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Sperm characteristics in the digenean *Diplodiscus amphichrus* (Paramphistomoidea, Diplodiscidae), a parasite of the Chinese edible frog *Hoplobatrachus rugulosus*

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Abstract

The ultrastructural characteristics of the mature spermatozoon of *Diplodiscus amphichrus* (Digenea, Paramphistomoidea, Diplodiscidae) and their ultrastructural organisation were examined by means of transmission electron microscopy. Live digeneans were collected from the Chinese edible frog *Hoplobatrachus rugulosus* in Thailand. The male gamete of *D. amphichrus* is a filiform cell with two axonemes of the 9+'1' trepaxonematan pattern, nucleus, one mitochondrion, parallel cortical microtubules, a well-developed lateral expansion, external ornamentation of the plasma membrane, spine-like bodies and granules of glycogen. These ultrastructural characteristics have already been described in other paramphistomoids studied so far except for the cladorchiids, which present some differences. Two characteristics of the male gamete of *D. amphichrus* were found for the first time in a digenean: (i) the appearance of the initial part of the lateral expansion before the axonemes in the anterior extremity and (ii) the separation between the second axoneme and the nucleus in the posterior tip. Our results are compared with the available data in the Digenea and particularly with other paramphistomoids.

Keywords: *Diplodiscus amphichrus*; Diplodiscidae; Paramphistomoidea; Digenea; Sperm characters; Ultrastructure

Introduction

Diplodiscus amphichrus Tubangui, 1949 is a digenean that belongs to the family Diplodiscidae. This family, included in the superfamily Paramphistomoidea, constitutes a cosmopolitan and small group of digeneans with only six valid genera, namely *Australodiscus*, *Catadiscus*, *Dermatemytrema*, *Diplodiscus*, *Progonimodiscus* and *Pseudodiplodiscus*. Representatives of Diplodiscidae are parasites of the digestive tract and they are predominantly found parasitizing amphibians but also recorded in reptiles and fish (see Jones, 2005).

The usefulness of sperm characters and their organisation in the male gamete for the interpretation of relationships between Platyhelminthes have been demonstrated for different groups by several authors (Justine 1991a, 1991b, 1998, 2001; Bâ and Marchand 1995; Miquel et al. 1999; Levron et al. 2010; Quilichini et al. 2010a, 2011; Bakhoum et al. 2017a; Justine and Poddubnaya 2018). Regarding the digenean superfamily Paramphistomoidea, spermatological studies have been done on seven species belonging to four families: the Cladorchiidae, Diplodiscidae, Gastrothylacidae and Paramphistomidae (Ashour et al. 2007; Seck et al. 2007, 2008a, 2008b; Swarnakar 2010; Bakhoum et al. 2011). In the Diplodiscidae, there is only an ultrastructural study of the spermatozoon of *Diplodiscus subclavatus* (Bakhoum et al. 2011). The present study aims to describe the sperm characters and their organisation along the mature spermatozoon of a second species of the family Diplodiscidae, *D. amphichrus*. Our results are also compared with the available data in other digeneans, particularly paramphistomoids, to highlight the most characteristic features and their phylogenetic importance.

Materials and methods

Specimens

Live adult specimens of *Diplodiscus amphichrus* Tubangui, 1949 were isolated from a Chinese edible frog *Hoplobatrachus rugulosus* (Wiegmann, 1834) collected in May 2014 by hand from a frog farm (N 17° 49.21'; E 102° 76.62'; 173m asl) in Udon Thani Province, Thailand. The collected amphibian was immediately transported alive to the laboratory at Udon Thani Rajabhat University. The frog was anesthetised and subsequently killed using MS222 (ethyl-4-aminobenzoate). After dissection, digeneans were isolated and fixed for transmission electron microscopy (TEM). This study was approved by the Udon Thani Rajabhat University Animal Care and Ethical Use Committee. Specimens of *D. amphichrus* were identified according to Sey (1985, 1991) and were previously reported in northeast Thailand (Wongsawad et al. 1998).

