Millipede (Diplopoda) identification characteristics viewed by scanning electron microscopy

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Introduction

During 2024 I had been undertaking some high magnification imaging of butterfly wing scales as part of a research project at the University of Sheffield. This involved taking images at magnifications up to x40,000 on a scanning electron microscope (SEM). When I finish working at the university, at the end of February 2025, I had the opportunity to continue accessing the SEM as an external user for a short time after my contract. During training in the use of the SEM I had used some invertebrate samples, such as millipede (Diplopoda) genitalia and harvestmen (Opiliones) palpal structures and found that they produced some interesting insights into their microscopic anatomy. In order to investigate this further and provide some valuable images to enhance existing identification material, I approached the British Myriapod & Isopod Group (BMIG) for some funding to pay for continuing access to the equipment.

Beyond the obvious differences in colour and size, the primary external features looked at for millipede identification include the shape of tail projections and jaw 'cheeks', eye arrangements, body striae and metazonite setae. However, as with many small invertebrates, the most diagnostic identification features for millipedes relate to structures associated with reproduction, such as those used for holding the couple together in the mating process or for transferring sperm. Although the actual genitalia are usually concealed, these secondary sexual characteristics—such as gonopods and other modified limb structures—are often more obvious and highly distinctive. However, these features can be challenging to observe under standard light microscopy. To help people to navigate through the descriptions in identification keys and to shed some light on the characters that point to species identity, I attempted to gather high magnification images of various millipede diagnostic characters using the electron microscope.

Method

The project aimed to capture images of key identification characteristics of as many British species as possible, at a resolution unattainable by standard light photography. These could show fine details of key ID features to enhance other current keys and identification information and be shared as an education and research resource for free via the BMIG website, social media and publications.

This use of the SEM hardly utilises its full potential for ultra-high magnification, as many of the characters used for identification rarely required magnification beyond x600. However, the capacity for creating high resolution images with a good depth of field means that the SEM reveals incredible detail of features only hinted at by most macro light photography. At SEM magnifications, unique and unimagined character details are revealed. However, there is little point in revealing the tiniest details if these are not visible to the majority of people trying to identify specimens under light microscopy. Such morphological species splitting may well be valuable once genetic studies suggest new taxonomic variations, but for this exercise, the aim is to support existing observable character recognition.

It was not possible to provide a standard set of images for all British species, due to time/cost constraints, specimen availability, preparation difficulties and the potential for imaging failures. However, representatives of 40 species of millipede known from Britain in 2025 were scanned, at least in part. This was achieved by the author utilising material from his own collection and from short-term

targeted field collecting. This provided sufficient samples for a limited number of preparations within the time restrictions to represent a reasonable spread of taxonomic categories, with some key similar species for comparative purposes where possible.

The main difficulty was in preparing undistorted specimens and mounting them successfully to show characters in the right orientation. Each structure needed to be dissected out and then slowly dried without distortion. Material must be absolutely dry before entering the SEM vacuum chamber. Ideally this can be achieved by using a critical point dryer or hexamethyldisilazane (HMDS) as a desiccating medium, but these options were not available for preparation as an external researcher. Dehydration was therefore undertaken by passing specimens through a series of progressively higher concentrations of alcohol over a period of a few days. Each day the concentration of alcohol is increased by 5-10% until the specimens are in 100% ethanol. This is renewed a couple of times to ensure that all water has been replaced. The specimen is then removed from the liquid and allowed to air dry for a few minutes. It is then carefully placed on to an aluminium SEM mounting stub with conductive double-sided tape, in the preferred orientation (Figure 1: left & centre). This is then allowed to thoroughly dry for another day or more before being scanned. For delicate specimens, the final air-drying stage can be undertaken in an atmosphere of 100% alcohol (in an air-tight box with a ball of cotton wool soaked in alcohol) to slow the drying even further. Most diagnostic features in millipedes tend to derive from chitinous exoskeletal structures, so can be dried in this way, without the distortion seen in softer tissues.

Figure 1: Specimens glued to aluminium stubs and coated with gold.

Before scanning, the dehydrated specimens on the stub are coated with a very thin (~20nm) conductive layer of gold (Figure 1). Because the specimens are fixed in this position on the stub, they can generally only be viewed from a limited perspective, dependent on how effectively they were orientated before being coated. Given the size and delicacy of a specimen, it is not always possible to mount it in a precise, preferred viewpoint.

Gonopods often needed to be dissected away from surrounding legs and body rings to reveal the structures in a way that could be viewed by the microscope. Using fine forceps, and pins, the post gonopod rings were pulled away to often allow the gonopods to be viewed in situ (Figure 1: right). Once dissected, the structures to be examined are initially kept in 70% alcohol (ethanol or IMS/IDA) until dehydrated.

The microscope used to generate most of the images was a JEOL JSM-6010LA (Figure 2) (some initial scans were undertaken on a Tescan Vega3). They use a beam of electrons to create an image of the surface of the specimen, rather than light illuminating a specimen, as used in conventional microscopy. A white-hot filament generates electrons which are focused in a beam of up to 20 kVolts on the object. This electron beam is scanned across the surface of the object in a vacuum and the interaction of the

beam with the conductive coating on the specimen releases secondary electrons. These are collected by a detector to produce a digital image of the specimen's surface. It should therefore be noted that a key feature of SEM imaging is that only images of physical surface structures are created, in monochrome, with minimal transparency, so colour patterns are not captured. Features that are highlighted by pigment, such as eye ocelli, are therefore more difficult to observe from only observing the surface cuticle (see Figure 3).

The focus, beam size and intensity, voltage, stigmatism, contrast and brightness can all be modified to generate the best image. How the specimen is mounted impacts how well it conducts and what views are possible. Due to the time restrictions on this project, the priority was to scan as many species as possible, rather than spending the limited time on achieving the very best image resolution at several magnifications. With an SEM it is very tempting to explore every tiny detail at high magnifications, but this does not provide material of use to anyone trying to separate species with a hand lens or under a light microscope.

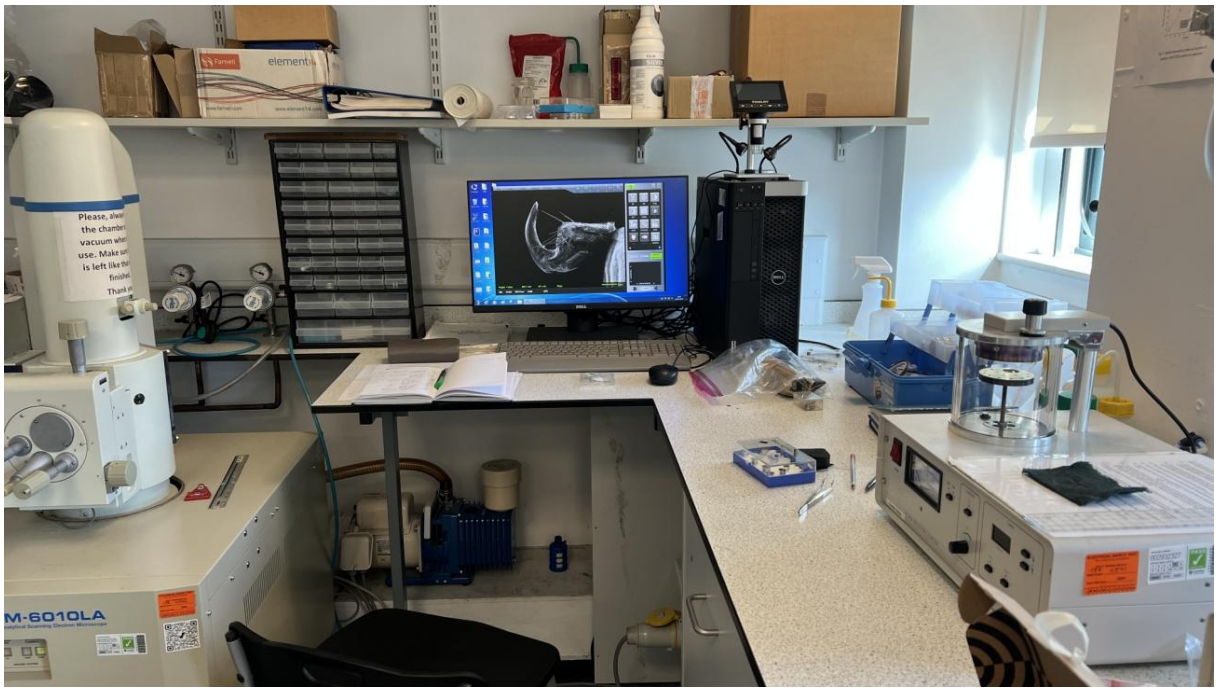

Figure 2 : The JEOL JSM-6010LA Scanning Electron Microscope (left), user interface and gold sputter-coater (right)

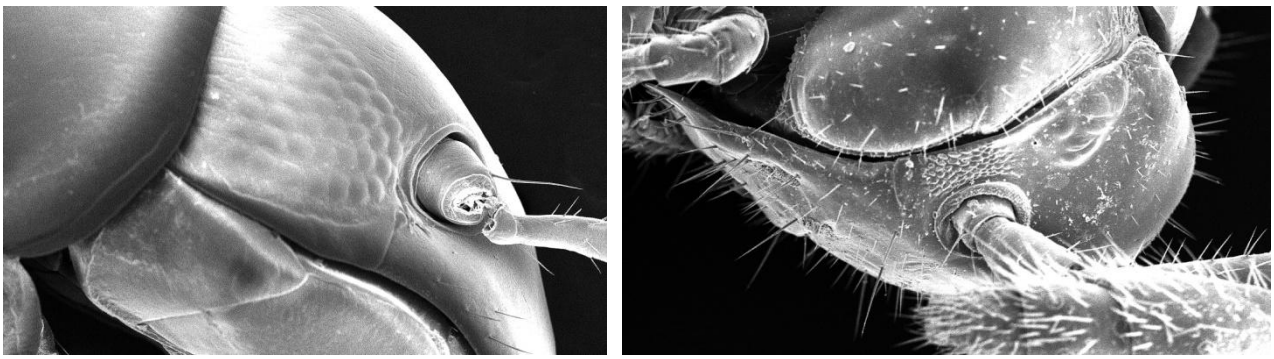

Figure 3: Ocelli of *Ophyiulus germanicus* and *Brachychaeteuma melanops*

Results

Preparation and scanning took 60 hours of which 17 hours was undertaken scanning on the SEM. Approximately 221 images were taken with examples of 40 British species. The primary aim was to capture images of tails, gonopods and other secondary sexual characters. Specific comparisons were also made of first leg modifications and coxal processes on the second leg in *Cylindroiulus* species and projections on cardo and stipes of the cheeks of species, where appropriate. Tails or whole bodies of 16 species were scanned and gonopods of 35 species. Samples of these are shown in galleries 1 and 2.

