the minor albeit existent differences between the species (Evans, 1901a,b; Kloss, 1926; Oliveira et al., 2012a). Thus, additional studies of the South-East Asian Peripatidae are required to clarify species diversity of this understudied onychophoran subgroup.

In this work, we describe a new species of *Eoperipatus*, which has been reported previously from Vietnam (Bai and Anh, 2010).

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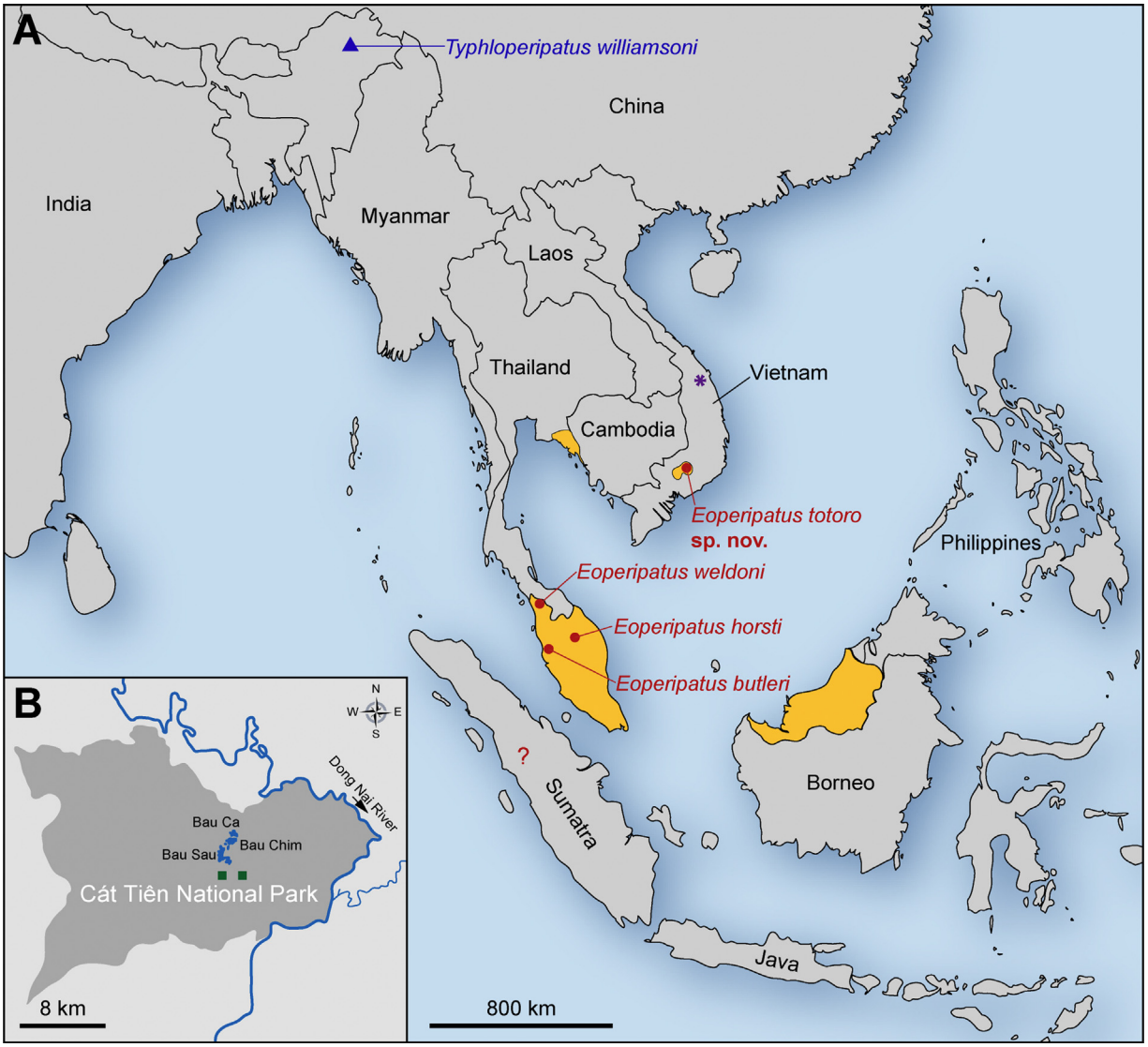


Fig. 1. Distribution of the South-East Asian species of Peripatidae. (A) Map showing the type localities of the species of *Eoperipatus* (red dots) and *Typhloperipatus* (blue triangle). Areas from which specimens of *Eoperipatus* have been recorded previously are highlighted in yellow. Question mark indicates the questionable type locality of *Eoperipatus sumatranus* in Sumatra. Purple asterisk indicates a recent record of an unidentified onychophoran in northern Vietnam (Moler et al., 2013). (B) Detailed map of the Cát Tiên National Park in Vietnam and the collecting sites of *Eoperipatus totoro* **sp. nov.** (green squares). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To gain insights into the morphological and genetic diversity of the group, we compared specimens of the new species to those of an additional species of *Eoperipatus* from Thailand (Baer and Mayer, 2012; Oliveira et al., 2012b). Our findings shed light on diversity and biogeography of *Eoperipatus* and highlight the need for more studies on this taxon.

2. Materials and methods

2.1. Animals

Specimens of *Eoperipatus totoro* **sp. nov.** were collected in the Cát Tiên National Park, province of Dong Nai, Vietnam (11°27′08”–11°27′32”N, 107°20′43”–107°22′04W, 109–135 m) (Fig. 1A and B). They were found under stones (Fig. 2A), mainly during the wet season (November to June 2007–2009). Specimens of *Eoperipatus* sp. ($n = 6$), an undescribed species from Thailand, were obtained as described previously (Baer and Mayer, 2012; Oliveira et al., 2012b). Unfortunately, this species cannot be described formally, as precise locality data are missing (Baer and Mayer, 2012). Syntypes of *E. horsti* (BMNH 1004909–1004914), *E. weldoni* (BMNH 1004884–1004894; 1004896–1004907) and *E. butleri* (BMNH 1004908), held in the Onychophora collection of the Natural History Museum of London (United Kingdom), were analysed for comparison. Information on *E. sumatranus* was obtained from the literature (Bouvier, 1905; Evans, 1901b; Horst, 1886; Sedgwick, 1888).

2.2. Morphological studies

Living specimens were photographed with digital camera and flashlight. Specimens preserved in 70% ethanol were analysed and photographed using a stereomicroscope as described previously (Oliveira et al., 2012b). For scanning electron microscopy, specimens of each sex were fixed in 4% formalin and dehydrated in an ethanol series. After dehydration, specimens were dried in a critical point dryer (CPD7501, Polaron Range, Quorum Technologies Ltd, East Sussex, United Kingdom), coated with gold in a

