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Research paper

Sperm characters of *Timoniella imbutiforme* (Digenea, Opisthorchioidea, Cryptogonimidae), a parasite of the European seabass *Dicentrarchus labrax*

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ABSTRACT

Spermatological characteristics of the digenean *Timoniella imbutiforme* (Cryptogonimidae) collected from *Dicentrarchus labrax* (Teleostei: Serranidae) collected at the Gulf of Gabès in Chebba (Tunisia) were investigated for the first time by means of transmission electron microscopy. The ultrastructural study reveals that the mature spermatozoon of *T. imbutiforme* is a filiform cell, tapered at both extremities. The sperm cell exhibits the characteristics of digenean spermatozoa type IV namely two axonemes of the 9 + '1' pattern of trepaxonematan Platyhelminthes, external ornamentation of the plasma membrane associated with cortical microtubules and located in the posterior part of the anterior region of the sperm cell, two bundles of parallel cortical microtubules, maximum number of cortical microtubules in the anterior part of the spermatozoon and presence of two mitochondria. The first mitochondrion of moniliform type is composed of a mitochondrial cord with joined mitochondrial bulges. In addition, the male gamete of *T. imbutiforme* shows spine-like bodies and a posterior extremity with only the second axoneme. The ultrastructural characters of the spermatozoon of *T. imbutiforme* are compared with those of other digeneans belonging to the superfamily Opisthorchioidea.

1. Introduction

The superfamily Opisthorchioidea consists of three well-known families, namely the Cryptogonimidae, Opisthorchiidae and Heterophyidae, including numerous species with similarities in their morphological traits and life cycles. However, the phylogenetic relationships between these families remain controversial (Bray, 2008; Olson et al., 2003). There are several studies that analyse the molecular affinities within the Opisthorchioidea (Kvach et al., 2017a,b; Le et al., 2017; Thaenkham et al., 2011, 2012), which demonstrate the paraphyly of the Heterophyidae and Opisthorchiidae and show that these two families form an inseparable single clade. Recently, Le et al. (2017) confirmed certain incongruences in the molecular placement of the genera *Cryptocotyle* and *Eurytrema* (placed within the Heterophyidae), which appeared to have more affinities with opisthorchiids. All the latter authors agree that a multidisciplinary approach, using both morphological and molecular data, is needed for a better knowledge of phyletic relationships within the Opisthorchioidea.

The ultrastructural study of the mature spermatozoon has proved to be useful for phylogenetic inference in parasitic Platyhelminthes. In fact, ultrastructural characteristics of sperm cells of monogeneans, cestodes and, recently, digeneans have contributed to a better knowledge of the interrelationships within these groups (Bâ and Marchand, 1995; Bakhoum et al., 2017a; Justine, 1991a, 1991b, 1998, 2001; Levron et al., 2010).

With respect to the Digenea, during the last years, studies about the ultrastructural characters of the spermatozoon have notably increased (for a review see Bakhoum et al., 2017a). However, for the Cryptogonimidae only four species have been studied even though

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the Cryptogonimidae is a large and cosmopolitan family, parasitizing a range of marine and freshwater host including teleosts, reptiles and, rarely, amphibians (Miller and Cribb, 2008). The four previously studied species are *Adlardia novaecaledoniae* (studied under the name "*Siphoderina elongata*" –see Miller et al., 2009; Quilichini et al., 2009), *Anisocoelium capitellatum* (Ternengo et al., 2009), *Aphallus tubarium* (Foata et al., 2012), and *Stemmatostoma pearsoni* (the spermatological results were published under the name "*Neochasmus* sp." –see Cribb, 1986; Jamieson and Daddow, 1982).

Thus, the aim of the present study is to produce the first complete description of the ultrastructure of another cryptogonimid *Timoniella imbutiforme*, contributing to the ultrastructural spermatological database on the Digenea. In order to highlight criteria that may be useful for phylogenetic purposes, results on *Timoniella imbutiforme* are also compared with available data on the Opisthorchioidea.

2. Materials and methods

2.1. Materials

Live adult specimens of *Timoniella imbutiforme* were collected during December 2015 from the digestive tract of the European seabass *Dicentrarchus labrax* captured in the Mediterranean Sea, off La Chebba (34°14′N, 11°06′E) (Tunisia). A voucher specimen, stained with Semichon's acetic carmine and mounted in Canada balsam, was deposited in the parasitological collection of the Muséum National d'Histoire Naturelle (Paris) (MNHN): one slide of *T. imbutiforme* ex. *D. labrax* (no. 2015121106) off La Chebba (Tunisia), 11 December 2015–accession number MNHN HEL733.

