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Ultrastructure of Spermatozoa in *Pungtungia herzi*

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돌고기, *Pungtungia herzi* 정자의 미세구조

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ABSTRACT : The fine structure of spermatozoa of *Pungtungia herzi* was examined with scanning and transmission electron microscopies. The spermatozoa of *P. herzi* are approximately 37.4 μm in length and a relatively simple cell with a spherical nucleus, a short midpiece and a tail. The acrosome is not present as in most teleost fishes. The ultrastructure of spermatozoa represents typical characteristics of cyprinid spermatozoa including the lateral insertion of flagellum, the organization of centriolar complex in shallow nuclear fossa, and the occurrence and asymmetrical arrangement of mitochondria. In the nuclear envelope and mitochondrion, however there were some morphological differences for their ultrastructure. The nuclear envelope is severely undulated and the shallow nuclear fossa contains two centrioles which are at the angle of some 130° each other. The most significant feature can be observed with the mitochondrion; five or more mitochondria, which are shown in primary spermatocyte, fuse to form a single one in the mature spermatozoon. The mitochondrial aspect is different from that of other cyprinid spermatozoa, where their mitochondria have a conventional aspect and never fuse to form a mitochondrial derivative. In terms of sperm evolution the fused mitochondria are regarded as the apomorphic character in comparison with the separate mitochondria. The single mitochondrion is not reported in cyprinid spermatozoon except the case of *Rhodeus*.

Key words: Ultrastructure, Anacrosomal spermatozoon, Nuclear envelope, Mitochondrion, Asymmetrical arrangement, *Pungtungia herzi*.

요 약 : 돌고기 *Pungtungia herzi* 정자의 미세구조를 주사 및 투과 전자현미경적 방법으로 연구하였다. 정자는 구형의 두부, 짧은 중편 및 꼬리로 구성되어 있으며 비교적 그 구조가 단순하고 그 길이는 약 37.4 μm이었다. 정자의 미세구조에서 핵에 대한 접선 방향으로의 편모 배열, 짧은 핵 그리고 미토콘드리아의 비대칭적 배열은 잉어과 정자의 공통된 특징과 마찬가지로 나타났으며, 대부분의 경골어류에서와 같이 첨체를 가지고 있지 않았다. 반면 돌고기 정자에서는 핵막의 구조와 미토콘드리아에서 현저한 특징을 나타내었다. 핵막은 매우 심하게 파동되어 있으며 얕은 핵와내의 기부 및 말단중심립은 약 130°의 각도로 배열되어 있었다. 미토콘드리아는 제 1 정모세포기에 5개 이상 관찰되었으나 성숙한 정자에서는 하나의 형태로 융합되어 있었다. 이러한 양상은 융합되지 않은 다른 잉어류 정자에서의 미토콘드리아와 비교할 때 현저한 차이가 있었다. 정자의 계통 진화적 견지에서, 돌고기에서와 같이 융합된 미토콘드리아는 융합되지 않고 분리되어 있는 미토콘드리아에 비해 원거리 형질(apomorphic character)로 간주된다. 한 개의 미토콘드리아를 가지는 정자는 현재까지 *Rhodeus* 이외의 다른 잉어류 정자에서는 보고된 바 없었다.

INTRODUCTION

The sperm morphology has contributed to a great extent both to phylogenetic relations within groups and to taxonomic classification (Billard, 1970; Mattei & Mattei, 1975; Baccetti & Afzelius, 1976; Baccetti, 1986a, b).

There is an enormous variety of forms and structures in fish spermatozoa, which reveals specific differences of wide species (Lahnsteiner & Patzner, 1996). The Neopterygii, though greatly diversified, bear a common character that distinguishes them from other fishes and even from vertebrates as a whole; they lack an acrosome (Mattei, 1991).

As for the Ostariophysi, they are distinguished into two groups by the number of mitochondria present in the gamete. The first group consists of the Cyprinidae, which have a small number of mitochondria per gamete: 2 to 10,

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with an average of 3 or 4 (Baccetti et al., 1984). The species *Rhodeus ocellatus* has even a single mitochondrion. The second group corresponds to the Siluriformes as a whole, which have rather more mitochondria.

