# Parasites infecting the cultured oyster *Crassostrea gasar* (Adanson, 1757) in Northeast Brazil

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SUMMARY

The oyster *Crassostrea gasar* is a species widely used as food and a source of income for the local population of the estuaries of Northeast Brazil. *Perkinsus marinus* and *Perkinsus olseni* are deleterious parasites for oyster farming and were recently detected in Brazil. In this study, a histopathologic survey of the oyster *C. gasar* cultured in the estuary of the River Mamanguape (Paraíba State) was performed. Adult oysters were collected in December 2011 and March, May, August and October 2012 and processed for histology and *Perkinsus* sp. identification by molecular analyses. Histopathological analysis revealed the presence of parasitic organisms including viral gametocytic hypertrophy, prokaryote-like colonies, protozoans (*Perkinsus* sp. and *Nematopsis* sp.) and metazoans (*Tylocephalum* sp. and cestodes). Other commensal organisms were also detected (the protozoan *Ancistrocoma* sp. and the turbellarian *Urastoma* sp.). The protozoan parasite *Perkinsus* sp. had the highest overall prevalence among the symbiotic organisms studied (48·9%), followed by *Nematopsis* sp. (36·3%). The other organisms were only sporadically observed. Only the protozoan *Perkinsus* sp. caused alterations in the oysters' infected organs. Molecular analyses confirmed the presence of *P. marinus, P. olseni* and *Perkinsus beihaiensis* infecting the oyster *C. gasar*. This is the first report of *P. beihaiensis* in this oyster species.

Key words: *Crassostrea gasar*, *Perkinsus* sp., histopathology, PCR–RFLP, rDNA.

## INTRODUCTION

Estuaries along the coast of Paraíba State are inhabited by several species of bivalves, including oysters *Crassostrea gasar* (Adanson, 1757) and *Crassostrea rhizophorae* (Guilding, 1828), clams *Anomalocardia brasiliana* (Gmelin, 1791) and mangrove mussels *Mytella falcata* (Orbigny, 1846) and *Mytella guyanensis* (Lamarck, 1819). In these ecosystems, the bivalves are used as food and a source of income for the local population. Such species are a promising economic alternative through a regular production system, as they grow fast (Lopes *et al.* 2013) due to suitable temperatures (25–30 °C) in the Northeastern region, in addition to the presence of an increasing consumer market.

Nowadays, the major impediment to oyster farming is the occurrence of parasites and diseases that hinder the growth and reproduction of cultured organisms and cause, in some cases, mortality events (Bower *et al.* 1994; Figueras and Novoa, 2011). Among the known pathogens, the most detrimental for oyster farming are bacteria and protozoa (Figueras and Novoa, 2011). Parasites from the genus *Perkinsus* are a constraint for bivalves cultured worldwide (see reviews of Choi and Park, 2010; Villalba *et al.* 2004, 2011). Two species, *Perkinsus marinus* Levine, 1978 and *Perkinsus olseni* Lester and Davis, 1981, are notifiable to the World Organisation for Animal Health (OIE). *Perkinsus marinus* is responsible for mass mortalities of eastern oysters *Crassostrea virginica* (Gmelin, 1791) in the United States and Mexico (Burreson and Ragone-Calvo, 1996; Gullian-Klanian *et al.* 2008) and *Crassostrea gigas* (Thunberg, 1793) in Mexico (Enríquez-Espinoza *et al.* 2010), whereas *P. olseni* has been reported in clams *Ruditapes decussatus* (Linnaeus, 1758) and *Ruditapes philippinarum* (Adams and Reeve, 1850) from several countries in Europe (France, Spain, Italy, Portugal), Asia and Australia (see reviews of Choi and Park, 2010; Villalba *et al.* 2011; Soudant *et al.* 2013).

In the last 15 years, in Brazil, several studies have been conducted to identify and study parasite species present in wild and cultured bivalve populations. Most studies were focused on cultured oysters, including the Japanese species *C. gigas* and the native species *C. rhizophorae*, and mussels *Perna perna* (Linnaeus, 1758) (da Silva *et al.* 2002, 2012, 2014; Suárez-Morales *et al.* 2010; Sabry *et al.* 2011;

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Brandão *et al*. 2013*a*, *b*; Queiroga *et al*. 2013). But it was only in 2012 that the Brazilian government (Ministry of Fisheries and Aquaculture) created a National Network of Reference Laboratories in order to perform diagnostic testing for diseases of aquatic animals and control disease spread and commercialization of molluscs. This measure was in part a result of the increasing countrywide production of bivalves.

So far, mortality events have been only sporadically reported in bivalves from Brazil, in Santa Catarina State (South Brazil). One occurred in a wild population of stout razor clam *Tagelus plebeius* (Lightfoot, 1786) associated with a sudden drop in salinity after a flood (da Silva *et al*. 2009); another affected 20% of a cultured mussel population of *P. perna* due to a rare infection in the mantle by a larva of the copepod *Monstrilla* sp. Dana, 1849 (Suárez-Morales *et al*. 2010) and, finally, *summer mortality* events that occur occasionally in cultured oysters *C. gigas*.

OIE notifiable parasite species for molluscs (OIE, http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2014/) were only recently reported in Brazil, *P. marinus* infecting wild oysters *C. rhizophorae* (da Silva *et al*. 2013) and *P. olseni* infecting cultured and wild populations of the economically important native oyster *C. gasar* (da Silva *et al*. 2014). In addition, *Perkinsus beihaiensis* Moss *et al*. 2008 was reported infecting the oyster *C. rhizophorae* (Sabry *et al*. 2009, 2013) and other *Perkinsus* spp. remain to be identified (Brandão *et al*. 2013*b*).

Since *C. gasar* is the most promising oyster species for husbandry in the northeast region of Brazil and the risk presented by *Perkinsus* spp., more attention should be given to this oyster species. Therefore, this study aimed to detect and describe the organisms and diseases affecting the oysters *C. gasar* cultured in the Mamanguape estuary as well as the host reactions and histological changes in the host.

## MATERIAL AND METHODS

### *Study area and experimental design*

The coastal region of Paraíba State 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 rainfall of 1800 mm and air temperature of 26 °C.

Oysters *C. gasar* over 70 mm in shell height were obtained from a suspended–fixed cultivated system located on the estuary of the Mamanguape River (S 06°47′082″; WO 34°59′467″).

