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Use of molecular markers for species identification of Korean *Perkinsus* sp. isolated from Manila clams *Ruditapes philippinarum*

Kyung-Il Park[1], Joong-Ki Park[2], Jehee Lee[1], Kwang-Sik Choi[1,*]

[1]School of Applied Marine Science, College of Ocean Science, Cheju National University, 1 Ara 1-Dong Jeju City, Jeju 690-756, Republic of Korea

[2]Department of Parasitology and Medical Research Institute, College of Medicine, Chungbuk National University, Chongju, Chungbuk 361-763, Republic of Korea

ABSTRACT: *Perkinsus* is the pathogen responsible for mass mortality of the Manila clam *Ruditapes philippinarum*. *Perkinsus* sp. isolated from Manila clams collected in Korean waters was assayed by polymerase chain reaction (PCR) to determine its phylogenetic affinity with other congeneric species. Regions of rRNA of *Perkinsus* sp. isolated from clam haemolymph were cloned and sequenced. Sequences of a non-transcribed spacer (NTS), internal transcribed spacers (ITS 1, 2) and 5.8S rRNA genes were compared to those available from other *Perkinsus* species. The NTS sequence of Korean *Perkinsus* was approximately 99.9 to 100% similar to that of *P. atlanticus* and 98.06 to 98.15% and 73.05 to 73.14% similar to those of *P. olseni* and *P. marinus*, respectively. The ITS 1, 5.8S rRNA and ITS 2 sequences of Korean *Perkinsus* showed 100% similarity to *P. atlanticus* and *Perkinsus* sp. reported from Japan. The ITS–5.8S rRNA sequences of Korean *Perkinsus* were 99.86 and 93.73% similar to those of *P. olseni* and *P. marinus*, respectively. The sporulation pattern and morphology of the Korean *Perkinsus* were very similar to those of *P. atlanticus*. Our data suggest that the *Perkinsus* sp. isolated from clams in Korean waters is *P. atlanticus*, which is currently synonymous with *P. olseni* reported from Australia. By considering that *P. olseni* has taxonomic priority, Korean *Perkinsus* sp. is accepted as *P. olseni* (*atlanticus*).

KEY WORDS: NTS · ITS · 5.8S rRNA · *Perkinsus olseni* (*atlanticus*) · *Ruditapes philippinarum* · Taxonomy · Korea

INTRODUCTION

The Manila clam (also known as the little-neck or short-neck clam) *Ruditapes philippinarum* is one of the most common marine animals on sandy beaches and tidal flats along the coast of Korea, Japan and China. Owing to its fast growth and high productivity, this clam has been cultured commercially on the western and southern coasts of Korea (Lee et al. 1996, Chung et al. 2001). Over the past decade, clam harvests in Korea have declined dramatically as a result of recurrent mass mortalities during late summer and early fall. Several studies of clam mortalities have reported a high level of *Perkinsus* sp. infection, suggesting that *Perkinsus* sp. is responsible for the mortalities, as has been observed in other marine molluscs (Mackin & Ray 1954, Da Ros & Canzonier 1985, Azevedo et al. 1990, Park & Choi 2001).

Since the first report of *Perkinsus marinus* (formerly *Dermocystidium marinum*) from the American oyster *Crassostrea virginica* in the Gulf of Mexico (Mackin et al. 1950), several species of *Perkinsus* have been identified in various marine molluscs, including *P. olseni* in the Australian black lip abalone *Haliotis rubra* (Lester & Davis 1981), *P. atlanticus* in the carpet shell clam *Ruditapes decussatus* in Portugal (Azevedo 1989),

*Corresponding author. Email: skchoi@cheju.ac.kr

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 in Chesapeake Bay, USA. Casas et al. (2004) recently reported a new species of *Perkinsus*, *P. mediterraneus*, from the European flat oyster *Ostrea edulis* from the Balearic Islands in the Mediterranean Sea.

Unidentified *Perkinsus* species have been isolated from *Ruditapes philippinarum* along the coasts of Korea and Japan (Choi & Park 1997, Choi et al. 1998, Hamaguchi et al. 1998, Park & Choi 2001, Choi et al. 2002), as well as along the north-eastern coast of China, in the Yellow Sea (Liang et al. 2001). Hamaguchi et al. (1998) analysed the nucleotide sequences of 2 internal transcribed spacers (ITS 1 and ITS 2) and 5.8S rRNA of *Perkinsus* isolated from clams in Kumamoto and Hiroshima, Japan. ITS sequences of *Perkinsus* from Japanese clams were almost identical to those of *P. atlanticus* and *P. olseni*, suggesting that *Perkinsus* isolated from clams in Japan may be conspecific with, or closely related to *P. atlanticus* in European waters. Size and microscopic features of hypnospores and trophozoites of *Perkinsus* isolated from the Manila clam in Korea were very similar to those of *P. atlanticus* (Park & Choi 2001), suggesting that *Perkinsus* found in Korea is also closely related to *P. atlanticus*.

