

Spermatogenesis and Ultrastructural Characteristics of Spermatozoa of Brackish Water Diploid Clam, *Corbicula japonica* (Bivalvia: Corbiculidae)

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기수산 2배체 재첩, *Corbicula japonica*(Bivalvia: Corbiculidae)의 정자형성과정 및 정자의 미세구조적 특징

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ABSTRACT : Spermatogenesis and ultrastructural characteristics of sperm of brackish water diploid *Corbicula japonica* were investigated by electron microscope observations. Based on the cytological studies, the spermatozoon of this species (brackish water diploid) *C. japonica* is approximately 55 μm in length. The sperm head (about 12 μm long) is elongated and tapers with a slight curve. Sperm nucleus is about 7.90 μm long, and the acrosome is about 2.70 μm long. The morphologies of the sperm nucleus type and the acrosome shape of this species are a long arrow-like type and long cone-like shape, respectively. The sperm head of this species (external fertilization, dioecious and oviparous species) is partially modified from that of the primitive type, as seen in triploid *Corbicula* species (internal fertilization, hermaphrodite and ovoviparous species), reported by some authors. However, this species produces uniflagellate spermatozoa, unlike freshwater triploid hermaphroditic clams being possessed of partially modified biflagellate spermatozoa. Diploid *C. japonica* is similar to those of other bivalves being possessed of a short midpiece containing four mitochondria surrounding the centrioles. The axoneme of the sperm tail flagellum consists of nine pairs of microtubules at the periphery and a pair at the center. The axoneme of the sperm tail shows a 9+2 structure, and from uniflagellate sperm cross sectioned, in particular, wing-like axonemal lateral fins are observed, as seen in external fertilization fishes.

Key words : *Corbicula japonica*, Diploid, Spermatogenesis.

요 약 : 기수산 2배체 일본재첩(*Corbicula japonica*)의 정자형성과정 및 정자의 미세 구조적 특징을 전자현미경 관찰에 의해 조사하였다. 세포학적 조사 결과, 기수산 2배체인 일본재첩의 정자 길이는 약 55 μm 이다. 정자 두부(길이 약 12 μm)는 길게 신장되어 있으며 약간 구부러져 있다. 정핵 길이는 7.90 μm , 첩체 길이는 약 2.70 μm 이다. 정자의 핵과 첩체의 형태는 각각 긴 화살 모양과 길다란 원추 모양을 나타낸다. 본 종(체외수정, 자웅이체, 난생종)의 정자 두부는 이미 몇몇 저자들에게 의해서 보고된 3배체 재첩류(체내수정, 자웅동체, 난태생종)의 정자 두부에서 나타나는, 원시형으로부터 부분적으로 변형된 형태를 나타내고 있다. 그러나 부분적으로 변형된 2개의 편모가 있는 정자를 가지는 담수산 3배체인 자웅동체 조개류와 달리 한 개의 편모를 갖는 정자를 본 종은

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2개의 편모가 있는 정자를 가지는 담수산 3배체인 자웅동체 조개류와 달리 한 개의 편모를 갖는 정자를 본 종은

생산한다. 2배체 일본재첩은 중심체를 둘러싸는 4개의 미토콘드리아를 가지고 있어, 짧은 중편을 가지는 다른 이매패류의 것들과 유사하다. 정자 미부 편모의 악소님은 중앙에 1쌍의 미세소관과 주변에 9쌍의 미세소관으로 구성되어 있다. 정자 미부의 악소님은 9+2구조를 가지며, 횡절단된 한 개의 편모를 갖는 정자에서는 특히, 체외수정 어류들에서 나타나는 날개 모양의 악소님 lateral fin들이 관찰되었다.

