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First report of *Perkinsus* sp. infecting mangrove oysters *Crassostrea rhizophorae* from the Brazilian coast

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ABSTRACT: Protozoan parasites of the genus *Perkinsus* are considered important pathogens responsible for mass mortalities in several mollusk species worldwide. In the present study we describe for the first time a parasite of the genus *Perkinsus* infecting the mangrove oyster *Crassostrea rhizophorae* from the Brazilian coast. Prevalence of this parasite was low in the Pacoti River estuary (Ceará, northeast Brazil) and absent in oysters from southern Brazil. Oyster gill and rectum tissues incubated in Ray's fluid thioglycollate medium (RFTM) revealed the presence of spherical hypnospores (5 to 55 µm diam.). Histological analysis showed the occurrence of typical signet-ring trophozoites and schizonts (3 to 6 µm diam.) infecting connective tissues of several organs and digestive epithelia. PCR assays specific to the genus *Perkinsus*, followed by cloning and sequencing of the internal transcribed spacer (ITS) region of the ribosomal ribonucleic acid (rRNA) gene complex, confirmed a close phylogenetic relationship between Brazilian *Perkinsus* sp. and *P. beihaiensis* infecting Chinese oysters.

KEY WORDS: *Crassostrea rhizophorae* · Mangrove oyster · *Perkinsus* sp. · Protozoan parasite · PCR-RFLP · rRNA · RFTM · Sporulation

INTRODUCTION

The state of Santa Catarina in southern Brazil is the country's largest producer (95%) of bivalve mollusks (11300 tons in 2007). This activity is of great social and economic importance for the region. The brown mussel *Perna perna* and the Pacific oyster *Crassostrea gigas* are the main cultured species, with scallop *Nodipecten nodosus* and mangrove oyster *C. rhizophorae* culture existing on a smaller scale. Northeast Brazil has an extensive system of estuaries and mangroves that are inhabited by several oyster populations (*C. brasiliana* and *C. rhizophorae*). These oysters are widely consumed by local peoples. Accordingly, several attempts have been made to develop native oyster aquaculture in that region, with modest results to date.

In 2003, the Brazilian government started the National Program for Aquatic Animal Health (PNSAA) to help prevent the introduction of exotic animal diseases and to eradicate those already present in the national territory. Guided by this initiative, in 2007 we began a research project to diagnose parasites and dis-

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eases affecting edible and cultured bivalve species from 2 Brazilian regions. The project was designed to survey parasites among wild and cultivated populations of oysters *Crassostrea rhizophorae* and *C. gigas* from Santa Catarina Island and the Pacoti River estuary (Fortaleza, in the state of Ceará, northeast Brazil). The results provide data on distributions of pathogens and on host susceptibilities to pathogens, which will qualify further monitoring programs, help avoid disease transfers from infected populations, and inform efforts to keep unaffected areas parasite-free.

The first described parasite of the genus *Perkinsus* was *P. marinus* (Mackin et al. 1950), which was identified as being responsible for mortality outbreaks among *Crassostrea virginica* oyster stocks from Louisiana, USA. It was first described as *Dermocystidium marinum*, based partly on characteristics of hypertrophic hypnospores and zoosporangia that enlarge among infected oyster tissues when they are incubated in a high-salt formulation of Ray's fluid thioglycollate medium (RFTM) (Ray 1952). Since that time, diverse *Perkinsus* spp. have been reported to infect many important commercially cultured mollusk species worldwide.

Perkinsus olseni was first described as a pathogen of Australian abalone *Haliotis ruber* (Lester & Davis 1981). Azevedo (1989) described the species *P. atlanticus* infecting the carpet shell clam *Ruditapes decussatus* in Portugal. *P. olseni*, which is currently synonymous with *P. atlanticus*, has wide geographic and host ranges. It has subsequently been reported in other bivalve species, such as *Pitar rostrata* from Uruguay (Cremonte et al. 2005) and *R. philippinarum*, *Venerupis pullastra*, and *Paphia aurea*, from the Atlantic and Mediterranean coasts of Spain (Navas et al. 1992, Sagristà et al. 1995, Ordás et al. 2001). However, many of the above studies were based on morphological characteristics, without molecular characterizations. Casas et al. (2002a) described the first use of molecular biology methods to characterize *P. olseni* infecting *R. decussatus* from Spain. Since then, molecular tools have been used to describe and confirm *P. olseni* infecting several bivalve species such as *R. decussatus* from the Mediterranean coast of Spain (Elandaloussi et al. 2009); *R. philippinarum* from Italy (Abollo et al. 2006), South Korea (Park et al. 2005), and China (Zhang et al. 2005); *Protothaca jedoensis* from South Korea (Park et al. 2006); *Austrovenus stutchburyi* from New Zealand (Dungan et al. 2007); and *Tridacna crocea* from Vietnam (Sheppard & Phillips 2008).

Since a recent review on perkinsosis (Villalba et al. 2004), in which 6 *Perkinsus* species (*P. marinus*, *P. olseni*, *P. qugwadi*, *P. chesapeaki*, *P. andrewsi*, and *P. mediterraneus*) were considered valid, data on the molecular genetics of the species has increased. As a result, improved molecular characterizations show that *P. chesapeaki* and *P. andrewsi* are synonymous (Burreson et al. 2005), and 2 additional new *Perkinsus* species were described: *P. honshuensis* infecting Manila clams in Japan (Dungan & Reece 2006) and *P. beihaiensis* infecting *Crassostrea hongkongensis*, *C. ariakensis*, and other bivalve hosts from Fujian to Guangxi provinces in southern China (Moss et al. 2008).

Among Brazilian mollusk hosts, *Crassostrea rhizophorae* is reported as susceptible to *Perkinsus marinus* after experimental infections (Bushek et al. 2002). Based on RFTM assay results, Littlewood (2000) reported natural infections by *P. marinus* among mangrove oysters *C. rhizophorae* from Jamaica, although molecular analyses were not used to confirm parasite identity. Therefore, a surveillance program for perkinsosis was initiated along the geographic distribution of this oyster species in Brazilian waters. The present study provides detailed characteristics on the first report of a *Perkinsus* sp. infection in oysters *C. rhizophorae* from Brazil. Infections were detected by RFTM assays; parasites were confirmed *in situ* and characterized by histology; and the pathogen was partially characterized by molecular-genetic methods.

