Assessment of the Northern Distribution Range of Selected Perkinsus Species in Eastern Oysters (Crassostrea virginica...
ASSESSMENT OF THE NORTHERN DISTRIBUTION RANGE OF SELECTED PERKINSSUS SPECIES IN EASTERN OYSTERS (CRASSOSTREA VIRGINICA) AND HARD CLAMS (MERCENARIA MERCENARIA) WITH THE USE OF PCR-BASED DETECTION ASSAYS

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ABSTRACT: Perkinsus species (Perkinsozoa, Alveolata) are the causative agent of perkinsiosis in a variety of mollusc species. For some host species, such as the eastern oyster C. virginica, Perkinsus species infections cause widespread mortality in both natural and farmed oyster populations, resulting in severe economic loss for the shellfishery, and detrimental effects on the environment (Andrews, 1955; Perkins, 1988; Coss, 2000). It is not yet clear whether P. chesapeaki and P. andrewsi are different species and, although their synonymization has been proposed, it remains an open question whether P. andrewsi and P. chesapeaki are equally pathogenic. Perkinsus species have been reported in C. virginica as far north as Maine, sometimes associated with high prevalence, but low mortality. Thus, we hypothesized that, in addition to P. marinus, Perkinsus species with little or no pathogenicity for C. virginica may be present. Accordingly, we investigated the distribution of Perkinsus species in C. virginica and Mercenaria mercenaria, collected from Maine to Virginia, by applying PCR-based assays specific for P. marinus, P. andrewsi, and a Perkinsus sp. isolated from M. mercenaria. DNA samples of M. mercenaria possessed potent PCR inhibitory activity, which was overcome by the addition of 1 mg/ml BSA and 5% (v/v) DMSO to the PCR reaction mixture. All 3 Perkinsus species were found in both host species throughout the study area. Interestingly, the prevalence of P. marinus in M. mercenaria was significantly lower than in C. virginica, suggesting that M. mercenaria is not an optimal host for P. marinus.

Perkinsus species (Perkinsozoa, Alveolata) are protistan parasites of molluscs. In Chesapeake Bay, Perkinsus marinus, Perkinsus chesapeaki, and Perkinsus andrewsi are sympatric, infecting oysters and clams. Although P. marinus is a pathogen for Crassostrea virginica, it remains unknown whether P. andrewsi and P. chesapeaki are equally pathogenic. Perkinsus species have been reported in C. virginica as far north as Maine, sometimes associated with high prevalence, but low mortality. Thus, we hypothesized that, in addition to P. marinus, Perkinsus species with little or no pathogenicity for C. virginica may be present. Accordingly, we investigated the distribution of Perkinsus species in C. virginica and Mercenaria mercenaria, collected from Maine to Virginia, by applying PCR-based assays specific for P. marinus, P. andrewsi, and a Perkinsus sp. isolated from M. mercenaria. DNA samples of M. mercenaria possessed potent PCR inhibitory activity, which was overcome by the addition of 1 mg/ml BSA and 5% (v/v) DMSO to the PCR reaction mixture. All 3 Perkinsus species were found in both host species throughout the study area. Interestingly, the prevalence of P. marinus in M. mercenaria was significantly lower than in C. virginica, suggesting that M. mercenaria is not an optimal host for P. marinus.

The development of culture methods for Perkinsus species (Gauthier and Vasta, 1993; Kleinschuster and Swink, 1993; La Peyre et al., 1993) greatly facilitated the development of specific PCR-based diagnostic assays. The first PCR-based assay was developed for P. marinus and was species-specific and more sensitive than the FTM assay (Marsh et al., 1995; Robledo et al., 1998). Subsequently, PCR-based assays specific for Perkinsus olseni (de la Herrán et al., 2000; Robledo et al., 2000), P. andrewsi (Coss, Robledo, Ruiz, and Vasta, 2001), and for other species of Perkinsus (Robledo et al., 2002) were also developed. Quantitative PCR assays for P. marinus (Yarnall et al., 2000; Gauthier et al., 2006), a multiplex PCR assay detecting P. marinus and Haplosporidium species (Penna et al., 2001), and modified PCR-based assays have been developed that can distinguish between P. marinus, P. olseni, Perkinsus mediterraneus, and P. andrewsi/P. chesapeaki or P. marinus and P. olseni, respectively (Elandalloussi et al., 2004; Abollo et al., 2006).

Prior to 2000, all surveys for Perkinsus species were conducted with the use of histology or FTM-based assays, and all Perkinsus infections observed in C. virginica were attributed to P. marinus, the only Perkinsus species described along the Atlantic coast of the Americas at that time. By 2001, 2 new species, P. chesapeaki and P. andrewsi, were described from clams (M. arenaria and M. balthica, respectively) in Chesapeake Bay (Coss, 2000; McLaughlin et al., 2000; Coss, Robledo, Ruiz, and Vasta, 2001). However, in addition to its type host, P. andrewsi was also found in C. virginica (the type host of P. marinus) and in the clams M. mercenaria and M. mitchelli (Coss, 2000; Coss, Robledo, Ruiz, and Vasta, 2001). Conversely, P. marinus was detected in M. arenaria, M. balthica, and Macoma mitchelli (Kotob et al., 1999; Coss, 2000; McLaughlin et al., 2000; Coss, Robledo, Ruiz, and Vasta, 2001), suggesting a broad host range for these Perkinsus species.

