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First report of *Perkinsus beihaiensis* in *Crassostrea madrasensis* from the Indian subcontinent

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ABSTRACT: Protozoan parasites of the genus *Perkinsus* are considered important pathogens responsible for mass mortalities in many wild and farmed bivalve populations. The present study was initiated to screen populations of the Indian edible oyster *Crassostrea madrasensis*, a promising candidate for aquaculture along the Indian coasts, for the presence of *Perkinsus* spp. The study reports the presence of *P. beihaiensis* for the first time in *C. madrasensis* populations from the Indian subcontinent and south Asia. Samples collected from the east and west coasts of India were subjected to Ray's fluid thioglycollate medium (RFTM) culture and histology which indicated the presence of *Perkinsus* spp. PCR screening of the tissues using specific primers amplified the product specific to the genus *Perkinsus*. The taxonomic affinities of the parasites were determined by sequencing both internal transcribed spacer (ITS) and actin genes followed by basic local alignment search tool (BLAST) analysis. Analysis based on the ITS sequences showed 98 to 100% identity to *Perkinsus* spp. (*P. beihaiensis* and Brazilian *Perkinsus* sp.). The pairwise genetic distance values and phylogenetic analysis confirmed that 2 of the present samples belonged to the *P. beihaiensis* clade while the other 4 showed close affinities with the Brazilian *Perkinsus* sp. clade. The genetic divergence data, close affinity with the Brazilian *Perkinsus* sp., and co-existence with *P. beihaiensis* in the same host species in the same habitat show that the remaining 4 samples exhibit some degree of variation from *P. beihaiensis*. As expected, the sequencing of actin genes did not show any divergence among the samples studied. They probably could be intraspecific variants of *P. beihaiensis* having a separate lineage in the process of evolution.

KEY WORDS: Edible oyster · *Crassostrea madrasensis* · Protozoan parasite · *Perkinsus beihaiensis* · Brazilian *Perkinsus* sp. · Indian subcontinent

INTRODUCTION

The edible oyster *Crassostrea madrasensis,* also known as the Indian backwater oyster, is the most dominant oyster species occurring in the estuaries, bays and backwaters along the southeast and southwest coasts of India. The species is well adapted to estuarine conditions and occurs as single individuals to small groups or dense beds forming oyster reefs (Narasimham & Kripa 2007). Successful hatchery breeding and seed production have made this species a promising candidate for aquaculture along the southeast and southwest coasts of India. Presently, culture of *C. madrasensis* in India is in its initial phase and practically no information is available on its pathogens or diseases from the Indian subcontinent.

Protozoan parasites of the genus *Perkinsus* are known to infect many species of marine molluscs including oysters, abalones, clams, scallops, pearl oysters, cockles and mussels (Villalba et al. 2004). The hypothetical life cycle of *Perkinsus* spp. involves 2 distinct phases with 4 stages, viz. trophozoite, hypnospore (prezoosporangia), zoosporangium and

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zoospore. Transmission of *Perkinsus* spp. does not require an intermediate host. In moribund hosts or when cultured in Ray's fluid thioglycollate medium (RFTM), the mature trophozoites transform into hypnospores. In sea water, these hypnospores develop into zoosporangia, undergo zoosporulation and produce infective zoospores. Trophozoites and hypnospores are also infective (Villalba et al. 2004).

Since the introduction of molecular techniques in disease diagnosis, new species belonging to the genus *Perkinsus* have been described from various molluscan hosts across the world (Dungan & Reece 2006, Moss et al. 2008). Through culture in RFTM, the standard diagnostic method for *Perkinsus* spp., the parasites can be identified up to the genus level. The broad host range and highly variable and overlapping morphologic and morphometric features of these parasites makes their species-level identification difficult (Goggin & Lester 1995, Perkins 1996, Coss et al. 2001). The enhanced sensitivity and specificity offered by the recent molecular diagnostic techniques have made them indispensible tools in the diagnosis of *Perkinsus* spp. Presently, these techniques, which use generic and species-specific primers along with sequencing, are employed for the species-level identification of *Perkinsus* spp.

The first described parasite of the genus *Perkinsus* was *P. marinus* (Mackin et al. 1950), the etiological agent causing mass mortalities in *Crassostrea virginica* along the Gulf of Mexico and Atlantic coast. *P. olseni* (= *P. atlanticus*) was first described by Lester & Davis (1981) in the Australian abalone *Haliotis ruber.* Azevedo (1989) described *P. atlanticus* from *Ruditapes decussatus* in Portugal, although it was later considered to be synonymous with *P. olseni* (Murrell et al. 2002). Blackbourn et al. (1998) reported *P. qugwadi* from *Patinopecten yessoensis* in Canada, but this species shows many important morphological, molecular, ecological and life cycle-related differences from the other existing species of the genus *Perkinsus* (Villalba et al. 2004). McLaughlin et al. (2000) described *P. chesapeaki* (= *P. andrewsi*) from the soft shell clams *Mya arenaria* in the Chesapeake Bay, mid-Atlantic region of USA. Coss et al. (2001) reported *P. andrewsi* from *Macoma balthica* from the Chesapeake Bay, but this parasite is presently considered as a synonym of *P. chesapeaki* (Burreson et al. 2005). *P. mediterraneus* was described from *Ostrea edulis* in the Mediterranean Sea (Casas et al. 2004) and *P. honshuensis* from *Ruditapes philippinarum* in Japan (Dungan & Reece 2006). Moss et al. (2008) described *P. beihaiensis* from *C. hongkongensis*, *C. ariakensis*, and other bivalve hosts from Fujian to Guangxi provinces in southern China. Sabry et al. (2009) identified a species of the genus *Perkinsus* that is phylogenetically close to *P. beihaiensis* in the mangrove oyster *C. rhizophorae* from the Brazilian coast.

Protozoan infections have been known to cause mass mortalities in many wild and farmed bivalves (Andrews 1988, Soniat 1996). Among them, *Perkinsus* spp. have been identified as the etiological agents of many such mass mortalities in bivalves, including those of the oysters in Chesapeake Bay and Gulf of Mexico and in the carpet shell clams *Ruditapes decussatus* in Europe (Villalba et al. 2004). Except for a preliminary report on *P. marinus* infection in *Crassostrea madrasensis* (Muthiah & Nayar 1988), the information on the Office International des Épizooties (OIE) listed pathogens of molluscs in the Indian subcontinent and south Asia is limited to the observation of *Perkinsus olseni* in the pearl oyster *Pinctada fucata* (Sanil et al. 2010). Against this background, considering the importance of *C. madrasensis* as a candidate species for mariculture, the present study was initiated to screen populations of *C. madrasensis* along the southeast and southwest coasts of India for the presence of *Perkinsus* spp.