MATERIALS AND METHODS

Sampling. Adult mangrove oysters *Crassostrea rhizophorae* were collected at Santa Catarina Island during March and April 2008, and at Fortaleza during August 2008. At Santa Catarina Island, samples were collected in summer, because of the increase in temperature that could favor parasite proliferation. At Fortaleza, samples were collected during winter, because seawater temperature there does not vary during the year (authors' pers. obs.). Oysters were sampled and maintained in tanks with aerated, raw seawater until analysis, for a maximum of 3 d.

At Santa Catarina Island, native oysters were sampled at 2 sites, located on the northern (Sambaqui; 27° 29′ 18″ S, 48° 32′ 1″ W) and on the southern (Ribeirão da Ilha; 27° 42′ 51″ S, 48° 34′ 6″ W) bays of the island (Fig. 1). Oysters from Ribeirão da Ilha and Sambaqui inhabited the natural rocky shores of the intertidal zone between 200 and 450 m, respectively, from *Crassostrea gigas* culture areas.

At Fortaleza, oysters were sampled at 3 different sampling sites located inside the Pacoti River estuary: Site 1 (3° 49′ 8″ S, 38° 25′ 9″ W), Site 2 (3° 49′ 15″ S, 38° 25′ 10″ W), and Site 3 (3° 49′ 19″ S, 38° 25′ 11″ W), where no *Crassostrea gigas* oyster culture exists (Fig. 1). The Pacoti River is the most important waterway flowing through the metropolitan region of Fortaleza. The estuary is characterized by the presence of a rich mangrove flora, including *Rhizophora mangle*,

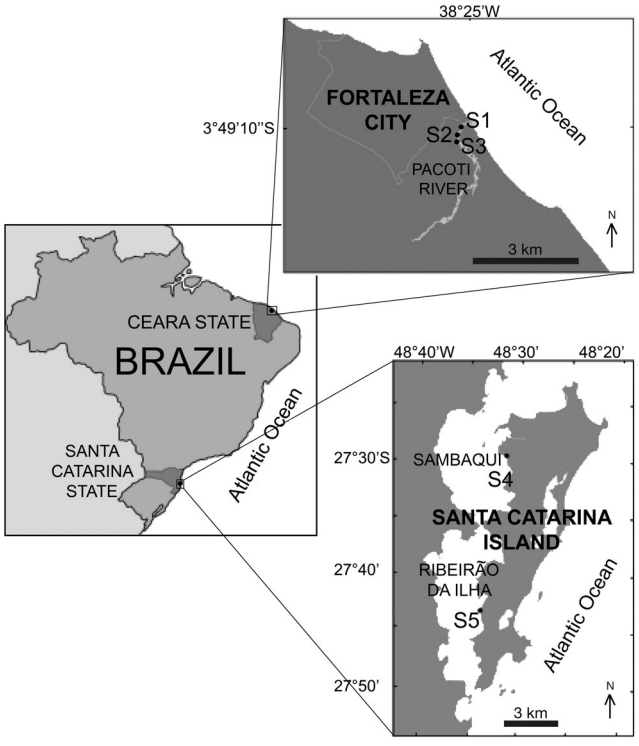


Fig. 1. Oyster sampling sites (S) at Pacoti River estuary (Fortaleza) Santa Catarina Island (Ribeirão da Ilha and Sambaqui)

whose roots form the substrate for natural oyster settlement, from which oysters were collected for the present study.

Seawater temperature and salinity were measured on the day of sampling at each site, near the surface (0.5 m), using an immersion thermometer and a refractometer, respectively. A total of 150 oysters were analyzed from each site. Oysters (n = 750) were opened, observed for gross abnormalities, and processed using the recommended Office International des Epizooties (OIE) technique for screening *Perkinsus* sp. using RFTM (OIE 2006). Additionally, tissues from various oysters were randomly or specifically selected for other diagnostic techniques, as follows.

Histological analysis. Among the 150 oysters sampled per site, 30 were randomly selected for histology. Oysters were shucked and a transverse section of 5 mm thickness including gills, gonad, and digestive tissues was excised and fixed in Davidson's fixative (Shaw & Battle 1957) for 48 h. Fixed tissues were embedded in paraffin, sectioned at 5 µm thickness, and stained with hematoxylin and eosin (H&E) (Howard et al. 2004).

Detection of *Perkinsus* sp. by RFTM assays. Oysters collected from each site (n = 150) were shucked and the 2 branchial lamellae from one side of the body and the rectum were excised and incubated in RFTM for 7 d in the dark at room temperature. Samples were prepared for microscopic analysis by individually chopping the RFTM-incubated tissues with a scalpel, adding drops of Lugol's solution on microscope slides, and examining the resulting preparations by light microscopy for dark-stained, spherical *Perkinsus* sp. hypnospores. The prevalences of *Perkinsus* sp. infections were estimated as the percentages of infected oysters in each sample (n = 150).

Each oyster was rated using the following infection intensity scale: 0 = no infection, no hypnospores observed on the whole slide; 1 = very light infection, up to 10 hypnospores observed on the whole slide; 2 = light infection, from 11 to 100 hypnospores observed on the whole slide; 3 = moderate infection, at least 40 hypnospores observed in each of 10 microscope fields (40×) scattered over the tissue; and 4 = heavy infection, more than 40 hypnospores observed in each of 10 microscope fields (40×) scattered over the tissue.

***In vitro* zoosporulation.** Two selected RFTM-positive tissues from an extra August 2008 sampling of Fortaleza oysters were washed twice with filtered sterilized seawater (FSSW) and transferred to culture plate wells containing 2 ml of FSSW. The plates were incubated for 48 to 96 h at room temperature (approx. 28°C) and examined periodically by light microscopy for zoosporulation.

