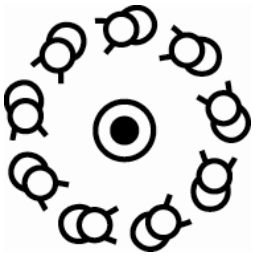
UNIVERSITY OF SOUTH BOHEMIA

FACULTY OF SCIENCE

SPERMATOLOGICAL CHARACTERS IN BOTHRIOCEPHALIDEA (CESTODA)

Master Thesis

Bc. Lenka ŠÍPKOVÁ



Supervisor: Céline Levron, PhD.

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Annotation

Spermiogenesis and ultrastructure of the spermatozoon of two bothriocephalidean cestodes, *Oncodiscus sauridae* and *Senga* sp., have been studied using transmission electron microscopy. The presence of a classical pattern for spermatological characters (spermiogenesis of type I with dense-material in early stages and sperm of type II with a characteristic ring of cortical microtubules in the anterior part) in Bothriocephalidea is discussed.

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Lenka Šípková

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Prohlašujeme, že se Lenka Šípková podstatným způsobem podílela na vložené publikaci.

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CONTENTS

1. INTRODUCTION

1.1. Model of study

1.1.1. Parasitic Platyhelminthes

Parasitic Platyhelminthes compose a monophyletic, highly diversified group of bilaterally symmetric and dorsoventrally flattened animals (Bilateria, Protostomia). Molecular phylogenetic analyses (Fig. 1) have shown that most of parasitic Platyhelminthes form a derived clade "Neodermata" and include members of three major classes – Cestoda (Gyrocotylidea, Amphilinidea and Eucestoda), Monogenea (Monopisthocotylea and Polyopisthocotylea) and Trematoda (Aspidogastrea and Digenea) (Baverstock et al. 1991; Blair 1993; Rohde et al. 1993; Littlewood et al. 1998, 1999; Litvaitis and Rohde 1999; Littlewood and Olson 2001; Littlewood 2008).

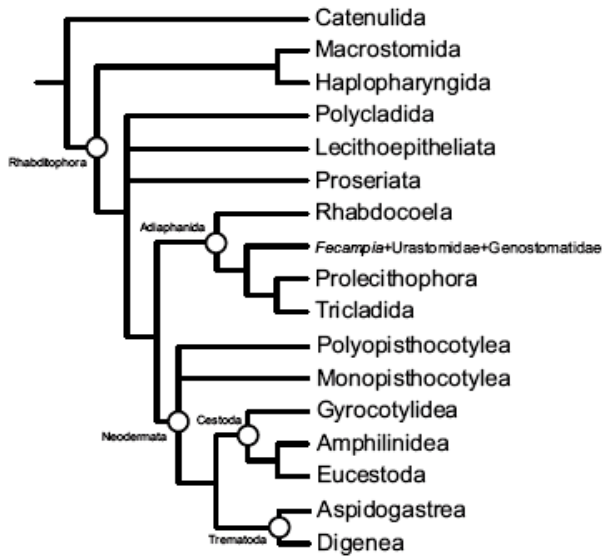

Fig. 1. Summary of major interrelationships among Platyhelminthes (Littlewood 2008).

Ehlers (1984) proposed the term Neodermata on the basis of the presence of a syncytial tegument in adult stages. Parasites of the taxon Neodermata do not possess a ciliary epidermis (except larvae), but they create a new epidermal syncytial epithelium of mesodermal origin, called neodermis, or tegument (Ehlers 1985). This syncytium serves for the digestion and transport of the nutrients, protection of the parasite against the environment, including components of the digestive and immunity system of the host. The cells and ducts participate in metabolism and transport of nutrients and metabolic products of metabolism, whereas function of the parenchyma is to provide the internal support (Roberts and Janovy 2005; Mehlhorn 2008). The typical character of Neodermata is the presence of neoblasts. These totipotent cells give rise to individual differentiated cells and tissues of Neodermata and are also responsible for reproduction (Roberts and Janovy 2005; Mehlhorn 2008).

Subtegumental muscular system is composed of circular, longitudinal and transversal muscles. Well developed musculature is also present in the suckers, pharynx and other organs. Excretory system consists of protonephridia. Flame cells filtre a body fluid from parenchymatous spaces through the system of canals. These canals unify and empty in the excretory bladder, which opens onto the surface of the body by excretory pore. Nervous system is composed of a furcated ganglion in the forebody, from which short and longer strands branch out to the anterior and posterior part of the body.

Parasitic Platyhelminthes are mainly hermaphrodites. Male genitalia include various numbers of testes. Spermatozoa are transported by vasa efferentia, which then unite to form vas deferens that enters the seminal vesicle. The seminal vesicle and duct of the prostatic glands empty into the cirrus, which is often enclosed by a muscular cirrus-sac (Roberts and Janovy 2005).

Female genitalia involve a single ovary, whose oviduct opens into the ootype (site of fertilization of oocytes and formation of eggs and their shells), together with the proximal end of the vagina (present in cestodes and monogeneans), which then usually enlarges to form a seminal receptacle. The ootype is surrounded by Mehlis glands, which secrete substance forming a membrane around the zygote, and it is associated with the vitelline cells. Vitellarium may be compact or follicular; cells of the vitellarium form yolk and eggshell components. As oocytes mature in the ovary, they pass through a single oviduct, which in general has a regulating sphincter, the oocapt. Vitelline follicles produce vitelline cells that surround and nourish oocytes. Eggs are polylecithal in many groups, which means that they contain a large amount of vitellocytes. Fertilization of the zygote proceeds in the ootype. The ootype continues as a uteroduct that then enters to the uterus, which can be tubular, often strongly coiled, or saccular, i.e. sac-like, and opens out onto the surface by a genital pore, which is usually on the ventral side of the body (Roberts and Janovy 2005).

1.1.2. Cestoda

The Cestoda, or tapeworms include about 5000 described species (Georgiev 2003). Adult tapeworms are parasites of all classes of vertebrates and use mainly arthropods as first intermediate hosts. Species of most tapeworm orders occur in water vertebrates (elasmobranchs and teleosts), but by far the most specious order, Cyclophyllidea, includes

parasites of birds and mammals, with a few species parasitizing amphibians and reptiles (Khalil et al. 1994).

Several species of cestodes are etiological agents of important diseases of men and domestic animals, for example *Diphyllobothrium latum* from the order Diphyllobothriidea (Muller 2002). Person becomes infected after consuming raw or undercooked fish. In many cases, the diphyllobothriasis is apparently asymptomatic, but anemia in infected persons has been reported. More dangerous parasites of humans and animals are some species of the order Cyclophyllidea, especially of the family Taeniidae, members of which can cause severe diseases, e.g. cysticercosis, echinococcosis and taeniosis (Kassai 1999; Roberts and Janovy 2005).

Since the time of ancient Greece, when cestodes were firstly recognized (Grove 1990), many things have changed (Olson and Tkach 2005). Morphological methods have been supplemented by utilization of molecular data, which threw new light upon problems between relationships and evolution of the Cestoda (Olson and Tkach 2005).

Tapeworms are characteristic with the absence of the intestine and presence of microtriches. Microtriches are highly specialized cylindrical tubular structures covering the entire surface of the tegument of cestodes. They play a role in absorption of nutrients, amplification of the absorptive surface area and attachment to the surface of the host intestinal tract (Chervy 2009).

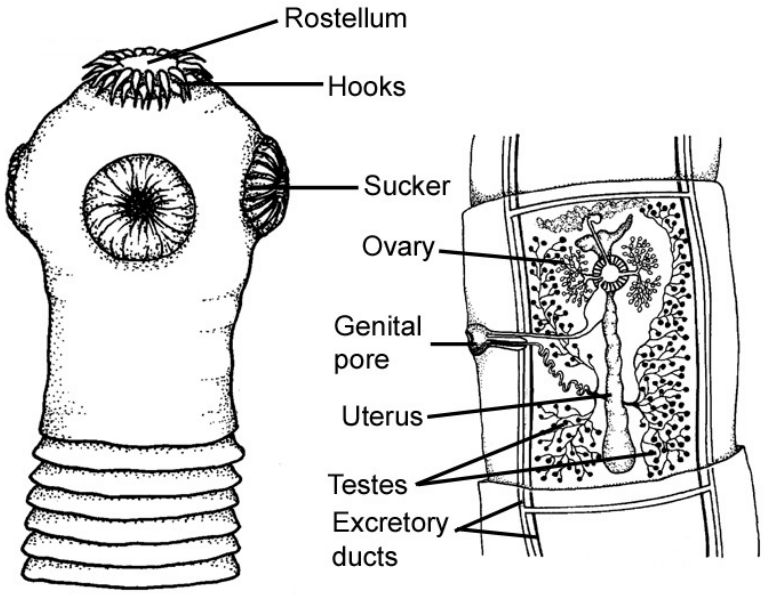

Fig. 2. The scolex (left) and a mature proglottid (right) of a typical cestode (from www.marlin.ac.uk/taxonomydescriptions.php#cestoda).

Between the scolex and the first segments of the strobila is often an undifferentiated zone, called the neck, which can be long, short or absent. The neck or the posterior part of the scolex contains germinal cells that have the potential for the formation of segments. This process is called strobilization. Depending on the group, the scolex (Fig. 2) can possess suckers, grooves, hooks, spines, tentacles, glandular areas or combinations of these. In a few species, the scolex is reduced, being replaced in function by the anterior extremity of strobila. The organ thus produced is called a pseudoscolex (Schmidt 1986).

The Eucestoda, except for the orders Caryophyllidea, Spathebothriidea and a few species of the order Bothriocephalidea, involves species with segmented strobila. If each segment overlaps the following one, the strobila is said to be craspedote, if not, it is called acraspedote (Fig. 3). Each segment contains usually one, rarely two or exceptionally more genital complexes – proglottids. Cestodes with one proglottid are monozoic, whereas these with more than one are polyzoic (Smyth and MacManus 1989). The absence of segmentation in groups such as the Caryophyllidea could be argued as an evidence of their "primitive" condition or they have lost it secondary (Olson and Tkach 2005).

Tapeworms comprise almost exclusively species with indirect (heteroxenous) life cycles. In the egg, the first larval stage of the cestodes develops and it is equipped with hooks, whose number reaches 10 (most basal cestodes Gyrocotylidea and Amphilinidea – larva is called decacanth) and 6 (Eucestoda with hexacanth).

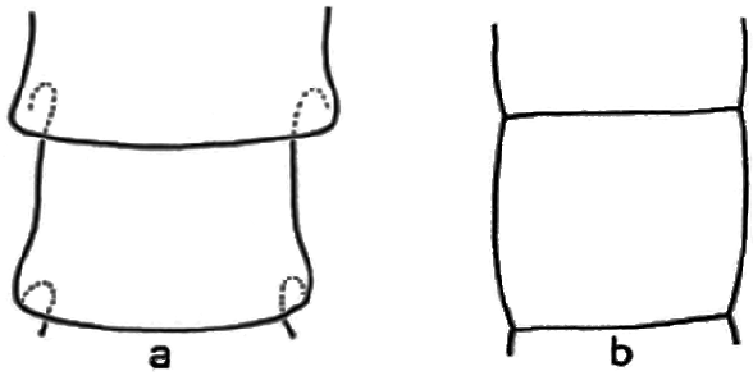

Fig. 3. Craspedote (a) and acraspedote (b) segments (from Schmidt 1986).

1.1.3. Bothriocephalidea

Recently, taxonomic and molecular studies have revealed that the order Pseudophyllidea should be suppressed, because it consists of two phylogenetically unrelated groups, for which the names Bothriocephalidea and Diphyllobothriidea were proposed (Kuchta et al. 2008). These orders were previously grouped together because they possess two dorsoventrally situated attachment organs, called bothria (Bray et al. 1994).

The order Bothriocephalidea includes mainly parasites of freshwater and marine fish, rarely in amphibians and it is considered to be a more derived clade than the order Diphyllobothriidea (Fig. 4 A; Kuchta et al. 2008). These tapeworms compose a sister group to the acetabular cestodes, which are considered to be a more derived group (Kuchta et al. 2008) and include also veterinary and medically important species of the order Cyclophyllidea (Khalil et al. 1994; Muller 2002). Bothriocephalidea comprises more then 40 genera, which are separated in 4 families, namely Bothriocephalidae, Echinophallidae, Philobythiidae and Triaenophoridae (Kuchta et al. 2008). Out of 305 nominal species of the order Bothriocephalidea, only about 125 species are thought to be valid (Kuchta and Scholz 2007).

Order Bothriocephalidea includes small to middle-sized tapeworms with one or two intermediate hosts. Their strobila possesses craspedote segments, which are usually wider than longer. They have complete or incomplete segmentation. Scolex is usually unarmed, but it is equipped with hooks in some taxa (*Triaenophorus*). Dorsal and ventral part of the scolex possesses two bothria of a different shape and depth. An apical disk and neck can be also present (Bray et al. 1994; Kuchta et al. 2008).

In Bothriocephalidea, genital pores (outer openings of the cirrus pouch and vagina) are lateral (Triaenophoridae, Philobythiidae, some Echinophallidae), sublateral (some Echinophallidae) or median on the dorsal part of the segment (Bothriocephalidea). A uterine pore is located ventrally, anterior to the genital pores (Kuchta et al. 2008).

Reproductive organs (Fig. 4 B) are single or duplicated in a segment and consist of numerous vitelline follicles and abundant testes. Vas deferens is twisted; cirrus pouch may comprise an internal seminal vesicle. The cirrus is usually unarmed, but it can be covered with spines. A bilobed, compact, follicular or dendritic ovary is located in the medulla near the posterior margin of a proglottid. A uterus of different shape is divided into the uterine duct, forming loops, and uterine sac. Eggs can be operculate or not. In the egg, larva

(oncosphere) can be formed either in the uterus (intrauterine eggs are embryonated) or in the water (unembryonated eggs). In some species, the surface of the oncosphere is covered with cilia that enable movement of the larva (coracidium) in the water (Beveridge 2001; Kuchta et al. 2008).

