Sperm characters of *Timoniella imbutiforme* (Digenea, Opisthorchioidea, Cryptogonimidae), a parasite of the European seabass *Dicentrarchus labrax*

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Abstract

Spermatological characteristics of the digenean *Timoniella imbutiforme* (Cryptogonimidae) collected from *Dicentrarchus labrax* (Teleostei: Serranidae) collected at the Gulf of Gabès in Chebba (Tunisia) were investigated for the first time by means of transmission electron microscopy. The ultrastructural study reveals that the mature spermatozoon of *T. imbutiforme* is a filiform cell, tapered at both extremities. The sperm cell exhibits the characteristics of digenean spermatozoa type IV namely two axonemes of the 9+'1' pattern of trepaxonematan Platyhelminthes, external ornamentation of the plasma membrane associated with cortical microtubules and located in the posterior part of the anterior region of the sperm cell, two bundles of parallel cortical microtubules, maximum number of cortical microtubules in the anterior part of the spermatozoon and presence of two mitochondria. The first mitochondrion of moniliform type is composed of a mitochondrial cord with joined mitochondrial bulges. In addition, the male gamete of *T. imbutiforme* shows spine-like bodies and a posterior extremity with only the second axoneme. The ultrastructural characters of the spermatozoon of *T. imbutiforme* are compared with those of other digeneans belonging to the superfamily Opisthorchioidea.

Keywords: *Timoniella imbutiforme*, Cryptogonimidae, Opisthorchioidea, Digenea, ultrastructure, spermatozoon.

1. Introduction

The superfamily Opisthorchioidea consists of three well-known families, namely the Cryptogonimidae, Opisthorchiidae and Heterophyidae, including numerous species with similarities in their morphological traits and life cycles. However, the phylogenetic relationships between these families remain controversial (Bray, 2008; Olson et al., 2003).

There are several studies that analyse the molecular affinities within the Opisthorchioidea (Kvach et al., 2017, in press; Le et al., 2017; Thaenkham et al., 2011, 2012), which demonstrate the paraphyly of the Heterophyidae and Opisthorchiidae and show that these two families form an inseparable single clade. Recently, Le et al. (2017) confirmed certain incongruences in the molecular placement of the genera *Cryptocotyle* and *Euryhelmis* (placed within the Heterophyidae), which appeared to have more affinities with opisthorchiids. All the latter authors agree that a multidisciplinary approach, using both morphological and molecular data, is needed for a better knowledge of phyletic relationships within the Opisthorchioidea.

The ultrastructural study of the mature spermatozoon has proved to be useful for phylogenetic inference in parasitic Platyhelminthes. In fact, ultrastructural characteristics of sperm cells of monogeneans, cestodes and, recently, digeneans have contributed to a better knowledge of the interrelationships within these groups (Bâ and Marchand, 1995; Bakhoum et al., 2017a; Justine, 1991a, 1991b, 1998, 2001; Levron et al., 2010).

With respect to Digenea, during the last years, studies about the ultrastructural characters of the spermatozoon have notably increased (for a review see Bakhoum et al., 2017a). However, for the Cryptogonimidae only four species have been studied even though the Cryptogonimidae is a large and cosmopolitan family, parasitizing a range of marine and freshwater host including teleosts, reptiles and, rarely, amphibians (Miller and Cribb, 2008). The four previously studied species are *Adlardia novaecaledoniae* (studied 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), and *Stemmatostoma pearsoni* (the spermatological results were published under the name "*Neochasmus* sp." –see Cribb, 1986; Jamieson and Daddow, 1982).

Thus, the aim of the present study is to produce the first complete description of the ultrastructure of another cryptogonimid *Timoniella imbutiforme*, contributing to the ultrastructural spermatological database on Digenea. In order to highlight criteria that may be useful for phylogenetic purposes, results on *Timoniella imbutiforme* are also compared with available data on the Opisthorchioidea.

2. Materials and methods

2.1. Materials

Live adult specimens of *Timoniella imbutiforme* were collected during December 2015 from the digestive tract of the European seabass *Dicentrarchus labrax* captured in the Mediterranean Sea, off La Chebba (34°14′N, 11°06'E) (Tunisia). A voucher specimen, stained with Semichon’s acetic carmine and mounted in Canada balsam, was deposited in the parasitological collection of the Muséum National d’Histoire Naturelle (Paris) (MNHN): one slide of *T. imbutiforme* ex *D. labrax* (no. 2015121106) off La Chebba (Tunisia), 11 December 2015 – accession number MNHN HEL733.

