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Molecular detection of the pathogenic protist *Perkinsus marinus* in farmed native and introduced oysters (*Crassostrea* spp.) in southern Brazil

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ABSTRACT. The parasitic protozoan *Perkinsus marinus* (Perkinsidae) is known to infect marine bivalves; unfortunately, treatment options are quite limited. The parasite is associated with mass mortalities worldwide and it requires notification to the World Organization for Animal Health (OIE). In Brazil, since the first report of *P. marinus* infecting *Crassosstrea rhizophorae* in the state of Paraíba in 2013, populations of oysters have been subject to continuous surveillance programs by the Ministry of Agriculture, Livestock and Food Supply for OIE-listed pathogens. Here, we report the first official case of *P. marinus* detection in native *Crassosstrea* sp. and in *Crassostrea gigas* from southern Brazil by PCR followed by sequencing of amplified fragments of the rDNA ITS region. For a better understanding of the epidemiology of *P. marinus*, we studied the parasite's phylogenetic intraspecific variability based on its rDNA NTS region by comparisons of our isolates with other isolates

from two Mexican regions on the Pacific coast. All Brazilian isolates clustered together with the Mexican isolates. As expected, high similarities were seen between all Brazilian isolates. Additional studies on *P. marinus* genotyping using new genomic target tools should be conducted for a better understanding of this parasite's epidemiology.

Key words: Protozoan; Bivalve; PCR; Diagnostic; Phylogeny

INTRODUCTION

Commercial bivalve mollusk farming is a recent activity in Brazil, dating from the late 80s, with Santa Catarina being responsible for more than 95% of the total production (EPAGRI, 2014). Oyster farming is dominated by culture of the Japanese oyster *Crassosstrea gigas*, followed by other species including mangrove oysters *C. rhizophorae* and *C. gasar* (EPAGRI, 2014 and Suhnel et al., 2016). The first introduction of *C. gigas* seeds in Santa Catarina occurred in 1987 for experimental purposes and until today *C. gigas* farming is being conducted successfully.

Populations of marine bivalve mollusks are being affected by disease epidemics caused by many pathogens, including protozoans of the genus *Perkinsus* (Villalba et al., 2004 and Queiroga et. al., 2015). This genus includes six species, but only two, *P. marinus* and *P. olseni*, are associated with mass mortalities worldwide; both require notification to the World Organization for Animal Health (Villalba et al., 2004, OIE, 2016). Detection of *Perkinsus* is normally conducted by cultivation in Fluid thioglycollate culture, followed by examination of the hypnospore with a light microscope (Suhnel et al., 2016). However, it is not possible to differentiate between *Perkinsus* species based on morphological analyses alone. The OIE recommends that *P. marinus* surveillance be conducted first using *Perkinsus* genus PCR assays, followed by a *P. marinus* specific assay for positive samples.

In the past few decades, Brazilian fisheries and aquaculture sectors have greatly improved in terms of regulatory policies and environmental sustainability with the introduction of new sanitary measures including the National Hygienic and Sanitary Control of Bivalve Mollusks (PNCMB) and the National Network of Laboratories (RENAQUA) for official diagnosis using molecular tools and the development of new analytical diagnostic methodologies for diseases, contaminants and residues. In Brazil, *P. marinus* was first reported infecting wild oysters, *C. rhizophorae*, in the northeastern state of Paraíba in 2013 (Da Silva et al., 2013), which lead to regulatory legislation by the Ministry of Agriculture, Livestock and Food Supply (MAPA) prohibiting the export of bivalve mollusks, their products and byproducts from the affected state (Brasil, 2013). Subsequently, other cases of *P. marinus* infections have been reported in *Crassostrea gasar* oysters from the estuaries of the São Francisco River, state of Sergipe (Da Silva et al., 2014), and in the Mamanguape River of the State of Paraíba (Queiroga et al., 2015). Consequently, oyster populations have been subject to continuous target surveillance programs for the OIE-listed pathogens by MAPA. Monitoring of farmed and wild oysters has been conducted since 2015 in the region of São Francisco do Sul (SC), after mortalities were reported by local farmers. This region produces native oysters (*Crassostrea* sp.) and recently started to cultivate *C. gigas* from allegedly "*Perkinsus*-free" seeds that were obtained from the Laboratory of Marine

