Journal of Invertebrate Pathology 113 (2013) 96–103

Contents lists available at SciVerse ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

First report of the protozoan parasite *Perkinsus marinus* in South America, infecting mangrove oysters *Crassostrea rhizophorae* from the Paraíba River (NE, Brazil)

Patricia Mirella da Silva [a,*], Rogério Tubino Vianna [b], Cristhiane Guertler [c], Liana Pinho Ferreira [d], Lucas Nunes Santana [a], Sergio Fernández-Boo [e], Andrea Ramilo [e], Asunción Cao [e], Antonio Villalba [e]

[a] Departamento de Biologia Molecular, Universidade Federal da Paraíba (UFPB), CEP 58051-900, João Pessoa, PB, Brazil
[b] Universidade Federal de Santa Catarina (UFSC), CEP 89520-000, Curitibanos, SC, Brazil
[c] Departamento de Biologia Celular, Embriologia e Genética, Universidade Federal de Santa Catarina (UFSC), CEP 88040-900, Florianópolis, SC, Brazil
[d] Instituto de Ciências do Mar, Universidade Federal do Ceará (UFC), CEP 60165-081, Fortaleza, CE, Brazil
[e] Centro de Investigacións Mariñas (CIMA), Consellería do Medio Rural e do Mar, Xunta de Galicia, 36620, Vilanova de Arousa, Spain

ARTICLE INFO

Article history:
Received 11 June 2012
Accepted 12 February 2013
Available online 22 February 2013

Keywords:
Perkinsus marinus
Mangrove oyster
Crassostrea rhizophorae
PCR
rDNA ITS
In vitro culture

ABSTRACT

The present work aimed to study the infection by *Perkinsus* sp. in the mangrove oysters *Crassostrea rhizophorae* from the estuary of the Paraíba River (Paraíba State, Brazil). Perkinsosis was detected by incubation of oyster gill pieces in Ray's fluid thioglycollate medium. The monthly prevalence values were all above 70%, thus infection was not likely to be a transient event. *Perkinsus* sp. parasites isolated from eight oysters were propagated *in vitro*. PCR–RFLP analysis of *in vitro* cultured cells as well as the sequences of the rDNA ITS region allowed the identification of the *in vitro* propagated parasites as *Perkinsus marinus*. Phylogenetic analyses using rDNA ITS region sequences strongly supported the *Perkinsus* sp. from Paraíba in a monophyletic group with *P. marinus*. Thus, the results confirmed the species affiliation of Paraíba *Perkinsus* sp. as *P. marinus*. This is the first report of *P. marinus* in Brazil and South America and the first report of *P. marinus* naturally infecting *C. rhizophorae*.

1. Introduction

Bivalve mollusc farming is a recent activity (since 1990) in Brazil. The state of Santa Catarina located in the Southern region, is the country's largest producer of bivalves (15,636 tonnes in 2010). In contrast, the Northeastern region of Brazil is the largest national producer of marine aquaculture (79.2%), which is based mostly on shrimp farms (Ministério da Pesca e Aquicultura, 2012). Nevertheless, bivalve production in this region was stagnant until recently because of the lack of aquaculture researchers, technical experts and government support. The coast of Paraíba has great potential for developing bivalve production, since there are two major estuaries, Paraíba and Mamanguape, inhabited by several bivalve species, including mangrove oysters, *Crassostrea gasar* (=*brasiliana*) and *C. rhizophorae*. Oyster farming occurs on a small scale in the estuary of the Mamanguape River (information from local oystermen).

Protozoan parasites of the genus *Perkinsus* infect marine molluscs worldwide. The genus includes seven valid species: *P. marinus*, *P. olseni* (=*atlanticus*), *P. qugwadi*, *P. chesapeaki* (=*andrewsi*), *P. mediterraneus*, *P. honshuensis* and *P. beihaiensis* (Villalba et al., 2004, 2011). Two species, *P. marinus* and *P. olseni*, receive more attention because they can dramatically affect the physiology of their hosts causing significant mortality in affected bivalve populations (Choi and Park, 2010; Villalba et al., 2004, 2011). Both species are listed as being notifiable pathogens by the World Organization for Animal Health.

