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Light and electron microscopical characteristics of *Perkinsus* sp. from Manila clam, *Ruditapes philippinarum*, in Korea

Kyoung Jin Ahn, Sung-Hoi Huh[1] and Ki Hong Kim*

Department of Aquatic Life Medicine, College of Fisheries Sciences and
[1]Department of Oceanography, College of Marine Scinces and Technology,
Pukyong National University, Pusan 608-737, Korea

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Light and electron microscopical characteristics of *Perkinsus* sp. parasitizing in Manila clam, *Ruditapes philippinarum*, in Korea were investigated. Trophozoite within the tissue was spherical or ovoid and ranged 2.5~10.5 μm (mean=6.2 μm) in diameter. Trophozoite had a nucleus with a prominent nucleolus and a large cytoplasmic vacuole within the cytoplasm. Single trophozoite was phagocytozed by host hemocyte and cluster cells were encapusulated by hemocytes aggregation within the host tissues. Hypnospores incubated in thioglycollate medium (FTM) for 1 to 15 days were also spherical or ovoid and ranged 10~132 μm (mean±S.D.: 44.25±7.91 μm) in diameter. Zoospores were spherical or ovoid, had a nucleus and two flagella. Zoospores contained apical complex, which consisted of conoid, subpellicular microtubules, rhoptries and rectilinear micronemes.

Key words: *Perkinsus* sp., *Ruditapes philippinarum*, morphological characteristics, light microscopy, electron microscopy

Introduction

Manila clam, *Ruditapes philippinarum*, is a commercially important shellfish species in Korea. Recently, the production of Manila clam has declined, and it become an economic concern. Increase of environmental pollution, reclamation of slime along the bank of inlets, and shortage of seedlings have contributed to the decline of Manila clam production, but a significant proportion might be attributed to diseases. Various pathogens, including rickettiae, bacteria and parasites have been recorded from cultured clams in the world. In Korea, however, little information is available on the diseases of Manila clam.

Recently, unidentified *Perkinsus* species infection in Manila clam (*Tapes philippinarum*) was reported from Korea (Choi and Park, 1997) and Japan (Maeno et al., 1999). Moreover, transplantations of Manila clam throughout European countries have resulted in the introduction of *Perkinsus* sp. to most clam culture areas of those countries (Cigarría et al., 1997).

Perkinsus species are apicomplexan parasites of shellfish, and have been associated with mass mortalities of commercially important molluscs (Mackin et al., 1950; Andrew, 1988; Bower et al., 1994). Five species of this genus *Perkinsus marinus*, *P. olseni*, *P. karlssoni*, *P. qugwadi* and *P. atlanticus* have been described parasitizing molluscs from various parts of the world (Mackin et al., 1950; Lester and Davis, 1981; McGladdery et al., 1991; Blackbourn et al., 1998; Azevedo, 1989).

The life cycle of *Perkinsus* consists of a pathogenic trophozoite stage that proliferates in molluscan tissues primarily by schizogony. Mature trophozoites enlarge to become prezoosporangia, which upon entering the water column sporulate to release large numbers of biflagellated zoospores. These motile zoospores presumably give rise to trophozoites, which can be detected in most host tissues including the hemocytes. These life stages of *Perkinsus* species have been extensively studied using light (McLaughlin and Faisal, 1998; García-Valero et al., 1996; Azevedo et al., 1989; Auzoux-Bordenave et al., 1995; Maeno, 1999) and electron microscopy (Perkins, 1976; Azevedo, 1989a and 1989b;

*Corresponding author: khkim@dolphin.pknu.ac.kr

McGladdery et al., 1991; Blackbourn et al., 1998). Morphological characteristics based on those observations have been used to classify *Perkinsus* spp. occurred in various molluscan species. However, there are little investigations on the morphological characteristics of each life stage of *Perkinsus* sp. parasitizing in Manila clam cultured in Korea. Therefore, in the present study, the general morphology and ultrastructures of *Perkinsus* sp. at each life stage were investigated with light and electron microscopes, and were compared with the other recorded *Perkinsus* species.

