Scanning electron microscopy in the taxonomical study of free-living marine nematodes

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Summary

Free-living marine nematodes are microinvertebrates composing one of the most diversified groups of the marine biota, with more than 7000 species. This means that only the 20% of the species is currently known. Several morphological features can help their taxonomical identification such as cephalic, cervical and body setae, amphids, cuticle, spicules and tail that may also have a functional role. Given the small size of these organisms, they differ in minute characters that can be detected more effectively by scanning electron microscopy (SEM). This study presents an overview of the use of SEM on some nematode species collected in the Maldivian archipelago, and highlights the importance of this technique in the taxonomical study of nematodes as well as its potentialities in the functional investigation of some of their structures.

Key words: nematodes, taxonomic identification, morphological characters, adaptations, scanning electron microscopy.

Introduction

Nematodes are cylindrical elongated worms with free-living, symbiotic or parasite life style. In particular, free-living species have a body length of about 1-2 mm and are one of the most abundant component of the meiofaunal assemblage: to date about 7000 species have been described, but this number has been estimated as only a small part of the real diversity of the phylum (Appeltans *et al.*, 2012).

Nematodes show different body shapes, from the most common cylindrical form to a short or thick body (*i.e.* order Desmoscolecida) or to a "S" and "ε"-shaped body (*i.e.* families Draconematidae and Epsilonematidae). Other nematodes are completely covered with bacterial ectosymbionts arranged in multiple layers (i.e. sub-family Stilbonematinae) or may present close relations with ectocommensals such as Suctoria (Semprucci and Balsamo, 2012; Ansari *et al.*, 2017).

These organisms have a pseudocoelic body cavity filled with a high-pressure fluid where the reproductive system lies (Platt and Warwick, 1983). Their body wall is formed by an external thick cuticular layer made of collagen. The cuticle usually bears sensory structures (sensillae) distributed along the body, but mainly present on the head region. Arrangement, shape, position and size of sensillae and lateral organs (amphids) are species-specific and consequently are diagnostic characters for the taxonomical identification. Among them, the amphid is the largest and most complex sensorial organ of the nematode cephalic region (Decraemer *et al.*, 2014).

Nematodes contribute to the benthic energy flows in different ways (Giere, 2009) and have a central role in the trophic chains of marine habitats (Danovaro *et al.*, 2008; Semprucci and Balsamo, 2012; Semprucci *et al.*, 2015). They have numerous trophic styles that may also be recognized by the different morphology of the buccal cavity (Wieser, 1953).

Habitats show a nematode fauna of different composition in species that are characterized by specific morphological and physiological adaptations. It is well known that both the general shape of nematode bodies and the structure of their organs may be correlated to environmental factors prevailing in the habitat for which they are adapted (Wieser, 1959).

Due to the small body size, light microscopy has some limits in effectively discerning minute morphological details. Several publications focused on the taxonomy and systematics of nematodes have applied ultrastructural techniques, but the latter are still underemployed. This paper is a review on the great variety of morphological features of nematodes that can be analyzed by scanning electron microscopy (SEM), like cephalic, cervical and body setae, amphids, cuticle, spicules and tail, and highlights the importance of this technique in the taxonomical study of nematode species.

Materials and Methods

Specimens were obtained from sediments collected from the Central part of the Maldivian Archipelago, mainly from Ari, South Malé, and Felidhoo atolls (Indian Ocean). Sediment samples were taken by a Scuba diver using a plexiglass corer (Ø2 cm). They were treated with a 7% $MgCl_2$ aqueous solution for narcotizing fauna, and then fixed with a neutralized formaldehyde solution (4% final concentration). Meiofauna were separated from sediment after decantation through a 42 µm mesh sieve and centrifugation in Ludox HS 30 (Pfannkuche and Thiel, 1988). All meiofaunal animals were counted and sorted by taxon level (mainly *phylum* and order) under a stereo-microscope (Leica G26). Some nematode specimens especially interesting from a morphological point of view were selected for an ultrastructural analysis by SEM. To apply this technique, the selected organisms were first rinsed in cacodylate buffer to remove formalin medium and possible foreign material and subsequently they were dehydrated in a graded ethanol series, 5 minutes for each solution. Lastly the specimens were transferred in hexamethyldisilazane (HMDS) and allowed to dry, according to Hochberg and Litvaitis (2000). Dry specimens were mounted on aluminum stubs, and sputter-coated with gold-palladium for 5 minutes. Observations were carried out under a SEM Philips 515, at the University of Urbino, and under a SEM Q200FEG, at the Agency of the Regional Environmental Protection of Pesaro.

