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Description of *Perkinsus andrewsi* n. sp. Isolated from the Baltic Clam (*Macoma balthica*) by Characterization of the Ribosomal RNA Locus, and Development of a Species-Specific PCR-Based Diagnostic Assay

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ABSTRACT. A *Perkinsus* species was isolated from the baltic clam *Macoma balthica* and an in vitro culture established under conditions described for *P. marinus.* As reported previously, morphological features remarkable enough to clearly indicate that this isolate is a distinct *Perkinsus* species were lacking. In this study, regions of the rRNA locus (NTS, 18S, ITS1, 5.8S, and ITS2) of this isolate were cloned, sequenced, and compared by alignment with those available for other *Perkinsus* species and isolates. Sequence data from the rRNA locus and species-specific PCR assays indicated not only that *Perkinsus* sp. from *M. balthica* was not *P. marinus,* but it was different from *P. atlanticus* and *P. olseni.* The degree of difference was comparable to or greater than differences between accepted *Perkinsus* species. In particular, NTS sequence and length were dramatically different from that of *P. marinus* and *P. atlanticus.* Therefore, we formally propose to designate the *Perkinsus* sp. from *M. balthica* as a separate species, *P. andrewsi* n. sp. Primers based on *P. andrewsi* NTS sequence were used to develop a PCR-based diagnostic assay that was validated for species-specificity and sensitivity. PCR-based assays specific for either *P. andrewsi* or *P. marinus* were used to test for their presence in bivalve species sympatric to *M. balthica.* Although isolated from *M. balthica, P. andrewsi* was also detected in the oyster *Crassostrea virginica* and clams *Macoma mitchelli* and *Mercenaria mercenaria,* and could coexist with *P. marinus* in all four bivalve species tested.

Key Words. *Crassostrea virginica,* diagnostic assay, ITS, *Mercenaria mercenaria,* NTS, PCR, SSU.

PERKINSUS marinus was initially described as a fungus (*Dermocystidium marinum)* (Mackin, Owen, and Collier 1950), later classified in the phylum Apicomplexa (Levine 1978). Most recently it was characterized as more closely related to the dinoflagellates (Fong et al. 1993; Goggin and Barker 1993; Reece et al. 1997; Siddall et al. 1997). However, based on ultrastructural features and phylogenetic studies of a new species, *Parvilucifera infectans,* and a critical review of the pertinent literature, Norén, Moestrup, and Rehnstam-Holm (1999) created the new phylum Perkinsozoa that includes *Perkinsus* species, and bridges the Dinoflagellata and Apicomplexa. *Perkinsus marinus* is a principal cause of mortality of the eastern oyster, *Crassostrea virginica,* along the Gulf of Mexico and Atlantic coast of North America (Burreson and Ragone-Calvo 1996; Ford 1996; Soniat 1996). Additional *Perkinsus* species have been identified, including *P. olseni* in Australia (Lester and Davis 1981; Lester, Goggin, and Sewell 1990), *P. atlanticus* in Europe (Azevedo 1989), and most recently *P. qugwadi* on the west coast of Canada (Blackbourn, Bower, and Meyer 1998). Based on the fluid thioglycollate medium assay (FTM) and/or microscopical examination, numerous presumptive *Perkinsus* species have been identified in various mollusk species around the world (Andrews 1954; Choi and Park 1997; Goggin and Lester 1987; Kleinschuster et al. 1994, Cigarría, Rodríguez, and Fernández 1997; Choi and Park 1997; Hamaguchi et al. 1998; McLaughlin and Faisal 1998), but detailed ultrastructural studies of most of these parasites are lacking. Initially developed for the detection of *P. marinus,* the FTM assay (Ray 1952, 1966) relies on the ability of the trophozoite to enlarge and mature into the hypnospore stage after 5–7 d of incubation in FTM, and stain with Lugol's iodine solution. Although this simple and inexpensive method has been routinely applied for the detection of *Perkinsus* species, its specificity for the genus *Perkinsus* has not been rigorously examined. Because enlargement in FTM and staining with Lugol's have been considered features common to all *Perkinsus* species described to date except *P. qugwadi* (Blackbourn, Bower, and Meyer 1998), the assay is not suitable for species identification.

The in vitro culture of *P. marinus* (Gauthier and Vasta 1993, 1995; Kleinschuster and Swink 1993; La Peyre, Faisal, and Burreson 1993) has facilitated the application of molecular technologies to the basic questions of detection, enumeration, physiology, and phylogeny of this parasite (Gauthier and Vasta 1994; Marsh, Gauthier, and Vasta 1995; Robledo et al. 1998; Robledo, Coss, and Vasta 2001). Ribosomal RNA sequence information has been applied to develop molecular diagnostic probes and to support further *Perkinsus* species designations (Fong et al 1993; Goggin 1994; Marsh, Gauthier, and Vasta 1995; Robledo et al. 1998, Robledo, Coss, and Vasta 2001). Based on similar criteria used to distinguish *Tetrahymena* species by comparing their small subunit (SSU) sequences (Sogin et al. 1986), Fong et al. (1993) suggested that *P. marinus* and a *Perkinsus* species from the blood cockle, *Anadara trapezia,* may constitute different species. The ITS (internal transcribed spacer) sequence of the rRNA genes has also been used as a reliable characteristic to distinguish between species of morphologically similar fungi and animals (Goodwin et al. 1995; Lee and Taylor 1992; Paul 2000; Porter and Collins 1991; Shaw 2000; Zahler et al. 1997). Goggin (1994) compared sequences of the ITS region from three currently accepted *Perkinsus* species and two *Perkinsus* isolates from Australia and concluded that *P. marinus* was clearly distinct from *P. atlanticus,* however, *P. atlanticus, P. olseni,* and the isolates from Australia may actually belong to a single species. Based on the rRNA locus, two different *Perkinsus* spp., one resembling *P. marinus* and the other described as a novel isolate (G117), have been reported in the clam, *Mya arenaria* (McLaughlin and Faisal, 1998).

