Natural and cultured populations of the mangrove oyster *Saccostrea palmula* from Sinaloa, Mexico, infected by *Perkinsus marinus*

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**A B S T R A C T**

The mangrove oyster *Saccostrea palmula* coexists with the pleasure oyster *Crassostrea corteziensis* in coastal lagoons of northwest Mexico. Recent discovery of *Perkinsus marinus* infecting the pleasure oyster in the region prompted evaluation of *S. palmula* as an alternative *P. marinus* host. An analysis to determine the possible presence of *P. marinus* in natural and cultured populations of *S. palmula* at four coastal lagoons in Sinaloa, Mexico was carried out during October–November 2010. Tissues from apparently healthy *S. palmula* were evaluated using Ray’s fluid thioglycollate method (RFTM), which revealed a *Perkinsus* sp. to be present in all four locations at 6.7–20.0% prevalence. Histopathological analysis of these specimens showed tissue alterations and parasite forms consistent with moderate *P. marinus* infection, which was confirmed by ribosomal non-transcribed spacer (NTS)-based PCR assays on DNA samples from oysters positive by RFTM and histology. DNA sequencing of amplified NTS fragments (307 bp) produced a sequence 98–100% similar to GenBank-deposited sequences of the NTS from *P. marinus*. Fluorescent in situ hybridization for *Perkinsus* spp. and *P. marinus* corroborated the PCR results, showing clear hybridization of *P. marinus* in host tissues. This is the first record of *P. marinus* infecting a species from genus *Saccostrea* and the first record of the parasite from coastal lagoons in Sinaloa, Mexico.

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1. Introduction

The mangrove oyster *Saccostrea palmula* is distributed from Laguna San Ignacio, Baja California, Mexico to Panama, the Galapagos Islands, Ecuador and Costa Rica. This species occurs on mangrove roots and on rocks exposed at low tides and it presents high morphological variability along the Panamic province (Cruz and Jiménez, 1994; Jiménez, 1994). *S. palmula* coexists with the pleasure oyster *Crassostrea corteziensis*, a larger species, which is distributed in mangrove zones from the Mar de Cortez, Mexico to Peru (Stuardo and Martínez 1975). Morphologically *Saccostrea* differs from *Crassostrea* in having the margin crenulated and from *Ostreia* by crenulation present all along the shell’s periphery (in *Ostreia* only in the anterior margin near the hinge) (Huber, 2010). Species are clearly distinguishable. In Mexico both species are valued as food by communities along coastal lagoons and support a regional fishery.

Reduction in natural populations of *C. corteziensis* has favored development its culture (Góngora-Gómez et al., 2007). Production of *C. corteziensis* now approaches 2000 metric tons/year (Cáceres-Martínez et al., 2010). While there have been some attempts to grow *S. palmula* (Baqueiro, 1984), culture of this oyster is still nascent and generally co-occurs with that of *C. corteziensis*.

Both species play important roles in the ecosystem as filter feeders and show a marked parallelism in life history traits. Their reproductive cycles, for example, are synchronous, with spawning starting in May for *C. corteziensis* and in June for *S. palmula* and continuing in both species to November (Cuevas-Guevara and Martínez-Guerrero, 1979). With the significant pathogen *Perkinsus marinus* recently having been detected infecting *C. corteziensis* in Nayarit (Cáceres-Martínez et al., 2008) and that this pathogen is under the watch of the World Organization of the Animal Health (OIE), the question arose as to whether *S. palmula* would share this particular attribute as well, particularly as *P. marinus* is transmitted horizontally among oysters via the water column (Andrews, 1996) and capable of infecting a range of oyster species (Meyers et al., 1991; Moss et al., 2006). *C. corteziensis* is transported within the region in the context of aquaculture as well as research projects (Chávez-Villalba et al., 2005; Arcos et al., 2009), and disease...
impacts of this activity on other species like *S. palmula* must be considered. Evaluation of the *P. marinus* infection status of natural and culture populations of *S. palmula* was the focus of this project.

