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First report of *Perkinsus beihaiensis* in wild clams *Anomalocardia brasiliana* (Bivalvia: Veneridae) in Brazil

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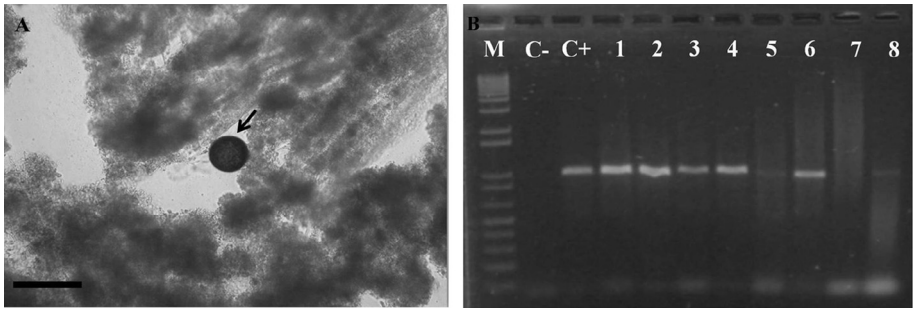
HIGHLIGHTS

- This work is the first report of this protozoan infecting *A. brasiliana* from Timonha River estuary, Ceará, Brazil.
- *Perkinsus* sp. was detected in low intensity of infection.
- The phylogenetic analyzes strongly indicated that the species of *Perkinsus* found in *A. brasiliana* is *Perkinsus beihaiensis*.

GRAPHICAL ABSTRACT



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ABSTRACT

This is the first report of *Perkinsus* sp. (Bivalvia: Veneridae) infecting wild clams of the species *Anomalocardia brasiliana* in Brazil. The gill lamellae and rectum of 150 specimens of *A. brasiliana* collected in the Timonha river estuary (Ceará, Northeastern Brazil) in March 2012 were incubated in Ray's fluid thioglycollate medium (RFTM) for detection of *Perkinsus* sp. In RFTM, the prevalence of *Perkinsus* sp. was 14.7% (22/150) and the intensity of infection ranged from very light (1–10 cells across the slide) to light (12–100 cells). The presence of *Perkinsus* sp. was confirmed by PCR in seven (31.8%) out of 22 RFTM-positive specimens. DNA sequencing confirmed the presence of the genus *Perkinsus* and the phylogenetic analysis strongly indicated *Perkinsus beihaiensis* as the species responsible for the infection.

1. Introduction

Located on the coast of Ceará, 300 km northwest of Fortaleza (Northeastern Brazil), the Timonha river estuary is home to a number of bivalves of commercial interest, including the mangrove oyster *Crassostrea rhizophorae*, the mussel species *Mytella falcata* and *Mordella guyanensis* and the clam species *Anomalocardia brasiliana*, locally known as "búzio". These bivalves are of socioeconomic and ecological importance as a source of food and income for local communities.

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Little has been published on diseases affecting bivalves in estuaries in Ceará. In fact, to our knowledge, only one Brazilian study is available on parasitic infection in *A. brasiliana*, involving the metazoon *Bucephalus* sp. (Trematoda: Bucephalidae). The infected specimens came from the estuary of the Jaguaribe River, 100 km southeast of Fortaleza (Araújo and Rocha-Barreira, 2004).

Parasitic protozoa of the genus *Perkinsus* are known for infecting marine mollusks worldwide, mainly bivalves (Villalba et al., 2004; Choi and Park, 2010). Only two of seven accepted species, *P. olseni* and *P. marinus*, have been associated with significant mortality in wild and farmed populations of mollusks, requiring notification to the World Organization for Animal Health (OIE, 2012).

