Morphological investigations of microdrile oligochaetes (Annelida, Clitellata) using scanning electron microscopy

Carlos CARAMELO, Enrique MARTÍNEZ-ANSEMIL*
Departamento de Biología Animal, Biología Vexetal e Ecoloxía, Universidade da Coruña, 15071 A Coruña - SPAIN

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Abstract: Over the past several years, the authors of this work have investigated external structures of microdrile oligochaetes using scanning electron microscopy (SEM). Both published and still-unpublished data have revealed new structures of interest, especially those associated with mating, ciliate sense receptors, chaetae, and dorsal pores. Mating systems can be classified in 3 categories: grasping, coupling, and embracing. Ciliate sense receptors can be classified as poorly defined (unelavated), sensory buds, or papillae, and are provided with blunt cilia and/or sharp cilia. They are present along the whole body (including clitellum, budding, and regeneration zones), scattered on the prostomium, peristomium, and pygidium, arranged in transversal rows and scattered in chaetal segments. Somatic chaetae show great variability when observed by SEM. Hair chaetae are at least potentially provided with denticulations. Dorsal pores were observed in aquatic microdriles. SEM proves to be an invaluable tool to discover important structures associated with functional anatomy in microdriles.

Key words: Functional anatomy, microdrile oligochaetes, morphology, SEM, ciliate sense receptors, mating structures, somatic chaetae, dorsal pores

Introduction

Before the year 2000, the observation by scanning electron microscopy (SEM) of external structures in microdriles was uncommon. Only a few studies have been published, most of them referring to observations on ciliate sense receptors (Chapman, 1979; Farnesi et al., 1982a, 1982b; Smith, 1983; Römbke and Schmidt, 1999) and somatic chaetae (Harman and McMahan, 1975; Milbrink, 1983; Smith, 1985; Chapman and Brinkhurst, 1986; Grimm, 1986, 1987, 1988; Finogenova and Poddubnaja, 1990; Römbke and Schmidt, 1990; Ohtaka, 1995, Bouché et al., 1999).

A descriptive study dealing with the external structures involved in attachment and sperm transfer in some freshwater microdriles using SEM was published by Cuadrado and Martínez-Ansemil (2001). More recently, Yáñez et al. (2006) and Caramelo and Martínez-Ansemil (2010) contributed to the knowledge of ciliate sense receptors in microdriles, providing a significant amount of new data as well as analyses of the typology and patterns of distribution in the main taxonomic groups.

The aim of the present work was to provide an overview of the functional anatomy of microdrile oligochaetes as revealed by SEM. New data are
provided mainly on systems related with mating, somatic chaetae, and dorsal pores.

Materials and methods

The biological material used in the present study was examined previously in the studies by Cuadrado and Martínez-Ansemil (2001), Yáñez et al. (2006), and Caramelo and Martínez-Ansemil (2010). Most of the samples were collected from habitats in Galicia (NW Iberian Peninsula); detailed information about each species discussed in this paper is distributed among the aforementioned articles and the current one (i.e. when dealing with individuals for which new data are provided). All the figures in this paper are original.

Material studied

The material studied comprises a total of 36 species belonging to the microdrile families Naididae (9 Naidinae, 2 Pristininae, 5 Rhyacodrilinae, 1 Phallodrilinae, and 9 Tubificinae), Phreodrilidae (1), Parvidrilidae (1), Lumbriculidae (4), and Enchytraeidae (4), as well as Eiseniella tetraedra (Savigny, 1826)—a lumbricid very common in freshwaters.

Material providing new data

Most specimens were collected in the 2 following streams of A Coruña (Galicia): Porto do Cabo stream at Moeche and Sar stream at Codesido (Rois). In the list below we will refer to them, respectively, as Porto do Cabo and Sar.


