Reinvestigation of the sperm ultrastructure of *Hypoderaeum conoideum* (Digenea: Echinostomatidae)

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Abstract

Spermatological characteristics of the digenean *Hypoderaeum conoideum* (Echinostomatidae) collected from *Anas platyrhynchos* in the Lac d'Annecy (France) were reinvestigated using transmission electron microscopy. The previous study on this species only describes the presence of two axonemes of unequal lengths, a mitochondrion, a posterior nucleus and the disposition of cortical microtubules. The present ultrastructural study reveals that the mature spermatozoon of *H. conoideum* is a filiform cell, tapered at both extremities. The sperm cell exhibits the characteristics of digenean spermatozoa type V 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 anterior part of the proximal region of the sperm cell, lateral expansions, two bundles of parallel cortical microtubules, maximum number of cortical microtubules in the anterior part of the spermatozoon and presence of two mitochondria. In addition, the sperm cell of *H. conoideum* shows spine-like bodies and a posterior extremity with only the nucleus. The ultrastructural characters of the spermatozoon of *H. conoideum* are compared with those of other digeneans belonging to the superfamily Echinostomatoidea.

Keywords: *Hypoderaeum conoideum*; Echinostomatidae; Digenea; Sperm characters; Ultrastructure.

Introduction

The Echinostomatidae constitutes a large family of digeneans currently including ten subfamilies and numerous genera basically due to the large range of vertebrates acting as definitive hosts and to their wide geographical distribution (Kostadinova 2005). *Hypoderaeum* is a genus included within the subfamily Echinostomatinae. Individuals in this subfamily are

mainly characterized by the presence of a circumoral head collar ventrally interrupted but uninterrupted at a dorsal level. The present study describes the sperm ultrastructure of *Hypoderaeum conoideum*, which is the type species of this genus, and parasitizes the intestine of birds and mammals (Kostadinova 2005).

The study of the sperm ultrastructure provides information on a large number of characters potentially useful to the interpretation of relationships within the Platyhelminthes. Thus, several researchers have largely studied these characters in different groups such as monogeneans or cestodes and more recently digeneans (Bâ and Marchand 1995; Justine 1991a, b, 1998, 2001; Levron et al. 2010; Quilichini et al. 2010a, 2011; Bakhoum et al. 2017; Justine and Poddubnaya 2018).

The sperm ultrastructure of Echinostomatoidea is one of the most poorly analysed among Digenean superfamilies. There are only four studies on the spermatozoon, two of them on the family Fasciolidae (*Fasciola gigantica* and *Fasciola hepatica* by Ndiaye et al. 2003, 2004), and the remaining on the Echinostomatidae (*Echinostoma caproni* by Iomini and Justine 1997 and *Hypoderaeum conoideum* by Chen et al. 1996). However, Chen et al. (1996) did not describe the full ultrastructural organization of *H. conoideum* spermatozoa, their results were poorly illustrated. In fact, Chen et al. (1996) describes only general characters such as the presence of two 9+'1' axonemes of unequal lengths, mitochondrion, nucleus and parallel cortical microtubules. Therefore, the aim of the present study is to provide the first complete study on the spermatozoon of *H. conoideum* through an accurate analysis of different ultrastructural characters and their organization.

Materials and methods

Materials

Live adult specimens of *Hypoderaeum conoideum* (Bloch, 1782) were collected from the digestive tract of a naturally infected mallard duck *Anas platyrhynchos* Linnaeus, 1758 (Anseriformes: Anatidae) captured in the Lac d'Annecy (Saint Jorioz, France) during November 2017.