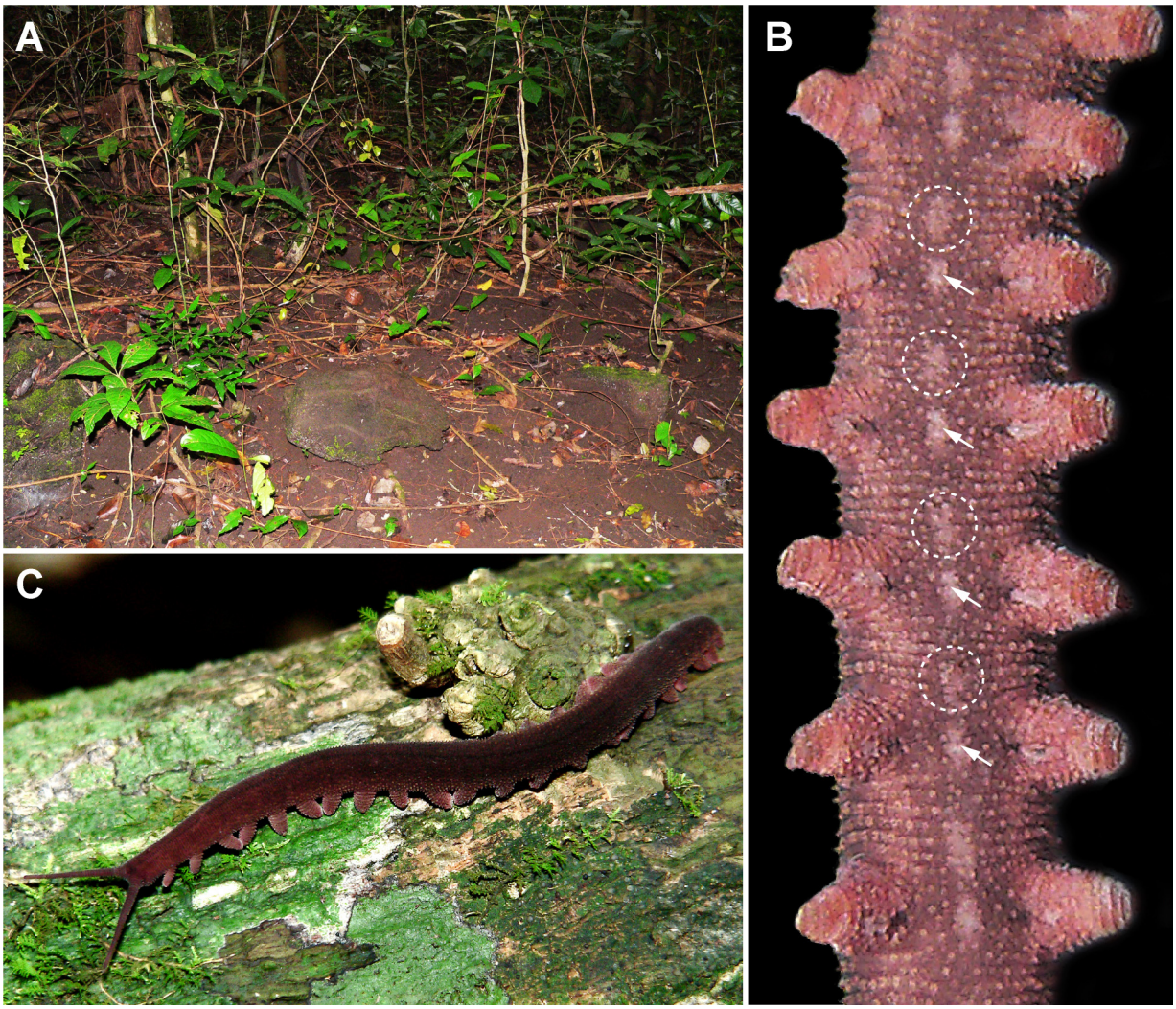


Fig. 2. Microhabitat and life colour of *Eoperipatus totoro* **sp. nov.** Photographs. (A) Typical habitat of *E. totoro* **sp. nov.** in the Cát Tiên National Park. Note the large stones under which the specimens are usually found. (B) Mid-body of a specimen in ventral view. Note the repeated bright spots along the ventral midline, which correspond to the ventral fields of modified scales (dashed circles) and to the ventral and preventral organs (arrows). Anterior is up. (C) Walking specimen in dorso-lateral view. Note the brown colour of dorsal integument without any pattern.

SCD 050 Sputter Coater (Balzers Union, Balzers, Liechtenstein) and examined in a scanning electron microscope (EVO 50, Carl Zeiss, Jena, Germany). The classification and terminology of morphological structures were used according to Oliveira et al. (2012b, 2010). Type specimens were deposited in the collections of the Zoological Museum of Moscow University, Russia (ZMMU) and the Zoological Museum of Berlin, Germany (ZMB). Nomenclatural acts suggested in this paper were registered in ZooBank following the recommendations of the International Commission of Zoological Nomenclature.

2.3. Molecular analyses

Total DNA was extracted from fresh muscle tissue of a single specimen of each species using a NucleoSpin® Tissue Kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's protocol. DNA was sheared with Covaris S2 Sonicator (Covaris Inc., Woburn, MA, USA). Starting at the blunt end repair step, a shotgun library for the entire genome was prepared and sequenced as described previously (Oliveira et al., 2012b). This library was sequenced according to the manufacturer's instructions for single read multiplex experiments with 76 cycles paired-end on the Genome Analyzer IIx platform (v4 sequencing chemistry and v4 cluster generation kit, Illumina, San Diego, CA, USA). The sequenced reads were assembled *de novo* using the CLC Genomics Workbench 4.7.2 (CLC bio, Aarhus, Denmark). The *COI* sequence was obtained from the whole genome shotgun library by BLAST searches (Altschul et al., 1997). The *12S rRNA* sequence was amplified using the primers SR-J-14233 and SR-N-14588 from Simon et al. (1994). Sequences were aligned using the online version of MAFFT (Katoh et al., 2005), applying the FFT-NS-i strategy. The obtained sequences were placed in GenBank under specific accession numbers (Table 1). The Maximum Likelihood inference method was used for phylogenetic analyses using the combined *12S rRNA* and *COI* datasets as described previously (Oliveira et al., 2012b). Twenty-eight additional sequences from ten species of Peripatidae (ingroup) and five species of Peripatopsidae (outgroup) were obtained from GenBank and from the literature and included in the phylogenetic analyses (Table 1). Translated amino acid alignments were verified *a priori* using DAMBE (Xia and Xie, 2001). The Maximum Likelihood analysis was conducted using RAxML 7.3.0 PTHREADS-SSE3 (Stamatakis, 2006), selecting the substitution models GTR+G+I for nucleotides and MTART for amino acids. The latter was derived from PROTTEST3 (Abascal et al., 2005)

Table 1
COI and *12S rRNA* sequences used for phylogenetic analyses with corresponding GenBank accession numbers and references.

Species name	Accession number (*COI*)	Reference (*COI*)	Accession number (*12S rRNA*)	Reference (*12S rRNA*)
Peripatidae (ingroup)				
Eoperipatus sp.	JX569005	Oliveira et al. (2012b)	JX568982	Oliveira et al. (2012b)
Eoperipatus totoro **sp. nov.**	KC139082	Present study	KC139083	Present study
Epiperipatus acacioi (Marcus and Marcus, 1955)	HQ404902–404905	Lacorte et al. (2011)	HQ404920–404923	Lacorte et al. (2011)
Epiperipatus adenocryptus Oliveira et al., 2011	HQ236113, 236114	Oliveira et al. (2011)	HQ236139, 236140	Oliveira et al. (2011)
Epiperipatus biolleyi (Bouvier, 1902)	NC_009082	Podsiadlowski et al. (2008)	NC_009082	Podsiadlowski et al. (2008)
Epiperipatus biolleyi	HM600781	Rota-Stabelli et al. (2010)	HM600781	Rota-Stabelli et al. (2010)
Epiperipatus diadenoproctus Oliveira et al., 2011	HQ236095–236097	Oliveira et al. (2011)	HQ236121–236123	Oliveira et al. (2011)
Epiperipatus machadoi (Oliveira and Wieloch, 2005)	HQ236089, 236090	Lacorte et al. (2011)	HQ236115, 236116	Lacorte et al. (2011)
Epiperipatus paurognostus Oliveira et al., 2011	HQ236104–HQ236106	Oliveira et al. (2011)	HQ236130–236132	Oliveira et al. (2011)
Principapillatus hitoyensis Oliveira et al., 2012b	JX568985, 568995	Oliveira et al., 2012b	JX568962, 568972	Oliveira et al., 2012b
Oroperipatus sp.	NC01589	Segovia et al. (2011)	NC015890	Segovia et al. (2011)
Peripatus solorzanoi Morera-Brenes and Monge-Nájera, 2010	[a]	Morera-Brenes and Monge-Nájera (2010)	–	–
Peripatopsidae (outgroup)				
Euperipatoides rowelli Reid, 1996	U62425	Gleeson et al. (1998)	AF338016	Rockman et al. (2001)
Metaperipatus inae Mayer, 2007	EF624055	Braband et al. (2010a)	EF624055	Braband et al. (2010a)
Opisthopatus cinctipes Purcell, 1899	NC014273	Braband et al. (2010b)	NC014273	Braband et al. (2010b)
Peripatopsis moseleyi (Wood-Mason, 1879)	EU855276	Daniels et al. (2009)	EU855469	Daniels et al. (2009)
Phallocephale tallagandensis Reid, 1996	U62407	Gleeson et al. (1998)	AF338015	Rockman et al. (2001)

[a] Sequences not found in GenBank but obtained from the original publication (Morera-Brenes and Monge-Nájera, 2010).

according to the Akaike information criterion (Akaike, 1973). Node support was calculated using 1000 bootstrap pseudoreplicates (Felsenstein, 1985).

3. Description of the new onychophoran species from Vietnam

Eoperipatus Evans, 1901b
urn:lsid:zoobank.org:act:3513CCF8-476E-41B8-BA5B-7B7852E6EAB7
Eoperipatus totoro, **sp. nov.**
urn:lsid:zoobank.org:act:E62AA5B7-77E2-4143-A516-9615BBC4FE17
(Figs. 1A and B, 2A–C, 3, 4A–F, 5A and B, 6A–C, 7A–C, 8A–C, 9A–F, 10A–E, 11A–F, 12, S1, S2)

3.1. Material examined

Holotype: male, in 70% ethanol, Vietnam, Dong Nai Province, Cát Tiên National Park, 107°20′43.7″E, 11°27′32.9″N, 119 m, May 2009, P. Geissler col. (ZMB 48506). Paratypes: two females, in 100% ethanol, locality as for the holotype, 11°27′08″, 107°22′04W, June 2008, P. V. Kvartalnov col. (ZMMU Z1, Z2). Additional material used for morphological and molecular analyses: one male and two females, locality as for the paratypes, June 2008, P. V. Kvartalnov col.; one female, locality as for the holotype, May 2009, P. Geissler col.

3.2. Etymology

Following the request of Pavel V. Kvartalnov, Eduard A. Galoyan and Igor V. Palko, the species is named after the main character of the cartoon movie "My Neighbour Totoro" by Hayao Miyazaki (1988, studio Ghibli), who uses a many-legged animal as a vehicle, which according to the collectors resembles a velvet worm.

