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Short Communication

First record of *Perkinsus chesapeaki* infecting *Crassostrea rhizophorae* in South America

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ABSTRACT

This study investigated *Perkinsus* spp. infecting *Crassostrea rhizophorae* from the Jaguaribe River estuary, Ceará, Brazil. Fragments of gills and rectum of the oysters (n = 150) were incubated in Ray's fluid thioglycollate medium (RFTM). Genus *Perkinsus*-specific PerkITS85/750 PCR assays were performed and their amplicons were sequenced by the Sanger method. The RFTM assays confirmed *Perkinsus* spp. The sequencing of the amplified fragments from the rDNA internal transcribed spacers (ITS) of *Perkinsus* spp. confirmed *Perkinsus chesapeaki*. Neighbor-Joining analyzes place *P. chesapeaki* identified in this study in a well-supported clade with other isolates of the same species. This is the first record of *P. chesapeaki* infecting *C. rhizophorae* in South America.

1. Introduction

The estuary of the Jaguaribe River (Ceará State, Northeast Brazil) has great importance both from an ecological point of view, as well as for supporting fisherman communities, as a mollusk collecting site for consumption and marketing. Mollusks from natural environments and also from cultivation can be affected by different groups of pathogens, including protozoa belonging to the genus *Perkinsus*. This genus currently has seven accepted species. Among them, *P. marinus* and *P. olseni* require mandatory reporting to the World Organization for Animal Health, due to the risk that they represent for mollusk populations (Villalba et al., 2004; Choi and Park, 2010). In Brazil, studies on perkinsosis have attracted attention since the first record of *Perkinsus* sp. was made in *Crassostrea rhizophorae* from the Pacoti River estuary, Ceará (Sabry et al., 2009). Sabry et al. (2013) confirmed that the species of *Perkinsus* affecting oysters in that estuary was *P. beihaiensis*. In this same year, the first South American records were published of *P. marinus* infecting *C. rhizophorae* from the estuary of Paraíba River (da Silva et al., 2013) and *Perkinsus* sp. infecting the same species of oyster from the coast of Bahia State (Brandão et al., 2013). Recently, *Perkinsus* sp. and *P. marinus* were reported infecting *Crassostrea gasar* from Paraíba State (Queiroga et al., 2013, 2015.), *P. marinus* and *P. olseni* were identified in *C. gasar* from the estuary of the São Francisco River, Sergipe State (da Silva et al., 2014) and *P. beihaiensis* was recorded in *Anomalocardia brasiliana* from the Timonha River estuary, Ceará State (Ferreira et al., 2015). This study investigated the presence and prevalence of *Perkinsus* spp. in *C. rhizophorae* from the estuary of the Jaguaribe River, Ceará, Brazil.

2. Materials and methods

The oysters were manually collected from the roots of *Rhizophorae mangle* at low tide in the estuary of the Jaguaribe River (04°27′39,21″S, 37°47′31,68″W), on the east coast of Ceará State. Collections were made in March 2012 (N = 150) during the rainy season (January-April) and October (N = 150) of the same year during the dry season (May-December). The temperature and salinity data were measured during the sampling. The oysters were transported to the laboratory alive in isothermal boxes. Prior to processing, the animals were measured for shell height, according to Galtsoff (1964). Two gill lamellae and the rectum of each oyster were incubated in Ray's fluid thioglycollate medium in the presence of antibiotics and antifungals (penicillin/streptomycin and nystatin) for 7 days in the dark at room temperature. After this

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period, the organ fragments were processed according to Ray (1954). The material was analyzed under light microscope in order to assess the presence/absence of *Perkinsus* cells, which presented spherical shape and blue or bluish black color. The prevalence of *Perkinsus* was calculated according to Bush et al. (1997) and the intensity of infection followed the Mackin scale (Ray, 1954), as modified by Sabry et al. (2009).

The extraction of total genomic DNA was performed on animals diagnosed as positive by the RFTM technique, according to the protocol described by Sambrook and Maniatis (1989). The genetic material was extracted from approximately 50 mg of gill tissue from each sampled oyster, which were previously preserved in 95% ethanol. Integrity of the extracted DNA was assessed by electrophoretic run on 1% agarose gel, prepared with TAE (1X) buffer. For application in the gel, 5.0 μL of each DNA sample was mixed with 3 μL of *Blue juice*™ dye 10X (Invitrogen) containing 1 μL *Gel Red* (20X) (BIOTIUM). After that, the gel was visualized in an ultraviolet transilluminator *SelectTMSeries* (ESPECTROLINE®) and photodocumented through a system of analysis and electrophoresis photodocumentation (Pizzonia, 2001).

