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# Spermatological characteristics of *Siphoderina aloysiae* (Digenea, Cryptogonimidae), an intestinal parasite of *Sciaena umbra* (Teleostei: Sciaenidae)

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## Abstract

The ultrastructural organization of the spermatozoon of *Siphoderina aloysiae* (Digenea, Cryptogonimidae) is described based upon examination by transmission electron microscopy (TEM). Live digeneans were collected from the digestive tract of Brown meagre *Sciaena umbra* (Teleostei: Sciaenidae), caught off the Gulf of Gabès in La Chebba (Tunisia). The male gamete of *S. aloysiae* is a filiform cell, tapered at both extremities, and exhibits typical characteristics of digenean sperm such as two axonemes showing the 9+'1' trepaxonematan pattern with different lengths. The first axoneme is disorganised and disappears near the anterior nuclear extremity. However, the second axoneme reaches the posterior extremity of the spermatozoon. The latter also contains a nucleus, two bundles of parallel cortical microtubules, external ornamentation of the plasma membrane, spine-like bodies and granules of glycogen. Cortical microtubules are absent in the anterior extremity of the spermatozoon and appear after the disappearance of the electron-dense material. In addition, the male gamete of *S. aloysiae* has two mitochondria. The first mitochondrion is associated with the external ornamentation of the plasma membrane and spine-like bodies. The second is more posteriorly located at the nuclear region after the disappearance of the first axoneme. The present study provides new data on the mature male gamete of *S. aloysiae* that may be useful for the understanding of digenean relationships and phylogenetic studies.

**Key words:** *Siphoderina aloysiae*, Cryptogonimidae, Digenea, ultrastructure, spermatozoon.

## Introduction

The superfamily Opisthorchioidea contains three families, the Cryptogonimidae, Opisthorchiidae and Heterophyidae. The Cryptogonimidae is a large and cosmopolitan family currently including over 93 genera and 370 species. Cryptogonimids inhabit the intestine or pyloric caeca of a large variety of poikilothermic vertebrates, both in fresh and marine waters (Miller and Cribb 2008a; Martínez-Aquino et al. 2017; WoRMS 2018). The Cryptogonimidae consists of 19 subfamilies, among them the subfamily Siphoderinae. The genus *Siphoderina* is currently considered as the largest group in the Cryptogonimidae family with 43 species, among them *Siphoderina aloysiae,* which has been reported worldwide (Miller and Cribb 2008b).

Miller and Cribb (2008a) were not convinced by the morphological characteristics used to justify subfamily-level divisions in the Cryptogonimidae, because several subfamilies were separated by few and often trivial characters. Due to many inconsistencies and the absence of a robustness in the existing classification of cryptogonimids, it would be important to combine data such as ultrastructure with morphological characters and molecular analyses for a better knowledge of relationships within this family of digeneans. It has been demonstrated that ultrastructural study of the mature spermatozoon is considered as a valuable tool in assessing phylogenetic and taxonomic problems of parasitic Platyhelminthes (Justine 1991a, 1998, 2001; Levron et al. 2010; Quilichini et al. 2010a, 2011; Bakhoum et al. 2017).

Ultrastructural studies of spermatozoa have been carried out on five species belonging to the superfamily Opisthorchioidea namely *Adlardia novaecaledoniae* (published under the name *Siphoderina elongata* –see Miller et al. 2009; Quilichini et al. 2009), *Anisocoelium capitellatum* (Ternengo et al. 2009), *Aphallus tubarium* (Foata et al. 2012), *Stemmatostoma pearsoni* (published under the name *Neochasmus* sp. –see Cribb 1986; Jamieson and Daddow 1982) and *Timoniella imbutiforme* (Kacem et al. 2017).

The present study brings for the first time a complete description of the ultrastructure of the mature spermatozoon of *S. aloysiae*. Our results are also compared with the available data on digenean spermatology, in particular with species belonging to the superfamily Opisthorchioidea in order to highlight the potential criteria useful for phylogeny.

## Materials and methods

Live adult specimens of *Siphoderina aloysiae* (Stossich, 1885) were collected during November 2015 and December 2016 from the digestive tract of Brown meagre *Sciaena umbra* Linnaeus, 1758 captured in the Mediterranean Sea, off La Chebba (34°14′N, 11°06'E) (Tunisia).