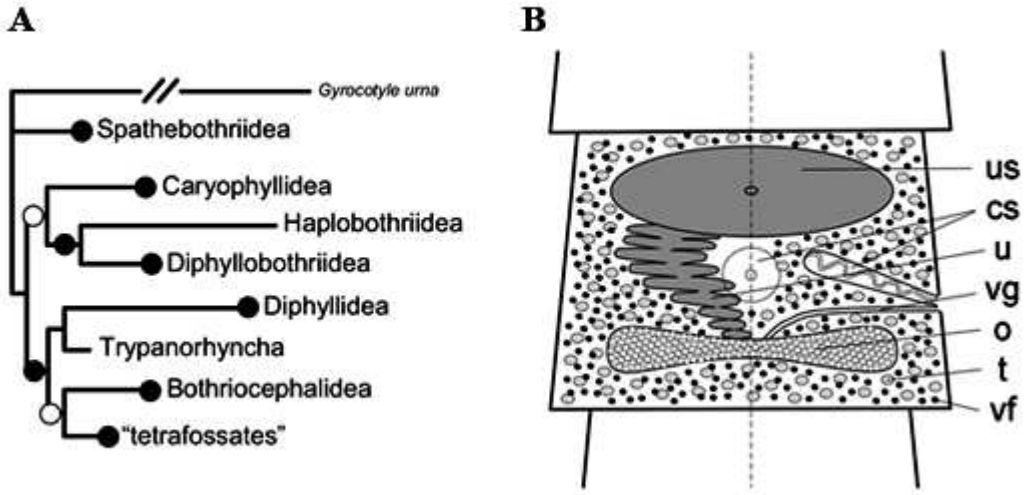

Fig. 4. A. Phylogenetic tree of basal tapeworms (Eucestoda) inferred from SSU + LSU data (sequences of the small and large subunits of the rRNA gene; from Kuchta et al. 2008). **B.** Schematic drawing of the proglottid of a cestode of the order Bothriocephalidea, ventral view. *Abbreviations*: cs, cirrus-sac; o, ovary; t, testes; u, uterus; us, uterine sac; vf, vitelline follicles; vg, vagina (from Kuchta et al. 2008).

1.2. Importance of spermatological characters

1.2.1. Parasitic Platyhelminthes

Spermiogenesis and sperm ultrastructure are important characters for comparative and phylogenetic studies of Platyhelminthes (Hendelberg 1986; Justine 1995, 1998, 2001; Levron et al. 2010). Hendelberg (1969) used, for the first time, spermatological characters for a study of phylogenetic relationships of the "Turbellaria". Ehlers (1984, 1985, 1986), Brooks (1989) and Brooks and McLennan (1993) included sperm characters for the definition of some major groups of helminths. Even a higher-level taxon, Trepaxonemata, was proposed on the basis of ultrastructural data, i. e. morphology of sperms (Ehlers 1984).

Parasitic Platyhelminthes (Neodermata) belong to Trepaxonemata, which also comprise Polycladida, Seriata, Prolecitophora, Typhloplanoida and Dalyellioida (Ehlers

1986). The name Trepaxonemata is derived from “axoneme in spiral” and it arose from the spermatozoon structure of the axoneme 9 + “1” (Fig. 5 A), in contrast to most axonemes of eukaryotic organisms, which are characteristic by a 9 + 2 axoneme pattern (Fig. 5 B). In the pattern 9 + “1”, the number 9 represents nine peripheral doublets of microtubules. The number “1” corresponds to the central core, which is a structure without tubulin. Indeed, immunocytochemical studies proved that the central core of trepaxonematans does not contain tubulin, the protein that characterizes microtubules (Iomini and Justine 1997; Iomini et al. 1998).

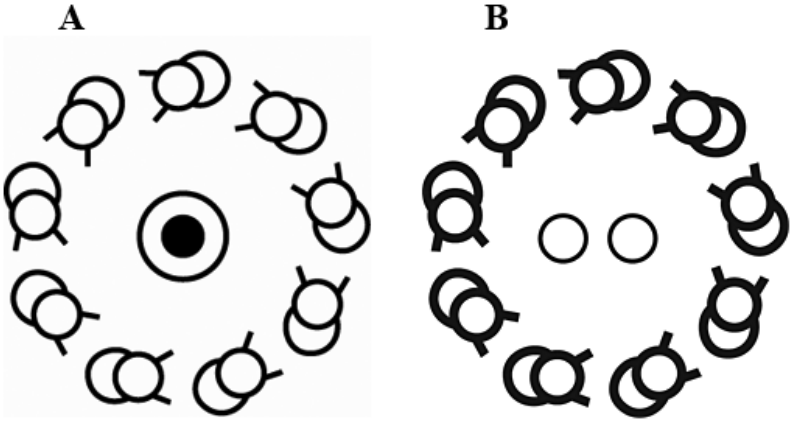

Fig. 5. A. Axoneme of the Trepaxonemata 9 + “1”. **B.** Axoneme of the most eukaryotic organisms 9 + 2.

Spermiogenesis in Neodermata is characterized by formation of an original structure called “zone of differentiation”, which is typically composed of a nucleus, an intercentriolar body, striated rootlets, two centrioles, two flagella and a cytoplasmic extension. Neodermata shares the synapomorphy of proximodistal fusion during spermiogenesis (Justine 2001, 2003). This particular pattern of spermiogenesis gives rise to a long and filiform spermatozoon, lacking a head and a tail as it is in most animals. In general, the spermatozoon contains two axonemes, a nucleus, mitochondria, cortical microtubules and different types of granules (Justine 2003). The presence of some characters in individual groups and their absence in others are useful for phylogenetic studies in Platyhelminthes. These characters include, among others, the length of axonemes and microtubules, displacements of axonemes, position of the nucleus, number of axonemes, etc. (Mollaret and Justine 1997; Justine 2001).

1.2.2. Eucestoda

Spermatological characters in the Cestoda represent an extensive source of information important for phylogenetic studies (Justine 1995, 1998, 2001; Levron et al. 2010). For the first time, Euzet et al. (1981) linked the ultrastructure of the spermatozoon of cestodes with their phylogeny. Later, Justine made principal contributions to evolutionary considerations of spermatological characters (Justine 1991, 1995, 1998, 2001, 2003). Characters of the sperms were used together with morphologic and molecular data to unravel the phylogeny of Eucestoda. A total of 33 characters were used in matrix, of which 11 characters were spermatological (Hoberg et al. 1997, 2001; Olson et al. 2001). Recently, data on spermiogenesis and ultrastructure of spermatozoa of Eucestoda have been summarized by Levron et al. (2010). Since 2001, when Justine's review article on cestodes appeared, about 50 species of the Eucestoda have been studied or reinvestigated, including members of previously poorly studied orders, such as Caryophyllidea, Spathebothriidea, Diphyllobothriidea, Bothriocephalidea, Trypanorhyncha, Tetraphyllidea and Proteocephalidea. Seven basic types of spermatozoa have been distinguished in the Eucestoda based on characters considered to be the most important for classification (Fig. 6) and a phylogenetic tree inferred from spermatological characters was constructed (Fig. 7; Levron et al. 2010). The following characters were used in the spermatological phylogenetic analysis: number of axonemes in zone of differentiation and in mature spermatozoa, presence/absence of an intercentriolar body, typical striated rootlet, flagellar rotation, proximodistal fusion, mitochondria in the mature spermatozoon, crested body, apical cone, shape of nucleus, etc. (Levron et al. 2010). A phylogenetic tree (Fig. 8) of Waeschenbach et al. (2007), inferred from molecular data, can be compared, with the column on the right side showing some ultrastructural spermatological characters. In the case of presence/absence mitochondria in the mature spermatozoon, molecular data correspond to those inferred from ultrastructural studies, because the absence of mitochondria in the spermatozoon is a character of evolutionary more derived cestodes (Justine 1995, 1998).

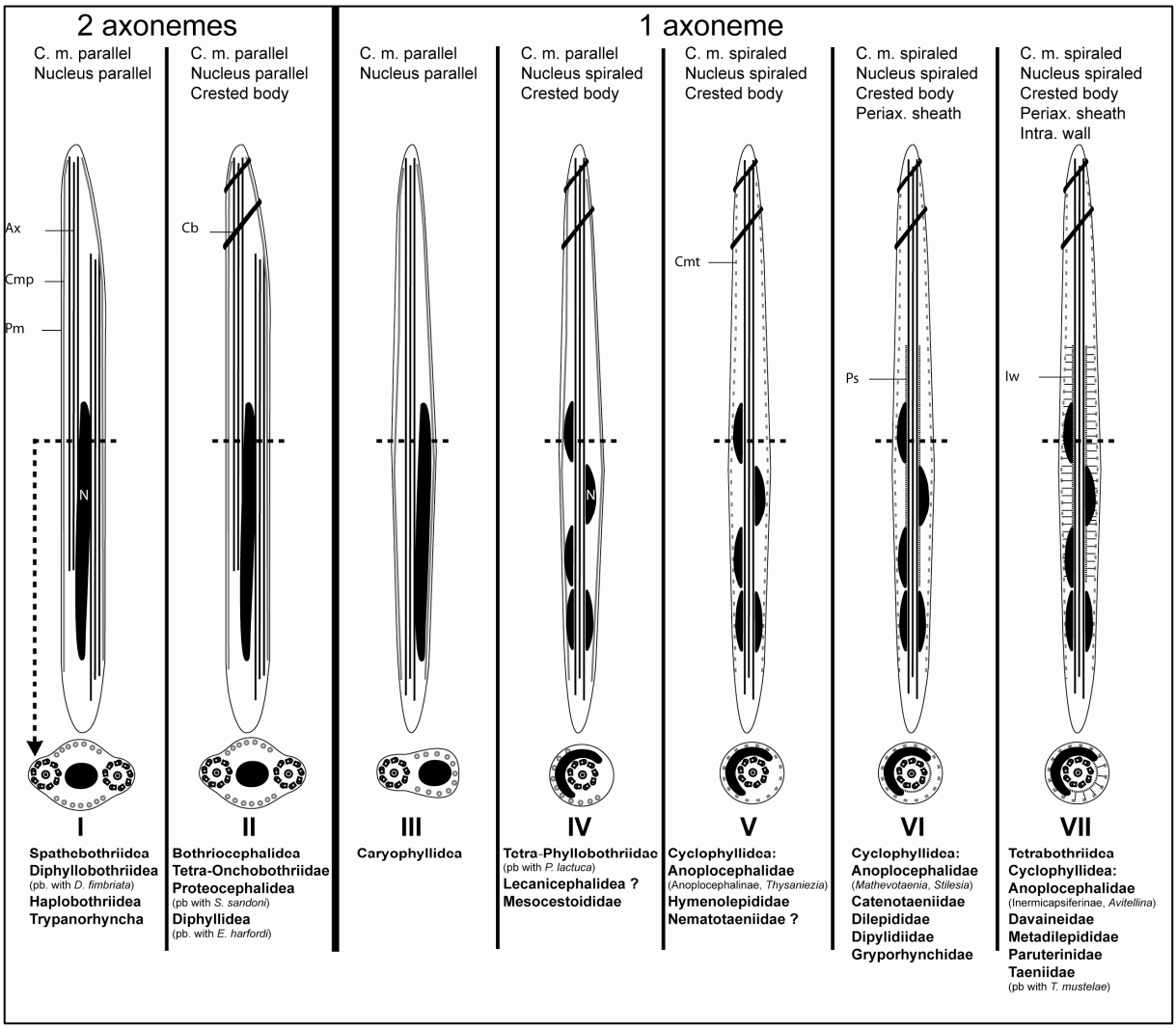

Fig. 6. Schematic representation of seven basic types of spermatozoa (longitudinal and cross sections) in the Eucestoda (from Levron et al. 2010). Abbreviations: Ax, axoneme; Cb, crested body; Cmp, cortical microtubules parallel; Cms, cortical microtubules spiraled; Iw, intracytoplasmic wall; N, nucleus; Pm, plasma membrane; Ps, periaxonemal sheath.

