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Ultrastructure of the body wall of three species of *Grania* (Annelida: Clitellata: Enchytraeidae)

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Abstract

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The body wall of three species of *Grania*, including the cuticle, epidermis and the musculature, are studied using TEM. The cuticle is similar to previously studied enchytraeids, with an orthogonal grid pattern of collagen fibers. This pattern is also seen in Crassiclitellata, which has been suggested as the sister taxon of Enchytraeidae. Variation of epicuticular and fiber zone patterns seen in Naididae (formerly Tubificidae and Naididae) seem to be lacking in Enchytraeidae. The fiber thickness, however, varies between *Grania* species and may be a phylogenetically informative character. The epidermis consists of supporting cells, secretory cells and sensory cells. Basal cells, typical for Crassiclitellata, were not observed. The clitellum of *Grania* seems to consist of two types of gland cells, which develop from regular epidermal tissue. It is possible that more cell types exist in different regions of the clitellum, however. The body wall musculature is arranged somewhat differently from that of closely related taxa; this refers to the reduction of circular and outer, triangular longitudinal muscle fibers, while the inner, ribbon-shaped longitudinal muscle fibers are well-developed. A search was conducted for the cause of the peculiar green coloration of *Grania galbina* De Wit and Erséus 2007, and it was concluded that neither cyanobacteria nor epidermal pigment granules were present in the fixed material.

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Introduction

The body wall of oligochaetous annelids consists of an external cuticle, an epidermis and an underlying muscle layer, internally bounded by a peritoneum which limits the coelomic cavity. The body wall acts together with the fluid-filled coelomic cavity as a hydrostatic skeleton, allowing the worm to move in various ways (Jamieson 1981, 1992). The actual locomotive pattern is related to several factors, including the size and shape of the musculature (Tzetlin and Filippova 2005). It is also likely that the thickness and flexibility of the cuticle affect the movement pattern.

The typical cuticle of oligochaetous annelids is described as an 'orthogonal grid' of collagen fibers arranged in layers, where each layer is perpendicular to the ones above and below it, all embedded in a mucopolysaccaride matrix. At the outermost part of the cuticle, there is a fiberless zone, the epicuticle. The surface of the epicuticle is covered with epicuticular projections, small membrane-bound bodies (Richards 1984).

The epidermis typically consists of a monolayer of epithelial cells, with supporting cells being the most numerous, and occasional gland and sensory cells present (Welsch *et al.* 1984). In some taxa, particularly crassiclitellates (i.e. earthworms with a multi-layered clitellum) and leeches, small basal cells are present between the supporting cells. The function of these is still rather unclear (Jamieson 1992). The clitellum is a region of the body where the epidermis is thickened and glandularized, in order to secrete cocoons during reproduction (Hess and Vena 1974; Richards 1977a; Welsch *et al.* 1984). In crassiclitellates the clitellar epidermis is also multilayered, a unique feature within the oligochaetous clitellates (Jamieson 1992).

The musculature consists of outer circular and inner longitudinal muscle fibers, as well as chaetal muscle fibers. Within the muscle fibers are packages of myofilaments, surrounded by cytoplasmic regions containing mitochondria (Jamieson 1981, 1992).

Enchytraeidae is a group of about 650 species of clitellate worms (Erséus 2005). They are typically long, slender and whitish in color. Their relatively thick cuticle allows the majority of enchytraeid species to inhabit terrestrial environments, although many species are found in aquatic and littoral habitats. Within Enchytraeidae only a few studies of the ultrastructure of the integumentary system have been undertaken (Hess and Menzel 1967; Goodman and Parrish 1971; Hess and Vena 1974; Richards 1977a).

The phylogenetic placement of the enchytraeids is still somewhat controversial. Historically, Enchytraeidae has been placed as closely related to Propappidae and Haplotaxidae, based on morphological studies (Coates 1987, 1989), and more recently on parsimony-based molecular analyses (Rousset *et al.* 2007). Other molecular studies, however, place it as a monophyletic group together with Crassiclitellata (Siddall *et al.* 2001; Bleidorn *et al.* 2003; Erséus and Källersjö 2004; Hall *et al.* 2004), whereas Propappidae groups with Haplotaxidae, Phreodrilidae and Naididae (*sensu* Erséus *et al.* 2008; formerly divided into Tubificidae and Naididae) (Erséus and Källersjö 2004).

Grania Southern, 1913, is a strictly marine genus with a number of specialized features, e.g. the fusion of segments I-IV into a 'head'. Some species of *Grania* possess the only multicellular sensory structure ever reported in enchytraeids, the 'head organ', thought to be a georeceptor (Rota and Erséus 1996; Rota *et al.* 1999; Locke 2000). As all clitellates, *Grania* has a clitellum. It is located from the anterior of segment XII to about 2/3 of XIII (PDW, personal observation).

In this study the integumentary system, as well as the body wall musculature, is studied using TEM (transmission electron microscopy) with focus on three species of *Grania*. Two of these, *G. postclitellochaeta* (Knöllner, 1935) and *G. americana* Kennedy, 1966, are North Atlantic species, whereas the third, *G. galbina* De Wit and Erséus 2007; is from the South Pacific. All three species inhabit heterogeneous shell sand, from the intertidal zone to 100 m depth or more. *Grania postclitellochaeta* seems to tolerate moderately brackish water, as it is also found in the western Baltic Sea, but usually all three species studied here inhabit water with full oceanic salinity. An interesting feature of *G. galbina* is that it is conspicuously colored greenish-yellow. As coloration of annelids most often arises from pigment in the epidermis (Welsch *et al.* 1984), or other tissues (Kennedy 1969), we search for a cause to the coloration within this study. Hypothesized causes are symbiotic cyanobacteria, or pigment granules within the epidermal tissue. The main purpose of this study, however, is to describe the body wall of *Grania*, to compare the structure of the body wall between different species of the genus, and to compare the body wall structure to other, closely related taxa. Representatives of *Grania* are marine interstitial worms, while most other enchytraeids are either terrestrial or aquatic burrowing forms. These kinds of environmental factors are likely to affect the animal morphology.