After their extraction, adult worms were immediately rinsed with a 0.9% NaCl solution and fixed in cold (4 ˚C) 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.4 for a minimum of 2 h, rinsed in a 0.1 M sodium cacodylate buffer at pH 7.4. They were then postfixed 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 resin and finally polymerized at 60 ˚C for 72 h. Ultrathin sections were obtained using a Reichert-Jung Ultracut-E ultramicrotome, placed on copper grids and double-stained with uranyl acetate and lead citrate according to Reynolds (1963) methodology. Finally, all stained grids were studied with a JEOL 1010 transmission electron microscope operated at 80 kV, in the ‘Centres Científics i Tecnològics de la Universitat de Barcelona (CCiTUB)’.

The Thiéry (1967) technique was used to locate glycogen. Gold grids were treated in periodic acid, thiocarbohydrazide and silver proteinate (PA-TCH-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.

## Results

The observations of numerous ultrathin sections in the seminal vesicle of *S. aloysiae* allow us to distinguish three regions (I–III) from the anterior to the posterior extremities of the male gamete (Figs. 1-4). The mature spermatozoon of *S. aloysiae* exhibits two axonemes of the 9+‘1’ trepaxonematan pattern, external ornamentation of the plasma membrane, spine-like bodies, nucleus, two mitochondria, two bundles of parallel cortical microtubules and granules of glycogen.

Region I (Figs. 1a-j and 4I) corresponds to the anterior region of the spermatozoon. The anterior tip forms a sharp point devoid of axonemes, exhibiting a submembranous and moderately electron-dense material (Figs. 1a and 4I). This anterior electron-dense material is present until the region where the two axonemes are still formed and is observed as a submembranous layer surrounding only the second axoneme (Figs. 1b, c and 4I). The two 9+'1' axonemes are slightly longitudinally displaced (Figs. 1b, c and 4I). In the middle part of this region, cortical microtubules appear as a continuous and submembranous layer composed of a maximum number of around 16 microtubules (Figs. 1e, 4I). When the four attachment zones appear, cortical microtubules become organized into two fields with a maximum of about 3+10 microtubules (Figs. 1f and 4I). In the distal part of the region I, the first mitochondrion appears and also an external ornamentation of the plasma membrane is observed in association with cortical microtubules and spine-like bodies (Figs. 1g-j and 4I). Moreover, also noticeable is the presence of granules of glycogen along this posterior part (Figs. 1i, j and 4I). The posterior extremity of the first mitochondrion marks the transition between regions I and II.

Region II (Figs. 1k and 4II) corresponds to the middle region of the mature spermatozoon. It is a transitional area anterior to the nuclear region. This region shows the simultaneous presence of two axonemes, two bundles of about 8+6 cortical microtubules and a large amount of glycogen (Figs. 1k and 4II).

Region III (Figs. 2a-l and 4III) corresponds to the nuclear and posterior spermatozoon region. In its proximal part, the nucleus presents a reduced section and is located between the two axonemes, granules of glycogen are abundant, and the two bundles of cortical microtubules are constituted by 8+6 elements (Figs. 2a and 4III). At a slightly distal level, the first axoneme initiates its disorganization and the central core remains present after the disappearance of doublets (Figs. 2b, c and 4III). When the first axoneme has disappeared, the number of cortical microtubules increases to 9+7 microtubules (Fig. 2d). Towards the posterior part of region III, the transition of characters is as follows: (i) appearance of the second mitochondrion and increase in the number of cortical microtubules (9+8) (Fig. 2e), (ii) disappearance of the second mitochondrion and progressive reduction in the number of microtubules (4+2; 3+0; 0+0) (Figs. 2g-i and 4III), (iii) disorganisation of the second axoneme (Figs. 2k, l and 4III), and finally (iv) disappearance of nucleus (Figs. 2l and 4 III). However, it is interesting to note that axonemal doublets remain present until the posterior spermatozoon tip (Figs. 2l and 4III).

The glycogenic nature of the granular material observed along the sperm cell was determined using the test of Thiéry (Fig. 3).

## Discussion

The mature spermatozoon of *S. aloysiae* presents the usual ultrastructural features described in some digeneans: two 9+'1' axonemes, nucleus, two mitochondria, granules of glycogen, external ornamentation of the plasma membrane, two bundles of parallel cortical microtubules, spine-like bodies in the ornamented area of the sperm cell and the anterior electron-dense material. Some of these characters as well as the morphology of both extremities could be potential candidates for phylogenetic analysis.

### Anterior spermatozoon region

Cross-sections in the anterior region of the *S. aloysiae* spermatozoon show a number of characters potentially useful for phylogenetic inference, arranged into two sets: principal and secondary characters. The principal characters are the type of axonemes, the location of the maximum number of cortical microtubules and their number of bundles. At the present state of knowledge, the secondary characters such as the anterior electron-dense material have not currently a clear usefulness (see Bakhoum et al. 2017).

