Ultrastructural organisation of the spermatozoon of *Allopodocotyle tunisiensis* Derbel and Neifar, 2009 (Digenea, Opecoelidae), an intestinal parasite of *Solea aegyptiaca* Chabanaud, 1927 (Teleostei, Soleidae)

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Abstract

The ultrastructure of the spermatozoon of *Allopodocotyle tunisiensis* (Digenea, Opecoelidae), an intestinal parasite of *Solea aegyptiaca* (Teleostei, Soleidae), is described by transmission electron microscopy (TEM). The mature spermatozoon is a filiform cell that exhibits two axonemes of different length with the 9+'1' pattern of trepaxonematan Platyhelminthes. In the anterior spermatozoon extremity, cortical microtubules are absent. They appear after the disappearance of an anterior electron-dense material, being initially in a continuous and submembranous layer. They surround only partially the sperm cell. Later, these cortical microtubules are distributed into two bundles. Additionally, the spermatozoon of *A. tunisiensis* shows two mitochondria, a nucleus, an external ornamentation of the plasma membrane, spine-like bodies, and a large amount of glycogen granules. According to the location of the external ornamentation, *A. tunisiensis* presents a Quilichini et al.'s type 2 spermatozoon. With respect to the posterior extremity, the sperm cell of *A. tunisiensis* corresponds to the Quilichini et al.'s opecoelid type. The morphology of the first mitochondrion with a U-shaped posterior extremity is described for the first time in a digenean spermatozoon.

Keywords: *Allopodocotyle tunisiensis*; Opecoelidae; Digenea; Sperm characters.

1. Introduction

The Opecoelidae Ozaki, 1925 is a cosmopolitan family which occurs in marine, freshwater teleost fish and sporadically in amphibians (Cribb, 2005). It is the most rich-species of all digenean families, with over 90 genera and nearly 900 species. It currently includes six subfamilies, namely the Helicometrinae, Opecoelinae, Opecoelininae, Plagioporinae, Polypapiliotrematinae, and Stenakrinae (Bray et al., 2016; Martin et al., 2018a).

Allopodocotyle tunisiensis belongs to the subfamily Plagioporinae which is presently the largest subfamily comprising over half of currently recognised genera of family Opecoelidae (Cribb, 2005; Gibson, 2014). However, recent phylogenetic analyses have demonstrated that the Plagioporinae is polyphyletic (Bray et al., 2016; Fayton and Andres, 2016; Martin et al., 2018a, b). These analyses suggest that plagioporines should be restricted to species mainly infecting Holarctic freshwater fish as adults and potentially species known from deep-sea fish (Fayton and Andres, 2016). Most Plagioporinae s. l. mature in epipelagic marine fish and these taxa now require new subfamilial classification (Martin et al., 2018a). Due to many inconsistencies and the absence of a robustness in the existing classification, a combined analysis approaching morphology, genetic data, life-cycle and ultrastructural traits would resolve ambiguities and generate more robust phylogenetic hypotheses. In this context, the study of spermatological characteristics of species belonging to the subfamily Plagioporinae would be of great importance to provide additional information useful for clarifying the systematic status and phylogenetic relationships within opecoelids as it has been demonstrated in diverse groups of parasitic Platyhelminthes (Justine, 1991, 1995, 1998; Levron et al., 2010; Quilichini et al., 2010a, 2011a; Bakhoum et al., 2017a; Justine and Puddubnaya, 2018).

To date, ultrastructural studies of spermatozoa have been performed on nine opecoelid species belonging to three subfamilies. These are the Helicometrinae *Helicometra epinepheli* (Quilichini et al., 2011b) and *Helicometra fasciata* (Levron et al., 2003), the Opecoelinae

Opecoeloides furcatus (Miquel et al., 2000) and *Poracanthium furcatum* (Levron et al., 2004), and the Plagioporinae *Allopodocotyle pedicellata* (Bakhoum et al., 2017b), *Macvicaria obovata* (Kacem et al., 2017), *Nicolla testiobliquum* (Quilichini et al., 2007a), *Nicolla wisniewskii* (Quilichini et al., 2007b) and *Podocotyloides magnatestis* (Diagne et al., 2016).

To increase database on the ultrastructural organisation of spermatozoa in the Opecoelidae, the present study provides for the first time a description of the sperm cell of *A. tunisiensis*. Our results are also compared with the available ultrastructural studies on digenean spermatology, in particular with those species belonging to Opecoelidae family in order to highlight the possible criteria useful for phylogeny.