Results and Discussion

Cephalic, cervical and body setae

Sensillae (also called setae) have generally a tactile function. For instance, long setae probably lead the worm in the interstitial habitat, especially in exposed sediments, or may be used as adhesive organs as in some representatives of the family Epsilonematidae (Wieser, 1959; Gad, 2002).

The description of the number, arrangement and length of the labial and cephalic setae in nematodes is a major character reported in all taxonomical descriptions of the species and is used also as an element of phylogenetic discussion (*e.g.* Jensen, 1979; Lorenzen, 1994; Leduc, 2013; Semprucci, 2015); however, this character is valid only in adults because setae may be subject to changes from juvenile to adults stage.

In the head region, the most common pattern of setae is represented by 3 circles around the mouth as reported in Figure 1A, where hair-like setae are showed and arranged following the scheme 6+6+4. The distinctive feature of this specimen (genus *Desmodorella*, family Desmodoridae) is the presence of rows of short setae along the whole body. Although there is no evidence that this specific feature of *Desmodorella* is a possible adaptation to the habitat conditions, the representatives of this genus are dominant in medium-coarse sands likely thanks to the body stoutness provided by the combination of a thick cuticle and the presence of these short setae along the whole body (Semprucci *et al.*, 2010). Another possible arrangement of the cephalic setae is in two circles with the scheme 6+10. This pattern is visible in Figure 1B that shows a specimen of the genus *Bathylaimus* (family Tripyloididae) with the mouth surrounded by 3 high, rounded lips deeply incised. The 6 labial setae are very short, while 6 longer and 4 shorter jointed setae lie at the same level. Jointed setae are rare, but they are a characteristic feature of the family Tripyloididae.

Also the presence of setae in the oesophageal region (cervical setae) and their position and length are of taxonomical importance. They are generally mechanoreceptors, possibly with also a chemoreceptive function (Decraemer *et al.*, 2014).

Figure 1C shows a nematode of the genus *Pseudosteineria* (family Xyalidae) with very long subcephalic setae that are subdivided in 8 groups at the same level of the amphids. As argued previously, so developed structures could be have an important function as an adaptation to the interstitial habitat.

The somatic setae on the general surface of the body may be arranged randomly or in a definite pattern. Figure 1D shows a particular example of stout somatic setae of a specimen of the genus *Tricoma* (family Desmoscolecidae). An important role of these somatic setae in the locomotion of Desmoscolecidae within the substratum has been documented by different authors (see Decraemer and Rho, 2014 for details).

Finally, setae may be present or absent in the tail region, and their length and number have a relevance in the specific identification. A well-developed caudal seta in a specimen of the genus *Pseudosteineria* is visible in Figure 1E.

Amphids

Amphids are bilateral and generally symmetrical structures situated on the head region and are the main sensory organs of the nematodes. They are generally recognized as chemoreceptors, but detailed ultrastructural analyses revealed a more complex role of these organs (Decraemer *et al.*, 2014). Their shape, size and position in relation to the cephalic end are relevant information in the process of identification of nematodes and form a standard part of all the taxonomical descriptions of the species.