The spermatozoon of *S. aloysiae* contains two axonemes exhibiting the 9+'1' trepaxonematan pattern constituted by nine peripheral doublets of microtubules disposed around a central core (Ehlers, 1984). This type of axonemes has been observed in all digeneans except for the species of *Schistosoma* genus 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).

All the studied cryptogonimids, namely *Adlardia novaecaledoniae*, *Anisocoelium capitellatum*, *Aphallus tubarium* and *Timoniella imbutiforme*, have a different length of axonemes. One of them disorganises and disappears at the level of the very anterior part of the nuclear region (Quilichini et al. 2009; Ternengo et al. 2009; Foata et al. 2012, Kacem et al. 2017). This is also the case for *S. aloysiae* showing the disorganisation of the first axoneme in the pre-nuclear region of the sperm cell, while the second axoneme reaches the posterior extremity of the spermatozoon. On the other hand, the remaining species belonging to the Heterophyidae (*Euryhelmis squamula*) and the Opisthorchiidae (*Opisthorchis felineus* and *Opisthorchis viverrini*) exhibit this disorganisation in the pre-nuclear region or at the very anterior end of the nuclear region of their spermatozoa (Bakhoum et al. 2009; Zhukova et al. 2014; Miquel et al. 2017).

It has been proposed that digenean parasites could be divided into two groups based on the location of the maximum number of cortical microtubules along the spermatozoon

(Quilichini et al. 2007; Bakhoum et al. 2017). This maximum number is located in the anterior part of the spermatozoon for the first group and in the middle or the more posterior part of the spermatozoon for the second one.

The maximum number of cortical microtubules of the cryptogonimids spermatozoa studied to date occurs in the middle region of the male gamete, except for *T. imbutiforme* and *S. aloysiae,* whose maximum number is located in the anterior part of the spermatozoon, which is also the case for heterophyids and opisthorchiids.

The number of bundles that cortical microtubules present along the mature spermatozoon is another crucial characteristic for the establishment of sperm models in digeneans (Bakhoum et al. 2017). Most digeneans exhibit an arrangement of cortical microtubules into two fields, as occurs in *S. aloysiae* as well as in the previously studied species belonging to the Opisthorchioidea. Other digeneans, mainly species belonging to the Hemiuroidea superfamily, do not follow this pattern. Thus, lecithasterids, hemiurids and sclerodistomids present a single field of cortical microtubules (Quilichini et al. 2010a; Ndiaye et al. 2013, 2014, 2017) and some didymozoids (*Didymocystis* and *Didymozoon* species) are devoid of cortical microtubules (Justine and Mattei 1983; Pamplona-Basilio et al. 2001).

The electron-dense material is considered as another particularity observed in the anterior part of the spermatozoon. It appears as a submembranous layer located laterally around the second axoneme. This organization is observed in *S. aloysiae* and also reported in numerous digenean species particularly the Aephnidiogenidae *Holorchis micracanthum* (Bâ et al. 2011), the Gyliauchenidae *Gyliauchen* sp. and *Robphildollfusium fratum* (Quilichini et al. 2011; Bakhoum et al. 2012), and the Lepocreadiidae *Hypocreadium caputvadum* and *Opechona bacillaris* (Kacem et al. 2012; Ndiaye et al. 2015).

## Mitochondrial region

Many characters in mitochondrial region of *S. aloysiae* spermatozoon have been considered as potentially informative for phylogenetic inference such as the number of mitochondria, spine-like bodies, the external ornamentation of the plasma membrane, its location, and its association or not with cortical microtubules.

The presence of mitochondria is a valuable character to discern phylogenetic relationships. It is always observed in digenean spermatozoa and the presence of a mitochondrion is considered as a plesiomorphic character whereas in other groups, such as the Eucestoda, the absence of mitochondria has been highlighted as a synapomorphy (Justine, 1991a). According to Burton (1972), numerous mitochondria of the spermatid accompany the nucleus migration along the median cytoplasmic process during spermiogenesis where they apparently fuse together to form a long mitochondrion in the mature spermatozoon. However, in order to establish a logical interpretation of cross-sections, the presence of more than one mitochondrion in the sperm cell has been proposed. In the present study, the male gamete of *S. aloysiae* possesses two mitochondria: the first one is located in the ornamented area, however, the second is observed more posteriorly in the nuclear region. Species of the family Cryptogonimidae exhibit a different number of mitochondria. One mitochondrion has been described in *Ap. tubarium* (Foata et al. 2012), two mitochondria in *Ad. novaecaledoniae* and *T. imbutiforme* (Quilichini et al. 2009; Kacem et al. 2017) and three mitochondria in *An. capitellatum* (Ternengo et al. 2009). Concerning the remaining opisthorchioidean families, *E. squamula* (Heterophyidae) contains three mitochondria and both opisthorchiids present two mitochondria (Bakhoum et al. 2009; Zhukova et al. 2014; Miquel et al. 2017).