In addition to *P. marinus, Perkinsus*-like organisms of unknown virulence have been detected in bivalve species sympatric with *C. virginica* (Andrews 1954). For example, production of zoospores in seawater without preincubation in FTM (Kleinschuster and Swink 1993; Perkins 1988), morphological features of the trophozoite in host tissue (Perkins 1988), and partial sequences of the SSU and ITS regions reported earlier (Coss et al. 1997) indicated that *Perkinsus* isolates from the baltic clam *Macoma balthica* may be a different species from *P. marinus.* The identity and host specificity of the various *Perkinsus* species described in sympatric mollusk species in Chesapeake Bay, and the possibility that these may constitute alternate hosts or reservoir species for *P. marinus* has received limited attention (Coss et al. 1997; McLaughlin and Faisal 1998; Coss, Robledo, and Vasta 2001). Accordingly, questions about the presence of *P. marinus* in non-oyster bivalves, as well as

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other *Perkinsus* species infecting *C. virginica,* are addressed in this study.

The study reported herein was designed to: (1) characterize the rRNA locus of *Perkinsus* sp. from *M. balthica,* hereafter referred to as *Perkinsus andrewsi* n. sp, for comparison with rRNA sequences reported for known *Perkinsus* species; (2) determine the range of intraspecific variability in regions of the rRNA of *P. andrewsi* n. sp.; (3) develop and validate a PCR-based assay based on the NTS (non-transcribed spacer) sequence for diagnostic of *P. andrewsi* n. sp.; (4) examine field samples of clams (*M. balthica, M. mitchelli,* and *M. mercenaria*) and oysters (*C. virginica*) for the presence of *P. andrewsi* n. sp. using the specific PCR assay; and (5) assess the presence of *P. marinus,* by use of a specific PCR-based assay (Marsh, Gauthier, and Vasta 1995; Robledo et al. 1998) in sympatric clams and oysters. This study complements the ultrastructural characterization of this in vitro clonally-propagated *Perkinsus* species from *M. balthica* and its comparison to the previously described *Perkinsus* species (Coss, Robledo, and Vasta 2001).

MATERIALS AND METHODS

DNA extraction. DNA from in vitro propagated *P. andrewsi* n. sp. and *P. marinus* (Gauthier and Vasta 1993), and *P. atlanticus* zoosporangia and zoospores was extracted using the QIAamp tissue kit (QIAGEN, Valencia, California) following the manufacturer's instructions. DNA used in the ''spike/recovery'' experiments (see below) was extracted from *M. balthica* and *C. virginica* tissues using the same methodology.

PCR amplification, cloning, and sequencing. The NTS, SSU, and ITS fragments were amplified following Robledo et al. (1999), Medlin et al. (1988), and Goggin (1994), respectively. At least three clones of each type from two amplification reactions for each region (NTS, SSU, and ITS1–5.8S-ITS2) were cloned, sequenced as reported elsewhere (Robledo et al. 1999), and deposited in GenBank™ (AF102171).

SSU rRNA sequences for comparison were obtained from GenBank™ including *P. marinus* (X75762), *P. atlanticus* (AF140295), *Perkinsus* sp. from *A. trapezia* (L07375), and *Perkinsus* sp. (G117) from *M. arenaria* (AF042707F) for comparison, rRNA ITS sequences were obtained from GenBank™ for *P. marinus, P. olseni, P. atlanticus, P. qugwadi,* and *Perkinsus* sp. (G117) from *Mya arenaria* (U07700, U07701, U07697, AF151528, and AF091541). Sequences were aligned using the ''pileup'' program of the Wisconsin GCG package (GCG, Madison, Wisconsin), and realignments were made by eye.

Assessment of intraspecific variability in rRNA of *P. andrewsi* n. sp. DNA from *M. balthica* (n = 10) collected from Chesapeake Bay (Maryland) and from *C. virginica* from Maryland (n = 5) and Maine (n = 5) infected with *P. andrewsi* n. sp. was used for assessment of intraspecific variability in selected regions of the rRNA. A 3.8-kb fragment comprised of part of the NTS, and the complete sequence of the SSU, ITS1–5.8S-ITS2 was generated using primer NTS 7, specific for *P. andrewsi* n. sp. (see below) and the ITSD primer of Goggin (1994). This fragment was used as a template for nested PCR with primers for the NTS [NTS 6/ NTS 7 (see below), expected product size 290-bp], SSU (UPRA/ UPRB, Medlin et al. 1988, expected product size 1808-bp), and ITS (ITSA/ ITSD, Goggin 1994, expected product size 775-bp). These PCR products were used for direct sequence in both directions as reported elsewhere (Robledo et al. 1999). The SSU product was sequenced using additional internal primers SSU3F (Sense−5′-AGT TGG ATT TCT GCC TTG GGC G-3′-) and SSU4F (Sense−5′-ACC AGG TCC AGA CAT AGG AAG G-3′-).

Development of a PCR-based diagnostic assay specific for *P. andrewsi* n. sp. Primers designated NTS 7 (Sense−5′-AAG TCG AAT TGG AGG CGT GGT GAC-3′-) and NTS 6 (Antisense−5′-ATT GTG TAA CCA CCC CAG GC-3′-) were designed based on the NTS sequence using the GeneJockey II program (Biosoft, Cambridge, UK) and amplifed a 290-bp fragment from *P. andrewsi* n. sp. DNA. The PCR reaction conditions were 3 min at 94 °C, 35 cycles of 94 °C for 1 min, 60 °C for 30 s, and 72 °C for 90 s, with a final extension at 72 °C for 7 min in PCR reaction mixtures as reported elsewhere (Robledo et al. 1998). Positive controls consisted of similar reaction mixtures, using 50 ng of DNA purified from cultured *P. andrewsi* n. sp. as template. In negative controls, template DNA was substituted by autoclaved Milli-Q-filtered water.