MATERIALS AND METHODS

Sampling

Samples of *Crassostrea madrasensis* were collected from the oyster beds in Tuticorin waters along the southeast coast, and Kollam and Ernakulam along the southwest coast of India during the period from January 2009 to December 2010. A total of 224 individuals of *C. madrasensis* were examined for the presence of *Perkinsus* spp. infection. Oysters were collected at random from natural oyster beds. Collections were made from 4 sampling sites (Veppalodai, Tuticorin Bay, Korampallam Creek and Punnakayal) at Tuticorin in the Gulf of Mannar. Samples were also collected from the brackish water habitats at Dalavapuram in Ashtamudi Lake at Kollam, and Sattar Island in Vembanad Lake at Ernakulam (Fig.1, Table 1).

Parameters like general appearance, fouling, shell damage, presence of abnormalities, gaping, retraction of mantle, wateriness of the tissues, abnormal coloration, and presence of abscesses, lesions, pustules and tissue discoloration were considered while assessing the condition of the oysters.

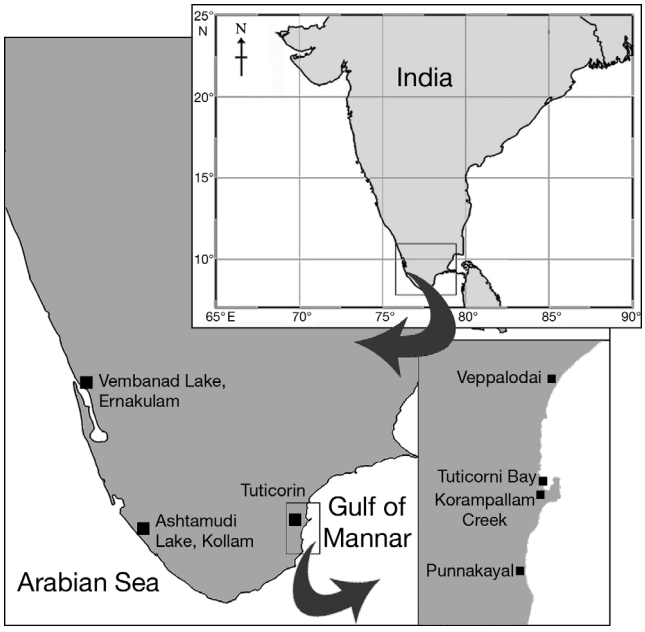

Fig. 1. The southeast and southwest coasts of India showing the sites of collection (■) of *Crassostrea madrasensis*

RFTM culture

Tissue samples from gill and mantle measuring approximately 5 to 10 mm were excised and processed as per standard procedures (Ray 1966, OIE 2006). Following incubation, the tissue fragments from each tube were placed on a glass slide and macerated along with a drop of Lugol's iodine solution. The preparation was covered with a cover-slip, allowed to sit for 10 min and examined under the microscope (Nikon Eclipse 80i). The intensity of infection was assessed based on the scale devised by Mackin (1962) and modified by Craig et al. (1989). The scale assigns numerical values to each oyster's degree of infection and ranges from 0 to 5 (0 = negative, 1 = light, 2 = light/moderate, 3 = moderate, 4 = moderately heavy and 5 = heavy infection).

Histology

Tissues were fixed in Davidson's fixative for 24 h, transferred to 70% alcohol, dehydrated in ethanol series, cleared, embedded in paraffin and cut into 5 µm thick sections using a Leica microtome. The sections were stained using Harris Haematoxylin & Eosin (H&E), examined under a light microscope and measured (Nikon Eclipse 80i).

PCR screening

Total DNA was isolated from the mantle/gill tissue of *Perkinsus*-infected *Crassostrea madrasensis*, using a standard phenol/chloroform protocol followed by ethanol precipitation (Sambrook et al. 1989). The ITS region of *Perkinsus* sp. was amplified using *Perkinsus* genus-specific ITS primers (Casas et al. 2002) and the PCR conditions followed by Sanil et al. (2010). *P. beihaiensis*-specific reverse primer PerkITS-430R (Moss et al. 2008) along with forward primer ITS-85 (Casas et al. 2002) were also used for PCR screening.

Table 1. Sampling details and prevalence of *Perkinsus* spp. in *Crassostrea madrasensis* based on Ray's fluid thioglycollate medium (RFTM) and PCR screening techniques. Prev.: prevalence

Location	Geographic co-ordinates	Total no. of oysters	RFTM		PCR		Sequencing		
			No. of oysters examined (positive)	Prev. (%)	No. of oysters examined (positive)	Prev. (%)	No. of oysters sequenced (sample ID)	GenBank accession nos. ITS	Actin
Veppalodai	8° 57′ N 78° 12′ E	47	47 (15)	32	44 (13)	30	1 (CmPb Ttn 1)	JN054741	JN807331
Tuticorin Bay	8° 46′ N 78° 09′ E	20	20 (1)	5	13 (10)	77	—	—	—
Korampallam Creek	8° 45′ N 78° 09′ E	20	20 (6)	30	5 (1)	20	1 (CmPb Ttn 4)	JN054744	JN807333
Punnakayal	8° 37′ N 78° 07′ E	17	15 (2)	13	17 (2)	12	2 (CmPb Ttn 2, CmPb Ttn 3)	JN054742 JN054743	— JN807332
Kollam	8° 56′ N 76° 33′ E	60	60 (9)	15	57 (1)	2	1 (CmPb Klm 1)	JN054740	—
Ernakulam	10° 11′ N 76° 11′ E	60	60 (5)	8	51 (3)	6	1 (CmPb Ekm 1)	JN054739	JN807334

An approximately 330 bp fragment of the Type 1 actin gene was amplified using PerkActin1 130F and PerkActin1 439R primers (Moss et al. 2008) for sequencing studies. Amplifications were performed with 30 to 80 ng of template DNA in 25 µl reactions containing PCR buffer (SIGMA) at 1× concentration with 1.5 mM $MgCl_2$, 5 pmol of each primer, 0.2 mM of each dNTP and 1.5 U *Taq* DNA polymerase (SIGMA). The thermocycler conditions followed an initial denaturation at 95°C for 3 min, then denaturation at 95°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 40 s, repeated for 39 cycles, followed by a final extension for 5 min at 72°C. Following amplification, 2 µl of the PCR products were visualized on 1.5% agarose gel.

Cloning and sequencing

PCR products of the ITS gene from 6 representative samples were purified using GenElute PCR Clean-Up Kit (SIGMA) and used for direct cycle sequencing employing both forward and reverse primers without cloning into a vector plasmid. An internal forward primer ITS-65 (5′-ACG ATG GAT GCC TCG GCT CG-3′) designed in the present study was also used for sequencing where the original forward primer ITS-85 (Casas et al. 2002) failed to amplify. Products of actin gene from 4 samples were purified by Min-Elute gel extraction kit (Qiagen). The purified products were cloned into pJET 1.2/blunt Cloning Vector using CloneJET PCR Cloning Kit (Fermentas) and transformed into chemically competent *Escherichia coli* cells (TOP10, Invitrogen) following the manufacturer's instruction. Candidate clones were screened by PCR using actin gene specific primers. The positive clones with expected size PCR amplifications were sequenced using pJET1.2 forward and pJET1.2 reverse primers supplied with the cloning kit to sequence the insert. Nucleotide sequencing was performed by the dideoxy chain-termination cycle sequencing method (Sanger et al. 1977).