Transmission electron microscopy

For the present TEM study, several flukes were rinsed with a 0.9% NaCl solution and fixed in cold (4 °C) 2.5% glutaraldehyde in a 0.1M sodium cacodylate buffer at pH 7.4 for a minimum of 2 h, rinsed in 0.1M sodium cacodylate buffer at pH 7.4, post-fixed in cold (4 °C) 1% osmium tetroxide with 0.9% potassium ferricyanide in the same buffer for 1 h, rinsed in Milli-Q water (Millipore Gradient A10), dehydrated in an ethanol series and propylene oxide, embedded in Spurr's resin and polymerised at 60 °C for 72 h. Ultrathin sections (60–90 nm thick) at the level of the seminal vesicle were obtained using a Reichert-Jung Ultracut E ultramicrotome. Sections were placed on 200-mesh copper and gold grids. Sections placed on copper grids were double-stained with uranyl acetate and lead citrate according to Reynolds (1963). Copper grids were examined in a JEOL 1010 transmission electron microscope operated at an accelerating voltage of 80 kV, in the 'Serveis Científics i Tècnics de la Universitat de Barcelona (CCiTUB)'.

Cytochemistry

Sections placed on gold grids were treated according to the specific cytochemical test of Thiéry (1967) to reveal the presence of glycogen. Thus, they were treated in periodic acid (PA), thiocarbohydrazide (TCH) and silver proteinate (SP) as follows: 30 min in 10% PA, rinsed in Milli-Q water, 24 h in TCH, rinsed in acetic solutions and Milli-Q water, 30 min in 1% SP in the dark, and rinsed in Milli-Q water. Sections were examined in a JEOL1010 transmission electron microscope at an accelerating voltage of 80 kV, in the CCiTUB.

Results

The observation of numerous ultrathin sections at the level of the seminal vesicle allowed us to distinguish three regions in the mature spermatozoon of *Diplodiscus amphichrus* **(**Figs. 1-3).

These three regions exhibit different ultrastructural characteristics and organisation as follows:

Region I or anterior region of the spermatozoon (Figs. 1a-i and 3I). The main characteristics of this region are the simultaneous presence of external ornamentation of the plasma membrane and a well-developed lateral expansion (Figs. 1a-i and 3I). The anterior spermatozoon extremity is formed by a large section with a continuous and submembranous layer of parallel cortical microtubules associated with the external ornamentation of the plasma membrane (Fig. 1a). The two axonemes of the 9+'1' pattern appear almost simultaneously (Fig. 1b-d). The maximum number of cortical microtubules (around 63) is observed in the part of the sperm cell exhibiting the maximum development of the lateral expansion (Fig. 1d-f). In the posterior part of region I the lateral expansion reduces and disappears (Fig. 1g, h) and the external ornamentation progressively reduces (Fig. 1h, i). The cortical microtubules form a continuous and submembranous layer along region I except in the posterior part where their arrangement into two bundles is observed (Fig. 1i). Another ultrastructural character of the region I is the presence of spine-like bodies irregularly distributed (Fig. 1d, f, i). Finally, electron-dense granules appear in the cytoplasm in the posterior part of region I (Fig. 1g-i). This granular material was identified as glycogen by the application of the specific cytochemical test of Thiéry (see Fig. 2l for the remaining regions).

Region II or middle region of the spermatozoon (Figs. 1j-k, 2l and 3II). It is mainly characterised by the absence of most of the above-mentioned characters. Thus, region II lacks the lateral expansion, the external ornamentation of the plasma membrane and spine-like bodies (Figs.1j-k and 3II). Thereby, the middle region just presents the two 9+'1' axonemes, two bundles of parallel cortical microtubules and a large amount of granules of glycogen (Fig. 1j-k).

Region III or posterior region of the spermatozoon (Figs. 2a-l and 3III). This region corresponds with the nuclear and also mitochondrial part of the sperm cell. The anterior part of region III only has the nucleus (Fig. 2a). Later, the mitochondrion appears when the nucleus is already present (Fig. 2b, c). After the disappearance of the mitochondrion, the first axoneme becomes disorganised and disappears (Fig. 2d-f). In the posterior part of region III, the second axoneme exhibits a lateral cytoplasmic protrusion (Fig. 2g, h). Finally, the posterior extremity of the sperm cell is characterised by a separation between the second axoneme and the nucleus by a plasma membrane cytokinesis (Fig. 2i, j). That way, the posterior tip contains only the nucleus (Fig. 2k).