Assessment of specificity and sensitivity of the PCR assay. Assay specificity was assessed on 50 ng of DNA from *P. marinus, P. atlanticus,* and *P. andrewsi* n. sp. Assay sensitivity was determined directly by PCR on decreasing DNA quantities (10 ng to 10 ag) of *P. andrewsi* n. sp., or in a spike/recovery format using decreasing quantities (10 ng to 10 ag) of *P. andrewsi* n. sp. DNA mixed with a constant amount (1 μg) of *M. balthica* DNA or *C. virginica* DNA negative for *P. andrewsi* n. sp. The PCR reaction mixtures and conditions were as above.

PCR assays on clam and oyster samples. *Macoma balthica* (n = 21) and *M. mitchelli* (n = 10) clams were collected from the Rhode River, Maryland. *Mercenaria mercenaria* clams were obtained from Indian River, Delaware (n = 5), Assateague Island, Maryland (n = 1), and from a local (Chesapeake Bay) seafood market (n = 3). *Crassostrea virginica* were obtained from Severn River, Maryland (n = 21), James River, Virginia (n = 9), Damariscotta River, Maine (Mook Sea Farms, Damariscotta, Maine) (n = 20), and from various sites around the Chesapeake Bay (n = 75). The DNA from these bivalves was extracted as described above, and 1 μg of DNA was tested with the PCR-based assays for *P. andrewsi* n. sp. and *P. marinus* (Marsh, Gauthier, and Vasta 1995; Robledo et al. 1998).

RESULTS

Sequence of the rRNA locus. PCR amplification of DNA from *P. andrewsi* n. sp. using primers based on conserved regions of 5S and SSU resulted in a single 1672-bp amplicon. Sequence analysis of several clones revealed the sequences of the 5S and SSU rRNA genes present at the 3′- and 5′- ends of the amplified fragment, confirming the amplification of the NTS region of the rRNA cluster of *P. andrewsi* n. sp. The NTS sequence of *P. andrewsi* n. sp. was 1545-bp long and contained a repeat element arranged in tandem (-5′-GCA ATT TTC GMA ATS Y-3′-) (Fig. 1). The NTS sequence of *P. andrewsi* n. sp. had 35.7% and 34.5% identity to *P. marinus* and *P. atlanticus,* respectively.

The 1804-bp SSU sequence from *P. andrewsi* n. sp. showed very high (97.5%–99.1%) sequence identity to that from *P. marinus* and *P. atlanticus* (Table 1A). Most of the sequence differences between it and *P. marinus* occurred in regions where the sequence was identical to that of *P. atlanticus* and the isolate from *A. trapezia.*

The 5.8S region of *P. andrewsi* n. sp. was 159 bases long, identical to that of *Perkinsus* sp. (G117) from *Mya arenaria,* differed in two positions from the sequences of *P. marinus, P. olseni,* and *P. atlanticus* (Fig. 2), and differed in 14 positions from the sequence for *P. qugwadi* (Fig. 2). The ITS1 region of *P. andrewsi* n. sp. was 185 bases long. Comparison of the ITS1 region (Table 1 B) among *Perkinsus* species and isolates showed that *P. andrewsi* n. sp. shared highest sequence identity to *Perkinsus* sp. (G117) from *Mya arenaria,* while the sequences of *P. atlanticus* and *P. olseni,* in this region were identical. The ITS2 sequence of *P. andrewsi* n. sp. was 369 bases long and had a similar level of high sequence identity to *Perkinsus*

Fig. **1**. Sequence of the NTS region (rRNA locus) from *Perkinsus andrewsi* n. sp. Underlined nucleotides indicate a repeat sequence motif. PCR primers for *Perkinsus* NTS amplification (NTS 1 and NTS 2) and for specific diagnosis of *Perkinsus andrewsi* n. sp. (NTS 7 and NTS 6) are indicated with solid arrows below the nucleotide sequence. Underlined Y or R: variable positions.

Table **1**. Percent identity of rDNA sequences among *Perkinsus* species and isolates.

A

	% Identity of SSU			
	P. marinus	*P. atlanticus*	*Perkinsus* sp. (*A. trapezia*)	*Perkinsus* sp. G117
P. andrewsi n. sp.	97.5	99.1	98.9	99.2
P. marinus		98	97.7	97.1
P. atlanticus			99.6	98.7
Perkinsus sp. (*A. trapezia*)				98.7

B

	% Identity of ITS1					
	P. marinus	*P. atlanticus*	*P. olseni*	*Perkinsus* sp. (*A. trapezia*)	*P. qugwadi*	*Perkinsus* sp. G117
P. andrewsi n. sp.	70.2	85.6	85.6	85.6	39.9	95.7
P. marinus		76.6	76.6	76.6	38.2	71.6
P. atlanticus			100	100	38.7	87.8
P. olseni				100	38.7	87.8
Perkinsus sp. (*A. trapezia*)					38.7	87.8
P. qugwadi						36.8

C

	% Identity of ITS2					
	P. marinus	*P. atlanticus*	*P. olseni*	*Perkinsus* sp. (*A. trapezia*)	*P. qugwadi*	*Perkinsus* sp. G117
P. andrewsi n. sp.	78.3	78.4	78.7	78.4	31.8	92.1
P. marinus		92.3	92.5	92.3	40	78.3
P. altanticus			99.7	99.5	40.7	76.8
P. olseni				99.7	40.4	77
Perkinsus sp. (*A. trapezia*)					40.4	77.4
P. qugwadi						37.7

sp. (G117) from *Mya arenaria* as is found between *P. marinus* and *P. atlanticus* or *P. olseni* (Table 1 C).

Intraspecific variation in regions of rRNA of *P. andrewsi* n. sp. Sequence of the ITS1–5.8S-ITS2 and the SSU regions of *P. andrewsi* n. sp. from infected oyster (n = 10) or clam (n = 10) samples revealed no variability. Sequence of the NTS amplicons, however, showed that there were base substitutions at two locations in the 290 bp indicating the presence of three putative sequence types (Fig. 1). Of the NTS amplicons sequenced from infected oyster (n = 10) and clam (n = 10) samples, 10 had bases T and A, 8 had bases C and A, and 2 had bases C and G in positions 514 and 573, respectively. In amplicons from both oysters and clams, all three sequence types were represented.

