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Prof. Dr. Rodrigo Hirata Willemart (orientador)

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”(…) *once only bacteria, but now petunias and people as well*-(…)” Stephen Jay Gould, in *Full House*, p146.

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SUMÁRIO

Contextualização e rumos do projeto

Opiliones é a uma das maiores ordens de aracnídeos em termo de número de espécies, atrás apenas das megadiversas ordens Acariformes, Parasitiformes (ambas na antiga ordem "Acari") e Araneae (Beccaloni, 2009). As cerca de 6600 espécies descritas (Kury, 2016) estão divididas em uma subordem fóssil, a recém descrita Tetrophthalmi (Garwood et al., 2014), e quatro subordens viventes: Cyphophthalmi, Eupnoi, Dyspnoi e Laniatores (Kury, 2013). Os Cyphophthalmi são conhecidos em inglês como "*mite harvestmen*" ("opiliões ácaros") devidos ao seu diminuto tamanho (1-2 mm) e inconspicuidade (Giribet et al., 2012); os Dyspnoi são opiliões com perna curta, alguns com belas ornamentações do oculário (tubérculo dos olhos) (Pinto-da-Rocha e Giribet, 2007); os Eupnoi variam desde grupos com pernas curtas como os Dyspnoi, até os delicados "*Daddy-long-legs*" (*Opa Langbein*, no alemão) com pernas bem finas e longas (Pinto-da-Rocha e Giribet, 2007); e os Laniatores, grupo de opiliões mais esclerotizados, que incluem a maior parte da variação de ornamentos e espinhos, colorações e comportamentos observados no grupo (Pinto-da-Rocha e Giribet, 2007; Buzatto e Machado, 2014; Giribet e Sharma, 2015).

Uma característica comum aos opiliões é o segundo par de pernas alongado, tradicionalmente considerado como os apêndices sensoriais. Também possuem função especialmente sensorial o primeiro par de pernas (nos Phalangida: Eupnoi + Dyspnoi + Laniatores) e o pedipalpo (nos Palpatores: Eupnoi + Dyspnoi) (referências em Willemart et al., 2009). No entanto, o conhecimento acerca das estruturas sensoriais - ou sensilla - dos opiliões é muito limitado quando comparado às outras ordens de aracnídeos (eg. Foelix e Chu-Wang, 1973a, 1973b; Barth e Blickhan, 1984; Foelix, 1985; Punzo, 1998; Coons e Alberti, 1999; Gaffin e Brownell, 2001; Barth, 2002; Talarico et al., 2005, 2006, 2007/08, 2008; Santer e Hebets, 2011), especialmente quanto a morfologia interna. Histologia é especialmente relevante, pois observar apenas a morfologia externa é geralmente insuficiente para determinar função de um receptor (Altner e Prillinger, 1980; Zacharuk, 1985). Na subordem Laniatores, que representa 2/3 das espécies de Opiliones, há apenas um único paper (Proud e Felgenhauer, 2013) mostrando um corte transversal de uma sensillum. Essa é uma grande lacuna do conhecimento, se considerarmos que a maioria das informações acerca da biologia sensorial de opiliões provém principalmente de espécies de Laniatores Neotropicais (Willermart et al., 2009).

O conhecimento sobre a morfologia e biologia sensorial de Opiliones foi recentemente revisado em Cyphophthalmi (Willemart e Giribet, 2010) e Phalangida (Eupnoi, Dyspnoi e Laniatores) (Willemart et al., 2009). Para os opiliões, a quimiorrecepção é uma modalidade sensorial muito importante em diversos contextos, um paradigma suportado por diferentes frentes de evidência. Evidências de estudos comportamentais demonstram a importância da quimiorrecepção no forrageamento (Willemart and Chelini, 2007; Costa and Willemart, 2013), aprendizado associativo (dos Santos et al., 2013), comunicação e reconhecimento intraespecífico (Machado et al., 2002; Donaldson e Grether, 2007; Grether e Donaldson, 2007; Willemart e Hebets, 2011; Teng et al., 2012). Na frente morfológica, há evidências de quimiorreceptores nas quatro subordens viventes de Opiliones: Cyphophthalmi, Eupnoi, Dyspnoi e Laniatores. Presentes em todas as subordens, as *sensilla chaetica* são consideradas até o momento os receptores táteis/gustatórios dos opiliões. Há evidência para dois receptores olfativos: as *solenidia* (não reportadas em Laniatores) e os *dorsal prosomal spines* (apenas em Dyspnoi) (referências em Willemart et al., 2009, Willemart e Giribet, 2010). Em Laniatores, no entanto, cerca de 2/3 das espécies de opiliões, não há evidência morfológica de receptores olfativos, apesar de evidências comportamentais de olfação existirem (Machado et al., 2002, Costa e Willemart, 2013, dos Santos et al. 2013, Hashimoto e Hayashi, 2014). Outras cerdas de função desconhecida foram reportadas, como as *falciform hairs* e as *sensilla basiconica* (Willemart et al., 2007, 2009). Outro aspecto relevante da biologia sensorial dos opiliões é a dependência de ambientes úmidos e temperaturas amenas, em especial as espécies Neotropicais (Santos, 2003, 2007). Apesar de ser sabido que os opiliões são capazes perceber mudanças de temperatura e umidade (Todd, 1949; Immel, 1954; Clingenpeel e Edgar, 1966; Santos, 2003, 2007), a identidade e a localização das sensilla responsáveis pela detecção é desconhecida.

Neste contexto, meu projeto inicial de mestrado se propunha a investigar a morfologia interna dos potencias receptores olfativos em uma espécie de Laniatores (*Heteromitobates discolor*; Gonyleptidae), focando nas cerdas *falciform hairs* e *sensilla basiconica*. Com meu estágio de 4 meses na Harvard University (EUA), no laboratório do Prof. Gonzalo Giribet obtive acesso ilimitado à microscopia eletrônica de varredura e uma das maiores coleções de Opiliones do mundo. Nesse estágio, coletei praticamente toda a informação de morfologia externa apresentada nessa dissertação. Com o estágio de 6 meses na Ernst-Moritz-Arndt Universität Greifswald (Alemanha) com o Dr. Peter Michalik, tivesse acesso a uma excelente infraestrutura de microscopia eletrônica de transmissão. Com esses dois estágios, pude investigar em mais detalhe a morfologia interna de todas as sensilla do tarso de *H. discolor*, expandindo a investigação para além dos quimiorreceptores, e amostrando a morfologia

externa do tarso de grande parte da diversidade de Opiliones, com ênfase em Laniatores. Esse é um trabalho inteiramente colaborativo, como fica evidente na lista de autores por capítulo.

No Capítulo 1 ("*Not so touchy after all: ultrastructure of chemoreceptive tarsal sensilla in an armored harvestman (Arachnida: Opiliones: Gonyleptidae) and evidence of olfaction in 17 families of Laniatores*"), me atenho aos objetivos iniciais do projeto: realizamos o primeiro estudo detalhado sobre a morfologia interna de sensilla em Laniatores, na espécie *Heteromitobates discolor* (Laniatores, Gonyleptidae), com foco nos quimioreceptores. No Capítulo 2 ("*Ultrastructure of putative hygro-thermoreceptive tarsal sensilla on a Neotropical armored harvestman (Arachnida, Opiliones, Laniatores, Gonyleptidae*"), fornecemos a primeira evidência morfológica de detectores de umidade e temperatura em opiliões, estudando as sensilla basiconica e uma nova sensilla, que chamamos de apical-hood sensilla. Os dois tipos de cerda formam uma tríade na ponta das pernas sensoriais, que estão intimamente associadas em sua morfologia interna. Por fim, no Capítulo 3 ("*Widespread tarsal sensilla in harvestmen (Arachnida, Opiliones): characters for multi-level phylogenetic relationships and implications for sensory biology*") investigamos a distribuição filogenética da tríade identificada nas pernas I e II, explorando o potencial dessas estruturas para a sistemática de Opiliones, botando em perspectiva os resultados do Capítulo 2.

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Chapter 1: Not so touchy after all: ultrastructure of chemoreceptive tarsal sensilla in an armored harvestman (Arachnida: Opiliones: Gonyleptidae) and evidence of olfaction in 17 families of Laniatores

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Abstract

Harvestmen (Arachnids, Opiliones) are especially dependent on chemical cues and are often regarded as dependent on touch to detect these stimuli. Information on harvestmen sensilla is scarce when compared to other arachnid orders, especially concerning internal morphology. Using scanning (SEM) and transmission (TEM) electron microscopy, we investigated tarsal sensilla on the distal tarsomeres (DT) of all leg pairs in *Heteromitobates discolor* (Laniatores, Gonyleptidae), and surveyed by means of SEM the sensilla on DT I and II of species from all main lineages of the suborder Laniatores, including members of 17 families. The DT I and II of *H. discolor* are equipped with wall-pored *falciform hairs* (two types), wall-pored *sensilla chaetica* (two types) and tip-pored *sensilla chaetica*, while DT III and IV are mainly covered in tip-pored *sensilla chaetica* and trichomes (non-sensory). Ultrastructure supports an olfactory function for all wall-pored sensilla and a dual gustatory/touch function for tip-pored *sensilla chaetica*. Wall-pored sensilla occur in all Laniatores

investigated, demonstrating the widespread occurrence in the suborder and highlighting the importance of both legs I and II as the sensory appendages of laniatorean harvestmen. Our results provide the first morphological evidence for olfactory receptors in Laniatores (which include 2/3rds of Opiliones species) and suggest that olfaction is more important for harvestmen than previously thought.

Keywords: Olfaction; Goniosomatinae; wall pores; sensory morphology; Chemoreceptors

1. Introduction

The sensory structures of arthropods are highly organized organs called sensilla, which have cuticular and cellular specializations related to their sensory modality (Altner & Prillinger, 1980; Zacharuk, 1985; Keil & Steinbrecht, 1984). Sensillar function can be inferred with behavioral ablation tests (e.g., Blumenthal, 1935; Gleeson, 1982), electrophysiology (e.g., Yokohari and Tateda, 1976; Tichy et al., 2001; Piersanti et al., 2011) and morphological evidence (e.g., Foelix and Chu-wang, 1973a, b; Altner et al., 1977; Steinbrecht and Müller, 1976, Talarico et al., 2006, 2008). Extensive morphological studies on the types of insect sensilla and their distribution on the body enabled behavioral tests and the development of electrophysiological protocols, which have provided great knowledge on mechano- chemo- hygro- and thermoreceptive sensilla (reviewed in Altner & Prillinger, 1980; Altner & Loftus, 1985; Zacharuk, 1985). Yet, for taxa in which basic information on sensillar types and distribution are scarce, such as the arachnid order Opiliones, morphology is usually the most suitable tool not only for understanding the basic organization of sense organs but also for proposing functional hypotheses.

Opiliones, commonly known as harvestmen or daddy-long-legs, is the third most speciose arachnid order after Acariformes and Araneae, with over 6600 accepted described species (Beccaloni, 2009; Kury, 2016). These are currently divided in one newly discovered fossil suborder, Tetrophthalmi, and four extant suborders: Cyphophthalmi, Eupnoi, Dyspnoi and Laniatores (reviewed in Giribet and Sharma, 2015). Pedipalps, legs I and legs II are considered the sensory appendages of Phalangida (Eupnoi + Dyspnoi + Laniatores), although in the suborder Laniatores the spiny pedipalps are mainly involved in prey capture and there is no evidence of a sensory function (Willemart et al., 2009; Wolff et al., 2016; Costa et al., 2016). Legs II are especially elongated in Phalangida, being held upwards laterally and slowly waving when the animal is not walking (Acosta and Machado, 2007). Harvestmen sensory capabilities and sensory structures have been reviewed recently (Willemart et al., 2009, Willemart and Giribet, 2010); unlike spiders, they lack trichobothria (wind current detectors), have

relatively few slit sense organs (which may function as substrate vibration detectors) and in general are thought to have poor visual acuity (Barth and Stagl, 1976; Willemart et al., 2009). They are also equipped with presumed chemoreceptive setae, such as *sensilla chaetica* (in all suborders) and *solenidia* (not reported in Laniatores) (Willemart et al., 2007, 2009).

Information on Opiliones sensilla is limited when compared to insects and other arachnid orders, especially concerning internal morphology (Insects: e.g., Altner and Prillinger, 1980; Zacharuk, 1985; Keil and Steinbrecht, 1984; Other arachnids: e,g., Barth and Blickhan, 1984; Foelix, 1985; Punzo, 1998; Coons and Alberti, 1999; Gaffin and Brownell, 2001; Talarico et al., 2005, 2006, 2007/08, 2008; Foelix, 2011; Santer and Hebets, 2011). Histological data are necessary, since observing only external morphology is usually insufficient for determining the function of a given receptor (Altner and Prillinger, 1980; Zacharuk, 1985). Histological studies on harvestmen sense organs are only available for the *eyes* (Cyphophthalmi: e.g., Alberti et al., 2008), *slit sensilla* (Luque, 1993), *dorsal prosomal spines* (Dyspnoi: Lopez et al., 1980; Juberthie et al., 1981) and *sensilla chaetica* (Eupnoi: Foelix, 1976; Guffey et al., 2000; Laniatores: Proud and Felgenhauer, 2013). Other sensilla, such as the *sensilla basiconica* and *falciform hairs* (Willemart et al., 2007, Willemart et al., 2009) have only been described with SEM and their function is unknown.

Herein, we provide an ultrastructural description of laniatorean sensilla on the terminal tarsomere of all legs of the species *Heteromitobates discolor* (Sørensen, 1884) (Laniatores, Gonyleptidae). This species belongs to the subfamily Goniosomatinae (Laniatores, Gonyleptidae), a group with 39 species that concentrates most behavioral studies conducted in harvestmen (DaSilva and Gnaspini, 2009; Buzatto and Machado, 2014; DaSilva, 2014; Silva and Willemart, 2015). With an SEM survey, we also investigate the sensilla on the last tarsomere of legs I and II in 17 families of Laniatores.

2. Material and methods

Adult individuals of *Heteromitobates discolor* were collected at Casa da Farinha (Corisco River or Fazenda River), Ubatuba, São Paulo, Brazil (S 23° 20' 18.5" W 44° 50' 15") between February and March, 2014 or on April 25th, 2015. Animals were hand collected while resting on or under rocks and leaves along the margins of the streams. Laniatores specimens for the SEM survey were obtained from the Invertebrate Zoology collection in the Museum of Comparative Zoology (Harvard University, USA) and the Museu de Zoologia da Universidade de São Paulo (Brazil). The list of species, voucher

specimens and localities can be found in Table 1. Additional information can be accessed in the MCZ data base (mcz-base.mcz.harvard.edu).

2.1 Scanning Electron Microscopy (SEM)

Tarsi were cut with micro scissors and submitted to three rounds of ultrasound cleansing (Branson 200 or Unique® Ultracleaner 800A): first with the specimens in distilled water, second with the specimens submerged in a 1:10 detergent solution (Alconox®) and third back in distilled water. The duration of each round was determined empirically. Samples were critical point dried (Tousimis 931 GL), being dehydrated in graded ethanol series. They were afterwards mounted on stubs with carbon bi-adhesive tabs (Electron Microscopy Science, Hatfield, PA, USA) and sputter coated with Pt-Pd targets (EMS 300T D Dual Head Sputter Coater). Photographs were taken in a Zeiss Ultra-Plus FESEM and Zeiss Supra FESEM (field emission scanning electron microscope, at the Center for Nanoscale Systems, Harvard University), or in a Zeiss DSM 940 (at Instituto de Biociências da Universidade de São Paulo).

2.2 Transmission Electron Microscopy (TEM)

The distal tarsomeres of females and males were dissected in cold NaH2PO4x2H2O buffer and then fixed in a mixture of 2,5% Glutaraldehyde and 2% Paraformaldehyde in 0,1M NaH2PO4x2H2O buffer (after Karnovsky 1965) and kept at 4ºC for approximately 1.5 months. Samples were rinsed in Siena PBS buffer, post-fixed in 2% Osmium Tetroxide for 3 hours and rinsed again in Siena PBS buffer. For embedding, the material was serially dehydrated from 50% to 100% pure ethanol and then gradually embedded in Spurr medium (Spurr 1969) with an alcohol/ resin series 2/1 (6 hours), 1/1 (overnight), 1/3 (6 hours) and pure resin (overnight). Longitudinal and transverse serial ultrathin sections were obtained with a diamond knife (Diatome-Biel, Switzerland) on a Leica Ultracut (Wetzlar, Germany). Semithin section were taken in between series and stained with Blue Methilene.

3. Results

3.1. External morphology of distal tarsomeres

The tarsus of most Opiliones (this is not the case of Cyphophthalmi or Sandokanidae) is subdivided in tarsomeres, which are always more numerous in leg II than in leg I. We focused on the distal-most tarsomere of each leg, for in arachnids they usually present the highest variety of integument structures (Foelix 1985). Two morphological groups can be identified on the distal tarsomeres (DT) of *Heteromitobates discolor*: the anterior DTs I and II and the posterior DTs III and IV. The anterior DTs have a single claw and are both thinner than the counterpart on the posterior legs. DT II is slightly thinner than DT I (Fig. 1). The posterior DTs III and IV have double claws and are similar in shape (Fig. 2a, b). Setal composition and distribution is similar within each group, but the anterior DTs have more sensillar types than the posterior. We detected no obvious differences between sexes, but no counting of sensilla was conducted. Dorsal and lateral surfaces of DTs III and IV are covered in trichomes (non-sensory, sections not shown) while on the same region of DTs I and II trichomes seldom occur. The ventral surface of all DTs has an aggregation of trichomes, which is denser on DTs III and IV.

Our SEM survey initially indicated that more than one type of *sensilla chaetica* existed, based on shaft length differences and the presence of wall pores in some of them. TEM analysis also showed subtypes, but correspondences between internal and external morphology could not always be established. Therefore, we propose the following classification, which still enables further refinement of subtypes in the future: *sensilla chaetica* with a terminal pore (Sc-tp) and *sensilla chaetica* with wall pores (Sc-wp), the later presenting at least 2 subtypes. We also investigated the *falciform hairs* (Fh), defining two subtypes.

3.2. Ultrastructure and distribution of types of sensilla

3.2.1.Tip-pored sensilla

Sensilla chaetica with a terminal pore (Sc-tp)

Sc-tp are present in DTs I, II, III and IV. They have a regular distribution pattern, inserted in longitudinal rows on the dorsal, lateral and ventral surfaces of the tarsomeres (Fig. 1, Fig. 2a, b). These sensilla are the longest setae on all DTs, measuring around 215µm on DT I, but being longer on DT II (283-422µm) and DTs III and IV (345-665µm). The shaft is sigmoidally curved and gradually tapers towards the apex (Fig. 3a, arrow). A terminal pore on the Sc-tp of DTs I-II was not always visible in the SEM (Fig. 3b), but in the ones on DTs III-IV the pore was delimited by a round margin (Fig. 3c, arrow). The thick shaft at the base (width: 8.4-18.5µm) inserts in a pronounced socket (Fig. 3d, asterisk) and forms an angle between 45º and 60º with the surface of the cuticle. The shaft wall has no pores, but has longitudinal ridges (Fig. 3e, arrows), which are more pronounced on the Sc-tp of DTs III-IV (not shown). In cross sections, this sensillum shows a single lumen filled with outer receptor lymph (Orl). The lumen is innervated by several outer dendritic segments (Od) surrounded by a thick dendritic sheath (Ds) and accompanied by an inner enveloping cell (E1) (Fig. 3f, g). Fixation of the Od along the shaft was poor when compared with other sensilla, which is probably related to the thick wall (0.5-1µm). The margins of the wall have a characteristic ring of minute longitudinal canals (10nm), which have a "honeycomb" appearance and are electron-lucid (Fig. 3h, black arrowhead). Narrow transversal canals are present in some sections, connecting the lumen to the outer parts of the wall (Fig. 3h, white arrowhead). However, no connection with the outside (pores) was visible (Fig. 3h, inset). Below the apical portion of the shaft, an inner receptor lymph (Irl) occurs between the Od and the dendritic sheath (Fig. 3g), and continues to the basal portion. Slightly below the socket, the Irl is reduced and numerous microvilli (Mv) invade the space in between the Od, forcing them to the periphery of Irl space. At this level 15 Od are discernible (Fig. 3i). The dendritic sheath then gradually disappears (Fig. 3j) and the Od display a typical ciliary pattern with 9x2+0 microtubules (Fig. 3j, k, inset). The Od are then immediately surrounded by the inner most enveloping cell (E1), which has sparse microtubules (Fig. 3k, inset). A middle enveloping cell and an outer enveloping cell surround the E1 and Od (Fig. 3j). The space between the cilia increases and is filled with more Irl and microvilli (Fig. 3k), immediately before the transition zone from the outer dendritic segments to the inner dendritic segments (Fig. 3l).

3.2.2. Wall-pored sensilla

The different types of wp-sensilla share some external and internal characteristics: (1) the shaft always presents external longitudinal ridges (Fig. 3-6, 8, white arrows), with rows of pores between

them (Fig. 4-6, 8); (2) pores starting at the base of the shaft, some micrometers away from the insertion (except in Sc-wp type 2) and follow to the apex (e.g., Fig. 4c); (3) external pores connected to the lumen by transversal canals (Fig. 4-6, 8, white arrowheads); (4) longitudinal canals in the wall (Fig. 4-6, 8, black arrowheads), radially aligned with the external ridges and adjacent to the transversal pore canals; (5) a sheath cell invading the lumen (Fig. 4h, i, 5f-h, 6h-j, 8g) along with the outer dendritic segments; (6) inner receptor lymph below the socket level filled with several microvilli (Fig. 4j, 5i, 7b, 8i, j). The different types differ in the shaft inclination, length, tapering degree, morphology of the insertion, wall and pore characteristics, and innervation.

3.2.2.1. *Sensilla chaetica* with wall pores (Sc-wp)

All *sensilla chaetica* with wall pores are restricted to DTs I and II, being distributed on the dorsal and lateral surfaces (Fig. 1). The only exception found is an isolated wall-pored sensillum occurring dorsally on DTs III and IV, adjacent to the small tarsal process (Fig. 2c, d).

Sc-wp type 1

These sensilla measure around 75-100µm in length, tapering toward the apex in a smaller degree than other wall-pored sensilla (Fig. 4a, white arrow). The tip is blunt (width: 1.7µm), presenting a large pore-like inward folding (Fig. 4b, black arrow). The basal shaft (width: 6.6µm) inserts in a socket with no dorsal hinge, but it seems to have some articulation ventrally (Fig. 4a, asterisk). It is inserted in an angle of 20-30º with respect to the cuticular surface, a smaller angle than the steeply inserted Sc-tp (section 3.2.1). The most conspicuous feature is the presence of many rows of wall pores in the shaft, clearly visible in the SEM (Fig. 4b, d, f). At the base, rows of 1 to 5 parallel pores are present (Fig. 4c), which then converge to double and single rows along the middle of the shaft (Fig. 4d). Small longitudinal ridges separate the rows of pores (Fig. 4b, d, white arrows). The longitudinal canals in the wall are electron-lucid and vary from circular (diameter: ~60nm) to ellipsoid profiles (~60/90nm) (Fig. 4e, h, black arrowheads). A broken shaft reveals an elaborate meshed structure (Fig. 4f). Transverse canals between the longitudinal canals connect the single lumen to the exterior through the meshed wall (Fig. 4f, g, arrowheads), by means of single or multiple pore openings (Fig. 4 g). The lumen is filled with Orl and is innervated by multiple branched Od (Fig. 4e, h, i) which are branched above the socket. The highest number of branches is found towards the middle shaft (Fig. 4h). The inner enveloping cell inside the shaft shows branching (Fig. 4h). At the level of the socket, the inner enveloping cell has no

branches and begins surrounding the Od (Fig. 4i). The Od are larger and show few or no branching at this level (not resolved), being surrounded by the dendritic sheath (Fig. 4i). Below the socket, dendrites reach the ciliary region, along with microvilli and are surrounded by the enveloping cells (Fig. 4j).

Sc-wp- type 2

Sensilla chaetica with wall pores type 2 are longer than Sc-wp type 1 (length: ~120µm) and have a high tapering degree (Fig. 5a, arrow), with the basal shaft (width: ~6µm) being ~10 times wider than the fine apical portion (width: ~0.6µm) (Fig. 5b, c). The socket is similar to Sc-tp and seems articulated (Fig. 5c, asterisk). The shaft emerges at an angle of ~40º with respect to the cuticular surface setting the fine tip above the other sensilla, except for Sc-tp. Longitudinal rows of minute pores (20-25µm) can be seen in very clean samples (Fig. 5b, d, inset). Broken shafts and cross sections reveal longitudinal canals with circular to ellipsoid profiles (width: ~110/75nm) (Fig. 5e, f, black arrowheads). Each pore connects to the lumen by transverse canals between two longitudinal canals (Fig. 5f, white arrowhead). Branched Od innervate the lumen and are accompanied by the inner enveloping cell (E1), which also has some microtubules (Fig. 5f). More basally, the Od are surrounded by an electron-dense substance (Fig. 6g). At the socket level, the Od are protected by a dendritic sheath (Ds) (Fig. 5h). Below the socket, the inner enveloping cell involves the Od, forming a compartment filled with microvilli. At least three enveloping cells are discernible around the Od (Fig. 5i).

3.2.2.2. Falciform hairs (Fh)

Falciform hairs are only found on DTs I and II, on the dorsal and lateral surfaces (Fig. 1). They have a small angle of insertion (25-30º) with respect to the cuticular surface and a downward curved shaft, which frequently appears shrunk in non critical-point dried samples. Two subtypes can be recognized on the basis of shaft length and wall perforations.

Fh type 1

The shaft measures 50-70µm in length, tapering towards the apex (Fig. 6a) and ending in a fine tip (width: 0.7-0.9µm) (Fig. 6b). The socket has no dorsal hinge, but an articulation groove exists between the cuticle and the ventral side of the shaft (Fig. 6c, asterisk). The outer wall has multiple rows of single perforations which start basally (Fig. 6d) and continue toward the end of the shaft (Fig. 6b, d-f). Each row of pores lays between two longitudinal ridges of the shaft (Fig. 6e, f, arrows), but

occasionally two rows may fuse in one. In some samples, the pores were externally covered by round plugs, which could be extruded hemolymph (Fig. 6e, dotted circles). The longitudinal canals of the wall vary from circular (~115nm diameter) to ellipsoid (145/65nm) and are electron-lucid, occasionally showing some electron-dense material (Fig. 6g, h; black arrowheads). These canals fuse in a diffused ring at the basal level of the shaft, where no pores are seen on the outside (Fig. 6j). Very narrow transversal canals (width: 12nm) connecting the outside pores to the internal lumen (Fig. 6g, i) are found between the longitudinal canals. At the apical portion of the shaft, the lumen is filled only with receptor lymph (Fig. 6g), while multiple Od are seen from the middle to inner parts of the sensillum (Fig. 6h-j; Fig. 7). At the level of the socket, a dendritic sheath is seen around the Od (Fig. 7a). Below the cuticle, the E1 and two other enveloping cells surround the Od, forming an inner receptor lymph space filled with multiple microvilli (Fig. 7b). More proximally, the sensory cells reach the ciliary region (Fig. 7c-e) and immediately enlarge and present organelles (Fig. 7c, f). At this level, at least 9 inner dendritic segments are discernible (Fig. 7f).

Fh type 2

Falciform hairs type 2 are shorter than type 1 (length: ~28um) (Fig. 8a), tapering less and ending in a tip with ~0.7µm (width) (Fig. 8b). The socket is similar to that of type 1, only with a ventral articulation groove. The surface of the shaft has rows of single pores, with more rows than that of type 1 (compare middle shaft in Figs. 6d and 8c). These sensilla have the thinnest wall of all types here described (0.2µm), which is visible in broken shafts (Fig. 8d) and cross sections (Fig. 8e-h). The longitudinal canals are minute (diameter: 20nm), but the transversal pore canals are slightly larger (width: 18-30nm) than in Fh type 1. The lumen is filled with outer receptor lymph (Fig. 8e-h) and innervated by multiple Od that reach approximately the second third of the shaft (Fig. 8g, h). The dendritic sheath appears at the level of the socket, remains until the inner enveloping and other two enveloping cells tightly surround the microvilli and Od (Fig. 8i), disappearing closer to the ciliary region (Fig. 8j).

3.3. Laniatores diversity survey

We observed wall-pored sensilla on the dorsal and lateral surface of DTs I and II in 17 species representing the following laniatorean families: Travuniidae, Synthetonychiidae, Trieanonychidae,

Pyramidopidae, Epedanidae, Petrobunidae, Podoctidae, Sandokanidae, Tithaeidae, Gonyleptidae, Phalangodidae, Stygnommatidae, Escadabiidae, Fissiphaliidae, Guasiniidae, Icaleptidae and Zalmoxidae (Fig. 9, black asterisks). The species of the families investigated are representative of all currently recognized superfamilies of Laniatores (i.e., Kury 2013), namely: Travunioidea, Triaenonychoidea, Phalangodoidea, Epedanoidea, Gonyleptoidea, Assamiodea, Samooidea and Zalmoxoidea. We observed variation in shape, length and characteristics of the shaft wall. All wp-sensilla had multiple perforations in the shaft, which always started on the basal portion of the shaft, but slightly above the insertion (Fig. 10b, e, i, n). In some of them, we observed a characteristic terminal constriction, which was not perforated as the rest of the shaft (Fig. 10a, j, k, n). They could be inserted in smooth basal membranes (Fig. 9a, b, e, f, h, i, n) or in more prominent and robust socket (Fig. 10c). More than one type of wp sensilla is clearly present even within the same individual. For example, in *Guasinia* sp. (Guasiniidae), two wp-sensilla were discernible on the basis of shaft diameter and presence of a terminal constriction (Fig. 10 n, constriction showed by black arrow). At present, we find it problematic to unambiguously assign the wp-sensilla of this survey to the types proposed for *H. discolor*, solely based on external morphology. However some similarities are noticeable. For example, the terminal constriction in some "sausage-like" wp-sensilla observed (Fig. 10a, j, k, n) resemble the morphology of Sc-wp type 1 of *H. discolor* (Fig. 4).

4. Discussion

We provided the first ultrastructural study on the tarsal sensilla of a laniatorean harvestman, describing ultrastructural features previously not investigated in any Opiliones species. We identified three *sensilla chaetica* and two *falciform hairs* subtypes in *Heteromitobates discolor* and reported the presence of wall-pored sensilla in 17 families of Laniatores.

4.1. General features

The sensilla of harvestmen are uniform in many characteristics. The subtypes of *sensilla chaetica* and *falciform hairs* all have dendrites innervating the shaft accompanied by the innermost enveloping cell, which are characteristic of chemoreceptive sensilla of arthropods (Altner and Prillinger, 1980; Foelix, 1985; Zacharuk, 1985; Tichy and Barth, 1992). All sensilla described herein are innervated by 9-15 neurons, which is a higher number than usually observed in insect chemoreceptors, but typical for

arachnids (Altner and Prillinger, 1980; Foelix, 1985). At the transition from inner to outer dendritic segments, the neurons emit one cilium with a microtubular pattern of 9x2+0 above the basal body (Figs. 3j-l; 7c-e). This is the usual conformation in arthropods (Keil and Steinbrecht, 1984; Foelix, 1985; Keil, 1997, 2012), although emission of two cilia (Crustacea: Grünert and Ache, 1988; Myriapods: Tichy and Barth, 1992) and alternative ciliary arrangements (Parasitiformes, Ixodida: Hess and Vlimant, 1982; Foelix, 1985) have been described. At least three enveloping cells are present in *Heteromitobates discolor*: an innermost, middle and outer enveloping cell (e.g., Fig. 7b). The organization of enveloping cells is uniform across arthropods (Keil and Steinbrecht, 1984; Keil, 1997, 2012), so these cells likely correspond to the thecogen (produces the dendritic sheath), trichogen (secretes the hair shaft) and tormogen (secretes basal parts of the shaft) cells. In arachnids, additional enveloping cells may be present (Araneae: Harris, 1977; Acari: Haupt and Coineau, 1978; Solifugae: Haupt, 1982), however, it is not clear whether additional enveloping cells occur in *H. discolor*. Microvilli invade the inner receptor lymph space at levels below the socket (Fig. 3i-l; 4i; 6b, c, f; 7i, j), but we could not determine their origin. This feature has also been reported in spiders and ticks, where these microvilli are projections of the enveloping cells, usually the innermost (thecogen) (Foelix and Axtell, 1971, 1972; Foelix and Chu-wang, 1972; Harris, 1977). Thus, the ultrastructure of harvestmen sensilla follows a basic groundplan for Arthropoda, sharing with other arachnids a multiple innervation of the shaft. Additionally, investigating whether more than three enveloping cells are present would be interesting to determine if they are similar to other arachnids in this respect.

Below, we discuss the specific function and distribution of chemoreceptors, and the implications of the widespread occurrence of wp-sensilla in Laniatores for their sensory biology.

4.2. Function of subtypes and distribution on distitarsi

4.2.1. Tip-pored sensilla: Sensilla chaetica with a terminal pore (Sc-tp)

Sensilla chaetica with a terminal pore present characteristics typical for touch-dependent sensory modalities, namely contact chemoreception (gustation) and contact mechanoreception (Foelix and Chu-Wang, 1973a, b; Altner and Prillinger, 1980). They have a steep angle of insertion and are the longest setae on the tarsi, making them prominent (Fig. 3) and the first integument structure to touch the surroundings while the animal is exploring. Moreover, these sensilla have a terminal pore, an innervated

thick walled lumen and a movable socket, which are characteristic of dual function contact chemoreceptors/mechanoreceptors of insects and arachnids (Altner and Prillinger, 1980; Zacharuk, 1985; Foelix, 1985). In the arachnid order Amblypygi, the dual function contact chemoreceptor/mechanoreceptor setae are regularly spaced in the tarsomeres, forming five aligned rows (Foelix et al., 1975; Igelmund, 1987; Santer and Hebets, 2011). Similarly, the Sc-tp of *H. discolor* are arranged in longitudinal rows in all surfaces of DTs I, II III and IV (Fig. 2a, b). Thus, we support a dual function for this subtype of *sensilla chaetica*, as has been generally assumed for general *sensilla chaetica* of Opiliones (Willemart et al., 2009).

Sc-tp outer dendritic segments remain protected by a dendritic sheath (Fig. 4i, j) and are presumably free for uptaking chemical stimuli only at the terminal pore (Fig. 4c). Therefore, the narrow transversal cuticular canals observed in the middle shaft (Fig. 4h) are probably not involved in the perception mechanism, since Od are not free at this level. Similar canals have been observed in tp-sensilla of a tick, where a sensory related function has also been disfavored (Chu-Wang and Axtell, 1973). The number of sensory cells (15) identified in *H. discolor* is very close to the 16 reported in the shaft of a *sensillum chaeticum* in *Siro* sp. (Opiliones, Cyphophthalmi) and in *Phalangium opilio* (Opiliones, Eupnoi) (Foelix, 1976, 1985). Multiple innervation is also typical for arachnids in general, in comparison with singly innervated sensilla in insects (Altner and Prillinger, 1980; Zacharuk, 1985; Foelix, 1985). While contact chemoreception is well supported, and mechanoreception seems likely based on external morphology, we found no histological evidence that Sc-tp functions as a mechanoreceptor. The mechanoreceptive unit of dual function sensilla is formed by at least one dendrite attached to the movable socket, forming structures called tubular bodies (McIver, 1985; Keil, 2012).We found no evidence of tubular bodies and we could not determine whether all 15 dendrites observed below the socket (Fig. 4i, j) penetrate the shaft, as the fixation of upper parts was not adequate. Since the only two other studies that investigated *sensilla chaetica* with histology also report no mechanoreceptive unit (Cyphophthalmi: Foelix, 1976; Eupnoi: Guffey, 2000), it remains an open question whether we have missed them in the sections or if they do not exist.

4.2.2. Wall-pored sensilla: Sensilla chaetica with wall pores (Sc-tp) and Falciform hairs (Fh)

Wall-pored sensilla are widespread among insects (Schneider and Steinbrecht, 1968; Zacharuk, 1985; Keil and Steinbrecht, 1984), but are much less common in arachnids, where they have only been reported in Acariformes and Parasitiformes (Alberti and Coons, 1999; Coons and Alberti, 1999; Krantz

and Walter, 2009), Amblypygi (Santer and Hebets, 2012), Araneae (a brief mention of a single sensillum in *Gradungula*; Foelix, 1985), Ricinulei (Talarico et al., 2006, 2008), and in non-laniatorean Opiliones (only SEM: Willemart et al., 2009; Willemart and Giribet, 2010). Wall-pores in the shaft are a compelling evidence of olfactory function, a correlation which has been extensively supported by numerous morphological and electrophysiological studies in arthropods, mostly insects (e.g., Altner and Prillinger, 1980; Zacharuk, 1985; Keil and Steinbrecht, 1984). Pore openings provide a direct pathway for the odorant molecules to reach the outer receptor lymph bathing the chemoreceptive dendrites inside the shaft (Steinbrecht, 1997). Sc-wp and Fh show clear wall pores that connect the outer dendritic segments to the exterior (Fig. 4-6, 8) and therefore are probably olfactory receptors. Wall-pored sensilla are divided in two categories: single walled (sw) sensilla, with pore tubules; and double-walled (dw) sensilla, with spoke channels (Altner et al., 1977; Altner and Prillinger, 1980; Keil and Steinbrecht, 1984; Zacharuk, 1985; Steinbrecht, 1997). A feature shared by all wall-pored sensilla found in *H. discolor* is the presence of longitudinal canals in the walls, rendering them a double-walled appearance (Fig. 4e, h; 5e-g; 6g-j; 8h). In Arachnida, dw-sensilla have only been reported on the first pair of legs of some mites and ticks (Parasitiformes), presenting typical vase-shaped canals (Foelix and Axtell, 1971, 1972; Coons and Axtell, 1973; Hess and Vlimant, 1982; Akkerhuis et al., 1985; Tichy and Barth, 1992). However, *H. discolor* wp-sensilla do not perfectly fit in this category. Dw-sensilla in arthropods typically show 3 characteristics: (1) longitudinal slits on the outer surface (2) spoke wheel channels, and (3) longitudinal canals (Altner and Prillinger, 1980; Keil and Steinbrecht, 1984; Zacharuk, 1985; Steinbrecht, 1997). In *H. discolor* wp-sensilla, slits on the outer surface are absent. The longitudinal canals can be minute (Fig. 8h) and in higher number than usually observed in dw-sensilla of insects, mites and ticks (Keil and Steinbrecht, 1984; Alberti and Coons, 1999; Coons and Alberti, 1999). Moreover, small longitudinal canals also exist in the wall of Sc-tp ("honey-comb" canals: Fig. 3h, inset), sensilla that we infer to be gustatory. Similar canals on a gustatory sensillum were presented in a drawing of a *sensillum chaeticum* of a harvestman of the suborder Cyphophthalmi (Foelix, 1985). Recently, glandular sensilla in Opiliones have been suggested to be modified *sensilla chaetica*, and the authors clearly show the presence of longitudinal "cuticular canals" in these setae (Wolff et al., 2016). Given that this feature is shared by gustatory, olfactory and modified glandular setae in Opiliones, we hypothesize that it may be a structural feature that reflects a common developmental program of harvestmen chemoreceptive sensilla, rather than being related with the sensory process as in dw-sensilla (Steinbrecht, 1997). In glandular sensilla, these canals are inferred to transport the secretions released at

the apex (Wachmann, 1970; Wolff et al., 2016), but for general chemoreceptors they may provide flexibility and economy of cuticular material in the secretion of the hair shaft. Unfortunately, little is known about the ontogeny of chemoreceptors in arachnids (Harris, 1977; Haupt and Coineau, 1978; Haupt, 1982) and nothing in Opiliones.

Sc-wp type 2 are inserted in an articulated socket (Fig. 5a, c), are long and prominent setae, with a steep angle (~40º) with respect to the cuticular surface. These external characteristics suggest that they might also be touch receptors (Foelix and Chu-Wang, 1973a, b; Altner and Prillinger, 1980), but we found no histological evidence of mechanoreception. Additional touch sensitivity is more common in gustatory sensilla, but mechanoreceptors have also been reported in olfactory sensilla of ticks (Parasitiformes, Ixodida), where a long dw-sensilla has mechanoreceptors at the base of the shaft (Hess and Vlimant, 1982).

Several wp-sensilla in our SEM survey in Laniatores showed an external morphology that resembles the wall-pored *solenidia* of Cyphophthalmi, Eupnoi and Dyspnoi (Willemart et al., 2009; Willemart and Giribet, 2010). For instance, in *Synthethonychia* sp. (Fig. 10a) and *Guasinia* sp. (Fig. 10n), some wall-pored sensilla have thick shafts ending in an obtuse end, similar to what has been reported for Cyphophthalmi (Willemart and Giribet, 2010). In *H. discolor*, wp-sensilla are much more elongated than the reported *solenidia*, however external morphology is frequently insufficient to establish homologies (Altner and Prilliner, 1980). Investigating the internal morphology of the *solenidia* in non-laniatorean harvestmen seems crucial to determine whether there is a correspondence with the subtypes here identified.

4.3. Sensory appendages

Laniatorean harvestmen typically support their bodies with leg pairs III and IV, while leg pairs I and II are mainly used for tapping and sensing their surroundings (Willemart et al. 2009; and references therein). Despite the traditional view of leg pair II as the sensory appendages, growing evidence has demonstrated that leg pair I is also very important for sensory perception. For instance, ingestion in *Iporangaia pustulosa* is only initiated after leg I touches the food (Willemart and Chelini, 2007). Morphologically, tarsi I and II have been shown to have sensillar types absent in legs III and IV (*sensilla basiconica* and *falciform hairs*) and to possess more *sensilla chaetica* than those of posterior appendages

(Willemart and Gnaspini, 2003; Willemart et al., 2009). We confirm this morphological evidence, showing that DTs III and IV have much simpler sensory equipment in terms of sensillar types and that DTs I and II share the sensillar types here identified. Therefore, we also found no evidence to attribute a special sensory status to leg pair II over leg pair I.

To summarize, the higher density of *sensilla chaetica* on the anterior appendages of Laniatores described in the literature (Willemart and Gnaspini, 2003; Willemart et al. 2009) accounts for the presence of wall-pored olfactory subtypes, which are largely absent on the posterior appendages except for a single wall-pored sensillum on DTs III and IV (Fig. 2c, d). Moreover, *falciform hairs*, which are restricted to DTs I and II, also show a typical olfactory morphology.

