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Polymorphism at the ITS and NTS Loci of *Perkinsus marinus* Isolated from Cultivated Oyster *Crassostrea corteziensis* in Nayarit, Mexico and Phylogentic Relationship to *P. marinus* along the Atlantic Coast

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Abstract:

Prevalence of the protozoan *Perkinsus* spp. in the gills of the pleasure oyster *Crassostrea corteziensis* from two estuaries in Nayarit, Mexico, was measured. The protozoan was identified by PCR amplification of the internal transcribed spacer (ITS) region of the rDNA of *Perkinsus* spp. The pathogen was found in 92% of oysters from Boca de Camichín and 77% of oysters from Pozo Chino. ITS sequences characterized from *C. corteziensis* showed 96–100% similarity to *Perkinsus marinus*. The most frequent ITS sequence (GenBank JQ266236) had 100% identity with the ITS locus of *P. marinus* from New Jersey, Maryland, South Carolina and Texas, and the second most frequent observed sequence (GenBank JQ266240) was 100% identical to ITS sequences of *P. marinus* from New Jersey, South Carolina, Louisiana, and Bahía Kino, Sonora, Mexico. The 14 sequences from the non-transcribed spacer (NTS) showed 98% similarity to *P. marinus* from Texas. The most frequent polymorphism identified was at nucleotide 446 of the ITS region; however, the NTS showed the highest nucleotide diversity, thereby suggesting that this region is suitable for genotype identification. Moreover, the most conserved ITS marker is better for species-specific diagnosis. Both the ITS and NTS sequences of *P. marinus* obtained from *C. corteziensis* were grouped in two clades, identifying two allelic variants of *P. marinus*.

Keywords: *Crassostrea corteziensis* ; *Perkinsus marinus* ; nternal transcribed spacer ; non-internal transcribed spacer ; aquatic disease

Introduction

Perkinsosis is a shellfish disease caused by protozoans of the genus *Perkinsus*, of which there are seven species. In North America, two species have been reported. *Perkinsus marinus,* which occurs along the Atlantic Coast from Maine, USA (Reece et al., 2001; Pecher et al., 2008) to Tabasco, in the Gulf of Mexico (Burreson et al., 1994; Gullian-Klanian et al., 2008) and on the west Coast of Mexico and offshore in Hawaii. *P. marinus* is a pathogen to oysters (*Crassostrea virginica, Crassostrea gigas, Crassostrea ariakiensis*, *Crassostrea rizhoporae,* and *Crassostrea corteziensis*), as well as several clam species (OIE, 2012). The other species in North America is *Perkinsus chesapeaki,* which infects clams along the east coast of the USA. Co-infection with both protozoans in wild *C. virginica* has been identified by molecular methods because the common method of tissue incubation in fluid thioglycolate medium (FTM) is not species-specific (Reece et al., 2008). *P. marinus* has been the most studied pathogen because it impacts oyster populations, causing large die-offs in *C. virginica* along the east coast of the USA (Ford, 1996).

P. marinus is included in the World Organization for Animal Health (OIE) list of disease organisms and is the most prevalent of *Perkinsus* species along the mid-eastern coast of the United States. However, differences in impact by *P. marinus* has been variable among *C. virginica* stocks, which is associated with: (1) Variations in environmental factors (Oliver et al., 1998), (2) Virulence among *P. marinus* genotypes (Bushek and Allen, 1996), and (3) Genetic variability among oyster stocks, which may lead to differences in resistance to *P. marinus* (Encomio et al., 2005).

Along the west coast of Mexico, *P. marinus* was initially detected in 2006 in the native oyster *C. corteziensis* (Cáceres-Martínez et al., 2008), which naturally grow in mangroves. Captured oyster seeds in March and April are cultivated in river estuaries in the State of Nayarit, Mexico. *P. marinus* was detected in *C. gigas* cultivated further north in the Gulf of California (Enríquez-Espinoza et al.,

64 2010). Historical data for the Nayarit estuaries indicate maximum prevalence in summer (Cáceres-
65 Martínez et al., 2008). Currently, mass die-offs of *C. corteziensis* from outbreaks of *P. marinus* has not
66 occurred in farmed oysters despite ideal water temperature (30 °C) for proliferation of *P. marinus*
67 (Gauthier and Vasta, 1995) from June through September 2007, however rainfall associated with
68 tropical storms cause fluctuations in salinity, from 19 to 31‰ (Cáceres-Martínez et al., 2008).
69 To date, it is difficult to morphologically differentiate between *Perkinsus* species; however,
70 molecular analyses, using rDNA markers, has identified novel *Perkinsus* species and defined
71 synonymous species. *P. marinus* genotypes have been identified by sequencing the internal transcribed
72 spacer (ITS) region ITS1-5.8S-ITS2 of rDNA flanked by the 18S and 28S genes (Brown et al., 2004).
73 Multiple copies of the ITS (Goggin, 1994) have revealed multiple polymorphic sites that can be used to
74 distinguish among *P. marinus* genotypes (Brown et al., 2004). Based on earlier genetic and phenotypic
75 (virulence) data for context, the analysis of the ITS1 and ITS2 and seven other loci identified 12 *P.*
76 *marinus* genotypes in *C. virginic*a populations from Massachusetts to Texas. Oysters from New Jersey
77 to Texas had three predominant genotypes, oysters from Virginia had a unique genotype; and oysters
78 from Massachusetts to Maryland and North Carolina to Texas had unique genotypes, but also shared
79 one genotype (Reece et al., 2001). Differences in virulence of *P. marinus* genotypes ranked the Virginia
80 genotype as the most virulent, followed by the moderately virulent genotype from New Jersey. The
81 least virulent were genotypes from Louisiana and Texas (Bushek and Allen, 1996).
82 Despite the high intraspecific variability in the non-transcribed spacer (NTS) from the rDNA of
83 *P. marinus*, a complete sequence has been defined only for the Texas isolate (Robledo et al., 1999). The
84 partial NTS sequence obtained from *C. corteziensis* collected from Nayarit show 98% similarity in a
85 307 bp to *P. marinus* from Texas. Six polymorphisms were identified (Cáceres-Martínez et al., 2008).
86 Polymorphism in *P. marinus* have been attributed to ploidy (Thompson et al., 2011), recombination,
87 and multi-copies of the NTS and ITS regions.

This study assayed polymorphism in two regions of rDNA of *P. marinus* detected in Nayarit oysters to determine *P. marinus* genetic variability and assess the relationship between *P. marinus* from the Pacific and the Atlantic, based on phylogenetic inferences. Molecular characterization of the parasite in different geographical locations would facilitate a better understanding of the epidemiology of *P. marinus*.

Materials and Methods

In July 2010, 25 adult *C. corteziensis* (6.06 ± 0.97 cm long) were collected from the Boca de Camichín estuary and 31 oysters (6.63 ± 1.09 cm) were collected from the Pozo Chino estuary (Fig. 1). A section of the gill from each oyster was fixed in 90% ethanol and DNA was extracted from 10 mg of this tissue, using a molecular diagnostic kit (QIAamp DNA, Qiagen, Hilden, Netherlands) according to the manufacturer's instructions. DNA quality and concentration were measured at 280 nm, using a UV-Vis spectrophotometer (Nanodrop 2000, Thermo Scientific, Waltham, MA).

