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# Ultrastructure of Dark Chub *Zacco temmincki* (Cyprinidae) Spermatozoa

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Mature spermatozoa of dark chub *Zacco temmincki* (Temminck and Schlegel), were examined under a scanning electron microscope (SEM) and a transmission electron microscope (TEM). The spermatozoa have a spherical, homogeneously electron-dense nucleus with an axial nuclear fossa containing two laterally oriented centrioles. The centrioles, which are arranged at about a 120° angle to each other, have the 9+2 microtubule structure typical of flagella. The mature spermatozoon is of the primitive anacrosomal aquasperm type. The nuclear envelope is strongly undulated and contains nuclear vacuoles of different sizes and positions. The midpiece contains six or more mitochondria and encircles the basal body of the flagellum with an axoneme covered by the plasma membrane. Cytoplasmic vesicles lie between the axonemal doublets and the plasma membrane, and encircle the anterior part of the tail. The plasma membrane of the flagellum extends laterally and forms a pair of side fins. The species showed minor differences in number and structure of mitochondria, the angle between centrioles, and total length and occurrence of the fins. These characters, especially the side fins, appear to be apomorphic and useful for determining phylogenetic relationships at the genus or family level.

Key words: Dark chub, Spermatozoal ultrastructure, *Zacco temmincki*

## Introduction

An organism's mode of reproduction (e.g., internal vs. external fertilization) is generally coupled with morphological and physiological adaptations of its gametes (Kweon et al., 1998). Adaptations of the male gamete involve modifications in size, shape, and ultrastructure to facilitate fertilization under particular environmental conditions (Nicander, 1970). Indeed, every animal species may have unique spermatozoa (Chawanji et al., 2005). Despite this diversity, the organization of the spermatic organelles is very conservative among members of a family or subfamily (Bacetti et al., 1984; Mattei, 1991; Gusmao et al., 2005). Therefore, it may be possible to use differences in sperm ultrastructure to discern not only phylogenetic relationships among related species (Wolenski and Hart, 1987; Kim and Park, 1996; Lee and Kim, 1998; Kim and Lee, 2000), but the effects of disease and/or environment (e.g., toxins) on a particular group of organisms (Gibbson et al., 1985; Kenzo, 1993).

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The family Cyprinidae comprises approximately 2,010 species of mainly freshwater fishes in 210 genera. The greatest diversity of cyprinids occurs in Southeast Asia (Nelson, 1994). Although the spermatozoan structure of about 300 fish species spanning more than 100 families (Mattei, 1991; Kweon et al., 1998) has been reported, relatively few cyprinids have been studied in this context; some examples include goldfish *Carassius auratus*, common carp *Cyprinus carpio*, and the zebrafish *Brachydanio rerio* (see also Billard, 1970; Fribourgh et al., 1970; Morisawa, 1979; Baccetti et al., 1984; Guan, 1990; Kim et al., 1998; Lee and Kim, 1998).

The dark chub, *Zacco temmincki*, is a highly diverse freshwater cyprinid found in East Asian countries such as Korea, Japan, and China (Kim et al., 2005). Descriptions of the species, however, have reported slightly conflicting phenotypic, genetic, and morphologic characteristics, as well as different geographic distributions (Lee et al., 1986; Yang and Min, 1987; Lee and Lee, 1988; Min and Yang, 1993). Min and Yang (1993) divided the species into A type (Mdh-$1^{MS}$) and B type (Mdh-$1^{MM}$), two sympatric

morphs categorized by the temperature of their preferred habitat. Kim et al. (2005) reclassified these types into two separate species, designating B type (Mdh-$1^{MM}$) *Z. temmincki* and A type (Mdh-$1^{MS}$) *Z. koreanus*, a new species.

Still, surprisingly little is known about the reproductive biology and spermatozoal ultrastructure of *Zacco*. Considering that ultrastructural differences can help distinguish between closely related taxa and clarify phylogenetic relationships (Jamieson, 1991), the spermatozoal ultrastructure presented in this paper will not only help determine the reproductive strategy of the dark chub, but also refine the relationship between *Z. temmincki* and *Z. koreanus*.

## Materials and Methods

Male dark chub *Z. temmincki* were collected from the upper Milyang river of Peongri-Ri, Kyeongsangnam-Do, Korea, using fish pots and a dip net (5.5 mm mesh) during the spawning period (20 June 2005), and kept in an aquarium with a controlled environment. Semen was hand-stripped from the urogenital opening of mature males, and gentle pressure was applied to prevent contaminating the semen with water, urine, feces, blood, or mucus.

