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A Scanning Electron Microscopy method to visualise the copulatory organ morphology of microturbellarian flatworms: *Trigonostomum* Schmidt, 1852 as a case study

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Abstract

Traditional methods for studying the morphology of microturbellarian flatworms rely on light microscopy, which often lacks the resolution necessary to capture fine structural details. Therefore, we present a protocol to improve the visualisation of structural morphological details in microturbellarians by means of Scanning Electron Microscopy (SEM). We demonstrate this method by imaging the sclerotised copulatory organs of three species of *Trigonostomum* (Rhabdocoela, Trigonostomidae): *T. venenosum*, *T. setigerum*, and *T. penicillatum*. Additionally, we successfully visualise the bursal appendage of *T. penicillatum*. SEM imaging offered new morphological insights for the genus, and corrected earlier interpretations made with light microscopy. The method requires precision and careful handling, especially during the isolation of the hard parts. However, it is cost-effective and straightforward to carry out in any standard laboratory setting.
Hence, our SEM protocol complements traditional light microscopy and opens new avenues for taxonomical research in microturbellarian taxa with hard parts.

Key words: Platyhelminthes, ultrastructure, SEM, sclerotised structure, taxonomy

1. Introduction

'Turbellaria' is a paraphyletic group of mainly free-living flatworms. Among these, microturbellarians are those ranging from 0.5-2 mm (Schockaert et al., 2008). They represent a highly diverse assemblage and are commonly found interstitially across a wide range of ecosystems. The morphological identification of microturbellarian flatworms is traditionally based on the observation of live specimens under a compound microscope, followed by permanent mounting of the entire animal on a microscopic slide to study the sclerotised parts,

particularly those of the male copulatory organs (Schockaert, 1996). Such morphological studies are typically carried out using traditional light microscopy on wholemounts and serial sections, differential interference contrast, or phase contrast. However, these methods may not fully capture the intricate details of more complex structures. Visualisation techniques such as electron microscopy (EM) could clarify important morphological features, aiding in the identification of microturbellarians and providing valuable new insights into possible homologies between these organs and/or their function.

EM has been widely used in studies of various groups of flatworms, with transmission electron microscopy (TEM) being the most frequently utilised technique. This technique has been widely applied to investigate detailed aspects of genital morphology of small free-living flatworms from sections (Brüggemann, 1984, 1985; Doe, 1982, 1986a, 1986b; Doe and Smith III, 2016). Serial block-face scanning electron microscopy (SBFSEM), a technique that combines serial sectioning with scanning electron microscopy (SEM), has also been used once to visualise the reproductive structures of *Macrostomum schareri* Brand, 2023 (Brand, 2023).

Classical SEM has been predominantly used to investigate the external morphology of different representatives of Neodermata, for example the scolex of various Cestoda (Mutti, Franzese, Ivanov, and Arredondo, 2023; and references therein) and the tegumental surface of different Digenea (Allam, Mashaly, and El-Naggar, 2023; and references therein). Additionally, SEM has been employed to study isolated copulatory organs and haptoral sclerites in parasitic monopisthocotylan and polyopisthocotylan monogenean flatworms (Dos Santos, Dzika, and Avenant-Oldewage, 2019a; Dos Santos, Maina, and Avenant-Oldewage, 2015, 2019b; Fannes, Vanhove, and Huyse, 2017; Fannes, Vanhove, Huyse, and Paladini, 2015; Harris, Cable, Tinsley, and Lazarus, 1999; Mo and Appleby, 1990; Shinn, Gibson, and Sommerville, 1993, 2001).

However, SEM investigations focusing on the sclerotised parts in microturbellarians have been conducted less frequently, except for a few studies on *Gieysztoria* Ruebush & Hayes, 1939 (Brusa, Damborenea, and Noreña, 2003, 2008; Damborenea, Brusa, and Noreña, 2005), *Temnocephala* Blanchard, 1849 (Damborenea and Brusa, 2008; Lenis, Ruiz, Muskus, Marcilla, and Vélez, 2020; Seixas, Amato, and Amato, 2010), *Macrostomum velastylum* Brusa, 2006 (Brusa, 2006), and the description of the egg capsules of the symbiotic *Syndesmis kurakaikina* Monnens, Vanhove & Artois, 2019 (Monnens et al., 2019). While these SEM-studies provide more resolution and detail, follow-up work has remained limited. We suspect this may be attributed to the variable success rates of currently available protocols for the isolation and visualisation of sclerotised structures. Further finetuning of these protocols holds much potential to extend their application to other microturbellarian taxa, and thus facilitate taxonomic research on these organisms.

In this study, we present a protocol, adapted from Fannes et al. (2015), for the isolation and SEM visualisation of the sclerotised structures in microturbellarian flatworms. We explore and illustrate the use of this protocol for *Trigonostomum* Schmidt, 1852, a cosmopolitan marine genus of microturbellarians which at present contains 22 species (Tyler, Artois, Schilling, Hooge, and Bush, 2006-2024). Mature specimens are typically 0.5–1.5 mm long and largely colourless, although some exhibit variations in pigmentation pattern. All species possess a sclerotised male copulatory organ and a sclerotised bursal appendage. The copulatory organ shows considerable interspecific differences, yet they are hypothesised to all have a uniform base structure consisting of an inner 'stylet' and an outer 'mantle,' forming a 'double-walled copulatory organ' (Willems, Artois, Vermin, and Schockaert, 2004). However,

this hypothesis is based solely on light microscopy (LM), which reaches its limits in resolving the finest structural details within this taxon. As these observations underpin our current understanding of comparative morphology in species of *Trigonostomum*, it serves as a good candidate not only to assess the effectiveness of our SEM protocol, but also to explore whether it allows us to uncover new important morphological insights. Therefore, the goal of this study is to develop a laboratory method to isolate and visualise the sclerotised parts of species of *Trigonostomum* with SEM, and to compare our morphological findings with those proposed by Willems et al. (2004).

