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ORIGINAL PAPER

Ultrastructural characters of the spermatozoon of the liver fluke *Opisthorchis viverrini* (Poirier, 1886) (Opisthorchiidae)

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Abstract The present study records the ultrastructural organization of the mature spermatozoon of *Opisthorchis viverrini* by means of transmission electron microscopy. The spermatozoon of *O. viverrini* is a filiform cell, tapered at both extremities. It exhibits the characteristics of type IV spermatozoon of digeneans, namely with two axonemes of the 9+'1' trepaxonematan pattern, external ornamentation of the plasma membrane associated with cortical microtubules that are in the posterior part of the anterior region of the sperm cell, and with two mitochondria. The maximal number of cortical microtubules is in the anterior part of the spermatozoon and arranged into two bundles. Other characteristics are spine-like bodies and a posterior extremity with only the second axoneme. Ultrastructural characters of the spermatozoon of *O. viverrini* are compared with those of other known digeneans belonging to the Opisthorchioidea, with particular emphasis on representatives of the family Opisthorchiidae. The main differences between *O. viverrini* and its congener *Opisthorchis felineus* are the spine-like bodies (present and absent, respectively) and the posterior spermatozoon character (axoneme and nucleus, respectively).

Keywords *Opisthorchis viverrini* · Opisthorchiidae · Digenea · Sperm characters · Ultrastructure

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Introduction

Among the Digenea, the family Opisthorchiidae includes numerous species with great importance in human health. In the genus *Opisthorchis*, there are agents of human opisthorchiasis caused by three fish-borne zoonotic species: *Opisthorchis felineus*, *Opisthorchis tenuicollis*, and *Opisthorchis viverrini*. The latter species is a liver fluke endemic and highly prevalent in the Southeast Asia region, particularly in the Mekong River basin. About 9 million people globally are estimated to be infected by *O. viverrini*. Moderate infections may be asymptomatic, but high-intensity and chronic infections are a critical risk factor for the development of several types of bile duct cancers or cholangiocarcinoma (Chai et al. 2005; Hung et al. 2013; Torgerson et al. 2014).

Ultrastructural characters of the spermatozoon and sperm development (spermatogenesis and spermiogenesis) have proved to be useful in systematics and phylogeny for several groups of Platyhelminthes (Justine 1991a, b, 1998, 2001; Bâ and Marchand 1995; Levron et al. 2010; Bakhoum et al. 2017). During recent decades, the study of ultrastructural characters and usefulness for phylogenetic inference in the Digenea has increased notably (Bakhoum 2012; Bakhoum et al. 2017). Researchers are focused on all the ultrastructural characters of the sperm cell of different families to evaluate their potential for phylogenetic inference (Bakhoum 2012; Quilichini et al. 2010a, 2011; Bakhoum et al. 2017). Of the family Opisthorchiidae, only the ultrastructure of spermatozoa

of *Clonorchis sinensis*, *Metorchis orientalis* and *O. felineus* is known (Jeong and Rim 1984; Liu and Pan 1990; Zhukova et al. 2014). The number of published TEM micrographs for *C. sinensis* and *M. orientalis* is not high. Furthermore, because of apparent misinterpretations of data, the ultrastructural organization of sperm cells of both species is not well resolved.

The aim of the present study is to provide demonstration and analysis of the ultrastructure of the mature spermatozoon of *Opisthorchis viverrini* and to compare results with the congener *O. felineus* and other digeneans, particularly those of the Opisthorchioidea.

Materials and methods

Sampling of specimens

In a study conducted in 2013 around Khon Kaen Lawa Lake wetland in the upper part of the Northeast of Thailand, cyprinid fish traditionally caught by local people were sampled for *Opisthorchis viverrini* metacercariae. The isolation of metacercariae was achieved by pepsin digestion. The fish were weighed and minced by electric blender before being immersed in an aqueous solution containing 0.25% pepsin, 1.5% HCl, and 0.85% NaCl. The mixture was then incubated at 37 °C for 2 h and the digested fish were filtered using three consecutive metal sieves (1100, 350 and 250 μm apertures). Filtered fish pellets were sedimented several times with normal saline in a sediment jar until the supernatant was clear (Pinlaor et al. 2013). Metacercariae in the sediment were then identified and counted using a stereomicroscope.