4.4. Not so touchy after all?

Chemoreceptors in arachnids are concentrated on the anterior appendages (Foelix, 1985; Hess and Vlimant, 1983, 1986; Igelmund, 1987; Santer and Hebets, 2011), and olfactory sensilla are usually restricted to them or occur in higher numbers (Acariformes and Parasitiformes, legs I: Alberti and Coons, 1999; Coons and Alberti, 1999; Amblypygi, legs I: Santer and Hebets, 2011; Ricinulei, legs I and II: Talarico et al., 2006). Some mites and ticks (Parasitiformes) often wave legs I, a behavior associated with the detection of several odors, such as aggregation and sexual pheromones and chemicals to find a host or prey (Sabelis and Baan, 1983; Coons and Alberti, 1999). In Amblypygi, the antenniform legs (leg I) are required for the location and acceptance of dead prey (Santer and Hebets, 2011), and ricinuleids use legs I and II to explore their surroundings (García et al., 2015). Similar to those arachnids, harvestmen explore the surroundings with the sensory appendages of legs I and II (Willemart et al., 2009; see 4.3), which we showed to possess many olfactory sensilla. Moreover; those olfactory sensilla show a diversity of types and relative abundance on the anterior appendages (Fig. 1). Different types of olfactory sensilla usually have different specificities (Chapman, 1998). For example, the *sensilla trichodea* on the antenna of male silkworm moth (*Bombyx mori*) are specific for perceiving female sex pheromones, while other olfactory sensilla respond to plant and other general odors (Steinbrecht, 1987, 1996). Therefore we expect Sc-wp and Fh subtypes to respond to odors in different contexts.

The occurrence of wp-sensilla in *Heteromitobates discolor* is not restricted to this species. S*ensilla chaetica* and *falciform hairs*, in which we found wall-pores, had already been observed in several harvestman species (Willemart et al., 2009). Moreover, subtypes of *sensilla chaetica* have been hypothesized in the literature for other suborders (Spicer, 1987; Willemart and Gnaspini, 2003; Willemart et al. 2009) which suggests that some may have wall pores and also be olfactory. Furthermore, our SEM survey sampling all the main lineages of Laniatores revealed the presence of wp-sensilla in members of 17 families (Fig. 9, 10). Even if we assume that the 15 remaining families not investigated do not have wp-sensilla, ancestral state reconstruction would still recover the presence of wp-sensilla in the last common ancestor of Laniatores (reconstruction not shown, based on a compiled phylogeny by Giribet and Sharma, 2015 and unpublished results from Fernández, Sharma, Tourinho, Giribet; Fig. 9).

These findings are especially relevant in the case of harvestmen, because mechanoreception at a distance but also vision are assumed to be less important than in most other arachnids, and chemoreception is arguably the most important sensory modality for Opiliones (Willemart et al., 2009). Chemoreception is important in different contexts, such as foraging (Willemart and Chelini, 2007; Costa and Willemart, 2013), associative learning (dos Santos et al., 2013), communication and intraspecific recognition (Machado et al., 2002; Donaldson and Grether, 2007; Grether and Donaldson, 2007; Willemart and Hebets, 2011; Teng et al., 2012). From most of these behavioral studies, their chemical perception seems restricted to contact chemoreception or to close range olfaction of strong odors, which has supported the idea of a “Touchy Harvestmen” (Macías-Ordóñez, 2000). With the evidence provided herein, however, we expect olfaction to be more important than previously considered (see also Hashimoto and Hayashi, 2014). We encourage more behavioral experiments dealing with olfaction in contexts which have not been studied in Opiliones, such as detection of conspecifics, chemicals from predators, finding roosting sites, and sexual pheromones. Pheromones are especially interesting, considering the widespread occurrence of sexually dimorphic glands in Laniatores (Willemart et al., 2010), which in at least two species are used for marking the substrate (Fernandes and Willemart, 2014; Murayama and Willemart, 2015). In light of the new morpho-anatomical evidence, addressing these behavioral questions seems a promising field of inquiry.

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TABLE AND FIGURES

Table 1: List of species of Laniatores used in our scanning electron microscopy survey, with taxonomy, voucher numbers, collection details and gender.

	Taxonomy	Voucher #	Locality	Coordinates	Sex
Erebomaster flavescens flavescens Cope, 1872	Cladonychiidae	MCZ DNA101444	USA: Patton Cave, Deam Wilderness Area, Indiana	n/a	?
Synthetonychia glacialis Forster, 1954	Synthetonychiidae	MCZ DNA101718	New Zealand: Minnehaha walk, Fox Glacier, South Island	43 28 7 S 170 1 5 E	Male
Larifuga cf. *capensis*	Triaenonychiidae	MCZ DNA100727	South Africa: Newlands Forest. Table Mountain, Cape Province	n/a	Male
cf. *Pyramidops* sp.	Pyramidopidae	MCZ DNA101432	Equatorial Guinea: Montaña Chocolate, Niefano District	1º45’25”N, 10º17’03”E	?
Pseudoepedanus doiensis Suzuki, 1969	Epedanidae	MCZ DNA101438	Thailand: Huaykhok Ma, Doi Suthep, Chiang Mai	n/a	Male
Petrobunus schwendingeri Sharma & Giribet, 2011	Petrobunidae	MCZ DNA 103572	Panay, Sibaliw, Phillipines	n/a	?
Austribalonius sp.	Podoctidae	MCZ DNA 106334	Australia: Ella Bay	17° 28´ 39.3´´ S 145° 4´ 22.2´´ E	Male
Sandokan truncatus Thorell, 1891	Sandokanidae	MCZ DNA101099	Singapore: Bukit Timah Nature Reserve, Jungle Fall Valley	10 20 53.3 N 103 46 35.4 E	Male
Tithaeus sp.	Tithaeidae	MCZ DNA104074	Malaysia: Terengganu state	5º53’52”N, 102º44’2”E	Male
Remyus sp.	Phalangodidae	MCZ DNA 102660	Madagascar: Toliara Prov., Parc National d'Andohahela, Foret d'Ambohibory	24 55' 48"S 46 38' 44" E	Female
Stygnomma bispinatum Goodnight & Goodnight, 1953	Stygnommatidae	MCZ DNA105636	Mexico: Chiapas. Sierra Morena	16 9 123 N 93 36 2.8 W	Male
Baculigerus milenae Kury, 2012	Escadabiidae	MCZ DNA100640	Brazil: Parque Ecológico de Cocó, Fortaleza	n/a	Male
Fissiphallius sp.	Fissiphaliidae	MCZ DNA104057	Colombia: Santuario de Fauna y Flora Iguaque, Departamento de Boyacá	5 42 43 N 73 27 44 W	Female
Guasinia sp.	Guasiniidae	MCZ DNA 105838	Cuba: Santiago de Cuba Prov., Rio la Mula	n/a	?
Icaleptes sp.	Icaleptidae	MCZ DNA101420	Colombia: Reserva Natural Río Ñambí, Município de Barbacoas	1 17 6 N 78 4 25 W	?
Zalmoxis sp.	Zalmoxidae	MCZ DNA106885	Espiritu Santo, Vanuatu	n/a	Female

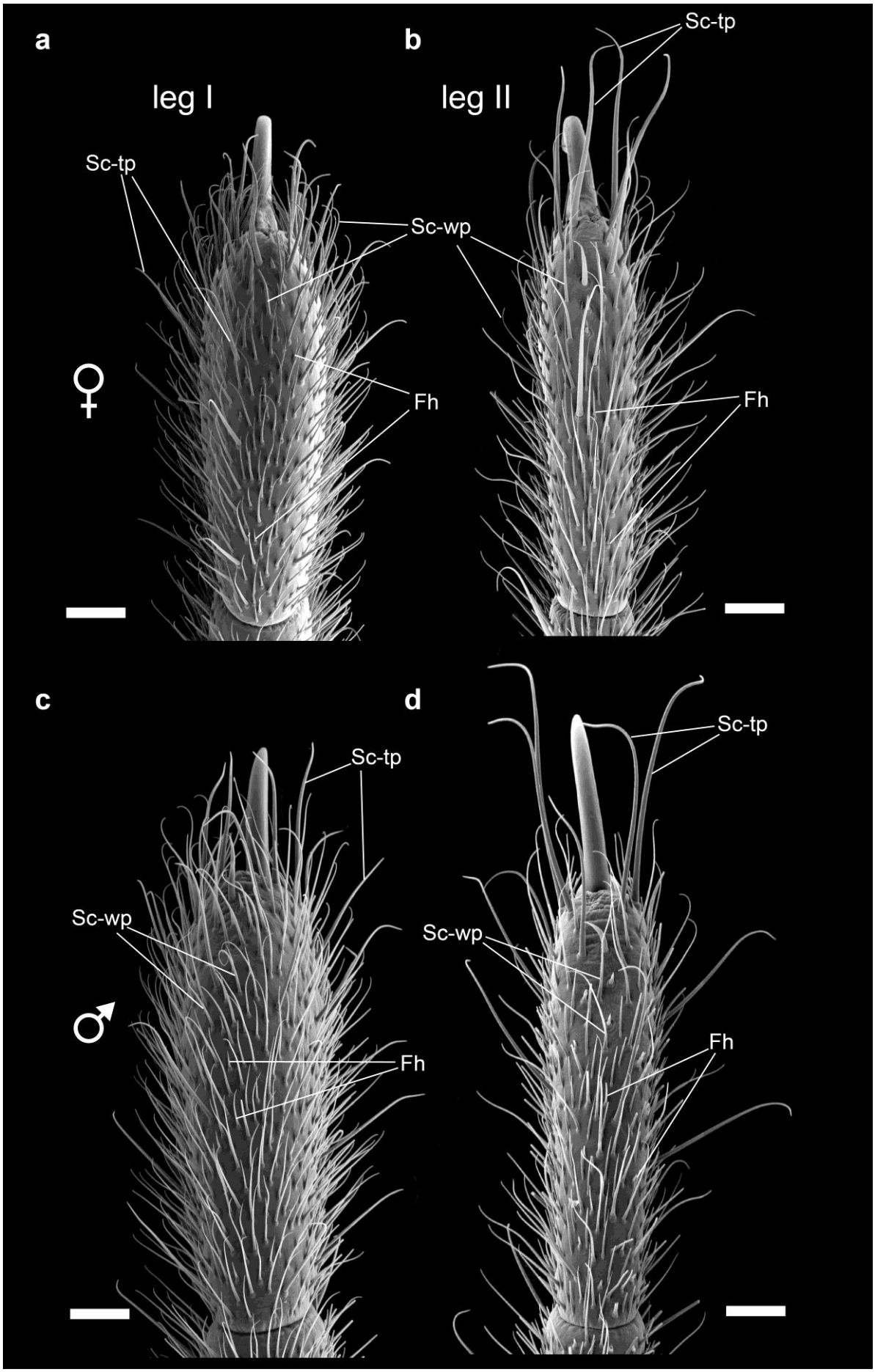

a
leg I
Sc-tp
Sc-wp
Fh
♀
b
leg II
Sc-tp
Sc-wp
Fh
c
Sc-tp
Sc-wp
Fh
♂
d
Sc-tp
Sc-wp
Fh

Figura 1: Distalmost tarsomeres I (first column) and II (second column) of female (a, b) and male (c, d) *Heteromitobates discolor*. Sc-tp: *Sensilla chaetica* tip-pored; Sc-wp: *Sensilla chaetica* with wall pores; Fh: *Falciform hairs*. Sub types of Sc-wp and Fh are not discriminated because unambiguous sorting is not possible at low magnifications. Scale bars: 100 μm.

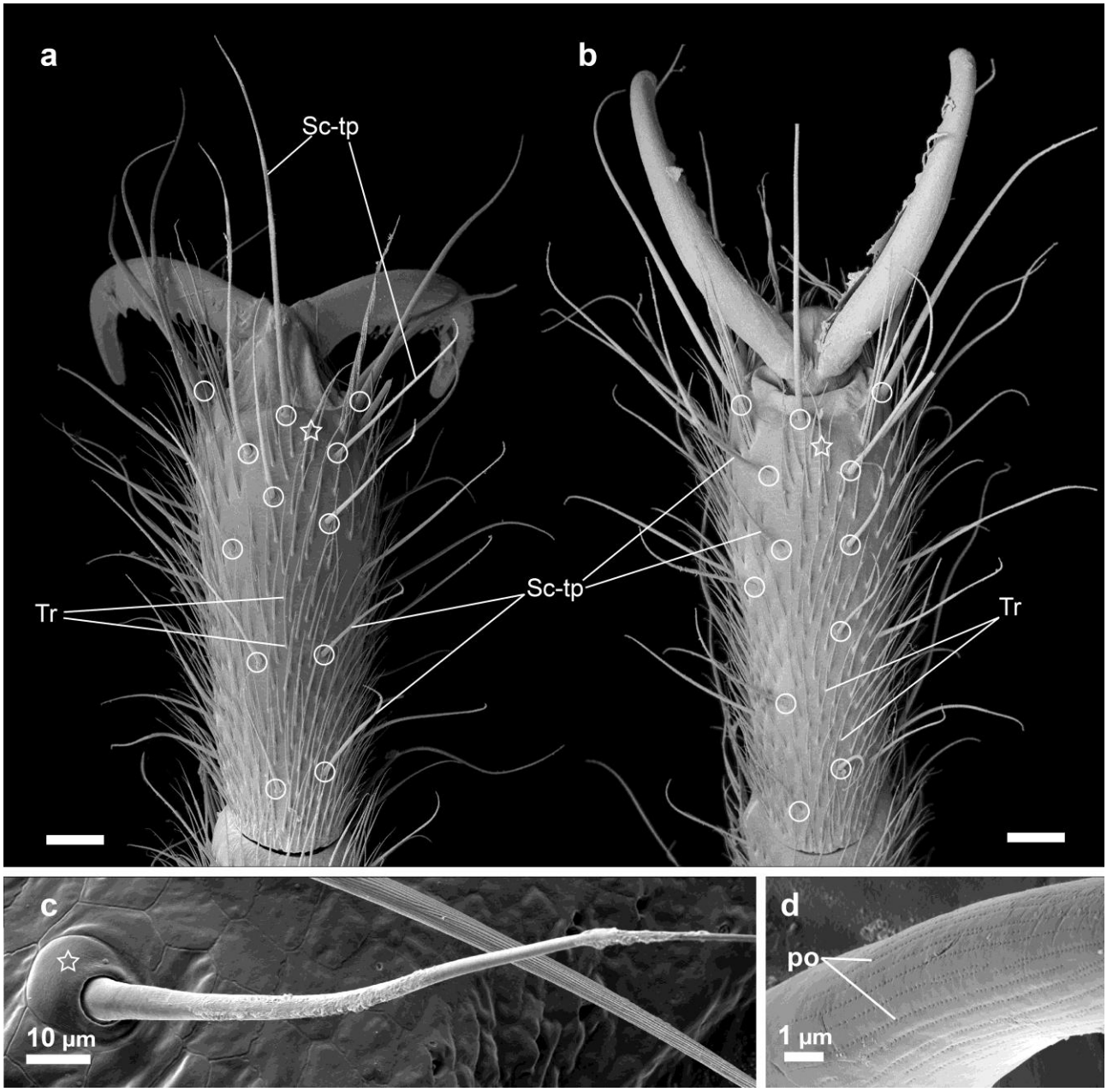
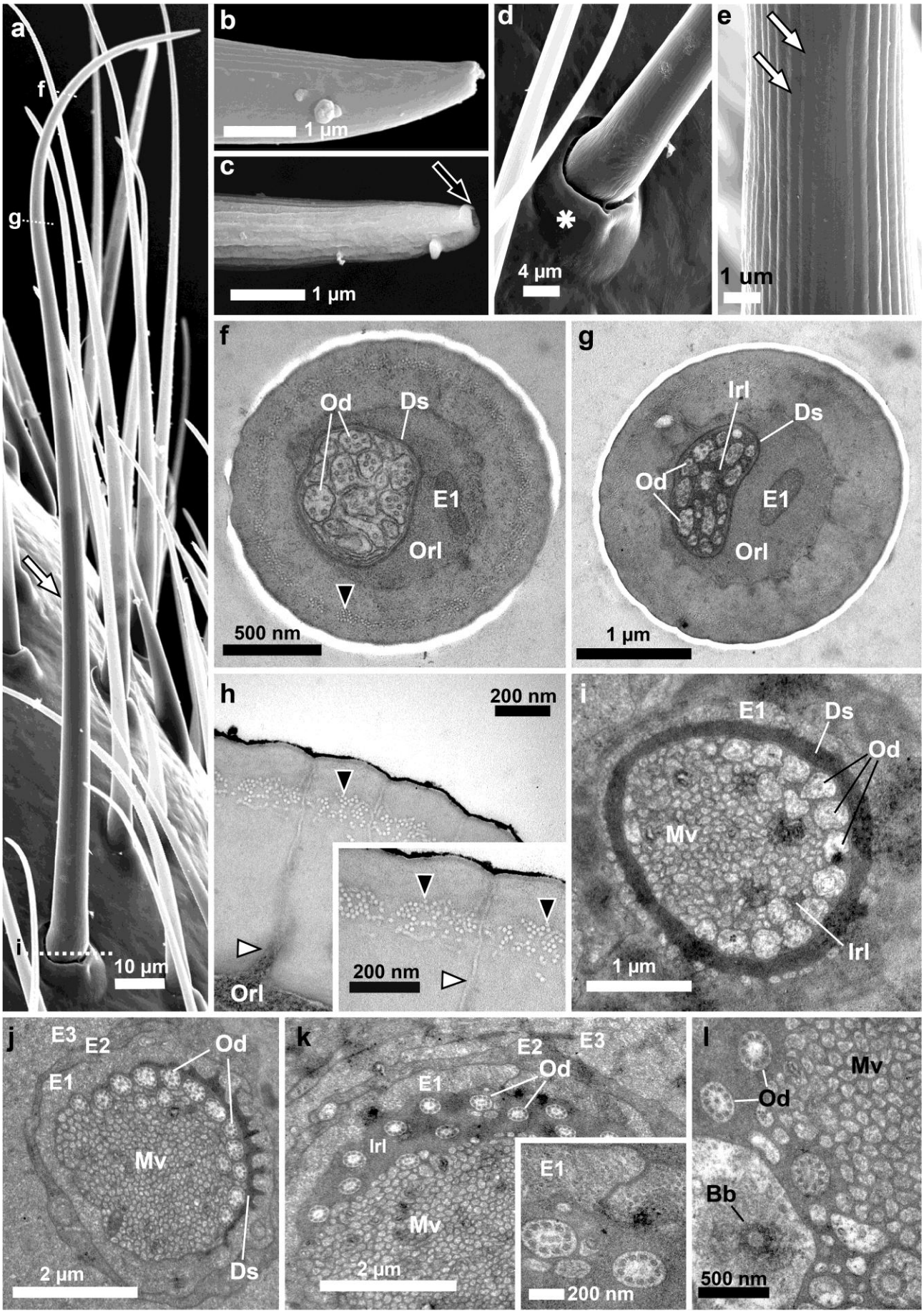


Figura 2: Distalmost tarsomeres III (a) and IV (b) of a male *Heteromitobates discolor*. Scale bars: 100 µm. Circles indicate the insertion of dorsal Sc-tp and star indicates a single wall-pored sensilla. c: A single wall-pored sensillum (same position as the star on "b"), on DT IV, dorsal surface of a male. Detail of the shaft on the correspondent sensilla on the DT III of a male. Sc-tp: *Sensilla chaetica* tip-pored; Po: pore; Tr: Trichome.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E1
200 nm
l
Mv
Od
Bb
500 nm

Figure 3: *Sensilla chaetica* tip-pored (Sc-tp). External morphology in SEM (a-e) and cross sections in TEM (f-l; last tarsomere I, female). a: Sc-tp on the lateral region of last tarsomere I (white arrow), male. b: Sc-tp apical portion on tarsomere I, male. c: Sc-tp apical portion on tarsomere III, male, with visible terminal pore (black arrow). d: Socket (asterisk). e:Middle shaft, with longitudinal ridges (white arrows). f: Shaft sectioned near the apex. g: Shaft sectioned on distal third towards the apex. h: Detail of the wall, showing transversal canal (white arrowhead) and "honey-comb" longitudinal canals (black arrowhead). i: Section imediatly below the socket. j-l: Serial sections below "i". Bb: basal body; Ds: dendritic sheath; E1: inner enveloping cell E2: midlle enveloping cell; E3: outer enveloping cell; Irl: inner receptor lymph; Mv: microvilli; Od: outer dendritic segment; Orl: outer receptor lymph.

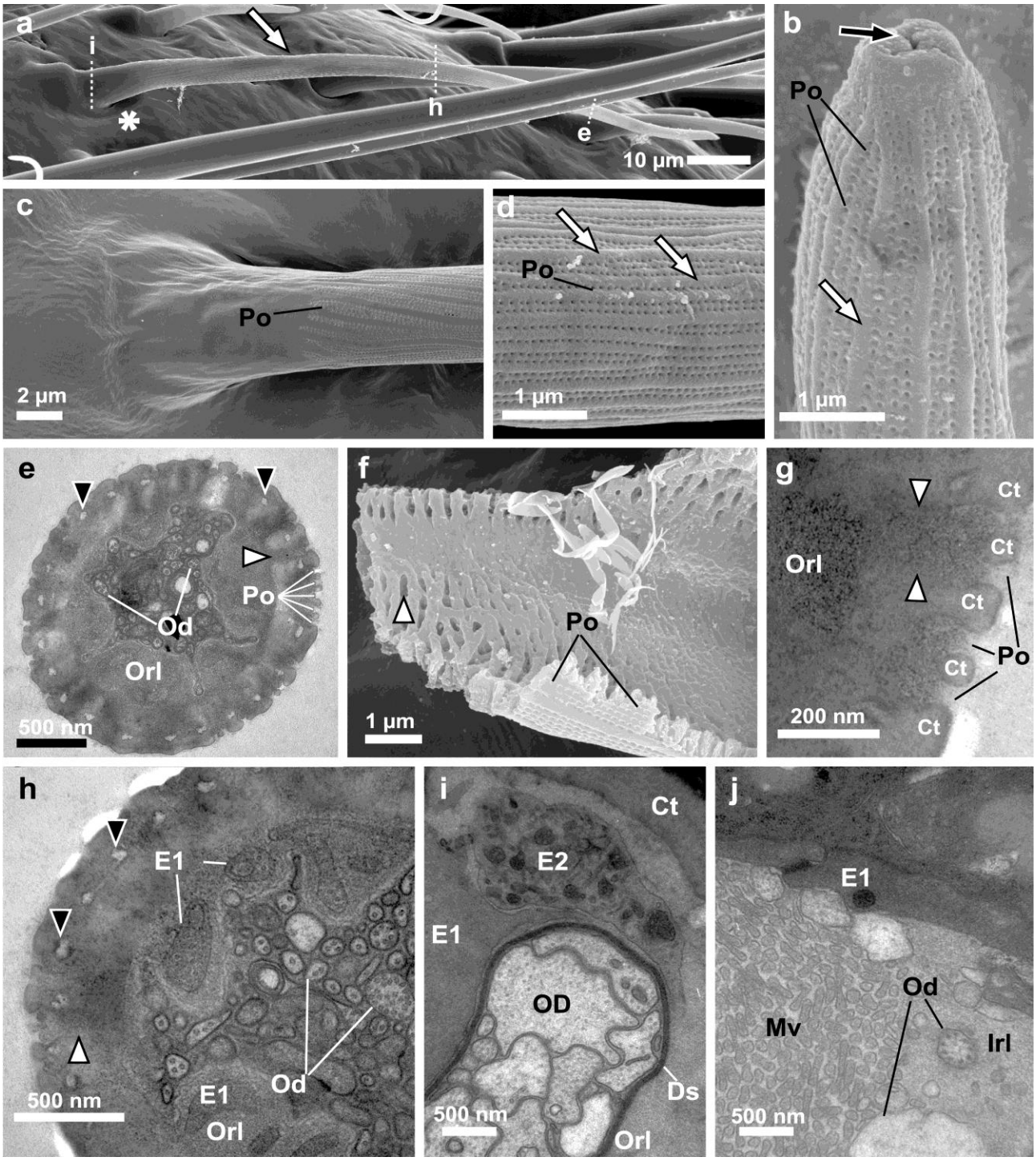
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Figure 4: *Sensilla chaetica* wall-pored (Sc-wp) type 1. External morhology in SEM (a-d, f) and internal morphology in TEM (e, g-I; last tarsomere I, female). a-d: male, last tarsomere I. a: Lateral view of the shaft, dorso/laterally inserted (white arrow), showing ventral articulation (asterisk). b: Apex, with terminal pore-like infolding (black arrow) and logitudinal ridges (white arrow). c: dorsal view of the insertion in the cuticle and basal shaft. d: Middle shaft, with longitudinal ridges (white arrows). e, g-i:Serial sections of the shaft. e: Section of apical shaft, with longitudinal canals in the wall (black arrowhead). f. Internal surface of a broken shaft, last tarsomere II, male. g: Section of the middle shaft. h: Detail of the wall in "g", showing pore canals (white arrowhead). i: Section at the socket level. Ds: dendritic sheath; E1: inner enveloping cell; E2: midlle enveloping cell; Irl: inner receptor lymph; Mv: microvilli; Od: outer dendritic segment; Orl: outer receptor lymph; Po: pore.

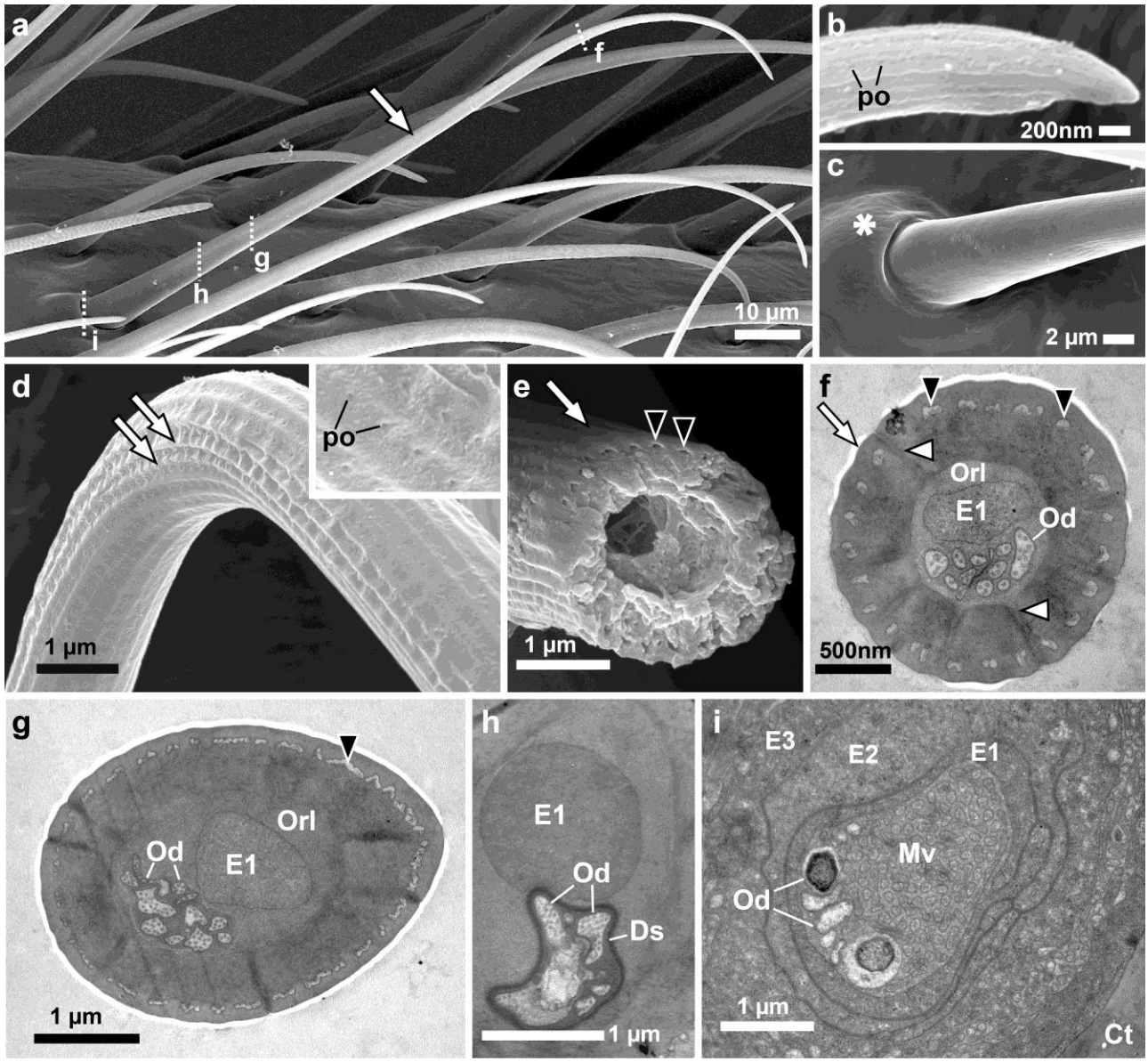

Figure 5: *Sensilla chaetica* wall-pored (Sc-wp) type 2. External morphology in SEM (a-e) and internal morphology in TEM (f-i; last tarsomere I, female). a-d: Last tarsomere I, male. a: Lateral view of the shaft (white arrow) on dorsal surface of the tarsomere. b: Apex. c: Socket (asterisk); d: Distal shaft, showing parallel longitudinal ridges (white arrowhead). Inset: detail of the pores. e: Broken distal shaft, revealing narrow lumen and longituginal canals in the wall (black arrowhead), leg II, male. f: Section at shaft's apical portion. White arrow indicates correspondent ridges seen on the external surface (d, e). g-i: Serial sections from "f". g: Section at the middle shaft. h: Detail of a section at the fisrt third of the shaft, above socket. i: Section below the socket. Ct: cuticle; Ds: dendritic sheath; E1: inner enveloping cell; E2: midlle enveloping cell; E3: outer enveloping cell; Mv: microvilli; Od: outer dendritic segment; Orl: outer receptor lymph; Po: pore.

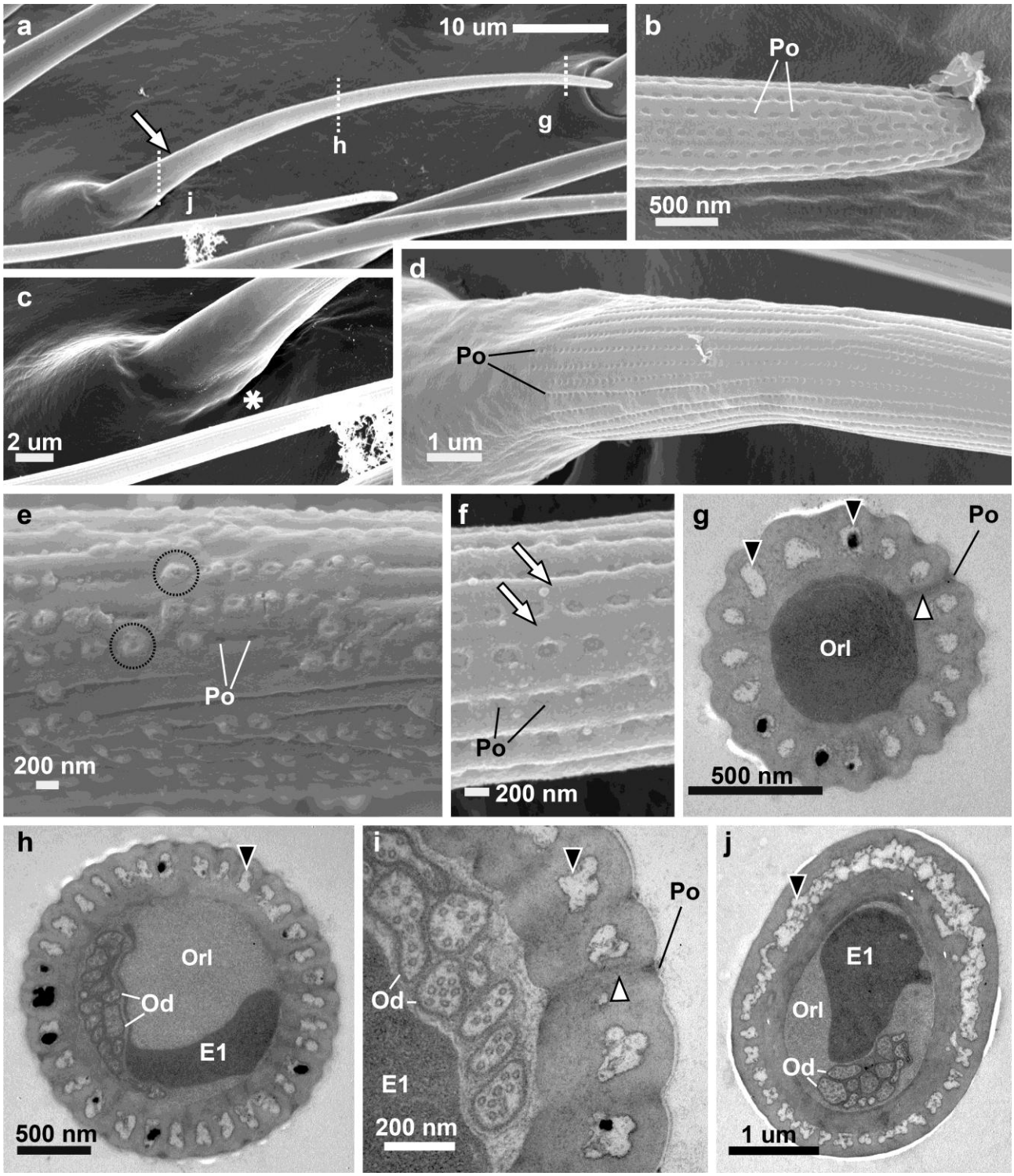
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Figure 6: *Falciform hairs* type 1. External morphology in SEM (a-f; last tarsomere I, male) and internal morphology in TEM (g-j; last tarsomere I, female). A: Lateral view of the shaft, dorsal surface of the tarsomere (white arrow). b: Apical portion. c: Basal shaft and insertion with ventral articulation (asterisk). d: Basal and middle shaft, showing transition from no-pored to the pored region (first pores indicated). e: Middle shaft with some pores plugged (dotted circles). f: Detail of the rows of pores in between ridges (white arrows) close to apex. g-j: Cross sections of the shaft, with longitudinal canals (black arrowhead) and transversal pore canals (white arrowhead). Images h and j are from the same series of Fig. 7. g: Close to the apex; h: Middle shaft. i: Detail of the shaft´s wall, at a level comparable to "h". j: Immediately above the insertion. Ds: dendritic sheath; E1: inner enveloping cell; Od: outer dendritic segment; Orl: outer receptor lymph; Po: pore.

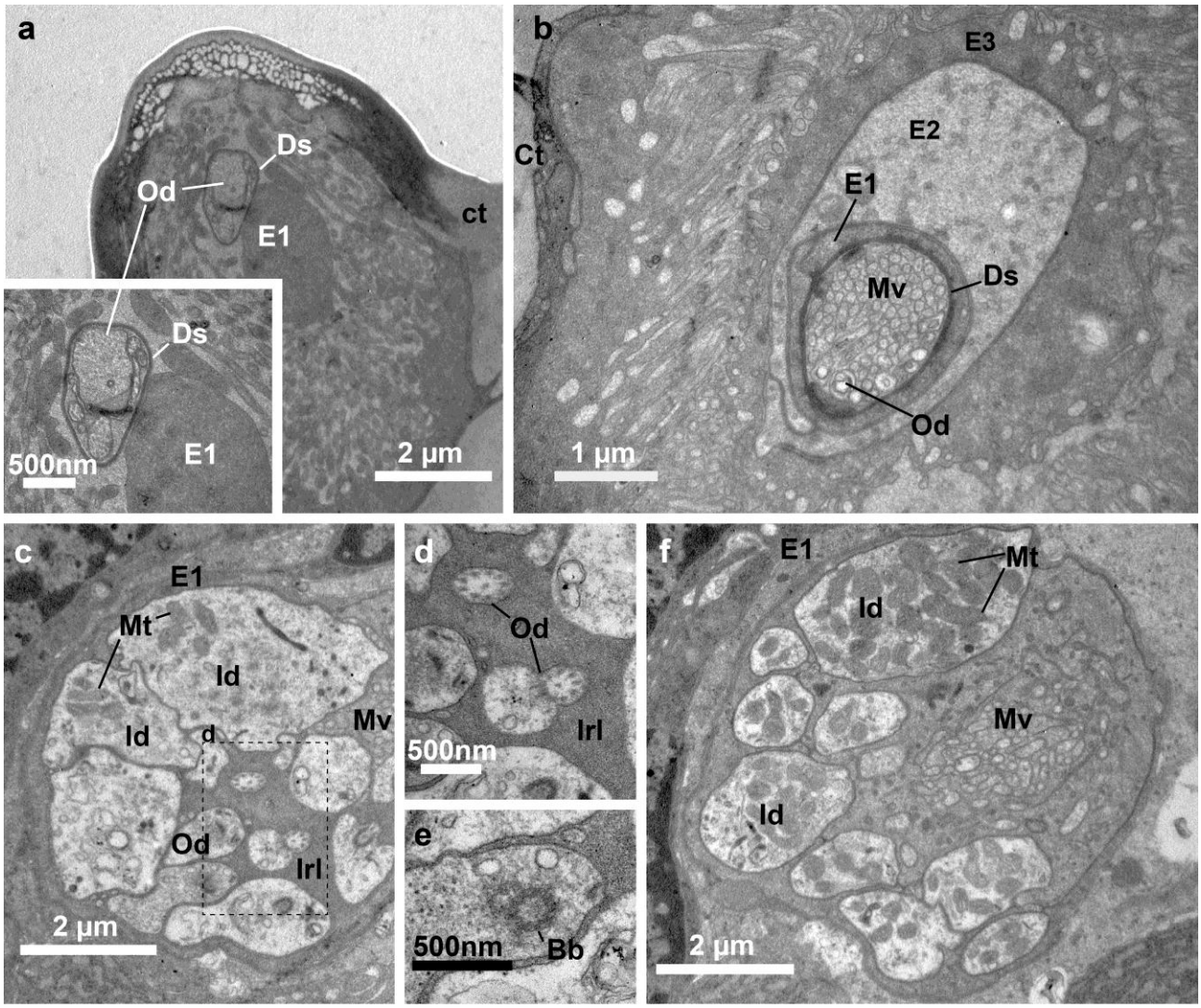

Figure 7: *Falciform hairs* type 1. Serial cross sections of the inner parts, in TEM. a: Insertion in the cuticle; b: Immediately below the insertion; At the ciliary region, showing transitions from outer to inner dendritic segments. d: Detail of the dashed area in "c", showing outer dendritic segments forming typical 9x2+0 ciliary patterm, immediately before transition to inner segment. e: Detail of a dendrite at the level of the basal body. f: Below the ciliary region. Bb: basal body; Ct: cuticle; Ds: dendritic sheath; E1: inner enveloping cell; E2: midlle enveloping cell; E3: outer enveloping cell; Id: inner dendritic segment; Irl: inner receptor lymph; Mv: microvilli; mt: mitochondria; Od: outer dendritic segment; Orl: outer receptor lymph.

