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Diagnosis, Pathology, and Taxonomy of *Perkinsus* sp. Isolated from the Manila clam *Ruditapes philippinarum* in Korea

Kwang-Sik Choi*, Kyung-Il Park†, Moonjae Cho[1] and Philippe Soudant[2]

School of Applied Marine Science, College of Ocean Science, Cheju National University, Jeju 690-756, Korea
[1]Department of Medicine, Cheju National University, Jeju 690-756, Korea
[2]LEMAR UMR 6539, IUEM-UBO, Place Nicolas Copernic, Plouzané, France

We report on the diagnosis, pathology, and taxonomy of *Perkinsus* sp. infection in Manila clams (*Ruditapes philippinarum*) from Korean waters. Amplimers were designed from internal portions of the non-transcribed spacer (NTS) of *P. atlanticus* for molecular diagnosis of *Perkinsus* infection. PCR-based identification methods and an *in situ* hybridization assay were developed for detection of *Perkinsus* sp. in live tissues as well as in histological preparations. Hybridization signals were observed around the nucleus of trophozoites. Positive results from PCR and *in situ* hybridization indicated that Korean *Perkinsus* sp. is genetically identical with *P. atlanticus* reported in Europe, which is currently synonymous with *P. olseni* reported from Australia. Microscopic morphological features of different life stages of *Perkinsus* sp. appeared very similar to those of *P. atlanticus*. Severely infected clams often exhibited white nodules on their mantles and gills as a consequence of inflammation. In lightly to moderately infected clams, *Perkinsus* sp. was mainly found in gill tissues, whereas the protozoan parasites were found in digestive tracts, gonadal tissues, and foot tissues of heavily infected clams. It is likely that the gills are the portal of the infection and that *P. olseni* spreads to other tissues as the infection advances. In conclusion, by considering the taxonomic priority of *P. olseni*, Korean *Perkinsus* sp. is accepted as *P. olseni*. *P. olseni* appears to be common on tidal flats on the western and southern Korean coasts and is considered to be a pathogen capable of causing mass mortality of clams.

Keywords: *Perkinsus olseni*, *Ruditapes philippinarum*, PCR, *in situ* hybridization, FTM, Korea

Introduction

Perkinsosis is a parasitic disease occurring among some commercially important shellfish including oysters, clams, and abalones (Perkins, 1996; Bondad-Reantaso, 2001; OIE, 2004). Perkinsosis is caused by several species of marine protozoa of the genus *Perkinsus*. Since the first report of *P. marinus* from the American oyster *Crassostrea virginica* in the Gulf of Mexico (Mackin et al., 1950), several species of *Perkinsus* have been reported from various marine mollusks. Lester and Davis (1981) reported *P. olseni*, the second species of *Perkinsus* to be identified, from the Australian black lip abalone, *Haliotis rubra*. Later another species, *P. atlanticus,* was isolated from the carpet shell clam, *R. decussates,* and the Manila clam, *Ruditapes philippinarum,* in Portugal (Azevedo, 1989). Recently there have been reports of several more *Perkinsus* species including *P. qugwadi* in the Japanese scallop, *Patinopecten yessoensis,* on the west coast of Canada (Blackbourn et al., 1998) and *P. andrewsi* in the Baltic clam, *Macoma balthica* (Coss et al., 2001), and *P. chesapeaki* in the soft shell clam, *Mya arenaria* (McLaughlin et al., 2000), both of which were found in the Chesapeake Bay, USA. Casas et al. (2004) isolated *P. mediterraneus* from the European flat oyster, *Ostrea edulis,* cultured around the Balearic Islands in Spain.