The external ornamentation is considered as another interesting criterion used for phylogenetic purposes in the Digenea and is of particular interest for the establishment of spermatozoa models (Quilichini et al. 2011; Bakhoum et al. 2017). According to Quilichini et al. (2011), digenean parasites could be divided into three types basing on the location of the external ornamentation along the spermatozoon: (i) type 1 presents an external ornamentation in the anterior extremity of the spermatozoon; (ii) type 2 presents an external ornamentation at a more posterior level, usually in the mitochondrial region; and (iii) type 3 lacks external ornamentation. In addition to the location, Bakhoum et al. (2017) added another consideration concerning the association or not of the external ornamentation with cortical microtubules along the mature spermatozoon. Most digeneans present spermatozoa that exhibit an external ornamentation associated with cortical microtubules. However, certain species contain an external ornamentation, usually located in the anterior extremity of the sperm cell, which is not associated with cortical microtubules (see Bakhoum et al. 2017). This type of ornamentation was described for the first time by Quilichini et al. (2007) in the spermatozoon of *Pronoprymna ventricosa*. The spermatozoon of *S. aloysiae* displays an external ornamentation of the plasma membrane associated with cortical microtubules and located posterior to the anterior extremity of the sperm cell, as occurs in the remaining Opisthorchioidea studied to date (see Table 1).

The mature spermatozoon of *S. aloysiae* shows the presence of spine-like bodies. Miquel et al. (2000) described this element for the first time in *Opecoeloides furcatus* as submembranous and prominent electron-dense structures that seem to contain a spherical vesicle. Since that time, many studies have described this organisation in numerous digeneans. Spine-like bodies are either present or absent in the Opisthorchioidea. Only four species belonging to the Cryptogonimidae, namely *Ad. novaecaledoniae, An. capitellatum*, *Ap. tubarium* and *T. imbutiforme* (Quilichini et al. 2009; Ternengo et al. 2009; Foata et al. 2012; Kacem et al. 2017) exhibit spine-like bodies as in the opisthorchiid *O. viverrini* (Miquel et al. 2017). On the other hand, the heterophyid *E. squamula* and the opisthorchiid *O. felineus* lack spine-like bodies in their male gametes.

In *S. aloysiae* spine-like bodies are associated with the external ornamentation as in all the opistorchioideans presenting this structure. To date, the apocreadiid *Neoapocreadium chabaudi* is the sole species that exhibits spine-like bodies in the non-ornamented area of the spermatozoon (Kacem et al. 2010).

## Posterior spermatozoon extremity

As occurs with the anterior spermatozoon tip, the posterior spermatozoon extremity presents a great variability that emphasizes the usefulness of this criterion when establishing spermatozoon models in the Digenea. Three models of posterior spermatozoon extremities in digenean spermatozoa, namely opecoelidean, fasciolidean and cryptogonimidean, were proposed by Quilichini et al. (2010b) considering on the sequence of disappearance of characters towards the posterior tip. However, the posterior spermatozoon extremities of certain digeneans exhibit numerous variations leading to unfitness with the three previous models. This is the principal reason argued by Bakhoum et al. (2017) to scrutinize the last spermatozoon character instead of the sequence of characters. The present study shows that *S. aloysiae* shows the axoneme as a terminal character of the male gamete as all studied opisthorchioideans except for *O. felineus* (Zhukova et al. 2014). In fact, these authors describe the nucleus as the last character, but an accurate observation of published TEM micrographs may indicate otherwise.

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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

**Fig. 1.** Spermatozoon of *Siphoderina aloysiae* (regions I and II). **a** Longitudinal section of the anterior spermatozoon tip. **b–d** Consecutive cross-sections of region I showing the progressive appearance of the second axoneme. Note the location of the anterior electron-dense material (*ADM*). **e–f** Cross-sections illustrating the progressive decrease in the number of cortical microtubules (*CM*). **g–j** Cross-sections showing the ornamented area of the sperm cell showing the presence of the first mitochondrion (*M1*) and spine-like bodies (*SB*). **k** Cross-section of the proximal area of region II. *ASE* anterior spermatozoon extremity, *Ax1* first axoneme, *C2* centriole of the second axoneme, *EO* external ornamentation of the plasma membrane, *G* granules of glycogen. *Scale bars* 300 nm.