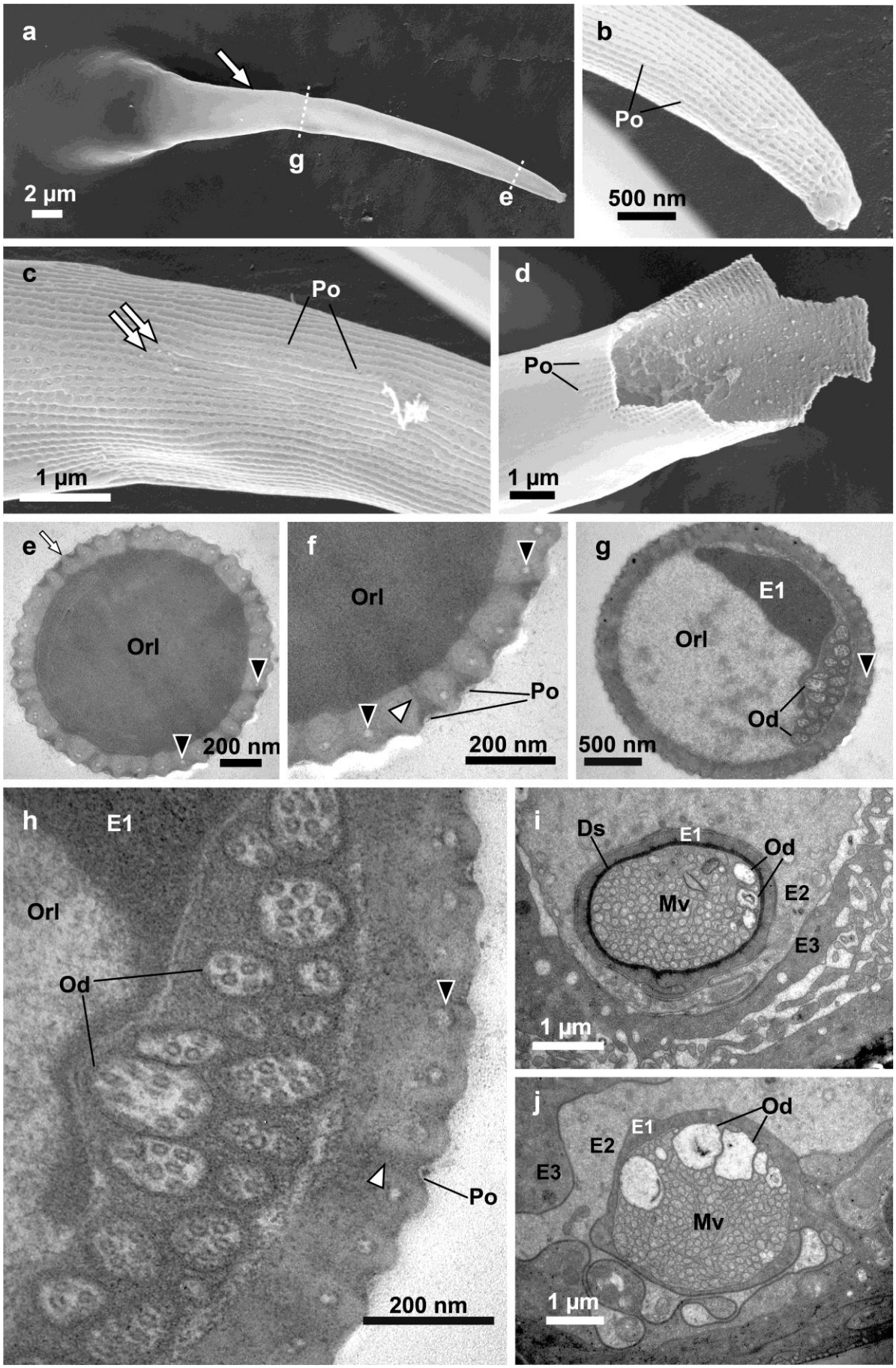
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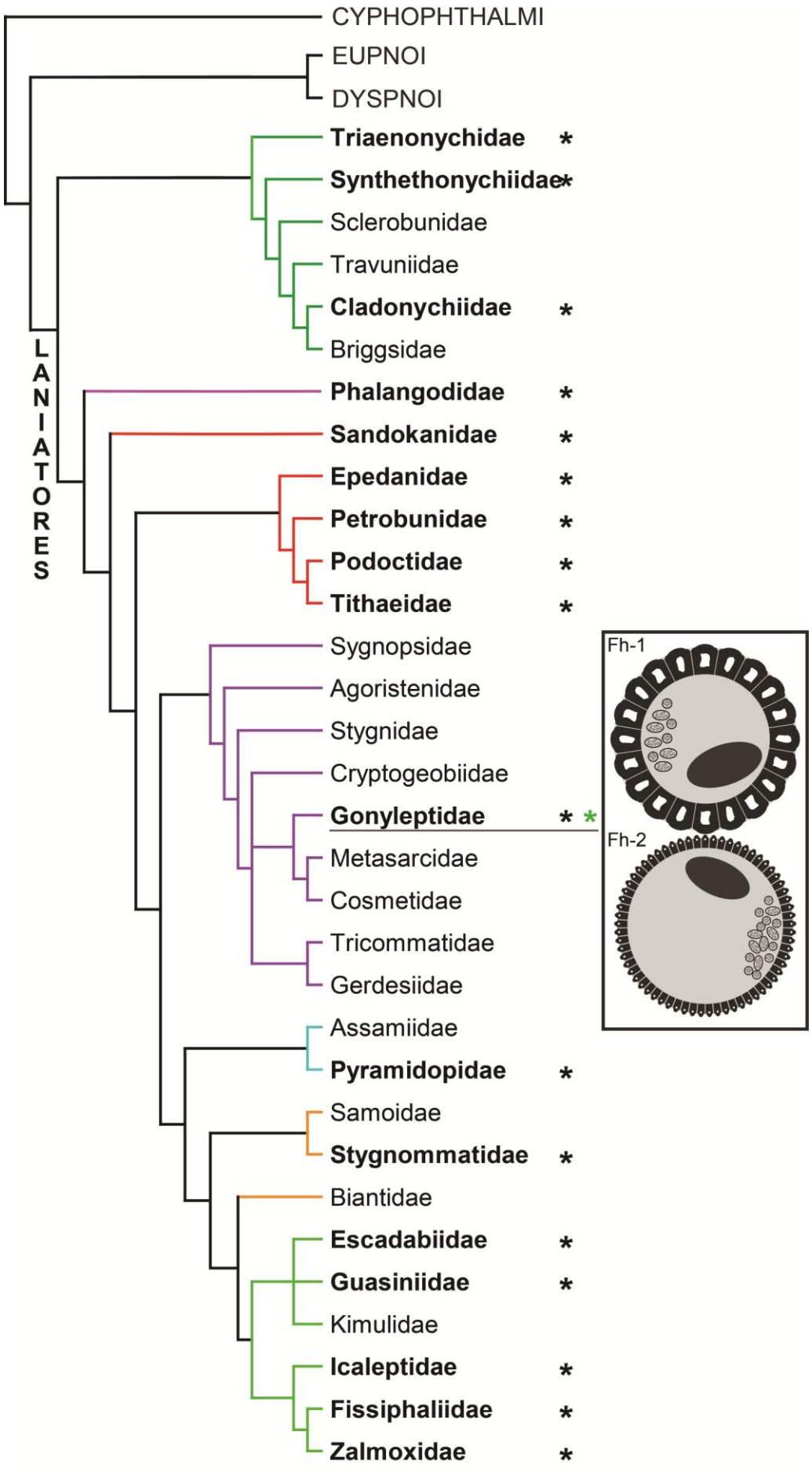
Figure 8: *Falciform hairs* type 2. External morphology in SEM and internal morphology in TEM. a-c: last tarsomere I, male. a:Dorso-lateral view of the shaft (white arrow), on the dorso-lateral surface of the tarsomere. b: Apex. c: Middle shaft, with longitudinal ridges (white arrows). d: Broken shaft ,last tarsomere II, male. First pores are indicated, showing transition from no-pored to the pored region. e-j: Cross sections. e: Apical portion of the shaft, showing longitudinal canals (black arrows). f: Detail of "e". the same longitudinal canals (black arrowhead) are indicated and transversal pore canals (white arrowhead) are also visible. g-j: serial sections. g: Middle shaft. h: Detail of "g", showing longitudinal (black arrowhead) and transersal canals (white arrowhead). i: Immeadiatly below the insertion of the shaft. j: section bellow the dedritic sheath zone. Ds: dendritic sheath; E1: inner enveloping cell; E2: midlle enveloping cell; E3: outer enveloping cell; Mv: microvilli; Od: outer dendritic segment; Orl: outer receptor lymph; Po: pore.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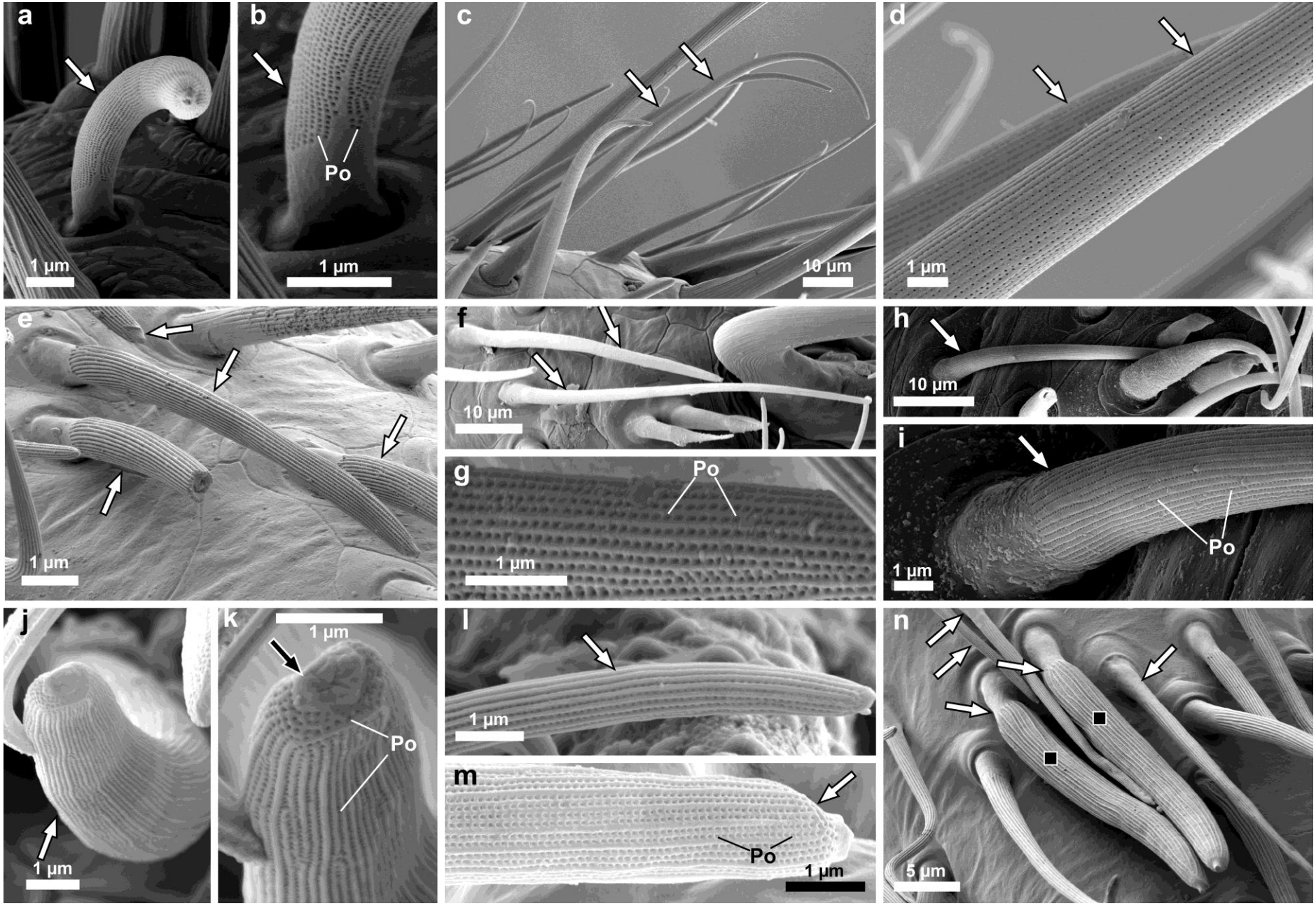
Travuniidae
Cladonychiidae *
Briggsidae
LANIATORES
Phalangodidae *
Sandokanidae *
Epedanidae *
Petrobunidae *
Podoctidae *
Tithaeidae *
Sygnopsidae
Agoristenidae
Stygnidae
Cryptogeobiidae
Gonyleptidae * *
Metasarcidae
Cosmetidae
Tricommatidae
Gerdesiidae
Assamiidae
Pyramidopidae *
Samoidae
Stygnommatidae *
Biantidae
Escadabiidae *
Guasiniidae *
Kimulidae
Icaleptidae *
Fissiphaliidae *
Zalmoxidae *
Fh-1
Fh-2

Figure 9: Families of Laniatores for which we report wall-pored sensilla on legs I and II (16 families, asterisks). Black asterisk: SEM evidence. Green asterisk: TEM evidence (*Heteromitobates discolor*). Phylogenetic relationships between 32 families of Laniatores is based on Giribet & Sharma (2015) and unpublished results from Fernández, Sharma, Tourinho & Giribet. Question mark in Sandokanidae indicates uncertain phylogenetic affinities. Dark green infraorder "Insidiatores". Light purple: superfamily Phalangodoidea. Red: superfamily Epedanoidea. Dark purple: superfamily Gonyleptoidea. Blue: superfamily Assamioidea. Yellow: superfamily Samooidea. Light green: Zalmoxoidea.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l
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m
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Figure 10: Wall-pored sensilla on DTs I or II (white arrows) of selected specimens representing the laniatorean families surveyed. a: *Sythetonychia glacialis* (Synthetonychiidae), DT I, male. b: Detail of "a". c: *Larifuga capensis* (Triaenonychiidae), DT I, male. d: Detail of "c". e: *Remyus* sp. (Phalangodidae), DT I, female. f: *Pseudoepedanus doiensis* (Epedanidae), DT I, male. g: Middle shaft of a sensilla on DT II of the same individual in "f". h: *Austribalonius* sp. (Podoctidae), DT II, male. i: Detail of the basal shaft of sensilla showed in "h". j: *Icaleptes* sp. (Icaleptidae), DT I, undetermined sex. k: Detail of the sensilla showed in "j", showing the terminal constriction at the apex (black arrow). l: Apex of a different type of wp-sensilla in *Icaleptes* sp., adjacent to the sensilla in " j". m: Apex of a sensilla in *Zalmoxis* sp., DT II, female. n: *Guasinia* sp. (Guasiniidae), DT II, undetermined sex. Black squares mark thick subtypes with an obtuse apex and terminal constriction (black arrow). Po: pore.

Chapter 2: Ultrastructure of putative hygro- thermoreceptive tarsal sensilla on a Neotropical armored harvestman (Arachnida, Opiliones, Laniatores, Gonyleptidae)

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Abstract

Most harvestman species are dependent on high humidity levels and amenable temperatures for homeostasis. While they are known to actively choose environments with these conditions, no hygro/thermoreceptor has yet been identified in the group. Using scanning electron microscopy and transmission electron microscopy, we investigated the fine morphology of two hair sensillar types of the armored harvestman *Heteromitobates discolor* (Laniatores, Gonyleptidae): sensillum basiconicum (Sb) and apical-hood sensillum (Ahs). Both structures occur in small numbers on the body (Sb: 28 units; Ahs: 4 units) and are distributed on the distal parts of the legs. On the distal-most tarsomeres I and II a pair of Sb and Ahs have a very close cellular association, forming a bundle which proceeds to tarsal nerve. The Sb is innervated by 3-4 dendrites and

has a sagittal slit that results in a shaft with two flaps, which probably allows evaporation of receptor lymph. The Ahs is innervated by two bundles of 3 dendrites, has two pore-like structures on its tip and unusual meshed structure of the shaft's wall. Cuticular structure, putative evaporation of receptor lymph and innervation support a hygro/thermoreceptive function for Sb, but the function of Ahs remains unclear. We discuss the specific morphological characteristics in support of these functional inferences, the adequacy of the evaporation system of Sb to specific hygroreception mechanisms and the cellular association between these two sensillar types on the tip sensory appendages.

1. Introduction

The arachnid order Opiliones comprises over 6600 known species, which are divided in four main extant lineages: the suborders Cyphophthalmi, Eupnoi, Dyspnoi and Laniatores (Giribet and Sharma, 2015; Kury, 2016). They occur worldwide from rain forests and temperate regions, to semi-arid and arid environments (Cokendolpher et al., 1993; Curtis and Machado, 2007). The greatest diversity is located in the tropics, including most families of the suborder Laniatores (2/3 of Opiliones species) (Curtis and Machado, 2007; Giribet and Kury, 2007). This common distributional pattern may be partially due to environmental restrictions, such as those imposed by temperature and humidity, which seems to be especially relevant for Opiliones (Wiens and Donoghue, 2004; Curtis and Machado, 2007). Temperature and humidity may also affect median richness of harvestmen communities, as suggested from generally smaller diversity in higher altitudes, which have colder and drier conditions (Almeida-Netto et al., 2006), and in open areas as compared with forested areas (Curtis and Machado, 2007). Mostly nocturnal, harvestmen seek shelter during the day under logs, leaf litter, palm sleeves, rocks and crevices, which provide microenvironments with higher humidity (Todd, 1949; Curtis and Machado, 2007; Proud et al., 2011). Harvestmen in the subfamily Goniosomatinae (Laniatores, Gonyleptidae), for instance, inhabit Atlantic rain forests from Brazil, being usually found on rocks and vegetation (humid microhabitats) close to water sources (Machado et al., 2000; Silva and Willemart, 2015). Some Goniosomatinae harvestmen regularly live in caves and forage in the forest (Gnaspini, 1995; Machado et al., 2000; Willemart and Gnaspini, 2004), or use them as temporary refuges (Chelini et al., 2011). They also actively drink water droplets or chew the edges of leaves for water uptake (Santos and Gnaspini, 2002). Being able to discern between dry and wet microenvironment is not only important to select suitable habitats, but also to choose good oviposition sites (Gnaspini, 1995; Machado and Oliveira, 1998). In *Heteromitobates discolor*, for instance, most of the eggs are found within 3 cm to wet vegetation, which can provide humidity for the eggs and water for egg-caring females (Silva and Willemart, 2015).

Laboratory experiments show that in general harvestmen have a low tolerance to dehydration and prefer humid environments (>60%), while temperature preferences may vary between species from temperate and tropical areas (Todd, 1949; Immel, 1954; Edgar, 1971; Santos, 2003). Susceptibility to dehydration has been related to the fact that they have a diluted hemolymph (unfavorable osmotic pressure) and opened spiracle apertures, both factors contributing to higher lymph evaporation (Santos, 2003, 2007).

While harvestmen are certainly capable of sensing humidity and temperature changes (Todd, 1949; Immel, 1954; Clingenpeel and Edgar, 1966; Santos, 2003, 2007), virtually nothing is known about which sensory structures, or sensilla, are responsible for the detection of these stimuli. Legs II are typically elongated in harvestmen, and, together with legs I, are considered the main sensory appendages (Willemart and Chelini, 2007; Willemart el., 2009). Immel (1954) verified that individuals of the Dyspnoi species *Paranemastoma quadripunctatum* with ablated legs I and II were still capable of selecting humid environments, implying that structures sensitive to light/moisture in this species must occur elsewhere.

Studying the internal morphology of sensilla is an effective approach to understand their functions (Altner and Prillinger, 1980; Keil and Steinbrecht, 1984), but unfortunately very few studies exist in harvestmen with this respect (Willemart et al., 2009, and references therein). Most of our knowledge on the function of harvestmen sensilla comes from data on the external morphology of laniatorean species (eg. Willemart et al., 2007, 2009). Four sensillar types have been identified: slit sensilla, sensilla chaetica, falciform hairs and sensilla basiconica. Slit sensilla are general substrate vibration detectors and/or proprioceptors that are also observed in other arachnids (Barth and Stagl, 1976; Luque, 1993; Barth, 2002). Sensilla chaetica in Opiliones are generally assumed to be dual function mechanoreceptive/gustatory sensilla, although recent evidence shows subtypes in Laniatores, which together with falciform hairs, have typical olfactory morphology (Gainett et al, Chapter I). Sensilla basiconica occur in small numbers on the distal parts of the legs (Willemart et al., 2007, 2009), and by exclusion (also due do their small size), have been hypothesize as chemo/hygro/thermoreceptive sensilla (Willemart et al., 2007). However, sensilla basiconica have never been ultrastructurally investigated, and their function remain unknown.

Complementing our studies on sensilla chaetica and falciform hairs in *Heteromitobates discolor* (Gonyleptidae, Goniosomatinae) (mechano- and chemoreceptors, see Gainett et al Chapter I), here we

investigate the ultrastructure of two putative hygro- thermoreceptive tarsal sensilla on the distal most tarsomeres of the legs: the sensilla basiconica, and a previously unknown sensillar type, here termed apical-hood sensilla. We describe internal morphology, cellular arrangement on the tarsi and distribution on the animals, discussing functional hypothesis for both sensillar types.

2. Methods

2.1. Collection and measurements

Heteromitobates discolor individuals were collected at Casa da Farinha (Corisco River or Fazenda River), Ubatuba, São Paulo, Brazil (S 23° 20' 18.5" W 44° 50' 15") between February and March, 2014 or on April 25th, 2015. Measurements of sensillar attributes were made using open source software ImageJ 1.48v (https://imagej.nih.gov/ij).

2.2 Scanning Electron Microscopy (SEM)

Appendages were dissected from alcohol fixated animals and cleaned in ultrasound following the same protocol described in Chapter 1. Samples were dehydrated in graded ethanol series, critical point dried (Tousimis 931 GL). They were mounted on stubs with carbon bi-adhesive tabs (Electron Microscopy Science, Hatfield, PA, USA) and sputter coated with Pt-Pd targets (EMS 300T D Dual Head Sputter Coater). Imaging was done with a Zeiss Ultra-Plus FESEM and Zeiss Supra FESEM (field emission scanning electron microscope, at the Center for Nanoscale Systems, Harvard University), or in a Zeiss DSM 940 (at Instituto de Biociências da Universidade de São Paulo).

2.3 Transmission Electron Microscopy (TEM)

Two females and one male were anesthetized for 5-10 minutes in a freezer (6 ºC) and had their distal tarsomeres dissected in cold NaH2PO4x2H2O buffer and then fixed in a mixture of 2,5% Glutaraldehyde and 2% Paraformaldehyde in 0,1M NaH2PO4x2H2O buffer (after Karnovsky 1965) and

kept at 4ºC for approximately 1.5 months. Post-fixation, embedding, sectioning and imaging as in Chapter 1.

3. Results

3.1. Distribution

The previously undescribed sensillar type, here termed apical-hood sensilla (Ahs), has a very conserved position on the dorsal surface of the distalmost tarsomeres I and II, always distal to a pair of sensilla basiconica (Sb) (Figs 1a, b, d). Sensilla basiconica are the shortest hair-sensilla on the body, and always occur as a pair on the distal third of the distalmost tarsomere of leg pairs I and II, dorsally (Figs. 1b). They are also present on the distalmost dorsal region of the metatarsi of all leg pairs, adjacent to the metatarsal paired slits (Willemart et al., 2009; Gainett et al., 2014). Additionally, we also found a single sensillum basiconicum on the retrolateral region of the distalmost tarsomere of leg pairs III and IV (Fig 1d). Therefore *H. discolor* has at least 28 units of sensilla basiconica on its body, and 4 units of the apical-hood sensillum. We observed no sexual dimorphism on the morphology the apical-hood sensilla and sensilla basiconica. There was some intraspecific variation in the disposition of the triad on the distalmost tarsomere I and II (Ahs+Sb), where the three sensilla could appear aligned along the sagittal axis, or the distal sensillum basiconicum (Sb1) could be slightly displaced laterally (Fig 1b).

3.2. Ultrastructure

3.2.1. Apical-hood sensilla

An apical-hood sensillum of *H. discolor* has a long shaft (~105µm) inserted in ~70º with the underlying cuticle, and can easily be mistaken for a sensillum chaeticum in the stereomicroscope. The general structure of this sensillum is schematically shown in figure 2b. The tip has a hood shape, with a smooth internal surface and a grooved external surface (Fig. 2a; hood length: ~7.5 µm). On the smooth internal surface, one pore-like structure (or possibly two, see discussion) can be seen with SEM. Cross sections through the hood reveal a connection between the structure and a dendritic profile, by a narrow canal in a spongy cuticular matrix (Figs. 2a1-a6). Immediately below the hood (we are calling it the

“neck”), two discrete bundles of dendrites appear tightly associated to the cuticular matrix, and no lumen occur in the shaft (Figs. 2c, d). We refer to the larger light bundle as B1 and to the smaller dark bundle as B2. Both bundles are composed of three dendrites surrounded by a dendritic sheath, but in B2 only one dendrite clearly follows up to the hood´s level (Fig. 2c). The dendritic sheath of both bundles is fused with the surrounding cuticle and the dendrites are surrounded by a dark inner receptor lymph (Figs.2c, d). Below the neck, a lumen in the shaft appears filled with outer receptor lymph and continues to the proximal parts of the sensillum (Figs.2e, f; Fig. 3a). Therefore, the bundles are bathed in outer receptor lymph along most of the shaft, except at the hood level. At the middle shaft, the dendrites become laterally flattened along a vertical axis, with some degree of folding (n=3 out of 3 sensilla observed; Figs. 2f, g). At the level of the socket, the dendrites have many microtubules and the bundles are progressively surrounded by an enveloping cell (Fig. 3a). One of the dendrites in B1 presents some vesicles of unknown composition (Fig. 3a, asterisk). Each bundle is then surrounded by an individual inner enveloping cell and by at least one more enveloping cell, at a point where the dendritic sheath starts to disappear (Fig. 3b). Numerous microvilli fill the inner receptor lymph space (Fig. 3b). The dendrites of B1 reach the ciliary region slightly before the ones in B2 (Figs. 3c, e). Each cilia display a typical 9x2+0 (Figs. 3c inset, f, g), but one of the dendrites in B2 presented a pair of central structures in one of the sections (Fig. 3f). The transition zone from inner to outer dendritic segment is marked by the presence of two basal bodies in tandem (Fig. 3d). Immediately below the basal bodies, ciliary rootlets project ~3µm proximally in the inner dendritic segment, and the cell has many organelles (eg. mitochondria) (Figs. 3d, e, h). At this point, the inner dendritic segments go to the corner of their bundles, progressively enlarge, and leave a middle space filled only with sparse microvilli (Fig. 3h).

The ultrastructure of the wall of shaft is unique among other tarsal sensilla we found in this study. Externally, the shaft bears longitudinal ridges, which are denser at basal parts of the shaft (Figs. 4a, b). Instead of a solid continuous wall, the procuticle of the shaft is arranged as the weaved fibers of a braided gas hose, forming a mesh (Figs. 4c-h). At the middle of the shaft, the thickness of this layer in between ~0.8-1 µm. The epicuticle forms a peripheral layer (~160-290nm) that is highly plicate, externally appearing as closely apposed knobs in cross sections (Figs. 4.f, g). Each knob corresponds to one longitudinal ridge of the external shaft (Figs.4a, b). No pores were detected in the wall, but small tubule-like structures occur at the transition from the epicuticular to the procuticular layer (Figs. 4g, e). At the socket level, the cuticle shows an articulation membrane (Fig. 4i).

3.2.2. Sensilla basiconica (Sb)

The ultrastructural description below is based on the pair of Sb on leg pairs I and II. The distal sensillum basiconicum (Sb1; length: ~23µm) has always a longer shaft than the proximal (Sb2, length: ~15 µm), but they have a very similar internal organization.

Each sensillum basiconicum is externally composed by a short conical shaft that is "sliced" along the sagittal axis, resulting in two flaps resembling the beak of a bird (Figs. 5a-c). Two opposite slits are externally visible in the laterals of the shaft, delimiting the two flaps (Figs. 5a-c). The slit-segment of the shaft varies between 1/3 to 1/2 of the total length. The shaft is inserted in a smooth circular basal membrane at an angle of ~ 50-60º (Figs. 5a-c). The sensillum is innervated by three (proximal Sb) to four dendrites (distal Sb), which are surrounded by a dendritic sheath and run to the tip of the shaft, in between the two flaps (Figs. 5d, e). An outer receptor lymph occurs between the cuticle of the flaps and the dendritic sheath of the dendrites (Figs. 5e, f). Along the slit-segment, the dendritic sheath is fused with the edges of the two flaps (Figs. 5f-h). This results in a lateral constriction of the sheath profile along a vertical axis, which assumes a narrow ellipsoid form (Figs. 5f-h). One of the slits appears plugged by a dark substance (Figs. 5f, g), while the other is sealed by a thin membrane continuous with the epicuticular layer of the flaps (Figs. 5f, h; arrow). In several sections a particulate material was observed outside the slits, which appears to be extruded liquid of the inner receptor lymph cavity (Figs. 5f, h; asterisk). Extruding material is also present at the tip (Fig. 5e; asterisk). In one sample this slit showed no protective layer, leaving the inner lymph readily exposed to the environment (not shown). The organization of the flaps and dendritic sheath is so that the outer receptor lymph is isolated from the slits by the dendritic sheath and only the inner receptor lymph is in direct contact with the slits (Figs. 5g, h). It is not clear if apically there is a direct connection between the environment and the outer and/or inner receptor lymph, but the dendritic sheath proceeds to the very tip of the shaft (Fig. 5e). Only two dendrites are seen along the slit-segment, which appear compressed in the ellipsoid profile of the dendritic sheath (Figs. 5f-h). The remaining dendrites only go up to the beginning of the slit-segment (Figs. 5i-k). At the transition from the slit-segment to the tube-segment of the shaft, the dendritic sheath detaches from the cuticle leaving it free in the outer receptor lymph (Fig. 5i). The dendritic profile assumes a more circular shape from this point to more proximal parts (Figs. 5j, k). A clear gradient of electron density exists on the other receptor lymph (Fig. 5d), which is dark at the tip and gradually lighter towards the proximal parts of the sensillum (Figs. 5f, j).

Below the insertion in the cuticle, processes of the innermost enveloping cell begin to surround the dendritic sheath (Fig. 6a). Proximally, at least two other enveloping cells appear around the innermost cell (Fig. 5b), but we did not investigate their morphology in detail. The outer receptor lymph cavity is extensive, occupying a large portion of the underlying cuticle (Figs. 8d, e).The inner receptor lymph space is unusual in that it greatly enlarges from the socket level to the proximal parts (Fig. 6d): (1) initially it is filled only with inner receptor lymph and loosely arranged dendrites (Fig. 6a); (2) Then, several microvilli appear invading the inner receptor lymph cavity interspersed with the dendrites (Fig. 6b); (3) microvilli increase in density and occupy a central part, while the dendrites occupy the periphery of the space when approaching the transition zone from outer to inner dendritic segment (Figs. 6c, d inset 1). The ciliary arrangement is a typical 9x2+0 (Figs. e-g). A flocculent material surrounds each cilium at this level (Figs. 6c, e-g); (4) below the basal body (Fig. 6i), the inner dendritic segments present several organelles (Figs. 6h, i), The membranes of the inner dendritic segments are then apposed to the innermost enveloping cell in the corners of the cavity, in a cross arrangement (Figs. 6h, j). Proximally, the innermost enveloping cell (E1) completely surrounds the inner dendritic segments (Fig. 6j). The innermost enveloping cell is possibly the source of the microvilli inside the inner receptor lymph cavity (Figs. d, j).

3.2.3 Ahs and Sb: cellular organization on the distitarsi I and II

Despite being only three hair sensilla externally emerging adjacent to each other, the internal components of these sensilla occupy a considerable space on the dorsal tissue of the last tarsomeres I and II and have a peculiar cellular disposition (Fig. 7). Since the apical-hood sensillum, the Sb1 and the Sb2 appear in sequence (distal to proximal, cross sections through the sagittal axis of the tarsi) (Figs. 7a-c), their sensory neurons achieve the transition to the inner dendritic segment and cell body in sequence along the longitudinal axis of the leg: (1) at the point where the six neurons of apical-hood sensillum have just passed the ciliary region (Figs. 3h; 7b; section plane d), the dendrites of Sb1 are entering the base of the shaft dorsally and already have a large outer receptor lymph (Fig. 7d, dotted area marks outer receptor lymph cavity); (2) then the inner dendritic segments of the apical-hood sensillum enlarge toward their cell bodies (ventrally), while the inner receptor lymph of the Sb1 begin to enlarge (Fig. 7b; section plane e). At this point, the dendrites of Sb2 are entering their shaft dorsal to the Sb1 and apical-hood sensillum (Fig. 7e). (3) When the dendrites of the apical hood sensillum reach their cell bodies and nuclei, the Sb1 have already become inner dendritic segments, and are at the corners of the very large

inner receptor lymph (7b; section plane f). The outer dendritic segments of Sb2 approach their ciliary region (Figs. 7f-i). The arrangement of the three sets of cells is so that the cell bodies of the apical-hood sensillum ventrally surround the inner dendritic segments of the Sb1, both groups of cells below the outer dendritic segments of the Sb2 (Figs. 7g-i). Therefore, the cells form a conspicuous bundle occupying a large portion of the dorsal tissue of the tarsomere (Fig. 7f; dotted square). The axons of those sensilla, as well as the adjacent sensilla, proceed to a dorsal nerve fiber, which starts to form around the end of the first third of the tarsomere (distal to proximal) (Figs 7a, j; asterisk marks the beginning of the nerve).

4. Discussion

We described the ultrastructure of the apical-hood sensillum and the sensillum basiconicum, two sensillar types that occur on the distal-most tarsomere I and II of the laniatorean harvestman *Heteromitobates discolor*. Below, we discuss general arguments supporting functional inferences for both types of sensilla, and specific arguments for each sensillar type. We also discuss the general cellular arrangement and innervation of these structures on the tarsi I and II.

4.1. Distribution and function

While in insects, crustaceans and myriapods the majority of sensillar structures occur on the antenna, in arachnids they are concentrated on the extremities of the legs (Altner and Prillinger, 1980; Foelix, 1985a). Harvestmen legs are mainly covered in sensilla chaetica (initially thought to be only gustatory/touch receptors), but also with sensilla of which function was unknown until recently, such as the falciform hairs (Willemart et al., 2009; Willemart and Giribet, 2010). The distal tarsomeres of the harvestman *H. discolor* (Gonyleptidae, Laniatores) have recently been studied with focus on the chemoreceptive tarsal sensilla and the characterization of sensillar types were further refined (Gainett et al., Chapter 1): gustatory/touch sensilla (sensilla chaetica tip-pored) are regularly spaced on the tarsomeres of all leg pairs; two types of olfactory sensilla, with two subtypes each, occur on pair of legs I and II (sensilla chaetica wall-pored and falciform hairs, largely absent on legs III and IV); and the apical-hood sensilla and sensilla basiconica, with a more restricted distribution (see below) (Fig. 9). The distal tarsomeres being well equipped with touch mechanoreceptors, gustatory and olfactory chemoreceptors weakens the idea that Ahs and Sb also have these functions, though not excluding it.

Another important point that weakens the idea that Ahs and Sb are mechano- or chemoreceptors is the internal morphology. Mechanoreceptive hair sensilla, (either detectors of touch, substrate-borne vibrations or airflow), always have at least one dendrite attached to the socket, forming structures called tubular bodies (McIver, 1985; Keil, 2012). In the sensilla we studied, however, there is no evidence of a mechanoreceptive unit at the socket and all dendrites observed leaving the cell bodies penetrate the shaft. Hair-like chemoreceptors typically show wall-pore structures (olfactory receptors) or tip-pores (gustatory receptors) (Altner and Prillinger, 1980; Zacharuk, 1985; Keil and Steinbrecht, 1984; Steinbrecht, 1997), but we observed no such structures in our ultrastructural investigation. One could argue that the pore-like structure on the tip of Ahs is a tip-pore connecting the dendrites to the exterior, a condition which would favor a contact chemoreceptive function (Altner and Prillinger, 1980; Zacharuk, 1985). However, serial-sectioning of this region does not reveal a clear connection between the pore and the bundle of dendrites, and show an electron-dense substance filling the narrow circular canal formed (Fig. 2a1-a6). This structure resembles a clogged molting pore, which is a scar in the outer surface that marks the point of connection between the old and new dendritic sheath after molting (Zacharuk et al., 1977; Zacharuk, 1985). Thus, we have no evidence at present to support a mechano or chemoreceptive functions for apical-hood sensilla.

A possible function for the apical-hood sensilla and sensilla basiconica could be as thermo-hygroreceptors. The external appearance of thermo/hygroreceptors is by far the most variable among all sensory modalities (Altner et al., 1983; Altner and Loftus, 1985; Steinbrecht, 1998), the most common feature being the absence of pore structures on the wall, which classifies them as non-pored sensilla (np-sensilla) (Altner and Prillinger, 1980; Altner et al., 1983). Although most described thermo-hygroreceptors are housed in np-sensilla, they have also been described in sensilla with wall pores (double-walled sensilla with spoke channels) (Foelix and Axtell, 1972; Altner et al., 1977; Altner et al., 1981; Rutchy et al., 2009) and even on small tip-pored pegs in the case of the tarsal organ of spiders (Foelix and Chu-Wang, 1973; Foelix, 1985a; Tichy and Loftus, 1996). Therefore, the fact that the apical-hood sensillum has a pore-like structure on the tip (Fig. 2a) (but see discussion above) and sensillum basiconicum has a long slit aperture (Fig. 5a) should not disfavor the thermo/hygroreceptive hypothesis. Long prominent shafts like that of the apical-hood sensillum are not the most common among thermo- hygroreceptors, which often have short and concealed shafts (Altner et al., 1983; Tichy and Loftus, 1996). However, long shafts have also been reported for some thermoreceptive sensilla in

beetles (e.g. *Notiophilus biguttatus*: Altner et al., 1983) and ticks (*Amblyomma variegatum*: Hess and Vlimant, 1983).

The thermo- hygroreceptive sensilla of insects occur mostly on the antennae, with usually more units on the distal segment (Altner et al., 1983; Altner and Loftus, 1985; but see Schmitz, 1997). In arachnids the only thermo/hygroreceptors confirmed with electrophysiology are located specifically on the distalmost tarsomere of the legs of spiders and ticks (Foelix and Axtell, 1972; Foelix and Chu-Wang, 1973; Hess and Loftus, 1984; Ehn and Tichy, 1994). The occurrence of thermo- hygroreceptors on a distal position of the appendages is arguably related to a demand of high exposure of these sensory modalities (Steinbrecht, 1989). Accordingly, the sensilla basiconica are restricted to the distal segments of the legs (metatarsi-tarsi junction and distalmost tarsomeres) and the apical-hood sensilla to the tip of pair of legs I and II. The very restricted distribution of the Ahs associated with a pair of Sb is interesting, because those legs are held as the sensory appendages of harvestmen, being constantly waved in the air or used to tap the surroundings (Willemart et al., 2009, and references therein). Thermo/hygroreceptive sensilla are usually the least abundant sensilla on the legs, occurring in relatively small numbers (Steinbrecht and Müller, 1976; Schaller, 1978; Altner and Loftus, 1985; Altner et al., 1978, 1981, 1983; Steinbrecht, 1989; Tichy and Loftus, 1996). We found 28 units of the sensilla basiconica in the whole body of *H. discolor*, and only 4 units in the case of the apical-hood sensillum (Results). These numbers are very small when compared with the high density of mechano- and chemoreceptors observed on the rest of the tarsomeres (Fig. 1d; Fig. 9) (Willemart and Gnaspini, 2003; Willemart et al., 2009; Gainett et al., Chapter I), and compatible with the numbers of thermo- hygroreceptors in arthropods (Steinbrecht and Müller, 1976; Schaller, 1978; Altner and Loftus, 1985; Altner et al., 1978, 1981, 1983; Foelix, 1985a; Steinbrecht, 1989; Tichy and Loftus, 1996).

Taken together, these evidences do not completely rule out that these sensilla may detect chemicals, but indicate that apical-hood sensilla and sensilla basiconica are good candidates for thermo-hygroreceptors. Below, we discuss other specific morphological characteristics of both sensilla types and their relation with the function hypothesis proposed.

4.2. Apical-hood sensillum

A sensillum is composed of sensory cells, at least three accessory cells, which secrete a dendritic sheath around the dendrites, and normally a cuticular apparatus (shaft and socket) (Altner and Prillinger, 1980; Keil and Steinbrecht 1984; Harris, 1977; Keil, 1997). The number of dendrites innervating a shaft varies depending on the sensory modality and taxonomic group, but normally one shaft is innervated by a single bundle of neurons with a dendritic sheath (Altner and Prillinger, 1980; Keil and Steinbrecht, 1984; Foelix, 1985a). The innervation of the Ahs is different from this pattern, because two dendritic sheaths penetrate the shaft, with three dendrites each (Figs. 2b-f). Therefore, it is a single shaft probably innervated by the fusion of two sensilla. The presence of two innervation bundles and the occasional observation of a second pore-like structure suggest that each bundle might be associated with one of these structures, which, as discussed above, are probably molting pores. Association of sensilla in a single shaft has also been observed in insects (Altner and Thies, 1978; Foelix et al., 1989). Most commonly, several hair sensilla can be clustered forming a sensory unit, or organ, with a similar function, such as the Haller's organ in ticks (Parasitiformes) and the tarsal organ in spiders (Foelix and Axtell, 1972; Foelix and Chu-Wang, 1973). The tarsal organ of the spider *Cupiennius salei*, for instance, is formed by the association of 8 nipple shaped tip-pored sensilla inside a pit on the distal segment of all leg pairs (Anton and Tichy, 1994), being shown to respond to temperature, humidity and chemical stimuli (Blumenthal, 1923; Dumpert, 1978; Ehn and Tichy, 1994). Although the pit is present in most spider groups (Blumenthal 1923), the nipple-shaped shafts may be exposed in various degrees (Platnick et al., 2012) and at least in some spiders of the genus *Amauropelma* (Ctenidae), and families Amaurobiidae and Gradungulidae the shafts can be fused in a single hair-like tarsal organ (Forster, 1980; Foelix, 1985a; Raven et al., 2001; Milledge 2011). The tarsal organ of the spiders *Otira* sp. (Amaurobiidae) and *Gradungula* sp. (Gradungulidae) are particularly similar to the external and internal structure of the apical-hood sensilla, possessing an elongated shaft that is innervated by 5 bundles of 3 dendrites, each one with a dendritic sheath (Fig. 11 in Foelix, 1985).

Along the middle of the shaft, both bundles of dendrites are laterally flattened along a vertical axis, and one of the dendrites in the larger bundle (B1) is folded (Figs. 2f, g), roughly resembling a lamellation. Lamellation in insects have first been described in np-sensilla and then electrophysiological evidence consistently assigned a thermoreceptive function to most of these sensilla (McIver, 1973;

Altner et al., 1978; Altner and Loftus, 1985; Haug, 1985; Foelix et al., 1989; Steinbrecht, 1989; Steinbrecht and Müller, 1976, 1991; Keil, 2012). Lamellation has also been observed in some odor (Lee et al 1985) and CO2 sensitive wall-pored sensilla (Keil, 1996; Stange and Stowe, 1999). However, the dendrite configuration in the Ahs differs from a typical lamellation in its small degree (but see Steinbrecht and Müller, 1976) and loose arrangement, besides the fact that it occurs only along a portion of the shaft. Therefore, we find problematic to interpret this feature at the moment.

The arthropod cuticle is composed of an outer thin procuticle and an inner procuticle (Nation, 2008). The wall of a sensillum´s shaft usually has a solid appearance, but may vary in having small channels and clefts (e. g. Steinbrecht and Müller, 1991; Talarico et al., 2005), different procuticular densities (e.g. Grünert and Ache, 1998), or in having specialized pore channels and spokes (wall-pored sensilla) (Altner and Prillinger, 1980; Keil and Steinbrecht, 1984; Steinbrecht, 1984, 1987, 1996, 1997). We observed no pore systems in the wall, but small tubular structures occur between the epicuticle and procuticle, which apparently do not connect the lumen with the outside (Figs. 4g a, e). A conspicuous feature of the Ahs revealed with the sectioning is the meshed structure of the procuticle (Fig. 4) covered by a highly plicate epicuticle (Figs. 4a, b, f, g), which is unlike any other shaft studied in the tarsi (Gainett et al Chapter I). We speculate that this complex structure might confer tenacity, and thus allow bending without breaking, in a way similar to a braided gas hose. Glandular sensilla in harvestmen, which are brushed against prey to glue them, have been recently hypothesized to be more flexible due to the presence of parallel annelations along the shaft (Wolff et al., 2016). In a similar way, the meshed procuticle and plicate epicuticle of the Ahs could be adaptations to protect a sensory structure which is not abundant (4 units) and exposed to mechanical damaging on the tip of tarsi I and II.

In sum, the morphological characteristics of the apical-hood sensillum are unique and of difficult interpretation; therefore precise functional inference is problematic. We presented arguments in favor of hygro-, thermoreception, but its function remains to be discovered by future morphological and electrophysiological studies.

