Germ Cell Development during Spermatogenesis and Taxonomic Values of Sperm Morphology in *Septifer* (*Mytilisepta*) *virgatus* (Bivalvia: Mytilidae)

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ABSTRACT : Spermatogenesis and taxonomic values of mature sperm morphology of in male *Septifer* (*Mytilisepta*) *virgatus* were investigated by transmission electron microscope observations. The morphologies of the sperm nucleus and the acrosome of this species are the cylinder shape and cone shape, respectively. Spermatozoa are approximately 45-50 ㎛ in length including a sperm nucleus (about 1.26 ㎛ long), an acrosome (about 0.99 ㎛ long), and tail flagellum (about 45-47 ㎛). Several electron-dense proacrosomal vesicles become later the definitive acrosomal vesicle by the fusion of several Golgi-derived vesicles. The acrosome of this species has two regions of differing electron density: there is a thin, outer electron-dense opaque region (part) at the anterior end, behind which is a thicker, more electron-lucent region (part). In genus *Septifer* in Mytilidae, an axial rod does not find and also a mid-central line hole does not appear in the sperm nucleus. However, in genus *Mytilus* in Mytilidae, in subclass Pteriomorphia, an axial rod and a mid-central line hole appeared in the sperm nucleus. These morphological differences of the acrosome and sperm nucleus between the genuses *Septifer* and *Mytilus* can be used for phylogenetic and taxonomic analyses as a taxonomic key or a significant tool. The number of mitochondria in the midpiece of the sperm of this species are five, as seen in subclass Pteriomorphia.

Key words : *Septifer* (*Mytilisepta*) *virgatus*, Germ cell, Spermatogenesis, Sperm Morphology

INTRODUCTION

The purplish bifurcate mussel, *Septifer* (*Mytilisepta*) *virgatus* in family Mytilidae comprise one of the more taxonomically important group of bivalve molluscs. To date, sperm morphologies and ultrastructures during spermatogenesis has been investigated in many species of bivalve molluscs using electron microscopy (Gaulejac et al., 1995; Chung & Ryou, 2000; Chung et al., 2007, 2010). Recently, it is well-known that the ultrastructure and morphological variations of the spermatozoon in bivalve groups appear to be correlated with the phylogenetic relationship and systematics of bivalves (Bacetti, 1979; Popham, 1979; Healy, 1989, 1986, 1995).

Within the sperm in bivalves there is little variation in the fine structure of the tail and midpiece but great variation in the form of the acrosome and the nucleus. Although the morphologies of sperm acrosome in many species of the family Mytilidae are similar, there are very distinct differences in the morphologies of the sperm acrosome in the family Mytilidae. In general, morphologies and ultrastructures of the sperm nuclei of most species in bivalves are very similar, however, their morphologies vary with the species in the same family. For that reason, the morphologies of sperm nuclei can not be a valuable tool in assessing taxonomic and phylogenetic problems within the bivalvia because the morphologies of the sperm nuclei vary with the genus names in the same family.

Previously, regarding Mytilidae species, there have been several studies on Mytilidae species, especially, *M. edulis* and *M. coruscus* on aspects of spermatogenesis, including the fine structure of spermatid differentiation (Kim et al., 2010b), spermatozoon morphology and bivalve phylogeny (Popham, 1979), ultrastructure of sperm and spermatogenesis

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(Longo & Dornfeld, 1967; Hodgson & Bernard, 1986).

Recently, regarding ultrastructural studies of the species in Mytilidae, there is only two reports on ultrastructure of spermatozoa of *S.* (*M.*) *virgatus* (Kim, 2001) and *M. coruscus* (Kim et al., 2010b). Although a report on spermatogenesis has been reported, there are still gaps in our knowledge regading spermatogenesis. Little information is available on ultrastructure of germ cell differentiations during spermatogenesis and its taxonomic values of mature sperm morphology related with bivalve phylogeny of this species.