2. Materials and methods

2.1. Oysters

Four coastal lagoons in Sinaloa, Topolobampo, La Bocanita, La Reforma, and Cospita (Fig. 1), were sampled in October–November 2010. In Topolobampo, where the aquaculture activity is most concentrated, 30 oysters were sampled from culture arrays. The number of oysters sampled from the culture area in La Bocanita was 15. At La Reforma, where natural populations occur, 15 animals were sampled. Finally, in Cospita, 10 oysters were sampled from natural beds. Oysters were measured by taking the total shell height from the umbo to the distal margin of the shell in mm. One-way ANOVA and Duncan’s test for multiple comparisons were used to compare size range values (Zar, 1974). Sample size and oyster size depended on the available organisms. Oysters were shipped alive in a cooler to the laboratory of the Instituto de Sanidad Acuícola for processing.

2.2. Oyster processing and visual diagnostics

All fouling organisms were removed with a hard brush and a stream of seawater. Oysters were placed in a Petri dish, opened, and soft tissues were examined for the presence of abnormalities. For *P. marinus* detection all oysters were screened through induction of hypnospore formation, pieces of rectum, gills and mantle of each oyster were excised and two subsamples were obtained. One subsample was placed in Ray’s fluid thioglycollate medium (RFTM, Ray, 1966; Fisher and Oliver, 1996; Kim et al., 2006) and the other was refrigerated for possible confirmation for PCR results. The tissues placed in RFTM were incubated for 8 days in the dark, after which they were stained with Lugol’s iodine and observed using light microscopy. The remaining visceral mass of each oyster was removed from the shell and fixed whole in Davidson’s fixative (Shaw and Battle, 1957) for at least 24 h. An anterior transverse section including stomach and intestine, digestive gland, gonad, mantle, and gills was processed using standard histological methods and embedded in paraffin. Samples were sectioned and stained with hematoxylin and eosin (Shaw and Battle, 1957). Histological positive controls for *Perkinsus* sp. and *P. marinus* were used for comparison with stained slides.

Fig. 1. Map showing sampling localities in the study area.
When present, infections with a *Perkinsus* sp. parasite were scored according to the associated tissue alterations, with three categories established for rating the intensity of infection (Cáceres-Martínez et al., 2008): light infection (1), when parasite stages occurred only in the epithelia of some areas of the gut and no hemocyte infiltration nor tissue destruction was observed; moderate infection (2), when parasite stages occurred in the epithelia of some areas of the gut and in the connective tissue with focal hemocyte infiltration; and severe infection (3), when generalized hemocyte infiltration occurred and the parasite invaded not only the gut but also connective tissue surrounding the digestive gland, gonad, mantle and gills. The numbers assigned to each category for intensity of infection were added and divided by the total number of infected animals in the sample each month to obtain a numerical value of mean intensity of infection. Prevalence of parasites was the number of infected oysters in the sample divided by the total number of oysters in the sample expressed in percentage.

2.5. DNA sequencing

PCR-amplified products were directly sequenced in triplicate and bi-directionally using the standard dideoxy termination method at the San Diego State University Research Foundation, CA, USA (CSUPERB Micro Chemical Core Facility). Chromatograms were evaluated using FinchTV version 1.4.0 (Geospiza Inc.). Sequence alignments were performed using Geneious version 5.4.4 (Drummond et al., 2010) and only one consensus sequence was obtained. Sequence similarity was searched against the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLASTN 2.2.25) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences determined in this study have been deposited in the GenBank database under accession number JN676160.

