

**The Opecoelidae sperm model and its contribution to phylogeny: spermatozoon  
ultrastructural particularities of *Allopodocotyle pedicellata* (Plagioporinae, Digenea,  
Platyhelminthes)**

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## Abstract

The ultrastructure of the spermatozoon of *Allopodocotyle pedicellata* (Digenea, Opascoelidae), an intestinal parasite of the teleost *Sparus aurata* (gilt-head bream), is described by means of transmission electron microscopy (TEM). The mature spermatozoon possesses two axonemes of the 9+“1” trepaxonematan pattern, an anterior electron-dense material, two mitochondria, a nucleus, external ornamentation of the plasma membrane, spine-like bodies and parallel cortical microtubules distributed into two bundles. The anterior electron-dense material and the morphology of the anterior mitochondrion are the noteworthy characters that distinguish the spermatozoon of *A. pedicellata* from those of most opascoelids. However, a comparative study with the remaining opascoelids described so far, reveals similarities in their ultrastructural organisation. Thus, the sperm model is established for the Opascoelidae and it is compared to mature spermatozoa of other digeneans, particularly those belonging to the family Opistholebetidae and the superfamilies Allocreadioidea and Brachycladioidea or their related sister groups. The morphology of the posterior spermatozoon extremity, the disposition of cortical microtubules and the association of external ornamentation with cortical microtubules and spine-like bodies appear as interesting elements with phylogenetic significance for the Opascoelidae.

**Keywords:** TEM; ultrastructure

## 1. Introduction

The Opcoelidae is a large family of digeneans currently including over 90 genera and 900 species (Bray et al., 2016). The vast majority of species are parasites of marine and freshwater teleosts (Cribb, 2005). Despite the complex structure, diverse content and substantial species richness of the family, the phylogenetic relationships and systematic position of the Opcoelidae have always been controversial. Cribb (2005) included this family within the Allocreadioidea and emphasized that the status of the superfamily was not stable. More recent molecular findings, place the Opcoelidae in either the superfamily Opcoeloidea (Littlewood et al., 2015) or in the Brachycladioidea (Curran et al., 2006). Finally, Bray et al. (2016) found the Opcoelidae as a monophyletic group, but considering the Plagioporinae, this subfamily is found to be paraphyletic.

This historical account emphasizes the need for other sets of characters to better understand the complex relationships between digenean families or superfamilies. Spermatological characteristics have been useful for clarifying the evolutionary and phylogenetic relationships within the Plathelminthes especially in Cestoda (Bâ and Marchand, 1995; Justine, 1998; Levron et al., 2010) but also in Monogenea (Justine, 1991, 1993).

In the Digenea, many studies dealing with spermatozoa have been carried out to assess the phylogenetic usefulness of their ultrastructural characteristics (Bakhoun et al., 2015a; Kacem et al., 2015a, b; Miquel et al., 2013; Quilichini et al., 2015). Spermatological data in Opcoelidae are available in seven species, i.e. *Helicometra epinepheli* (Quilichini et al., 2011), *Helicometra fasciata* (Levron et al., 2003), *Nicolla testiobliquum* (Quilichini et al., 2007a), *Nicolla wisniewskii* (Quilichini et al., 2007b), *Opcoeloides furcatus* (Miquel et al., 2000), *Podocotyloides magnatestis* (Diagne et al., 2016) and *Poracanthium furcatum* (Levron et al., 2004a).

1 In the present study we compare the structure of the spermatozoon of *Allopodocotyle*  
2 *pedicellata* with those of other opecoelids in order to establish the sperm pattern in the family.  
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4 Moreover, the sperm model of the Opecoelidae is compared with mature spermatozoa from  
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6 the Opistholebetidae, the Allocreadioidea and Brachycladioidea and its phylogenetic  
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8 significance is discussed.  
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## 11 12 13 14 **2. Materials and methods** 15

