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Spermatozoa ultrastructure of two *osmerid* fishes in the context of their family (Teleostei: Osmeriformes: Osmeridae)

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Summary

Systematics of the Osmeridae remains controversial, and may benefit from detailed examination of sperm biology. Sperm morphology and ultrastructure of two osmerids were analyzed, one from Tribe Salangini (*Mallotus villosus*) and another from Tribe Osmerini (*Osmerus mordax*) to try to clarify some of the observations previously made by other authors in the context of the Osmeridae family. In both species there is a bullet-shape head with a deep nuclear fossa where one finned flagellum is deeply inserted, and there is only one mitochondrion. A general schematic model for Osmeridae sperm is proposed that excludes the Salangini tribe but includes *M. villosus*. *M. villosus* ultrastructure is more similar to the Osmerini than the Salangini where it is currently placed.

Introduction

Most members of the Osmeridae (Northern Hemisphere smelts) are anadromous or freshwater residents such as rainbow smelt (*Osmerus mordax*), though some spawn in the marine environment sub tidally or on beaches, such as capelin (*Mallotus villosus*) (Martin and Swiderski, 2001). Currently they are considered part of the Osmeriformes order and the Osmeridae family (Nelson, 2006), and throughout this paper we follow Nelson's (2006) taxonomic classification. These fishes were initially classified within the Salmonidae (Cuvier, 1817), and still after several revisions based on morphometric characteristics and phylogenetics their systematics is debatable (Fu et al., 2005; Nelson, 2006; Ilves and Taylor, 2009). As an example *M. villosus* placement inside of the family is unclear (Ilves and Taylor, 2009).

Spermatozoa ultrastructure can represent a valuable tool in deciphering fish systematics and evolutionary history (reviewed by Jamieson, 1991). Several Osmeridae species have been evaluated, but unfortunately only European smelt (*Osmerus eperlanus*) (Kowalski et al., 2006) and ayu (*Plecoglossus altivelis*) (Gwo et al., 1994) sperm ultrastructure descriptions are available in English, while *Hypomesus nipponensis*, *Hypomesus japonicas*, *Spirinchus lanceolatus*, *O. eperlanus mordax*, *M. villosus*, *Salangichthys microdon*, *Salangichthys ishikawae*, *Salanx ariakensis* and *Neosalanx reganius* descriptions are published in Japanese (Hara, 2009). The genus *Hypomesus* belongs to the Hypomesinae subfamily. The genera *Salangichthys, Salanx, Neosalanx, Mallotus*, *Osmerus* and *Spirinchus* belong to the Osmerinae subfamily but the first three were previously considered a different family, Salangidae. Salangidae are now part of the Salangini tribe together with *Mallotus* while the remainder of the Osmerinae form the Osmerini tribe. *Plecoglossus* belong to the Plecoglossinae subfamily; also previously considered a separate family, Plecoglossidae. All Osmeridae sperm (except the Salangini but including *Mallotus*) seem to have an ovoid bullet-shape head and one finned flagellum that is deeply inserted into the nucleus (Gwo et al., 1994; Kowalski et al., 2006; Hara, 2009). In all species but the Salangini (excluding *Mallotus*) only one mitochondrion is present along the base of the flagellum (Gwo et al., 1994; Hara, 2009) except *O. eperlanus* where three spermatozoa were observed with two mitochondria (Kowalski et al., 2006), see discussion. For the two species with descriptions available in the English literature, the cytoplasmic canal is absent in *P. altivelis* (Gwo et al., 1994) but present in *O. eperlanus* (Kowalski et al., 2006).

In the present work we analyze the sperm morphology and ultrastructure of anadromous *O. mordax* and marine *M. villosus* and compare it with other Osmeridae. We clarify some of the observations previously made by other authors and discuss our results in the context of this family.

Materials and methods

Spawning fish were captured in the second week of June and the third of July 2012 for *O. mordax* and *M. villosus* respectively. *O. mordax* were captured in the Salmonier River (47°19′N, 53°39′W) and *M. villosus* on Bellevue beach (47°64′N, 53°78′W), both on the Avalon Peninsula, Newfoundland, Canada. Fish were transported to Memorial University and kept alive for a maximum of 2 days in 7–10°C holding tanks until sampling was complete.