For that reason, recently, the acrosomal morphology of the sperm has been used to organize bivalve subclasses (Popham, 1979). In association with the acrosomal morphology, Healy (1989) reported that different subclasses of bivalves each have unique acrosomal morphologies. Therefore, the acrosomal morphology of the sperm in *S.* (*M.*) *virgatus* should be compared with other genuses in the same Mytilidae in subclass Pteriomorphia because *S.* (*M.*) *virgatus* taxonomically belongs to the species of Mytilidae in subclass Pteriomorphia.

In association with the acrosomal morphology, Healy (1989) reported that different subclasses of bivalves each have unique acrosomal morphologies. Therefore, the acrosomal morphology of the sperm in *S.* (*M.*) *virgatus* should be compared with other species of the subclass Pteriomorphia.

In addition, the number of mitochondria in the sperm midpiece tends to be stable within any given family or superfamily varying from a maximum of 14 (Dorozdov & Reunov, 1986) to a minimum of 4 (common to many bivalve families) (Healy, 1989, 1995). Therefore, the number of mitochondria in sperm midpiece of this species should be investigated and compared with the same family Mytilidae.

Thus, it is very important to study that sperm ultrastructures of bivalves are now widely used in taxonomic analyses (Healy, 1995).

If some characteristics showing taxonomic values are obtained or found from sperm ultrastructure during spermiogenesis, the results of the ultrastructural studies on bivalve spermatozoa will provide information needed for the elucidation of relationship patterns among several families in subclasses Pteriomorphia (Popham, 1979). Therefore, the purpose of the present study is to describe the ultrastructures of germ cells during spermatogenesis, and to clarify morphological, structural differences of spermatozoa in genuses *Septifer* and *Mytilus* in the same Mytilidae in subclass Pteriomorphia by phylogenetic analysis.

MATERIALS AND METHODS

1. Sampling

Specimens of *Septifer* (*Mytilisepta*) *virgatus* were collected monthly in the intertidal zone of Gyeokpo, Korea, for one year from January to December, 2006. A total of 127 male clams ranging from 40.6 ㎜ to 46.7 ㎜ in shell length were used for the studies of spermatogenesis by electron microscopic observations.

2. Ultrasructure of Germ Cells and Sperm Morphology

For electron microscopical observations, excised pieces of the gonads 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 hours at 4℃. After prefixation, the specimens were washed several times in the buffer solution and then postfixed in a 1% osmium tetroxide solution in 0.2 M phosphate buffer (pH 7.4) for 1 hour at 4℃. 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 ㎚. Tissue sections were mounted on collodion-coated copper grids, doubly stained with uranyl ace tate followed by lead citrate, and observed with a JEM 100 CX-Ⅱ(80-KV) electron microscope.

RESULTS

1. Ultrastructure of Germ Cells during Spermatogenesis

Based on the testicular development and morphological characteristics of germ cells by electron microscopic observation. Spermatogenesis occurs in the acini of the testis. The process of spermatogenesis appears to be similar to other bivalve species. Spermatogenesis occurs in numerous acini of the testis and can be divided into four stages as follows: (1) spermatogonia, (2) spermatocytes, (3) spermatids, and (4) spermatozoa.

2. Process of Spermatogenesis

1) Spermatogonia

Spermatogonia (about 6.0-7.0 ㎛ diameter) are appear near the acinus wall and are characterized by a large ellipsoidal nucleus. The nucleus (approximately 4.5 ㎛ diameter) contains small clumps of electron-dense chromatin, which are often associated with the inner nuclear envelope, and the cytoplasm contains several small mitochondria and vacuoles. In general, they are confined to the outer region of the acinus. Their cytoplasm is largely devoid of organelles except for scattered mitochondria. Thus, the gonad developmental stage of spermatogonia are very weak (Fig. 1A).