DNA extraction and PCR assays. Gill tissues from selected oysters were preserved in 95% ethanol for subsequent extraction of their preserved DNA for use as PCR template samples. Among 150 oysters collected at each of the 2 Santa Catarina Island sample sites, DNA from the gill tissues of the same 30 oysters randomly selected for histological assays were also preserved for PCR assays (n = 60). From the 3 samples collected at sites in the Pacoti River estuary near Fortaleza, gill tissues from all 150 oysters from Sites 1 and 3 were preserved for PCR assays, while only the 30 oysters from Site 2 selected for histology were preserved for PCR assays (n = 330 total).

DNA of 29 oysters from Fortaleza was analyzed for *Perkinsus* sp. by PCR, including 21 that were positive by RFTM assays, 6 that were randomly selected among oysters that were RFTM-negative (2 from each site), and 2 whose tissues were used to confirm zoosporulation induction after RFTM incubation. No samples from Santa Catarina Island were analyzed by PCR, because all of their RFTM assays were negative.

DNA extraction was performed with DNAzol® reagent (Invitrogen) following the manufacturer's protocol. For PCR assays we used PerkITS 85/750 primers that specifically hybridize at conserved regions of the internal transcribed spacer (ITS) region of the ribosomal ribonucleic acid (rRNA) gene complex that are unique to members of the genus *Perkinsus* (except for *P. qugwadi incertae sedis*) (Casas et al. 2002a). A posi-

tive control used *P. olseni* cells isolated *in vitro* from Galicia, Spain (provided by Dr Antonio Villalba). Negative controls used nuclease-free water instead of template DNA.

PCR reactions were performed in 25 µl reactions containing 1 µl (50 to 100 ng) of genomic sample DNA, PCR buffer at 1× concentration, $MgCl_2$ at 1.5 mM, nucleotides at 0.2 mM each, primers at 0.8 µM, and 1 unit of *Taq* DNA polymerase (Invitrogen). The cycling protocol included template DNA denaturation at 94°C for 10 min; 35 amplification cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; followed by a 72°C final extension for 10 min. PCR products were separated on a 1.5% agarose gel made and bathed in 1× Tris-EDTA buffer (TE) and stained with ethidium bromide.

PCR-RFLP assays. *Perkinsus* spp. identification was conducted using restriction fragment length polymorphism (RFLP) assays based on PCR amplification and restriction endonuclease digestions of ITS-region PCR amplicons (Abollo et al. 2006). PCR amplifications were performed as described above, and the restriction reactions were carried out with the enzymes *Rsa*I or *Hin*fI, following the manufacturer's directions (Promega). Briefly, restriction reactions were carried out in a final volume of 20 µl containing 3 µl of PCR products, 2 µl of enzyme buffer, 0.2 µl of BSA, and 0.5 µl of restriction enzyme (*Rsa*I or *Hin*fI) diluted in sterilized distilled water. The digestions were performed during 2 h at 37°C, followed by endonuclease inactivation for 20 min at 65°C. To improve visualization of the restriction fragment patterns, 10 µl of endonuclease-digested PCR products were resolved on a 6% non-denaturing polyacrylamide gel stained with ethidium bromide. Restriction profiles from the *Perkinsus* sp. infection of mangrove oyster *Crassostrea rhizophorae* (n = 7) were compared with those described for other *Perkinsus* species (Abollo et al. 2006) as well as with those from *P. olseni* isolate DNA. Restriction profiles expected by digestion of the ITS region of *Perkinsus* sp. DNA with *Rsa*I or *Hin*fI were determined using Restriction Mapper v.3.0 software (http://www.restrictionmapper.org).

Molecular cloning, DNA sequencing, and phylogenetic analysis. Freshly amplified PCR products of the ITS region from the *Perkinsus* sp. of 2 infected oysters *Crassostrea rhizophorae* were cloned into a pCR 2.1 TA cloning vector, using a TA cloning kit (Invitrogen). The positive recombinant clones were identified by colony PCR with the M13 vector primers (Invitrogen), and 1 bacterial colony of each sample was sequenced in both directions. Sequencing was performed in an automated MegaBace 1000 DNA Analysis System, using the DYEnamic ET Dye Terminator kit (GE Healthcare).

The *Perkinsus* sp. ITS sequences obtained were subjected to BLASTN searches (Altschul et al. 1997) against sequences from the National Center for Biotechnology Information (NCBI). The sequences were aligned with previously reported ITS sequences obtained from the GenBank database, using the Clustal X program (Thompson et al. 1997). GenBank sequences included in the ITS region analysis are given in Table 1. The phylogenetic tree was computed using MEGA 4 software (Tamura et al. 2007) using the maximum parsimony method. Bootstrap values (%) were calculated from 1000 replicates, and the cut-off value used for a condensed tree was 65%.

RESULTS

The mean shell heights (±SD) of oysters collected from Sambaqui, Ribeirão da Ilha, and Sites 1, 2, and 3 from the Pacoti River estuary were 57 ± 4.5 mm, 55 ± 4.4 mm, 58 ± 8.4 mm, 50 ± 5.4 mm, and 50 ± 5.2 mm, respectively. The temperatures (and salinities) at those sites were 24.2°C (33.5‰), 22.0°C (26.5‰), 30.8°C (32‰), 29.0°C (29‰) and 29.8°C (23.3‰), respectively.