16 Specimens of *Allopodocotyle pedicellata* (Stossich, 1887) Pritchard, 1966 were  
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18 collected alive from the digestive tract of *Sparus aurata* L., caught in the Gulf of Gabès off  
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20 Chebba (Tunisia) during October 2015. Adult digeneans were rinsed with a 0.9% NaCl  
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22 solution and fixed in cold (4 °C) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at  
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24 pH 7.4, rinsed in 0.1 M sodium cacodylate buffer at pH 7.4, post-fixed in cold (4 °C) 1%  
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26 osmium tetroxide with 0.9% potassium ferricyanide in the same buffer for 1 h, rinsed in Milli-  
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28 Q water, dehydrated in ethanol series and propylene oxide, embedded in Spurr resin and  
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30 polymerized at 60 °C for 72 h. Ultrathin sections (60–90 nm) of the seminal vesicle were cut  
31  
32 on a Reichert-Jung Ultracut E ultramicrotome. Several sections placed on 200 mesh copper  
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34 grids were stained with uranyl acetate and lead citrate according to Reynolds' methodology  
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36 (1963). Ultrathin sections placed on gold grids were stained with periodic acid,  
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38 thiocarbohydrazide, and silver proteinate according to the cytochemical technique established  
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40 by Thiéry (1967) for evidencing granules of glycogen. Finally, all stained grids were  
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42 examined on a Jeol 1010 transmission electron microscope, operating at an accelerating  
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44 voltage of 80 kV, in the “Centres Científics i Tecnològics” of the University of Barcelona  
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46 (CCiTUB).  
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## 58 **3. Results** 59 60 61 62 63 64 65



1 The interpretation of several cross- and longitudinal sections of the mature  
2 spermatozoon of *Allopodocotyle pedicellata* enable us to describe its ultrastructural  
3 organization by distinguishing three regions from the anterior to the posterior spermatozoon  
4 extremity (Figs 1–4).  
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9 Region I (Figs 1a–h and 4I) constitutes the anterior spermatozoon extremity. It exhibits  
10 in its anterior tip the presence of one axoneme of the 9+“1” trepaxonematan pattern and an  
11 electron-dense material (Figs 1a and 4I). In a more posterior zone, the centriole of the second  
12 axoneme appears in the area containing the electron-dense material (Figs 1b and 4I). When  
13 both axonemes are present, cross-sections show about 22 cortical microtubules (Fig. 1c). This  
14 is the maximum number of cortical microtubules in the sperm cell. Cortical microtubules  
15 surround one of the axonemes (Figs 1c and 4I) before their distribution into two bundles (Figs  
16 1d–f, h and 4I). In a posterior area of region I, cross-sections show the presence of external  
17 ornamentation of the plasma membrane, the anterior mitochondrion and spine-like bodies.  
18 The external ornamentation is located on the ventral side of the sperm cell (mitochondrial  
19 side) and is associated with cortical microtubules and spine-like bodies (Figs 1e–g and 4I).  
20 The spine-like body contains a sort of electron-dense vesicle (Fig. 1g). The anterior  
21 mitochondrion consists in a mitochondrial cord with mitochondrial bulges (Figs 1e–h and 4I).  
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41 Region II (Figs 1i, j and 4II) corresponds to the middle part of the mature  
42 spermatozoon. It is a transitional area before the nuclear region. There are two axonemes in its  
43 proximal part and parallel cortical microtubules arranged into two bundles (Figs 1i and 4II).  
44 The distal part of region II shows the posterior mitochondrion (Figs 1j and 4II). It is  
45 interesting to note the decrease in the number of cortical microtubules from 13 (Fig. 1i) to 11  
46 (Fig. 1j).  
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56 Region III (Figs 2a–h and 4III) is the nuclear and posterior area of the spermatozoon. It  
57 is characterized by the presence of nucleus, two axonemes, parallel cortical microtubules  
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distributed into two submembranous bundles and granules of glycogen (Figs 2a–c and 4III). In its proximal area, the posterior mitochondrion, two axonemes, nucleus, granules of glycogen, and cortical microtubules are present (Figs 2a and 4III). Towards the extremity the posterior mitochondrion disappears and the nucleus increases its size (Figs 2b, c and 4III). The first axoneme disorganizes (Figs 2c, d and 4III) and the number of cortical microtubules decreases to about 7 (Fig. 2d). In a more posterior area of region III, the second axoneme disorganizes (Figs 2e and 4III) and the nucleus progressively reduces its size and disappears (Figs 2e–g and 4III). Finally, cortical microtubules disappear and thus, only a few granules of glycogen are present in the posterior tip of the male gamete (Figs 2g–h and 4III).

