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Spermiogenesis and Taxonomical Values of Sperm Ultrastructures in Male *Mercenaria stimpsoni* (Heterodonta: Veneridae)

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ABSTRACT

Spermatid differentiations during spermiogenesis and sperm ultrastructures in male *Mercenaria stimpsoni* were investigated by transmission electron microscopic observations. In the early stage of the spermatid during spermiogenesis, a few granules and a proacrosomal granule, which is formed by the Golgi complex, become a proacrosomal vesicle. Consequently, it becomes an acrosome by way of the process of acrosome formation. The morphologies of the sperm nucleus type and the acrosome of this species have a curved cylindrical type and cap shape, respectively. The spermatozoon is approximately 48-51 μm in length including a curved cylinderical sperm nucleus (about 4.18 μm long), an acrosome (about 0.52 μm in length) and tail flagellum (42-45 μm long). As some ultrastructural characteristics of the acrosomal vesicle, the peripheral parts of two basal rings show electron opaque part (region), while the apex part of the acrosome shows electron lucent part (region). These charateristics of the sperm belong to the family Veneridae in the subclass Heterodonta, unlike 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 acrosome structures. Exceptionally, In particular, a cylinder-like nucleus of the sperm is curved (the angle of the nucleus is about 80°), as seen in some species of Veneridae (range from 0° to 80°). The number of mitochondria in the midpiece of the sperm of this species are four, as one of common characteristics appeared in most species except for a few species in Veneridae in the subclass Heterodonta. Cross-sectioned axoneme of the sperm tail flagellum shows a 9+2 structure.

Key words: *Mercenaria stimpsoni*, Spermiogenesis, Sperm Ultrastructures, Heterodonta

INTRODUCTION

To date, many studies on spermatid differentiations during spermiogenesis and mature sperm ultrastructures have been documented to varying degrees in many species of bivalves using electron microscopy (Dorange and Le Pennec, 1989; Healy, 1989, 1995; Gaulejac, 1995; Chung and Ryou, 2000; Chung *et al*., 2005, 2006, 2007; 2010; Chung, 2006).

Previously there have been afew report on aspect of reproduction including reproduction and spermatogenesis (Kim, 2001). To date, little information is available on spermatid differentiation during spermiogenesis and mature sperm morphology and ultrastructures of *M. stimpsoni*. In the Mollusca, sperm ultrastructure is considered a valuable tool in assessing taxonomic and phylogenetic problems within the Bivalves (Franzen, 1970, 1983; Daniels *et al*., 1971; Popham, 1979; Healy, 1989, 1995; Koike, 1985; Hodgson and Bernard, 1986; Eckelbarger *et al*., 1990; Eckelbarger and Davis, 1996; Kim *et al*., 2010; Chung *et al*., 2010) and is especially

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useful when comparing closely related species (Popham *et al*., 1974; Popham, 1979).

In particular, it is well-known that acrosomal morphologies and ultrastructures of sperms have been used to organize bivalve subclasses (Popham, 1979), the number of mitochondria in the sperm midpiece tends to be stable within any given family or superfamily. For that reason, it needs to study acrosomal morphology of the sperm and the number of mitochondria in the sperm midpiece for taxonomic analyses of this specie with many species in the families or superfamilies in the subclass Hetrodonta.

Therefore, the main aim of the present study is to describe the ultrastructural characteristics of spermatids by developmental stages, and to confirm sperm type with sperm ultrastructure by phylogenetic analysis of *Mercenaria stimpsoni* in Veneridae in the subclass Heterodonta.

MATERIALS AND METHODS

1. Sampling

A total of 75 male specimens of *M. stimpsoni* were collected monthly in the subtidal zones of coastal waters of Daejin, Gosung-gun, Korea, from January to December, 2007. The specimens were transported to the laboratory where they were maintained in seawater at 20°C. The specimens were used for ultrastructural study of germ cells and mature spermatozoa by transmission electron microscopy.