Perkinsus sp. diagnosis by RFTM assays

The RFTM assay was used as the first and the primary diagnostic technique for *Perkinsus* sp. All oysters from Sambaqui and Ribeirão da Ilha were negative for perkinsosis in RFTM assays. In contrast, the Pacoti River estuary (Fortaleza) had oysters infected by a *Perkinsus* sp. Enlarged parasite cells were spherical,

Table 1. GenBank sequences included in the *Perkinsus* sp. internal transcribed spacer (ITS) region analysis

Species	Accession nos.
P. beihaiensis	EF204019, EF204020, EF204021, EU068087, EU068091, EU068092
P. chesapeaki	AY876302, AY876304, AY876305, AY876311, AY876314
P. honshuensis	DQ516696, DQ516697, DQ516698, DQ516701, DQ516702
P. marinus	AY295180, AY295188, AY295189, AY295194, AY295199
P. mediterraneus	AY487834, AY487837, AY487842, DQ370491, DQ370492
P. olseni	AF441207, AF441209, AF441213, AY435092, AY820757
P. qugwadi (outgroup taxon)	AF151528

had 5 to 55 µm diameters, and were light- or dark-stained, depending on the depth of penetration of oxidizing iodine into infected tissue samples (Fig. 2). The mean prevalence of infected oysters from samples from the Pacoti River estuary was 5.78% (26 of 450) with a range among oyster samples from different sites of 5.3 to 6.7% (Table 2). Among 26 RFTM-positive oysters, 22 showed very light infection, and 4 (from Sites 1 and 3) showed heavy infection (Fig. 2).

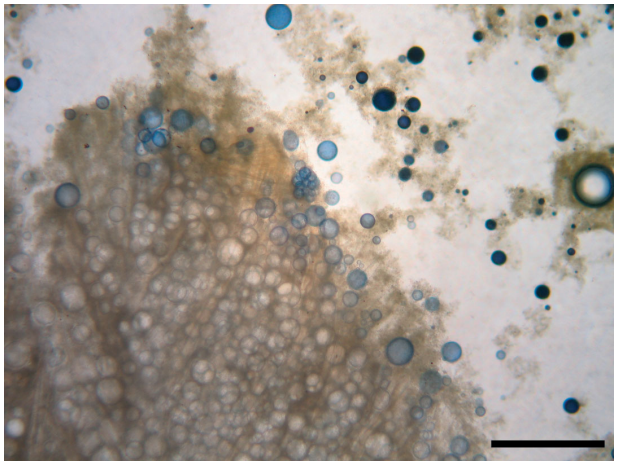


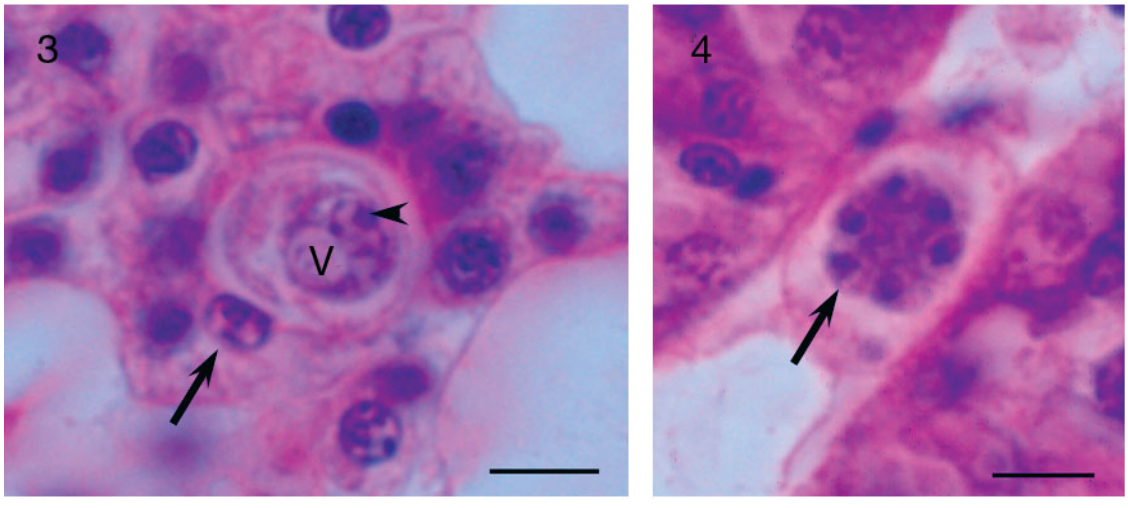
Fig. 2. Low magnification image of numerous enlarged *Perkinsus* sp. hypnospores from and within gill tissues of a heavily infected *Crassostrea rhizophorae* after incubation in Ray's fluid thioglycollate medium (RFTM) and staining with Lugol's solution. Numerous hypnospores confined within gill tissues did not come in contact with iodine and remained unstained. Scale bar = 200 µm

Histological analysis

Oysters from Santa Catarina Island did not show any trophozoites of *Perkinsus* sp. infection in histological tissue samples. Samples of oysters from the Pacoti River estuary contained 26 oysters with enlarged hypnospores of *Perkinsus* sp. after RFTM incubation, but tissues of only 7 of those oysters were fixed and embedded in paraffin for histological analysis (Table 2). Of those 7 oysters, only one (from Site 3; heavily infected by *Perkinsus* sp. by RFTM) was positive by histological sections, and the remaining oysters (with very light infection by RFTM) did not show any parasite in histological sections. The histologically confirmed *Perkinsus* sp. infection was characterized by the presence of trophozoites (3 to 6 µm diam.) and actively proliferating schizonts (4 to 6 µm diam.) among the connective tissues (Figs. 3 & 4) of mantle, labial palps, and digestive gland; although some epithelial cells of digestive tubules were also infected in that oyster. Trophozoites of *Perkinsus* sp. presented typical signet-ring morphology, with an eccentric vacuole occupying a large area of the cytoplasm, and an

Table 2. Prevalence (%) of *Perkinsus* sp. infection among *Crassostrea rhizophorae* from the Pacoti River estuary (Fortaleza) based on Ray's fluid thioglycollate medium (RFTM) assays and histology. Numbers in parentheses represent positive oysters per total number of samples analyzed by each technique

Sample site	RFTM	Histology
1	5.3% (8 of 150)	3.3% (1 of 30)
2	5.3% (8 of 150)	10.0% (3 of 30)
3	6.7% (10 of 150)	10.0% (3 of 30)