Material and Methods

Specimens of the three species of *Grania* were collected at different times and locations. *Grania galbina* was collected by C. Erséus on Lifou, Loyalty Islands, New Caledonia at about 20°55′S, 167°18′E in November 2000, *G. postclitellochaeta* was collected by dredge in the Koster area on the west coast of Sweden by C. Erséus in August 2004, and *G. americana* was collected near Fort Pierce on the east coast of Florida at 27°51 24.3′N, 80°27 03.8′W by P. De Wit in April 2005. At all occasions, subtidal sediment samples of about 3–4 l were repeatedly stirred in seawater, and the suspensions obtained were decanted through a 250 μm mesh sieve. The sieved fractions were studied under a dissecting microscope, where *Grania* specimens were sorted out and identified alive to the species level in a drop of seawater under a coverslip on a slide, using a compound microscope.

Four specimens of *G. galbina*, two of *G. postclitellochaeta* and one of *G. americana* were fixed in a mixture of paraformaldehyde, glutaraldehyde, sucrose and picric acid (Ermak and Eakin 1976) in a 0.15 M sodium cacodylate buffer at pH 7.2 and transported to the Swedish Museum of Natural History (SMNH), Stockholm, Sweden. They were then rinsed in a 0.1 M sodium cacodylate buffer overnight, postfixed in 1% osmium tetroxide in the same buffer for 1 h, and rinsed in buffer. The specimens were dehydrated in a graded ethanol series and then embedded in Epon 812, using propylene oxide.

Ultrathin sections were obtained using an Ultracut UCT WS microtome, triple stained with lead citrate and uranyl acetate (Daddow 1983), and finally studied in a LEO 912 AB TEM at 80 kV. Semithin sections (0.5–1 μm) for light microscopy were stained with 1% toluidin blue in 1% borax solution.

In *G. postclitellochaeta*, the sections studied were from the region just anterior of the clitellum. In *G. americana*, the sections were from the clitellum and the region just posteriorly of the clitellum. In *G. galbina*, they were from the region just posteriorly of the clitellum, and also from a region near the posterior end of the worm.

To address the possibility of symbiotic cyanobacteria in specimens of *G. galbina*, PCR reactions were conducted with cyanobacterial 16S rRNA primers CYA359F and CYA781R (Nübel *et al.* 1997). As no DNA-extract of *G. galbina* was available at this time, however, an extract of an undescribed species from Lizard Island, Australia, was used instead. This as yet undescribed species is also colored greenish-yellow, and has by mitochondrial sequence data been suggested to be a close relative to *G. galbina* (De Wit, unpublished data).

Results

The body wall of *Grania* consists of an outer cuticle, an epidermal layer, and a muscle layer consisting of circular and longitudinal muscle fibers. In *Grania*, however, the circular muscle fibers do not form a continuous layer. A peritoneum borders the muscle layers toward the coelomic cavity. The observations below are arranged to follow the body wall from the outer surface inward to the coelomic cavity, ending with the musculature. The clitellum section is separated from the one on epidermis, to allow more detailed notes on the clitellar region. Each section describes one species in detail, followed by observed similarities and differences in the other two species.

Cuticle

The cuticle of *G. postclitellochaeta* consists of a fiber zone which is characterized by unbranching collagen fibers arranged in layers, where each layer is perpendicular to the ones above and below it (an 'orthogonal grid') and at about 45° angle to the body axis (Table 1, Fig. 1A).

The outermost part of the cuticle (the epicuticle) is devoid of fibers and is divided into two distinct zones based on electron density; there is one thick, less electron-dense inner layer, and a thin outer electron-dense layer (arrows in Fig. 1B). On the outer surface of the epicuticle there are numerous oval projections, which are about four times as long (perpendicular to the epicuticular surface) as they are wide. These epicuticular projections are bounded by double membranes (not seen in figure), and contain a homogeneous material. Within the cuticle, microvilli stretch from the epidermis to the epicuticular surface. They seem to have a similar content, and are of the same width as the epicuticular projections (Fig. 1B).

Grania galbina and *G. americana* differ from *G. postclitellochaeta* in the fiber diameter and the depth of the fiber zone (Table 1). All three species possess microvilli, but those of *G. galbina* are thicker than those of *G. postclitellochaeta* (and considerably thicker than *G. galbina*'s epicuticular projections) (Fig. 1C). The microvilli of *G. americana* are distally of similar width as the epicuticular projections; proximally they are about twice as wide. They also seem to contain more electron-dense material than the projections (Fig. 1D). The epicuticle of all three species is structurally similar.

Table 1 Cuticular characters of the *Grania* species observed

	G. americana	*G. postclitellochaeta*	*G. galbina*
Worm length (mm)	8.7–12.6	4.5–7.4	6.4–7.8
Worm diameter (mm)	0.14–0.20	0.07–0.13	0.16–0.22
Cuticle thickness (nm)	1500–1800	700–1050	900–1050
Epicuticle thickness (nm)	85–125	80–110	80–115
Fiber zone thickness (nm)	1375–1715	590–970	785–970
Fiber diameter (nm)	90–95	40–50	50–60
Number of fiber layers	15–17	15–17	14–16
Microvilli diameter (nm)	30–40	30–40	60–70
EP length (nm)	130	150	150
EP diameter (nm)	24	30	30
EP number per μm	18	16	16

Worm length and diameter data were obtained from literature (Erséus and Lasserre 1976; Locke and Coates 1999; Rota and Erséus 2003; De Wit and Erséus 2007). EP, epicuticular projections.

Epidermis

The epidermis of *G. postclitellochaeta* consists of one cell layer, only 2–3 μm thick, between the cuticle and the basal lamina. The thickness decreases to 0.8–0.9 μm where the circular muscle cells are located (Fig. 2A; see 'Musculature' below). *Grania galbina* is similar to *G. postclitellochaeta*, while the epidermis of *G. americana* is only 0.4–0.5 μm thick at its thinnest point (Fig. 2B). The epidermis is composed of three different cell types: supporting cells, sensory cells and gland cells, where the supporting cells by far outnumber the other cell types. Basal cells are visible in none of the specimens (see Jamieson 1992: p 223).