3.3. Species diagnosis

Ventral integument showing an alternating pattern of bright spots along midline, which correspond to ventral fields of modified scales (found in interpedal regions) and to ventral and preventral organs (found between each leg pair) (Figs. 2A, 3, 4). Ventral fields

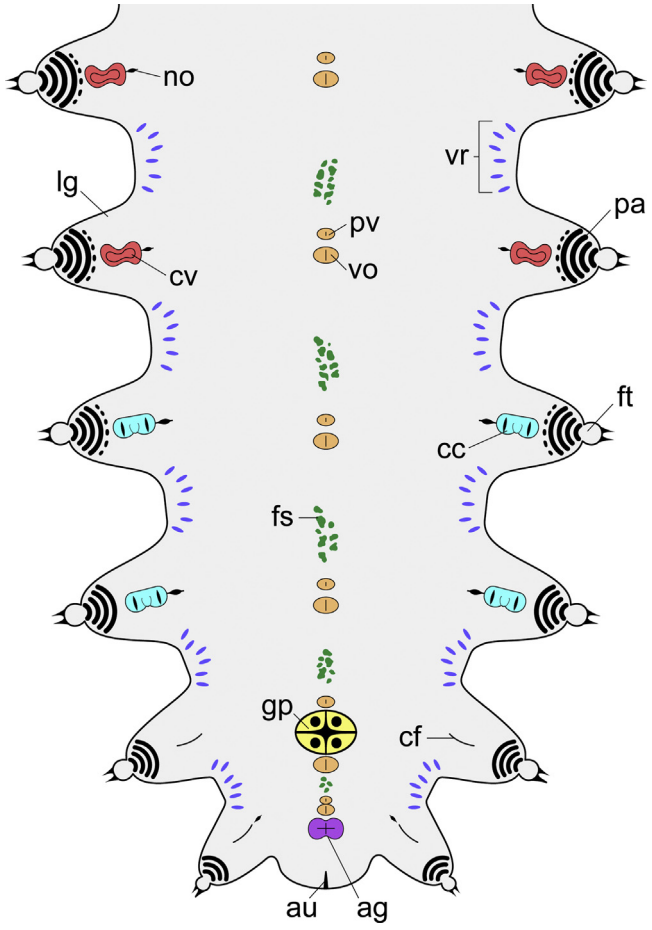


Fig. 3. Diagram of the posterior end of a male of *Eoperipatus totoro* **sp. nov.** in ventral view showing the position of structures studied. Anterior is up. *Abbreviations*: ag, anal gland opening; au, anus; cc, crural complex; cf, coxal furrow; cv, coxal vesicles; fs, ventral fields of modified scales; ft, foot; gp, genital pad; lg, leg; no, nephridial opening; pv, preventral organ; vo, ventral organ; vr, ventral row of type II crater-shaped papillae.

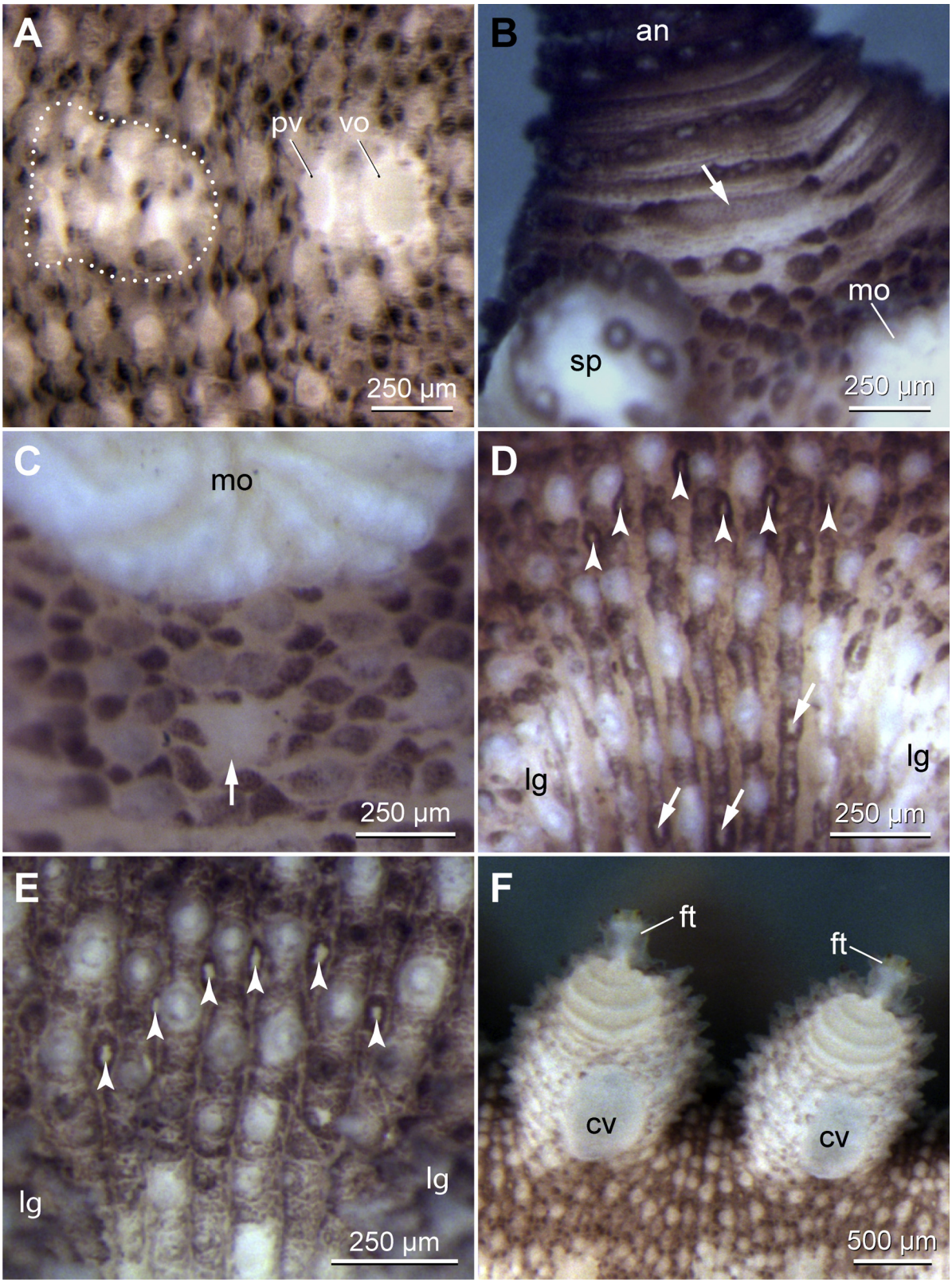


Fig. 4. Morphology of *Eoperipatus totoro* **sp. nov.** Light micrographs; anterior is left in A and D–F and up in B and C. (A) Detail of a ventral field of modified scales (dotted line) and the ventral and preventral organs. (B) Ventral view of the antennal basis showing a frontal organ (arrow). (C) Detail of the postoral pit (arrow). (D) Ventral row of type II crater-shaped papillae (arrowheads). Note the presence of type I crater-shaped papillae laterally on the plicae between two subsequent leg pairs (arrows in D). (E) Ventro-lateral row of type II crater-shaped papillae. (F) Two legs from the mid-body region in ventral view showing everted coxal vesicles. *Abbreviations*: an, antenna; cv, coxal vesicle; ft, foot; lg, leg; mo, mouth; pv, preventral organ; sp, slime papilla; vo, ventral organ.