Species-specificity of the PCR-based assay for *P. andrewsi* n. sp. To test the species-specificity of the PCR-based assay for *P. andrewsi* n. sp., we performed the diagnostic test on DNA from *P. andrewsi* n. sp., *P. marinus,* and *P. atlanticus.* Only *P. andrewsi* n. sp. yielded the expected 290-bp amplicon **(**Fig. 3A, 3B). In addition, DNA from *P. andrewsi* n. sp. was not amplified using the *P. marinus* specific primers (Fig. 3C).

Sensitivity of the PCR-based assay for *Perkinsus* sp. (*M.b.*) using spike/recovery. The lowest limit of detection of the PCR-based assay for *P. andrewsi* n. sp. DNA, as determined EtBr-stained amplicons resolved by electrophoresis, was 100 fg of DNA (Fig. 4A). As assessed by spike/recovery experiments, the presence of host (*M. balthica*) DNA in the reaction mixture did not affect the specificity of amplification (Fig. 4B) but only slightly modified (1 pg of *C. virginica* DNA) the sensitivity of the PCR assay (data not shown).

Presence of *P. andrewsi* n. sp. and *P. marinus* in sympatric bivalve species. The presence of *P. andrewsi* n. sp. and *P. marinus* in bivalve species (*M. mitchelli, C. virginica,* and *M. mercenaria*) sympatric with the baltic clam was tested by applying the specific PCR-based diagnostic assays. *P. andrewsi* n. sp. and *P. marinus* were detected in all four bivalve species examined, even simultaneously in many individuals (Table 2, Fig. 5).

DISCUSSION

The isolation and establishment of an in vitro culture of a *Perkinsus* species from the baltic clam *M. balthica,* a study of the gross morphology and fine structure reported elsewhere (Coss, Robledo, and Vasta 2001), and the characterization of its rRNA locus described in the present study, have enabled a comparative analysis of morphological and molecular aspects of this isolate with those of other accepted species, *P. marinus, P. atlanticus, P. olseni,* and *P. qugwadi.* A preliminary screening for *P. marinus* infections in *M. balthica* by both the FTM and *P. marinus*-specific PCR assays revealed that, as reported elsewhere for eastern oysters (Robledo et al. 1998), it is possible to identify individuals that are FTM positive/ PCR negative (Coss et al. 1997). Therefore, in order to understand the basis of these results, we characterized an in vitro *Perkinsus* culture established from a FTM positive/ PCR negative individual.

Several *Perkinsus*-like organisms have been detected in mollusks using gross morphology of the parasite and/or the outcome of the FTM assay (Andrews 1954; Goggin and Lester 1987; Cigarría, Rodríguez, and Fernández 1997; Choi and Park 1997; Hamaguchi et al. 1998). The FTM assay, however, is clearly not sufficient to identify a parasite as a species within

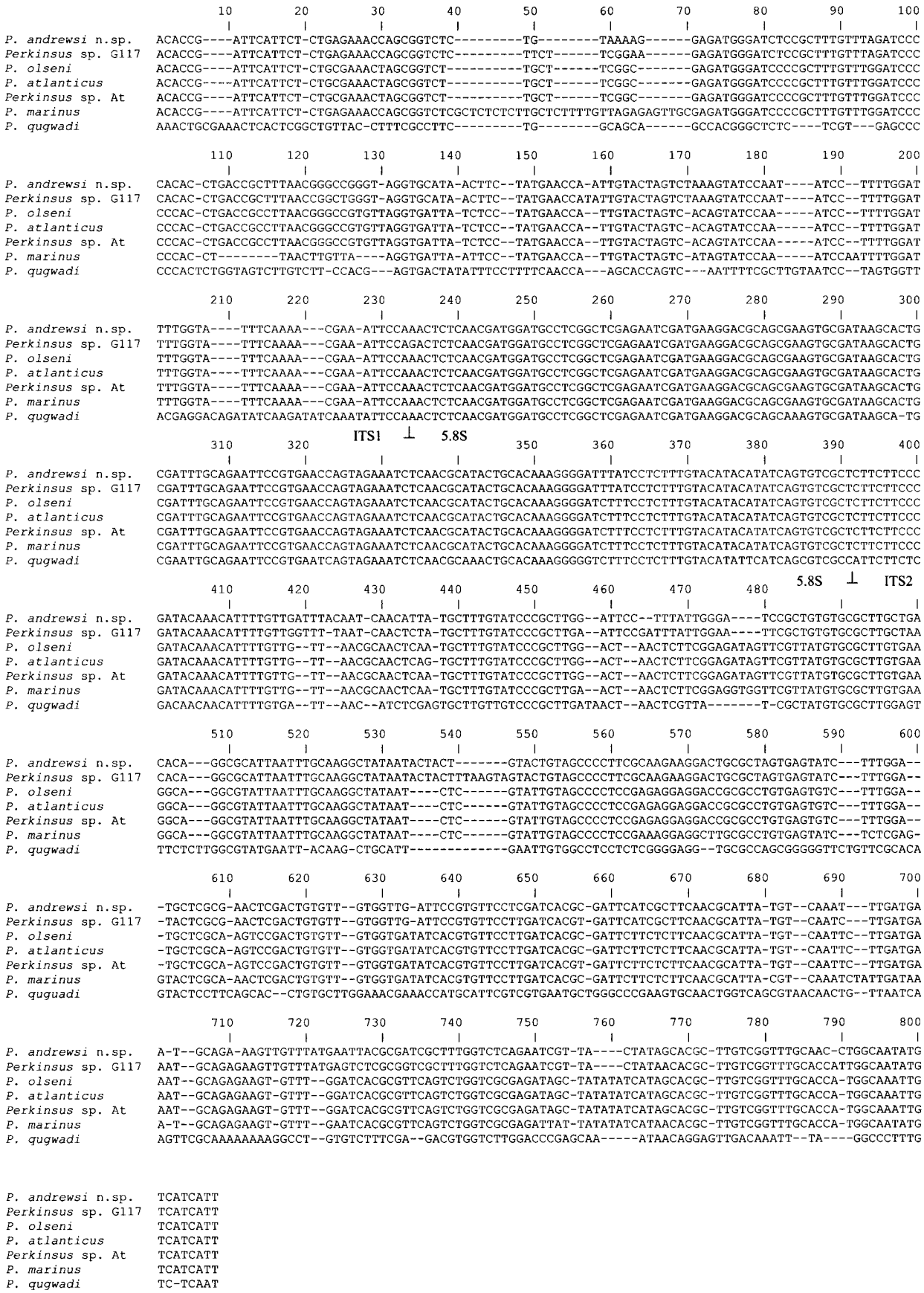

Fig. **2**. Alignment of ITS1, 5.8S, and ITS2 of *Perkinsus andrewsi* n. sp., *Perkinsus* sp. G117, *Perkinsus olseni*, *Perkinsus atlanticus*, *Perkinsus* sp. from *Anadara trapezia*, *Perkinsus marinus*, and *P. qugwadi*.