The supporting cells of *G. postclitellochaeta* are cuboidal in shape, 10–12 μm wide, with large nuclei situated basally. They comprise the main part of the epidermis, and are characterized by the absence of granular inclusions and the presence of tonofilaments that stretch from the basal lamina through the cells to the cuticle (Fig. 2A). At the cuticular border, the tonofilaments form hemidesmosomal 'knobs' which are attached to the cuticle, so that the tonofilament bundles connect the cuticle to the basal lamina (Fig. 2A,B). Cytoplasmal microvilli extend into the cuticle from the epidermis, seemingly independent from the tonofilamentous 'knobs'. Apically in the supporting cells there are occasional granular vesicles (probably lysosomal in nature) as well as mitochondria, the latter being 100 nm thick and 400 nm long (Fig. 2B). The Golgi apparatuses are generally well-developed. The supporting cells of *G. americana* and *G. galbina* are indistinguishable from those of *G. postclitellochaeta*.

In *G. postclitellochaeta*, gland cells occur occasionally, and have a cytoplasm with a highly developed vacuolar system, which can be either full of rounded electron-dense inclusions, 0.15–0.45 μm in diameter, or empty (Fig. 2C,D). The nuclei are located basally, bordered by a well-developed Golgi complex. The rest of the cells are occupied by the vacuolar system. It is not uncommon to find gland cells with inclusions only at the basal portion of the vacuolar system. Gland cells are 2–2.5 μm wide and are seldom cuboidal in shape; rather they seem to fill the space between the supporting cells. In *G. galbina*, the empty gland cells are a prominent feature (Fig. 2E); no gland cells containing inclusions were observed. No gland cells were seen in *G. americana*.

Sensory cells were observed only in *G. galbina* (Fig. 2F,G). They possess numerous cilia that stretch through the cuticle. The basal bodies of the cilia are surrounded by microvilli

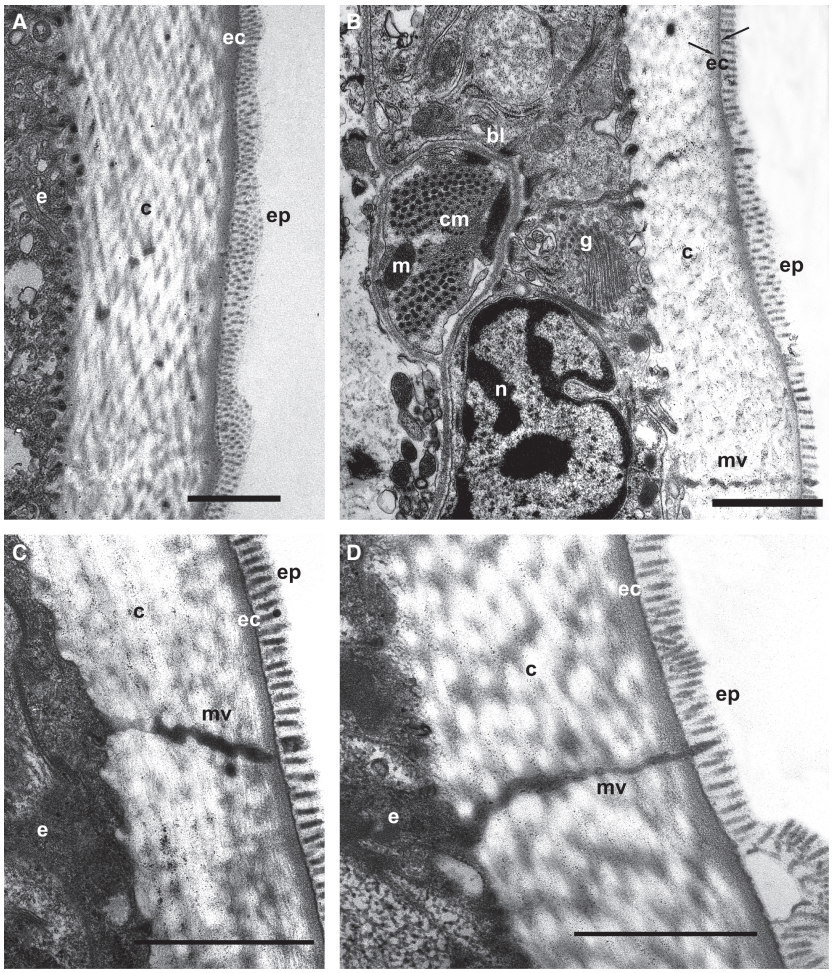

Fig. 1—Cuticle. (A) Cuticular collagen fiber pattern in *Grania postclitellochaeta* (Cross-section). (B) Cuticle with microvilli in longitudinal section of *G. postclitellochaeta*, together with an epidermal supportive cell containing a nucleus and Golgi apparatus, and a circular muscle fiber in cross-section. Arrows are pointing to the two layers of the epicuticle. Also note the basal lamina. (C, D) Cuticle with microvilli of *G. galbina* (C) and *G. americana* (D) (Both in cross-section of the worm). bl, basal lamina; c, cuticle; cm, circular muscle fiber; e, epidermis; ec, epicuticle; ep, epicuticular projections; g, Golgi apparatus; m, mitochondrion; mv, microvillus; n, nucleus. Scale bars represent 1 μm.

(Fig. 2G). Internally, the cells consist of highly folded double membranes that compartmentalize them into many small regions containing multivesicular bodies. As in the other epidermal cell types, the nucleus of the sensory cells is located basally. There are no granular inclusions present. No nerve fiber was observed.

In all three species, neighboring epidermal cells are bordered by cell membranes that stretch from the basal lamina to the cuticle. At the basal end, these membranes are folded, creating an interdigitative pattern (e.g. Fig. 2C). At the apical end, near the cuticle, the membranes are attached to each other with a zonula adhaerens (Fig. 2C). The intercellular gap is narrow, about 15 nm. The basal lamina consists of an extracellular matrix and is 50–60 nm thick (Fig. 1B). It stretches between the epidermis and the muscle tissue, and also surrounds all muscle fibers.