2) Spermatocytes

The spermatogonium develops into primary spermatocytes by the mitotic division. At this stage, two stages of primary and secondary spermatocytes are observed in the acinus wall. Primary spermatocytes (approximately 4.5 to 5.2 ㎛ diameter) are smaller cells that are distinguished by nuclei with highly condensed chromatin materials. The nucleus (about 3.4-3.7 ㎛ diameter) of the primary spermatocyte is similar in size and shape to that of the spermatogonium, At this stage, however, the nucleolus is no longer prominent, and the chromatin is in the form of a patchwork. In the stage of first meiotic prophase, primary spermatocytes are distinguished within the germinal layer of the testis. Zygotene/patchtene spermatocytes contain nuclei with more highly condensed chromatin and synaptonemal complexes. The synaptonemal complexes in the nucleus appear in the prophase during the first meiotic division. Cellular outlines are oval in shape. The cytoplasm contains a similar complement of organelles to the spermatogonia (Fig. 1B). Primary spermatocytes differentiate into secondary spermatocytes by the first meiotic division of the primary spermatocytes. At this stage the secondary spermatocytes are rarely observed because of the rapidity of the first meiotic division of the primary spermatocytes. They are irregular in shape and range from about 4.2-4.7 ㎛ diameter in size. Spherical nucleus possess scattered heterochromatin forming a network. In particular, several mitochondria, vacuoles and glycogen particles are present in the cytoplasm of the secondary spermatocytes. Secondary spermatocytes are frequently observed undergoing meiotic division, and the sizes of secondary spermatocytes become smaller than those of primary spermatocytes (Fig. 1C).

3) Spermatids

During the testicular development, the secondary spermatocytes develop into the spermatids by the secondary meiotic division. For convenience, spermiogenesis divided arbitrary into two stages: the early and late stage.

In the early stage of spermatid development, the nucleus (about 3.0 ㎛ diameter) showed spherical or oval in shape and occupies the center of the cell. The cytoplasm contains numerous mitochondria, the Golgi complex, and small proacrosomal granules. As the spermatid matures (midspermatid), the cytoplasm is lost by sloughing, Nuclei (2.2-2.5 ㎛ diameter) of spermatids become smaller, and the nuclear contents continue to condense (Fig. 1D).

In the middle stage of spermatid development, proacrosomal granules, which are formed from the Golgi complex, appear near the acrosomal vesicle. At this time, a proacrosomal vesicle migrate to the presumptive anterior end of the spermatid where they coalesce to form a single electron-dense vesicle. At the same time the mitochondria become reduced in number but increase in size. In some cells the mitochondria are in close proximity to one another, suggesting that reduction in number and increase

in size may be by mitochondrial fusion. The larger mitochondria form a close association with the nucleus and in many cases appear tightly apposed to the nuclear envelope.

As futher development occurs, the mitochondria come to occupy the end of the cell opposite the acrosome, thus forming the sperm midpiece (Fig. 1E). After a proacrosomal vesicle attached to the membrane of the nucleus, a small subacrosomal space appear between the proacrosomal vesicle and the nucleus, and then this vesicle lie horizontal on the anterior apical part of the nucleus (Fig. 1F), at the same time, the nuclei are slightly elongated and nuclear materials are condensed into longitudinally. the mitochondria come to occupy the end of the cell opposite the acrosome, thus forming the sperm midpiece.