For sperm morphology, fresh samples from eight fish of each species were observed from photos taken with a Leica microscope (DM IL LED) using a 60 × phase contrast lens and a Leica DFC420 camera (5 megapixel). For each sample at least 50 spermatozoa were photographed and measured with ImageJ (Schneider et al., 2012).

For the ultrastructure analysis, sperm samples were prediluted (5 μl of semen: 45 μl of 0.1 M Sodium Cacodylate buffer) and fixed by adding Karnovsky solution (50 × the volume of our sample, 2.5 ml), for 10–24 h at 4°C. Samples

were centrifuged for 5 min at room temperature to pellet the cells and washed with buffer solution. Samples were then processed following the Hyam (1981) protocol. Briefly, samples were dehydrated (1% Osmium Tetroxide for 15 min, 0.1M buffer solution for 5 min, 70% ethanol for 10 min two times, 95% ethanol for 10 min two times, absolute ethanol for 10 min two times and absolute acetone for 10 min two times) and infiltrated with resin (50 : 50, acetone:resine) for 15 min. Samples were then embedded in BEEM capsules and polymerized overnight at 80°C and a series of ultrathin sections were cut using a Reichert Ultracut S. The ultrathin sections were counterstained with uranyl acetate and lead citrate and viewed in a JEOL 1200 EX transmission electron microscope (JEOL, Ltd) operated at 80 kV and attached to a digital camera (SIA L3C side mounted – 2048 × 2048 pixels). Many images were saved to allow the identification of the different sperm structures.

Statistics were conducted using R 2.15.1 (R Development Core Team, 2012). Significant differences ($P < 0.05$) between the two species in terms of spermatozoa morphology were detected with a MANOVA followed by *post-hoc t*-test for each measurement. Results are expressed as means ± SEM.

Results

In both *M. villosus* and *O. mordax* the spermatozoa has a bullet-shape head, a short midpiece and one long flagellum. While there are significant differences in the spermatozoa sizes (Pillai's = 0.978, $F_{4,11} = 127.88$, $P < 0.001$) between the two species (see below) the ultrastructure is typically similar.

Sperm head

The *M. villosus* sperm head is longer and wider than that of *O. mordax* ($F_{1,14} = 89.62$, $P < 0.001$ and $F_{1,14} = 155.54$, $P < 0.001$ respectively), Table 1. In both species the nucleus has a U shape in the longitudinal plane and is surrounded by a membrane, and the chromatin is nuclear dense (Figs 1a, 2a and 3).

Centriolar complex

Two types of centrioles (proximal and distal centrioles) are deeply inserted in the anterior portion of the deep nuclear fossa that has a cylindrical shape (Figs 1a, 2a and 3). The proximal centriole has the classic (9 + 0) nine triplets of microtubules (Fig. 1a). The two centriolar axes are in different planes, the proximal centriole appears inclined in relation to the distal centriole more than 90° in *M. villosus* and more than 120° in *O. mordax* along the sagittal plane. The proximal centriole is close to the nuclear envelope and at least partially embedded in osmiophilic material. Different shaped nuclear dense structures (pericentriolar material) link the two centrioles to each other and anchor the proximal centriole to the nuclear envelope and the distal centriole in the transitional region (Fig. 2a). The distal centriole forms the basal body of the flagellum. Its anterior portion is embedded in osmiophilic material and in the transitional region has the classical (9 + 0) nine doublets of microtubules. These doublets are continuously connected to each other by an inner ring (Figs 1b and 2b). At the end of the transitional region an additional central singlet of tubules is present (9 + 2), the inner ring is no longer present and the doublets project radial spokes toward the singlets (Figs 1c and 2c).

Midpiece

The midpiece is also significantly larger in *M. villosus* than *O. mordax* ($F_{1,14} = 140.31$, $P < 0.001$), (Table 1). This structure contains the centriolar complex and the mitochondrion with irregularly arranged cristae (Fig. 3). Only one mitochondrion is present in each species which surrounds the base of the flagellum, in *M. villosus* the mitochondrion almost encloses the flagellum (Fig. 1d) while in *O. mordax* it has a C-shape (Fig. 2d). Neither in *M. villosus* nor *O. mordax* is there evidence of a cytoplasmic canal separating the flagellum from the mitochondrion. Nonetheless, the cytoplasmatic membrane was partially destroyed during sample preparation causing difficulties in the identification of the presence or absence of the cytoplasmic canal.