Clitellum

The clitellum is conspicuous in *G. americana*, forming a monolayered glandular thickening of the epidermis to 17–18 μm. In cross-section through the anterior of segment XII, this thickening occurs all around the body wall except mid-ventrally where no gland cells are present; this was also clearly visible in the overview sections of XII. At the border between normal epidermis and clitellar epidermis in this mid-ventral interruption of the clitellum, there is a rapid increase in epidermal thickness (Fig. 3A).

The clitellar epidermis of *G. americana* consists of two types of gland cells which were not seen elsewhere in the epidermis – the granular gland cells and the globular gland cells (Fig. 3B). The glandular cells normally seen in the epidermis are not visible. Supporting cells are present, but not in as high a proportion as elsewhere. The granular gland cells (type 1 *sensu* Hess and Vena 1974) contain rounded or oval microtubule-containing inclusions, ranging in size from 0.7 to 1.8 μm (Fig. 3B). The surrounding cytoplasm contains high levels of rough ER, and apically in the cells there is an inclusion-free space, containing numerous small vesicles (Fig. 3B,C). The globular gland cells (type 2 *sensu* Hess and Vena 1974), on the other hand, have amorphous globules containing a heterogeneous product. These inclusions occupy most of the intracellular space (Fig. 3B). In both cell types the nuclei are located basally along with well-developed Golgi apparatuses.

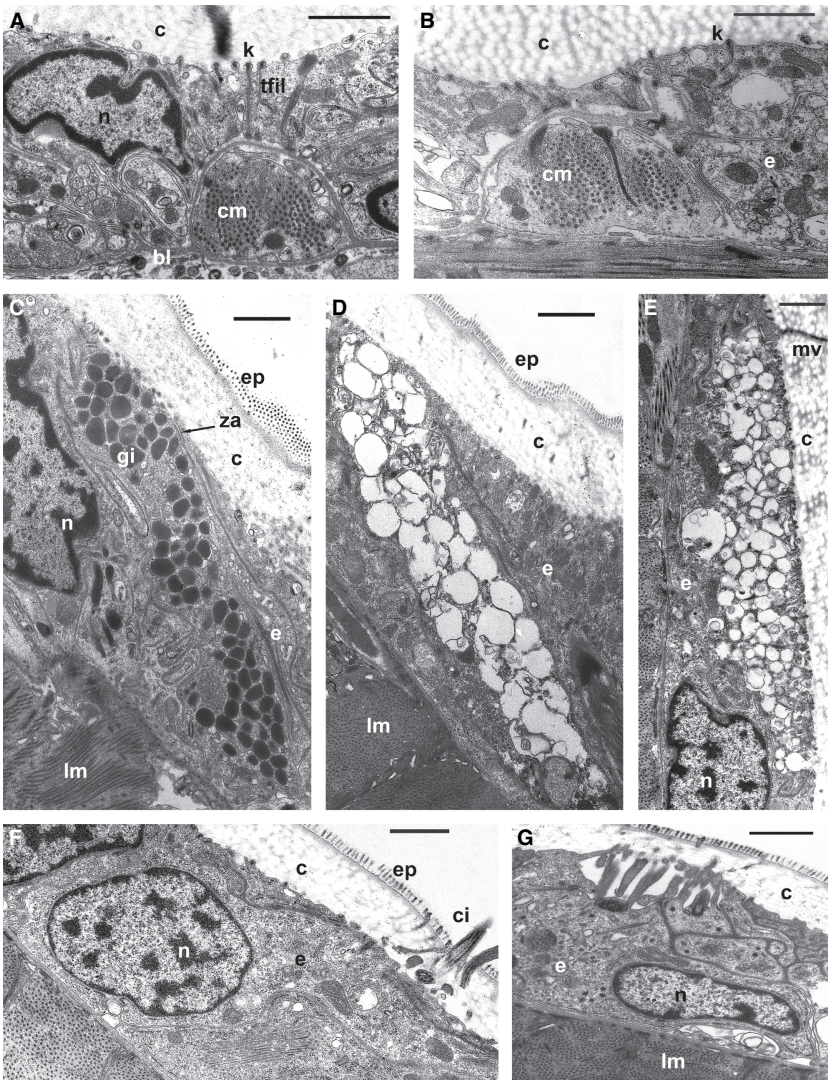

Fig. 2—Epidermis. (A, B) Epidermis and circular muscle fiber in a longitudinal section of *Grania postclitellochaeta* (A) and *G. americana* (B). Note tonofilaments in (A) and knobs in (A) and (B). (C-E) Epidermal gland cells with (C) and without (D) granular inclusions in *G. postclitellochaeta*, and without inclusions in *G. galbina* (E). (F, G) Sensory cell of *G. galbina*. Notice cilia extending through cuticle (F) and compartmentalized cytoplasm with multivesicular bodies (G). bl, basal lamina; c, cuticle; ci, cilia; cm, circular muscle fiber; e, epidermis; ep, epicuticular projections; gi, granular inclusions; k, knobs; lm, longitudinal musculature; mv, microvillus; mvb; multivesicular body; n, nucleus; tfil, tonofilament; za, zonula adhaerens. Scale bars represent 1 μm.

The clitellum of *G. galbina* was not studied, but the one of *G. postclitellochaeta* was indistinguishable from that of *G. americana*.

Musculature

The body wall musculature of *G. americana* consists of circular and longitudinal muscles, which are single-obliquely and double-obliquely striated, respectively. The circular musculature, however, is reduced and consists of single muscle fibers that surround the body, with a 6 μm distance between the fibers. When viewed in a longitudinal section of the worm, the circular muscle fibers are shaped like half ellipses rounded toward the epidermis, 2.5 μm wide if measured parallel to the body axis, and 1.5 μm thick if measured perpendicular to the body axis (Fig. 2B). Each muscle fiber consists of a contractile part of four bundles of 30–50 myofilaments each, separated laterally by sarcoplasmic reticulum and z-rods, and a cytoplasmic region containing nuclei and elongated mitochondria. The cytoplasmic region of the circular fiber is located basally and centrally when viewed in a longitudinal section. The circular muscle fibers of *G. postclitellochaeta* and *G. galbina* are distributed and shaped just as those of *G. americana*, with the exception that they seem to be more half-circular in shape, with a radius of about 2.0 μm (Figs 1B, 2A and 4A). The myofilamental bundles are also less distinctly delineated in *G. postclitellochaeta* and in *G. galbina*.