Discussion

The mature spermatozoon of *Diplodiscus amphichrus* exhibits many ultrastructural characters previously described in most digenean species. These are two axonemes of the 9+'1' trepaxonematan pattern (Ehlers 1984), a nucleus, a mitochondrion, parallel cortical microtubules, a lateral expansion, external ornamentation of the plasma membrane, spine-like bodies and granules of glycogen. The presence/absence and organisation of these features along the sperm cell show many similarities with the remaining studied paramphistomoids when they are compared (see Table 1). However, the spermatozoon of *D. amphichrus* exhibits two ultrastructural particularities: (i) the anterior extremity formed by the proximal part of the lateral expansion before the appearance of the two axonemes and (ii) the posterior extremity showing a separation between the second axoneme and the nucleus by a plasma membrane cytokinesis. To our knowledge, the present study constitutes the first finding of such characteristics.

Anterior spermatozoon region: lateral expansion, external ornamentation and spine-like bodies

The most characteristic features present in the anterior region of the spermatozoon of *D. amphichrus* are a well-developed lateral expansion, the external ornamentation of the plasma membrane and spine-like bodies.

Lateral expansions are present in the sperm cell of diverse digeneans belonging to the Bucephalidae, Echinostomatidae, Fasciolidae, Mesometridae and Troglotrematidae (Ndiaye et al. 2003; Miquel et al. 2006, 2018; Bakhoum et al. 2013; Kacem and Miquel 2018). In the Paramphistomoidea, all the studied species exhibit this feature. So, a lateral expansion has been reported by Ashour et al. (2007) in *Basidiodiscus ectorchis* and *Sandonia sudanensis* (Cladorchiidae), by Bakhoum et al. (2011) in *Diplodiscus subclavatus* (Diplodiscidae), by Seck et al. (2008a) in *Carmyerius endopapillatus* (Gastrothylacidae), and by Seck et al. (2007, 2008b) and Swarnakar (2010) in *Paramphistomum microbothrium*, *Cotylophoron cotylophorum* and *Orthocoelium scoliocoelium* (Paramphistomidae). These lateral expansions, as in *D. amphichrus* (present study) are associated with external ornamentation of the plasma membrane and submembranous cortical microtubules (see Table 1). However, the morphology of lateral expansion is not similar in all these digeneans. Among digeneans the morphology of lateral expansions is variable; e.g. there is reduced lateral expansions or hook-shaped lateral expansions. Only *D. subclavatus* (Bakhoum et al. 2011) presents a well-developed lateral expansion as its congener *D. amphichrus*. To our knowledge only aspidogastreans and some monogeneans exhibit in their spermatozoa lateral expansions so developed (Justine and Mattei 1985; Giese et al. 2020). Both spermatozoa of monogeneans and digeneans have numerous cortical microtubules in their lateral expansions. In contrast, in the lateral expansions of aspidogastreans there is also internal microtubules additionally to the peripheral ones.

The external ornamentation of the plasma membrane is usually associated with cortical microtubules and are frequent in digenean spermatozoa (see Bakhoum et al. 2017a). This is the case for *D. amphichrus* and also for all the currently studied species of the Paramphistomoidea (see Table 1). However, in other cases, the ornamentation of the plasma membrane is not observed in association with cortical microtubules, e.g. in *Pronoprymna ventricosa* (Faustulidae) and hemiuroideans (Quilichini et al. 2007; Kacem et al. 2020).

Recently, both lateral expansion and external ornamentation have been considered useful criteria to establish different models of spermatozoa in the Digenea (see Bakhoum et al. 2017a). These authors consider the type V of spermatozoon as the characteristic for the Paramphistomoidea, but also present in other digenean species such as brachylaimoideans, echinostomatoideans, microscaphidioideans or pronocephaloideans.

Additionally, the majority of paramphistomoids also exhibit spine-like bodies in this region. Only the two studied cladorchiids (Ashour et al. 2007) lack these ultrastructural elements (see Table 1). Spine-like bodies, described for the first time in *Opecoeloides furcatus* (Opecoelidae) by Miquel et al (2000), are prominent electron-dense structures usually present in the ornamented region of the spermatozoon. Only the apocreadiid *Neoapocreadium chabaudi* has spine-like bodies not associated with the external ornamentation of the plasma membrane (Kacem et al. 2010).