Flagellum

The flagellum is inserted centrally in the nucleus and has similar length in both species ($F_{1,14} = 1.80$, $P = 0.201$) (Table 1). In both cases the flagellum (axoneme) is composed of a 9 + 2 microtubular doublet structure enclosed by the plasma membrane. The nine doublets are formed by subtubules A and B and two dynein arms arise from each subtubule A and extend toward the next doublet tubule B (Figs 1e and 2e). The plasma membrane forms two lateral extensions in line with the two central tubules similar to fins.

Table 1
Sperm cell morphology comparison of *M. villosus* and *O. mordax* (this study) with *O. eperlanus* (Kowalski et al., 2006) and *P. altivelis* (Gwo et al., 1994). Significant differences between *M. villosus* and *O. mordax* indicated by *. Values for *M. villosus* and *O. mordax* are means for eight males ± SEM

Species	*M. villosus*	*O. mordax*	*O. eperlanus*	*P. altivelis*[2]
Head length[1] (μm)	1.82 ± 0.02*	1.56 ± 0.04	1.42 ± 0.05	2.1
Head width (μm)	1.20 ± 0.02*	0.91 ± 0.01	1.32 ± 0.07	1.2
Midpiece width (μm)	0.59 ± 0.02*	0.35 ± 0.01	0.54 ± 0.02	–
Flagellum length (μm)	30.51 ± 0.65	31.42 ± 0.19	27.72 ± 0.61	21.2

[1]Refers to length without the mid-piece.
[2]SEM values not available.