INTRODUCTION

The family Corbiculidae is a group of relatively small Asiatic clams belonging to the bivalve order Veneroida. The genus *Corbicula* is one of the most well-known invasive and taxonomically complicated. In Korea, six species, *C. leana*, *C. fluminea*, *C. fenouilliana*, *C. papyracea*, *C. colorata* and *C. patentosa*, are recognized based on shell form (Kwon et al., 1993). Of these species mentioned above, *C. colorata*, *C. fenouilliana*, *C. patentosa* and *C. sandai* are freshwater diploid clams. In particular, *C. japonica* is a brackish water diploid clam. However, *C. leana*, *C. fluminea* and *C. papyracea* are freshwater triploids, with a chromosomal complement of 54 (Okamoto & Arimoto, 1986; Park et al., 2000).

Recently, several authors (Kramer, 1983; Komaru & Konish, 1996; Park et al., 2002) reported that freshwater triploid clams (*C. leana* and *C. fluminea*) have two flagella per spermatozoon (biflagellate sperm), as one of special ultrastructural characteristics.

The brackish water clam *C. japonica* is one of the important edible bivalves in East Asia, including Korea, China and Japan. In Korea, this species is mainly found in silty sandy bed up to 5~8 m in water depth in the brackish waters of Korea (Kwon et al., 1993).

Previously, there have been many studies on *C. japonica* in Korea and Japan on aspects of reproduction, including reproductive cycle and larval development (Kim et al., 2003; Choi et al., 2007), reproductive cycle and the sex ratio (Kim et al., 2003), on aspects of ecology, including distribution and ecology (Jo et al., 1998) and environmental factors and population density (Kim et al., 2002), on aspect of genetics, including karyotypes (Okamoto & Arimoto, 1986; Park et al., 2000) and molecular phylogenetics (Park

& Chung, 2003), on aspect of systematic study (Lee & Kim, 1997). Although there have been several studies on ultrastructural function of biflagellate sperm of freshwater triploid *Corbicula* species (*C. leana* and *C. fluminea*) by some authors, little information is available on ultrastructural differentiation of germ cells during spermatogenesis and structural, functional characteristics of partially modified uniflagellate sperm associated with reproductive biology of brackish water diploid *C. japonica*. In particular, it is necessary to study the same and different structures of spermatozoa between brackish water diploid *C. japonica* and freshwater triploid *C. leana*. Understanding of sperm morphology of this species will provide information needed for classification of family Corbiculidae. Recently, sperm morphology of mollusca has been used increasingly in assessing long-standing taxonomic problems (Popham, 1979; Healy, 1988; 1996).

Therefore, the purpose of the present study is to describe spermatogenesis and ultrastructural, functional characteristics of uniflagellate sperm of *C. japonica*, compared with ultrastructural characteristics of biflagellate sperm of freshwater triploid *Corbicula* species which was already reported by some authors.

MATERIALS AND METHODS

1. Sampling

Male specimens of *C. japonicus* were collected by the dredge monthly in the brackish water of Jujincheon, Gochang-gun, Jeollabuk-do, Korea, from January to December, 2006. A total of 78 male clams ranging from 30.0 mm to 35.0 mm in shell length were used for the study. After the clams transported alive to the laboratory, the sizes of the specimens were recorded using a Vernier caliper.

2. Ultrastructural Study of Germ Cells during Spermatogenesis by Electron Microscopic Observation

For transmission electron microscope (TEM) observations, excised specimens of the testes were cut into small pieces and fixed immediately in 2.5% paraformaldehyde-glutaraldehyde in 0.1 M phosphate buffer solution (pH 7.4) for 2 h at 4°C. After prefixation, the specimens were washed several times in the buffer solution and then postfixed in 1% osmium tetroxide solution in 0.2 M phosphate buffer (pH 7.4) for 1 h at 4°C. Specimens then were dehydrated in increasing concentrations of ethanol, cleared in propylene oxide, and embedded in an Epon-Araldite mixture. Ultrathin sections of Epon-embedded specimens were cut with glass knives on a Sorvall MT-2 microtome and LKB ultramicrotome at a thickness of about 80~100 nm. Tissue sections were mounted on collodion-coated copper grids, doubly stained with uranyl acetate followed by lead citrate, and observed with a JEM 100 CX-II (80-KV) electron microscope.