Cortical microtubules: number of bundles, the maximum number and their location

Another interesting characteristic concerns cortical microtubules. These submembranous ultrastructural elements are present in the sperm cells of the majority of digeneans with a parallel disposition in contrast with the more evolved cestodes (Justine et al. 2001). Only some didymozoids lack cortical microtubules in their spermatozoa (Justine and Mattei 1983; Pamplona-Basilio et al. 2001). When present, cortical microtubules are normally arranged into two fields in the principal region of the spermatozoon (mitochondrial and nuclear regions). However, species of the Hemiuridae and the faustulid *P. ventricosa* have male gametes with cortical microtubules arranged into a single bundle (Quilichini et al. 2007; Kacem et al. 2020). An interesting aspect is that related to the high number of cortical microtubules in *D. amphichrus* (63 elements). The remaining studied paramphistomoids also present a high number (about 50 or more). Other taxa with a comparably high number of cortical microtubules (around 40 to 50) are included in the Mesometridae, Notocotylidae, Pleurogenidae or Pronocephalidae (Ndiaye et al. 2012a, 2015a; Bakhoum et al. 2013; Miquel et al 2013). The location along the spermatozoon of the maximum number of cortical microtubules has also been considered as an interesting aspect in the above-mentioned review of Bakhoum et al. (2017a). As occurs in *D. amphichrus,* all the paramphistomoids present the maximum number of these structural elements in the anterior region of the spermatozoon, specifically in the lateral expansion.

Mitochondria: number and morphology

The mitochondrion is another structure present in the spermatozoon of the Digenea. The number and morphology of the mitochondria vary according to the species. The number of mitochondria varies between one and three (see Bakhoum et al. 2017a). In the Paramphistomoidea, all the species analysed until now have one mitochondrion except the cladorchiids *B. ectorchus* and *S. sudanensis* (Ashour et al. 2007) in which they are three mitochondria (see Table I). The majority of digeneans have one or two mitochondria in the spermatozoon and only two species, namely *Euryhelmis squamula* (Heterophyidae) and *Anisocoelium capitellatum* (Cryptogonimidae) contain three mitochondria in their spermatozoa (Bakhoum et al. 2009; Ternengo et al. 2009).

Considering the morphology, variability is also described in the sperm cells of digenean species. A filiform mitochondrion has been reported in the majority of species (see Bakhoum et al. 2017a). A second type, a moniliform mitochondria constituted by several mitochondrial bulges joined by a mitochondrial cord, was described for the first time in *Holorchis micracanthum* by Bâ et al. (2011) and posteriorly it has been reported in the male gamete of some digeneans such as the cryptogonimids *Aphallus tubarium* and *Timoniella imbutiforme* (Foata et al. 2012; Kacem et al. 2017a), the acanthocolpid *Stephanostomoides tenuis* (Bakhoum et al. 2015), the opecoelids *Allopodocotyle pedicellata* and *Macvicaria obovata* (Bakhoum et al. 2017b; Kacem et al. 2017b), the lepocreadiid *Opechona bacillaris* (Ndiaye et al. 2015b), the plagiorchiid *Enodiotrema reductum* (Ndiaye et al. 2012b) and the sclerodistomoidid *Sclerodistomoides pacificus* (Bâ et al. 2020). Finally, Kacem et al. (2019) have recently described in the opecoelid *Allopodocotyle tunisiensis* a U-shaped mitochondrion characterised by the presence of a circular fold in its posterior part. In the Paramphistomoidea all the species exhibit a filiform type of mitochondrion.

Posterior spermatozoon extremity

As the anterior extremity, the posterior one shows variable characters depending on the species. Thus, several authors have proposed the use of posterior spermatozoon morphology as phylogenetic characters (Quilichini et al. 2010; Bakhoum et al. 2017a). Quilichini et al. (2010) considered the sequence of the disappearance of principal characters (nucleus, second axoneme and cortical microtubules) in the posterior extremity of the spermatozoon. They distinguished three types of posterior spermatozoon extremities: type 1 or opecoelidean type characterised by the sequence 'second axoneme, nucleus and cortical microtubules', type 2 or fasciolidean type with the sequence 'cortical microtubules, second axoneme and nucleus' and type 3 or cryptogonimidean type with the sequence 'cortical microtubules, nucleus and second axoneme'. However, several years later, due to several inconsistencies with these three types (Quilichini et al. 2010) in some digeneans, Bakhoum et al. (2007a) proposed the consideration of only the last spermatozoon character. Thus, as occurs in *D. amphichrus,* the posterior character in the sperm cells of the majority of paramphistomoids is the nucleus (see Table 1).