In Brazil, *Perkinsus* sp. was first recorded in *C. rhizophorae* from the estuary of the Pacoti River, 7 km southeast of Fortaleza (Sabry et al., 2009). When submitted to DNA sequencing, the specimens from the Pacoti River presented high molecular similarity to *Perkinsus beihaiensis* which infects oysters in China (Moss et al., 2008) and India (Sanil et al., 2012). The similarity was confirmed in later studies by Sabry et al. (2013). *Perkinsus* sp. has also been found infecting the same oyster species in two important estuaries (Marau and Graciosa) along the coast of Bahia, Brazil (Brandão et al., 2013).

Recently, da Silva et al. (2013) published the first Brazilian report of *P. marinus*. The species was found in *C. rhizophorae* from the Paraiba River estuary (Northeastern Brazil). Considering the risk posed by this parasite, continuous monitoring of *Perkinsus* in wild and farmed bivalve populations along the Brazilian coast is highly advisable.

This is the first report of *Perkinsus* sp. infecting wild clams of the species *Anomalocardia brasiliana* in Brazil.

2. Materials and methods

2.1. Àrea de sampling of animals

Specimens ($n = 150$) of the clam species *A. brasiliana* (shell length: 17–20 mm) were sampled in March 2012 in the estuary of the Timonha river (03°00′55.8″ S, 041°15′09.8″ W), in the northwestern corner of Ceará, Brazil (Fig. 1). The mangrove of Timonha's River has an area of 5011 km^2, it being the major and more conserved mangrove area of the Ceará State. In the region, predominates the semi-arid climate, with average temperature of 28 °C, average year precipitation of approximately 1300 mm and without mollusks farming activity. The achievement of the collection on March was random and had no direct relationship with the seasonal peaks of occurrence of *Perkinsus* sp., because it dealt just a preliminary investigation.

The animals were collected manually from the muddy substrate at a depth of 10–15 cm, transported to our laboratory and kept in tanks with water from the sampling site under continuous aeration for 24 h before processing. At the sampling site, the temperature and salinity of the water were 29 and 35 °C, respectively.

2.2. Detection of Perkinsus in Ray's fluid thioglycollate medium (RFTM)

Two gill lamellae and the rectum were retrieved from each clam specimen ($n = 150$) and incubated individually in Ray's fluid thioglycollate medium (RFTM) (Ray, 1966) containing antibiotics (penicillin G/streptomycin, 100 U ml^{-1}/µg ml^{-1}; Sigma) and an antifungal (Nystatin, 100 U ml^{-1}; Sigma) (Ray, 1966). After 7 days of