Mollusks of the Federal University of Santa Catarina (LMM). Diagnostic analyses were conducted using PCR and sequencing of amplified fragments (oysters and oyster seeds) from ribosomal DNA internal transcribed spacers. Here we report several cases of *P. marinus* in *Crassostrea* sp. and in adult *C. gigas* from the state of Santa Catarina. Furthermore, we studied the parasite phylogenetic intraspecific variability based on its rDNA non-internal transcriber spacer (NTS) region that is suggested to be a marker for *P. marinus* genotyping (Escobedo-Fregoso et al., 2012). A molecular characterization of *P. marinus* in different geographical locations would facilitate a better understanding of the parasite's epidemiology.

MATERIAL AND METHODS

Three samplings of adult farmed (N = 111; ~ nine months old) and wild (N = 38) *Crassostrea* sp. with suspected mortalities (~10%) and without any evident clinical signals were conducted from May 2015 to February 2016 in the region of Laranjeiras, São Francisco do Sul, SC (Figure 1; Table 1). *Crassostrea gigas* seeds from the LMM were collected for a preliminary evaluation of the presence of *P. marinus*, before being introduced to the farming site (Table 1). From July 2016 to January 2017, five samplings were conducted of juvenile (N = 50; ~ three months old) and adult (N = 59; ~ nine months old) stages of *C. gigas* (Table I). All samplings were conducted by inspectors of the Santa Catarina State Agency for Animal Health (CIDASC). Oysters were transported in insulated boxes containing ice packs and oyster seeds were placed in 10-mL tubes containing 96% ethyl alcohol. All samples were sent to the Laboratory for Aquatic Animals Diseases (AQUACEN) situated at the Federal University of Minas Gerais, Belo Horizonte, MG, for OIE-listed pathogen detection.

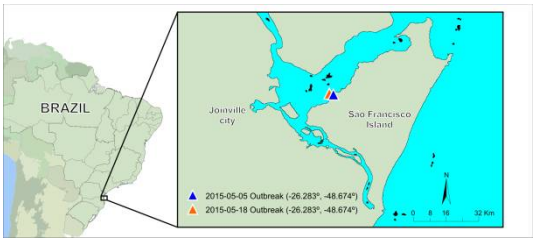

Figure 1. Map of Brazil showing the southern state of Santa Catarina, indicating two samplings of outbreaks of *Perkinsus marinus* in Laranjeiras, São Francisco do Sul, SC.

Table 1. Results of *Perkinsus marinus* detection by PCR in oyster tissues that were sampled in the region of Laranjeiras, São Francisco do Sul, SC.

Date of sampling	Oyster species (rearing or life stage)[1]	Wild/Cultivated	PCR Results	Pos /Tot[2] (%)
2015-05-05	*Crassostrea* sp. (AO)	Cultivated	59/59	100
2015-05-18	*Crassostrea* sp. (AO)	Cultivated	50/52	96
2016-02-25	*Crassostrea* sp. (AO)	Wild	21/38	55
2016-04-19	*C. gigas* (OS)	Cultivated	0/*	0
2016-07-28	*C. gigas* (JO)	Cultivated	0/50	0
2017-01-18	*C. gigas* (AO)	Cultivated	4/59	7

[1] Rearing or life stages were as follows: adult oysters: ~ 9 months (AO); oyster seeds (OS); juvenile oysters: ~ 3 months (JO).
[2] Number of positive samples divided by the total number of samples.
* *C. gigas* seeds (50 mg) were sent to the lab fixed in 95 % ethyl alcohol.