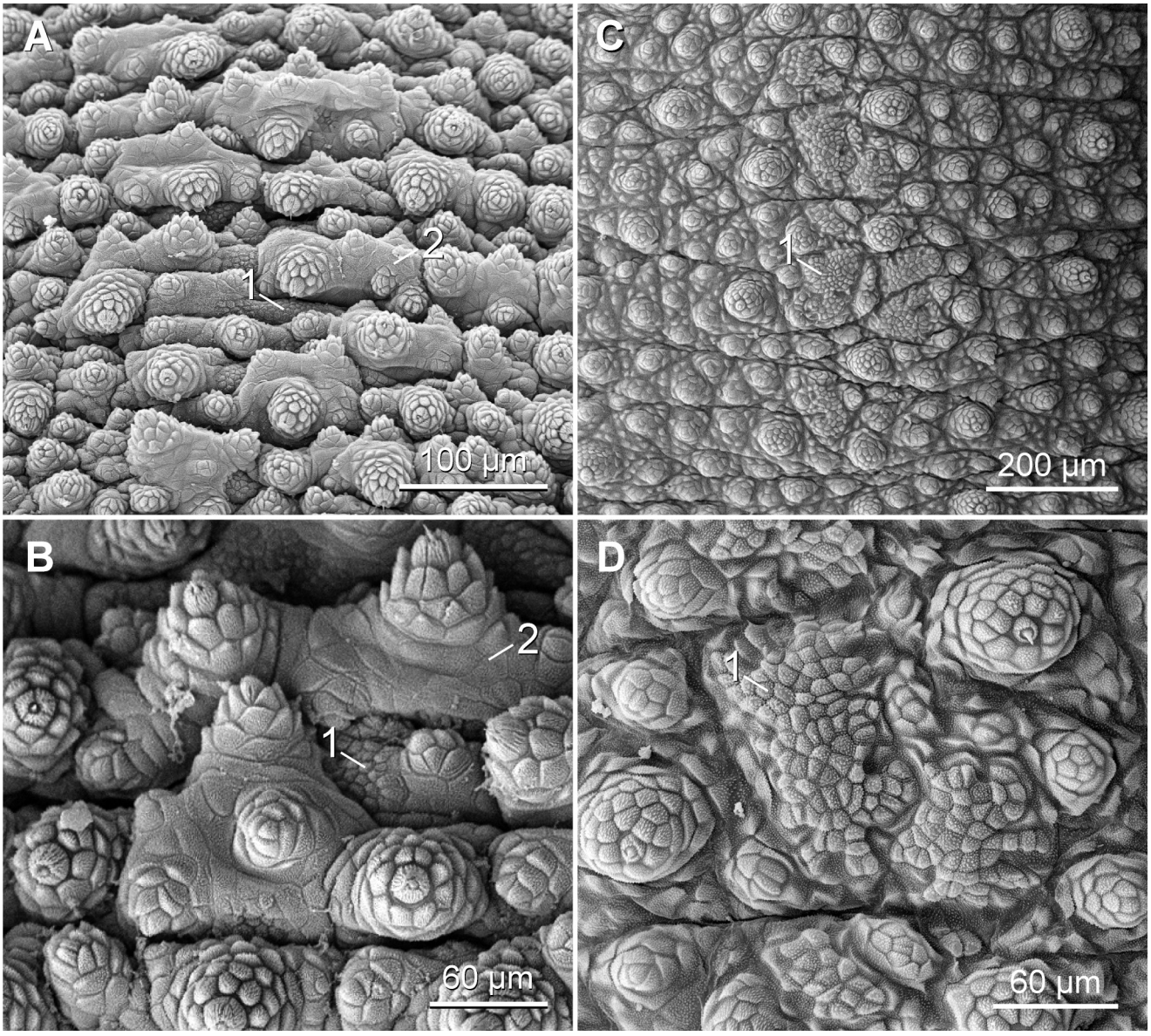


Fig. 5. Features of the ventral body surface in the two species of *Eoperipatus* studied. Scanning electron micrographs. (A, B) Overview and detail of a ventral field of modified scales in *Eoperipatus totoro* **sp. nov.** from Vietnam. (C, D) Overview and detail of a ventral field of modified scales in *Eoperipatus* sp. from Thailand. Note that two types of modified scales are present in *E. totoro* **sp. nov.** (numbered), whereas only one type occurs in *Eoperipatus* sp.

characterised by irregular regions with two different types of modified scales: large flattened scales and small granular scales (Fig. 5A and B). Scales covering genital and anal gland pads slender and with pointy tips (Figs. 6A and B and 7A–C). Inner wall of circular pits in the male genital pad covered with scales; floor of pits perforated with minute holes, which might represent tracheal openings (Fig. 6C). Anal cone reduced in size in males; anal gland pad situated between last pair of legs (Fig. 7A). Crural complexes present in two pregenital leg pairs; each complex with an anterior field of modified, wart-like and widely spaced scales and smooth cuticle (Figs. 3 and 8A–C). Apical pieces of dorsal primary papillae spherical, with asymmetrically arranged scales and posteriorly located sensory bristle (Fig. 9A and B). Males with 23, females with 24 leg pairs.

3.4. Description

The following data complement those in the species diagnosis.

Measurements. Maximum body size after fixation: length 65.0 mm, width 6.6 mm, height 3.8 mm.

Colour pattern. Colour of dorsal integument *in vivo* dark-brown without pattern; dorsal midline darker than surrounding integument (Fig. 2B). Ventral body surface brownish-pink, brighter than dorsal integument, with repeated bright spots; ventral surface of legs pink (Fig. 2A).

Antennae. Antennal tip composed of terminal button and 13 rings; 8th, 10th and 12th rings thinner than others (Fig. 10A); number of rings in antennal body variable (~40). Type I sensilla restricted to antennal body; type II sensilla restricted to antennal tip. Chemoreceptors roundish, surrounded by scales and arranged in anterior rows on each ring of antennal tip, except for thinner rings that lack chemoreceptors altogether (Fig. 10A); scattered chemoreceptors found dorso-laterally on alternated rings of proximal antennal portion. Spindle-shaped sensilla oval in shape and with short, blunt-ended bristles (Fig. 10B).

Head. No distinct head pattern. Eyes pigmented, diameter ~110 µm (Fig. 10C). Frontal organs present (Fig. 4B). Mouth

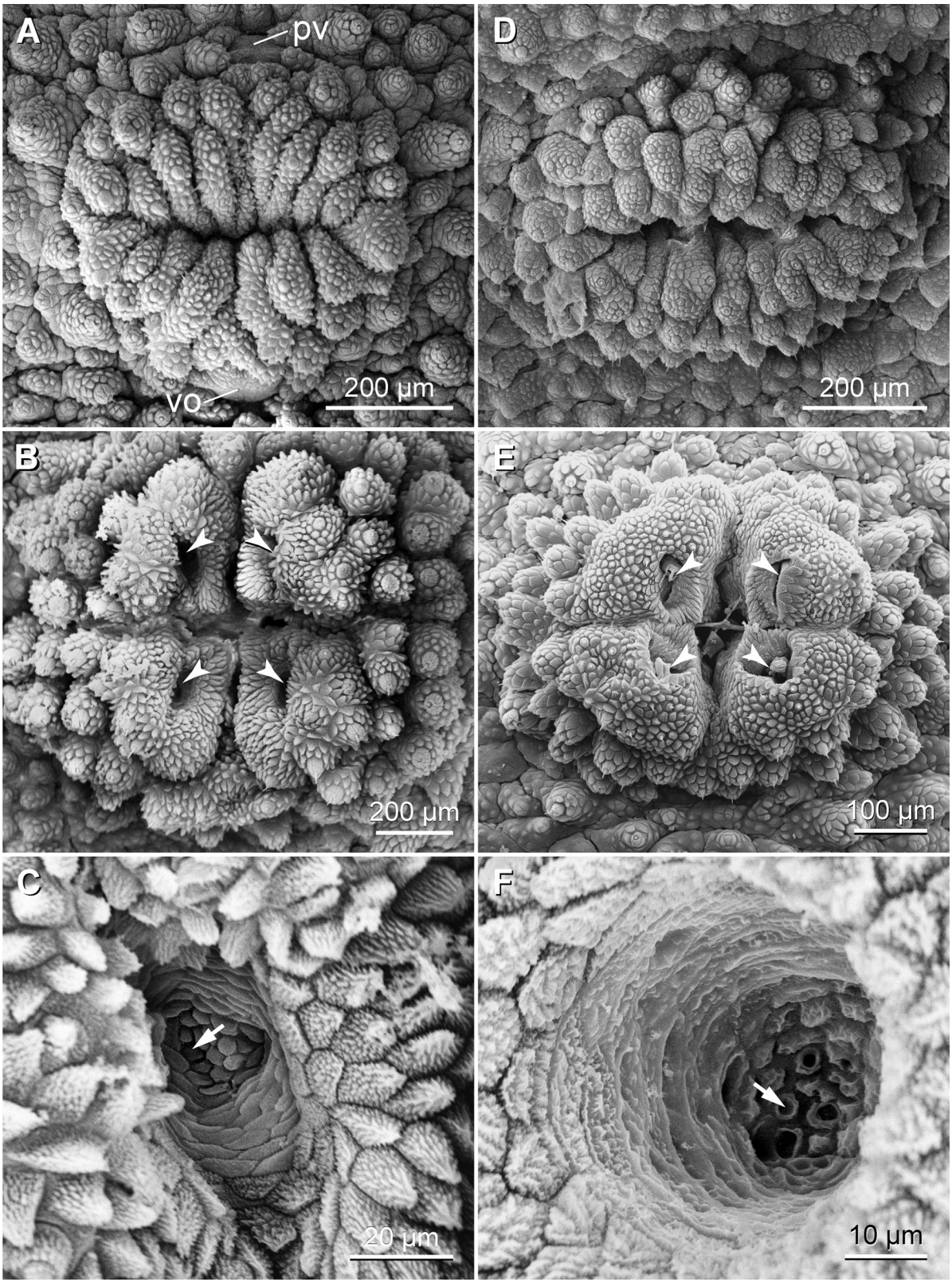


Fig. 6. Features associated with the genital pad in the two species of *Eoperipatus* studied. Scanning electron micrographs; anterior is up in all images. (A–C) *Eoperipatus totoro* **sp. nov.** (D–F) *Eoperipatus* sp. (A, D) Genital pad in females. (B, E) Genital pad in males. Arrowheads point to the circular pits. Note the inter-specific differences in the shape of scales covering the genital pads. (C, F) Inner structure of a circular pit. Arrows point to the holes at the floor of each pit. *Abbreviations*: pv, preventral organ; vo, ventral organ.