Five golden syrian hamsters (*Mesocricetus auratus*) were experimentally infected with 50 *O. viverrini* metacercariae using intragastric intubation. The animals were housed under standard conditions and fed a stock diet and water ad libitum. All procedures complied with National Experimental Animal Centre guidelines and the Animal Unit, Faculty of Medicine, Khon Kaen University (Thailand). The hamsters were sacrificed after 3 months post-infection and adult flukes were recovered from the liver and biliary system before fixing for transmission electron microscopy.

Transmission electron microscopy

Live adult flukes 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 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 procedure (Reynolds 1963). Stained ultrathin sections 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).

Cytochemical test of Thiéry

Sections placed on gold grids were treated according to the Thiéry test (Thiéry 1967) to reveal the ultrastructural localisation of glycogen. Thus, sections 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. Ultrathin sections were also examined in a JEOL 1010 transmission electron microscope in the CCiTUB.

Results

The mature spermatozoon of *Opisthorchis viverrini* is a filiform cell, tapered at both extremities and exhibiting the main characteristics or structures of digenean spermatozoa: two axonemes of the 9+'1' pattern of trepaxonematan Platyhelminthes, parallel cortical microtubules, mitochondrion, nucleus, external ornamentation of the plasma membrane, 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. 1, 2, and 3), a male gamete was divided into three regions (I to III).

Region I (Figs. 1a–k and 3I) corresponds to the anterior extremity of the spermatozoon. This region is mainly characterized by the presence of external ornamentation of the plasma membrane, spine-like bodies, and the first mitochondrion. The anterior tip of the spermatozoon is sharp and filiform (Figs. 1a–c and 3 (I)). The two axonemes of the 9+'1' trepaxonematan pattern are slightly longitudinally displaced one to another (Fig. 1a, b). In the anterior area of Region I, the two axonemes are surrounded by a continuous layer of submembranous cortical microtubules lacking attachment zones (Fig. 1d). Posteriorly, they become arranged in two fields and the four attachment zones appear (Fig. 1e). The principal area of this region exhibits external ornamentation of the plasma membrane and spine-like bodies (Figs. 1f–h and 3 (I)). In the posterior area of Region I, the first mitochondrion appears and spine-like bodies are still present (Figs. 1h–k and 3 (I)).

Region II (Figs. 1l, m; 2a, j; and 3 (II)) corresponds to the middle region of the spermatozoon and is mainly characterized

