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ORIGINAL ARTICLE

Diuronotus aspetos (Gastrotricha): new morphological data and description of the spermatozoon

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Abstract The genus *Diuronotus* (Gastrotricha Chaetonotida) includes two species, *D. aspetos* and *D. rupperti*; its morphological affinity with the genus *Musellifer* has been pointed out. Here, new morphological data from light and electron microscopy and the description of the spermatozoon of *D. aspetos* are reported, with the aim of clarifying the phylogenetic position of the genus. The mouth cavity has a wreath of stout, protrusible processes. The two secondary furcal tubes are inserted ventrally and are covered with elongate scales. All the caudal tubes contain a duo-gland adhesive system. Three kinds of ciliated sensory receptors are described for the first time in *Diuronotus*. The filiform spermatozoon consists of an acrosome, a nuclear-mitochondrial complex, and a flagellum. The acrosome including two long and different cones, the single, giant mitochondrion surrounding the nuclear base, and the axoneme arising from a deep nuclear 'fossa' appear as autapomorphic characters. The keeled, solid cuticular body scales and the spermatozoon with a supernumerary membrane are features shared with *Musellifer delamarei*. The structure of the accessory fibres is a strong spermatological similarity between the families Muselliferidae and Xenotrichulidae. Thus morphological and spermatological characters support the inclusion of *D. aspetos* and *M. delamarei* into the family Muselliferidae recently described. The comparative spermatology also suggests that Xenotrichulidae may be the sister group of Muselliferidae.

Keywords *Diuronotus aspetos* · Gastrotricha · Chaetonotida · Ultrastructure · Spermatozoon

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Introduction

The phylum Gastrotricha includes over 700 meiobenthic-sized species, traditionally grouped into the two orders Macrodasyida and Chaetonotida, different in a number of morphological, ecological and reproductive features. All 450 species of Chaetonotida Paucitubulatina share a uniform tenpin-shaped body plan, with two caudal adhesive tubes, and a pharynx with an inverted Y-shaped lumen lacking pores (Remane 1961). Within the Chaetonotida, the families Muselliferidae and Xenotrichulidae are marine, the Chaetonotidae include both marine and freshwater species, while Dasydytidae, Dichaeturidae, Neogosseidae and Proichthydiidae are exclusively freshwater. The monophyly of the strictly freshwater families has never been questioned, and has gained support from an inclusive cladistic analysis of the Gastrotricha based on 81 morphological and reproductive traits (Hochberg and Litvaitis 2000). On the contrary, the phylogenetic relationships within Chaetonotidae and between Chaetonotidae and Xenotrichulidae are still unclear, due in part to the uncertain status of the genus *Musellifer,* originally assigned to the Chaetonotidae. A sister-group relationship between

Xenotrichulidae and *Musellifer* was hypothesized based on the similarity of the spermatozoa ultrastructure (Guidi et al. 2003; Marotta et al. 2005). However, the muscular architecture of *Musellifer* greatly differs from species of both Chaetonotidae and Xenotrichulidae (Leasi and Todaro 2008). The close affinity of the two genera *Diuronotus* and *Musellifer,* both marine and hermaphroditic, is supported by numerous traits of the external morphology, which has prompted Leasi and Todaro (2008) to create the new family Muselliferidae. The genus *Diuronotus* includes only two species, *D. aspetos* and *D. rupperti,* which have been mainly described on the basis of optical microscopy (Todaro et al. 2005). Here we provide ultrastructural details of several morphological traits of *D. aspetos,* and a full description of the spermatozoon. The aim of this study is to describe additional details on the anatomy of a species of *Diuronotus*, and to find possible additional autapomorphies useful to support the family Muselliferidae and its phylogenetic relations.

Methods

Sampling sites

The sediment samples were taken by a mini van Veen grab (225 cm^2) at 2.0–2.5 m water depth in the small bay of Itersla (69°26.989′N; 52°19.688′W), near the settlement of Skansen, Disko Island, West Greenland. The samples were taken on 25th August 2006 during the Arctic Biological Field Course 2006. In the subtidal sandy ripples, the sediment consists of fine, well-sorted sand with detritus. Further information can be found in Kristensen and Niilonen (1982), Kristensen and Nørrevang (1982) and Todaro et al. (2005).