Perkinsus species infections have been observed in oysters from Tabasco, Mexico, to Maine (reviewed in Burreson and Ragone Calvo, 1996; Ford, 1996; Soniat, 1996). In some areas of the northeastern United States, mortalities in oyster populations were low to moderate, despite high prevalence and infection densities of Perkinsus species (Ford, 1996; Karolus et al., 2000). This observation led us to hypothesize that, in addition
to *P. marinus*, other *Perkinsus* species are present in the northeastern regions that may be less virulent towards *C. virginica*. We therefore surveyed *C. virginica* and *M. mercenaria* obtained from selected sites from Maine to Virginia for the presence of *Perkinsus* species, and specifically for *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) using specific PCR-based assays. This is the first study that assesses the distribution of sympatric *Perkinsus* species in 2 economically important molluscan hosts.

**MATERIALS AND METHODS**

**Collection of tissue specimens and DNA extraction**

*Crassostrea virginica* (size: 44–142 mm) and *M. mercenaria* (size: 40–73 mm) specimens, collected monthly from June 2002 to September 2002, were obtained from shellfish farmers and academic institutions from 8 sites along the Atlantic coast of the United States as follows: *C. virginica* were obtained from Walpole (Maine), Martha’s Vineyard (Massachusetts), Narragansett Bay (Rhode Island), Oyster Bay (New York), Delaware Bay (New Jersey), and Sandy Point (Maryland); *M. mercenaria* were obtained from Eliot (Maine), Martha’s Vineyard (Massachusetts), and Cheriton (Virginia) (Fig. 1). Upon arrival, the shellfish were stored up to 72 hr at 4°C until further processing.

Eighteen to 60 specimens from each sampling site and collection date were individually dissected. From each individual, gut, gill, and mantle tissues were collected and pooled (50–100-mg wet weight of total tissue/pool), and DNA was extracted with the use of a commercially available kit (DNeasy, 96-well format, QIAGEN, Valencia, California). DNA concentration and purity were estimated by spectrophotometry at wavelengths of 260 and 280 nm. The DNA samples were stored at −20°C until testing.

**PCR assays**

PCR-based assays specific for the genus *Perkinsus*, and the species *P. marinus*, *P. andrewsi*, and *P. olseni* (syn. *Perkinsus atlanticus*), were used according to Marsh et al. (1995), Coss, Robledo, Ruiz, and Vasta (2001), and Robledo et al. (2000, 2002).

**Development of a PCR-based diagnostic assay specific for the genus *Perkinsus* isolated from *Meridia mercenaria***

Primers designated M6L (sense, 5′-GGGCGGCAAAATTACATCATCTTG AG-3′) and M5 (antisense, 5′-AACCTATCGGACTTACCCTG GG-3′) were designed based on the intergenic spacer of the rRNA gene cluster of *Perkinsus* sp. (*M. mercenaria*) with the use of an Oligo Calculator v3.07 (http://www.basic.northwestern.edu/biotools/oligocalc.html). Thermocycler conditions were 94°C for 4 min, 35 cycles of 94°C for 1 min, 65°C for 30 sec with an extension of 1 sec per cycle, 72°C for 1 min, with a final extension at 72°C for 7 min.

Three different PCR reaction mixtures (A–C) were used. PCR reaction mixture A consisted of 1× QIAGEN PCR Master mix (contains Taq DNA Polymerase (250 µl), KCl, Tris-Cl, (NH₄)₂SO₄, 1.5 mM MgCl₂, and 200 µM of each dNTP) (QIAGEN), and 40 nM of each primer. To obtain PCR reaction mixture B, heat-treated bovine serum albumin (BSA) (New England Biolabs, Ipswich, Massachusetts) and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, Missouri) were added to a final concentration of 1 mg/ml BSA and 5% (v/v) DMSO to PCR reaction mixture A. PCR reaction mixture C consisted of TaKaRa Ex Taq® DNA Polymerase (250 µl/µl) (TaKaRa Bio, Inc., Otsu, Shiga, Japan), 1× of the proprietary Ex Taq reaction buffer (contains 2 mM MgCl₂), 200 µM of each dNTP (TaKaRa Bio), 1 mg/ml BSA, 5% (v/v) DMSO, and 40 nM of each primer.

**Assessment of the specificity and sensitivity of the PCR assays**

To assess the specificity of each PCR assay, 50 ng of DNA from *P. marinus* (ATCC 50489), *P. andrewsi* (ATCC 50807), and *Perkinsus* sp. (*M. mercenaria*) were used as templates in the PCR reactions. Sensitivities of the species-specific assays were assessed with the use of decreasing amounts of genomic DNA (100 pg to 1 fg) from the respective *Perkinsus* species. For the genus-specific assay, the sensitivity was assessed with the use of *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) genomic DNA, and for the *Perkinsus* sp. (*M. mercenaria*)–specific assay genomic DNA of *Perkinsus* sp. (*M. mercenaria*) and *P. andrewsi* genomic DNA were used. Assay sensitivities were assessed in the presence or absence of 500 ng of host (*C. virginica* or *M. mercenaria*) genomic DNA. Sensitivity of the PCR assays in the presence of *C. virginica* genomic DNA was assessed with the use of PCR reaction mixture A. For assessment of the specificity in the presence of *M. mercenaria* genomic DNA, PCR reaction mixtures A and C were used. Negative controls contained similar PCR reaction mixtures, except that the template was replaced by sterile double-distilled H₂O.

**PCR-based detection of selected *Perkinsus* species in oyster and clam samples**

Five hundred nanograms of DNA extracted from *C. virginica* and *M. mercenaria* were tested for *Perkinsus* species, *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*). For *C. virginica* DNA samples, PCR reaction mixture A were used and for *M. mercenaria* DNA samples, PCR reaction mixture C. Positive controls consisted of similar PCR reaction mixtures, except that 1 ng of genomic DNA extracted from cultured *Perkinsus* species was used as a template. In negative PCR controls, the DNA template was substituted by sterile double-distilled H₂O.

