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Geographic distribution of *Perkinsus marinus* genetic strains along the Atlantic and Gulf coasts of the USA

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Abstract *Perkinsus marinus* (Mackin, Owen and Collier) is a major pathogen of the eastern oyster *Crassostrea virginica* (Gmelin). Elucidating the spatial distribution of genetic variation within the species is critical to an examination of potential virulence differences among strains and to understanding the implications of transferring infected oyster stocks. The genetic similarity of *P. marinus* in vitro cultures and their clonal composition was examined using isolates from individual oysters collected from 1991 through 1999 along the Atlantic and Gulf of Mexico coasts of the USA. *P. marinus* has been endemic to the Atlantic coast from the Virginia portion of Chesapeake Bay southward and into the Gulf of Mexico since its initial description in 1949, and over the last 10–15 years there has been a range expansion of this parasite into the Maryland portion of Chesapeake Bay and northward along the Atlantic coast from New Jersey to Maine. DNA purified from 76 primary (parental) cultures and 86 clonal cultures derived from the parental isolates was examined at eight polymorphic loci by restriction fragment length polymorphism analysis. Comparison of clonal and parental culture genotypes supported previous observations that isolate cultures initiated from a single oyster can be polyclonal, providing evidence that an individual oyster can be infected with multiple strains. Allelic and genotypic frequencies differed significantly among three regions of the USA; the Northeast Atlantic (Maine to Maryland), the Southeast Atlantic (Virginia to Florida's east coast), and the Gulf of Mexico (Florida's west coast to Texas). Overall, 12 different composite genotypes were detected in this study, with >88% of isolates possessing one of three predominant genotypes. One of the major composite genotypes was unique to Gulf coast isolates, while the genetic strain of *P. marinus* detected most frequently in oysters from the Northeast was not found in Gulf coast oysters. *Perkinsus marinus* is ubiquitous along the Atlantic and Gulf coasts, but different regions possess unique assemblages of genetic strains. Apparently, the historically common practice of oyster transplantation between regions has not significantly altered the population genetic structure at the regional level. The data suggest a founder event in the Northeast region, corresponding to the recent range expansion. Previously documented differences in virulence are consistent with genotypic differences, indicating the potential to identify strain virulence with molecular genetic tools.

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Introduction

Perkinsus spp. are protozoan parasites of marine and estuarine molluscs (Bower et al. 1994). Along much of the eastern and Gulf coasts of the USA, *Perkinsus marinus* has had devastating effects on populations of the eastern oyster *Crassostrea virginica* (Andrews and Ray 1988; Burreson and Ragone Calvo 1996; Ford 1996; Soniat 1996). Several studies have indicated that strains of *P. marinus* exist, which vary in virulence and other physiological traits (Bushek 1994; La Peyre and Faisal 1995; La Peyre et al. 1995, 1996, 1998; Bushek and Allen 1996a; Chu et al. 1998). Few studies, however, have examined genetic variation within the species or the geographic distribution of genetic strains (Marsh et al. 1995; Reece et al. 1997; Kotob et al. 1999a,b).

Historically, the parasite's range in the USA was primarily confined to the Virginia portion of Chesapeake Bay, southward along the Atlantic coast and into the Gulf of Mexico. *P. marinus* has undergone a recent range extension, beginning around 1988, when outbreaks began to occur in the Maryland portion of Chesapeake Bay. It has since been found infecting oysters in a wide range of salinities along the US coastline from Maine to Texas (Ford and Tripp 1996). It has long been known that *P. marinus* can be transmitted directly between hosts through the water column (Ray 1954), and this is probably the predominant mechanism of transfer between oysters (Andrews and Hewatt 1957; Ellin 2000). Theoretically, *P. marinus* cells could be transported long distances by ocean currents resulting in mixing of genetic strains. The duration of planktonic parasite survival remains unknown, but this time period has important implications for population structure as well as transmission dynamics (Bushek and Allen 1996b). Specifically, shorter survival times in the plankton provide more opportunity for the development of genetically structured populations, whereas longer survival times provide more opportunity for strains to disperse and mix, reducing genetic structure in the populations. Dispersal distances of several kilometers have been suggested (Mackin 1962; Ford 1996), yet separation distances as short as 15 m have been shown to dramatically reduce transmission (Andrews and Hewatt 1957). If natural dispersal distances are on the order of tens of meters or less, then the level of gene flow among populations of *P. marinus* is likely to be very low. As a result, populations may have differentiated genetically over time due to drift or natural selection.