Specimens were identified according to previous descriptions of *T. imbutiforme* (see Brooks and Holcman, 1993; Maillard, 1973). Later, this identification was corroborated by molecular techniques.

2.2. Molecular analyses

Total genomic DNA was isolated from one specimen following Lopez et al. (2015). A PCR reaction in order to amplify the large subunit ribosomal RNA gene was carried out following Tkach et al. (2000). Sequences were obtained in Macrogen (Korea). In order to confirm the identity of the specimens, a BLAST search was carried out.

A fragment of 972 bp of the 28S rRNA was obtained and deposited in GenBank under the accession number MF983699. The BLAST search showed a 99% of identity with *T. imbutiforme* (accession number: MF491865.1), obtained from *Neogobius melanostomus* in Ukraine, Black Sea (Kvach et al., 2017). Only three variable nucleotide positions were observed in the alignment of both sequences. When comparing to the MF491865.1 sequence, the variable nucleotides in our sequence were A:G in the position 391, C:T in the position 487, and G:T in the position 597.

2.3. Transmission electron microscopy

For the present TEM study, several worms were 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 0.1 M sodium cacodylate buffer at pH 7.4, post-fixed in cold (4 °C) 1 % osmium tetroxide with 0.9 % potassium ferricyanide [$K_3Fe(CN)_6$] 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 polymerized 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 the Reynolds (1963) procedure. Copper grids were examined in a JEOL 1010 transmission electron microscope operated at an accelerating voltage of 80 kV, in the "Centres Científics i Tecnològics" of the University of Barcelona (CCiTUB).

2.4. Cytochemistry

Sections placed on gold grids were treated according to the Thiéry (1967) test 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 JEOL 1010 transmission electron microscope at an accelerating voltage of 80 kV, in the CCiTUB.

3. Results

The interpretation of numerous cross- and longitudinal sections of the mature spermatozoon of *T. imbutiforme* allow us to establish three distinctive regions exhibiting different ultrastructural characteristics, from the anterior to the posterior extremities of the male gamete (Figs. 1-4). The mature spermatozoon of *T. imbutiforme* exhibits the usual structures found in most digeneans. Indeed, it contains two axonemes of the 9+'1' trepaxonematan pattern, external ornamentation of the plasma membrane, spine-like bodies, nucleus, mitochondria, two bundles of parallel cortical microtubules and granules of glycogen.

Region I (Figs. 1a-h and 4I) corresponds to the anterior region of the spermatozoon. Cross-sections through the anterior tip show both centrioles and axonemes, and a few cortical microtubules arranged in two discontinuous and submembraneous layers composed of a maximum number of 9+10 microtubules (Fig. 1a, b). In a more posterior area, the number of cortical microtubules increases, forming a continuous layer of about 24 microtubules, but they do not surround the sperm cell completely (Fig. 1c, d). It is possible to observe two attachment zones at this level (Fig. 1d). In the posterior part of region I, there is a moniliform mitochondrion, composed of a mitochondrial cord with joined mitochondrial bulges (Figs. 1e-h and 4I). Moreover, in this area the cortical microtubules are arranged into two fields with a maximum number of cortical microtubules of 1+14 (Fig. 1e-h). It is also possible to observe an external ornamentation of the plasma membrane associated with cortical microtubules and spine-like bodies along this posterior part of region I (Figs. 1e-h and 4I). The four attachment zones are observed in these sections (Fig. 1e-g) compared to previous cross-section (Fig. 1d). The transition toward region II is marked by the disappearance of the first mitochondrion and the external ornamentation.

Region II (Figs. 2a-d, 3 and 4II) corresponds to the middle region of the spermatozoon, which is mainly characterized by the presence of the second mitochondrion. The anterior area of this region shows the simultaneous presence of both axonemes, two bundles of about 4+6 cortical microtubules and a large amount of granular material characterized as glycogen according to the Thiéry's test (Figs. 2a and 3). In the middle and posterior areas of this region, the second mitochondrion appears and the maximum number of cortical microtubules is 6+6 (Figs. 2b, c and 4II). In the distal part of this region, the first axoneme disorganises and disappears (Fig. 2d).