Samples preparation for optical and electron microscopical analysis

The sediment samples were sorted out at Arctic Station, Qeqertarsuaq, immediately after collection. They were next treated with isotonic magnesium chloride at 4°C; the meiofauna was retained in a 32 μm mesh net and thereafter was observed with an Olympus stereomicroscope at 40–80× magnification. Five adult specimens were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), and stored in 0.1 M sucrose/sodium cacodylate buffer until the post-fixation in 1% osmium tetroxide buffered with 0.1 sodium cacodylate. They were next prepared for EM analysis. For TEM, after washing in the same buffer, three of them were dehydrated in a graded alcohol series, stained en bloc in uranyl acetate in 70% acetone, and embedded in Araldite. Semi-thick and ultrathin sections were cut with a LKB Ultrotome 2,088 V, contrasted with toluidine blue and lead citrate respectively. The semi-thick sections were observed in transmission light under VANOX AHBT3 Olympus optical microscope, whereas the ultrathin sections were observed under a Philips CM10 transmission electron microscope. For SEM study, two specimens were dehydrated in a graded alcohol series, then critical point-dried using CO_2, mounted on aluminium stubs, sputter coated with gold palladium and finally observed with a Philips 515 scanning electron microscope. Three specimens (two adult and one subadult) fixed in 2% glutaraldehyde were observed under Nomarski differential interference contrast (DIC) microscope, and photographed with a Nikon Coolpix 990 digital camera. Measurements were taken on one of the two adult specimens by an ocular micrometer.

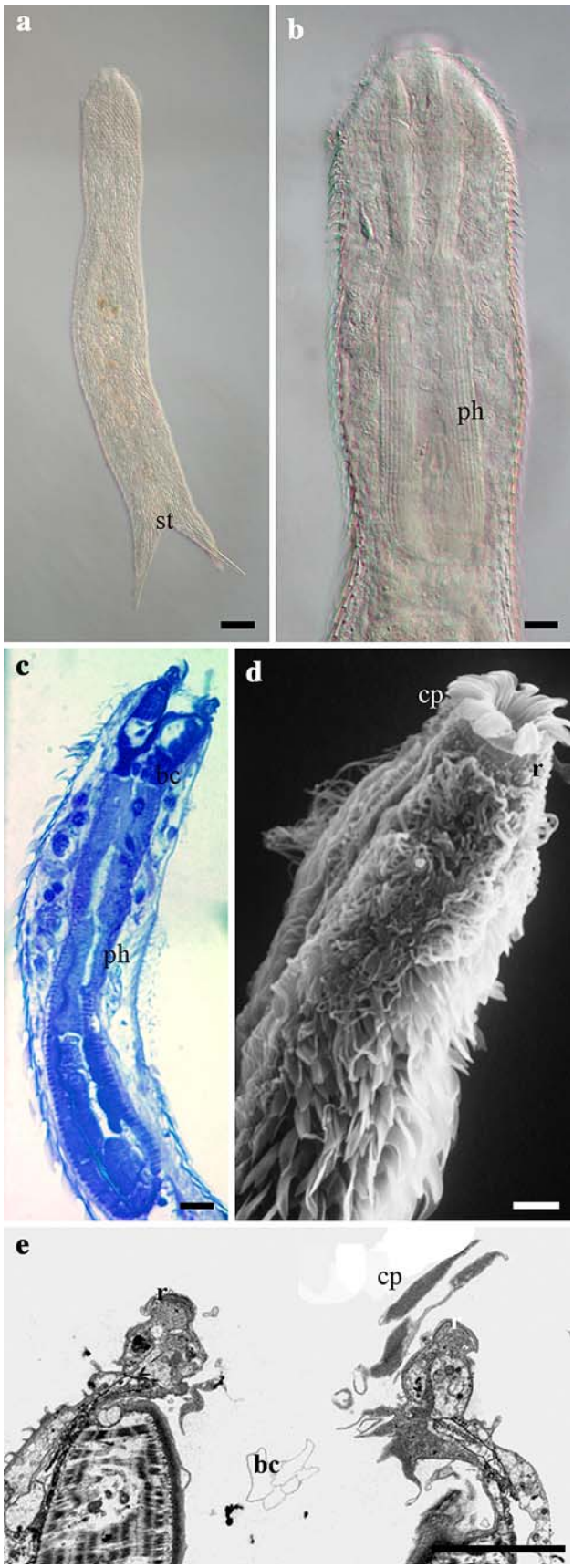
Results

The interstitial gastrotrich fauna of Skansen appeared very rich, including seven species of gastrotrichs in addition to *Diuronotus aspetos,* of which the present is the second absolute finding since 1979 (see Todaro et al. 2005). Two more species of chaetonotidans (*Chaetonotus atrox, Halichaetonotus* sp.), and five species of macrodasyidans (*Mesodasys* sp., *Paradasys* sp., *Tetranchyroderma* sp., *Thaumastoderma* sp., *Turbanella* sp.) were found. All these seven species, as well as two undeterminated others were reported by Danish students during an Arctic field course at Qaamassoq, Disko (Ehrhardt and Svendsen 1994). However, *Diuronotus aspetos* was not found, even if Qaamassoq is only twenty kilometres far from its type locality.

In the present paper new features of the general morphology and of the structure of the spermatozoon are described.

