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Article *in* Journal of Parasitology · May 2008

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J. Parasitol., 94(2), 2008, pp. 410–422

ASSESSMENT OF THE NORTHERN DISTRIBUTION RANGE OF SELECTED *PERKINSUS* 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 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*.

Perkinsus species (Perkinsozoa, Alveolata) are the causative agent of perkinsosis 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, 1988; Ford, 1996; Villalba et al., 2004). Currently, 3 *Perkinsus* species are recognized along the Atlantic coast of the United States, i.e., *P. marinus*, isolated from the eastern oyster *C. virginica* (Mackin et al., 1950), *P. chesapeaki* from the soft shell clam *Mya arenaria* (McLaughlin et al., 2000), and *P. andrewsi* from the Baltic clam *Macoma balthica* (Coss, Robledo, and Vasta, 2001). In addition, various *Perkinsus* isolates have been reported, including an isolate from the hard clam *M. mercenaria* [hereafter referred to as *Perkinsus* sp. (*M. mercenaria*)] that appears to be closely related to *P. andrewsi* (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 (Burreson et al., 2005), because of the limited evidence available at present time, we consider *P. andrewsi* as a distinct *Perkinsus* species in the present study.

The standard diagnostic method for *Perkinsus* spp. infections has been the fluid thioglycollate medium (FTM) assay (Ray, 1952, 1966), which is considered to be more sensitive than histological diagnosis (McLaughlin and Faisal, 1999). However, neither method is able to discriminate among *Perkinsus* species (reviewed in Villalba et al., 2004); diagnostic assays based on anti-*Perkinsus* sp. antibodies (Choi et al., 1991; Dungan and Robertson, 1993; Ottinger et al., 2001; Montes et al., 2002) have not been rigorously validated, and may exhibit cross-reactivity with dinoflagellates (Dungan et al., 1993; Bushek et al., 2002; Villalba et al., 2004).

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

Received 2 April 2007; revised 8 August 2007; accepted 6 August 2007.

* L. Roth and S. T. Berg participated in the project as student interns through the Technology Magnet Program, Howard County Public School System, Ellicott City, Maryland, and the Ingenuity Research Practicum, Baltimore Polytechnic Institute, Baltimore, Maryland.

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 *Perkinsus* species isolated from *Mercenaria mercenaria*

Primers designated M6L (sense, 5′-GCGGCGCAAATTCATCACT TG AG-3′) and M5 (antisense, 5′-AACCATCCCGACTACCATCT 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 mU/μl), KCl, Tris-Cl, $(NH_4)_2SO_4$, 1.5 mM MgCl, 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 mU/μl) (TaKaRa Bio, Inc., Otsu, Shiga, Japan), 1× of the proprietary Ex Taq reaction buffer (contains 2 mM $MgCl_2$), 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 sensitivity 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 H_2O.

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 H_2O.

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 the use of the PCR mixtures A, B, and C. PCR amplification controls consisted of 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 AY305326), *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