Materials and Methods

Diagnosis and culture of *Perkinsus* sp. using fluid thioglycollate medium (FTM)

The presence of *Perkinsus* sp. in Manila clam was determined by fluid thioglycollate medium (FTM) method. FTM was prepared by mixing 20 g of NaCl and 30 g of dehydrate fluid thioglycollate media in 1 L distilled water. The mixture was heated until the solution became transparent golden-yellow color. After cooling, 30 mL of the solution poured into a 50 mL culture tube, then was autoclaved and sealed with parafilm to keep anaerobic condition. The tubes were kept in dark until used. Just before culturing the tissues of Manila clam in this medium, antibiotics (penicillin G and streptomycin sulfate, Sigma) and antifungal agent (Nystantin or Mycostatin, Sigma) were added at 200 U/mL for each agent. The FTM culture tubes containing tissues of clam were incubated at 30℃ for 4~5 days, then, the presence of hypnospores were examined under a phase contrast microscope.

Artificial seawater

The composition of artificial seawater used in the present experiments was as follows; sodium hydrochloride 27.5 g was dissolved completely in 1 L distilled water, then, magnesium chloride 5 g, magnesium sulfate 2 g, potassium chloride 1 g, calcium chloride 0.5 g, and ferrous sulfate 0.001 g were serially added. The mixture was autoclaved and filtered with 0.22 μm filter paper and adjusted to 30 ‰.

Preparation of trophozoite for light microscope

Manila clams collected from southern coastal areas in Korea were dissected in the laboratory, and nodules that were macroscopically observed on the diverse organs including gills, mantle, digestive gland and foot of heavily infected clams, were biopsied with scalpels. The biopsies were squashed on the slide and the presence of trophozoites was examined under the light microscope. In histological sections, the tissues containing nodules were fixed in Davidson's fixative, embedded in paraffin, and sections were stained with Harris' hematoxylin and eosin.

Isolation of trophozoites

In order to get pure isolates of trophozoites from infected clam tissues, the following method (Chu et al., 1994) was used; visceral mass of clam was excised and minced by razor blender for 1 min in artificial seawater containing antibiotics and antifungal agents (each of penicillin G, streptomycin sulfate, and Nystantin at 100 U/mL). The suspension was maintained at 4℃ for 24 hours, then, supernatant was gently aspirated with a pasteur pipette. Subsequently, the supernatant was serially filtered using 40, 20, 10 μm millipore gauze. The filtrate was centrifuged at 50 g for 10 min in order to pellet any remaining clam cells and tissue fragments. Then, the supernatant was centrifuged at 800 g for 20 min 3~4 times to settle the trophozoites on bottom. The pellet was resuspended in 30 ‰ artificial seawater and washed 3~4 times to reduce the number of bacteria. Trophozoites were stained with neutral red, and their sizes were measured under the light microscope with an ocular micrometer.

Hypnospore

Whole tissue of clam was incubated in FTM for 4~5 days at 30℃ in dark condition. After incubation, the tissue was centrifuged 3~4 times at 4000 rpm for 10 min. The pellets were washed at the same speed 3~4 times with artificial seawater containing antibiotics (200 U/mL penicillin G and streptomycin sulfate) and antifungal agent (Nystantin 200 U/mL), then, filtrated with gauze. To know the sizes of hypnospores according to incubation time, the tissues of clams that had been incubated at 30℃ from 1 to 15 days in FTM were treated everyday as above method, and the sizes of the hypnospores were measured under the light microscope with an ocular micrometer.

Preparation of trophozoite and hypnospore for transmission electron microscope (TEM)

For preparation of TEM, small pieces of gill showing small nodules and hypnospores obtained by FTM culture were fixed in 2.5% glutaraldehyde in 0.1 m phosphate buffer, pH 7.4, for 1 hour at 4℃. Specimens were washed in the same buffer and post-fixed in buffered 1% osmium tetroxide for 2 hours at the same temperature. After a wash in the same buffer, dehydration in a graded series of alcohol solution was carried out at room temperature, and specimens were embedded in low viscosity epoxy resin (Epon 812). Semithin sections made using a microtome (Reichert-Jung 820, Leica) were stained with toluidine blue. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined by a transmission electron microscope (TEM, 1200EX-II, JEOL, Japan).