In Brazil, Sabry et al. (2009) reported the first case of a representative of the *Perkinsus* genus infecting mangrove oysters, *C. rhizophorae* from the Pacoti estuary, Ceará State, NE Brazil. The sequences of the ITS region of the rRNA gene complex of the *Perkinsus* sp. reported by Sabry et al. (2009, 2013) showed high identity to that of *P. beihaiensis*, infecting oysters in China (Moss et al., 2008) and India (Sanil et al., 2012). Recently, our group detected *Perkinsus* sp. infecting *C. gasar* from the São Francisco Estuary, Sergipe State, NE Brazil (da Silva et al., in prep.). *Perkinsus olseni* was found infecting the clam *Pitar rostrata* in Uruguay (Cremonte et al., 2005), 4500 km away from the Ceará coast, where *Perkinsus* sp. was reported for the first time in Brazil. Interestingly, in the coast of Santa Catarina State closer (1000 km) to Uruguay no case

* Corresponding author. Address: Universidade Federal da Paraíba – Campus I, Centro de Ciências Exatas e da Natureza, Departamento de Biologia Molecular, Jardim Universitário s/n, Bairro Castelo Branco, CEP 58051-900, João Pessoa, PB, Brazil. Fax: +55 83 32167787.
E-mail addresses: mirella_dasilva@hotmail.com (P.M. da Silva), r.t.vianna@ufsc.br (R.T. Vianna), villalba@cimacoron.org (A. Villalba).

0022-2011/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jip.2013.02.002

of perkinsosis had been found in cultured oysters *C. gigas* or natural oyster *C. rhizophorae* populations (da Silva et al., 2012; Sabry et al., 2009, 2011).

Littlewood (2000) reported the occurrence of *Perkinsus* cf. *marinus* in cultured oysters *C. rhizophorae* from Jamaica, although the infection was diagnosed by the Ray's fluid thioglycolate medium, (RFTM; Ray, 1966), a poor technique for discrimination between species of the genus *Perkinsus*. Bushek et al. (2002) confirmed the susceptibility of the oyster *C. rhizophorae* to *P. marinus* through experimental infection.

This article reports the occurrence of perkinsosis in mangrove oysters *C. rhizophorae* from the coast of Paraíba State, NE Brazil. Induction of *in vitro* propagation and molecular analysis identified *P. marinus* as the causative agent, which is the first detection of this parasite species in Brazil and South America.

2. Materials and methods

2.1. Studied area and sampling

The coastal region of Paraíba has a tropical hot-humid climate, with rain in the autumn and winter (May–September) and dry conditions in the spring and summer (October–April), with an annual average of 1800 mm rainfall and of 26 °C of air temperature. The estuary of the Paraíba River comprises an area of about 260 km^2 and is covered on both sides by mangrove vegetation.

Samples of mangrove oysters were taken in July ($N = 40$), August ($N = 55$), September ($N = 15$), and December ($N = 20$) 2011 from the estuary of the Paraíba River (06°58′16.6″S and 34°51′45.1″W) (Fig. 1). The oysters were collected directly from the rhizophores of the red mangrove tree *Rhizophora mangle* and were taken to the laboratory facilities. The height of the shells (longer axis) were measured, and they were kept in 40 l tanks in a closed system with raw seawater and aeration, for 24–48 h before processing, except in August, when the oysters were held longer (7–10 days) to be used to isolate *Perkinsus* sp.