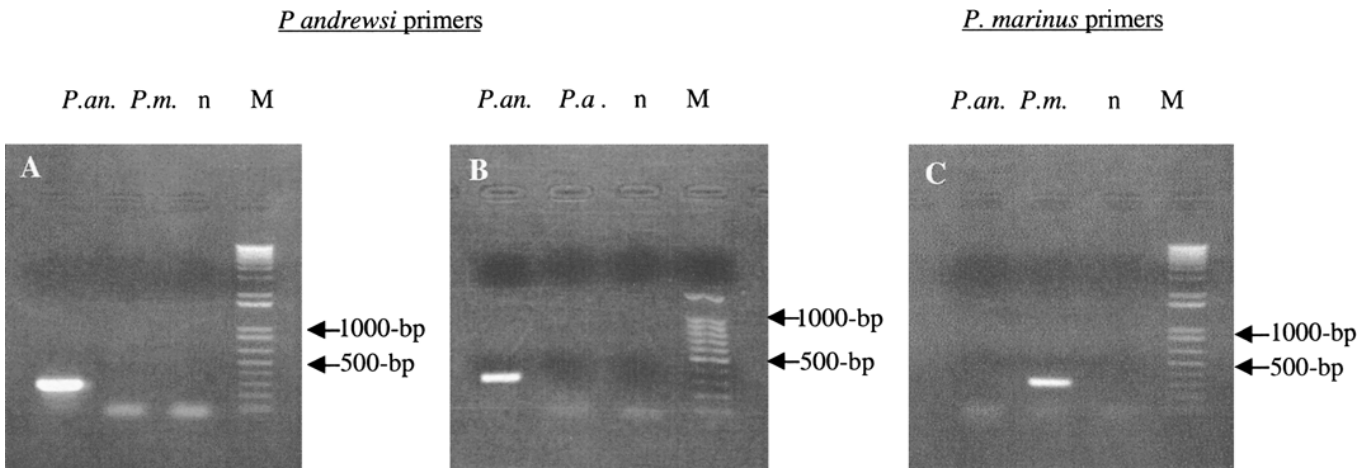

Fig. **3**. Agarose gel electrophoresis of amplified products of PCR assays with *Perkinsus andrewsi* n. sp. diagnostic primers (**A** and **B**) and *Perkinsus marinus* diagnostic primers (**C**); *P. andrewsi* n. sp. DNA (P.an.); *Perkinsus marinus* DNA (P.m.); *P.a., Perkinsus atlanticus* DNA (P.a.); n, Mili-Q-filtered water as negative control; M, 1 Kb DNA ladder (A and C) and 100-bp DNA ladder (B).

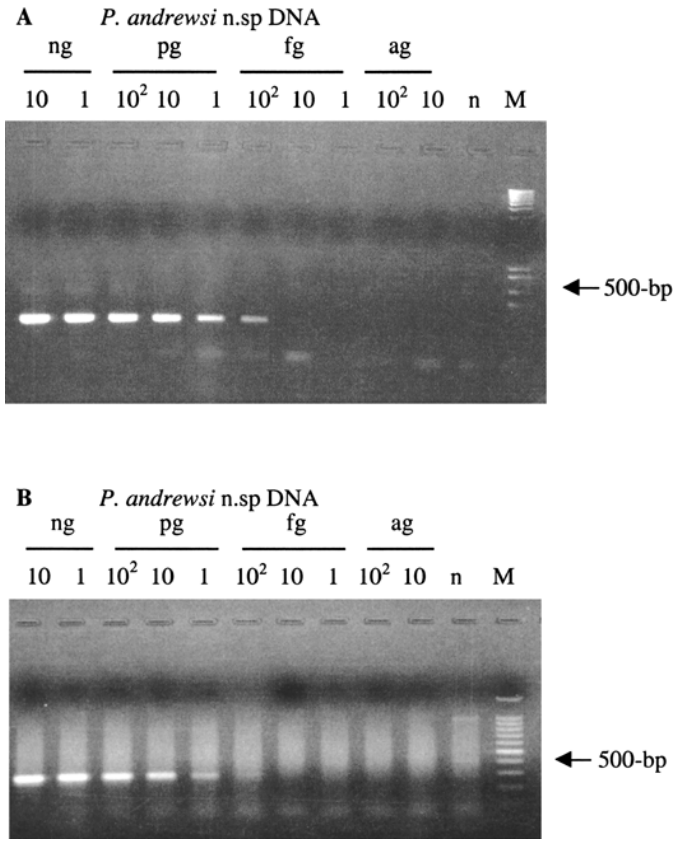

Fig. **4**. Agarose gel electrophoresis of amplified products of the spike/recovery experiments for PCR-based diagnostic assay for *Perkinsus andrewsi* n. sp. Decreasing amounts of *P. andrewsi* n. sp. DNA (10 ng–10 ag) alone or spiked in 1 μg bivalve DNA. **A.** PCR products of *P. andrewsi* n. sp. DNA samples. **B.** PCR products of *Macoma balthica* DNA samples spiked with DNA of *P. andrewsi* n. sp. n, Mili-Q-filtered water as negative control; M, 1-Kb DNA ladder (A) and 100-bp DNA ladder (B).

the genus *Perkinsus* (Robledo et al. 1998). In addition, *P. quagwadi* tests negative by the FTM assay (Blackbourn, Bower, and Meyer 1998), suggesting that the FTM assay not only may give false positive results (Goggin et al. 1996), but also false negative results with some true *Perkinsus* species. Reliance on assays that lack specificity may not only result in misidentification of a parasite species, but may even hinder the understanding of host range and geographic distribution of an epizootic event.

