Korean J. Malacol. 27(2): 149-157, 2011

Spermiogenesis and Taxonomic Value of Sperm Morphologies of Two Species in Veneridae (Bivalvia: Heterodonta)

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ABSTRACT

Some characteristics of the formations of acrosomal vesicles during the late stage of spermatids during spermiogenesis and taxonomical charateristics of sperm morphology in male two species (*Saxidomus purpurata* and *Meretrix petechialis*) in the family Veneridae were investigated by electron microscope observations. In two species, the morphologies of the spermatozoa have the primitive type and are similar to those of other bivalves in that it contains a short midpiece with five mitochondria surrounding the centrioles. The morphologies of the sperm nuclear types of *S. purpurata* and *M. petechialis* in Veneridae have the curved cylindrical and cylinderical type, respectively. And the acrosome shapes of two species are the same cap-shape type. In particular, the axial filament is not found in the lumen of the acrosome of two species, however, subacrosomal material are observed in the subacrosomal spaces between the anterior nuclear fossa and the acrosomal vesicle of two species. The spermatozoon of *S. purpurata* is approximately 46-52 μm in length, including a curved sperm nucleus (about 3.75 μm in length), a long acrosome (about 0.40 μm in length),and a tail flagellum (about 45-47 μm long). And the spermatozoon of *M. petechialis* is approximately 47-50 μm in length including a slightly curved sperm nucleus (about 1.50μm in length), an acrosome (about 0.56 μm in length) and tail flagellum (44-48 μm in length). In two species, the axoneme of the sperm tail flagellum of each species consists of nine pairs of microtubules at the periphery and a pair of cental doublets at the center. Therefore, the axoneme of the sperm tail flagellum shows a 9 + 2 structure. In particular, taxonomically important some charateristics of sperm morphologies of two species in the family Veneridae are acrosomal morphology of the sperm, The axial filament is not found in the acrosome as seen in a few species of the family Veneridae in the subclass Heterodonta. The acrosomal vesicle is composed of right, left basal rings and the apex part of the acrosomal vesicle. In particular, right and left basal rings show electron opaque part (region), while the apex part of the acrosomal vesicle shows electron lucent part (region). These charateristics belong to the subclass Heterodonta, unlikely a characteristic of the subclass Pteriomorphia showing all part of the acrosome being composed of electron opaque part (region). Therefore, it is easy to distinguish the families or the subclasses by the acrosomal structures. The number of mitochondria in the midpiece of the sperm of *S. purpurata* and *M. petechialis* in Veneridae are five. However, the number of mitochondria in the midpiece of the sperm in most species of Veneridae in the subclass Heterodonta are four. Therefore, the number of mitochondria of the sperm midpiece of two species are exceptionally 5, and it is only exceptional case in the species in Veneridae in the subclass Heterodonta. Except these cases, the number of mitochondria in the sperm midpiece in all families in the subclass Heterodontaare are 4, and now widely used in taxonomic analyses.

Key words: *Saxidomus purpurata*, *Meretrix petechialis*, spermiogenesis, sperm morphology

INTRODUTION

Spermatogenesis and sperm morphology has been reported in many species of bivalve molluscs using both light and electron microscopy (Eckelbarger *et al*., 1990; Eckelbarger and Davis, 1996; Gaulejac *et al*.,

Received June 5, 2011 ; Accepted June 26, 2011
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1225-3480/24389

1995; Chung and Ryou, 2000; Chung *et al*., 2007, 2010; Kim *et al*., 2010a,b). Recently, in the mollusca, sperm ultrastructure is considered a valuable tool in assessing taxonomic and phylogenetic problems within the bivalvia (Franzén, 1970, 1983; Popham, 1979; Healy, 1989, 1995; Hodgson and Bernard, 1986; . Thus, sperm ultrastructures of bivalves are now widely used in taxonomic analyses (Healy, 1995), and is especially useful when comparing closely related species (Popham *et al*., 1974; Popham, 1979).

To date, there are several ultrastructural studies on spermatogenesis of the family Veneridae in Korea: *Cyclina sinensis* (Chung *et al*., 1991), *Meretrix lusoria* (Kim, 2006), *Gomphina melanaegis* (Lee *et al*., 1999), *Gomphina veneriformis* (Park *et al*., 2003; Chung *et al*., 2010). *Protothaca* (*Notochione*) *jedoensis* (Kim *et al*., 2010c).