Molecular analyses and specific diagnosis

The specific identification of the adult worms studied herein was performed by molecular analysis as described previously by Georgieva et al. (2014). Genomic DNA was isolated from an individual worm stored in absolute ethanol using Tteh Realpure Spin Kit (Durviz, Valencia, Spain), according to the manufacturer's instructions. A partial sequence of the NADH dehydrogenase subunit 1 (ND1) gene was amplified using the primers NDJ11 (forward; 5'-AGA TTC GTA AGG GGC CTA ATA-3') and NDJ2A (reverse; 5'-CTT CAGCCT CAG CAT AAT-3') (Morgan and Blair 1995; Kostadinova et al. 2003). The PCR thermocycling profile comprised initial denaturation at 95 °C for 5 min, followed by 35 cycles (30 s denaturation at 94 °C, 20 s primer annealing at 48 °C, and 45 s at 72 °C for primer extension), with a final extension step of 4 min at 72 °C as described by Georgieva et al. (2014). All PCR reactions were performed with a T100™ Thermal Cycle (Biorad, Hercules, California, USA). PCR products were visualized on a 1% agarose gel. Sanger sequencing was performed, and chromatograms were inspected visually to resolve any ambiguities. Search of similarities was performed using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) and forward and reverse sequences were aligned using EMBOSS needle tool (https://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html).

Transmission electron microscopy (TEM)

For the present TEM study, several live 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 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 (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).

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 in the CCiTUB.

Results

The general morphological and morphometric data of the adult worms collected were consistent with previous reports of *Hypoderaeum conoideum* (Toledo et al. 1996) and molecular analysis confirmed this diagnosis (Fig. 1). The 419 bp obtained herein (GenBank accession number:

MH282580) showed a 99.8% of homology with the previously published GenBank 497 bp long NADH dehydrogenase subunit 1 (ND1) gene of *H. conoideum* (Accession number: AY168949.1), with a score of 2086.0 and without gaps.

The mature spermatozoon of *H. conoideum* is a filiform cell, tapered at both extremities and exhibiting two axonemes of the 9+'1' pattern of trepaxonematan Platyhelminthes, parallel cortical microtubules, two mitochondria, nucleus, external ornamentation of the plasma membrane, two lateral expansions, spine-like bodies, and a large amount of glycogen granules. Based on the organization and location of these structures from the anterior to the posterior sperm extremities (Figs. 2, 3, and 4), the sperm cell was divided into three regions (I to III).

Region I (Figs. 2a-m, 4I) corresponds to the anterior region of the spermatozoon. The anterior tip of the spermatozoon is filiform and sharp (Fig 2a). Cross-sections of the anterior area of region I exhibit the presence of parallel cortical microtubules and the two axonemes that seem to appear simultaneously (Fig. 2b-e). The ornamented area is observed when the two 9+'1' axonemes are already formed (Fig. 2f-m). The ornamented area also surrounds two differently shaped lateral expansions (Fig. 2g, h). One of them consists in a simple lateral cytoplasmic expansion that progressively reduces and disappears before the second one. The second expansion is hook-shaped and in a dorsolateral position (Fig. 2h, i). Thus, there is an area of the spermatozoon exhibiting two lateral expansions (Fig. 2g, h) and another with only the hook-shaped expansion (Fig. 2i-k). The first mitochondrion appears in the posterior part of region I (Fig. 2h, i). The transition toward the posterior area of region I is marked by the appearance of spine-like bodies (Fig. 2j, l), the reduction and disappearance of the hook-shaped dorsolateral expansion (Fig. 2j-l) and the progressive increase of granular material (Fig. 2j-m). Cross-sections in the posterior part of region I show the reduction of external ornamentation to only cover one side of the male gamete and the arrangement of cortical microtubules in two fields (Fig. 2m).

Region II (Figs. 2n, o, 3a, b and 4II) is the middle region of the spermatozoon and exhibits the two axonemes, two fields of parallel cortical microtubules and granular material (Fig. 2n, o), whereas posterior areas are characterized by the presence of the second mitochondrion (Fig. 3a, b).