Although fine structural characterization, description of gross morphological features, and in vitro proliferation behavior provide indispensable contributions to the rigorous identification and description of protistan parasites, such as *Perkinsus* species, the molecular characterization of loci recognized as applicable to taxonomic analysis clearly provides relevant information. Sequence analysis of the rRNA gene locus, consisting of the 5.0S, NTS, SSU, ITS1, 5.8S, ITS2 and large subunit, is useful for taxonomic and phylogenetic studies because it encompasses both highly conserved and variable regions, such as the SSU and NTS, respectively (Hillis and Dixon 1991). Sequence identity of the SSU among species within a genus is usually high. For example, sequence identity values ranging from 98.0% to 99.9% were found among 13 *Tetrahymena* species (Sogin et al. 1986), 94.6% to 99.2% for *Naegleria* species (Baverstock et al. 1989), and 99.8% for two species of *Leishmania* (Field, Landfear, and Giovannoni 1991). Based on this information, the 97.5% SSU sequence identity between a *Perkinsus*-like organism isolated from *A. trapezia* and *P. marinus* supported the

Table **2**. Presence of *Perkinsus marinus* and *Perkinsus andrewsi* n. sp. in *Macoma balthica, Macoma mitchelli, Crassostrea virginica,* and *Mercenaria mercenaria* individuals using the *P. marinus*- and *P. andrewsi*-specific PCR assays.

	P. marinus PCR assay	*P. andrewsi* PCR assay	
		Positive	Negative
M. balthica	positive	12	4
n = 21	negative	4	1
C. virginica	positive	43	37
n = 125	negative	39	6
M. mitchelli	positive	3	4
n = 10	negative	3	0
M. mercenaria	positive	1	1
n = 9	negative	1	6

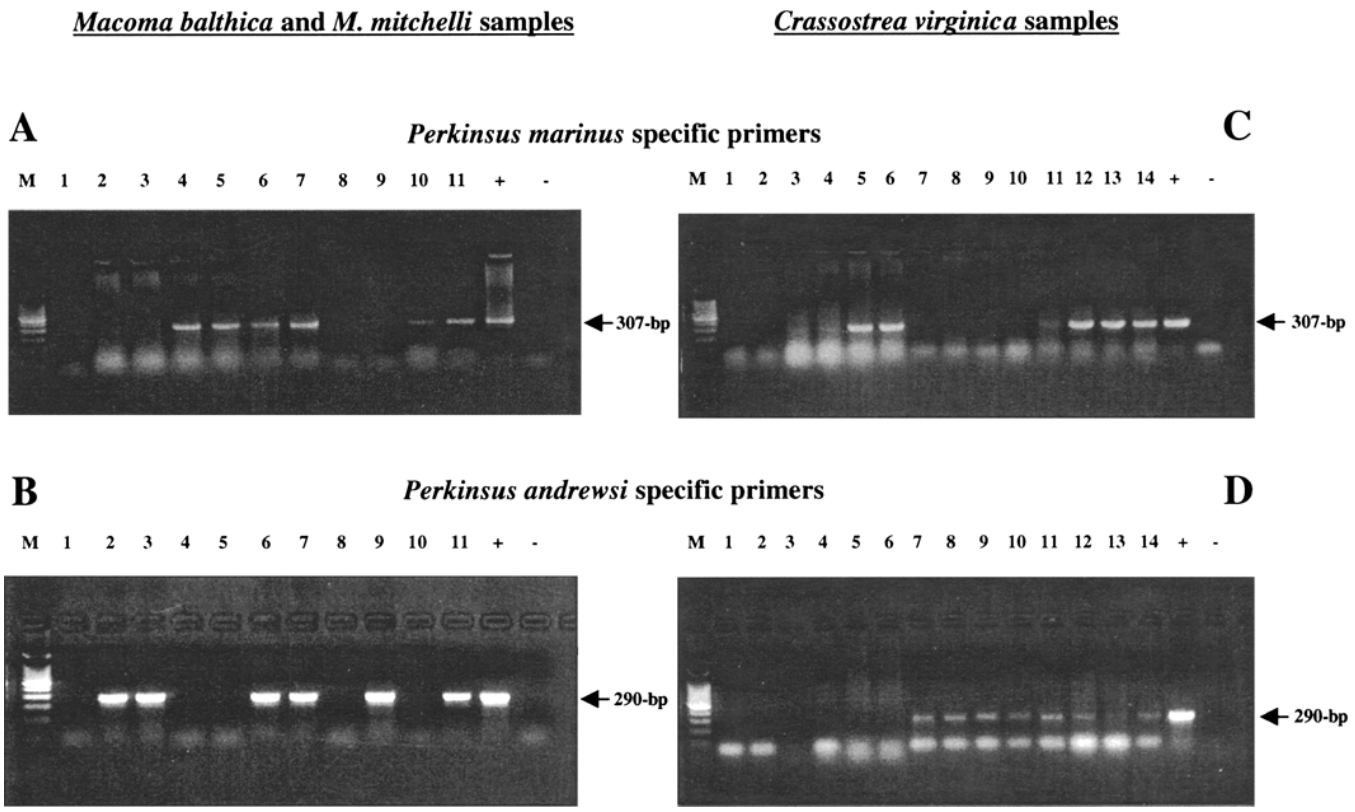

Fig. **5**. Representative agarose gels of amplified products of PCR assays of *Perkinsus* species. *Perkinsus marinus*-specific diagnostic primers (**A** and **C**) and *Perkinsus andrewsi*-specific diagnostic primers (**B** and **D**) were used to assess duplicate samples of *Macoma balthica* (A and B, lanes 1–7) and *Macoma mitchelli* (A and B, lanes 8–11) from Rhode River and *Crassostrea virginica* from Chesapeake Bay and Maine (C and D, lanes 1–14). Note that bivalves 6, 7, and 11 (A, B) and 11, 12, 13, and 14 (C, D) were infected with both *Perkinsus* species. M, 100-bp DNA ladder; +, *Perkinsus andrewsi* n. sp. as positive control; −, Mili-Q-filtered water as negative control