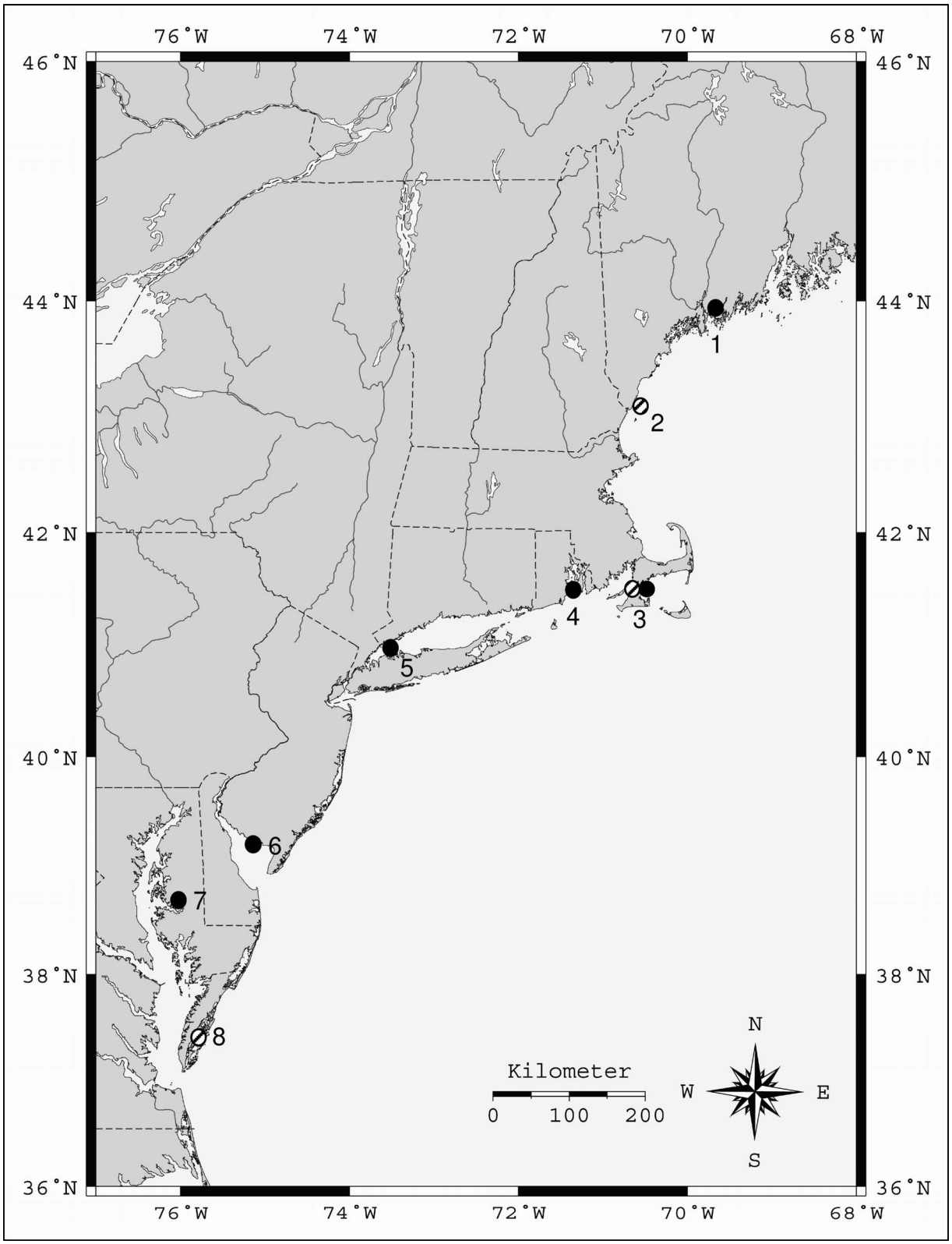

FIGURE 1. Sample site locations. *Crassostrea virginica* (●) and *Mercenaria mercenaria* (⊘) specimens were received from shellfish providers each month from June 2002 to September 2002. 1: Walpole (Maine); 2: Eliot (Maine); 3: Martha's Vineyard (Massachusetts); 4: Narragansett Bay (Rhode Island); 5: 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).