2.2. Diagnosis of Perkinsus sp. by RFTM

Oysters ($N = 130$) were shucked by cutting the adductor muscle and two gill demibranchs were excised and placed individually in tubes containing Ray's fluid thioglycollate medium (RFTM; Ray, 1966) plus antibiotics (penicillin G, streptomycin and nystatin; Park et al., 2006). Tissues were incubated in this medium for 7 days at room temperature (20–25 °C) in the dark. Then the gills were partially crushed on a slide using a scalpel, stained with Lugol's solution and observed under light microscopy to estimate the intensity of infection with *Perkinsus* sp., according to the following scale of Ray (1954) modified according to Sabry et al. (2009):

- Null infection (0): no *Perkinsus* sp. detected in the whole slide (100×).
- Very light infection (1): up to 10 *Perkinsus* sp. hypnospores observed in the whole slide (100×).
- Light infection (2): 11–100 *Perkinsus* sp. hypnospores observed in the whole slide (100×).
- Moderate infection (3): up to 40 *Perkinsus* sp. hypnospores observed in a total of 10 random fields (400×), scattered throughout the preparation.
- Heavy infection (4): more than 40 *Perkinsus* sp. hypnospores observed in a total of 10 random fields (400×) scattered throughout the preparation.

The prevalence of *Perkinsus* sp. was calculated as the percentage of infected oysters in each sample. The mean intensity was calculated as the average intensity among infected oysters of each sample, while the mean abundance was calculated as the average intensity among all the oysters of each sample (Bush et al., 1997).

2.3. In vitro Perkinsus sp. propagation

Induction of *in vitro* propagation of the *Perkinsus* sp. parasite infecting the oysters was performed using the protocol adapted from that described by Casas et al. (2002a), although using two different culture media, separately: JL-ODRP-2A (Casas et al., 2002a) and Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM:HAM F12, 1:1; Sigma) (Gauthier and Vasta, 1995) with antibiotics (nystatin, 100 U/ml; gentamicin, 100 mg/ml; penicillin, 200 U/ml, and streptomycin, 200 μg/ml).

Briefly, oysters collected in August 2011 ($N = 55$) were distributed into three groups of 15, 25 and 15 oysters to be used in separated *in vitro* culture propagation attempts. One gill demibranch from all oysters was removed from the oyster body and cut into fragments of 1 cm^2, which were introduced into a tube with filtered sterilised seawater (FSSW). After various rinses in FSSW, the gill fragments were decontaminated in an antibiotic solution

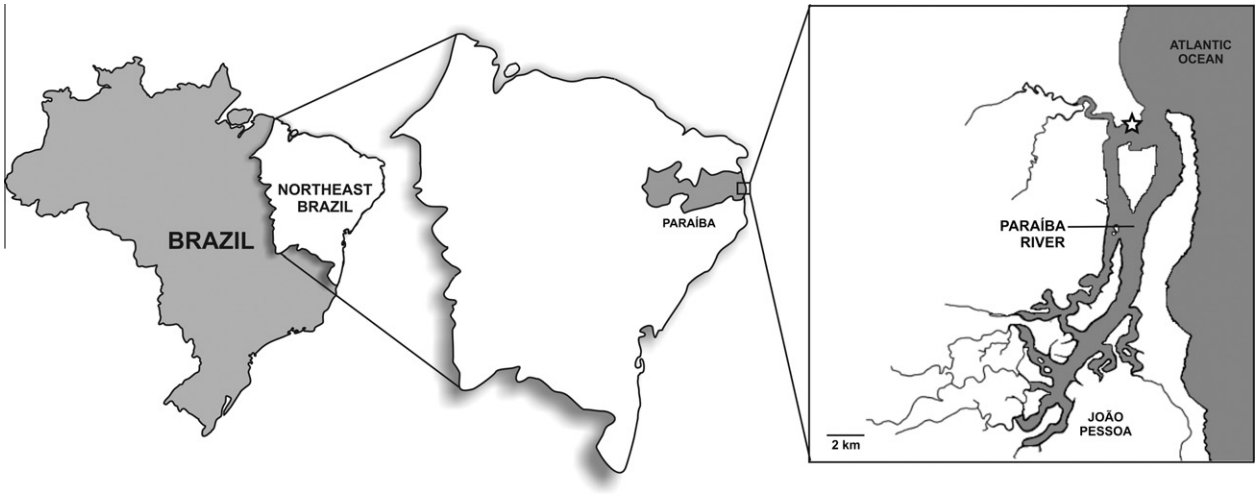

Fig. 1. Map of Brazil showing the Northeastern region, the estuary of the Paraíba River and the sampling site (∗).