To minimize false negatives, the small subunit rRNA gene (SSU) was amplified from all *M. mercenaria* samples with the use of the universal primers UPRA and UPRB from Medlin et al. (1988), which are designed to amplify the SSU of all eukaryotes. For *C. virginica*, 45 of 226 samples that were negative for the presence of *Perkinsus* species were tested for the amplifiability of the SSU. PCR reaction mixtures were identical to those used to detect *Perkinsus* infections in *C. virginica* and *M. mercenaria*. Positive PCR amplification controls consisted of similar PCR reaction mixtures, except that 500 ng of genomic DNA extracted from either *C. virginica* or *M. mercenaria* was used that was known to be amplifiable. Cycling conditions were 94°C for 4 min, 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, with a final extension at 72°C for 7 min.

**Attenuation of inhibitory effects on the PCR amplification**

To establish and optimize PCR conditions that would attenuate the observed inhibition of PCR amplification, experiments were conducted by spiking *M. mercenaria* genomic DNA (500 ng) with 10 pg and 1 pg *P. olseni* genomic DNA. These mixtures were tested for *P. olseni* as described elsewhere (Robledo et al., 2000), with do et al., 2000) with all PCR reaction mixtures A, B, and C. PCR amplification controls consisted of similar PCR reaction mixtures containing only *P. olseni* genomic DNA. *Perkinsus olseni* DNA was used because infections with this species have not been reported in the United States. Therefore, it is unlikely that the clams or oysters carried *P. olseni*, allowing us to control accurately for the amount of target DNA added to the sample.

**Sequencing**

Forty amplicons generated by the *Perkinsus* genus–specific PCR assay of samples that tested negative with all of the *Perkinsus* species–specific assays were cored from agarose gels and reamplified with the use of the genus-specific assay as described above. The amplified products were separated on 1.5% agarose gels, purified from the gels (QIAquick, QIAGEN) and sequenced from both directions with the primers PER1 and PER2 (Robledo et al., 2002). Sequencing services were provided by the Bioanalytical Services Laboratory at the Center of Marine Biotechnology, Baltimore, Maryland. Fragment assembly was performed with the use of the Staden Package v1.6.0 on a Mac OS X (Apple Computer, Inc., Cupertino, California) or Linux Fedora® Core 5 (Red Hat, Inc., Raleigh, North Carolina)–based computer.

**Ribosomal RNA sequences for assay design and sequence comparisons**

Sequences of rRNA genes and intergenic spacers of the rRNA sequences of *P. andrewsi* (Genbank AF102171 and AF305326), *P. marinus* (AF497479), *P. olseni* (syn. *P. atlanticus*, AF140295), and *Perkinsus* sp. (*M. mercenaria*) (deposited as *Perkinsus* sp. CCA2001, AF252288) were obtained from GenBank®. Sequence alignments were
FIGURE 1. Sample site locations. *Crassostrea virginica* (●) and *Mercenaria mercenaria* (○) specimens were received from shellfish providers each month from June 2002 to September 2002. 1: Walpole (Maine); 2: Eliot (Maine); 3: Martha’s Vineyard (Massachusetts); 4: Narragansett Bay (Rhode Island); 5: Oyster Bay (New York); 6: Delaware Bay (New Jersey); 7: Sandy Point (Maryland); 8: Cheriton (Virginia). The map was generated with the Generic Mapping Tools, v.4.1.4 (Smith and Wessel, 1990; Wessel and Smith, 1998).
performed with the use of the Needleman–Wunsch global alignment algorithm within EMBOSS (Rice et al., 2000). Sequence alignments were used to design the Perkinsus sp. (M. mercenaria)–specific assay and to identify possible new Perkinsus sp. strains in the study area.

Statistical analysis

The main focus of this article was to assess and compare infection frequencies of Perkinsus species collected from 2 hosts at several sampling sites over a relatively short sampling period (4 mo). Therefore, the χ² test and, for pairwise comparison of the sampling site and the 2 host species, the Fisher’s exact test were used. Statistical analyses were performed with the R software suite (R Development Core Team, 2006).

Climatologic data

Temperature and precipitation data were obtained from COOP Data/Record of Climatological Observations Forms from selected weather stations made available to the public online by the National Climatic Data Center, U.S. Department of Commerce (http://www7.ncdc.noaa.gov/IPS).

RESULTS

Specificity of the diagnostic assays

To assess the specificity of the 4 PCR diagnostic assays used in this study, 50-ng genomic DNA from clonal cultures of P. marinus, P. andrewsi, and Perkinsus sp. (M. mercenaria) were tested. The genus-specific assay amplified a fragment of approximately 300 bp from each DNA preparation, whereas the assays designed to be specific for P. marinus and P. andrewsi amplified fragments of expected size (approximately 300 bp) only from genomic DNA preparations of the respective Perkinsus species (Fig. 2). The assay designed for P. andrewsi showed intense low molecular bands when P. marinus and Perkinsus sp. (M. mercenaria) were used as templates. Because this band also appeared with lesser intensity in the negative PCR control, they may represent primer dimers. The PCR assay designed for Perkinsus sp. (M. mercenaria) amplified a fragment of expected size (approximately 300 bp) from Perkinsus sp. (M. mercenaria). However, it also amplified a 300-bp fragment from P. andrewsi (Fig. 2).