One of the most conventional ways to differentiate species in the genus *Perkinsus* is to use several phenotypic keys, including host utilities, ultrastructure of the zoospores and pathologic attributes (Perkins 1969, Azevedo 1989). Recently, phylogenetic analysis of nucleotide sequence data has been widely used for species-level taxonomy in a number of living organisms, including *Perkinsus.* In particular, the NTS, ITS and 5.8S rRNA regions of *Perkinsus* spp. have become the most frequently used molecular marker genes for species discrimination among *Perkinsus* species (Goggin & Barker 1993, Reece et al. 1997, Kotob et al. 1999a,b, Robledo et al. 1999, Figueras et al. 2000, Coss et al. 2001, Casas et al. 2002).

In the present paper, we report the species identity of Korean *Perkinsus* found in *Ruditapes philippinarum*, based on a comparative analysis of NTS, ITS 1, ITS 2 and 5.8S rDNA sequence datasets, in addition to the zoosporulation patterns of the parasite.

MATERIALS AND METHODS

***In vitro* culture of *Perkinsus* and DNA extraction.** For genomic DNA preparation, *Perkinsus* was extracted from the haemolymph of Manila clams, *Ruditapes philippinarum*, collected from Wando Island (WAN) and Gomso Bay (GOM) on the southern and western coasts of Korea, respectively, where a high prevalence of *Perkinsus* infection has been reported (Park & Choi 2001). *Perkinsus* trophozoites in the haemolymph were propagated for 1 wk in Dulbecco's Modified Eagle Medium (DMEM):Ham's F-12 (1:2), with Hepes buffer and 5% foetal bovine serum (FBS) according to Ordas & Figueras (1998). Total genomic DNA was then extracted from *in vitro*–cultured *Perkinsus* using a commercially available DNA extraction kit (QIAGEN).

DNA sequencing and data analysis. The NTS, ITS 1, ITS 2 and 5.8S rRNA gene regions of Korean *Perkinsus* sp. were PCR-amplified using 2 primer sets: PKnts for the NTS region and PKits for the ITS–5.8S region (Fig. 1). The primer sequences used for PCR amplification are shown in Table 1. For PCR amplification, the reaction mixtures contained 5 µl of reaction buffer (10 mM Tris-HCl [pH 9.0], 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 1 µl of 10 mM dNTP, 1 µl of each primer, 1 µl of *Taq* DNA polymerase (Promega) and 3 µl of DNA template in a total volume of 50 µl. The reaction was carried out in a MiniCycler (MJ Research) as follows: 2 min at 94°C, 30 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were electrophoresed in 1 × TAE buffer and visualised under long-

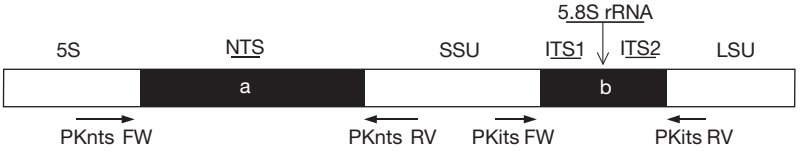


Fig. 1. Representation of the rRNA locus of *Perkinsus* sp. showing location of the non-transcribed spacer (NTS) domain (Region a), locations of the internal transcribed spacer (ITS 1, 2) domain and the 5.8S rRNA domain (Region b), and locations of primers (horizontal arrows) used in amplification of complete sequences of NTS and ITS with 5.8S rRNA (PKnts FW, forward primer for amplification of Region a; PKnts RV, reverse primer for amplification of Region a; PKits FW, forward primer for amplification of Region b; PKits RV, reverse primer for amplification of Region b; LSU, large subunit; SSU, small subunit)

Table 1. Oligonucleotide primers used to amplify NTS and ITS 1, ITS 2 and 5.8S rRNA regions of *Perkinsus* sp. isolated from *Ruditapes philippinarum*

Name	Primers	Target region
PKnts	FW: 5′-AAGTCCTTAGGGTGCTGCTGGCT-3′	NTS
	RV: 5′-ACTACTGGCAGGATCAACCAGGT-3′	NTS
PKits	FW: 5′-CTTAGAGGAAGGAGAAGTCGTAACA-3′	ITS, 5.8S rRNA
	RV: 5′-GCTTALTTATATGCTAAATTCAGCG-3′	ITS, 5.8S rRNA

wavelength UV light on a 1% agarose gel (w/v) containing ethidium bromide (EtBr). Products were then purified using a QIAEX II gel extraction kit (QIAGEN) for cloning and sequencing. The sequencing reactions were accomplished using a Thermo Sequenase Cycler 5.5 (Amersham Pharmacia Biotech) according to the manufacturer's directions. Reaction products were purified using the ethanol precipitation method and electrophoresed on an ABI 3100 automated DNA sequencer. Sequence comparison and multiple sequence alignment for each of the NTS and ITS–5.8S rRNA regions with homologous sequences found in other *Perkinsus* species (Fig. 2, Table 2) were performed separately using CLUSTAL X (Thompson et al. 1994). Interspecific length heterogeneity of eukaryotic rDNA gene clusters is very common due to the high frequency of indel events during anagenetic evolutionary changes among taxa. This is especially true for the NTS and ITS regions.