Semen samples (0.5 mL) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 to 4 h at 4°C and post-fixed in 1% osmium tetroxide in the same buffer for 2 h, then prepared for TEM and SEM. For TEM, samples were dehydrated in a graded ethyl alcohol series (50% → absolute alcohol) and embedded in Epon 812 resin. Sections were made with a glass knife on an LKB ultramicrotome and stained with 4% uranyl acetate followed by lead citrate for 30 min. Preparations were examined with a Hitachi H-600 TEM (Japan) at 75 kV. For SEM, samples were attached to coverslips coated with poly-L-lysine. Samples were fixed and dehydrated as described above, then critical-point dried and coated with gold. Preparations were examined with a Hitachi S-4100 SEM, and measurements were made on both SEM and TEM electron micrographs.

## Results

The mature spermatozoon of *Z. temmincki* is approximately 44.0 $\mu$m long (Fig. 1A), and free spermatozoa fill the testis (Fig. 1B). The spermatozoon consists of a small spherical head, a short midpiece, and a single flagellum (Fig. 2 A). According to Mattei (1970), it is a simple, primitive type of aquasperm. The spherical head, which lacks an acrosome, is approximately 2.0 $\mu$m in diameter. The species seems to show a Type II nuclear rotation, according to Mettei (1970). The basal nuclear fossa, which extends distally from the posterior nuclear invagination about one-third the length of the nuclear diameter, is 0.4-0.5 $\mu$m deep, hollow, and not well developed (Fig. 2B). The chromatin is homogeneous and condensed, and the axial body extends into the nucleus. The nuclear envelope and plasma membrane, which wrap tightly around the anterior region of the nucleus (Fig. 2B), are strongly undulated and contain nuclear vacuoles of different sizes and positions (Fig. 2C, E).

The proximal centriole is located at the anterior end of the nuclear fossa, whereas the distal centriole, which forms the basal body of the axoneme, extends

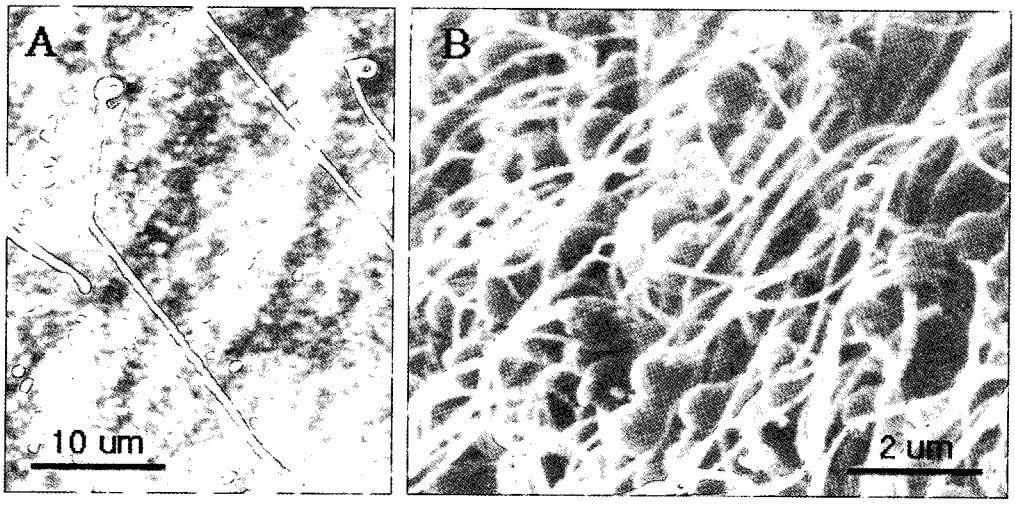


Fig. 1. Scanning electron micrographs of dark chub *Zacco temmincki*, spermatozoon (A), and spermatozoa in testis (B).