Region III (Figs. 2e-i, 3 and 4III) corresponds to the nuclear and posterior spermatozoon extremity. In the anterior area of this region, the second mitochondrion is still present (Figs. 2e-f and 4III). The transition of characters toward the posterior tip is as follows: (i) disappearance of the second mitochondrion (Figs. 2g and 4III), (ii) cortical microtubules no longer present (Figs. 2h and 4III), and (iii) disappearance of nucleus during the disorganisation of the second axoneme (Figs. 2i and 4III). With respect to cortical microtubules, a progressive reduction in their number is observed (5+7 –Fig. 2d, 4+5 –Fig. 2e, 2+3 –Fig. 2f, and 0+2 –Fig. 2g). Then, near the posterior spermatozoon tip only doublets of the second axonemes and a few granules of glycogen are still present (Fig. 2i).

The granular material observed along the mature spermatozoon is evidenced as glycogen using the test of Thiéry (Fig. 3).

4. Discussion

The mature spermatozoon of *T. imbutiforme* exhibits the usual ultrastructural characteristics as most digeneans described so far: two axonemes, nucleus, mitochondria, parallel cortical microtubules and granules of glycogen. Additionally, spine-like bodies and external ornamentation of the plasma membrane are also present.

Bakhoum et al. (2017a) analysed the ultrastructure of several characters of the mature spermatozoon of digeneans to evaluate their potential for phylogenetic purposes and to establish different models of spermatozoa in the Digenea. Thus, two sets of characters have been considered: principal (used to define the five digenean sperm cell types) and secondary characters. The principal characters include the type of axoneme, the lateral expansion, the external ornamentation (associated or not with cortical microtubules) and its location, the location of the maximum number of cortical microtubules and the number of cortical microtubule bundles, and the number of mitochondria. According to Bakhoum et al. (2017a), the usefulness of secondary characters such as spine-like bodies or the terminal sperm character is not currently clear.

4.1. Axonemes

The spermatozoon of *T. imbutiforme* contains two axonemes showing the 9+‘1’ *trepaxonematan* pattern (Ehlers, 1984). This is the typical structure of axonemes observed in all digeneans except for the species of the genus *Schistosoma* with a special 9+‘1’ pattern (Jamieson and Justine, 2017; Justine et al., 1993) and species of *Didymozoon* with a 9+0 pattern (Justine and Mattei, 1983).

Both axonemes are of different length; in all the studied cryptogonimids, namely *Adlardia novaecaledoniae*, *Anisocoelium capitellatum* and *Aphallus tubarium,* one of the axonemes disorganises and disappears at the level of the very anterior part of nuclear region (Foata et al., 2012; Quilichini et al., 2009; Ternengo et al., 2009). This is also the case for

Timoniella imbutiforme showing the disorganisation of the first axoneme in the pre-nuclear region of the sperm cell. For the remaining Opisthorchiodea families, both *Euryhelmis squamula* (Heterophyidae) and *Opisthorchis* spp. (Opisthorchiidae) exhibit this disorganisation in the pre-nuclear region or at the very beginning of the nuclear region of their spermatozoa (Bakhoum et al., 2009; Miquel et al., 2017; Zhukova et al., 2014).

4.2. External ornamentation

The mature spermatozoon of most digeneans displays ornamentation in the external surface of its membrane. Quilichini et al. (2011) considered the presence/absence of external ornamentation and its location if present, as a useful characteristic for phylogenetic inference within digeneans. Recently, Bakhoum et al. (2017a) reinforced the usefulness of this character to establish different types of spermatozoa in digeneans and added another criterion concerning the external ornamentation: the association or lack of association with cortical microtubules. Most digeneans present spermatozoa that exhibit external ornamentation associated with cortical microtubules, but 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., 2017a). This type of ornamentation was found for the first time by Quilichini et al. (2007) in the spermatozoon of the faustulid *Pronoprymna ventricosa.*

In the Opisthorchioidea, all the studied species present spermatozoa exhibiting external ornamentation of the plasma membrane associated with cortical microtubules and located posteriorly to the anterior sperm extremity (see Table I).

4.3. Cortical microtubules

The location of the maximum number of cortical microtubules along the sperm cell has been considered a useful criterion for phylogenetic inference within the Digenea (Bakhoum et al., 2017a; Quilichini et al., 2007). Sperm cells of digeneans could be classed according to the occurrence of the maximum number of cortical microtubules either in the anterior part or in the middle area of the gamete. For species belonging to the superfamily Opisthorchioidea the location of the maximum number of cortical microtubules is variable. In the spermatozoa of cryptogonimids, the maximum number of cortical microtubules occurs in the middle region of the male gamete, whereas in heterophyids and opisthorchiids, as well as in *T. imbutiforme,* it occurs in the anterior part of the gamete (see Table I).