Perkinsus-like organisms also have been observed in Korean waters. In 1997, the National Fisheries Research and Development Agency of Korea reported the first incidence of *Perkinsus* sp. infection in Manila clams collected from Gomso Bay on the west coast (NFRDA, 1997). Infected clams had inflamed tissues, and massive hemocyte infiltration was observed around *Perkinsus*-like cells. Choi and Park (1997, 2001) and Choi et al. (1998) investigated *Perkinsus* sp. infection among several Korean populations of clams. Their studies revealed that most clams on the western and southern coasts were infected to varying degrees with *Perkinsus* sp. Choi and Park (1997) also investigated other marine bivalves including

*Corresponding author: skchoi@cheju.ac.kr
†Current address: Institute of Aquaculture, University of Santiago de Compostela, Santiago de Compostela 15706, Spain

several species of veneriid clams, oysters and pearl oysters for the presence of *Perkinsus* sp. using a fluid thioglycollate medium (FTM) assay. Only *R. philippinarum* were found to be *Perkinsus* positive, indicating that this clam was the only host of *Perkinsus* sp. in Korean waters. *Perkinsus* sp. has also been reported from Hiroshima and Kumamoto, Japan and Dalian, China (Hamaguchi et al., 1998; Maeno et al., 1999; Liang et al., 2001; Choi et al., 2002). More recently, *Perkinsus* sp. has also been isolated from undulate clams, *Paphia undulata,* in Thai Bay, Thailand (Leethochavalit et al., 2003).

Several key phenotypic characteristics, including ultrastructural features of trophozoites and zoospores, host organisms and pathology have been used to identify *Perkinsus* (Perkins, 1969; Lester and Davis, 1981; Azevedo, 1989). *Perkinsus* sp. have also been discriminated on the basis of nucleotide sequences of rRNA genes. PCR assays have been particularly useful for phylogenetic studies as well as in the diagnosis of *Perkinsus* infection (Hamaguchi et al., 1998; Coss et al., 2001; Park et al., 2002). In addition to PCR methods, *in situ* hybridization can provide high specificity and sensitivity in diagnosis, enabling localization of target nucleic acid sequences in cells and tissues (Antonio et al., 2000; Lipart and Renault, 2002).

We report the molecular diagnosis of *Perkinsus* infection using a combination of PCR methods and *in situ* hybridization assays. We also investigated the taxonomic affiliations of Korean *Perkinsus* sp. based on microscopic morphological traits of different life stages.

Materials and Methods

Histological preparation

Clams used in histological analyses were collected from Wando Island, Mokpo, and Gomso Bay, where they are commercially cultivated. Park and Choi (2001) reported that clams in these areas were heavily infected with *Perkinsus* sp. To serve as negative controls, *Perkinsus*-free clams were collected from a sand beach on the northwestern coast of Jeju Island. Entire clams were fixed in Davidson's solution for 24-48 h. Cross-sectional tissue blocks were made, dehydrated in ethanol, and embedded in paraffin. Sections (6 μm thick) were stained with Harris' hematoxylin and eosin Y, and examined under a light microscope. Various clam tissues were examined for pathological signs of *Perkinsus* infection. Cell diameters of trophozoites were measured using image analyzing software.

Zoospore development

A modified FTM method was used to induce hypnospores from infected tissues, according to Wilson-Ormond et al. (1993). Gills of heavily infected clams collected from Wando, Korea were excised and incubated in FTM for 2 d. Hypnospores formed during incubation were then transferred into a Petri dish filled with filtered (Whatman GF/C), aerated seawater (30 psu, pH 7.5) fortified with antibiotics (mycostatin and chloromycetin). The Petri dish was then incubated in a dark chamber for 48-72 h at 25°C. Zoosporulation patterns and the development of zoospores were observed using a light microscope.

Species-specific PCR

For amplification of *Perkinsus* species-specific nucleotides, a primer pair was designed from the NTS region of *P. atlanticus* (GenBank accession number AF140295) after comparison with sequences from *P. marinus* (AF150986) and *Perkinsus* sp. (AF522321, reported from Thailand). The upstream and downstream primers were 5'-CATTATCGAGGTCTGTGGTGACG-3' and 5'-ACGATAGGTCTGCTGAGCAAGC-3' (Park et al., 2002). The expected size of the amplified DNA product using these primers was 661 bp. Genomic DNA was extracted from gill tissues of heavily infected clams collected from Wando Island, Gomso Bay, and Mokpo, Korea. PCRs were performed using ExTaq polymerase (TaKaRa ExTaq, RR001PS, Korea) for 30 cycles of denaturation (30 sec, 94°C), annealing (30 sec, 55°C), and extension (1 min, 72°C), followed by a final 5-min extension at 72°C. Amplification products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide.