4.3. Sensilla basiconica

Sensillum basiconicum has 3 to 4 neurons innervating the shaft, but only 2 dendrites penetrate the region between the slits, appearing compressed by the electron-dense lymph and cuticle (Figs. 5f-h),

while the other dendrites stop at the base of the slit (Figs. 5i-k; 8). This cellular arrangement is similar to what has been observed in hygro- and thermoreceptors in other arthropods: normally there is a triad of cells, including a moist and a dry receptor tightly packed inside the shaft (hygroreceptors) and one cold receptor ending at the socket (thermoreceptors) (Foelix and Axtell, 1972; Altner et al., 1978, 1981; Yokohari, 1981, 1983; Yokohari et al., 1982; Tominaga and Yokohari, 1982; Hess and Vlimant, 1983; Steinbrecht, 1984, 1998; Haug, 1985; Steinbrecht and Muller, 1976, 1991; Altner and Loftus, 1985; Tichy and Loftus, 1996; Rebora et al., 2008; Rutchy et al., 2009). The number of neurons (3) may slightly vary to up to 5 in some insect species (Steinbrecht, 1998) and sometimes across sensilla in an individual (e. g. Lee et al 1985). Because we were not interested in intra-specific variation and therefore did not analyze many individuals, it is not possible to say if the variation observed in the number of dendrites of Sb1 and Sb2 is consistent or due to individual variability. The main differences between Sb and a typical thermo-hygroreceptive sensilla is that the presumable thermoreceptive dendrites penetrate the shaft to the transition from the tube-segment to the slit-segment (Figs. 5f, i) and the comparable hygroreceptive units are only tightly packed along the slit-segment. However, we argue that these differences in innervation and shaft morphology are possibly related to the transduction mechanisms involved in hygroreception (below).

4.3.1. Models of hygroreception for sensillum basiconicum

The shaft of sensillum basiconicum is sagittaly divided in two flaps (Figs. 5a-c), which are held together by an electron-dense substance in one face (Fig. 5g) and a very thin membrane on the other (Fig. 5f, h; arrow; Fig.8). This arrangement is so that inner receptor lymph bathing the dendrites is exposed along the slits, while the outer lymph is isolated in this region by the dendritic sheath (Figs. 5f-h, Fig. 8). Therefore, evaporation of lymph possibly takes place along the slit-segment (inner lymph) and apically on the tip (inner and/or outer lymph) (Fig. 8). Accordingly, we observed in all samples an extruded material adjacent to the slit with the thin membrane (Fig. 5f), and also on the tip of the shaft (Fig. 5e). Also, there is a very clear gradient of increasing electron-density in the outer receptor lymph, which is lucid in the proximal parts of the sensilla and achieves the darkest tone on the distal-most tip of the shaft (Fig. 5d). The outer receptor lymph is produced by the auxiliary cells below the cuticle (Altner and Prillinger, 1980; Keil and Steinbrecht, 1984) and the gradient is probably explained by diluted lymph in the internal parts and a local higher concentration of substances on the tip maybe due to apical evaporation. Evaporation is a key feature in some transduction models of hygroreception in arthropods

(Tichy and Loftus, 1996; Steinbrecht, 1998). Taken together with innervation pattern, distribution and relative small numbers of sensilla basiconica, the occurrence of an evaporative mechanism supports a thermo- hygroreceptive function for this sensillar type.

Three main transduction models of hygroreception have been proposed for arthropods (after Tichy and Loftus, 1996): (1) the mechanical hygrometer (MH) model assumes that changes in humidity causes volume changes on the hygroscopic cuticle of the shaft, which compresses mechanosensitive dendrites, generating stimuli; (2) the evaporimeter (EM) model, which assumes that humidity affects evaporation rate of receptor lymph and that the resulting change in lymph concentration is perceived by chemosensitive dendrites; and (3) the psychrometer (PY) model, in which temperature depression caused by evaporation is used to measure humidity by thermosensitive dendrites. Properties of the cuticle are involved in MH model, while evaporation is required in the EH and PY models. Below, we discuss in sequence how the morphology of sensillum basiconicum conforms to each model.

Under the mechanical hygrometer model, air dryness would cause flaps to hygroscopically shrink, decreasing internal pressure in the slit-segment, causing widening of the dendritic sheath profile and decompression of the dendrites. This model has traditionally been favored in insect hygroreceptors (Yokohari, 1978, Itoh et al., 1984; Haug, 1985), but direct observations supporting this model are lacking in the literature. Attempts to assess hygroscopic properties of the cuticle and to quantify the effect of mechanical forces on hygroreceptive sensilla of insects did not find support for the model (Resch et al., 1998; Tichy and Kallina, 2010, 2013).

Under the evaporimeter model, dryness would cause outer and/or inner lymph to evaporate, changing the concentration of substances in the lymph, which would be perceived by chemosensitive dendrites. The electron-density gradient in the outer receptor lymph observed demonstrates that at least a differential lymph concentration in the slit-segment exists (Fig. 5d; Fig. 8), which fits this model. The evaporimeter model has received less attention in the literature (Steinbrecht, 1998), only recently receiving some support in the cockroach hygroreceptor (Tichy and Kallina, 2013).

Under the psychrometer model, one of the dendrites inside the slit-segment would measure the real temperature ("dry cell") and the other would measure the temperature depression due to evaporation of outer and/or inner receptor lymph. The psychrometer model has received support from a

morphological study with the silkmoth (Steinbrecht and Müller, 1991) and more recently from studies exploring humidity parameters in the cockroach (Tichy and Kallina, 2010, 2013).

An alternative model could be mechanical perception of evaporation rate by changes in the shaft conformation directly caused by evaporation, and not by hygroscopic uptake of water. The two flaps of the shaft in some air-dried SEM samples were occasionally wide-opened (not shown), revealing that there is some mobility of the flaps. Therefore, evaporation of inner and/or outer lymph could cause slight opening and closing of the flaps. Although the flaps are sealed in the live animal, a small approach of the two flaps could increase the pressure of the cavity, mechanically narrowing the dendritic sheath and the two dendrites. If this transduction mechanism is correct, we predict that in dry conditions (higher evaporation) the volume of liquid in the apical portion of the shaft will be smaller, the flaps will be tightly apposed against each other (by forces such as capillarity) and the dendritic sheath and dendrites will display a narrower profile. It is worth noting that these morphological changes are exactly the opposite of what would be predicted under the mechanical hygrometer model (see above). Testing the opposing models could be done with freeze-fixation of dry and moist adapted animals, with subsequent sectioning and TEM analysis. A similar experiment has been performed with the thermo/hygroreceptive sensilla of the silkmoth *Bombyx mori*, in which it was demonstrated that dryness causes a reversible increase in the size of the lumen around the dendrites and retraction of the dendrites (Steinbrecht and Müller, 1991).

In the models involving evaporation, especially the evaporimeter model, the lymph is kept within controlled limits, in constant unidirectional flow to the outside. Otherwise, keeping a viable lymph concentration would be a problem (Tichy and Loftus, 1994). Keeping evaporation low has been proposed to occur by keeping thin layers of lymph around the dendrites and by accumulating substances in the evaporation area (Tichy and Loftus, 1994). In the sensillum basiconicum of *H. discolor*, the presumable evaporation surface along the slits and tip is extensive (Fig. 8), which probably implies in high evaporation rates. An alternative way to compensate the high lymph flow would be increasing the source of receptor lymph. In line with this idea, the outer receptor lymph of sensillum basiconicum occupies a large portion of the distal part of the distal tarsomeres I and II, despite having the smallest shaft of all tarsal sensilla types (Figs. 7d, e, dotted lines show outer lymph cavity). Moreover the inner receptor lymph cavity is unusually large and expands below the transition from outer to inner dendritic segment (Figs. 6d, j; Figs. 7f-i; Fig. 8). This morphological observation suggests that this sensillum has

relatively more outer and inner receptor lymph than other tarsal sensilla. Similarly, quantitative measurements of the auxiliary cells of the silkmoth hygro/thermoreceptor showed that the volume of tormogen and thecogen cells is larger in sensilla styloconica (hygro/thermoreceptor) than in olfactory sensilla (sensilla trichodea) (Steinbrecht et al., 1989). Moreover, these authors showed that cell membrane of the inner-most enveloping cell (thecogen) is more than 100 times larger than of an olfactory sensilla and that the volume of inner receptor lymph is more than 30 times larger (Steinbrecht et al., 1989). Therefore, having large inner receptor lymph volume may be a characteristic of hygro/thermoreceptors, which we argue could be related to the evaporative transduction mechanism. Comparatively measuring the volume of sensillum basiconicum and other sensillar types in *H. discolor* and studying in detail the secretory activity of the accessory cells could provide further insights into this subject. For instance, a prediction of this relationship is that species living in habitats with different dehydration stresses should present differences in receptor lymph volume and/or rate of lymph secretion. Therefore, species like *H. discolor*, which commonly rests under rocks in small streams during the day (very high humidity, low dehydration stress) and forages in the forest during the night (Silva and Willemart, 2016), may have sensilla basiconica with smaller lymph volumes and/or lymph secretion rates than that of harvestmen species living in drier environments.

4.4. Innervation and cellular association

As a rule for arthropods, synapses of the sensillar neurons occur directly on the central nervous system (Chapman, 1998). Still, contacts between the sensory cells, including hygro/thermoreceptors, have been observed in insects, and it was hypothesized that peripheral interactions between them might be relevant for the stimulus uptake (Steinbrecht, 1989 and references therein). Arachnids are remarkable among arthropods in this respect, in that some groups present regular peripheral synapses between the sensory cells of tarsal sensilla (Foelix 1985b). This has been observed on the legs of Uropygi, Scorpiones, Amblypygi and Opiliones (Foelix 1975, 1985b; Foelix & Troyer 1980; Foelix et al 2002). In the triad Ahs+Sb of the harvestman here studied, the inner dendritic segments of sensillum basiconicum are in direct contact with each other at some points of the “cross” configuration (Fig. 6j, asterisk) and the bundle organization of the sensory cells of the apical-hood sensillum and sensilla basiconica (Figs. 7 g-i) implies that some points of contact between cell body/inner dendritic segment/axons of Ahs, Sb1 and

Sb2 might exist. Therefore, it would be interesting to study in detail the organization of the sensory cells of Ahs and Sb , as well as fully resolving the disposition of their enveloping cells.

Conclusion

We described a new sensillum type, the apical-hood sensillum, and provided the first morphological evidence of hygro- thermoreceptors in a harvestman. Apical-hood sensillum and sensillum basiconicum are restricted to the distal parts of the legs, the apical-hood sensillum being restricted to the sensory appendages (legs I and II). Although our data provides only indirect evidences of function, it has raised testable hypothesis which can now be interpreted in light of the ultrastructural data. It would be of interest to test the hygro- thermoreceptive function of these sensillar types both with electrophysiology and behavioral essays. Studying the putative hygroreceptive transduction mechanisms of sensillum basiconicum has the potential to provide insights into the mechanisms of hygroreception in arachnids, a field which has received relatively little attention in the literature.

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FIGURES

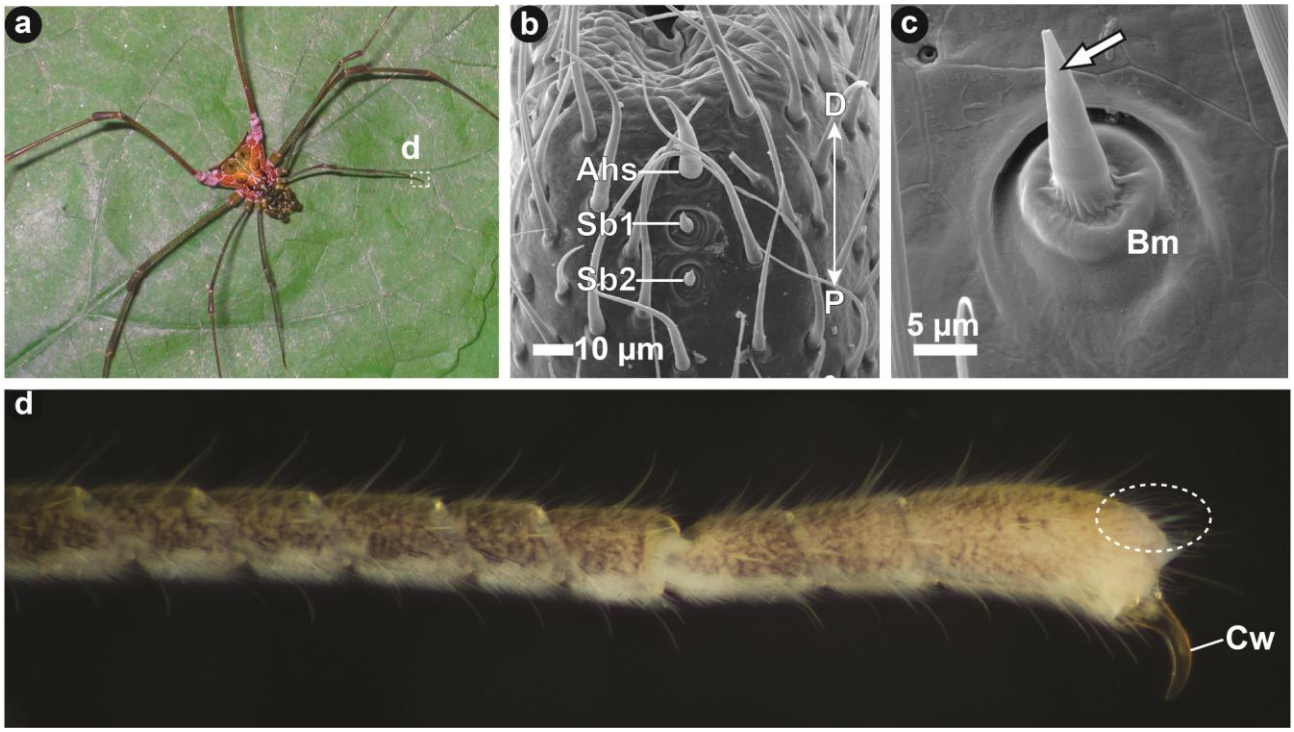
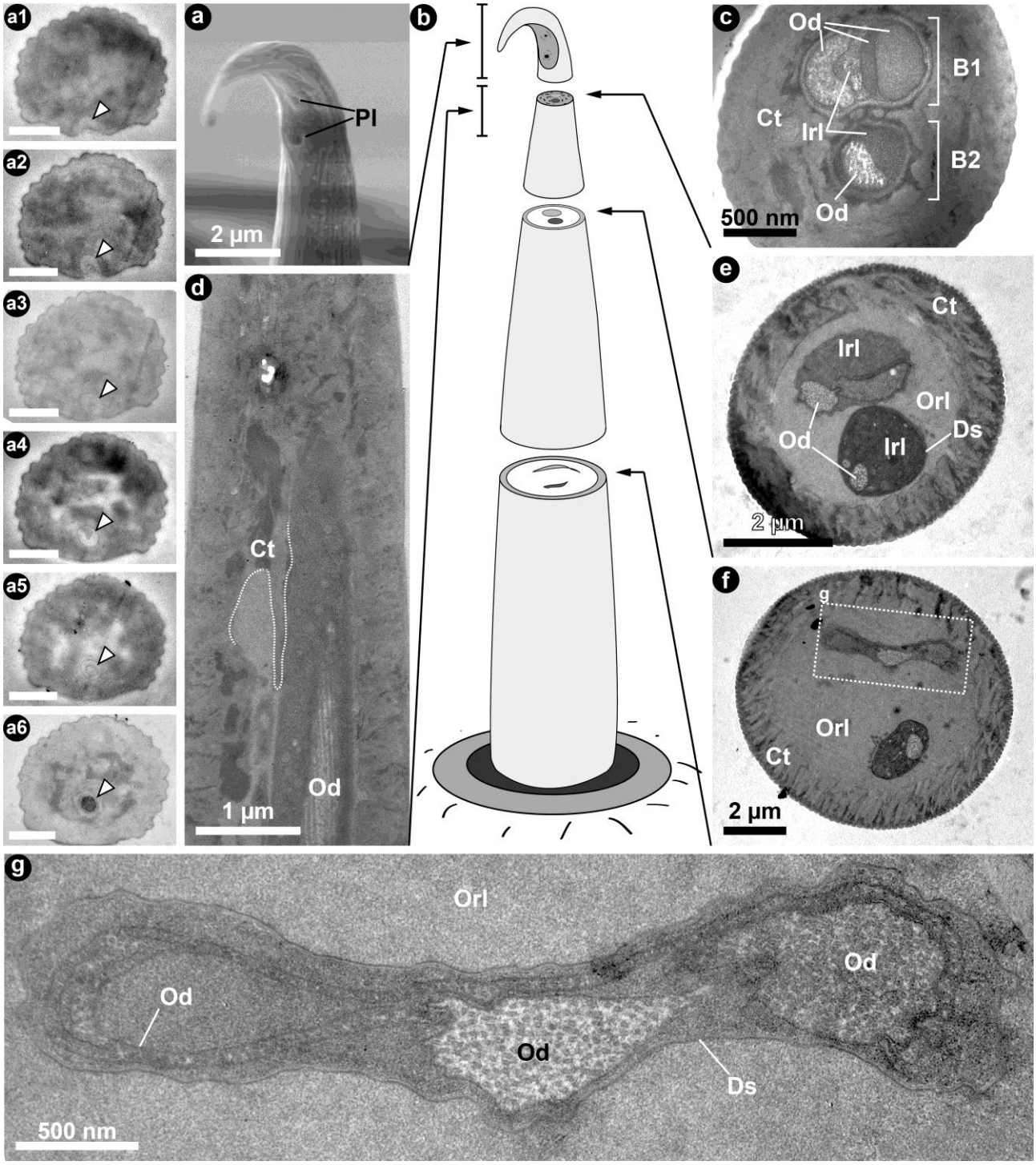


Figure 1: Apical hood sensilla (Ahs) and sensilla basiconica (Sb) on the legs of *Heteromitobates discolor*. a: Adult female *Heteromitobates discolor*. b: Superior view of the distal third of the distal-most tarsomere I (female), with an apical-hood sensillum, and two sensilla basiconica. c: Detail of an isolated sensillum basiconicum on the lateral region of the distalmost tarsomere IV (male). d: Automontage photograph of the distal tarsomere I (female). Dotted circle shows the region where Ahs and Sb occur. Ahs: apical-hood sensillum; Bm: Basal membrane; Cw: Claw; Sb1: Distal sensillum basiconicum; Sb2: proximal sensillum basiconicum; White arrow: slit openning.


a1
a2
a3
a4
a5
a6
a
Pl
2 µm
b
c
Od
B1
Ct
Irl
B2
Od
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d
Ct
Od
1 µm
e
Ct
Irl
Orl
Od
Irl
Ds
2 µm
f
g
Orl
Ct
2 µm
g
Orl
Od
Od
Od
Ds
500 nm

Figure 2: Apical-hood sensillum (Ahs), shaft ultrastructure. External morphology in scanning electron microscopy (a) and internal morphology in transmission electron microscopy (a1-a6, c-g). a: Apical portion of Ahs (hood), showing the smooth surface with two pore-like structures. a1-a6: Serial cross sectioning through the hood. Note the formation of the pore-like structure (arrowhead), which ends in a electron-dense substance. Scale bars: 500 nm. b: Schematic representation of the external appearance of the shaft, indicating correspondence with figures a, c-f. c: Cross section immediately bellow the hood, showing the two dendritic bundles, B1 and B2. Note the absence of outer receptor lymph. d: Longitudinal section immediately bellow the hood, at the point where the two bundles enter the outer-receptor-lymph-free region of the shaft. e,f: Cross sections toward the base. e: Distal part of the shaft. f: Middle part, at a point where the larger bundle (B1) is flattened and dendrites fold. g: Detail of B1 shown in "f". Note folding of outer dendritic segments. B1: larger bundle of dendrites; B2: smaller bundle of dendrites; Ct: cuticle; Ds: dendritic sheat; Irl: inner receptor lymph; Od: outer dendritic segment; Orl: outer receptor lymph; Pl: pore-like structure.

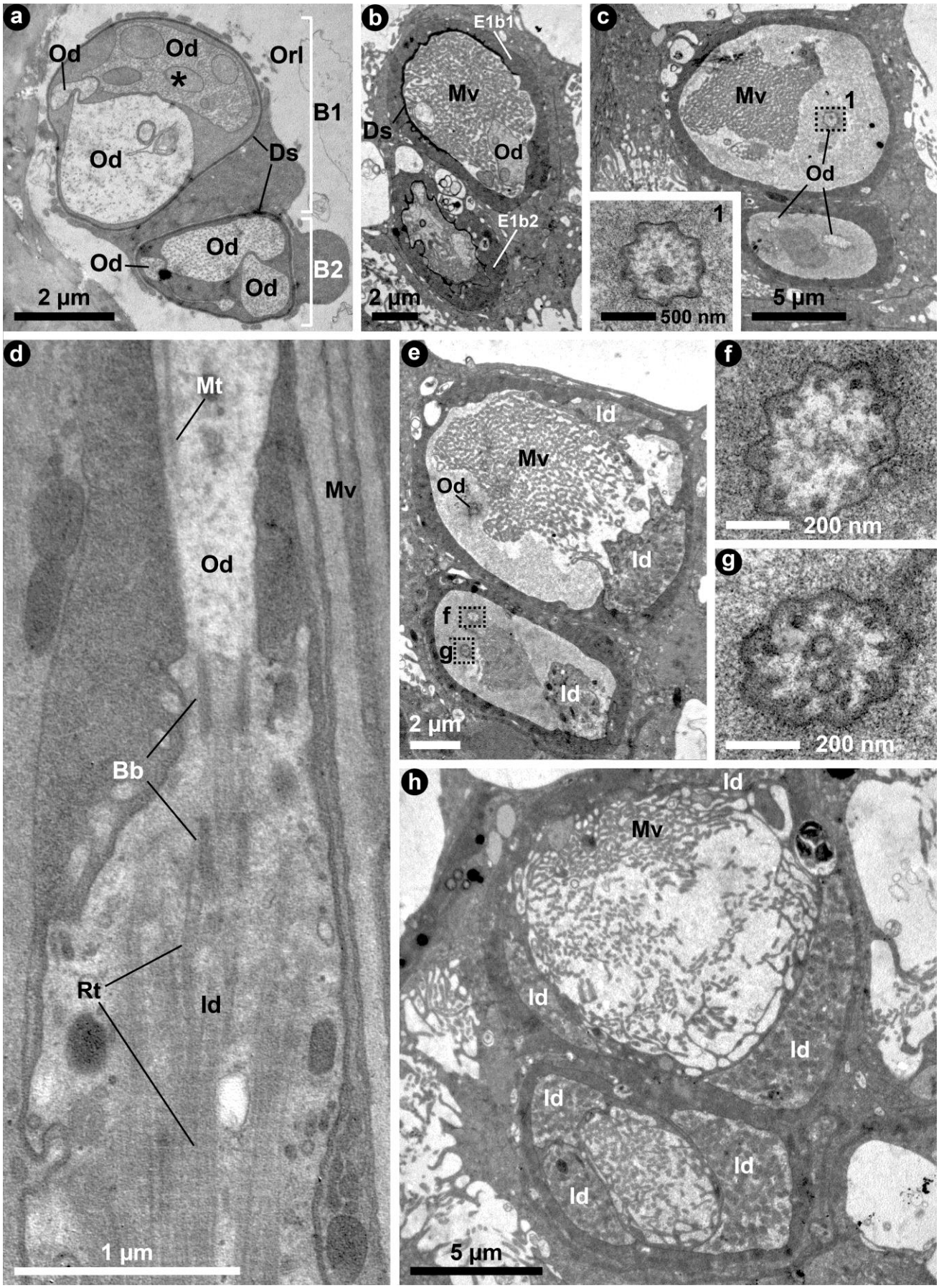

a
Od
Od
Orl
*
B1
Od
Ds
Od
Od
B2
Od
2 µm
b
E1b1
Mv
Ds
Od
E1b2
2 µm
c
Mv
1
Od
1
500 nm
5 µm
d
Mt
Mv
Od
Bb
Rt
Id
1 µm
e
Id
Mv
Od
Id
f
g
Id
2 µm
f
200 nm
g
200 nm
h
Id
Mv
Id
Id
Id
Id
Id
5 µm

Figure 3: Apical-hood sensillum, ultrastructure of inner parts in transmission electron microscopy. a-c, e-h: Serial cross sections (distal to proximal) of the two bundles of dendrites, B1 (upper) and B2 (lower). a: Slightly below the insertion of the shaft. Bundles are not yet fully envolved by envelopping cells. Note that one of the outer dendritc segments (Od) of B1 have round vesicles (asterisk). b: Bundles are surronded by individual inner enveloping cell, and gradually loose dendritic sheath (Ds). Not numerous microvilli (Mv) c: At the ciliary transition. Insight: outer dendictic segment immediately before transition to inner dendritic segment. d: Longitudinal section of a dendrite at the transition from inner to outer dendritic segment. Note the two basal bodies and ciliary rootlets. e: At the ciliary transition. Note that some dendrites achieved inner dendritic segments (Id). f,g: Ciliary profile of two dendrites in B2, showing 9 periferal microtubules (doublets distinguishble), Note that "g" has two central ring elements. H: Section through the bundles, with inner dendritic segments at the periphery of the inner cavity. Bb: basal body; B1: larger bundle of dendrites; B2: smaller bundle of dendrites; Ds: dendritic sheat; E1b1: inner enveloping cell of B1; E1b2: inner enveloping cell of B2; Id; Inner dendritic segment; Mv: microvilli; Mt: microtubule; Od: outer dendritic segment; Orl: outer receptor lymph; Rt: ciliary rootlets.

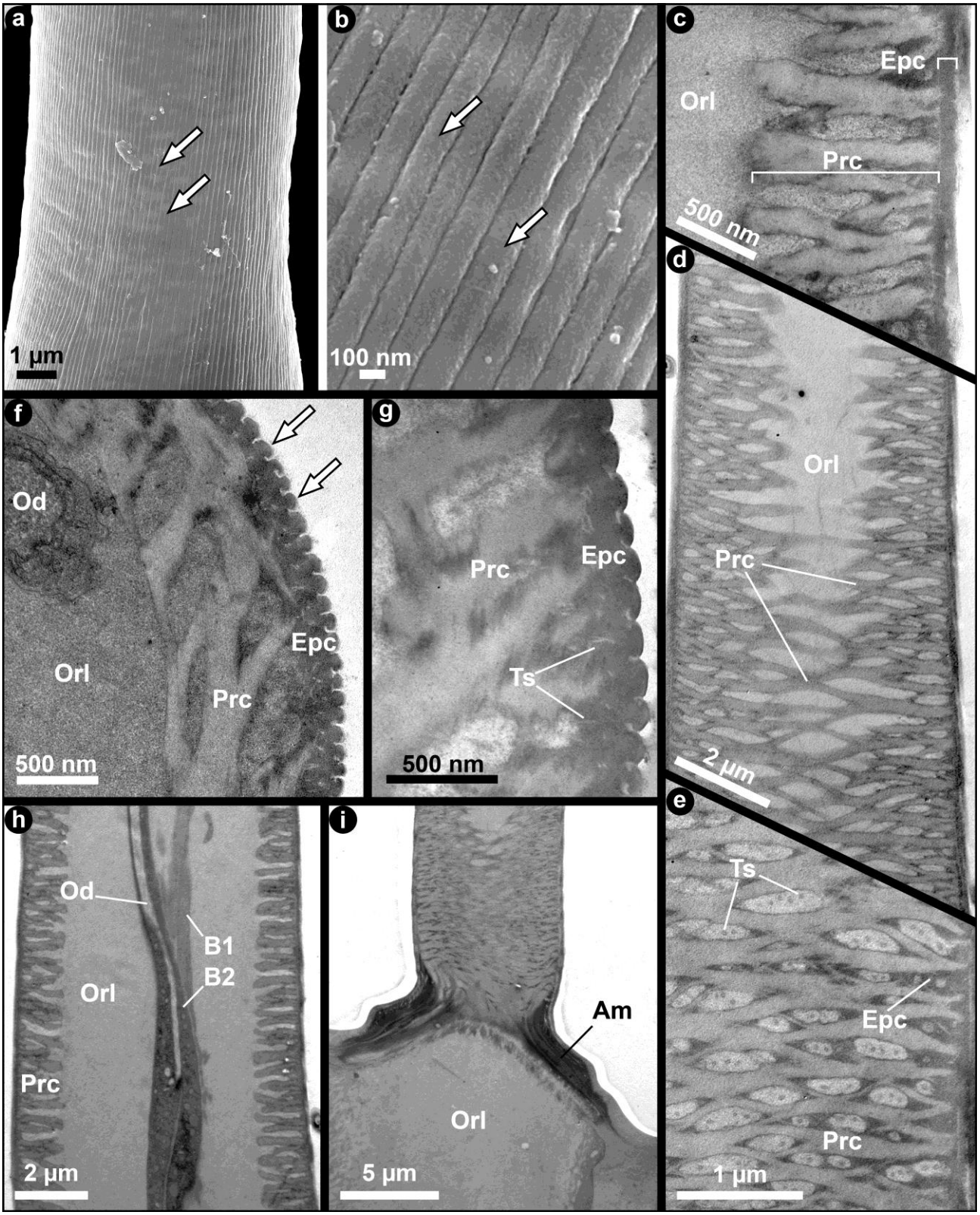
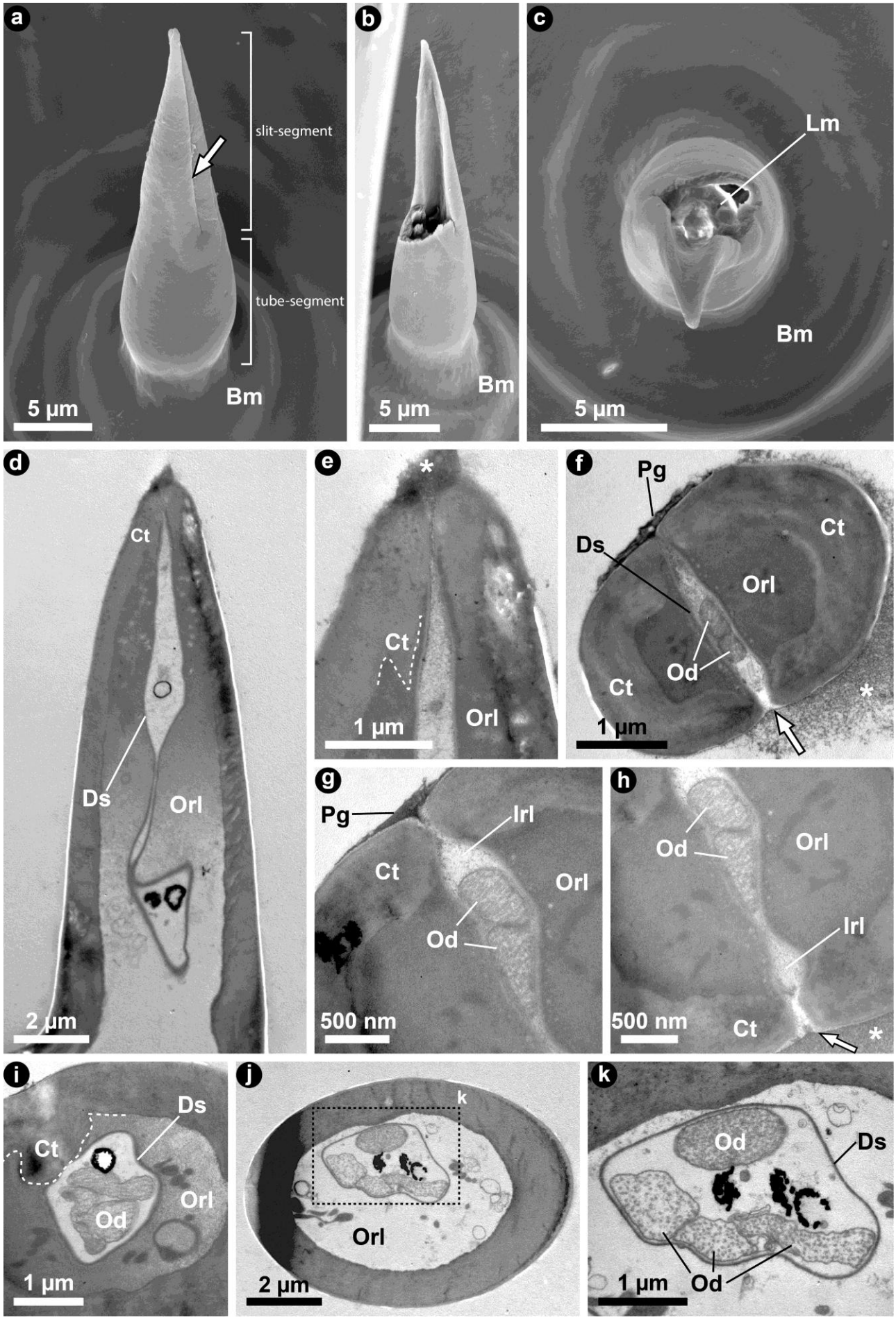

a
1 µm
b
100 nm
c
Epc
Orl
Prc
500 nm
d
Orl
Prc
2 µm
e
Ts
Epc
Prc
1 µm
f
Od
Orl
Epc
Prc
500 nm
g
Prc
Epc
Ts
500 nm
h
Od
B1
B2
Orl
Prc
2 µm
i
Am
Orl
5 µm

Figure 4: Apical-hood sensillum, ultrastructure of the shaft´s wall. External morphology in scanning electron microscopy (a, b) and internal morphology in transmission electron microscopy (b-i). a: Mid shaft. b: Detail of the mid shaft showns in “a”. c-e: Longitudinal sections. c: Sagittal section of the shaft in high magnification, showing part of the lumen (Orl) and wall. D: Oblique section through the mid shaft, from outer part (lower) to central part of the shaft, towards the lumen (upper). Note the “gradual formation” of the meshed structure. e: Higher magnification of the edge of the wall and enviroment. Note the presence of epicuticular spots and small tubular structures (Ts) inside procuticular clefts. f, g: Cross sections. f: Quarter of the mid shaft section shown in “Fig. 2f”. g: Detail of the wall, showing tubular structures (Ts) between cuticular layers. h,i: Longidutinal sections. h: Sagital section of the mid shaft. Note the two bundles of dendrites; i: Basal part of the shaft and insertion on the tegument. Arrow: external ridge; Am: articulation membrane; B1: larger bundle of dendrites; B2: smaller bundle of dendrites; Epc:epicuticle; Od: outer dendritic segment; Orl: outer receptor lymph; Prc: procuticle; Ts: tubular structure.


a
slit-segment
tube-segment
Bm
5 µm
b
Bm
5 µm
c
Lm
Bm
5 µm
d
Ct
Ds
Orl
2 µm
e
*
Ct
Orl
1 µm
f
Pg
Ds
Ct
Orl
Od
Ct
*
1 µm
g
Pg
Irl
Ct
Orl
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h
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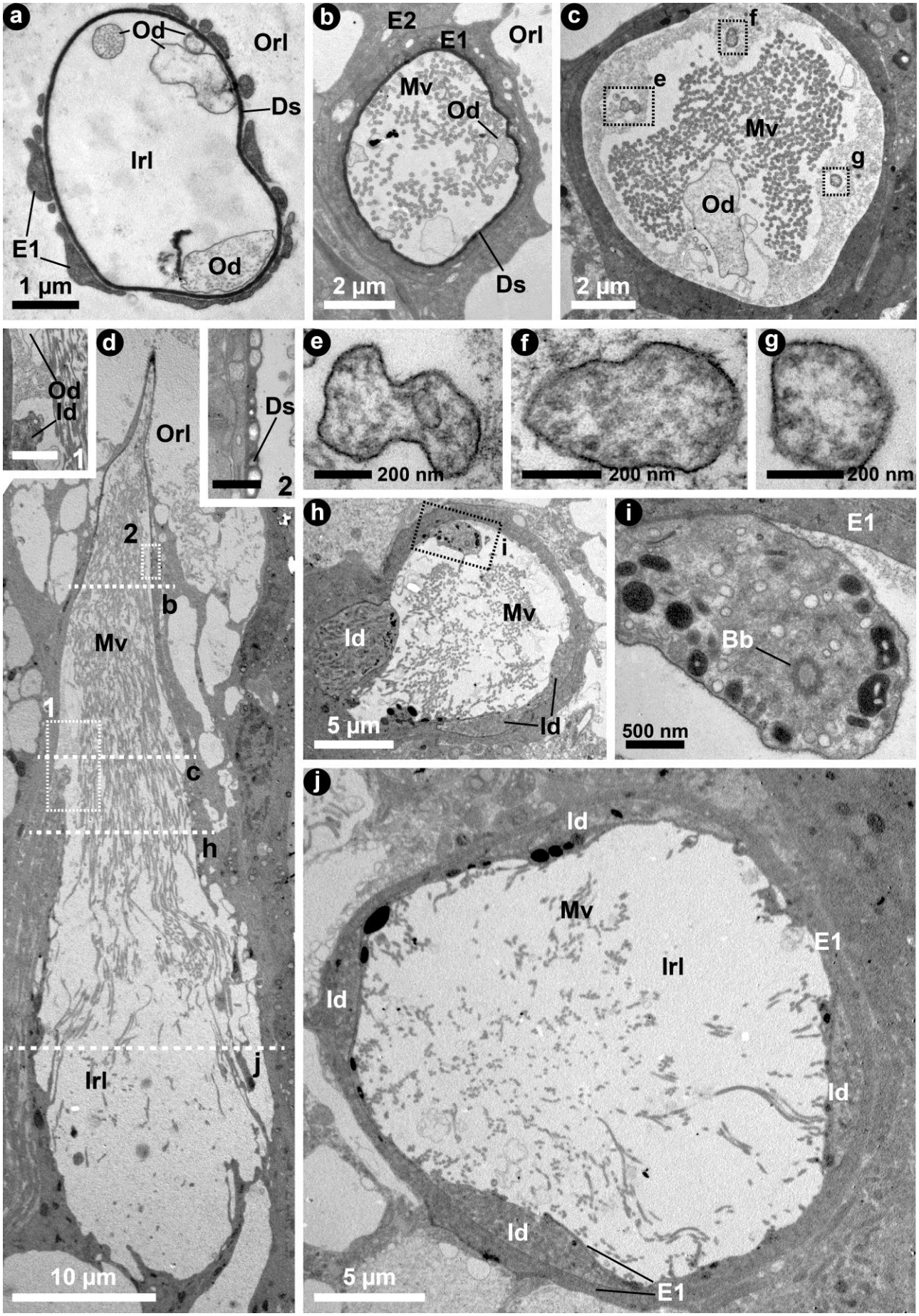
Orl
Irl
Ct
*
500 nm
i
Ds
Ct
Od
Orl
1 µm
j
k
Orl
2 µm
k
Od
Ds
Od
1 µm

Figure 5: Sensilla basiconica on the distal-most tarsomere of pair of legs I (a, d-k) and II (b,c), ultrastructure of the shaft. External morphology in scanning electron microscopy (a-c) and internal morphology in transmission electron microscopy (d-k). a: Distal sensilla basiconicum (Sb2) in lateral view. Note the longitudinal slit (arrow) and two flaps. b: Proximal sensillum basiconicum (Sb1) in lateral view, with one of the flaps removed. c: Same shaft as in "b" superior view. d: Sagittal section through the shaft of Sb2. Note the electron-density gradient in the longitudinal axis. e: Detail of the tip in "d". Note extruding lymph on the tip (asterisk). f-k: Cross section of the shaft, outer to inner parts. f: Slit-segment of the shaft. g: Detail of the slit pluged by a dark substance (Pg). h: Same section as "g", showing detail of the opposite slit opening (arrow). i: At the transition from slit-sement to the tube-segment. Note the contact between the cuticular wall (Ct) and dendritic sheath (Ds). j: Tube segment slightly above insertion on the tegument. k: Detail of dotted area in "j". Note four outer dendritic segments (Od). Arrow: slit; Asterisk: extruded lymph; Bm: basal membrane; Ct: cuticle; Ds: dendritic sheat; Irl: inner receptor lymph; Lm: lumen; Od: outer dendritic segment; Orl: outer receptor lymph; Pg: plug.


a
Od
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E1
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Mv
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Od
2 µm
Od
Id
1
d
Orl
Ds
2
e
200 nm
f
200 nm
g
200 nm
2
b
Mv
1
c
h
Irl
j
10 µm
h
i
Mv
Id
Id
5 µm
i
E1
Bb
500 nm
j
Id
Mv
E1
Irl
Id
Id
Id
5 µm
E1

Figure 6: Sensilla basiconica, ultrastructure of inner parts in transmission electron microscopy. a-c, e-j: Serial cross sections from outer to inner parts, same series as in “Fig 5”. a: Slightly below the insertion of the shaft; b: Envelopping cells (E1, E2) surround the dendritic sheath (Ds). c: At the ciliary transition, with three outer dendritic segments immediately before achieving inner dendritic segment (e-g). d: Longitudinal section of inner parts. See dotted lines for correspondences to “b,c,h” and “j”, and “Fig. 8” for general orientation. Insight 1: transition from outer (Od) to inner dendritic segment (Id). Insight 2: Electron lucid vesicle on the dendritic sheath (Ds). e-g: Detail of the cilia constriction at the transition. See dotted areas in “c” for correspondence. h: The four inner dendritic segments (Id) at the periphery of the expanding inner cavity. i: Detail of the dendrite in “h”, showing a basal body (Bb). j: Section of a level with large expansion from the inner receptor lymph cavity (irl), with dendrites at the periphery. Bb: basal body; Ds: dendritic sheat; E1, E2: enveloping cells; Id; Inner dendritic segment; Irl: inner receptor lymph; Mv: microvilli; Od: outer dendritic segment; Orl: outer receptor lymph.