During the late spermatid stage there is continued loss of cytoplasm by sloughing, decrease in nuclear size, and

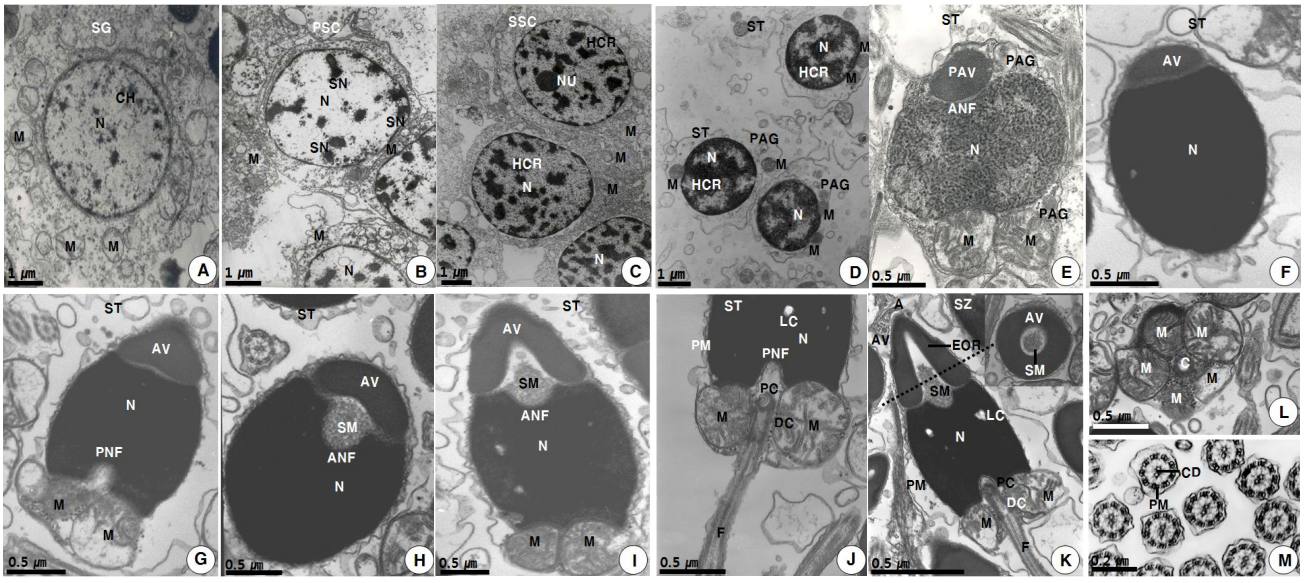

Fig. 1. Transmission electron micrographs of spermatogenesis in male *Septifer* (*Mytilisepta*) *virgatus* (A-M). A, A spermatogonia (SG). Note chromatins in the nucleus (N) and mitochondria (M) and vaculoes (V) in the cytoplasm; B, Primary spermatocyte (PSC). Note several synaptonemal complexes (SN) in the nucleus (N) and a number of mitochondria (M) in the cytoplasm; C, Secondary spermatocytes (SSC). Note heterochromatin (HCR) in the nucleus and several mitochondria (M) and vacuoles in the cytoplasm; D, A spermatid (ST) snd a proacrosomal vesicle. Note heterochromatin (HCR) in the nucleus (N) and a proacrosomal granule (PAG) near the nucleus; E, A spermatid (ST) during the proacrosomal vesicle (PAV) formation. Note the appearances of a proacrosomal vesicle (PAV) and proacrosomal granules on the anterior fossa (ANF) of the nucleus (N) with spherical mitochondria (M); F, A spermatid during the acrosomal vesicle formation. Note initial variation of an acrosomal vesicle (AV) on the nucleus (N); G, A spermatid in the late stage during the acrosome formation. Note an acrosomal vesicle (AV) on the nucleus (N) and spherical mitochondria (M) beneath the posterior nuclear fossa (PNF); H, A spermatid in the late stage with an acrosomal vesicle (AV). Note subacrosomal material (SM) in the anterior nuclear fossa (ANF); I, A spermatid in the same stage and the acrosome formation. Note subacrosomal material (SM) in the anterior nuclear fossa (ANF) of the nucleus (N) near mitochondria (M); J, A spermatid in the same stage. Note plasma membrane, a lacunae in the nucleus, the proximal centriole (PC) and distal centriole (DC), spherical mitochondria (M), posterior nuclear fossa (PNF) in the midpiece of sperm and a flagellum (F); K, A completed spermatozoon with the acrosmal vesicle (AV) being composed of electron-opaque region (EOR) and electron-lucent region (ELR). Note subacrosomal material (SM) in the acrosomal space on the nucleus (N), lacunae (LC) and the proximal (PC) and distal centriole (DC), mitochondria (M) and a flagellum (F); L, The sperm midpiece. Note 5 mitochondria (M) surrounding a proximal centriole (C) in the sperm midpiece; M, Cross sectioned tail flagellum of mature sperm. Note the axoneme showing a 9+2 structure (a pair of central doublets (CD) and nine pair of peripheral microtubules (PM)).