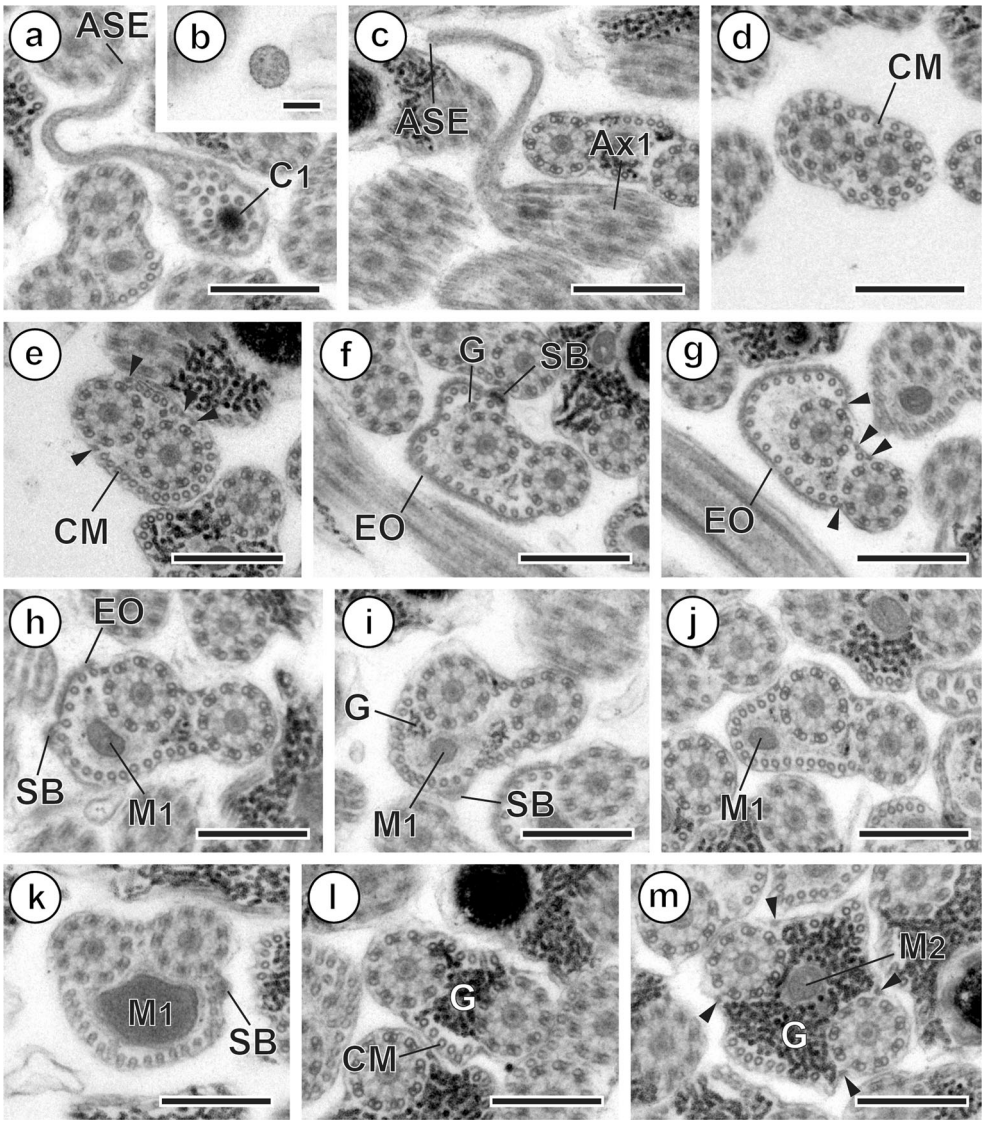

Fig. 1 TEM of *Opisthorchis viverrini* spermatozoon (regions I and II). **a**–**c** Cross- and oblique sections of the anterior spermatozoon extremity (*ASE*). **d** Continuous layer of submembranous cortical microtubules (*CM*). **e** Cross-section showing the disposition of cortical microtubules into two fields and the presence of four attachment zones (*arrowheads*). **f**–**k** Consecutive sections of region I from the appearance of the ornamentation of the plasma membrane *(EO*) to the posterior area containing the first mitochondrion (*M1*). Note the presence of spine-like bodies (*SB*) along this area. **l**, **m** Region II at the level of anterior part showing large amount of glycogen granules (*G*) and at the level of the second mitochondrion (*M2*). *Ax1* first axoneme, *C1* centriole of the first axoneme. *Scale bars* (a, c–m) = 0.3 μm; (b) = 0.1 μm

by having the second mitochondrion. The external ornamentation of the plasma membrane and spine-like bodies are no longer present. A large amount of glycogen appears as granular material along this region (Figs. 1l, m and 2a). The presence of the second mitochondrion characterizes the posterior area of Region II (Figs. 1m, 2a, and 3 (II)). One of the axonemes initiates its disorganization in the transition area between regions II and III (Figs. 2a, b and 3 (II, III)).

Region III (Figs. 2b–j and 3 (III)) corresponds to the nuclear and posterior extremity of the spermatozoon. The presence of the nucleus is the main characteristic of this region (Figs. 2b–g and 3 (III)). The transition toward the posterior extremity of the sperm cell is marked by (i) the disorganization of the first axoneme (Fig. 2b, c), followed by (ii) the disappearance of the second mitochondrion (Fig. 2d, e), by (iii) the disappearance of cortical microtubules (Fig. 2e, f), and by (iv) the disorganization of the second axoneme (Fig. 1g–i). During the progressive disorganization of the second axoneme, there is a reduction in nucleus size before its disappearance (Fig. 2g, h). It is noticeable that the four attachment zones are still present after the disappearance of the second axoneme (Fig. 2d). The posterior spermatozoon tip is characterized by the presence of doublets of the second axoneme (Fig. 2i).