Region III (Figs. 3c-h) is the posterior and nuclear region of the spermatozoon. The main characteristic of this region is the presence of the nucleus from its anterior area to the posterior extremity of the sperm cell (Fig. 3c-h). The transition of characters along this region is: (a) the disappearance of the first axoneme (Fig. 3c), (b) the disappearance of one of the bundles of cortical microtubules (Fig. 3c, d), (c) the disappearance of the second mitochondrion and the remaining cortical microtubules (Fig. 3d, e), and (d) the disorganization and disappearance of the second axoneme (Fig. 3f-h). A particularity of this region is the presence of the nucleus throughout its length (Fig. 3h).

All the granular material observed in the spermatozoon of *H. conoideum* was identified as glycogen by means of the test of Thiéry (Fig. 3i).

Discussion

Adult *H. conoideum* specimens were accurately identified by molecular and morphology-based studies. The ultrastructural study of their mature spermatozoa revealed the main characters usually found in most digeneans, namely two axonemes of the 9+'1' pattern of the Trepaxonemata (Ehlers 1984), parallel cortical microtubules arranged in two fields, external ornamentation of the plasma membrane, mitochondria, nucleus and granules of glycogen. However, it also presents other less frequent characters in digeneans such as the lateral expansions and spine-like bodies.

Lateral expansion

The lateral expansion is a character present in the anterior area of the spermatozoa of certain digeneans (see Bakhoum et al. 2017 for a review). The lateral expansion is usually associated with other characters, namely cortical microtubules, external ornamentation of the plasma membrane and spine-like bodies. The presence/absence of a lateral expansion in the spermatozoon was recently considered a criterion for the establishment of sperm models in the Digenea (Bakhoum et al. 2017). The latter authors consider spermatozoa exhibiting a lateral expansion as the type V spermatozoon. To date, all digeneans presenting spermatozoa with lateral expansion exhibited only one expansion except for the mesometrid *Mesometra brachycoelia,* which shows two lateral expansions (Bakhoum and Miquel 2011). In the present study, we describe the second digenean species presenting two expansions in the male gamete.

In the Echinostomatoidea, both fasciolids and echinostomatids have lateral expansions in their spermatozoa (Iomini and Justine 1997; Ndiaye et al. 2003, 2004). Moreover, the morphology of the lateral expansions in these groups is quite similar: they consist in the so-called hook-shaped dorsolateral expansion. This type of lateral expansion is also present in the sperm cell of the troglotrematid *Troglotrema acutum* (Miquel et al. 2006). In *H. conoideum* (present study), one of the lateral expansions is also hook-shaped, while the other consists in a simple lateral elongation of cytoplasm.

External ornamentation and cortical microtubules

The external ornamentation of the plasma membrane is present in the spermatozoon of most digeneans. Considering the data on the external ornamentation of the plasma membrane available at the time, Quilichini et al. (2011) established three types of anterior regions of spermatozoa in the Digenea; the anterior region of the spermatozoon either presented (i) ornamentation more anteriorly, (ii) more posteriorly or (iii) no ornamentation. More recently, Bakhoum et al. (2017) considered that (i) the presence/absence of external ornamentation and

its location, along with (ii) its association or not with cortical microtubules are potentially useful for clarifying relationships within digeneans. Although the external ornamentation of the plasma membrane is usually associated with cortical microtubules (see Bakhoum et al. 2017 for a review), in some cases ornamentation was not found to be associated with cortical microtubules. This is the case of the sperm cell of the Faustulidae *Pronoprymna ventricosa* (Quilichini et al. 2007) and other digeneans such as hemiurids, lecithasterids and sclerodistomids (Quilichini et al. 2010b; Dione et al. 2016; Ndiaye et al. 2017).

In the Echinostomatoidea, all species studied to date have external ornamentation of the plasma membrane associated with cortical microtubules and located in the anterior region of the male gamete. Moreover, this ornamentation occurs in the sperm area presenting lateral expansion or expansions and the maximum number of cortical microtubules.