The granules of glycogen are irregularly distributed along the mature spermatozoon (from the anterior mitochondrion zone to the posterior spermatozoon tip), as evidenced by the cytochemical test of Thiéry (Fig. 3).

#### 4. Discussion

The ultrastructural organization of the mature spermatozoon of *Allopodocotyle pedicellata* follows the architecture reported in other opecoelids, presenting two axonemes of the 9+“1” pattern of Trepaxonemata (Ehlers, 1984), external ornamentation of the plasma membrane, two mitochondria, a nucleus, parallel cortical microtubules and spine-like bodies (Diagne et al., 2016; Levron et al., 2003, 2004a; Miquel et al., 2000; Quilichini et al., 2007a,b, 2011).

##### 4.1. Spermatological organization in the Opecoelidae

The location of ultrastructural characteristics along the mature spermatozoon of all opecoelids described until now, allow distinguishing three types of characters useful to establish the sperm model in this family:

#### 4.1.1. First type of characters: anterior characters

Anterior spermatozoon characteristics such as the association “external ornamentation + cortical microtubules”, the posterior location of the external ornamentation and spine-like bodies are present in *A. pedicellata* and in all opistholebetid and opecoelid species except in *Helicometra fasciata* (Levron et al., 2003) (Table 1). A similar organization of these anterior characters has been described in the Cryptogonimidae (Foata et al., 2012; Quilichini et al., 2009; Ternengo et al., 2009), Dicrocoeliidae (Agostini et al., 2005; Bakhoun et al., 2015b) and Lepocreadiidae (see Quilichini et al., 2015). On the other hand, there are digenean spermatozoa containing the above mentioned anterior characters with a different location of the external ornamentation. This is the case of the apocreadiid *Neoapocreadium chabaudi* (Kacem et al., 2010) or the notocotylids *Notocotylus neyrai* and *N. noyeri* (Ndiaye et al., 2003a, 2015a).

#### 4.1.2. Second type of characters: cortical microtubules and mitochondria

In the male gamete of *A. pedicellata*, two bundles of cortical microtubules are clearly observed as in other opecoelid and opistholebetid species (Table 1). Within the Digenea, mature spermatozoa exhibiting two bundles of parallel cortical microtubules are frequently reported. This is the case of the allocreadiid *Crepidostomum metoecus* (Quilichini et al. 2007c), the opistholebetid *Heterolebes maculosus* (Quilichini et al., 2010) or the acanthocolpids *Stephanostomum murielae* and *Stephanostomoides tenuis* (Bakhoun et al., 2015a). However, it is interesting to remark that only one field of cortical microtubules has been described in species belonging to the Hemiuroidea (Dione et al., 2016; Ndiaye et al., 2012a, 2013a, 2014). Moreover, the absence of cortical microtubules has been reported in the mature spermatozoon of some didymozoids (Justine and Mattei, 1983; Pamplona-Basilio et

al., 2001). The character cortical microtubules (presence or absence, number and arrangement into one or two bundles) may be of great phylogenetic interest.

Another character that could characterize the sperm model of the Opecoelidae is the number of mitochondria. In the male gamete of *A. pedicellata* two mitochondria were observed. The first one is located in the ornamented zone and the second one in the transitional area of the spermatozoon (before the nuclear region) but also in the anterior part of the nuclear area. Among the spermatozoa of the other opecoelid species, two mitochondria have also been reported in most species although their position along the spermatozoon is variable (Diagne et al., 2016; Levron et al., 2004a; Quilichini et al., 2007a,b, 2011). Two opecoelids, namely *Opecoeloides furcatus* and *Helicometra fasciata* contain only one mitochondrion in the sperm cell (Table 1). Two mitochondria have also been reported in the mature spermatozoon of the opistholebetid *Heterolebes maculosus* (Quilichini et al., 2010).

#### 4.1.3. Third type of characters: posterior characters

The morphology of the posterior spermatozoon tip characterizes the sperm model of the Opecoelidae. According to the diagram established by Quilichini et al. (2010), *A. pedicellata* exhibits a type 1 spermatozoon since the posterior spermatozoon part presents the following sequence: posterior extremity of the second axoneme, posterior extremity of the nucleus and cortical microtubules in the posterior extremity. The type 1 or Opecoelidean type has been reported in mature spermatozoa of all opecoelid species described until now, with the exception of *Podocotyloides magnatestis* (Diagne et al., 2016), which exhibits the type 2 or Fasciolidean type. Nevertheless, in the character transition towards the posterior extremity of the sperm cell of *P. magnatestis*, it is interesting to remark that the second axoneme disappears before cortical microtubules (Diagne et al., 2016).