Methods

Specimens collected in the field were brought alive to the laboratory and identified using a light microscope equipped with differential interference contrast (mounted in a drop of water). Individuals were anesthetized with 7.5% magnesium chloride added progressively to water or with ethanol and fixed with 2% glutaraldehyde and 1% paraformaldehyde in 0.1M cacodylate buffer for 2 h. After fixation, specimens were washed 3 times in 0.1M cacodylate buffer for 10 min, postfixed with 1% osmium tetroxide for 2 h in the same buffer, and dehydrated through increasing acetone series. All specimens were mounted on stubs, and sputter-coated with gold. Observations were carried out using a Jeol JSM-6400 SEM at the SAI (Universidade da Coruña).
Results and discussion

Systems related with mating (Figures 1 and 2)

In observations conducted using SEM, Cuadrado and Martínez-Ansemil (2001) described some structures and mechanisms used by naidids for attachment and sperm transfer. Some of these systems appear to be similar to those already known in earthworms (see Jamieson, 2006) whereas others represent anatomical novelties that could be found exclusively in microdriles. All mating systems revealed by Cuadrado and Martínez-Ansemil (2001) imply the apposition of male and spermathecal pores. After observing by SEM several other species belonging to the families Enchytraeidae, Lumbriculidae, and Phreodrilidae, and after comparing these observations with previous results, we now propose that the way to achieve the apposition of genital pores and to maintain the attachment of partners during the sperm transfer in microdriles can be included in 1 of the following 3 categories:

Grasping. The most elaborate system described by Cuadrado and Martínez-Ansemil (2001) belongs to this category. It is the system utilized by the rhyacodriline Peristodrilus montanus (Figure 1A-C), in which the combined action of a complex system of muscles allow the penial chaetae (placed between male pores) to enter the lateral sides of a singular ‘anchorage bridge’ located between the spermathecal pores. The apposition of the male and the spermathecal pores, and the firm holding of the 2 partners, would easily allow sperm transfer to occur. A dense ciliated area on the prominent outer part of the tegument surrounding the penial chaetal bundles may facilitate the finding of the correct anchorage place.

According to Cuadrado and Martínez-Ansemil (2001), similar systems could be present in at least 4 of the 5 species of the tubificine genus Krenedrilus Dumnicka, and in the phalldriline Bathynucidrilus rohdei (Jamieson, 1977). In Krenedrilus the bundles of penial chaetae are arranged with the tips close together and directed towards each other, and a small epidermal papilla is present in the mid-ventral line of the spermathecal segment. B. rohdei is provided with an ‘X-shaped mid-ventral slit’ that, according to Erséus (1981), corresponds to the location of the tips of the penial chaetae of the mate. Many aquatic naidids have similar penial chaetae that—based on form, location and orientation—might also have an anchorage function. Although they could anchor directly on the body wall, new anchorage structures can be expected in these species when more extensive observations through SEM are performed.

Coupling. Many microdriles are provided with complementary mating structures that fit each other and are used both for attachment and sperm transfer. This seems to be the case for Rhyacodrilus falciformis, whose falciform penial chaetae fit into the lateral oblong spermathecal pores. Similarly, in Protuberodrilus tourenqui, the male porophore brings the 2 male pores (close to the tip) to fit with the 2 spermathecal pores of the partner, which are placed near the median ventral line of the body in a depression flanked by prominent chaetophores (Cuadrado and Martínez-Ansemil, 2001). The prominent pendant penes of Stylodrilus heringianus, oriented towards the rear part of the worm, could also help in holding partners when they penetrate deeply into the large spermathecal pores (Figure 1E).

Erséus (1979) and Erséus and Baker (1982) interpreted the giant penial chaetae of Adelodrilus Cook and Inanidrilus Erséus as structures involved in sperm transfer. Cuadrado and Martínez-Ansemil (2001) consider their probable participation also in the holding of the partners. When protracting penes are long and surrounded by cuticular sheaths (e.g., Limnodrilus Claparède, Aktedrilus Knöllner), their intrusion far into the spermathecal ducts might help, not only in the sperm transfer, but also in the attachment of the mates (Cuadrado and Martínez-Ansemil, 2001). Whether or not attachment is the primary function, anatomically all these structures of the male genitalia somehow seem to play a role in the holding of the partners, especially when they are strongly curved and the spermathecal pores occupy a lateral or a dorsal position (e.g., Aktedrilus). However, besides their eventual participation in sperm transfer, another possible role could be associated with sperm competition (i.e. the emptying of full spermathecae from a previous mating—prior to sperm transfer by the new mate), a phenomenon already known in other taxonomical groups (e.g., Cordero et al., 2004; Velando et al., 2008).
Some of the coupling systems also seem to be aided by gland secretions, such as in *P. tourenqui* (see Cuadrado and Martínez-Ansemil, 2001).