Oyster samplings ($N = 40$) were performed in the following months: December 2011, March, May, August and October 2012. After each sampling, oysters were distributed into tanks containing seawater from the site in a closed system under constant aeration for a maximum of 48 h. The oyster capture and experimental procedures were authorized by ICMBio (Chico Mendes Institute for Biodiversity Conservation/Authorization under the code: 30718-1).

### *Histological sections*

Oysters were opened (~40/sample) by severing their adductor muscles. A transverse histological sample approximately 5 mm in thickness was excised, fixed in Davidson's solution for 48 h, and embedded in paraffin. Histological sections (5 $\mu$m) were stained with Mayer's haematoxylin and eosin (Howard *et al*. 2004) for examination by light microscopy. Some oysters were lost during histological procedures, and a total of 182 oysters were analysed (December 2011, $N = 40$; March, $N = 39$; May, $N = 40$; August, $N = 44$ and October 2012, $N = 19$). Prevalences of parasites and commensal associates were calculated as the proportion of oysters affected in each sample month. The sizes of organisms are shown as Mean±S.E.

### Perkinsus *sp. diagnosis by Ray's fluid thioglycollate medium (RFTM)*

To select oysters infected by *Perkinsus* sp. for further molecular analyses, 2 gill demibranchs were excised from each oyster for incubation in RFTM (Ray, 1966) and a small piece of gill tissue was preserved in 96% ethanol. After 7 days of incubation in RFTM at room temperature (~25 °C) in the dark, the gills were used to determine the presence and intensity of infection by *Perkinsus* sp. (Ray, 1966; da Silva *et al*. 2013).

### *PCR assays*

Gill samples (50–80 mg) were submitted to DNA extraction with DNAzol (Invitrogen) reagent according to manufacture instructions.

To identify the species of *Perkinsus*, the internal transcriber spacer (ITS) regions of *Perkinsus* spp. ribosomal RNA gene complexes were amplified from isolated DNAs, using the genus-specific primers PerkITS-85 and PerkITS-750 (Casas *et al*. 2002). For PCR, 25 $\mu$L reactions containing 1 $\mu$L of genomic DNA (50–200 ng), 1 × PCR buffer, $MgCl_2$ at 1·5 mM, 0·2 mM nucleotides, 0·4 $\mu$M of each primer and 0·04 U $\mu L^{-1}$ of Taq DNA polymerase (da Silva *et al*. 2013). DNA samples from *in vitro* cell cultures of *P. olseni*, from *P. marinus* (GenBank JX144335; da Silva *et al*. 2013), and from *P. beihaiensis*-infected oysters (GenBank FJ472346; Sabry *et al*. 2009) were used as controls for PCR and PCR–RFLPs analyses.

To identify the species of *Crassostrea*, PCR reactions were performed as previously described (da

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Silva *et al*. 2013) in a total volume of 25 μL, containing 1 μL of genomic DNA (50 ng), PCR 1 × buffer, $MgCl_2$ at 1·5 mM, 0·2 mM nucleotides, 0·4 μM of each primer and 0·04 U $\mu L^{-1}$ of Taq DNA polymerase. The primer pair (16SAR – 16SBR) is targeted to amplify a region of the mitochondrial DNA of the small subunit of ribosomal RNA (16S) (Kessing *et al*. 1989). DNA samples of oysters *C. gasar, C. rhizophorae* and *C. gigas* were used as controls for PCR and PCR–RFLPs analyses.

PCR products were electrophoresed on 1·5% agarose gels, stained with ethidium bromide and visualized by UV illumination.

### *Restriction fragment length polymorphism (RFLP) analyses*

Freshly amplified *Perkinsus* sp. rDNA ITS-region PCR products from DNA samples of 25 *Perkinsus* sp.-infected oysters and control DNA samples (*P. olseni, P. marinus* and *P. beihaiensis*) were digested separately with the enzymes *Rsa*I and *Hinf*I following manufacturer's protocols (Fermentas) (Abollo *et al*. 2006). Similarly, PCR–RFLP analysis was performed to identify oyster species among the 39 *Perkinsus* sp.-infected (34) and uninfected (5) specimens. The freshly amplified 16S mtDNAs were digested with the enzyme *Alu*I (Fermentas). Restriction patterns were compared with those obtained from the control (*Perkinsus* ssp. and *Crassostrea* ssp.).

Ten microlitres of digestion products were resolved on an 8% polyacrylamide gel and stained with ethidium bromide.

### *DNA sequencing and phylogenetic analyses*

Freshly amplified *Perkinsus* sp. rDNA ITS-region PCR products (60 ng) from 15 oyster DNA samples were mixed separately with primers (4·5 pmol), and then dried at 60 °C and send for sequencing at the ACTGene *Análises Moleculares* Ltd. (*Centro de Biotecnologia*, UFRGS, Porto Alegre, RS, Brazil). Briefly, samples were labelled with 3 μL of BigDye Terminator v3.1 Cycle Sequencing RR-100 in a final volume of 10 μL. Labelling reactions were performed in a GeneAmp PCR System 9700 thermocycler with an initial denaturing step of 96 °C for 3 min followed by 25 cycles of 96 °C for 10 s, 55 °C for 5 s and 60 °C for 4 min. Labelled samples were analysed using ABI-PRISM 3100 Genetic Analyzer automatic sequencer (Applied Biosystems).

The resulting sequences were compared to those deposited in GenBank using basic local alignment search tool (BLAST; Altschul *et al*. 1990) of the National Center for Biotechnology Information (NCBI) database. Sequences of rDNA-ITS were aligned using ClustalW (Thompson *et al*. 1994) in BioEdit 5.0.9 (Hall, 1999). Parsimony bootstrap analysis was done with PAUP*4.0b10 (Swofford, 2002), with 50% deletion and 100 random additions of 1000 replicates. The ITS trees were rooted with the *Perkinsus qugwadi* Blackbourn *et al*. 1998 (GenBank no. AF151528) and *Parvilucifera infectans* Norén *et al*. 1999 (GenBank no. KF359485) ITS regions sequences. The GenBank accession numbers for the other *Perkinsus* spp. sequences used in the analyses are annotated in the phylogenetic tree figure (Fig. 7).

Nucleotide sequences reported in this paper are available in GenBank under the accession numbers KP160919 - KP160933

## RESULTS

### *Histopathological analysis*

In the present study, histological sections of 182 oysters collected at different times of the year were analysed. Forty-two oysters showed no histopathological changes, whilst the remaining 140 showed different parasitic and commensal organisms.