To collect more informative and conserved sequence blocks from each of the NTS and ITS regions of various *Perkinsus* species, we also used Gblock software (Castresana 2000) with the following options for multiple alignment: a minimum of 5 sequences for a conserved position, a minimum of 7 sequences for a flank position, a maximum of 8 contiguous non-conserved positions and a minimum block length of 10 after gap cleaning for NTS. For ITS alignment, the scores 7, 11, 8 and 10 were set as each of the block parameters in the Gblock program. To determine the taxonomic affiliation of Korean *Perkinsus* with other congeneric species, phylogenetic analyses for each of the NTS and ITS datasets of *Perkinsus* species were separately performed using the neighbour-joining (NJ) and maximum-parsimony (MP) methods in PAUP, version 4.0b8 (Swofford 1998).

***In vitro* sporulation.** Gills of heavily infected clams collected from Wando Island were incubated in FTM for 2 d to induce zoospores *in vitro*. Hypnospores formed in FTM were harvested by centrifuging at 100 × *g*. Hypnospores were then transferred into aerated and filtered seawater fortified with antibiotics (mycostatin and chloromycetin). Hypnospores were incubated in a dark chamber at 25°C for 48 to 72 h. Different cell-division stages and the occurrence of motile zoospores were examined under a light microscope. Zoospore size was measured using image-analysis software.

RESULTS

Nucleotide sequences of NTS, ITS 1, ITS 2 and 5.8S rRNA of Korean *Perkinsus*

The sequences of the NTS and the ITS 1, 5.8S and ITS 2 rRNA genes of *Perkinsus* sp. isolated from clams collected on Wando Island, Korea, were 1147, 183, 371