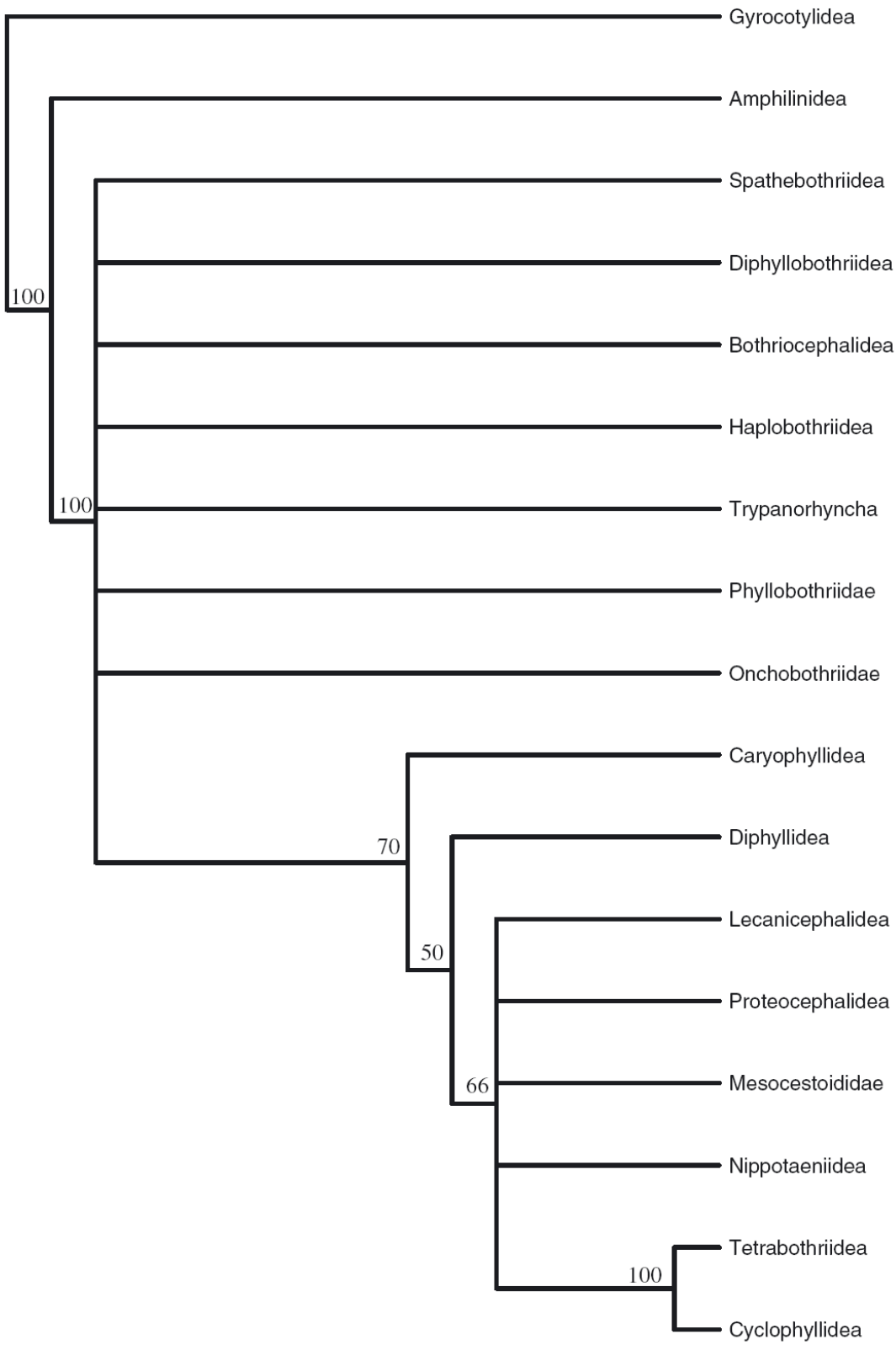

Fig. 7. A majority rule (50%) consensus tree derived from 785 equal-length (most-parsimonious) trees based on the analysis of spermatological data. Numbers at the nodes show majority rule values (percentages) (from Levron et al. 2010).

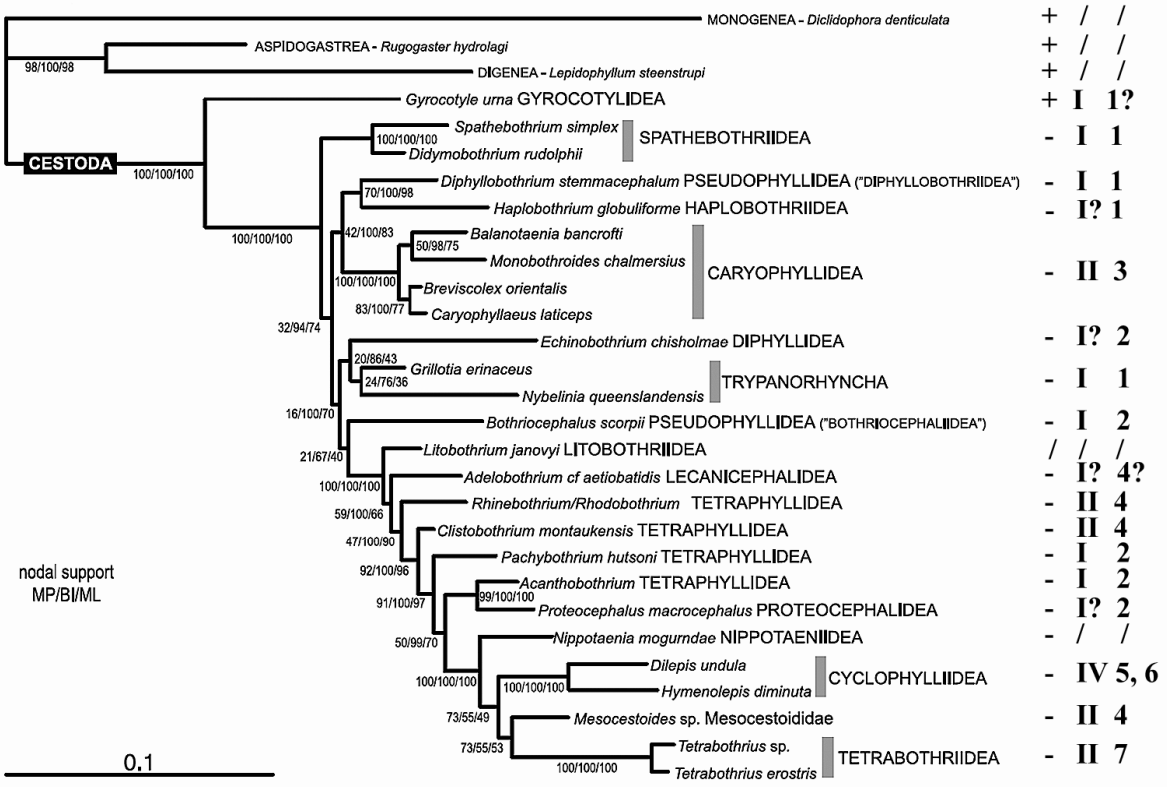

Fig. 8. Bayesian consensus phylogram based on analyses of data partitions, in order to estimate ordinal level relationships within the Cestoda; complete ssrDNA+complete lsrDNA. Nodal support is indicated for BI (posterior probabilities), MP (bootstrap, n = 1000) and ML (bootstrap, n = 100). The branch length scale is number of substitutions per site (from Waeschenbach 2007). *Abbreviations*: +/-, presence/absence of the mitochondria in the mature spermatozoon; I–IV, type of spermiogenesis; 1–7, type of the mature spermatozoon; ?, supposition of the presence of the structure; /, absence of data.

Spermatozoon of cestodes differs substantially from those of other parasitic flatworms, i.e. trematodes (Aspidogastrea and Digenea) and monogeneans (Monopisthocotylea and Polyopisthocotylea), because it lacks a mitochondrion. Cestodes possess glycogen, which serves as a source of energy, and thus do not need mitochondria in their mature spermatozoon (Euzet et al. 1981; Justine 1995). Among characters of potential importance for phylogenetic studies belong, for example, the presence of apical electron-dense material in the early stages of spermiogenesis, ring and semi-ring of cortical microtubules surrounding the axoneme in the anterior part of the spermatozoon, and angle between the flagella and cytoplasmic extension more than 90° (Levron et al. 2010).

1.2.3. Bothriocephalidea

Since 1980, spermiogenesis and sperm ultrastructure of eight species currently placed in the order Bothriocephalidea have been examined. Studied species belong to the families Bothriocephalidae, Echinophallidae and Triaenophoridae (Świderski and Mokhtar–Maamouri 1980; Bruňanská et al. 2001, 2002, 2010; Levron et al. 2005, 2006a, b; Bâ et al. 2007; Šípková et al. 2010). In all species studied the process of spermiogenesis corresponds to the type I of Bâ and Marchand (1995) and the spermatozoon to the type II of Levron et al. (2010). This indicates that these characters are typical of all species of the order, but members of some families (Philobythiidae) and those parasitic in important groups (marine teleosts) and different geographical regions were not studied at all or just in a limited number of taxa.

Electron-dense material in the apical region of the zone of differentiation in the early stages of spermiogenesis was found in four species studied (Bruňanská et al. 2001, 2002; Levron et al. 2005, 2006a; Šípková et al. 2010). The presence/absence of the intercentriolar body and its structure are characters used in phylogenetic analyses of the Eucestoda (Justine 1998; Levron et al. 2010). In Bothriocephalidea, it is usually composed of three electron-dense plates and two electron-lucent layers (Bruňanská et al. 2001, 2002; Levron et al. 2005, 2006a; Šípková et al. 2010).

The mature spermatozoon of bothriocephalidean cestodes corresponds to the type II of Levron et al. (2010) and usually contains two axonemes, one crested body, glycogen, nucleus and the ring of cortical microtubules surrounding the axoneme in the anterior part of the spermatozoon. The presence of the crested body and ring of cortical microtubules was revealed in seven members of the order Bothriocephalidea and may be typical for this cestode group (Bruňanská et al. 2010; Levron et al. 2010).

2. OBJECTIVES OF THE STUDY

The objectives of the study were as follows:

1. Based on search of literary data, to summarize the information about phylogenetic importance of spermatological characters in parasitic Platyhelminthes (Neodermata), Eucestoda and in the Bothriocephalidea in particular.
2. Using transmission electron microscopy, to obtain new data on spermiogenesis and spermatozoon ultrastructure of two bothriocephalidean tapeworms, *Senga* sp. from a freshwater fish in India and *Oncodiscus sauridae* Yamaguti, 1934 from a marine fish in New Caledonia.
3. To compare newly obtained spermatological data with those on other species of the order Bothriocephalidea and to describe characteristics typical of these tapeworms.

3. MATERIALS AND METHODS

3.1. Materials

As model species, two bothriocephalidean tapeworms of the family Bothriocephalidae were used. *Oncodiscus sauridae* Yamaguti, 1934 is a parasite of a marine fish that has wide distribution (Tropics of the Indian and Pacific Ocean), whereas another model tapeworm, a species of *Senga* Dollfus, 1934, most probably *Senga lucknowensis* Johri, 1956 (see Kuchta and Scholz 2007), occurs in freshwater fish and its distribution is probably limited to tropical Asia (Indomalyan region).

3.1.1. *Oncodiscus sauridae* Yamaguti, 1934

Adults of *Oncodiscus sauridae* (Bothriocephalidea, Bothriocephalidae) (Figs. 9 A–D) were collected from the intestine of lizardfish *Saurida nebulosa* (Valenciennes, 1850) (Aulopiformes, Synodontidae) (Fig. 9 E) off New Caledonia in 2009 (Kuchta et al. 2009) by Jean-Lou Justine (Muséum National d'Histoire Naturelle, Paris, France).

A total of three specimens were collected: one of them was used for identification, whereas two remaining specimens were used for an ultrastructural study.

3.1.2. *Senga* sp.

An unidentified species of the genus *Senga* Dollfus, 1934 (Bothriocephalidea, Bothriocephalidae), most likely *Senga lucknowensis* (see Kuchta and Scholz 2007) (Figs. 10 A–F), was obtained from the intestine of freshwater zig-zag eel *Mastacembelus armatus* (Lacépède, 1800) (Synbranchiformes, Mastacembelidae) (Fig. 10 G) from a fish market in Aurangabad, Maharashtra, India (in 2008), by Mikuláš Oros (Parasitological Institute, Slovak Academy of Sciences).

Three specimens were used for transmission electron microscopy study, whereas others have been used for morphological and molecular studies, including identification.