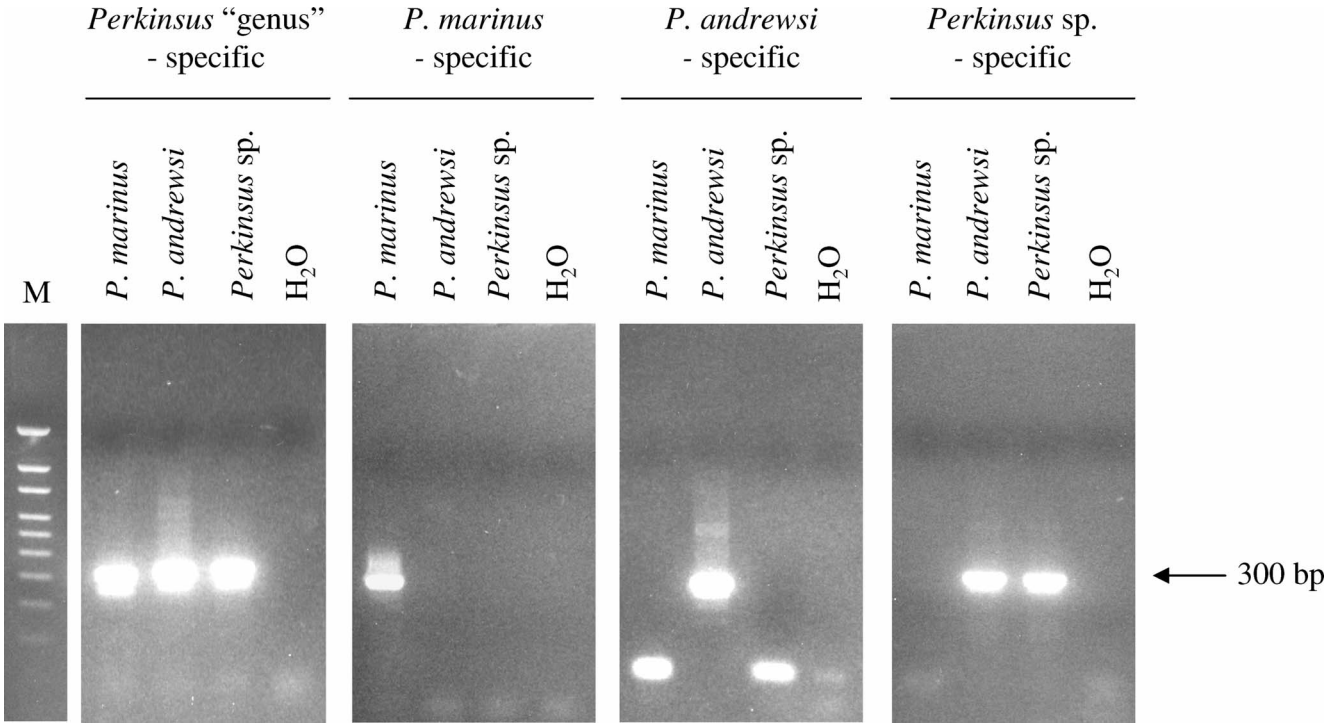

FIGURE 2. Specificity of the PCR-based assays. Fifty nanograms of genomic DNA from *Perkinsus marinus*, *Perkinsus andrewsi*, and *Perkinsus* sp. (*Mercenaria mercenaria*) were tested with PCR-based assays specific for the *Perkinsus* genus, *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) with the use of the PCR reaction mixtures A. *Perkinsus* sp.: *Perkinsus* sp. (*M. mercenaria*); H_2O: negative control.

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 χ^2 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

