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Ultrastructure of spermatozoa and spermatogenesis in *Octopus minor* (Sasaki, 1920) (Cephalopoda: Octopoda)

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ABSTRACT

Octopus minor is widely distributed along the coastal areas of the west Pacific Ocean. This paper investigates spermatozoa, spermiogenesis from the testes, and spermatophores using light and electron microscopy. Mature spermatozoa are about 650 µm long. The head includes mainly the acrosome and nucleus. The acrosome consists of a striated cone surrounded by a single helix. The nucleus is cylindrical, homogeneous and of high electron density. The neck is short and connected with the head through the internal nuclear fossa. The axoneme connects the head, neck and tail. The tail is divided into middle, principal and final pieces. The '9 + 9 + 2' structure is surrounded by a mitochondrial sheath, which includes 9–11 mitochondria in transverse section. The sperm morphology is compared with the ultrastructure of other cephalopod spermatozoa, and taxonomic and phylogenetic implications are discussed.

Introduction

Octopus minor (Sasaki, 1920) is widely distributed along the coastal areas of the west Pacific Ocean, including from the Bohai Sea to the South China Sea, Korean Peninsula, as well as south of Sakhalin to the whole of Japan (Okutani et al. 1987), with *Octopus variabilis* (Sasaki, 1929) as a frequent synonym used in Japan and China (Yamamoto 1942; Roper et al. 1984; Dong 1988; Okutani 2000; Lu et al. 2012). Because it is economically important, many studies have been conducted on the ecology, reproductive biology, physiology and genetics of this cephalopod species (Taki 1944; Iwakoshi et al. 2000; Seol et al. 2007; Zuo et al. 2011; Cheng et al. 2012; Qian et al. 2013). However, its sperm morphology and spermatogenesis have yet not been investigated.

The morphology and formation of cephalopod spermatozoa have considerable potential in systematics (Healy 1989), and much research has been conducted on sperm morphology and spermiogenesis of cephalopods (Franzén 1955, 1967; Maxwell 1974, 1975, 1983; Fields and Thompson 1976; Arnold and Williams 1978; Healy 1990a, 1990b, 1993; Selmi 1996; Giménez-Bonafé et al. 2002; Ribes et al. 2002; Martínez et al.

2007; Roura et al. 2009, 2010a, 2010b). However, only a few *Octopus* species have been investigated regarding sperm ultrastructure (e.g. *Octopus vulgaris* Cuvier, 1797: Galangau and Tuzet 1968a; 1968b; Giménez-Bonafé et al. 2002; Ribes et al. 2002; *O. bimaculatus* Verrill, 1883: Longo and Anderson 1970). To provide further elements to the discussion of anatomical homologies within octopodid spermatozoa, the present study provides a detailed description of sperm ultrastructure in *O. minor*.

Materials and methods

Octopus minor specimens were caught in the coastal waters of Qingdao, Yellow Sea, in January 2010. Four live mature males were anesthetised using 5–10% magnesium chloride ($MgCl_2$), and then dissected. The range of total weight and dorsal mantle length of the samples was 393–468 g and 86–92 mm, respectively.

The spermatophores from three individuals were removed from the spermatophore storage sac, and then photographed under a light microscope with a digital camera (Olympus C-5050). The spermatophores were put into fresh filtered seawater at 19°C. After about 15 min, the sperm mass was released with the eversion of the ejaculatory apparatus. Sperm were then observed and measured under a light microscope at 200× magnification.

For scanning electron microscopy (SEM), the sperm mass of the spermatophore from one male was fixed in 3% glutaraldehyde for 4 h at 4°C, washed in 0.1 mol/L phosphate-buffered saline, dehydrated in a graded ethanol series followed by isoamyl acetate, and then critical point dried and finally sputter-coated with gold. The samples were observed under a JEOL JSM-840 SEM operated at 5 kV.

For transmission electron microscopy (TEM), testis tissue and the sperm mass were diced into 1 mm³ pieces and fixed in 3% glutaraldehyde for 4 h at 4°C, rinsed for 30 min in 0.1 mol/L phosphate buffered saline, then placed into a 1% osmium tetroxide solution for 1 h, dehydrated in a graded ethanol series and embedded in Spurr's resin. Ultrathin sections were cut on an Ultracut Eultramicrotome, double stained with uranyl acetate and lead citrate, then observed and photographed using a JEOL JEM-1200EX TEM microscope with an accelerating voltage of 120 kV.