Cyprinidae are known mainly as freshwater fishes which have their greatest diversity in Southeast Asia. They form a large group with about 2,010 species in 210 genera (Nelson, 1994) and are of considerable importance for commercial value. Cyprinidae possess spermatozoa that are very close to the ancestral model of the Neopterygii, from which it differs by the lateral implantation of the flagellum on the nucleus (Mattei, 1991).

All of spermatozoa of cyprinid species belong to the type called primitive sperm. This sperm type is characterized by a spherical head, a short midpiece with mitochondria, and a long tail (Baccetti & Afzelius, 1976; Guan & Afzelius, 1991). Cyprinidae, with simple anacrosomal aquasperm, are also characterized by a head eccentrically placed on the tail, two variously oriented centrioles, and a tail with no lateral fins (Baccetti et al., 1984; Jamieson, 1991; Gwo et al., 1995).

The ultrastructure of cyprinid spermatozoa has been examined in the goldfish, *Carassius auratus* (Fribourgh et al., 1970; Guan, 1990), *Cyprinus carpio* (Billard, 1970; Morisawa, 1979), the zebrafish, *Brachydanio rerio* (Kessel et al., 1983), two species of *Squalidus* (Lee & Kim, 1998; Kim et al., 1998), and others (Baccetti et al., 1984). However, there are no data on sperm organization of *Pungtungia herzi* yet. The animal belongs to the subfamily Gobioninae. All genera of Gobioninae except for *Gobio* are restricted within Eastern Asia including Korea (Nelson, 1994). The Gobioninae are freshwater teleost fishes that contain the most traditional species in Korea. Recently there has been reports on the fine structure of spermatozoa in Korean cyprinid species (Lee & Kim, 1998; Kim et al., 1998).

This paper describes the ultrastructure of the mature spermatozoa of *P. herzi* and is discussed in connection with sperm evolution. The phylogenetic relationships of Gobioninae will be considered in a subsequent study.

MATERIALS & METHODS

Adult *Pungtungia herzi* were collected during the spawning period from Naktong river of Korea and kept in a controlled environment. Male fishes were judged to be mature when their semen could be handstripped from the urogenital opening. For the experiment, mature spermatozoa were obtained by pressing both sides of the abdomen and kept in a small petri dish with physiological saline. And then, living spermatozoa were examined with a phase contrast microscope.

For transmission electron microscopy (TEM), the semen and pieces of testis were dissected and fixed in 2.5 to 5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in the same buffer. They were then dehydrated in a graded ethanol series and embedded in Epon 812. The samples were sectioned on a LKB ultramicrotome, stained with 4% aqueous uranyl acetate, poststained with lead citrate and finally examined with a Hitachi H-600 transmission electron microscope.

For scanning electron microscopy (SEM), the testes went through the same fixing and dehydrating procedures as for TEM. They were followed by isoamylacetate and subjected to critical point dryer. They were coated with gold by ion-sputter and observed under Hitachi S-4100 scanning electron microscope.

RESULTS

The spermatozoon of *Pungtungia herzi*, having a length of approximately 37.4 μm, is a relatively simple cell composed of a spherical head, a short midpiece and a tail (Fig. 1A). There is thus no acrosome.

1. Head

The nucleus is spherical, measuring about 1.9 μm. The nuclear envelope and the plasma membrane are tightly apposed over the anterior two thirds of the sperm head (Fig. 1B). In this region they are strongly undulated (Figs. 1C-1E). The chromatin has an almost uniform electron density, but in some instances there are nuclear vacuoles with different position and size in it (Fig. 1B). The centriolar