Although there have been several studies on sperm ultrastructure during spermatogenesis of two species (*S. purpurata* and *M. petechialis*) have been investigated by some authors, there are still gaps in our knowledge on the use of taxonomic characteristics of mature sperm morphology as taxonomic tools to solve the taxonomic problems. Little information is available on the ultrastructural studies associated with spermatogenesis by electron microscopic observation.

Recently, sperm ultrastructures of bivalves are now widely used in taxonomic analyses (Healy, 1995). In particular, it is well-known that acrosomal morphology of sperms has been used to organize bivalve subclasses (Popham, 1979), Therefore, it need to study acrosomal morphology of the sperm and the number of mitochondria in the sperm midpiece for taxonomic analyses of this species. If some characteristics obtained from sperm ultrastructure and the process of spermiogenesis are phylogenetically analyzed, the results of the ultrastructural studies on bivalve spermatozoa will provide information needed for the elucidation of relationship patterns among several bivalve subclasses (Popham *et al*., 1974, Popham, 1979; Healy, 1989, 1995). Therefore, the main aim of the present study is to describe some taxonomic characteristics for classification from the spermatid and mature sperm morphologies of two species (*Saxidomus purpurata* and *Meretrix petechialis* in Veneridae (Heterodonta) by electron microscope observation.

MATERIALS AND METHODS

1. Sampling

For collection of two species (*Saxidomus purpurata* and *Meretrix petechialis*), a total of 126 male individuals were collected at the intertidal and subtidal zones of Simpo, Jollabuk-do, Korea, and used for transmission / scanning electron microscope observations.

2. Transmission electron microscope observations

For transmission electron microscope observations, excised pieces of the gonads were cut into small pieces and fixed immediately in 2.5% paraformaldehydeglutaraldehyde in0.1 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 1hour 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 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.

3. Scanning electron microscope observations

A drop of sperm suspension was placed on a cover glass, prefixed with 2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M carcodylate buffer (pH 7.5) at 4°C for 15 min, and post fixed with 1% OsO_4 for 10 min, before rinsing with carcodylate buffer. The specimens were dehydrated in a graded ethanol series, critical point dried, coated with gold, and observed under a scanning electron microscope (ISI-SS4D). In addition, after dehydration, some testes were

freeze-fractured in liquid nitrogen, and then submitted to the same procedure described above.

RESULTS

1. Spermiogenesis and sperm morphology of *Saxidomus purpurata* and *Meretrix petechialis*

In general, the process of spermiogenesis of spermatids and mature process of spermatozoa appear to be similar to those of other bivalve species. In this study, to get some taxonomic characteristics for classification from the spermatid and mature sperm morphologies, morphological and ultrastuctural characteristics of spermatids and mature spermatozoa in male *Saxidomus purpurata* and *Meretrix petechialis* in Veneridae (Bivalvia: Heterodonta) are investigated by electron microscope observation. Studies on the morphological and ultrastructural characteristics of two species in Veneridae are as follows.

1) *Saxidomus purpurata*

Spermatids: For convenience, spermiogenesis has been divided arbitrarily into two stages: the early and late stages. Of them, in the late stage of spermiogenesis, the morphology of the spermatid nucleus changes gradually. At this time, small granules are formed by the Golgi complex in the cytoplasm move to a position just in front of the nucleus, while mitochondria move to a position just behind the nucleus. After all, the morphologies of the spermatid nuclei are gradually elongated, and one or a few granules in the cytoplasm of the spermatid become a proacrosomal vesicle. The proacrosomal vesicle migrate to the presumptive anterior end the spermatid, where they coalesce to form a single electron-dense vesicle. The mitochondria become reduced in number but increase in size by mitochondrial fusion. The larger mitochondria form a close association with the nucleus and in many cases appear tightly apposed to the nuclear envelope. However, the shape of the nucleus is modified and becomes greatly elongated (Fig. 1A).