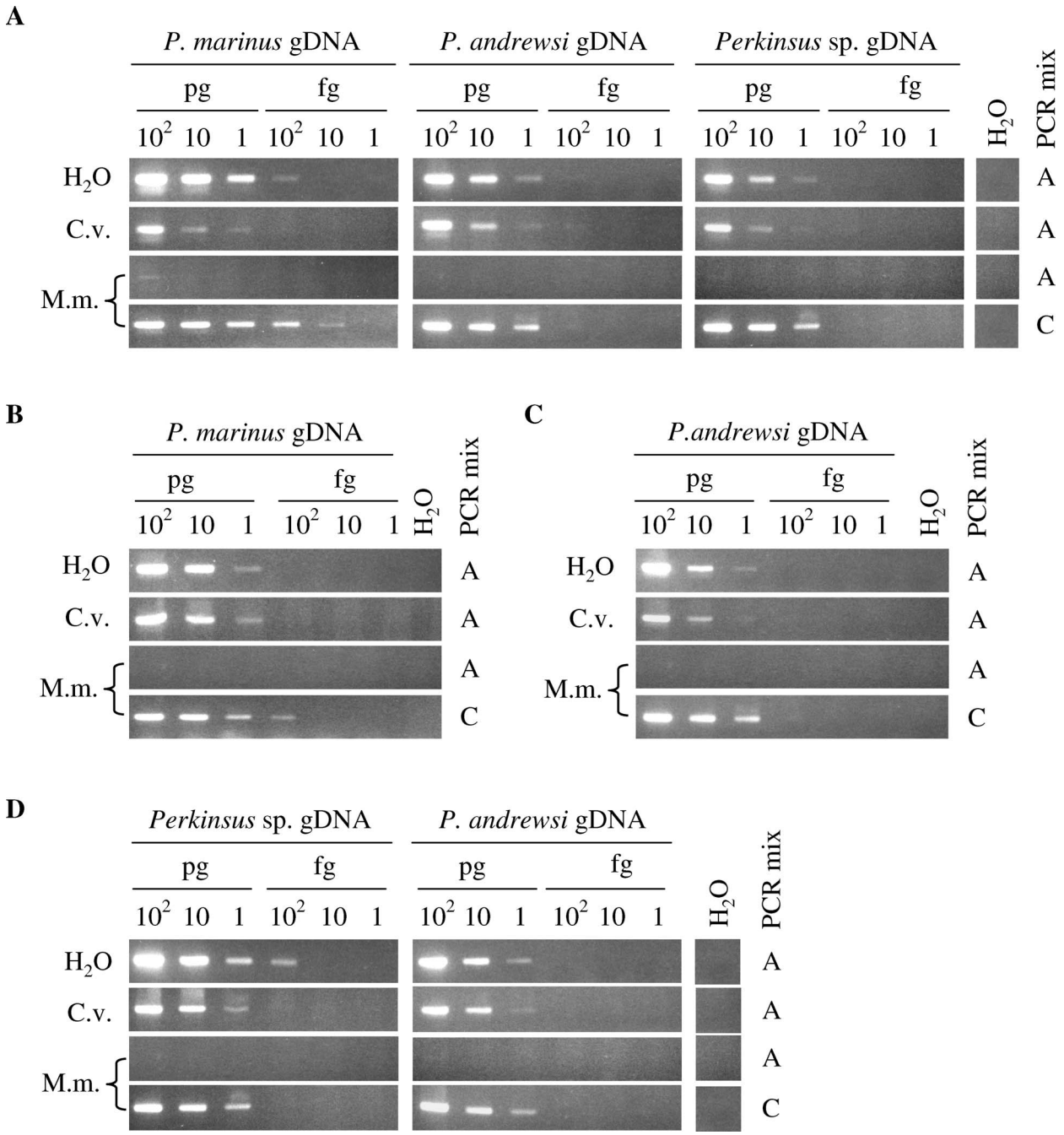

FIGURE 3. Sensitivity of the PCR-based assays with the use of standard PCR reaction conditions (PCR reaction mixture A) and PCR reaction conditions optimized for *Mercenaria mercenaria* (PCR reaction mixture C). Decreasing amounts of genomic DNA (100 pg to 1 fg) from *Perkinsus marinus*, *Perkinsus andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) were tested with the respective PCR-based assays using the PCR reaction mixture A. Assays were performed in the absence or presence of 500 ng *Crassostrea virginica* (C.v.) or *M. mercenaria* (M.m.) genomic DNA. (**A**) Sensitivity of the genus-specific assay. (**B**) Sensitivity of the *P. marinus*–specific assay. (**C**) Sensitivity of the *P. andrewsi*–specific assay. (**D**) Sensitivity of the *Perkinsus* sp. (*M. mercenaria*)–specific assay. C.v.: *C. virginica*; M.m.: *M. mercenaria*; *Perkinsus* sp.: *Perkinsus* sp. (*M. mercenaria*); H_2O: negative control; PCR mix: PCR reaction mixture.