2. Material and methods

Specimen selection

Three species of the genus *Trigonostomum* from Hasselt University (HU) collections were studied with SEM. The sclerotised parts of the copulatory organ of two specimens of *T. venenosum* (Uljanin, 1870) Meixner, 1924 (collection numbers: HU-ST1, HU-ST5), three specimens of *T. setigerum* Schmidt, 1852 (collection numbers: HU-ST2, HU-ST3, HU-ST4), and one specimen of *T. penicillatum* (Schmidt, 1857) Graff, 1905 (collection number: HU-ST7) were visualised. This selection included one species from each group as delineated by Willems et al. (2004), which will be introduced later. Specimens were primarily collected in Sardinia in 2010, except for one specimen of *T. setigerum*, which was collected in Tuscany in 2019. All specimens were preserved individually in 90% ethanol post-field collection.

The copulatory organs of other available specimens of each included species were visualised with Differential Interference Contrast microscopy (DIC, referred to as LM for the remainder of the study), using a Leica DM 2500 LED microscope. The images were captured via the accompanying LAS X software (version 3.6.0.20104), and they were stacked with the auto-blend feature in Adobe Photoshop (version 26.0.0). The wholemounts used for this purpose are part of the Hasselt University reference collection, listed under the following collection numbers: HU-IX.1.14 (*T. venenosum*), HU-II.2.41 (*T. penicillatum*), HU-II.3.12 (*T. setigerum*).

Isolation of the sclerotised structure

Our protocol builds further on the protocol of Fannes et al. (2015).

Specimens preserved in ethanol were first transferred to an embryo dish (Fig. 1A). Using a glass pipette, ethanol was removed as much as possible (Fig. 1B), after which the specimen was immersed in distilled water (Fig. 1B). After approximately 10 min, a 2 µl droplet of water was placed onto a microscope slide and covered by a round glass coverslip (Ø = 10 mm, VWR, 631-0170). Excess water was siphoned off with filter paper, affixing the coverslip to the slide. Using a glass pipette coated with Sigmacote® (Sigma-Aldrich, VWR), the specimen was transferred to the coverslip (Fig. 1C) and carefully moved out of the water with a fine needle. Filter paper was used to remove as much water as possible by holding it on the edge of the droplet (Fig. 1D). This step must be executed with caution to prevent the specimen from being absorbed by the filter paper.

Subsequently, the specimen was covered by 2 µl of Proteinase K:TNES buffer (1:1) (Proteinase K from Thermo Scientific, 10181030: in-house prepared TNES lysis buffer) (Fig. 1E). The enzymatic digestion was allowed to proceed for approximately 10 min,

while being monitored periodically under a stereomicroscope. If the digestion was incomplete after 10 min, 2 µl of distilled water was added to dissolve any crystals, followed by the addition of another 2 µl of proteinase K:TNES buffer (Fig. 1E–F), with regular checks under the stereomicroscope. Digestion was considered complete when the sclerotised copulatory organ became clearly visible, and the remainder of the specimen had (almost) fully disappeared.

A few minutes after the start of the digestion process, the enzymatic solution begins to crystallise. Fannes et al. (2015) address this problem by adding water and allowing the slide to dry repeatedly. However, this method is, in our experience, time-consuming and often ineffective, possibly resulting in specimen loss. Therefore, we extracted the sclerotised structure from the buffer as soon as digestion was (nearly) complete. Using a fine needle, and working under a stereomicroscope, the structure was carefully moved to a dry section of the cover glass (Fig. 1G–H), and excess water was carefully siphoned away with filter paper (Fig. 1I). The slides were then left to dry overnight, covered by a lid, cap, or small petri dish to prevent dust from falling on the sample.

In this state, prepared specimens can be stored at room temperature for several weeks. However, it is preferable to continue the protocol promptly to avoid contamination by dust particles. The proteinase K:TNES buffer can be re-used when stored at -20 °C or even at 4 °C, yet we recommend using freshly prepared buffer solution as digestion proceeds faster with fresh reagent.

Next, the samples were examined under a stereomicroscope and compound microscope to locate the sclerotised structure and screen for any remaining attached crystals, which could impair clear visualisation under the SEM later. Eventual crystals were carefully dislodged using a needle under a compound microscope. If not all crystals could be detached with the needle, a tiny droplet (1–2 µl) of distilled water was applied directly to the sclerotised structure, after which the structure was carefully dragged away with the needle from the water and left to dry. Any residual water on the coverslip was carefully wiped off before proceeding. The drying process could take a few days, and the sample was checked under a compound microscope daily until it was fully dried up.

SEM visualisation

Once the coverslip was completely dry, it was carefully detached from the slide with a needle or fingernail and mounted on a small aluminium stub (Ø = 12.7 mm, Micro To Nano, Haarlem, The Netherlands) using conductive double-sided adhesive carbon tape (Ø = 12 mm, Micro To Nano, Haarlem, The Netherlands). Next, the stub was sputter coated with gold for 15 s at 30 mA (Jeol JFC 1300) and loaded into a benchtop SEM (Phenom ProX) for visualisation.

3. Results

The method introduced in this study was applied to 16 specimens of *Trigonostomum*, successfully isolating and visualising the sclerotised part of the male copulatory organ in six specimens from three different species (Fig. 2A–C, 3A, 4A–D), with each species representing one of the groups delineated by Willems et al. (2004). Additionally, the bursal appendage of one specimen of *T. penicillatum* was successfully isolated and visualised (Fig. 3B). All sclerotised structures generally display morphological

characteristics consistent with descriptions based on DIC light microscopy (LM), though certain details differ.
To facilitate a comparison of imaging techniques, LM images of other specimens from the same species were included. While a detailed description of the LM findings has been previously provided by Willems et al. (2004) and will be further discussed below, these results focus on observations from the SEM images, emphasising the most notable structural differences identified between LM and SEM images for the respective species.