Concluding remarks

All the currently studied paramphistomoids share several features in their sperm cells that follow the type V of Bakhoum et al. (2017a). This model of spermatozoon is mainly characterised by (i) two 9+'1' axonemes, (ii) a lateral expansion, (iii) external ornamentation associated with cortical microtubules and located in the anterior part of the anterior region, (iv) the parallel cortical microtubules arranged into two bundles, (v) the maximum number of cortical microtubules located in the anterior part, and (vi) generally one mitochondrion.

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Competing interests

The authors declare that they have no competing interests.

Compliance with Ethical Standards

This article does not contain any studies with animals performed by any of the authors.

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Legends to figures

Fig. 1 Spermatozoon of *Diplodiscus amphichrus*: regions I and II. **a-g** Correlative cross-sections of the anterior part of region I showing the lateral expansion. Note the presence of external ornamentation of the plasma membrane associated with cortical microtubules and spine-like bodies. **h, i** Cross-sections of the posterior part of region I lacking lateral expansion. **j, k** Cross-sections of the region II lacking external ornamentation of the plasma membrane. *Ax1* first axoneme, *C1* and *C2* centrioles of the first and second axonemes, *CM* cortical microtubules, *EO* external ornamentation of the plasma membrane, *G* granules of glycogen, *LE* lateral expansion, *SB* spine-like bodies. *Scale bars* 300 nm.

Fig. 2. Spermatozoon of *Diplodiscus amphichrus*: region III. **a-d** Correlative cross-sections of the anterior part of nuclear region before the disorganisation of the first axoneme. Note the presence of the mitochondrion in this part. **e** Cross-section at the level of the posterior extremity of first axoneme. **f-h** Correlative cross-sections showing the appearance of a cytoplasmic extension near the second axoneme. **i** Cytokinesis of plasma membrane (arrows); the sperm cell shows two separate sections containing the second axoneme and the nucleus. **j** Disorganisation of the second axoneme. **k** Posterior spermatozoon tip presenting only the nucleus. **l** Positive result for glycogen using the test of Thiéry. *Ax2* second axoneme, *CC1* central core of the first axoneme, *CM* cortical microtubules, *G* granules of glycogen, *M* mitochondrion, *N* nucleus, *S1* and *S2* singlets of the first and second axonemes. *Scale bars* 300 nm.

Fig. 3. Schematic reconstruction of the mature spermatozoon of *Diplodiscus amphichrus*. The sperm cell is organised in three different regions (*I*, *II* and *III*). To make the diagram clearer, granules of glycogen are not shown in longitudinal sections. *ASE* anterior spermatozoon extremity, *Ax1* and *Ax2* first and second axonemes, *CC1* central core of the first axoneme, *CM* cortical microtubules, *EO* external ornamentation of the plasma membrane, *G* granules of glycogen, *LE* lateral expansion, *M* mitochondrion, *N* nucleus, *PM* plasma membrane, *PSE* posterior spermatozoon extremity, *S1* singlets of the first axoneme, *SB* spine-like bodies.