The glycogenic nature of the granular material observed in regions II and III has been determined by means of the Thiéry test (Fig. 2j).

Discussion

Anterior spermatozoon extremity

The anterior extremity of the spermatozoon of *O. viverrini* is filiform and exhibits the two trepaxonematan axonemes (Ehlers 1984) of different lengths, which are slightly longitudinally displaced one to another. In fact, some microtubules of the

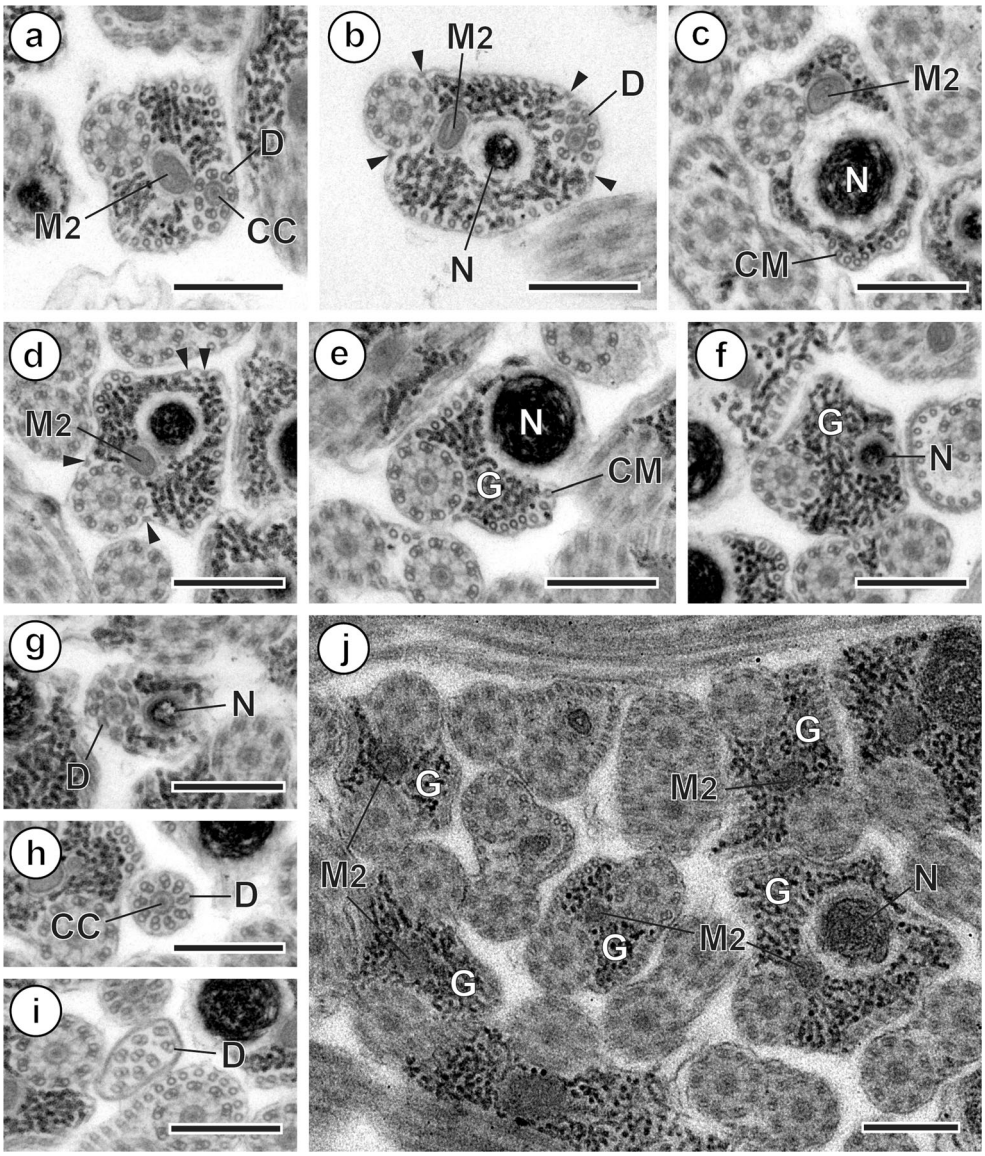

Fig. 2 TEM of *Opisthorchis viverrini* spermatozoon (regions II and III). **a**, **b** Disorganization of the first axoneme in the transition area of regions II and III. **c**–**i** Consecutive cross-sections toward the posterior spermatozoon tip showing progressive disappearance of the second mitochondrion (*M2*), cortical microtubules (*CM*), the nucleus (*N*), and the second axoneme. Note the presence of the four attachment zones after the disappearance of the second axoneme (*arrowheads*). **j** Glycogen labelling by means of Thiéry test. *CC* central core, *D* doublets, *G* granules of glycogen. *Scale bars* = 0.3 μm

second centriole are already present at the level of the first centriole. In the case of *O. felineus*, Zhukova et al. (2014) described an anterior extremity not filiform and exhibiting clearly shifted axonemes.