Results

Octopus minor spermatophores are approximately 50 mm long (Figure 1A) and are composed of a sperm mass, a cement body and an ejaculatory apparatus (Figure 1B–D). There is a long cap thread (about 150 mm long) at the back of the ejaculatory apparatus (Figure 1E–F). There is a connective complex between cement body and ejaculatory apparatus (Figure 1G). Initially, sperm mass expelled from the everted spermatophore shows a net-like structure and is inactivated. Activated by seawater, sperm begin swimming, and then gradually separate. Finally, they are found intertwined with each other by their long tails at a certain level (Figure 1H–I).
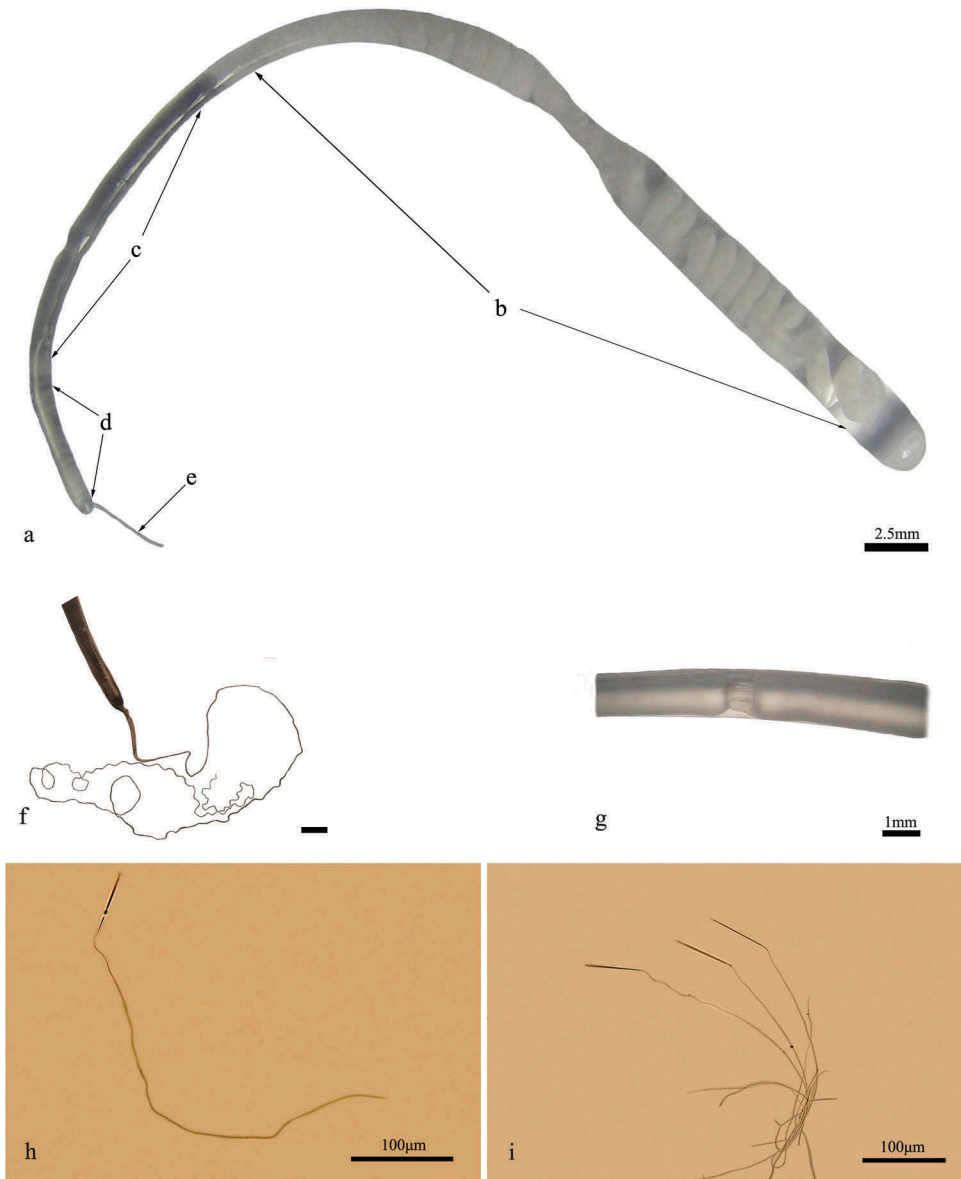
Figure 1. Spermatophore and spermatozoa of *Octopus minor* under the light microscope. (A) Spermatophore; (B) sperm mass; (C) cement body; (D) ejaculatory apparatus; (E) the cap thread; (F) enlarged ejaculatory apparatus and cap thread structure; (G) region of connection of cement body and ejaculatory apparatus; (H) spermatozoon; (I) spermatozoa with entangled flagella.