2. Materials and methods

Specimens of *Allopodocotyle tunisiensis* Derbel and Neifar, 2009 were collected alive from the digestive tract of the Egyptian sole *Solea aegyptiaca* Chabanaud, 1927 (Teleostei, Soleidae), caught in the Gulf of Gabès off Chebba (Tunisia) during May 2018. Adult digeneans were rinsed with a 0.9% NaCl solution and fixed in cold (4 °C) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4, rinsed in 0.1 M 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, dehydrated in ethanol series and propylene oxide, embedded in Spurr resin and polymerized at 60 °C for 72 h. Ultrathin sections (60–90 nm) of the seminal vesicle were cut on a Reichert-Jung Ultracut E ultramicrotome. Several sections placed on 200 mesh copper grids were stained with uranyl acetate and lead citrate according to Reynolds' methodology (1963). Ultrathin sections placed on gold grids were stained with periodic acid, thiocarbohydrazide, and silver proteinate according the cytochemical technique established by Thiéry (1967) for evidencing granules of glycogen. Finally, all stained grids were examined on

a Jeol 1010 transmission electron microscope, operating at an accelerating voltage of 80 kV, in the "Centres Científics i Tecnològics" of the University of Barcelona (CCiTUB).

3. Results

The observation and the interpretation of numerous ultrathin cross- and longitudinal sections in the seminal vesicle of *A. tunisiensis* allow us the distinction of three different regions (I–III) from the anterior to the posterior extremities of the male gamete (Figs. 1-4). The sperm cell of *A. tunisiensis* exhibits the usual structures found in most of digeneans. Indeed, it contains two axonemes of the 9+'1' trepaxonematan pattern, external ornamentation of the plasma membrane, spine-like bodies, nucleus, mitochondrion, two bundles of parallel cortical microtubules, and granules of glycogen.

Region I (Figs. 1a–k, and 4I) corresponds to the anterior spermatozoon extremity of *A. tunisiensis*. The anterior tip forms a sharp point (Fig. 1a). The anterior extremity is characterized by the presence of one axoneme of the 9+'1' trepaxonematan pattern and an electron-dense material that persists in this region until the appearance of singlets of the second axoneme (Figs. 1b–d and 4I). When both axonemes are present, cross-sections show about 23 cortical microtubules surrounding the second axoneme (Fig. 1e). This is the maximum number of cortical microtubules in the sperm cell before their distribution into two bundles (Figs. 1f–h and 4I). In the middle part of this region, the number of cortical microtubules decreases and gets organised into two fields with around 13 (7+6) microtubules surrounding partially the second axoneme (Fig. 1f, g). In the posterior part of region I, cross-sections show the presence of the first mitochondrion with a U-shaped posterior extremity (Figs. 1h–k and 4I). Moreover, this area exhibits spine-like bodies and external ornamentation of the plasma membrane, located on the ventral side of the sperm cell or mitochondrial side, and associated with cortical microtubules (Figs. 1h–j and 4I). The number of cortical microtubules reduces progressively

from 12 (6+6) to 7 (1+6) (Fig. 1h–j). The transition toward region II is marked by the disappearance of the first mitochondrion, spine-like bodies, and the external ornamentation.

Region II (Figs. 2a–d, and 4II) corresponds to the middle part of the mature spermatozoon. It is a transitional area located in front of the nuclear region. There are two axonemes in its proximal part and parallel cortical microtubules arranged into two bundles (Figs. 2a, b and 4II). The distal part of region II shows the second mitochondrion (Figs. 2c, d and 4II). It is interesting to note the increase of cortical microtubules from 11 (2 + 9) (Fig. 2a) to 22 (10 + 12) (Fig. 2d).

Region III (Figs. 3a–j, and 4III) corresponds to the nuclear region and posterior spermatozoon extremity. In its proximal part, we notice the appearance of the nucleus with the simultaneous presence of the second mitochondrion, two axonemes, granules of glycogen, and cortical microtubules (Figs. 3a, b and 4III). Later, the nucleus increases its size and it is interesting to note a variability in the disappearance of the second mitochondrion; it disappears at a different level, before and after the disorganisation of the first axoneme (Figs. 3c–f and 4III). Posteriorly, the second axoneme disorganises (Fig. 3g), the nucleus progressively reduces its size and disappears (Fig. 3h, i) and the number of cortical microtubules also decreases. Finally, only a few granules of glycogen and cortical microtubules are present in the posterior tip of the male gamete (Fig. 3i, j).

The granules of glycogen are irregularly distributed along the mature spermatozoon from the first mitochondrial zone to the posterior spermatozoon tip. This granular material is evidenced as glycogen by means of the cytochemical test of Thiéry (Fig. 2e).

4. Discussion

4.1. General characters in the Digenea spermatozoon

The mature spermatozoa of *A. tunisiensis* are filiform cells tapered at both ends, and show the usual characteristics of most digeneans. Thus, several aspects concerning characters such as axonemes, cortical microtubules, mitochondrion, external ornamentation, spine-like bodies, and morphology of both sperm extremities are briefly discussed below.