The DNA of the oysters was analyzed for *Perkinsus* spp. by PCR with the primers set *Perk* ITS 85/750 (Casas et al., 2002) which specifically hybridize with conserved regions of the rDNA internal transcribed spacers (ITS) for members of the genus *Perkinsus*. The positive control used in the PCR assays was a local sample of *P. beihaiensis* previously confirmed by DNA. For negative control, DNA was replaced by nuclease-free water. Each reaction of 25 μL contained 50–100 ng of template DNA, 1x PCR buffer, $MgCl_2$ 1.5 mM, 0.2 mM of each dNTP, 0.8 mM of each primer and one unit of *Taq* DNA polymerase (Invitrogen). The thermocycling conditions included a DNA denaturation at 94 °C for 10 min, and 35 cycles of amplification at 94 °C (1 min), 55 °C (1 min) and 72 °C (1 min), followed by a final extension at 72 °C for 10 min. PCR products were electrophoresed and visualized as described above.

PCR products of approximately 700 bp for members of the genus *Perkinsus* were directly sequenced through the *BigDye Terminator v3.1 Cycle Sequencing kit* (Applied Biosystems) following the manufacturer's directions. The sequencing reaction products were purified by precipitation in isopropanol/ethanol and read in an ABI 3500 capillary automated sequencer (Applied Biosystems). MEGA 6.0 software (Tamura et al., 2013) was used for elaboration of the phylogenetic tree, consensus sequences, and alignment. Representative *Perkinsus* spp. sequences available from GenBank were added to dataset to allow the analysis. The phylogenetic tree was produced through the Neighbor-Joining algorithm (Saitou and Nei, 1987), using the Kimura 2 parameter distance (Kimura, 1980), missing sites pairwise deletion and 1000 bootstrap replicates (Felsenstein, 1985).

3. Results

The average height (±SD) of oysters collected in the estuary was 56 ± 12 mm and 60.1 ± 6.1 mm in the rainy and dry season respectively. The observed water temperature and salinity at the time of collection were 28 °C and 23‰ (rainy season) and 30 °C and 35‰ (dry season). RFTM assays revealed the presence of *Perkinsus* sp. The spherical cells ranged from 4.11 μm to 45.31 μm in diameter. The prevalences of oysters infected with *Perkinsus* spp. were 3.3% (5/150) and 14% (21/150), for the rainy and dry season respectively. The mean intensities of infection for these two studied periods were 1.14 ± 0.38 and 1.87 ± 0.89, on a scale from 1 to 4.

All 5 samples collected during the rainy season that were positive by genus-specific RFTM assays, were also positive by genus-specific PerkITS85/750 PCR assays. From 21 samples collected during the dry season positive in RFTM, only 5 were positive in PCR. Sequencing of fragments from the ITS region of *Perkinsus* spp. amplified by PCR resulted in sequences with 505 to 645 bp. Four of the sequences showed high homology to publicly available

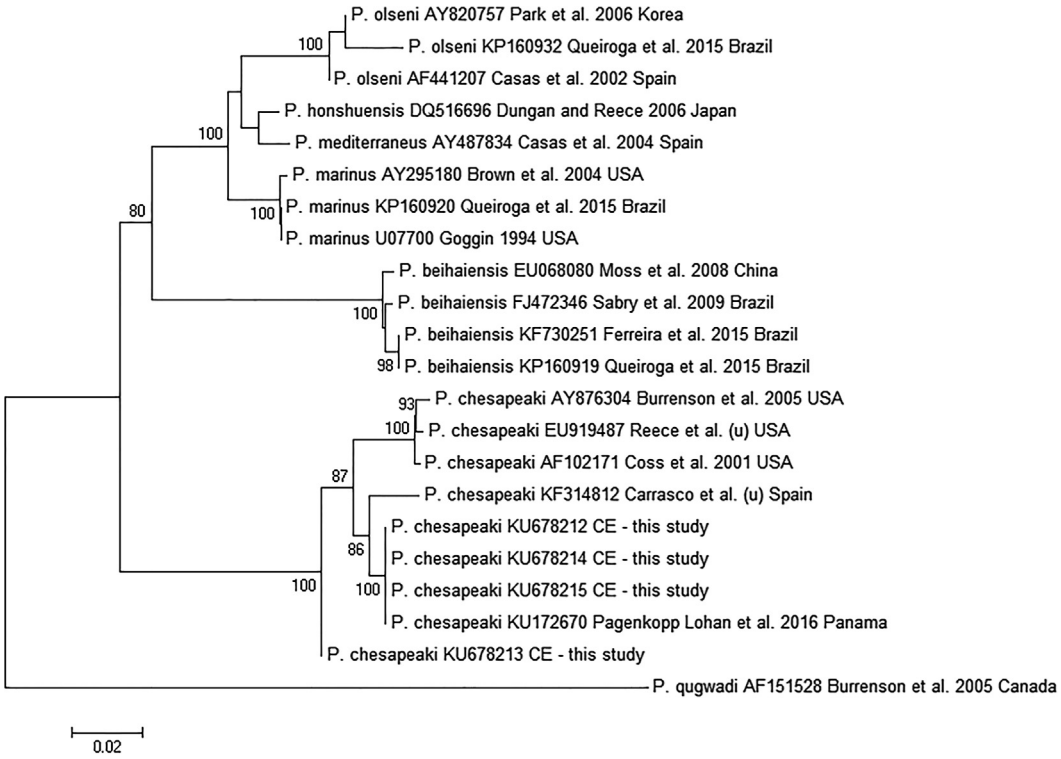

Fig. 1. Neighbor-Joining tree based on uncorrected distances among *Perkinsus* species, including representative GenBank sequences for all species previously recorded for the Brazilian coast. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). Numbers next to branches represent bootstrap support after 1000 replicates.