Prevalence of *Perkinsus* sp.

Detection of *Perkinsus* was performed by PCR targeting a 532 bp of the ITS of *Perkinsus* sp. The PCR reactions were carried out in 25 μL containing 5 μL 5× buffer, 2.5 mM $MgCl_2$, 10.5 μg BSA (Promega, R396E), 0.25 mM dNTP, 0.2 μM primers PerkITS1F (5′-GAG ATG GGA TCY CCG CTT TGT TT-3′) and PerkITS1R (5′-GAA TCG CGT GAT CRA GGA ACA CG-3′), which were described by Park et al. (2006), 1 U Go*Taq* Flexi DNA polymerase (Promega, M8295), and 100 ng genomic DNA. Positive controls consisted of DNA from cultures of *P. olseni* provided by the European Union Reference

111 Laboratory for Mollusc Diseases of IFREMER at La Tremblade, France. Amplification was performed
112 in a Peltier thermal cycler (PTC-100, MJ Research, Waltham, MA) with an initial denaturation step at
113 95 °C for 4 min followed by 40 cycles (95 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min), and a
114 final extension at 72 °C for 5 min. PCR products were electrophoresed in 1.2% agarose gels, stained
115 with ethidium bromide, and visualized with UV light.
116
117 **RFLP from *Perkinsus* ITS**
118
119 The ITS amplicons (10 μL) were digested for 2 h at 37 °C in a final volume of 20 μL of 25 U μL^{-1}
120 *Rsa*I (Abollo et al., 2006) or *Hinf*I (Promega) and 2 μL 10× buffer (Promega). Restriction patterns
121 were obtained with DNA structural analyzer software (Restriction Mapper 3.0,
122 http://www.restrictionmapper.org/). Digested products were electrophoresed in 2% agarose gels, and
123 digestion patterns were compared to *P. marinus*, *P. olseni*, and *P. chesapeaki* (Table 1).
124
125 **NTS amplification**
126
127 The NTS of *P. marinus* collected from three oysters from Boca de Camichín and two oysters from
128 Pozo Chino was amplified by PCR, using the following primer set: PKnts-FW (5′-AAG TCC TTA
129 GGG TGC TGC TGG CT-3′) and PKnts-RV (5′-ACT ACT GGC AGG ATC AAC CAG GT-3′)
130 described by Park et al. (2005). The PCR reaction was carried out in a final volume of 20 μL with the
131 following components: 10 μL Go*Taq* Master Mix (Promega), 1 μM of each primer, and 100 ng DNA.
132 PCR cycling conditions were: initial denaturation at 95 °C for 4 min, 30 cycles at 94 °C for 1 min each,
133 annealing at 57 °C for 1 min, extension at 70 °C for 1 min; and a final extension at 70 °C for 5 min.

PCR products were electrophoresed in 1.2% agarose gels, stained with ethidium bromide, and visualized under UV light.

Cloning and sequencing

The ITS PCR products obtained from ten oysters from Boca de Camichín and seven oysters from Pozo Chino were cloned. The NTS PCR products were cloned from three oysters from Boca de Camichín and two oysters from Pozo Chino. All products were ligated in a cloning vector pCR2.1 Topo TA, (Invitrogen, Carlsbad, CA) and transformed in chemically competent cells. For each PCR product, ten clones were verified by colony PCR for insert of PCR products and then cultured in LB medium containing ampicillin medium for isolating plasmids. Plasmid DNA was extracted with a spin miniprep kit (QIAprep, Qiagen). At least three clones for each PCR product were bi-directionally sequenced using universal vector primers, SP6 and T7. Sequencing was performed at the University of Valencia, Spain.

Sequence analyses

The chromatograms were converted from an ABI format to a FASTA format; low quality bases were trimmed using Phred software (Ewing et al., 1998; Ewing and Green, 1998b). Vector sequences were trimmed using Seqclean script (http://seqclean.sourceforge.net/) and the UniVec database (NCBI). Polymorphic sites within the consensus sequences were also visually verified in chromatograms using 4Peaks (Mekentosj). ITS and NTS sequences were aligned using CLUSTAL W software, and nucleotide diversity was calculated using DnaSP 5 software (Librado and Rozas, 2009). Phylogenetic distances were calculated with MEGA5 software, using the obtained models Kimura-2-Parameter (K2)

for the ITS and Tamura-3-Parameter (T92) for the NTS (Tamura et al., 2011). The robustness of neighbor-joining trees was tested with 10,000 bootstrap repetitions, and parsimony tree analyses were generated using a heuristic search with 1,000 bootstraps. DNA sequences were also subjected to the BLAST searches (Altschul et al., 1997) to confirm their identity to rDNA markers of *Perkinsus*. The listed accession numbers are sequences in the NCBI GenBank. Our nucleotide sequence data were submitted to the GenBank database under accession numbers: JQ266231 through JQ266264.

Results

Prevalence of *Perkinsus* and RFLP-PCR

PCR analysis provided 23 positive results from 25 samples of *C. corteziensis* (92%) from Boca de Camichín and 24 of 31 samples from Pozo Chino (77.4%). Digestion of the 47 ITS PCR products with *Rsa*I yielded fragments of 74 and 195 bp, and digestion with *Hinf*I, resulted in fragments of 161 and 368 bp. These restriction profiles were the same as those observed in *P. marinus* (Table 1). However, some ITS products provided differences in the restriction profile that were likely from polymorphisms at the restriction sites. These clones, as well as clones with equals fragment length, were selected for sequencing. Digestion with *Rsa*I and *Hinf*I of *P. olseni* and *P. chesapeaki* ITS PCR products (used as the controls), yielded restriction fragments of the expected size (Table 1); the digestion pattern of *P. olseni* and *P. chesapeaki* were not found in *C. corteziensis.*

Polymorphism in the *Perkinsus marinus* ITS region

The PerkITS1 primer set amplified the 532 bp of the ITS region of *P. marinus* obtained from *C.*