In situ hybridization

DNA probes were produced by PCR using *P. atlanticus* DNA as a template (10 ng per reaction) and the primer pair 5'-CATTATCGAGGTCTGTGGTGACG-3' and 5'-ACGATAGGTCTGCTGAGCAAGC-3' designed for the NTS (Park et al., 2002). PCR conditions were similar to those described previously except digoxigenin-11-dUTPs (Roche, Germany) were included in the reaction mixture. Manila clam tissue sections (5 μm thick) were placed on Poly-prep slides (Sigma, USA) and dried at 37°C overnight. Sections were deparaffinized, rehydrated, and treated with proteinase K (100 μg/ml in distilled water) at 37°C for 15 min. Pre-hybridization in hybridization buffer (50% formamide, 10% dextran sulfate, 4×SSC (Sigma, USA), 250 g/ml yeast tRNA, and 10% Den-

hart's solution) occurred for 30 min at 42°C in a humid chamber. The solution was replaced with hybridization buffer containing digoxigenin-labeled probe (10 ng/ml). Both the target DNA and digoxigenin-labeled probes were denatured at 95°C for 5 min, and the hybridization was performed overnight at 42°C. Sections were washed in 1 × SSC at room temperature (2×5 min). Detection of *P. atlanticus*-specific probe was performed using a DIG Nucleic Acid Detection kit (Roche, USA) according to the manufacturer's instructions. Specifically bound probe was detected using an alkaline phosphatase-conjugated sheep IgG antibody against digoxigenin and a NBT/BCIP color reaction. The antibody was omitted during the detection step as a negative control.

Results

Pathologic traits of *Perkinsus* sp. infection

White nodules (0.5-1.0 mm diameter) were commonly observed on the surface of mantle, gill, and foot tissues of heavily infected clams (arrows in Fig. 1A). These nodules were formed by inflammation as a consequence of heavy hemocyte infiltration around the trophozoites (asterisk in Fig. 1C). Trophozoites undergoing schizogony were commonly distributed in gills and digestive glands of lightly to moderately infected clams, indicating that the gills are a portal for *Perkinsus* infection (Fig. 1A-C). *Perkinsus* trophozoites were also observed in digestive tracts, gonads, and foot tissues of heavily infected clams. Mature trophozoites ranged in diameter from 7 to 15 μm. Characteristically, each trophozoite appeared as a "ring" formed by a large vacuole and a nucleus, 5.4 μm in diameter, with a 2.3-μm nucleolus (Fig. 1B). Numerous trophozoites were often packed in a capsule (Fig. 1B), surrounded by massive clam hemocyte infiltration (Fig. 1C). The presence of daughter cells of *Perkinsus* sp. trophozoites (Fig. 1C) indicates that the protozoa might reproduce in the host by binary fission.

In vitro sporulation

After 48-72 h of incubation in FTM, hypnospores (i.e., prezoosporangia) with cell diameters of 67.9±21.3 μm were observed in the incubated clam tissues. Hypnospores were characterized by thick cell walls (Fig. 1D) which stained dark blue or brown with Lugol's iodine. Hypnospores placed in aerated seawater underwent successive bi-partitioning of nuclei, producing numerous zoospores inside the hypnospore (Fig. 1E). A discharging tubule formed on the hypnospore cell surface as early as the 2-cell stage (Fig. 1F). Three to 5 days after incubation, bi-flagellated motile zoospores were released via the discharging tubule (Fig. 1F). Zoospores had a mean head diameter of 4.2 μm, and each zoospore had two flagella with a mean length of 9.6 μm, comparable to those of *Perkinsus* sp. (Fig. 1G).