All sequences generated were searched for similarity using the basic local alignment search tool (BLAST) (Altschul et al. 1990) available through the National Center for Biotechnology Information (NCBI) website (www.ncbi.nih.gov/BLAST/).

Phylogenetic analysis

ITS sequences of *Perkinsus* spp. obtained from *Crassostrea madrasensis* (GenBank accession nos. JN054739, JN054740, JN054741, JN054742, JN054743 and JN054744) were aligned with the various available *Perkinsus* spp. ITS sequences (GenBank accession nos. GQ896506, GQ896507, AF441207, AF441209, AY435092, AY820757 [*P. olseni*]; AY487835, AY487834, DQ370492, DQ370491 [*P. mediterraneus*]; AY295194, AY295199, AY295180, AY295188 [*P. marinus*]; DQ516696, DQ516697, DQ516698, DQ516699 [*P. honshuensis*]; EF 204068, EU068095, EU068080, EF204015 [*P. beihaiensis*]; FJ472346, FJ472347 [Brazilian *Perkinsus* sp.]; AF151528 [*P. qugwadi*] and AY876302, AY876311, AY876304, AY876314 [*P. chesapeaki*]). Similarly actin gene sequences of *Perkinsus* spp. obtained from *C. madrasensis* (GenBank accession nos. JN807331, JN807332, JN807333 and JN807334) were aligned with actin gene sequences of *Perkinsus* spp. from GenBank (accession nos. DQ019936, AY876352, AY876355, EF204111, EF204110, EF204109 [*P. olseni*]; EF204112–EF204115 [*P. mediterraneus*]; DQ516686–DQ516689 [*P. honshuensis*] and EF526411–EF526427 [*P. beihaiensis*]). Alignment was done using the CLUSTAL-W algorithm (Thompson et al. 1994) in Bioedit 7.0 (DNA Sequence Analysis Software package). Pairwise genetic distances (GDs) between the present samples of the genus *Perkinsus* and other *Perkinsus* spp. were calculated based on the Kimura 2 parameter model. Phylogenies were constructed using maximum parsimony, maximum likelihood and neighbor joining analyses. All the analyses were carried out using the software Molecular Evolutionary Genetics Analysis (MEGA) version 5 (Tamura et al. 2011).

RESULTS

RFTM assay

Enlarged, blue-black hypnospores characteristic of *Perkinsus*-like organisms were observed in the RFTM assay of the *Crassostrea madrasensis* tissues (Fig. 2). The hypnospores were circular in appearance and measured 18.75 to 98.65 µm in size. In moderate/heavy infections, the hypnospores appeared as aggregations in tissues while in lighter infections, appeared isolated and scattered.

Samples of *Crassostrea madrasensis* from Veppalodai showed very light to moderately heavy levels of infections with a prevalence of 32%, while at Tuticorin Bay the intensity was very low with a prevalence of 5%. At Korampallam Creek the level of infection varied from very light to moderate with a

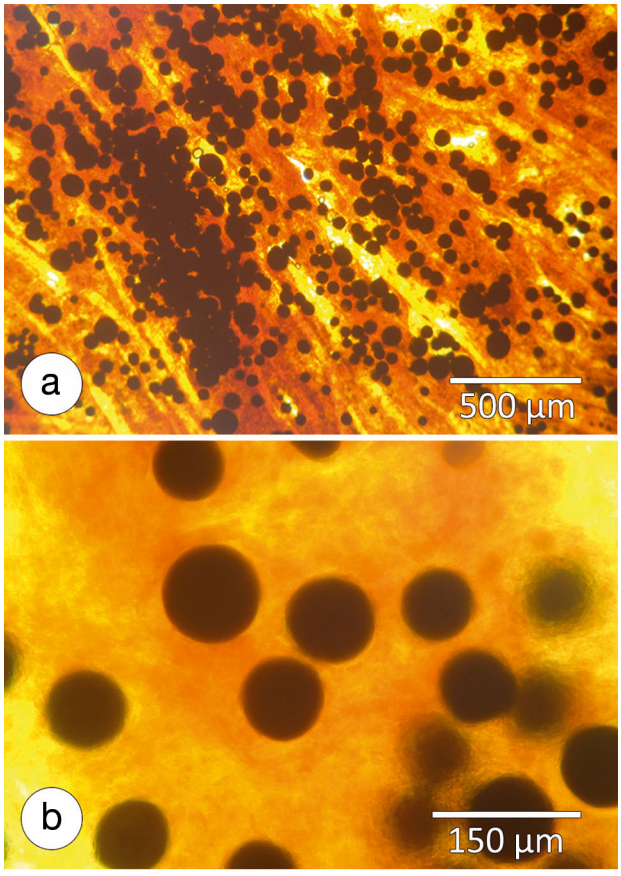

Fig. 2. *Perkinsus beihaiensis* in *Crassostrea madrasensis*. (a) Hypnospores characteristic of *Perkinsus* spp. in oyster gill tissues (note the heavy level of infection) as determined through Ray's fluid thioglycollate medium (RFTM) assay. (b) Magnified view of hypnospores in mantle tissues

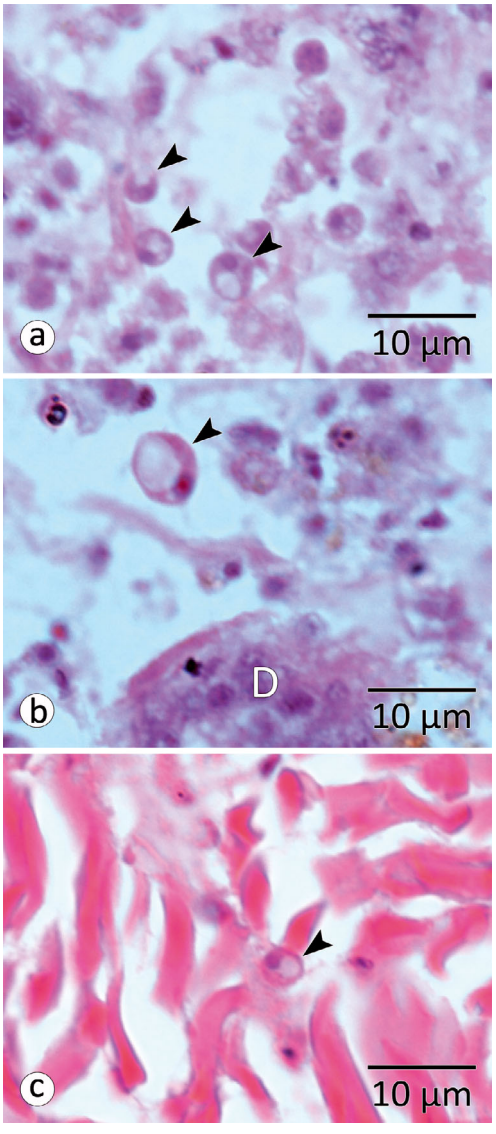

Fig. 3. *Perkinsus beihaiensis* in *Crassostrea madrasensis*. (a) Trophozoites of *P. beihaiensis* identified based on rDNA internal transcribed spacer (ITS) region sequencing in the oyster tissues (black arrows). (b) Trophozoite adjacent to the digestive tubule (D). (c) Trophozoite in the muscle fibres (haematoxylin and eosin stain)

prevalence of 30 %, while at Punnakayal the intensity of infection was light with a prevalence of 13 %. Samples collected from Kollam and Ernakulam showed very light levels of infections with a prevalence of 15 and 8 % respectively (Table 1).