The anterior spermatozoon area of both opisthorchiid species exhibits the maximum number of cortical microtubules. A continuous submembranous layer of 26 cortical microtubules completely surrounds the male gamete of *O. viverrini*. In the case of *O. felineus*, the maximum number of cortical microtubules seems to be around 26–28, but their disposition differs from those observed in its congener; although they also occupy a single field but they do not surround the sperm cell completely (Zhukova et al. 2014). The location of the maximum number of cortical microtubules along the spermatozoon has recently been considered a potential character of different sperm models in the Digenea. Bakhoum et al. (2017) argued that two locations are possible for the maximum number of cortical microtubules: (a) in the anterior part or (b) in the middle or more posterior part (non-nuclear region) of the spermatozoon. In both *Opisthorchis* species examined thus far, the maximum number of cortical microtubules is in the anterior part of the male gamete.

External ornamentation

The external ornamentation of the plasma membrane is usually present in the spermatozoon of most digeneans. There are two aspects of the external ornamentation that have a potential for showing relationships within digeneans: (1) the presence/absence of ornamentation and location, if present, and (2) its association or not with cortical microtubules (Bakhoum et al. 2017). Three types of spermatozoa exist in digeneans according to the presence/absence and location of ornamentation. Though some species lack ornamentation, others have it in the anterior extremity, and most species present this character