182 *corteziensis*; however, only 485 bp aligned were considered for the analysis. The ITS region included
183 108 bp of the ITS1, 159 bp of the 5.8S ribosomal subunit, and 218 bp of the ITS2. From Boca de
184 Camichín oysters, 25 ITS sequences of *P. marinus* were obtained and 20 ITS sequences from Pozo
185 Chino oysters. Of the 45 ITS sequences, 20 were different at one or more nucleotide sites, and
186 polymorphisms were identified at 25 nucleotide sites, including 18 transitions and five transversions
187 (Table 2). The ITS1 showed only one transversion at the 3rd position, and the ITS2 had four
188 transversions and nine transitions. No transversions were identified at the 5.8S ribosomal subunit,
189 thereby confirming that this gene region is more conserved than the spacers.
190 The most frequent sequences were JQ266236 and JQ266240. The JQ266236 sequence was
191 obtained from six clones of Pozo Chino oysters and seven clones of Boca de Camichín oysters. The
192 JQ266240 sequence was obtained from five clones of Pozo Chino oysters and eight clones of Boca de
193 Camichín oysters. The most frequent (A/C) transversion, which occurred at position 446 (JQ266236
194 versus JQ266240), was found in three oysters (2C, 3C, and 4C) from Boca de Camichín and two
195 oysters (6P and 10P) from Pozo Chino (Table 2).
196
197 **_Perkinsus_ ITS region homology**
198
199 The JQ266236 sequence characterized from *C. corteziensis* had 100% identity and coverage with the
200 ITS of *P. marinus* from South Carolina [SC2-4-8 (AY295197)], New Jersey [NJ3-1-6 (AY295188)],
201 Maryland [MA2-11-3 (AY295184) and MA1-1-12 (AY295182)], Texas [TXsc (AF497479) and
202 TCMD-1 (AF150990)], and Wright Island, Maryland [WIMD-2 (AF150989)]. The similarity of the
203 JQ266236 sequence to *P. olseni* ITS (GenBank U07701) was 96%. The JQ266240 sequence was
204 identical to the ITS of *P. marinus* from Bahía Kino, Sonora, Mexico [Kino0806 (GQ861511)], South
205 Carolina [SC3-2-8 (AY295199) and SC2-4-7 (AY295196)], New Jersey [NJ3-1-6 (AY295188)], and

206 Louisiana [LA8-11-1B (AY295178) and UBMD-3 (AF150986)]. The similarity of the GenBank
207 JQ266240 sequence to *P. olseni* ITS (U07701) was 95%.
208
209 **Phylogeny of the ITS of *Perkinsus marinus***
210
211 Based on the neighbor-joining analysis, the ITS sequences of *P. marinus* from *C. corteziensis* were
212 grouped into two main clades (A and B) with 66% bootstrap support (Fig. 2). Clade A included 13 ITS
213 sequences from Boca de Camichín oysters and 11 sequences from Pozo Chino oysters, and Clade B
214 included 12 ITS sequences from Boca de Camichín oysters and 9 sequences from Pozo Chino oysters.
215 Clade A was grouped with the ITS sequences of *P. marinus* genotypes from Texas (TXsc), Maryland
216 (MA and MD), Parsons Island, MD (EBPIC), and Broomes Island, MD (PXBIC). Clade B included
217 ITS sequences of *P. marinus* from Bahía Kino, Sonora (Kino0806), North Carolina (NC), and Upper
218 Bay, MD (UBMD). The ITS sequences of *P. marinus* from South Carolina (SC), LA, and NJ were
219 present in both clades. A divergent clade without ITS sequences of *P. marinus* from Nayarit was
220 grouped with ITS sequences of *P. marinus* from Virginia (P1), Mobjack Bay, VA (MBVA); Kedges,
221 MD (KS), and Upper Hackets, MD (UBHA). An ITS parsimony tree was prepared; however, it was not
222 reported because the bootstraps values were low supported, as explained by the low nucleotide
223 substitutions.
224 Unique sequences of Clade A were obtained from three oysters from Boca de Camichin (30%),
225 three unique sequences of Clade B (30%), and four oysters with both Clades A and B (40%). From
226 Pozo Chino, two oysters had unique sequences of Clade A (28.6%), one oyster had a unique sequence
227 of Clade B (14.3%), and four oysters with both Clades A and B (57.1%) (Fig. 3). Whether the presence
228 of multiple sequences represents infection by multiple *P. marinus clones* or sequence variability among
229 ITS loci within single clone requires further investigation.

NTS polymorphism and homology

In total, eight *Perkinsus* NTS sequences were obtained from Boca de Camichín oysters and six NTS sequences were obtained from Pozo Chino oysters. 14 different NTS sequences with 1165 bp were obtained, showing 32 polymorphic sites with 21 transitions and 11 transversions (Table 3).

The 14 NTS sequences showed 98% similarity to *P. marinus* TXsc, obtained from Texas (AF497479), with coverage between 97% and 100%. Lower levels of similarity were linked to *P. olseni* (AF466527 and AF590333), with 81% similarity and 64% coverage and to *P. chesapeaki* (AF102171), with 77% similarity and 37% coverage.

NTS phylogeny

The identified NTS sequences of *P. marinus* were grouped into two clades according to neighbor-joining analysis (Fig. 4). Both clades included NTS sequences of *P. marinus* from Pozo Chino and Boca de Camichín oysters. Additionally, Clade B included the NTS sequence of *P. marinus* from Texas; Clade A consisted only of sequences obtained in our assays. Both *P. marinus* clades diverged from *P. olseni* (GenBank AF466527 and AF509333) and *P. chesapeaki* (GenBank AF102171).

Nucleotide diversity

Nucleotide diversity for the ITS2-5.8S-ITS1 region was 0.00326 (Fig. 5). However, the nucleotide diversity inside the ITS region was 0.00161 for ITS1, 0.00222 for 5.8S, and 0.00486 (Table 4) for

ITS2. The nucleotide diversity in the NTS region (0.00968) was higher than the ITS region.

Discussion

The increasing spread of diseases in cultivated species is a major concern because economic productivity is at risk. Global warming is the greatest environmental challenge, where spreading pathogens have been associated with an increase in ocean temperature (Marcos-López et al., 2010). Equally important is the globalization of international trade of aquatic animals and products, giving rise to emerging diseases around the world (Oidtman et al., 2012; Alborali, 2006). *P. marinus* in Nayarit (Cáceres-Martínez et al., 2008) is a disease in a new area and in a new species; therefore, the origins of this protozoan and its virulence must be evaluated, and the intensity and prevalence of infection must be monitored.

The prevalence of *P. marinus* in *C. corteziensis* cultivated in Nayarit estuaries in 2006–7 ranged from 1–6% (Cáceres-Martínez et al., 2008). For our study area, 5 and 3%, respectively, occurred in oysters harvested Boca de Camichín and Pozo Chino in March 2008, and 13% in Boca de Camichín oysters harvested in June 2009 and 10% in Pozo Chino oysters in February 2009 (CESANAY, 2010). We report *P. marinus* prevalences of 92 in Boca de Camichín and 77% in Pozo Chino oysters. However, the diagnostic method and sample size since 2006 have been different.

Furthermore, there is high cultivation density at Boca de Camichín than at Pozo Chino (CESANAY, 2010), which should also influence infection rates, since transmission occurs at least in part via oyster feces and increases with oyster density (Ford et al., 2002; Park et al., 2010).