None of the RFTM-positive samples of *Crassostrea madrasensis* showed any apparent macroscopic clinical signs of parasitic infection, but their general condition varied widely from healthy to pale watery.

Histology

Perkinsus-like organisms with the typical 'signet ring' configuration and measuring 3.19 to 7.66 µm (mean 4.03 ± 0.93 µm; n = 20) in diameter were observed in the histological preparations from only 2 oysters from Veppalodai (Fig. 3). Developing stages (schizonts) with up to 12 nuclei and measuring 3.09 to 7.41 µm (mean 5.28 ± 1.03 µm; n = 20) (Fig. 4a) and groups of sibling trophozoites measuring 1.86 to 3.06 µm (mean 2.28 ± 0.36 µm; n = 10) (Fig. 4b) were also observed in large numbers. The parasite stages were mostly observed in the connective tissue, especially adjacent to the epithelial lining of the stomach, among the digestive tubules and in the muscles. Although no apparent lesions were observed in the tissues, destruction of digestive tubules was evident in the samples examined. Irrespective of the status of

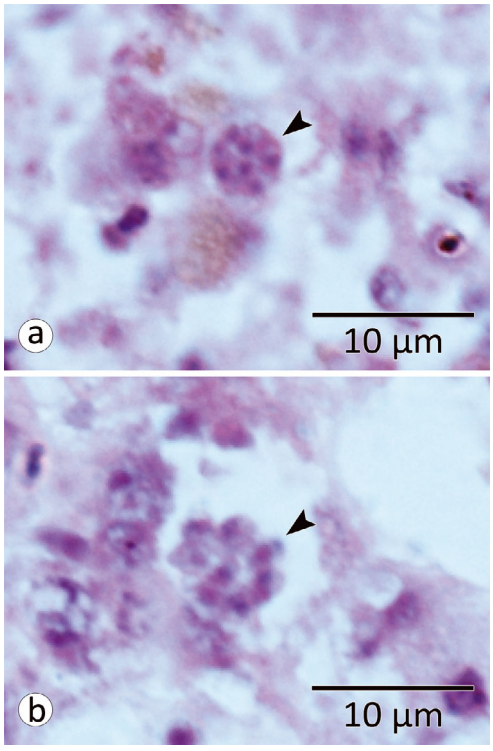

Fig. 4. *Perkinsus beihaiensis* in *Crassostrea madrasensis*. (a) Schizont of *P. beihaiensis* identified based on rDNA internal transcribed spacer (ITS) region sequencing in the oyster tissues (black arrows). (b) Young/developing trophozoites (haematoxylin and eosin stain)

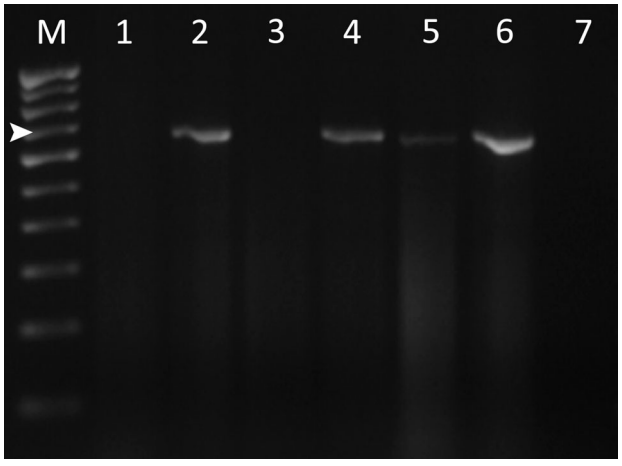

Fig. 5. Agarose gel electrophoresis of the amplified products of the PCR using *Perkinsus* genus-specific internal transcribed spacer (ITS) 85 & ITS 750 primers (ca. 700 bp). Lane 1: negative tissue control; Lanes 2–6: RFTM-positive DNA of oysters from Korampallam Creek, Veppalodai, Tuticorin Bay, Kollam and Ernakulam, respectively; Lane 7: negative control of distilled H_2O; M: molecular size marker (100 bp ladder, arrow head indicates 700 bp position)

infection, damage to digestive tubules was observed in 90.91% of the samples from Tuticorin but only in 50% of the samples from Kollam.

PCR screening

PCR screening of *Crassostrea madrasensis* DNA using the *Perkinsus* genus-specific ITS 85 & ITS 750 primers amplified the product specific to *Perkinsus* spp. (ca. 700 bp) confirming the presence of *Perkinsus* spp. infection (Fig. 5). The prevalence of *Perkinsus* spp. infection observed in samples from Veppalodai, Tuticorin Bay, Korampallam Creek and Punnakayal was 30, 77, 20 and 12% respectively, while the prevalence at Kollam and Ernakulam was 2 and 6% respectively (Table 1).

Sequencing

The sequence information on the ITS region of the 6 samples (CmPb Klm 1–475 bp, CmPb Ekm 1–520 bp, CmPb Ttn 1–665 bp, CmPb Ttn 2–521 bp, CmPb Ttn 3–528 bp and CmPb Ttn 4–529 bp) and the actin gene of the 4 samples (CmPb Ekm 1–329 bp, CmPb Ttn 1–330 bp, CmPb Ttn 3–330 bp and CmPb Ttn 4–330 bp) was generated and analysed using BLAST. The results for the ITS region showed 98 to 100% identity to *Perkinsus* spp. including *P. beihaiensis* and the Brazilian *Perkinsus* sp. with 96 to 100% query coverage (E value = 0). In the case of the actin sequence, the similarity observed was 99 to 100% with *P. beihaiensis*. The sequence information generated was submitted to NCBI database (Table 1).

Screening of samples using *Perkinsus beihaiensis*-specific primers gave multiple bands along with the targeted product (ca. 460 bp), indicating cross amplifications with host DNA.

