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Infection prevalence and phylogenetic analysis of *Perkinsus olseni* in *Ruditapes philippinarum* from East China

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ABSTRACT: The prevalence of *Perkinsus* sp. infection in Manila clam *Ruditapes philippinarum* was investigated in the coastal areas of east China. Thirteen groups of clams were collected from 5 sites: Dandong and Qingdao Bays (Yellow Sea), Weifang Bay (Bohai Sea), and Ningbo and Fuzhou Bays (East China Sea). The clams were tested for perkinsosis infection using Ray's fluid thioglycollate medium culture assay. *Perkinsus* sp. was found in samples from all 5 sites from May 2008 to May 2009. Infection prevalence ranged from 43.75 to 95.83%, and was significantly higher in October than in May. The only 3 uninfected groups of clams were collected from Weifang Bay, the site farthest from the ocean. There was no difference in the prevalence of infection among the remaining 4 sites. The conserved internal transcribed spacer regions of the ribosomal RNA gene complex in each of the *Perkinsus* sp. isolates were amplified by PCR. The resulting amplicons were sequenced and phylogenetically analyzed. All the *Perkinsus* isolates were identified as *Perkinsus olseni*.

KEY WORDS: *Perkinsus olseni* · *Ruditapes philippinarum* · Infection prevalence · Phylogenetic analysis · China

INTRODUCTION

Filter-feeding bivalves such as clams and oysters are an important component of marine ecosystems and play a critical role in maintaining water quality. In addition, they form the basis of several valuable fisheries. For example, the Manila clam *Ruditapes philippinarum* is commonly found on sandy beaches and tidal flats along the coast of China (Park et al. 2006). The intensive culture of these clams plays an important role in the supply of live seafood to local markets and for export. The total area under culture is 967 000 ha with an annual production of 10.08 Mt in 2008 in China, which is up to 75% of the world total production. In recent years, the annual production of clams has declined due to changes in environment quality and diseases. In particular, parasitic diseases such as those caused by *Perkinsus* pose a serious threat to the health of the coastal ecosystem and the supply of shellfish worldwide (Fernández-Robledo et al. 2008).

Perkinsus is an intracellular parasite of mollusks (Sheppard & Phillips 2008). Within the genus *Perkinsus*, *P. olseni* (synonymised with *P. atlanticus* by Murrell et al. 2002) is distributed over a wide range, including the Pacific Ocean around Japan (Choi & Park 1997, Hamaguchi et al. 1998), Korea (Choi & Park 1997), New Zealand, Australia (Lester & Davis 1981), and regions of the European Atlantic and Mediterranean (Dungan & Reece 2006). *Perkinsus* spp. are spread by inadvertent transportation of infected clams on ships, by importation of untested and unquarantined ornamental clams (Sheppard & Phillips 2008), or by the introduction of exotic clam species for intensive cultivation (Elandaloussi et al. 2009). *P. olseni* infections have been identified in many types of mollusks throughout the world, including carpet shell clams

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Ruditapes decussatus in Spain (Ordas et al. 2001), undulated surf clams *Paphia undulata* in Thailand (Leethochavalit et al. 2004), venus clams *Protothaca jedoensis* in Korea (Park et al. 2006), and cockles *Austrovenus stutchburyi* in New Zealand (Dungan et al. 2007).

The occurrence of perkinsosis in the Republic of Korea, Japan, and China represents a significant threat to the viability of intensive clam culture. Over the past decade, clam harvests in Korea have declined dramatically as a result of recurrent mass mortalities caused by *Perkinsus* sp. (Park & Choi 2001). The majority of Manila clam populations in Japan are highly infected with *P. olseni*, resulting in population declines and some of the lowest population levels since the 1980s (Hamaguchi et al. 1998, Shimokawa et al. 2010). Unidentified *Perkinsus* species have been isolated from the clam *Ruditapes philippinarum* along the northeast coast of China in the Yellow Sea (Liang et al. 2001). However, the species was not confirmed in any subsequent studies.