4.1.1. Anterior extremity: anterior dense material

The anterior extremity of the mature spermatozoon of *A. tunisiensis* presenting only the first axoneme exhibits a continuous and submembraneous layer of an electron-dense material. This electron-dense material is located on the opposite side of the axoneme and disappears when the second axoneme is already present. A similar organisation is reported in numerous digenean species, particularly those belonging to the family Opecoelidae, namely *A. pedicellata*, *H. epinepheli*, *H. fasciata*, *M. obovata*, *Pod. magnatestis* and *Por. furcatum* (Levron et al., 2003, 2004; Quilichini et al., 2011b; Diagne et al., 2016; Bakhoum et al., 2017b; Kacem et al., 2017). In contrast, other opecoelids do not exhibit any electron-dense material in the anterior extremity of the sperm cell. Thus, according (Quilichini et al., 2007a, b), spermatozoa of *Nicolla* species exhibit two axonemes in their anterior extremities. However, it is important to remark that sections showing centriole/s from axoneme/s are not shown and the presence of two axonemes in this extremity of gamete should be considered with caution. On the other hand, Miquel et al. (2000) described in *O. furcatus* an anterior extremity constituted by only the first axoneme.

4.1.2. Axonemes

The structure of axonemes with 9+'1' trepaxonematan pattern constituted by nine peripheral doublets of microtubules disposed around a central core (Ehlers, 1984) has been observed in the spermatozoon of *A. tunisiensis* as in all digeneans, except for the *Schistosoma*

species with a special 9+'1' pattern (Justine et al., 1993; Jamieson and Justine, 2017) and *Didymozoon* species with a 9+0 pattern (Justine and Mattei, 1983).

4.1.3. Cortical microtubules

The presence of cortical microtubules, their parallel disposition with respect to the hypothetical longitudinal axis of the spermatozoon, the location of their maximum number, and the number of bundles are considered as interesting criteria used for phylogenetic inference. These parallel tubular structures underlying the plasma membrane have been described in most mature sperm of parasitic flatworms such as digeneans (except *Didymocystis* and *Didymozoon* species which have no cortical microtubules -see Justine and Mattei, 1983; Pamplona-Basilio et al., 2001) and monogeneans (except in certain monopisthocotyleans -see Justine et al., 1985; Justine, 1991). In cestodes, cortical microtubules are also present in a parallel disposition except for the tetrabothiideans and cyclophyllideans (excluding mesocestoidids), which have spiralled cortical microtubules (Stoitsova et al., 1995; Miquel et al., 1999, 2007).

On the other hand, when present, the arrangement of cortical microtubules is usually in two bundles in most digeneans as occurs in *A. tunisiensis* and also in the remaining studied opecoelideans (see Table 1). However, only one field has been described in some species belonging to three different families of the Hemiuroidea (Quilichini et al., 2010b; Ndiaye et al., 2013; Dione et al., 2016).

Another interesting character of great phylogenetic interest is the location of the maximum number of cortical microtubules along the spermatozoon. Indeed, based on this last character, Quilichini et al. (2007c) and Bakhoum et al. (2017a) proposed that the spermatozoon of digenean parasites could be divided into two groups: (i) a first one in which maximum number is located in the anterior part of the spermatozoon and (ii) a second one with the maximum number located in a middle or more posterior part of the spermatozoon. In all

opecoelids studied until now, the maximum number of cortical microtubules is located in the middle region of the sperm cell except for *M. obovata* that exhibits this maximum number in a very posterior region, after the disappearance of the two axonemes and near the posterior extremity of the spermatozoon (Kacem et al., 2017), and *Allopodocotyle* species that present the maximum number in the anterior part of the spermatozoon (Bakhoum et al., 2017b and present study).

4.1.4. Mitochondrial region: mitochondrion, external ornamentation and spine-like bodies

Many spermatological characters in the mitochondrial region may provide some potential information in phylogenetic inference. These are: (i) the number, the morphology and the location of mitochondria, (ii) the presence/absence of spine-like bodies, and (iii) the external ornamentation of the plasma membrane, its location and its association or not with cortical microtubules. The mature male gamete of *A. tunisiensis* contains two mitochondria. The first mitochondrion is located at the level of the external ornamentation of the plasma membrane while the second one is located in the middle region of the sperm cell and it usually overlaps with the anterior part of the nucleus. Species of the family Opecoelidae exhibit a different number of mitochondria. One mitochondrion has been described in *H. fasciata* (Levron et al., 2003) and *O. furcatus* (Miquel *et al.* 2000), while the remaining opecoelids exhibit two mitochondria in their spermatozoa (see Table 1). It is interesting to note that recent spermatological studies have highlighted the variability in the morphology of the first mitochondrion according to species. In fact, a moniliform mitochondrion, which has been described for the first time in *Holorchis micracanthum* by Bâ et al. (2011), has been reported in the male gamete of some digeneans such as *Aphallus tubarium* (Foata et al., 2012), *Stephanostomoides tenuis* (Bakhoum et al., 2015), *M. obovata* (Kacem et al., 2017) or *Opechona bacillaris* (Ndiaye et al., 2015) and the opecoelid *A. pedicellata* (Bakhoum et al.,

2017b). In the present study, we describe for the first time another morphological type observed for the first mitochondrion, characterised by the presence of a circular fold in its posterior part making a characteristic U-shaped posterior extremity. Concerning the second mitochondrion, it is also interesting to remark the existing variability in the level of its disappearance. Thus, in *A. tunisiensis*, the end of the second mitochondrion occurs both before and after the disorganisation of the first axoneme.