External morphology

Total body length 670 μm (369 μm in the subadult); pharynx length 190 μm (subadult 175 μm); pharingeo-intestinal junction at U28. The ciliary band surrounding the head edge is 22 μm wide. Widths of head/neck/trunk/caudal base are as follows: 60/65/75/70 μm at U07/U28/U60/U90, respectively (Fig. 1a,b). The caudal furca indents at U83. Each furcal branch measures 93 μm in length (50 μm in the subadult), and is made of a fleshy portion, 72 μm long and a primary adhesive tube, 21 μm. The secondary adhesive tubes are much longer than the primary ones (62 vs 21 μm), and arise from a conical base, 42 μm, which is inserted on the intrafurcal edge of the furcal branches (Fig. 1a).



◄ **Fig. 1** External morphology, mouth and buccal cavity of *Diuronotus aspetos*. **a** *Habitus* of an adult specimen; note the secondary tubes (light microscopy). Mouth, buccal cavity and pharynx. (**b**, **c** light microscopy; **d** SEM; **e** TEM). Note the evident cuticular mouth ring. Twelve couples of stout, double, cuticular processes protrude from the buccal cavity. *bc* Buccal cavity, *cp* cuticular processes, *ph* pharynx, *r* mouth ring, *st* secondary tubes. *Scale bars* **a** = 42 μm; **b**, **c** = 14 μm; **d**, **e** = 4 μm

Mouth and buccal cavity

The mouth is a circular opening supported by a thick cuticular ring, 4 μm thick, inner and external diameters 7.5 and 10 μm respectively. Twenty-four longitudinal, cuticular ridges run along the wall of the wide mouth cavity. Twelve stout, double, cuticular processes are inserted to internal base of the mouth ring and protrude 2 μm outside (Fig. 1b–e). Each process appears to be made of two very thin sheets, overlapping each to the other, the upper one narrower and shorter (Fig. 1d, e).

Cuticular armature

The body is covered with 41 alternate columns (25 dorsal and 8 ventrolateral per side) of about 55 scales each. The scales are hemielliptic, keeled and measure in length from 2.25 μm on the head to 10.25 μm on the trunk (Fig. 2a, b). The ventral interciliary field posteriorly to the PhIJ is covered with scales 6 μm in length, arranged into 18–23 alternate columns. They are similar in morphology to the dorsal ones (Figs. 2c, 3b).

The fleshy base of each primary furcal tube is entirely covered with 3–4 rings of 7–8 scales similar to the dorsal ones. The tube is enveloped by 3–4 rings of 7–8 very thin scales tightly adhering to it (Fig. 2d, g, h), and longer (11 μm) than the other furcal scales (Fig. 2h). Each secondary tube is covered with 14 rings of 4–5 scales each, looking like the dorsal ones, and decreasing in length from 8 up to 4 μm in the top 2–3 rings (Fig. 2e, f). Both the primary and the secondary tubes show a bare apex (Fig. 2f, h) and contain a duo-gland adhesive system (Fig. 2i, j).

The cuticle is composed of two layers: the thin exocuticle is formed by only one 'bilayer', while the endocuticle is thicker (0.2 μm) and fibrous (Fig. 3a). The single exocuticular 'bilayer' extends to cover the whole surface of the scale (Fig. 3c). Scales are solid, and made of two strongly electron-dense, homogeneous layers, closely tight each to the other. In transverse section the boundary line between the two layers appears strongly electron-dense and striated (Fig. 3a). The prominent keel of each dorsal scale greatly increases posteriorly its height taking the shape of a talon (Fig. 3b). In each scale column, scales adhering to the cuticle regularly alternate to pedunculated scales (Fig. 3c–e). For a