A proacrosomal vesicle is modified and becomes the cap-shaped acrosomal vesicle on the nucleus. At this time, an acrosomal vesicle is composed of the apex part of the acrosomal vesicle, right and left basal rings, in paticular, right and left basal rings are electron dense opaque parts (region), while the apex part of acrosomal vesicle is electron lucent part (region). In general, these characteristics are one of the ultrastructures of the acrosomal vesicle in the acrosome. At this time, subacrosomal material are present between the anterior part of nuclear fossa and the acrosomal vesicle. However, the axial filaments, which are existed in *Ruditapes philippinarum* and *Gomphina veneriformis* in Veneridae, are not found in the subacrosomal material (Fig. 1B). Thereafter, a cap-shaped acrosomal vesicle becomes an acrosome. And then the acrosome lying on the sperm nucleus become a cone in shape. Therefor, the completed acrosome has two regions of differing electron density. It is composed of two long electron-opaque parts (right and left basal rings) and electron-lucent part (apex part). At the same time, the subacrosomal material are present between acrosomal vesicle and the anterior nuclear fossa of the nucleus (Fig. 1C). In the basal part of the nucleus, the mitochondria become reduced in number but increase in size by mitochondrial fusion. Posterior to the nucleus is the midpiece. This region consists of five spherical mitochondria surrounding a pair of triplet substructure centrioles. The cristae of each mitochondrion are randomly arranged. Larger mitochondria form a close association with the nucleus and in many cases appear tightly apposed to the nuclear envelope. At this time, of the two centrioles lying in the midpiece of the spermatozoon, the two centrioles, at right angles, show the classic nine triplets of microtubles (Figs. 1D, E). The proximal centriole laying the posterior fossa of the nucleus lies at 90° to the sperm longitudinal axis. However, it appears to be unconnected to the nuclear envelope. The distal centriole lies parallel to the sperm longitudinal axis and forms the point of origin for flagellar axoneme (Figs. 1D). During the late spermatid stage, the sperm nucleus is long curved (the angle of the nucleus is about 15°), and the cytoplasm is greatly reduced, and so the rate of nucleo-cytoplasm is high. After the sperm nucleus is