2.6. Fluorescent in situ hybridization (FISH)

Fluorescent in situ hybridization (FISH) with specific probes was used to link the parasite cells observed in histological sections to the *P. marinus* DNA sequenced from those sections. The protocol described here was adapted from Carnegie et al. (2006). Tissue sections (6-μm) were placed on positively charged slides (Fisher Scientific), deparaffinized with xylene (3 × 2 min), and rehydrated in an ethanol series (100%, 2 × 1 min; 80%, 30 s; 50%; 30 s; 30 s) into dH2O (1 min). Sections were equilibrated in P buffer (50 mM Tris, pH 7.5; 5 mM EDTA) for 1 min, then subjected to partial proteolysis with pronase (125 μg/μl in P buffer) for 30 min at 37 °C. Pronase was removed by 2 × 5 min washes in P buffer, then sections were acetylated with acetic anhydride (5% [v/v]) in 0.1 M triethanolamine-HCl for 10 min at room temperature in a fume hood and washed in PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na2HPO4; 2 mM KH2PO4; pH 7.4) for 5 min. Sections were equilibrated in 5X SET (750 mM NaCl; 6.4 mM EDTA; 100 mM Na2HPO4; 2 mM KH2PO4; pH 7.4) for 5 min, then 50 μl hybridization solution (200 μg/ml bovine serum albumin, 0.025% [w/v] sodium dodecyl sulfate in 5X SET) containing the appropriate probe (see below) or dH2O (no probe control) was applied. Final concentrations of probes were 12 ng/μl, 8 ng/μl and 10 ng/μl hybridization solution for PmarLSU181, specific for *P. marinus* (Reece et al., 2008), PerkspSSU700, generic for *Perkinsus* spp. (Elston et al., 2004), and OE309, a positive control probe “universal” for molluscan hosts (5’-TCATGCTCCTCTCGCC-3’), respectively. Oligonucleotide probes used in this study were commercially synthesized and 5’-end labeled with AlexaFluo 488 green-fluorescent labels (Invitrogen). Slides were covered with plastic in situ coverslips and hybridized overnight at 42 °C in a humid chamber. The next morning cover slips were removed and sections washed with 0.2× SET (3 × 1 min) at 42 °C, air dried, and covered with Fluoromount (Sigma Life Sciences) and glass cover slips. Hybridization was evaluated using an Olympus Provis epifluorescence microscope with a dual red-green filter. A positive control sample (*P. marinus*-infected *C. virginica* from Virginia, USA) was included for both *Perkinsus* probes.

3. Results

The mean sizes of oysters in the four samples varied from 48.3 ± 0.7 mm (mean ± S.D.) in the wild sample from La Reforma to 90.0 ± 0.9 mm in the wild sample from Cospita. Mean sizes of oysters from aquaculture at Topolobampo and La Bocanita were 56.0 ± 0.8 mm and 59.5 ± 0.8 mm, respectively, statistically similar to each other but distinct from the samples at La Reforma and Cospita (p < 0.001, Duncan’s Test). Examinations of fresh tissues showed no gross signs of pathology in any oyster from any sample. Incubation of target tissues in RFTM showed that oysters from all
four localities were infected by a *Perkinsus* sp. In Fig. 2A a positive result using RFTM is shown. Its prevalence was 13.3% at Topolobampo, 6.7% at La Bocanita, 13.3% at La Reforma, and 20.0% in Cospita. Histological analysis of infected oysters revealed light to moderate infections with parasite stages in the epithelia of some areas of the gut and in connective tissues, with focal to systemic hemocyte infiltration produced in response by the host (Fig. 2B and C). Observed cells were typically small (<5 μm) unicellular trophonts. While some meronts were present, none were observed with nuclear counts greater than four.

Three positive samples by FTM and histology for *Perkinsus* sp. were used for species specific *P. marinus* PCR analysis, indicating the presence of this particular parasite. Sequencing of amplified NTS fragment (307 nt.) produced an identical sequence to one originally generated for *P. marinus* from *C. corteziensis* from Nayarit (GenBank accession number EU617394.1; Cáceres-Martínez et al., 2008) and 98% identical to the NTS region of *P. marinus* strain TXsc, characterized from Texas (AF497479.1; Robledo et al., 1999). FISH using both the PmarLSU181 probe (specific for *P. marinus*) and Perksp700 (generic for *Perkinsus* spp.) produced similar patterns of hybridization to cells in oyster gut epithelia and underlying connective tissues, as well as in gill epithelia. Hybridization with the "universal" Oe309 positive control probe produced the expected broad pattern of fluorescence (representing hybridization to host) and the no-probe negative control probe revealed no substantial auto fluorescence in the green wavelengths. The cells to which the probes hybridized in the experimental treatments conformed in size and morphology to the *Perkinsus* sp. cells observed histologically (Fig. 2D). These FISH results confirm that *P. marinus* was indeed present in the oysters from which the presumptive *P. marinus* sequence was amplified. The similarity in hybridization patterns between the PmarLSU181 (*P. marinus*-specific) treatment and the Perksp700 (*Perkinsus* spp.-generic) treatment indicate that, as the sequencing suggested, *P. marinus* was the only *Perkinsus* sp. present.