Figs. 3 & 4. *Perkinsus* sp. infecting connective tissues of the mangrove oyster *Crassostrea rhizophorae*. Fig. 3. Engulfed trophozoite showing typical signet-ring appearance, a large vacuole (V), and an eccentric nucleus with prominent nucleolus (arrowhead). Note: nucleus (arrow) of hemocyte that has phagocytosed the trophozoite. Fig. 4. *Perkinsus* sp. schizont (arrow) containing small daughter cells in the connective tissue between 2 digestive gland tubules. Hematoxylin and eosin. Scale bars = 5 µm

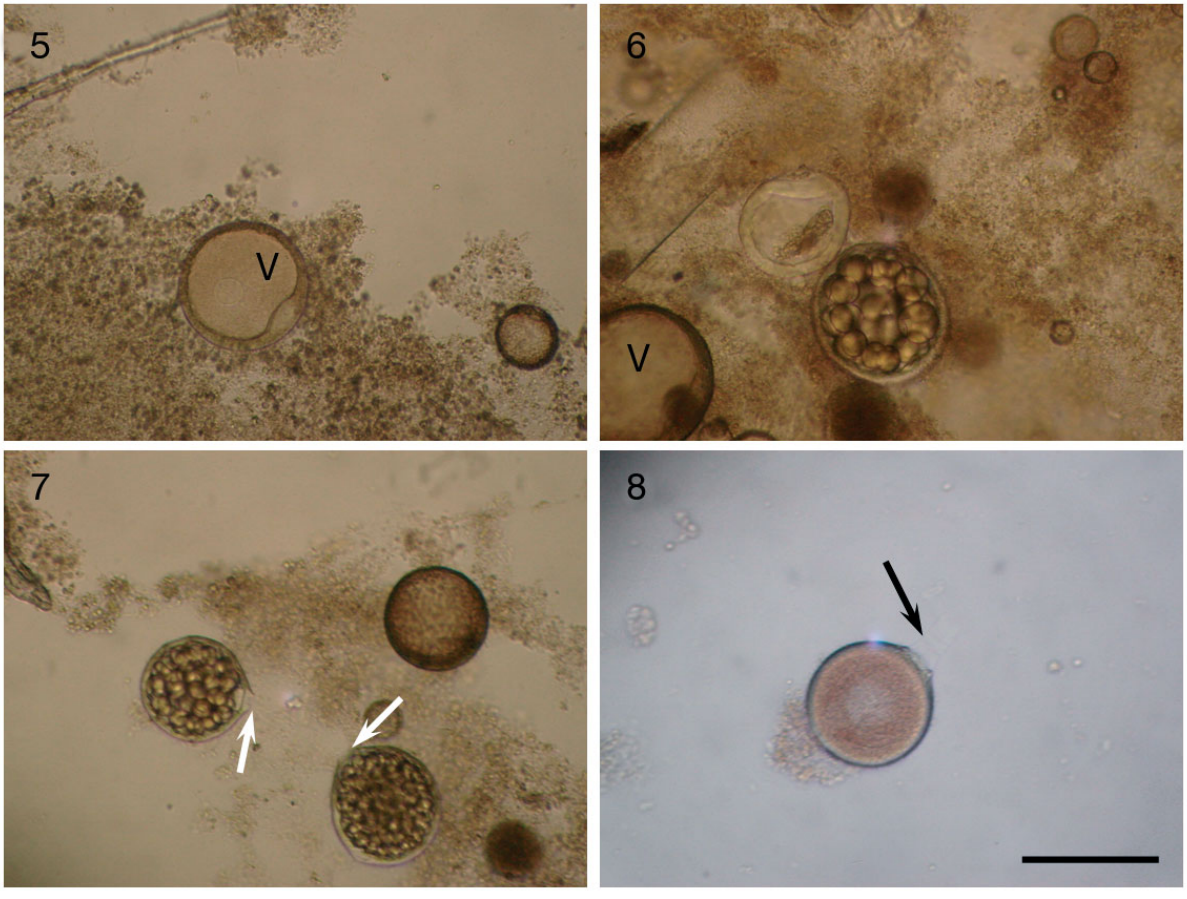
eccentric nucleus with a patent nucleolus (Fig. 3). Trophozoites and schizonts were systemically seen engulfed by hemocytes (Figs. 3 & 4), surrounded by an acellular acidophilic matrix, and apparently healthy. Hemocytic infiltration was not associated with parasites. Observations on additional infected oysters are required to comprehensively ascertain the effects of *Perkinsus* sp. on *Crassostrea rhizophorae* host tissues.

Parasite zoosporulation

After 48 to 96 h of FSSW incubation of RFTM-enlarged *Perkinsus* sp. hypnospores, zoosporulation took place. Various life-stages of parasite were observed, including mature, pre-sporulation hypnospores, zoosporangia showing progressive and proliferative reductive subdivisions of zoosporonts, clusters of sibling zoosporont cells, and mature zoosporangia containing numerous motile zoospores (Figs. 5 to 8). Only one discharge tube occurred on each zoosporangium (Figs. 7 & 8). Zoospores (2 µm) were observed leaving the discharge tube.

Detection of *Perkinsus* sp. by PCR

A total of 26 oysters tested positive for *Perkinsus* sp. by RFTM assays: 8 from Site 1; 8 from Site 2; and 10 from Site 3. Among those 26 RFTM-positive oysters, 21 had tissue DNA preserved for PCR analysis: 8, 3, and 10 oysters from Sites 1, 2, and 3, respectively. The PerkITS primer pair yielded amplicons of the expected size (687 bp) from 16 of 21 (76%) samples (Fig. 9), and 5 were negative (2 from Site 1 and 3 from Site 3). Assays were repeated twice for negative samples. Oysters that were RFTM-positive and PCR-negative for *Perkinsus* sp. presented gills with low intensities of infection by RFTM assays. Six RFTM-negative samples (2 from each site of the Pacoti River estuary) were also negative by PCR. Two extra samples used for parasite



Figs. 5 to 8. *In vitro* sporulation of *Perkinsus* sp. in filtered sterilized seawater. Fig. 5. Hypnospore with a large central vacuole (V) and a peripheral nucleus bulging into that vacuole. Fig. 6. Zoosporangium containing approximately 32 subdividing zoosporont cells. Fig. 7. Two zoosporont cells undergoing advanced zoosporulation stages and showing zoosporangium discharge tubes (arrows). Fig. 8. Zoosporangium with distended discharge tube (arrow) and numerous zoospores. Scale bars = 50 µm

zoosporulation experiments were analyzed by PCR, and both produced positive amplicons.