Spermatogenesis

Spermatogenesis in *O. minor* is a continuous process according to TEM analysis of the testis (Figure 2A). Although primary and secondary spermatocytes were observed in our preparations (Figure 2B–D), the description herein is focused on spermiogenesis. The
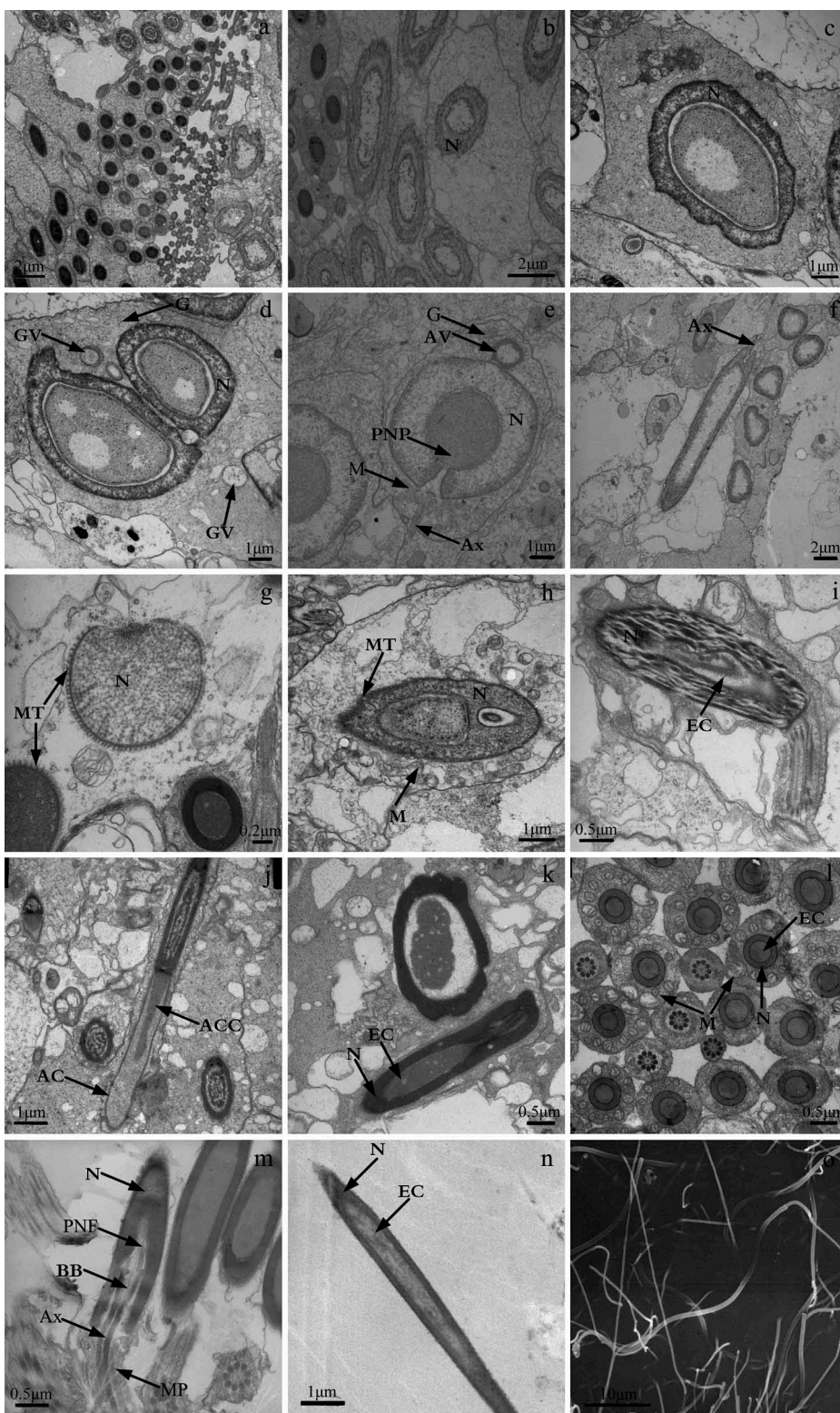
spermatogenesis process is divided into six stages, according to the processes of nuclear reorganisation and elongation, organelle change and electron density. As will be seen, nuclear reorganisation is the most conspicuous aspect of spermiogenesis in *O. minor*.