Sensitivity of the diagnostic assays

The sensitivity of the Perkinsus genus– and species–specific assays was assessed by performing the respective assays on serially diluted genomic DNA (100 pg to 1 fg) with the use of the standard PCR reaction mixture A. The Perkinsus genus–specific assay amplified 100 fg of P. marinus and 1 pg of P. andrewsi and Perkinsus sp. (M. mercenaria) genomic DNA (Fig. 3A). The P. marinus– and the P. andrewsi–specific assays amplified 1 pg of P. marinus and P. andrewsi genomic DNA (Fig. 3B, C). The assay designed for the Perkinsus sp. (M. mercenaria) amplified 100 fg Perkinsus sp. (M. mercenaria) and 1 pg of P. andrewsi genomic DNA (Fig. 3D). In the genus-specific assay, the addition of 500 ng C. virginica DNA had no effect on the detection limit of P. andrewsi and Perkinsus sp. (M. mercenaria), but reduced the sensitivity by about 10-fold for P. marinus. No effects of 500 ng C. virginica DNA were observed on the P. marinus–, and P. andrewsi–specific assays (Fig. 3B, C). In the Perkinsus sp. (M. mercenaria)–specific assay, although the 500 ng of C. virginica DNA had no effect on the detection limit of P. andrewsi, it reduced the sensitivity by about 10-fold for Perkinsus sp. (M. mercenaria) (Fig. 3D). The addition of 500 ng of M. mercenaria genomic DNA to the PCR reactions reduced the sensitivity by at least 1,000-fold in all 4
assays for all *Perkinsus* species tested (Fig. 3A–D), suggesting that the *M. mercenaria* genomic DNA preparations possessed potent PCR inhibitory activity.

**Attenuation of the inhibition of the PCR amplification**

To obtain PCR conditions that attenuate the inhibition of the PCR amplification, 500 ng of *C. virginica* and *M. mercenaria* DNA were spiked with 10 pg and 1 pg *P. olseni* genomic DNA. These mixtures were tested for *P. olseni* with the use of PCR mixtures A, B, and C. PCR reaction mixture A did not amplify *P. olseni* in the presence of *M. mercenaria* genomic DNA, confirming PCR-amplification inhibition by DNA extractions from *M. mercenaria* (Fig. 4). The use of PCR reaction mixture B, which contains BSA (1 mg/ml) and DMSO (5% v/v), alle-
viated PCR inhibitory effects in most clam DNA preparations (Fig. 4). However, DNA extracted from some individual clams was not amplified even in mixture B. With the use of PCR reaction mixture C (containing TaKaRa Ex Taq®, 1× of the proprietary Ex Taq reaction buffer, 1 mg/ml BSA, and 5% (v/v) DMSO), no PCR amplification inhibition was observed in any of DNA extractions tested (Fig. 4). Under these conditions, the detection limits of the genus- and the species-specific assays were 1 pg to 10 fg, respectively (Fig. 3).

False-negative PCR results analysis

To exclude false-negative PCR results from our analysis, the SSU of DNA samples was amplified with the use of primers that anneal in conserved regions of the SSU (Medlin et al., 1988). Forty-five of 226 C. virginica that tested negative with the diagnostic PCR assays were examined. In all samples tested, the SSU was amplified (data not shown). With the use of PCR reaction mixture C, the SSU in 225 out of 244 M. mercenaria samples could also be amplified (data not shown). The 19 M. mercenaria samples (7.8%) for which no amplification was observed were excluded from further analysis.

Prevalence of Perkinsus species in Crassostrea virginica

In total, 625 C. virginica collected monthly from June to September 2002 from Walpole (Maine), Martha’s Vineyard (Massachusetts), Narragansett Bay (Rhode Island), Oyster Bay (New York), Delaware Bay (New Jersey), and Sandy Point (Maryland) (Fig. 1) were tested for the presence of P. marinus, P. andrewsi, and Perkinsus sp. (M. mercenaria) (Table I). Overall, by using the genus-specific assay, 449 (66.5%) C. virginica tested positive for Perkinsus species as far north as Maine. The differences in prevalence between the sampling sites were statistically significant (P < 0.001). Generally, prevalences of Perkinsus species infections increased from north (Walpole; 10.3%) to south (Sandy Point; 96.5%), with the exceptions of Martha’s Vineyard, which had a significantly higher prevalence compared to Narragansett Bay (Fisher’s exact test, P < 0.001), and Oyster Bay, which had lower prevalence compared to Delaware Bay (Fisher’s exact test, P < 0.001) and Narragansett Bay (Fisher’s exact test, P < 0.001) (Table I). There were no significant differences between prevalences at Sandy Point and Martha’s Vineyard, and Sandy Point and Delaware Bay.

A seasonal trend in Perkinsus sp. infection prevalences was observed in Narragansett Bay, Oyster Bay, and Delaware Bay, where infections were lower in early summer (June), as compared to mid- (August; Delaware Bay, Narragansett Bay) or late summer (September; Oyster Bay). In Walpole, prevalences remained low (0–19.4%; Fisher’s exact test, P ≥ 0.237) during the sampling period. In Martha’s Vineyard, prevalences were high (86.7–100%; Fisher’s exact test, P ≥ 0.173) throughout

<table>
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<tr>
<th>C. virginica</th>
<th>M. mercenaria DNA</th>
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<tr>
<td>P. olseni DNA (pg)</td>
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Figure 4. Attenuation of PCR amplification inhibition. Five hundred nanograms genomic DNA from 1 individual of Crassostrea virginica and 2 Mercenaria mercenaria specimens were spiked with 10 pg and 1 pg Perkinsus olseni genomic DNA. Samples were tested with a P. olseni-specific PCR-based assay with the use of PCR reaction mixtures A, B, and C. In the positive control, host DNA was omitted. H₂O: negative control.
the entire sampling period. Samples from Sandy Point were not available for the months of June and September. Prevalences at this site did not differ between July and August (Fisher’s exact test, \( P = 1 \)) (Fig. 5A).