Rather than the sequence of characters toward the posterior spermatozoon we propose in the present study, to use only the posterior spermatozoon character in order to minimize the variation of characters toward the posterior spermatozoon extremity. Moreover, in some spermatological descriptions it is difficult if not nearly impossible to establish the sequence of characters in the spermatozoon posterior part because some characters disappear at the same level (Bakhoun et al., 2015b). Hence, the single character in the posterior spermatozoon tip of *A. pedicellata* is cortical microtubules. Its description in the male gamete of *A. pedicellata* confirms the particularity of this character for the Opecoelidae and Opistholebetidae (Table 1).

Among the digenean spermatozoa two other morphologies of the posterior spermatozoon extremity have been described. For example, digenean mature spermatozoa containing nucleus as the posterior character were reported in the Fasciolidae (Ndiaye et al., 2003b, 2004), Fellodistomidae (Kacem et al., 2015a), Mesometridae (Bakhoun et al., 2012, 2013a,b), Pronocephalidae (Ndiaye et al., 2011, 2012b) or Zoogonidae (Levron et al., 2004b; Kacem et al., 2015b), as well as in the recent study of the opecoelid *P. magnatestis* (Diagne et al., 2016). On the other hand, the presence of one axoneme was identified as the posterior character in the male gamete of species belonging to the Allocreadiidae (Quilichini et al., 2007c), Collyriclidae (Bakhoun et al., 2014), Pleurogenidae (Bruňanská et al., 2014; Miquel et al., 2013) or the Sclerodistomidae (Ndiaye et al., 2013b).

#### 4.2. Particularity of the mature spermatozoon of *A. pedicellata*

An ultrastructural character that allows distinguishing the mature spermatozoon of *A. pedicellata* is the anterior electron-dense material. It is located around the second axoneme arranged as a discontinuous submembranous layer. This particularity is reported for the first time in the mature spermatozoon of the Opecoelidae. In fact, among the Opecoelidae a

continuous layer of electron-dense material seems to be present in the mature spermatozoon of *Helicometra epinepheli* (Fig. 1c in Quilichini et al., 2011) and *H. fasciata* (Fig. 11 in Levron et al., 2003) although authors have not mentioned its presence. However, in *H. epinepheli* an electron-dense material in the centre of the apical cone of the spermatozoon was described (Quilichini et al., 2011). In the case of *Podocotyloides magnatestis*, the anterior electron-dense material was observed not only in the periphery of the sperm cell, but also in the central cytoplasm (Diagne et al., 2016). With respect to remaining opecoelid and opistholebetid species described until now, anterior electron-dense material has never been reported (Table 1). In other digenean spermatozoa, a continuous layer of electron-dense material has been reported under different names (Bâ et al., 2011; Bakhoum et al., 2015b; Ndiaye et al., 2015b; Quilichini et al., 2015).

Another interesting character found in *A. pedicellata* that distinguishes its mature sperm cell from those of the remaining opecoelids studied until now is the morphology of the anterior mitochondrion. In the present study we describe for the first time in the Opecoelidae a mitochondrion constituted by a mitochondrial cord and several mitochondrial bulges. This type of mitochondrion was previously reported in the spermatozoa of the cryptogonimid *Aphallus tubarium* (Foata et al., 2012), and the lepecoreadiids *Holorchis micracanthum* (Bâ et al., 2011) and *Opechona bacillaris* (Ndiaye et al., 2015b).

#### 4.3. Contribution of spermatological characteristics in opecoelidean phylogeny

As stated in the introduction, the systematic and phylogenetic status of opecoelids remains controversial and a multidisciplinary approach is recommended in order to elucidate the complex relationships of this large digenean family. Based on molecular studies, the Opecoelidae was placed in the Allocreadioidea (Olson et al., 2003), the Brachycladioidea (Curran et al., 2006) or the Opecoeloidea (Littlewood et al., 2015). However, the close

relationship between Opecoelidae and Opistholebetidae is widely accepted (Bray et al., 2016; Olson et al., 2003).