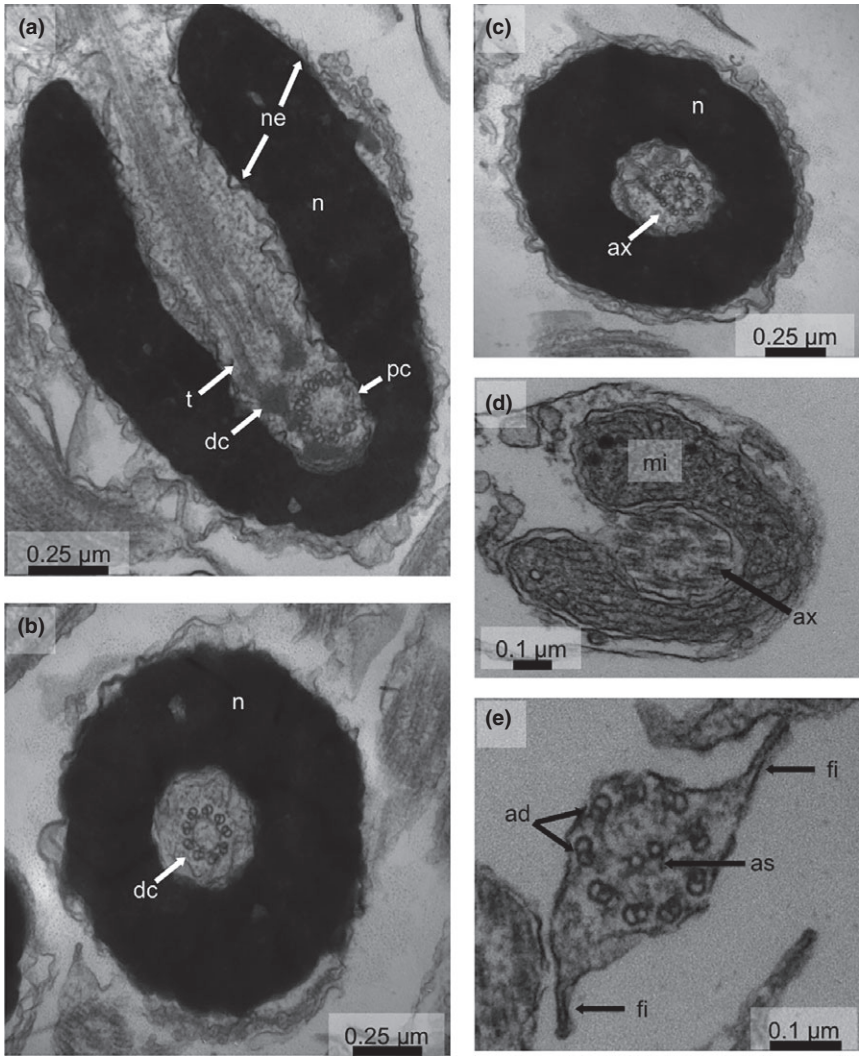

Fig. 1. Transmission electron micrographs, *M. villosus* sperm. (a) Longitudinal section of the head showing nucleus condensed chromatin (n) enclosed in nuclear envelope (ne), proximal centriole with triplets of microtubules (pc), distal centriole with the osmiophilic material (dc), and distal centriole transitional region (t). (b) Cross-section of the head in the distal centriole transitional region showing 9 + 0 microtubular doublet connected to each other by an inner ring. (c) Cross-section of the head in the basal region of the axoneme (ax) showing 9 + 2 configuration with the microtubular doublets projecting radial spokes. (d) Cross-section of mitochondrion (mi) with diverse cristae; the mitochondrion almost encloses the axoneme. (e) Cross-section of the flagellum showing details of axonemal structure with the plasma membrane lateral extensions, fins (fi), the 9 + 2 microtubular structure with the axonemal singlet (as) and axonemal doublets (ad) with the dynein arms

Discussion

The Osmeridae spermatozoa fine structure is the typical aquasperm type (Jamieson, 1991) similar to other teleosts with external fertilization; lacking an acrosome, the midpiece is small and only one long flagellum is present. In general the spermatozoa are small in comparison with other teleost families; head lengths between 2.1 μm for *P. altivelis* (Gwo et al., 1994), 1.82 $\pm$ 0.02 μm for *M. villosus* (the larger spermatozoa in this study) and 1.42 $\pm$ 0.05 μm for *O. eperlanus* (Kowalski et al., 2006), and flagella between 31.42 $\pm$ 0.19 μm for *O. mordax* (this study) and 21.2 *P. altivelis* (Gwo et al., 1994). Several common features are present between these two species and the other described Osmeridae (Gwo et al., 1994; Kowalski et al., 2006) and a general schematic model for this family is suggested in Fig. 4. *P. altivelis* have a larger sperm head but smaller flagellum (Gwo et al., 1994) compared to the other three described Osmeridae (this study and Kowalski et al. (2006)). Nonetheless all Osmeridae species (this study; Gwo et al., 1994; Kowalski et al., 2006; Hara, 2009) but the Salangini, have an ovoid bullet-shape head with a U shape nucleus surrounded by a membrane, and a very deep nuclear fossa that encloses both the centriolar complex (proximal and distal centriole) and the initial portion of the flagellum formed by the distal centriole. The Salangini tribe, excluding the genus *Mallotus*, presents a more spherical head and a moderately deep nuclear fossa. The angle formed by the two centrioles seems to be variable between the Osmeridae species since our observations point to higher angle in *O. mordax* compared to *M. villosus*, close to the 135°C observed in *P. altivelis* (Gwo et al., 1994), yet