sequences of *P. chesapeaki* and one showed high homology to *Perkinsus beihaiensis*. The mean prevalences of *P. chesapeaki* and *P. beihaiensis* in the oysters collected during the rainy season were 2.6% and 0.66% respectively. During the dry season, the oysters were infected by *P. beihaiensis* only with mean prevalence of 3.3%.

The sequences of *P. chesapeaki* identified in this study were deposited in GenBank under access numbers KU678212, KU678213, KU678214 and KU678215. The phylogenetic tree produced by the Neighbor-Joining method (Fig. 1) grouped the sequences obtained in this study in highly supported clades, showing clearly *P. chesapeaki* in a different group.

4. Discussion

This paper reports the occurrences of *Perkinsus beihaiensis* and, for the first time, *P. chesapeaki* infecting *Crassostrea rhizophorae* in South America. The RFTM assays revealed the presence of hypnospores of *Perkinsus* spp. infecting the tissues of *C. rhizophorae*. The pathogen cells enlarged during incubation in RFTM, according to the description made by Choi and Park (2010) and similar to those observed in other studies with mollusks from the Brazilian coast (Sabry et al., 2009; Brandão et al., 2013; da Silva et al., 2013). Molecular-genetic analyses revealed that 4/5 of the detected infections were caused by *Perkinsus chesapeaki*, species for which there are no previous records in South America. The prevalences of *P. chesapeaki* (2.6%) and *P. beihaiensis* in oysters were low (0.6 and 3.3%) in both dry and rainy seasons. These values were lower than those observed for *Perkinsus* sp. (5.8%) in this same species of oyster from the Northeastern region of Brazil (Sabry et al., 2009) and also lower than the prevalences observed in *C. rhizophorae* from Bahia infected with *Perkinsus* sp. (92%) (Brandão et al., 2013) and from Paraíba infected by *P. marinus* (100%) (da Silva et al., 2013). It is lower as well than the prevalence observed for *C. gasar* from Paraiba infected with *Perkinsus* sp. (93.3%) (Queiroga et al., 2013) and for *C. rhizophorae* from the Pacoti River estuary (located 100 km to the west from the collection site of this study), in 2010, infected by *P. beihaiensis* (4%) (Sabry et al., 2013). *Perkinsus chesapeaki* was recorded in *Tagelus plebeius* from Delaware Bay with prevalences of 75% in PCR assays (Bushek et al., 2008), in *Mya arenaria* (Mclaughlin et al., 2000) and in *Mya arenaria* and *Macoma balthica* from United States (Burreson et al., 2005). Later on, this protozoan was observed in the bivalve *Cerastoderma edule* from the Mediterranean coast of Spain (Carrasco et al., 2014).

In this study, the discrepancy between the results of PCR and RFTM possibly is related to the amount of preserved tissue for these two techniques or due to the low intensity of infection, suggesting that the fragment of gills used for PCR maybe not present the pathogen cells. The occurrence of false negative by PCR has been reported by Burreson (2008).

In the present study, the intensity of infection by RFTM was very light (level 1) and similar to those observed in *C. rhizophorae* (null to very light) (Sabry et al., 2013) and in *Anomalocardia brasiliana* (very light to light) from the Timonha River estuary (extreme west of Ceará) (Ferreira et al., 2015). On the other hand, Brandão et al. (2013) reported high intensities of infection (2.56 ± 0.83), on a scale from 1 to 4, by *Perkinsus* sp. in *C. rhizophorae* from Bahia.

Researches carried out with BLAST tool indicated that the obtained sequences from the ITS region of *Perkinsus beihaiensis* showed high identities (99%) with the same *Perkinsus* species investigated in Brazil by Queiroga et al. (2015) and by Ferreira et al. (2015). *Perkinsus chesapeaki* shared high identity (96–100%) with *P. chesapeaki* from United States (Burreson et al., 2005), showing clear differentiation from other *Perkinsus* species, including those already reported for the Brazilian coast.

In conclusion, the results of this study represent a new advance in research about perkinsosis in Brazil, enlarging the area of distribution of this parasite on the Brazilian coast while reporting the occurrence of *P. beihaiensis* and for the first time the occurrence of *P. chesapeaki* infecting the oyster *C. rhizophorae* from the Jaguaribe River estuary, Ceará, Brazil. This is also the first detection of this species infecting bivalves in South America.

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