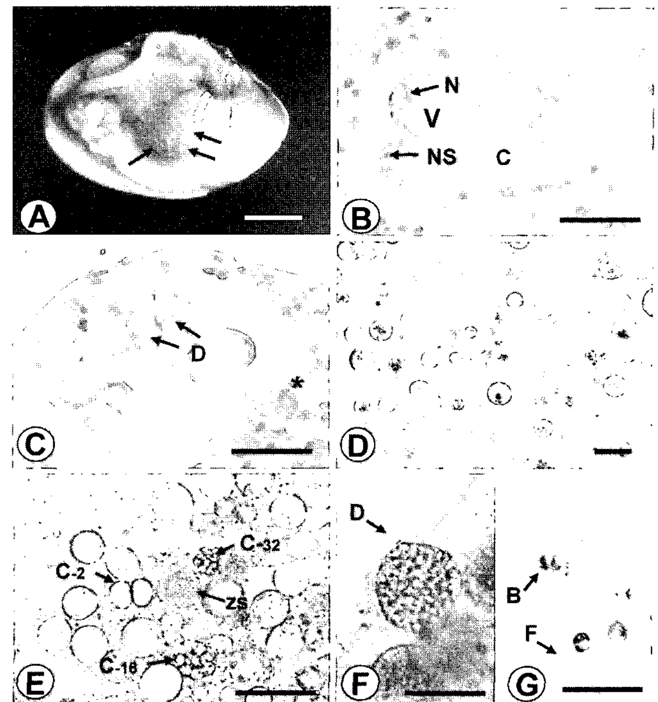

Fig. 1. The life forms of *Perkinsus* sp. A: Nodules on the surface of clam tissues caused by *Perkinsus* infection. Arrows indicate nodules. Bar=1 cm. B: Mature trophozoites containing a vacuole. V, vacuole; N, nucleus; NS, nucleolus; C, capsule. Bar=10 μm. C: Daughter cells within a trophozoite. D, daughter cell. Bar=10 μm. Asterisk indicates infiltrated hemocytes. D: Hypnospores developed in FTM. Bar=100 μm. E: Zoosporulation in various cell division stages. C_2, two-cell stage; C_{16}, 16-cell stage; C_{32}, 32-cell stage; ZS, zoospores. Bar=100 μm. F: A hypnospore with a discharge tube (D). Bar=50 μm. G: Motile zoospores discharged from hypnospores. Each zoospore has 2 flagella. B, body; F, flagella. Bar=10 μm.

PCR diagnosis

A single *P. atlanticus*-specific DNA amplicon of 661 bp resulted from PCR with infected clams collected from Wando Island, Gomso Bay, and Mokpo (Fig. 2). This positive PCR result indicates that the *Perkinsus* cells in tissues of these Korean clams are *Perkinsus atlanticus*.

In situ hybridization

The DIG-labeled PCR products obtained after the amplification of genomic *Perkinsus* sp. DNA enabled visualization of the parasite in fixed clam tissues. Positive reactions, char-

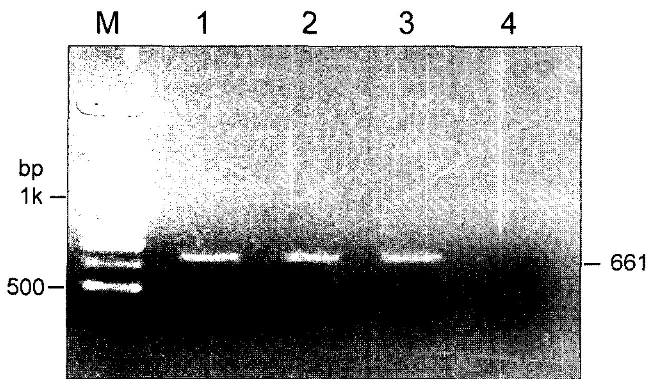

Fig. 2. Results of PCR using *P. atlanticus*-specific primers. Amplicons had the predicted size of 661 bp. Lane M: 100 bp DNA ladder; Lane 1-3: DNA extracted from infected clams from Gomso Bay, Mokpo, and Wando Island, respectively; Lane 4: no template DNA.