The 3 Perkinsus species, *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*), were detected in *C. virginica* from Maryland to Maine. *Perkinsus marinus* was the dominant species, with 394 (58.4%) *C. virginica* testing positive. In contrast, only 15 (2.2%) and 9 (1.3%) *C. virginica* tested positive for *Perkinsus* sp. (*M. mercenaria*) and *P. andrewsi*, respectively. This trend holds true for all sampling sites with the exception of the sampling site in Walpole, where *P. marinus* and *P. andrewsi* were both found at low prevalences (Table I). Coinfections with *P. marinus* and *P. andrewsi* or *P. marinus* and *Perkinsus* sp. (*M. mercenaria*) species were also observed (Table I). However, none of the oysters that tested positive for *P. andrewsi* was positive for *Perkinsus* sp. (*M. mercenaria*).

Fifty-two oysters that tested positive for infection with a *Perkinsus* species were negative for *P. marinus*, *P. andrewsi*, or *Perkinsus* sp. (*M. mercenaria*) (Table I). Sequence analysis of amplicons obtained by the genus-specific PCR assay from 22 of the 52 oysters suggests that 13 oysters carried *P. marinus*, 3 *P. andrewsi*, and 2 *Perkinsus* sp. (*M. mercenaria*). Four samples showed extensive sequence ambiguities, possibly due to infections with more than 1 *Perkinsus* species.

### Prevalence of Perkinsus species in Mercenaria mercenaria

To assess the prevalence of *Perkinsus* species infections in the hard clam *M. mercenaria*, 225 specimens were tested with the PCR-based diagnostic assays described above. The specimens tested were collected monthly from June 2002 to August 2002 from Eliot (Maine), Martha’s Vineyard (Massachusetts), and July 2002 to September 2002 from Cheriton (Virginia) (Fig. 1).

Overall, by using the genus-specific PCR-based assay, a total of 72 (32%) specimens tested positive for a *Perkinsus* species (Table II). Infection prevalences differed significantly between sites (\( \chi^2 \text{test}, P < 0.001 \)), increasing from north (Eliot) to south (Cheriton) (Table II). A seasonal trend in infection prevalence was only observed in Cheriton, where prevalence was lowest in July and increased over the sampling period. In Martha’s Vineyard, prevalence peaked in July. In Eliot, *Perkinsus* species infections were not observed in June. Prevalence observed in July and August did not differ considerably (Fisher’s exact test, \( P = 0.765 \)) (Fig. 5B).

Overall, 10 (4.4%) clam specimens tested positive for *P. marinus*, and 3 (1.3%) and 17 (7.6%) tested positive for *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*), respectively. Similar to infections in *C. virginica*, coinfections in individual host specimens with *P. marinus* and *Perkinsus* sp. (*M. mercenaria*) were observed (Table II). None of the clams that tested positive for *P. andrewsi* was positive for *Perkinsus* sp. (*M. mercenaria*). *Perkinsus marinus* infections were observed at all 3 sampling sites. *Perkinsus andrewsi* infections were observed solely in Martha’s Vineyard, and *Perkinsus* sp. (*M. mercenaria*) were observed in Eliot and Cheriton (Table II).

Forty-four specimens tested positive for a *Perkinsus* species infection, but negative for any of the *Perkinsus* species or isolates tested. Eighteen of the 44 amplicons generated by the *Perkinsus* genus–specific PCR were sequenced. Fourteen of the obtained sequences were highly similar or identical to the sequence of *P. marinus* and 3 sequences to *P. andrewsi*. One sequence showed extensive ambiguities, suggesting an infection with more than 1 *Perkinsus* species.

### Comparison of Perkinsus species prevalences in Crassostrea virginica and Mercenaria mercenaria

Prevalence of *Perkinsus* species and *P. marinus* in *M. mercenaria* was significantly lower compared to prevalence in *C. virginica* (Fisher’s exact test, \( P < 0.001 \)). *Perkinsus andrewsi* prevalence was similar in both host species (Fisher’s exact test, \( P = 1 \)). Prevalence of *Perkinsus* sp. (*M. mercenaria*) was significantly higher in *M. mercenaria* (Fisher’s exact test, \( P < 0.001 \)).

Martha’s Vineyard provides a very useful side-by-side comparison of prevalence in both clams and oysters because both hosts were collected from proximal locations. Here, 96.1% of the *C. virginica* were infected with a *Perkinsus* species, compared to 26.2% of the *M. mercenaria* (Fig. 6; Tables I, II). All infected *C. virginica* specimens carried *P. marinus*, and *P. andrewsi* infected 1.1% and *Perkinsus* sp. (*M. mercenaria*) 3.9% of *C. virginica*. Each of the oysters infected with *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*) were dually infected with *P. marinus*. In *M. mercenaria*, *P. marinus* and *P. andrewsi* were present in low prevalences (3.3% and 4.9%, respectively). *Per-
Figure 5. Monthly Perkinsus infection prevalence in Crassostrea virginica and Mercenaria mercenaria. Percent prevalences of Perkinsus species (detected by the genus-specific assay) (■), Perkinsus marinus (□), Perkinsus andrewsi (◎), Perkinsus sp. (M. mercenaria) (●), dual infections with P. marinus and P. andrewsi (□), as well as P. marinus and Perkinsus sp. (M. mercenaria) (○), and infections with Perkinsus species only (□) are shown for all sampling sites. (A) Percent prevalence in C. virginica. (B) Percent prevalence in M. mercenaria. Jun: June; Jul: July; Aug: August; Sep: September.
Table II. Percent prevalence of Perkinsus species infections in Mercenaria mercenaria collected from June 2002 to September 2002. 2: Eliot, Maine; 3: Martha’s Vineyard, Massachusetts; 8: Cheriton, Virginia; N: number of individuals; n: number of infected individuals; (%): prevalence in percent; P. m.: Perkinsus marinus; P. a.: Perkinsus andrewsi; P. sp.: Perkinsus sp. (M. mercenaria); P. spp.: Perkinsus infections detected with the generic PCR-based assay.