RESULTS

1. Morphology of the Testis

The general morphology of the testis of *C. japonica* is similar to those for the testes in bivalves. The testis of the brackish water clam *C. japonica* is located between the digestive diverticula and the outer fibromuscular layers, which are compacted by the fibrous connective tissues and muscle fibers. The testis is composed of a number of acini, and it is a diffuse organ consisting of branching acini containing differentiating sperm in a variety of stages. Spermatogenic germ cells are distributed in a centripetal pattern from the acinus wall to the lumen.

2. Ultrastructure of Spermatogenesis

Based on the testicular development and morphological characteristics of germ cells, spermatogenesis can be classified into five phases: (1) spermatogonial phase, (2) primary spermatocyte phase, (3) secondary spermatocyte phase,

(4) spermatid phase, and (5) spermatozoon phase.

1) Spermatogonial Phase

The spermatogonia, which are located on the acinus wall, are somewhat oval in shape. Each of the spermatogonium (about 9~10 μm in diameter), contains a large nucleus and heterochromatin showing low electron density in the nucleus. At this phase, a number of oval, small mitochon-

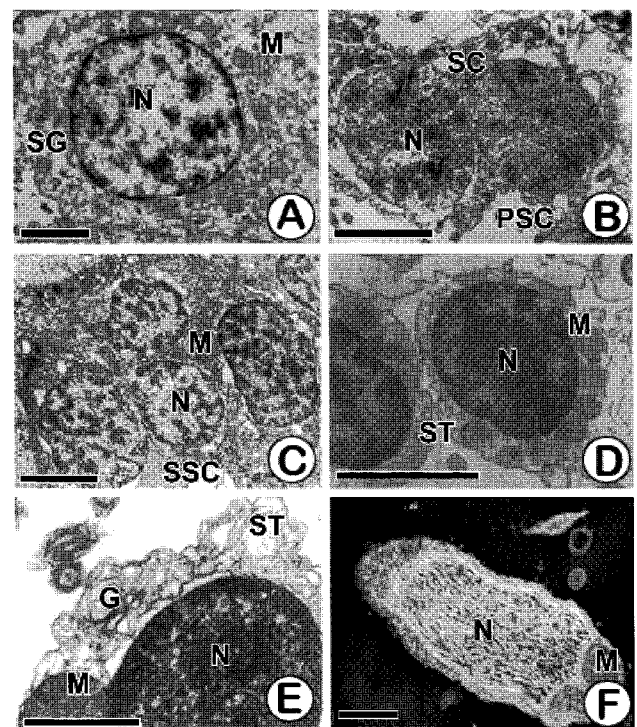


Fig. 1. Electron micrographs of spermatogenesis of *Corbicula japonica* (A~F). A, spermatogonium in the spermatogonial phase, with mitochondria in the cytoplasm.; B, primary spermatocytes in the spermatocyte phase, with synaptonemal complexes in the nucleus; C, secondary spermatocytes, with heterochromatin in the nucleus; D, spermatids in the spermatid phase, with high electron dense heterochromatin in the nucleus and mitochondria in the cytoplasm; E, spermatid during spermiogenesis, with the Golgi complex and mitochondria; F, spermatid during spermiogenesis, with mitochondria in the cytoplasm. Abbreviations: G, Golgi complex; M, mitochondrion; N, nucleus; PSC, primary spermatocyte; SC, synaptonemal complex; SG, spermatogonium; SSC, secondary spermatocyte; ST, spermatid. Scale bars = 2 μm .

dria appear in the cytoplasm (Fig. 1A).

2) Primary Spermatocyte Phase

The spermatogonia develop into primary spermatocytes by multiplication. The nucleus of the primary spermatocyte (approximately $7\sim 8\ \mu\text{m}$ in diameter) contains slightly electron dense chromatin than that of the spermatogonium. In particular, the synaptonemal complexes in the nucleus appear in the prophase during the first maturation division. At this time, several mitochondria appear in the cytoplasm (Fig. 1B).