Regarding cortical microtubules, two characteristics are important for phylogenetic inference: (i) the presence/absence and arrangement in one or two bundles and (ii) the location of their maximum number (see Bakhoum et al. 2017). Only certain didymozoids lack cortical microtubules (Justine and Mattei 1983; Pamplona-Basilio et al. 2001). With respect to the arrangement in one/two bundles, as most digeneans, all the echinostomatids and fasciolids studied to date present two bundles of parallel cortical microtubules (see Table 1). There are only a few digeneans with a single bundle of cortical microtubules, the Faustulidae *P. ventricosa* and some hemiuroideans (Quilichini et al. 2007, 2010b; Dione et al. 2016). The location of the maximum number of cortical microtubules is anterior in all the studied Echinostomatoidea, concretely in the area of expansion/expansions (see Table 1).

Spine-like bodies

These structures have been generally found in the male gamete of numerous digeneans (see Bakhoum et al. 2017 for a review). However, according to some authors (Bakhoum et al. 2017)

its usefulness for phylogenetic inference within the Digenea is unknown since in some older studies these bodies were probably misinterpreted as artefacts in the descriptions of sperm cell (Justine and Mattei 1982; Orido 1988).

The spine-like bodies are prominent, submembranous electron-dense structures that contain a sort of vesicle (Miquel et al. 2000). For some digeneans, a periodicity between spine-like bodies has been described, while in other species these ultrastructural elements are irregularly distributed along the sperm cell (see Miquel et al. 2006 and Kacem et al. 2010 for a review). Moreover, spine-like bodies are observed as isolated structures in most digeneans that exhibit these elements in their spermatozoa. Nevertheless, some bucephalids show numerous spine-like bodies at a same level in cross-sections (Miquel et al. 2017; Kacem and Miquel 2018).

According to the available data on the Echinostomatoidea these structures have been described in the two fasciolids studied to date (*Fasciola gigantica* and *Fasciola hepatica*, Ndiaye et al. 2003, 2004), whereas in the echinostomatids there is variation in the presence/absence of this character. Spine-like bodies have been observed in the present study whereas *Echinostoma caproni* lacks these elements in its sperm cells (Iomini and Justine 1997).

Transition of characters toward the posterior spermatozoon extremity

The transition sequence of cortical microtubules, second axoneme and nucleus toward the posterior extremity of the spermatozoon was proposed as a potential feature to evaluate interrelationships within digeneans establishing three models of posterior spermatozoon extremities in the Digenea, namely opecoelidean, fasciolidean and cryptogonimidean (Quilichini et al. 2010a). Nevertheless, incongruences found in some species, e.g. *Scaphiostomum palaearcticum* or *Aponurus laguncula* (Ndiaye et al. 2002; Quilichini et al. 2010b), led several authors to propose using exclusively the terminal character instead of the

sequence of characters towards the posterior spermatozoon tip as a potential feature to evaluate interrelationships within digeneans (Bakhoum et al. 2017). Thus, in the Echinostomatoidea both fasciolids (Ndiaye et al. 2003, 2004) and *H. conoideum* (present study) exhibit a transition of characters in the posterior extremity of their spermatozoa coincident with the fasciolidean type, characterized by the following sequence of characters: posterior end of cortical microtubules followed by the disappearance of the second axoneme and finally the nucleus. The sole doubtful data concerns *E. caproni* (Iomini and Justine 1997), species for which published TEM micrographs do not show clearly the posterior spermatozoon character (see Table 1).

Concluding remarks

Mature spermatozoa of *H. conoideum* correspond to type V of Bakhoum et al. (2017) and this is also the model for all the studied species of the Echinostomatoidea. According to these authors, this type is characterised by the following principal characters: (i) the presence of two axonemes of the 9+'1' trepaxonematan pattern, (ii) lateral expansion, (iii) external ornamentation of the plasma membrane associated with cortical microtubules that are located in the anterior part of the proximal region of the sperm cell, (iv) maximum number of cortical microtubules in the anterior part of the spermatozoon, (v) organisation of cortical microtubules into two bundles and (vi) presence of one mitochondrion in general. The sperm cell of *H. conoideum* presents two lateral expansions and two mitochondria, while fasciolids and *E. caproni* have only one lateral expansion and a single mitochondrion. A secondary character (not considered for the establishment of digenean sperm models of Bakhoum et al. 2017) such as the presence/absence of spine-like bodies shows variability between the two studied species of the family Echinostomatidae.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Legends of figures