Available spermatological data from Opecoelidae emphasises several similarities between Opecoelidae and Opistholebetidae. These similarities are: (i) the association “external ornamentation + cortical microtubules”, (ii) the position of the external ornamentation, (iii) the spine-like bodies, (iv) the two bundles of parallel cortical microtubules, (v) the presence of two mitochondria, and (vi) the posterior spermatozoon tip exhibiting only cortical microtubules.

These ultrastructural findings confirm the close relationship between the Opecoelidae and Opistholebetidae as indicated in recent molecular results in which the Opistholebetidae is reduced to a subfamily status (Bray et al., 2016).

With regards to relationships between Opecoelidae and Allocreadiidae, spermatological characters observed in species belonging to both families reveal several differences between their respective spermatozoa. The major differences between these families concern the morphology of the anterior and posterior spermatozoon extremities. Thus, spermatological studies reveal that there are no close relationships between Opecoelidae and Allocreadiidae.

On the other hand, spermatological differences were also reported between opecoelid + opistholebetid species and the acanthocolpids *Stephanostomum murielae* and *Stephanostomoides tenuis* (Bakhoun et al., 2015a). Considering the Acanthocolpidae, a member of the Brachycladioidea (see Curran et al., 2006), the spermatological findings do not support the close relationships between Opecoelidae + Opistholebetidae and Brachycladioidea.

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## Legends to figures

**Fig. 1.** Spermatozoon of *Allopodocotyle pedicellata*. (a and b) Cross-section of region I showing the anterior spermatozoon extremity with the first axoneme (Ax1), the anterior electron-dense material (ADM) and the centriole of the second axoneme (C2). Note the discontinuous layer of the anterior electron-dense material. (c) Cross-section showing the appearance of cortical microtubules (CM) forming a continuous layer of about 22. (d) Cross-section showing two bundles of cortical microtubules (1+10) and external ornamentation of the plasma membrane (EO). (e and f) Posterior part of region I characterized by the presence of the anterior mitochondrion and the external ornamentation (EO) associated with cortical microtubules and spine-like bodies (SB). The mitochondrion is constituted by a mitochondrial cord (Mc) and mitochondrial bulges (Mb). The maximum number of cortical microtubules is around 14 (2+12). (g) Detail of a spine-like body showing a vesicle under the plasma membrane. Note the morphology of the anterior mitochondrion. (h) Posterior extremity of the anterior mitochondrion. The number of cortical microtubules is about 20 (7+13). (i and j) Region II or transitional area before nuclear region showing two axonemes, the posterior mitochondrion (M2) and two bundles of cortical microtubules, which maximum number decrease from 13 (6+7) in (i) to 11 in (j). G, granules of glycogen. Scale bars (a–f, h–j): 300 nm, (g): 100 nm.

**Fig. 2.** Spermatozoon of *Allopodocotyle pedicellata*. (a) Region III or nuclear area showing the simultaneous presence of the posterior mitochondrion (M2) and the nucleus (N). (b and c) Cross-sections where the posterior mitochondrion disappears while the nucleus, both axonemes (Ax1, Ax2) and cortical microtubules (CM) are still present. Note the disorganization of the first axoneme exhibiting doublets (D). (d) Cross-section showing only the nucleus, the second axoneme and cortical microtubules (about 8). (e and f) Cross-sections showing the disorganization and posterior disappearance of the second axoneme. (g and h)



1 Cross-sections where only cortical microtubules and granules of glycogen (G) are observed in  
2 the posterior spermatozoon tip. S, singlets. Scale bars: 300 nm.  
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4 **Fig. 3.** Spermatozoon of *Allopodocotyle pedicellata*. Granules of glycogen (G) evidenced by  
5 the cytochemical test of Thiéry in different regions of the sperm cell. CM, cortical  
6 microtubules; M1, anterior mitochondrion; M2, posterior mitochondrion; N, nucleus. Scale  
7 bar: 500 nm.  
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10 **Fig. 4.** Schematic organisation of the spermatozoon of *Allopodocotyle pedicellata*. ADM,  
11 anterior electron-dense material; ASE, anterior spermatozoon extremity; Ax1, first axoneme;  
12 Ax2, second axoneme; C1, centriole of the first axoneme; C2, centriole of the second  
13 axoneme; CM, cortical microtubules; D, doublets; EO, external ornamentation of the plasma  
14 membrane; G, granules of glycogen; M1, anterior mitochondrion; M2, posterior  
15 mitochondrion; Mb, mitochondrial bulge; Mc, mitochondrial cord; N, nucleus; PSE, posterior  
16 spermatozoon extremity; S, singlets; SB, spine-like body.  
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**Table 1.** Comparative spermatological characters in the Opecoelidae and Opistholebetidae.