3) Secondary Spermatocyte Phase

The primary spermatocytes develop into secondary spermatocytes (approximately $5\sim 6\ \mu\text{m}$ in diameter) by the first maturation division. The heterochromatin materials in the nucleus of the secondary spermatocyte are denser and more highly concentrated than those of the primary spermatocytes. At this time, several mitochondria are present in the cytoplasm of the secondary spermatocytes (Fig. 1C).

4) Spermatid Phase

The secondary spermatocyte is transformed into the spermatids (about $4\ \mu\text{m}$ in diameter) by the secondary maturation division. At this time, electron-dense heterochromatin materials appear in the nucleus, and the Golgi complex, acrosomal granule and several mitochondria are found in the cytoplasm around the nucleus of the spermatid (Figs. 1D, E). The morphology of the spermatid changes gradually during the differentiation of the spermatid. During this phase, the mitochondria move to a position just behind the elongated nucleus (Fig. 1F). And the morphology of the spermatid nucleus is elongated, and an acrosomal vesicle appear just before the elongated nucleus (Fig. 2A). At this time, of the two centrioles lying in the midpiece of the sperm, the proximal centriole appears near the distal centriole, giving rise to the axial filament of the flagellum of the sperm (Fig. 2B). The mass of four mitochondria, forming the paranucleus (surrounding the centrioles), is

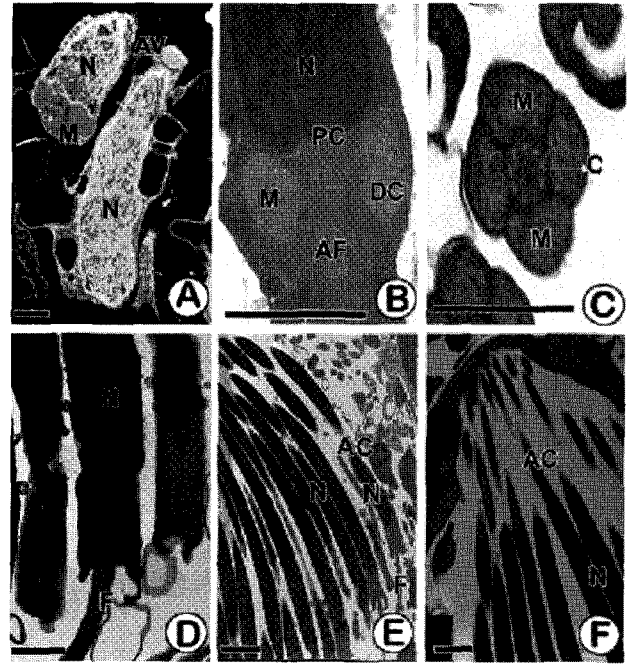


Fig. 2. Electron micrographs of spermiogenesis of *Corbicula japonica* (A~F). A, spermatids during spermiogenesis, with the elongated nucleus and the acrosomal vesicle; B, the middle piece of the sperm, with the proximal centriole, distal centriole and axial filament; C, cross section of paranucleus composed of the centrosome surrounded with 4 mitochondria; D, spermatozoa in the spermatozoon phase; with sperm nucleus and a flagellum; E, F, a completed spermatozoa, with acrosome, nucleus and a flagellum. Abbreviations: AC, acrosome; AF, axial filament; AV, acrosomal vesicle; C, centrosome; DC, distal centriole; F, flagellum; M, mitochondrion; N, nucleus; PC, proximal centriole. Scale bars = $2\ \mu\text{m}$.

localized in a short midpiece of the spermatid (Fig. 2C).