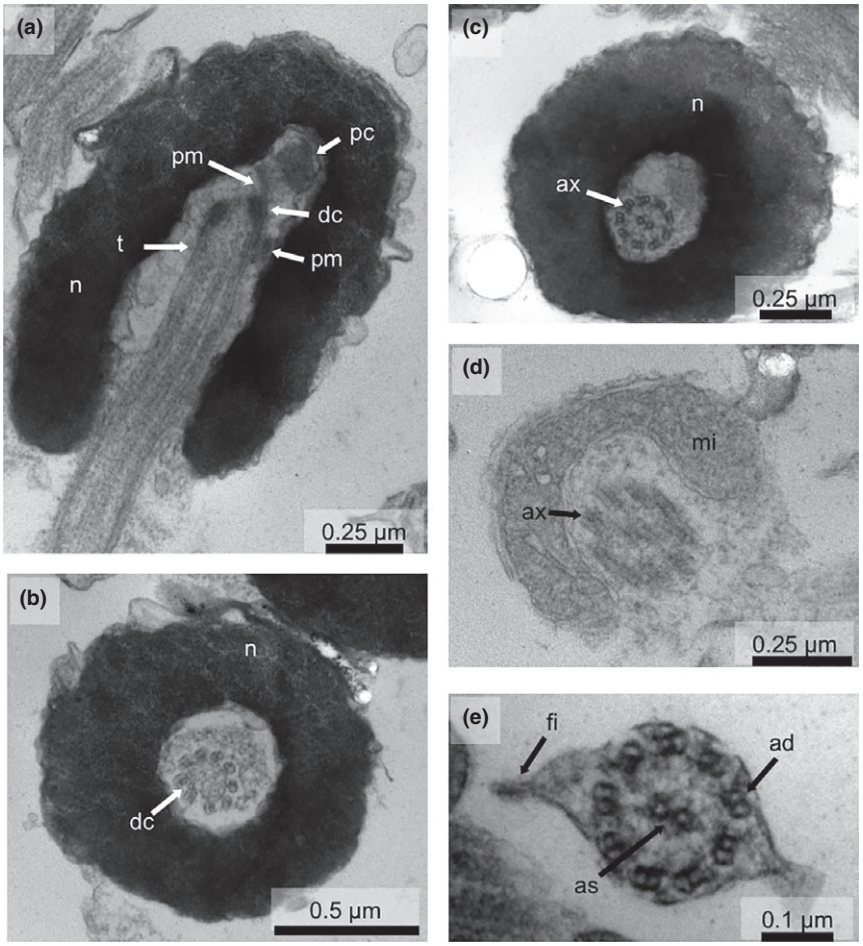

Fig. 2. Transmission electron micrographs, *O. mordax* sperm. (a) Longitudinal section of the head showing nucleus condensed chromatin (n), proximal centriole (pc), distal centriole with the osmiophilic material (dc) and pericentriolar material (pm) and distal centriole transitional region (t) with pericentriolar material anchoring the axoneme to nuclear envelope. (b) Cross-section of the head in distal centriole transitional region showing 9 + 0 microtubular doublet connected to each other by an inner ring. (c) Cross-section of the head in basal region of the axoneme (ax) showing 9 + 2 configuration with the microtubular doublets projecting radial spokes. (d) Cross-section of mitochondrion (mi) in a C-shape with axoneme in the centre. (e) Cross-section of the flagellum showing details of axonemal structure with the plasma membrane lateral extensions, fins (fi), the 9 + 2 microtubular structure with the axonemal singlet (as) and axonemal doublets (ad) with the dynein arms

this feature has been observed to be highly variable within other families (see, for example, Sinipercidae by Luo et al., 2011). Osmeridae sperm typically have a deep nuclear fossa (this study; Gwo et al., 1994; Kowalski et al., 2006), but it is shallower in the Salangini tribe (excluding *M. villosus*) (Hara, 2009), which may be related to the spherical as opposed to oval head shape. Although the deep nuclear fossa can also be found in other teleosts, such as the Soleidae (Medina et al., 2000), its presence clearly differentiates sperm of Osmeridae from the related Salmonidae, which have a shallow nuclear fossa (Billard, 1983; Jamieson, 1991). Whereas the placement of the centriolar complex inside of the nuclear fossa is a plesiomorphic characteristic (Jamieson, 1991), the function of such a deep nuclear fossa is not clear, but Gwo et al. (1994) speculated that it could be related with securing the sperm flagellum to the nucleus, yet these authors did not observe in *P. altivelis* the presence of pericentriolar material linking the centrioles to each other and anchoring them to the nuclear envelope as we observed in both *M. villosus* and *O. mordax*. Also in all Osmeridae species the distal centriole in the transitional region has the classical (9 + 0) nine doublets of microtubules and at the end of the transitional region an additional central singlet of tubules is present (9 + 2). However in the case of *P. altivelis* (Gwo et al., 1994) there is a ring structure surrounding the anterior end of the distal centriole that does not seem to be present in the other species.