Trigonostomum venenosum

The SEM images of the male copulatory organ of *T. venenosum* reveal a complex structure that poses challenges in terms of interpretability (Fig. 2A–C). The morphology of the stylet is relatively straightforward (Fig. 2A–C, st): it begins proximally with an enlarged part that contains the stylet opening (Fig. 2A–C, so), then extends in a 270° curve, narrowing distally into a slender, nearly straight part. In Figure 2B, this slender, straight segment of the stylet is not visible. There is an unknown structure at the top of the image (Fig. 2B, x) which could be a remnant of a broken off stylet or a part of the mantle.
The mantle (Fig. 2A–C, m) attaches proximally at the stylet's opening, fully enveloping the stylet up to approximately halfway along its length, just beyond the curvature. Here, it forms a prominent, tubular structure that runs alongside the straight portion of the stylet. This tubular structure appears to have an opening adjacent to the stylet's opening (Fig. 2A–C, mo), leading downward into a channel. It remains uncertain whether this channel is open or closed at its distal end. The mantle structure located between the stylet opening and the opening of the tubular structure of the mantle is more challenging to interpret and seems to vary slightly among the specimens.

Compared with LM (Fig. 2D), SEM imaging offers enhanced resolution, greater magnification, and improved structural clarity. The stylet is identifiable in the LM image (Fig. 2D, st), though with less precision, while the mantle structure is nearly indistinguishable with LM. Despite the higher resolution of SEM, however, some intricate details of the mantle structure remain too complex to interpret with complete certainty.

Trigonostomum penicillatum

The sclerotised copulatory organ of *T. penicillatum* is readily interpretable from the SEM image (Fig. 3A). Proximally, the stylet (Fig. 3A, st) features a large opening (Fig. 3A, so) with a broad gutter-like structure (Fig. 3A, gu). The stylet curves at approximately 90° (Fig. 3A, indicated angle), tapering distally into a narrower straight segment.
The mantle attaches at the lower end of the gutter-like structure near the proximal stylet opening (Fig. 3A, m). It is likely that the mantle attaches to both sides of the gutter-like structure, as the opposite side of the mantle appears visible, parallel to the front side. Both sides of the mantle converge and encase the distal, straight portion of the stylet. At the distal end, the mantle produces a protrusion that aligns with the stylet (Fig. 3A, pr). Furthermore, there appears to be a plate-like structure attached to the underside of the proximal end of the stylet (Fig. 3A, x). It is unclear whether this structure is part of the stylet proper, or of the mantle, as the precise morphology remains obscured by the overlapping part of the mantle.

The LM image (Fig. 3C) generally aligns with the SEM image in depicting the stylet structure (Fig. 3C, st), though the proximal region containing the stylet opening and gutter-like structure is less distinct than in the SEM image. The morphology of the mantle is notably less defined in the LM image (Fig. 3C, m); while both sides of the mantle are visible, they could be misinterpreted as two spines oriented distally along the stylet (Fig. 3C, sp). The proximal portion of the mantle is also unclear in the LM image.

On the SEM image (Fig. 3B), the bursal appendage of *T. penicillatum* reveals densely packed tubular structures (Fig. 3B, tu). Near the top, a fold or collar-like feature is visible (Fig. 3B, co), followed by a region covered with granules (Fig. 3B, gr) that culminates in a crown-like structure (Fig. 3B, cr). The LM image (Fig. 3D) reveals the same characteristics, although the collar (Fig. 3D, co) and granular region (Fig. 3D, gr) are less distinct, whereas the crown-like structure is more distinguishable (Fig. 3D, cr).

Trigonostomum setigerum

The SEM images of the sclerotised male copulatory organ of *T. setigerum* are quite straightforward to interpret. Seen from the proximal end (Fig. 4A–B, D), the stylet (Fig. 4A–D, st) displays a broad opening (Fig. 4A–B, D, so), which then narrows and spirals into approximately 2.5 clockwise turns. The number of turns is only discernible on the images taken from the proximal end, as the proximal opening of the stylet is obscured by the structure in the image taken from the distal end (Fig. 4C). Towards the distal region, the stylet straightens and terminates in a narrow aperture (Fig. 4A, ap). The mantle (Fig. 4A–D, m) is attached at the proximal portion of the stylet, completely encasing the spiral and terminating distally into two closely connected hooked spines (Fig. 4A–C, sp). A pronounced thickening of the mantle wall is observed near the centre of the structure (Fig. 4A–D, tw), which is seen both from the proximal and distal side, with distinct radial wrinkles (Fig. 4A–D, rw) connecting this thickened wall and the stylet. In fig. 4A, the distal portion of the stylet is detached from the two terminal spines of the mantle. In fig. 4C, one of the mantle spines is detached from the stylet.
The LM image (Fig. 4E) reveals most features observed in the SEM images, but with less resolution. The spiralling stylet and mantle (Fig. 4E, st, m), radial wrinkles (Fig. 4E, rw), and two distal hooks (Fig. 4E, sp) on the mantle are identifiable, but with much less clarity. Certain structural details are more apparent in SEM, such as the proximal opening of the stylet (Fig. 4A–B, D, so) and the thickened mantle wall (Fig. 4A-E, tw) in the centre of the spiral. The latter may give the wrong impression of a double structure of the mantle at the centre of the spiral, on the opposite side of the stylet, when looking at the LM image.

4. Discussion

Our protocol proved effective for imaging the sclerotised copulatory organs of three species of *Trigonostomum*. The resulting images reveal morphological details that are difficult to discern using light microscopy (LM) and provide new insights into the morphological structure of the copulatory organ of these *Trigonostomum* species. The protocol is straightforward and cost-effective, using materials and reagents commonly available in standard laboratory settings.