Results and Discussion

Gross and histological observations

Clams heavily infected with *Perkinsus* sp. gaped valves, and showed many small milky-white nodules on various tissues including gill, gonad, mantle, and foot. Trophozoites masses were observed within the nodules (Fig. 1). Similar characteristics have been reported in *P. atlanticus* infecting *Ruditapes decussatus* (Azevedo, 1989; Almeida et al., 1999), *R. semidecussatus* (Azevedo, 1989; Sagristà et al., 1995). Abalones (*Haliotis rubber*) infected with *P. olseni* also showed soft cream or brown swellings on tissues (Lester and Davis, 1981).

In histological observations, trophozoites masses were encapsulated by aggregated hemocytes of host or surrounded by amorphous eosinophilic materials originated from the host (Fig. 2.1~2.2). Sometimes single parasite cell was observed within a hemocyte. This inflammation-like response of the host against *Perkinsus* sp. infection was also reported in other bivalves infected with other *Perkinsus* species (Mackin, 1951; McGladdery et al., 1991; Whyte et al., 1994; Sagristà et al., 1995; McLaughlin and Faisal, 1998).

Characteristics of trophozoite stage

Trophozoites were spherical or ovoid, contained an eccentric nucleus with a prominent nucleolus and a large vacuole (Fig. 2.3). Trophozoites were ranged 2.5~10.5 μm (mean=6.2 μm) in diameter

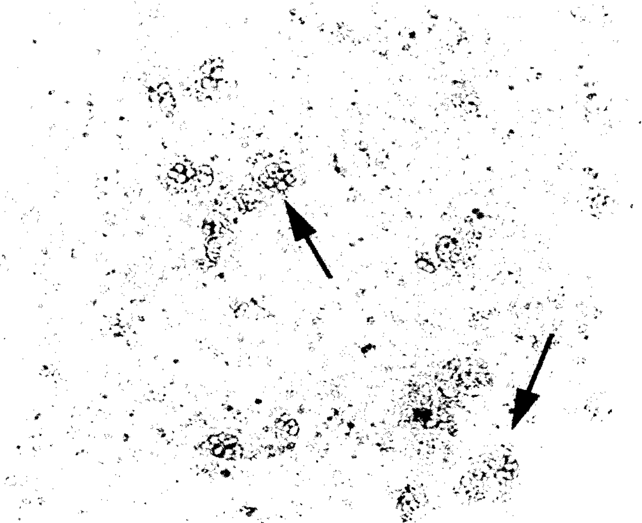

Fig. 1. Biopsied gill tissue. Most of trophozoites were clustered each other, and showed rosette-form (arrows).

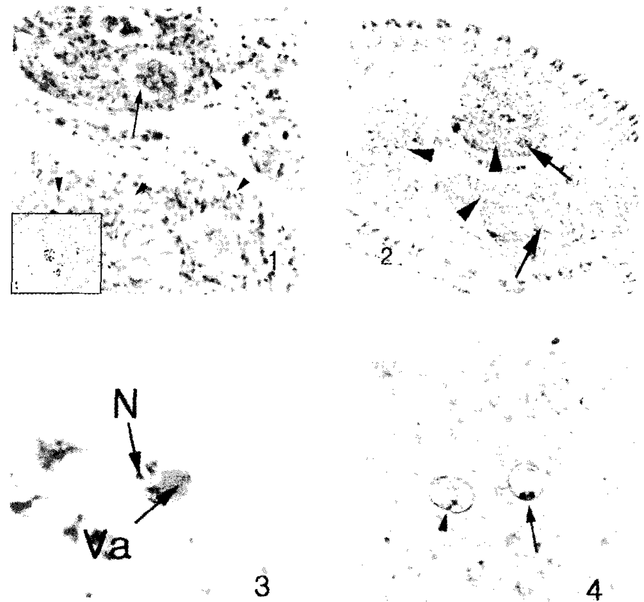

Fig. 2. Trophozoites of gill tissue in heavily infected clams. H & E stain. 2.1. Trophozoites (arrow) were surrounded by infiltiated hemocytes (arrow head). Isolated trophozoites from host tissue were indicated in inset. Inflammation-like signs were observed in surroundings of trophozoites. 2.2. Trophozoites masses (arrow) were surrounded by eosinophilic hemocytes (arrow head) of host. 2.3. Trophozoites stained with neutral red. N; Nucleus, V; Vacuole. 2.4. Dividing trophozoites by binary fission (arrow).

and observed as a single cell or cluster cells. Single cells were signet ring-like shaped, and cluster cells were rosette-shaped. The viability of trophozoites could be confirmed by the uptake of neutral red. The vacuole of live trophozoites took up neutral red and colored a pale red or pink (Fig. 2.3). The divisions of trophozoites by binary fission were observed (Fig. 2.4).