Spermatid I (Figure 2E): oval or anomalous. Round acrosomal vesicle is close to karyotheca, and is filled with a substance of low electron density. The posterior nuclear pocket is opposite to the acrosomal vesicle. The outer region of the nucleus is more electron dense than the inner core. The electron density of the outer nucleus is lower than the inner. Spermatid II (Figure 2F): The distribution of chromatin looks granular. Centriole starts to develop from the centrosome at the posterior nuclear pocket. Microtubules are observed at the karyotheca periphery. Spermatid III (Figure 2G): the cell and its nucleus keep elongating. Microtubules are more obvious. Granular chromatin becomes much larger and electron density of the nucleus increases. Spermatid IV (Figure 2H, I, J): chromatin within the nucleus is slender and fibrous. Acrosome is cone-shaped. The surrounding cytoplasm contains numerous small vesicles. Mitochondria are irregularly distributed around the nucleus but they start to recede. Spermatid V (Figure 2K, L): the degree of aggregation of nuclear chromatin strengthens further. The electron-lucent uniform endonuclear channel can be observed in the centre of the nucleus. Mitochondria are distributed around the nucleus, and there are nine to 11 in transverse section. Spermatid VI (Figure 2M–O): the nucleus is uniform, electron dense. Electron density of endonuclear channel is lower than that of nucleus, and acrosome is tapered. Electron density of acrosomal vesicle and its lacuna are lower than that of nucleus. There is a mitochondrial sheath in the middle piece of the tail, and the microtubule disappears at the karyotheca periphery.

Sperm structure

The mature spermatozoon of *Octopus minor* is about 650 μm long. The head is about 25 μm and consists of a helical acrosome (Figure 3A–D) and a long straight nucleus (Figure 3F). The neck is about 2 μm (Figure 3G), and the tail is about 620 μm (Figure 1B).

The entire head is surrounded by an irregular skirt membrane (Figure 3A–E). The acrosome (Figure 3A–D) is about 5.5 μm long, has six to nine whorls, and consists of an electron-dense acrosomal vesicle surrounded by an electron-lucent acrosomal vesicle lacuna. The diameters of the acrosomal vesicle and complex (acrosomal vesicle+ lacuna) are about 400 nm and 1.2 μm, respectively. The widest part of the straight nucleus is 550–700 nm (Figure 3F). In sagittal section, the acrosomal vesicle bears outer protuberances that form the spiral whorls, and internally several equidistant striations spaced 60 nm apart (Figure 3A, B). There is a sub-acrosomal lacuna between the base of the acrosome and the nucleus (Figure 3A). The nucleus is straight, cylindrical, and about