5) Spermatozoon Phase

The distal centriole gives rise to the axial filament of a flagellum of the spermatozoon, and the differentiation of the spermatozoa is completed (Fig. 2D). During the acrosomal formation the acrosomal filament makes contact with morphologically modified and elongated nucleus, and a well-developed acrosome just before the elongated nucleus is then formed by way of morphological changes of the acrosomal vesicle. The formation of the acrosome is completed at this phase. The morphologies of the sperm

nucleus type and the acrosome shape of this species have a long arrow-like type and long cone-like shape, respectively.

The spermatozoon is approximately about 55 μm in length. The sperm head (about 12 μm long) is elongated and tapers with a slight curve. Sperm nucleus is about 7.90 μm long, and the acrosome is about 2.70 μm long (Figs. 2E, F). There is a little interval between the acrosome and the sperm nucleus, the acrosome is filled with subacrosomal materials which are granular substances showing electron density (Fig. 3A). Subacrosomal materials appear in the acrosome cross sectioned (Fig. 3B). The morphology of the elongated nucleus cross sectioned shows oval in shape (Fig. 3C). From the flagellum cross sectioned, in particular, wing-like axonemal lateral fins of the flagellum are

observed, as seen in teleost fishes occurring external fertilization in the brackish water and seawater (Fig. 3D). And the axoneme of the sperm tail flagellum consists of nine pairs of microtubules at the periphery and a pair at the center. The axoneme of the sperm tail shows a 9+2 structure (Fig. 3E).

DISCUSSION

In general, most of the bivalves have a primitive type of spermatozoa with a head (containing a small acrosome and nucleus), a short midpiece (with four to five mitochondria surrounded the centrioles) and a long flagellum (Longo & Dornfield, 1967; Chung, 2006; Chung et al., 2007). Franzén (1970) divided molluscan sperm morphology into two types: 1) the primitive type found in species that performs external fertilization, and 2) the modified type found in internal fertilization species. Also, Verdonk et al. (1983) divided sperm morphology into four types: 1) primitive, 2) modified, 3) biflagellate, and 4) aflagellate types.

The primitive type is seen in most bivalves, and the modified type is found in most gastropods that perform internal fertilization. In addition to the primitive type and partially modified type of molluscan sperm, the biflagellate type is seen in the triploid *C. fluminea* and *C. leana* in natural populations (Komaru & Konishi, 1996; Komaru et al., 1997; Park et al., 2002). The aflagellate type is hard to find in molluscan sperm, but this type is only found in a few crustaceans (Kim, 2001).

According to Franzén (1956), spermatozoa in virtually all species in bivalvia studied were classified as the primitive type. Recently the modified type spermatozoa in bivalvia have been reported in *Lasaea subviridis* (Ó Foighil, 1985), *Scrobicularia plana* (Sousa et al., 1989), and *Codakia orbicularis* (Mouëza & Frenkiel, 1995).

Komaru and Konish (1996) reported that sperm morphology in *C. leana* is highly modified from that of the primitive type described for most bivalves (Franzén, 1956). The modification of sperm morphology in these species