The amphids consist of an internal cavity, called *fovea*, filled with a gelatinous substance (*corpus gelatum*), opening at the cuticle surface. Several sensory filaments are inserted into the *corpus gelatum* and are generally more numerous in the class Adenophorea than in Secernentia. Lorenzen (1994) subdivided amphids in two major groups: spiral e non-spiral. In the first, the *fovea* can turn ventrally or dorsally so that amphids appear loop-shaped, with only one turn, or multi-spiral (with numerous turns). The same group includes also amphids with other shapes such as circular and transverse. The non-spiral amphids are pocket-like structures. Figure 2 shows all examples of the first group of amphids.

In particular, the first two pictures show circular amphids of different size, in the genus *Pseudosteineria* (family Xyalidae) (Figure 2A) and in the genus *Monoposthia* (family Monoposthiidae), respectively (Figure 2B). A rounded amphid with a vesicular (blister-like) *corpus gelatum* that covers the whole head region was documented in the genus *Desmoscolex* (Figure 2C): this is the typical amphid of the family Desmoscolecidae. In Figure 2D a multi-spiral amphid of the genus *Dorylaimopsis* (family Comesomatidae) is visible. In the multi-spiral amphids, the number of turns may be very important from a taxonomical point of view. Figure 2E shows

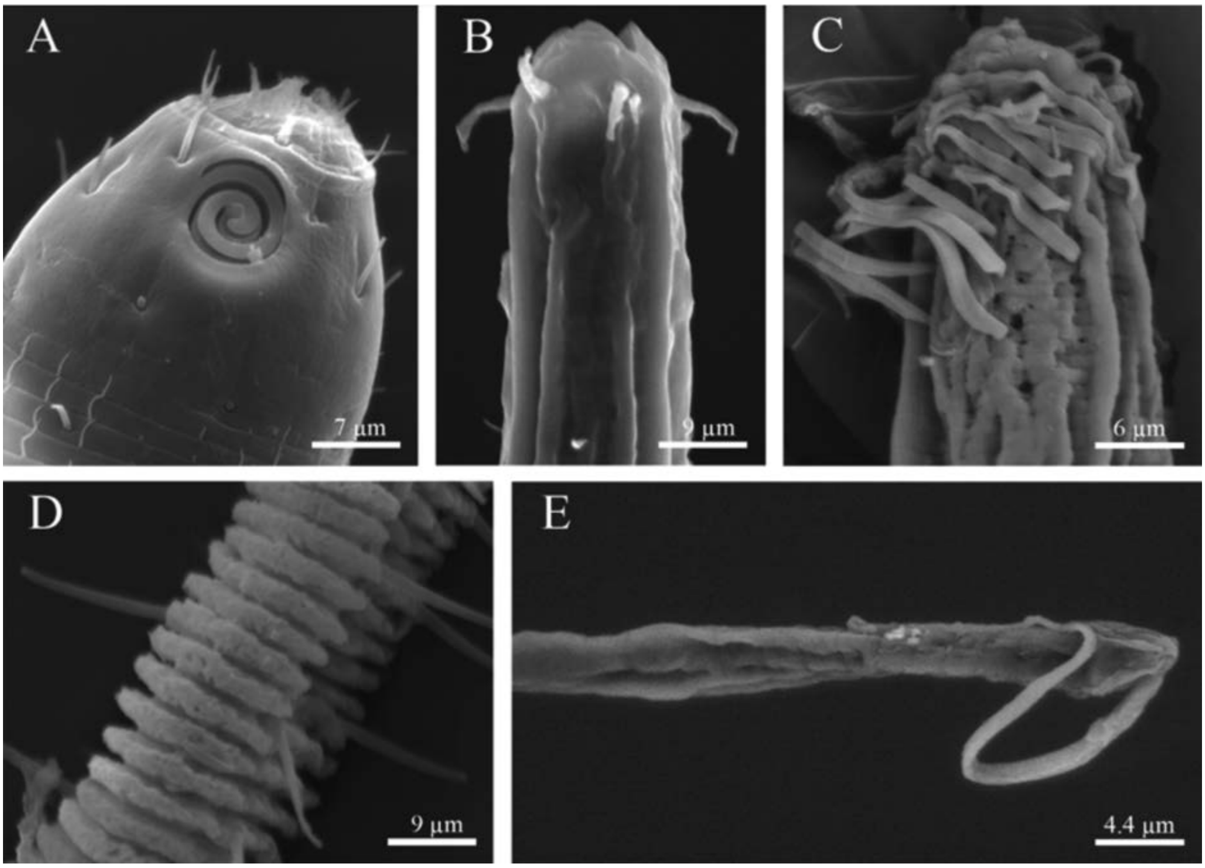

Figure 1. A) Hair-like setae of the genus *Desmodorella* (fam. Desmodoridae); B) Jointed setae, genus *Bathylaimus* (fam. Tripyloididae); C) Subcephalic setae, genus *Pseudosteineria* (fam. Xyalidae); D) Somatic setae, genus *Tricoma* (fam. Desmoscolecidae); E) Caudal seta, genus *Pseudosteineria* (fam. Xyalidae).

an elongated loop-like amphid typical of the genus *Ceramonema* and the relative family Ceramonematidae.

Available information about a possible differential function of the amphids in different types of habitats is scarce. However, it seems that amphids may perform a different role in soil and in open fresh waters. In the first case, the microhabitat is richer in substances and chemical information travels a short distance, while open freshwaters are usually less rich in dissolved matter, and chemical information needs to travel long distances (Zullini A., personal communication). Accordingly, soil nematode species seem to have small and even punctiform amphids, while the

