

The unusual spermatozoa of *Dolichodasys* sp. (Gastrotricha, Macrodasysida)

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Abstract.—The spermatozoon of an undescribed species of *Dolichodasys* (Cephalodasyidae) from the Pacific coast of Panama was studied at structural and ultrastructural levels. Under optical microscopy, it appears as a short and wide cell with pointed extremities but without a flagellum. The cell body is made up of two well distinct regions: an anterior region with a homogeneous appearance, and a posterior region containing an evident rod-like nucleus. Under TEM, a peripheral layer of microtubules densely arranged extends for the whole cell length. In the anterior cell region, microtubules surround many tubular cisternae of smooth endoplasmic reticulum (SER), and a thin layer of vesicles with a probable acrosomal function lies just beneath the plasma membrane. The rod-shaped nucleus fills up the posterior cell region and forms a pouch that hosts a single large, irregular mitochondrial mass. A hypothesis about the motility of this aflagellate cell is advanced, on the basis of the coexistence of singlet microtubules and SER. The general architecture of *Dolichodasys* sp. spermatozoon departs from the Macrodasysida sperm basic model, consisting of a filiform cell with a corkscrew-shaped acrosome, a spring-shaped nucleus surrounding a mitochondrial axis and an ordinary flagellum. The unusual morphology of the *Dolichodasys* sperm seems to be unique in the family Cephalodasyidae: the data available for 6 species belonging to the other 4 genera of the family report spermatozoa perfectly matching the basic sperm plan of the Macrodasysida. A sister-taxon relationship between *Dolichodasys* and *Cephalodasys*, two genera drastically different in sperm shape, emerged from recent phylogenetic molecular studies, but it needs confirmation due to the still limited number of molecular data and the likely polyphyletic nature of the family Cephalodasyidae.

Keywords: *Dolichodasys*, Macrodasysida, Gastrotricha, aflagellate sperm, ultrastructure

The cosmopolitan phylum Gastrotricha includes about 830 meiobenthic species divided into two orders: Macrodasysida and Chaetonotida. Macrodasysida includes 356 species, mostly marine, distributed in 10 families and 35 genera. The members of

this order are hermaphroditic and reproduce by internal cross-fertilization (e.g., Balsamo et al. 2014, 2015, Kieneke & Schmidt-Rhaesa 2015). The current in-group systematization is mainly based on morphology but, due to the poor description in early studies and the insufficient knowledge of the microscopic anatomy of

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numerous key taxa, it is far from being stable. Indeed, modern cladistics analyses have found several taxa, especially at family level, to be non-monophyletic, with major contrasts between the phylogenetic reconstructions based on morphology and molecules (Hochberg & Litvaitis 2000, Kieneke et al. 2008, Todaro et al. 2011, 2012a, Kånneby et al. 2013).

Recently, a re-evaluation of known morphological features along with the acquisition of new anatomical and biological characteristics has led to the re-systematization of selected taxa (families and genera) reducing some of the conflicts between the morphological and molecular analyses (Todaro et al. 2006a, 2012a, Leasi & Todaro 2008, Hummon & Todaro 2010, Guidi et al. 2014). It became clear that precise information on the reproductive system lay-out and especially on the male gamete ultrastructure is of particular relevance in the process of natural systematization, principally at family level (Balsamo et al. 2002, Guidi et al. 2004, 2014, Marotta et al. 2005, 2011, Todaro et al. 2011).

Here we describe the unusual, spermatozoon of an undescribed species of *Dolichodasys* from the Pacific coast of Panama, and provide hypotheses about its functioning. It is worth mentioning that previous information of spermatozoa of *Dolichodasys* species are based exclusively on light microscopy observation. Details on the general morphology of the new species and of its reproductive system will be reported in the description of the new species that is currently in progress.