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**Water temperature and precipitation**

Water temperature and salinity are the main environmental factors affecting Perkinsus species infections (Andrews, 1988; Burreson and Ragone Calvo, 1996). Air temperature is often used as a substitute for water temperature, because it is more frequently recorded and correlates with the temperature of nearby water bodies (Jeffries and Johnson, 1976; Sauriau, 1991). Salinity of coastal water bodies is influenced by freshwater inflow from major rivers and precipitation. Thus, to compare prevalences to an earlier study on the distribution of Perkinsus species in C. virginica and M. mercenaria were statistically significant for Perkinsus species and P. marinus infections (Fisher’s exact test, P < 0.001), but not for P. andrewsi (Fisher’s exact test, P = 0.105) or Perkinsus sp. (M. mercenaria) (Fisher’s exact test, P = 0.208).

The aim of the present study was to assess the presence of P. marinus, P. andrewsi, and Perkinsus sp. (M. mercenaria) in 2 economically important bivalves, C. virginica and M. mercenaria, with the use of PCR-based assays. Along the Gulf of Mexico and Atlantic coast of the United States, 3 Perkinsus species have been described, i.e., P. marinus (Mackin et al., 1950; type host C. virginica), P. chesapeaki (McLaughlin et al., 2000; type host M. arenaria), and P. andrewsi (Coss, Robledo, Ruiz, and Vasta, 2001; type host M. balthica).

The heterospecificity of P. andrewsi and P. chesapeaki has been controversial and synonymization has been suggested (Burreson et al., 2005). According to the original description, P. chesapeaki is a distinct morphotype (McLaughlin et al., 2000). In contrast, P. andrewsi cannot be distinguished from other Perkinsus species based on morphology, but sequences of the rRNA genes and intergenic regions of P. andrewsi differ from other Perkinsus species (Coss, Robledo, and Vasta, 2001; Coss, Robledo, Ruiz, and Vasta, 2001). The Perkinsus isolate that was analyzed to clarify the relationship of P. andrewsi and P. chesapeaki has been designated as P. chesapeaki because it has been isolated from the appropriate type host. However, this isolate appears to be morphologically identical to P. andrewsi and, thus, may not be the P. chesapeaki originally described. Therefore, until additional evidence is obtained, we retain the P. andrewsi designation for the present study.

The standard diagnostic assay for Perkinsus species that is based on the FTM method does not distinguish between the sympatric Perkinsus species found along the Atlantic coast (Ray, 1952; Coss, 2000; McLaughlin et al., 2000; Coss, Robledo, Ruiz, and Vasta, 2001). However, several Perkinsus species–specific assays are available (Marsh et al., 1995; Yarnall et al., 2000; Coss, Robledo, Ruiz, and Vasta, 2001; Robledo et al., 2002). No PCR-based assay has been developed for P. chesapeaki, mainly due to the lack of a bona fide type culture that would allow design and validation of such an assay.

For the purpose of the present study, we used available PCR-based assays specific for the genus Perkinsus, and the species P. marinus and P. andrewsi (Marsh et al., 1995; Coss, Robledo, Ruiz, and Vasta, 2001; Robledo et al., 2002), and developed a new PCR-based assay for a Perkinsus species isolated from M.
The assay designed for Perkinsus sp. (M. mercenaria) also amplified P. andrewsi genomic DNA extracted from the P. andrewsi type culture. This is to be expected, for we have previously shown that P. andrewsi has 2 distinct rRNA gene units (types A and B) (Pecher et al., 2004). Sequence analysis of the rRNA gene unit of the Perkinsus sp. (M. mercenaria) revealed only 1 rRNA gene unit that is very similar in sequence to the rRNA-B gene unit of the P. andrewsi type culture (W. T. Pecher and G. R. Vasta, unpubl. obs.). In particular, the IGS of Perkinsus sp. (M. mercenaria) is 98.9% identical to the IGS of the P. andrewsi rRNA-B gene unit (W. T. Pecher and G. R. Vasta, unpubl. obs.), explaining the cross amplification. On the other hand, the P. andrewsi–specific assay does not detect Perkinsus sp. (M. mercenaria), because it has been developed based on the IGS of the rRNA-A gene unit that is only 71.3% identical to the IGS of Perkinsus sp. (M. mercenaria).

The sensitivities of each species–specific assay observed in our study (0.1–1 pg genomic DNA of the respective Perkinsus species) is similar to the sensitivities for the P. marinus and P. andrewsi diagnostic assays reported by Marsh et al. (1995) and Coss, Robledo, Ruiz, and Vasta (2001). Our data suggest that the sensitivity of the genus–specific assay (10 fg to 1 pg genomic Perkinsus species DNA) is equal, or greater, compared to the respective species–specific assays, allowing us to identify low-intensity Perkinsus infections.