2. Transmission electron microscope observation

For transmission electron microscope observations, excised pieces of the gonads were cut into small pieces and fixed immediately in 2.5% paraform-aldehydeglutaraldehyde in 0.1 M phosphate buffer solution (pH 7.4) for 2 hours at 4°C. 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°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. Ultrastructure of spermatids and spermatozoa during spermiogenesis

Based on the testicular development and morphological characteristics of germ cells by electron microscopic observations, in general. spermatogenesis occurs in the acini of the testis, and for convenience, the developmental stage of germ cells during spermiogenesis can be divided into two stages: (1) spermatids, and (2) spermatozoa. In general, the process of spermiogenesis of this species appears to be similar to those of other bivalve species.

Spermatids: For convenience, spermiogenesis of spermatid has been divided arbitrarily into two stages: the early and late stages of spermatids.

In the early stage of spermatids during spermiogenesis, spermatids are oval in shape and range from approximately 3-4 μm in diameter. Their round nuclei (3.0-4.0 μm in diameter) contain scattered marginal heterochromatin and several mitochondria appear in the cytoplasm of the spermatid. The morphology of the spermatid nucleus changes gradually during the differentiation of the spermatid. 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. At this time, the morphologies of the spermatid nuclei were gradually elongated, and one or a few granules appear on the nucleus of spermatid (Fig. 1A). Thereafter, they become a proacrosomal vesicle on the nucleus (Fig. 1B). In the late stage of spermatids during spermiogenesis, spermatids The acrosomal vesicle migrates to the presumptive anterior end of the spermatid nucleus, where, they coalesce to form a single electron-dense vesicle (Fig. 1C). A cone-like proacrosomal vesicle gradually becomes a round