In the ultrastructural level, trophozoite had an eccentric nucleus with a prominent nucleolus, a large vacuole, and several lipid droplets (Fig. 3.1). In semithin and ultrathin sections of the trophozoites, the cleaving of the trophozoites by binary fission was clearly observed and daughter trophozoites originated from a same mother trophozoite were showed asynchronous division pattern (Fig. 3.2).

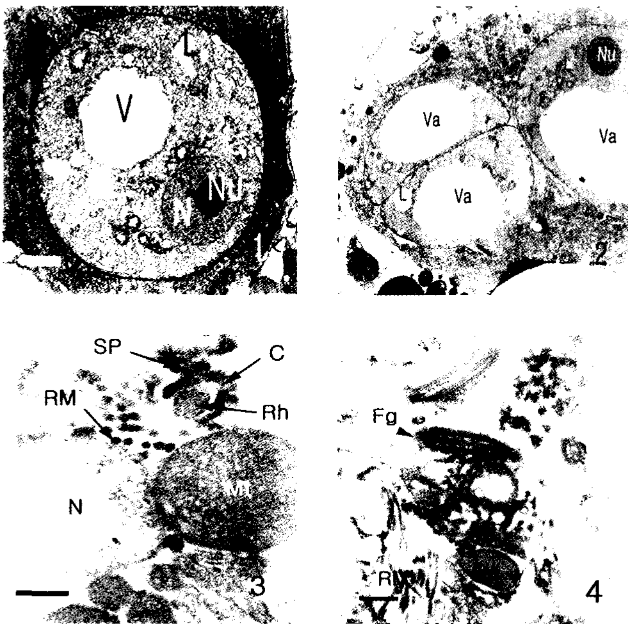

Fig. 3. Ultrastructural characteristics of trophozoite and zoospore. 3.1. Trophozoite had an eccentric nucleus (N) with a prominent nucleolus (Nu), a large vacuole (V) and several lipid droplets (L). A trophozoite phagocytosed by host hemocyte. Scale bar=1 μm. 3.2. Dividing trophozoites. Scale bar=1 μm. 3.3. Ultrastructure of zoospore. Rhoptry (Rh) was observed inside of conoid (C). Subpellicular microtubule (SP) surrounded periphery of conoid. Retilinear microneme (RM) was observed just beneath the conoid. N; nucleus, Mt; mitochondria. Scale bar=2 μm. 3.4. Flagella (Fg) and rectilinear micronemes (RM) were observed. Scale bar=2 μm.

Characteristics of hypnospore stage

The hypnospores were spherical or ovoid and ranged 10~132 μm (44.25±7.91 μm) in diameter. The sizes of hypnospores gradually increased according to the increase of incubation time in FTM, and reached the maximum size at 12 days post-incubation (Fig. 4). The hypnospores had a very refringent characteristics, and were seemed healthy when the hypnospore were incubated in FTM for 4~5 days, and stained black or blue-dark color by Lugol's iodine.

Hypnospores of other *Perkinsus* spp. were also spherical or ovoid in shape (Azevedo, 1989; McGladdery et al., 1991; Auzoux-Bordenave et al., 1995; Perkins, 1996), and contained numerous eccentric droplets (Lester and Davis, 1981). Trophozoites of all recorded *Perkinsus* species except *P. qugwadi* enlarged in size and transformed into hypnospores when tissue containing trophozoites placed into FTM (Lester and Davis, 1981; Navas et al., 1995; McLaughlin et al., 1999; Almeida et al., 1999).