Species within the genus *Perkinsus* are typically differentiated using several key phenotypic characters, including host species, pathogen shape and size, and the ultrastructure of the zoospores (Perkins 1969). Similarly, *Perkinsus* spp. are typically detected by histological examination of sampled tissue and diagnosis by hypnospore culture in fluid thioglycollate medium (FTM). However, ultrastructural observation of the zoospores using transmission electronic microscopy (TEM) has little value for taxonomic distinction, as it is difficult to distinguish *Perkinsus* sp. from other protists (e.g. *Colpodella* sp.) based on morphological features alone (Siddall et al. 2001). The development of molecular methods has facilitated the taxonomic identification of *Perkinsus* sp. Furthermore, the analysis of molecular sequences has permitted the study of the phylogenetic relationship of *Perkinsus* sp. with other protists. The most common molecular markers for *Perkinsus* sp. discrimination include the 5.8S sequence of the internal transcribed spacer (ITS) region and the nontranscribed spacer (NTS) region of ribosomal DNA (rDNA). Comparison of the ribosomal sequence with reference sequences constitutes the only confirmed method for the identification of *Perkinsus* sp. (Kotob et al. 1999, Robledo et al. 1999, Casas et al. 2002, Burreson et al. 2005, Park et al. 2005).

In this study, the prevalence and intensity of *Perkinsus* sp. infection in *Ruditapes philippinarum* cultured in east China were investigated using the Ray's fluid thioglycollate medium (RFTM) culture assay (Ray 1952) and DNA sequencing to report the phylogenetic relationship of *Perkinsus* sp. isolates from this study with other *Perkinsus* spp.

MATERIALS AND METHODS

Sample collection. Manila **c**lams were collected from 5 bays on the east coast of China: Dandong, Qingdao, Weifang, Ningbo, and Fuzhou (Fig. 1) from May 2008 to May 2009.

Ray's fluid thioglycollate medium (RFTM) culture assay. Samples of the clams were collected randomly by diagonal method for hypnospore culture in the laboratory. The total number of clams selected for diagnosis is shown in Table 1. After the bivalve was opened, part of the gill was excised separately and stored at –20°C for DNA extraction and PCR. The remaining whole tissue of each clam, including the gill, mantle, and muscle, was processed for incubation of hypnospore using the quantitative method described by Ray (1952). In brief, a tissue sample from each clam (1 to 3 mm^3 in size) was placed in fluid thioglycollate medium (Fluka) supplemented with 2.5% chloromycetin (Amresco) and 1% nystatin (Amresco). After incubation at 22 to 25°C in the dark for 7 d, the cellular pellet was harvested by centrifugation at 1500 × *g* for 5 min and further digested in 1 ml of 2 M sodium hydroxide for 2 h. The digested cellular material was

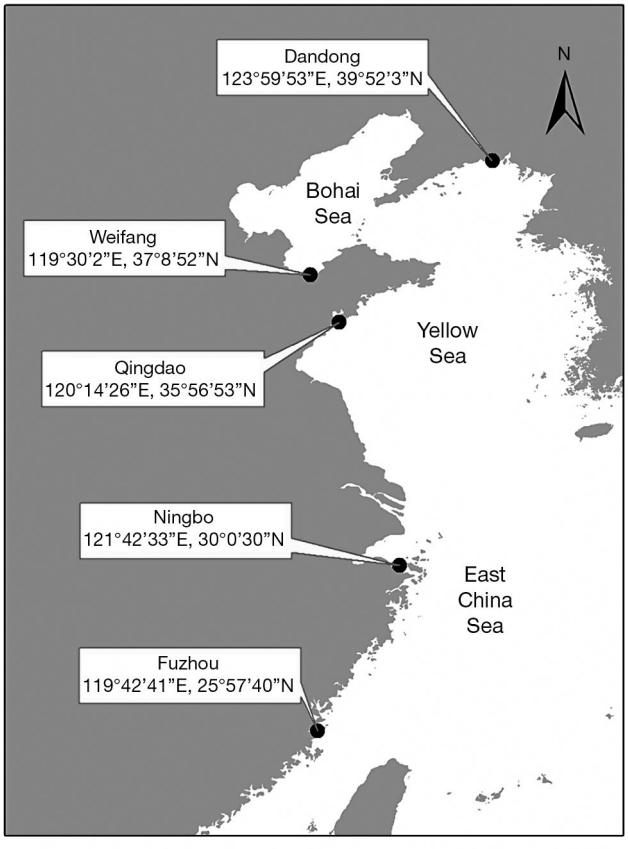

Fig. 1. *Ruditapes philippinarum*. Collection sites in east China coastal bays

Table 1. *Ruditapes philippinarum*. Prevalence of *Perkinsus* infection in east China coastal bays