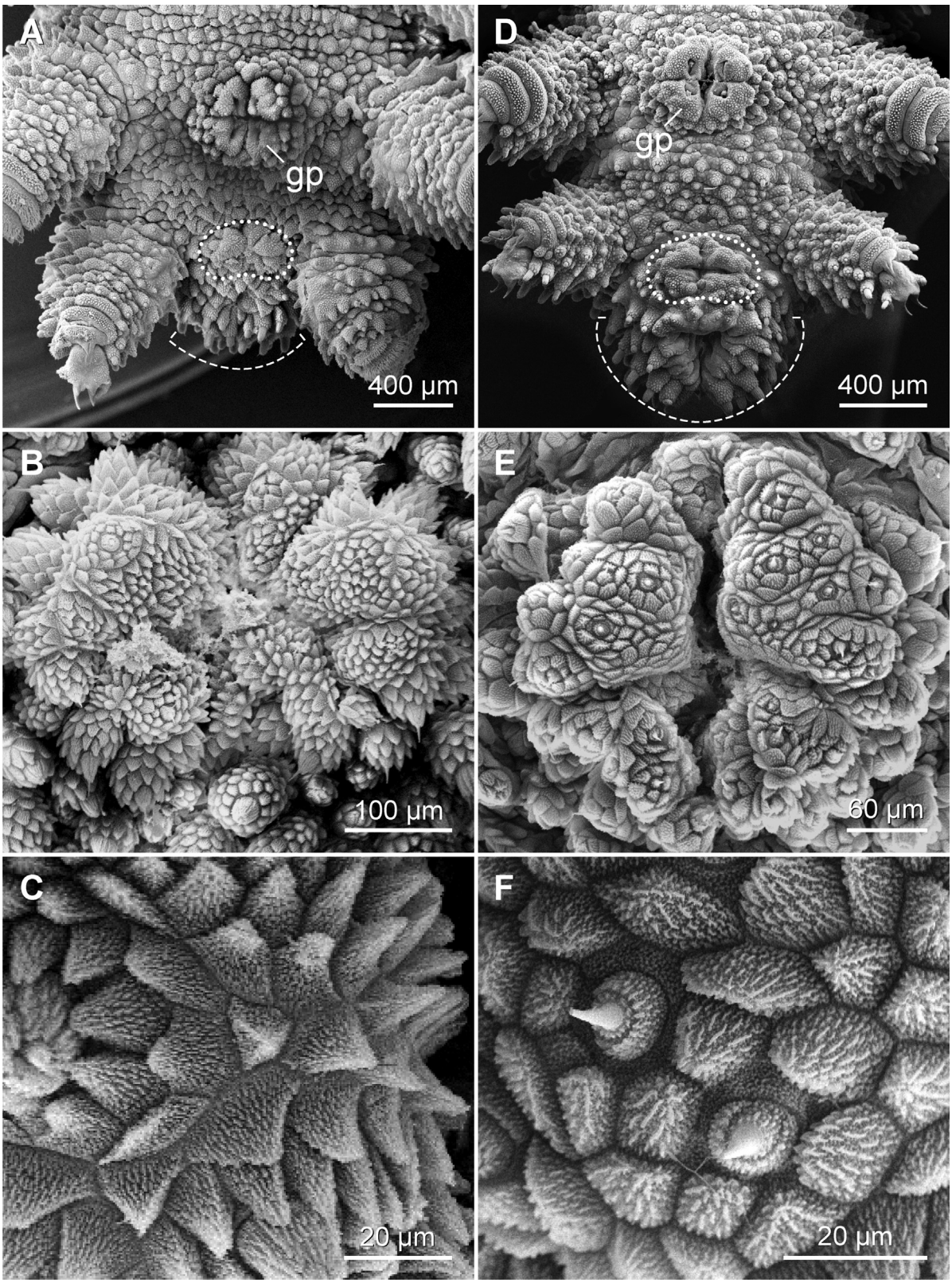


Fig. 7. Features associated with the male anal gland pad in the two species of *Eoperipatus* studied. Scanning electron micrographs; anterior is up in all images. (A–C) *Eoperipatus totoro* **sp. nov.** (D–F) *Eoperipatus* sp. (A, D) Position of the anal gland pad. Note that the anal cone (dashed line) in *Eoperipatus totoro* **sp. nov.** is relatively smaller than in *Eoperipatus* sp. Note also different position of the anal gland pad between the two species. (B, E) Anal gland pad. Observe the inter-specific variation of this structure regarding its size, shape and type of scale. (C, F) Detail of the scales covering the anal gland and genital pads. *Abbreviation*: gp, genital pad.

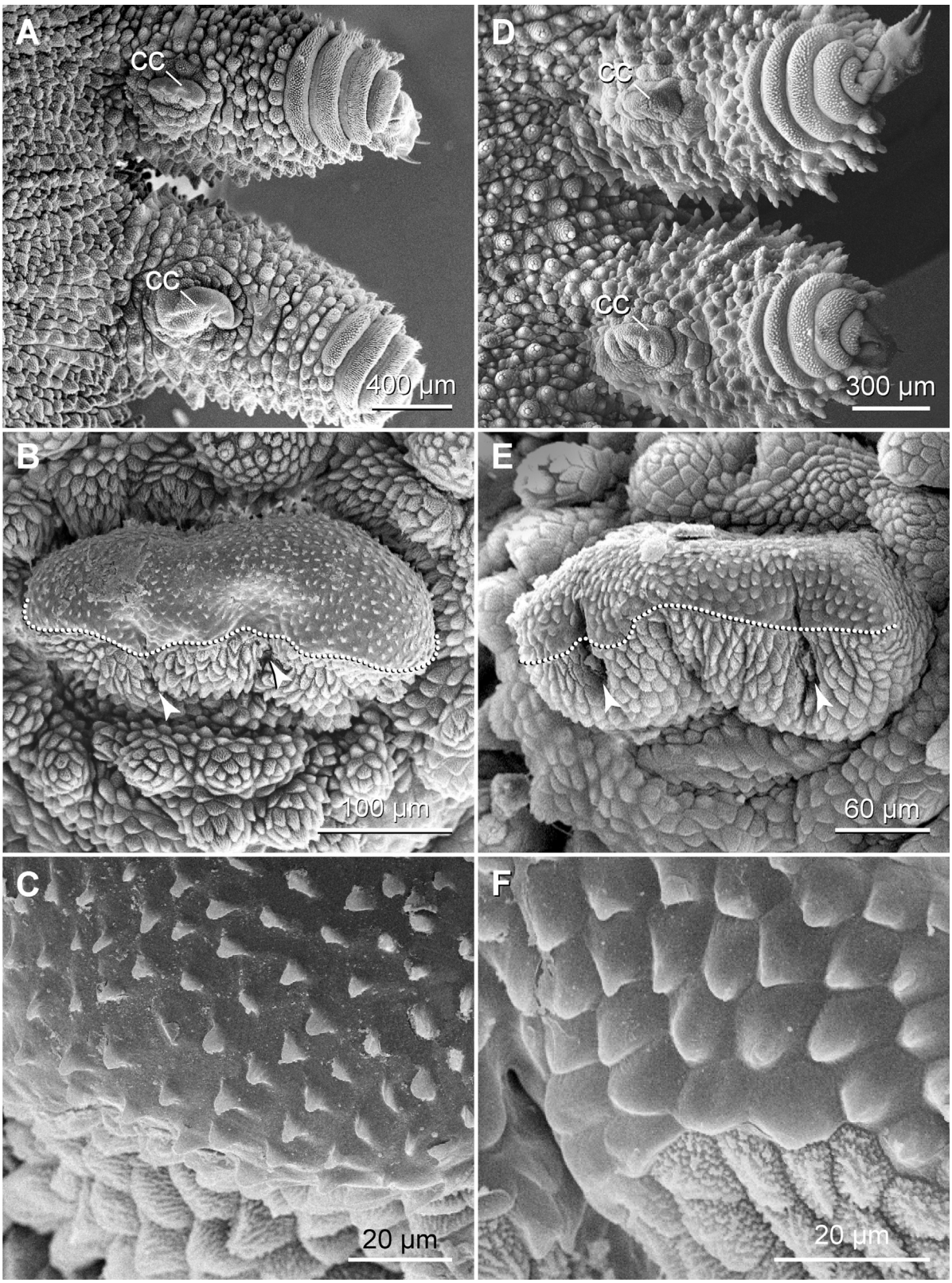


Fig. 8. Features associated with the male crural complexes in the two species of *Eoperipatus* studied. Scanning electron micrographs; anterior is up in all images. (A–C) *Eoperipatus totoro* **sp. nov.** (D–F) *Eoperipatus* sp. (A, D) Overviews. Note that the crural complexes lie in the same position as the coxal vesicles but have a different structure. (B, E) Detail of a single crural complex. Arrowheads point to the openings of the crural glands. Dotted lines demarcate the anterior area with modified scales. (C, F) Detailed views of scales from each anterior area with modified scales. Abbreviation: cc, crural complex.