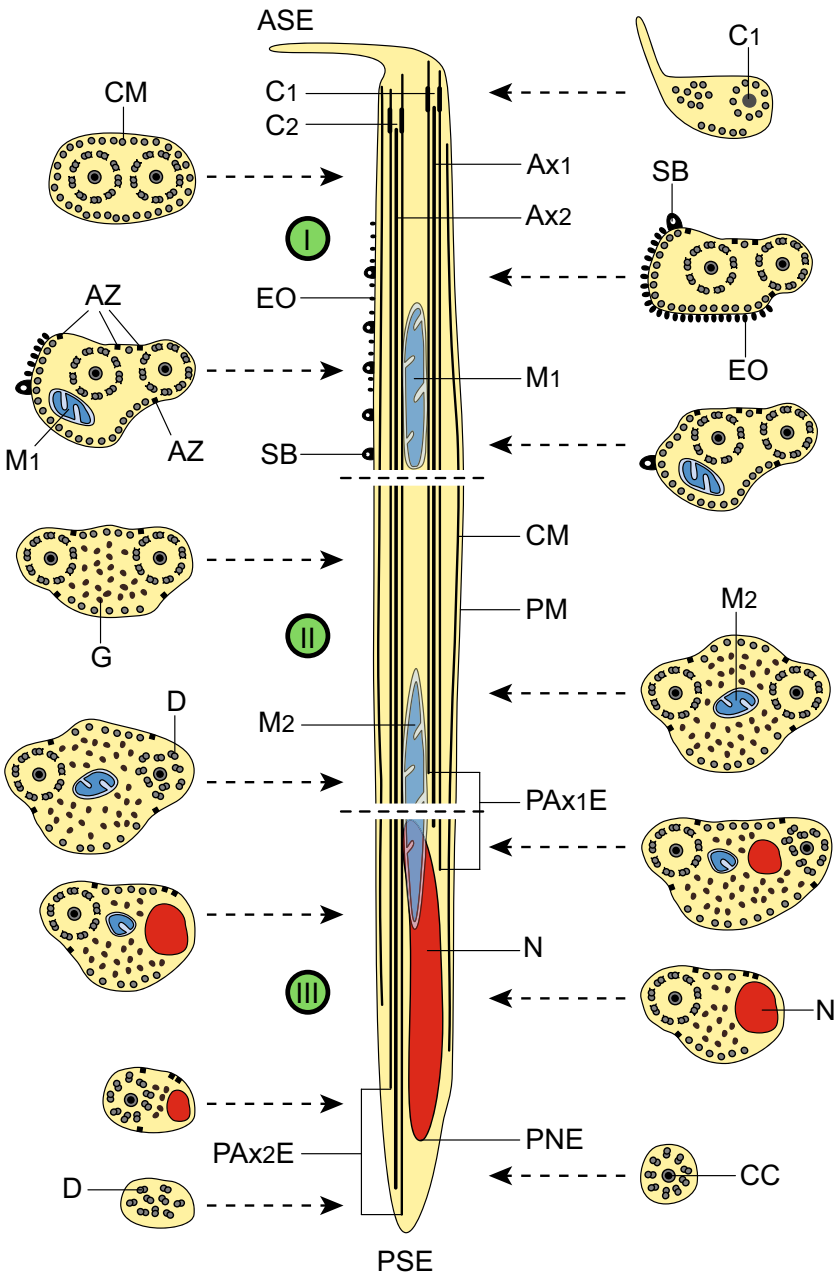

Fig. 3 Schematic reconstruction of the spermatozoon of *Opisthorchis viverrini*. In order to make the diagram clearer, granules of glycogen were omitted in the longitudinal section. *ASE* anterior spermatozoon extremity, *Ax1* first axoneme, *Ax2* second axoneme, *AZ* attachment zones, *C1* centriole of the first axoneme, *C2* centriole of the second axoneme, *CC* central core, *CM* cortical microtubules, *D* doublets, *EO* external ornamentation of the plasma membrane, *G* glycogen, *M1* first mitochondrion, *M2* second mitochondrion, *N* nucleus, *PAx1E* posterior extremity of the first axoneme, *PAx2E* posterior extremity of the second axoneme, *PM* plasma membrane, *PNE* posterior nuclear extremity, *PSE* posterior spermatozoon extremity, *SB* spine-like bodies

in the posterior part of the anterior region of the sperm cell (Quilichini et al. 2007, 2011). The external ornamentation of the plasma membrane is usually associated with cortical microtubules. However, a different type of ornamentation not associated with cortical microtubules was first described in *Pronoprymna ventricosa* (Faustulidae) and subsequently found in the male gamete of hemiurids, lecithasterids, and sclerodistomids (Quilichini et al. 2007, 2010b; Ndiaye et al. 2014, 2017). These two kinds of external ornamentation are considered for sperm models for digeneans (Bakhoum et al. 2017). Both *O. felineus* (Zhukova et al. 2014) and *O. viverrini* have external ornamentation associated with cortical microtubules and located in the anterior extremity of the spermatozoon. Authors do not mention the status of ornamentation for *Clonorchis sinensis* and *Metorchis orientalis* sperm, however (Jeong and Rim 1984; Liu and Pan 1990). Other species of the superfamily Opisthorchioidea (cryptogonimids and heterophyids) exhibit the ornamentation in a more posterior area of the spermatozoon (Bakhoum et al. 2009; Quilichini et al. 2009; Ternengo et al. 2009; Foata et al. 2012).

Spine-like bodies

Spine-like bodies, described for the first time in the opecoelid, *Opecoeloides furcatus* (Miquel et al. 2000), have since been generally found in the mature spermatozoon of numerous

digeneans (Bakhoum et al. 2017). However, in recent times, the usefulness of these spine-like bodies in making phylogenetic inference within the Digenea is unknown since older studies made no mention of these bodies in descriptions of sperm (Justine and Mattei 1982; Orido 1988; Bakhoum et al. 2017).

These spine-like bodies are prominent, submembranous electron-dense structures that contain a sort of vesicle (Miquel et al. 2000). Various authors analysed the periodicity between spine-like bodies; for some digeneans, a periodicity has been described, while in other species these ultrastructural elements are irregularly distributed along the sperm cell (Miquel et al. 2006; Kacem et al. 2010).