Hopefully these images will offer some clarification in the identification of similar species and new insights, particularly into the anatomy of millipede genitalia and other characters which are described in keys. Alongside existing macro images and drawings, it is hoped that they will bring some clarity for beginners and experienced researchers alike.

The gonopods of five different specimens of *Brachychaeteuma bradeae* were scanned to try to capture some of the variation known for this species (Clegg & Richards, 2007). Anterior median and lateral horns differed slightly, but did not show the variation that was hoped for (Gallery 1.)

One pleasing result was the undistorted images of the ectoparasitic fungus *Rickia laboulbenioides* on *Cylindroiulus latestriatus* (Figure 4.) It was expected that the dehydration process would create distortion in the presumed softness of the fungal fruiting bodies, but they seemed to have dried very successfully (Reboleira *et al*., 2018).

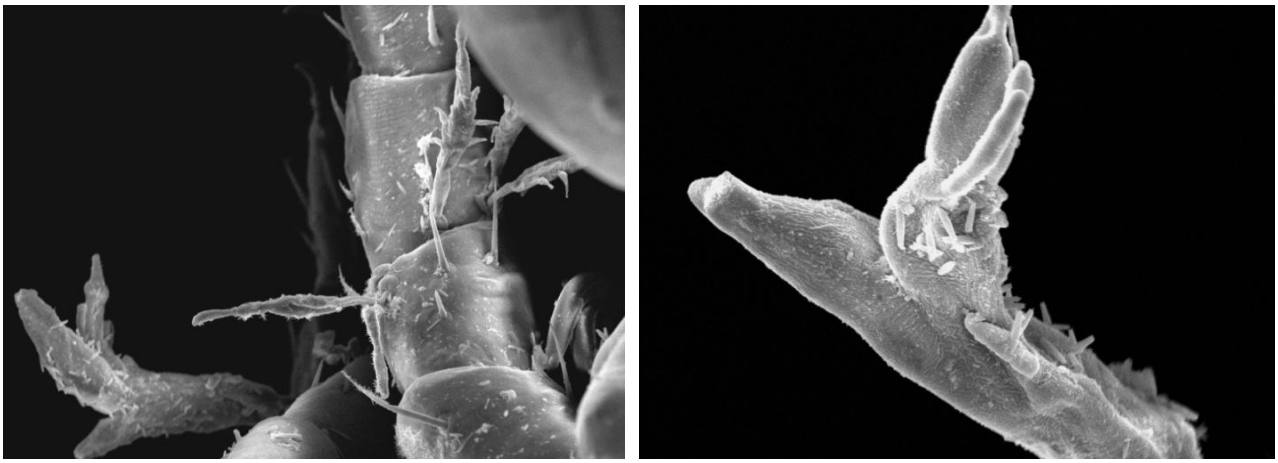

Figure 4: Fore legs of *Cylindroiulus latestriatus* with *Rickia laboulbenioides* fungus x400 and fungus detail x1000

The SEM showed the dorsal glands on the 16th ring of a male *Chordeuma proximum* (Gallery 1) which can be used to distinguish between this species and *Chordeuma sylvestre* (no image).

Although the orientation of the *Ophyiulus pilosus* specimen differs from that of *Ophyiulus germanicus*, there is still a noticeable difference in the hooked second leg structure. There is some indication of subtle differences in the shape and micro-sculpture, but the process that is only found at the base of the second leg of *Ophyiulus germanicus* is much more obvious (Gallery1.)

The variations in tail/telson/caudal projection structures speak for themselves (Gallery 2.) The SEM images also show the basal attachment points for setae on the anal valves even where these have been broken off, for example in *Cylindroiulus parisiorum* with at least 5 setae per valve.

The various differences shown across the scans are likely to be genuine inter-species variations, but it is also possible that the small sample size presents atypical features and artificial abnormalities created by

the SEM preparation methods. Gonopods are presented as they were dissected, as intact as possible, but often with lost flagellae, or indistinct positioning between brachit, prosomerite, mesomerite, solenomerite and other structures. Each image should therefore be viewed with some caution, incase the individual in question may not offer the most typical representation for the species. However, the ability for revealing these potentially distinguishing and undescribed details shows the value that SEM imaging has in helping to identify seemingly similar species.

If future access to SEM resources was secured, it would be advantageous to develop a more systematic approach to imaging multiple specimens per species. This would be valuable for creating further reference images to clarify and reinforce the revelations seen through the initial somewhat opportunistic approach of this project.

The image files are available to the BMIG committee for use as required. It is anticipated that the images will be made more available as reference material within the species galleries on the BMIG website (https://bmig.org.uk/checklist/millipede-checklist). They may also appear on the Isopods and Myriapods of Britain and Ireland Facebook group if helpful for recorders with identification

The most complicated aspect of the project was the dissection and preparation of specimens and their mounting on stubs. As this has now been undertaken, the gold-coated material can be re-scanned if opportunity arises. In particular it would be interesting to investigate if these or additional specimens could be used to create three-dimensional views with rotational scanning electron micrography (rSEM) to enhance the insights further (Reboleira *et al*., 2018, Cheung *et al*., 2013).

For all their value in showing accurate images of species micro-anatomy for identification, there is no doubt that scanning electron microscopes also offer inspirational images and insight that the general public, as well as myriapod enthusiasts find fascinating. Hopefully these images will help to advance the wider understanding and appreciation of millipedes and provide new detailed information for training anyone wanting to become more involved or proficient in identification and recording. Such SEM images offer an innovative, high-quality addition to enhance any future teaching or publications in this field when presented alongside other species illustrations and photographs.

Acknowledgements

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References

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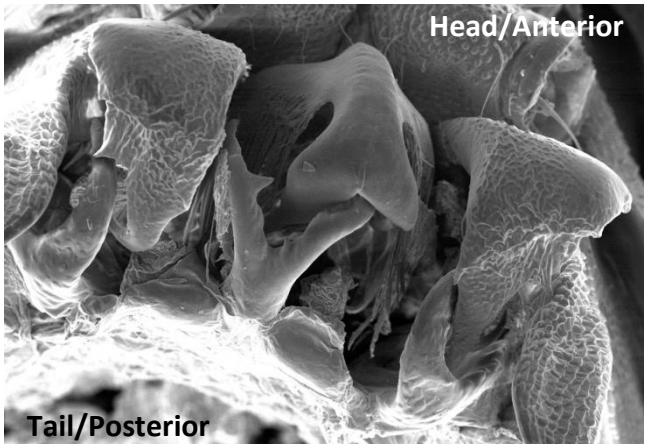
Clegg, P & Richards, JP. (2007) Street Safari: recording myriapods and isopods as part of a community project in Sheffield, UK. *Bulletin of the British Myriapod and Isopod Group* **22**: 36-43. https://bmig.org.uk/sites/default/files/bulletin/BullBMIG22%20p36-43%20Clegg%26Richards%20Street%20safari.pdf

Reboleira ASPS, Enghoff H, Santamaria S. (2018) Novelty upon novelty visualized by rotational scanning electron micrographs (rSEM): Laboulbeniales on the millipede order Chordeumatida. PLoS ONE 13(11): e0206900. https://doi.org/10.1371/journal.pone.0206900

Richards, JP. (2011) Introduction to Myriapods & Centipedes British Myriapod & Isopod Group/Nature Bureau. https://bmig.org.uk/sites/default/files/docs/Millipedes.pdf

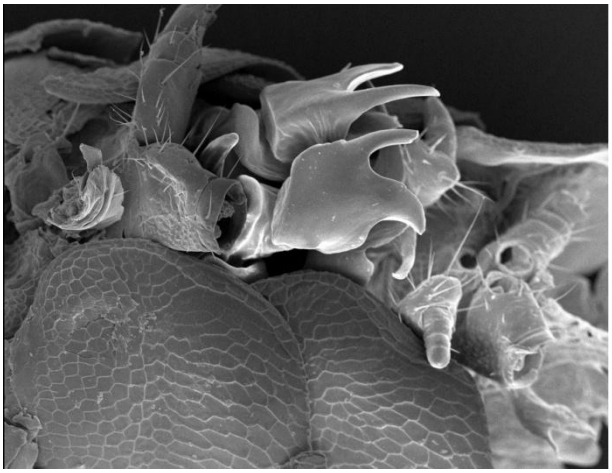
Gallery 1: Comparison of millipede gonopods and secondary sexual characters on SEM

All captions indicate original microscope magnification.