Family	Species	Anterior region				Middle region		Posterior region	Reference
		ADM	EO+CM	LEO	SB	BCM	M	PSC	
Opecoelidae	<i>Allopodocotyle pedicellata</i>	+	+	Post	+	2	2	CM	Present study
	<i>Helicometra epinepheli</i>	+	+	Post	+	2	2	CM	Quilichini et al. (2011)
	<i>Helicometra fasciata</i>	-	-	NA	-	2	1	CM	Levron et al. (2003)
	<i>Opecoeloides furcatus</i>	-	+	Post	+	2	1	CM	Miquel et al. (2000)
	<i>Poracanthium furcatum</i>	-	+	Post	+	2	2	CM	Levron et al. (2004a)
	<i>Nicolla testiobliquum</i>	-	+	Post	+	2	2	CM	Quilichini et al. (2007a)
	<i>Nicolla wisniewskii</i>	-	+	Post	+	2	2	CM	Quilichini et al. (2007b)
	<i>Podocotyloides magnatestis</i>	+	+	Post	+	2	2	N	Diagne et al. (2016)
Opistholebetidae	<i>Heterolebes maculosus</i>	-	+	Post	+	2	2	CM	Quilichini et al. (2010)

ADM, anterior electron-dense material; BCM, number of bundles of cortical microtubules; CM, cortical microtubules; EO+CM, association external ornamentation and cortical microtubules; LEO, location of external ornamentation; M, number of mitochondria; N, nucleus; NA, not applicable; Post: posterior location of the external ornamentation; PSC, posterior spermatozoon character; SB, spine-like body; ?, unknown data; +/-, presence/absence.