Figure 1

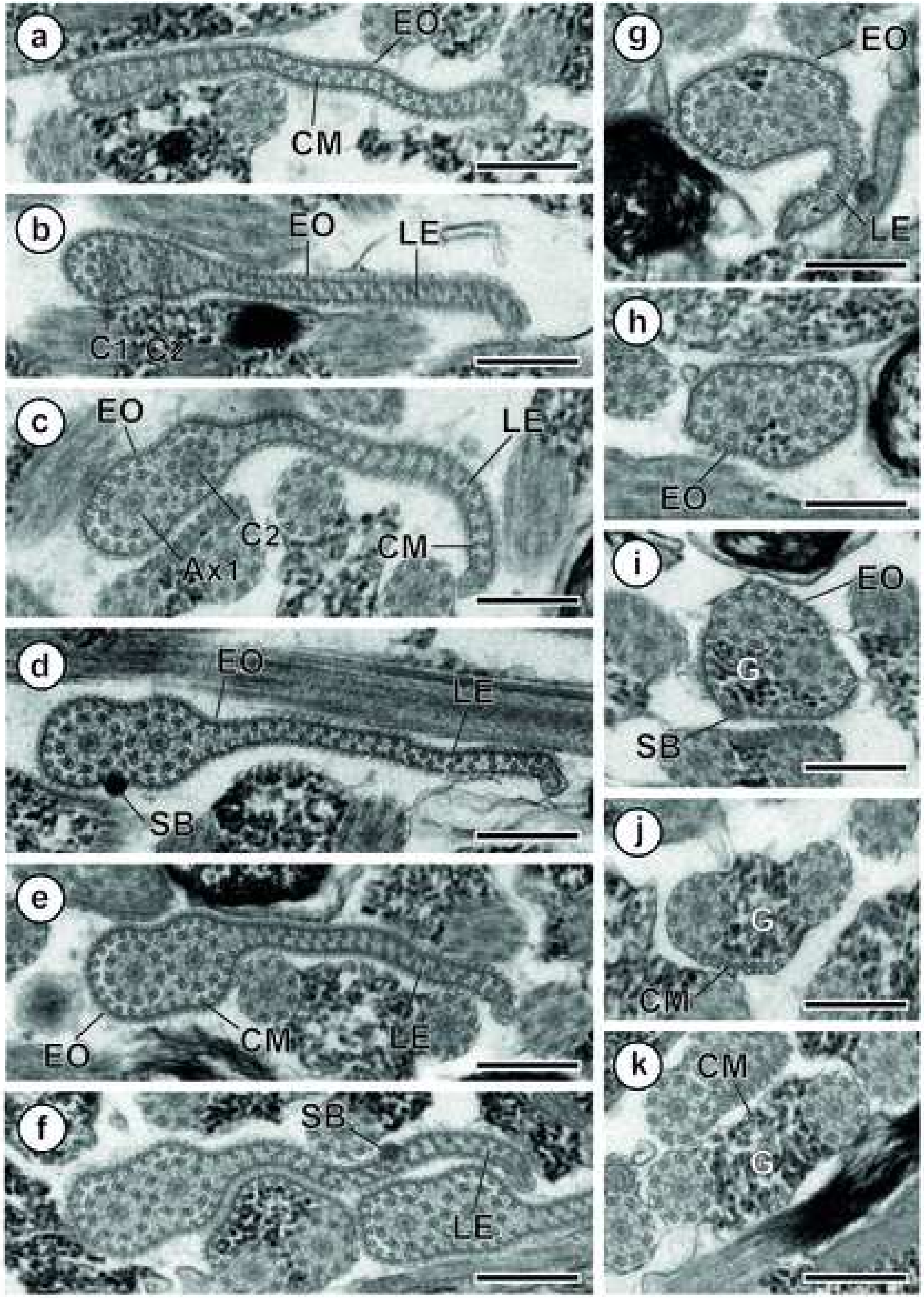
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Figure 2