The occurrence of viral gametocytic hypertrophy was characterized by gametes with markedly hypertrophied nuclei (31·0±0·71 μm; range: 28·8 to 31·8 μm; $N = 4$) in the epithelium of male gonad follicles. The hypertrophied nuclei were basophilic with peripheral heterochromatin (Fig. 1A). This pathology was identified in only 2 oysters; 1 of them showed 3 hypertrophied gametes and the other only 1.

Prokaryotic colonies were observed as basophilic inclusions in the cytoplasm of epithelial cells of the digestive gland tubules (Fig. 1B). The colonies had a granular appearance and a circular shape of variable size (8·6±0·49 μm; range: 3·8–19·6 μm; $N = 79$) (Fig. 1C). Infected oysters contained 1–74 colonies per histological section (24±9·1).

The *Ancistrocoma* sp. Chatton and Lwoff, 1926 (Ciliophora: Ancistrocomidae) had a fusiform shape and measured 8·6–40·6 μm (24·9±7·11 μm; $N = 16$) (Fig. 1F). One to three (1·0±0·03) *Ancistrocoma* sp. were observed in the lumen of the digestive tubules and in 1–18 cells (3±0·7) per histological section.

Oocysts of the protozoan *Nematopsis* sp. Schneider, 1892 (Apicomplexa: Porosporidae) were observed (1–6 oocysts; Fig. 1D) in the connective tissue of all organs of the oysters. The oocysts had a diameter ranging from 8·1 to 13·7 μm (11·7±1·23 μm; $N = 23$) and contained a basophilic and ellipsoid sporozoite (Fig. 1D and E). The mean intensity of infection was 2·5±0·65 (range: 1–16) oocysts per histological section.

Trophozoites of the protozoan *Perkinsus* sp. (Perkinsozoa: Perkinsidae) were observed infecting the digestive epithelia of the stomach, intestine and oesophagus. The trophozoites were spherical (3·4

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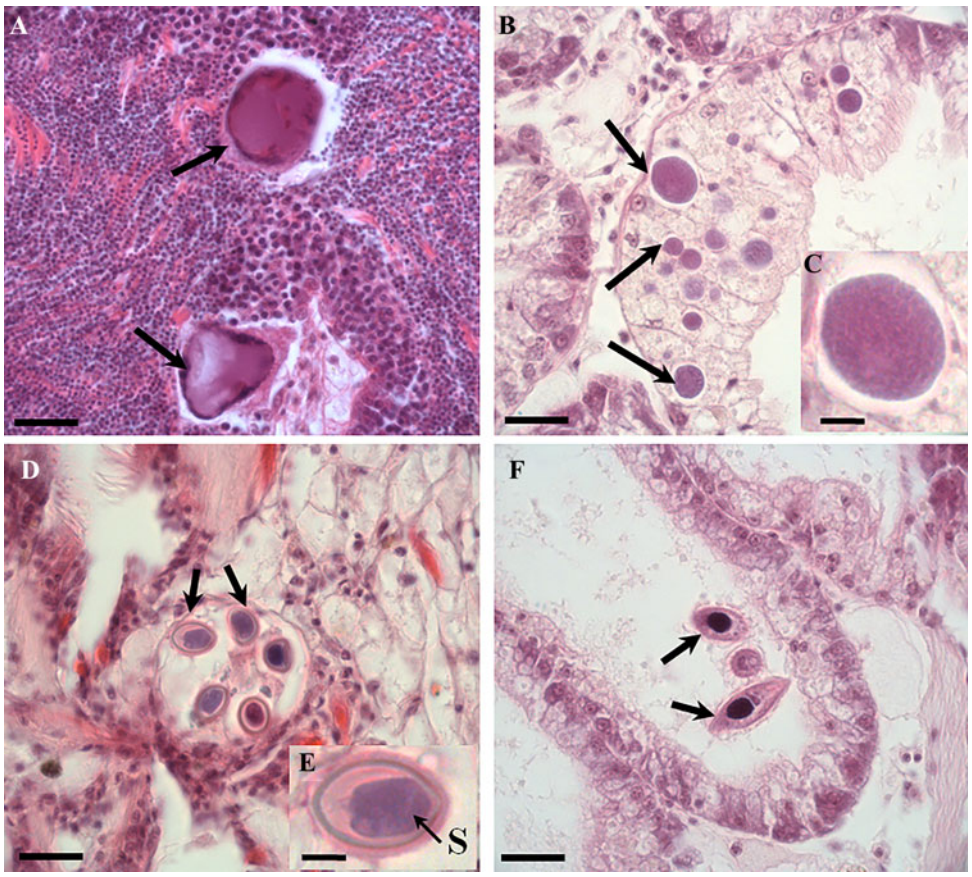


Fig. 1. Histological sections of the oyster *C. gasar* showing parasites and commensals. (A) Male gonad follicle containing 2 altered cells. Note the hypertrophied nuclei and condensed heterochromatin (arrows) at the periphery; (B) epithelial cells of a digestive gland tubule containing various prokaryotic colonies (arrows); (C) magnification of the colony; (D) 5 oocysts (arrows) of *Nematopsis* sp. in the connective tissue; (E) magnification of 1 oocyst showing the basophilic sporozoite (S); (F) the ciliated *Ancistrocoma* sp. (arrows) in the lumen of a digestive tubule. Bar = 20 $\mu$m for Figures A, B, D and F and 5 $\mu$m for Figures C and E.

$\pm$0·12 $\mu$m; range: 1·7 to 7·1 $\mu$m; $N = 87$), containing a large vacuole in the cytoplasm, and an eccentric nucleus with a prominent nucleolus (Fig. 2A–C). Occasionally, basophilic schizonts were observed (Figs. 2A and 3D). The trophozoites and, less often, schizonts, were found engulfed by infiltrated haemocytes, which contained 1–9 parasites. The cytoplasm of phagocytic haemocytes occasionally contained a brownish substance (Fig. 2A). Most oysters were lightly infected (1–3 trophozoites in the epithelia), and only 11·2% of the oysters showed a more severe infection (several trophozoites and schizonts engulfed by haemocytes) (Fig. 2A). Those cases were observed in the months of March (10·25%), May (12·5%) and August (2·3%).

The turbellarian *Urastoma* sp. Dörler, 1900 (Platyhelminthes) was identified by its round or ellipsoid shape (150·3$\pm$23·68 $\mu$m; range: 68·0 to 208·7 $\mu$m; $N = 5$) and its surface covered with cilia (Fig. 3A). *Urastoma* sp. was observed in external spaces between gill lamellae or near gill filaments. Only 1 specimen was observed per histological section, except in 2 cases, in which 2 were observed.