At present the genus *Dolichodasys* is affiliated with the family Cephalodasyidae (Hummon & Todaro 2010), which recent phylogenetic analyses based mostly on molecular data found to be a polyphyletic taxon (e.g., Guidi et al. 2014, Todaro et al. 2015). Therefore, in a larger framework the new information may provide new data to help clarify the relationships among the five genera of the family.

Material and Methods

Samples were collected in February 2016 during a three-week workshop on the meiofauna of Pacific Panama held at the Anchotine Laboratory (Los Santos, Panama; collection and export permits n. SE/A-2-16 and SEX/A-32, respectively). About 15 specimens of *Dolichodasys* sp. were found in sandy samples collected at 1.2–2.0 m water depth, 07°25'51"N and 80°11'44"W, from Playa Venado (Pedasi, Panama). Gastrotrichs were extracted from the sediment by the narcotization decantation technique, using an isosmotic (7%) magnesium chloride solution (Todaro & Hummon 2008). The fauna containing supernatant was then poured directly into 5-cm diameter Petri dishes and scanned for specimens under a Wild M3 dissecting microscope set at 50× magnification. For optical microscopy, gastrotrichs were removed with a micropipette from the Petri dish, fresh-mounted on slides and observed using a Leitz 20 Dialux microscope equipped with Differential Interference Contrast (Nomarski). During observation, the animals were photographed with a DS-5M Nikon digital camera and measured with the Nikon NIS software. For the transmission electron microscopy, 10 specimens were fixed and stored until further processing in a 0.1 M cacodylate buffer paraformaldehyde-glutaraldehyde mixture in a saturated solution of picric acid with sucrose added (SPAFG, Ermak & Eakin 1976). They were subsequently stored in a 0.1 M sodium cacodylate buffer and then post-fixed in 1% osmium tetroxide in the same buffer, repeatedly washed in the same buffer, dehydrated in a graded ethanol series and embedded in Araldite. Semi-thin (2 µm thick) and ultra-thin sections (70 nm thick) were cut with an LKB Ultratome 2088V. The semi-thin sections were stained with toluidine blue while the ultra-thin sections were contrasted with a saturated solution of uranyl acetate in ethanol 50%, followed by lead

citrate solution (Reynolds 1963). The ultra-thin sections were observed under a Philips CM10 transmission electron microscope. The locations of some morphological characteristics along the body are given in percentage units (U) of the total body length measured from anterior to posterior.

Results

Under optical microscopy, the spermatozoa of *Dolichodasys* sp. appear scattered along the two sides of the intestine from the pharyngo-intestinal junction (U20) to the posterior part of body (U85) (Fig. 1A, B). They are relatively short and wide cells with pointed extremities, approximately 25 μm in length and 4.5 μm in width, without a discernible flagellum (Figs. 1C, 2A). Each sperm cell show two well distinct parts: the anterior 1/3 is of homogenous appearance, while the posterior 2/3 contains an evident rod-like nucleus (Figs. 1C, 2A).

Under TEM, on the inner side of the plasma membrane, is a peripheral layer made of densely arranged filaments, that run parallel to each other along the cell main axis for its whole length (Figs. 2A–D, 3B, F). They are 30 nm in thickness and highly electron-dense. In the anterior cell region, the filaments surround numerous tubular cisternae of smooth endoplasmic reticulum (SER) that appear highly convoluted and packed into a conical structure (Figs. 2A–B, 3B, F). They are in turn surrounded by a thin layer of vesicles that is delimited by two membranes, one adhering to the cell membrane and the other to the filaments. These vesicles are ampulla-shaped, with a flask-like, electron-transparent base and a long, highly electron-dense neck (Figs. 2A–B, 3C–D).