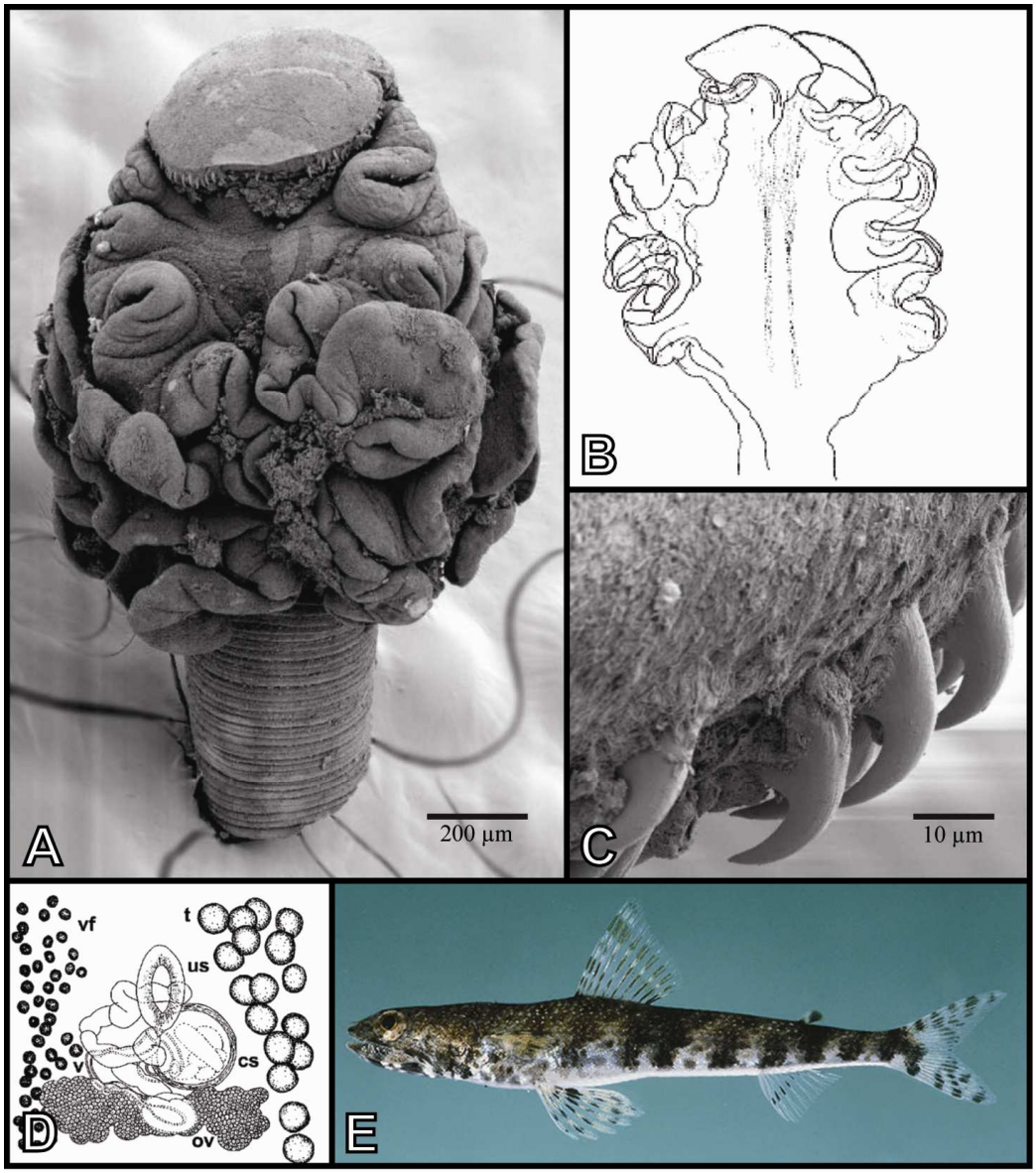

Fig. 9 A–D. *Oncodiscus sauridae*. **A.** Scanning electron (SEM) photomicrograph of the scolex (courtesy of R. Kuchta). **B**. Drawing of the scolex (from Kuchta et al. 2009). **C.** Detail of hooklets of apical disc, SEM photomicrograph (courtesy of R. Kuchta). **D.** Detail of genital organs ex *Saurida tumbil*; vitelline follicles illustrated only on the left side and testes only on the right side, ventral view. Abbreviations: cs, cirrus-sac; ov, ovary; t, testes; us, uterine sac; v, vagina; vf, vitelline follicles (from Kuchta et al. 2009). **E.** *Saurida nebulosa* (Valenciennes, 1850) (from FishBase - www.fishbase.org).

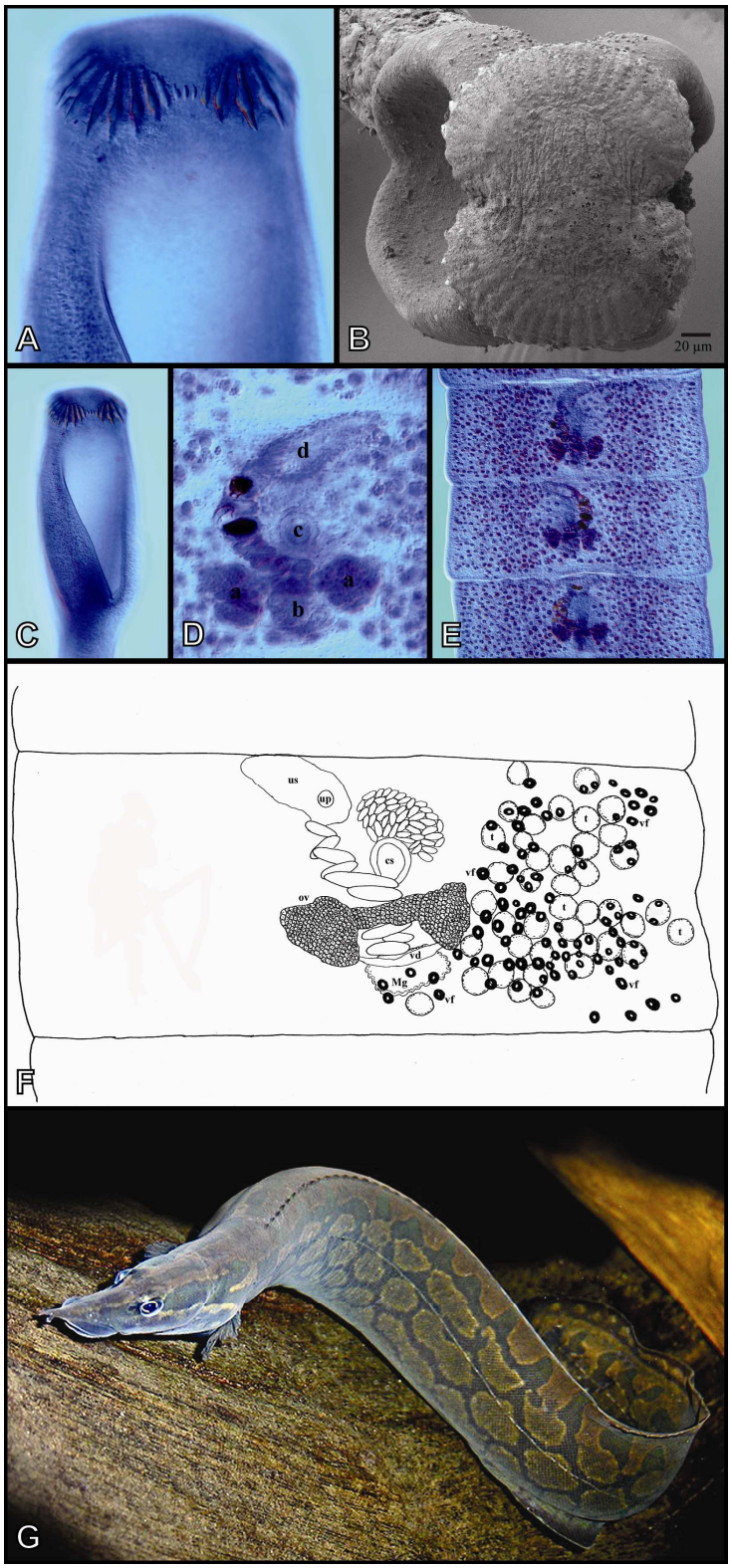

Fig. 10 A–F. *Senga* sp. **A.** Scolex with hooks, permanent preparation (original). **B.** SEM photomicrographs of the scolex (courtesy of R. Kuchta). **C.** Scolex, permanent preparation (original). **D.** Detail of the gravid proglottid of *Senga* sp., permanent preparation (original). Abbreviations: a, ovarium; b, Mehlis glands; c, cirrus sac; d, uterus (original). **E**. Gravid segments (original). **F.** Mature segment. Abbreviations: cs, cirrus-sac; Mg, Mehlis glands; ov, ovary; t, testes; up, uterine pore; us, uterine sac; vd, viteloduct; vf, vitelline follicles (original). **G.** *Mastacembelus armatus* (Lacépède, 1800). (from http://flickriver.com/photos/cyprinoid/2266226979/).

3.2. Methods – Transmission Electron Microscopy (TEM)

All the samples were processed according to the protocol used in the Laboratory of Electron Microscopy (Institute of Parasitology, BC AS CR, České Budějovice), which is briefly described below.

3.2.1. Fixation

Alive worms (two *Oncodiscus sauridae* specimens and three specimens of *Senga* sp.) were rinsed in 0.9% NaCl solution. Mature and gravid proglottids were separated and fixed with cold (4°C) 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer at pH 7.4 during 10 days. After fixation, the material was sent to the Laboratory of Helminthology (Institute of Parasitology, BC AS CR, České Budějovice). The proglottids were washed overnight in 0.1 M sodium cacodylate buffer (pH 7.4) and postfixed in cold (4°C) 1% OsO_4 in the same buffer for 1 h. After fixation with OsO_4, the specimens were dark and thus became better visible, which made manipulation with samples easier.

3.2.2. Dehydration

After fixation, the specimens were dehydrated in a graded series of acetone. In every concentration of acetone (30%, 50%, 70%, 80%, 90%, 95% and 100%), the specimens were placed for 15 minutes. Acetone causes a high extraction of the cell material, especially of the lipids, and it is directly soluble with all used resins.

3.2.3. Embedding

After dehydration, the material was dried up in 100% acetone. The specimens were embedded in Epon resin with acetone. Concentration of the resin increased gradually (1 : 2, 1 : 1 and 2 : 1). In every concentration of Epon resin and acetone, the specimens were placed for 1 hour. Afterwards, the specimens were left in pure resin without air to the next day. Then the polymerized samples were embedded in mold with Epon resin.

A total of 15 and 12 blocks of mature proglottids of *Oncodiscus sauridae* and *Senga* sp., respectively, were obtained and prepared for transmission electron microscopy.

3.2.4. Semithin and ultrathin sections

First, semithin sections (about 400 nm in thickness) were cut on Leica Ultracut UCT ultramicrotome with glass knife. Subsequently, semithin sections (Figs. 11, 12) were coloured with toluidine blue and observed under the light microscope.

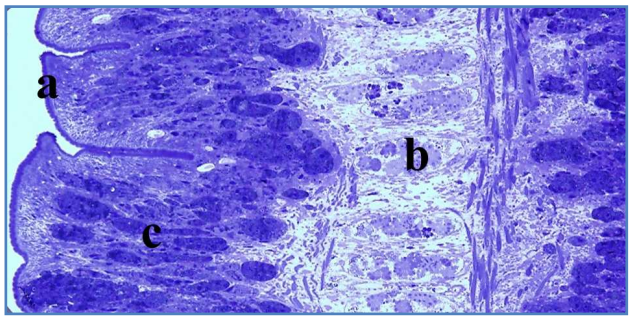

Fig. 11. Detail of a semithin section of the gravid proglottid of *Senga* sp. a) tegument, b) testes, c) vitelline follicles (original).

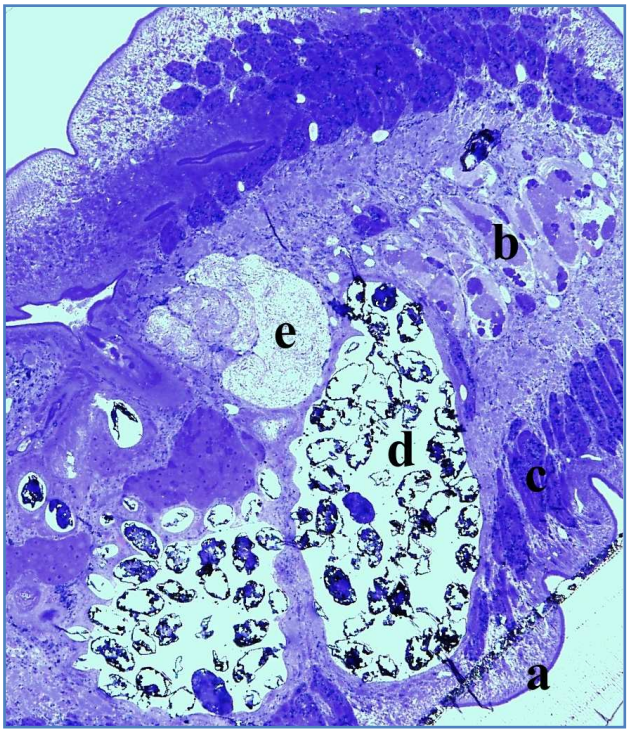

Fig. 12. Semithin section of the gravid proglottid of *Senga* sp. a) tegument, b) testes, c) vitelline follicles, d) eggs, e) canal with the mature spermatozoa (original).

Thereafter, ultrathin sections (60-90 nm in thickness) were cut from each sample on Leica Ultracut UCT ultramicrotome with diamond knife and placed on copper grids. This procedure was done by a technician Petra Masařová from the Laboratory of Electron Microscopy.

3.2.5. Contrasting

Ultrathin sections were contrasted with uranyl acetate and lead citrate according to Reynolds (1963). Uranyl acetate reacts particularly with nucleic acids and proteins. Lead citrate increases contrast of membranes, proteins, nucleic acids and glycogen.

Ultrathin sections were contrasted in darkness. Drops of uranyl acetate were applied onto parafilm in an ethanol atmosphere. Grids with sections were placed on the drops for 30 minutes. Then they were washed in 30% ethanol and dried-up on the filter paper. Drops of lead citrate were applied onto parafilm in an atmosphere with reduced percentage of CO_2. Grids with ultrathin sections were placed in the drops for 20 minutes. Afterwards the grids were washed in distilled H_2O and dried.

3.2.6. Observation and software

Sections with testes and mature spermatozoa were examined using a JEOL 1010 transmission electron microscope (Fig. 13) operated at 80 kV. About 10 sessions of observations of *Senga* sp. and 8 sessions of *Oncodiscus sauridae* were carried out using transmission electron microscope.

Pictures showing the process of spermiogenesis and sperms were taken by the CCD camera. Subsequently, they were processed with computer softwares Adobe Photoshop and Adobe Illustrator.

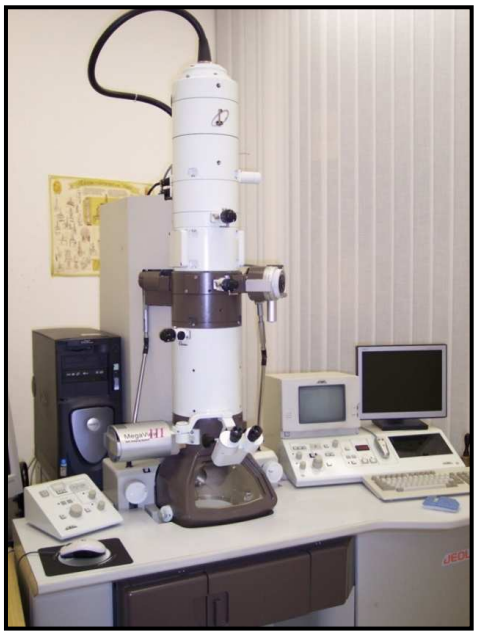

Fig. 13. Transmission electron microscope JEOL 1010.