Phylogenetic analysis

Maximum parsimony, maximum likelihood and neighbor joining (not shown) analyses showed that the nucleotide sequences of the ITS region of the present 6 samples were grouped under 2 closely related sister clades (Fig. 6). The sequences of the 2 samples from the southeast coast (CmPb Ttn 1 and CmPb Ttn 4) grouped with the *Perkinsus beihaiensis* clade (Group A), while the other 4 samples, 2 from the southeast coast (CmPb Ttn 2 and CmPb

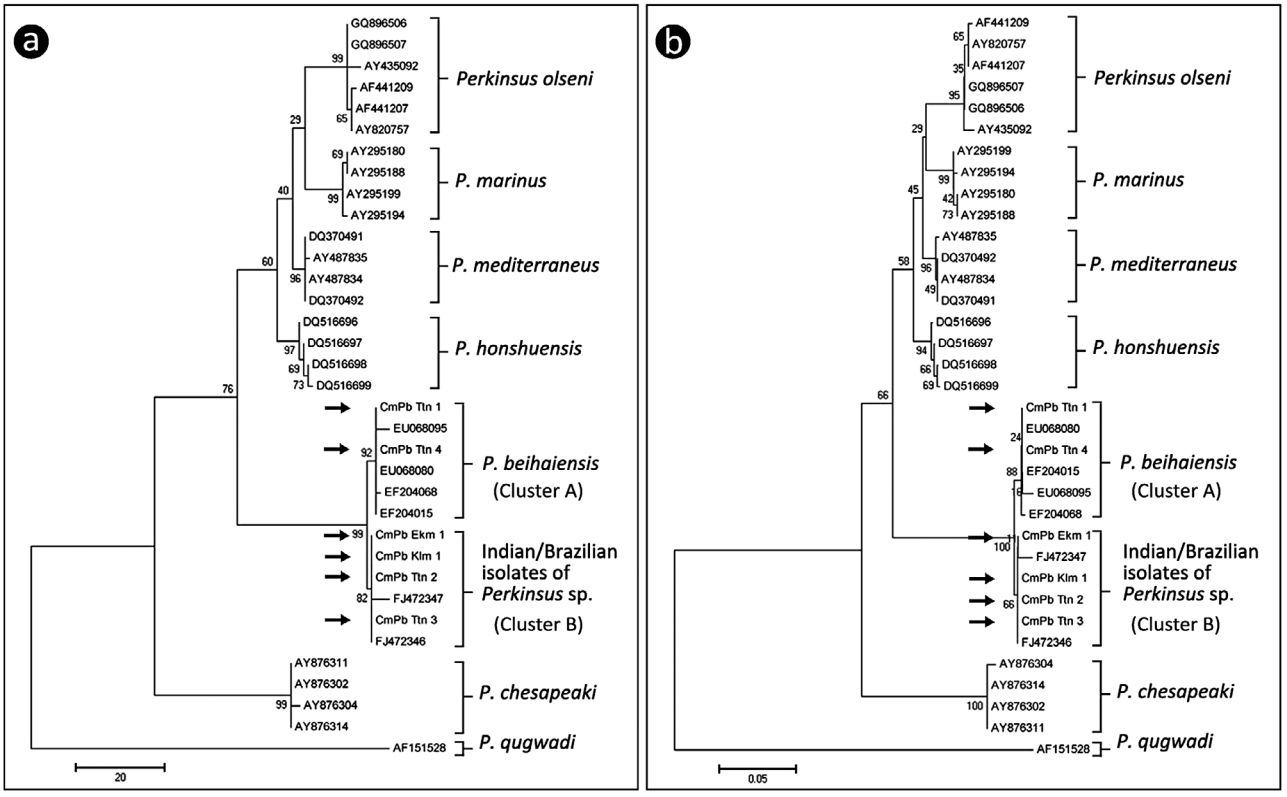

Fig. 6. Phylogenetic analysis of the rDNA internal transcribed spacer (ITS) region sequences of different *Perkinsus* species with (a) maximum parsimony and (b) maximum likelihood analyses. Numbers at nodes show bootstrap values (%) for 1000 replicates. Arrows indicate the samples from the present study

Ttn 3) and 2 from the southwest coast (CmPb Klm 1 and CmPb Ekm 1) grouped with the Brazilian *Perkinsus* sp. clade (Group B) with 99 to 100% bootstrap support during the phylogenetic analyses. The topologies of the trees generated with all 3 analyses were similar. The mean Kimura 2 parameter distance (0.0101) observed between the typical *P. beihaiensis* cluster (Cluster A) and the Brazilian *Perkinsus* sp. cluster (Cluster B) was found to be considerably higher than the GD values (0.0031) observed among the individuals of the above groups. The pairwise GD between individuals of *P. beihaiensis* and Brazilian *Perkinsus* sp. to that of the other valid *Perkinsus* spp. along with the mean

Table 2. Mean pairwise genetic distances between the various species of the genus *Perkinsus* based on the internal transcribed spacer (ITS) sequences. The mean pairwise genetic distances estimated within the species are given in **bold** across the diagonal

	Table ID	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Present *Perkinsus* sp.A	(1)	**0.0**	0.0070	0.0023	0.0117	0.1099	0.1110	0.1266	0.1366	0.1841	0.4553
Present *Perkinsus* sp.B	(2)		**0.0**	0.0094	0.0047	0.1070	0.1082	0.1236	0.1335	0.1776	0.4500
P. beihaiensis	(3)			**0.0047**	0.0141	0.1126	0.1138	0.1294	0.1390	0.1856	0.4587
Brazilian *Perkinsus* sp.	(4)				**0.0093**	0.1125	0.1137	0.1293	0.1393	0.1838	0.4520
P. mediterraneus	(5)					**0.0012**	0.0303	0.0359	0.0369	0.1405	0.3825
P. honshuensis	(6)						**0.0039**	0.0390	0.0545	0.1321	0.3803
P. marinus	(7)							**0.0027**	0.0481	0.1477	0.3637
P. olseni	(8)								**0.0045**	0.1541	0.3730
P. chesapeaki	(9)									**0.0023**	0.4141
P. qugwadi	(10)										—

GD values within each species observed in the present study are presented in Table 2.

Phylogenetic analyses of the actin gene sequences grouped the present 4 samples, 3 from the east coast (CmPb Ttn 1, CmPb Ttn 3 & CmPb Ttn 4) and 1 from the west coast (CmPb Ekm 1) along with *Perkinsus beihaiensis* in a single cluster (Fig. 7). No genetic divergence (K2P) was observed among the above samples (Table 3).