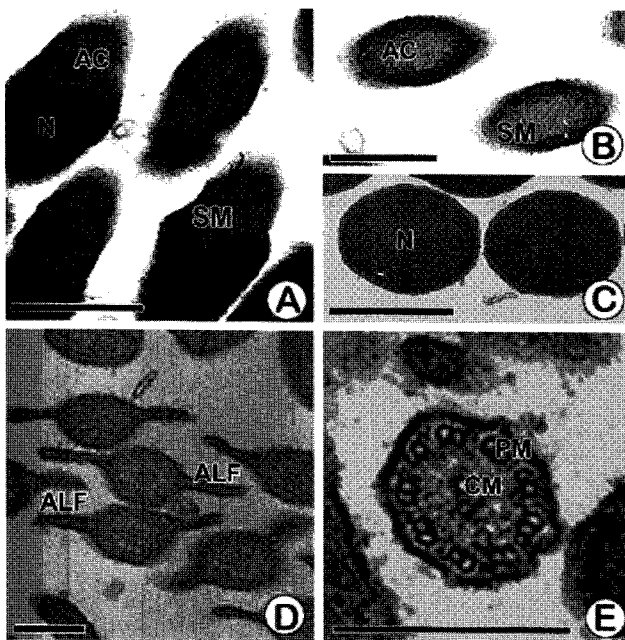


Fig. 3. Electron micrographs of spermiogenesis of *Corbicula japonica* (A~E). A, sperm nucleus, with an acrosome and nucleus; B, cross section of the acrosome; C, cross section of the sperm nucleus; D, cross section of the flagellum, with axonemal lateral fin (wing-like structure); E, cross section of the axoneme of the spermatozoon, with 9+2 structure. Scale bar = 0.01 μm . Abbreviations: AC, acrosome; ALF, axonemal lateral fin; CM, central microtubule; PM, peripheral microtubule; SM, subacrosomal material. Scale bars = 1 μm .

may related to their specialized mode of reproduction (e.g., hermaphroditism, brooding and possibly self-fertilization). In this study, although *C. japonica* is dieocious and external fertilization species, sperm morphology of this species is highly modified from that of the primitive type, as seen in hermaphroditic triploid *Corbicula* species (internal fertilization and viviparous). Therefore, it may be used as a character of classification of family Corbiculidae.

In the class bivalvia spermatozoa with two flagella have only been found in hermaphroditic *C. fluminea* (Kraemer, 1983) and *C. leana* (Komaru & Konishi, 1996; Park et al., 2002). The elongated nucleus and the lack of distinct mid-piece are characteristics of the spermatozoa in *C. sandai* (Hachiri & Higashi, 1970), *S. plana* (Sousa et al., 1989), and *Codakia orbicularis* (Mouëza & Frenkiel, 1995). In the present study, diploid *C. japonica* has an elongated sperm nucleus as one of the same morphological characteristics, as seen in freshwater triploid hermaphroditic *Corbicula* species. It is assumed that an elongated sperm head in these bivalves may be more efficient than that of primitive type such as rounded or conical type for penetration into the jelly coat and cytoplasm of larger oocytes.

Popham (1979) reported that the acrosome morphology of the sperm head differs markedly among the species: the acrosomal shape can be classified into four types: cone, cap, elongate modified cone, and modified cap types. In the present study, the morphology of acrosomal shape of *C. japonica* is of a cone-like shape, as seen in freshwater triploid and diploid *Corbicula* spp.

Regarding the morphology of the sperm nucleus, sperm nuclei of triploid freshwater *C. leana* are arrow-shaped. In this study, the morphology of the sperm nucleus of *C. japonica* shows an elongated nucleus (arrow-shaped). Therefore, this character may be used as a key for classification of family Corbiculidae.

C. japonica is possessed of relatively distinct midpiece containing 4 mitochondria, in contrast, Komaru and Konishi (1996) reported that *C. leana* is possessed indistinct mid-piece containing 5 or over 5 mitochondria. From these

structural differences of midpiece of sperm, it is assumed that the lack of distinct midpiece of spermatozoa of freshwater triploid *C. leana* may be related to increase the dynamic efficiency of internal fertilization in the inner demi-branch, however, it is assumed that brackish water diploid *C. japonica* is possessed of a distinct short midpiece of sperm, because fertilization occurs in the brackish water, as seen in most external fertilization species.

The membranous wing-like structure along the flagellum was observed in the flagellum of spermatozoa of *C. japonica*, as seen in *C. sandai* (Hachiri & Higashi, 1970) and *Codakia orbicularis* (Mouëza & Frenkiel, 1995) by electron microscopy. In addition, wing-like axonemal lateral fins of the flagellum were observed, as easily seen in fish species occurring external fertilization in the brackish water and seawater. Regarding this structure, Hachiri and Higashi (1970) suggested that this structure may increase the fluid dynamic efficiency of flagellation. We agree to opinion suggested by Hachiri and Higashi (1970).

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