Metazoan *Tylocephalum* sp. Linton, 1890 (Cestoda: Tetragonocephalidae) larvae were observed encapsulated by haemocytes in the connective tissue near the oesophagus, stomach and intestine (Fig. 3B). Their size ranged from 95·7 to 108·6 $\mu$m (103·1$\pm$3·74 $\mu$m; $N = 3$). In all cases, there was only 1 encapsulation per histological section.

Throughout the study period, the protozoans *Perkinsus* sp. and *Nematopsis* sp. were the organisms with the highest overall prevalence, 48·9 and 36·3%, respectively. The remaining organisms were detected with a low mean prevalence: *Ancistrocoma* sp. (14·8%); *Urastoma* sp. (9·3%), prokaryotic colonies (4·9%), *Tylocephalum* sp. (1·6%) and viral gametocytic hypertrophy (1·1%). The monthly prevalence of all organisms is shown in Fig. 4.

### *Identification of oysters* Crassostrea *spp. by PCR–RFLP*

All oysters ($N = 39$) analysed showed a fragmentation pattern of their 16S mtDNA sequence with the endonuclease *Alu*I identical to the species *C. gasar*. This pattern had a larger fragment of ~250

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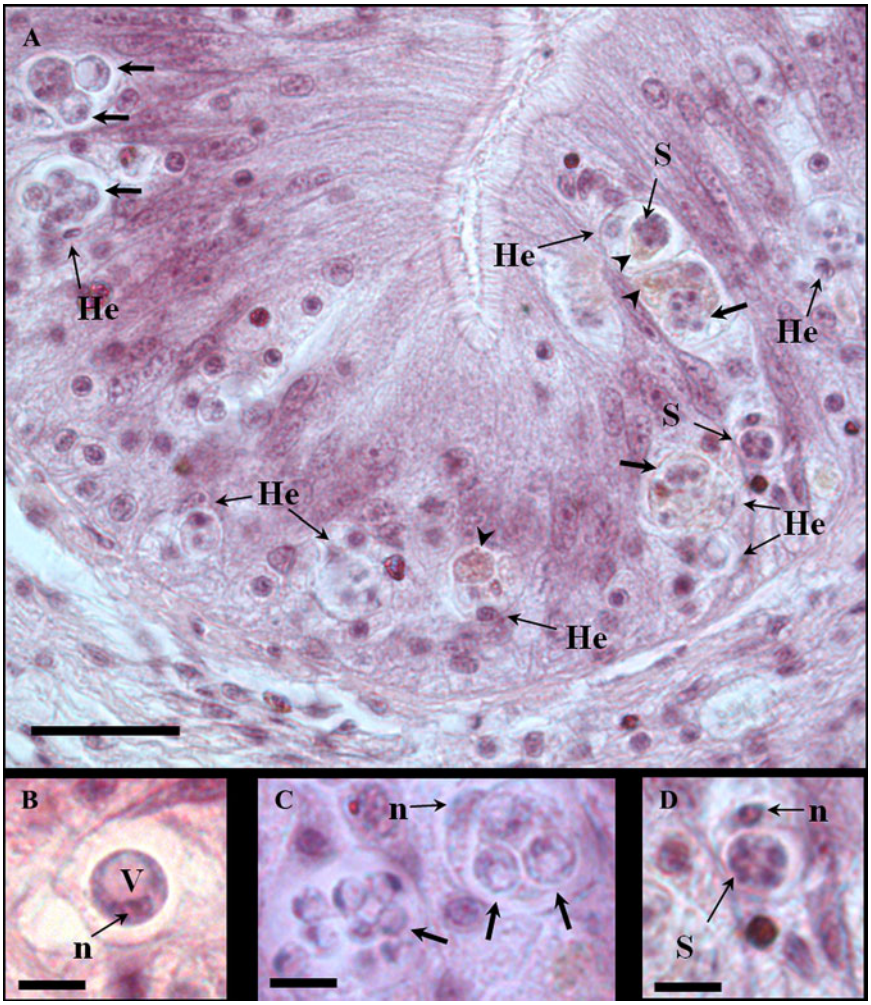


Fig. 2. Histological sections of a *Perkinsus* sp.-infected oyster *C. gasar*. (A) Trophozoites (arrows) and schizonts (S) phagocytosed by haemocytes in the epithelium of the intestine. Note the infiltration by infected haemocytes (He) and the presence of a brownish substance (arrowhead). (B), (C) and (D) Magnification of *Perkinsus* spp. cells; (B) trophozoite showing a large vacuole (V) and eccentric nuclei with a prominent nucleolus (n); (C) detail of trophozoites of different sizes. Note the eccentric nucleus of the haemocyte (n); (D) phagocytosed schizont showing 4 daughter cells arranged in rosettes. Note the eccentric nucleus of the haemocyte (n). Bar = 20 $\mu$m for figure A and 5 $\mu$m for figures B, C and D.

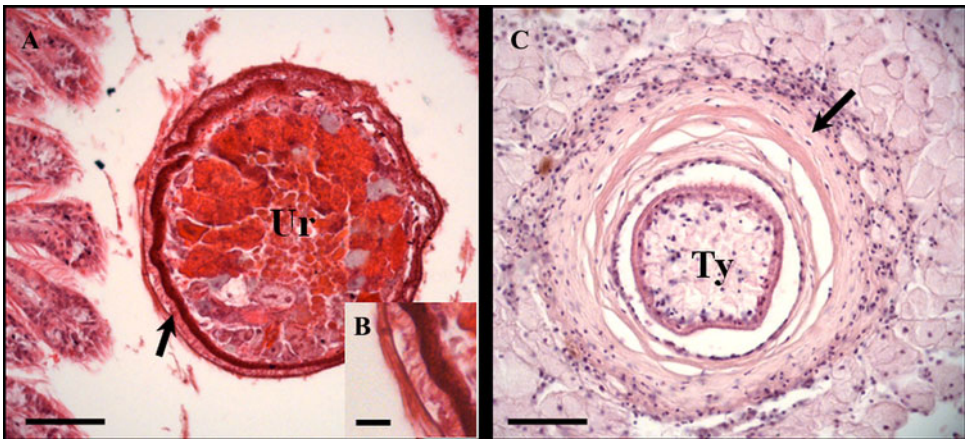


Fig. 3. Histological sections of the oyster *C. gasar* showing metazoans. (A) A cross-section of the turbellarian *Urastoma* sp. (Ur). Note the ciliated surface (arrow); (B) detail of ciliary margin; (C) cross-section of encapsulated larvae of *Tylocephalum* sp. (Ty) (arrow). Bar = 50 $\mu$m for figures A and C and 10 $\mu$m for figure B.