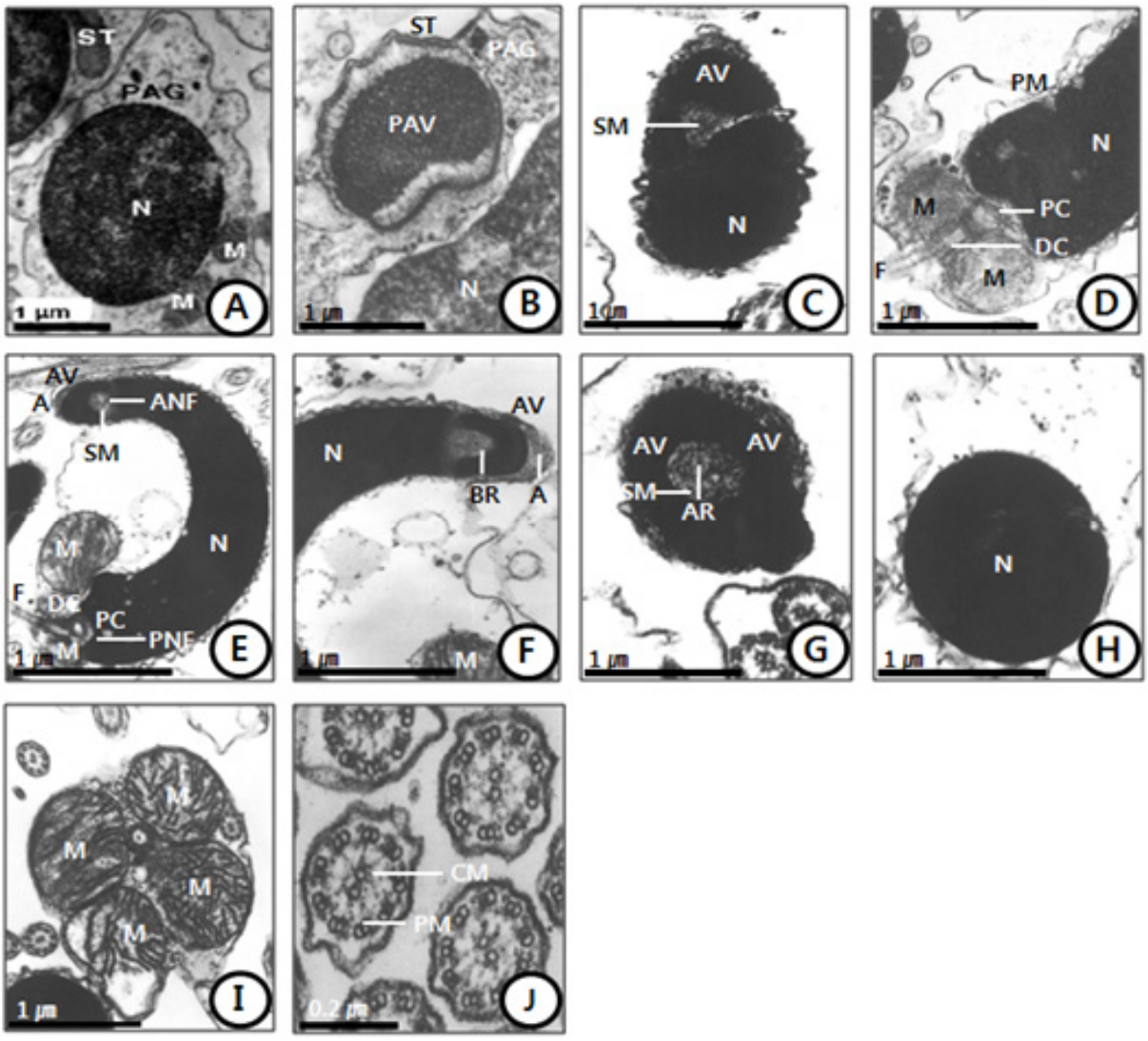

Fig. 1. Electron micrographs of spermiogenesis and completed spermatozoon in male *Mercenaria stimpsoni* (**A**-**J**). **A**, Spermatids (ST) in the early stage of spermiogenesis. Note granules, proacrosomal granules (PAG) on the nucleus (N) and mitochondria of the spermatid; **B**, A spermatid (ST) in the same stage of spermiogenesis. Note a proacrosomal vesicle (PAV) on the nucleus (N) and mitochondria (M) in the early stage of .the spermatid; **C**, Acrosomal vesicle and the nucleus of a spermatid (ST) in the late stage of spermiogenesis. Note a acrosomal vesicle (AV) and subacrosomal materials (SM) on the anterior nuclear fossa of the elongated nucleus (N); **D**, An elongated nucleus (N) with of plasma membrane of a spermatid (ST) in the late stage of spermiogenesis; Note proximal centriole (PC) and distal centriole (DC) are surrounded with two spherical mitochondria in the sperm midpiece and a tail flagellum (F); **E**, A completed spermatozoon (SZ) with an acrosome (A), curved sperm nucleus (N), the sperm midpiece and a flagellum (F). Note an acrosomal vesicle (AV) of the acrosome being composed of electron dense part and electron lucent part subacrosomal materials (SM), the anterior (ANF) and posterior nuclear fossas (PNF), the proximal (PC) and distal centrioles (DC), two spherical mitochondria (M), and a flagellum (F); **F**, The head part of the completed sperm in the spermatozoon stage. Note the acrosomal vesical (AV) (or acrosome (A)) being compose of right and left basal rings (high electron dense opaque part (materials) and the appex part (electron lucent part (materials); **G**, A cross sectioned sperm head part (the acrosomal vesical and the nucleus). Note a axial rod in the subacrosomal materials (SM); **H**, A cross sectioned sperm head part (the nucleus). Note the nucleus of the sperm head part; **I**., A cross sectioned sperm midpiece. Note a pair of centrioles (C) surrounding with 4 mitochondria (M); **J**, A cross sectioned sperm tail flagellum. Note the axoneme of the sperm tail flagellum showing a 9+2 structure (nine peripheral microtubules (PM) and a pair of central microtubules (CM)).