inclusion of this isolate within the genus *Perkinsus* (Fong et al. 1993). The SSU sequence identity (of 98%) between *P. marinus* and *P. andrewsi* n. sp. is in the same range. There is higher sequence identity between *P. andrewsi* n. sp. and *Perkinsus* sp. from *A. trapezia* (99%) or *P. atlanticus* (99.2%). This is attributable to the number of insertions and base substitutions that are shared by *P. andrewsi* n. sp., *Perkinsus* sp. from *A. trapezia*, and *P. atlanticus*, but not by *P. marinus.*

The sequence of the ITS region of the rRNA cluster has been used as a reliable character to distinguish between species of morphologically similar fungi and animals (Goodwin et al. 1995; Lee and Taylor 1992; Paul 2000; Porter and Collins 1991; Shaw 2000; Zahler et al. 1997). In the ITS2 region, Goggin (1994) reported a 99.8% sequence identity between *P. atlanticus, P. olseni,* and the two unidentified *Perkinsus* isolates (the ''*P. atlanticus* group''), and 92% to 93% sequence identity between those four isolates and *P. marinus.* However, in the ITS1 region, Goggin (1994) reported that while the sequences from the *P. atlanticus* group were 100% identical, they were only 77% identical to the ITS sequence of *P. marinus.* The ITS1 sequence identity found between *P. andrewsi* n. sp. and *P. qugwadi* was very low. In contrast, sequence alignment of the ITS2 region showed little difference in sequence identity between *P. andrewsi* n. sp. and either the *P. atlanticus* group (78.4% to 78.7%) or *P. marinus* (78.3%), but the identity to *P. qugwadi* was very low (31.8%).

The sequences reported to date in the 5.8S region for most accepted *Perkinsus* species and an unidentified *Perkinsus* isolate (*P. marinus, P. olseni, P. atlanticus,* and *Perkinsus* sp. from *A. trapezia*) are identical (Goggin 1994). In this region, however, *P. andrewsi* n. sp. differs from them in 2 positions. Interestingly, the 5.8S sequence deposited in GenBank™ for *P. qugwadi* is different from *P. marinus* in 12 positions and from *P. andrewsi* n. sp. by 14 positions. This, in combination with the overall low sequence identity of *P. qugwadi* to the other *Perkinsus* species including our isolate, suggests either that *P. qugwadi* is not as closely related to the other *Perkinsus* species as they are to each other or that the material used for sequencing may have been contaminated.

The NTS region of *P. andrewsi* n. sp. is dramatically different in both length and sequence from the NTS regions of *P. marinus* and *P. atlanticus.* In contrast to the conserved SSU, ITS and 5.8S regions, the NTS is not transcribed (Dover and Coen 1981) and therefore has the potential to accumulate a high degree of sequence variability, even between closely related species (Henriques et al. 1991; Marsh, Gauthier, and Vasta 1995). In *Xenopus laevis* and *Drosophila melanogaster* the NTS regions contain sequence motifs associated with putative RNA polymerase A promoters (Challoner et al. 1985; Long, Rebbert, and Dawid 1981) and repeats of unknown function have also been found in *Saccharomyces cerevisiae* and *Saccharomyces carlbergensis* (Skryabin et al. 1984). Interestingly, the NTS sequence of *P. andrewsi* n. sp. contained a repeat element that is not present in the NTS sequences of either *P. marinus* or *P. atlanticus.* Although the value of the NTS region as a taxonomic marker has not been formally established to date, in *P. marinus* the sequence variability of the NTS region is not random. The frequency of two *P. marinus* NTS sequence types in sam-

ples from the Atlantic and Gulf Coasts varies with their geographic origin (Robledo et al. 1999). We found that similar variation in the NTS sequence occurs in *P. andrewsi* n. sp. with base substitutions in two positions in the 290-bp NTS fragment corresponding to three NTS sequences types.

The differences revealed by the gross morphological and ultrastructural studies alone were not striking enough to indicate that *P. andrewsi* n. sp. was a distinct *Perkinsus* species (Coss, Robledo, and Vasta 2001). The molecular studies reported herein revealed differences that clearly indicate that this *Perkinsus* isolate from *M. balthica* is neither *P. marinus* nor any of the species from the *P. atlanticus* group, nor *P. qugwadi*. Thus, supplementing the gross morphological and ultrastructural studies with the molecular analysis provide higher taxonomic resolution. Accordingly, we formally propose that this *Perkinsus* isolate from *M. balthica* be designated a new species, *Perkinsus andrewsi* n. sp. after Dr. Jay D. Andrews, who made significant contributions to fundamental work on *Perkinsus* species. It is not clear, however, whether this species designation, *P. andrewsi*, also applies to the *Perkinsus* species earlier described in this host by Perkins (1968) and Kleinschuster et al. (1994). *Perkinsus andrewsi* n. sp. is characterized by the ability to zoosporulate in culture, the zoospores being slightly larger than *Labyrinthomyxa* sp. (Perkins 1968) and more homogeneous in size (Coss, Robledo, and Vasta 2001), and their ability to lose their flagella and give rise to trophozoites that continue to proliferate by budding, palintomy, or zoosporulation (Coss, Robledo, and Vasta 2001).