In the laboratory, mantle and gill tissues of oysters (10 - 40 mg) and oyster seeds (50 mg) were collected and transferred to -80ºC, until further analysis. DNA was individually extracted from each sample using Maxwell 16 Tissue DNA purification kits (Promega, Madison, WI, USA) according to manufacturer's instructions. To detect *Perkinsus* spp., PCR assays were conducted in a Veriti 96-well thermal cycler (Life Technologies, Carlsbad, CA, USA) using a pair of *Perkinsus* genus-specific primers, PerkITS85/PerkITS750 (Casas et al., 2002). These primers specifically hybridize to the conserved regions of the ribosomal ribonucleic acid gene, including internal transcribed spacer 1 (ITS), the 5.8S gene and ITS2. Briefly, each reaction of 25 µL contained 2µL of genomic DNA (50 – 200 ng), PCR buffer at 1x concentration, 3.0 mM $MgCl_2$, 0.2 mM nucleotides, 0.1 µM of each primer and 2 U/µL Hot Start Taq DNA polymerase. The thermocycling conditions included a DNA denaturation at 95ºC for 15 min, and 30 cycles of amplification at 95ºC (1 min), 60ºC (1 min) and 72ºC (1 min), followed by a final extension at 72º C for 10 min. The expected product size was 703 bp. The PCR amplification products were stained with Blue Green Loading Dye I (LGC Biotecnologia, Brazil) and resolved via electrophoresis in 1.5% agarose gels. PCR products were purified with Agencourt AMPure XP (Beckman Coulter, Pasadena, CA, USA) and subjected to sequencing using a BigDye™ Terminator Cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA) and an ABI 3500 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). Species determination was evaluated using BLAST webserver (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Similarities of 99 - 100% were considered for *P. marinus* affiliation.

Phylogenetic analyses were conducted using nucleotide sequences of rRNA gene-complex NTS regions of six representative isolates from two sampling dates described in Table 1 from the Atlantic shorelines together with 14 sequences of *P. marinus* isolated from *C. corteziensis* cultivated in two neighboring regions of the Mexican Pacific coast: Pozo Chino (PC) and Boca de Camichin (BC) (Escobedo-Fregoso et al., 2012). One sequence of *Perkinsus olseni* (Genbank accession number: U07701.1) was also included as an outgroup. Sequences were aligned on CLC Genomic Workbench (Qiagen, USA) using cost parameters equals to 9 (open), 5 (extension), and Free (end). A phylogenetic tree was constructed with CLC Genomic Workbench using the UPGMA Juckes-Cantor construction method, for nucleotide distance measure, and 1000 replicates in a bootstrap analysis. In the tree view, a bootstrap of less than 50% was not shown.

RESULTS AND DISCUSSION

This case report is part of a surveillance program carried out by MAPA that monitors the presence of the OIE-listed pathogens, including *P. marinus* in bivalve mollusks in Brazil. Target surveillance was conducted in an oyster producing site of São Francisco do Sul, SC. Results of genus *Perkinsus*-specific PCR followed by sequencing by the Sanger method are shown in Table 1. Sequencing of amplified fragments from the rDNA internal transcribed spacers (ITS) of *Perkinsus* spp. confirmed the identity of *P. marinus* with 99 - 100% of similarity in all native oysters (*Crassostrea* sp.) and adult *C. gigas*. *Perkinsus marinus* was detected in all samples of *Crassostrea* sp. at higher frequencies (Table I). However, no evident pathology was observed (data not shown). Results of the PCR analyses of *C. gigas* seeds confirmed their status as being *Perkinsus*-free. Seeds were then cultivated until they reached adult size. The parasite was only

detected in *C. gigas* at later rearing stages (~ nine months old) and at low frequencies (7%) when compared to native species (55 - 100%). Furthermore, no mortality of *C. gigas* was recorded by the farmers.