Inhibition of PCR amplification is frequently observed in environmental and biological samples (reviewed by Wilson, 1997). Inhibitory substances include organic and phenolic compounds, humic acids, heavy metals, fats, and polysaccharides. In molluscs, PCR inhibition has been attributed to glycogen (Hill et al., 1991; Andersen and Omiecinski, 1992; Atmar et al., 1993). Modified DNA extraction protocols (Atmar et al., 1993), and inclusion of additives in the PCR reaction mixture is commonly used to attenuate the effects of the interfering substances (reviewed in Wilson, 1997). In addition, commercially available kits have been developed that can be used to extract DNA from plants, animals, and fungi from complex sources such as the soil and other environmental samples.

We did not observe PCR inhibition in C. virginica DNA extracts. However, PCR inhibition was dramatic in DNA from M. mercenaria, and has been observed in scallop DNA (Argopecten irradians) extracted with a commercial tissue kit (W. T. Pecher and G. R. Vasta, unpubl. obs.). We succeeded in attenuating the PCR amplification inhibition in M. mercenaria samples by adding 1 mg/ml BSA in combination with 5% (v/v) DMSO to the PCR reaction mixture. While using regular Taq DNA polymerase with a standard PCR buffer, we were able to PCR amplify the SSU from 70% of all M. mercenaria samples (data not shown). The use of a specialty Taq DNA polymerase with its optimized buffer system designed to amplify large DNA fragments increased the success rate to 92%. However, similar reaction conditions failed to amplify scallop DNA samples (W. T. Pecher and G. R. Vasta, unpubl. obs.). These findings underline the importance of validation of PCR conditions for each sample type used, including template “amplifiability” vis-à-vis inhibition. Once DNA is extracted from samples and PCR conditions are optimized, the PCR-based assays enable detection of any Perkinsus species and different Perkinsus species in the same sample. Thus, the application of the genus– and species–specific assays presents a valuable alternative to the FTM assay.

Studies based on the FTM assay documented the distribution of Perkinsus species in oysters from the Yucatán Peninsula, Mexico, to Maine (Burreson et al., 1994; Ford, 1996; Soniat, 1996). These infections have been attributed to P. marinus. However, the discovery of additional Perkinsus species and the development of specific PCR assays for them have provided tools to test this assumption. In the present study, commercially harvested C. virginica and M. mercenaria populations were tested for the presence of Perkinsus species, P. marinus, P. andrewsi, and a Perkinsus sp. isolated from M. mercenaria with the use of specific PCR–based assays.

In accordance with the studies identified above, with the use of the genus–specific assay in both bivalve host species, Perkinsus species, infections were observed as far north as Maine. The intensity of the amplicons obtained by the PCR–based assays suggested, in the majority of the positive samples, the presence of 10 pg or more of Perkinsus spp. DNA (data not shown), which is roughly equivalent to more than 100 Perkinsus spp. cells (see below). However, it cannot be ruled out that in some specimens that yielded low-intensity amplicons, these actually reveal only the presence of parasite rather than true infections.

With the use of the genus–specific assay, prevalences of Perkinsus species in C. virginica appeared lower in early summer (June) than in mid– (August) or late summer (September) in Delaware Bay, Oyster Bay, and Narragansett Bay. Similar trends were observed in the Chesapeake Bay and other regions. When compared to C. virginica, significantly fewer M. mercenaria specimens tested positive for the genus Perkinsus and P. marinus. Prevalences of Perkinsus species (as assessed by the genus–specific method) differed from site to site in both host species. Generally, prevalences of Perkinsus species decreased from south to north, with the exception of Martha’s Vineyard (Massachusetts) and Oyster Bay (New York) in C. virginica. In Martha’s Vineyard, prevalences were surprisingly high (86.7–100.0% over the 4–mo study period) compared to those in Narragansett Bay and Oyster Bay and are in contrast to observations by Russell et al. (2004). These authors did not observe P. marinus infections in C. virginica specimens that were collected in September 2000 and tested by a multiplex PCR–based assay, suggesting that Martha’s Vineyard may have experienced a localized Perkinsus epizootic in 2002.

Prevalences in Oyster Bay were significantly lower compared to Narragansett Bay. However, the observed prevalences in Oyster Bay in 2002 were higher than those reported by Russell et al. (2004), who observed 0% and 3% P. marinus prevalence in C. virginica collected in June and August 2000, respectively, from a site in Oyster Bay. Similarly, we observed no P. marinus
infections in Oyster Bay in June 2002. In August 2002, however, 17% of C. virginica were infected with P. marinus. These differences may be due to higher temperatures and drier conditions in 2002 compared to 2000, as judged by monthly mean air surface temperatures and precipitation recorded by a nearby weather station (Mineola, New York, COOP ID 305377). Higher temperatures and drier conditions may result in higher water temperatures and higher salinity, both conditions favorable to P. marinus infections (Andrews, 1988; Burreson et al., 1996). Furthermore, differences in the sensitivities of the PCR assays may have contributed to the observed differences. The PCR assay used in the current study detects 1 pg genomic DNA of P. marinus. Based on the following calculation, the PCR-based assay used in the present study is about 2.5-fold more sensitive than the one used by Russell et al. (2004). Based on available sequences, P. marinus has an estimated genome size between 70 and 80 Mb (N. M. El-Sayed, J. A. Fernandez-Robledo, and G. R. Vasta, unpubl. obs.; http://www.tigr.org/tdb/e2k1/pmg/), and a single rRNA gene unit is approximately 7 kb long (J. A. Fernandez-Robledo and G. R. Vasta, unpubl. obs.). If a genome size of 80 Mb is assumed, then 1 pg genomic DNA represents 13 genome equivalents. If it is furthermore assumed that, similar to P. olseni (syn. P. atlanticus) (de la Herrán et al., 2000), P. marinus rRNA genes are encoded by 5% of its genome, then about 570 copies of a single rRNA gene unit are present per genome equivalent. Thus, the PCR assay used in the present study would detect 7,500 copies of the rRNA gene unit, or, as each gene unit contains 1 IGS, roughly 7,500 copies of the IGS. The multiplex PCR assay used by Russell et al. (2004) detects 100 fg of plasmid DNA containing the P. marinus IGS (Russell et al. 2004). Because the plasmid with the IGS sequence is approximately 4.5 kb, 100 fg plasmid DNA represents about 20,000 copies of the IGS.