4. RESULTS (Manuscript in press in *Parasitology Research*)

Results of MSc thesis are presented in form of manuscript that has been accepted for publication in international scientific journal and it is now in press (published on-line). A brief summary of this article is provided below.

Title of the article: Spermatological characters of bothriocephalideans (Cestoda) inferred from an ultrastructural study on *Oncodiscus sauridae* and *Senga* sp.
Autors: Lenka Šípková, Céline Levron, Mikuláš Oros, Jean-Lou Justine
Journal: *Parasitology Research*
Submitted: 13 October 2010
Accepted: 2 December 2010
Online first: 22 December 2010

Spermiogenesis and spermatozoon ultrastructure of *Oncodiscus sauridae* and *Senga* sp. from the order Bothriocephalidea have been studied using transmission electron microscopy. These tapeworms of dissimilar morphology were found in different hosts and habitats. In *O. sauridae* from the marine lizardfish *Saurida nebulosa* (Valenciennes, 1850) and *Senga* sp. from the freshwater eel *Mastacembelus armatus* (Lacépède, 1800), the same ultrastructure characters have been found. Spermiogenesis corresponds to the type I in both species and spermatozoon to the type II. The results have been presented in the article (below), which is to be published in *Parasitology Research* within next few months.

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Parasitol Res
DOI 10.1007/s00436-010-2215-1

ORIGINAL PAPER

Spermatological characters of bothriocephalideans (Cestoda) inferred from an ultrastructural study on *Oncodiscus sauridae* and *Senga* sp.

Lenka Šípková · Céline Levron · Mikuláš Oros · Jean-Lou Justine

Received: 13 October 2010 / Accepted: 2 December 2010

Abstract Spermiogenesis and ultrastructure of the spermatozoon of two bothriocephalidean cestodes, *Oncodiscus sauridae* from the lizardfish *Saurida nebulosa* Valenciennes, 1850 and *Senga* sp. from the eel *Mastacembelus armatus* (Lacepède, 1800), have been studied using transmission electron microscopy. Spermiogenesis included the formation of a zone of differentiation, where two centrioles associated with the striated rootlets occur. An intercentriolar body composed of one thick central electron-dense plate and two thinner plates on each side appears between two centrioles. Two flagella of unequal length grow and undergo a vertical rotation and proximodistal fusion with the median cytoplasmic process. Subsequently, the nucleus penetrates into the median cytoplasmic extension. The electron-dense material in the early stages of spermiogenesis is characteristic for the apical region of the differentiation zone. This electron-dense material is typical for basal tapeworms, e.g., Bothriocephalidea, Caryophyllidea, Diphyllobothriidea, and Spathebothriidea. The mature spermatozoon of *O. sauridae* and *Senga* sp. is filiform and possesses two axonemes of the 9+"1" trepaxonematan pattern, a nucleus, cortical microtubules, and electron-dense granules. The anterior part of the gamete contains a single electron-dense crested body. The most interesting character found is the presence of a ring of cortical microtubules encircling the axoneme in the anterior part of the spermatozoon. This feature has been detected only for species of the order Bothriocephalidea and may represent a synapomorphy of these tapeworms. A classical pattern for spermatological characters (spermiogenesis of type I with dense material in early stages and sperm of type II with a characteristic ring of cortical microtubules in the anterior part) in Bothriocephalidea is discussed.

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Introduction

Spermatological characters are important elements for studying the phylogenetic relationships within Platyhelminthes (Euzet et al. 1981; Hoberg et al. 1997, 2001; Justine 1991, 1995, 1998, 2001, 2003; Levron et al. 2010). Tapeworms (Cestoda) have been the subject of numerous studies, with more than 100 species investigated. Recently, Levron et al. (2010) mapped the main gaps in the current knowledge of spermatological characters among the orders of the Eucestoda, i.e., the major, more evolved branch of tapeworms.

Until recently, families Bothriocephalidae and Diphyllobothriidae composed a part of the order Pseudophyllidea (Bray et al. 1994), but it was suppressed on the basis of molecular and morphological studies and two separate

orders, Bothriocephalidea and Diphyllobothriidea, were proposed (Kuchta et al. 2008).

The order Bothriocephalidea includes mainly intestine parasites of teleost fish (Kuchta et al. 2008) and is composed by four families. To date, spermiogenesis and sperm ultrastructure of seven species belonging to three families, have been examined, namely, *Bothriocephalus clavibothrium* (Świderski and Mokhtar-Maamouri 1980), *Bothriocephalus claviceps* (Bâ et al. 2007), *Bothriocephalus scorpii* (Levron et al. 2006a) (Bothriocephalidae), *Parabothriocephalus gracilis* (Šípková et al. 2010), *Paraechinophallus japonicus* (Levron et al. 2006b) (Echinophallidae), *Eubothrium crassum* (Bruňanská et al. 2001, 2002), and *Triaenophorus nodulosus* (Levron et al. 2005) (Triaenophoridae). In all taxa studied, the spermiogenesis corresponds to the type I of Bâ and Marchand (1995) and the spermatozoon to the type II of Levron et al. (2010).

The present contribution aimed to study the spermiogenesis and sperm ultrastructure of two bothriocephalidean tapeworms, namely *Oncodiscus sauridae* and *Senga* sp. (Bothriocephalidae), using transmission electron microscopy. Newly obtained spermatological data are compared with

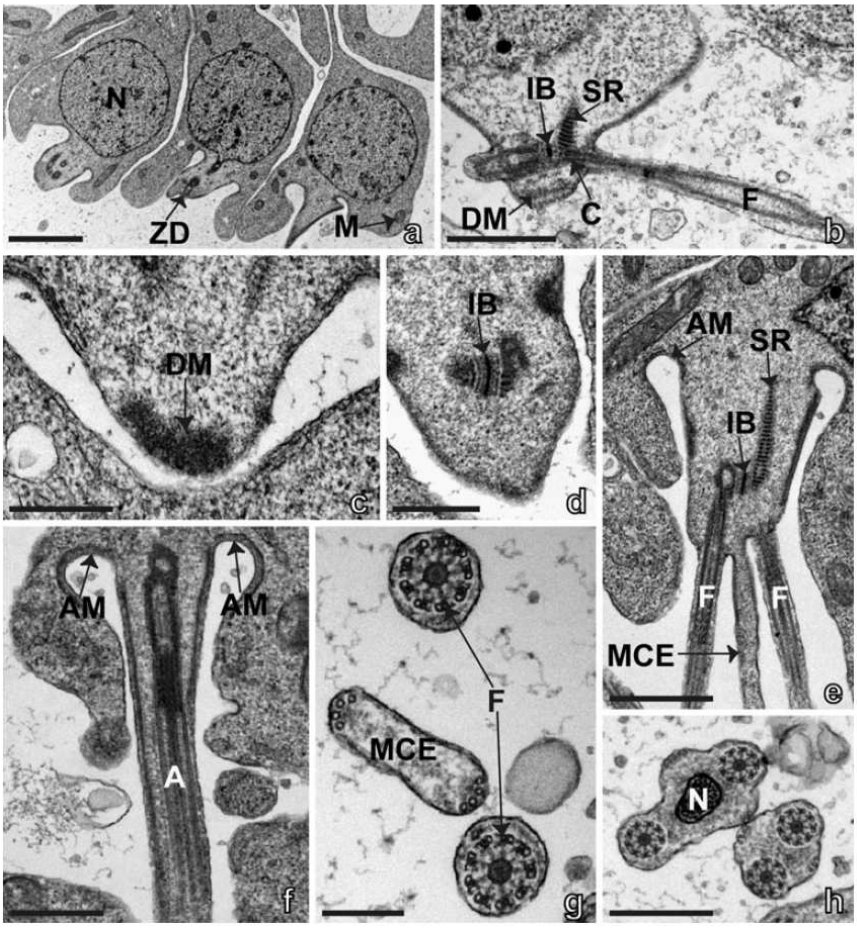

Fig. 1 Spermiogenesis of *Oncodiscus sauridae*. **a** Longitudinal section of the spermatids showing the zone of differentiation, nucleus, and mitochondria. *Bar* 2 μm. **b** Longitudinal section of the zone of differentiation with centrioles, intercentriolar body, striated rootlets, electron-dense material, and two flagella. *Bar* 1 μm. **c** Electron-dense material visible in the first stages of spermiogenesis. *Bar* 300 nm. **d** Intercentriolar body. *Bar* 500 nm. **e** Longitudinal section showing the rotation of the flagella and the elongation of the median cytoplasmic extension. *Bar* 1 μm. **f** Fusion of two flagella with the median cytoplasmic extension, longitudinal section. *Bar* 600 nm. **g** Cross-section of free flagella and median cytoplasmic extension. *Bar* 200 nm. **h** Cross-sections of the young spermatozoon with two axonemes, nucleus with not-well condensed chromatin, and two rows of cortical microtubules. *Bar* 600 nm. *A* axoneme, *AM* arched membranes, *C* centriole, *DM* electron-dense material, *F* flagellum, *IB* intercentriolar body, *M* mitochondria, *MCE* median cytoplasmic extension, *N* nucleus, *SR* striated rootlets, *ZD* zone of differentiation

those on other bothriocephalideans. Based on previously published data and new observations, a general pattern for spermiogenesis and sperm ultrastructure of bothriocephalidean tapeworms is described.

Materials and methods

Adults of *O. sauridae* Yamaguti, 1934 (Bothriocephalidea, Bothriocephalidae) were collected from the intestine of clouded lizardfish *Saurida nebulosa* Valenciennes, 1850 (Aulopiformes, Synodontidae) off New Caledonia (Kuchta et al. 2009) by one of the authors (J.-L. J.). Tapeworms of the genus *Senga* Dollfus, 1934 (Bothriocephalidea, Bothriocephalidae), which could not be identified to the species level due to doubtful taxonomic status of *Senga* spp. (R. Kuchta, personal communication), were obtained from the intestine of zig-zag eel *Mastacembelus armatus* (Lacepède, 1800) (Synbranchiformes, Mastacembelidae) in Maharashtra State, India (2008), by another author (M. O.). Living worms were rinsed in 0.9% NaCl solution, mature and gravid proglottids were separated and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 during 10 days. Then, they were washed overnight in 0.1 M sodium cacodylate buffer (pH 7.4), postfixed in cold (4°C) 1% OsO_4 in the same buffer for 1 h, dehydrated in graded series of acetone and embedded in Epon resin. Ultrathin sections (60–90 nm in thickness) were cut on a Leica Ultracut UCT ultramicrotome, placed on copper grids, and stained with uranyl acetate and lead citrate according to Reynolds (1963). The sections were examined in a JEOL 1010 transmission electron microscope operated at 80 kV.

Results

Spermiogenesis

Spermiogenesis of *O. sauridae* and *Senga* sp. starts in the testes where spermatids are grouped in rosettes. Each spermatid contains a nucleus and mitochondria (Fig. 1a). At the beginning, the zone of differentiation is formed at the periphery of the spermatid (Fig. 1a). The differentiation zone includes two centrioles associated with the intercentriolar body and the striated rootlets of cone-shaped structure (Figs. 1b–d, 2a, and 3a). The intercentriolar body consists of one thick central electron-dense plate and two thinner plates on each side (Figs. 1d, 2a, and 3a). The thick electron-dense plate is separated from the thinner ones by two electron-lucent layers (Figs. 1d, 2a, and 3a). Electron-dense material appears in the apical part of the zone of differentiation in the first stages of spermiogenesis (Figs. 1b, c, 2a, and 3a, b). Centrioles give rise to free flagella. Initially, both centrioles are oriented in the same

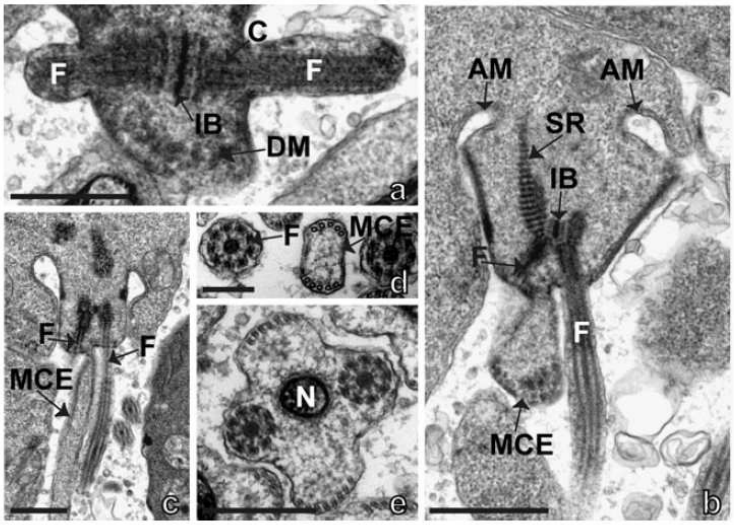

Fig. 2 Spermiogenesis of *Senga* sp. **a** Longitudinal section of the differentiation zone showing centrioles, intercentriolar body, flagella, and electron-dense material in the apical region of the median cytoplasmic extension. *Bar* 600 nm. **b** Longitudinal section pointing to the rotation of the flagella and the formation of the median cytoplasmic extension. *Bar* 1 μm. **c** Longitudinal section showing the fusion of two flagella with the median cytoplasmic extension. *Bar* 1 μm. **d** Cross-section of the flagella parallel to the median cytoplasmic extension. *Bar* 200 nm. **e** Cross-section of the early spermatozoon with two axonemes and nucleus. *Bar* 500 nm. *A* axoneme, *AM* arched membranes, *C* centriole, *DM* electron-dense material, *F* flagellum, *IB* intercentriolar body, *MCE* median cytoplasmic extension, *N* nucleus, *SR* striated rootlets

plane (Figs. 2a and 3b). Afterwards, the median cytoplasmic extension is formed in the posterior part of the differentiation zone and becomes longer (Figs. 1e, 2b, and 3c). The zone of differentiation is bounded by arching membranes in the anterior extremity (Figs. 1e, f, 2b, c, and 3b–e). Then, the flagella of different length undertake a rotation of 90°, and subsequently, they fuse with the median cytoplasmic extension in the proximodistal direction (Figs. 1e–g, 2b–d, and 3c–e). The nucleus penetrates into the median cytoplasmic extension (Figs. 1h, 2e, and 3d, e). Finally, the region of the arching membranes strangulates, and the sperm is detached from the residual cytoplasm (Figs. 1h, 2e, and 3e). At the end of spermiogenesis, the median part of the young spermatozoon contains two axonemes, two fields of cortical microtubules opposite each other, and a nucleus (Figs. 1h, 2e, and 3e).