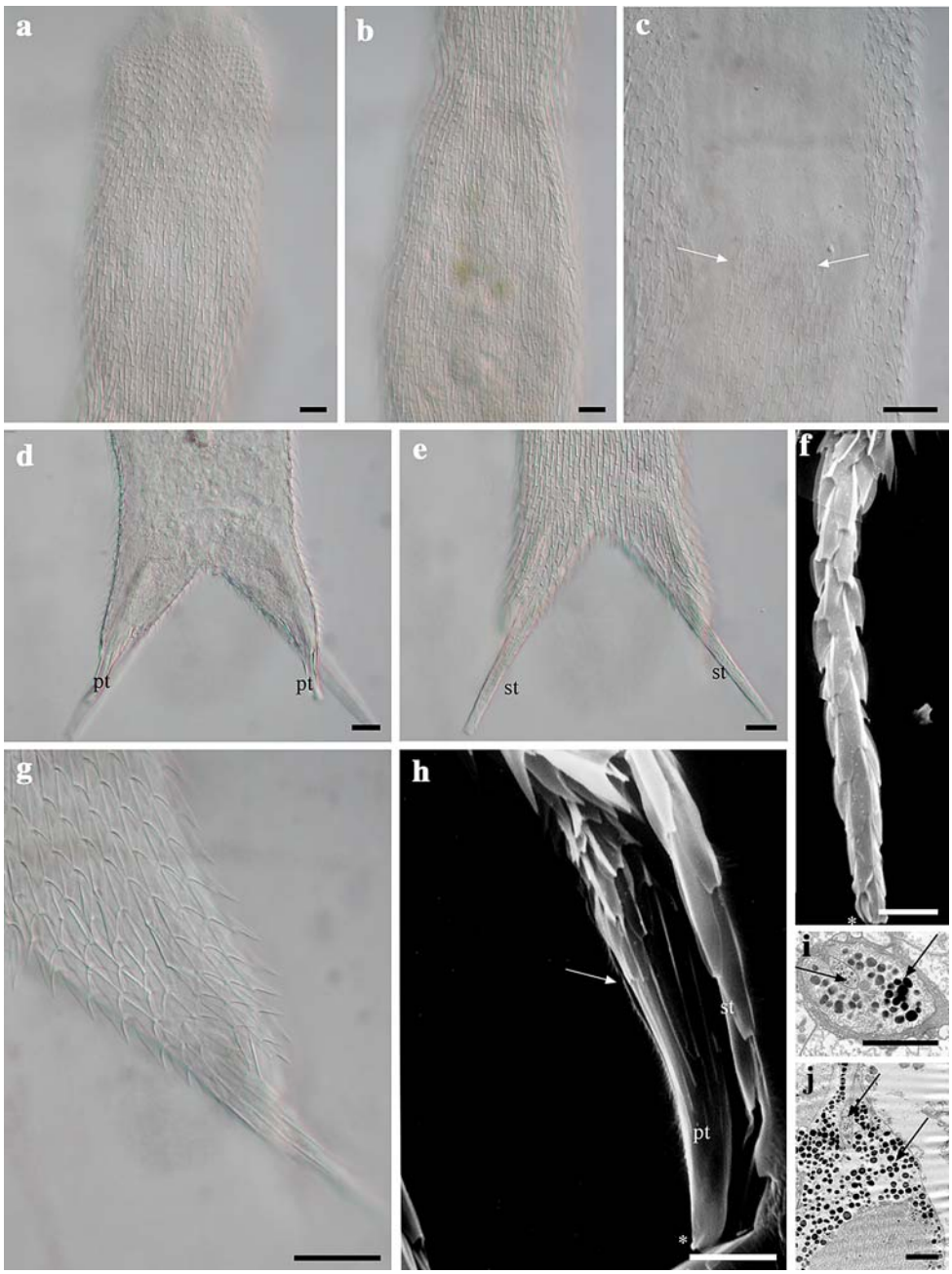


Fig. 2 Cuticular armature and furca. **a**, **b** Dorsal view showing the scales on the head and the trunk. **c** Detail of the ventral scales (*arrows*) in the interciliary field in the trunk region. **d** Dorsal posterior end, with the primary tubes. **e** Dorsal posterior end, showing the secondary tubes (light microscopy). **f** Reconstruction of the whole secondary tube: scales shape and arrangement and the free apex (*asterisk*) are visible (SEM). **g**, **h** Close-up of the dorsal posterior end showing the primary and the secondary tube of one furcal branch (**g** light microscopy; **h** SEM). Note the very thin scales (*arrow*) tightly adhering to the primary tube and its free apex (*asterisk*). **i**, **j** TEM images showing the duo-gland adhesive system (arrows) of the furcal tubes. *pt* Primary tube, *st* secondary tube. *Scale bars* **a**–**e**, **g** = 16 μm; **f**, **h** = 8 μm; **i**, **j** = 3 μm

comparison we have also studied the cuticle and the scales of *Musellifer delamarei*. The cuticle of this species is formed by a thin, single-'bilayered' exocuticle and a thicker (0.2 μm), fibrous endocuticle; scales are solid and homogeneous and adhere to the cuticle (Fig. 3f).

Sensory organs

The head bears dorsally and laterally two kinds of ciliary sensory organs. Scattered, single cilia, about 20 μm long, with the base protected by a small, cuticular cup, surround the mouth ring: they are probably mechanical receptors (Fig. 3g). Some other sensory receptors, with a putative chemioreceptive function, look like small bulges; each of them includes at least five short cilia arising from a single epidermal cell and slightly protruding outside (Fig. 3h). A third type of sensory, possibly mechanoreceptive, organ is represented by single, scattered, and very short cilia, which lie beneath the dorsal pedunculated scales, in touch with their lower side (Fig. 3i). Each cilium rises from a modified monociliated epidermal cell and is surrounded by a ring of microvilli. The complex cilium-microvilli is at the centre of a rounded, small (6 μm in diameter), bump-like, electron-dense protrusion.