Little information exists regarding the population structure of *P. marinus*. Based on the historical distribution of *P. marinus* it is likely that the Northeast Atlantic coast from Maryland to Massachusetts contains a distinct population of strains resulting from the recent establishment of the parasite in this region. Many molecular genetic studies also indicate a break in population structure for coastal species between the Atlantic and Gulf coasts (Avise 1996). Thus, it is likely that the Southeast Atlantic (Virginia to Florida's east coast) and the Gulf of Mexico (Florida's west coast to Texas) may represent two additional distinct populations.

Bushek and Allen (1996a) previously found differences in virulence between in vitro *P. marinus* isolates derived from mid-Atlantic and Gulf coast oysters. Specifically, two mid-Atlantic isolates produced heavier infections in a shorter period of time than two from the Gulf coast. Other studies have shown that in vitro *P. marinus* isolates differ in the production and activity of some extracellular proteins, e.g., in the production of proteases (La Peyre et al. 1995, 1998). In addition, differences in acid phosphatase and superoxide dismutase activity were observed among six isolates from oysters collected at different sites (Chu et al. 1998).

A previous molecular genetic study based on restriction fragment length polymorphism (RFLP) analysis of four variable loci demonstrated genetic differences among *P. marinus* isolates (Reece et al. 1997). In that study, 14 parental isolates collected from Connecticut to south Texas and 12 clonal cultures derived from four of the parental isolates were examined for genetic variation at four polymorphic loci. Five different genetic "strains" (4-locus genotypes) of the parasite were identified. Low sample sizes provided little power for regional comparisons, but the study suggested genetic differentiation with greatest diversity in the Southeast. In addition, RFLP analysis indicated that cultured *P. marinus* cells are diploid, and that genetically different "strains" of *P. marinus* could be found in oysters collected from a single site and even within an individual oyster. The present study incorporates 20 additional sampling sites ($n = 30$) and four additional polymorphic loci ($n = 8$) to expand geographic sampling and genetic resolution. Overall, isolate cultures were established from 76 *P. marinus*–infected oysters collected from the Atlantic and Gulf coasts of the USA. In addition, genotypes of 86 clonal cultures from 20 of the primary (or parental) isolates were determined.

Materials and methods

Parasite culturing and cloning

A total of 76 in vitro–cultured isolates of *Perkinsus marinus* (Mackin, Owen and Collier), representing the parasite's range in the USA, was obtained for analysis (Table 1). These isolates were established between 1991 and 1999 and came from infected oysters during the present or previous studies (Bushek 1994; Reece et al. 1997), or were acquired from researchers at other laboratories. Regardless of source, each isolate was derived from a single oyster. Up to ten clonal cultures were produced from each of 20 isolates as described by Bushek et al. (2000), which yielded a total of 86 clonal cultures. Many of the isolates (cloned or not) have been deposited with the American Type Culture Collection (ATCC) in Manassas, Virginia, USA (Appendix 1, electronic supplementary material).

DNA extraction and PCR amplification with "universal" primers

Genomic DNA was isolated from each uncloned parental and clonal isolate culture as previously described in Reece et al. (1997). Using "universal" PCR primers for ATPase 6 (J. Quattro, personal communication), serine protease genes (Sakanari et al. 1989), and the plastid ribosomal protein12 gene (Denny et al. 1998), several non-specific amplification products were obtained. These products were cloned into a plasmid vector: either pBluescript-SK$^+$ (Stratagene, La Jolla, Calif., USA) or pCR2.1 (Invitrogen, Carlsbad, Calif., USA) using standard cloning protocols (Sambrook et al. 1989) or the TA cloning kit (Invitrogen) following the manufacturer's protocol.