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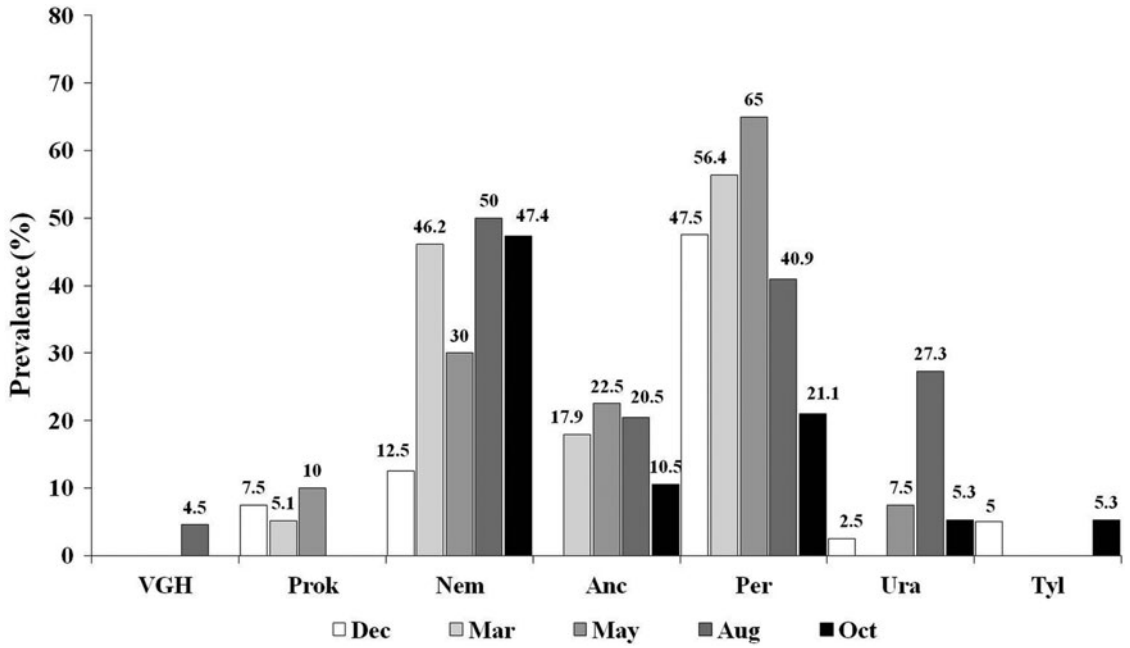


Fig. 4. Monthly prevalences (%) of parasitic organisms and commensals detected in oysters *C. gasar* in the months of December 2011, March, May, August and October 2012. VGH: viral gametocytic hypertrophy; Prok: prokaryotic colonies; Nem: *Nematopsis* sp.; Anc: *Ancistrocoma* sp.; Per: *Perkinsus* spp.; Ura: *Urastoma* sp.; Tyl: *Tylocephalum* sp.

bp and a group of smaller fragments less than 100 bp (Fig. 5).

### Perkinsus *sp. diagnosis by RFTM*

*Perkinsus* sp. infections were detected by RFTM assays in all (4) samples, at prevalences ranging from 80 to 100% (mean = 93·3%) and with sample mean infection intensities of 1·84–2·45 (mean = 2·0), which reflected predominately low and moderate infection intensities.

### *Identification of protozoans* Perkinsus *spp. by PCR–RFLP*

The rDNA ITS segments amplified by genus *Perkinsus*–PCR produced 2 different fragmentation patterns when cut with either endonuclease *Hinf*I or *Rsa*I, and those patterns closely corresponded with those of either *P. marinus* or *P. beihaiensis* (Fig. 6). In the case of *Rsa*I, for the species *P. marinus* digestion yielded a group of 3 fragments of very similar size, ~200 bp (triplet), and 1 fragment ~ 60 bp, whereas *P. beihaiensis* showed 3 well distinguished bands of ~400, ~200 and~ 70 bp (Fig. 6A). In the case of *Hinf*I, for *P. marinus* the pattern obtained corresponded to 4 easily distinguishable bands of ~400, ~150, ~100 and ~60 bp, whereas *P. beihaiensis* had a group of 4 fragments with sizes ranging from 150 to 200 bp and 1 smaller of ~10 bp (Fig. 6B).

Among 25 RFTM-positive oysters whose DNAs were analysed by PCR–RFLP, 12 were infected by *P. marinus* and 13 were infected by *P. beihaiensis* (Fig. 6). The modal (5/12) intensity rank for oysters infected by *P. marinus* was 2 (light), while the modal (6/13) intensity rank for oysters infected by *P. beihaiensis* was 3 (moderate) (Table 1).

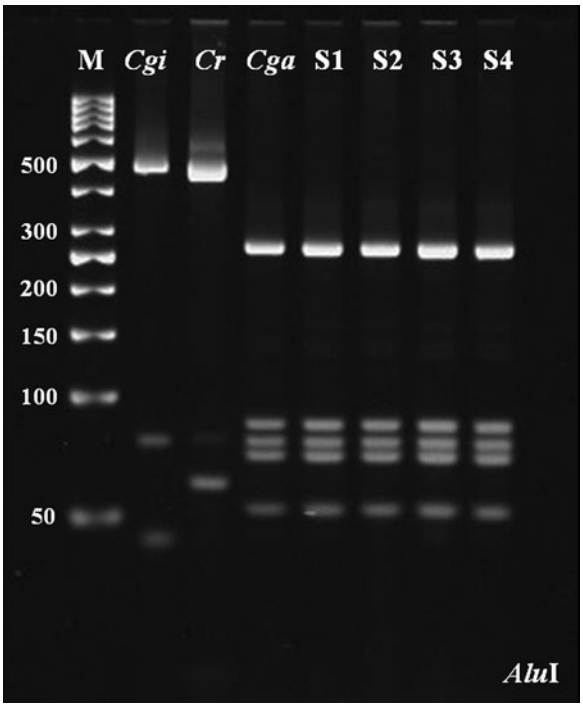


Fig. 5. PCR–RFLP for differential diagnosis of *Crassostrea* spp.. Fragmentation patterns of PCR-amplified products of the mtDNA 16S region from controls *C. gigas* (*Cgi*), *C. rhizophorae* (*Cr*), *C. gasar* (*Cga*) and the *Crassostrea* sp. samples (S) after digestion with *Alu*I endonuclease. M: 50 bp molecular size marker. All oysters S1–S4 showed fragmentation pattern of *C. gasar*.