The number of bundles that cortical microtubules exhibit along the mature spermatozoon is another interesting characteristic for the establishment of sperm models in digeneans (Bakhoum et al., 2017a). Most digeneans show an arrangement of cortical microtubules into two fields, as occurs in *T. imbutiforme* and also in the remaining opisthorchioideans. Other digeneans, particularly species belonging to the superfamily Hemiuroidea do not follow this pattern. In fact, cortical microtubules are arranged in a single field in lecithasterids, hemiurids and sclerodistomids (Ndiaye et al., 2013, 2014, 2017; Quilichini et al., 2010a), and didymozoids lack cortical microtubules (Justine and Mattei, 1983).

4.4. Mitochondria

The presence of mitochondria in the mature spermatozoon is considered a plesiomorphic character in the Digenea (Bakhoum et al., 2014) whereas in other groups, such as the Eucestoda, the absence of mitochondria has been highlighted as a synapomorphy (Justine, 1991a). While the presence or absence of mitochondria is a valuable character to discern phylogenetic relationships, the number of mitochondria is also an important character.

Our work highlights the presence of two mitochondria in the mature spermatozoon of *T. imbutiforme*. The first mitochondrion is located at the level of the ornamented area, and the second one is located more posteriorly in the nuclear area. The first mitochondrion of *T. imbutiforme* is moniliform. A moniliform mitochondrion was described for the first time by Bâ et al. (2011) in the spermatozoon of the lepocreadiid *Holorchis micracanthum*. Since then, this type of mitochondrion has been found in the spermatozoon of other species such as the acanthocolpid *Stephanostomoides tenuis* (Bakhoum et al., 2015), the lepocreadiid *Opechona bacillaris* (Ndiaye et al., 2015), the opecoelids *Allopodocotyle pedicellata* and *Macvicaria obovata* (Bakhoum et al., 2017b; Kacem et al., 2017), the plagiorchiid *Enodoitrema reductum* (Ndiaye et al., 2012), and the cryptogonimid *Aphallus tubarium* (Foata et al., 2012).

Cryptogonimidae species present different numbers of mitochondria. One mitochondrion has been described in *A. tubarium* (Foata et al., 2012), two mitochondria in *A. novaecaledoniae* and *T. imbutiforme* (Quilichini et al., 2009; present study) and three mitochondria in *A. capitellatum* (Ternengo et al., 2009). In the remaining families of the Opisthorchioidea, the heterophyid *E. squamula* presents three mitochondria and both studied opisthorchiids present two mitochondria (Bakhoum et al., 2009; Miquel et al., 2017; Zhukova et al., 2014).

4.5. Spine-like bodies

Spine-like bodies were described for the first time in the opecoelid *Opecoeloides furcatus* as prominent and submembraneous electron-dense structures that contain a sort of vesicle (Miquel et al., 2000). These ultrastructural elements are usually present in the ornamented area of the spermatozoon, with the sole exception of *Neoapocradium chabaudi*, which exhibits spine-like bodies not associated with ornamentation (Kacem et al., 2010).

Because of their probable misinterpretation or omission in older papers, Bakhoum et al., (2017a) considered spine-like bodies as a secondary character. However, since their first description, spine-like bodies have been found in the male gamete of numerous digeneans. In the Opisthorchioidea, there is variation in the presence/absence of spine-like bodies in the spermatozoon. These structures are described in the spermatozoon of the cryptogonimids *A. novaecaledoniae, A. capitellatum* and *A. tubarium*, and in the opisthorchiid *O. viverrini* (Foata et al., 2012; Miquel et al., 2017; Quilichini et al., 2009; Ternengo et al., 2009). On the other hand, the heterophyid *E. squamula* and also *O. felineus* lack spine-like bodies in their male gametes. As in all the opistorchioideans, the spermatozoon of *T. imbutiforme* also contains spine-like bodies, which are located in the ornamented region of the gamete.

4.6. Posterior spermatozoon extremity

Quilichini et al. (2010b) proposed three models of posterior spermatozoon extremities in digenean spermatozoa: opecoelidean, fasciolidean and cryptogonimidean types. These types were established according to the sequence of characters such as axoneme, nucleus and cortical microtubules toward the posterior tip of the sperm cell. However, certain digeneans exhibit posterior spermatozoon extremities that do not fit any of these models. According to Bakhoum et al. (2017a), this is the principal reason why only the posterior spermatozoon character should be considered instead of the sequence of the above-mentioned characters toward the posterior extremity of the spermatozoon. Moreover, along with spine-like bodies, the latter authors considered the posterior spermatozoon character to be another secondary character.