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. mercencaria* 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-

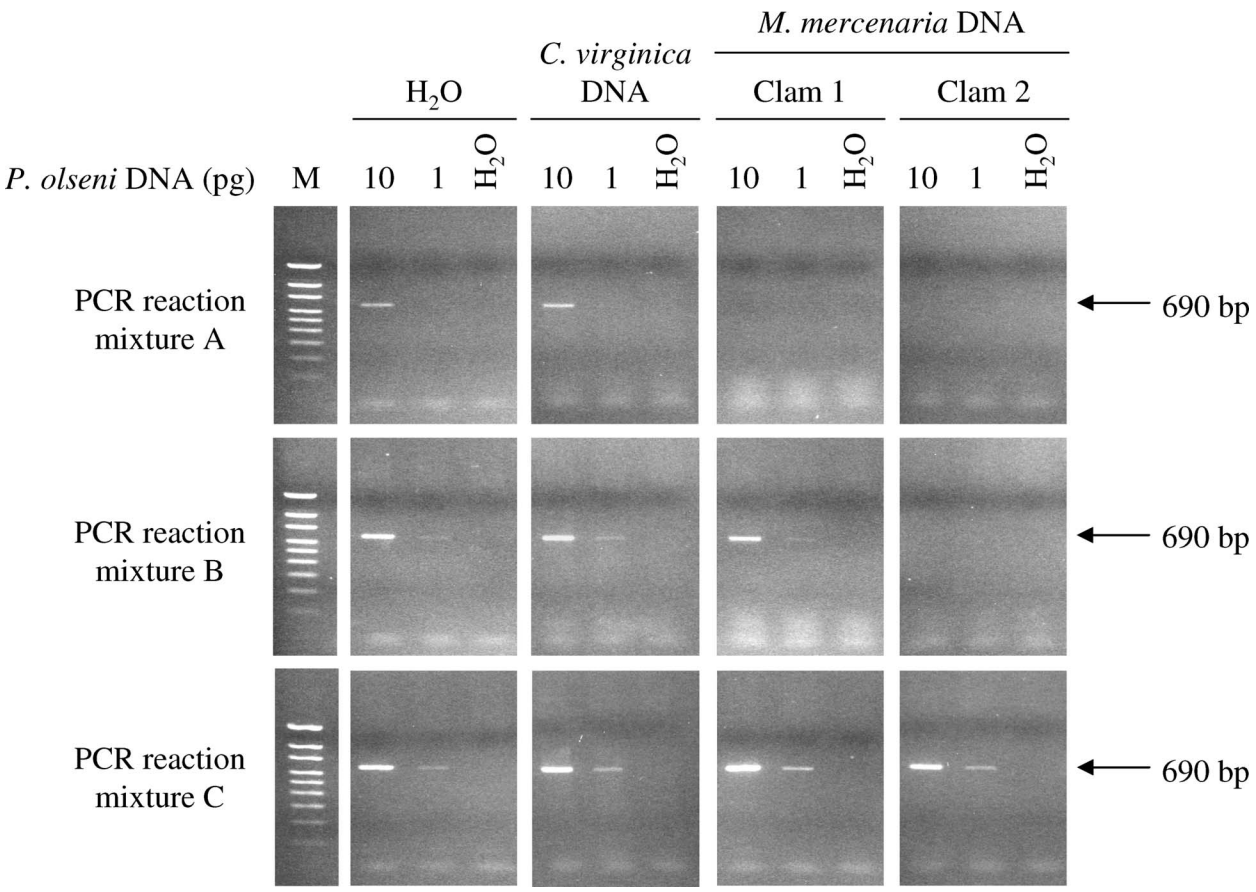

FIGURE 4. Attenuation of PCR amplification inhibition. Five hundred nanograms genomic DNA from 1 individual of *Crassostrea virginica* and 2 *Mercenaria mercenaria* specimens were spiked with 10 pg and 1 pg *Perkinsus olseni* genomic DNA. Samples were tested with a *P. olseni*–specific PCR-based assay with the use of PCR reaction mixtures A, B, and C. In the positive control, host DNA was omitted. H_2O: negative control.