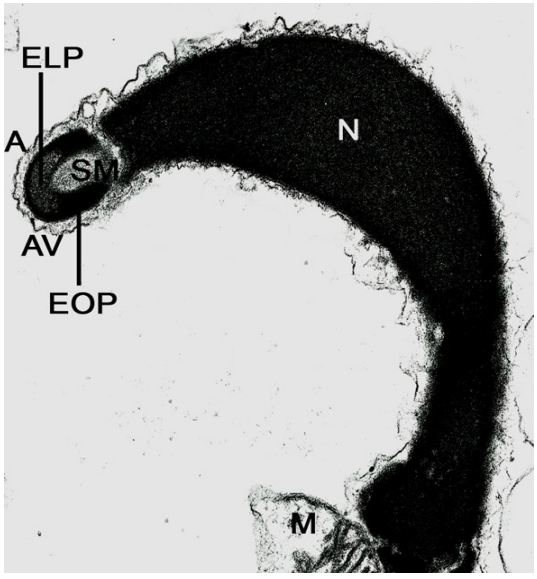

Fig. 2. A diagram of an acrosomal vesicle being composed of electron opaque part (EOP) and electron lucent part (ELP) of an acrosome on the anterior nuclear fossa of the curved nucleus in male *Phacosoma japoni* (Kim *et al*., 2011).

acrosome. Thereafter, a proacrosome lying on the sperm nucleus becomes round cone in shape, and several mitochondria are reduced its number at the posterior part of the nucleus. However, the nucleus of the spermatid is slightly curved (Fig. 1D). Thereafter, A round acrosomal vesicle lied on the anterior nuclear fossa.

Spermatozoa: Particularly, in the spermatozoon stage especially, two large mitochondria at the midpiece part appear beneath the nucleus (Fig. 1E).

At this stage, the acrosomal vesicle of spermatozoa is completed and the acrosomal vesicle is composed of electron dense part (region) and electron lucent part (region): especially, the basal and lateral parts of the basal rings of the acrosomal vesicle show electron dense part (region), while the anterior apex part of the acrosomal vesicle shows electron lucent part (Fig . 1F).

The axial filaments are not found in the acrosomal lumen, while subacrosomal granular materials are present in the subacrosomal space between the anterior nuclear fossa and the acrosomal vesicle of the acrosome. At this time, the acrosomal vesicle is composed of well developing basal rings, and the curved nucleus (angle of the nucleus: 80°) is covered with the plasma membrane. The anterior nuclear fossa exist in front of an acrosomal vesicle and the posterior centriole with some spherical mitochondria in the sperm midpiece.(Fig. 1I)

At this time, of the two centrioles at right angles, show the classic nine triplets of microtubles. The proximal one lies in the posterior nuclear fossa and is perpendicular to the axoneme (Fig. 1E). It appears unconnected to the nuclear envelope. The distal centriole occupying the basal portion of the flagellum constitutes the basal rings of the flagellum. The cytoplasm is greatly reduced, and so the nucleus-cytoplasm is high (Fig.1J).

As shown in Fig. 2, Kim *et al*. (2011) reported that complete sperm morphology of *Phacosoma Japonicus* in Verneridae in the subclass Heterodonta showed a curved cylindrical (angle of the nucleus is 45°) sperm nucleus, as found in several Veneridae species. As some characteristics of the acrosomal vesicle structures in an acrosome of *P. japonicus*, the basal and lateral parts of the basal rings show electron dense part (region), while the rings show electron dense part (region), while the anterior apex part of the acrosomal vesicle shows electron lucent part (region). These characteristics of the acrosomal vesicle were found in Veneridae in the subclass Heterodonta (Fig. 2). Therefore, sperm ultrastructures and morphologies of *P. japonicus* is very simila with those of *M. stimpsoni* (Fig. 3).