In all opisthorchioideans studied to date, including *T. imbutiforme*, the axoneme is described as the terminal character of the gamete (see Table I). However, in the case of *O.*

felineus (Zhukova et al., 2014), a thorough observation of the published micrographs may indicate otherwise.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

The authors are grateful to 'Centres Científics i Tecnològics' of the University of Barcelona (CCiTUB) for their assistance in the preparation of samples.

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Legends

Fig. 1. Spermatozoon of *Timoniella imbutiforme* (region I). (a) Cross-section of the anterior spermatozoon extremity showing the two centrioles (C_1 and C_2). (b–d) Cross-sections illustrating the progressive increase in the number of cortical microtubules (CM). (e–g) Cross-sections showing the ornamented area of region I. Note the presence of the first mitochondrion (M_1) and spine-like bodies (SB). (h) Longitudinal section of the ornamented area. Note the presence of prominent and submembranous spine-like body. Arrowheads,

attachment zones; EO, external ornamentation of the plasma membrane; MB, mitochondrial bulge; MC, mitochondrial cord. Scale bars: 300 nm.

Fig. 2. Spermatozoon of *Timoniella imbutiforme* (regions II and III). (a) Cross-section of the proximal area of region II. (b, c) Two cross-sections showing the appearance of the second mitochondrion (M$_2$). (d) Cross-section at the level of the disorganisation of the first axoneme showing its central core (CC). (e–i) Consecutive cross-sections of region III, from the appearance of the nucleus (N) to the posterior spermatozoon extremity. Arrowheads, attachment zones; CM, cortical microtubules; 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; M$_2$, second mitochondrion; N, nucleus. Scale bar: 300 nm.

Fig. 4. Schematic reconstruction of the mature spermatozoon of *Timoniella imbutiforme*. 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. ASE, anterior spermatozoon extremity; Ax$_1$, first axoneme; Ax$_2$, second axoneme; AZ, attachment zones; C$_1$, centriole of the first axoneme; C$_2$, centriole of the second axoneme; CC, central core; CM, cortical microtubules; D, doublets; EO, external ornamentation of the plasma membrane; G, granules of glycogen; M$_1$, first mitochondrion; M$_2$, second mitochondrion; MB, mitochondrial bulge; MC, mitochondrial cord; N, nucleus; PAx$_1$E, posterior extremity of the first axoneme; PAx$_2$E, posterior extremity of the second axoneme; PM, plasma membrane; PSE, posterior spermatozoon extremity; SB, spine-like bodies.

Table

Table I. Spermatological characters in the superfamily Opisthorchioidea.

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; 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 micrographs.

Data from *Stemmatostoma pearsoni* (Cryptogonimidae), *Cryptocotyle lingua* (Heterophyidae), and *Clonorchis sinensis* and *Metorchis orientalis* (Opisthorchiidae) are not shown due to the limited or incomplete observations published for these species.

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)
Timoniella imbutiforme	IV	9+‘1’	-	+	+	PostA	2	AntS	2	+	Ax	Present study
Heterophyidae												
Euryhelmis squamula	IV	9+‘1’	-	+	+	PostA	2	AntS	3	-	Ax	Bakhoum et al. (2009)
Opisthorchiidae												
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)