The presence/absence and location of the external ornamentation of the plasma membrane along the mature spermatozoon and its association or not with cortical microtubules are considered as other interesting criteria used for phylogenetic purposes in the Digenea and of particular interest for the establishment of spermatozoa models (Bakhoum et al., 2017a; Quilichini et al., 2011a). The sperm cell of *A. tunisiensis* displays the association of external ornamentation of the plasma membrane and cortical microtubules located in the anterior area of the spermatozoon and placed only in the mitochondrial side of gamete, corresponding to the side with a high number of cortical microtubules as occurs in most opecoelids, namely *A. pedicellata*, *H. epinepheli*, *M. obovata*, *N. testiobliquum*, *N. wisniewskii*, *O. furcatus*, *Pod. magnatestis*, *Por. furcatum* and *M. obovata* (Miquel et al., 2000; Levron et al., 2004; Quilichini et al., 2007a, 2007b, 2011b; Diagne et al., 2016; Bakhoum et al., 2017b; Kacem et al., 2017). Among the Opecoelidae, only sperm cells *H. fasciata* lack ornamentation (Levron et al., 2003).

The spine-like bodies, which consist of a submembraneous and prominent electron-dense structures that seem to contain a spherical vesicle, are considered as another particularity observed in the anterior part of the spermatozoon. Since his first description in the opecoelid *O. furcatus* by Miquel et al. (2000), the presence of spine-like bodies has been reported in numerous digeneans (for a review see Bakhoum et al. 2017a). In *A. tunisiensis* and in most opecoelids described so far, spine-like bodies are present and located in the ornamented area

(see Table 1). The sole exception within representatives of this family is *H. fasciata*, which lacks spine-like bodies in their male gametes (Levron et al., 2003).

4.1.5. Posterior spermatozoon extremity

The morphology of the posterior spermatozoon tip is another crucial characteristic for the establishment of sperm models in digeneans. Quilichini et al. (2010a) have established three types of posterior spermatozoon extremities (Opecoelidean or type 1, Fasciolidean or type 2 and Cryptogonimidean or type 3) according to the succession of characters towards the posterior tip. In *A. tunisiensis* the posterior extremity of the male gamete corresponds to the type 1 or Opecoelidean type characterised by the following sequence: "posterior axoneme extremity", "posterior nucleus extremity" and "cortical microtubules". This succession of ending characters has been reported in mature spermatozoa of all opecoelids studied until now, with the sole exception of *Pod. magnatestis* (Diagne et al., 2016), which exhibits the type 2 or Fasciolidean type (see Table 1).

4.2. Concluding remarks: typical characters and spermatozoon model in the Opecoelidae

Table 1 shows the most interesting characters that exhibit spermatozoa of the representatives of the family Opecoelidae studied up to date. The sperm cell structure and organisation are considerably homogeneous in opecoelids. Concerning the external ornamentation, except *H. fasciata* (Levron et al., 2003), all the species follow the pattern 2 of Quilichini et al. (2011a). As for the posterior extremity, except *Pod. magnatestis* (Diagne et al., 2016), the remaining studied species exhibit the Opecoelid type of posterior extremity stablished by Quilichini et al. (2010a), being cortical microtubules the posterior spermatozoon character. Other discrepancies concern the anterior dense material; three species, namely *N. testiobliquum*, *N. wisniewski* and *O. furcatus* lack this character (Miquel et al., 2000; Quilichini

et al., 2007a, b). Thus, diverse characters can be remarked as constitutive of the usual ultrastructural organisation of the opecoelid sperm cell. These are the 9+'1' type of axonemes, the anterior dense material, the absence of lateral expansion, the presence of an external ornamentation of the plasma membrane associated with cortical microtubules and placed in the posterior area of the anterior spermatozoon region, the maximum number of cortical microtubules in a median region of the spermatozoon, the presence of spine-like bodies, two mitochondria, and cortical microtubules as a posterior spermatozoon character.

Some of these characters might be useful for phylogenetic purposes. Bakhoum et al. (2017a) proposed diverse models for spermatozoa of digeneans and the above-mentioned characteristics allowed them to stablish the model III as the typical type of sperm cell in the Opecoelidae. However, species of *Allopodocotyle* (Bakhoum et al., 2017b; present study) do not follow this pattern due to the presence of the maximum number of cortical microtubules in a median region of spermatozoon. Their gamete should be included in type IV.

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Conflict of interest

The authors declare that they have no conflict of interest.