DNA sequencing and PCR primer design

The cloned *P. marinus* DNA inserts are listed in Table 2. The ITS, ACFLNK, ATAN, and SPD5 clones were manually sequenced as previously described in Reece et al. (1997). The DNA inserts SPD50, SPNC11, RPAN30, and RPAN33 were sequenced on an automated sequencer by simultaneous bi-directional cycle-sequencing using the Thermo Sequenase sequencing kit (Amersham Life Science, Piscataway, N.J., USA) and infrared labeled primers (LI-COR,

Table 1 *Perkinsus marinus*. Isolates from *Crassostrea virginica* collected along the East and Gulf coasts of the USA. Genotypes detected, total number of oysters/parental isolates, and clonal isolates from each collection site. Maryland isolates are from upper Chesapeake Bay; Virginia isolates are from lower Chesapeake Bay

Region/State/Site	Genotypic composition	No. of oysters/ parental cultures	No. parental cultures cloned	Total no. clonal cultures
Northeast				
Massachusetts				
Cotuit Bay	1, 2	2	2	7
Wareham R.	3	1	1	5
Connecticut				
Long Island Sound	1	1	0	0
Rhode Island				
Charleston Pond	3	1	0	0
New Jersey				
Delaware Bay	1, 3, 5	3	1	4
Cape Shore, Delaware Bay	1	2	1	1
Maryland				
Townsends Wharf, Choptank R.	1	1	0	0
Oyster Shell Point, Choptank R.	1	1	0	0
Pope's Creek, Potomac R.	6	1	0	0
Chicken Cock, St. Mary's R.	1	1	0	0
Oxford Laboratory	1	5	0	0
Western Island	1	3	0	0
Kedges Strait	1, 7	6	0	0
Fog Point, S. Kedges Strait	1, 12	6	0	0
Southeast				
Virginia				
Mobjack Bay	8	1	1	3
York R.	9	1	0	0
James R.	3	2	0	0
Lynnhaven Inlet	8	1	0	0
North Carolina				
Neuse R.	3, 10	3	0	0
South Carolina				
Crabhaul Creek	3	1	1	3
Old Man Creek	3	1	0	0
North Inlet	1, 3, 11	2	1	4
Georgia				
Skidaway Island	3	2	0	0
Florida (eastern coast)				
Ft. Pierce	3	3	0	0
Gulf of Mexico				
Florida (western coast)				
Cedar Key	3	7	0	0
Louisiana				
Mozambique Point	3, 4	7	5	20
Grande Terre	3, 4, 12, 9	5	4	23
Bay Tambour	3,4	4	3	16
Texas				
Galveston Bay	4	1	0	0
Laguna Madre	3	1	0	0
Total		76	20	86

Lincoln, Neb., USA) following the manufacturer's protocol. Sequencing reactions were electrophoresed and detected on a LI-COR automated sequencer (model 4200L). The DNA sequences were subjected to BLAST searches (Altschul et al. 1990) of the National Center for Biotechnology Information (NCBI) GenBank database, and none, except the ITS sequences, matched deposited sequences. PCR primers were designed with the aid of MacVector DNA sequence analysis software (Oxford Molecular, Madison, Wis., USA) and were used to amplify these regions of the *P. marinus* genome. PCR amplification parameters and primers for the loci ITS, ACFLNK, ATAN, and SPD5 were already available (Reece et al. 1997). Amplifications of four new loci SP50, SPNC11, RPAN30, and RPAN33 were accomplished with 10–50 ng of genomic DNA in 25 μl reactions using the reaction conditions recommended for BRL PCR reagent system (Life Technologies, Gaithersburg, Md., USA) with the addition of 20% BSA (1 mg ml^{-1}). The primers for all eight loci are shown in Table 2. The following cycling conditions were used for each locus: an initial denaturation of 4 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at an annealing temperature that varied with primer pair (see Table 2), and 3 min at 65°C, with a final extension of 5 min at 65°C.

A subset of the amplification products was screened with up to 40 different restriction endonucleases to identify enzymes that demonstrated intraspecific polymorphisms for each locus. Amplification products (3–6 μl of the PCR product) from each parental and clonal culture DNA were digested with the informative enzyme selected for each locus (see Reece et al. 1997; and Table 2). Digestion products were subjected to electrophoresis in 2.5% agarose gels (1.5% NuSieve, FMC Biochemical, Rockland, Me., USA; and 1% BRL Ultrapure agarose, Life Technologies). Fragment lengths were compared to each other and to a size standard (1 kb ladder, Life Technologies) to assess polymorphisms. Amplifications and digestions were done in triplicate to ensure the reproducibility of results.