While LM remains the most rapid and accessible technique for observing sclerotised organs in microturbellarians, the method presented here is a valuable complement to traditional LM. The SEM images provide a higher resolution and the possibility to visualise the structure at a greater magnification than acquired with LM. Once one has discovered and properly understands the structural morphology as seen with SEM, it is much easier to understand and recognise those morphological features with LM. However, the protocol requires some skill and patience, as tracking the sclerotised structure under a stereomicroscope during isolation can be challenging. We chose to isolate the sclerotised structure from the enzymatic solution using a needle, rather than following the method of Fannes et al. (2015), which involves repeated cycles of drying and adding distilled water to remove the crystals. The needle technique is more time-efficient, but it requires careful handling of the structure to avoid damaging it. Furthermore, we observed that the specimen does not always fully dissolve, which makes gently extracting the copulatory organ with a needle more practical. Nevertheless, isolating very small and delicate structures, such as the bursal appendage in *Trigonostomum*, remains challenging due to the risk of damage or loss of the structure during the process. Furthermore, the orientation of the structure during SEM imaging is difficult to determine beforehand, and excessive manipulation with needles may damage the structure. Therefore, it is advisable to refrain from further adjustments once successful isolation is achieved. Many modern SEM devices allow to adjust the viewing angle, providing greater flexibility than the SEM used in this study. 16 specimens of different species of *Trigonostomum* were included in this study, but the sclerotised part of the copulatory organ of only six specimens was visualised successfully. It's important to note that the success rate improves significantly with increased practice.

Despite these limitations, with sufficient experience, imaging multiple specimens per species from different angles can readily be achieved, providing insights into the morphological structure of sclerotised copulatory organs. Therefore, we are optimistic that implementing this protocol will enhance the utility of SEM as a method for detailed morphological and taxonomic studies of microturbellarians.

Refining previous morphological descriptions of copulatory organs of Trigonostomum *with SEM imaging*

Using LM, Willems et al. (2004) described the general morphological structure of the copulatory organ of representatives of *Trigonostomum* as consisting of an inner stylet surrounded by a mantle (Willems et al., 2004: figure 4A). As such, they identify two major groups of copulatory organs. In group 1, the stylet is a narrow tubular structure with a broad, curved, proximal opening, and a straight distal part. The mantle does not follow the curvature of the stylet and envelops only part of its straight distal portion, where it terminates in spiny plates (Willems et al., 2004: figures 4B, 6, 7). The first group is further divided into two subgroups. In group 1A (Willems et al., 2004: figure 6), the long and narrow stylet makes a proximal turn of 270°, while its mantle forms a narrow ring with a long spine. In group 1B (Willems et al., 2004: figure 7), the shorter and wider stylet makes a turn of about 90° or 180°, depending on the species. The mantle is divided into one, two, or three spines, depending on the species. In group 2, the stylet is peripheral, with the mantle forming a, according to Willems et al. (2004) but see further, double plate towards the centre of the spiral, displaying radial wrinkles. The distal part of the stylet and

mantle is straighter, with the mantle ending in two spiny plates, which are equipped with hooks oriented towards the convex side of the coil (Willems et al., 2004: figures 4C, 9, 10).

In this study, we were able to visualise one species of *Trigonostomum* for each type of copulatory organ described by Willems et al. (2004). From group 1A, we imaged the copulatory organ of *T. venenosum* (Fig. 2A-C). Willems et al. (2004) describe it as having a long, narrow stylet with a proximal part that turns 270°, and a mantle forming a narrow ring bearing a long, spoon-like spine (Willems et al., 2004: figure 6 A_1, A_3). While the SEM images confirm the morphology of the curved stylet in more detail (Fig. 2A–C, st), they uncover a mantle structure that is more complex than described with LM (Fig. 2A–C, m). They reveal a tube-like mantle structure with an opening adjacent to the stylet opening (Fig. 2A–C, mo). This tube is referred to as 'the spoon-like spine' by Willems et al. (2004). Furthermore, Willems et al. (2004) suggest that the mantle surrounds only the distal (straight) portion of the stylet. However, our SEM images reveal a different configuration: the mantle attaches at the proximal opening of the stylet, enveloping it fully until halfway along the stylet, just behind the curve, where it forms the tube-like structure next to the straight part of the stylet. Fig. 2B also shows an unknown structure at the top left of the organ (Fig. 2B, x). This might be the distal part of the stylet that broke off, or it might be a detached part of the mantle. Despite the increased detail and resolution provided by the SEM images, certain aspects of the mantle structure remain challenging to interpret, especially in the region between the stylet opening and mantle opening. Imaging additional copulatory organs from different angles from this species would be valuable to better understand the mantle's intricate structural composition.

The copulatory organ of *T. penicillatum* belongs to group 1B (Fig. 3A). The stylet is wider than that of group 1A, and proximally turns approximately 90° (Fig. 3A, st, indicated angle). The SEM image conforms largely to previous LM descriptions, with the proximal opening of the stylet curving and transitioning into the straight distal part. However, on the drawing of Willems et al. (2004, figure 7 C_1), it appears that the actual opening of the stylet points downward, while our SEM image shows that this opening is very large, contains a long gutter-like structure, and opens on top (Fig. 3A, so, gu). The mantle attaches to the lower end of the gutter-like structure and, with a fold on both sides, encloses the straight distal part of the stylet, terminating with a protrusion (Fig. 3A, pr). In contrast to the findings reported by Willems et al. (2004), the mantle does not terminate in three spiny plates. These observed spines may be an artefact, or they may have been misinterpreted, as on our LM image (Fig. 3C, sp), the two mantle folds fusing together around the stylet could readily be interpreted as the existence of similar spiny plates. The discrepancy could also be due to the spines breaking off during the isolation process of the sclerotised structure. Nevertheless, no damage is apparent in the visualisation, rendering this an unlikely explanation. Another possibility might be that the spines are hidden behind the mantle fold and that they comprise the distal part of the structure attached to the underside of the proximal part of the stylet, positioned between the two mantle folds (Fig. 3A, x). This is highly speculative, as it is unclear whether this unknown structure is a plate-like extension of the stylet or if it is indeed part of the mantle. Since one of the mantle folds obscures this structure, its precise morphology cannot be determined. Given that we had only one specimen of this species, additional images of the sclerotised copulatory organ from different angles are needed to resolve these uncertainties.