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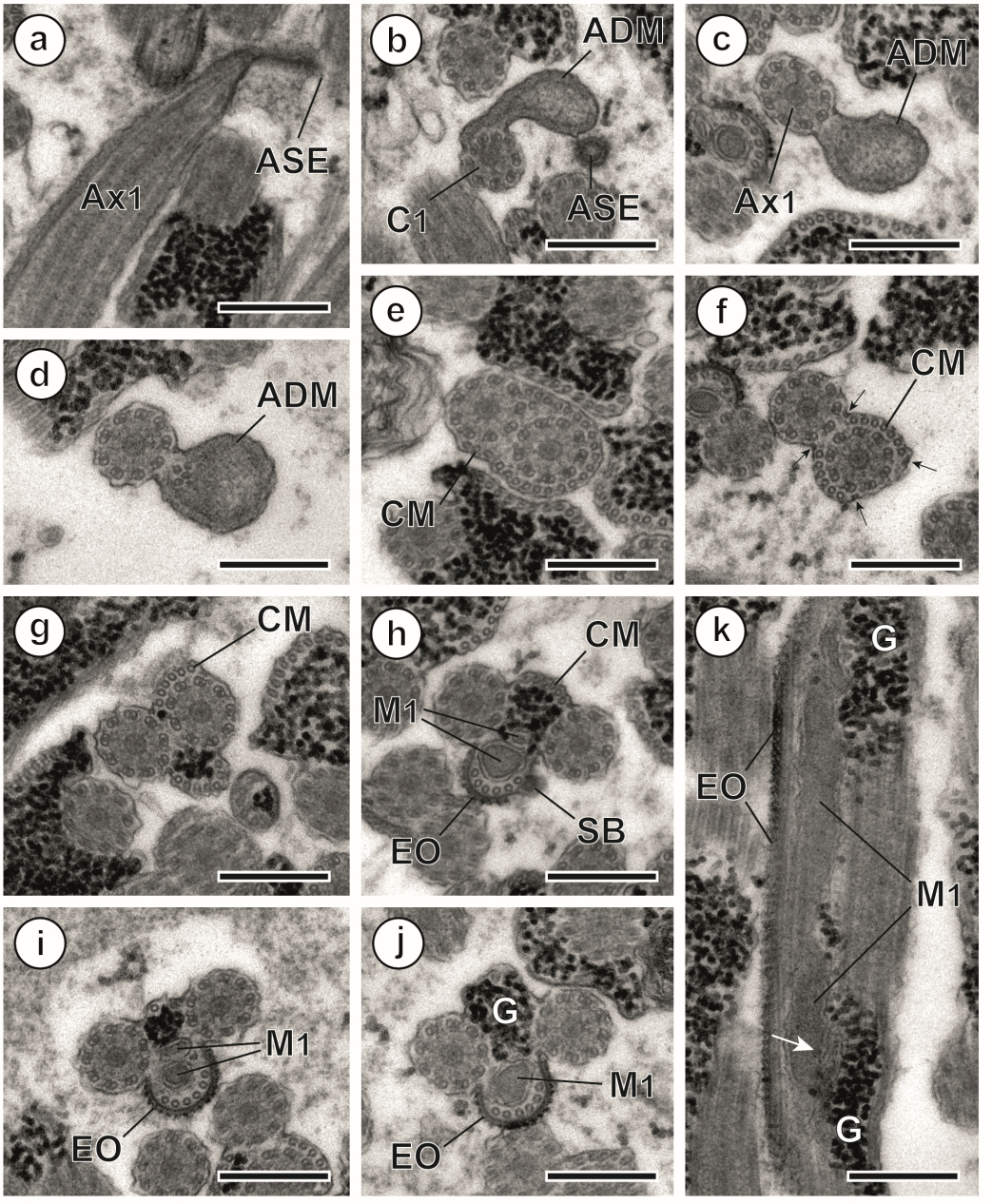
Legends to figures

Fig. 1. Region I or anterior region of the spermatozoon of *Allopodocotyle tunisiensis*: (a) longitudinal section in the anterior tip showing a sharp point; (b–j) consecutive cross-sections in the anterior region of the spermatozoon showing (1b, c) the first axoneme and (1d) the appearance of singlets of the second axoneme. Note the presence of an anterior electron-dense material at this level. In the posterior part of region I it is remarkable the presence of the first mitochondrion and the external ornamentation associated with cortical microtubules and spine-like bodies (1h–j); (k) longitudinal section showing the first mitochondrion with an U-shaped posterior extremity. ADM, anterior electron-dense material; ASE, anterior spermatozoon extremity; Ax1, first axoneme; Ax2, second axoneme; C1, centriole of the first axoneme; CM, cortical microtubules; EO, external ornamentation of the plasma membrane; G, granules of glycogen; M1, first mitochondrion; SB, spine-like body. Scale bars = 300 nm.