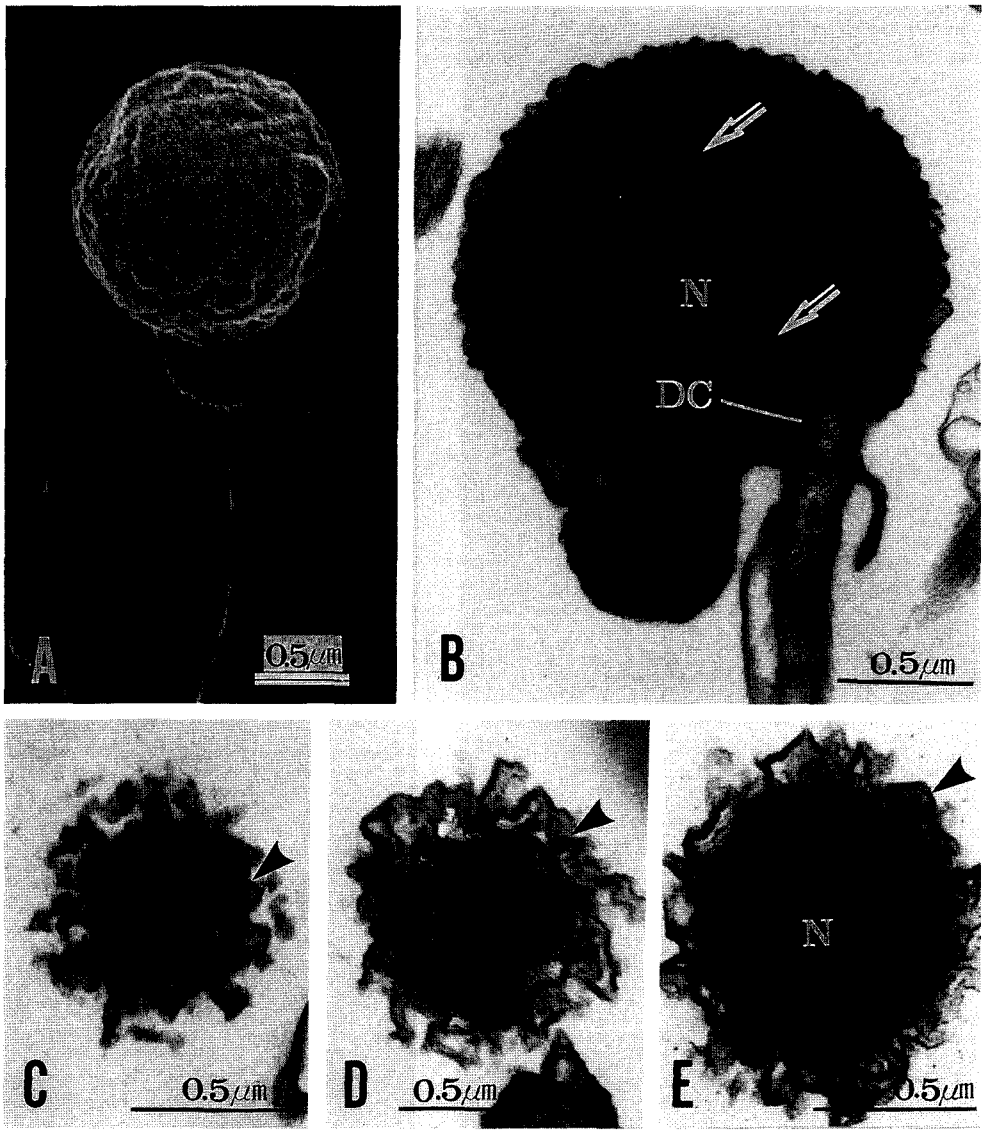

Fig. 1. Electron micrographs of spermatozoa of *Pungtungia herzi*.
A. Scanning electron micrograph of a spermatozoon showing a spherical head (H), a short midpiece (MP) and a tail (T). B. Longitudinal section through a spermatozoon showing the chromatin containing nuclear vacuoles (arrows) and the single mitochondria (M) in the juxtanuclear pocket of the postnuclear cytoplasm. Note the lateral implantation of the flagellum with respect to the nucleus (N) and that the anterior portion of the distal centriole (DC) is inserted in the nuclear fossa. C-E. The nuclear envelope and the plasma membrane are strongly undulated (arrowheads).

complex is located at the lateral side of the nucleus in a 0.3 to 0.4 μm deep invagination, a nuclear fossa. No acrosome is found. The anterior portion of distal centriole and whole proximal centriole are inserted in the nuclear fossa (Figs. 1B, 2A).