The nucleus fills up the posterior region of the spermatozoon. It is rod-shaped, about 13 μm long (Fig. 1C), and forms a pouch in which a single large, irregular

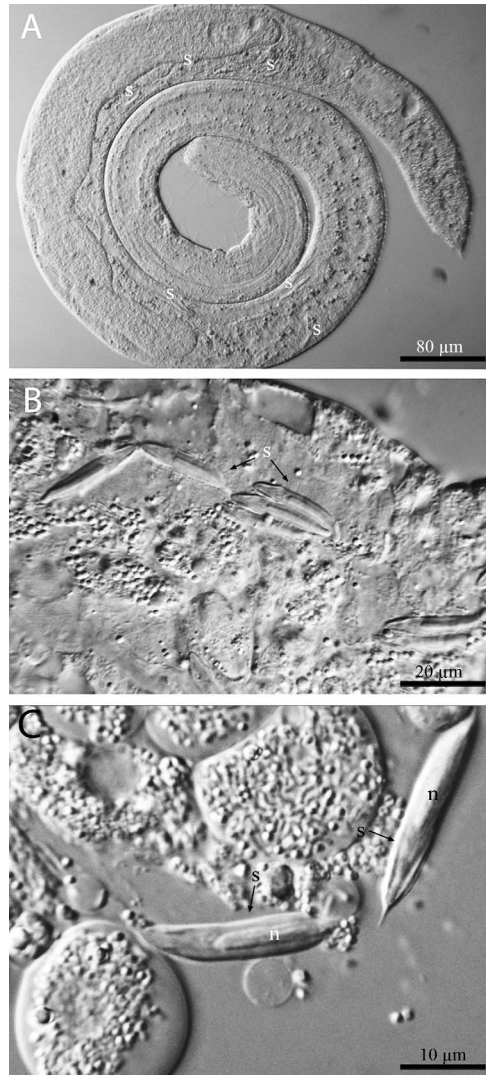


Fig. 1. A. Habitus of *Dolichodasys* sp. The spermatozoa (s) are scattered on both sides of the intestine. B. Some spermatozoa (s) lateral to the intestine. C. Close-up of two pod-shaped spermatozoa (s): note the rod-shaped nucleus (n), (DIC Nomarsky microscopy).

mitochondrial mass is located. In cross section, the mass shows a characteristic star shape with four to six arms (Figs. 2A, C, D, 3A, 4A–B).

During the spermatogenesis the mitochondrial mass arises from the coalescence of small mitochondria with a conventional appearance. It coils around the nucleus