4. Discussion

The methods employed in this study collectively provide definitive evidence that *P. marinus* infects *S. palmula* along the Pacific coast of Mexico. The histology revealed parasite cells conforming in morphology and tissue tropism to published observations of the parasite in *C. virginica* (Mackin, 1951) and *C. corteziensis* (Cáceres-Martínez et al., 2008). While sizes of the cells were not measured systematically, they were generally comparable to the *P. marinus* cells observed in *C. corteziensis* by Cáceres-Martínez et al. (2008) and to those observed routinely infecting *C. virginica* today (Carnegie, unpublished observations). Intensity of infection at the histological level was light to moderate, generally lighter than has been observed in other, seriously affected species (Mackin, 1951; Cáceres-Martínez et al., 2008), though this should not be interpreted as a clear sign that *S. palmula* is less susceptible to *P. marinus* than *C. virginica* and *C. corteziensis* are. The relatively low infection levels observed may also reflect the season of sampling, sample size (heavier infections not having been captured), the oyster size profile, or a combination of these and other factors. Specific studies must be carried out to determine the course of perkinsosis in *S. palmula* of different sizes over the course of a year.

*P. marinus* has primarily been known as a parasite of oysters in the genus *Crassostrea*. Its devastating effects on *C. virginica* in the US mid-Atlantic since the 1980s have been well documented (e.g., Burreson and Ragone Calvo, 1996), and the parasite continues to be a major pathogen of *C. virginica* in the Gulf of Mexico (Soniat, 1996). The Caribbean oyster *Crassostrea rhizophorae* may be nearly as susceptible (Bushel et al., 2002), and we now recognize *C. corteziensis* to be at least moderately susceptible to *P. marinus* as well (Cáceres-Martínez et al., 2008). The more distantly related Asian species *C. gigas* and *Crassostrea ariakensis* have been infected by *P. marinus* in the field (Calvo et al., 1999; Calvo et al., 2001) but appear to be much less susceptible, displaying in some cases, reduced prevalences relative to *C. virginica* and seldom (though occasion-
ally: e.g., Barber and Mann, 1994; Moss et al., 2006) developing advanced infections. Potential infection of C. gigas in particular could be highly relevant to the Pacific states of Mexico, where C. gigas aquaculture is an important industry. There is a recent record of infection of C. gigas by P. marinus associated with mortalities in cultured populations in Sonora, Mexico (Enríquez-Espinoza et al., 2010). However, histological images of the presumptive parasite from Sonora appear not to correspond to P. marinus development stages, but to ascidicidic infections; there was no clear evidence from the Sonora case that the parasite was prevalent and causing intense, lethal infections. While some intense P. marinus infections are possible, particularly under stressful conditions (as for C. ariakensis; Moss et al., 2006), it is unlikely that significant transmission of P. marinus within a C. gigas population would occur absent a significant better source of infectious material, such as C. virginica, given the relative resistance of C. gigas to P. marinus. P. corteziensis may be such a source, however, and the question now emerges whether S. palmula may be as well. While the Perkinsioid disease risk to C. gigas may be low to non-existent where neither C. corteziensis nor S. palmula occurs, it may be higher where its commercial distribution overlaps with C. corteziensis, and possibly S. palmula. It is unlikely that C. gigas would be a significant vector by which P. marinus may be introduced to C. corteziensis and S. palmula populations that are not already parasitized, but care should be taken to ensure the biosecurity of transfers of any of these species. Understanding the distribution of P. marinus in them is essential in this regard, as a better appreciation in general of the physical and biological factors that control Perkinsioid in the Pacific waters of Mexico.

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