The NTS sequence differences observed between *P. marinus*, *P. atlanticus*, and *P. andrewsi* n. sp. provided the basis for the development of a species-specific diagnostic PCR assay for *P. andrewsi* n. sp. The high variability of the NTS sequence made it an ideal target for the detection of subtle differences among intraspecific strains or types and the detection of probably greater differences between species and higher taxonomic categories (Henriques et al. 1991; Marsh, Gauthier, and Vasta 1995). The PCR-based diagnostic assay for *P. andrewsi* n. sp. reported here performs similarly to that of the *P. marinus*-specific PCR-based assay. Moreover, the presence of *M. balthica* DNA or *C. virginica* DNA did not have a major influence on its specificity or sensitivity. More modern techniques such as the fluorogenic 5′-nuclease assay (TaqMan) (Gibson, Heid, and Williams 1996; Heid et al. 1996) could be used for the development of quantitative PCR assays based on this region or other regions of the rRNA locus.

Until recently, the question of host specificity and the possibility of alternate hosts or reservoir species for *P. marinus* has received little attention. The detection of *P. marinus* in non-oyster Chesapeake Bay mollusk species, including *M. balthica*, was initially carried out by Andrews (1954) using the FTM assay. Coss et al. (1997) detected *P. marinus* in non-oyster bivalve species, including *M. balthica, M. mitchelli, M. mercenaria, Geukensia demissa,* and *Ischadium recurvum*, using the PCR-based assay (Marsh, Gauthier, and Vasta 1995; Robledo et al. 1998). However, the potential pathogenicity of *P. marinus* for these bivalve species remains to be determined. The problem of host specificity has also been addressed with other experimental approaches with variable results depending on the parasite and host species. Goggin, Sewell, and Lester (1989) were able to successfully cross-infect nine mollusk species using zoospores induced from *Perkinsus* sp. prezoosporangia isolated from six different host species. Most attempts to cross-infect North American bivalve species with *Perkinsus* species, however, have not been successful (Andrews and Hewatt 1957; Ray 1954), suggesting that these species may exhibit some degree of host-specificity. Nevertheless, zoospores obtained from the *Perkinsus* sp. in *M. balthica* can infect *C. virginica* (Perkins 1996). In the present study, both *P. andrewsi* n. sp. and *P. marinus* were detected in *M. balthica, M. mitchelli, M. mercenaria,* and *C. virginica*. A considerable number of individuals (15% to 50%) of each bivalve species were coinfected with both *Perkinsus* species. Interestingly, 90% of the oysters that were negative for *P. marinus* and positive for *P. andrewsi* n. sp. had been collected in either low salinity sites (7–10‰) of the Chesapeake Bay or from the Damariscotta River (29–31‰) in Maine. This suggests that although the two *Perkinsus* species are sympatric, environmental factors, such as salinity, may determine their overlapping, yet distinct geographic distributions. While the sample size and the number of locations assayed in this study were low, in light of the recent reports on the expansion of the range of *P. marinus* into the upper estuary regions of Chesapeake Bay (Burreson and Ragone-Calvo 1996) and northward along the Atlantic coast (Ford 1996), these findings warrant further investigation.

In conclusion, PCR-based assays specific for *P. andrewsi* n. sp. and *P. marinus* (Marsh, Gauthier, and Vasta 1995; Robledo et al. 1998) indicate that *P. andrewsi* n. sp. can be present in bivalve species sympatric to *M. balthica*, and that in all four bivalve species examined *P. andrewsi* n. sp. and *P. marinus* can be detected simultaneously. While the potential pathogenicity of *P. andrewsi* n. sp. for *C. virginica* or *M. balthica* is unknown, its presence in the oyster is of particular concern because the FTM assay, used routinely by most laboratories for determining *P. marinus* infections, cannot discriminate between the two *Perkinsus* species. The number of false positives by FTM could be considerable, confounding the accurate assessment of *P. marinus* epizootics. Therefore, in order to ensure a rigorous assessment of *P. marinus* infection intensities and disease prevalence, future studies should incorporate the species-specific PCR assays currently available (Marsh, Gauthier, and Vasta 1995; Robledo et al. 1998). Furthermore, we have preliminary results that show that the short amplicon resulting from the specific PCR-based assay for *P. marinus* can be amplified from paraffin-embedded bivalve tissues. Thus, archival samples can now be analyzed for specific identification of *Perkinsus* species in past epizootics or routine samplings that have been diagnosed by the FTM assay. Such initiatives should benefit both the scientific community by generating accurate data for the epizootiological mapping of ''Dermo'' disease, and the shellfish farmers by enabling improved management strategies.

DESCRIPTION

Perkinsus andrewsi n. sp. Coss, Robledo, Ruiz, and Vasta.

Morphological aspects. Trophozoites are uninucleate, spherical, surrounded by a thick wall, 9.8 ± 4.2 μm (n = 30) in diameter having an eccentric nucleus with a prominent central nucleolus and a large eccentric vacuole that displaces the nucleus against the membrane producing the typical ''signet ring'' appearance of *Perkinsus* species. Cells divide by budding or palintomy. In immature trophozoites the nucleus is often spherical and occupies a larger proportion of the cell than in mature trophozoites and there are multiple vacuoles of various sizes spread throughout the cytoplasm. Zoosporulation is spontaneous and continuous in in vitro culture. Zoosporangia are spherical, variable in size, 28–82 μm (n = 21) in diam., and give rise to numerous zoospores by successive cycles of karyokinesis and cytokinesis. Zoospores are uninucleated, biflagellated, ovoid, 4.4 ± 0.6 μm in length and 2.0 ± 0.5 μm (n = 10) in width.

Molecular aspects. Nucleotide sequences from the rRNA locus are accessible through GenBank™ (AF102171). The 5.8S

sequence differs by two bases, at base positions 127 and 129, from *P. marinus* and all other *Perkinsus* species available to date. The NTS sequence (1545 bp) is longer than the NTS from both *P. marinus* (1150 bp) and *P. atlanticus* (1147 bp) (Robledo, Coss, and Vasta 2001).

Type host. Baltic clam, *Macoma balthica.*

Type locality. Fox Point, Rhode River, Edgewater, MD, USA (38°51′N, 76°32′W).

Material deposited. A monoclonal culture of *Perkinsus andrewsi* has been deposited in the American Type Culture Collection, Rockville, MD as ATCC 50807.

Etymology. This species is named in honor of Dr. Jay D. Andrews for his fundamental contributions to the study of *Perkinsus* species.

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