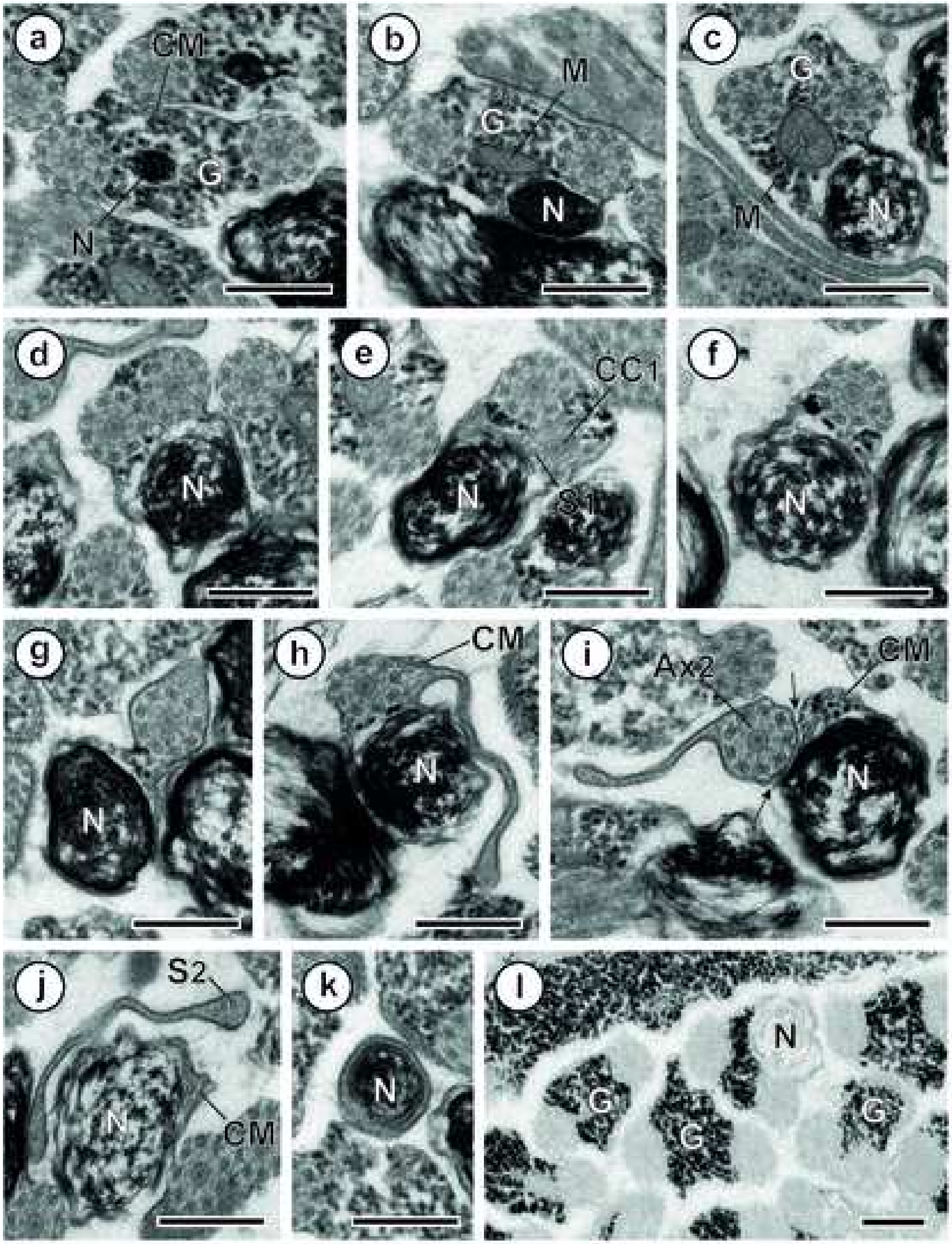
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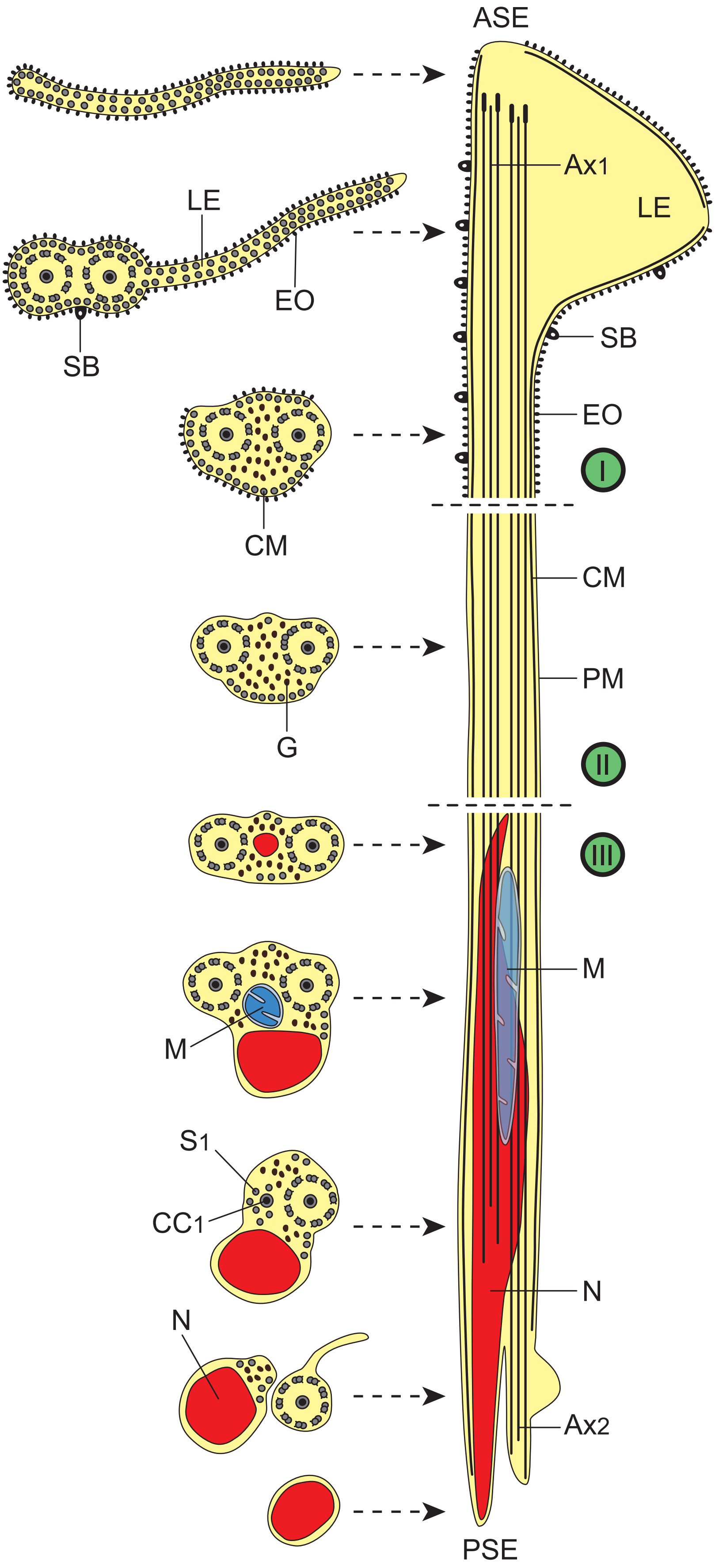
Figure 3