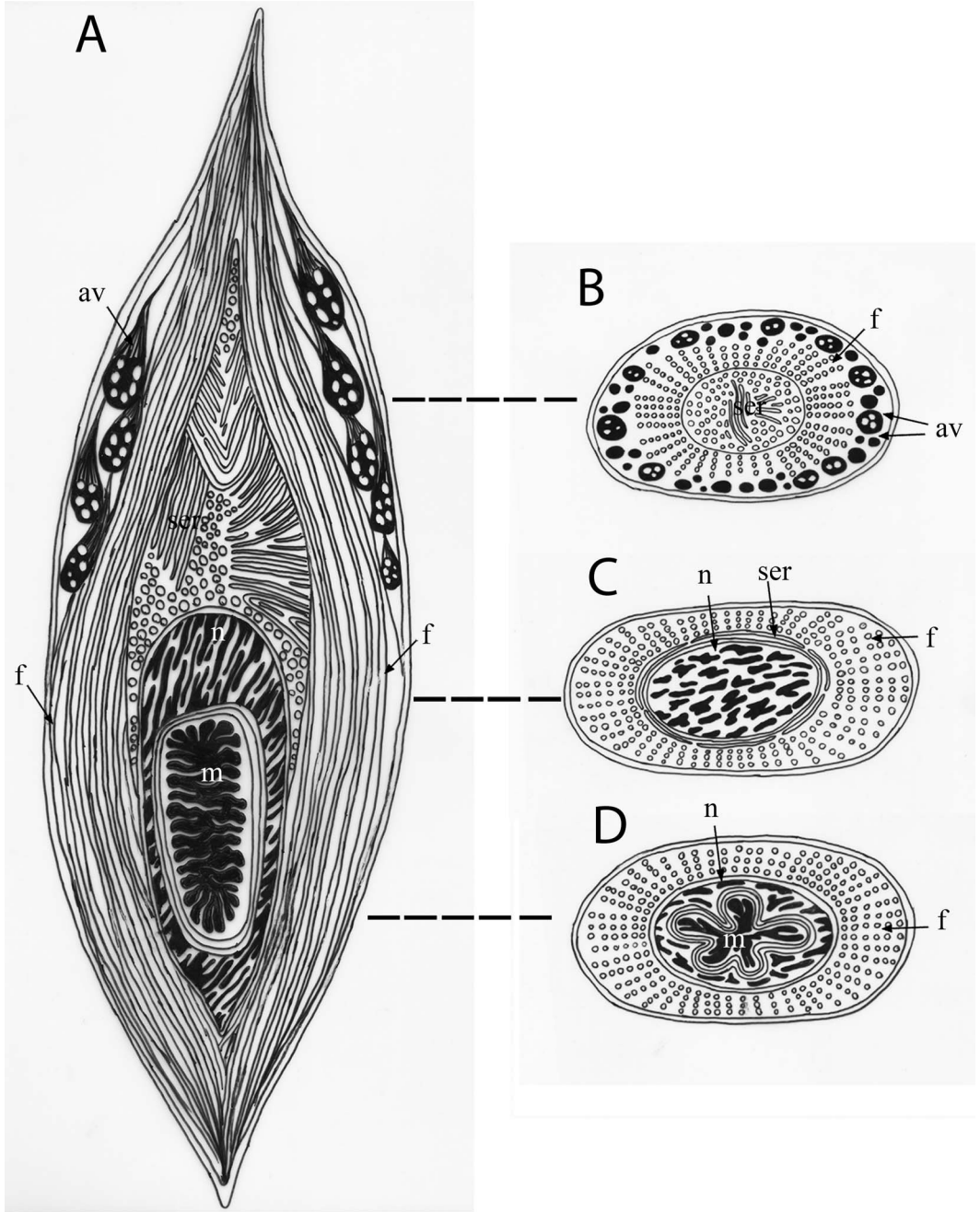


Fig. 2. A–D. Schematic drawing of the mature spermatozoon of *Dolichodasys* sp. based on light and TEM data in longitudinal (A) and cross-section (B–D). The ampulla-shaped vesicles (av), the cisternae of smooth endoplasmic reticulum (ser), the filaments (f), the nucleus (n) and the mitochondrial mass (m) are highlighted by labels.