Zoosporulation of hypnospores was occurred when the hypnospores were placed in the 30‰ artificial seawater at 30℃ (Fig. 5). The hypnospores attached on the bottom of the well plates and underwent further development. In 24~48 hours, cytoplasm of most hypnospores was shrunken (Fig. 5.1), then, a discharge pore or a discharge tube was formed on the side of the hypnospore wall (Fig. 5.2). Sub-

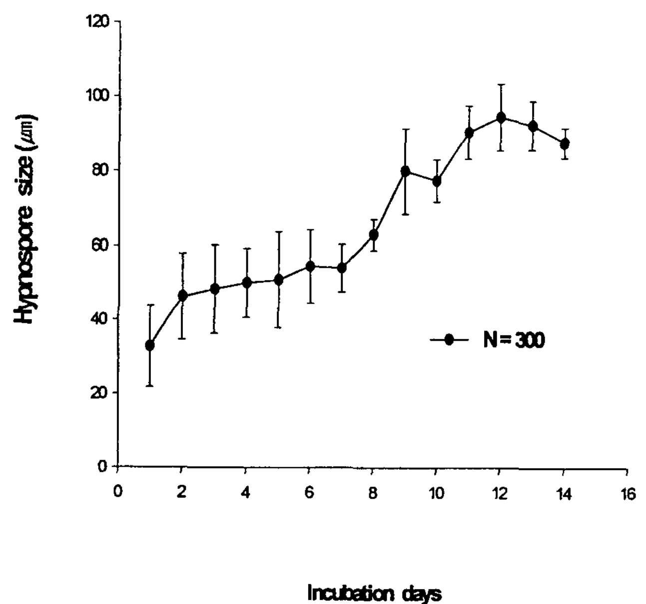

Fig. 4. Increase of hypnospore sizes according to FTM incubation period.