The longitudinal muscle layer of *G. galbina* is well-developed and thick (12–14 μm), and consists of about 100 fibers stacked next to each other along the body circumference. There are two different types of longitudinal muscle fibers, outer triangular fibers and inner, ribbon-shaped fibers (Fig. 4B,C). The outer fibers are shaped as right isosceles triangles with hypotenuses facing the basal lamina (4–4.5 μm), from which they taper about 3 μm toward the coelom, when studied in cross-section. Myofilaments are arranged laterally

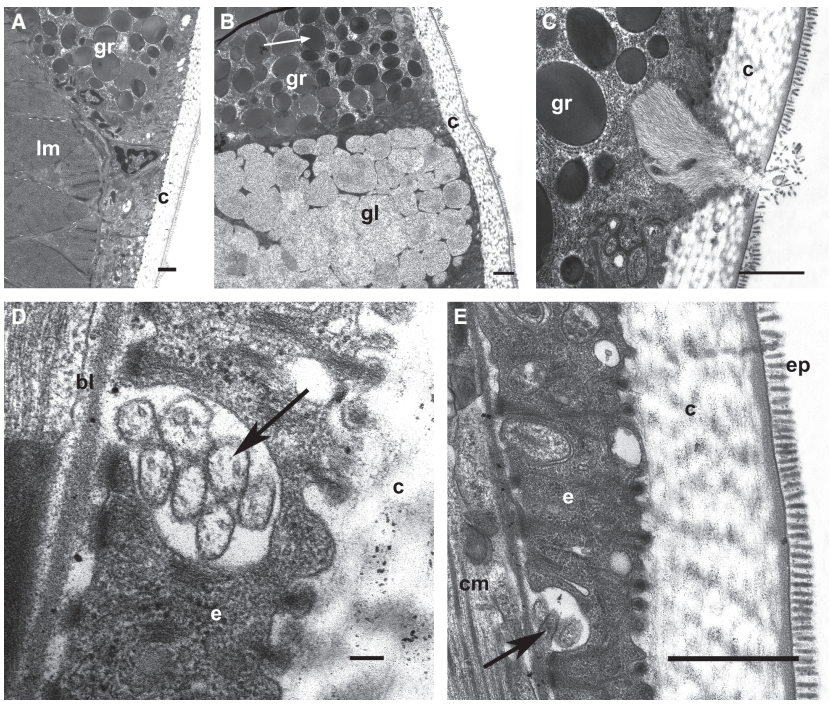

Fig. 3—(A-C) Clitellum of *Grania americana*. (A) Thinning of the clitellar epidermis along the midventral line. (B) Type 1 (granular) and type 2 (globular) clitellar gland cells. Arrow points to a granular inclusion. (C) Ejection of contents of a granule through the cuticle, notice numerous small vesicles disassembling the cuticle. Scale bars represent 1 μm. (D, E) Epidermal compartments seeming to contain small bacteria in *G. galbina* (arrows). Scale bars represent 0.1 μm in (D) and 1 μm in (E). bl, basal lamina; c, cuticle; cm, circular musculature; e, epidermis; ep, epicuticular projections; gl, globular gland cell; gr, granular gland cell; lm, longitudinal musculature.

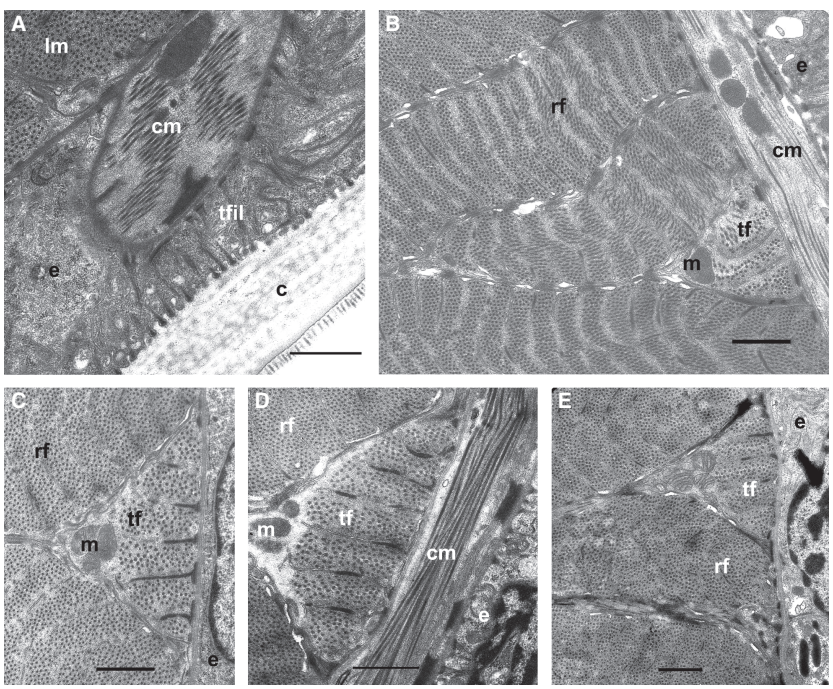

Fig. 4—Musculature. (A) Circular muscle fiber in a longitudinal section of *Grania galbina*. (B, C) Cross-section of longitudinal muscle layer of *G. galbina* with circular muscle (B); close-up of outer triangular and ribbon-shaped muscle fibers (C) (note myofibrillar bundles). (D, E) Cross-section of longitudinal muscles of *G. postclitellochaeta* (D) and *G. americana* (E). c, cuticle; cm, circular musculature; e, epidermis; lm, longitudinal musculature; m, mitochondrion; rf, ribbon-shaped longitudinal muscle fiber; tf, triangular longitudinal muscle fiber; tfil, tonofilament. Scale bars represent 1 μm.

in 7 bundles, separated by sarcoplasmic reticulum and z-rods. The mitochondria are located in a non-contractile part of the muscle fibers, facing the coelomic cavity. The triangular outer fibers are wedged in between every two or three ribbon-shaped muscle fibers.