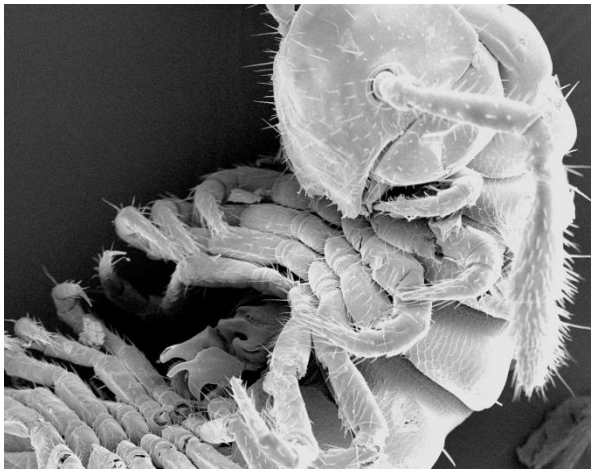


***Nanogona polydesmoides* gonopods x220**

***Brachychaeteuma melanops* x90**

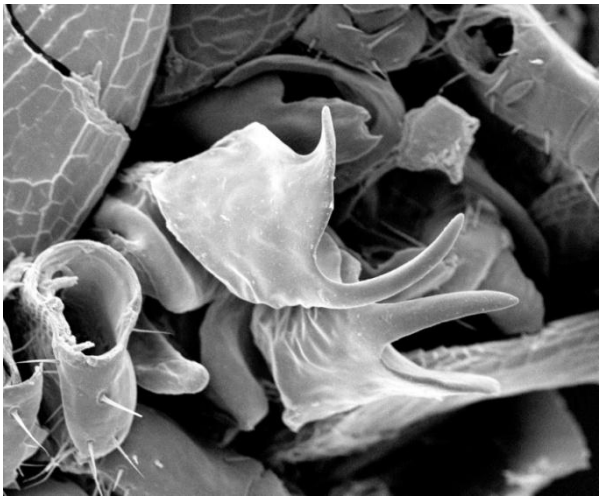


***Brachychaeteuma bradeae* gonopods x170**



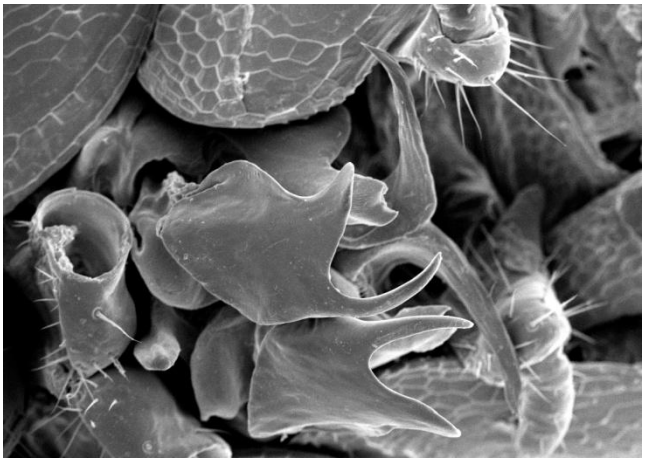
***Brachychaeteuma bradeae* gonopods x55**

***Brachychaeteuma bradeae* gonopods x300**

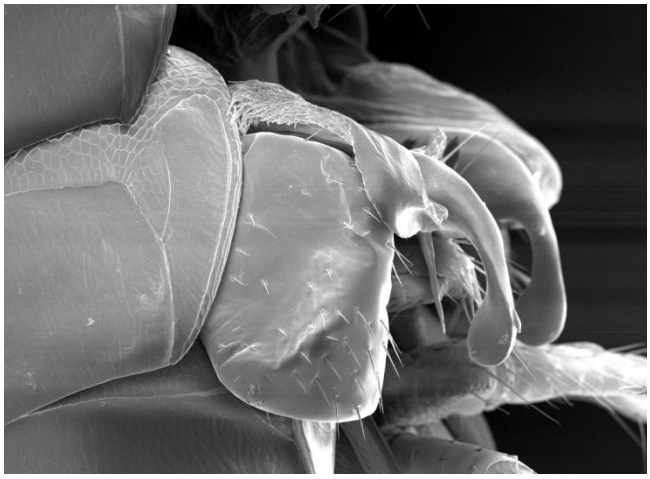


***Brachychaeteuma bradeae* gonopods x300**

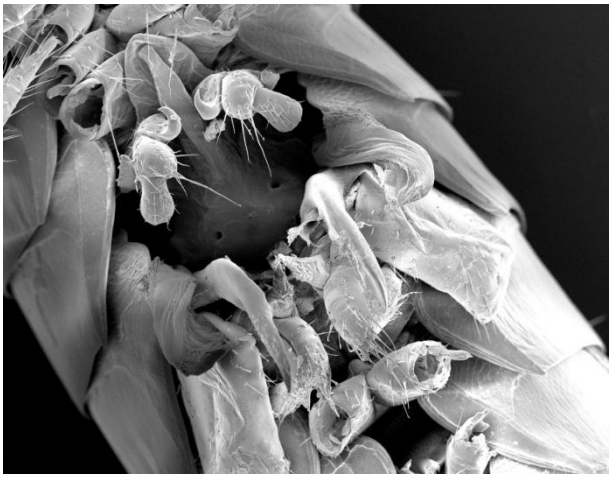
***Brachychaeteuma bradeae* gonopods x300**



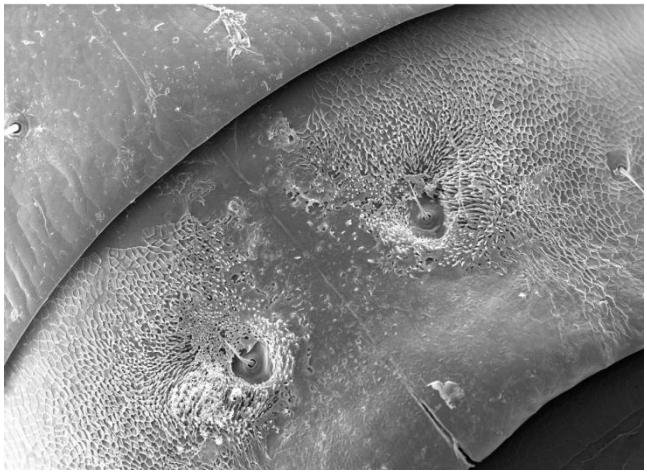
***Brachychaeteuma bradeae* gonopods x300**



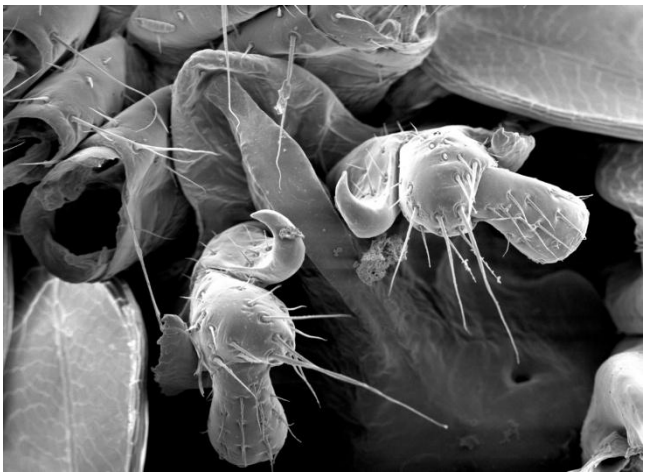
***Chordeuma proximum* gonopods x150** (anterior upper left)



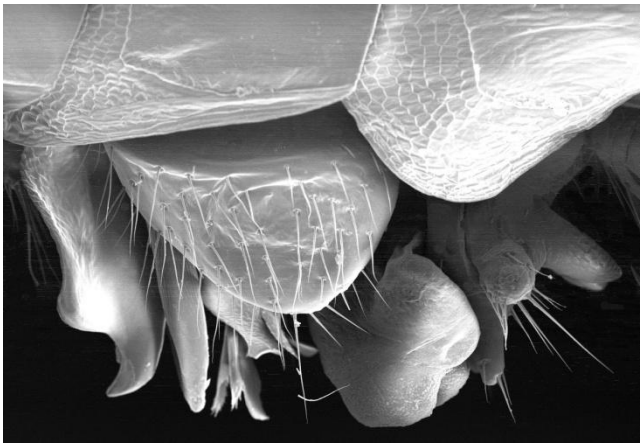
***Chordeuma proximum* gonopods x100** (anterior upper left)



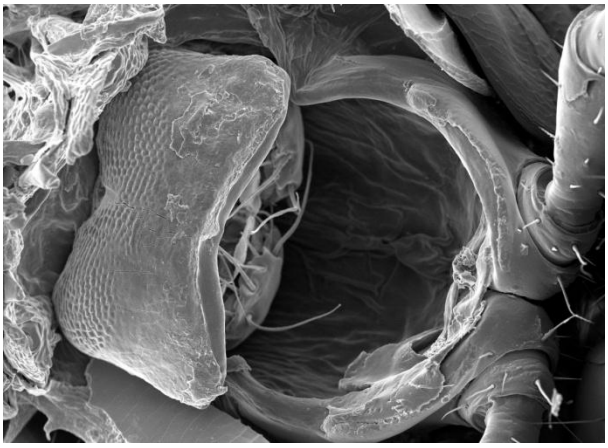
***Chordeuma proximum* male ring XVI glands x170** (anterior upper left)



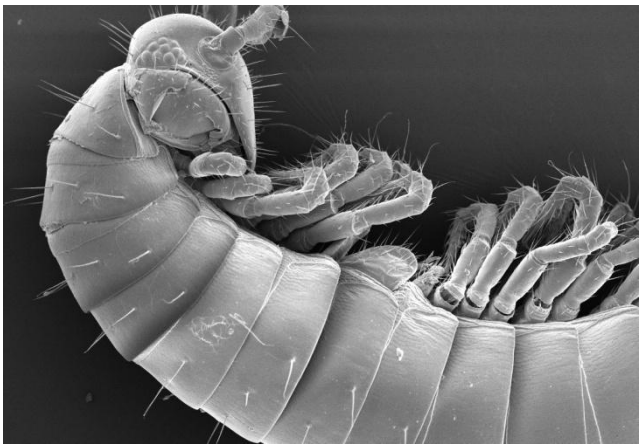
***Chordeuma proximum* gonopods x230** (anterior upper left)