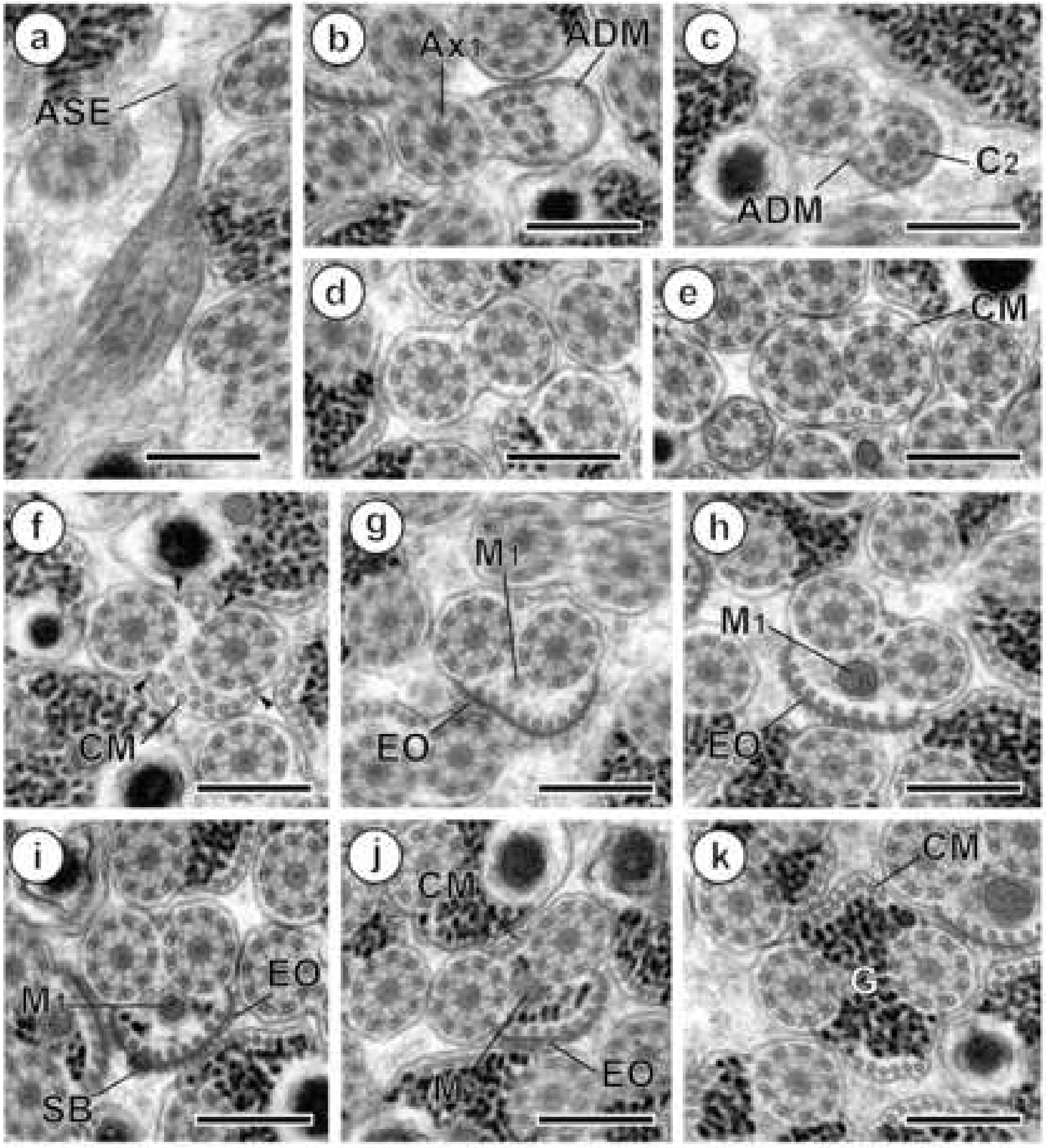
**Fig. 2.** Spermatozoon of *Siphoderina aloysiae* (region III). **a–d** Cross-sections of the region III (nuclear region) showing the disappearance of the first axoneme. **e–f** Cross-sections illustrating the appearance of the second mitochondrion (*M2*). **g–l** Cross-sections showing the disorganisation of the second axoneme and disappearance of cortical microtubules (*CM*) and nucleus (*N*). *CC1* central core of the first axoneme, *D* doublets, *G* granules of glycogen. *Scale bars* 300 nm.

**Fig. 3.** Transmission electron micrograph showing the positive test of Thiéry. *G* granules of glycogen, *M2* second mitochondrion, *N* nucleus. *Scale bar* 300 nm.