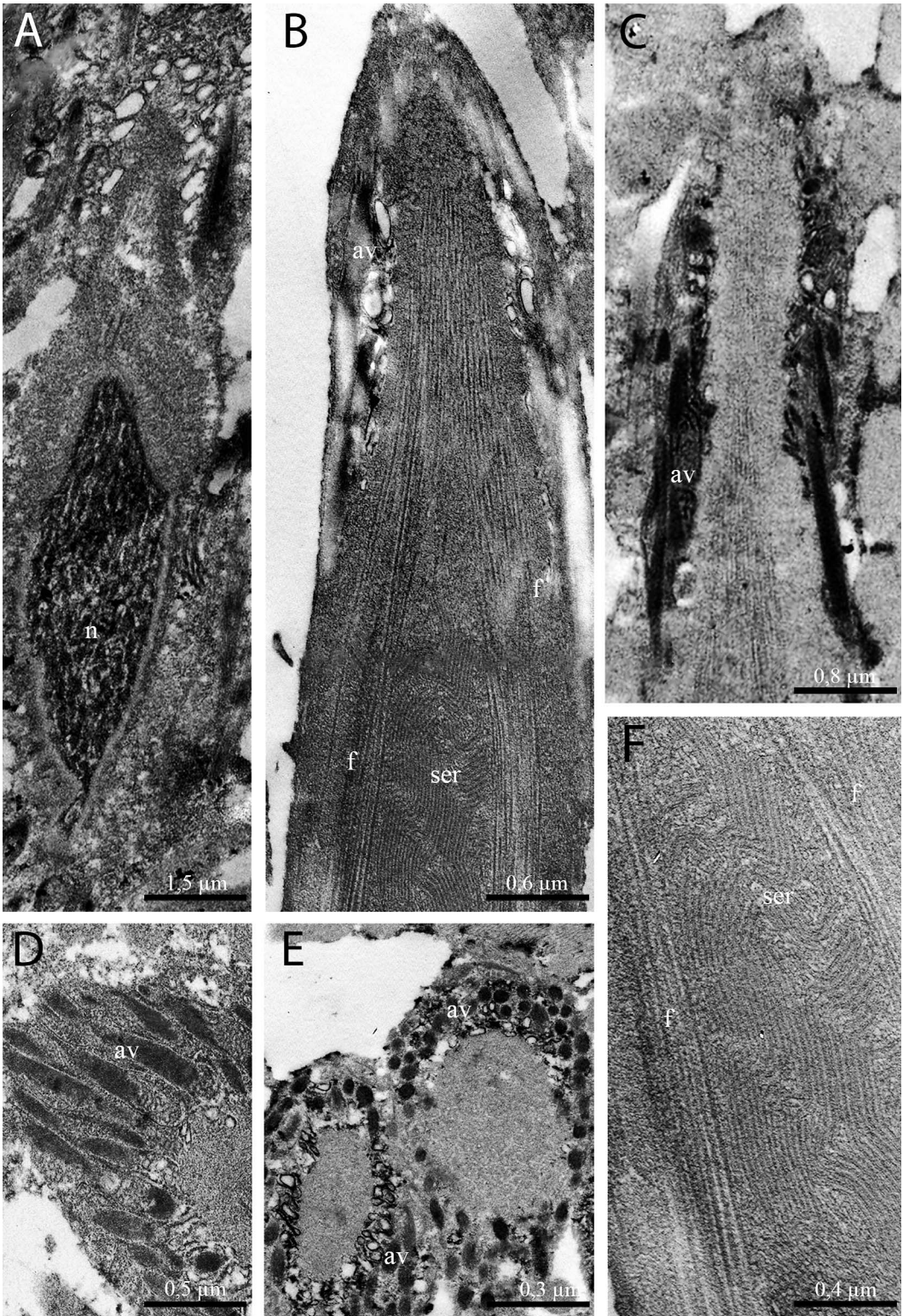


Fig. 3. A. TEM micrograph of a mature spermatozoon in longitudinal section. B. Sperm anterior region in longitudinal section: the convoluted cisternae of smooth endoplasmic reticulum (ser), the layers of filaments

and then sinks into it (Fig. 4C, D). At the same time, a large number of filaments appear that fill the whole cytoplasm in an unorganized way. Furthermore, numerous vesicles are produced in the anterior part of the cell. Neither basal body nor developing flagellum have been observed during spermatogenesis (Fig. 4C, D). A flagellum is not present in the mature cell.

Discussion

The spermatozoon of *Dolichodasys* sp. is uncommon within Macrotrichida in lacking a flagellum. Data on the fine morphology of the male cell of the studied species substantiate the uncertain information of Ruppert & Shaw (1977) on the spermatozoon of *Dolichodasys carolinensis*, the only other species of the genus observed in some detail. It may be inferred that also the spermatozoon of *Dolichodasys delicatus* very likely lacks a flagellum, as previously suggested by Ruppert & Shaw (1977) based only on light microscopy data. Small cell size and absence of a flagellum in the spermatozoa of these three species strongly calls for a re-examination of the sperm cell of *Dolichodasys elongatus* for which a total length of over 50 μm , including an over 25 μm long tail, has been reported (Gagne 1977). An investigation of the sperm morphology of the latter species is needed in order to use its characters for phylogenetic inferences.