The main interspecies difference seems to be the mitochondria shape and number. In *O. mordax* and *M. villosus* (this study) and in *P. altivelis* (Gwo et al., 1994) only one is present. Hara (2009) studying the ultrastructure of an additional seven Osmeridae also found one mitochondrion in all species, except the Salangini tribe (excluding *M. villosus*) that he refers to have multiple mitochondria. On the other hand, the number in *O. eperlanus* is uncertain; Kowalski et al. (2006) had admitted problems with sample preparation, losing most of the mitochrondria among cells, rarely finding one, and in three spermatozoa they concluded that there were two. When

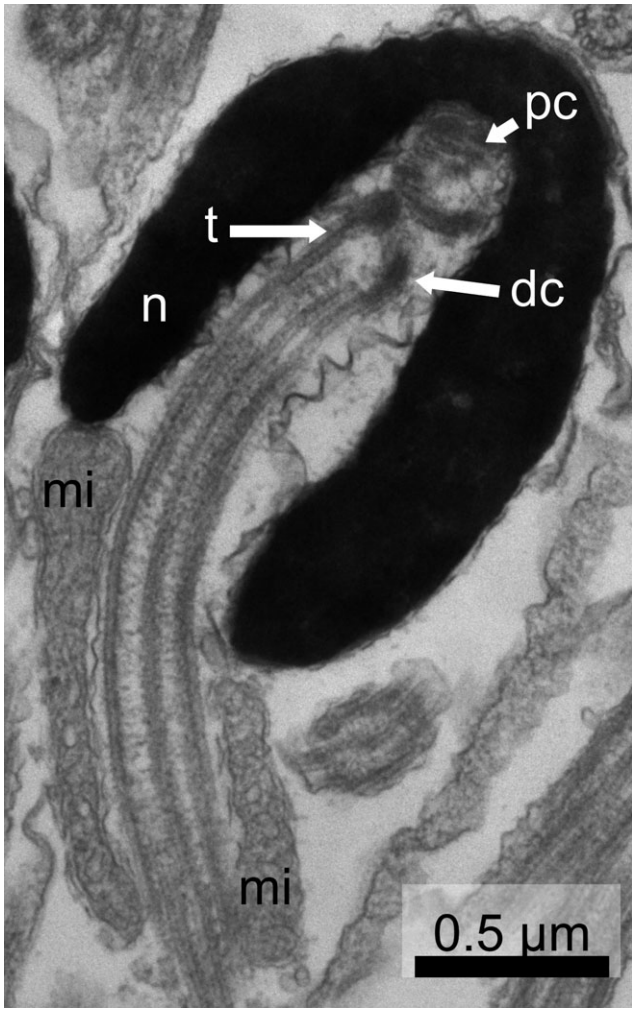

Fig. 3. Transmission electron micrograph of *M. villosus* spermatozoon longitudinal section of the head showing nucleus condensed chromatin (n) proximal centriole (pc), distal centriole (dc) and distal centriole transitional region (t) and mitochondrion (mi) with cristae membranes irregularly distributed