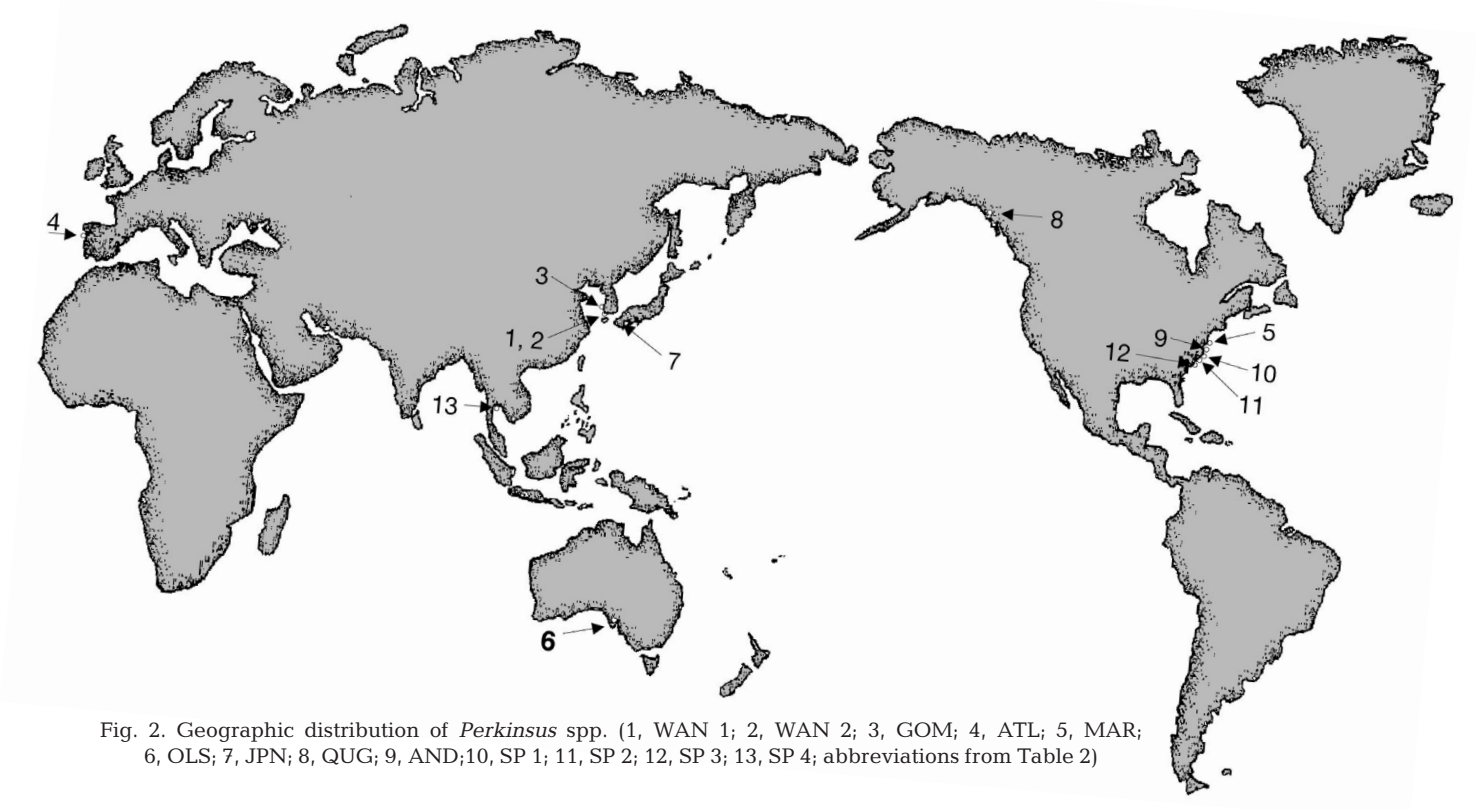


Fig. 2. Geographic distribution of *Perkinsus* spp. (1, WAN 1; 2, WAN 2; 3, GOM; 4, ATL; 5, MAR; 6, OLS; 7, JPN; 8, QUG; 9, AND;10, SP 1; 11, SP 2; 12, SP 3; 13, SP 4; abbreviations from Table 2)

Table 2. Abbreviations, host species, geographic origins and GenBank accession numbers of *Perkinsus* spp. WAN1: Wando Island 1; WAN2: Wando Island 2; GOM: Gomso Bay; ATL: *P. atlanticus*; MAR: *P. marinus*; OLS: *P. olensi*; JPN: *Perkinsus* sp. from Japan QUG: *P. qugwadi*; AND: *P. andrewsi*; SP1: *Perkinsus* sp. 1; SP2: *Perkinsus* sp. 2; SP3: *Perkinsus* sp. 3; SP4: *Perkinsus* sp. 4

Organisms	Abbreviation	Host	Order of host	Location	Accession Number/Source
Perkinsus sp.	WAN 1	*Ruditapes philippinarum*	Veneroida	Korea	AF473840, AF438150
Perkinsus sp.	WAN 2	*R. philippinarum*	Veneroida	Korea	Present study
Perkinsus sp.	GOM	*R. philippinarum*	Veneroida	Korea	Present study
P. atlanticus	ATL	*R. decussatus*	Veneroida	Spain	AF140295
P. marinus	MAR	*Crassostrea virginica*	Pterioida	USA	AF150986
P. olseni	OLS	*Haliotis laevigata*	Archaeogastopoda	Australia	U07701, AF466527
Perkinsus sp.	JPN	*R. philippinarum*	Veneroida	Japan	Hamaguchi et al. (1998)
P. qugwadi	QUG	*Patinopecten yessoensis*	Pterioida	Canada	AF151528
P. andrewsi	AND	*Macoma baltica*	Veneroida	USA	AF102171
Perkinsus sp.	SP 1	*Mercenaria mercenaria*	Veneroida	USA	AF252288
Perkinsus sp.	SP 2	*Mya arenaria*	Veneroida	USA	AF091541
Perkinsus sp.	SP 3	*Mya arenaria*	Veneroida	USA	AF091542
Perkinsus sp.	SP 4	*Paphia undulata*	Veneroida	Thailand	AF522321

and 159 bp in length, respectively. Two NTS genotypes (WAN 1, WAN 2) were detected from 2 geographical isolates (GOM, WAN 1), which differed from each other by a single substitution. Sequence divergences between Korean *Perkinsus* and other *Perkinsus* species for each of the NTS and ITS–5.8S loci are shown in Tables 3 & 4, respectively. The sequences reported here have been deposited in GenBank (Accession Numbers AF473840 & AF438150 for NTS and ITS–5.8S, respectively).

The NTS sequence of the Korean *Perkinsus* showed approximately 99.91 to 100% similarity to *P. atlanticus*, with only a single base substitution, and 99.91% sequence similarity. The sequence was also very similar to that of *P. olseni*; the mean pairwise sequence comparison showed 98.12% similarity. However, the NTS sequence of Korean *Perkinsus* showed pronounced sequence divergence from those reported in other *Perkinsus* lineages; we observed mean sequence divergence of 26.94% between Korean isolates and *P. marinus* (Table 3). The degree of sequence divergence among various *Perkinsus* lineages in the ITS–5.8S rRNA region was relatively low, compared to the NTS region. Nucleotide sequences of Korean *Perkinsus* for each of the ITS 1, ITS 2 and 5.8S rRNA regions were 100% identical to those of *P. atlanticus* and Japanese *Perkinsus* isolates. The ITS–5.8S rRNA sequence of Korean *Perkinsus* also showed high similarity to those of *P. olseni* (99.86%) and Thai *Perkinsus* species (SP 4) (99.72%). In contrast, the sequence divergence of the ITS–5.8S region between Korean *Perkinsus* and *P. qugwadi* was pronounced (35.04%), while the sequence difference between Korean *Perkinsus* and *P. marinus* was 6.27% (Table 4).