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, $P \geq 0.237$) during the sampling period. In Martha's Vineyard, prevalences were high (86.7–100%; Fisher's exact test, $P \geq 0.173$) throughout

TABLE I. Percent prevalence of *Perkinsus* species infections in *Crassostrea virginica* collected from June 2002 to September 2002. 1: Walpole, 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; N: number of individuals; n: number of infected individuals; (%): prevalence in percent; P. m.: *Perkinsus marinus*; P. a.: *Perkinsus andrewsi*; P. sp.: *Perkinsus* sp. (*Mercenaria mercenaria*); P. spp.: *Perkinsus* infections detected with the generic PCR-based assay.

		P. spp.		P. m.		P. a.		P. sp.		P. m. and P. a.		P. m. and P. sp.		P. spp. only	
Site	N	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
1	116	12	10.3	1	0.9	3	2.6	0	0.0	0	0.0	0	0.0	8	6.9
3	180	173	96.1	173	96.1	2	1.1	7	3.9	2	1.1	7	3.9	0	0.0
4	112	81	72.3	64	57.1	0	0.0	0	0.0	0	0.0	0	0.0	17	15.2
5	99	30	30.3	18	18.2	0	0.0	0	0.0	0	0.0	0	0.0	12	12.1
6	111	98	88.3	84	75.7	4	3.6	2	1.8	4	3.6	2	1.8	14	12.6
7	57	55	96.5	54	94.7	0	0.0	6	10.5	0	0.0	6	10.5	1	1.8
All	675	449	66.5	394	58.4	9	1.3	15	2.2	6	0.9	15	2.2	52	7.7

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 (χ^2 test, $P < 0.001$), increasing from north (Elliot) 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-*