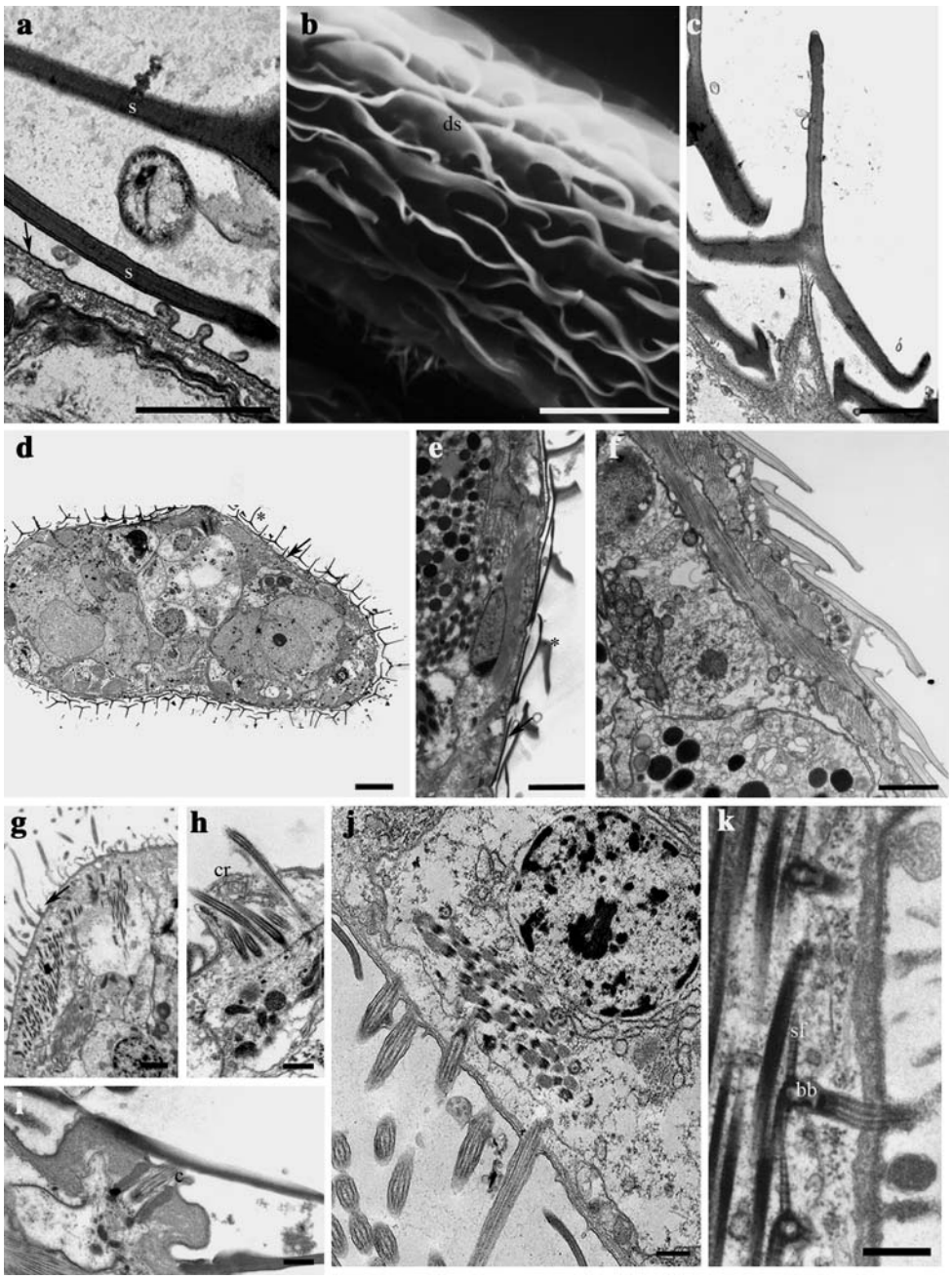


Fig. 3 Ultrastructure of the body cuticle and of the scales. **a** The cuticle is made of two layers, a thin exocuticle (*arrow*) and a thicker endocuticle (*asterisk*). The scales are solid and homogeneous in structure (TEM). **b** Shape and arrangement of the dorsal scales (SEM). **c** Cross section of a pedunculate scale (TEM). **d**, **e** Cross and longitudinal sections of the trunk showing the arrangement of the scales. Note the sequence of scales fully adhering to the cuticle (*arrow*) and others linked to the cuticle through a peduncle (*asterisk*) (TEM). **f** Longitudinal section of the trunk of *Musellifer delamarei*. The ultrastructure of the cuticle and scales look like in *Diuronotus aspetos*. All the scales are fully adhering to the cuticle (TEM). **g** Single cilium surrounding the mouth ring (TEM). **h** Sensory cephalic receptor with about five short cilia (TEM). **i** A cilium lying under the dorsal body scales (TEM). **j** An epidermal multiciliated cell (TEM). **k** Basal ciliary structures: a single basal body and two striated fibres are present (TEM). *bb* Basal body, *c* cilium, *cr* cephalic receptors, *ds* dorsal scales, *s* scales, *sf* striated fibres. *Scale bars* **a**, **c**, **g**–**k** = 1 μm; **b**, **d**, **e**, **f** = 10 μm