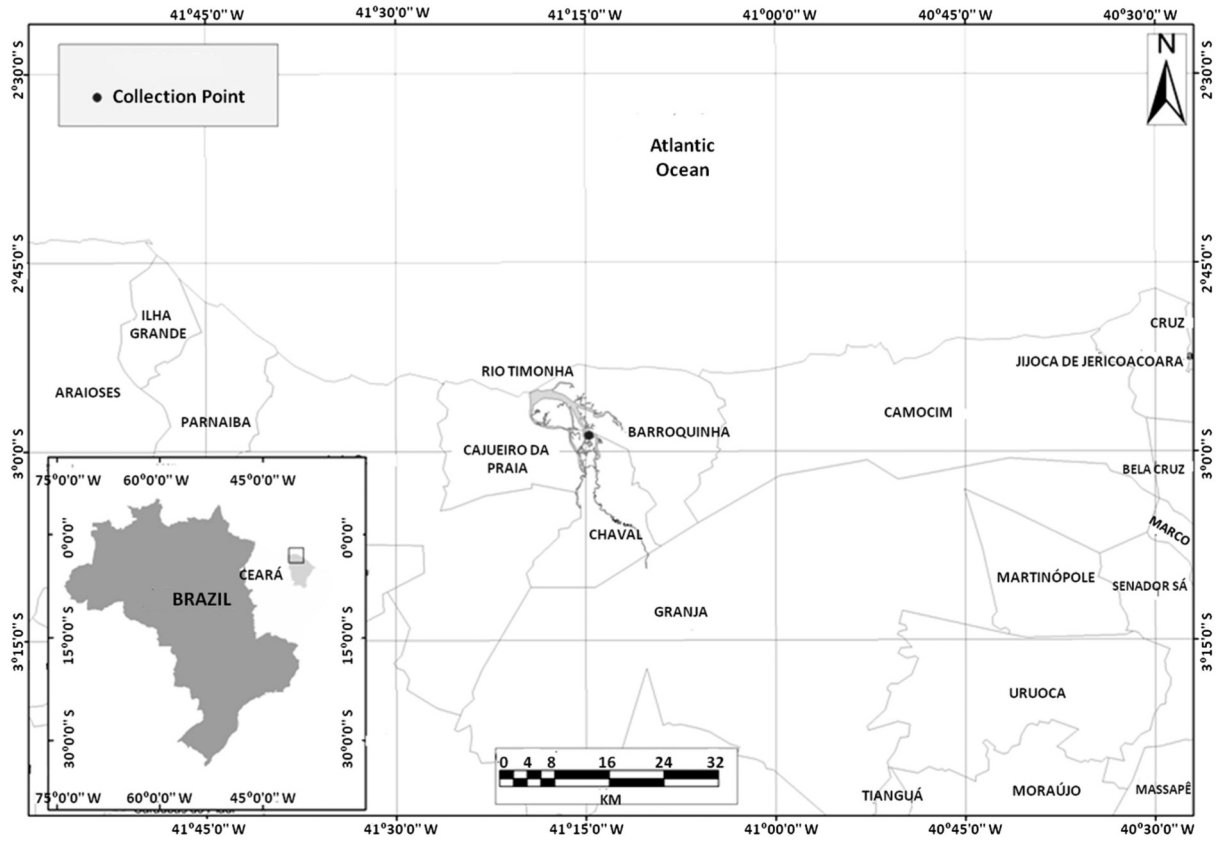

Fig. 1. Brazil Map indicating Ceara State and collection point (•) at Timonha River estuary.

incubation in the dark at room temperature, the tissues were macerated on a slide, stained with Lugol's iodine (3%) and examined under a light microscope for *Perkinsus* sp. hypnospores.

The prevalence of infection by *Perkinsus* was expressed as the number of infected animals over the total number of sampled animals (Bush et al., 1997), while the intensity of the infection was estimated using the Mackin scale (Ray, 1954) modified by Sabry et al. (2009).

2.3. *Diagnosis of* Perkinsus *by PCR*

Two demibranchs of each animal were excised and immediately fixed in 95% ethanol. The DNA extraction was performed with DNAzol® reagent (Invitrogen) following the manufacturer's protocol. Only RFTM-positive animals ($n = 22$) were used. The amplification of the gene sequences of *Perkinsus* sp. was performed with the primers PerkITS 85/750 specific for the ribosomal RNA gene complex (Casas et al., 2002), following the protocol published by Sabry et al. (2009). PCR reactions were carried out in volumes of 12.5 µL containing 1 µL (50–100 ng) sample genomic DNA, 1× proprietary PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer and 1 unit of Taq DNA polymerase (Invitrogen). The positive control used in the PCR assays was a local sample of *P. beihaiensis* previously confirmed by DNA.

2.4. *Sequencing and phylogenetic analysis*

The PCR products three RFTM-positive clams were directly sequenced with the BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems®), following the manufacturer's instructions. The sequencing reaction products were purified by precipitation in isopropanol and were read on an ABI 3100 automatic sequencer. The sequences generated were deposited in GenBank (NCBI, www.ncbi.nlm.nih.gov) under accession numbers KF730251, KF730252 and KF730253. Neighbor-joining trees and distance matrixes were produced using the software MEGA 5 (Tamura et al., 2011) from an alignment including sequences publicly available in GenBank.