condensation of nuclear materials. This stage is characterized by further development of the acrosomal vesicle. The acrosomal vesicle then begins to invaginate on its nuclear surface, an acrosomal vesicle showing temporarily triangular shape is formed from a proacrosomal vesicle showing an oval shape, and then the posterior nuclear fossa is formed by invasination in the midpiece part near spherical mitochondria with well developed cristae (Fig. 1G). Thereafter, the morphology of an acrosomal vesicle is transformed from trianglular shape to the curved line shape, and then the posterior acrosomal fossa is formed by invagination of the acrosomal vesicle, and also anterior nuclear fossa is formed by invagination of the nucleus (Fig. 1H). As the invagination of the acrosomal vesicle continuously progress, consequently it bcomes a conical-shaped acrosome which is formed the characteristic hollow, and also the anterior nuclear fossa is formed on the nucleus.

At this time, in particular, the acrosome has two regions of differing electron density. There is a thin and outer electron-dense opaque region at the anterior end, behind which is a thicker, more electron-lucent region. In particular, these characteristics of the acrosomal vesicle are related to the characteristics of the subclass for classification. The plasma mebrane and a few lacunae are found in the nucleus, and large spherical mitochondria are present at the posterior part of the nucleus. At this time, the invagination of the posterior nuclear fossa appear at the posterior part of the nucleus, and the nucleus becomes oval in shape.

At this time the proximal centriole lied at 90° to the sperm longitudinal axis or the distal centriole near the basal invagination of the nucleus. The distal centriole lied parallel to the sperm longitudinal axis and forms the point of origin of flagellar axoneme. In particular, the satellite fibers are not found, unlike the sattelite fibers linked to distal centriole in the genus *Mytilus* species in Mytilidae. The tail developing from the distal centriole appears during the late spermatid stage.

The midpiece includes five spherical mitochondria, which has the typical 9+2 arrangement of microtubules, originates from the distal centriole and is surrounded at its base by a collar of thickened plasma membrane (Figs. 1I, J).

4) Spermatozoa

The mature spermatozoon is the primitive type and about 45-50 ㎛ long. As shown in Fig. 1K, the structure of the sperm comprises cylinder-shape and electron dense nucleus (1.26 ㎛ long) with an anterior nuclear fossa, and an acrosome (0.99 ㎛ long). the acrosome contains two materials: an outer electron-dense material and a more electron-lucent substance that occurs predominantly in the basal region. And the acrosome has two regions of differing electron density. There is a thin, outer electron-dense opaque region (part) at the anterior end, behind which is a thicker, more electron-lucent region (part). The subacrosomal space between the anterior nuclear fossa and the part of two basal rings is filled subacrosomal material. The central hollow of the acrosome contains a granular subacrosomal material and one straight line of the axial rod does not appear in the subacrosomal space of the acrosomal vesicle, and also one straight line (as a substructure in the nucleus in Mytilidae) does not find in the nucleus (Fig. 2A). The size of the sperm head (the nucleus and acrosome) is about 2.70 ㎛ long. The midpiece includes five spherical mitochondria (Fig. 1L), which has the typical 9+2 arrangement of microtubules, originates from the distal centriole and is surrounded at its base by a collar of thickened plasma membrane (Fig. 1M).