Data analysis

Composite genotypes were generated for each culture by assigning genotypes at each locus based on RFLP patterns and assuming diploidy (Reece et al. 1997). Genotypic profiles of clonal cultures were compared to those of the parental cultures from which they were derived to assess the level of multiple strain infectivity. Genotypic data were used to examine the geographic distribution and regional composition of *P. marinus* strains. The Atlantic coast of the USA was divided into three regions for this analysis based on the historical and current range of the parasite. The Northeast region extended from Maryland northward, including the Maryland portion of Chesapeake Bay. This area was impacted by the relatively recent northward expansion of *P. marinus* (Ford 1996). The Southeast region included the Virginia portion of Chesapeake Bay and the Atlantic coast from Virginia south to the eastern coast of Florida. This region was found to harbor *P. marinus* at the time it was first described from the Gulf of Mexico (Andrews and Hewatt 1954). The Gulf samples were collected from the western coast of Florida to South Bay Laguna Madre near the United States–Mexican border in southern Texas. Genetic relationships among the 12 composite genotypes were assessed. Neighbor-joining analysis based on the total number of pairwise character differences and maximum-parsimony bootstrap analysis with 1000 replicates of 100 random additions were done using PAUP*4.0 (Swofford 2000). Allelic and genotypic distributions were compared among the Northeast, Southeast, and Gulf coast regions. An unbiased estimate of the *P*-value of a log-likelihood (*G*) based exact test (Goudet et al. 1996) was performed for each locus, based on the frequency that each strain was detected in individual oysters, using the population genetic analysis program GENEPOP (Raymond and Rousset 1995).

Results

Based on RFLP analysis using the enzymes listed in Table 2, no more than two alleles were observed at any

Table 2 *Perkinsus marinus*. Primer sequences and related information. Loci are as follows: internal transcribed spacer sequence (*ITS*) of rRNA locus; flanking region of actin gene (*ACFLNK*); anonymous loci (*ATAN*, *SPD5*, *SPNC11*, *SPD50*, *RPAN30* and *RPAN33*)

Locus	Primer name	Annealing temp. (°C)	Amplified product size (bp; approx.)	Informative restriction enzyme	Primer sequence 5′–3′
ITS (Goggin 1994)	ITS-5	55	750	*Cla*I – size polymorphism visualization	CGT AGG TGA ACC TGC GGA AGG ATC
	ITS-3				TAT GCT TAA ATT CAG CGG GT
ACFLNK	ACFLNK-5	54	1400	*Msp*I	GCT TGA GTT TAC AAG TAT GGT GGT AGG G
	ACFLNK-3				CCA GAA ATT CCC AAT AGT TGA CC
ATAN	ATAN-5	54	450	*Msp*I	GAT GAG TTA CGC CGT AGG GCA GCT C
	ATAN-3				TAT CCA ACG ACG AGG TAG TGA GGG GCA C
SPD5	SPD5-5	52	270	*Msp*I	CTC AGT GGG CAT CCG AGA TAT TGT CCA TG
	SPD5-3				GTG TCA CCG CTC ACC TTC GCA TC
SPNC11	SPNC11-5	56	200	*Hin*cII	TTT GTC AGG AGA GAG CGA CTT GAG GC
	SPNC11-3				CAA TTT TCC ATG TGG CGG CTA TTC TAG TAG
SPD50	SPD5-5-3	47	600	Size polymorphism	ATC CGT GCT GAA GTC TGC
	SPD5-3				GTG TCA CCG CTC ACC TTC GCA TC
RPAN33	E33B-5	53	200	*Hae*III	TAC AAC GAG GTC AAG ACG AGG C
	E33B-3				TCA CAA CGC TCA TCA TCC CC
RPAN30	E30B-5	57	450	*Msp*I	GGT AGC CTC ACA ATG ATT CAC AG
	E30B-3				AGC ATC TGC GTC AAC CTG C

locus (Table 3). At each locus, the more common allele was designated "A" and the other "B". At the seven presumed single-copy loci (all except ITS), restriction fragment patterns of the amplified products from several clonal culture DNAs indicated diploidy. Therefore, with three possible genotypes at each of eight loci, the potential number of different composite genotypes was 3^8 or 6561. Despite this, only 12 different composite genotypes were observed among the 162 *Perkinsus marinus* cultures examined (Table 3).