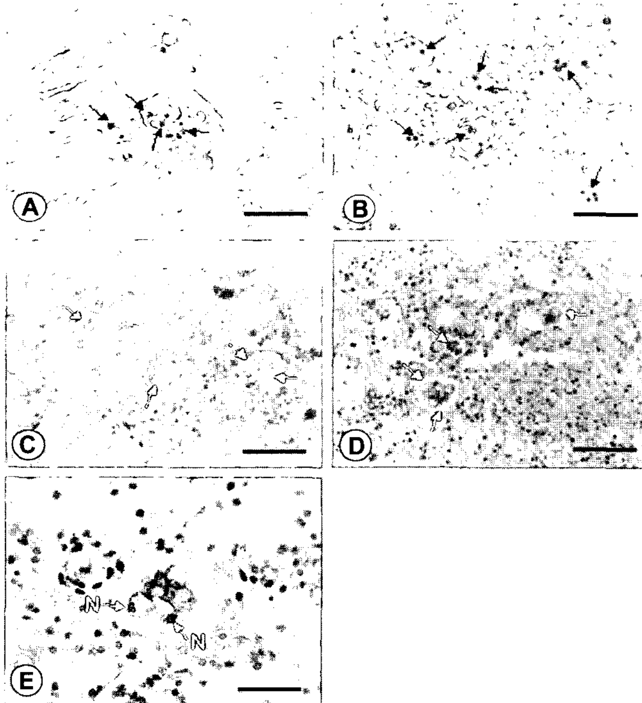

Fig. 3. Detection of *P. atlanticus* in the Manila clam *R. philippinarum* using *in situ* hybridization. *In situ* hybridization was performed using a digoxigenin-11-dUTP-labeled, *P. atlanticus*-specific probe and resulted in a distinct positive signal for the *P. olseni* NTS region in the nucleus of *P. atlanticus* trophozoites parasitizing clam gills (A, ×400; scale bar=50 μm) and the connective tissues of the visceral mass (B, ×400; scale bar=50 μm). Control (C, ×400; scale bar=50 μm); hematoxylin-eosin stained *P. atlanticus* cells (D, ×400; scale bar=50 μm); hematoxylin-eosin stained *P. atlanticus* cells (E, ×1,000; N=nucleus; scale bar=20 μm).

acterized by dark brown precipitate, were detected in trophozoites distributed in the visceral mass, mantle and gills of Manila clams, indicating that perkinsosis in these Korean Manila clam is caused by *P. atlanticus* (Fig. 3A-B). Examination under higher magnification following staining with hematoxylin-eosin revealed that the hybridization signal localized in the nucleus of each trophozoite (Fig. 3D-E). No signal was detected from *Perkinsus* sp.-infected tissue when the antibody was omitted at the staining step (Fig. 3C).

Species identification of Korean *Perkinsus* sp.

PCR and *in situ* hybridization results indicated that the *Perkinsus* sp. in Manila clams from Korean waters was the same as *P. atlanticus* reported from Portugal. Morphological observations of the different life history stages support the genetic evidence. Recently Murrell et al. (2002) proposed the synonymy of *P. atlanticus* and *P. olseni* based upon the similarity of DNA sequences of the two species. As *P. olseni* has taxonomic priority, we accordingly conclude that Korean *Perkinsus* sp. is *Perkinsus olseni.* In summary, taxonomic position of *P. olseni* new to Korean water is established according to Norén et al. (1999);

Kingdom: Protozoa 원생동물계
Phylum: Perkinsozoa Norén et al. (1999) (퍼킨서스편모충 문, 신칭)
Class: Perkinsea Levine 1978 (퍼킨서스편모충 강, 신칭)
Order: Perkinsida Levine 1978 (퍼킨서스편모충 목, 신칭)
Family: Perkinsidae Levine 1978 (퍼킨서스편모충 과, 신칭)
Genus: *Perkinsus* Levine 1978 (퍼킨서스편모충 속, 신칭)
Perkinsus olseni (퍼킨서스편모충, 신칭)

Discussion

Perkinsus species identification has been problematic in the past. Mackin et al. (1950) first reported the "yeast-like" haplosporidian pathogen responsible for the mass mortalities of American oysters along the coast of the Gulf of Mexico (see review of Mackin, 1961) as *Dermocystidum marinum.* All species in the genus *Dermocystidium* are fungal parasites found in muscle, gills, and skin of amphibians and fishes (Perez, 1907, 1913) and are characterized by trophozoite stages with large eccentric vacuoles containing a vacuoplast. Mackin and Ray (1966) later moved the oyster parasite from *Dermocystidium* to *Labyrinthomyxa,* a fungal parasite of aquatic plants, after finding amoeba-like plasmodia with rhizoid-like "mucoid-tracks" in an *in vitro* culture of *D. marinum* in blood agar. However, Perkins (1973) argued that the cells observed by Mackin and Ray (1966) were *Labyrinthuloides* sp. and were contaminants in their culture. *Labyrinthomyxa marina* then reverted to *D. marinum* (Perkins, 1976a).