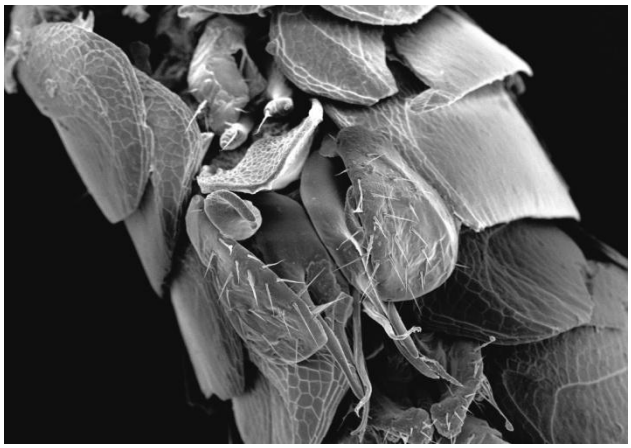
***Melogona gallica* gonopods x190**
(lateral, anterior left)



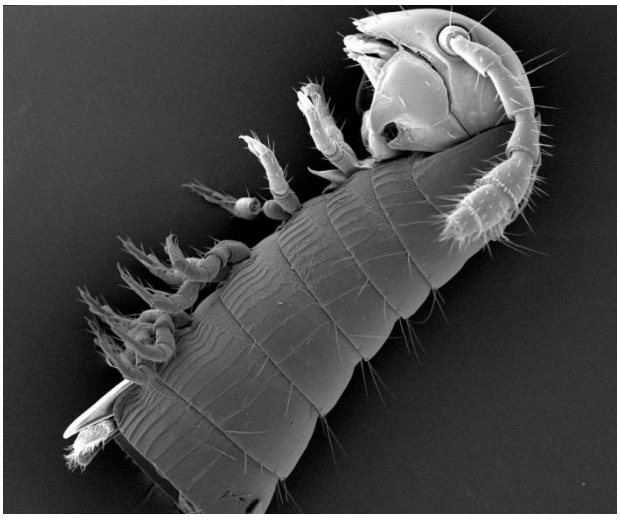
***Melogona gallica* vulva x230**



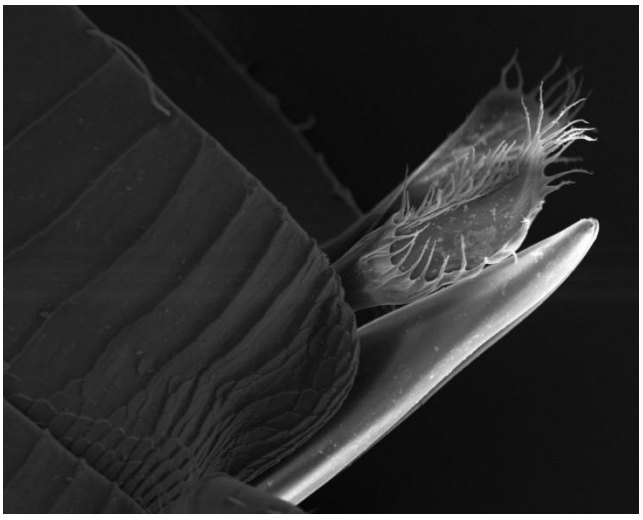
***Melogona scutellaris* male x65**



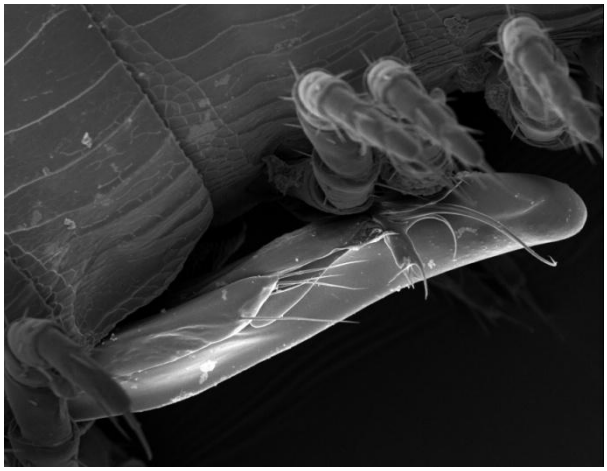
***Melogona scutellaris* gonopods x160**
(anterior upper left)



***Choneiulus palmatus* male x65**

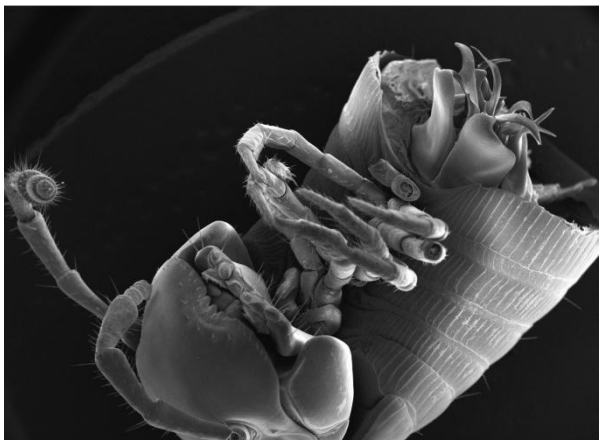


***Choneiulus palmatus* gonopods x350**



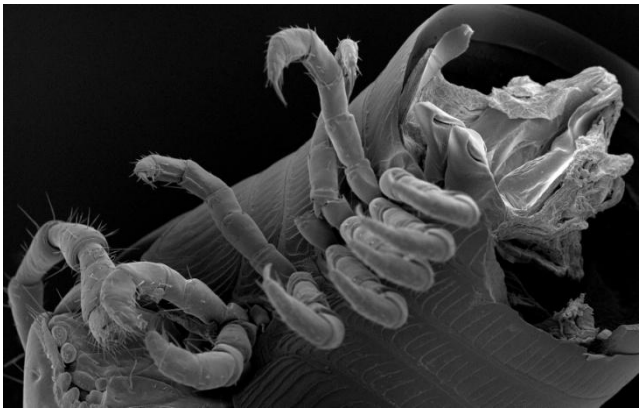
***Blaniulus guttulatus* gonopods x180**

***Archiboreoiulus pallidus* gonopods x60**

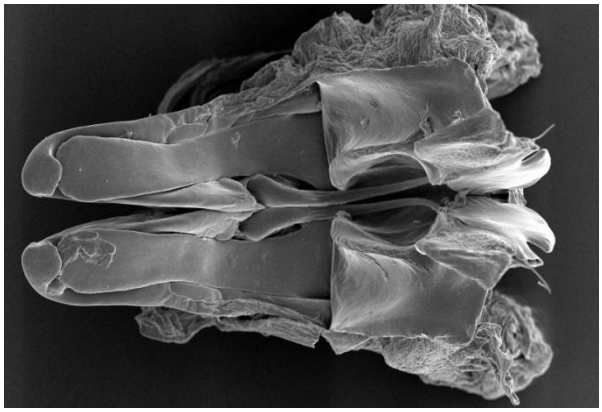


***Ommatoiulus moreleti* gonopods x30**

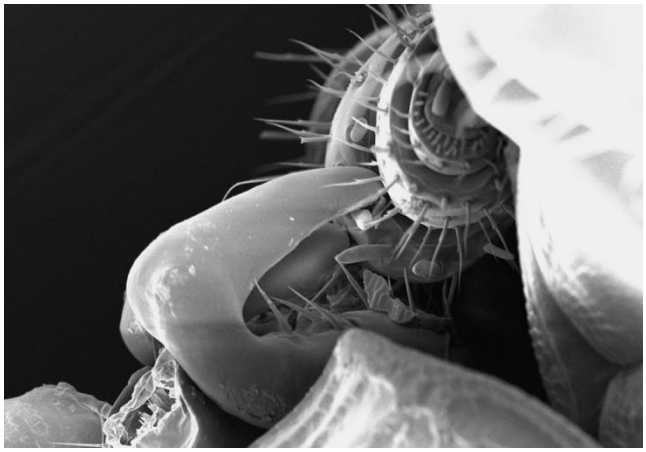
***Tachypodoiulus niger* male x33**



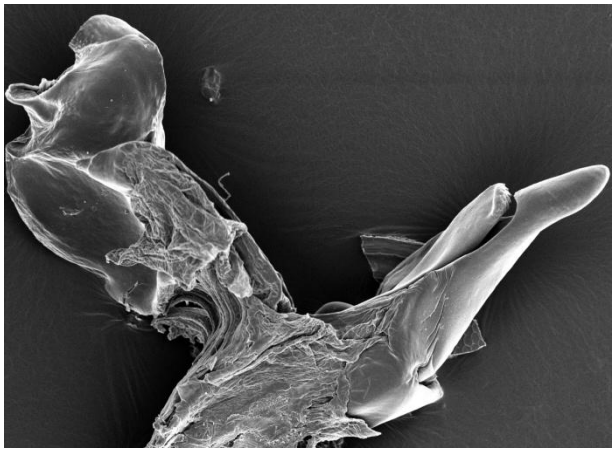
***Cylindroiulus caeruleocinctus* gonopods x33**