Location	Sampling date	No. of clams examined	No. of positive samples	Prevalence (%)
Yellow Sea, Qingdao	May 2008	78	36	46.00
	Oct 2008	120	115	95.83
	Oct 2008	120	114	95.00
	Oct 2008	120	106	88.33
Yellow Sea, Dandong	Mar 2009	80	35	43.75
Bohai Sea, Weifang	May 2008	30	0	0
	Jul 2008	23	0	0
	Oct 2008	23	12	52.17
	Oct 2008	23	7	30.43
	May 2009	120	0	0
East China Sea, Fuzhou	Nov 2008	40	36	90.00
	Nov 2008	27	25	92.59
East China Sea, Ningbo	May 2009	23	13	56.52

then centrifuged and the pellet was rinsed 3 times with 0.1 M phosphate-buffered solution (PBS) containing 0.1% bovine serum albumin (BSA). The pellet was suspended in 2 ml of deionized water and the suspension was mounted on a glass slide, stained with Lugol's iodine solution according to Choi et al. (1989), and then examined under a microscope for the presence of hypnospores.

DNA extraction. Genomic DNA was extracted from the excised gill tissue using a DNeasy Tissue Kit (Qiagen), following the manufacturer's protocol with some modifications. The gill mixture of 5 clams collected during one round of diagonal sampling procedure was frozen in liquid nitrogen for 5 min then ground into powder. The powder was then digested with proteinase K (600 mAU ml^{-1}, Merck) at 55°C overnight. Following extraction, the final genomic DNA was eluted with 50 µl deionized water and stored at –20°C until use. Three genomic DNA extracts were collected for each batch of sample.

PCR, cloning, and sequence analysis. The *Perkinsus*-specific ITS-1 to ITS-2 ribosomal DNA primers PerkITS85 and PerkITS750 (Casas et al. 2002), which were expected to amplify a 673 base pair (bp) DNA fragment, were chosen to amplify the *Perkinsus*-specific ITS-1 to ITS-2 ribosomal DNA fragment. For the negative control, the template DNA was substituted with deionized, nuclease-free water. The PCR reaction solution consisted of 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 2.5 U rTaq polymerase (TaKaRa), and 5 µl of the purified genomic DNA. The thermal program consisted of an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and a final elongation step at 72°C for 5 min. The PCR product (5 µl) was examined by electrophoresis in a 1% agarose gel, stained with SyBr Green (Invitrogen), and visualized under UV light.

The amplified DNA product with the expected size was excised from the gel and purified using the Gel Extraction Kit (Omega Bio-Tek). After quantification, the amplicon was ligated to the pGEM-T easy vector (Promega) and transformed to *E. coli* JM109 competent cells (TaKaRa). Positively transformed bacterial colonies were initially screened using the blue and white colony selection method, followed by screening for inserts using the PCR protocol described above. The PCR product was sequenced using the T7 and SP6 sequencing primer pairs on an ABI 377 DNA Sequencer. The three DNA sequences amplified each time were compared and the universal sequence was analyzed using the basic local alignment search tool (BLAST) online (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned to available sequences for *Perkinsus* spp. deposit in the GenBank database using the CLUSTAL-W algorithm (Thompson et al. 1994). To determine the taxonomic affiliation of the *Perkinsus* isolates with other congeneric species, phylogenetic analyses of all the isolates were performed using maximum-parsimony (MP) methods in MEGA version 4 (Tamura et al. 2007) with 1000 replicates for bootstrap values.

RESULTS

Prevalence of perkinsosis in clams

Hypnospores were identified in 10 out of the 13 batches and from all 5 sites. The rate of infection ranged from 43.75 to 95.83% (Table 1). The only 3 uninfected batches were all collected at Weifang Bay in the Bohai Sea, the site farthest from the ocean. The hypnospores were spherical with diameter 30 to 80 µm, and the cell wall was stained bluish-black with Lugol's iodine solution revealing the vesicles internally (Fig. 2).