DISCUSSION

Spermatogenesis of this speciece showed similar phenomenona to those of other bivalves (Eckelbarger et al., 1990; Eckelbarger & Davis, 1996; Chung & Ryou, 2000, Jun et al., 2009; Kim, 2001; Kim et al., 2010a, b; Chung 2007, 2010).

In particular, however, the ultrasructure and morphology of the nucleus and acrosomal vesicle in an acrosome of

mature spermatozoon of *S.* (*M.*) *virgatus* were quite different, unlike those of sperm of the species of Mytilidae in subclass Pteriomorphia.

In this study, during germ cell differentiation, the synaptonemal complexes in the nucleus of the primary spermatocyte appeared in the patchytene stage in the prophase during the first maturation division. In general, it was easy to observe that the patchyte stage in the primary spermatocyte was characterized by the presence of synaptonemal complexes in the nucleus.

Recently, sperm ultrastructure of bivalves is considered a valuable tool in assessing taxonomic and phylogenetic problems within the bivalves (Franzen, 1970, 1983; Popham, 1979; Eckelbarger et al., 1990), and it is now widely used in taxonomic analyses (Healy, 1995): for example, 1) acrosomal morphology, and 2) the number of mitochondria in the sperm midpiece.

Regarding the modes of acrosomal development, Healy (1989) stated that acrosomal development in the Mollusca can be classified into three modes as follows: The first mode of the acrosomes involves that numerous electron-dense proacrosomal vesicles, which are at first formed by the Golgi complex, become later the definitive acrosomal vesicle by the fusion of several Golgi-derived vesicles (Longo & Dornfeld, 1967; Longo & Anderson, 1969; Bernard & Hodgson, 1985; Healy, 1989). In general, this mode is observed in numerous other externally-fertilizing invertebrate (Bacetti & Afzelius, 1976).

The second mode of acrosomal development involves that after the initial definitive acrosomal vesicle is formed by the Golgi complex of a large receptacle vesicle, and then the growth of which is achieved through fusion of small vesicles budded from the Golgi cisternae, or from materials channeled directly from the cisternae. In general, this mode occurs in internally fertilizing molluscs (higher prosobranch gastropods, opisthobranch and pulmonate gastropods; cephalopods (Healy, 1995, 1989) and in many other internally fertilizing animal groups (Baccetti & Afzelius, 1976; Bacetti, 1979).

The third mode of acrosomal development indicated that in case of freshwater clam *Neotrigonia* (Unionoidea), acrosome formation is formed through production of multiple proacrosomal vesicles which do not fuse into a single acrosomal vesicle and are not associated with any recognizable subacrosomal deposit (either diffuse or organized as an axial rod). Alternatively, this pattern could be viewed as a variation on the first mode of acrosomal development.

1. Taxonomic Values of Mature Sperm Morphology and Ultrastructure

Observations on the ultrastructure of the sperm of *S.* (*M.*) *virgatus* and *Mytilus corscus* showed that differences in acrosome and nuclear morphology exist between these two Korean Mytilidae species. As shown in Figures 2A, B, these species have clear differences in acrosomal and nuclear morphologies. Thus, we support the use of sperm ultrastructure as an aid to bivalve identification (Hodgson & Bernard, 1986).

Compared the sperm morphology and ultrastructure of *S.* (*M.*) *virgatus* with those of *Mytilus coruscus*, we can find several differences between two species as follows. In this study, in Figure 2A, *S.* (*M.*) *virgatus* showed that the sperm of this species has a cone-like acrosome, and the acrosomal vesicle has two regions of differing electron density: that is, a thin, outer electron-dense opaque region (part) at the anterior end, behind which is a thicker, more electron-lucent region (part). Relatively wide central hollow of the acrosome contains a granular subacrosomal material, instead of a mid-central line of the axial rod does not appear in the nucleus. Compared with *M. coruscus*, this species has some special differences in sperm morphology and structures.