The increasing dissemination of infectious agents in cultivated species due to the globalization of aquatic animal trade is a major concern. Production of mollusks is at risk since there are limited options for disease control. For a better understanding of the epidemiology of *P. marinus* throughout the Americas, phylogenetic analyses using an rDNA NTS region were conducted by comparisons of our isolates with Mexican isolates that are deposited in GenBank. Based on Escobedo-Fregoso et al. (2012), the NTS region is considered to be a better marker for *P. marinus* genotype identification due to its higher nucleotide diversity when compared to the ITS region. Consequently, the more conserved region of the ITS can be used for species discrimination (Escobedo-Fregoso et al., 2012).

Based on the UPGMA analysis (Figure 2), all Brazilian NTS sequences from both samplings (49 and 51) clustered together with Mexican sequences from both regions of Pozo Chino and Boca de Camichin. Another clade was formed with only Mexican NTS sequences from the two Mexican regions.

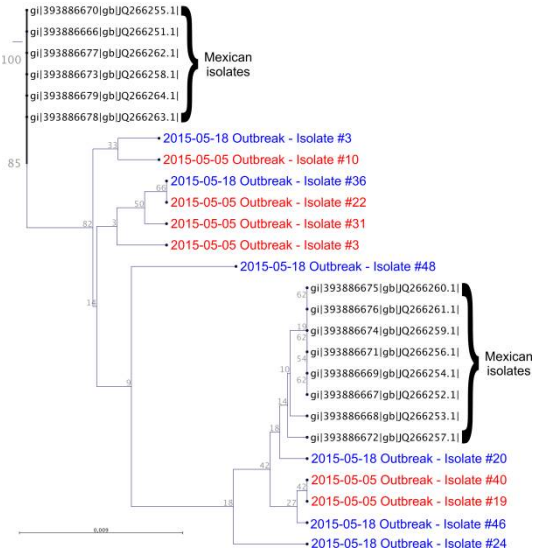

Figure 2. Phylogenetic tree of the NTS region of Brazilian and Mexican isolates of *Perkinsus marinus*. Six representative isolates of each outbreak in Brazil are exemplified in red and blue colors. Mexican isolates are represented by 14 isolates of two regions on the Pacific coast. The sequences were analyzed by UPGMA, Juckes-Cantor as nucleotides distance measure, and 1000 replicates in bootstrap analysis. In the tree view, bootstrap support values for each branch are given when above 50%.

All Brazilian and Mexican sequences of *P. marinus* diverged from *P. olseni*, which was considered as the outgroup species. As was expected, no allelic variants of *P. marinus* were found in the two samplings in Brazil, indicating a homogeneous distribution of these isolates. Despite geographical isolation, high similarities of our NTS sequences of the Atlantic shores with genotypes along the Pacific coast of Mexico suggest the introduction of *P. marinus* by transport of mollusks from the Pacific to the Atlantic. In the last decade, advancement in sequencing technologies has deeply impacted eukaryote genotyping

practices. Since information on the *P. marinus* NTS region genotype is scarce, additional surveillance studies using new genomic target methods for *P. marinus* genotyping should be conducted to confirm this assumption.

Perkinsus marinus is believed to be transmitted directly from infected to uninfected oysters thorough filter feeding of infective particles (Perkins, 1993 and Villalba et al., 2004). Live and dead infected oysters release *P. marinus* cells through feces and predation and by means of tissue decay, scavenging or vector transfer (Villalba et al., 2004 and Bidegain et al., 2017). In our investigation, transmission of *P. marinus* to *C. gigas* appears to have occurred horizontally from close contact with native species since the former were positive for *P. marinus* in all previous analyses. However, additional transmission studies should be conducted to confirm this hypothesis. To date, this report describes the first official cases of *P. marinus* detection in native *Crassosstrea* sp. and in *C. gigas* in Brazil.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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