**Embracing.** The embrace seems to be a common procedure to achieve and maintain the apposition of male and spermathecal pores during the sperm transfer in many of the microdriles, especially those that are devoid of grasping penial chaetae or elaborated coupling structures. Embrace allows the alignment of male and spermathecal pores even if the latter have a lateral position and it can be performed by at least 2 different mechanisms. The first mechanism involves a great flexibility of the body wall and a complex muscular system at the genital region, that permit the protrusion and retraction of different areas to ensure the apposition of the pores during sperm transfer, as has been described by Cuadrado and Martínez-Ansemil (2001) for some tubificines. Similarly, it has now been clearly shown for the enchytraeid *Enchytraeus albidus* (Figure 2A, B) and presumed for *Insulodrilus* sp. (Figure 2C, D).

The second mechanism, described by Schmelz (2003) for enchytraeids in the genus *Fridericia* Michaelsen, consists of the eversion of the bursae, embracing the partner as 2 narrow claspers, thus allowing the contact of male and lateral spermathecal pores during mating. According to Schmelz (2003), most of the glandular secretions of the male copulatory organs are probably adhesive slimes. Figure 2E-G illustrates this embrace system.

According to Cuadrado and Martínez-Ansemil (2001), the typical gutter-shaped spermathecal chaetae (Figure 1D), present in many aquatic microdriles, would not participate directly in the attachment and sperm transfer, but would act as piercing chaetae involved in some mechanical or chemical stimulation during sperm transfer. If some mechanical stimulation occurs, the secretions of the large glands associated with these genital chaetae would serve to firmly attach the partners while they protract and retract. Until biochemical and physiological studies are performed, the exact
role of these structures remains unknown. Thus, Koene et al. (2005) proposed that the chaetal glands in earthworms may produce an allohormone that manipulates the reproductive physiology of the mating partner.

Ciliate sense receptors (Figure 3)

A summary of the typology and distribution of the external ciliated sense receptors according to Yáñez et al. (2006) and Caramelo and Martínez-Ansemil (2010) is provided below. The only novelties
are some micrographs helping in the interpretation of the different categories and patterns of distribution proposed by the aforementioned authors.

**Typology**

The observations by SEM allow identification of different types of receptors according to the shape of the cilia and to the shape of the receptors.

Types according to the shape of the cilia

*Receptors of blunt cilia*, generally multi-ciliated and shorter than 6 μm long (Figure 3A-C);

*Receptors of sharp cilia*, with 1-5 cilia more than 6 μm long (mostly 8-14 μm long) (Figure 3E);

*Composed receptors*, with a variable number of blunt and sharp cilia together (Figure 3D, F).

Both blunt and sharp cilia are present in all microdriles studied, with the only exception in the amphibiotic family Enchytraeidae. Out of the 8 species studied in this family (Römbke and Schmidt, 1999; Caramelo and Martínez-Ansemil, 2010), only *Cognettia sphagnetorum* seems to have sharp cilia in addition to the characteristic receptors of very short blunt cilia of this family.

Types according to the shape of the receptor

*Poorly defined* (unelevated) (Figure 3A, E, F, I, J)

*Sensory buds* (epithelial bumps) (Figure 3B, C, G, H) *Papillae* (Figure 3D)

The most common type of sense receptor in microdriles is the poorly defined one. Sensory buds are common among the Enchytraeidae. Ciliate papillae appear to be restricted to the chaetal segments of a few species of Naididae. The characteristic multi-ciliated receptors of blunt cilia of the enchytraeids deserve special mention (Figure 3C). Papillae are always composed receptors, with the sharp cilia occupying the inner part. Many sensory buds of *Eiseniella tetraedra*, the most common lumbricid found in freshwaters, are also composed receptors, but here the sharp cilia are located at the periphery of the organ (Figure 3F).