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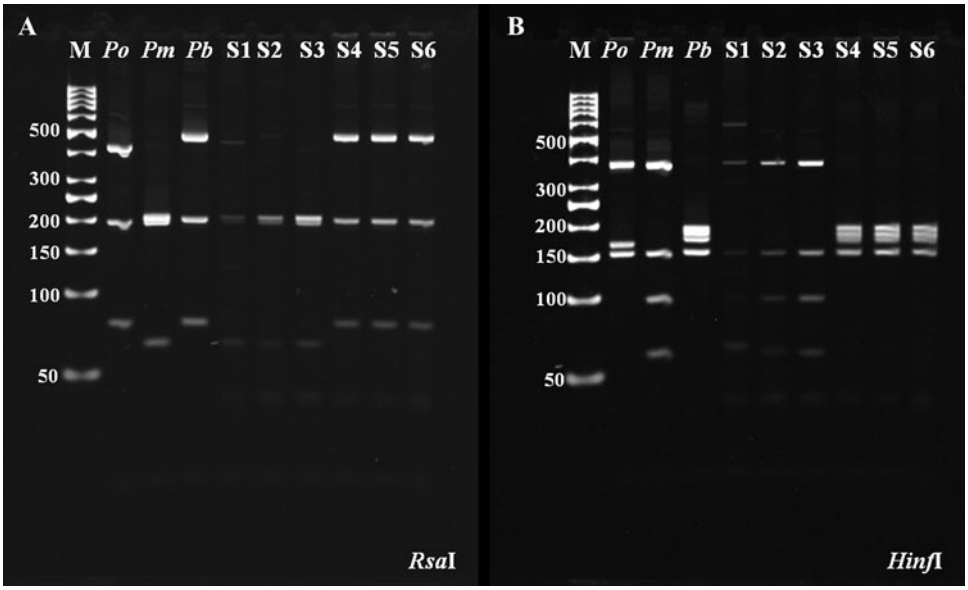


Fig. 6. PCR–RFLP for differential diagnosis of *Perkinsus* spp. Fragmentation patterns of PCR amplicons of the rDNA ITS region from *P. olseni* (*Po*), *P. marinus* (*Pm*), *P. beihaiensis* (*Pb*) and *Perkinsus* sp.-infected oysters (S) after digestion with *Rsa*I (A) and *Hinf*I (B) endonucleases. M: 50 bp molecular size marker. PCR amplicons from oysters S1–S3 showed the fragmentation patterns of *P. marinus*; while those from oysters S4–S6 showed the patterns of *P. beihaiensis*.

### *Phylogenetic analysis*

A total of 15 ITS amplicons of *Perkinsus* spp. were sequenced from 15 individual host samples with different intensities of infection. BLAST analysis of the ITS sequences indicated that *P. marinus* DNAs had been amplified from 6 oysters (CB-MA43; 44; 79; 126; 235; 239) with 99–100% identity; that *P. beihaiensis* DNAs had been amplified from 8 oysters (CB-MA33; 84; 90; 92; 120; 121; 142; 287) with 99% identity, and that *P. olseni* sequence was obtained from 1 oyster (CB-MA245) with 99% identity.

The parsimony topology consisted of 3 clades, one including *P. beihaiensis* (100%), another (91%) including *Perkinsus chesapeaki* McLaughlin *et al.* (2000) (=*Perkinsus andrewsi*) (100%), and the third containing *P. marinus* (100%), *P. olseni* (=*Perkinsus atlanticus*) (100%), *Perkinsus honshuensis* Dungan and Reece, 2006 (93%) and *Perkinsus mediterraneus* Casas *et al.* 2004 (69%) (Fig. 7). Results of phylogenetic analyses of the ITS sequences were consistent with the species identification from BLAST analyses. The *P. marinus* (6), *P. beihaiensis* (8) and *P. olseni* (1) sequences grouped in clades, with bootstrap support values of 100% (Fig. 7).

Table 1. Number of cases of each of *Perkinsus* species determined by PCR–RFLP within each intensity of infection (1–4; by RFTM)

| | Intensity of infection by *Perkinsus* spp. | | | | |
|---|---|---|---|---|---|
| | 1-Very Light | 2-Light | 3-Moderate | 4-Heavy | *N* |
| *Perkinsus marinus* | 2 | 5 | 3 | 2 | 12 |
| *Perkinsus beihaiensis* | 1 | 3 | 6 | 3 | 13 |
| Total | 3 | 8 | 9 | 5 | 25 |

## DISCUSSION

### *Histopathological analysis*

This study describes the organisms found in the oyster *C. gasar* cultured in the Mamanguape estuary (NE, Brazil). The most important finding was infection by the protozoan *Perkinsus* spp., although viral gametocytic hypertrophy (VGH), prokaryotic colonies and other protozoa (*Nematopsis* sp. and *Ancistrocoma* sp.) and metazoa (*Urastoma* sp. and *Tylocephalum* sp.) were also detected.

The hypertrophy of male gametes, possibly the nuclei, with a basophilic inclusion and peripheral heterochromatin suggests a disease caused by a virus of the Papillomaviridae and Polyomaviridae families that affects bivalve gonads (Garcia *et al.* 2006; Cheslett *et al.* 2009). Viral gametocytic hypertrophy, as the disease is called, has been reported in both gametes of *C. gigas* (Garcia *et al.* 2006; Cáceres-Martínez *et al.* 2010), but only in males of *C. rhizophorae* (da Silva *et al.* 2012).