These ribbon-shaped muscle fibers are by far the largest fibers, in cross-section thickening from a thickness of 1.5–2 μm at the end bordering the epidermis to a thickness of 4–5 μm at the end facing the coelomic cavity; they are 12–14 μm wide from the epidermal to the coelomic end (in the radial plane of the worm). The myofilaments are divided by sarcoplasmic reticulum and z-rods into v-shaped bundles. Each fiber contains 20–25 bundles of 80–100 myofilaments. The mitochondria supplying energy to the ribbon-shaped

muscle fibers are located in a cytoplasmic space facing the coelomic cavity. *Grania americana* and *G. postclitellochaeta* also possess these two types of longitudinal muscle fibers (Fig. 4D,E), although in both species only 50–70 fibers line the body circumference. The outer triangular fibers of *G. americana*, however, only contain 5–6 myofilamental bundles.

Coloration

No pigment granules were found in *G. galbina* that could be responsible of the greenish-yellow coloration. It seems, however, that there is a system of extracellular spaces located basally between epidermal cells in *G. galbina*, which at some occasions possess inclusions resembling small bacteria in size and shape, 0.1–0.2 μm in diameter (although cell walls are not visible) (Fig. 3D,E). The PCR reaction with cyanobacterial 16S rRNA primers and DNA extracted from a closely related undescribed greenish-yellow species of *Grania* gave negative results, failing to corroborate the presence of cyanobionts.

Discussion

The overall structure of the body wall is similar in the three species studied. The differences lie in the size and detailed design of each component. *Grania americana* has the thickest cuticle, and also the thinnest epidermis, in the latter case where the circular muscle fibers are located. The musculature of all of the species studied is characterized by large, ribbon-shaped longitudinal muscle fibers and reduced circular fibers, which do not form a continuous layer. This will be discussed in more detail below.

Cuticle

In the *Grania* species under study here, the cuticle thickness ranges from 0.9 to 1.1 μm in *G. postclitellochaeta* and *G. galbina*, to 1.5–1.8 μm in *G. americana* (Table 1). This seems to be the typical thickness of the enchytraeid cuticle, ranging from 0.5 μm in *Enchytraeus fragmentosus* (Hess and Menzel 1967), 0.9 μm in *Mesenchytraeus solifugus* (Goodman and Parrish 1971) to almost 2 μm in several species of *Lumbricillus* (Richards 1977b).

The morphology of the cuticle has recently been shown to differ significantly in Naididae (*sensu* Erséus *et al.* 2008), with larger variations occurring in the pattern of the collagen fibers (Richards 1974, 1984; Jamieson 1988a; Gustavsson and Erséus 2000; Gustavsson 2001) than previously described for lumbricids (Richards 1974, 1978; Jamieson 1992). In Naidinae, for example, collagen fibers seem to be completely absent (Gustavsson 2001). The variation in naidids discussed by Gustavsson (2001) does not seem to exist in enchytraeids, at least not from the evidence gathered to date (e.g. Hess and Vena 1974; Richards 1984; Jamieson 1992); *Enchytraeus*, *Mesenchytraeus* and *Lumbricillus* all have their collagen fibers arranged in an orthogonal grid pattern. Our results corroborate those of previous studies on enchytraeids, since the pattern of the fiber zone of all *Grania* species studied here also had fibers arranged in an orthogonal grid. It is also noteworthy that previous studies have shown that the collagen fibers of crassiclitellate cuticles also are arranged in an orthogonal grid (Coggeshall 1966; Burke 1974a; Jamieson 1981).

Variation in fiber thickness has been reported between genera in enchytraeids, with comparably narrow fibers in *Mesenchytraeus* and thick fibers in *Fridericia* (Richards 1977b), but in *Grania* there seems to exist variation also between species within the genus. The slight differences seen in the thickness of fibers in the cuticular fiber zone could possibly be used as a phylogenetic character within *Grania*, but additional species need to be studied to enable more generalized conclusions.

The epicuticle of naidids has also recently been shown to vary significantly in the number of layers (Sjölin and Gustavsson 2008). In *Grania*, however, no variation is evident, neither in structure nor in thickness.

The abundance and shape of microvilli and epicuticular projections have also been shown to differ between clitellate taxa (Richards 1974, 1984; Gustavsson 2001). No variation was found concerning the epicuticular projections in *Grania*, but the microvilli of *G. galbina* were found to be significantly thicker than those of the other species studied. It has been suggested that different kinds of microvilli might exist, with some being thicker than others (Gustavsson 2001). As all microvilli seen in *G. galbina* were thicker than those of the other species, however, we cannot conclude that different kinds of microvilli exist in *Grania*. In other clitellates, they have been observed to 'pinch off' epicuticular projections (Hess and Menzel 1967; Goodman and Parrish 1971), but this was not confirmed in this study. It is still likely that the microvilli and epicuticular projections are connected at some point, and participate in transporting substances from the epidermal cells through the cuticle to the outside or vice versa. The microvilli are also thought to play a role in the formation of the pattern of the collagen fibers (Burke 1974b).

Epidermis

The epidermis of *Grania* is thinner than that of most other annelid species (see e.g. Goodman and Parrish 1971; Giere *et al.* 1988; Hausen 2005). Apart from this, it is similar to previously described epidermal epithelia, containing supporting cells as well as occasional sensory and mucous cells. Several different types of mucous cells have been reported in clitellates, most notably three different types in the genus *Lumbricillus* (Richards 1977b); granular acid mucous cells, globular, non-acid mucous cells and small granular cells. In *Enchytraeus*, Burke and Ross (1975) found four different types, which they named 'glandular cell types I–IV'. In *Grania*, we found two types: the granule-containing and the

'empty' ones. The granule-containing ones are structurally similar to Burke and Ross' 'type IV' cells and Richards' 'acid mucous' cells, with some notable differences: the acid mucous cells of Richards had a fingerprint or honeycomb pattern within the granules, while the granules in *Grania*'s cells have a homogeneous electron density. In Burke and Ross (1975), granules only occupy the most apical part of the cytoplasm in the type IV cells, while in *Grania* the granules occupy the majority of the cell space. The 'empty' cells seen in *Grania* remind of *Enchytraeus*' type II cells (Burke and Ross 1975) and *Lumbricillus*' non-acid mucous cell (Richards 1977b). The two cell types found in *Grania* could, however, represent the same cell type in different stages of development, as was suggested by Burke and Ross (1975).