Amplification of *Perkinsus* ITS DNA and phylogenetic analysis

Electrophoresis of the PCR product revealed an approximate 673 bp amplicon. The sequences of the isolates were subjected to phylogenetic analysis

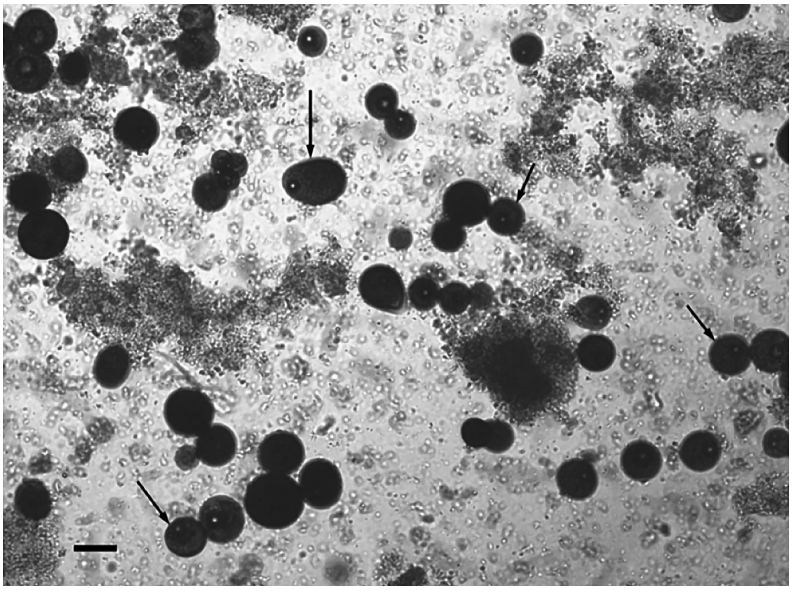

Fig. 2. *Perkinsus* sp. after FTM culture and staining with Lugol's solution (200×). Hypnospores are indicated by arrows. Scale bar = 50 µm

against *Perkinsus* sequences of the known species deposited in GenBank by maximum parsimony method using the software MEGA 4.0. All the sequences of the isolates were grouped in the same clade as other known *P. olseni* sequences isolated from Japan (DQ516712), Korea (AF473840), and Australia (U07697) and *P. atlanticus* sequences isolated from Japan (AF509333). Conversely, the isolate sequences were distinct from several other *Perkinsus* species, including *P. marinus* (AF126022, AF295179), *P. chesapeaki* (DQ370493, AY876304), *P. mediterraneus* (AY487834, EU068097), *P. honshuensis*, and *P. qugwadi* (AF151528) (Fig. 3).

DISCUSSION

Perkinsus olseni infections were widespread in Manila clams along the east coast of China. The only uninfected clams were collected at Weifang Bay of the Bohai Sea. The low infection intensity and prevalence of *P. olseni* at this site is likely related to the relative lack of tidal and oceanic influences when compared with the other sites. The salinity is relatively constant in Weifang Bay, ranging from 28 to 38 ppt throughout the year (Wang et al. 2010). A number of studies have shown that the abundance and prevalence of *Perkinsus* sp. is strongly influenced by salinity. For example, Park & Choi (2001) demonstrated that perkinsosis was absent at sites where salinity remained constant throughout the year. Similarly, Dang et al. (2010) noted that perkinsosis was absent from Arguin (Arcachon Bay, France), an area characterized by relatively constant salinity

The prevalence of *Perkinsus olseni* was significantly greater in October than in May suggesting that prevalence varies seasonally. This is consistent with Park et al. (2006) who reported relatively low infection intensity during summer in Korean waters. Similarly, Uddin et al. (2010) reported lower infection intensity and prevalence levels from March to August and higher levels from September to January for *P. olseni* in Manila clams. Villalba et al. (2005) reported on the seasonality of *P. olseni* infection intensity and prevalence in the carpet shell clam *Ruditapes decussatus* in Galicia, Spain. Over 5 yr of monitoring, the infection intensity and prevalence were lower in winter and higher from spring to autumn, with 2 main annual peaks in spring and late summer/early autumn. The temporal variation in infection intensity and prevalence is closely linked with seasonal changes in seawater temperature. The high prevalence and intensity of infection observed at high temperatures and salinity during the summer is likely associated with increases in parasite multiplication rates and decreases in host defense capabilities and physiological condition (Burreson & Calvo 1996).

The significantly higher infection prevalence during fall (September to November) compared with other seasons may be related to the post-spawning condition of the clams. As the clams exhaust themselves during spawning, they may become more susceptible to *Perkinsus olseni* infection. Park et al. (2006) also observed higher levels of *P. olseni* infection during the post-spawning period in *Ruditapes philippinarum* in Gomso Bay on the west coast of Korea. Immune parameters such as the number and viability of hemocytes also decrease after spawning in Manila clams, which may increase their susceptibility to *P. olseni* infection (Soudant et al. 2004).