Figure 1

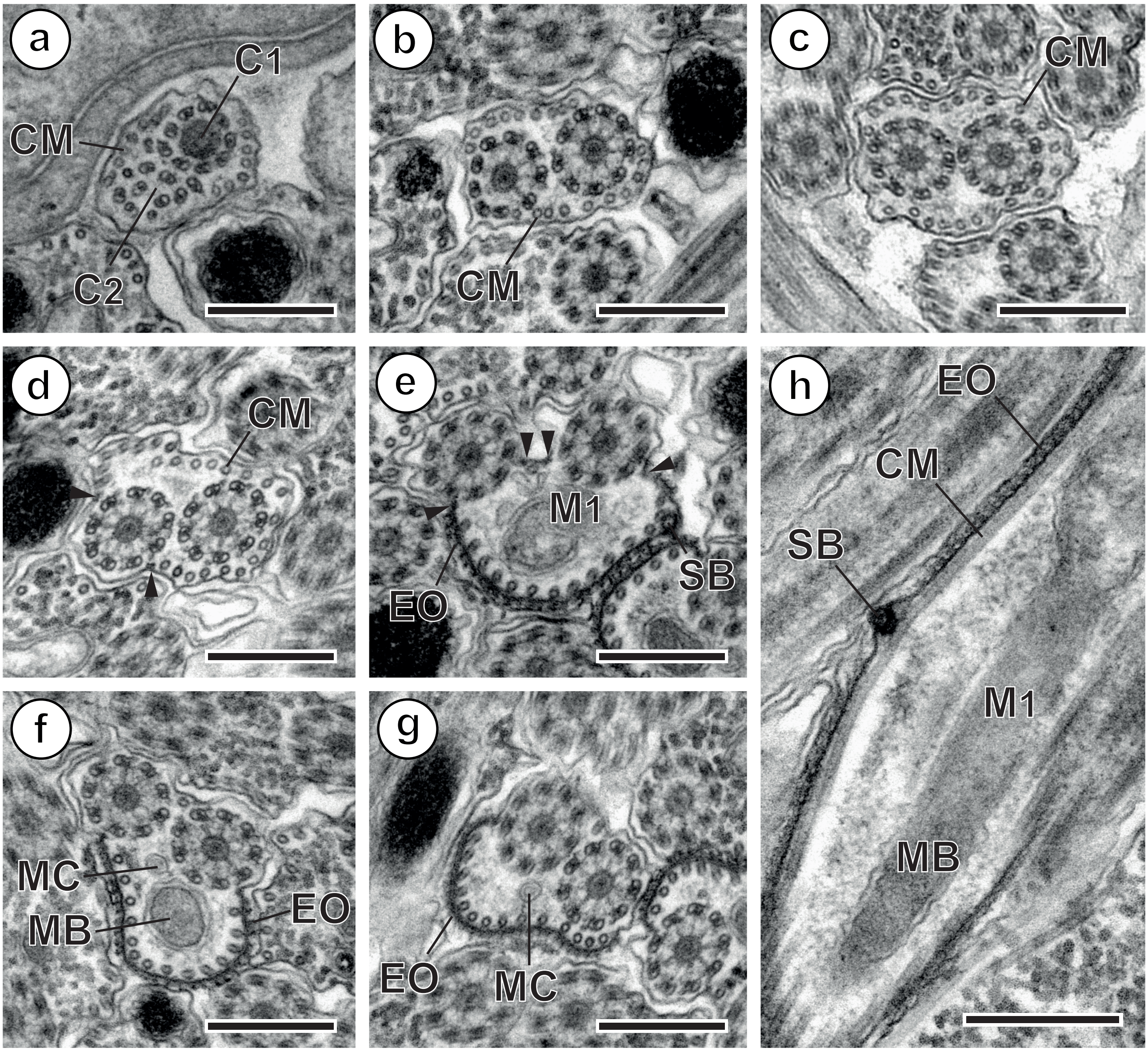


Figure 2

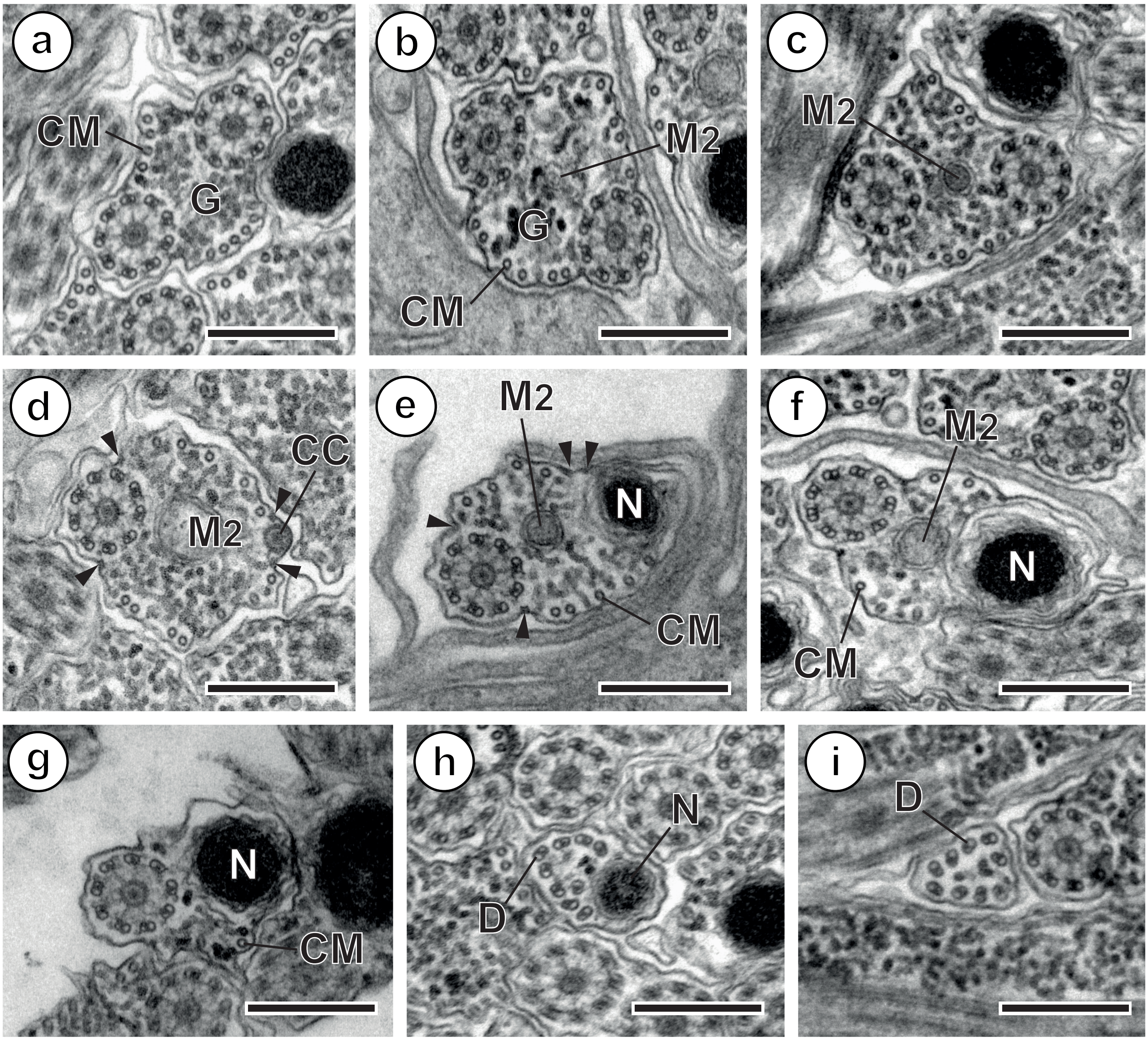


Figure 3