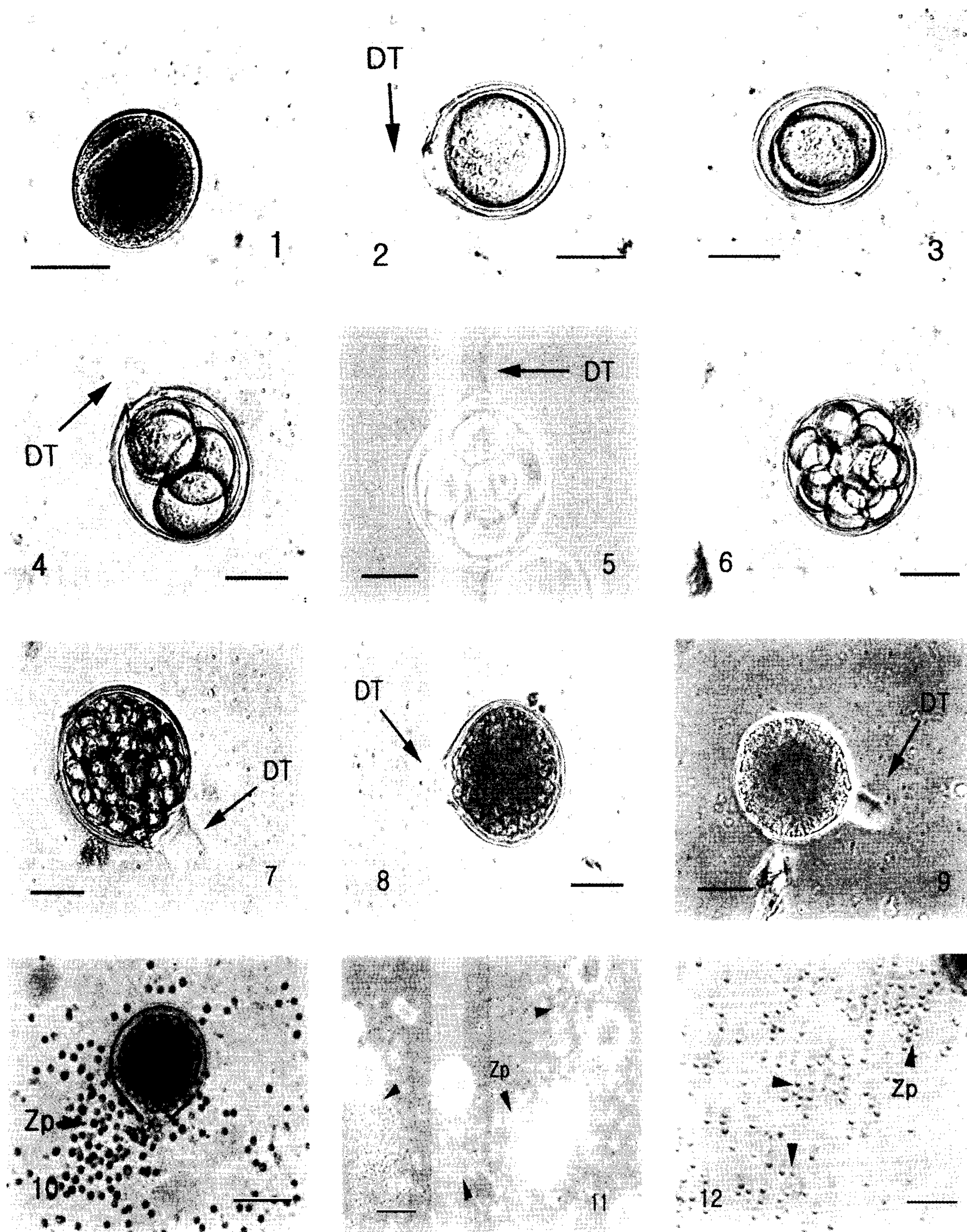

Fig. 5. Development of *Perkinsus* sp. hypnospore. DT, discharge tube. Scale bar=30 μm. 5.1. Prominently eccentric cytoplasm at early stage. 5.2. Shrinkage of cytoplasm and isolation from cell wall, and discharge tube (arrow) was observed 1~2 days after exposure 30‰ artificial seawater at 30℃. 5.3. 2-cell stage by bipartition of cytoplasm. 5.4. 4-cell stage of hypnospores. 5.5~5.8. Developmental stage (8~128 cell) of hypnospore 5.9. Hundreds of motile biflagellated zoospores were observed within hypnospore. 5.10. Motile biflagellated zoospores (arrow head) were released into seawater through discharge tube. Zp; zoospore. Fig. 5.11. Biflagellalted zoospores (arrow head) swam freely and actively. 5.12. Freely swimming zoospores were observed in artificial sea water.

sequently, the hypnospores that were undergone central invagination of cytoplasm as like mitosis of animal cell were divided into 2 cells (Fig. 5.3). The divisions of the cell showed unique pattern; the two cells, which were dividing to the next 4-cell stage, crossed each other, then, produced independent 4 cells (Fig. 5.4). The subsequent divisions of the hypnospore after 4 cell stage was occurred as the same strategy above (Fig. 5.5~5.8). About 30~40 minutes were needed for dividing to each next stage.

In 72~96 hours, small, numerous and actively moving zoospores were observed within the hypnopspore (Fig. 5.9), and in a few hours from this stage, zoospores were released into ambient seawater through discharge tube of hypnospore (Fig. 5.10). A hundreds of zoospores left mother cell (hypnospore) in a few min and they swam very actively in seawater (Fig. 5.11~5.12).

In semithin sections, development of hypnospores was performed by successive bipartition of protoplast, in which karyokinesis was followed by cytokinesis (Fig. 6). In the early stage of development, almost of the dividing cells within the hypnospores were irregular-shaped, but most of cells in the later in the later developmental stages gradually became regular and spherical in shape. The uninucleated cells within the hypnospore contained 2~4 large vacuoles, and sometimes had 1~2 electron dense vacuoplasts and lipid droplets.

Characteristics of zoospores

Zoospores were spherical or ovoid, measuring about 2 μm (n=20). Zoospores had an eccentric nucleus at the posterior end, and tapered at the anterior end, and possessed two flagella.

In ultrastructural observation, zoospores possessed an apical complex similar to that seen in apicomplexans. The apical complex consisted of a conoid, subpellicular microtublues, rhoptries and rectilinear micronemes (Fig. 3.3). A nucleus and two flagella were observed (Fig. 3.3~3.4).

The presence of apical complex and two flagella in zoospores has been demonstrated also in other *Perkinsus* species by ultrastructural studies (Perkins, 1976, 1987 and 1996; Azevedo, 1989a and 1989b; McGladdery et al., 1991).