Heterozygous AB and homozygous AA genotypes were detected at all loci, but at three loci, homozygous BB genotypes were not observed. Genotypes 1, 3, and 4 infected >90% of the oysters from which the isolates were derived (Table 4). All other genotypes were relatively rare: five were each found in only a single oyster and another four *P. marinus* genotypes were isolated from only two individuals each. Two genotypes (5 and 11) were unique to clonal cultures. Most clonal culture genotypes (79 of 86) matched the parental isolate cultures from which they were cloned, indicating that isolates are dominated by one clone. Five of the 20 parental cultures that were cloned, however, contained more than one genetic type of *P. marinus* cell (Appendix 2, electronic supplementary material). The genotypes of at least two clonal cultures always matched the parental genotype indicated by RFLP analysis of the parental culture DNA. Clonal cultures with three distinct genotypes were isolated from NJ-3. This observation is consistent with that of Reece et al. (1997), demonstrating that multiple *P. marinus* genetic strains can infect a single oyster. Of the five parental isolates in which more than one strain was detected, one was from the Northeast, one was from a site in the Southeast, and three were from two nearby sites in the Gulf.

Figure 1a shows the unrooted neighbor-joining tree that was generated based on the genetic relatedness of composite genotypes. The analysis indicated that the predominant genotypes (1, 3, and 4) were not closely related. Genotype 1 was heterozygous at all eight loci, while genotype 3 was homozygous for the dominant allele at all loci. Genotype 4 was heterozygous at one locus, homozygous at four of eight loci for the common allele, and was homozygous for the alternate allele at three loci. Genotypes 2, 5, 6, 7, 9,11, and 12 grouped in a cluster with the major genotype 1. Genotypes 5, 7, and 12 each possessed only one homozygous locus (Table 3) and grouped most closely to genotype 1. Genotypes 5 and 12 were only observed in clonal cultures whose sister clones, and the parental cultures from which they were derived, were genotype 1 (Appendix 2, electronic supplementary material). Genotype 10 had a single heterozygous locus and was closely related to genotype 3 (Table 3; Fig. 1). Genotypes 4 and 8 were not closely related to any other genotypes (Fig. 1a). Parsimony bootstrap analysis resulted in a tree with little resolution (Fig. 1b). Major composite genotypes 1 and 4 grouped in an unresolved cluster with genotypes 2, 5, 6, 7, 9, 11, and 12. Genotypes 3 and 10 grouped together, and genotype 8 did not group with any other genotype, which was consistent with results of the neighbor-joining analysis.

Genotypic and allelic frequency data were used to examine the geographic distribution and regional composition of *P. marinus* strains (Table 4). The null hypotheses tested were that there were no differences in allelic and genotypic frequencies among the Northeast, Southeast, and Gulf coast regions. Regional distribution of *P. marinus* genotypes among the oyster samples is shown in Table 4. Significant differences in the observed allelic and genotypic frequencies were found among the three regions at all eight loci. Seven different strains were present in the Northeast and six strains were found in the Southeast region, while only four strains were observed in the Gulf. The diversity of strains was not related to the number of oysters sampled, nor to the number of different sampling sites (14 Northeast, 10 Southeast, 6 Gulf; Table 1). A total of 82% of the oysters from the Northeast was infected by genotype 1, which was absent in the Gulf and found in only one oyster from the Southeast (Table 4). The only *P. marinus* strain found in oysters from all three regions was genotype 3. This genotype was relatively rare in the Northeast (8.8% of oysters sampled), but was prevalent in oysters from the Southeast and Gulf regions (65% and 64%, respectively). Genotype 3 occurred at 3 of 14

Table 3 *Perkinsus marinus*. Twelve composite genotypes resulting from RFLP analysis of 76 primary (parental) and 86 clonal cultures at eight polymorphic loci (*A* more common allele; *B* less common allele)

Genotype no.	ITS	ACFLNK	ATAN	SPD5	SPNC11	SPD50	RPAN33	RPAN30
1	AB	AB	AB	AB	AB	AB	AB	AB
2	AB	AA	AA	AB	AB	AB	AB	AB
3	AA	AA	AA	AA	AA	AA	AA	AA
4	AB	AA	AA	AA	BB	BB	BB	AA
5	AA	AB	AB	AB	AB	AB	AB	AB
6	AB	AA	AB	AA	AB	AA	AB	AB
7	AB	BB	AB	AB	AB	AB	AB	AB
8	AA	BB	AA	BB	AA	AA	BB	AA
9	AB	AA	AB	AB	AB	BB	AB	AB
10	AA	AA	AA	AB	AA	AA	AA	AA
11	AB	AB	AB	AA	AB	AB	AB	AB
12	AB	AA	AB	AB	AB	AB	AB	AB