In our study, higher *P. marinus* prevalences were found in Nayarit in *C. corteziensis* collected in July 2010 compared to those detected in 2006 (Cáceres-Martínez et al., 2008) and in Sonora in *C. gigas* (Enríquez-Espinoza et al., 2010). Detection of *Perkinsus* in our study was done by PCR, while

278 detection in 2006 was done by histological examination, which is less sensitive (Navas, et. al., 2007).
279 Prevalence of *P. marinus* in July 2010 in Nayarit was similar to those found in the first *P. marinus*
280 recorded in *C. virginica* (60–100%) in coastal Tabasco in the Gulf of Mexico (Burreson et al., 1994)
281 and in *C. virginica* (98–100%) from Florida, Virginia, and New York (Oliver et al., 1998). Along the
282 east coast of the United States, four year-old *C. virginica* were used to determine the prevalence of the
283 protozoan in *C. virginica* (Karolus, 2000), whereas five month-old oysters were used in our study
284 (oysters seed captured in March and collected in July), indicating that the spread in *C. corteziensis*
285 farming occurs in young oysters.
286 In spite of the presence of *P. marinus* in our samples, no deaths were recorded at oyster farms in
287 Nayarit. This may be related to the intensity and virulence of *Perkinsus* and the resistance of *C.*
288 *corteziensis* to infection or *C. corteziensis* reaching market size in less than 13 months, whereas *C.*
289 *virginica* from New York (Oliver et al., 1998) reach harvest size (7.5 cm) when they are three years old.
290 The rapid growth of *C. corteziensis* may limit mortality because the intensity of perkinsosis in oysters
291 and clams increases (Calvo et al., 1996; Villalba et al., 2005) until their second year of growth, when
292 mortality follows (Park et al., 2010).
293 The digestion patterns obtained after RFLP-PCR analysis of the ITS region allowed us to
294 distinguish between *P. marinus* and the closely related species *P. olseni* and also to distinguish *P.*
295 *chesapeaki* from the Atlantic (Table 1). While generic primers are commonly used to amplify the ITS
296 region of most *Perkinsus* species, RFLP-PCR analysis is a faster diagnostic method that can
297 discriminate between closely related species according to the digestion pattern. This method will be
298 used in future studies to increase the identification of possible *Perkinsus* co-infections, as happens in
299 bivalves in Chesapeake Bay (Reece et al., 2008).
300 Based on 12 cultured genotypes obtained along the US east coast, the ITS of *P. marinus* showed
301 20 variant sequences at 25 polymorphic sites, compared to earlier reports that identified 12 genotypes

302 of 27 allelic variants at 14 polymorphism sites (Brown et al., 2004). Our study was based on sequences
303 obtained from infected oysters from two neighbouring sites; therefore, the higher genetic variation is an
304 unexpected result that warrants further investigation. Studies in different hosts show genetic variability
305 in *Perkinsus* ITS sequences. Even multiple variants have been found in a single clonal culture (Dungan
306 et al., 2002).

307 The ITS phylogenetic analysis showed that *P. marinus* infection of *C. corteziensis* clusters in
308 two clades that could be separated by the most frequent transversion, A/T at position 446. This suggests
309 the existence of two variants in the ITS of *P. marinus*. Variants A and B were found in oysters at both
310 sites and presence of both ITS variants were found in tree oysters from Camichín, and two oysters from
311 Pozo Chino (Table 2). Thompson et al. (2011) explains intraspecific polymorphism by multi-copy
312 regions and genetic recombination.

313 Regardless of geographic isolation of genotypes along the east coast of the United States, the
314 variant A had high similarity to *P. marinus* from Texas (TXsc) and Maryland (MA, MD, and EBPIC),
315 and variant B was similar to *P. marinus* from North Carolina (NC), and Maryland (UBMD). High
316 similarity of ITS sequences in our study to genotypes along the Atlantic shoreline suggests the possible
317 introduction of *P. marinus* by transport of mollusks from the Atlantic to the Pacific, as suggested by
318 Cáceres-Martínez et al. (2008).

319 The ITS of *P. marinus* from Nayarit showed high similarity to isolates classified as genotype 1
320 in New Jersey and Maryland and genotype 3 from North Carolina, South Carolina, Louisiana, and
321 Texas (Reece et al., 2001). *P. marinus* genotypes with virulence were classified as follows: genotype 1
322 is moderately virulent and genotype 3 is mildly virulent (Bushek and Allen, 1996). Genotype 8 from
323 Virginia was classified as the most virulent and we grouped it in a divergent clade of *P. marinus* from
324 Nayarit. In summary, our results suggest that *P. marinus* in Nayarit have high similarity to genotypes
325 with low to moderate virulence. However, the virulence of *P. marinus* in Nayarit must be evaluated by

challenge infection tests (Reece et al., 2001).

NTS phylogenetic analysis grouped the sequences into two clades, which were similar to those obtained with the ITS region. The Clade B were grouped with *P. marinus* from Texas, but Clade A could not be grouped with reported genotypes because the information about the NTS region genotypes is limited. Still, both clades diverged from *P. olseni.*

The nucleotide diversity of ITS and NTS markers was similar between *P. marinus* in both estuaries, thereby indicating a geographically homogeneous polymorphism. Within the ITS region, nucleotide diversity showed higher variability for ITS2, followed by 5.8S, and then by ITS1. From an analysis of 12 *P. marinus* genotypes from the Atlantic shoreline (Brown et al., 2004), nucleotide diversity for the 485 bp of the ITS region was higher (0.00712) than in *P. marinus* types from Nayarit (0.00326). The interspecies nucleotide diversity of *P. marinus* (GenBank JQ266231) and *P. olseni* (GenBank U07701) was one order of magnitude higher (0.04487). The highest nucleotide diversity in the NTS suggests that this region can be used for genotype identification and the more conserved ITS marker is better for species-specific diagnosis.

Conclusions

From the phylogenetic analysis of the ITS and NTS regions of the parasite *P. marinus* collected from *C. corteziensis,* we recognized two clades. Both parasite allelic variants were found in oysters from two estuaries, which indicate a homogeneous distribution. High similarity of *P. marinus* from Nayarit to Atlantic genotypes suggests that *P. marinus* may have been introduced to the Pacific Ocean by transport of mollusks from the Atlantic Ocean. Based on our ITS analyses, *P. marinus* from Nayarit had high similarity to genotypes from Maryland to Texas, but excluded Virginia genotypes. Despite the presence of *P. marinus* in Nayarit estuaries, no die-offs of *C. corteziensis* were recorded, which may be

explained by some combination of intensity levels and virulence of the *P. marinus*; rapid growth of the oysters, compared to the Atlantic oysters; and resistance of *C. corteziensis* to *P. marinus.* Intensity level analysis and challenge tests must be carried out to clarify the cause for the resistance of the oysters.

Diseases are sometimes not recognized in regions with important aquaculture or fishery production because surveillance is inadequate or diagnosis protocols are not properly validated. Therefore, studies to characterize the variability of molecular markers used as template DNA for diagnosis is recommended.

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References

Abollo, E., S. M. Casas, G. Ceschia, and A. Villalba, 2006: Differential diagnosis of *Perkinsus* species by polymerase chain reaction-restriction fragment length polymorphism assay. *Mol. and Cell Probes* 20, 323–329.

Alborali, L., 2006: Climatic variations related to fish diseases and production. *Vet. Res. Commun.* 30, 93–97.