The term 'commaform' was used by Ruppert & Shaw (1977) to define the shape of the sperm of *D. carolinensis*. The same term has also been used for the short and compact spermatozoa of *Neodasys* (e.g., Remane 1936, Ruppert 1991). However, as the spermatozoon of *Neodasys* has a flagellum (Guidi et al. 2003), the use of

the same term for the sperm of *Dolichodasys* may be ambiguous and a source of confusion. Considering the astonishing resemblance between the sperm of *Dolichodasys* sp. and the fruit of the Leguminosae plants, we propose the term pod-shaped for the unusual spermatozoa of this genus.

Our TEM analysis of the sperm of *Dolichodasys* sp. yielded several ultrastructural details that may help to re-interpret corresponding structures in the sperm of *D. carolinensis* that was only studied by light microscopy. Moreover, our study allows to increase the number of spermatozoological traits known for the genus.

In *D. carolinensis* a high number of filaments are described as forming a capsule of unclear origin surrounding the entire sperm (Ruppert & Shaw 1977). Very similar filaments lie on the internal side of the cell membrane of *Dolichodasys* sp., running for the whole cell length, and under TEM analysis, they appear as conventional microtubules. Since TEM clearly shows their intracellular position, it is incorrect to define these spermatozoa as 'encapsulated' (see Ruppert & Shaw 1977). In the anterior region, the sperm of *D. carolinensis* is reported to have a truncated cone-shaped acrosome located on one side of the nucleus (Ruppert & Shaw 1977). In *Dolichodasys* sp. the thin layer of vesicles lying just beneath the plasma membrane forms a sort of mantle covering the microtubules and most likely have an acrosomal function. The vesicles are full of proteinaceous contents and are surrounded by membranes, which suggests that the vesicles have an enzymatic function and the two membranes may be the external and internal acrosomal membranes, usually visible in the sperm acrosome. Further-

(f) and of ampulla shaped vesicles (av) are visible. C, D. Close-up of the layer of vesicles (av) in longitudinal section. E. Cross-section of the anterior end of the spermatozoon showing the thin vesicle layer (av). F. Detail of the cisternae of smooth endoplasmic reticulum (ser) and the filaments (f).