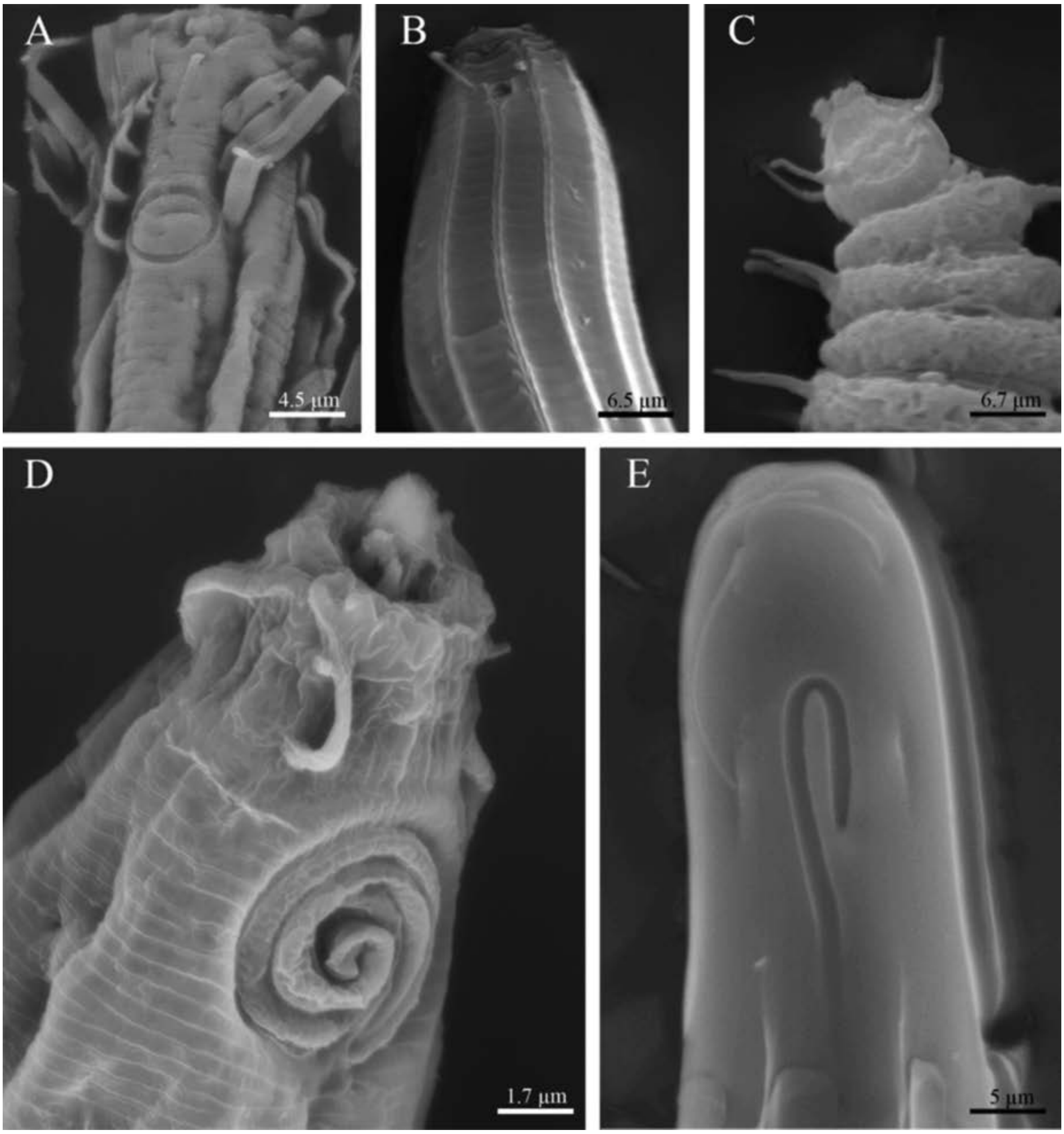

Figure 2. A) Circular amphid, genus *Pseudosteineria* (fam. Xyalidae); B) Small circular amphid, genus *Monoposthia* (fam. Monoposthiidae); C) Rounded amphid with a vesicular (blister-like) *corpus gelatum*, genus *Desmoscolex* (fam. Desmoscolecidae); D) Multi-spiral amphid, genus *Dorylaimopsis* (fam. Comesomatidae); E) Elongated loop-like amphid, genus *Ceramonema* (fam. Ceramonematidae).

amphids of freshwater nematodes appear larger. Marine species show a greater variety of morphologies of the amphids than in the terrestrial or freshwater habitats that likely reflects the comparatively higher heterogeneity of habitats in marine ecosystems. However, a possible relation between the amphid morphologies and the habitat features has not yet been explored in marine ecosystems.

Cuticle

Nematode cuticle represents a barrier between the animal and the environment, confers the body shape, and supports locomotion in synergy with body muscles and pseudocoel.

Nematodes show different types of cuticles: smooth, dotted, transversely annulated, covered with rows of spines or desmens (thick transversal rings consisting of sedimentary particles and concretions). Figure 3 shows some examples of these patterns: a smooth cuticle of the genus *Halalaimus* (family Oxystominidae, Figure 3A), and two different types of annulations with lateral bands (Figure 3B,C). Figure 3B shows a band in the lateral field along the whole body length (genus *Leptolaimus*,