The copulatory organ of *T. setigerum* belongs to the second group (Willems et al., 2004). Our SEM images (Fig. 4A–D) corroborate previous observations by Willems et al. (2004, figures 9 C, 10 C_1), but they show the structure in higher resolution, which provides additional details of the different features such as the radial wrinkles (Fig. 4A–D, rw), the thickened wall (Fig. 4A–D, tw) at the centre of the spiral, the proximal opening of the stylet (Fig. 4A–B, D, so), and the two distal spines of the mantle (Fig. 4A–C, sp). However, it is slightly more difficult to discern with SEM how many spirals the copulatory organs contain, as the structures are seen from the proximal or distal side and the spirals are tightly coiled up, rather than flattened as seen in wholemounts. As the number of spirals is a taxonomically important trait, it shows why this technique should be a complement to LM, rather than a replacement. Furthermore, Willems et al. (2004) describe a mantle that forms a double plate towards the centre, but our SEM images do not confirm this. Instead, the thickened mantle wall (Fig. 4A–E, tw) at the centre of the spiral was apparently misinterpreted as a double structure. This further demonstrates the value of SEM images for gaining more clarity about structural features that might be misinterpreted with LM.

Visualising a bursal appendage with SEM provides new morphological details

According to Meixner (1924), there are two primary types of bursal appendages in *Trigonostomum*: (1) the '*setigerum* type' which consists of two slightly coiled narrow tubes attached to a funnel- or ring-like structure at the bursal wall, and (2) the '*penicillatum* type' which is characterised by a bundle of tubules with a 'barrel-like' casing near the top. Willems et al. (2004) identified a third type, comprising 'a funnel with two highly coiled tubes'.

Although the main goal of this study was to visualise the copulatory organs of species of *Trigonostomum*, we additionally succeeded in visualising the bursal appendage of *T. penicillatum* (Fig. 3B), which is of type 2 described by Meixner (1924) and Willems et al. (2004). The SEM image provides a complete view of the bursal appendage, clearly depicting the bundle of tubules (Fig. 3B, tu), a fold or collar-structure (Fig. 3B, co), and a crown-like feature at the top (Fig. 3B, cr). The 'barrel-like' casing described by Willems et al. (2004, figure 7 C_2) most likely corresponds to the granular region we observed on the SEM image, which extends from the fold up to the crown (Fig. 3B, gr). The 'brush-like appearance' of the crown, as seen in LM and illustrated by Willems et al. (2004, figure 7 C_2), seems to have been flattened during the imaging procedure, or it may have been an artifact caused by squeezing the animals, as suggested by Willems et al. (2004) themselves.

5. Conclusions

The protocol presented in this study proved successful in isolating and imaging the sclerotised copulatory organs of different species of *Trigonostomum*. By offering greater magnification than LM, SEM yielded images of these taxonomically important traits with higher resolution. The images revealed morphological details that are difficult to discern using LM, providing new insights into the structural morphology of the sclerotised structure. The stylet region of the copulatory organs in the three species examined was relatively straightforward to describe and largely aligned with previous LM descriptions,

except for an unknown structure observed in one of the images for both *T. venenosum* and *T. penicillatum*. The mantle structure, however, remains difficult to interpret with SEM, especially in *T. venenosum* and, to a lesser extent, in *T. penicillatum*. Additional specimens are required to resolve current uncertainties.

While LM remains an essential and accessible tool, SEM provides additional detail that enhances our understanding and is a valuable complement to LM. Visualising multiple specimens per species at different angles with SEM, together with the traditional LM, will provide robust data for future morphological studies. The protocol can readily be extended to other species and could be a useful tool for other researchers studying the morphology of microturbellarians, with significant potential to advance morphological and taxonomic investigations.

Author contributions

Laura Vanstraelen: methodology, data curation, investigation, validation, visualisation, writing−original draft, writing−review & editing. **Tom Artois:** Conceptualisation, supervision, funding acquisition, project administration, resources, writing−review & editing. **Thierry Backeljau:** supervision, writing−review & editing. **Nikol Kmentová:** methodology, writing−review & editing. **Mare Geraerts:** methodology, writing−review & editing. **Marlies Monnens:** conceptualisation, methodology, supervision, funding acquisition, project administration, resources, writing−review & editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting the findings of this study consists of images created of wholemounts and SEM samples mounted on stubs. This material can be found in the reference collection of Hasselt University.