Fig. 2. Region II or middle region of spermatozoon of *Allopodocotyle tunisiensis*: (a, b) cross-sections in the proximal part; (c, d) cross-sections in the distal part showing the second mitochondrion; (e) granules of glycogen evidenced by the cytochemical test of Thiéry in different regions of the sperm cell. CM, cortical microtubules; G, granules of glycogen; M1, first mitochondrion; M2, second mitochondrion; N, nucleus; R-I, anterior spermatozoon region; R-II, middle spermatozoon region; R-III, posterior spermatozoon region or nuclear region. Scale bars = 300 nm.

Fig. 3. Region II, nuclear or posterior region of the spermatozoon of *Allopodocotyle tunisiensis*: (a-j) Consecutive cross-sections in the nuclear region from the anterior part with the simultaneous presence of the nucleus and the second mitochondrion (3a, b) to the posterior tip showing only granules of glycogen and cortical microtubules (3i, j). CC1, central core of the first axoneme; C2, centriole of the second axoneme; CM, cortical microtubules; D2, doublets of the second axoneme; G, granules of glycogen; M2, second mitochondrion; N, nucleus; S1, singlets of the first axoneme. Scale bars = 300 nm.

Fig. 4. Schematic organisation of the spermatozoon of *Allopodocotyle tunisiensis*. The sperm cell is organised in three different regions: region I or anterior part, region II or middle part and region III or posterior part. In order to make the diagram clearer, granules of glycogen are not shown in longitudinal sections. The use of a continuous line or a discontinuous line for the second mitochondrion would to represent the existing variability at the level of its disappearance. ADM, anterior electron-dense material; ASE, anterior spermatozoon extremity; Ax1, first axoneme; Ax2, second axoneme; C1, centriole of the first axoneme; C2, centriole of the second axoneme; CM, cortical microtubules; D2, doublets of the second axoneme; EO, external ornamentation of the plasma membrane; G, granules of glycogen; M1, first mitochondrion; M2, second mitochondrion; N, nucleus; PM, plasma membrane; PSE, posterior spermatozoon extremity; S1, singlets of the first axoneme; SB, spine-like body.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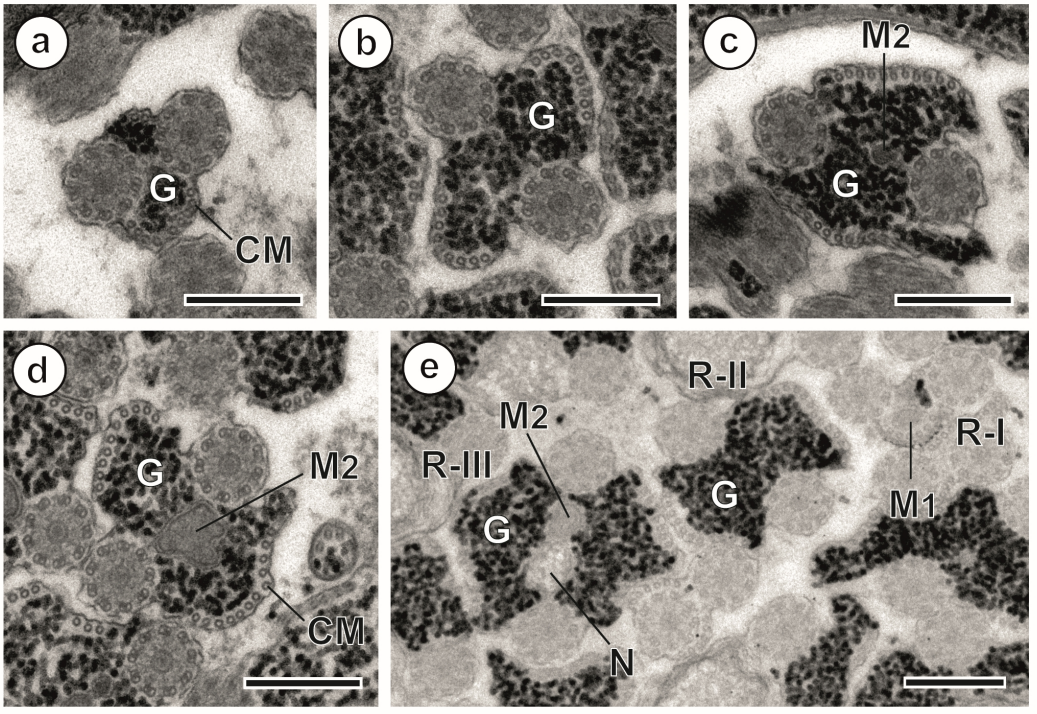
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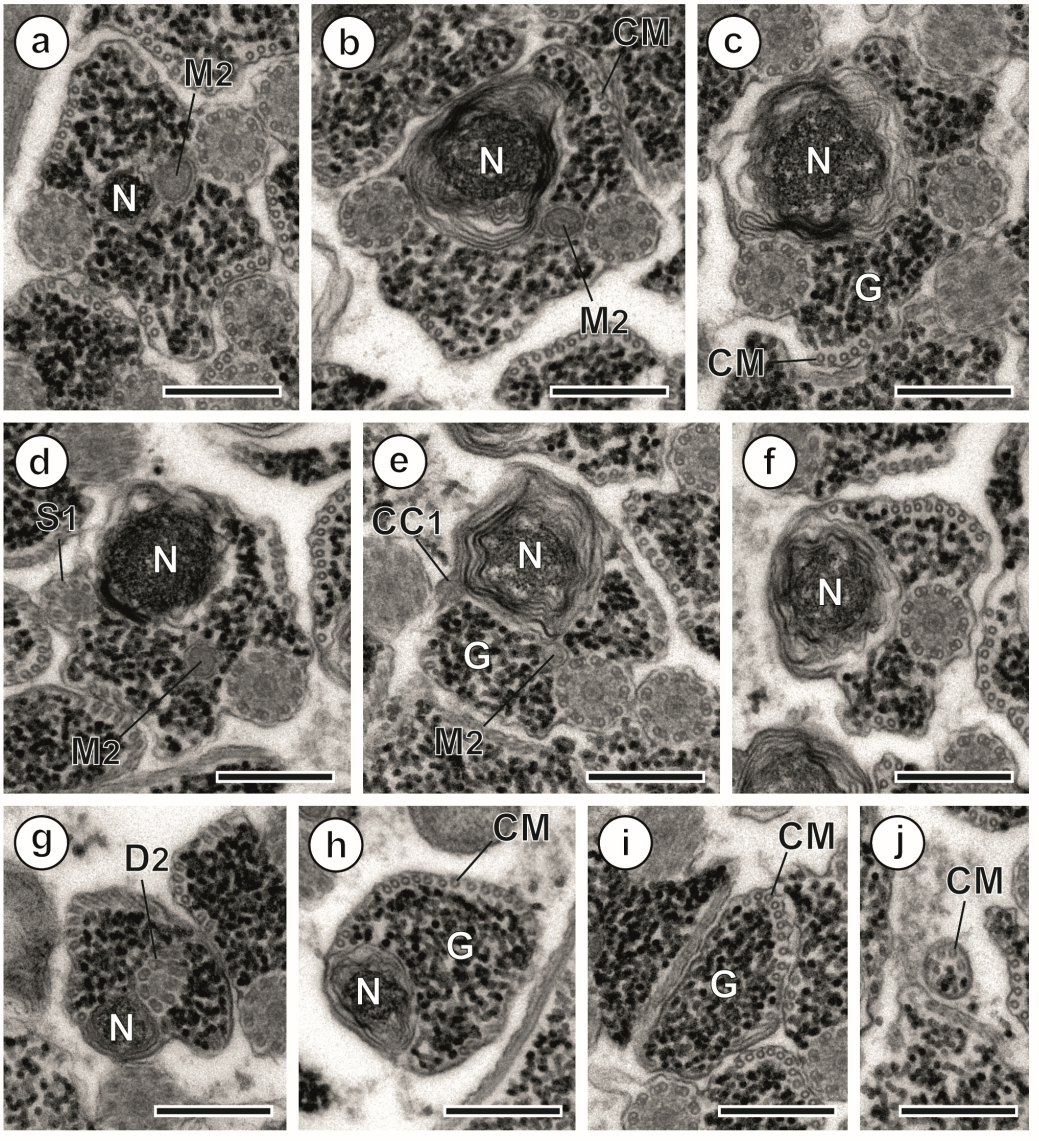
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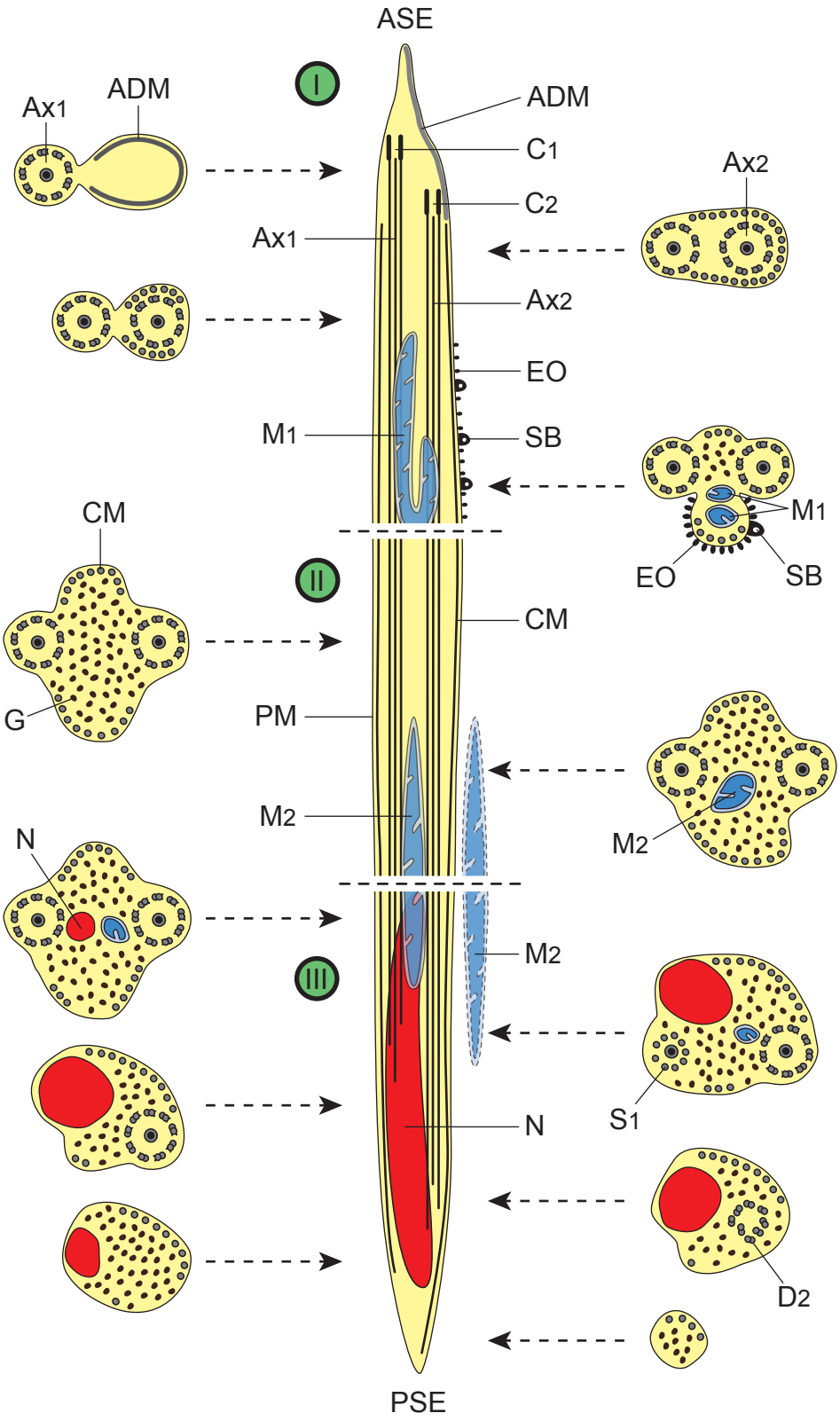
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PM
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D2
PSE