Table 3. Mean pairwise genetic distances between the various species of the genus *Perkinsus* based on the actin gene sequences. The mean pairwise genetic distances estimated within the species are given in **bold** across the diagonal

	Table ID	(1)	(2)	(3)	(4)	(5)	(6)
Present *Perkinsus* sp. A	(1)	**0.0**	0.0000	0.0016	0.2037	0.1871	0.1702
Present *Perkinsus* sp. B	(2)		**0.0**	0.0016	0.2037	0.1871	0.1702
P. beihaiensis	(3)			**0.0032**	0.2048	0.1887	0.1713
P. mediterraneus	(4)				**0.0076**	0.0707	0.1209
P. honshuensis	(5)					**0.0**	0.1423
P. olseni	(6)						**0.0192**

DISCUSSION

The presence of enlarged, blue-black hypnospores in the RFTM assay and the observations of typical *Perkinsus*-like cells in the histological preparations clearly indicated the presence of *Perkinsus* spp. in the *Crassostrea madrasensis* samples examined. Subsequent studies using molecular diagnostic tools showed that the samples were positive for PCR, and specific amplicons of *Perkinsus* spp. were obtained in the samples studied. Of the 224 samples collected, 185 samples were subjected to both RFTM and PCR assays. A comparison of these results showed sharp variations with 30 samples appearing RFTM positive but PCR negative and 22 samples RFTM negative but PCR positive. Generally larger tissue samples (5 to 10 mm) are used for RFTM assays while small samples are used for DNA extraction and PCR screening. Low intensity and/or localized

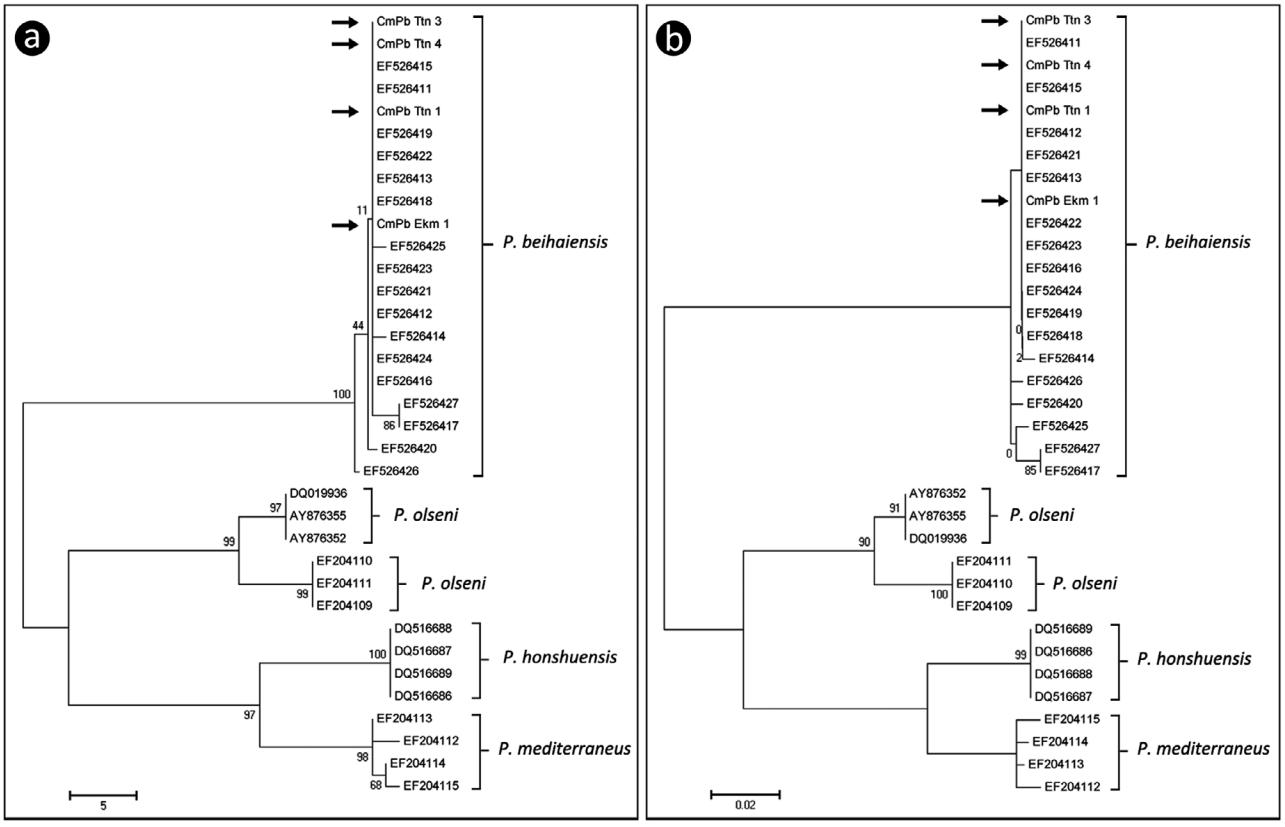

Fig. 7. Phylogenetic analysis of the actin gene sequences of different *Perkinsus* species with (a) maximum parsimony and (b) maximum likelihood analyses. Numbers at nodes show bootstrap values (%) for 1000 replicates. Arrows indicate the samples from the present study

infections coupled with the small quantity of template DNA must have produced negative results for many of the samples that were positive in the RFTM assay. Instances of discrepancies between the results of RFTM and PCR assays and the sensitivity of first step PCR over RFTM have been discussed by many authors (Burreson 2008, Reece et al. 2008, Sabry et al. 2009).

Further phylogenetic analysis based on ITS sequences grouped them under distinctly different but closely related sister clades of *Perkinsus beihaiensis* and the Brazilian *Perkinsus* sp. described by Sabry et al. (2009). The pairwise genetic distance between 2 of the present Group A Indian samples (CmPb Ttn 1 and CmPb Ttn 4) and other members of the *P. beihaiensis* group studied was very low, indicating its affiliation to the *P. beihaiensis* clade. On the other hand, Group B Indian samples of *Perkinsus* sp. (CmPb Ttn 2, CmPb Ttn 3, CmPb Klm 1 and CmPb Ekm 1) indicated variations with *P. beihaiensis.* Moreover, in the maximum parsimony, maximum likelihood and neighbor joining analysis, Group A Indian samples were also positioned along with the members of the *P. beihaiensis* clade, while the Group B Indian samples of *Perkinsus* sp. formed a distinct clade along with the Brazilian *Perkinsus* sp. The mean pairwise GD value (0.0031) observed within the *P. beihaiensis* and Brazilian *Perkinsus* sp. clusters (Clusters A & B, Fig. 7) was in accordance with the GD values observed within other *Perkinsus* spp. (which ranged from 0.0012 to 0.0045; Table 2). The mean GD values between the different *Perkinsus* spp. in the present study ranged from 0.0303 (between *P. mediterraneus* and *P. honshuensis*) to 0.4587 (between *P. beihaiensis* and *P. qugwadi*). Although the mean GD value (0.0101) observed between Clusters A and B (Fig. 7) of *Perkinsus* sp. was lower when compared to other *Perkinsus* spp., it was found to be significantly higher than that observed within all *Perkinsus* spp. The GD value (0.0047) between the Group B Indian samples of the genus *Perkinsus* and the Brazilian *Perkinsus* sp. samples falls in the range of that observed between the samples of the same species. The pattern of genetic divergence observed and the formation of 2 distinct sister clades with high bootstrap support (99 to 100%) suggests that the present Group B Indian samples (CmPb Ttn 2, CmPb Ttn 3, CmPb Klm 1 and CmPb Ekm 1) of *Perkinsus* sp. varies slightly from *P. beihaiensis*. Excepting *P. qugwadi* (outgroup), *P. chesapeaki* appeared to be the most distant one, followed by *P. olseni*, *P. marinus, P. honshuensis* and *P. mediterraneus* respectively for both the Indian groups (Group A & B) of *Perkinsus* sp. The pairwise genetic distance values and the phylogenetic analysis of the ITS sequences show that the present samples of the genus *Perkinsus* from *Crassostrea madrasensis* forms 2 slightly different groups.