Spermatozoon

Mature spermatozoa from the seminal vesicle of mature proglottids in *O. sauridae* and *Senga* sp. were investigated. They contain two axonemes, crested body, nucleus, cortical microtubules, and are divided into five characteristic regions from the anterior to posterior extremities. Except for the region V, the organization of spermatozoon of *O. sauridae* and *Senga* sp. is almost similar.

Region I (Figs. 4a, b, 5a, b, and 6I) constitutes the anterior extremity of the spermatozoon. The anterior tip of the spermatozoon is characterized by the presence of the centriole, accompanied by a single electron-dense crested body and few cortical microtubules (Figs. 4a and 5a). The helicoidal, 150 nm thick crested body corresponds to a lateral electron-dense projection of the spermatozoon. The centriole gives rise to an axoneme of the 9+"1" trepaxonematan pattern. At the posterior extremity, the crested body disappears and about 30 cortical microtubules form a ring encircling the axoneme (Figs. 4b and 5b). The cortical microtubules are characterized by a thick membrane and an electron-lucent center. At this stage, the diameter of the spermatozoon is about 270 nm.

Region II (Figs. 4c–e, 5c–e, and 6II) is distinguished by the presence of two axonemes and cortical microtubules. The ring of cortical microtubules disappears, and the second centriole is present forming the second axoneme

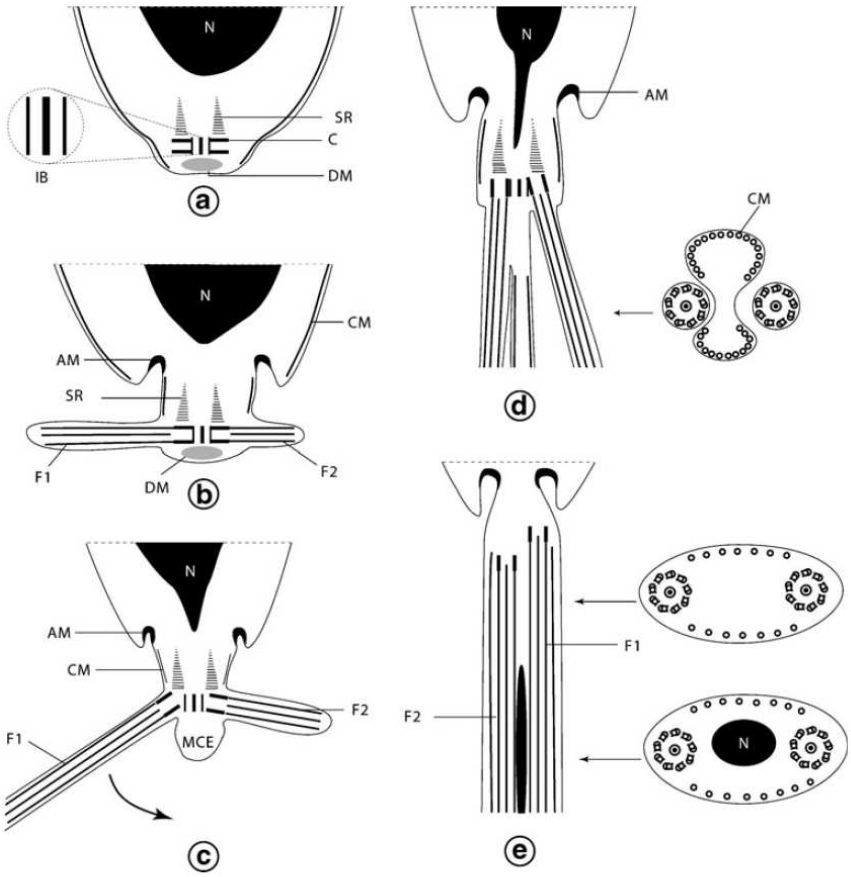

Fig. 3 a–e Schematic illustration of the main stages of spermiogenesis of *Oncodiscus sauridae* and *Senga* sp. *AM* arched membranes, *C* centriole, *CM* cortical microtubules, *DM* electron-dense material, *F* flagellum, *IB* intercentriolar body, *MCE* median cytoplasmic extension, *N* nucleus, *SR* striated rootlets

in the anterior part of this region (Figs. 4c and 5c). The two axonemes are situated very close to each other with only one or two cortical microtubules between them. The longest diameter of the cell is about 400 nm (Figs. 4d and 5d). Cortical microtubules are different from those in the first region because they are thin-walled and their center is electron-lucent. The attachment zones, remains of the fusion of the flagella with the cytoplasmic extension during spermiogenesis, are present (Figs. 4d and 5d). The diameter of the spermatozoon increases toward the middle part of the gamete to reach about 700 nm. The number of cortical microtubules increases between axonemes to form two opposite fields, each composed of four to seven cortical microtubules (Figs. 4e and 5e).

Region III (Figs. 4f, 5f, and 6III) is characterized by the presence of two axonemes, cortical microtubules, nucleus,

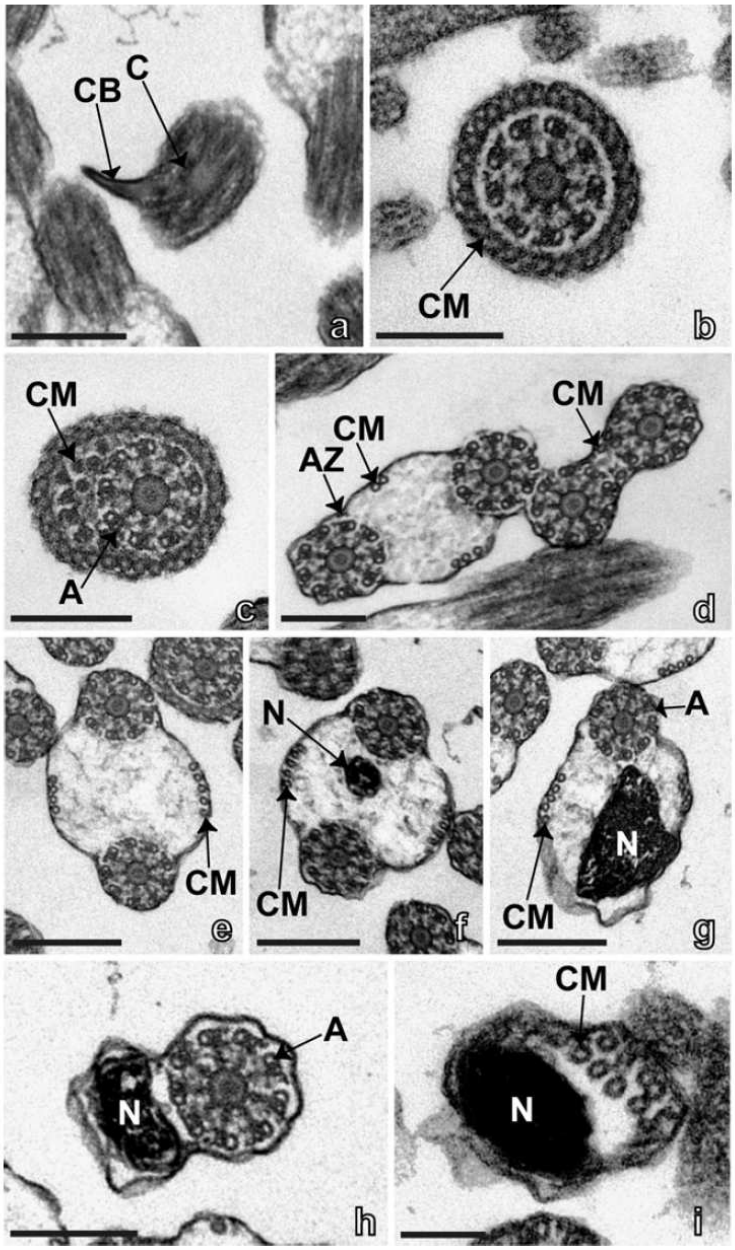

Fig. 4 Mature spermatozoon of *Oncodiscus sauridae*. All cross-sections. **a** Anterior part of region I showing the crested body and the centriole. *Bar* 300 nm. **b** Region I with ring of cortical microtubules. *Bar* 200 nm. **c** Region II with the axoneme and cortical microtubules. *Bar* 200 nm. **d** Region II showing two axonemes with attachment zones separated by few cortical microtubules. *Bar* 200 nm. **e** Region II containing two axonemes and two lateral rows of cortical microtubules. *Bar* 300 nm. **f** Region III with the appearance of the nucleus and two axonemes. *Bar* 300 nm. **g** Region IV with the nucleus, axoneme, and cortical microtubules. *Bar* 300 nm. **h** Region V without cortical microtubules. *Bar* 200 nm. **i** Posterior part of region V with the nucleus and axonemal doublets and singlets. *Bar* 100 nm. *A* axoneme, *AZ* attachment zone, *C* centriole, *CB* crested body, *CM* cortical microtubules, *N* nucleus

and electron-dense granules. The nucleus is electron-dense with fibrillar patches of chromatin. The diameter of the nucleus increases progressively. Each field of cortical microtubules consists of five to eight units. The diameter of the spermatozoon is 800 nm in this region (Figs. 4f and 5f). At the posterior part of the region III, one of the two axonemes is disorganized and disappears.

Region IV (Figs. 4g, h, 5g, h, and 6IV) contains one axoneme, nucleus, electron-dense granules, and cortical microtubules. The diameter of the nucleus is the largest in this region and decreases in the direction of the posterior part of the spermatozoon. Two fields of cortical microtubules are still present and disappear at the posterior part of region IV (Figs. 4g and 5g). The diameter of the spermatozoon is approximately 600–700 nm.

Region V (Figs. 4i, 5i, and 6V) corresponds to the posterior extremity of the spermatozoon. The axoneme becomes disorganized, the central core disappears, and the doublets lose their arms (Figs. 4i and 5i). The ultrastructure of the spermatozoon of *O. sauridae* and *Senga* sp. is different in the terminal part of this region: in *O. sauridae*, the nucleus and axonemal doublets and singlets are present

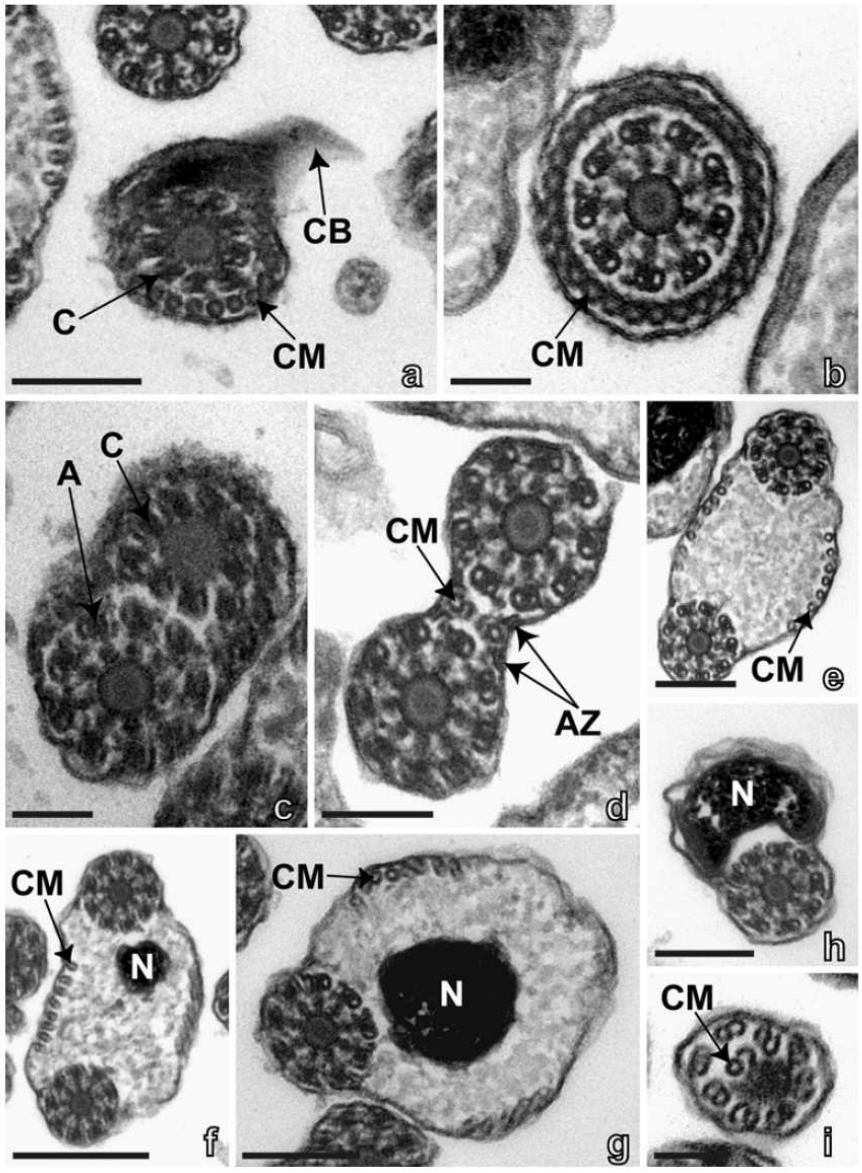

Fig. 5 Mature spermatozoon of *Senga* sp. All cross-sections. **a** Region I with the crested body, centriole and cortical microtubules. *Bar* 200 nm. **b** Ring of cortical microtubules in region I. *Bar* 100 nm. **c** Anterior extremity of region II showing the appearance of the second axoneme and disorganization of the ring of cortical microtubules. *Bar* 100 nm. **d** Region II with two axonemes close to each other, attachment zones, and cortical microtubules. *Bar* 200 nm. **e** Distal part of region II. *Bar* 200 nm. **f** R egion III with two axonemes and nucleus. *Bar* 500 nm. **g** Region IV with one axoneme, cortical microtubules, and increasing nucleus. *Bar* 300 nm. **h** Anterior part of region V showing nucleus and axoneme. *Bar* 300 nm. **i** Region V with disintegrating doublets of axoneme. *Bar* 100 nm. *A* axoneme, *AZ* attachment zone, *C* centriole, *CB* crested body, *CM* cortical microtubules, *N* nucleus

(Fig. 4i), whereas in *Senga* sp., only few singlets and doublets of axoneme are found (Fig. 5i). The diameter of this part in both species is about 200 nm.