***Cylindroiulus caeruleocinctus* gonopods x70**



***Cylindroiulus vulnerarius* male 1st leg x270**



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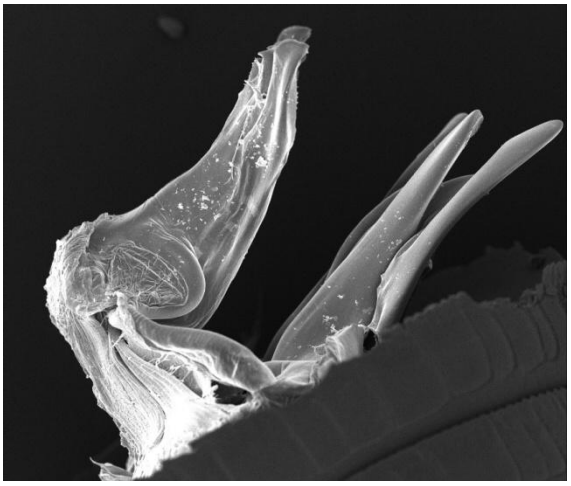
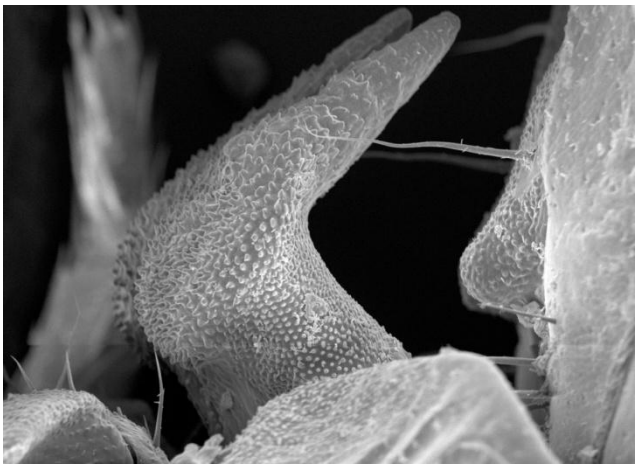
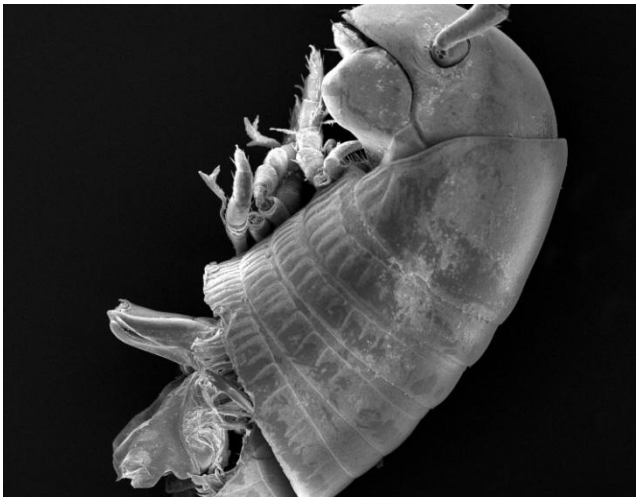


***Allajulus nitidus* gonopods x95**



***Allajulus nitidus* male 1st leg x300**

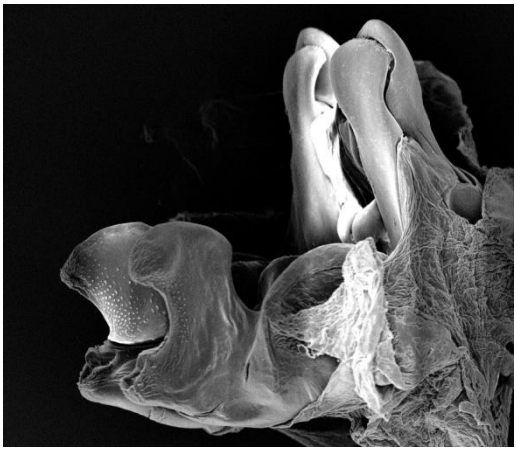
***Cylindroiulus latestriatus* gonopods x130**



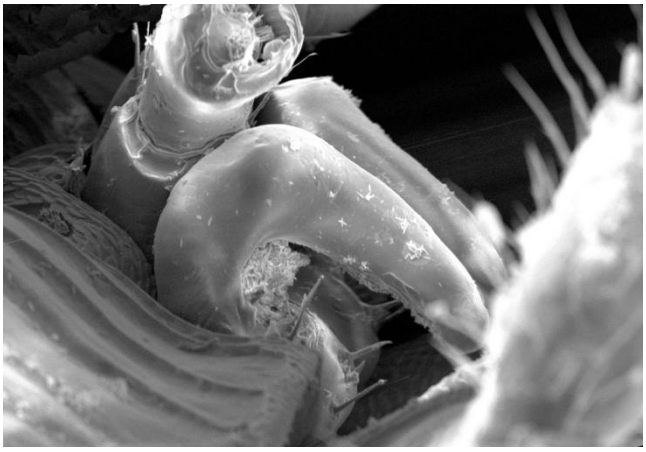
***Cylindroiulus latestriatus* male x40**
(with *Rickia* fungus on fore legs)

***Cylindroiulus britannicus* male x75**

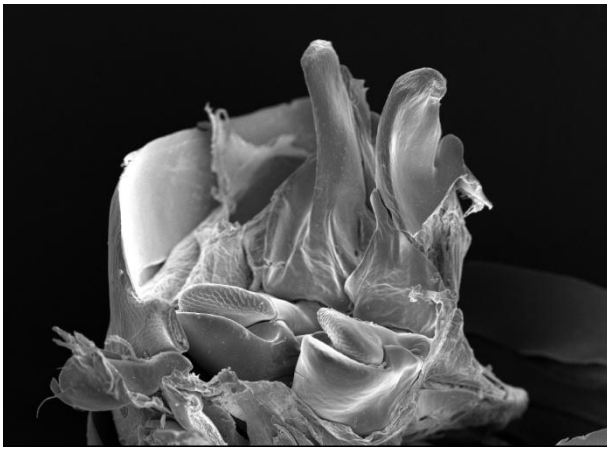
***Cylindroiulus britannicus* gonopods x130**



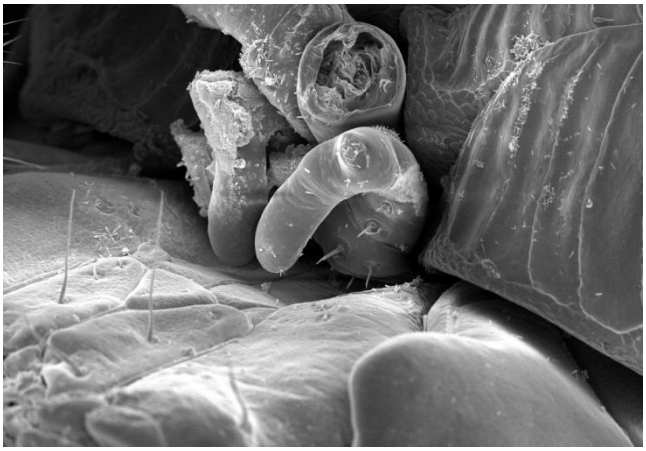
***Cylindroiulus apenninorum* gonopods x120**



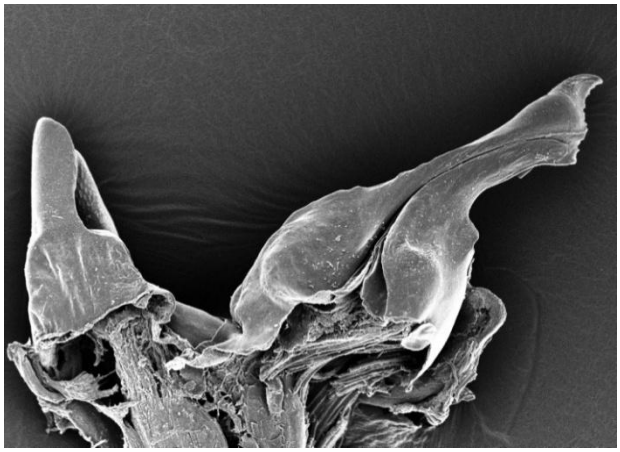
***Cylindroiulus apenninorum* 1st leg x270**



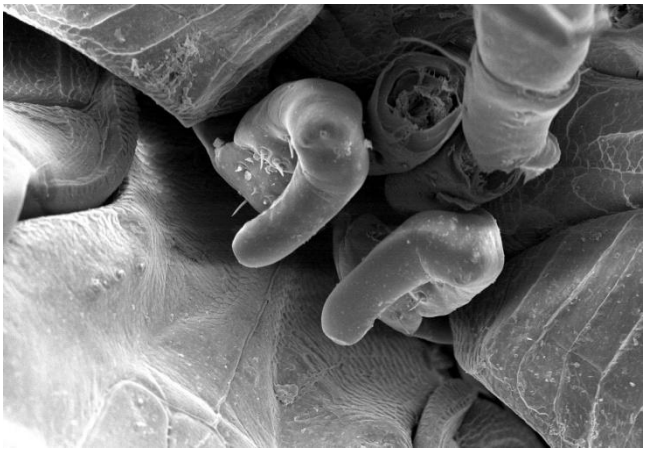
***Cylindroiulus pyrenaicus* gonopods x140**



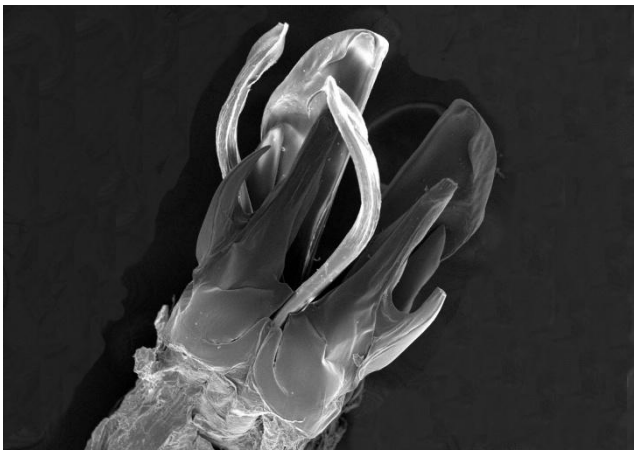
***Cylindroiulus pyrenaicus* 1st leg x190**