The ultrastructure of the *D. marinum* zoospore was first

investigated by Perkins (1969) using transmission electron microscopy (TEM). He found an apical complex in the zoospore, including subpellicular membranes, micropores and conoids, which are characteristics of apicomplexan protozoa (Perkins, 1969). Perkins (1976a) further reported a conoid, polar ring, subpellicular microtubules, rhoptries, and micronemes in the zoospore. Based on TEM studies, Perkins (1976b) concluded that *D. marinum* was not a fungus but a protozoan, belonging to the phylum Apicomplexa. Accordingly, Levine (1978) renamed *D. marinum* as *Perkinsus marinus* with its own class (Perkinsea), order (Perkinsida), and family (Perknisidae) in the phylum Apicomplexa.

Siddall et al. (1997) analyzed the phylogeny of various species of *Perkinsus* based on rRNA sequences. They concluded that *Perkinsus* was taxonomically closer to dinoflagellates than apicomplexans. Subsequently, Norén et al. (1999) established the new phylum Perkinsozoa, basal to Dinozoa and Apicomplexa. This new classification system has been accepted by several subsequent studies involving the classification of *Perkinsus*-like organisms (Brugerolle, 2002; Ahmed et al., 2003; Moreira and López-García, 2003). Accordingly, we follow the classification suggested by Norén et al. (1999) for the *Perkinsus* species found in Korean waters.

Several studies have reported molecular phylogenetic relationships among different species of *Perkinsus* based on DNA sequences of the internal transcribed spacer (ITS), the small subunit (SSU) and the non-transcribed spacer (NTS) (Goggin, 1994; Hamaguchi et al., 1998; Kotob et al., 1999). Robledo et al. (2000) also compared NTS and ITS DNA sequences of *P. atlanticus* and *P. olseni*. They observed very high similarity between *P. atlanticus* and *P. olseni* sequences, although they were both distinctly different from other *Perkinsus* species. Recently, Murrell et al. (2002) analyzed the ITS and NTS of *P. olseni* and compared them with other *Perkinsus* species. They concluded that the genetic similarity of *P. olseni* and *P. atlanticus* is high enough to synonymize *P. atlanticus* as *P. olseni* (which has taxonomic priority); we have adopted this suggestion of the synonymy of *P. atlanticus* and *P. olseni*.

We obtained strongly positive results in our PCR and *in situ* hybridization assays diagnostic for the NTS region of *P. olseni* in clams collected from the southern coast of Korea. This indicates that the *Perkinsus* sp. infecting Korean clams is *P. olseni*. It is remarkable that the NTS region of *Perkinsus* sp. found in Korea is identical to those of *P. olseni* in Australia and Europe, despite the huge geographical distance between the populations. This co-occurrence of *P. olseni* in Korea and Europe as well as in Australia has no clear explanation, although the extensive exchange of commercial vessels and fisheries products among the regions could rapidly transfer *P. olseni* from one place to another.

In the *in situ* hybridization assays, staining was limited to trophozoite nuclei as the probe was designed from NTS. *In situ* hybridization assays as employed in this study have some advantages over conventional diagnostic methods for *Perkinsus* spp. using FTM or histology. First, *in situ* hybridization assays are species-specific, whereas FTM and histology methods are genus-specific. *In situ* hybridization assays can be used to identify different species of *Perkinsus*, at which task immunological assays have been unsuccessful (Dungan and Roberson, 1993; Romestand et al., 2001).