2. Midpiece

The proximal centriole is inclined at an angle of 130° with respect to the distal one (Fig. 2A) which is bound to the nuclear envelope by satellite fibrils (Fig. 2C). The distal centriole is quite laterally inserted into the nucleus and serves as basal body of the flagellum (Figs. 1B, 2A).

The midpiece region has the shape of an asymmetrical truncated cone and is pervaded by the cytoplasmic canal, an invagination of the plasma membrane where the flagellum is located. The cytoplasmic canal is approximately 0.8 μm long and 0.6 μm wide. In the posterior region of the head, the postnuclear cytoplasm encircles the flagellum and

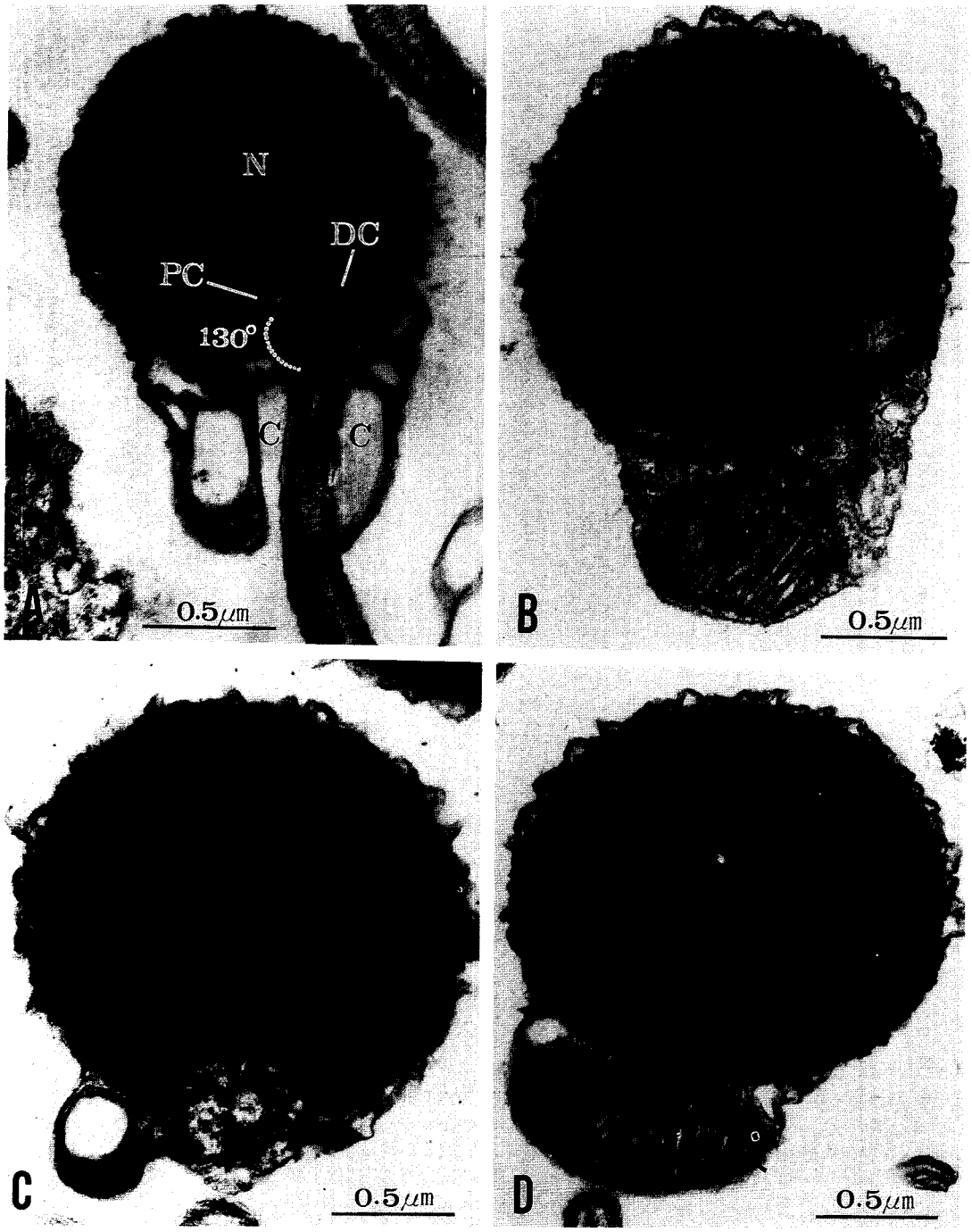

Fig. 2. Electron micrographs of spermatozoa of *Pungtungia herzi*.
A. Longitudinal section through a spermatozoon showing the proximal (PC) and the distal centrioles (DC) forming an angle of 130° to each other. Note that the postnuclear cytoplasm surrounds the axoneme (Ax) and is separated from it by the cytoplasmic canal (C). B. Satellite fibrils (arrowheads) connecting the distal centriole to the nuclear envelope lining the nuclear fossa. C-D. Longitudinal sections of the mature spermatozoon showing a single mitochondrion (M) in the postnuclear cytoplasm. Note that the mitochondrial cristae (arrows) are parallely arranged.

is completely separated from it by the cytoplasmic canal (Fig. 2A). The midpiece is about 0.6 μm in length and 1.4 μm in width. The postnuclear cytoplasm of the mature spermatozoon contains a single mitochondrion (Figs. 1B, 2B, 2D). However, the cytoplasm of spermatocyte has five mitochondria or more (Fig. 3A). The cytoplasm surrounding cytoplasmic canal is asymmetrically distributed with respect to the axoneme (Fig. 3B) and contains the mitoch-