3. Results and discussion

This is the first report of *Perkinsus* sp. in the clam species *A. brasiliana* in Brazil. Following incubation in RFTM, *Perkinsus* sp. was identified in samples of gill lamellae and rectum of *A. brasiliana*. The cells of the parasite were spherical in shape and stained bluish black in Lugol's iodine (Fig. 2A). The prevalence of *Perkinsus* sp. in clams was 14.7% (22/150) and the intensity ranged from very light ($n = 18$) to light ($n = 4$). The prevalence of *Perkinsus* sp. was higher in this bivalve species *A. brasiliana* than in the oysters *C. rhizophorae* from another Ceará estuary (Pacoti river) (<6.7% in 2008 and <7.3% in 2009) (Sabry et al., 2009, 2013), but considerably lower than the prevalences found in oysters from other regions of Brazil. In *C. rhizophorae* from Bahia state, the mean prevalence of *Perkinsus* sp. was 66.7% in summer 2010 (Brandão et al., 2013) while it reached in *Crassostrea gasar* the highest mean prevalence (93.3%) ever seen so far in Brazil (Queiroga et al., 2013). The *Perkinsus* sp. prevalence recorded in the current investigation was also lower than the prevalences observed by *P. marinus* in *C. rhizophorae* (up to 100% in spring 2011; da Silva et al., 2013). Nevertheless, it is important to note that recently it was demonstrated that the dynamic of infection by *P. marinus* and *P. olseni* in oyster's *C. gasar* can vary seasonally in tropical climate and be associated with culture practices (da Silva et al., 2014).

In view of the observed differences in prevalence and intensity of infection by *Perkinsus* sp. in bivalves from the Brazilian coast, further studies are necessary to better understand the impact of this parasite on aspects of bivalve production performance, such as growth and mortality rates. In addition, it would interesting to submit clam tissues infected with *Perkinsus* sp. to histological analysis in order to confirm the infection of the parasite as well as any pathological effects on the host.

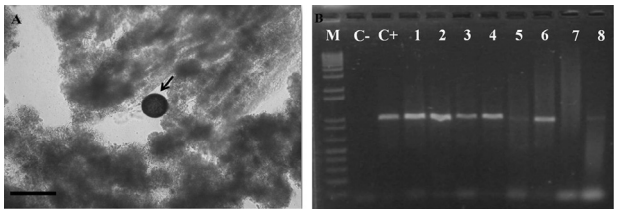

Fig. 2. *Perkinsus* sp. in *Anomalocardia brasiliana* Timonha River estuary, Ceará. (A) Hypnospores from *Perkinsus* sp. (arrow) infecting the gills of *A. brasiliana* after incubation in Ray's fluid thioglycollate medium (RFTM). Scale bar = 50 µm and (B) Agarose gel (1%) for the molecular diagnosis of *Perkinsus* sp. Detection of rDNA ITS region of *Perkinsus* sp. by PCR. M: molecular marker (1 Kb Plus Invitrogen); C–: negative control (water); C+: positive control; Animals 1, 2, 3, 4, 5, 6 and 8: positive samples; Animals 7: negative sample.