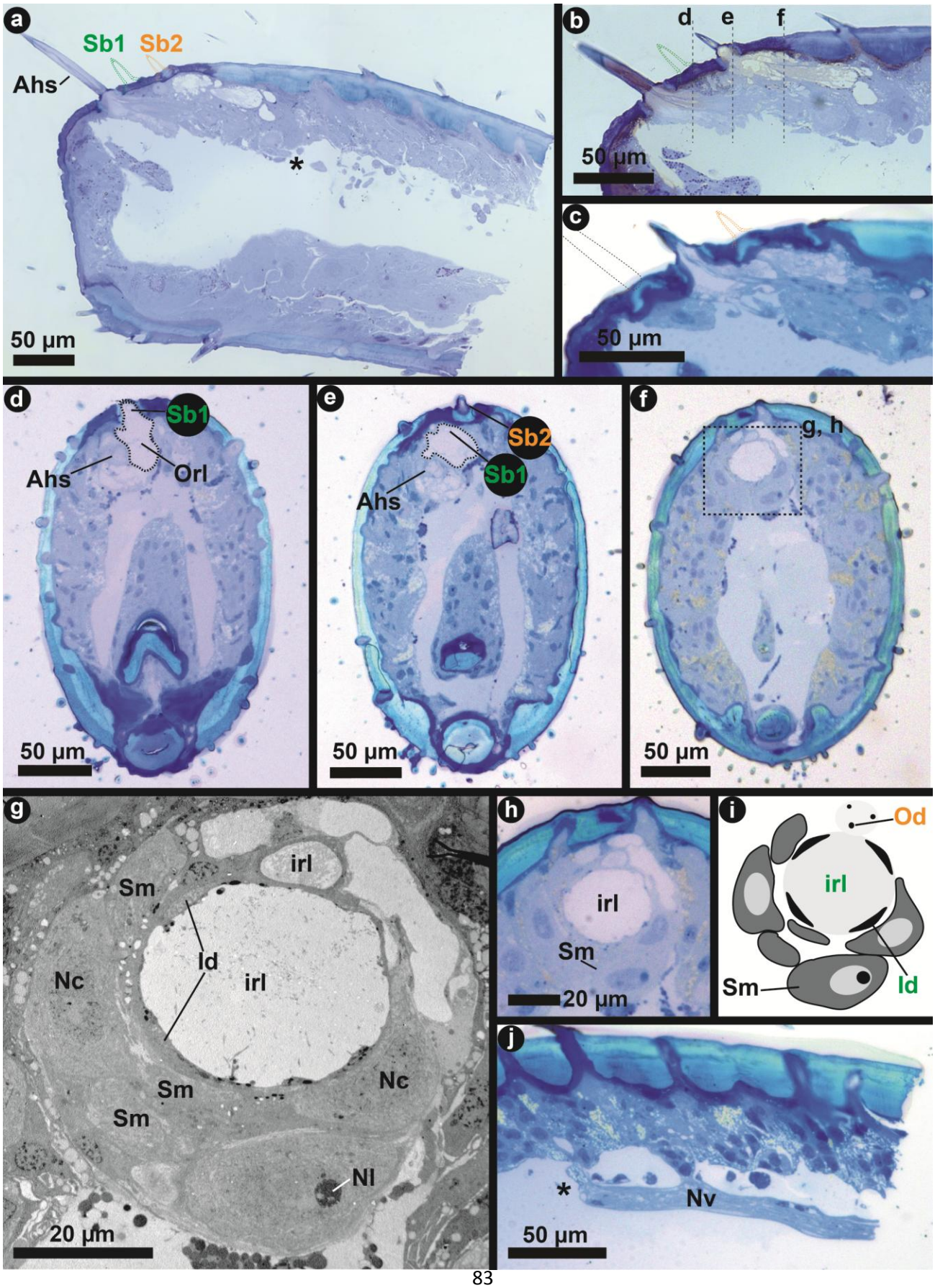
a
Sb1
Sb2
Ahs
*
50 µm
b
d
e
f
50 µm
c
50 µm
d
Sb1
Ahs
Orl
50 µm
e
Sb2
Sb1
Ahs
50 µm
f
g, h
50 µm
g
irl
Sm
Id
irl
Nc
Sm
Nc
Sm
Nl
20 µm
h
irl
Sm
20 µm
i
Od
irl
Sm
Id
j
*
Nv
50 µm

Figure 7: Cellular arrangement of apical-hood sensillum (Ahs) and Sensillum basiconicum (Sb) on the distal tarsomere I, in light microscopy (a-f, h, j) and transmission electron microscopy (g). a-c: Serial sections. a: Sagittal section of the distal-most tarsomere. Sb1 (green) and Sb2 (orange) are out of section plane b: Detail of the distal third of the dorsal region, with Ahs and Sb2. Sb1 (green) is out of section plane. c: Detail of the same region in “b”, showing Sb1. Ahs (black) and Sb2 (orange) are out of the section plane. d-f: Cross sections of the tarsomere, from distal to proximal. See dotted lines in “b” for correspondence. Ahs, Sb1 and Sb2 indicate position of the dendrites. g: Bundle of sensory cells formed by the somata (Sm) of Ahs, inner dendritic segments (Id) of Sb1 and outer dendritic segments (Od) of Sb2. See dotted area in “f”for correspondence. h: Same as in “g”, but in light microscopy. i: Schematic representation of the bundle of cells in “g”and “h”. Black, green end orange colors correspond, respectively, to Ahs, Sb1 and Sb2. j: Dorsal cellular tissue of the tarsomere, showing nerve (asterisk). Id; Inner dendritic segment; Irl: inner receptor lymph; Od: outer dendritic segment; Orl: outer receptor lymph.

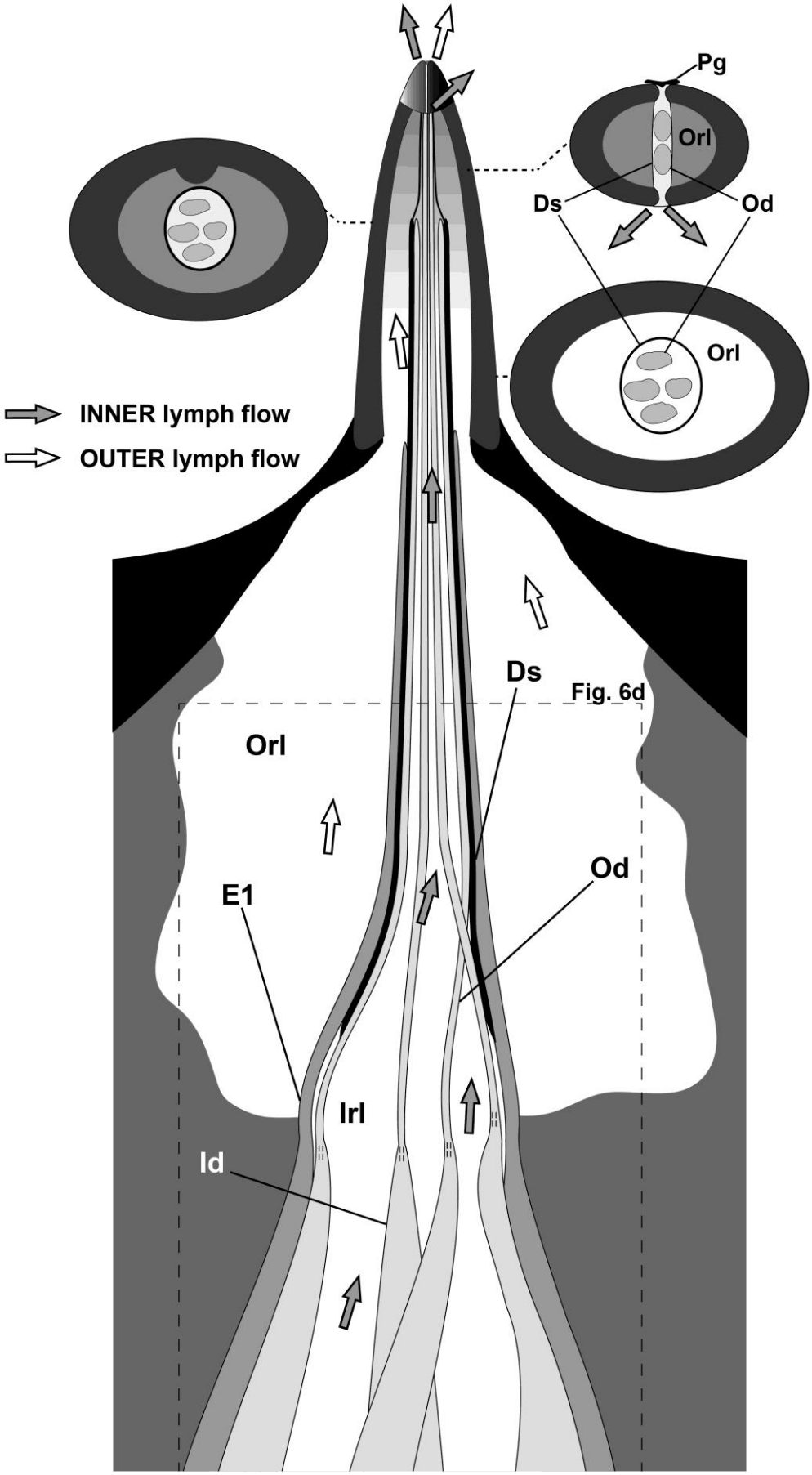
Pg
Orl
Ds
Od
Orl
INNER lymph flow
OUTER lymph flow
Ds
Fig. 6d
Orl
Od
E1
Irl
Id

Figure 8: Schematic representation of a longitudinal section of sensillum basiconicum, with correspondent cross sections of the shaft. and presumable routes of evaporation of inner (grey arrows) and outer receptor lymph (white arrows). Ds: dendritic sheat; E1: inner enveloping cell; Id; Inner dendritic segment; Irl: inner receptor lymph; Od: outer dendritic segment; Orl: outer receptor lymph; Pg: plug.

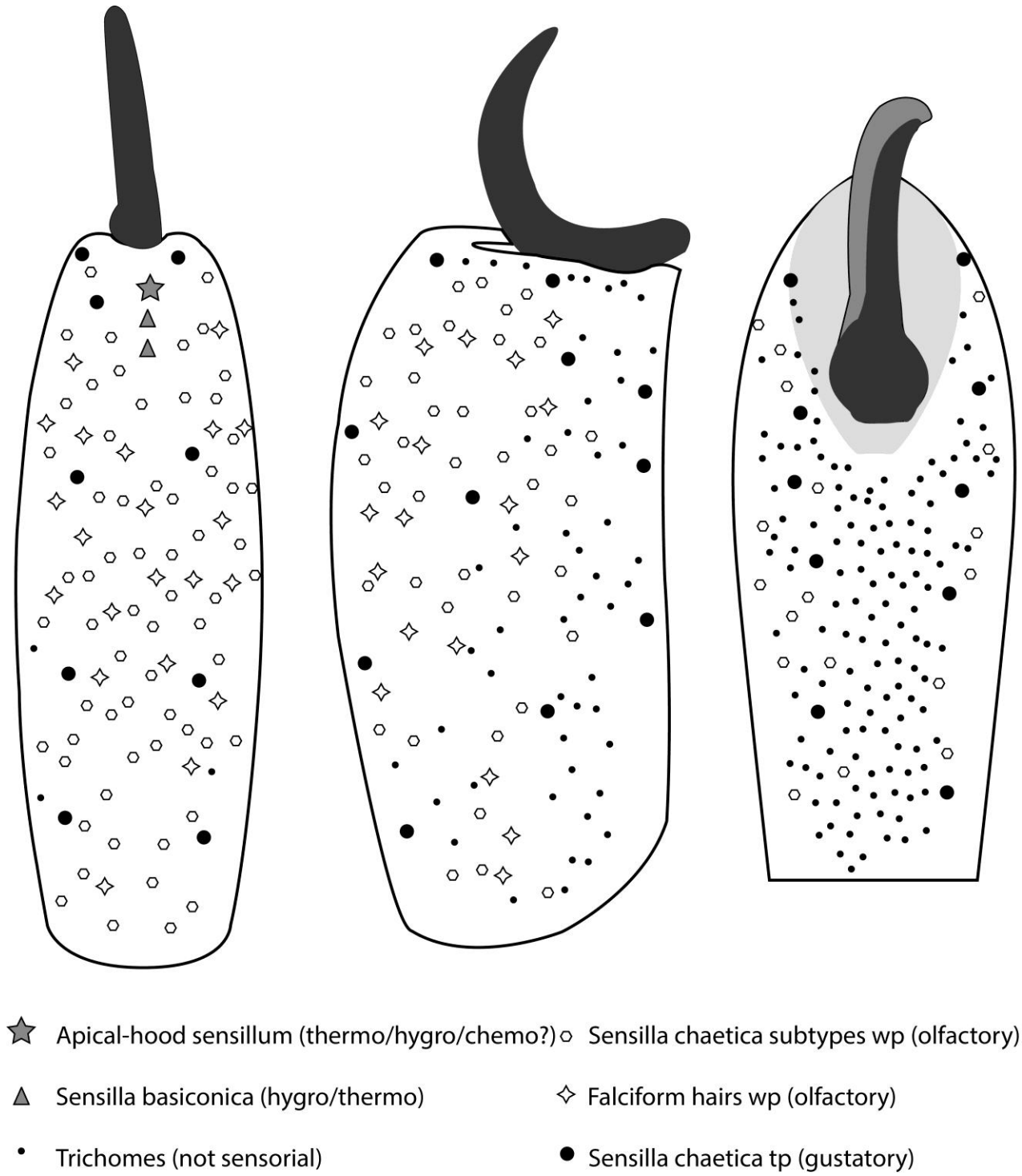

Figure 9: Schematic representation of the distribution of sensillar types on the distal-most tarsomere I of *Heteromitobates discolor* (based on a female), and their putative function.

Chapter 3: Widespread tarsal sensilla in harvestmen (Arachnida, Opiliones): characters for multi-level phylogenetic relationships and implications for sensory biology

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Abstract

The arachnid order Opiliones is composed of four main lineages: the suborders Cyphophthalmi, Eupnoi, Dyspnoi and Laniatores, the later with more than 2/3 of the described species. Phylogenetic relationships between and within these suborders have greatly improved in recent years, mainly due to advances molecular systematics. Still, identifying new character system is important as it increases the

evaluation power of cladistic analysis and further tests newly proposed relationships. Here, we investigated (with SEM) a promising character system: the sensory hairs (sensilla) on the distal-most segment of legs I and II. We identified five new discrete characters and scored species in nearly all families of Laniatores (28 families, 44 species), and also in 3 species of Dyspnoi, 2 of Eupnoi and 2 of Cyphophthalmi. With an ancestral state reconstruction using a compiled phylogeny of Opiliones, we traced the evolution of these sensilla across these lineages, to evaluate their potential for systematics. We show a widespread occurrence of three sensilla (a pair of sensilla basiconica and one apical-hood sensilla) on the distal-most tarsomere of the anterior legs occur in all families of Laniatores, and that comparable structures occur in the other suborder of Opiliones. Ancestral state reconstruction shows that this sensory field provides phylogenetic information on several levels of phylogenetic relationships. We discuss the implications of the widespread occurrence of these sensilla in Opiliones, which have recently been hypothesized to be hygro- thermoreceptors.

1. Introduction

Harvestmen, arachnids of the order Opiliones, comprise four well defined lineages with considerable morphological disparity: the suborder Cyphophthalmi includes diminutive inconspicuous animals (1-2mm) popularly known as “mite-harvestmen” (Giribet et al., 2012); Dyspnoi include generally small short-legged animals, with characteristic fusion pattern of opisthosomal tergites (Pinto-da-Rocha and Giribet, 2007); Eupnoi species range from short-legged, to the very long-legged “daddy-long-legs”(Pinto-da-Rocha and Giribet, 2007); and the suborder Laniatores includes more sclerotized animals (“armored harvestmen”) with typical raptorial pedipalps (Sharma and Giribet, 2011). Since the first numerical cladistic analysis of Opiliones (Shultz, 1998), the clade Phalangida (Eupnoi+Dyspnoi+Laniatores) is consistently recovered as the sister group to the suborder Cyphophthalmi (Fig. 1) (Giribet et al., 1999, 2002, 2010; Garwood et al., 2014; Sharma and Giribet 2014), in contrast to the Cyphopalpatores hypothesis (Cyphophthalmi+Eupnoi+Dyspnoi) (eg. Martens et al., 1981). Inside Phalangida, the clade Palpatores (Eupnoi + Dyspnoi) as sister to Laniatores was first supported by a parsimony analysis with 26 morphological characters Shultz (1998). Subsequently, Giribet et al. (2002) compiled 253 morphological characters and two molecular markers, in a total evidence approach that supported the Dyspnolaniatores clade (Dyspnoi+Laniatores) (Fig. 2). This result

had also been supported by a previous study with a smaller morphological matrix and the same molecular markers (Giribet et al., 1999). Since then, phylogenetic approaches exclusively with molecular sequence data, or with additional genes analyzed with morphological data, have consistently corroborated the Palpatores clade (Giribet et al., 2010; Garwood et al., 2014; Sharma and Giribet, 2014; Fernandez, Tourinho, Sharma, Giribet, in prep) and disfavored the placement of Dyspnoi as sister to Laniatores.

Laniatores represents more than two thirds of the described harvestmen species, comprising ~30 families, depending on the classification scheme employed (Kury, 2013; Giribet and Sharma, 2015) (Fig. 1). Relationships between the families and superfamilies have greatly improved in recent years, mainly due to the improvement of molecular based approaches (Giribet et al., 2010; Sharma and Giribet, 2011). Still, identifying independent sources of characters is important, as it increases the evaluation power of cladistic analysis and further tests newly proposed relationships (Sharma and Giribet, 2011, Pinto-da-Rocha et al., 2014; Kury, 2014; Kury and Villarreal, 2015).

In Arthropoda, the employment of sensilla occurrence and distribution as characters (chaetotaxy) and other integumental structures is a common practice that wields sinapomorphies in different categories of relationship, from genus to broader groups (Insecta: Brozek and Bourgoin, 2013; Crustacea: Karanovic and Kim, 2014; Arachnida: Tomasiewicz and Framenau, 2005; Botero-Trujillo and Flórez, 2011; Platnick et al., 2012). In Opiliones various cuticular structures have been suggested as promising for systematics, such as microstructure of the integument (Rodriguez et al., 2014a, b), glandular openings (Willemart and Giribet, 2010; Gainett et al., 2014; Rodriguez and Townsend, 2015), and sensilla occurrence and distribution (Willemart and Giribet, 2010; Gainett et al., 2014; Kury and Villareal, 2015; Townsend et al., 2015; Wolff et al., 2016). While appendicular characters seems to be a promising source of characters (Gainett et al., 2014), the potential of tarsal sensilla has never been rigorously investigated under a phylogenetic approach.

The tarsi (last segment of the leg) of harvestmen concentrate most sensillar units: in the case of Laniatores, legs I and II are used as sensory legs, and their tarsi have higher density and diversity of sensory structures (Willemart and Gnaspini, 2003; Willemart et al., 2009; Gainett et al., Chapter 1). Willemart et al. (2007) first described a pair of sensilla basiconica on the distal-most tarsomeres I and II of *Iporangaia pustulosa* (Mello-Leitão, 1935) and *Neosadocus maximus (*Giltay, 1928) (Laniatores, Gonyleptidae), inserted “on an irregular depression of the cuticle”. Gainett et al. (Chapter 2) then described a third sensillum on this irregular depression, the apical-hood sensilla, always distal to the pair

of sensilla basiconica. A preliminary survey on the family Gonyleptidae showed that these sensillar units were conserved in position and morphology in these legs, being good candidates for new characters.

Therefore, we searched for similar structures occurring on the correspondent region of the distal-most tarsomeres I and II of species in other laniatorean families to investigate the phylogenetic distribution of the triad (apical-hood sensillum and pair of sensilla basiconica). Additionally, we sampled the correspondent region on representatives of the suborders Dyspnoi, Eupnoi and Cyphophthalmi. To reconstruct the morphological evolution of these structures, we proposed and optimized characters in a compiled phylogeny of Opiliones.

2. Methods

2.1. Species sampling

The specimens were obtained from the frozen tissue collection at the Museum of Comparative Zoology (Harvard University, USA) and the Museu de Zoologia da Universidade de São Paulo (Brazil). We sampled the tarsi I and II of 44 species representatives of 28 families of Laniatores (60 individuals), 3 species of Dyspnoi, 2 species of Eupnoi and 2 of Cyphophthalmi. Absence of the studied structures in tarsi III and IV for some laniatorid species was inferred from the SEM database generated by Gainett et al (2014) and the study of the laniatorid *Heteromitobates discolor* (Gainett et al, in prep.). The list of specimens can be found in the Supplementary Material S1. Additional information can be accessed in the MCZ data base (mcz-base.mcz.harvard.edu).

2.2. Scanning Electron Microscopy

Tarsi were cut with micro scissors and submitted to three rounds of ultrasound cleaning (Branson 200): in distilled water, a 1:10 detergent solution (Alconox®) and distilled water. The duration of each round was determined empirically. Most specimens were critical point dried (Tousimis 931 GL), being dehydrated in graded ethanol series. Otherwise, they were immersed in 100% acetone and then air dried. The samples were then mounted on stubs with carbon adhesive tabs (Electron Microscopy Science,

Hatfield, PA, US) and sputter coated with Pt-Pd targets (EMS 300T D Dual Head Sputter Coater). Photographs were taken in a Zeiss Ultra-Plus FESEM and Zeiss Supra FESEM (field emission scanning electron microscope, at the Center for Nanoscale Systems, Harvard University), or in a Zeiss DSM 940 (at Instituto de Biociências, Universidade de São Paulo).

2.3 Coding and ancestral state reconstruction

Characters and character states were proposed following a reductive coding scheme, as to explore the informativeness of the hierarchal variations observed in less inclusive clades (Strong and Lipscomb, 1999; Brezeau, 2011). Ancestral state reconstructions were performed in Mesquite ver. 2.75 (Maddison and Maddison, 2011) under equal weights parsimony. The tree topology used was a compiled phylogeny of Opiliones based on Giribet and Sharma (2015), with updated relationships of most inclusive clades of Laniatores, after a transcriptome based phylogeny by Fernandez, Sharma, Tourinho and Giribet (unpublished) (Fig. 1). The main outcome of adding the data of Fernandez et al. is in Laniatores, with the reinstatement of Insidiatores as a monophyletic group sister to Grassatores and placement of the family Sandokanidae as early branching Grassatores (Fig.). The clade Palpatores (Eupnoi + Dyspnoi) sister to the suborder Laniatores is recovered, corroborating recent phylogenetic studies (Giribet et al., 2010; Sharma and Giribet, 2014). In ambiguous optimizations, we did not favor specific transformation schemes, discussing both ACCTRAN and DELTRAN reconstructions (Agnarsson and Miller, 2008). For characters 3 and 5 we also conducted ancestral state reconstructions in a modified topology with Dyspnoi as sister group of Laniatores, the Dyspnolaniatores hypothesis (Fig. 2) (Giribet et al., 2002), and discussed the different outcomes of both scenarios.

3. Results

3.1.SEM survey

Laniatores

All species of Laniatores investigated, in 28 families, possess three sensilla on the distal third of the distal-most tarsomere of leg pairs I and II, roughly aligned along the sagittal axis (Fig. 3). The only

exception is *Sandokan truncatus* (Sandokanidae), in which only the distal sensilla was identified in the correspondent position. The distal sensillum has been termed the *apical-hood sensillum* (Gainett et al., Chapter 2), and the middle and proximal sensilla have been termed *sensilla basiconica* (after Willemart et al 2007, 2009). Below, we describe the variation observed in the morphology and topology of these three sensilla on the distal-most tarsomere I and II.

Distal sensillum: apical-hood sensilla

In all species studied for both males and females (15 species), the apical-hood sensilla (Ahs) were sexually monomorphic. Moreover, no difference in shaft morphology was detected between legs I and II. It is inserted dorsal to dorsolaterally in the tarsi, in a flat and oval-shaped basal membrane (Figs. 4b-d). The basal membrane has no protuberance, which is different from the socket of surrounding sensilla chaetica (see Fig. 4b for comparison with sensillum chaeticum). The seta wall bears continuous longitudinal grooves along the shaft (Figs. 5; 6; 7). The basal portion of the shaft is much wider than the apex, tapering gradually (Fig. 4-7). Due to the angles of the photos across species, we could not make reliable measurements of shaft length. Still, Ahs is always longer than the sensilla basiconica (Figs. 3; 4). In *Erobomaster flavescens flavescens* (Travuniidae) it appeared to be the longest setae in the distal-most tarsomere (Fig. 3a). The apical portion has the shape of a hood, with variation in the length and shape of the hood between groups. One of the sides of the hood is morphologically similar to the rest of the shaft, bearing grooves (Figs. 5b, d, h, l; 6b). The opposite side has an oval sub apical concavity with a smooth surface, with a pore-like structure that frequently appears clogged with some material (Figs. 5a, c, e, g, i, j, k, m; 6a, c, e; 7b, d). Some samples appear to have two pore-like structures, the most distal one being smaller in diameter (Figs. 5a; 6a, e), but in the majority of samples only one was seen (Figs. 5c, g, i). This difference might be artefactual, probably related to clogging and dirt particles. Three types of hood morphology can be recognized: terminal swelling, with no tapering after swelled region (spoon-shaped) (Figs. 5, e, l, m); sub-terminal swelling, with tapering after swelled region ("death's hood" shape) (Figs. 5a, b, c, f, g, k; Figs. 4a-e; Figs. 5b-e); and gradual tapering, with no swelling (Fig. 5h-j) (Table 1).

Middle and proximal sensilla: sensilla basiconica

Two sensilla basiconica occur proximal to the distal sensilla (apical-hood sensilla), to which we refer to as the middle and proximal sensilla (numbers 2 and 3 in Fig. 4). In all species studied for males and females (15 species), they were sexually monomorphic. The middle sensillum is always longer than the proximal one, both being shorter than the distal apical-hood sensillum (Figs. 3; 4). They are inserted in a flat basal membrane, with no prominent socket. Although first described in two gonyleptid species as being short and conic (Willemart et al 2007), there was variation in length in Laniatores. Sensilla basiconica could be peg-like (Fig. 3a, d) or elongated (Figs. 3b; 7a), but they were always the shortest seta in this region of the tarsi (Fig. 3). The shaft appears smooth in most species, but some ridges were present in the basal portion of *Synthetonychia glacialis* (Synthetonychiidae) (Fig. 7a), a feature that is possibly an artifact of the drying process. No wall pores were detected on the shaft's wall. A ubiquitous feature is the apical portion with an opening to the exterior by means of a longitudinal slit that result in two terminal flaps (Fig. 4a, b). The slit-segment of the shafts occupies approximately the last third of the shaft (Fig. 4a, b) from. We detected no differences in shaft morphology between leg pairs I and II.

In most species, the two sensilla basiconica are inserted in the same basal membrane, with no cuticular-polygon lines in between the two shafts (Fig. 4a, b) (Table 1). In some species, they can be inserted individually, with cuticular-polygon lines in between them (Fig. 4d). We also observed species in which the two sensilla basiconica and the apical-hood sensillum shared the same basal membrane (Fig. 4c). Therefore, three conformations occur in Laniatores: (1) the proximal and middle shafts with basal membranes fused, separated from the distal shaft; (2) the three sensilla with isolated basal membranes; and (3) all three shafts with basal membranes fused. In all, except two species of the family Agoristenidae and the Nomoclastidae species, the basal membrane configuration is the same in legs I and II. In those above-mentioned three species, pair of leg II has the three shafts separated (2), but leg I has two sensilla basiconica clustered (1) (*Agoristenus haitensis* and *Avima octomaculata*; Agoristenidae), or sensilla basiconica and apical-hood sensillum clustered (*Poassa limbata*; Nomoclastidae) (3).

Dyspnoi

In a correspondent position of the distal-most tarsomere I and II the three species of Dyspnoi studied show three sensilla aligned along the sagittal axis, which differ from the surrounding sensilla chaetica and trichomes in having a flat basal membrane (Figs. 8; 9). The proximal two sensilla are short

and conic, being also termed sensilla basiconica. The distal sensillum has a very similar morphology to the apical-hood sensilla in Laniatores, and therefore we refer to it using the same name.

The apical-hood sensillum is longer than both sensilla basiconica (Figs. 8a, d; 9a, d). The shaft has longitudinal ridges and gradually tapers into a fine tip (Fig. 8c; Fig. 9a). The apical portion has two pore-like structures in a region without ridges, but shows no sub-terminal swelling (see *Ps* in Fig. 8d; 9d). The distal pore-like structure is smaller than the proximal (Fig. 8d). The side opposite to the pore-like structures is similar to the rest of the shaft, with ridges.

The sensilla basiconica are thin pegs, being the shortest sensilla in the tarsomere (Figs. 8a, b; 9a-c). The proximal sensillum in *Anelasmocephalus* sp.(Trogulidae) is shorter than the middle, both being shorter than the distal apical-hood sensillum (Fig. 9a), but in the other species we could not access their relative length. The shaft is bent forward in its middle portion, in an angle of almost 90º (Figs. 6b; 7a-c). Apically, it bears a sub terminal pore with a short slit, which was facing down in *Nemastoma bimaculatum* (Nemastomatidae) (Figs. 8a, b), or up in *Anelasmocephalus* sp. (Figs. 9a-c).The slit apparently does not divide the shaft in two flaps as in Laniatores.

We observed no basal membrane fusion between the three sensilla, and three shafts were more interspaced than in Laniatores (Figs. 8a; 9a).

Eupnoi

The tarsi I and II of the Eupnoi species also have three sensilla with short conic shafts and flat basal membranes in the correspondent position to Laniatores, which we refer to as sensilla basiconica. These structures differ from the ones in Laniatores in their disposition and morphology of the shaft opening. In the undetermined sp. (Phalangiidae), both legs I and II have a triad of sensilla basiconica in the distal third of the tarsomere (Figs. 10a-d). In its proximal third, the dorsal surface of the last tarsomere also has 2 and 4 additional isolated sensilla basiconica on legs I and II, respectively (Fig. 10a). In *Astrobunus grallator* (Sclerosomatidae), a similar triad of sensilla basiconica occurs in the distal third of legs I, but legs II shows only isolated sensilla basiconica in dorsal position, numbering 7. Legs I also have 2 isolated sensilla basiconica (Figs. 11a, b) in the proximal third of the tarsomere, and thus the total number in the tarsomere is 5 sensilla (Fig. 11c). The apical portion of the sensilla basiconica of the undetermined sp. (Phalangiidae) has a pore-like structure (Figs. 10b-d), while in *A. grallator* there is a pore opening with a slit below it (Fig. 11c). This second condition is similar to the morphology observed

in *Anelasmocephalus* sp. (Dyspnoi, Fig. 9c). Even though the three shafts in the triad are similar in shape, the three shafts are progressively larger in the undetermined sp. (Phalangiidae), with the most distal being the longest and thickest (Figs. 10c, d). In *A. grallator* the three shafts have a similar length (Fig. 11c).

Cyphophthalmi

The distal third of the last tarsomeres I and II of the cyphophthalmid species studied shows a structure called sub-apical process (Fig. 12). Juberthie (1979, 1988, 2000) first described the sub-apical process, which occurs isolated on the distal third of the tarsomeres I and II only, of males and females of some Cyphophthalmi species. Willemart and Giribet (2010) later found this structure to be widespread in Cyphophthalmi. Our investigation of 2 Cyphophthalmi species, belonging to one family each (Troglosironidae and Pettalidae), confirmed the occurrence of this structure in the correspondent position of the apical-hood sensillum and sensilla basiconica found in the other sub-orders. In the species here investigated, this single sensillum occurred among other types of sensilla (solenidia, sensilla chaetica), but no short and conic sensilla, such as sensillum basiconicum, is present in the tarsomere (Fig. 12a). The shaft is wide at the base (~6.5 µm), and tappers to a fine rounded tip (Fig. 12c), possessing a high density of longitudinal ridges (Fig. 12b, insight). The apical portion has no sub-terminal swelling and no pore-like structures.

3.2.Characters and coding

In the previous section we comparatively described the occurrence of sensilla with external similarities in the shaft and basal membrane, with a restricted distribution to the distal third of the distal-most tarsomeres of legs I and II of representatives of all suborders of Opiliones. This conserved sensory field shows variation in number, basal membrane association, composition of sensillar types and shaft morphology. In order to trace how this sensory field has been modified across Opiliones evolution, we propose the following characters and character states: (1) Number of sensilla: (1a) One, (1b) Three; (2) Basal membrane association: (2a) Distal, middle and proximal sensilla not fused (Fig. 13a); (2b) Distal sensilla isolated, middle and proximal fused sensilla (Fig. 13b); (2c) Distal, mid and proximal sensilla

fused (Fig. 13c); (3) Distal sensilla, sensillar type: (3a) Apical-hood sensilla, (3b) Sensilla basiconica, (3c) Sub-apical process; (4) Apical-hood sensilla, hood morphology: (4a) Terminal swelling (spoon-shaped), (4b) Sub-terminal swelling ("death's hood"), (4c) No swelling (regular); (5) Middle and proximal sensilla, apical opening: (5a) Pore-like, (5b) Complete slit.

Table 1 contains the list of characteristics organized by characters and character states for all species studied. States listed are representative of the morphology of leg pairs I and II, unless otherwise stated in the few cases of serial polymorphisms observed. In the two species of Agoristenidae (Laniatores), *Poassa limbata* (Laniatores, Nomoclastidae) and *Astrobunus grallator* (Eupnoi, Sclerosomatidae) legs I and II have different character states, indicating that each leg may have different evolutionary histories. Apart from that, species in which we could access the character in both legs always showed the same character state and morphology (29 species). In order to ensure comparability, we choose to code leg pairs I for our analysis. For 4 species in which legs I could not be accessed properly, the sensilla on leg pair II was used for coding as representative of leg pair I (species marked with asterisk in Table 1).

3.3.Ancestral state reconstruction

Since we were interested in variation within Opiliones, our topology is rooted in Cyphophthalmi and thus ancestral state inferences at the root of Opiliones were always ambiguous. Variation in the number of sensilla composing the sensory field (distal third, distal-most tarsomeres I and II) (character 1) has two equally parsimonious optimizations (cost=2): either having one sensillum (1a, white) is the ancestral state of Opiliones, which has changed for three sensilla (1b, black) in Phalangida (Eupnoi+Dyspnoi+Laniatores), or ancestral three sensilla (1b, black) has transformed to a state with one sensillum (1a, white) in Cyphophthalmi and maintained in Phalangida (Fig. 14). Reversion from three (1b, black) to one sensillum (1a, white) is unambiguously recovered in the laniatorean family Sandokanidae (*Sandokan truncatus*) (Fig. 14).

Association between the basal membranes of the three sensilla (character 2) is inapplicable for Cyphophthalmi species and ambiguous at the most recent common ancestor of Phalangida, Palpatores and Laniatores (Fig. 15). Several transformations occur in the equally parsimonious reconstructions (cost=7), with untraceable ancestral states in most cases. The transition from fused middle and proximal sensilla (2b, green) to all sensilla fused (2c, black) is unambiguous in the family Podoctidae

(superfamily Epedanoidea). Three sensilla separated (2a, white) was acquired independently in Dyspnoi and a clade inside the laniatorean superfamily Gonyleptoidea (Metasarcidae+Cometidae+Gonyleptidae) (Fig. 15).

The morphological type of the distal sensilla of the triad (character 3) observed in Phalangida presents a single most parsimonious local optimization in Phalangida (Fig.16), with a transformation in Eupnoi (3b, green). The ancestral state of Phalangida is recovered as having an apical-hood morphology, but it is not possible to resolve if it first appeared in Phalangida or at the root of Opiliones (Fig. 16). We alternatively optimized this character in a topology which favors the placement of Dyspnoi and Laniatores as sister clades (Dyspnolaniatores, Giribet et al 2002), to evaluate the changes in evolutionary scenarios. In this scenario, the character state at the root of Phalangida also becomes ambiguous, and additionally makes the first occurrence of the sensilla basiconica shape of Eupnoi unresolved (3b, green) (Fig. S2).

The tip of the distal sensilla with apical-hood morphology (inapplicable in Cyphophthalmi and Eupnoi) (character 4) has undergone several changes in the phylogeny and is ambiguously optimized (cost=8). The ancestral state of less inclusive clades (Phalangida, Palpatores, Laniatores and Grassatores) is ambiguously reconstructed, but inside Grassatores four transformation events are unambiguous (Fig. 17): Terminal swelling (4a, white) has independently evolved from Sub-terminal swelling (4b, green) in the laniatorid families Podoctidae, Biantidae and Guasiniidae; and from Sub-terminal swelling(4b, green) or No swelling (4c, black) in the family Phalangodidae. No swelling (4c, black) has independently evolved two times: in the family Stygnopsidae (Gonyleptoidea) and in a clade inside Gonyleptoidea including the families Metasarcidae, Cosmetidae and Gonyleptidae (Fig. 17).

Finally, type of apical opening of the middle and proximal sensilla (character 5, inapplicable for Cyphophthalmi) is ambiguously reconstructed in Phalangida (cost=1) (Fig. 18), but still having a pore-like opening (5a, white) is homologous in Palpatores (Eupnoi+Dyspnoi) and a having complete slit (5b) is homologous in Laniatores. Under the Dyspnolaniatores hypothesis (Giribet et al 2002), there is only one most parsimonious optimization (cost=1): pore-like opening (5a) represents the ancestral state of Phalangida, which has undergone a single change in Laniatores into a complete-slit opening (Fig. S3).

4. Discussion

We investigated the phylogenetic distribution of sensilla occurring on the distal third of the

distal-most tarsomeres of legs I and II of harvestmen, providing comparative morphological data on the variability of these structures in all four extant suborders of Opiliones. In the first session, we discuss the arguments in favor of the correspondence between these sensilla observed in species in all four suborders. In the second session, we discuss the contributions of these structures for systematics. In the last session, we discuss the implications of the widespread occurrence of these structures for Opiliones sensory biology.

4.1.The triad in Laniatores, Dyspnoi, Eupnoi and Cyphophthalmi

We have shown that apical-hood sensilla and a pair of sensilla basiconica on the sensory appendages are morphologically conserved across Laniatores families, leaving little doubt that they are homologous structures in this group (see also discussion in next session). The triad shows four marked characteristics in Laniatores: (1) occurrence on the distal third of the distal-most tarsomere I and II, dorsally; (2) the distal sensilla is of a different type than the middle and proximal sensilla; (3) an ascending length of the shafts, from proximal to distal sensilla; and (4) the distal sensilla (apical-hood sensilla) occurs only on this region of pair of legs I and II. We assumed a topographic/positional homology, and listed characteristics that support this assumption in Laniatores. With some variations, these criteria are also met by species in the suborders Dyspnoi, Eupnoi and Cyphophthalmi.

The Dyspnoi species here investigated meet all these four criteria. Besides, the distal sensilla in Dyspnoi have remarkable similarity with the external morphology of laniatorid apical-hood sensilla, having a hood with two pore-like openings (Figs. 8c, d).

In the Eupnoi species, at least characteristics 1, 3 and 4 are met. We did not investigate in detail the morphology of each sensillum in the triad, so for now we treat them as the same type of sensillum, leaving criteria 2 to be investigated in the future. In the Phalangiidae species, the three shafts have a increasing length (proximal to distal; Figs. 10c, d), which follows criteria 3 of Laniatores. Even though sensilla basiconica occur in other body regions in Eupnoi species (Willemart et al., 2009; Wijnhoven, 2013), the triad (or “trident”, sensu Wijnhoven, 2013) occurs only on legs I and II (undetermined sp., Phalangiidae) or only on legs I (*Astrobunus gralattor*, Sclerosomatidae), meeting our criteria 4.

Finally, the Cyphophthalmi species show no sensilla basiconica (see also Willemart and Giribet 2010) and have a sub-apical process, a bottle-shaped sensilla on the distal-third of the tarsi of pair of legs I and II only (Fig.12; Juberthie, 1979, 1988, 2000; Willemart and Giribet, 2010). Since sensilla basiconica do not occur in this group, criteria 2 and 3 are inapplicable. Still, the positional criteria (1)

and the characteristic occurrence on sensory appendages (2) support our hypothesis that the sub-apical process is homologous to what is observed in the other suborder. Since no sensilla basiconica occur, we assume, adhering to a positional criteria, the sub-apical process to correspond to the distal sensilla of the triad in Eupnoi, Dyspnoi and Laniatores.

Given this positional/topographic congruence and the list of similarities, we find it reasonable to trace a correspondence between these structures and consider it to be a conserved sensory field on the sensory appendages, an idea further supported by the available ultrastructural information on a laniatorean species (Gainett et al., Chapter 2; see discussion in last session). Using a compiled phylogeny of Opiliones, we conducted an ancestral state reconstruction to test the homology of these structures and to investigate how specific attributes been modified during Opiliones evolution. In the next session, we discuss the potential of the characters here identified for the systematics of broad clades in Opiliones, with emphasis in Laniatores.

4.2.Ancestral state reconstruction and new characters for systematics

We recognized the variation observed as belonging to five discrete characters, pertaining number of sensilla (character 1), association of basal membranes (character 2), sensillar type of the distal sensillum (character 3), shape of the hood (character 4) and shape of the apical portion of mid and proximal sensilla (character 5). Since our topology is rooted in Cyphophthalmi, our power of inference is limited to our Phalangida and less inclusive clades. Below, we address the contribution of each character in different levels of Opiliones phylogeny, with focus in the ingroup Laniatores.

Sub-ordinal level

Presence of three sensilla on the sensory field of legs I and II (character state 1b, black) only occur on species of the Phalangida clade (Fig. 14). The number of sensilla on the sensory field (character 1) has undergone at least two changes in the phylogeny, but the reconstruction of this character is ambiguous (Fig. 14). However, it should be stressed that while having three sensilla is homologous in all Phalangida species, it is not possible to assert if this is a synapomorphy of Phalangida, or of Opiliones, in the later case with loss of two sensilla in Cyphophthalmi.

Considering the hypothesis with Palpatores (Fig. 1), having an apical hood sensillum as the distal sensilla of the sensory field (character 3; character state 3a, white), is a homologous structure in

Phalangida, which could either be a retained character state from Opiliones ancestral, or a synapomorphy for Phalangida (Fig. 16). The most parsimonious optimization reveals that the condition observed in Eupnoi (distal sensilla as sensillum basiconicum; character state 3b, green) is an autapomorphy, deriving from the apical-hood sensilla morphology and reducing in size. In the alternative topology with the Dyspnolaniatores clade (Fig. S2), having apical-hood sensilla as the distal sensilla is also homologous in Phalangida, but it is not possible to determine if this shared character state is plesiomorphic or apomorphic. Therefore, despite Laniatores and Dyspnoi sharing this character state due to common ancestry, it would be incorrect, with our present knowledge, to assert that the shared condition suggests a Dyspnolaniatores clade (Giribet et al., 1999, 2002).

The apical opening of the middle and proximal sensilla (character 5, inapplicable for Cyphophthalmi) also shows variation at the level of suborders, with a pore-like opening in Eupnoi and Dyspnoi, and a complete slit opening in Laniatores (Fig. 18). Considering the Palpatores hypothesis, a pore-like opening (5a, white) and a complete slit opening (5b, black) are homologous character states in Palpatores and Laniatores, respectively. However, it is ambiguous which character state is plesiomorphic or apomorphic. Considering the Dyspnolaniatores clade, the scenario changes: the pore-like opening is recovered as plesiomorphic, and the complete slit is an unambiguous synapomorphy of Laniatores (Fig. S3).