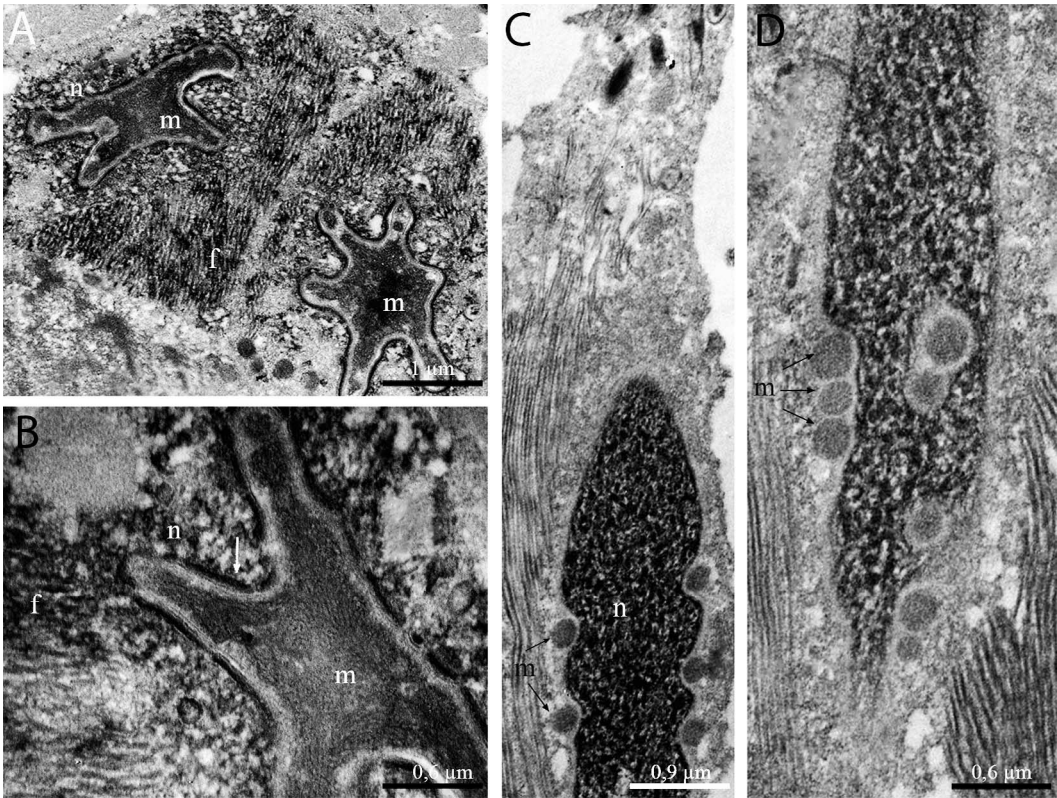


Fig. 4. A, B. TEM micrographs of the posterior region of a mature spermatozoon in cross-section. The nucleus (n) includes the internal star-shaped mitochondrial mass (m). Filaments are attached to the nucleus (f). The white arrow highlights a particular of the double membranes of the nucleus and of the mitochondrial mass. C. Small mitochondria (m) are regularly arranged around the nucleus (n). D. The mitochondria (m) are moving into the nucleus (n).

more, the 'slightly acidophilic sphere' reported by Ruppert & Shaw (1977; Fig. 1F) in the anterior part of the sperm of *D. carolinensis* may match the conical mass of smooth endoplasmic reticulum in the sperm cell of *Dolichodasyis* sp. The possible correspondence between these two structures is supported by their location and the well-known acidophilic property of SER (Bancroft & Gamble 2008). The nucleus is rod-shaped and located in the posterior part of the cell in both species of *Dolichodasyis*. In *Dolichodasyis* sp., TEM observations have highlighted a large mitochondrial mass inside a pouch of the nucleus, and the absence of flagellum; no information regarding mitochondria are available for *D. carolinensis*.

Thus, the general architecture of spermatozoa of species of *Dolichodasyis* differs from the Macrodasysida basic sperm model, which is represented by a filiform cell with a corkscrew-shaped acrosome, a spring-shaped nucleus surrounding a mitochondrial axis and an ordinary flagellum (Teuchert 1976, Fisher 1994, Ferraguti & Balsamo 1995, Balsamo et al. 2002, Guidi et al. 2004, 2010, 2014, Todaro et al. 2012b). On the contrary, the sperm of *Dolichodasyis* is not filiform, has a mantle-shaped acrosome, a rod-shaped nucleus and lacks a flagellum.

The absence of a flagellum may raise the question about how this cell can move. Sperm motile systems of singlet microtubules are reported for aflagellate sperma-

tozoa of coccid insects (Baccetti et al. 1982) and of some platyhelminths (Newton 1980, Ehlers 1981, Justine et al. 1985). Newton (1980) demonstrated the presence of ATPase at the level of the singlet microtubules of the aflagellate motile sperm of the turbellarian *Macrostomum*. Moreover, Baccetti et al. (1982) described links between singlets of a row and singlets of two different rows and demonstrated the presence of dynein in the insects' spermatozoa by electrophoretic methods. Therefore, the motility would be the result of dynein-singlet interaction, as it occurs in the axoneme between the doublets.

The SER appears particularly developed in the sperm of *Dolichodasys* sp. unlike in flagellate spermatozoa examined so far. A close association of the SER with singlet microtubules has been documented in aflagellate turbellarian and monogenean sperm (i.e., *Pseudostomum*, Ehlers 1981; *Diplozoon*, Justine et al. 1985). The coexistence of singlet microtubules and SER in the aflagellate spermatozoa may be related to the cell motility. In fact, it is well known that the Ca^{2+} stores are membrane-bound organelles, such as smooth endoplasmic and sarcoplasmic reticula, and that the elevation of cytoplasmic Ca^{2+} concentration is indispensable to activate the mobility of the sperm (Fliegert et al. 2007). The increase of the concentration of Ca^{2+} can occur by its entry into cells through the plasma membrane (Catterall 1995, Pitcher et al. 1998, Dutta 2000, Wiesner et al. 1998) or release of Ca^{2+} from membrane-bound internal stores (Lytton et al. 1991). Therefore, it is possible that the SER observed in aflagellate sperm serve as a Ca^{2+} store, which may be used in addition to extracellular Ca^{2+} , to activate the mitochondrial apparatus to produce a greater amount of ATP; a sperm motile system organized in singlet microtubules could require more ATP than the one organized in doublets.