Discussion

Spermiogenesis

Spermiogenesis of the type I (Bâ and Marchand 1995) is typical for *O. sauridae* and *Senga* sp., and it is characterized by flagellar rotation and proximodistal fusion of two flagella. This type of spermiogenesis has been found also in other tapeworms of the orders Bothriocephalidea, Diphyllobothriidea, Spathebothriidea, "Tetraphyllidea"—Onchobothriidae, Trypanorhyncha, and Proteocephalidea (Justine 1998; Levron et al. 2010), and it is regarded as the most primitive of existing types of spermiogenesis (Bâ and Marchand 1995).

In *O. sauridae* and *Senga* sp., the intercentriolar body is composed of three electron-dense plates and two electron-lucent layers. The same character is present also in other bothriocephalideans (Table 1), namely *B. scorpii* (Levron et al. 2006a) (Bothriocephalidae), *E. crassum* (Bruňanská et al. 2001) (Triaenophoridae), *P. gracilis* (Šípková et al. 2010) (Echinophallidae), *T. nodulosus* (Levron et al. 2005) (Triaenophoridae), but also *Cyathocephalus truncatus* of the order Spathebothriidea (Bruňanská et al. 2006). The intercentriolar body is a character used in phylogenetic analyses of Eucestoda (Justine 1998; Levron et al. 2010). Reduction of the layers of the intercentriolar body is observed in the "higher", i.e., evolutionary more evolved cestodes (Justine 2001). In the Diphyllobothriidea, the intercentriolar body consists of five electron-dense plates and four electron-lucent layers (Levron et al. 2006c, 2009), which suggests a position of this group among eucestodes more basal than the Bothriocephalidea.

Electron-dense material appears in the first stages of spermiogenesis in the apical region of the differentiation zone of both taxa studied, i.e., *O. sauridae* and *Senga* sp. This character was also reported in all studied members of the order Bothriocephalidea, except *B. clavibothrium* and in the tapeworms of orders Diphyllobothriidea, Caryophyllidea, and Spathebothriidea (Świderski and Mokhtar-Maamouri 1980; Bruňanská et al. 2001, 2002, 2006; Levron et al. 2005, 2006a, c, 2009; Bruňanská and Poddubnaya 2006; Gamil 2008; Miquel et al. 2008; Šípková et al. 2010).

Spermatozoon

Presently, seven basic types of spermatozoa are distinguished in the Eucestoda (Levron et al. 2010). The first and second types are typified by the possession of two axonemes, whereas the others contain only one axoneme. The mature spermatozoon of the type II of *O. sauridae* and *Senga* sp. is characterized by the presence of two axonemes of the 9+"1" trepaxonematan pattern (Ehlers 1984). Two axonemes occur in Bothriocephalidea, Diphyllobothriidea, Spathebothriidea, Haplobothriidea, Trypanorhyncha, Tetraphyllidea (Onchobothriidae), Proteocephalidea, and Diphyl-

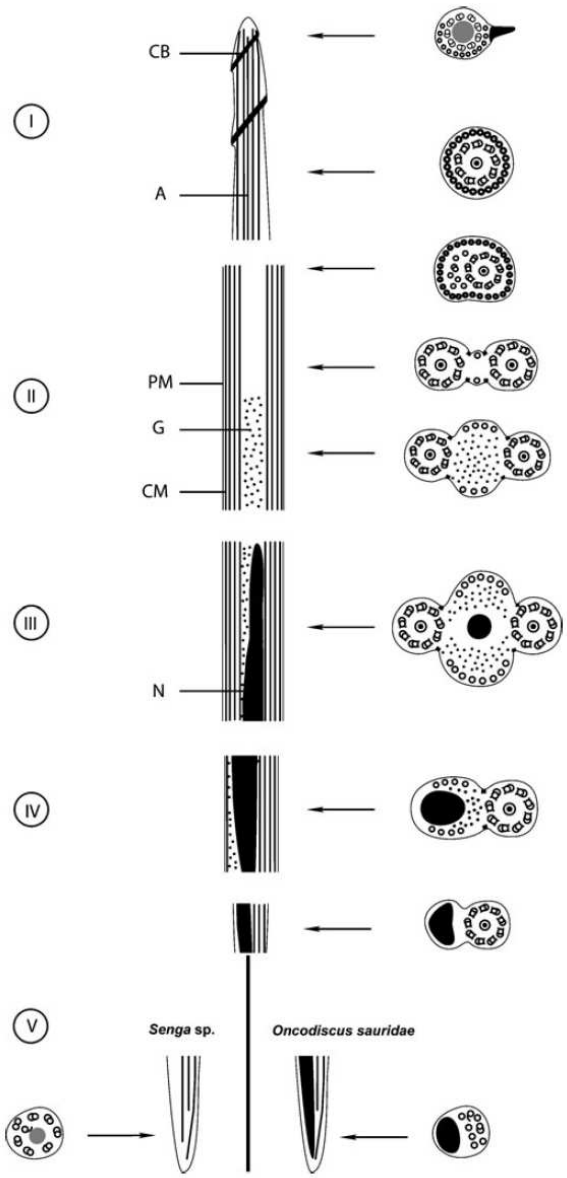

Fig. 6 I–V Schematic illustration of the mature spermatozoon of *Oncodiscus sauridae* and *Senga* sp. *A* axoneme, *CB* crested body, *CM* cortical microtubules, *G* glycogen, *N* nucleus, *PM* plasma membrane

lidea (Levron et al. 2010). This feature seems to be plesiomorphic for the Eucestoda (Justine 1998) in comparison with the presence of one axoneme in secondary more evolved cestodes, for example in the order Cyclophyllidea (Justine 1998; Levron et al. 2010). It is also plesiomorphic for the Neodermata (Justine 1995).

The crested body appears in the anterior extremity of the spermatozoon of *O. sauridae* and *Senga* sp. A crested body was reported in all species of the order Bothriocephalidea, except *B. clavibothrium* (Świderski and Mokhtar-Maamouri 1980). It occurs in almost all Eucestoda, with the exception of the orders Caryophyllidea, Spathebothriidea, Haplobothriidea, and Trypanorhyncha (MacKinnon and Burt 1985; Świderski and Mackiewicz 2002; Bruňanská et al. 2006; Miquel and Świderski 2006; Miquel et al. 2007; Gamil 2008; Bruňanská 2009). In Diphyllobothriidea, the crested body is present only in *Duthiersia fimbriata* (Justine 1986). This feature may be a structure of phylogenetic importance (Justine 1998; Levron et al. 2010) because its absence is typical for presumably most basal Eucestoda (MacKinnon and Burt 1985; Świderski and Mackiewicz 2002; Bruňanská et al. 2006; Miquel and Świderski 2006; Miquel et al. 2007, 2008; Gamil 2008; Bruňanská 2009).

One of the most important characters observed in *O. sauridae* and *Senga* sp. is the presence of a ring of cortical microtubules surrounding the axoneme in the anterior part of the spermatozoon. This character was described in all studied species of the order Bothriocephalidea (Świderski and Mokhtar-Maamouri 1980; Bruňanská et al. 2002; Levron et al. 2005, 2006a, b; Šípková et al. 2010). Unlike other bothriocephalideans, a partial ring of cortical microtubules is present in *B. claviceps* (Bâ et al. 2007). A complete ring of electron-dense tubular structures has been reported for all other species of the order Bothriocephalidea and may represent an autapomorphy of this cestode group. A semi-ring of cortical microtubules in the forepart of the spermatozoon was found in species of the orders Spathebothriidea, Trypanorhyncha, Tetraphyllidea, Proteocephalidea, and Mesocestoididae (Levron et al. 2010).

The posterior part of the spermatozoon differs in *O. sauridae* from and *Senga* sp. In *O. sauridae*, this region contains the nucleus and axonemal doublets and singlets ("decomposed" axoneme), but in *Senga* sp., only few singlets and doublets of axoneme are present. The terminal part of the spermatozoon of species of the order Bothriocephalidea and Diphyllobothriidea exhibits some variation in the number of axonemes and nucleus. In *B. clavibothrium* (Świderski and Mokhtar-Maamouri 1980), *E. crassum* (Bruňanská et al. 2002), *T. nodulosus* (Levron et al. 2005) from the order Bothriocephalidea, and *Ligula intestinalis* (Diphyllobothriidea; Levron et al. 2009), only one axoneme occurs in the end region of the spermatozoon. In *B. scorpii* (Bothriocephalidea; Levron et al. 2006a) and *Diphyllobothrium latum* (Diphyllobothriidea; Levron et al. 2006c), the nucleus is present. In the bothriocephalideans *Paraechinophallus japonicus* (Levron et al. 2006b) and *P. gracilis* (Šípková et al. 2010), the posterior spermatozoon extremity contains the nucleus and cortical microtubules.

Table 1 Data on the ultrastructure of the spermiogenesis and spermatozoon of the order Bothriocephalidea

Species	Spermiogenesis			Spermatozoon					References
	Type	IB	DM	Type	Nax	CB	R	PSE	
Bothriocephalidea									
Bothriocephalidae									
Bothriocephalus clavibothrium	I	+	−	II	2	−	+	Ax	Świderski and Mokhtar-Maamouri (1980)
Bothriocephalus claviceps				II	2	1	Partial	Ax	Bâ et al. (2007)
Bothriocephalus scorpii	I	2	+	II	2	1	+	N	Levron et al. (2006a)
Oncodiscus sauridae	I	2	+	II	2	1	+	N+CM	Present study
Senga sp.	I	2	+	II	2	1	+	CM	Present study
Echinophallidae									
Paraechinophallus japonicus				II	2	1	+	N+CM	Levron et al. (2006b)
Parabothriocephalus gracilis	I	2	+	II	2	1	+	N+CM	Šípková et al. (2010)
Triaenophoridae									
Eubothrium crassum	I	2	+	II	2	1	+	Ax	Bruňanská et al. (2001, 2002)
Triaenophorus nodulosus	I	2	+	II	2	1	+	Ax	Levron et al. (2005)

Ax axoneme, *CB* crested body, *CM* cortical microtubules, *DM* electron-dense material, *IB* intercentriolar body (number of electron-lucent layers), *N* nucleus, *Nax* number of axonemes in the mature spermatozoon, *PSE* posterior extremity, *R* ring of cortical microtubules, +/− presence/absence

Conclusions

Spermiogenesis and spermatozoon ultrastructure of *O. sauridae* and *Senga* sp. include typical characters of the order Bothriocephalidea (see Table 1) and distinguish them from other orders. Until now, nine species of the order Bothriocephalidea belonging to three different families have been examined for spermiogenesis and ultrastructure of the spermatozoon. Since similar features have been observed almost consistently, it is possible to draw a general pattern for spermatological characters in the Bothriocephalidea as follow: spermiogenesis of the type I with dense material in the apical region of the differentiation zone in early stages and sperm of the type II with a characteristic ring of cortical microtubules in the anterior part. However; no data are available for members of one family (Phylobythiidae), parasite of deep-sea teleosts.

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5. DISCUSSION

Spermiogenesis

Spermiogenesis of the two bothriocephalideans studied, *Oncodiscus sauridae* and *Senga* sp., follows the type I of Bâ and Marchand (1995). It is characterized by the presence of two flagella, flagellar rotation and proximodistal fusion. This type of spermiogenesis has been found also in other tapeworms of the orders Bothriocephalidea, Diphyllobothriidea (Table I), Spathebothriidea, "Tetraphyllidea" – Onchobothriidae, Trypanorhyncha, Proteocephalidea, Amphilinidea and Gyrocotylidea (Justine 1998; Levron et al. 2010). Spermiogenesis of the type I is considered to be the most primitive (plesiomorphic) of four existing types of spermiogenesis (Bâ and Marchand 1995). The second type of spermiogenesis is distinguished by the formation of one flagellum and abortion of the second one, a flagellar rotation and proximodistal fusion of the flagellum. It occurs in the "Tetraphyllidea" – Phyllobothriidae, Caryophyllidea and Tetrabothriidea. The third type appears in some species of Cyclophyllidea, i. e. the most evolved group of tapeworms, and it is distinguished by the presence of proximodistal fusion of one flagellum and absence of flagellar rotation. The spermiogenesis of the type IV without flagellar rotation and proximodistal fusion is characteristic for most of the members of the order Cyclophyllidea (Justine 1998).