Fig. 1. Nucleotide alignment of *Hypoderaeum conoideum* partial NADH dehydrogenase subunit 1 (ND1) gene sequence (Accession number: MH282580) with the previously GenBank published (Accession number: AY168949.1).

Fig. 2. Spermatozoon of *Hypoderaeum conoideum* (Regions I and II). **a** Longitudinal section of the anterior spermatozoon extremity (*ASE*). **b–e** Correlative cross-sections showing the progressive appearance of axonemes. **f–m** Correlative cross-sections of the ornamented area of Region I. Note the presence of lateral expansions (*LE1* and *LE2*), spine-like bodies (*SB*) and the first mitochondrion (*M1*) along this area. A large amount of glycogen (*G*) also appears in the posterior area of Region I. **n, o** Cross-sections of Region II showing the decrease in the number of cortical microtubules (*CM*). *Ax* axoneme, *Ax1* first axoneme, *C1* and *C2* centrioles of the first and second axonemes, *EO* external ornamentation of the plasma membrane. Scale bars = 300 nm.

Fig. 3. Spermatozoon of *Hypoderaeum conoideum* (Regions II and III). **a, b** Cross-sections of Region II showing the appearance of the second mitochondrion (*M2*). **c-h** Correlative cross-sections of the nuclear region or Region III showing the transition of characters toward the posterior extremity of the spermatozoon. **i** Positive test of Thiéry for glycogen (*G*). *CM* cortical microtubules, *D* doublets, *N* nucleus, *S* singlets. Scale bars = 300 nm.

Fig. 4. Schematic reconstruction of the spermatozoon of *Hypoderaeum conoideum*. In order to make the diagram clearer, granules of glycogen were omitted in the longitudinal section. *ASE* anterior spermatozoon extremity, *Ax1* and *Ax2* first and second axoneme, *C1* and *C2* centrioles of the first and second axoneme, *CM* cortical microtubules, *D* doublets, *EO* external ornamentation of the plasma membrane, *G* glycogen, *LE1* and *LE2* lateral expansions, *M1* and *M2* first and second mitochondrion, *N* nucleus, *PM* plasma membrane, *PSE* posterior spermatozoon extremity, *S* singlets, *SB* spine-like bodies.

Table 1. Available data on the ultrastructure of the spermatozoon in the Echinostomatoidea.

Families and species	Spermatozoon characters											References
	TS	TAx	LE	EO	EO+CM	LEO	BCM	LMCM	M	SB	PSC	
ECHINOSTOMATIDAE												
Echinostoma caproni	V	9+'1'	+	+	+	AntA	2	AntS	1	-	Ax/N?	Iomini and Justine (1997)
Hypoderaeum conoideum	V	9+'1'	+	+	+	AntA	2	AntS	2	+	N	Present study
FASCIOLIDAE												
Fasciola gigantica	V	9+'1'	+	+	+	AntA	2	AntS	1	+	N	Ndiaye et al. (2004)
Fasciola hepatica	V	9+'1'	+	+	+	AntA	2	AntS	1	+	N	Ndiaye et al. (2003)

AntA, anterior part of the anterior region; AntS, anterior region of the spermatozoon; Ax, axoneme; BCM, number of bundles of cortical microtubules; EO, external ornamentation of plasma membrane; EO+CM, association of external ornamentation with cortical microtubules; LE, lateral expansion; LEO, location of external ornamentation; LMCM, location of maximum number of cortical microtubules; M, number of mitochondria; N, nucleus; PSC, posterior spermatozoon character, SB, spine-like bodies; TAx, type of axoneme; TS, type of spermatozoon; +/-, presence/absence of considered character; ?, doubtful data.