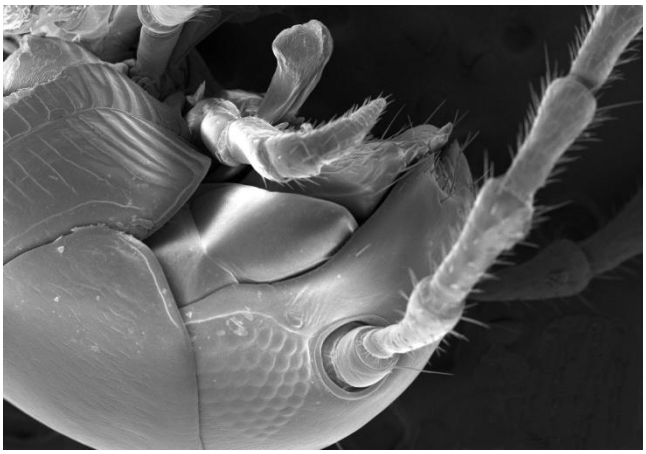
***Cylindroiulus sagittarius* gonopods x150**



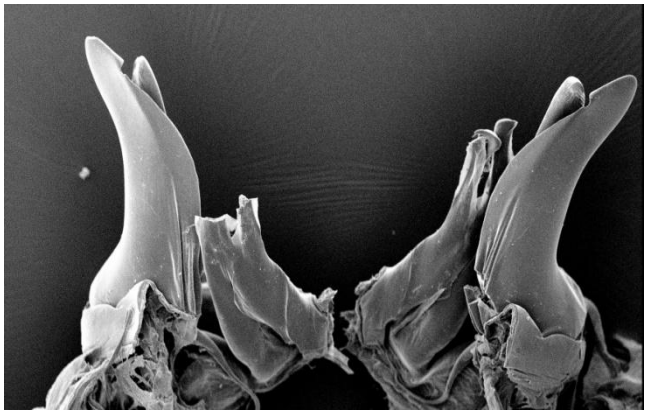
***Cylindroiulus sagittarius* 1st legs x190**



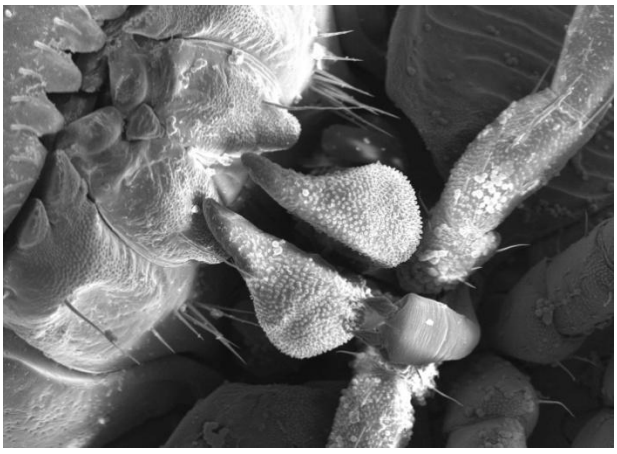
***Julus scandinavius* gonopods x70**
(posterior view)



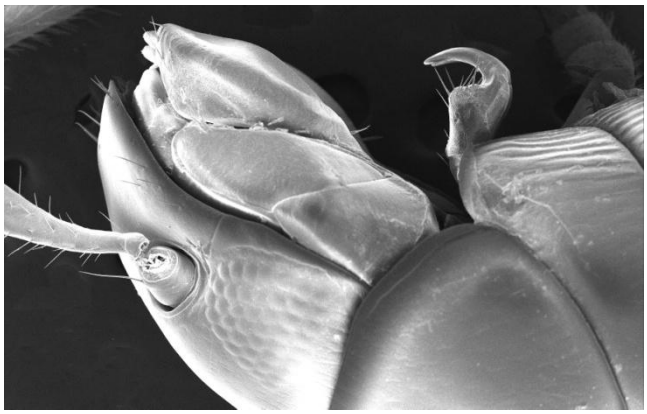
***Julus scandinavius* male 2nd leg pair x55**



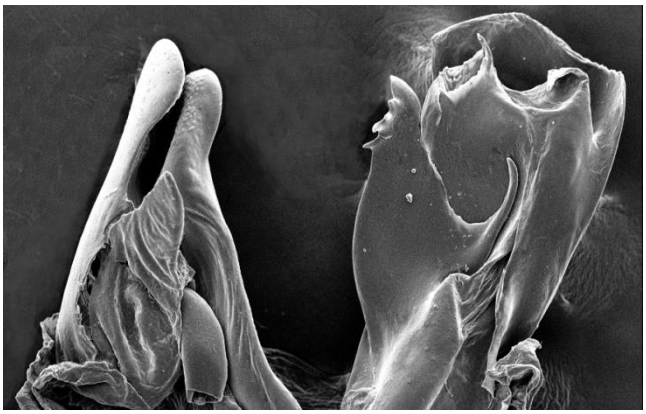
***Haplopodoiulus spathifer* gonopods x60**
(anterior view)



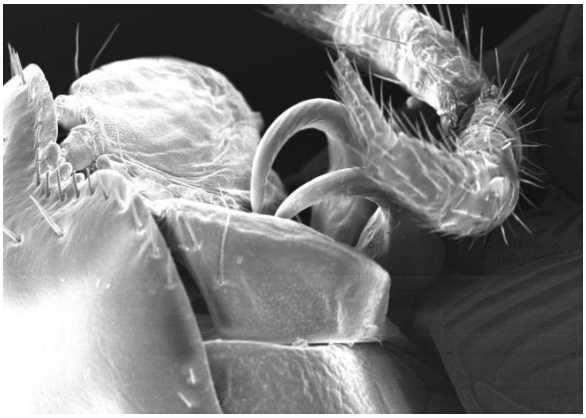
***Haplopodoiulus spathifer* male 2nd legs and penis x140**



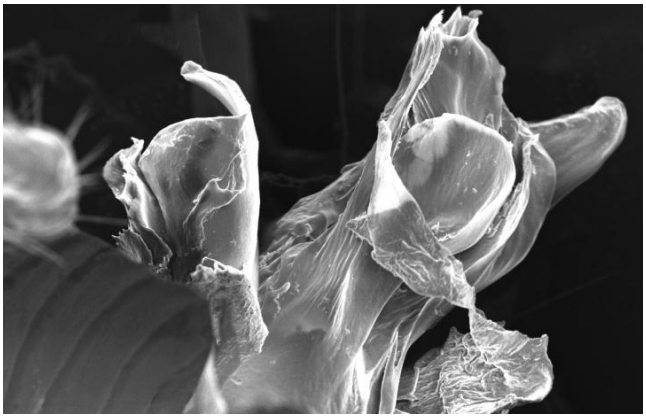
***Ophyiulus germanicus* male 1st leg x50**