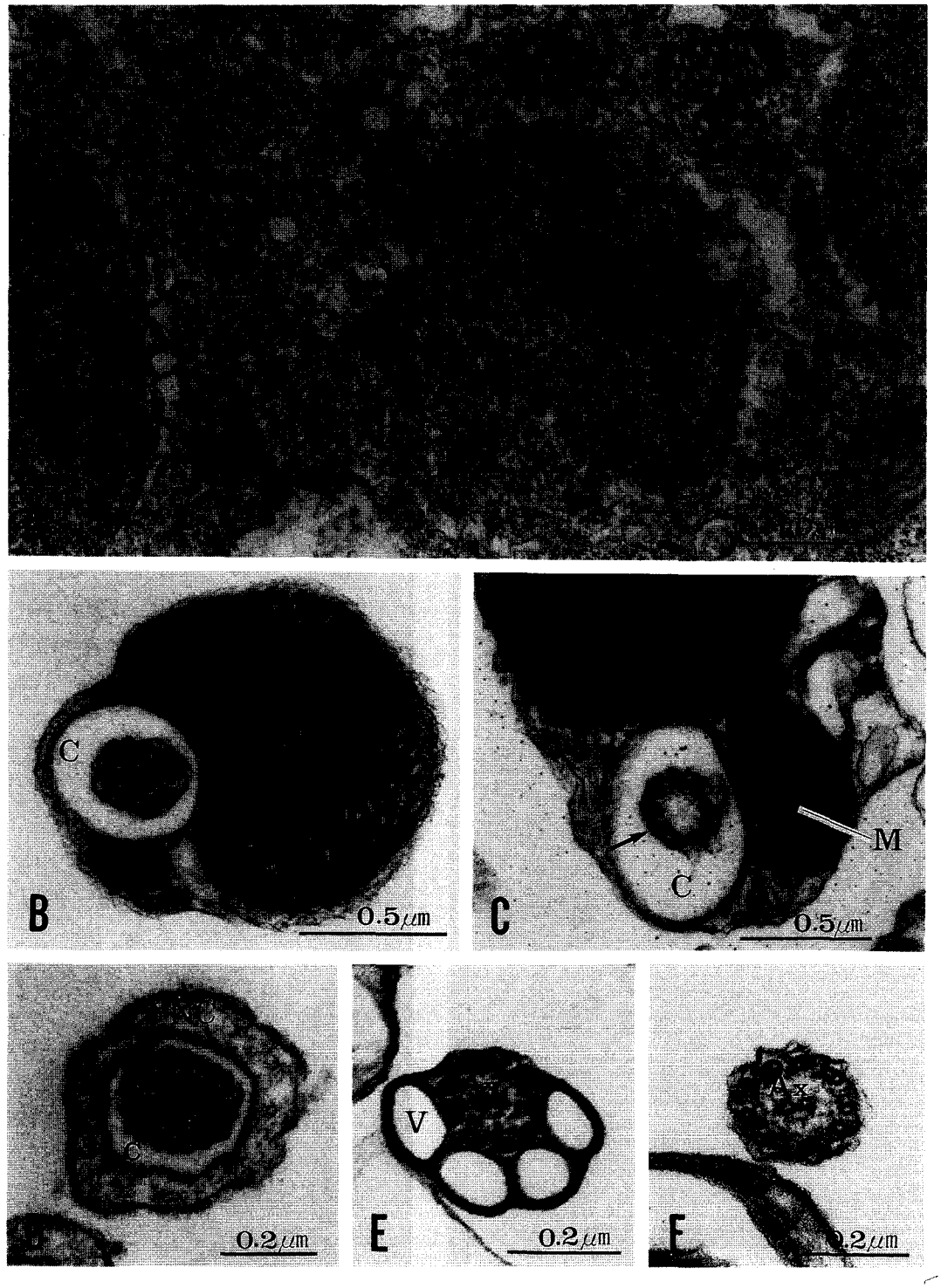

Fig. 3. Electron micrographs of spermatozoa of *Pungtungia herzi*.

A. The spermatocyte showing five mitochondria (M) in the cytoplasm. B. Transverse section through the midpiece showing the single C-like shaped mitochondrion (M) surrounding a half of the cytoplasmic canal (C). Note asymmetrical arrangement of the mitochondria with respect to the axoneme and that the mitochondrial matrix is moderately electron-dense. C. Transverse section through the posterior end of the midpiece showing the ring-shaped postnuclear cytoplasm (PNC) surrounding the axoneme (Ax) and the cytoplasmic canal (C). D. Transverse section through the midpiece showing the axoneme (arrow) consisting of nine double outer tubules in the transition region. Note that the mitochondrion (M) is located only in the area adjacent to the nucleus. E. Transverse section of the anterior part of the sperm tail containing vesicles (V) in cytoplasm surrounding the axoneme (Ax). Note the absence of lateral fins in the flagellum. F. Transverse section of the posterior part of the sperm tail showing only the axoneme (Ax) with the classic 9+2 microtubular doublet construction.

ondrion in the juxtanuclear pocket of the postnuclear cytoplasm (Figs. 1B, 3C). Distal to the mitochondrion, the postnuclear cytoplasm forms a ring surrounding the axoneme in a transverse section (Fig. 3D).

The single mitochondrion is unilaterally located posterior to the nucleus and surrounds a half of the cytoplasmic canal in cross section of midpiece (Fig. 3B). One C-shaped mitochondrion is located in the area adjacent to the nucleus, but not in the opposite area (Figs. 1B, 3C). It measures about 0.8 μm in width and about 1.2 μm at its longest axis. The mitochondrial matrix is moderately electron-dense and the cristae are irregularly or sometimes parallelly arranged (Figs. 2B, 2D, 3B).

3. Tail

The sperm tail is approximately 35.2 μm long and contains an axoneme covered by the plasma membrane. Between the base of the axoneme and the distal centriole is the transition region, where the axoneme consists of nine double outer tubules and no central tubules (Fig. 3C). The axoneme has the classic 9+2 microtubular doublet construction (Figs. 3E, 3F).

The cytoplasmic vesicles are inserted between axonemal doublets and plasma membrane and encircle the anterior part of the tail (Fig. 3E), but are not observed toward the posterior part (Fig. 3F). They are right under the plasma membrane of the flagellum, and reveal several clear spaces with various sizes (Fig. 3E). The lateral fins (sidefin) are not observed in the flagellum.