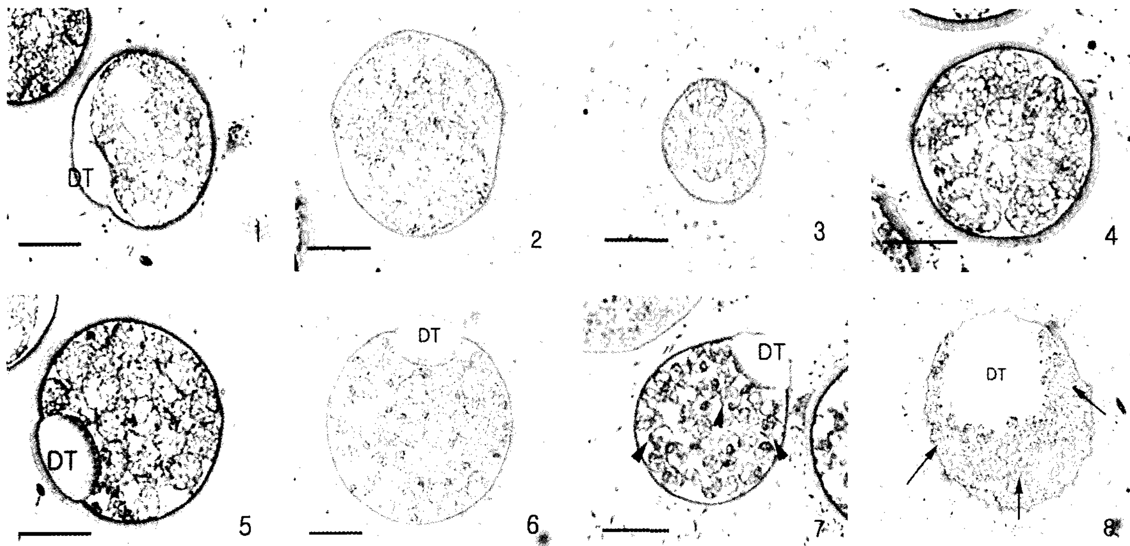

Fig. 6. Developmental stage of hypnospore stained with toluidine blue in semithinsection. Scale bar=30 μm. 6.1. Discharge tube (DT) was appeared within 24 hours. Shrinkage of cytoplasm was appeared after discharge tube formation. 6.2. 4-cell was formed by bipartition. 6.3. 8-cell stage of hypnospore. 6.4. Dividing cell was observed within a thick wall. Each dividing cell was very irregular-shaped and diverse in size. 6.5. Stage of 8~16 cell transformation was observed. 6.6. 16~32 cell stage of hypnospore. 6.7. Discharge tube and zoospores within hypnospore were observed. Zoospores were contained a eccentric nucleus and vacuole (arrow head). Fig. 6.8. Hundreds of zoospores (Zp) were occupied a hypnospore.

Table 1. Comparative measurements of trophozoites and hypnospores

Species	Host	Trophozoite (μm)	Hypnospore (μm)	References
Perkinsus sp.	*Ruditatapes philippinarum*	2.5~10.5	10~132	Present study
Perkinsus sp.	*Tapes philippinarum*	5.7~11.4	13~60	Maeno et al. (1999)
P. marinus	*Crassostrea virginica*	3~10	15~100	Mackin et al. (1950); Perkins (1976)
P. olseni	*Haliotis ruber*	13~16	56~94	Lester and Davis (1981)
P. atlanticus	*Ruditapes decussatus*	30~40	25~100	Azevedo (1989)
P. karlssoni	*Agropecten irridians*	4.3~7.6	80~138	McGladdery et al. (1991)
P. qugwadi	*Patinopecten yessoensis*	10.8	12.57	Blackbourn et al. (1998)

Comparison of morphometrics with other *Perkinsus* species

Morphometrics of trophozoites and hypnospores of *Perkinsus* spp. are shown in Table 1. From these results, distinguishing species by only size ranges is considered as improper, because of large variations in the size ranges in *Perkinsus* spp. Therefore, more scrutinized investigation on more various characters and molecular biological studies are needed to elucidate species identity of *Perkinsus* spp.

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