PCR-RFLP assays

The *Rsa*I endonuclease produced 3 fragments from PCR amplicons for all 7 of the *Crassostrea rhizophorae* oysters infected by *Perkinsus* sp. (Fig. 10). Sizes of those fragments corresponded to approximately 74, 195, and 418 bp, as predicted by subsequent sequence analysis. The *in vitro* isolate of *P. olseni* produced 3 fragments with similar, but not identical, band sizes (Fig. 10).

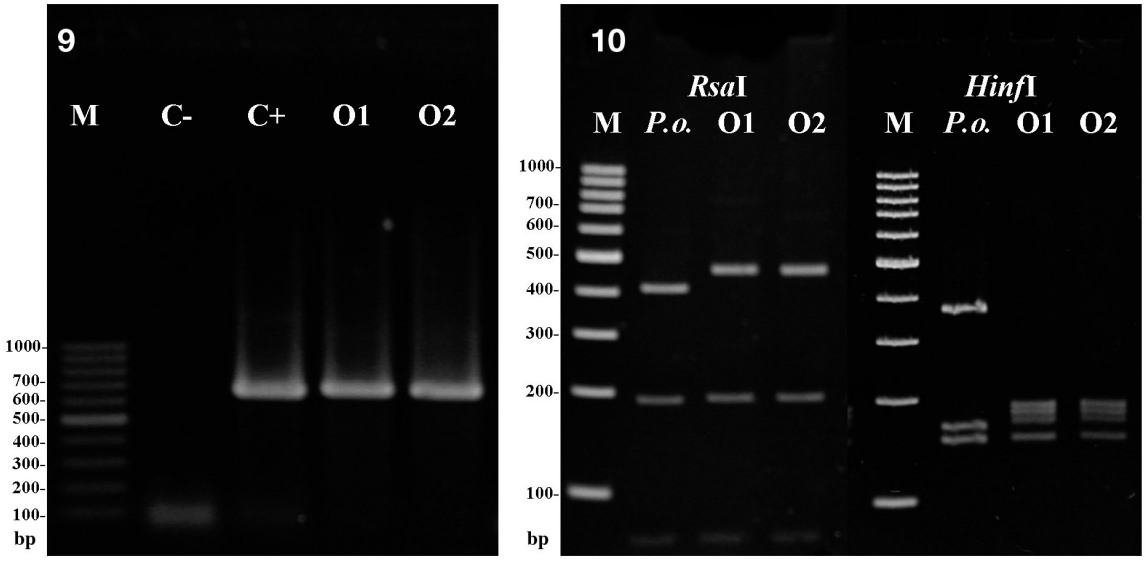
RFLP patterns from *Hin*fI digestions of PCR amplicons from the *Perkinsus* sp. that infected Brazilian *Crassostrea rhizophorae* oysters all contained 5 fragments. Four of those were individually resolved on the polyacrylamide gel at 189, 173, 161, and 153 bp. An additional, non-visualized, very small fragment (Fig. 10) of 11 bp was predicted by subsequent sequence analysis. In contrast, by similar analysis with the same endonuclease, *P. olseni* showed only 3 fragments (Fig. 10).

Phylogenetic analysis of rDNA ITS region

Phylogenetic analysis of the DNA sequences of parasite ITS region were performed to evaluate the taxonomic affinities of the Brazilian *Perkinsus* sp. The expected ITS region products of 687 bp amplified from 2 PCR-positive *Crassostrea rhizophorae* oysters were sequenced and compared with corresponding sequences reported for other *Perkinsus* species. Nucleotide sequences of the ITS region of the Brazilian *Perkinsus* sp. were deposited in GenBank under accession numbers FJ472346 and FJ472347.

BLASTN searches indicated that the ITS region nucleotide sequences of both Brazilian PCR isolates displayed greatest identity (97%) and also showed close similarities to sequences from the ITS regions of other *Perkinsus* species. Both of the sequences from the Brazilian *Perkinsus* sp. shared high nucleotide identities (~98%) with those of the recently described *P. beihaiensis* infection in Chinese oyster *Crassostrea ariakensis* and *C. hongkongensis* (Moss et al. 2008). They were also moderately similar to the ITS region sequences of *P. mediterraneus* (~87%), *P. olseni* (~86%), *P. honshuensis* (~86%), *P. marinus* (~82%), and *P. chesapeaki* (~82%); whereas *P. qugwadi* (outgroup) showed the lowest homology (<40%).

Maximum parsimony analysis indicated a close phylogenetic relationship between both Brazilian *Perkinsus* sp. PCR isolates and *P. beihaiensis*. The ITS region sequences obtained were grouped with strong support in a monophyletic clade close to *P. beihaiensis*, which is sister to another monophyletic clade containing *P. olseni*, *P. marinus*, *P. mediterraneus*, and *P. honshuensis*. The most distant group from the Brazilian PCR isolates, apart from the *P. qugwadi* outgroup, was *P. chesapeaki* (Fig. 11).