***Ophyiulus germanicus* gonopods x130**

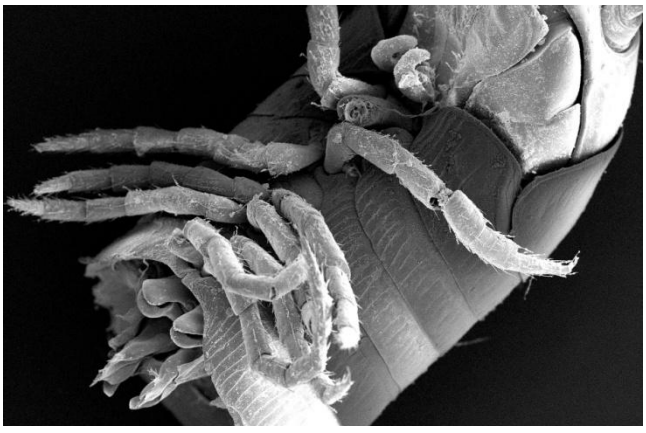


***Ophyiulus pilosus* male 1st leg x140**

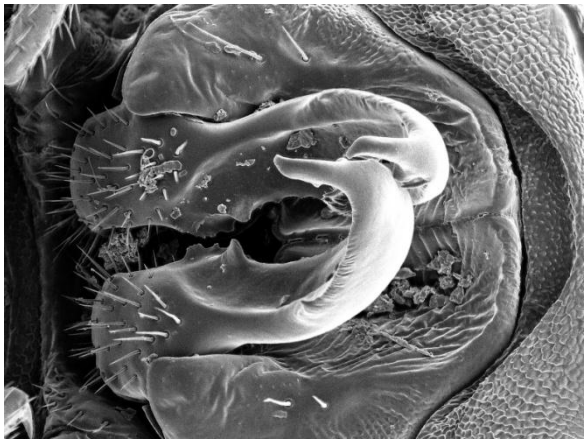


***Ophyiulus pilosus* gonopods x230**

***Ophyiulus germanicus* male 2nd leg process x330**

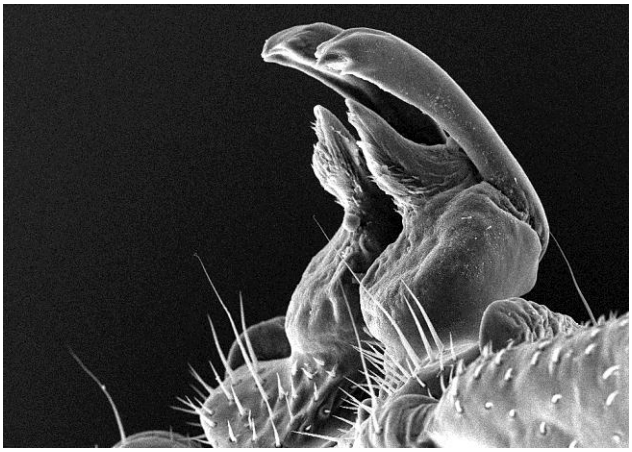


***Leptoiulus belgicus* male x50**



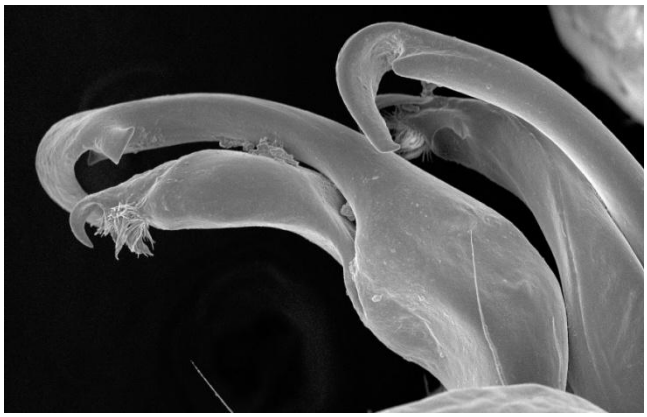
Brachydesmus superus **gonopods x120**

Polydesmus angustus **gonopods x95**

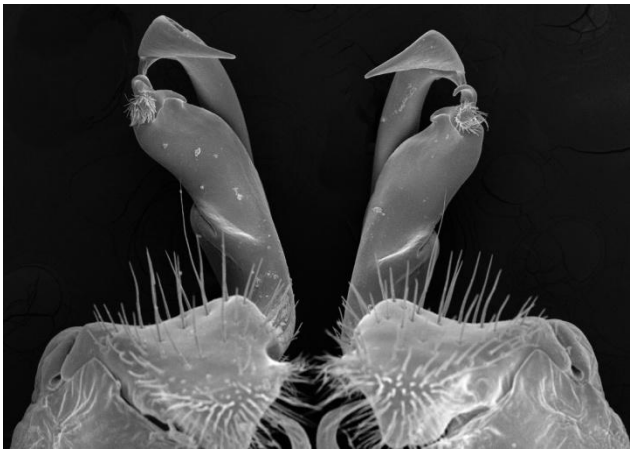


Polydesmus asthenestatus **gonopods x500**

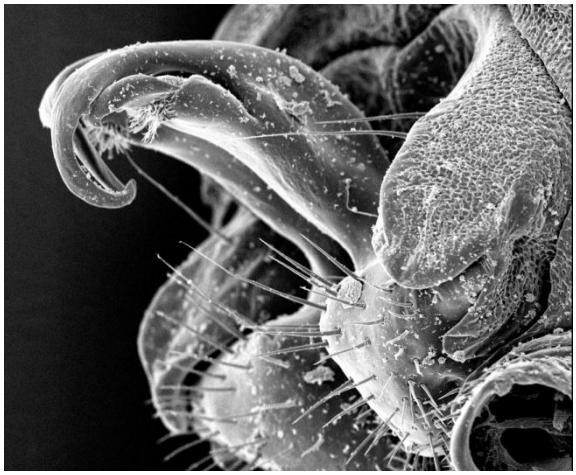
Polydesmus denticulatus **gonopods x110**



Polydesmus coriaceus **gonopods x110**

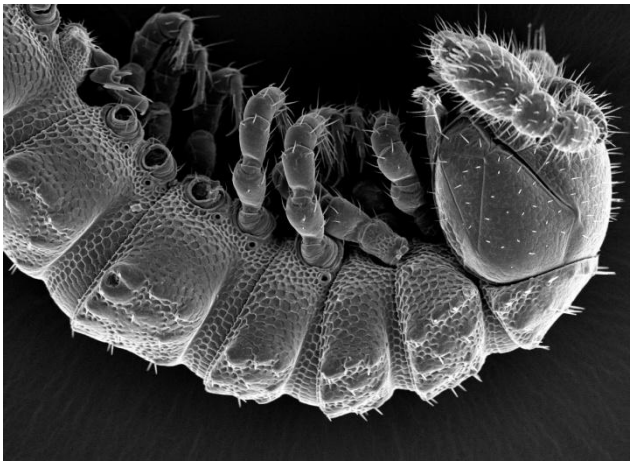


Polydesmus coriaceus **gonopods x85**
(posterior view)

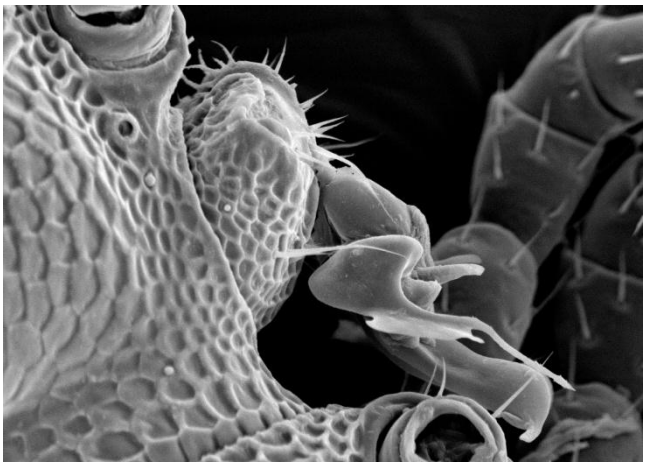


***Polydesmus inconstans* gonopods x130**

***Turdulisoma helenreadae* gonopods x200**

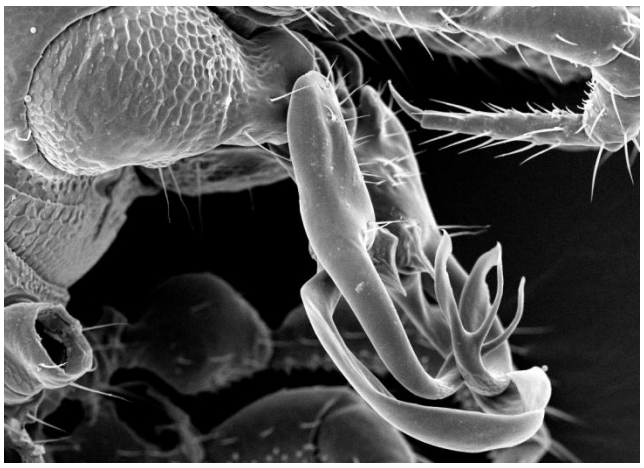


***Macrosternodesmus palicola* male x170**



***Macrosternodesmus palicola* gonopods x650**

***Ophiodesmus albonanus* male x 70**

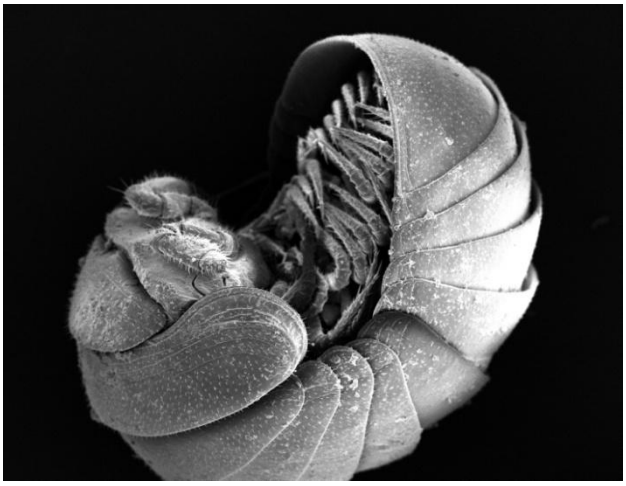


***Ophiodesmus albonanus* gonopods x270**

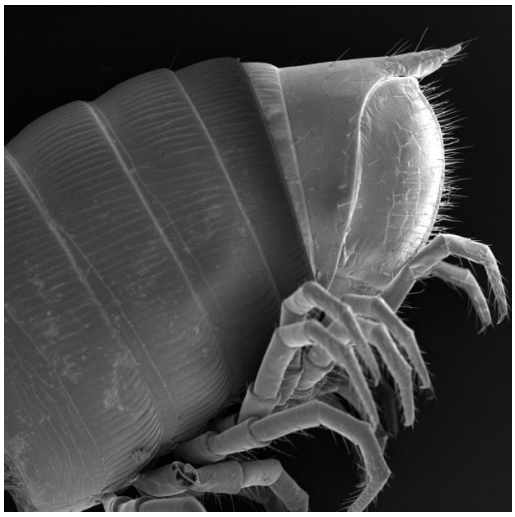
Gallery 2: Comparison of millipede tails/caudal projections on SEM