As shown in Fig. 3, mature spermatozoa of *M. stimpsoni* measure approximately 48-51 μm long, and consist of cap-like acrosome positioned at the top of an elongated nucleus, a pair of centrioles surrounded by a ring of four spherical mitochondria, and a flagellum. The morphology of the sperm nuclear type and the acrosomal shape of this species are of a deep bend cylindrical type and a cap shape, respectively. The sperm head is about 5.15 μm long and comprises a long, electron-dense nucleus (about 4.18 μm long), with a anterior nuclear fossa and an acrosome. The acrosomal vesicle is about 0.52 μm long membrane-bound, and deeply invaginated. The acrosomal vesicle is a cap shape. The morphology of

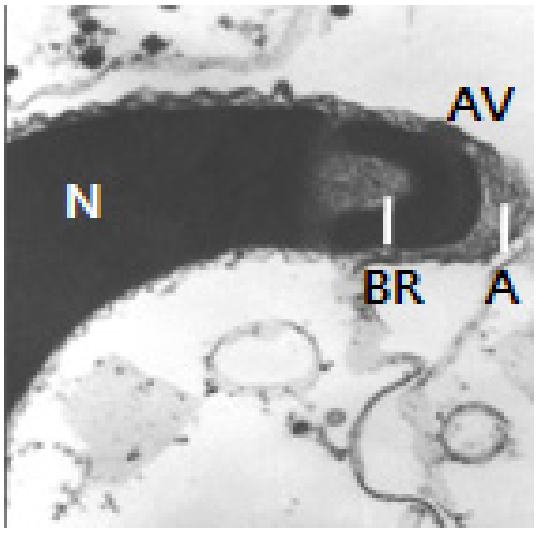

Fig. 3. A diagram of an acrosomal vesicle being composed of electron opque part and electron lucent part of an acro some on the anterior nuclear fossa of the curved nucleus in male *Mercenaria stimpsoni*.

the sperm nucleus and the acrosomes of this species are of a curved cylindrical type (angle of the nucleus is 80°) and a cap shape, respectively (Fig. 1E). The proximal centriole and distal centriole surrounded with four spherical mitochondria with well-defined cristae appear in the sperm midpiece (Fig. 1I). However, the axial filaments or an axial rod and satellite fibers are not found in the ultrastructure of mature spermatozoa of this species. And the tail flagellum is approximately 42-45 μm in length. A cross-sectioned tail flagellum shows that the axoneme is composed of a classic 9+2 microtubular substructure (nine pairs of peripheral microtubules at the periphery surrounding a pair of central doublets at the center) enclosed by a plasma membrane (Fig. 1J).

DISCUSSION

1. Spermatogenesis

In general, the testis of bivalves is composed of a number of acini, and spermatogenesis occurs in acini of the testis. The processes of germ cell differentiations and mature sperm ultrastucture of *M. stimsoni*, were similar to those in the species in bivalves (Eckbarger and Davis, 1996; Chung *et al*., 2010). Therefore, the processes of spermatogenesis of bivalves was similar to those in the species in bivalves (Sakker, 1984; Bernard and Hodgson, 1985; Chung, 2006; Chung *et al*., 2007, Kim *et al*., 2010 a,b).

Yasuzumi (1974) reported that the nuclei of spermatids can be classified into three morphological types according to the concentration morphology and degree of chromatins in the nuclei of spermatids: (1) granular, (2) fibrous, and (3) layer types. In general, in case of bivalves, it was a granular type in the early stage of chromatin concentrations, however, as the concentration of chromatins progresses, it was changed to be the fibrous type.

In particular, the morphologies of the sperm nuclei, the types of the sperm nuclei are of the cylinder type in *M. lusoria*, *C. sinensis* and Notochione jedoensis, the long cylinder type in *Gomphina veneriformis*, and the curved cylinder type in *Ruditapes philippinarum*, *Saxidomus purpuratus*, *Phacosoma Japonicus* and *M. stimpsoni* (Kim, 2001). In this study, the type of the sperm nucleus of *M. arenaria oonogai* belongs to the curved cylinder type.

This species belongs to external fertilization species and the type of spermatozoon is the primitive type. In particular, Kim (2001) reported that several species of Veneridae in subclass Heterodonta have three kinds of sperm nuclear types according to the angle of curved sperm nucleus: (1) no curved nuclear type (angle of the nucleus: 0°), (2) slightly curved sperm nuclear type (angle of the curved sperm nucleus: 5°-10°), and (3) largely curved sperm nuclear type (angle of the curved nucleus: 15°-80°). He reported that the angles of the sperm nucleus of the species in family Veneridae ranged from 0° (*Notochione jedoensis*) to 80° (*M. stinpsoni*).