**Fig. 4**. Schematic reconstruction of the mature spermatozoon of *Siphoderina aloysiae*. 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. *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, *CC1* central core of the first axoneme, *CM* cortical microtubules, *D* doublets, *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, *SB* spine-like bodies.

Figure 1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b
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ADM
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C2
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f
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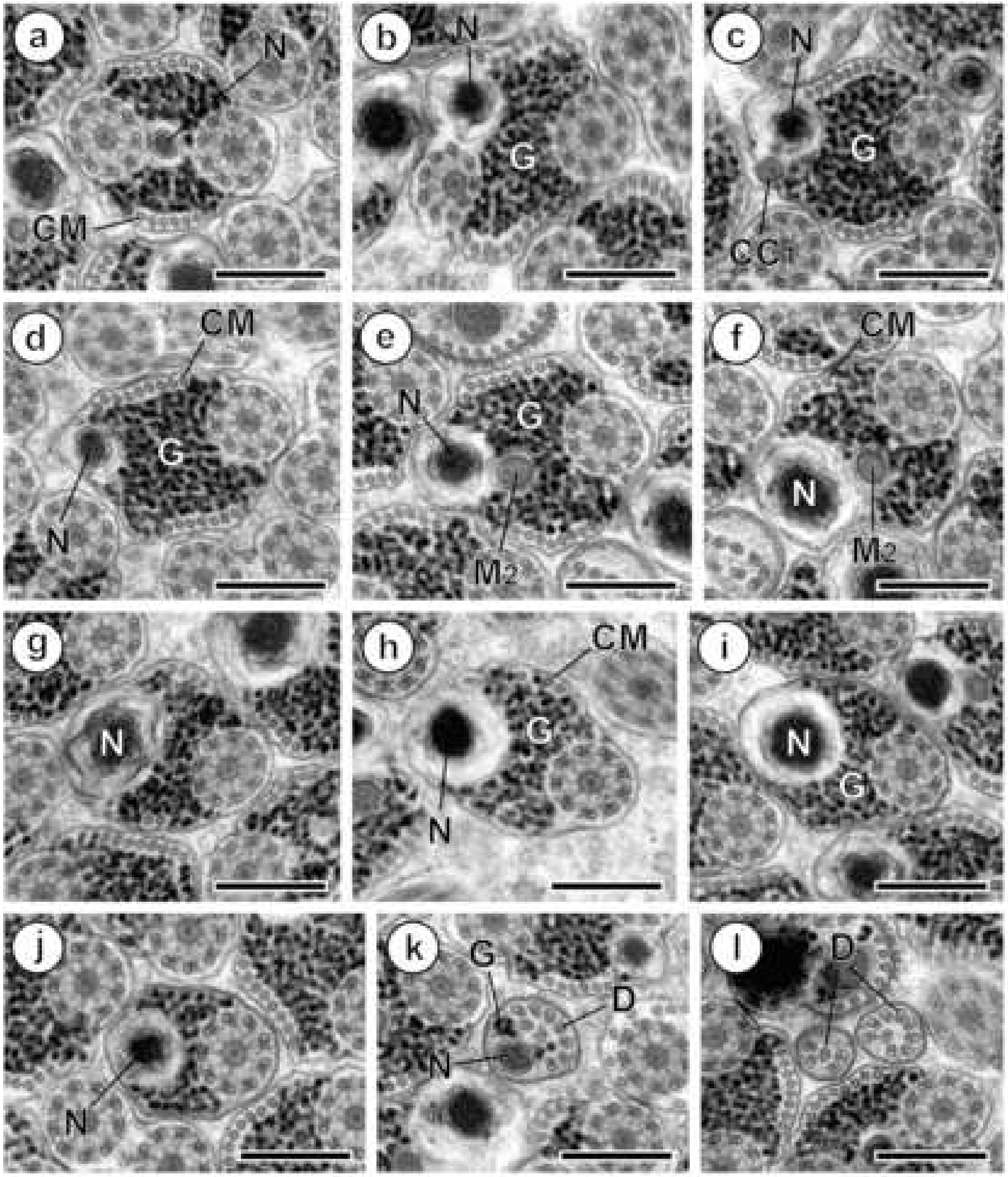
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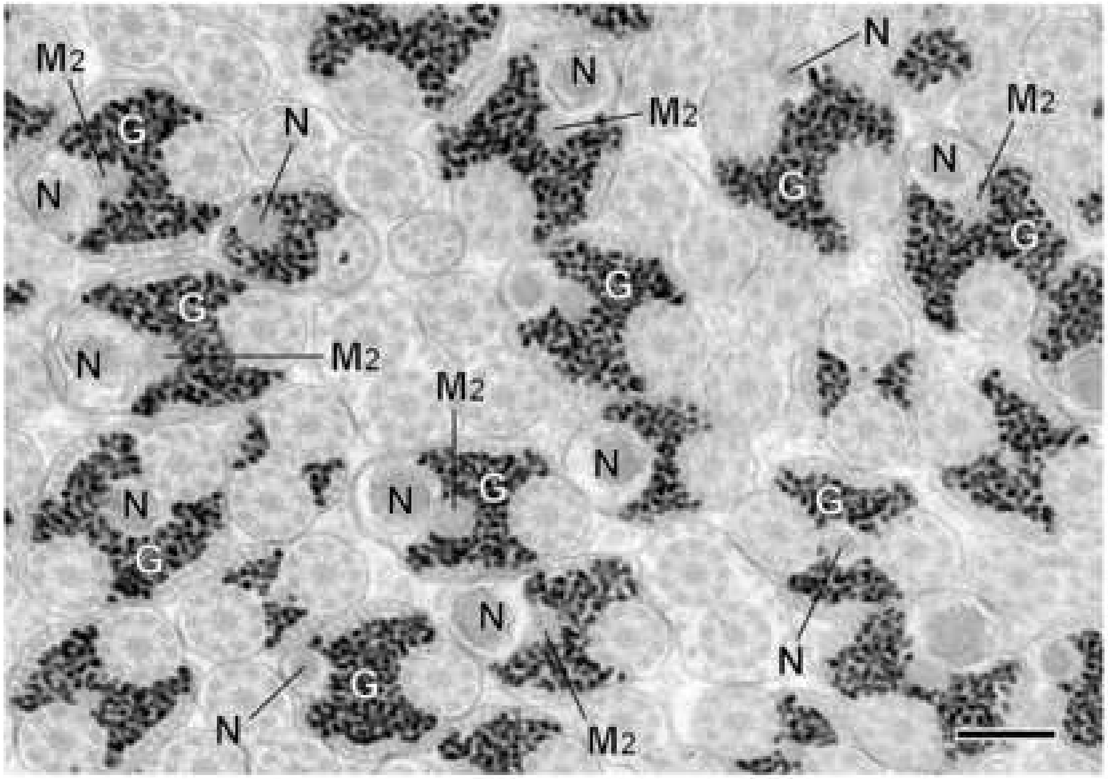
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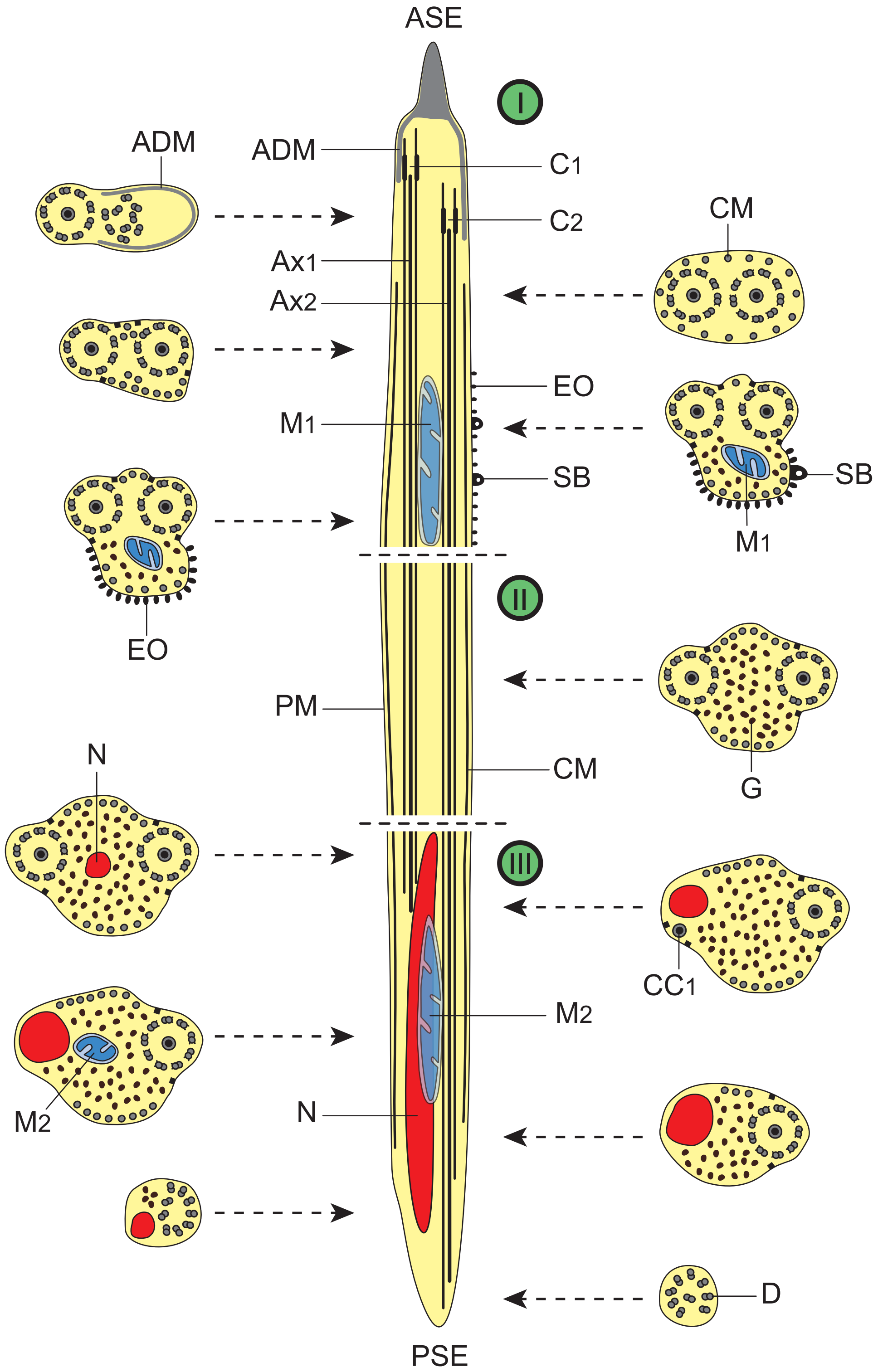
Figure 4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I
ADM
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C1
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Ax1
Ax2
EO
M1
SB
SB
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II
PM
CM
G
N
III
CC1
M2
M2
N
D
PSE