Table

Table I: Ultrastructural characteristics of the spermatozoon in the Paramphistomoidea

Families/Species	ASE	LE	EO	EO+CM	LEO	MCM	BCM	LMCM	M	SB	PSC	References
Cladorchiidae												
Basidiodiscus ectorchus	1Ax?	+	+	+	AntA	?	2	AntS	3	-	N?	Ashour et al. (2007)
Sandonia sudanensis	1Ax?	+	+	+	AntA	≈ 90?	2	AntS	3	-	N?	Ashour et al. (2007)
Diplodiscidae												
Diplodiscus subclavatus	1Ax	+	+	+	AntA	75	2	AntS	1	+	N	Bakhoum et al. (2011)
Diplodiscus amphichrus	2Ax-LE+CM+EO	+	+	+	AntA	63	2	AntS	1	+	N	Present study
Gastrothylacidae												
Carmyerius endopapillatus	1Ax	+	+	+	AntA	≈ 50?	2	AntS	1	+	N	Seck et al. (2008a)
Paramphistomidae												
Cotylophoron cotylophorum	1Ax	+	+	+	AntA	?	2	AntS	1	+	N	Seck et al. (2008b)
Orthocoelium scoliocoelium	?	+	+	+	AntA	?	2	AntS	1	?	1Ax?	Swarnakar (2010)
Paramphistomum microbothrium	1Ax	+	+	+	AntA	≈ 50?	2	AntS	1	+	N	Seck et al. (2007)

AntA anterior part of the anterior region, *AntS* anterior region of the spermatozoon, *ASE* anterior spermatozoon extremity, *Ax* axoneme, *BCM* number of bundles of cortical microtubules, *EO* external ornamentation of the plasma membrane, *EO+CM* association between the external ornamentation and cortical microtubules, *LE* lateral expansion, *LEO* location of the external ornamentation, *LMCM* location of maximum number of cortical microtubules, *M* number of mitochondria, *MCM* maximum number of cortical microtubules, *N* nucleus, *NA* not applicable, *PSC* posterior spermatozoon character *SB* spine-like bodies, *+/-* presence/absence of the considered character, *?* doubtful or unknown data.