Ventral ciliature

The ventral ciliature arises from cuboidal, multiciliated, epidermal cells (10 cilia per cell). Each cilium stands out from a basal body with two striated rootlet fibres. An accessory centriole is absent (Fig. 3j, k).

Spermatozoon

The spermatozoon of *Diuronotus aspetos* is a filiform cell composed by an acrosomal region, a nucleus, and a very long flagellum. A single, giant mitochondrion surrounds the basal half of the nucleus and a supernumerary external membrane surrounds the whole cell (Fig. 4a).

The acrosome, about 18 μm long, is formed by two very elongated cones, which run parallel to each other and gradually reduce their diameter from the base, about 350 nm, to the apex, about 75 nm. The two cones have a different length, and a different texture of their contents. The larger cone is very electron-dense and uniform in aspect, whereas the smaller one shows several microfilaments (Fig. 4b–d). The two cones originate separately from different points of the apical surface of the nucleus: the larger one begins at the apex of the nucleus, whereas the

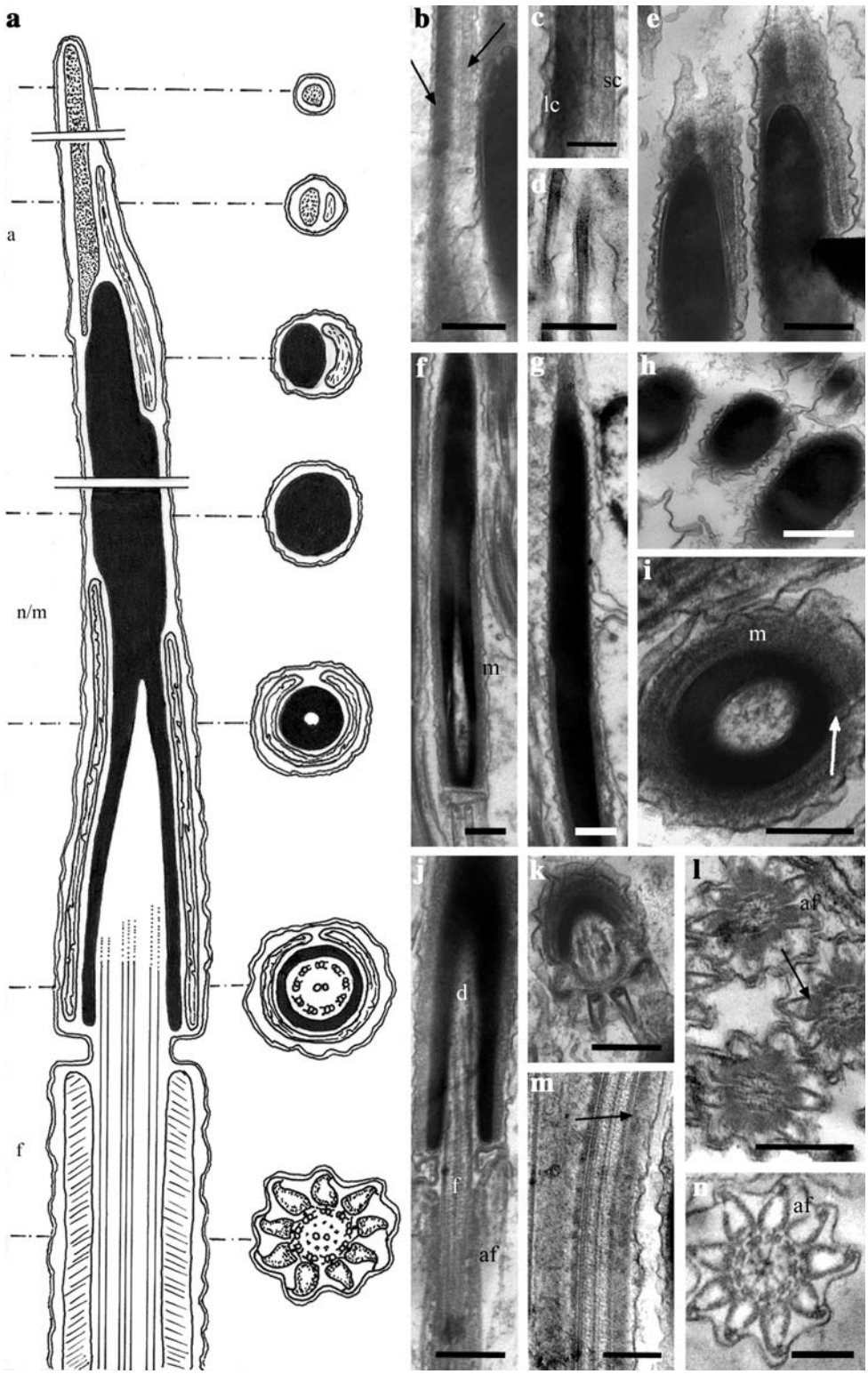


Fig. 4 The spermatozoon of *Diuronotus aspetos*. **a** Schematic drawing of the filiform spermatic cell. **b**, **c** The two elongated cones forming the acrosome are visible (*arrows*): the larger one is very electron-dense and uniform in aspect, whereas the smaller one shows several microfilaments. **d** Apical portion of the small cone. **e** Detail of the insertion of the two acrosomial cones on the nucleus. The larger cone starts just at the nuclear apex, whereas the smaller one slightly behind. **f**, **g** The two nuclear regions are shown: the basal region is almost completely surrounded by a single, giant mitochondrion, the apical one is slightly larger and tapers only at the top (*asterisk*). **h** Oblique sections of the apical portion of the nucleus. **i** Cross section of the basal nuclear region. Note that the mitochondrion does not completely surround the nucleus, leaving a narrow space (*arrow*). **j** The flagellar axoneme sinks into a deep depression of the nuclear base, and it is surrounded by an outstanding system of nine accessory fibres. **k** Oblique section of the piece connecting head and tail. **l** A slightly oblique section of the flagellum. Note the moderately electron-dense contents (*arrow*) into the areas toward the doublets. **m** Longitudinal