The basophilic inclusions with granular appearance in the epithelial cells of the digestive tubules resembled those of prokaryotic colonies, which are commonly found infecting several bivalve species worldwide (Romalde and Prado, 2011). In some cases, this infection induced haemocytic infiltration into organs of oysters *Crassostrea corteziensis*

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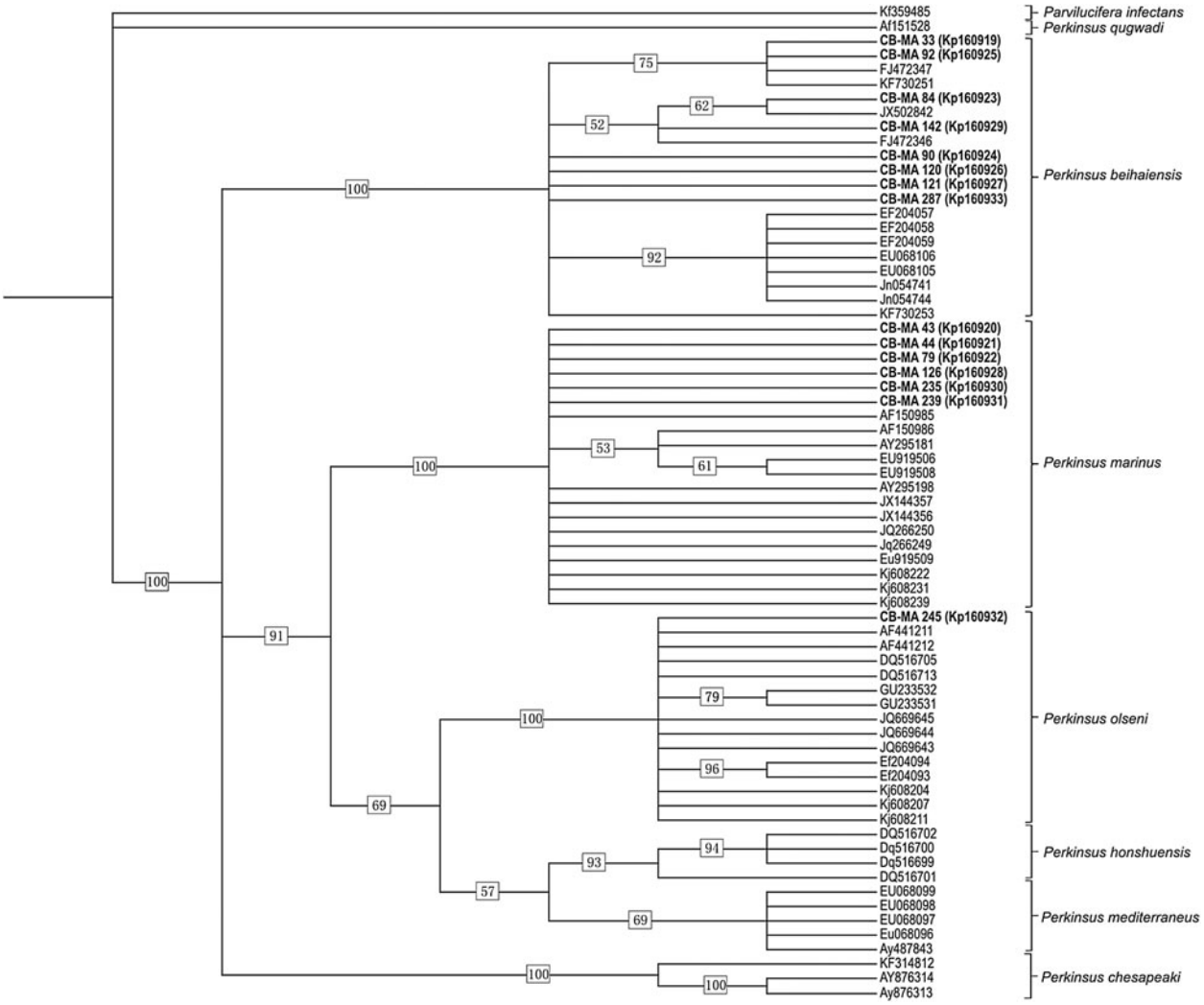


Fig. 7. Cladogram showing results of maximum parsimony analyses on *Perkinsus* spp. ITS region sequences. Bootstrap support values are indicated at nodes. Previously determined sequences are indicated by Genbank accession numbers only. Sequences from this study are indicated in bold, with the oyster sample number followed by the Genbank accession number.

(Hertlein, 1951) (Cáceres-Martínez *et al*. 2010). Sporadically, associated mortalities are reported, such as in oysters *Crassostrea ariakensis* (Fujita, 1913) from China (Sun and Wu, 2004) and *C. rhizophorae* from Brazil (Azevedo *et al*. 2005). Sabry *et al*. (2011) reported a low intensity of infection (1–5 colonies/histological section) in oysters *C. rhizophorae* and *C. gigas* from southern Brazil, contrasting with the more intense cases (up to 74 colonies/histological section) diagnosed in *C. gasar* from the current investigation.

Ciliates *Ancistrocoma* sp. are common commensals of oysters and usually do not cause deleterious effects on bivalves (Bower *et al*. 1994).

*Nematopsis* sp. was the second most prevalent protozoan found, but showed low intensities of infection. *Nematopsis* sp. has also been identified with varying prevalences throughout the year (20–100%) in oysters *C. rhizophorae* from Bahia (NE Brazil). The lowest prevalences could be associated with the decreased salinity that occurs during periods of high rainfall in winter (Brandão *et al*. 2013*a*). In the present study, the occurrence of *Nematopsis* sp. also followed this pattern, with the highest prevalence in May, the period with high rainfall. However, the low prevalence in December 2011 could not be similarly explained. Thus, a longer survey is recommended to better understand the influence of environment parameters in the occurrence of this protozoan.

Typical trophozoites of the protozoan parasite *Perkinsus* sp. were observed phagocytosed by haemocytes in the epithelia of the oesophagus, stomach and intestine of the oyster *C. gasar*. Interestingly, other tissues showed no cells of the parasite. Digestive epithelia are a common *Perkinsus* spp. infected site in its hosts, including Brazilian oysters (*C. rhizophorae* and *C. gasar*) (Moss *et al*. 2008; Sabry *et al*. 2009; Brandão *et al*. 2013*b*; da Silva *et al*. 2014). The location of this

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parasite in host tissue may be associated with the mechanism of entry *via* food particle capture (Mackin, 1951). Indeed, granulocytes are able to cross the digestive epithelium reaching the lumen of gut where they capture, digest food particles and cross back transporting nutrients to oyster tissues (Kennedy, 1996). Accordingly, granulocytes of *C. virginica* produce and secret galectins (CvGal), that interacts with algal food and strongly with *P. marinus* in the gut lumen, function as opsonin and leading to phagocytosis, contributing to spreading the pathogen into host (Tasumi and Vasta, 2007). However, mucus and pseudofaeces may also be considered an entrance for *P. marinus* to cross the epithelial layer of the mantle (Allam *et al*. 2013).