Figs. 9 & 10. Molecular diagnosis of *Perkinsus* sp. infecting the mangrove oyster *Crassostrea rhizophorae*. Fig. 9. rDNA ITS-region PCR for *Perkinsus* sp. detection. M: 100 bp molecular size markers; C–: negative control (water); C+: positive control (*P. olseni* isolate DNA); O1: oyster 1 (*Perkinsus* sp.); O2: oyster 2 (*Perkinsus* sp.). Fig. 10. PCR-RFLP for differential diagnosis of *Perkinsus* spp. Fragmentation patterns of PCR-amplified products of the rDNA ITS region from *P. olseni* (*P.o.*) and the *Perkinsus* sp. (O1 and O2) after digestion with *Rsa*I and *Hin*fI endonucleases. See Fig. 9 for abbreviations

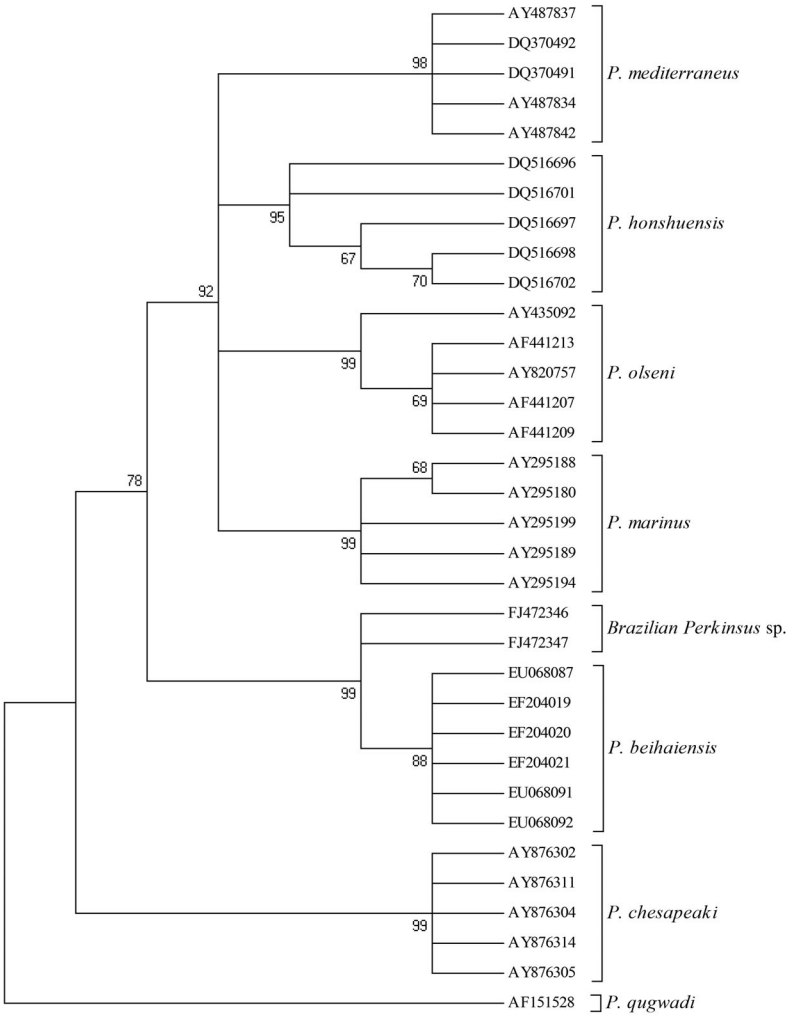


Fig. 11. Maximum parsimony analysis of the rDNA ITS region sequences of different *Perkinsus* species. Numbers at nodes show bootstrap values (%) for 1000 replicates

DISCUSSION

This study describes, for the first time in a Brazilian mollusk, the presence of a protozoan *Perkinsus* sp. infection in mangrove oysters *Crassostrea rhizophorae* from the Pacoti River estuary and not detected in oysters from southern Brazil. The parasite was detected by RFTM assays that are reported as a sensitive and reliable diagnostic technique for *Perkinsus* sp. screening (Reece & Dungan 2006) and confirmed histologically. PCR assays specific to the genus *Perkinsus*, followed by cloning and sequencing of ITS region amplicons conserved among known *Perkinsus* spp. (except *P. qugwadi* s*incertae sedis*) (Casas et al. 2002a), further confirmed and qualified these findings.

The differences in size between the trophozoites observed in histological sections and the hypnospores observed after incubation of infected tissues in RFTM demonstrate enlargement of trophozoites in RFTM, which is characteristic of *Perkinsus* spp. (Ray 1954, 1966), except *P. qugwadi* (Blackbourn et al. 1998). The timing and morphological changes throughout the process of zoosporulation and the morphological characteristics of zoospores are all consistent with observations from other *Perkinsus* spp. (Sunila et al. 2001, Casas et al. 2002b, Dungan & Reece 2006, Dungan et al. 2007, Moss et al. 2008).

In histological sections, cells of the *Perkinsus* sp. infecting *Crassostrea rhizophorae* were small (3 to 6 µm) and showed the signet-ring morphology typical for members of the genus. Moreover, this *Perkinsus* sp. showed some similarities with *P. beihaiensis* infection in Chinese oysters (Moss et al. 2008), such as small size, prevalent low-intensity infections, and infection of digestive epithelia. In contrast, epithelia of Japanese clams *Ruditapes philippinarum* were not typically infected by *P. honshuensis* or *P. olseni* (Dungan & Reece 2006), which have nearly exclusive distributions among connective tissues (Dungan et al. 2007). However, pathological consequences of infections among *C. rhizophorae* from northeastern Brazil may have been underestimated due to the low number of heavily infected oysters (1 of 26, 3.85%) that were available for histological analysis. Here we report histological results from one heavily infected oyster. *Perkinsus beihaiensis* caused necrotic lesions among stomach, intestine, and digestive gland epithelia of infected Chinese oysters (Moss et al. 2008).

Phagocytosis is the most important cellular defense mechanism in bivalves, and *Perkinsus* sp. cells, due to their small size, were found phagocytosed by haemocytes of *Crassostrea rhizophorae*. However, the response might have been ineffective in killing the parasite, since integral schizonts and trophoites were observed inside phagocytic hemocyte vacuoles. This response is common among oysters infected by *P. marinus* (La Peyre et al. 1995, Villalba et al. 2004) and was observed in oysters heavily infected by *P. beihaiensis* (Moss et al. 2008). In contrast, *P. olseni*, whose trophozoites are larger, often elicits encapsulation instead of phagocytosis as host reactions against the parasite (Montes et al. 1995, Park & Choi 2001, Dungan & Reece 2006).