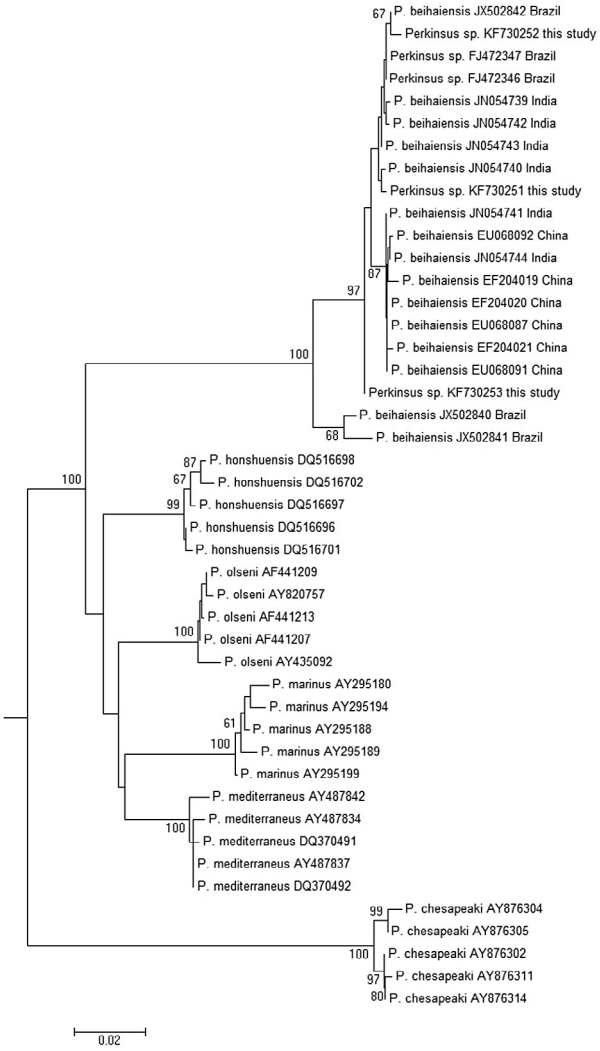

Fig. 3. Neighbor-joining tree based on sequences including partial ITS1, complete 5.8S and partial ITS2 regions, for *Perkinsus species*. Numbers near branches represent support after a bootsrap of 1000 replicates; only bootstrap values above 60 are shown. GenBank accession numbers for each sequence are shown; for *P. beihaiensis* sequences, the country of origin is indicated as well."

Our PCR analyses confirmed only seven (31.8%) of the 22 specimens identified by RFTM. The PCR products presented larger amplicons (1 kb) than those expected from the primer pair used (700 pb) (Fig. 2B). This result may be due to the low intensity of infection as observed by RFTM, suggesting that some of the tissues submitted to PCR contained no cells of the pathogen. This suggests that even if a sample contains cells of the pathogen, the techniques may produce false-negative result even if the host is infected.

Differences in the ability of PCR and RFTM to detect infection by *Perkinsus* sp. have previously been reported (Reece et al., 2008; Brandão et al., 2013; Sabry et al., 2009, 2013).

The samples were sequenced to confirm that the amplified products corresponded to the genus *Perkinsus*. Sequences were produced from three PCR-positive samples, covering a total of 629 bp of the ribosomal RNA gene complex, including partial ITS1, complete 5.8S rRNA, and partial ITS2 regions. The neighbor-joining tree grouped these sequences consistently in a clade including all *P. beihaiensis* sequences (Fig. 3). Presence of *P. beihaiensis* was confirmed for *C. rhizophorae* from an estuary over 300 km from the Timonha river estuary (Sabry et al., 2013). Finally, the average *p*-distance observed between Brazilian samples and Chinese type species samples is 1.6%, while the average *p*-distance among Chinese samples is 0.2%, suggesting that Brazilian strains constitute a distinct lineage. Our analysis actually shows two distinct clades (maybe populations) among samples from India. However, we consider the sampling insufficient to draw any relevant conclusions on that point at the moment. We know very little about the presence of *Perkinsus* in Brazil yet and we would rather refrain from any further conclusions, which would be highly speculative.

This is the first report of *Perkinsus* sp. in wild clams of the species *A. brasiliana* in Brazil. The phylogenetic analysis strongly indicated *P. beihaiensis* as the present species. Further studies are necessary to determine the impact of this parasite on the host.

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References

Araújo, M.L.R., Rocha-Barreira, C.A., 2004. Occurrence of *Bucephalus* sp. (Trematoda: Bucephalidae) in *Anomalocardia brasiliana* (Gmelin, 1791) (Mollusca: Veneridae) at Canto da Barra Beach, Fortim, Ceará State, Brazil. Arq. Ciênc. Mar. 37, 35–37.

Brandão, R.P., Boehs, G., Sabry, R.C., Ceuta, L.O., Luz, M.S.A., Queiroga, F.R., da Silva, P.M., 2013. *Perkinsus* sp. infecting oyster *Crassostrea rhizophorae* (Guilding, 1828) on the coast of Bahia, Brazil. J. Invertebr. Pathol. 112, 138–141.