While discussing the taxonomic affinities of the Brazilian *Perkinsus* sp. Sabry et al. (2009) have observed that a *Perkinsus* sp. found in a Brazilian mollusc expressed close taxonomic affinities with *P. beihaiensis* of Chinese oysters, rather than those (*P. marinus* and *P. olseni*) from the neighboring regions, which was surprising. Although close to *P. beihaiensis* reported from China, the Brazilian *Perkinsus* sp. stands out as a separate clade in the phylogenetic analysis based on the ITS sequence. One of the reasons for this could be the geographical separation between the habitats; however, in spite of the absence of any geographical separation, and being in the same host species, the Group B samples from India continued to form a separate cluster (Cluster B; Fig. 6), exhibiting high similarity to the Brazilian *Perkinsus* sp. Thus, the pattern of distribution of the Cluster B individuals of *Perkinsus* sp. shows a very wide range from the east coast of South America to the Indian subcontinent. Presently, no information is available on the status of *Perkinsus* spp. infections in bivalves from the entire African continent, which separates the Asian and American land masses. Similarly no information on *Perkinsus* spp. is available from western Asia. The pattern of geographic distribution, genetic divergence and phylogenetic data based on ITS sequences indicates that the Cluster B individuals could be an intraspecific variant of *P. beihaiensis* having a separate lineage and in the process of evolution.

Phylogenetic analysis based on the actin gene, an additional marker, gave a different picture. The analysis did not show any divergence among the 4 samples studied, which were earlier grouped under different clusters in the phylogenetic analysis based on the ITS region. The mean genetic distance between the present samples and *Perkinsus beihaiensis* was 0.0016, which was lower than the GD values observed within the *P. beihaiensis* group (0.0032) (Table 3). Even though the results based on actin gene sequence analysis did not show any variation among the samples studied, the possibility of multiple infection by both of the strains could not be eliminated. A single positive clone derived from the direct amplification of the infected host tissues has been selected from each sample for sequencing. Further, the unavailability of actin data of typical Brazilian *Perkinsus* sp. for comparison and direct amplifi-

cation of actin gene from the infected host tissue were also limiting factors in arriving at conclusions based on actin gene sequence analysis. Being a nuclear protein coding gene and highly conserved within species, actin has fewer intraspecific variations that can be expected, unlike in the case of ITS, which is a non-coding region. Although actin genes have been used for studying interspecific variations in many organisms, its use as a phylogenetic marker has been confined to the analyses of distantly related taxa (Carlini et al. 2000) and hence it may not be logical to use this gene for resolving intraspecific variations. Further detailed studies with increased sample size and distribution are required to obtain a conclusive picture regarding the genetic diversity within *P. beihaiensis.*

Efforts to establish the species-level identity of the parasites using *Perkinsus beihaiensis* specific primers in *Crassostrea madrasensis* produced multiple bands along with the targeted band (ca. 460 bp), creating difficulties in identifying the specific band. Even though several PCR optimization trials were carried out, non-targeted bands were consistently appearing which may be due to cross reactions with regions of host DNA.

In the present study, macroscopic clinical signs or pathology was not apparent in any of the animals examined and except in a few cases, the infections were of low intensity, which usually does not lead to visible pathological manifestations. The general health of the animals examined during the present study varied widely and the pale watery appearance of some of the samples observed could be taken as an indicator for poor health. In *Perkinsus beihaiensis*-infected *Crassostrea madrasensis* from Veppalodai, *Perkinsus*-like organisms with the typical 'signet ring' configuration were observed in the histological preparations. The size of the trophozoites seen in the tissues was in accordance with that of *P. beihaiensis* as reported by Moss et al. (2008). Generally, the genus *Perkinsus* is known to occur as spherical clusters of trophozoites eliciting varying degrees of host responses in tissues. During the present study, trophozoites were observed in the mantle and visceral connective tissues and in digestive gland and muscle tissues along with schizonts in various stages of development and clusters of sibling trophozoites. Since the histologically-positive samples observed during the present study were of *P. beihaiensis*, the pathology of the Group B samples of *Perkinsus* sp. in *C. madrasensis* cannot be commented upon. Moss et al. (2008) have observed necrotic lesions among stomach, intestine, and digestive gland epithelia of infected Chinese oysters. During the present study, destruction of digestive tubules was observed in both RFTM- and PCR-positive and negative samples; hence this cannot be ascribed to perkinsosis. So far no serious mortalities have been reported from *C. madrasensis* populations along the Indian coasts. The absence of regular monitoring programmes for bivalve health enhances the chances of under-reporting diseases in the region and in such instances mortalities in wild populations can be overlooked. Pollution is known to enhance the effect of many pathogens in bivalves (Winstead & Couch 1988). In sublethal *Perkinsus* spp. infections, interference with host energy fluxes may reduce the growth, resulting in poor condition and potential reduced reproduction (Park & Choi 2001). Sanil et al. (2010) have suggested that along with pollution and other anthropogenic factors *P. olseni* might have played a role in the depletion of the pearl oyster populations at Tuticorin. The prevalence and intensity of *Perkinsus* sp. infection observed at Kollam and Ernakulam were less than that at Tuticorin. Compared to Ernakulam and Kollam, the waters around Tuticorin are more polluted due to various industrial and anthropogenic activities (Edward et al. 2005, Jayaraju et al. 2009). High prevalence of digestive tubule destruction observed in oysters from Tuticorin also supports this. The higher prevalence and intensities of *Perkinsus* spp. coupled with pollution may pose a threat to the *C. madrasensis* populations at Tuticorin.

Sanil et al. (2010) have viewed the earlier report on *Perkinsus marinus* infection in *Crassostrea madrasensis* by Muthiah & Nayar (1988) as a case of misidentification because *P. marinus* is known only from North America and has a very limited host range. According to the RFTM assay, the present study suggests that the *P. marinus* reported by Muthiah & Nayar (1988) could be either *P. beihaiensis*, the Indian/Brazilian (Group B) samples of *Perkinsus* sp. or even *P. olseni*, since all of the above 3 species coexist in the Gulf of Mannar ecosystem. The host range and host–parasite interactions of these species in the region remain to be explored.

The present study forms the first report of *Perkinsus beihaiensis* in *Crassostrea madrasensis* from the Indian subcontinent and South Asia. Considering the mariculture potential of the host species, more studies on the pathology, host–parasite interactions, pattern of infection and epidemiology of the parasites are required.