Richards (1977b) noted that the gland cells in small clitellates are constricted to the epidermal space between circular muscle fibers, where the epidermis is thicker. This observation is further supported by our data, as gland cells never were seen between the cuticle and circular muscle fibers. The multiciliate sensory cells are similar to previously described sensory cells in lumbricids (Burke 1974a; Jamieson 1981). Interestingly, basal cells are absent in *Grania*; this cell type is otherwise typical for members of Crassiclitellata (Welsch *et al.* 1984), which has been suggested as the sister taxon of Enchytraeidae (Erséus and Källersjö 2004; Erséus 2005).

The epidermal supporting cells are by far the most abundant, being similar to what has previously been described (Welsch *et al.* 1984; Jamieson 1992). The supporting cells are thought to secrete the collagenous fibers forming the cuticle (Richards 1978; Welsch *et al.* 1984). There are no such fibers present in the epidermal cells, but there are numerous small vesicles in the apical part of the cells, and the collagen subunits are secreted from these vesicles and polymerize within the basal part of the cuticle (Richards 1978). Our results support this view, as we found no collagen fibers inside the epidermal cells of *Grania*. The small vesicles and mitochondria seen apically in the epidermal supporting cells suggest that there is secretion of subunits occurring from the epidermis into the basal cuticle.

Tonofilaments stretch from the basal lamina to the cuticle, and they have frequently been described in oligochaetes as continuing inside microvilli through the cuticle (Richards 1977b, 1984; Jamieson 1981), but in our study they were never seen inside microvilli. Rather they seemed to be separate from the microvilli, only anchoring the cuticle to the basal lamina. This seems to be the case also in Naididae (Gustavsson and Erséus 2000; Gustavsson 2001).

Clitellum

In the clitellar region of clitellates, large gland cells are the most prominent feature (Hess and Vena 1974; Richards 1977a; Suzutani 1977; Suzutani-Shiota 1980). Two types of gland cells exist in the clitellum of enchytraeids, one associated with secretion of the cocoon wall, and the other with secretion of the nutritive material contained within the cocoon (Hess and Vena 1974; Richards 1977a). Historically, these two glandular cell types have been named in different ways. Hess and Vena (1974) called them 'type 1' and 'type 2' cells, while Richards (1977a) named them 'granular clitellar cells' and 'globular clitellar cells'. In *Grania*, they have also previously been called 'granular cells' and 'hyaline cells' because of their appearance in a light microscope (e.g. Rota and Erséus 2000, 2003; De Wit and Erséus 2007). We consider it likely that type 1 cells correspond to the granular gland cells, and the type 2 cells to the globular and the hyaline gland cells. The granular, type 1 cells are characterized by round or oval electron-dense structures which are surrounded by rough ER, while the globular, type 2 cells possess amorphous vesicles (called 'secretion droplets' by Hess and Vena 1974) which appear lighter in TEM and occupy practically all intracellular space. As it may be confusing to use different terms for the same cell type, it is desirable to agree on one single term for each cell type. Preferring more descriptive names for these cell types, we choose to use Richards' terms of 'granular' (type 1), and 'globular clitellar gland cells' (type 2 or hyaline gland cells). In *Tubifex*, four cell types have been found, occurring in different regions of the clitellum (Suzutani 1977; Suzutani-Shiota 1980; Fleming and Baron 1982). Richards (1977a,b) also noted the presence of different-looking cells in the boundary regions of the clitellum. In the present study, two cell types were seen, which seem to correspond to the granular and globular gland cells described for enchytraeids, but as only one part of the clitellum was studied, it is possible that there might be other cell types in other regions. In fact, recent light-microscopical studies have indicated that there might be more cell types present in specific regions of the clitellum of some species of *Grania* (Rota *et al.* 2007).

Regular epidermal cell types, such as sensory, supporting and mucous cells have also been reported from within the clitellum, wedged in between the gland cells (Hess and Vena 1974; Suzutani 1977; Fleming and Baron 1982). In this study, only a few wedged-in supporting cells were noted (except for in the mid-ventral line where the supporting cells were ubiquitous); no sensory or mucous cells were seen (although they are likely to be present). As the gland cells dominate the clitellar epidermis, we conclude that the epidermal supporting cells are likely to be transformed into gland cells when the clitellum is formed.

The mid-ventral absence of gland cells has also been reported from *Enchytraeus fragmentosus* (Hess and Vena 1974) (Enchytraeidae). Many naidids, however, possess gland cells all around the body circumference (LMG, personal observation). It might be interesting to further study this as a possible phylogenetic character.

The numerous small grains seen near the cuticle of *Grania*'s gland cells (Fig. 3C) seem to have a role in forming pores though the cuticle to allow secretion. No clitellum observed here, however, seemed to be in a state of secreting a cocoon at the moment. Hess and Vena (1974) reported

secretion occurring through groups of microvilli stretching through the cuticle. In this study, however, no such groups of microvilli were seen.