Figure 1

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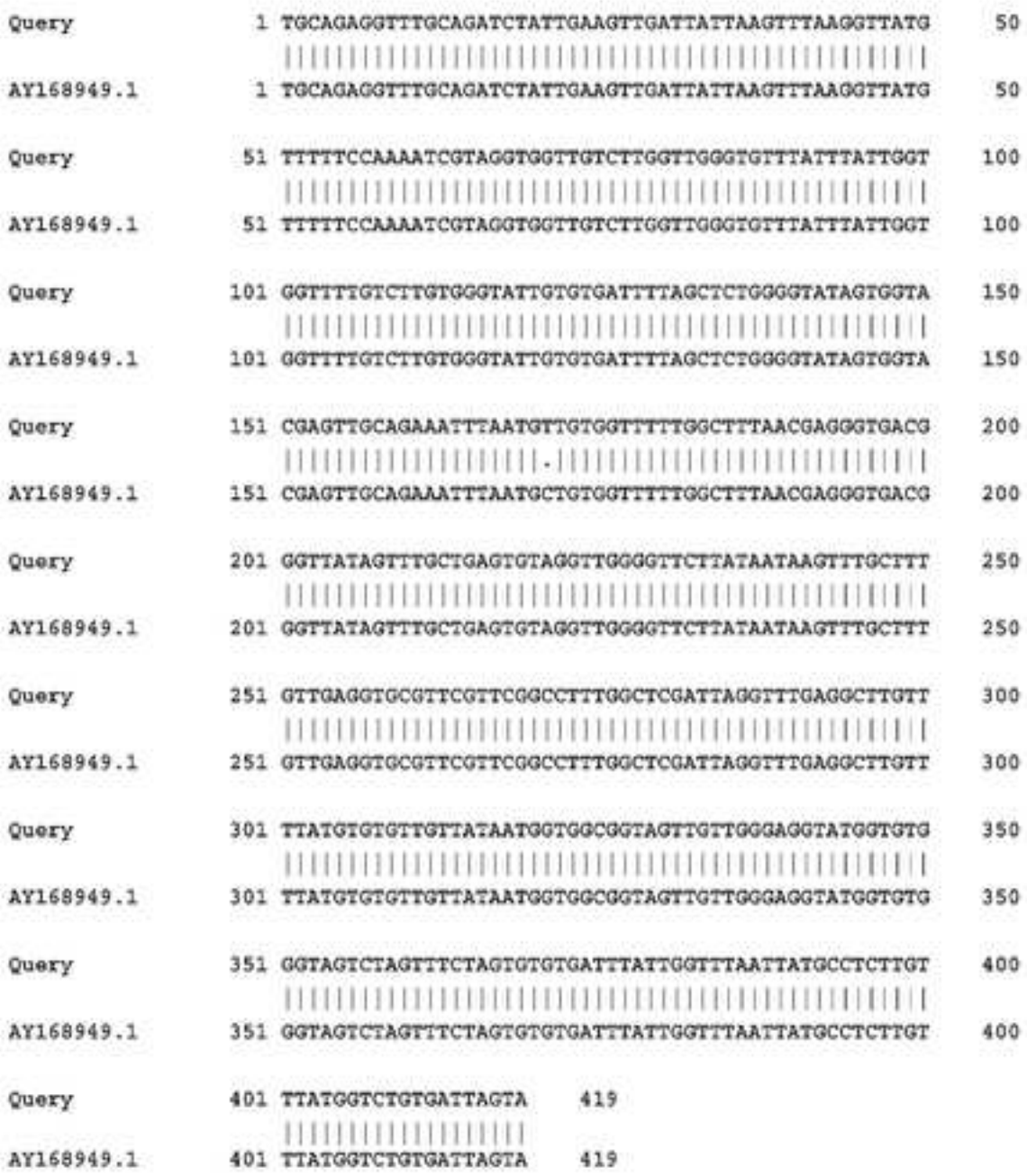