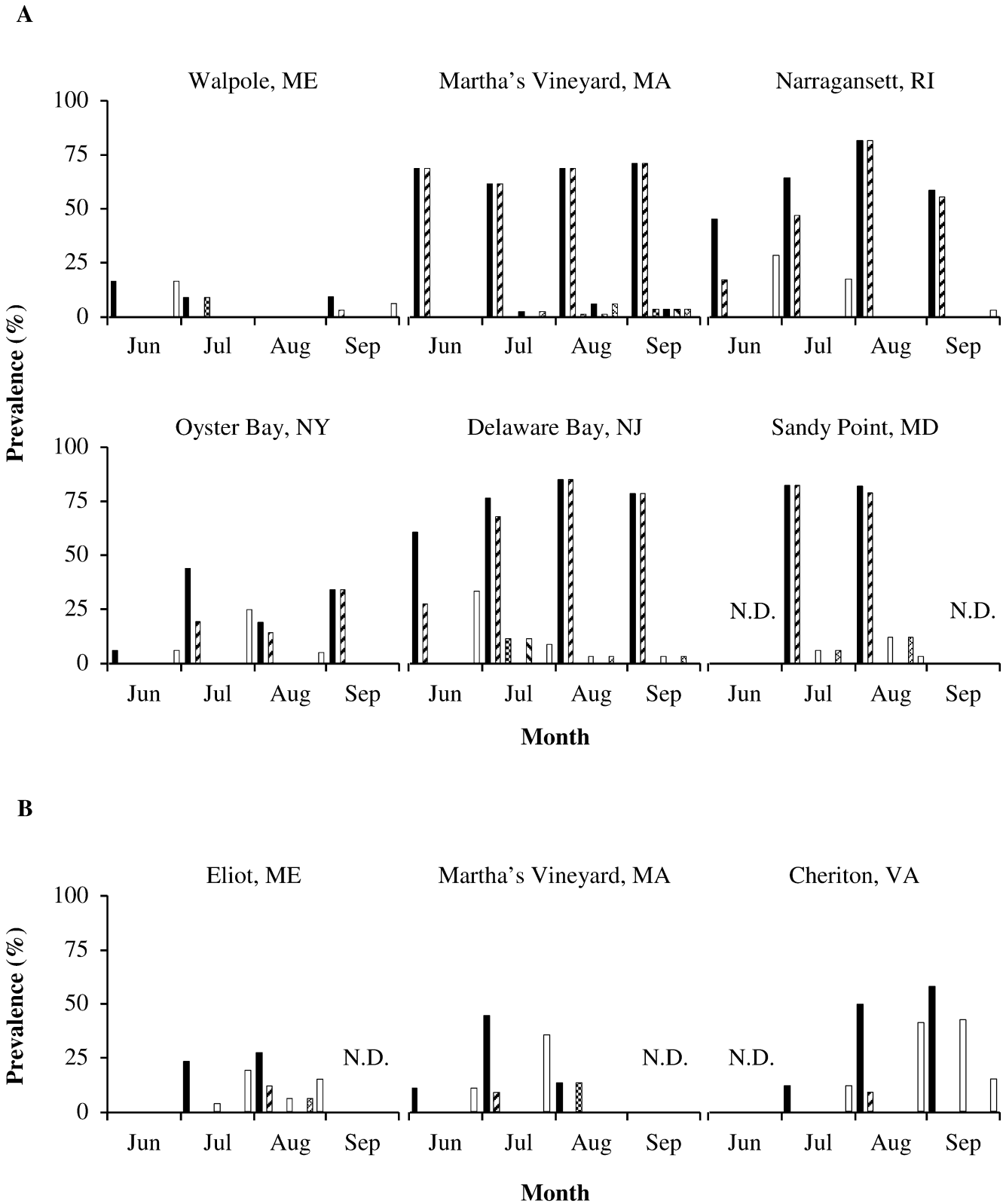

FIGURE 5. Monthly *Perkinsus* infection prevalence in *Crassostrea virginica* and *Mercenaria mercenaria*. Percent prevalences of *Perkinsus* species (detected by the genus-specific assay) (■), *Perkinsus marinus* (▨), *Perkinsus andrewsi* (▩), *Perkinsus* sp. (*M. mercenaria*) (⬚), dual infections with *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.

		P. spp.		P. m.		P. a.		P. sp.		P. m. and P. a.		P. m. and P. sp.		P. spp. only	
Site	N	N	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
2	79	15	19.0	4	5.1	0	0.0	3	3.8	0	0.0	2	2.5	10	12.7
3	61	16	26.2	2	3.3	3	4.9	0	0.0	0	0.0	0	0.0	11	18.0
8	85	41	48.2	4	4.7	0	0.0	14	16.5	0	0.0	0	0.0	23	27.1
All	225	72	32.0	10	4.4	3	1.3	17	7.6	0	0.0	2	0.9	44	19.6

kinsus sp. (*M. mercenaria*) was not detected (Fig. 6; Tables I, II). Differences in prevalence between *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, $P = 0.208$).

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 influx from major rivers and precipitation. Thus, to compare prevalences to an earlier study on the distribution of *P. marinus*, and 2 *Haplosporidium* species in *C. virginica* collected in 2000 (Russell et al., 2004), temperature and precipitation data were obtained for May to August 2000 and 2002, from Mineola (New York) (COOP ID 305377), a weather station near Oyster Bay (New York). Mean monthly air temperatures in 2000 were 16.9, 20.4, 21.2, and 21.9 C in May, June, July, and August, respectively. Monthly precipitation was 108.2, 111.3, 137.7, and 61.2 mm over the same time period, respectively. In 2002, mean monthly temperatures were 15.2, 21.0, 25.9, and 24.8 C from May to August. Monthly precipitation (May–August) was 93.0, 95.8, 22.9, and 183.9 mm.