In this study, one straight line (as a substructure appeared in the nucleus of *Mytilus* species in Mytilidae) does not find in the nucleus (Kim et al., 2010b). The relatively wider subacrosomal space between the anterior nuclear fossa and the part of two basal rings is filled subacrosomal material. The central hollow of the acrosome contains a

granular subacrosomal material. And also the satellite fibres does not find in *S.* (*M.*) *virgatus* in Mytilidae.

However, as shown in Figure 2B, *Mytilus coruscus* in the genus *Mytilus* in the same Mytilidae showed that the acrosome of the sperm is a modified cone-like shape. the acrosomal vesicle has two regions of differing electron density: that is, a thin, outer electron-dense opaque region (part) at the anterior end, behind which is a thicker, more electron-lucent region (part). An axial rod is found in a mid-central line hole (from the posterior nuclear fossa to the anterior nuclear fossa parts of the nucleus), and also exisist at the upper-central parts of the acrosomal vesicle. Thus, the axial rod is found two places: the acrosome and the sperm nucleus (Kim et al., 2010b). In particular, the satellite fibres exist near the distal centriole.

Despite the subtle differences in acrosome morphology

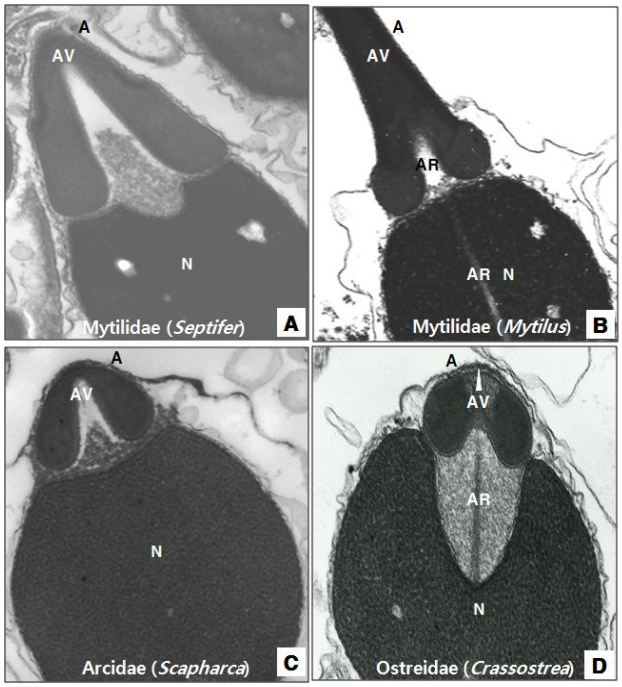

Fig. 2. A comparison of transmission electron micrographs of 4 species in 4 families in subclass Pteriomorphia. A, *Septifer* (*Mytilisepta*) *virgatus* in Mytilidae; B, *Mytilus coruscus* in Mytilidae; C, *Scapharca subcrenata* in Arcidae; D, *Crassostrea gigas* in Ostreidae. Abreviations: A, acrosome; AR, axial rod; AV, acrosomal vesicle; N, nucleus.

that exist between closely related genera, an examination of published micrographs of sperm structure from five subclasses of bivalve reveals that each subclass is characterized by a basic acrosomal morphology or position (Hodgson & Bernard, 1986). Popham reported that the Pteriomorpia and Heterodonta have very similar acrosomes, while our investigations that lead us to conclude that the Pteriomorphia and Heterodonta can be separated according to acrosome morphology. The Pteriomorphia all have acrosomes that are in the shape of a cone, albeit of varying dimensions, and that contain electron-dense material from the base to tip. By contrast, the acrosomes of the Heterodonta (Hodgson & Bernard, 1986) are characterized by restriction of the electron-dense material to the base or lateral regions, with such area joined by the acrosome membrane only.