Patterns of distribution

External ciliate sense receptors are scattered on the prostomium (including proboscis when present), on the peristomium and pygidium, and arranged in a transversal chaetal row and scattered or organized in other transversal rows in each chaetal segment (Figure 3G-I). Ciliate sense receptors persist in the clitar region at maturity and are present in budding and regeneration zones (Figure 3J-M). The density of ciliate sense receptors is greater at the anterior region (especially on the prostomium and peristomium), less in the subsequent chaetal segments, and increases again in the posterior segments.

Except in the case of the composed receptors, the aquatic families and subfamilies of microdriles show the same types of ciliated sense receptors and with a similar distribution along the body. However, a longer size in blunt and sharp cilia and a smaller number of cilia in the multi-ciliated receptors of blunt cilia could be related to a more epibentic way of life (see Caramelo and Martínez-Ansemil, 2010, Table 1). The number of cilia per receptor, at least in the anterior body region, is generally greater in the amphibiotic family Enchytraeidae and especially in the lumbricid *E. tetraedra*. Higher numbers have been observed in terrestrial megadriiles (see Aros et al., 1978; Moment and Johnson, 1979).

Although different kinds of cilia and ciliate sense receptors are presumed to play different functions, additional ultrastructural and physiological data are needed to accurately assign a role to a particular kind of sense receptor. The limited information available to date generally suggests that the penetrative uniciliate sensory cells act as mechanoreceptors and the multiciliate sensory cells with emergent cilia act as chemoreceptors (see Welsch et al., 1984). Nonetheless, penetrative multiciliate sense receptors can be produced by uniciliated and/or multiciliated sensory cells. According to Yáñez et al. (2006), the distribution in rows of most of the multiciliated receptors of blunt cilia at the chaetal segments could be related to a tactile function in relation to locomotion, whereas the presence of receptors with long cilia in freshwater families of microdriles together with their absence in earthworms could indicate that they act as rheoreceptors. The recent observation of long sharp cilia in aquatic or semiaquatic worms of terrestrial origin (*E. tetraedra* and probably also *C. sphagnetorum*) is interpreted by Caramelo and Martínez-Ansemil (2010) as the expression of an ancient character.
Figure 3. Ciliate sense receptors. (A) Scarcely defined sense receptor of blunt cilia: *Lumbricus variegatus*; (B, C) Sensory buds: B, *Protuberodrilus tourenqui*; C, *Lumbricillus* sp.; (D) Papilla: *Ophidonais serpentina*; (E) Sense receptor of sharp cilia: *Insulodrilus* sp.; (F) Composed receptor: *Eiseniella tetraedra*; (G) Sense receptors on pro stomium and peristomium: *Pristina longiseta*; (H) Sense receptors on pygidium: *Enchytraeus albidus*; (I) Transversal chaetal row: *Peristodrilus montanus*; (J) Cilia at the clitellum: *Stylodrilus heringianus*; (K) Sense receptors at a budding zone: *P. longiseta*; (L, M) Sense receptors on a regeneration zone: *Lumbriculus variegatus*, L, general view; M, detail of sense receptors. dp, dorsal pore; ec, emerging chaeta.
Somatic chaetae (Figure 4)

Somatic chaetae are generally used to identify microdriles at the family level, and sometimes also at the subfamily, generic, or specific levels. Thus, the type of the chaetae, their particular shape, and number in dorsal and ventral bundles are always included in the description of oligochaete taxa. Most of the information about the somatic chaetae in microdriles has been provided by optical microscopy. Our observations by SEM, combined with the available literature, reveal particular traits that we briefly summarize and discuss in the following 3 sections devoted to hair chaetae, crotchets, and needles. The presence or absence of denticulations in hair chaetae and the presence or absence of intermediate teeth in those chaetae currently considered to be bifid crotchets are the most relevant questions.

Hair chaetae (Figure 4A-H)

Hair chaetae are present in many aquatic oligochaetes, commonly in dorsal bundles and, rarely, in ventral ones (Capilloventridae, Parvidrilidae). Traditionally, the hair chaetae of microdriles were reported to be smooth; thus, with only a few exceptions, the mentions of denticulations in hair chaetae were limited to the Naidinae (Ripistes Dujardin, Stylaria Lamarck, and Vejdovskyella Michaelsen), and Pristininae (Pristina Ehrenberg), but even in these groups (the formerly family Naididae) smooth hair chaetae were considered the general rule (Sperber, 1948).