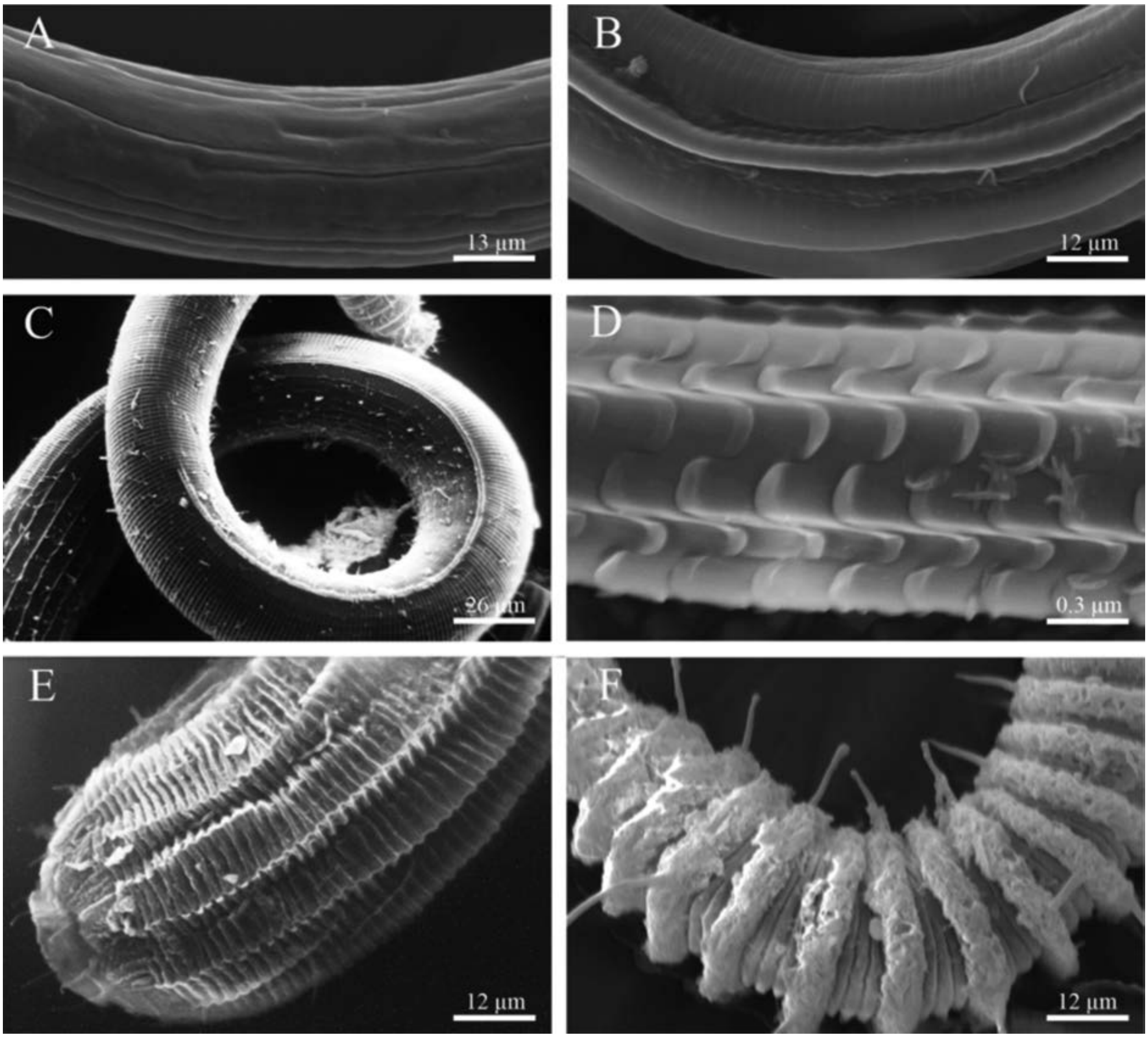

Figure 3. A) Smooth cuticle, genus *Halalaimus* (fam. Oxystominidae); B-C) Different types of annulated cuticle, genera *Leptolaimus* (fam. Leptolaimidae) and *Pseudochromadora* (fam. Desmodoridae); D-E) Strongly sculptured cuticle, fam. Ceramonematidae and fam. Selachnematidae; F) Desmens, genus *Desmoscolex (*Desmoscolecidae).

family Leptolaimidae), while Figure 3C focuses on the end of the oesophageal region (genus *Pseudochromadora*, family Desmodoridae). The genus *Ceramonema* (family Ceramonematidae) has wide body annules and longitudinal ridges (Figure 3D), the genera of the family Selachnematidae show a strongly sculptured cuticle of various morphologies (Figure 3E), and the genus *Desmoscolex* (family Desmoscolecidae) show the characteristic desmens (Figure 3F).