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Figure legends

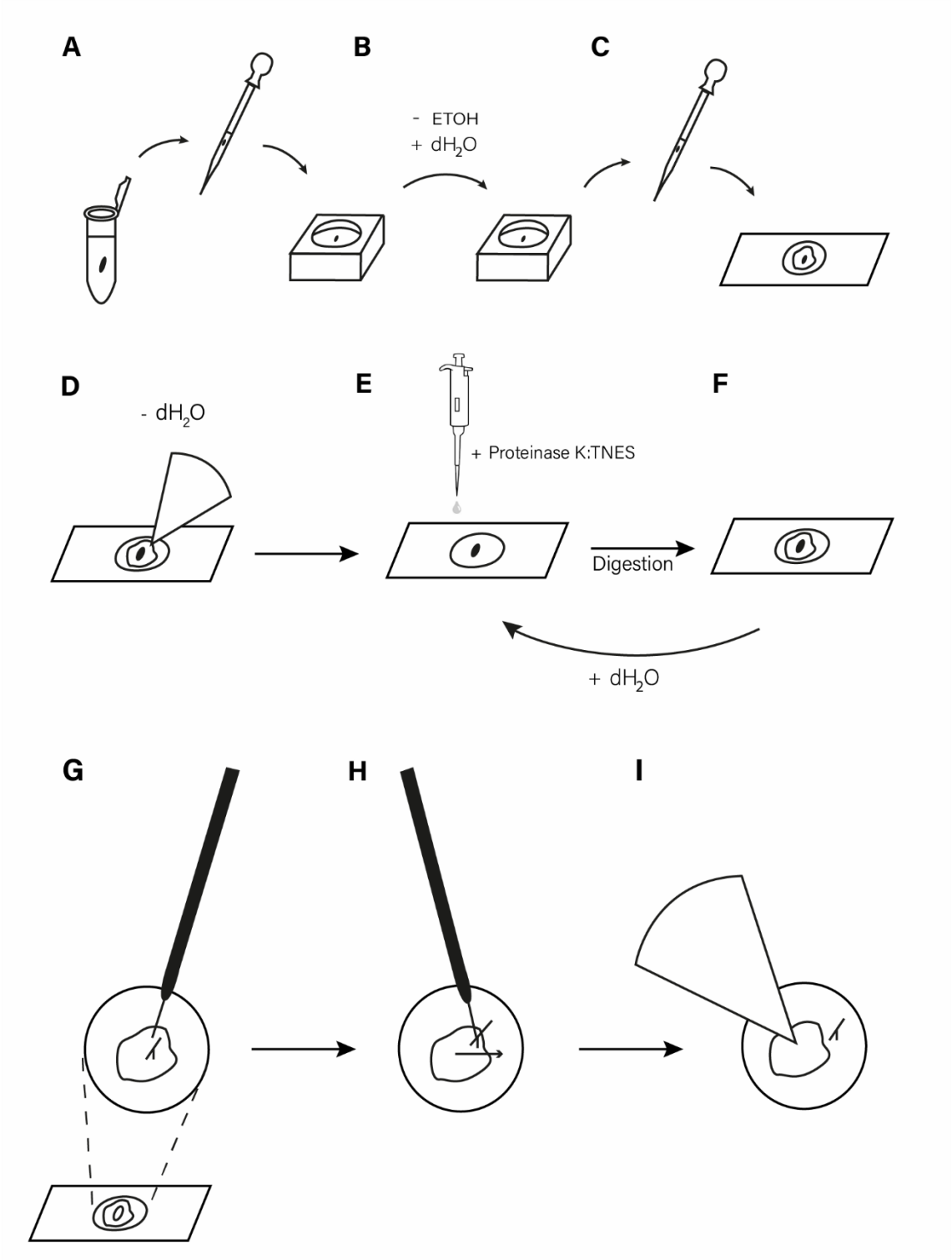

Fig. 1: Schematic overview of the protocol. **A:** The specimen is transferred to an embryo dish using a glass pipette. **B:** Ethanol is removed from the embryo dish and distilled water is added. **C:** Using a (coated) glass pipette, the specimen is transferred to the coverslip on the glass slide. **D:** Water is removed from the specimen with a filter paper. **E:** The specimen is covered by 2 µl of proteinase K:TNES buffer. **F:** The digestion process takes place. If the digestion is incomplete after 10 min, 2 µl of distilled water is added and step **E** is repeated. **G**, **H:** A needle is used to swipe the sclerotised structure out of the water on the coverslip. **I:** The excess water is removed with a filter paper.