Figure 2. Observation of spermatogenesis of *Octopus minor* under TEM and SEM. (A) Testis; (B) primary spermatocyte; (C) secondary spermatocyte; (D) secondary spermatocyte of binucleate phase; (E) spermatid; (F) spermatid; (G) spermatid III; (H–J), spermatid IV; (K) longitudinal section of spermatid V; (L) transverse section of spermatid V; (M) longitudinal section of spermatid VI at neck; (N) longitudinal section of spermatid VI at nucleus; (O) mature spermatozoon; Ax: axoneme; AC: acrosome; ACC: acrosomal cone; AV: acrosomal vesicle; BB: basal body; EC: endonuclear channel; G: Golgi bodies; GV: Golgi vesicle; M: mitochondria; MP: middle piece; MT: microtubules; N: nucleus; PNF: posterior nuclear fossa; PNP: posterior nuclear pocket.
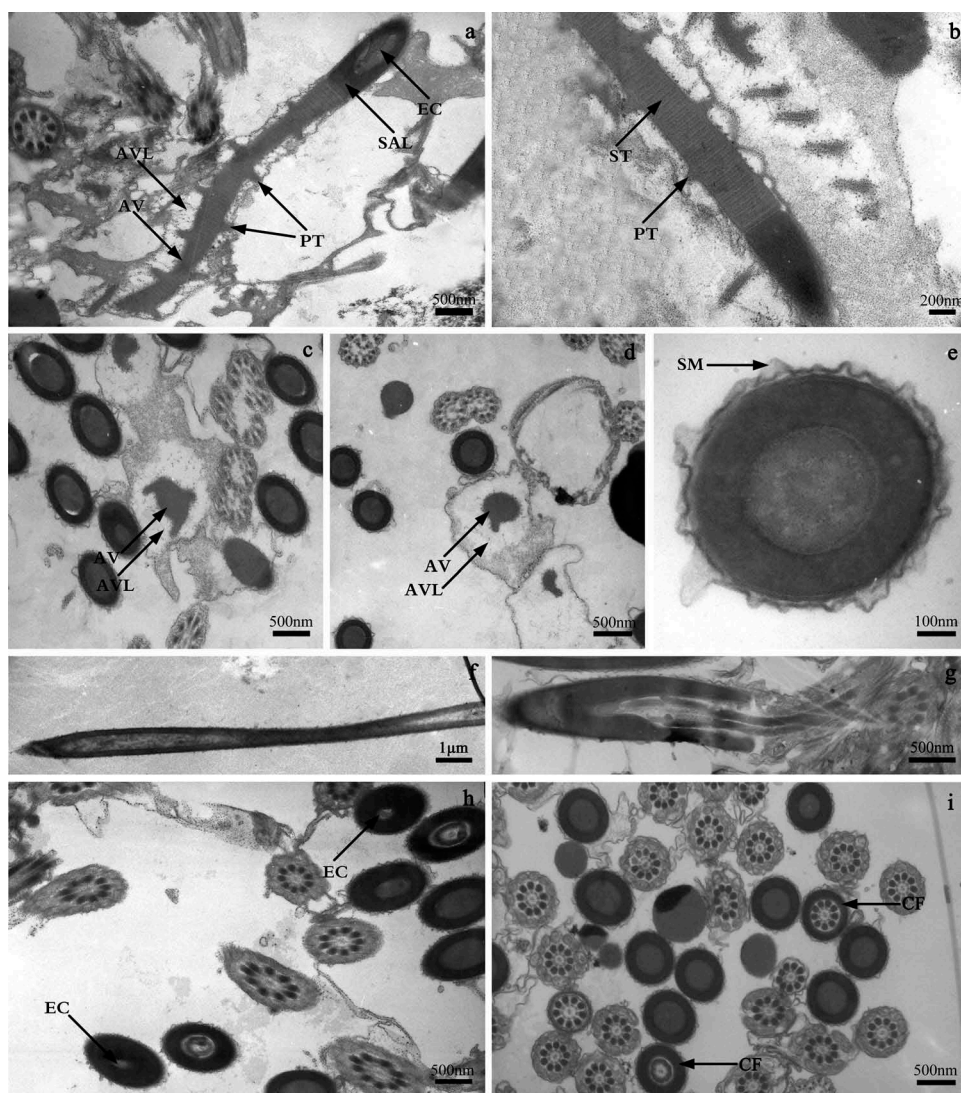
Figure 3. Observation of head and neck of spermatozoon of *Octopus minor* under TEM. (A–B) Longitudinal section of spermatozoon at acrosome and anterior nucleus; (C–D) transverse section of acrosome; (E–G) longitudinal section of spermatozoon at nucleus, endonuclear channel and neck; (H) transverse section of spermatozoon at anterior nucleus; (I) transverse section of posterior nucleus. AV: acrosomal vesicle; AVL: acrosomal vesicle lacuna; PT: protuberance; SAL: sub-acrosomal lacuna; ST: striation; SM: skirt membrane; EC: endonuclear channel; CF: coarse fibre.

550-700 nm in width. The chromatin of the nucleus is uniformly electron dense, and there is an electron-lucent endonuclear channel at the centre of the nucleus (Figure 3B–D, G, H), which apparently does not communicate with the acrosomal vesicle (Figure 3A).

At the posterior region of the nucleus (i.e. neck), the nucleus bears an indentation (the centriolar fossa) that accommodates the centriole (Figure 3G). Anteriorly, the

centriolar fossa connects with the nuclear fossa, and posteriorly the centriole connects with the axoneme (Figure 3G). A transverse section through the neck reveals that the axoneme is located at its centre and is surrounded by nine bundles of equidistant outer coarse fibres that lie parallel to it and extend into the tail.