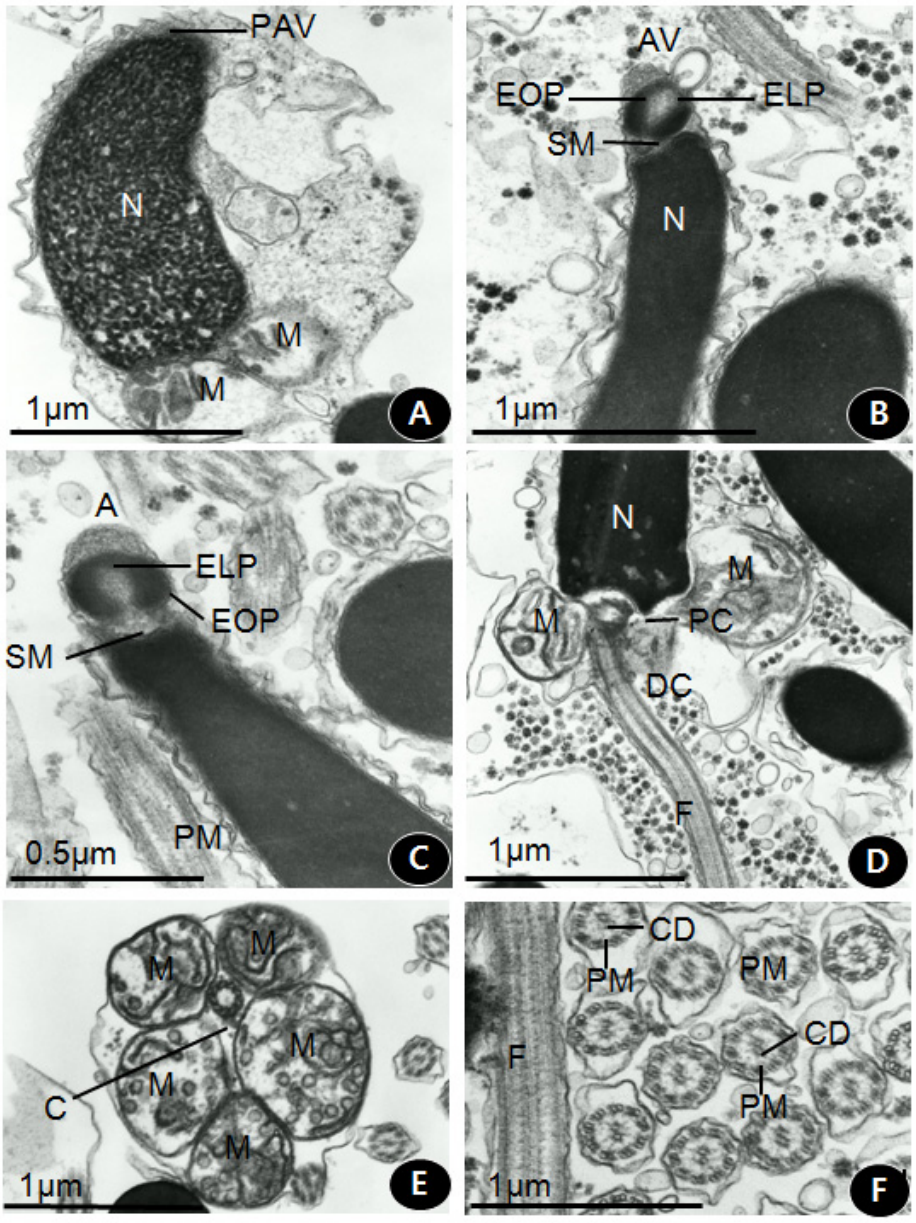

Fig. 1. Transmission electron micrographs of spermiogenesis and spermatozoa in male *Saxidomus purpurata* (A-F). **A**, A spermatid (ST) in the late stage of spermatid during spermiogenesis. Note proacrosomal vesicle (PAV) just before the nucleus (N) of the spermatid and the mitochondria (M) near the posterior nuclear fossa; **B**, An acrosomal vesicle (AV) on the elongated, curved spermatid nucleus (N). Note an acrosomal vesicle (AV) being composed of basal rings showing the electron dense opaque part (EOP), electron lucent part (ELP) and subacrosomal material (SM) on the elongated spermatid nucleus; **C**, An acrosome on a sperm nucleus. Note a modified acrosome consisting of basal rings of the acrosomal vesicle showing electron dense opaque part (EOP), electron lucent part (ELP) and subacrosomal material (SM) on the anterior nuclear fossa of the spermatozoon nucleus surrounded with the plasma membrane; **D**, Sperm midpiece beneath the curved nucleus (N) and a flagellum. Note the proximal centriole (PC) and distal centriole (DC) with spherical mitochondria (M) beneath the posterior nuclear fossa and a tail flagellum (F); **E**, Cross sectioned part of the sperm miedpiece. Note five mitochondria surrounding a pair of centrioles; F, Cross section of the tail flagella of mature sperm. Note the axoneme showing a 9+2 structure (a pair of central doublets (CD) and nine pair of peripheral microtubules (PM)).

long elongated, an acrosomal vesicle is also gradually very elongated (average 0.40 μm long).

Spermatozoa: After spermiogenesis, differentiation of the spermatozoon is completed. Sperm morphology is the primitive type, as found in most bivalve species that undergo external fertilization. Mature sperm are approximately 46-52 μm long. The head is about 4.60 μm long and comprises a long, curved electron-dense nucleus (about 3.75 μm), with a posterior nuclear fossa, and an acrosome about 0.40 μm long. The morphology of the sperm nucleus and the acrosomes of this species are of a curved cylindrical type and the cap-shape, respectively (Figs. 1C, D). At this time, a cross- sectioned tail flagellum shows that the axoneme is composed of a 9 + 2 substructure (nine peripheral doublets surrounding a central pair of singlet microtubules) enclosed by a plasma membrane (Figs. 1D, F),

2. ***Meretrix petechialis***.