Therefore, exceptionally, of the species in families in subclass Heterodonta, it is assumed that only Myidae and Veneridae species have the curved sperm nucleus.

In this study, the size of sperm nucleus of this species in Veneridae was 4.18 μm long. Kim (2001) reported that the sizes of sperm nuclei in Veneridae species ranged from 2.13 μm (*C. sinensis*) to 7.80 μm in diameter (*G. veneriformis*). Therefore, he described that the morphologies of the sperm nucleus can not be used in taxonomic analyses because of irregular morphological characteristics of the nuclei (Healy, 1995).

It is well-known that the acrosome is formed by various granular secreations secreted by the Golgi complex (Longo and Dornfeld, 1967; Sastry, 1979). In *Mytilus coruscus*, several small proacrosomal vesicle are formed by the Golgi complexes, and these vesicles were mixed with each other in the acrosomal vesicle ckelbarger and Davis (1996) reported that commonly proacrosomal vesicles were found in C. virginica spermatocytes. In this study, however, in *M. stimpsoni* a proacrosomal vesicle appeared spermatid stage and this vesicle developed to an acrosomal vesicle, and became an acrosome. The mechanism of acrosomal vesicle formation in mollusc sperm are diverse and that no single mechanism characterizes bivalve sperm. during spermiogenesis (Kim *et al*., 2010a).

2. Taxonomic values in mature sperm morphology ultrastructure

Sperm ultrastructure of bivalves is considered a valuable tool in assessing taxonomic and phylogenetic problems within the bivalvia (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. Of sperm ultrastructures of bivalves, acrosomal morphology are now widely used in taxonomic analyses (Healy, 1995) because its morphological characteristics has been used to organize bivalve subclasses (Popham, 1979). Regarding acrosomal morphologies, Healy (1989) reported that different subclasses of bivalves each have unique acrosomal morphologies.

According to the acrosomal morphologies of the sperms, Kim (2001) reported that the acrosome can be classified into four shapes (cone, long cone, modified cone, cap, modified cap shape). The acrosomal morphologies of the sperms in Veneridae were the cone shapes in *C. sinensis* and *P. japonicus*, and a modified cone shape in *R. philippinarum*, and the cap shapes in *Saxidomus ponicus*, *M. lusoria*, *Mercenaria stinpdoni*, *Notochione jedoensis*. In this study, the acrosomal morphology of this species in Vernaridae has a cap shape, as seen in the family Veneridae.

In particular, regarding a common structural characteristics of the acrosomal vesicles of the subclasses Heterodonta and Pteriomorphia in the bivalves, Hodgson and Bernard (1986) reported that all species in subclass Heterodonta in the bivalves have a common structural characteristics of the acrosomal vesicles showing the cap shape, and being composed of electron high density (opaque) material and electron lucent material. Hodgson and Bernard (1986) described that 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. However, the subclass Pteriomorphia in the bivalves have a common structural characteristics of the acrosomal vesicles showing the cap shape, and being composed of electron high density (opaque) materials in all parts (Hodgson and Bernard, 1986). The acrosomal vesicles of this species is composed of electron high density (opaque) part (from the basal ring part to the lateral parts) and electron lucent part (the apex part (region)) as reported by Hodgson and Bernard (1986). Therefore, from morphologies of the acrosomal vesicles of the acrosome mentioned above, this species belongs to subclass Heterodonta reported by Hodgson and Bernard (1986).

Accordingly, these phenomena of this species showed remarkably similar patterns to various kinds of species of Mactridae, Veneridae, Tellinidae, Solenidae, Hiatellidae in subclass Heterodonta.

Regarding the number of mitochondria in the sperm midpiece, some authors (Hodgson and Bernard, 1986; Healy, 1989) stated that the number of mitochondria in the sperm midpiece tends to be stale within any family or superfamily varying from a maximum of 14 in the mytilord *Modiolus difficilis* (Drozdov and Reunov, 1986) to a minimum of 4 (Healy, 1989, 1995).

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