Since the first investigations of Teuchert (1976), spermatozoa of at least 26 Macro-

dasyida species have been studied ultrastructurally (Ruppert 1978, Ferraguti & Balsamo 1994, 1995, Fisher 1994, 1996, Fregni et al. 1999, Balsamo et al. 2002, Guidi et al. 2004, 2009, 2010, 2014, Pierboni & Kristensen 2007, Todaro et al. 2012b). These studies support the general filiform sperm type, which however reveals an unexpected ultrastructural cell complexity. Major discrepancies from the basic sperm plan can be found e.g. in *Macrodasys* where a twisted mitochondrion enwraps the nucleus (Marotta et al. 2005) and in *Urodasys* where the mitochondria are missing from the mature sperm cell (Balsamo et al. 2007).

Within Macrodasysida, a completely different sperm morphology and ultrastructure was reported only in *Dactylopodola baltica*. The spermatozoon of this species, probably paradigmatic for the whole genus, is filiform but consists of a long, thin, and rod-shaped nuclear region and an adjacent rod-like compartment in which small disc-shaped and piled mitochondria alternate with dense bodies of unknown function (Fisher 1996). Acrosome and flagellum were not observed, but it is worth mentioning that TEM observations only focused on penetrated spermatozoa, and thus need confirmation on the fine morphology of testicular spermatozoa. In all cases, the differences emerged so far in the ultrastructure of the spermatozoa of *Dactylopodola* and *Dolichodasys*, seem to exclude a common origin of the two phyletic lines.

Within the family Cephalodasyidae, spermatological data are available for *Cephalodasys maximus*, *Mesodasys adenotubulatus* and *M. laticaudatus* (Ferraguti & Balsamo 1994, Fisher 1994, Fregni et al. 1999). The ultrastructural morphology of these spermatozoa perfectly matches with the basic sperm plan of the Macrodasysida. As for the other two genera of Cephalodasyidae, no report on the morphology of sperm of *Pleurodasys* is available, but a new species recently discovered in South

Africa shows, under DIC optics, filiform sperm with some corkscrew like portions, thus similar to the basic sperm plan (Todaro et al. this volume). Concerning *Paradasys*, data on sperm morphology are available for *P. littoralis* only: a filiform, flagellate sperm with a corkscrew head (Rao & Ganapati 1968). However, the affiliation of this species to genus *Paradasys* is doubtful (see Ruppert & Shaw 1977). Due to the absence of reliable data on *Paradasys*, it is not yet clear whether the unusual morphology of the *Dolichodasys* sperm is unique within the Cephalodasyidae. Phylogenetic reconstructions of Macrodasyida based on molecular data show the family Cephalodasyidae to be non-monophyletic, with the genus *Dolichodasys* clustering together with the genus *Cephalodasys* (e.g., Todaro et al. 2014, 2015). These results suggest that closely related genera might show drastically different forms of spermatozoa, which contrasts with previous studies that find a close similarity of spermatozoa at the family level (e.g., Marotta et al. 2011, Todaro et al. 2012b, Guidi et al. 2014). On the other hand, it is worth noting that the closeness of *Cephalodasys* and *Dolichodasys* needs to be confirmed, due to the relatively poor taxonomic sampling of Cephalodasyidae in the phylogenetic studies based on molecular markers performed so far (Todaro et al. 2006b, 2014, 2015).

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