The external ornamentation of the cuticle may be observed in a different way by light and electron microscopy. Indeed, some fine details are not immediately visible even under SEM. For instance, the cuticle with dots of the Comesomatidae species appears only annulated using SEM (Muthumbi *et al.*, 1997), as well as do some complex cuticles of Chromadoridae species. However, the inner structure of the cuticle and important additional details may be captured by SEM simply by breaking the sur-

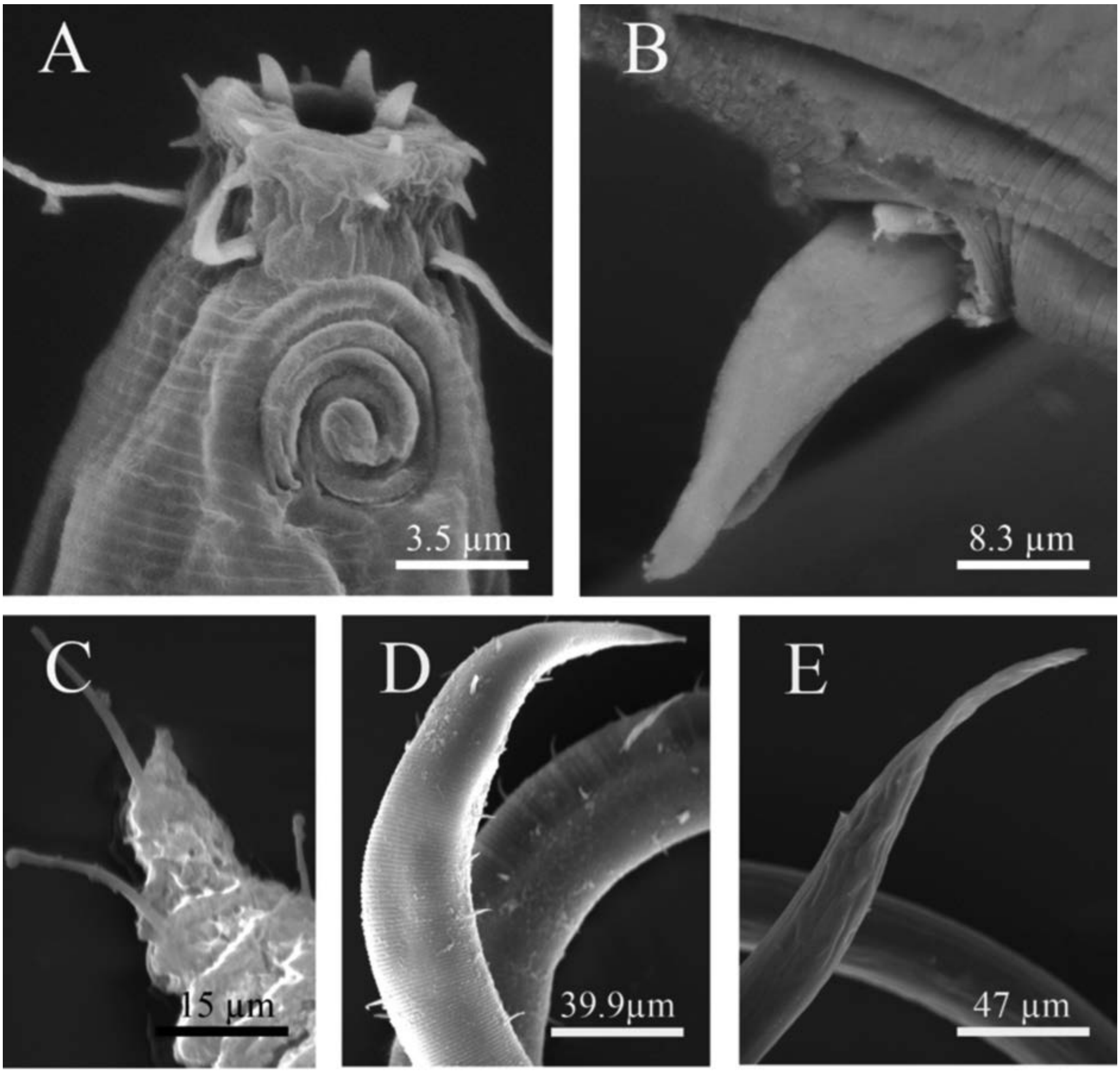

Figure 4. A) Teeth, genus *Dorylaimopsis* (fam. Comesomatidae). B) Tip of the spicule of a *Dorylaimopsis* species (fam. Comesomatidae). C) Short tail, genus *Desmoscolex* (fam. Desmoscolecidae); D) Conical tail, genus *Croconema* (fam. Desmodoridae); E) Elongate tail, genus *Halalaimus* (fam. Oxystominidae).

face of the nematode cuticle and observing the deeper layers (Gourbault and Vincx, 1994).