The tail originates from the centriole located in the centriolar fossa, and its main structure is the axoneme and nine outer coarse fibres. The tail can be divided into three regions: middle, principal and end pieces. The middle piece is composed of the axoneme, outer coarse fibres and the mitochondrial sheath (Figure 4A–E). The mitochondrial sheath is composed of nine to 11 elliptical mitochondria surrounding and parallel to the axoneme-coarse fibre complex. There is a fibrous sheath (Figure 4A, B) surrounding the middle piece. The principal piece is the longest part of the tail (Figure 4F–H), and the coarse fibres within it taper gradually. There is some electron-lucent discontiguous matter between the membrane and the coarse fibres (Figure 4G), observed at the principal piece. In addition, there are some digitiform tubers on the membrane (Figure 4B, C), some of which are twice as long as the sperm diameter. The nine outer coarse fibres are absent at the end piece (Figure 4I).

Discussion

Sperm length in cephalopods is very variable, the average length being 35 μm in *Nautilus* (Arnold and Williams 1978), 54 μm in *Loligo* (Maxwell 1975), 115–120 μm in *Spirula* (Healy 1990a), 124 μm in *Sepia* (Maxwell 1975), 156 μm in *Rossia* (Fields and Thompson 1976) and 139.5–144.5 μm in *Vampyroteuthis* (Healy 1989). Sperm size ranges from 280 μm to 1130 μm in the reported species of Octopodidae (Maxwell 1974; Healy 1993; Selmi 1996; Roura et al. 2009, 2010a, 2010b).