Figure 2

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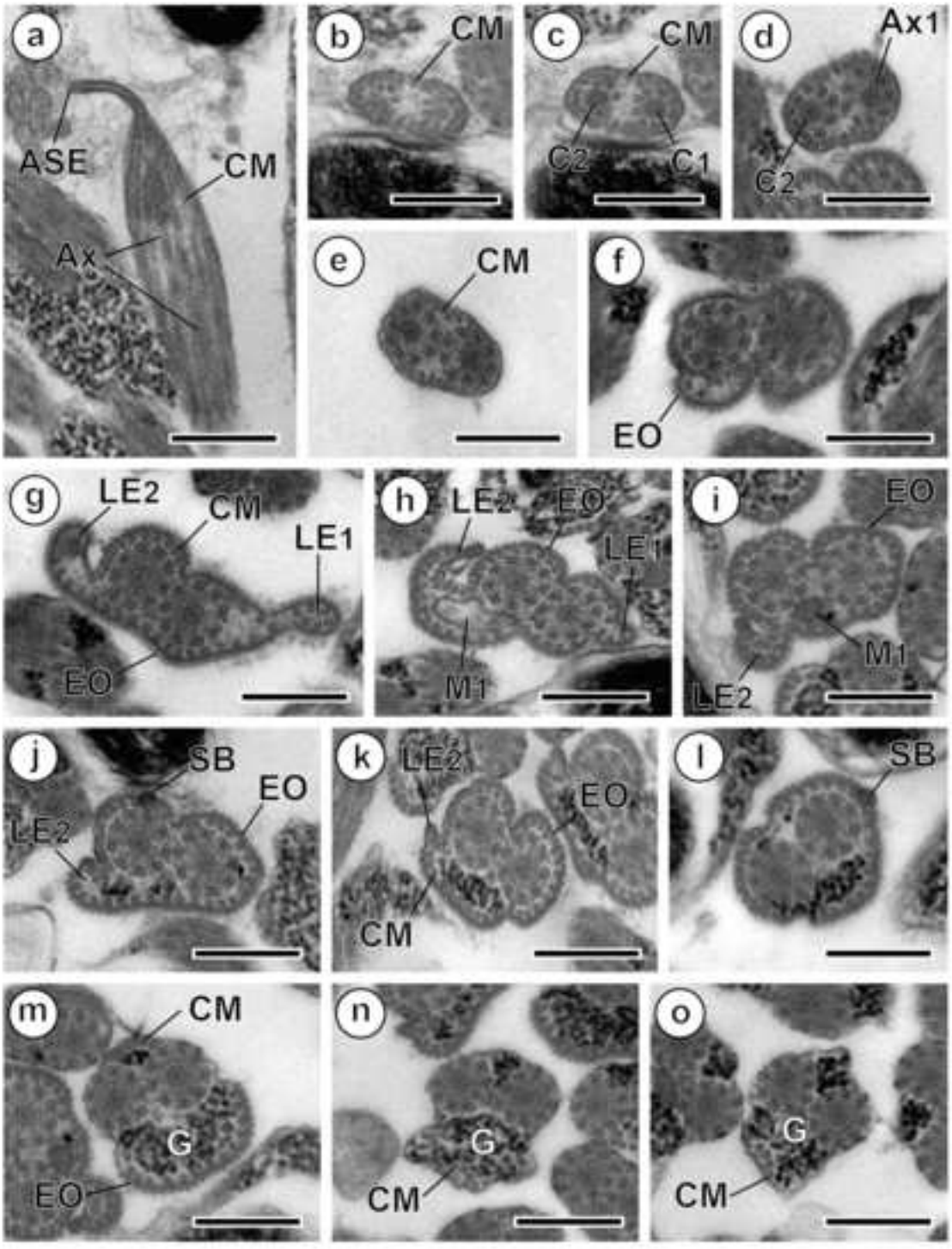