The sequence data from the *Perkinsus* isolates were within the range of intraspecific variance observed for *P. olseni*. Thus, the isolates were classified as *P. olseni*. The phylogenetic groupings derived from these results were similar to those of Park et al. (2005) who showed a high degree of sequence divergence between Korean *Perkinsus* isolates and other congeneric species, including *P. marinus* and *P. andrewsi*, but only minor sequence differentiation between Korean *Perkinsus* and Japanese *Perkinsus*.

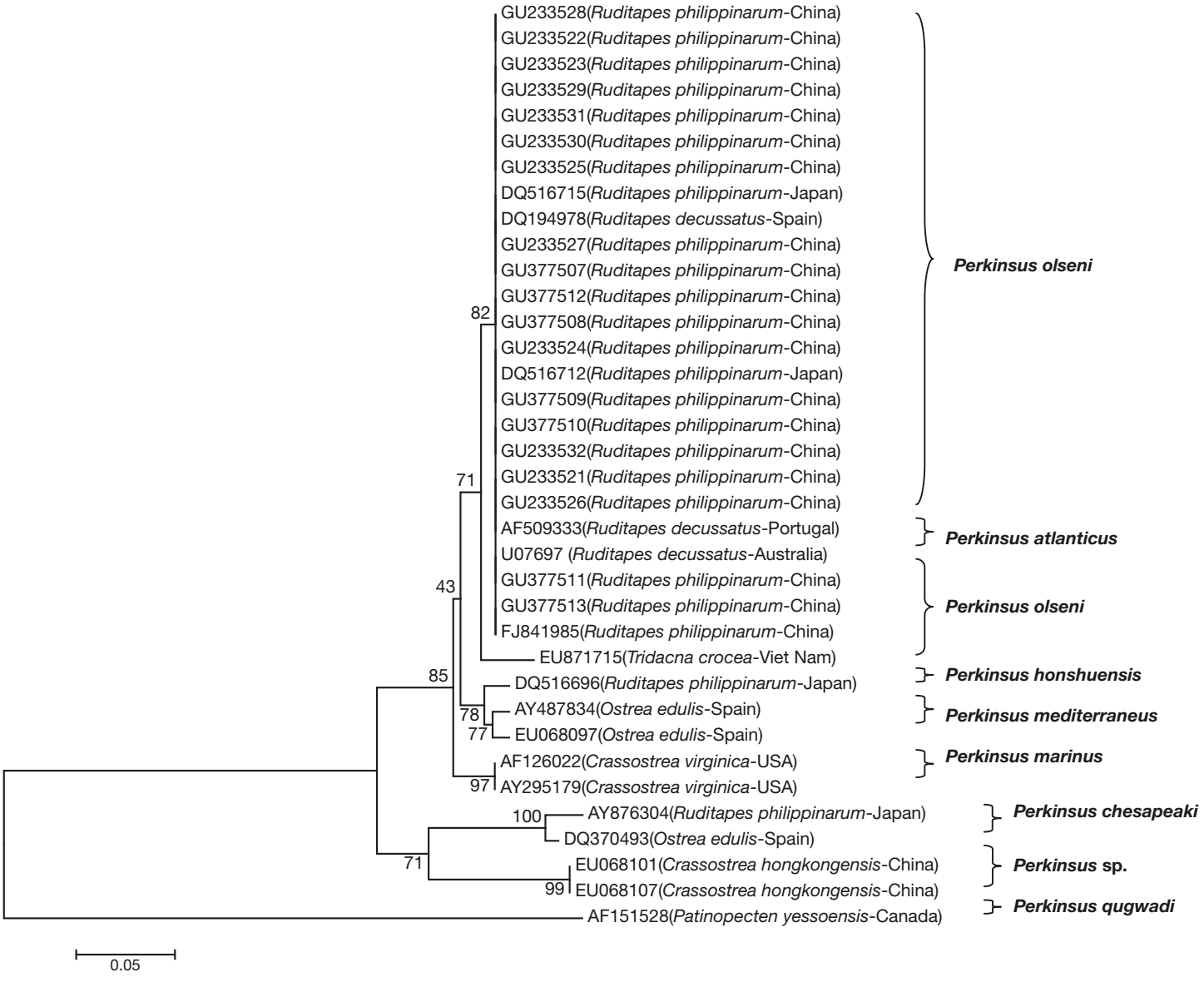

Fig. 3. *Perkinsus* spp. Phylogenetic analysis of isolates based on the ITS region sequences. (Nucleotide sequence data reported in this paper are available in the GenBank database under the accession numbers FJ841985, GU377507–13)

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