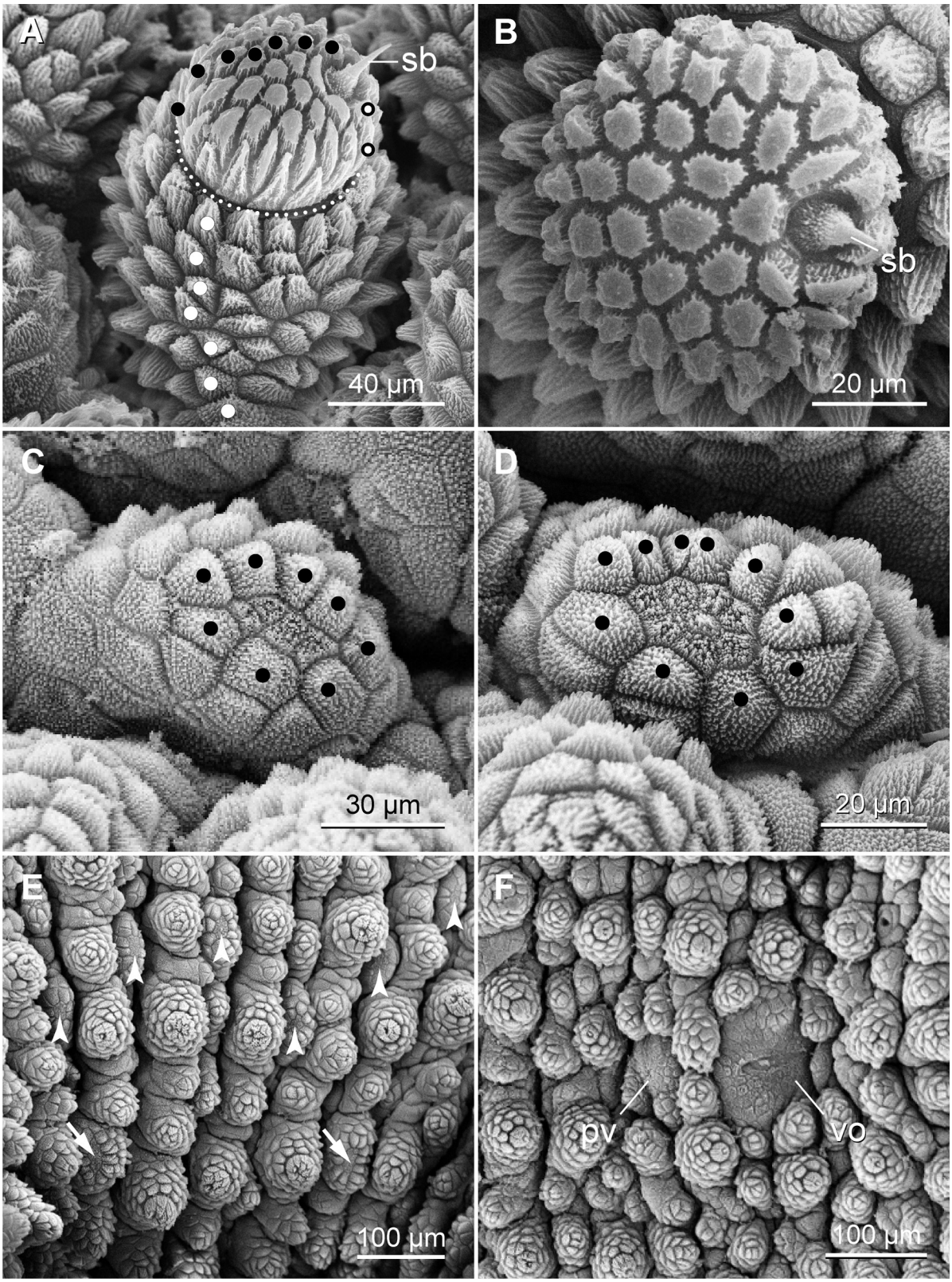


Fig. 9. Features associated with the integument in *E. totoro* **sp. nov.** Scanning electron micrographs; anterior is left in all images. (A) Primary papilla from dorsal integument. Note the spherical shape of the apical piece and the posteriorly placed sensory bristle. White dots indicate the number of scale ranks in the basal piece, black dots indicate those in the anterior region of the apical piece and black dots with a white centre those in the posterior region of the apical piece. Dotted line demarcates the constriction between the basal and apical pieces. (B) Detail of apical piece in dorsal view. (C) Type I crater-shaped papilla from ventral integument. (D) Type II crater-shaped papilla from ventral integument. Black dots in C and D indicate the number of scales in the apical collar. (E) Ventral row of type II crater-shaped papillae (arrowheads). Note that the type I crater-shaped papillae occur on the plicae (arrows), whereas the type II papillae are found in plical furrows. (F) Detail of ventral and preventral organs. *Abbreviations*: pv, preventral organ; sb, sensory bristle; vo, ventral organ.

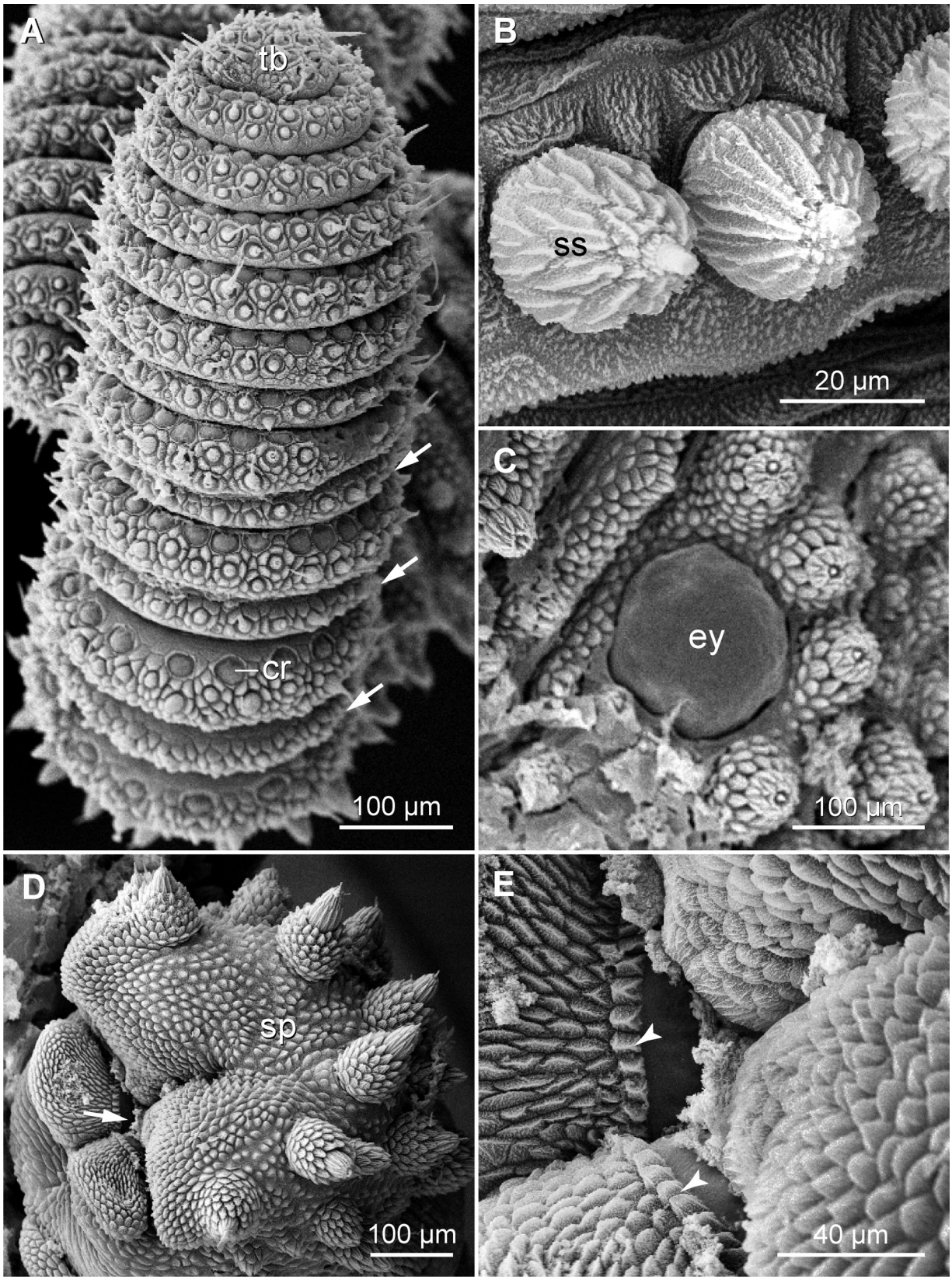
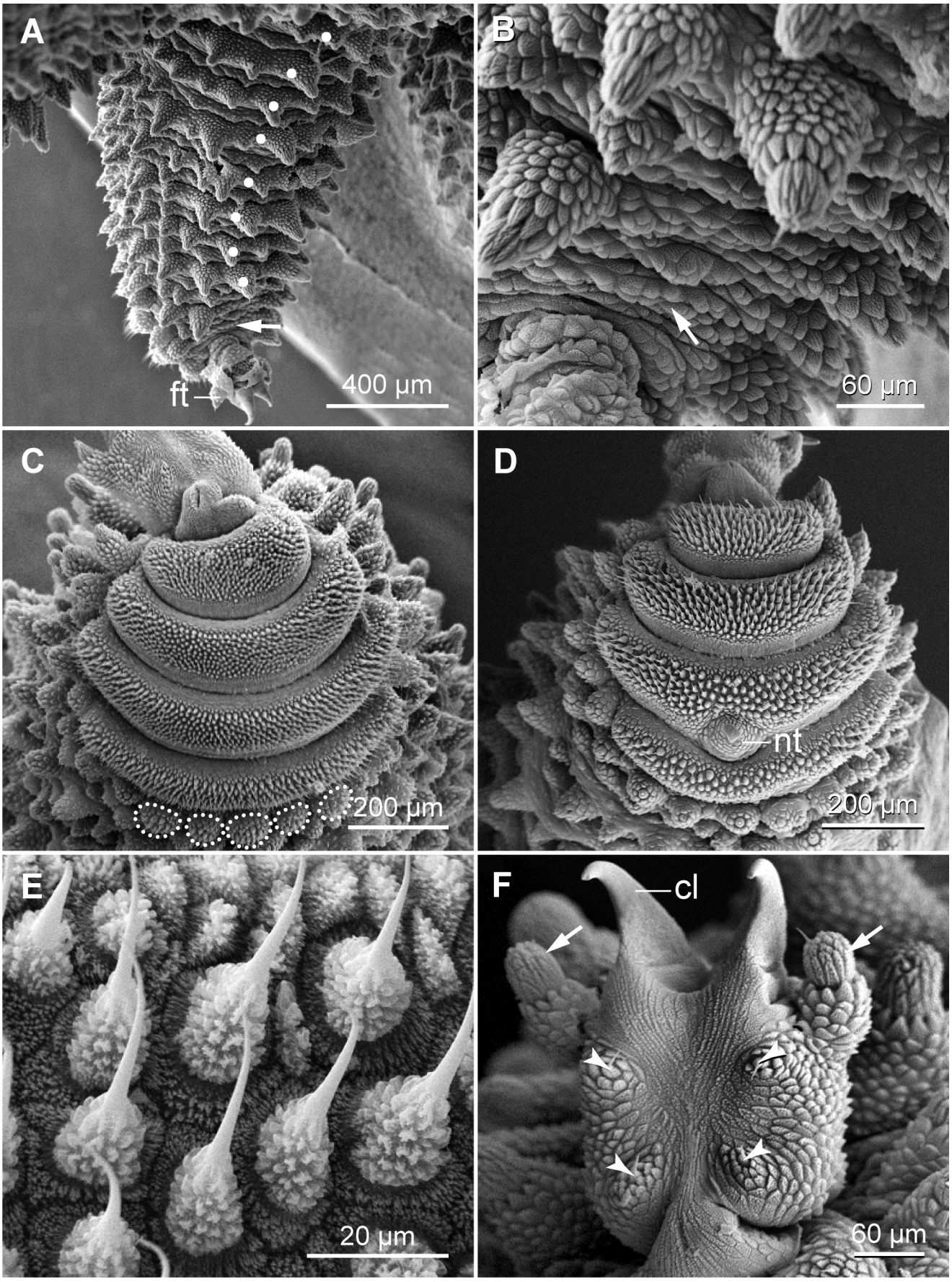