Table 4 *Perkinsus marinus*. Composite genotype distribution among individual oysters (*Crassostrea virginica*). *Bold type* indicates the three most common genotypes. Note: total number of oysters with a particular genotype can be greater than the number examined, and percentage of oysters containing *P. marinus* of a particular genotype can be >100% due to multiple infectivity

Genotype no.	No. of oysters, Northeast region	% Containing genotype	No. of oysters, Southeast region	% Containing genotype	No. of oysters, Gulf region	% Containing genotype	No. of oysters, overall	% Containing genotype
1	**28**	**82.0**	**1**	**5.9**	**0**	**0**	**29**	**38.2**
2	1	2.9	0	0	0	0	1	1.3
3	**3**	**8.8**	**11**	**65.0**	**16**	**64.0**	**30**	**39.5**
4	**0**	**0**	**0**	**0**	**10**	**40.0**	**10**	**13.2**
5	1	2.9	0	0	0	0	1	1.3
6	1	2.9	0	0	0	0	1	1.3
7	1	2.9	0	0	0	0	1	1.3
8	0	0	2	11.8	0	0	2	2.6
9	0	0	1	5.9	1	4.0	2	2.6
10	0	0	2	11.8	0	0	2	2.6
11	0	0	1	5.9	0	0	1	1.3
12	1	2.9	0	0	1	4.0	2	2.6
Total no. oysters examined	34		17		25		76	

sites in the Northeast, 7 of 10 sites in the Southeast, and 5 of 6 sites in the Gulf. Genotype 3 was only found at sites along the Atlantic coast in the Northeast (in 3 of 10 oysters) and not in the Maryland portion of Chesapeake Bay (in 0 of 24 oysters). Genotype 4 occurred at four of six sites along the Gulf coast (40% of all Gulf oysters), but was not found along the Atlantic coast.

At three locations, samples were collected during multiple years, providing a preliminary evaluation of the temporal variation in strain composition. Oysters from New Jersey and Maryland were consistently infected by genotype 1. Over 4 years, only two clonal cultures of the five parental and five clonal isolates from Delaware Bay, N.J., were not genotype 1. One of the clonal cultures was genotype 5, which is closely related to genotype 1 (see above), and the other clonal culture was genotype 3. Of 24 oysters sampled from 1992 through 1996 in Maryland, only three contained *P. marinus* genotypes other than genotype 1. In contrast, three different strains were found in five oysters collected in Virginia over the 5 years that oysters were sampled between 1991 and 1999.

Discussion and conclusions

While *Perkinsus marinus* is presently ubiquitous in oysters from the Atlantic and Gulf coasts, this study demonstrates significant structure in the geographic distribution of genetic strains of the parasite. Most