**Table I.** Spermatological characters in the superfamily Opisthorchioidea

| Families and species | TS | TAx | LE | EO | EO+CM | LEO | BCM | LMCM | M | SB | PSC | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cryptogonimidae** | | | | | | | | | | | | |
| *Adlardia novaecaledoniae* | III | 9+‘1’ | - | + | + | PostA | 2 | MedS | 2 | + | Ax | Quilichini et al. (2009) |
| *Anisocoelium capitellatum* | III? | 9+‘1’ | - | + | + | PostA | 2 | MedS? | 3 | + | Ax | Ternengo et al. (2009) |
| *Aphallus tubarium* | III | 9+‘1’ | - | + | + | PostA | 2 | MedS | 1 | + | Ax | Foata et al. (2012) |
| *Siphoderina aloysiae* | IV | 9+‘1’ | - | + | + | PostA | 2 | AntS | 2 | + | Ax | Present study |
| *Stemmatostoma pearsoni* | ? | 9+‘1’ | -? | + | + | ? | 2 | ? | 2? | ? | Ax | Jamieson and Daddow (1982) |
| *Timoniella imbutiforme* | IV | 9+‘1’ | - | + | + | PostA | 2 | AntS | 2 | + | Ax | Kacem et al. (2017) |
| **Heterophyidae** | | | | | | | | | | | | |
| *Cryptocotyle lingua* | ? | 9+‘1’ | -? | -? | NA | NA | 2 | MedS? | 1? | ? | Ax? | Rees (1979) |
| *Euryhelmis squamula* | IV | 9+‘1’ | - | + | + | PostA | 2 | AntS | 3 | - | Ax | Bakhoum et al. (2009) |
| **Opisthorchiidae** | | | | | | | | | | | | |
| *Clonorchis sinensis* | ? | 9+‘1’ | ? | ? | ? | ? | 2 | AntS? | 1? | ? | ? | Jeong and Rim (1984) |
| *Metorchis orientalis* | ? | 9+‘1’ | ? | ? | ? | ? | ? | ? | ? | ? | ? | Liu and Pan (1990) |
| *Opisthorchis felineus* | IV | 9+‘1’ | - | + | + | PostA | 2 | AntS | 2 | - | N/Ax? | Zhukova et al. (2014) |
| *Opisthorchis viverrini* | IV | 9+‘1’ | - | + | + | PostA | 2 | AntS | 2 | + | Ax | Miquel et al. (2017) |

*AntS* anterior region of the spermatozoon, *Ax* axoneme, *BCM* number of bundles of 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, *PSC* posterior spermatozoon character, *SB* spine-like bodies, *TAx* type of axoneme, *TS* type of spermatozoon, *+/-* presence/absence of considered character, *?* unclear data.