section of the flagellum: the electron-dense contents of the accessory fibres and their thin oblique striation (*arrow*) are clearly visible. **n** Cross section of the posterior region of the flagellum where the electron-dense contents of the accessory fibres disappears. *d* Depression, *f* flagellum, *lc* large acrosomal cone, *m* mitochondrion, *sc* small acrosomal cone. *Scale bars* **b**, **d**, **e**–**h**, **j**, **k**, **l** = 0.5 μm; **c**, **i**, **m**, **n** = 0.25 μm

other one starts at about 0.5 μm posterior to the apex of the nucleus (Fig. 4e). The whole acrosome extends anteriorly to the nuclear apex: its length is at least three-times as that of the nucleus, but the small cone is long about a half of the large one (Fig. 4a).

The nucleus is a cylinder 15 μm long, approximately 0.5 μm in diameter, with a completely condensed chromatin. It can be divided in two regions of roughly equal length: the basal one, 8 μm long, is almost completely surrounded by a single, giant mitochondrion; the apical

region, 7 μm, appears slightly larger, and tapers only at the top (Fig. 4g–i). The axoneme sinks into a deep (ca 4 μm) depression of the nuclear base for about 2 μm (Fig. 4j, k). A conventional basal body was never found. The bases of the nuclear-mithocondrial complex and of the flagellum are spaced out by a deep groove of the double membrane. The flagellum, about 0.5 μm in diameter, is at least five times longer than the nucleus; it has a conventional axoneme surrounded by an outstanding system of nine external accessory fibres. Each accessory fibre is connected to the subfibres A and B of the corresponding doublet as well as to the subfibre B of the following doublet. The outline of the accessory fibres is denser than their internal contents, which appears electron-transparent. In grazing longitudinal sections, the dense material of the accessory fibres shows a thin (19 nm) oblique striation. The moderately electron-dense contents of the areas toward the doublets (Fig. 4l,m) tends to disappear in the posterior region of the flagellum (Fig. 4n).

Discussion

The objective of this description is to provide increased morphological resolution on the enigmatic chaetonotidan *Diuronotus aspetos*. Our study reveals important anatomical details not present in the original description, including data on the ultrastructure of the body wall and the spermatozoon.