Super-familial level

The degree of association of the basal membrane of the three sensilla (character 2, inapplicable for Cyphophthalmi) is not unambiguously synapomorphic for any broad clade, since Eupnoi, Dyspnoi and Laniatores don´t share character states at this higher level (Fig 15). However, having the sensilla separated (2a, white) is synapomorphic for a few families on the superfamily Gonyleptoidea, namely Gonyleptidae, Comestidae, and Metasarcidae (Fig. 15). The relationship of this families is further suggested by the fact that they share an apical-hood sensillum without swelling (character 4, character state 4c, black), which is recovered as unambiguous synapomorphy of this clade (Fig. 17). Gonyleptoidea is the largest superfamily of Laniatores, including the second most diverse harvestmen family, the family Gonyleptidae (Caetano and Machado, 2013; Pinto-da-Rocha et al., 2014). Relationships within Gonyleptoidea have recently been addressed by studies using molecular markers (Sharma and Giribet, 2011; Pinto-da-Rocha et al., 2014; Fernandez, Sharma, Tourinho and Giribet, in

prep.). Considerable changes in the topology have been made since the first cladistic hypothesis by Kury (1993), such as the erection of the families Cryptogeobiidae (Kury, 2014), Gerdesiidae (Bragagnolo et al., 2015) and Metasarcidae (Benedetti, 2012; Pinto-da-Rocha et al., 2014). Therefore, further investigating this characters in this superfamily is promising, both for testing newly proposed relationships and providing new diagnostic features for its suprafamilial clades.

Familial level

The loss of two sensilla basiconica associated with the apical-hood sensilla (character 1, character state 1a, white) is recovered as an unambiguous synapomorphy of the family Sandokanidae (Laniatores, Grassatores) (Fig. 14). This family placement in Laniatores phylogeny remained elusive in recent attempts to reconstruct its history (Schwendinger, 2007, Sharma and Giribet 2009, 2011, Giribet et al., 2010), but a transcriptome based approach has recovered it as an early branching Grassatores (Fig. 1) (Fernandez, Sharma, Tourinho and Giribet, in prep.) This family has several morphological autapomorphies, such as the complete fusion of the carapace and opisthosomal tergites (scutum completum, a cyphophthalmid condiction), reduced tarsi, and is the only family without a laniatorean synapomorphic slit sense organ, the Metatarsal Paired Slits (Schwendinger, 2007, Sharma and Giribet, 2009, Gainett et al. 2014). Together with those, the absence of the pair of sensilla basiconica adjacent to the apical-hood sensillum on tarsi I and II, a widespread condition in Laniatores, makes them a very interesting group to be studied under a sensory morphology perspective. Very few SEM data on their cuticular morphology has been published and little is known about their natural history and behavior (Schwendinger and Martens, 2004). New data on their general sensory morphology will be shown in a separate paper (Gainett et al., in prep.).

Three sensilla clustered (character 2, character state 2c, black) have been independently acquired in the laniatorean families Podoctidae, Stygnommatidae, Biantidae Cryptogeobiidae, although ambiguous optimization confuses if the character states in Stygnommatidae and Biantidae are homologous (Fig. 15). At least for Podoctidae (Epedanoidea), this conformation is certainly derived from partial fusion of the three sensilla (character state 2b, green), which is the general condition for Laniatores. The association of the three shafts in Cryptogeobiidae is interesting because this group was previously known as part of the subfamily Tricommatinae, inside the family Gonyleptidae (Pinto-da-Rocha & Giribet 2007, Kury 2014). Therefore, the autapomorphic condition in Cryptogeobiidae is in

accordance with the recent erection of this family as a group outside Gonyleptidae (Kury, 2014; Pinto-da Rocha et al., 2014); with the later family having all three shafts separated (character state 2a) (Fig. 15).

The shape of the hood of the apical-hood sensillum may also prove useful. Despite having undergone several changes in the evolution of the group (Fig. 17), some families have synapomorphic character states: A terminal swelling (4a, white) was independently acquired in Podoctidae, Biantidae and Guasiniidae, being putative autapomorphies (Fig. 17).

Other sources of characters

The only laniatorean species in which we found different character states (character 2) were *Avima octomaculata*, *Agoristenus haitensis* (Agoristenidae) and *Poassa limbata* (Nomoclastidae). Thus, it is possible that having a serial dimorphism in the degree of fusion of the three shafts (character 2) is a characteristic restricted to these families. Similarly, serial dimorphism has also been observed in the tarsal organ of spiders, in which a dimorphism in the shape of the sensilla of the tarsal organs of anterior and posterior legs has been suggested as a synapomorphy of the family Oonopidae (Platnick et al., 2012). A similar, but independent condition occurs in the Eupnoi *Astrobunus grallator* (Sclerosomatidae), in which legs I have the three sensilla clustered and legs II have isolated ones (Fig. 11). Interestingly, Wijnhoven (2013) reported three clustered sensilla (or "trident") on the distal-most region of the last tarsomere I and last segment of the pedipalps of *Dicranopalpus ramosus* (Phalangiidae). Therefore, it would be interesting to investigate if the same condition occurs in *A. grallator*, and if it has any taxonomic value.

In conclusion, this sensory field on the sensory appendages is widespread in harvestmen and constitutes a new source of information to be explored in several levels of phylogenetic relationships.

4.3.A conserved "tarsal organ"

Relatively few studies have been conducted on the sensory structures of harvestmen in comparison with most arachnid orders (Foelix, 1985; Willemart et al., 2009). Most of what is known about the function of specific sensillar types in harvestmen comes from studies in the suborder Laniatores, including data on the function of sensilla basiconica and the apical-hood sensilla (Willemart et al., 2009; Gainett et al., Chapter 2). Therefore, we base our discussion on the triad observed in this

suborder. We showed that the pair of sensilla basiconica and the apical-hood sensillum are phylogenetically conserved in this suborder and with correspondent structures in Eupnoi, Dyspnoi and Cyphophthalmi. This conserved association indicates that they might be functionally associated, possibly functioning as a sensory unit, or organ. In *Heteromitobates discolor* (Laniatores, Gonyleptidae), the dendrites innervating the three shafts are concentrically arranged, forming a conspicuous bundle that proceeds to the tarsal nerve (Gainett et al., Chapter 2). The three shafts are inserted in a relatively thinner area of the dorsal cuticle, and their inner parts occupy a considerable space of the distal-third of the last tarsomeres I and II, which is unusual when compared with the remaining tarsal sensilla. Moreover, in several species, the three shafts even share the same basal membrane (Character state 2c; Fig. 15). Therefore, the widespread phylogenetic conservatism and morphological evidence of association suggest that they are may function as a joint sensory organ at the tip of the sensory legs.

Ultrastructural data (*H. discolor*) supports a hygro/thermoreceptive function for sensilla basiconica, although the function of the apical-hood sensilla remains unclear, due to its unique ultrastructural characteristics, such as a double innervation of the shaft, two pore-like structures at the tip of the shaft (on the hood; eg. Fig. 5) and unusual meshed structure of the shaft´s wall (Gainett et al., Chapter 2). The complete slit opening of sensilla basiconica has been argued to be related to the transduction mechanism involved in hygroreception (Gainett et al., Chapter 2). Interestingly, this feature is conserved in all Laniatores species studied, but is different is Eupnoi and Dyspnoi. Expanding the distribution of this triad to the whole suborder Laniatores provides a unique opportunity of generalizing these functional inferences for a large portion (2/3) of Opiliones species. Given the variations of the external morphology of the three sensilla in Eupnoi and Dyspnoi, and the occurrence of a single sensillum in Cyphophthalmi, it remains imperative to investigate ultrastructure in these suborders, both to test the homology hypothesis here proposed, and to investigate if they are functionally similar to what has been observed in Laniatores.

A phylogenetically conserved group of sensilla on distal tarsomeres of the legs have also been observed in other arachnid orders. These so called “tarsal organs” have been reported in Araneae, Amblypygi, Scorpiones, Parasitiformes (Haller’s organ), and Ricinulei (pore organ) (Blumenthal, 1935; Foelix and Axtell, 1972; Foelix and Chu-Wang, 1973; Foelix et al., 1975; Foelix and Schabronath, 1983; Anton and Tichy, 1994; Tichy and Loftus, 1996; Talarico et al., 2005). In Parasitiformes, the Haller’s organ sensilla contain olfactory and thermo- hygroreceptors (Foelix and Axtell, 1972; Hess and Vlimant, 1983; Hess and Loftus, 1984). In the spider *Cupiennius salei*, tip-pored sensilla of the tarsal organ

respond to humidity, temperature and chemical stimuli (Foelix and Chu-Wang, 1973; Ehn and Tichy, 1994). The tarsal organs of Ricinulei, Amblypygi and Scorpiones have never been studied with electrophysiology and their function is unclear, although ultrastructural data suggests at least an olfactory function in Ricinulei (Talarico et al., 2005). Even though some of these tarsal organs were suggested as homologous structures due to their common position (dorsal region, distal-most tarsomere, close to the claw) (Foelix et al., 1975; Foelix and Schabronath, 1983; Talarico et al., 2005), they occur on different leg pairs, have different shapes and may occur exposed or inside invaginated portions of cuticle (capsules) (Table 2). While comparing these aggregations of sensilla across arachnid orders seems problematic under a phylogenetic perspective, it is still notable that they all show conserved multifunctional association of sensilla on the distal-most part of the legs, including confirmed or putative hygro- thermoreceptors. Relatively little is known about hygro- and thermoreceptors in arachnids, apart from the studies with the tarsal organ of Araneae (Blumenthal, 1935; Anton and Tichy, 1994; Ehn and Tichy, 1994; Tichy and Loftus, 1996) and the Haller´s organ in Parasitiformes (eg. Foelix and Axtell, 1972). Studying the previously known tarsal organs and the new structures here revealed on the sensory appendages of harvestmen may shed some light on the patterns of hygro- and thermoreceptor occurrence in arachnids, and reveal if these sensillar associations on distal parts of the leg are functional convergences, or derived from a common developmental program.

Conclusion

We showed that a pair of sensilla basiconica and one apical-hood sensillum, putative hygro-thermoreceptive sensilla, are a phylogenetically widespread sensory field on the sensory appendages of laniatorean species, with comparable structures in the remaining suborders of Opiliones. These structures show variation in several levels of harvestmen phylogeny and likely constitute a promising source of characters for the systematics of the group. Further investigating the ultrastructure and physiological responses of these structures in different harvestmen suborders is imperative both for supporting their use as character and understanding their importance for the biology of Opiliones.

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TABLES

Table 1: List of characteristics organized by characters and character states for the species investigated. Species sorted by family. Character states showed represent legs I and II, unless otherwise indicated. In these cases, character state on the left indicates leg I and on the right indicates leg II. Species marked with an asterisk have missing information on legs I, and the character state observed on legs II was used in the analysis. Number 1, 2 and 3 on character (2) represent, respectively, the distal, middle and proximal sensilla. Parentheses indicate association.

Species	**Taxonomy**	**(1) Number of sensilla**	**(2) Basal membrane configuration**	**(3) Morphology of distal sensilla**	**(4) Apical-hood sensiilum, tip motphology**	**(5) Middle and proximal sensilla, tip morphology**
Erebomaster flavescens flavescens Cope, 1872	Travuniidae	3	1 (2 3)	apical-hood sensilla	subterminal	complete slit
Synthetonychia glacialis Forster, 1954	Synthetonychiidae	3	1 (2 3)	apical-hood sensilla	subterminal	complete slit
Larifuga cf. *capensis*	Triaenonychidae	3	1 (2 3)	apical-hood sensilla	regular	complete slit
Montalenia sp.	Assamidae	3	1 (2 3)	apical-hood sensilla	subterminal	complete slit
Neopygoplus siamensis	Assamidae	3	1 (2 3)/ ?	apical-hood sensilla	?	complete slit
cf. *Pyramidops* sp.	Pyramidopidae	3	1 (2 3)	apical-hood sensilla	subterminal	complete slit
Pseudoepedanus doiensis Suzuki, 1969	Epedanidae	3	1 (2 3)	apical-hood sensilla	subterminal	complete slit

Petrobunus spinifer Sharma & Giribet, 2011	Petrobunidae	3	?	apical-hood sensilla	?	complete slit
Petrobunus torosus Sharma & Giribet, 2011	Petrobunidae	3	?	apical-hood sensilla	subterminal	complete slit
Austribalonius sp.*	Podoctidae*	3	1 (2 3)	apical-hood sensilla	terminal dilatation	complete slit
Sandokan truncatus Thorell, 1891	Sandokanidae	3	x	apical-hood sensilla	regular	complete slit
Tithaeus sp.*	Tithaeidae*	3	1 (2 3)	apical-hood sensilla	subterminal	complete slit
Avima octomaculata Roewer, 1963	Agoristenidae	3	1 (2 3) / 1 2 3	apical-hood sensilla	subterminal	complete slit
Agoristenus haitensis Šilhavý, 1973	Agoristenidae	3	1 (2 3) / 1 2 3	apical-hood sensilla	subterminal	complete slit
Poassa limbata Roewer, 1943	Agoristenidae	3	1 (2 3) / 1 2 3	apical-hood sensilla	?	complete slit
Gryne perlata	Cosmetidae	3	Separated	apical-hood sensilla	?	complete slit
Phareicranaus hermosa Pinto-da Rocha & Kury 2003	Cranaidae	3	Separated	apical-hood sensilla	regular	complete slit
Cobania picea Bertkau, 1880	Gonyleptidae	3	Separated	apical-hood sensilla	?	complete slit

Ampheres luteus Giltay, 1928	Gonyleptidae	3	?	apical-hood sensilla	?	complete slit
Cryptogeobius sp.	Cryptogeobiidae	3	(1 2 3)	apical-hood sensilla	subterminal	complete slit
Camarana flavipalpi B. Soares, 1945	Cryptogeobiidae	3	(1 2 3)	apical-hood sensilla	?	complete slit
Gonycranaus pluto Bragagnolo et al, 2015	Gerdesiidae	3	?	apical-hood sensilla	?	complete slit
Cajamarca spinigera Roewer, 1957	Metasarcidae	3	Separated	apical-hood sensilla	?	complete slit
Mischonyx cuspidatus Roewer, 1913	Gonyleptidae	3	Separated	apical-hood sensilla	?	complete slit
Promitobates ornatus Roewer, 1931	Gonyleptidae	3	?	apical-hood sensilla	?	complete slit
Sodreana sodreana Mello-Leitão, 1922	Gonyleptidae	3	1 2 3	apical-hood sensilla	?	complete slit
Iporangaia pustulosa Mello-Leitão, 1935	Gonyleptidae	3	1 2 3	apical-hood sensilla	?	complete slit
Pseudopucrolia mutica (Perty, 1833)	Gonyleptidae	3	1 2 3	apical-hood sensilla	?	complete slit
Heteromitobates discolor Sørensen, 1884	Gonyleptidae	3	1 2 3	apical-hood sensilla	regular	complete slit
Discocyrtoides	Gonyleptidae	3	?	apical-hood	?	complete slit

nigricans Mello-Leitão, 1922				sensilla		
Saramacia lucasae Jim & Soares, 1991	Manaosbiidae	3	?	apical-hood sensilla	?	complete slit
Stygnus multispinosus Piza, 1938	Stygnidae	3	?	apical-hood sensilla	?	complete slit
Hoplobunus sp.	Stygnopsidae	3	1 (2 3)	apical-hood sensilla	regular	complete slit
Remyus sp.	Phalangodidae	3	1 (2 3)	apical-hood sensilla	terminal dilatation	complete slit
Martibianta virginsulana Silhavy, 1973	Biantidae	3	?	apical-hood sensilla	terminal dilatation	complete slit
Metabiantes sp.*	Biantidae*	3	1 (2 3)	apical-hood sensilla	terminal dilatation	complete slit
Neoscotolemon sp.	inserta sedis	3	?	apical-hood sensilla	x	complete slit
Stygnomma bispinatum Goodnight & Goodnight, 1953	Stygnommatidae	3	1 (2 3)	apical-hood sensilla	subterminal	complete slit
Baculigerus milenae Kury, 2012	Escadabiidae	3	1 (2 3)	apical-hood sensilla	subterminal	complete slit
Fissiphallius sp.	Fissiphaliidae	3	1 (2 3)	apical-hood	subterminal	complete slit

				sensilla		
Guasinia sp.	Guasiniidae	3	1 (2 3)	apical-hood sensilla	terminal dilatation	complete slit
*Icaleptes sp.**	Icaleptidae*	3	1 (2 3)	apical-hood sensilla	?	complete slit
Minuides rudicoxa Roewer, 1949	Kimulidae	3	1 (2 3)	apical-hood sensilla	?	complete slit
Zalmoxis sp. (Vanuatu)	Zalmoxidae	3	1 (2 3)	apical-hood sensilla	subterminal	complete slit
*Ortholasma pictipes*Banks, 1911	Ortholasmatidae	3	1 2 3	apical-hood sensilla	?	pore-like
Nemastoma bimaculatum Fabricius, 1775	Nemastomatidae	3	1 2 3	apical-hood sensilla	regular	pore-like
Anelasmocephalus sp.	Trogulidae	3	1 2 3	apical-hood sensilla	regular	pore-like
Astrobunus grallator Simon, 1879	Sclerosomatidae	3	(1 2 3)	basiconic-like	not applicable	pore-like
Undet. Sp.	Phalangiidae	3	(1 2 3) / 1 2 3	basiconic-like	not applicable	pore-like
Troglosiro sp.	Troglosironidae	1	not applicable	sub-apical process	not applicable	not applicable
Aoraki longitarsa Forster, 1952	Pettalidae	1	not applicable	sub-apical process	not applicable	not applicable

Table 2: Comparison of morphology, distribution and function between the reported tarsal organs in the arachnid orders Araneae, Amblypygi, Scorpiones, Parasitiformes, Ricinulei and Opiliones.

	Araneae	**Amblypygi**	**Scorpiones**	**Parasitiformes**	**Ricinulei**	**Opiliones**
Appendages	Legs I-IV	Legs I	Legs I-IV	Legs I	Legs I & II	Legs I & II
Position	Last tarsomere, close to the claw, dorsal	Last tarsomere, close to the claw, dorsal	Last tarsomere, close to the claw, dorsal	Last tarsomere, close to the claw, dorsal	Last tarsomere, close to the claw, dorsal	Last tarsomere, close to the claw, dorsal
Cuticular invagination?	Most cases in capsule, but may be exposed	No capsule	No capsule	Anterior capsule and posterior setae cluster	Capsule	Setae cluster
External appearance of the cuticular apparatus	Pore/small peg/button or setae	Button	Button/pore	Setae	Setae	Setae
Function	Thermo/hygro/chemo	?	?	Thermo/hygro/chemo	Chemo; Thermo/hygro?	Thermo/hygro; Chemo?
References	Blumenthal 1923; Foelix & Chu-Wang 1973b; Dumpert 1978; Tichy & Barth 1992; Ehn & Tichy 1993; Anton & Tichy 1994; Tichy & Loftus 1996	Foelix et al 1975; Igelmund 1987; Santer & Hebets 2011	Foelix & Schabronath 1983	Foelix & Axtell 1972	Talarico et al 2005	Willemart et al 2007, 2009; Gainett et al Chapter 2; this study

FIGURES

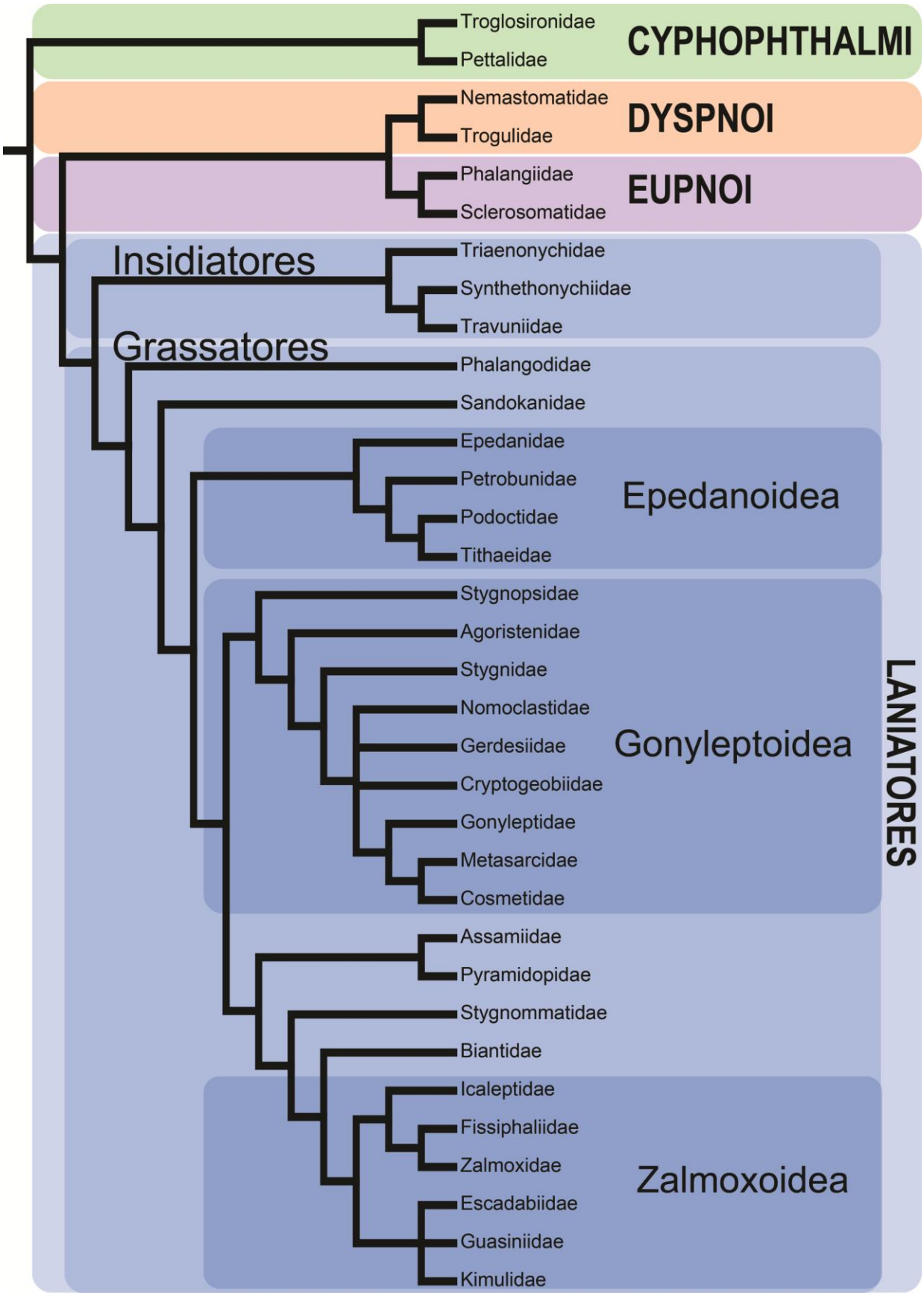

Troglosironidae
Pettalidae
CYPHOPHTHALMI
Nemastomatidae
Trogulidae
DYSPNOI
Phalangiidae
Sclerosomatidae
EUPNOI
Insidiatores
Triaenonychidae
Synthethonychiidae
Travuniidae
Grassatores
Phalangodidae
Sandokanidae
Epedanidae
Petrobunidae
Podoctidae
Tithaeidae
Epedanoidea
Stygnopsidae
Agoristenidae
Stygnidae
Nomoclastidae
Gerdesiidae
Cryptogeobiidae
Gonyleptidae
Metasarcidae
Cosmetidae
Gonyleptoidea
Assamiidae
Pyramidopidae
Stygnommatidae
Biantidae
Icaleptidae
Fissiphaliidae
Zalmoxidae
Escadabiidae
Guasiniidae
Kimulidae
Zalmoxoidea
LANIATORES

Figure 1: Classification of Opiliones, showing the most supported hypothesis of relationship among the suborders Cyphophthalmi, Eupnoi, Dyspnoi and Laniatores, and relationships between the families used in this study. Compiled phylogeny is based on Giribet & Sharma (2015), with updated relationships of most inclusive clades of Laniatores, after a transcriptome based phylogeny by Fernandez, Sharma, Tourinho and Giribet (unpublished).

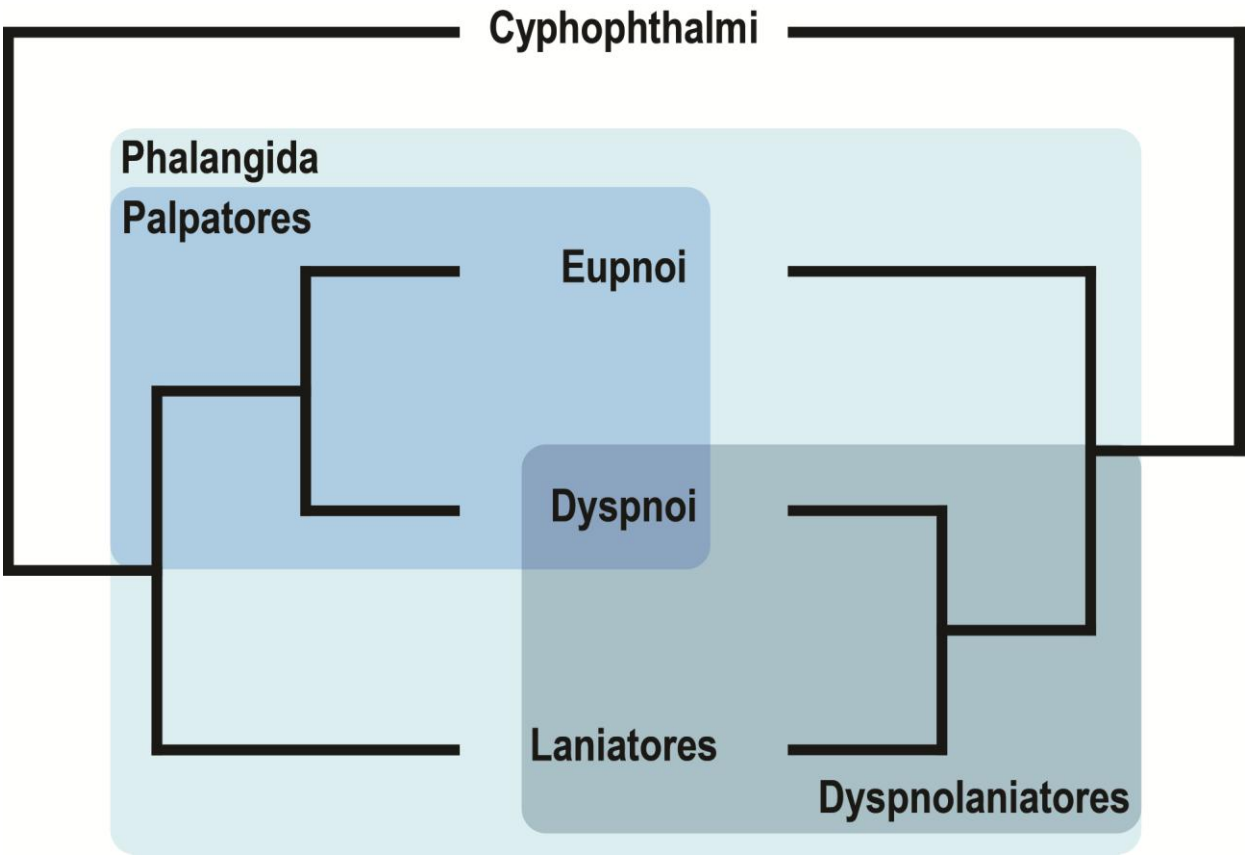


Figure 2: Alternative hypothesis of relationships between the four suborders of Opiliones. Topology on the left shows the Phalangida hypothesis (Giribet et al., 2010; Sharma & Giribet, 2014) and topology on the right shows the Dyspnolaniatores hypothesis (Giribet et al 1999, 2002).

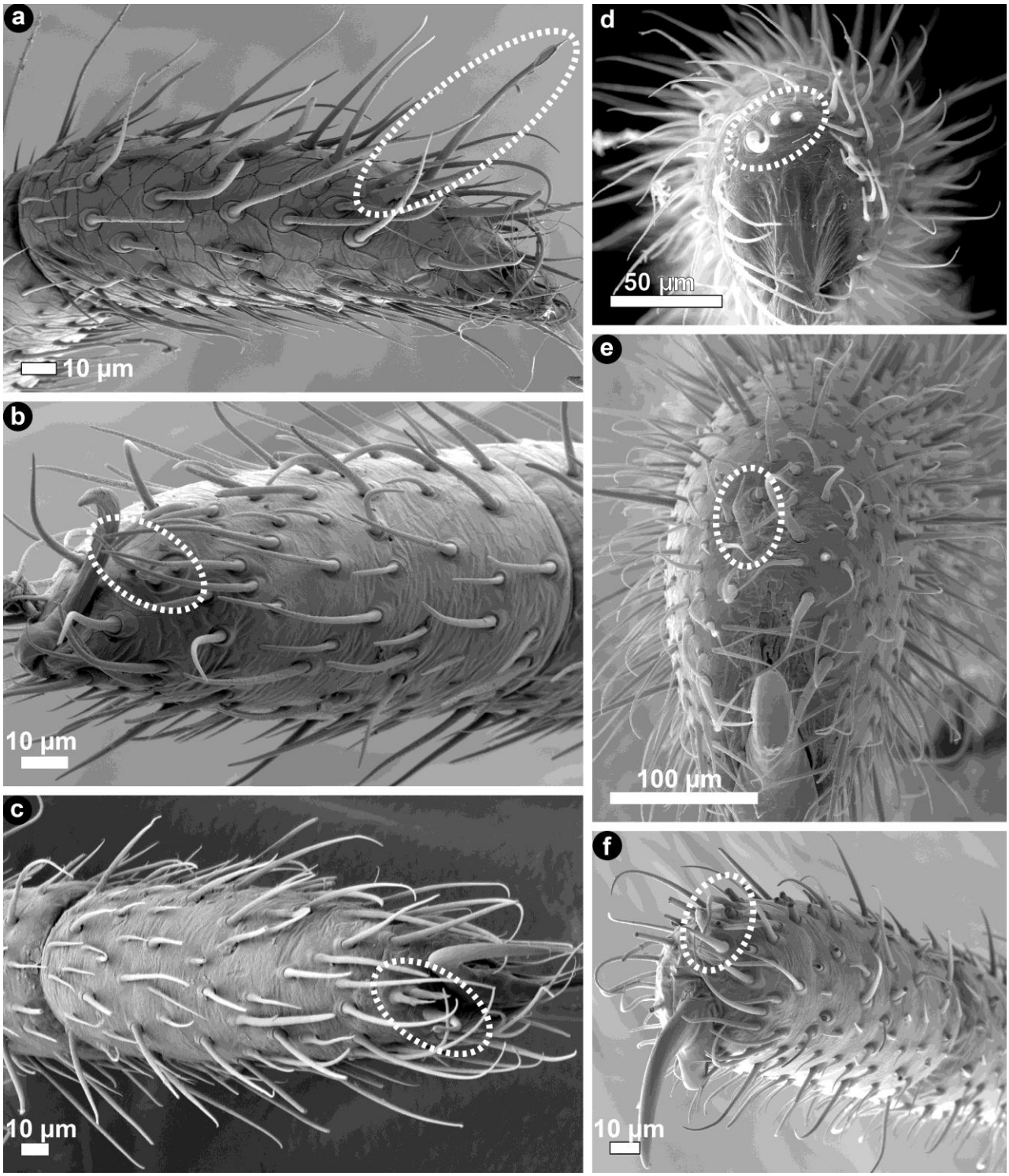


Figure 3: Laniatores. Triad on the sensory field formed by two sensilla basiconica (proximal and middle sensilla) and one apical hood sensillum (distal sensillum) (dotted area) on the distal-most tarsomeres I of selected families. a: *Erebomaster flavescens flavescens* (Travuniidae), undetermined sex. b: *Remyus* sp. (Phalangodidae), female. c: *Pseudoepedanus doiensis* (Epedanidae), male. d: *Avima octomaculata* (Agoristenidae), male. e: *Heteromitobates discolor* (Gonyleptidae), male. f: *Stygnomma bispinatum* (Stygnommatidae), male.

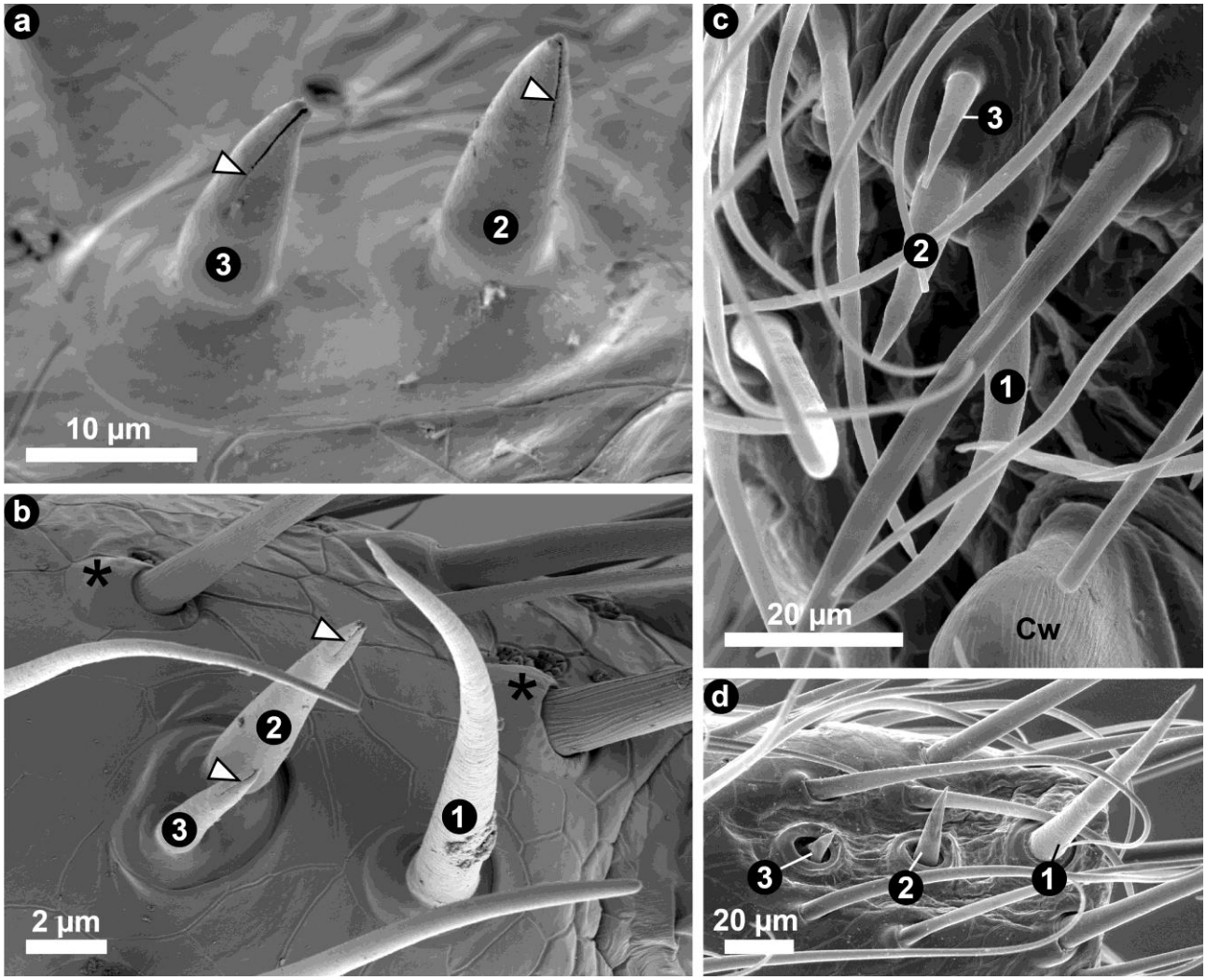


Figure 4: Laniatores. Different associations between the basal membrane of the three sensilla on the sensory field of the distal-most tarsomeres I (c) and II (a, b, d). a: Proximal (3) and middle sensilla (3) of *Hoplobunus* sp. (Stygnopsidae), female. Distal sensilla not shown. b: *Larifuga* cf. *capensis* (Trieanonychiidae), male. Proximal (3) and middle (2) sensilla have fused basal membranes that are isolated from the basal membrane of the distal sensillum (1). c: *Camarana flavipalpis* (Cryptogeobiidae), female. Note that the three sensilla are associated in the same basal membrane. d: *Sodreana sodreana* (Gonyleptidae), male. The three sensilla have individualized basal membranes, with no external association. Asterisk: sensillum chaeticum, socket; Cw: claw; White arrowhead: slit opening.

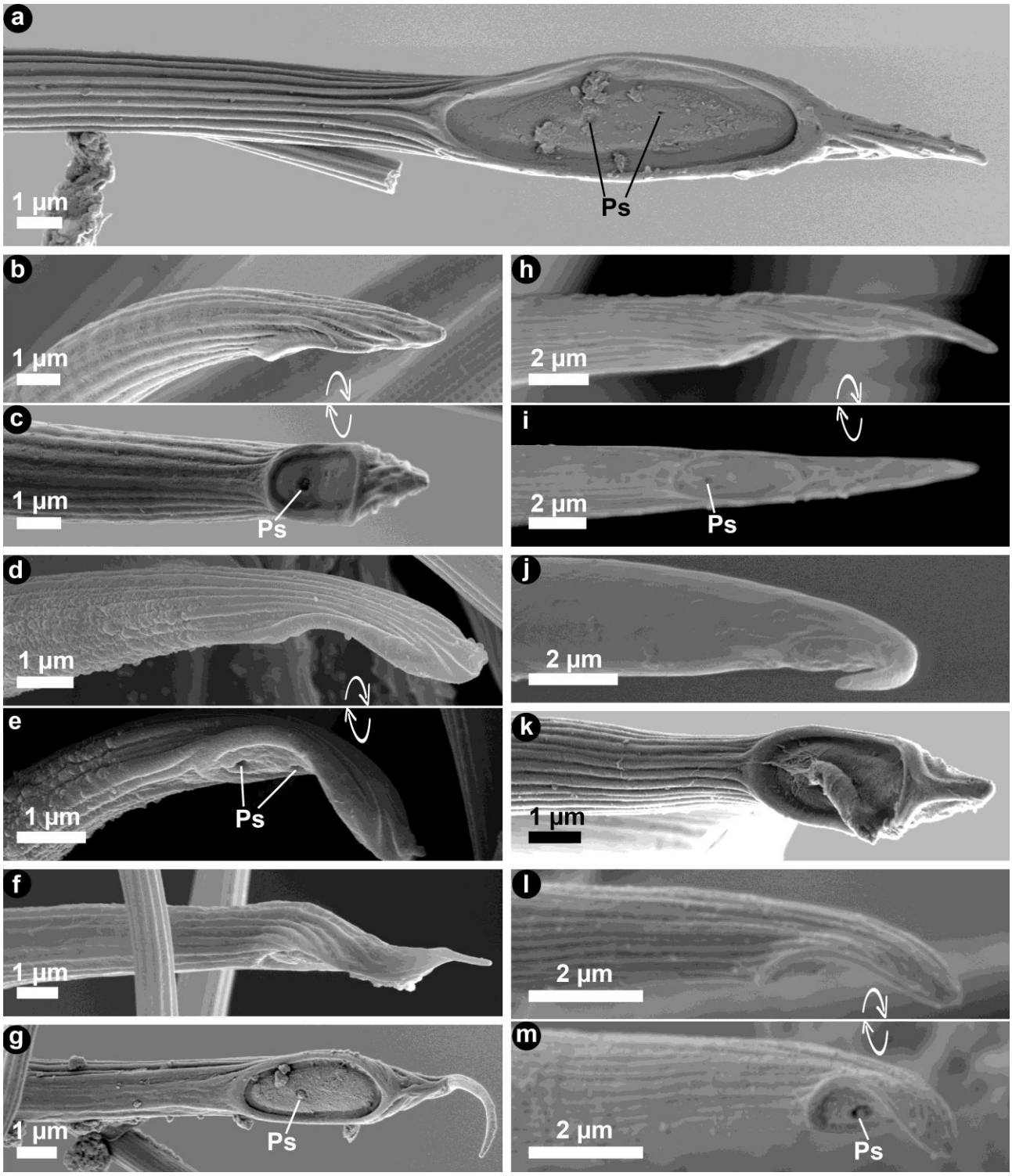

a
1 µm
Ps
b
1 µm
c
1 µm
Ps
d
1 µm
e
1 µm
Ps
f
1 µm
g
1 µm
Ps
h
2 µm
i
2 µm
Ps
j
2 µm
k
1 µm
l
2 µm
m
2 µm
Ps

Figure 5: Laniatores. Tip of the distal sensilla (apical-hood sensillum) on the sensory field in selected families, leg pairs I (a- c, f, g, j) and II (d, e, h, i, k-m) a: *Erebomaster flavescens flavescens* (Travuniidae), undetermined sex. b,c: *Larifuga* cf. *capensis* (Triaenonychidae), male. d,e: *Austribalonius* sp. (Podoctidae), male. f: *Pseudoepedanus doiensis* (Epedanidae), male. g: *Tithaeus* sp. (Tithaeidae), male. h, i: *Hoplobunus* sp.(Stygnopsidae), female. j: *Phareicranaus hermosa* (Gonyleptidae, Cranainae), male. k: *Stygnomma bispinatum* (Stygnommatidae), male. l, m: *Martibianta virginsulana* (Biantidae), female. Circular arrows indicate different views of the same unit. Ps: pore-like structure.