A limited number of studies have dealt with the observation of hair chaetae by means of SEM, but, interestingly, they generally report the observation of denticulations in hair chaetae of taxa that were formerly considered devoid (e.g., Smith, 1985; Chapman and Brinkhurst, 1986; Grimm, 1987; Ohtaka, 1995). We have also found some kind of denticulations (Figure 4A-H) in at least some chaetae in most of the genera studied (Nais Müller, Stylaria, Vejdovskylia, Pristina, Peristodrilus Baker and Brinkhurst, Krenedrilus, Potamothrix Vejdovský and Mrázek, Psammoryctides Hrabě, Spiroperma Randolph, and Parvidrilus Erésus). Although the treatment of the samples might sometimes influence the appearance of denticulations, it seems now that probably all hair chaetae have at least the potential to develop denticulations according to a more or less defined pattern (Figure 4A-H). Thus, the view of the hair chaetae as generally smooth structures must be challenged by that of hair chaetae as structures at least potentially provided with some kind of denticulations.

Crotchets (Figure 4K-O)

A number of more recent references have reported the presence of intermediate teeth in chaetae that historically were considered bifid (mostly ventral crotchets) (e.g., Brinkhurst, 1963; Brinkhurst and Jamieson 1971; Smith, 1985; Martínez-Ansemil and Giani, 1986; Chapman and Brinkhurst, 1987; Grimm, 1987). Similarly, our observations (Figure 4K-O) confirm that pectination is more widespread than had been previously thought. Currently, pectination in bifid chaetae has already been documented in at least one species in each of the following genera: Nais, Dero Oken, Branchiura Beddard, Peristodrilus, Rhyacodrilus Bretscher, Potamothrix, Psammoryctides, Tubifex Lamarck, and Varichaetadrilus Brinkhurst and Kathman.

In most cases, the presence of intermediate teeth in typical bifid chaetae is not characteristic of a particular species but rather appears to occur under certain environmental conditions (see Chapman and Brinkhurst, 1987). In our opinion, the development of intermediate teeth in typical bifid crotchets reveals a certain degree of plasticity in the phenotypic expression of the chaetoblasts, particularly in the transition between typical bifid crotchets and typical pectinate chaetae when following the evolutionary models shown in Brinkhurst (1984).

Needles (Figure 4H, I)

Needles are acicular dorsal chaetae whose bifid or simple-pointed condition is sometimes difficult to ascertain. Yet, when observed using SEM, minute teeth can show remarkable differences (Figure 4H, I). Some variability in the shape of the chaetae was generally accepted in the literature, but after the insightful study by Loden and Harman (1980) “Ecophenotypic variation in setae of Naididae (Oligochaeta)”, the shape of the chaetae as a valid criterion to distinguish species from one another was seriously questioned. Furthermore, the environment influences not only the shape but also the presence and number of a particular kind of chaetae as has
been demonstrated by Chapman and Brinkhurst (1987) in their work eloquently entitled “Hair today, gone tomorrow: induced chaetal variation in tubificid oligochaetes”. The SEM could help to reveal some morphological differences between taxa, as could be the case of different types of denticulations in hair chaetae. However, due to the high plasticity and homoplasy concerning the expression and the
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The evolution of the chaetae among microdriles, SEM would probably be a more useful tool to detect abnormalities produced by environmental stresses as shown by Milbrink (1983) than to reveal phylogenetic relationships.

Dorsal pores (Figures 3I and 5)

In many oligochaetes, the coelomic cavity communicates with the exterior through small pores located in the mid-dorsal line of the body. These unpaired structures are named dorsal or coelomic pores. Until now, dorsal pores were generally considered present only in earthworms (megadriles) and the enchytraeids of the genus Fridericia (Stephenson, 1930; Brinkhurst and Jamieson, 1971; Bouché, 1972; Schmelz, 2003). Nevertheless, the presence of dorsal pores had already been reported in 2 aquatic species of microdriles: the rhyacodrilines Monopylephorus auklandicus (Benham, 1909) and Bothrioneurum vejdovskyanum (see Chekanovskaya, 1962; Timm, 1979).