Spermatids: In the late stage of spermiogenesis, the morphology of the spermatid nucleus gradually elongated during the differentiation of the spermatid. At this time, one or a few granules, which are formed by the Golgi complex in the cytoplasm of the spermatid, form a proacrosomal vesicle, while mitochondria move to a position just behind the nucleus. The nuclei of spermatids are about 2.9 μm long and are cylinderical shape. At this stage, the nucleus shows a granular type. A proacrosomal vesicle migrates to the presumptive anterior end of the spermatid, where they coalesce to form a single electron-dense acrosomal vesicle. A single acrosomal vesicle locates at the presumptive anterior pole of the spermatids, and the subacrosomal material appear between the acrosomal vesicle and the anterior nuclear fossa of the nucleus (Fig. 2A). The acrosomal vesicle is initially oval in shape, but gradually assumes a cap-like form with invaginated posterior face. Two components of the acrosomal vesicle can be recognized: the acrosomal vesicle and an extensive deposit of subacrosomal (extravesicular) material. The acrosomal vesicle is membrane bound, become cap-shaped form by way of various morphorphological changes, and its size is about 0.56 μm long. Contents of the acrosomal vesicle are finely granular, moderately electron dense. At this stage, after mitochondria move to a position just behind the nucleus, several small mitochondria are fused each other and become larger ones near the posterior nuclear fossa of the nucleus. The morphologies of many cristae of mitochondria are irregular (Fig. 2B).

As the late stage of spermatid develops gradually, granular nuclei of spermatids change into fibrous ones, and then rapidly they change to narrow and slightly elongated curved nucleus (the angle of the nucleus is 10°). A curved acrosomal vesicle is composed of right, left basal rings and subacrosomal materials in the space between the anterior nuclear fossa and the basal rings. At this time, in particular, right and left basal rings are electron dense opaque part (region), while the apex part of acrosomal vesicle is electron lucent part (region). Subacrosomal material are electron moderate part.

In the basal part of the nucleus, the mitochondria become reduced in number but increase in size by mitochondrial fusion. Posterior to the nucleus is the midpiece. This region consists of five spherical mitochondria surrounding a pair of triplet substructure centrioles. The cristae of each mitochondrion are randomly arranged. Larger mitochondria appear tightly apposed to the nuclear envelope (Fig. 2C). At this time, of the two centrioles lying in the midpiece of the spermatozoon, the two centrioles, at right angles, show the classic nine triplets of microtubles. The proximal centriole laying the posterior fossa of the nucleus lies at 90° to the sperm longitudinal axis. However, it appears to be unconnected to the nuclear envelope. The distal centriole lies parallel to the sperm longitudinal axis and forms the point of origin for flagellar axoneme . During the late spermatid stage, the sperm nucleus is slightly curved (the angle of the nucleus is about 10°), and the cytoplasm is greatly reduced, therefore, the rate of nucleo-cytoplasm is high. After the sperm nucleus is elongated, an acrosomal vesicle is also gradually elongated (about 0.56 μm long, Fig. 2D).