Figure 1

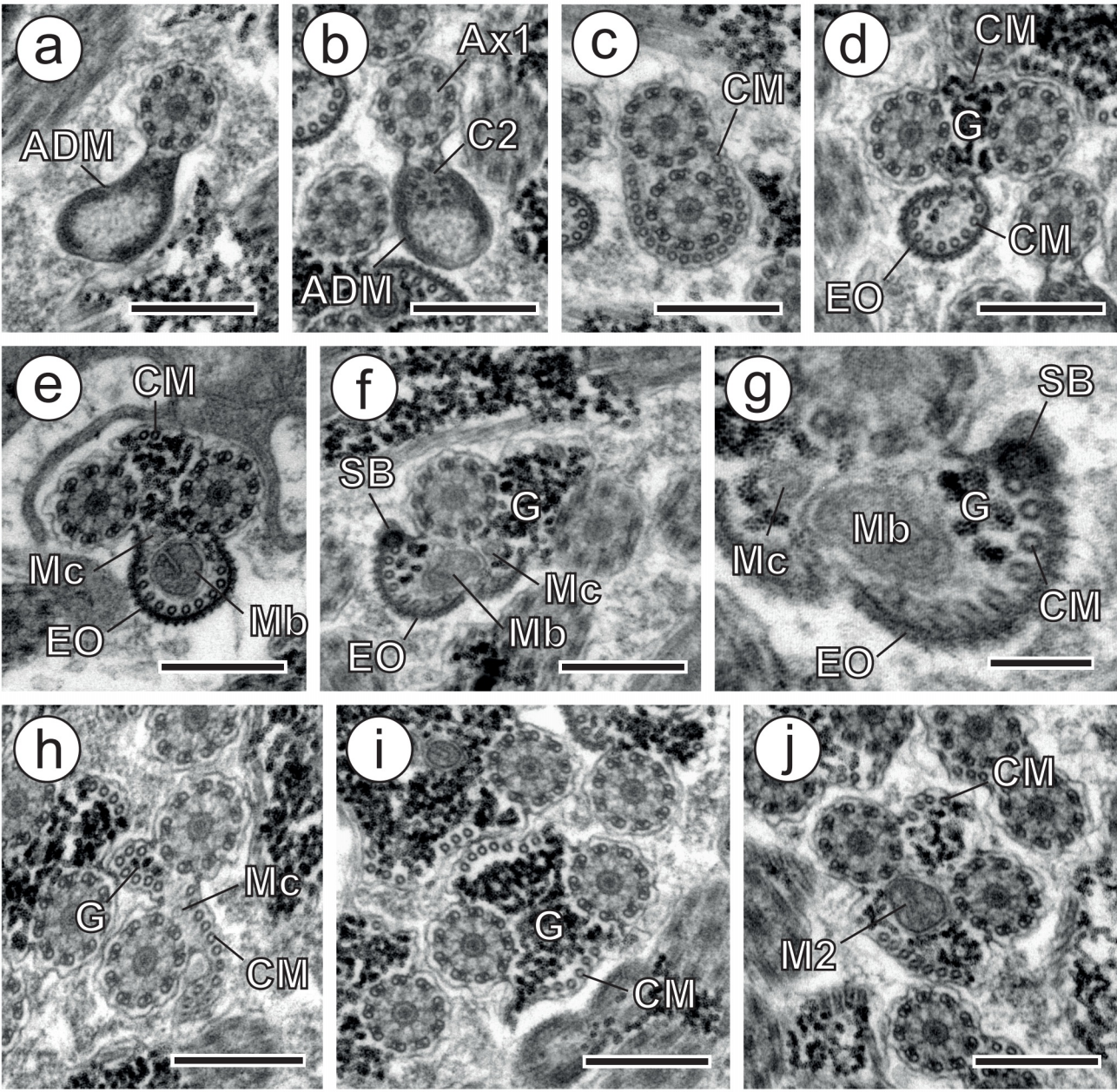




Figure 2

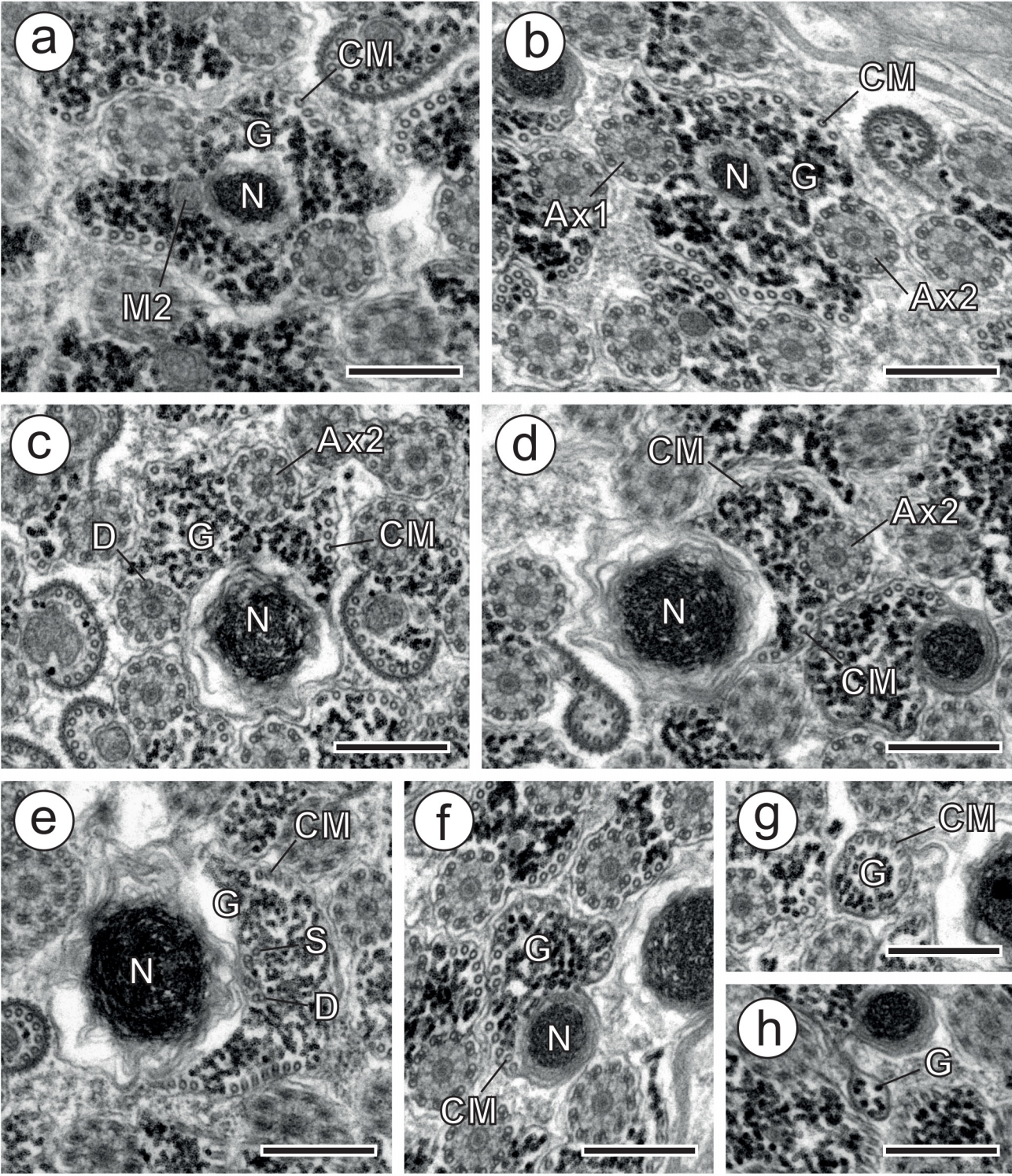