Our data enabled us to extend the observed range of P. andrewsi and Perkinsus sp. (M. mercenaria) from Chesapeake Bay to Maine. It is noteworthy that the prevalence of Perkinsus sp. (M. mercenaria) was highest in the south (Cheriton, Virginia), suggesting that this Perkinsus isolate may prefer warmer waters. However, until investigations on the southern distribution range of this Perkinsus isolate are conducted, this remains speculative. Interestingly, we did not detect P. andrewsi in farmed oysters in Chesapeake Bay. This finding is in contrast to reports of Coss, Robledo, Ruiz, and Vasta (2001) that indicate 65% of 125 C. virginica collected from natural populations throughout Chesapeake Bay were infected with P. andrewsi, 64% with P. marinus, and 34% with both. In our study, 94.7% of oysters were infected with P. marinus and 10.5% with Perkinsus sp. (M. mercenaria). A possible explanation is that the conditions at the particular location were favorable for a P. marinus infection. Alternatively, and not mutually exclusively, it is conceivable that once a P. marinus infection has been established in an oyster population, it may outgrow other Perkinsus infections. To address these questions, further investigations need to be conducted on the infection dynamics of different Perkinsus species.

Perkinsus marinus appears to be the most prevalent Perkinsus species in C. virginica. Therefore, discrepancies between high infection density, prevalence, and low mortality observed in other studies in C. virginica (Ford, 1996; Karolus et al., 2000) cannot be attributed to the presence of a different Perkinsus species with less pathogenicity toward the oyster. Alternative hypotheses that will require further study include the notion of variable P. marinus strains with different pathogenocities, rather than different Perkinsus species, and, perhaps, differences in environmental factors such as cooler summer peak temperatures that could influence the outcome of an infection (Ford, 1996). Of course, the presence of host populations with different genetic backgrounds could be another component leading to lower host mortality. This is exemplified by the observation that in M. mercenaria Perkinsus sp. (M. mercenaria) was the most prevalent Perkinsus species.

Because of sequence similarities of the second rRNA gene unit (rRNA-B) of the P. andrewsi haplotype to the Perkinsus sp. isolated from M. mercenaria (W. T. Pecher and G. R. Vasta, unpubl. obs.), the P. andrewsi haplotype is detected by the Perkinsus sp. (M. mercenaria)—specific assay with a 10-fold lower sensitivity. In our study, none of the clams and oysters that tested positive for P. andrewsi also tested positive for Perkinsus sp. (M. mercenaria). Certainly, the lower sensitivity of the Perkinsus sp. (M. mercenaria) toward P. andrewsi may partially explain this observation, but the presence of P. andrewsi isolates that contain only the rRNA-A gene unit and thus are not detected by the Perkinsus sp. (M. mercenaria) assay cannot be ruled out. Because the rRNA unit of Perkinsus sp. (M. mercenaria) and the P. andrewsi rRNA-B unit share high sequence similarities, Perkinsus sp. (M. mercenaria) could be considered a variant of P. andrewsi that possesses only the rRNA-B gene unit. Similar observations have been reported for Trypanosoma cruzi, where isolates have been identified that possess either 2 distinct rRNA gene units (rRNA unit 1 and 2), or one of the 2 rRNA gene units (Souto et al., 1996; Zingales et al., 1999; Stolf et al., 2003).

Application of the genus-specific PCR-based assay to both C. virginica and M. mercenaria specimens resulted in the detection of Perkinsus infections that could not be attributed to P. marinus, P. andrewsi, or Perkinsus sp. (M. mercenaria) by the species-specific PCR assays. However, sequence analysis of selected amplicons generated by the genus-specific PCR failed to reveal novel sequences, which could suggest the presence of yet-undescribed Perkinsus species or strains. Further, it rather suggested that the density of Perkinsus infections in these specimens was below the detection limit of the species-specific assays, but high enough to be detected by the genus-specific assay.

Although Perkinsus species appear to lack strict host specificity, they may have adapted best to the hostile environment of one particular host species. Studies on the effects of plasma of different mollusc species on the in vitro proliferation of P. marinus show that parasite growth is reduced by plasma or sera from bivalve molluscs (Andara ovalis, Geukensia demissa, M. mercenaria, and Mytilus edulis) that are naturally exposed to the parasite as compared to plasma or sera from C. virginica (Anderson, 2001; Gauthier and Vasta, 2002). These observations suggest a preference of P. marinus for its type host C. virginica. Results from our study provide further evidence for a possible host preference of P. marinus. Although P. marinus was detected in M. mercenaria and C. virginica, the prevalence in M. mercenaria was significantly lower. Further studies aimed at elucidating the molecular mechanisms behind this host preference are ongoing.
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LITERATURE CITED