Specimens were identified according to previous descriptions of *T. imbutiforme* (see Brooks and Holcman, 1993; Maillard, 1973). Later, this identification was corroborated by molecular techniques.

2.2. Molecular analyses

Total genomic DNA was isolated from one specimen following Lopez et al. (2015). A PCR reaction in order to amplify the large subunit ribosomal RNA gene was carried out following Tkach et al. (2000). Sequences were obtained in Macrogen (Korea). In order to confirm the identity of the specimens, a BLAST search was carried out.

A fragment of 972 bp of the 28S rRNA was obtained and deposited in GenBank under the accession number MF983699. The BLAST search showed a 99% of identity with *T. imbutiforme* (accession number: MF491865.1), obtained from *Neogobius melanostomus* in Ukraine, Black Sea (Kvach et al., 2017b). Only three variable nucleotide positions were observed in the alignment of both sequences. When comparing to the MF491865.1 sequence, the variable nucleotides in our sequence were A:G in the position 391, C:T in the position 487, and G:T in the position 597.

2.3. Transmission electron microscopy

For the present TEM study, several worms 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 a minimum of 2 h, rinsed in 0.1 M sodium cacodylate buffer at pH 7.4, post-fixed in cold (4 °C) 1% osmium tetroxide with 0.9% potassium ferricyanide [$K_3Fe(CN)_6$] 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 (1963) procedure. Copper grids were examined in a JEOL 1010 transmission electron microscope operated at an accelerating voltage of 80 kV, in the "Centres Científics i Tecnològics" of the University of Barcelona (CCiTUB).

2.4. Cytochemistry

Sections placed on gold grids were treated according to the Thiéry (1967) test to reveal the presence of glycogen. Thus, they were treated in periodic acid (PA), thiocarbohydrazide (TCH) and silver proteinate (SP) as follows: 30 min in 10% PA, rinsed in Milli-Q water, 24 h in TCH, rinsed in acetic solutions and Milli-Q water, 30 min in 1% SP in the dark, and rinsed in Milli-Q water. Sections were examined in a JEOL 1010 transmission electron microscope at an accelerating voltage of 80 kV, in the CCiTUB.

3. Results

The interpretation of numerous cross- and longitudinal sections of the mature spermatozoon of *T. imbutiforme* allow us to establish three distinctive regions exhibiting different ultrastructural characteristics, from the anterior to the posterior extremities of the male gamete (Figs. 1–4). The mature spermatozoon of *T. imbutiforme* exhibits the usual structures found in most digeneans. Indeed, it contains two axonemes of the 9 + '1' trepaxonematan pattern, external ornamentation of the plasma membrane, spine-like bodies, nucleus, mitochondria, two bundles of parallel cortical microtubules and granules of glycogen.

Region I (Figs. 1a–h and 4I) corresponds to the anterior region of the spermatozoon. Cross-sections through the anterior tip show both centrioles and axonemes, and a few cortical microtubules arranged in two discontinuous and submembraneous layers composed of a maximum number of 9 + 10 microtubules (Fig. 1a and b). In a more posterior area, the number of cortical microtubules increases, forming a continuous layer of about 24 microtubules, but they do not surround the sperm cell completely (Fig. 1c and d). It is possible to observe two attachment zones at this level (Fig. 1d). In the posterior part of region I, there is a moniliform mitochondrion, composed of a mitochondrial cord with joined mitochondrial bulges (Figs. 1e–h and 4I). Moreover, in this area the cortical microtubules are arranged into two fields with a maximum number of cortical microtubules of 1 + 14 (Fig. 1e–h). It is also possible to observe an external ornamentation of the plasma membrane associated with cortical microtubules and spine-like bodies along this posterior part of region I (Figs. 1e–h and 4I). The four attachment zones are observed in these sections (Fig. 1e–g) compared to previous cross-section (Fig. 1d). The transition toward region II is marked by the disappearance of the first mitochondrion and the external ornamentation.

Region II (Figs. 2a–d, 3 and 4II) corresponds to the middle region of the spermatozoon, which is mainly characterized by the presence of the second mitochondrion. The anterior area of this region shows the simultaneous presence of both axonemes, two bundles of about 4 + 6 cortical microtubules and a large amount of granular material characterized as glycogen according to the Thiéry's test (Figs. 2a and 3). In the middle and posterior areas of this region, the second mitochondrion appears and the maximum number of cortical microtubules is 6 + 6 (Figs. 2b and c and 4II). In the distal part of this region, the first axoneme disorganises and disappears (Fig. 2d).

Region III (Figs. 2e–i, 3 and 4III) corresponds to the nuclear and posterior spermatozoon extremity. In the anterior area of this region, the second mitochondrion is still present (Figs. 2e–f and