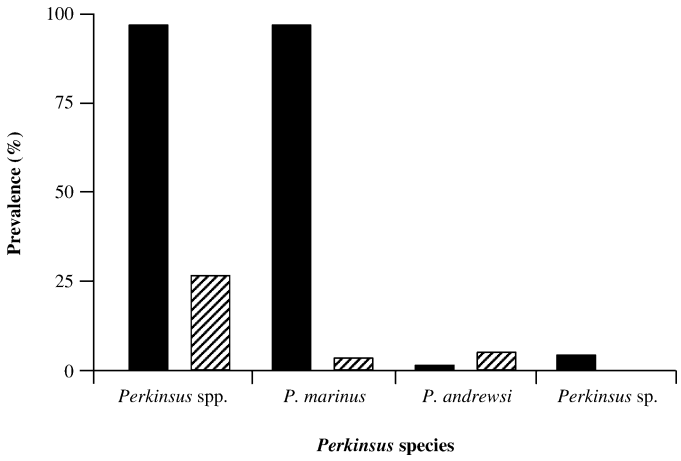

FIGURE 6. Comparison of the *Perkinsus* infection prevalence in *Crassostrea virginica* and *Mercenaria mercenaria*. Percent prevalences of *Perkinsus* species, *Perkinsus marinus*, *Perkinsus andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) in *C. virginica* (■) and *M. mercenaria* (▨) collected from June 2002 to September 2002 in Martha's Vineyard, Massachusetts are shown. *Perkinsus* sp.: *Perkinsus* sp. (*M. mercenaria*).

DISCUSSION

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.*

mercenaria [*Perkinsus* sp. (*M. mercenaria*)]. The species-specific assays are based on sequence differences within the intergenic spacer (IGS) of the rRNA gene locus of *Perkinsus* species that links the 5S and SSU genes, whereas the genus-specific assay is based on a conserved region at the 3′ end of the IGS (Marsh et al., 1995; Coss, Robledo, Ruiz, and Vasta, 2001; Robledo et al., 2002). As expected, the genus-specific assay detected all *Perkinsus* species tested in this study, including *Perkinus* sp. (*M. mercenaria*). *Perkinsus* sp. (*M. mercenaria*) was not detected by the *P. marinus*–specific or the *P. andrewsi*–specific assays, demonstrating the capacity of the genus-specific *Perkinus* assay to detect new *Perkinsus* species or strains.

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 sensitivity 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 of 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 pathogenicities, 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* hapantotype to the *Perkinsus* sp. isolated from *M. mercenaria* (W. T. Pecher and G. R. Vasta, unpubl. obs.), the *P. andrewsi* hapantotype 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.

ACKNOWLEDGMENTS

This study was supported by grants 556804 from NRAC, USDA, and SA7528035-D and SA7528068-H from the Maryland Sea Grant, NOAA. The authors are indebted to Gary Arnold (Mook Sea Farm Inc., Walpole, Maine), Kenneth LaValley (Spinney Creek Shellfish Inc., Eliot, Maine), Richard C. Karney (Martha's Vineyard Shellfish Group, Oaks Bluff, Massachusetts), Robert B. Rheault (Moonstone Oysters, Wakefield, Rhode Island), Braun Seafood Co. (Cutchogue, New York), Susan E. Ford (Rutgers University, Haskins Shellfish Research Laboratory, Port Norris, New Jersey), Donald W. Merritt (University of Maryland Center for Environmental Science, Horn Point Laboratory, Cambridge, Maryland), and Mike Peirson (Cherrystone Aquafarms, Cheriton, Virginia) for providing oysters (*C. virginica*) and clams (*M. mercenaria*), and to Harmony A. Hancock for assistance with shellfish dissection.

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