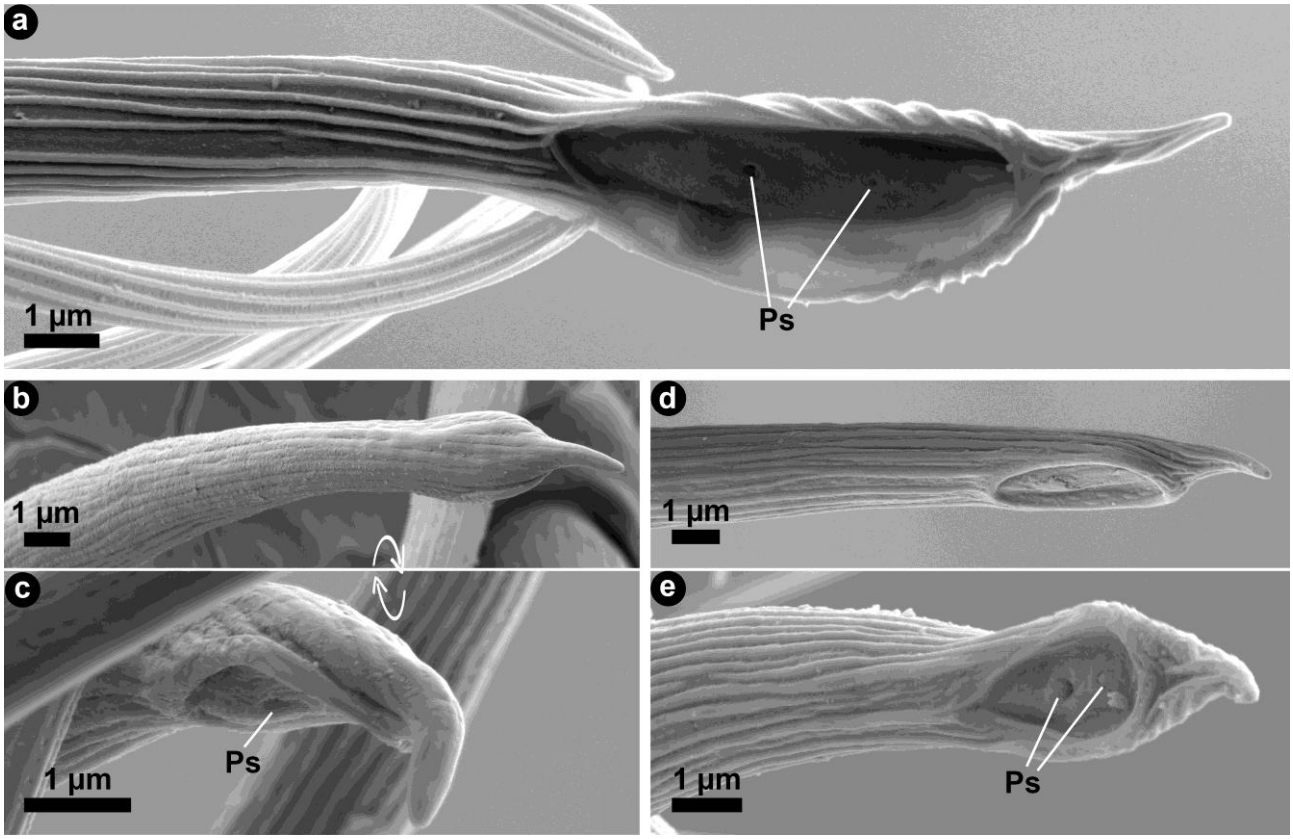


Figure 6: Laniatores. Tip of the distal-most sensilla of the triad (apical-hood sensilla) in selected families of Laniatores, leg pairs I (a-c, d) and II (e). a: cf. *Pyramidops* sp. (Pyramidopidae), undetermined sex. b, c: *Montalenia* sp. (Assamidae), male. d, e: *Baculigerus milenae* (Escadabiidae), male. Circular arrows indicate different views of the same unit. Ps: pore-like structure.

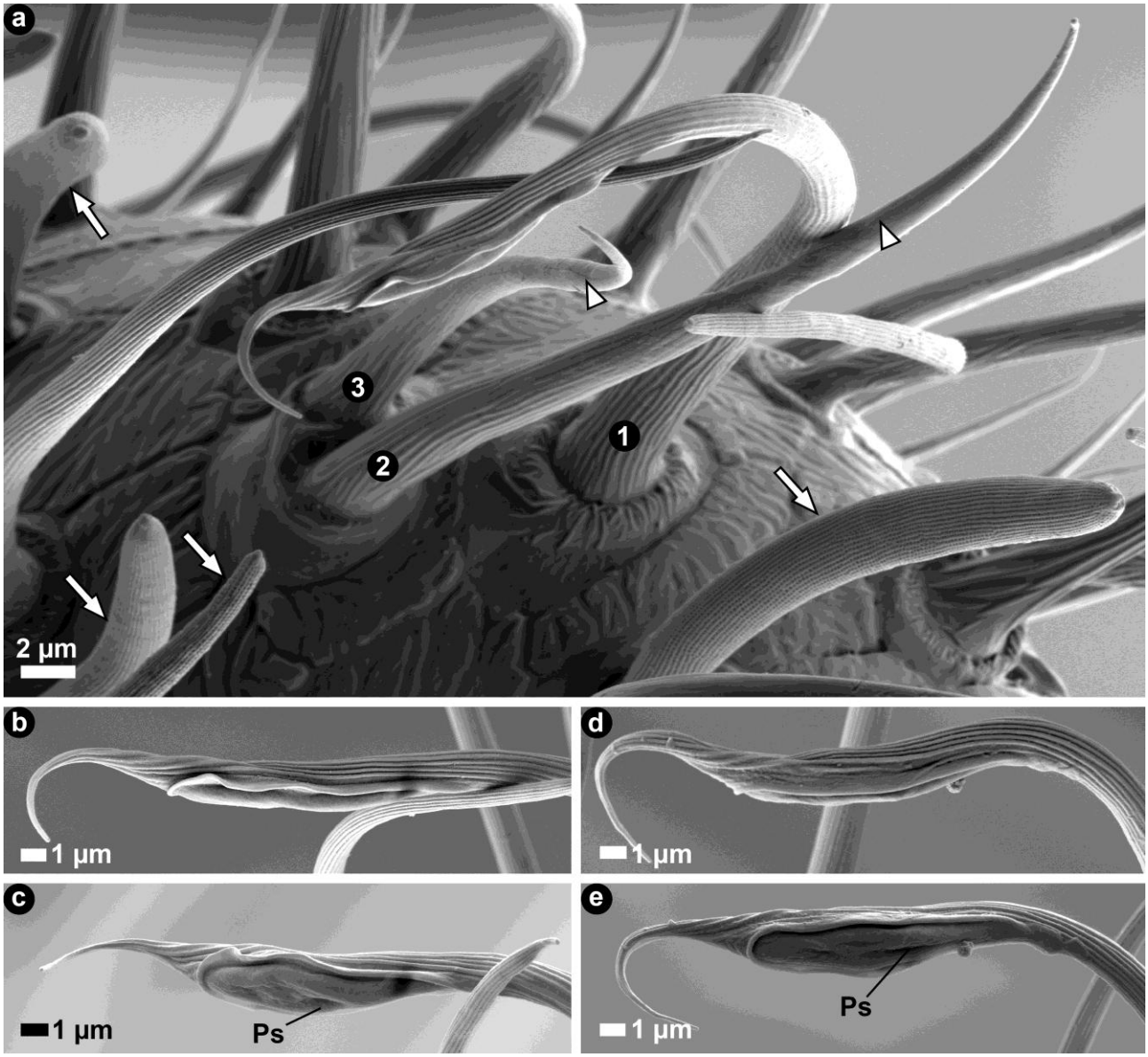


Figure 7: Laniatores. Triad on the sensory field of *Synthetonychia glacialis* (Sythetonychiidae) (male). a: Overview of the three sensilla (1-3), on the distal third of the distal-most tarsomere I, dorsal region. b-e: comparison of the "hood" of the distal sensilla between legs I (b, c) and II (d, e). Ps: pore-like structure; White arrow: wall-pored sensilla chaetica; White arrowhead: slit opening.

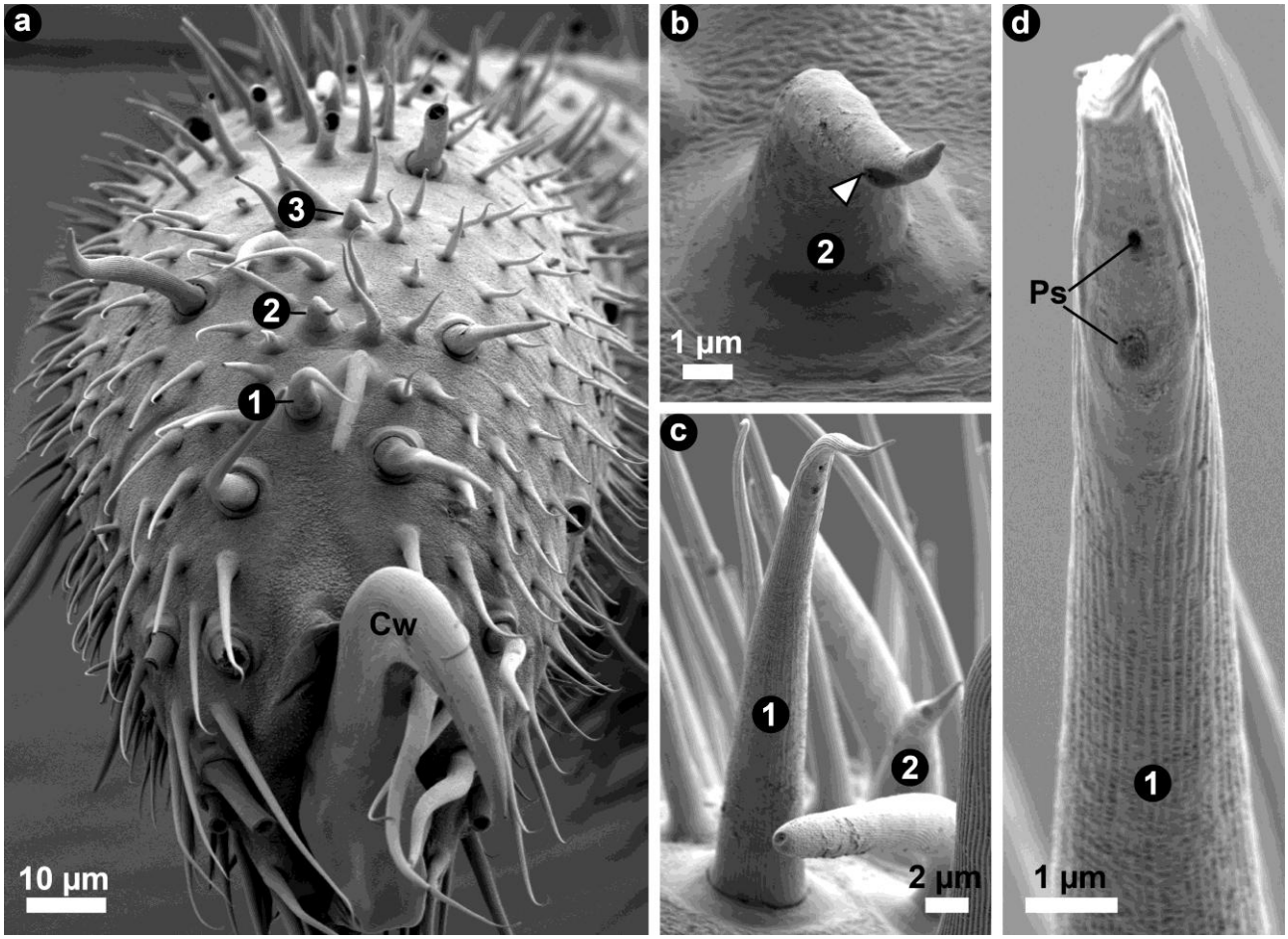


Figure 8: Dyspnoi. Distal-most tarsomere of leg pair II of *Nemastoma bimaculatum* (Nemastomatidae), undetermined sex. a: frontal view of the tarsomere. b: Detail of the middle sensillum in “a” (2). c: The distal sensilla marked in “a” (1), frontal view. d: Detail of the tip of the distal sensilla in “c”. Cw: claw ; Ps: pore-like structure; White arrowhead: slit opening.

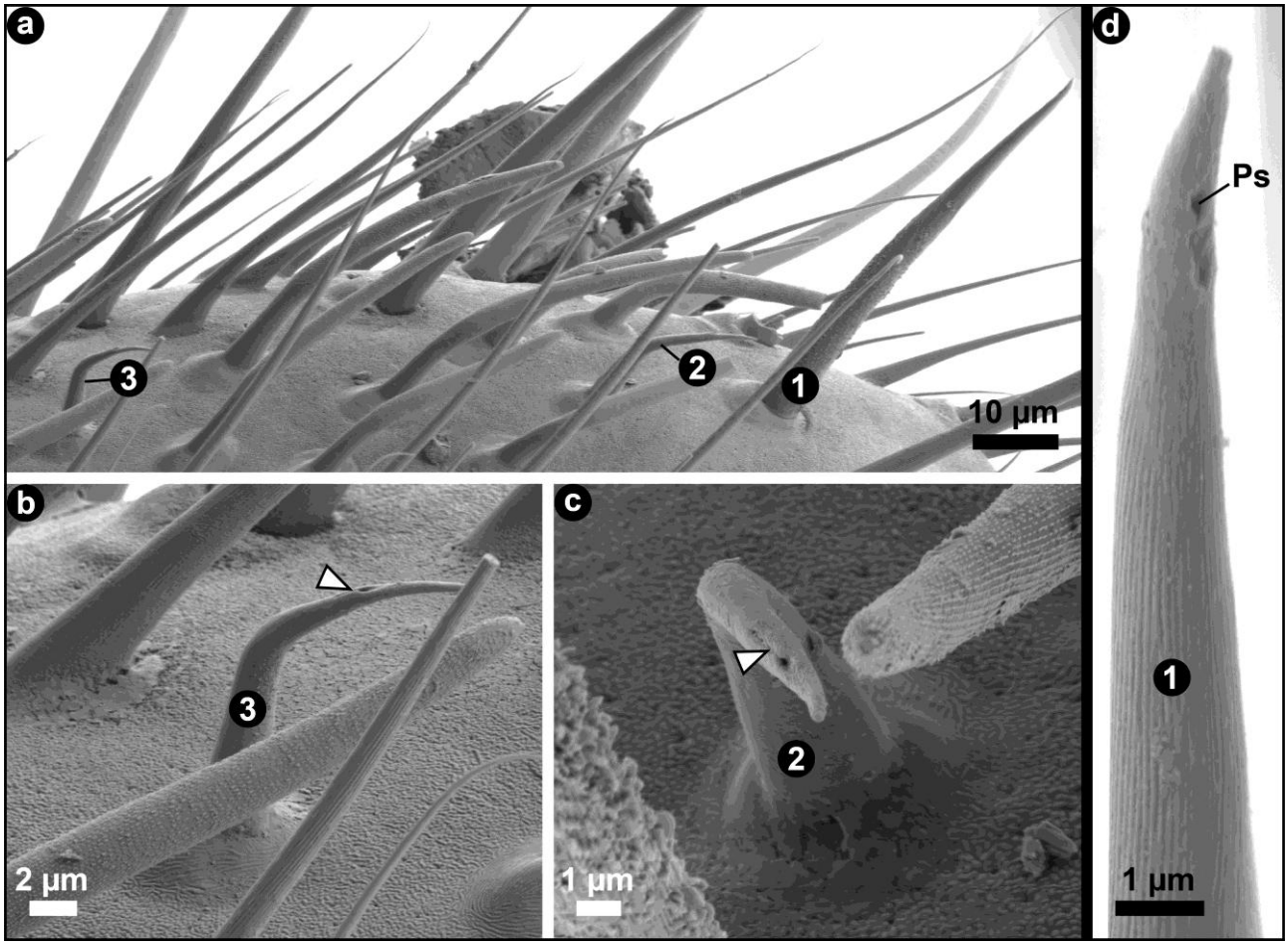


Figure 9: Dyspnoi. *Anelasmocephalus* sp, undetermined sex. a: Dorsal region of the distal third of the distal-most tarsomere II, showing the three sensilla with flat basal membranes (see text for explanation), lateral view. b: Proximal sensilla (3) of the three marked in "a", lateral view. c: Middle sensilla (2) on the same region of leg I, frontal-superior view. d: Detail of the tip of the distal sensilla (1) in "a", lateral view. Ps: pore-like structure; White arrowhead: slit opening.

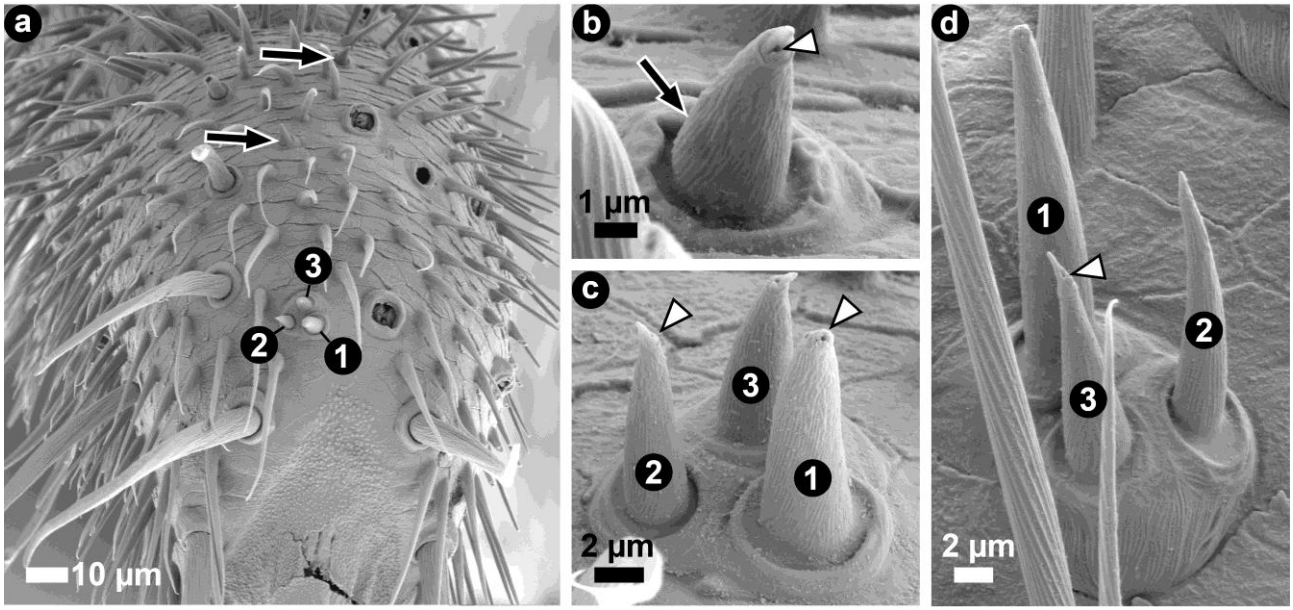


Figure 10: Eupnoi. Undetermined sp. (Phalangiidae), female. a: Frontal view of the last tarsomere I. b: Detail of an isolated sensillum basiconicum proximal to the triad. c: Detail of the triad (1-3) on the distal third of the tarsomere I shown in "a". Note the all thee sensilla share the same basal membrane. d: Detail of the same region on leg II, showing the triad (1-3). Note that the three sensilla have ascendant length of the shaft, from proximal to distal (3 to 1). Black arrow: proximal sensilla basiconica; Ps: pore-like structure; White arrowhead: apical opening.

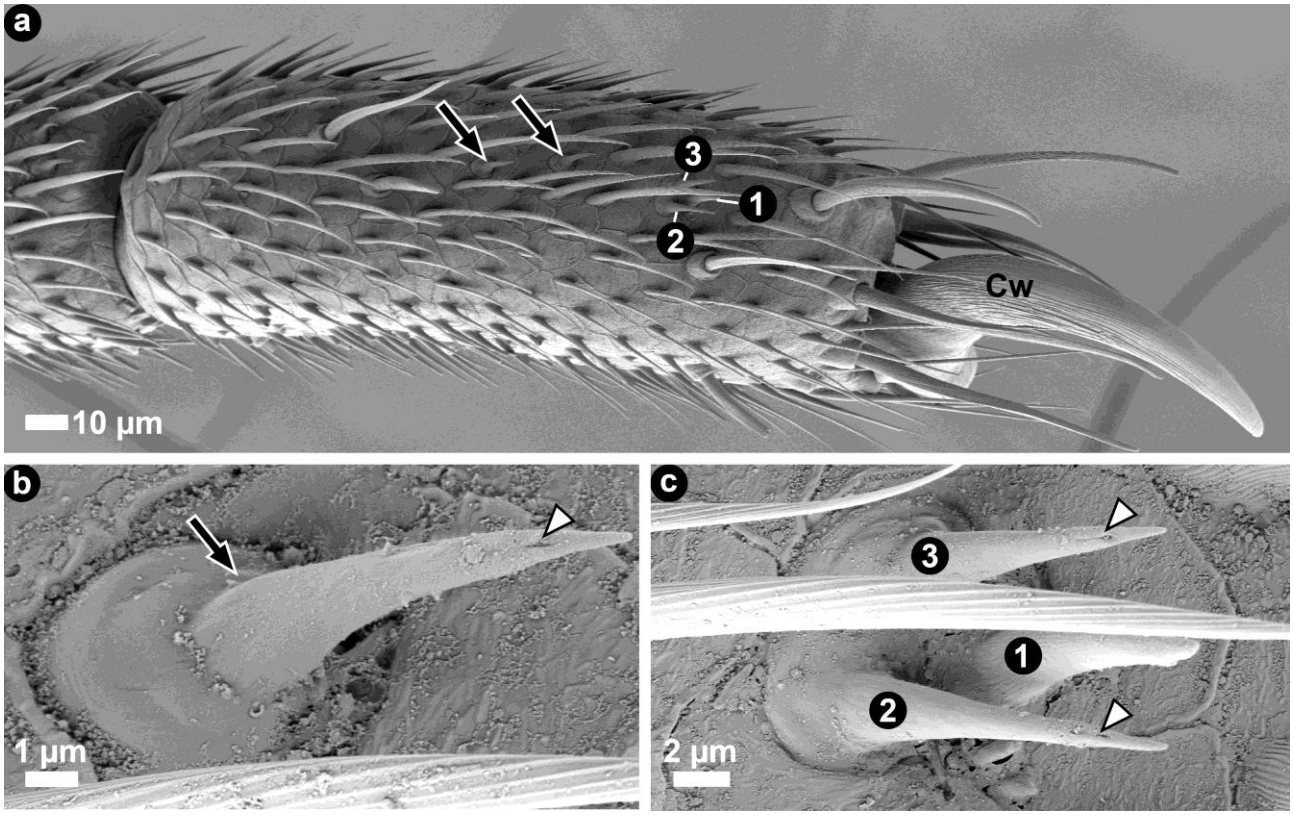


Figure 11: Eupnoi. *Astrobunus grallator* (Sclerosomatidae), undetermined sex. a: Lateral view of the last tarsomere I, indicating three sensilla (1-3) clustered on the distal third region, and two isolated sensilla basiconica on the middle of the tarsomere. b: Detail of an isolated sensillum basiconicum on the last tarsomere I, dorsal region. c: Detail of the three sensilla clustered on "a". Black arrow: proximal sensilla basiconica; Cw: claw; White arrowhead: apical opening.

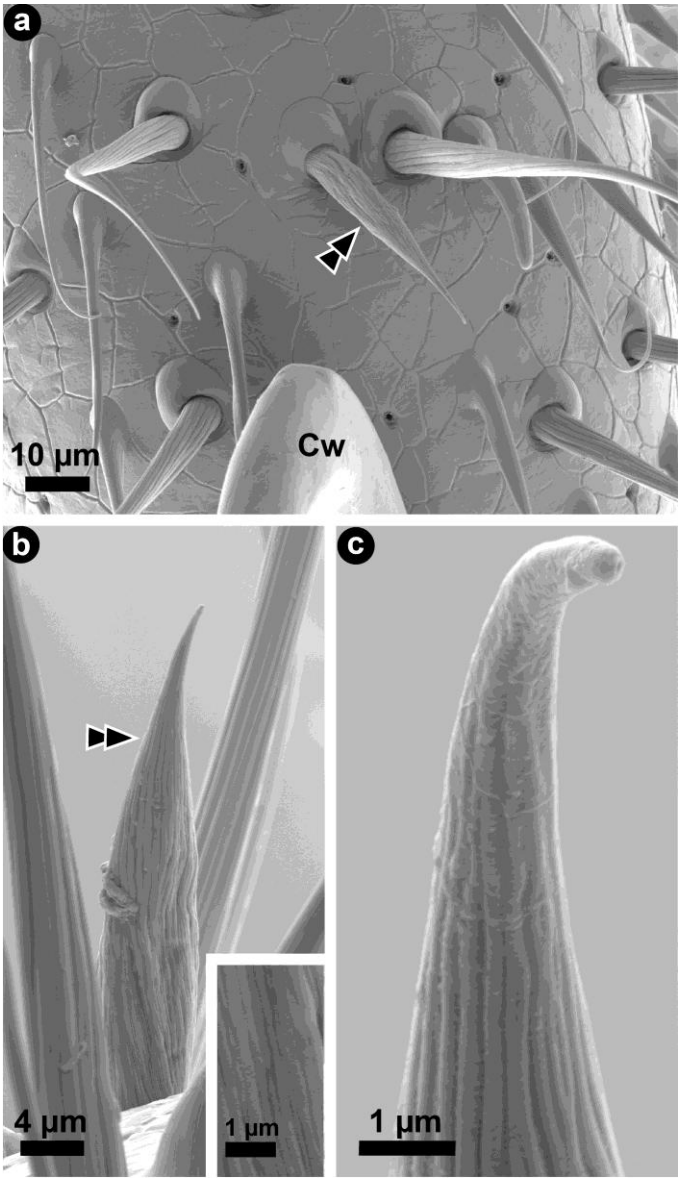


Figure 12: Cyphophthalmi. *Aoraki longitarsa* (Pettalidae), male. a: Frontal view of the tarsi II, showing the sub-apical process. b: Frontal view of the sub-apical process. Insight: detail of the shaft's wall, showing longitudinal ridges. c: Detail of the tip of the sub-apical process in "b". Cw: cla; Double arrowhead: sub-apical process.

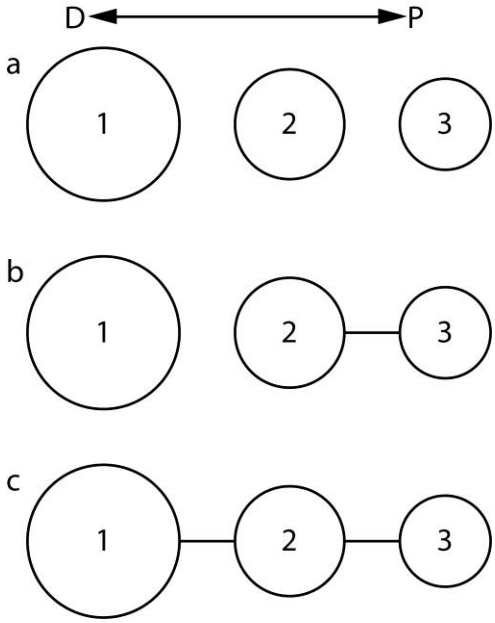


Figure 13: Character 2-Basal membrane association. Schematic representation of character states. a: Character state (2a). Distal, middle and proximal sensilla not fused. b: Character state(2b). Distal sensilla isolated, middle and proximal sensilla fused. c: Character state (2c). Distal, mid and proximal sensilla fused.

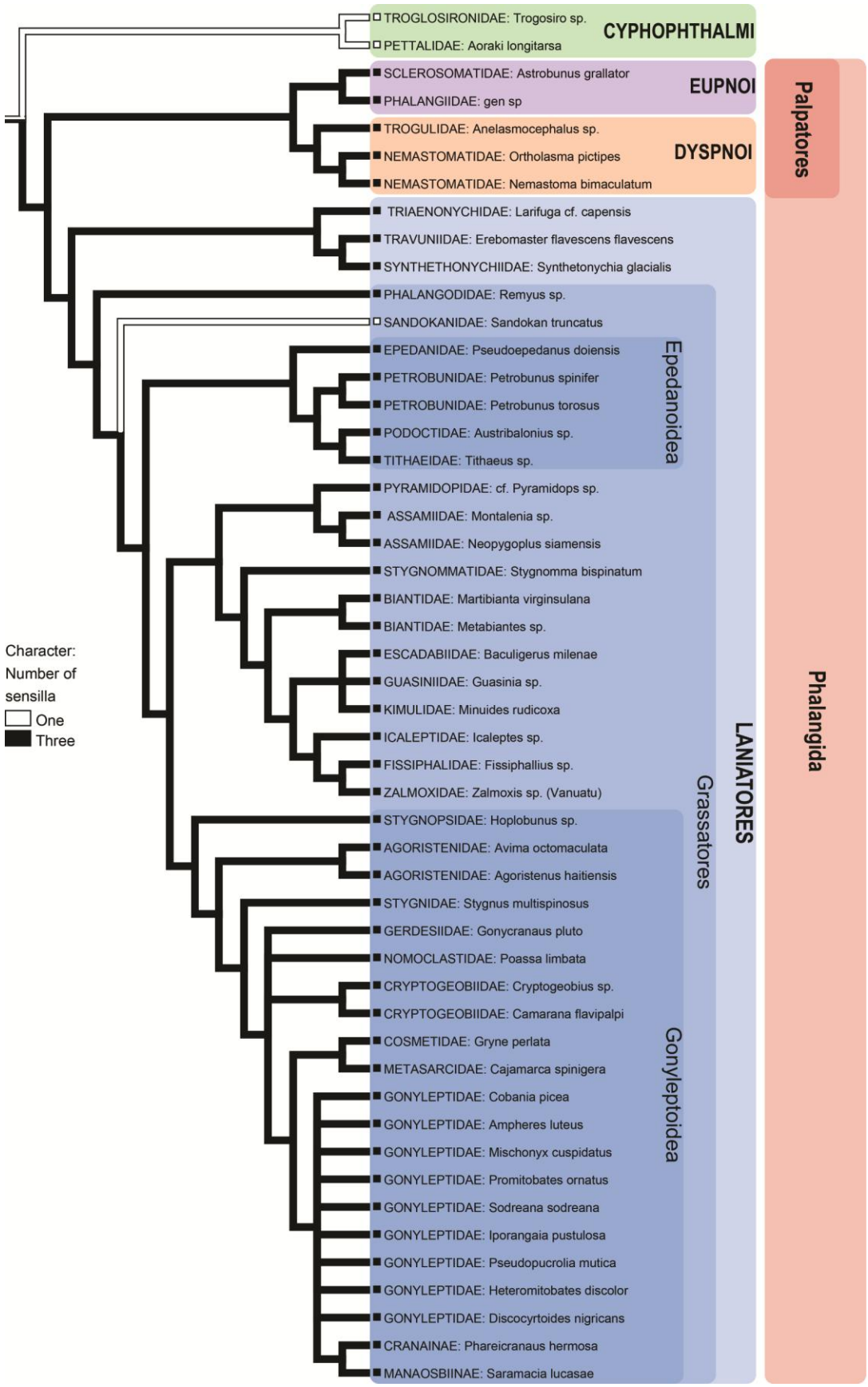
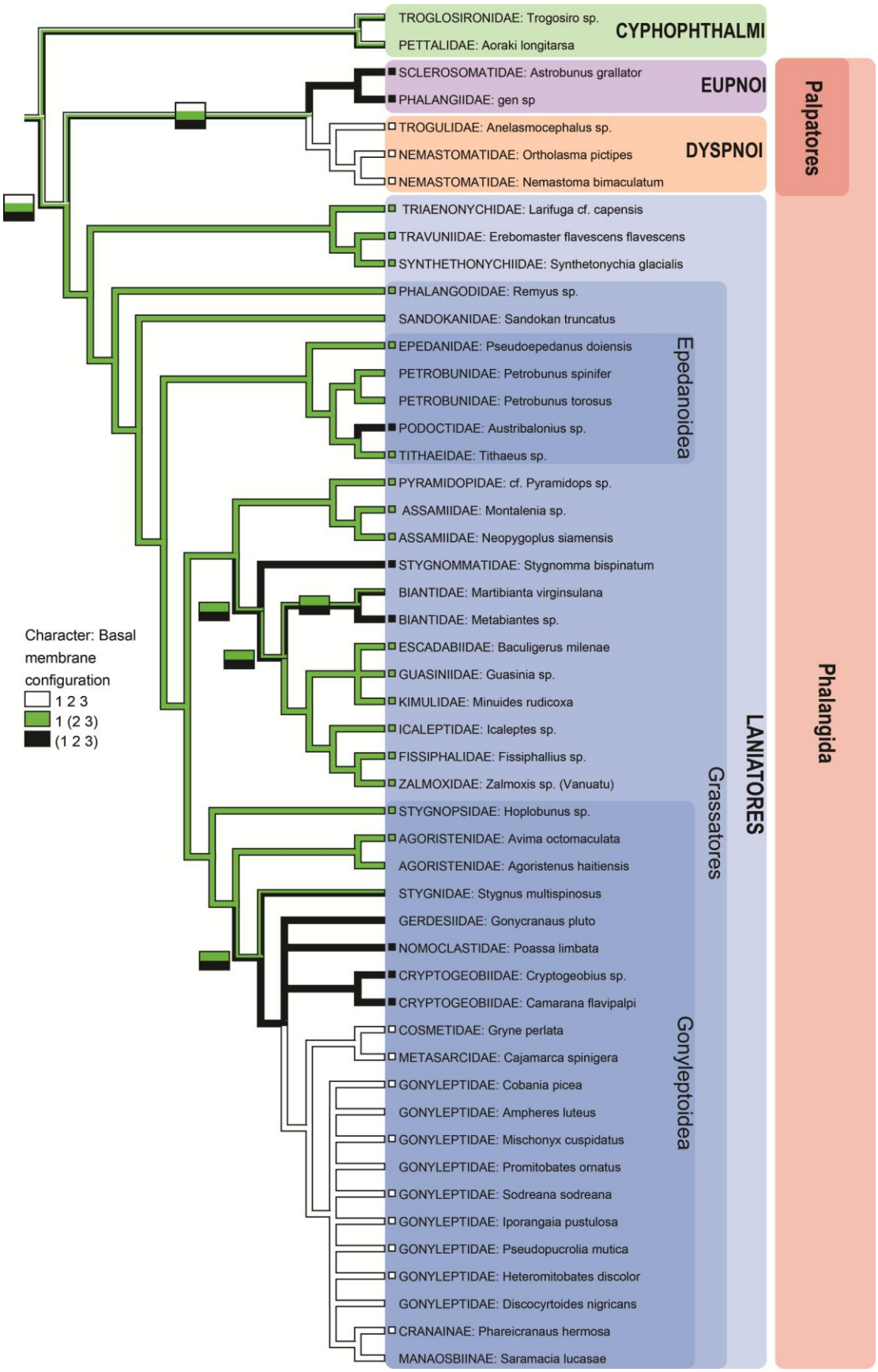

TROGLOSIRONIDAE: Trogosiro sp.
PETTALIDAE: Aoraki longitarsa
CYPHOPHTHALMI
SCLEROSOMATIDAE: Astrobunus grallator
PHALANGIIDAE: gen sp
EUPNOI
TROGULIDAE: Anelasmocephalus sp.
NEMASTOMATIDAE: Ortholasma pictipes
NEMASTOMATIDAE: Nemastoma bimaculatum
DYSPNOI
Palpatores
TRIAENONYCHIDAE: Larifuga cf. capensis
TRAVUNIIDAE: Erebomaster flavescens flavescens
SYNTHETHONYCHIIDAE: Synthetonychia glacialis
PHALANGODIDAE: Remyus sp.
SANDOKANIDAE: Sandokan truncatus
EPEDANIDAE: Pseudoepedanus doiensis
PETROBUNIDAE: Petrobunus spinifer
PETROBUNIDAE: Petrobunus torosus
PODOCTIDAE: Austribalonius sp.
TITHAEIDAE: Tithaeus sp.
Epedanoidea
PYRAMIDOPIDAE: cf. Pyramidops sp.
ASSAMIIDAE: Montalenia sp.
ASSAMIIDAE: Neopygoplus siamensis
STYGNOMMATIDAE: Stygnomma bispinatum
BIANTIDAE: Martibianta virginsulana
BIANTIDAE: Metabiantes sp.
ESCADABIIDAE: Baculigerus milenae
GUASINIIDAE: Guasinia sp.
KIMULIDAE: Minuides rudicoxa
ICALEPTIDAE: Icaleptes sp.
FISSIPHALIDAE: Fissiphallius sp.
ZALMOXIDAE: Zalmoxis sp. (Vanuatu)
Grassatores
LANIATORES
Phalangida
STYGNOPSIDAE: Hoplobunus sp.
AGORISTENIDAE: Avima octomaculata
AGORISTENIDAE: Agoristenus haitiensis
STYGNIDAE: Stygnus multispinosus
GERDESIIDAE: Gonycranaus pluto
NOMOCLASTIDAE: Poassa limbata
CRYPTOGEOBIIDAE: Cryptogeobius sp.
CRYPTOGEOBIIDAE: Camarana flavipalpi
COSMETIDAE: Gryne perlata
METASARCIDAE: Cajamarca spinigera
GONYLEPTIDAE: Cobania picea
GONYLEPTIDAE: Ampheres luteus
GONYLEPTIDAE: Mischonyx cuspidatus
GONYLEPTIDAE: Promitobates ornatus
GONYLEPTIDAE: Sodreana sodreana
GONYLEPTIDAE: Iporangaia pustulosa
GONYLEPTIDAE: Pseudopucrolia mutica
GONYLEPTIDAE: Heteromitobates discolor
GONYLEPTIDAE: Discocyrtoides nigricans
CRANAINAE: Phareicranaus hermosa
MANAOSBIINAE: Saramacia lucasae
Gonyleptoidea
Character:
Number of
sensilla
One
Three

Figure 14: Ancestral state reconstruction of “Character (1) Number of sensilla” on a compiled phylogeny of Opiliones after Giribet & Sharma (2015) and Fernandez, Sharma, Tourinho and Giribet (unpublished). White: (1a) One. Black (1b) Three. Branches with more than one color represent equally parsimonious reconstructions.


TROGLOSIRONIDAE: Trogosiro sp.
PETTALIDAE: Aoraki longitarsa
CYPHOPHTHALMI
SCLEROSOMATIDAE: Astrobunus grallator
PHALANGIIDAE: gen sp
EUPNOI
TROGULIDAE: Anelasmocephalus sp.
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NEMASTOMATIDAE: Nemastoma bimaculatum
DYSPNOI
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PETROBUNIDAE: Petrobunus torosus
PODOCTIDAE: Austribalonius sp.
TITHAEIDAE: Tithaeus sp.
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ASSAMIIDAE: Montalenia sp.
ASSAMIIDAE: Neopygoplus siamensis
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NOMOCLASTIDAE: Poassa limbata
CRYPTOGEOBIIDAE: Cryptogeobius sp.
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METASARCIDAE: Cajamarca spinigera
GONYLEPTIDAE: Cobania picea
GONYLEPTIDAE: Ampheres luteus
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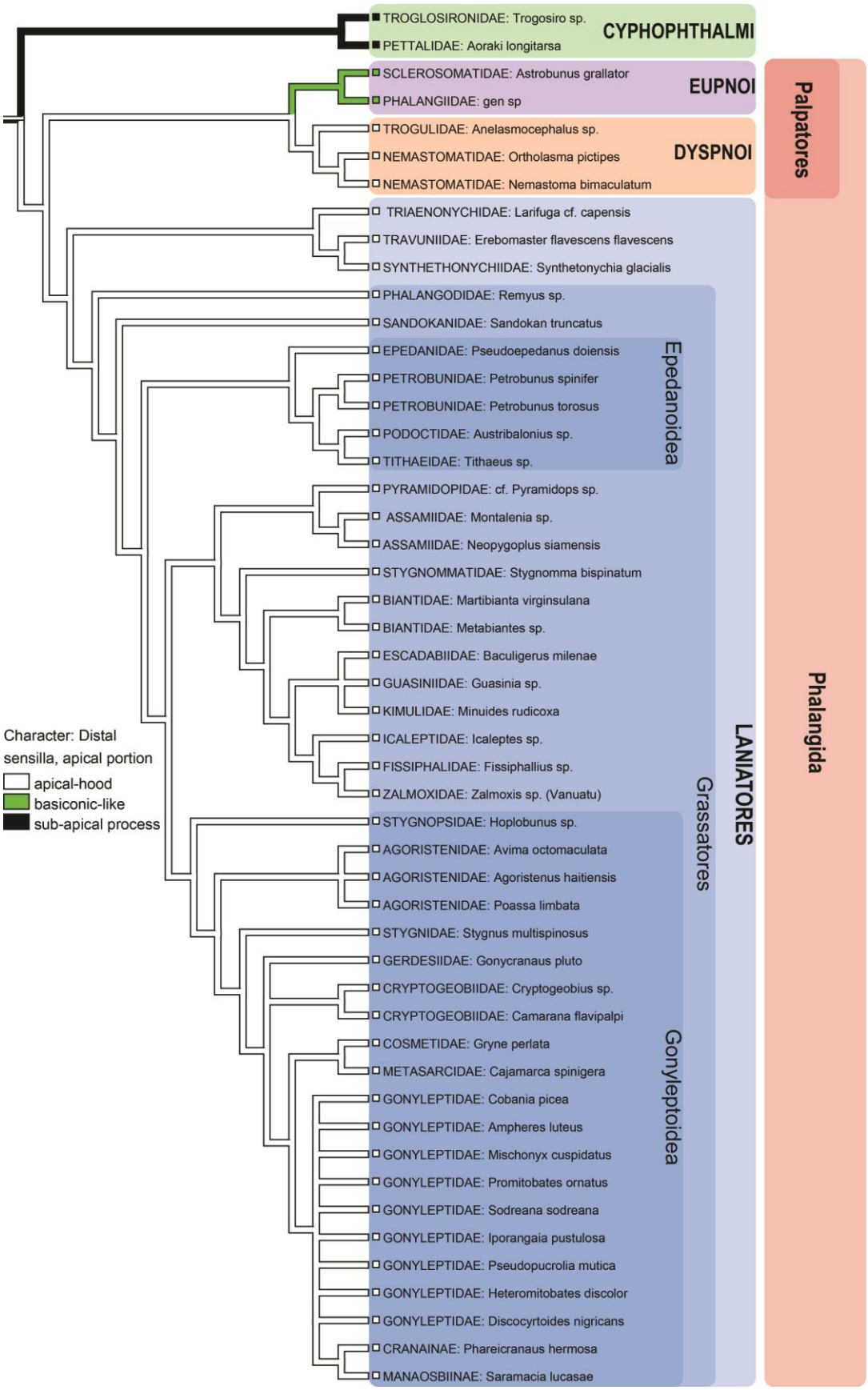
GONYLEPTIDAE: Pseudopucrolia mutica
GONYLEPTIDAE: Heteromitobates discolor
GONYLEPTIDAE: Discocyrtoides nigricans
CRANAINAE: Phareicranaus hermosa
MANAOSBIINAE: Saramacia lucasae
Grassatores
Gonyleptoidea
LANIATORES
Phalangida
Character: Basal membrane configuration
1 2 3
1 (2 3)
(1 2 3)

Figure 15: Ancestral state reconstruction of “Character (2) Basal membrane association” on the same topology as in Figure 13. White: (2a) Distal, middle and proximal sensilla not fused. Green: (2b) Distal sensilla isolated, middle and proximal fused sensilla. Black: (2c) Distal, mid and proximal sensilla fused. Branches with more than one color represent equally parsimonious reconstructions. This character is not applicable to the terminals in Cyphophthalmi and Sandokanidae (Laniatores). Other terminals without squares are missing data.