Musculature

It has recently been noted that a significant number of annelid taxa lack circular muscles, and instead some of them have 'bracing muscles' acting as a counterforce to the longitudinal fibers (Tzetlin and Filippova 2005). Needless to say, the size of the circular musculature is important to the locomotory pattern. Lumbricids have well-developed circular muscles which form a continuous layer, an adaptation to their pattern of movement in peristalsis through a dense medium (Jamieson 1992). Other enchytraeids also seem to have a more well-developed circular musculature than *Grania*. *Enchytraeus* sp. have fibers that form a continuous layer, which in larger specimens forms 'Napoleon hat' shapes, since the contractile part of the fiber surrounds the cytoplasmic region at regular intervals (de Eguilor *et al.* 1987; Valvassori *et al.* 1989). In *Lumbricillus mirabilis*, the circular musculature is somewhat reduced, forming separate triangular fibers at regular intervals (Richards 1977b). The circular muscle fibers of *Grania*, however, are still much more reduced than those of *L. mirabilis*. Rota (2001) also noted this pattern in undescribed *Grania* specimens from Sardinia, forming discrete bands along the body wall clearly visible in light microscopy. In polychaetes, it has been noted that the circular muscles in many cases do not span around the entire body (Tzetlin and Filippova 2005). In *Grania*, however, as in other clitellates studied (Jamieson 1992; Rota 2001) the fibers do seem to circle the entire body.

In a few clitellate taxa, diagonal muscle fibers have also been reported, but not in enchytraeids (Jamieson 1992), and none were visible in the *Grania* species of this study.

Within the longitudinal musculature, the two cell types have been shown to have different roles (de Eguilor *et al.* 1989; Valvassori *et al.* 1989; Lanzavecchia *et al.* 1994). The outer triangular fibers function in the maintenance of the body shape, whereas the ribbon-shaped fibers are used in movement. It is worth noting that the only muscle type involved in movement are the ribbon-shaped longitudinal muscle fibers, as the circular muscles of enchytraeids also histochemically have been demonstrated to function tonically to maintain body shape (Lanzavecchia *et al.* 1994).

The outer triangular fibers of enchytraeids have been described as forming an almost continuous layer outside of the ribbon-shaped fibers (Lanzavecchia *et al.* 1994). This description is based on marine intertidal species of *Enchytraeus* (*E. minutus* and *E. albidus*) and *Lumbricillus* (*L. lineatus* and *L. mirabilis*). In *Grania*, however, the triangular fibers are wedged in only between every two or three ribbon-shaped fibers, something which is more similar to the organization seen in lumbriculids or naidids (Lanzavecchia *et al.* 1994). Most notably, similar muscular patterns are seen in interstitial naidids, such as species of *Aktedrilus*, *Thalassodrilus*, *Olavius* and *Bathydrilus* (Giere 1983; personal observation).

The enchytraeid species studied by Lanzavecchia *et al.* (1994) are also shallow-water species, but they are significantly larger, and burrow through the medium in a peristaltic motion, while the small *Grania* spp. move interstitially, by bending at choice 'joints' in a serpent-like fashion (Giere and Pfannkuche 1982; Rota 2001). This movement pattern, combined with the slender body form and the stout chaetae, allow *Grania* spp. to move between the grains of sand. As there is no peristaltic motion involved, there is less need for well-developed tonic musculature. There is, however, need for strong longitudinal musculature, which is well-developed in *Grania*. The same pattern is seen, however, in larger mud-dwelling naidids (Lanzavecchia *et al.* 1994), which makes is difficult to conclude that this is an adaptation to interstitial life without further enquiries.

Coloration

In *G. galbina*, no pigment granules were discovered in the epidermal tissues. The network of channels basally in the epidermis might host such granules, but they were not seen in this study. Still, this seems as the most likely cause of the coloration at this point. It is possible that G. *galbina* receives pigment from its diet, as it lives in an environment full of green algae. It is also possible that bacteria other than cyanobacteria are present in the epidermal tissues. In fact, there are structures in the epidermal tissue of *G. galbina* which resemble the small bacteria described in gutless tubificids (Giere and Langheld 1987; Giere *et al.* 1995; Giere and Erséus 2002; Blazejak *et al.* 2005). To further investigate this, more material is needed.

Phylogenetic relationships and conclusions

Based on recent phylogenetic studies (e.g. Siddall *et al.* 2001; Erséus and Källersjö 2004), Enchytraeidae is a monophyletic group and forms a monophyletic group together with the Metagynophora, which includes Crassiclitellata (with multilayered clitellum), Moniligastridae and Alluroididae (Jamieson 1988b). The latter two 'families' are thought to be closely related to Crassiclitellata, but possess a single-layered clitellum (Jamieson 1988b; Erséus 2005), similar to that of enchytraeids. The phylogenetic affinities of these two taxa have not been molecularly studied to date, however (Erséus 2005).

The body wall of *Grania* largely resembles those of other studied Enchytraeidae. The cuticle seems to host little variation within this family (Hess and Vena 1974; Richards 1984; Jamieson 1992), as opposed to the cuticle of Naididae (*sensu* Erséus *et al.* 2008) which has been shown to vary both in the structure of the epicuticle (Sjölin and Gustavsson 2008) and the fiber zone (Gustavsson and Erséus 2000; Gustavsson 2001). Within *Grania*, as in other enchytraeids, the fiber zone is arranged in an orthogonal grid, similar to the pattern seen

in Crassiclitellata (Richards 1974). The thickness of the individual fibers seems to vary between species, however, and might be a phylogenetically informative character.

The epidermis does not contain any basal cells, a cell type typical for Crassiclitellata (Welsch *et al.* 1984). It would be interesting to search other closely related taxa, such as Moniligastridae and Alluroididae, for basal cells, to further investigate the relations within the Enchytraeidae + Metagynophora group.

The clitellum is clearly monolayered, as in other enchytraeids (Hess and Vena 1974), and we conclude that the clitellar gland cells are converted from regular epidermal cells as the clitellum develops, as few regular epidermal cells were visible in the clitellum. In this study, we found two different clitellar gland cell types, which we prefer to call 'granular' and 'globular gland cells' (see Richards 1977a). To further investigate how many different cell types there actually are in the clitellum, it is necessary to section and study the entire region, as there might exist different cell types in different parts of the clitellum.

The tonic musculature (circular and triangular longitudinal fibers) is reduced in *Grania*. This is most likely an adaptation to interstitial life, as a similar muscular pattern can be seen in interstitial naidids (Giere 1983), but not in larger, burrowing, enchytraeids (Lanzavecchia *et al.* 1994). The number of myofilamental bundles within the muscle fibers (both the circular and longitudinal ones) also seems to differ between species, another character which could be phylogenetically informative.

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