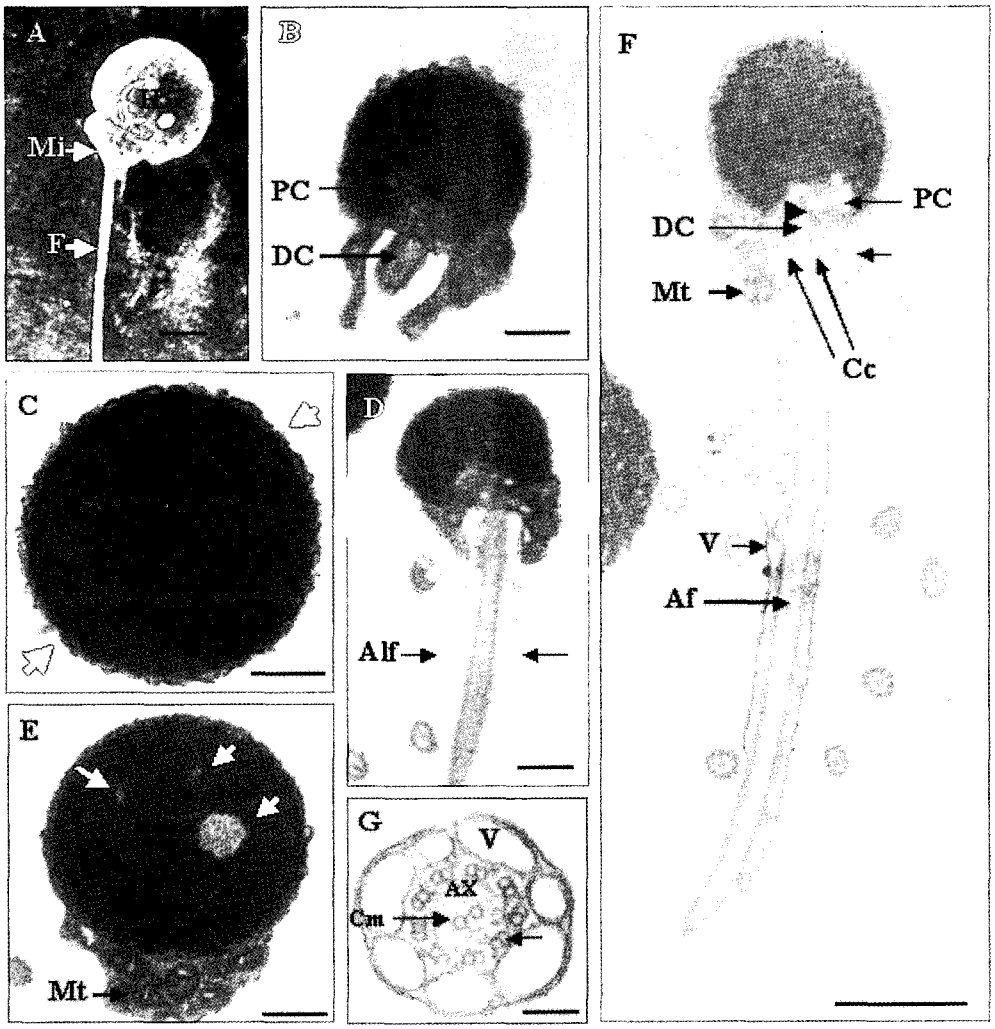


Fig. 2. Transmission electron micrographs of the spermatozoon of *Zacco temmincki*. Bars indicate 0.5 $\mu$m. A: External morphology of spermatozoon showing small spherical head (H), short midpiece (Mi) and single flagellum (F). Note an acrosom is abscent: B, Nuclear fossa containing the proximal centriole (PC), distal centriole (DC) and cytoplasmic canal: C, Transverse section of the nucleus (N) showing electron-dense chromatin material. Note the nuclear envelop and the plasma membrane are strongly undulated (arrows): D, Longitudinal section of flagellum. Note the presence of cytoplasmic collar in head part and axonemal lateral fin (Alf) (arrows): E, Longitudinal section of the spermatozoon showing mitochondria (Mt) in the postnuclear cytoplasm and nuclear vacuoles with different position and size in this size (arrows): F, Longitudinal section of flagellum. The proximal centriole is connected by electron-dense filament (arrowhead) to the distal centriole: G, Transverse section of anterior region of the sperm tail containing vesicles (V) in cytoplasm surrounding the axoneme (Ax) with the classic 9+2 microtubular doublet construction. Cm, Central microtubules; Pm, Peripheral microtubules; Af, Axial filament; Alf, Axonemal lateral fin; Cc, Cytoplasmic canal; Cpc, Cytoplasmic collar; V, Vacuoles.

from the anterior end of the cytoplasmic canal to the basal nuclear fossa (Fig. 2B). The two centrioles are oriented laterally at about a 120° angle to each other (Fig. 2B, D), and both have a typical 9+2 microtubule structure (Fig. 2G). Electron-dense filaments surround the anterior end of the distal centriole, suggesting that the two centrioles are laterally fastened by microfibrils (Fig. 2F, arrowhead).

The midpiece contains six or more generally spherical mitochondria tightly packed together (Fig. 2E). The mitochondrial collar encircles the base of the flagellum, and is separated from the flagellum by

the cytoplasmic canal (Fig. 2B, D, F), which is about 1.0 $\mu$m long, 0.8 $\mu$m wide, and extends far into the nucleus. The mitochondria are located posterior to the nucleus and possess irregularly arranged tubular cristae and a well developed matrix with granular materials (Fig. 2D-F). The mitochondria do not fuse into a mitochondrial derivative. All cell organelles except the proximal centriole are oriented symmetrically around the axis of the flagellum, which is parallel to the nucleus (Fig. 2A-F). The flagellum, which has an axoneme covered by the plasma membrane, is approximately 42 $\mu$m long and composed of the typical 9+2 microtubular doublet structure (Fig. 2G). The cytoplasmic vesicles lie between the axonemal doublets and the plasma membrane, and encircle the anterior part of the tail (Fig. 2G). The plasma membrane of the flagellum extends laterally and forms a pair of side fins that line up with the two central microtubules (Fig. 2D, F).