Perkinsus sp. is widely observed among clams distributed on tidal flats and beaches along the coasts of the Yellow Sea (Choi and Park, 1997; Hamaguchi et al., 1998; Choi and Park, 2001; Liang et al., 2001). According to Choi and Park

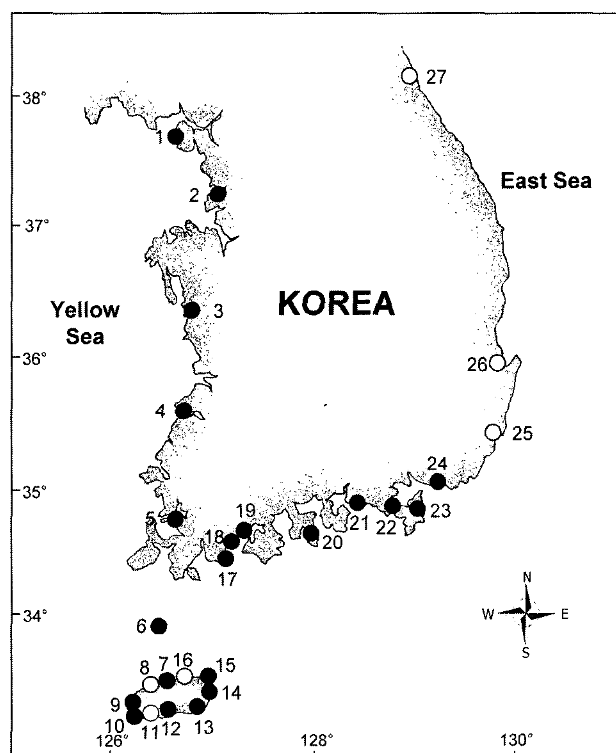

Fig. 4. Map showing spatial distribution of *Perkinsus* sp. identified from Korean Manila clams. Open circle indicates *Perkinsus* free area, solid circle indicates *Perkinsus* infected area. (modified from Choi and Park 2001, Park and Choi 2001).
1, Gangwha; 2, Jebu Island; 3, Boryeong; 4, Gomso; 5, Mokpo; 6, Chuja Island; 7, Waido; 8, Iho; 9, Kumneong; 10, Moslpo;11, Yongmeri; 12, Seogwipo; 13, Pyoson; 14, Sungsan; 15, Jongdalri; 16, Gimnyong; 17, Wando Island; 18, Gangjin; 19, Jangheung; 20, Yeosu; 21, Sachon; 22, Tongyeong; 23, Geoje Island; 24, Jinhae; 25, Ulsan; 26, Pohang; 27, Sokcho.

(2001) and Park and Choi (2001), *Perkinsus* is epizootic along the coast of Korea: FTM diagnosis revealed that clams collected from 21 of 27 locations were infected with *Perkinsus*, while clams from the eastern coast and some beaches on Jeju Island were uninfected (Fig. 4). It is notable that the prevalence (i.e., the percentage of clams infected with *Perkinsus*) and the infection level (in terms of the number of *Perkinsus* cells per clam or grams of tissue) vary from place to place (Park and Choi, 2001). The prevalence and infection intensity appear to be much higher in most commercial clam beds in the west and south, indicating that *Perkinsus* infection is associated with high densities of clams as well as physico-chemical parameters such as salinity and temperature (Burreson and Ragone Calvo, 1996; Park and Choi, 2001).

The different life stages of *P. olseni* observed in this study (Fig. 5) are comparable to the life stages of *P. atlanticus* suggested by Auzoux-Bordenave et al. (1995). The vegetative stage with trophozoites is observed in histological preparations of live clam tissues. This stage is known to be pathogenic and often induces a host cellular defense response such as hemocyte infiltration and subsequent inflammation (Chagot et al., 1987; Park and Choi, 2001; Choi et al., 2002). Trophozoite size as measured in our study is similar to that reported for other species from other areas: 7.6-11.4 µm for *Perkinsus* sp. in the Manila clam in Japan (Maeno et al., 1999), 3-15 µm for *P. atlanticus* (Ordas et al., 2001), and 5.8-9.6 µm for *P. marinus* (Perkins, 1996). Under anaerobic conditions (such as in FTM or an anaerobic chamber), trophozoites develop into hypnospores or prezoosporangia (Mackin, 1961; Choi et al., 1991); this is called the growth stage (Auzoux-Bordenave et al., 1995). The hypnospore cell wall is composed of polysaccharides, which stains dark blue or brown with iodine (Perkins, 1966). Iodine-staining of hypnospores developed in FTM has been most frequently used for the diagnosis of *Perkinsus* infection (see review of Fisher and Oliver, 1996). The mean size of hypnospores developed in FTM in our study was 67.9±21.3 µm, which is somewhat similar to that reported for *P. atlanticus* (30-70 µm; Azevedo, 1989) and somewhat smaller than that reported for *P. marinus* (20-80 µm; Perkins, 1996). The proliferative stage is initiated when the hypnospore is placed under aerobic conditions. After incubation in aerated seawater for a few days, successive bipartitioning of the hypnospore protoplast occurs. At the 4 to 8-cell stage, a discharging tubule develops on the surface of the hypnospore (Fig. 5). Finally, biflagellated zoospores are released from the hypnospore through the discharge tubule. The released zoospores are assumed to be free-swimming in the water column, where they infect new host clams. This is called the infective stage, and the swimming zoospores may penetrate host tissues by means of the apical complex