In vitro sporulation pattern

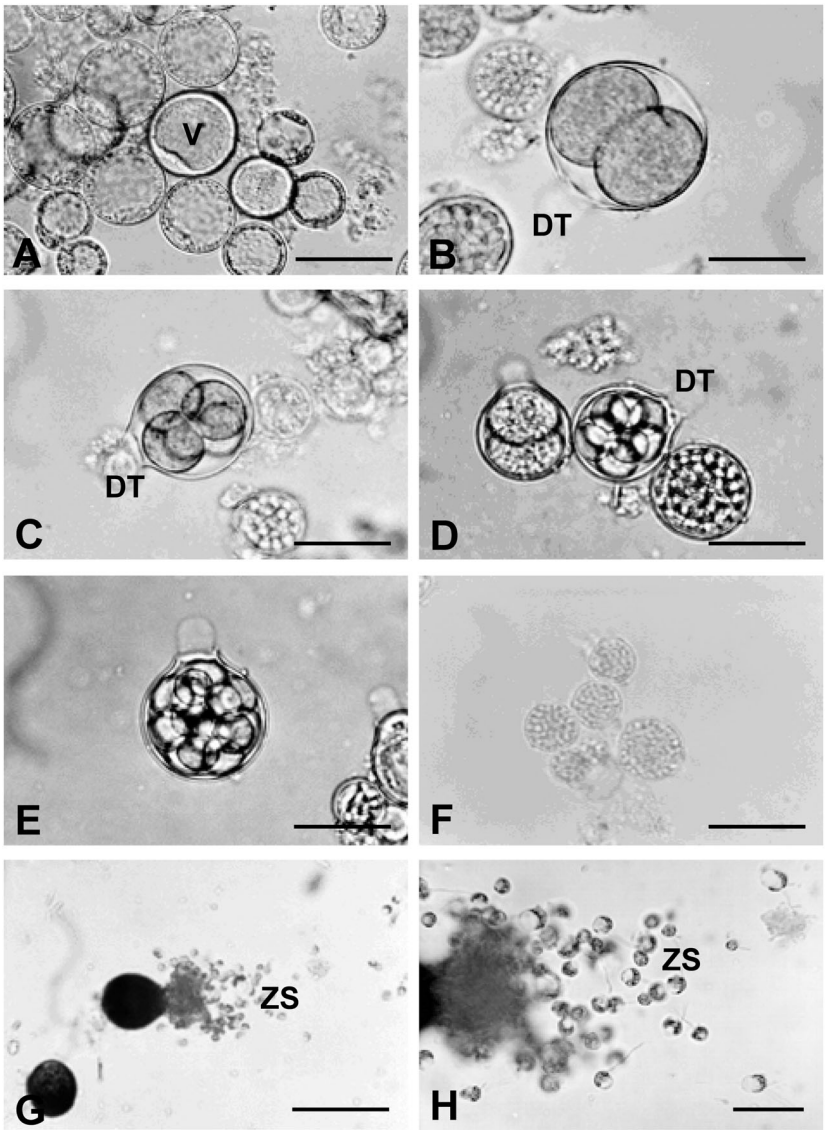
The zoosporulation pattern observed in Korean *Perkinsus* isolates is illustrated in Fig. 3. After 2 d in aerated seawater at 25°C, large lipid droplets that were commonly observed among hypnospores had disappeared and the nucleus had become extremely enlarged. Successive karyokinesis and cytokinesis resulted

Table 3. Mean uncorrected pairwise distance (*P*; below diagonal) and percent similarity (above diagonal) of non-transcribed spacer (NTS) sequences among *Perkinsus* spp. (for abbreviations, see Table 2)

	WAN 1	WAN 2	GOM	ATL	OLS	MAR	AND	SP 1	SP 4
WAN 1		99.91	100.00	100.00	98.15	73.14	61.88	62.62	97.03
WAN 2	0.0009		99.91	99.91	98.06	73.05	61.8	62.53	96.94
GOM	0.0000	0.0009		100.00	98.15	73.07	61.91	62.56	97.03
ATL	0.0000	0.0009	0.0000		98.15	73.07	61.91	62.56	97.03
OLS	0.0185	0.0194	0.0185	0.0185		73.31	61.51	62.39	97.65
MAR	0.2686	0.2695	0.2693	0.2693	0.2669		58.12	57.82	72.85
AND	0.3812	0.382	0.3809	0.3809	0.3849	0.4188		81.08	59.96
SP 1	0.3738	0.3747	0.3744	0.3744	0.3761	0.4218	0.1892		62.31
SP 4	0.0297	0.0306	0.0297	0.0297	0.0235	0.2715	0.4004	0.3769	