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LITERATURE CITED

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Andrews JD (1988) Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effects on the oyster industry. Spec Publ Am Fish Soc 18:47–63

Azevedo C (1989) Fine structure of *Perkinsus atlanticus* n. sp. (Apicomplexa, Perkinsea) parasite of the clam *Ruditapes decussates* from Portugal. J Parasitol 75: 627–635

Blackbourn J, Bower SM, Meyer GR (1998) *Perkinsus qugwadi* sp. nov. (incertae sedis), a pathogenic protozoan parasite of Japanese scallops, *Patinopecten yessoensis*, cultured in British Columbia, Canada. Can J Zool 76: 942–953

Burreson EM (2008) Misuse of PCR assay for diagnosis of mollusc protistan infections. Dis Aquat Org 80:81–83

Burreson EM, Reece KS, Dungan CF (2005) Molecular, morphological, and experimental evidence support the synonymy of *Perkinsus chesapeaki* and *Perkinsus andrewsi.* J Eukaryot Microbiol 52:258–270

Carlini DB, Reece KS, Graves JE (2000) Actin gene family evolution and the phylogeny of coleoid cephalopods (Mollusca: Cephalopoda). Mol Biol Evol 17:1353–1370

Casas SM, Villalba A, Reece KS (2002) Study of perkinsosis in the carpet shell clam *Tapes decussatus* in Galicia (NW Spain). I. Identification of the aetiological agent and *in vitro* modulation of zoosporulation by temperature and salinity. Dis Aquat Org 50:51–65

Casas SM, Grau A, Reece KS, Apakupakul K, Azevedo C, Villalba A (2004) *Perkinsus mediterraneus* n. sp., a protistan parasite of the European flat oyster *Ostrea edulis* from the Balearic Islands, Mediterranean Sea. Dis Aquat Org 58:231–244

Coss CA, Robledo JAF, Ruiz GM, Vasta GR (2001) Description of *Perkinsus andrewsi* n. sp. isolated from the Baltic clam (*Macoma balthica*) by characterization of the ribosomal RNA locus, and development of a species-specific PCR-based diagnostic assay. J Eukaryot Microbiol 48: 52–61

Craig A, Powell EN, Fay RP, Brooks JM (1989) Distribution of *Perkinsus marinus* in Gulf Coast oyster populations. Estuaries 12:82–91

Dungan CF, Reece KS (2006) In vitro propagation of two *Perkinsus* spp. parasites from Japanese Manila clams *Venerupis philippinarum* and description of *Perkinsus honshuensis* n. sp. J Eukaryot Microbiol 53:316–326

Goggin CL, Lester RJG (1995) *Perkinsus*, a protistan parasite of abalone in Australia: a review. Mar Freshw Res 46: 639–646

Jayaraju N, Sundara Raja Reddy BC, Reddy KR (2009) Metal pollution in coarse sediments of Tuticorin coast, Southeast coast of India. Environ Geol 56:1205–1209

Lester RJG, Davis GHG (1981) A new *Perkinsus* species (Apicomplexa, Perkinsea) from the abalone *Haliotis ruber.* J Invertebr Pathol 37:181–187

Mackin JG (1962) Oyster disease caused by *Dermocystidium marinum* and other microorganisms in Louisiana. Publ Inst Mar Sci Univ Tex 7:132–229

Mackin JG, Owen HM, Collier A (1950) Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassostrea virginica* (Gemlin). Science 111:328–329

McLaughlin SM, Tall BD, Shaheen A, Elsayed EE, Faisal M (2000) Zoosporulation of a new *Perkinsus* species isolated from the gills of the softshell clam *Mya arenaria.* Parasite 7:115–122

Moss JA, Xiao J, Dungan CF, Reece KS (2008) Description of *Perkinsus beihaiensis* n. sp., a new *Perkinsus* sp. parasite in oysters of southern China. J Eukaryot Microbiol 55: 117–130

Murrell A, Kleeman SN, Barker SC, Lester RJG (2002) Synonymy of *Perkinsus olseni* Lester & Davis, 1981 and *Perkinsus atlanticus* Azevedo, 1989 and an update on the phylogenetic position of the genus *Perkinsus.* Bull Eur Assoc Fish Pathol 22:258–265

Muthiah P, Nayar KN (1988) Incidence of *Perkinsus marinus* in *Crassostrea madrasensis.* CMFRI Bull 43:232–235

Narasimham KA, Kripa V (2007) Textbook of oyster biology and culture in India. Directorate of Information and Publications of Agriculture, New Delhi

OIE (Office International des Épizooties) (2006) Manual of diagnostic tests for aquatic animals, 5th edn. Office International des Épizooties, Paris

Park KI, Choi KS (2001) Spatial distribution of the protozoan parasite *Perkinsus* sp. found in the Manila clams, *Ruditapes philippinarum*, in Korea. Aquaculture 203: 9–22

Patterson Edward JK, Patterson J, Mathews G, Wilhelmsson D (2005) Status of coral reefs of the Tuticorin coast, Gulf of Mannar, southeast coast of India. In: Souter D, Lindén O (eds) Coral reef degradation in the Indian ocean, status report 2005. CORDIO, University of Kalmar, Kalmar, p 119–127

Perkins FO (1996) The structure of *Perkinsus marinus* (Mackin, Owen and Collier 1950) Levine, 1978 with comments on taxonomy and phylogeny of *Perkinsus* spp. J Shellfish Res 15:67–87

Ray SM (1966) A review of the culture method for detecting *Dermocystidium marinum* with suggested modifications and precautions. Proc Natl Shellfish Assoc 54:55–69

Reece KS, Dungan CF, Burreson EM (2008) Molecular epizootiology of *Perkinsus marinus* and *P. chesapeaki* infections among wild oysters and clams in Chesapeake Bay, USA. Dis Aquat Org 82:237–248

Sabry RC, Rosa RD, Magalhães ARM, Barracco MA, Gesteira TCV, da Silva PM (2009) First report of *Perkinsus* sp. infecting mangrove oysters *Crassostrea rhizophorae* from the Brazilian coast. Dis Aquat Org 88: 13–23

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Sanil NK, Vijayan KK, Kripa V, Mohamed KS (2010) Occurrence of the protozoan parasite, *Perkinsus olseni* in the wild and farmed pearl oyster, *Pinctada fucata* (Gould) from the Southeast coast of India. Aquaculture 299:8–14

Soniat TM (1996) Epizootiology of *Perkinsus marinus* disease of Eastern oysters in the Gulf of Mexico. J Shellfish Res 15:35–43

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Villalba A, Reece KS, Camino Ordás M, Casas SM, Figueras A (2004) Perkinsosis in molluscs: a review. Aquat Living Resour 17:411–432

Winstead JT, Couch JA (1988) Enhancement of protozoan pathogen *Perkinsus marinus* infections in American oysters *Crassostrea virginica* exposed to the chemical carcinogen n-nitrosodiethylamine (DENA). Dis Aquat Org 5:205–213

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