In the present study, the prevalence of *Perkinsus* sp. infections was relatively low, and consistent with the fact that no epizootic mortalities occurred among oys-

ters in the affected populations that were surveyed during this investigation. *P. marinus*, one of the most harmful species for its host, the eastern oyster *Crassostrea virginica* in the United States, regularly causes high prevalences of high-intensity infections that result in epizootic seasonal oyster mortalities (Ragone-Calvo et al. 2003). In contrast, *P. marinus* infecting *C. corteziensis* in Mexico showed very low prevalences (1 to 6%) of typically low-intensity infections (Gullian-Klanian et al. 2008). *P. beihaiensis*, which is widespread along the southern China coast, infects at least 2 oyster species at variable prevalences, depending on the site (Moss et al. 2008).

In the present study, PCR assays did not confirm (5 of 21, 25%) RFTM-positive cases. The most likely reason for this discrepancy was the generally low intensities of most infections and the small volumes of gill tissues from which the template DNA for PCR assays were extracted and amplified. In our protocol, relatively large volumes of gill tissues were assayed by RFTM assays; while relatively small sub-samples of DNA from those tissues were tested by PCR assays. Similar discrepancies between results of RFTM and PCR assays have been described by others (Reece et al. 2009), and diverse sources of sampling error artifacts that qualify the accuracies of PCR assays have been reviewed, including the effects of focal or localized lesions (Burreson 2008).

Despite the fact that *Perkinsus olseni* infections are reported in clams *Pitar rostrata* from neighboring Uruguay (Cremonte et al. 2005), no *Perkinsus* sp. infections were found among oysters *Crassostrea rhizophorae* sampled at our survey sites in the major oyster-producing region of southern Brazil, which is located 1360 km from the Uruguayan capital. The allocation of the Brazilian parasite to the genus *Perkinsus* was further confirmed when the ITS region was amplified through PCR with genus-specific primers (Casas et al. 2002a). The restriction patterns of the amplified region obtained through digestion with 2 endonucleases allow discrimination between the *Perkinsus* sp. infection in *C. rhizophorae* from the Brazilian coast from at least 4 *Perkinsus* spp. (*P. marinus*, *P. chesapeaki*, *P. olseni*, and *P. mediterraneus*) whose PCR-RFLP profiles are available for comparison (Abollo et al. 2006). Those results indicate that the *Perkinsus* sp. infecting *C. rhizophorae* does not belong to either the *Perkinsus* species previously reported in Uruguay (*P. olseni*) (Cremonte et al. 2005), or from the same oyster species in Jamaica (*Perkinsus* cf. *marinus*) (Littlewood 2000), although the latter study did not use molecular tools for parasite species identification.

The *Rsa*I endonuclease RFLP pattern of the Brazilian *Perkinsus* sp. differed slightly from that of *P. olseni* and *P. mediterraneus,* and consistently differed from that of *P. chesapeaki* and *P. marinus* (Abollo et al. 2006). In contrast, the *Hin*fI RFLP pattern of the *Perkinsus* sp. was only similar with that of *P. mediterraneus*, which also showed 4 fragments (149, 162, 179, and 192 bp) (Abollo et al. 2006). Subtle differences between RFLP band patterns obtained from the Brazilian *Perkinsus* sp., *P. olseni* (*Rsa*I), and *P. mediterraneus* (*Hin*fI), were more easily resolved on 6% polyacrylamide gels than on agarose gels (Abollo et al. 2006). Accordingly, we recommend the use of non-denaturing polyacrylamide gels to improve the sensitivity for differential diagnosis of *Perkinsus* spp. by PCR-RFLP. However, RFLP alone cannot be used to ascertain parasite identification, especially for a possible new member of the *Perkinsus* genus. Phylogenetic analysis based on nucleotide sequences of its rDNA ITS region consistently placed the Brazilian PCR isolates within the genus *Perkinsus*, and revealed high homology with sequences from the recently described *P. beihaiensis* infection of Chinese oysters (Moss et al. 2008). Unfortunately the RFLP pattern for *P. beihaiensis* and *P. honshuensis* and 2 more recently described *Perkinsus* species could not be included in our analysis, since that DNA was not available for testing.

The maximum parsimony analysis showed that the 2 sequences from the Brazilian *Perkinsus* sp. formed a distinct monophyletic clade close to the *P. beihaiensis* clade. Nevertheless, to rigorously evaluate apparent phylogenetic relationships that may exist between *P. beihaiensis* and the *Perkinsus* sp. infection of mangrove oyster *Crassostrea rhizophorae* will require extended analysis of nucleotide sequences from additional genetic loci.

We concluded that the morphological characteristics observed by light microscopy, such as enlargement in RFTM, the signet-ring appearance of trophozoites, as well as our diverse and consistent molecular genetic results, conclusively support the inclusion of the parasite infecting *Crassostrea rhizophora*e as a member of the genus *Perkinsus*. Moreover, some similarities between this *Perkinsus* sp. and *P. beihaiensis*, based on histological and molecular-genetic characteristics, suggest a taxonomic affinity. It is surprising that a *Perkinsus* sp. found in a Brazilian mollusk host would show potential taxonomic affinities with a parasite found in Chinese oysters, rather than with *Perkinsus* spp. from neighboring regions, including *P. marinus* infecting *C. virginica* in the Gulf of Mexico (Gullian-Klanian et al. 2008) and *P. olseni* infecting *Pitar rostrata* off the coast of Uruguay (Cremonte et al. 2005). Future challenges include a full description of the *Perkinsus* sp. infection in Brazilian oyster *C. rhizophorae*; establishment of *in vitro* isolate cultures for definitive identification and experimental work; and determinion of the host range, pathological effects, and epidemiology of infections.

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