Fig. 10. Anterior structures in *E. totoro* **sp. nov.** Scanning electron micrographs; anterior is up in all images. (A) Characteristics of the antennal tip. Arrows indicate the thinner rings without chemoreceptors. (B) Detail of spindle-shaped sensilla from the ventral surface of the antennal basis. (C) Detail of the eye. (D) Distal portion of a slime papilla. Arrow points to the opening of the slime gland. Note the lack of dermal papillae on the distal-most surface of the slime papilla. (E) Detail of the slime gland opening. Arrowheads point to denticle-like scales surrounding the opening. *Abbreviations*: cr, chemoreceptor; ey, eye; sp, slime papilla; ss, spindle-shaped sensillum; tb, terminal button.


A
ft
400 µm
B
60 µm
C
200 µm
D
nt
200 µm
E
20 µm
F
cl
60 µm

surrounded by an internal row with seven pairs of lips and an external row with eight pairs of lips; anterior unpaired lip absent. Outer and inner jaw blades with one principal and two accessory teeth; inner jaw blade with 11–13 denticles; accessory and principal teeth sickle-like and similar in shape. Condensed fields of tracheal openings found posteriorly to each eye and anteriorly and posteriorly to mouth; postoral pit present (Fig. 4C). Distal-most surface of slime papilla devoid of dermal papillae; slime gland opening surrounded by denticle-like scales (Fig. 10D and E).

Dorsal integument. Twelve complete plicae per segment, seven of which pass between adjacent leg pairs to ventral side. Dorsomedian furrow distinct along entire body length; hyaline organs not evident. Primary and accessory papillae distributed in a random fashion along dorsal midline. Dorsal primary papillae of one type, cylindrical, predominant in number and with apical and basal pieces separated by a constriction (Fig. 9A). Basal piece cylindrical, larger than apical piece and with eight to nine antero-posterior and seven lateral scale ranks (Fig. 9A). Apical piece covered with scales arranged in five to seven anterior and one or two posterior scale ranks; apical-most scales smooth (Fig. 9A and B). Sensory bristle of apical pieces thorn-shaped and varying in size (Fig. 9A). Accessory papillae small and low in number (usually none, one or two accessory papillae between each two primary papillae).

Ventral integument. Type I and type II crater-shaped papillae present (Fig. 9C and D). Type I crater-shaped papillae roundish, arranged in a random fashion close to legs and with six to eight scales in apical collar (Fig. 9C); type II crater-shaped papillae with elongated bases, with seven to ten scales in apical collar and arranged in an irregular ventral and ventro-lateral row of six papillae between subsequent leg pairs (Figs. 4D and E and 9D and E); rudimentary apical piece missing in both types of crater-shaped papillae. Ventral and preventral organs roundish, separated by genital pad in genital segment and by single plica in remaining trunk segments (Figs. 3, 6A and 9F); ventral organs larger than preventral organs (Fig. 9F).

Legs. Males with 23, females with 24 leg pairs; first and last leg pairs reduced in size; legs of last pair not rotated. Dorsal leg surface with eight transverse rings (Fig. 11A); bean-shaped papilla absent (Fig. 11A and B). Four arched and proximally increasing spinous pads, occupying an area of nearly half leg length (Figs. 4F and 11C and D); fifth pad fragmented but present in most legs except for two anterior-most and three posterior-most leg pairs (Fig. 11C and D); only three pads in last leg pair (Fig. 3). Nephridial tubercle in fourth and fifth leg pairs situated between third and fourth spinous pads, indenting third pad (Fig. 11D). Spines on spinous pads densely distributed, needle-shaped and with textured basis (Fig. 11E). Eversible coxal vesicles present in all legs, except for fourth and fifth leg pairs (Figs. 3 and 4F); coxal furrows extending from proximal border of fourth spinous pad to leg basis. Two distal foot papillae per leg: one anterior and one posterior (Fig. 11F). One or two bristles on each proximal and distal setiform ridges. Embryonic foot projections absent.

Posterior region. Genital pad situated between penultimate leg pair (Figs. 3 and 7A); genital opening cruciform in males but appearing as a transverse slit in females (Fig. 6A); male genital pad with four circular pits (Fig. 6B). Male anal glands open in a single orifice on a large pad (=anal gland pad) (Fig. 7A and B). Crural complexes restricted to two pregenital leg pairs; each complex covered by an anterior field of modified scales; posterior half of crural complex covered with scales similar to those found on dermal papillae (Fig. 8A–C). Anal cone well-developed in females; anus terminal (Fig. 3).

Mitochondrial data. COI and *12S rRNA* sequences as for specimen with GenBank accession numbers KC139082 and KC139083 (Table 1).

3.5. Differential diagnosis

Unfortunately, there are no data from the remaining species of *Eoperipatus* on most characters described herein, and we were not allowed to analyse the type specimens of these species by our destructive methods. Hence, we have to restrict the differential diagnosis to characters that have been described previously. Our literature survey (Bouvier, 1905; Evans, 1901a,b; Horst, 1886; Sedgwick, 1888) and analyses of syntypes placed in the Natural History Museum of London revealed that *E. totoro* **sp. nov.** can be distinguished from the remaining described species of *Eoperipatus* by the shape of apical pieces on dorsal primary papillae, the position of the nephridial tubercle in the fourth and fifth leg pairs, and the ventral colour pattern. While the apical pieces are cylindrical in *E. sumatranus*, conical and relatively small in *E. butleri* and variable in shape in *E. horsti* and *E. weldoni* (either conical, cylindrical or spherical), they are spherical in *E. totoro* **sp. nov.** In addition, the nephridial tubercle, which lies between the third and fourth spinous pads in *E. totoro* **sp. nov.**, occurs in the middle of the fourth pad in *E. weldoni*, *E. butleri* and *E. sumatranus* (thus, dividing this pad in two halves) but at the proximal border of the fourth pad in *E. horsti* (thus, leaving this pad undivided) (Evans, 1901a,b). Moreover, the repeated bright spots, which are clearly visible in the interpedal regions of the ventral integument in *E. totoro* **sp. nov.**, have not been reported from any other species of *Eoperipatus* (Bouvier, 1905; Evans, 1901a,b; Horst, 1886; Sedgwick, 1888). The bright spots along the ventral midline in *E. totoro* **sp. nov.** were misinterpreted by Bai and Anh (2010) as preventral and ventral organs. However, our results clearly show that the anterior spot in each interpedal region corresponds to a ventral field of modified scales, whereas the posterior spot between each leg pair corresponds to a ventral and a preventral organ lying next to each other (cf. Figs. 2B and 3).

3.6. Molecular analyses and phylogenetic relationships

The final alignment of the *COI* fragments contained 627 bp. The translation of the *COI* nucleotide sequences into amino acid sequences revealed no stop codons, suggesting the sequences belong to functional mitochondrial protein-coding genes. The final alignment of the *12S rRNA* fragments contained 372 bp. Both *COI* and *12S rRNA* sequences are A + T biased. The Maximum Likelihood analyses revealed congruent topologies using either nucleotide or translated amino acid sequences of *COI* (Figs. 12 and S1). As expected, *E. totoro* **sp. nov.** clusters with *Eoperipatus* sp. from Thailand forming a well-supported clade, which is the sister group to the clade containing the neotropical species of Peripatidae (Fig. 12).