Most trophozoites and schizonts were found phagocytosed by haemocytes of the oyster *C. gasar* infiltrated in digestive epithelia. This finding suggests phagocytosis is an important defence mechanism of the oyster against the parasite *Perkinsus* sp. In the current investigation, *Perkinsus* sp. infections were predominantly light. This might be a result of an efficient intracellular lytic mechanism triggered by the host after the phagocytic defence process, which therefore acts to control the spread of disease. The appearance of a brown substance inside infected haemocytes may indicate the involvement of phenoloxidase enzyme (PO). This enzyme is activated by proteolytic cleavage initiated by a cascade of serine proteases that produce various cytotoxic compounds, including quinones (Cerenius and Söderhall, 2004). The implication of phenoloxidase as a defence mechanism has been suggested in clams *R. decussatus* infected by *P. olseni* (Muñoz *et al*. 2006) and in oysters *C. gasar* infected by *P. marinus/olseni* (da Silva *et al*. 2014). In addition, there is some evidence indicating PO mediates phagolysosomal killing of the protozoan *Marteilia sydneyi* Perkins and Wolf, 1976, which is phagocytosed by haemocytes of the oyster *Saccostrea glomerata* (Gould, 1850) (Kuchel *et al*. 2010). Nevertheless, some oysters (11·2%) harbouring advanced intensities of *Perkinsus* sp. were also identified. We believe the parasite may also be able to evade the host defence mechanism. Several evasion mechanisms are known for the *P. marinus–C. virginica* model, such as secretion of serine proteases that act by inhibiting haemocyte function (mobility, agglutination, lysozyme, etc.) and antioxidant enzyme activities (acid phosphatase, superoxide desmutases and dependent ascorbate peroxidase) (see review of Soudant *et al*. 2013). Some of these mechanisms could be occurring in the present model and must be elucidated.

The prevalence of *Perkinsus* sp. in *C. gasar* determined by histological sections was high during the whole studied period, except in October (21·1%). May was the month with the highest prevalence and frequency of specimens with severe infection. Although May is the month that marks the beginning of the rainy season (April, 5·6 mm against May 116·4 mm), the sampling was taken early (6th) in this month. Thus, the high prevalence in this month reflects the cumulative effect of the dry season, when the highest water temperatures and salinities are recorded. It was in May that these oysters started to suffer an impairment of the immune system (Queiroga *et al*. 2013). *Perkinsus marinus* and *P. olseni* are strongly associated with high temperatures and salinities (see reviews of Villalba *et al*. 2004, 2011; Choi and Park, 2010). Studies conducted to explore the influence of such abiotic parameters on the prevalence of *Perkinsus* sp., have been performed only for the latter species (see review of Villalba *et al*. 2011). In Brazil, recently, a seasonal pattern of *Perkinsus* sp. prevalence and intensity of infection was demonstrated in cultured and wild oyster *C. gasar* populations from Sergipe State, which is located 450 km south of Paraíba State. An abrupt drop in prevalence was observed in winter (July), when salinity was the lowest (24‰) compared to summer's 34‰ (da Silva *et al*. 2014). However, more confident environmental data (daily and continuous temperature and salinity monitoring) must be collected in order to study their influence on the dynamics of infection by *Perkinsus* spp. in tropical hosts.

The turbellarian *Urastoma* sp. showed a very low prevalence and intensity in the oyster *C. gasar*, as observed for oysters in Brazil (Sabry *et al*. 2011, 2013; da Silva *et al*. 2012). *Urastoma* sp. are free-living organisms and can inhabit the body cavities and gills of bivalves, not penetrating the tissues. As a result of environmental changes, they may become abundant and over-inhabit, causing disruption of oysters *C. corteziensis* gills (Cácerez-Martínez *et al*. 2010).

Cestode larvae of *Tylocephalum* sp. were observed encapsulated with no damage to the host tissue, as reported before (Sabry *et al*. 2011, 2013; da Silva *et al*. 2012; Dang *et al*. 2013).

### *Identification of the* Perkinsus *sp*.

The PCR–RFLP analysis confirmed oysters cultivated in the estuary of the Mamanguape River belong to the species *C. gasar*. The oysters were infected by *P. marinus* and *P. beihaiensis*, both parasites having already been reported on the northeastern coast of Brazil (Sabry *et al*. 2009; da Silva *et al*. 2013). Although *P. marinus* is considered a dangerous species for oyster health, here the majority of *P. marinus*-infected oysters showed light infection (level 2). In contrast, the impact of *P. beihaiensis* on its hosts has not yet been studied. Our results showed *P. beihaiensis* mostly occurred with moderate infection (level 3). Perhaps this explains why, despite the high prevalence *Perkinsus* sp. achieved in the *C. gasar* population, its impact seemed to be very low and no mortality event was reported (data

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from the oyster producer). This result provides insights for future studies on the differential susceptibility of the oysters *C. gasar* to *Perkinsus* spp. A previous study showed *P. olseni, P. marinus* and dual-infection among oysters *C. gasar* from Sergipe State (da Silva *et al*. 2014).

Phylogenetic analyses based on nucleotide sequences of the ITS region consistently placed all sequences within the genus *Perkinsus*. Sequences studied corresponded to 3 known *Perkinsus* spp. It was not a surprise to find *P. marinus* infecting oysters in the estuary of the Mamanguape River, which is located around 40 km from the estuary of Paraíba do Norte, where *P. marinus* was recently reported for the first time infecting *C. rhizophorae* in Brazil (da Silva *et al*. 2013). Concerning the host studied, *P. marinus* was also recently reported in this oyster *C. gasar* species, from Sergipe State (da Silva *et al*. 2014). However, to date, *P. beihaiensis* has been reported in the oyster *C. rhizophorae* (Sabry *et al*. 2009) and the clam *A. brasiliana* (Ferreira *et al*. in press) from Brazil, *Crassostrea madrasensis* Preston, 1916 from India (Sanil *et al*. 2012), and *Crassostrea hongkongensis* Lam and Morton, 2003 and *C. ariakensis* from China (Moss *et al*. 2008).

## *Concluding remarks*

The majority of organisms found in the cultured oyster *C. gasar* were rare or occasionally observed and showed low pathological potential. Only the protozoan *Perkinsus* sp. and *Nematopsis* sp. occurred with moderate monthly prevalence. The protozoan *Perkinsus* sp. showed light intensity in the tissue; thus, the host defence response might control the spread of the parasite. Three species of *Perkinsus* were identified in oyster *C. gasar: P. marinus* and *P. beihaiensis* were more abundant than *P. olseni*. Some evidences suggest that this oyster species might be differentially susceptible to *P. marinus* and *P. beihaiensis*.

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