The intercentriolar body in *O. sauridae* and *Senga* sp. consists of three electron-dense plates and two electron-lucent layers. This character is present also in other members of the Bothriocephalidea (Table I): *Bothriocephalus scorpii* (Bothriocephalidae; Levron et al. 2006a), *Eubothrium crassum* (Triaenophoridae; Bruňanská et al. 2001), *Parabothriocephalus gracilis* (Echinophallidae; Šípková et al. 2010), and *Triaenophorus nodulosus* (Triaenophoridae; Levron et al. 2005). It has also been observed in the spathebothriidean *Cyathocephalus truncatus* and the caryophyllidean *Wenyonia virilis* (Bruňanská et al. 2006; Miquel et al. 2008). The presence or absence of an intercentriolar body is used as a character of phylogenetic importance in phylogenetic studies (Justine 1998; Levron et al. 2010). Reduction of the layers of the intercentriolar body or its complete disappearance has been observed in the "higher", i.e. evolutionary more evolved cestodes, because this body is absent in Tetrabothriidea and Cyclophyllidea (Justine 2001; Levron et al. 2010). In *Diphyllobothrium latum*, studied by Bonsdorff and Telkkä (1965) and Levron et al. (2006c), and *Ligula intestinalis*, observed by Levron et al. (2009), both of

the order Diphyllobothriidea, the intercentriolar body is composed of five electron-dense plates and four electron-lucent layers, which suggests a more basal position of these tapeworms compared to that of the order Bothriocephalidea. Actually, molecular data support this assumption (Brabec et al. 2006; Waeschenbach et al. 2007).

In *O. sauridae* and *Senga* sp., an electron-dense material appears in the first stages of spermiogenesis in the apical region of the differentiation zone. This material was observed for the first time in *Eubothrium crassum* (Triaenophoridae) by Bruňanská et al. (2001) and then has been reported for all studied members of the order Bothriocephalidea, except for *Bothriocephalus clavibothrium* (see Table I). However, the latter species should be re-investigated to confirm this exception. An electron-dense material is also present in *D. latum* and *L. intestinalis* of the order Diphyllobothriidea and in tapeworms of the orders Caryophyllidea and Spathebothriidea (Bonsdorff and Telkkä 1965; Świderski and Mokhtar–Maamouri 1980; Bruňanská et al. 2001, 2002, 2006; Levron et al. 2005, 2006a, b, c, 2009; Bruňanská and Poddubnaya 2006; Gamil 2008; Miquel et al. 2008; Šípková et al. 2010). The presence of electron-dense material has been recently considered as a character of potential phylogenetic interest (Levron et al. 2010). However, its suitability for the assessment of relationships of cestodes is not yet clear and more comparative studies are required.

Table I. Data on spermiogenesis and ultrastructure of spermatozoa of tapeworms of the orders Bothriocephalidea and Diphyllobothriidea, previously grouped together as Pseudophyllidea. Type of spermiogenesis according to Bâ and Marchand (1995) and type of spermatozoon according to Levron et al. (2010).

Species	Definitive host	Habitat	Spermiogenesis			Spermatozoon					References
			Type	IB	DM	Type	Nax	CB	R	PSE	
Bothriocephalidea											
Bothriocephalidae											
Bothriocephalus clavibothrium	*Arnoglossus laterna* (teleost)	marine	I	+	–	II	2	–	+	Ax	Świderski and Mokhtar-Maamouri (1980) – reinvestigation needed
Bothriocephalus claviceps	*Anguilla anguilla* (teleost)	freshwater	/	/	/	II	2	1	incomplete	Ax	Bâ et al. (2007)
Bothriocephalus scorpii	*Neogobius* sp. (teleost)	marine	I	2	+	II	2	1	+	N	Levron et al. (2006a)
Oncodiscus sauridae	***Saurida nebulosa* (teleost)**	**marine**	**I**	**2**	**+**	**II**	**2**	**1**	**+**	**N+CM**	**Present study**
***Senga* sp.**	***Mastacembelus armatus* (teleost)**	**freshwater**	**I**	**2**	**+**	**II**	**2**	**1**	**+**	**CM**	**Present study**
Echinophallidae											
Paraechinophallus japonicus	*Psenopsis anomala* (teleost)	marine	/	/	/	II	2	1	+	N+CM	Levron et al. (2006b)
Parabothriocephalus gracilis	*Psenopsis anomala* (teleost)	marine	I	2	+	II	2	1	+	N+CM	Šípková et al. (2010)
Triaenophoridae											
Eubothrium crassum	*Salmo trutta* m. *fario* (teleost)	marine	I	2	+	II	2	1	+	Ax	Bruňanská et al. (2001, 2002, 2010)
Eubothrium rugosum	*Lota lota* (teleost)	freshwater	/	/	/	II	2	1	+	Ax	Bruňanská et al. (2010)
Triaenophorus nodulosus	*Esox lucius* (teleost)	freshwater	I	2	+	II	2	1	+	Ax	Levron et al. (2005)
Diphyllobothriidea											
Diphyllobothriidae											
Diphyllobothrium latum	*Felis silvestris f. catus* (mammal)	terrestrial	I	4	+	I	2	–	–	N	Bonsdorff and Telkkä (1965), Levron et al. (2006c)
Ligula intestinalis	*Podiceps cristatus* (bird)	terrestrial	I	4	+	I	2	–	–	Ax	Levron et al. (2009)
Scyphocephalidae											
Duthiersia fimbriata	*Varanus niloticus* (reptile)	terrestrial	/	/	/	I?	2	1	incomplete	/	Justine (1986)

Abbreviations: Ax, axoneme; CB, crested body; CM, cortical microtubules; DM, electron-dense material; IB, intercentriolar body (number of electron-lucent layers); N, nucleus; Nax, number of axonemes in the mature spermatozoon; PSE, posterior extremity; R, ring of cortical microtubules; +/-, presence/absence of the structure; /, absence of data.

Spermatozoon

Recently, Levron et al. (2010) distinguished seven basic types of spermatozoa (Fig. 6) in the Eucestoda. They are divided on the basis of the number of axonemes (one or two), the parallel/spiraled pattern of cortical microtubules and the nucleus, the absence/presence of a crested body, periaxonemal sheath and intracytoplasmic walls. The first and second types are characterized by the possession of two axonemes, whereas the others contain only one axoneme.

In *O. sauridae* and *Senga* sp., the mature spermatozoon corresponds to the type II and contains two axonemes of the 9 + "1" trepaxonematan pattern (Ehlers 1984). Two axonemes are also observed in other species of Bothriocephalidea, Diphyllobothriidea, Spathebothriidea, Haplobothriidea, Trypanorhyncha, Tetraphyllidea (Onchobothriidae), Proteocephalidea and Diphyllidea (Bruňanská et al. 2010; Levron et al. 2010). This feature seems to be a plesiomorphic condition for the Eucestoda (Justine 1998), because only one axoneme is present in more evolved cestodes, for example in the orders Cyclophyllidea and Tetrabothriidea (Justine 1998; Levron et al. 2010). The presence of only one axoneme in these cestodes is probably a result of secondary reduction, because two axonemes are present in more basal Neodermata (Justine 1995).

The crested bodies of the spermatozoon of *O. sauridae* and *Senga* sp. characterize the anterior extremity of the spermatozoon. This feature has been reported for all species of the order Bothriocephalidea (Table I), except for *B. clavibothrium*, the spermatozoon of which should be reinvestigated (Świderski and Mokhtar–Maamouri 1980). The crested body is present in almost all Eucestoda, with the exception of the orders Caryophyllidea, Spathebothriidea, Haplobothriidea and Trypanorhyncha (MacKinnon and Burt 1985; Świderski and Mackiewicz 2002; Bruňanská et al. 2006, 2010; Miquel and Świderski 2006; Miquel et al. 2007; Gamil 2008; Bruňanská 2009). The presence or absence of crested bodies in species of Diphyllobothriidea is still unclear, because a crested body was not observed in *Diphyllobothrium latum*, *Ligula intestinalis* and *Schistocephalus solidus* (Levron et al. 2006b, 2009, unpublished data). In contrast, Justine (1986) reported a unique crested body in the anterior part of the spermatozoon in *Duthiersia fimbriata* (Scyphocephalidae), a parasite of monitors (Varanidae). This character is considered to be a structure of phylogenetic importance (Justine 1998; Levron et al. 2010), because its absence is typical for presumably most basal Eucestoda. The presence of crested bodies is considered as a synapomorphic character for a part of the Eucestoda (Bothriocephalidea,

Diphyllidea, Tetraphyllidea (Onchobothriidae and Phyllobothriidae), Lecanicephalidea, Proteocephalidea, Tetrabothriidea, Mesocestoididae and Cyclophyllidea) (Justine 2001; Levron et al. 2010). Usually, a single crested body is reported. However, in hymenolepidids (Cyclophyllidea), as many as 12 crested bodies have been observed (Bâ and Marchand 1992), which may indicate a trend of increasing numbers of crested bodies during evolution of most evolved groups, i.e. members of Cyclophyllidea.

One of the most interesting features observed in the spermatozoon of *O. sauridae* and *Senga* sp. is the presence of a ring of cortical microtubules surrounding the axoneme in the anterior part. This character has been found in all studied species of the order Bothriocephalidea (Table I), except *Bothriocephalus claviceps*, in which a partial ring of cortical microtubules was observed (Świderski and Mokhtar–Maamouri 1980). A ring of tubular structures may occasionally encircle two fully formed axonemes, as observed in the spermatozoa of *Eubothrium rugosum* (Bothriocephalidea – Bruňanská et al. 2010). This new character has been observed for the first time in the Eucestoda and it was not found in other bothriocephalideans.

In most Diphyllobothriidea, a ring of cortical microtubules is absent. The exception is *D. fimbriata* from reptiles, which possesses a partial ring of cortical microtubules. A complete ring of electron-dense tubular structures encircling one axoneme has been reported for all other species of the order Bothriocephalidea and may represent an autapomorphy of this cestode group (Bonsdorff and Telkkä 1965; Justine 1986; Levron et al. 2006c, 2009). A semi-ring of cortical microtubules in the anterior part of the spermatozoon was found in species of the orders Spathebothriidea, Trypanorhyncha, Tetraphyllidea, Proteocephalidea and Mesocestoididae (Levron et al. 2010).

The posterior part of the spermatozoon of *O. sauridae* differs from that of *Senga* sp. In *O. sauridae*, this region contains the nucleus and axonemal doublets and singlets (‘decomposed’ axoneme), whereas only a few singlets and doublets of axonemes are present in *Senga* sp. The terminal part of the spermatozoon of species of the orders Bothriocephalidea and Diphyllobothriidea exhibits remarkable diversity in the number of axonemes and nucleus (Table I). In bothriocephalideans *E. crassum* and *E. rugosum*, only singlets of the axoneme appear in the posterior spermatozoon extremity (Bruňanská et al. 2002, 2010). In *B. clavibothrium*, *B. claviceps*, *T. nodulosus* (all Bothriocephalidea) and *L. intestinalis* (Diphyllobothriidea), only one axoneme is present in the end region of the spermatozoon (Świderski and Mokhtar–Maamouri 1980; Levron et al. 2005, 2009; Bâ et

al. 2007). In *B. scorpii* (Bothriocephalidea) and *D. latum* (Diphyllobothriidea), only the nucleus characterizes this part (Levron et al. 2006a, 2006c), but in the bothriocephalideans *Paraechinophallus japonicus* and *Parabothriocephalus gracilis*, studied by Levron et al. (2006b) and Šípková et al. (2010), the posterior spermatozoon extremity contains the nucleus and cortical microtubules. Thus the posterior extremity of spermatozoon does not appear to be suitable character for the differentiation of the orders Bothriocephalidea and Diphyllobothriidea, because its ultrastructure is variable in both orders and between families and genera of the same order (see Table I).

6. CONCLUSIONS AND PERSPECTIVES

In *Oncodiscus sauridae* and *Senga* sp. from the order Bothriocephalidea, spermiogenesis follows the same pattern as in other bothriocephalideans. The spermatozoon of these two cestodes also exhibits ultrastructural characters typical of members of this cestode order. Until now, ten species of the order Bothriocephalidea, belonging to three families (Bothriocephalidae, Echinophallidae, Triaenophoridae), have been examined for the spermiogenesis and/or spermatozoon ultrastructure. These tapeworms are dissimilar in their morphology, especially shape of the scolex (see Kuchta et al. 2008) and were found in unrelated teleost fish from markedly different habitats (brackish, freshwater and marine environments). In all these species, similar features have been observed, which made it possible to describe a general pattern characteristic for tapeworms of the order Bothriocephalidea, inferred from ultrastructural characters. These characters are spermiogenesis of the type I with an electron-dense material in the apical region of the differentiation zone in early stages, and spermatozoon of the type II with a ring of cortical microtubules surrounding one axoneme in the anterior part of the sperm.

Considering the fact that all studied species belong to three families of the order Bothriocephalidea and all exhibited almost identical ultrastructural features, it is questionable to study using TEM other representatives of these families. In contrast, no data are available for the members of the family Philobythiidae, which are parasites of deep-sea teleosts. It would thus be interesting to examine some species of this group of tapeworms that adapted to life in extreme conditions of deep-seas.

Ultrastructural information on spermiogenesis and spermatozoa morphology appeared to be valuable for differentiation of higher taxonomic groups (orders), but it does not seem to provide too many suitable characters for lower-level systematics and comparative studies. Nevertheless, there still remain numerous gaps in our knowledge before more generalizations are taken and several specious groups of tapeworms, which have been poorly studied, should be examined in the future.

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