The body size and shape, and the number, the dimensions and the shape of the scales of these specimens are in line with the data taken on the single adult specimen originally described (Todaro et al. 2005). The mouth cavity shows a very complex structure: in fact, the stout, protrusible processes arise from the cuticular ridges; the latter are more numerous than previously reported (24 vs. 19–20) (Todaro et al. 2005). The muzzle characteristic of the family Muselliferidae appears much more developed in species of *Diuronotus* than in *Musellifer* (Hummon 1969). The fine structure of the body cuticle of both *D. aspetos* and *M. delamarei* agrees with that of the other Chaetonotida Paucitubulatina in lacking the apical zone of the endocuticle, and showing the exocuticle made of a single 'bilayer' in contrast with all the macrodasyidans in which it is made of at least two bilayers (Rieger and Rieger 1977, Ruppert 1991). Both the keeled scales of *Diuronotus* and the spined scales of *Musellifer* have a solid, homogeneous structure unlike those of all the other Chaetonotida. The presence of pedunculated scales in addition to the ones fully adhering to the cuticle distinguishes *Diuronotus* from *Musellifer*. Among Chaetonotida pedunculated scales are present only in *Aspidiophorus* and *Xenotrichula*, two genera greatly differing in morphology from the Muselliferidae, and belonging to separate evolutionary lines (Hochberg and Litvaitis 2000).

The secondary tubes on the furca are the most striking feature of the genus *Diuronotus*. They have been proved to arise from the ventral side of the furcal base. The furcal scales are not restricted to the fleshy base of the primary tubes but extend to cover their whole length as well as that of the secondary tubes. The presence of a well-developed duo-gland system in all the tubes proves with certainty that they are adhesive tubes, and not simple cuticular structures like those present at the furcal end of other chaetonotidans (e.g., species of *Dichaetura*).

Of the three kinds of sensory organs detected in *D. aspetos*, the two cephalic ones fall within the structure of typical mechanoreceptors represented by individual or grouped cilia and described for a number of Gastrotricha (see Marotta et al. 2005 for references). The third type, which is placed in an unusual location, has a very complex structure reminiscent to that of the sensory cilia of *Lepidodermella squamata* (Hochberg 2001, Fig. 4): it is likely a mechanoreceptor too.

The multiciliated condition of ventral epidermal cells of *Diuronotus* is in line with that of all the other Chaetonotida Paucitubulatina. *Diuronotus aspetos* is similar to Xenotrichulidae in having a single centriole per cilium unlike most gastrotrichs which have two centrioles (Rieger 1976).

Sperm Discussion

Species of three families of Chaetonotida Paucitubulatina have been spermatologically studied: *Heteroxenotrichula squamosa, Xenotrichula punctata* and *X. intermedia* (Xenotrichulidae; Ferraguti et al. 1995), *Musellifer delamarei* (Muselliferidae; Guidi et al. 2003), *Lepidodermella squamata* and *Chaetonotus maximus* (Chaetonotidae; Hummon 1984; Balsamo 1992). The spermatozoa of the latter two species consist of nothing but rods of condensed chromatin enclosed in a cellular membrane, so they are very different from the filiform and complex spermatozoa of the other Chaetonotida. *L. squamata* and *C. maximus* are parthenogenic species, thus their aberrant sperm are generally interpreted as relictual structures and will not be discussed here. The spermatozoa of *Diuronotus aspetos* show an assemblage of plesiomorphic and apomorphic character states, and may be classified among the modified spermatozoa (*sensu* Franzén 1955). The acrosome including two long, different cones, the completely condensed chromatin, the single, giant mitochondrion around the nuclear base, and the very deep nuclear fossa hosting the axoneme base are all peculiar characters of *Diuronotus* spermatozoa. However, it may be reminded that *Heteroxenotrichula squamosa* shows two conical paracrosomal bodies in a

position similar to that of the double acrosome of *Diuronotus*. The bodies observed in *H. squamnosa,* however, were not interpreted as acrosomes since (a) they are not surrounded by a plasma membrane and (b) there is a conventional acrosome. A correct interpretation of the paraacrosomal bodies in xenotrichulids and the parallel, long, double acrosomes of *Diuronotus* awaits the study of spermiogenesis. The presence of supernumerary membranes is a feature shared by *D. aspetos* and *M. delamarei*, while the structure of the accessory fibres and a similar geometry of the connections to the doublets are synapomorphies of *D. aspetos*, *M. delamarei* and the Xenotrichulidae. The thin, oblique striation of the dense material of the accessory fibres is similar in *D. aspetos* and the Xenotrichulidae (Ferraguti et al. 1995; Guidi et al. 2003).

Conclusions

A number of additional morphological features of *D. aspetos* and the ultrastructure of its spermatozoon are described with the aim of integrating the original description, and clarifying the phylogenetic position of the genus. Summarizing, the solid structure of the cuticular scales, and the presence of supernumerary membranes in the spermatozoon appear to be synapomorphies shared by *D. aspetos* and *M. delamarei*, supporting the assumption that the two species group in the same clade. The sharing of the peculiar structure of the flagellum between the Musellideridae and the Xenotrichulidae suggests their sister group relationship.

Adding these new data to the morphological and spermatological sets of autapomorphies will be very useful for a phylogenetic analysis at low taxonomic level and a better resolution of systematics and taxonomy of Gastrotricha Chaetonotida.

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