Table 4. Mean uncorrected pairwise distance (*P*; below diagonal) and percent similarity (above diagonal) of internal transcribed spacer (ITS) and 5.8S rRNA sequences among *Perkinsus* spp. (for abbreviations, see Table 2)

	WAN 1	WAN 2	GOM	ATL	JAP	OLS	MAR	AND	QUG	SP 1	SP 2	SP 3	SP 4
WAN 1		100.00	100.00	100.00	100.00	99.86	93.73	87.37	64.96	87.33	87.48	93.01	99.72
WAN 2	0.0000		100.00	100.00	100.00	99.86	93.73	87.37	64.96	87.33	87.48	93.01	99.72
GOM	0.0000	0.0000		100.00	100.00	99.86	93.73	87.37	64.96	87.33	87.48	93.01	99.72
ATL	0.0000	0.0000	0.0000		100.00	99.86	93.73	87.37	64.96	87.33	87.48	93.01	99.72
JAP	0.0000	0.0000	0.0000	0.0000		99.86	93.73	87.37	64.96	87.33	87.48	93.01	99.72
OLS	0.0014	0.0014	0.0014	0.0014	0.0014		93.87	87.53	65.11	87.47	87.62	93.15	99.86
MAR	0.0627	0.0627	0.0627	0.0627	0.0627	0.0613		87.37	65.60	87.61	87.76	99.31	93.72
AND	0.1263	0.1263	0.1263	0.1263	0.1263	0.1247	0.1263		63.15	95.93	96.06	86.65	87.39
QUG	0.3504	0.3504	0.3504	0.3504	0.3504	0.3489	0.3440	0.3685		63.63	63.48	64.85	64.96
SP 1	0.1267	0.1267	0.1267	0.1267	0.1267	0.1253	0.1239	0.0407	0.3637		99.72	86.89	87.33
SP 2	0.1252	0.1252	0.1252	0.1252	0.1252	0.1238	0.1224	0.0394	0.3652	0.0028		87.03	87.48
SP 3	0.0699	0.0699	0.0699	0.0699	0.0699	0.0685	0.0069	0.1335	0.3515	0.1311	0.1297		93.01
SP 4	0.0028	0.0028	0.0028	0.0028	0.0028	0.0014	0.0628	0.1261	0.3504	0.1267	0.1252	0.0699	



in the formation of 2, 4, 8, 16, or 32 cells inside the hypnospore. After 3 to 4 d of incubation, a pore was observed on the surface of the hypnospores as early as during the 2-cell stage, which later formed a discharge tube. Although some hypnospores required 3 to 4 d of incubation prior to successive cell divisions, most of them started cell division within 2 to 3 d in aerated seawater. Swimming zoospores released via the discharge tube from mature hypnospores were observed 4 to 5 d after incubation. Zoospores were estimated as having a mean (±SD) head diameter of 4.55 ± 0.90 µm and a flagellar length of 8.12 ± 1.03 µm (Table 5).

DISCUSSION

Due to its sub-lethal impacts on some commercially important marine molluscs, *Perkinsus* has received much attention since its first discovery in the early 1950s. Secretion of extracellular

Fig. 3. *In vitro* sporulation of *Perkinsus* sp. in GF/C-filtered seawater: (A) beginning of eccentric vacuole subdivision (400×); (B) 2-cell stage (400×); (C) 4-cell stage (400×); (D) 8-cell stage (400×); (E) 16-cell stage (400×); (F) stage with hundreds of zoospores (200×); (G) releasing zoospores (200×); (H) releasing zoospores (1000×). V: vacuole; DT: discharging tube; ZS: zoospores. Scale bars: A to E = 50 µm, F and G = 100 µm, H = 10 µm

Table 5. Zoospore size (mean ± SD) among *Perkinsus* spp.