According to the present study and available data on the Opisthorchioidea, there is variation as to the presence/absence of spine-like bodies in the male gamete of representatives of this superfamily. These structures are described in the spermatozoon of cryptogonimids (Quilichini et al. 2009; Ternengo et al. 2009; Foata et al. 2012) and in the present study in the opisthorchiid *O. viverrini*. On the other hand, the sperm cells of heterophyids and *O. felineus* lack spine-like bodies (Bakhoum et al. 2009; Zhukova et al. 2014).

Mitochondria

The presence of mitochondria in the sperm cell is a consistent character for the Digenea. This has been the consensus since Burton (1972) reported the fusion of multiple mitochondria to form a long mitochondrion during spermiogenesis in the mature spermatozoon of a frog lung fluke (*Haematoloechus* sp.). Nevertheless, more than one mitochondrion has been described in spermatozoa of numerous digeneans to correlate with other characters such as cortical microtubules or the nucleus (Bakhoum et al. 2017). The observation of two parallel mitochondria in the mature spermatozoon of *Dicrocoelium hospes* strongly supports the possibility of the existence of more than one mitochondrion in some digenean sperm cells (Agostini et al. 2005). In the Opisthorchioidea, the number of mitochondria is variable between the species. One and two mitochondria have been observed in spermatozoa of cryptogonimids (Quilichini et al. 2009; Ternengo et al. 2009; Foata et al. 2012) and three in the heterophyid, *Euryhelmis squamula* (Bakhoum et al. 2009). In the opisthorchiids, *O. felineus* and *O. viverrini*, spermatozoa contain two mitochondria, one in the ornamented area and another in the nuclear region (Zhukova et al. 2014).

Posterior spermatozoon extremity

The posterior extremity of the spermatozoon with the second axoneme is similar in all the Opisthorchioidea with the sole exception of *O. felineus*, which presents the nucleus as the terminal structure (Bakhoum et al. 2009; Quilichini et al. 2009; Ternengo et al. 2009; Foata et al. 2012; Zhukova et al. 2014). Considering the transition of some characters (cortical microtubules, second axoneme, and nucleus) toward the posterior tip, Quilichini et al. (2010a) established three types of posterior spermatozoon extremities in the Digenea, namely opecoelidean, fasciolidean, and cryptogonimidean types. All the opisthorchioids ultrastructurally studied until now exhibit the cryptogonimidean type, with the above mentioned exception of *O. felineus* (Zhukova et al. 2014).

Conclusions

Mature spermatozoa of Opisthorchiidae species correspond to type IV of Bakhoum et al. (2017). This type is characterized by (i) two axonemes of the 9+'1' trepaxonematan pattern, (ii) an external ornamentation of the plasma membrane associated with cortical microtubules that are located in the posterior part of the anterior region of the sperm cell, (iii) maximum number of cortical microtubules in the anterior part of the spermatozoon, (iv) the organization of cortical microtubules into two bundles, and (v) two mitochondria. Two secondary characters such as the posterior spermatozoon extremity and the presence/absence of spine-like bodies allow us to distinguish *O. felineus* from *O. viverrini*. Future ultrastructural studies on other species of this family are needed to determine what other secondary characters, not considered in forming models of spermatozoa in the Digenea, might have use at the infrafamily level in making phylogenetic inferences in the Digenea. Available spermatological data on the Opisthorchioidea are concordant with results of molecular studies that show the paraphyletic relationships within this superfamily (see Olson et al. 2003; Thaenkham et al. 2011, 2012; Kvach et al. 2017; Le et al. 2017). Thus, spermatozoa of cryptogonimids are of type III, while in both heterophyids and opisthorchiids, the model of their sperm cells is of the type IV. The main difference between these two types of male gametes concerns the location of the maximal number of cortical microtubules: situated in the anterior (type IV) or in the middle region (type III) of the sperm cell.

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Compliance with ethical standards All procedures complied with National Experimental Animal Centre guidelines and the Animal Unit, Faculty of Medicine, Khon Kaen University (Thailand).

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