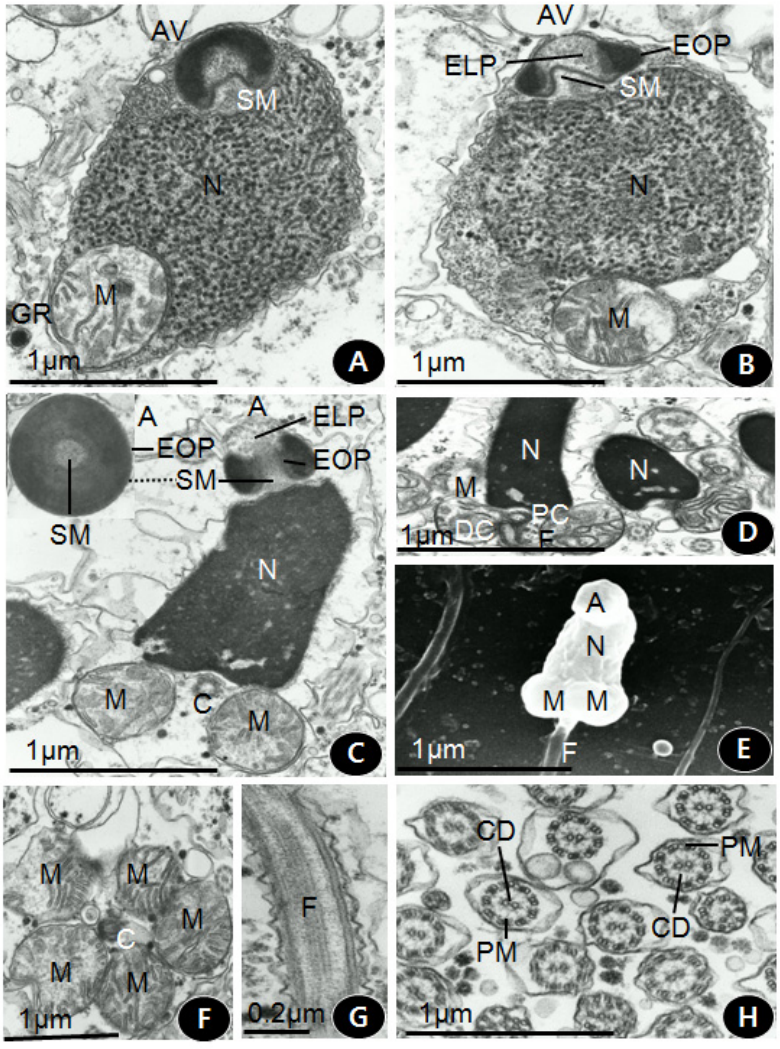

Fig. 2. Transmission / scanning electron micrographs of spermiogenesis and spermatozoa in male *Meretrix petechialis* (A-H). **A**, A spermatid (ST) in the late stage of spermatid during spermiogenesis. Note granules (GR), acrosomal vesicle (AV) and subacrosomal material just before the spermatid nucleus (N) of the spermatid and the mitochondria (M) near the posterior nuclear fossa; **B**, An acrosomal vesicle (AV) on granular spermatid nucleus (N). Note an acrosomal vesicle (AV) being composed of basal rings showing the electron dense opaque part (EOP), electron lucent part (ELP) and subacrosomal material (SM) on the spermatid nucleus; **C**, An acrosome on a sperm nucleus and the midpiece of the sperm. Note a modified acrosome consisting of the basal rings of acrosomal vesicle showing electron dense opaque part (EOP), electron lucent part (ELP) and subacrosomal material (SM) on the anterior nuclear fossa of the sperm nucleus (N) and the centrioles and large mitochondria (M) in the midpiece of the sperm. Cross sectioned an acrosome being compose of acrosomal vesicle showing e lectron dense opaque parts and subacrosomal material (SM); **D**, Sperm midpiece and a flagellum beneath the nucleus (N). Note the proximal centriole (PC) and distal centriole (DC) with spherical mitochondria (M) beneath the posterior nuclear fossa and a tail flagellum (F); **E**, Scanning electron micrograph. Longitudinal sectioned a sperm. A completed spermatozoon is composed of the head part including acrosome (A) and nucleus (N), the midpiece part, and the tail flagellum part (F); **F**, Cross sectioned part of the sperm midpiece. Note five mitochondria surrounding a pair of centrioles; **G**, **H**, Longitudinal section (G) and cross section (H) of the tail flagella of mature sperm. Note the axoneme showing a 9 + 2 structure (a pair of central doublets (CD) and nine pair of peripheral microtubules (PM)).

Spermatozoa: In the mature stage, the morphology of the spermatozoon has a primitive type and is similar to those of other bivalves. The spermatozoon of *M. petechialis* is approximately 47-50 μm in length including a slightly curved sperm nucleus (N) (about 1.50 μm in length), an acrosome (A) (about 0.56 μm in length) and tail flagellum (44-48 μm in length). At this time, several lacunae are found in the nucleus (Fig. E).

At this time, in particular, an acrosome on th nucleus is composed of the acrosomal vesicle (which comprises both right and left basal rings) and subacrosomal material (Figs. 2C, D). In the midpiece of the spermatozoon, this region consists of five equal-sized, spherical mitochondria surrounding a pair of triplet substructure centrioles (Fig. 2F). The cristae of each mitochondrion are randomly arranged., The initial portion of the flagellum attached to the plasma membrane (Fig. 2G). Transverse sections reveal that the triplets of the distal centriole posteriorly transform into doublet are continuos with the nine doublets of the flagellar axoneme. The flagellum is composed of a 9 + 2 substructure axoneme (that is, nine peripheral microtubules surrounding a pair of central doublets enclosed by the plasma membrane (Figs. 2G, H).