All captions indicate original microscope magnification

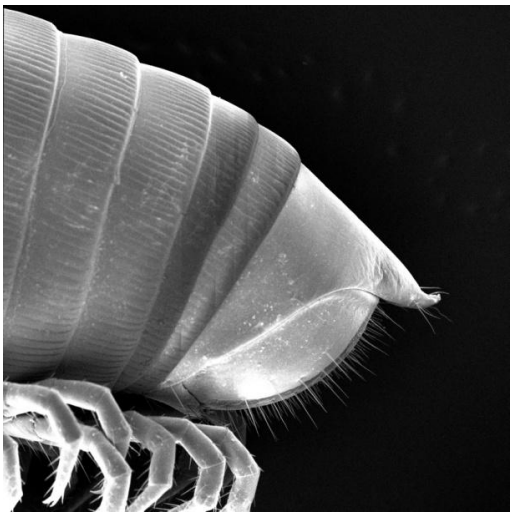
***Adenomeris gibbosa* x55**



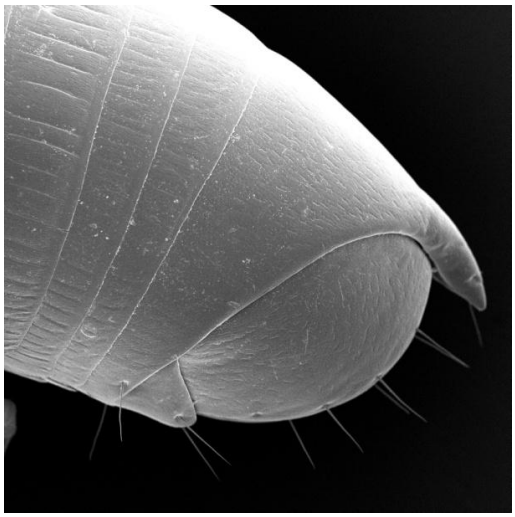
***Geoglomeris subterranea* x65**



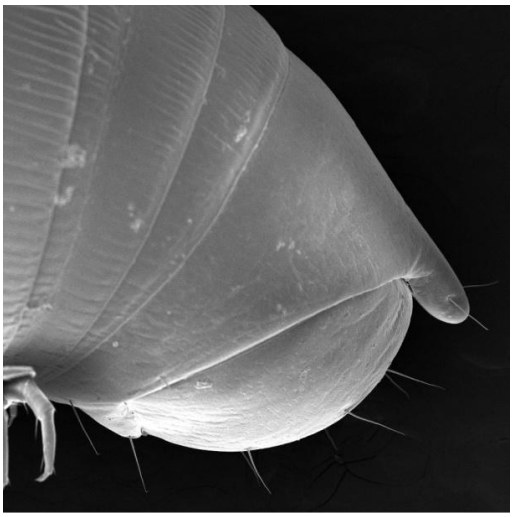
***Tachypodoiulus niger* x33**



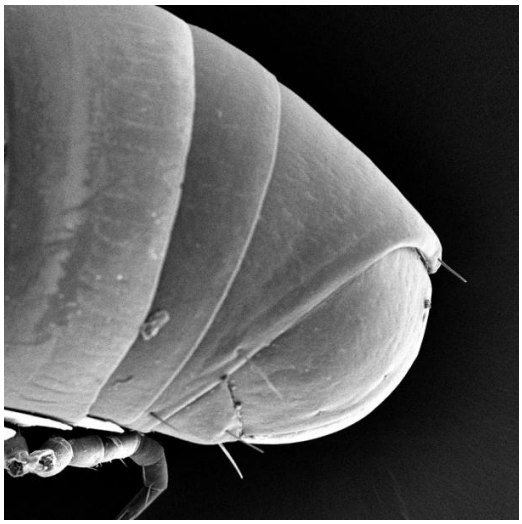
***Ommatoiulus moreleti* x37**



***Cylindroiulus vulnerarius* x80**

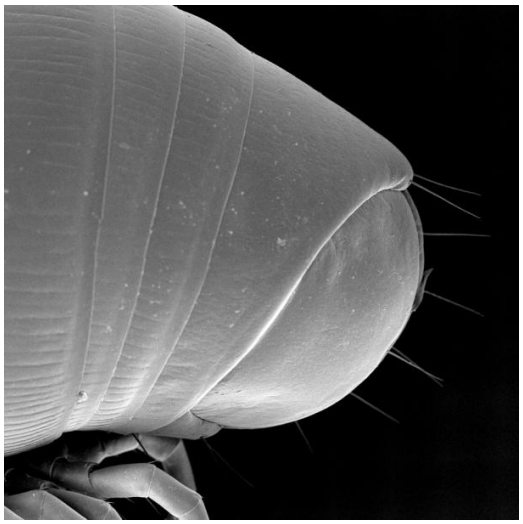


***Cylindroiulus punctatus* x75**

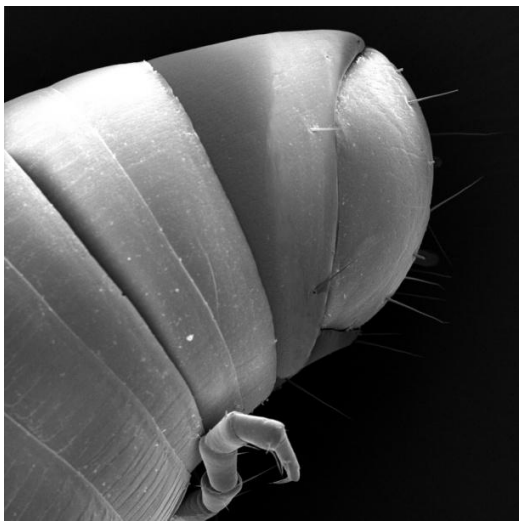


***Cylindroiulus latestriatus* x100**

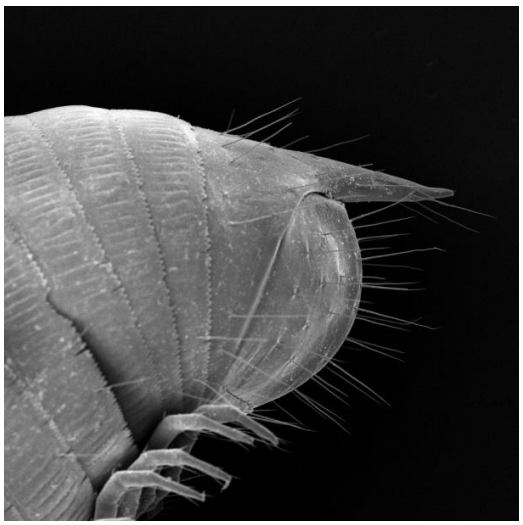
(Anal spines absent)



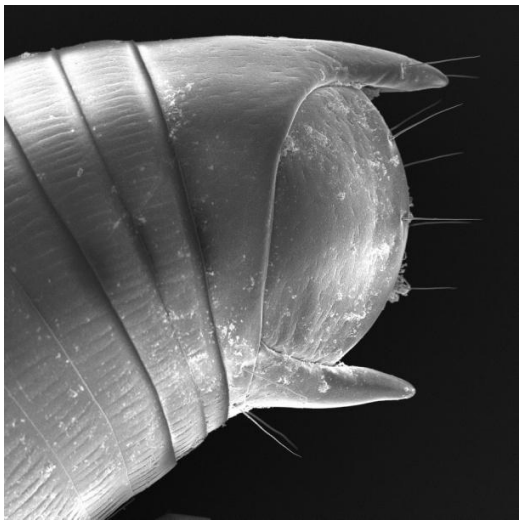
***Cylindroiulus caeruleocinctus* x55**



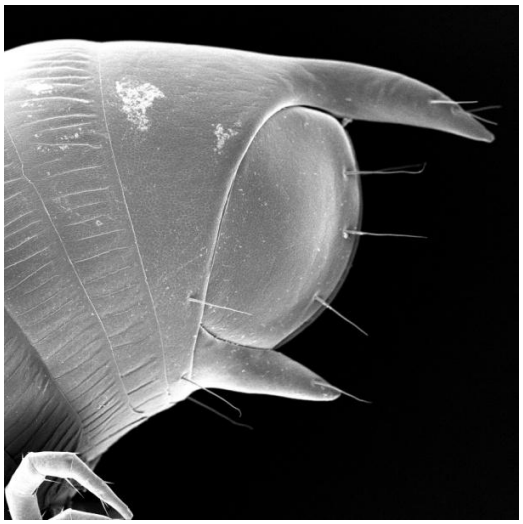
***Cylindroiulus parisiorum* x95**



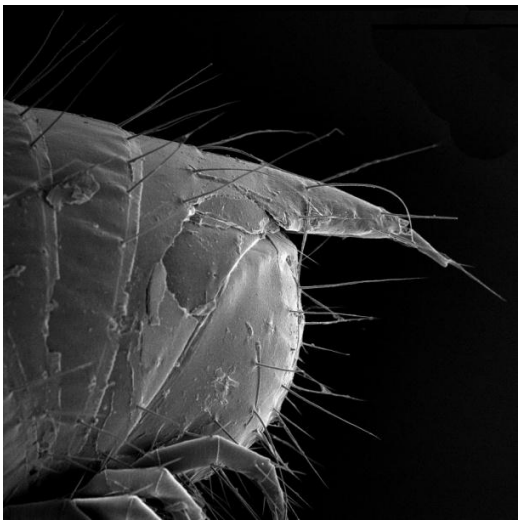
***Allajulus nitidus* x60**



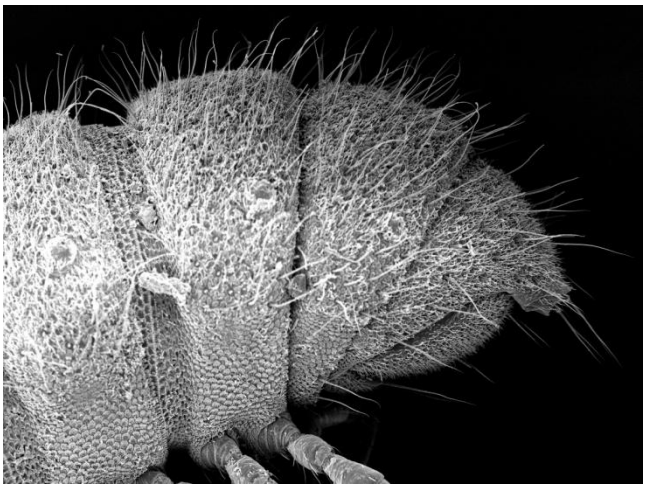
***Cylindroiulus apenninorum* x80**



***Cylindroiulus pyrenaicus* x85**

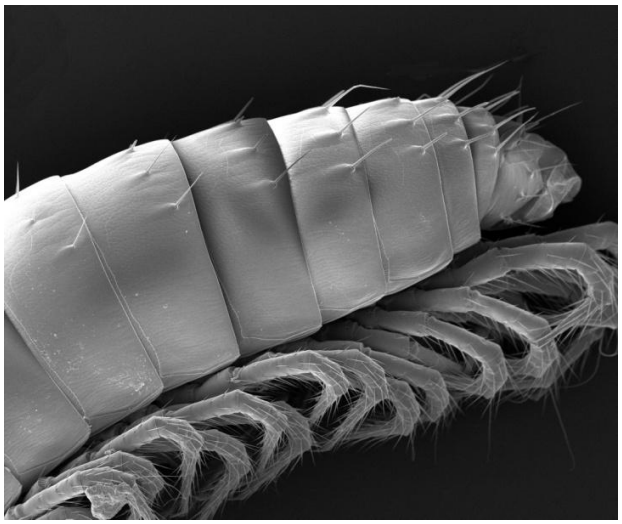


***Ophyiulus pilosus* x90**



***Cylindrodesmus hirsutus* x140**

***Haplopodoiulus spathifer* x65**



***Melogona gallica* male x60**