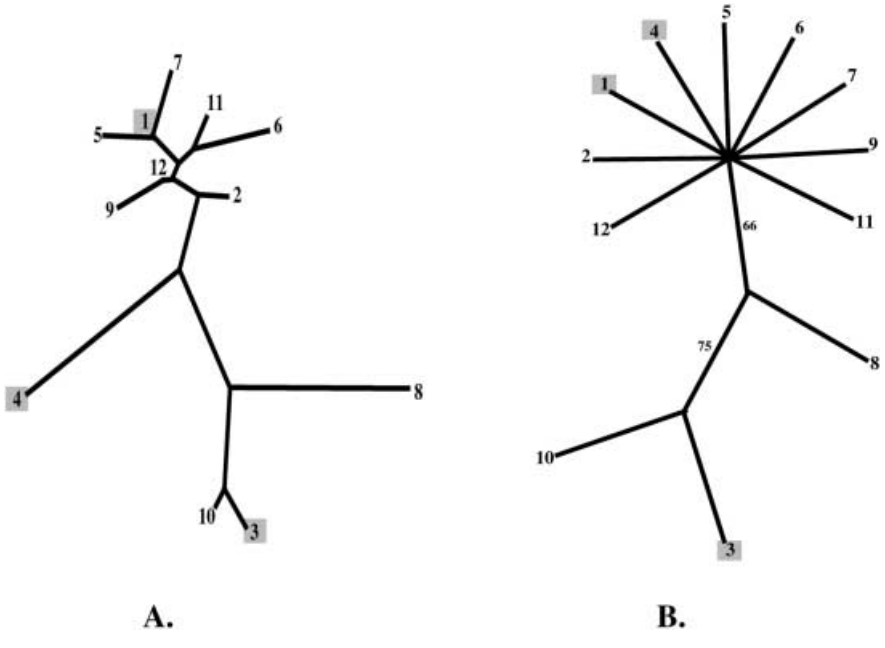

Fig. 1A, B *Perkinsus marinus*. Unrooted neighbor-joining (**A**) and parsimony bootstrap (**B**) trees showing genetic relationships among the *P. marinus* genetic strains based on composite genotypes. The major genotypes 1, 3 and 4 are shown *shaded*. Boostrap support values are shown on the branches of the parsimony tree

oysters (28 of 34) collected from the northeastern Atlantic coast were infected with *P. marinus* cells of composite genotype 1, although there were seven composite genotypes present in this region. The 17 oysters collected off the southeastern coast had six genetically different strains of the parasite. In fact, the five samples collected from Virginia waters of Chesapeake Bay were infected by three different genetic strains of *P. marinus* (Table 1). Almost all infected oysters in the Gulf were harboring *P. marinus* cells of genotypes 3 or 4 (Tables 1, 4), and analysis of clonal cultures showed that several oysters were infected by both strains (Appendix 2, electronic supplementary material).

A preliminary analysis of the *P. marinus* genotypes in oysters collected during different years from New Jersey and Maryland waters did not demonstrate major changes in genotypic composition over time. In Virginia waters, although three different genetic strains were detected in five oysters, genotype 8 *P. marinus* were found in 1993 and again in 1999. At this level of analysis, the results suggest that there is little temporal variation in the overall distribution of genotypes. A more deliberate and intensive sampling effort will be required to address temporal variation at specific sites.

In addition to the possibility of natural dispersal through current transport, the relatively common practice of oyster transplantation could move different genetic strains of *P. marinus* to other locales. These movements may have mixed historically isolated populations of the parasite. However, the large genetic divergence among regions observed in this study would suggest that transplantation has not substantially altered the overall population genetic structure of *P. marinus* at this regional scale unless, as discussed below, it was a significant factor in causing the northward expansion of the parasite in recent years.

Population genetic structure of the *P. marinus* host, *Crassostrea virginica*, does not appear to have been heavily impacted by transplantation either. A number of studies have examined the population genetic structure of *C. virginica* (Buroker 1983; Hedgecock and Okazaki 1984; Reeb and Avise 1990; Karl and Avise 1992; King et al. 1994; Hare et al. 1996; Small and Chapman 1997). Allelic frequencies among oyster populations along the Atlantic and Gulf coasts have been found to differ significantly at both mitochondrial (Reeb and Avise 1990) and nuclear loci (Karl and Avise 1992; Hare et al. 1996), with a major break in genetic structure near Cape Canaveral along the Atlantic coast of Florida (Hare et al. 1996). Several other coastal species also have been found to show significant genetic differences among Atlantic and Gulf populations (reviews in Avise 1992, 1996).

Many factors can influence the impact of *P. marinus* infections on oyster populations. Differences in environmental conditions, including temperature and salinity, affect transmission patterns, disease prevalence and intensity (Soniat 1985; Burreson and Andrews 1988; Soniat and Gauthier 1989; Ragone Calvo and Burreson 1993; Burreson and Ragone Calvo 1996; Powell et al. 1996; Chu et al. 1998). Host populations may vary in overall susceptibility to *P. marinus* infection and pathogenicity, but Bushek and Allen (1996a) did not observe "race-specific" resistance of the oysters to different *P. marinus* isolates. In other words, the most virulent *P. marinus* isolate in one oyster population was the most virulent across all oyster populations. This suggests that the relative virulence of isolates is independent of oyster population.

The present study indicates that oysters from the Northeast and Southeast Atlantic coasts and along the Gulf of Mexico can be exposed to different "strains" of *P. marinus* depending on their geographic location. Since previous studies have suggested differences in virulence among isolates (Bushek and Allen 1996a), it is possible that the disease commonly referred to as "Dermo" could have varying impacts on oyster populations. Bushek and Allen (1996a) found that of the parental isolate cultures they tested, VA-1 (genotype 8) was the most virulent. NJ-1 (genotype 1) was moderately virulent and LA-1 (genotype 7) and TX-1 (genotype 3) had relatively low virulence. Genetic differences in the parasite may reflect variations in virulence, but disease challenge studies using clonal isolates of the different genotypes must be done to determine if there is a correlation between genetic strain and virulence.