The observation of species belonging to the families Enchytraeidae, Naididae, Lumbriculidae, Lumbricidae, Phreodrilidae, and Parvidrilidae revealed the presence of dorsal pores—not only in taxa where they should be expected (the lumbricid Eiseniella tetraedra and the enchytraeid Fridericia monochaeta) (Figure 5A-D) but also in some species of typical freshwater microdriles. Although not all the material was observed in dorsal view, it must be pointed out that the only freshwater microdriles where we have found dorsal pores are the rhyacodrilines B. vejdovskyanum, Peristodrilus montanus, and Protuberodrilus tourenqui. The study of a poorly preserved specimen of Parvidrilus spelaeus (Parvidrilidae) also revealed the presence of pores in the mid-dorsal line of the body, but these are probably not truly dorsal (coelomic) ones but the pores of the mid-dorsal glandular pouches characteristic of the family (see Martínez-Ansemil et al., 2002). The distribution and diameter of the dorsal pores that we observed in the 3 species of rhyacodrilines are as follows:

Bothrioneurum vejdovskyanum. (Figure 5G-I). Dorsal pores present from 3/4 to the posterior end. Diameter, 8-13 μm.

Peristodrilus montanus. (Figure 3I, Figure 5J, K). Dorsal pores observed only at 3/4 and 4/5. Diameter, 13-20 μm.

Protuberodrilus tourenqui. (Figure 5E, F). Dorsal pores present from 5/6 to at least 7/8. Diameter, 8-12 μm.

SEM proves to be a useful tool for the observation of dorsal pores in microdriles. Interestingly, all 4 aquatic species of microdriles showing dorsal pores belong to the Rhyacodrilinae, an ancient subfamily of naidids. Historically, it has been accepted that the functions of dorsal pores were connected with their presence in species from terrestrial habitats and their absence in aquatic species (i.e. protection against desiccation, keeping the surface wall moist for respiratory exchanges, protection against bacteria and parasites) (see Stephenson, 1930). Before introducing new biological hypotheses related to the presence of dorsal pores in aquatic microdriles, we think it appropriate to investigate their presence in other microdriles, especially other rhyacodriline genera and their closest relatives. It is worth highlighting the different number and distribution of dorsal pores in the 4 species of rhyacodrilines, and that the first dorsal pore is located in a more anterior segment than had been reported previously in megadriles and Fridericia.

Other structures (Figure 6)

In addition to the structures described above, we have occasionally observed some structures that could also provide additional information from a taxonomical or a biological perspective if they were systematically observed. Thus, it is noteworthy that the accurate position and diameter of the nephridial pores is easily observed by SEM and could be informative (Figures 6A, B). Furthermore, it is interesting to note the porosity observed at the body wall (e.g., Lumbriculus variegatus: Figures 6C, D). Finally, we note the observation of female marks (depressions in the body wall) with different shapes in mature (well developed male pores and clitellum) enchytraeids (Fridericia monochaeta) (Figures 6E, F) and naidids (Limnodrilus udekemianus) (Figure 6G); these depressions correspond with the position of the female pores. Perhaps the shape of female marks is highly variable and consequently not very useful as a taxonomical character (e.g., comparison of the
different shape of the 2 marks of the same individual in *L. udekemianus*: Figure 6H, I). Regardless, these female marks—devoid of female pores when male pores and clitellum are well developed—provide us with insight into how protandric worms likely avoid self-fertilization.
Conclusion

SEM is an invaluable tool that has increased the knowledge of the functional anatomy on microdriles, and has resulted in the broader understanding and discovery of several important structures. However, additional studies and observations using SEM are still
needed, especially with regard to mating structures. Furthermore, the results obtained during this study encourage us to pursue new investigations using other techniques (e.g., CLSM, TEM, microscopic sections) in order to broaden our knowledge on the muscular complex and glands involved in the different mating systems, the relationships between the ciliate sense receptors and the nervous system, and the muscular and nervous systems that control the opening of the dorsal pores.

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