TROGLOSIRONIDAE: Trogosiro sp.
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SCLEROSOMATIDAE: Astrobunus grallator
PHALANGIIDAE: gen sp
EUPNOI
TROGULIDAE: Anelasmocephalus sp.
NEMASTOMATIDAE: Ortholasma pictipes
NEMASTOMATIDAE: Nemastoma bimaculatum
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PETROBUNIDAE: Petrobunus torosus
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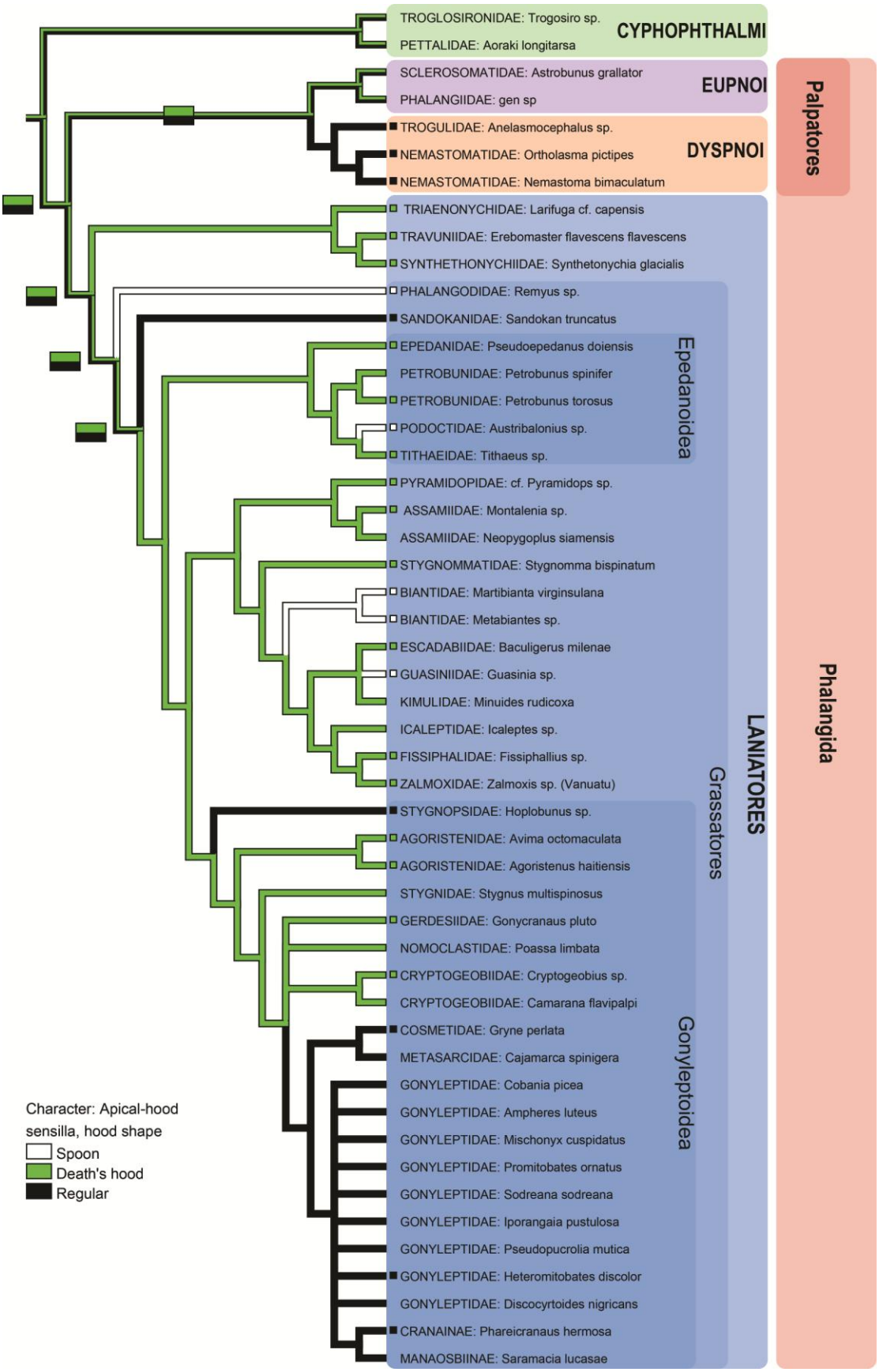
TITHAEIDAE: Tithaeus sp.
Epedanoidea
PYRAMIDOPIDAE: cf. Pyramidops sp.
ASSAMIIDAE: Montalenia sp.
ASSAMIIDAE: Neopygoplus siamensis
STYGNOMMATIDAE: Stygnomma bispinatum
BIANTIDAE: Martibianta virginsulana
BIANTIDAE: Metabiantes sp.
ESCADABIIDAE: Baculigerus milenae
GUASINIIDAE: Guasinia sp.
KIMULIDAE: Minuides rudicoxa
ICALEPTIDAE: Icaleptes sp.
FISSIPHALIDAE: Fissiphallius sp.
ZALMOXIDAE: Zalmoxis sp. (Vanuatu)
Grassatores
STYGNOPSIDAE: Hoplobunus sp.
AGORISTENIDAE: Avima octomaculata
AGORISTENIDAE: Agoristenus haitiensis
AGORISTENIDAE: Poassa limbata
STYGNIDAE: Stygnus multispinosus
GERDESIIDAE: Gonycranaus pluto
CRYPTOGEOBIIDAE: Cryptogeobius sp.
CRYPTOGEOBIIDAE: Camarana flavipalpi
COSMETIDAE: Gryne perlata
METASARCIDAE: Cajamarca spinigera
GONYLEPTIDAE: Cobania picea
GONYLEPTIDAE: Ampheres luteus
GONYLEPTIDAE: Mischonyx cuspidatus
GONYLEPTIDAE: Promitobates ornatus
GONYLEPTIDAE: Sodreana sodreana
GONYLEPTIDAE: Iporangaia pustulosa
GONYLEPTIDAE: Pseudopucrolia mutica
GONYLEPTIDAE: Heteromitobates discolor
GONYLEPTIDAE: Discocyrtoides nigricans
CRANAINAE: Phareicranaus hermosa
MANAOSBIINAE: Saramacia lucasae
Gonyleptoidea
LANIATORES
Phalangida
Character: Distal sensilla, apical portion
apical-hood
basiconic-like
sub-apical process

Figure 16: Ancestral state reconstruction of “Character (3) Distal sensilla, sensillar type” on the same topology as in Figure 13. White: (3a) Apical-hood sensilla. Green: (3b) Sensilla basiconica. Black: (3c) Sub-apical process.


TROGLOSIRONIDAE: Trogosiro sp.
PETTALIDAE: Aoraki longitarsa
CYPHOPHTHALMI
SCLEROSOMATIDAE: Astrobunus grallator
PHALANGIIDAE: gen sp
EUPNOI
TROGULIDAE: Anelasmocephalus sp.
NEMASTOMATIDAE: Ortholasma pictipes
NEMASTOMATIDAE: Nemastoma bimaculatum
DYSPNOI
Palpatores
TRIAENONYCHIDAE: Larifuga cf. capensis
TRAVUNIIDAE: Erebomaster flavescens flavescens
SYNTHETHONYCHIIDAE: Synthetonychia glacialis
PHALANGODIDAE: Remyus sp.
SANDOKANIDAE: Sandokan truncatus
EPEDANIDAE: Pseudoepedanus doiensis
PETROBUNIDAE: Petrobunus spinifer
PETROBUNIDAE: Petrobunus torosus
PODOCTIDAE: Austribalonius sp.
TITHAEIDAE: Tithaeus sp.
Epedanoidea
PYRAMIDOPIDAE: cf. Pyramidops sp.
ASSAMIIDAE: Montalenia sp.
ASSAMIIDAE: Neopygoplus siamensis
STYGNOMMATIDAE: Stygnomma bispinatum
BIANTIDAE: Martibianta virginsulana
BIANTIDAE: Metabiantes sp.
ESCADABIIDAE: Baculigerus milenae
GUASINIIDAE: Guasinia sp.
KIMULIDAE: Minuides rudicoxa
ICALEPTIDAE: Icaleptes sp.
FISSIPHALIDAE: Fissiphallius sp.
ZALMOXIDAE: Zalmoxis sp. (Vanuatu)
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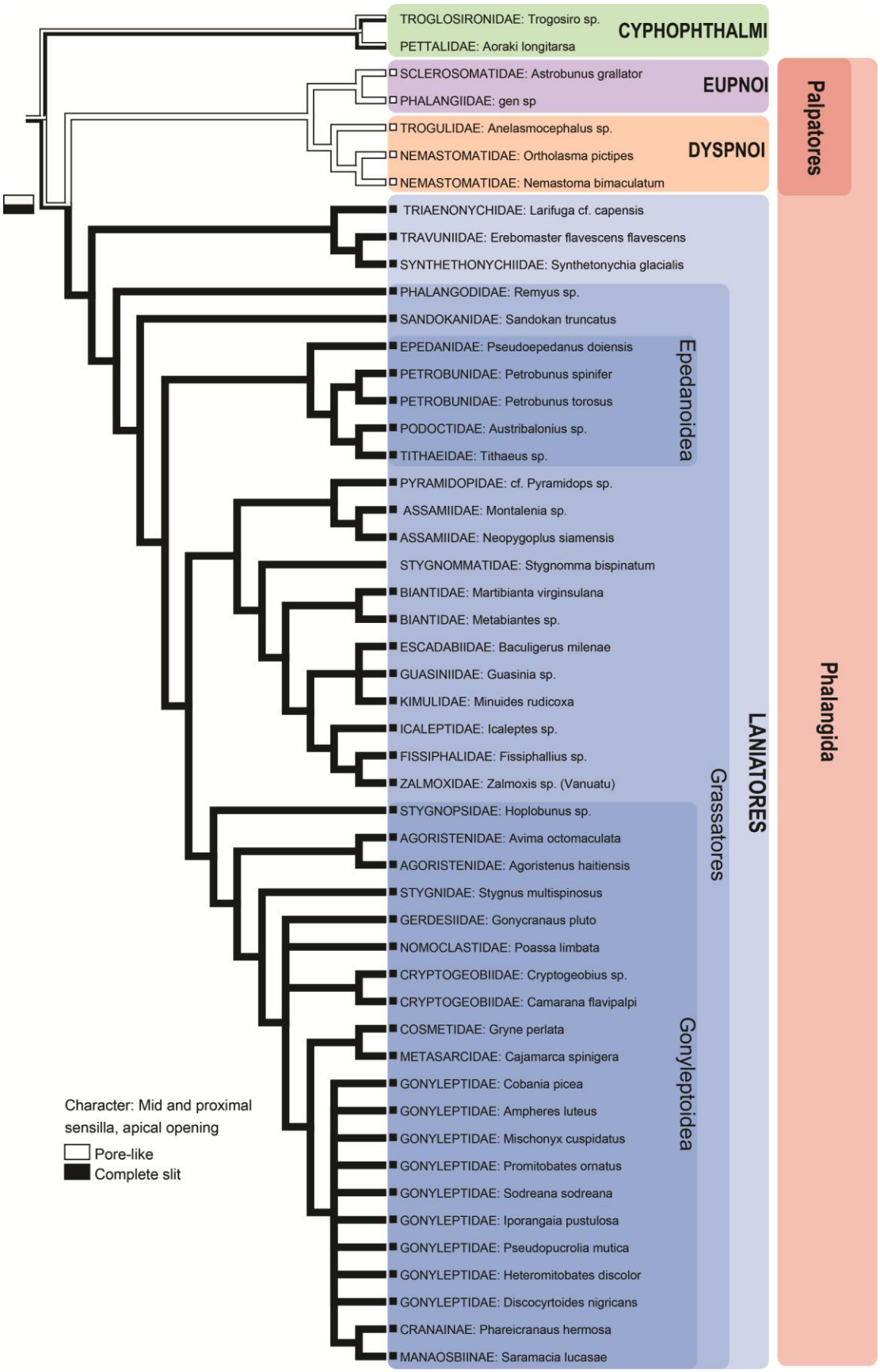
AGORISTENIDAE: Agoristenus haitiensis
STYGNIDAE: Stygnus multispinosus
GERDESIIDAE: Gonycranaus pluto
NOMOCLASTIDAE: Poassa limbata
CRYPTOGEOBIIDAE: Cryptogeobius sp.
CRYPTOGEOBIIDAE: Camarana flavipalpi
Grassatores
COSMETIDAE: Gryne perlata
METASARCIDAE: Cajamarca spinigera
GONYLEPTIDAE: Cobania picea
GONYLEPTIDAE: Ampheres luteus
GONYLEPTIDAE: Mischonyx cuspidatus
GONYLEPTIDAE: Promitobates ornatus
GONYLEPTIDAE: Sodreana sodreana
GONYLEPTIDAE: Iporangaia pustulosa
GONYLEPTIDAE: Pseudopucrolia mutica
GONYLEPTIDAE: Heteromitobates discolor
GONYLEPTIDAE: Discocyrtoides nigricans
CRANAINAE: Phareicranaus hermosa
MANAOSBIINAE: Saramacia lucasae
Gonyleptoidea
LANIATORES
Phalangida
Character: Apical-hood sensilla, hood shape
Spoon
Death's hood
Regular

Figure 17: Ancestral state reconstruction of “Character (4) Apical-hood sensilla, hood morphology” on the same topology as in Figure 13. White: (4a) Terminal swelling (spoon-shaped). Green: (4b) Sub-terminal swelling (“death’s hood”). Black: (4c) No swelling (regular). Branches with more than one color represent equally parsimonious reconstructions. This character is not applicable to the terminals in Cyphophthalmi and Eupnoi. Other terminals without squares are missing data.


TROGLOSIRONIDAE: Trogosiro sp.
PETTALIDAE: Aoraki longitarsa
CYPHOPHTHALMI
SCLEROSOMATIDAE: Astrobunus grallator
PHALANGIIDAE: gen sp
EUPNOI
TROGULIDAE: Anelasmocephalus sp.
NEMASTOMATIDAE: Ortholasma pictipes
NEMASTOMATIDAE: Nemastoma bimaculatum
DYSPNOI
Palpatores
TRIAENONYCHIDAE: Larifuga cf. capensis
TRAVUNIIDAE: Erebomaster flavescens flavescens
SYNTHETHONYCHIIDAE: Synthetonychia glacialis
PHALANGODIDAE: Remyus sp.
SANDOKANIDAE: Sandokan truncatus
EPEDANIDAE: Pseudoepedanus doiensis
PETROBUNIDAE: Petrobunus spinifer
PETROBUNIDAE: Petrobunus torosus
PODOCTIDAE: Austribalonius sp.
TITHAEIDAE: Tithaeus sp.
Epedanoidea
PYRAMIDOPIDAE: cf. Pyramidops sp.
ASSAMIIDAE: Montalenia sp.
ASSAMIIDAE: Neopygoplus siamensis
STYGNOMMATIDAE: Stygnomma bispinatum
BIANTIDAE: Martibianta virginsulana
BIANTIDAE: Metabiantes sp.
ESCADABIIDAE: Baculigerus milenae
GUASINIIDAE: Guasinia sp.
KIMULIDAE: Minuides rudicoxa
ICALEPTIDAE: Icaleptes sp.
FISSIPHALIDAE: Fissiphallius sp.
ZALMOXIDAE: Zalmoxis sp. (Vanuatu)
STYGNOPSIDAE: Hoplobunus sp.
AGORISTENIDAE: Avima octomaculata
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STYGNIDAE: Stygnus multispinosus
GERDESIIDAE: Gonycranaus pluto
NOMOCLASTIDAE: Poassa limbata
CRYPTOGEOBIIDAE: Cryptogeobius sp.
CRYPTOGEOBIIDAE: Camarana flavipalpi
COSMETIDAE: Gryne perlata
METASARCIDAE: Cajamarca spinigera
GONYLEPTIDAE: Cobania picea
GONYLEPTIDAE: Ampheres luteus
GONYLEPTIDAE: Mischonyx cuspidatus
GONYLEPTIDAE: Promitobates ornatus
GONYLEPTIDAE: Sodreana sodreana
GONYLEPTIDAE: Iporangaia pustulosa
GONYLEPTIDAE: Pseudopucrolia mutica
GONYLEPTIDAE: Heteromitobates discolor
GONYLEPTIDAE: Discocyrtoides nigricans
CRANAINAE: Phareicranaus hermosa
MANAOSBIINAE: Saramacia lucasae
Grassatores
Gonyleptoidea
LANIATORES
Phalangida
Character: Mid and proximal sensilla, apical opening
Pore-like
Complete slit

Figure 18: Ancestral state reconstruction of “Character (5) Middle and proximal sensilla, apical opening” on the same topology as in Figure 13. White: (5a) Pore-like. Black: (5b) Complete slit. Branches with more than one color represent equally parsimonious reconstructions. This character is not applicable to the terminals in Cyphophthalmi and Sandokanidae (Laniatores). Other terminals without squares are missing data.

Conclusão

As informações de ultraestrutura de sensilla aqui apresentadas revelam características histológicas do sistema sensorial nunca antes investigadas nesse grupo, como padrões de inervação periférica e organização básica de uma sensilla. Além disso, fornecem evidência de olfação em uma grande parte das famílias de Laniatores e a primeira evidência morfológica de higro- e termorreceptores em opiliões. Essas informações seviram de base para comparação de sensilla entre grupos de opiliões e fomentar seu uso como caracteres para sistemática. As informações sobre a distribuição filogenética das sensilla na ponta das pernas I e II (candidatos à higro/termorreceptores) são uma primeira tentativa de usar cerdas tarsais como caracteres, as quais possuem demonstrável potencial para elucidar relações filogenéticas em vários níveis de relacionamento. Além disso, colocam em perspectiva os resultados obtidos com a espécie *Heteromitobates discolor*, permitindo generalização de algumas informações de ultraestrutura para um maior número de espécies de Opiliones e contribuindo para o conhecimento da biologia sensorial do grupo.

Por fim, esse estudo evidencia diversas características nas sensilla de opiliões - como canais longitudinais em diversas cerdas olfativas de parede simples, fendas longitudinais nas sensilla basiconica e sua evaporação, a dupla inervação e estrutura emaranhada da parede da apical-hood sensilla – que são de difícil comparação com a literatura de sensilla de insetos. Defendo que o fato dessas características aparentemente fugirem do padrão e serem incomuns é na verdade devido à falta de conhecimento sobre sensilla em aracnídeos em geral. A literatura em Insecta é vasta e muito avançada em relação ao que se sabe sobre aracnídeos. Isso fica ainda mais evidente se considerarmos o fato de que ao menos quatro eventos independentes de colonização do meio terrestre ocorreram no filo Arthropoda (Insecta, crustáceos Isopoda, Myriapoda e Chelicerata) (Lozano-Fernandez et al., 2016). Ao menos quatro vezes essas linhagens foram selecionadas para sentir cheiro, perceber mudanças de temperatura e de umidade num ambiente com ar, o que, apesar da incrível universalidade do plano básico de uma sensilla (eg. Altner e Prillinger, 1980; Keil e Steinbrecht, 1984), nos levaria a esperar soluções ligeiramente diferentes. Portanto, é de se esperar que novos estudos com aracnídeos cada vez mais encontrem excentricidades no funcionamento das sensilla. Um promissor campo de investigação é a ontogenia de uma sensillum em aracnídeos, que conta com apenas 4 publicações (Araneae: Harris 1977; Acariformes: Haupt e Coineu, 1978;Solifugae: Haupt, 1982;Uropygi: Haupt, 1996), comparadas com mais de um século com muitos estudos em insetos. Investigações nessa linha poderiam responder

perguntas como:“Double-walled sensilla em aracnídeos são formadas ontogeneticamente da mesma forma que as de insetos? (eg. Ameismeier 1985); “Quais similaridades do plano básico de uma sensilla de um inseto e de um aracnídeo são homologias e quais são convergências?; “Se são convergências morfológicas, seriam os genes envolvidos no desenvolvimento homólogos ou exclusivos de cada grupo? Espero que algumas das informações aqui levantadas fomentem o interesse por perguntas em qualquer um desses níveis de indagação: desde a anatomia do cílio sensorial da apical-hood sensilla e seu uso na sistemática, até, quem sabe, a evolução dos sistemas sensoriais em Arthropoda.

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Haupt, J., Coineau, Y., 1978. Moulting and morphogenesis of sensilla in a prostigmate mite (Acari, Actinotrichida, Actinedida: Caeculidae). Cell Tissue Res. 186, 63–79. doi:10.1007/BF00219655

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SUPPLEMENTARY MATERIAL

Chapter 3

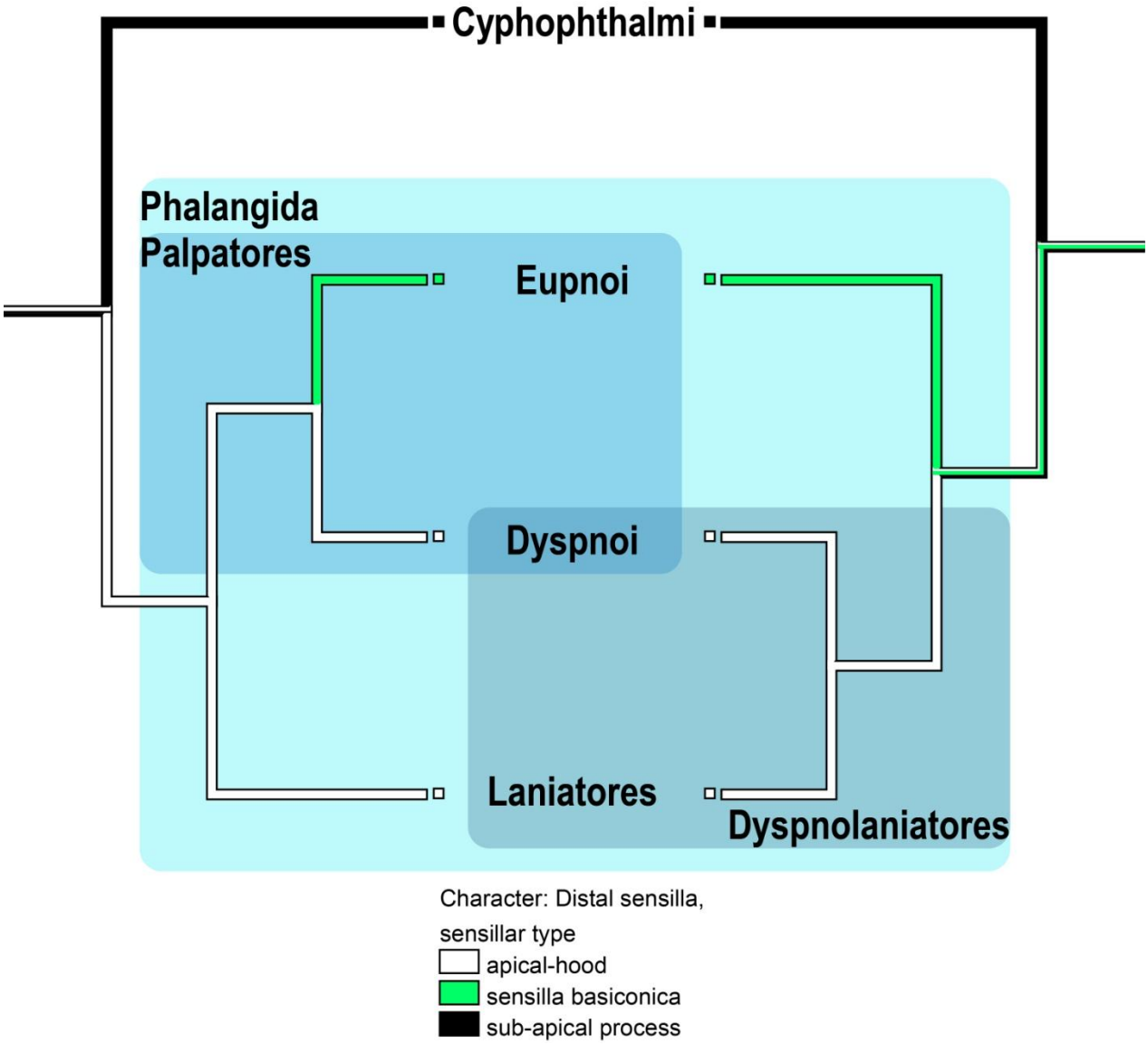
Supplementary material 1, table: List of species used in this study, with accession numbers and gender when available. *Cajamarca spinigera* is a new synonym of *Palcares spiniger* (Bennedeti 2012, unpublished master´s thesis.

	Taxonomy	Voucher #	Locality	Coordinates	Individuals #	Male	Female
Erebomaster flavescens flavescens Cope, 1872	Travuniidae/Cladonychinae	MCZ DNA101444	USA: Patton Cave, Deam Wilderness Area, Indiana	n/a	1	?	?
Synthetonychia glacialis Forster, 1954	Synthetonychiidae	MCZ DNA101718	New Zealand: Minnehaha walk, Fox Glacier, South Island	43 28 7 S 170 1 5 E	1	X	
Larifuga cf. *capensis*	Triaenonychiidae	MCZ DNA100727	South Africa: Newlands Forest. Table Mountain, Cape Province	n/a	1	X	
Montalenia sp.	Assamidae	MCZ DNA105667	Cameroon: Campo Reserve	2º 44.465' 0"N 9º 52.908' 0" E	1	X	
Neopygoplus siamensis	Assamidae	MCZ DNA 104858	Thailand: Kanchanaburi	14º 38.136' 0" N 98º 59.837' 0" E	1	X	
cf. *Pyramidops* sp.	Pyramidopidae	MCZ DNA101432	Equatorial Guinea: Montaña Chocolate, Niefano District	1º45’25”N, 10º17’03”E	1	?	?
Pseudoepedanus doiensis Suzuki, 1969	Epedanidae	MCZ DNA101438	Thailand: Huaykhok Ma, Doi Suthep, Chiang Mai	n/a	1	X	
Petrobunus spinifer Sharma & Giribet, 2011	Petrobunidae	MHNG PAL 09/04	Philippines: El Nido, Palawan, Bulalacao Waterfall	11º13' 41" N 119º28’00" E	1	?	
Petrobunus torosus Sharma & Giribet, 2011	Petrobunidae	MHNG PAL 09/07	Philippines, Palawan, Puerto Princesa Region, Sabang Underground National Park, Daylight Hole	10º09'06"N, 118º53'10"E	1	X	

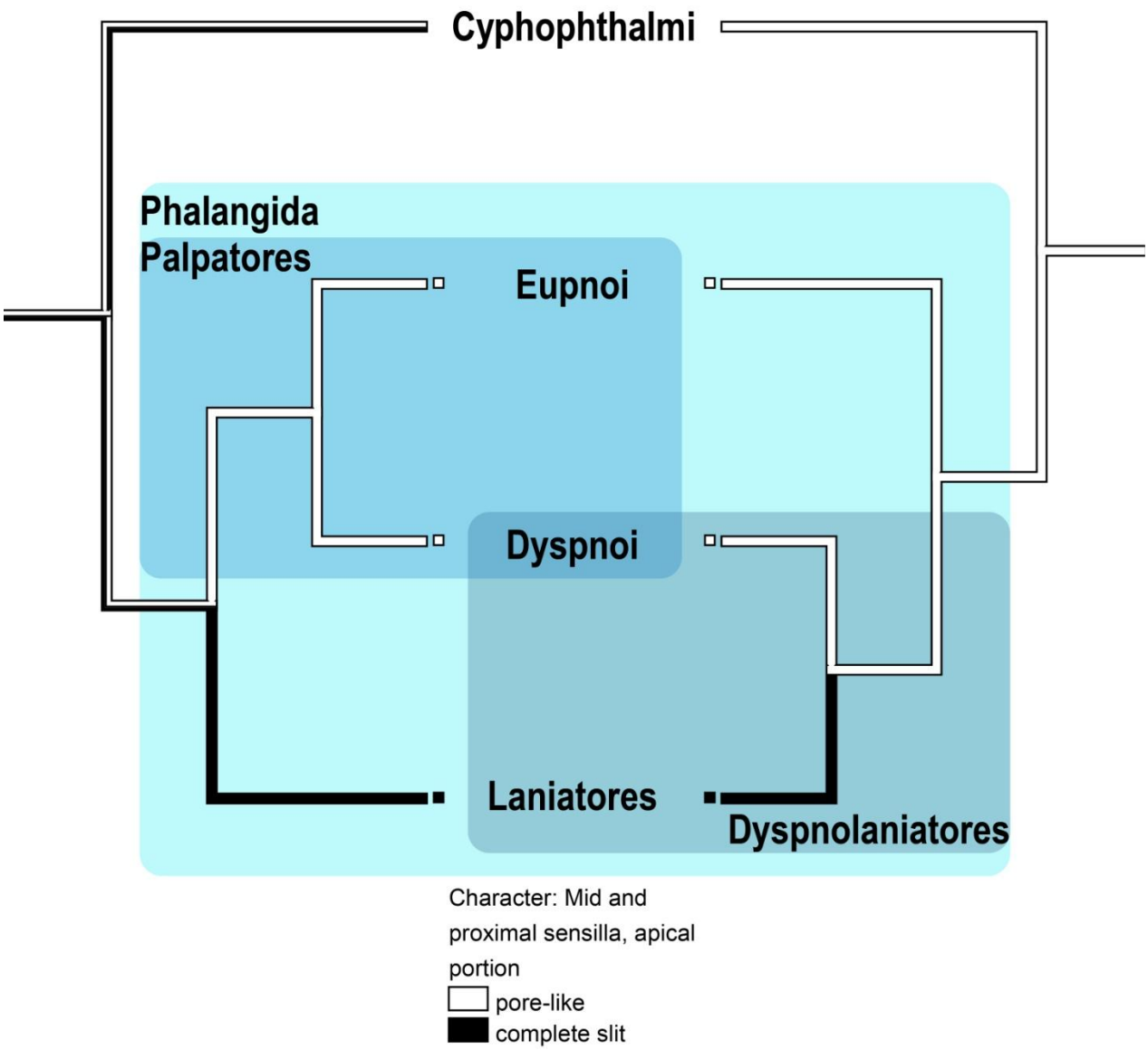
			and Lions Cave				
Austribalonius sp.	Podoctidae	MCZ DNA 106334	Australia: Ella Bay	17° 28´ 39.3´´ S 145° 4´ 22.2´´ E	1	X	
Sandokan truncatus Thorell, 1891	Sandokanidae	MCZ DNA101099	Singapore: Bukit Timah Nature Reserve, Jungle Fall Valley	10 20 53.3 N 103 46 35.4 E	1	X	
Tithaeus sp.	Tithaeidae	MCZ DNA104074	Malaysia: Terengganu state	5º53’52”N, 102º44’2”E	1	X	
Avima octomaculata Roewer, 1963	Agoristenidae	MZUSP	Peru, Cajamarca, Parque Nacional Cutervo (near Cueva San Andrés)	6º13'49"S 78º44'49"W	2	X	X
Agoristenus haitensis Šilhavý, 1973	Agoristenidae	2927X P	República dominicana, La Vega	n/a	1	X	
Poassa limbata Roewer, 1943	Nomoclastinae	1318X P; to be deposited	Costa Rica, Limon, Liverpool	n/a	1		
Gryne perlata	Cosmetidae	?nathi	?nathi	n/a	2	X	X
Phareicranaus hermosa Pinto-da Rocha & Kury 2003	Gonyleptidae: Cranainae	1319-G* and OP-999*	Equador, Napo, Archidona, Rio Holin (Sacha Wagra Lodge)	0º 57' S 77º 41'W	2	X	X
Cobania picea Bertkau, 1880	Gonyleptidae: Cobaniinae	MZUSP 21709	?	n/a	2	X	X
Ampheres luteus Giltay, 1928	Gonyleptidae: Caelopyginae	MZUSP-27692; MZUSP-15163	?	n/a	2	X	X
Cryptogeobius sp.	Cryptogeobiidae	3055X G	Brazil, RJ, Rio de Janeiro	n/a	1	X	
Camarana flavipalpi B. Soares, 1945	Cryptogeobiidae	MZUSP-30630	?	n/a	2	X	X
Gonycranaus pluto Bragagnolo et al, 2015	Gerdesiidae	1598X P	Brazil, MG, Morro do Pilar	n/a	1	X	
Cajamarca spinigera Roewer, 1957	Metasarcidae	1076X P; to be deposited	Peru	n/a	1	X	
Mischonyx cuspidatus Roewer, 1913	Gonyleptidae: Gonyleptinae	MZUSP31970; MZUSP-31979	?	n/a	2	X	X
Promitobates ornatus Roewer, 1931	Gonyleptidae: Mitobatinae	MZUSP-3977; MZUSP-320	?	n/a	2	X	X

Sodreana sodreana Mello-Leitão, 1922	Gonyleptidae: Sodreaninae	MZUSP-32604; MZUSP-32603	?	n/a	2	X	X
Iporangaia pustulosa Mello-Leitão, 1935	Gonyleptidae: Progonyleptoideliinae	MZUSP 16709	?	n/a	2	X	X
Pseudopucrolia mutica (Perty, 1833)	Gonyleptidae	MZUSP 26934	?	n/a	2	X	X
Heteromitobates discolor Sørensen, 1884	Gonyleptidae: Goniosomatinae	to be deposited	?	n/a	2	X	X
Discocyrtoides nigricans Mello-Leitão, 1922	Gonyleptidae	MZUSP 29455	?	n/a	2	X	X
Saramacia lucasae Jim & Soares, 1991	Manaosbiidae	P500; to be deposited	Brazil: Manaus (Reserva do Km 41)-AM	n/a	2	X	X
Stygnus multispinosus Piza, 1938	Stygnidae	MZUSP 15166	?	n/a	2	X	X
Hoplobunus sp.	Stygnopsidae	MZUSP 29077	Mexico: 25 km of Valle Nacional on Highway 125 Ooixaca-Tuxtepec, Ooaxaca	n/a	2	X	X
Remyus sp.	Phalangodidae	MCZ DNA 102660	Madagascar: Toliara Prov., Parc National d'Andohahela, Foret d'Ambohibory	24 55' 48"S 46 38' 44" E	1		X
Martibianta virginsulana Silhavy, 1973	Biantidae	MZUSP-16816	Tortola-BVI Sage Mt. Nat. Park	n/a	1		X
Metabiantes sp.	Biantidae	MCZ DNA100704	Swaziland: Sarah campsite (old archaeological site), Mlabula Nature Reserve	26 11 44 S 31 59 24 E	1	?	?
Neoscotolemon sp.	Insertae sedis	MZUSP-56000	Puerto Rico, Rio Grande, 4km,	n/a	1	?	?
Stygnomma bispinatum Goodnight & Goodnight, 1953	Stygnommatidae	MCZ DNA105636	Mexico: Chiapas. Sierra Morena	16 9 123 N 93 36 2.8 W	1	X	
Baculigerus milenae Kury, 2012	Escadabiidae	MCZ DNA100640	Brazil: Parque Ecológico de Cocó, Fortaleza	n/a	1	X	
Fissiphallius sp.	Fissiphaliidae	MCZ	Colombia:	5 42 43 N 73	1		X

		DNA104057	Santuario de Fauna y Flora Iguaque, Departamento de Boyacá	27 44 W			
Guasinia sp.	Guasiniidae	MCZ DNA 105838	Cuba: Santiago de Cuba Prov., Rio la Mula	n/a	1	?	?
Icaleptes sp.	Icaleptidae	MCZ DNA101420	Colombia: Reserva Natural Río Ñambí, Município de Barbacoas	1 17 6 N 78 4 25 W	1	?	?
Minuides rudicoxa Roewer, 1949	Kimulidae	MZUSP-31317	Brazil: Caruaru-PE, Serra dos cavalos	8 22' 00" S 36 01' 22" W	1	?	?
Zalmoxis sp. (Vanuatu)	Zalmoxidae	MCZ DNA106885	Espiritu Santo, Vanuatu	n/a	1		X
*Ortholasma pictipes*Banks, 1911	Ortholasmatidae	MCZ DNA 101879	USA: MacDonald Sate Forest, Benton Co, Oregon	n/a	1	?	?
Nemastoma bimaculatum Fabricius, 1775	Nemastomatidae	MCZ DNA 100709	France:	44 5 18N; 3 34 54 E	1	?	?
Anelasmocephalus sp.	Trogulidae	MCZ DNA 103940	Spain: Font del vidre, camí a Vilada, Berga, Barcelona	42 91 41N; 1 55,896'E	1	?	?
Astrobunus grallator Simon, 1879	Sclerosomatidae	MCZ DNA 100311	Spain: Serralada del Montseny, Barcelona	n/a	1	?	?
Undet. Sp.	Phalangiidae	MCZ 126999	South Africa, M Pulamanca, Mount Sheba, Private Nature Reserve, Waterfall trail	(-) 24,9380610321 30,7133980002	1		X
Troglosiro sp.	Troglosironidae	Prashant5-2 (stub)- MCZ DNA101694	?	?	1		X
Aoraki longitarsa Forster, 1952	Pettalidae	MCZ 35659	New Zealand: South Island, WD, Chancellor Hut	43 30,62 S; 170 6,509 E	3	X	X



Supplementary material 2, figure: Comparison between ancestral state reconstructions of "Character (3) Distal sensilla, sensillar type" under the classic Palpatores hypothesis (left) and under the Dyspnolaniatores hypothesis (right).



Supplementary material 3, figure: Comparison between ancestral state reconstructions of "Character (5) Middle and proximal sensilla, apical opening" under the classic Palpatores hypothesis (left) and under the Dyspnolaniatores hypothesis (right). Character not applicable for Cyphophthami.

Protocols

Protocol for fixing, embedding and staining harvestmen appendages for TEM

Dissection of live animals and primary fixation

For this study, we used Karnovsky fixative (see recipe in the end)

1. Plan well the amount of samples needed, to avoid unnecessary animal sacrifices: all you need is ONE well fixed animal, so follow the next steps carefully. Consider dissecting in the end of the day, to leave samples fixing overnight. Anesthetize the animal cooling it for ~5 min. in the freezer
2. Fill a petridish with the desired primary fixative (cool). Plan the number of samples and label corresponding eppendorfs. Fill them with cool fixative and place them in a styrofoam with crushed ice.
3. While cutting, always try to submerge the part of interest under the fixative. If not possible, use at least a large droplet of fixative in a glass slide. For small specimens, submerge the entire animal for the dissection. Use sharp razor blades and make clean cuts (Do not break cuticle, slice it). For sensilla, try not to cut pieces larger than 1mm, otherwise fixation will not be sufficient.
4. After dissecting a part (eg. tip of the tarsus), transfer it for the eppendorf with cool fixative. Repeat the procedure for all desired parts.
5. Leave it overnight (4ºC). Samples can be left in fixative for a long time (months?) before the next steps of processing.

Post-Fixation

Osmium Tetroxide aqueous solution 2%

1. Rinse samples with buffer (**PBS Siena** or NaH2PO4x2H2O buffer) (3x15min). Tip for all liquid exchanges: do not remove all the liquid from the eppendorfs, always leave samples submerged.
2. Handle Osmium with care, it is very toxic. Exchange the buffer in the eppendorf for the Osmium solution, enough to submerge it.
3. Let it fix for 3 hours in the refrigerator (4ºC)
4. Rinse in buffer again (3x 15min), keeping in the refrigerator (4ºC). Proceed to embedding.

Embedding in Spurr resin

1. Dehydration series with Ethanol/distilled water: 50%, 60%, 70%, 80%, 90%, 96%, 100%, letting 1 hour in each step. The pure ethanol stage should be changed two times to ensure absolute dehydration.

2. Gradual embedding: check the Kit´s specifications for the embedding. Prefer hard mixtures for the resin. I proceeded as follows. Ethanol/resin 2:1 for 6 hours, 1:1 overnight; 1:3 6h, pure resin overnight. If possible, leave samples rotating slowly to enhance penetration. For the results presented here, samples were not agitated and were always kept in the refrigerator. Some researchers recommend that in the 1:3 phase the eppendorf should be left open, for the ethanol to evaporate
3. Fill the block forms with freshly prepared resin and orient your samples. Check kit for curing time. Usually ~18 hours at 60ºC or 8 hours at 70ºC.
4. After curing, the blocs are ready to be cut.

Staining with grid-sticks

Lead citrate and Uranil acetate aqueous solution

1. Centrifuge the solutions in an eppendorf to remove impurities. When using it, do not take from the bottom of the eppendorf, otherwise the centrifugation is useless!
2. We used grid-sticks (Electron Microscopy Sciences) to help staining the grids.
3. Load the grids with sections in the grid-stick. Insert in the modified pipette.
4. Fill it with distilled water to wet the grids (3x)
5. Discard water and fill it with lead citrate solution. Let it stain for 3 min. Return the chemical to the eppendorf, it can be used several time (the same applies below). Rinse it carefully with distilled water (5x). Discard the rinsing water in an appropriate container.
6. Discard water and fill it with uranyl acetate solution. Let it stain for 3 min. Rinse it carefully with distilled water (5x). Discard the rinsing water in an appropriate container.
7. Remove the grid-stick from the pipette and let it dry in a paper filter. Cover it with a petri dish to keep it free of particles.
8. Store the grids in your grid box. They are ready to be imaged.

Chemical Recipes

Karnovsky fixative

After Karnovsky (1965); This protocol is used at Gabriele Uhl´s laboratory at Ernst Moritz Arndt Universität Greifswald.

Components:

-NaH2PO4x2H2O buffer (sodium dihydrogen orthophosphate) or cacodylate buffer (if available)

-Paraformaldehyde

-Glutaraldehyde

-NaOH

-Glucose or fructose

Making of solution A: 11.3 g (2.6 %) of NaH2PO4x2H2O (sodium dihydrogen orthophosphate, monobasic), dissolve and make up to 500 ml with distilled water.

Making of solution B: 2.52 g (2.52%) of NaOH, dissolve and make up to 100 ml with distilled water

Making of buffer solution: take 83 ml of Solution A plus 17 ml of Solution B, bring to pH value of 7.3 with HCl or more drops of NaOH as necessary. Note that the buffer (even if kept permanently in the fridge) has a short life-time, maximally 1-2 weeks.

Prefixation procedure: Dissolve 2 g of paraformaldehyde in 17 ml of solution B in a water bath, with temperature not exceeding 70 degrees. When completely dissolved add 83 ml of solution A and contents of 10 ml of 25 % glutaraldehyde, adjust to pH value of 7.3 and afterwards add 1.5 g of d-glucose or fructose per 100 ml for osmolarity matching.

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Reference: Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. The Journal of Cell Biology 27, p. 1A-149A