DISCUSSION

In the present study, morphology and the ultrastructure of spermatozoa during spermatogenesis in two species (*S. purpuratus* and *M. petechialis*) are similar to those of ther bivalves, In particular, spermiogenesis of two species also shows similar phenomena to those of other bivalves (Hodgson and Bernard, 1986; Eckelbarger*et al*., 1990; Healy and Lester, 1991; Chung *et al*., 1991; Eckelbarger and Davis, 1996; Chung and Ryou, 2000; Park *et al*., 2006; Chung *et al*., 2007; Kim *et al*., 2010 a,b,c,d)

Regarding the morphologies of the sperm nuclei in Veneridae species, Kim (2001) reported that the angles of the sperm nuclei in the family Veneridae ranged from 0° (*Notochione jedoensis*) to 80° (*Mercenaria stinpsoni*). In this study, the angles of the sperm nuclei of *S. purpurata* was slightly curved (15°), however, exceptionally, that of this species was smaller than those of *Dosinorbis japonicus* (45°) and *G. melanaegis* (9.54°). Thus, the morphologies of sperm nuclei vary with the species. Therefore, morphologies of the sperm nuclei can not be used for classifications of the species, genuses, families and subclasses as taxonomic tool.

Regarding the morphology of sperm acrosome, *S. purpuratus* and *M. petechialis* are the cap shape (Kim, 2001). Compare two species with the morphology of the acrosomal vesicles in species of other families, the morphological, phylogenetical characteristics of acrosomal vesicles in *S. purpurata* and *M. petechialis* are the cap shape during spermatogenesis. Therefore, we assume that the presence of a special acrosomal vesicle during spermatogenesis can be used as a key characteristic for identification of species of two genuses *Saxidomus* and *Meretrix*, as seen in the family Veneridae.

1. Taxonomic value of sperm morphology and ultrastructure

Recently, sperm ultrastructure of bivalves is considered a valuable tool in assessing taxonomic and phylogenetic problems within the Bivalvia (Franzén, 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.

From the results investigated, we have confirmed that the acrosomes can be distinguisable those of the genuses, families and subclasses (Pteriormorphia and Heterodonta) by the morphologies, positions and characteristics of the acrosomal vesicle. Family Veneridae belongs to the subclass Heterodonta.

According to Hodgson and Bernard (1986), in general, all species in the subclass Heterodonta in the bivalves have a common structural characteristics of the acrosomal vesicles showing the cap-like or cone-like in shape, being composed of electron high density (opaque) materials (right and left basal rings) and electron lucent materials (the apex part of the acrosomal vesicle).

In addition, Hodgson and Bernard, 1986 reported that the subclass Pteriomorphia in the bivalves have a common structural characteristics of the acrosomal vesicles showing the cone-like in shape, being composed of the electron high density (opaque) part in all parts.

In this study, two species (*S. purpurata* and *M. petechialis*) that belong to the family Veneridae in the subclass Heterodonta, have the acrosomal vesicles showing the cap in shape, being composed of electron high density (opaque) materials (right and left basal rings) and electron lucent materials (the apex part of acrosomal vesicle) as reported by Hodgson and Bernard (1986). Therefore, *S. purpurata* and *M. petechialis* in Veneridae belong to the subclass Heterodonta because our results coincided with the opinion of Hodgson and Bernard (1986).

Regarding the number of mitochondria in the midpiece of the sperms, Kim (2001) reported that the number of mitochondria in the midpiece of the sperms of the species in the family Veneridae are four in n most specie, while exceptionally, five in *S. purpurata*, *M. lusoria* and *C. sinensis*. Exceptionally, within one species, the number of mitochondria in the midpiece of the sperm show slight differences in number. Regarding the number of mitochondria in the sperm midpiece, Healy (1995) reported that the number of mitochondria tends to be stable within any given family or superfamily. Therefore, we agree with the opinion of Healy (1995).

ACKNOWLEDGEMENTS

The authors are grateful to Professor Emeritus Ee-Yung Chung, Kunsan National University, for helpful comments on the manuscript.

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