Species	Host	Body (µm)	Flagellum (µm)	Source
Perkinsus sp.	*Ruditapes philippinarum*	4.55 ± 0.90	8.12 ± 1.03	Present study
P. atlanticus	*R. decussatus*	4.5 ± 0.6	12.7 ± 2.4	Azevedo (1989)
P. qugwadi	*Patinopecten yessoensis*	4.5 ± 1.0	9.67 ± 2.07	Blackbourn et al. (1998)
Perkinsus sp.	*Macoma balthica*	4–6	–	Perkins (1996)
Perkinsus sp.	*Tagelus plebeius*	4	–	Dungan et al. (2002)
P. atlanticus	*R. decussatus*	3.69 ± 0.05	–	Casas et al. (2002)

products and continuous energy drain by *Perkinsus* degrade defence activity (Choi et al. 1989, Garreis et al. 1996, Chu et al. 2000), retard the growth and reproduction and increase the mortality of the host (Ray 1954, Menzel & Hopkins 1955, Paynter & Burreson 1991, Choi et al. 1994). Perkinsosis, a disease caused by *Perkinsus* spp., has been classified by the Office International des Epizooties (OIE) as a disease that warrants notification.

Perkinsus-like parasitic organisms were first reported in Korean waters in 1997 from Manila clams (*Ruditapes philippinarum*); hypnospore development in FTM and microscopic features of the trophozoite indicated that these parasites were a species of *Perkinsus* (Choi & Park 1997). According to Park & Choi (2001), *Perkinsus* sp. is epidemic along the western and southern coasts of Korea, and its prevalence and infection intensity vary from approximately 0 to 100% and 12 to 3 924 309 cells g^{-1} tissue, respectively. Heavily infected clams often exhibit nodules on their gills and mantle tissues; these clams also show necrosis in various organs, suggesting that the mass mortalities of clams observed in Korean waters are closely associated with high levels of infection (Park & Choi 2001).

In the present study, sequence analyses of the NTS and the ITS–5.8S rRNA genes provided significant insight into the determination of species identity and phylogenetic affinity of Korean *Perkinsus*. A high degree of sequence divergence was observed between Korean *Perkinsus* isolates and other congeneric species, including *P. marinus* and *P. andrewsi*, while minor sequence differentiation was found between Korean *Perkinsus* and Japanese *Perkinsus*, *P. atlanticus* and *P. olseni*. The mean uncorrected pairwise distance (*P*) of the NTS sequence of Korean *Perkinsus* deviated from those of *P. marinus*, *P. andrewsi* and SP 1 (North American isolate, see Table 2) by 26.94, 37.98 and 37.33%, respectively. Several studies on the molecular phylogenetic affinities of *Perkinsus* spp. have reported similar results (Hamaguchi et al. 1998, Kotob et al. 1999a, Robledo et al. 2000, Murrell et al. 2002, Leethochavalit et al. 2003). The ITS–5.8S rRNA sequence of Korean *Perkinsus* was 100% identical to that of *Perkinsus* sp. isolated from *Ruditapes philippinarum* collected in Kumamoto and Hiroshima, Japan (Hamaguchi et al. 1998).

To infer phylogenetic relationships among various *Perkinsus* species, we performed comparative phylogenetic analysis. A bootstrapped, unrooted NJ phylogram was constructed based on the NTS sequences of *Perkinsus.* The phylogram showed close phylogenetic ties between Korean *Perkinsus*, *P. atlanticus* and *P. olseni* (Fig. 4). In the phylogram, Korean *Perkinsus* isolates and European *P. atlanticus* formed a robust clade, with a bootstrap support value of 100%. Monophyletic grouping between the Korean–European *Perkinsus* clade and the *P. olseni*–SP 4 clade was evident in the phylogram, with a bootstrap value of 100%. Use of the MP criterion did not alter the tree topology constructed using NJ (Fig. 4). A close phylogenetic relationship between *P. atlanticus* and Korean isolates was also demonstrated by NJ and MP phylogenetic methods (not shown), using an independent ITS dataset of *Perkinsus* species. These results strongly suggest that the *Perkinsus* sp. extracted from Manila clams in Korean waters is *P. atlanticus*, which is present in European waters and/or is closely related to *P. olseni*, which is infecting a wide

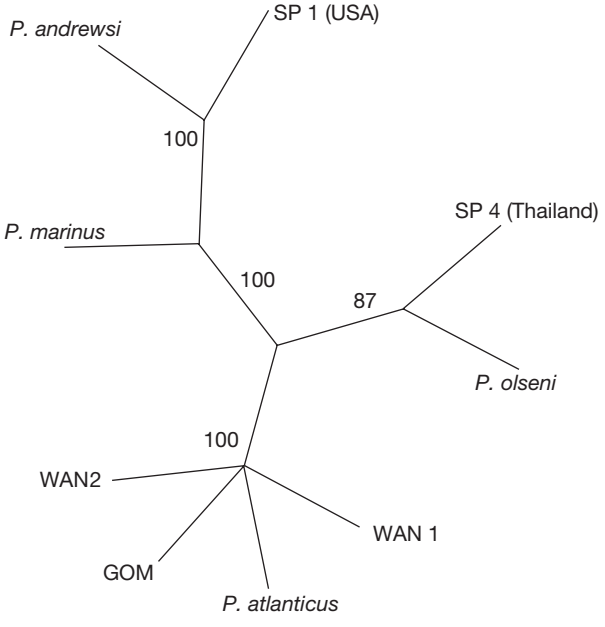


Fig. 4. Unrooted neighbour-joining (NJ) tree (based on HKY distances), bootstrapped with 1000 replicates for the *Perkinsus* NTS dataset. Numbers on branches represent bootstrap values for each node