Figure 3

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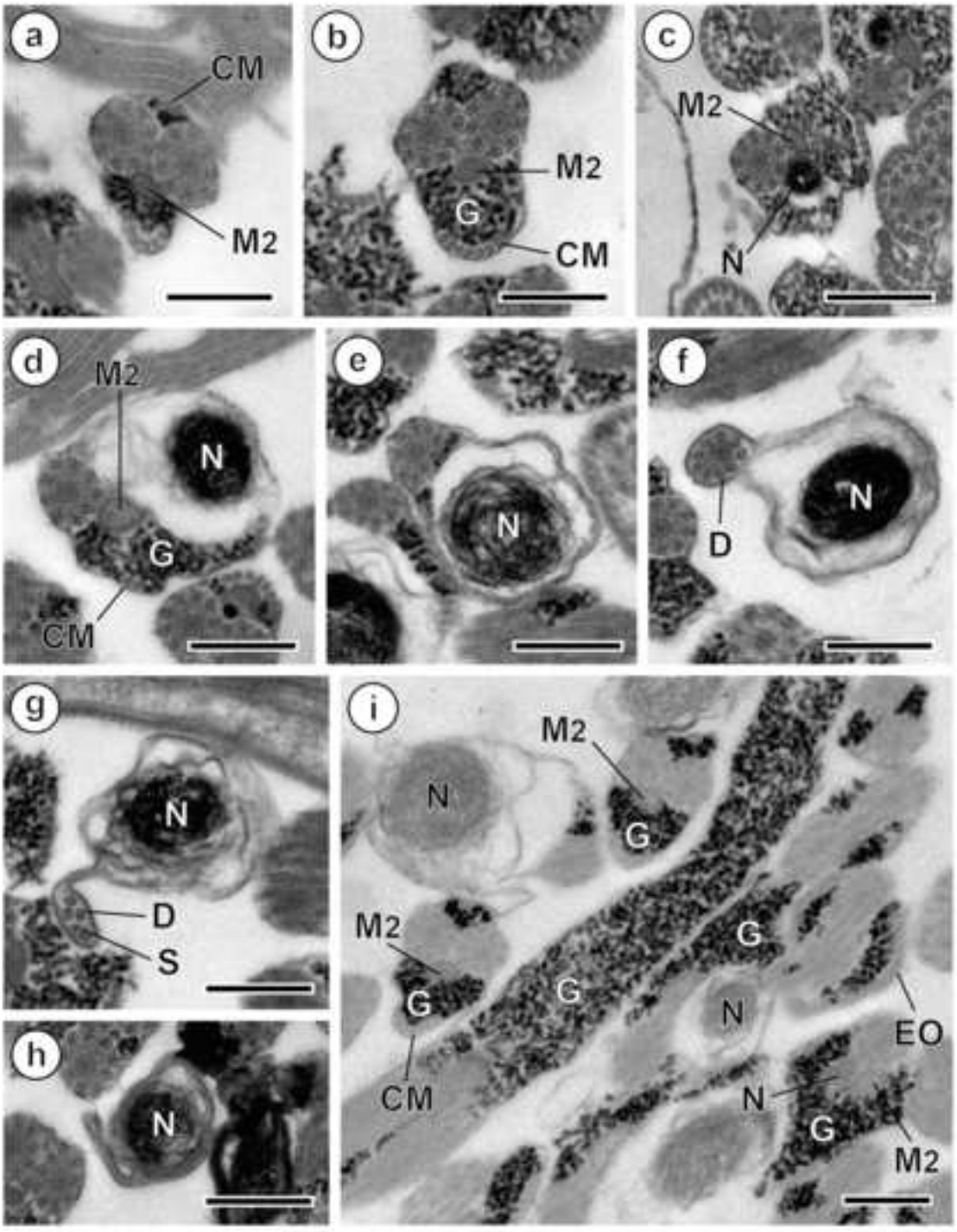

Figure 4

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