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman, 1997:

374 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic*
375 *Acids Res*. 25, 3389–3402.
376 Brown, G. D., K. L. Hudson, and K. S. Reece, 2004: Multiple polymorphic sites at the ITS and ATAN
377 loci in cultured isolates of *Perkinsus marinus. J. Eukaryot. Microbiol.* 51, 312–320.
378 Burreson, E., R. Sima-Alvarez, V. Vidal-Martinez, and L. Aguirre-Macedo, 1994: *Perkinsus marinus*
379 (Apicomplexa) as a potential source of oyster *Crassostrea virginica* mortality in coastal lagoons of
380 Tabasco, Mexico. *Dis. Aquat. Org.* 20, 77–82.
381 Bushek, D., and S. Allen, 1996: Host-parasite interactions among broadly distributed populations of the
382 eastern oyster *Crassostrea virginica* and the protozoan *Perkinsus marinus*. *Mar. Ecol. Prog. Ser.*
383 139, 127–141.
384 Calvo, G. W., R. J. Fagan, K. N. Greenhawk, G. F. Smith, and S. J. Jordan, 1996: Spatial distribution
385 and intensity of *Perkinsus marinus* infections in oyster recovery areas in Maryland. *J. Shellfish*
386 *Res.* 15, 381–389.
387 CESANAY, 2010: Comité de Sanidad Acuícola de Nayarit. Farm oyster of Nayarit. Available at:
388 http://www.cesanay.com/ (accessed 24 January 2012).
389 Cáceres-Martínez, J., R. Vásquez-Yeomans, G. Padilla-Lardizábal, and M. A del Río Portilla, 2008:
390 *Perkinsus marinus* in pleasure oyster *Crassostrea corteziensis* from Nayarit, Pacific coast of
391 México. *J. Invertebr. Pathol.* 99, 66–73.
392 Dungan, C. F., R. M. Hamilton, K. L. Hudson, C. B. McCollough, and K. S. Reece, 2002: Two
393 epizootic diseases in Chesapeake Bay commercial clams, *Mya arenaria* and *Tagelus plebeius*. *Dis.*
394 *Aquat. Org.* 50, 67–78.
395 Encomio, V., S. Stickler, S. A. Allen Jr., and F. L. Chu, 2005: Performance of "natural dermo-resistant"
396 oyster stocks-survival, disease, growth, condition and energy reserves. *J. Shellfish Res.* 24, 143–
397 155.

Enríquez-Espinoza, T. L., J. M. Grijalva-Chon, R. Castro-Longoria, and J. Ramos-Paredes, 2010: *Perkinsus marinus* in *Crassostrea gigas* in the Gulf of California. *Dis. Aquat. Org.* 89, 269–273.

Ewing, B., L. Hillier, M. C. Wendl, and P. Green, 1998: Base-calling of automated sequencer traces using Phred I. *Accuracy Assessment Genome Res.* 175–185.

Ewing, B., and P. Green, 1998b: Base-calling of automated sequencer traces using Phred II Error Probabilities. *Genome Res.* 186–194.

Ford, S. E., 1996: Range extension by the oyster parasite *Perkinsus marinus* into the northeastern United States: Response to climate change? *J. Shellfish Res.* 15, 45–56.

Ford, S. E., M. M. Chintala, and D. Bushek, 2002: *Perkinsus marinus.* III. Fecal elimination and its role in transmission. *Dis. Aquat. Org.* 51, 217–225.

Gauthier, J. D., and G., Vasta, 1995: *In vitro* culture of the eastern oyster parasite *Perkinsus marinus*: optimization of the methodology. *J. Invertebr. Pathol.* 66, 156–168.

Goggin, C. L., 1994: Variation in the two internal transcribed spacers and 5.8 S ribosomal RNA from five isolates of the marine parasite *Perkinsus* (Protista, Apicomplexa). *Mol. Biochem. Parasitol.* 65, 179–182.

Gullian-Klanian, M., J. A. Herrera-Silveira, R. Rodriguez-Canul, and L. Aguirre-Macedo, 2008: Factors associated with the prevalence of *Perkinsus marinus* in *Crassostrea virginica* from the southern Gulf of Mexico. *Dis. Aquat. Org.* 79, 237–247.

Karolus, J., 2000: Prevalence of *Perkinsus marinus* (Dermo) in *Crassostrea virginica* along the Connecticut shoreline. *Aquaculture* 183, 215–221.

Librado, P., and J. Rozas, 2009: DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451–1452.

420 Marcos-López, M., P. Gale, B. C. Oidtmann, and E. J. Peeler, 2010: Assessing the impact of climate
421 change on disease emergence in freshwater fish in the United Kingdom. *Transbound. Emerg. Dis.*
422 57, 293–304.
423 Navas J.I., R. de la Herrán, I. López-Flores, M.C. Quintero, I.M. Prado, M. Ruiz-Rejón, and J.C.
424 Manzano, 2007: Evaluation of the sensitivity and specificity by latent class analysis of Ray's
425 method, histological assay, and PCR diagnosis of *Perkinsus olseni* in *Ruditapes decussatus*.
426 Workshop for the analysis of impact of perkinsosis to the European shellfish industry, Vigo, Spain.
427 Oidtmann B., C. Johnston, K. Klotins, G. Mylrea, P. T. Van, S. Cabot, P. Rosado Martin, L. Ababouch,
428 and F. Berthe**,** 2012: Assessment of the safety of aquatic animal commodities for international
429 trade: The OIE Aquatic Animal Health Code. *Transbound. Emerg. Dis*. DOI: 10.1111/j.1865-
430 1682.2012.01315.x.
431 OIE, 2012: World Organisation for Animal Health. OIE listed diseases 2012 Available at:
432 http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2012/ (accessed 15 April 2012).
433 Oliver, L. M., W. S. Fisher, S. E. Ford, L. M. Calvo, E. M. Burreson, E. B. Sutton, and J. Gandy, 1998:
434 *Perkinsus marinus* tissue distribution and seasonal variation in oysters *Crassostrea virginica* from
435 Florida, Virginia and New York. *Dis. Aquat. Org.* 34, 51–61.
436 Park, K. I., T. T. T. Ngo, S. D. Choi, M. Cho, and K. S. Choi, 2006: Occurrence of *Perkinsus olseni* in
437 the Venus clam *Protothaca jedoensis* in Korean waters. *J. Invertebr. Pathol.* 93, 81–87.
438 Park, K. I., J. K. Park, J. Lee, and K. S. Choi, 2005: Use of molecular markers for species identification
439 of Korean *Perkinsus* sp. isolated from Manila clams *Ruditapes philippinarum*. *Dis. Aquat. Org.*
440 66, 255–263.
441 Park, K. I., H. S.Yang, H. S. Kang, M. Cho, K. J. Park, and K. S. Choi, 2010: Isolation and
442 identification of *Perkinsus olseni* from feces and marine sediment using immunological and
443 molecular techniques. *J. Invertebr. Pathol.* 105, 261–269.