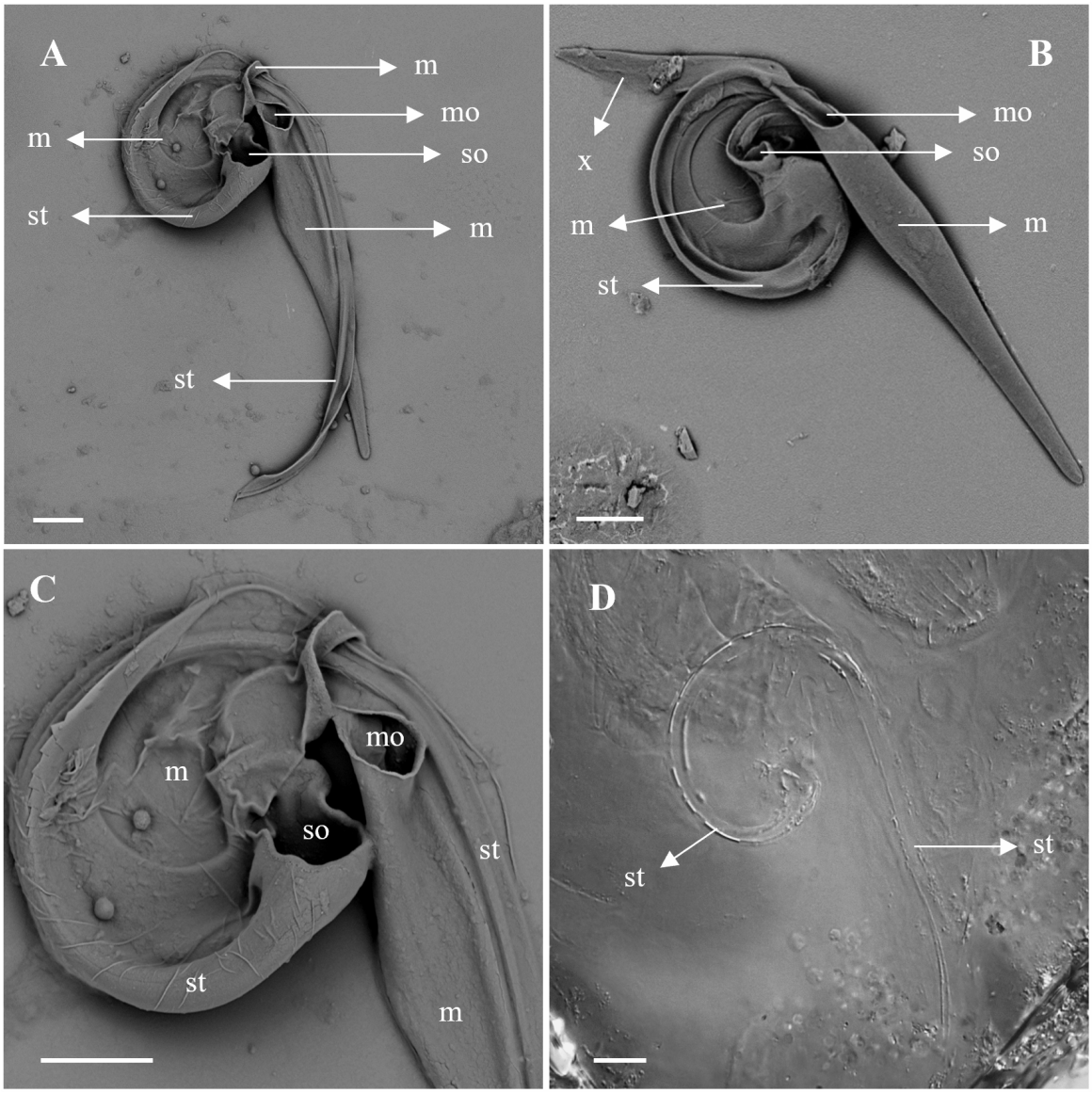

Fig. 2: Images of sclerotised copulatory organs of *T. venenosum*. **A** and **B:** SEM image of the complete structure of two specimens from Sardinia (collection numbers HU-ST5 and HU-ST1)**.** In image B, the distal part of the stylet is missing. **C:** More detailed SEM image showing the proximal curved part of the stylet with the mantle (collection number HU-ST5). **D**: Stacked LM image of the copulatory organ of a specimen from Sardinia (collection number IX.1.14). Scale bars = 10 µm. Abbreviations: m = mantle, mo = mantle opening, so = stylet opening, st = stylet, x = unknown structure.