Figure 3

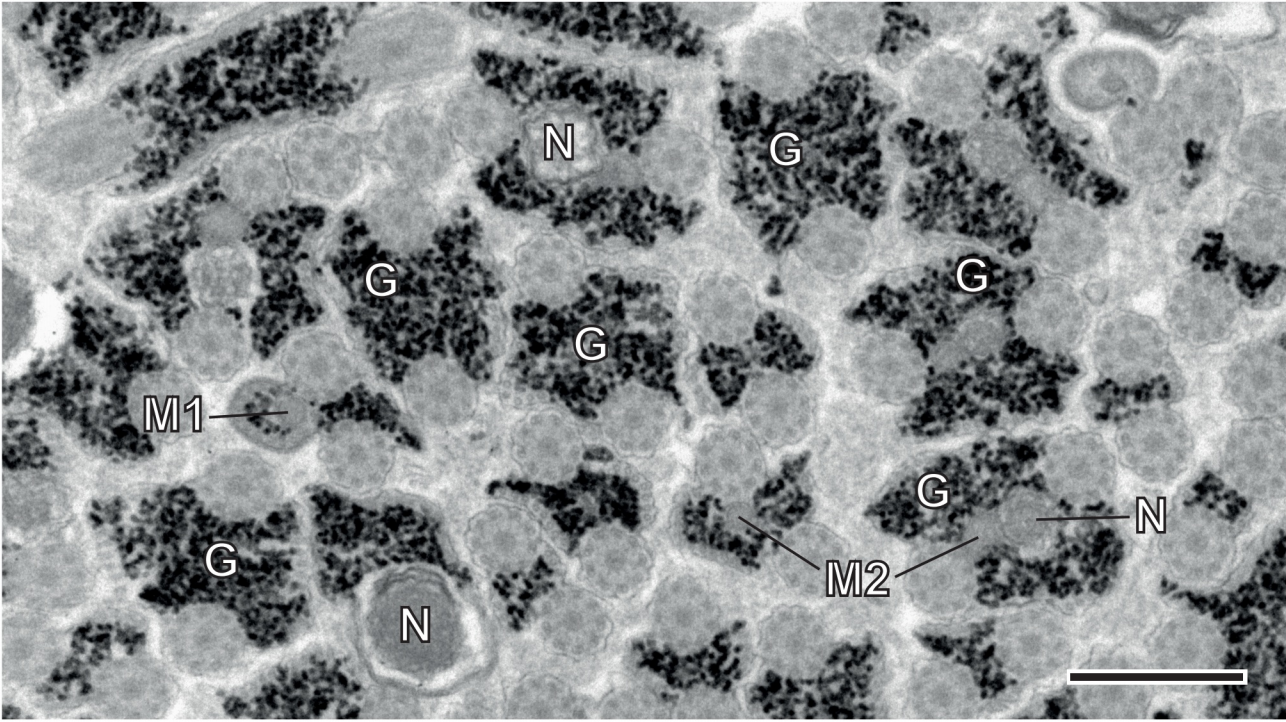


Figure 4