444 Pecher, W. T., M. R. Alavi, E. J. Schott, J. A. Fernandez-Robledo, L. Roth, S. T. Berg, and G. R. Vasta,
445 2008: Assessment of the northern distribution range of selected *Perkinsus* species in eastern
446 oysters (*Crassostrea virginica*) and hard clams (*Mercenaria mercenaria*) with the use of PCR-
447 based detection assays. *J. Parasitol.* 94, 410–422.
448 Reece, K., C. F. Dungan, and E. M. Burreson, 2008: Molecular epizootiology of *Perkinsus marinus* and
449 *P. chesapeaki* infections among wild oysters and clams in Chesapeake Bay, USA. *Dis. Aquat. Org.*
450 82, 237–248.
451 Reece, K. S., D. Bushek, K. L. Hudson, and J. E. Graves, 2001: Geographic distribution of *Perkinsus*
452 *marinus* genetic strains along the Atlantic and Gulf coasts of the USA. *Marine Biology* 139, 1047–
453 1055.
454 Robledo, J. A. F., A. C. Wright, A. G. Marsh, and G. R.Vasta, 1999: Nucleotide sequence variability in
455 the nontranscribed spacer of the rRNA locus in the oyster parasite *Perkinsus marinus*. *J. Parasitol.*
456 85, 650–656.
457 Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar, 2011: MEGA5: Molecular
458 evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum
459 parsimony methods. *Mol. Biology and Evol.* 28, 2731–2739.
460 Thompson, P. C., B. M. Rosenthal, and M. P. Hare, 2011: An evolutionary legacy of sex and clonal
461 reproduction in the protistan oyster parasite *Perkinsus marinus*. *Infect. Genet. Evol.* 11, 598–609.
462 Villalba, A., S. M. Casas, C. López, and M. J. Carballal, 2005: Study of perkinsosis in the carpet shell
463 clam *Tapes decussatus* in Galicia (NW Spain). II. Temporal pattern of disease dynamics and
464 association with clam mortality. *Dis. Aquat. Org.* 65, 257–267.

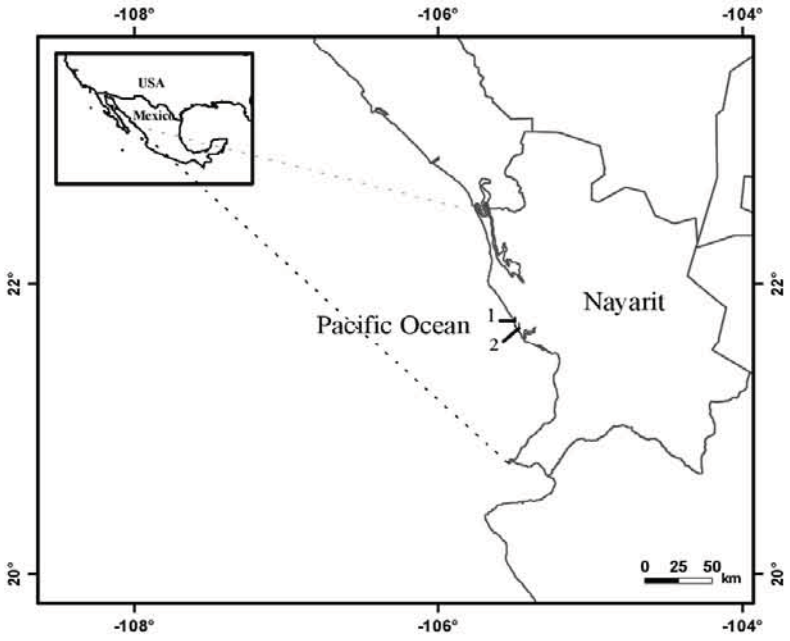
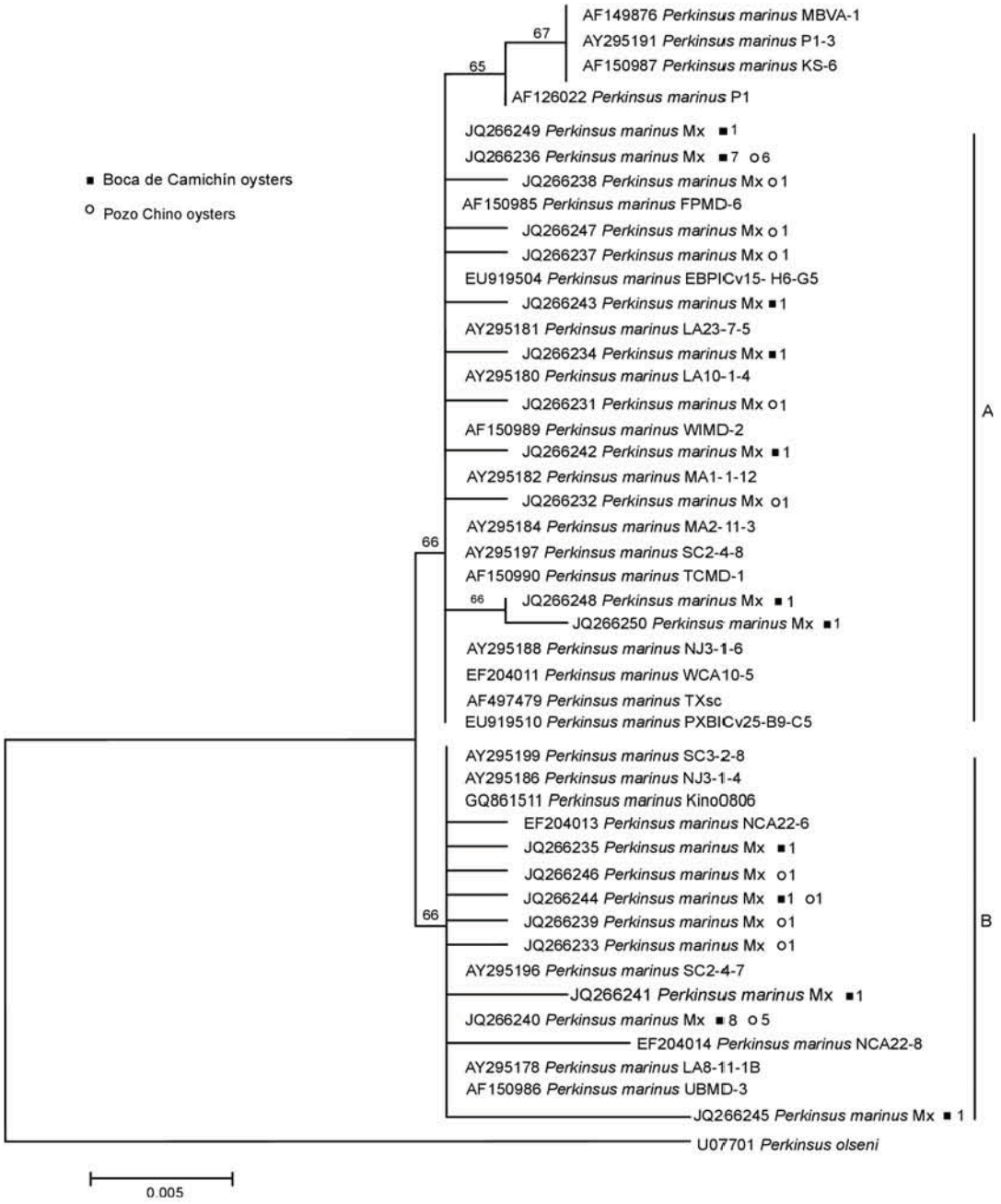