## Discussion

Fish spermatozoa are widely divergent in form and structure. They range from tailless to two-tailed, and vary greatly in shape, size, and number (Jamieson, 1991; Kweon et al., 1998). The spermatozoa of externally fertilizing cyprinids are generally primitive anacrosomal aquasperm (Jamieson, 1991; Mattei, 1998) characterized by a spherical nucleus, shallow nuclear fossa, short midpiece containing a few mitochondria, and a long flagellum positioned tangentially to the nucleus (Lee and Kim, 1998). This basic morphology, the result of reproductive adaptations to external fertilization, enables the fish to produce large amounts of spermatozoa and save time and energy during spermatogenesis (Kweon et al., 1998; Mattei, 1998). Internally fertilizing fish species typically produce modified spermatozoa with elongated nuclei and midpieces (Mattei, 1991). However, several exceptions exist of internally fertilizing fish species with primitive aquasperm (Kweon et al., 1998). In Neopterygii, the acrosome has disappeared, and during spermiogenesis, the vestigial remains of the acrosome-like vesicle also disappears (Guan, 1988); this trait appears to be apomorphic, whereas the acrosomal aquasperm of other teleosts is generally considered plesiomorphic (Jamieson, 1991; Mattei, 1991; Park and Kim, 1996).

Spermatozoon types are classified according to whether nuclear rotation occurs (Type I) or not (Type II) during spermatogenesis (Mattei, 1970). Because the spermatozoa of *Z. temmincki* did not show rotation, they fall into the Type II category. The chromatin is homogeneous and condensed. Chromatin condensation depends on the type of nuclear proteins associated with the DNA (Saperase et al., 1993) and results from different condensation processes during spermiogenesis (Gusmao et al., 2005).

The midpiece is short, similar to that of most other cyprinid fishes. The number of mitochondria in the spermatozoa is similar to that of many other types of fishes. There are 8-10 mitochondria in Nile tilapia (Cichlidae) and *Scopthalmus maximus* (Pleuronectidae) (Lou and Takahashi, 1989), 5-8 in *Cobitis striata* (Cobitidae) (Kim and Park, 1996), 7-8 in *Limanda yokohamae* (An et al., 1999) and *Eopsetta grigorjewi* (An et al., 1999) (Pleuronectidae), and 2-10 in several other species of Cyprinidae (2 in *Barbus* and *Alburnus*, 5 or more in *Pungtungia herzi*, 10 in *Carassius auratus*). Dark chub showed 6 or more mitochondria, which were not fused. In general, the mitochondria of mature spermatozoa in cyprinids do not fuse, although some species will show fusion in the postnuclear cytoplasm of the mature spermatozoon (Ohta and Iwamatsu, 1983: Lee and Kim, 1998). The presence or absence of fused mitochondria is considered an apomorphic character (Lee and Kim, 1998). The number of mitochondria, which supply energy for sperm motility, is closely related to the shape and size of the cytoplasmic collar, the depth of the cytoplasmic canal (Baccetti et al., 1984), and sperm motility duration and activity (An et al., 1999). The mitochondria of dark chub documented in this study were asymmetrically distributed, similar to other cyprinid species.

The relative positions of the centrioles in teleost spermatozoa vary among species, and a perpendicular arrangement seems to be plesiomorphic in Neopterygii (Jamieson, 1991; Mattei, 1991). The angle between the two centrioles is species-specific among cyprinid fish, ranging from 40 to 140° (Lee and Kim, 1998; Gwo et al., 2005); dark chub is within that range at 120°. In several Mullidae species, however, the two centrioles are parallel (Gwo et al., 2004). Although the spermatozoal ultrastructure of dark chub is very similar to that of other cyprinids, we demonstrated minor differences in the number and structure of mitochondria, the angle between the centrioles, and the total length and occurrence of flagellar fins.

Flagellar fins have been reported in the spermatozoa of many other fishes, but not in all cyprinids (Lee and Kim, 1998). This absence in some cyprinid fishes implies apomorphic loss (Jamieson, 1991). Further comparative studies of sperm morphology will highlight the potential application of spermatozoal ultrastructure to fish phylogeny.

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