Since it was first described in 1950 and until the mid-1980s, *P. marinus* was found infecting oysters from the lower portion of Chesapeake Bay southward along the Atlantic and Gulf of Mexico coastlines. There were only sporadic epizootics of *P. marinus* in the coastal waters north of this range, and these were usually associated with oyster importations. The epizootic events did not generally result in high mortalities, and there did not appear to be an established parasite population in this region (Newman 1971; Meyers 1981; Cooper and Durfee 1982; Ford 1996). Beginning in the mid- to late-1980s, however, there has been a progressive northward extension of the *P. marinus* range into the more northern reaches of Chesapeake Bay and along the Atlantic coast. This expansion has had devastating effects on oyster populations in the upper Chesapeake Bay, Delaware Bay, and in Long Island Sound. Ford (1996) proposed several explanations for the expansion, including a general climate warming over the past few decades that has allowed the parasite to survive and proliferate. In addition, she suggested that there could have been a specific introduction of infected oysters to the region that resulted in establishment of the parasite. Several previous introductions, however, had not resulted in persistent prevalence of the parasite, and several significant epizootic events in the 1990s did not appear to be associated with particular transplants of infected oysters. Protozoan parasites can, however, be introduced to a region by transplantation of host species (Hoffman 1970; Elston et al. 1986; Hudson and Hill 1991; Burreson et al. 2000), and there is strong genetic evidence that the parasite *Haplosporidium nelsoni* was introduced to the Atlantic coast by importation of the non-native

oyster *Crassostrea gigas* (Burreson et al. 2000). While less virulent to its native host *C. gigas*, the parasite *H. nelsoni*, like *P. marinus*, has heavily impacted *C. virginica* populations along the Atlantic coast of the USA. In addition, Ford also discussed the possibility that changes in the distribution of either strains of *P. marinus* or populations of its host *C. virginica* may have increased the parasite's ability to invade the Northeast.

In the present study *P. marinus* of composite genotype 1 were found in 82% of infected oysters from the Northeast region, the area defined by the parasite's recent range expansion. Seven of ten oysters from the Atlantic coast and 21 of 24 from the Maryland portion of Chesapeake Bay carried this strain of *P. marinus*. Along the southeastern Atlantic coast *P. marinus* of genotype 1 were found in only about 6% of oysters (Table 4). In addition, genotype 1 *P. marinus* were not found in any Gulf coast oysters. Strains found in the Northeast, with the exception of genotype 3, are relatively closely related to the heterozygous genotype 1, with only one (genotypes 5 and 7) or two (genotype 2) homozygous loci (Fig. 1). It is worth noting that *P. marinus* genotype 3 was found at three of the six Northeast Atlantic coast sites (in 3 of 10 oysters), while it was absent from upper Chesapeake Bay sites. Since genotype 3 is dominant in the Southeast and Gulf regions and genotype 1 is rare, but present, in the Southeast, it is possible that transplantation of oysters (most likely from the Southeast) resulted in transport of parasite cells to the Northeast.

Overall the results of this study are consistent with a founder event and bottleneck in the Northeast resulting from oyster transplantation or natural dispersal, while a combination of factors may have contributed to the northward expansion of *P. marinus*. These factors include the introduction and proliferation of parasite genotypes that were more tolerant of cooler temperatures and a "recent warming trend that has made the environment suitable for parasite development" as suggested by Ford (1996). It is possible that some *P. marinus*, in particular those of genotype 1, are more cold tolerant and/or that there was a specific introduction of these strains with oysters from the Southeast at a time when environmental conditions were favorable for establishment and propagation of the parasite. Likewise, it is possible that at least one genetic strain of *P. marinus* (genotype 4) cannot tolerate the relatively cooler waters of the Atlantic coast, since it was found only in oysters along the Gulf coast. Tolerance of different *P. marinus* genetic strains to diverse environmental conditions needs to be investigated. Studies to examine variations in virulence and environmental tolerance among genetic strains and to explore more directly host/parasite interactions with genetically different oyster stocks and *P. marinus* strains, will promote a better understanding of the mechanisms of Dermo disease.

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