The acrosome can be classified as an important distinguishing character in cephalopods which varies by length and the number of helices and striations as well as by the inner cone. Comparison of the acrosome lengths in Octopodidae studied to date reveals that of *Graneledone* to be the longest ever reported (*G. gonzalezi* Guerra et al. 2000: 9.89 ± 0.46 μm, Roura et al. 2009). Morphologically, the sperm of *O. minor* resembles the other species in Octopodidae, Eledoninae and Graneledoninae (Galangau and Tuzet 1968a; Leik 1970; Longo and Anderson 1970; Giménez-Bonafé et al. 2002; Ribes et al. 2002; Roura et al. 2009), because it consists of a single helix surrounding the acrosome (Figure 2O). This feature distinguishes it from *Bathypolypus* species in Bathypolypodinae with a double helix surrounding the acrosome (Roura et al. 2010a). By comparison with *Eledone* spp., Maxwell (1974) described an internal ladder-like structure in the mature helical acrosome of *Octopus*. From a phylogenetic point of view, Franzén (1967) proposed that the sperm of *Eledone* could be derived evolutionarily directly from that of *Octopus*. The presence of the acrosomal periodic striations is evidence of a close relationship between the two genera, and the structure might be a peculiarity of octopod spermatozoa (Selmi 1996). Furthermore, incirrate octopods have an inner cone with striations oriented perpendicularly to the long axis of the spermatozoon (Galangau and Tuzet 1968a; Leik 1970; Longo and Anderson 1970; Selmi 1996; Ribes et al. 2002; Roura et al. 2009, 2010a, 2010b). Cirrate octopods lack this structure, although two or three striations in *Opisthoteuthis persephone* (Berry 1918) are observed (Healy 1993).
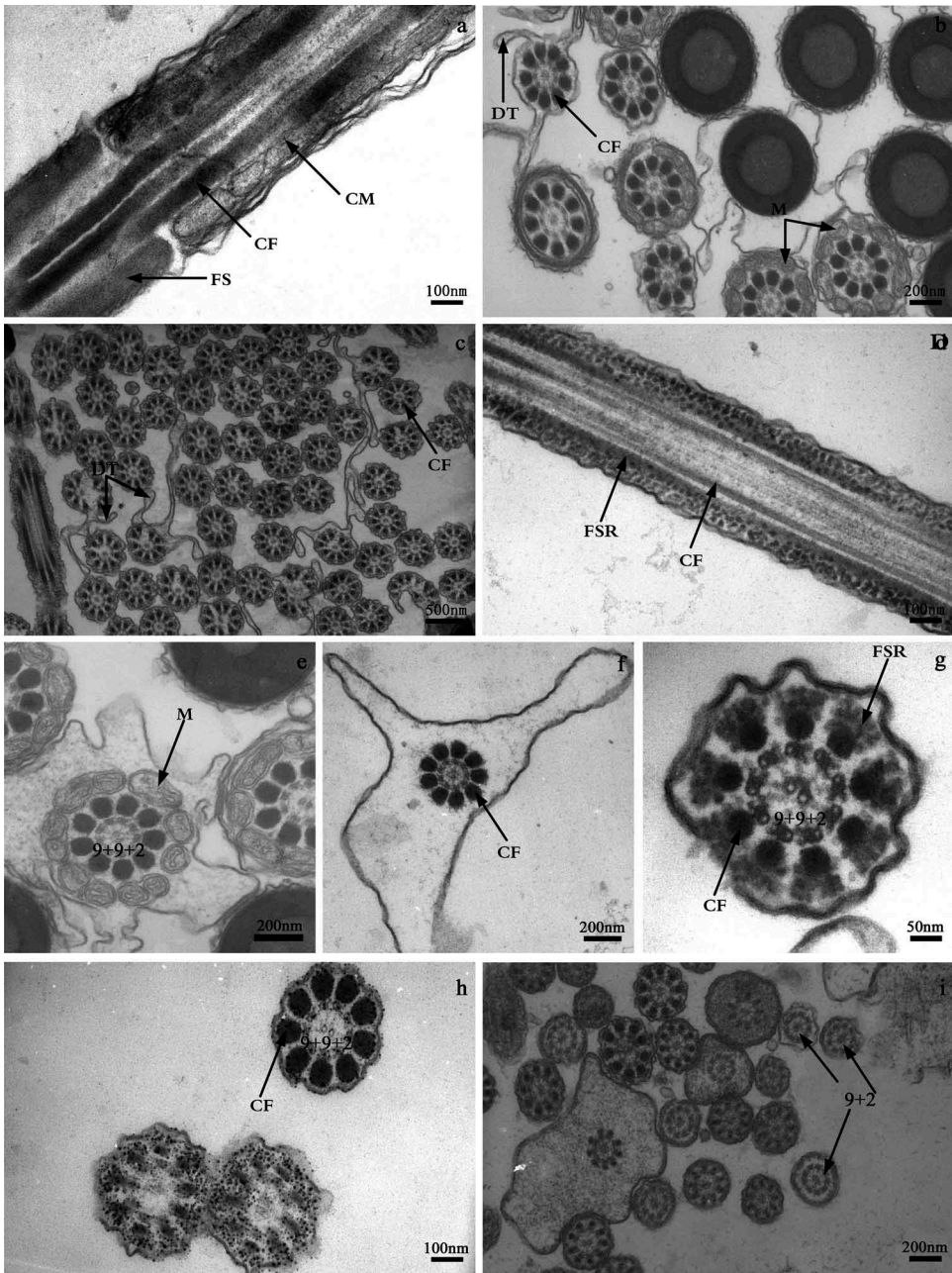
Figure 4. Observation of tail of spermatozoon of *Octopus minor* under TEM. (A) Longitudinal section of the mitochondria and fibrous sheath at middle piece of tail; (B) transverse section of the mitochondria and fibrous sheath at middle piece of tail; (C) transverse section at principal piece; (D) longitudinal section at principal piece; (E) transverse section of the mitochondria sheath at middle piece of tail; (F–H) different transverse sections at principal piece; (I) transverse section at end piece of tail. CM: chondriosomal mantle; CF: coarse fibre; DT: digitiform tuber; FS: fibrous sheath; M: mitochondria; FSR: fibrous sheath remnant.

The spermatozoa middle pieces of *Bathypolypus bairdii* (Verrill 1873) and *B. sponsalis* (Fischer and Fischer 1892) also contain the mitochondrial sheath, which have respectively 11 and nine bean-shaped mitochondria in transverse section, parallel to the coarse fibres (Roura et al. 2009). However, the arrangement of mitochondria in octopods is remarkably different from that in sepiolids, teuthoids and nautiloids. *Vampyroteuthis* spermatozoa have a triangular cluster of three or four mitochondria surrounding both centrioles (Healy 1989), and a mitochondrial sleeve is observed in most sepiolids and teuthoids (Maxwell 1975; Fields and Thompson 1976). *Nautilus pompilius* has two big extended mitochondria around the centriolar complex (Arnold and Williams 1978). All *Sepia* spp. (Sepiida), *Loligo* spp. and *Alloteuthis* spp. (both genera in Teuthoida) have a middle piece composed of a cylindrical mitochondrial sheath around a '9 + 9 + 2' arrangement (Maxwell 1975), but *Octopus* and *Eledone* species possess the complete mitochondrial sheath and true midpiece (Healy 1990a).

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