Fig. 11. Features associated with legs in *E. totoro* **sp. nov.** Scanning electron micrographs; anterior is right in all images. (A) Arrangement of transverse rings (white dots) on the dorsal surface of a leg. Note the absence of a bean-shaped papilla in the distal leg portion (arrow). (B) Detail of a distal leg portion, which lacks a bean-shaped papilla but shows an area covered with scales instead (arrow). (C) Spinous pads in a leg from the midbody in ventral view. Note the presence of a fragmented fifth spinous pad (dotted lines). (D) Spinous pads in the fourth leg in ventral view showing the position of the nephridial tubercle. (E) Detail of bristles from the surface of a spinous pad. (F) Foot in ventral view. Note a single anterior and a single posterior distal foot papilla (arrows). Arrowheads indicate bristles on the proximal and distal setiform ridges. *Abbreviations*: cl, claw; ft, foot; nt, nephridial tubercle.

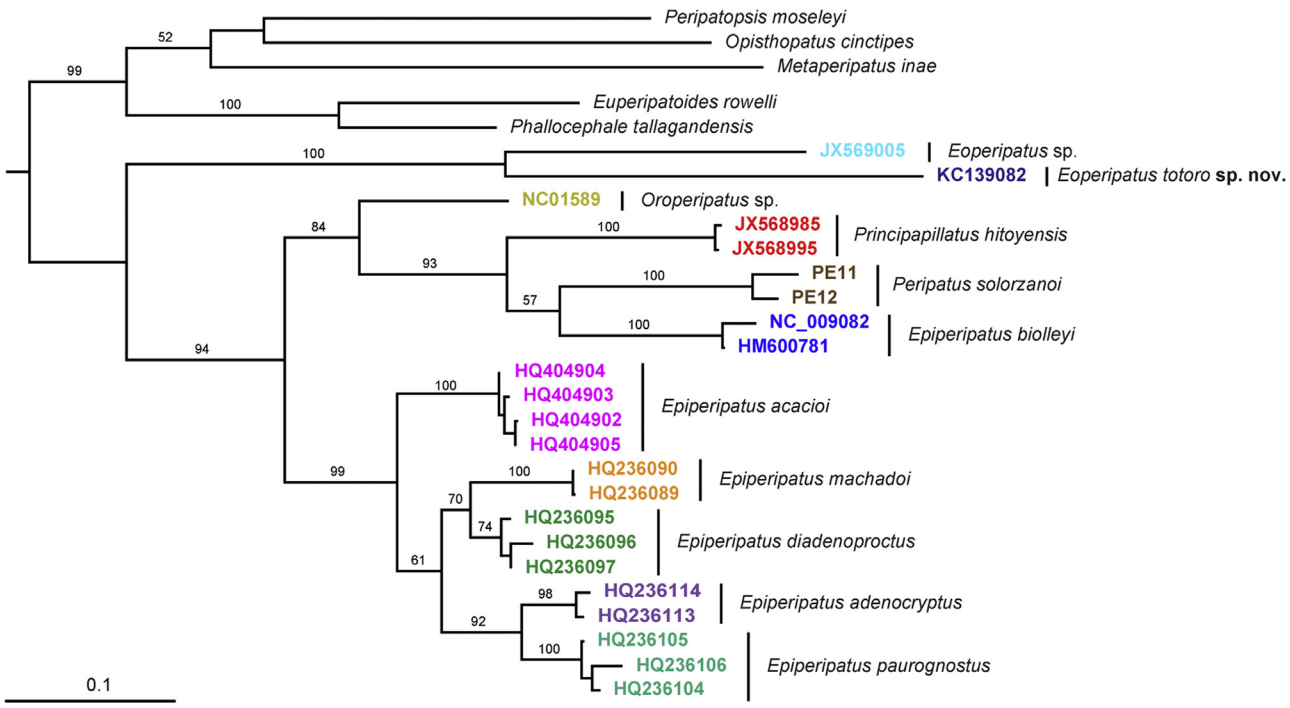


Fig. 12. Maximum Likelihood topology combining the *COI* and *12S rRNA* nucleotide sequences. Five species of Peripatopsidae were used as outgroup taxa. Bootstrap values lower than 50 are not shown. Abbreviations in different colours correspond to the accession numbers of the *COI* sequences in GenBank.

4. Novel, species-specific characters for *Eoperipatus*

A morphological comparison of *E. totoro* **sp. nov.** from Vietnam with *Eoperipatus* sp. from Thailand using scanning electron microscopy revealed novel characters and character states useful for distinguishing the species of *Eoperipatus*. For example, the ventral fields of modified scales contain a single type of scales in *Eoperipatus* sp., whereas there are two types in *E. totoro* **sp. nov.** (Fig. 5A–D). Likewise, the characteristics of scales that cover the genital and anal gland pads differ between the two species. In *E. totoro* **sp. nov.**, these scales are slender and show pointy tips, whereas they are bulky and have blunt ends in *Eoperipatus* sp. (Figs. 6A–F and 7A–F).

Additional differences concern male reproductive structures, such as different types of modified scales covering the anterior region of each crural complex (Fig. 3). In *Eoperipatus* sp., the modified scales are large, flat and densely packed, whereas they are small and widely spaced in *E. totoro* **sp. nov.** (Fig. 8A–F). Moreover, in *E. totoro* **sp. nov.** the space between the scales is covered with a smooth cuticle, which is not evident in *Eoperipatus* sp. from Thailand (Fig. 8C and F).

The male genital pad in both species of *Eoperipatus* shows a circular pit in each of the four pad quarters separated from each other by the cruciform genital opening (Figs. 6B and E and 7A and D). The internal structure of the pits in *E. totoro* **sp. nov.** shows flattened scales in the inner wall of the pit, whereas no scales are evident inside the pits in *Eoperipatus* sp. from Thailand (Fig. 6C and F). Moreover, the minute holes (tracheal openings?) on the floor of each pit are fewer in number and less evident in *E. totoro* **sp. nov.** (Fig. 6C and F).

Notably, the anal cone is relatively small in males of *E. totoro* **sp. nov.**, as it does not exceed the length of the last leg pair, whereas the anal cone is considerably larger in males of *Eoperipatus* sp. (Fig. 7A and D). Moreover, the position of the anal gland pad differs between the two species, as it lies between the last leg pair in *E. totoro* **sp. nov.**, whereas it is situated further posteriorly in *Eoperipatus* sp. (Fig. 7A and D). The relative size of the anal gland pad is also different between the two species, as it is larger in *Eoperipatus* sp. from Thailand (Fig. 7B and E).

5. Discussion

5.1. Hitherto unexplored morphological diversity within *Eoperipatus*

Eoperipatus totoro **sp. nov.**, is the first onychophoran species described from Vietnam and it differs from other representatives of *Eoperipatus* in the position of the nephridial tubercle, the shape of apical pieces and the ventral colour pattern (Bouvier, 1905; Evans, 1901a,b; Horst, 1886; Sedgwick, 1888). Our scanning electron microscopy data from *E. totoro* **sp. nov.** and from an undescribed species of *Eoperipatus* from Thailand revealed novel species-specific characters, which might be useful for comparative morphological studies in representatives of *Eoperipatus*. These characters include different types of modified scales in the ventral integument, on the crural complexes and on the anal gland pads. Additional characteristic features include the relative position and size of the anal gland pads and the inner structure of the circular pits on the male genital pads. Although some of these features, including the structure of the genital and anal gland pads in males, were regarded as a diagnostic feature of *Eoperipatus* (Evans, 1901b; Oliveira et al., 2012b), their inter-specific variation has remained unexplored.

The discovery of novel morphological features described herein suggests that a detailed analysis of the remaining species of *Eoperipatus* using scanning electron microscopy might reveal additional characters, which would help clarify species diversity and phylogeny of the South-East Asian Peripatidae (Oliveira et al., 2011, 2012b). Unfortunately, scanning electron microscopy could not be applied to syntypes from the museums' collections and topotypes are unknown for all species of *Eoperipatus* described previously (Evans, 1901a,b; Oliveira et al., 2012a; Sedgwick, 1888). Thus, new

collecting efforts from each type locality are required to enable a thorough revision of this taxon.

5.2. Distribution of Eoperipatus in South-East Asia

Recent discovery of *Eoperipatus* in Thailand and Vietnam (Baer and Mayer, 2012; Bai and Anh, 2010) extends the distribution range of this taxon to nearly 1000 km further north-east (Fig. S2). We have shown here that specimens from Thailand and Vietnam belong to two separate species, which is supported by our morphological and molecular data. Both species occur in remote habitats (Chanthaburi mountain range in Thailand and Cát Tiên National Park in Vietnam) and, therefore, it is unlikely that they were introduced to these areas accidentally by humans. We rather assume that they represent relics of past speciation events. According to the current pattern of distribution of *Eoperipatus* and a recent report of an unidentified onychophoran species from northern Vietnam (Moler et al., 2013), it would not be surprising if onychophorans were found in Cambodia, Laos and Myanmar, given that our knowledge of the fauna in these countries is limited (Sterling and Hurley, 2005; Woodruff, 2010).

Nonetheless, we caution that records of Onychophora from South-East Asia should be interpreted with care, given the known cases of accidentally introduced onychophoran species to this region, e.g., by importing or exporting plant material and soil (van der Lande, 1991). A thorough revision and analysis of phylogenetic relationships of the South-East Asian Peripatidae will help clarify the evolutionary history of this interesting, albeit largely understudied onychophoran subgroup.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcz.2013.01.001.

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