-108°
-106°
-104°
22°
20°
USA
Mexico
Pacific Ocean
Nayarit
1
2
0 25 50
km

Fig 1.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o Pozo Chino oysters
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AF150987 Perkinsus marinus KS-6
AF126022 Perkinsus marinus P1
JQ266249 Perkinsus marinus Mx ■1
JQ266236 Perkinsus marinus Mx ■7 o6
JQ266238 Perkinsus marinus Mx o1
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JQ266237 Perkinsus marinus Mx o1
EU919504 Perkinsus marinus EBPICv15- H6-G5
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EU919510 Perkinsus marinus PXBICv25-B9-C5
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AY295186 Perkinsus marinus NJ3-1-4
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EF204013 Perkinsus marinus NCA22-6
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U07701 Perkinsus olseni
67
65
66
66
66
A
B
0.005

Fig 2.

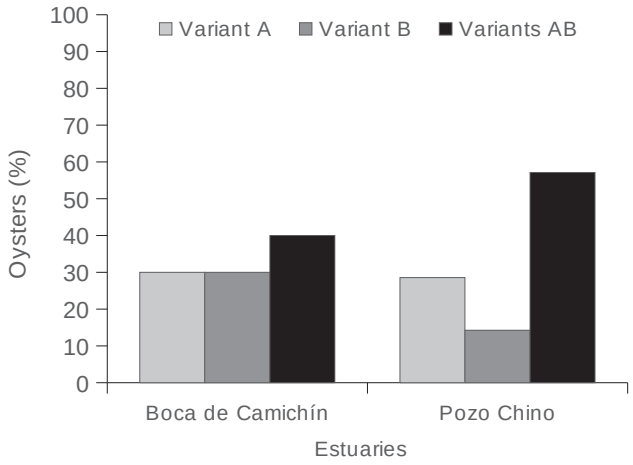
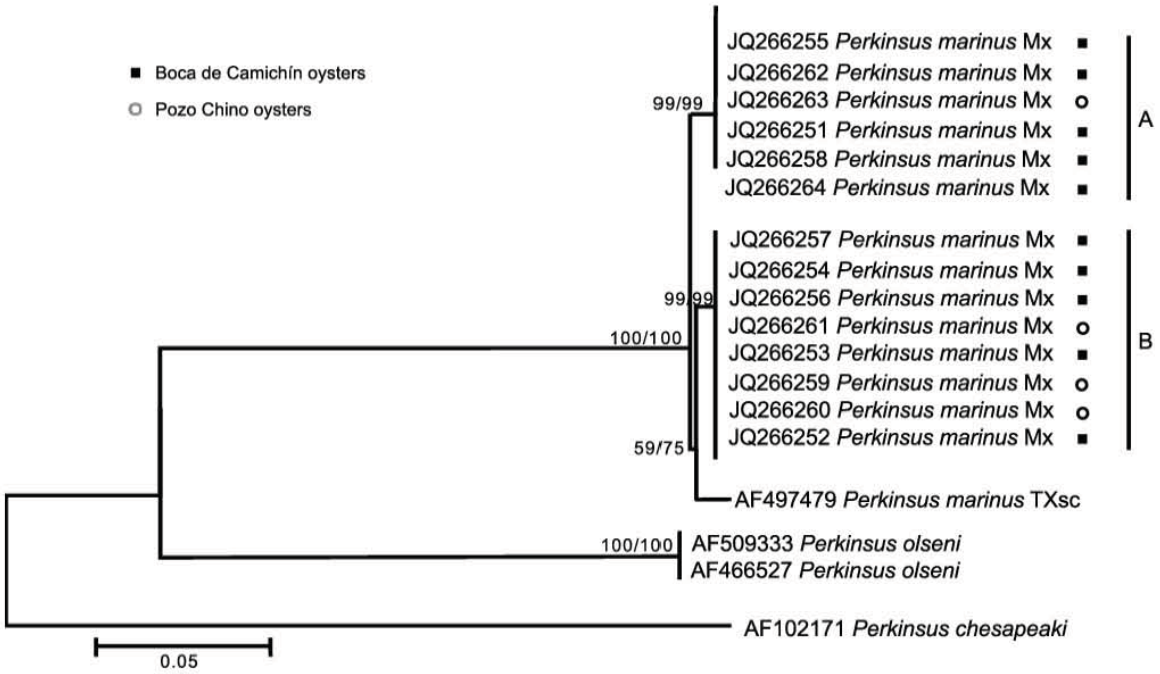

Variant A
Variant B
Variants AB
100
90
80
70
60
50
40
30
20
10
0
Oysters (%)
Boca de Camichín
Pozo Chino
Estuaries

Fig 3.


Boca de Camichín oysters
Pozo Chino oysters
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JQ266258 Perkinsus marinus Mx
JQ266264 Perkinsus marinus Mx
JQ266257 Perkinsus marinus Mx
JQ266254 Perkinsus marinus Mx
JQ266256 Perkinsus marinus Mx
JQ266261 Perkinsus marinus Mx
JQ266253 Perkinsus marinus Mx
JQ266259 Perkinsus marinus Mx
JQ266260 Perkinsus marinus Mx
JQ266252 Perkinsus marinus Mx
AF497479 Perkinsus marinus TXsc
AF509333 Perkinsus olseni
AF466527 Perkinsus olseni
AF102171 Perkinsus chesapeaki
99/99
99/99
100/100
59/75
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A
B
0.05

Fig 4.

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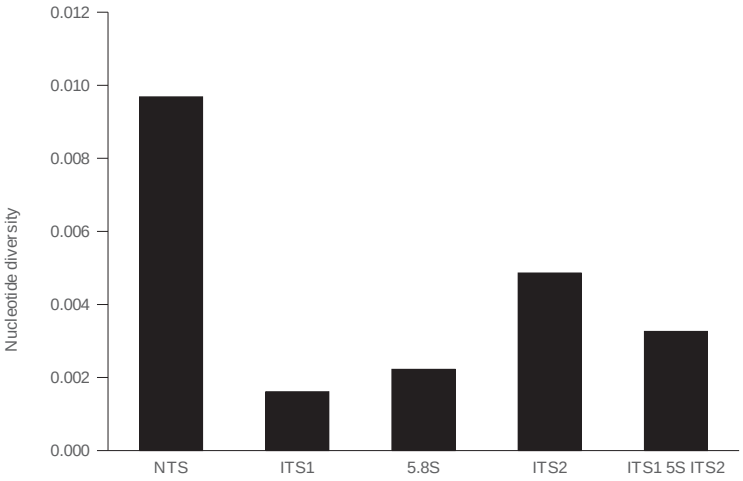

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591 Fig 5.