Buccal cavity

Nematodes show different levels of feeding specialization related to the adaptive plasticity of the buccal cavity structure: they may be omnivorous, predators, plant-feeding, or detritivorous (Semprucci and Balsamo, 2012).

Wieser (1953) proposed a distinction of nematodes in four trophic groups based on the morphology of their buccal cavity: selective deposit feeders (1A), non-selective deposit feeders (1B), epigrowth feeders (2A) and predators and omnivorous (2B).

The group 1A is formed by species that mainly feed on small detritus particles and bacteria, and thus show a small and non-cuticularized buccal cavity because they use the muscular pharynx to pump up the food. Species of the group 1B feed on bacteria, diatoms cells and organic particles and have a well-developed buccal cavity, but weakly cuticularized because they ingest mainly by lips. Instead, teeth of various size and denticles characterize the buccal cavity of species of the trophic group 2A. These structures allow them to feed on benthic diatoms, and also to scrape bacteria from the surface of the sand granules. Finally, the group 2B include species showing a buccal cavity with strongly cuticularized teeth and even mandibles used for predating various organisms, also other nematodes. A detail of the lips in the genus *Bathylaimus* (family Tripyloididae, trophic group 1B) is reported in Figure 1B and a close-up of the teeth in the genus *Dorylaimopsis* (family Comesomatidae, trophic group 2A) is visible in Figure 4A.

Spicules

Spicules are one or two cuticular copulatory male organs that are inserted into the female vulva for widening it during mating and allowing the sperm transfer. Nematode males generally have spicules with very complex structure and differing in shape and size in various species. The spicules are slightly curved and lie in the cloacal area within their specific pouches; they can be protruded and retracted by erector and protractor muscles. An accessory piece, the *gubernaculum*, may be present in some species and serves as a guide during the protrusion of the spicules. Spicules may be partially visible using SEM only if they are protracted outside the body of the animal; a complete observation of their structure may be possible after excision (Rammah and Hirschmann, 1987). In Figure 4B, it is possible to observe the tip of the spicule of a *Dorylaimopsis* species (family Comesomatidae) that reveals fine morphological details undetectable by light microscopy that can be used as a diagnostic taxonomical character (Semprucci *et al.*, 2016).

Tail

Tail shape is an important character in species identification: in fact it can be round, conical, clavate (i.e. conico-cylindrical with swollen tip) or elongated, and can change during the development phases (Schmidt-Rhaesa, 2014). Figure 4C shows a short tail of a specimen of the genus *Desmoscolex* (family Desmoscolecidae), a conical tail of a nematode of the genus *Croconema* (family Desmodoridae) (Figure 4D), and an elongate tail of an individual of the genus *Halalaimus* (subfamily Oxystominidae) (Figure 4E). Thistle and Sherman (1985) pointed out the importance of tail as a functional character like the trophic life style, and in particular as an adaptation to the habitat due to its great relevance in the locomotion. Glands can be also found in the caudal region where they produce an adhesive substance that is released outside through the tail tip (Figure 4C-E).

Conclusions

Nematodes have a small-sized body and generally differ in minute morphological characters. Although several publications focused on the taxonomy and systematics of nematodes have employed ultrastructural techniques, the use of these techniques is still limited even if they can allow to show details that light microscopy may hardly detect. Thus, we would like to point out the importance of SEM analysis in taxonomical studies as well as its potentialities in ecological surveys.

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