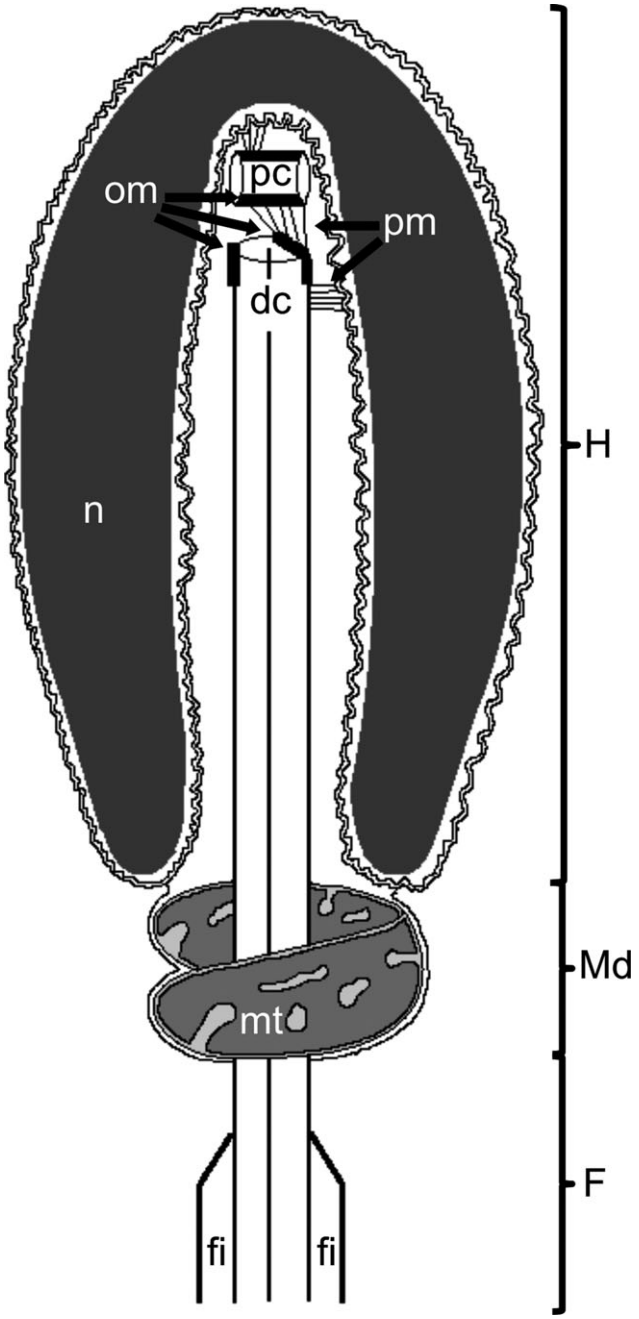

Fig. 4. Schematic model of Osmeridae sperm. H – head with the nucleus (n), proximal centriole (pc), distal centriole (dc), osmiophilic material (om) in the proximal centriole and in anterior part of distal centriole, and pericentriolar material (pm) linking both centrioles and anchoring them to the nuclear envelope. Md – midpiece with mitochondrion (mt) enclosing the axoneme. F – flagellum with fins (fi)

one mitochondrion is present (Salangini unknown to us, but may appear in Japanese), the shape varies between an incomplete ring that almost encloses the flagellum in *M. villosus* (this study) and a curved shape situated laterally to the flagellum in *P. altivelis* (Gwo et al., 1994). The presence of only one or two mitochondria could explain the short motility period of these species' sperm, 22 s in the case of *O. eperlanus* at 6°C (Kowalski et al., 2006), less than 1 min in the case of the *P. altivelis* (Utsugi, 1993) and about 20 s for *M. villosus* and 25 s for *O. mordax* both at 10°C (Lewis, 2013). The duration of the motility period is normally related with the temperature (Purchase et al., 2010) and with the amount of ATP available to the axonemal beating, which is proportionally related to the number of mitochondria (Alavi et al., 2013).

In the studied Osmeridae species (this study; Gwo et al., 1994; Kowalski et al., 2006; Hara, 2009) the flagellum is composed of a 9 + 2 microtubular doublet structure that is typical of eukaryotic flagellar organization (Jamieson, 1991) and enclosed in a plasma membrane with lateral extensions in line with the two central tubules, similar to fins. These fins are common to several other teleost groups such as Salmoniformes (Billard, 1983), Pleuronectiformes (Medina et al., 2000) and Perciformes (Luo et al., 2011) and most likely act

to stabilize sperm swimming (Stoss, 1983), thus enhancing the chances to achieve fertilization; nevertheless its efficiency was questioned by Lahnsteiner et al. (1995) who did not find an improved motility in species with this fin like structure.

In conclusion the Osmeridae spermatozoa could be regarded as type I sperm, which are the most primitive sperm within teleosts (Mattei, 1970). Type I sperm are characterized by the centrioles in right angles located in the nuclear fossa structure (Jamieson, 1991). In addition the deep nuclear fossa (excluding the Salangini tribe) clearly differentiate this family from the phylogenetically close Salmonidae (Jamieson, 1991). Furthermore, the *M. villosus* ultrastructure is closer to the Osmerini tribe than the Salangini tribe, where it is currently placed. The results of the present study highlight the importance of sperm morphology in the analysis of fish phylogenetic relationships and studies on other Osmeridae sperm could help solve some of the controversies about this group's classification.

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