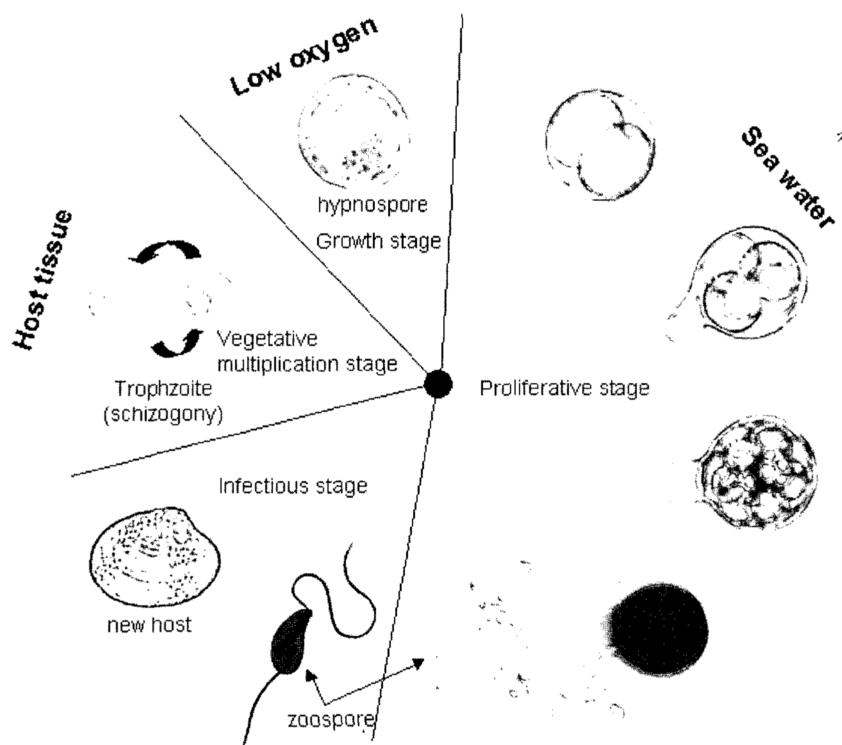

Fig. 5. Life cycle of *P. olseni* parasitizing the Manila clam *R. philippinarum*.

located in the head (Leander and Keeling, 2003). Based upon microscopic observation of 4 different life cycle stages, a hypothetical life cycle of Korean *P. olseni* can be summarized as (Fig. 5): 1) vegetative multiplication stage in infected clam tissues, with palintomy; 2) resting stage during which trophozoites transform into hypnospores under anaerobic conditions, such as in FTM; 3) proliferative stage in which hypnospores produce motile biflagellate zoospores by successive bipartitioning of nuclei; and 4) Infective stage with free-swimming zoospores capable of infecting new hosts (although this stage is yet to be observed in nature).

In conclusion, morphological features such as life history stages, zoosporulation patterns, and size of zoospores, as well as molecular methods including PCR and *in situ* hybridization assays, confirm that the *Perkinsus* sp. found in Korean *R. philippinarum* is *P. olseni*, which has previously been reported from Australia and Portugal (Lester and Davis, 1982; Azevedo, 1989). *P. olseni* appears to be common on tidal flats and small bays on the western and southern Korean coasts, and is considered to be one of the major pathogens disturbing physiology of the Manila clam.

Acknowledgments

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