Table I. Spermatological characters in the Opecoelidae.

Subfamilies and species	Principal characters								Secondary characters			References
	TAx	LE	EO	EO+CM	LEO	BCM	LMCM	M	ADM	SB	PSC	
HELICOMETRINAE												
Helicometra epinepheli	9+'1'	-	+	+	PostA	2	MedS	2	+	+	CM	Quilichini et al. (2011b)
Helicometra fasciata	9+'1'	-	-	NA	NA	2	MedS	1	+	-	CM	Levron et al. (2003)
OPECOELINAE												
Opecoeloides furcatus	9+'1'	-	+	+	PostA	2	MedS	1	-	+	CM	Miquel et al. (2000)
Poracanthium furcatum	9+'1'	-	+	+	PostA	2	MedS	2	+	+	CM	Levron et al. (2004)
PLAGIOPORINAE												
Allopodocotyle pedicellata	9+'1'	-	+	+	PostA	2	AntS	2	+	+	CM	Bakhoum et al. (2017b)
Allopodocotyle tunisiensis	9+'1'	-	+	+	PostA	2	AntS	2	+	+	CM	Present study
Macvicaria obovata	9+'1'	-	+	+	PostA	2	PostS	2	+	+	CM	Kacem et al. (2017)
Nicolla testiobliquum	9+'1'	-	+	+	PostA	2	MedS	2	-	+	CM	Quilichini et al. (2007a)
Nicolla wisniewski	9+'1'	-	+	+	PostA	2	MedS	2	-	+	CM	Quilichini et al. (2007b)
Podocotyloides magnatestis	9+'1'	-	+	+	PostA	2	MedS	2	+	+	N	Diagne et al. (2016)

ADM, anterior electron-dense material; AntS, anterior region of the spermatozoon; Ax, axoneme; BCM, number of bundles of cortical microtubules; CM, cortical microtubules; EO, external ornamentation of the plasma membrane; EO+CM, association 'external ornamentation-cortical microtubules'; LE, lateral expansion; LEO, location of external ornamentation; LMCM, location of maximum number of cortical microtubules; M, number of mitochondria; MedS, median region of the spermatozoon; N, nucleus; NA, not applicable; PostA, posterior part of anterior region; PostS, posterior region of the spermatozoon; PSC, posterior spermatozoon character; SB, spine-like bodies; TAx, type of axoneme; +/-, presence/absence of considered character; ?, doubtful or unknown data.

AUTHORS AGREEMENT

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