DISCUSSION

Cyprinid spermatozoa are characterized by a round nucleus with a shallow nuclear fossa, a midpiece containing mitochondria and a flagellum positioned tangentially to the nucleus (Gwo et al., 1995). Besides, the spermatozoa of *P. herzi* are similar to those of cyprinid because of the organization of centriolar complex in shallow nuclear fossa, the occurrence of spherical mitochondria in postnuclear cytoplasm, and the asymmetrical arrangement of mitochondria. However, the ultrastructure of *P. herzi* spermatozoa shows another interesting features. Especially, *P. herzi* spermatozoon has a single mitochondrion in the postnuclear cytoplasm, which is not reported in cyprinid spermatozoon with exception of two species of *Rhodeus* (Ohta & Iwamatsu, 1983; Ohta, 1991; Guan & Afzelius, 1991).

P. herzi show five or more mitochondria in the primary spermatocyte and a single form in the mature spermatozoon. This aspect is different from that of the mitochondria in other cyprinid spermatozoa which have a conventional aspect and never fuse to form a mitochondrial derivative. From the aspect of sperm evolution it can be said that the fused mitochondria are considered as an apomorphic character in comparison with the separate mitochondria (Kim et al., 1998).

The mitochondrial number varies from two in *Barbus* and *Alburnus* to ten in *Carassius auratus* of cyprinid species (Baccetti et al., 1984), with a higher frequency of three to four. For any spermatozoon, the number is the only character closely linked with phylogeny (Baccetti et al., 1984). They suggested that the number and size of mitochondria determine the depth of cytoplasmic canal in cyprinid spermatozoa.

The mitochondria are asymmetrically distributed in the area adjacent to the nucleus and do not surround the axoneme. This asymmetrical distribution is a general pattern of cyprinid spermatozoa, and thus, this may be considered to be common character of cyprinid species.

The midpiece is less developed in teleost spermatozoa than in other vertebrate group (Billard, 1970). The midpiece of *P. herzi* is rather short, which has been observed in teleost fishes utilizing external fertilization. *Blennius pholis* spermatozoa do not even have midpiece. In this case the mitochondria are in the anterior part of the nucleus (Silveira et al., 1990). The elongation of the midpiece is found in the teleosteans that employ internal fertilization, but this cannot be said to be general (Mattei, 1991).

Two centrioles vary considerably in their relative position in teleost fish spermatozoa. While the angle of the two centrioles in cyprinid species is variable from 40° to 140°, that of proximal centriole is rarely perpendicular to the distal centriole only in *Alburnus* and *Barbus*. In most case, proximal and distal centrioles are arranged in a right angle each other. These variations in centriolar geometry are

clearly correlated with the position of the nucleus with respect to the axis of tail in cyprinid fishes (Baccetti et al., 1984).

The flagellar apparatus of spermatozoa exhibits a great diversity among species (Afzelius, 1982). Lateral fins have been observed in spermatozoa of various fishes (Billard, 1970; Afzelius, 1978; Stein, 1981), but not for all cyprinid sperm reported. Jamieson (1991) suggested that in view of the widespread occurrence of flagellar fins throughout the Osteichthyes, the absence in cyprinids appears to be an apomorphic loss and an ostariophysian synapomorphy.

The cytoplasmic vesicles are also observed in many cyprinid species but their locations are differing each other; the vesicles of most cyprinids including *P. herzi* are located in the anterior portion of the axoneme, while in *Rutilus* (Baccetti et al., 1984) they are located along almost whole length of axoneme; and in *Alburnus* (Baccetti et al., 1984) they only appear in the intracanal portion. Kudo (1980) stated that the cytoplasmic vesicles of carp are composed of tubular smooth endoplasmic reticulum and some of their membranes even fuse with flagellar plasmalemma and communicate with external environment.

The flagellum of *P. herzi* is mediolaterally positioned to the nucleus, and consequently, the sperm reveals an asymmetrical organization. Such asymmetrical spermatozoa have been observed in most cyprinids, Trachinidae (Lahnsteiner & Patzner, 1996) and Esocidae (Billard, 1970).

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