In this study, our observations add further support to the theories that the single acrosomal vesicle, produced in the early spermatid, is formed by fusion of several small pro-acrosomal vesicles, and the five or six large mitochondria of the sperm are formed by fusion of several mitochondria.

The structure of the spermatozoon of *M. coruscus* (Kim et al., 2010b), which is a close relative of *S.* (*M.*) *virgatus*, has been included in Figure 2B to show that these species also differ slightly in acrosmal and nuclear morphology. Thus, we support the use of sperm ultrastructure as an aid to bivalve identification.

Popham shows that the Pteriomorphia and Heterodonta have very simmilar acrosomes, while our investigations lead us to conclude that the Pteriomorphia and Heterodonta can be separated according to acrosome morphology.

By contrast, the acrosomes of the Heterodonta are characterized by restriction of the electron-dense material to the base or lateral regions, with such area joined by the acrosome membrane only. In this study, the acrosomal vesicle has two regions of differing electron density: that is, a thin, outer electron-dense opaque region (part) at the anterior end, behind which is a thicker, more electron-lucent region (part). Therefore, this species belongs to subclass Pteriomorphia.

As shown in Figure 2C, in case of the acrosomal vesicle structures of S. subcrenata in Arcidae, the right and left basal rings shows electron opaque region (part), and also the anterior apex part of the acrosomal vesicle shows electron opaque region (Chung et al., 2011). These characteristics of the acrosomal vesicle were found in Arcidae in subclass Pteriomorphia. These common characteristics of the acrosomal vesicle in subclass Pteriomorphia can be used for phylogenetic and taxonomic analyses as a taxonomic key or a significant tool.

As shown in Figure 2D, a few tranverse bands (whorled substructure) appeared at the anterior part of the acrosomal vesicle were only found in Ostreidae species such as *Crassostrea gigas*. This special substructure is not found in the acrosomal vesicle, but found at the anterior part of the acrosomal vesicle. Commony, of Ostreidae species, *C. gigas* (Kim et al., 2010 a) contain 2-3 electron dense and electron lucent tranverse bands (special substructure associated with fertilization), while, exceptionally, *Saccostrea commercialis* contained 3-4 transverse bands (special substructure associated with cross incompatibility between *C. gigas* and *Saccostrea commercialis*) at the anterior region (part) of the acrosomal vesicle (Healy & Lester, 1991; Kim et al., 2010a). Compared *S.* (*M.*) *virgatus* with *C. gigas* in this study, however, we could not find a few tranverse bands (whorled substructure) at the anterior part of the acrosomal vesicle of *S.* (*M.*) *virgatus* in subclass Pteriormorphia. Therefore, this special substructure can be used for phylogenetic and taxonomic analyses as a taxonomic key or a significant tool (Fig. 2D) because this special structural characteristic was not found in the acrosomal vesicle in the species of Mytilidae and Arcidae in subclass Pteriomorphia (Figs. 2A, B, C).

Healy (1995) reported that of sperm ultrastructures of bivalves, the number of mitochondria in the sperm midpiece are now widely used in taxonomic analyses. That is the reason that the number of mitochondria in the sperm midpiece tends to be stable within any given family or superfamily (Healy, 1989, 1995).

Recently, some authors (Chung & Ryou, 2000; Kim, 2001; Kim et al., 2010b; Chung et al., 2007; 2010) described that the number of mitochondria at the midpiece of the spermatozoon were five in Mytilidae, Arcidae, Pinnidae, and Pteriidae in subclass Pteriomorphia, exceptionally, the numbers are four in only families Ostreidae in the subclass Pteriomorphia, Although it is the species in the same Family, the number of mitochondria at the sperm midpiece may be some different. Therefore, the number of mitochondria in the sperm midpiece were not concerned with the subclasses, however, their numbers were concerned with family or superfamily (Healy, 1995). Therefore, our results on the number of mitochondria coincide with opinions of Healy (1995).

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