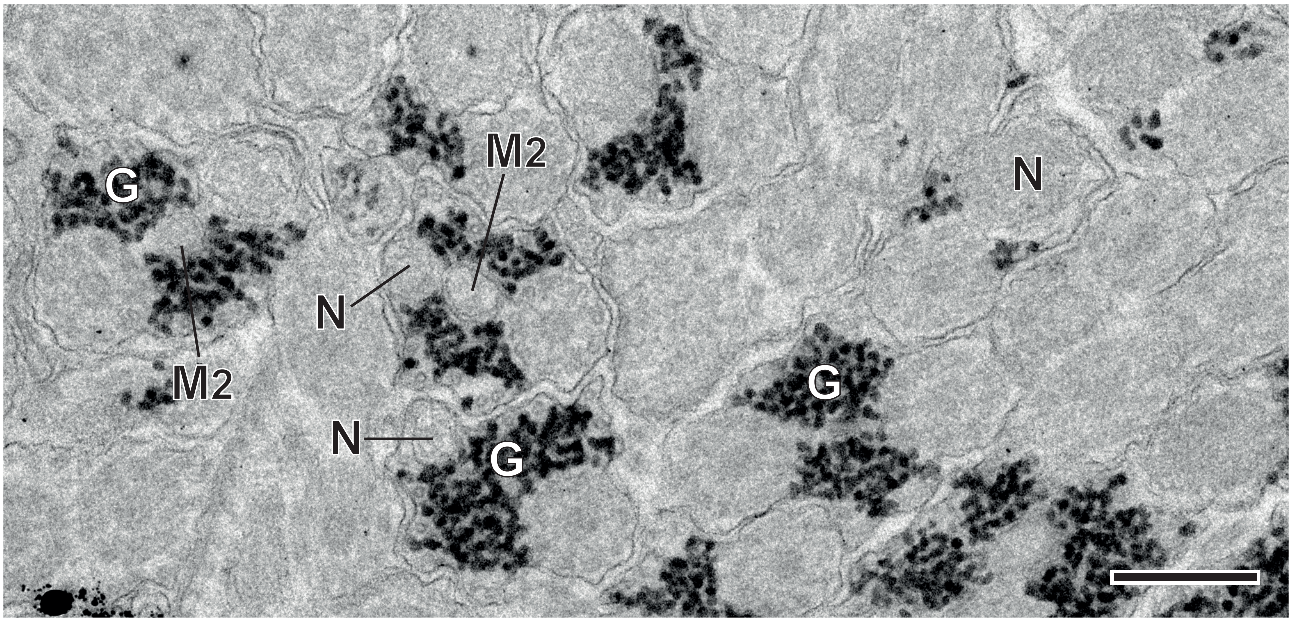
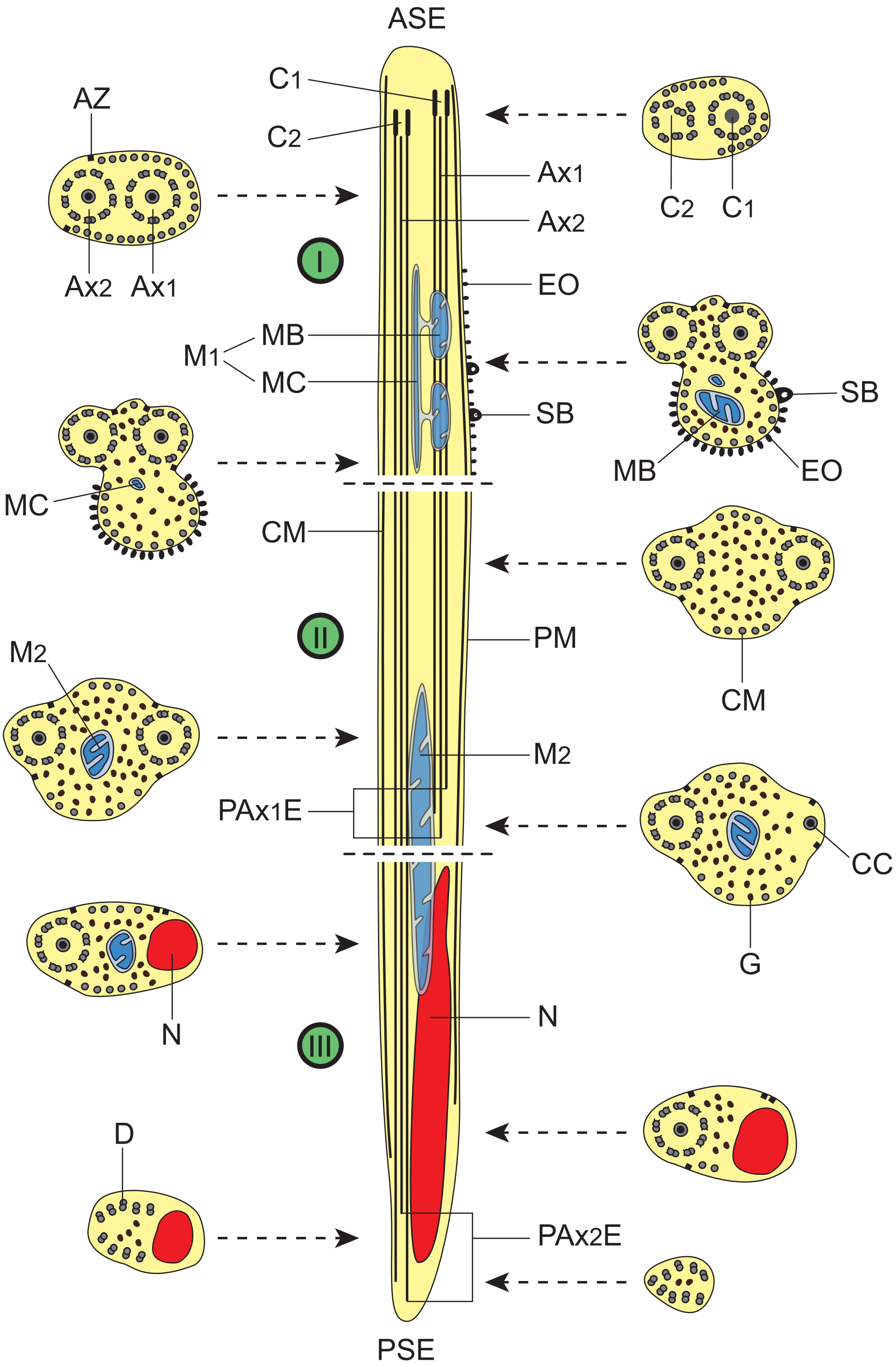


Figure 4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AZ
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EO
MC
CM
II
PM
CM
M2
M2
PAx1E
CC
G
N
N
III
D
PAx2E
PSE