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Figure legends

Fig. 1. Collection sites of oysters in the estuaries Boca de Camichín (1) and Pozo Chino (2) in the State of Nayarit, Mexico.

Fig. 2. Phylogenetic tree of the ITS region of *Perkinsus* sp. from Boca de Camichín (■) and Pozo Chino oysters (○). The sequences were analyzed by a neighbor-joining algorithm supported by 10,000 bootstraps based on the Kimura-2-parameter model. Supported probabilities are shown at the nodes. Clades A and B included *P. marinus* from the State of Nayarit, Mexico. The number beside the symbol indicates the total clones of the same sequence.

Fig. 3. Percentage of oyster with different variants of *Perkinsus marinus* ITS found in *Crassostrea corteziensis* oysters in the Boca de Camichín and Pozo Chino estuaries.

Fig. 4. Phylogenetic tree of the NTS region of *Perkinsus* sp. from Boca de Camichín (■) and Pozo Chino oysters (○). The sequences were analyzed by neighbor-joining and parsimony algorithms s based on the Tamura-3-parameter model. Supported probabilities are shown at the nodes, the first bootstrap value indicates neighbor-joining followed by the parsimony bootstrap value. *Perkinsus marinus* from the State of Nayarit, Mexico were grouped into clades A and B.

Fig. 5. Nucleotide diversity (π) of the ITS and NTS of *Perkinsus marinus* from the State of Nayarit, Mexico.

Table 1

Enzyme	*P. marinus*	*P. olseni*	*P. chesapeaki*
*Rsa*I	204	259[b]	195
	195[a]	193	166
	74[a]	86	103
	56	—	86
*Hinf*I	368[a]	367	212[b]
	161[a]	171	151

[a]Identified fragments used in this study.

[b]Fragments that lead to *P. marinus* differentiation.

Table 2

Accession number	ITS1			5.8S							ITS2															No. *	P	C	N
	3	63	104	117	132	147	160	178	195	248	270	273	274	275	276	277	307	319	321	330	382	433	446	447	479				
JQ266231[A]	A	T	T	A	G	A	A	A	A	C	C	-	C	C	C	G	-	C	G	A	T	T	C	T	G	7P	1	-	1
JQ266232[A]								G				-					-								A	7P	1	-	1
JQ266233[B]										T		-					-						A		A	5P	1	-	1
JQ266234[A]												-					-			G					A	2C	-	1	1
JQ266235[B]									G			-					-						A		A	14C	-	1	1
JQ266236[A]												-					-								A	+	6	7	13
JQ266237[A]											T	-					D								A	9P	1	-	1
JQ266238[A]												-					-							C	A	9P	1	-	1
JQ266239[B]												-					-	T					A		A	15P	1	-	1
JQ266240[B]												-					-						A		A	++	5	8	13
JQ266241[B]		C										-					-				C		A		A	11C	-	1	1
JQ266242[A]							G					-					-								A	11C	-	1	1
JQ266243[A]						G						-					-								A	11C	-	1	1
JQ266244[B]					A							-					-						A		A	6P, 17C	1	1	2
JQ266245[B]												I	A	A	A	A	-						A		A	17C	-	1	1
JQ266246[B]				G								-					-						A		A	13P	1	-	1
JQ266247[A]	C											-					-		A						A	13P	1	-	1
JQ266248[A]												-					-					C			A	12C	-	1	1
JQ266249[A]	C											-					-								A	12C	-	1	1
JQ266250[A]			C									-					-					C			A	8C	-	1	1

*Protozoan identifier from Boca de Camichín (C) and Pozo Chino (P). The number in parentheses indicates the number of clones with the same sequence.

+1C (2), 2C (2), 3C, 4C, 5P (2), 6P, 7P, 8C, 9P, 10P.

++2C, 3C (2), 4C (2), 6P, 10P, 13P, 14C (2), 15P (2), 16C.

A and B indicate the phylogenetic variant.

Table 3

Accession number	16	24	25	29	30	31	45	62	68	69	87	89	109	119	127	131	141	232	284	424	448	533	548	572	602	675	704	802	818	930	932	978	1009	1066	1131	No. *
JQ266251	T	T	T	C	A	A	C	T	G	T	T	T	T	C	A	A	A	G	I	A	T	-	A	T	T	G	T	A	T	T	G	-	G	G	G	14C
JQ266252	C	G	C	G	T	C	T	A	C	A	C	A	C			G			-			D	G					C			T	-		A	A	14C
JQ266253	C	G	C	G	T	C	T	A	C	A	C	A	C		G	G			-			-						C			T	-			A	14C
JQ266254	C	G	C	G	T	C	T	A	C	A	C	A	C			G		A	-			-						C			T	-			A	2C
JQ266255																	T		-			-										-			A	2C
JQ266256	C	G	C	G	T	C	T	A	C	A	C	A	C			G			-		C	-						C			T	I			A	18C
JQ266257	C	G	C	G	T	C	T	A	C	A	C	A	C	T		G			-			-						C		C	T	-			A	18C
JQ266258																			-	G		-										-	A		A	18C
JQ266259	C	G	C	G	T	C	T	A	C	A	C	A	C			G			-			-					C	C			T	-			A	7P
JQ266260	C	G	C	G	T	C	T	A	C	A	C	A	C			G			-			-			C			C			T	-			A	7P
JQ266261	C	G	C	G	T	C	T	A	C	A	C	A	C			G			-			-		C				C			T	-			A	7P
JQ266262																			-			-										-			A	10P
JQ266263																			-			-							C			-			A	10P
JQ266264																			-			-				A						-			A	10P

*Identifier organism from Boca de Camichín (C) and Pozo Chino (P)

.

642 **Table 4**

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Marker	Site	NS	Length	si	sv	Ratio si/sv	π
ITS	C	25	485	18	5	3.6	0.00355
	P	20					0.00315
NTS	C	8	1165	20	10	2	0.01020
	P	6					0.01019

Table titles

Table 1. Digestion patterns for *Perkinsus marinus*, *Perkinsus olseni*, and *Perkinsus chesapeaki* restricted by *Rsa*I and *Hinf*I.

Table 2. Polymorphic nucleotide positions identified in the ITS1-5.8S-ITS2 sequences of *Perkinsus marinus* from oysters collected at Pozo Chino (C) and Boca de Camichín (C) in the State of Nayarit, Mexico. The first sequence was used as the base, insertions (I) and deletions (D) are indicated in the columns. Transversions are marked as grey columns and transitions in white.

Table 3. Polymorphic nucleotide positions in the NTS sequence of *Perkinsus marinus* extracted from *Crassostrea corteziensis* at Pozo Chino (P), and Boca de Camichín (C). The first sequence was used as the base, and insertions (I) and deletions (D) are indicated in the columns. Transversions are marked as grey columns and transitions in white.

Table 4. Nucleotide diversity (π), transitions (si), and transversions (sv) on the ITS and NTS of *Perkinsus marinus* obtained from oysters from Boca de Camichín (C) and Pozo Chino (P). NS = number of sequences analyzed.