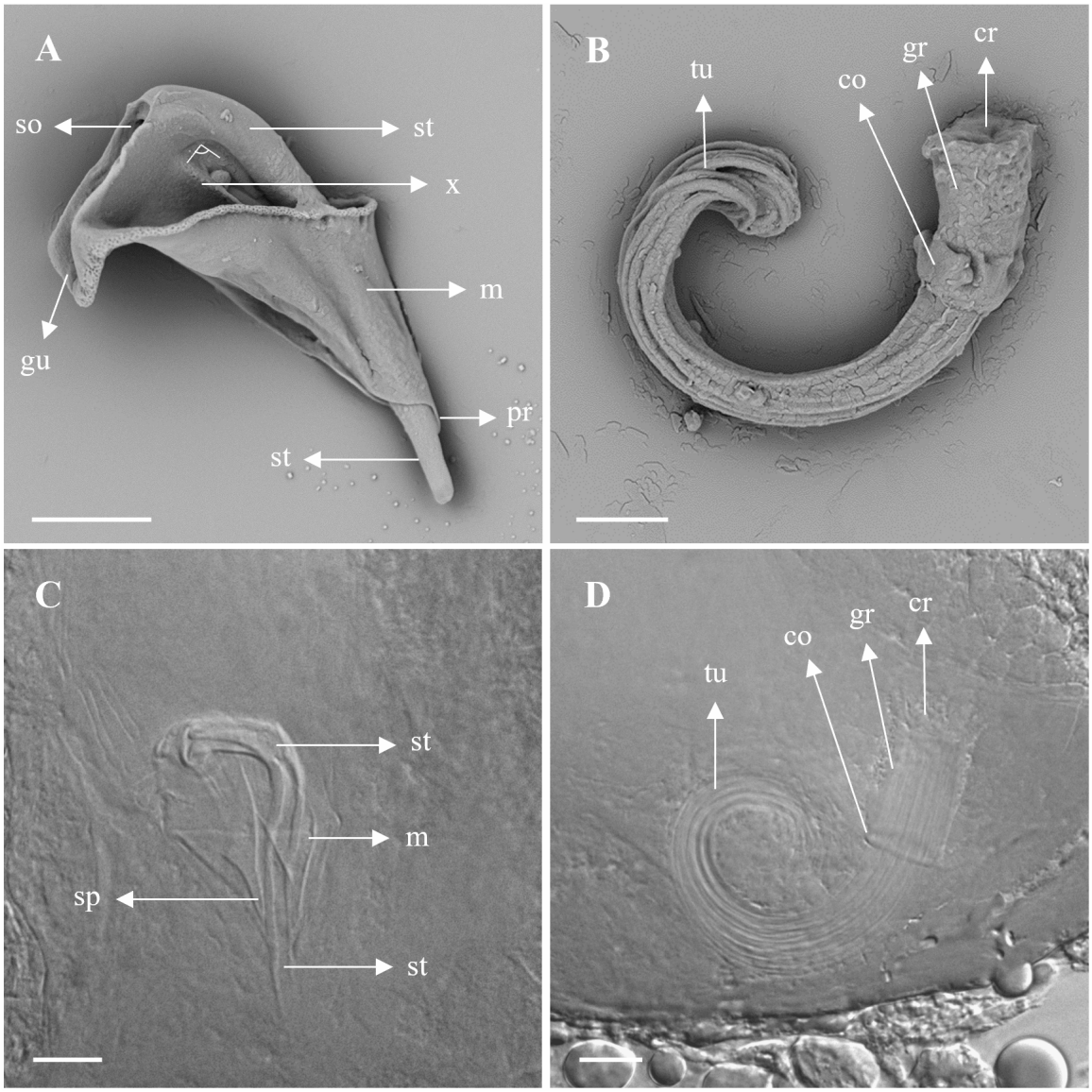

Fig. 3: Images of a sclerotised copulatory organ and a bursal appendage of *T. penicillatum* **A:** SEM image of the sclerotised copulatory organ of a specimen from Sardinia (collection number HU-ST7). The stylet forms an approximate 90° angle, as indicated. **B:** SEM image of the bursal appendage of the same specimen. **C:** Stacked LM image of the sclerotised copulatory organ of a specimen from France (collection number II.2.41). **D:** Stacked LM image of the bursal appendage of the same specimen. Scale bars = 10 µm. Abbreviations: co = collar or fold-like structure, cr = crown-like structure, gr = granular region, gu = gutter-like structure, m = mantle, pr = protrusion, so = stylet opening, st = stylet, tu = tubular structures, x = unknown structure.

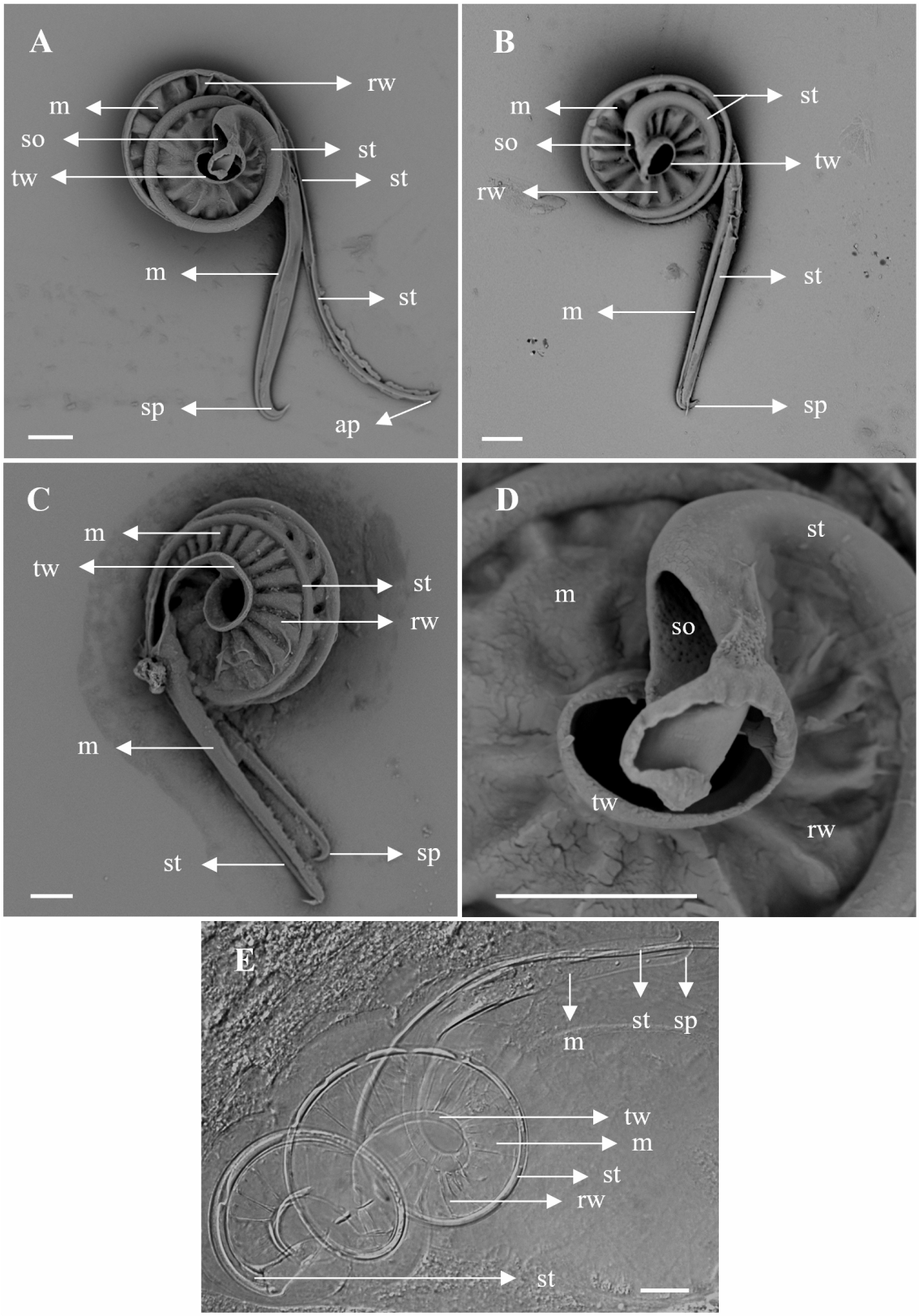

Fig. 4: Images of sclerotised copulatory organs of *T. setigerum*. **A** and **B:** SEM images of the complete structure of specimens from Sardinia and Tuscany, seen from the proximal end (collection numbers HU-ST4 and HU-ST2). **C:** SEM image of the complete structure of a specimen from Sardinia, seen from the distal end (collection number HU-ST3). **D:** Detailed SEM image of the structure seen from the proximal end (collection number HU-ST4). **E**: Stacked LM image of the copulatory organ of *T. setigerum* seen from the side (collection number II.3.12). Scale bars = 10 µm. Abbreviations: ap = aperture, m = mantle, rw = radial wrinkles, so = stylet opening, sp = hooked spines, st = stylet, tw = thickened wall.