range of abalones and marine bivalves on the south and the west coasts of Australia and the Gulf of Thailand (Goggin & Lester 1987, 1995, Leethochavalit et al. 2004). Comparing nucleotide sequences of the ITS–5.8S rRNA region, Hamaguchi et al. (1998) reported that the *Perkinsus* sp. found in Japan may be *P. atlanticus* or a closely related species. Based on the sequence similarity between *P. atlanticus* and Australian isolates (i.e. *P. atlanticus*–*P. olseni*, *P. atlanticus*–unidentified *P. atlanticus*–like isolate), Robledo et al. (2000) suggested that these 3 geographic isolates constitute a single species or a subgroup of *Perkinsus* species with a wide geographic distribution. Murrell et al. (2002) sequenced NTS loci of *P. olseni* and compared these sequences to known *Perkinsus* sequences. Since the NTS sequences of *P. olseni* were very close to those of *P. atlanticus*, they proposed that *P. atlanticus* should be synonymised with *P. olseni*.

In addition to the ITS regions (ITS 1 and ITS 2), the NTS region is among the most common molecular markers used to characterise the genetic structure of a population or to determine the taxonomic position of morphologically complex cryptic species, including *Perkinsus* species. Based on sequence variations in the NTS region among different species, a novel PCR-based molecular marker for correct species identification is available for some *Perkinsus* groups. Robledo et al. (1998) and Park et al. (2002) developed PCR-based NTS markers for *P. marinus*- and *P. atlanticus*-specific diagnosis, respectively. The NTS region is known to accumulate a high degree of sequence variability, even between closely related species, since the nucleotides are not transcribed (Marsh et al. 1995). Robledo et al. (1999) reported that NTS sequences of *P. marinus* varied with geography, even though the ITS regions were identical. Interestingly, the nucleotide sequence of the NTS region of the Korean *Perkinsus* sp. is 99.91 to 100% identical to that of *P. atlanticus* found in European waters, despite the wide geographical distance between these 2 isolates.

Incidences of *Perkinsus* in European waters date back to the late 1960s. Alderman & Gras (1969) reported the occurrence of a *Perkinsus*-like organism in oysters from the Atlantic and Mediterranean coasts of France. Da Ros & Canzonier (1985) reported *Perkinsus* infections in the carpet clam *Venerupis decussata* (=*Ruditapes decussatus*) and the flat oyster *Ostrea edulis* collected from Italy in 1978. Based upon transmitted electron microscopy (TEM), Azevedo (1989) identified *Perkinsus* isolated from the carpet clam *R. decussatus* in Portuguese waters as *P. atlanticus*. Subsequently, *P. atlanticus* infections among venerid clams have been reported from Portugal, Spain, France, Italy and Ireland (Azevedo 1989, Figueras et al. 1992, Almeida et al. 1999, Canestri-Trotti et al. 2000). However, it is uncertain whether *Perkinsus* is endemic to the Atlantic or the Mediterranean, or whether it was introduced from elsewhere. According to Flassch & Leborgne (1992), Manila clams in European waters were originally transplanted from the Pacific coast of North America in the early 1970s. Manila clams are endemic to the Yellow Sea (Goulletquer 1997), and they were accidentally introduced to the Pacific coast of North America in the 1930s from Japan, when the Pacific oyster *Crassostrea gigas* was transplanted for commercial purposes (Quayle 1964). However, there have been no reports of the occurrence of *Perkinsus* infections in Manila clam populations along the north-eastern Pacific coast. Thus, it is unlikely that *P. atlanticus* in Europe was introduced from the Pacific coast of North America. Alternatively, we speculate that the high degree of affinity between the NTS and the ITS–5.8S rRNA genes of *P. atlanticus* in Europe and *Perkinsus* in Korea may be coupled with anthropogenic activities between Europe and Asia, such as ballast-water discharge from maritime vessels or transfer of infected molluscs from one place to another, although evidence to support these hypotheses is currently unavailable.

In conclusion, our molecular sequence data (NTS, ITS 1, ITS 2 and 5.8S) in addition to morphological features, such as life stage, zoosporulation pattern and zoospore size suggest that the *Perkinsus* sp. found in Manila clams (*Ruditapes philippinarum*) in Korean waters is *P. atlanticus* and/or *P. olseni*. However, considering the taxonomic priority of *P. olseni*, we conclude that Korean *Perkinsus* sp. is *P. olseni* (*atlanticus*).

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