Bush, A.O., Lafferty, A.D., Lotz, J.M., Shostak, A.W., 1997. Parasitalogy meets ecology on its own terms: Margoli et al. revisited. J. Parasitol. 83, 575–583.

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

Choi, K.S., Park, K.I., 2010. Review on the protozoan parasite *Perkinsus olseni* (Lester and Davis 1981) infection in Asian waters. In: Ishimatsu, A., Lie, H.-J. (Eds.), Coast Environ. Ecos. Issues East China Sea. pp. 269–281.

da Silva, P.M., Vianna, R.T., Guertler, C., Ferreira, L.P., Santana, L.N., Fernández-Boo, S., Ramilo, A., Cao, A., Villalba, A., 2013. First report of the protozoan parasite *Perkinsus marinus* in South America, infecting mangrove oysters *Crassostrea rhizophorae* from the Paraiba River (NE, Brazil). J. Invertebr. Pathol. 113, 96–103.

da Silva, P.M., Scardua, M.P., Viana, R.T., Mendonça, R.C., Vieira, C.B., Dungan, C.F., Scott, G.P., Reece, K.S., 2014. Two *Perkinsus* spp. infect *Crassostrea gasar* oysters from cultured and wild populations of the Rio São Francisco estuary, Sergipe, northeastern Brazil. J. Invertebr. Pathol. 119, 62–71.

Moss, J.A., Xiao, J., Dungan, C.F., Reece, K.S., 2008. Description of *Perkinsus beihaiensis* n. sp., a new *Perkinsus* sp. parasite in oysters of southern China. J. Eukaryot. Microbiol. 55, 117–130.

OIE, 2012. Código Sanitario para los Animales Acuáticos 2012. Disponível em <http://www.oie.int/esp/normes/fcode/es_sommaire.htm>. Acesso em 10 de Agosto de 2013.

Queiroga, F.R., Marques-Santos, L.F., Hégaret, H., Soudant, P., Farias, N.F., Schlindwein, A.D., da Silva, P.M., 2013. Immunological responses of the mangrove oysters *Crassostrea gasar* naturally infected by *Perkinsus* sp. in the Mamanguape Estuary, Paraiba State (Northeastern, Brazil). Fish Shellfish Immunol. 35, 319–327.

Ray, S.M., 1954. Biological Studies of *Dermocystidium marinum*, a Fungus Parasite of Oysters. Rice Institute pamphlet (Monogr Biol Spec Ser Iss), Rice Institute, Washington, DC.

Ray, S.M., 1966. A review of the culture method for detecting *Dermocystidium marinum*, with suggested modifications and precautions. Proc. Natl. Shellfish Assoc. 54, 55–69.

Reece, K.S., Dungan, C.F., Burreson, E.M., 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, R.C., Rosa, R.D., Magalhães, A.R.M., Barracco, M.A., Gesteira, T.C.V., 2009. First report of *Perkinsus* sp. infecting mangrove oysters *Crassostrea rhizophorae* from the Brazilian coast. Dis. Aquat. Org. 88, 13–23.

Sabry, R.C., Gesteira, T.C.V., Magalhães, A.R.M., Barracco, M.A., Guertler, C., Ferreira, L.P., Vianna, R.T., da Silva, P.M., 2013. Parasitological survey of mangrove oyster, *Crassostrea rhizophorae*, in the Pacoti River Estuary, Ceará State, Brazil. J. Invertebr. Pathol. 112, 24–32.

Sanil, N.K., Suja, G., Lijo, J., Vijayan, K.K., 2012. First report of *Perkinsus beihaiensis* in *Crassostrea madrasensis* from the Indian subcontinent. Dis. Aquat. Org. 98, 209–220.

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. 28, 2731–2739.

Villalba, A., Reece, K.S., Ordás, M.C., Casas, S.M., Figueras, A., 2004. Perkinsosis in molluscs: a review. Aquat. Living Resour. 17, 411–432.